An Abstract of the Thesis of

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Title : Water Stress, Fertilization and Light Effects on the Growth of Nodulated Mycorrhizal Red Alder Seedlings.

Signature redacted for privacy.

Abstract approved by : _____

Randolph J. Molina

Red alder (*Alnus rubra* Bong.) seedlings fertilized with NH_4NO_3 or inoculated with a pure culture *Frankia* and inoculated with live or dead spores of the mycorrhizal fungus *Alpova diplophloeus* (Zeller & Dodge) Trappe & Smith were grown in a growth chamber or in a greenhouse for six months. *Frankia* inoculation and subsequent nodulation and N_2-fixation increased seedling growth more than N-fertilization. *Alpova* inoculation significantly increased seedling growth in some parameters by increments of 6 to 16 % but only when the seedlings were also inoculated with *Frankia*. Water stress significantly decreased nodule and *Alpova* ectomycorrhiza development, N_2-fixation, growth and photosynthesis of red alder seedlings. *Alpova* inoculation did not improve water relations of red alder seedlings. Heavy N-fertilization with 5 ml of 50 mM NH_4NO_3 per seedling three times a week significantly increased mycorrhiza formation and N and P concentration in leaves but decreased N-fixation, shoot growth and P concentration in nodules. P fertilization with 5 ml of 5mM KH_2PO_4 per seedling three times a week significantly increased total N-fixation. Light intensities below photosynthetic photon flux density of 220 $\mu mol/m^2/s$ significantly decreased N_2-fixation, total plant growth and photosynthesis, but increased leaf area, shoot to root

ratio and N and P concentrations in plant tissues. Reduced light significantly decreased *Alpova* mycorrhiza formation after three-weeks shading in Experiment 1 but not after ten-weeks shading of Experiment 2. During the long shade exposure of Experiment 2, alder seedlings adapted morphologically to low light intensity, thus moderating negative effects on mycorrhiza formation; plants were unable to do this in the 3-week shade period of Experiment 1.

Results suggest that *Frankia* is more important for *Alpova* mycorrhiza formation and growth of red alder seedlings than *Alpova* is for nodule formation and growth. Alder seedlings apparently adapt to a certain point of light stress by increasing leaf area and shoot growth and maintain balanced symbiont development and growth. N-fertilization does not affect nodulated alder plant growth. The importance of P fertilization and mycorrhizae to plant growth increases with plant size and age.

WATER STRESS, FERTILIZATION AND LIGHT EFFECTS ON THE GROWTH OF NODULATED, MYCORRHIZAL RED ALDER SEEDLINGS

by

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I dedicate this thesis to two humble Korean women, my mother, Soon Bok Son and my wife, Gill Soon Kwon who have everlasting love and faith in me. I would like also to express deep gratitude to my brothers' and brothers-in-law families for their financial support, encouragement and love.

TABLE OF CONTENTS

	Page
WATER STRESS, FERTILIZATION AND LIGHT EFFECTS ON THE GROWTH OF NODULATED, MYCORRHIZAL RED ALDER SEEDLINGS	1
INTRODUCTION	1
CHAPTER 1 : EFFECTS OF WATER STRESS ON ECTOMYCORRHIZA AND NODULE FORMATION, N_2-FIXATION AND GROWTH OF RED ALDER SEEDLINGS	3
ABSTRACT	4
INTRODUCTION	5
MATERIALS AND METHODS	9
RESULTS	14
DISCUSSION	18
REFERENCES	29
CHAPTER 2 : EFFECTS OF N AND P FERTILIZATION ON ECTOMYCORRHIZA AND NODULE FORMATION, N_2-FIXATION AND GROWTH OF RED ALDER SEEDLINGS	34
ABSTRACT	35
INTRODUCTION	37
MATERIALS AND METHODS	39
RESULTS	45
DISCUSSION	58
REFERENCES	62
CHAPTER 3 : LIGHT EFFECTS ON ECTOMYCORRHIZA AND NODULE FORMATION, N_2-FIXATION AND GROWTH OF RED ALDER SEEDLINGS	64
ABSTRACT	65
INTRODUCTION	66
MATERIALS AND METHODS	70
RESULTS	76
DISCUSSION	91
REFERENCES	98
CONCLUSION	102
BIBLIOGRAPHY	103
APPENDIX	112

LIST OF FIGURES

Fig. Pages

1.1 A simple apparatus for determining nitrogenase activity of intact red alder seedling-soil system 13

1.2 Physiological activity changes during 30 hr drought cycle of 6-month-old nodulated nonmycorrhizal (F) and *Alpova diplophloeus* mycorrhizal (FA) *Alnus rubra* seedlings grown in a walk-in growth chamber 26

1.3 Physiological activity changes of 6-month-old nodulated *Alnus rubra* seedlings during cyclic water stress development 27

2.1 A simple apparatus for determining nitrogenase activity of intact red alder seedling-soil system 42

2.2 Experiment 1. N- and P-fertilization effects on the mycorrhiza formation (A), height (B), diameter (C), nodule dry weight (D), total nitrogenase activity (E) and specific nitrogenase activity (F) of 23-week-old *Alnus rubra* seedlings grown in a growth chamber 47

2.3 Experiment 1. N- and P-fertilization effects on the shoot dry weight (A), root dry weight (B), shoot/root ratio (C) and CO_2 exchange rate (D) of 23-week-old *Alnus rubra* seedlings grown in a growth chamber 48

2.4 Experiment 2. N- and P-fertilization effects on the mycorrhiza formation (A); open bars show *Alpova diplophloeus* mycorrhiza percentage, height (B), diameter (C), nodule dry weight (D), total nitrogenase activity (E) and specific nitrogenase activity (F) of 20-week-old *Alnus rubra* seedlings grown in a greenhouse 55

2.5 Experiment 2. N- and P-fertilization effects on the leaf dry weight (A), leaf area (B), specific leaf dry weight (C), shoot dry weight (D), root dry weight (E) and shoot/root ratio (F) of 20-week-old *Alnus rubra* seedlings grown in a greenhouse 56

2.6 Experiment 2. N- and P-fertilization effects on the CO_2 exchange rate (A), stomatal conductance (B), transpiration rate (C) and internal CO_2 concentration (D) of 20-week-old *Alnus rubra* seedlings grown in a greenhouse 57

3.1 A simple apparatus for determining nitrogenase activity of intact red alder seedling-soil system 73

3.2 Experiment 1. Three-week shading effects on mycorrhizal development, growth, and N-fixation of 23-week-old red alder seedlings grown in a walk-in growth chamber 81

3.3 Experiment 1. Three week shading effects on the growth of 23-week-old red alder seedlings grown in a walk-in growth chamber 82

3.4 Experiment 1. Three week shading effects on the growth of 23-week-old red alder seedlings grown in a walk-in growth chamber 83

3.5 Experiment 2. Ten-week shading effects on mycorrhiza and nodule development, growth, and N-fixation of 20-week-old red alder seedlings grown in a greenhouse 88

3.6 Experiment 2. Ten-week shading effects on the growth of 20-week-old red alder seedlings grown in a greenhouse 89

3.7 Experiment 2. Ten-week shading effects on photosynthesis, stomatal conductance, transpiration and internal CO_2 concentration of 20-week-old red alder seedlings grown in a greenhouse 90

A.1 Height growth of *Alnus rubra* seedlings inoculated with *Frankia* and dead spores of *Alpova diplophloeus* (F), and *Frankia* and live spores of *Alpova* (FA) 113

LIST OF TABLES

Table Page

1.1 Experiment 1. Mean growth of 6-month-old nodulated *Alnus rubra* Bong.seedlings grown in a walk-in growth chamber, inoculated with live or dead *Alpova diplophloeus* spores 24

1.2 Mean growth of 6-month-old nodulated *Alnus rubra* Bong. seedlings in a greenhouse under 5 days cyclic drought stress (WS) or daily watered (W), with live (FA) or dead (F) *Alpova diplophloeus* spore inoculation 25

2.1 Comparison of N-, and P-fertilization and *Frankia* and *Alpova diplophloeus* spore inoculation treatments by analysis of variance of data from 23 weeks old *Alnus rubra* seedlings grown in a walk-in growth chamber (Experiment 1) 46

2.2 Experiment 2. *Alnus rubra* seedling growth for ten weeks in a greenhouse prior to beginning fertilization treatments 52

2.3 Comparison of N- and P-fertilization and *Alpova diplophloeus* spore inoculation treatments by analysis of variance of data from 20-week-old *Alnus rubra* seedlings grown in a greenhouse (Experiment 2) 53

2.4 N and P concentration in the leaf, root and nodule tissues of 20-week-old *Alnus rubra* seedlings fertilized for 10 weeks 54

3.1 Growth data of 20-week-old red alder seedlings grown in a walk-in growth chamber before shading treatment with and without *Frankia* and *Alpova diplophloeus* spore inoculation 78

3.2 Growth of 23-week-old red alder seedlings grown in a walk-in growth chamber at full light intensity with and without *Frankia* and *Alpova diplophloeus* spore inoculation 79

3.3 Comparison of treatment by analysis of variance of data from Experiment 1 80

3.4 Comparison of treatment by analysis of variance of data from Experiment 2 86

3.5 Ten-weeks shading effects on the concentrations of N and P of 20-week-old nodulated red alder seedlings grown in a greenhouse 87

A.1 Delayed *Frankia* inoculation effect on 3-month-old *Alnus rubra* Bong. seedling growth and mycorrhiza development by contaminating fungi in a greenhouse 112

PREFACE

This thesis is composed of three chapters written for separate publication. Titles are; Chapter 1: Effects of water stress on ectomycorrhiza and nodule formation, N_2-fixation and growth of red alder seedlings. Chapter 2: Effects of N and P fertilization on ectomycorrhiza and nodule formation, N_2-fixation and growth of red alder seedlings. Chapter 3: Light effects on ectomycorrhiza and nodule formation, N_2-fixation and growth of red alder seedlings. References cited in the text are listed at the end of each chapter. These references are collected into a comprehensive biblography at the end of the thesis.

In chapter 1 to 3, Randolph J. Molina and Steven L. Miller are listed as co-authors because the former contributed to the chapter as an adviser and editor and the latter helped establish experimental designs and provided the critically important symbiont. In chapter 2, Ching Y. Li is also listed as a co-author because he helped in establish the experimental design and provided the symbiont and techniques to measure *in situ* N_2-fixation activity.

WATER STRESS, FERTILIZATION AND LIGHT EFFECTS ON THE GROWTH OF NODULATED, MYCORRHIZAL RED ALDER SEEDLINGS

INTRODUCTION

Red alder (*Alnus rubra* Bong.) is an ecologically and economically important tree species in Pacific Northwest forests (Tarrant and Trappe, 1971; Resch, 1988). It significantly enhances soil fertility through large inputs of N symbiotically fixed by *Frankia* in root nodules (actinorrhizae). The hardwood market for red alder is also steadily increasing, and silviculturists continue to show interest in its rotational use or for interplanting with conifers.

Often dominating riparian zones, red alder is an N_2-fixing plant demanding high P nutrition, intolerant to shade and often limited to mesic habitats. Moisture stress limits N_2-fixation in legumes directly by reducing gas diffusion through shrinking nodules (Weisz et al., 1985), or indirectly by affecting photosynthetic activity of host plants through stomatal closure (Huang et al., 1975b). Forest soils supporting alder are higher in N and lower in P concentration than soils lacking alder (Koo et al., unpublished data). N addition can reduce nodule formation and nitrogenase activity (Hughes et al., 1968; Burgess and Peterson, 1987) while P addition enhances nodule formation (Seiler and McCormick, 1982). Intercropping N_2-fixation and non-N_2-fixation plants can maximize crop yield per unit land area with minimum use of N fertilizer (Ofori and Stern, 1987), but in this system slowly growing plants become shaded. Low irradiance reduces nodule formation and nitrogenase activity of European black alder (Gordon and Wheeler, 1978).

Perry et al. (1979) suggested that, although N-fixation usually requires symbioses adapted to relatively temperate forest environments, symbiotic genotypes should be sought for silvicultural use for N-fixation under water stress, shade and in cold soils. Before we begin selecting for such genotypic adaptation, however, the ecophysiological interaction between the symbioses must be better understood.

Alders form tripartite symbioses of both mycorrhizae and N_2-fixing root nodules. Mycorrhizae improve water relations and P nutrition of non-N-fixing as well as N-fixing plants (Nelsen, 1987; Harley and Smith, 1983; Bethlenfalvay et al., 1981; Subba Rao et al., 1986) but can reduce growth of some hosts under deep shade (Hayman, 1974). Alders form mycorrhizae with host-specific fungi (Molina, 1981). Miller et al.(unpublished data) found that *Alpova diplophloeus* (Zeller & Dodge) Trappe & Smith was the dominant mycorrhizal fungus in mesic and clear cut sites.

The objectives of my studies were to determine how 1) water stress, 2) N and P fertilization and 3) shade affect photosynthesis, N_2-fixation, mycorrhiza and nodule development and growth of red alder seedlings inoculated with a pure culture of *Frankia* and *Alpova* spores in a controlled environment.

CHAPTER 1

EFFECTS OF WATER STRESS ON ECTOMYCORRHIZA AND NODULE FORMATION, N_2-FIXATION AND GROWTH OF RED ALDER SEEDLINGS

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ABSTRACT

Red alder (*Alnus rubra* Bong.) seedlings inoculated with *Frankia* pure cultures were grown in a walk-in growth chamber and in a greenhouse for six months. Half were inoculated with live or dead spores of the ectomycorrhizal fungus *Alpova diplophloeus* (Zeller & Dodge) Trappe & Smith. In the growth chamber *Alpova* - inoculated seedlings were significantly larger than nonmycorrhizal plants in diameter and nodule and shoot dry weight. These parameters increased by 6 to 16 %. *Alpova* mycorrhizal effects on water relations of red alder seedlings were explored in a 2 x 7 factorial (mycorrhiza x water stress) experiment in a completely randomized design. Mycorrhizal and nonmycorrhizal seedlings did not significantly differ in leaf water potentials, CO_2 exchange rates or N_2-fixation rates during a 30-hour drought cycle.

In the greenhouse, effects of water stress on growth, symbiosis development and physiological activities of red alder seedlings were explored in a randomized block design with blocks having two levels of watering regimes (every day and every fifth day watering) and with treatments of live vs. dead *Alpova* spore inoculation. Water stress significantly decreased mycorrhiza formation, nodule dry weight and seedling growth. Inoculated plants did not differ significantly from noninoculated in the growth parameters measured. Cyclic drought stress hindered *Alpova* mycorrhiza formation. As a result, *Alpova* seedlings and noninoculated seedlings did not significantly differ in leaf water potential, CO_2 exchange rate, stomatal conductance, transpiration rates, internal CO_2 concentration or N_2-fixation rates. In both experiments N_2-fixation rates were less sensitive than CO_2 exchange rates to water stress. Our results suggest that in the growth chamber, *Alpova diplophloeus* mycorrhizae increase red alder seedling growth only under well watered conditions and do not significantly affect water relations of the plants.

INTRODUCTION

Red alder (*Alnus rubra* Bong.) is an ecologically and economically important tree species in Pacific Northwest forests. It significantly enhances soil fertility through large inputs of N symbiotically fixed by *Frankia* actinomycetes in root nodules (actinorrhizae). The hardwood market for red alder is also steadily increasing, and silviculturalists continue to show interest in its rotational use or interplanting with conifers. Red alder is widespread in disturbed mesic habitats of the coastal and Cascade mountains, and is often the dominant tree in riparian zones. Not surprisingly, its range is limited by severe moisture stress. Similarly, moisture stress is an important limiting factor for N_2-fixation in legumes, directly affecting N_2-fixation by reducing gas diffusion through shrinking nodules (Weisz *et al.*, 1985), or indirectly by affecting photosynthetic activity of host plants through stomatal closure (Hung *et al.*, 1975b).

Red alder also forms ectomycorrhizae that likely function in nutrient uptake as shown for *Alnus viridis* (Chaix) D.C. ectomycorrhizae (Mejstrik and Benecke, 1969). Mycorrhizae can also affect plant water relations, but such interactions with alder are unexplored. Several studies show that mycorrhizae can increase a plant's drought tolerance (Nelsen and Safir, 1982), increase plant growth under water stress (Sweat and Davies, 1984; Busse and Ellis, 1985), and even improve plant survival in the field after short periods of exposure to drought (Allen and Boosalis, 1983; Goss, 1960). Safir *et al.* (1971) presented the following four hypotheses to explain improved plant water relations by vesicular-arbuscular (VA) mycorrhizae : (1) increase in total soil absorptive surface area by external hyphae, (2) low-resistance water pathway to the endodermis by hyphal penetration into root cortex, (3) decrease in water transport resistance within the roots by nutrient uptake enhancement, and (4) larger root systems of mycorrhizal plants. Of these, mycorrhizal enhancement of P uptake was primarily responsible for greater

root conductivity in legumes (Safir *et al.*, 1972) and *Citrus* root stocks (Graham and Syvertsen, 1984), whereas increased surface area for water absorption provided by external hyphae was the most important in other studies (Hardie, 1985; Hardie and Leyton, 1981; Allen, 1982: Read and Boyd, 1986). Mycorrhizae can also lower stomatal resistance through regulation of abscisic acid/cytokinin levels (Allen, 1982; Levy and Krikun, 1980), increase water absorption by bridging the gap between soil and root that occurs when they shrink away from each other upon drying (Graham *et al.*, 1987) and enable plants to maintain leaf turgor and conductance at great tissue water deficits and low leaf and soil water potentials by lowering the osmotic potential of leaves (Auge *et al.*, 1986b).

Experimentally validating these hypotheses, however, has been difficult due to the complex nature of mycorrhiza-plant-water relationships. For example, under well-watered conditions, leaf conductance of VA mycorrhizal rose plants was not related to phosphorus nutrition (Auge *et al.*, 1986a). Water transport through hyphal entry points was considered insignificant for *Citrus* (Graham and Syvertsen, 1984) and sunflower (Koide, 1985). During drought stress and recovery periods, VA mycorrhizal *Citrus* had comparable whole-plant transpiration rates and leaf water potentials to nonmycorrhizal plants, but mycorrhizae reduced root hydraulic conductivity of plants when they were comparable in size, P sufficiency, and relative growth rate (Graham, et al, 1987). On the other hand, larger mycorrhizal geraniums stressed more rapidly due to their greater water demands than nonmycorrhizal control plants, but more efficiently recovered from water deficit (Sweat and Davies, 1984).

Water relations of ectomycorrhizal plants have received less attention than VA mycorrhizal plants, but ectomycorrhizal *Pinus radiata* seedlings resist summer drought better than nonmycorrhizal seedlings (Theodorou and Bowen, 1970), and ectomycorrhizal *Pinus ponderosa* seedlings recover more rapidly when rewatered after

limited drought even though the mycorrhizal plants are more water stressed than nonmycorrhizal controls (Goss, 1960). The response to water stress varies with ecologically different fungal strains (Harley and Smith, 1983; Read and Boyd, 1986) and between fungal species (Mexal and Reid, 1973; Theodorou, 1978; Parke *et al.*, 1983; Mudge *et al.*, 1987). Mudge *et al.* (1987) propose four mechanisms for ectomycorrhiza-related effects on host plant water relations similar to those listed previously for VA mycorrhizae. Of these mechanisms, increased absorptive surface of external mycelium and water transport through vessel hyphae in rhizomorphs or hyphal strands have been well supported (Read and Malibari, 1979; Duddridge *et al.*, 1980). Mudge *et al.* (1987) also propose that ectomycorrhizal fungi can affect plant water relations by fungal osmoregulation of water uptake.

The influence of mycorrhizae on growth and N_2-fixation of legumes has received considerable attention. In general, enhanced nutrient status of mycorrhizal plants is often responsible for increased plant growth or N_2-fixation, although the interactions of the two symbioses can be complex. For example, VA mycorrhizae can improve legume growth by increasing phosphorus uptake and N_2-fixation rates (Mosse *et al.*, 1976; Asimi *et al.*, 1980; Bethlenfalvay and Yoder, 1981) and even by increasing N and P nutrient-use efficiency in photosynthesis (Brown and Bethlenfalvay, 1987; 1988). Such mycorrhizal effects were more pronounced under water stress, influencing nodule development and activity, transpiration, leaf conductance, fresh and dry weight of tissues and P nutrition (Busse and Ellis, 1985). In a recent review, Bethlenfalvay *et al.* (1987a) concluded that the legume tripartite association is more likely an interdependent C-N-P supply/demand relationship. An increase in the product by one of the symbionts such as carbohydrate, N or P enhances the output by the others, thus forming an autocatalytic cycle rather than a source-sink one. VA mycorrhizal plants were higher than non-VA mycorrhizal in water use efficiency (Bethlenfalvay *et al.*,

1987b) measured as the ratio of net CO_2 uptake vs. transpiration and in CO_2 fixation (Brown and Bethlenfalvay, 1988), although N and/or P concentration were lower in VA mycorrhizal plant leaf tissues.

The interactions of ectomycorrhizae, actinorrhizae and host plants are relatively unexplored compared to the legume-mycorrhizae-*Rhizobium* complex. Actinorrhizal nodules differ strongly from legume nodules because they are perennial and their endophytes are *Frankia* spp. with vesicles for N_2-fixation. Similarly, ectomycorrhizae differ strongly from VA mycorrhizae in form and functions. It is likely that the tripartite symbioses of alder may operate differently from that in the legume symbioses. The objectives of this study therefore were to examine the interactions of ectomycorrhizae and actinorrhizae on growth, photosynthesis and N_2-fixation rate of moisture stressed red alder seedlings.

MATERIALS AND METHODS

Two experiments were conducted to examine the effects of ectomycorrhizae on water stressed, actinorrhizal red alder seedlings. In Experiment 1, seedlings were watered daily and then stressed and assessed during one continuous dry down cycle. In Experiment 2, seedlings were grown under cyclic drought conditions. Specific procedures were as follows:

Experiment 1

Biological materials: Red alder seeds (seed zone 251, Brown Seed Company, 12101 N.E. 28th St. Vancouver, Washington 98668) were selected for uniform size by dry sieving, and surface-sterilized with 30 % H_2O_2 for 15 min prior to planting. *Frankia* was isolated by filtration method (Benson, 1982) from nodules on 1-year-old red alder seedlings collected at the U.S. Forest Service Cascade Head Experimental Forest in the Oregon coast range and cultured on N free BAP liquid medium (Murry *et al.*, 1984) for one month. Sporocarps of *Alpova diplophloeus* (Zeller & Dodge) Trappe & Smith, a hypogeous, ectomycorrhizal fungus specific to alder (Molina, 1981) were collected under young alder trees at the Cascade Head Experimental Forest in November 1987 and stored at -18° C until used.

Growth conditions: Surface sterilized seed were planted into 3.2 cm diameter x 20 cm long leach tube containers (Ray Leach Conetainers, 1500 N. Maple Canby, Oregon 97101) filled with a 2:1:1 mixture of sandy loam soil collected at Willamette Valley in Oregon : sphagnum peat moss : coarse vermiculite and misted daily. After seed germination, 2 ml of water diluted *Frankia* inoculum containing ca. 1 µl packed cell volume were inoculated into each leach tube. Ten million *Alpova* spores suspended in 5 ml water were similarly inoculated into each leach tube. The nonmycorrhizal treatment received the same amount of autoclaved spores. Alder seedlings were grown in a walk-

in growth chamber with day/night regimes of 14/10 hr light period, 25/17° C temperature and 60/80 % relative humidity, with photosynthetic photon flux density of ca. 570 μmol/m^2/s measured at soil surface. Seedlings were irrigated daily with tap water to saturation and never fertilized. Fifteen weeks after the inoculation, 21 seedlings of similar height were selected from each mycorrhizal and nonmycorrhizal treatment. Water was then withheld to begin the moisture stress period and seedling measurements taken.

Experimental design and data collection : This was a 2 x 7 factorial experiment arranged in a completely randomized design with three replications measured twice at a three day interval. A total of 42 seedlings (14 treatments x 3 seedlings/treatment) were randomly distributed within holding trays. The first factor was fungus inoculation at two levels: live *Alpova* spore and dead spore inoculation. The second factor was water stress measured at seven time intervals four hours apart beginning two hours after the final morning watering (seven measurements during a 30-hour dry cycle). Water stress treatment was generated by withholding water until the seedlings wilted. Physiological parameters; nitrogenase activity, photosynthetic activity, stomatal conductance and leaf water potential; were measured for three seedlings from each treatment . Nitrogenase activity was measured by acetylene reduction assay of intact root systems entirely enclosed in a plastic (PVC) tube 5.2 cm diameter x 25 cm deep; stems and leaves extended above the PVC tube (Fig. 1.1). Photosynthetic rate and stomatal conductance were measured on the middle (16 cm^2) of the 4th leaf of each seedling with a portable LI6000 photosystem (LI-COR, Inc, Lincoln, Nebraska). Leaf water potential was measured on the 5th or 6th leaf by a pressure bomb. At the end of the dry down cycle (30 hours duration), seedlings were watered daily to saturation for three days. A second identical dry down cycle was then conducted and the same parameters measured. After this second replication, growth parameters; i.e. height and root collar diameter,

were also measured in addition to the physiological parameters. Soil water contents were also calculated from the dry weight of one-third volume of the pot substrate. All nodules were collected from whole root systems and dried at 65° C to constant weight and measured. Degree of ectomycorrhizal development was calculated from three root subsamples collected at 2-5, 7-10 and 12-15 cm depth of each root mass and expressed as percent of total short roots colonized. Data were analyzed for the physiological parameters using the means based on three seedlings from each sampling time. Mean values for the nonmycorrhizal and mycorrhizal seedlings were tested individually using Tukey's test ($p \leq 0.05$).

Experiment 2

Biological materials : Red alder seeds, *Frankia* isolate culture and *Alpova diplophloeus* spores were the same as in Experiment 1.

Growth conditions : Seedlings were grown in a greenhouse shaded during summer with day/night temperature regime of 25/20 °C, and photosynthetic photon flux density of 200 to 600 $\mu mol/m^2/s$ supplemented with sodium vapor lamps. Seed surface sterilization, growth containers, potting substrate and inoculation with *Frankia* and spores of *Alpova* were the same as in Experiment 1. During the first three months, seedlings were irrigated with tap-water to saturation each morning. Three months after inoculation the drought cycling was initiated. Stressed plants were watered to field capacity every five days; non-stressed plants were watered daily. Effects on leaf expansion and height growth of water stressed seedlings were apparent one month following initiation of stress.

Experimental design and data collection : The experiment was a randomized block design with blocks by two levels of watering regimes and with treatments of live or dead *Alpova* spore inoculation with 18 replications each. Watering regimes were either every day watering or every fifth day watering. Thirty six tubes (2 treatments x

18 replication) were randomly distributed within a block (watering regime) and rotated weekly. Ten weeks after beginning the water stress treatment, nitrogenase activity, photosynthetic acitvity, stomatal conductance and leaf water potentials were measured for 6 seedlings of each treatment on the first, third, and fifth day of the watering cycle; measurement methods were as described in Experiment 1. Soil water contents were calculated in three tubes from each day measurement. Height and diameter growth, dry weights of shoots, roots, nodules and leaves, and leaf areas were also measured. Mycorrhizal development was calculated as in Experiment 1. Effects of two main factors (watering and fungus inoculation) and interactions within a sampling day were analyzed by ANOVA. When those effects were significant at $p \leq 0.05$ level, mean values of treatment combinations were compared by Tukey's test.

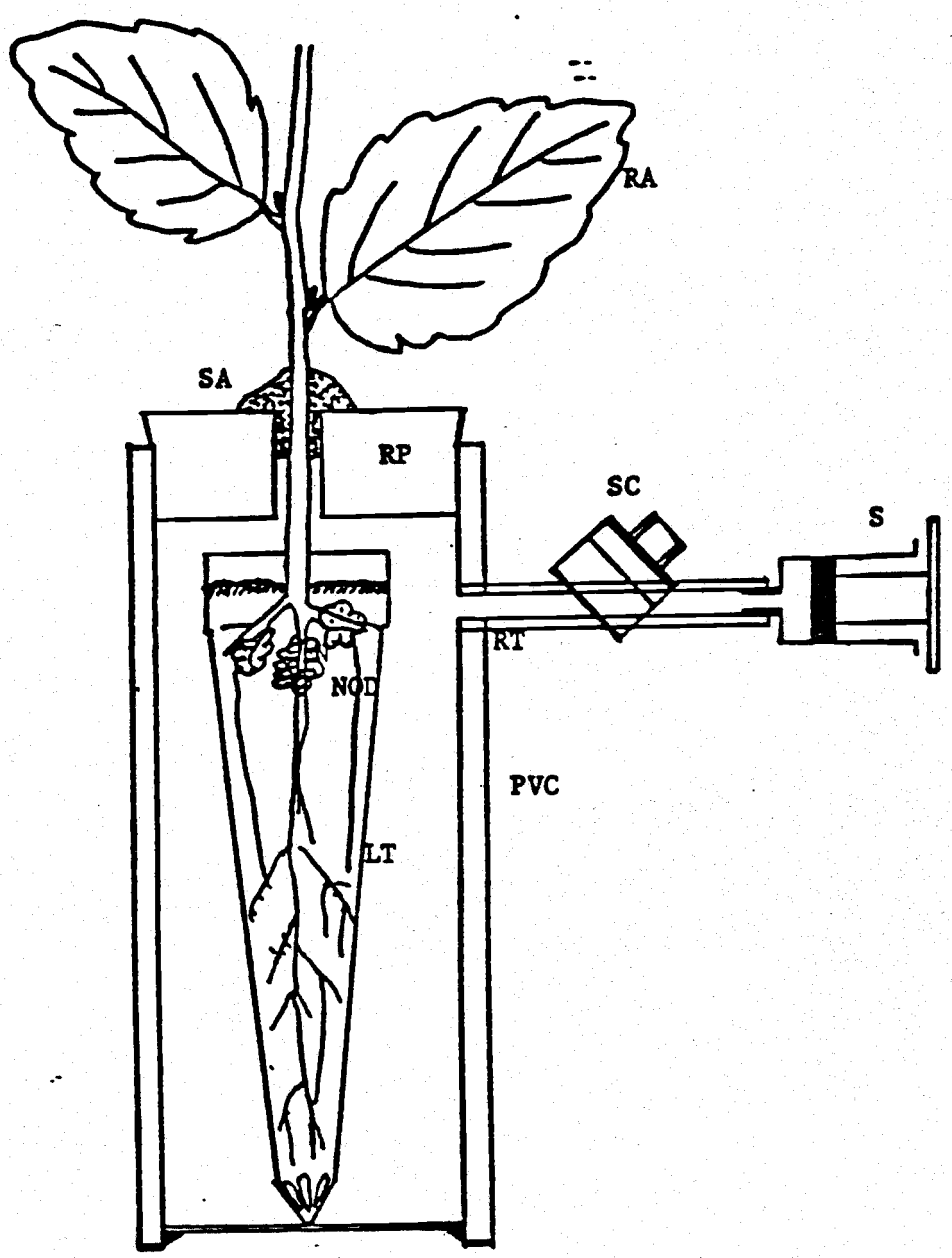


Fig. 1.1 A simple apparatus for determining nitrogenase activity of intact red alder seedling-soil system.

RA=red alder seedling; SA=sealing agent, Roma Italian Pastilina #2 grade; RP=120o splitted #9 holed rubber plug; RT=rubber tubing 0.5 x 10 cm; SC=spring clip; S=syringes for injecting and sampling gas; Nod=nodules; LT=leach tube, 3.2 x 20 cm, 165 ml; PVC=5.2 x 27 cm, 573 ml PVC tube.

RESULTS

Experiment 1.

Growth : Nodulated *Alpova diplophloeus* mycorrhizal seedlings were significantly larger than nodulated nonmycorrhizal ones in diameter, shoot dry weight and nodule dry weight (Table 1). Heights did not differ significantly between inoculation treatments.

Physiological response to rapid drought stress : In general, *Alpova* mycorrhizal seedlings did not differ significantly from non-mycorrhizal ones in physiological activity during rapid drought stress (Fig. 1.2). Leaf water potentials of both mycorrhizal and nonmycorrhizal seedlings decreased slightly from ca. -6.0 bars two hours after watering in the morning to -8.0 to -9.0 bars the next morning, then rapidly dropped to wilting point around -14.0 bars the next afternoon (Fig. 1.2 A) as soil water content dropped from ca. 65 through 40 to 22 % (Fig. 1.2 B). During the night leaf water potentials were kept at ca. -1.0 bars. Most mycorrhizal seedlings started to wilt ca. 30 hours after last watering at -14.2 bars, at which time nonmycorrhizal ones were at ca. -13.5 bars and had not begun to wilt.

CO_2 exchange rates (CER) of both mycorrhizal and nonmycorrhizal plants were constantly maintained at ca. 0.45 mg $CO_2/m^2/s$ until the next morning, then rapidly decreased to ca. 0.2 mg $CO_2/m^2/s$ as water stress developed during the afternoon (Fig. 1.2 C). Stomatal conductance of both mycorrhizal and nonmycorrhizal plants also remained stable at ca. 0.7 cm/s until the next morning, although the rates of mycorrhizal plants were higher than nonmycorrhizal at the first day, and then rapidly dropped to ca. 0.3 cm/s (Fig. 1.2 D). The pattern was very similar to that of CER.

Total nitrogenase activity (TNA) continuously increased from ca. 10.2 in the morning to 19.5 μmol C_2H_2 reduced/plant/hr in the evening, rapidly dropped to ca. 10.5 μmol C_2H_2 reduced/plant/hr during the night, and then again increased to the pre-

evening rate the following morning (Fig. 1.2 E). N_2-fixation rates also dropped to ca. 13.5 μmol C_2H_2 reduced/plant/hr during afternoon water stress. The changes in TNA were not directly related to CER and stomatal conductance during the first three measurements, i.e. TNA gradually increased without changes in CER. TNA remained relatively high during the night, and decreased less under water stress (ca. 22%) compared to the other physiological activities (55% in CER and 57 % in stomatal conductance). Specific nitrogenase activity showed a similar pattern to that of TNA (Fig. 1.2 F).

Experiment 2.

Symbiosis development and seedling growth : Unfortunately, most seedlings became contaminated with mycorrhizal fungi in the greenhouse environment (mostly *Thelephora* species). Total mycorrhizal formations on both *Alpova diplophloeus* inoculated and noninoculated seedlings were ca. 90 % under daily watering and 73 % under the fifth day watering conditions. Non-water stressed seedlings inoculated with *Alpova* spores formed more total percentage mycorrhizae (92 %) than either water stressed treated seedlings (ca. 73 %). *Alpova* colonized the same percentage of short roots regardless of water stress treatment. However, 81 % of the *Alpova* mycorrhizae of stressed seedlings had long, uncolonized tips protruding from the mycorrhizal base; on daily-watered plants *Alpova* rapidly colonized growing apical tips, so that only 3 % had uncolonized tips protruding from the mycorrhizal base. This root tip phenonmenon was not observed on *Thelephora* mycorrhizae regardless of water treatment. Spore inoculation with *Alpova*, in general, however, did not significantly affect red alder seedling growth regardless of watering regimes except that *Alpova* inoculated plants were significantly larger in shoot/root ratio under daily watered condition and their diameter growth was significantly smaller than noninoculated seedlings under water

stress (Table 1.2). Overall, the water stress significantly reduced seedling growth in shoot and root dry weight, diameter, leaf area, and nodule dry weight. Water stressed plants were also significantly lower in shoot/root ratios, and heavier in specific leaf dry weight than nonstressed plants.

Physiological response to five-day-cyclic drought stress : Overall, physiological responses to cyclic drought stress were not significantly affected by *Alpova* inoculation or total mycorrhizal colonization (Table 1.2 and Fig. 1.3). Leaf water potentials during the drought cycle decreased from ca. -7.0 bars on the first day, to -9.2 bars at the third day, and to -15.0 bars by the fifth day for all water stressed seedlings. Daily watered seedlings maintained leaf water potentials at ca. -7.0 bars (Fig. 1.3 A). As water stress developed, CO_2 exchange rate (CER), stomatal conductance and transpiration rates decreased, and internal CO_2 concentration increased (Figs. 1.3 C, D, E and F). Soil water content was maintained at ca. 30 % in daily watered soil, but it decreased from ca. 55 to 10 % in cyclic drought treated soil (Fig. 1.3 B).

CER remained at ca. 0.4 mg $CO_2/m^2/s$ on daily watered plants, but decreased from 0.25 mg $CO_2/m^2/s$ to zero or negative values on the cyclic water stressed plants (Fig. 1.3 C). On the first days, stomatal conductances were 0.29 and 0.36 cm/s on dead and live spore inoculated seedlings, respectively, but did not differ significantly from the ca. 0.40 cm/s of the daily watered seedlings. Stomatal conductances decreased to 0.29 and 0.22 cm/s on the third day and then to 0.07 and 0.04 cm/s on the fifth day on dead and live spore inoculated plants, respectively, during the drought cycle. Transpiration rate of the cyclic stressed plants decreased from ca. 43 on the first day to 10 mg $H_2O/m^2/s$ by the fifth day. Daily watered plants remained ca. 50 mg $H_2O/m^2/s$ (Fig. 1.3 E).

Internal CO_2 concentration in leaf cells increased during the water stress cycle from ca. 280 to 470 ppm. This increment indicates that photosynthetic enzyme activity in mesophyll cells decreased with water stress development while cells continued maintenance respiration (Fig. 1.3 F).

Total nitrogenase activity (TNA) of stressed plants, ca. 6.5 μmol C_2H_2 reducedplant/hr at the first three days, was significantly lower than that of well watered plants, ca. 13.4 μmol C_2H_2 reduced/plant/hr (Fig. 1.3 G), due to significantly less development of nodules in the stressed plants (Table 1.2). But specific nitrogenase activity (SPNA) did not differ significantly between watering regimes at the first two measurements (Fig. 1.3 H). TNA increased from ca. 4.5 to 6.5 μmol C_2H_2 reduced/plant/hr while the leaf water potentials of stressed plant decreased from ca. -6.5 to -9.0 bars (Fig. 1.3 A), then rapidly decreased to 1.7 μmol C_2H_2 reduced/plant/hr as leaf water potentials decreased to ca. -14.6 bars at the fifth day. The initial increase was reproducible in both experiments (Figs. 1.2 E, 1.2 F, 1.3 G and 1.3 H), but it was not related to changes in leaf water potential (Figs. 1.2 A and 1.3 A), photosynthesis (Figs. 1.2 C and 1.3 C), stomatal conductance (Figs. 1.2 D and 1.3 D), or transpiration (Fig. 1.3 E).

DISCUSSION

These experiments showed that ectomycorrhiza formation with *Alpova diplophloeus* can increase red alder seedling growth in some aspects, such as, diameter, nodule dry weight and tissue dry weight when well watered (Table 1.1). But *Alpova* ectomycorrhizae neither affected the water relations of red alder nor influenced leaf water potential, photosynthesis, stomatal conductance, transpiration and N_2-fixation under rapidly developed water stress (Fig. 1.2). Nor did ectomycorrhizae of *Thelephora, Alpova+Thelephora*, or total percentage of mycorrhiza formation affect seedling physiological responses to cyclic drought stress in a greenhouse (Table 1.2 and Fig. 1.3). These findings support previous conclusions that mycorrhizae may not affect water relations of the host plant under certain drought stressed conditions (Mudge *et al.*, 1987; Graham *et al.*, 1987; Hetrick *et al.*, 1984; Fitter, 1987) and that not all fungi confer drought tolerance (Parke *et al.*, 1983).

Several possible reasons may account for the lack of mycorrhizal affects on drought stress. Noninoculated seedlings also formed two kinds of mycorrhizae in the greenhouse, *Thelephora terrestris* and an unidenfied brown type, so treatment comparisons in the greenhouse study are between mycorrhizal seedlings rather than between mycorrhizal and nonmycorrhizal seedlings. However, in the growth chamber experiment, wherein control seedlings only formed a trace of ectomycorrhizae, *Alpova* inoculated seedlings with ca. 50 % ectomycorrhizal short roots still failed to enhance seedling response to rapid drought stress. We hypothesize that *Alpova* functions less under drought stress because we observed the tips of these mycorrhizae to remain uncolonized as they protruded from the mantles during drought stress. These results can not be applied to the hypothesis (Nelsen, 1987) that VA mycorrhizae are more important to plant growth under dry conditions and that mycorrhizae necessarily

improve phosphorus nutrition under conditions of limited soil moisture. Our results would rather mean that moderate soil moisture encourages *Alpova* ectomycorrhizal formation and that dry conditions or drought discourage the mycorrhizae.

Several studies shown that plant responses and mycorrhizal development under water stess are often inconsistent and depend on plant species, fungal species and growth conditions. For example, greenhouse corn inoculated with *Glomus mosseae*, did not show benefit from mycorrhizae when exposed to cyclic drought stress, even though root colonization under drought was greater than that for adequately watered plants at reduced soil phosphorus levels (Hetrick *et al.*, 1984). Conversely, *G. fasciculatum* enhanced soybean drought tolerance, even though mycorrhiza colonization was significantly reduced by water stress (Busse and Ellis, 1985). In another soybean study, *G. mosseae* increased N_2-fixation, transpiration, leaf conductance, tissue dry weight, and P nutrition of host plants without changing root colonization under cyclic water stress (Bethlenfalvay *et al.*, 1987). For little bluestem grown in an unsterilized soil, mycorrhizal plants grew slower than nonmycorrhizal and root colonization decreased as soil water availability decreased (Cerligione *et al.*, 1988). Inoculation of rose with, *G. deserticola* enabled plants to maintain leaf turgor and conductance at greater tissue water deficits, and at lower leaf and soil water potentials than nonmycorrhizal plants (Augé et al., 1986b). Fewer examples are available for ectomycorrhizal interactions. However, in one important study of Douglas-fir seedlings grown in a greenhouse, an unidentified ectomycorrhizal fungus and *Rhizopogon vinicolor* improved plant water relations under cyclic water stress but other tested fungi did not (Parke *et al.*,1983). Unfortunately, no root colonization rates were reported in that study after the drought stress treatment.

The failure of plants to respond to mycorrhizal fungus inoculation under water stress has often been attributed to the rapid and severe water stress that develops when

large plants are confined to a small volume of potting substrate in a greenhouse (Hetrick *et al.*, 1984; Hardie and Leyton, 1981; Levy *et al.*, 1983; Graham and Syvertsen, 1984). Even in large pots, however, drought stress can develop rapidly (Graham *et al.*, 1987; Hetrick *et al.*, 1987) and mycorrhizal effects are still not evident. For example, water stress developed in 10 days on mycorrhizal citrus grown in large pots and no mycorrhizal benefit occurred (Graham *et al.*, 1987). Similarly, severity of stress, plant size, or soil type could not explain the lack of mycorrhizal benefit in corn and sudan grass grown in large containers (Hetrick *et al.*, 1987).

Although container size, low light intensity and the greenhouse environment may have been factors in our experiments, it is also possible that mycorrhizae may not benefit red alder under conditions of drought stress. Given the mesic habitat preference of red alder, a need for drought stress avoidance via a mycorrhizal mechanism may not be strongly selected for. In our experiments we did not systematically count lateral root formation, but we observed that alder did not differentially produce short lateral roots before mycorrhizal formation. Nonmycorrhizal lateral roots typically grew long and formed second order lateral roots. *Alpova* ectomycorrhizae were monopodial or simple pinnate structures (Miller *et al.*, 1989). Without mycorrhiza formation roots produced profuse root hairs 1 to 2 mm long (Koo, personal observation). Such profuse root hair production may serve the same function as mycorrhizae in increasing absorptive surface area. In natural forest settings, however, Miller *et al.* (1989) have observed that most red alder short roots are ectomycorrhizal and root hairs are confined to long lateral roots.

Lack of mycorrhizal benefit during water stress also draws attention to the direct effect of water stress on plant physiological activity and mycorrhizal dependence on host carbohydrate. Photosynthate translocation, measured with ^{14}C, from source leaves to roots decreased as water stress level increased in loblolly pine seedlings (Kuhns and Gzerstad, 1988). Reduced ^{14}C translocation out of exposed leaves with increased water

stress was also reported in *Liriodendron tulipifera* L. (Roberts, 1964) and in sugar cane (Hartt, 1967). In the Kuhns and Gzerstad study, sugars were the major souce of the ^{14}C, and exposed leaves had 88% of the total ^{14}C in a seedling at the highest water stress level. However, of the total carbohydrate in the roots, the proportion of sugars increased to 84 % as water stress level increased, even though total root sugar decreased due to less export from source leaves. These sugars produced under higher stress conditions can also be used as substrates for increased respiration (Kramer, 1983), or as solutes for osmotic adjustment in leaves and roots (Osonubi and Davies, 1978). Although we did not measure sugar levels in roots, nonmycorrhizal root tip development on *Alpova* mycorrhizae in our study may indicate a reduction of sugar export to roots. Such a result would also support Bjorkman's carbohydrate theory (1970) for mycorrhiza formation. However, Fellows *et al.* (1987) reported significantly increased sucrose concentration in soybean root and in nodules as water stress developed and so one would expect mycorrhizal development to increase if such were the case. Clearly, specific studies on changes in root sugar content of plants under water stress and consequent effects on mycorrhiza development is needed.

Water stress also directly affects N_2-fixation. The N_2-fixation process in legumes is thought to be more sensitive to water stress than photosynthesis, because the shrinkage of nodules due to water deficit reduces oxygen transport to ATP production sites (Sprent, 1976; Pankhurst and Sprent, 1975). In support of this hypothesis, Bennett and Albrecht (1984) found that N_2-fixation was closely correlated with nodule water potential, which was more sensitive to drought stress than leaf water potential or diffusive conductance. A reduction in N_2-fixation was also reported for nodules water-stressed on a separated root system without decreasing supply of photosynthate (Khanna-Chopra *et al.*, 1984). Thus reduced N_2-fixation under water stress can be directly caused by a decrease in nodule gas permeability, that is, limited

oxygen flux, followed by a decrease in nodule surface area (Weisz *et al.*, 1985). Although we did not directly test such direct effects, our studies indicate that alder nodules may be less sensitive to water stress than legume nodules (Figs. 1.2 E, 1.2 F, 1.3 G and 1.3 H). Several observations by researchers show that actinorrhizae have adaptible mechanisms to oxygen problems. For example *Casuarina* nodule cell walls *Frankia* infected become impregnated with hydrophobic suberinlike structural compounds (Berg, 1983) and the *Frankia* endophyte can alter the wall thickness of vesicles, the site of N_2-fixation, in response to an O_2 concentration (Po_2) of ambient (Parsons *et al.*, 1987). Silvester *et al.* (1988) found that N_2-fixation in alder nodules also has a wide optimum range of 10 to 21 Po_2, when the nodules were grown at 21 Po_2. Low Po_2 in the range may mean oxygen deficiency by low nodule gas permeability due to water flooding or water stress. Water stress shrinks nodule according to studies on legume nodules dicussed above.

On the other hand, our data indirectly show that N_2-fixation in alder nodules is reduced by low gas peameability due to flooding. N_2-fixation (Figs. 1.2 E, 1.2 F, 1.3 G and 1.3 H) increased with decreasing soil water content at the beginning of the water stress treatment (Figs. 1.2 B and 1.3 B), but leaf water potential, photosynthesis, stomatal conductance and transpiration remained relatively unchanged (Fig. 1.2) or slightly decreased (Fig. 1.3), i.e. N_2-fixation was lowest immediately after the substrate was saturated and increased as the substrate dried until the water stress became limiting. Huang *et al.*, (1975a) also observed this increase in soybean at various times after water was withheld. They found that acetylene reduction decreased when more water was added to the soil of a well watered plant and increased by draining the water without changing photosynthesis or transpiration. Schwintzer (1985) also reported this negative flooding effect on N_2-fixation in actinorrhizal *Myrica gale* .

In summary, although we did find *Alpova diplophloeus* mycorrhizae able to enhance growth of red alder seedlings, it did not influence on plant water stress under our experimental conditions. *Alpova* mycorrhizae apparently ceased functioning under drought stress. More detailed field analysis of red alder root growth patterns are needed to define natural adaptations to soil moisture status. For example, Koo (unpublished data) has observed red alder seedlings growing on exposed slopes in clearcuts to produce deeper tap root systems than seedlings growing in riparian habitats. As moisture decreases in the clearcut soil, the fibrous root system including ectomycorrhizae is present in the deep soil profile. Future investigations need to consider natural root dynamics to understand water stress adaptations by red alder. N_2-fixation by red alder nodules decreased with increasing water stress but is considered less sensitive to water stress than is typical of leguminous N_2-fixation. This is likely due to differences in nodule morphology and the ability of alder nodules to function within a wide range of Po_2.

Table 1.1 Experiment 1. Mean growth of 6-month-old nodulated *Alnus rubra* Bong. seedlings grown in a walk-in growth chamber, inoculated with live or dead *Alpova diplophloeus* spores.

Parameter	*Frankia* [1)]	*Frankia* + *Alpova*
Height (cm)	52.1±1.4[a]	53.8±1.3[a]
Diameter (mm)	5.2±0.1[b]	5.5±0.1[a]
Shoot dry weight (g)	2.4±0.1[b]	2.8±0.1[a]
Alpova ectomycorrhizae (%)	0.3±0.2[b 2)]	51±5[a]
Nodule dry weight (mg)	105±5[b]	123±7[a]

Values are means of 21 seedlings ± standard error. Values within a row followed by a different letter are significantly different at the $P \leq 0.05$ level according to Tukey's test.

1) : inoculated with dead *Alpova diplophloeus* spores.
2) : formed with contaminating fungus but not *Alpova*

Table 1.2 Mean growth of 6-month-old nodulated *Alnus rubra* Bong. seedlings in a greenhouse under 5 day cyclic drought stress (WS) or daily watered (W), with live (FA) or dead (F) *Alpova diplophloeus* spore inoculation.

Measurement	F		FA	
	W	WS	W	WS
Alpova ectomycorrhizae (%)	0b	0b	37±5a	36±8a
Nonmycorrhizae on *Alpova* ectomycorrhizae (%) [1)]	0	0	3±1a	81±4b
Total ectomycorrhizae (%)	88±4ab	70±7b	92±3a	76±5b
Nodule dry weight (mg)	121±6a	59±5b	119±5a	55±3b
Height (cm)	37.3±1.6a	18.2±0.6b	39.6±1.0a	19.6±1.0b
Diameter (mm)	6.17±0.13a	3.85±0.05b	5.90±0.13a	3.48±0.05b
Shoot dry weight (g)	3.11±0.15a	0.96±0.05b	3.22±0.09a	0.94±0.05b
Root dry weight (g)	2.42±0.22a	0.82±0.07b	2.06±0.16a	0.70±0.02b
Shoot/root ratio	1.34±0.07b	1.24±0.08b	1.63±0.10a	1.21±0.06b
Leaf area (cm^2)	285±17a	66±3c	321±11a	78±4b
Leaf dry weight (g)	1.55±0.10a	0.41±0.02b	1.63±0.07a	0.47±0.03b
Specific leaf dry weight	5.42±0.11a	6.23±0.22b	5.08±0.10a	6.12±0.22b

Values are means of 18 seedlings ± standard error.

Values within a row followed by the same letter do not differ significantly at the $P \leq 0.05$ level by Tukey's test.

1) : Percentage of *Alpova diplophloeus* ectomycorrhizae with uncolonized tips protruding from the fungus mantle.

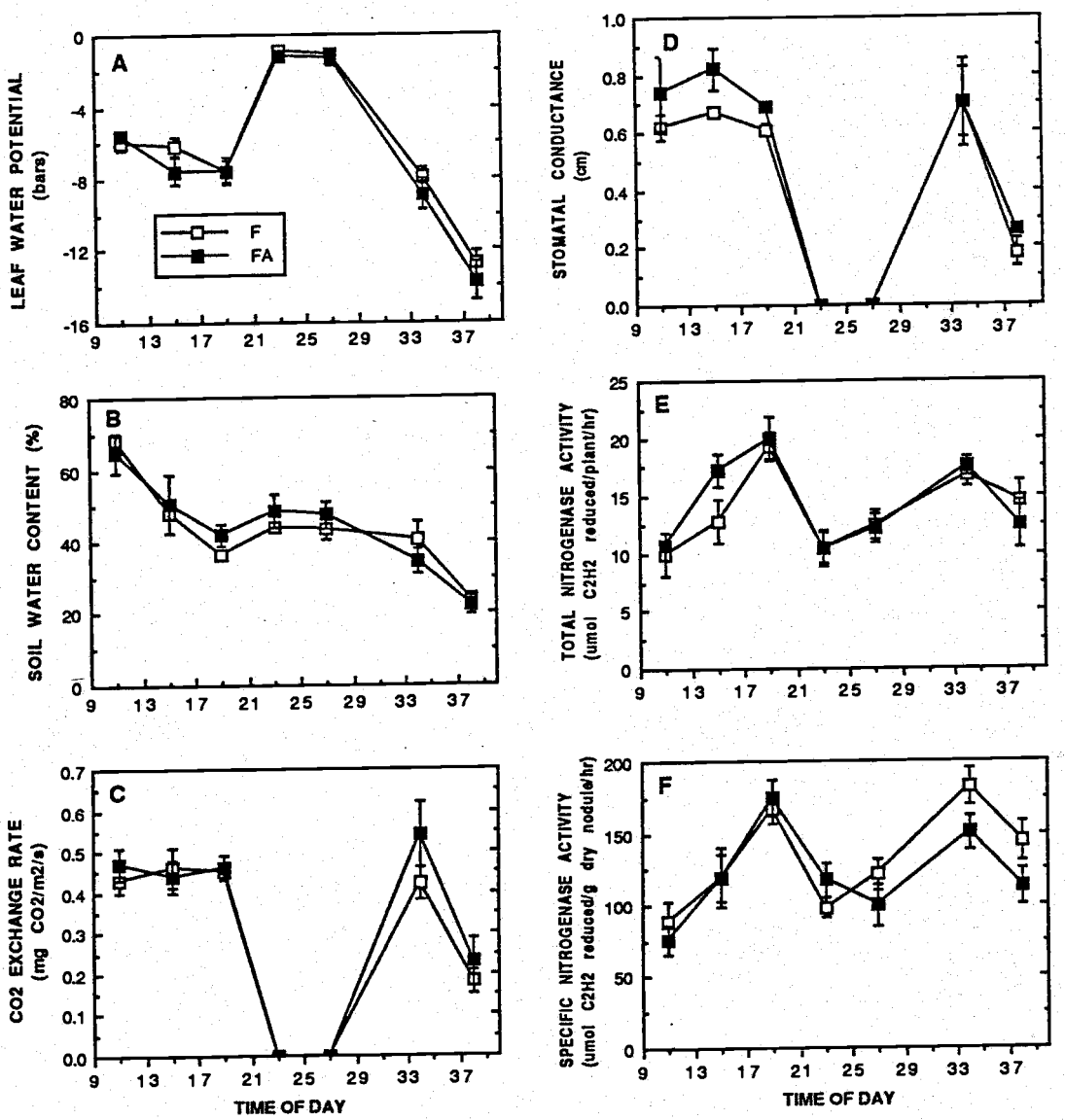
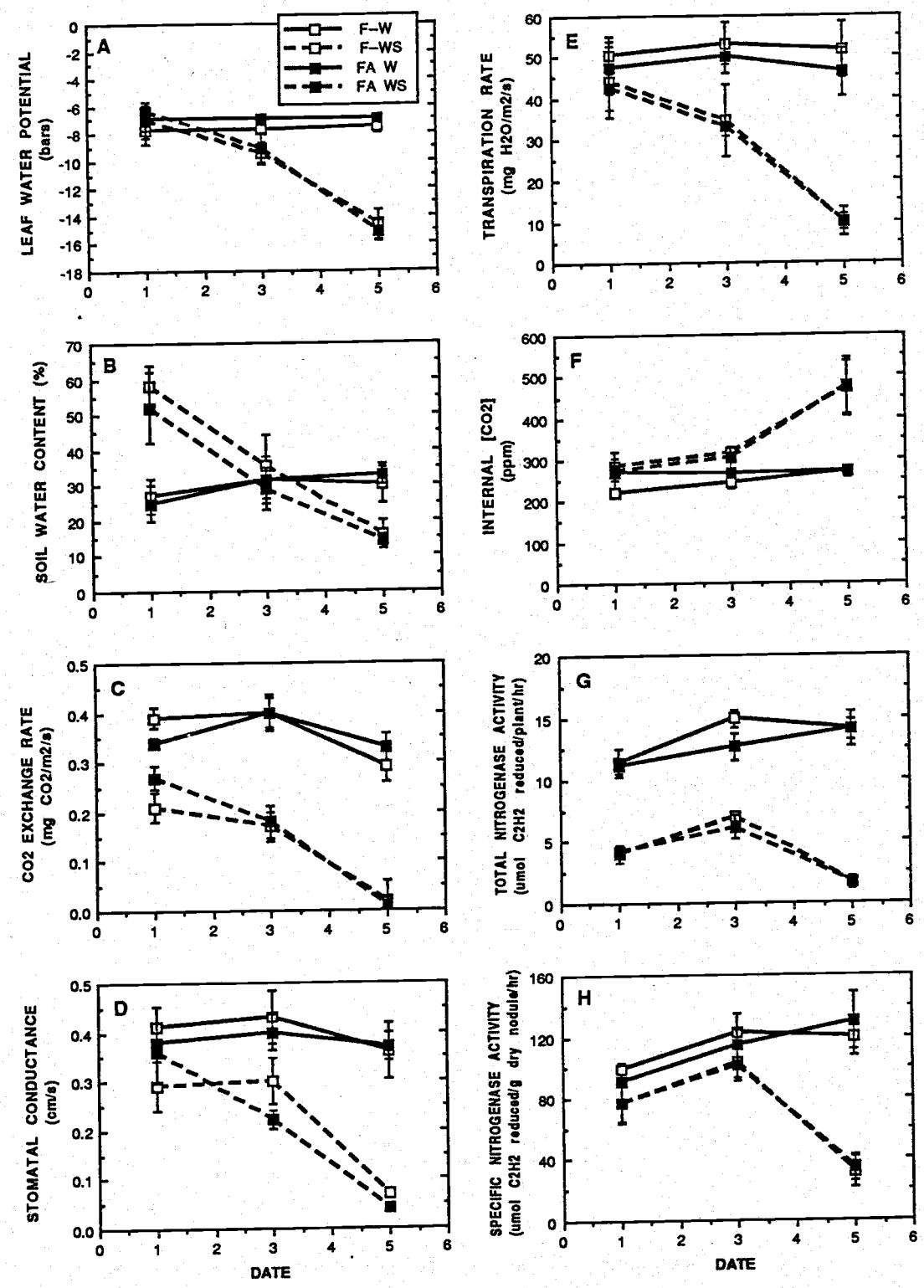


Fig. 1.2 Physiological activity changes during 30-hr drought cycle of 6-month-old nodulated nonmycorrhizal (F) and *Alpova diplophloeus* mycorrhizal (FA) *Alnus rubra* seedlings grown in a walk-in growth chamber. Each point is a mean of two replications of three seedlings each. Standard errors of treatments are shown on each point. (A): leaf water potential; (B): soil water content; (C): CO_2 exchange rate; (D): stomatal conductance; (E): total nitrogenase activity; (F): specific nitrogenase activity.

Fig. 1.3 Physiological activity changes of 6-month-old nodulated *Alnus rubra* seedlings during cyclic water stress development. F=noninoculated (mycorrhizae with *Thelephora*); FA=*Alpova diplophloeus* mycorrhizal; W=daily watered; WS=five day cyclic watered. Each line is a combination of the treatments. Each point is a mean of six replicated seedlings. Standard errors of treatments are shown on each point. (A): leaf water potential; (B): soil water content; (C): CO_2 exchange rate; (D): stomatal conductance; (E): transpiration rate; (F): internal CO_2 concentration; (G): total nitrogenase activity; (H): specific nitrogenase activity.


A
F-W
F-WS
FA W
FA WS
LEAF WATER POTENTIAL (bars)
B
SOIL WATER CONTENT (%)
C
CO2 EXCHANGE RATE (mg CO2/m2/s)
D
STOMATAL CONDUCTANCE (cm/s)
DATE
E
TRANSPIRATION RATE (mg H2O/m2/s)
F
INTERNAL [CO2] (ppm)
G
TOTAL NITROGENASE ACTIVITY (umol C2H2 reduced/plant/hr)
H
SPECIFIC NITROGENASE ACTIVITY (umol C2H2 reduced/g dry nodule/hr)
DATE

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CHAPTER 2

EFFECTS OF N AND P FERTILIZATION ON ECTOMYCORRHIZA AND NODULE FORMATION, N_2-FIXATION AND GROWTH OF RED ALDER SEEDLINGS

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ABSTRACT

Red alder (*Alnus rubra* Bong.) seedlings were grown in a walk-in growth chamber with either *Frankia* inoculation or N-fertilization and live or dead spore inoculation of the ectomycorrhizal fungus *Alpova diplophloeus* (Zeller & Dodge) Trappe & Smith, or in a greenhouse with *Frankia* inoculation and either live or dead spore inoculation of *Alpova*. In the growth chamber, 20-week-old seedlings were grown under four fertility regimes (CO: no fertilization, N: 1 mM NH_4NO_3, P: 1 mM KH_2PO_4, or NP: N+P fertilization) for three weeks. N fertilization significantly decreased total nitrogenase activity but increased CO_2 exchange rate. P-fertilization did not significantly affect any growth or physiological parameter. *Frankia* inoculation significantly increased *Alpova* mycorrhiza formation and seedling growth. *Alpova* inoculation did not affect any growth or physiological parameter.

In the greenhouse, ten-week-old seedlings were grown under six fertility regimes (CO: no fertilization, N1: 10 mM NH_4NO_3, N2: 50 mM NH_4NO_3, P: 5 mM KH_2PO_4, N1P: N1+P fertilization, and N2P: N2+P fertilization) for ten weeks. N fertilization significantly increased total mycorrhiza formation, N concentrations in leaf and root tissues and P concentration in leaf tissues, but decreased N_2-fixation, leaf and shoot growth and P concentration in nodule tissues. P fertilization significantly increased nodule and shoot dry weight and CO_2 exchange rate and P concentration in plant tissues, but decreased specific nitrogenase activity. *Alpova* inoculation significantly increased growth parameters, CO_2 exchange rate and P concentration in leaf tissues, but decreased specific nitrogenase activity, leaf development, N concentration in root tissues and P concentration in nodule tissues. Our results suggest that N-fertilization is not needed for nodulated red alder seedlings, that importance of

Alpova mycorrhiza formation increases as N accumulation increases in plant tissues, and that the role of *Alpova* mycorrhizae was similar to P-fertilization.

INTRODUCTION

Red alder (*Alnus rubra* Bong.) is an important N-fixing tree in Pacific Northwest forests and is receiving renewed attention for use in reforestation programs (Resch, 1988). Growth of red alder seedlings is highly responsive to nodule formation (actinorrhizae). Crushed nodules (Akkerman, 1979) or alder forest soil (Hilger and Myrold, personal communication) have been used as an inoculum to improve seedling growth in nurseries. Selected, beneficial strains of the nodule endophyte (*Frankia*) grown in pure culture offer an advanced technology to further improve alder rearing programs (Perinet *et al.*, 1985). Red alder also forms ectomycorrhizal symbioses, typically with a limited group of host specific fungi (Molina, 1979; 1981). Because mycorrhizae are well known to function in mineral uptake, any seedling inoculation program should include the use of both root symbionts.

The actinorrhizal and mycorrhizal symbioses of alder have usually been studied separately. To better understand their ecological interactions in forest soils or to implement a comprehensive seedling inoculation program, we must explore both root symbioses together, particularly in regard to nutrient uptake in soils of variable fertility. In general, both symbioses are sensitive to fertilization, especially with high N and P. For example, N addition can reduce nodule formation (Hughes *et al.*, 1968), nitrogenase activity (Burgess and Peterson, 1987) and even N-fixation in *Frankia* pure cultures (Tjepkema *et al.*, 1981) whereas P addition can enhance nodule formation (Seiler and McCormick, 1982). Mycorrhizae are well known for enhancing P uptake in infertile soils, and P fertilization often reduces mycorrhiza formation and symbiotic effectiveness (Thomas *et al.*, 1982). But, given the co-evolved nature of the *Alnus*-mycorrhiza-actinorrhiza tripartite symbioses, it is possible that the three organisms interact synergistically to enhance the performance of the union. For example, Koo *et*

al. (1989, chapter 3 in this thesis) report that *Frankia* inoculation of red alder seedlings significantly enhances ectomycorrhizal development compared to comparably sized, N-fertilized non-*Frankia*-inoculated seedlings.

Given the propensity for fertilizing with high levels of soluble N and P in seedling nurseries, the effects of these fertilizers on the two symbioses must be thoroughly evaluated in developing inoculation programs. The objective of this study was to examine the effect of N and P fertilization on mycorrhiza formation, nodulation, nitrogenase activity and growth of red alder seedlings inoculated with *Frankia* and the mycorrhizal fungus, *Alpova diplophloeus* (Zeller & Dodge) Trappe & Smith.

MATERIALS AND METHODS

To test N and P fertilization effects on nodule and mycorrhiza development, N-fixation and growth of red alder seedlings, two experiments were conducted, one in a walk-in growth chamber and the other in a greenhouse.

Experiment 1

Biological materials : Red alder seeds (seed zone 251, Brown Seed Company (12101 N. E. 28th st. Vancouver, Washington)) were selected for uniform size by dry sieving. *Frankia* was isolated by the filtration method (Benson, 1982) from nodules of one-year-old red alder seedlings collected at the U. S. Forest Service Cascade Head Experimental Forest near the Oregon coast. Isolates were cultured for one month in N-free BAP liquid medium (Murry *et al.*, 1984). Sporocarps of *Alpova diplophloeus* , a hypogeous ectomycorrhizal fungus specific to alder (Molina, 1981), were collected under young red alder trees at the Cascade Head Experimental Forest and stored at -18° C until used.

Seedling growth conditions : Red alder seeds surface-sterilized with 30 % H_2O_2 for 15 min were planted in a tray with fine-granule vermiculite, covered with autoclaved coarse sand 0.2 cm deep, gently mist-irrigated, and covered with a clear plastic tent until germination was complete. After two weeks, germinants were transplanted to growth tubes, 3.2 cm diameter x 20 cm long plastic super cell, 165 cc capacity (Ray Leach Conetainer, 1500 N. Maple Canby, Oregon 97101). Potting substrate was a mixture of 1:1:2 peatmoss, coarse vermiculite and sandy loam soil collected at Willamette valley in Oregon. The soil mixture was autoclaved for 120 min. Nutrients in the mixture were 0.068 % N, 620 ppm total P, 6 ppm available P, 583 ppm K, 1799 ppm Ca, and 430 ppm Mg after autoclaving. Plants were grown in a walk-in growth chamber at a day/night temperature of 25/17° C, 14/10 hr light regime, and 60/95 % relative

humidity. Photosynthetic photon flux density (PPFD) was 680±26 μmol/m^2/s as measured with a LICOR quantum radiometer/photometer located 50 cm above the surface of the growth tubes.

Experimental design : This was completely randomized block design blocking with four levels of soil fertilities and treatments were *Frankia* inoculation or N fertilization and live or dead spore inoculation of *Alpova*. Within a fertilization regime four replicated seedlings per treatment combination were used. *Frankia* inoculated seedlings received 1 μl packed cell volume (3020 x g for 10 min.) of one month old *Frankia* culture. Because non-*Frankia* inoculated seedlings remain stunted, we applied additional N to produce comparably sized seedlings. Noninoculated seedlings received 10 ml of 2 mM NH_4NO_3 twice a week for two weeks, 10 ml of 8 mM for next 10 weeks and 10 ml of 10 mM every other day for the final 7 weeks. Mycorrhizal seedlings received 10 million *Alpova* spores suspended in 10 ml of water per tube; Nonmycorrhizal seedlings received 10 million autoclaved spores. Soil fertility levels were combinations of application vs. absense of N-fertilization and applicaton vs. absense of P-fertilization. The N-fertilized seedlings received 10 ml of 1mM NH_4NO_3 every other day. The P-fertilized seedlings received 10 ml of 1 mM KH_2PO_4 every other day. Non-P-fertilized seedlings received 10 ml of 1 mM KCl every other day to remove K-fertilization effect. *Frankia* and mycorrizal fungus treatments were applied when seedlings were 4 weeks old and the N- and P-fertilization treatments began at 20 weeks. Seedlings were repositioned every week to reduce location effects in the growth chamber.

Data collection : Three weeks after fertilization treatments, photosynthetic activity, nitrogenase activity, seedling growth, and symbiosis development were measured for each seedling. Apparent photosynthetic activity was measured as CO_2 exchange rates (CER) with a LI6000 portable photosynthesis system (LI-COR, Inc, Lincoln,

Nebraska) for 16 cm^2 leaf area on the 4th leaf from the top. At the same time stomatal conductance, initial transpiration rate, and initial internal CO_2 concentration were obtained from the equation stored in the system. During the measurements a respiration mask connected to a vacuum was used to remove CO_2 respired by the experimentor.

To measure *in situ* acetylene reduction rates, we used the closed system diagramed in Fig. 1.1 An entire seedling root system was placed inside a 5.2 cm inside diameter x 27 cm long PVC tube. A hole was made on the PVC tube 5 cm below the top to remove air, inject acetylene, and collect gas samples. A rubber tube, ca. 0.5 cm diameter x 10 cm long was connected to the hole with a syringe directly attached at the other end to expedite gas injection and sampling. Gas flow was controlled with a spring clip on the tube. A split #9 holed rubber plug was used to seal the seedling growth tube in the PVC tube. Space around the seedling stem was sealed with Roma Italian Plastilina, #2 degree. The air volume in the closed PVC tube after seedling base insertion was calculated to be ca. 400 ml. After removing 40 ml of air (10 % of the air volume) from a sealed PVC tube with a 60 ml syringe, an equal amount of acetylene gas was injected into the tube and syringe pumped ten times to mix the gas. The rubber tube was then clamped shut with the spring clip. After one hour incubation, a gas sample was collected in a two-ml vacutainer with a five-ml syringe through the rubber tube after pumping 15 times to mix the air. The gas sample was analyzed for acetylene and ethylene with a gas chromatograph (Helwett Packard, Model HP5830A) equipped with a hydrogen flame ionization detector and a Porapak R (80-100 mesh) filled column (1.8m long x 2mm inside diameter) by injecting a 0.2 ml sample gas with a 1 cc tuberculin syringe. The oven temperature was adjusted to 70 ° C. The temperatures of injection port and detector were adjusted to 70 ° C. Flow rate of N carrier gas was 40 ml/min. Acetylene reduction rates were calculated as a percent value of produced ethylene to injected acetylene per plant and per unit nodule dry weight. The acetylene

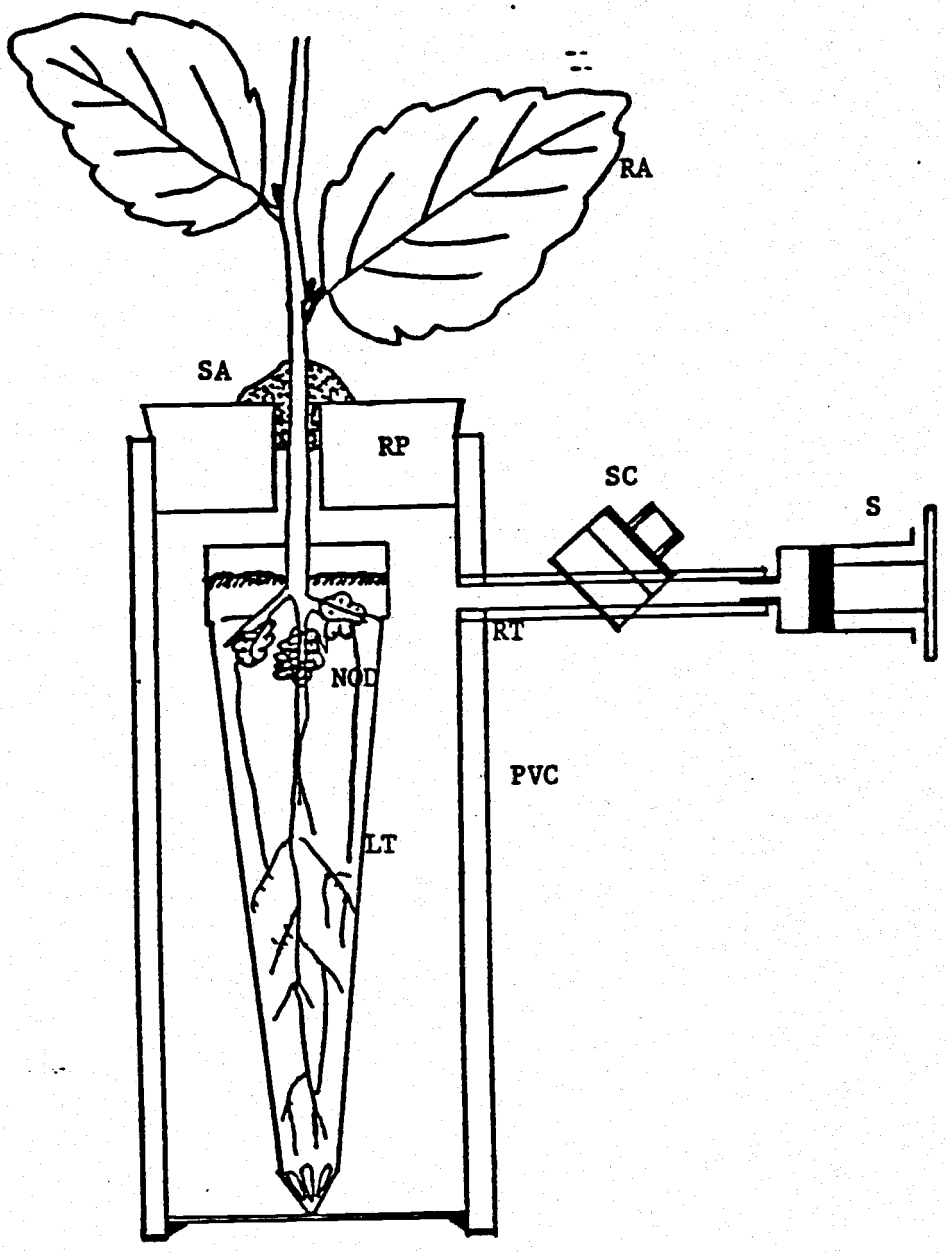

Fig. 2.1 A simple apparatus for determining nitrogenase activity of intact red alder seedling-soil system.
RA=red alder seedling; SA=sealing agent, Roma Italian Pastilina #2 grade; RP=120° splitted #9 holed rubber plug; RT=rubber tubing 0.5 x 10 cm; SC=spring clip; S=syringes for injecting and sampling gas; Nod=nodules; LT=leach tube, 3.2 x 20 cm, 165 ml; PVC=5.2 x 27 cm, 573 ml PVC tube.

reduction assay with the intact root nodules showed that the amount of the reduction had a linear relationship with incubation time up to four hours.

Height, diameter, dry weight of shoot and root, area of ten top leaves, and dry weight of the leaves were determined for each seedling. Dry weight was obtained after drying the tissues at 65 ° C to constant weight. Leaf area was measured with a LI3100 Area meter (LI-COR, Inc, Lincoln, Nebraska). Specific leaf dry weight was calculated by dividing the leaf dry weight with the leaf area of each plant. A mean value of mycorrhiza formation for each plant was calculated from three root subsamples collected at 2.5 to 5 cm, 7.5 to 10 cm, and 12.5 to 15 cm along the length of the root plug. From each subsample 50 to 100 short roots were counted (mycorrhizal and nonmycorrhizal) and mycorrhizal formation was calculated as percentage.

Data analysis : Data were analyzed by GLM (General Linear Models) procedures in SAS® (SAS Institute Inc. Cary, North Carolina) to test the treatment effects for each parameter.

Experiment 2.

Biological materials : Red alder seeds, the *Frankia* isolate and *Alpova diplophloeus* spores were the same as in Experiment 1.

Seedling growth conditions : Growth containers were the same as in Experiment 1. Rooting substrate was a 2:1:1 mixture of ca. 60-year-old alder forest soil, coarse vermiculite and peatmoss. Nutrients in the mixture were 0.252 % N, 80 ppm total P, 11 ppm available P, 352 ppm K, 1570 ppm Ca and 400 ppm Mg after autoclaving for 120 min. Seeds were prepared as in Experiment 1; seeds were directly planted in the tubes and grown in a greenhouse with day/night temperatures of ca. 24/18 ° C with supplemental light from sodium vapor lamps for 16 hours a day. Light intensity was PPFD of 510±26 $\mu mol/m^2/s$ at noon on a clear day. Seeds started to germinate in five days and seedlings were thinned to one per tube after two weeks. After thinning, all

seedlings were inoculated with one-month-old *Frankia* culures by injecting 1μl packed cell volume per seedling. Half of the seedlings also received five million spores of *Alpova* per tube. Seedlings were watered to saturation every morning during the experiment.

Experimental design : The experiment was completely randomized block design blocking with six fertilization regimes (three N levels x two P levels) and treatments were live vs. dead spore inoculation of *Alpova*. All seedlings were inoculated with a pure culture of *Frankia*. Each treatment was replicated with eight seedlings within a fertility regime. N-fertilization was three levels: no fertilization, 5 ml of 10mM NH_4NO_3 and 5 ml of 50 mM NH_4NO_3 for each seedling. P-fertilization was two levels of no fertilization and 5ml of 5mM KH_2PO_4. The fertilizations were applied three times a week since the 10th week. Seedlings were repositioned every other week to even out location effects in the greenhouse.

Data collection and data analysis : After ten weeks of N- and P-fertilization data were collected and analyzed as in Experiment 1. To determine the concentrations of total N and P in leaves, feeder roots (<ca. 2 mm diameter) and nodules, samples from two seedlings within a treatment were combined and determined by autoanalyzer after Kjeldahl digestion. Feeder root samples were collected by carefully rubbing the dried root system.

RESULTS

Experiment 1. *Alpova* inoculation did not significantly affect seedling growth, nodule development or N-fixation; P-fertilization did not affect any variable (Table 2.1). However, *Frankia* inoculation significantly affected seedling growth and mycorrhiza formation, even though non-nodulated (and N-fertilized) seedlings grew to similar height as did *Frankia*-inoculated seedlings (Figs. 2.2 A and B). N-fertilization significantly decreased total nitrogenase activity, but not specific nitrogenase activity (Figs. 2.2 E and F).

Alpova formed ectomycorrhizae with all inoculated seedlings, but the percentage was significantly lower without nodule formation at ca. 14% compared with 58 to 69 % with nodulation (Fig. 2.2 A). Neither N, P, nor N+P fertilization significantly affected mycorrhiza development. Seedlings inoculated with dead *Alpova* spores remained nonmycorrhizal.

No treatments significantly affected height growth (Fig. 2.2 B). Diameter growth was significantly increased only by *Frankia* inoculation (Fig. 2.2 C).

Neither fertilization nor fungus inoculation affected nodule development (Fig. 2.2 D), but N-fertilization significantly decreased total nitrogenase activity (Fig. 2.2 E). Specific nitrogenase activity was not significantly affected by any treatment (Fig. 2.2 F).

Frankia inoculation significantly increased shoot growth but the effect disappeared when seedlings were fertilized with both N and P (Fig. 2.3 A). *Frankia* inoculation also significantly increased root growth (Fig. 2.3B) but significantly decreased shoot/root ratio (Fig. 2.3 C) .

Table 2.1 Comparison of N-, and P-fertilization and *Frankia* and *Alpova diplophloeus* spore inoculation treatments by analysis of variance of data from 23 weeks old *Alnus rubra* seedlings grown in a walk-in growth chamber (Experiment 1).

Parameter	N	P	F	A	N*P	N*F	N*A	N*P*F	F*A
Mycorrhizae	--	--	**	**	--	--	--	--	**
Height	--	--	--	--	--	--	--	--	--
Diameter	--	--	**	--	--	--	--	--	--
Nodule dry weight	--	--	**	--	--	--	--	--	--
TNA [1)]	**	--	**	--	--	**	--	--	--
SPNA [2)]	--	--	--	--	--	--	--	--	--
Shoot dry weight	--	--	**	--	--	--	--	*	--
Root dry weight	--	--	**	--	--	--	--	--	--
Shoot/root ratio	--	--	**	--	--	--	--	--	--
CO_2 exchange rate	**	--	--	--	--	--	--	*	--

N : nitrogen; P : phosphorus; F : *Frankia* inoculation only; A : *Alpova diplophloeus* inoculation only; FA : F + A..
1) : total nitrogenase activity; 2) : specific nitrogenase acitvity.
* and ** are significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

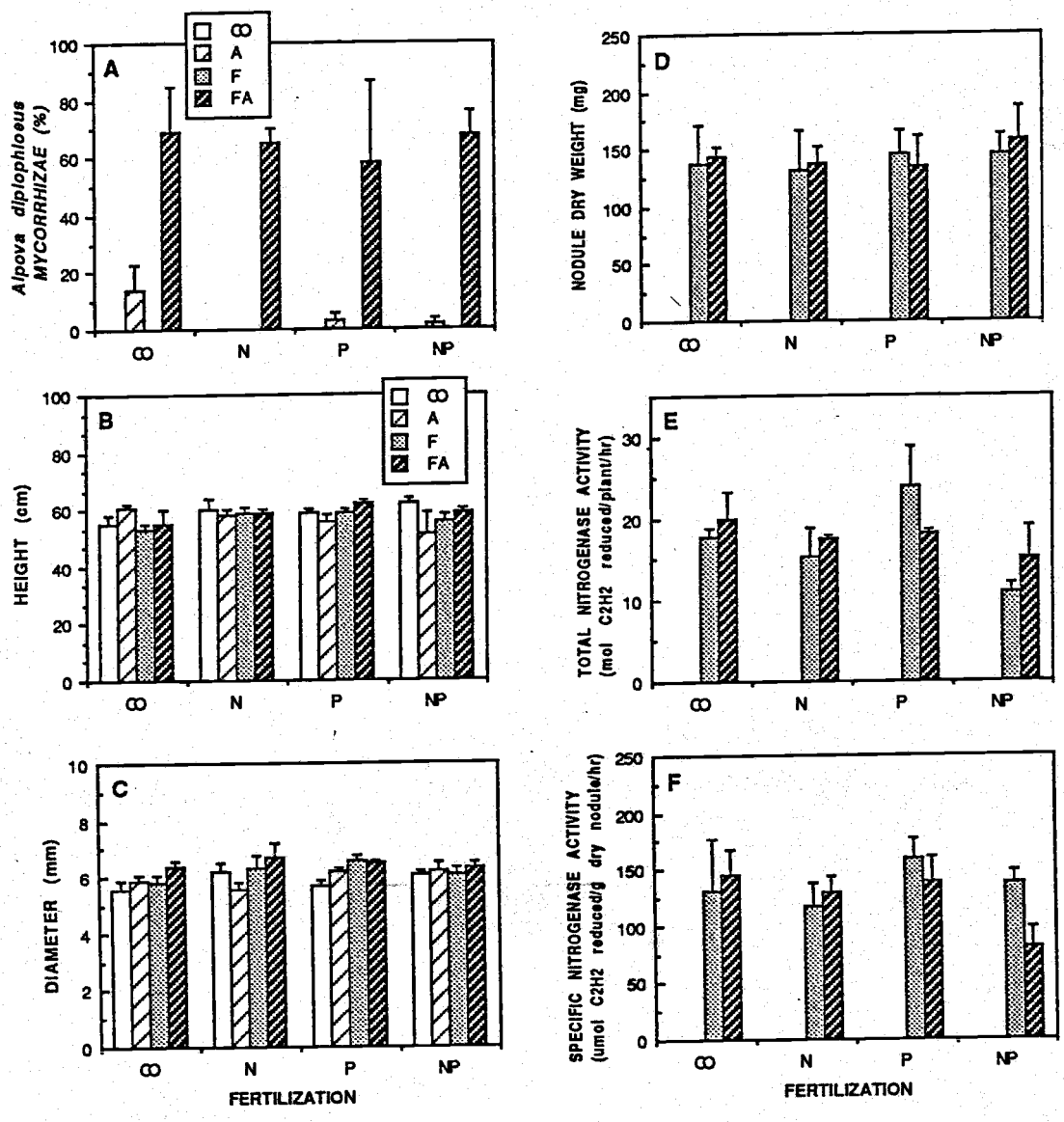

Fig. 2.2 Experiment 1. N- and P-fertilization effects on the mycorrhiza formation (A), height (B), diameter (C), nodule dry weight (D), total nitrogenase activity (E) and specific nitrogenase activity (F) of 23-week-old *Alnus rubra* seedlings grown in a growth chamber. In the legend, CO=control, no inoculation; A=*Alpova diplophloeus* spore inoculation; F=*Frankia* pure culture inoculation; FA=both F and A treatments. No *Frankia* inoculated seedlings received NH_4NO_3 by increasing the amount as plant grew to obtain similar size to the *Frankia* inoculated. No spore inoculated seedlings received dead spores of the fungus. On the horizontal, CO=no fertilization; N=10 ml of 1mM NH_4NO_3; P=10 ml of 1mM KH_2PO_4; NP=combination of N and P. Seedlings received the fertilizer every other day for 3 weeks.

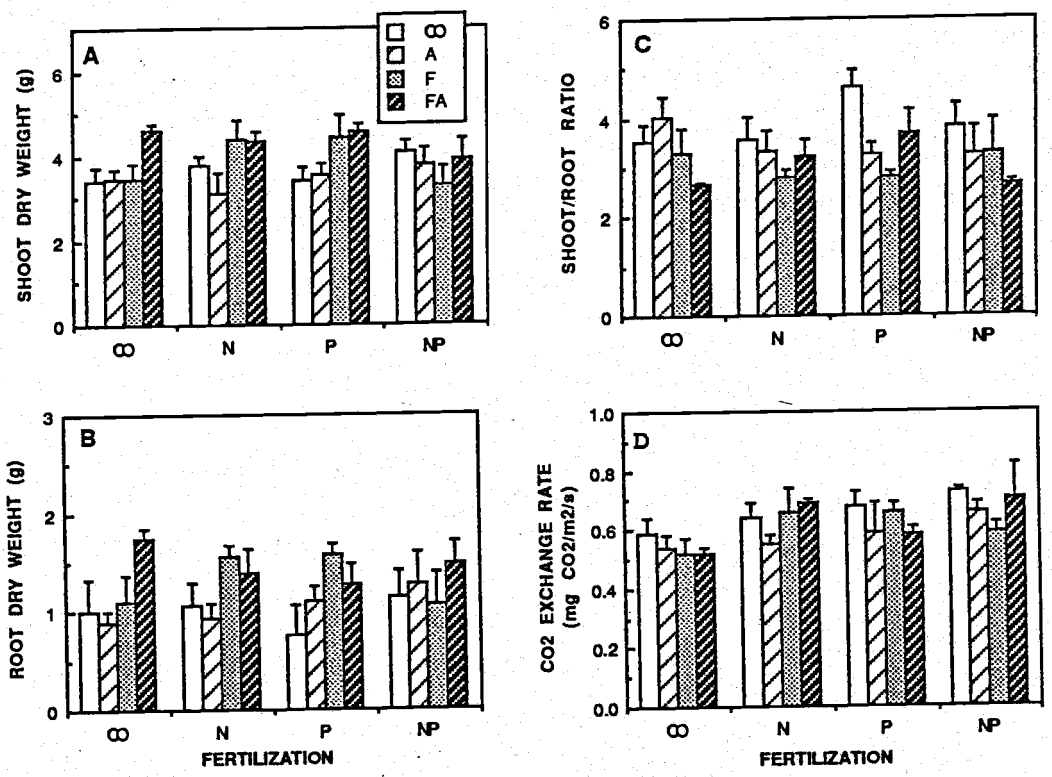

Fig. 2.3 Experiment 1. N- and P-fertilization effects on the shoot dry weight (A), root dry weight (B), shoot/root ratio (C) and CO_2 exchange rate (D) of 23-week-old *Alnus rubra* seedlings grown in a growth chamber. In the legend, CO=control, no inoculation; A=*Alpova diplophloeus* spore inoculation; F=*Frankia* pure culture inoculation; FA=both F and A treatments. No *Frankia* inoculated seedlings received NH_4NO_3 by increasing the amount as plant grew to obtain similar size to the *Frankia* inoculated. No spore inoculated seedlings received dead spores of the fungus. On the horizontal, CO=no fertilization; N=10 ml of 1mM NH_4NO_3; P=10 ml of 1mM KH_2PO_4; NP=combination of N and P. Seedlings received the fertilizer every other day for 3 weeks.

CO_2 exchange rate was significantly increased only by the N-fertilization, but this effect was disappeared by N- and P-fertilization and *Frankia* interaction (Fig. 2.3 D).

Experiment 2. After 10 weeks of growth, but prior to beginning fertilization treatments, *Alpova*-inoculated seedlings were significantly taller and showed greater total nitrogenase activity than noninoculated seedlings (Table 2.2). This was true even though all seedlings became mycorrhizal with contaminant greenhouse fungi (mostly *Thelephora* spp.), ca. 50 % on uninoculated seedlings (Table 2.2). At the end of the experiment, all seedling were mycorrhizal to a high percentage with either contaminant fungi or combination of a *Alpova*+ contaminant fungi. Nevertheless, the ANOVA showed *Alpova* inoculation continued to significantly affect total mycorrhiza formation, stem diameter, specific nitrogenase activity, leaf area and leaf dry weight, root dry weight, shoot/root ratio, and photosynthesis (Table 2.3). N-fertilization was the second most influential treatment, with significant effects on mycorrhiza formation, nodulation, N-fixation, leaf parameters, and shoot dry weight; P-fertilization affected nodulation, specific nitrogenase activity, shoot dry weight, and CO_2 exchange rate while a N-P interaction affected mycorrhiza development, nodulation, total nitrogenase activity and leaf area (Table 2.3).

Total mycorrhiza formation, 58 to 96 % depending on N-fertilization levels (Fig. 2.4 A), was significantly increased by *Alpova* inoculation and N-fertilization but not by P-fertilization. *Alpova* mycorrhiza formation, 15 to 62 %, was significantly decreased by the highest N-fertilization. However, when P was added, *Alpova* mycorrhiza percent remained similar regardless of N levels (Fig. 2.4A).

Height growth was not significantly affected by any treatment (Fig. 2.4 B), whereas the means of all fertilization treatments diameter growth was significantly increased by *Alpova* inoculation from 6.2 to 6.5 mm (Fig. 2.4 C).

N-fertilization at the highest rate significantly decreased nodule dry weight by ca. 57 % (Fig. 2.4 D). On the other hand, P-fertilization increased nodule dry weight by 50 %, but this P effect was reduced by adding N-fertilization. Total nitrogenase activity was significantly and drastically reduced by the highest N-fertilization from ca. 9.8 to ca 1.7 μmol C_2H_2 reduced/plant/hr (Fig. 2.4 E). *Alpova* inoculation, and N- and P-fertilization significantly decreased specific nitrogenase activity without significant interaction between these treatments (Fig. 2.4 F). Thus, N-fertilization affected N-fixation differently from other two treatments, reducing both nodule development and specific nitrogenase activity, while P-fertilization and the fungus inoculation increased nodule development and decreased specific nitrogenase activity.

Leaf development measured as leaf area and leaf dry weight was significantly decreased by *Alpova* inoculation and N-fertilization (Figs. 2.5 A and B). Specific leaf dry weight was significantly decreased only by the highest N-fertilization (Fig. 2.5 C).

Shoot dry weight was significantly decreased by N-fertilization and increased by P-fertilization (Fig. 2.5 D). Root dry weight was significantly increased by *Alpova* inoculation (Fig. 2.5 E), resulting in significantly reduced shoot /root ratio (Fig. 2.5 F).

CO_2 exchange rate (CER) (Fig. 2.6 A), stomatal conductance (Fig. 2.6 B) and transpiration rate (Fig. 2.6 C) were significantly increased by *Alpova* inoculation. CER was also significantly increased by P-fertilization. However, internal CO_2 concentration was not significantly affected by any treatment (Fig. 2.6 D).

N and P contents of leaves, roots, and nodules, and analysis of variance are shown in Table 2.4. N concentration in roots was significantly decreased by *Alpova* inoculation and increased by the highest N-fertilization. Leaf N concentration was

significantly increased by the highest N-fertilization as well as by the the interaction effect of *Alpova* inoculation and P-fertilization (Table 2.4); it was not affected by either treatment alone but significantly increased by both treatments together. Root N concentration was affected by the interaction effect of N-and P-fertilization; it was increased by the highest N-fertilization but the difference was reduced by adding P. Nodule N was not affected by any treatment.

P concentration was significantly increased in leaf but decreased in nodule tissues by both *Alpova* inoculation and N-fertilization, and significantly increased in leaf, root and nodule tissues by P-fertilization. P in roots was increased 100 to 150 % by the P-fertilization, whereas in leaves and nodules P was increased by 8 to 80 %. Leaf P concentration was significantly increased by combined N- and P-fertilization; it was significantly increased by N-fertilization and increased even more by adding P. Nodule P concentration was significantly decreased by N-fertilization and the degree of the decrease was greater when P was added than when P was not.

Table 2.2 Experiment 2. *Alnus rubra* seedling growth for ten weeks in a greenhouse prior to beginning fertilization treatments. Seedlings were inoculated with *Frankia* only (F) or *Frankia* and *Alpova diplophloeus* spores (FA) when they were two weeks old.

Parameter	Inoculation	
	F	FA
Alpova diplophloeus mycorrhizae.(%)	0.0	19.0±5.0
Total mycorrhizae (%)	54.0±7.2	39.6±6.8
Height (cm)	23.7±0.8	28.8±0.9 **
Diameter (mm)	4.0±0.2	4.1±1.0
Shoot dry weight (g)	1.09±0.09	1.25±0.06
Root dry weight (g)	0.43±0.05	0.47±0.05
Shoot/Root ratio	2.82±0.28	2.80±0.19
Leaf dry weight(g)	0.50±0.03	0.56±0.04
Leaf area(cm2)	119.0±8.0	128.0±10.0
Nodule dry weight(mg)	38.3±3.8	41.5±2.1
Total nitrogenase activity (μmol C_2H_2 reudced/plant/hr)	7.7±0.3	10.1±0.9 *
Specific nitrogenase activity (μmol C_2H_2 reduced/g dry nodule/hr)	233.0±41.0	245.0±24.0
CO_2 exchange rate (mg $CO_2/m^2/s$)	0.22±0.03	0.23±0.02

Values are means of 9 or 10 samples ± standard error.

* and ** are significantly different at $p \leq 0.05$ and $p \leq 0.01$ by Duncan's test, respectively.

Table 2.3 Comparison of N- and P-fertilization and *Alpova diplophloeus* spore inoculation treatments by analysis of variance of data from 20-week-old *Alnus rubra* seedlings grown in a greenhouse (Experiment 2).

Parameter	N	P	A	N*A	N*P	P*A	N*P*A
Total mycorrhizae(M)	**	--	**	**	--	--	--
A. diplophloeus M.	--	--	--	--	**	--	--
Height	--	--	--	--	--	--	--
Diameter	--	--	**	--	--	--	--
Nodule dry weight	**	**	--	--	**	--	--
TNA [1]	**	--	--	--	*	--	--
SPNA [2]	**	*	*	--	--	--	--
Leaf area	*	--	**	--	*	--	--
Leaf dry weight	**	--	**	--	--	--	--
Specific leaf dry weight	**	--	--	--	--	--	--
Shoot dry weight	**	**	--	--	--	--	--
Root dry weight	--	--	*	--	--	--	--
Shoot/root ratio	--	--	*	--	--	--	--
CO_2 exchange rate	--	*	**	--	--	--	--
Stomatal conductance	--	--	**	--	--	--	--
Transpiration rate	--	--	**	--	--	--	--
Internal [CO_2]	--	--	--	--	--	--	--

N : nitrogen ; P : phosphorus; A : *Alpova diplophloeus* spore inoculation.

[1] : total nitrogenase activity; [2] : specific nitrogenase acitvity.

* and ** are significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

Table 2.4 N and P concentration in the leaf, root and nodule tissues of 20-week-old *Alnus rubra* seedlings fertilized for 10 weeks.

Fertilization	Leaf		Root		Nodule	
	F	FA	F	FA	F	FA
			N(%)			
CO	1.82±0.05	1.73±0.05	1.79±0.10	1.59±0.03	2.58±0.21	2.73±0.14
N1	1.80±0.03	1.74±0.09	1.81±0.14	1.83±0.03	2.41±0.13	3.06±0.20
N2	1.93±0.02	2.03±0.08	2.88±0.04	2.33±0.14	2.80±0.11	2.77±0.17
P	1.69±0.03	1.87±0.04	1.60±0.02	1.63±0.09	2.52±0.04	2.79±0.20
N1P	1.80±0.05	1.78±0.09	1.94±0.02	1.82±0.06	3.07±0.21	2.55±0.23
N2P	1.94±0.03	2.13±0.04	2.24±0.04	2.38±0.08	2.91±0.17	3.06±0.18
				P(%)		
CO	0.10±0.01	0.11±0.01	0.15±0.01	0.14±0.01	0.16±0.01	0.15±0.01
N1	0.09±0.01	0.12±0.01	0.12±0.01	0.15±0.01	0.13±0.01	0.14±0.01
N2	0.11±0.01	0.12±0.01	0.14±0.01	0.13±0.01	0.12±0.01	0.11±0.01
P	0.14±0.01	0.14±0.01	0.32±0.03	0.28±0.01	0.19±0.01	0.18±0.01
N1P	0.15±0.01	0.15±0.01	0.34±0.03	0.32±0.01	0.20±0.01	0.18±0.01
N2P	0.18±0.01	0.21±0.01	0.29±0.01	0.33±0.02	0.13±0.01	0.13±0.01

F : *Frankia* only inoculated.
FA : *Frankia* and *Alpova diplophloeus* spores inoculated.
CO : no fertilization; N1 : 5 ml of 10 mM NH_4NO_3;
N2 : 5 ml of 50 mM NH_4NO_3; P : 5 ml of 5 mM KH_2PO_4
N1P : N1 and P; N2P : N2 and P
Values are the means of three to four samples ± standard error.

Comparison of treatment by analysis of variance

Source	N			P		
	Leaf	Root	Nodule	Leaf	Root	Nodule
Alpova(A)	--	*	--	*	--	*
N	**	**	--	**	--	**
P	--	--	--	**	**	**
A x N	--	--	--	--	--	--
A x P	*	--	--	--	--	--
N x P	--	*	--	**	--	**
A x N x P	--	--	--	--	--	--

* : significant at $p \leq 0.05$; ** : significant at $p \leq 0.01$.

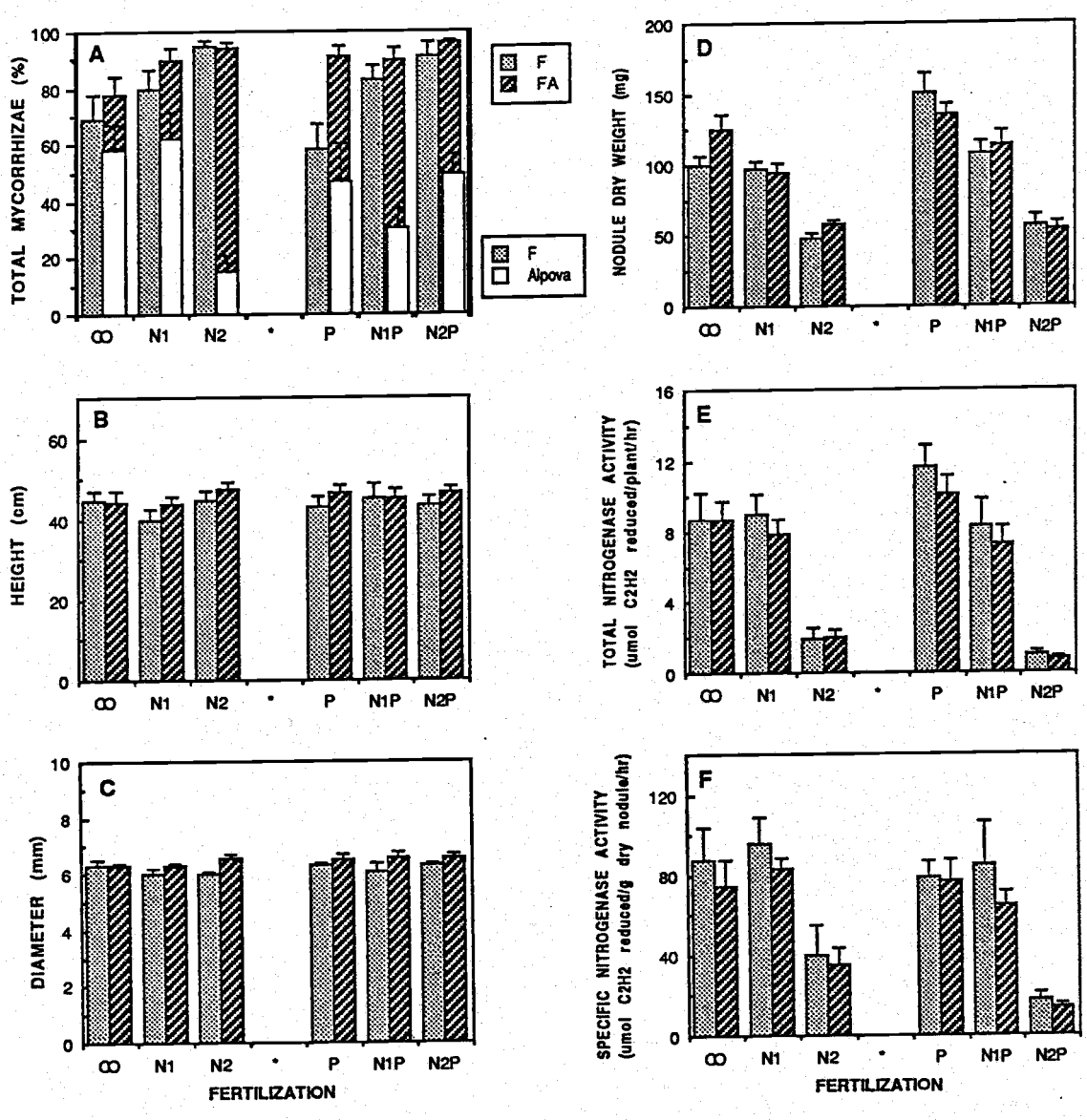

Fig. 2.4 Experiment 2. N- and P-fertilization effects on the mycorrhiza formation (A); open bars show *Alpova diplophloeus* mycorrhiza percentage, height (B), diameter (C), nodule dry weight (D), total nitrogenase activity (E) and specific nitrogenase activity (F) of 20-week-old *Alnus rubra* seedlings grown in a greenhouse. In the legend, F=*Frankia* pure culture and dead *Alpova* spore inoculation; FA=*Frankia* and live *Alpova* spore inoculation. On the horizontal, CO=no fertilization; N1=5 ml of 10 mM NH_4NO_3; N2=5 ml of 50 mM NH_4NO_3, P=5 ml of 5 mM KH_2PO_4 , N1P=combination of N1 and P treatments, N2P=combination of N2 and P treatments. Seedlings received the fertilizer three times a week for 10 weeks.

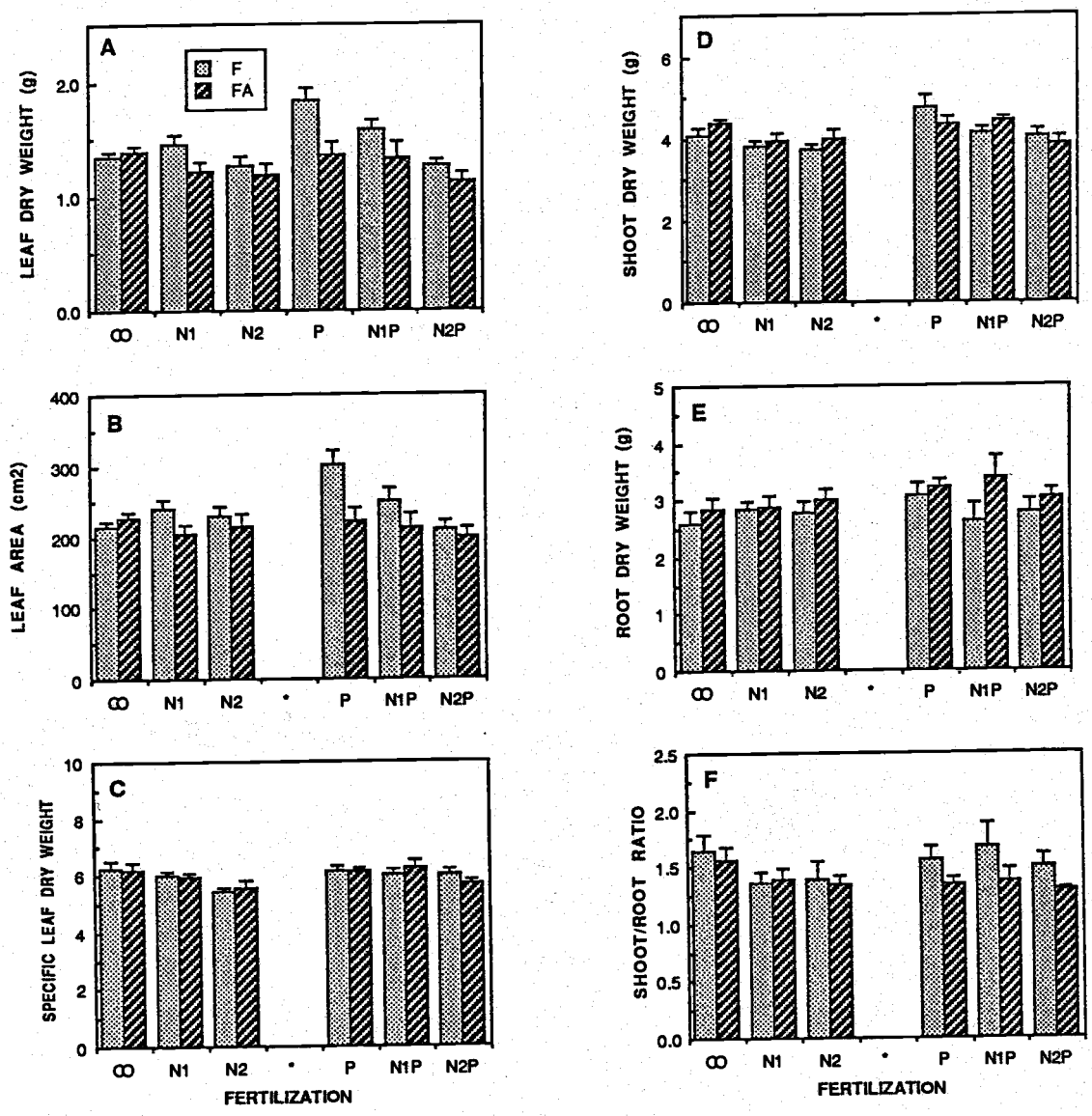

Fig. 2.5 Experiment 2. N- and P-fertilization effects on the leaf dry weight (A), leaf area (B), specific leaf dry weight (C), shoot dry weight (D), root dry weight (E) and shoot/root ratio (F) of 20-week-old *Alnus rubra* seedlings grown in a greenhouse. In the legend, F =*Frankia* pure culture and dead *Alpova diplophloeus* spore inoculation; FA=*Frankia* and live *Alpova* spore inoculation. On the horizontal, CO=no fertilization; N1=5 ml of 10 mM NH_4NO_3; N2=5 ml of 50 mM NH_4NO_3, P=5 ml of 5 mM KH_2PO_4 , N1P=combination of N1 and P treatments, N2P=combination of N2 and P treatments. Seedlings received the fertilizer three times a week for 10 weeks.

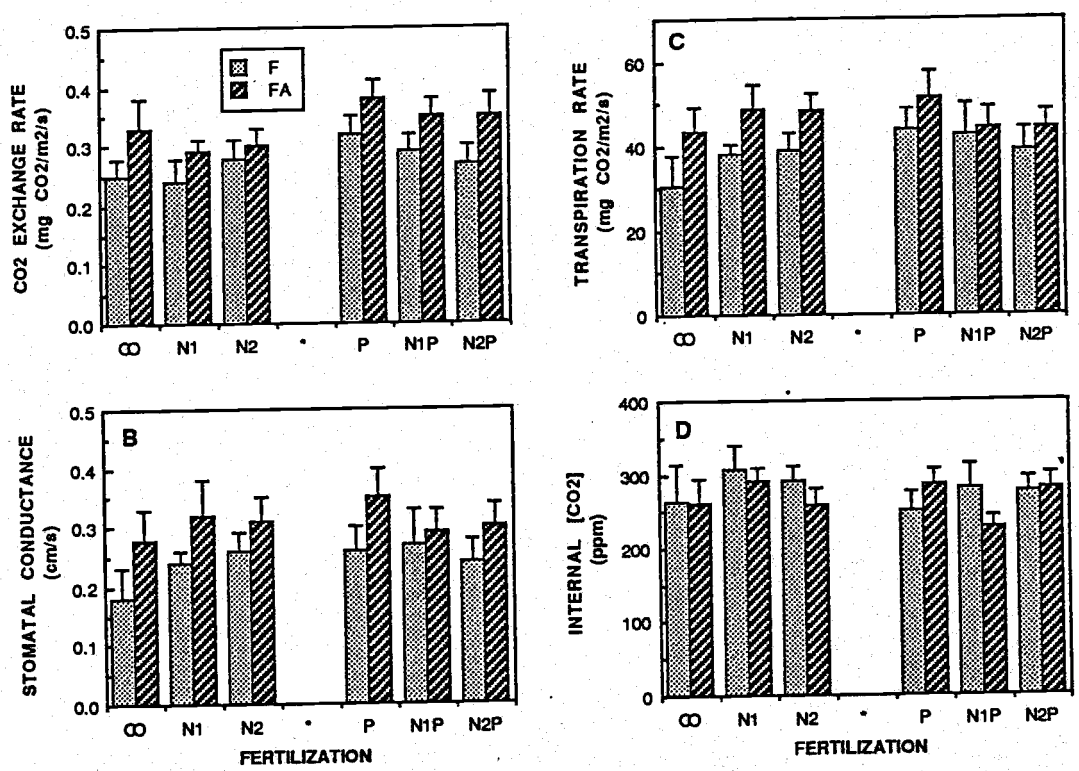

Fig. 2.6 Experiment 2. N- and P-fertilization effects on the CO_2 exchange rate (A), stomatal conductance (B), transpiration rate (C) and internal CO_2 concentration (D) of 20-week-old *Alnus rubra* seedlings grown in a greenhouse. In the legend, F : *Frankia* pure culture and dead *Alpova diplophloeus* spore inoculation; FA =*Frankia* and live *Alpova* spore inoculation. On the horizontal, CO=no fertilization; N1=5 ml of 10 mM NH_4NO_3; N2=5 ml of 50 mM NH_4NO_3, P=5 ml of 5 mM KH_2PO_4 , N1P=combination of N1 and P treatments, N2P=combination of N2 and P treatments. Seedlings received the fertilizer three times a week for 10 weeks.

DISCUSSION

These results support other studies showing that N-fixation can be reduced by N-fertilization and increased by P-fertilization and that mycorrhizae improve plant growth through enhanced P-nutrition. However, under our experimental conditions the ectomycorrhizal effect provided by *Alpova diplophloeus* is not as large for red alder seedlings as VAM is for legumes (Asimi,*et al.*, 1980; Suba Rao, *et al.*, 1983; Piccini, *et al.*, 1988) or actinorrhizal *Ceanothus velutinus* Doug. (Rose and Youngberg, 1981) and *Hippophäe* (Gardner *et al.*, 1984). In the growth chamber experiment, three-week-long N-fertilization with 10 ml of 1mM NH_4NO_3 did not significantly affect growth or physiological activities except for total nitrogenase activity (Fig. 2.2 E). Specific nitrogenase activity was reduced less than total nitrogenase activity, because nodule dry weight was also slightly decreased by the N-fertilization. These data indicate that red alder assimilates the same total amount of N from soil and N-fixation combined, regardless of soil N-content: low soil N is compensated by higher fixation rates and vice-versa. On the other hand, the ten-week-long and 25 times stronger N-fertilization treatment in Experiment 2 greatly reduced nodule formation, total nitrogenase activity, specific nitrogenase activity and shoot growth (Figs. 2.4 D, E and F).

As seen in a previous study (Koo *et al.*, 1989, chapter III in this thesis), *Alpova* ectomycorrhizae did not affect nodulation or N-fixation of red alder, but *Frankia* inoculation strongly enhanced ectomycorrhiza formation compared to the comparably sized N-fertilized, non-nodulated seedlings in Experiment 1 (Fig. 2.2 A). In preliminary *Frankia* inoculation trials, Koo(unpublished data) also observed that ectomycorrhizae on non-nodulated red alder seedlings had thinner mantles than on nodulated seedlings. Furthermore, when *Frankia* inoculation is delayed, mycorrhiza

formation remains low until after nodulation begins, and even then the length of feeder root colonized by mycorrhizal fungi is less than on early nodulated seedlings (Table A.1). We have also observed that non-nodulated, non-N-fertilized red alder seedlings remain stunted and *Alpova* ectomycorrhizae either fail to form after spore inoculation or only trace of mycorrhizae form. As seen in Experiment 1, N-fertilization of non-nodulated seedlings can enhance *Alpova* mycorrhiza formation to ca. 15 % but never to the levels on nodulated seedlings. Further investigation is needed on how *Frankia* nodulation mediates ectomycorrhiza development on red alder seedlings in this regard.

P-fertilization or high soil P levels can substitute for beneficial mycorrhizal effects on plant growth in ectomycorrhiza (Thomas *et al.*, 1982) and in VAM symbioses (Pacovsky *et al.*, 1986; Asimi *et al.*, 1980). But mycorrhiza response to P-fertilization differs depending on soil characteristics, host and fungus species. For example, Tyminska *et al.* (1986) found that *Thelephora terrestris* and *Laccaria laccata* mycorrhizae on *Pinus silvestris* were not significantly changed but *Hebeloma crustuliniforme* mycorrhizae were increased by high P-fertilization in a greenhouse. Thomas *et al.* (1982) observed that *Thelephora terrestris* mycorrhizae on *Picea sitchensis* even increased slightly in high P-soil, although the fungus enhanced host growth only in low P-soil. P-fertilization effects on mycorrhizal formation may depend on the degree of the demand by the symbionts or on the N/P ratio in the soil. For example, Hughes *et al.* (1968) found that P deficiency was aggravated by N-fertilization. P deficiency occurs as an N-fixing plant grows: as total plant N increases, P is depleted in the soil. Thus, high soil P effects on mycorrhiza formation can diminish as the plant grows. This was observed in nodulated VAM soybean. Fredeen and Terry (1988) reported that rapid VAM infection in the high P-treatment was delayed by 14 days compared to the low P-treatment. After two additional weeks, both high and low P treated seedlings had similar root P concentration and mycorrhiza formation. In both of our experiments,

even though P-fertilization increased the P concentration in feeder root tissues by over 100 %, it did not significantly decrease mycorrhiza formation.

However, N- and P-fertilization differently affected N and P concentrations in plant tissues. Table 2.4 shows that P concentration in leaf and nodules tissues was also affected by the interaction of N- and P-fertilization : P concentration in leaves gradually increased when N and P were added, whereas it gradually decreased when N was added without P, or when the highest N was added together with P. On the other hand, P concentration in roots was not changed by N-fertilization. Burgess and Peterson (1987) also reported similar increases in P concentration in leaves by adding inorganic N to both nodulated and nonnodulated *Alnus japonica* (Thunb.) seedlings. However, they did not report P changes in the nodules. In our studies, the low P concentration in nodules is not related to P deficiency of the plant but to the lowered activity of nodules due to easily available N. Nodules activity declines when soil N is readily available. Negative effects of N-fertilization on N-fixation are reduced by high P-fertilization. Huss-Danell *et al.* (1982) found that inorganic N-fertilization with NH_4Cl extensively damaged *Frankia* vesicles within alder nodules. Burgess and Peterson (1987) also found that increased addition of inorganic N reduced the numbers of endophytic vesicles per unit area of infected cells.

Jakobsen (1985) found that supplying P to P-deficient plants increased nodule dry weight, specific nitrogenase activity and P concentration in the shoot relatively faster than it increased shoot dry weight and P concentration in nodules. P-fertilization also substituted for mycorrhizae in N-fixing soybeans (Asimi, *et al.*, 1980).

Alpova-inoculated seedlings were smaller in leaf growth but larger in shoot and root growth and photosynthetic activities. This can be explained by the roles of mycorrhizae in nutrient exploitation, effects of limited rooting substrate and mechanisms of photosynthate sink demand. Before starting the fertilization treatment, *Alpova*-

inoculated seedlings were generally larger in all the parameters (Table 2.2). But height growth difference gradually decreased with age (Fig. A.1), probably due to depletion of soil nutrients. As a result, inoculated plants showed similar height and leaf development at the end as noninoculated ones. However, the *Alpova*-inoculated plants maintained larger shoot and root dry weight than noninoculated ones. Maintaining this larger biomass of non-photosynthetic tissues may induce compensating higher photosynthetic rates in smaller leaves. Internal CO_2 concentration would be maintained by coupling high CO_2 exchange rates and high stomatal conductance. These higher photosynthesis rates shows that plant leaves have an unexpected photosynthetic capacity suggested by Lauer and Shibles (1987) . Fredeen and Terry (1988) demonstrated that CO_2 exchange rates did not differ between mycorrhizal and nonmycorrhizal soybean plants and suggested that VA mycorrhizal colonization increased production of photosynthate due to an increase in the rate of leaf surface expansion .

In conclusion, N-fertilization inhibits N_2-fixation in red alder but promotes *Alpova* mycorrhiza formation in the absense of nodulation. In contrast, P-fertilization promotes N_2-fixation but inhibits mycorrhiza formation. However, this P-inhibiting effect may disappear as the plants grow and deplete nutrients in the soil. In that case, the importance of mycorrhizae in alder will increase as the plants grow. Thus, for growing red alder seedlings in nurseries, early *Frankia* and specific mycorrhizal fungus inoculation is recommended but N-fertilization is not. Further investigation is needed to determine how mycorrhizal function may change from the early seedling stage to mature alder trees.

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CHAPTER 3

LIGHT EFFECTS ON ECTOMYCORRHIZA AND NODULE FORMATION, N_2-FIXATION AND GROWTH OF RED ALDER SEEDLINGS

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ABSTRACT

Red alder (*Alnus rubra* Bong.) seedlings were grown in a walk-in growth chamber with either *Frankia* inoculation or N-fertilization and live or dead spore inoculation of the ectomycorrhizal fungus *Alpova diplophloeus* (Zeller & Dodge) Trappe & Smith, or in a greenhouse with *Frankia* inoculation and either live or dead spore inoculation of *Alpova*. In the growth chamber, 20-week-old seedlings were grown under three levels of light intensities [photosynthetic photon flux density (PPFD) of 680, 320, and 220 $\mu mol/m^2/s$] for three weeks. PPFD of 220 significantly decreased growth and some physiological parameters. PPFD of 320 and 220 significantly decreased CO_2 exchange rates. *Frankia* inoculated seedlings were significantly greater in mycorrhiza formation, growth and physiological activities than N-fertilized seedlings. *Alpova* inoculated seedlings had significantly greater growth but not N_2-fixation. None of the symbionts affected CO_2 exchange rates.

In the greenhouse, ten-week-old seedlings were grown under three levels of light intensities (PPFD of 510, 250 and 120 $\mu mol/m^2/s$) for ten weeks. PPFD of 120 significantly increased parameters related to light harvesting, and photosynthate use efficiency, and decreased parameters related to total biomass and photosynthesis. Light stress did not significantly affect mycorrhiza formation. *Alpova* inoculation did not significantly affect seedling growth and physiological activities. Our results suggest that *Frankia* is more important to seedling growth and *Alpova* mycorrhiza formation than *Alpova* is for growth and nodule formation, that red alder plants adapt to light stress by increasing photon harvesting structures, and that PPFD of 250 is a minimum light intensity for balanced symbiosis development and growth of red alder seedlings.

INTRODUCTION

Red alder (*Alnus rubra* Bong.) is an important N_2-fixing tree species in Pacific Northwest forests. In addition to being a dominant riparian tree, it rapidly colonizes exposed mineral soil recently disturbed by logging or land slide. Because it has less economic value than Douglas-fir (*Pseudotsuga menziesii* (Mirb) Franco) or western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) and competes with conifers, alder has often been treated more as a weed than as a manageable or a profitable species for forest productivity. However, the importance of red alder in Pacific Northwest forestry is gradually increasing in annual sawtimber production (Resch, 1988), in use to improve soil fertility by adding N and organic matter into the forest ecosystem (Franklin *et al.*, 1968; Bormann and DeBell, 1981), and in as an alternative species in sites infected with *Phellinus weirii* root rot of conifers (Hansen, 1979).

Intercropping systems have been intensively researched in agriculture, because they can maximize crop yield per unit land area with minimum use of N-fertilizer (Ofori and Stern, 1987). With this in mind, forest scientists have tried systems for growing mixtures of red alder and conifers, but most involve manipulating growth of the alder. Miller and Murray (1978) found that controlling the density of red alder trees to maintain them as dominants or codominants improved N_2-fixation and growth of associated conifers. Alternatively, red alder can be introduced to conifer forests after precommercial thinning (Helgerson *et al.*, 1984). Mixed plantings of poplar and alder for short-rotation biomass production have also shown promising results (Cote and Camire, 1984).

Photosynthesis is recognized as a key factor in N_2-fixation and plant growth (Hardy and Havelka, 1976), because the N_2-fixing process demands high energy input. Most N_2-fixing plants, particularly pioneering species, are not shade tolerant. Shading

generally reduces both nodule number and size in legumes (Chu and Robertson, 1974; Dart and Mercer, 1965; Sprent, 1973; Lawn and Brun, 1974; Wahua and Miller, 1978; Trang and Giddens, 1980; and Antoniw and Sprent, 1978). In European alder (*Alnus glutinosa*) (Gordon and Wheeler, 1978), low irradiance reduced nodule formation and nitrogenase activity. To refine red alder-conifer intercropping system light effects on its growth and N_2-fixation must be better understood. Red alder forms ectomycorrhizae (Molina, 1981; Miller *et al.*, 1989) which are important in nutrient uptake and also possibly responsive to light effects.

In legume symbioses, vesicular-arbuscular mycorrhizae (VAM) are essential for the N_2-fixing plants (Daft and El-Giahmi, 1974), because the plants need an extraordinarily high P supply to energize the N_2-fixing process; VAM enhance P uptake of N_2-fixing plants (Barea *et al.*, 1987; Mosse *et al.*, 1976; Bethlenfalvay *et al.*, 1987; Bethlenfalvay and Yoder, 1981). In addition, VAM can increase CO2 fixation per unit shoot weight (Kucey and Paul, 1982) of host plants by increasing sink strength (Koch and Johnson, 1984), or increase CO2 fixation per unit leaf P content (Brown and Bethlenfalvay, 1987; 1988). Ectomycorrhizae are vital in P uptake of the host plant (Harley and Smith, 1983). Mejstrik and Benecke (1969) found enhanced P-uptake in excised ectomycorrhizae of *Alnus veridis*. Red alder mycorrhizae probably function similarly, so they may directly influence nodule formation and N_2-fixation.

Photosynthesis directly affects mycorrhiza formation. Plant growth response to VAM infection generally decreases when photon irradiation is reduced (Bethlenfalvay and Pacovsky, 1983; Tester *et al.*, 1985; Haymen, 1974; Son and Smith, 1988). Growth reduction was due more to an increase of carbohydrate use by the fungus (Tester *et al.*, 1985) rather than to a P starvation of plants as suggested by Hayman (1974). Tester *et al.*(1986), however, found that the main effect of decreased irradiance was on the growth of the root system; the numbers of first- and second-order lateral

roots decreased and that reduced VAM colonization via less available entry points for VAM initiation. Son and Smith (1988) concluded that growth and physiological activity of both of the plant and fungus symbionts are limited by carbohydrate supply at low irradiance as evidenced by the increase of fresh to dry shoot ratio, decrease of root to shoot ratio, and reduced fungus infection level and P inflow.

Light generally limits tripartite symbioses in the same manner as with dual symbioses. However, the responses of three symbionts can vary tremendously depending on the kinds and degrees of the stress. Daft and El-Giahmi (1978), for example, found in alfalfa that short day length (5 hr) produced more small nodules and slightly increased N_2-fixation rate/plant, whereas whole plant dry weight, mycorrhizal colonization and plant growth response to mycorrhizal infection decreased. They concluded that the supply of photosynthate is an important factor controlling the development of VAM on alfalfa. On the other hand, Bayne *et al.* (1984) found that limiting photosynthate availability by defoliation severely reduced nodulation and nodule activity of soybean, but root colonization by the VAM fungus in the tripartite symbiosis was less affected. They concluded that the fungal endophyte was more competitive than the N_2-fixing endophyte for host carbohydrate as photosynthetic products became limiting. Low light intensities also reduce mycorrhiza formation in conifers (Björkman, 1970; Wenger, 1955) and beech (Harley and Waid, 1955). Although red alder has been reported to form both VAM and ectomycorrhizae, effects of mycorrhizae on the alder growth and nitrogen fixation are poorly known.

Perry *et al.* (1979) suggested that, because N_2-fixation requires symbioses adapted to relatively temperate forest environments, genotypic symbioses should be selected for silvicultural ability to survive and fix N in shade, cold soils, and under water stress. However, before we begin selecting for such genotypic adaptations, the ecophysiological interaction between the symbioses must be better understood. Our

objective, therefore, was to examine the responses of red alder seedlings, to shading in terms of photosynthetic rates, plant growth, nodule and mycorrhiza formation, and N_2-fixation.

MATERIALS AND METHODS

Two experiments were conducted. In experiment 1, seedlings were grown in a walk-in growth chamber for 20 weeks and harvested after shading for three additional weeks. In experiment 2, the plants were grown in a light supplemented naturally-lit greenhouse for ten weeks and harvested after shading for ten additional weeks.

Experiment 1.

Biological materials : Red alder seeds (seed zone 251, Brown Seed Company (12101 N. E. 28th st. Vancouver, Washington)) were selected for uniform size by dry sieving. *Frankia* was isolated by filtration method (Benson, 1982) from nodules of one-year-old red alder seedlings collected at the U. S. Forest Service Cascade Head Experimental Forest near the Oregon coast. Isolates were cultured for one month on N-free BAP liquid medium (Murry *et al.*, 1984). Sporocarps of *Alpova diplophloeus* (Zeller & Dodge) Trappe & Smith, a hypogeous ectomycorrhizal fungus specific to alder (Molina, 1981), were collected under young red alder at the Cascade Head Experimental Forest and stored at -18° C until used.

Seedling growth conditions : Red alder seeds were surface-sterilized with 30 % H_2O_2 for 15 min, planted in a tray with fine-granule vermiculite, covered with autoclaved coarse sand 0.2 cm deep, and mist-irrigated. The tray was then covered with a clear plastic tent to maintain moist conditions until germination was complete. When the seedlings produced their first real leaves in two weeks, they were transplanted to Conetainer super cell plastic tubes, 3.2 cm diameter x 20 cm long (165 ml capacity). Potting substrate was a 1:1:2 mixture of peatmoss, vermiculite and sandy loam soil collected at Willamette valley in Oregon. The soil mixture was autoclaved for 120 min. Nutrients in the mixture were 0.068 % N, 620 ppm total P, 8 ppm available P, 583 ppm K, 1799 ppm Ca, and 430 ppm Mg after autoclaving. Plants were grown at a day/night

temperature of 25/17° C, 14/10 hr light regime, and 60/95 % relative humidity. Photosynthetic photon flux density (PPFD) was 680±26 μmol/m^2/s as measured with a LICOR quantum radiometer/photometer located 50 cm above the surface of the growth tubes.

Experimental design : This was a 2 x 2 factorial design blocked with three light levels and with four or eight replicated seedlings per treatment combination. The first factor was N-fertilization vs. *Frankia* inoculation. Because plant growth was stunted without *Frankia* inoculation, nonnodulated red alder seedlings received 10 ml of 10 mM NH_4NO_3 every other day. The second factor was live spore inoculation vs. autoclaved spore inoculation of *Alpova diplophloeus* (Zeller & Dodge) Trappe & Smith. Ten million spores suspended in 5 ml sterilized distilled water were irrigated into each seedling four weeks. The blocking factor was light at three respective intensities: PPFD of 680±26, 320±25, and 220±15 μmol/m2/s measured at 50 cm height from the tube surface. These intensities were obtained by top and side shading with grey colored window shield set at 90 cm height from the tube surface. Shading treatments started at the 20th week (when seedlings had formed mycorrhizae with *Alpova*) and were continued for three weeks before harvesting. Seedling growth at week 20 is shown in Table 3.1.

A total of 16 seedlings (four inoculation treatments with four replicated seedlings each) were randomly arranged within the shade rack (30 x 60 cm for 98 super cells). The nonshaded treatment had a total of 32 seedlings (eight replicated seedlings for each inoculation treatment). Seedlings in the rack were randomly rearranged and racks were repositioned every week to even out location effects.

Data collection : At the 20th week, before shading, and then three weeks after shading began, photosynthetic rates, nitrogenase activity, seedling growth, and symbiosis development were measured for each seedling. Apparent photosynthetic rate

was measured as CO_2 exchange rates (CER) with a LI6000 portable photosynthesis system (LI-COR, Inc, Lincoln, Nebraska) for 16 cm^2 leaf area on the 4th leaf from the top under each shading treatment. At the same time stomatal conductance, initial transpiration rate, and initial internal CO_2 concentration were obtained from the equation stored in the LI-COR system. During the measurements a breathing mask connected to a vacuum was used to remove CO_2 input from the investigator.

To measure *in situ* acetylene reduction rates, we used the closed system diagrammed in Fig. 3.1. An entire seedling root system was placed inside a 5.2 cm inside diameter x 27 cm long PVC tube. A hole was made on the PVC tube 5 cm below the top to remove air, inject acetylene, and collect gas samples. A rubber tube 0.5 cm diameter x 10 cm long was connected to the hole and a syringe directly attached at the other tube end to expedite gas injection and sampling. Gas flow into the tube was controlled with a spring clip. A split #9 holed rubber plug was used to seal the seedling growth tube in the PVC tube. Space around the seedling stem was sealed with Roma Italian Plastilina, #2 degree. The air volume in the closed PVC tube after seedling base insertion was calculated to be ca. 400 ml. After removing 40 ml of air (10 % of the air volume) from a sealed PVC tube with a 60-ml syringe, an equal amount of acetylene gas was injected into the tube and syringe pumped ten times to mix the gas. The rubber tube was then clamped shut with the spring clip. After one hour incubation under the shading treatment, a gas sample was collected in a two ml vacutainer with a five ml syringe through the rubber tube after pumping 15 times to mix the air. The gas sample was analyzed for acetylene and ethylene with a gas chromatograph (Helwett Packard, Model HP5830A) equipped with a hydrogen flame ionization detector and a Porapak R (80-100 mesh) filled column (1.8 m long x 2 mm inside diameter) by injecting 0.2 ml sample gas with 1 cc tuberculin syringe. The oven temperature was adjusted to 70 ° C. The temperature of injection and detection was adjusted to 70 ° C. Flow rate of N

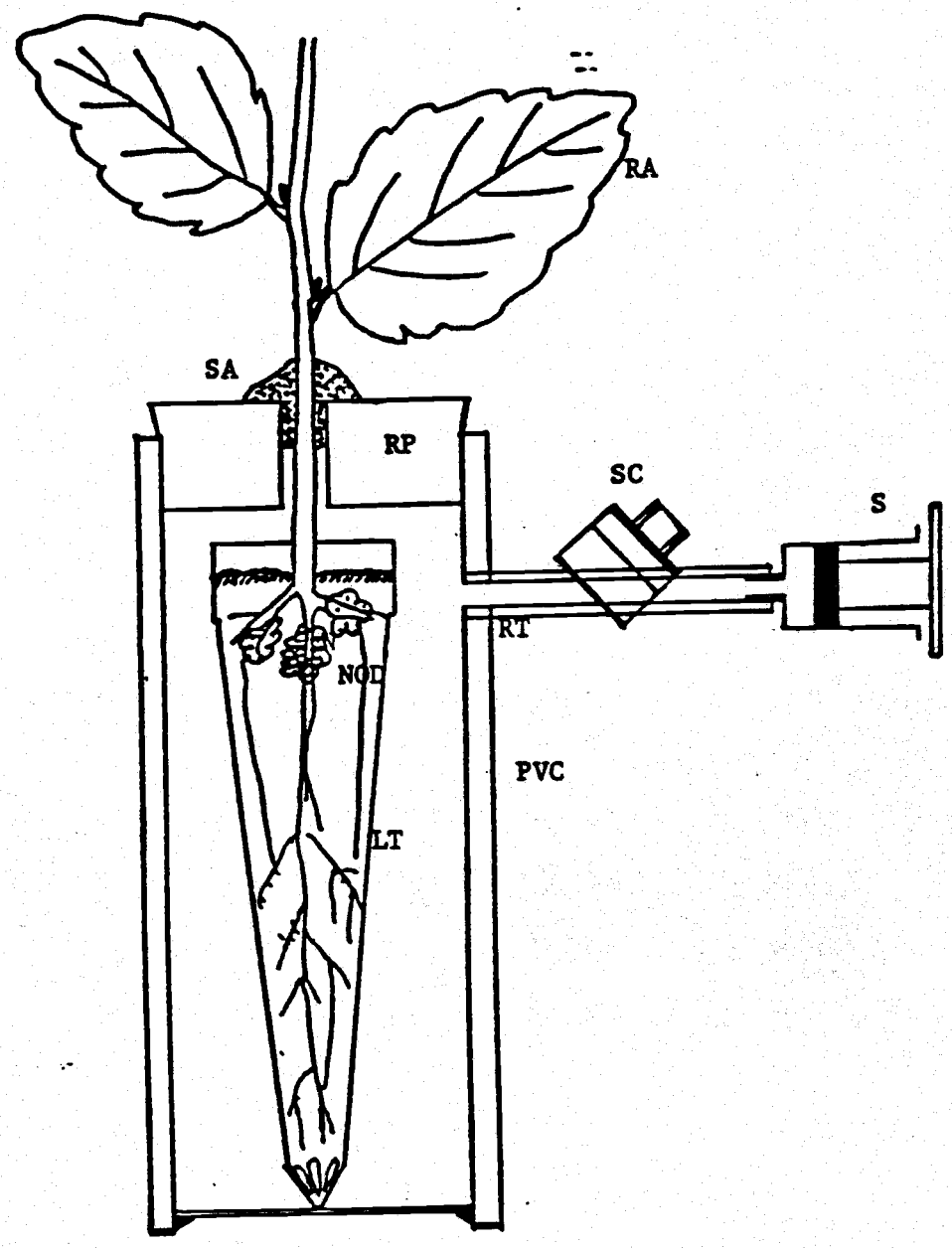

Fig. 3.1 A simple apparatus for determining nitrogenase activity of intact red alder seedling-soil system.

RA=red alder seedling; SA=sealing agent, Roma Italian Pastilina #2 grade; RP=120° splitted #9 holed rubber plug; RT=rubber tubing 0.5 x 10 cm; SC=spring clip; S=syringes for injecting and sampling gas; Nod=nodules; LT=leach tube, 3.2 x 20 cm, 165 ml; PVC=5.2 x 27 cm, 573 ml PVC tube.

carrier gas was 40 ml/min. Acetylene reduction rates were calculated as a percent value of produced ethylene to injected acetylene per plant and per unit nodule dry weight. The acetylene reduction assay with the intact root nodules showed that the amount of the reduction had a linear relationship with incubation time up to four hours.

Height, diameter, dry weight of shoot and root, area of the ten top leaves, and dry weight of the leaves were determined for each seedling. Dry weight was obtained after drying the tissues at 65 ° C to constant weight. Leaf area was measured with LI3100 Area meter (LI-COR, Inc, Lincoln, Nebraska). Specific leaf dry weight was calculated by dividing the leaf dry weight by the leaf area of each plant. A mean value of mycorrhiza formation for each plant was calculated from three root subsamples collected at 2.5 to 5 cm, 7.5 to 10 cm, and 12.5 to 15 cm deep along the length of the root plug. From each subsample 50 to 100 root tips were counted (mycorrhizal or nonmycorrhizal) and mycorrhiza formation calculated as percentage.

Data analysis : Data were analyzed by the general linear models (GLM) procedures in SAS® (SAS Institute Inc, Cary, North Carolina) to test the treatment effects for each parameter.

Experiment 2

Biological materials : Red alder seeds, *Frankia* isolates and *Alpova diplophloeus* spores were the same as in Experiment 1.

Seedling growth conditions : Seeds and soil mixture were prepared as in Experiment 1; seeds were directly planted in the tubes and grown in a greenhouse with day/night temperatures of ca. 24/18 ° C with supplemental light from sodium vapor lamps for 16 hours a day. Light intensity was PPFD of 510±26 $\mu mol/m^2/s$ at noon on a clear day. Seeds started to germinate in five days and seedlings were thinned to one per tube in two weeks. After thinning, all seedlings were inoculated with one month old

Frankia cultures by adding 1µl packed cell volume per seedling. Seedlings were watered to saturation every morning during the experiment.

Experimental design : The experiment was ramdomized block design blocking with light levels and treatments were live or dead spore inoculation of *Alpova diplophloeus* with eight replicated seedlings for each combination. In this experiment three levels of light intensities measured at 50 cm height from tube surface were PPFD of 510±26, 250±17 and 120±6 µmol/m^2/s. These light intensities were obtained by using gray window shields as in Experiment 1. Shading treatments began at 10 weeks and continued for another 10 weeks. Seedlings were arranged as in Experiment 1.

Data collection and analysis : Data were collected and analyzed as in Experiment 1 except that N and P were analyzed in plant tissues. To determine the concentrations of total N and P in leaves, feeder roots (<ca. 2 mm diameter) and nodules, samples from two seedlings within a treatment were combined and N and P determined by autoanalyzer after Kjeldahl digestion. Feeder root samples were collected by carefully rubbing them off the dried root systems.

RESULTS

Experiment 1.

Frankia inoculated seedlings grew significantly better than the N-fertilized seedlings. *Frankia* + *Alpova diplophloeus* spore inoculated seedlings performed the best in some parameters (Fig. 3.2, 3.3 and 3.4, and Table 3.1 and 3.2). *Frankia* inoculation and shading have affected mycorrhiza formation (Fig. 3.2 A). *Frankia* inoculation significantly increased *Alpova* mycorrhiza percentage at all light levels. Between light levels photosynthetic photon flux density (PPFD) of 220 $\mu mol/m^2/s$ significantly decreased mycorrhiza formation after three weeks; *Alpova* formed more than twice the amount of ectomycorrhizae at 680 or 320 PPFD (65 %) than at 220 PPFD (25 %). At full light *Frankia* +*Alpova* formed six times as many mycorrhizae as non-*Frankia*, N-fertilized seedlings. Seedlings in N-fertilized + *Alpova* alone treatment formed no mycorrhiza at 220 PPFD.

Height growth of N-fertilized seedlings resembled the nodulated ones and, overall, shading did not significantly affect height growth (Fig. 3.2 B and Table 3.2). *Alpova* treated seedlings were consistently taller than those treated with killed spores at 320 and 220 PPFD. Height was significantly increased by the *Frankia* + *Alpova* inoculation (Table 3.3). Although diameter growth was significantly reduced by shading under 320 PPFD, *Frankia* + *Alpova* significantly enhanced diameter over all other treatments (Fig. 3.2 C).

Nodule formation measured as dry weight was significantly decreased only at 220 PPFD and ranged from 80 to 110 mg for nonmycorrhizal seedlings and from 89 to 125 mg on the mycorrhizal plants (Fig. 3.2 D). *Frankia* + *Alpova* did not significantly increase nodule formation over *Frankia* alone at any light level. Total nitrogenase activity per plant (TNA) was also significantly decreased by shading but unaffected by

Alpova treatment (Fig. 3.2 E). Specific nitrogenase activity per unit nodule dry weight (SPNA) was not affected by shading (Fig. 3.2 F).

In general, *Frankia* and *Frankia* + *Alpova* treatments increased leaf dry weight, leaf area, and specific leaf dry weight (Fig. 3.3 A to F), and leaf dry weight and leaf area were increased by the combination of *Frankia* and *Alpova* inoculation. Shading decreased leaf dry weight and specific leaf dry weight and, less so, leaf area, but the interaction of shade with *Frankia* + *Alpova* increased leaf dry weight and leaf area over N-fertilized or either symbiont inoculated treatment (Figs. 3.3 A, B and C; Table 3.3). Despite significant interactions between light and inoculation treatments, shading overall decreased shoot dry weight (Figs. 3.3 D; Table 3.3); *Frankia* + *Alpova* increased shoot dry weight, particularly at 320 PPFD (Fig. 3.3 D). Root dry weight was similarly enhanced by *Frankia* + *Alpova* and decreased overall by shading (Fig. 3.3 E). Conversely, the shoot/root ratio was generally decreased by *Frankia* alone and *Frankia* + *Alpova* treatments compared to N-fertilized seedlings (Fig. 3.3 F). Apparent photosynthetic rate measured as CO_2 exchange rate significantly decreased with shading but there was little difference between inoculation treatments (Fig. 3.4).

Experiment 2.

In this greenhouse experiment, all seedlings formed nodules when inoculated with a pure culture of *Frankia*. Seedlings also formed mycorrhizae with noninoculated contaminant fungi, primarily *Thelephora* species, but also with an unknown brown fungus. However, *Alpova diplophloeus* only formed mycorrhizae on live-spore inoculated seedlings; no *Alpova* mycorrhizae were seen on dead-spore inoculated seedlings. It's also important to note that *Alpova* formed from 77 to 87 % of the total mycorrhizae on live-spore inoculated seedlings (Fig. 3.5 A). Thus, for this experiment, the treatment comparison is *Frankia* + greenhouse fungi (*Thelephora* and brown type)

Table 3.1 Growth data of 20-week-old red alder seedlings grown in a walk-in growth chamber before shading treatment with and without *Frankia* and *Alpova diplophloeus* spore inoculation.

Parameter	N	NA	F	FA
Mycorrhizae (%)	0a	2±2a	0a	23±7b
Nodule dry weight (mg)	0a	0a	85±14b	78±8b
Total nitrogenase activity (μmol C_2H_2 reduced/plant/hr)	0a	0a	14.1±1.8b	14.0±2.2b
Specific nitrogenase activity (μmol C_2H_2 reduced/g dry nodule /hr)	0a	0a	186±39b	176±18b
Height (cm)	43.6±0.9ab	41.5±0.7a	43.0±1.7ab	46.0±0.8b
Diameter (mm)	4.2±0.1a	4.5±0.2a	5.1±0.2b	5.3±0.2b
Leaf dry weight (g)	1.02±0.07a	1.11±0.05ab	1.41±0.13bc	1.60±0.15c
Shoot dry weight (g)	1.78±0.12a	1.91±0.06a	2.48±0.25b	2.81±0.25b
Root dry weight (g)	0.43±0.05a	0.42±0.04a	0.79±0.12b	0.80±0.08b
Shoot/root ratio	4.41±0.39a	4.70±0.34a	3.14±0.17b	3.50±0.27b

Values are the means of eight samples ± standard error.

N = N-fertilized only; NA = N-fertilized and *Alpova* spore inoculated; F = *Frankia* inoculated only; FA = *Frankia* and *Alpova* spore inoculated.

Different letters within a row show significant difference between treatments at $p \leq 0.05$ by Duncan's multiple range test.

Table 3.2 Growth of 23-week-old red alder seedlings grown in a walk-in growth chamber at full light intensity with and without *Frankia* and *Alpova diplophloeus* spore inoculation.

Parameter	N	NA	F	FA
Mycorrhizae (%)	0a	10±4b	0a	65±7c
Nodule dry weight (mg)	0a	0a	110±14b	125±10b
Total nitrogenase activity (μmol C_2H_2 reduced/plant/hr)	0a	0a	14.5±2.1b	15.1±1.7b
Specific nitrogenase activity (μmol C_2H_2 reduced/g dry nodule /hr)	0a	0a	135±23b	117±8b
Height (cm)	50.1±1.9 a	52.0±1.9 a	48.1±2.4 a	51.7±1.8 a
Diameter (mm)	5.18±0.24a	5.61±0.16ab	5.79±0.15b	5.96±0.14b
Leaf dry weight (g)	1.68±0.11a	1.72±0.08a	1.87±0.10ab	2.13±0.14b
Shoot dry weight (g)	3.07±0.22a	3.15±0.15a	3.41±0.16a	4.04±0.26b
Root dry weight (g)	0.86±0.08a	0.81±0.06a	1.06±0.05ab	1.28±0.12b
Shoot/root	3.74±0.17ab	4.06±0.18a	3.26±0.16 b	3.38±0.20b

Values are the means of eight samples ± standard error.

N = N-fertilized only; **NA** = N-fertilized and *Alpova* spore inoculated; **F** = *Frankia* inoculated only; **FS** = *Frankia* and *Alpova* spore inoculated.

Different letters within a row show significant difference between treatments at $p \leq 0.05$ by Duncan's multiple range test.

Table 3.3 Comparison of treatment by analysis of variance of data from Experiment 1.

Parameter	Shade(S)	Frankia(F)	Alpova(A)	S x F	S x A	F x A	S x F x A
Alpova diplophloeus Mycorrhizae	**	**	**	--	**	**	--
Height	--	--	*	--	--	*	*
Diameter	**	**	**	--	--	--	--
Nodule dry weight	**	**	--	**	--	--	--
Total nitrogenase activity	*	**	--	--	--	--	--
Specific nitrogenase activity	--	--	--	--	--	--	--
Leaf dry weight	**	**	*	--	--	*	*
Leaf area	--	--	**	--	--	**	*
Specific leaf dry weight	**	**	--	--	--	--	--
Shoot dry weight	**	**	**	--	*	*	*
Root dry weight	**	**	--	--	--	--	--
Shoot/Root ratio	**	**	**	--	--	--	--
CO_2 exchange rate	**	--	--	--	--	--	--

* and ** are significant at $p<0.05$ and $p<0.01$, respectively.
-- means not significant at $p<0.05$.

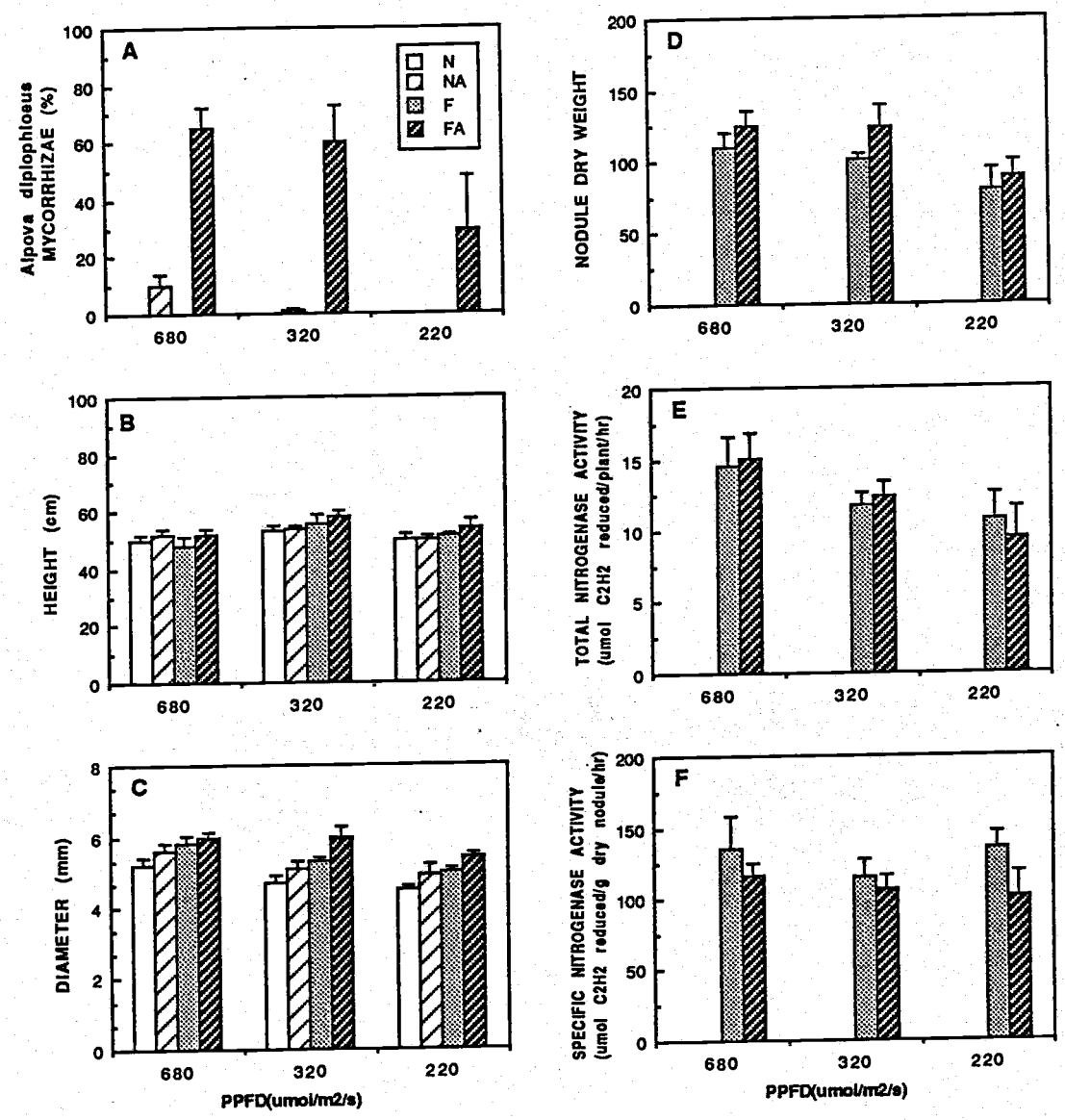

Fig. 3.2 Experiment 1. Three-week shading effects on mycorrhizal development, growth, and N-fixation of 23-week-old red alder seedlings grown in a walk-in growth chamber. N=N-fertilized only, NA=N-fertilized and *Alpova* spore inoculated, F=*Frankia* alone, and FA=*Frankia* and *Alpova* spore inoculated. The values at PPFD of 680 were from Table 2. PPFD=Photosynthetic photon flux density. Standard error bar shows on each treatment.

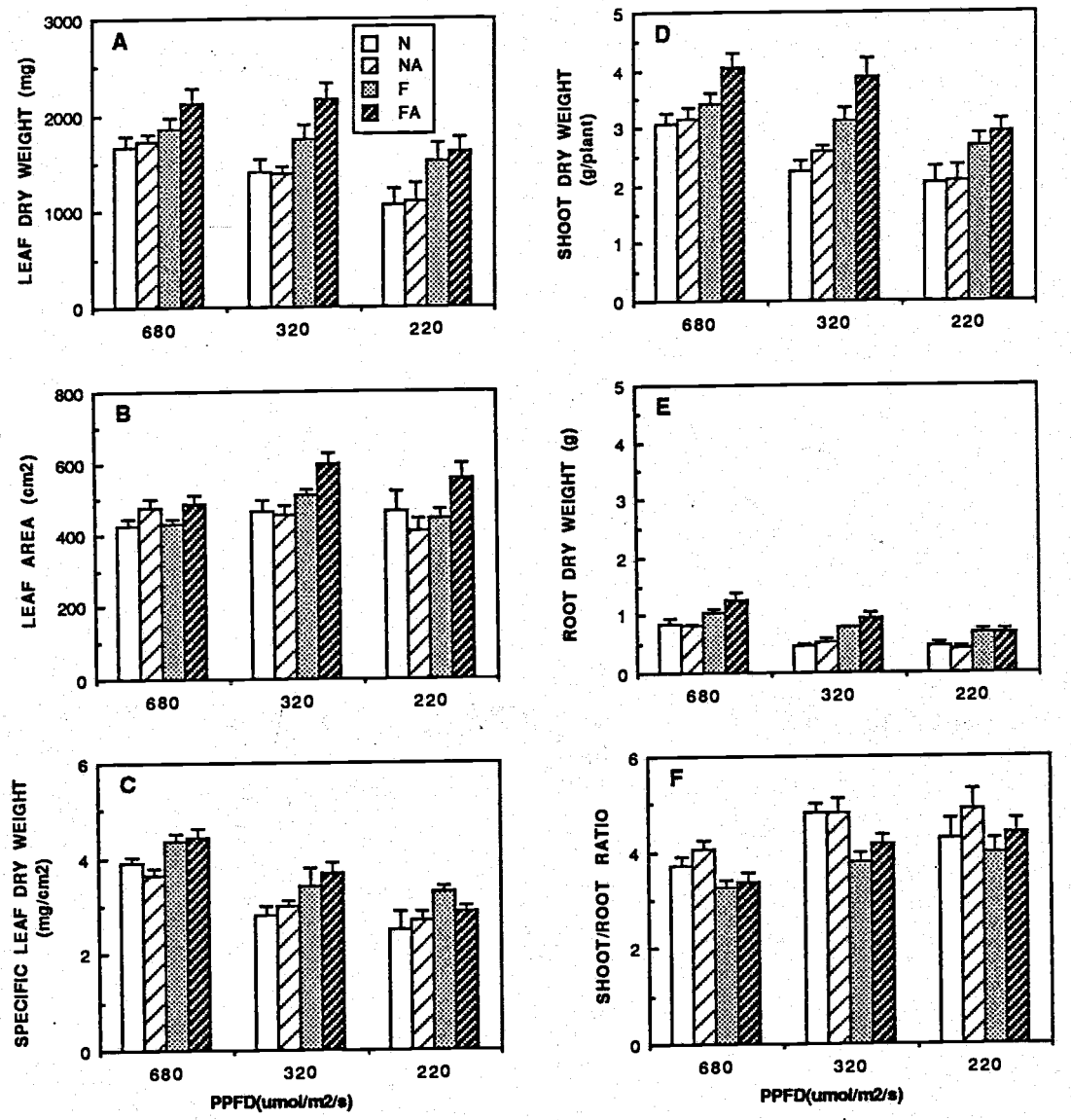

Fig. 3.3 Experiment 1. Three week shading effects on the growth of 23-week-old red alder seedlings grown in a walk-in growth chamber. N=N-fertilized only, NA=N-fertilized and *Alpova* spore inoculated, F=*Frankia* alone, and FA=*Frankia* and *Alpova* spore inoculated. The values at PPFD of 680 were from Table 2. PPFD=Photosynthetic photon flux density. Standard error bar is shown on each treatment.

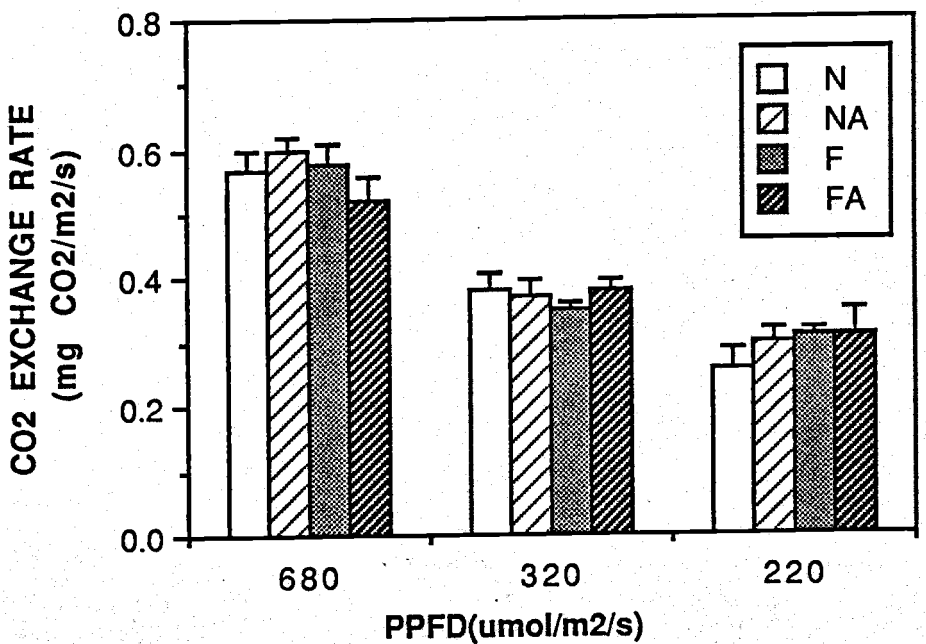

Fig. 3.4 Experiment 1. Three week shading effects on the CO_2 exchange rate of 23-week-old red alder seedlings grown in a walk-in growth chamber. N=N-fertilized only, NA=N-fertilized and *Alpova* spore inoculated, F=*Frankia* alone, and FA=*Frankia* and *Alpova* spore inoculated. The values at PPFD of 680 were from Table 2. PPFD=Photosynthetic photon flux density. Standard error bar is shown on each treatment.

vs. *Frankia* + predominately *Alpova.* Total mycorrhiza percent of live-spore inoculated seedlings was greater than dead-spore inoculated seedlings, and was unaffected by shading. *Alpova* mycorrhiza percent was not significantly affected by shading, either.

Both shading treatments significantly increased height growth over no shade. *Alpova* did not affect height (Fig. 3.5 B). Stem diameter significantly decreased at 120 PPFD and was significantly increased by *Alpova* treatment only at 250 PPFD (Fig. 3.5 C).

Nodule formation measured as dry weight was significantly decreased at 120 PPFD; *Alpova* did not significantly affect nodule formation compared to *Frankia* alone (Fig. 3.5 D). Total nitrogenase activity per plant (TNA) was also significantly decreased at 120 PPFD compared to 510 PPFD grown plants; *Alpova* treatment did not affect N_2-fixation rates (Fig. 3.5 E). TNA was affected by the interaction of *Alpova* inoculation and shading, i.e. it was reduced much more in *Alpova* inoculated seedlings (Fig. 3.5 E and Table 3.4). On the other hand, specific nitrogenase activity per unit nodule dry weight was not affected by shading (Fig. 3.5 F).

Leaf area development significantly increased under shade (Fig. 3.6 A), specific leaf dry weight significantly decreased at 120 PPFD (Fig. 3.6 B). *Alpova* treatment did not affect these leaf parameters.

Shoot dry weight was significantly decreased only at 120 PPFD, while root dry weight significantly and proportionally decreased at 250 and 120 PPFD; neither was affected by *Alpova* treatment (Fig. 3.6 C). Shoot to root ratios significantly increased with shading, but were not affected by *Alpova* treatment (Fig. 3.6 D).

CO_2 exchange rate significantly decreased with shading at 120 PPFD, but less on *Alpova* treated seedlings under the shade (Fig. 3.7 A); CO_2 exchange rate proportionally decreased with light intensities. Stomatal conductance also significantly decreased with increasing shade, but was not affected by fungus treatment (Fig. 3.7 B).

Transpiration rate was significantly decreased only at 120 PPFD and unaffected by *Alpova* treatment (Fig. 3.7 C). Initial internal CO_2 concentration, an indicator of photosynthetic enzyme activity in mesophyll cells, was significantly increased by shading at 120 PPFD, i.e. the enzyme activity was decreased by shading (Fig. 3.7 D). The CO_2 concentration was lower on *Alpova* treated seedlings under all three light treatments, but differences were not significant.

Shading significantly increased N concentration in leaves and roots, and P in leaves, roots and nodules (Table 3.5). *Alpova* treatment significantly decreased N concentration in leaves and increased P in leaves at 510 PPFD.

Table 3.4 Comparison of treatment by analysis of variance of data from Experiment 2.

Parameter	Shade(S)	*Alpova*(A)	S x A
Total mycorrhizae	--	**	--
Alpova diplophloeus mycorrhizae	--	**	--
Height	**	--	--
Diameter	**	--	--
Nodule dry weight	**	--	--
Total nitrogenase activity	*	--	**
Specific nitrogenase acitvity	*	--	--
Leaf dry weight	*	--	--
Leaf area	**	--	--
Specific leaf dry weight	*	--	--
Shoot dry weight	*	--	--
Root dry weight	**	--	--
Shoot/root ratio;	**	--	--
CO_2 exchange rate	**	--	--
Stomatal conductance	**	--	--
Internal CO_2 concentration	*	*	--
Transpiration rate	*	--	--

* and ** are significant at $p<0.05$ and $p<0.01$, respectively.
-- means not significantly different.

Table 3.5 Ten-weeks shading effects on the concentrations of N and P of 20-week-old nodulated red alder seedlings grown in a greenhouse. Photosynthetic photon flux density (**PPFD**) of 510, 250 and 120 μmol/m²/s. **F**=*Frankia* only inoculated; **FA**=*Frankia* and *Alova* live spore inoculated. Values are the means of three or four samples ± standard error.

PPFD	Treatment	Leaf	Root	Nodule
			N(%)	
510	F	2.24+0.05	1.59+0.06	2.54+0.07
	FA	2.04+0.05	1.57+0.06	2.85+0.10
250	F	2.53+0.07	1.74+0.07	2.98+0.17
	FA	2.24+0.05	1.69+0.03	2.83+0.21
120	F	2.71+0.07	1.95+0.07	3.06+0.09
	FA	2.59+0.06	1.77+0.03	3.05+0.14
			P(ppm)	
510	F	1330+90	1780+180	2000+100
	FA	1530+30	1800+110	1870+90
250	F	1570+70	1630+80	2200+50
	FA	1620+50	1730+50	2030+90
120	F	1730+110	1970+70	2400+110
	FA	1930+70	2000+20	2360+50

Comparison of treatments by analysis of variance

Source	N			P		
	Leaf	Root	Nodule	Leaf	Root	Nodule
Shade (S)	**	**	ns	**	*	**
Alpova (A)	**	ns	ns	*	ns	ns
(S) x (A)	ns	ns	ns	ns	ns	ns

* and ** are significantly different at $p \leq 0.05$ and $p \leq 0.01$ by Duncan's test, respectively.

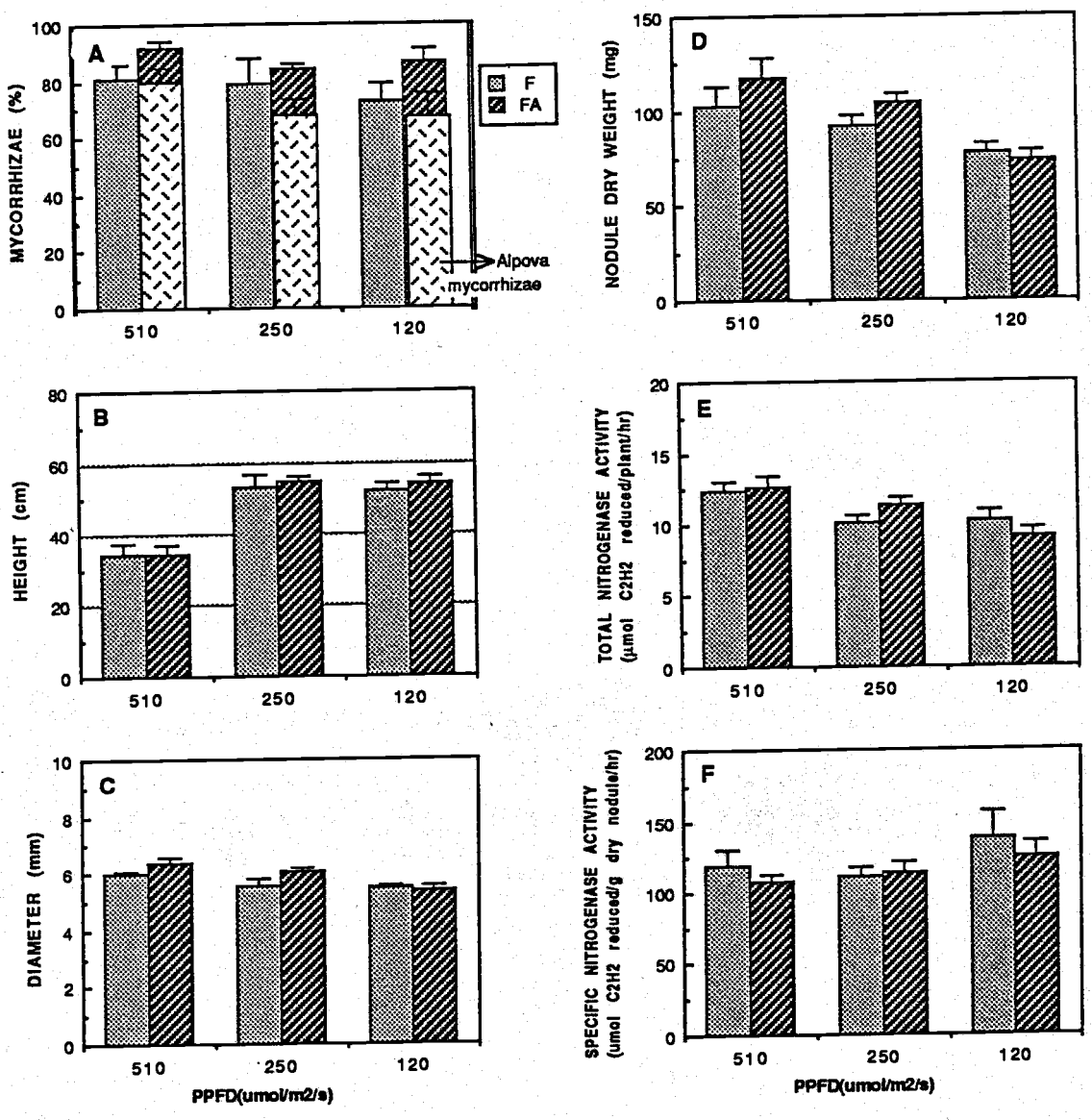

Fig. 3.5 Experiment 2. Ten-week shading effects on mycorrhiza and nodule development, growth, and N-fixation of 20-week-old red alder seedlings grown in a greenhouse. F=*Frankia* alone; and FA=*Frankia* and *Alpova* spore inoculated. 'F' seedlings formed mycorrhizae with contaminating *Thelephora terrestris* and unidentified brown fungus. PPFD=Photosynthetic photon flux density. Standard error bar is shown on each treatment.

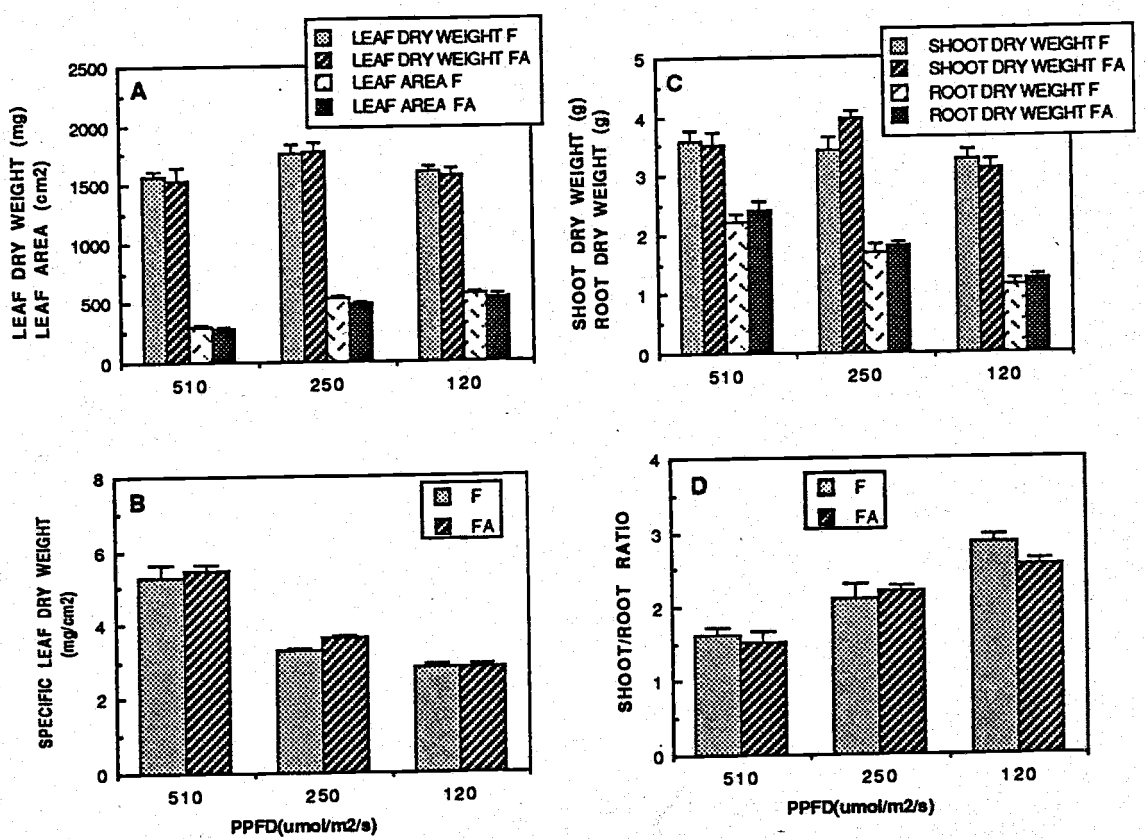

Fig. 3.6 Experiment 2. Ten-week shading effects on the growth of 20-week-old red alder seedlings grown in a greenhouse. F=*Frankia* alone; and FA=*Frankia* and *Alpova* spore inoculated. 'F' seedlings formed mycorrhizae with contaminating *Thelephora terrestris* and unidentified brown fungus. PPFD=Photosynthetic photon flux density. Standard error bar is shown on each treatment.

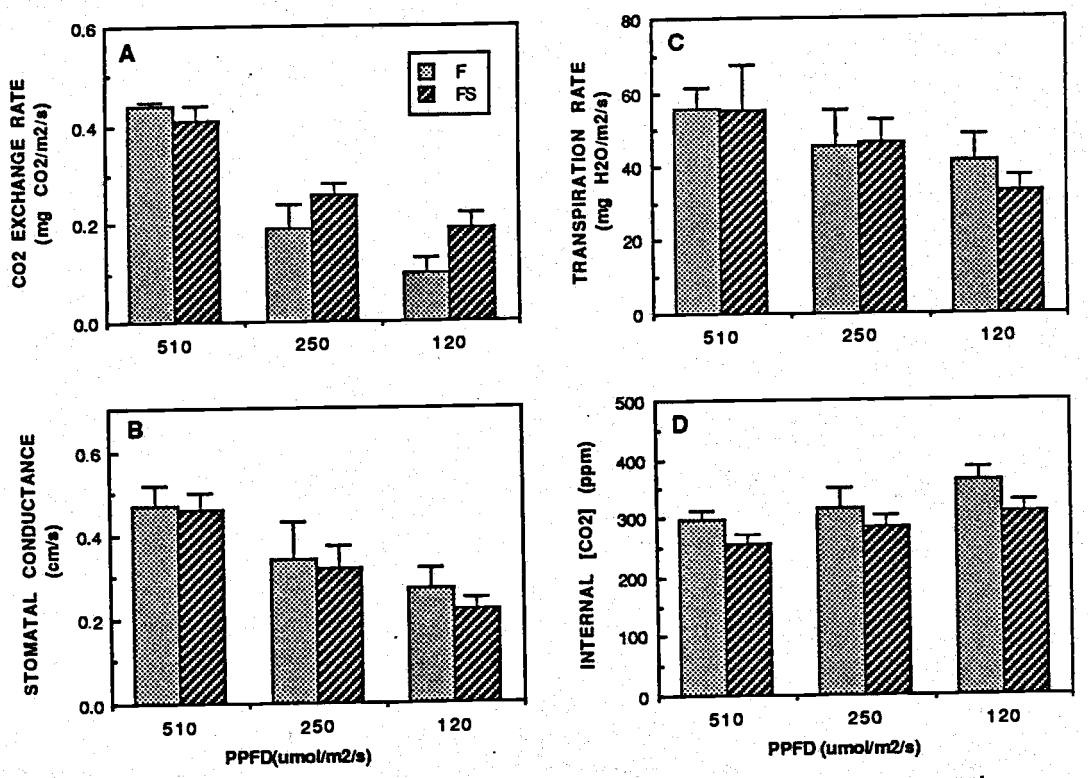

Fig. 3.7 Experiment 2. Ten-week shading effects on CO_2 exchange rate, stomatal conductance, transpiration and internal CO_2 concentration of 20-week-old red alder seedlings grown in a greenhouse. F=*Frankia* alone; and FA=*Frankia* and *Alpova* spore inoculated. 'F' seedlings formed mycorrhizae with contaminating *Thelephora terrestris* and unidentified brown fungus. PPFD=Photosynthetic photon flux density. Standard error bar is shown on each treatment.

DISCUSSION

Mycorrhizae formed with *Alpova diplophloeus* benefited red alder growth little in these experiments and was strongly overshadowed by the impact of the N_2-fixing symbiosis. Overall, nodulated *Alpova* -mycorrhizal seedlings grew better than most other treated seedlings, particularly compared to the non-nodulated, nonmycorrhizal seedlings in Experiment 1. However, the extra growth benefit from *Alpova* mycorrhizal formation was small compared to *Frankia* nodulated seedlings alone, and only significantly different in increasing shoot dry weight. This small benefit occurred in spite of the fact that approximately 65 % of the feeder roots were mycorrhizal with *Alpova* in both experiments. For non-nodulated seedlings (Experiment 1), 10 % *Alpova* mycorrhizae on inoculated seedlings provided no benefit to seedling growth.

The two microbial symbionts did interact significantly. *Frankia* inoculation increased *Alpova* mycorrhiza formation from 10 % on N-fertilized seedlings to 65 % on nodulated seedlings; the seedlings were similar in height and stem diameter but nodulated seedlings had greater leaf, shoot and root dry weights (Table 3.2). This result immediately raises the question of whether such differences in mycorrhiza formation were due to the presence of *Frankia*, either via a direct interaction between microorganisms or as mediated through the nodulated root system, or an inhibition by the N-fertilization. Given the large accretion of N in red alder stands, it is likely that a common alder mycorrhizal fungus like *Alpova* is adaptable to functioning at high soil N levels. Furthermore, in a recent study, Koo *et al.* (1989, chapter 2 in this thesis) found that high N fertilization (5 times higher than used in Experiment 1) did not inhibit *Alpova* mycorrhiza formation when the seedlings were nodulated. They also present evidence that early nodule formation on red alder seedlings promotes more rapid and abundant ectomycorrhiza formation than does delayed nodule formation (Table A.1).

Such a pattern of root symbiosis development also follows the natural sequence on wild red alder seedlings. Koo (unpublished data) followed the development of nodules and ectomycorrhizae on red alder in a variety of disturbed forest soils and observed that nodulation always preceded mycorrhiza development. This sequence raises the possibility that *Frankia* nodulation enhances the receptivity of roots to mycorrhizal fungi, perhaps through biochemical or morphological changes in the tissue or release of attractant root exudates. Such helper microbial interactions have been hypothesized in other root symbioses and merit further investigation in the alder-*Frankia*-mycorrhiza interaction.

Further work is also needed to elucidate the symbiotic role of mycorrhizal fungi for N_2-fixation of red alder. One major difficulty we encountered in these experiments and preliminary trials was in separating effects of the two root symbionts. Red alder germinants remained stunted and chlorotic when *Frankia* inoculation was withheld; when inoculated with *Alpova*, few or no mycorrhizae formed and the seedlings remained stunted. When non-nodulated seedlings were fertilized with N, they grew normally but mycorrhiza development remained low. When mycorrhiza enhancement of seedling growth occurred in combination with nodulation, it was rarely over 20 % of what nodulation alone provided and never affected N_2-fixation rates. These results contrast sharply with mycorrhizal benefits seen in VAM-rhizobial legumes (Asimi *et al.*, 1980; Bethlenfalvay and Yoder, 1981; Piccini *et al.*, 1988; Subba Rao *et al.*, 1986) and in VAM-actinorrhizal snowbrush (Rose and Youngberg, 1981). In those tripartite symbioses VAM significantly increased N_2-fixation, N and P nutrition, and plant growth by 50 % to several times. In our Experiment 2, *Alpova* mycorrhizal inoculation did increase P concentration in leaves at full light over noninoculated but mycorrhizal (*Thelephora* + brown type) seedlings. Regretably, we did not record P tissue contents in Experiment 1 to compare mycorrhizal and non-mycorrhizal seedlings. Mejstrick and

Benecke (1969) found that excised *Alnus viridis* ectomycorrhizae absorbed more P than nonmycorrhiza roots. Koo (unpublished data) found total P levels to be quite low in red alder soils. If red alder mycorrhizae are important in mineral nutrition, particularly for P, then further experimental study of a variety of alder mycorrhizal fungi at low P levels common to alder sites is needed.

The supply of photosynthate from the host is important for nodule and mycorrhiza formation. Reducing photosynthate by shortening photoperiod or shading reduces the formation of nodules (Sprent, 1973; Chu and Robertson, 1974) and mycorrhizae (Hayman, 1974; Björkman. 1970). However, this generalization was not fully supported by our results, because the two experiments showed different results depending on degree and duration of shading and symbiotic endophyte. Nodule formation and total nitrogenase activity significantly decreased only under the heaviest shading in both experiments; mycorrhiza formation was not affected by the ten weeks of shading in Experiment 2 (Fig. 3.5 A), but was significantly reduced after three weeks of heaviest shading in Experiment 1 (Fig. 3.2 A). It is important to note, however, that in Experiment 1 nodulated mycorrhizal seedlings had about 23 % mycorrhiza formation at week 20 just prior to initiating shade treatments. Percentage mycorrhiza formation increased to ca. 60 % for both 680 and 320 PPFD but remained unchanged in 220 PPFD. In Experiment 2, we had better success in early inoculation with *Alpova* . At 10 weeks just prior to shading, *Alpova* inoculated (and nodulated) seedlings already had ca. 65 % mycorrhiza formation. This percentage was maintained regardless of shading for 10 additional weeks. This result may be partially explained by the plant's adaptation to the 10 weeks of reduced light by increasing leaf area and maintaining photosynthetic rate and mycorrhiza development. But in Experiment 1, three weeks of shading may not have been long enough to allow the plants to adjust to the reduced CO_2 exchange rate and so mycorrhiza formation could not increase beyond initial levels.

Results from our 10-week-long shading treatment are similar to those cited by Bethlenfalvay and Pacovsky (1983) for soybeans. Under moderate shading, seedling vigor recovers by increasing leaf area development to compensate for low CO_2 exchange rate (Fig. 3.6A and 3.7 A). CO_2 exchange rate reduction in a soybean line is thought to be due to abnormally low leaf chlorophyll content per unit leaf area (Buttery and Buzzell, 1977). This reduction may be also associated with low specific leaf dry weight (Lugg and Sinclair, 1979; Dornhoff and Shibles, 1976) and thin leaves (Charles-Edwards, 1978). An inverse relationship between area per leaf and maximum CO_2 exchange rate is partly a reflection of the inverse relation of leaf thickness and leaf area (Gifford and Evance, 1981). In our experiment, specific leaf dry weight significantly decreased under shading (Fig. 3.6 B); we did not measure individual leaf area and leaf thickness. Increased leaf area with little change in leaf dry weight may explain why total plant photosynthesis was not reduced much under shading.

In addition, shading remarkably changed seedling growth patterns in terms of morphology, i.e. reduced diameter and root growth, and increased leaf area and shoot/root ratio. Shading had less reducing effects on shoot dry weight (Fig. 3.6 C). Our data generally agreed with Chu and Robertson (1974), who found that shading elongated height growth, reduced specific leaf weight and increased leaf area per unit plant weight. Plants may need comparable amounts of N for photosynthetic enzyme formation and cell division under shading as in full light. This may explain why total nitrogenase activity was only reduced by 30 % and specific nitrogenase remained unaffected under the heaviest shading, even though CO_2 exchange rate was significantly and proportionally decreased (50 to 75 %) by shading. These minimum effects of shading on specific nitrogenase activity agree well with results from legumes by Sprent (1973) and Eriksen and Whitney (1984).

It is important to realize how these structural changes may operate in a natural forest setting and how they may be useful in a management context. The pattern of structural adaptation under shading can make the plant vulnerable to environmental stress, especially water stress, due to high shoot/root ratio (Fig. 3.6 D) and fresh to dry weight ratio (Son and Smith, 1988; Tester *et al.*, 1986). Plants producing more shoot than root under heavy shade easily lose their large leaves during water stress. This sets up a negative feedback loop wherein; when water stress is eased, plants must struggle with less photosynthesis for recovery. One result is decreased root production. The shaded trees will gradually weaken and be unable to accumulate enough carbohydrate for regrowth after stress damage or during the following year. By controlling interplant density, the amount of shading on the N_2-fixing intercrop species can be adjusted to allow adequate growth and N_2-fixation while minimizing competition effects. Thus, balanced growth of red alder under moderate shading can be an option for management of conifer-alder mixed forests.

Tester *et al.*, (1985) explained decreased growth response of VAM plants to low irradiation as due to carbohydrate drain by the fungus. In experiment 2, our results (Table 3.6) showed both N and P accumulation in leaves, roots and nodules under shade which may be due to the limited supply of photosynthate to these organs or by the plants' strategy to harvest limited photons by forming more photon harvesting pigments to assimilate carbon. These accumulations also meant that P was not limiting under shade (Tester *et al.*, 1985) and that light has a direct effect on both symbionts by affecting the availability of photosynthate (Bethlenfalvay and Pacovsky, 1983). Carbohydrate drain by the fungus was likely not a major factor for the decreased growth response in Experiment 1 because mycorrhiza development was significantly decreased after shading for three weeks (Fig. 3.2 A). On the other hand, in Experiment 2, the high percentage of mycorrhiza formation and N and P accumulation under shading may

not support Björkman's carbohydrate theory for mycorrhiza formation. The relationship between carbohydrate supply and tissue nutrient content is still difficult to explain. Severe defoliation causes accumulation of N and P in soybean tissues and increase in photosynthetic efficiency (μmol CO_2/unit leaf area/hr), but little affect on VAM colonization (Bayne *et al.*, 1984).

In conclusion, *Frankia* was more critical for seedling growth and mycorrhiza development than a mycorrhizal fungus was for seedling growth and nodule formation. Nodulated mycorrhizal plants were consistently larger than nodulated nonmycorrhizal plants in growth parameters, but rarely significantly different. The role of *Alpova* mycorrhizae in influencing seedling growth is not clear, and further experimentation with *Alpova* and other alder fungi is needed to address mycorrhizal mediation of mineral uptake, particularly under P-limited conditions. Such experimentation is needed to shed further light on possible mycorrhizal effects on N_2-fixation. Although VAM have had large positive effects on N_2-fixation of legumes, no similar ectomycorrhizal effects were evident in our alder studies.

Shade can be an overwhelming environmental factor influencing growth of N_2-fixing plants by reducing photosynthesis. In our study, plants adapted their structure to maintain carbohydrate production by increasing leaf area and shoot/root ratio. Specific N_2-fixation rates also remained constant but nodule development and total nitrogenase activity decreased with shading. Even though plant photosynthate is a key influence on both microsymbionts, we found no evidence that the symbionts significantly increased photosynthetic rate per unit leaf area. For balanced red alder seedling growth at least PPFD of 250 μmol/m^2/s is suggested so that root growth is not much reduced. To improve intercropping forest management with shaded alder, we need to further understand how these plants adapt their morphology and biochemistry to reduced light

intensity and still maintain normal symbioses and growth. Genetic improvement of the shade tolerance of alder is another possibility for investigation.

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CONCLUSION

Frankia actinorrhizae are more important for mycorrhiza formation and growth of red alder seedlings than *Alpova diplophloeus* ectomycorrhizae are for nodule formation and seedling growth. Although *Alpova* mycorrhizae increased seedling growth 6 to 16 %, these values were relatively less than those from VAM-legume, or VAM-actinorrhiza plants of other studies. Water stress decreased both symbionts' development. *Alpova* ectomycorrhiza did not improve water relations of host plants. Heavy N fertilization inhibited N_2-fixation but increased mycorrhiza development. P fertilization increased N_2-fixation. Importance of P fertilization or mycorrhiza formation increased as plant N is accumulated. Seedlings adapted to certain low light intensities by increasing light harvesting structures and maintained balanced symbiosis development and plant growth. Light stress decreased nodule formation but not ectomycorrhiza development after ten weeks. Plant physiological and biochemical changes under water stress, different soil fertilities and light stress need to be studied for further understanding of mycorrhizal roles in red alder plant growth.

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APPENDIX

Table A.1 Delayed *Frankia* inoculation effect on 3-month-old *Alnus rubra* Bong. seedling growth and mycorrhiza development by contaminating fungi in a greenhouse.

Parameter	*Frankia* inoculation			
	F0	F3	F6	CO
Height (cm)	35.8 a	32.3 b	18.0 c	3.5 d
Diameter (mm)	4.6 a	4.4 a	3.2 b	1.9 c
Shoot dry weight (g)	1.62 a	1.28 b	0.51 c	0.09 d
Root dry weight (g)	0.57 a	0.43 b	0.18 c	0.09 c
Shoot/root ratio	3.3 a	3.4 a	3.5 a	1.1 b
Nodule dry weight (mg)	48 a	51 a	20 b	0 c
Nodule formation sites	18 a	42 b	68 c	0 d
TNA (μmol C_2H_2 reduced/plant/hr)	7.0 a	10.4 a	7.8 a	0 b
Mycorrhizae(M) (%)	95 a	88 a	78 ab	65 b
M. length/short root length ratio	0.96 a	0.83 a	0.49 b	0.32 c

F0, F3 and F6 mean that *Frankia* was inoculated when seedlings were 0, 3, and 6 weeks old, respectively.

CO: no inoculation.

TNA : total nitrogenase activity.

Values are the means of 12 to 24 samples.

Different letters show significant difference between treatments at $p \leq 0.05$.

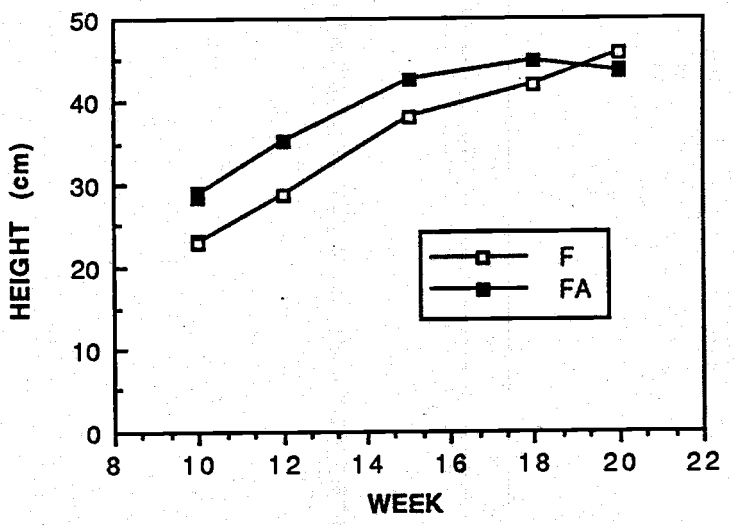

Fig. A.1 Height growth of *Alnus rubra* seedlings inoculated with *Frankia* and dead spores of *Alpova diplophloeus* (F), and *Frankia* and live spores of *Alpova* (FA). The values are means of 48 seedlings including fertilization treatments. Standard errors are less than 1.0 cm.