XEROTOLERANCE IN *FRANKIA*

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The experiments described herein contribute to the development of a model system for studying resistance to water limitation stress in dinitrogen fixation by actinorhizal plants. Actinorhizal plants are certain dicotyledonous plants which can form root nodules with bacteria of the genus Frankia. These symbiotic root nodules are the site of dinitrogen fixation, the biological process by which atmospheric molecular nitrogen is combined with hydrogen to form ammonium ions (Brill, 1975; Burns, 1979; Postgate, 1982).

An experimental model for quantitative evaluation of the contributions of host, endosymbiont, and nodule physiology to xerotolerance (the ability to withstand drying) in actinorhizal symbiotic dinitrogen-fixing systems (Alnus plus Frankia) is being sought cooperatively by the Department of Forestry, and the Department of Botany and Microbiology. This model could be valuable in the development of plants for use in the many areas where dry, nitrogen-poor soils are prevalent, for plant development research is generally an expensive, long-term project.

Under the direction of Thomas C. Hennessey, water

stress effects on unnodulated cloned host plants have been studied (Hennessey et al., 1985). They compared the effects of water stress on the growth of cloned hosts possessing contrasting xerotolerances which were either nodulated, unnodulated, or nitrogen fertilized. The effects of nodulation with Frankia of high or low xerotolerance will later be compared using hosts which have comparatively low or high water stress resistance.

The actinorhizal plants produced using water stress resistance model may be able to grow in soils which have limited nitrogen and moisture availability better than most other plants. They may aid in the development of these marginal soils and the reclamation of damaged ones into productive agricultural or silvicultural areas in order that the ever-growing demands for food, fiber, and timber can be satisfied. Such plants have obvious advantages over others. They may be able to grow with minimal or no irrigation, and they may have no need for additions of expensive chemical fertilizers to supply nitrogen.

The research presented here is limited to work concerning the microbiological aspects of this shared project: Frankia medium development, isolation of Frankia from Alnus nodules collected from experimentally inoculated or wild hosts, quantitative determination of the xerotolerances of selected Frankia strains, and development of methods for bacterial inoculations of Alnus cuttings.

CHAPTER II

REVIEW OF LITERATURE

Soil Nitrogen and Nitrogen Fixation

Nitrogen is needed in large quantities by plants, largely for protein synthesis; only carbon, oxygen, and hydrogen are more abundant in plants. Because the adequately watered plant can fix carbon from the atmosphere, nitrogen is often the limiting factor in its growth. Nitrogen is usually absorbed from the soil, mainly as salts of nitrate (NO_3^-) ions by root tissues. The nitrate must be reduced to ammonium before it can be used in biosynthetic processes (Salisbury and Ross, 1985).

The major forms of nitrogen in soils are organic nitrogen associated with humus, ammonium nitrogen adsorbed by certain clay minerals, and soluble organic ammonium and nitrate. Most soil nitrogen is associated with the organic matter; this nitrogen is slowly released by microbial mineralization, and therefore not readily available to the plant. Clay-adsorbed ammonia is also slowly released. Soluble ammonia and nitrate in unfertilized soils are rarely more than one to two percent of the total nitrogen present. These soluble compounds are subject to rapid loss by leaching and volatilization, and they are readily removed by plants and soil

microbes (Brady, 1974).

The main commercial source for replacement or supplementary nitrogen necessary for intensive agricultural methods is ammonia in chemical fertilizer. The ammonia is produced by the Haber-Bosch process by which gaseous dinitrogen and dihydrogen molecules are catalytically reacted at elevated temperature and pressure (as described in Sweeney, 1974). Because large quantities of petroleum products are commonly used as fuel for this process, supplies are potentially limited, and costs are subject to variation. Additional costs are added since the fertilizers must be transported, stored, and applied. To make matters worse, only about half of the nitrogen applied as fertilizer is actually taken up by plants (Salisbury and Ross, 1985). Alternative nitrogen sources are being explored, and one promising source is biologically fixed nitrogen (Cromack et al., 1979; DeBell, 1978; Rehfuess, 1978; Wantanabe, 1981; Cote and Camire, 1985).

Nitrogen fixation is the process by which combined nitrogen compounds are formed from dinitrogen (N_2) molecules. A total of about 2×10^8 metric tons of nitrogen are fixed per year globally by biological or non-biological means. About 30% (6×10^7 metric tons) of the total dinitrogen fixed is chemically fixed for fertilizers industrially. Approximately 5×10^6 metric tons (2.5%) of atmospheric nitrogen per year are washed into the soil as nitrate by rainfall. Biologically fixed nitrogen constitutes 67.5% of the earth's total yearly supply (Postgate, 1982).

The nitrate in rainfall is formed from atmospheric nitrogen oxides produced by fossil fuel-burning furnaces and modern engines, and to a much smaller extent, by lightning. Ammonia is formed by biological nitrogen fixation, which occurs only in procaryotes.

The biological nitrogen fixation process requires enzyme systems involving the nitrogenase complex and a hydrogenase, and electron transport molecules such as ferredoxins, flavodoxins, quinones, and cytochromes (Brill, 1975; Postgate, 1982). These systems require at least fifteen moles of ATP per mole of dinitrogen fixed, and eight protons and eight electrons to reduce the nitrogen to ammonia. Six electrons and six protons are combined with the nitrogen atoms, and the remaining two protons and electrons are released as one molecule of hydrogen (Postgate, 1982).

Bacteria (including cyanobacteria) are the only biological dinitrogen fixers, and they do so either in a free-living state or in symbiotic associations with plant hosts (reviewed by Sprent, 1979). (A report has been made concerning a green alga which can fix dinitrogen, but its properties and taxonomic position require further investigation (Postgate, 1982).) Endosymbiotic dinitrogen fixers such as Rhizobium and Frankia which cause nodulation in host roots are of interest because they directly exchange combined nitrogen for photosynthate so that the nitrogen compounds are not taken by other soil microorganisms or leached away, as much of fertilizer nitrogen can be.

Rhizobium-legume systems are known to fix large amounts of nitrogen. They can add several hundred kilograms of combined nitrogen per hectare in a year (Parker, 1977), and constitute a major natural source of combined nitrogen in soil.

The contributions of the symbionts in *Frankia*-host interactions have not been studied as extensively as those of *Rhizobium* and legumes, primarily because pure cultures were not available until 1978 (Callaham, et al., 1978). Field studies have shown that pure or mixed plantings of nodulated alder can result in the accretion of nitrogen in the soil (Radwan et al., 1984; Cote and Camire, 1985), and that alders can grow well in soils which are no longer suitable for agricultural uses (Pregent and Camire, 1985). Actinorhizal plants appear to be capable of fixing nitrogen under greater water stress than most legumes (Sprent, 1976; Helgerson et al., 1979; Dalton and Zobel, 1977; McNabb et al., 1979).

Actinorhizal Plants

Non-leguminous plants which form diazotrophic nodules have been known since before the turn of the century. Postgate (1982) cites from W. D. P. Stewart's *Nitrogen Fixation in Plants* (1966) that Nobbe and coworkers published one of the early reports of nodulation in non-legumes in 1892. The endosymbiont causing nodulation in almost all non-legumes is the actinomycete *Frankia*. (The single exception to date is *Trema cannabina* var. *scabra*, which is nodulated by a

Rhizobium species which was infective for certain legumes (Becking, 1977).) Currently 199 plant species in 21 genera (Table I) are listed as known actinorhizal plants (a non-legume nodulated with *Frankia*) by M. Lechevalier (1983). The list is likely to grow as more species are examined for the presence of nodules. The genus *Alnus* (alder) contains the largest number (41) of species known to be nodulated by *Frankia* (Lechevalier, 1983).

Actinorhizal host plant genera vary widely in habitat, morphology, and commercial usefulness. The choice of *Alnus* as a model host plant was influenced by several factors. *Alnus* includes species which are of direct commercial value as a fast-growing wood and pulp sources (Resch, 1978), and *Alnus* was, at the time of this investigation, the only host genus which could be cloned by reliable methods (T. C. Hennessey, personal communication). (Since then, methods for cloning *Eleagnus* have been developed by Bertrand and Lalonde, 1985). Finally, a large number of *Frankia* isolates infective for *Alnus* are available (e. g. Lechevalier and Lechevalier, 1979; Tisa et al., 1983; Zhang et al., 1984).

Frankia Taxonomy

The study of *Frankia* began over a century ago. A microorganism was first shown to be the cause of root nodules in European black alder (*Alnus glutinosa*) by Woronin in 1866, although he could neither isolate nor identify the type of

TABLE I

KNOWN ACTINORHIZAL PLANT GENERA

Alnus
Casuarina
Ceanothus
Cercocarpus
Chamaebatia
Colletia
Comptonia
Coriaria
Cowania
Datisca
Discaria
Dryas
Eleagnus
Hippophae
Kentrothamnus
Myrica
Purshia
Rubus
Shepherdia
Talguenea
Trevoa

From Lechevalier, 1983

microorganism present (Hawker and Fraymouth, 1951, as cited by Lechevalier and Lechevalier, 1979). Many investigators since Woronin made proposals about the nature of the endophyte; it has been called a filamentous fungus, parasitic slime mold, bacterium, and an actinomycete (reviewed by Lechevalier and Lechevalier, 1979). Not until 1964 was the prokaryotic nature of Frankia ascertained by electron microscopy of nodule sections (Silver, 1964; Becking et al., 1964). Presumptive endosymbionts had been isolated and grown in pure laboratory culture (Uemura, 1952, 1964; Quispel, 1960; Lalonde, 1975), but not until 1978 was Frankia proven to be the causative agent of actinorhizal root nodules according to Koch's postulates by Callaham, del Tredici, and Torrey (1978).

Frankia is a bacterial genus placed in the family Frankiaceae, order Actinomycetales. These microorganisms form a true mycelium (branched and septate), irregularly shaped spores in specialized sporangia, and vesicles which may contain crosswalls (Becking, 1970, in Bergey's Manual of Determinative Bacteriology, 8th ed., 1974). The Pommer diagram (Figure 1) represents the endophyte of Alnus glutinosa and gives a schematic of Frankia structure Pommer, 1959). A fourth structure, chlamydospore-like bodies called reproductive torulose hyphae (RTH), result from hyphal swelling and closely-spaced septation (Diem and Dommergues, 1985). Nitrogen fixation activity was associated early with vesicle formation (Becking, 1974; Callaham et al., 1978). Noridge

Figure 1. The Pommer Diagram

The Pommer diagram represents the structures characteristic of the bacterium Frankia. (from Pommer, 1959.)

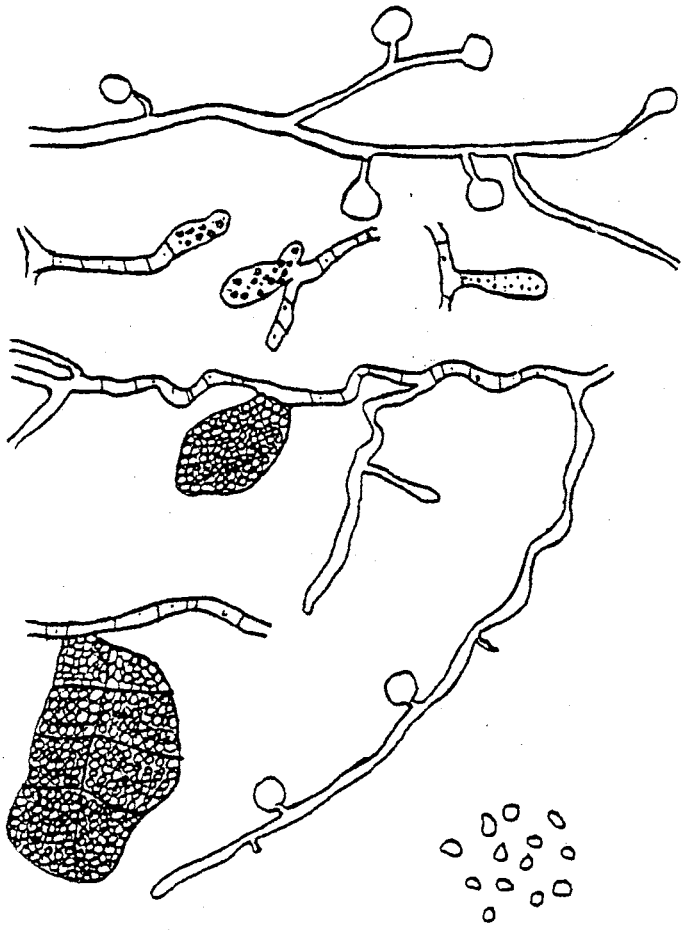
A Vesicles

B Immature sporangia

C Maturing sporangium on branched, septate hyphae; vesicles on lower branch

D Mature sporangium

E Free spores



and Benson (1986) have shown that acetylene reducing activity is associated solely with isolated, intact vesicles. Frankia are able to fix dinitrogen both in vitro (Callaham et al., 1978; Tjepkema et al., 1980) and in a mutualistic symbiosis with at least 21 non-leguminous dicotyledonous plant genera (as listed in Table I).

An unknown can now be identified as a member of the genus Frankia by several methods which vary in usefulness. These methods include morphological characters such as hyphal structure, sporangia, and (when possible) vesicles (Becking, 1974; Baker et al., 1979); cell chemistry such as whole cell sugar patterns (Lechevalier et al., 1982; Lechevalier and Ruan, 1984); or the presence of an unusual "marker" sugar, 2-o-methyl-D-mannose (Mort et al., 1983). Other methods include the demonstration of host plant infectivity and/or effectivity (Becking, 1974; Zhang, et al., 1984); serology (Baker et al., 1981); and DNA restriction analysis pattern comparisons (An et al., 1985a, 1985b). But a Frankia species is not yet clearly defined.

Becking suggested in 1970 in Bergey's Manual of Determinative Bacteriology (8th ed., 1974) that species names be assigned in the genus Frankia on the basis of the plant genus for which the bacterium is infective. However, some strains have since been shown to infect more than one host genus (Zhang et al., 1984).

Lechevalier (1984) lists nine species criteria sug gested by many workers and objections to each method. The

methods include 1) cell chemistry patterns such as proteins or whole cell sugars; 2) DNA homology; 3) 16s RNA catalogs; 4) physiology, such as carbon metabolism or extracellular enzyme production; 5) ecology (host of origin); 6) host range; 7) mycelial and colony morphology; 8) serology; and 9) phage grouping. However, the first three techniques are too complicated for routine taxonomy, and the next three are not useful when considered alone. Mycelial and colony morphologies are unstable. Serological methods are considered by some workers to be too sensitive to differentiate between strains, and too insensitive by others. Phage grouping is not useful because no phages have yet been isolated from Frankia.

Lechevalier and colleagues (1983) propose that two groups of Frankia strains exist. Type A strains have pigmented cells, are aerobic, grow rapidly on various carbohydrates at a concentration of 0.5%, and are chemically and serologically diverse. Type A strains may produce lipases, and media containing Tween 80 may inhibit growth due to accumulations of released fatty acids. Only one isolate in this group is known to be infective and effective on its original host plant. Type B strains have non-pigmented cells (but may release pigments into media), are microaerophilic, and do not import carbohydrates supplied at a concentration of 0.5%. Type B strains typically have a characteristic growth response which indicates that Tween 80 facilitates transport of certain carbohydrates into the cells.

Type B strains are chemically homogeneous, and they have been shown to be serologically related (Baker et al., 1981). All but one produces effective nodules on its host of origin. (See also Lechevalier and Ruan, 1984.)

There is currently no system for the characterization of Frankia species, but several thousand strains are available in pure culture worldwide. Most isolates are assigned a strain acronym unique to the isolating laboratory and meaningful mainly to the workers in that particular laboratory. In order to unify and simplify strain designations and to organize information concerning all of these strains, a committee to devise a cataloging system was chosen. M. P. Lechevalier, A. Quispel, M. Lalonde, and D. Baker have compiled the data available for most strains and have devised a standard cataloging system. Trivial acronyms are still used for the strains already used widely in studies, but are to be used in conjunction with the standard strain number. Table II lists the information requested for each catalog entry (Lechevalier, 1983).

Frankia Isolation and Cultivation

According to Koch's postulates, proof that an agent is responsible for an infection must always include 1) association of the agent with the infection, 2) growth of the pure agent apart from the source, 3) production of the infection in susceptible hosts using the pure culture as inoculum, and 4) reisolation of the agent from these experimental hosts.

TABLE II

STRAIN INFORMATION FOR
STANDARDIZED CATALOG

1)	Strain acronym; consists of collection designation (three letters) and numbers which specify host and strain (up to ten)
1a)	Parent strain
2)	Previous synonyms
3)	Primary reference (where first described)
4)	Secondary reference (where best described)
5)	Plant host of origin; host native or exotic to location
6)	Soil of origin, if applicable
7)	Locale of collection -- latitude, longitude, name
8)	Year and season of collection
9)	Plant passages prior to isolation (secondary hosts)
10)	Isolation from fresh, frozen, or dry nodules
11)	Type of nodule, if known (spore + = P; spore - = N)
12)	Whether nitrogen is fixed <u>in vitro</u>
13a)	Infective for b) Non-infective for
14a)	Effective for b) Non-effective for
15)	Whole cell sugar pattern
16)	Availability to others
17)	Other persons from whom the strain may be obtained
18)	Name, address, and telephone number of person reporting
19)	Other information the reporter would like to have recorded

From Lechevalier, 1983.

Callaham, del Tredici, and Torrey were credtted in 1978 with the first successful fulfillment of Koch's postulates using Frankia isolated from Comptonia peregrina. Other isolations from nodules had been made before, but the investigators were unable to achieve reinfection of host plants. (Uemura, 1952 and 1964; Lalonde et al., 1975; Baker and Torrey, 1979).

There are a number of obstacles in isolation of pure Frankia. Isolation requires that the ratio of contaminants to Frankia fragments and/or spores be reduced greatly and that a medium suitable if not selective for Frankia be used. Electron microscopic examination of soils reveals that soil contains 10^9 to 10^{10} bacteria per gram of dry soil (Nikitin, 1973; Brady, 1974). Perhaps 10^2 of these may be Frankia fragments or disseminules in rhizosphere soil (roughly estiimating using information from Baker and O'Keefe, 1984). The recent success of Baker and O'Keefe in isolating Frankia from two of six soil samples in spite of this unfavorable ratio was remarkable. Soil suspensions were prepared and treated with 0.7% phenol to reduce contaminant numbers. Small samples of the treated soil suspension were layered onto discontinuous sucrose density gradients, which were then ultracentrifuged. The lower interface (between 45 and 60% sucrose) was collected and used to inoculate pour plates. Cycloheximide was added to inhibit fungal growth.

Nodules contain concentrated populations of Frankia. Until Baker and O'Keefe obtained isolates from soil, all of the available isolates were taken from preparations of fresh,

frozen, or dried root nodule tissue. However, actinorhizal nodules are commonly coralloid in form and rough-surfaced. They have numerous places where minuscule soil particles, which carry most soil microorganisms, can cling. Soil microbes can adhere to and colonize the nodule surface to take advantage of the rich microhabitat. Callaham and coworkers (1978) used aeroponically grown Comptonia peregrina to avoid having clinging soil particles or microbe populations associated with water-grown nodules. They also used washing and nodule surface sterilization to minimize contaminantion. Surface sterilization was accomplished by incubating washed nodules in 0.1% mercuric chloride in 0.5% hydrochloric acid (Callaham et al., 1978). Other investigators who have successfully isolated Frankia have employed other surface sterilization methods. Chloramine T (1%) (Diem et al., 1982), and sodium hypochlorite (1%, as diluted laundry bleach) (Benson, 1982) have been used. Callaham and coworkers ensured that nodule lobes were not contaminated by incubation in rich liquid medium (M-3 medium, Table III); cultures which became turbid during a six week incubation period were discarded.

The next step in isolations from nodules is to release the Frankia. Baker and Torrey (1979) list a number of nodule disruption techniques used by investigators. One method uses enzymatic digestion of nodule sections with cellulase and pectinase in nutrient medium (BD medium, Table IV), followed by teasing apart the nodule lobes with dissecting

needles (Callaham et al., 1978). Other methods include microdissection (eg. Pommer, 1959), homogenization (Benson, 1982), or crushing nodules (eg. Lalonde, et al., 1979). Of these techniques, crushed nodule suspensions are least time consuming, tedious, and expensive to prepare.

Some investigators chose to further purify their preparations at this point to further select for Frankia. Callaham and coworkers (1978) filter the suspension prepared from enzymatically treated and mechanically disrupted nodules through nylon screen, and pelleted and washed the filtrate by centrifugation. Benson (1982) filtered homognized nodule suspensions through nylon screens differing in mesh sizes to remove particles larger and smaller than Frankia clusters. Baker and colleagues have used sucrose density gradient fractionation (Baker et al., 1979).

Finally, the preparation which hopefully contains mainly viable Frankia must be cultured in some medium. Enrichments for Frankia may include the use of certain antibiotics, low nutrient media, or pretreatment of the suspensions with heat, antibiotics, or centrifugation (Baker and Torrey, 1979). H. G. Diem has successfully used agar containing no added nutrients at all for Frankia isolations; this technique selects against fast-growing contaminants (personal communication). Obviously, nutrient media will eventually be required. Callaham and colleagues (1978) plated two milliliter aliquots of resuspended pellet on 6.0 centimeter plates of supplemented BD medium (Table IV). Other investigators use solidified

TABLE III

M-3 MEDIUM

Component	Concentration	
Sucrose	20.0	g per liter
Mannitol	1.0	
Edamin	1.0	
Yeast extract, Difco	0.5	
$CaCO_3$	0.5	
K_2HPO_4	0.5	
$MgSO_4 \cdot 7H_2O$	0.2	
NaCl	0.1	
H_3BO_3	0.1	
$MnSO_4 \cdot H_2O$	25	mg per liter
$ZnSO_4 \cdot 7H_2O$	10	
$Na_2MoO_4 \cdot 2H_2O$	2.5	
$CuSO_4 \cdot 5H_2O$	0.25	
Nicotinic acid	0.5	
Thiamin hydrochloride	0.1	
Pyridoxine hydrochloride	0.1	
Agar (optional)	10.0	g

pH adjusted to 7.0

Table IV

SUPPLEMENTED BD MEDIUM

Chemical	Concentration
Sucrose	40 g per liter
$Ca(NO_3)_2 \cdot 4H_2O$	242 mg
$MgSO_4 \cdot 7H_2O$	42
KNO_3	85
KCl	61
KH_2PO_4	20
$FeCl_3 \cdot 6H_2O$	2.5
Thiamine HCl	0.1
Nicotinic acid	0.5
Pyridoxine HCl	0.5
H_3BO_3	1.5
$ZnSO_4 \cdot 7H_2O$	1.5
$MnSO_4 \cdot H_2O$	0.25
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.04
Naphthleneacetic acid	2.0
Zeatin	0.001
L-glutamic acid	1.0 mM
L-aspartic acid	1.0 mM
Glycine	1.0 mM
L-arginine	1.0 mM
L-asparagine	1.0 mM
L-glutamine	1.0 mM
Urea	1.0 mM

Lalonde's QMOD B (Tables V and VI) (Lalonde and Calvert, 1979; Diem, et al., 1982). Diem and colleagues suggest that a thin layer of 1.5 % agar spread over a plate containing the same agar underneath will enhance Frankia growth by making conditions more uniform and by increasing spatial separation over that afforded by a moist agar surface.

Media

Lalonde and Calvert's (1979) QMOD B (Table V) was the medium most widely used to culture Frankia strains at the time this study was begun. The Frankia broth of Baker and Torrey (1979) (Table VII) was thought to lack some nutrients required for continued culture. Lalonde and Calvert (1979) had reported that moderate levels of lecithin stimulated growth. Frankia has now been cultured in a variety of complex media. Unfortunately QMOD, a complex medium containing insoluble components and available at the start of this investigation, was not suitable for further defining nutritional requirements or substrate utilization. In addition, the insoluble ingredients of QMOD interfered with estimation ,of growth either by visual approximation, by dry weight, or by harvesting by centrifugation. More recently, defined media have been used by other investigators (Blom, 1980, 1981, 1982; Tjepkema, 1980; Burggraaf and Shipton, 1982; Murray et al., 1984) to test nutritional requirements. Blom's medium (1982) is listed in Table VIII. The carbon sources used as substrates by some Frankia strains are listed

TABLE V

QMOD B

Component	per liter
K_2HPO_4	300 mg
NaH_2PO_4	200 mg
$MgSO_4 \cdot 7H_2O$	200 mg
KCl	200 mg
Yeast extract (BBL)	500 mg
Bacto-peptone (Difco)	5 g
Glucose	10 g
Ferric Citrate (Citric acid and ferric citrate, 1% sol)	1 mL
Trace Mineral Solution, Table	1 mL
Deionized H_2O to	1 L
Adjust pH to 6.8-7.0	
then add:	
$CaCO_3$	100 mg
Lipid supplement*	0.5-50 mg
Agar (optional)	15 g

* Prepared as 500 mg L-α-lecithin (commercial grade from soybeans, 22% phosphatidyl choline, Sigma Chemical Co., St. Louis, Missouri) in 100 mL 50% ethanol.

TABLE VI

TRACE MINERAL SOLUTION FOR QMOD B

Component	Concentration
H_3BO_3	1.5 g/L
$MnSO_4 \cdot 7H_2O$	0.8
$ZnSO_4 \cdot 7H_2O$	0.6
$CuSO_4 \cdot 7H_2O$	0.1
$(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$	0.2
$CoSO_4 \cdot 7H_2O$	0.01

TABLE VII

FRANKIA BROTH (YD MEDIUM)

Component	Concentration
Yeast extract (Difco)	0.5% (w/v)
Dextrose	1.0% (w/v)
Casamino acids	0.5% (w/v)
Cyanocobalamin	1.6 mg/L
H_3BO_3	1.5 mg/L
$ZnSO_4 \cdot 7H_2O$	1.5 mgL
$MnSO_4 \cdot H_2O$	4.5 mg/L
$Na_2MoO_4 \cdot 2H_2O$	0.25 mg/L
$CuSO_4 \cdot 5H_2O$	0.04 mg/L
Agar (optional)	0.8% (w/v)

final pH adjusted to 6.4

TABLE VIII

BLOM'S MEDIUM

Compound	Concentration
K_2HPO_4	300 mg/L
NaH_2PO_4	200
KCl	200
$MgSO_4 \cdot 7H_2O$	200
$CaCO_3$	100
Iron citrate	10
$ZnSO_4$	1
Riboflavin	0.1
Thiaminium dichloride	0.1
Biotin	0.1
Nicotinic acid	0.1
Pyridoxine hydrochloride	0.1
Calcium Pantothenate	0.1
Folic acid	0.1
H_3BO_3	1.5
$MnSO_4 \cdot 7H_2O$	0.8
$CuSO_4 \cdot 7H_2O$	0.1
$(NH_4)_6 Mo_7O_{24}$	0.2
$CoSO_4 \cdot 7H_2O$	0.01

in Table IX. Glucose is utilized by only one strain. Sugars, the favored substrates of many microbes, are rarely utilized by Frankia strains. Salts of organic acids are, however, commonly utilizable.

Although Frankia strains are capable of fixing nitrogen, they may not do so in laboratory media. Therefore, sources of combined nitrogen are commonly added to media to be used to culture Frankia. Ammonium ions are taken up by Frankia cells (Blom, 1981) and are utilized as nitrogen sources (Blom, 1982; Shipton and Burggraaf, 1982a). Nitrate is also used by some strains (Shipton and Burggraaf, 1982a). Organic nitrogen sources utilized by some Frankia include glutamic acid, aspartic acid, and urea.

Some media for growing Frankia include vitamins. Blom adds a mixture of seven vitamins to his medium (Table VIII), apparently to make certain that the common required vitamins were present in order to determine substrate and nitrogen source requirements (Blom, 1982). He does not study growth in relation to vitamin content of the medium. Shipton and Burggraaf (1982b) added two mixtures of vitamins to media in order to obtain information about growth factor requirements for Frankia. They added nicotinic acid, thiamin, pyridoxine, and cyanocobalamine to one set of media; and nicotinic acid, thiamin, pyridoxine, calcium pantothenate, riboflavin, folic acid and biotin to another. They found that biotin alone could account for most of the stimulation of growth seen in the second medium.

TABLE IX

CARBON SOURCES USED BY SOME *FRANKIA* STRAINS

Strain	Carbon Sources	Reference
AvcIl	Acetate	Blom, 1982,
	Tween 40 80 Tween 85 Caprylic acid Propionic acid	Blom, 1981 Tween
CpIl	Succinate Fumarate Malate Acetate	Blom, 1982
	Tween 80 Propionic acid	Tisa et al., 1983
Ag + l	Tween 80 Acetate	Blom, 1982
Eanl pec	Acetate Succinate Malate Mannitol Sorbitol Fructose Sucrose Trehalose Cellobiose	Tisa et al., 1983
EuIl c	Cellobiose Trehalose Galactose Glucose Propionate	Tisa et al.,

Burggraaf and Shipton (1982) found that strains LDAgp1, AvcI1, and CpI1 have a pH optimum between 6.5 and 7.0 units at 28.5°C, with most strains preferring the lower values. Growth yields at 13 days for the three respective strains by dry weight evaluations were between 0.125-0.195 mg/mL, 0.075-0.168 mg/ml, and 0.125-0.175 mg/mL. A pH above 8 units was found not to favor growth (Burggraaf and Shipton, 1982). Callaham and coworkers (1978) state that CpI1 grew optimally at pH 6.4 at 25-30°C.

Other Conditions of Culture

Factors other than medium composition were considered for routine culturing of Frankia. Among these were aeration, temperature, and method of inoculation.

Frankia was considered microaerophilic (Blom et al., 1980; Callaham et al., 1978). Frankia grows slowly under any conditions, but even more slowly on agar surfaces and in shaken liquid culture, and not at all under anaerobic conditions (Callaham et al., 1978; Blom et al., 1980). It has been found to grow best in liquid cultures without shaking, although it is now routinely grown with shaking or sparging with air for acetylene reduction assays (Murray et al., 1984).

Depending on the strain, Frankia grows optimally in the temperature range of 25 to 35°C. Many strains grow best at about 30°C (Callaham, et al., 1978; Burggraaf and Shipton, 1982). Lalonde and Calvert incubated Frankia cultures

in QMOD B at 27°C (Lalonde and Calvert, 1979).

Frankia cultures are commonly inoculated and propagated from concentrated homogenates made by one of two methods. Some researchers repeatedly draw up and expel mycelial fragments through a small hypodermic needle using a syringe (Burggraaf and Shipton, 1982; Normand and Lalonde, 1982). Berry and Torrey (1979) used a a Potter-Elvehjem tissue grinder with a Teflon pestle to prepare homogenates.

Xerotolerance

Xerotolerance is the term applied to an organism's ability to withstand desiccation. For bacteria, osmotolerance can be used to measure xerotolerance. Xerotolerance and osmotolerance are related in that both refer to the non-availability of water. Xero- refers to the absence of water while osmo- refers to the fact that the water is not available to the organisms due to strong non-covalent chemical bonding. Osmotic stress can be induced by adding a soluble chemical to the culture medium which water molecules in the medium will surround to form a shell of hydration.

Water potential, which is one measure of the quantity of water bound by molecules in solution, consists of two components: the pressure potential and the osmotic potential. The pressure potential component is generated by hydrostatic pressure. When concerned with water potential at atmospheric pressure, the pressure potential component is defined as zero. The osmotic potential factor refers to the degree to

which the water molecules are held by chemical interaction with molecules in solution (osmotic potential) and/or by adsorption to particles such as clay (matrix potential). Because the components are additive for total water potential, at atmospheric pressure water potential is equal to osmotic potential when one is concerned with liquid media. These two terms are sometimes used interchangeably where conditions such as those for this research are involved (Salisbury and Ross, 1978).

Some compounds used as osmotica ionize when dissolved in water. Salts such as sodium chloride can severely affect metabolic processes such as enzyme function, and thus inhibit growth. Ions can act as perturbing solutes, which interact with ligands and active sites of enzymes to perturb enzyme function, and they can affect the hydration, solubility, and charge interactions of the protein to disrupt its structure (Yancey et al., 1982). Sugar interactions with proteins are much more complex, and no definite pattern of effects is yet certain. Generally, hexoses are less inhibitory of enzyme function than other sugars. Fructose produces inihibition slope patterns similar to glycerol, which is a common natural osmolyte, for yeast isocitrate dehydrogenase (Brown, 1976). Fructose, glucose, mannitol, and sucrose are sometimes themselves found to be the principal osmolytes in non-animal systems (Yancey et al., 1982).

Furthermore, ionic and non-ionic solutes both effect water potentials by affecting the osmotic potential compo-

nent, whereas solutions of high molecular weight polyols, such as polyethylene glycol (PEG) exert water stress mainly by increasing matrix potentials (Shipton and Burggraaf, 1982b). They believe that a PEG-controlled system is more like the natural situation in the soil than the osmotically (NaCl) controlled system, although there were only small differences in growth responses they observed for a single strain in either system. These investigators believed that the Frankia strains they tested showed greater sensitivity to PEG than to sodium chloride. In addition, certain lots of PEG may contain some unknown toxic factor while other lots do not (Shipton and Burggraaf, 1983).

Monosaccharides and other small polyols are capable of exerting osmotic potential in media; they do not ionize significantly in solution, and they are often found as intracellular osmolytes (Yancey, et. al, 1982).

An osmoticum for determining xerotolerance should not affect the cell except to bind extracellular water. Such osmotica are "compatible solutes" in that they are not required for metabolic function, nor are they injurious to the cell; they function as osmotically active substances and as enzyme activity protectors (Brown, 1976). In addition, the osmoticum should not be imported or utilized in large quantities by the organism, for it would no longer exert the environmental stress as was intended.

Approximate osmolalities of solutions containing various concentrations of many different osmotica can be calcu-

lated using the tables provided by Wolf and coworkers (1980).

The effects of water stress on legume root nodules have been explored (reviewed by Sprent in 1976). Some Rhizobium-legume nodules may be irreversibly damaged by water stress. Although Rhizobium-nodulated cowpea (Vigna unguiculata) can grow in unwatered soil for 42 days in semi-arid field conditions, nitrogen fixation capacity is decreased by about 75% (Zablotowicz and Focht, 1981). Cowpea is exceptional in its water stress resistance for a legume; most leguminous systems are unsuitable as nitrogen fixers in dry soils. Scotch broom (Cytisus scoparius) is unable to fix nitrogen at a soil water potential of -5 bars (Helgerson et al., 1979) (1 bar = 0.986 atmosphere).

However, in field studies, nodules of some actinorhizal hosts have been shown to be more water stress resistant than leguminous associations. Nodulated Purshia from a dry site was able to continue appreciable nitrogen fixation at -24 bars (Dalton and Zobel, 1977); Ceanothus velutinus in another study continued to fix measurable dinitrogen at -20 bars (McNabb et al., 1979). (Seawater has a water potential of about -28 bars at atmospheric pressure (Salisbury and Ross, 1978.))

Isolated Rhizobium japonicum strain USDA191 is a recently isolated salt-tolerant strain infective for soybean and cowpea. Yelton et al. (1983) have shown that it can grow at sodium chloride concentrations up to 0.4 molar. This strain of Rhizobium is quite exceptional, as it is able to grow at

water potentials of about -16.7 bars. This potential is almost as low as that produced by 12% (w/v) glucose.

Shipton and Burggraaf (1982b, 1983) have investigated the osmotolerance of Frankia strains LDAgpl, AvcIl, CpIl, and LDMgl. They used potassium chloride, sodium chloride, and polyethylene glycol 4000 (PEG) as osmotica; their Frankia medium is described in Table X. The first three isolates were able to grow in media of water potentials to between -2 and -5 bars when sodium chloride was used as osmoticum. LDMgl was able to grow at water potentials down to -13 bars when PEG 4000 was used as osmoticum.

Many growth yields formerly were commonly given as dry weight of total culture per volume of medium. Currently most Frankia growth yield data are given as protein yield by weight per volume of culture. The major problem associated with protein determinations is releasing the proteins from the mycelium. Burggraaf and Shipton (1982) presented a comparison of total culture dry weight, total protein and ATP content data (for the latter two mycelia were disrupted by sonication for one minute at 150 watts with a Bronson sonifier). Digestion for 14 hours at 40° C with 0.1N sodium hydroxide has also been used to prepare mycelia for total protein determinations (Vogel and Dawson, 1985).

Host Plant Inoculations

Host plants have been inoculated by several methods. Baker and Torrey (1979) have achieved nodulation by adding

TABLE X

SHIPTON AND BURGGRAAF'S MEDIUM

Component	Concentration
Propionic acid	0.5 grams per liter
$CaCl_2 \cdot 2H_2O$	0.1
$MgSO_4 \cdot 7H_2O$	0.2
NH_4Cl	0.096
K_2HPO_4	1.0
$NaH_2PO_4 \cdot 2H_2O$	0.67
EDTA-Fe III Na chelate	0.01
$CoCl_2$	0.05
H_3BO_3	0.0015
$ZnSO_4 \cdot 7H_2O$	0.0015
$MnSO_4 \cdot H_2O$	0.0045
$Na_2MoO_7 \cdot 2H_2O$	0.00025
$CuSO_4 \cdot 5H_2O$	0.00004

pH adjusted to 6.8

liquid media containing Frankia mycelial fragments to liquid growth media in which host plants were growing. Lalonde and Calvert (1979) reported that nodulation would occur when approximately 0.25 milliliter of a suspension of homogenized Frankia which had an optical density of 0.03 was applied to ungerminated seeds, or to the liquid growth medium of 30 or 40 days old seedlings.

Frankia inocula remain infective for several months, or as long as eighteen months. Dr. van Dijk (1978) reported that axenic root nodule homogenates stored at 4-6° C were infective after several months of storage. Quispel (1960) observed no loss, and even an increase of infectivity of nodule suspensions which had been stored at room temperature when peat or alder root extracts were added to the preparations.

Conclusions

At the time at which this investigation was begun the construction of a model system for investigating effects of water stress on the actinorhizal system Alnus plus Frankia required 1) development of a medium which would allow estimation of growth yields, 2) search for non-metabolizable compatible osmotica suitable for each available Frankia strain, 3) determination of osmotolerance differences between available strains and their effectiveness in the Alnus clones used, 4) isolation of Frankia from relatively xerotolerant Alnus (Hennessey et al., 1985), and 5) development of routines for inoculating experimental Alnus clones.

CHAPTER III

MATERIALS AND METHODS

Frankia Strains

The *Frankia* strains used in this study are listed in Table XI by both trivial acronym and (where known) standard catalog number (according to Lechevalier, 1983). Trivial acronyms typically derive from the host of origin and isolate number; a few others contain other information. The origin and any interesting characteristics of these strains are listed below.

Most *Frankia* colonies in liquid media have the same general appearance. They are typically cream-colored, and form rounded, flocculent colonies. If a culture is heavily inoculated, a loose mat is formed which is easily disrupted into individual pieces by mild agitation.

PtIl was isolated and generously provided by D. Baker, C. F. Kettering Research Laboratory, Yellow Springs, Ohio. PtIl was isolated from *Purshia tridentata* nodules collected in Laramie, Wyoming. It forms large colonies in liquid culture. A light brown pigment is found in the hyphae and medium. A large portion of the colonies adhere tightly to the culture flask, often forming a rough mat along the submerged surface of the flask. Large, smooth, rounded colonies are

TABLE XI

STRAIN ACRONYMS

Trivial Acronym	New Standard Number
PtI1	DDB 170110
MP1	--
ARgP5 AG	ULQ 0132105009
ARbN4 β i	--
AVP3f	--
ACN1 11m	--
ACN1 11q	--
ACN1 AG	ULQ 0102001007
AGN1 $\frac{AG}{EXO}$	--

-- indicates that the standard is not yet published.

also free in the medium.

MPl was isolated by H. S. Vishniac in this laboratory. Alnus rubra root nodules collected on Mary's Peak, Corvallis, Oregon served as source material. MPl forms a typical colonies and excretes a yellow pigment which diffuses into the medium.

All of the remaining strains were gifts of M. Lalonde, Universite Laval, Quebec, Canada. The strain acronyms usually include a two or three letter abbreviation of the host of origin, a letter indicating whether the nodules contained sporogenous endophytes upon microscopic examination prior to isolation, and other information.

ARgP5 AG was isolated from Alnus rugosa. The nodules used for isolation were found to contain spore-forming Frankia, and five is the isolate number. The strain was isolated, grown in pure culture, inoculated into Alnus glutinosa, and reisolated from the resulting nodules. The "AG" indicates that the isolate was passed through a secondary host, and the underlining signifies that the secondary host was inoculated using a purified isolate rather than a crushed nodule suspension. ARgP5 AG mycelia form typical colonies, and do not form pigments under routine culture conditions.

ARbN4 β i was subcultured from a parent strain ARbN4b, an isolate from Alnus rubra. ARbN4 β i is a morphological variant. The colonies are usually cream-colored and rounded, but are smaller and more compact than the typical Frankia colony. Occasionally, a light brown pigment is present in

both medium and mycelium, but less than is made by PtIl. ARbN4 β i also adheres to the culture flask in some cases, but in smaller proportions than does PtIl. When examined under bright field and phase contrast light microscopy, the colonies appear to be more densely packed than those of the other strains maintained in this laboratory.

AVP3f is a spore-positive isolate from Alnus viridis nodules. No pigments have been observed.

ACNl llm, ACNl llq, and ACNl AG are all isolates from Alnus crispa (Alnus viridis subspecies crispa). ACNl AG was used to inoculate Alnus glutinosa as a secondary host in a crushed nodule suspension prior to isolation and in vitro culture.

AGNl $\frac{AG}{EXO}$ was isolated from Alnus glutinosa. This host served as both primary and secondary hosts, and the inoculum for the secondary host was a crushed nodule suspension. The meaning of the subscript "EXO" is not known.

Chemicals and Media

The chemical ingredients used in media preparations were obtained from Sigma Chemical Company, St. Louis, Missouri. Reagent grade chemicals were used. Peptone and yeast extract were obtained from Difco Laboratories, Detroit, Michigan. Yeast extract was also obtained from BBL Laboratories, Cockeysville, Maryland. Water was purified using a Sybron/Barnstead Nanopure ion exchange system.

Strain Maintenance

All strains from Alnus nodules used or isolated in this study were maintained in ten milliliter cultures in 3AP/2 (Tables XII, XIII, and XIV) or in modified QMOD B (Tables XIII and XV). PtIl was maintained in 3AP/2 or modified Frankia broth (Tables XIV and XVI). Minor modificatons in media were made in order to make use of available chemicals, or to simplify preparation. Two sets of stock cultures were kept: one unused flask per strain from the previous conservation transfer, and two flasks per strain made by dividing the colonies from an older duplicate flask equally between flasks of fresh medium. The colonies were transferred by Pasteur pipet, and a minimum of spent medium was transferred. Transfers were made approximately once every three to four months.

One hundred milliliter cultures were grown routinely for the production of inoculum stocks. These cultures were grown in 3AP/2 for one month before being homogenized. The concentrated homogenized mycelia were stored refrigerated (4° C in a series 69021 Kenmore frost-free refrigerator-freezer) in phosphate buffered saline (Table XVII).

Conditions of Culture

When this study was begun, cultures were incubated at room temperature out of direct sunlight; room temperature varied between 18 and 32° C. Cultures were later incubated in a Precision Model 815 low temperature incubator at 28° C.

TABLE XII

3AP/2

Component	Concentration
$MgSO_4 \cdot 7H_2O$	100 mg per liter
KCl	100 mg
* K_2HPO_4	150 mg
* NaH_2PO_4	100 mg
Lalonde trace mineral solution, modified (Table XIV)	0.5 mL
@ Iron citrate solution	0.5 mL
Difco Bacto-peptone	1.0 g
Yeast extract (BBL or Difco)	250 mg
Frankia vitamin mix, 100X (Table XIII)	5.0 mL
NH_4Cl	0.5 mM
NaH glutamate, pH 6.8	0.2 mM
# Na acetate, pH 6.8	2.5 mM
# Glucose	5.0 g

pH 6.8

* Prepared as one solution 100X for convenience
@ Added as a solution containing 10.9 g citric acid and 47.1 g ferrous sulfate (heptahydrate) per liter.
\# Aseptically added as separately autoclaved solutions.

TABLE XIII

FRANKIA VITAMIN MIX

Component	Final Concentration
Biotin	2 mg
Calcium pantothenate	400 mg
Cyanocobalamin	1.6 mg
Folic acid	2 mg
Nicotinic acid	400 mg
p-Aminobenzoic acid	200 mg
Pyridoxine HCl	400 mg
Riboflavin	200 mg
Thiamine HCl	400 mg

TABLE XIV

MODIFIED LALONDE TRACE MINERAL SOLUTION

Component	Concentration
H_3BO_3	1.5 g per liter
$MnSO_4 \cdot 7H_2O$	800 mg
$ZnSO4 \cdot 7H_2O$	600 mg
$CuSO_4 \cdot 5H_2O$	100 mg
$CoCl_2 \cdot 6H_2O$	8.5 mg
$Na_2 MoO_4 \cdot 2H_2O$	3.9 mg

TABLE XV

MODIFIED QMOD B

	Component	Concentration
*	K_2HPO_4	300 mg per liter
*	NaH PO	200 mg
	$MgSO_4 \cdot 7H_2O$	200 mg
	KCl	200 mg
	BBL yeast extract	500 mg
	Difco Bacto-peptone	5 g
@	Iron citrate solution	1 mL
	Lalonde trace minerals solution (Table XIV)	1 mL
	$CaCO_3$	100 mg
#	Glucose	10 g
**	Lipid supplement	0.5-50 mg

* Prepared as one solution 100X for convenience.
@ Added as a 1% solution of ferric acid and ferric citrate.
\# Aseptically added as separately autoclaved solutions.
** In solution as 500 mg L-α-lecithin (phosphatidyl choline) in 100 mL 47% ethanol.

TABLE XVI

MODIFIED *FRANKIA* BROTH

Component	Concentration
BBL yeast extract	5.0 g per liter
Difco casein hydrolysate	5.0 g
Lalonde trace mineral solution (modified, Table XIV)	1.0 mL
Glucose (added aseptically after autoclaving)	10.0 g

TABLE XVII

PHOSPHATE BUFFERED SALINE
(PBS)

Component	Concentration
NaCl	9.0 g per liter
K_2HPO_4	300 mg
NaH_2PO_4	200 mg
pH 6.8	

Cultures were incubated under aerobic conditions without agitation. Batch cultures except those of one liter in volume were prepared in Erlenmeyer or microfernback flasks to contain two-fifths of the total flask volume. One liter cultures were made in two liter Erlenmeyer flasks or in 2.8 liter Fernback flasks. The surface to volume ratios for flask-grown cultures varied betwwen 0.3 and 0.9 square centimeters per milliliter.

Because Frankia grows slowly, cultures must be incubated for longer periods than most bacteria. Evidence of growth may take as long as two weeks to appear, and maximum yields may require four to five weeks under usual conditions. Most incubations were continued for four weeks, or about 30 days before final evaluations of growth were made.

The long incubations dictate that every precaution be taken against contamination. Flasks with volume capacities exceeding 25 milliliters were closed with cheesecloth-covered cotton wool plugs. The plugs and the mouth of the flasks were covered with aluminum foil skirts that extended below the bottom level of the plug, ideally to where the flask diameter begins to increase for ease in handling. This arrangement allows for easy aseptic manipulations of the medium or culture and prevents most contamination due to microbes which would fall on the otherwise exposed portions of the plug or on the flask lip. Erlenmeyer or microfernback flasks with a 25 milliliter capacity were capped with loosely fitting flat-top glass or fluted-top aluminum caps (20 milli-

meter aluminum test tube closures). These small flasks were arranged in sets in Pyrex baking dishes, using one identical dish inverted over the other as a cover. Media were autoclaved in the flasks inside the dishes; after cooling, all sides were sealed with inch wide cellophane tape to minimize air flow and to stabilize the arrangement. Aseptic manipulations were usually carried out in a Germfree Laboratories Bioflow Chamber laminar flow hood. Media were incubated at room temperature for at least three days after aseptic additions in order to insure sterility.

Cultures were inoculated either by transferring colonies by pipet or by adding 0.1 milliliter (typically) of homogenized mycelia per 100 milliliters medium. Inoculum sizes as measured by colony- forming units varied with age and density of the individual homogenate from which the sample was taken.

Homogenization Procedure

Large cultures were first placed in a tilted position to allow the colonies to settle along one side. When space permitted, the flasks were supported by test tube racks in the laminar flow hood. Flasks containing coherent mats did not require the settling step. Approximate twelve milliliter aliquots of settled culture or mats, or entire ten milliliter cultures were transferred to 16 milliliter polyallomer centrifuge tubes which were cheesecloth and cotton wool plugged and aluminum foil skirted (as described for culture flasks).

Centrifugation in an International Equipment Company

Model CL centrifuge was used to pellet (or concentrate in some cases) the cells. The medium was decanted aseptically by pouring, and ten milliliters of phosphate-buffered saline (PBS, Table XVII) was added. The resuspended cells were recentrifuged; tubes containing the same strain or treatment were combined, and the wash steps were repeated. The washed mycelia were then transferred to sterile, plugged and skirted Potter-Elvehjem tissue grinder tubes; centrifuge tubes were then rinsed with a small amount of PBS to complete the transfer. The colonies were sheared using three to six strokes (as necessary) of the sterilized ground glass or Teflon pestle so that the colonies were well disrupted. Occasionally colonies were still visible in the suspension; in order to avoid killing too many of the other mycelial fragments, these were often left intact. The concentrated homogenates were then traansferred to sterile 18 millimeter screw-capped test tubes. Final volumes were adjusted with PBS to approximately one-tenth of the original culture volume. If more homogenate was required the volume was increased slightly; if culture yield was especially low, less PBS was added Homogenates were not diluted after preparation, nor were homogenates of the same strain mixed. All homogenates except those intended for plant inocula were labelled and stored refrigerated (4° C) until needed.

Large cultures of Frankia were not found to centrifuge well in a large ultracentrifuge rotor because during the time

it took for the rotor to stop, the colonies had resuspended. In order to proceed quickly, the settling step was included in the harvesting procedure. The fact that Frankia naturally settle was useful in concentrating them, and it was useful in designing procedures where gentle handling is essential.

The time required for settling depends on the strain and on the size of the culture. For example, PtIl adheres to the flask; they must first be loosened with a sterile pipet or other instrument. Small, compact colonies such as those of ARbN4 β i take longer to settle and are easier to resuspend. On the other hand, cultures in which a "mat" has formed are easily removed with a large bore pipet.

Visual Growth Evaluations

Serial dilutions or "titrations" of inocula were made in zgrowth medium in order to determine the number of colony-forming units (viable mycelial fragments plus spores or other structures) present in homogenized inocula. One milliliter of homogenate was pipetted into ten milliliters of medium. Ten-fold serial dilutions were then made to the order of 10^{-6} to 10^{-8}. At the end of the incubation period, the endpoint was determined by finding the last flask containing approximately ten colonies, or by the dilution which had about as many colonies as the next higher dilution. The number of colonies was multiplied by the inverse of the dilution factor, and the titer was recorded as colony-forming units per milliliter of homogenate (CFU/mL). Inoculum sizes were

calculated by multiplying the inoculum titer by the inoculum volume in milliliters to yield colony-forming units.

During the month long incubation periods needed for most experiments, growth yields were monitored by visual estimations at approximate one week intervals. Discrete colonies were counted, or their equivalent numbers estimated from mycelial mass. The counts were recorded and translated into a grade system using pluses and zeros. One plus symbol is given for every fifty colonies. Table XVIII lists the symbols used for ten milliliter cultures and their interpretations. Other experiments are given individual keys.

The visual estimation system is not as precise as other methods of growth evaluations. The colonies could not always be counted accurately, especially in early stages of the investigation when insoluble medium components could be mistaken for colonies, or when there were more than approximately seventy-five colonies in a flask. Estimations were also subjective, and varied between experiments. These comparisons are most reliable between cultures of the same size containing the same inoculum. When comparing the growth yields between strains in a single experiment, the inoculum size must also be considered. Visual evaluations are not useful for comparisons in growth yields between separate experiments; each experiment must be considered separately. The evaluations were simplified by the use of homgenized inocula, which allowed colony development and growth to be more easily judged. Visual evaluations were adequate for

TABLE XVIII

VISUAL EVALUATION GRADE SYSTEM
FOR TEN MILLILITER CULTURES

Grade	Interpretation
++++	200 or more colonies, or mat formed on flask bottom
+++	Approx. 150 colonies
++	Approx. 100 colonies
+	Approx. 50 colonies
$\pm$	Poor growth, or indistinguishable from precipitate in medium
0	No growth
C	Contaminated

most of this work.

Host *Alnus* Clones

All host trees, seedlings, and cuttings were prepared and cared for by Department of Forestry personel under the direction of Dr. T. C. Hennessey. This work is primarily concerned with inoculation of the host plants to determine optimum inoculation method, and to determine host ranges of the *Frankia* strains. The *Alnus* species and clone numbers are listed in Table XIX along with the location of the seed sources. The numbers following the seed source numbers indicate the clone number of the particular tree which served as the source of cloned cuttings.

Longevity of Homogenates

To determine how well homogenized *Frankia* can survive in low nutrient media at refrigerator temperatures, homogenates of MPl and PtIl were prepared as for routine homogenates and stored in phosphate buffered saline at approximately 4°C. At approximate one month intervals, one milliliter of each homogenate was diluted in nutrient media (PtIl in modified *Frankia* broth, and MPl in modified QMOD or 3AP/2) as described for "titrations." Visual estimations of colony numbers were made at regular intervals until all of the homogenate was used.

TABLE XIX

ALNUS CLONES

Clone	Location of Seed Source
A. glutinosa	
2-58	Unknown
4x09-11	German Seed Orchard, Germany
4x23-49 4x23-54	German Seed Orchard, Germany
8022-05 8022-14 8022-16 8022-24	Astaneh-Ashrafieh, Iran
8023-05	Astaneh-Ashrafieh, Iran
A. maritima	Johnston County, Oklahoma
A. rubra	Idaho County, Idaho
A. serrulata	McCurtain County, Oklahoma

Medium Development

Most of the strains used in this study are isolates from Alnus species. These isolates were first cultured in this laboratory in modified QMOD B medium. PtIl, and isolate from Purshia tridentata, was first maintained in modified Frankia broth. (These media are described above.) The isolates from different host genera were maintained in separate media until a medium was devised which would support the growth of all strains.

The present work began with medium development experiments in order to devise a medium suitable for osmotolerance tests. The first phase of medium development was to find a medium in which water-insoluble and undefined ingredients were eliminated.

Two growth medium bases were formulated for Alnus isolates (Tables XX AND XXI), using QMOD B (Lalonde and Calvert, 1979) and the work of Blom et al., (1980), Blom, (1981: 1982), and the work of Tisa and coworkers (personal communication to H. S. Vishniac, published in 1983) as guides. To these bases, one of three combinations of substrates and supplements was added such that six separate media were prepared. The combinations were (final concentrations given):

1) 0.5% peptone,

2) 1.0 mM ammonium chloride,
   0.4 mM sodium glutamate.

3) 1.0 mM ammonium chloride,
0.4 mM sodium glutamate,
5.0 mM sodium acetate.

Triplicate 10 milliliter flasks aliquots of media were inoculated with 0.3 milliliter of MP1 homogenate (inoculum size unknown) for each variation. After 37 days at room temperature, final estimates of growth were recorded.

The requirement of MP1 for water-insoluble ingredients was tested by preparing a base medium which contained those ingredients listed for "A" base except calcium carbonate, lecithin, and peptone; ammonium chloride and sodium glutamate were added to each flask in the same concentrations as for the third set of additions. Two sets of additions were devised which resulted in sixteen different test media. Two different carbon sources, sodium acetate (5 mM) and Tween 80 (polyoxyethylene [20] sorbitan mono-oleate, 0.2%) were tested, both with and without added peptone (0.5%). To each of these four combinations of carbon sources was added water only, calcium carbonate (1 mg/mL), lipid supplement (0.5 µg/mL), or calcium carbonate plus lipid supplement. Each flask contained ten milliliters of medium, and received a nonhomogenized inoculum of 0.1 mL of MP1 cultured in QMOD B. Because the inoculum for each flask contained small amounts of both lecithin and calcium carbonate, a continuation of this experiment was necessary. In the first series of media, the maximum amount of calcium carbonate added in the inoculum was calculated to be 0.01 mg, and the maximum amount of lipid was 0.05 µg. After 27 days of incubation, the flask

TABLE XX

BASE EXPERIMENT MEDIA :
BASE "A"

Component	Concentration
* K_2HPO_4	300.0 mg per liter
* NaH_2PO_4	200.0 mg
$MgSO_4 \cdot 7H_2O$	200.0 mg
Lalonde trace mineral solution, modified (Table XIII)	2.0 mL
@ Iron citrate solution	0.5 mL
Wickerham's vitamin mix, modified, 100X (Table XXII) (van der Walt and Yarrow, 1984)	10.0 mL
BBL yeast extract	50.0 mg
KCl	200.0 mg
$CaCO_3$	100.0 mg
# Glucose	10.0 g
** Lipid supplement	5.0 mg

* Prepared as one solution 100 X for convenience @ Added as a solution containing 10.9 g citric acid and 47.1 g ferrous sulfate (heptahydrate) per liter.

\# Aseptically added as separately autoclaved solution

** In solution as 500 mg L-α-lecithin in 100 mL 47% ethanol.

TABLE XXI

BASE EXPERIMENT MEDIA
BASE "B"

Component	Concentration
* K_2HPO_4	300.0 mg
* NaH_2PO_4	200.0 mg
$MgSO_4 \cdot 7H_2O$	0.2 mM
BBL yeast extract	500.0 mg
Vishniac and Santer trace mineral solution (Table XXIII) (Vishniac and Santer, 1957)	1.0 mL
KCl	200.0 mg
Wickerham's vitamin mix, modified (Table XXII) (van der Walt and Yarrow, 1984)	10.0 mL
# Glucose	1.0 g

* Prepared as one solution 100 X for convenience

\# Aseptically added as separately autoclaved solution

TABLE XXII

MODIFIED WICKERHAM'S VITAMIN MIX,

(van der Walt and Yarrow, 1984)

Component	Concentration
Biotin	20 g per liter
Calcium pantothenate	2 g
Inositol	10 g
Nicotinic acid	400 g
p-Aminobenzoic acid	200 g
Pyridoxine HCl	400 g
Riboflavin	200 g
Thiamin HCl	400 g

TABLE XXIII

VISHNIAC AND SANTER TRACE MINERAL SOLUTION

(Vishniac and Santer, 1957)

Component	Concentration
EDTA, free acid	50.00 g/L
$ZnSO_4 \cdot 7H_2O$	22.00
$CaCl_2$	5.54
$MnCl_2 \cdot 4H_2O$	5.06
$FeSO \ 7H \ O$	4.99
$CuSO_4 \cdot 5H_2O$	1.87
$CoCl_2 \cdot 6H_2O$	1.16
$(NH_4)_6 Mo_7 O_{24} \cdot 4H_2O$	1.10

containing acetate as carbon source and none of the above growth factors was homogenized and used to inoculate a second series of the same media as described. The inocula then contained maximum quantities of 0.5 µg calcium carbonate and 2.5 ng lipid. Data for the first series were compiled after 44 days incubation at room temperature. After 31 days incubation at room temperature, final data were recorded.

A Frankia medium was formulated based on the results of the above described medium experiments. It was called 3AP because it consisted of the third set of growth factor additions in base "A", plus peptone. The composition of 3AP is given in Table XXIV.

All strains, including PtIl, were inoculated into fresh 3AP medium in order to ensure that they could grow in it. Only one strain could not grow in 3AP. AVP3f was able to grow only if 3AP was diluted by half when incubation conditions remained unchanged. The other Alnus strains grew as well or better in half-strength 3AP, which was designated 3AP/2. All routine transfers and subculturings for further experiments were done in 3AP/2 or a modification of 3AP/2 (Tables XII, XIII, and XIV).

The final adjustment made in the formula for 3AP/2 involved adjustment of the pH before autoclaving and aseptic additions. Three aliquots of medium were prepared as usual; pH values of the solutions were adjusted to 6.8, 7.0, and 7.2 before sterilization. The pH values of the aseptically added solutions were adjusted as for the base solutions. The

TABLE XXIV

3AP

	Component	Concentration	
	$MgSO_4 \cdot 7H_2O$	200	mg per liter
	KCl	200	mg
	Frankia vitamin mix, (Table XIII)	10	mL
*	K_2HPO_4	300	mg
*	NaH_2PO_4	200	mg
	Lalonde trace mineral solution, modified (Table XIV)	1	mL
@	Iron citrate solution	1	mL
	BBL yeast extract	500	mg
	Difco Bacto-peptone	2	g
	NH_4Cl	1	mM
	NaH glutamate, pH 7.0	0.4	mM
#	Na acetate, pH 7.0	5.0	mM
#	Glucose	10	g

* Prepared as one solution 100X for convenience.

@ Added as a solution containing 10.9 g citric acid and 47.1 g ferrous sulfate (heptahydrate) per liter.

\# Aseptically added as separately autoclaved solutions.

flasks were inoculated with homogenates of strains. The final pH values of the media were also recorded.

Substrate Utilization Experiments

Medium experiments using Alnus and Purshia isolates were carried out separately at first. PtIl was tested in ten milliliter cultures of Purshia strain base (Table XXV). This medium is based on the Frankia broth of Baker and Torrey (1978). Three sets of media were prepared; either 0.025% or 0.05% each casein hydrolysate and yeast extract added to one third of the base prepared, and none was added to the remaining third. Several substrates were tested for utilization, including sodium acetate, Tween 80, glucose, sucrose, fructose, mannitol, sodium succinate, and 0.5% each casein hydrolysate and yeast extract. Each flask was inoculated with approximately 1×10^4 CFU/mL; incubation was for 27 days at room temperature.

MPl was first tested for substrate utilization in 3AP base. Ten milliliter aliquots of medium were prepared, and substrates were added aseptically after autoclaving. Substrates included glucose, sucrose, fructose, mannitol, Tween 80, sodium acetate (10 mM or 5 mM), BBL yeast extract Difco yeast extract, and Difco Bacto-peptone. Incubation was for 30 days at room temperature.

Frankia strains were tested for growth in 3AP/2 medium containing one of several selected sole carbon sources prior to osmotolerance screening. Ten milliliter aliquots

TABLE XXV

PURSHIA STRAIN BASE

Component	Concentration
$MgSO_4 \cdot 7H_2O$	0.2 mM
NH_4Cl	1.0 mM
K_2HPO_4	300 mg per liter
NaH_2PO_4	200 mg
Vishniac and Santer trace mineral solution (Table XXIII)	1.0 mL
Wickerham's vitamin mix modified (Table XXII)	10.0 mLs

of medium were prepared with no added peptone, yeast extract, nor sodium glutamate; sodium acetate and glucose were added to only those flasks in which they were being tested. Carbon sources were chosen on the basis of availability and potential as osmotica; these included glucose, sucrose, fructose, galactose, lactose, and mannitol. Polyethylene glycol was not included because preliminary work done in this laboratory by other workers indicated toxicity (see Shipton and Burggraaf, 1983). Acetate and Tween 80 were included as positive controls. Control flasks were also prepared with no added carbon source; sterile distilled water was added to these flasks to adjust the volume. Flasks were inoculated with homogenates and incubated at 28°C for 43 days.

Isolations

Material for the isolation from Alnus maritima was obtained from a site in southern Oklahoma near Tishomingo in Johnston County in November, 1983. Young runners three to five feet tall were checked for nodules at the site and time of collection to insure the presence of the endophyte. The runners were transported to the greenhouse maintained by the Department of Forestry, where they were potted in nitrogen-free potting soil. Because the trees were collected in autumn just before leaf drop, they were wintered in the greenhouse. In June, 1984, the budding alders were uprooted to look for fresh-looking creamy tan-colored nodules, which were present. Some were removed by hand and taken back to

the laboratory in the Department of Botany and Microbiology.

Nodules from seedlings inoculated from pure cultures of ARgP5 AG were obtained by taking one Alnus maritima and one A. glutinosa at the termination of a Forestry experiment and uprooting it and removing nodules by hand. Alnus glutinosa nodules were reddish orange in color; Alnus maritima nodules were brownish orange.

For all isolations, the nodules were used immediately upon collection. They were washed under running tap water to remove all soil, and any remaining portions of root were removed. The nodules were then drained on paper towelling, weighed to the nearest milligram, and placed in a sterile ten millimeter screw-capped test tube. Weights of nodules used were for experimental plants: A. maritima, 339 mg; A. glutinosa, 293 mg; for field collected A. maritima: 205 mg. Ten milliliters of a solution of commercial liquid dish-washing detergent ("Joy", Proctor and Gamble) diluted one part detergent in three parts distilled water was added to the tube, and it was mechanically vortexed in short bursts for two minutes. The copious amounts of foam which resulted were carefully rinsed out with running tap water. The nodules were rinsed twice with ten milliliters of sterile distilled water and four times with ten milliliters of sterile PBS. The final PBS was decanted, and ten milliliters sodium hypochlorite (NaOCl, as freshly opened commercial bleach diluted by half in distilled water) was added, and the tube was shaken for five minutes. The bleach solution was decant-

ed, and the nodules were rinsed twice with ten milliliters of sterile distilled water and thrice with ten milliliters of sterile PBS.

Washed nodules were transferred aseptically to a sterile wire Millipore filter support which was inverted so that the metal ring formed a containing rim; the wire support was supported by bent glass rods in a petri dish. This arrangement allowed the nodules to be rinsed with a stream of sterile PBS from a pipet but be separated from the pool of PBS.

Nodules were then carefully transferred from the filter support to a sterilized ceramic mortar using flamed forceps. Ten grams of sterile white sand was added, along with enough PBS to form a slurry. The nodules were then ground with a sterile ceramic pestle until they were well disrupted.

Ten milliliters of PBS was added to the ground nodule slurry, and the mixture was stirred carefully with the mortar tilted. The sand was allowed to settle for a moment, and the crushed nodule suspension was collected sterile screw-capped tube. This suspension was labelled the 10 or undiluted preparation. Serial dilutions were made from this stock in sterile PBS to 10^{-3} by powers of ten. Pour plates were made in duplicate using 0.1 milliliter of each suspension in 3AP/2 medium without the yeast extract, peptone, ammonium chloride, but with 0.8% agar; sodium glutamate was present in half of the plates. Bilayered agar plates were also made using 0.1 milliliter in two milliliters of soft agar (same as media

above, with or without sodium glutamate) which was spread on 1.5% agar plates of the same nutrient content and the soft agar. Disposable plastic petri dishes were used in all cases. Solidified plates were incubated at 28°C. Plates were examined for the presence of microcolonies using a light microscope at 30 magnifications with very high light intensity at 11 days for reisolations and at 16 days for the isolation using naturally nodulated A. maritima. Frankia colonies were found at the above times for both isolation experiments as judged by the presence of branched hyphal colonies which were too small to be fungi. The microcolonies were transferred by marking the position of the Frankia in areas well separated from contaminants and scooping out the agar with a flamed wire loop. The agar was placed in ten milliliter cultures of liquid 3AP/2 medium, incubated at 28° C, and watched for the appearance of colonies in the agar or liquid medium; contaminated or non-productive flasks were discarded.

When colonies were easily visible in the liquid medium after approximately six weeks incubation, flasks with one small colony present were reserved. Such flasks would be more likely to contain an isolate which resulted from the smallest colony-forming unit, hence most likely to be genetically homogeneous. In addition, the chance of transfer of spores or fragments of other colonies was reduced. One of each of the strains from experimentally inoculated plants and four from the wild plant were chosen. These have been

propagated by homogenizing the colony in approximately one milliliter of PBS and culturing. These strains are maintained in the manner described above for other Frankia.

Measuring Osmolalities of Media

Osmolality was measured indirectly on a Precision osmometer, the "Osmette," the use of which was generously demonnstrated and permitted by R. Ortez. This instrument measures in arbitrary units the freezing point depression (FPD) of a solution relative to that of an osmotic standard. The standard used was 100 milliosmolal sodium chloride. Using the average of three osmometer readings, the freezing point depression of a solution is calculated by solving the equation of the ratio of the solution reading divided by the standard reading to the unknown FPD divided by the FPD of the standard (interpolated from in Wolf et al., 1980).

$$\frac{\text{average solution value}}{\text{average standard value}} = \frac{X}{\text{standard FPD}}$$

The calculated freezing point depression is then multiplied by 12.2 to convert to osmotic potential in units of bars. The units of osmolality are defined to be negative in this case because the water is being "pulled" from the cell by the hypertonic solution.

Preliminary Osmotolerance Tests

MP1 was grown in ten milliliter cultures of 3AP plus glucose, or glucose plus one percent sucrose as osmotica.

One percent sucrose was added to some of the flasks in order to determine whether a mixture of osmotica resulted in different growth yields than did glucose alone. Glucose and sucrose were added aseptically to each set of medium in amounts which would produce approximately the same osmotic potentials. This first osmotolerance experiment would also give a range of the osmotic potentials which would be useful in screening isolates for relative osmotolerance.

Approximately 800 CFU/mL were added to each flask, and incubation was carried out at room temperature for 34 days.

PtIl was also tested separately for osmotolerance. Purshia strain base (Table XXV) was prepared, and mannitol or mannitol plus sucrose were added as osmotica, as for the pretest for MPl. Incubation was for 33 days at room temperature. In this instance, PtIl was harvested from the both of the flasks with osmotic potentials of about -7, -18, and -23 bars (5, 12, and 15% osmotica). The mycelia were homogenized and used as inocula for a second set of osmotic media. This second set consisted of Purshia base plus 2, 5, 8, 11, or 14% (w/v) mannitol (higher percentages tended to crystalize out of the media). In this manner the viability of the mycelia could be determined, and adaptation to osmotic stress might be detected. Incubation was for 40 days at room temperature.

Osmotolerance Screens

Alnus strains were screened for differences in osmo-

tolerance using ten milliliter cultures of 3AP plus lipid supplement and $CaCO_3$, and using glucose as osmoticum. This medium was used because the medium experiments were still in progress. 10, 12, and 14% (w/v) glucose were chosen based on earlier test results using MP1.

Quantitative Osmotolerance Measurments

Dry weight determinations for osmotolerance were made for four strains. Each culture was grown in 100 milliliters of 3AP/2 medium plus 1, 8, 12, or 14% (w/v) glucose. 100 milliliter cultures would allow sufficient media for measurable biomass to be grown. The glucose concentrations were chosen for low stress and very high stress (1 and 14%), and the two intermediate values were chosen to monitor response at intermediate stress. One percent glucose added to 3AP/2 produces an osmotic potential (measured value) of about -1.8 bars, while 14% produces about -19.7 bars. Intermediate percentages of 8% and 12% glucose were used because they were easily prepared concentrations which produced water potentials about five times or ten times the minimum value, or 12.8 bars and -17.8 bars, respectively. Cultures were prepared in triplicate except for ARgP5 AG. Cultures were incubated for 30 days at 28 C.

To begin dry weight determinations, aluminum weighing pans were inscribed on the tabs with an identifying number, and a Gelman Type A/E glass fiber filter (pore sizes 0.1 to 0.4 µm, effective retention size 0.3 µm) is placed in each

pan except those which will serve as empty pan controls. The pans plus filters were dried in a foil-covered Pyrex baking dish overnight in a Fisher Isotemp over, series 200, at 95° C. The pans were handled with forceps when removed from the dish and placed in a large desiccator. The pans and filters were cooled for 30 minutes to two hours, and then were weighed on an analytical balance to 0.01 mg (accuracy of the balance is to 0.1 mg). The pans and filters were dried and weighed thrice.

One hundred milliliter cultures were harvested by gentle vacuum filtration using a 47 millimeter Buchner funnel for support. Culture flasks were rinsed with one milliliter of distilled water five times. The pans and used filters were redried and reweighed three times as described above. The sets of three weights are then averaged and the difference between the averages found. Standard deviations were also calculated were applicable.

Inoculation of Host Plants

To ensure that the best practical method of inoculation of host plants was used for all phases of research of interest to both foresters and microbiologists, four methods of inoculation were tested. The first method tested was to prepare a large quantity of homogenate and dip rooted cuttings into the suspension. The second was to add aliquots of inoculum to plastic pouches containing alder cuttings in liquid media. A third method was to inoculate rooted cut-

tings which were established in individual pots containing nitrogen free potting mix by adding 0.5 milliliter of homogenate to the soil near the stem. Finally, cuttings which were rooted in a bed in a mist chamber were uprooted, and 0.5 milliliters of concentrated homogenate applied using a mechanical pipet. The inoculum was distributed over as much of the root system as possible.

The fourth method was used for all plant inoculations for the joint purposes of determining host range of the Frankia and for producing nodulated plants for water stress resistance evaluation tests of the host plants.

CHAPTER IV

RESULTS

Longevity of Homogenates

Frankia homogenates remained viable for at least one year at 4° C in PBS (Table XXVI, Figures 2 and 3). There was an increase in apparent viability during the first thirty days at 4°C, possibly due to spore germination. Subsequently, the titers gradually declined over the test period.

Medium Development

The initial medium experiment appeared to confirm that Frankia require lecithin and calcium carbonate for growth (Table XXVII). Growth was seen only in base "A" (Table XX), which was formulated using modified QMOD B (Table XV). Growth did not occur in base B, which does not contain lecithin or calcium carbonate (Table XXI). Peptone was not required for growth.

The requirement of MP1 for lecithin and calcium carbonate was again tested in base "A." Subculture in media lacking lecithin and/or calcium carbonate, however, indicated that neither was required (Table XXVIII). Small quantities of these water-insoluble components are transferred with the inocula. The maximum amount of lecithin present in each

TABLE XXVI

VIABILITY OF STORED *FRANKIA* HOMOGENATES

Days Stored	MP1	PtI1
0	4×10^4 CFU/mL	17×10^5 CFU/mL
30	25×10^6	
33		40×10^6
61	30×10^6	
63		60×10^5
94	20×10^4	
106		30×10^6
122	10×10^5	
147		50×10^6
153	10×10^5	
175		50×10^6
214		5×10^6
235	30×10^4	
245		30×10^5
266	5×10^4	
391		20×10^2

Figure 2. Longevity of Homogenized *Frankia* strain PtIl

The graph presents a plot of the logarithm of colony forming units present as measured by visual estimation versus number of days stored.

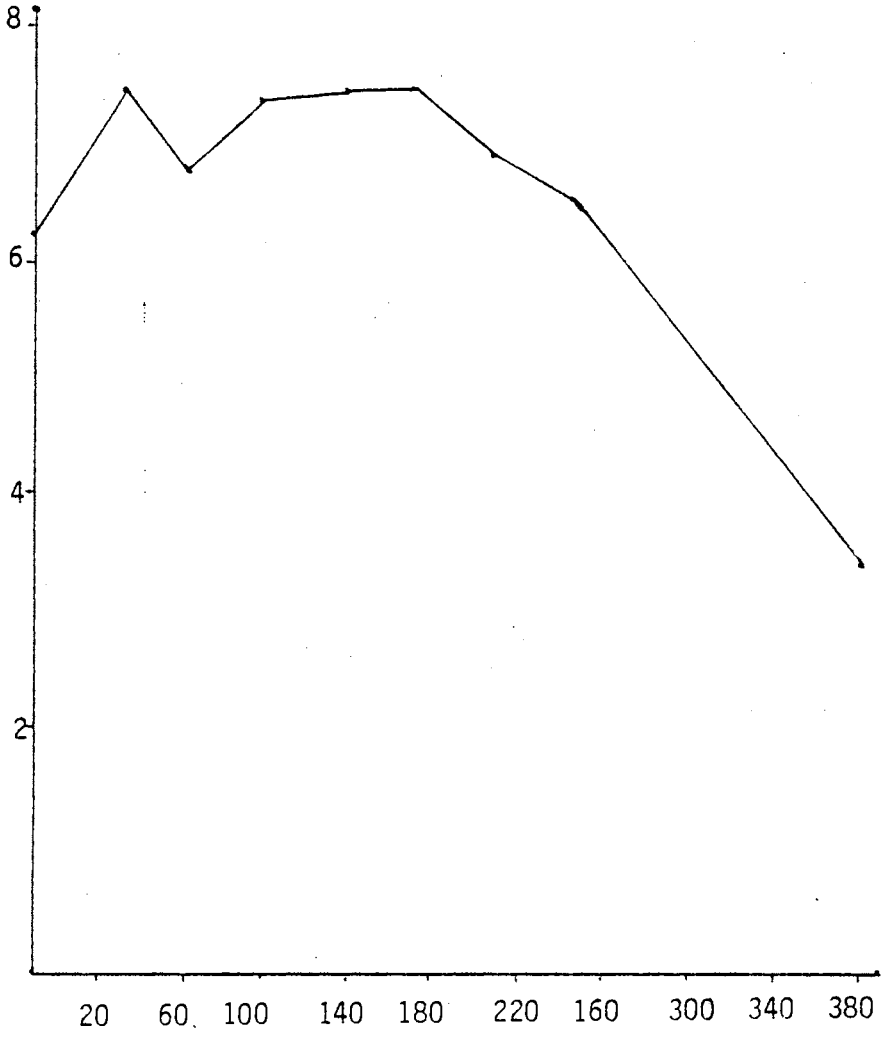

8
6
4
2
20
60
100
140
180
220
160
300
340
380

Figure 3. Longevity of Homogenized *Frankia* strain MP1

The graph presents a plot of the logarithm of colony forming units present as measured by visual estimation versus number of days stored.

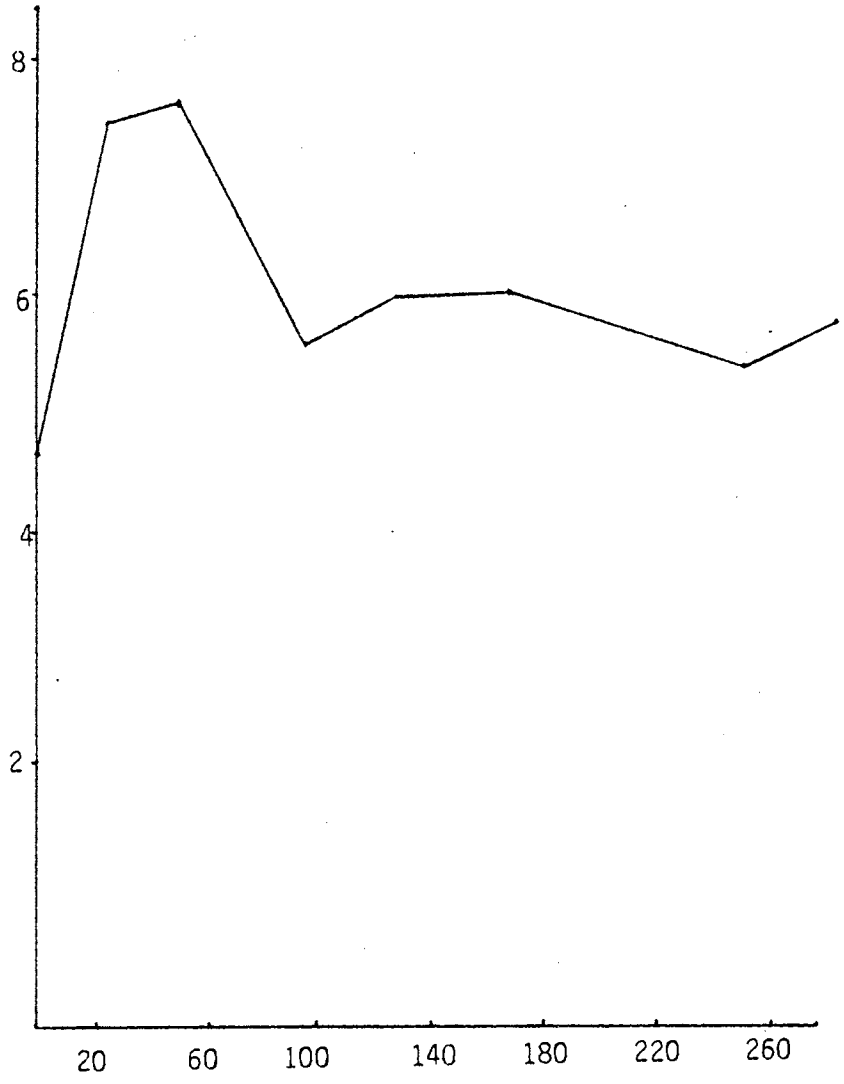

8
6
4
2
20
60
100
140
180
220
260

TABLE XXVII

GROWTH OF MP1 IN EXPERIMENTAL BASE MEDIA

Additions	in Base "A"	in Base "B"
1) 0.5% Difco Bacto-peptone	±	0
2) 1.0 mM NH Cl, 0.4 mM NaH glutamate	±	0
3) 1.0 mM NH Cl 0.4 mM NaH glutamate 5.0 mM Na Acetate	++	0

Plusses indicate approximate biomass yield in each culture; see Table XVIII.

TABLE XXVIII

GROWTH OF MP1 IN LIPID/$CaCO_3$ TEST MEDIA

First Series

Additives:	None	$CaCO_3$ 1 mg/mL	Lipid 0.5 ug/mL	$CaCO_3$ + lipid
Substrates:				
Na acetate, 5.0 mM	*	++	+	++
Tween 80, 0.2%	+	+	+	++
Na acetate + peptone, 0.2%	+	+	+	+
Tween 80 + peptone, 0.2%	±	±	±	±

* flask used as inoculum for second series at 27 days.

Second series

Additives:	None	$CaCO_3$ 1 mg/mL	Lipid 0.5 ug/mL	$CaCO_3$ + lipid
Substrates:				
Na acetate, 5.0 mM	++	++	++	++
Tween 80, 0.2%	++	++	++	++
Na acetate peptone, 0.2%	++	++	++	++
Tween 80 peptone, 0.2%	++	++	++	++

Plusses indicate approximate biomass yield in each culture; see Table XVIII.

inoculum for the first series of tests was 50 ng, and the maximum amount of calcium carbonate was 0.01 mg. The inocula for the second subculture contained maximum quantities of 2.5 ng lecithin and 0.5 µg calcium carbonate. Peptone, again, was not required. The Frankia strains used in this study have been transferred routinely in media without insoluble additives at least fifteen times with no apparent ill effects.

The pH of QMOD B (Table V) was adjusted to 7.2. The addition of calcium carbonate is intended as additional buffer in the medium. The calcium ions could disassociate from the carbonate portion of the molecule to associate with and neutralize protons; the carbonate portion could react with hydroxide ions. However, when initial pH values of 6.8-7.2 were used for 3AP/2, not only were there no differences in visible growth, but the medium became more alkaline (Table XXIX).

Substrate Utilization Experiments

Frankia strains were tested for substrate utilization in order to improve growth yields and to find suitable osmotica.

PtI1 was able to use glucose, sodium acetate, and Tween 80 as carbon sources in the absence of casein hydrolysate and yeast extract (Table XXX). For PtI1, mannitol, sucrose, or fructose are suitable osmotica.

TABLE XXIX

GROWTH OF FIVE STRAINS AT VARIED pH

Strain	Initial pH	Grade	Final pH
MP1	6.8	+++	8.20
	7.0	+++	8.20
	7.2	+++	8.25
ARgP5 AG	6.8	+++	8.15
	7.0	C	NA
	7.2	+++	8.22
ARbN4βi	6.8	++	8.23
	7.0	++	8.21
	7.2	++	8.29
AVP3f	6.8	++	7.61
	7.0	++	7.76
	7.2	++	7.78
PTI1	6.8	+++	7.87
	7.0	+++	7.80
	7.2	+++	7.83

Inocula: MP1, 6×10^5 cfu;

ARgP5 AG, 1×10^3 cfu

ARbN4βi, 700 cfu

AVP3f and PTI1, unknown.

Plusses indicate approximate biomass yield in each culture; see Table XVIII.

TABLE XXX

PtI1 SUBSTRATE UTILIZATION IN EXPERIMENTAL BASES

Carbon Source	Base Only	0.025% CH 0.025% YE	0.05% CH 0.05% YE
Na acetate, 5.0 mM pH 7.0	+	+	+
Tween 80, 0.2%	++	+	+
Glucose, 10 mM	++	++	++
Sucrose, 10 mM	±	±	+
Fructose, 10 mM	±	±	C
Mannitol, 10 mM	±	+	+
Succinate, 10 mM pH 7.0	±	±	±
CH & YE, 0.5%	±	±	±
None added	±	±	+

CH = Difco casein hydrolysate

YE = Difco yeast extract

Plusses indicate approximate biomass yield in each culture; see Table XVIII.

MP1 was first tested for substrate utilization in 3AP base (Table XXIV). MP1 apparently uses sodium acetate and Tween 80 as substrates in 3AP, which contains other possible substrates (Table XXXI). Glucose, mannitol, sucrose, and fructose were not used, and merited further testing to determine their suitability as osmotica.

The results of testing all Frankia strains used in this study except OSU 01 (Am1) for substrate utilization using defined 3AP/2 base (excluding yeast extract, peptone, and sodium glutamate) are presented in Table XXXII. Incubation was for 43 days at 28° C. (OSU 01 was not tested for substrate utilization due to lack of sufficient inoculum.) No visible growth was present for AVP3f at all; inoculum for this strain was scarce at the time of testing, and the inocula for the flasks was too small. AVP3f has, however, been successfully cultured in media containing sodium acetate as substrate.

Glucose was chosen as primary osmoticum for the Alnus isolates that grew because it was not utilizable as sole carbon source and allowed the preparation of solutions of the highest osmotic potentials. Glucose was utilized by the Purshia isolate, and could not be used as osmoticum. The suitability of glucose as osmoticum for AVP3f was uncertain.

Isolations

Many isolated Frankia colonies were produced in each of the three isolation attempts using the procedure de-

TABLE XXXI

MP1 SUBSTRATE UTILIZATION

Substrate	Grade
None added	±
BBL yeast extract, 0.2%	±
Difco yeast extract, 0.2%	±
Na acetate, 10 mM	++
Na acetate, 5 mM	++
Fructose, 10 mM	±
Sucrose, 10 mM	±
Mannitol, 10 mM	±
Tween 80, 0.2%	++
Difco Bacto-peptone, 0.2%	±

Inoculum, approx. 68 cfu per flask.

Plusses indicate approximate biomass yield in each culture; see Table XVIII.

TABLE XXXII

CARBON SOURCE UTILIZATION BY *FRANKIA* STRAINS

Strain	PtI1	MP1	ARgP5 AG	ARbN4βi	AVP3f	ACN1 11m	ACN1 11q	ACN1 AG	AG AGN1 EXO
Carbon Source									
Acetate, 5 mM	++	++	++	++	0	+	+	++	++
Tween 80, 0.2%	++	++	++	++	0	+	++	++	++
Glucose, 1%	++	C	0	0	0	0	0	0	0
Sucrose, 1%	0	0	0	+	0	0	0	0	+
Fructose, 1%	0	0	0	0	0	0	0	0	0
Galactose, 1%	0	0	0	0	0	0	0	0	0
Lactose, 1%	0	0	0	0	0	0	0	0	0
Mannitol, 1%	0	0	0	0	C	0	0	0	C
None added	0	0	0	0	0	0	0	0	0

Plusses indicate approximate biomass yield in each culture; see Table XVIII.

scribed. One isolation was made from nodules from a greenhouse tended Alnus maritima taken from a natural site and nodulated by "wild" Frankia. The new isolate has been given the standard strain designation OSU 01180101; it is generally referred to as OSU 01 or Aml. The standard catalog data is listed in standard format (as described in Table II) in Table XXXIII. Two isolations were made using nodules produced in the greenhouse by experimental inoculation of A. glutinosa and A. maritima with ARgP5 AG. These strains were labelled AG-PI-P5 and AM-PI-P5 at the time of isolation, respectively, in order to differentiate them from unpassaged ARgP5 AG. The abbreviations refer to the host through which the Frankia was passaged by the initials of the generic names, "PI" for "passage isolate", and P5 to indicate the parent strain.

For each of the three isolates, colonies were identified fied by morphological characters. For the isolate of the new strain from A. maritima, colonies were transferred from plates inoculated with undiluted nodule preparation to liquid 3AP/2. For both isolations from experimentally inoculated host plants, colonies were transferred from plates inoculated with ten-fold diluted and with undiluted nodule preparations to liquid 3AP/2.

Measuring Osmotic Potentials of Media

Table XXXIV lists the calculated osmolalities of media consisting of 3AP/2 plus selected percentages of glucose

TABLE XXXIII

STRAIN INFORMATION FOR OSU 01180101

1) Strain acronym: OSU 01180101
Parent strain: none

2) (Previous) synonyms: OSU 01; Aml

3) Primary reference (where first described): none

4) Secondary reference (where best described): none

5) Plant host origin: 01-18 (*Alnus maritima*); native

6) Soil of origin: NA

7) Locale of origin: 34° 20′ N, 96° 43′ W;
near Tishomingo, Oklahoma

8) Year and season of collection:
Fall 1983; Summer 1984

9) Plant passages prior to isolation: none

10) Isolation from fresh nodules

11) Type of nodule: unknown

12) Nitrogen fixed *in vitro*: unknown

13a) Infective for: 01-07; 01-18
b) Non-infective for: unknown

14a) Effective for: 01-07; 01-18
b) Non-effective for: unknown

15) Whole cell sugar pattern: unknown

16) Available to others

17) Strain may be obtained from
Dr. H. S. Vishniac
Department of Botany and Microbiology
318 Life Science East
Stillwater, OK 74078

18) J. C. Williams, address same as above

19) Plants taken from collection site in the fall of 1983 to winter in greenhouse. Fresh new nodules collected in the spring of 1984.

TABLE XXXIV

MEASURED OSMOTIC POTENTIALS OF MEDIA

% Glucose	FPD (°C)*	Water Potential in bars
3AP/2 + 0%	0.027	-0.33
3AP/2 + 1%	0.107	-1.80
3AP/2 + 2%	0.214	-3.41
3AP/2 + 4%	0.433	-6.25
3AP/2 + 6%	0.668	-9.89
3AP/2 + 8%	0.913	-12.81
3AP/2 + 10%	1.167	-14.94
3AP/2 + 12%	1.443	-17.81
3AP/2 + 14%	1.731	-19.72

*FPD = freezing point depression calculated from osmometer readings

ranging from 0 to 14 percent. The table also lists the measured freezing point depression values from which the osmolalities were calculated.

Preliminary Osmotolerance Tests

The growth evaluations for the MP1 osmotolerance preliminary experiment are given in Table XXXV. This strain was able to grow in media containing glucose as osmoticum at osmotic potentials of about -15.3 bars; using one percent sucrose plus glucose, growth occurred in all flasks, with osmotic potentials down to -22 bars. This difference in water stress resistance may indicate that MP1 exoenzymes are less sensitive or less accessible to sucrose than they are to glucose.

Tables XXXVI and XXXVII present the preliminary osmotolerance data using for PtI1. Substantial decreases in apparent growth occurred between -2 and -5 bars, and between -5 and -10 bars, but an endpoint for osmotolerance was not found in media containing mannitol or in sucrose plus mannitol. PtI1 was capable of limited growth at osmotic potentials down to at least -22.6 bars (estimated osmolality). The data in Table XXXVII show that PtI1 was alive at these low potentials, but no adaptation is apparent.

The Alnus isolates from Lalonde's laboratory were tested for osmotolerance in 3AP-based media at osmotic potentials which appeared to encompass the maximum value for MP1 (Table XXXVIII). AVP3f was not tested due to lack of

TABLE XXXV

GROWTH OF MP1 IN OSMOTIC MEDIA

	% Osmoticum	Water Potential in bars (est.)	Grade
Glucose	1	-1.40	+
	2	-2.81	+
	4	-5.73	+
	8	-11.95	+
	10	-15.28	+
	12	-18.70	±
	14	-22.40	0
1% sucrose plus glucose, total %	1	-1.48	+
	2	-2.88	+
	4	-5.73	+
	8	-11.82	+
	10	-15.07	+
	12	-18.47	+
	14	-22.03	+

Inoculum 800 cfu per flask.

Plusses indicate approximate biomass yield in each culture; see Table XVIII.

TABLE XXXVI

GROWTH OF PTI1 IN OSMOTIC MEDIA

	% Osmoticum	Water Potential in bars	Grade
Mannitol	0	0.00	++++
	5	-7.16	+++
	10	-15.12	+
	12	-18.54	+
	13	-20.32	+
	14	-22.13	+
	15	-23.99	+
1% sucrose plus mannitol, total %	2	-1.48	++++
	5	-7.14	+++
	10	-14.93	+
	12	-18.27	+
	13	-20.02	+
	14	-21.80	+
	15	-23.61	+

Inoculum size unknown.

Plusses indicate approximate biomass yield in each culture; see Table XVIII.

TABLE XXXVII

GROWTH OF PtIl IN OSMOTIC MEDIA, SUBCULTURED

% Mannitol	Water Potential in bars	-7	-18	-23
2	-2.76	ND	+	++
5	-7.16	+	±	±
8	-11.82	+	±	±
11	-16.79	±	±	±
14	-22.13	±	±	±

Inocula per flask: from: -7 bars: 63 x 10 5 cfu;

-18 bars: 20 x 10 4 cfu;

-23 bars: 14 x 10 4 cfu.

Plusses indicate approximate biomass yield in each culture; see Table XVIII.

sufficient inocula at that time; AVP3f did not grow well in 3AP. AGN1 $\frac{AG}{EXO}$ and ARgP5 AG both were capable of excellent growth at all tested potentials. These two isolates were best choices for the xerotolerant strain to be used for experimental host plant inoculations. The other strains were judged to have lower xerotolerances; the growth of ACN1 AG and ACN1 11m decreased between -17.8 and -19.7 bars. ARbN4βi was not able to grow well in any of the media.

Quantitative Osmotolerance Measurements

Quantitative evaluations of osmotolerance were made for selected strains ARgP5 AG (more osmotolerant) ARbN4βi, (less osmotolerant), AVP3f (untested), and PtI1. Table XXXIX gives the growth evaluations and dry weight measurements (with standard deviation values where applicable) for these four strains grown for 30 days at 28°C in 100 milliliter cultures of osmotic media. Figures 4, 5, 6, and 7 present the dry weight data graphically for each strain.

PtI1 alone shows a decline in biomass yield with increasing glucose content. The visual evaluations seem to correspond to dry weight data for PtI1, but not for other strains. ARgP5 AG and ARbN4βi show peaks in dry weight at -12.8 bars. At -19.7 bars the dry weight yield for ARgP5 AG is in conflict with visual estimation. ARbN4 i clearly has a decrease growth yield at -19.7 bars, and it appears to be less osmotolerant. AVp3f produced little biomass in any media, and the dry weights indicate a growth

TABLE XXXVIII

VISUAL EVALUATIONS FOR OSMOTOLERANCE SCREEN OF SIX *FRANKIA* STRAINS

	% glucose	10%	12%	14%
	osmotic potental (bars)	-14.94	-17.81	-19.72
Strain	Inoculum (CFU)			
AGN1 AG EXO	15×10^2	++++	++++	++++
ACN1 AG	20×10^2	++++	++++	+
ARbN4 β i	unknown	+	+	+
ARgP5 AG	25×10^3	++++	++++	++++
ACN1 11m	25×10^3	++++	++++	++
ACN1 11q	2×10^4	+++	+++	+

Plusses indicate approximate biomass yield in each culture; see Table XVIII. Comparisons were made within and between the differing strains without regard to inoculum size.

TABLE XXXIX

MYCELIAL DRY WEIGHT YIELDS OF FOUR STRAINS IN OSMOTIC STRESS MEDIA

osmotic potential in bars	-1.80	-12.81	-17.81	-19.72
Strain				
PTI1	34.56 mg ±2.26	16.67 mg ±3.30	12.62 mg ±5.47	8.48 mg ±2.89
grade	++++	+++	++	+
ARgP5 AG	9.00	7.62	6.13	10.41
grade	++++	+++	+	+
ARbN4 β i	10.87 ±0.27	19.82 ±0.74	14.96 ±0.68	9.78 ±0.33
grade	++++	++++	+++	+++
AVP3f	5.71 ±0.28	4.55 ± 0.34	4.62 ±0.55	4.77 ±0.22
grade	+++	++	+	+

Inocula: PtIl, unknown;

ARgP5 AG, unknown;

ARbN4 $^{\beta i}$, 20 x 10^6 cfu per flask;

AVP3f, 12 x 10^5 cfu per flask.

Plusses indicate relative quantities of biomass in each flask as judged visually. Comparisons were made within and between the differing strains; flasks containing the same medium and the same strain did not have differing apparent growth yields. No individual colonies were countable in any flask due to overinoculation..

Figure 4. Growth Response of PtIl to Decreasing Water Potential

The graph represents the growth pattern obtained when PtIl is subjected to media of increasing water stress. Bars represent standard deviations for data points.

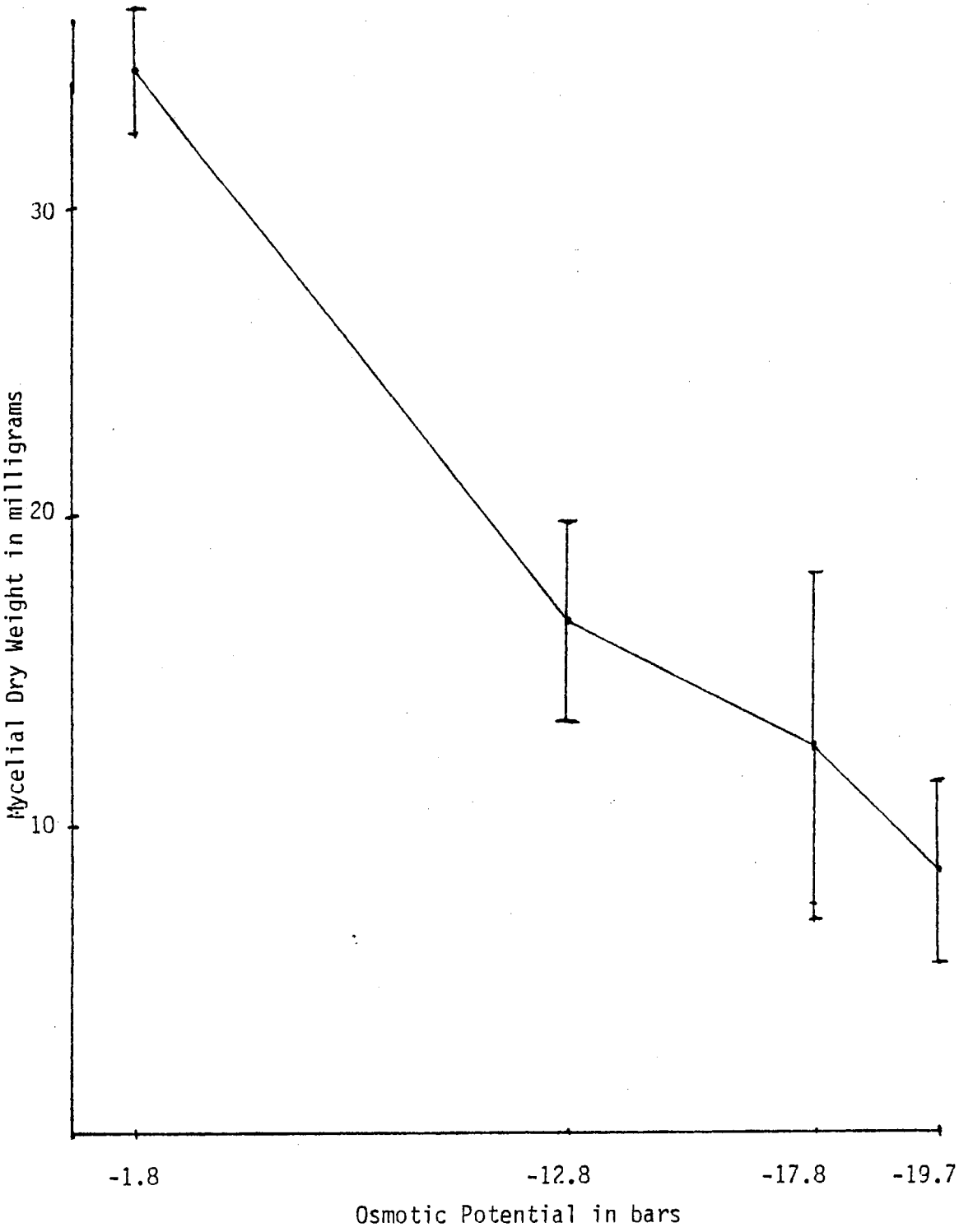

Mycelial Dry Weight in milligrams
30
20
10
-1.8
-12.8
-17.8
-19.7
Osmotic Potential in bars

Figure 5. Growth Response of ARgP5 AG to Decreasing Water Potential

The graph represents the growth pattern obtained when ARgP5 AG is subjected to media of increasing water stress.

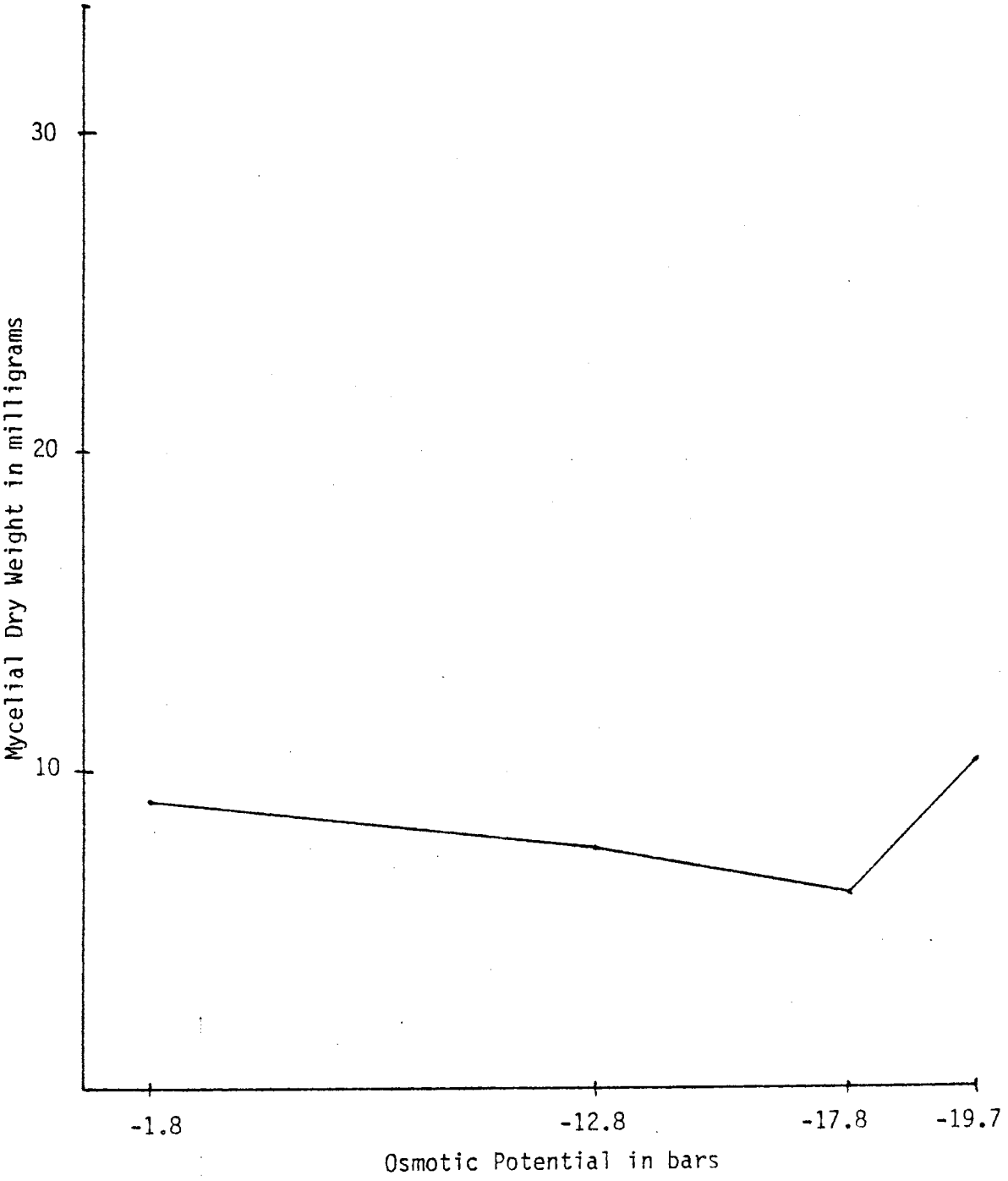

30
20
10
Mycelial Dry Weight in milligrams
-1.8
-12.8
-17.8
-19.7
Osmotic Potential in bars

Figure 6. Growth Response of ARbN4 i to Decreasing Water Potential

The graph represents the growth pattern obtained when ARbN4 i is subjected to media of increasing water stress. Bars represent standard deviations for data points.

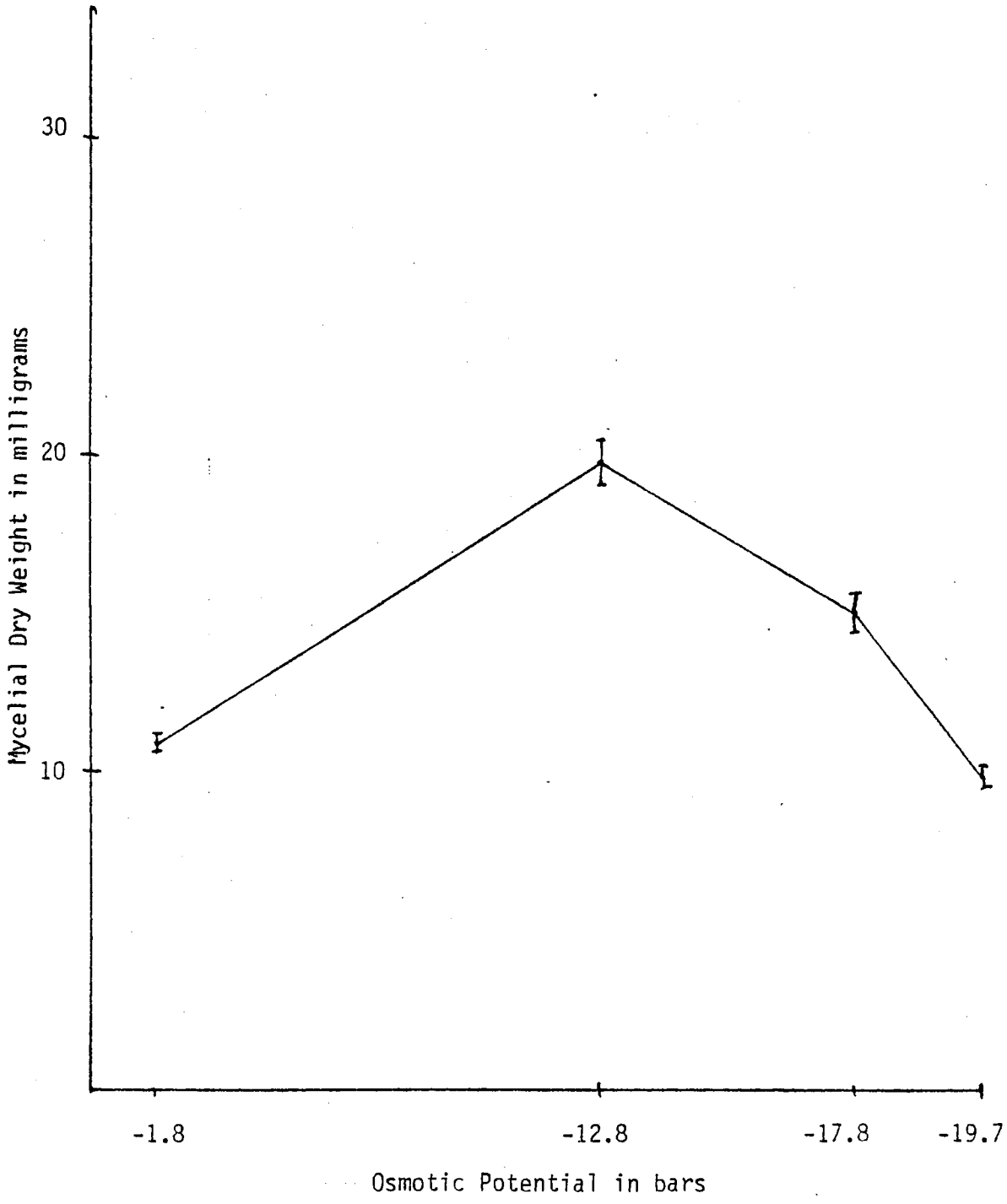
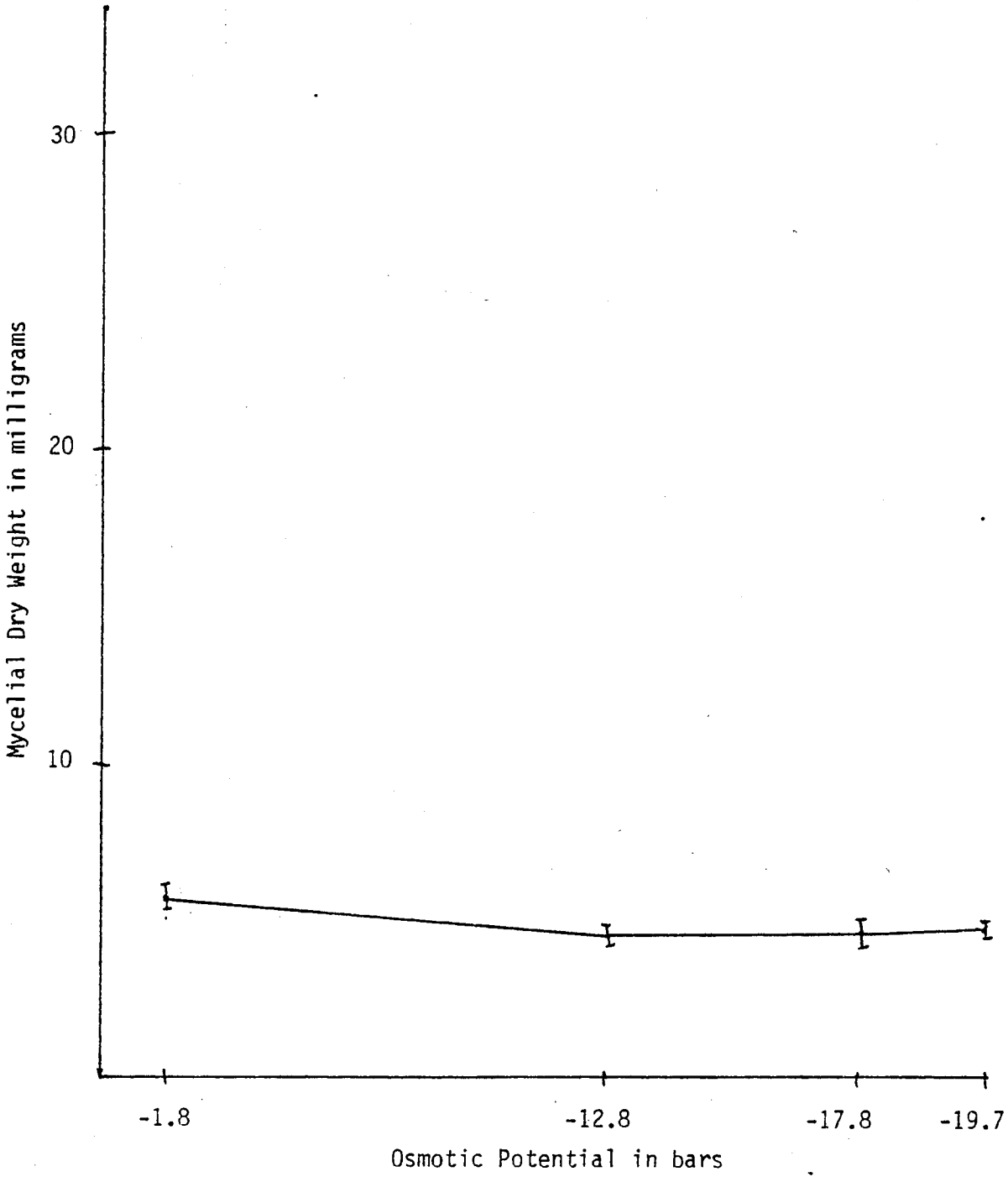

30
20
10
Mycelial Dry Weight in milligrams
-1.8
-12.8
-17.8
-19.7
Osmotic Potential in bars

Figure 7. Growth Response of AVP3f to Decreasing Water Potential

The graph represents the growth pattern obtained when AVP3f is subjected to media of increasing water stress. Bars represent standard deviations for data points.


30
20
10
Mycelial Dry Weight in milligrams
-1.8
-12.8
-17.8
-19.7
Osmotic Potential in bars

curve which is essentially flat. Visual estimations again differ from dry weight data.

Visual estimations are useful for relative differences in growth yields. Dry weights of cultures were intended to determine the exact differences in water stress resistance between strains, and were expected to correlate with visual data. Unfortunately, there are problems inherent in the method, especially when dealing with sugar solutions. It is easily concieved that sugar may be trapped within the dried hyphal mass. Often a browning of the filtered hyphal mass occurred, possibly indicating reactions between sugars and amino acids such as those which cause the browning of foods.

Inoculation of Host Plants

Host plants were inoculated with Frankia homogenates as described in Chapter III. Table XL is a compilation of the infectivity data for all Frankia strains on host rooted cuttings. Most strains, with the exception of PTI1 and ACN1 11q for A. glutinosa 8022-16, were infective and effective for all hosts tested. Most other failures of the Frankia to nodulate the host rooted cuttings were due to the death of the host plant before nodulation could occur.

TABLE XL

INFECTIVITY OF *FRANKIA* STRAINS IN *ALNUS* CLONES

Strain / Clone:	AG AGN1 EXO	MP1	ACN1 AG	ACN1 11m	ACN1 11q	ARgP5 AG	ARbN4ai	AVP3f	PtI1	OSU 01
A. glutinosa 2-58	3/3	16/16		6/6		10/10	1/1			
4x09-11			3/3							
4x23-49					3/3	2/3		3/3		
4x23-54	7/7		3/3	2/2			2/3			
8022-05	3/3	5/5	5/5	4/4		3/3	4/4	4/4		
8022-14	8/8	3/3	3/3	6/6	5/5	3/3	5/5		1/3	3/3
8022-16	3/3	5/5	8/8	3/3	0/3	9/12	4/7	8/12	0/4	4/4
8022-24		8/8	5/5		5/5	7/9	9/9	10/10	4/5	5/5
8023-05	8/8	5/5	7/7	5/5	5/5	10/11	9/9	8/9	3/4	4/4
A. maritima		29/29	5/5	4/4	5/5	5/5	4/5	3/3		
A. rubra			3/3		4/4	4/4		5/5		
A. serrulata		6/6								

Fractions indicate the number of plants that greened after inoculation over the total number of plants inoculated. Spaces indicate that the data is not yet available.

CHAPTER V

DISCUSSION

Introduction

The goals of this project were to develop a medium in which growth yields were improved and in which osmotolerance experiments could be successfully carried out, to isolate a new strain from a xerotolerant host and from experimentally inoculated host plants, to quantitatively measure the osmotolerance of selected strains, and to develop a method for inoculating host plants.

When this research was begun, little was known about Frankia, for it had been available in pure culture for only about four years. Many aspects concerning Frankia were the subjects of much active research, especially methods and conditions of isolation, culture, and host plant inoculation.

Longevity of Homogenates

Frankia can remain viable for over a year in phosphate buffer at 4°C. PtIl was viable for over one year under the same conditions. The vegetative cells probably are not be alive after such extended periods, but spores could conceivably remain viable. Other preparations of Frankia have been reported to be long-lived. Van Dijk (1979) reports that

refrigerated nodule homgenates remain infective for several months; air dried nodules were still able to provide very low levels of infectivity after seven years.

It is not known whether the increase in titer is due to the germination of spores or reproductive torulose hyphae or both. Some preparations and/or storage conditions may promote spore germination. J. C. Ensign has been unable to induce spore germination when he tried cold storage or low nutrient media, as well as other means such as heat shock (personal communication). However, the combination of cold storage and low nutrient availability could have provided the necessary activation (as Diem and Dommergues, 1985, propose is required). Quispel (1960) observed an increase in infectivity of nodule homogenates stored in peat or nodule extract at room temperature. Whether the increase in infectivity was due to differences in plant response, more bacterial infective units, or other factor(s) is unsure. At that time, no Frankia had been purified and cultured outside of the host so that further investigations were not possible.

Medium Development

The 3AP/2 medium is similar to QMOD B (Lalonde and Calvert, 1979). It lacks the lecithin and calcium carbonate of QMOD B, but contains yeast extract, peptone, growth factors, and added nitrogen sources. It also contains a carbon source commonly utilized by Frankia strains. The lack of requirements by Frankia for lipid supplement and calcium carbonate

are now well substantiated by this work and by the work of other laboratories. Media reported since QMOD B (Lalonde and Calvert, 1979) include neither lecithin nor calcium carbonate (e. g. Blom, 1981, 1982; Burggraaf and Shipton, 1982). Ammonium chloride is a favored nitrogen source for Frankia strains (Blom, 1982; Shipton and Burggraaf, 1982b). The addition of vitamins, specifically biotin, to Frankia media has been found to stimulate growth (Shipton and Burggraaf, 1982a).

In the experiments in which pH was varied, the strains did not appear to favor any of the pH's, but only a few values in a narrow range were tested.

All strains used for this study are able to grow in 3AP/2. Biomass yields as measured by dry weights are improved compared to those in QMOD B, in which the "Comptonia isolate" (CpI1) produced 0.83 mg dry mycelium per 100 mL medium in four weeks (0.1 mg in 12 mL) (Lalonde and Calvert, 1979). The yields in 3AP/2 are comparable to those produced in Burggraaf and Shipton's medium (1982). Depending on the particular strain, 3AP/2 plus 1% glucose yielded 9.0 to 34.5 mg dry mycelium per 100 mL medium in 30 days; Burggraaf and Shipton's medium (1982, Table X) produced 9.0 to 17.8 in 13 days.

Substrate Utilization Experiments

All strains except OSU 001 (Am1) and Avp3f were successfully tested for substrate utilization in defined media. The

results were similar to those obtained by other laboratories (Blom, 1981, 1982; Shipton and Burggraaf, 1982a; Tisa et al., 1983) in that all strains were able to utilize acetate (see Table X). Other laboratories prefer to use propionic acid as substrate in their media based on growth responses (Blom, 1981; Shipton and Burggraaf, 1982a). It is also the preferred substrate for nitrogen fixation studies (Murray et al., 1984).

The medium experiments described constitute the work leading to the realization of the first objective in these studies, namely, the development of a medium which is suitable for osmotolerance testing.

Isolations

Serial dilution of suspensions of crushed nodules is a simple but effective method for isolating Frankia, provided that surface contaminants are removed or prevented from overgrowing the Frankia. This technique (with minor variations) is often used for isolating Frankia because it is rapid, simple, and requires no special equipment (Baker and Torrey, 1979).

Xerotolerance in Frankia

Frankia are not exceptional among microbes for their osmotolerance. They grow well within the range of -30 to 0 bars; most bacteria are able to grow within the range of -140 to 0 bars (water activity = 0.9 to 1.00) (Pirt, 1975;

Brown, 1976).

The preliminary, non-quantitative data for PtIl using mannitol as osmoticum show that the limit for its osmotolerance exceeds -24 bars and the osmotic potential which can be produced by mannitol in solution. (Tables XXXV and XXXVI). This is not surprising, because nodulated Purshia shrubs have been found to fix nitrogen when soil water potentials reach -24 bars. Subculturing selected cultures showed that the Frankia were able to survive the initial osmotic shock, even at -24 bars; growth, if it occurred, was slight. MPl appears to have a limit slightly below -22 bars using glucose (Table XXXIV).

Shipton and Burggraaf (1982b) report that the limits of growth may be close to -16 bars for Frankia isolates CpIl, AvcIl, LDAgpl, and LDMgl when sodium chloride or polyethylene glycol 4000 were used as osmotica; they measured growth yields by protein yields by the Coomassie brilliant blue method. The differences in limiting osmotic potentials are not unexpected. The non-ionic sugar and sugar alcohol have very different effects on cellular components than do inorganic ions.

The screen for osmotolerant strains (Table XXXVII) revealed variability in the osmotolerance of Frankia strains. These results agree with those of Shipton and Burggraaf (1982b), who did not note significant strain differences, and three of the four strains seemed to have similar growth patterns. There are, however, strains with relatively higher

and lower osmotolerances.

Quantitative measurements of growth yields and visual evaluations of growth in media of varying osmotic potentials generally did not correlate (Table XXXVIII). Errors may have resulted from the inherent inaccuracy in estimating large numbers of colonies, deliberate overinoculation of cultures to insure measureable growth at lower potentials which resulted in mat formation at higher potentials, insufficient washing of mycelia, or inadequate drying of the mycelia. The results given, however, are from trials using filtered cultures; harvesting by centrifugation was also tried, and similar but less precise results were obtained (as judged by smaller standard deviations).

Figures 4-7 reveal that there are three patterns of response to osmotic stress for the Frankia strains tested. The first is a simple inhibition curve demonstrated by PtIl. This Frankia strain is capable of removing the osmoticum and utilizing it as a substrate. It is unlikely that PtIl is able to remove more than one percent of the glucose from the medium; the initial osmotic potentials are correct upon inoculation, and the strain was able to withstand the osmotic shock. This graph does not represent a proper osmotolerance test, but is instead a growth curve showing the response to greatly increased levels of an osmotically active substrate. The growth curve for PtIl is similar to those generated by the addition of sodium chloride as osmoticum to cultures of other Frankia strains (Shipton and Burggraaf, 1982b). The

limit of osmotolerance was not reached for PtIl.

ARgP5 AG also shows a decline in growth yield as osmotic potentials are decreased. The increase in growth yield at -19.7 bars can be attributed to osmoticum which was trapped on the filter.

The second pattern is that which is seem in strain ARbN4βi. There is a peak in growth yield at -12.8 bars. Lower water potentials are generally considered to be more favorable to filamentous microbes than to bacteria (Shipton and Burggraaf, 1982b). These investigators recorded a small peak for strain LDAgpl when PEG was used as osmoticum to produce a water potential of -6 bars. The slope of the curve for AvcIl between the same points were not as steep as the other portions of the curve, and CpIl was able to grow equally well at -2 to -6 bars when PEG was used as osmoticum. The pattern seems to result from stimulation of growth when water potentials approach optimal values for the individual strains under the various conditions.

ARbN4βi may be equally or superior in osmotolerance to ARgP5 AG. The osmotolerance limits of these were not found.

The third pattern is that of AVP3f. No significant differences between any of the growth yields was found. AVP3f appears to be limited by a factor other than or in addition to water potential. Concentrations of other substances in 3AP/2 may yet be too high, for this strain was apparently unable to grow at all in 3AP.

The data collected for osmotolerance shows that Frankia

strains do differ measurably in their osmotolerances. This information is useful in developing a model for water stress resistance in actinorhizal plants because strains with relatively high (ARgP5 AG and ARbN4 βi) and low (AVP3f) have been identified.

Plant Inoculations

All isolates originating from Alnus nodules were infective and apparently effective for all Alnus clones inoculated, with one single exception. ACN1 11q was not infective for A. glutinosa 8022-16. In addition, the same Alnus clone did not nodulate when inoculated with PtI1, the isolate from Purshia. The bacterium and plant may not have been compatible due to host-endosymbiont specificities. The number of plants inoculated is low, and the possibility that the plants were not yet receptive to infection exists. The two inoculum preparations were infective for other clones inoculated concurrently.

The new isolate OSU 01 is infective and effective for four clones of A. glutinosa, and this information is included in the catalog entry, Table XXXIII.

CHAPTER VI

SUMMARY, CONCLUSIONS, AND SUGGESTIONS FOR FURTHER STUDY

Frankia was first demonstratably grown in pure culture approximately five years before the undertaking of this study. Because the media published at that time were unsuitable for determining xerotolerance (measured as osmotolerance), a suitable medium was developed. The new medium (3AP/2) was used to find osmotica which were not used as sole carbon sources and then to screen eight *Frankia* strains for osmotolerance. Nine strains were tested for infectivity and effectivity in a wide range of *Alnus* clones. Successfully effective strains of contrasting osmotolerance were: high-- ARgP5 *AG* and ARbN4 βi, which produced averages of 6.13 mg and 14.96 mg dry weight of biomass respectively in 100 mL cultures in 30 days at an osmotic potential of -17.8 bars (12% glucose); and low-- AVp3f, which was unable to grow at potentials of less than -12.8 bars (8% glucose) and produced an average of 4.55 mg dry weight in 100 mL cultures in 30 days at that potential. Osmotic potentials were measured by osmometer. Because these *Frankia* isolates came from hosts of relatively low xerotolerance, novel strains were isolated from *A. maritima* following the discovery of the superior

water-stress resistance of this species in T. C. Hennessey's laboratory. This isolate could prove to be more osmotolerant than ARgP5 AG.

Inoculation with 0.5 mL of homogenized culture containing at least 106 mycelial fragments plus spores per mL (as measured by serial dilution; OD620 = 0.16) applied directly to bare roots proved to produce well-nodulated plants. The homogenates can be stored at 4°C in phosphate-buffered saline for at least 13 months; an initial increase in colony-forming units (presumably due to germination of spores) was followed by a slow decline in viability. Strain ARgP5 AG was reisolated from experimentally inoculated hosts.

From this research, five conclusions may be drawn.

1) Homogenates of Frankia are long-lived, and may be relied upon as ready sources of inocula, although the strength of any inoculum must be determined at the time of use.

2) The water-insoluble components of QMOD B (Lalonde and Calvert, 1979), calcium carbonate and lecithin are not required for growth of Frankia strains.

3) Isolations of Frankia strains may be made easily by serially diluting suspensions of crushed nodules and plating the suspensions on 3AP/2 medium.

4) The Frankia strains tested for osmotolerance show variation in response to osmotic stress. They will be useful in determining a model for water stress resistance in actinorhizal plants.

5) The Frankia strains used in this study are generally

infective and effective for the host clones tested.

These investigations are part of continuing work. Unfinished business may therefore include the discovery of *Frankia* strains of higher osmotolerance for more critical tests of the influence of each symbiotic partner, examination of the influence of increased water potentials on nitrogen fixation *in vitro*, development of methods to positively identify *Frankia* strains isolated from wild actinorhizal plants or recovered from laboratory-inoculated plants, and the responses of laboratory-inoculated *Alnus* under field conditions.

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