THE INTERACTION BETWEEN *FRANKIA* AND ECTOMYCORRHIZAE ON A SHARED *ALNUS RUBRA* HOST

BY

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A Thesis submitted to
the Faculty of Graduate Studies
In Partial Fulfillment of the Requirement for the Degree of

MASTER OF SCIENCE

Department of Botany
University of Manitoba
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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Abstract

Most studies examining tripartite mutualisms examine whether adding a second endosymbiont increases host plant benefit. My thesis uses two experiments to examine the relationship between *Frankia* and ectomycorrhizae on a shared *Alnus rubra* host. The first involved manipulating both mutualisms with fertilisers of varying phosphorus and nitrogen concentrations. *Alnus rubra* plants were inoculated with *Frankia, Paxillus involutus*, or both *Frankia* and *P. involutus* and fertilised with one of five fertilisers. Increasing nitrogen concentrations had a positive effect on growth while increasing phosphorus concentrations had no effect. *Frankia* increased plant growth while mycorrhizae had no effect on plant growth due to a lack of mycorrhizal development. There was a non-significant trend for mycorrhizae to increase the specific nitrogenase activity of the nodules; however, this trend did not translate into an increase in plant growth. It is possible that either the *Frankia* or the mycorrhizae used the nitrogen. In my experiment, *P. involutus* has a beneficial effect on *Frankia* but the effect of *Frankia* on *P. involutus* is still unclear.

The second experiment was designed to examine the interaction between the two endosymbionts on endosymbiont colonisation. *Alnus rubra* plants were inoculated with various combinations of spore positive *Frankia*, spore negative *Frankia*, *Paxillus involutus*, and *Hebeloma crustuliniforme*. Spore positive *Frankia* were more infective on uninoculated plants and equally infective as spore negative *Frankia* on plants inoculated with spore positive *Frankia,* spore negative *Frankia*, or both spore positive and negative *Frankia*. All plants formed mycorrhizae in treatments inoculated with *H. crustuliniforme* but not in treatments inoculated with *P. involutus*. Mycorrhizae had no effect on the

colonisation of either *Frankia* type. Mycorrhizae did, however, decrease plant shoot dry mass compared to the shoot dry mass of non mycorrhizal plants. Both shoot and nodule masses were higher in plants inoculated with one type of *Frankia* compared to plants inoculated with both types of *Frankia*. Neither mycorrhizae nor *Frankia* had an effect on colonisation of the other endosymbiont. Mycorrhizae had a beneficial effect on the *Frankia* mutualism by increasing nitrogen fixation, but *Frankia* do not appear to have a beneficial effect on the mycorrhizal mutualism.

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1 Introduction

The relationship between *Alnus rubra*, *Frankia*, and ectomycorrhizae is described as a tripartite mutualism, a mutualism between three organisms. Although we know the relationship between *A. rubra* and *Frankia* is a mutualism and the relationship between *A. rubra* and ectomycorrhizae is a mutualism, we do not know the nature of the relationship between *Frankia* and ectomycorrhizae on the same host. As such, we cannot say that this system is a true tripartite mutualism. This thesis examines the interaction between *Frankia* and ectomycorrhizae on a shared *A. rubra* host in an attempt to determine if the relationship between the *Frankia* and ectomycorrhizae is, in fact, a mutualism.

In order to examine the relationship between the two endosymbionts, I designed two experiments. The first examined the hypothesis that *Frankia* and *Paxillus involutus* on the same host form a mutualism because each alleviates the nutrient deficiency of the other organism. My experiment used a factorial design of five fertilisers of varying phosphorus and nitrogen concentrations (0 mM P, 5 mM N; 5 mM P, 5 mM N; 10 mM P, 5 mM N; 5 mM P, 0 mM N; and 5 mM P, 10 mM N) and four endosymbiont treatments (no endosymbiont, inoculated with *Frankia*, inoculated with *P. involutus*, and inoculated with both *Frankia* and *P. involutus*).

The second experiment examined three hypotheses. The first was that *Frankia* and ectomycorrhizae benefit the host by increasing plant growth by providing nitrogen and phosphorous to the host plant. The second was that when two similar endosymbionts (e.g. two strains of mycorrhizal fungus) colonise the host plant, the presence of a second endosymbiont (e.g. a nitrogen-fixing bacterium) would alter the colonisation rates by

interacting with the first endosymbiont. The third was that the presence of one endosymbiont would benefit the mutualism of a second endosymbiont by providing nutrients to that mutualism. I tested these hypotheses by inoculating *A. rubra* plants with various combinations of spore positive *Frankia*, spore negative *Frankia*, *P. involutus*, and *Hebeloma crusuliniforme*.

The main objective of this thesis was to understand the relationship between *Frankia* and ectomycorrhizae on the same host. More specifically, the objective in the first experiment was to discover if the relationship between *Frankia* and *P. involutus* on a shared host was mutualistic. The objective in the second experiment was to discover if *Frankia* and ectomycorrhizae have an effect on each other's colonisation rates.

2 Literature Review

2.1 Definition of terms

This thesis examines the symbiotic relationships between *Alnus rubra*, *Frankia*, and ectomycorrhizae. The term symbiosis, first used by de Bary (1879) (in Lewis, 1985), refers to an intimate physical relationship between two or more unrelated organisms (Francis and Read, 1995). Symbioses are categorised based on how both organisms in the relationship are affected and can be beneficial, neutral, or detrimental to the organisms involved. However, these effects exist on a continuum with one extreme being relationships in which both organisms are beneficially affected and the other extreme being relationships in which both organisms are detrimentally affected. Lewis (1985) proposes six categories of symbioses: 1) both organisms are detrimentally affected – competition; 2) one organism is detrimentally affected while the other is not affected – amensalism; 3) one organism is detrimentally affected while the other benefits – agonism; 4) both organisms are not affected – neutralism; 5) one organism benefits while the other is not affected – commensalism; and 6) both organisms benefit – mutualism. Although the categories of amensalism and neutralism are included in the categories of symbioses, they are academic rather than actual categories because these relationships do not appear to occur in nature.

2.1a Mutualism *vs.* symbiosis

Despite their different definitions, in recent years the terms mutualism and symbiosis have come to be used interchangeably. In fact, some authors use the term symbiosis to refer only to mutualisms (see Starr, 1975; Goff, 1982; Lewin, 1982; Lewis,

1985; Douglas and Smith, 1989) leading to considerable confusion regarding the terms (Starr, 1975; Goff, 1982; Lewis, 1985; Douglas and Smith, 1989; Francis and Read, 1995). As Starr (1975) points out, it is impractical to have the same word refer to both a natural phenomenon as well as a subset of that phenomenon. For my thesis, I will follow Starr (1975), Lewis (1985), and Francis and Read (1995) in using the term symbiosis to refer to a close living association between two or more organisms and the term mutualism to refer solely to a relationship in which both organisms benefit.

2.1b Host and endosymbiont

The definitions of symbioses and mutualisms are not the only definitions related to mutualisms that are debated by researchers. Consider the definition of the word host. There are three general ways to delineate host and endosymbiont, each with its own problems. The first definition of host and endosymbiont is by size. Generally, the host is considered the larger of the two organisms. This definition can be problematic, though, as mycorrhizal fungi in the soil may actually be larger than the host plant they colonised in terms of total biomass and area occupied. The second definition of host and endosymbiont is based on the outcome of the relationship. If one organism parasitises another organism, the organisms that is doing the parasitising is regarded as the endosymbiont while the organism being parasitised is regarded as the host. This definition can be problematic because the outcome of a relationship is dynamic. Although mycorrhizae are generally considered to be mutualistic, there are times during the life of the plant during which the plant may not benefit. In addition, some mycorrhizal fungi have been shown not to benefit the host at all and could be said to be parasitic. Plants are generally considered to be hosts for mycorrhizal fungi. In orchid

mycorrhizae, it is the plant that parasitises the fungi (Singh and Varma, 2000), blurring the definition of host and endosymbiont. The third definition of host and endosymbiont is based on habitat. Under this definition, the endosymbiont is the organism that lives inside the host. Mycorrhizae, however, live both inside and outside the host so there is some disagreement as to whether mycorrhizae can be classed as endosymbionts. Douglas and Smith (1989) do not consider mycorrhizae to be endosymbiotic since the fungus is not entirely within the roots while Law and Lewis (1983) consider mycorrhizae to be endosymbiotic. In a further complication, vesicular arbuscular mycorrhizae (VAM) could be considered more endosymbiotic than ectomycorrhizae since the VAM penetrate the host root cells while ectomycorrhizae do not. For my thesis, I will follow the usage of Law and Lewis (1983) and refer to mycorrhizal fungi as endosymbionts, *Frankia* as endosymbionts, and *Alnus rubra* as the host.

2.1c Benefit

The term benefit is crucial to classifying a symbiosis and there are two main ways of defining benefit. Since mycorrhizae and *Frankia* symbioses involve the transfer of carbon and nutrients between host and endosymbiont, several authors (e.g. Mejstrik and Benecke, 1969; Fitter, 1985; Keeler, 1985; Rytter *et al.*, 1991; Thomas *et al.*, 2000) define benefit as an increase in the nutrients or carbon the organism receives. Following their logic, if, for example, plant nitrogen concentrations increase because of a *Frankia* mutualism, the plant has benefited from the relationship. Similarly, if a plant must increase its photosynthetic output as a result of providing carbon to a *Frankia* symbiont, the plant is said to have paid a cost as a result of the relationship. Overall, the relationship is considered mutualistic if the benefit outweighs the cost. It is difficult to

determine whether an increase in nitrogen outweighs a decrease in photosynthates. Since the two are not comparable, there is no practical scale for equating a given amount of nitrogen or phosphorus to an equivalent amount of photosynthates. For example, if a nitrogen-fixing bacterium increases a plant's nitrogen content by 10% but takes 5% of the plant's photosynthates, does this constitute a benefit to the plant? It is difficult to determine, using photosynthates and nutrients as currency, whether the host plant has benefited. The ultimate benefit of any relationship is an increase in fitness and as such, it is more intuitive to define the benefit of a given mutualism as an increase in fitness. This definition solves the problem of comparing nutrients gained with carbon lost to determine overall benefit. Several authors have defined benefit in this manner (Gates and Wilson, 1974; Law, 1985; Douglas and Smith, 1989; Gange, 1999) and for my thesis, I will follow these authors in defining benefit as an increase in fitness. The fitness of plants in this thesis will be measured indirectly by measuring the shoot dry mass of the plants, which is correlated with plant fitness (Johnson, 1993).

Although the definition of benefit as an increase in fitness is intuitive, it is not always easily applicable. The question of how to measure benefit for an endosymbiont has not been resolved and is still being debated (Douglas and Smith, 1989). Several authors (e.g. Nutman, 1963; Sprent *et al.*, 1987; Douglas and Smith, 1989) state that the endosymbiont benefits from being provided with a safe habitat in which to live. An endosymbiont only benefits if the endosymbiont's fitness increases as a result of the mutualism; however, determining fitness can be problematic. *Frankia* is a slow-growing bacterium that is difficult to culture. As such, it is difficult to determine the fitness of free-living *Frankia* and, consequently, the benefit (increase in fitness due to the

symbiosis) *Frankia* receives from the host is difficult to calculate. Douglas and Smith (1989) point out that when working with obligate endosymbionts, such as VAM, it is difficult to distinguish between benefit and dependence. An endosymbiont is dependent on the host when the endosymbiont cannot live outside of the host. According to Douglas and Smith (1989), dependence is widely accepted to be beneficial to the endosymbiont because the fitness outside the host is zero. They argue that the endosymbiont's dependence is actually a cost because if the two organisms are separated, the endosymbiont will die. In addition, the current means of determining benefit cannot distinguish between benefit and dependence (Douglas and Smith, 1989).

One of the few studies to examine the benefit of a mutualism to the endosymbiont was conducted by Bever and Simms (2000). The model they created was based on the legume-*Rhizobium* system but is applicable to other endosymbiotic mutualisms. The overall conclusion was that rhizobia benefit from a mutualism via the process of kin selection (Bever and Simms, 2000). An organism's reproductive fitness is determined by the production of offspring by that organism and by that organism's kin. Rhizobia cells in nodules have low to zero fitness but they increase the fitness of related free-living rhizobia by increasing carbon exudates from the nodule. The model proposed by Bever and Simms (2000) demonstrates that an increase in spatial structure, resulting in an increase in mixing in the population, increases the likelihood that non-nodulating kin benefit from nodulation by rhizobia through an increase in plant exudates. Additionally, their work demonstrates that habitat is not the only positive effect for endosymbionts as is commonly assumed (Douglas and Smith, 1989). It is widely assumed that the endosymbiont benefits from a relationship with the host because the host provides a

habitat for the endosymbiont that is free from predators and nutrient rich and, as such, is assumed to increase the endosymbiont's fitness.

The model proposed by Bever and Simms (2000) only applies to endosymbionts that live entirely within the host, such as rhizobia. Unlike rhizobia, when endosymbionts, such as fungi, colonise the host, they retain their reproductive ability. These organisms, though, exist both inside and outside the host, making it possible for them to reproduce outside the host. Although *Frankia* are nitrogen-fixing bacteria like rhizobia, they do not have the same drastic reduction in fitness that *Rhizobia sp.* have when they colonise the host. *Rhizobia sp.* differentiate into bacterioids in the nodule, which prevents them from reproducing. *Frankia* do not differentiate into bacterioids as rhizobia do. *Frankia* are capable of producing spores in the nodule and these spores allow the *Frankia* to reproduce (Benson and Silvester, 1993). For my thesis, I will be focusing purely on the benefit to the host and measuring that benefit by measuring the shoot dry mass of the alder plants. I will not be considering the benefit the endosymbiont may receive from the host plant.

2.1d Plant – soil microbe mutualisms

Plants form many relationships with soil microbes, two of the most important being the mutualisms involving nitrogen-fixing bacteria and mycorrhizal fungi. Nitrogen-fixing bacteria, in symbiosis with vascular plants, produce the majority of biologically usable nitrogen in terrestrial ecosystems by reducing atmospheric nitrogen (N_2) to ammonium (NH_4^+) (Postgate, 1982). The rhizobia-legume mutualism adds a large amount of usable nitrogen to the soil, 90Tg of nitrogen per year globally (Vance, 1997), and as such, is vital to agriculture. Actinorhizal mutualisms form between *Frankia*

and members of the Betulaceae, Casuarinaceae, Coriariaceae, Datiscaceae, Elaeagnaceae, Myricaceae, Rhamnaceae, and Rosaceae, all of which, with one exception, are trees and shrubs (Huss-Danell, 1997). In non-agricultural settings, these actinorhizal plants are responsible for producing anywhere from less than 1 kg/ha/year to over 300kg/ha/year of nitrogen (Silvester, 1983). Global totals for actinorhizal nitrogen fixation are not available.

Mycorrhizae are the other important mutualism that plants enter into with soil microbes. Mycorrhizal fungi colonise host plant roots and form hyphal projections that extend into the soil and absorb nutrients that are then transferred to the host plant in exchange for photosynthates. There are many classification systems for mycorrhizae; however, generally all classification systems agree that there are at least four main types of mycorrhizae: ericoid mycorrhizae, vesicular arbuscular mycorrhizae (VAM), ectomycorrhizae, and orchid mycorrhizae. Ericoid mycorrhizae form between members of the Ericales and members of the Ascomycota and Basidiomycota and involve penetration of the hyphae into host root cells (Harley and Smith, 1983). VAM also involve penetration of the hyphae into host root cells although the hyphae form arbuscules and vesicles in the root cells, which ericoid mycorrhizae lack (Harley and Smith, 1983). VAM form between the majority of terrestrial plants and members of the Glomales. Unlike ericoid mycorrhizae and VAM, ectomycorrhizae do not involve penetration of the host cells; instead, the fungus surrounds the host cortical cells forming a Hartig net (Harley and Smith, 1983). Ectomycorrhizae form between fungi in the Agaricales, Russulales, Gautieriales, Hymenogastrales, Phallales, Lycoperdales, Melanogastrales, Sclerodermatales, Aphyllophorales, Eurotiales, Pezizales, Tuberales,

Endogonales (Lakhanpal, 2000) and plants in the Betulaceae, Fagaceae, Pinaceae, Rosaceae, Mimosaceae, and Salicaceae (Gupta *et al.*, 2000). Orchid mycorrhizae form between members of the Orchidaceae and the fungal form genus *Rhizoctonia sp.*, which has members in the Ascomycota and Basidiomycota (Singh and Varma, 2000). Orchid mycorrhizae involve the penetration of host root cells (Singh and Varma, 2000), similar to VAM and ericoid mycorrhizae. Orchids are achlorophyllous for at least part of their life and depend on the mycorrhizae to provide them with carbon while they are achlorophyllous (Smith and Read, 1997).

Most mycorrhizae are considered to be mutualisms. It is difficult to determine whether VAM fungi benefit from the relationship, though, since the fungus is an obligate endosymbiont. Mutualisms, like any other type of symbioses, are not static. Although they are generally beneficial to both organisms, changing environmental conditions or genetic variation can result in some mutualisms being more beneficial than others. In addition, there is always the possibility that the endosymbiont could start to parasitise the host and make the mutualism detrimental to the host, possibly to the extent that the relationship is no longer a mutualism but rather a parasitism. Ectomycorrhizae, such as those used in this thesis, provide mineral nutrients, specifically phosphorus, and water to the host plant and receive photosynthates in return (Manoharachary and Reddy, 2000). Gupta *et al.* (2000) suggest additional beneficial roles that mycorrhizae may play in the host including pathogen protection and tolerance to drought. These effects are not universally associated with mycorrhizae, however.

2.1e Tripartite symbioses

Work on symbioses has generally focused on symbioses composed of two organisms even though typically symbioses do not occur between isolated pairs of organisms. A single host plant can simultaneously have two or more endosymbionts. Alder, for example, can form mutualisms with the actinomycete *Frankia*, vesicular-arbuscular mycorrhizae (VAM), and ectomycorrhizae. These tripartite (symbioses involving three organisms) and tetrapartite (symbioses involving four organisms) symbioses can form between different endosymbionts (*e.g. Frankia* and ectomycorrhizae) or similar endosymbionts (*e.g.* VAM and ectomycorrhizae). The idea of mutualisms existing as isolated pairs of organisms is further complicated by the fact that different strains of *Frankia* or fungi may simultaneously colonise the host plant. Recently, researchers have begun to investigate these tripartite and tetrapartite relationships. The majority of this research has tended to focus on the legume, rhizobia, and VAM mutualisms (Bethlenfalvay *et al.*, 1997; Robson *et al.*, 1981; Duc *et al.*, 1989) since legumes are importantly agriculturally; however, some work has been done on tripartite mutualisms involving actinorhizal plants such as *Alnus spp.* (Carling *et al.*, 1978; Rose and Youngberg, 1981; Russo, 1989).

Research on tripartite mutualisms has tended to follow one of two paths. The first comprises studies examining whether the host plant benefits more from the tripartite relationship than relations with a single endosymbiont (Gardner *et al.,* 1984; Chatarpaul *et al.*, 1989; Wheeler *et al.*, 2000). The second comprises studies investigating how the tripartite mutualism affects nitrogen fixation in nodules (Carling *et al.*, 1978; Asimi *et al.*, 1980; Bethlenfalvay and Yoder, 1981; Robson *et al.,* 1981; Rose and Youngberg, 1981; Russo, 1989; Wheeler *et al.*, 2000). Although all these studies examine the interaction

between the two endosymbionts, they only examine the effects of the interaction on the host and occasionally one endosymbiont. Little research has been done to determine the nature of the interaction between the endosymbionts.

My thesis focuses on the tripartite mutualism between *Alnus rubra*, *Frankia*, and the ectomycorrhizal fungi *Paxillus involutus* and *Hebeloma crustuliniforme*, specifically investigating the interaction between the two endosymbionts on the shared alder host. My thesis is comprised of two experiments designed to study this interaction. The first experiment was designed to test the hypothesis that *Frankia* and ectomycorrhizae on the same host would have a mutualistic relationship because both *Frankia* and ectomycorrhizae provide nutrients for the other organism to use, alleviating the nutrient deficit of the other organism in the mutualism. The second experiment was designed to test three hypotheses. The first hypothesis was that *Frankia* and ectomycorrhizae would be beneficial to the host by providing nitrogen and phosphorus to the plant and thus increasing plant fitness. The second hypothesis was that when two similar endosymbionts were used to inoculate the host, the presence of a second type of endosymbiont would alter the colonisation rates of the first type of endosymbiont by increasing colonisation of one endosymbiont at the expense of the other. The third hypothesis was that the presence of one endosymbiont would have a beneficial effect on the mutualism involving the other endosymbiont by providing nutrients to that mutualism.

2.2. The interaction between soil nutrient concentrations, nitrogen-fixing bacteria, and mycorrhizae on a tripartite mutualism.

2.2a Introduction

A mutualism is a close physical relationship between two organisms in which both organisms benefit from the interaction (Francis and Read, 1995). A mutualism between a host and an endosymbiont is a direct mutualism; that is, the host and endosymbiont interact directly with each other. Plants can form many mutualisms simultaneously and when actinorhizal plants, for example, are colonised by *Frankia* and ectomycorrhizal fungi, three relationships are possible. A direct relationship exists between the host and each of the endosymbionts and a third relationship exists between the two endosymbionts. Cluett and Boucher (1983) suggest that there are four possible outcomes for this relationship. The two endosymbionts could have an indirect, competitive relationship (such as those seen in exploitation competition); an indirect, mutualistic relationship; a direct competitive relationship (such as those seen in interference competition); or a direct mutualistic relationship (Cluett and Boucher, 1983). An indirect relationship occurs when two organisms interact via a third organisms. In this case, the two endosymbionts would interact via the host plant. Indirect relationships are also known as diffuse relationships.

Symbioses are not static; as conditions change, the benefit gained from the interaction also changes. Two factors that might be expected to have a large impact on a tripartite mutualism involving plants and soil microbes are the soil environment (primarily the soil nutrient concentrations) and the presence of a second endosymbiont. Soil nutrient concentrations are known to affect plant growth and both nitrogen-fixing bacteria and mycorrhizae help alleviate deficiencies in soil nutrients for the host plant.

As such, it is intuitive that soil nutrient concentrations will have an impact on tripartite mutualisms.

Under field conditions, plant growth is often limited by nitrogen (Stilling, 2002) or phosphorus (Ricklefs, 1997). As such, adding nitrogen (Stewart and Bond, 1961; Gates and Wilson, 1974; Hera, 1976; Sistachs, 1976; Bethlenfalvay *et al.*, 1978; Johnson, 1993) or phosphorus (Hayman and Mosse, 1979; Roldan-Fajardo *et al.*, 1982; Pacovsky *et al.*, 1986-a; Johnson, 1993; Ekblad *et al.*, 1995; Uliassi *et al.*, 2000) to the soil will increase plant growth. Not all of the research suggests that increased nitrogen and plant fitness are positively correlated. Ekblad *et al.* (1995) found that increasing nitrogen had no effect on *Alnus incana* total plant biomass while Thomas *et al.* (2000) found that nitrogen did not have an effect on the dry mass of *Gliricidia sepium*. Ekblad *et al.* (1995) used a low dosage of ammonium nitrate for their high nitrogen treatment (54.0 mg N/kg soil, which is equivalent to 0.675 mM), which could account for their results; however, Stewart and Bond (1961) used an even lower dosage and still found nitrogen had an effect. As the nitrogen concentration in the fertiliser increased, nitrogen fixation decreased while nitrogen absorption from the soil increased (Thomas *et al.*, 2000). It is likely that the increase in nitrogen applied to the plants did not translate into an increase in plant nitrogen content. As such, plant growth might not show a response to increasing fertiliser concentrations since although the method of nitrogen procurement changes, the overall nitrogen available to the plant remains constant.

Several researchers have found that there is an interaction between phosphorus and nitrogen on the host plant. Gates and Wilson (1974) found that as the concentration of phosphorus in the fertiliser increases, the concentration of nitrogen in the fertiliser has

more of an impact on the plants. Huss-Danell (1997) states that providing plants with phosphorus can reverse the negative effect nitrogen has on nodulation but the mechanism of this reversal is unclear. Nitrogen inhibits nodulation in *Alnus spp.* by preventing the production of root hairs (Huss-Danell, 1997). Since *Frankia* infect *Alnus spp.* via root hairs, the lack of root hairs prevents nodulation. The ability of phosphorus to reverse the inhibitory effect of nitrogen is most likely due to the fact that nitrogen fixation and nodulation have a high phosphorus requirement (Asimi *et al.*, 1980) and when the phosphorus requirement is satisfied, nodule growth increases and as such, more nitrogen is needed to have the same level of inhibition of nitrogenase activity. Ekblad *et al.* (1995) performed an experiment on the effects of high and low concentrations of nitrogen, potassium, phosphorus, calcium, magnesium, and sulphur in various combinations on *Pinus sylvestris* and *Alnus incana* inoculated with *Paxillus involutus*. They found that the application of both nitrogen and phosphorus to *Alnus incana* caused the mycorrhizal plants to have larger total biomass than non-mycorrhizal plants, whereas applying only phosphorus or nitrogen did not cause the two to differ (Ekblad *et al.*, 1995). The addition of both nitrogen and phosphorus decreased mycorrhizal colonisation more than application of either phosphorus or nitrogen alone (Ekblad *et al.,* 1995).

2.2b Nitrogen-fixing bacteria

The effect of nitrogen-fixing bacteria on host benefit

Research shows that a symbiosis with nitrogen-fixing bacteria tends to increase plant benefit compared to plants that are not in a symbiosis with nitrogen-fixing bacteria. Sanginga *et al.* (1989) examined the effects of fertiliser and *Frankia* on *Casuarina equisetifolia, Allocasuarina littoralis*, and *Allocasuarina torulosa*. *Frankia* inoculation

increased the shoot dry mass of *C. equisetifolia* plants compared to uninoculated *C. equisetifolia* plants. The shoot masses of both *Allocasuarina* species was not significantly different between inoculated and uninoculated plants. Ham *et al.* (1976) examined four soybean isolines inoculated with three strains of *Rhizobium japonicum.* They found that the non-nodulating isolines had 20-41% of the yield of nodulating isolines (Ham *et al.*, 1976). Subba Rao (1976) grew inoculated and uninoculated *Cicer arsetinum* plants in field experiments around India and found that the yield increased when plants were inoculated with *Rhizobium sp.*

Nitrogen-fixing bacteria do not universally increase host plant fitness. The benefit received by the host plant depends, to some extent, on the strain or species of nitrogen-fixing bacteria and the species of the host. While there was a difference in shoot mass for inoculated and uninoculated *Casuarina equisetifolia*, there was no difference in the shoot mass between inoculated and uninoculated *Allocasuarina littoralis* and *Allocasuarina torulosa* (Sanginga *et al.*, 1989). Ham *et al.* (1976) found that rhizobia could be beneficial or detrimental to the host depending on the variety of *Rhizobium japonicum* used. Subba Rao (1976) found that the benefit the host *Cicer arietinum* received varied depending on the strain of *Rhizobium sp.* used and the location of the experiment. It appears that the genetic variation of the host plants and the nitrogen-fixing bacteria is responsible for the variation in benefit to the host. However, there do not appear to be any trends (e.g. taxonomic, geographic) as to which host/strain combination will be the most beneficial to the host plant.

The interaction of nitrogen and symbiotic nitrogen-fixing bacteria on host benefit

The effect of nitrogen-fixing bacteria on the host is altered by the nitrogen concentration in the fertiliser that is applied to the plant. Bethlenfalvay *et al.* (1978) examined the effect of applying fertiliser with either 0, 2, 4, 8, or 16 mM $(NH_4)_2CO_3$ to *Pisum sativum* plants. They found that inoculation with *Rhizobium leguminosarum* was only beneficial to the host at 0 or 2 mM nitrogen levels. At 4, 8, and 16 mM nitrogen concentrations, inoculation with *R. leguminosarum* was not beneficial to the host plant. Pacovsky *et al.* (1986) examined the effect of 0.0, 1.0, 2.0, and 4.0 mM NH_4NO_3 fertiliser and inoculation with *Glomus fasciculatum* and *Bradyrhizobium japonicum* on *Glycine max.* They found that there was no difference in shoot mass between plants receiving the highest rates of nitrogen application (2.0 and 4.0 mM) and plants that were inoculated with *B. japonicum.* Inoculation with *B. japonicum* was only beneficial to the plant in comparison to plants inoculated with 0.0 or 1.0 mM nitrogen.

The inoculated and uninoculated *P. sativum* responded differently to the application of various nitrogen fertiliser concentrations (Bethlenfalvay *et al.*, 1978). The inoculated plant dry mass increased at low concentrations and then slowly decreased whereas the uninoculated plant dry mass increased until it reached a plateau. Bethlenfalvay *et al.* (1978) suggest that the nitrogenase rates are responsible for these results. Nitrogenase activity peaked at a nitrogen fertiliser concentration of 2 mM and decreased at nitrogen concentrations higher than 2 mM (Bethlenfalvay *et al.*, 1978). When the nitrogen fixation rates are high, the presence of *R. leguminosarum* benefits the host. They suggest that at the higher nitrogen fertiliser concentrations, the presence of nodules on the plant inhibits NH_4^+ absorption by the roots so that the plant cannot switch from fixing nitrogen to absorbing it (Bethlenfalvay *et al.*, 1978). Because nitrogen-fixing

bacteria act as a carbon drain on the host, the shoot mass of inoculated plants will be less than uninoculated plants that can absorb nitrogen because the uninoculated plants will not be diverting photosynthates from growth to their endosymbionts.

The effect of phosphorus on symbiotic nitrogen-fixing bacteria

In general, phosphorus has a positive effect on nitrogen-fixing bacteria. Hayman and Mosse (1979) performed a field test examining the responses to *Trifolium repens* plants inoculated with *Rhizobium trifolii* to 0, 22.5, and 90 kg P/ha fertiliser. They found that as the phosphorus concentration applied to inoculated *Trifolium repens* plants increased from 0 to 22.5 to 90 kg P/ha, the nodule dry mass increased. Israel (1987) performed a pot experiment examining the effect of 0, 0.1, 0.25, 0.5, 1.0, and 2.0 mM phosphorus fertilisers on inoculated and nitrate fertilised *Glycine max* plants. He found that found that as the phosphorus fertiliser concentration applied to *Glycine max* inoculated with *Bradyrhizobium japonicum* increased, nodule fresh weight per plant, the number of nodules per plant, the fresh weight/nodule, nitrogenase activity per plant, and the specific nitrogenase activity (the amount of ethylene produced per hour per gram of nodule). Robson *et al.* (1981) performed a pot experiment examining the effects of 0.1, 0.2, 0.6, and 1.2 g P per pot and inoculation with *Glomus monosporus* on *Trifolium subterraneum* plants inoculated with *Rhizobium trifolium.* They found that the nitrogenase activity per pot for *R. trifolii* inoculated *T. subterraneum* plants increased as phosphorus fertiliser concentrations increased. Asimi *et al* (1980) performed a pot experiment to examine the effects of 0, 0.25, 0.5 g P/kg soil and inoculation with *Glomus mosseae* on *Glycine max* plants inoculated with *Rhizobium japonicum.* They found that the acetylene reduction per plant and nodule dry mass of uninoculated plants and plants

inoculated with *G. mosseae* increased as the concentration of phosphorus applied to the plant increased. Jha *et al.* (1992) examined the effects of 0, 30, 60, 120, and 240 kg P/ha on *Alnus nepalensis* plants inoculated with *Glomus mosseae* and *Frankia.* They found that as the phosphorus applied to the plants increased, the specific nitrogen fixation rates (nitrogen fixed/g nodule) increased.

Phosphorus does not always have a positive effect on nitrogen-fixing bacteria, though. Russo (1989) examined the effect of 10, 50, and 100 ppm phosphorus fertiliser concentrations and inoculation with *Glomus intra-radices* on *Alnus incuminata* inoculated with *Frankia.* He found that specific acetylene reduction did not change between 10 ppm and 50 ppm phosphorus, but dropped when 100 ppm phosphorus was given to *A. acuminata* inoculated with *Frankia.* Robson *et al* (1981) examined the effect of inoculation with *Glomus monosporus* and 0.1, 0.2, 0.6, and 1.2 g P/pot on *Trifolium subterraneum* plants inoculated with *Rhizobium trifolli.* Uliassi *et al.* (2000) examined the effects of poplar and alder soil, the presence and absence of phosphorus fertiliser, and *Frankia* inoculation on *Alnus tenuifolia.* Robson *et al.* (1981) and Uliassi *et al.* (2000) found that, while nitrogenase activity per plant increased with increasing phosphorus concentrations, the specific nitrogenase activity was unaffected. This lack of a phosphorus effect is most likely due to the fact that phosphorus increases both nitrogenase activity (Israel, 1987; Asimi *et al.*, 1980; Uliassi *et al.*, 2000; Robson *et al.*, 1981) and nodule mass (Israel, 1987; Hayman and Mosse, 1979). While the nitrogenase activity per plant increases, the corresponding increase in nodule mass means that specific nitrogenase activity remains constant.

The effect of nitrogen on symbiotic nitrogen-fixing bacteria

Nitrogen applications tend to have two different effects on nitrogen-fixing bacteria. At low concentrations, nitrogen tends to increase nitrogenase activity while at high concentrations, it tends to decrease nitrogenase activity (Huss-Danell, 1997). Bethlenfalvay *et al.* (1978) examined the effects of 0, 2, 4, 8, and 16 mM $(NH_4)_2CO_3$ and inoculation with *Rhizobium leguminosarum* on *Pisum sativum* plants. They found that nitrogenase activity peaked at a concentration of 2 mM and increasing the nitrogen concentration past 2 mM caused a decrease in nitrogenase activity. Stewart and Bond (1961) examined the effect of 0, 10, 50, and 100 mg nitrogen/L on *Alnus glutinosa* and *Myrica gale* inoculated with *Frankia.* They found nitrogenase activity per plant increased when the applied nitrogen concentration was increased from 0 mg/L to 10 mg/L but increasing the nitrogen concentration beyond 10 mg/L actually decreased nitrogen fixation per plant. *Myrica gale* inoculated with *Frankia* experienced a decrease in nitrogenase activity as the concentration of nitrogen applied to the plants increased from 0 to 100 mg nitrogen/L (Stewart and Bond, 1961). Ekblad and Huss-Danell (1995) found that nitrogenase activity in *Alnus incana* inoculated with *Frankia* decreased with the addition of nitrogen to the soil. Thomas *et al.* (2000) examined the effect of CO_2 and 0, 1, and 10 mM nitrogen fertiliser on *Gliricidia sepium* inoculated with *Rhizobium.* They found that there was no difference in nitrogenase activity per plant between plants given 0 and 1 mM nitrogen but nitrogenase activity per plant increased as the nitrogen concentration increased from 1 to 10 mM. Nitrogenase activity was not the only aspect affected by increasing nitrogen levels. Increasing nitrogen levels also decreased nodule number (Thomas *et al.*, 2000) and the proportion of the total plant biomass that nodules occupied (Ekblad and Huss-Danell, 1995).

Nitrogen has the opposite effect to phosphorus on nodule mass. Nitrogen-fixing bacteria require phosphorus to form nodules and fix nitrogen (Asimi *et al.*, 1980) so as the phosphorus status of the soil and the plant increases, there will be more phosphorus available for nodule growth and nitrogen fixation. When plants are given nitrogen, though, they switch to absorbing nitrogen from the soil rather than fixing it in the nodules (Stewart and Bond, 1961). As a result, increasing the nitrogen levels in the soil and the plant will reduce the nodule dry mass.

As the soil nitrogen level increases the rate of nitrogen fixation has been found to decrease while the rate of nitrogen absorption increase in *Myrica gale* given 0, 10, 50, and 100 mg nitrogen/L (Stewart and Bond, 1961) and *Gliricidia sepium* given 0, 1, and 10 mM nitrogen (Thomas *et al.*, 2000). Bethlenfalvay *et al.* (1978) found that even though the amount of fixed nitrogen used by the *Pisum sativum* plants given 0, 2, 4, 8, and 16 mM nitrogen decreases, the total nitrogen content in the plant remains constant, indicating that the plant relies more on absorbed nitrogen to supply its nitrogen demands. Both ammonium and nitrate appear to inhibit nitrogen fixation (Huss-Danell, 1997; Gulden and Vessey, 1998; Vessey and Luit, 1999). There is no energetic difference between absorbing nitrogen and fixing nitrogen (Postgate, 1982) so it is likely that when plants switch from fixing nitrogen to absorbing it, they do so because the absorbed ammonium has inhibited the nitrogen fixation and so the plant must now absorb the nitrogen but not because it is energetically cheaper to do so.

2.2c Mycorrhizae

The effect of mycorrhizae on host benefit

Overall, the presence of mycorrhizae increases the shoot dry mass of the host plant. In other words, the host benefits from the mycorrhizae. Shoot mass is often used asan indirect measure of fitness. Michelsen and Sprent (1994) examined several species of *Acacia* that were inoculated with five VAM fungi and found that, in general, mycorrhizae increased shoot dry mass. Ianson and Linderman (1993) examined the effect different VAM species had on *Cajanus cajan* and found that shoot dry mass generally increases with the presence of mycorrhizae. Gange's (1999) hypothesis regarding mycorrhizal benefit suggest that, although host benefit increases with mycorrhizal colonisation, there is a maximum degree of colonisation that will be beneficial to the host. If colonisation increases beyond that level, then the benefit to the host will decrease. Asimi *et al.* (1980) examined the effect of 0, 0.25, and 0.5 g KH_2PO_4/ kg soil and inoculation with *Glomus mosseae* on *Glycine max* and found that mycorrhizae can increase the yield of *G. max* at the lower phosphorus levels. Roldan-Fajardo *et al.* (1982) examined the effect of different VAM species and 0, 75, and 150 kg P/ha had on *Prunis dulcis* and found that mycorrhizal *Prunis dulcis* had higher shoot dry mass than non-mycorrhizal *P. dulcis* at all phosphorus levels.

Mycorrhizae are not always beneficial to the host, however. Isopi *et al.* (1994) examined the effect of inoculating *Alnus cordata* with *Glomus fasciculatum*, *Glomus mosseae*, and *Frankia* and found that mycorrhizae provided no benefit to the host. Although Ianson and Linderman (1993) found that, in general, mycorrhizae were beneficial to *Cajanus cajan*, three out of the seven VAM fungi did not have a significant effect on the host plant or decreased the shoot mass when compared with the control

plants. Some mycorrhizal fungal species are more beneficial to the host plant than others. In Ianson and Linderman's (1993) experiment, the lack of benefit could be due to a lack of colonisation as one out of the seven VAM species did not colonise the host at a rate of significantly different from the control. Lack of colonisation was not a factor in other VAM species in this experiment, though, so it is likely that the results are due to variation among fungal species. Michelsen and Sprent (1994) found that some VAM species did significantly increase the host shoot mass while others did not. Again, there must be some genetic variation among the endosymbionts with regards to host benefit. Additionally, the results did depend on which species of *Acacia* was used as the host, indicating that some of the variation in benefit was due to the host species.

The mycorrhizal relationship, like all symbioses is not a static relationship. The benefit to the host is dynamic and, as such, the endosymbiont may be considered mutualistic under some conditions and parasitic under others. Because the majority of mycorrhizal fungi benefit the host plant, mycorrhizal relationships are considered to be mutualisms. That does not mean, however, that some mycorrhizal fungal species may not be parasitic. It is possible that in some of the above experiments, the fungi used were closer to parasitic than mutualistic and hence little or no benefit to the host was seen.

The interaction of phosphorus and mycorrhizae on host benefit

Often, there is an interaction between the mutualisms and the nutrient the mutualism provides. Hayman and Mosse (1979) examined the effect of mycorrhizal infection and 0, 22.5, and 90 kg P/ha on *Trifolium repens*. They found that the shoot dry mass of mycorrhizal *Trifolium repens* increased with increasing concentrations of phosphorus applied to the plants but non-mycorrhizal *T. repens* appeared unaffected by

phosphorus application. Asimi *et al.* (1980) examined the effect of 0, 0.25, and 0.5 g KH_2PO_4/kg soil and mycorrhizal inoculation on *Glycine max*. They found that mycorrhizal *G. max* only had significantly higher dry masses only at a fertiliser concentration of 0 g KH_2PO_4/kg soil (Asimi *et al.*, 1980). When 0.25 and 0.5 g KH_2PO_4/kg soil were applied, there was no difference between mycorrhizal and non-mycorrhizal dry masses (Asimi *et al.*, 1980). Bethlenfalvay and Yoder (1981) had similar results in their experiment looking at the effect of 4, 20, 100, and 500 μM phosphorus and mycorrhizal inoculation on *Glycine max*. They found that at 4 μM and 20 μM phosphorus, mycorrhizal plants had higher dry masses than non-mycorrhizal plants but when the phosphorus level rose to 100 μM and 500 μM , there was no difference in the shoot masses of mycorrhizal and non-mycorrhizal *G. max* (Bethlenfalvay and Yoder, 1981).

These three experiments used different units of concentration (kg P/ha, g KH_2PO_4/kg soil, μM), making a comparison difficult. It is possible that once the phosphorus concentrations in the soil reach a certain level, the plant roots are able to provide enough phosphorus for plant growth without the plant having mycorrhizae. When plants are mycorrhizal, the fungi act as a carbon drain. If the fungi provide enough phosphorus to encourage growth despite the drain on the plant, then there will be a net increase in plant growth. If the plant can obtain enough phosphorus with just its roots, then there will be a net decrease in growth. In comparison, the non-mycorrhizal plant will not have the mycorrhizae to drain carbon from the plant and, as such, will experience greater growth.

Ekblad *et al.* (1995) found that, not only was there an interaction between phosphorus and mycorrhizae, but the effect of phosphorus depended on the other nutrients provided. With no additional nutrients, non-mycorrhizal *Alnus incana* were larger than mycorrhizal *A. incana* when phosphorus was not added (Ekblad *et al.*, 1995), suggesting that under these conditions, the mycorrhizal fungus was parasitic. Adding phosphorus made the symbiosis less parasitic as there was no difference between mycorrhizal and non-mycorrhizal total dry masses when phosphorus was provided. Adding potassium as well as phosphorus returned the mycorrhizae to their parasitic state. When nitrogen was added alone and in combination with potassium, the addition of phosphorus caused mycorrhizal plants to grow enough that they had significantly higher shoot dry masses than non-mycorrhizal *A. incana* and the mycorrhizae were no longer parasitic but were instead beneficial. This experiment illustrates the dynamic nature of the symbiosis between plants and mycorrhizal fungi as the relationship in Ekblad *et al.*'s experiment went from parasitic to mutualistic depending on the soil nutrient conditions.

The effect of phosphorus on mycorrhizae

The addition of a small amount of phosphorus to the soil causes a decrease in mycorrhizal colonisation. As the phosphorus concentration in the soil increased from 0 to 0.25 to 0.5 g KH_2PO_4/kg soil, *Glycine max* plants inoculated with *Glomus mosseae* showed a decrease in mycorrhizal colonisation (Asimi *et al.*, 1980). Bethlenfalvay and Yoder (1981) found that when *Glycine max* plants were inoculated with *Glomus fasciculatus*, there was an increase in colonisation when the level of phosphorus applied to the plants increased from 4 μM KH_2PO_4 to 20 μM and a decrease in colonisation when the phosphorus concentration was increased to 100 μM and 500 μM KH_2PO_4. Roldan-

Fajardo *et al.* (1982) examined the effect of 0, 75, and 150 kg P/ha and inoculation with VAM on *Prunus dulcis*. Jha *et al.* (1993) examined the effects of inoculation with *Glomus mosseae* and 0, 30, 60, 120, and 240 kg P/ ha on *Alnus nepalensis*. Both experiments found a decrease in colonisation as the phosphorus concentration applied to the plants increased. Armstrong *et al.* (1992) found that VAM colonisation of *Aristidia armata, Cenchus cilaris, Digitaria ammophilla*, and *Thyridolepsis mitchelliana* decreased as phosphorus levels applied to the plants increased from 0 to 7.6 to 15.3 to 30.4 mg P/pot.

Ectomycorrhizae show the same response to increasing phosphorus levels as VAM do. Jones *et al.* (1990) examined the colonisation of *Salix viminalis* by *Laccaria proxima* and *Thelephora terrestris* under soil phosphorus concentrations of 0, 6, 10, 21, 60, and 90 mgP/kg soil and found that colonisation decreased as the phosphorus concentration applied to the plants increased. Newton and Pigott (1991) examined the effects of the presence and absence of nutrients on the ectomycorrhizal colonisation of *Quercus robur* and *Betula pendula.* They found that ectomycorrhizal colonisation of both *Q. robur* and *B. pendula* decreased when phosphorus was applied. Baum and Makeschin (2000) examined the effect of the presence and absence of nutrients on ectomycorrhizal colonisation of *Populus trichocarpa* and *Populus tremula x tremuloides.* They found that colonisation on both species decreased as phosphorus applied to the host increased. Ekblad *et al.* (1995) found that colonisation of *Alnus incana* by *Paxillus involutus* decreased when phosphorus was applied alone, in conjunction with nitrogen, and in conjunction with nitrogen and potassium. When phosphorus was added in

conjunction with potassium, there was no effect on colonisation (Ekblad *et al.*, 1995) so it appears as though potassium is able to decrease the inhibitory effects of phosphorus.

Roldan-Fajardo *et al.* (1982) found that when *Prunis dulcis* was inoculated with native VAM endophytes, the colonisation did not decrease with increasing phosphorus levels but rather increased. They suggest that the native endophytes are better adapted to the host than *Glomus mosseae* and that the native endophytes are used to the phosphate applications to the experimental plots. Roldan-Fajardo *et al.* (1982) did not suggest a mechanism by which the native VAM endophytes would be used to phosphorus application.

The effects of nitrogen on mycorrhizae

Nitrogen appears to have mixed effects on mycorrhizae. Johnson (1993) found that adding nitrogen to soils that had been previously fertilised and soils that had not been previously fertilised increases the VAM colonisation of *Andropogon gerardii.* Nitrogen fertilisation decreased the ectomycorrhizal colonisation of *Populus trichocarpa* and *Populus tremula x tremuloides* (Baum and Makeschin, 2000), *Quercus robur*, and *Betula pendula* (Newton and Pigott, 1991). The reason for the difference in these findings could be due to the different types of mycorrhizae used in the experiments. It is possible that VAM mycorrhizae respond in one manner to nitrogen in the soil while ectomycorrhizae respond in a different manner.

Ekblad *et al* (1995) examined the effects of the presence and absence of nutrients on mycorrhizae in *Pinus sylvestris* and *Alnus incana.* They included tests for interactions between nitrogen, phosphorus, and potassium. They found that the effect of nitrogen on *A. incana* and *P. sylvestris* mycorrhizae was dependant on what nutrients were added in

conjunction with the nitrogen. When nitrogen was added alone or in conjunction with potassium and phosphorus, there was no difference in colonisation rates on *A. incana*. When added in conjunction with potassium, nitrogen application increased colonisation on *A. incana*. When added in conjunction with phosphorus, nitrogen application decreased mycorrhizal colonisation on *A. incana*. Adding nitrogen, phosphorus, or potassium alone decreased *P. sylvestris* colonisation. Adding nitrogen and potassium concurrently or phosphorus and potassium concurrently had no effect on *P. sylvestris* mycorrhizal colonisation. Adding nitrogen and phosphorus concurrently decreased *P. sylvestris* mycorrhizal colonisation. Adding all three nutrients increased *P. sylvestris* mycorrhizal colonisation. As with phosphorus, it appears as though the effect of nitrogen on mycorrhizae can be altered by the addition of other nutrients in the fertiliser.

2.2d Tripartite mutualisms

The effect of tripartite mutualisms on the host

The general consensus is that the benefit a plant receives increases when it forms relationships with both nitrogen-fixing bacteria and mycorrhizae as compared to when it forms relationships with just nitrogen-fixing bacteria or mycorrhizae. Tian *et al.* (2002) found that *Hippophae tibetana* had higher shoot masses when inoculated with both *Frankia* and VAM fungi than when inoculated with either *Frankia* or VAM fungi. Chatarpaul *et al.* (1989) examined the effect of inoculating *Alnus incana* with *Glomus fasciculatus, Paxillus involutus*, and *Frankia*. They found that the shoot mass of *A. incana* was lowest when the host only had a single mutualism. A tripartite mutualism increased shoot mass of *A. incana* compared to individual mutualisms and a tetrapartite mutualism increased the shoot mass in comparison with a tripartite mutualism. Jha *et al.*

(1993) found that the shoot dry mass of *Alnus nepalensis* was higher when inoculated with both *Frankia* and VAM fungi than when inoculated with either *Frankia* or VAM. Dry masses of *Casuarina cunninghamia, Casuarina equisetifolia,* and *Casuarina junghuniana* were higher when inoculated with both *Frankia* and *Glomus fasciculatus* than when inoculated with just *Frankia* (Wheeler *et al.*, 2000). *Ceanothus velutinus* had higher shoot masses when inoculated with both *Frankia* and *Glomus gerdemanii* than when inoculated with just *Frankia* or *G. gerdemanii* (Rose and Youngberg, 1981). *Glycine max* plants inoculated with *Glomus fasciculatus* and *Rhizobium japonicum* (=*Bradyrhizobium japonicum*) had higher shoot masses than plants inoculated with just *G. fasciculatus* or *R. japonicum* (Carling *et al.*, 1978; Pacovsky *et al.* 1986-b).

One of the reasons that tripartite mutualisms benefit host plants more than singular mutualisms is that tripartite mutualisms have a higher diversity of endosymbionts than singular mutualisms and the diversity of nutrient supplied to the tripartite host is therefore higher. Plants inoculated with *Frankia* receive nitrogen while plants inoculated with mycorrhizae receive phosphorus. Plants that are inoculated with both *Frankia* and mycorrhizae receive both nitrogen and phosphorus, giving them a nutritional advantage over plants inoculated with just *Frankia* or just mycorrhizae. Rose and Youngberg (1981) suggest that a possible synergistic response of the endosymbionts could maximise host benefit, presumably by an increase in the number of nutrients provided to the host plant. Plant growth is usually limited by one particular nutrient, for example, nitrogen. When plants can overcome this limitation, by forming a symbiosis with nitrogen-fixing bacteria and fixing nitrogen, then a second nutrient, for example, phosphorus, will becoming the limiting factor. Plants in tripartite mutualisms are able to overcome this

second limiting nutrient by having mycorrhizae that aid in phosphorus uptake in addition to nitrogen-fixing bacteria. Additionally, phosphorus increases nitrogen fixation (Rose and Youngberg, 1981), which increases plant growth, which would presumably increase phosphorus uptake from the soil. In this way, the two endosymbionts work synergistically to increase plant growth.

Not all of the research shows that plants benefit from the increases in the number of mutualisms. Bethlenfalvay *et al.* (1997) examined the effect of inoculating *Pisum sativum* with *Glomus mosseae* and rhizobia. They found that *P. sativum* plants inoculated with *G. mosseae* had the highest shoot dry mass, followed by *P. sativum* inoculated with rhizobia., *P. sativum* inoculated with *G. mosseae* and rhizobia, and finally control *P. sativium* plants. Isopi *et al.* (1994) found that the benefit from a tripartite mutualism can depend on the species of endosymbionts involved. They examined the effect of inoculating *Alnus cordata* with combinations of *Glomus fasciculatum*, *Glomus mosseae*, and *Frankia.* Plants inoculated with *G. fasciculatum* and *Frankia* had shoot masses that were not significantly different than plants inoculated with just *G. mosseae*, *G. fasciculatum*, or *Frankia* whereas plants inoculated with *G. mosseae* and *Frankia* had higher shoot masses than any other inoculation treatment (Isopi *et al.*, 1994). Michelsen and Sprent (1994) found that the benefit of a tripartite mutualism depended on the strain of endosymbiont and the species of host involved. They examined the effect of inoculating four *Acacia* species with five different species of VAM fungi. For *Acacia abyssinica*, the tripartite mutualism was beneficial only when one strain of VAM fungi was used. *Acacia sieberiana* did not benefit when grown with two of the four strains of VAM fungi used. In the same study, *Eucalyptus globulus* did

not benefit from a tripartite mutualism regardless of the strain of VAM fungi used. The plants were inoculated using the rhizobia in native soil, so it is possible that different species of *Rhizobium* colonised different hosts. Smith *et al.* (1979) found that *Trifolium subterraneum* inoculated with both *Rhizobium trifolii* and *Glomus mosseae* did not have significantly different shoot dry mass than *T. subterraneum* inoculated with just *R. trifolii.*

There is a natural variation in the benefit provided by endosymbionts. This variation occurs because in some environments, there is no selective pressure for the endosymbiont to benefit the host. For example, two types of *Frankia* are delineated on the basis of spore production in the nodule. Those that produce spores in the nodules are referred to as spore positive and those that do not produce spores in the nodule are referred to as spore negative. Although these two types of *Frankia* are genetically distinct, they do not form two separate species. Spore negative are said to be more efficient in terms of nitrogen fixation and provide more benefit to the host than spore positive (Schwintzer, 1990). The spore positive *Frankia* do not provide as much benefit to the host because the production of spores coincides with a reduction in nitrogenase activity. In the case of the spore positive *Frankia*, the decrease in nitrogenase efficiency (and consequently a reduction in benefit to the host) is selected for because it increases the fitness of *Frankia.*

Because of this variation in benefit, it is intuitive that the benefit of a tripartite mutualism can change depending on the endosymbionts or host plant involved. It is likely that those researchers that found there was no benefit to a host as the number of endosymbionts increased used a less beneficial endosymbiont. The variability of benefit

is a well known phenomenon and as such, a large portion of the literature on legumes is devoted to finding the most beneficial match of host and endosymbiont (see Ham *et al.*, 1976; Roughley, 1976; Roughley *et al.*, 1976; Subba Rao, 1976; and Mytton and de Felice, 1977).

The effect of nitrogen-fixing bacteria on mycorrhizae in a tripartite mutualism

Symbiotic nitrogen-fixing bacteria are generally indirectly beneficial to mycorrhizal colonisation. The presence of *Frankia* increased VAM colonisation in *Hippophae tibetana* (Tian *et al.*, 2002). The presence of *Frankia* increased colonisation by both *Glomus fasciculatus* and *Paxillus involutus* on *Alnus incana* (Chatarpaul *et al.*, 1989). *Frankia* nodules increased *Glomus gerdemannii* colonisation on *Ceanothus velutinus* (Rose and Youngberg, 1981). Pacovsky *et al.* (1986-b) found that *Rhizobium* increased the VAM colonisation of *Glycine max*; although, the increase depended on the strain of *Rhizobium* used to inoculate the plants. Nitrogen-fixing bacteria have also been shown to affect phosphorus concentrations in mycorrhizal plants. Carling *et al* (1978) found that the presence of *Rhizobium japonicum* increased the percent phosphorus in host *Glycine max* that were also inoculated with *Glomus fasciculatus.*

Nitrogen-fixing bacteria do not always increase mycorrhizal colonisation or plant phosphorus concentrations. Isopi *et al.* (1994) examined the effects of inoculating *Alnus cordata* with *Glomus fasciculatum, Glomus mosseae*, and *Frankia* and found that for *A. cordata* plants inoculated with *G. fasciculatum*, *Frankia* decreased the mycorrhizal colonisation. When *G. mosseae* colonised *A. cordata* though, *Frankia* increased the mycorrhizal colonisation. Bethlenfalvay *et al.* (1997) found that colonisation of *Pisum sativum* by *Glomus mosseae* was lower in plants inoculated with *Rhizobium*

leguminosarum than in plants given nitrogen fertiliser. It is possible that the nitrogen fertiliser provided more nitrogen and allowed the plant to be colonised more without the mycorrhizae being a drain on the plant. It appears as though the effect of nitrogen-fixing bacteria depend on the species involved in the tripartite mutualism.

The effect of mycorrhizae on nitrogen-fixing bacteria in a tripartite mutualism

Mycorrhizae appear to have the same beneficial effects on nitrogen-fixing bacteria as phosphorus. VAM have been shown to indirectly increase nodule weight in *Pisum sativum* inoculated with *Rhizobium leguminosarum* (Bethlenfalvay *et al.*, 1978), *Trifolium repens* inoculated with *Rhizobium trifolii* (Hayman and Mosse, 1979), *Alnus cordata* inoculated with *Frankia* (Rose and Youngberg, 1981), *Alnus incana* inoculated with *Frankia* (Chatarpaul *et al.*, 1989), *Alnus nepalensis* inoculated with *Frankia* (Jha *et al.,* 1993), and *Hippophae tibetana* inoculated with *Frankia* (Tian *et al.*, 2002). Ectomycorrhizae have also been shown to increase nodule mass of *Alnus incana* inoculated with *Frankia* (Chatarpaul *et al.*, 1989).

There appears to be variation in the effect of mycorrhizae on nodule mass. Some mycorrhizal strains/species are more beneficial than others are. Michelsen and Sprent (1994) found that some VAM strains increased nodule mass while others did not. Pacovsky *et al.* (1986-b) found that VAM infected *Glycine max* plants had smaller nodules than *Glycine max* plants fertilised with phosphorus fertiliser. Nodulation and nitrogen fixation require large quantities of phosphorus (Asimi *et al.*, 1980) and it is likely that mycorrhizae help provide the needed phosphorus to the nitrogen-fixing bacteria and as such, increase nodule dry mass (Rose and Youngberg, 1981).

The presence of mycorrhizae in plant roots tends to increase the total nitrogenase activity of nitrogen-fixing bacteria although the mycorrhizal effect depends on the host and endosymbiont species in the relationship. Smith *et al.* (1979) found that the presence of VAM increased the rate of ethylene production per plant in *Trifolium subterraneum* inoculated with *Rhizobium trifolii*. Rose and Youngberg (1981) found that the presence of VAM increased acetylene reduction/plant and specific acetylene reduction in *Ceanothus velutinus* inoculated with *Frankia*. Michelsen and Sprent (1994) found that three of four VAM strains used increased the percentage of nitrogen fixed by *Acacia abyssinica* inoculated with rhizobia. One of the four VAM strains decreased the percent nitrogen fixed (Michelsen and Sprent, 1994). Wheeler *et al* (2000) also found that the effect of mycorrhizae on nitrogen fixing bacteria depends on the species involved. One strain of *Frankia* inoculating four *Casuarina* species decreased specific nitrogenase activity when the host had mycorrhizae. The other two strains of *Frankia* increased specific nitrogen fixation when the host *Casuarina* plant was infected with VAM (Wheeler *et al.*, 2000). The increase in nitrogenase activity is thought to be due to the ability of the mycorrhizae to provide phosphorus to satisfy the high phosphorus requirements of nitrogen-fixation (Robson *et al.*, 1981; Rose and Youngberg, 1981;Gardner *et al.*, 1984; Ianson and Linderman, 1993).

Robson *et al.* (1981) found that although *Glomus monosporus* increases nitrogen fixation per plant for *Trifolium subterraneum* inoculated with *Rhizobium trifolii*, the specific nitrogenase activity actually decreased when the host was inoculated with *G. monosporus*. Although the total nitrogen fixation increased, the mycorrhizae increased the nodule mass of the plants, so specific nitrogen fixation actually decreases with the

presence of mycorrhizae. These results could be the result of natural variation of nitrogen fixation in nitrogen-fixing bacteria. Carling *et al.* (1978) found that up until week 12, *Glycine max* plants inoculated with *Rhizobium* had increased nitrogen fixation/plant when inoculated with *Glomus sp.* On week 14, though, nitrogen fixation in the tripartite plants decreased to the level of *Glycine max* not inoculated with *Glomus sp.* (Carling *et al.*, 1978). Carling *et al.* do not suggest why this decrease occurs.

The effects of nitrogen and phosphorus on a tripartite mutualism

The effect of one symbiont on the other symbiont or on the host plant can be complicated by the addition of nitrogen or phosphorus fertiliser. Bethlenfalvay and Yoder (1981) found that at low (20 μM, 100 μM) phosphorus fertiliser concentrations, mycorrhizal *Glycine max* plants had more nodule mass than non-mycorrhizal plants but at higher phosphorus concentrations (500 μM), there was no difference in nodule mass between the mycorrhizal and non-mycorrhizal plants. These results suggest that providing phosphorus fertiliser is not equivalent to mycorrhizae. At the 500 μM phosphorus concentration, there was little mycorrhizal colonisation (Bethlenfalvay and Yoder, 1981), possibly because the high phosphorus levels replaced the mycorrhizae. As such, at 500 μM, the mycorrhizal plants were almost non-mycorrhizal and would then be expected not to be significantly different from non-mycorrhizal plants. Russo (1989) found that even though increasing phosphorus concentrations applied to *Alnus acuminata* increased nitrogenase activity, mycorrhizal plants still had higher nitrogenase activity than non-mycorrhizal ones. At low (10 ppm) and high (100 ppm) concentrations of phosphorus, leaf dry mass of *A. acuminata* did not differ. At 50 ppm, though, *A. acuminata* inoculated with both *Frankia* and *Glomus* had higher leaf masses (Russo,

1989). At low (10ppm) phosphorus, *A. acuminata* had higher specific nitrogenase activity when inoculated with both *Frankia* and *Glomus* (Russo, 1989). As the phosphorus concentration increased, *A. incuminata* inoculated with just *Frankia* had higher specific nitrogenase activity (Russo, 1989).

2.2e Conclusion

Phosphorus, nitrogen, mycorrhizae, and nitrogen-fixing bacteria all appear to be beneficial to plants, mycorrhizae, and nitrogen-fixing bacteria because they increase phosphorus and nitrogen available to the plant. The combination of phosphorus and mycorrhizae appears to both increase and decrease host plant benefit. Nitrogen-fixing bacteria increase host plant benefit at low nitrogen fertiliser concentration but decrease host plant benefit at high nitrogen fertiliser concentrations. Plants inoculated with nitrogen-fixing bacteria have a different response to increasing nitrogen concentrations than uninoculated plants. A host that is in a relationship with both mycorrhizae and nitrogen-fixing bacteria will benefit more than a host that has a relationship with mycorrhizae or nitrogen-fixing bacteria alone. In general, phosphorus appears to decrease mycorrhizal colonisation while the application of nitrogen has mixed effects. Nitrogen-fixing bacteria appear to increase mycorrhizal colonisation on the same host. Phosphorus tends to increase nitrogenase activity and nodule mass for plants inoculated with nitrogen-fixing bacteria. In general, low phosphorus concentrations are indirectly detrimental to nitrogen-fixing bacteria. The presence of mycorrhizae tends to benefit nitrogen-fixing bacteria on the same host.

2.3 The interaction of nitrogen-fixing bacteria and mycorrhizae on host colonisation

2.3a Introduction

There are two key components to the formation of a successful endosymbiotic mutualism: I) infectivity, or the ability of the endosymbiont to colonise host roots; and II) effectivity, the ability of the endosymbiont to fix nitrogen or provide phosphorus and increase plant benefit. Both infectivity and effectivity are generally viewed from the plant's perspective (e.g. which endosymbiont is more likely to colonise a given host or which bacterial strain is more effective). Rarely is the endosymbiont's point of view considered (e.g. which plant is more susceptible to colonisation by a given endosymbiont or which plant provides more carbon for the endosymbiont). Regardless of which perspective infectivity or effectivity are viewed from, variation in either results in variation in the benefit of the host plant.

2.3b Colonisation

Colonisation of red alder roots by *Frankia* involves a series of complex interactions between host roots and bacteria. Among actinorhizal hosts, root hair curling is unique to alder and plants in the Casuarinaceae and Myricacaeae (Huss-Danell, 1997). Unlike the rhizobial system, plant exudates do not stimulate production of root hair curling compounds by *Frankia* (Provorov *et al.*, 2002); instead, these compounds are produced continuously by free-living *Frankia*. In response to the exudation of root hair curling compounds, the alder root hair begins to branch (Huss-Danell, 1997). A single *Frankia* hypha then penetrates the epidermal cell walls where the root hair is crooked or sharply folded (Huss-Danell, 1997). Once the hypha has penetrated the root hair, the hypha continues growing until it has passed through the root epidermal cells and into the

cortex of the root (Huss-Danell, 1997). This penetration induces the hypodermis and cortical root cells to divide and form a prenodule (Benson and Silvester, 1993). The true nodule develops from division of pericycle cells and becomes infected as it grows through the prenodule (Huss-Danell, 1997). True nodule development is similar in development to that of a lateral root (Benson and Silvester, 1993).

Mycorrhizal colonisation is less complicated than nitrogen-fixing bacteriaa colonisation. For mycorrhizal colonisations, root exudates stimulate the germination of fungal spores (Anderson, 1992) and flavanoids and isoflavanoids attract the hyphae to the root (Barker *et al.*, 1998). Fungal hyphae then exude hyphorine (an indolic compound) causing changes in root hair development (Barker *et al.*, 1998). The hyphae branch and attach to the root surface forming an appressorium, after which, it penetrates through the root cell wall (Anderson, 1992). Since ectomycorrhizae do not penetrate the cell membrane, ectomycorrhizal appressoria only penetrate the cell wall. Once the hyphae have penetrated the cell walls, they branch out to form the Hartig net. The early stages of mycorrhizal development are characterised by a burst of chitinase activity in the plant root that quickly falls below that found in non-mycorrhizal plants (Gianinazzi-Pearson *et al.*, 1996). Chitinase is an enzyme that degrades the chitin in the fungal cell wall and thus prevents fungal colonisation. When the chitinase activity decreases, the fungi are then able to colonise the roots.

2.3c Host choice

Both partners in a mutualism enter into the relationship to derive the maximum benefit possible from the relationship. As such, researchers have suggested that the host would actively choose which endosymbionts would colonise its roots. Vincent and

Waters (1953) suggest their findings that *Rhizobium trifolii* strains do not colonise *Trifollium sp.* roots in the same ratio as found in the inoculant support the idea that hosts select which endosymbionts colonise their roots. However, if the host were able to select the strains colonising the host, the ineffective strains should not form nodules. Effective *Rhizobium trifolii* formed a greater percentage of nodules on *Trifolium sp.* than was expected from the ratio of effective to infective strains in the inoculant (Robinson, 1969-a). These results were interpreted to mean that the host plant is able to select which strains colonise its roots and the suggestion was put forth that selection occurs at or just after colonisation (Robinson, 1969-a). *Trifolium pratense* and *Trifolium subterraneum* formed more nodules with strains of *Rhizobium trifolli* isolated from their roots than from roots of the other species (Robinson, 1969-b), which Robinson suggested was further evidence supporting the hypothesis that plants are able to select which endosymbionts colonise their roots. It is possible that instead of host selection, bacterial selection determines which endosymbionts colonise the host plant. Little research has been done on bacterial choice so the role bacteria play in which endosymbiont colonises the host is still unclear.

Later experiments involving *Medicago sativa* and *Rhizobium meliloti* found that the percentage of effective nodules did not change regardless of the ratio of effective to infective strains in the inoculant but did change based on which host the symbiont was isolated from (Amarger, 1981-a). The benefit received by *Medicago sativa* from a mutualism with *Rhizobium meliloti* and *R. leguminosarum* was, however, proportional to the percent of effective nodules, indicating that hosts would receive a greater benefit if they were able to select the most effective strains (Amarger, 1981-a). To date, no

relationship has been found between effectivity and infectivity in any system (Russell and Jones, 1975; Amarger, 1981-a; Amarger, 1981-b; Weber *et al.*, 1987; Anand and Dogra, 1997;), suggesting that plants have no precolonisation method for determining the benefit they will receive from a given endosymbiont.

The intricate and specific recognition system that developed between plants and nitrogen-fixing bacteria would seem predisposed to allow hosts to select for the most effective bacteria. Because of its specific nature, this recognition system limits the number of strains of bacteria with which the host can form a mutualism. Only bacteria that release a given compound will stimulate the release of a particular compound, thus allowing a mutualism to form. However, the chemicals involved in the recognition system do not give the plant an indication of symbiont effectiveness (Denison, 2000). Moreover, an effective bacterium and a closely related parasitic bacterium will both be able to colonise the host (Denison, 2000). Some strains of rhizobia, for example, do not fix nitrogen but still form nodules on plant roots. They still obtain carbon from the host plant although they do not supply nitrogen to the host in return; these endosymbionts are known as cheaters. In some instances, the host plant will impose sanctions on the cheaters in the hopes that the sanctions will prevent endosymbionts from becoming cheaters. Sanctions against nitrogen-fixing bacteria could include attacking bacterioids that are not fixing nitrogen, limiting the carbon provided the bacterioids, or limiting the oxygen supply to the nodule (Denison, 2000). If plants were able to detect effective bacteria pre-colonisation, there would be no need for the sanctions taken by the plant against rhizobia with its roots (see Denison, 2000).

Actinorhizal plants appear to be able to select the *Frankia* strain that colonises their roots. Weber (1986) found that *Alnus glutinosa* nodules were predominantly spore negative while *Alnus incana* nodules were predominantly spore positive. Normand and Lalonde (1982) found that spore positive nodules were associated with *Alnus rugosa* while spore negative nodules were associated with *Alnus crispa*. It appears as if the host alder is able to select which strain of *Frankia* (spore positive or spore negative) colonises its roots. There is a problem with this hypothesis, however. The production of spores is correlated with a decrease in nitrogen fixation (Torrey, 1987) and as such, it is unclear why plants would choose to form a relationship solely with a less effective bacterial strain. It is believed that all individuals of Frankia have the genetic capability to produce spores (Schwintzer, 1990) so perhaps it is not a question of which type of *Frankia* colonises the host, but what causes *Frankia* strains on certain hosts to produce spores. The host itself has been proposed as a means of regulating spore production (Schwintzer, 1990). In that case, it would not be the host choosing to allow only spore positive or spore negative bacteria to colonise its roots but rather the host promoting or inhibiting spore production once the nodule has already formed. Other factors that have been suggested as regulating spore production include soil pH and the length of time a host has been at a particular site (Schwintzer, 1990).

In contrast to nitrogen-fixing bacteria, though, there is direct evidence that plants have the ability to select which fungi colonise their roots. Mutualistic and parasitic fungi both penetrate host roots in a similar manner, yet the plant defense pathways are not activated or only minimally activated by mutualistic fungi (Gianinazzi-Pearson *et al.*, 1996; Kapulnik *et al.* 1996; Barker *et al.*, 1998; Provorov *et al.*, 2002). Parasitic fungi,

on the other hand, activate the plant defense pathways and thus are prevented from penetrating any further into the host plant. It is not clear, however, whether these defense pathways are inhibited by symbiosis related plant genes (i.e., by the plant) or by fungal suppressors (i.e. by the fungus) (Kapulnik *et al.*, 1996).

A further difference between the fungi and *Frankia* in my thesis is that the two fungi are different species, while the *Frankia* are different types within the same genus. It is possible that the variation between kinds of endosymbionts is only present at the species level or only at the variant level. Because the variation occurs at different levels in the two kinds of endosymbionts in my thesis, it is possible that the two will react in different ways.

2.3d Endosymbiont similarities

Nitrogen-fixing bacteria and mycorrhizal fungi form what appear to be two very different symbioses. Nitrogen-fixing bacteria form visible growths of plant tissue on plant roots while mycorrhizae can be less visible to the naked eye. Mycorrhizae have a simpler genetic system for colonisation (Provorov *et al.*, 2002) than nitrogen-fixing bacteria. Fungi and bacteria belong to two different Kingdoms. Yet despite these differences, there are many similarities between the two symbioses that suggest that host plants may react to both symbioses in a similar manner and that the two symbioses may have evolved in a similar manner.

Morphologically, both types of microbes colonise root cortical cells and induce the development of sub-cellular compartments (Provorov *et al.*, 2002). These sub-cellular compartments are surrounded by the peribacteroid membranes in nitrogen-fixing mutualisms and periarbuscular membranes in VAM mutualisms [although

ectomycorrhizae appear to have a similar barrier (Harley and Smith, 1983)]. In addition, the development of the infection tube of the nitrogen-fixing bacteria is similar to the development of the infection hyphae of mycorrhizae (Provorov *et al.,* 2002).

Another similarity between the two symbioses is the ability of both nitrogen-fixing bacteria and mycorrhizal fungi not to activate plant defenses. When an organism damages a plant through herbivory or parasite penetration, biochemical pathways are activated to reduce the damage done to the plant. Since both bacterial and fungal colonisation involve host root penetration, it is logical to assume that plant defenses would be activated. Mycorrhizal fungi only weakly, if at all, activate the defense mechanisms (Perotto *et al.*, 1993; Gianinazzi-Pearson *et al.*, 1996; Kapulnik *et al.*, 1996; Barker *et al.*, 1998). The main defense mechanism used against fungi is chitinase. Gianinazzi-Pearson *et al.* (1996) found that mycorrhizae actually decrease chitinase production when they colonise the host roots. Rhizobia are associated with an accumulation of antigens recognised by antibody MAC 265 that are thought to be associated with plant defense pathways (Perotto *et al.*, 1993). Although mycorrhizal fungi are not associated with an accumulation of MAC 265, both the fungi and bacteria are associated with an increase of the antigen MAC 266 (Perotto *et al.*, 1993). The function of MAC 265 is unknown, but it is thought that it has some role to play in the events leading to the senescence of the microbes (Perotto *et al.*, 1993). The host plant produces the antibodies, which attach to the endosymbiont and probably result in deactivating the host defense system.

The peribacteroid and periarbuscle membranes provide evidence that the bacteria and fungi colonising the host plant at one time triggered plant defense mechanism. The

endosymbiont organelle in nitrogen-fixing mutualisms resembles a lysosome, an organelle that would have been involved in plant defense (Mellor, 1989). Mellor (1989) suggests that the organelle membrane surrounding the nitrogen-fixing bacteria is similar to that surrounding the mycorrhizae. When the plant is invaded, the lysosome would be sent to the infection site to surround the invading organism and digest it to prevent colonisation. The fact that peribacteroid and periarbuscle membranes resemble lysosomes suggests that at one time, both mycorrhizal fungi and nitrogen-fixing bacteria triggered the plant defense system and were engulfed by lysosomes. Over time, the endosymbionts became engulfed not because they were parasitic, but because they were mutualistic.

Both nitrogen-fixing bacteria and mycorrhizae produce similar recognition compounds, a factor that may play in role in the host plants not differentiating between nitrogen-fixing bacteria and mycorrhizal symbioses. Nitrogen-fixing bacteria produce nodulins, compounds that stimulate nodulation in the host plant (Chabot *et al.*, 1992). Hilbert and Martin (1988) found that ectomycorrhizae produce polypeptides called ectomycorrhizins, which were later found to be identical to nodulins (Wyss *et al.*, 1990; Provorov *et al.*, 2002). Some flavanoid compounds released by plants that are known to stimulate colonisation by nitrogen-fixing bacteria have been found to stimulate mycorrhizal growth (Chabot *et al.*, 1992). Chabot *et al.* (1992) postulate that bacteria release Nod factors causing the plant to release flavanoids that both nitrogen-fixing bacteria and mycorrhizal fungi use as recognition signals and as a result, colonise the host plant. These findings suggest that bacteria and fungi have functionally similar flavanoid recognition systems (Chabot *et al.*, 1992). Since both fungi and bacteria respond to the

compounds produced by the plant and produce the same polypeptides (nodulins or ectomycorrhizins), it is likely that plants do not differentiate between the two endosymbionts and as such, treat both in the same manner in terms of colonisation.

Further evidence that host plants view the endosymbionts as similar comes from the link between nod^- plants (those that do not form nodules due to mutation) and myc^- plants (those that do not form mycorrhizae due to a mutation). Duc *et al.* (1989) found that nod^+fix^- plants (those that formed nodules but do not fix nitrogen) always formed mycorrhizae but nod^- plants did not always form mycorrhizae. Shirtliffe and Vessey (1996) suggest that the infection process is the same for both mycorrhizae and nitrogen-fixing bacteria and that depending on where in the process the mutation occurs, plants can be $nod^-fix^-myc^-$ (do not form nodule or mycorrhizae or fix nitrogen), $nod^-fix^-myc^+$ (do not form nodules or fix nitrogen but form mycorrhizae), $nod^+fix^-myc^-$ (form nodules but do not fix nitrogen or form mycorrhizae), or $nod^+fix^-myc^+$ (form nodules and mycorrhizae but do not fix nitrogen).

2.3e Colonisation and the Second Mutualism

Host plants do not always form mutualisms with the most beneficial endosymbionts. Because there is no way to determine pre-colonisation which endosymbionts will be most beneficial, it is unlikely that host selection of endosymbionts occurs. Consequently, there must be some other factor that determines which nitrogen-fixing bacterium or mycorrhizal fungus colonises the host. Although the host plant treats both mycorrhizae and nitrogen-fixing bacteria in a similar manner, it can distinguish between the two kinds of endosymbionts. As such, it is possible that one endosymbiont

could play a role in determining which strain of the other endosymbiont colonises the host.

Mycorrhizae and *Frankia* do not compete for colonisation sites on host plant roots (Sempavalan *et al.*, 1995). *Casuarina equisetifolia* plants were inoculated with *Glomus* or *Frankia* and then, after a delay of 0, 20, 40, or 60 days, inoculated with the other endosymbiont. Only a delay of 60 days had an effect on colonisation. There are two possible explanations for this lack of competition: I) *Frankia* infect via root hairs (Huss-Danell, 1997) while mycorrhizae infect via the epidermis (Harley and Smith, 1983) and II) not all available infection sites are colonized (Sempavalen *et al.*, 1995). Since the two endosymbionts do not infect the host in the same place on the root, they do not compete for the same sites. Since not all potential infection sites are colonized, there is no need for the endosymbionts to compete for infection sites. If there is an interaction between the two endosymbionts in terms of colonisation, it is not a competitive relationship.

Once both endosymbionts have colonised the plant, though, the relationship between the two could become competitive. Both endosymbionts derive their carbon from the host and could end up competing for photosynthates. If the photosynthates were in such short supply that the endosymbionts are forced to compete for them, the host plant would be detrimentally affected. Research has shown, though, that as the number of endosymbionts increases, plant biomass increases (Chatarpaul *et al.*, 1989), indicating that the nitrogen and phosphorus the endosymbionts supply increases plant photosynthesis enough to overcome the carbon drain the endosymbionts represent.

Some non-symbiotic bacterial species have been shown to increase mycorrhizal colonisation. Bending *et al.* (2002) found that seven of nine species in one *Bacillus*

subgroup and both species in a second *Bacillus* subgroup promoted mycorrhizal colonisation while *Serratia sp.*, *Burkholderia sp.*, and *Pseudomonas sp.* decreased colonisation. Antibodies produced by the bacteria as well as competition between the bacteria and fungi were suggested as reasons why these three species decreased colonisation. No explanation was given as to why the two *Bacillus* subgroups increase colonisation, but it is likely that bacterial exudates are responsible for the increase in colonisation as well as the decrease. The bacterial exudates might stimulate the plant to release flavanoids that are involved in mycorrhizal colonisation (Xie *et al.*, 1995).

Symbiotic bacteria have also been shown to have a positive effect on VAM colonisation. *Bradyrhizobium japonicum* was found to increase VAM colonisation on *Glycine max* (Xie *et al.*, 1995). *Frankia* was found to increase VAM colonisation on *Ceanothus velutinus* (Rose and Youngberg, 1981) and VAM and ectomycorrhizae colonisation on *Alnus incana* (Chatarpaul *et al.*, 1989). Plants incapable of forming nodules (nod^- plants) showed an increase in VAM colonisation when inoculated with both *Bradyrhizobium* and *Glomus mosseae* (Xie *et al.*, 1995) suggesting that it is not the presence of nodules that stimulates mycorrhizal colonisation but the interaction between plant and bacteria. Xie *et al.* (1995) used entire bacteria in their experiment; however, they also tests nod factors isolated from the bacteria. While one nod factor, NodNGR-V (MeFuc, S) (Methylfucose, sulfated), did not affect colonisation, another nod factor, NodNGR-V (MeFuc, Ac) (Methylfucose, acetylated), increased colonisation. Xie *et al.* (1995) concluded that Nod factors stimulate flavanoid release, which in turn stimulates mycorrhizal colonisation.

VAM have also been found to increase nodulation in *Trifolium subterraneum* (Smith *et al.*, 1979). In this experiment, the mycorrhizal *T. subterraneum* had higher percent phosphorus, nodule volume, and acetylene activity, suggesting that increasing phosphorus is what caused the increase in *Rhizobium trifolii.* Nodulation has a high phosphorus requirement (Smith *et al.*, 1979) so any means of increasing phosphorus, such as mycorrhizae, for example, should result in an increase in nodulation. Sanginga *et al.* (1989) found that increased phosphorus levels also increased nodulation. As such, it does not appear that the mycorrhizae themselves are responsible for increasing nodulation but rather the phosphorus they provide that increases nodulation.

The presence of a second symbiosis does not always increase colonisation by the first endosymbiont though. VAM did not increase nodulation on *Ceanothus velutinus* (Rose and Youngberg, 1981) and *Glycine max* had higher VAM colonisation with nitrogen fertilizer as opposed to rhizobia inoculation (Bethlenfalvay *et al.*, 1985). Bethlenfalvay *et al.* (1985) have found that delaying inoculation of the second endosymbiont increases the colonisation by that endosymbiont. It is possible that if Rose and Youngberg had delayed inoculation of the *Frankia* until after the VAM had been applied to the hosts, the VAM might have had a positive effect on nodulation. One of the reasons why Rose and Youngberg (1981) might not have found an increase in nodulation due to VAM is the fact that *Frankia* colonisation differs from rhizobial colonisation in that plant exudates do not stimulate production of root hair curling factors by *Frankia* (Provorov *et al.*, 2002). Mycorrhizal colonisation increases the exudation of flavanoids by host roots (Chabot *et al.*, 1992). These flavanoids stimulate the production of nod factors by rhizobia, a vital step for colonisation. The increase in flavanoid production

should increase rhizobial colonisation. Since flavanoids do not stimulate a similar increase in *Frankia* exudates, an increase in flavanoid production by VAM colonisation would not increase *Frankia* colonisation.

2.3f Conclusion

Intuitively, it would be to the advantage of the host if it were able to select the most beneficial endosymbionts; however, host plants have been found to form mutualisms with endosymbionts that do not provide the host with the maximum benefit. As such, plants do not have a means of determining the benefit a given endosymbiont will provide at the time of colonisation. Plants do have the ability to differentiate between parasitic and mutualistic fungi since the plant defense system only activates when parasitic fungi colonise the host, but this differentiation occurs after colonisation.

Despite the obvious differences between nitrogen-fixing bacteria and mycorrhizal fungi, the symbioses have a number of similarities. Morphologically, the infection tube and infection hyphae are similar as well as the colonisation location (cortical cells). In addition, both symbioses appear to be able to turn off or avoid activating the plant defense mechanisms. To the host plant, both mutualisms are physiologically similar in terms of colonisation. Nitrogen-fixing bacteria and mycorrhizae produce similar polypeptides, known as nodulins or ectomycorrhizins, that stimulate plant flavanoid release. Both endosymbionts also have a similar uptake mechanism for flavanoids released by the host. In addition, there is evidence that the presence of one endosymbiont increases the colonisation of the other endosymbiont. This increase is achieved by stimulating the host to release flavanoids, the chemical signal that attracts both nitrogen-

fixing bacteria and mycorrhizal fungi. As well, root phosphorus levels may play a role in the increase of colonisation.

This evidence suggests that since the plant cannot differentiate between endosymbionts based on benefit, if selection does occur, it is by an alternate mechanism. Given the similarities between the two symbioses from the perspective of the plant and the fact that the endosymbionts have a positive effect on colonisation, perhaps the presence of one endosymbiont is the factor driving selection of the other endosymbiont.

2.4Species in the study

My thesis involves five organisms: red alder (*Alnus rubra*), a spore positive strain of *Frankia*, a spore negative strain of *Frankia*, the ectomycorrhizal fungus *Paxillus involutus*, and the ectomycorrhizal fungus *Hebeloma crustuliniforme*.

2.4a *Alnus rubra*

Alnus rubra (Bong.), red alder, is a tree species that grows along the west coast of North America, ranging from Alaska to California (Furlow, 1979). It is considered a lowland species, preferring elevations below 750 m (Harrington *et al.*, 1994). *Alnus rubra* prefers wet environments and grows near the ocean (Harrington *et al.*, 1994) as well as along stream banks, flood plains, and lakes (Furlow, 1979). It copes with the wet environments, and the periodic flooding associated with such environments by producing adventitious roots (Harrington *et al.*, 1994).

Alnus rubra is a short-lived pioneer species (Harrington, 1990) and the mutualisms it forms are the primary reason it is one of the first species to inhabit an area. The mutualism primarily allowing *A. rubra* to colonise areas involves the nitrogen-fixing actinomycete *Frankia*. This actinorhizal relationship is responsible for adding nitrogen to

the soil. In fact, *A. rubra* is the only common species west of the Rocky Mountains that can fix nitrogen (Harrington, 1990). *Alnus rubra* also forms mycorrhizal associations, both the ubiquitous VAM and the less prevalent ectomycorrhizae. *Alnus rubra* is extremely specialised in the ectomycorrhizal fungi it forms associations with, however (Molina, 1979). Only four of 28 fungal species tested formed mycorrhizae with *A. rubra* (Molina, 1979). *Alnus rubra* primarily forms mycorrhizae with the alder-specific *Alpova diplophloeus* (Harrington, 1990) although *A. rubra* will form mycorrhizae with some more generalist fungi such as *Paxillus involutus* (Molina, 1979).

2.4b *Frankia*

Frankia is an actinomycetous bacterium in the family Frankiaceae in the order Actinomycetales (Benson and Silvester, 1993). The bacterium produces fungal-like hyphae, spore-producing sporangia, and vesicles that contain nitrogenase and are the site of nitrogen fixation (Benson and Silvester, 1993). The thick walls of the vesicles decrease oxygen flux into the vesicles (Molina *et al.*, 1994), creating a low-oxygen environment necessary for nitrogen fixation.

A phylogeny of species within the genus *Frankia* has been difficult to construct (Benson and Silvester, 1993). Several researchers have used several means of delineating species within the genus (e.g. whole cell proteins, hosts colonised, fatty acid analysis); however, these means were discarded due to the complexity of variation in *Frankia* (Benson and Silvester, 1993). As a result, bacteria in the genus *Frankia* are generally referred to by the genus name only.

There are two types of *Frankia*: the free-living saprophytic *Frankia* and the endosymbiotic *Frankia* that forms nodules on host plants in 24 genera, 8 families, and 7

orders (Benson and Silvester, 1993). Endosymbiotic *Frankia* form more nodules than free-living *Frankia* (Lechevalier and Lechevalier, 1984). In the nodules, *Frankia* reduce N_2 to NH_4^+, which is exchanged for photosynthates from the host plant.

Endosymbiotic *Frankia* have been divided into two types based on spore production (Schwintzer, 1990): those that produce spores (spore positive) and those that do not produce spores (spore negative). Although all *Frankia* are capable of producing spores in culture, not all *Frankia* produce spores in nodules (Schwintzer, 1990), leading to the suggestion that there is a genetic basis for spore production. Spore negative *Frankia* are more common in the soil than spore positive and spore positive *Frankia* are found only in association with *Alnus spp.* and *Myrica gale* (Schwintzer, 1990). Spore positive *Frankia* are more infective than spore negative *Frankia* but spore negative *Frankia* have a higher nitrogenase activity than spore positive *Frankia* (Schwintzer, 1990).

2.4c *Paxillus involutus*

Paxillus involutus (Batsch: Fries) Fries, poison Paxillus, is an ectomycorrhizal fungus in the family Paxillaceae, order Boletales, class Basidiomycotina. The sporocarps of *P. involutus* are approximately 5 cm tall with marbled brown caps 20 cm in diameter and a yellow-brown spore print (Schalkwijk-Barendsen, 1991). This species is found across North America and Europe (Schalkwijk-Barendsen, 1991). On potato dextrose agar (PDA), *P. involutus* forms circular to irregularly circular colonies that sometimes produce drops of brown liquid (Hutchison, 1991). These colonies are dull white to light brown and can exhibit great morphological variability (Hutchison, 1991).

Paxillus involutus produces long and tortulous mycorrhizae with globose sclerotia found loosely in the hyphae (Ingleby *et al.*, 1990). The mantle is smooth (Miller *et al.*, 1991) and the mycorrhizae are silver-white when young, darkening to silver-buff, then silver bay with age (Ingleby *et al.*, 1990). The mycorrhizae are easily bruised with the bruises forming darker patches (Ingleby *et al.*, 1990). *Paxillus involutus* is a generalist fungus, forming ectomycorrhizae on a wide range of hosts (Molina *et al.,* 1992).

2.4d *Hebeloma crustuliniforme*

Hebeloma crustuliniforme (Bulliard: St Amans) Quelet, poison pie, is an ectomycorrhizal fungus in the family Cortinariaceae, order Agaricales, class Basidiomycotina. The sporocarps of *H. crustuliniforme* are approximately 6 cm tall with a buff cap 7 cm in diameter and a medium dull brown spore print (Schalkwijk-Barendsen, 1991). On PDA, *H. crustuliniforme* forms white circular to irregularly circular colonies (Hutchison, 1991). Sporocarps of *H. crustuliniforme* have not been found in association with *Alnus rubra*, but the fungus has formed mycorrhizae with *A. rubra* under laboratory conditions (Miller *et al.*, 1991). *Hebeloma crustuliniforme* forms mycorrhizae that are white, creamy white, or pale grey with a smooth mantle and do not bruise (Miller *et al.,* 1991). *Hebeloma crustuliniforme* is a generalist fungus, forming associations with many hosts (Molina *et al.*, 1992).

3 The interaction between *Frankia* and *Paxillus involutus* and between nitrogen and phosphorus on *Alnus rubra*

3.1 Introduction

Although the *A. rubra/Frankia/P. involutus* system is referred to as a tripartite mutualism, little is known about the relationship between *Frankia* and *P. involutus* on the shared host. As such, I designed an experiment to investigate the relationship between *Frankia* and *P. involutus* on a shared *A. rubra* host using nitrogen and phosphorus fertiliser concentrations to manipulate the mutualisms. The experiment involved a factorial design of five fertiliser treatments (0 mM P, 5 mM N; 5 mM P, 5 mM N; 10 mM P, 5 mM N; 5 mM P, 0 mM N; 5 mM P, 10 mM N) and four endosymbiont treatments (not inoculated with endosymbionts, inoculated with *Frankia*, inoculated with *P. involutus*, inoculated with *Frankia* and *P. involutus*). The objective of this experiment was to determine if the relationship between *Frankia* and *P. involutus* on *A. rubra* was a mutualism.

This experiment was designed based on two key ideas I) more than one mutualism can simultaneously form on a single host and II) varying soil nutrient levels alters the effect the endosymbiont has on host benefit (Fitter, 1985; Roldan-Fajardo *et al.*, 1982; Johnson, 1993; Armstrong *et al.*, 1992). Research done on tripartite mutualisms has mainly focused on the benefit the host receives from the endosymbionts. This research has shown that as the number of different types of endosymbionts on a single host increases, the host benefit increases (Chatarpaul *et al.*, 1989). Few studies have examined the interaction between the two endosymbionts on the same host. This experiment was designed to test the hypothesis that *Frankia* and ectomycorrhizae on the

same host would have a mutualistic relationship due to the fact that nutrients provided by one organism will alleviate the nutrient deficit of the other.

Colonisation in this experiment was defined differently for *Frankia* colonisation and mycorrhizal colonisation. Plants in this experiment were considered to have been colonised by the *Frankia* if they produced nodules. Plants in this experiment were considered to have been colonised by the mycorrhizal fungi if a single fungal hypha was found in the examined root sections. The colonisation status of the plants was used to determine which plants were included in the analysis. Plants were inoculated with both *Frankia* and mycorrhizal fungi – the inoculation treatment. Whether or not plants formed nodules/mycorrhizae was the colonisation status of the plant. If the colonisation status matched the inoculation treatment, then the plants were included in the analysis.

3.2 Materials and Methods

3.2a Plant material

Alnus rubra plants were grown from seed (Ministry of Forestry, British Columbia) in trays of sterilised 1:1 vermiculite:turface mixture. The seeds germinated 13 weeks prior to the application of the treatments. Prior to the experiment, the seeds were watered daily and fertilised weekly with Flora Gro and Flora Micro (General Hydroponics, San Rafael, California) (12.43 mM N, 1.41 mM P, 21.24 mM K, 2.06 mM Mg, 1.25×10^{-3} mM Ca, 0.170 mM Co, 0.179 mM Fe, 0.0910 mM Mn , 8.34×10^{-4} mM Mo). The seeds were then transplanted to yellow Ray Leach Conetainers (3.7 cm diameter, 13.6 cm height, Stuewe and Sons, Inc., Corvallis, Oregon, USA) with black electrical tape around the rim of the tubes to reduce algal growth. The electrical tape prevented the light from passing through the plastic and allowing algae to grow on the

inside surface of the tube. The tubes were filled with a sterilised 1:1 vermiculite:turface mixture and after the treatments were applied, a layer of stones was placed on top of the vermiculite/turface to prevent algal growth.

3.2b Experimental Design

The treatments were applied to the host plants in a 5x4 factorial design consisting of five nutrient treatments and four endosymbiont treatments. The five nutrient treatments were a combination of three phosphorus and three nitrogen concentrations (Table 1). The fertilizer was modified from the Rorison nutrient solution (Booth *et al.*, 1993) (Table 2). The four endosymbiotic treatments were control (no endosymbiont), Frankia (inoculated with spore positive *Frankia*), Paxillus (inoculated with *Paxillus involutus*), and tripartite (inoculated with both spore positive *Frankia* and *P. involutus*). The fertiliser treatments are referred to by the concentration of phosphorus and nitrogen in the fertiliser. The microbial treatments are referred to by the inoculant. Control plants received no inoculant. Frankia plants were inoculated with *Frankia.* Paxillus plants were inoculated with *P. involutus.* Tripartite plants were inoculated with *Frankia* and *P. involutus*. Sample size calculations (using a 0.05 alpha value, a 0.20 beta value, a smallest measurable difference of 30% (Krebs, 1999), and a shoot dry mass variance of 1.36) showed that, in order to determine a difference in shoot biomass, 35 replicates were needed for a total of 700 plants. Of the 700 plants planted, though, only 353 survived and were used for analysis. Analysing the remaining plants in this experiment by the fertiliser treatment reveals that the treatment with the smallest number of replicates (plants given 5 mM phosphorus, 0 mM nitrogen) had 21 replicates. If a beta value of 0.40 is used, then only 22 replicates are needed to determine a least significant difference

of 30%. Analysing the remaining plants in this experiment by the microbial treatments reveals that the treatment with the smallest number of replicates (plants inoculated with both *Frankia* and *P. involutus*) had four replicates. This number of replicates is not enough to determine a least significant difference of 30%. Removing data points to even out the number of replicates would not have been feasible because some of the 20 treatments had no replicates at all. Instead of the original 700 plants, only 353 were used for the analyses and caution was taken in interpreting the results in light of the fact that not enough replicates were present.

Table 3.1. Nitrogen and phosphorus concentration combinations used in the experiment. Combinations with a * were used as treatments while combinations with a – were not applied to plants. All other nutrients remained constant between the five nutrient treatments.

	0 mM phosphorus	5 mM phosphorus	10 mM phosphorus
0 mM nitrogen	-	*	-
5 mM nitrogen	*	*	*
10 mM nitrogen	-	*	-

3.2c Inoculation

Cultures of *P. involutus* (UAMH 5871, University of Alberta Mycological Herbarium, Edmonton, Alberta) were grown on Modified Melin-Norkran's agar (MMN) (Marx, 1969). Blocks 1 cm^2 of agar containing the fungus were added to liquid MMN and shaken daily to break up hyphal growth. One block was added per 50 mL of liquid MMN. After 2-3 weeks, the agar blocks were ground up with a mortar and pestle and returned to the medium and mixed thoroughly. This mixture was applied as the inoculant and contained a packed cell count of 0.1mL for the 5 mL volume that was applied to each

Table 3.2. The nutrient concentrations for the fertilisers applied in this experiment. The Full Basic solution is from Booth *et al.* (1993) and is shown as the reference fertiliser. The five fertilisers (0 mM P, 5 mM N; 5 mM P, 5 mM N; 10 mM P, 5 mM N; 5 mM P, 0 mM N; 5 mM P, 10 mM N) used in this experiment are variations of the full basic solution as shown and are shown under "variations" in the chart.

				Amount of stock solution added for the fertiliser (mL stock/L water)					
Element	mg/L element in stock	mass of compound in stock (mg)	Compound in stock solution	Full Basic	0 mM P, 5mM N	5 mM P, 5mM N	10 mM P, 5 mM N	5 mM P, 0 mM N	5 mM P, 10 mM N
Ca/N	80/56	476.1	$Ca(NO_3)_2x4\ H_2O$	1	------	------	------	------	------
Mg	24	248	$MgSO_4x7\ H_2O$	1	1	1	1	1	
K/P	78/31	230.7	$K_2HPO_4x3\ H_2O$	1	------	------	------	------	------
Fe	3	25	FeEDTA	1	1	1	1	1	1
Mn	0.5	2.028	$MnSO_4x4\ H_2O$						
B	0.5	2.8963	H_3BO_3						
Mo	0.1	0.184	$(NH_4)_6Mo_7O_{24}x4\ H_2)$	1	1	1	1	1	1
Zn	0.1	0.44	$ZnSO_4x7\ H_2O$						
Cu	0.1	0.393	$CuSO_4x5\ H_2O$						
Variations									
Ca/N (5mM)	200/70.05	590.45	$Ca(NO_3)_2x4\ H_2O$	------	1	1	1	------	------
Ca/N (10mM)	400.75/140.1	1180.9	$Ca(NO_3)_2x4\ H_2O$	------	------	------	------	------	1
Ca	80	447	$CaCl_2x\ 6H_2O$	------	------	------	------	1	------
K/P (5mM)	195.5/154.85	1141.2	$K_2HPO_4x3\ H_2O$	------	------	1	------	1	1
K/P (10mM)	391/309.7	2282.4	$K_2HPO_4x3\ H_2O$	------	------	------	1	------	------
K	78	175	K_2SO_4	------	1	------	------	------	------

plant. The packed cell counted was determined by placing 5 mL of the inoculant in an centrifuge tube and then centrifuging it to determine the volume cellular debris in the inoculant. The *Frankia* inoculant was prepared by grinding 0.130g of spore positive *Alnus incana* nodule and adding it to 2 L of distilled water. Five mL of inoculant was used to inoculate each host plant and plants were inoculated twice, three weeks apart, to ensure the endosymbiont colonised the host plant.

3.2d Growing conditions and harvest

The plants were grown in a growth chamber (Conviron, Winnipeg, Manitoba) with a 16 hour light: 8 hour dark photoperiod. The light was gradually increased and decreased over a 24 hour period to mimic natural conditions rather than a sharp change to light or dark. The light in the growth chamber when all the lights were on was 180 μmol photons/m^2/s. The temperature ranged from 18 °C during the night to 24 °C during the day over the course of twenty four hours and again gradually increased and decreased to mimic natural conditions. Relative humidity was set at 60% but the watering cycle caused the humidity to fluctuate from 75% to 85%. These parameters where chosen to mimic natural conditions and prevent desiccation. Before the treatments were applied, plants were watered for ten minutes every four hours to maintain field capacity in the soil. For the first four weeks after the treatments were applied, plants were watered for five minutes every eight hours. During the last four weeks the treatments were applied, plants were watered for six minutes every four hours. The length of watering was varied to strike a balance between desiccation (due to lack of water) and algal growth (due to overabundance of water). The position of the plants in the growth chamber was rotated

every two weeks to prevent the position in the growth chamber from having an influence on the performance of the plants.

Alnus rubra plants were 13 weeks old at the start of the experiment and were grown for 13 weeks under experimental conditions. Ten mL of fertilizer was applied three times a week to eliminate rapidly increasing the nutrient concentration in the rooting medium immediately after fertiliser application and then drastically decreasing the nutrient concentration between fertiliser applications. Height from the soil surface to the shoot apical meristem was measured weekly and diameter at the base of the shoot was measured weekly after the first four weeks once the stems had started to harden. The Control and Paxillus plants were separated into roots and shoots on harvest and weighed immediately to determine fresh mass. The Frankia and Tripartite plants were harvested and acetylene reduction assays were immediately performed on these plants. After the acetylene reduction assay had been completed, Frankia and Tripartite plants were separated into roots, shoots, and nodules, and weighed to determine fresh mass.

Shoots and nodules were dried at 65°C for 14 days immediately after harvesting. Roots were stored in water for two weeks to prevent root desiccation until the roots could be examined for mycorrhizae. To determine mycorrhizal colonisation, the roots were cleared and then stained. Clearing the roots removes the cytoplasm and cellular contents from the roots increasing the visibility of the cell walls. The clearing and staining procedure was adapted from Phillips and Hayman (1970). Roots were cleared by heating the roots to 65 °C in 3M KOH for 1.5. Roots were rinsed and then heated for 1 hour to 65 °C in 1M HCl to acidify them. After rinsing, the roots were briefly immersed in trypan blue stain (200 mL lactic acid, 200 mL glycerol, 200 mL distilled water, 0.2 g

trypan blue stain) and immediately rinsed in water. Two or three roots per sample were hand sectioned and examined under the microscope to determine mycorrhizal status. Plants were said to be mycorrhizal if a single fungal hypha was present in the roots. Because the hypha increased the cell wall thickness, once the roots had been cleared and stained, the thickened cell walls due to the presence of mycorrhizal hyphae were easily identifiable. Once mycorrhizal status was determined, the roots were dried at 65 °C for 14 days. After drying for 14 days, the shoot, root, and nodule dry masses were determined.

3.2e Chemical Assays

Acetylene reduction assays were performed on plants inoculated with *Frankia* (Granhall *et al.*, 1983). The plants were placed in 60 mL, 120 mL, or 250 mL glass jam jars with screw top lids depending on the size of the plant. Plant roots were inside the jar while the shoots were outside the jar. The neck of the jar was sealed with Tak 'N Stick (Ross Products, Toronto, Ontario) to prevent gas exchange. Plastic syringes were used to remove 6, 12, or 25 mL of air from the 60, 120, or 250 mL jar respectively and replace it with the same volume of acetylene to create a 10% acetylene mixture in the jars. After one hour, a 1.5 mL gas sample was drawn off from the jar and injected into a CP 3800 gas chromatograph (Varian 3800, Mississauga, Ontario) to determine ethylene concentrations in the gas. The gas chromatograph had an oven temperature of 180°C, a flame ionisation detector temperature of 200°C, a flow rate of helium carrier gas of 30 mL/min, and a 1 mL sample loop. A closed acetylene system was used rather than a flow through system because a relative acetylene reduction rate was sufficient to compare between treatments (Vessey, 1994). Once the acetylene reduction assays had been

performed, plants were separated into roots, shoots, and nodules, and the fresh masses of all three were determined. Nodules were separated from the roots so the nodule dry mass and the specific acetylene reduction rates (ethylene produced/ g nodule/hr) could be calculated.

Ethylene production was determined mathematically by first multiplying the area under the peak on the gas chromatograph display by the volume of the container used for the acetylene reduction (60 mL, 120 mL, or 250 mL). The area under the ethylene peak was then subtracted from that value to remove the background ethylene and give just the ethylene that was produced by the nitrogenase enzyme. The background ethylene was considered the ethylene found when a sample of the acetylene gas was injected into the gas chromatograph. The ethylene produced by the nitrogenase enzyme was then converted from area under a peak to a concentration using the formula: area under the curve = 119560 * the concentration of ethylene. This formula for determining the concentration of ethylene in the gas sample was developed from a standard curve. The ethylene concentration was then divided by the total nodule dry mass of the plant to give the specific acetylene reduction rate per hour.

3.2f Nutrient determination

Foliar nitrogen and phosphorus levels were determined for select treatments. All the leaves of all plants within a given fertiliser/microbial combination were ground in a Wiley mill (Arthur H. Thomas, Co., Philadelphia, Pennsylvania) and passed through a screen with 1 mm^2 holes so that all the particles used in the nutrient analyses were less than 1 mm^2. The leaves for all plants within a given fertiliser/microbial combination were combined and only one measurement was performed for that combination rather

than each plant being tested separately. Combining the leaves was done for two purposes. The first was to reduce the cost of the foliar nitrogen and phosphorus tests. The second was that 0.200 g of leaves were needed for the nutrient analyses and it was only by combining all of the leaves of all of the plants that the 0.200 g was achieved. Once ground, the leaves were mixed to homogenise the sample used for the analyses. Total Kjeldhal nitrogen (Bradstreet, 1965) was determined for control plants, nodulated Frankia plants, non-nodulated Frankia plants, nodulated Tripartite plants , and non-nodulated Tripartite plants. Foliar phosphorus was determined for the Control plants, Paxillus plants, nodulated Tripartite plants, and non-nodulated Tripartite plants. Each of these treatments was further divided into the five fertiliser treatments. Not all of the fertiliser treatments had Tripartite plants that formed nodules, so a total of 17 treatments were examined for foliar phosphorus content. These five groups were then further divided into the five fertiliser categories. *Frankia* and Tripartite plants did not form nodules in all fertiliser treatments so only twenty samples were tested. The foliar nitrogen concentrations were determined by Department of Animal Science (University of Manitoba, Winnipeg, Manitoba) (Leco CNS 2000).

Foliar phosphorus was determined using Roland and Grimshaw's (1985) wet oxidation method. A sulphuric acid-hydrogen peroxide mixture was used to digest 0.200 g of dried, ground leaves. Some samples did not have 0.200 g of leaves and as such, a smaller volume of digestion mixture was used and the digestions were adjusted accordingly. As the lack of plant matter was adjusted for in the nutrient analysis, the values were treated exactly the same as values for samples with the requisite 0.200 mg of plant matter in the data analysis. The plant material in the digestion mixture was heated

for 3 hours to 350°C. The mixture was then diluted to 50 mL and then diluted five fold. Molybdenum blue was added to colour the solution and colour intensity was measured in a spectrophotometer at a 882 μm wavelength (Roland and Grimshaw, 1985). The foliar phosphorus concentrations were determined in the Department of Soil Science (University of Manitoba, Winnipeg, Manitoba).

3.2g Data Analysis

Not all of the plants in the Frankia and Tripartite treatments formed nodules and not all of the of the plants in the Paxillus treatment formed mycorrhizae. There were also some Control plants that formed mycorrhizae. As such, only those plants that actually matched the inoculation treatment were included in the analysis. That is, Control plants were only included if they had no endosymbionts, Frankia plants were only included if they formed nodules, Paxillus plants were only included if they formed mycorrhizae, and Tripartite plants were only included if they formed both nodules and mycorrhizae. As a result the lack of colonisation or nodulation, only 188 of the 353 living plants were analysed. Shoot dry mass and root dry mass were examined and found not to be normally distributed. As a result, shoot dry mass and root dry mass were log transformed. Relative growth rate was determined using the formula (log $height_{t2}$-log $height_{t1}$)/(14 days). One way analyses of variances (ANOVAs) were performed on the data to determine differences among the four mycorrhizal treatments and the five nutrient treatments (JMP version 4.04, SAS Institute). *Post hoc* Tukey's tests were performed on all significant ANOVAs (JMP version 4.04, SAS Institute).

3.3 Results

3.3a Colonisation

Mycorrhizal colonisation levels were high, ranging from 83% to 96% colonisation depending on the treatment (Table 3.3). The three fertiliser nitrogen levels , the three fertiliser phosphorus levels, and *Frankia* colonisation had no effect on mycorrhizal colonisation (Table 3.3). In total, only nine plants formed *Frankia* nodules. *Frankia* colonisation was considerably lower than mycorrhizal colonisation levels (Table 3.4). Mycorrhizae and fertiliser phosphorus levels had no effect on *Frankia* colonisation (Table 3.4). Plants with 10 mM nitrogen fertiliser had lower colonisation rates (χ^2=11.445, p=0.0220), although colonisation levels were low in all treatments (Table 3.4). Of the nodulated plants given 0 mM P, 5 mM N fertiliser, 3 were in the Frankia treatment and 1 was in the Tripartite treatment. Of the nodulated plants given 5 mM P, 5 mM N fertiliser, 3 were in the Tripartite treatment and 1 was in the Frankia treatment. The nodulated plant given 5 mM P, 10 mM N fertiliser was in the Frankia treatment. Due to the low levels of *Frankia* colonisation, this experiment was repeated, but the levels of *Frankia* colonisation were again low in the repeat experiment. For the colonisation analysis of *Frankia* and *Paxillus involutus*, all plants were used. For the rest of the analyses, plants were only included if their colonisation status matched treatment they were inoculated with (e.g. plants were used for the Tripartite treatment only if they had both nodules and mycorrhizae).

3.3b Plant size

Nitrogen concentrations in the fertiliser had an effect on plant size. Plants fertilised with 10 mM nitrogen had approximately twice as much shoot mass as plants in the three treatments that were fertilised with 5 mM nitrogen fertiliser (F=13.46,

Table 3.3. The *Paxillus involutus* mycorrhizal colonisation rates of plants in the fertilizer and microbial treatments. Paxillus = plants inoculated with *Paxillus involutus*; Tripartite = plants inoculated with *Frankia* and *Paxillus involutus*.

Treatments	Total number of plants	Percent colonisation (%)
Inoculant treatments		
Paxillus	94	91
Tripartite	89	92
Nutrient treatments		
0 mM P, 5 mM N	37	92
5 mM P, 5 mM N	39	92
10 mM P, 5 mM N	34	91
5 mM P, 0 mM N	23	83
5 mM P, 10 mM N	40	96

Table 3.4. The *Frankia* colonisation rates of plants in the fertilizer and microbial treatments. Frankia = plants inoculated with *Frankia*; Tripartite = plants inoculated with *Frankia* and *Paxillus involutus*.

Treatments	Total number of plants	Percent colonisation (%)
Inoculant treatment		
Frankia	71	7
Tripartite	89	4.5
Fertiliser treatment		
0 mM P, 5 mM N	33	12
5 mM P, 5 mM N	31	13
10 mM P, 5 mM N	36	0
5 mM P, 0 mM N	14	0
5 mM P, 10 mM N	46	2

p<0.0001) (Figure 3.1). Plants fertilised with 10 mM nitrogen fertiliser (0.353 g +/- 0.324) had root masses that were approximately 1.5 times that of plants in the three treatments fertilised with 5 mM nitrogen fertiliser (0.212 g +/- 0.239) (F=7.1302, P<0.0001) (data not shown). Plants fertilised with 10 mM nitrogen fertiliser (8.99 cm +/- 4.27) were approximately 1.3 times taller than times that of plants in the three treatments that were fertilised with 5 mM nitrogen fertiliser (6.46 cm +/- 3.67) (F=12.52, p<0.0001) (data not shown). Plants fertilised with 10 mM nitrogen fertiliser (1.99 mm +/- 0.97) had diameters that were approximately 1.3 times larger than diameters of plants in the three treatments that were fertilised with 5 mM nitrogen fertiliser (1.50 mm +/- 0.83) (F=8.2594, p<0.0001) (data not shown). Plants fertilised with 0 mM nitrogen fertiliser (0.0181 g +/- 0.042) had shoot masses that were approximately 10% less than plants in the three treatments that were given 5 mM nitrogen fertiliser (0.149 +/- 0.125) (Figure 3.1). The roots masses of plants given 0 mM nitrogen fertiliser (0.002 g +/- 0.002) were approximately 1% that of plants in the three treatments that were given 5 mM nitrogen fertiliser (0.212 g +/- 0.239) (F=7.1302, p<0.0001) (data not shown). Plants given 0 mM nitrogen fertiliser (2.07 cm +/- 0.85) were approximately one third as tall as plants in the three treatments that were given 5 mM nitrogen fertiliser (6.46 cm +/- 3.67) (F=12.5233, p<0.0001) (data not shown). Phosphorus did not have an effect on shoot mass (Figure 3.1), root mass, shoot diameters, or height in this experiment (data not shown).

Nodulated plants in the Frankia and Tripartite treatments had a tendency to have higher shoot masses than plants in the Control and Paxillus treatments (F=2.5327, p=0.0585) (Figure 3.2) but the nodulated plants in the Frankia and Tripartite treatments did not differ from plants in the Control and Paxillus treatments in terms of root mass

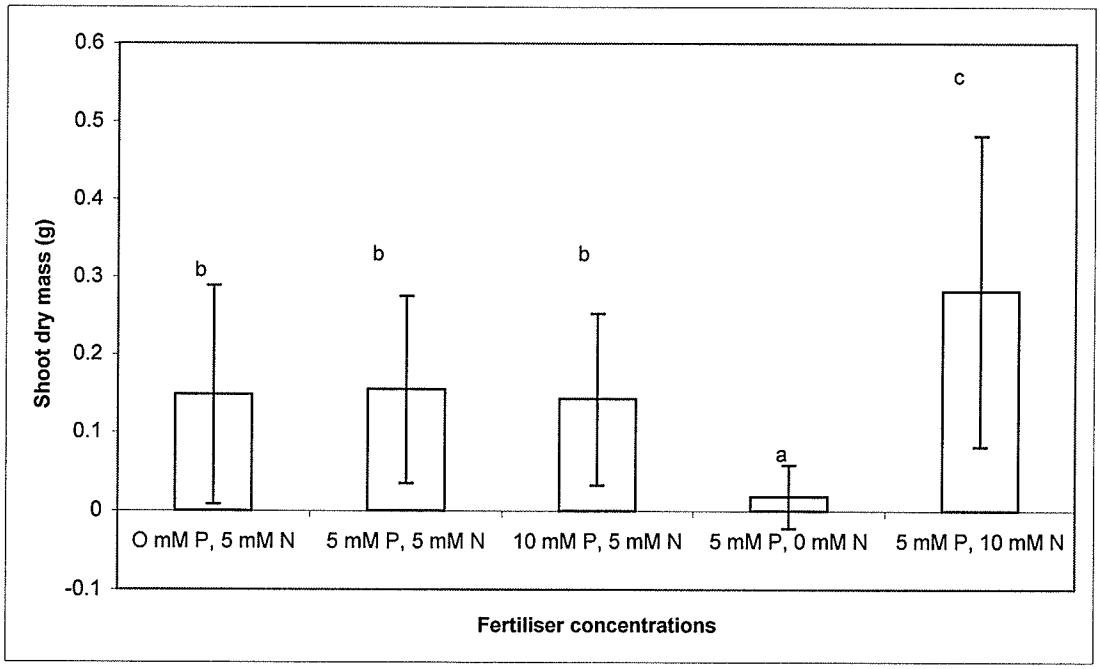

Figure 3.1. Shoot dry masses of *Alnus rubra* plants colonised by microbes in five fertiliser treatments (0 mM P, 5 mM N; 5 mM P, 5 mM N; 10 mM P, 5 mM N; 5 mM P, 0 mM N; 5 mM P, 10 mM N). Microbial combinations (Control, Frankia, Paxillus, and Tripartite) are combined within the fertiliser treatments because all four microbial treatments follow the same trend. Values given are means plus or minus standard deviations. Treatments with different letters are significantly different at the 0.05 level according to a Tukey's *post hoc* test. There were 48 0 mM P, 5 mM N plant ; 41 5 mM P, 5 mM N plants; 34 10 mM P, 5 mM N plants; 21 5 mM P, 0 mM N plants; and 44 5 mM P, 10 mM N plants.

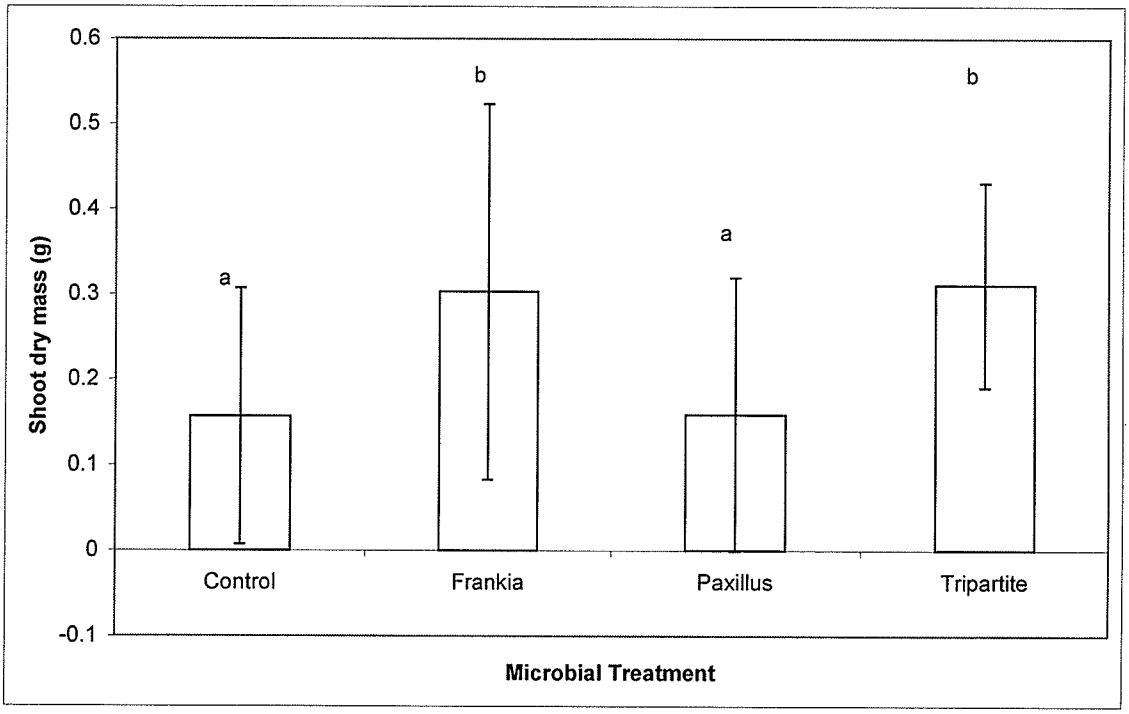

Figure 3.2. The shoot dry masses of *Alnus rubra* plants colonised by microbes in the microbial treatments. Control plants were not inoculated with any endosymbionts; Frankia plants were inoculated with *Frankia*; Paxillus plants were inoculated with *Paxillus involutus*; Tripartite plants were inoculated with *Frankia* and *Paxillus involutus*. Values given are means plus or minus standard deviations. Fertiliser treatments (0 mM P, 5 mM N; 5 mM P, 5 mM N; 10 mM P, 5 mM N; 5 mM P, 0 mM N; 5 mM P, 10 mM N) are combined within microbial treatments. Treatments with different letters were significantly different at the 0.05 level according to a Tukey *post hoc* test.

(Figure 3.3), height, or shoot diameter. The presence of mycorrhizae had no effect on shoot mass (Figure 3.2), root mass (Figure 3.3), height, or diameter. Within treatments inoculated with *Frankia*, there was no difference between nodulated and non-nodulated plants in terms of root mass (n=151, 0.173 g +/- 0.192 for non-nodulated plants *vs.* n= 9, 0.219 g +/- 0.129 for nodulated plants), diameter (n=148, 1.706 mm +/- 0.938 for non-nodulated plants *vs.* n= 9, 1.971mm +/- 0.535 for nodulated plants) or height (n=148, 7.236 cm +/- 3.807 for non-nodulated plants *vs.* n= 9, 8.750 cm +/- 2.941 for nodulated plants). There was a non-significant trend for nodulated plants to have higher shoot masses than non-nodulated plants in treatments where plants were inoculated with *Frankia* (n=5, 0.303 g +/- 0.223 for nodulated plants *vs.* n=66, 0.196 g +/- 0.160 for non-nodulated plants) (F=1.9772, p=0.1642) and with both *Frankia* and *P. involutus* (n=4, 0.311 g +/- 0.118 for nodulated plants *vs.* n=84, 0.184 g +/- 0.155)(F=2.6298, p=0.1085) (F=2.6298, p=0.1085). There was no difference in shoot mass between the Control, Paxillus, non-nodulated Frankia, and non-nodulated Tripartite treatments; however, there was a non-significant trend for nodulated plants in the Frankia and Tripartite plants to have larger shoot masses than the Control, Paxillus, non-nodulated Frankia, and non-nodulated Tripartite treatments (Figure 3.4).

The fertiliser treatments had an earlier noticeable effect on plant growth (five weeks after inoculation) than microbial treatments (eight weeks after inoculation) (Figures 3.5 and 3.6). The fertiliser treatments caused plant relative growth rates to be later in the experiment while the microbial treatments caused plant relative growth rates to be higher earlier in the experiment (Figures 3.7 and 3.8). Plants given 0 mM nitrogen fertiliser had significantly lower relative growth rates compared to plants given 10 mM

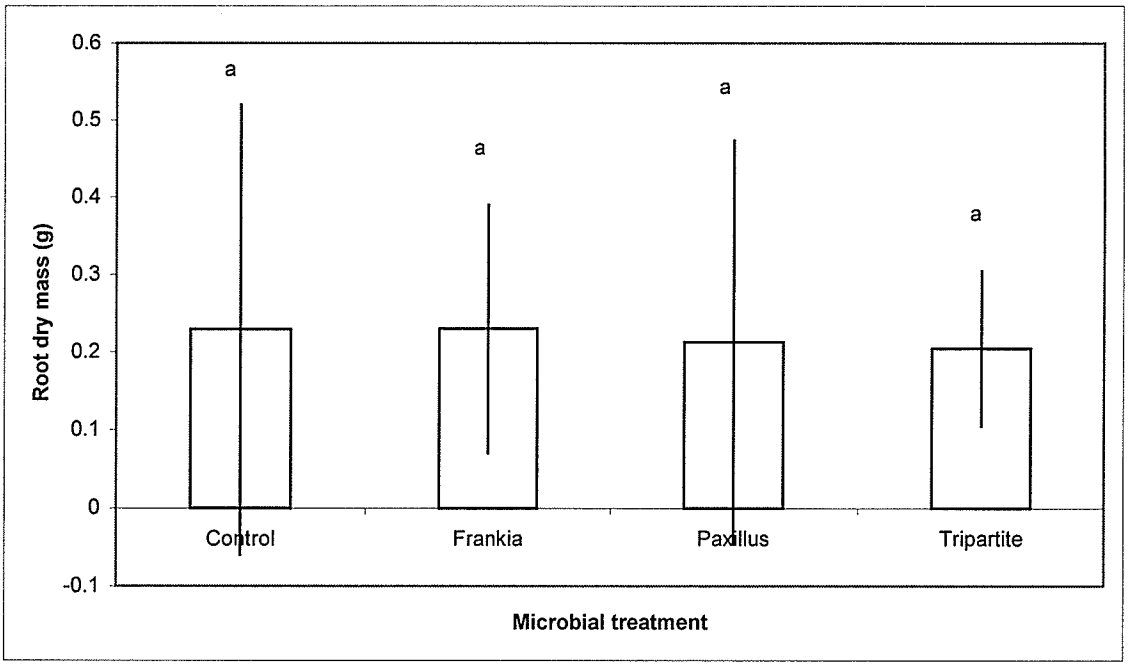

Figure 3.3. The root dry masses of *Alnus rubra* plants colonised by microbes in the microbial treatments. Control plants were not inoculated with any endosymbionts; Frankia plants were inoculated with *Frankia*; Paxillus plants were inoculated with *Paxillus involutus*; Tripartite plants were inoculated with *Frankia* and *Paxillus involutus*. Fertiliser treatments (0 mM P, 5 mM N; 5 mM P, 5 mM N; 10 mM P, 5 mM N; 5 mM P, 0 mM N; 5 mM P, 10 mM N) are combined within microbial treatments. Values given are means plus or standard deviations. Treatments with the same letter are not significantly different at the 0.05 level according to an ANOVA test.

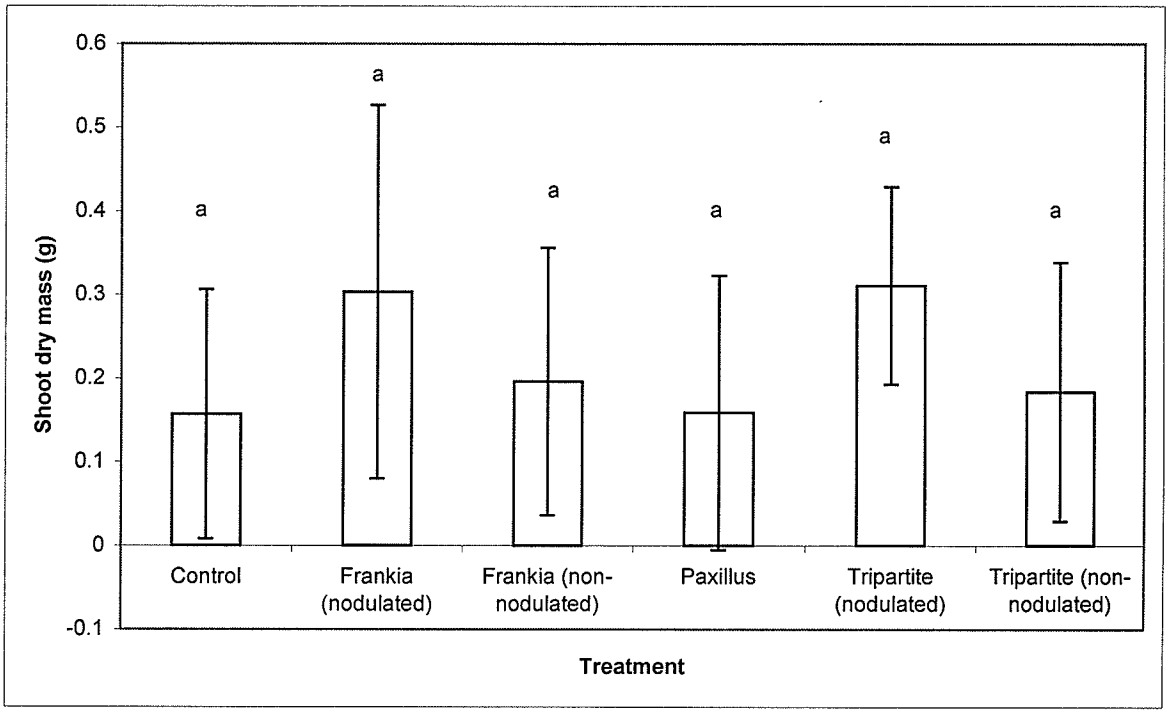

Figure 3.4. Shoot dry masses of *Alnus rubra* plants colonised by microbes in the microbial treatments including non-nodulated Frankia and Tripartite plants. Control = plants inoculated with no endosymbionts; Frankia = plants inoculated with *Frankia*; Paxillus = plants inoculated with *Paxillus involutus*; Tripartite = plants inoculated with both *Frankia* and *Paxillus involutus*. The five fertiliser treatments (0 mM P, 5 mM N; 5 mM P, 5 mM N; 10 mM P, 5 mM N; 5 mM P, 0 mM N; 5 mM P, 10 mM N) were combined within the microbial treatments. Values given are means plus or minus standard deviations. There were 93 Control plants, 5 nodulated Frankia plants, 66 non nodulated Frankia plants, 86 Paxillus plants, 4 nodulated Tripartite plants, and 85 non nodulated Tripartite plants. Treatments with different letters are not significantly different at the 0.05 level according to a Tukey *post hoc* test.

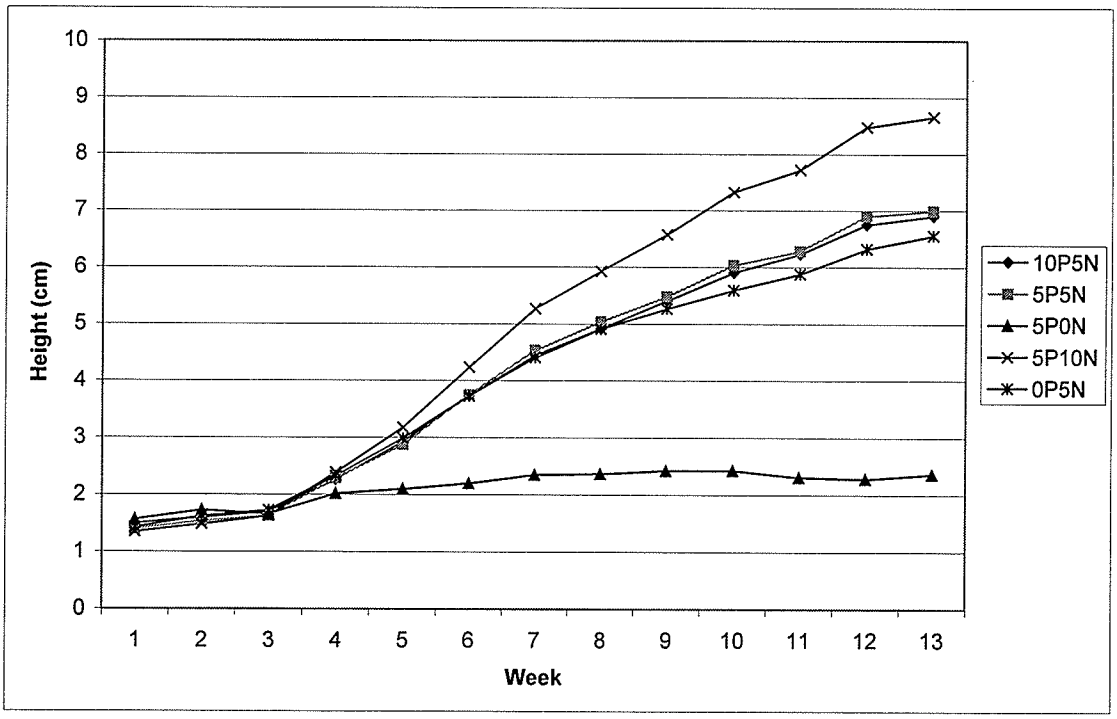

Figure 3.5. Change in height of *Alnus rubra* plants colonised by microbes in the five fertiliser treatments (0 mM P, 5 mM N; 5 mM P, 5 mM N; 10 mM P, 5 mM N; 5 mM P, 0 mM N; 5 mM P, 10 mM N) for each week during the experiment after inoculation. All microbial treatments (Control, Frankia, Paxillus, and Tripartite) are combined in the fertiliser treatment. 10P5N = 10 mM P, 5 mM N; 5P5N = 5 mM P, 5 mM N; 5P0N = 5 mM P, 0 mM N; 5P10N = 5 mM P, 10 mM N; 0P5N = 0 mM P, 5 mM N. Values given are means for the fertiliser treatments. There were 48 0 mM P, 5 mM N plants; 41 5 mM P, 5 mM N plants; 34 10 mM P, 5 mM N plants; 21 5 mM P, 0 mM N plants; and 44 5 mM P, 10mM N plants.

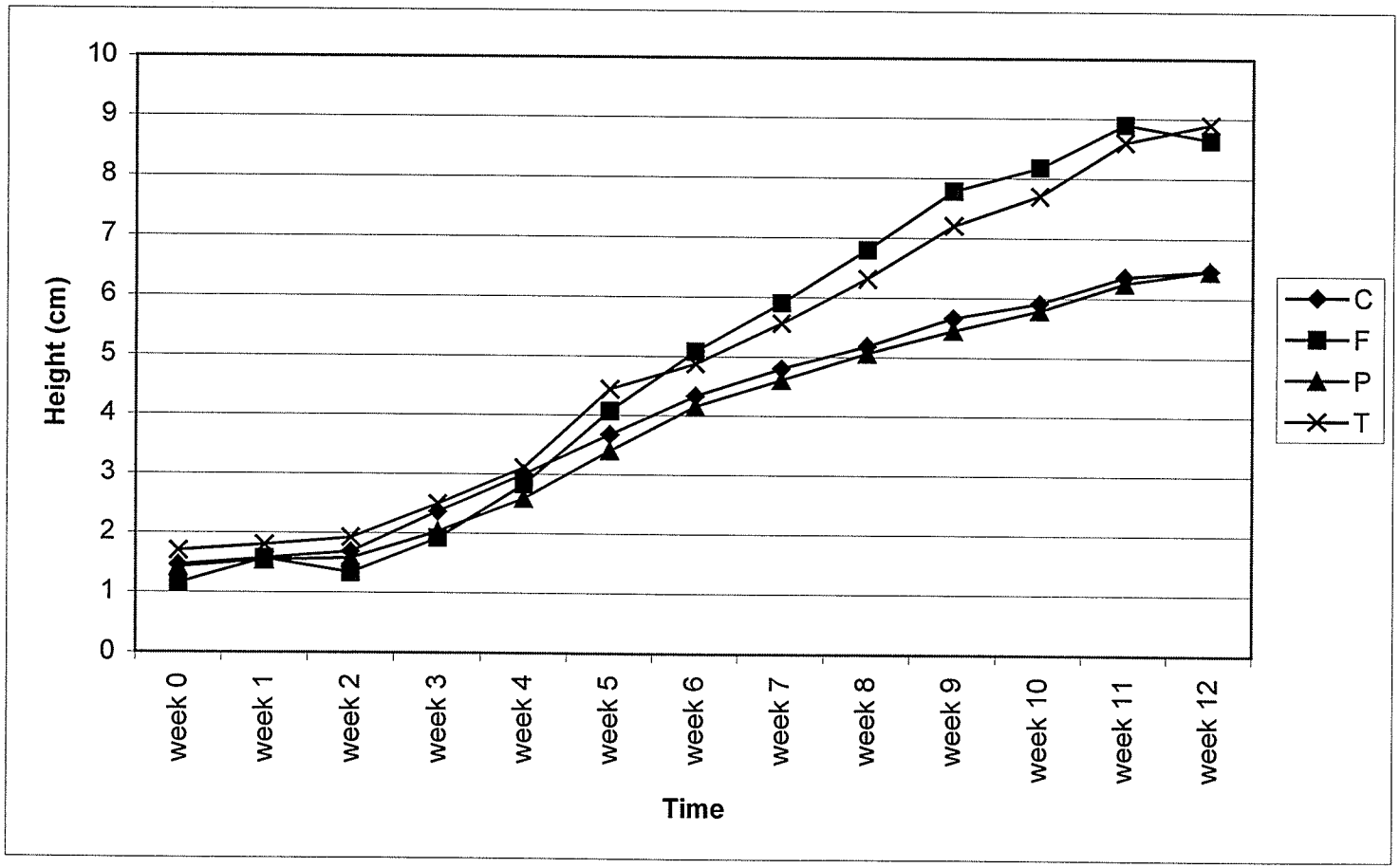

Figure 3.6. The height of *Alnus rubra* plants colonised by microbes in the four microbial treatments (Control, Frankia, Paxillus, Tripartite) over the course of the experiment after inoculation. All fertiliser treatments (0 mM P, 5 mM N; 5 mM P, 5 mM N; 10 mM P, 5 mM N; 5 mM P, 0 mM N; 5 mM P, 10 mM N) are combined in the microbial treatments. C = plants not inoculated with endosymbionts; F = plants inoculated with *Frankia*; P = plants inoculated with *Paxillus involutus*; T = plants inoculated with *Frankia* and *Paxillus involutus*. Values given are means for the fertiliser treatments. There were 93 Control plants, 5 Frankia plants, 86 Paxillus plants, and 4 Tripartite plants.

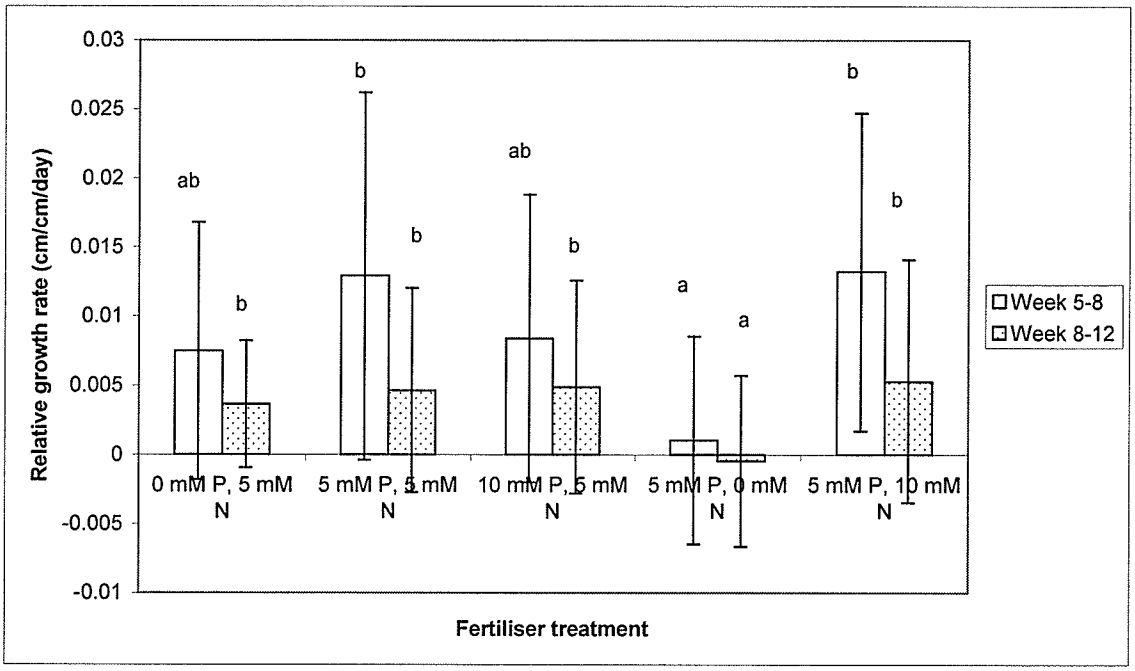

Figure 3.7. Relative growth rates for weeks 5-8 and 8-12 for *Alnus rubra* plants colonised by microbes in the five fertiliser treatments (0 mM P, 5 mM N; 5 mM P, 5 mM N; 10 mM P, 5 mM N; 5 mM P, 0 mM N; 5 mM P, 10 mM N). The four microbial treatments (Control, Frankia, Paxillus, and Tripartite) are combined within the fertiliser treatments. Week 5-8 represents the middle of the experiment just after the fertiliser treatment began to take effect while week 8-12 represents the end of the experiment. Values given are means plus or minus standard deviations. Within one time period, treatments with different letters are significantly different at the 0.05 level according to a Tukey's *post hoc* test. There are 48 0 mM P, 5 mM N plants; 41 5 mM P, 5 mM N plants; 34 10 mM P, 5 mM N plants; 21 5 mM P, 0 mM N plant ; and 44 5 mM P, 10 mM N plants.

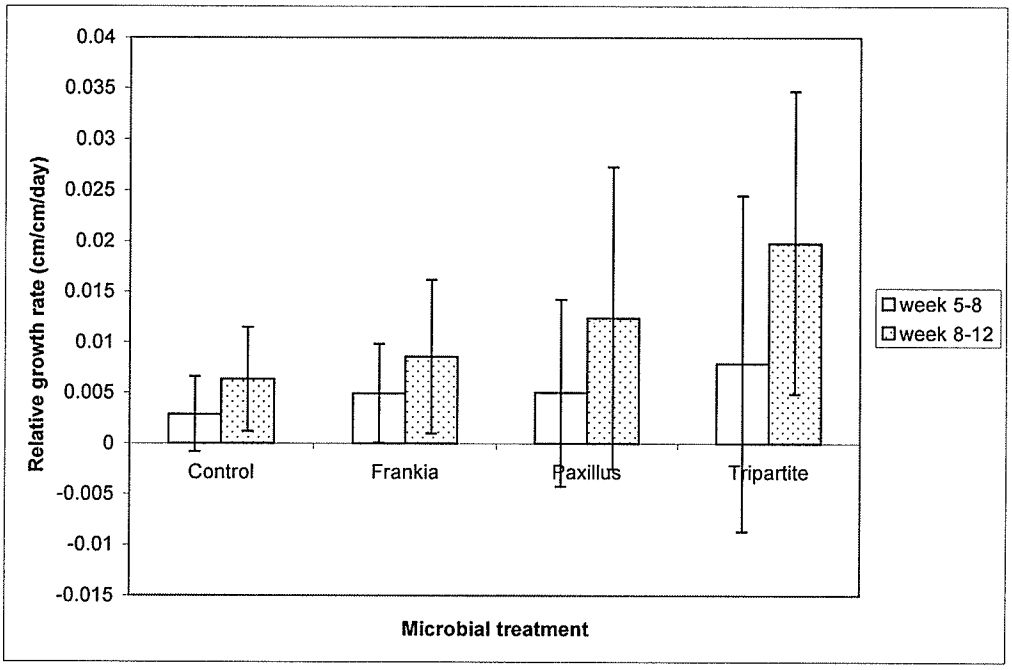

Figure 3.8. Relative growth rates for weeks 5-8 and 8-12 for *Alnus rubra* plants colonised by microbes in the four microbial treatments (Control, Frankia, Paxillus, and Tripartite). The five fertiliser treatments (0 mM P, 5 mM N; 5 mM P, 5 mM N; 10 mM P, 5 mM N; 5 mM P, 0 mM N; 5 mM P, 10 mM N) are combined within the microbial treatments. Week 5-8 represents the middle of the experiment just after the fertiliser treatment began to take effect while week 8-12 represents the end of the experiment. Values given are means plus or minus standard deviations. Values given are means plus or minus standard deviations. Although the treatments were found to be significantly different during week 8-12 according to an ANOVA test, there was no difference found in the Tukey *post hoc* test. There are 93 Control plants, 5 Frankia plants, 86 Paxillus plants, and 4 Tripartite plants.

nitrogen or 5 mM phosphorus, 5 mM nitrogen fertiliser during the early part of the experiment (F=5.9790, p=0.0002) (Figure 3.7). During the early part of the experiment, when plants were give nitrogen (either 5 mM or 10 mM), plants given 5 mM phosphorus fertiliser had the highest growth rates (Figure 3.7). During the latter part of the experiment, plants given 0 mM nitrogen fertiliser had significantly lower growth rates compared to all the rest of the treatments (F=2.7188, p=0.0312) (Figure 3.7). Phosphorus fertiliser did not have an effect on plant growth rates in the latter part of the experiment (Figure 3.7). The microbial treatments did not affect plant growth rates during the early part of the experiment (Figure 3.8). During the latter part of the experiment, the presence of mycorrhizae and nodules increases plant growth rates while the absence of mycorrhizae and nodules decreases plant growth rates (F=5.6641, p=0.0010) (Figure 3.8).

The presence of mycorrhizae or *Frankia* did not have an effect on the root:shoot ratios of plants (Figure 3.9). The presence and absence of phosphorus and the absence of nitrogen in the fertiliser given to the plants had an effect on the root:shoot ratios (F=2.9550, p=0.0214) (Figure 3.9). Plants given 0 mM or 10 mM phosphorus fertiliser had the highest root:shoot ratios while plants given 0 mM nitrogen fertiliser had the lowest root:shoot ratios (Figure 3.9). None of the endosymbionts had an effect on the root:shoot ratios.

3.3c Nitrogen fixation

Neither the phosphorus concentration nor the nitrogen concentration in the fertiliser had an effect on specific acetylene reduction rates (F=0.2748, p=0.7688) (Figure 3.10) or the acetylene reduction rates per plant. There was a non significant trend for the presence of mycorrhizae (n=4, 60.736 mmol ethylene/g nodule/h) to increase the specific

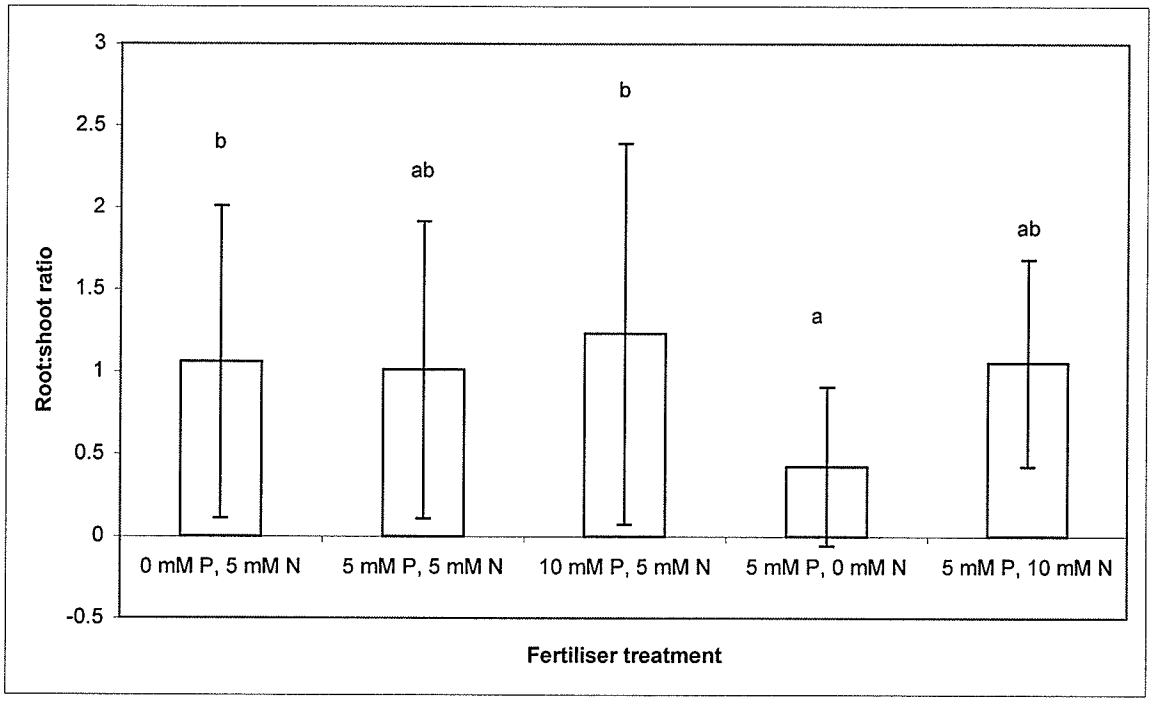

Figure 3.9. The root:shoot ratio of *Alnus rubra* plants colonised by microbes in five fertiliser treatments (0 mM P, 5 mM N; 5 mM P, 5 mM N; 10 mM P, 5 mM N; 5 mM P, 0 mM N; 5 mM P, 10 mM N). Microbial treatments (Control, Frankia, Paxillus, and Tripartite) were combined within the fertiliser treatments because all four microbial treatments followed the same trend. Values given are means plus or minus standard deviations. Treatments with different letters are significantly different at the 0.05 level according to a Tukey *post hoc* test.

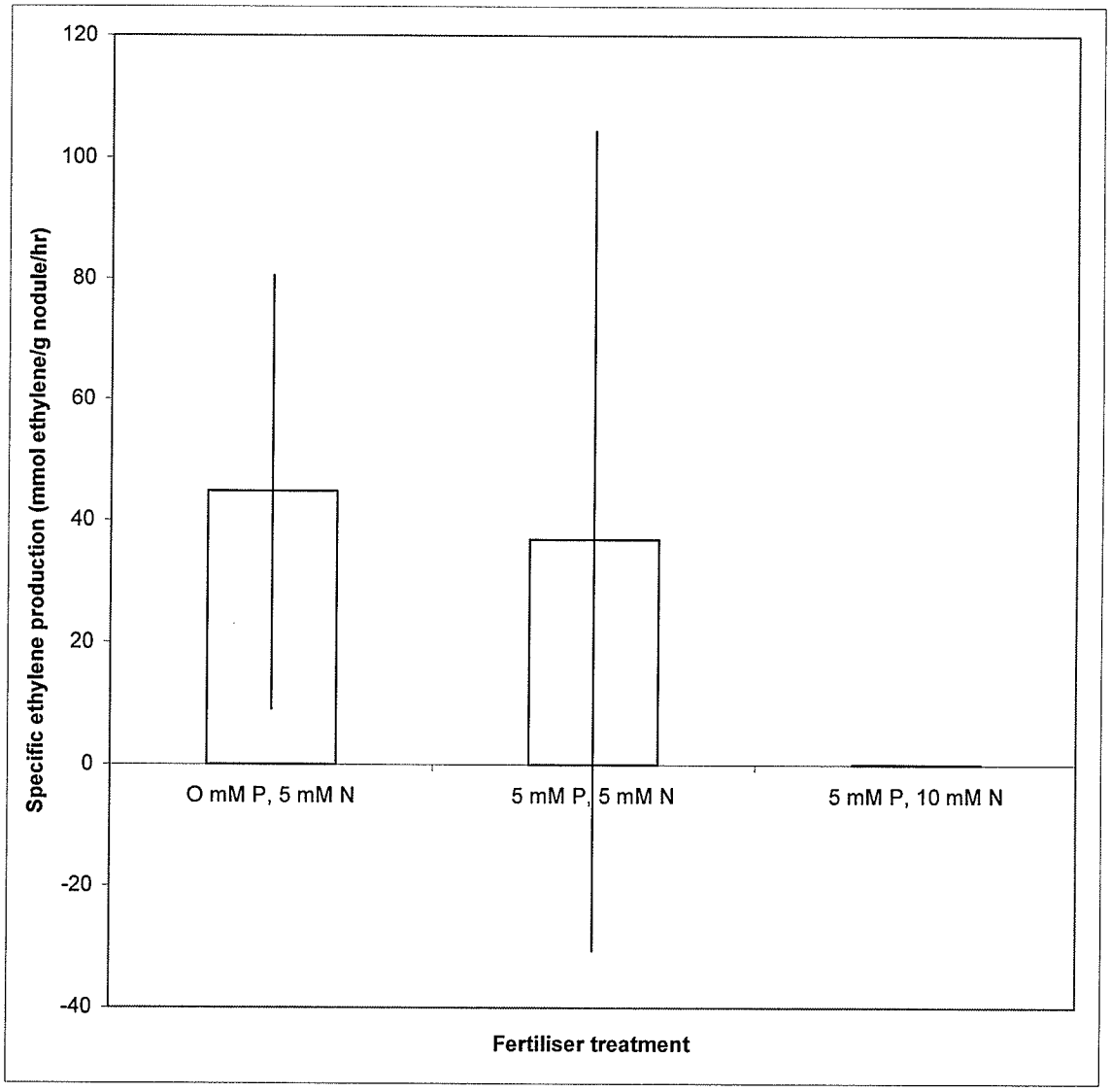

Figure 3.10. Specific ethylene production reflecting nitrogenase activity for nodulated *Alnus rubra* plants in the different fertiliser treatments (0 mM P, 5 mM N; 5 mM P, 5 mM N; 5 mM P, 10 mM N). Only fertiliser treatments with nodulated plants are shown. Plants in the Frankia and Tripartite treatments are combined in the fertilizer treatments. Values given are means plus or minus standard deviations. There were 4 0 mM P, 5 mM N plants; 4 5 mM P, 5 mM N plants; and 1 5 mM P, 10 mM N plant.

acetylene reduction of plants inoculated with *Frankia* in comparison to plants inoculated with both *Frankia* and *P. involutus* (n=5, 16.713 mmol ethylene/g nodule/h) (F=2.0402, p=0.1963); however, there were only four plants that formed nodules and mycorrhizae and only five plants that formed just nodules. Specific acetylene reduction was not related to shoot dry mass, root dry mass, diameter, height, foliar nitrogen, nodule dry mass, or foliar phosphorus (data not shown). Acetylene reduction per plant was not related to shoot dry mass, root dry mass, diameter, or height (data not shown).

Increasing fertiliser nitrogen levels increased nodule dry mass (Figure 3.11); however, there was only one plant in the 10 mM nitrogen fertiliser treatment that produced nodules. Neither the presence of mycorrhizae (n= 5, 0.0134 g +/- 0.0217 for Frankia plants; n=4, 0.0105 g +/- 0.00794 for Tripartite plants) nor any of the three phosphorus fertiliser levels (Figure 3.11) did not had an effect on nodule dry mass although again, there were only nine nodulated plants in this experiment.

Nodule dry mass was positively related to shoot dry mass ($R^2 = 0.799204$, $p = 0.0012$), root dry mass ($R^2 = 0.729846$, $p = 0.0034$),and total nitrogen (Figure 3.12). Nodule dry mass was not related to foliar nitrogen, height (Figure 3.13), diameter, or foliar phosphorus (data not shown).

Mycorrhizae had no effect on the percent nodule dry mass (Frankia plants n=5, 1.877% +/- 1.653, Tripartite plants n=4, 1.858 % +/- 0.993; F=0.0004, p=0.9843). Plants that were given 10 mM nitrogen had higher percent nodule dry mass than plants that were given 5 mM nitrogen (Figure 3.14). Phosphorus had no effect on percent nodule dry mass (Figure 3.14).

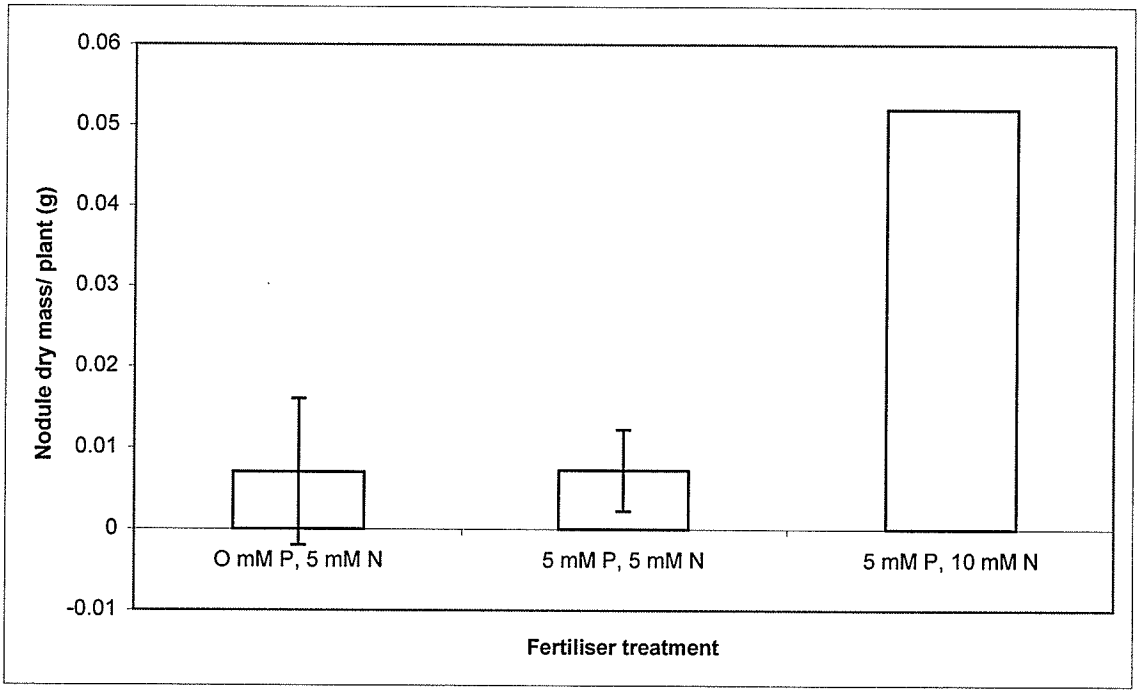

Figure 3.11. Nodule dry mass for *Alnus rubra* plants in the fertiliser treatments (0 mM P, 5 mM N; 5 mM P, 5 mM N; 5 mM P, 10 mM N) that formed nodules. Only fertiliser treatments with nodulated plants are shown. Microbial combinations (Control, Frankia, Paxillus, and Tripartite) are combined within the fertiliser treatments because all four microbial treatments follow the same trend. Values given are means plus or minus standard deviations. There were 4 0 mM P, 5 mM N plant ; 4 5 mM P, 5 mM N plants; and 1 5 mM P, 10 mM N plant.

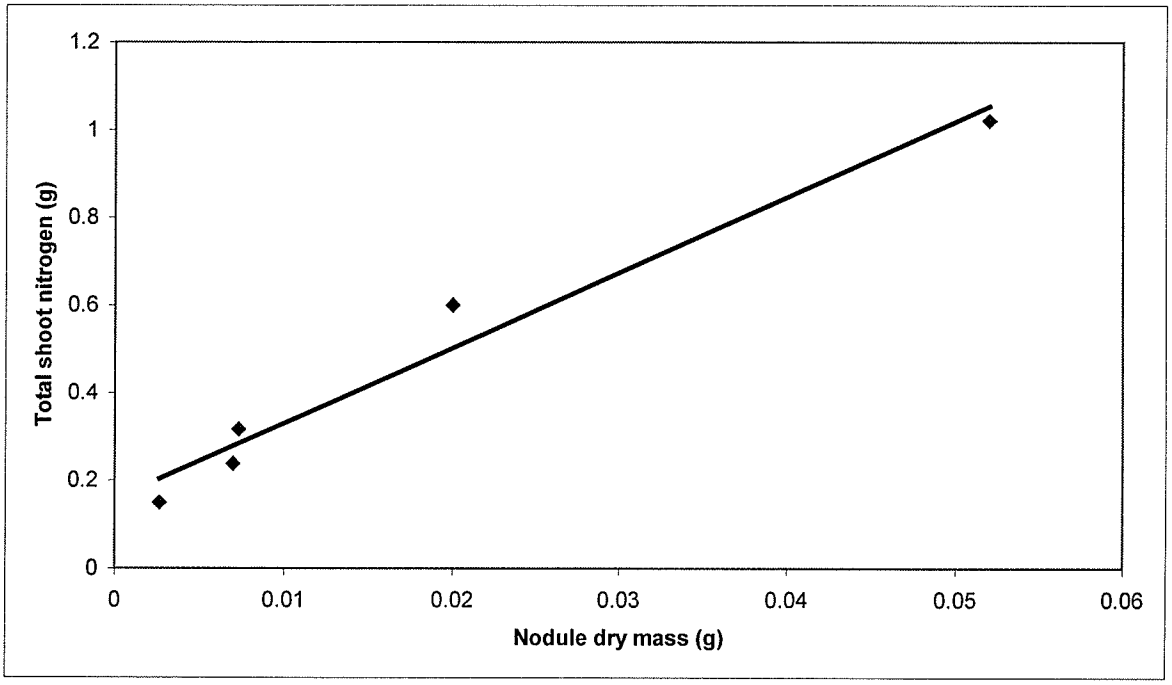

Figure 3.12. The relationship between total shoot nitrogen (g) and nodule dry mass (g) for *Alnus rubra* plants. Values shown are means for a given fertiliser/microbial treatment. The sample size for the treatments ranges from 1 to 3. The R^2 value was 0.94378 and the p value was <0.0001.

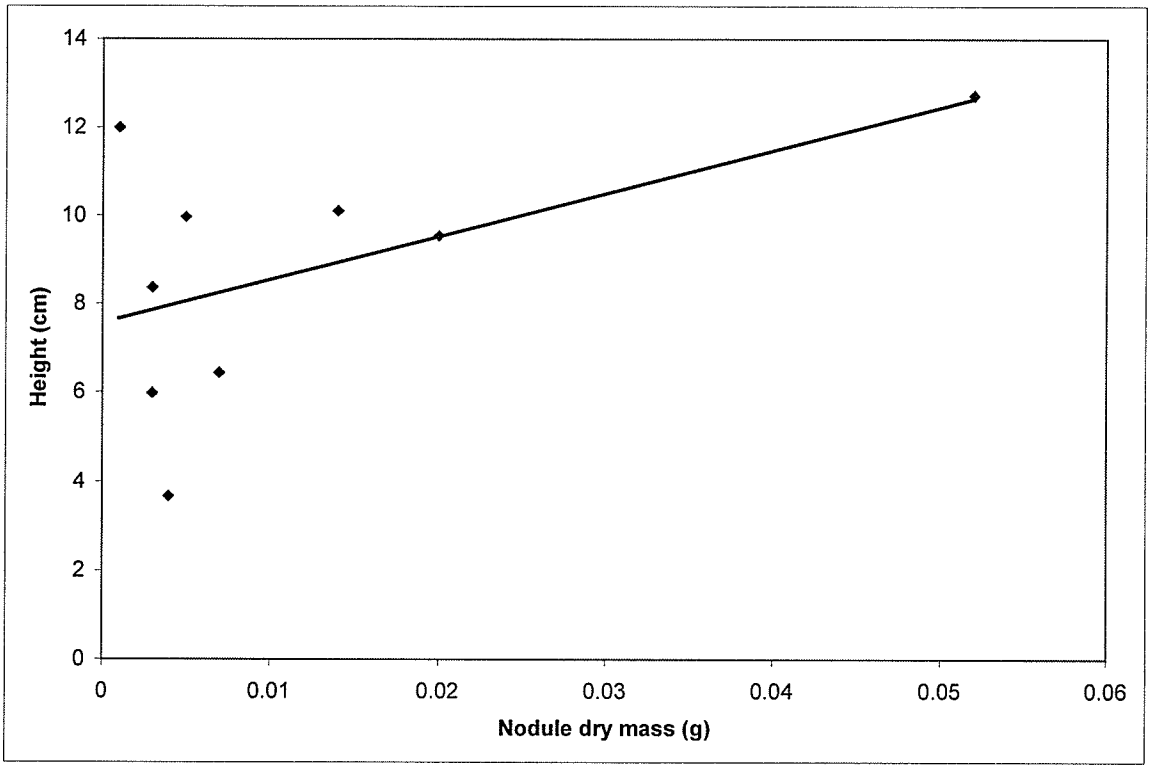

Figure 3.13. The relationship between height (cm) and nodule dry mass (g) of *Alnus rubra* plants. Values shown are means for a given fertiliser/microbial treatment. The sample size for the treatments ranges from 1 to 3. The R^2 value was 0.288454 and the p value was 0.112.

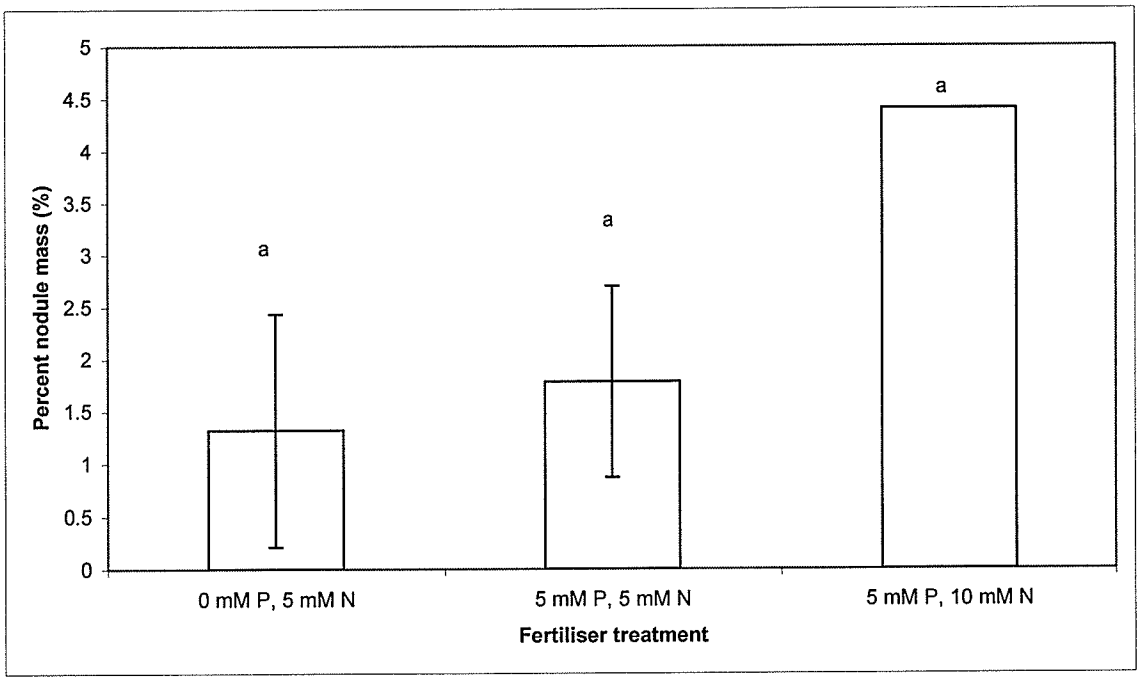

Figure 3.14. The percent nodule dry mass of total plant dry mass of *Alnus rubra* plants colonised by microbes in the fertilser treatments (0 mM P, 5 mM N; 5 mM P, 5 mM N; 5 mM P, 10 mM N) that formed nodules. Microbial treatments (Control, Frankia, Paxillus, Tripartite) are combined within the fertiliser treatments because all four microbial treatments follow the same trend. Values given are means plus or minus standard deviations. There were 4 0 mM P, 5 mM N plants, 4 5 mM P, 5 mM N, and 1 5 mM P, 10 mM N plant.

3.3d Foliar nitrogen and phosphorus

None of the three nitrogen fertiliser concentrations or the three phosphorus fertiliser concentrations had an effect on foliar nitrogen concentrations (Figure 3.15). There was a non significant trend for nodulated plants to have higher foliar nitrogen concentrations compared to non nodulated plants($F=2.9079$, $p=0.1204$) but the presence of mycorrhizae had no effect on foliar nitrogen concentrations (Figure 3.16). Foliar nitrogen is positively related to shoot mass (Figure 3.17) and diameter (diameter = 0.606 + 1.107 *foliar nitrogen, $R^2=0.380137$, $p=0.0576$) but is not related to root mass, height, or foliar phosphorus (data not shown).

Phosphorus fertiliser concentrations of 0 mM reduced foliar phosphorus concentrations ($F=4.4200$, $p=0.0426$) to approximately one third of the foliar phosphorus concentrations of the other treatments but fertiliser nitrogen concentrations did not have an effect on foliar phosphorus levels (Figure 3.18). Neither mycorrhizae nor *Frankia* had an effect on foliar phosphorus levels ($F=1.0370$, $p=0.3933$) (Figure 3.19). Foliar phosphorus was not related to shoot dry mass, root mass, height, diameter, specific acetylene reduction, or nodule dry mass (data not shown).

3.4 Discussion

3.4a Colonisation

Mycorrhizae

Neither fertiliser nitrogen nor phosphorus had an effect on mycorrhizal colonisation. These findings support the findings of Johnson (1993) that fertiliser phosphorus had no effect on mycorrhizal colonisation. However, the majority of research shows that fertiliser nutrients do have an effect on mycorrhizal colonisation. Fertiliser phosphorus has been shown to decrease mycorrhizal colonisation at concentrations of 0.5

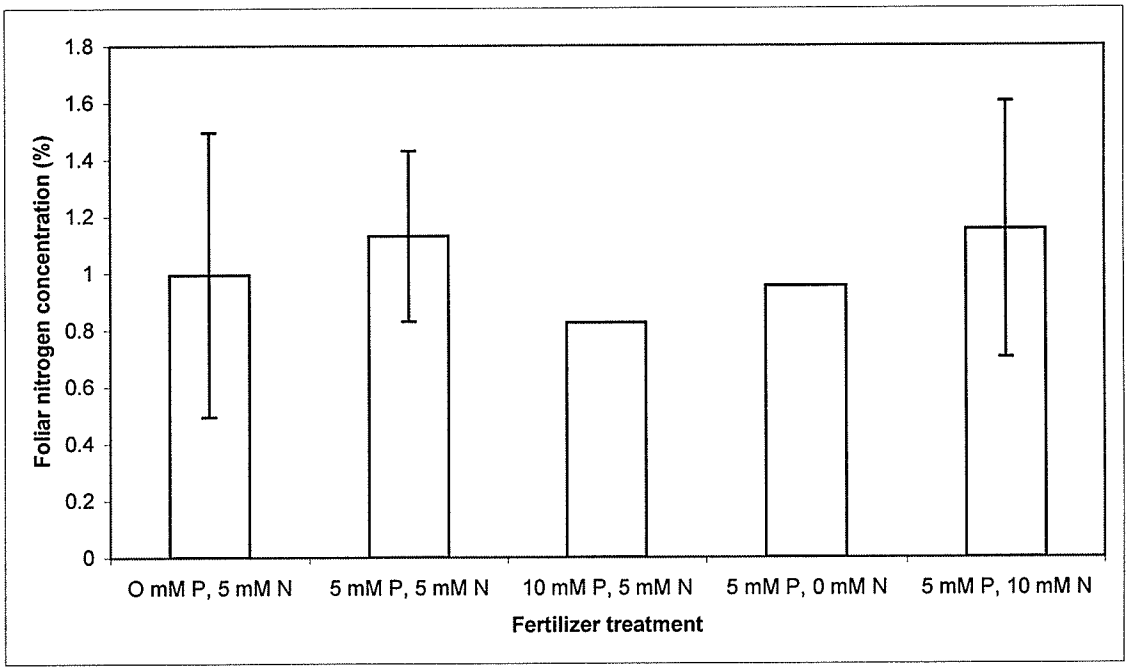

Figure 3.15. The foliar nitrogen concentrations of *Alnus rubra* plants colonised by microbes in the five fertiliser treatments (0 mM P, 5 mM N; 5 mM P, 5 mM N; 10 mM P, 5 mM N; 5 mM P, 0 mM N; 5 mM P, 10 mM N). Microbial combinations (Control, Frankia, Paxillus, and Tripartite) are combined within the fertiliser treatments because all four microbial treatments follow the same trend. Values given are means plus or minus standard deviations. There were 3 0 mM P, 5 mM N plant ; 3 5 mM P, 5 mM N plants; 1 10 mM P, 5 mM N plants; 1 5 mM P, 0 mM N plants; and 2 5 mM P, 10 mM N plants.

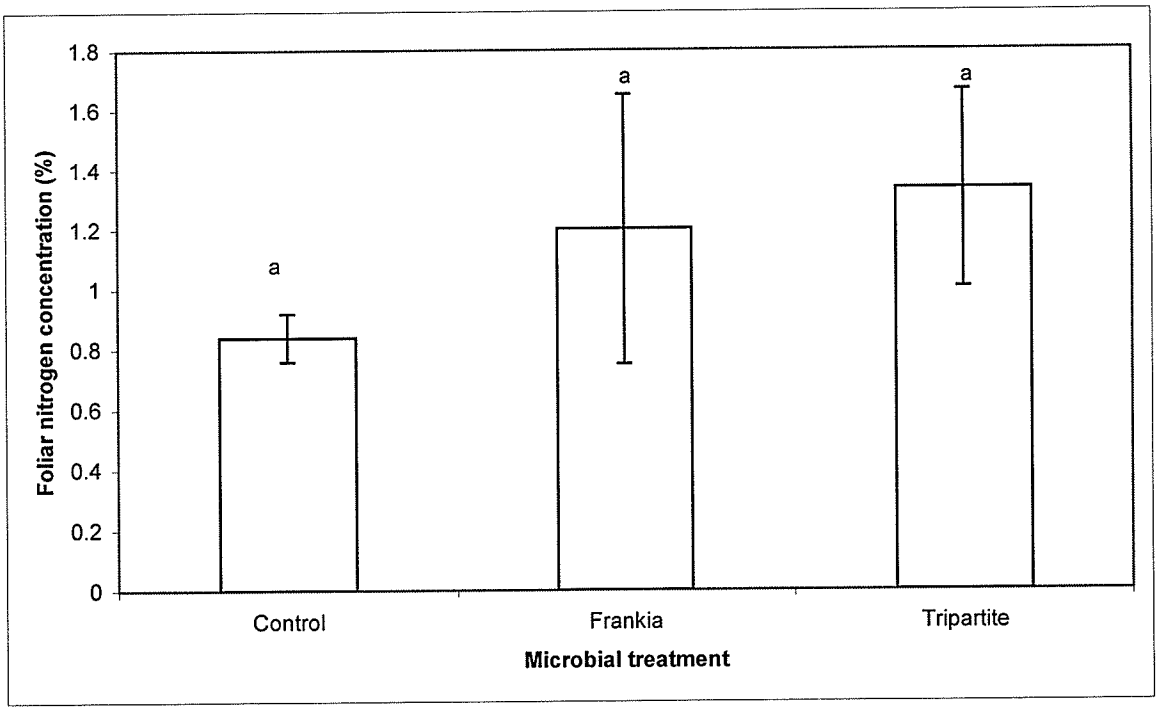

Figure 3.16. The foliar nitrogen concentrations of inoculated *Alnus rubra* plants colonised by microbes in the microbial treatments. Control = plants not inoculated with endosymbionts; Frankia = plants inoculated with *Frankia*; Tripartite = plants inoculated with *Frankia* and *Paxillus involutus*. Values given are means plus or minus standard deviations. Fertiliser treatments (0 mM P, 5 mM N; 5 mM P, 5 mM N; 10 mM P, 5 mM N; 5 mM P, 0 mM N; 5 mM P, 10 mM N) are combined within microbial treatments. Treatments with the same letters were not significantly different at the 0.05 level according to a Tukey *post hoc* test. There were 5 Control plants, 3 Frankia plants, and 2 Tripartite plants.

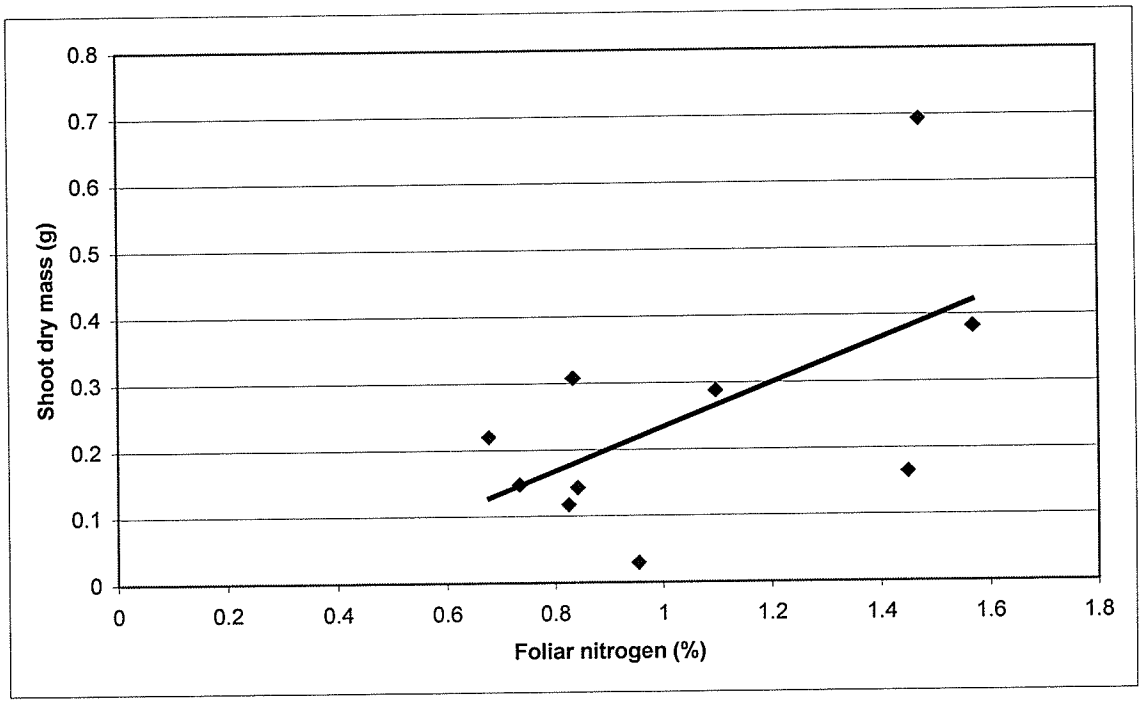

Figure 3.17. The relationship between foliar nitrogen concentrations and shoot masses (g) of nodulated *Alnus rubra* plants in treatments inoculated with *Frankia*. Data points shown are the means for fertiliser/microbial treatments. The sample size for the treatments ranges from 1 to 27. The equation for the regression line is $y = 0.3293x - 0.0953$. The R^2 value was 0.342 and the p value was 0.0754. There were 10 treatments included in this analysis.

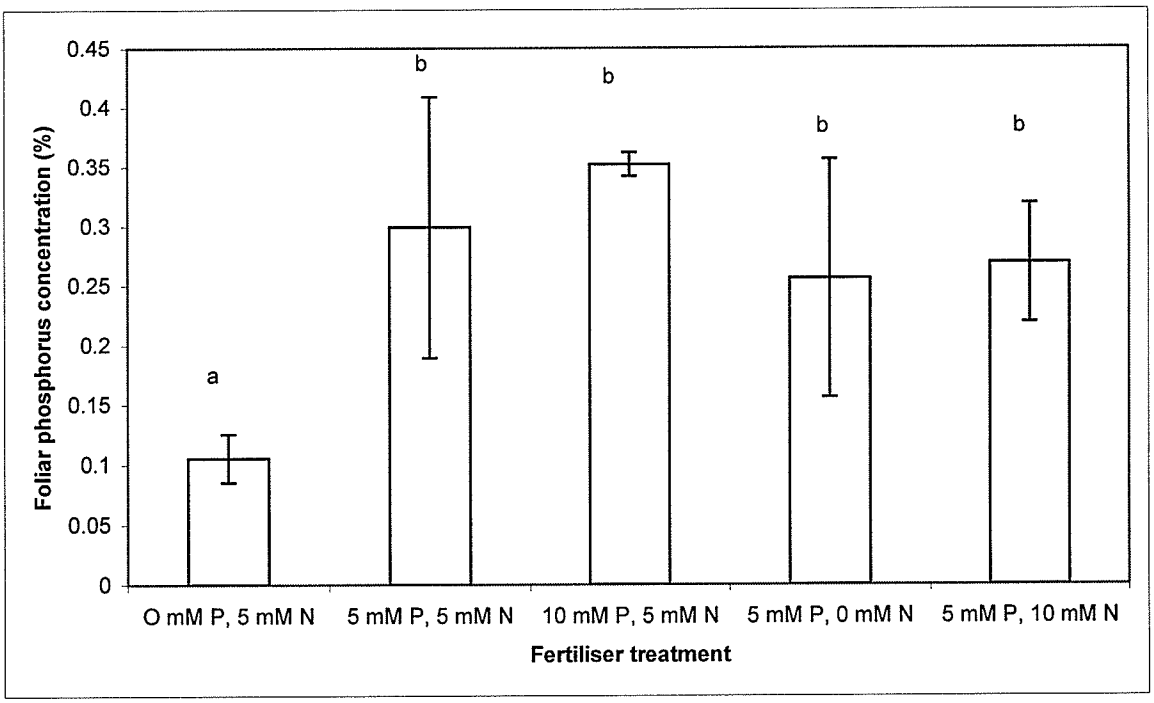

Figure 3.18. The foliar phosphorus concentrations of *Alnus rubra* plants colonised by microbes in the five fertiliser treatments (0 mM P, 5 mM N; 5 mM P, 5 mM N; 10 mM P, 5 mM N; 5 mM P, 0 mM N; 5 mM P, 10 mM N). Microbial treatments (Control, Frankia, Paxillus, and Tripartite) are combined within the fertliser treatments. Treatments with different letters are significantly different at the 0.05 level according to a Tukey *post hoc* test. Values given are means plus or minus standard deviations. There were 3 0 mM P, 5 mM N plants; 3 5 mM P, 5 mM N plants; 2 10 mM P, 5 mM N plants; 2 5 mM P, 0 mM N plants; and 2 5 mM P, 10 mM N plants.

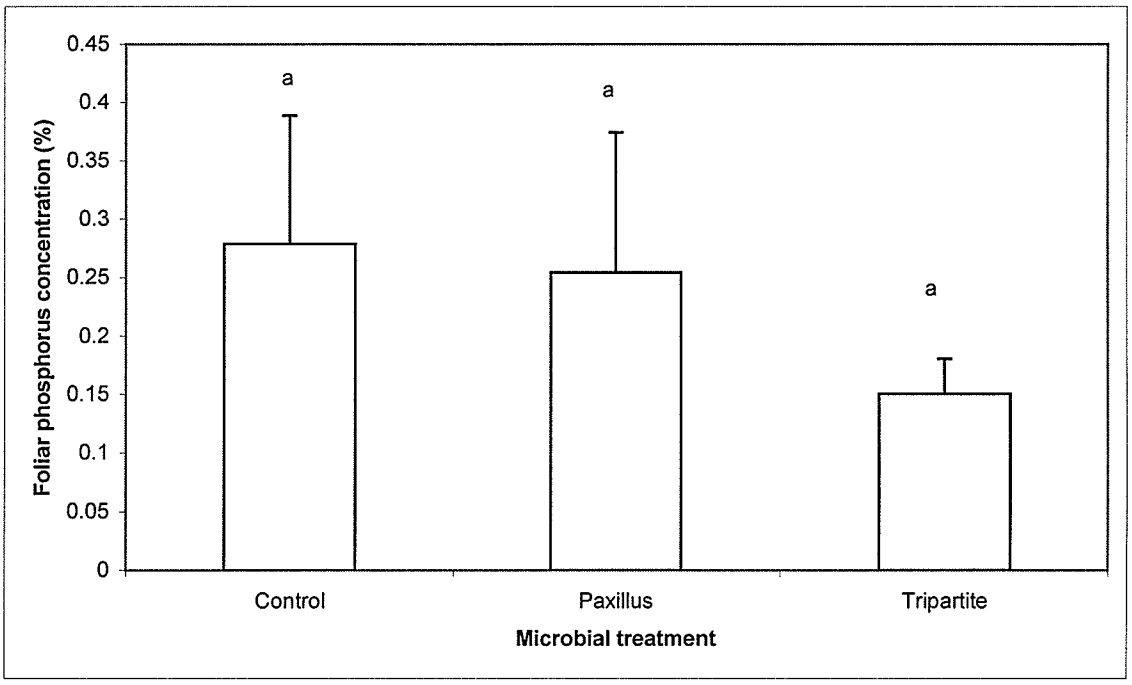

Figure 3.19. Foliar phosphorus concentrations of *Alnus rubra* plants in the treatments inoculated with *Paxillus involutus*. Control = plants not inoculated with endosymbionts; Paxillus = plants inoculated with *Paxillus involutus*; Tripartite = plants inoculated with *Frankia* and *Paxillus involutus*. Fertiliser treatments (0 mM P, 5 mM N; 5 mM P, 5 mM N; 10 mM P, 5 mM N; 5 mM P, 0 mM N; 5 mM P, 10 mM N) are combined within microbial treatments. Values given are means plus or minus standard deviations. There were 5 Control plants, 5 Paxillus plants, and 2 Tripartite plants. Treatments with the same letters are not significantly different at a 0.05 level according to a Tukey *post hoc* test.

and 1.0 g KH_2PO_4/ kg soil (Asimi *et al.*, 1980), 0.6 and 1.2 g P per pot (Robson *et al.*, 1981), 75 and 150 kg P/ha (Roldan-Fajardo *et al.*, 1982) 15.2 and 30.4 mg P/pot (Armstrong *et al.*, 1992), 60, 120, and 240 kg P/ha (Jha *et al.*, 1993), 54.0 mgP/kg soil (Ekblad *et al.*, 1992) and 50 kg P/ha (Baum and Makeschin, 2000). In all these studies, the phosphorus levels that decreased the mycorrhizal colonisation were the higher levels of fertiliser phosphorus applied to the plants in the experiment. Nitrogen fertiliser has been shown to both increase (Johnson, 1993) and decrease (Baum and Makeschin, 2000) mycorrhizal colonisation. The lack of fertiliser phosphorus or nitrogen effects in this experiment could be due to the lack of mycorrhizal development. Plants were considered mycorrhizal if hyphae were seen in the root. The mycorrhizae did not form mantles or Hartig nets, but cell wall thickening was visible within the roots. It is possible that mycorrhizal fungi can invade plant roots at all phosphorus and nitrogen levels but that the nutrient levels in this experiment prevent the full development of mycorrhizae. If that were the case, phosphorus and nitrogen would have no effect on colonisation by individual hyphae, which was consistent with observations made in my experiment, but they would still decrease colonisation by well developed mycorrhizae, which is consistent with observations made in the above experiments.

The lack of well developed mycorrhizae suggests two possible explanations for a lack of mycorrhizal effect. First, the mycorrhizae may not have been developed enough to have an effect because the three-month duration of the experiment might not have been sufficiently long enough to allow for mycorrhizal development. Robson *et al.* (1981) found that the effect of mycorrhizae over time increases, presumably due to an increase in mycorrhizal development. Given more time for the mycorrhizae to develop, a

mycorrhizal effect may have been seen. Repeating this experiment using plants with fully developed mycorrhizae or quantifying the mycorrhizal colonisation would likely strengthen conclusions drawn from this experiment. Second, mycorrhizae may not have had an effect on the particular plant measurement used in this study (e.g. shoot diameter). Research has shown that mycorrhizae do not always have an effect on all plant measurements (Asimi *et al.*, 1980; Bethlenfalvay and Yoder, 1981; Rose and Youngberg, 1981; Jones *et al.*, 1990; Armstrong *et al.*, 1992). Further work is needed to determine at what stage of development mycorrhizae have an effect on host plants.

The mycorrhizae in my experiment were not well developed. Chatarpaul *et al.* (1989) inoculated *Alnus incana* with *Frankia*, VAM fungi, and *Paxillus involutus*. After 10 weeks, they found mycorrhizae in the roots of the *A. incana* plants. Molina (1979) examined mycorrhizal formation by various fungal species on *Alnus rubra* and found mycorrhizae after six months. My experiment ran for 12 weeks, after which time, I found no mantle or Hartig net formation. It is possible that *A. rubra* requires more time than *A. incana* to form mycorrhizae and as such, had I let my experiment run for a longer period of time, I would have seen more well-developed mycorrhizae.

In my experiment, *Frankia* did not have an effect on mycorrhizal colonisation. This finding contradicts other research, which has shown that *Frankia* increase mycorrhizal colonisation (Rose and Youngberg, 1981; Chatarpaul *et al.*, 1989; Jha *et al.*, 1993; Tian *et al.,* 2000). There were nine nodulated plants in my experiment and as such, it is not surprising that there was no *Frankia* effect on mycorrhizal colonisation. Repeating this experiment with more nodulated plants and allowing the experiment to run

longer would allow the *Frankia* to have an effect on the mycorrhizal colonisation if an effect were present.

Frankia

Frankia colonisation rates in this experiment were low and as there were very few nodulated plants, drawing any concrete conclusions regarding the nodulated plants is difficult. Cusato and Tortosa (1998) found that nodulation rates can vary widely depending on the species of host involved in the experiment. It is possible that the *Frankia* nodule used as inoculant in my experiment has a low infectivity on the host used. In my experiment, 0, 5, and 10 mM nitrogen concentrations were used. It is possible that the plants receiving 0 mM nitrogen were too nitrogen stressed to form nodules, while the plants receiving 5 and 10 mM nitrogen had a sufficient or overabundant amount of nitrogen eliminating the need to form nitrogen-fixing nodules. Nitrogen fertiliser has been shown to decrease *Frankia* colonisation (Stewart and Bond, 1961; Huss-Danell, 1997; Thomas *et al.*, 2000). Stewart and Bond (1961) used nitrogen levels that were equivalent to 0, 0.5, 2.8, and 5.5 mM nitrogen and found that at the equivalent of 2.8 and 5.5 mM nitrogen, nodulation decreases. It is possible that the nitrogen concentration in my experiment decreased the nodule formation at the 5 and 10 mM concentrations to the point where very few nodules formed. As such, it is possible that it appears as though there was no nitrogen effect on colonisation when in fact, the 5 mM nitrogen had such an inhibitory effect that any inhibitory effect at 10 mM was not noticeable. In addition, it is possible that the nitrogen concentrations in my experiment were high enough that the plants did not require fixed nitrogen to supply their nitrogen requirements. The low nodulation rates in this experiment make drawing conclusions from the data difficult.

Repeating this experiment with more nodulated plants would help determine if 5 mM nitrogen prevents colonisation to the scale it appears to in this experiment or whether that effect is just a result of the overall lack of colonisation. Repeating this experiment with lower nitrogen concentrations may increase nodulation by eliminating the inhibitory effect of high nitrogen on nodulation.

3.4b Plant size

From the plant responses to fertiliser, it is clear that 10 mM nitrogen has a positive effect on alder growth while 0 mM nitrogen has a negative effect on alder growth. Nitrogen has been shown to increase the dry mass of *Pisum sativum* (Bethlenfalvay *et al.*, 1978) and *Alnus glutinosa* (Stewart and Bond, 1961) and to have no effect on the shoot mass of *Myrica gale* (Stewart and Bond, 1961) and *Alnus incana* (Ekblad *et al.*, 1995). The lack of a nitrogen effect on *M. gale* is due to the ability of the host to switch to using fixed nitrogen when nitrogen fertiliser concentrations are low (Stewart and Bond, 1961). Fertiliser containing 10 mM nitrogen significantly increased shoot mass of *Gliricidia sepium* but had no effect on root mass (Thomas *et al.*, 2000). The application of nitrogen fertiliser appears to cause a shift in plant allocation patterns as nitrogen stimulates shoot growth but not root growth (Salisbury and Ross, 1992). In my experiment, plants that were given 0 mM nitrogen had the lowest root:shoot ratio while there was no difference in root:shoot ratio between plants given 5 mM and 10 mM nitrogen. When plants receive a high nitrogen fertiliser, more resources are allocated to shoot growth because the abundance of nitrogen means less root mass is required to obtain the requisite level of nitrogen. As shoot mass increases, though, root mass has to increase to support the shoot growth, but overall, the percent allocation of resources to

the roots will not increase. Johnson (1993) found that nitrogen fertiliser had an effect on mycorrhizal *Andropogon gerardii* but not on non-mycorrhizal *A. gerardii.* In my experiment, nitrogen fertiliser may not have had an effect on mycorrhizal plants because the mycorrhizae were not well developed.

Phosphorus levels in this experiment did not have an effect on plant size but it did have an effect on root:shoot ratio. Plants given 10 mM or 0 mM phosphorus had higher root:shoot ratios than plants given 5 mM phosphorus, suggesting that high or low phosphorus concentrations increase root mass. Increasing phosphorus concentrations increase root mass (Salisbury and Ross, 1992), but it is unclear why low phosphorus concentrations would increase the root:shoot ratio. The findings of my experiment support the findings of Sanginga *et al.* (1989) who found that 0, 30, 60, and 90 mg phosphorus/kg soil had no effect on *Allocasuarina littoralis* and *Allocasuarina torulosa.* The findings of my study regarding the effect of phosphorus on plant size contradict the majority of the research, though. Increasing phosphorus fertiliser concentrations have been shown to increase total *Alnus incana* biomass (6.00 and 54.0 mg P/kg soil) (Ekblad *et al.*, 1995), *Trifolium repens* shoot dry mass (0, 22.5, and 90 kg P/ha) (Hayman and Mosse, 1979), *Glycine max* shoot dry mass (4, 20, 100, and 500 μM phosphorus) (Bethlenfalvay and Yoder, 1981), *Glycine max* shoot dry mass (0, 0.2, 0.4, and 1.0 mM phosphorus) (Pacovsky *et al.*, 1986-a), and *Sorghum bicolor* (0, 0.2, 0.4, and 1.0 mM phosphorus) (Pacovsky *et al.,* 1986-a). Increasing phosphorus fertiliser concentrations have also been shown to increase the root and shoot dry mass of *Glycine max* (0, 0.25, 0.5, and 1.0 g KH_2PO_4/kg soil) (Asimi *et al.*, 1980), *Prunus dulcis* (0, 75, and 150 kgP/ha) (Roldan-Fajardo *et al.*, 1982), and *Alnus tenuifolia* (Uliassi *et al.*, 2000).

The lack of a phosphorus effect in my experiment is a result of the phosphorus concentrations that were used. Phosphorus fertiliser concentrations have been shown to increase shoot dry mass at low concentrations but not at high concentrations in *Trifolium subterraneum* (0.1, 0.2, 0.6, and 1.2 g P/pot) (Robson *et al.,* 1981), *Glycine max* (0, 0.1, 0.25, 0.5. 1.0, and 2.0 mM P) (Israel, 1987), *Salix viminalis* (4, 6, 10, 21, 60, and 90 mg P/kg soil) (Jones *et al.*, 1990), *Aristidia armata* (0, 4, 8, and 12 kg P/ha) (Armstrong *et al.*, 1992), *Cenchrus ciliaris* (0, 6, 12, and 24 kg P/ha) (Armstrong *et al.*, 1992), *Digitaria ammophilla* (0, 4, 8, and 12 kg P/ha) (Armstrong *et al.*, 1992), and *Thyridolepis mitchelliana* (0, 4, 8, and 12 kg P/ha) (Armstrong *et al.*, 1992). There are several different ways of measuring phosphorus application, making a comparison between studies difficult. There were three studies that used the same units of concentration (mM) as this experiment and of those studies, two (Bethlenfalvay and Yoder, 1981; Pacovsky *et al.*, 1986-a) demonstrated a positive effect of phosphorus fertiliser on shoot mass while the remaining one (Israel, 1987) demonstrated that increasing phosphorus beyond 0.8 mM had no effect on shoot mass. Both the studies that showed a positive effect of phosphorus were below the 0.8 mM maximum found by Israel. It appears as though increasing phosphorus concentrations up to 0.8 mM have a positive effect on shoot mass; therefore, studies that examine phosphorus concentrations below 0.8mM will find that phosphorus has a positive effect while those above 0.8 mM will find phosphorus has no effect. The three phosphorus fertiliser concentrations used in this study were 0, 5mM, and 10mM, two of which were greater than the 0.8 mM maximum concentration Israel (1987) found. As such, it possible that the concentration of the fertiliser was high enough that it had no effect on plant size in my experiment. The phosphorus concentration in the

conetainers was not constant, however, because the application of fertiliser three times a week increased the phosphorus concentration in the root material and the watering system washed the phosphorus out of the rooting medium.

In my experiment, there was no significant decrease in the treatment with 0 mM phosphorus. Since 0 mM phosphorus is below the 0.8 mM threshold limit Israel (1987) found, it should have decreased the plant mass. The plants in my experiment were fertilised with fertiliser containing 1.41 mM P and 12.43 mM N prior to inoculation. It is possible that the phosphorus provided in that fertiliser was enough to sustain the plants throughout the experiment and as such, a lack of phosphorus during the experiment would not have had an effect. Once these previously-fertilised plants were given fertiliser without phosphorus, it is possible that they were transporting the phosphorus from the older leaves to the younger leaves in response to the lack of phosphorus. Plants given 0 mM phosphorus had less foliar phosphorus than plants in any of the other fertiliser treatments so either the plants were transporting what little phosphorus they had within the plant and consequently growth was not affected or the lack of phosphorus was enough to decrease the foliar phosphorus levels but not sufficient to decrease growth.

In this experiment, plants with *Frankia* nodules were larger than plants without *Frankia* nodules. The growth increase in nodulated plants was a result of the nitrogen fixed in the nodules. Plants with nodules were able to fix nitrogen and since nitrogen increases plant growth, plants with nodules were bigger than plants without nodules. Free-living *Frankia* do not fix nitrogen (Sprent *et al.*, 1987) and so only plants that are inoculated with *Frankia* and form nodules will experience an increase in growth because only these plants experience an increase in available nitrogen due to the nitrogen-fixing

bacteria in the nodules. The presence of nodules was shown to increase plant growth for *Glycine max* (Carling *et al,* 1978), *Alnus glutinosa* (Houwers and Akkermans, 1981), *Ceanothus velutinus* (Rose and Youngberg, 1981), *Casuarina equisetifolia* (Gardner, 1986), *Alnus incana* (Chatarpaul *et al.,* 1989), and *Alnus cordata* (Isopi *et al.*, 1994). The nitrogen provided by the *Frankia* seems to cause an increase in leaf mass only since shoot mass increased in inoculated plants but height, diameter, and root mass did not increase. Dawson and Gordon (1979) demonstrated nitrogen and plant growth are related in a positive feedback loop. Leaf area increases nitrogen fixation, which increases the accumulated nitrogen per plant, which then increases plant photosynthesis, which in turn increased plant growth.

In this experiment, mycorrhizal colonisation did not have an effect on plant growth. As has been previously stated, there are two possible explanations for this lack of mycorrhizal effect: either the mycorrhizae were not developed enough to have an effect or there is no mycorrhizal effect. The lack of a mycorrhizal effect in this study supports the findings of Jones *et al.* (1990) and Armstrong *et al.* (1992). The majority of studies contradict the findings of this experiment, though, as mycorrhizae have been shown to increase the mass of *Trifolium repens* (Hayman and Mosse, 1979), *Glycine max* (Asimi *et al.*, 1980), *Trifolium subterraneum* (Robson *et al.*, 1981), *Prunus dulcis* (Roldan-Fajardo *et al.*, 1982), *Andropogon gerardii* (Johnson, 1993), and *Casuarina sp.* (Wheeler, 2000). Research has also shown that as the number of endosymbionts increases, the host mass increase (Rose and Youngberg, 1981; Gardner *et al.*, 1984, Chatarpaul *et al.*, 1989; Jha *et al.*, 1993; Tian, 2002). Chatarpaul *et al.* (1989) found that *Paxillus involutus* does increase the growth of *Alnus rubra.* Given the overwhelming

evidence that mycorrhizae do increase plant growth, and the fact that a mycorrhizal effect has been found in the same host/fungal system, the mycorrhizae in this experiment were more likely not developed enough to have an effect on plant growth.

In this experiment, the lag time between treatment application and noticeable effects of the treatment was larger for the microbial treatments than for the fertiliser treatments. The microbial treatments had a longer lag time because the microbe had to colonise the host and then start supplying the host with either the nitrogen or the phosphorus, which would take longer to occur than increased growth due to an increase in soil nutrients. The lag times between treatment application and treatment effect explain the differences seen in the relative growth rates. The growth rates of the five fertiliser treatments were higher in the earlier part of the experiment than in the later part while the growth rates of the four microbial treatments were higher in the later part of the experiment. The fertiliser treatments had an earlier effect than the microbial treatments and as such, would have higher growth rates earlier in the experiment than the microbial treatments. Ingestad (1981) found that as the nitrogen levels applied to *Betula verrucosa* and *Alnus incana* increased, the relative growth rate also increased. He also found that as the experiment progressed, the relative growth rates for the plants decreased. My experiment concurs with Ingestad (1981) in that when plants received a large amount of nitrogen (whether by the nitrogen-fixing bacteria or by the fertiliser), relative growth rates were increased. Plants in my experiment that were fertilised with the 5 mM phosphorus, 5 mM nitrogen fertiliser had high relative growth rates during the early part of the experiment, suggesting that 5 mM phosphorus is the optimum concentration in this experiment for plant growth.

3.4c Nitrogen fixation

Ethylene production

This experiment found that neither phosphorus nor nitrogen fertiliser concentrations had an effect on specific acetylene reduction. Nitrogen has been shown to have no effect on the specific acetylene reduction of *Myrica gale* (0, 10, 50, 100 mg NH_4/pot) (Stewart and Bond, 1961) while increasing nitrogen concentrations have been found to decrease specific acetylene reduction of *Alnus glutinosa* (0, 10, 50, 100 mg NH_4/pot) (Stewart and Bond, 1961) and *Gliricidia sepium* (0, 1, and 10 mM nitrogen) (Thomas *et al.*, 2000). Stewart and Bond (1961) used small concentrations of nitrogen, which could explain why there was no effect on *Myrica gale* since Thomas *et al.* (2000) found low concentrations of nitrogen (e.g. 1 mM) do not actually have an effect on nitrogenase activity.

My experiment found there was no phosphorus effect on specific acetylene reduction rates. This finding agrees with the findings that phosphorus has no effect on the specific acetylene reduction rates of *Pisum sativum* (0, 15, 30, 60, and 120 mg P/kg growth medium) (Jakobsen, 1985), and *Casuarina equisetifolia*, *Allocasuarina littoralis,* and *Allocasuarina torulosa* (0. 30. 60, and 90 mg P/kg soil) (Sanginga *et al.*, 1989). Most other research, however, shows that phosphorus increases specific nitrogenase activity. Jha *et al.* (1993) found that as phosphorus fertiliser concentrations increased through 0, 30, 60, 120, and 240 kg P/ha, specific acetylene reduction also increased. Russo (1989) found that *Alnus acuminata* inoculated with *Frankia* had low acetylene reduction at high (100 ppm P) and low (10ppm P) fertiliser phosphorus concentrations and *A. acuminata* inoculated with both *Frankia* and *Glomus intra-radices* had decreased acetylene reduction as phosphorus fertiliser concentrations increased from 10 ppm to 50

ppm. The concentrations of phosphorus in these two studies are not comparable (kg/ha *vs.* ppm), which makes explaining the different phosphorus effects difficult. It is likely that the difference in response is due to differences in the concentrations of phosphorus used in the two experiments. Work by Israel (1987) suggests that above 0.8 mM, phosphorus has no effect on plants. It is possible that experiments showing a negative phosphorus effect were applying less than 0.8 mM phosphorus. Again, this experiment did not have enough nodulated plants to confirm these conclusions.

In this experiment, there was a non-significant trend where mycorrhizae increased specific ethylene production. Carling *et al.* (1978), Robson *et al.* (1981), Rose and Youngberg (1981), Gardner *et al.* (1984), Gardner (1986), and Jha *et al.* (1993) found that the presence of mycorrhizae increase nitrogenase activity. Wheeler *et al.* (2000) found that, in general, mycorrhizae increase acetylene reduction but there were host/endosymbiont combinations that did not follow this trend. The increase in nitrogenase activity is attributed to the increase in phosphorus provided to the nodules (Robson *et al.*, 1981; Rose and Youngberg, 1981;Gardner *et al.*, 1984; Ianson and Linderman, 1993).

Despite the non-significant increase in acetylene reduction rates in plants inoculated with both *P. involutus* and *Frankia*, there was no difference between the mycorrhizal and non-mycorrhizal *Frankia* nodulated treatments in terms of nodule dry mass. Because the total fixed nitrogen is a function of the nitrogen fixed/g nodule and the dry mass of the nodules, plants inoculated with both endosymbionts fixed more nitrogen than plants inoculated with just *Frankia*. The increase in nitrogen fixation did not result in an increase in either foliar nitrogen or host shoot mass though. The nitrogen

most likely went to the fungus or the nitrogen-fixing bacteria. Further studies using isotopes to trace the fate of the fixed nitrogen would be useful in determining which of the two endosymbionts benefits from this increase in available nitrogen. Gardner *et al.* (1984) also found that an increase in nitrogenase activity due to mycorrhizae did not translate into an increase in plant mass. They suggest that the increase in nitrogenase activity was not accompanied by an increase in photosynthates so growth would not increase. The other explanation is that the mycorrhizae increase nitrogen fixation but the *Frankia* or the mycorrhizae themselves benefit from the increase in nitrogen fixation. Further studies using isotopes to trace the fate of the fixed nitrogen would be useful in determining which of the two endosymbionts benefits from this increase in available nitrogen. Specific nitrogen fixation rates can be variable (Schwintzer, 1990), which could explain why there was no relationship between specific acetylene reduction and any other variables.

Nodule mass

This experiment found that increasing nitrogen fertiliser levels increased nodule mass. This finding contradicts the findings of Bethlenfalvay *et al.* (1978) and Thomas *et al.* (2000) who found that as the concentration of nitrogen fertiliser applied to the plant increases through 1, 2, 4, and 8 mM nitrogen (Bethlenfalvay *et al.*, 1978) and 0, 1, and 10 mM nitrogen (Thomas *et al.*, 2000), nodule dry mass decreases. Phosphorus was found to have no effect on nodule dry mass in this study although previous research has shown a positive relationship between phosphorus fertiliser concentrations and nodule dry mass (Hayman and Mosse, 1979; Asimi *et al.*, 1980; Bethlenfalvay and Yoder, 1981;Robson *et al.*, 1981; Jakobsen, 1985; Sanginga *et al.*, 1989; Uliassi *et al.*, 2000). Since the

nodulation rate for plants in this experiment was low, it is difficult to draw conclusions from this experiment about the effects of fertiliser on nodule dry mass. The reason generally given for the increase in nodulation is that nodulation and nitrogenase activity require a large amount of phosphorus. As the phosphorus concentration in the fertiliser increases, *Frankia* are able to meet that phosphorus requirement and as a result, nodule mass will increase.

Mycorrhizae did not have an effect on nodule dry mass in this experiment and there are three possible reasons for this lack of effect. The first explanation is that mycorrhizae may not be developed enough to have an effect. The second explanation is that mycorrhizae do not have an effect on nodule dry mass. The third explanation is that some unknown environmental parameter was influencing the system. Research has shown that mycorrhizae increase nodule dry mass (Carling *et al.,* 1978; Hayman and Mosse, 1979; Asimi *et al.*, 1980; Rose and Youngberg, 1981; Jha *et al.*, 1993). Ianson and Linderman (1993) and Chatarpaul *et al.* (1989) found that the effect of mycorrhizae on nodule dry mass is species specific. Chatarpaul *et al.* (1989) used *Alnus incana* and *Paxillus involutus*, the same mycorrhizal fungus used in my experiment, and found that mycorrhizae increased the nodule dry mass. Based on the fact that other research has shown that mycorrhizae has an effect and the fact that the mycorrhizal fungus used in my study has been shown to affect nodule dry mass, it seems likely that the mycorrhizae in this experiment were not developed enough to have an effect. Chatarpaul *et al.* (1989) inoculated *Alnus incana* with *Frankia* and *P. involutus* and found mycorrhizal formation after 10 weeks. Molina (1979) found mycorrhizae six months after colonising *Alnus rubra* with *P. involutus*. It is possible that by manipulating the nutrient concentrations in

the fertiliser, the plants were nutrient stressed and as a result, did not fully develop mycorrhizae. Repeating this experiment with less nutrient-stressed plants or letting the experiment run for a longer period of time might have allowed full development of mycorrhizae.

Nodule dry mass is this experiment is related to foliar nitrogen because nodule dry mass is indicative of total nitrogen available to the host if nodule efficiency is constant. Nodule dry mass is also positively related to shoot dry mass and root dry mass. The total nitrogen fixed by the plant is a product of the nodule efficiency (nitrogenase activity/g nodule) and total nodule dry mass. The specific nitrogenase activity in my experiment was constant among treatments. As such, plants with more nodule dry mass will fix more nitrogen than plants with less nodule dry mass. Because nitrogen increases plant growth, plants with more nodule mass will be larger than plants with less nodule mass. This finding supports the finding of Robson *et al.* (1981), who found that shoot mass and nodule mass were positively correlated. Nodule dry mass, and the total nitrogen it represents, increases foliar nitrogen concentrations. It appears, though, as if there is a maximum nitrogen concentration that can be found in the leaves. Beyond that, any additional nitrogen is used to increase plant growth rather than foliar nitrogen.

In my experiment, there was no relationship between nitrogenase activity per plant and any other plant variable (i.e. shoot dry mass, height). The nutrient concentrations in the fertiliser were a confounding variable. Plants that received 10 mM nitrogen fertiliser would have high shoot masses or root masses while at the same time having low nodule dry masses because the nitrogen would increase plant growth while at the same time decreasing nodule mass. As a result, the shoot mass is not a result of the

nitrogenase activity per plant but rather a result of the nitrogen available from the fertiliser. With so few nodulated plants, making conclusions regarding ethylene produced per plant was difficult.

3.4d Foliar nitrogen and phosphorus

Foliar nitrogen

Neither nitrogen nor phosphorus has an effect on foliar nitrogen concentrations in my experiment. Research has shown, however, that both phosphorus (Asimi *et al.*, 1980; Robson *et al.*, 1981; Huss-Danell, 1997; Jakobsen, 1985; Uliassi *et al.*, 2000) and nitrogen (Gates and Wilson, 1974; Bethlenfalvay *et al.*, 1978) increase foliar nitrogen. Foliar nitrogen concentration is expressed as a percent of the leaf mass, so as the nitrogen increased plant growth, the total nitrogen in the shoot may have increased, but the concurrent increase in shoot mass results in a constant foliar nitrogen concentration.

In this experiment, *Frankia* nodules increased foliar nitrogen but mycorrhizae had no effect on foliar nitrogen. There appears to be some disagreement in the literature over whether nodules have an effect on foliar nitrogen concentrations. Rose and Youngberg (1981) found that nodules increased foliar nitrogen while Sanginga *et al.* (1989) found that nodules did not increase foliar nitrogen levels. There is also some disagreement over the effect of mycorrhizae on foliar nitrogen concentrations. Asimi *et al* (1980) found that mycorrhizae do not increase foliar nitrogen while Carling *et al.* (1978) and Robson *et al.* (1981) found that mycorrhizae increased foliar nitrogen. The use of different units and different applications rates of phosphorus application makes comparing the concentrations between the studies difficult. The presence of nodules increases the nitrogen available to nodulated plants and, as a result, nodulated plants will be larger and

have higher total nitrogen levels. Because the increase in total shoot nitrogen in nodulated plants is concurrent with an increase in shoot mass, the foliar nitrogen concentration remains constant. The low numbers of plants with nodules in my experiment makes drawing a conclusion difficult.

Increased fertiliser nitrogen concentrations caused an increase in shoot mass. As such, a positive relationship between shoot mass, diameter, and foliar nitrogen is expected. The lack of a relationship between foliar nitrogen and root mass could be due to the fact that the nitrogen measurements was from leaves and there may not be a relationship between root mass and foliar nutrient concentrations.

The lack of a relationship between foliar nitrogen and foliar phosphorus suggests that the host plant is not switching from one nutrient to the other when one nutrient becomes limited and that the uptake of nitrogen is not related to the uptake of phosphorus. These findings disagree with Israel (1987) who found that whole plant nitrogen increased with whole plant phosphorus but eventually began to plateau.

Foliar phosphorus

A fertiliser concentration of 5 mM appears to be a threshold level of phosphorus in terms of any effect phosphorus has on plants in this experiment. Fertiliser concentrations below that decrease foliar phosphorus levels but beyond that, increasing fertiliser phosphorus does not increase foliar phosphorus. There were only three levels of phosphorus in my experiment, though. Other studies have worked with smaller concentrations of phosphorus and have found that there does indeed appear to be a threshold limit for phosphorus. Israel (1987) found that 0.8 mM was the threshold level of fertiliser phosphorus for *Glycine max*. Jones *et al.* (1990) found that the threshold

fertiliser phosphorus for *Salix viminalis* was less than 1 mM. Hayman and Mosse (1979) found that only the highest level of phosphorus fertiliser, 90 kg P/ha, had any effect on uninoculated plants. It appears as though the concept of a maximum phosphorus level is a robust idea but further research must be carried out in order to determine the maximum level.

Neither *Frankia* nor *P. involutus* had an effect on foliar phosphorus. This finding disagrees with the literature; however, there is disagreement in the literature as to whether *Frankia* and mycorrhizae increase or decrease foliar phosphorus. Frankia (Carling *et al.*, 1978; Sanginga *et al.*, 1989) and mycorrhizae (Asimi *et al.*, 1980; Bethlenfalvay and Yoder, 1981; Rose and Youngberg, 1981) have both been found not to have an effect on foliar phosphorus concentrations. Other studies have shown that both *Frankia* (Rose and Youngberg, 1981) and mycorrhizae (Carling *et al.*, 1978; Jones *et al.*, 1990) increase foliar phosphorus. Bethlenfalvay and Yoder (1981) found that although mycorrhizae have no effect on foliar phosphorus, they did increase total phosphorus. In their experiment, the phosphorus increased plant mass so that the increase in phosphorus was accompanied by a mass increase, meaning that the percent phosphorus remains the same. In this experiment, mycorrhizae did not increase plant mass or the total phosphorus. As a result, foliar phosphorus was not affected by the presence or absence of mycorrhizae. Given that the majority of the research has found both a mycorrhizal and a *Frankia* effect on foliar phosphorus (although whether the effect is positive or negative is still to be determined), it is most likely that the mycorrhizae in my study were not developed enough to have an effect on the foliar phosphorus concentrations.

3.4e Conclusions

Nitrogen increased the growth of *Alnus rubra* and the *A. rubra* seemed to respond the same way regardless of whether the nitrogen came from fertiliser or from *Frankia* nodules. Fertiliser phosphorus did not have an effect on *Alnus rubra*. Mycorrhizae in my experiment did not have an effect on plant size, probably because the mycorrhizae were not well developed. Despite not being well developed, plants with mycorrhizae had a tendency to have higher specific acetylene reduction rates although this difference was not significant. Nodule mass was constant regardless of the presence of mycorrhizae leading to an increase in nitrogen available to the plant. This nitrogen did not, however, result in any increase in plant size. *Frankia* colonisation in this experiment was low, making conclusions regarding nodule measurements (nodule dry mass, nitrogenase activity etc.) difficult.

Work involving isotopes would help ascertain whether the Frankia or the ectomycorrhizae is using the fixed nitrogen. Further work is needed to determine at which stage of development the mycorrhizae begin to have an effect on the host plant. Work involving different phosphorus levels would aid in determining the maximum phosphorus concentration at which phosphorus has an effect. Finally, repetition of this experiment with plants that have nodules and fully developed mycorrhizae would allow more conclusions to be drawn about the effect of the two endosymbiont on the host.

4 The interaction between *Frankia* and ectomycorrhizae on the colonisation and host benefit of *Alnus rubra*

4.1 Introduction

This experiment was designed based on two important ideas. First, when host plants form mutualisms with endosymbionts, they do not always enter into relationships with the most beneficial endosymbiont (Russell and Jones, 1975). One of the reasons why plants do not always choose the most beneficial endosymbionts is that there does not appear to be a precolonisation mechanism for determining the benefit an endosymbiont will provide (Rusell and Jones, 1975; Armarger, 1981-a; Armarger, 1981-b; Weber *et al.*, 1987; Anand and Dogra, 1992). Second, the presence of one type of mutualism increases the likelihood of another type of mutualism forming on the same host (Xie *et al.*, 1995). Since the host plant has no pre-colonisation mechanism of determining which endosymbiont is more beneficial there must be some other factor at work that has an effect on determining which endosymbionts colonise the host plant. Therefore, I designed an experiment to study the effects of *Frankia* and ectomycorrhizae on *Frankia* and ectomycorrhizal colonisation rates on *A. rubra*.

This experiment was designed to test three hypotheses. The first hypothesis was that *Frankia* and ectomycorrhizae would be beneficial to the host by increasing plant growth by providing nitrogen and phosphorus to the host. The second hypothesis was that when two similar endosymbionts (i.e. two strains of nitrogen-fixing bacteria) inoculate the host, the presence of a second kind of endosymbiont (i.e. mycorrhizae) would alter the colonisation rates of the first type of endosymbiont by increasing colonisation of one type of endosymbiont at the expense of the other. The third hypothesis was that the presence of one endosymbiont would have a beneficial effect on

the mutualism involving the other endosymbiont by providing nutrients to the first mutualism. The overall objective of this experiment was to determine if *Frankia* and mycorrhizae can alter the host colonisation rates by different endosymbionts.

The definition of colonisation was different for *Frankia* than for mycorrhizae. Plants were considered to have been colonised by *Frankia* if nodules had formed on the roots. Plants were considered to be mycorrhizal if fungal hyphae were found in the roots. Neither species of mycorrhizae formed mantles or Hartig nets although cell wall thickening could be observed for both species. For the plants that had nodules, plants were considered contaminated if one of two conditions were met I) the plants formed nodules but were not inoculated or II) the plants formed nodules that were a different type than the *Frankia* type they were inoculated with.

This experiment used two categories of *Frankia* – spore positive and spore negative. These two categories are referred to as 'types' in my thesis because spore positive and spore negative *Frankia* do not represent two taxonomically defined strains. It is believed that all *Frankia* possess the genes for spore production (Schwintzer, 1990), so the spore producing genes do not divide the spore positive and spore negative *Frankia* into two strains; however, only spore positive *Frankia* form spores in nodules. In my thesis, I will use the word 'type' to refer to either spore positive or spore negative *Frankia* as used in my experiment. When I am reporting the findings of other researchers, I will be consistent with the author's use of strain or type. If they author refers to the bacterium as a strain, I will refer to it as a strain in reference to their research.

4.2 Materials and Methods

4.2a Plant material

Three hundred ninety individuals of *Alnus rubra* were grown from seed (Ministry of Forestry, British Columbia) in plastic trays in a sterilised 1:1 turface:vermiculite mixture. The plants were fertilised weekly with Flora Gro and Flora Micro (General Hydroponics, San Rafael, California) (12.43 mM N, 1.41 mM P, 21.24 mM K, 2.06 mM Mg, 1.25×10^{-3} mM Ca, 0.170 mM Co, 0.179 mM Fe, 0.0910 mM Mn , 8.34×10^{-4}mM Mo). The plants were transplanted into yellow Ray Leach Conetainers (3.7 cm diameter, 13.6 cm length) (Stuewe and Sonds, Inc., Covallis, Oregon) with black electrical tape around the rim to prevent algal growth. The electrical tape prevented the light from penetrating the plastic tube and prevented algal growth. The tubes were filled with the same sterilised turface:vermiculite mixture as the trays the seeds were grown in.

4.2b Experimental design

Fifteen treatments were set up for this experiment (Table 4.1). Plants were inoculated with one endosymbiont to observe its effect on the host. The single endosymbiont treatments also to acted as a control so that the results from the mixed endosymbiont treatments could be compared. Plants were inoculated with both *Frankia* types (spore positive and spore negative) or both mycorrhizal species (*Paxillus involutus* and *Hebeloma crustuliniforme*) to observe if there was competition between two endosymbionts of the same type. Plants were inoculated with both *Frankia* types and one fungal species or both fungal species and one *Frankia* type to determine whether the presence of another kind of endosymbiont had an effect on the interactions between two similar endosymbionts. Plants were inoculated with one endosymbiont (e.g. one *Frankia* strain) and another endosymbiont (e.g. one fungal species) to see if the presence of a

second endosymbiont had an effect on the first endosymbiont. Sample size calculations (using a 0.05 alpha value, a 0.20 beta value, a smallest measurable difference of 30% (Krebs, 1999), and a shoot dry mass of 1.36) indicated that 26 replicates were needed for a total of 390 plants.

Table 4.1. The *Frankia* strains and fungal species combinations used in this experiment.

Treatment	*Frankia* strain	Fungal species
Control	None	None
F+	spore positive	None
F+F-	spore positive, spore negative	None
F+P	spore positive	*Paxillus involutus*
F+H	spore positive	*Hebeloma crustuliniforme*
F+PH	spore positive	*Paxillus involutus, Hebeloma crustuliniforme*
F-	spore negative	*None*
F-P	spore negative	*Paxillus involutus*
F-H	spore negative	*Hebeloma crustuliniforme*
F-PH	spore negative	*Paxillus involutus, Hebeloma crustuliniforme*
P	None	*Paxillus involutus*
PH	None	*Paxillus involutus, Hebeloma crustuliniforme*
PF+F-	spore positive, spore negative	*Paxillus involutus*
H	None	*Hebeloma crustuliniforme*
HF+F-	spore positive, spore negative	*Hebeloma crustuliniforme*

4.2c Inoculation

The host plants were inoculated twice, with three weeks between inoculations, to increase the chance of colonisation. Cultures of *Paxillus involutus* (UAMH 5871, University of Alberta, Edmonton, Alberta) and *Hebeloma crustuliniforme* (Lakehead University Mycological Herbarium 079, Lakehead University, Thunder Bay, Ontario) were grown on modified Melin Norkran's medium (MMN) (Marx, 1969). Blocks $1cm^2$ of agar containing fungal mycelium were added to liquid MMN to create the inoculant. One block was added per 50mL of liquid MMN. After 2-3 weeks, the agar was ground up with a mortar and pestle, returned to the liquid, and thoroughly mixed. The spore positive and spore negative *Frankia* inoculant was made by grinding 0.217 g of the spore

positive or spore negative *Alnus incana* nodule with a mortar and pestle and adding it to 2 L of water. Grinding the nodules released the actinomycete hyphae and, in the case of the spore positive nodule, spores.

4.2d Growth and chemical analysis

See Materials and Methods 1 for a description of the growing conditions during the experiment. Shoot height was measured every two weeks and diameter was measured every two weeks starting four weeks after treatment application. The diameter measurements were delayed four weeks to prevent the calipers from crushing the young stems. After 12 weeks, the plants were harvested and the shoot dry mass was determined. Acetylene reduction assays were performed on plants inoculated with *Frankia*. See Materials and Methods 1 for a description of how the acetylene reduction assay was performed and how the final ethylene concentration was determined.

4.2e Harvest

The number of nodules was counted and the strain of *Frankia* in the nodule was determined by examining cross sections of the nodules for *Frankia* spores. Nodules were hand-sectioned and three sections per nodule were stained with Fabil's stain (lactophenol, 0.2% aniline blue, 0.05% basic fuschin, 1.5 g/L iodine, 3 g KI) and examined for the presence of spores. Percent colonization in terms of number of nodulated plants was then calculated for each *Frankia* strain. Roots inoculated with one species of mycorrhizal fungi were examined under the dissecting microscope for mycorrhizal colonisation. See the Materials and Methods section for the first experiment for a description of how the roots were cleared.

4.2f DNA extraction and PCR

The presence of mycorrhizae in plants inoculated with both species of mycorrhizal fungi was determined by polymerase chain reaction (PCR) (Mullis and Faloona, 1987). The DNA extraction was a modified version of Jeong and Myrold's (1999) protocol. Samples of root (20-25mg) were ground by mortar and pestle in 700μL 2x CTAB (2% Hexadecyltrimethyl Ammonium Bromide, 100mM Tris, 20mM ethylenediaminetetraacetic acid, 1.4M NaCl, pH =8). The mixture was incubated at 65°C for 60 minutes. The DNA was extracted twice with 24:1 chloroform:isoamyl alcohol and then precipitated with 100μL 5M NaCl and 1000 μL 95% ethanol. After 30 minutes at 9°C, the DNA was spun for 10 minutes in a microcentrifuge to form a pellet and the salt/ethanol mixture poured off. The pellet was washed once with 1 mL of cold 70% ethanol, once with 300μL PEG/NaCl (20% PEG 8000, 2.5 M NaCl), and twice with 500μL of 80% ethanol. The pellets were dissolved in 30μL 0.1 TE (10mM Tris, 0.1mM ethylenediaminetetraacetic acid) and stored at –20°C until the PCR was performed.

PCR was performed on 5-50 mg of the DNA extract mixed with 13μL of distilled water, 1x PCR Buffer (10 mM Tris-HCl, 50 mM KCl)(Invitrogen, Burlington, Ontario), 0.2 mM dATP, dCTP, dTTP, dGTP, 2 mM $MgCl_2$ (Invitrogen, Burlington, Ontario), 10% DMSO (v/v), 25pmol internal transcribed spacer (ITS) primer 1 (White *et al.*, 1990) (5' TCCGTAGGTGAACCTGCGG 3'), 25 pmol ITS primer 2 (White *et al.*, 1990) (5' GCTGCGTTCTTCATCGATGC 3'), and 5 units of Taq. The two primers amplified the ITS region of the nuclear ribosomal DNA. The mixture was initially denatured for 2 min at 95°C and then run through 30 cycles of 1minute at 95 °C, 30 seconds at 62 °C, and 1 minute at 72 °C with a final extension of 10 minutes at 72 °C (White *et al.*, 1990). Because very few mycorrhizae were present in the roots, it was felt that a second PCR

performed on the product of the first PCR would help to amplify the small quantity of fungal DNA present in the roots. A second PCR, using the same protocol, was performed using 1μL of the PCR mix in place of the DNA extract. The PCR products were run on a 1.5% agarose gel with a confirmed sample of *Paxillus involutus* and *Hebeloma crustuliniforme* extracted from fungal cultures acting as controls. The bands of DNA were separated on a 1.5% agarose gel in TBE (Tris-Borate-EDTA) buffer at 80 V for approximately 1 hour. The gel was stained with ethidium bromide and the bands were photographed on a UV transilluminator. Fungal DNA was confirmed as *P. involutus* or *H. crustuliniforme* based on the size comparison of the bands with the confirmed samples.

4.2g Nutrient determinations

Leaves of all plants in a treatment were ground in a Wiley mill (Arthur H. Thomas, Co., Philadelphia, Pennsylvania) and passed through a $1mm^2$ screen. The leaves for all plants in a treatment were combined and tested as one so there was no variation in the foliar nitrogen or phosphorus data. Foliar nitrogen and phosphorus treatments were determined for select treatments. Nitrogen was analysed in the control treatment and treatments inoculated with *Frankia.* Phosphorus was analysed in the control treatment and treatments inoculated with mycorrhizal fungi. See Materials and Methods 1 for a description of how the foliar nitrogen and phosphorus concentrations were determined.

4.2h Data analysis

When the colonisation rates (number of plants in a treatment forming nodules or mycorrhizae) were analysed, all plants, colonised and non-colonised, were analysed using χ^2 tests (JMP version 4.04, SAS Institute). When all other analyses were performed, only

those plants that had the required colonisation status for a treatment were used. For example, plants inoculated with spore positive *Frankia* and *P. involutus* were only included if they formed spore positive nodules and mycorrhizae. Shoot dry mass and root dry mass were examined and found to be not normally distributed. As a result, shoot and root dry masses were log transformed. One way analyses of variances (ANOVAs) were performed to determine the differences between the inoculation treatments (JMP version 4.04, SAS institute). *Post hoc* Tukey's tests were performed on all significant ANOVAs (JMP version 4.04, SAS institute). The number of nodules and percent colonised plants were analysed using G tests (JMP version 4.04, SAS institute). Relative growth rate was determined using the formula (log $height_{t2}$ –log $height_{t1}$)/(14 days).

4.3 Results

4.3a Colonisation

Frankia nodule formation did not differ among the treatments either in terms of number of plants colonised (Figure 4.1) or number of nodules per plant (Figure 4.3). Mycorrhizae did not have an effect on *Frankia* colonisation rates either in terms of number of plants colonised (Figure 4.1), number of nodules per plant (Figure 4.3), or colonisation by different *Frankia* types (Figures 4.1 and 4.4). Plants inoculated with both species of mycorrhizal fungi had lower colonisation rates than plants inoculated with no mycorrhizae or one species of mycorrhizae (Figure 4.2) (χ^2=8.969, p=0.0297). Inoculating plants with both types of *Frankia* resulted in nodules of both types in approximately equal proportions (Figure 4.4). All spore positive nodules were clearly spore positive and all spore negative nodules were clearly spore negative as all spore

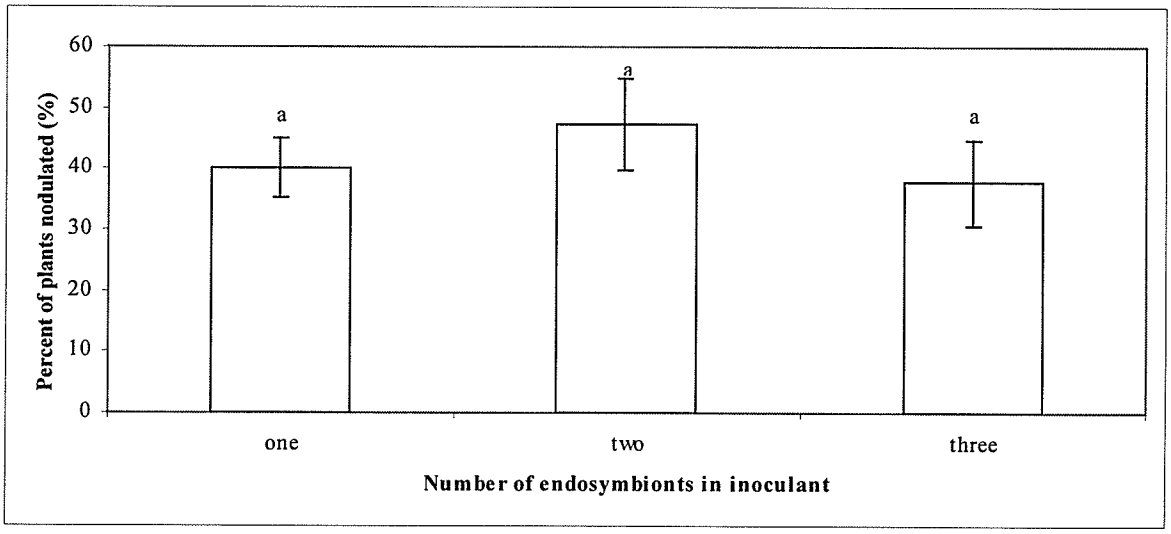

Figure 4.1. The percent of inoculated *Alnus rubra* plants in *Frankia* inoculated treatments that formed nodules. The treatments are grouped according to the number of endosymbionts they were inoculated with. One = plants inoculated with one endosymbiont, Two = plants inoculated with two endosymbionts, Three = plants inoculated with three endosymbionts. There were 2 treatments inoculated with one endosymbiont, 5 treatments inoculated with two endosymbionts, and 4 treatments inoculated with three endosymbionts. Values given are the means plus and minus one standard deviation. Values given are the means of the treatment means included in the categories. Treatments with the same letter are not significantly different at the 0.05 level according to a one way ANOVA test.

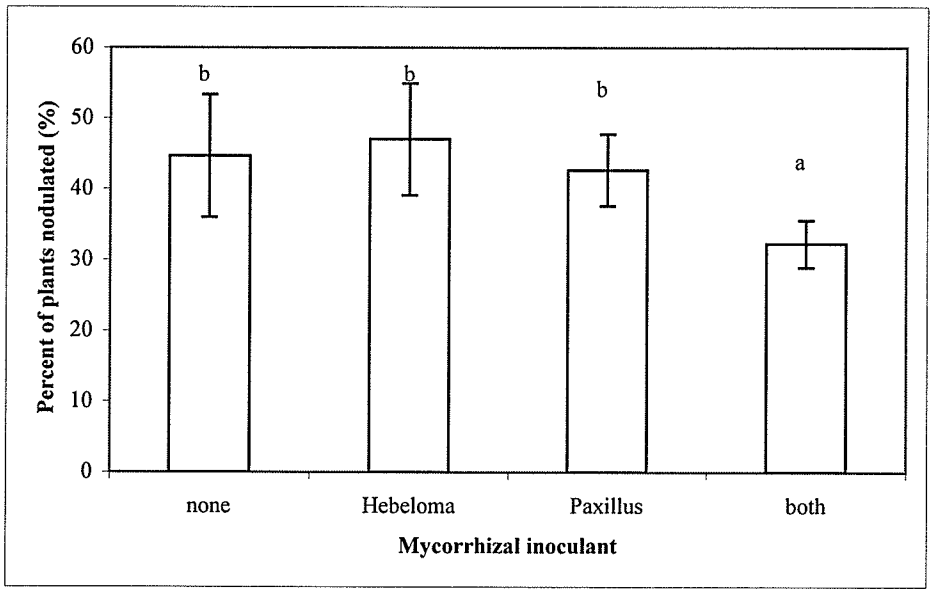

Figure 4.2. The percent of nodulated *Alnus rubra* plants in treatments inoculated with mycorrhizae. None = plants inoculated with no mycorrhizae, Hebeloma = plants inoculated with *Hebeloma crustuliniforme*, Paxillus = plants inoculated with *Paxillus involutus*, Both = plants inoculated with both *Paxillus involutus* and *Hebeloma crustuliniforme*. Values shown are means plus and minus standard deviations. There were 3 none treatments, 3 Hebeloma treatments, 3 Paxillus treatments, and 2 both treatments. Treatments with the same letter are not significantly different at the 0.05 level according to a one way ANOVA test.

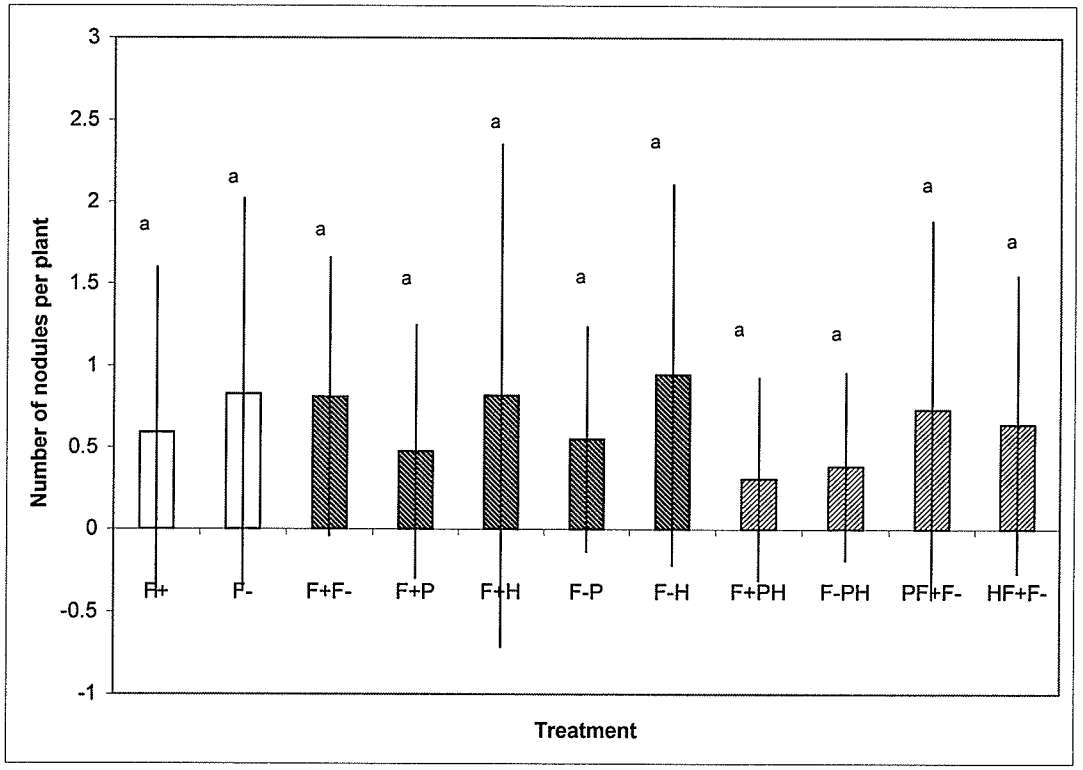

Figure 4.3. The number of nodules on *Alnus rubra* plants inoculated with *Frankia*. F+ = plants inoculated with spore positive *Frankia*; F- = plants inoculated with spore negative *Frankia*; P = plants inoculated with *Paxillus involutus*; H = plants inoculated with *Hebeloma crustuliniforme*. Values given are means plus or minus standard deviations. There were 22 F+ plants, 26 F+F- plants, 19 F+P plants, 22F+H plants, 26 F+PH plants, 23 F- plants, 20 F-P plants, 18 F-H plants, 26 F-PH plants, 26 PF+F- plants, and 25 HF+F- plants. Plants with the same letter are not significantly different at the 0.05 level according to a G test. White bars are treatments inoculated with one endosymbiont. Dark striped bars are treatments with two endosymbionts. Light striped bars are treatments inoculated with three endosymbionts.

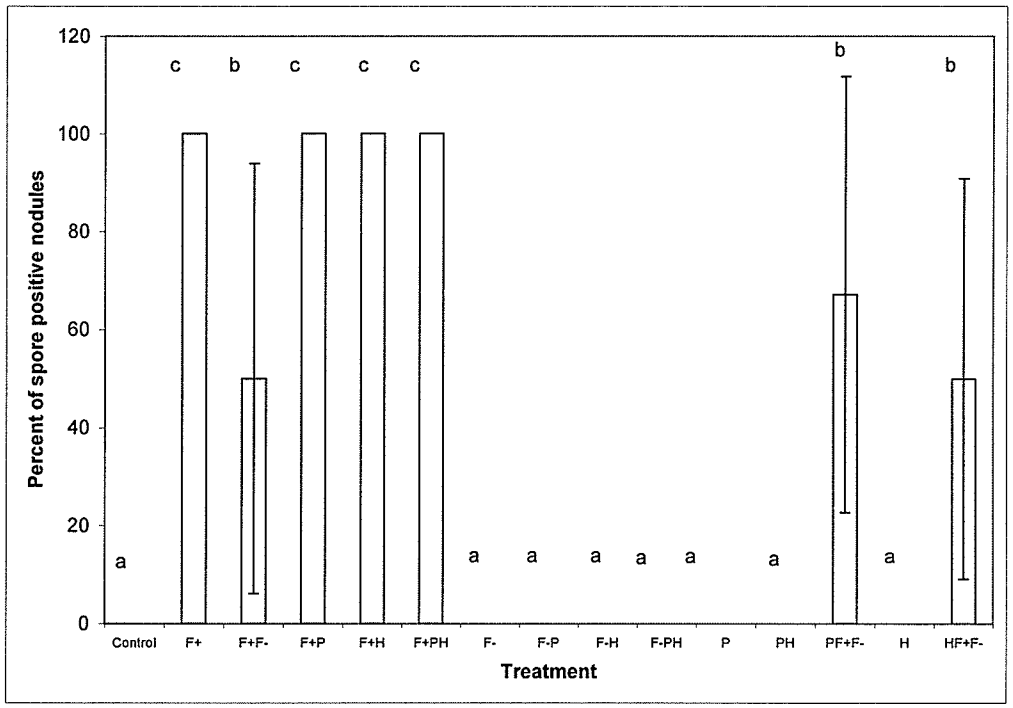

Figure 4.4. The percent of spore positive nodules on *Alnus rubra* plants. Values given are means plus or minus standard deviations. Treatments with different letters are significantly different at a 0.05 level according to the Tukey's *post hoc* test. See Figure 4.3 for an explanation of treatment abbreviations. There were 12 control plants, 8 F+ plants, 14 F+F- plants, 7 F+P plants, 10 F+H plants, 6 F+PH plants, 9 F- plants, 9 F-P plants, 10 F-H plants, 7 F-PH plants, 13 P plants, 20 PH plants, 12 PF+F- plants, 17 H plants, and 10 HF+F- plants.

positive nodules clearly had spores present when examined under the microscope while spore negative nodules did not. Uncolonised plants were included in the analyses of the number of colonised plants in a treatment. For all other analyses, plants were only included in the analysis if they had the correct colonisation status for that treatment (e.g. plants in the spore positive *Frankia*, *Hebeloma crustuliniforme* inoculation treatment were only included if they were colonised by fungi and spore positive *Frankia*).

Mycorrhizae did not form on all of the plants in treatments inoculated with *Paxillus involutus* but did when *Hebeloma crustuliniforme* was used as the inoculant (χ^2=13.810, p=0.0182) (Figure 4.5). Neither type of *Frankia* had an effect on *H. crustuliniforme* colonisation (Figure 4.5). Colonisation by *P. involutus* did not change when plants were inoculated with spore negative *Frankia* (Figure 4.5). Colonisation by *P. involutus* was lower when plants were inoculated with spore positive *Frankia* compared to when plants were inoculated with just *P. involutus* although this difference was not significant (χ^2=1.975, p=0.1599) (Figure 4.5). The DNA amplification distinguished between the two control samples of *P. involutus* and *H. crustuliniforme* that were developed from fungal colonies (data not shown). Although the analysis determined the presence of fungi in the roots, it was unable to determine whether the fungi on the roots were *P. involutus* or *H. crustuliniforme* (Figure 4.6). As such, it was impossible to determine mycorrhizal colonisation rates for plants that were inoculated with both fungal species.

All four treatments that were not inoculated with *Frankia* had plants that produced nodules through contamination (Table 4.2). Of the non-inoculated plants that formed nodules, only one plant inoculated with *P. involutus* had spore negative nodules

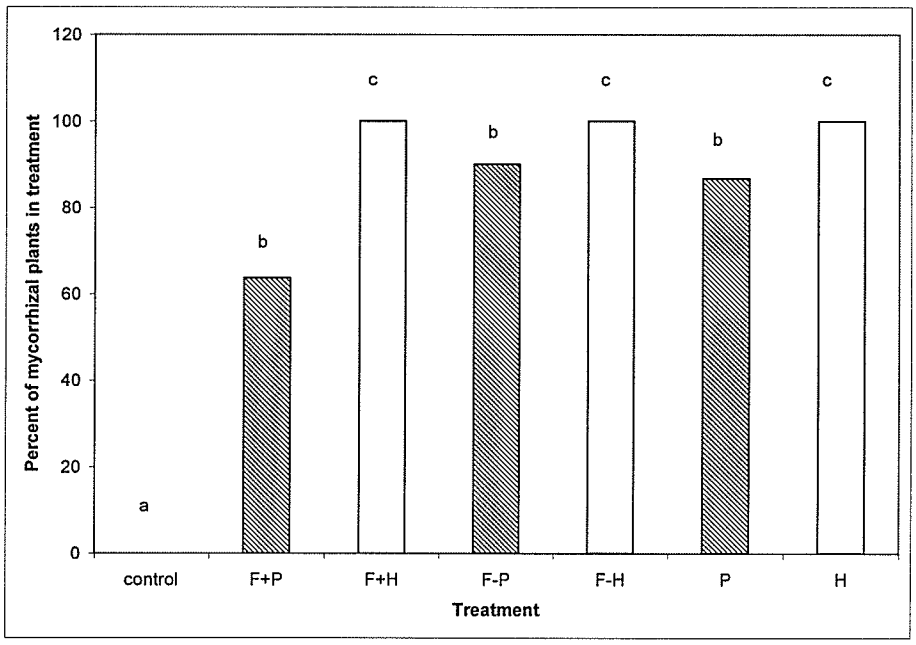

Figure 4.5. The percent of *Alnus rubra* plants inoculated with mycorrhizal fungi that were mycorrhizal See Figure 4.3 for an explanation of the treatment codes. Treatments with different letters are significantly different at the 0.05 level according to a G test. There were 12 control plants, 7 F+P plants, 10 F+H plants, 9 F-P plants, 10 F-H plants, 13 P plants, and 17 H plants. White bars are treatments inoculated with *Hebeloma crustuliniforme*. Striped bars are treatments inoculated with *Paxillus involutus*.

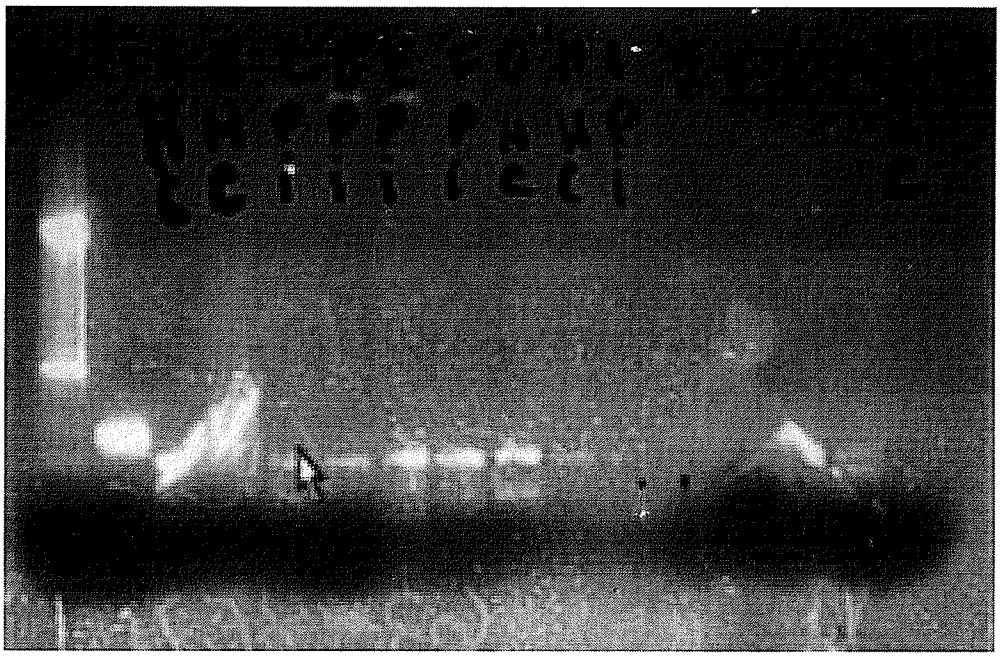

Figure 4.6. A 1.5% agarose gel showing the 100 kbp ladder (extreme left well), the control sample that was extracted from the fungal colony (Pi = *Paxillus involutus*) and 14 labeled wells. (A = sample 13 F+H, B = sample 7 H, C = sample 22 F-P, D = sample 22 F+P, E = sample 24 P, F = sample 7 PF+F-, G = sample 25 H, H = sample 14 H, I = sample 18 F+P, J = sample 23 HF+F-, K = sample 17 F-P, L = sample 15 F-H, M = sample 16 P, N = sample 19 F-H). See Figure 4.3 for an explanation of the inoculant codes.

Table 4.2. The number of plants in treatments not inoculated with *Frankia* that produced nodules, the number of total nodules produced in each treatment, and the breakdown of spore positive and negative nodules. Control = plants not inoculated with endosymbionts, P = plants inoculated with *Paxillus involutus*, H = plants inoculated with *Hebeloma crustuliniforme*.

Treatment	number of plants	number of nodules for all plants	number of spore positive nodules
control	12	15	15
P	10	12	11
H	8	11	11
PH	6	7	7

while all the rest of the plants had spore positive nodules. In total, there were 55 nodules present on plants that were not inoculated with *Frankia* and of these 55 nodules, only one was spore negative (Table 4.2). Plants inoculated with both species of mycorrhizae had fewer nodules than plants inoculated with one species of mycorrhizae when the plants were not inoculated with *Frankia* (Table 4.2). Out of the treatments inoculated with *Frankia* that produced nodules not of the type they were inoculated with, only one plant inoculated with spore positive *Frankia* and *H. crustuliniforme* had spore negative nodules while all of the rest of the plants had spore positive nodules when they were inoculated with spore negative *Frankia* (Table 4.3). There were only seven nodules of the wrong type on plants inoculated with *Frankia*; of these seven nodules, one was spore negative (Table 4.3). Plants that formed nodules that were not of the *Frankia* type they were inoculated with were included for the above analysis but were excluded for the rest of the analyses, including the colonisation analysis.

4.3b Plant size

Based on a one way ANOVA test, there was a non-significant trend for plants with *Frankia* nodules to have approximately twice as much shoot dry mass ($F=1.5007$, $p=0.2161$) (Figure 4.7), approximately 1.8 times as much root dry mass ($F=2.2370$, $p=0.0857$) (Figure 4.8), and larger shoot diameters ($F=5.0630$, $p<0.0001$) than plants without *Frankia* nodules. There was an non-significant trend for plants with *Frankia* nodules to be taller ($F=6.0723$, $p<0.0001$) than plants without *Frankia* nodules. There was a non-significant trend for plants without endosymbionts to have less shoot mass than plants with one, two, or three endosymbionts (Figure 4.9). There was a non significant trend for plants with spore positive *Frankia* nodules to have greater plant

Table 4.3. The number of plants in treatments inoculated with *Frankia* that produced nodules of a different strain than the strain of *Frankia* they were inoculated with. See Figure 4.3 for an explanation of treatment abbreviations

Treatment	F+P	F+H	F-P	F-H	F+	F-	F+PH	F-PH
number of plants with nodules of a different strain	0	1	1	2	0	1	0	2

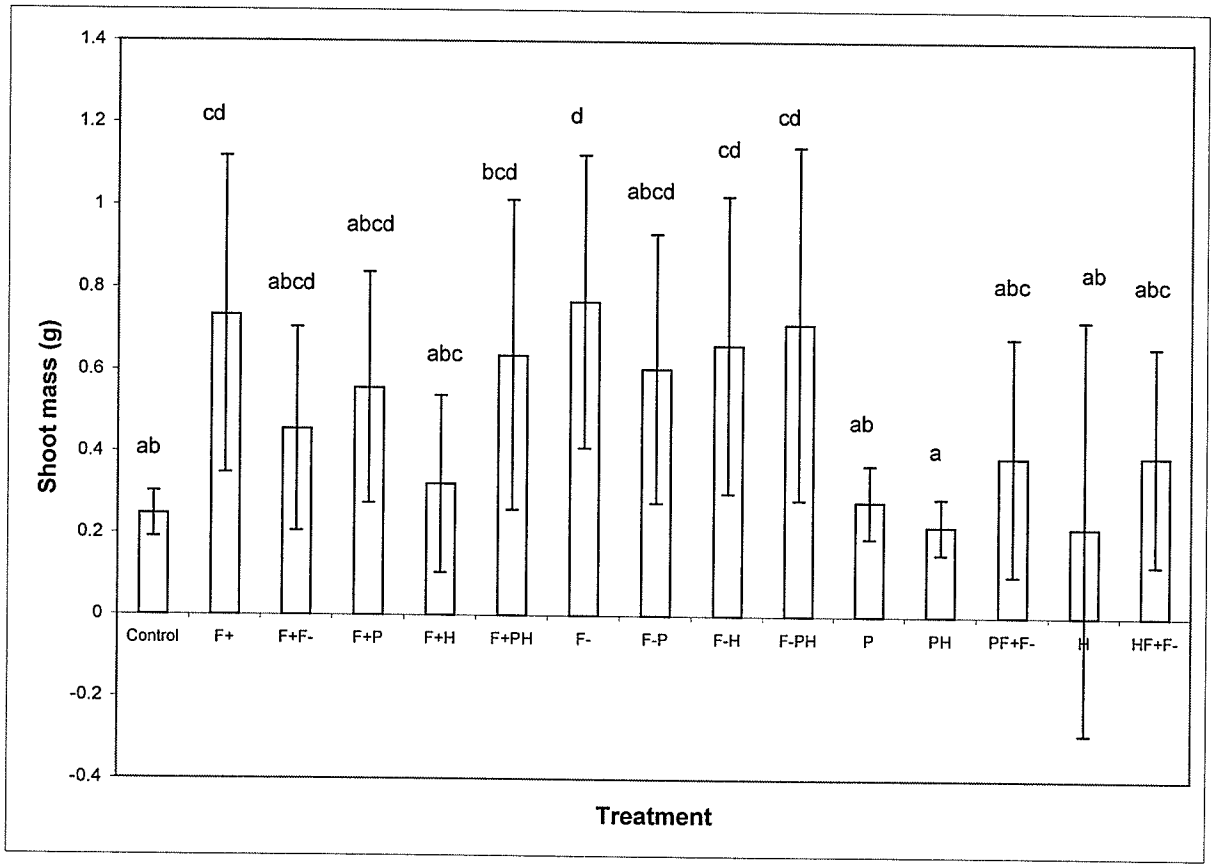

Figure 4.7. Shoot dry mass of *Alnus rubra* plants inoculated with different combinations of *Frankia* and mycorrhizal fungi. Values given are means plus or minus standard deviations. Treatments with different letters are significantly different at the 0.05 level according to a Tukey *post hoc* test. See Figure 3.1 for an explanation of the treatment abbreviations. There were 12 control plants, 8 F+ plants, 12 F+F- plants, 7 F+P plants, 10 F+H plants, 6 F+PH plants, 9 F- plants, 9 F-P plnats, 10 F-H plants, 7 F-PH plants, 12 P plants, 20 PH plants, 12 PF+F- plants, 17 H plants, and 10 HF+F- plants.

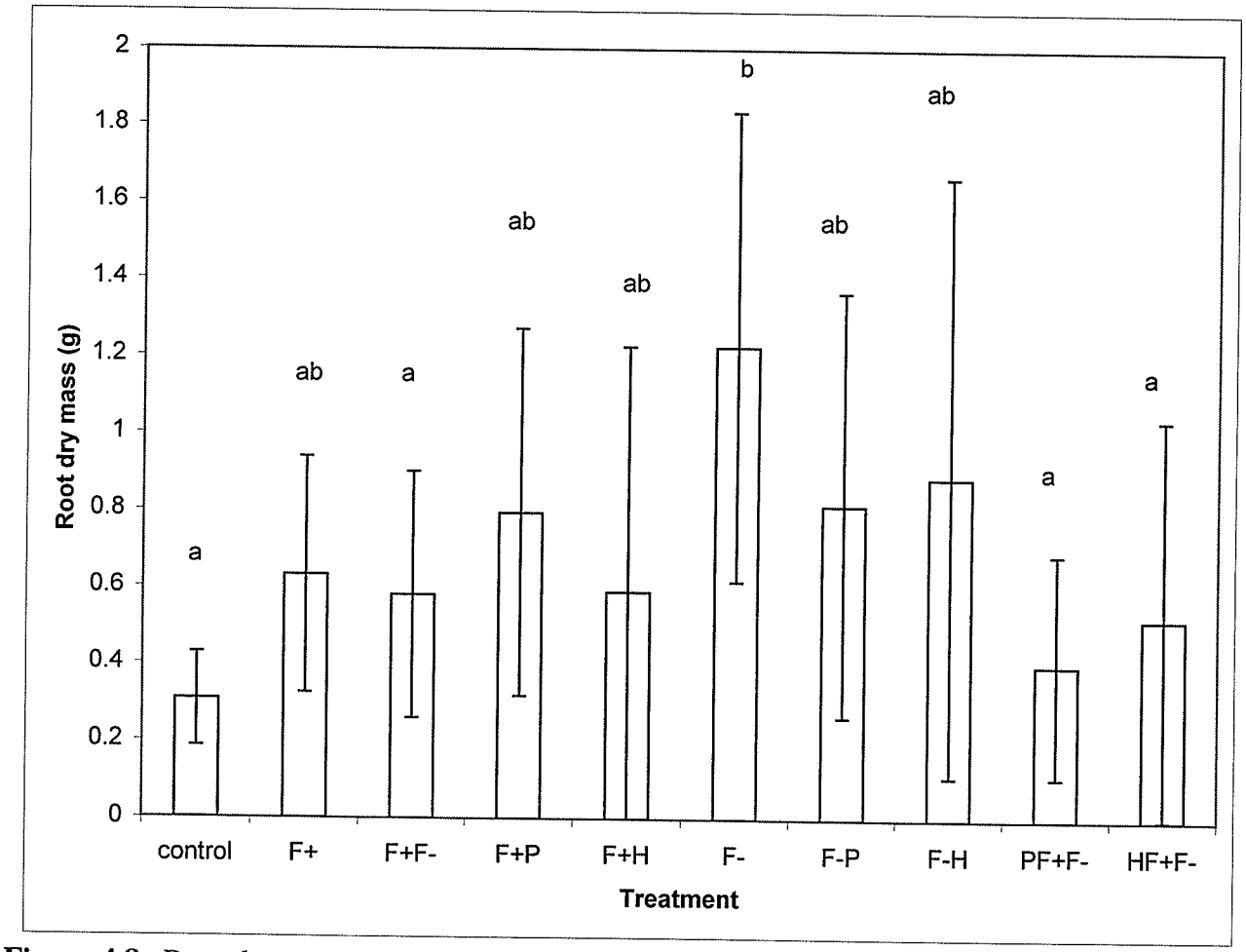

Figure 4.8. Root dry mass for *Alnus rubra* plants inoculated with *Frankia*. Treatments with different letters are significantly different at the 0.05 level according to a Tukey *post hoc* test. Values given are means plus or minus standard deviations. There were 12 control plants, 8 F+ plants, 12 F+F- plants, 7 F+P plants, 10 F+H plants, 6 F+PH plants, 9 F- plants, 9 F-P plnats, 10 F-H plants, 7 F-PH plants, 12 P plants, 20 PH plants, 12 PF+F- plants, 17 H plants, and 10 HF+F- plants.

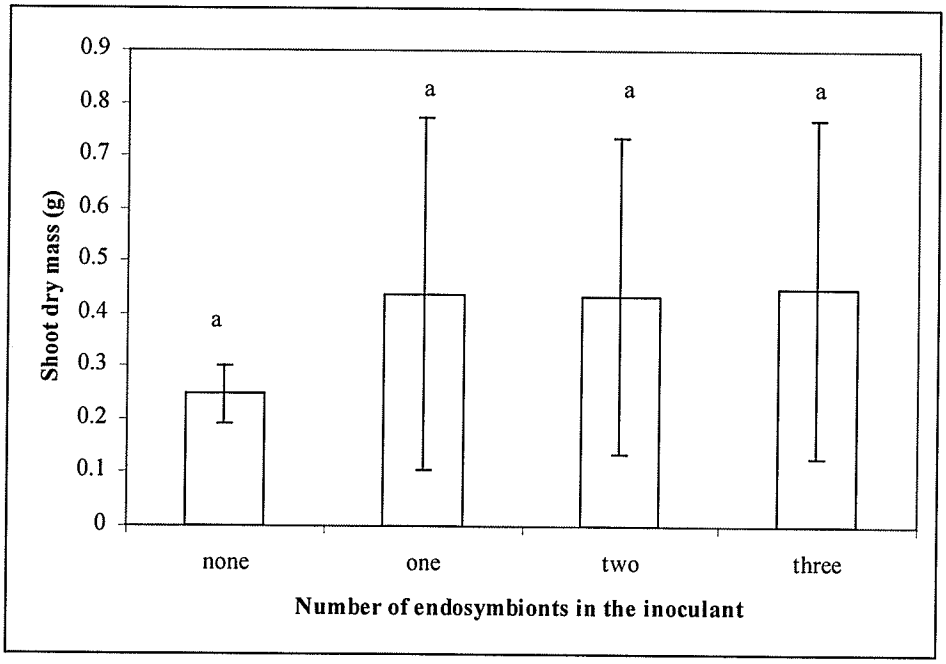

Figure 4.9. Shoot dry mass of *Alnus rubra* plants inoculated with different numbers of endosymbionts. None = plants not inoculated with endosymbionts, one = plants inoculated with one endosymbiont, two = plants inoculated with two endosymbionts, three = plants inoculated with three endosymbionts. Values given are means plus or minus standard deviations. Treatments with different letters are significantly different at the 0.05 level according to the Tukey's *post hoc* test. There were 12 none plants, 48 one plants, 54 double plants, and 64 triple plants.

growth compared to control plants when spore positive *Frankia* was the only inoculant the plants received or when spore positive *Frankia* was applied in combination with both fungal species (Figure 4.7). Plants with spore negative *Frankia* had greater plant growth compared to the control when spore negative *Frankia* was the only inoculant, when spore negative *Frankia* was applied in combination with either of the fungal types, or when spore negative *Frankia* was applied in combination with both fungal types. Plant growth was not significantly different from control plants when plants were inoculated with both *Frankia* types (Figure 4.7). Plants inoculated with either *Frankia* type had similar shoot dry masses (Figure 4.7) and heights, both of which were greater than plants inoculated with both *Frankia* types although this difference was not significant. There was a non-significant trend for plants inoculated with no endosymbionts or with three endosymbionts to have less root mass than plants inoculated with one or two endosymbionts (Figure 4.10) Plants inoculated with spore negative *Frankia* had significantly higher roots masses compared to plants inoculated with both spore positive and spore negative *Frankia* (Figure 4.8). Plants inoculated with spore positive *Frankia* did not have significantly different root masses from either plants inoculated with spore negative *Frankia* or plants inoculated with both types of *Frankia* (Figure 4.8). Non mycorrhizal plants had higher shoot masses than mycorrhizal plants ($F=3.0287$, $p=0.0512$) (Figure 4.11). Plants inoculated with just one species of mycorrhizal fungi or with both species of mycorrhizal fungi did not experience a significant increase in growth when compared to the control treatments (Figure 4.7).

Plants inoculated with *Frankia* had higher relative growth rate compared to plants not inoculated with *Frankia* ($F=2.6755$, $p=0.0014$ (Figure 4.12). Relative growth rates

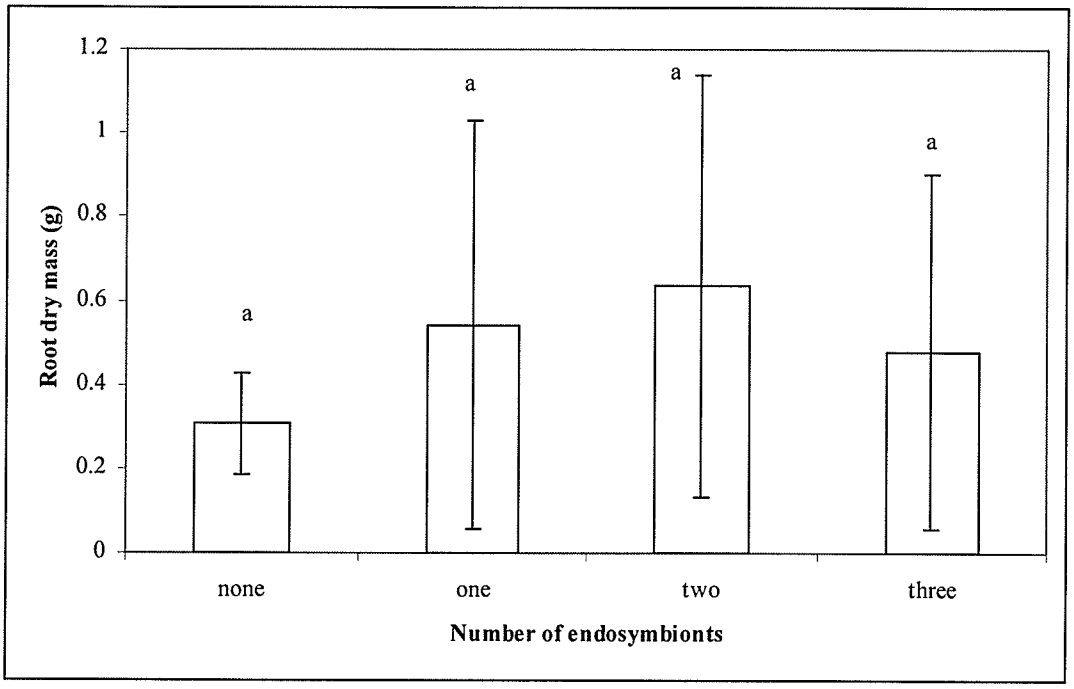

Figure 4.10. Root dry mass for *Alnus rubra* plants inoculated with numbers of endosymbionts. None = plants not inoculated with endosymbionts, one plants inoculated with one endosymbiont, two = plants inoculated with two endosymbionts, three = plants inoculated with three endosymbionts.Treatments with different letters are significantly different at the 0.05 level according to the Tukey's *post hoc* test. Values given are means plus or minus standard deviations. There were 12 none plants, 48 one plants, 54 double plants, and 64 triple plants.

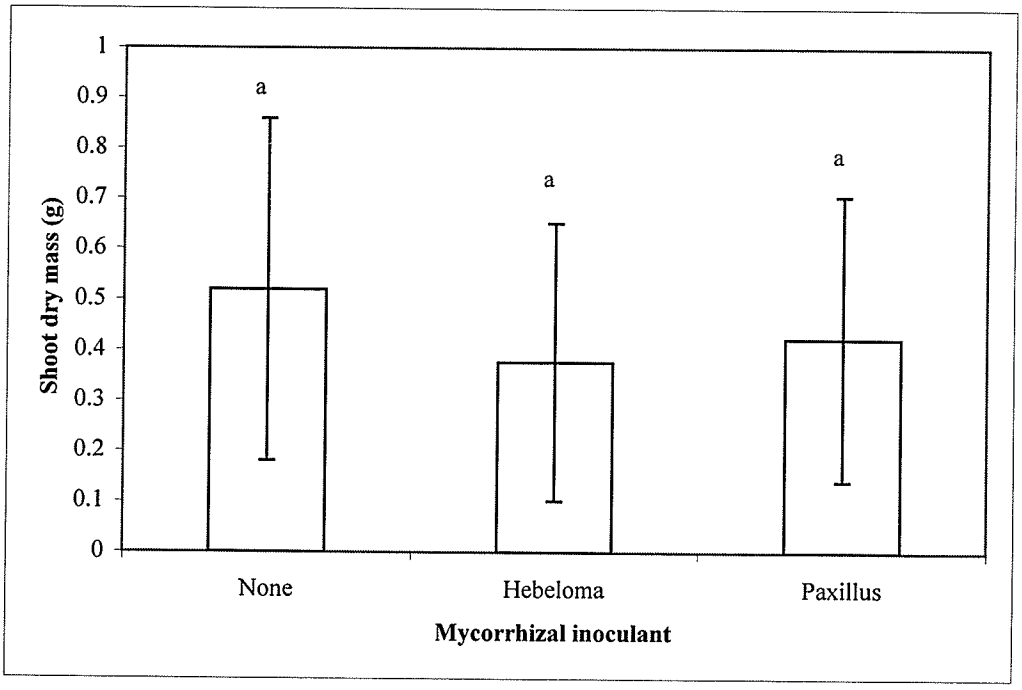

Figure 4.11. Shoot dry mass of *Alnus rubra* plants inoculated with mycorrhizal fungi. None = plants not inoculated with mycorrhizae. Hebeloma = plants inoculated with *Hebeloma crustuliniforme*. Paxillus =plants inoculated with *Paxillus involutus*. Values given are means plus or minus standard deviations. Treatments with the same letter are not significantly different at the 0.05 level according to an ANOVA test. There were 44 none plants, 62 Hebeloma plants, and 56 Paxillus plants.

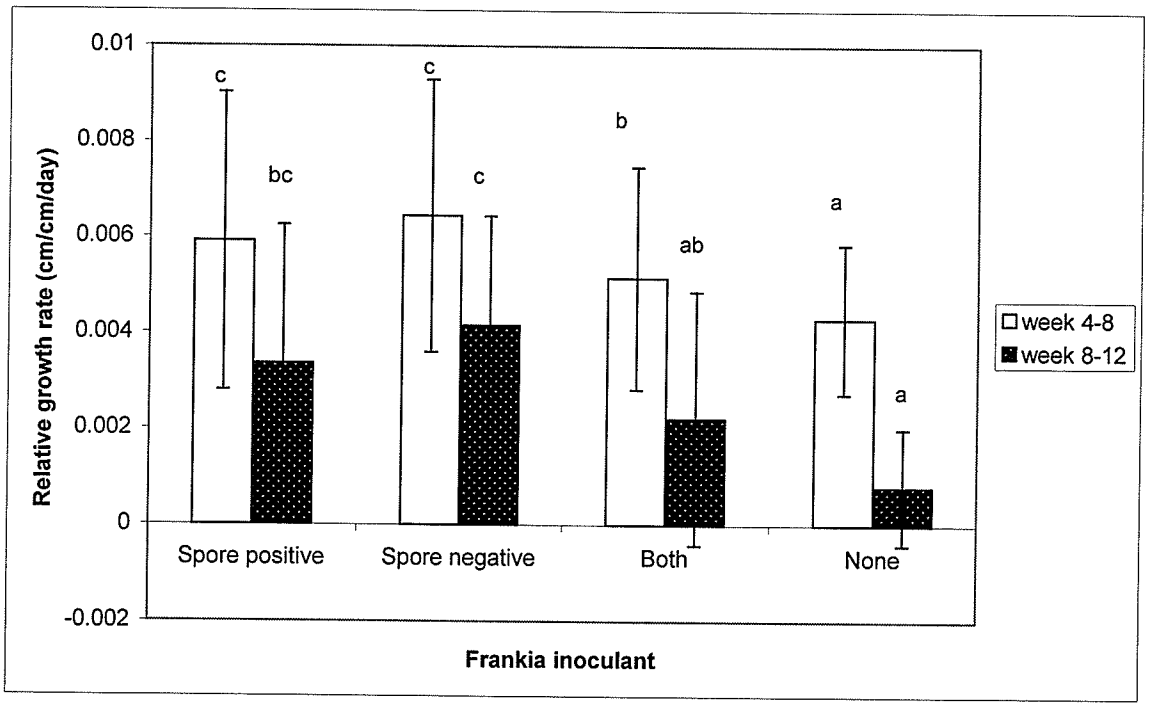

Figure 4.12. The relative growth rates for *Alnus rubra* with different types of *Frankia* used as the inoculant. Spore positive = plants inoculated with spore positive *Frankia*; Spore negative = plants inoculated with spore negative *Frankia*; Both = plants inoculated with both spore positive and spore negative *Frankia*; None = plants inoculated with no *Frankia*. Week 4-8 represents the time in the experiment just after the treatments have taken effect. Week 8-12 represents the later part of the experiment. Within the two time periods, treatments with different letters are significantly different at the 0.05 level according to a *post hoc* Tukey test. Values given are means plus or minus standard deviations. There were 31 Spore positive plants, 37 spore negative plants, 65 both plants, and 62 none plants.

for all plants were higher in the early part of the experiment compared to later in the experiment (Figure 4.12). When plants were analysed based on the type of *Frankia* in the inoculant, the growth rates were higher in the earlier part (weeks four through eight) of the experiment than in the later part (weeks eight through twelve) of the experiment (Figure 4.12). In the early part of the experiment, plants inoculated with one strain of *Frankia* had higher growth rates than plants inoculated with both strains ($F=7.3476$, $p<0.0001$) (Figure 4.12). Plants inoculated with either one or both types of *Frankia* had significantly higher growth rates than the control plants in the early part of the experiment ($F=7.3476$, $p<0.0001$) (Figure 4.12). In the later part of the experiment, plants inoculated with spore negative *Frankia* had significantly higher growth rates than plants inoculated with both types of *Frankia* although neither were significantly different from the growth rates of plants inoculated with spore positive *Frankia* ($F=19.7166$, $p<0.0001$) (Figure 4.12). Plants inoculated with one strain of *Frankia* had significantly higher growth rates than the control plants in the later part of the experiment although plants inoculated with both types of *Frankia* did not have significantly different growth rates from the control plants ($F=19.7166$, $p<0.0001$) (Figure 4.12).

4.3c Nitrogen fixation

There was no difference between the two *Frankia* types in terms of specific acetylene reduction ($F=0.9856$, $p=0.4617$) (Figure 4.13). There was no difference between the four *Frankia*/mycorrhizal combinations in terms of specific acetylene reduction ($F=0.9856$, $p=0.4617$) (Figure 4.13). Specific acetylene reduction rates were not related to shoot dry mass, height, diameter, nodule dry mass, foliar phosphorus, or foliar nitrogen. There was a significant negative relationship between specific acetylene

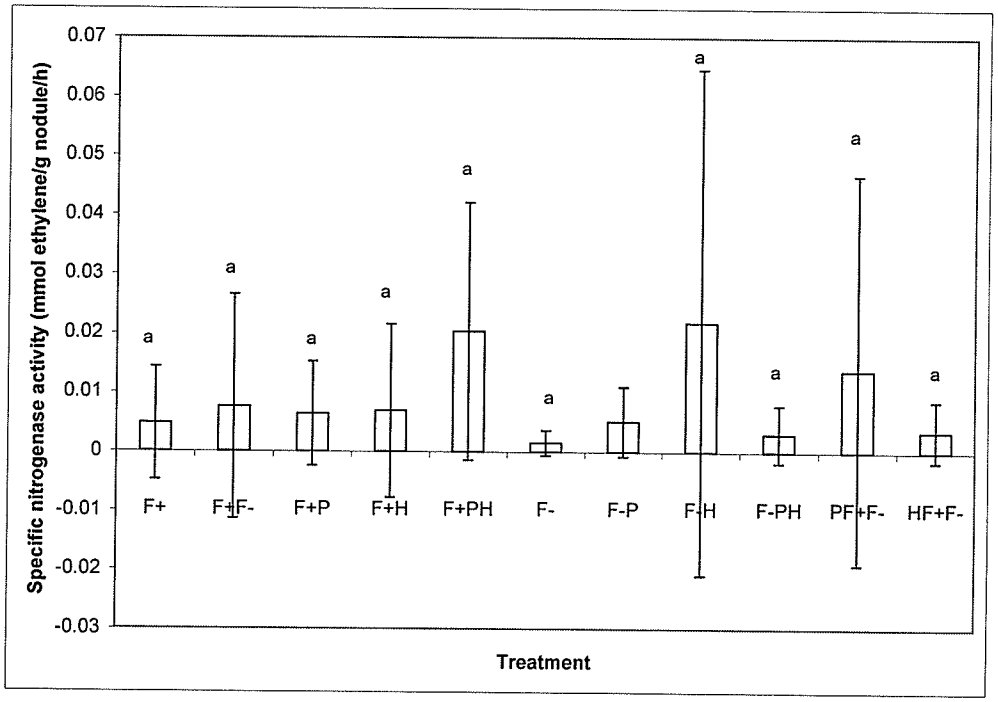

Figure 4.13. Specific ethylene production representing specific nitrogenase activity for *Alnus* plants inoculated with *Frankia*. Values given are means plus or minus standard deviations. See Figure 4.3 for an explanation of treatment abbreviations. Treatments with the same letters are not significantly different at the 0.05 level according to a Tukey *post hoc* test. There were 12 control plants, 8 F+ plants, 14 F+F- plants, 7 F+P plants, 10 F+H plants, 6 F+PH plants, 9 F- plants, 9 F-P plants, 10 F-H plants, 7 F-PH plants, 13 P plants, 20 PH plants, 12 PF+F- plants, 17 H plants, and 10 HF+F- plants.

reduction rates and root dry mass although the relationship explained little of the variation (Figure 4.14).

Plants inoculated with spore positive *Frankia* had less nodule dry mass than plants inoculated with spore negative *Frankia* (Figure 4.15). When either of the *Frankia* types was inoculated with *P. involutus*, the dry mass of spore positive and spore negative nodules was approximately equal (Figure 4.15). When plants were inoculated with *H. crustuliniforme* and one type of *Frankia*, nodule dry mass was lower when the *Frankia* in the inoculant was spore positive than when it was spore negative (Figure 4.15) although this difference was not significant. When either of the *Frankia* types was inoculated in combination with both fungal species, the spore positive nodules had less nodule dry mass than spore negative nodules (Figure 4.15) although this difference was not significant. Plants inoculated with spore positive *Frankia* had less nodule dry mass when they were inoculated with either species of mycorrhizal fungi compared to when plants were inoculated with just spore positive *Frankia* (Figure 4.15) although this difference was not significant.. Plants inoculated with both *Frankia* types had less nodule dry mass when inoculated with *P. involutus* and the same nodule dry mass when inoculated with *H. crustuliniforme* compared to plants just inoculated with both *Frankia* types (Figure 4.15) although this difference was not significant.

Nodule dry mass was positively related to shoot dry mass (Figure 4.16). Nodule dry mass was negatively related to foliar phosphorus (Figure 4.17). Nodule dry mass was not related to foliar nitrogen or specific acetylene reduction (data not shown). Plants inoculated with spore positive *Frankia* and both fungal species and plants inoculated with spore negative *Frankia* and *H. crustuliniforme* had approximately the same acetylene

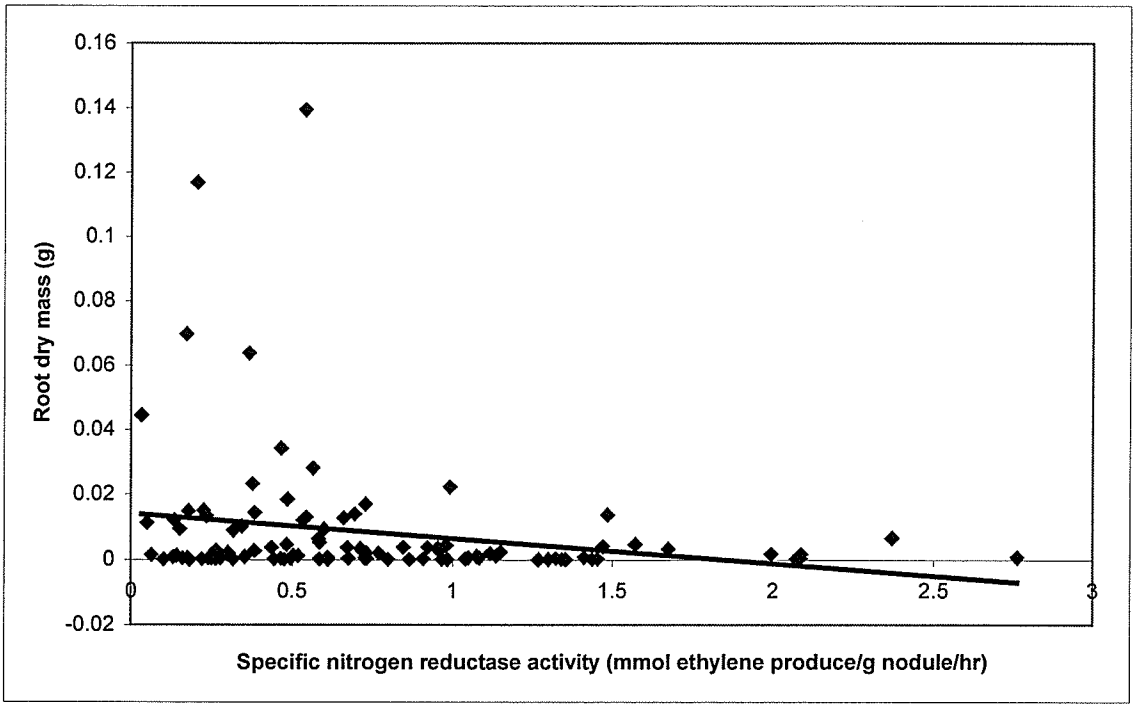

Figure 4.14. The relationship between specific ethylene production (mmol ethylene produced/g nodule/h) representing nitrogenase activity and root dry mass (g) for all *Alnus rubra* plants with nodules. The equation for the regression line is y= -0.0076x - 0.0141. The R^2 value was 0.043023 and the p value was 0.0415.

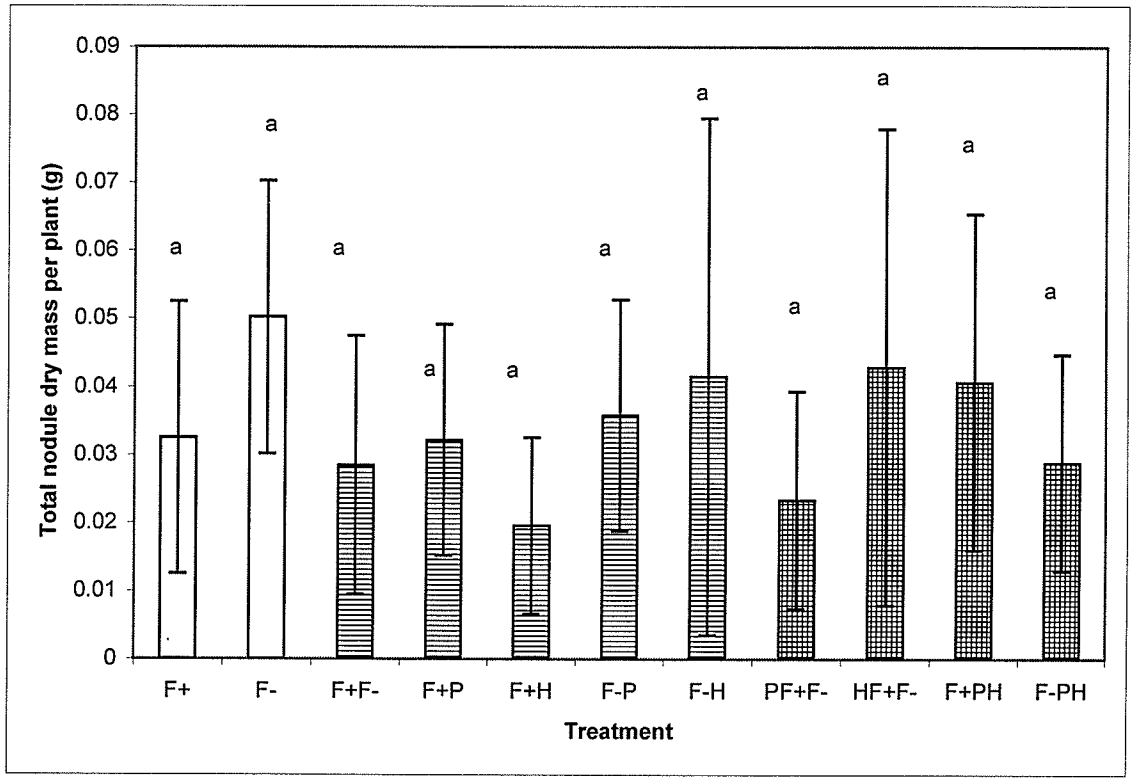

Figure 4.15. Nodule dry mass of *Alnus rubra* plants in treatments inoculated with *Frankia*. Values given are means plus or minus standard deviations. See Figure 4.3 for an explanation of the treatment abbreviations. There were 12 control plants, 8 F+ plants, 14 F+F- plants, 7 F+P plants, 10 F+H plants, 6 F+PH plants, 9 F- plants, 9 F-P plants, 10 F-H plants, 7 F-PH plants, 13 P plants, 20 PH plants, 12 PF+F- plants, 17 H plants, and 10 HF+F- plants. The treatments are different at the 0.05 level according to an ANOVA test but there is n difference between the treatments at the 0.05 level according to a Tukey *post hoc* test. White bars were treatments inoculated with one endosymbiont. Bars with horizontal stripes were inoculated with two endosymbionts. Checkered bars were inoculated with three endosymbionts.

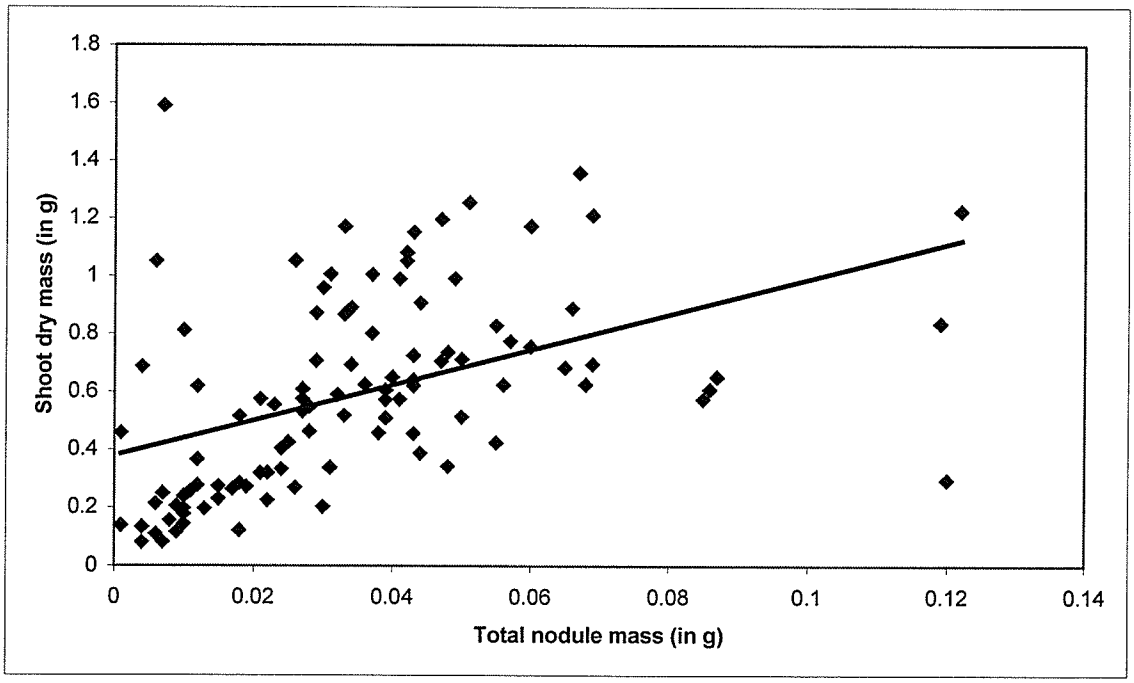

Figure 4.16. The relationship between total nodule dry mass (g) and shoot dry mass (g) for all nodulated *Alnus rubra* plants. The equation for the regression line is shoot dry mass = 0.379 + 6.113 * nodule dry mass. The R^2 value was 0.204939 and the p value was <0.0001.

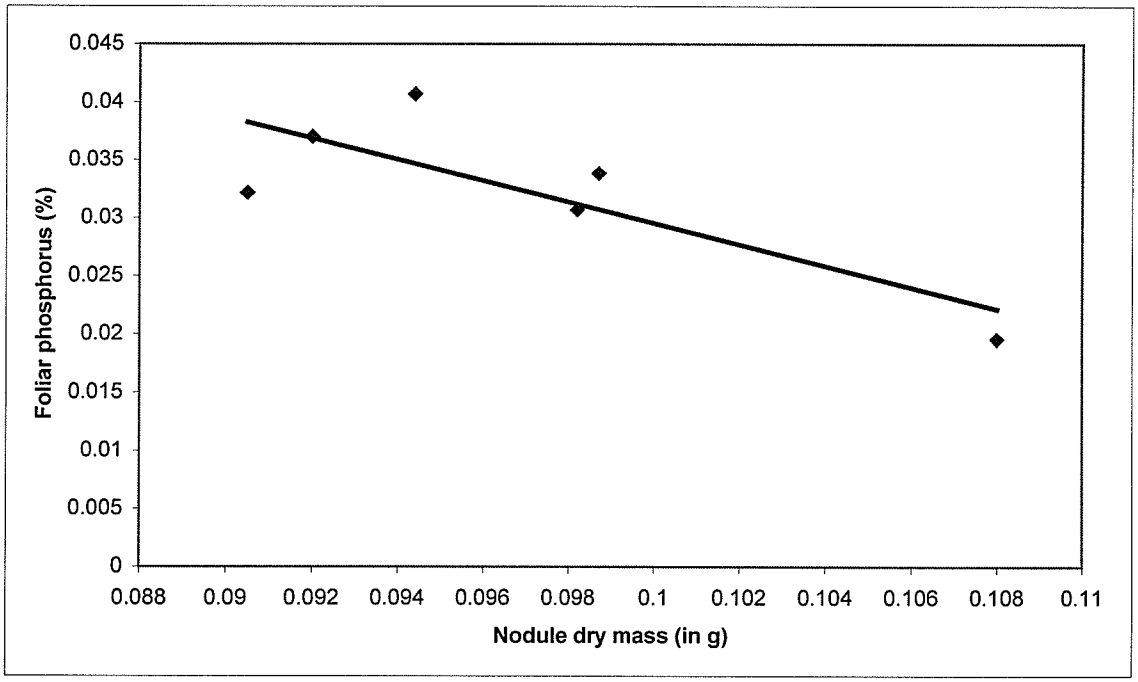

Figure 4.17. The relationship between foliar phosphorus concentration (%) and total nodule dry mass (g) of plants inoculated with *Frankia*. Data points shown are the means of all *Alnus rubra* plants in a given treatment. The sample sizes for the treatments range from 6 to 10. The equation of the regression line is $y = 0.120 - 0.709\ x$. The R^2 value was 0.52242 and the p value was 0.0520.

reduction rate per plant and were higher than the rates for other treatments although the difference was not significant (F=1.876, p=0.0586) (Figure 4.18).

4.3d Foliar nitrogen and phosphorus levels

Plants inoculated with *Frankia* had higher total shoot nitrogen levels than the control treatment while plants with three endosymbionts had less total shoot nitrogen levels than other treatments (Table 4.4). There was no difference among any of the treatments in terms of either shoot mass/g nodule (Figure 4.19) or ethylene production/g shoot mass (Figure 4.20). Plant with *Frankia* nodules had approximately twice the foliar nitrogen concentrations compared to the control treatments (Table 4.4). Plants inoculated with both spore positive *Frankia* and *H. crustuliniforme* had higher foliar nitrogen concentrations compared to all other treatments while plants inoculated with *P. involutus* and both types of *Frankia* had less foliar nitrogen concentrations compared to the rest of the *Frankia*-inoculated treatments (Table 4.4).

Total shoot nitrogen was positively related to shoot dry mass (shoot dry mass = 0.0591 + 0.785 * total shoot nitrogen, R^2=0.929738, p<0.0001), root dry mass (root dry mass = 0.115 + 0.959 * total shoot nitrogen, R^2=0.694427, p=0.0027), height (Figure 4.21), and diameter (diameter = 1.245 + 1.467 * total shoot nitrogen, R^2=0.772634, p=0.0008). Foliar nitrogen concentration was not related to shoot dry mass, root dry mass, nodule dry mass, specific acetylene reduction rates, or foliar phosphorus (data not shown).

Neither *Frankia* nor mycorrhizae had an effect on the foliar phosphorus concentrations (Table 4.5). Plants with *Frankia* nodules had higher total shoot phosphorus levels compared to the control treatment while plants inoculated with mycorrhizal fungi did not differ from the control plants in terms of total shoot phosphorus

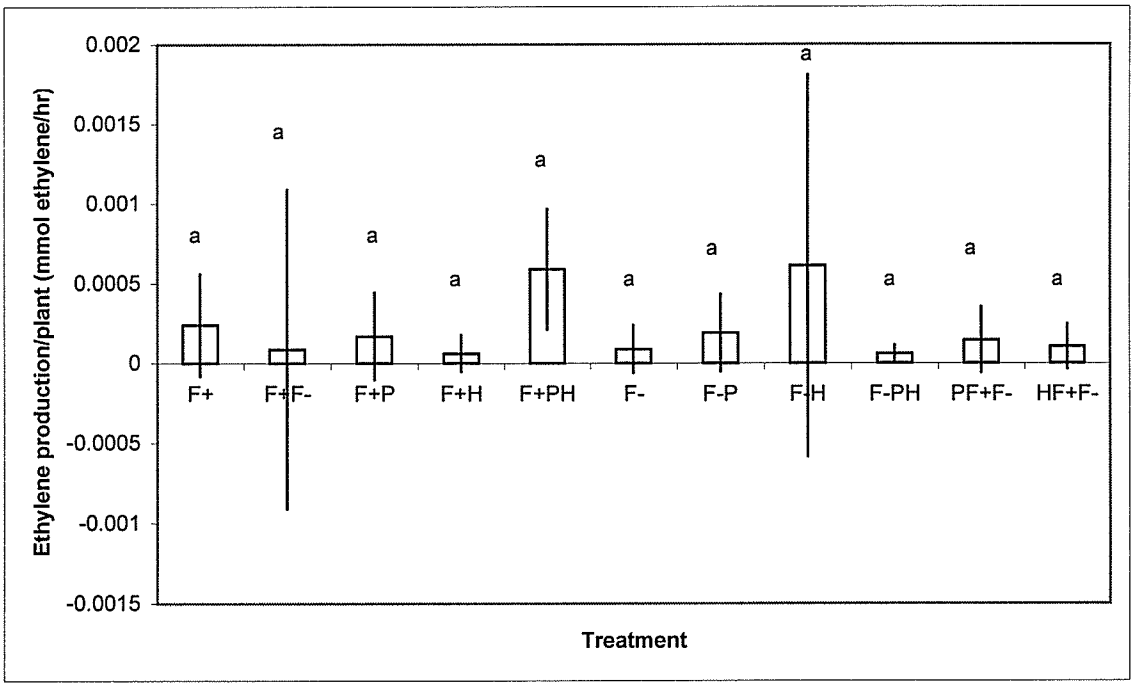

Figure 4.18. Ethylene production per plant for treatments inoculated with *Frankia*. Values given are means plus or minus standard deviations. See Figure 4.3 for an explanation of the treatment abbreviations. Treatments with the same letter are not significantly different at the 0.05 level according to a Tukey's *post hoc* test. There were 12 control plants, 8 F+ plants, 14 F+F- plants, 7 F+P plants, 10 F+H plants, 6 F+PH plants, 9 F- plants, 9 F-P plants, 10 F-H plants, 7 F-PH plants, 13 P plants, 20 PH plants, 12 PF+F- plants, 17 H plants, and 10 HF+F- plants.

Table 4.4. The foliar nitrogen concentration and total shoot nitrogen of plants inoculated with *Frankia*. Values given are for all plants combined in a treatment. See Figure 3.1 for an explanation of treatment abbreviations.

Treatment	Foliar Nitrogen (%)	Total Nitrogen (g)
Control	0.624	0.152
F+	1.097	0.805
F+F-	1.174	0.534
F+P	1.161	0.646
F+H	1.408	0.452
F-	1.124	0.826
F-P	1.173	0.663
F-H	1.123	0.683
PF+F-	1.006	0.392
HF+F-	1.174	0.461

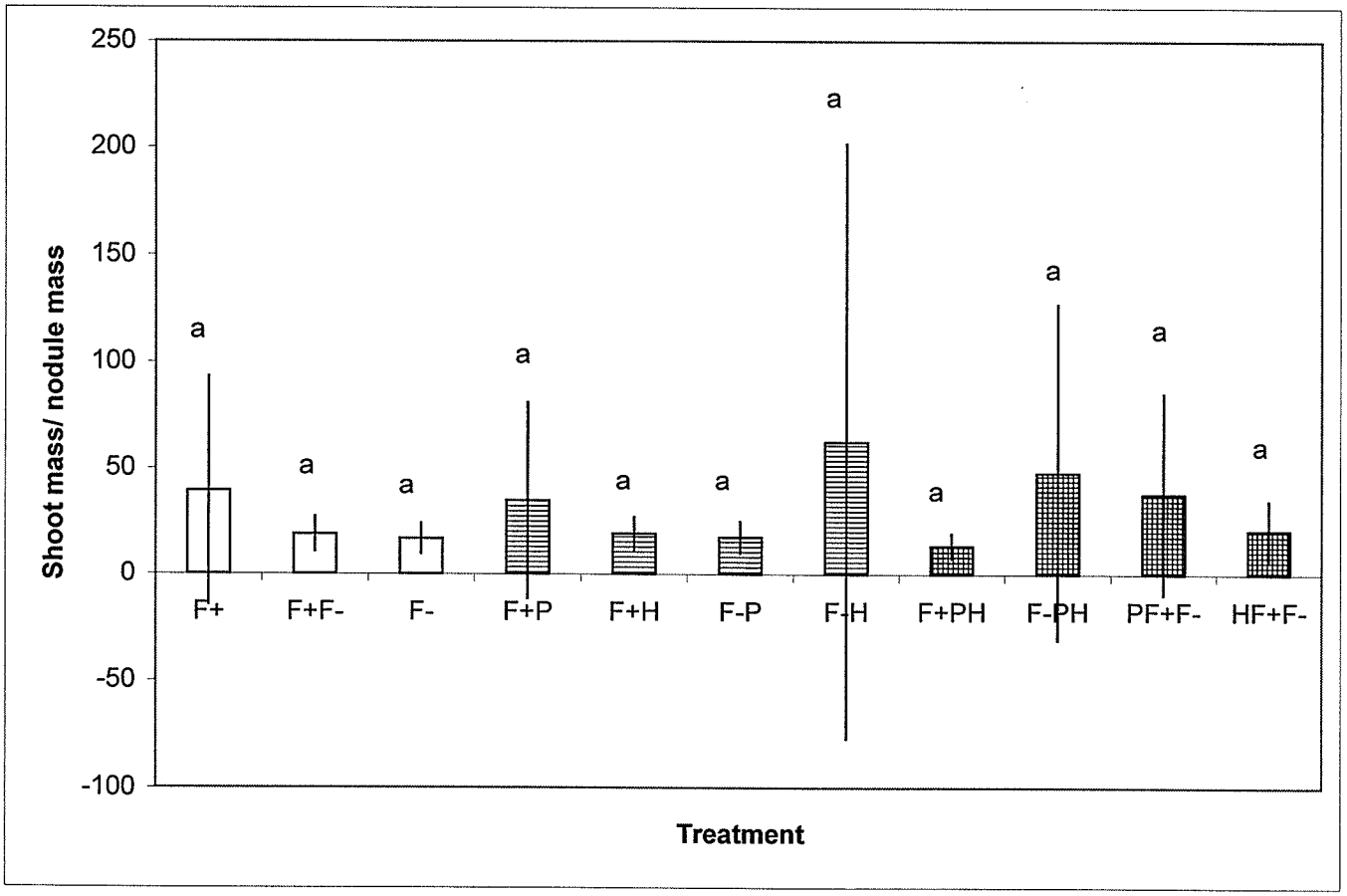

Figure 4.19. The shoot mass/ gram nodule of treatments inoculated with *Frankia.* Values shown are means plus or minus standard deviations. See Figure 4.3 for an explanation of treatment abbreviations. Treatments with the same letter are not significantly different at a 0.05 level according to a Tukey *post hoc* test. There were 12 control plants, 8 F+ plants, 14 F+F- plants, 7 F+P plants, 10 F+H plants, 6 F+PH plants, 9 F- plants, 9 F-P plants, 10 F-H plants, 7 F-PH plants, 13 P plants, 20 PH plants, 12 PF+F- plants, 17 H plants, and 10 HF+F- plants. White bars are treatments inoculated with no mycorrhizae. Horizontally striped bars are treatments inoculated with one species of mycorrhizae. Checkered bars are treatments inoculated with both species of mycorrhizae.

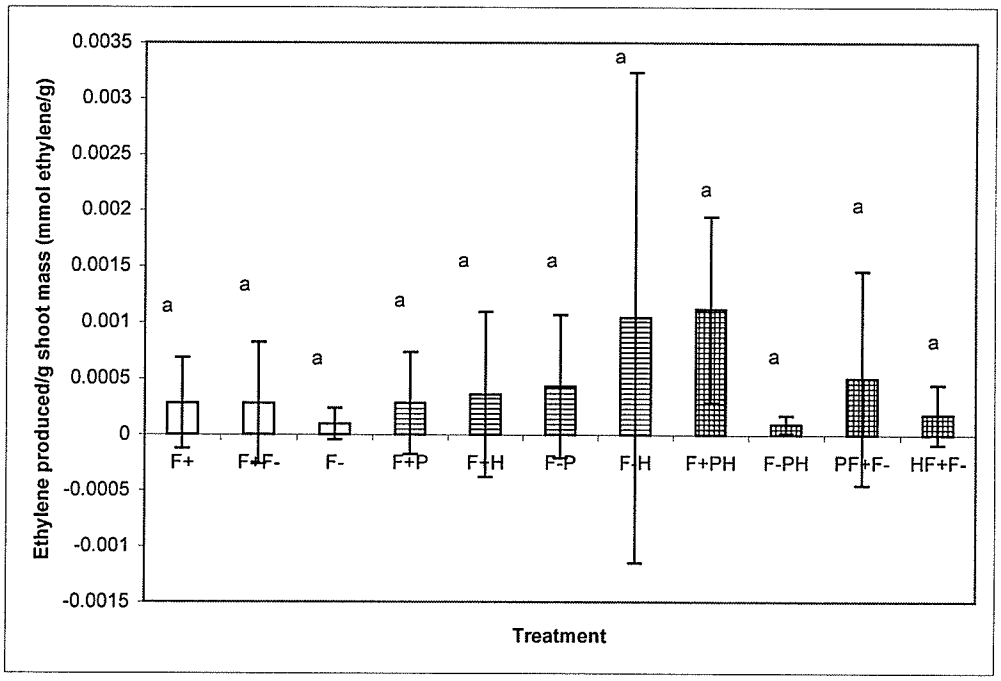

Figure 4.20. The ethylene produced representing nitrogenase activity per gram shoot mass of *Alnus rubra* inoculated with *Frankia*. Values given are means plus or minus standard deviations. See Figure 4.3 for an explanation of treatment abbreviations. There were 12 control plants, 8 F+ plants, 14 F+F- plants, 7 F+P plants, 10 F+H plants, 6 F+PH plants, 9 F- plants, 9 F-P plants, 10 F-H plants, 7 F-PH plants, 13 P plants, 20 PH plants, 12 PF+F- plants, 17 H plants, and 10 HF+F- plants. White bars were not inoculated with mycorrhizae. Horizontally striped bars were inoculated with one species of mycorrhizae. Checkered bars were inoculated with both species of mycorrhizae.

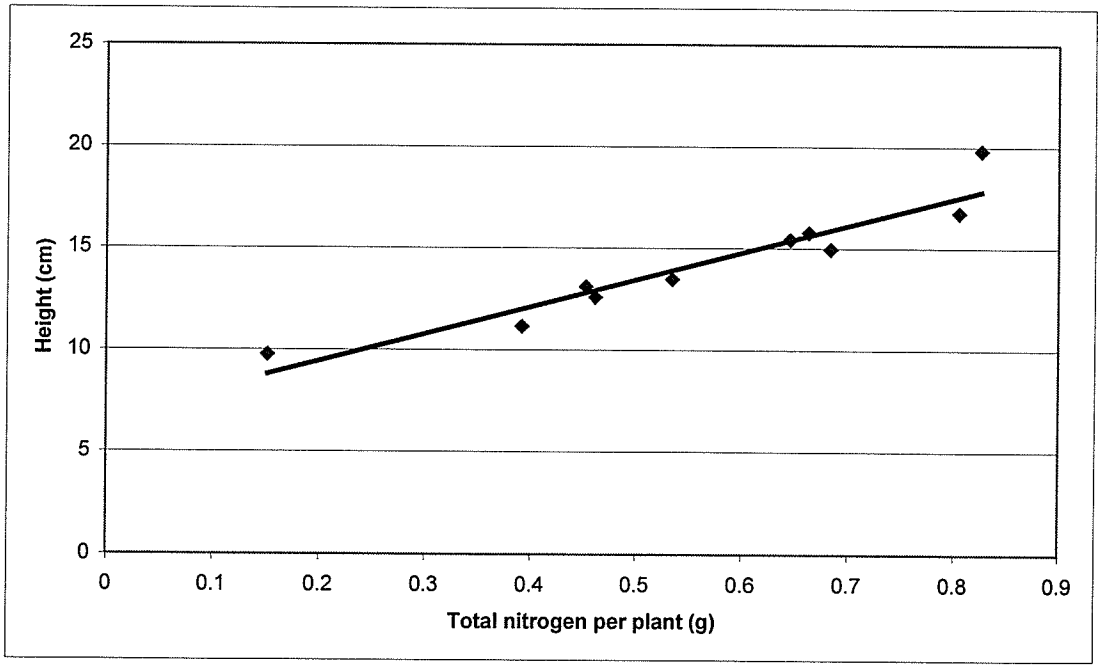

Figure 4.21. The relationship between total shoot nitrogen (g) and height (cm) of *Alnus rubra* plants inoculated with *Frankia*. All data points shown are means for the treatment. The sample sizes for the treatments range from 7 to 26. The equation for the regression line is $y = 13.354x + 6.7625$. The R^2 value was 0.9001 and the p value was <0.0001.

Table 4.5. The foliar phosphorus concentration and total shoot phosphorus of plants inoculated with mycorrhizal fungi. Values given are for all plants combined in a treatment. See Figure 3.1 for an explanation of treatment abbreviations.

Treatment	Foliar Phosphorus (%)	Total Phosphorus (g)
Control	0.0995	0.0242
F+P	0.0905	0.0503
F+H	0.1080	0.0347
F+PH	0.0944	0.0600
F-P	0.0987	0.0558
F-H	0.0920	0.0660
F-PH	0.0982	0.0673
P	0.1138	0.0318
PH	0.1310	0.0288
H	0.1036	0.0220

(Table 4.5). Plants inoculated with spore positive *Frankia* and *H. crustuliniforme* had lower total shoot phosphorus levels than any other *Frankia*-inoculated treatment (Table 4.5).

4.4 Discussion

4.4a Colonisation

Frankia

The results of my experiment indicated that there was no difference in colonisation rates between spore positive and spore negative *Frankia*; that is, one type was not better than the other at colonising *Alnus rubra.* These findings contradict the findings of Akkermans and van Dijk (1976) and Houwers and Akkermans (1981) who found that spore positive *Frankia* are orders of magnitude more infective than spore negative *Frankia* and form more nodules per plant. Hall *et al.*, (1979) also found that spore positive *Frankia* are more infective than spore negative *Frankia,* although they did not find the difference between the infectivity of the two types of *Frankia* to be as extreme as did Akkermans and van Dijk (1976) and Houwers and Akkermans (1981) did. Akkermans and van Dijk (1976) attribute this increase in colonisation to what they term 'infective particles.' These infective particles appear to be analogous to spores, which would suggest that spore negative *Frankia* should not colonise any plants at all. In this experiment, spore production is not related to infective ability.

Although only certain treatments were inoculated with *Frankia*, there were uninoculated treatments that formed nodules. None of the non-inoculated treatments was immune to *Frankia* colonisation. When the plants were inoculated with *Frankia*, there was no difference in colonisation rates between the two types, but when plants were not

inoculated with *Frankia,* spore positive *Frankia* were more infective than spore negative *Frankia*. These findings agree with the findings of Akkermans and van Dijk (1976), Hall *et al.*, (1979), and Houwers and Akkermans (1981) that spore positive *Frankia* are more infective than spore negative *Frankia*. These studies only looked at plants that were inoculated with different strains of nitrogen-fixing bacteria, however. There were no reports found in the literature about plants that were not inoculated with nitrogen-fixing bacteria but formed nodules.

From the results in this experiment, it appears as though very little inoculant was needed to colonise the contaminated plants since it is most likely that they were inoculated from inoculant that splashed into the conetainers while inoculating other plants. Houwers and Akkermans (1981) found that nodules formed on plants with as little as 5×10^{-6} g of spore positive nodule used as inoculant. One plausible explanation is that uninoculated plants were inadvertently subjected to small amounts of inoculant. In my experiment, the percentage of colonised plants in any treatment (regardless of whether the treatments were inoculated or contaminated) did not differ between treatments. It is unclear as to why spore positive nodules should be more infective on plants that were not inoculated with any type of *Frankia*. The accepted view of colonisation is that spore positive *Frankia* are more infective than spore negative *Frankia*; however, very few studies have actually tested this claim. In my experiment, plants not inoculated with *Frankia* followed the accepted view regarding *Frankia* infectivity. Plants inoculated with *Frankia* did not follow the accepted view regarding *Frankia* infectivity. It is clear from these contradictory results that the idea of spore

positive *Frankia* being more infective than spore negative *Frankia* needs to be reexamined.

There are two explanations why inoculating plants with a single species of mycorrhizal fungus in my experiment did not increase *Frankia* colonisation. Either the mycorrhizae were not developed enough to have an effect on *Frankia* colonisation or mycorrhizae did not actually have an effect. In terms of *Frankia* colonisation, it is more plausible that mycorrhizae did not actually have an effect. Rose and Youngberg (1981) found that VAM did not affect *Frankia* colonisation on *Ceanothus velutinus*. Studies showing a positive effect of mycorrhizae on the colonisation of nitrogen-fixing bacteria are typically done on rhizobial symbioses. Rhizobial colonisation requires flavanoid exudates from the host roots. This flavanoid exudation can be stimulated by mycorrhizal colonisation (Chabot *et al.*, 1992). *Frankia* do not need these root exudates to colonise the host (Provorov, 2002) and as such, may not be affected by mycorrhizal colonisation.

Inoculating plants with one type of *Frankia* and both species of mycorrhizae had lower colonisation rates in terms of number of nodules formed and percentage of plants colonised when compared to plants inoculated with *Frankia* alone or *Frankia* and one species of fungus. It is possible that at this stage of their development, the mycorrhizae were parasitic rather than mutualistic. As such, they could be draining carbon resources from the plants. The host plant may be able to compensate for one type of parasitic endosymbiont but not two and, as such, decreases colonisation of all endosymbionts. More work is needed to determine the relationship between three endosymbionts colonising the same host.

Inoculating plants with both types of *Frankia* decreased the percentage of spore positive or spore negative nodules in comparison with plants inoculated with just one type. Inoculating plants with one *Frankia* type results in every nodulated plant in that treatment, by definition, having nodules of that type. When plants are inoculated with a mixture of two infective types, there are now two types colonising the host plant. As such, the percent colonisation by any given type will decrease because there are now two types trying to inoculate the host. The number of nodules per plant did not differ among the treatments but the percentage of spore positive and spore negative nodules did, indicating that the types might be competing for a fixed number of colonisation sites.

Other studies have investigated colonisation rates of plants when two or more strains are used to inoculate the host plant. Vincent and Waters (1953) found that nodule colonisation rates of five strains of *Rhizobium trilfolium* ranging from ineffective to effective were not related to the ratio of strains present in the inoculum. The ratio of the strains in relation to one another did not change in their experiment but the total concentration of all strains differed in the treatments (Vincent and Waters, 1953). Treatment one, for example, had ten times the total concentration of cells in the inoculant as treatment two. Because the ratios of the strains in the inoculum did not change between treatments, the colonisation rates would not be expected to differ between the treatments. Robinson (1969-b) and Amarger (1981-a) found that as the proportion of effective rhizobia in the inoculum increased, the number of effective nodules increased. All three of these experiments look at ineffective *vs.* effective bacterial strains while my experiment looked at two effective types of *Frankia*. Russell and Jones (1975) found that when red and white clover were inoculated with two effective strains of *Rhizobium*

trifolium (Aberystwyth strain 7a and Rothamsted strain A121111), the percent colonisation by the two *R. trifolium* strains depended on the host. One strain of red clover (S123) and one strain of white clover (S100) had more nodules of Aberystwyth strain 7a *R. trifolium* while in the other two red clover and two white clover strains, the nodules were evenly distributed between the two strains.

In this experiment, the proportion of *Frankia* types was halved when the inoculum was applied as a mixture but each plant received the same amount of one type regardless of whether the plant was inoculated with the other type. Each plant that was inoculated with spore positive *Frankia* received 0.543 mg of crushed nodule of that inoculant. If that plant was also inoculated with spore negative *Frankia*, 0.543 mg of crushed nodule of that inoculant was also injected into the soil. When the plant was inoculated with both types though, each type of *Frankia* only made up half of the total inoculant applied to the plant. Since the mixed inoculum treatment received twice as much total *Frankia* as the pure inoculum, the number of nodules per plant should have been higher for the mixed treatment. This finding suggests that the host plant limits colonisation once it has enough nodules to obtain sufficient nitrogen for growth.

Mycorrhizae

In treatments inoculated with *Hebeloma crustuliniforme*, all plants formed mycorrhizae while in treatments inoculated with *Paxillus involutus*, only approximately 80% of plants formed mycorrhizae. The mycorrhizal colonisation rates in this experiment demonstrate that different species have different abilities to colonise *Alnus rubra*. This finding supports the findings of Molina (1979), who examined 28 species of mycorrhizal fungi and found that only four formed mycorrhizae and that those four had

varying success at colonising this host. *Paxillus involutus* has been found to colonise a number of *Alnus* species; however, it does not fully develop a Hartig net (Molina, 1979). The findings of my experiment contradict the findings of Miller *et al.* (1991) who found that *P. involutus* and *H. crustuliniforme* colonised *A. rubra* equally well, although field collection of either was low. Miller *et al.* (1991) did not examine the colonisation rates of the two species in quantitative terms, though, but rather summerised field findings categorically with ++++, +++, ++, + representing the degree of colonisation (+ being the least colonised and ++++ being the most colonised). Under their classification system, both mycorrhizal species were in the same category, but there was no indication whether one species was a better coloniser than the other within that category.

The mycorrhizae in my study were not well developed. Plants were classified as having mycorrhizae if fungal hyphae were observed in the roots; no mantle or Hartig net was seen although *Paxillus involutus* only forms a fragmented mantle and a weakly penetrating Hartig net in *Alnus rubra* (Molina, 1979). Because mycorrhizal colonisation was weak in my experiment, there are two explanations for any lack of a mycorrhizal effect. The first is that the mycorrhizae were not developed enough to have an effect. Robson *et al.* (1981) found that the effect of mycorrhizae on *Trifolium subterraneum* dry mass increased over time, presumably because mycorrhizal development in his experiment increased over time. Chatarpaul *et al.* (1989) inoculated *Alnus incana* with *Frankia* and *P. involutus* and found mycorrhizal formation after 10 weeks. Molina (1979) found mycorrhizae six months after colonising *Alnus rubra* with *P. involutus*. My experiment lasted for 12 weeks, more than Chatarpaul *et al.*'s experiment but less than Molina's experiment. It is possible that *Alnus rubra* needs more time than *Alnus incana*

to develop mycorrhizae, in which case, allowing my experiment to run for a period of time closer to Molina's six months rather than Chatarpaul *et al.*'s 10 weeks would have allowed time for more mycorrhizal development. The second explanation is that mycorrhizae have no effect on certain host measurements. Further work is needed to determine at what developmental stage mycorrhizae have an effect. Further work repeating this experiment with truly mycorrhizal plants would be useful in determining which of these two explanations is valid.

There was a non-significant trend for the presence of spore positive *Frankia* nodules to decrease *P. involutus* colonisation in my experiment. This decrease contradicts the findings of other researchers, who found that *Frankia* increased colonisation by VAM in *Ceanothus velutinus* (Rose and Youngberg, 1981), *Paxillus involutus* in *Alnus incana* (Chatarpaul *et al.*, 1989), and VAM in *Hippophae tibetana* (Tian *et al.*, 2002). Xie *et al.* (1995) found that *Bradyrhizobium japonicaum* increases mycorrhizal colonisation because the *B. japonicum* released nodulins that stimulate mycorrhizal colonisation. *Frankia*, however, do not release nodulins (Provorov *et al.*, 2002) and as such, the mechanism of *Frankia* increasing mycorrhizal colonisation is unknown. The mechanism behind the decrease in mycorrhizal colonisation in plants colonised with spore positive *Frankia* is also unknown. More work is needed to determine the precise nature of the interaction between spore positive *Frankia* and *P. involutus.*

The DNA analysis of mycorrhizal species was unable to distinguish between the two mycorrhizal species on the same plant. The cultures of *P. involutus* and *H. crustuliniforme* had bands that were easily distinguishable from each other. The band

from the ITS region of *P. involutus* was approximately 500 kbp long while the band from the ITS region of *H. crustuliniforme* was approximately 400 kbp long. When the double PCR was performed on the fungal DNA extracted from the roots, the bands produced were not the same size as the bands for either *P. involutus* or *H. crustuliniforme*. As such, it was not possible to tell what fungal species colonised plants that were inoculated with both *P. involutus* and *H. crustuliniforme*. The PCR technique used to amplify the DNA was repeated, using the PCR mixture with the amplified DNA in place of the extracted DNA in the second PCR. This method can create false positive results by the production of primer-dimers, which could explain why fungal DNA was found in the samples but was neither *P. involutus* nor *H. crustuliniforme*. It is possible that other fungal species could have grown in the turface:vermiculite mixture once it was in the growth chamber. Additionally, after harvest, the roots were sitting exposed to the air until the DNA extraction was performed. As such, it is possible that contamination by other fungal species occurred and the bands seen in the electrophoresis are from the fungal contamination rather than from the mycorrhizae.

4.4b Plant size

The presence of *Frankia* nodules increased plant size. Chatarpaul *et al.* (1989), Gardner (1986), Rose and Youngberg (1981), and Houwers and Akkermans (1981) also found that nodulation increases plant size. *Frankia* fix nitrogen and nitrogen increases plant growth. Plants with *Frankia* nodules will have more nitrogen available for growth than plants without *Frankia* nodules. Mycorrhizae, on the other hand, did not have an effect on plant size in my experiment. These findings contradict other research that found mycorrhizae have a positive effect on plant size (Asimi *et al.*, 1980; Roldan-

Fajardo *et al.*, 1982; Ianson and Linderman, 1993; and Michelsen and Sprent, 1994). Given that other researchers have found a mycorrhizal effect on plant size, it is likely that the mycorrhizae in this experiment were not developed enough to have an effect.

In my experiment, there was a nonsignificant trend for plants with one, two, or three endosymbionts to have higher root and shoot masses than plants not inoculated with endosymbionts. These findings suggest that as the number of different endosymbionts colonising a host increases, the benefit to the host does not increase. This finding contradicts the findings of Chatarpaul *et al.* (1989), who found that as the number of different endosymbionts colonising the host increases, the host benefit increases. The mycorrhizae in Chatarpaul *et al.*'s study were well developed, however, while the mycorrhizae in my experiment were not well developed. In my study, the presence of mycorrhizae did not benefit the host plant because they were not well developed. As such, increasing the number of endosymbionts did not benefit the host plant because the mycorrhizae did not benefit the host and as such the number of mutualistic endosymbionts did not increase.

In my experiment, non mycorrhizal plants had higher shoot masses than mycorrhizal plants. The fact that non mycorrhizal plants had higher shoot masses than mycorrhizal plants combined with the lack of mantle and Hartig net development suggests that at this stage in their development, the mycorrhizae were carbon drains on the host plant and were parasitic. Bethlenfalvay *et al.* (1982) examined the effect of *Glomus fasciculatus* on *Glycine max* and found that until week nine of a 20 week experiment, the mycorrhizae were actually carbon sinks draining carbon away from the

host plant. Mycorrhizae are generally considered beneficially, but it appears that during the early stages of mycorrhizal development, they can be parasitic.

Plants inoculated with spore positive *Frankia* and *H. crustuliniforme* had similar shoot masses to the control plants, which was less than any other *Frankia*/mycorrhizae combination. Plants inoculated with *Frankia* and *H. crustuliniforme* also had less total phosphorus and total nitrogen than any other treatment, which could be explain why they were not as large as plants in other treatments. Nitrogen and phosphorus are required for plant growth and as such, any plant with lower nitrogen and phosphorus levels will have reduced growth. Plants inoculated with both spore positive *Frankia* and *H. crustuliniforme* also had less nodule mass. Since nodule mass reflects how much nitrogen a plant receives (if specific nitrogenase activity is constant), the lack of nodule mass, and nitrogen available to the plant, could explain the decrease in plant mass.

When examining plants inoculated with *Frankia* alone, spore positive plants had the same shoot dry mass as spore negative plants, both of which were greater than plants inoculated with both strains of *Frankia*. This finding contradicts the findings of Hall *et al.* (1979) and VanderBosch and Torrey (1984) who found that plants inoculated with spore negative *Frankia* had higher shoot masses than plants inoculated with spore positive *Frankia*. Schwintzer (1990) suggests that spore negative *Frankia* increase growth of the host plant more than spore positive *Frankia* because they have higher specific nitrogenase activity. In this experiment, plants with spore negative nodules had the same rates of specific nitrogenase activity as plants with spore positive nodules, which accounts for the fact that plants with spore positive nodules have similar dry masses to plants with spore negative nodules. Plants with both types of *Frankia* nodules

did have lower total nitrogen levels, which could explain the reduced plant growth. Nitrogen increases plant growth and so with less nitrogen available, the plant growth would be low. There was no difference in either specific or per plant nitrogen fixation rates among the three treatments. As such, it is unclear why inoculation of plants with a combination of spore positive and spore negative *Frankia* produced a decrease in shoot mass.

Few studies that examine colonisation competition have looked at the shoot mass of the plants. Rojas *et al* (1992) found that plants with *Frankia* alone had higher shoot masses than plants inoculated with a combination of *Frankia* and non-*Frankia* actinomycetes, indicating that perhaps it is the mixture of the two types that caused the decrease. Russell and Jones (1975) examined the colonisation rates of two strains of *Rhizobium trifolii* (Aberystwyth strain 7a and Rothamsted strain A121111) on strains of red and white clover. Some clover strains had a higher shoot mass when inoculated with the Aberystwyth strain 7a of *R. trifolii*. Other clover strains had similar shoot masses when inoculated with a mixture of the two *R. trifolii* strains as when inoculated with *R. trifolii* (Aberystwyth strain 7a) (Russell and Jones, 1975). Some strains of red and white clover inoculated with a mixture of the two strains had shoot masses similar to plants inoculated with *Rhizobium trifolii* (Aberystwyth strain 7a) (Russell and Jones, 1975). With some strains of red and white clover, all three treatments had approximately the same shoot dry masses (Russell and Jones, 1975). Mytton and de Felice (1977) found that when strains of *Rhizobium trifolli* were mixed and used to inoculate white clover, the mixed inoculum plants generally had the same dry yield as plants of inoculated with one

strain alone, both of which had higher yields than plants inoculated with the other *R. trifolii* strain alone.

In terms of height and root dry mass, alders with spore negative *Frankia* nodules were larger than alders with spore positive nodules. Normand and Lalonde (1982) found that spore negative plants were taller than spore positive plants. The spore negative plants had higher nitrogen levels than the spore positive plants (Normand and Lalonde, 1982), which could explain the increase in growth. In my study, spore negative and spore positive *Frankia* had similar nitrogen levels, which does not explain why the two treatments had different heights and root masses. It is unclear why plants with spore negative nodules have similar shoot dry masses but have more root dry mass and were taller in comparison to plants with spore positive nodules.

Plants with *Frankia* nodules had higher relative growth rates than plants without *Frankia* nodules, although this difference was only significant later (weeks 8-12) in the experiment. *Frankia* nodules supply nitrogen to the host plant, which increases plant growth compared to plants without nodules. The relative growth rates for all treatments were higher later in the experiment, supporting the findings of Ingestad (1981), who found that growth rates increase with time. In the later part of the experiment, plants with spore negative nodules had higher relative growth rates than plants with both spore positive and negative nodules, although neither treatment was significantly different from plants with spore positive nodules. The trend in relative growth rate mirrors the trend evident in final shoot dry masses with the exception that both plants with spore positive nodules and plants with spore negative nodules had significantly different shoot dry masses than plants with both spore positive and spore negative nodules.

4.4c Nitrogen Fixation

Ethylene production

In my experiment, there was no difference between plants with spore negative nodules and plants with spore positive nodules in terms of nitrogenase activity. Several researchers, though, have found that spore negative plants have higher specific acetylene reduction activity then spore positive plants (Normand and Lalonde, 1982; VandenBosch and Torrey, 1984; Schwintzer, 1990). Yelton *et al.* (1993) found that a difference in nitrogenase activity did not translate into a difference in shoot mass. In my experiment, there was no difference between the specific nitrogenase activity of plants with spore positive nodules and plants with spore negative nodules, which could explain the lack of difference in shoot dry mass. There was no difference in the amount of nitrogen available to plants with spore positive nodules and plants with spore negative nodules so there would be no difference in shoot mass. Schwintzer (1990) and Sellstadt *et al.* (1986) state that there is considerable variation in the nitrogenase activity of both spore positive and spore negative nodules, so it is possible that there was no difference in the nitrogenase activity of the two *Frankia* types. Schwintzer (1990) states that spore negative nodules in *Alnus rubra* have substantially higher nitrogenase rates than spore positive nodules. My study differs from all these studies in that the nodules used to inoculate the host *Alnus rubra* were nodules from *Alnus rugosa.* It is possible that the nitrogenase activity could be different in plants inoculated with nodules from plants that are the same species and plants inoculated with nodules from plant that a different species from the host. Several authors have found that the effect of nitrogen-fixing bacteria on the host plant varies depending on the host/endosymbiont combination used (Ham *et al.*, 1976; Subba Rao, 1976; Sanginga *et al.*, 1989).

Mycorrhizae in my study had no effect on the specific nitrogenase activity. Other studies (Carling *et al.* 1978; Robson *et al.* 1981; Rose and Youngberg, 1981; Gardner *et al.* 1984; Gardner 1986; and Jha *et al.* 1993) have found that mycorrhizae increase nitrogenase activity. Nitrogen fixation has a high phosphorus requirement and since mycorrhizae increase the available phosphorus, the presence of mycorrhizae increases nitrogen fixation rates (Robson *et al.*, 1981; Rose and Youngberg, 1981;Gardner *et al.*, 1984; Ianson and Linderman, 1993). It is likely in my study that the mycorrhizae were not developed enough to have an effect on nitrogenase activity.

Specific acetylene reduction activity was negatively related to root mass in my experiment. As the acetylene reduction activity increases, the nitrogen available to the plant increases. Plant allocation to roots decreases when soil nutrient levels are high (Smith and Smith, 2001) because the plant does not need to expend as many resources to obtain the same amount of nutrients when the soil nutrient levels are high than when they are low. When plants have nodules that fix nitrogen, as the specific nitrogenase activity increases, the plant requires less root mass to acquire the nitrogen it needs because the nitrogen-fixing bacteria are supplying the nitrogen to the plant.

Nodule dry mass

Plants with just spore negative nodules had higher nodule dry mass than plants with spore positive nodules and plants with both spore positive and spore negative nodules. The trend for nodule dry mass follows the trend for plant shoot mass, suggesting that the nodule dry mass (and the total nitrogen represented by the nodule mass) is related to the trend in shoot mass. As nodule mass increases, the amount of nitrogen available to the plant increases (if specific nitrogenase activity is constant).

Nitrogen increases plant growth and so an increase in nodule mass will increase shoot mass. In addition, an increase in shoot mass could also increase nodule mass. There was no difference between treatments, however, in the shoot mass/ nodule mass. Under high nitrogen conditions, plants allocate more resources to the shoots because the plant does not need as much root mass to obtain the same amount of nitrogen (Smith and Smith, 2001). When the plants have *Frankia* nodules, the *Frankia* provide nitrogen to the plant and so the plant does not need as much root mass to obtain nitrogen. As such, the shoot mass would be expected to increase. As nodule size increases, though, the total nitrogen available to the plant increases and so plant growth increases. As such, any increase in shoot mass is accompanied by a corresponding increase in nodule mass and so the shoot mass/nodule mass ratio remains constant.

Plants inoculated with *H. crustuliniforme* and *Frankia* had a non-significant trend to have less nodule dry mass compared to plants inoculated with *Frankia*, regardless of which type of *Frankia* was in the inoculant. Plants inoculated with *P. involutus* and *Frankia* had a non-significant trend to have less nodule dry mass compared to plants inoculated with spore negative *Frankia*. Different mycorrhizal species have been shown to have different effects on different plant measurements (Rose and Youngberg, 1981; Robson *et al.*, 1981; Gardner *et al.*, 1984; Ianson and Linderman, 1993). Those that found that mycorrhizae increase nodule mass suggest that the increase in nodule mass is due to mycorrhizae providing phosphorus to fill the large phosphorus requirement for nodulation and nitrogenase activity (Rose and Youngberg, 1981; Robson *et al.*, 1981; Gardner *et al.*, 1984; Ianson and Linderman, 1993). A difference in foliar and total phosphorus concentrations was not seen in plants inoculated with *P. involutus* and plants

inoculated with *H. crustuliniforme* in my experiment so an increase in phosphorus must not be responsible for the difference in nodule mass in this experiment. Further research is needed to determine the exact effect of mycorrhizae on nodule formation.

Plants inoculated with both *Frankia* types had less nodule dry mass when inoculated with *P. involutus* and the same nodule dry mass when inoculated with *H. crustuliniforme* when compared to plants that were inoculated with just both *Frankia* types, although this trend was not significant. These findings suggest that despite not being well developed, *H. crustuliniforme* and *P. involutus* may still have had a small effect on *Frankia*. These findings contradict the findings of Rose and Youngberg (1981), Robson *et al.* (1981), Gardner *et al.* (1984), and Ianson and Linderman (1993) who found that nodule dry mass increased with mycorrhizal infection. Repeating this experiment with plants that have well developed mycorrhizae would help determine if these two species of mycorrhizal fungi have an effect on nodule dry mass.

Nodule dry mass was positively related to shoot dry mass because a plant with larger nodules will have more nitrogen fixed per plant (if specific nitrogenase activity is constant) and more available nitrogen, which increases plant growth. Robson *et al.* (1981) also found that nodule dry mass was positively related to shoot dry mass. Yelton *et al.* (1983) found that an increase in nodule dry mass resulted in an increase in ethylene production per plant. This finding suggests that nodule dry mass can be used as an indicator of nitrogenase activity in plants.

Several researchers (Rose and Youngberg, 1981; Robson *et al.*, 1981; Gardner *et al.*, 1984; Ianson and Linderman, 1993) suggest that nodule growth has a high phosphorus requirement. As such, foliar phosphorus would be expected to be positively

related to nodule dry mass because plants with high phosphorus concentrations would have enough phosphorus to meet the nodule phosphorus requirements. In my experiment, foliar phosphorus was negatively related to nodule dry mass, suggesting that the nodules may have been a phosphorus drain on the plants. Foliar nitrogen is not correlated with nodule mass because of how the foliar nitrogen is calculated. Foliar nitrogen is calculated as a percent of the total shoot mass. Larger nodules increase the amount of nitrogen that is available to the plant, which increases the plant growth. The total amount of nitrogen in the leaves also increases, but because of the concurrent leaf mass increase, the foliar nitrogen concentration actually remains constant. Specific acetylene reduction is standardised per gram of nodule, so it would not be expected to be related to nodule mass.

4.4d Plant nitrogen and phosphorus levels

Foliar nitrogen

Foliar nitrogen levels were higher in plants that had *Frankia* nodules because *Frankia* nodules provide nitrogen for the plant. As the *Frankia* fix nitrogen, the available nitrogen increases, and so the shoot mass and foliar nitrogen concentrations will increase. The *Frankia* inoculated treatments had higher shoot masses and higher foliar nitrogen concentrations than the control treatment. In general, the larger plants had higher foliar nitrogen concentrations; however, the treatment with the highest level of foliar nitrogen, plants inoculated with spore positive *Frankia* and *H. crustuliniforme* has the lowest shoot mass. The *Frankia* nodules would increase the nitrogen content of plants in this treatment while the mycorrhizae, which were not well developed, may have been a drain on the host carbon supply, which would explain the low shoot dry masses. It is not clear

why *H. crustuliniforme* in combination with spore negative *Frankia* and *P. involutus* did not also act as such a carbon drain on the host.

Total nitrogen

Plants with spore negative nodules and plants with spore positive nodules had similar levels of total nitrogen, indicating that both *Frankia* types were able to provide the same amount of nitrogen to the host. Anand and Dogra (1997) found that plants with the highest nitrogenase activity per plant had the highest shoot mass, nodule mass, and number of nodules. As nitrogenase activity increases, more nitrogen is available to the plant and so plant growth increases. Smith *et al.* (1979) found that mycorrhizae increase the ethylene production per plant. Similar results were not found in my experiment. From Smith *et al.*, it would be expected that plants with *Frankia* and mycorrhizae should have the highest total nitrogen levels. In fact, plants with three endosymbionts had the lowest total nitrogen levels of plants in *Frankia*-inoculated treatments, suggesting that the mycorrhizae might be a nitrogen drain on the plant, at least at this stage of my experiment. The mycorrhizae in this experiment were not well developed. As such, they may have represented a significant carbon drain on the plant without providing phosphorus in return. It is possible that the mycorrhizae acted as a nitrogen drain on the plant as well. The *Frankia* may not have been able to produce enough nitrogen to compensate for this drain, and as such, the nitrogen concentration in the host would decrease when the host was inoculated with both mycorrhizal species.

The trends seen in specific acetylene reduction and acetylene reduction per plant do not correspond with the trends seen in the total nitrogen of the plant so the host is not receiving the nitrogen that has been fixed. Further work using nitrogen isotopes could

trace the path of the fixed nitrogen to determine where it is going if it is not going to the host plant.

In my experiment, total shoot nitrogen was positively related to plant size. This finding supports Normand and Lalonde's (1982) finding that nitrogen content in plants were correlated with height and dry mass. There are two reasons why total shoot nitrogen was positively related to plant size. First, total nitrogen was found by multiplying the percent nitrogen by shoot mass. As a result, the bigger a plant was, the more total nitrogen the plant has. Second, nitrogen increases plant growth. As a result, when a plant has more nitrogen available, it will absorb more nitrogen, increasing both plant nitrogen levels and plant growth.

Foliar phosphorus

In this experiment, the mycorrhizae were developed enough to increase the phosphorus levels of the plant. Plants inoculated with mycorrhizal fungi had higher foliar phosphorus concentrations but lower total phosphorus levels than plants inoculated with *Frankia*. *Frankia* inoculated plants had larger shoot masses and since total phosphorus levels were calculated based on shoot masses, the *Frankia* plants with the higher shoot masses would have higher total phosphorus levels. Plants inoculated with spore positive *Frankia* and *H. crustuliniforme* had lower total phosphorus levels and lower shoot masses. Given that total phosphorus levels were calculated based on shoot masses, those plants with lower shoot masses would have lower total phosphorus levels. In addition, phosphorus is needed for growth, so those plants that had lower phosphorus levels would have less growth than plants with higher phosphorus levels.

4.4d Conclusions

There were no differences in colonisation rates between plants inoculated with spore positive *Frankia* and plants inoculated with spore negative *Frankia* in plants that were inoculated with *Frankia*. In plants not inoculated with *Frankia* and in plants with nodules not of the *Frankia* type they were inoculated with, spore positive *Frankia* were more infective than spore negative *Frankia*. All of the plants in treatments inoculated with *H. crustuliniforme* formed mycorrhizae while not all of the plants in treatments inoculated with *P. involutus* formed mycorrhizae. *Frankia* colonisation decreased only when plants were inoculated with *Frankia* and both species of mycorrhizal fungi. The molecular analysis was unable to distinguish between *P. involutus* and *H. crustuliniforme* in the roots of plants that were inoculated with both mycorrhizal fungi species.

Plants with *Frankia* nodules were larger than plants without *Frankia* nodules. Plants inoculated with spore positive *Frankia* and *H. crustuliniforme* had less shoot mass than other *Frankia*/mycorrhizae combinations. Plants with spore positive nodules had similar shoot masses to plants with spore negative nodules, both of which had larger shoot masses than plants with both spore positive and spore negative nodules. Plants with spore positive nodules had similar heights and root dry masses to plants with both spore positive and spore negative nodules, both of which were less than plants with spore negative nodules. Non mycorrhizal plants had higher shoot masses than mycorrhizal plants. Relative growth rates were higher in the later part of the experiment. Plants with *Frankia* nodules had higher relative growth rates than plants without *Frankia* nodules, a trend also found in the shoot dry masses.

The trend in nodule dry mass mirrored that seen in shoot dry masses. Plants inoculated with *H. crustuliniforme* had higher nodule dry masses than plants inoculated

with *P. involutus*. Plants with *Frankia* nodules had higher foliar nitrogen and total nitrogen concentrations than plants without *Frankia* nodules. Plants inoculated with one type of *Frankia* and both mycorrhizal had the lowest total nitrogen levels. Plants inoculated with mycorrhizae had higher foliar phosphorus concentrations but lower total phosphorus levels than plants inoculated with *Frankia*.

Further research would be helpful in determining the infectivity of spore positive and spore negative *Frankia*. Work is needed to determine when during development mycorrhizae have an effect on the host plant. Repeating this experiment with plants that have well developed mycorrhizae will help to clear up whether the lack of mycorrhizal effects are due to the mycorrhizae not being well developed or mycorrhizae just not having an effect. Further works is also needed to determine the mechanism by which spore positive *Frankia* decrease colonisation by *P. involutus* and how mycorrhizae decrease nodule dry masses. In addition, isotope work would aid in tracing the path of fixed nitrogen to determine which partner in the tripartite mutualism receives the nitrogen.

5 Conclusions

It cannot be concluded that *Frankia* and ectomycorrhizae on the same host form a mutualistic relationship. However, from my experiment, it is clear that ectomycorrhizae have an indirectly beneficial effect on the *Frankia*. I was unable to determine whether *Frankia* have a beneficial effect on ectomycorrhizae. In my experiments, *Frankia* had a directly beneficial effect on the host plant by providing nitrogen to the plant, which increases plant growth. Mycorrhizae did not have an effect on the host plant in the first experiment and decreased plant fitness in the second experiment. When *Alnus rubra* plants were inoculated with two types of *Frankia*, inoculating the plants with mycorrhizae as well did not affect the colonisation rates of the two *Frankia* types. Molecular analysis could not distinguish between *Paxillus involutus* and *Hebeloma crustuliniforme* in roots of plants that were inoculated with both mycorrhizal fungi. As such, my experiment was unable to determine whether the presence of one type of *Frankia* had an effect on the colonisation rates of both mycorrhizal species. Mycorrhizae had no effect on plant growth of hosts inoculated with *Frankia* while *Frankia* increased plant growth of hosts inoculated with mycorrhizae.

Nitrogen, whether from fertiliser or from *Frankia* nodules, increased plant growth while phosphorus had no effect on plant growth. Mycorrhizae did, however, have a non-significant trend to increase specific nitrogenase activity. This non-significant increase in specific nitrogenase activity did not translate into an increase in plant growth, suggesting that the Frankia or the mycorrhizae used the nitrogen. Relative growth rates were higher earlier in the experiment (weeks 4-8) for the microbial treatments. Relative growth rates for the fertiliser treatments were higher later in the experiment (weeks 8-12).

Spore positive *Frankia* were not more infective than spore negative *Frankia* on plants that were inoculated with *Frankia*. On plants that were not inoculated with *Frankia*, spore positive *Frankia* were more infective than spore negative *Frankia*. Mycorrhizae formed on all plants in treatments inoculated *Hebeloma crustuliniforme* but not on all plants in treatments inoculated with *Paxillus involutus*. Neither species of mycorrhizal fungi had an effect on the colonisation of either type of *Frankia*. Relative growth rates were higher later in the experiment. Plants with *Frankia* nodules had higher relative growth rates than plants without *Frankia* nodules. The presence of mycorrhizae had no effect on relative growth rates. Nodule dry mass followed the same trend as shoot mass. Plants with spore negative *Frankia* nodules had higher shoot and nodule dry masses than plants with spore positive *Frankia* nodules, which had higher shoot and nodule dry masses than plants inoculated with both spore positive and spore negative *Frankia*. When the specific nitrogenase activity is constant, nodule mass is a good indication of the nitrogen available to the host plant.

Plants with *Frankia* nodules had higher foliar nitrogen concentrations and total shoot nitrogen levels. Mycorrhizae had no effect on foliar nitrogen concentrations or total shoot nitrogen levels. The presence of mycorrhizae increased foliar nitrogen concentrations but not total phosphorus levels. Plants with *Frankia* nodules had more shoot mass than plants without *Frankia* nodules. Because of total phosphorus levels were calculated by multiplying shoot mass by foliar phosphorus levels, the increased shoot mass of plants with *Frankia* nodules resulted in higher total phosphorus levels.

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