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HOST-SYMBIONT INTERACTIONS AMONG FRANKIA STRAINS AND ALNUS OPEN-POLLINATED FAMILIES

Iowa State University PH.D. 1980

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Host-symbiont interactions among *Frankia* strains and *Alnus* open-pollinated families

by

Charles Alvin Maynard

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
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INTRODUCTION: NITROGEN IN FORESTRY

Nitrogen is often the most limiting essential element in ecosystems (Black, 1968). Under present conditions in the earth's biosphere, the prevailing configuration for nitrogen is in the dimeric form, N≡N. In this form nitrogen is energetically stable but biologically unavailable to higher plants. Dinitrogen, however, is present in vast quantities, making up approximately 78 percent by volume of the atmosphere. The primary reason nitrogen is limiting is the small number of organisms capable of transforming this dinitrogen molecule into more easily utilized forms (MacNeil *et al.*, 1978).

The first major increase in the supply of mineralized nitrogen to agricultural crops came about through the industrial conversion of N_2 into NH_3 through the Haber process (Bridger *et al.*, 1979; Finneran and Czuppon, 1979; Sweeney, 1976). The three- to five-fold increase in agricultural yields per acre over the last century is due in large part to this fertilizer made from industrial nitrogen (Gutschick, 1978). Industrial nitrogen will undoubtedly continue to play a vital role in intensive agriculture (Pimentel, 1976).

In forestry the situation is more complex. Because many forest ecosystems are limited by the amount of available nitrogen in the soil, yield responses to the addition of nitrogen are often substantial. The addition of one unit of energy in the form of combined nitrogen may yield over 10 units of energy in the form of increased capture of solar radiation (Miller and Flight, 1979). Some authorities question the accuracy of this estimate (Beuter, 1979). Others indicate that even a 10-fold energy gain

is poor when compared with the yields available from other silvicultural options (Smith and Johnson, 1977). Even if biological and economic yields are attractive, political considerations may be a deciding factor. In a severe shortage, agricultural production would, quite justifiably, have a higher priority than forestry for either industrial nitrogen or the liquid fuels necessary to apply the nitrogen (Beuter, 1979).

In a world where price stability of all forms of fossil fuels appears to be a thing of the past and where national and international politics have a dominant role in both price and availability, the development of alternate strategies to the use of large quantities of energy-dependent industrial nitrogen is vital. In forestry, one alternative would be a return to extensive management, where rather low per acre yields are offset by the huge land areas harvested. However, in almost all regions of the United States competing uses are removing forest land from active production (Josephson and Hair, 1973; Vaux, 1973; Dawson and Pitcher, 1970). Therefore, this option seems impossible.

On the demand side of the equation, the picture is one of potentially explosive increases. Projected demand for all wood products is rising, with the largest increases expected in fiber and composite products (Fahey and Starostovic, 1979; Tenwolde and Stone, 1978). Most of these projections assumed fairly stable price relationships between wood products and substitutes. These relationships, however, are not stable. A smaller component of the cost of manufacturing wood building material is devoted to energy than in the manufacture of any other building material (Tenwolde and Stone, 1978). Therefore, a given change in the cost of energy will be reflected in a smaller increase in the cost of wood products, all other

things remaining equal. This shift in relative price is likely to increase the demand for wood construction materials.

Products utilizing fossil carbon directly in manufacture, plastics being the prime example, also will increase in price. Many items historically manufactured from wood or wood fiber are now being manufactured from oil-derived plastics--egg cartons, vinyl wallpaper, and plastic laminated paneling being examples. As price increases or shortages of oil become severe enough, economics may dictate a return to wood products.

If forestry begins utilizing large quantities of industrial nitrogen which in turn depends on large quantities of economically and politically expensive fossil fuel, another major industry will be added to the list already dependent on foreign energy supplies. Currently there is a major effort to substitute waste wood for fossil fuel in many wood processing plants (Fahey and Starostovic, 1979). It would be ironic to free the processing component of forestry from foreign energy dependence only to increase the foreign dependency of the raw material component.

There is another option. Biological nitrogen fixation has served plant ecosystems for aeons and continues to provide most of the nitrogen in forest ecosystems (Evans and Barber, 1977). Expanding biological nitrogen fixation rather than substituting industrial nitrogen has another advantage in addition to price and availability. Biologically fixed nitrogen is supplied continuously to the plant any time conditions are good for growth, rather than at long intervals as is the common practice with applied nitrogen.

There are two major systems of symbiotic biological nitrogen fixation, the legume-Rhizobium association and the nonlegume or actinorhizal

association. Herbaceous legumes can have a role in enhancing forest tree growth early in the development of a plantation or after thinning operations (Sprent and Silvester, 1973; Gadgil, 1971), but these plants will die out fairly soon after the tree crowns close (Rehfuess, 1979; Silvester et al., 1979). To provide a sustained input of nitrogen throughout a rotation, the nitrogen fixing species must maintain at least a codominant position in the forest canopy. The only major North American legume tree species known to fix nitrogen and adapted to forest conditions is black locust, Robinia pseudoacacia L. The actinorhizal species include many shrubs adapted to forest understories and one native tree form species, red alder, Alnus rubra Bong. In addition, the European tree, black alder, Alnus glutinosa (L.) Gaertn. (Figure 1) has been planted extensively and has become naturalized in some parts of the eastern United States (Schopmeyer, 1974). A. glutinosa has a much larger natural range than R. pseudoacacia, thus black alder probably has more genetic variability in its population. The range of A. glutinosa also extends to within 5^{o}N latitude of the Arctic Circle (Robison et al., 1979), approximately 20^{o} of latitude further north than the range of R. pseudoacacia (Roach, 1965). Therefore, some A. glutinosa selections would be expected to be superior in frost hardiness. The Department of Forestry, Iowa State University, has several ongoing projects dealing with A. glutinosa and an extensive provenance collection of A. glutinosa seed. For these reasons, A. glutinosa and its symbionts were chosen for this study.

Figure 1. A well-nodulated Alnus glutinosa tree growing on the edge of a small pond in The Netherlands

COMPONENT I: INITIAL STUDY OF HOST-SYMBIONT INTERACTION

Objectives

The primary objective of this component was to evaluate how much of an effect different available endophytes might have on growth rates of A. glutinosa so that an effective inoculum could be used in a provenance study of A. glutinosa being conducted by the Department of Forestry, Iowa State University (Robison et al., 1979). The secondary objective was to determine weaknesses in the endophyte evaluation procedures so that improvements could be made for use in further studies.

Background

Research has established patterns of response among the many genera and species of the Leguminosae family to different strains of Rhizobium (Wilson, 1939). This research has been summarized and presented in the form of cross-inoculation groups (Alexander, 1977). Not all researchers agree, however, with the groupings so other alternative plans have been proposed (Grahm, 1976). To a large degree, strains of Rhizobium do behave as Alexander (1977) indicated, inducing effective nodulation on members within a group and not among groups (Burton, 1964). Exceptions do arise often enough to make testing necessary for any new strain (Grahm, 1976).

Patterns of compatibility and incompatibility also have been summarized for actinorhizal plants (Becking, 1974), and the results for this system are even less clear. Actinorhizal nitrogen fixation is found in at least 160 species of woody trees and shrubs from 15 genera and seven families (Torrey, 1978; Bond, 1976). It is thus a more taxonomically diverse system than the single family legume-Rhizobium association.

Three levels of cross-infectivity have been studied in the actinorhizal system: a) among different families, b) among genera within a single family, and c) among species within a single genus (Miguel et al., 1978). A fourth level among different genotypes within a single species has been of considerable importance in selecting vigorous host-endophyte combinations in legume-Rhizobium symbiosis (Burton, 1964). This aspect has been ignored in actinorhizal symbiosis. This fourth level of cross-infectivity and effectivity among provenances within black alder will be the primary subject of this dissertation.

A review of the findings of the first three levels of cross-compatibility will demonstrate the wide range of results that may be expected. At all levels, Alnus has been the most widely studied genus. Therefore, references deal primarily with interaction between Alnus hosts and inocula from other sources or the performance of Alnus-derived endophytes on other host species.

Level A: Cross-inoculation studies among families

Betulaceae (Alnus) - Myricaceae Early tests between Alnus and Myrica indicated that their endophytes were sufficiently different that they would not cross-inoculate in either direction (Bond et al., 1954). Later it was reported that Myrica gale L. seedlings could be successfully inoculated with an Alnus endophyte, but the reverse was not possible. The authors indicated that positive results could have been caused by the endophyte of Myrica surviving as a surface contaminant on the Alnus nodules used to make the inoculum (Rodriguez-Barrueco and Bond, 1976). The A. glutinosa endophyte was reported to be compatible as a source of

inoculum for Myrica faya Ait. seedlings, but the combination was incompatible if M. faya inoculum was used on A. glutinosa seedlings (Miguel et al., 1978).

In another series of studies from the same group, 100 habitat soils, not supporting Myrica host plants, were used as inocula for both M. gale and A. glutinosa seedlings. The correlation between percentage nodulated plants for each species was 0.82, suggesting that the two plant families shared a common Frankia endophyte (Miguel et al., 1978).

In the most recent test, M. faya inoculum was used to initiate effective nodulation of A. glutinosa seedlings (Rodriguez-Barrueco and Miguel, 1979). In this study, two inocula derived from hydroponically grown M. gale plants were also included. One of the inocula came originally from field collected M. gale nodules, but the other was originally derived from soil collected in an A. glutinosa stand not containing any Myrica plants. There was a four-fold increase in dry weight of A. glutinosa plants treated with the inoculum originally derived from the A. glutinosa stand over plants treated with M. gale crushed nodule inoculum (Rodriguez-Barrueco and Miguel, 1979).

Two explanations are possible. The superior endophyte may actually have been a normal associate of A. glutinosa, indicating that normal combinations produce superior plant growth. The second possibility is that major differences exist within Frankia endophytes derived from a single species of host plant.

Betulaceae (Alnus) - Elaeagnaceae A. glutinosa will cross-inoculate with Elaeagnus angustifolia L. as host or endophyte source but is

incompatible with Hippophaë rhamnoides L. (Miguel et al., 1978; Rodriguez-Barrueco and Miguel, 1979).

Betulaceae (Alnus) - Coriariaceae (Coriaria) Alnus viridis (Chaix) D.C. seedlings are incompatible with Coriaria sarmentosa Forst. f. endophyte (Benecke, 1969). A. glutinosa was found to be incompatible as host or inoculum source with Coriaria myrtifolia L. (Miguel et al., 1978), but when the same combination was repeated the next year, the combination was compatible (Rodriguez-Barrueco and Miguel, 1979).

Level B: Cross-inoculation studies among genera within a family

A second level of compatibility is among genera within the same family. At this level only the Elaeagnaceae family has been thoroughly studied, Alnus being the only known member of the Betulaceae family to form actinorhizal nodules.

Hippophaë, Elaeagnus, and Shepherdia have generally been shown to easily cross-inoculate in all combinations (Gardner and Bond, 1957; Gardner, 1958; Moore, 1964). An exception was noted in Japan where Shepherdia canadensis Nutt.[1] and H. rhamnoides remained nodule free in habitat soil supporting prolific nodulation of Elaeagnus (Uemura, 1971; Uemura and Sato, 1975). Another exception was noted in the set of tests showing Elaeagnus angustifolia L. to be compatible with Hippophaë inocula but incompatible with Elaeagnus pungens Thunberg inocula (Gardner, 1958).

---

[1]This species was referred to in the text as S. americanus but in an accompanying table as S. canadensis. I interpreted the author to mean S. canadensis (Uemura and Sato, 1975).

Level C: Cross-inoculation studies among species within a genus

Casuarina The earliest study of cross-inoculation among species within a single genus was reported in 1932. Parker used Casuarina equisetafolia L. inoculum on four other nonnodulated and chlorotic species of Casuarina growing in the field. Three of the eight plants improved immediately, the rest gradually. This study indicated that cross-inoculation was possible within the Casuarina genus but also indicated that some degree of incompatibility also might exist (Parker, 1932). However, in a field study, numerous microsite differences could have caused unequal distribution of viable endophyte to the individual plants, causing what appeared to be incompatibility responses.

Coriaria Coriaria japonica Sieb and Zucc. was found to be incompatible with C. myrtifolia endophyte when tested with habitat soil from C. myrtifolia stands (Bond, 1962).

Myrica Partial incompatibility exists in the Myrica genus. M. gale inoculum will cause sparse nodulation on Myrica cerifera L. and Myrica cordifolia L. after a delay of four to six weeks (Gardner and Bond, 1966; Bond, 1967; Mackintosh and Bond, 1970). M. gale inoculum will cause rapid nodulation but little nitrogen fixation on M. faya seedlings (Mian et al., 1976).

Alnus Alnus has been the most extensively examined genus. Generally cross-inoculations with species from the same continent or major region have proven effective (Becking, 1966). Cross-inoculation tests among species from more geographically separated regions may take longer to form nodules (Rodriguez-Barrueco and Bond, 1968), may form few, ineffective

nodules (Becking, 1966), or may occasionally form large numbers of minute, ineffective nodules (Rodriguez-Barrueco, 1966).

Discussion

A cross-inoculation attempt may fail because the Frankia strain and host plant genotype are truly incompatible. A cross-inoculation attempt may also fail if the endophyte cells are unrecognizable to the new host because of surface changes caused by a growth cycle in the nodules of another host species. Positive results also are not irrefutable. With crushed nodule inoculum, especially if collected from wild stands, the possibility exists that a Frankia endophyte of one species may exist saprophytically on the surface of nodules of some other actinorhizal host. When these nodules are used as crushed nodule inoculum, the previously saprophytic Frankia strain may nodulate the new hosts species (Rodriguez-Barrueco and Bond, 1976).

As pure cultures of Frankia become available, these cross-inoculation groups are being retested. On the basis of pure culture tests, isolate CpI1 from Comptonia peregrina (L.) Coult. has nodulated numerous Alnus species (Lalonde, 1979; Lalonde and Calvert, 1979). Isolate ArI3 from A. rubra nodulated all Alnus species tested as well as M. gale and C. peregrina. The M. gale and C. peregrina cross-inoculations would not be expected to nodulate on the basis of previous reports (Becking, 1974). There seemed to be a barrier between Alnus and Ceanothus as the ArI3 isolate failed to nodulate Ceanothus americanus L. (Berry and Torrey, 1979).

One major criticism of all previously reported studies is that each cross-inoculation was attempted on a very narrow sampling of possible

genotypes within either the host species or the endophyte (van Dijk, 1979). A. glutinosa, for example, has a geographic range from the northern tip of Africa to near the Arctic Circle and from the west coast of Great Britain, well into Siberia (Robison et al., 1979). Over this vast area black alder occurs as many small semi-isolated populations along river banks and around lakes (Schopmeyer, 1974). These semi-isolated populations often form many distinct ecotypes (Wright, 1976).

The Frankia microorganism is under similar pressures. It was originally thought to be an absolute obligate symbiont (Becking, 1974). Later a free living stage of Frankia was observed in a zone of mucilage on the surface of roots and root hairs of A. glutinosa seedlings (Becking, 1976). No naturally occurring free living stage completely away from a host plant has been reported. Thus, Frankia also might be expected to develop a myriad of distinct strains, each adapted only to a narrow range of hosts, temperature ranges, or other environmental factors (van Dijk, 1979).

Specialization between hosts and associate organisms can reach surprising proportions. Some semi-sessile insect pests inhabiting long-lived forest tree species have evolved to the point where a strain of pest is able to live only on a single host genotype (Edmunds and Alstad, 1978). One reason such specialization within a species has never been reported for the actinorhizal system may be that researchers have never looked for it. In almost all cases where cross-inoculation trials have been conducted, a single, usually undefined, seed source has been used to represent each host species and a single crushed nodule inoculum source used to represent each endophyte source.

In the legume-Rhizobium system, dozens of single-cell derived strains of Rhizobium and inbred lines of host plant are available from stock culture collections and commercial seed dealers, respectively. A natural step was to test a number of strains of Rhizobium on a number of commercially useful varieties of legume hosts (Erdman and Burton, 1938; Burton, 1964). Researchers working with actinorhizal symbionts had to gather seed from wild stands and laboriously dig root nodules or depend on cooperators to do the same for them. Faced with these problems, most authors have assumed for the sake of simplicity that, within a plant species, all plants were infected by one homogeneous population of Frankia or at least that differences were less important than those occurring between species, genera, and families. They also tacitly assumed that little variation existed in the host plant species in its ability to form an effective association with a given source of Frankia. For the purpose of sketching in broad outlines of compatibility, these assumptions were reasonable, but if the objective is to improve the symbiotic efficiency of the association, variation within host species and among endophytes collected from the same species must be examined as well (Dawson and Gordon, 1979).

The first hint of variability within the Frankia endophytes of a single host species was reported for the A. glutinosa endophyte (Schaede, 1933, as cited in Akkermans, 1978). Schaede noted that there appeared to be two distinct types of Frankia found in alder root nodules. One produced structures that have been variously called spores, granula, or bacteroids while the other type was free of such structures. The development of this spore stage was studied by light and electron microscopy to determine where and how the spores form (van Dijk and Merkus, 1976). Van Dijk collected

seed and nodules separately from A. glutinosa trees bearing spore-forming nodules and from trees bearing nonspore-forming nodules. These two lots of seed and two nodule types were tested in all four possible combinations to determine if spore formation was host or endophyte controlled. He found that the endophyte was responsible (van Dijk, 1978).

No differences between the two strains in the acetylene reduction assay were found, but the coefficient of variation for the two strains was 26 percent for the spore (+) nodulated plants and 49 percent for the spore (-) nodulated plants. Under these conditions, a very large difference would have been required to achieve statistical significance (van Dijk, 1978).

The second work reporting variability between strains from a single host species was a comparison between Frankia strain EuI1 isolated from Elaeagnus umbellata Thunb. and a crushed nodule inoculum from the same host. The isolate induced normal nodulation but was incapable of forming vesicles or fixing atmospheric nitrogen (Baker et al., 1979). The authors did not speculate whether the ineffective properties of the isolate were induced in culture or had existed in the wild population.

The objective of the present work was to expand this area of knowledge at the fourth level by testing for differences in symbiotic efficiency among different provenances of host plant and sources of endophyte derived primarily from A. glutinosa.

Experimental Design

This study was conducted as a 4 x 6 complete factorial design in randomized blocks with four seed sources, four inoculum types, two controls,

and one replication for each of four blocks. Three of the seed sources were European black alder, A. glutinosa, from Wales, The Netherlands, and Poland. The fourth seed source was red alder, A. rubra, from Idaho (Table I.1).

Table I.1. Alnus spp. host plants used to study actinorhizal cross-inoculations

Seed source	Host species	Origin
AR-0110	A. rubra	Idaho, U.S.A.
AG-1724	A. glutinosa	Wales, U.K.
AG-6131	A. glutinosa	The Netherlands
AG-5413	A. glutinosa	Poland

The inoculum types were a spore (+) and a spore (-) strain collected in The Netherlands by Dr. Maurice Lalonde, an A. rubra derived endophyte from Oregon, provided by Dr. John Gordon, and a central Iowa endophyte of unknown natural association (Table I.2). Two controls were included, a no-nitrogen, no-inoculum treatment to check for uncontrolled nodulation and a combined-nitrogen, no-inoculation treatment.

Procedure

Ninety-six Leonard jars (Leonard, 1943) filled with silica sand were top-watered with 250 ml of van der Crone's nitrogen-free nutrient solution (Crone's solution) (Hewitt, 1966). Each bottom jar was 3/4 filled with distilled water, the upper vessel covered with aluminum foil, and the entire assembly autoclaved for 90 minutes.

Table I.2. *Frankia* endophytes used to study actinorhizal cross-inoculations

Inocula	Original host species	Geographical source	Cooperator
Spore (+)	*A. glutinosa*	The Netherlands	Dr. Maurice Lalonde
Spore (-)	*A. glutinosa*	The Netherlands	Dr. Maurice Lalonde
Red alder	*A. rubra*	Oregon, U.S.A.	Dr. John Gordon
Local Iowa	Unknown	Iowa, U.S.A.	Collected locally

Seedlings were grown in metal flats. After filling the flats with sand and watering with Crone's solution, the flats were autoclaved for 60 minutes at 115°C. After cooling, each flat was planted with several hundred surface sterilized seeds (Neal *et al.*, 1967), and a 0.75 cm mulch of autoclaved sand was applied. Flats were transferred to a growth chamber. Seedlings were inoculated and transplanted to the Leonard jars when they reached the two-leaf stage and began showing signs of chlorosis.

Crushed nodule inocula were prepared in phosphate buffer (Lalonde and Knowles, 1975). Three or four seedlings were dipped in the appropriate inoculum and transplanted to a Leonard jar. When all jars assigned to a single inoculum type had been filled, one ml of the inoculum was applied to the sand around each seedling. All equipment was then sterilized in household bleach (5.25% NaOCl) and the lab countertop similarly washed down. The no-inoculum, no-nitrogen treatment and the no-inoculum, combined-nitrogen treatments were transplanted last to serve as a check on the decontamination technique.

All Leonard jars were transferred to a growth chamber and covered with small plastic bags for the first two weeks after transplanting to maintain high relative humidity. After four and one-half weeks, jars were thinned to one plant by pulling up extra seedlings. The plants removed were checked for nodulation, chlorosis, height, and survival. After an additional three months, three blocks were harvested. Block four was reserved to use as a source of inoculum for further studies.

Block one was prepared for acetylene reduction assay by removing the lower jar and enclosing the upper sand-containing jar and the whole plant in a plastic bag. The air was evacuated from the bag, refilled with 200 ml of air, and injected with 20 ml acetylene (C_2H_2). This was incubated for approximately two hours before testing for ethylene (C_2H_4) production. The total time of incubation was recorded to use as a covariate in later analysis. A Varian model 2700 gas chromatograph with a Poropack R. 100/120 mesh column with 100°C detector temperature and 60°C column temperature was used to measure ethylene.

A simpler sampling method was tried on a subsample of plants from block one. After the initial assay described above, each plant was cut at the soil level and the sand removed. The roots with attached nodules were then inserted into 100 ml test tubes. The test tubes were sealed with serum stoppers, 10 ml of air was extracted, and 10 ml of acetylene injected. Incubation and assay procedures were the same. There was a 0.97 correlation in C_2H_4 assay between whole plants in the jar and root systems incubated alone. Therefore, the simpler severed-root procedure was adopted for blocks two and three.

Results

Four and one-half week thinning

Highly significant differences between treatments were evident at the 4.5 week thinning (Table I.3). None of the plants removed from control treatments had nodulated, indicating decontamination procedures were effective. These control plants also remained nodule-free throughout the remainder of the study. The spore (+) strain produced the most nodules, followed by the spore (-) and the red alder inoculum. The central Iowa strain was notably poor; approximately one plant in 10 nodulated (Table I.4).

Table I.3. Analysis of variance for the number of nodules per Alnus spp. seedling 4.5 weeks after inoculation (R^2 = 0.92)

Sources of variation	Degrees of freedom	Sums of squares	F	Prob. > F[a]
Host	3	138.6	13.59	0.0001
Inoculum (Inoc)	5	1574.0	92.59	0.0001
Host x Inoc	15	249.0	4.87	0.0001
Error	53	180.2		
Corrected total	76	2141.8		

[a]The probability of an F value of this size occurring by chance.

Final harvest

At the end of four and one-half months (three months for height measurements), treatment differences were more obvious than at the initial

Table I.4. Mean number of nodules per Alnus spp. seedling at 4.5 weeks, as affected by Frankia inocula

Inocula	N	Number of nodules
Spore (+)	13	12.33 [a]
Spore (-)	10	6.25
Red alder	14	2.00
Local Iowa	12	0.00
Control	14	0.00
Nitrogen supplement	14	0.00

[a]Values connected by the same bracket are not significantly different at the 0.05 level, Duncan's multiple range test (Steel and Torrie, 1960).

thinning (Table I.5). Many of the control plants were dead, as were most of the plants inoculated with the central Iowa endophyte. Final results were based on 27 survivors: 10 spore (+) inoculum, eight spore (-) inoculum, and nine red alder inoculum treated plants. Host plants and inocula both had a highly significant effect (beyond the 0.01 level) on final plant height (Table I.5). The plants treated with spore (-) inoculum averaged 20.5 cm, the plants treated with spore (+) inoculum averaged 16.3 cm, and the plants treated with red alder endophyte averaged 7.4 cm in height (Table I.8).

The A. rubra plants, from a seed source collected in Idaho, grew to an average height of 7.5 cm when treated with spore (-) inoculum and 3.5 cm when treated with spore (+) inoculum but only 2.0 cm when treated with the red alder inoculum from Oregon (Table I.6).

Table I.5. Analysis of variance for mean height of Alnus spp. seedlings three months after transplanting (R^2 = 0.85)

Sources of variation	Degrees of freedom	Sums of squares	F	Prob. > F[a]
Host	3	985.5	21.1	0.0001
Inoc	5	3328.0	42.7	0.0001
Host x Inoc	15	568.5	2.4	0.0085
Error	56	873.6		
Corrected total	79	5755.6		

[a]The probability of an F value of this size occurring by chance.

Table I.6. Mean height of Alnus spp. seedlings three months after transplanting as affected by seed source and inoculum

Host	Seed source	Inoculum				
		Spore (+)	Spore (-)	Red alder	Local Iowa	Control
A. rubra	AR-0110	3.5	7.5	2.0	1.5	1.7
A. glutinosa	AG-1724	22.3	24.5	11.5	2.5	3.7
A. glutinosa	AG-6131	16.5	23.7	9.3	6.0	2.5
A. glutinosa	AG-5413	16.5	20.6	3.5	2.3	2.0

For the acetylene reduction assay, the coefficient of variation of acetylene reduced per gram of nodules was 94 percent of the mean, even larger than that cited earlier (van Dijk, 1978). Because of this high variation, differences among the three endophytes tested in fixation per gram nodule were only significant at the 0.05 level (Table I.7). The difference between the two A. glutinosa endophytes was not sufficiently large to be significant at the 0.05 level (Table I.8) but was in the same direction and of approximately the same magnitude as cited earlier (van Dijk, 1978).

Between the three and one-half week thinning and the final harvest, the number of nodules on the spore (-) inoculum treated plants increased slightly over three-fold, the number of nodules on spore (+) inoculum treated plants increased almost 11-fold, and the number of nodules on red alder inoculum treated plants increased 24-fold (Table I.9).

Discussion

The poor nodulation and ineffective nitrogen fixation of the local Frankia source was in agreement with reports for other unusual cross-inoculations (Becking, 1966).

Another explanation for the poor nodulation may have been low viability of the endophyte in the nodules. Both the local Iowa inoculum and the A. rubra inoculum were derived from nodules collected from the field. The spore (+) and spore (-) inocula came from hydroponically grown plants. Although all nodules appeared healthy and received the same processing treatment in making up the inocula, the field collected nodules may have lost viability between the time of collection and use or may have

Table 1.7. Analysis of variance for acetylene reduction per gram of nodules three months after transplanting (C.V. 94%; $R^2 = 0.58$)

Sources of variation	Degrees of freedom	Sums of squares ($X10^{-4}$)	F	Prob. > F[a]
Block	2	49	3.23	0.0702
Host	3	3	0.12	0.9451
Inoc	2	60	3.93	0.0441
Host x Inoc	6	35	0.07	0.6143
Error	14	107		
Corrected total	27	254		

[a]The probability of an F value of this size occurring by chance.

Table 1.8. Relative nodular efficiency and plant height of *Alnus* spp. seedlings as affected by *Frankia* inoculum type

Inocula	N	Relative efficiency per g nodules (%)	N	Plant height (cm)
Spore (-)	8	100 [a]	12	20.5
Spore (+)	11	66	14	16.3
Red alder	9	25	13	7.4

[a]Values connected by the same bracket are not significantly different at the 0.05 level, Duncan's multiple range test (Steel and Torrie, 1960).

Table I.9. Changes in average nodule number per Alnus spp. seedling between the 4.5 week thinning and the final plant harvest, as affected by Frankia inoculum type

Inocula	Average nodule number per seedling 4.5 weeks	4 months	Increase over time
Spore (-)	6	19	3.2 X
Spore (+)	12	130	10.8 X
Red alder	2	48	24.0 X

been of lower viability when collected. More collections should be tested from this stand. The delayed nodulation and moderately effective nitrogen fixation for plants treated with the A. rubra endophyte was also in agreement with earlier reports (Rodriguez-Barrueco and Bond, 1968). But, the better performance of the A. rubra plants with both strains of A. glutinosa than with its own endophyte was contrary to expectations. The distinct differences in height growth of plants treated with the two types of A. glutinosa inoculum was contrary to what has been reported (Akkermans, 1978).

The nodulation rates of the A. glutinosa inocula tested and the change in nodule number over time made interpretation difficult. Height measurements closely parallel the relative fixation per gram nodule results. However, it was possible that the large numbers of nodules induced by the spore (+) inoculum were overtaxing the ability of the host plant to supply photosynthate. The study was conducted in a growth chamber supplied with approximately 1000 footcandles of illumination. Under these conditions, photosynthate may have been more limiting to plant growth than the ability

to fix nitrogen. Future studies will be conducted under higher light intensities.

Conclusions

1) The local Iowa Frankia endophyte appears to be a poor symbiont in association with A. glutinosa or A. rubra seedlings.
2) The significant difference in plant growth and the parallel, but not significant difference (0.05 level), in relative efficiency of acetylene reduction indicates that there may be considerable variation in efficiency among different Frankia symbionts that naturally associate with A. glutinosa.
3) A method of controlling initial nodule number must be developed.

COMPONENT II: INOCULUM-TYPE STUDY

Objective

The objective of this study was to detect differences in patterns of nodulation between crushed nodule and pure culture inocula using two strains of endophytes, two seed sources of A. rubra, and two seed sources of A. glutinosa host plants.

Background

Until the isolation of Frankia strain CpI1, all research on actinorhizal nitrogen fixation had to depend on crushed nodule inocula (Callaham et al., 1978). Today, with more than a dozen strains of Frankia available in pure culture (Baker and Torrey, 1979), the situation has changed.

Such cultures require less space and expense than the pot cultures required to maintain Frankia symbiotically on host plants. A Frankia endophyte can be multiplied rapidly in culture, and inoculum concentration can be controlled by measuring the optical density of harvested spores and mycelium in sterile buffer. Commercial production of such Frankia inocula has been proposed (Lalonde and Calvert, 1979).

Commercial Rhizobium inoculants have been available for many years. The normal procedure for selecting a strain of Rhizobium to be included in an inoculum is to isolate a large number of strains from healthy nodules growing on plants of unusual vigor, grow them in pure culture, and test them extensively with the many varieties of legume they will be used to inoculate (Burton, 1964; Date, 1976; Bergersen et al., 1971). However, Frankia isolation technology has yet to reach the stage where all endophyte

selections can be cultured at will. For this reason, the evaluation of some field selections of Frankia still must be based on inoculation with crushed nodule preparations.

If pure culture inocula are used for those strains already isolated and crushed nodule preparations used for new populations to be tested, the comparison of most interest, that between the genetic potential of the pure culture strains and the genetic potential of the wild populations, is confounded statistically with an unintended comparison between inoculum types. If the pattern of nodulation of these two types of inoculum is identical or nearly so, differences due to this factor can be ignored. If, however, large differences exist, some method must be adopted to adjust for this effect. This study was conducted to determine if substantial differences do exist because of the type of inoculum used to initiate nodules.

Methodology

Two pure culture strains of Frankia were available when this study was initiated: 1) an A. rubra strain isolated by Allison Berry at Harvard, isolate ArI3 and 2) an A. crispa strain isolated by Maurice Lalonde at Kettering Research Laboratory, isolate AcN1. Both strains were tested for infectivity by inoculating A. glutinosa seedlings of seed source AG-4X11 growing in Growth Pouches® (pouches) (Scientific Products, McGraw Park, Illinois). The strains also were subcultured on a medium recommended by Dr. John Torrey (Baker and Torrey, 1979). After both sources of the endophyte had developed sufficiently, inocula were harvested. The experimental design consisted of a 2 x 2 x 2 x 2 factorial with two strains, two cultural treatments, and two seed sources from each of the two Alnus

species (Table II.1). The study was replicated twice. Eight uninoculated control pouches also were included as a check for contamination.

Table II.1. Alnus host plant species and seed sources used to test nodulation of pure culture and crushed nodule inocula

Host species	Seed source and description	
A. rubra	AR-3410	Lincoln City, Oregon, U.S.A.
A. rubra	AR-Clat#4	Seedlot from Dr. Torrey
A. glutinosa	AG-4X11	German seed orchard selection
A. glutinosa	AG-6523	Nance, France, wild stand

Pure culture inocula were prepared by removing visible clumps of mycelium and then crushing and resuspending the hyphae in sterile buffer. Each inoculum was centrifuged and resuspended in buffer three times to remove all medium and adjusted to an optical density of 0.03 at 620 nm (Lalonde, 1979). Each crushed nodule inoculum was prepared by removing 85 mg of nodules from the pouch plants previously inoculated to test for infectivity. Nodules were surface sterilized for four minutes in commercial bleach, rinsed three times in sterile 0.06 percent saline solution, and crushed in sterile tissue grinders with cold phosphate buffer plus 10 g/l polyvinylpyrrolidone (PVP) (Loomis and Battaile, 1966). Cell debris was allowed to settle 20 minutes, then the supernatant was transferred to sterile tubes and centrifuged at medium speed on a table-top centrifuge for 10 minutes. The supernatant was removed and the pellet resuspended in chilled buffer plus PVP and centrifuged again for 10 minutes. The

procedure was repeated until the supernatant was clear. Then the pellet was resuspended in phosphate buffer and stored in an ice bath.

Ten pouches, prepared as outlined in Component III, of each seed source with 11-week-old seedlings were numbered, labeled, randomly assigned a treatment combination, and inoculated with 1.0 ml of Frankia endophyte suspension. Pouches were placed in rack-crisper assemblies described in Component III and transferred to a growth chamber.

The factors of interest in this study were time of nodulation, total number of nodules produced, and the survival of plants. The variables chosen to estimate these factors were the number of new nodules produced at each observation date, the total number of nodules produced over the entire period, and the number of live seedlings per pouch at the end of the experiment.

Results

Nodulation varied with each of the isolates of Frankia, the cultural treatments, and the host species. None of the interactions were significant at the 0.05 level, but the three-way interaction approached this significance level (Table II.2).

The two host species responded differently. The A. rubra seedlings nodulated normally with the pure culture inocula (Table II.3) but failed to nodulate when inoculated with the crushed nodule inoculum of either strain (Table II.4). The A. glutinosa seedlings nodulated normally with the AcN1 derived crushed nodule inoculum (Table II.5) and at a reduced rate with the ArI3 derived crushed nodule inoculum. For both seed sources of A. glutinosa seedlings, survival was greater with the two crushed nodule

Table II.2. Analysis of variance for the total number of nodules per pouch (R^2 = 0.77)

Sources of variation	Degrees of freedom	Sums of squares	F	Prob. > F[a]
Inoc	2	897	5.65	0.011
Treatment	1	983	11.64	0.003
Host	3	1445	6.28	0.004
Inoc x Treatment	1	237	3.08	0.094
Inoc x Host	6	683	1.49	0.232
Treatment x Host	3	283	1.23	0.325
Inoc x Treat x Host	3	659	2.86	0.062
Error	20	1534		
Corrected total	39	6694		

[a]The probability of an F value of this size occurring by chance.

Table II.3. Cumulative number of nodules produced over time by Alnus spp. seedlings for pure culture Frankia inocula

Host	Days after inoculation		
	15	83	106
AR-3410	4.0	6.5	8.0
AR-Clat#4	4.3	7.5	7.8
AG-4X11	7.3	15.0	18.8
AG-6523	13.3	30.5	30.8

Table II.4. Cumulative number of nodules produced over time by Alnus spp. seedlings for crushed nodule Frankia inocula

Host	Days after inoculation		
	15	83	106
AR-3410	0.0	0.0	0.0
AR-Clat#4	0.0	0.0	0.0
AG-4X11	11.0	13.3	18.5
AG-6523	7.0	10.0	13.3

Table II.5. Average number of nodules produced per pouch for each seed source

Host	Inocula			
	Pure culture		Crushed nodule	
	Alnus crispa	Alnus rubra	Alnus crispa	Alnus rubra
AR-3410	11	5	0	0
AR-Clat#4	6	10	0	0
AG-4X11	25	14	21	6
AG-6523	19	44	19	2

inocula than with the two pure culture inocula (Table II.6). The differences, however, were not significant at the 0.05 level.

Patterns of nodulation over time show different responses between the two A. glutinosa seed sources. Seed source AG-6523 nodulated more quickly with the two pure culture inocula and developed over twice as many nodules compared with the crushed nodule inocula. Seed source AG-4X11 had slightly

Table II.6. Average number of surviving plants per pouch for each seed source

Host	Inocula			
	Pure culture		Crushed nodule	
	Alnus crispa	Alnus rubra	Alnus crispa	Alnus rubra
AR-3410	2.5	0.5	0.0	0.0
AR-Clat#4	0.0	2.0	0.0	0.0
AG-4X11	6.0	1.5	8.0	1.5
AG-6523	3.5	--[a]	5.5	1.0

[a]Plants were killed by moisture stress not lack of nodulation; therefore, a missing value is more appropriate than 0 survivors.

higher initial nodulation with crushed nodule inocula and ended with nearly identical total numbers of nodules from both crushed nodule and pure culture inocula (Figure II.1).

Discussion

The failure of the A. rubra seedlings to nodulate with the ArI3 derived crushed nodule endophyte was surprising. At least two explanations are possible. When a strain of Frankia is growing symbiotically within a host's nodules, it is encapsulated by host-derived pectic materials (Lalonde et al., 1975; Lalonde and Devoe, 1976; Lalonde and Knowles, 1975). This layer of A. glutinosa derived pectic material may have blocked the A. rubra recognition system. There is also a possibility that selection took place and that after one cycle of growth on A. glutinosa seedlings, a new strain had developed with increased symbiotic affinity for A. glutinosa

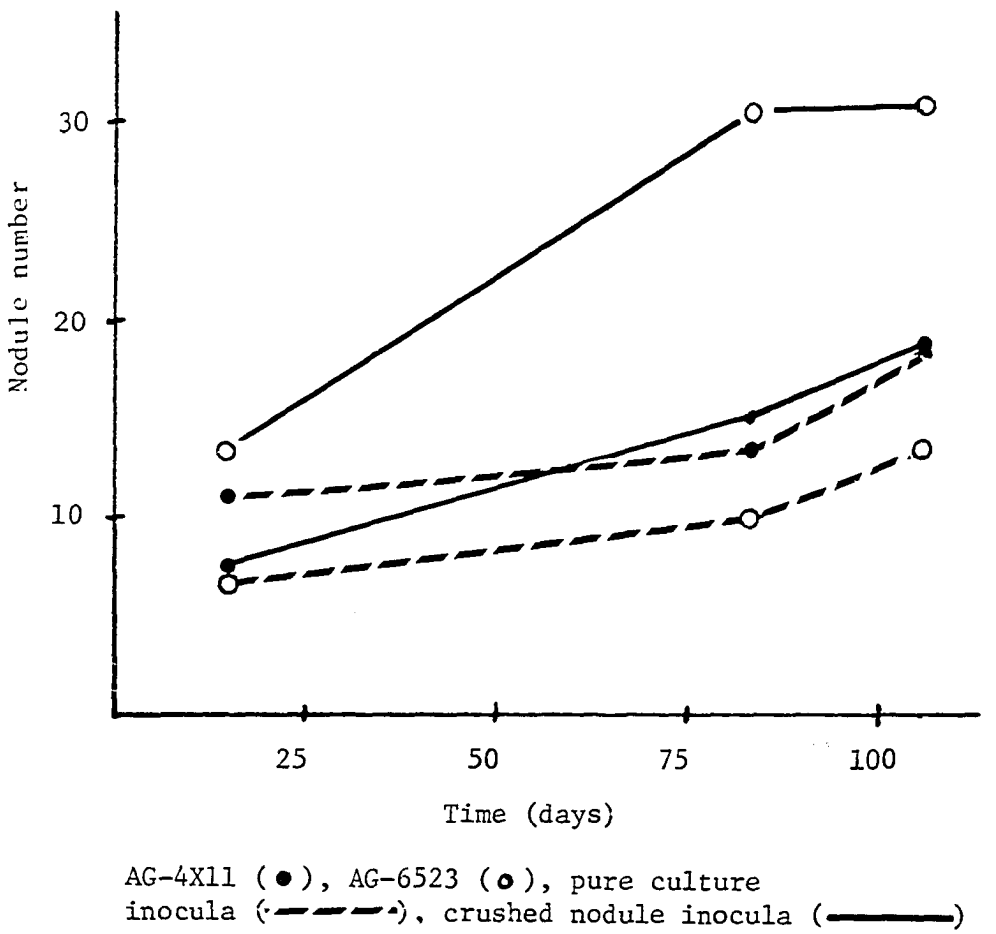

Figure II.1. Nodulation over time for Alnus glutinosa seed sources

and decreased affinity for the A. rubra host. Such selection for nodulating ability on new hosts has been demonstrated for legumes (Wilson, 1939).

The slower nodulation of seed source AG-6523 and the complete absence of nodulation on both seed sources of A. rubra with crushed nodules from AG-4X11 host plants indicated that much of the incompatibility reported in previous cross-inoculation trials may have been due to rejection of previous host surface contaminants. If Frankia strains are grown in pure culture before cross-inoculation studies are conducted, many barriers between groups may fall. However, a note of caution should be raised. Nodulation is only one criterion of successful symbioses. Of greater importance is the long-term efficiency of the interaction between the two organisms. If too much reliance is placed upon initial nodulation or total number of nodules, or even short-term plant growth parameters, potentially superior symbiont strains may be ignored. The relationship between initial nodulation and longer term plant growth responses are examined in Component III.

Conclusions

1) Significant differences exist in nodulation associated with the type of inoculum used.
2) A pure culture inoculum may induce nodulation over a broader host range than the same Frankia strain derived from crushed nodules.
3) Pure culture inocula should be used for unusual host-endophyte cross-inoculation tests so that previous-host pectic materials do not interfere with nodulation.

4) In a study utilizing both pure culture inocula and crushed nodule inocula, there may be some bias in favor of the crushed nodule inocula if the nodules are derived from the same species to be inoculated.

COMPONENT III: EXPANDED HOST-SYMBIONT INTERACTION STUDY

Objectives

The objectives of this study were the following:

1) To estimate the amount of variability in the rate of plant growth associated with endophytes collected from a single host species, A. glutinosa, over a wide geographic range;
2) To determine the types of interactions occurring between different endophyte strains and host seed sources; and
3) To determine if sufficient variability exists to warrant further collection of endophyte germplasm from the native range of A. glutinosa.

Background

Between 1977 when the initial study reported in Component I was conducted and the initiation of the second study in 1979, the number of available endophytes substantially increased. As discussed in Component II, pure cultures of Frankia isolated from A. rubra and A. crispa and later from C. peregrina were received. During the summer of 1978, I studied tree improvement in The Netherlands under Ir. Hans M. Heybroek. While in Europe, I collected root nodules of A. glutinosa. Viable endophytes were recovered from the nodules collected near Gohrenberg and Bad Soden, Germany, and from Amance, France. The spore type of each selection was determined by examining stained sections under a light microscope (van Dijk, 1978) (Table III.1).

On the basis of Component II, experiments with both pure cultures and crushed nodules used for some endophytes should be avoided, but the choice

Table III.1. *Frankia* inocula used in symbiont evaluations

Inoculum code no.	Other designations	Spore type	Inoculum type	Original host spp.	Geographic source	Cooperators
1	control	N.A.[a]	sterile buffer	N.A.	N.A.	N.A.
2	control	N.A.	sterile buffer	N.A.	N.A.	N.A.
3	AcN1	(-)	pure culture	*Alnus crispa*	United States	M. Lalonde, Kettering Research Laboratory
4	ArI3	(-)	pure culture	*A. rubra*	United States	A. Berry, Harvard Univ.
5	CpI1	(-)	pure culture	*Comptonia peregrina*	United States	J. Torrey, Harvard Univ.
6	#21[b]	(-)	crushed nodules	*A. glutinosa*	Amance, France	M. Aubert, Station D'Amelioration des Arbres
7	#37[b]	(-)	crushed nodules	*A. glutinosa*	Bad Soden, Germany	H. Weisgerber, Institute fuer Forstpflanzenzuchtung
8	#33[b]	(+)	crushed nodules	*A. glutinosa*	Gohrenberg, Germany	"
9	N.L.[c](+)	(+)	crushed nodules	*A. glutinosa*	The Netherlands	M. Lalonde, Kettering Research Laboratory
10	N.L.(-)	(-)	crushed nodules	*A. glutinosa*	The Netherlands	"

[a]Not applicable.

[b]The original code number from collection records.

[c]The Netherlands.

of host plant species to produce nodules was also shown to influence the results. A cycle on plants from the original host species of each isolate, AcN1 on A. crispa, ArI3 on A. rubra, and CpI1 on C. peregrina, would have eliminated the possibility of selection for A. glutinosa host preference. However, it would also have left the endophyte cells coated with foreign-host pectic material which could have blocked the A. glutinosa recognition system. If the isolates had gone through a cycle on A. glutinosa host plants, selection might have changed the actual strain being evaluated. Because no clear choice existed that would not bias the results in some way, and because the comparisons among endophytes naturally associating with A. glutinosa were of primary interest, all A. glutinosa endophytes were derived from crushed nodule inocula, and the three isolates were derived from pure cultures.

Plant culture conditions used in the initial study were reexamined and many changes were made. In the initial host-symbiont study, the Leonard jar system was satisfactory for growing plants (Figure III.1). The control plants remained free of nodules, jars were convenient to handle, and watering problems were minimized. However, some problems did occur. Evaporation from the surface of the silica sand caused a crust to form, damaging some of the trees. The clear glass sides of the Leonard jars allowed sufficient light to provide ideal conditions for growth of blue-green algae. The greatest problem was the impossibility of insuring equally well-nodulated plants from each endophyte source.

Many systems have been used to study biological nitrogen fixation. Plants have been inoculated while growing under field conditions (Mowry, 1933), in containers with habitat soil as both inoculant and support medium

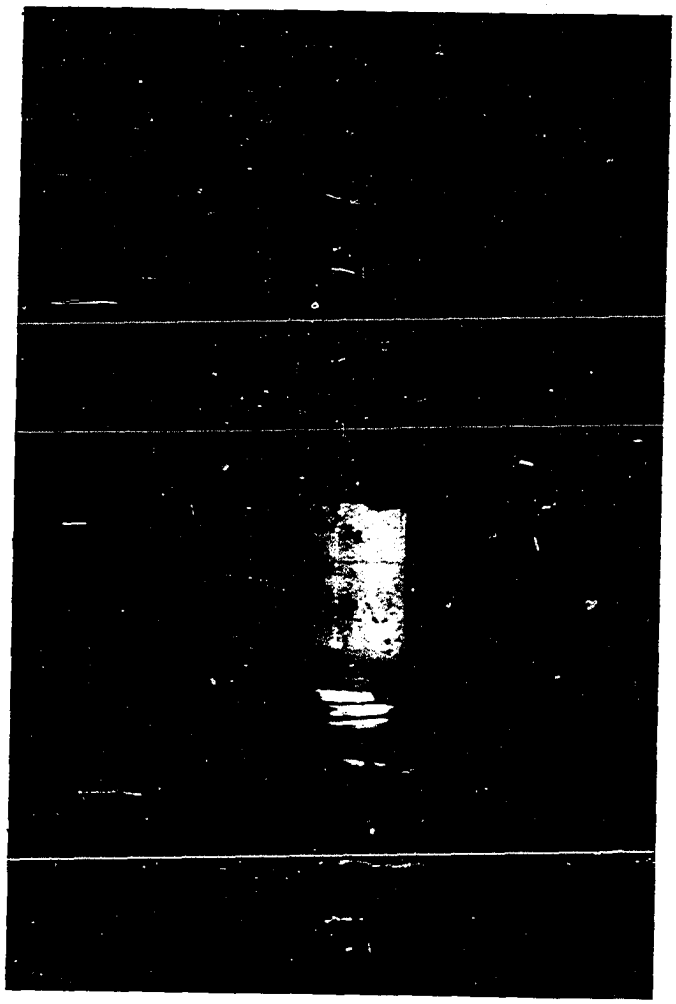

Figure III.1. Unpainted Leonard jar with Alnus glutinosa plant

(Bond, 1962), in hydroponic solution (Bond et al., 1954; Rodriguez-Barreuco and Bond, 1976; Quispel, 1954), in air with roots bathed in nutrient mist (Callaham et al., 1978), and in commercial semi-rigid clear plastic Growth Pouches® (Lalonde, 1979). Three of the Frankia endophytes to be tested were brought into the United States under special plant quarantine permits which do not allow field testing. Therefore, field tests were not considered. A hydroponic system was rejected because, in pilot studies, many plants died when transplanted from solid germination medium into liquid medium, and survivors often suffered extensive dieback of the root systems. Root mist chambers, or aeroponic chambers, were seriously considered, and a prototype was constructed. The method was abandoned when the collection of Frankia selections grew in number because only one endophyte could be evaluated in each chamber.

As mentioned earlier, the greatest difficulty was the production of plants of each host-endophyte combination with approximately equal numbers of nodules. In Component I and in a previous paper (Hall et al., 1979), superior growth of plants inoculated with the spore (-) strain over the spore (+) was reported, but because of greater numbers of nodules on the spore (+) strain-treated plants, the possibility existed that the spore (+) inoculated plants grew slower because of a temporary "sink effect" caused by increased photosynthate requirements of the nodules.

After evaluating the results of the Component I experiment, a number of different parameters were considered important in developing an understanding of endophyte and host development. Information was needed on rates of nodule formation which could be obtained best with the Growth Pouch® system. Plant responses to different endophytes, given a constant

level of nodulation, could be studied better in the Leonard jar system in a high light intensity environment. No simple system could be found that would solve all these problems, so a more complex two-stage experiment was developed (summarized in Table III.2). Another potential procedural problem was the scheduling of acetylene reduction assays. In a preliminary study, I found that 1.0 ml disposable syringes could be used to collect gas samples from the incubation chambers. After collecting a sample of gas, the syringe was inserted into a rubber stopper for short-term storage. Gas loss was acceptably small if samples were stored for less than one day. If all plants included in this study had been assayed for acetylene reduction at the same time, nine hours would have been required for the assays alone and at least an additional five hours for handling the plant materials. For this reason, the plants were divided into blocks for the acetylene reduction assay and the following steps. Each block of approximately 16 plants could then be assayed during a single day.

Methodology

Experimental design

The experiment began as a 10 x 5 x 2 factorial design in randomized blocks. There were 10 inocula, including controls (Table III.1), five levels of each inoculum, two A. glutinosa seed sources (Table III.3), and two complete blocks. Each host-inoculum-level combination was replicated once per block. The experimental unit was the individual pouch.

The factors of interest were rate of nodulation and total number of nodules formed. The variables chosen to measure these factors were the number of new nodules formed between each of the three evaluation dates,

Table III.2. Time schedule of activities in *Frankia* evaluation study

Activity	Date
Planted *Alnus* spp. seedlings	May 3, 1979
Subcultured pure culture inocula[a]	May 22, 1979
Inoculated *Alnus* seedlings	June 21, 1979
Counted nodules	July 6, 1979
Counted nodules	July 24, 1979
Counted nodules	August 13, 1979
Counted nodules	August 24, 1979
Transplanted seedlings	August 29, 1979
Moved seedlings to greenhouse	September 24, 1979
Measured plant heights	October 8, 1979
Measured plant heights	October 22, 1979
Measured plant heights	November 6, 1979
Measured plant heights	November 21, 1979
Measured $N_2(C_2H_4)$, Block 1	November 29, 1979
Measured $N_2(C_2H_4)$, Block 2	December 1, 1979
Measured $N_2(C_2H_4)$, Block 3	December 5, 1979
Measured $N_2(C_2H_4)$, Block 4	December 8, 1979

[a]Provided by Ms. Linda Johnston, Department of Bacteriology, Iowa State University.

Table III.3. *Alnus glutinosa* seed sources used to test *Frankia* endophytes

Seed source	Origin
AG-0X04	Plantation in central Iowa, European provenance unknown
AG-4X08	Seed orchard in central Germany

the total number of nodules formed, and the number of surviving seedlings in each pouch at the final evaluation date. It was assumed that germination was not affected by the inoculum treatment but only by the viability of the seedlots and the number of seeds planted per pouch. Any significant difference in the number of surviving seedlings at the end of 17 weeks among the inoculum treatments would thus be due to differences in the symbiotic ability of the endophytes. Observations on nodulation over time were expressed on a per-pouch basis. Final nodule number was adjusted for the number of surviving seedlings in each pouch.

For greenhouse growth evaluations, the experimental design was a completely randomized 9 x 2 factorial design with nine inocula and two seed sources. Replication varied with the number of available plants, but each treatment combination was replicated approximately five times. The experimental unit was the individual tree. The factor of interest was plant growth. The variables chosen to measure this factor were initial height at transplanting and height at each of four different growth periods. Height was measured from the surface of the sand to the center of the topmost unopened leaf.

For nitrogen fixation determinations, the design was an 8 x 2 factorial design in randomized blocks with eight inocula, two seed sources, and four blocks. Because of unequal numbers of plants for each combination, not all blocks were complete. The experimental unit was a single tree. The factor of interest was the amount of atmospheric nitrogen being fixed. The variable chosen to measure this factor was the amount of acetylene converted to ethylene as measured by gas chromatography (Hardy et al., 1973).

Depending upon the stage of the experiment, there were between seven and nine degrees of freedom available for testing the effects of inocula in the analysis of variance. These degrees of freedom were subdivided into five single degree of freedom comparisons (Table III.4). The five comparisons were tested for independence using orthogonal polynomials and found to be independent (Cochran and Cox, 1957). Each comparison was chosen to answer a question about the symbiotic effects of a subset of the inocula tested. Comparison one was between the three pure culture-derived inocula and the five crushed nodule inocula. Comparison two was between the two new endophytes from Germany, one a spore (+) strain, the second a spore (-) strain. Comparison three was between the spore (+) and spore (-) strains from The Netherlands. Comparison four was between the C. peregrina isolate and the two Alnus spp. isolates. Comparison five was between the three new collections of Frankia and the two strains collected by Maurice Lalonde in The Netherlands.

Equipment and nutrient solution preparation

Two hundred Growth Pouches® were refolded so that the seed trough was 0.64 cm deep, and four to five 1.3 cm slits were cut in the bottom of the trough to allow root penetration. Pouches were wrapped in aluminum foil in groups of 10 and treated with dry-heat for 60 minutes at 110°C to kill any Frankia contaminants.

Pouch racks were made from 0.13 x 9.5 x 45 cm clear acrylic sheets by cutting 10 parallel slits approximately 16 cm long in each end 0.75 to 1.0 cm apart, heating the plastic, and bending the assembly into a "U" shape (Figure III.2). Racks were placed in standard clear plastic, laboratory

Table III.4. Single degree of freedom comparisons used in the analysis of variance

Comparison code	Inocula compared	Question of interest
Q1	(3,4,5 vs. 6,7,8,9,10)	Are pure culture inocula significantly different from crushed nodule inocula?
Q2	(8 vs. 7)	Are the spore (+) and spore (-) strains from Gohrenberg and Bad Soden, Germany, significantly different?
Q3	(9 vs. 10)	Are the spore (+) and spore (-) strains from The Netherlands significantly different?
Q4	(5 vs. 3,4)	Is the Comptonia peregrina strain significantly different from the two Alnus spp. pure culture strains?
Q5	(6,7,8 vs. 9,10)	Are the three recently collected endophyte inocula significantly different from the two Alnus glutinosa strains already in our collection?

crispers, and each assembly was disinfected by spraying with a solution of 10 ml per liter of Zephiran®, a commercial disinfectant containing 17 percent alkylbenzyldimethyl-ammonium chloride as the active ingredient.

One-half strength Crone's solution plus micronutrients (Murashige and Skoog, 1962) and 28 mg/l iron as Fe-EDTA was sterilized by filtering through a number of 42 ashless paper filter and then through a Nalgene 0.2 µm filter. Fifteen ml of the nutrient solution were added to each pouch prior to seed planting.

Leonard jars were prepared by painting the exteriors with white latex paint, inserting a 17 cm wick, and packing the neck of the upper jar with

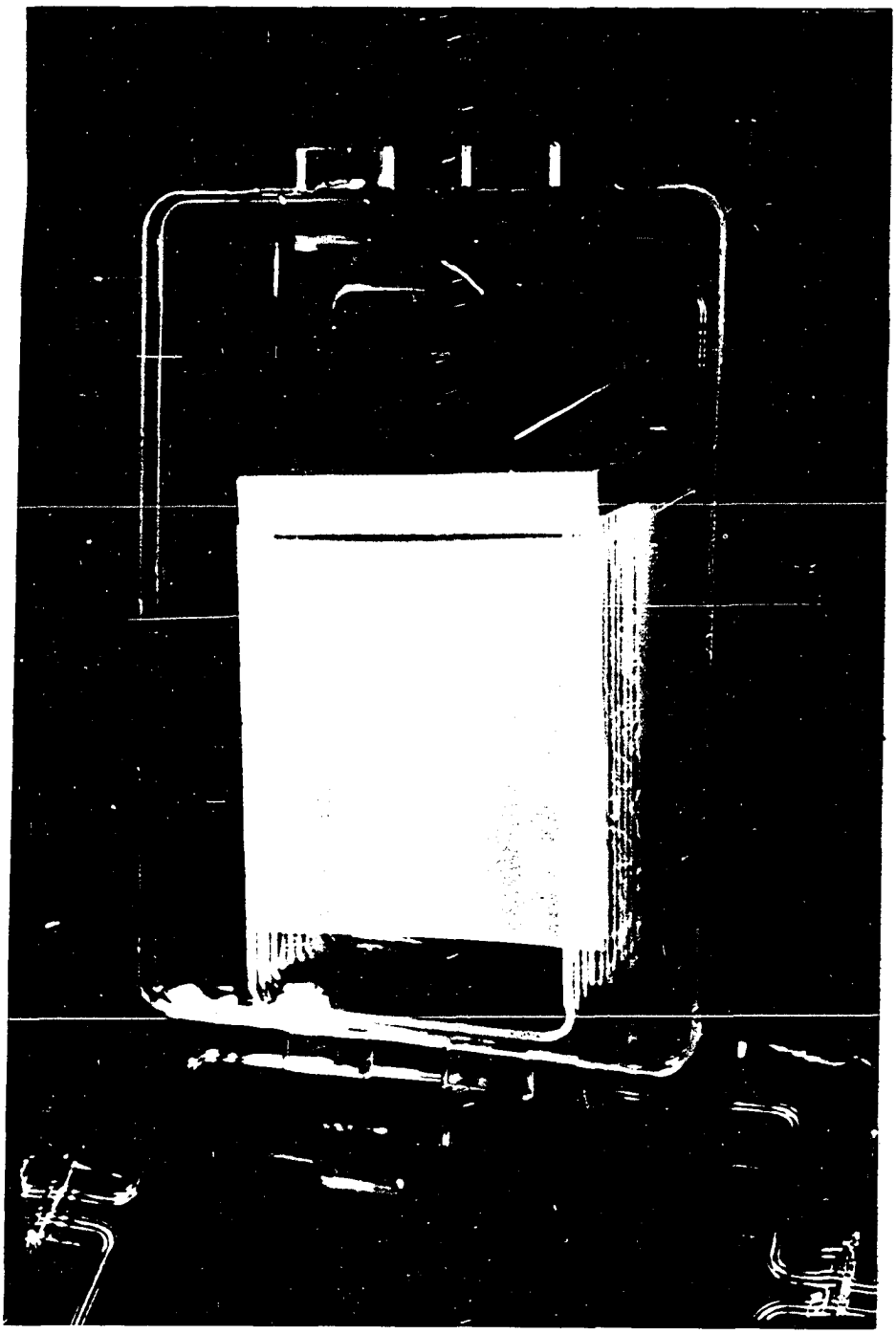

Figure III.2. Rack-crisper assembly[a] used for holding Growth Pouches®

[a]Final design by Dr. Chris Walker, former Research Assistant, Department of Forestry, Iowa State University.

cotton to hold the wick in place (Leonard, 1943). The jars were connected, and the top jar was covered with aluminum foil. All jars were autoclaved at 115°C for 30 minutes.

Oil Dry®, a fired, dolomitic clay medium (Oil Dry Corp. of America, Chicago, Illinois), was used instead of the silica sand used in Component I as a support medium in the Leonard jars because of its superior water-holding capacity (Silsbury, 1979). Thirteen liters of a new nitrogen-free nutrient solution (Table III.5) were poured over 20 l of Oil Dry®, mixed with an equal volume of dry Oil Dry®, transferred to wide-mouth jars, and autoclaved at 115°C for 2.5 hours. A mixture of dry and saturated Oil Dry® was used because, when dry, Oil Dry® is highly hydrophilic and may dessicate fine roots, but when saturated Oil Dry® will not pour.

Hydrophobic sand was added as a mulch to the surface of the Oil Dry® after the seedlings were transplanted. Any water droplets that landed on the sand remained on the surface until evaporation. Thus, airborne Frankia contaminants were not washed into the rooting zone. The hydrophobic sand was prepared by dissolving 10 g of paraffin in 1.0 l of benzene and coating 10 kg of fine silica sand with the solution. The benzene was allowed to evaporate under an exhaust hood, and the coated sand was heat-sterilized at 100°C for nine hours (Vincent, 1970).

Two 50-jar racks for holding Leonard jars were built using 0.64 cm plywood and 2.5 x 5.1 cm framing lumber. Holes 8.5 cm in diameter were drilled on a spacing of 15 by 18 cm. The frame of each rack fitted tightly inside the sides of a greenhouse bench while the plywood sheet rested on top of the side boards. Racks were held firmly in place approximately

Table III.5. Nutrient solution used in Leonard jars for Alnus glutinosa growth trial evaluations of Frankia endophytes[a]

Stock solution	Compound	100X stock solution (g/l)	Dilute solution mM	Dilute solution elemental ppm
I	$MgSO_4 \cdot 7H_2O$	36.5	1.48	36/47
II	$CaCl_2 \cdot 2H_2O$	31.8	2.16	87/153
III (pH adjusted to 5.5 with KOH)	KH_2PO_4	22.1	1.62	63/50
	K_2SO_4	20.7	1.19	93/38
IV	$FeSO_4 \cdot 7H_2O$	1.39	0.050	2.8
	Na_2EDTA	1.87	0.050	1.4 (N)
	$MnCl_2 \cdot 4H_2O$	0.198	0.010	0.55
	$CuSo_4 \cdot 5H_2O$	0.0250	0.001	0.064
	$ZnSO_4 \cdot 7H_2O$	0.0143	0.0005	0.033
	H_3BO_3	0.309	0.05	0.54
	$Na_2MoO_4 \cdot 2H_2O$	0.0121	0.0005	0.048
	$CoCl_2 \cdot 6H_2O$	0.0048	0.0002	0.012

[a]Modifications of Benecke (1970): NaH_2PO_4 replaced with KH_2PO_4, Zn reduced by 50 percent, Cl^- reduced, Ca reduced to same Ca/Mg ratio as Crone's solution; provided by Mr. Greg Miller, Department of Forestry, Iowa State University.

15 cm above the bench surface. Leonard jars were held upright and could easily be removed for watering or examination (Figure III.3).

A 20 l Pyrex® carboy, with a watering hose attachment and a hand-operated pump, was filled with 15 l of distilled water and autoclaved for 60 minutes at 115°C. This assembly was used to water Leonard jars while they were in the greenhouse. The carboy was refilled with redistilled

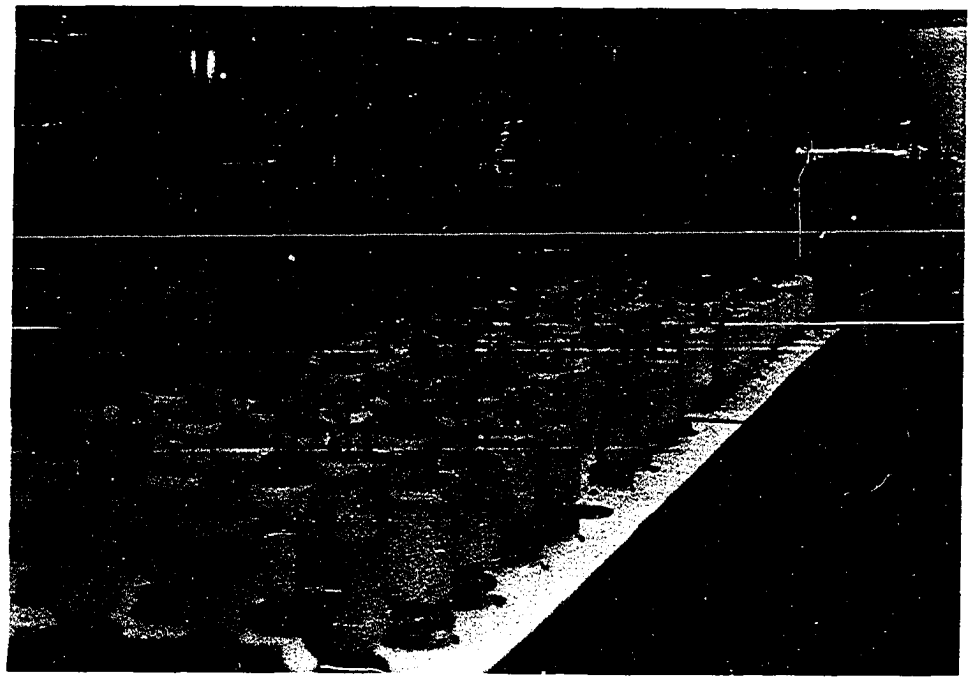

Figure III.3. Leonard jar rack mounted on a greenhouse bench with painted Leonard jars

water but not sterilized after each filling. Frankia cannot withstand temperatures in excess of 60°C (Lalonde, 1979). The steam recaptured by the distillation process is, of course, heated to approximately 100°C and should be free of Frankia contaminants. The water reservoir of the redistillation apparatus would not be sterile, but due to the countless gallons of Frankia-free wa r running through it since installation, the chances of viable Frankia being introduced into the Leonard jars from this source would be near zero. Leonard jars are not designed as aseptic systems, nor are the Growth Pouches®. Therefore, the added difficulty of obtaining autoclaved water for each watering was not warranted. None of the uninoculated control plants nodulated; therefore, the procedure was adequate.

For the nitrogen fixation evaluations, a Varian Series 2700 gas chromotograph, equipped with a flame ionization detector and 1.0 m 0.125" o.d. column packed with Durapack® phenylisocyanate/Porasil C 80/100 mesh range (Alltech Associates, Inc.), connected to a Spectra Physics integrator and a Varian Model A-25 chart recorder, was used for detecting ethylene in the acetylene reduction assay (Hardy et al., 1973). Machine parameters were a column temperature of 30°C and a detector temperature of 80-100°C. Under these conditions, retention times were approximately 29 seconds for ethylene and 50 seconds for the acetylene. The detector temperature remained stable within a range of approximately 1.0°C during the two to four hours required to assay all gas samples of one block. Among blocks, detector temperature did vary. Samples of a known concentration of ethylene were included at the start and end of each block as external standards. For this reason, variability in response due to differences in detector temperature should not have affected the results.

Pure culture inocula preparation

Frankia grows in clumps of hyphae and sporangia in the bottom of culture tubes (Callaham et al., 1978; Quispel, 1979; Lalonde and Calvert, 1979). This growth habit allows for easy harvest. Cultures growing in Torrey's stock solution plus 50 mg/l of lecithin and 500 mg/l of peptone (Johnston, 1980) were drawn into a Pasteur pipette and expelled into screw cap centrifuge tubes containing sterile phosphate buffer. To standardize inoculum concentration, all three pure culture strains were adjusted to an optical density of 0.03 at 620 nm (Lalonde, 1979) using a Bausch and Lomb Spectronic 20® spectrophotometer.

Crushed nodule inocula preparation

Although Quispel has isolated an endophyte from A. glutinosa (Quispel and Tak, 1978; Quispel, 1979), subcultures were not available when this study was initiated. All A. glutinosa endophyte inocula thus had to be derived from crushed nodules. The three sources collected in France and Germany during the summer of 1978 had been increased in Growth Pouches® on A. glutinosa seedlings. Each source was kept in a separate rack-crisper assembly. Several uninoculated control pouches were included in each rack to detect contamination. (No control plants developed nodules.) The spore (+) and spore (-) endophytes collected in The Netherlands by Maurice Lalonde had been increased on plants growing in hydroponic culture. To prevent cross-contamination, each spore type was kept in a separate growth chamber.

Nodules were collected from the hydroponically-grown plants, labeled, and refrigerated. Nodules were collected from the plants grown in pouches

by surface sterilizing the exterior of each pouch then cutting a small hole around each nodule with a flamed scalpel. The nodules of each strain were cut into single lobes, weighed (Table III.6), and surface disinfected for five minutes with continuous agitation in commercial bleach (Lalonde and Knowles, 1975). They were then rinsed three times in sterile chilled H_2O and transferred to an autoclaved Virtis homogenization flask with dual septum ports (Virtis Co., Inc., Gardiner, New York). Fifteen ml of sterile chilled phosphate buffer with 10 g/l PVP were added, and the entire assembly was sealed by the rotor and cap. Nitrogen gas (N_2) was passed through an Acrodisc® disposable syringe filter 0.2 µm (Gelman, Inc., Ann Arbor, Michigan) into the blender chamber through the upper septum port. Air and excess N_2 escaped around the rotor shaft and cap. After all air had been displaced, the nodules were crushed at high speed for two minutes. After debris was allowed to settle for two to three minutes, the supernatant was drawn with a sterile pipette and transferred into two, 5.0 ml sterile centrifuge tubes with screw caps. These tubes were centrifuged for five minutes. The supernatant was decanted and the pellet resuspended in sterile phosphate buffer. This procedure was repeated three times. The resulting inoculum was refrigerated. The procedure was repeated for each endophyte source extracted from nodules.

The stainless steel shaft and cap of the Virtis flasks were soaked in 95 percent alcohol, and the shaft was flamed three times between inoculum sources. Sufficient glass jars were available so that a freshly autoclaved jar was available for each inoculum. All extraction and transfer steps in the above procedure were conducted within a sterile hood to prevent contamination.

Table III.6. Weight of Alnus glutinosa nodules harvested for each crushed nodule inoculum

Inoculum code no.	Spore type	Fresh wt. of nodules (g)
6	(-)	82
7	(-)	367
8	(+)	112
9	(+)	100[a]
10	(-)	1000[a]

[a]The nodules from hydroponic plants were collected and weighed at a separate time, and the information was lost. Approximately 1000 mg of The Netherlands spore (-) and 100 mg of The Netherlands spore (+) strain were used.

Dilution series preparation

An attempt was made to produce equally well-nodulated plants by setting up a dilution series of each inoculum. The full-strength inoculum from each source of crushed nodules and pure cultures was used for the first level of each series. An equal-step 1:5 dilution series was prepared for each crushed nodule inoculum. Pure culture inocula have been shown to be highly infective, often several-fold more infective than crushed nodule inocula (Callaham et al., 1978). The three pure culture inocula, therefore, were diluted 1:100 to produce level two; then a 1:5 dilution series was used for levels three through five.

Study implementation

A. glutinosa seeds were soaked 60 minutes in H_2O, the last 15 minutes in vacuum to thoroughly wet the seed coat. The seeds were then soaked

15 minutes in 30 percent H_2O_2 to disinfect the surface (Neal et al., 1967). Ten to 15 surface-disinfected seeds were aseptically transferred to each pouch. Ten pouches were placed in plexiglass racks, the racks placed in clear plastic crispers, and the crispers placed on end in a Percival model PGC-78 growth chamber set at 18 hour, 21°C days and six hour, 15°C nights. Illumination was approximately 1,000 footcandles provided by 16 72-inch Coolwhite® fluorescent bulbs and 10 50-watt incandescent bulbs.

After seven weeks of growth, seedlings were chlorotic, and the root systems had grown to the bottom of the pouches. Pouches were removed from the crisper-rack assemblies, labeled, numbered, and randomly assigned an inoculum-level combination and a random position within the block. Seedlings were inoculated by opening the pouch and allowing 1.0 ml of inoculum to drain from a pipette over the roots growing in the pouch. Inoculum was dispersed further by squeezing gently on the pouch allowing the free water in the bottom to wash over the root system. Nodules were counted 15 days after the seedlings were inoculated and periodically recounted for the next 10 weeks.

After growing in the pouches for 17 weeks, seedlings were ready to transplant. Pouches were removed from the growth chamber and regrouped by inoculum and seed source so that chances of cross-contamination would be minimized. All pouches receiving a single source of inoculum were examined, and approximately five well-nodulated plants of each host-endophyte combination were selected. The exterior of the pouch was disinfected with Zephiran®, and the entire clear plastic front panel was cut away with a flamed scalpel. The selected seedling was carefully removed, and its nodules were counted and recorded. The seedling was transferred to a

sterile Leonard jar and held gently at the root collar while 350 to 400 ml of moist Oil Dry® was poured slowly around the root systems, followed by 150 ml of nutrient solution. As soon as the surface of the Oil Dry® was free of standing nutrient solution, a layer of approximately 1.0 cm of hydrophobic sand was added. All steps outlined were conducted within a sterile hood. After all 10 plants nodulated by one endophyte source had been transplanted to Leonard jars, all Zephiran®-soaked paper towels were replaced, and all interior surfaces of the sterile hood were resprayed with Zephiran® to reduce the possibilities of cross-contamination. Disposable rubber gloves were worn, and these, too, were sprayed whenever the operator's hands were removed from the hood.

Plant height was measured from the surface of the hydrophobic sand to the middle of the topmost unopened leaf. Seedlings were returned to the high-moisture growth chamber to minimize transplant shock. After three and one-half weeks in the growth chamber, plants were transferred to the greenhouse. Height measurements were taken periodically for the next eight weeks. Water in the lower reservoir of the Leonard jars was replenished weekly.

When the largest plants were of sufficient size to lower the water reservoirs quickly, the jars were assigned randomly to one of four blocks for acetylene reduction assay and dry weight measurements. Extra plants were kept for other experiments. Each block was transferred to a growth chamber for 12 to 18 hours before being assayed. This treatment subjected each block to nearly identical, if perhaps suboptimal, conditions immediately prior to $N_2(C_2H_4)$ assays.

After preconditioning, plants were removed carefully from the jars, Oil Dry® and sand were washed from the root system, and the plants were inserted into 250 ml Erlenmyer flasks. After all plants in a block had been processed to this point, the plants were severed at the root collar, and the flasks containing the root systems were sealed with large septum stoppers. Two empty flasks were included in each block as controls to obtain estimates of background levels of ethylene in the industrial grade acetylene used for the assays. Twenty-five ml of air were extracted from each flask, and the same volume of acetylene was injected. Flasks were incubated at room temperature for 60 minutes. After this period, four 1.0 ml samples were removed from each flask with disposable syringes and stored by inserting the needle into a labeled #8 rubber stopper. Gas samples were injected into the gas chromatograph and peak areas for ethylene and acetylene were recorded.

While the flasks were incubating, the top of each plant was divided into stems and leaves. Each component was placed in a labeled paper sack and transferred to a drying oven set at 36°C. After the acetylene reduction assays, root systems were removed from the flasks and separated into nodules and roots. The nodules were separated arbitrarily into large, medium, and small nodules. The number of nodules in each group was recorded, and all materials were placed in drying tins and transferred to a drying oven with the other plant components. After a minimum of 24 hours of drying, all plant components were weighed on a Mettler H54AR balance to the nearest 0.001 g.

Results

Nodulation

Highly significant differences in nodulation occurred between the two A. glutinosa seed sources (Table III.7). Trees of the local Iowa seed source, AG-0X04, averaged 23.6 nodules per plant, and the central Germany seed source, AG-4X08, averaged 11.7 per plant over all inocula-level combinations (Tables III.8 and III.9). The effect of inoculum, also highly significant (Table III.7), was examined more closely. Most of the variation in nodulation was because of differences between the two strains from central Germany. The spore (+) strain, inoculum eight, averaged 34.5 nodules per plant, and the spore (-) strain, inoculum seven, averaged 14.3 nodules per plant (Table III.10), in spite of the fact that the initial spore (-) inoculum had been over three times as concentrated as the spore (+) inoculum (Table III.5). There was no significant difference at the 0.05 level in mean number of nodules between the spore (+) and the spore (-) strains from The Netherlands, inocula 9 and 10 (Table III.10), presumably due to the higher initial concentration of the spore (-) inoculum (Table III.5). The C. peregrina strain, inoculum five, induced apparently normal nodulation on both host seed sources (Table III.10), confirming earlier reports that such wide cross-inoculations are possible (Berry and Torrey, 1979; Lalonde, 1979; Lalonde and Calvert, 1979). The nodulation response was linear over the range tested, indicating that not even the most concentrated inoculum saturated the capacity of the plants to form nodules.

The significant interaction between inoculum and level was also examined more closely. Most of the variation in nodulation was due to a

Table III.7. Analysis of variance for average number of nodules per Alnus glutinosa plant after 10 weeks of growth (R^2 = 0.83)

Sources of variation	Degrees of freedom	Sums of squares	F	
Block	1	3.29	2.20	NS[a]
Host	1	64.07	43.29	***
Inoc	7	151.60	14.63	***
I Crushed Nodule	(4)	135.79	22.94	***
Q_2	[1]	46.91	31.70	***
Q_3	[1]	2.28	1.54	NS
Q_5	[1]	73.65	49.76	***
II Pure Cultures	(2)	13.28	4.49	*
Q_4	[1]	1.68	1.14	NS
III Pure Culture vs. Crushed Nodules (Q_1)	(1)	1.50	1.01	NS
Level	4	178.85	30.21	***
Linear	(1)	172.26	116.39	***
Lack of Fit	(3)	6.59	1.49	NS
Inoc x Level	28	79.95	1.93	**
Inoc x Linear	(7)	71.65	6.92	***
I Crushed Nodule x Linear	[4]	12.27	2.07	NS
II Pure Culture x Linear	[2]	11.01	3.72	*
I vs. II x Linear	[1]	48.37	32.68	***
Inoc x Lack of Fit	(21)	8.30	0.27	NS
Host x Inoc	7	3.18	0.30	NS
Host x Level	4	20.67	3.49	**
Host x Inoc x Level	28	42.62	1.03	NS
Error	77	114.19		
Corrected Total	157	658.44		

[a]NS = No significant difference at the 0.05 level.

* = Significant difference at the 0.05 level.

** = Significant difference at the 0.01 level.

*** = Significant difference at the 0.005 level.

Table III.8. Cumulative nodule formation for seed source AG-0X04 over all levels of inocula

Inoculum code no.	Days after inoculation 15	33	53	64
control	0.0	0.0	0.0	0.0
3	6.2	10.6	16.0	18.6
4	9.6	18.0	21.8	31.6
5	6.6	17.0	21.2	27.0
6	2.3	14.8	21.8	23.8
7	2.0	10.8	13.2	18.4
8	27.0	38.8	42.0	43.4
9	5.4	10.4	11.4	12.8
10	1.0	4.8	8.6	13.4
				$\bar{X}$ 23.6

Table III.9. Cumulative nodule formation for seed source AG-4X08 over all levels of inocula

Inoculum code no.	Days after inoculation 15	33	53	64
control	0.0	0.0	0.0	0.0
3	1.4	2.8	6.8	7.6
4	2.0	5.6	11.6	17.0
5	1.6	6.4	8.4	12.4
6	0.2	2.6	7.6	10.4
7	0.0	6.0	8.4	12.6
8	7.8	13.2	15.0	18.4
9	1.0	4.6	7.2	8.2
10	0.0	1.8	5.2	6.8
				$\bar{X}$ 11.7

Table III.10. Cumulative nodule formation for each Frankia inoculum over both seed sources of Alnus glutinosa and all levels of inocula

Inoculum code no.	Days after inoculation			
	15	33	53	64
control	0.0	0.0	0.0	0.0
3	3.4	5.5	7.9	10.8
4	5.8	10.8	13.3	18.5
5	5.5	11.5	13.0	19.0
6	1.7	8.4	11.1	17.5
7	1.3	7.3	8.5	14.3
8	21.3	29.7	30.9	34.5
9	4.1	7.6	8.5	9.6
10	0.8	2.5	4.3	8.0

highly significant change in response of the pure culture inocula versus the crushed nodule inocula between levels one and two. The 1:100 dilution between level one and level two for the pure culture inocula could be expected to produce different results from the 1:5 dilution used for the crushed nodule inocula (Figure III.4). There was also a differential response between the two seed sources and the dilution series because of the unexplained increase in nodulation between levels two and three for seed source AG-4X08 (Figure III.5). The treatments and interactions accounted for 83 percent of the total variation observed in nodule number per pouch, an unusually high percentage for biological systems (see the value for R^2, Table III.7), indicating that the dilution series technique and uniform growing conditions in the pouches can produce uniformly nodulated plants of the desired host-endophyte combination.

In addition to the overall nodulation rate previously summarized, the nodule counts obtained throughout the time of nodule formation also

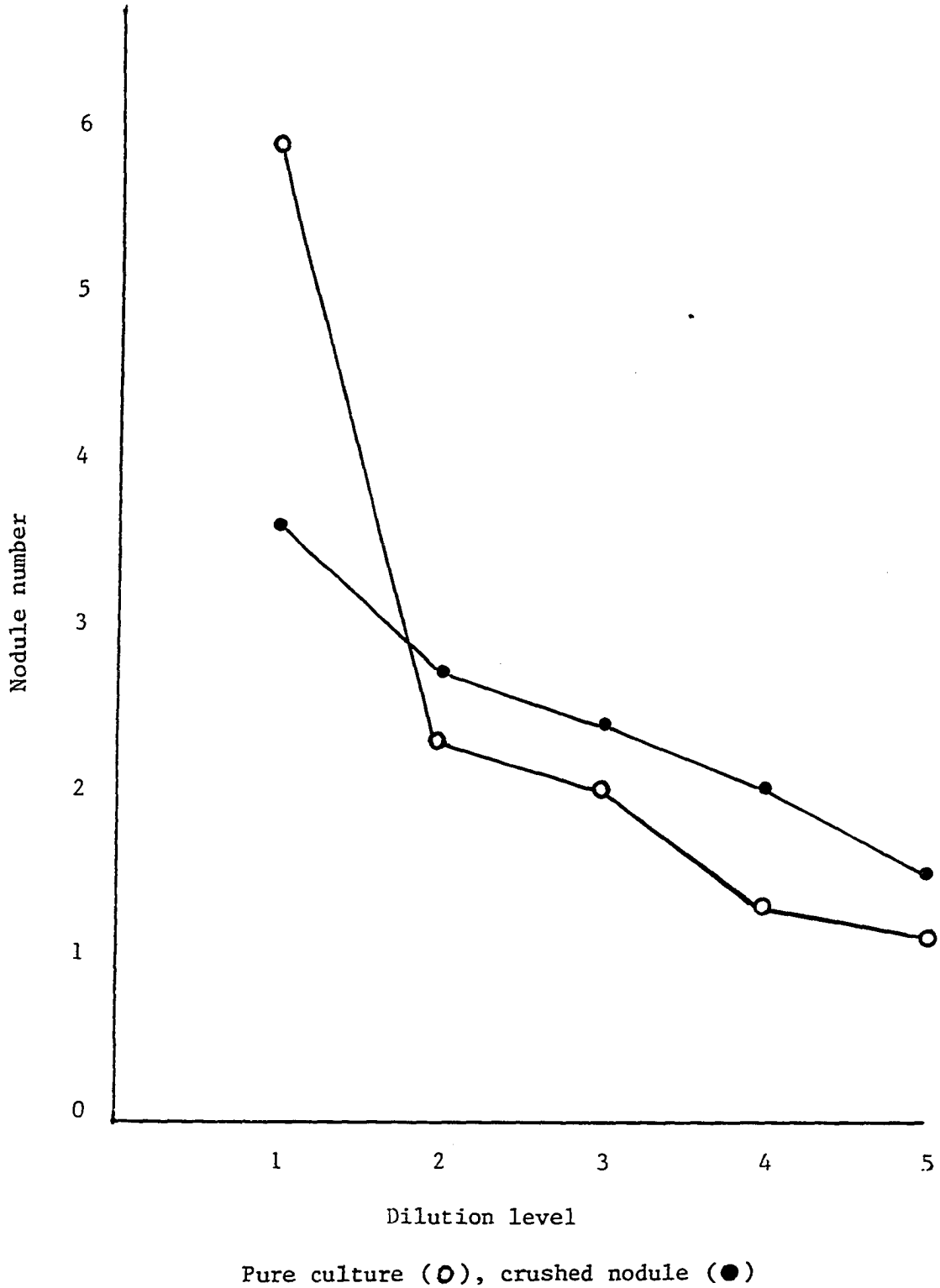

Figure III.4. Nodule number for pure culture and crushed nodule inocula across both seed sources, as affected by dilution level

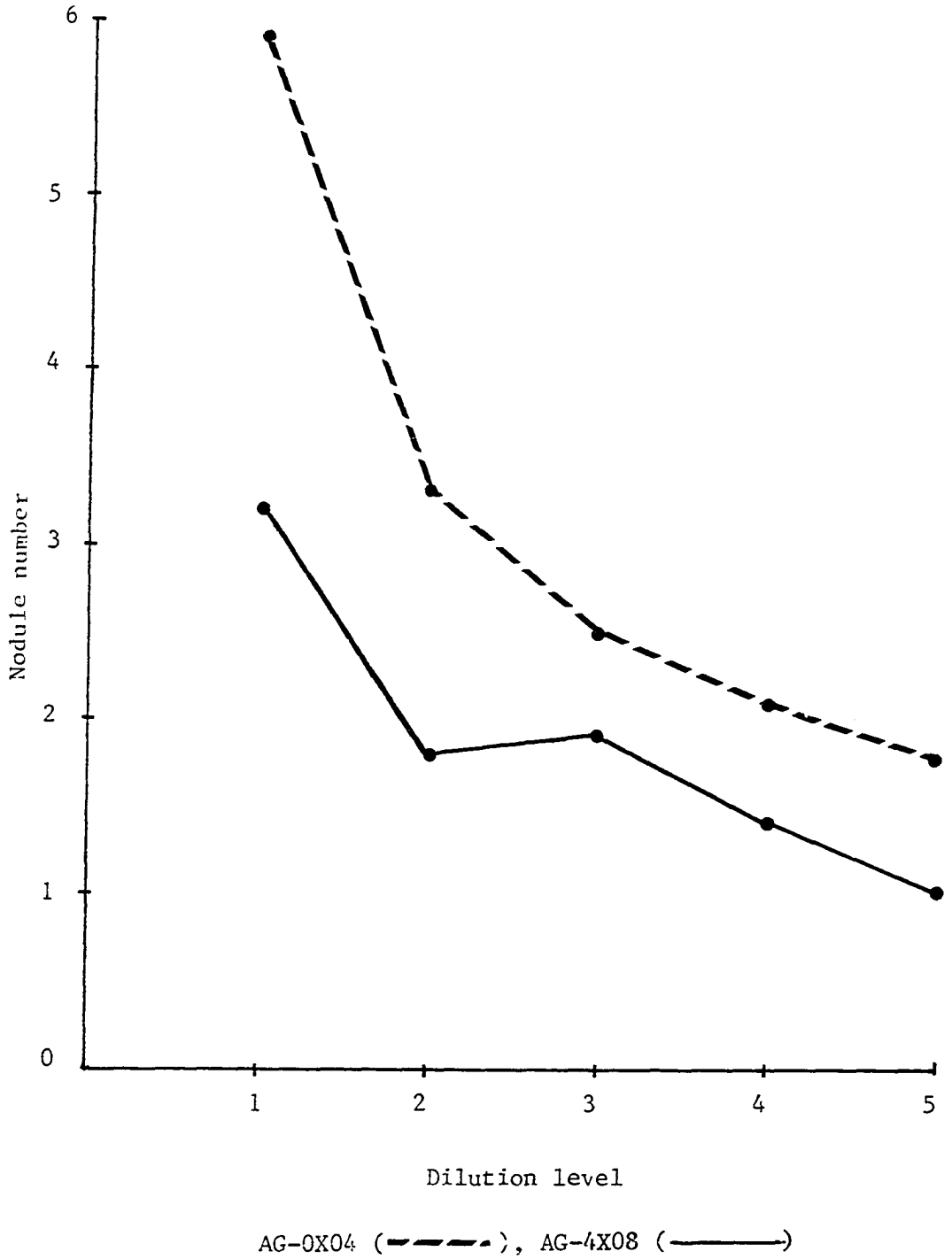

Figure III.5. Nodule number for Alnus glutinosa seed sources across all inocula, as affected by dilution level

reflected interesting patterns. Inoculum eight, from Gohrenberg, Germany, was unusual because of the early burst of nodulation over the first 15 days. At first nodule count, inoculum eight had already induced an average of 27.0 nodules per pouch on seed source AG-0X04, nearly three-fold as many as the next most potent inoculum (Table III.8, Figure III.6).

In addition to developing information on nodulation effects of different inocula, the pouch stage was intended to supply equally well-nodulated seedlings of all 16 host-inoculum combinations. Only partial success was achieved. Inocula seven and ten induced poor nodulation even at the strongest inoculum levels, while inoculum eight produced prolific nodulation over the 600-fold concentration range tested (Table III.11). By carefully selecting the best nodulated plants at the strongest inoculum level for most inocula and plants from dilution levels three, four, and five for inoculum eight, most of the effects of inoculum on nodule number were eliminated.

Height growth

After three months in Leonard jars, the largest plants had grown to a height of approximately 45 cm, and all the water in the lower jar was being transpired between watering intervals. Therefore, the largest plants were subjected to more water stress than the smaller plants. At this point the plants in the study were harvested.

All potential cofactors recorded at the start of the growth stage were rejected from the final analysis of variance model. Initial plant height was rejected because it was already strongly influenced by inoculum source. Nodule number at transplanting was rejected because, surprisingly, it did

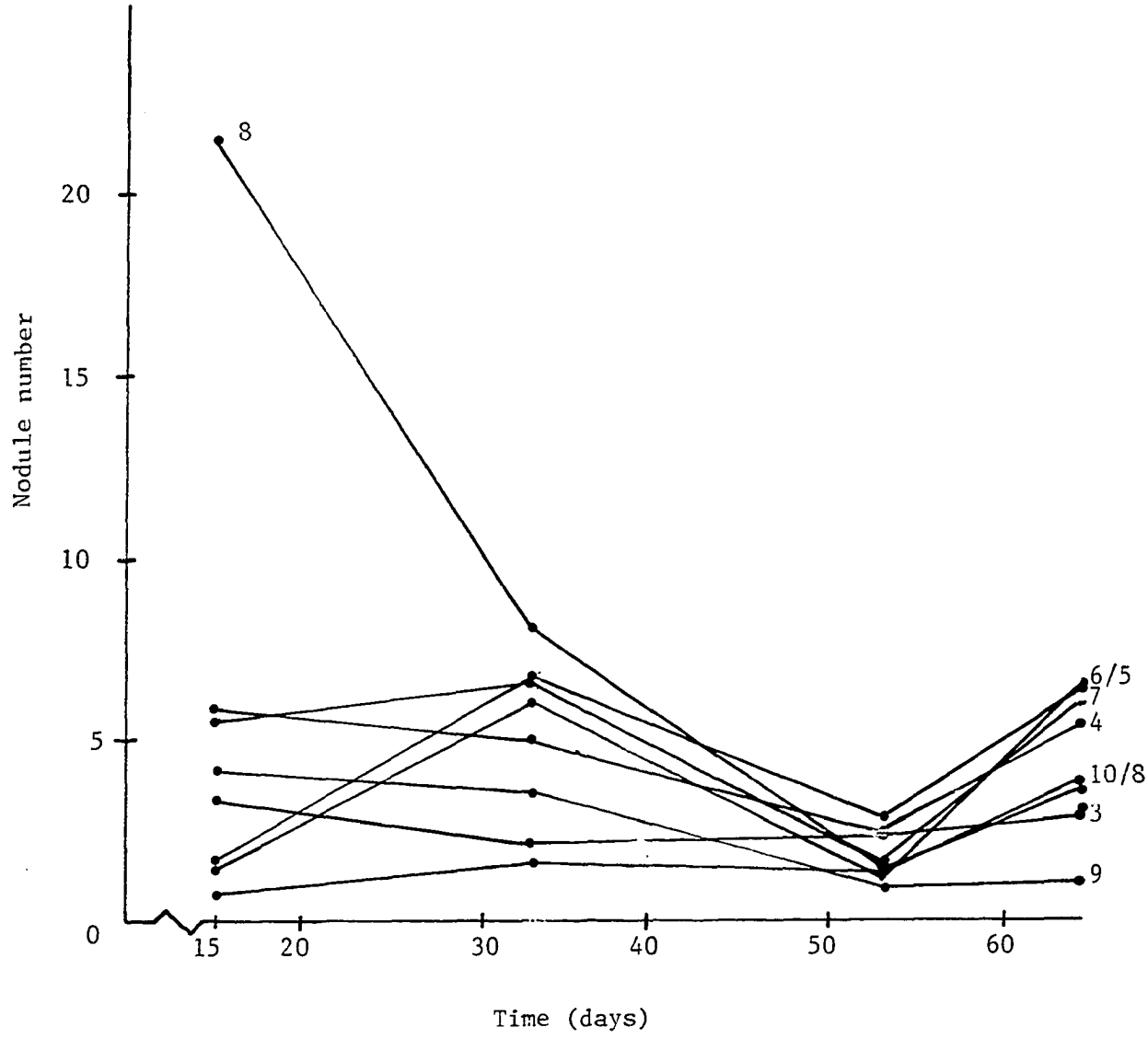

Figure III.6. Number of new nodules produced over time across both seed sources, as affected by Frankia inocula

Table III.11. Total number of nodules per pouch for each Frankia inoculum over both Alnus glutinosa seed sources

Inoculum code no.	Dilution level 1	2	3	4	5
control	0.0	0.3	0.0	0.0	0.0
3	25.5	10.5	9.5	3.5	4.8
4	27.8	17.8	22.0	13.3	11.8
5	54.0	16.0	11.8	6.3	7.0
6	19.5	20.3	13.0	14.8	21.0
7	17.8	13.3	15.5	13.5	11.5
8	44.0	37.0	37.3	30.8	23.0
9	15.5	10.8	8.3	8.3	5.0
10	17.3	8.0	6.8	6.5	4.3

not significantly influence any of the growth parameters measured. Level of inoculum, a strong influence upon initial nodulation rates, was rejected because it was so closely correlated with inoculum and host that it added little new information to the model. The final model selected contained only the treatment effects: host, inoculum, the host x inoculum interaction, and the five single degree of freedom comparisons (Table III.12).

As with nodulation results, host plant seed source had a strong influence on growth rate. Trees from seed source AG-0X04 grew 15.9 cm while those from seed source AG-4X08 grew 10.4 cm averaged over all inocula. The difference was significant beyond the 0.0001 level (Table III.12).

The source of inoculum also had a highly significant effect on height growth (Figures III.7 and III.8) and so was examined more closely. The single degree of freedom comparisons within the inoculum treatment were all highly significant (Table III.12). The crushed nodule inoculated plants grew 17.2 cm, and the pure culture inoculated plants grew 10.6 cm. Most of

Table III.12. Analysis of variance for three-month height growth of Alnus glutinosa seedlings treated with Frankia inocula ($R^2 = 0.69$)

Sources of variation	Degrees of freedom	Sums of squares	F	Prob. > F[a]
Host	1	968	21.24	0.0001
Inoc	8	5887	16.15	0.0001
Q_1	(1)	790	17.32	0.0001
Q_2	(1)	1110	24.35	0.0001
Q_3	(1)	1612	35.39	0.0001
Q_4	(1)	731	16.05	0.0001
Q_5	(1)	65	1.43	0.235
Host x Inoc	8	389	1.07	0.396
Error	72	3282		
Corrected total	89	10526		

[a]The probability of an F value of this size occurring by chance.

the reduction in growth of this group was because of the poor performance of the CpIl endophyte. The A. glutinosa endophyte from Bad Soden, inoculum seven, outperformed the second German endophyte from Gohrenberg, inoculum eight, by nearly three-fold. Plants treated with The Netherlands spore (-) strain maintained slightly over a three-fold advantage over The Netherlands spore (+) strain, thus confirming my earlier reports of a plant growth advantage associated with this strain (Robison et al., 1979).

The one between-family cross-inoculation included in the study, inoculum CpIl, performed poorly. Plants inoculated with this endophyte grew an average of only 4.8 cm. Plants inoculated with two Alnus spp. pure culture endophytes grew an average of 14.8 cm, approximately a three-fold difference. The poor growth of A. glutinosa plants inoculated with CpIl

Figure III.7. Alnus glutinosa plants after three months of growth in Leonard jars, arranged from left to right by Frankia inoculum code number

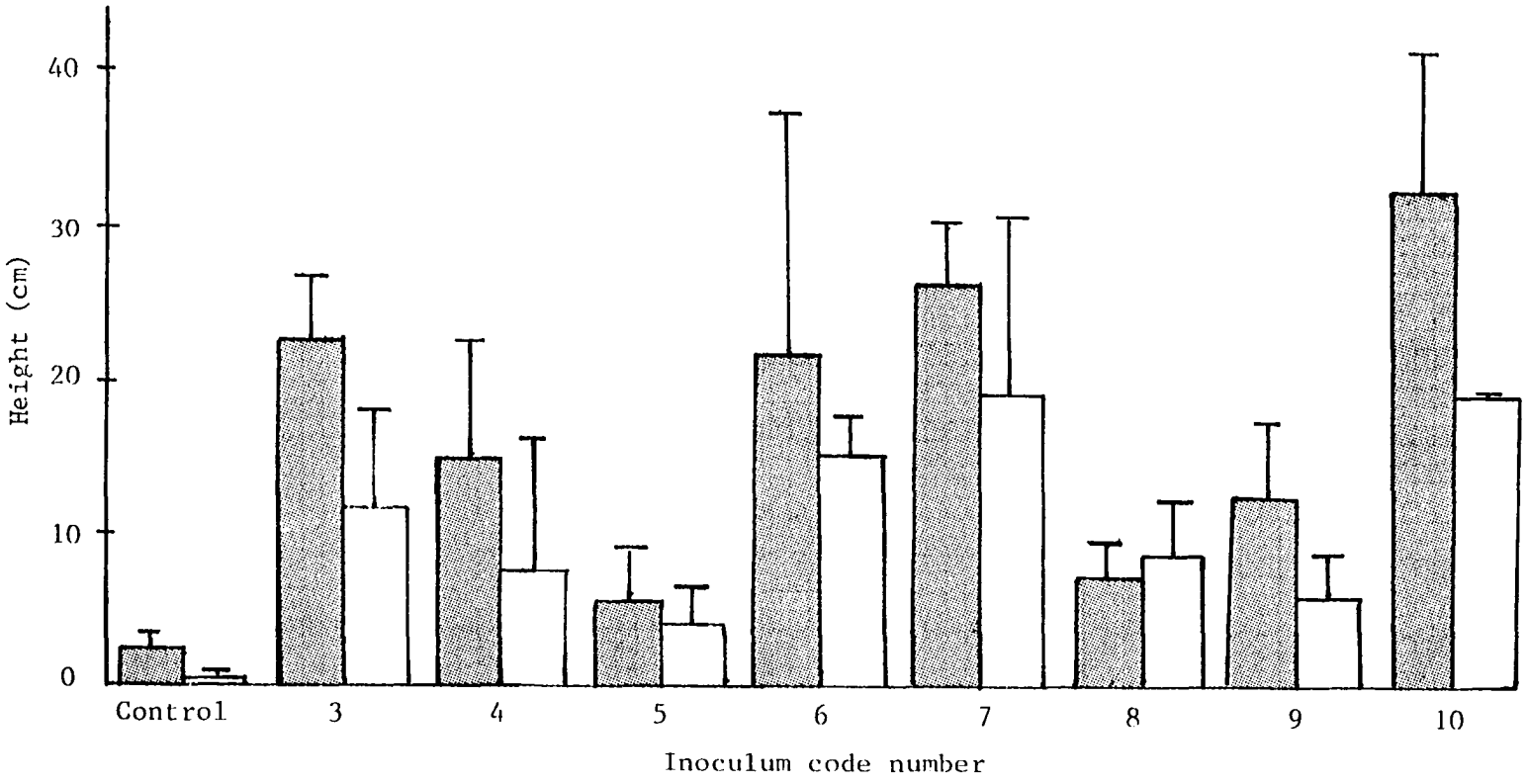

Figure III.8. Three-month height growth for both Alnus glutinosa seed sources, as affected by Frankia inocula; seed source AG-0X04 is indicated by shading, seed source AG-4X08 is indicated by open bars, the line above each bar is one standard deviation in length

Table III.13. Height growth three months after transplanting for Alnus glutinosa plants, treatment means for single degree of freedom comparisons

Code	Treatments	Growth (cm)	Treatments	Growth (cm)
Q_1	All pure culture inocula	10.6	All crushed nodule inocula	17.2 ***
Q_2	A. glutinosa spore (+) Gohrenberg, Germany	7.8	A. glutinosa spore (-) Bad Soden, Germany	22.7 ***
Q_3	A. glutinosa spore (+) The Netherlands	9.2	A. glutinosa spore (-) The Netherlands	29.5 ***
Q_4	C. peregrina pure culture from U.S.A.	4.8	Alnus spp. pure cultures from U.S.A.	14.8 ***
Q_5	A. glutinosa three recent collections	16.2	A. glutinosa two standard inocula	18.8 N.S.[a]

[a] N.S. = No significant difference at the 0.05 level.

*** = Significant difference at the 0.005 level.

was in disagreement with reports of successful cross-inoculation with this isolate (Lalonde, 1979; Lalonde and Calvert, 1979). Lalonde's report was based on acetylene reduction assays on detached nodules. The detached nodule technique has been criticized for causing decreased rates of acetylene reduction as well as increased variability among samples (Wheeler et al., 1978). The growth comparisons were based on plants growing in pouches. In the present study, no major differences in plant growth were observed until the seedlings had been transplanted and allowed to grow several weeks in the Leonard jars. Lalonde's study was conducted in a growth chamber under approximately the same conditions used in the study

reported in Component I. Under these comparatively low light conditions, growth differences would be expected to be small.

In the study reported by Lalonde and Calvert, no control plants inoculated with A. glutinosa crushed nodule inocula are reported (Lalonde and Calvert, 1979). Plants appeared healthy and vigorous when inoculated with the CpI1 endophyte, but no comparison can be made between the CpI1 endophyte and a normal A. glutinosa endophyte.

As in the nodulation results reported earlier, there were interesting differential responses over time. Inoculum 10, The Netherlands spore (-) strain, was slower to initiate nodules. Plants treated with this inoculum started out in seventh place in initial height. However, by the end of the study, plants of seed source AG-0X04 treated with inoculum 10 were the tallest. Plants of seed source AG-4X08 grew equally well when treated with inocula seven or 10 (Figures III.9 and III.10). With inoculum 10, nodule numbers increased steadily after transplanting from the pouches. Inoculum eight ranked fifth and fourth in initial height at transplanting on seed sources AG-0X04 and AG-4X08, respectively. On seed source AG-0X04, a seed source from central Iowa of unknown natural origin, inoculum eight performed poorly, plant height dropped to seventh place only slightly ahead of the plants treated with C. peregrina inoculum. The slope of the growth curve also leveled off (Figure III.9). After harvesting it was found that the AG-0X04 plants treated with inoculum eight had actually lost nodules from the time of transplanting (Table III.14). On AG-4X08, a seed source from Germany, inoculum eight performed better. Plants dropped only one rank between the initial and final height measurements (Figure III.9). The

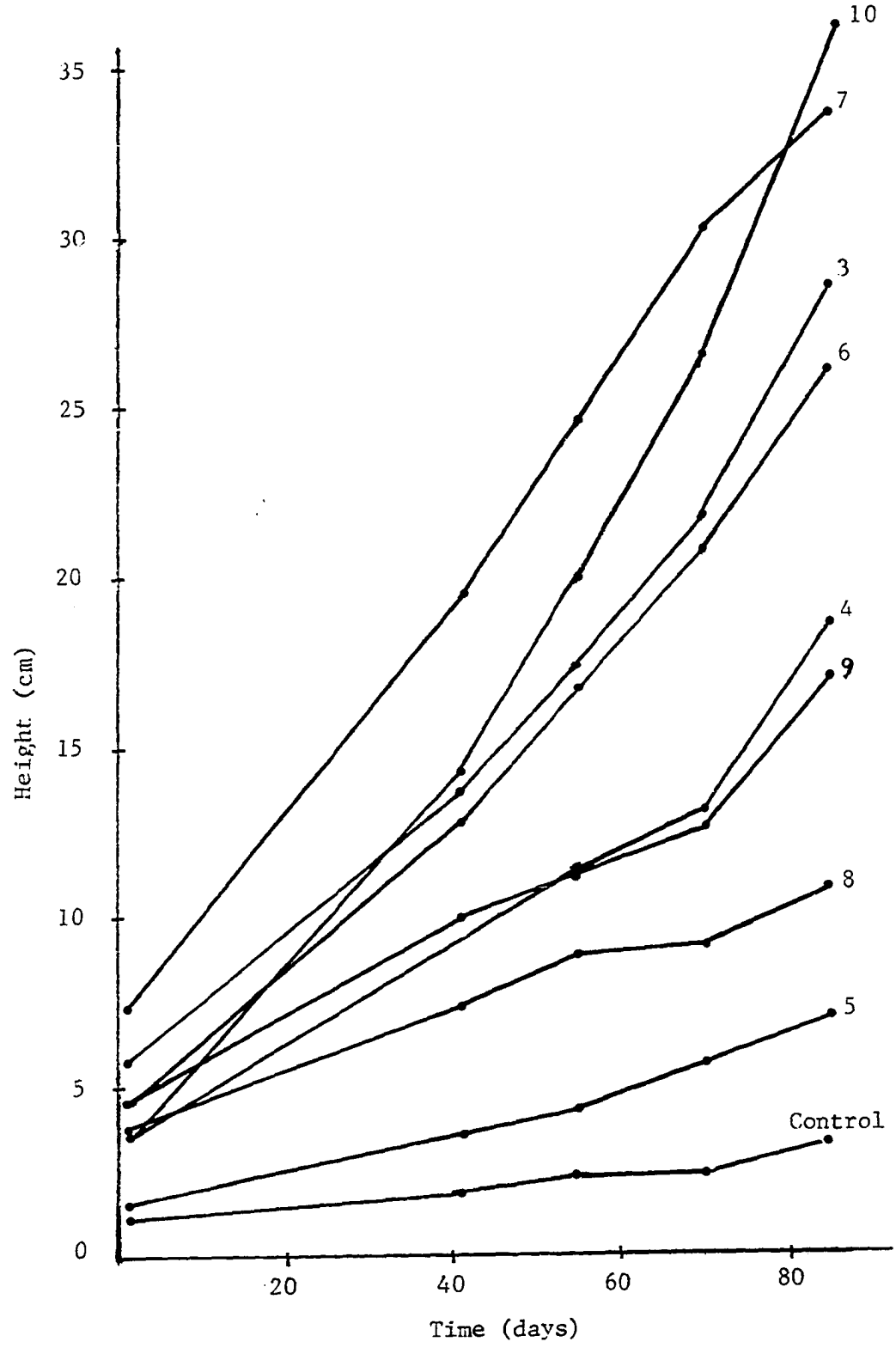

Figure III.9. Height over time for Alnus glutinosa seed source AG-0X04, as affected by Frankia inocula

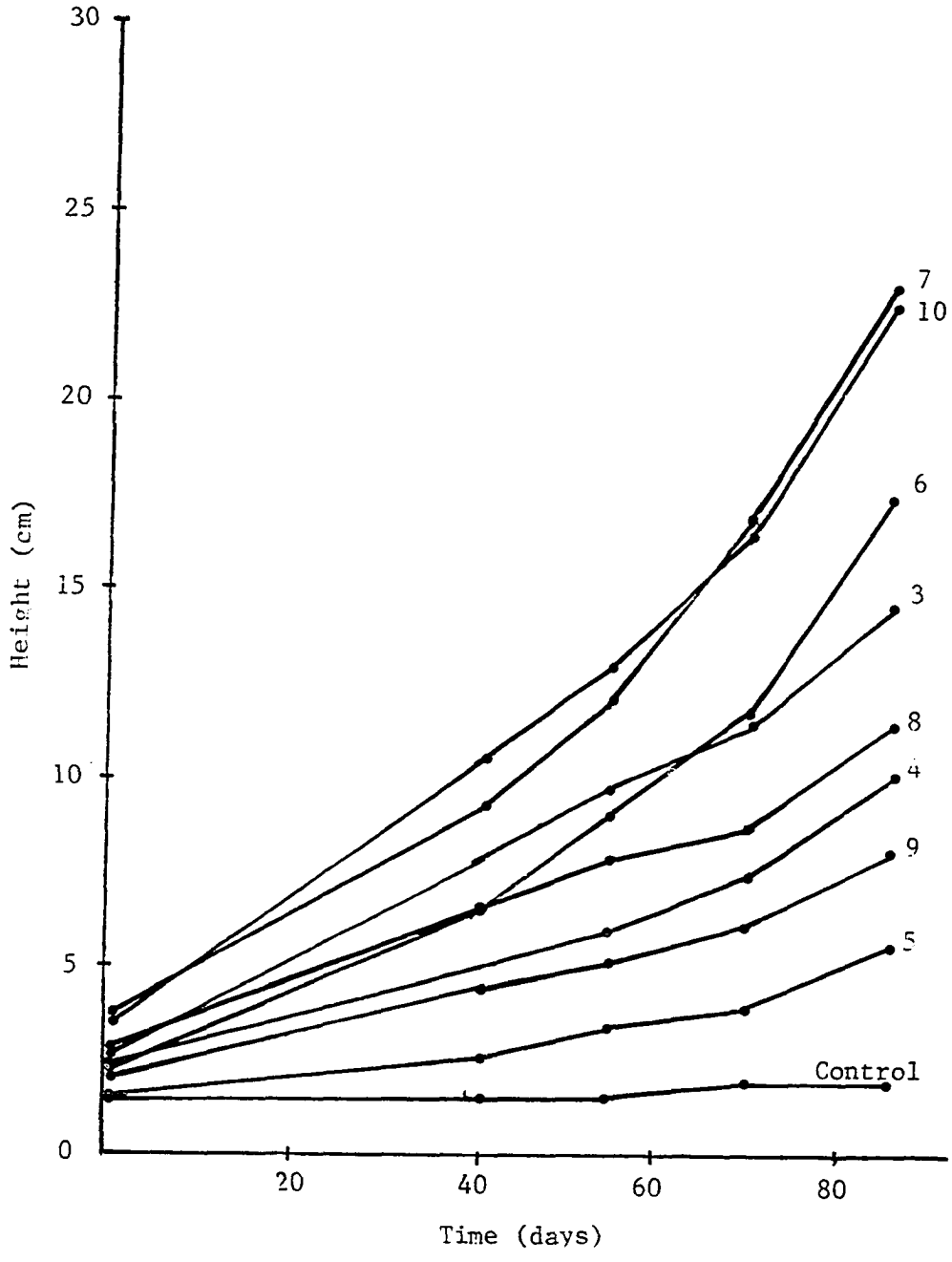

Figure III.10. Height over time for Alnus glutinosa seed source AG-4X08, as affected by Frankia inocula

Table III.14. Change in nodule number per seedling for seed source AG-0X04 during the growth period in the greenhouse

Inoculum code no.	Number of nodules at transplanting	Number of nodules at harvest	Change in number of nodules
3	7.6	17.8	+ 10.2
4	5.7	12.3	+ 6.7
5	10.5	9.3	- 1.2
6	4.8	45.3	+ 40.5
7	4.8	33.0	+ 28.3
8	7.5	7.0	- 0.5
9	5.3	11.3	+ 6.0
10	6.8	40.0	+ 33.2

growth curve did not level off, and nodule number increased slightly (Table III.15).

Acetylene reduction assay

The results from the acetylene reduction assay were surprising. Expressed on a per plant basis, the differences between the two hosts and among the seven inocula were significant at the 0.05 level, but only two of the single degree of freedom comparisons were significant at this level (Table III.16). Inoculum eight, from Gohrenberg, Germany, fixed an estimated 2.6 μM of ethylene per hour. Inoculum seven, from Bad Soden, Germany, fixed 6.1 μM ethylene per hour, over twice as much as inoculum eight. Inoculum five, the pure culture isolate from C. peregrina, fixed only 0.8 μM of ethylene per minute compared with an average of 3.6 μM for plants treated with the two Alnus spp. pure culture inocula (Tables III.17 and III.19). These estimates were on a per plant basis. When nitrogen fixation estimates were expressed on a per gram of nodules basis,

Table III.15. Change in nodule number per seedling for seed source AG-4X08 during the growth period in the greenhouse

Inoculum code no.	Number of nodules at transplanting	Number of nodules at harvest	Change in number of nodules
3	9.0	13.8	+ 4.8
4	6.5	11.3	+ 4.8
5	5.4	9.4	+ 4.0
6	6.5	23.0	+ 18.5
7	3.8	39.5	+ 35.8
8	9.5	11.5	+ 2.0
9	3.3	3.3	0.0
10	16.0	23.5	+ 7.5

Table III.16. Analysis of variance for acetylene reduction per Alnus glutinosa plant (R^2 = 0.50)

Sources of variation	Degrees of freedom	Sums of squares ($X10^{-5}$)	F	Prob. > F[a]
Block	3	288	0.93	0.4324
Host	1	693	6.75	0.0128
Inoc	7	2232	3.10	0.0097
Q_1	(1)	134	1.30	0.2603
Q_2	(1)	469	4.57	0.0383
Q_3	(1)	96	0.94	0.3385
Q_4	(1)	507	4.93	0.0317
Q_5	(1)	19	0.18	0.6724
Host x Inoc	7	1229	1.71	0.1324
Error	43	4416		
Corrected total	61	8858		

[a]The probability of an F value of this size occurring by chance.

Table III.17. Acetylene reduction rates per Alnus glutinosa plant, treatment means for single degree of freedom comparisons

Code	Treatments	µM/plant/hour	Treatments	µM/plant/hour
Q_1	All pure culture inocula	2.7	All crushed nodule inocula	3.9 N.S.[a]
Q_2	A. glutinosa spore (+) Gohrenberg, Germany	2.6	A. glutinosa spore (-) Bad Soden, Germany	6.1 *
Q_3	A. glutinosa spore (+) The Netherlands	4.4	A. glutinosa spore (-) The Netherlands	3.0 N.S.
Q_4	C. peregrina pure culture from U.S.A.	0.8	Alnus spp. pure cultures from U.S.A.	3.6 *
Q_5	A. glutinosa three recent collections	4.0	A. glutinosa two standard inocula	3.7 N.S.

[a] N.S. = No significant difference at the 0.05 level.

* = Significant difference at the 0.05 level.

differences were much smaller (Table III.19). The coefficient of variation was 59 percent of the mean. With this amount of unexplained variation, differences could not be shown to be significant at the 0.05 level (Table III.18). Large variations in acetylene reduction rates have been common in actinorhizal and Rhizobium research. A 50-fold variation in nitrogen fixation (C_2H_4) per gram of nodules has been reported for apparently healthy nodules of A. glutinosa (Akkermans and van Dijk, 1976).

If all the endophyte sources reduced similar amounts of acetylene per gram of nodules, some other explanation must be found for the large differences in plant growth reported. Endophytes may differ in efficiency of host photosynthate utilization. The garden pea (Pisum sativum L.)

Table III.18. Analysis of variance for acetylene reduction per gram dry weight of nodules for Alnus glutinosa plants (C.V. = 59%, R^2 = 0.44)

Sources of variation	Degrees of freedom	Sums of squares	F	Prob. > F[a]
Block	3	140750	4.21	0.0107
Host	1	65	0.01	0.9392
Inoc	7	138136	1.77	0.1178
Q_1	(1)	1575	0.14	0.7086
Q_2	(1)	64	0.01	0.9400
Q_3	(1)	17871	1.61	0.2120
Q_4	(1)	29600	2.66	0.1103
Q_5	(1)	299	0.03	0.8707
Host x Inoc	7	96754	1.24	0.3019
Error	43	478734		
Corrected total	61	854441		

[a]The probability of an F value of this size occurring by chance.

Table III.19. Acetylene reduction rates for Alnus glutinosa seedlings as affected by Frankia inocula

Inoculum code number	µM C_2H_4/ plant/hour	µM C_2H_4/ g nodules/hour
3	6.3	87.0
4	0.9	34.3
5	0.8	41.5
6	3.2	61.6
7	6.1	55.9
8	2.6	54.6
9	4.4	70.9
10	3.0	50.1

transported 32 percent of all newly fixed photosynthate to the root nodules (Minchin and Pate, 1973). If an endophyte utilizes large quantities of photosynthate to manufacture cell components, spores for example, and comparatively little in the fixation of atmospheric nitrogen, plant growth will suffer. Another possible source of variation is the difference in endophyte growth rate within the nodule tissue. Nitrogen fixation has often been expressed on the basis of nodule weight, because nodule weight is a convenient estimate for the amount of endophyte tissue present. In using this measure, it is assumed that there is approximately the same amount of endophyte tissue present per gram of nodules, that the same pro proportion of endophyte tissue is composed of vesicles, and further that all vesicles contain the same proportion of nitrogenase. If a single source of inoculum is used, all the nodules are of approximately the same age, and all host plants are genetically identical, these assumptions are likely to be valid. In this study, the two seed sources differed strongly in all characters measured, a wide assortment of endophytes were tested, and nodule formation and decay occurred throughout the growth period. For these reasons, there may have been a wide range in the amount of endophyte tissue present in nodules induced by different inocula. Costs to the plant in terms of milligrams of photosynthate expended per milligram of nitrogen fixed may have differed greatly.

Still another possible source of variation lies in the assay procedure. In addition to converting dimeric nitrogen into ammonia, nitrogenase converts hydrogen ions into H_2 which is then lost as a gas (Hardy et al., 1973). As much as 60 percent of the total energy expended by the nitrogenase enzyme may be lost to this side reaction (Schubert and Evans, 1976).

Some legume and actinorhizal associations have a hydrogenase system that recycles this hydrogen, increasing the overall efficiency (Ruiz-Argüeso et al., 1978). Rhizobium strains differed in their ability to recycle this hydrogen (Ruiz-Argüeso et al., 1978). Although no within-species differences in hydrogenase activity have been reported for actinorhizal associations, many-fold differences among species have been reported (Evans et al., 1979). When reducing acetylene rather than dinitrogen, hydrogen evolution is eliminated. Thus, the acetylene reduction assay may mask substantial differences in hydrogen evolution among different symbiotic associations (Hardy et al., 1973).

Dry weight production

Both host and endophyte had a strong influence on dry matter production (Table III.20). The interaction between host and inoculum type was not significant at the 0.05 level, indicating that the main effects of host and inoculum explained most of the observed variation. For this reason, mean values for dry weight were not presented for all 16 inoculum-host combinations.

As in the height growth and nodulation results, inoculum eight was noteworthy. In association with all other inocula, the Iowa seed source AG-0X04 outperforms AG-4X08 by as much as a factor of five, but with inoculum eight from Germany, the German seed source AG-4X08 averaged slightly over 25 percent greater dry weight production than the Iowa seed source (Figure III.11). A 25 percent difference between means was not large enough to attain significance at the 0.05 level, but taken together with earlier differences noted in nodulation rates and height growth, the

Table III.20. Analysis of variance for total dry weight of Alnus glutinosa seedlings (R^2 = 0.66)

Sources of variation	Degrees of freedom	Sums of squares	F	Prob. > F[a]
Block	3	8.2	0.78	0.5123
Host	1	50.4	14.43	0.0005
Inoc	7	214.4	8.77	0.0001
Q_1	(1)	33.6	9.63	0.0034
Q_2	(1)	98.6	28.23	0.0001
Q_3	(1)	44.2	12.65	0.0009
Q_4	(1)	18.5	5.31	0.0261
Q_5	(1)	0.04	0.01	0.9182
Host x Inoc	7	22.7	0.93	0.4947
Error	43	150.2		
Corrected total	61	445.8		

[a]The probability of an F value of this size occurring by chance.

difference in dry weight supported the idea of coadaptation in the Frankia-Alnus association.

Plants inoculated with spore (-) strains outgrew plants inoculated with spore (+) strains. In the present study, growth was increased by a factor of three in a comparison between the two strains from The Netherlands and by a factor of slightly over five in a comparison between the two strains from Germany. These large differences in dry weight production indicated that not only did considerable variation exist in endophytes normally associated with a single host species but that this variation was found over a narrow geographic range.

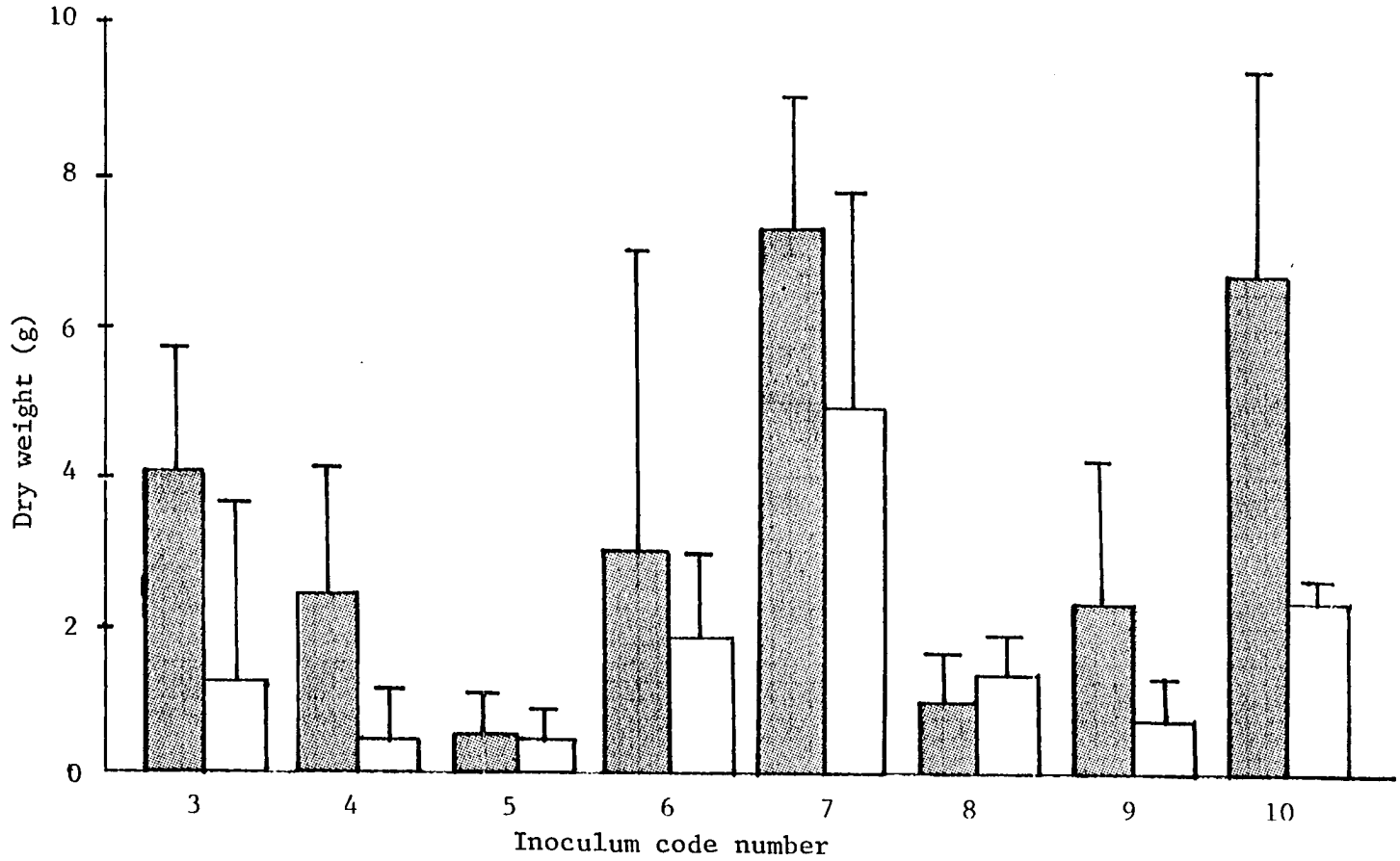

Figure III.11. Whole plant dry weight values for each inoculum-host combination; Alnus glutinosa seed source AG-0X04 is indicated by shading, seed source AG-4X08 is indicated by open bars, the line above each bar is one standard deviation in length

Table III.21. Whole plant dry weight values after a total of five months' growth, treatment means for single degree of freedom comparisons

Code	Treatments	Dry weight (g)	Treatments	Dry weight (g)
Q_1	All pure culture inocula	1.65	All crushed nodule inocula	3.23 **
Q_2	*A. glutinosa* spore (+) Gohrenberg, Germany	1.15	*A. glutinosa* spore (-) Bad Soden, Germany	6.11 **
Q_3	*A. glutinosa* spore (+) The Netherlands	1.53	*A. glutinosa* spore (-) The Netherlands	5.27 **
Q_4	*C. peregrina* pure culture from U.S.A.	0.46	*Alnus* spp. pure cultures from U.S.A.	2.31 *
Q_5	*A. glutinosa* three recent collections	3.29	*A. glutinosa* two standard inocula	3.13 N.S.[a]

[a] N.S. = No significant difference at the 0.05 level.

* = Significant difference at the 0.05 level.

** = Significant difference at the 0.01 level.

The two *Frankia* pure culture strains derived from *Alnus* spp. native to the United States ranked at almost opposite ends of the scale on *A. glutinosa*. Plants treated with AcN1, inoculum three, grew well, while plants treated with ArI3, inoculum four, grew comparatively poorly (Figures III.8, III.9, and III.10). This comparison between the two *Alnus* symbionts from the United States and the natural *A. glutinosa* symbionts from Europe is biased because all *A. glutinosa* endophytes were derived from crushed nodule inocula, and both of the *Frankia* selections from the United States were derived from pure cultures. On the basis of Component II, the bias is

probably in favor of the crushed nodule inocula because they were derived from A. glutinosa nodules.

Discussion

The actinorhizal plant is in a dynamic balance with its Frankia partner. If too many nodules are formed and too much photosynthate is required by the endophyte tissue in those nodules, the overall growth of the plant may suffer. Evolution will, therefore, select for plant genotypes capable of limiting the number and mass of nodules to that necessary to supply sufficient nitrogen for vigorous plant growth. In environments where soil nitrogen is plentiful, this optimum nodulation level may be zero.

From the standpoint of the Frankia endophyte, the maximum amount of host photosynthate should be devoted to production of endophyte tissue and more specifically to reproductive tissue. In highly nitrogen deficient habitats, selection for optimum symbiotic combinations would act strongly, eliminating those host plants forming too many or too few nodules. Selection would also act indirectly on those Frankia genotypes that "demanded" excessive photosynthate for reproductive tissue formation by eliminating their hosts. Where soil nitrogen is not limiting, however, selection pressures may actually be away from a symbiotic relationship. Host plants could derive adequate nitrogen from the soil and need not form nodules. Endophytes could become quasi-parasitic, devoting most or all of the host photosynthate to reproductive tissue formation and fixing little or no atmospheric nitrogen.

Van Dijk questioned what selective advantage would allow two strains of Frankia to exist in apparent equilibrium when both occupy the same ecological niche (van Dijk, 1979). Based on the findings in my studies, one possible explanation would be that if such a stand were progressing through various successional stages and gradually accumulating greater quantities of soil nitrogen, the presumably greater symbiotic ability of the spore (-) types would be losing its selective advantage in favor of the greater reproductive ability of the spore (+) types. In van Dijk's (1979) studies, the only discernible change in frequency of occurrence was an expansion of the spore (+) strain into an area previously occupied by the spore (-) strain (van Dijk, 1979). This is in agreement with my hypothesis. In my study, in both cases where spore (+) and spore (-) strains from the same geographic area were tested, the spore (-) strain induced greater plant growth, while the spore (+) type exhibited greater infectivity per gram of nodules.

If inoculum eight is examined as a symbiont, its performance is puzzling. It formed large numbers of nodules initially, but later most of these died, and it conveyed comparatively little net benefit to the plants. Although performing better on a host from the same geographic area, inoculum eight was still the poorest symbiont among those collected from A. glutinosa hosts. However, if inoculum eight is examined as a weak pathogen, another picture emerges. Quick invasion of host tissues would allow inoculum eight to form nodules and begin forming spores sooner than a less aggressive strain. The rapid loss of nodules would mean a quicker release of infective particles into the soil. The growth of plants associated with this endophyte was poor compared with growth of plants inoculated

with other symbionts but was still better than the nodule-free control plants. In an environment where soil nitrogen was not severely deficient, plant growth would probably not be noticeably affected.

In focusing on nitrogen fixation, researchers studying the actinorhizal system may have ignored the possibility that Frankia exacts a price from its host and that this price may be sufficient to push the relationship from symbiosis to parasitism. The endophyte in inoculum eight may be considered on the border between these states.

The large differences in plant growth and comparatively small differences in fixation per gram of nodules makes it important to seek other measures of endophyte efficiency. If nodule respiration and acetylene reduction can be monitored simultaneously, fixation per gram of host carbohydrate can be calculated. Hydrogen evolution of plants inoculated with the different endophytes should also be measured. This may provide a new dimension on efficiency.

The question of diversity among endophytes of a single host species has been clearly answered. There is a great deal of diversity. The question now becomes, how many groups to subdivide the endophyte into. On the basis of spore type, van Dijk proposed a two-strain model (van Dijk, 1978). On the basis of both spore type characters and plant growth responses, the five selections tested in the present work can be divided into a minimum of three groups. Between the two spore (+) types tested, the selection from Gohrenberg, Germany, can be distinguished from the selection from The Netherlands because of its rapid nodulation followed by nodule deterioration pattern. Among the three spore (-) types, none of the differences in

nodulation or growth are sufficiently striking to warrant further subdivisions, but as more host plants are tested, new divisions may emerge.

Conclusions

1) The source of endophyte used to inoculate A. glutinosa seedlings has a strong influence on both nodulation and plant growth.
2) Of the eight Frankia sources tested in this study, the spore (-) endophytes from A. glutinosa nodules collected in The Netherlands and Bad Soden, Germany, and the spore (-) A. crispa pure culture isolate from the United States were superior.
3) Nodulation alone is a poor predictor of the overall symbiotic efficiency of a particular endophyte. The two spore (+) endophytes induced the most rapid nodulation but ranked last among the natural associates of A. glutinosa in plant growth parameters.
4) Specialization between endophyte and host plant may exist, but among those selections tested, most of the variation was accounted for by the main effects of host and inoculum and only a small part was accounted for by their interaction.

SUMMARY

The three studies reported in this dissertation have provided evidence that the Frankia strain associating with an A. glutinosa host plant can have a profound effect upon the growth of that plant. In Component I, the endophyte source had a strong influence on the plant survival as well as growth. Contrary to expectation, A. rubra plants grew better with either of the two strains of A. glutinosa endophyte than with an A. rubra endophyte from another part of the United States.

In Component II, pure culture inocula nodulated a broader range of host species and induced more rapid nodulation than crushed nodule inocula. However, A. glutinosa plants formed slightly more nodules and had higher survival when inoculated with crushed nodule inocula. The question was raised concerning a labeling effect or selection for a change in host preference resulting from the passage through a cycle on a new host species.

In Component III, much variation was found among natural Frankia associates of A. glutinosa. A quasi-parasitic spore (+) strain was evaluated. In independent comparisons between spore (+) and spore (-) strains from The Netherlands, reported in Components I and III, and a second comparison of a spore (+) and spore (-) strain from Germany, reported in Component III, plants of two species and a total of six seed sources grew better when inoculated with the spore (-) strains.

RECOMMENDATIONS

1) The A. glutinosa spore (-) inocula should be tested with the spore (-) strains from the United States in further greenhouse trials on a wider range of host plants.
2) If further testing substantiates that the spore (-) strains selected from natural stands of A. glutinosa are superior, permission should be sought for field tests.
3) The spore (+) strain from Gohrenberg, Germany, should be tested under carefully controlled conditions until more is known about its quasi-parasitic development.
4) The spore (+) strain from The Netherlands should be eliminated from the standard inoculant used on Alnus field plantings, and the spore (-) AcN1 endophyte should be substituted.
5) In view of the superiority of the spore (-) strains, renewed efforts should be made to isolate this organism from A. glutinosa.

LITERATURE CITED

Akkermans, A. D. L. 1978. Root nodule symbioses in non-leguminous N_2-fixing plants. Pages 335-371 in Y. R. Dommergues and S. V. Krupā, eds. Interactions between non-pathogenic soil microorganisms and plants. Developments in agricultural and managed-forest ecology. Vol. 4. Elsevier Scientific Publishing Co., New York.

Akkermans, A. D. L. and C. van Dijk. 1976. The formation and nitrogen-fixing activity of the root nodules of Alnus glutinosa under field conditions. Pages 511-520 in P. S. Nutman, ed. Symbiotic nitrogen fixation in plants, IBP #7. Cambridge University Press, London.

Alexander, M. 1977. Introduction to soil microbiology. 2nd ed. John Wiley and Sons, New York. 307 pp.

Baker, D. and J. G. Torrey. 1979. The isolation and cultivation of actinomycetous root nodule endophytes. Pages 38-56 in J. C. Gordon, C. T. Wheeler, and D. A. Perry, eds. Symbiotic nitrogen fixation in the management of temperate forests, proceedings. Oregon State University, Corvallis, Oregon.

Baker, D., W. Newcomb, and J. G. Torrey. 1979. Ultrastructural differences between effective and ineffective nodule endophytes of Elaeagnus. Page 471 in J. C. Gordon, C. T. Wheeler, and D. A. Perry, eds. Symbiotic nitrogen fixation in the management of temperate forests, proceedings. Oregon State University, Corvallis, Oregon.

Becking, J. H. 1966. Interactions nutritionelles plantesactinomycetes. Annales de L'institut Pasteur 111(Supplement 3):211-264.

Becking, J. H. 1974. Frankiaceae. Pages 701-706 in R. E. Buchanan and N. E. Gibbons, eds. Bergey's Manual of Determinative Bacteriology. 8th ed. Williams and Wilkins, Baltimore, Maryland.

Becking, J. H. 1976. Actinomycete symbiosis in non-legumes. Pages 581-591 in W. E. Newton and C. J. Nyman, eds. Proceedings of the first international symposium on nitrogen fixation. Vol. 2. Washington State University Press, Seattle, Washington.

Benecke, U. 1969. Symbionts of alder nodules in New Zealand. Plant and Soil 30:145-149.

Benecke, U. 1970. Nitrogen fixation by Alnus viridis (Chaix) DC. Plant and Soil 33:30-48.

Bergersen, F. J., J. Brockwell, A. H. Gibson, and E. H. Schwinghamer. 1971. Studies of natural populations and mutants of Rhizobium in the improvement of legume inoculants. Pages 3-16 in T. A. Lie and E. G. Mulder, eds. Biological nitrogen fixation in natural and agricultural habitats. Special volume of Plant and Soil. Martinus Nijhoff, The Hague, The Netherlands.

Berry, A. and J. G. Torrey. 1979. Isolation and characterization in vivo and in vitro of an actinomycetous endophyte from Alnus rubra Bong. Pages 69-83 in J. C. Gordon, C. T. Wheeler, and D. A. Perry, eds. Symbiotic nitrogen fixation in the management of temperate forests, proceedings. Oregon State University, Corvallis, Oregon.

Beuter, J. H. 1979. Forest fertilization and the economics of perpetual motion machines. Pages 4-13 in J. C. Gordon, C. T. Wheeler, and D. A. Perry, eds. Symbiotic nitrogen fixation in the management of temperate forests, proceedings. Oregon State University, Corvallis, Oregon.

Black, C. A. 1968. Soil-plant relationships. 2nd ed. John Wiley and Sons, Inc., New York. 792 pp.

Bond, G. W. 1962. Fixation of nitrogen in Coriaria myrtifolia. Nature 193:1103-1104.

Bond, G. W. 1967. Nitrogen-fixation in some non-legume root nodules. Phyton 24(2):57-66.

Bond, G. W. 1976. Observations on the root nodules of Purshia tridentata. Proceedings of the Royal Society of London B, 193:127-135.

Bond, G. W., W. W. Fletcher, and T. P. Ferguson. 1954. The development and function of the root nodules of Alnus, Myrica, and Hippophae. Plant and Soil 4:309-323.

Bridger, G. W., R. E. Godsly, and D. E. Ridler. 1979. High-pressure ammonia synthesis and plant operation. Pages 291-332 in R. W. F. Hardy, ed. A treatise on dinitrogen fixation. Sections I and II. Inorganic and physical chemistry and biochemistry. John Wiley and Sons, New York.

Burton, J. C. 1964. The Rhizobium-legume association. Pages 107-134 in C. M. Gilmour and O. N. Allen, eds. Proceedings of the 1964 biological colloquium. Oregon State University, Corvallis, Oregon.

Callaham, D., P. Del Tredici, and J. G. Torrey. 1978. Isolation and cultivation in vitro of the actinomycete causing root nodule in Comptonia. Science 199(24):899-902.

Cochran, W. G. and G. M. Cox. 1957. Experimental designs. 2nd ed. John Wiley and Sons, Inc., New York. 612 pp.

Date, R. A. 1976. Principles of Rhizobium strain selection. Pages 137-150 in P. S. Nutman, ed. Symbiotic nitrogen fixation in plants. Cambridge University Press, London.

Dawson, D. H. and J. A. Pitcher. 1970. Tree improvement opportunities in the north central states related to economic trends. USDA Forest Service Research Paper NC-40, North Central Forest Experiment Station, St. Paul, Minnesota. 10 pp.

Dawson, J. O. and J. C. Gordon. 1979. Nitrogen fixation in relation to photosynthesis in Alnus glutinosa. Botanical Gazette 140(Supplement): 70-75.

Edmunds, G. F. and D. N. Alstad. 1978. Coevolution in insect herbivores and conifers. Science 199:941-945.

Erdman, L. W. and J. C. Burton. 1938. Strain variation and host specificity of Rhizobium leguminosarum on new pea varieties. Proceedings, Soil Science Society of America 3:169-175.

Evans, H. J. and L. E. Barber. 1977. Biological nitrogen fixation for food and fiber production, what are some immediately feasible possibilities? Science 197:332-339.

Evans, H. J., D. W. Emerich, R. J. Maier, F. J. Hanus, and S. A. Russell. 1979. Hydrogen cycling within the nodules of legumes and non-legumes and its role in nitrogen fixation. Pages 196-202 in J. C. Gordon, C. T. Wheeler, and D. A. Perry, eds. Symbiotic nitrogen fixation in the management of temperate forests, proceedings. Oregon State University, Corvallis, Oregon.

Fahey, T. and E. Starostovic. 1979. Changing resource quality: impact on the forest products and construction industries. Forest Products Journal 29(4):39-43.

Finneran, J. A. and T. A. Czuppon. 1979. Economics of production and consumption of ammonia. Pages 333-379 in R. W. F. Hardy, ed. A treatise on dinitrogen fixation. Sections I and II. Inorganic and physical chemistry and biochemistry. John Wiley and Sons, New York.

Gadgil, R. L. 1971. The nutritional role of Lupinus arboreus in coastal sand dune forestry. I. The potential influence of undamaged lupin plants on nitrogen uptake by Pinus radiata. Plant and Soil 34:357-367.

Gardner, I. C. 1958. Nitrogen fixation in Elaeagnus root nodules. Nature 181:717-718.

Gardner, I. C. and G. Bond. 1966. Host plant-endophyte adaptation in Myrica. Naturwissenschaften 53(6):161.

Gardner, I. C. and G. Bond. 1957. Observations on the root nodules of Shepherdia. Canadian Journal of Botany 35:305-314.

Grahm, P. H. 1976. Identification and classification of root nodule bacteria. Pages 99-112 in P. S. Nutman, ed. Symbiotic nitrogen fixation in plants, IBP #7. Cambridge University Press, London.

Gutschick, V. P. 1978. Energy and nitrogen fixation. Bioscience 28(9): 571-575.

Hall, R. B., H. S. McNabb, Jr., C. A. Maynard, and T. L. Green. 1979. Toward development of optimal Alnus glutinosa symbioses. Botanical Gazette 140(Supplement):120-126.

Hardy, R. W. F., R. C. Burns, and R. D. Holsten. 1973. Applications of the acetylene-ethylene assay for measurements of nitrogen fixation. Soil Biology and Biochemistry 5:47-81.

Hewitt, E. J. 1966. Sand and water culture methods used in the study of plant nutrients. Commonwealth Agricultural Bureaux, Farnham Royal, England. 547 pp.

Johnston, L. S. 1980. Nitrogen-fixing endophytes of Alnus glutinosa and Alnus rubra. M.S. Thesis. Iowa State University Library, Ames, Iowa. 148 pp.

Josephson, H. R. and D. Hair. 1973. Timber resources. Pages 22-46 in W. A. Duerr, ed. Timber! problems prospects policies. Iowa State University Press, Ames, Iowa.

Lalonde, M. 1979. Immuniological and ultrastructural demonstration of nodulation of the European Alnus glutinosa (L.) Gaertn host plant by an actinomycetal isolate from the North American Comptonia peregrina (L.) Coult. root nodule. Botanical Gazette 140(Supplement):35-43.

Lalonde, M. and H. E. Calvert. 1979. Production of Frankia hyphae and spores as an infective inoculant for Alnus species. Pages 95-110 in J. C. Gordon, C. T. Wheeler, and D. A. Perry, eds. Symbiotic nitrogen fixation in the management of temperate forests, proceedings. Oregon State University, Corvallis, Oregon.

Lalonde, M. and I. W. Devoe. 1976. Origin of the membrane envelope enclosing the Alnus crispa var mollis Fern. root nodule endophyte as revealed by freeze-etching microscopy. Physiological Plant Pathology 8:123-129.

Lalonde, M. and R. Knowles. 1975. Ultrastructure, composition, and biogenesis of the encapsulation material surrounding the endophyte in Alnus crispa var mollis root nodules. Canadian Journal of Botany 53: 1951-1971.

Lalonde, M., R. Knowles, and J. Fortin. 1975 . Demonstration of the isolation of non-infective Alnus crispa var. mollis Fern. nodule endophyte by morphological immunolabelling and whole cell composition studies. Canadian Journal of Microbiology 21:1901-1920.

Leonard, L. T. 1943. A simple assembly for use in the testing of cultures of Rhizobia. Journal of Bacteriology 45:523-526.

Loomis, W. D. and J. Battaile. 1966. Plant phenolic compounds and the isolation of plant enzymes. Phytochemistry 5:423-438.

Mackintosh, A. H. and G. Bond. 1970. Diversity in the nodular endophytes of Alnus and Myrica. Phyton 27:79-90.

MacNeil, D., T. MacNeil, and W. J. Brill. 1978. Genetic modifications of N_2-fixing systems. Bioscience 28:576-679.

Mian, S., G. Bond, and C. Rodriguez-Barrueco. 1976. Effective and ineffective root nodules in Myrica faya. Proceedings of the Royal Society of London B, 194:285-293.

Miguel, C., A. Costa, and C. Rodriguez-Barrueco. 1978. Some aspects of the Alnus-type root nodule symbiosis. Pages 121-133 in J. Doberener, R. H. Burris, and A. Hollander, eds. Limitations and potentials for biological nitrogen fixation in the tropics. Plenum Press, New York.

Miller, R. E. and R. O. Flight. 1979. Fertilizing douglas-fir forests. USDA Forest Service General Technical Report, PNW-83. Pacific Northwest Forest and Range Experiment Station, USDA Forest Service. 29 pp.

Minchin, F. R. and J. S. Pate. 1973. The carbon balance of a legume and the functional economy of its root nodules. Journal of Experimental Botany 24(79):259-271.

Moore, A. W. 1964. Note on non-leguminous nitrogen-fixing plants in Alberta. Canadian Journal of Botany 42:952-955.

Mowry, H. 1933. Symbiotic nitrogen fixation in the genus Casbarina. Soil Science 36:409-421.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiologica Plantarum 15: 473-497.

Neal, J. L., J. M. Trappe, K. C. Lu, and W. B. Bollen. 1967. Sterilization of red alder seed coats with hydrogen peroxide. Forest Science 13:104-105.

Parker, R. M. 1932. Casuarina root-nodules. Indian Forester 58:362-364.

Pimentel, D. 1976. Food, nitrogen, and energy. Pages 656-673 in W. E. Newton and C. J. Nyman, eds. Proceedings of the first international symposium on nitrogen fixation. Vol. 2. Washington State University Press, Seattle, Washington.

Quispel, A. 1954. Symbiotic nitrogen-fixation in non-leguminous plants. I. Preliminary experiments on the root-nodule symbiosis of Alnus glutinosa. Acta Botanica Neerlandica 3(4):494-510.

Quispel, A. 1979. Growth and infectivity of the endophyte of Alnus glutinosa. Pages 57-68 in J. C. Gordon, C. T. Wheeler, and D. A. Perry, eds. Symbiotic nitrogen fixation in the management of temperate forests, proceedings. Oregon State University, Corvallis, Oregon.

Quispel, A. and T. Tak. 1978. Studies on the growth of the endophyte of Alnus glutinosa (L.) Vill. in nutrient solutions. New Physiologist 81:587-600.

Rehfuess, K. E. 1979. Underplanting of pines with legumes in Germany. Pages 374-387 in J. C. Gordon, C. T. Wheeler, and D. A. Perry, eds. Symbiotic nitrogen fixation in the management of temperate forests, proceedings. Oregon State University, Corvallis, Oregon.

Roach, B. A. 1965. Robinia (locusts). Pages 641-648 in H. A. Fowells, ed. Silvics of forest trees of the United States. Ag handbook No. 271. Division of Timber Management Research, Forest Service, USDA, Washington, D.C.

Robison, T. L., C. A. Maynard, J. Thomas, and R. B. Hall. 1979. A germplasm collection and evaluation program for Alnus glutinosa. Pages 73-85 in K. C. Steiner, ed. 26th Northeastern forest tree improvement conference proceedings. Northeastern Forest Experiment Station, Durham, New Hampshire.

Rodriguez-Barrueco, C. 1966. Fixation of nitrogen in root nodules of Alnus jorullensis H. B. and K. Phyton 23(2):103-110.

Rodriguez-Barrueco, C. and G. Bond. 1968. Nodule endophytes in the genus Alnus. Pages 185-192 in J. M. Trappe, J. F. Franklin, R. F. Tarrant, and G. M. Hansen, eds. Biology of alder, Northwest Scientific Association. Fortieth Annual Meeting, Symposium Proceedings, 1967. USDA Forest Service, Portland, Oregon.

Rodriguez-Barrueco, C. and G. Bond. 1976. A discussion of the results of cross inoculation trials between Alnus glutinosa and Myrica gale. Pages 561-565 in P. S. Nutman, ed. Symbiotic nitrogen fixation in plants, IPB #7. Cambridge University Press, London.

Rodriguez-Barrueco, C. and C. Miguel. 1979. Host plant-endophyte specificity in actinomycete-nodulated plants. Pages 143-159 in J. C. Gordon, C. T. Wheeler, and D. A. Perry, eds. Symbiotic nitrogen fixation in the management of temperate forests, proceedings. Oregon State University, Corvallis, Oregon.

Ruiz-Argüeso, T., J. Hanus, and H. J. Evans. 1978. Hydrogen production and uptake by pea nodules as affected by strains of Rhizobium leguminosarum. Archives of Microbiology 116:113-118.

Schopmeyer, C. S. 1974. Alnus B. Ehrh. (Alder). Pages 206-211 in C. S. Schopmeyer, ed. Seeds of woody plants in the United States. Ag Handbook No. 450. USDA Forest Service, Washington, D.C.

Schubert, K. R. and H. J. Evans. 1976. Hydrogen evolution: a major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. Proceedings of the National Academy of Science 73:1207-1211.

Silsbury, J. H. 1979. Growth, maintenance and nitrogen fixation of nodulated plants of subterranean clover (Trifolium subterraneum L.). Australian Journal of Plant Physiology 6:165-176.

Silvester, W. B., D. A. Carter, and J. I. Sprent. 1979. Nitrogen input by Lupinus and Coriaria in Pinus radiata in New Zealand. Pages 253-265 in J. C. Gordon, C. W. Wheeler, and D. A. Perry, eds. Symbiotic nitrogen fixation in the management of temperate forests, proceedings. Oregon State University, Corvallis, Oregon.

Smith, D. M. and E. W. Johnson. 1977. Silviculture: highly energy-efficient. Journal of Forestry 75:208-210.

Sprent, J. I. and W. B. Silvester. 1973. Nitrogen fixation by Lupinus arboreus grown in the open and under different aged stands of Pinus radiata. New Phytologist 72:991-1003.

Steel, R. G. and J. H. Torrie. 1960. Principles and procedures of statistics, with special reference to the biological sciences. McGraw-Hill Book Co., Inc., New York. 481 pp.

Sweeney, G. C. 1976. Technology and economics of ammonia production. Pages 648-655 in W. E. Newton and C. J. Nyman, eds. Proceedings of the first international symposium on nitrogen fixation. Vol. 2. Washington State University Press, Seattle, Washington.

Tenwolde, A. and R. N. Stone. 1978. Consistent energy accounting in production of wood products and competing materials. Resource Recovery and Conservation 3:249-259.

Torrey, J. G. 1978. Nitrogen fixation by actinomycete-nodulated angiosperms. Bioscience 28:586-592.

Uemura, S. 1971. Non-leguminous root nodules in Japan. Pages 349-360 in T. A. Lie and E. G. Mulder, eds. Biological nitrogen fixation in natural and agricultural habitats. Special volume of Plant and Soil. Martinus Nijhoff, The Hague, The Netherlands.

Uemura, S. and T. Sato. 1975. Non-leguminous root nodules in Japan (a supplementary report). Pages 17-24 in H. Takahashi, ed. JIBP Synthesis. Vol. 12. Nit en fixation and nitrogen cycle. University of Tokyo Press, Tokyo, Japan.

van Dijk, C. 1978. Spore formation and endophyte diversity in root nodules of Alnus glutinosa (L.) Vill. New Phytologist 81:601-615.

van Dijk, C. 1979. Endophyte distribution in the soil. Pages 84-94 in J. C. Gordon, C. T. Wheeler, and D. A. Perry, eds. Symbiotic nitrogen fixation in the management of temperate forests, proceedings. Oregon State University, Corvallis, Oregon.

van Dijk, C. and E. Merkus. 1976. A microscopical study of the development of a spore-like stage in the life cycle of the root nodule endophyte of Alnus glutinosa (L.) Gaertn. New Phytologist 77:73-91.

Vaux, H. J. 1973. Timber resource prospects. Pages 92-134 in W. A. Duerr, ed. Timber! problems prospects policies. Iowa State University Press, Ames, Iowa.

Vincent, J. M. 1970. A manual for the practical study of the root nodule bacteria. IBP Handbook #15. International Biological Programe. Blackwell Scientific Publications, Oxford and Edinburgh.

Wheeler, C. T., E. M. Cameron, and J. C. Gordon. 1978. Effects of handling and surgical treatments on nitrogenase activity in root nodules of Alnus glutinosa, with special reference to the application of indole-acetic acid. New Phytologist 80:175-178.

Wilson, J. K. 1939. Leguminous plants and their associated organisms. Cornell University Agricultural Experiment Station Memoir 221. 48 pp.

Wright, J. W. 1976. Introduction to forest genetics. Academic Press, New York. 463 pp.

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