The Effect of *Frankia* spp. and Ectomycorrhizal Fungi on *Alnus viridis* ssp. *crispa* Growing in Low Fertility and Saline Soil

By

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Abstract

I examined the effect of *Frankia* spp. and ectomycorrhizal fungi on green alder (*Alnus viridis* ssp. *crispa*), growing in nutrient-poor soil and saline conditions. The first experiment involved inoculating green alder growing in low fertility soil with three species of ectomycorrhizal fungi (*Lactarius torminosus*, *Lactarius theiogalus*, *Hebeloma crustuliniforme*) alone or in combination, with and without *Frankia* spp. on. *Frankia* spp. inoculation significantly increased plant performance compared to non-*Frankia* treatments. However, nodulated plant total biomass decreased with an increasing number of fungi. The second experiment examined the effect of *Hebeloma crustuliniforme* and *Frankia* spp. on green alder exposed to 0, 50 and 100 mM NaCl. *Frankia* spp. inoculation showed significant increase on plant performance but *Hebeloma crustuliniforme* did not. Plant mass, root:shoot ratio, nodule allocation and total nitrogen fixation decreased with NaCl exposure. A decrease in root:shoot ratio caused by salt was more moderate in nodulated plants compared to non-nodulated plants.

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Chapter 1. GENERAL INTRODUCTION

The relationship between *Alnus viridis* ssp. *crispa*, *Frankia* spp., and ectomycorrhizal fungi is a tripartite symbiosis. Plant interactions with single symbiont are fairly understood but we do not know a lot about how the tripartite interactions work. The general concepts are nitogen-fixing bacteria and ectomycorrhizal fungi display the synergistic effect on plant performance while the competition between bacteria and single and multiple fungal combinations on the same host plant is still unclear. Firstly, we do not know whether single or multiple ectomycorrhizal fungus inoculation improves plants growth or reduces growth. Secondly, we do not know whether dual symbiosis between *Frankia* spp. and ectomycorrhizal fungi could benefit plants nutrients uptake. Thirdly, we do not know whether *Alnus viridis* ssp. *crispa* has tolerance to salt stress when in symbiosis with *Frankia* spp. and/or ectomycorhizal fungi. This thesis examines the interaction between *Frankia* spp. and single or multiple ectomycorrhizal fungi on a shared host (*Alnus viridis* ssp. *crispa*) in low fertility soil as well as under saline conditions to determine which combination increases plant performance and are tolerant to salt stress.

In order to examine the effect of *Frankia* spp. and ectomycorrhizal fungi growing in low fertility and saline soil, I designed two experiments. The first experiment examined two hypotheses. The first was that mycorrhizal fungal species show niche complementarity. Therefore multiple fungal specicies will provide more access to nutrients. Secondly, mycorrhizal fungi will increase plant nodulation by releasing more phosphatase and mobilizing P in the soil, which in return will increase fungal

colonization on host plants. To test this I inoculated plants with 3 fungal species alone or in mixture, with and without *Frankia* spp.

The second experiment examined the hypothesis that the tripartite symbiosis among *Frankia* spp., mycorrhizal fungi and a host plant (*Alnus viridis* ssp. *crispa*) increases plant salt tolerance. I tested this hypothesis by inoculating *Alnus viridis* ssp. *crispa* with *Frankia* spp. and (or) an ectomycorrhizal fungus and then exposed plants up to 100 mM NaCl.

Objectives

The main objective of this thesis was to understand the interaction between *Frankia* spp. and ectomycorrhizal fungi on a shared host as well as growing in low fertility and saline soil. More specifically, the aim of the first experiment was to look at the interaction among different fungal species with *Frankia* spp. and to compare single ECMF species inoculation with a mix of species inoculated on the seedlings. The aim of second experiment was to determine if actinorhizal and (or) ectomycorrhizae plants increased tolerance to salt stress. This is an important step in selecting the potential candidates to use for revegetation of salt-affected land.

Chapter 2. LITERATURE REVIEW

2.1 Soil nitrogen deficiency and limitation to plant growth

Previous research has indicated that primary production in terrestrial ecosystems is limited by nitrogen or phosphorous (Elser et al., 2007). While nitrogen is abundant in the form of N_2, plants cannot use N_2 directly (Kraiser et al., 2011). Plants primarily use inorganic nitrogen (NO_3^- and NH_4^+) and in some cases small amount of organic nitrogen in the form of amino acids (Näsholm et al., 2009). Most (98%) of the nitrogen in the soil is in an organic form, most of which cannot be taken by plants (http://soilquality.org.au/factsheets/mineral-nitrogen). Even if a plant can take up organic nitrogen, only a small amount of N is used by plants due to the greater competitive ability of soil microbes (Lipson and Näsholm, 2001; Neff et al., 2003; Wilkinson et al., 2015). Although microbes can decompose organic nitrogen into inorganic forms, the mineralization process is slow (Powlson, 1993).

Given a lack of available soil N in some environments, nitrogen fixation could be a major N source for plants. Globally, N_2 enters nitrogen cycle in four ways: through biological nitrogen fixation by prokaryotes (between 90-140 T g of N per year), by non-biological fixation including lightning (about 10 T g of N per year), industrial fixation by the Haber process (about 80 T g of N per year) and the production of nitrous oxides through the burning of fossil fuel (about 20 T g of N per year) (Vitousek et al., 1997).

2.2 Nitrogen fixation

2.2.1 Taxonomy of nitrogen fixing bacteria

Only prokaryotes have the ability to use N_2 through biological nitrogen fixation (BNF), which converts N_2 to ammonia (Stacey et al., 1992). Nitrogen-fixing bacteria are

called diazotrophs. Various diazotrophs can interact with plants, providing them with fixed nitrogen. Diazotrophs associated with plants can be divided into those forming nodules (endosymbiotic bacteria) and non-nodular (rhizospheric, endorhizospheric or endophytic, epiphytic bacteria) (Okon and Kapulnik, 1986; You and Zhou, 1989; Santi, et al., 2013). Previous studies have described rhizospheric or endorhizospheric associations like maize (*Zea.*) and wheat (*Triticum.*) with *Azospirillum* sp.; rice (*Oriza.*), *Alcaligenes faecalis* or *Azoarcus* sp. (You and Zhou, 1989; Santi, et al., 2013). Cyanobacteria are a diverse group of aerobic photosynthetic prokaryotes, some of which have the ability to have epiphytic or endophytic associations with a wide range of plants including Bryophytes (liverworts, hornworts, and moss), Pteridophyta (the genus *Azolla*), gymnosperms (family *Cycadaceae*) and angiosperms (family *Gunneraceae*) (You and Zhou, 1989; Santi, et al., 2013). The diazotroph bacteria involved in endosymbiotic interactions also include *Rhizobium*, two classes of proteobateria - alpha and beta proteobacteria which associate with ca. 80% of members of the Legume family as well as a non-legume genus, *Parasponia* (Pawlowski and Demchenko, 2012). *Frankia* spp. (a genus of actinomycete), are aerobic filamentous bacteria that can form an endosymbiosis with actinorhizal plants - a group of over 200 species belonging to 3 orders and 8 families. The striking difference between cyanobacteria-plant association and nodular bacteria-plant association, are that cyanobacteria (photoautotrophs) develop and fix nitrogen independently and infect not only roots, but also leaves, stems and gametophytes (Sprent and Parsons, 2000; Santi, et al., 2013).

Non-nodular bacteria associated with plants fix a low quantity of nitrogen compared to the nodular-bacteria. You and Zhou (1989) first showed that host plant (rice) root cells

inoculated with a non-nodular bacteria fixed nitrogen ranging from 18.5 to 38.5 µg N fixed /day/g dry weight. In comparison, legume host plants (*Trifolium subterraneum*) inoculcated with *Rhizobium* could fix nitrogen up to 198 µg N/day/mg nodule (Gibson, 1969).

Although non-nodular diazotrophs also can fix nitrogen on the surface of plants, in their leaves, on leaf litter, decaying wood and in the soil, they have a low rate of nitogen fixation per area of land. It is estimated that the free-living nitrogen fixation rate is 0.1-60 kg N/ha/year in tropical evergreen forests compared to >150 kg N/ha/year in plant nodule systems (Reed et al., 2011). The nitrogen from endosymbiotic relationships can therefore play a dominant role in the nitrogen buedget of ecosystems.

2.2.2 Actinorhizal plants distribution and their major role in nitrogen fixtion

Actinorhizal plants have been found on all continents except for Antarctica (Schwintzer, 2012). All the actinorhizal plants are trees and shrubs, except for the genus *Datisca*, which is an herbaceous. Actinorhizal plants are distributed among eight families belonging to three orders: Fagales (Betulaceae, Casuarinaceae and Myricaceae), Cucurbitales (Datiscaceae and Coriariaceae), and Rosales (Rosaceae, Eleagnaceae and Rhamnaceae) (Wall, 2000). Arctinorhizal plants have been found growing in marginally fertile soil and play an important role as pioneer plants early in plant community development. Actinarhizal plants have been found in a variety of climates and ecosystems including arctic tundra (*Shepherdia*), coastal dunes (*Casuarina*, *Hippophae*, *Myrica*, and *Elaeagnus*), riparian (*Alnus* and *Myrica*), glacial till (*Alnus* and *Dryas* species), forest (*Alnus*, *Casuarina*, *Coriaria*, and), chapparal (*Purshia*, *Ceanothus*), xeric (*Casuarina*, *Purshia*, *Ceanothus*, *Cercocarpus*, and *Cowania* species), and alpine (*Alnus, Dryas*)

(Benson and Silvester, 1993; Swensen, 1996). Some actinorhizal plants like *Alnus glutinosa* and *Myrica gale* show morphological adaptation to waterlogged soils. They grow well in such conditions and still have functional nodules (Dixon and Wheeler, 1983). Some actinorhizal plants show more tolerance to drought than legumes. Also, actinorhizal plants may rely more heavily on nitrogen fixation than legumes. Andrews et al. (2011) concluded that the percentage of total plant N derived from N_2 fixation/the atmosphere (%Ndfa) by actinorhizal plants was up to 98%, which was greater than legumes (88%).

Hundreds of *Frankia* spp. strains have been isolated from actinorhizal families since the first successful *Frankia* spp. isolation (Aroca, 2013). However, isolation is not highly successful and not all of the successful isolated *Frankia* spp. can re-infect the corresponding plants (Ramirez-Saad et al., 1998), and only one strain (CpI1, from *Comptonia peregrina*) is commonly. Among the *Frankia* spp. isolates, only about 19 genomic species have been defined and 7 genomic species are *Alnus*-compatible strains (Aroca, 2013). These data demonstrate that *Frankia* that form a symbiotic association with actinorhizal plants have a small population diversity.

Most *Alnus* species are distributed widely throughout the temperate region of northern hemisphere (http://web.uconn.edu/mcbstaff/benson/Frankia spp./Betulaceae.htm). To date, over 40 species of *Alnus* can bear nodules and grow in a wide range of N-poor habitates (Schwencke and Carú, 2001). In the temperate forest, the nitrogen fixation rate measured for a tree alder species (*Alnus rubra*) is as high as 300 kg of N_2 ha^{-1} $year^{-1}$, close to the nitrogen fixation rate reported in legumes with an estimate rate of 240-350 kg ha^{-1} $year^{-1}$, which is beneficial for plant growth, especially in N-

limiting area (Wall, 2000; Serraj, 2004; Pawlowski, 2008). The N inputs by shrub alders (*Alnus incana* ssp. *rugosa*) was found to be up to 43 kg ha^{-1} $year^{-1}$ in wetlands in New York (Andrews et al., 2011; Hurd et al., 2001)

In areas with low availability of inorganic nitrogen, alders rely heavily on N fixation, which not only meets their N requirement but also increases the fertility of the soil (Vogel and Gower, 1998). In addition to low N availability, some actinorhizal plants can survive under a number of environmental stresses, including high pH, low fertility, flooded and arid lands. Due to these properties, they have been used in land reclamation (Santi et al., 2013). For example, *Casuarina* species that grow in drought prone and saline lands have been used extensively for land afforestation (Sayed, 2011). A *Frankia* spp. inoculation experiment on *Casuarina cunninghamiana* carried out in the hot dry river valley in Yuanmou, Yunnan Province, China, showed survival of inoculated seedlings increased by 10.0 – 20.6% compared with un-inoculated seedlings (Zhong et al., 2010). Another study on black alder (*Alnus glutinosa*), showed that alder roots were not damaged by waterlogging (McVean, 1956). Seasonal flooding increased speckled alder (*Alnus incana ssp. rugosa*) foliar N absorption by 140% compared with non-flooded plants (Kaelke and Dawson, 2003). These studies show that alders can potentially grow in flooded or drought areas, but nitrogen fixation can be reduced.

Green alder (*Alnus viridis* ssp. *crispa*), a native woody plant widely distributed across the boreal North America (http://www.efloras.org/florataxon.aspx?flora_id=1&taxon_id=233500042), has been tested for use in land re-vegetation and stabilization work (Perinet et al., 1985; Pietrzykowski et al., 2015). From 1976 to 1984, more than 6 million green alder (*Alnus*

viridis ssp. *crispa*) were successfully used for land reclamation for a hydro-electric project in James Bay Quebec Canada. The fixed nitrogen input allowed a quick natural plant community establishment in the disturbed area (Perinet et al., 1985). A recent finding also confirmed that nodulated *Alnus viridis* ssp. *crispa* dry biomass was greater than those of non-nodulated when grown in pure oil sand process-affected materials (OSPM) with high pH, high salt and low nutrient content (Bissonnette et al., 2014).

2.3 Mycorrhizal fungi symbiosis

2.3.1 Types of Mycorhizal fungi

In mycorrhizal associations, fungi colonizing host plant roots have been classified into several groups: arbuscular mycorrhizas, ectomycorrhizas, ectendomycorrhizas, ericoid mycorrhizas, arbutoid mycorrhizas, monotropoid mycorrhizas, and orchid mycorrhizas (Peterson et al., 2004). Arbuscular mycorrhizal fungi (AMF) and ectomycorhzal fungi (ECMF) have been defined as two major categories by Brundrett (2004). There are some different phases in the life histories of different mycorrhizal fungi and corresponding aspects of plant-fungus associations. Most ECMF have the capacity to live and spread without hosts, but AMF long-time survival requires hosts.

2.3.2 ECMF-host plants symbiosis

Unlike nitrogen fixing symbionts, where the diversity of nodules forming bacteria is quite limited, there are many species of fungi that can form mycorrhizae with a host. Over 80% of all land plants form symbiotic associations with mycorrhizal fungi. Among the ectomycorrhizal fungi associations, there are as many as 6000 species, which increase N, P and other mineral nutrient uptake by plant roots (Landeweert et al., 2001). Ectomycorrhizae are characterized by the presence of three structures: 1). A mantle - a

sheath of fungi tissue cover on the root surface. 2) A hartig net - fungal hyphae penetrating the intercellular space between the epidermal and cortical root cells. 3) an extraradical mycelium - hyphae that develop from the outer mantle branching into the surrounding soil (Peterson et al., 2004). Most ECMF associations are mutualistic. There are however some ectomycorrhizal fungi considered to be parasitic since their net cost exceeds net benefits to the host plants (Johnson et al., 1997). By colonizing plant roots, ECMF fungal hyphae absorb nutrients and water and then transfer them to host plants. In return, they get plant photosynthates (Landeweert et al., 2001). Ectomycorrhizal fungi increase plants nutrient uptake in three ways. Firstly, via enzyme production, mycorrhizal fungi utilize organic nitrogen or inorganic nitrogen and phosphorus and then transfer them to host plants (Antibus et al., 1997; Chalot and Brun, 1998; Hobbie and Hobbie, 2006). Secondly, via organic acid production, fungal hyphae can mobilize the mineral nutrients from solid substrates (like biotite and microcline) and stimulate plant growth (Wallander and Wickman, 1999). Thirdly, a mantle of fungal material around the root tips and external hyphae grow into soil, increasing the root surface area in contact with soil and exploited soil volume, which is also a mechanism for ECMF increasing plant nutrient uptake (Landeweert et al., 2001). ECMF hyphae can also transfer nutrients from one plant to another (Brundrett, 2004).

In non-actinorhizal plants there can be a large degree of variation in the effect of ectomycorrhizal fungi species on plant growth. It has also been shown that plants can get increased benefit from being colonized by more than one species of fungi. Jonsson et al. (2001) showed that 8 ectomycorrhizal species could form symbioses with two non-actinorhizal tree species (*Pinus sylvetris* and *Betula pendula*). Their results indicated that

Betula pendula growth was higher when inoculated with all eight fungi compared to those inoculate with a single fungi in low fertility soil. This is likely due to the fungi being able to access resources in the soil under different conditions and therefore increase the uptake of nutrients by plants. There is no research comparing the effects between multiple ectomycorrhizal fungi and single ectomycorrhizal fungi on actinorhizal plants.

2.4 *Frankia* spp.-ECMF-host plants symbiosis

2.4.1 *Frankia* spp.-mycorrhizal fungi-actinorhizal plants symbiosis

In addition to nodule formation, actinorhizal plant roots can also have symbioses with ectomycorrhizal fungi. The major ectomycorrhizal fungi members belong to Basidiomycete phylum (Landeweert et al., 2001). Gardner (1986) has described actinorhizal mycorrhizae plants and listed a number of ectomycorrhizal fungi associated with them. All families of actinorhizal plants, except for the Rhamnacese and Rosaceae, have been found to be symbiotic with ectomycorrhizal fungi. The most common ectomycorrhizal fungi included *Alpova* spp., *Hebeloma* spp., *Paxillus involotus* and *Pisolithus* spp. (Gardner, 1986; Markham, 2005; Becerra et al., 2009). Gentili and Huss-Danell (2003) indicated that nodule initiation, nodule growth and function require a high level of P. It has been shown that mycorrhizal fungi colonization promoted P absorption five times more than non-mychorhizal inoculated *Alnus viridis* (Mejstrik and Benecke, 1969). These results suggest that mycorrhizal fungi enable nodulated actinorhizal plants to meet their additional phosphorus requirements. In addition, the presence of mycorrhizal fungi and *Frankia* spp. on the same root has been shown to stimulate nitrogen fixation, mineral acquisition and plant growth (Yamanaka et al., 2003).

While mycorrhizae may benefit plant growth and nitrogen fixation, the fungi may also compete with nitrogen fixing bacteria for host colonization and resources. By inoculating the two symbionts simultaneously and at different times, Bâ et al. (1994) found that ectomycorrhizal hyphae could modify the root morphology and inhibit nodule initiation and infection thread development. It is currently unknown how multiple ectomycorrhizal fungi would affect the nodule formation and plant performance.

2.4.2 Tripartite symbiosis among *Frankia* spp.-ECMF-*Alnus*

To date, the genus of *Alnus* is the most intensively studied actinorrhizal species for nutrient-poor soil restoration. Alders associate with a relatively restricted community of ECM fungi, which are highly host-specific. Among thousands of ectomycorrhizal fungi species, 50-60 species have been identified that associate with the *Alnus* genus (Hibbs et al., 1994, Tedersoo et al., 2009). Godbout and Fortin (1983) listed 46 ectomycorrhizal fungi species tested on *Frankia* spp. inoculated seedlings green alder (*Alnus viridis* ssp. *crispa*) and speckled alder (*Alnus incan* ssp. *rugosa*), of which only 10 formed mycorrhizae on both *Alnus* species. Eleven ectomycorrhizal fungi species have been characterized and identified on red alder (*Alnus rubra*) roots collected from the field, including *Alpova diplophloeus*, *Lactarius obscuratus*, *Cortinarius bibulous*, *Thelephora terrestris*, *Paxillus involutus*, *Laccaria laccata*, *Laccaria bicolor*, *Hebeloma crustuliniform* (Molina, et al., 1994). Among these ectomycorrhizal fungi species, *Alpova diplophloeus* and *Lactarius obscuratus* are by far the most widespread species in *Alnus*.

2.5 Soil contamination

There are various types of land contamination including heavy metal (e.g. Cu, Pb, Zn), and persistent organic pollutants, e.g. polyhalogenated biphenyls, polyaromatic

hydrocarbons, chlorinated phenols, and pesticides (Meharg and Cairney, 2000; Pulford and Watson, 2003). Another land contamination problem is salinity, which is considered one of the major environmental stresses worldwide limiting plant growth and productivity (Parida and Das, 2005). The FAO estimated that about 400 million hectare of land has been salt-affected, which consists of over 3% of the total land area (http://www.fao.org/soils-portal/soil-management/management-of-some-problem-soils/salt-affected-soils/more-information-on-salt-affected-soils/en/). There are different reasons causing this problem, such as weathering of rocks, land clearing and irrigation (Munns and Tester, 2008). With the increasing population and industrialization in many parts of the world, reclaimed water has been encouraged to be used for irrigating field crops, which causes a potential salt problem to the sensitive plants (Niu and Cabrera, 2010). Development of the oil sand industry in Alberta Canada, including the extraction of bitumen and the production of consolidated tailings also result in sodium and chloride accumulation (Renault et al., 2004; Renault, 2005b). High levels of salt dramatically inhibit a plant root's ability to extract water and impact native vegetation growth as well as agriculture. High concentrations of salt can be toxic for plant growth. It has been shown that increasing salt could reduce the weight of roots and shoots, plant height, leaf expansion, chlorophyll and total carotenoid contents of leaves (Parida and Das, 2005). Exposure to salt stress would be therefore a large problem for plant growth, productivity and the environment.

2.6 Restoration of salt contaminated land

2.6.1 ECMF in vitro pure culture or symbiosis tolerant to salt

Many ECMF isolates show high salt tolerance when they are grown in *vitro*.

Pisolithus tinctorius and *Suillus luteus* were found to be tolerant of NaCl (120 mM) (Dixon et al., 1993). Some *Laccaria bicolor* isolates showed tolerance to saline-alkaline conditions (200 mmol/L NaCl, Na_2SO_4, $CaSO_4$, pH 7.8) (Kernaghan et al., 2002). Tang et al. (2009) examined three different ECMF species and found *Boletus luridus* had the highest biomass at 300 mM NaCl compared to other 2 species. *Paxillus involutus* can even survive and start growth after 3 weeks acclimation in the medium containing 500mM NaCl (Langenfeld-Heyser et al., 2007).

Although we know little about the exact mechanisms of fungal resistance to salt stress in pure culture, the fungi have been applied to different plant species as a way of increasing their salt tolerance. Nguyen et al. (2006) tested 3 species of conifers, black spruce (*Picea mariana*), white spruce (*Picea glauca*), and jack pine (*Pinus banksiana*) seedlings inoculated with *Hebeloma crustuliniform* or *Laccaria bicolor* and subjected them to 25 mM NaCl and Na_2SO_4. The results showed that ECMF reduced Cl^- concentration in spruce tissue in NaCl-treated conditions.

Plant growth mostly responds positively when associated with ECMF by increasing biomass under salt stress compared to non-mycorrhizal salt-treated treatments. However, the growth responses vary from plant species to species and with the ECMF sepcies. Yi et al. (2008) tested two species, aspen (*Populus tremuloides*) and paper birch (*Betula papyrifera*) inoculated with *Hebeloma crustuliniforme* or *Laccaria bicolor* and treated with 25 mM NaCl for 6 weeks. The results showed that only aspen associated with *H. crustuliniforme* had significantly higher plant biomass and root hydraulic conductance compared to non-mycorrhizal seedlings under saline conditions. Langenfeld-Heyser et al. (2007) investigated a salt sensitive hybrid poplar associated with *Paxillus involutus* in

NaCl-stress conditions. The results showed *Paxillus involutus* could benefit the hybrid poplar growth under both 150 mM NaCl and non-NaCl condition compared to non-ECMF treatments. The ECMF inoculated plants also showed fewer symptoms of leaf injury that are characteristic of salt stress compared to non-mycorrhizal seedlings. However, ECMF did not prevent plant oxidative stress compared to non-ECMF, salt stress treatments (Langenfeld-Heyser et al., 2007).

2.6.2 *Frankia* spp. tolerance to salt

Previous studies have isolated *Frankia* spp. strains and *Rhizobium* strains for testing their nitrogen fixation ability on host plants. Fauzia (1999) found under in *vitro* culture, *Frankia* spp. strain CcOI isolated from *Casuarina* species showed NaCl tolerance up to 50 mM and 3 *Rhizobium* strains showed tolerance up to 300 mM NaCl. Tani and Sasakawa (2000) found that *Frankia* spp. strain Ema1 isolated from *Eurybia macrophylla* showed tolerance to 50 mM NaCl, but the cell growth was inhibited at 100 mM NaCl in *vitro* culture. The authors found that *Frankia* spp. hyphae became thick and short under 100 mM NaCl. Tani and Sasakawa (2003) tested *Casuarina equisetifolia* seedlings inoculated with *Frankia* spp. Ceq1 strain. The plants were tolerant to 300 mM NaCl. This *Frankia* spp. strain isolated from *Casuarina equisetifolia* exhibited salt tolerance up to 100 mM NaCl, and the hyphae became thick and short when the salt concentration was above 150 mM NaCl. Hyphae were deformed but the *Frankia* spp. cell salt concentration was less than 30 mM NaCl even in the 500 mM NaCl medium. The results strongly proved that both *Frankia* spp. strain and *Casuarina equisetifolia* seedlings were highly tolerant of a high salt environment and suggests this symbiosis could be used for revegetation in the salt contaminated areas. However, nitrogen fixation can be affected by

salt. Oshone et al. (2013) isolated *Frankia* spp. strain CcI6 from *Casuarina cunninghamiana* and found *Frankia* spp. vesicle production and nitrogenase activity was inhibited by NaCl even though this strain could grow in 300 mM NaCl.

2.6.3 Restoration of salt contaminated soil

Alders have been used for re-vegetation of contaminated lands in several previous studies. Pietrzykowski et al. (2015) successfully tested green alder growing in fly ash disposal sites for 6 years. The author found that the treatment of fly ash mixed with lignite and fertilizer improved the seedling growth compared to control (fly ash only fertilization). Markham (2005) showed that dual symbiosis of *Alnus incana* ssp. *rugosa* with ECMF and *Frankia* spp. had positive effects on host plants when they grew in peat and mine tailings (containing Mn, Bi, Zn, Br and low P, N availability). This might be because the inoculation of *Frankia* spp. and (or) ECMF increased root thickness and absorbed more nutrients (N, P). However, the mechanism for the presence of mycorrhizae benefit to plant performance exposed to mine tailings is still unclear. A review paper concluded that most *Alnus* species inoculated with *Frankia* spp. or/and mycorrhizal fungi could avoid heavy metal contamination (Cu, Zn, Pb, Mn) and the symbionts have positive effect when growing in lignite mine spoil or degraded forest soil (Roy et al., 2007). Although these symbioses have been found to promote host plant growth in contaminated soil, the real mechanism of microbial-host plant interactions need further study. A range of ECMF can play an important role in degrading persistent organic compounds, suggesting ECMF is a good choice for soil remediation (Meharg et al., 2000). Microorganisms in the soil stimulate the rhizodegradation of organic contamination by means of increase microbial activity on plant roots, utilizing contaminants as their source

of energy and nutrients (Meharg et al., 2000; Roy et al., 2007). The hydrolytic enzymes and proteases produced by fungal mycelium and the fungi secondary metabolism could be the major reason for mineral mobilization and organic contaminant degradation (Cairney and Burke, 1994)

Casuarina is another actinorhizal genus that has been used in land reclamation. These plants are naturally found in marine costal areas. Some *Casuarina* species naturally grow in saline areas as well as waterlogged conditions, which is important for plants used in land reclamation due to their salt tolerance (Van Der Moezel et al., 1989). *Casuarina* trees grow fast and are resistant to salt, drought stress and are adapted to live in nutrient poor or salt-affected soil due to their association with mycorrhizal and *Frankia* spp. (Zhong et al., 2010; Diagne et al., 2013a).

A very important goal for choosing actinorhizal plants for soil remediation and decontamination is that the continuous and long-term growth could deliver N fertilizer into soil and promote soil microbial activity. Mycorrhizal fungi associated with actinorhizal alders can be used for reclamation and revegetation in other contaminated areas like saline soil. However, few studies have focused on tripartite associations between plants, mycorrhizal fungi and nitrogen fixing bacteria in low fertility as well as saline soil. In my thesis, I will test the interaction between ECM fungi and *Frankia* on the same host plants (green alder) growing in low fertility and/or saline soil.

Chapter 3. TRIPARTITE ASSOCIATIONS IN ALDER: THE EFFECT OF *FRANKIA* SPP. AND SINGLE OR MULTIPLE ECTOMYCORRHIZAL FUNGI ON *ALNUS VIRIDIS* SSP. *CRISPA* GROWING IN LOW FERTILITY SOIL

3.1 Introduction

Although *Alnus viridis* ssp. *crispa*, *Frankia* spp. and ectomycorrhizal fungi are referred as a tripartite symbiosis, little is known about the relationship between *Frankia* spp. and different types of ectomycorrhizal fungi on a shared host as well as the difference between single and multiple ectomycorrhizal fungi on *Alnus*. I designed an experiment to investigate the effect of dual symbiosis of *Frankia* spp. and ECMF and compared the effect between single and multiple ectomycorrhizal fungi species on a shared host, *Alnus viridis* ssp. *crispa*. The experiment involved in 16 treatments, eight of which were inoculated by both *Frankia* spp. and (single or multiple) ectomycorrhizal fungi and the other eight of which were inoculated by single or multiple ectomycorrhizal fungi but without *Frankia* spp. The objective of this experiment was to determine if dual symbiosis of *Frankia* spp. and mutilple ectomycorrhizal could benefit *Alnus viridis* ssp. *cripsa* growing in low fertility soil.

This experiment was designed to test two key hypotheses. The first is that mycorrhizal fungi help meet P requirement of nodulation, increasing the effectiveness of nitrogen fixing symbiosis. This in turn increases the nutritional status of the plant and increases the fungal colonization rate (Mejstrik and Benecke, 1969; Chatarpaul et al., 1989). The overall result is that a tripartite symbiosis among ectomycorrhizal fungi, *Frankia* spp. and *Alnus viridis* ssp. *crispa* would have more benefits compared to single inoculation by just *Frankia* spp. or ECMF. The second hypothesis is that mycorrhizal fungi species show niche complementary (Jonsson, 2001). Therfore multiple

ectomycorrhizal fungi will increase plant growth compared to single ectomycorrhizal fungal species.

3.2 Material and Methods

3.2.1 Plant material

Seeds of *Alnus viridis* ssp. *crispa* collected from the Sandilands Provincial forest were sterilized with Hurek Seed Sterilization Solution (composition: 300mL of commercial sodium hypochlorite, 1 g Na_2CO_3, 30 g NaCl, 1.5 g NaOH per liter distilled water) for 15 min and rinsed with sterile distilled water twice. Surface-sterilized seeds were then placed on moist autoclaved Turface and stratified in a fridge for at least 2 weeks. The seeds were sown into trays with sterilized peatmoss and perlite (3:1 V/V). The trays were watered daily and fertilized weekly with a modified Rorison nutrient solution (1 mM $Ca(NO_3)_2$, 1 mM $CaCl_2$, 1 mM K_2HPO4, 0.0534 mM Fe-EDTA, 0.009 mM $MnSO_4$, 0.0045 mM H_3BO_3, 0.001 mM Na_2MoO_4, 0.0015 mM $ZnSO_4$, 0.0015 mM $CuSO_4$) (Hendry and Grime, 1993). After 2 months, the seedlings were then transplanted to D40H Deepots (6.4 cm diameter, 25.4 cm height, Steuwe and Sons, Oregon) containing 500 mL of field-collected sterilized soil.

3.2.2 Soil material

Mineral soil was collected from the Sandilands Provincial Forest in southeast Manitoba, from a *Pinus banksiana* forest that has *Alnus viridis* ssp. *crispa* growing in the understory. Soil samples were air-dried and then sieved to 2mm. The inorganic N (ammonium and nitrate), available phosphate, pH and soil texture were measured on air-dried samples. I used the micro diffusion technique to measure soil inorganic nitrogen (Khan et al. 2000). Briefly, 100 mL of 2 M NaCl were used to extract NH_4^+ and NO_3^-

from 10 g of soil. Devarda's alloy (0.2g) was used to reduce nitrate to ammonium and the ammonium was volatilized to ammonia with 0.2 g MgO. The amount of ammonia diffusing into a boric acid indicator was then determined by titration with 0.0025 M H_2SO_4. Soil particle-size was analyzed using the hydrometer method (Kalra, 1991). Phosphate was extracted using Bray and Kurtz No. 1 solution and then filtered through #42 Watman filter paper and measured using the Murphy Riley technique (Kalra, 1991). Soil pH was measured in a distilled water suspension at a ratio of 25 mL water to 10 g soil (Huang et al., 2011). Soil used to grow the plants was sterilized in an autoclave at 121 °C for 1 h.

3.2.3 ECMF and *Frankia* spp. material

Fungal strains were obtained from University of Alberta mycological Herbarium (UAMH). Three fungal strains were used: *Lactarius tomimosus* (Lto) (UAMH 6077), *Lactarius theiogalus* (Lth) (UAMH 5920), and *Hebeloma crustuliniforme* (Hcr) (UAMH 6064)) were selected as they had all been isolated from sporocarps found under *Alnus rugosa*. The fungal strains were maintained at 25 °C in the dark for over 2 months on plates containing Modified Melin Norkrans (MMN) agar medium (composition: glucose 2.5 g, malt extract 2.0 g, yeast extract 1.0 g, potassium phosphate monobasic (KH_2PO_4) 0.5g, ammonium phosphate dibasic ($(NH_4)_2HPO_4$) 0.25 g, magnesium sulphate ($MgSO_4$) 0.15g, calcium chloride ($CaCl_2$) 0.05 g, sodium chloride (NaCl) 0.025 g, ferric cholide ($FeCl_3$) 0.012 g, agar 15 g, per liter distilled water).

Since it is difficult to isolate *Frankia* from host plants (Pawlowski, 2009), I used crushed nodules as a source of *Frankia*, hereafter referred to as *Frankia* spp. The *Frankia* spp. inoculum came from freeze-dried and frozen spore negative nodules harvested from

Alnus roots. They were surface sterilized for 5min with Hurek Seed Sterilization Solution. They were then crushed in a phosphate buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4).

3.2.4 Experiment design

The aim of the experiment was to look at the interaction among different fungal species with *Frankia* spp. and to compare single ECMF species with a mix of species inoculated on the seedlings. The treatments are summarized in the table 3.1. The 8 combinations of ECMF treatments were inoculated with and without *Frankia* spp. Control plants received no inoculant. *Frankia* spp. inoculated plants received 4 mg of crushed nodules at a concentration of 2 mg nodules mL^{-1} PBS Buffer. Each plant was inoculated by injecting 2 mL of well-fragmented inoculum suspension on the surface of the soil. For the ECMF inoculated plants, three 3.9 mm diameter by ca. 7 mm height MMN agar plugs with actively growing hyphae were placed next to the upper roots of the plant. Each plant received the same number of plugs. A treatment with one fungal species got three plugs of the same fungus, a treatment with two fungal species got one and half plugs of each fungus, and a treatment with three fungal species got one plug of each fungus. Control treatments got three MMN plugs without any fungal hyphae. Tripartite plants were inoculated with *Frankia* spp. and ECMF at the same time when transplanted.

Table 3.1: Fungal species combinations with and without *Frankia* spp.

Non-*Frankia* spp.	*Frankia* spp.
non ECMF	non ECMF
Lactarius tomimosus (Lto)	Lto
Lactarius theiogalus (Lth)	Lth
Hebeloma crustuliniforme (Hcr)	Hcr
Lto+Lth	Lto+Lth
Lto+Hcr	Lto+Hcr
Lth+Hcr	Lth+Hcr
Lto+Lth+Hcr	Lto+Lth+Hcr

3.2.5 Growing conditions and harvest

The plants were grown with a 16 hour light: 8 hour dark photoperiod. The light intensity was gradually increased through lowering the height of fluorescence lights above the plants in the first two weeks after transplanting. The light ranged from 160 to 200 μmol photons m^{-2} s^{-1}. The plants were switched in terms of position on the bench every week to prevent the position in the room and variation in light from having an influence on the performance of the plants. The temperature ranged from 20 °C during the night to 24 °C during the day. For the first 4 weeks after transplanting, plants were watered with 15 mL distilled water every day. One month later, plants were watered with 25 mL distilled water every second day.

The plants were grown for 15 weeks after inoculation. Height from soil surface to the shoot apex and shoot basal diameter were measured weekly, starting the 6^{th} week after transplanting i.e., once the stems had started to harden. The ruler and the caliper used for measurement were sterilized with 70% alcohol to prevent contamination between measurements. At harvest, the plants were separated into roots, leaves and stems. Roots with attached nodules were cleaned of soil with room temperature water. Root respiration was measured with their nodules attached followed by measurements on the detached nodules alone. The process was started by filling a bag with fresh air at room temperature (22 °C) to provide a constant CO_2 concentration. The air was pumped from the bag and flowed at 0.2 L min^{-1} over the roots and/or nodules sealed in a 50 mL syringe. The air stream was then passed over a desiccant and the CO_2 concentration measured using a CO_2 analyzer (Qubit S151 CO_2 Analyzer). Typically, it took 5 minutes for the CO_2 levels to stabilize in the system. Respiration rates were calculated by taking

the difference in the CO_2 concentrations before and after the samples were added to syringe. CO_2 levels were converted from ppm to μmol CO_2 h^{-1} g^{-1} tissue dry mass using the following formula: (($CO_{2final} - CO_{2initial}$)*0.2 L min^{-1} *60)/(22.413 L/mol*((273+22)/273)* tissue dry mass g). CO_{2final} represents the final stable CO_2 level. $CO_{2initial}$ represents the fresh air CO_2 level in the bag. After measuring respiration, acetylene reduction assays were immediately performed on the nodules following Markham and Zekveld (2007). The fresh nodules were placed in a 50 mL bottle and sealed with a rubber septa. Then 5 mL of acetylene gas was injected to the bottle in exchange for 5 mL air to make a 10% acetylene atmosphere. The bottles were incubated for 1 h at 22 °C. After 1 h incubation, a 5 mL gaseous sample from each bottle was collected, and analyzed for acetylene and ethylene with gas chromatograph (Varian 450) with a Haysep T column, FID and 0.25 ml gas-sampling valve. The specific nodule activity was expressed as the ethylene production rate per nodule dry mass (μmol ethylene g^{-1} nodule h^{-1}). Nodule allocation was calculated as the nodules percentage of total dry mass. Root acid phosphatase activity was measured according to Treseder and Vitousek (2001). Root samples were placed with 2.5 mL of 3.5 mM para-nitrophenyl phosphate (p-NPP) with 100 mM modified universal buffer (pH 6.5) (buffer composition: 1.26 g boric acid, 2.8 g citric acid, 2.32 g maleic acid, 2.42 g Trizma base, 62 mL of 1 M NaOH, modified universal buffer pH adjusted with 37% HCl, and made up to 1L volume). Roots were incubated in the dark at room temperature for 1 h, after which 1 mL 1M NaOH was added to stop the reaction. A blank contained the same amount of p-NPP and NaOH without roots. The roots were removed from solution and the solution was frozen. After thawing, the solution was filter through No. 1 filter paper and the

absorbance measured at 410 nm on a spectrophotometer (U-2100 pro). The results were compared to a standard curve of para-nitrophenyl (p-NP). The phosphatase enzyme activity was expressed as μmol p-NP per hour per gram of dried root. The roots were divided into two halves, half for ECMF colonization rate measurement (stored in 70% alcohol) and half for root fresh and dry mass measurements. The ratio of ½ fresh to ½ dry root mass and total root fresh mass were used to calculate total root dry mass.

Stems, leaves, nodules and half roots were freeze dried after harvesting. Since the ECMF colonization rate was so low and much of it was superficial, quantifying the colonization rate per root length was not practical. So I only examined roots for the presence or absence of mycorrhizae, indicated by the presence of a Hartig net. The roots were observed under a dissecting microscope and up to 10 pieces of potential mycorrhizal roots were mounted on the slide and examined at 400X. Roots were considered potentially mycorrhizae when they had light to dark brown flexuous fine roots or succulent fibrous root or root tips (according to Molina et al., 1994; Becerra et al., 2009; Montoya et al., 2015).

3.2.6 Data analysis

Some control plants (without *Frankia* spp. inoculum) that formed nodules were excluded from the analysis. Plant relative growth rate was determined using the formula $(\text{Log Height}_{t2} * \text{Diameter}_{t2}^{2} - \text{Log Height}_{t1} * \text{Diameter}_{t1}^{2}) / (\text{days})$ (Hunt, 1979). According to residual plots there was a large difference in variation between treatments and the data were therefore log transformed. The data were analyzed using a two-factor ANOVA model followed by Tukey's HSD test (a post-hoc test) with the combination of fungal species as one treatment and *Frankia* spp. inoculation as the other treatment. A second

model used the number of fungal species inoculated on the plants as a continuous variable to determine if plant performance increased with the number of fungal species increasing, regardless of the fungal species identity. The relationship between different parameters was tested by linear regression analysis. The proportion of plants forming Hartig net was analyzed using chi-square test.

3.3 Results

3.3.1 Soil properties

Soil inorganic N and P were low, 10.2±0.6 mg/kg and 0.98±0.33 mg/kg, respectively. Almost 99.8% of the soil was made up of sand. Soil pH was 6.39±0.12.

3.3.2 Colonization

3.3.2.1 Nodulation

All the plants inoculated with *Frankia* spp. formed nodules (Table 3.2). There was no significant difference on mean nodule numbers per root system ($F=1.68$, $P=0.13$) (Table 3.2), nodule number per plant total biomass ($F=1.17$, $P=0.33$) (Table not shown), and mean nodule dry mass per nodule ($F=1.08$, $P=0.39$) (Table not shown) between ECMF treatments. Different types of ECMF had significant effects on nodules dry mass per plant ($F=5.567$, $P<0.0001$) and nodule allocation ($F=2.4785$, $P=0.0254$) (Table 3.2). On plants inoculated by a combination of *Lactarius torminosus* and *Hebeloma crustuliniforme*, nodule dry mass was decreased by 47.45% compared to control. Plants with just *Hebeloma crustuliniforme* inoculation had a significantly higher nodule allocation than plants inoculated with a combination of *Lactarius torminosus* and *Hebeloma crustuliniforme.*

Table 3.2: The effect of different ECMF types on nodule numbers, nodule dry mass and allocation (mean ± SE)

ECMF Type	Nodule dry mass per root system (g)	Nodule number per root system	Nodule allocation per plant (%)
No ECMF	0.059 ± 0.005 [a b]	20.4 ± 3.4	2.07 ± 0.15 [a b]
Lto	0.066 ± 0.005 [a]	21.0 ± 3.1	2.28 ± 0.14 [a]
Lth	0.052 ± 0.005 [a b c]	10.3 ± 3.2	1.98 ± 0.14 [a b]
Hcr	0.059 ± 0.005 [a b]	17.5 ± 3.1	2.22 ± 0.14 [a]
Lto+Lth	0.059 ± 0.005 [a b]	16.0 ± 3.4	2.15 ±0.15 [a b]
Lto+Hcr	0.031 ± 0.005 [c]	9.7 ± 3.2	1.55 ±0.14 [b]
Lth+Hcr	0.050 ± 0.005 [a b c]	15.3 ± 3.1	2.05 ±0.14 [a b]
Lto+Lth+Hcr	0.040 ± 0.005 [b c]	17.3 ± 3.1	2.01 ±0.14 [a b]

Note: Different letters within a column shows significant difference ($P<0.05$)

3.3.2.2 Mychorrhizae

The ECMF inoculated plants had only superficial colonization, without visible mantle (Figure 3.0). The proportion of plants that showed Hartig net formation was significantly higher in *Frankia* spp. inoculation treatments than in non-*Frankia* spp. treatments (Pearson $X^2 = 10.139$, P=0.0015) (Table 3.3). The proportion of plants having a Hartig net in *Frankia* spp. and ECMF treatments was around 50%, which was nearly twice as much as the ECMF treatments. ECMF species also had a significant effect on Hartig net formation (Pearson $X^2 = 22.40$, P=0.0022) (Table 3.4). Treatments that included *H. crustuliniforme* in the inocula had higher level of Hartig net formation (around 50%) compared to non-*H. crustuliniforme* treatments (22.6%) ($X^2 = 22.40$, P=0.0022) (Table 3.4). *Lactarius torminosus*+*Hebeloma crustiliniform* inoculated treatments had the highest percentage of plants forming Hartig nets among different ECMF types ($X^2 = 22.40$, P=0.0022) (Table 3.4)

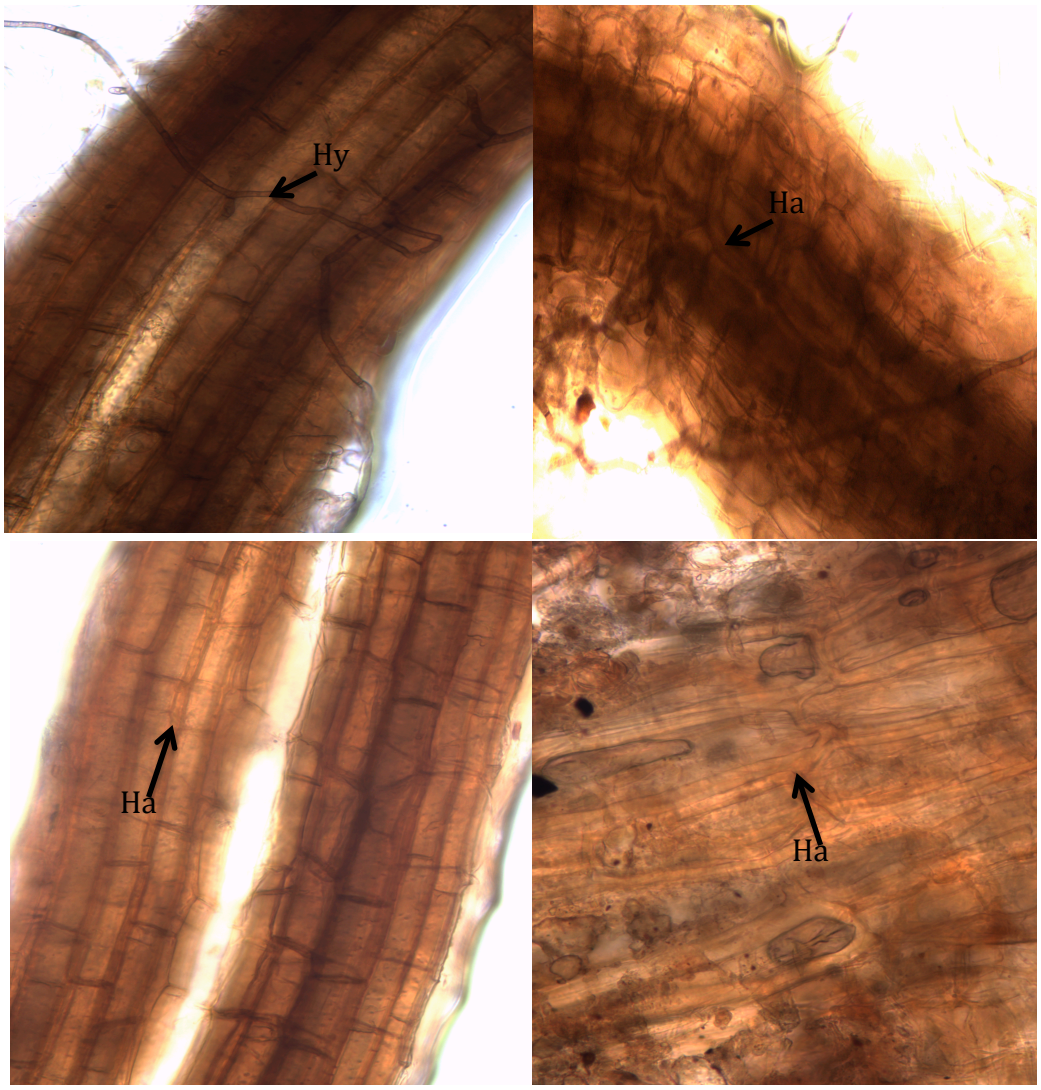

Figure 3.0: Examples of host plant roots with Hartig net formation.
Hy: Hyphae. Ha: Hartig net.

Table 3.3: Proportion ECMF inoculated plants with Hartig net formation in the two *Frankia* spp. inoculation treatments. Treatments with a different letter were significantly different according to a chi-square test. P=0.0015

Treatment	Hartig net formation (%)
Non-*Frankia* spp.	21 b
Frankia spp.	52 a

Table 3.4: Proportion of plants having Hartig net formation in the different ECMF treatments, averaged by the two *Frankia* spp. inoculation treatments. P=0.0022 (according to a chi-square test).

ECMF type	Hartig net formation (%)
Lto	11
Lth	39
Hcr	44
Lto+Lth	19
Lto+Hcr	61
Lth+Hcr	47
Lto+Lth+Hcr	47

3.3.3 The relationship between mycorrhizae formation and nodulation

The presence of a Hartig net showed significant negative relationship with nodule number per root system (F=13.03, P=0.0006) (Figure 3.1), and nodule allocation (F=5.74, P=0.02) (Figure 3.2). Plants with a Hartig net showed a decrease in nodule number almost by half compared with plants with no Hartig net formation (Figure 3.1). There was also a decrease in terms of nodule allocation in plants with a Hargtig net (Figure 3.2). There was no relationship between Hartig net presence and nodule dry mass per root system (F=3.94, P=0.0511) (Figure 3.3).

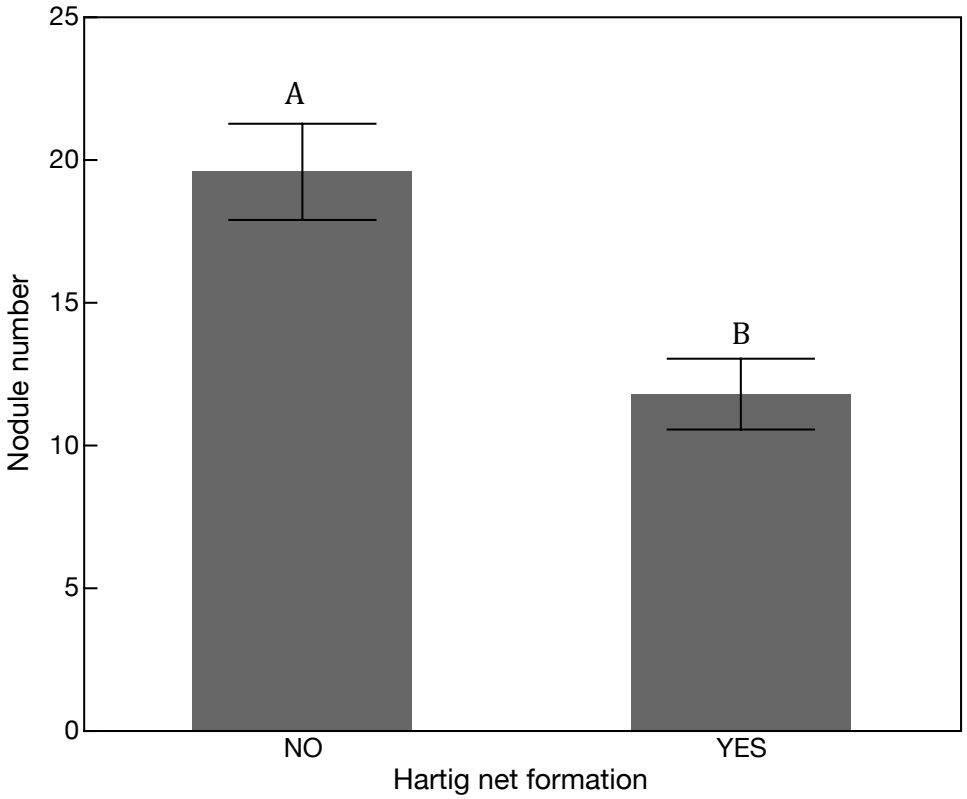

Figure 3.1: The relationship between Hartig net presence and nodule number per root system. Treatments with different letter were significantly different at the 0.05 level according to two-way ANOVA.

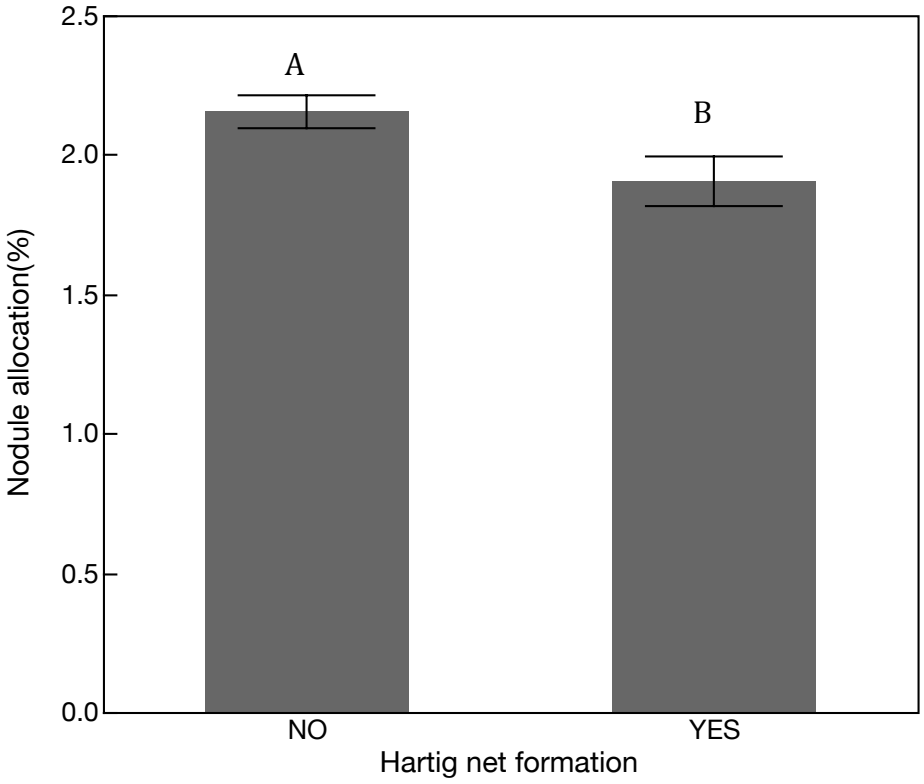

Figure 3.2: The relationship between Hartig net presence and nodule allocation as a percent of total biomass. Treatments with different letter were significantly different at the 0.05 level according to a two-way ANOVA.

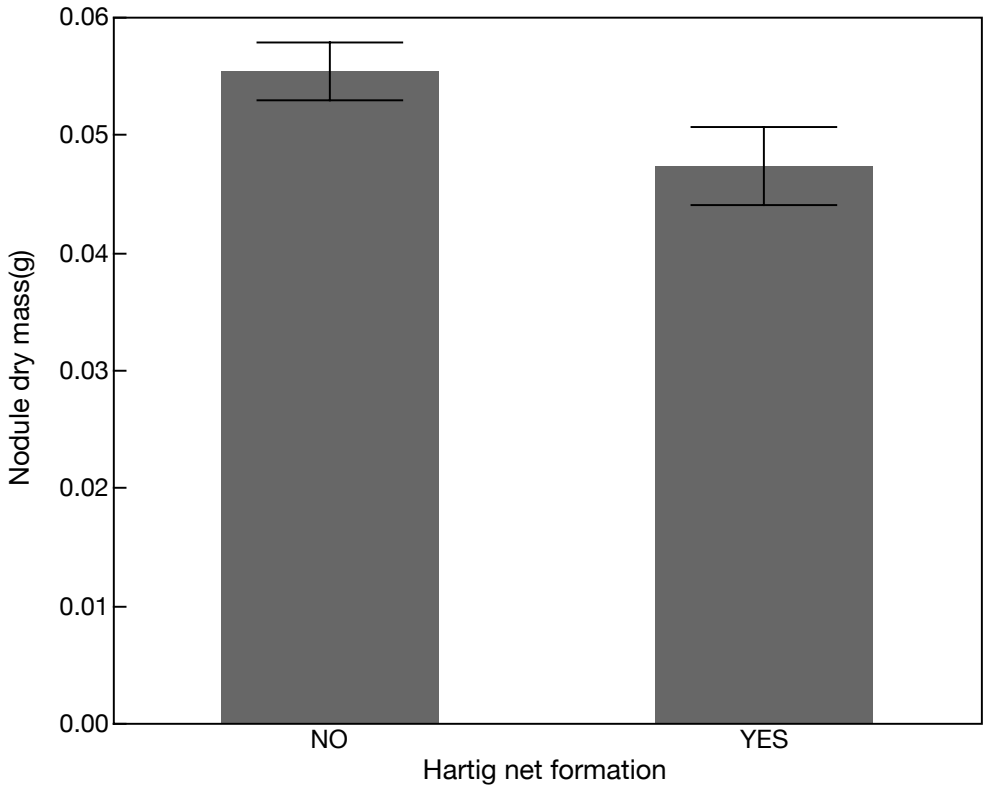

Figure 3.3: The relationship between Hartig net presence and nodule dry mass per root system.

3.3.4 Plant size

3.3.4.1 The effect of *Frankia* spp. and ECMF type on plant performance

Frankia spp. inoculation significantly increased plants total biomass ($F=82.1191$, $P<0.0001$). There was also a significant difference in plant total biomass among different ECMF type treatments ($F=4.8315$, $P<0.0001$). However, there were no interactive effects between ECMF type and *Frankia* spp. on plant total biomass ($F=1.1007$, $P=0.3675$) (Figure 3.4, Figure 3.5).

The mean total biomass for *Frankia* spp. inoculated plants was 2.51±0.06 g, a significantly increase in plant total biomass by 48.5% compared to non-*Frankia* spp. treatments (Figure 3.4). Whenever *Hebeloma crustuliniforme*, in comination with any of other two ECMF species was inoculated on plants, total biomass was significantly decreased by 22.13% - 30.03% compared to plants inoculated with *Lactarius torminosus* treatment (Figure 3.5). Comparing different ECMF treatments to the control, there was no significant difference in terms of plant total biomass. Overall, ECMF had less effect on plant size than *Frankia* spp. The increase in total biomass due to *Frankia* spp. inoculation (48.5% increase compared to controls) was greater than the reduction (28.05%) in total biomass resulting from ECMF inoculation. The presence of a Hartig net did not affect plant total biomass ($F=0.44$, $P=0.51$), 2.21±0.10 g plant^{-1} with Hartig net presence and 2.12±0.08 g^{-1} plant without Hartig net presence (Figure not shown).

The trends in terms of shoot dry mass affected by ECMF was the same as that in total biomass discussed above. Shoot dry mass varied significantly among the different ECMF treatents ($F=5.7992$, $P<0.0001$) (Figure 3.6). Whenever *Hebeloma crustuliniforme* was inoculated together with other 2 ECMF species, shoot dry mass was significant

decreased by over 20.0% compared to plants inoculated by *Lactarius torminosus* (Figure 3.6). *Frankia* spp. inoculation had a positive effect on plants shoot dry mass ($F=143.5702$, $P<0.0001$) (Figure 3.7). *Frankia* spp. inoculated plants had a significant higher shoot dry mass, 1.8±0.04 g^{-1} plant, which was 1.70 times greater than non-*Frankia* spp. inoculated plants (Figure 3.7). There was no ECMF and *Frankia* spp. interaction on shoot dry mass ($F=1.6015$, $P=0.1419$).

Frankia spp. inoculated plants root dry mass (excluding nodules) was 0.66±0.03 g, and did not differ from root dry mass of non-*Frankia* spp. inoculated plants (0.63±0.03 g) ($F=0.41$, $P=0.52$) (Figure 3.8). The ANOVA model showed a significant effect of the ECMF treatment ($F=2.1312$, $P=0.046$) on plant root dry mass (excluding nodules) but the post hoc test showed no differences between any of the treatments (Figure 3.9). There was no interaction between ECMF and *Frankia* spp. treatments on root mass ($F=0.54$, $P=0.80$).

Nodule dry mass was positively related to total biomass ($R^2 = 0.62$, $P<0.0001$), shoot dry mass ($R^2 = 0.67$, $P<0.0001$), root dry mass ($R^2 = 0.22$, $P<0.0001$) (Figure 3.10). There was a positive correlation between nodule allocation and shoot dry mass ($R^2 = 0.06$, $P = 0.03$) (Figure not shown).

ECMF and *Frankia* spp. had an interactive effect on plant root to shoot ratio ($F=2.65$, $P=0.0141$) (Figure 3.11). When plants were not inoculated with *Frankia* spp., only *Lactarius theiogalus* inoculated plants had a higher proportion of roots than non-ECMF, *Lactarius torminosus*, and the 2 ECMF species inoculated treatments (*Lactarius torminosus* + *Lactarius theiogalus*, *Lactarius torminosus* + *Hebeloma crustuliniforme*, *Lactarius theiogalus* + *Hebeloma crustuliniforme*). When plants were inoculated with

Frankia spp., ECMF type had no effect on the proportion of roots. *Frankia* spp. inoculation decreased root : shoot ratio only when plants were inoculated with *Lactarius theiogalus*, *Hebeloma crustuliniforme*, or all three ECMF species.

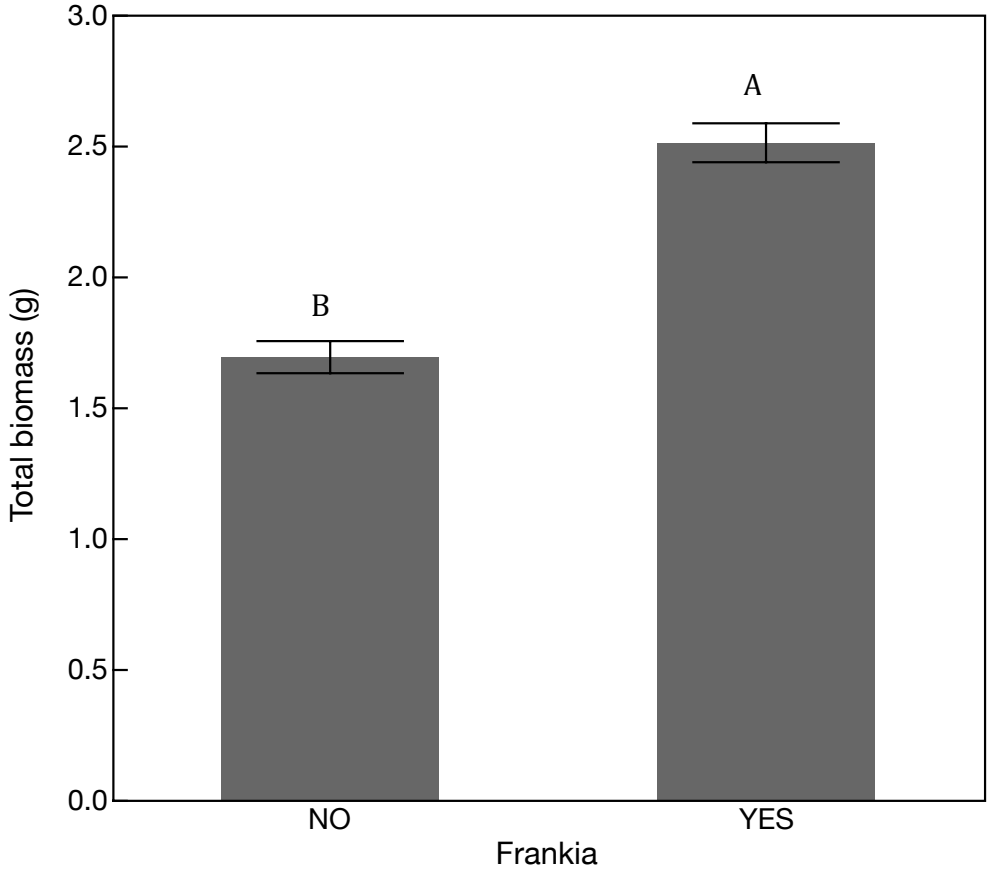

Figure 3.4: The effect of *Frankia* spp. on total biomass of *Alnus viridis* ssp. *crispa* averaged across all ECMF treatments. Treatments with different letter were significantly different at the 0.05 level according to a two-way ANOVA.

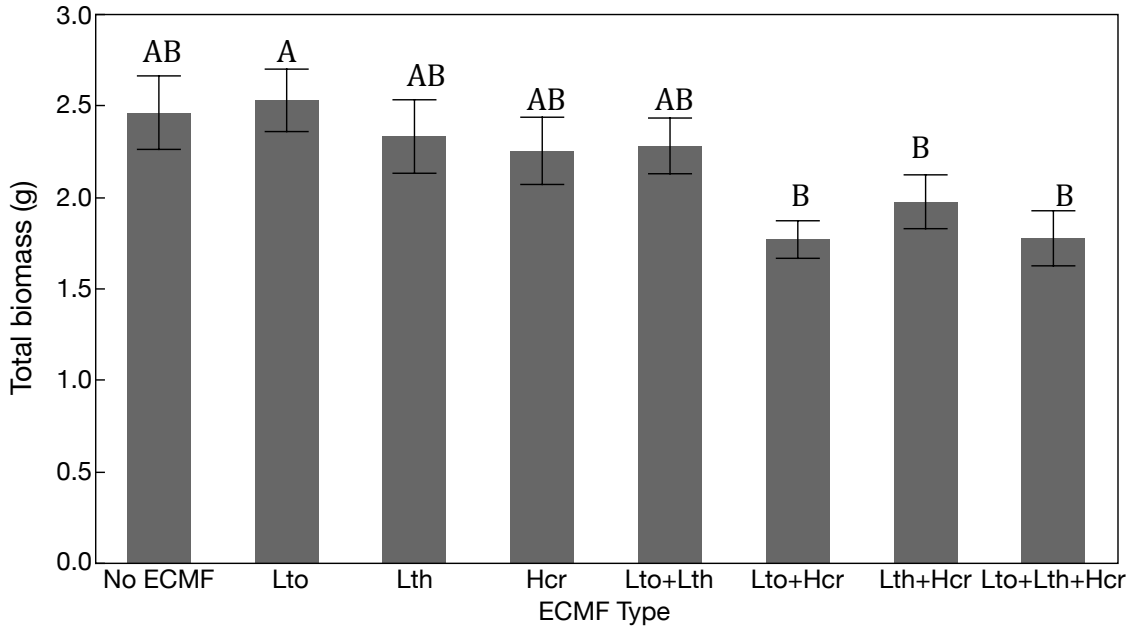

Figure 3.5: The effect of different ECMF type on total biomass of *Alnus viridis* ssp. *crispa* plants averaged among the two *Frankia* spp. inoculation treatments. No ECMF - plants were not inoculated with any ECMF. Lto- *Lactarius torminosus*. Lth - *Lactarius theiogalus*. Hcr- *Hebeloma crustuliniforme*. Treatments with different letters were significantly different at the 0.05 level according to a Tukey HSD test.

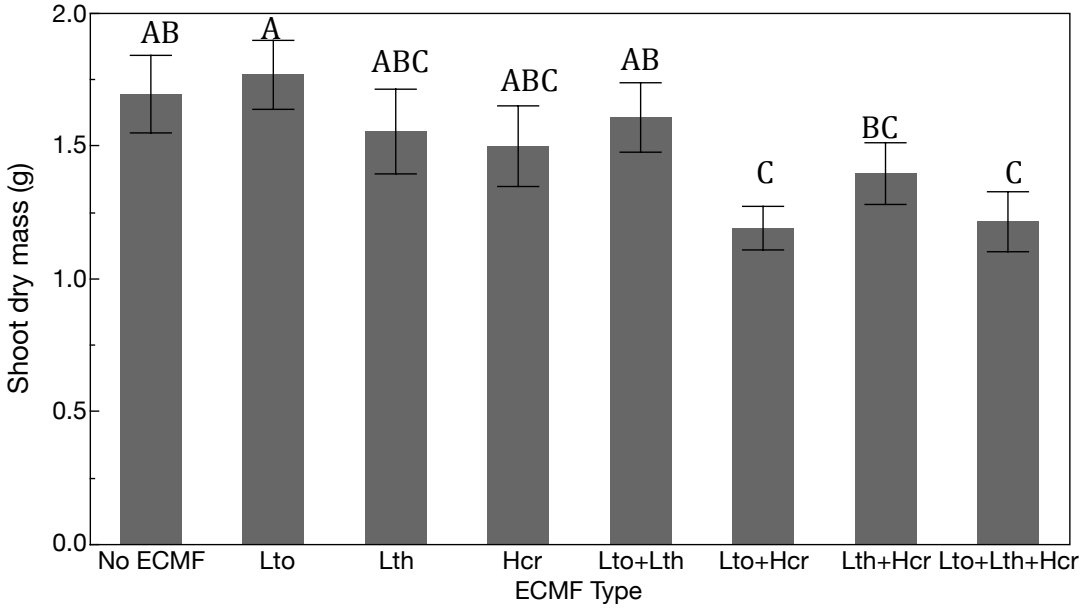

Figure 3.6: The effect of different ECMF type on shoot dry mass of *Alnus viridis* ssp. *crispa* colonized by different ECMF treatments averaged among the two *Frankia* spp. inoculation treatments. No ECMF - plants were not inoculated with any ECMF. Lto- *Lactarius torminosus*. Lth - *Lactarius theiogalus*. Hcr- *Hebeloma crustuliniforme*. Treatments with different letters were significantly different at the 0.05 level according to a Tukey HSD test.

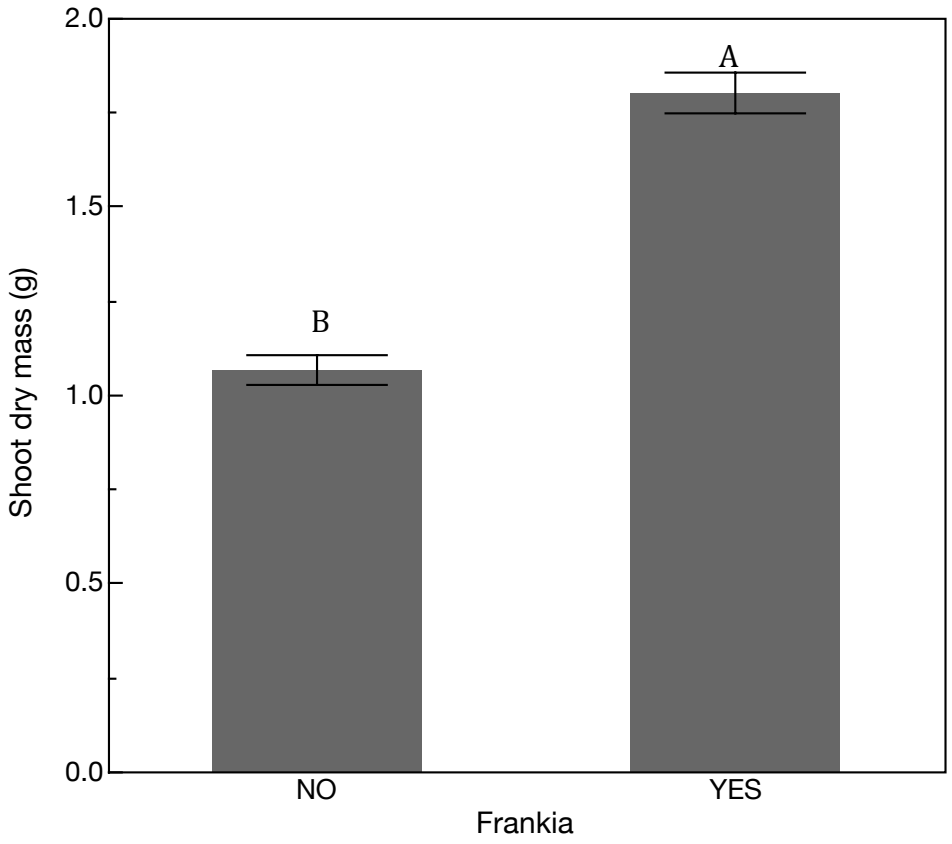

Figure 3.7: The effect of *Frankia* spp. on shoot dry mass of *Alnus viridis* ssp. *crispa* colonized by *Frankia* spp. averaged across all ECMF inoculation treatments. No-*Frankia* spp. = plants were not inoculated with *Frankia* spp. YES-*Frankia* spp. = plants were inoculated with *Frankia* spp.

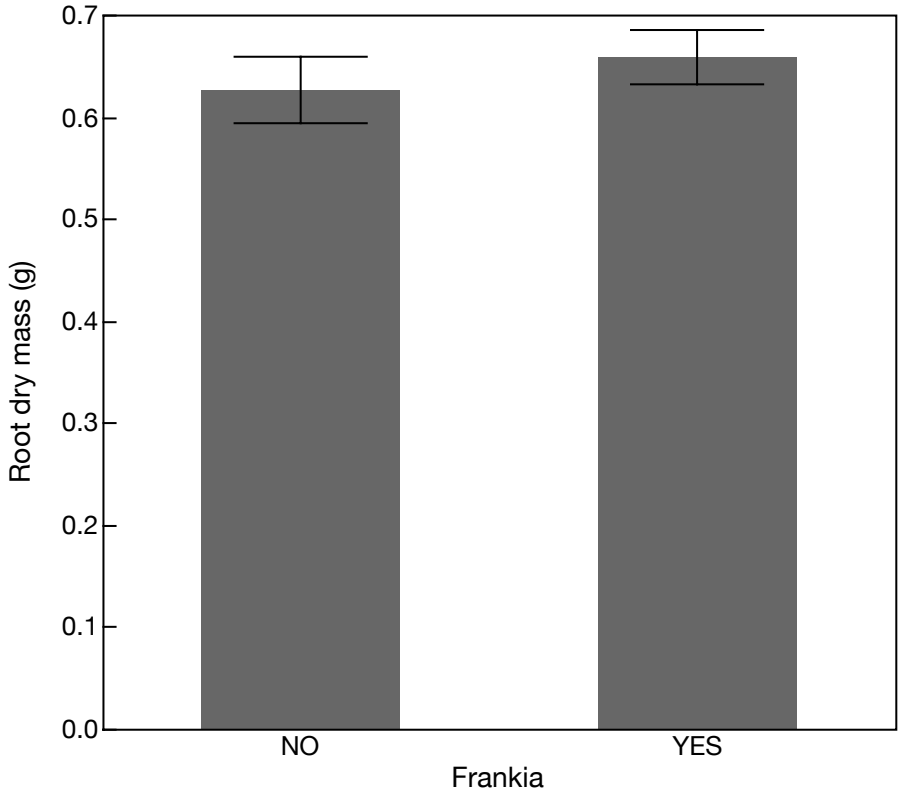

Figure 3.8: The effect of *Frankia* spp. on root dry mass of *Alnus viridis* ssp. *crispa* averaged across all ECMF treatments.

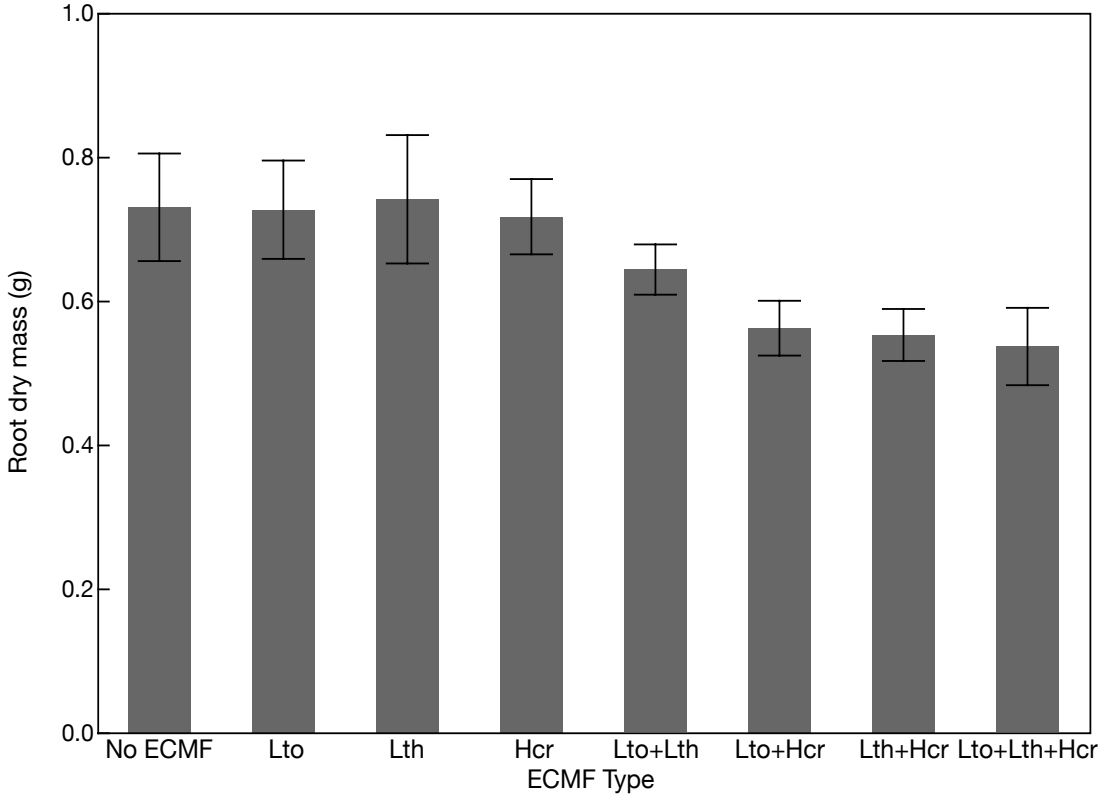

Figure 3.9: The effect of different ECMF type on root dry mass of *Alnus viridis* ssp. *crispa* averaged among the two *Frankia* spp. inoculation treatments. No ECMF - plants were not inoculated with any ECMF. Lto- *Lactarius torminosus*. Lth - *Lactarius theiogalus*. Hcr- *Hebeloma crustuliniforme*.

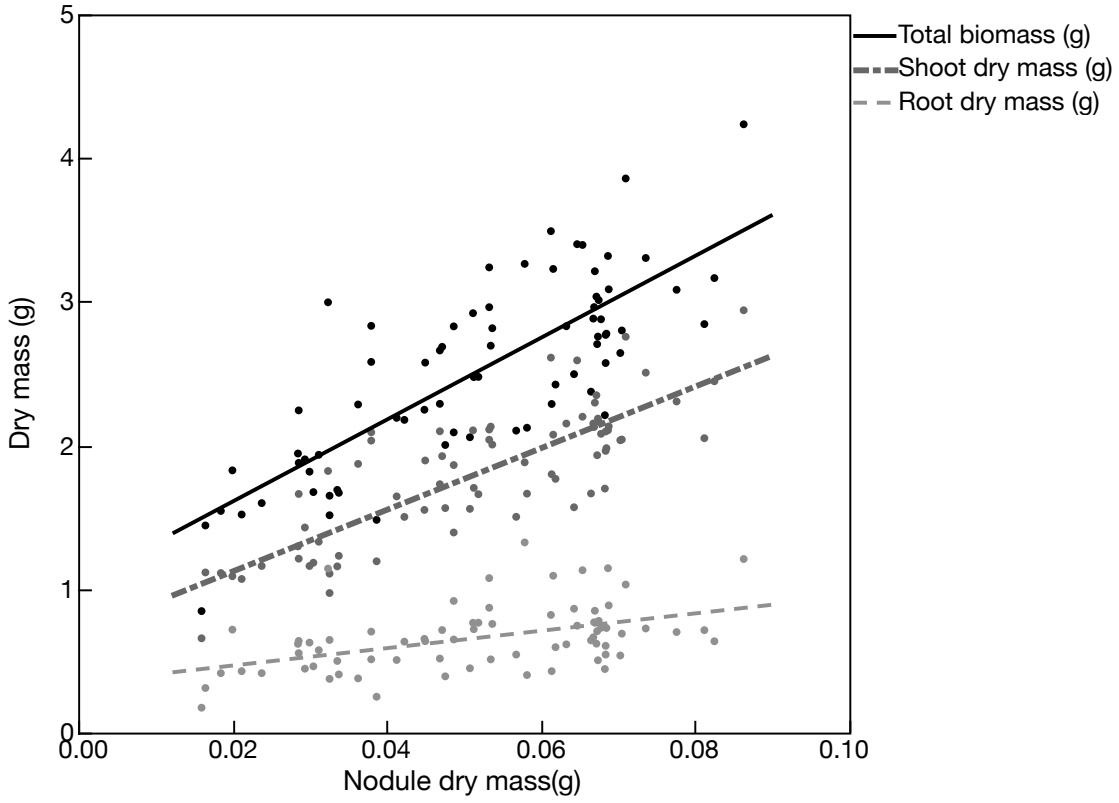

Figure 3.10: The relationship between nodule dry mass and total ($R^2 = 0.62$, $P<0.0001$), shoot ($R^2 = 0.67$, $P<0.0001$), root dry mass ($R^2 = 0.22$, $P<0.0001$) of *Alnus viridis* ssp. *crispa.*

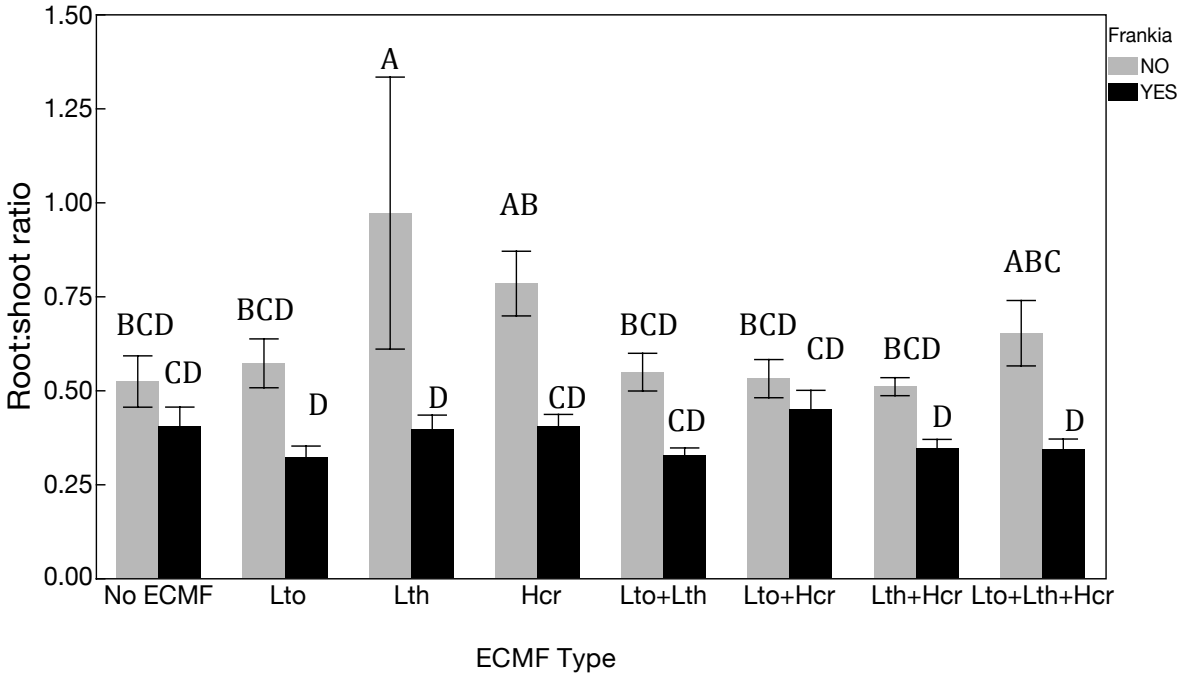

Figure 3.11: The effect of *Frankia* spp. and different ECMF type interaction on root:shoot ratio of *Alnus viridis* ssp. *crispa.*

3.3.5 Nitrogen fixation

Different types of ECMF inoculation did not have a significant effect on specific nodule activity i.e., ethylene production per mass of nodule tissue (F=0.96, P=0.4651) (Figure 3.12), (F=0.92, P=0.50) (Figure 3.13), or total nodule activity i.e., ethylene production per plant (F=1.30, P=0.27) (Figure 3.14).

Nodule number per plant was positively correlated with specific nodule activity (R^2=0.11, P=0.005) (Figure not shown) and total nodule activity (R^2=0.20, P=0.0001) (Figure 3.15).

Nodule dry mass per plant was not related to specific nodule activity (μmol h^{-1} g^{-1} nodule) (R^2=0.001, P=0.7784) (Figure not shown) but was positively correlated to total nodule activity (nmol h^{-1} $plant^{-1}$) (R^2=0.12, P=0.0042) (Figure 3.16).

Nodule allocation was not related to specific nodule activity (R^2=0.03, P=0.14) (Figure not shown) but was positive correlated to total nodule activity (R^2=0.17, P=0.0005) (Figure 3.17).

There was a strong liner relationship between specific nodule activity and total nodule activity (R^2=0.82, P<0.0001) (Figure 3.18), ethylene production per plant mass and total nodule activity (R^2=0.91, P<0.0001) (Figure 3.19).

Specific nodule activity was not related to total biomass (R^2=0.009, P=0.44) (Figure not shown), Shoot dry mass (R^2=9.3*e^{-6}, P=0.98) (Figure not shown), but was negatively correlated to root dry mass (R^2=0.07, P=0.03) (Figure 3.20).

Total nodule activity was not related to total biomass (R^2=0.02, P=0.24) (Figure not shown), root dry mass (R^2=0.02, P=0.22) (Figure not shown), However, total nodule activity was positive correlated to shoot dry mass (R^2=0.07, P=0.03) (Figure 3.21).

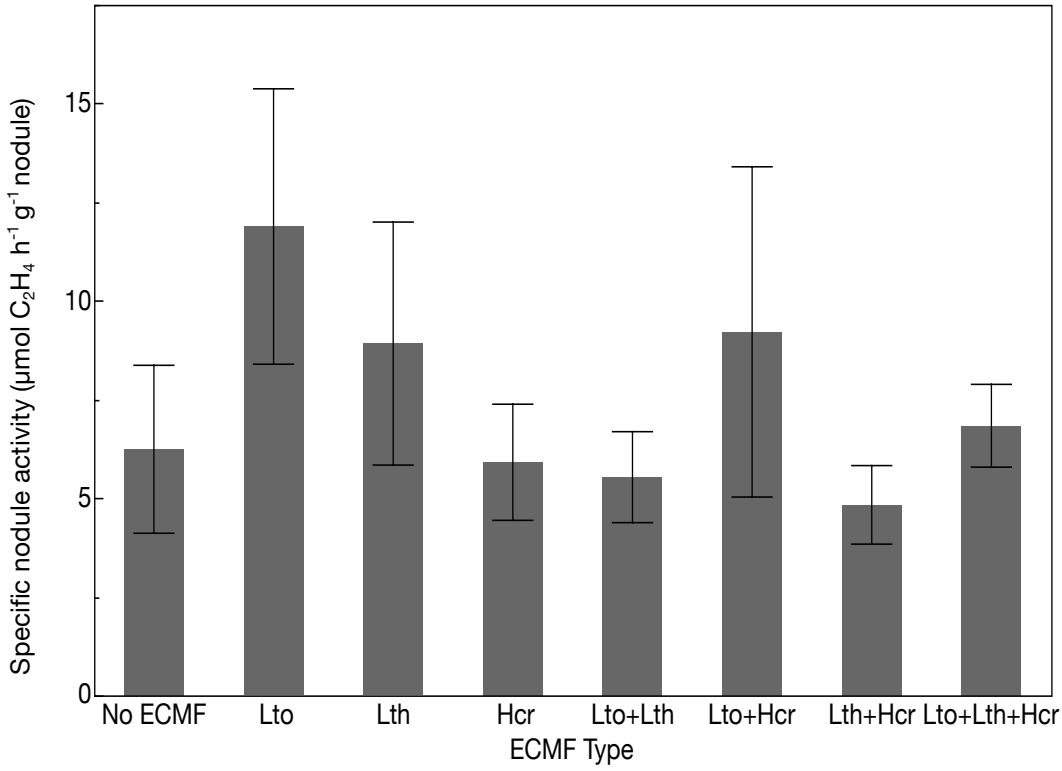

Figure 3.12: Specific nodule activity reflecting nitrogenase activity for nodualted plants *Alnus viridis* ssp. *crispa* in different ECMF treatments. There was no significant difference in specific nodule activity among different types of ECMF.

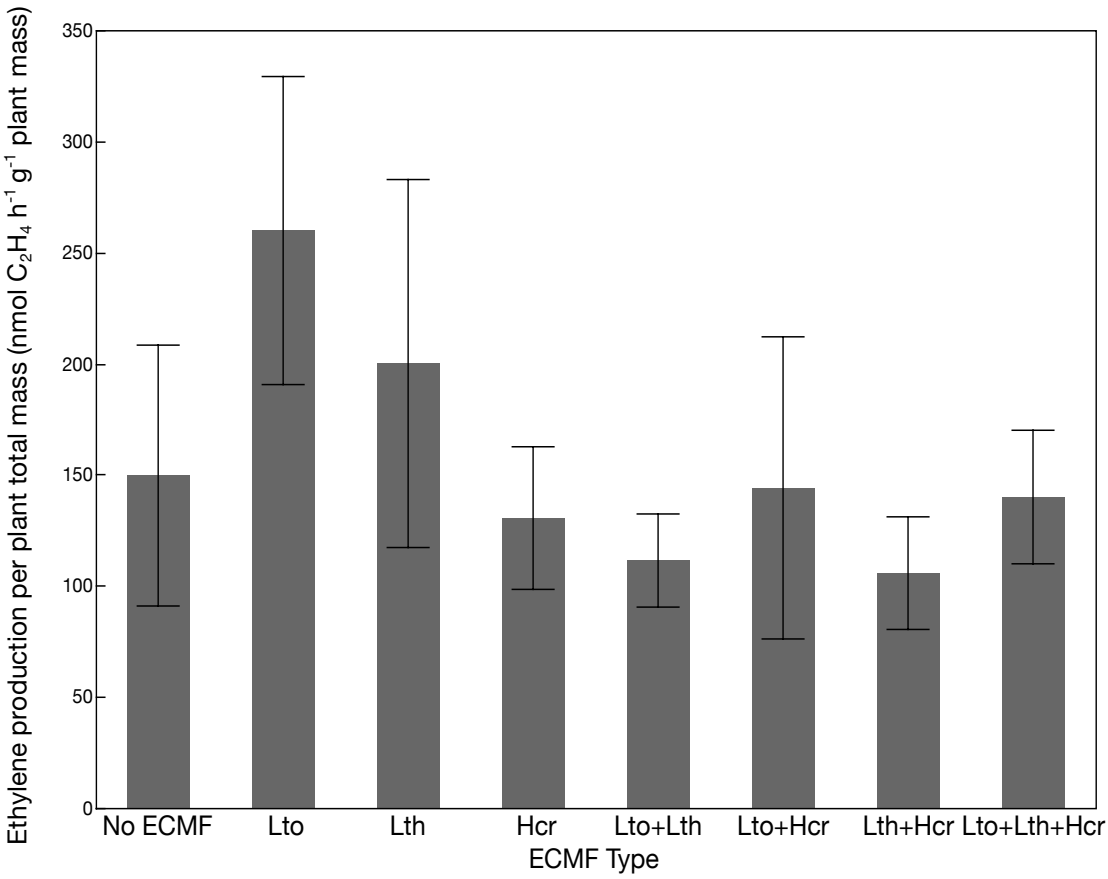

Figure 3.13: Ethylene production rate per plant mass for nodualted plants *Alnus viridis* ssp. *crispa* in different ECMF treatments. There was no significant difference in ethylene production per total plant mass among different ECMF treatments.

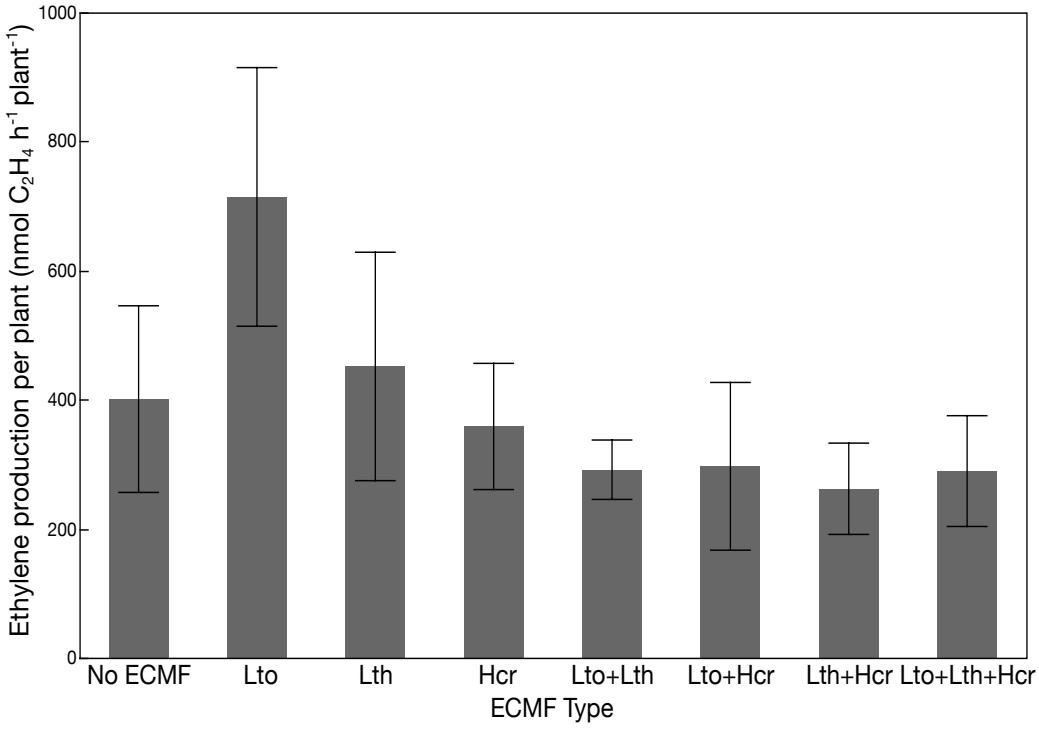

Figure 3.14: Ethylene production rate per plant for nodualted plants *Alnus viridis* ssp. *crispa* in different ECMF treatments. There was no significant difference in total nodule activity among different ECMF treatments.

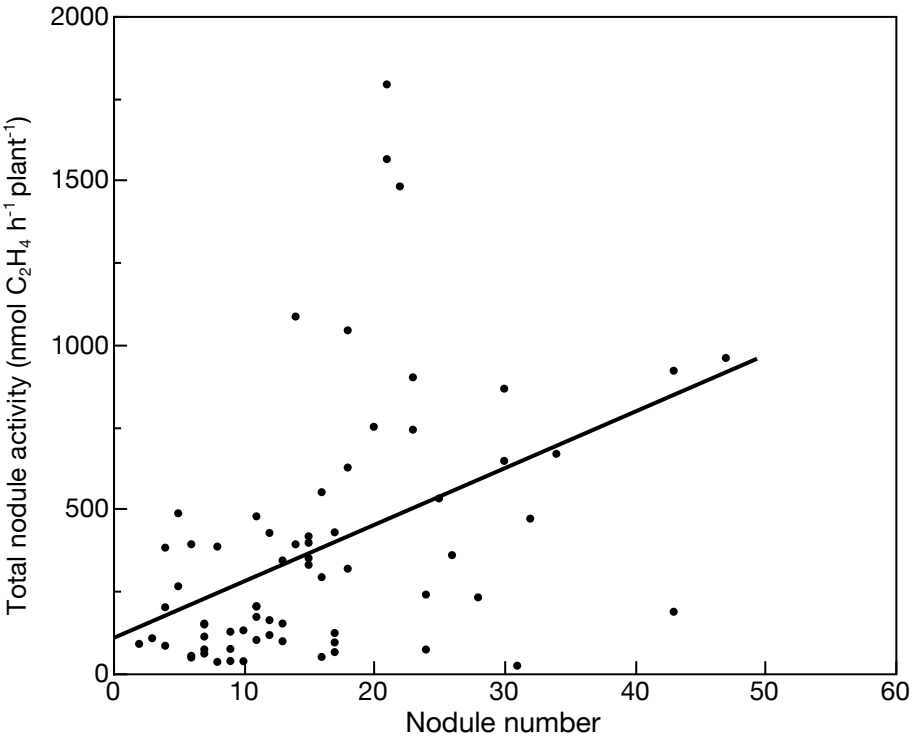

Figure 3.15: The relationship between total nodule activity (nmol h^{-1} $plant^{-1}$) and mean nodule number per plant. R^2=0.20, P=0.0001.

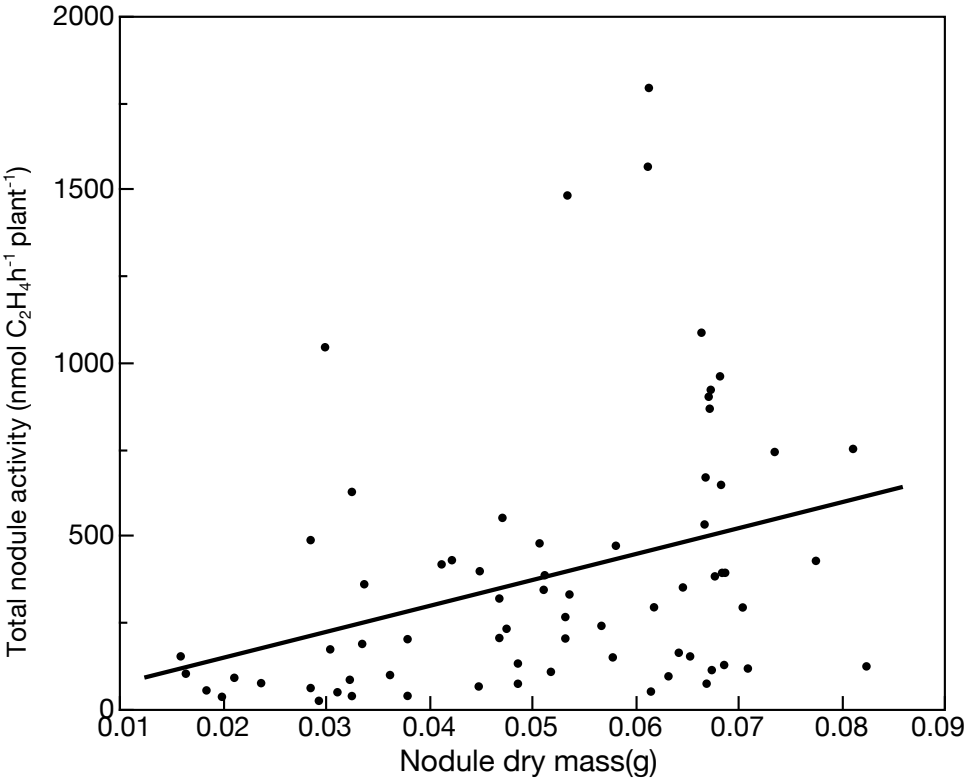

Figure 3.16: The relationship between total nodule activity (nmol h^{-1} $plant^{-1}$) and nodule dry mass (g) of *Alnus viridis* ssp. *crispa* plants. R^2=0.12, P=0.0042.

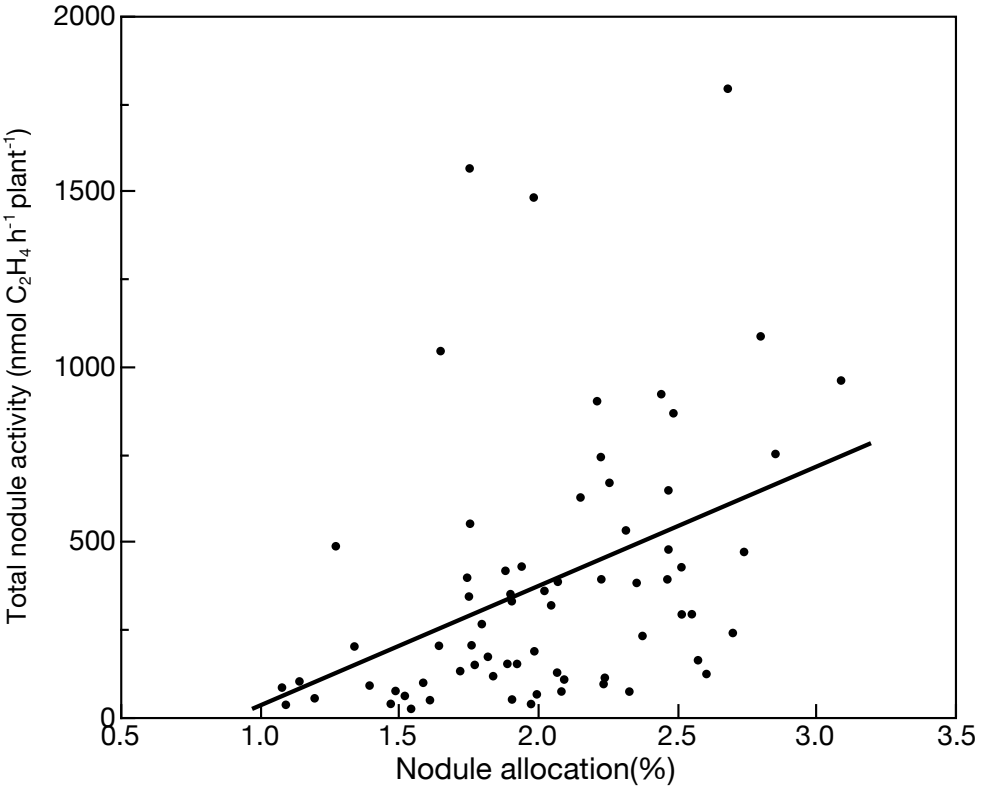

Figure 3.17: The relationship between and nodule allocation (%) of *Alnus viridis* ssp. *crispa* plants. R^2=0.17, P=0.0005.

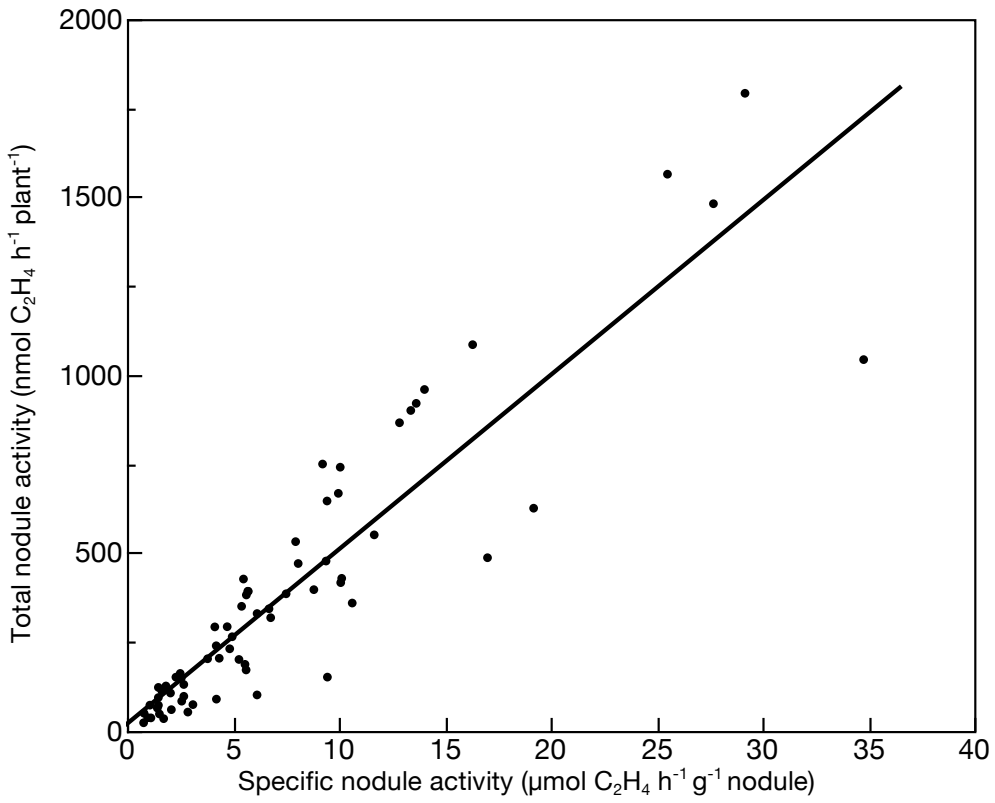

Figure 3.18: The relationship between specific nodule activity and total nodule activity measured by ethylene production rate per plant. R^2=0.82, P<0.0001.

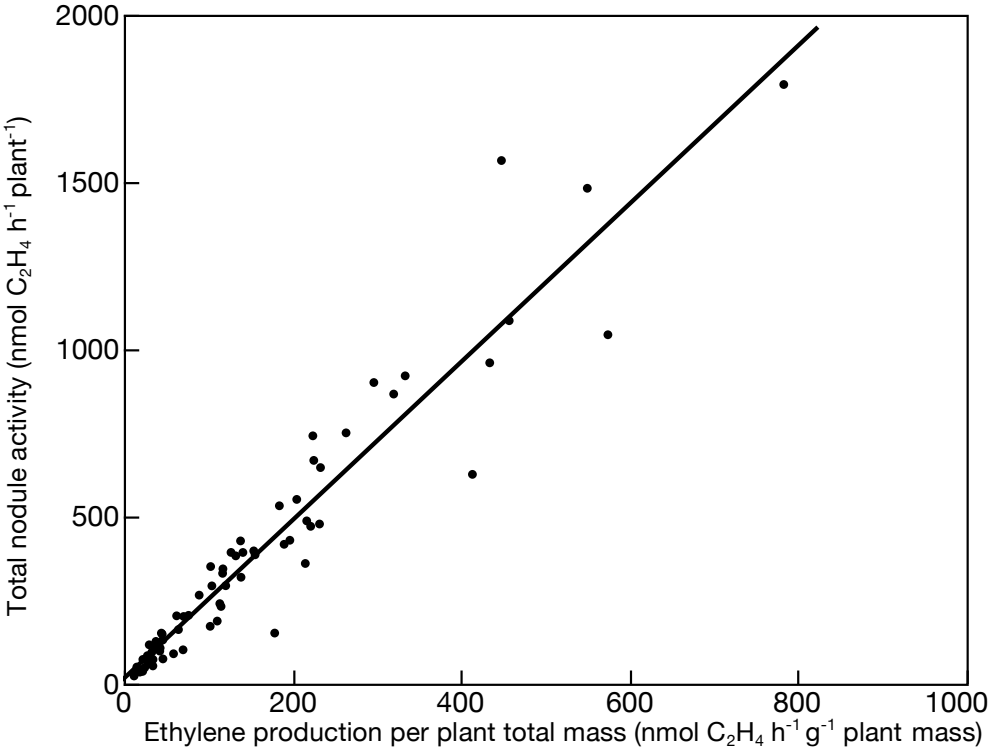

Figure 3.19: The relationship between ethylene production per plant total mass and total nodule activity measured by ethylene production rate per plant. R^2=0.91, P<0.0001.

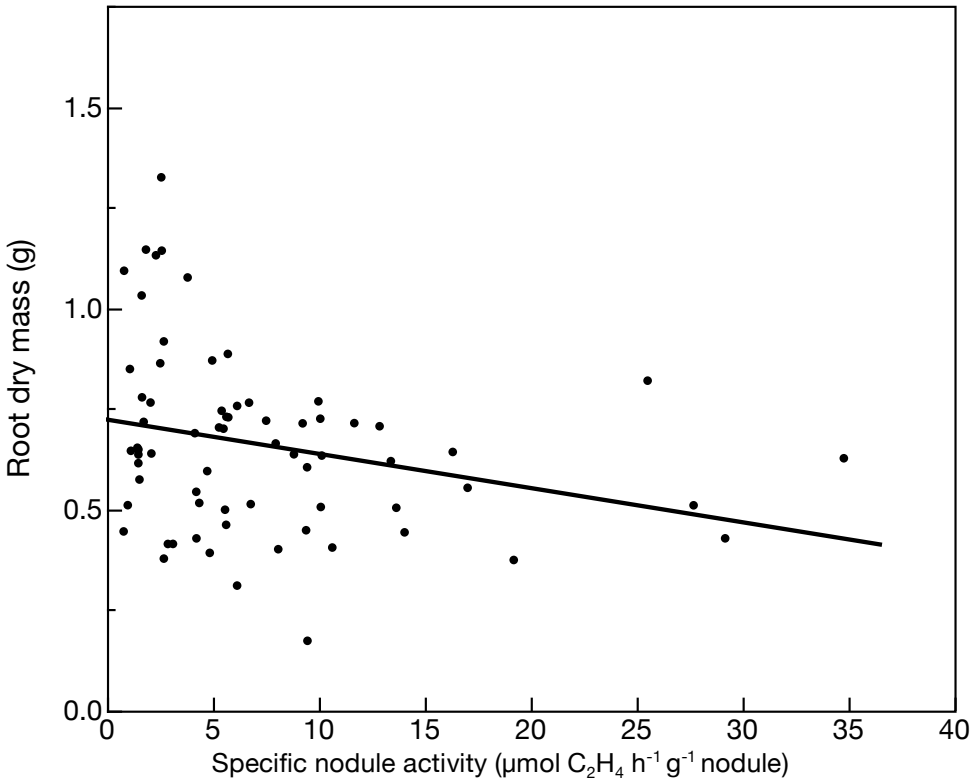

Figure 3.20: The relationship between specific nodule activity and root dry mass (g) of *Alnus viridis* ssp. *crispa* plants. (R^2=0.07, P=0.03)

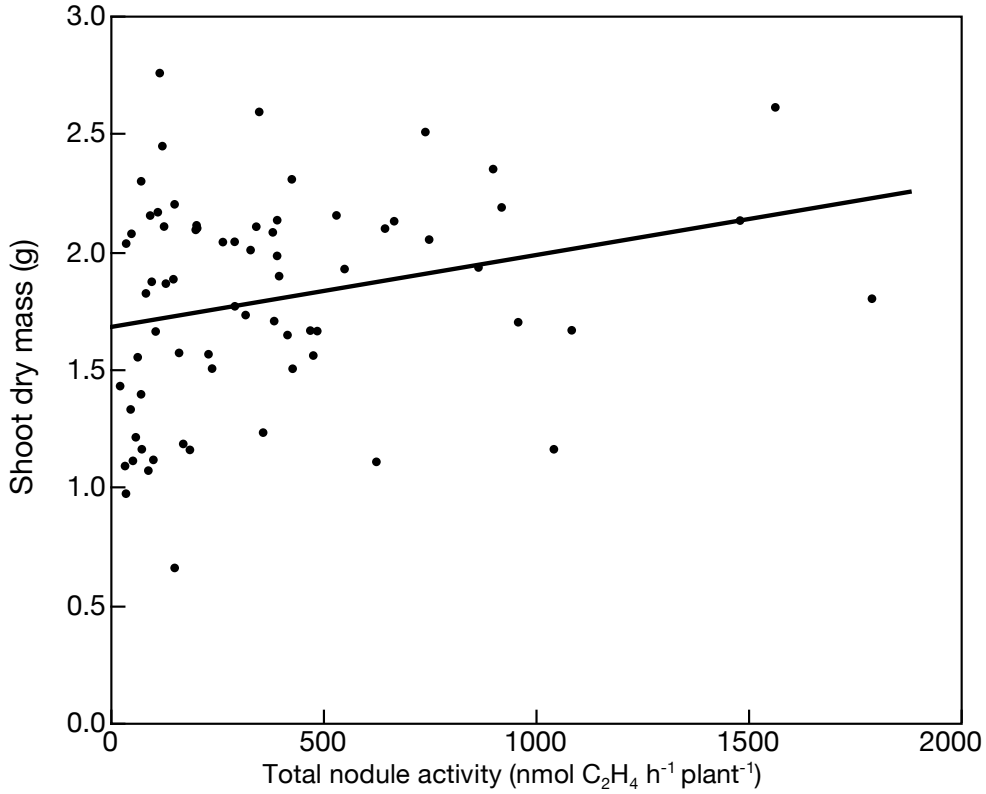

Figure 3.21: The relationship between total nodule activity and shoot dry mass (g) of *Alnus viridis* ssp. *crispa* plants. (R^2=0.07, P=0.03)

3.3.6 Nodule respiration, root respiration and their relationship to nitrogen fixation

Inoculation with different ECMF treatments (F=1.31, P=0.25), *Frankia* spp. (F=0.39, P=0.53) and their interaction (F=1.57, P=0.15) did not have a significant effect on plant root respiration rate (nodule removed) (Figures not shown). Different ECMF species did not have a significant effect on nodule respiration (F=1.5362, P=0.1741) (Figure not shown). There was no correlation between root respiration rate and nodule respiration rate (R^2=0.008, P=0.49) (Figure not shown).

There was positive correlation between specific nodule activity and nodule respiration rate per nodule dry mass (R^2=0.07, P=0.0492) (Figure 3.22). While the coefficient of determination was so low, in general, nodules with a high rate of acetylene reduction did not have a low respiration rate.

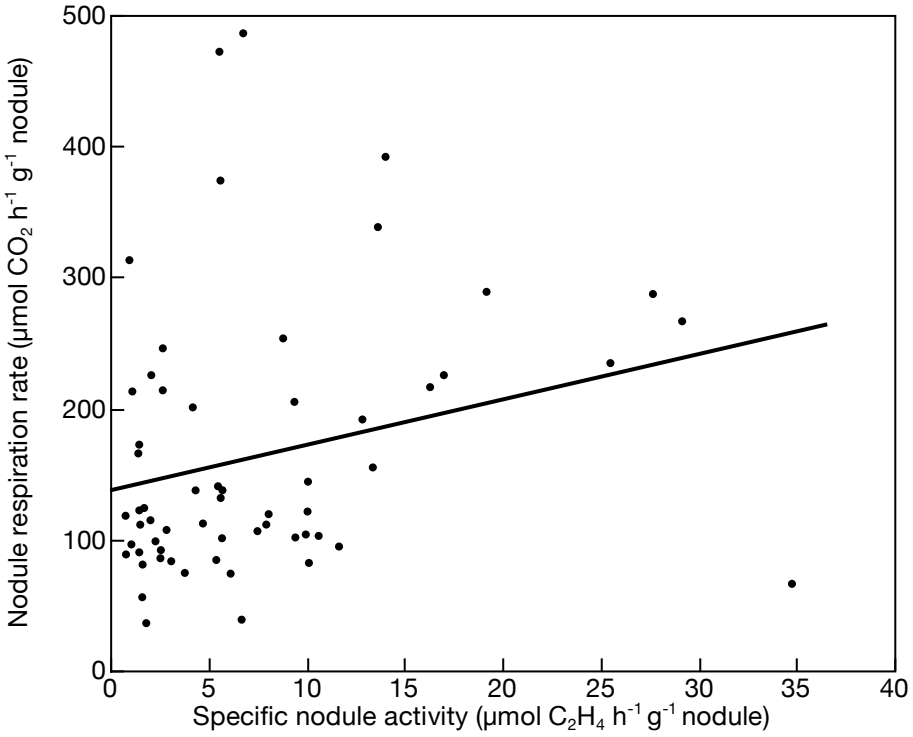

Figure 3.22: The correlation between specific nodule activity and nodule respiration rate. (R^2=0.07, P=0.0492)

3.3.7 Root phosphatase activity

Extracellular phosphatase activity results were Log transformed in order to homogenize the data variation between treatments. There were no interactive effects between ECMF and *Frankia* spp. treatments on plant roots extracellular phosphatase activity (F=1.32, P=0.245). *Frankia* spp. inoculation significantly increased the plant's root extracellular phosphatase activity from 5.59 ± 0.49 μmol p-NP h^{-1} g^{-1} root in non-*Frankia* spp. treatments to 6.08 ± 0.42 μmol p-NP h^{-1} g^{-1} root (F=4.3148, P=0.0400) (Figure 3.23). The ANOVA model showed a significant effect of the ECMF treatment (F=2.2766, P=0.0331) but the post hoc test showed no differences between any of the treatments on extracellular phosphatase activity of *Alnus viridis* plants roots (Figure 3.24).

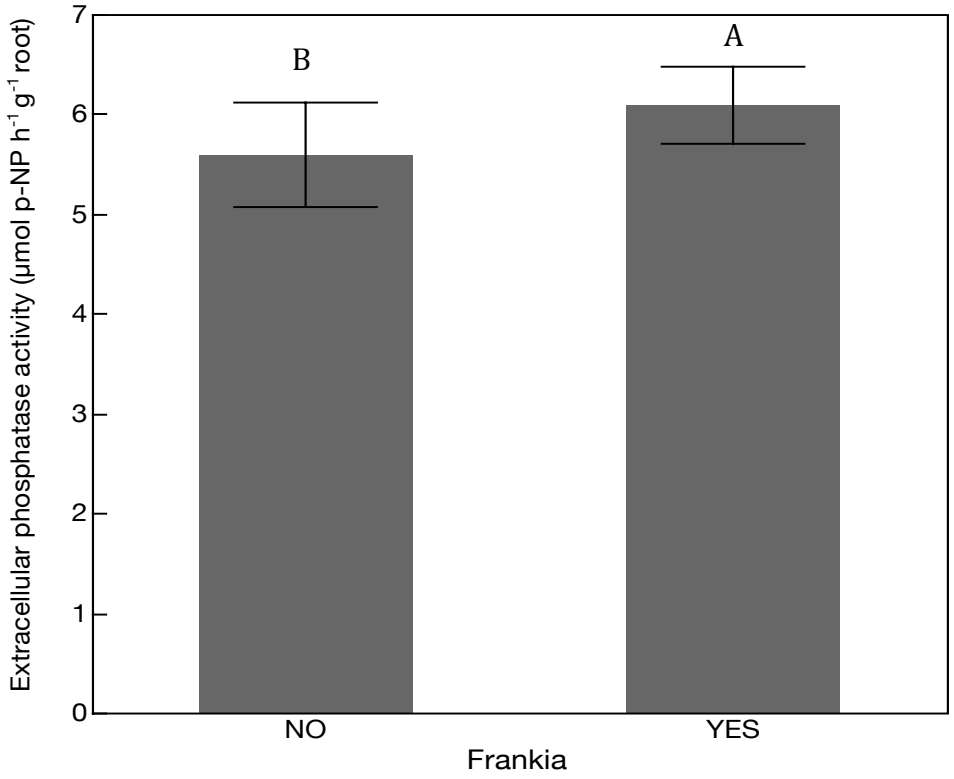

Figure 3.23: The effect of *Frankia* spp. on root extracellular phosphatase activity of *Alnus viridis* ssp. *crispa* averaged across all ECMF treatments. NO-*Frankia* spp. - plants were not inoculated with *Frankia* spp. YES-*Frankia* spp. - plants were inoculated with *Frankia* spp. Treatments with different letters are significantly different at the 0.05 level according to a two-way ANOVA.

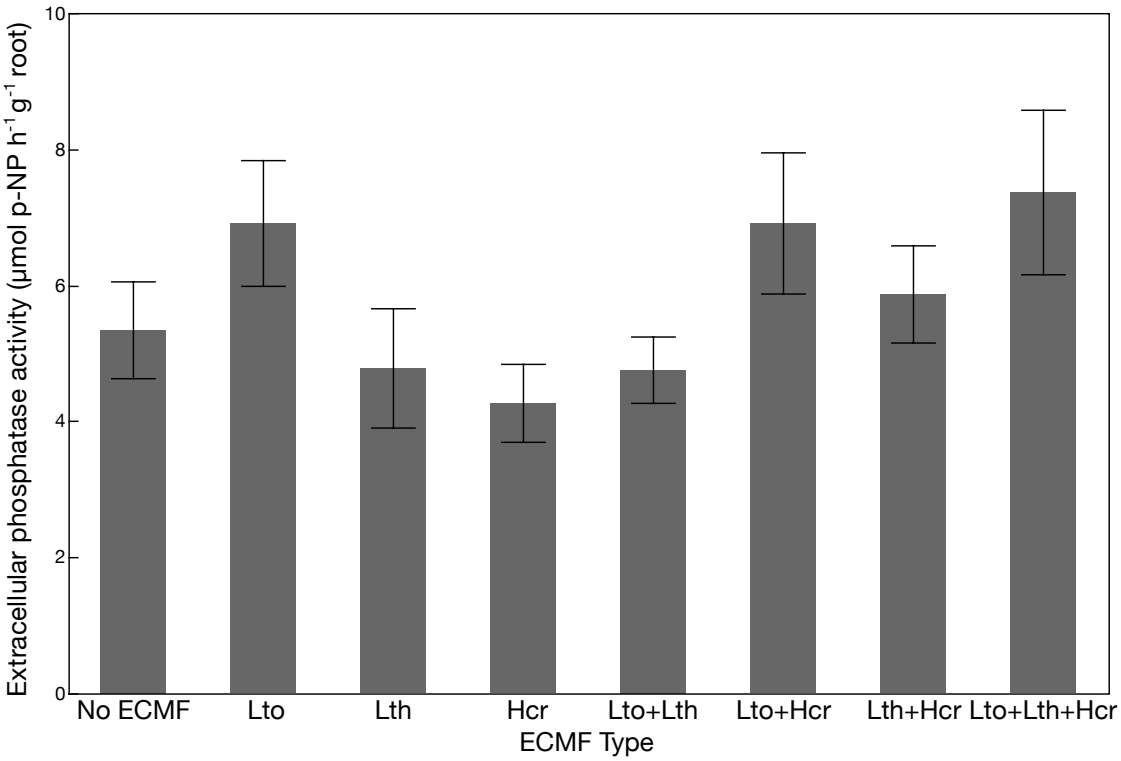

Figure 3.24: Extracellular phosphatase activity of *Alnus viridis* ssp. *crispa* plant roots inoculated by different types of ECMF averaged by the two *Frankia* spp. treatments.

3.3.8 Relationship between Hartig net formation and nodule activity, nitrogen fixation and phosphatase activity

Total nodule activity was decreased by 39.6% when plants had a Hartig net compared with those that did not, where the nitrogen fixation per plant was 464.91±61.1 nmol C_2H_4 h^{-1} $plant^{-1}$ (F=4.2, P=0.04) (Figure 3.25). This trend also occurred in ethylene production per plant mass where plants that had a Hartig net had a rate of 188.56±24.64 nmol C_2H_4 h^{-1} g^{-1} total plant mass compared to a rate of 113.97±26.50 nmol C_2H_4 h^{-1} g^{-1} total plant mass in plants that did not (F=4.25, P=0.04) (Figure not shown). However, there was no relationship between specific nodule activity and Hartig net formation (F=2.28, P=0.14) (Figure not shown). Extracellular phosphatase activity was positively correlated to specific nitrogen fixation rate but the coefficient of determination was low (R^2=0.06, P=0.04) (Figure 3.26).

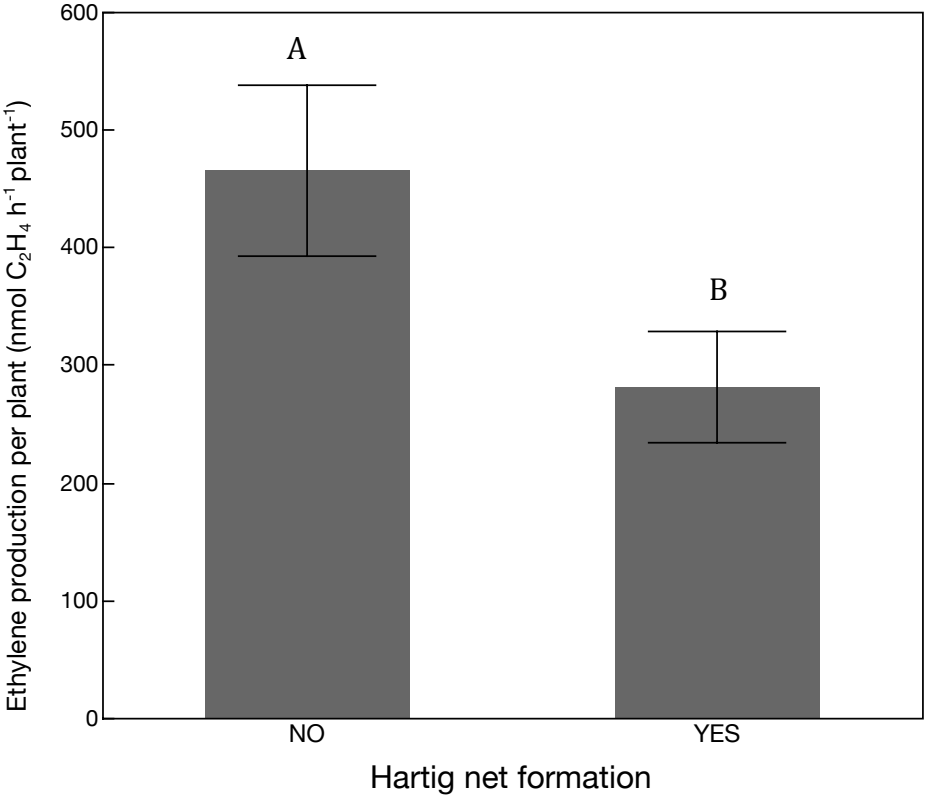

Figure 3.25: Relationship between ethylene production rate per plant and the presence of a Hartig net.

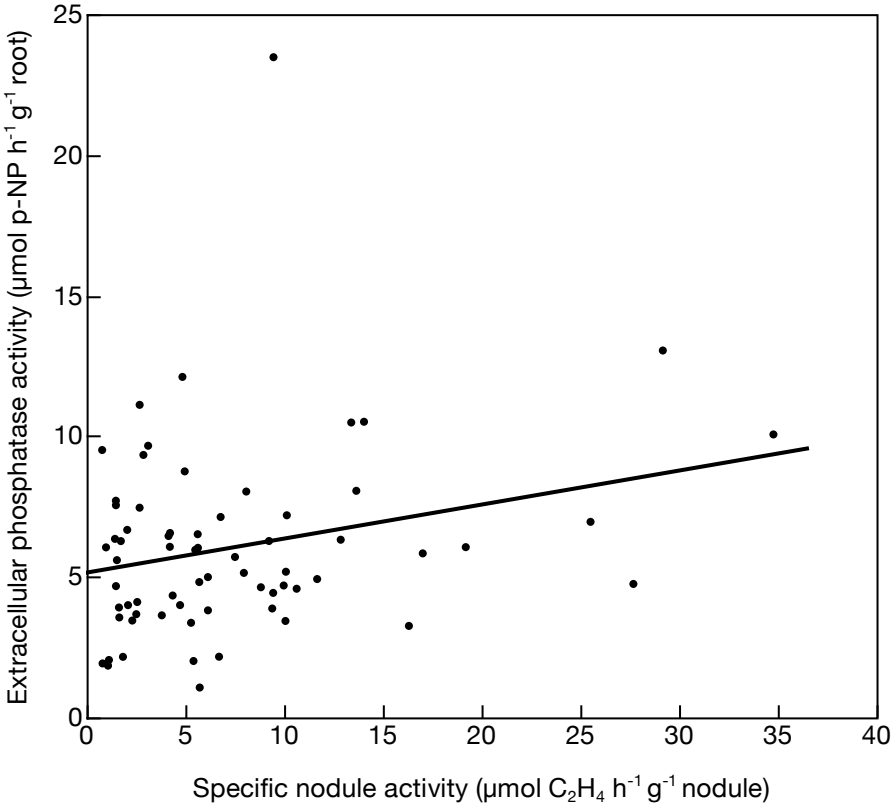

Figure 3.26: The relationship between extracellular phosphatase activity and specific nodule activity (R^2=0.06, P=0.04).

3.3.9 Effect of the Number of ECMF species on plant total biomass

The number of ECMF species and *Frankia* spp. inoculation had interactive effect on plant total biomass (F=4.45, P=0.0368) (Figure 3.27). Plant total biomass was decreased with increasing numbers of ECMF species when plants were also inoculated with *Frankia* spp. Plants colonized by 3 ECMF species total biomass was 1.93±0.23 g, a decreased of 32.99% compared to non-ECMF treatments. However, plant total biomass did not change with an increasing number of ECMF species in the absence of *Frankia spp.* inoculation (F=2.24, P=0.14).

The effect of the number of species on plant total biomass was not due to a sampling effect i.e., increasing number of species from a fixed pool increasing the chance of a plant being inoculated by a species that has a strong effect on plant performance. Inoculating plants with *Hebeloma crustuliniforme* did have a significant decrease on plant total biomass (F=22.83, P<0.0001) (Figure 3.28), but plants receiving only *H. crustuliniforme* as their ECMF did not differ from non ECMF inoculated plants – the reduction in growth associated with *H. crustuliniforme* only occurred when another fungal species was inoculated on the plants (Figure 3.4). The presence of *Lactarius torminosus* (F=0.20, P=0.65) and the presence of *Lactarius theiogalus* (F=2.99, P=0.09) did not display significant effect on plant total biomass (Figure not shown).

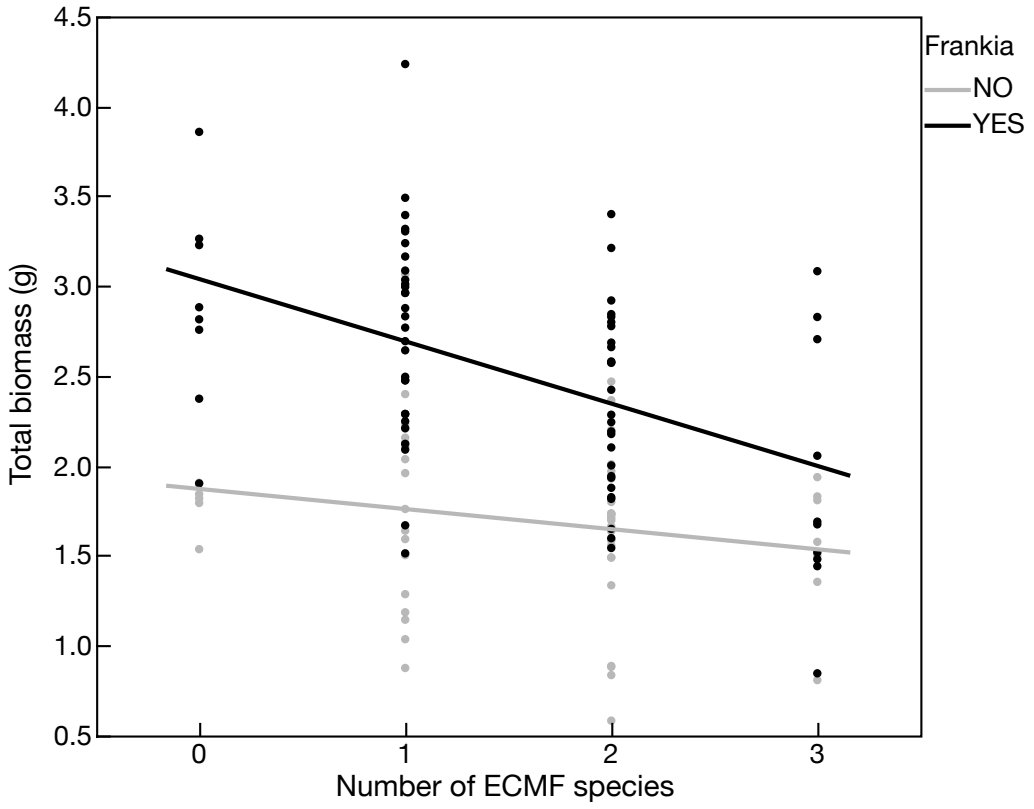

Figure 3.27: Total biomass of *Alnus viridis* ssp. *crispa* colonized by different number of ECMF species. The least square fit line for the presence of *Frankia* spp. is: Y=3.039-0.347X. The least square fit line for the absence of Frankia spp. only inoculated with different number of ECMF: Y=1.872-0.112X.

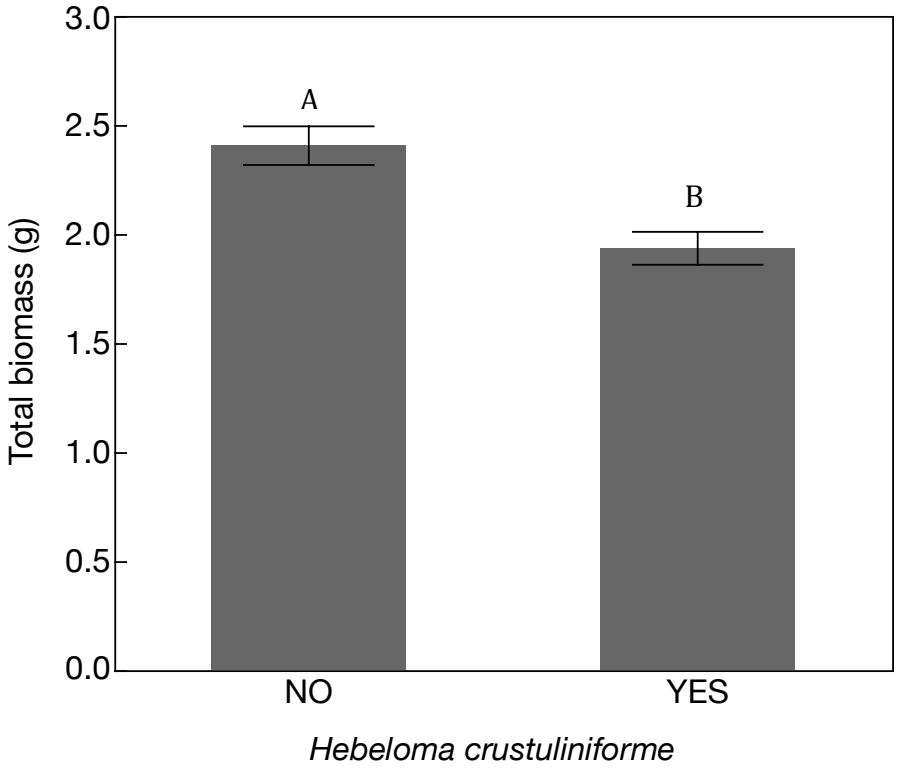

Figure 3.28: The effect of *Hebeloma crustuliniforme* on total biomass of *Alnus viridis* ssp. *crispa* averaged among the two *Frankia* spp. inoculation treatments

3.4 Discussion

Frankia spp. inoculation significantly increased plant shoot and total biomass whereas the different ECMF treatments had no effect. One reason for this lack of effect may have been the superficial colonization by the fungi, which is known to occur in *Alnus* species (Molina, 1979; Molina, 1981). Superficial ECMF associations are defined by the presence of thin Hatig net and sparse mantle (Brundrett, 2004). The *Alnus viridis* ssp. *crispa* mycorhizae in our experiment did not form mantles but did form Hartig nets. In conifers, a mantle and Hartig net are usually formed in few weeks. For example, Eastern white pine (*Pinus strobus* L.) was inoculated with *Pisolithus tinctorius* forming a Hartig net and mantle in 5 days, and after 6 weeks an extensive network of hyphal strands were found (Fortin et al., 1980). In contrast, mycorrhizal formation in *Alnus* can be much slower. Molina (1979) tested 28 ECMF species inoculated on red alder, and found only 4 out of 28 forming characteristic mycorrhizae with a clearly colored mantle after 6 months. Another study showed that *Hebeloma crustuliniforme* could develop both a mantle and Hartig net after one month on *Alnus viridis* spp. *crispa* (Godbout and Fortin, 1983). Godbout and Fortin (1983) concluded that *Alpova diplophloeus* was the only ECMF species that penetrate rapidly between alder root cell and form a Hartig net, while other species need a longer period because the other fungi had a relative broad host range and low specificity. It is unlikely that my superficial colonization was due to the limited 15-week duration of the experiment, since this is the normal growing season for *Alnus viridis* ssp. crispa.

For my study, the three ectomycorhizal fungi were collected under *Alnus rugosa*. *Alnus* has a very narrow range of ECMF types and *Alnus*-ECMF associations are known

to be highly specific with only a few exceptions (Tedersoo et al., 2009). Up to date, 50-60 ECMF species are known to associate with the entire *Alnus* genus worldwide (Godbout and Fortin, 1983; Molina et al., 1994; Tedersoo et al., 2009; Põlme et al., 2013). Ten out of 46 ectomycorrhizal fungi species formed mycorrhizae on green alder (*Alnus viridis* ssp. *crispa*) and speckled alder (*Alnus rugosa*), and only 11 ECMF species are suspected for red alder (*Alnus rubra*) (Godbout and Fortin, 1983; Molina et al., 1994). Godbout and Fortin (1983) and Gardner (1986) listed only10 ECMF species forming mychorrhzae on *Alnus viridis* ssp. *crispa* and *Alnus rugosa*: *Alpova diplophloeus*, *Cenococcum geophilum*, *Cortinarius cf subporphyropus*, *Hebeloma crustuliniforrne*, *Laccaria laccata*, *Leccinurn holopus*, *Leccinurn subleucophaeum*, *Paxillus involutus*, *Pisolithus tinctorius*, *Scleroderma citrinum*. This is not comparable to ECMF richness on conifer trees. Baar et al. (2000) concluded that 135 ECMF types were observed on *Pinus sylvestris* L. trees, 25 mycorrhizal types on *Picea abies* (L.) Karst. trees and 20 mycorrhizal types on *Pinus muricata* D. Don trees.

Besides the fact that *Alnus* mycorrhizae are known to show poor development, there are two possible reasons for the superficial colonization in my experiment. Firstly, the low fertility soil and young age of plants might not allow plants to meet the need for increasing carbon demand of ECMF colonization. Fungal establishment increases the demand of carbohydrate for fungal maintenance and growth. Hampp et al. (1999) found that the reduction of hexose on the host-fungus interface would adversely affect ECMF development. There is substantial overlap in ECMF community between mature trees and young seedlings, however, 40% of ECMF colonizing mature trees were not present in young seedling (Jones et al., 2003). This suggested that the age of seedlings play a minor

role in ECMF community between young and mature tress. Secondly, these three ECMF species were collected and isolated from sporocarps beneath *Alnus rugosa* shrubs, but it is hard to say that these species truely colonized *Alnus*. Brundrett (2004) has concluded that ectomycorrhizal fungi fruiting body near a potential host plants can not be putatively used as ECMF associations. In my experiment, the fungi fruiting body was growing near *Alnus* where conifers were also found. The putative association with *Alnus* might be incorrect if the fungi fruit body were found near both *Alnus* and conifers. *Cenococcum geophylum* has been identified as an ectomycorrhizal symbiont of *Alnus* in year 1964 and 1971, but failed to repeatedly form mycorrhiza in year 1981 (Gardner, 1986). That is because the latter *Cenococcum* were isolated from *Picea* host rather than *Alnus*.

It is commonly known that when ectomycorrhizal fungi colonize roots, they increase plant growth by mobilizing and increasing nutrients uptake, especially for P (Bolan, 1991). However, some fungi do not show an effect on plant growth although they develop extensive mycorrhizae (Thomson et al., 1994). Similarly, our results showed different ECMF types did not show positive effect on plant performance compared to the control treatment. Nguyen et al. (2006) tested *Hebeloma crustuliniforme* and *Laccaria bicolor* on spruce (*Picea maciana* and *picea glauca*) and jack pine (*Pinus banksiana*), and found that ectomycorrhizal fungi reduced jack pine dry mass, but there was a small increase in white spruce dry mass. Similarly, Ekblad et al. (1995) found ectomycorrhizal fungus (*Paxillus involutus*) had a negative effect on *Alnus incana* growth, but showed a greater total biomass of *Pinus sylvestris* compared to non-ectomycorrhizal treatments. It seems that *Paxillus involutus* was regarded as parasite for *Alnus* but not *Pinus*.

In my study, although different ECMF types did not have any effect on plant performance compared to controls, plant total biomass decreased linearly with an increasing number of ECMF in the presence of *Frankia* spp. However, there was no change with an increasing number of ECMF without *Frankia* spp. in terms of plant total biomass. Some studies have shown that a combination of fungal species may result in synergistic effect on plant performance, which may be due to niche complementarity (Parladé and Alvarez, 1993; Jonsson et al., 2001). Diagne et al. (2013b) tested a gradient of ectomycorrhizal fungi diversity (ranging from one to six fungal isolates) on nodulated Australian acacia (*Acacia mangium*) seedlings growing in P-deficiencient soil (N: 0.02%, total P: 39 mg kg^{-1}, available P: 4.8 mg kg^{-1}). The authors found that mycorrhizal richness had a greater impact on plant parameters (total, shoot and root dry mass) as well as nutrients acquisition (N, P) compared to single inoculation. The results of my study did not show consistent advantage of multiple ectomycorrhizal fungi species. Rather, the increasing number of ECMF species decreased plant mass in nodulated plants. The reduced growth with increased fungal species was not due to a "sampling effect" i.e., the chance of having one fungal species that has a strong effect on plants (Fargione et al., 2007; Diagne et al., 2013b). In this experiment, there was no strong effect of a single fungal species on plant growth, but when *Hebeloma crustuliniforme* was combined with other species, it seemed to be have stronger effect on plants. This was related to the Hartig net formation. The treatments with a higher proportion of plants with Hartig net formation had less plant total biomass. Since these treatments with higher proportion of plants with Hartig net had lower nodule number and nodule allocation, this affected total nitrogen fixation. Other experiments have shown that multiple species varing effects on

plant performance compared to single ECMF treatments. Onwuchekwa et al. (2014) inoculated 3 species of ectomycorrhizal fungi on jack pine (*Pinus banksiana*) and white spruce (*Picea glauca*) singly and multiply. The single ECMF and double ECMF inoculation significantly increased jack pine biomass, but three ECMF species treatment had slightly lower plant performance than single inoculation. Also none of the ECMF treatments had an effect on white spruce growth. The authors did not find clear and consistent advantage of multiple ECMF species, since some measured parameters like survival rate was lower in the multiple ECMF treatments compared to the single ECMF treatments. Kennedy et al. (2007) tested *Pinus muricata* inoculated with three ectomycorrhizal species in single-, double, and triple species treatment. The authors found that multiple ECMF treatments had lower shoot biomass than single ECMF treatments. Clearly, single versus multiple fungal species application in the field needs further investigation.

There was little effect of ECMF on root allocation. I found that only *Lactarius theiogalus* increased root allocation in the absence of *Frankia* spp. A study found that *Hebeloma crustuliniforme* increased the root allocation of paper birch (*Betula papyrifera Marsh*.) but did not have effect on trembling aspen (*Populus tremuloides Michx*.) (Yi et al., 2008). Another study showed that the ectomycorrhizal fungus (*Paxillus involutus*) did not have effect on root:shoot ratio of speckled alder (*Alnus incana*) regardless of *Frankia* spp. inoculation (Chatarpaul, 1989). It seems that root allocation affected by mycorrhizae varies from species to species.

Frankia spp. inoculation increased the proportion of plants with Hartig net formation (45.9%) compared with non-*Frankia* spp. treatments (19.3%). This result

supports the previous findings of Markham (2005) that nodulated plants were three times more likely to develop mycorrhizae than non-nodulated plants, and Oliveira et al. (2005) that the presence of *Frankia* spp. increased AMF spores density and extraradical mycelium vitality. This increased colonization may be caused by increased nitrogen nutrition of nodulated plants. Jonsson et al. (2001) found ectomycorrhizal colonization is higher under high fertility soil than those in low fertility soil. Another study showed that N fertilizer increased the amount of hyphae and AMF colonization (Johnson, 1993).

My results showed that different types of ECMF did not have effect on nodule number per root system, but had a slight decrease in nodule dry mass and nodule allocation compared to controls. Since plants were inoculated with fungi and *Frankia* spp. at the same time it may be that the fungi wouldn't have much of an effect on initial infection but once the fungi had started to colonize, the mycorrhizae had an effect on nodule development. Numerous studies have found that mycorrhizae could increase nodule number and nodule dry mass by enabling plant P uptake (Duponnois and Plenchette, 2003; Duponnois et al., 2007; Diagne et al., 2013). Koo (1989) found that ectomycorrhizae did not affect nodulation and nitrogen fixation of red alder, but *Frankia* spp. inoculation strongly increased mycorrhize formation.

Since not every plant receiving ECMF inoculum formed mycorrhizae, I also examined the relationship between Hartig net formation and nodulation, and found that the presence of Hartig net on a plant was associated with lower number of nodules and nodule allocation. Bâ (1994) found that the nodule number was not affected when ECMF and *Bradyehizobium* sp. were inoculated on *Acacia holosericea* seedlings simultaneously. However, the nodule meristem and infection threads were absent in ectomycorrhizal

zone, and nitrogen fixing bacterial were absent in the Hartig net zone, suggesting fungal hyphae might directly or indirectly modify the recognition factors leading to nodule initiation. I found that the inoculation treatment had the highest proportion of plants with Hartig net formation (inoculation by both *Lactarius torminosus* and *Hebeloma crustuliniforme*). It was also the treatment with the lowest nodule dry mass, nodule number and nodule allocation per plant compared to other treatments.

My results suggested that *Frankia* spp. inoculation increased both total biomass and shoot mass but not root mass compared to non-*Frankia* spp. treatment. *Frankia* spp. inoculated plants have been found to significantly increase biomass compared to non-inoculated plants only when soil N was limited (Markham and Zekveld, 2007). My results also confirm that nodulation reduces root allocation. Previous studies have shown that plants invest more in aboveground parts other than root mass when plants are nodulated (Bissonnette et al., 2014, Markham and Zekveld, 2007) and nodulated plants increase plant shoot mass much greater than root mass (Jeong and Myrold, 2001; Markham and Zekveld, 2007). This is likely due to nitrogen fixation meeting the plant's N requirement so nodulated plants would not spend more energy on root growth to forage for N in the soil. Additionally there is an increased photosynthate requirement to maintain enough energy for nitrogen fixing bacteria, which should shift biomass allocation from roots to shoots.

My study suggests that different ECMF did not have an effect on specific nodule activity, ethylene production rate per plant mass as well as ethylene production per plant. This might be due to the superficial colonization of ECMF. Many studies have shown that mycorrhizal fungi inoculation benefit nodulation and nitrogen fixation by increasing

P availability. Chatarpaul et al. (1989) found simultaneous inoculation of *Frankia* spp. and *Paxillus involutus* on *Alnus glutinosa* stimulated biomass production and nitrogen fixation. Ectomycorrhizal fungal symbiosis (with *Pisolithus albus*) can also improve nitrogen fixation of *Acacia holosericea* (André et al., 2005). AMF can enhance nodulation and nitrogen fixation of *Medicago arborea*, but these effects varied from fungal species to species (Valdenegro et al., 2001). Although many studies have found that mycorrhizal fungi and nodule forming bacteria act synergistically on infection rate and mineral acquisition, there are some exceptions. Specific nodule activity of *Alnus incana* was not affected by ectomycorrhizal fungus (*Paxillus involutus*) (Ekblad and Huss‐Danell, 1995; Ekblad et al., 1995). Since in my study not every plant receiving ECMF inoculum formed mycorrhizae, I examined the relationship between Hartig net and nitrogen fixation. These results showed that plants forming Hartig net had a lower level of total nodule activity but not specific nodule activity. This was due to the lower number of nodules and nodule allocation in the mycorrhizal plants, decreasing total nodule activity.

My results showed that total nodule activity was strongly linked to specific nodule activity, nodule allocation, nodule dry mass as well as nodule number. Increases in total N_2 fixation have mainly been attributed to increasing the specific nodule activity and total nodule dry mass (Pacovsky et al., 1986; Bona et al., 1991). My data also suggests that there is no correlation between specific nodule activity and plant growth, but plant growth (total, shoot, root dry mass) is positively related to nodule dry mass, and shoot dry mass is positively correlated to total nitrogen fixation. This likely occurred because the fixed N was mostly shifted to aboveground growth rather than root growth. Other studies

have confirmed that fixed nitrogen was correlated to nodule dry mass in both mycorrhizal and non-mycorrhizal *Alnus incana* (Ekblad and Huss‐Danell, 1995). Wheeler et al. (2000) also found that the greater biomass of *Casuriana equisetifolia* was accompanied by the greater mass of nodules per plant rather than the greater specific nodule activity. Bissonnette et al. (2014) also confirmed that shoot dry mass of *Alnus* was strongly correlated to total nodule mass. It has been suggested that insufficient nodule dry mass is the main limiting factor for N-fixing plant growth (Troelstra et al., 1987). However, Markham (2008) found that plant growth was positively correlated to mean mass per nodule and specific nodule activity, but negatively correlated with nodule number per plant when plants of a similar size to mine formed large numbers of nodules.

In my experiment, there was no difference among different ECMF types in terms of root and nodule respiration. Mycorrhizal colonization by VAM fungi has been shown to increase root and root with attached nodule respiration rate under severe P deficiency compared to non-mycorrhizal root (Valentine and Kleinert, 2007; Mortimer et al., 2008). Also, a positive linear relationship between nitrogenase activity and (nodule) respiration is known to occur (Mahon, 1977; Hurek et al., 1994; Lundquist, 2005; Ruess et al., 2013). While I found that specific nodule activity was related to nodule respiration, the relationship was not strong - a high level of nodule respiration did not always mean the high nitrogen fixation. This was consistent to previous study when nitrogen fixation rate was below 1 μmol N h^{-1} g^{-1} nodule, there was no correlation between nitrogen fixation and nodule respiration, and high level of nodule respiration did not result in high rates of nitrogen fixation (Ruess et al., 2013).

I found that ECMF did not have any effect on extracellular phosphatase activity due to the superficial colonization, which was not consistent to previous work showing the mycorrhizal colonization promote phosphatase activity (Conn and Dighton, 2000; Walker et al., 2014). Like organic N in the soil, organic P constitutes of a large amount of the total soil P (Schneider et al., 2000). Intracellular or secreted acid phosphatase activity has also been found to increase when plants were exposed to P-deficiency conditions (Schneider et al., 2000; Yun and Kaeppler, 2001). My experiment showed that while ECMF treatments had no effect on extracellular phosphatase avtivity, *Frankia* spp. inoculation increased extracellular phosphatase activity compared to non-*Frankia* spp. treatment. Houlton et al. (2008) found that soil phosphatase activity was significantly higher in the presence of N_2-fixing plants compared to non-N_2 fixers treatments. This indicated that N fixing bacteria could stimulate phosphatase production and increase P mineralization rate, therefore meeting the P requirement of nodulation. Walker et al. (2014) also confirmed that acid phosphatase activity of ECMF root tips from nitrogen fixing plants (*Alnus rubura*) was significantly higher than that from non-nitrogen fixing host (*Pseudotsuga menziesii*). This suggested that symbiosis between *Frankia* spp. and ECMF on host plant enhanced inorganic and organic acquisition ability for *Frankia* spp. associated plants. Phosphatase, as a protein, has relatively high N concentration and may represent a significant investment of N. Therefore, *Frankia* spp. inoculation may raise the acid phosphatase activity of plants (Treseder and Vitousek, 2001).

3.5 Conclusions

In my experiment, *Frankia* spp. inoculation increased plant performance in the nutrient-poor soil. ECMF did not colonize very well but plant total biomass decreased

with an increasing number of ECMF and with the presence of *Frankia* spp. Overall, Tripartite symbiosis among (single or multiple) ECMF, *Frankia* spp. and actinorhizal plants should be investigated in fieldwork under conditions of high plant and microorganism diversity and complex soil characteristics.

Chapter 4. THE EFFECT OF *FRANKIA* SPP. AND *HEBELOMA CRUSTULINIFORME* ON *ALNUS VIRIDIS* SSP. *CRISPA* GROWING IN SALINE SOIL

4.1 Introduction

I designed this experiment to study the effect of *Frankia* spp. and *Hebeloma crustuliniforme* symbiotic on *Alnus viridis* ssp. *crispa* exposed to 0, 50, 100 mM NaCl medium. In chapter 3, ectomycorrhizal fungal richness did not show positive effect on plant performance. So I choose single fungal species, *Hebeloma crustuliniforme*, which gave highest proportion mycorrhizal formation, as the fungal inoculum.

This experiment was designed to test three hypotheses. The first is that when plants are nodulated, the fixed N can balance the nutrient deficiency that occurs under salt stress. Therefore, *Frankia* spp. inoculated plants should show higher salt tolerance by maintaining a certain level of fixed N compared to the plants dependent on inorganic N from soil. The second is that under salt stress ectomycorrhizae can maintain a higher water content in plant tissue (Bois et al., 2006 a), therefore increasing plant salt tolerance compared to non-mycorrhizal plants. The third hypothesis is that ectomycorrhizal fungi and *Frankia* spp. will display a synergistic effect on plants growing saline soil compared to single symbionts.

4.2 Methods and materials

Alnus viridis ssp. *crispa* seeds collected from the Sandilands Provincial forest were sown in the same condition as described in chapter 3. Four-month-old *Alnus viridis* seedlings were transferred to D40H Deepots. Seedlings were inoculated with and without *Frankia* spp. combined with or without ECMF (*Hebeloma crustiliniforme*). Control plants received no inoculant. *Frankia* spp. inoculated plants received 4 mg of crushed

nodules at a concentration of 2 mg nodules mL^{-1} PBS Buffer. Each plant was inoculated by injecting 2 mL of well-fragmented inoculum suspension on the surface of the soil. The crushed nodules came from the chapter 3 experiment *Alnus viridis* ssp. *crispa* root nodules and were surface sterilized as chapter 3. For the ECMF inoculated plants, three 3.9 mm diameter by ca. 7 mm height MMN agar plugs growing with hyphae were placed next to the upper roots of the plant. Before transplanting, autoclaved soil was mixed with distilled water containing 1 mM K_2SO_4 (around 18.7 mg K kg^{-1} soil). (A side experiment showed that *Alnus viridis* ssp. *crispa* had K^+ deficiency when grown in this field soil). The plants were grown in a growth room with a 16 hour light: 8 hour dark photoperiod. The light ranged from 200 to 270 μmol photons m^{-2} s^{-1}, the relative humidity (RH) ranged from 30% to 50% and the temperature was 20 °C/25 °C (night/day). NaCl was added at the 6th week after inoculation. Each of 10 replicates were treated with 0 mM, 50 mM, or 100 mM NaCl. For control treatments: plants were treated with only distilled water. For the 50 mM NaCl treatments, plants received 25 mL of 50 mM NaCl twice (once a day) (totaling 50 mL). For the100 mM NaCl treatments, plants received 25 mL of 50 mM NaCl four times (once a day). After adding NaCl, plants were watered with about 50 mL distilled water (to about saturation) every two days. Each plug was provided a small cup underneath to collect the leaching solution from the soil and then poured back onto the soil surface. Plants were exposed to NaCl conditions for 6 to 7 weeks and then harvested. All of the parameters measured were the same as in chapter 3. Additionally, plant leaves chlorophyll fluorescence was measured every two weeks starting from the 6th week using a fluorometer (Opti-Science OS-30P). A random mature leaf in each plant was kept in the dark for 30 min prior to measurement. Dark-adapted minimum fluorescence (Fo) was

obtained with a dim modulated light (<1 μmol photons m^{-2} s^{-1}). A Saturation light (1800 μmol photons m^{-2} s^{-1}) was applied to obtain dark-adapted maximal fluorenscence (Fm). Measurements were conducted from 10:00 am to 1:00 pm. (Fm-Fo)/Fm was calculated and used to determine the efficiency of photosystem II.

4.2.1 Data analysis

Some non-inoculated plants (without *Frankia* spp. or (and) ECMF) formed nodules or (and) Hartig net. They were excluded from the analysis. Plant relative growth rate was determined using the formula (Log Height $_{t2}$* Diameter$_{t2}$ ^{2}- Log Height $_{t1}$* Diameter$_{t1}$^{2}) /(days). According to residual plots there was a large difference in variation between treatments and the data were therefore log transformed. The data analysis used a three-factor least squares model with *Frankia* spp. inoculation, ECMF inoculation and NaCl application as three factors. The NaCl treatment was considered as continuous variable modeling type. When there was a significant interacation between NaCl and the other factors, the effect of salt on the response variable was analyzed using linear regressions for each level of the interacting factor.

4.3 Results

4.3.1 Plant size

Frankia spp. inoculation significantly increased plant total biomass (F=12.23, P=0.0008) by 37.5% compared with non-*Frankia* spp. treatments (1.52±0.11 g) (Figure 4.1). NaCl significantly decreased plant total biomass (F=32.99, P<0.0001) with a plant biomass of 2.35±0.11 g at 0 mM NaCl level with a decrease of 45.96% at 100 mM NaCl level (Figure 4.2). ECMF did not have an effect on plant total biomass (F=0.12, P=0.73). There was no significant difference on plant total biomass due to the *Frankia* spp. and

ECMF interaction (F=0.12, P=0.73), *Frankia* spp. and NaCl interaction (F=1.01, P=0.32), ECMF and NaCl interaction (F=0.10, 0.76), or the interaction of all three factors (F=0.61, P=0.44) (Table 4.1).

Root:shoot ratio significantly decreased with increasing NaCl level but was dependent on whether or not the plants were inoculated with *Franknia* (*Frankia* spp. by NaCl interaction: F=17.78, P<0.0001). The root : shoot ratio was higher without the presence of *Frankia* spp. compared to *Frankia* spp. inoculation treatments when plants were not exposed to NaCl. For non-inoculated plants, there was sharp decrease in root:shoot ratio with an increasing level of NaCl from 1.53±0.20 at 0 mM NaCl to 0.47±0.06 at 100 mM NaCl, while *Frankia* spp. inoculated plant root:shoot ratio had a moderate decrease with increasing level of NaCl from 0.57±0.05 at 0 mM NaCl to 0.34±0.05 at 100 mM NaCl (Figure 4.3). All other factors and their interaction did not have effect on plant root:shoot ratio (Table 4.1).

With an increasing level of NaCl, plant leaf:stem ratio significantly increased (F=36.15, P<0.0001) (Figure 4.4). All other factors and their interaction did not affect leaf:stem ratio (Table 4.1).

Table 4.1: P values of the *Frankia* spp. inoculation treatment, ECMF inoculation treatment, salt treatment and their intercations from least squares models for different plant parameters. Significant effects are shown in red.

	Frankia	ECMF	Frankia *ECMF	NaCl	Frankia *NaCl	ECMF *NaCl	Frankia *ECMF*NaCl
Total mass	**<0.0008**	0.734	0.727	**<0.0001**	0.317	0.755	0.436
Root:Shoot ratio	**<0.0001**	0.938	0.453	**<0.0001**	**<0.0001**	0.651	0.922
Leave:stem ratio	0.097	0.687	0.836	**<0.0001**	0.173	0.436	0.732
Nodule number	NA	0.060	NA	0.117	NA	0.332	NA
Nodule allocation	NA	0.759	NA	**0.040**	NA	0.491	NA
Specific nodule activity	NA	0.644	NA	0.076	NA	**0.047**	NA
Ethylene production per plant mass	NA	0.696	NA	**0.008**	NA	**0.019**	NA
Total nodule activity	NA	0.788	NA	**0.0006**	NA	0.243	NA
Root respiration (nodule removed)	**0.007**	0.524	0.542	**0.003**	0.261	0.703	0.327
Nodule respiration	NA	0.396	NA	**0.007**	NA	0.660	NA
Acid phosphatase activity	**0.0001**	0.240	0.182	**<0.0001**	0.759	0.661	0.833
Plant relative growth (week 6-8)	0.262	0.151	0.520	**0.018**	0.158	0.394	0.141
Plant relative growth (Week 8-10)	0.445	**0.024**	0.763	0.777	0.066	0.135	0.844
Plant relative growth (week10-12)	**0.002**	0.107	0.830	0.092	0.054	0.810	0.133
Chlorophyll fluorescence	**0.003**	0.122	0.740	0.218	0.258	0.208	**0.005**

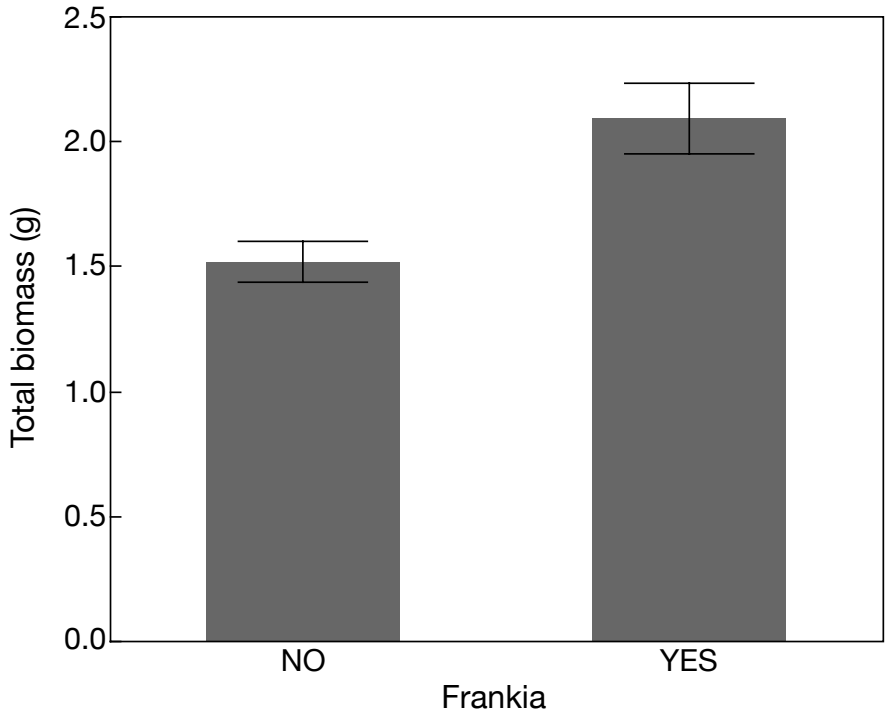

Figure 4.1: The effect of *Frankia* spp. inoculation on total biomass averaged across ECMF and NaCl treatments. Bars are mean with standard errors (SE). P=0.0008 came from a 3 factor least squares model.

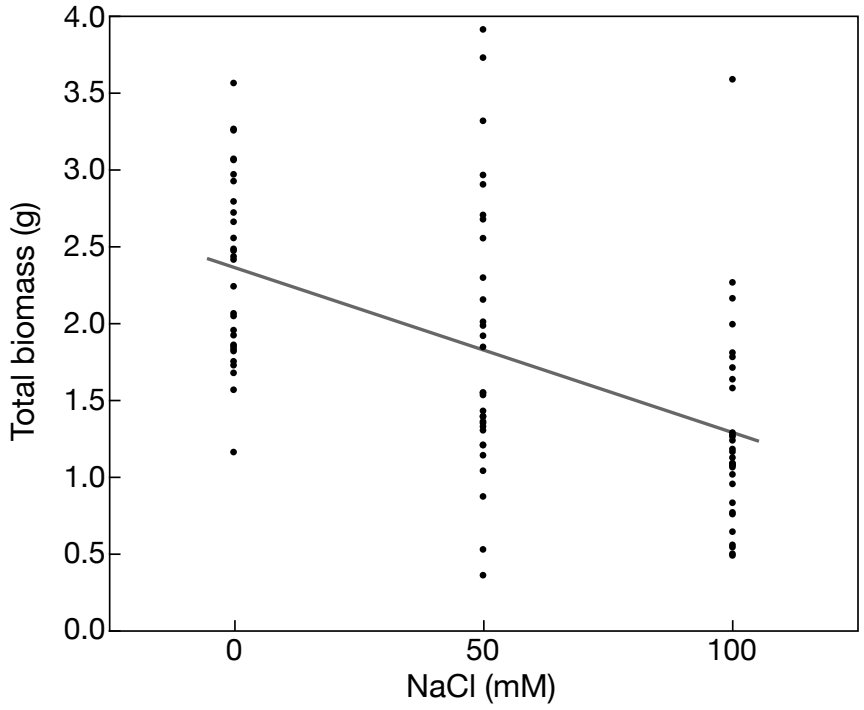

Figure 4.2: The effect of NaCl on total biomass of *Alnus viridis* ssp. *crispa*.
The least square fit line and R^2: Y=2.36-0.01*X, R^2=0.27. P<0.0001 came from a 3-factor least squares model.

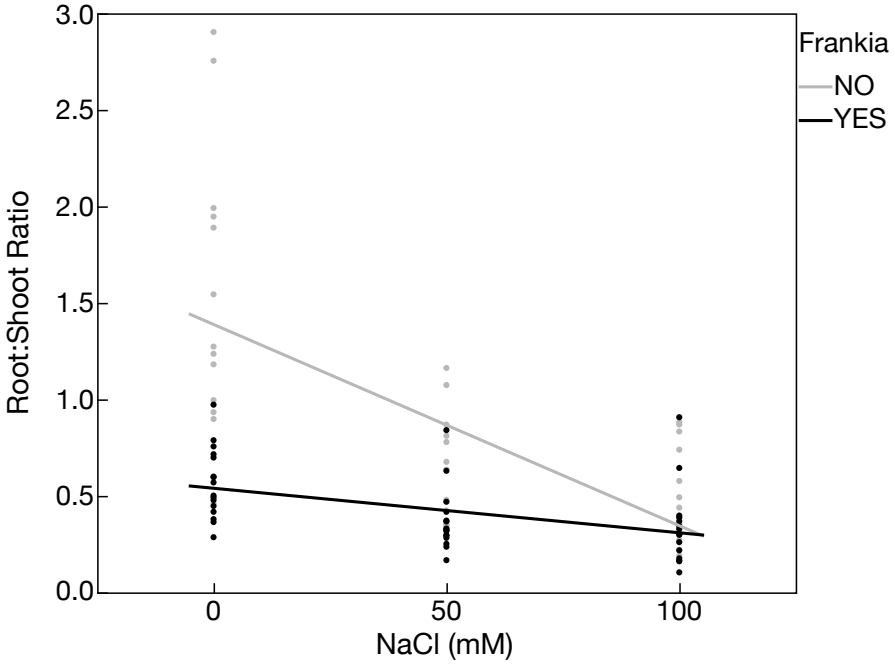

Figure 4.3: The effect of NaCl and *Frankia* spp. on root:shoot ratio of *Alnus viridis* ssp. *crispa*. The least square fit line, R^2 for *Frankia* spp. and non-*Frankia* spp. treatments respectively, were: Y (*Frankia* spp.) = 0.54-0.0024*X, R^2 (YES-*Frankia* spp.) = 0.21, Y(No-*Frankia* spp.) = 1.39-0.01*X, R^2 (No-*Frankia* spp.) = 0.44.

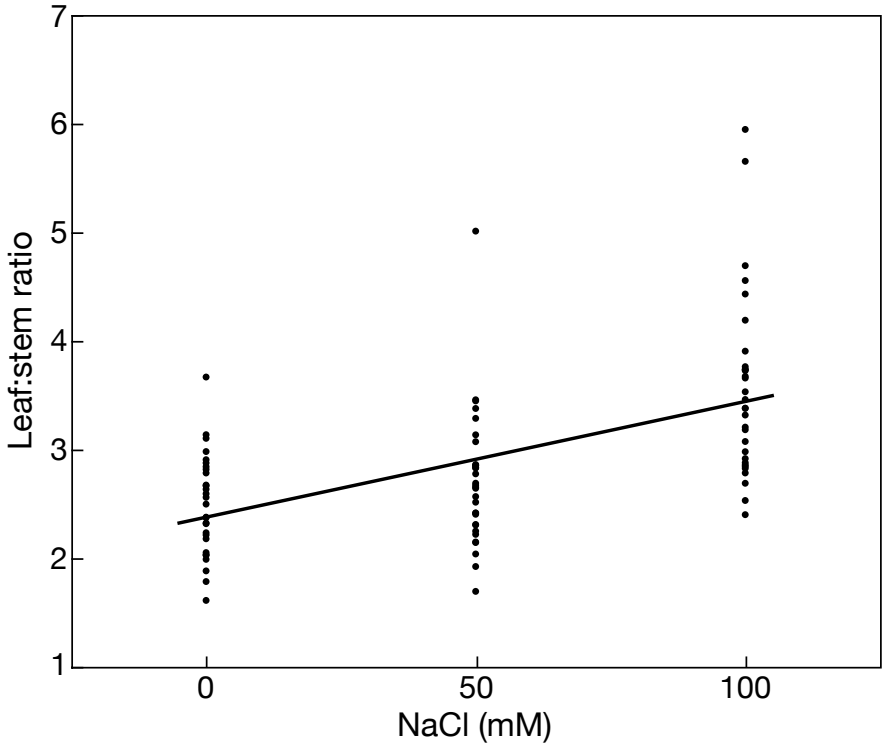

Figure 4.4: The effect of NaCl on leaf:stem ratio of *Alnus viridis* ssp. *crispa*. The least square fit line and R^2: Y=2.38+0.01*X, R^2=0.30.

4.3.2 Hartig net formation

The proportion of Hartig net formation in ECMF inoculation treatments was 96.4%. Neither *Frankia* spp. (X^2 =2.15, P=0.14) nor NaCl (X^2 = 1.19, P= 0.55) had significant effect on plant root Hartig net formation (Table 4.2, Table 4.3). Most of ECMF colonization was superficial.

Table 4.2: The effect of *Frankia* spp. on the proportion of plants forming Hartig net in ECMF treatment. P=0.14 (from chi-square test).

Treatment	Hartig net proportion of plants (%)
Non-*Frankia* spp.	100
Frankia spp.	93

Table 4.3: The effect of different level of NaCl on the proportion of plants forming Hartig net in ECMF treatments. P=0.55 (from chi-square test).

Treatment	Hartig net proportion of plants (%)
0 mM NaCl	94
50 mM NaCl	100
100 mM NaCl	94

4.3.3 Nodule formation and nitrogen fixation

Root nodule number was not significantly affected by the different level of NaCl (F=2.50, P=0.12), or the ECMF treatments (F=3.62, P=0.06) (Table 4.4, Table 4.5). Increasing NaCl levels decreased nodule allocation (F=4.47, P=0.04), from 2.49±0.14 % at 0 NaCl to 1.81±0.27% when plants were exposed to 100 mM NaCl (Figure 4.5). All other factors and their interactions did not significantly affect nodule allocation (Table 4.1).

ECMF and NaCl had interactive effect on specific nodule activity (F=4.20, P=0.0472) (Figure 4.6). ECMF inoculated plants specific nodule activity was higher than the non-ECMF treatment when plants were not exposed to NaCl. With an increasing level of NaCl, ECMF inoculated plants specific nodule activity decreased from 16.6±2.62 C_2H_4 h^{-1} g^{-1} nodule at 0 NaCl to 5.35±2.05 μmol C_2H_4 h^{-1} g^{-1} nodule to 100 mM NaCl, while the non-ECMF treatment specific nodule activity was not affected by NaCl. Similarly, ethylene production rate per plant mass was also affected by ECMF and NaCl interaction (F=5.97, P=0.02) (Figure 4.7). This followed the same trend as specific nodule activity.

Ethylene production rate per plant decreased by 68.5% when plants were exposed to 100 mM NaCl compared to non-NaCl treatments (F=14.02, P=0.0006) (Figure 4.8).

Table 4.4: The effect of ECMF inoculation on nodule number of *Alnus viridis* ssp. *crispa.*

Treatment	Nodule number
Non-ECMF	5.3±0.8 [a]
ECMF	3.4±0.6 [a]

Table 4.5: The effect of NaCl level on nodule number of *Alnus viridis* ssp. *crispa*.

Treatment	Nodule number
0 mM NaCl	5.1±1.0 [a]
50 mM NaCl	3.9±0.9 [a]
100 mM NaCl	3.4±0.8 [a]

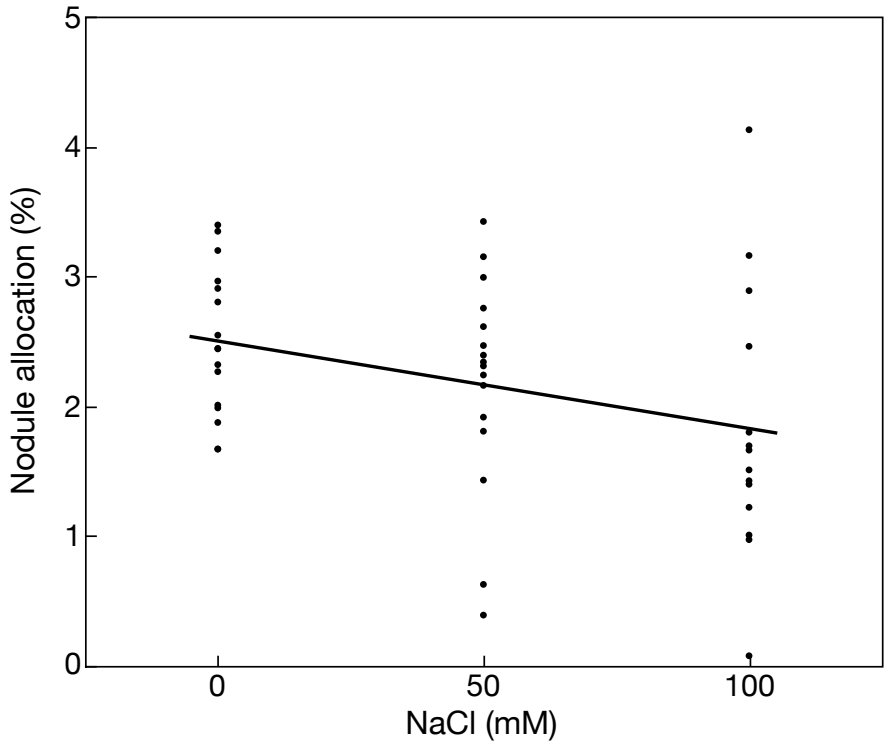

Figure 4.5: The effect of NaCl on nodule allocation of *Alnus viridis* ssp. *crispa* averaged by ECMF inoculation treatments. The least square fit line and R^2: Y=2.5-0.0067*X, R^2=0.11.

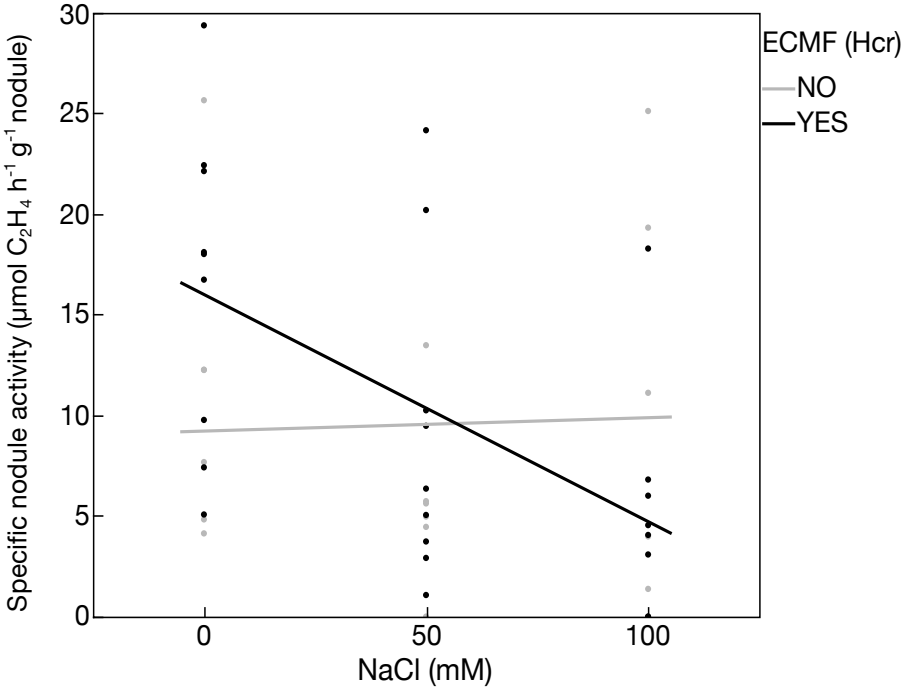

Figure 4.6: The effect of NaCl and ECMF on specific nodule activity. The least square fit line, R^2 for ECMF and non-ECMF treatments respectively, were: Y=16.01-0.11*X, R^2 = 0.3,Y = 9.22+0.0067*X, R^2 = 0.0013

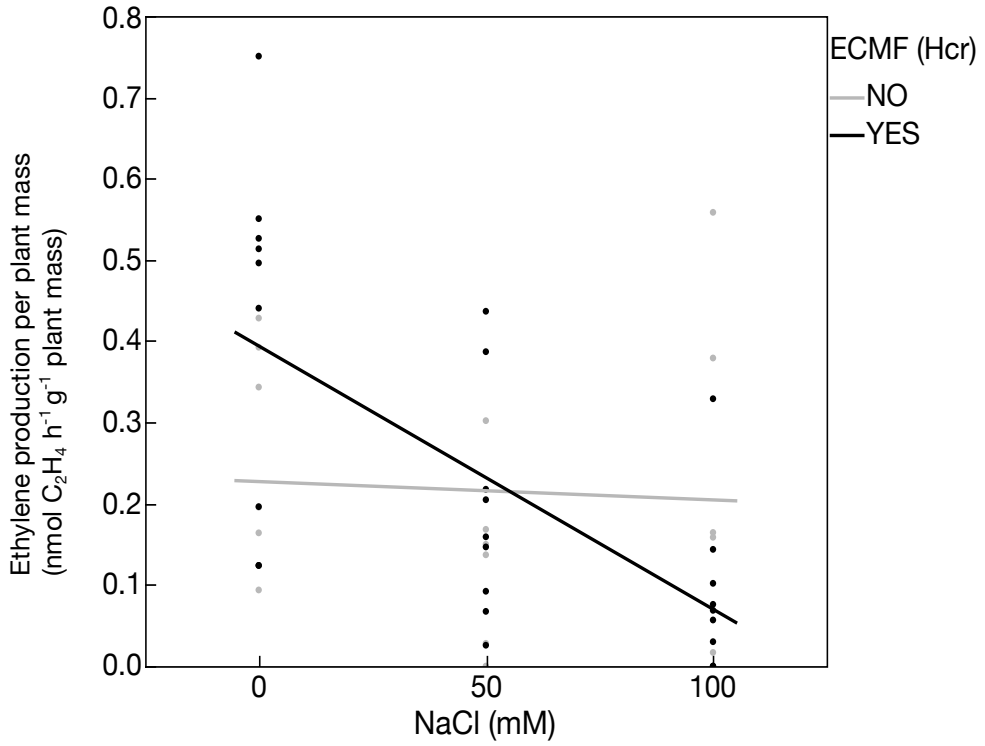

Figure 4.7: The effect of NaCl and ECMF on ethylene production rate per mass of *Alnus viridis* ssp. *crispa*. The least square fit line, R^2 for ECMF and non-ECMF treatments respectively, were: Y=0.39-0.0032*X, R^2 = 0.43, Y=0.23-0.00022*X, R^2 = 0.0035.

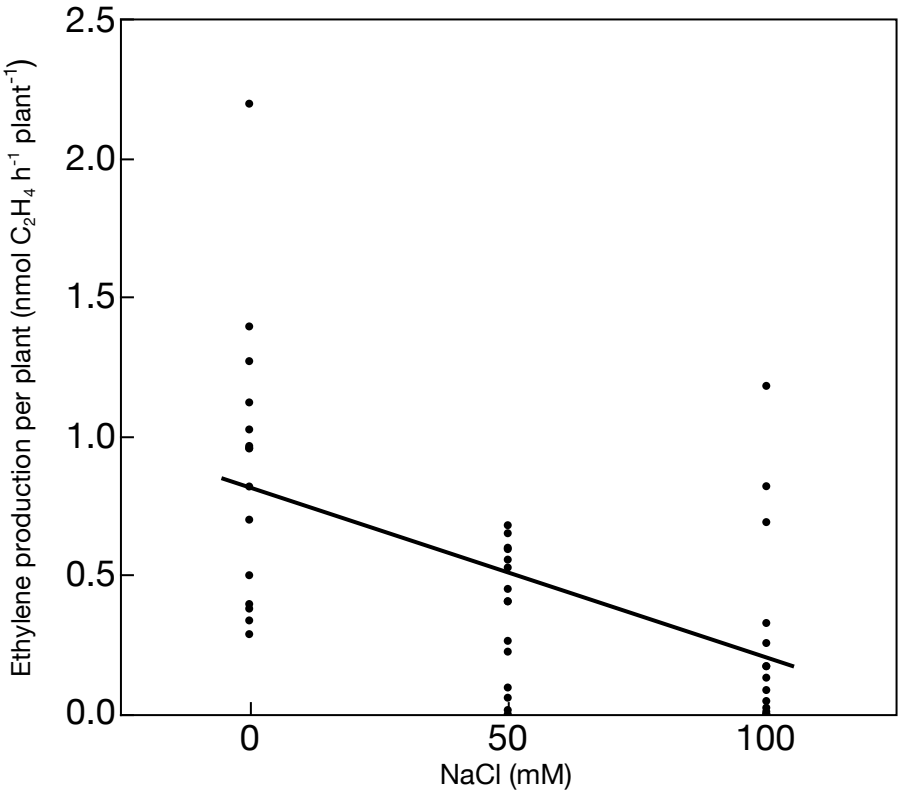

Figure 4.8: The effect of NaCl on ethylene production rate per plant of *Alnus viridis* ssp. *crispa*. The least square fit line was and R^2 were: Y = 0.82-0.006*X, R^2 = 0.29.

4.3.4 Root and nodule respiration

Frankia spp. inoculated plant root respiration rate (with nodules removed) was significantly increased by 42.4% compared with non-*Frankia* spp. treatments (F=7.70, P=0.007) (Figure 4.9). Increasing the level of NaCl also significantly increased the root respiration rate (with nodules removed) by 62.2% at 100 mM NaCl compared with non-NaCl treatments (F=9.20, P=0.005) (Figure 4.10). All other factors and their interaction did not have significant effects on root respiration rate (Table 4.1).

Increasing NaCl level also significantly increased nodule respiration rate (F=7.97, P=0.007). At 0 mM NaCl, nodule respiration rate was 198.18±21.41 μmol CO_2 h^{-1} g^{-1} nodule, which increased by 80% when plants were exposed to 100 mM NaCl (Figure 4.11). There was no significant effect on nodule respiration rate due to ECMF inoculation (F=0.74, P=0.40) and the interaction between ECMF and NaCl (F=0.20, P=0.66) (Table 4.1).

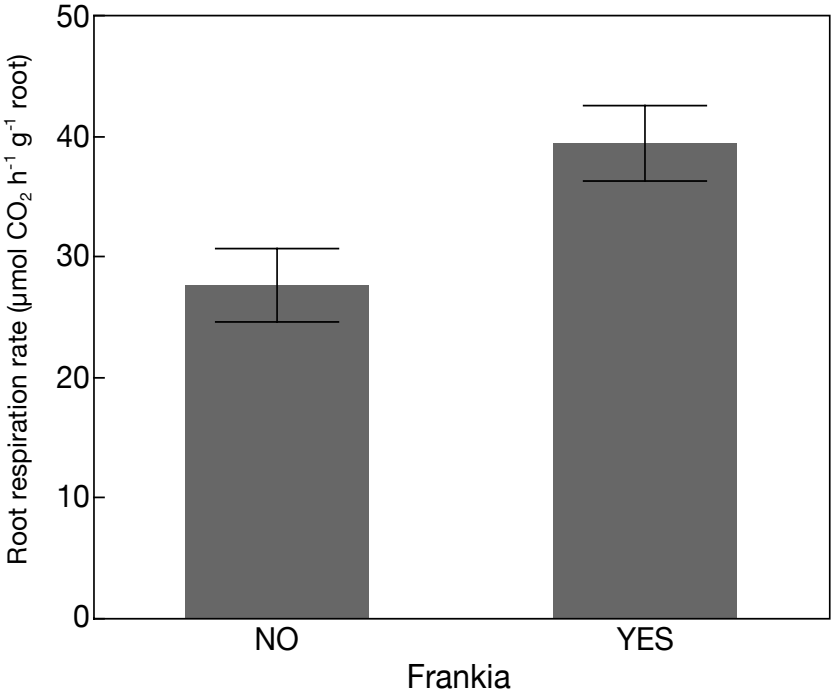

Figure 4.9: The effect of *Frankia* spp. on Root respiration rate (with nodules removed) of *Alnus viridis* ssp. *crispa*. Bars are mean with standard errors (SE). P=0.007 from 3 factor least squares model.

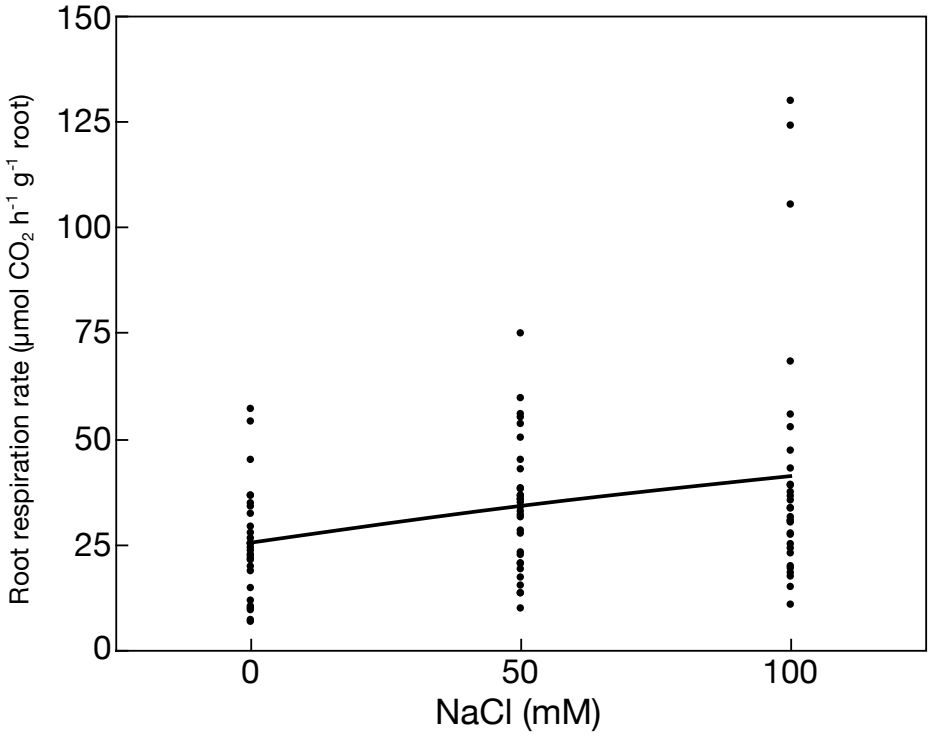

Figure 4.10: The effect of NaCl on root respiration rate (with nodule removed) of *Alnus viridis* ssp. *crispa.* The least square fit line and R^2 were: Y=25.63+0.16*X, R^2=0.09.

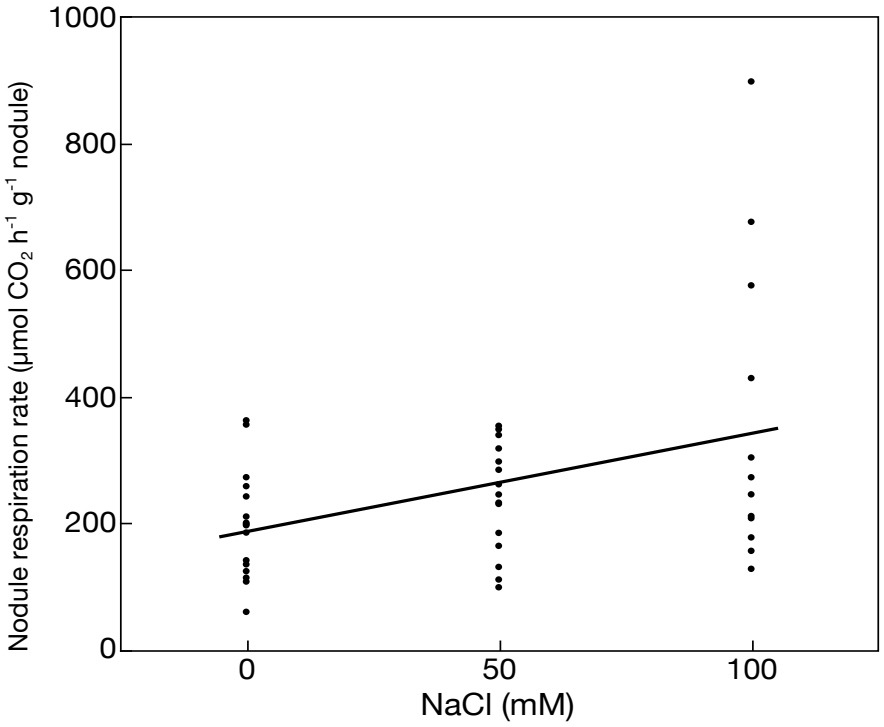

Figure 4.11: The effect of NaCl on nodule respiration rate of *Alnus viridis* ssp. *crispa*. The least square fit line and R^2 were: Y=186.9+1.55*X, R^2=0.16.

4.3.5 Root acid phosphatase activity

Frankia spp. inoculation positively affected root acid phosphatase activity (F=16.11, P=0.0001) (Figure 4.12). *Frankia* spp. inoculated plant root , which was nearly twice as much as the non-*Frankia* spp. treatments (Figure 4.12). Root acid phosphatase activity was increased with an increasing level of NaCl (F=18.12, P<0.0001) (Figure 4.13). Root extracellular phosphatase activity was 4.13±0.41μmol p-NP h^{-1} g^{-1} root in the 0 mM NaCl treatment, and increased to 8.90±0.86μmol p-NP $h^{-1}g^{-1}$ root at 100 mM NaCl. All other factors and their interaction did not significantly affect root acid phosphatase activity (Table 4.1).

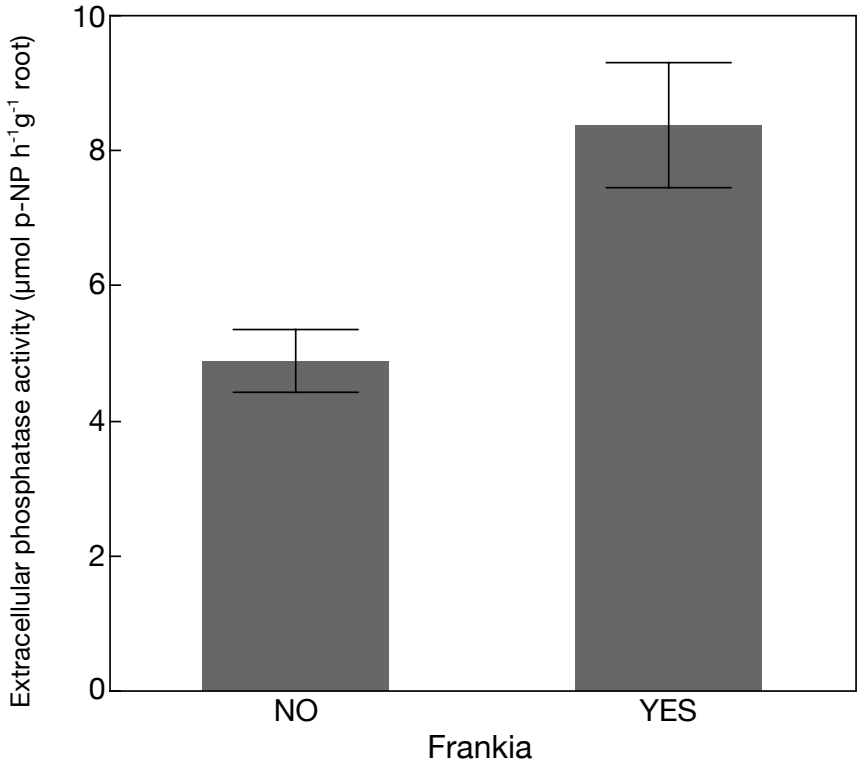

Figure 4.12: The effect of *Frankia* spp. on root extracellular phosphatase activity of *Alnus viridis* ssp. *crispa*. P=0.0001 came from 3 factor least squares model.

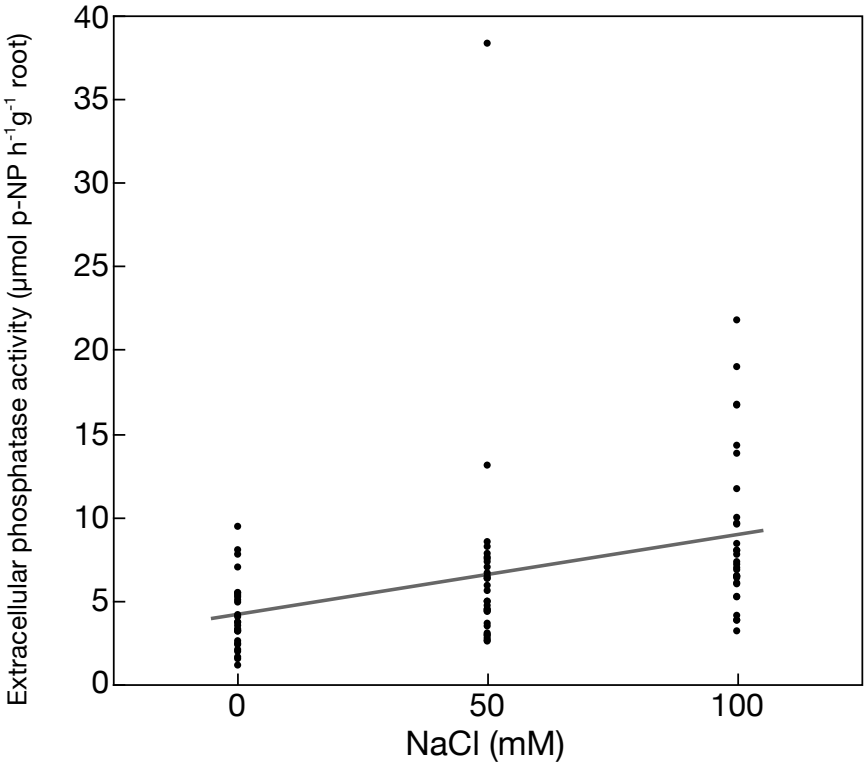

Figure 4.13: The effect of NaCl on root extracellular phosphatase activity of *Alnus viridis* ssp. *crispa*. The least square fit line and R^2 were: Y=4.18+0.05*X, R^2=0.14.

4.3.6 Plant relative growth rate

During week 6 to week 8, NaCl significantly decreased plant relative growth (F=5.87, P=0.02) (Figure 4.14). All other factors did not have any effect on the plant relative growth rate during this period (Table 4.1).

During week 8 to week 10, ECMF significantly increased plant relative growth rate (F=5.32, P=0.02), from 0.012 mm/mm/day in plants without ECMF inoculation to 0.017 mm/mm/day in plants with ECMF inoculation, an increase of 41.7% (Figure 4.15). All other factors did not affect plant relative growth rate during week 8 to week 10 (Table 4.1).

During week 10 to week 12, *Frankia* spp. inoculation significantly increased the plant relative growth rate (F=10.65, P=0.0018) (Figure 4.16). Plant relative growth rate increased from 0.08 mm/mm/day in plants without *Frankia* spp. to 0.09 mm/mm/day in plants with *Frankia* spp. All other factors did not affect plant relative growth rate during this period (Table 4.1).

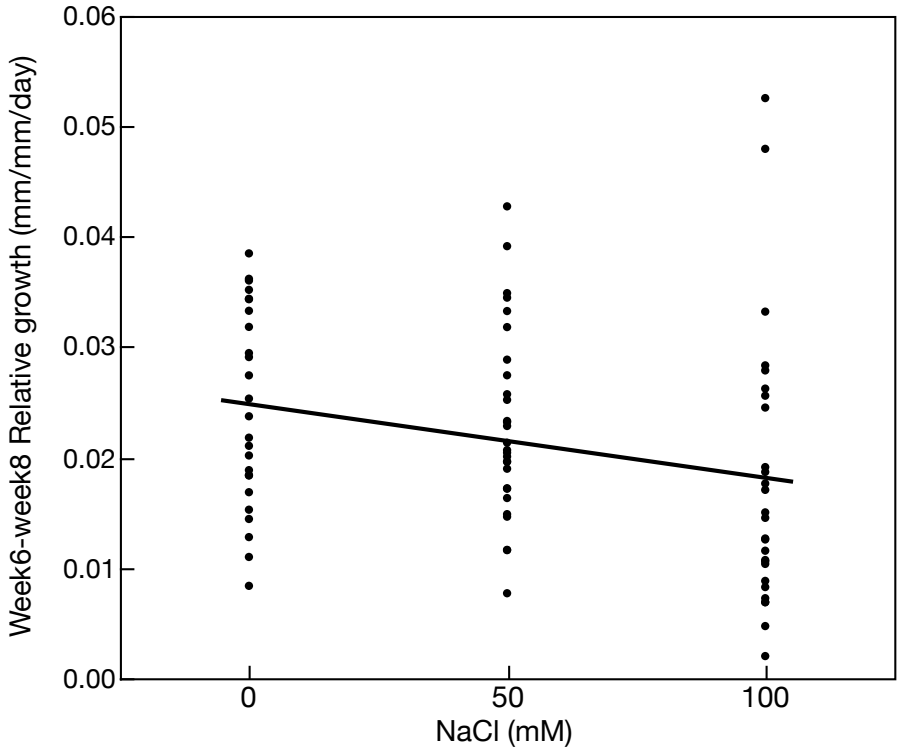

Figure 4.14: The effect of NaCl on plant relative growth rate during week 6 to week 8. The least square fit line and R^2 were: $Y=0.02-6.67e^{-5}*X$, $R^2=0.06$.

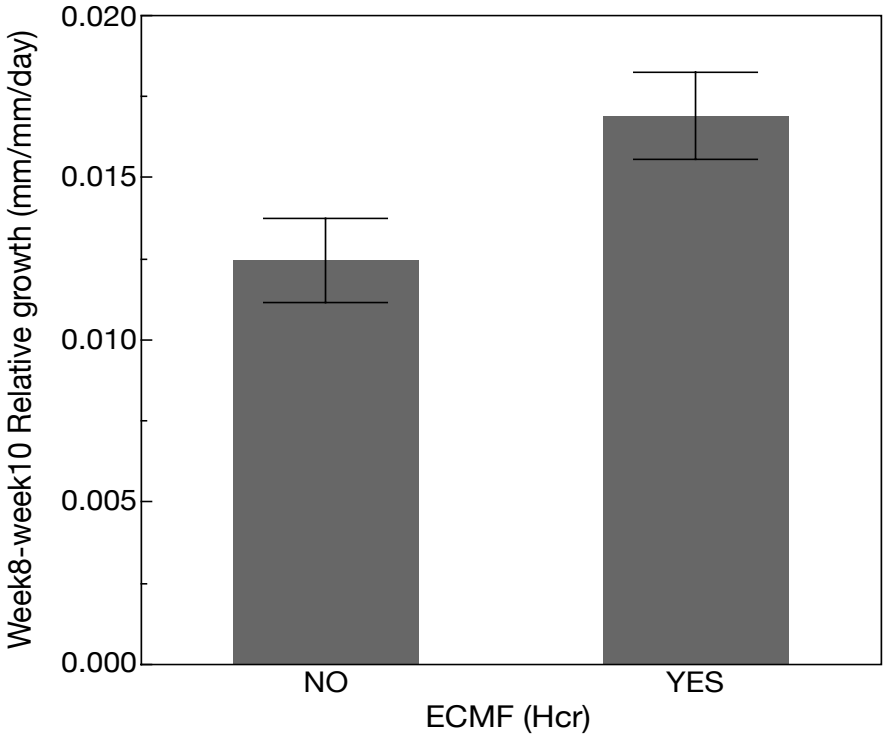

Figure 4.15: The effect of ECMF on plant relative growth rate during week 8-10. P=0.02 came from 3 factor least square moedel.

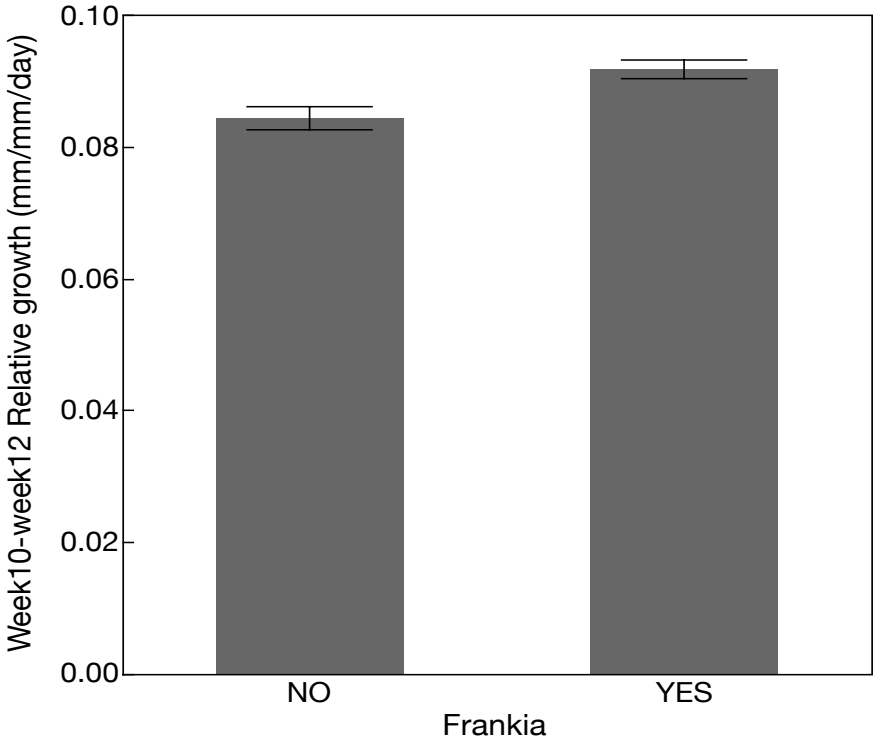

Figure 4.16: The effect of *Frankia* spp. on plant relative growth rate during week 10-12. P=0.0018 from 3 factor least square model.

4.3.7 Leaf chlorophyll fluorescence

There was no significant difference in terms of plant leaves chlorophyll fluorescence (Fv/Fm) the day before adding NaCl (Figure not shown). At week 12, *Frankia* spp., ECMF and NaCl had an interactive effect on plant chlorophyll fluorescence ($F=8.44$, $P=0.005$) (Figure 4.17). With an increasing level of NaCl, there was a decrease in plant chlorophyll fluorescence when plants were not inoculated with either symbiont. However, plant chlorophyll fluorescence did not decrease with the increasing level of NaCl when plants were inoculated with ECMF or (and) *Frankia* spp.

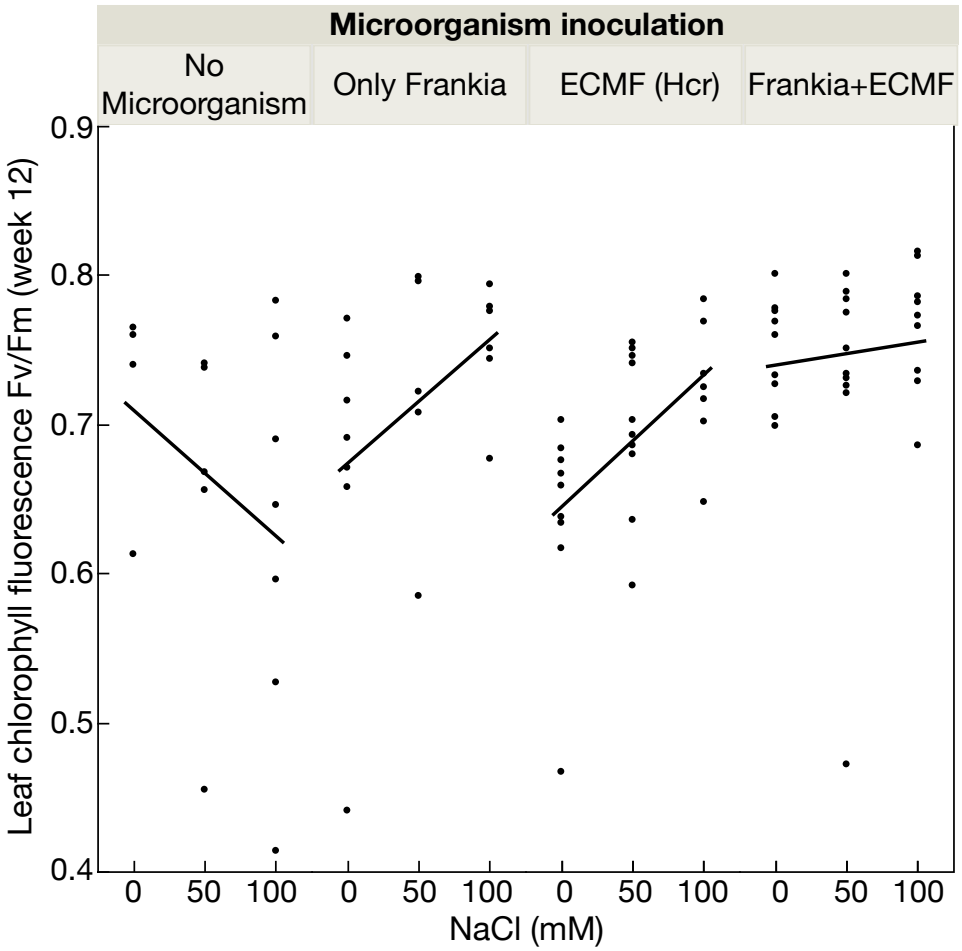

Figure 4.17: The effect of ECMF and (or) *Frankia* spp. on plant leaf chlorophyll fluorescence at week 12. The least square fit line, R^2 for without any microorganisms, with ECMF, with *Frankia* spp., with both *Frankia* spp. and ECMF respectively, were: Y=0.71-0.0008*X, R^2=0.10; Y=0.64+0.0009*X, R^2=0.29; Y=0.67+0.0008*X, R^2=0.17; Y=0.74+0.0002*X, R^2=0.01.

4.4 Discussion

Frankia spp. inoculated plants had significantly higher biomass compared to non-*Frankia* spp. treatments. This is consistent with the result of chapter 3. Also, as in the previous chapter, ECMF did not have any positive effect on plant performance, possibly due to *Hebeloma crustuliniforme* having superficial colonization on the roots.

The decreasing plant performance caused by NaCl in my experiment agreed with previous studies showing that increasing NaCl results in a significant decrease in plant total dry mass (Meloni et al., 2001; Tuna et al., 2008; Hajlaoui et al., 2010). Plant growth limitation by NaCl is known to be caused by two major factors: osmotic stress and ionic toxicity (Renault, 2005 a and b; Parida and Das, 2005). Osmotic effects of NaCl on plants are due to lower soil water potential caused by an increasing solute content (Na^+, Cl^-) in the root zone (Ahmad et al., 2013). This inhibits plants from extracting not only water but also nutrients form soil, especially for NO_3^- and K^+ (Peuke and Jeschke, 1999; Ahmad et al., 2013). A high concentration of NaCl in the rhizosphere impairs cell growth and cell elongation and decreases cell volume by reducing cell osmotic potential and turgor pressure (Yeo, 1983; Zhu, 2002). Under osmotic stress, plants tend to increase their active cell solutes (such as amino acids, sugars, sugar alcohol, betaines and cyclic polyhydric alcohols) to compensate salt stress by lowering the water potential in the cytoplasm, defined as osmotic adjustment (Yeo, 1983; Ahmad et al., 2013). The adverse effects of salinity on plants result also from ionic stress caused by the accumulation of ions (Na^+ and Cl^-) in the plant tissues (Hasegawa et al., 2000). The high concentration of Na^+ in the cytosol can alter biochemical reactions and affect most enzyme activities (Serrano, 1999). Salt tolerante plants generally rely on osmotic adjustment and ionic

compartmentalization to exclude or partition excess Na^+ (Yeo, 1983; Hasegawa et al., 2000).

A number of nitrogen fixation actinorhizal plants have been found to be salt tolerant. Some *Casuarina* species (*Casuarina glauca*, *Casuarina obesa, Casuarina cristata*) have been found growing in up to 5600 mS m^{-1} NaCl affected sand substrates, or 600 mM NaCl in hydroponic cultures (Van Der Moezel et al., 1989; Batista-Santos et al., 2015). *Elaegnus angustifolia* and *Elaegnus oxycarpa* also persisted in saline areas (300 mM NaCl) and even in P-deficiency conditions (Khamzina et al., 2009; Maimaiti et al., 2014). Another species, *Shepherdia argentea* possessed high salt tolerance up to 400 mM NaCl (Qin et al., 2010 a; Qin et al., 2010 b). The salinity tolerance of these actinorhizal plant species makes them potential candidates for reforestation and phytoremediation of saline areas. For my experiment, although *Alnus viridis* ssp. *crispa* total biomass was reduced with increasing NaCl, *Frankia* spp. inoculation increased plant performance at all levels of NaCl tested in the study.

Unlike some actinorhizal plants, the growth of most nitrogen fixing legumes that have been tested are sensitive to salt stress like all plants (Manchanda and Garg, 2008; Bruning and Rozema, 2013). Bruning and Rozema (2013) concluded that the legumes nodulation could be altered at four stages under salt stress: 1) decreased root hair number, 2) signal exchange between partners was negatively affected 3) disturbed infection threads 4) decreased nodule number and dry weight. This finding agreed with Bouhmouch et al. (2005) who found that nitrogen-fixing plants were more sensitive to salt stress than those depending on mineral N. This contradicts my experiment in that there was no interaction between *Frankia* spp. and NaCl on plant total biomass.

In my experiment, salt stress decreased Fv/Fm ratio with the absence of any microorganism, while Fv/Fm ratio did not decrease with salt stress when plants were inoculated with both ECMF and *Frankia* spp. and these ratios were within the normal range established by Björkman and Demmig (1987). The ratio of variable to maximum chlorophyll fluorescence (Fv/Fm) represents the maximum photochemical efficiency of photosystem II (PSII) (Favaretto et al., 2011; Lucena et al., 2012). Björkman and Demmig (1987) found that the typical range of Fv/Fm ratio of healthy vascular plants is 0.75-0.85. It has been reported that salt stress decreased photochemical efficiency of photosystem II (Fv/Fm) (Kalaji et al., 2011; Lucena et al., 2012). Under salt stress, older leaves were negatively affected first due to having higher Cl^- concenterations than young leaves. The loss of chlorophyll in older leaves might result in a decrease in the variable chlorophyll fluorescence, therefore decreased PSII efficiency (Smillie and Nott, 1982). According to Lucena et al. (2012), salt tolerant cultivars showed less reduction in PSII efficiency. My results suggest that ECMF and *Frankia* spp. inoculation could alleviate salt stress in term of photosynthesis. However, the real mechamism need further research.

It is commonly found that root growth is less sensitive to salt stress than shoot growth (Cheeseman, 1988; Muhsin and Zwiazek, 2002), resulting in increasing plant root:shoot ratios. The root:shoot ratio of spruce increased under 25 mM NaCl stress (Muhsin and Zwiazek, 2002). Qin et al. (2010 b) found the root:shoot ratio of *Shepherdia argentea* was significantly increased when treated with 400 and 600 mM NaCl compared to control. Plants maintain a higher root: shoot ratio under salinity stress in order to maintain water balance. Salt tolerant trees can also sequester Na in their roots when grown in a salt medium. It is known that larger root systems have a higher capacity

to deposit Na, therefore increasing resistance to salinity (Yi et al., 2008). In my study, the root:shoot ratio decreased with an increasing level of NaCl in both *Frankia* spp. and non-*Frankia* spp. treatments. *Alnus viridis* has a shallow root system (https://en.wikipedia.org/wiki/Alnus_viridis), which easily results in exposure to environment fluctuation (water stress, salt stress, temperature). This root morphology can increase a plant's sensitivity to soil salinity (Bernstein et al., 2004). It has been reported that avocado root system was also more sensitive to salinity than shoot due to the few root hairs and shallow root system (Bernstein et al., 2004). The authors confirmed that under salt stress, root growth was inhibited and therefore reduced the young root surface area and hence reduced the nutrients and water uptake capacity of root system. This in return, decreased the nutrient supply to shoots. These results also agreed with Renault (2005) who found that salt stress affected root growth of red-osier dogwood (*Cornus stolonifera*) to a greater extent compared to shoot growth. Similarly, Maize (Zea mays L. cv., DK647 F1) root:shoot ratio was also shown to decrease under 100 mM NaCl (Tuna et al., 2008).

Although *Alnus viridis* ssp. *crispa* showed reduction in root:shoot ratio under salt stress, the reduction rate was more moderate in *Frankia* spp. inoculated plants compared to non-*Frankia* spp. treatments. This suggested that salt interferers more severely on those plant dependent on inorganic N than nitrogen-fixing plants. *Frankia* spp. inoculated plants had less root allocation compared to non-*Frankia* spp. treatments at any level of NaCl. This was the same result as we found in chapter 3 and is likely due to the nodulated plants shifting more nutrients to shoot development rather than to roots in order to meet the carbon requirement of nitrogen fixing bacteria.

My results showed that root (with nodule removed) and nodule respiration rate increased with an increasing level of NaCl. It has been observed that salinity increased root respiration in many studies. Bloom and Epstein (1984) confirmed that root respiration of barley increased at 10 mM NaCl. The authors found that the more salt-sensitive variety (Arivat barley) showed higher root respiration compared to the more salt-tolerant variety (California Mariout barley) under salt stress. Salt stimulated respiration can also occur in salt tolerant species (*Tamarix ramosissima*), since the more energy was used for salt pumping (Kleinkopf and Wallace, 1974). Burchett et al. (1984) found that grey mangrove (*Avicennia marina*) root respiration increased under 25% seawater, but declined above 25% seawater. However, Kalir and Poljakoff-Mayber (1976) found a decreasing trend in root respiration of *Tamarix tetragyna* with increasing NaCl. The reason for respiration increase is not clear but may relate to the energy required to exclude Na^+ and nutrients uptake (K^+) under stress condition. Bernstein (1975) also suggested that high respiratory activity might be needed to maintain the solute concentration required for osmotic adjustment under salt stress.

Both NaCl and ECMF did not affect nodule number, but NaCl decreased nodule dry mass and allocation. For my experiment, NaCl was applied after nodulation, so salt stress did not affect nodule initiation but reduced nodule growth and establishment. Previous studies have shown that salt stress inhibited nodule initiation and nodule growth. A saline solution (>28.8 dS/m) reduced nodule number of *Medicago sativa* even under P-supplement or arbuscular mycorrhizal inoculated treatments (Azcon and El-Atrash, 1997). Soussi et al. (1999) found that nodule number of two cultivars of *Cicer arietinum* decreased at 100 mM NaCl conditions. Nodule dry weight of Pedrosillano (a salt-

sensitive cultivar) increased at low salinity level (50mM NaCl) but decreased in 100 mM NaCl, while in ILC1919 (a salt-tolerant cultivar) this parameter rose at all salt concentrations, compared to control. Aydi et al. (2008) investigated three *Medicago truncatula* varieties under salt stress and found that nodule number and nodule dry weight decreased in TN6.18 (a salt sensitive cultivar), while nodule dry weight increased but nodule number had no difference in TN8.20 (a salt tolerant cultivar) under 75 mM NaCl compared to control. El-Akhal et al. (2013) found that nodule number and nodule fresh weight of two peanut cultivars increased under salt stress.

ECMF inoculation increased plant specific nodule activity and ethylene production rate per plant mass under non-NaCl. However, this positive effect was inhibited by an increasing level of NaCl. This suggests that *Hebeloma crustuliniforme* was sensitive to salt stress. In my study, increasing NaCl decreased total nodule activity. Previous studies have tried to explain this negative effect. Aydi et al. (2008) suggested that the decreased nitrogen fixation was due to an increasing accumulation of Na^+ in leaves under a 75 mM NaCl exposure. Reddell et al. (1986) found that nitrogen fixation decreased when *Casuarina obesa* were exposed above 0.15 mg g^{-1} NaCl soil due to a decrease in nodule dry weight. It has been concluded that salt stress inhibited nitrogenase activity and photosynthate supply to nodule bacteria and increased the O_2-diffusion barrier in nodules, therefore reducing nitrogen fixation in stressed plants (Bouhmouch et al., 2005). Delgado et al. (1994) reported that the specific nodule activity decreased by salt might be mainly due to the dropping respiration of nodule bacteriod.

In chapter 3, I showed that biological nitrogen fixation was driven by the respiration of host plants i.e., there was linear relationship between nitrogen fixation and nodule

respiration. However, in this experiment, we found that salt stress decreased nitrogen fixation but stimulated nodule respiration, so nitrogen fixation was not linked to nodule respiration. In symbiotic systems, the enegetic cost of nitrogen fixation can be expressed as the ratio of nodule respiration assocated with nitrogenase activity relative to the amount of nitrogen fixed (Mahon, 1977). Under high salt level (6000 mg kg^{-1} NaCl), this cost was shown to increase in alfalfa but the respiration associated with plant growth decreased (Ikeda et al., 1992). However, some studies reported that salt stress limits both nitrogen fixation and respiration, and the decreased nitrogen fixation by salt stress might be due to the limitation of nodule respiration (Delgado et al., 1994; Aydi et al., 2008).

Increasing soil NaCl level increased root acid phosphatase activity. Increases in acid phosphatase activity caused by salt and osmotic stress have been investgated in many previous studies (Flasiński et al., 1989; Olmos and Hellin, 1997; Ehsanpour and Amini, 2003; Sharma et al., 2004). Under salt stress, water and nutrients uptake are inhibited, therefore phosphate delivery is impaired. Plants compensate by releasing more acid phosphatase to increase free phosphate uptake (Sharma et al., 2004). These changes have been found to be associated with an osmotic adjustment (Yeo, 1983; Flasiński et al., 1989; Ahmad et al., 2013). The present investigation on phosphatase activity agreed with the results obtained in chapter 3, which showed that *Frankia* spp. inoculation increased root extracellular phosphatase activity.

Hebeloma crustiluniforme did not alleviate the salinity stress on plant growth in this experiment, perhaps due to its poor colonization. Most ectomycorrhizal fungi, especially the basidiomycete species, have been shown to be salt sensitive (Dixon et al., 1993; Bois et al., 2006 b). However, there are still some species showing salt tolerance, like

Hebeloma crustuliniforme and *Pisolithus* species, which have been found to be growing in 200 mM NaCl medium by maintaining higher level of water content in the mycelium (Chen et al., 2001; Kernaghan et al., 2002; Bois et al., 2006 b). White spruce inoculated by *Hebeloma crustuliniforme* showed some salt tolerance (at 25 mM NaCl), with a slightly increased plant dry weight (around 20%) under saline conditions compared to non-ectomycorrhizal treatments (Nguyen et al., 2006). This result agrees with Muhsin and Zwiazek (2002) who found that white spruce symbiosis with *Hebeloma crustuliniforme* and treated with 25 mM NaCl showed reduced sodium both in shoots and roots. Mycorrhizal roots increased N and P uptake, and shoots maintained high rates of transpiration under salt stress. However, the benefits from ectomycorrhizal fungi for plant salt tolerance vary with both fungal and plant species. Bois et al. (2006 a) tested three ectomycorrhizal fungal species, found to be salt tolerant in vitro, on greenhouse-grown conifers. The authors found that white spruce seedlings inoculated with *Suillus tomentosus* had the best growth even at 200 mM NaCl. While jack pine seedling inoculated with *Hebeloma crustuliniforme* showed less biomass than the other two ECMF species inoculated seedlings, but were more tolerant to osmotic and ionic stress. Both of the host species showed higher water content in shoots and roots at any level of NaCl (0, 50, 100, 200 mM NaCl) when inoculated with *Hebemoma crustulimiforme*. Onwuchekwa et al. (2014) demonstrated that ectomycorrhizal fungi (*Hebeloma crustuliniforme*, *Suillus tomentosus* and *Laccaria bicolor*) inoculation increased jack pine (*Pinus banksiana*) growth and white spruce (*Picea glauca*) survival rate growing in oil sands reclamation areas. Although ectomycorrhizal fungi can show benefits to host plants grown in saline conditions, there are some exceptions. Nguyen et al. (2006) found that *Hebeloma*

crustuliniforme did not have any effect on dry weight as well as Cl^- concentration in jack pine under salinity conditions.

It has been suggested that mycorrhizal fungi increase salinity tolerance by increasing root allocation (Yi et al., 2008). Bois et al. (2006 a) reported that higher root:shoot ratio in jack pine helped minimize the toxic effect of Na^+ on photochemistry. This might be due to the ECMF enhacing sugar (pinitol) accumulation in response to NaCl. *Hebeloma crustuliniforme* inoculated white spruce (*Picea glauca*) showed higher root hydraulic conductance than non-mycorrhizal seedlings under salt stress (Muhsin and Zwiazek, 2002). It is possible that the ectomycorrhizal hyphae link between soil and roots reduced the hydraulic resistance under osmotic stress since the water flow must go through plasma membrane (Steudle and Peterson, 1998).

Mycorrhizae might also resist the ionic imbalance in the root zone caused by salt stress. Bledsoe and Rygiewicz (1986) found that *Hebeloma crustuliniforme* inoculation decreased Na^+ uptake of Douglas-fir seedlings and helped maintain an ionic balance compared with non-ectomycorrhizal seedlings. This could be due to *Hebeloma crustuliniforem* showing salt tolerance in *vitro* and accumulating higher sodium and chloride compared with other ECMF species by producing trehalose and mannitol in response to NaCl (Kernaghan et al., 2002; Bois et al., 2006 a).

In addition to fungal inoculation having no effect on plant salt tolerance, salt had no effect on mycorrhizal formation in my experiment. Dixon et al. (1993) found that ectomycorrhizal fungi colonization was reduced under salt stress (> 80 mM NaCl). In my study, since the NaCl solution was applied 6 weeks after inoculation, mycorrhizae should

have already been formed before adding salt. Thus, the proportion of plants forming a Hartig net was not affected by salt stress.

In this study, plant relative growth rate decreased in the first two-week after applying salt. While ECMF did not have an overall effect on plant performance, it did increase the plant relative growth rate later in this experiment. This suggested that ECMF might improve the plant growth over a longer growth period.

4.5 Conclusion

In my study, it was concluded that ECMF did not have any positive effect on overall plant performance but *Frankia* spp. did. However, ECMF did increase specific nodule activity under non-NaCl condition. Although ECMF inoculation did not show any benefits in plant biomass, ECMF and *Frankia* spp. inoculated plants displayed normal range of PSII efficiency and higher relative growth rate under salt stress compared to non-microorganism treatments.

Chapter 5. GENERAL CONCLUSION

Frankia spp. inoculation increased plant performance both in low fertility and saline soil, but ectomycorrhizae did not. It cannot be concluded that *Frankia* spp. and ectomycorrhizae had a synergistic effect on the growth of the same host plant (*Alnus viridis* ssp. *crispa*). However, the dual inoculation of *Frankia* spp. and *Hebeloma crusuliniforme* increased plant relative growth rate as well as leaf chlorophyll fluorescence 4 weeks after applying NaCl. This suggests that tripartite symbiosis among ECMF, *Frankia* spp. and *Alnus viridis* ssp. *crispa* might benefit host plant growth under saline condition over a longer period. To confirm this, it will be nessary to monitor seedlings response to long-term exposure to salt conditions in the field.

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