Biology of the *Frankia-Alnus maritima* subsp. *maritima* symbiosis

by

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A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Plant Physiology

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Ames, IA

2004

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To Ryan and Nathan

TABLE OF CONTENTS

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Alnus maritima (Marsh.) Muhl. ex Nutt (seaside alder) is an attractive shrub that is unique among species of *Alnus* Miller in its restriction to waterlogged soils. This species is indigenous to only three disjunct areas of North America and is listed as a threatened species in the states in which it occurs. As an actinorhizal species, *A. maritima* develops a root-nodule symbiosis with the nitrogen-fixing soil bacterium *Frankia* Brunchorst (Actinomycetales). The unusual habit of *A. maritima* for flowering in early autumn, when few other woody taxa are in bloom, also makes it promising for use in horticultural landscapes. Establishment of an effective symbiosis is sensitive to the concentration of oxygen in the root zone, however, and the limitation of this plant to low-oxygen soils of wetlands provides a challenge to both symbiotic partners. The overall goal of my dissertation work was to understand the factors that may foster or limit the survival of this species both in its native wetland habitat and in managed settings, where its capacity to fix atmospheric nitrogen may add significantly to the nitrogen status of the landscape and reduce the need for chemical fertilization. My research was conducted with plants grown from seeds collected on the Delmarva Peninsula. I chose to study this population, recently named *A. maritima* subsp. *maritima* Schrader & Graves, because of the interest expressed by the Plant Materials Center of the U.S. Dept. of Agriculture – Natural Resources Conservation Service (Cape May, N.J.) in using this subspecies for conservation plantings.

Dissertation Organization

This dissertation is organized into eight chapters, which include this chapter (General Introduction) and the final chapter (General Conclusions). The second chapter is a review that has been published both as a journal article and as a book chapter. It serves as the literature review for this dissertation. The fourth chapter is a techniques paper that has been submitted for publication and describes an apparatus that I designed in cooperation with my major professor and a committee member. The remaining four chapters consist of original research papers that are published, submitted, or ready for submission to appropriate journals. This dissertation includes two appendices: APPENDIX A contains additional data from Chapter VI, and APPENDIX B describes the preliminary results of ongoing work that is not yet ready for publication.

CHAPTER 2. NITROGEN FIXATION AS A STRESS-AVOIDANCE STRATEGY AMONG ACTINORHIZAL (NONLEGUME) TREES AND SHRUBS

A paper published in *Journal of Crop Improvement* and in R. Arora (ed.) *Adaptations and Responses of Woody Plants to Environmental Stresses*[1]

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ABSTRACT. Actinorhizal species of trees and shrubs are a diverse group of plants that may survive in poor soils by virtue of their associations with the actinomycete *Frankia*. These species include several important woody plants that are well suited for horticultural use in temperate climates. The symbiosis between *Frankia* and actinorhizal species shows some similarity to symbioses between rhizobia and woody legumes, and a common ancestor has been proposed for the predisposition to root-nodule symbiosis. Despite their probable common origin, important differences exist between actinorhizal and leguminous symbioses; characteristics of the microsymbiont, nodule architecture, and mechanisms controlling oxygen relations of the nodule are among the ways the two systems differ. If nitrogen fixation is sustained under unfavorable conditions, woody plants that associate with nitrogen-fixing organisms may show enhanced tolerance of environmental stress; species of plants capable of nitrogen-fixing symbioses are known to have comparatively strong resistance to invasion by pathogens.

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[1]Reprinted with permission of Haworth Press: J. of Crop Improvement, 2004, 10, 281-304; R. Arora (ed.) Adaptations and Responses of Woody Plants to Environmental Stresses. Haworth Press, 2004.

Expansion of the capacity to form nitrogen-fixing symbioses to novel species is a goal of those concerned with the economic and ecological impact of chemical fertilizers. Small in-roads have been made in this regard, but much remains to be discovered about introducing nitrogen fixation to additional species. Herein we review biological aspects of actinorhizal symbioses; consider the horticultural potential of temperate, woody species that form these symbioses; and discuss how nitrogen-fixing symbiosis may impact the stress resistance and use of actinorhizal species as horticultural crops.

KEYWORDS. Actinorhizal, nodulation, nitrogen fixation, stress tolerance, nursery crops

INTRODUCTION

Actinorhizal species are diverse dicotyledonous plants capable of developing root nodules that house nitrogen-fixing bacteria in the genus *Frankia* (Actinomycetales). These plants, almost all of which are woody, formerly were known as non-leguminous nitrogen-fixing plants to distinguish them from the subset of legumes that forms symbioses with nitrogen-fixing rhizobia. "Actinorhizal" was adopted in 1978 to reflect the symbiotic nature of the plant-microbial interaction (Tjepkema and Torrey, 1979). "Actino-" refers to the actinomycete *Frankia*, and "-rhizal" specifies the location of the symbiosis on the plant root. Because *Frankia* fix atmospheric dinitrogen, actinorhizal plants often occur as pioneer species on nitrogen-poor soils and thereby play particularly important roles in the course of ecological succession. Actinorhizal plants can add significantly to the nitrogen economy of the soil in which they grow (Morris et al., 1974; Lepper and Fleschner, 1977; Dawson, 1990; Paschke, 1997) and pave the way for subsequent establishment of non-nodulating species. In managed landscapes, where ornamental plants are introduced to soils that are or become low

in nitrogen, actinorhizal species often are regarded as unusually resilient by arborists and horticulturists who may not be aware that nitrogen fixation contributes to the success of certain woody species.

Promoting greater recognition of actinorhizal plants that are or have the potential to become important in horticultural commerce is one of our goals for this paper. We will highlight selected actinorhizal taxa with unusual horticultural merit and will contrast actinorhizal plants and nitrogen-fixing legumes, focusing on the unusual features of actinorhizal symbioses. Recognizing actinorhizal taxa with commercial potential is important, in part, because of their prevalence. There appear to be more temperate, actinorhizal species with horticultural importance or promise than there are temperate woody legumes that are common in the nursery industry and that associate with rhizobial bacteria. For example, several genera of temperate, leguminous trees that often are produced in nurseries, e.g., *Gleditsia*, *Cercis*, *Gymnocladus*, *Styphnolobium*, and *Cladrastis*, are comprised only of species that do not develop nitrogen-fixing symbioses (Allen and Allen, 1981; Foster, Horner, and Graves, 1998). Our final goal is to consider whether actinorhizal associations may confer enhanced stress resistance to the plant partner in the symbiosis. Although relatively little evidence has been collected on this subject, our discussion will be based on the hypothesis that actinorhizal species directly avoid stresses due to low soil nitrogen and also may avoid adverse effects of other biotic and abiotic stressors indirectly via increases in nitrogen content.

Phylogenetics and geographic distribution

A feature that sets actinorhizal plants apart from legumes is the occurrence of actinorhizal symbioses within multiple taxonomically distinct groups. In contrast to legumes

in which symbioses occur within one family, Leguminosae (Fabaceae), actinorhizal plants represent eight plant families and 24 genera (Benson and Silvester, 1993). According to this conventional classification system, which is based on comparative anatomy and morphology (e.g., Cronquist, 1981), the evolution of actinorhizal symbioses seems to have occurred a number of times in largely unrelated species (Sprent, 1979). However, more recent molecular systematic studies based on DNA sequence comparisons of the chloroplast gene *rbc*L imply a closer relationship among actinorhizal species than was previously thought (Swenson and Mullin, 1997). Studies of relationships among taxa at the molecular level have allowed for discrimination of the more subtle aspects of plant classification and provide an opportunity to make inferences about the evolutionary relatedness between such groups. In this light, a single origin for the predisposition for root nodule symbiosis (both legume and actinorhizal) has been proposed (Soltis et al., 1995), with evidence for the multiple occurrence of nitrogen-fixing symbiosis within each group (Swenson, 1996). Non-symbiotic relatives exist within taxonomically related genera, but the precise set of conditions that favors the development of novel symbioses within the actinorhizal plants has yet to be determined.

There are around 200 known plant species that interact with *Frankia* (Swenson and Mullin, 1997; Huss-Danell, 1997), all of them woody except two species within the genus *Datisca* that have herbaceous shoots (Paschke, 1997). The family Betulaceae, one of the earliest to appear in the pollen fossil records (Muller, 1981), is also the family that has been the most extensively investigated and is considered by some to be a "model" plant for the study of actinorhizal symbioses in temperate regions (Bousquet and Lalonde, 1990). It includes both non-symbiotic and symbiotic plants, including the nitrogen-fixing genus *Alnus* (alders). *Alnus* spp. are widely distributed in wet to wet-mesic soils throughout the temperate zone,

with representatives occurring in both North and South America, Europe and Asia, and in Oceania (Australia, New Zealand), where it has been introduced (Baker and Schwintzer, 1990; Dawson, 1990). Members of the family Myricaceae (*Myrica* and *Comptonia* spp.) appear about the same time in the fossil records and are native to the above geographic regions, plus Africa and Australia, where they occur under a wide range of conditions, from sandy soils to woodlands and coastal bogs (Morris et al., 1974; Wheeler and Miller, 1990). Next to appear in the fossil records, the Casuarinaceae family includes plants in the genera *Casuarina*, *Allocasuarina*, *Ceuthostoma*, and *Gymnostoma*. These species have a much narrower natural distribution range, native only to Australia, Malaysia, and Polynesia, but occur widely in both the northern and southern hemisphere as an economically important, introduced species (Diem and Dommergues, 1990). Members of this family are unique in that they are angiosperms yet possess conifer-like, reduced leaves and photosynthetic, deciduous branchlets (Torrey and Berg, 1988). They grow primarily in warm and humid environments, but selected species can be adapted to a wide variety of conditions (Diem and Dommergues, 1990).

Among actinorhizal plants for which pollen fossil records exist, the families Rosaceae, Elaeagnaceae, and Coriariaceae have appeared most recently. Symbiotic members of the Rosaceae family (*Cercocarpus, Chamaebatia, Cowania, Dryas, and Purshia* spp.) are native primarily to Europe (Wheeler and Miller, 1990) and North America (Baker and Schwintzer, 1990), where a number of species are ecologically important in the harsh chaparral and desert environments of the rangelands in western United States (Paschke, 1997), and other species (Dryas spp.) survive as far north as the arctic tundra (Benson and Silvester, 1993). The distribution range of the Elaeagnaceae family (*Elaeagnus, Hippophae, Shepherdia* spp.), like Casuarinaceae, has been expanded greatly by economic activity, the natural distribution

being limited to the temperate areas of North America and Europe (Baker and Schwintzer, 1990). Coriariaceae (*Coriaria* spp.) exist in disjunct populations in dry woods, hedges, and rocky places on every continent except Africa and Antarctica (Silvester, 1977; Baker and Schwintzer, 1990; Wheeler and Miller, 1990).

Fossil records do not exist for the Rhamnaceae *(Ceanothus, Colletia, Discaria, Kentrothamnus, Retanilla, Talguenea, Trevoa* spp.) and Datiscaceae (*Datisca* spp.). The natural distribution of members of the family Rhamnaceae appears to be limited to North and South America, and Oceania, where they are often exposed to extreme temperature and drought stress (Baker and Schwintzer, 1990). A number of species can be found only in South America (*Colletia, Retanilla, Talguenea, Trevoa* spp.), where they are limited to the xeric environment of the Chilean matorral (Silvester, Balboa, and Martinez, 1985). *Datisca* occurs in North America and Eurasia, where it grows along the banks of streams (Baker and Schwintzer, 1990; Wheeler and Miller, 1990).

Another nonlegume species *Parasponia* (family Ulmaceae) forms nitrogen-fixing nodules, but with the legume-colonizing *Rhizobium* rather than the actinomycete *Frankia* (Trinick, 1973). It is, therefore, not considered a true actinorhizal species, even though nodules of *Parasponia* are anatomically and ontogenetically similar to actinorhizal nodules (Trinick and Galbraith, 1976). There have been no other reports of rhizobial infection of nonlegume species. A common origin for the predisposition to nodulation has been proposed (Soltis et al., 1995; Doyle, 1998), and legume symbioses are thought to be more recent on an evolutionary scale than actinorhizal symbioses (Pawlowski and Bisseling, 1996). Further, bacterial nodulation genes appear to be transmissible to diverse groups of bacteria (Sullivan and Ronson, 1998). Taken together, it is feasible that the *Rhizobium-Parasponia* symbiosis represents the actinorhizal missing link that connects the evolution of legume symbioses to

the earlier appearance of actinorhizal associations. It is equally likely, however, that root nodule symbioses arose separately in these two groups (Swenson, 1996), with the *Rhizobium-Parasponia* symbiosis representing a broadening of the host range of the microsymbiont. Such taxonomical paradoxes serve as a reminder that organisms often defy human classification systems. Further phylogenetic studies of nodulation genes and their expression in diverse species (Doyle, 1998) are needed to surmise the evolutionary history of root nodule symbioses.

Selected actinorhizal species with horticultural merit for temperate climates

Horticultural features of selected temperate, actinorhizal taxa found in commerce are summarized below.

***Alnus* spp. (alders)**

All of the approximately 30 species of alders are considered actinorhizal. Plants in this genus (family Betulaceae) grow at rapid rates and develop into shrubs or trees that typically occupy riparian or wetland niches in their native habitats in North America and elsewhere (Figure 1). A few species have been introduced to the horticultural trade. As would be expected based on their ecology in the wild, alders generally are used in managed landscapes with heavy or wet soils, although at least certain species also are resistant to drought (Graves, Kroggel, and Widrlechner, 2002; Hennessey, Bair, and McNew, 1985). The species are monoecious, and all but three bloom early in the season. Staminate flowers are in catkins that may be considered subtly ornamental. Pistillate flowers develop into woody strobili, a recognizable marker for the genus. Several rather obscure species have not been evaluated thoroughly for use in horticulture but appear promising (Graves, Kroggel, and Widrlechner, 2002).

***Ceanothus* spp. (e.g., redroot, New Jersey tea)**

There are over 50 deciduous and evergreen species in this genus of shrubs in the Rhamnaceae family. Numerous hybrids and cultivars of North American species have been developed; many of these are widely used in European gardens, where they are valued for attractive foliage and colorful flowers. *Ceanothus americanus* (New Jersey tea) is a widely distributed native of the United States with a growing, favorable reputation among native-plant enthusiasts. It forms showy, white flowers in panicles after many other shrubs in gardens have finished blooming. Whereas seasonal low temperatures restrict the use of many taxa of *Ceanothus*, New Jersey tea is cold-hardy to USDA hardiness zone 4 and may be the most suitable member of its genus for cold climates (Dirr, 1998).

***Hippophae rhamnoides* (common seabuckthorn)**

Common seabuckthorn, an attractive member of the Elaeagnaceae family, is native to western China and is commonly used in European landscapes. Although it is less common in North America, the species is considered hardy to USDA zone 4 and could be used in poor, sandy soils or in landscapes where plants are exposed to salt spray (Dirr, 1998). The foliage and fruits of this small tree are prominent and unusual. Leaves are linear to lanceolate and are covered with silvery scales. The fine-textured and lightly colored foliage is a striking backdrop for the bright, glossy, orange drupes that form in large masses on female plants (the species is dioecious) and persist from the autumn through early spring. Although they are acidic, the fruits are edible and can be processed into juice or other products that may be unusually beneficial to human health (Wheeler and Miller, 1990).

***Elaeagnus* spp. (e.g., Russian olive)**

This genus of the Elaeagnaceae family contains about 40 species, most (Dirr, 1998) or all of which are actinorhizal. *Elaeagnus angustifolia* (Russian olive) is the most commonly

planted species of this genus. It is native to Europe and Asia but has been widely used in North American landscapes with harsh conditions. Like common seabuckthorn, this species is considered salt tolerant and is frequently planted in coastal landscapes and along northern highways on which de-icing salts are applied. While its silvery leaves, fruits, and twigs provide an interesting color in the landscape, the irregular form of adult trees, the structural weakness of trees, and the susceptibility of the species to disease limit the value of Russian olive; the species is best planted in masses where trees will be viewed from a considerable distance. Other members of the Elaeagnaceae (e.g., *Shepherdia* spp.) also are actinorhizal but are plagued by many of same problems associated with *Elaeagnus* spp.

***Myrica* spp. (e.g., bayberries, wax myrtles)**

Three of the many species in this genus of the Myricaceae family have received particular attention from horticulturists. *Myrica pensylvanica* (bayberry) is an outstanding shrub species for cold climates. Plants grow 2 to 3 m tall and colonize soils of poor quality with upright twigs that bear pest-resistant, fragrant leaves and attractive fruit (female plants only of this dioecious species). *Myrica cerifera* (southern bayberry or wax myrtle) is taller than bayberry (up to about 11 m) and evergreen, but unfortunately, the species is difficult to grow in areas colder than USDA zone 7. Several cultivars of *Myrica cerifera* have been selected. They vary in foliar qualities, plant habit, and cold hardiness but as a group are rather intolerant of dry, alkaline soils. *Myrica gale* (sweet gale), a fragrant shrub of about 1 m in height, is at least as cold-hardy as *Myrica pensylvanica* but is best restricted to uniformly moist, acidic soils.

***Comptonia peregrina* (sweet fern)**

This genus of the Myricaceae contains only one species, sweet fern. Unlike some other species in its family, sweet fern is deciduous and monoecious. But, like *Myrica* spp., its

leaves are fragrant and attractively dissected (pinnatifid) in a manner similar to many true ferns. The primary use for this cold-hardy (USDA zone 2) species is as a large-scale ground cover. Plants colonize most rapidly on acidic soils and develop fine-textured stems that reach about 1 m tall.

Benefits that result from the use of these and other actinorhizal taxa often are most evident at planting sites where low nitrogen content in the soil limits plant growth or prevents survival of plants not associated with nitrogen-fixing microsymbionts. Establishment of highly effective actinorhizal symbioses, however, is dependent on many factors. If we are to capitalize on the potential of actinorhizal taxa, it is important to understand the biology of both symbiotic partners, the formation and structure of nodules, and how two prevalent components of the environment, oxygen and nitrogen, regulate symbiosis.

Characterization of the microsymbiont

Essential to gaining insight into nodule evolution is consideration of the coevolutionary patterns between the actinorhizal plant and its *Frankia* microsymbiont. *Frankia* spp. are genetically diverse, slow-growing, gram-positive bacteria widely distributed in the soil rhizosphere and occurring in both symbiotic and free-living states (Benson, 1982; Lechevalier and Lechevalier, 1990). Research into actinorhizal associations has lagged behind that of legume symbioses because of the difficulty in isolating and maintaining *Frankia* in culture. Only in the last 25 years have attempts to isolate *Frankia* from actinorhizal nodules been successful (Callaham, Del Tredici, and Torrey, 1978). To date, *Frankia* strains from 20 of the 24 actinorhizal genera have been isolated, but these isolates represent only a small number of actinorhizal species. In addition, strains from only four of the eight families are able to reinfect the source plant (Benson and Silvester, 1993), and

ineffective (nonnitrogen-fixing) nodules are common. The events leading to the formation of ineffective nodules are unknown, but may be related to host incompatibility and/or environmental factors (Benson and Silvester, 1993; Quispel, 1958), and the potential for competitive interactions between effective and ineffective *Frankia* strains exists (Van Dijk and Sluimer-Stolk, 1990). Nevertheless, the genus *Frankia* is morphologically distinct and has been described.

The genus *Frankia* is placed in the order Actinomycetales and the family Frankiaceae. Attempts to differentiate the genus further into species, either by phenotypic characteristics or by host infectivity groups, have been confounded by the lack of strain isolates from a number of known host genera and the apparent promiscuity of some actinorhizal species and *Frankia* strains for their symbiotic partners. Fortunately, with the availability of molecular phylogenetic techniques for comparing 16S rRNA gene sequences (Normand et al., 1996), relationships between *Frankia* strains, even those that have so far resisted cultivation, can be discerned (Clawson, Caru, and Benson, 1998). In this way, meaningful groupings of related strains may eventually lead to the establishment of taxonomically sound *Frankia* species. The clustering of *Frankia* strains based on DNA sequence homology is consistent with similar grouping for actinorhizal plants, which speaks to the fidelity of the available technologies for discerning such relationships and suggests the potential for coevolution between *Frankia* and their actinorhizal hosts (Normand et al., 1996; Swensen and Mullin, 1997; Clawson, Caru, and Benson, 1998).

In culture, *Frankia* spp. are branching, filamentous bacteria. They can be discriminated from other actinomycetes by the presence of mulitlocular sporangia, containing non-motile spores, at the terminus of or sometimes intercalary to branched septate hyphae. These hyphae eventually differentiate into variably thick-walled, lipid-encapsulated

structures called vesicles. (See Lechevalier and Lechevalier (1990) and Benson and Silvester (1993) for an extensive review of *Frankia* systematics and morphology). Other distinguishing taxonomic features of *Frankia* include their specificity for actinorhizal hosts, unique cell wall composition, presence of the polysaccharide 2-*O* -methyl-D-mannose, and their growth characteristics in culture (Lechevalier, 1994). The presence and shape of *Frankia* vesicles and the morphology of symbiotic nodules are largely determined by the host plant (Huss-Danell, 1997).

Nodule Development and Morphology

Interactions between symbiotic partners begin with an exchange of chemical signals that triggers novel gene expression and allows the bacterial microsymbiont to over-ride the pathogen defense response normally elicited in the plant host (reviewed by: Berry and Sunell, 1990; Pawlowski and Bisseling, 1996; Huss-Danell, 1997; Franche et al., 1998; Wall, 2000). In some hosts (Betulaceae, Casuarinaceae, Myricaceae), *Frankia* invades host cells intracellularly by way of deformed root hairs, and invading hyphae are encapsulated by a host-derived membrane. In other actinorhizal hosts (Elaeagnaceae, Rhamnaceae, Rosaceae, Coriariaceae, and Datiscaceae), infection occurs intercellularly. Hyphae of *Frankia* are not encapsulated within a host membrane during intercellular infection. But electron-dense materials rich in pectins and proteins are secreted by neighboring epidermal and cortical cells into the intercellular space as *Frankia* invades. *Frankia* strains capable of infecting multiple hosts may infect via the intercellular or the intracellular route, depending on the host plant (Miller and Baker, 1986). Concurrent with the infection process, early chemical signals induce divisions of cells of the pericycle, resulting in the formation of a nodule primordium,

which ultimately is infected by *Frankia* hyphae and grows outward toward the root epidermis to form a mature nodule.

The morphology of actinorhizal root nodules varies widely among species (Table 1) and reflects the prevailing environment in which the plant evolved (Silvester, Harris, and Tjepkema, 1990). In general, actinorhizal nodules are perennial, coralloid structures comprised of one to multiple lobes, depending upon the age of the nodule (Figure 2). Each nodule lobe is discrete and develops in a way that is similar to lateral root development, the vascular cylinder of the nodule continuous with the vascular tissue of the root. An area of *Frankia*-infected cortical tissue interspersed with non-infected tissue surrounds the central vascular bundle, although in *Coriaria* and *Datisca*, the infected tissue is asymmetrically localized to one side of the vascular bundle (Berg, Langenstein, and Silvester, 1999). A periderm with one (*Coriaria* spp.) to many (*Alnus* spp.) lenticels surrounds each nodule lobe. The periderm in *Coriaria* and *Datisca* encloses the nodule lobe but also follows closely and nearly surrounds the localized area of infected cortical cells; the thickness and degree of suberinization of the periderm are sensitive to the oxygen environment in the root zone (Silvester and Harris, 1989). In *Casuarina*, *Datisca*, *Myrica*, *Comptonia*, and *Gymnostoma* spp., a nodule root arises from the nodule lobe apex and grows up towards the soil surface, substituting for the lenticel in facilitating gas diffusion into the nodule. Consistent with their lateral root-like development, actinorhizal nodule lobes are perennial structures having an indeterminate growth pattern, although active nitrogen fixation has only been demonstrated within the current season's growth (Schwintzer, Berry, and Tjepkema, 1980). Each nodule lobe exhibits a gradient of cell development, ranging from the uninfected, actively dividing meristematic cells at the tip and the infected cells of the nitrogen-fixation zone, to the

senescence zone toward the base of the nodule, in which infected cells and their contents are being degraded (described in Ribeiro et al., 1995).

In contrast to nodules of legumes and *Rhizobium*-infected *Parasponia*, in which infected cells are bounded by a diffusion-limiting cell layer that acts as a barrier to oxygen, most actinorhizal nodules are relatively well-aerated, allowing oxygen to diffuse freely throughout *Frankia*-infected tissues. The mechanics of the control of gas diffusion in actinorhizal nodules varies with, and is likely the driving force for the diversity in, actinorhizal nodule structure. Because nitrogenase, the enzyme involved in symbiotic nitrogen fixation, is sensitive to oxygen concentration, the mechanism of protection of nitrogenase from oxygen is an area of active research in actinorhizal species (Parsons et al., 1987; Rosendahl and Huss-Danell, 1988; Silvester and Harris, 1989; Tjepkema and Murray, 1989; Kleeman et al., 1994; Zeng and Tjepkema, 1994; Alskog and Huss-Danell, 1997; Lundquist, 2000).

Oxygen and nitrogenase

The relationship between *Frankia* and actinorhizal plants is mutualistic. *Frankia* is capable of fixing atmospheric nitrogen, providing a usable form of nitrogen for its actinorhizal host. In turn, the plant releases enough photosynthate to sustain the microsymbiont. Nitrogen fixation is an energy-intensive process, requiring that large amounts of photosynthate be processed via respiration by *Frankia* to fuel the function of nitrogenase. Respiration requires the presence of oxygen, but oxygen inactivates the nitrogenase enzyme. So, there is a paradoxical relationship between nitrogenase and oxygen; oxygen is both inhibitory to but indirectly needed for the activity of nitrogenase. This conundrum is resolved in nitrogen-fixing organisms by the compartmentalization of

nitrogenase such that an oxygen diffusion gradient is established across the compartmental barrier (Silvester, Harris, and Tjepkema, 1990).

The barrier to the diffusion of oxygen can be either intrinsically or extrinsically provided (Gallon, 1981). Extrinsic protection of nitrogenase is furnished by either the host plant or by ambient environmental conditions. An example of host-provided protection is the legume-rhizobial symbiosis, in which the *Rhizobium*-infected cells are enclosed within a diffusion-resistant layer of cortical cells, resulting in a low internal oxygen environment. *Rhizobium* is unable to express nitrogenase when free-living in air. In the majority of actinorhizal species, however, the oxygen-diffusion barrier is intrinsic; the protection of nitrogenase from inactivation by oxygen is provided by the microsymbiont. In this case, a vesicle confers oxygen protection. Vesicles, which are derived from *Frankia*, act as envelopes and form at the tip of hyphae (Figure 3). *Frankia* strains that form hyphae can express the nitrogenase enzyme and fix nitrogen while free-living in air (Callaham, Del Tredici, and Torrey, 1978).

The vesicle is a multilaminate structure composed of one to many lipid monolayers that function as a barrier to the diffusion of oxygen. Although free-living strains of *Frankia* are capable of forming vesicles, the presence and thickness of the vesicle is dependent upon the oxygen environment in which the strain is grown (Parsons et al., 1987). *Frankia* cells grown in a high-oxygen medium develop thicker vesicles than do *Frankia* cells grown in a low-oxygen medium, and cells grown in an oxygen-free medium do not form a vesicle (Murray, Zhongze, and Torrey, 1985). Within the symbiosis, however, the presence and morphology of the vesicle is determined by the host plant (Huss-Danell, 1997). This was demonstrated using cross-inoculation studies with strains of *Frankia* capable of infecting multiple genera (St-Laurent and Lalonde, 1987; Torrey and Racette, 1989). Further, the strain of *Frankia*

within nodules of *Casuarina* does not form a vesicle in symbiosis but does vesiculate when cultured on a high-oxygen medium (Murray, Zhongze, and Torrey, 1985). Heavily suberized cells of the periderm may provide oxygen protection of nitrogenase in the *Frankia* of *Casuarina*.

It has not been determined whether these phenomena result from host-plant regulation of the symbiosis, or whether they are responses of the microsymbiont to ambient conditions within the nodule. Indeed, it is often by effecting changes within the nodule environment that signals between symbiotic partners are executed. Further, changes in plant-growth conditions can trigger such changes in the nodule environment and affect the symbiotic relationship and, therefore, nodule function (Quispel, 1958). Markham (1996) demonstrated that *Alnus rubra* inoculated with crushed-nodule inoculum from parent plants were smaller and showed lower rates of nitrogen fixation when grown at elevations that differed from the parent than plants grown at the same elevation as their parents. This observation suggests coevolution between *Frankia* and *Alnus rubra* and demonstrates a change in the relationship between *Frankia* and *Alnus rubra* triggered by environmental change. For this reason, any attempt to establish an actinorhizal species within a particular environment should involve careful consideration of the source and/or the strain of *Frankia* chosen for inoculation.

Response to nitrogen environment – Autoregulation

A well-documented example of the regulation of nitrogen-fixing symbioses in response to changes in the nodule environment was first observed in legumes (MacConnell and Bond, 1957; Parsons et al., 1993) and was later demonstrated in actinorhizal species (Arnone, Kohls, and Baker, 1994; Wall and Huss-Danell, 1997). Autoregulation is a feedback mechanism in which increasing nitrogen concentrations in plant tissues result in a

localized inhibition of nitrogen fixation and in a systemic response that inhibits the formation of new nodules in younger parts of the plant. Autoregulation is hypothesized to be a way for the plant to regulate nodule numbers and to prevent excessive export of photosynthate. As plant nitrogen concentrations rise, dependence upon the microsymbiont to meet nitrogen needs decreases, and the potential for parasitism by the microsymbiont in meeting its own energy needs increases. The first observable effect of high nitrogen is a decreased rate of nitrogen fixation. This nitrogen-induced inhibition of nitrogen fixation appears to result in altered activities and concentrations of stress-related molecules, such as reactive oxygen species and reactive oxygen-quenching enzymes, and the inhibition triggers the premature senescence of developing nodules (Swaraj, Laura, and Bishnoi, 1993).

This effect can be induced by externally applied nitrogen as well as microsymbiont-fixed atmospheric nitrogen and, in some cases, can be eliminated by correction of phosphate deficiency in the plant (Huss-Danell, 1997). Indeed, earlier studies have demonstrated a stimulatory effect of phosphorus on nodulation (Quispel, 1958; Rhigetti et al., 1982). It is hypothesized that the ratio of nitrogen to phosphorus concentration in the plant is crucial to nodule development (Huss-Danell, 1997).

These observations have implications for the growth of actinorhizal species in controlled environments. For example, as little as 0.7-mM exogenous nitrogen (as NH_4NO_3) significantly inhibited nodulation in *Cowania mexicana* (Righetti, Chard, and Munns, 1982). Similarly, only 1-mM nitrate inhibited nodule number in *Alnus glutinosa*, *Casuarina cunninghamiana*, and *Myrica cerifera* (Kohls and Baker, 1989). Although low-level nitrogen amendment of the soil during germination can prevent the premature death of young seedlings, an excess of nitrogen during later growth and development of actinorhizal species can inhibit nodulation and result in dependence upon applied fertilizers to meet nitrogen

needs. It may also promote lush green shoot growth at the expense of root growth and ultimately limit the overall growth potential of the plant (Benoit and Berry, 1990). However, small amounts of nitrogen applied in increasing doses that did not exceed the nitrogen consumption of the plant enhanced both nitrogen fixation and plant growth in *Alnus incana* (Ingestad, 1980). So it appears that the plant can adapt to changing nitrogen conditions as long as the capacity of the plant to use the nitrogen is not exceeded. Further work with the same alder species demonstrated that this species uses fixed atmospheric nitrogen more efficiently than it does applied ammonium (Sellstedt and Huss-Danell, 1986). In this study, NH_4NO_3 was provided to nonsymbiotic plants at the same rate that the symbiotic plants accrued fixed nitrogen. The symbiotic plants consistently exhibited greater biomass production and contained more total plant nitrogen than did nonsymbiotic plants (133 mg vs. 73 mg total plant nitrogen after 56 d). No difference in biomass production was found between symbiotic and nonsymbiotic plants, however, when nitrate was used as the source of applied nitrogen (Sellstedt, 1986).

Nitrogen-fixing symbioses and stress avoidance

Other abiotic factors that may affect nodulation success include soil moisture (Hopmans et al., 1983; Hennessey, Bair, and McNew, 1985; Hawkins and McDonald, 1993; Batzli and Dawson, 1997; Sayed et al., 1997), temperature (Reddell, Bowen, and Robson, 1985; Hawkins and McDonald, 1993; Sayed et al., 1997), pH (Quispel, 1958; Crannell, Tanaka, and Myrold, 1994), salinity (Hopmans et al., 1983; Reddell, Foster, and Bowen, 1986; Sande and Young, 1992), and levels of other essential minerals like iron (Aronson and Boyer, 1994), molybdenum and cobalt (Huss-Danell, 1997), and calcium (Crannell, Tanaka, and Myrold, 1994). Also important to the plant, however, is whether the capacity to fix

nitrogen will protect against stress-induced constraints in growth and development. That is, is there a selective advantage, under stressful environmental conditions, to have the capacity to form nitrogen-fixing relationships?

Under low-nitrogen conditions, it is obviously an advantage to a capable plant to interact with nitrogen-fixing microsymbionts; otherwise, there would have been no evolutionary pressure for the development of such symbioses. This fact forms the basis for the observation that actinorhizal plants are often a dominant understory species in the early stages of plant succession after ecological disturbances and are prevalent on nitrogen-poor soils (Dawson, 1990). The situation is not so clear when nitrogen is not a limiting factor to growth and reproduction.

It is reasonable to hypothesize that a plant that is optimally nitrogen-nourished will be more resistant to other abiotic stresses than a plant that is nitrogen malnourished. Indeed, Reddell, Bowen, and Robson (1985) demonstrated that plants of *Casuarina cunninghamiana* provided with supplemental nitrogen (NH_4NO_3) were less susceptible to chilling (15 °C) than were uninoculated plants that were not supplied with supplemental nitrogen. However, the same study showed an increase in susceptibility to both low and high (30 °C) temperatures in *Frankia*-inoculated plants as compared with plants grown in nitrogen-amended soil. This increase in susceptibility of inoculated plants was attributed to poor nodulation and inhibition of nitrogen fixation at these temperatures. Interestingly, at moderate temperatures (20 vs. 25°C), differences in root and shoot dry weights and in total nitrogen content between inoculated and nitrogen-supplemented plants were found. At 20°C, total nitrogen content and root/shoot dry weight were higher in plants grown with applied nitrogen, while at 25°C, they were higher in inoculated plants. These results demonstrate a susceptibility of the microsymbiont to environmental factors, but suggest enhanced growth and nitrogen content

of inoculated plants over nitrogen-amended plants when grown at microsymbiont-compatible temperatures.

Given what is known about actinorhizal plants, it may be safe to conclude that under low-nitrogen conditions, and without access to an alternative source of nitrogen, plants that form nitrogen-fixing symbioses may be less susceptible to abiotic stress than their nonnitrogen fixing counterparts, provided the stress does not prevent the function of *Frankia*. A more strongly supported case may be made regarding resistance of plants to biotic stress. There is evidence that infection and nodulation by *Frankia* may provide the plant with resistance to pathogens (Baker, Newcomb, and Torrey, 1980). Many researchers have demonstrated the effects of environmental stressors on nodulation and nitrogen fixation, but interactions between *Frankia* and its actinorhizal symbiont are delicate and complex. The tolerance of either organism to a particular stress may conflict with that of the other. In any symbiosis, a balance must be struck between the partners such that one does not exist at the expense of the other. If this should occur, interactions between the two break down, and defense mechanisms are triggered in one or both partners. More work is needed to clarify the interactions between actinorhizal symbionts and how environmental changes might affect the function of such relationships at the ecosystem level.

CONCLUSIONS

Actinorhizal woody plants, because of their nitrogen-fixing capability, have the potential for broad commercial use, both in land-reclamation projects and in sustainable agronomic and horticultural environments, where the capacity to fix atmospheric nitrogen can add significantly to the productivity of poor soils. There is great interest in expanding the

nitrogen-fixing habit to nonnitrogen-fixing species to reduce dependence on use of chemical fertilizers to replenish the nitrogen content of depleted soils. Previous efforts to accomplish this goal have focused on agronomically important grasses (Bennett and Ladha, 1992), by using rhizobia as the potential microsymbiont. However, such attempts have been only marginally successful, probably because of the genetic dissimilarity between grasses and legumes. It is postulated that introduction of the nitrogen-fixing habit to novel species may meet with less resistance in more closely related plants that share a genetic predisposition to form root nodule symbioses (Swensen and Mullin, 1997).

In this spirit, an attempt was made to graft the rootstock of the actinorhizal species, *Cowania mexicana,* with the scion of *Fallugia paradoxa*, a nonnitrogen-fixing species of the same family, Rosaceae (Kyle et al., 1986). Reciprocal grafts were also made. This group used an in vitro micrografting technique to minimize environmentally introduced stress and seedling-source rootstock as a way to avoid potential incompatibility that could arise in older, more differentiated tissue. Although their results were modest (25% of grafts were successful), and the formation of effective nitrogen-fixing nodules has yet to be demonstrated, this work represents an important step in advancing research on the nodulation process and may have commercial applications for many actinorhizal crop species.

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Table 1. Nodulation characteristics of various actinorhizal plant taxa, which are listed in order of their appearance in the pollen fossil record. A clade refers to a lineage branch that results from splitting in an earlier lineage, producing two distinct taxa. This information is based on reports by Newcomb and Heisey (1984), Miller and Baker (1986), Balboa, Avila, and Arce (1988), Silvester et al. (1990), Normand et al (1996), Huss-Danell (1997), Swensen and Mullin (1997), and Wall (2000).

Plant host	Nodule anatomy	Infection route	Microsymbiont
Betulaceae (*Alnus*)	Infected cells surround central vascular bundle; oxygen diffusion via multiple periderm lenticels	Intracellular	*Frankia* isolates Clade I
Myricaceae	Infected cells surround central vascular bundle; oxygen perception/diffusion via nodule root	Intracellular	*Frankia* isolates Clades I & II
Casuarinaceae	Infected cells surround central vascular bundle; oxygen perception/diffusion via nodule root	Intracellular	*Frankia* isolates Clade I; (Gymnostoma - Clades I & II)
Elaeagnaceae	Infected cells surround central vascular bundle; route of oxygen diffusion not investigated; no nodule root	Intercellular	*Frankia* isolates Clade II
(Rhamnaceae)	Infected cells surround central vascular bundle; route of oxygen diffusion not investigated; no nodule root)	Intercellular	*Frankia* isolates Clades II & III
Rosaceae	Infected cells surround central vascular bundle; route of oxygen diffusion not investigated; no nodule root	Intercellular	*Frankia* isolates Clade III
Coriariaceae	Asymmetric localization of infected cells nearly enclosed by diffusion-limiting cell layer and surrounding vascular bundle; oxygen diffusion via one periderm lenticel	Intercellular	*Frankia* isolates Clade III
(Datiscaceae)	Asymmetric localization of infected cells nearly enclosed by diffusion-limiting cell layer and surrounding vascular bundle; oxygen perception/diffusion via nodule root	Intercellular	*Frankia* isolates Clade III
Parasponia	Infected cells entirely enclosed by diffusion-limiting cell layer and surrounding vascular bundle; oxygen diffusion via leghemoglobin	Route of infection not investigated	*Rhizobium*
Legumes	Infected cells entirely enclosed by diffusion-limiting cell layer and surrounded by vascular bundles; oxygen diffusion via leghemoglobin	Intra/Intercellular	*Rhizobium*

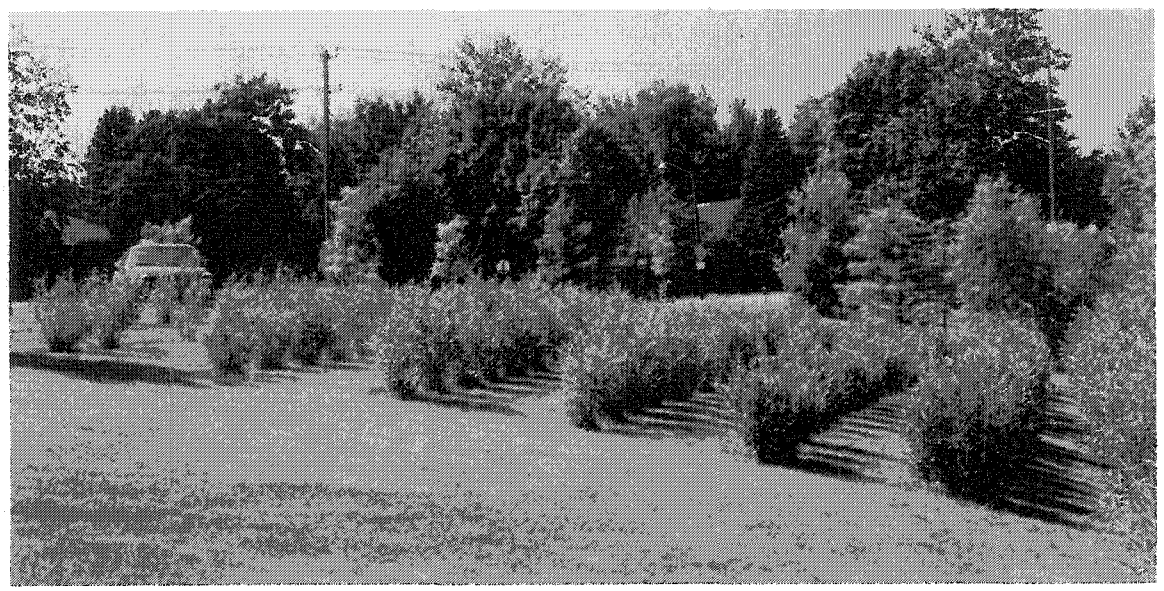

Fig. 1. Evaluation of the potential to use a rare species of alder in managed landscapes. Seedlings of *Alnus maritima* (seaside alder) were grown in rows along a slope in Ames, Iowa, USA. Rows were at various distances from a constant source of water at the bottom of the slope. Objectives of this trial included testing the cold hardiness of this species when planted north of its natural distribution, and determining whether plants could survive on drained soils; plants in the wild are found exclusively on saturated or continuously flooded soils. Commercialization of this attractive species is being pursued because plants proved hardy throughout the northern United States and have been shown capable of growth in dry soils.

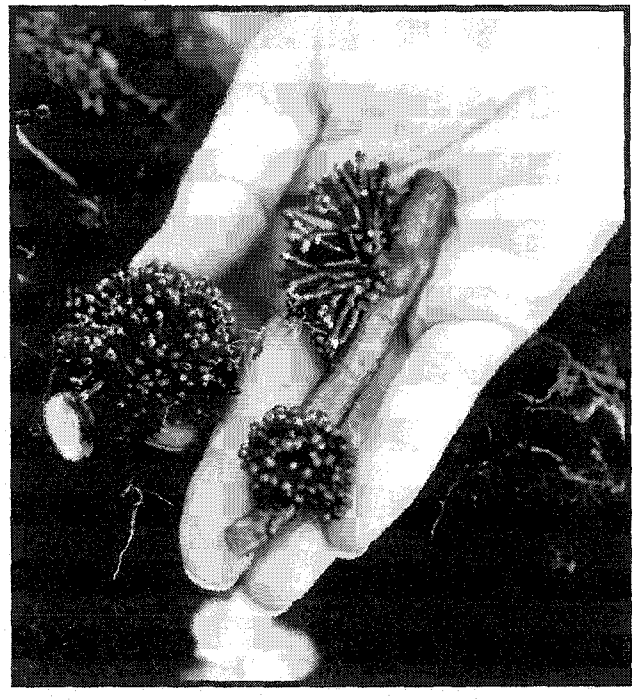

Fig. 2. External appearance of nodules on roots of *Alnus maritima* (seaside alder) dug on the Eastern Shore of Maryland, USA. Note the formation of multiple nodular lobes.

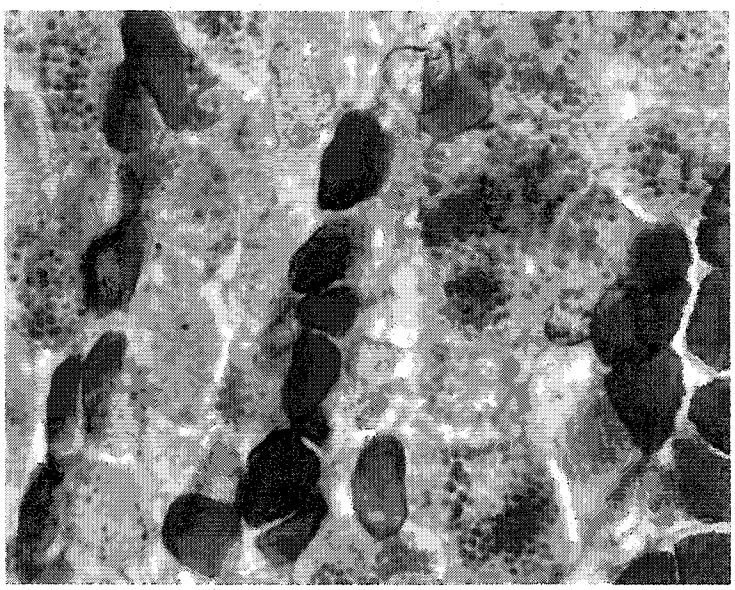

Fig. 3. Light micrograph of *Frankia* vesicles in cortical cells of a nodular lobe from *Alnus maritima* (seaside alder). The red-stained vesicles in this longitudinal section are uniformly spherical in shape and are thought to act as a barrier to oxygen diffusion within the lobe. Note the darkly stained cells, which contain tannins and phenolics. Bar represents 20 μm.

CHAPTER 3. ULTRASTRUCTURE OF NODULES FROM *ALNUS MARITIMA*

A paper published in *Acta Horticulturae (ISHS)*[1]

Heidi A. Kratsch, William R. Graves

Keywords: nitrogen fixation, alder, *Frankia*, hyphae, vesicles, symbiosis

Abstract

Alnus maritima (Marsh.) Muhl. ex Nutt (seaside alder) is an attractive woody perennial, is the only North American alder that flowers in autumn, and is considered threatened in its three small, disjunct natural habitats. An understanding of the conditions that foster or limit growth of seaside alder is crucial to its use and, perhaps, to its existence. Establishment of effective symbioses between *Alnus* and *Frankia* bacteria is sensitive to the concentration of oxygen in the root zone. *A. maritima*, in contrast to other alders, is restricted in its native range to flooded soils. Our hypothesis is that low oxygen in flooded soils affects nodule structure and function and may be essential to symbiotic compatibility. The objective of this work was to describe the morphology and structure of nodules from indigenous plants as a first step in testing our hypothesis. Roots of *A. maritima* subsp. *maritima* Schrader & Graves were excavated from saturated, sandy soils of Sussex County, Delaware, and Dorchester County, Maryland. Nodules were excised from the roots and processed for microscopy. The nodules are coralloid structures from 1-4 cm in diameter and comprised of one to many nodule lobes.

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[1]Reprinted with permission of Acta Hort. (ISHS), 2004, 630, 231-226.

Areas of *Frankia*-infected tissue within a lobe surround the vascular cylinder, which grows out from, and is continuous with, the vascular bundle of the plant root. Phenolic-containing cells delimit areas of *Frankia* infection in a way that hints at the eventual differentiation of the nodule into discreet lobes. *Frankia* hyphae invade new tissues acropetally and differentiate into septate multilaminar vesicles at their terminus. The ultrastructure of nodules from *A. maritima* has never been described and will serve as a basis for comparison in later work on oxygen relations in the *Frankia-Alnus maritima* symbiosis.

INTRODUCTION

Alnus maritima is a rare species that is capable of growth in poor soils by virtue of its capacity to form nitrogen-fixing symbioses with filamentous actinomycetes of the genus *Frankia*. However, its distribution is limited to only three disjunct areas in the United States (Schrader and Graves, 2002). In addition, this species is unique among *Alnus* spp. in that, in the wild, its roots seem to require waterlogged soils. Given the slow diffusion of oxygen through water, these soils tend to be low in oxygen. The bacterial microsymbiont, *Frankia*, is sensitive to oxygen and is unable to fix nitrogen at high oxygen levels. We hypothesize that the low oxygen concentration of its native wetland habitat is essential to the symbiosis between *Frankia* and *A. maritima*. Characterization of the factors affecting this relationship may be crucial to the survival and horticultural adaptation of this species. Our goal is to describe the ultrastructure of nodules of *A. maritima* and its bacterial microsymbiont to facilitate investigation of the oxygen relations between *Frankia* and its actinorhizal host.

Nodules of *Alnus* spp. are perennial and develop on the root in response to infection by compatible bacteria when plants are low in nitrogen. The nodule represents the physical

manifestation of a symbiosis between the plant and the nitrogen-fixing *Frankia* bacteria. Bond (1956) first demonstrated that nodules of field-grown *Alnus* spp. were capable of fixing and utilizing atmospheric nitrogen for plant growth. Since then, the structure of the nodules and the bacterial microsymbiont associated with a number of *Alnus* spp. have been characterized (Baker and Schwintzer, 1990; Benson and Silvester, 1993). Stibolt (1978) examined nodules of *A. maritima*, but a thorough description that includes ultrastructural details has not been reported.

MATERIALS AND METHODS

Nodules were collected in July 2001 from indigenous trees of *Alnus maritima* subsp. *maritima* Schrader & Graves (Schrader and Graves, 2002) at sites along the Delmarva Peninsula in Sussex County, Delaware, and in Dorchester County, Maryland. The roots on which nodules were found were in inundated soils of a creek and a river, and water was present above the soil surface. Although both waterways were influenced by tides, salinity in the area where the trees were found was very low, and adjacent vegetation was typical of a fresh-water wetland habitat. Large portions of root systems were transported to a local laboratory and held in cool, moist conditions. Nodules were excised from roots within the next 24 h, placed under running tap water to clear away remaining soil and debris, and immediately immersed in fixative.

Electron Microscopy

Tissues were fixed in a solution of 2% glutaraldehyde and 2% paraformaldehyde in a 0.1-M sodium cacodylate buffer, pH 7.2. While in fixative, individual nodule lobes were separated and dissected into three regions: apical, middle, and basal. Other lobes were

divided in half longitudinally, and smaller lobes were kept whole. All tissues were stored at 4 °C overnight in fresh fixative. After rinsing with plain buffer, the tissue was postfixed in 1% aqueous osmium tetroxide, dehydrated through a graded series of ethanols, and embedded in Spurr's resin. Thin sections (55 nm) were stained with aqueous uranyl acetate and lead acetate and viewed in a JEOL 1200Ex (JEOL USA, Inc., Peabody, MA, USA) scanning transmission electron microscope operating at 80 kV.

Light Microscopy

Whole nodules were sliced longitudinally, fixed in FAA (formalin-acetic acid-alcohol) buffer, and stored overnight at 4 °C. Tissues were dehydrated through a graded series of ethanols to xylene, embedded in paraffin, and sectioned into thick sections (10 μm) by using a Spencer "820" rotary microtome (Aloe Scientific, St. Louis, MO, USA). Paraffin-embedded sections were stained with Safranin O and Fast Green, and resin-embedded thick sections (1 μm) were stained with Toluidine Blue. These were viewed using an Olympus Bx40 (Olympus Optical Co. Ltd., Japan) compound light microscope.

RESULTS AND DISCUSSION

Nodules occurred on all trees we sampled and were from 1 cm to over 4 cm in diameter. The oldest nodules were large with long, distinct lobes. They were often found on the primary root, just below the root collar. Younger nodules were distributed throughout the root system, most often within the top 15 cm of soil/water. The lobes on the youngest nodules were compact, the distinction between individual lobes often barely discernible (Fig. 1a). Lobes from young nodules had multiple vascular connections to the vascular cylinder of the plant root from which they were excised. Numerous lenticels were observed around the

outer periderm of the nodule. Inside a nodule lobe were one to many vascular cylinders (Fig. 1b). Areas of pink were observed in the cortical tissue surrounding the vasculature. These areas represented the zone of *Frankia* infection. The pink color was presumably due to the presence of plant hemoglobin.

At the light microscopic level, areas of *Frankia* infection were bordered by cells containing tannins and phenolics. Layers of these phenolic-containing cells formed the outline of a single nodule lobe. We presume that these cells develop before the establishment of distinct nodule lobes because they can be seen in nodule lobes that have not yet differentiated (Fig. 2a). It is likely that these cells function as a barrier to the unchecked spread of *Frankia* bacteria throughout the nodule lobe. Indeed, *Frankia* hyphae infect new cells acropetally, but do not spread laterally through the nodule. Hyphal strands were noted to cross the cell walls readily in nodule longitudinal sections (not shown), but they were never observed to penetrate the cell wall in nodule cross-sections (Figs. 3a and b).

Another notable feature of the nodule lobe of *A. maritima* is the manner in which the vasculature branches and differentiates. At the base of a young nodule, there are numerous vascular connections to the plant root. The vascular bundles grow and develop along with the developing nodule lobe, and they begin to branch before a single nodule lobe has differentiated into two distinct lobes (Fig. 2a). It is not known what signals the vascular bundle to branch, but it is to the plant's advantage to have vasculature close to *Frankia*-infected cells because the nitrogen fixed by the bacteria is transported to the plant through the xylem (Huss-Danell, 1990). Access to the vasculature is also crucial to *Frankia*, which relies upon phloem-transported carbon compounds as its energy source. Therefore, it is conceivable that either or both organisms may cooperate in inducing vascular branching within a nodule lobe.

Within a single lobe, we identified four zones that correspond to the developmental stages observed in all actinorhizal nodules (Baker and Schwintzer, 1990) (Fig. 2b). The apex of the nodule lobe was free of vasculature and consisted of small dividing cells, which were void of *Frankia*. The apex represents the zone of cell division within a nodule. It is responsible for the elongation of the lobe as the nodule grows and differentiates into multiple lobes. Below the apical zone is an area of cells that are being penetrated acropetally by *Frankia* hyphae (Fig. 3a). The hyphae are encapsulated in cell wall matrix of the host (Fig. 3b and c). Surrounding the area of *Frankia* invasion within a cell are numerous vacuoles (or many manifestations of a single vacuole). Uninfected cells are singly vacuolate and contain plastids with large starch granules. This area represents the infection zone. While the invasion of this area by *Frankia* is of obvious importance, the role of this area as the site where new vasculature develops also should be recognized.

About 2 mm from the apex of a nodule lobe is an area of *Frankia*-infected cells in which the *Frankia* hyphae have differentiated into uniformly spherical vesicles (Fig. 4a). This area represents the nitrogen-fixation zone, as the vesicle is the main site of nitrogen fixation by *Frankia*. In this zone, many cells are completely filled with *Frankia* vesicles, and vacuoles have disappeared. The vesicles are multi-septate and surrounded by a host-derived capsule (Fig. 4b). The void space between the vesicle wall and the host-derived capsule (Fig. 4c) has been described (Berry and Sunell, 1990) and is likely to represent loss of the multilaminar lipid vesicle envelope during fixation of tissue for transmission electron microscopy. Also present are numerous mitochondria and variably shaped plastids, which lack starch granules (Fig. 3b). The presence of amoeba-like plastids in *Frankia*-infected tissue has also been reported by Gardner et al. (1989). They hypothesized that the appearance of this morphologically unique plastid only in cells containing *Frankia* vesicles

suggests a shift in function from storage to a metabolically active organelle. At the base of the nodule is an area of disorganized cells, in which each cell has plasmolyzed and the vesicles are clustered in the middle of the cell (Figs. 5a and b). This area, called the senescence zone, is the oldest part of the nodule lobe. These cells and their components are being degraded for recycling of nitrogen and other essential compounds.

An oft-cited feature of the nodules of *Alnus* spp. is the presence of air spaces between cortical cells within the nodule lobe that are continuous with the lenticels along the outer nodule periderm. Silvester et al. (1988) demonstrated a difference in the size and distribution of air spaces between *Frankia*-infected cortical cells in response to growth under various root-zone oxygen concentrations. We have noted a similar difference in the size of nodule cortical air spaces between root nodules excised from plants in the wild and those excised from nursery-grown plants (Figs. 6a and b). The presence of large, prominent air spaces in nodules of nursery-grown plants suggests a low oxygen concentration in the root zone of seedlings grown in pots. This could have implications for the horticultural production of this species, and the phenomenon is currently under investigation.

In summary, nodules of *A. maritima* have a perennial growth habit that is reflected by the existence of four distinct zones of development. As a nodule grows out from the apical tip, *Frankia* hyphae invade the newly developed cells, eventually differentiating into an actively nitrogen-fixing form manifested by a terminal spherical vesicle. At the end of a growing season, these cells begin to degrade and nitrogen fixation ceases. Nitrogen fixation resumes with the onset of a new growing season and subsequent growth and infection of new nodule tissue. Our future work is directed at further characterization of the senescence zone in an effort to understand the environmental cues that lead to the senescence of nodule tissue.

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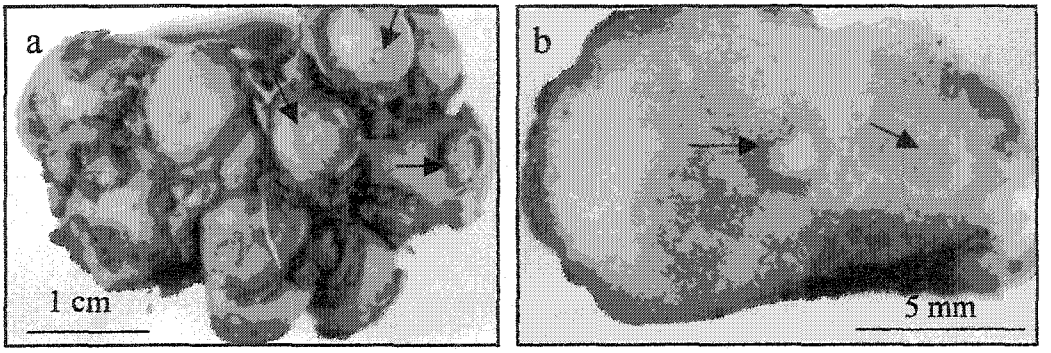

Fig. 1. Light microscopic view of a young nodule. (a) Whole nodules are from 1 to 4 cm. in diameter and consist of multiple lobes. Each lobe has numerous lenticels around the periderm (arrows). (b) A cross-section of a nodule lobe reveals multiple vascular cylinders (arrows).

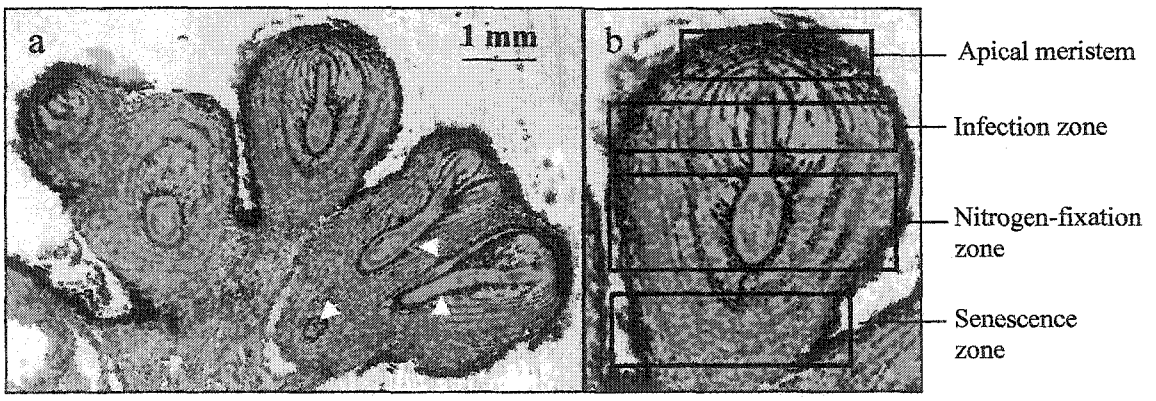

Fig. 2. Cross-section of a whole nodule. (a) Darkly stained cells contain tannins and phenolics and delimit regions of *Frankia* infection. Vasculature branches before individual lobes differentiate into multiple lobes (white arrowheads). (b) Individual nodule lobes consist of four distinct developmental zones.

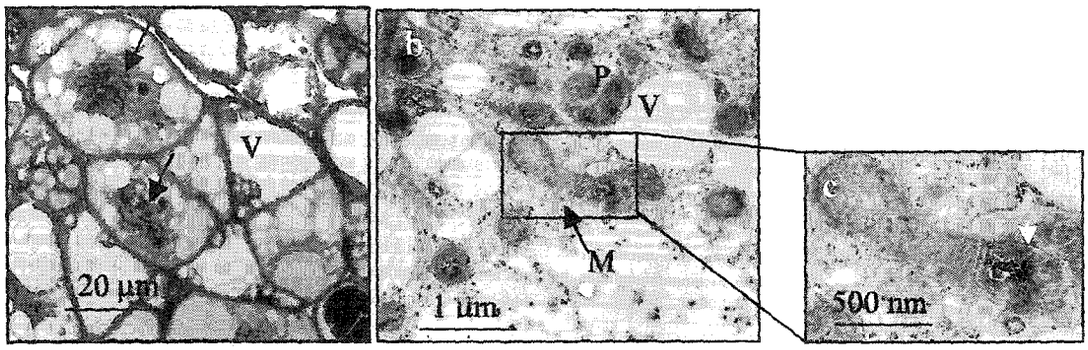

Fig. 3. *Frankia* hyphae in the infection zone. (a) LM image of *Frankia* hyphae invading new cells acropetally (arrows). (b) TEM image of infected cells. They are metabolically active and contain numerous mitochondria and variably shaped plastids with a reduced number of starch granules. (c) *Frankia* hypha with vesicle forming at the terminal end (white arrowhead). (M, mitochondrion; P, plastid; V, vacuole)

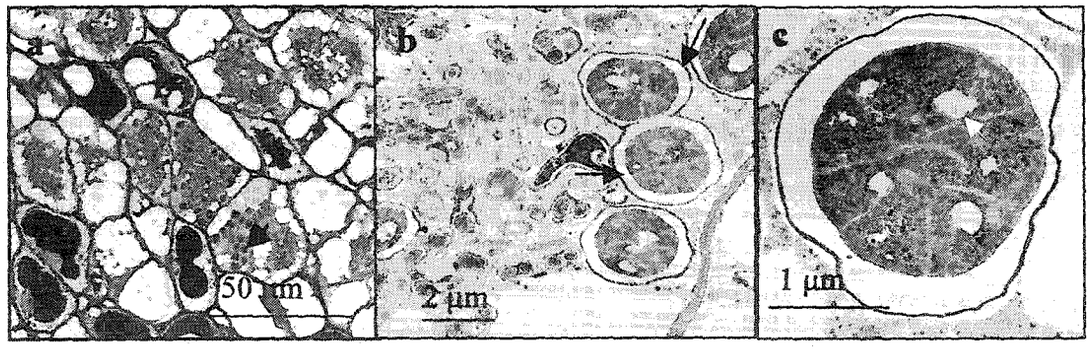

Fig. 4. *Frankia* vesicles in the nitrogen-fixation zone. (a) LM image of *Frankia* vesicles (arrow). As vesicles develop, vacuoles break up and disappear. (b) TEM image of *Frankia* vesicles, which along with the hyphae, are encapsulated in host cell wall matrix (arrows). (c) Close-up view of a vesicle. Note the multiple septa within the vesicle, which is characteristic of *Alnus* spp (white arrowhead).

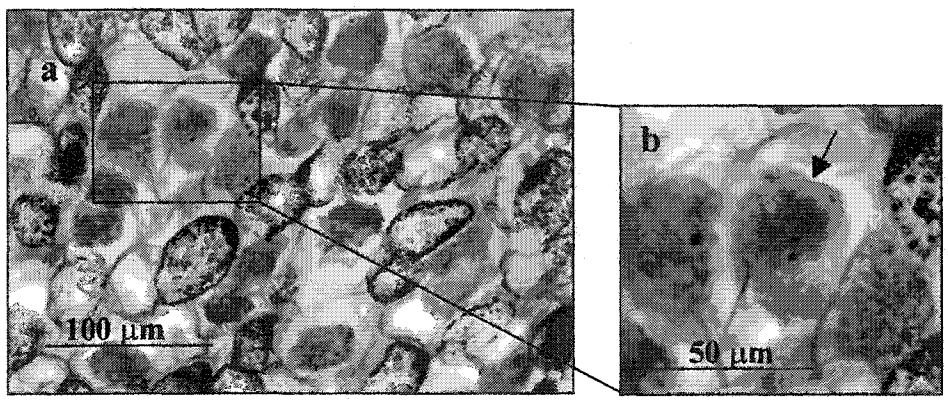

Fig. 5. Cells in the senescence zone (LM). (a) Cells in the senescence zone are plasmolyzed and *Frankia* vesicles are clustered in the middle of the cell. (b) Close-up view of plasmolyzed cells. Arrow points to the plasma membrane, which has pulled away from the cell wall due to shrinking of the cytoplasm.

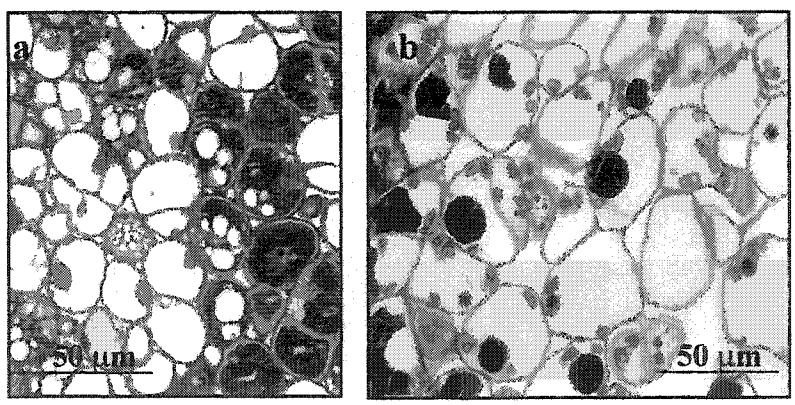

Fig. 6. Comparison of air spaces between cortical cells from nodules of indigenous *A. maritima* (a) with those from nodules of nursery plants grown in plastic pots (b). Airspaces in nursery-grown nodules are large and prominent, which suggests growth under low oxygen concentrations.

CHAPTER 4. LOCATION AND ANATOMY OF NODULES ON *ALNUS MARITIMA* SUBJECTED TO FLOODING

A paper accepted by the *Journal of the American Society for Horticultural Science*

Heidi A. Kratsch, William R. Graves

ADDITIONAL INDEX WORDS. seaside alder, *Frankia*, flood tolerance, symbiosis, oxygen, wetland, actinorhizal

ABSTRACT. Although many species of *Alnus* Miller grow in wet soils, none is as closely associated with low-oxygen, waterlogged soils as *Alnus maritima* (Marsh.) Muhl. ex Nutt. (seaside alder). An actinorhizal species with promise for use in horticultural landscapes, land reclamation, and sustainable systems, *A. maritima* associates with *Frankia* Brunchorst, thereby forming root nodules in which gaseous nitrogen is fixed. Our objective was to determine how root-zone moisture conditions influence the occurrence, location, and anatomy of nodules on *A. maritima*. Plants of *Alnus maritima* subsp. *maritima* Schrader and Graves were established in root zones with compatible *Frankia* and subjected to four moisture regimens (daily watered/drained, partially flooded, totally flooded, and totally flooded with argon bubbled through the flood water) for eight weeks. Oxygen content of the root zone, number and location of nodules on root systems, and dry weight and nitrogen content of shoots were determined. Root-zone oxygen content ranged from 17.3 kPa for daily watered/drained plants to 0.9 kPa for argon-treated plants. Across all treatments, 87% of the nodules were within the upper one-third (4 cm) of the root zone. Although shoot dry weights of daily watered/drained and partially flooded plants were not different, daily

watered/drained plants had more nitrogen in their leaves (25.3 vs. 22.1 $mg \cdot g^{-1}$). Nodulation occurred in all treatments, but nodules on totally flooded roots (with or without argon) were limited to a single lobe; in contrast, multilobed nodules were prevalent on partially flooded and daily watered/drained plants. *Frankia* infection within these submerged nodule lobes was limited to one or two layers of cortical cells. Submerged nodules developed large air spaces between cortical cells, and phenolic-containing cells appeared to inhibit *Frankia* expansion within the nodule. These data suggest that access to root-zone oxygen is critical to the *Frankia-A. maritima* subsp. *maritima* symbiosis, and that plants of this supspecies in the drained soils of managed landscapes may benefit more than plants in native wetland habitats from nodulation and nitrogen fixation.

Introduction

Alnus maritima is a rare shrub unique among *Alnus* spp. for its natural occurrence exclusively in waterlogged soils (Schrader and Graves, 2002). This species is indigenous to only three disjunct locations in North America. It is found in and along rivers and streams in Oklahoma and in Delaware and Maryland on the Delmarva Peninsula, and in one swamp in Georgia (Schrader and Graves, 2003). Like other *Alnus* spp. (Bond, 1956), *A. maritima* forms symbioses with the nitrogen-fixing bacterium *Frankia* (Stibolt, 1978). Symbiotic interactions culminate in formation of root nodules, a developmental process that is susceptible to irradiance (Hughes et al., 1999), temperature, pH, and nutrient status of the root zone (Quispel, 1958).

Alnus maritima tolerates several abiotic stressors, including cold (Schrader and Graves, 2003), drought (Graves et al., 2002; Schrader et al., 2004), and to a limited extent, salt (Graves and Gallagher, 2003). The species shows great potential for use in land

reclamation, sustainable systems, and other applications through which its capacity to improve soil by fixing atmospheric nitrogen can be especially beneficial. Understanding the factors that foster or limit growth of *A. maritima* would not only be of practical value but would facilitate efforts to preserve the species in its native habitat. All states where *A. maritima* is native consider the species rare, threatened, or imperiled (Schrader and Graves, 2002).

Hypoxia created by waterlogged soils presents a challenge for *A. maritima* roots and for *Frankia* because they both require oxygen to respire. Nodule occurrence (Schwintzer and Lancelle, 1983) and anatomical adjustments (Silvester et al., 1988) to low oxygen have been studied in *Myrica* spp., another actinorhizal genus of plants that occupies oxygen-poor soils. Whole-plant responses to flooding have been described for the more common *Alnus* spp. and include adventitious rooting and hypertrophied nodule and stem lenticel formation (Batzli and Dawson, 1997; Gill, 1975; McVean, 1956). The impact of flood-induced hypoxia on nodule occurrence in *Alnus* spp. and on nodule anatomy in *A. maritima* has not been investigated. Our objective was to determine whether flooding affects the location of nodules within the root system of potted *A. maritima*, the anatomy of nodules, and *Frankia* survival. We conducted this research with plants grown from seeds collected on the Delmarva Peninsula. This population, recently named *A. maritima* subsp. *maritima* (Schrader and Graves, 2002), was studied because the Plant Materials Center of the U.S. Dept. of Agriculture – Natural Resources Conservation Service (Cape May, N.J.) has expressed an interest in using it for conservation plantings.

Materials and Methods

Plant growth and development

PLANT MATERIALS. *Alnus maritima* subsp. *maritima* was propagated from seed collected in Dorchester Co., Md. Seeds were surface-sterilized in 10% commercial bleach for 2 min; rinsed in distilled, deionized water; and cold-stratified at 4 °C for three weeks. Seeds were sown in plastic pots (top diameter = 11.5 cm, height = 9.5 cm, volume = 929 cm^3) filled with an unpasteurized mix of 1 soil (sandy clay loam obtained in Iowa and confirmed in preliminary trials to contain *Frankia* compatible with *A. maritima*) : 2 perlite : 2 sphagnum peat (by volume). The pH of the substrate was 6.0. The pots were held on a greenhouse bench where 16-h photoperiods were provided by using incandescent lamps. Seedlings were irrigated with tap water daily and fertilized three times per week with Peters Excel All-Purpose and Cal-Mag® (17N–2.2P–13.3K) (Scotts Sierra Horticultural Products, Marysville, Ohio), providing nitrogen at 11 mM to prevent nodulation before treatments began. Nitrogen was 13.8% NH_4^+, 73.7% NO_3^-, and 12.5% urea. Three weeks after germination, seedlings were transferred to individual plastic pots (top diameter = 12.5 cm, height = 12.5 cm, volume = 1406 cm^3).

TREATMENTS AND EXPERIMENTAL DESIGN. Forty-four uniform seedlings that were four months old were considered experimental units and assigned randomly to one of four treatments (n = 11): daily watered/drained (positive control), partial flood, total flood, and total flood/argon (negative control). The pot of each seedling was placed in an opaque plastic container (top diameter = 14 cm, height = 15 cm, volume = 2520 cm^3). Daily watered/drained plants were irrigated daily with 400 mL of tap water, and pots were allowed to drain outside their external container such that no free water accumulated within the external container. Partially flooded conditions were imposed by maintaining 1.5 cm of tap

water in the bottom of the external container. Totally flooded conditions were maintained by keeping the external container filled with tap water and a water column 2 cm above the solid matrix. Total flood/argon treatment consisted of bubbling argon into totally flooded containers by using a gas-dispersion stone at the bottom of the external container. Argon was chosen over nitrogen as an inert gas for displacement of oxygen because of the potential for nitrogen to confound nodulation data. The gas-dispersion stones were connected to a cylinder of compressed argon via small-bore tubes attached to a copper manifold. Plants were held on a greenhouse bench where 16-h photoperiods were achieved by using two 400-W, high-pressure sodium lamps. Treatments were initiated 5 June 2002, and plants were harvested eight weeks later. We used a data logger (CR23X, Campbell Scientific, Logan, Utah) equipped with appropriate sensors to monitor the environment during treatments and found air temperature averaged 24 °C (range 21 to 35 °C), mean relative humidity was 67% (range 32 to 95%), and the maximum photosynthetically active radiation on cloudless days was 690 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Weights of all leaf blades, stems, and petioles were determined after tissues dried for 3 d at 67 °C. Dried leaf blades were ground in a tissue homogenizer and analyzed for nitrogen content by the Kjehldahl method. Oxygen content of each root zone was measured, after four and eight weeks of treatment, with a needle oxygen electrode connected to a chemical microsensor (Diamond General Development Corp., Ann Arbor, Mich.). Measurements were taken within 1 h after irrigation of the plants, and the electrode was placed 4 cm below the surface of the solid matrix (6 cm below the surface of the water for flooded plants). We used the general linear models (GLM) procedure of SAS/STAT®, Version 8.1 to examine the effect of irrigation treatments on the dependent variables, and

when appropriate, Fisher's least significant difference (LSD) test was used at $P \leq 0.05$ to separate irrigation treatment means.

Nodule occurrence and location

We recorded the number of nodules on each root system as plants were harvested. Each nodulation event, whether it resulted in a single nodule lobe or a cluster of lobes, was counted as one nodule. Roots of each plant were washed thoroughly and placed against a grid to determine the location of each nodule within the vertical profile of each root system (Fig. 1). Area 1 on the grid represented the lowest 1.5 cm of each root zone and corresponded to the saturated portion of root zones of the partially flooded plants. The upper boundary for area 2 represented the vertical middle of the root zone for partially flooded and daily watered/drained plants. Area 3 corresponded to the upper 4 cm of the solid matrix in each root zone, and area 4 represented the zone above the solid surface, which was filled with either water or air depending upon the treatment. We also used GLM and Fisher's LSD to examine variation in the number of nodules that formed.

Microscopy

All nodules from the three most morphologically representative plants from each treatment were harvested for chemical fixation. Nodules were fixed in a solution of 2% glutaraldehyde and 2% paraformaldehyde in a 0.1-M sodium cacodylate buffer at pH 7.2. While in fixative, nodule clusters were separated into individual lobes. Large-diameter lobes were dissected longitudinally, and smaller lobes were left whole to obtain a uniform sample size of ≤ 3 mm^3. All tissues were stored at 4 °C overnight in fresh fixative. After rinsing with buffer, the tissue was post-fixed in 1% aqueous osmium tetroxide and dehydrated through a graded series of ethanols. The tissue was stained *en bloc* with 5% aqueous uranyl acetate and embedded in Spurr's resin (Spurr, 1969). Thick sections (1 μm) were stained

with toluidine blue and viewed with an Olympus Bx40 (Olympus Optical, Tokyo) compound light microscope. Thin sections (55 nm) were stained with lead acetate and viewed with a JEOL 1200Ex (JEOL USA, Peabody, Mass.) scanning transmission electron microscope operating at 80 kV. Sections from at least three nodules from each treatment were observed. *Frankia* viability was assessed by using microscopy to verify the presence of intact *Frankia* hyphae and/or vesicles within the nodule cortical cells.

Results

Plant growth and development

After eight weeks, the leaves of all totally flooded plants (with and without argon) appeared mottled and chlorotic, and shoots of plants in those two treatments were multi-stemmed and stunted (Fig. 2A and B). No dieback of shoots or treatment-induced defoliation was noted. Proliferation of lenticels occurred at the root collar of plants in the total-flood treatments with and without argon; the lenticel tissue on the stems of argon-treated plants was long (2 cm), brittle, and easily broken (Fig. 2C). Nodules from these plants also developed hypertrophied lenticels along the surface of the periderm (not shown). Partially flooded plants were tall and single-stemmed, and the shoot tissue appeared healthy and green. Shoots of plants that were watered and drained daily were morphologically similar to shoots of partially flooded plants (Fig. 2A), except that shoots of drained plants appeared darker green than those of partially flooded plants. Root-zone oxygen content ranged from < 1 kPa for the argon-treated plants to > 17 kPa for the daily watered/drained plants (Table 1).

Nitrogen accumulation in leaves of daily watered/drained plants was 14% greater than that of partially flooded plants, despite the fact that partially flooded plants formed over 70% more nodules (Table 1). Totally flooded plants accumulated just over half as much

nitrogen as daily watered/drained plants despite a similar number of nodules among plants in the two treatments. Daily watered/drained and partially flooded plants had similarly high shoot dry weight. The combined mean shoot dry weights of plants in those two treatments was nearly twice that of plants in the two total-flood treatments (Table 1).

Nodule occurrence and location

Nodules of partially flooded and daily watered/drained plants appeared consistently larger than those from totally flooded and argon-treated plants. Nodules as large as 1 cm diameter were found on roots of partially flooded and daily watered/drained plants. Such nodules were often multilobed. Nodules on the roots of totally flooded and argon-treated plants were rarely multilobed and were not > 3 mm diameter. Some nodules that occurred in area 4 showed green and red pigmentation around the apex of nodule lobes.

Analysis of nodule-count data showed an interaction between irrigation treatment and root-zone area. Eighty-seven percent of the total number of nodules occurred within the upper 4 cm of the soil/air/water interface, primarily within area 3 (Fig. 1). Area 3 was the only zone in which totally flooded and argon-treated plants produced a statistically significant number of nodules (data not shown). Within area 4, the uppermost portion of the root zone, plants that were watered daily and drained developed more nodules than did plants in all other treatments. Within areas 3 and 2, however, partial flooding led to more nodules than did daily watering. Nodulation in area 1 was sparse; no nodules formed in area 1 for plants in both total flood treatments, and nodule development on plants in the other two treatments was so restricted that it was not significant statistically. Across all treatments, nodules frequently developed at the periphery of the root zone. They were also noted near drainage holes of the pots in the root zone of daily watered/drained plants.

Microscopy

Representative mature *Frankia* cells within nodules from all four treatments were intact and often surrounded by uniformly spherical vesicles (Fig. 3). Cortical cells in nodules from both partially flooded (Fig. 3A) and daily watered/drained (not shown) plants were densely arranged, with few air spaces between them. Infected cells were large, often multivacuolate, and filled with *Frankia* hyphae and/or mature *Frankia* vesicles. Uninfected cells were smaller and entirely filled with a single vacuole; starch grains were often present in these cells (not shown).

Air spaces between cortical cells in totally flooded (Fig. 3B) and argon-treated (Fig. 3C) nodules were large and prominent. Areas of *Frankia* infection were only one or two cell layers thick in these nodules, and they were bounded by rows of uninfected cells, the vacuoles of which were filled with darkly staining phenolic compounds. Phenolic compounds were particularly prominent in argon-treated nodules, and this often interfered with obtaining intact tissue sections. The viability of *Frankia* cells within totally flooded nodules was confirmed by electron microscopy (Fig. 4). In addition to observing intact *Frankia* vesicles and hyphae, the cytoplasm of infected cortical cells in totally flooded nodules was turgid and filled with mitochondria (Fig. 4A and B), ribosomes, and other organelles (not shown).

Discussion

Our results indicate that plants of *A. maritima* subsp. *maritima* grown under waterlogged root-zone conditions typical of native habitats are limited in their capacity to fix nitrogen. Further, these results suggest that nodulation and nitrogen fixation by this subspecies may be increased in managed environments where soils are not as frequently

waterlogged and are likely to contain more oxygen than do soils where plants are indigenous. The potential use of *A. maritima* subsp. *maritima* as a nitrogen-fixing actinorhizal subspecies should not be restricted to soils that drain well, however, because nodule formation and *Frankia* survival are not inhibited by flooding. Our data show that the number of nodules that develop on a plant of *A. maritima* subsp. *maritima* is not a reliable indicator of the amount of foliar nitrogen that can accumulate (Table 1). For other actinorhizal species, total nodule mass, but not total nodule number, per plant remains relatively constant (Nelson, 1983; Tjepkema et al., 1986), and development of ineffective (nonnitrogen-fixing) nodules has been reported (Berry and Sunell, 1990). Therefore, nodule counts should be used only in conjunction with other measures as an indicator of nitrogen fixation.

Our data on how flooding affects shoot dry weight are consistent with data of Schrader et al. (2004), who found that *A. maritima* survives extended periods of total root-zone inundation and thrives with a portion of its root zone flooded. The flood-induced symptoms of stress we observed were similar to those of other *Alnus* spp., but they were delayed relative to those species and occurred only under severely flooded conditions. McVean (1956) reported adventitious rooting in native stands of *Alnus glutinosa* (L.) Gaertn., and the appearance of "powdery lenticellular tissue" on the stem base and on the outer periderm of nodules on trees in wet soils that were not inundated. Many trees in this stand died when the roots were entirely submerged during a flooding event (Clarke, 1925), and nodules were not observed on roots that were submerged. Batzli and Dawson (1997) observed hypertrophied lenticel tissue after flooding *Alnus rubra* Bong. and *Alnus viridis* ssp. *sinuata* (Regel) Löve and Löve for 4 and 5 d, respectively; adventitious rooting of *A. rubra* was observed after 8 d. Conversely, two-year-old *A. rubra* saplings were killed by < 1 week of static flooding with water levels to or above the soil surface (Ewing, 1996). In contrast, *A.*

maritima had not developed observable symptoms after at least four weeks of total flooding. In a recent comparison with several other *Alnus* spp., *A. maritima* was among the most tolerant of soil-moisture extremes (Schrader et al., 2004).

Myrica gale L. occurs in waterlogged soils much like those in the native habitats of *A. maritima*. *M. gale* shows a growth response to flooding similar to that of *A. maritima* subsp. *maritima*. Shoot height and dry weight of *M. gale* were greatest when root zones were subjected to partial flooding (Schwintzer and Lancelle, 1983). Partially flooded plants of *M. gale* also had the greatest nodule biomass. We did not determine nodule biomass, but plants of partially flooded *A. maritima* subsp. *maritima* formed the most nodules (Table 1), and many of these nodules were as large as those on roots of daily watered/drained plants. Both *M. gale* and *A. maritima* subsp. *maritima* are capable of developing nodules on submerged roots, and these two taxa may have evolved some of the same mechanisms for controlling the oxygen environment within roots and nodules. We are confident that plants of *A. maritima* subsp. *maritima* were without nodules before flooding was imposed because we repeatedly observed complete inhibition of nodulation by nitrogen provided at the concentrations we applied before treatments began.

We observed that most nodules occurred within the upper one-third of the root zone. This is consistent with observations of nodule formation in *A. glutinosa* (McVean, 1956), and in some other actinorhizal species: *Colletia* Comm. ex Juss. (Rhamnaceae) (Bond and Becking, 1982) and the western North American shrubs *Cowania stansburiana* Torr., *Purshia tridentata* (Pursh) DC., *Purshia glandulosa* Curran, and *Cercocarpus ledifolius* Nutt. (Rosaceae) growing in native stands (Nelson, 1983). These workers found nodulation primarily within the upper part of the root system and close to the soil surface. On the other hand, Schwintzer and Lancelle (1983) reported differences in the distribution of nodules

within the root zone of *M. gale* depending on whether plants were grown in peat or sand at varying water-table depths. Nelson (1983) found uniform nodule distribution throughout root systems of containerized *C. stansburiana* grown in 1 native soil (texture not reported) : 1 silica sand-vermiculite (by volume). Such differences may be due to the various root-zone conditions to which plants in these experiments were exposed. The interaction between irrigation treatment and root-zone area for our nodule-count data illustrates that root-zone moisture conditions may be among the important determinants of nodule distribution. It remains uncertain whether our irrigation regimens affected nodule formation directly. Differences in pH or other root-zone conditions associated with the four treatments we applied may have influenced nodule distribution indirectly via influences on root growth in the four areas we defined. We did not partition root dry weight in the four areas, but compared to plants in other treatments, partially flooded plants appeared to have more roots distributed in the lower portion of the root zone and relatively few roots in area 4. This may explain the relatively poor nodulation in area 4 of partially flooded plants (Fig. 1), and the poor nodulation where roots appeared to proliferate in area 1 suggests that nodule number was not solely a function of root distribution.

Adjustments to low-oxygen peat soils of *M. gale* include upward-growing nodule roots (Schwintzer and Lancelle, 1983), extensive air spaces between cortical cells (Silvester et al., 1988), and the occurrence of hemoglobin within infected cortical cells (Pathirana and Tjepkema, 1995). Likewise, we found that totally flooded and argon-treated nodules had large air spaces between cortical cells (Fig. 3B and C). These, in combination with the lenticels on the outer periderm, could act as a channel for the diffusion of oxygen into the symbiosis. Interestingly, these adjustments in *M. gale* are not sufficient to support optimal levels of nitrogen fixation when nodules are flooded (Tjepkema et al., 1986). We suspect

this also is the case for *A. maritima* subsp. *maritima* because totally flooded plants accrue less nitrogen than do daily watered/drained and partially flooded plants (Table 1).

The fact that *Frankia* proliferation is limited within the nodules of totally flooded and argon-treated plants of *A. maritima* subsp. *maritima* supports this conclusion. These nodules accumulated greater amounts of phenolic compounds, and the areas of *Frankia* infection appeared to be restricted by closely arranged rows of cells containing these compounds (Fig. 3B and C). Flavonoids, a class of phenolic compounds, were extracted from *A. rubra* seeds and influenced nodulation of *A. rubra* by *Frankia* (Benoit and Berry, 1997), and phenolic-containing cells compartmentalized areas of *Frankia* infection within nodules of *Casuarina glauca* Sieber (Laplaze et al., 1999). These studies suggest a role for flavonoids in regulating interactions between *Frankia* and its plant host. It is feasible that, in our study, phenolic compounds may have limited the spread of *Frankia* within totally flooded and argon-treated nodules, presumably to prevent *Frankia* from becoming parasitic by consuming excessive carbon under growth-limiting conditions (Markham, 1996).

We have provided the first evidence that nodules with viable *Frankia* can form in partially flooded and totally flooded root zones. Yet our data also indicate that nodule formation and function are sensitive to inundation. Therefore, future workers should focus on defining the optimal root-zone oxygen concentration for nitrogen fixation by the symbiosis of *Frankia-A. maritima* subsp. *maritima*. Despite restrictions over their spread, *Frankia* and the cells they inhabit were viable under flooded conditions. This suggests that these plants retained the capacity to recover from their flooded state, and that plants in their native habitat may have mechanisms for coping with periods of flooding. Indeed, some *A. maritima* subsp. *maritima* occur in waterways that are subject to tidal changes in water depth (Graves and Gallagher, 2003). Nodules on these plants may be exposed periodically to

concentrations of oxygen that facilitate nitrogen fixation. Future studies could examine the impact of changing water levels on nodule occurrence and *Frankia* nitrogen fixation.

Acknowledgments

This project was supported by the Iowa Agriculture and Home Economics Experiment Station and State of Iowa funds through the Hatch Act. We would like to express our appreciation to Richard Gladon for his assistance with experimental design; and to Harry Horner, Tracey Pepper, and Rosanne Healy for assistance with preparation of materials for microscopy.

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Table 1. Root-zone oxygen (O_2), leaf nitrogen (N) accumulation, number of root nodules per plant, and shoot dry weight of *Alnus maritima* subsp. *maritima* grown in a greenhouse for eight weeks in pots with four irrigation regimens. Germinated seedlings were watered daily, and nitrogen fertilizer was provided three times weekly for four months before treatment. Treatments began 5 June 2002.

Root-zone moisture treatment[z]	Root-zone O_2 (kPa)[y]	Total leaf [N] ($mg \cdot g^{-1}$)	No. of nodules/plant	Shoot dry weight (g)
Watered daily, drained	17.3 a[x]	25.3 a	4.8 b	2.55 a
Partial flood	13.1 b	22.1 b	8.3 a	2.70 a
Total flood	1.2 c	14.1 c	3.9 bc	1.40 b
Total flood with argon	0.9 c	13.4 c	1.3 c	1.33 b

[z]n = 11 seedlings per treatment.

[y]Values represent the average of two measurements per plant, after four and eight weeks of treatment.

[x]Means within columns followed by the same letter are not different at $P \leq 0.05$ according to Fisher's least significant difference test.

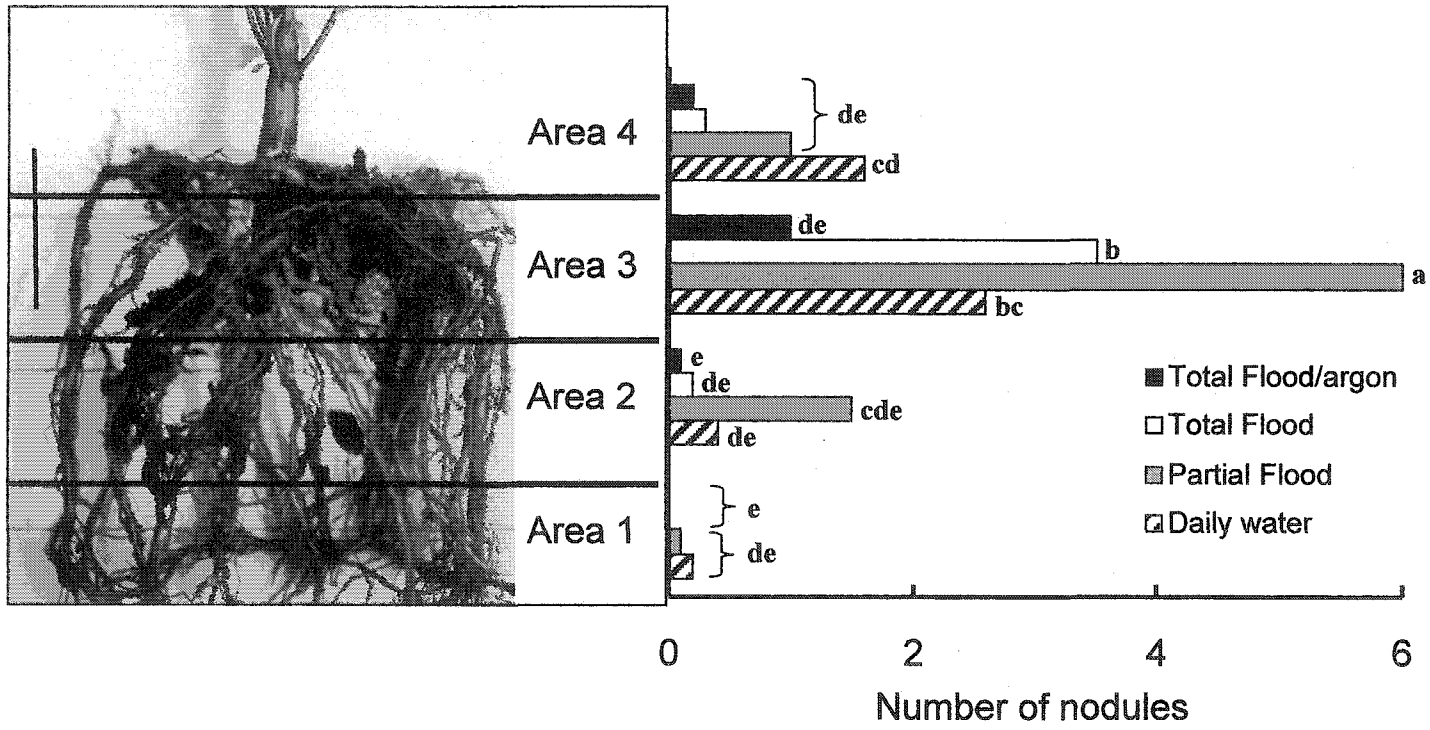

Fig. 1. Number and location of nodules on *Alnus maritima* subspecies *maritima* subjected to various degrees of flooding. A nodule placement grid was used to categorize nodule position into four areas along the vertical axis of the root zone. Area one represented the submerged part of the root zone in partially flooded plants. The line between areas two and three represents the vertical mid-point of the root zone. Area four included the submerged zone between the surface of the solid matrix and the air in totally flooded and argon-treated plants. Most nodules occurred in area three. The number of nodules that occurred in area one was not different from zero. Bars within each area followed by the same letter are not different at $P \leq 0.05$ according to Fisher's least significant difference test. Sidebar is 4 cm.

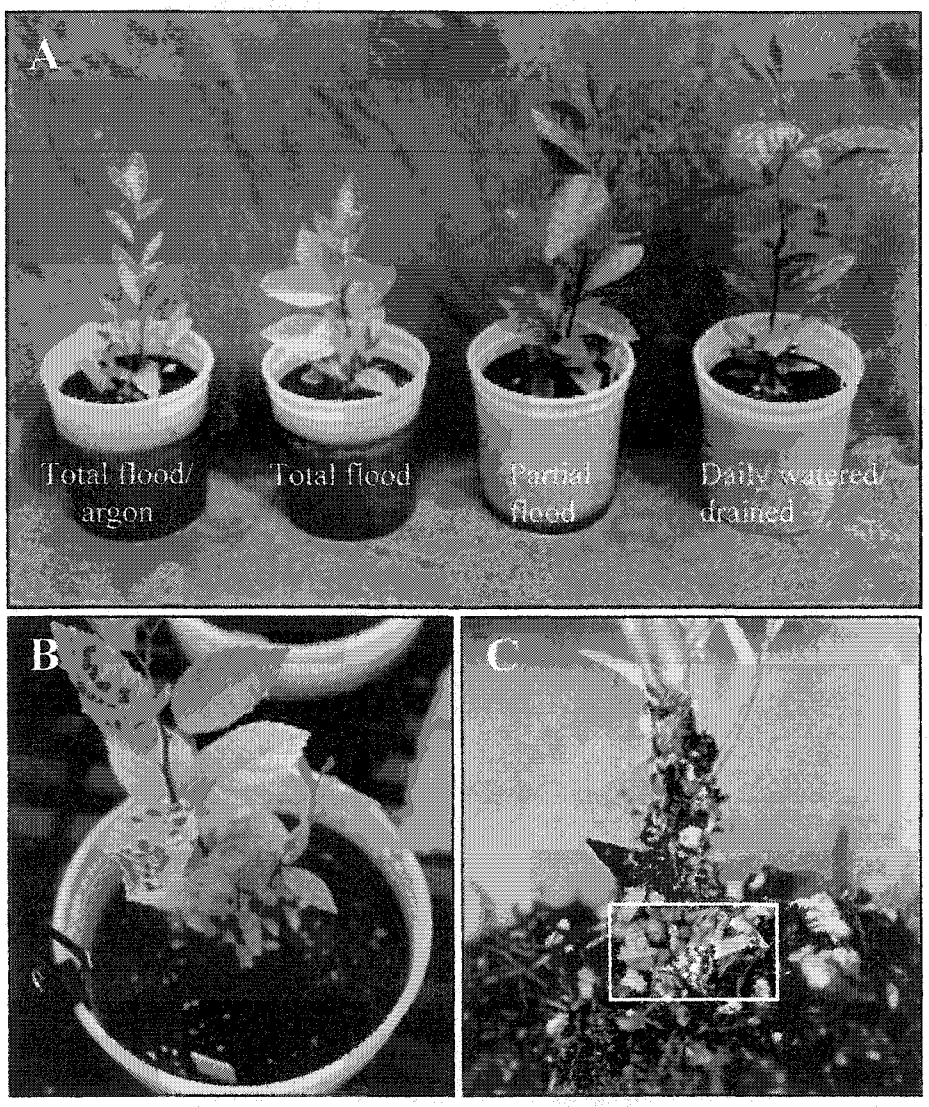

Fig. 2. Effects of various degrees of flooding on seedlings of *Alnus maritima* subsp. *maritima*. (A) Shoots of partially flooded plants were similar in size to shoots of plants that were watered daily and drained. (B) Shoots of plants with roots that were totally flooded in water gassed with argon were stunted, and the leaves appeared mottled and chlorotic. (C) Long (2 cm), fragile protuberances grew out from the lenticels at the base of argon-treated plants.

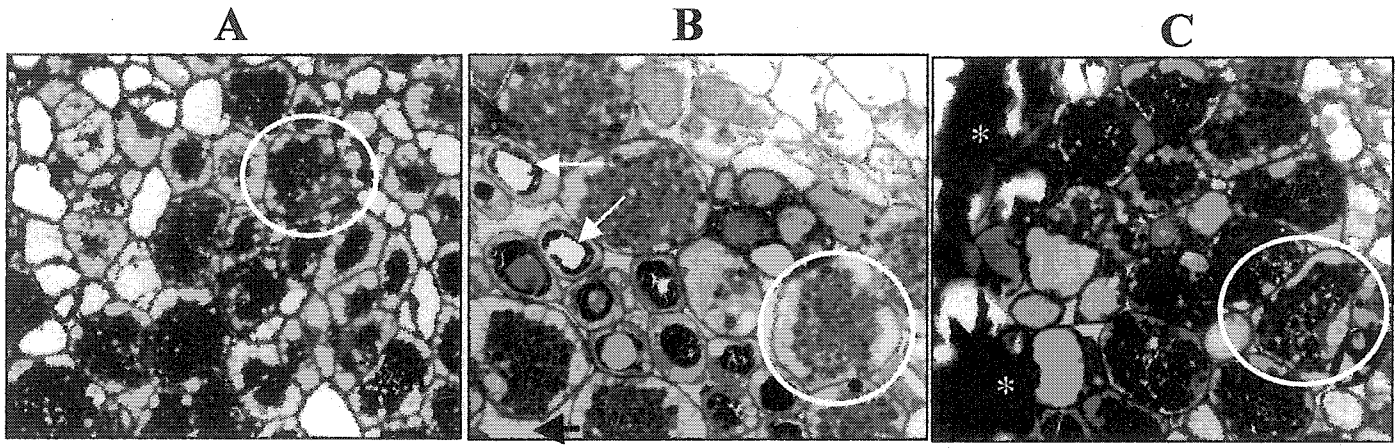

Fig. 3. Anatomy of nodules from flooded seedlings of *Alnus maritima* subsp. *maritima*. (A) Cortical cells of partially flooded nodules were densely arranged, and many cells were infected with mature *Frankia* vesicles (circle around infected cell). (B) Cortical cells of totally flooded nodules were separated by large air spaces (black arrow). The vacuoles of uninfected cells were often filled with phenolic compounds, which tended to be displaced during sectioning, leaving irregularly shaped holes in their place (white arrows). (C) Darkly staining, irregularly shaped objects (asterisks) in cross-sections of argon-treated nodules are phenolic compounds that became dislodged during sectioning. *Frankia* infection throughout the nodule was inhibited in totally flooded and argon-treated plants. Bar is 50 μm.

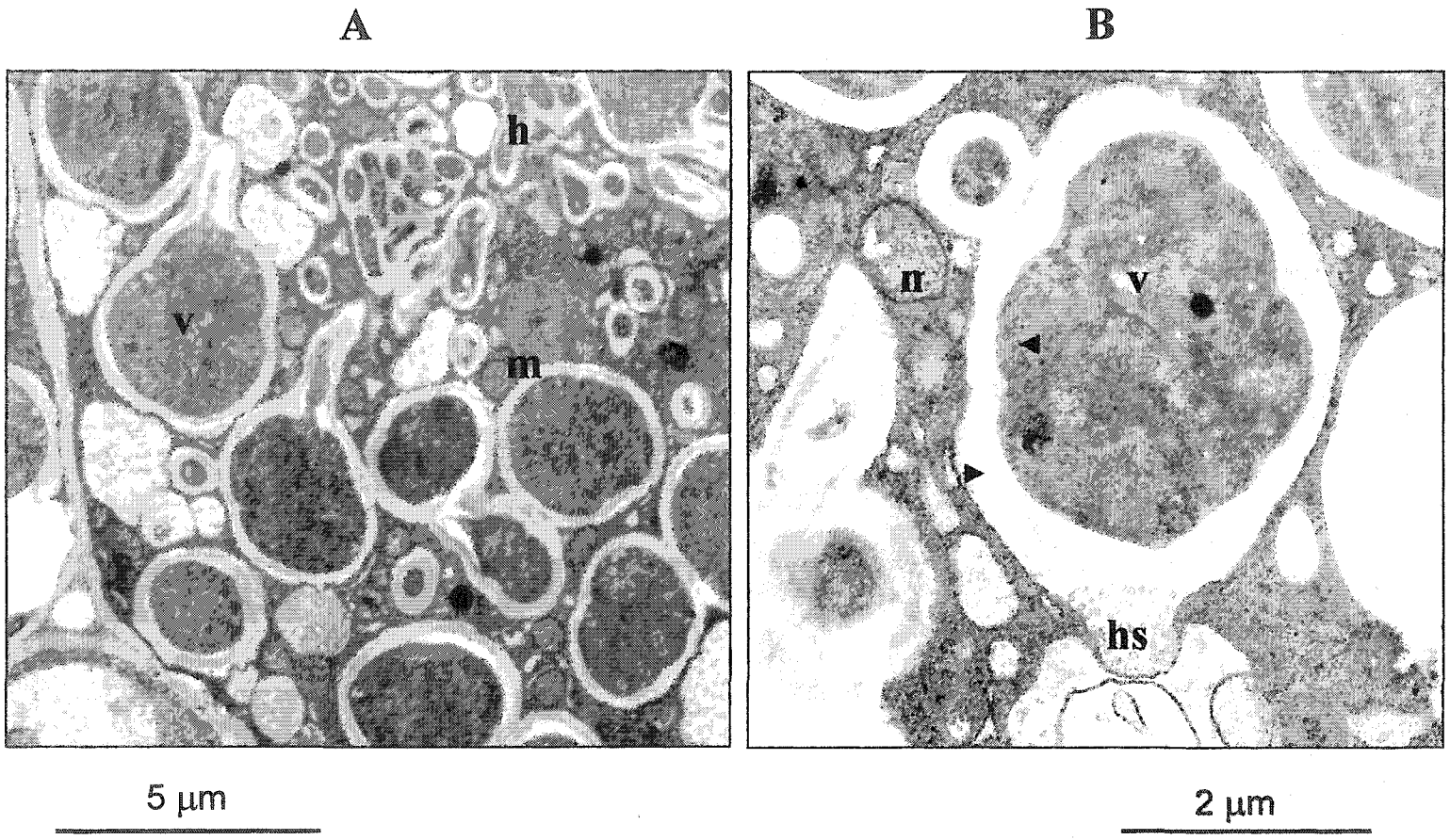

Fig. 4. Intact *Frankia* cells were found within nodules of totally flooded seedlings of *Alnus maritima* subspecies *maritima*. (A) The cytoplasm of totally flooded nodules was filled with *Frankia* hyphae (h), vesicles (v), and mitochondria (m). (B) This multiseptate *Frankia* vesicle was surrounded by a void space (arrowhead) and was within close vicinity of a mitochondrion. A hyphal strand (hs) was emerging from the bottom of the vesicle.

CHAPTER 5. AEROPONIC SYSTEM FOR CONTROL OF ROOT-ZONE ATMOSPHERE

A paper submitted to *Environmental and Experimental Botany*

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Abstract

Control of rhizospheric gases can be critical to research. Culturing plants with roots in liquid is a common component of protocols described previously for controlling the root-zone atmosphere, and liquid culture is routine during assays of symbiotic N_2 fixation. However, spurious data may result from gases becoming trapped within the boundary layer of solution-cultured roots and nodules. Aeroponics is a viable alternative to other soilless culture systems for maintaining plants with a controlled root-zone atmosphere. We have designed an aeroponic gas-delivery system that allows statistical treatment of individual plants as discrete experimental units. Roots of intact plants were held in closed 1-L Mason jars, with one plant per jar and the stem emerging from a hole drilled in the dome lid. Extra-fine fog nozzles controlled by a programmable timer sprayed roots periodically with nutrient solution. Gases were mixed and delivered to Mason jars at a prescribed rate by using a system of capillary tubes. During a study of O_2 effects on formation of root nodules, 100% survival of plants and profuse nodulation resulted after four weeks. The pO_2 within each jar remained within ± 1.0% of its prescribed value, and pre-and post-experimental O_2 measurements within individual jars did not differ ($P < 0.0001$). The system is easy to construct with inexpensive materials, has many applications for rhizosphere biology, and is unique among systems used previously.

Keywords: Rhizosphere; Cultural systems; Gas-delivery mechanisms; Hydroponics; Oxygen; Acetylene-reduction assay

1. Introduction

Achieving dependable results during biological research on roots requires accurate and precise regulation of gases in the root zone (Zobel, 1989). Root biologists have used various soilless culture systems, and aeroponics can provide the best control of the rhizosphere of intact plants (Weathers and Zobel, 1992). Aeroponics has been used successfully in many experiments (Zobel et al., 1976; Callaham et al., 1979; Hubick et al., 1982; Nir, 1982; Peterson and Krueger, 1988; Martin-Laurent et al., 1997; Burgess et al., 1998). However, none of the aeroponic systems used in these experiments allows direct control of gases within individual root zones, and gas-delivery mechanisms designed for this purpose in nonaeroponic systems are costly and complex. Aeroponic systems are commercially available, but they are constructed for the mass culture of crops and do not allow manipulation of individual root systems. We have designed an aeroponic system that, combined with an inexpensive gas-dispersal mechanism, facilitates accurate and precise control of the concentrations of root-zone gases delivered to individual plants. This permits randomization of replicates and greater statistical power of experiments.

Many applications for our aeroponic system are possible, but in this report, we describe how the system was used to determine the optimal O_2 tension for nodulation and N_2 fixation in actinorhizal species. Many workers culture plants in liquid during assays of N_2 fixation. However, liquid cultures may impede gas diffusion, thereby confounding efforts to control gas composition around N_2-fixing nodules. Further, the limited solubility of ethylene, the product of nitrogenase-reduced acetylene in the acetylene-reduction assay, may

result in trapped ethylene within wet roots (Weathers and Zobel, 1992) and nodules of liquid-cultured plants. Underestimation of the ethylene evolved during the assay could result. Our aeroponic system proved to be an effective way of avoiding that problem.

2. Materials and Methods

2.1. Plant containers

Plants were installed in closed 1-L wide-mouth Ball® Mason jars (Alltrista Zinc Products, Greeneville, Tenn.) for treatment periods of up to four weeks. Each jar held one root system, and the shoot emerged from a 2-cm-diameter hole drilled in the dome lid (Fig.1A). Each stem was mounted by using a no. 2 neoprene stopper (Fisher Scientific, Pittsburgh, Pa.), through which a hole was drilled in the center. A transverse cut was made from the outer surface of the stopper to the central hole to permit insertion and removal of the seedling. This transverse cut in the stopper also allowed radial growth of the plant stem. A slit that was 7 cm long by 1.8 cm wide from the outer edge of the dome lid to the 2-cm hole in the center of the dome lid facilitated insertion and removal of the plant with its stopper. Another 2-cm-diameter hole drilled in the dome lid accommodated a no. 2 two-hole rubber stopper, into which were inserted two glass tubes that were open at both ends and had an inside diameter (i.d.) of 3 mm. An 18-cm length of glass tubing served as a port for inflow of gases, and a 7-cm length permitted outflow of gases from each jar. A 28-cm length of Tygon® tubing with an i.d. of 5 mm was attached to the glass outflow tube to help prevent back flow of ambient air into the jar. Nutrient solution that accumulated at the bottom of the jar was aspirated by using a pipette bulb attached to a 30-cm length of 1.5-mm-i.d. polyethylene tubing that was inserted through the 28-cm length of Tygon® tubing attached to the outflow tube (Fig. 1B). A 1.3-cm-diameter hole drilled in the center of the bottom of the

Mason jar fit over a Baumac® fog nozzle (Extra Fine) (Hummert International, St. Louis, Mo.) (Fig. 1C). Frost King® rope caulk (Thermwell Products, Mahwah, N.J.) was used to seal openings in the dome lid and between the Baumac® nozzle and the hole drilled in the jar. These seals prevented ambient gases from contaminating the atmosphere within the jar. The jar was wrapped in aluminum foil to keep the roots in darkness and prevent algal growth.

2.2. Aeroponics system

Polyvinyl chloride (PVC) pipes and fittings were used. The apparatus was rectangular, 60 cm wide by 4.5 m long. Eighty Baumac® fog nozzles in PVC reducing tees were spaced 20 cm apart in four rows and were connected by PVC pipe (Fig. 2). A garden hose connected to the municipal water supply was attached to a length of PVC pipe positioned centrally within the apparatus, such that the apparatus was split into two connected blocks of four rows of 10 nozzles. A GEWA® portable fertilizer injector (Loos & Co., Naples, Fla.) was connected to the garden hose and delivered quarter-strength, N-free Hoagland's solution (Hoagland and Arnon, 1950) during irrigation of plant roots. The system was wired to a single-channel programmable timer (Phytotronics, Earth City, Mo.) adjusted to activate intermittently so that plant roots were maintained in saturated humidity without accumulation of liquid droplets on the roots. Expanded metal sheets (Hummert International, St. Louis, Mo.), laid over the aeroponic apparatus, provided a flat surface on which the Mason jars rested (Fig. 2). Individual Mason jars were stabilized over the fog nozzles by using duct tape to attach them to 1.5-m-long stakes inserted vertically through openings in the expanded metal sheet and the bench.

2.3. Gas-Delivery system

We modified a system described by Diesburg et al. (1989). Defined atmospheres within each jar were maintained by mixing the gases from cylinders of O_2, N_2, and argon (Ar). This was accomplished by using a system of capillary tubes (Ace Glass, Vineland, N.J.) of fixed diameter and length to control flow rates. Gases were mixed and delivered to individual Mason jars through the following sequence: compressed-gas cylinder, gas regulator, mixing barostat tower, mixing capillary tubes, flow-control barostat tower, and flow-control capillary tubes (Fig. 3). Connections between the gas regulators, barostat towers, and capillary tubes were made with 5-mm-i.d. Tygon® tubing and Y-connectors.

The mixing barostat tower consisted of three (one for each gas) tubes that were open at both ends and had an i.d. of 7 mm. These were placed within a larger, transparent glass tube that was sealed at the bottom and filled with water to provide head pressure. The flow-control barostat tower contained several (one for each gas composition) glass tubes (i.d. = 7 mm) within a larger glass tube sealed at the bottom and filled with water to a height equal to one-half the height of the water in the mixing barostat. The 7-mm tubes were connected to the gas lines, and the internal gas pressures were indicated by the depth to which water was forced down and out the tubes. The pressure across the system was indicated by the difference between the heights of the water in the two barostat towers. Flow rates through individual lines were controlled by a combination of the i.d. and length of the capillary tubes, as defined by Poiseuille's Law:

$$L = \frac{\pi d^4 (P1 - P2)}{128 \eta Q}$$

where: L = capillary tube length (cm)

d = capillary tube i.d. (cm)

$P1$ = upstream (before capillary tube) pressure

$P2$ = downstream (after capillary tube) pressure

η = gas viscosity ($g \cdot cm^{-1} \cdot s^{-1}$)

Q = flow rate ($cm^3 \cdot s^{-1}$).

The difference in height of the water columns within each tower was converted into pressure terms by multiplying the height of the barostat water column by the conversion factor 9.80638×10^2. This equation then becomes:

$$L = \frac{\pi d^4 (9.80638 \times 10^2)(B1 - B2)}{128 \eta Q}$$

where: $B1$ = upstream barostat water-column height (cm)

$B2$ = downstream barostat water-column height (cm).

When determining desired flow rates, a factor of 10% was added to the volume of each jar to ensure that adequate pressure across the system was achieved (Fig. 3). After the flow rate was determined, length of capillary tubes was calculated using this equation. Capillary tubes with a wide range, but a limited number, of defined diameters are commercially available, and as demonstrated by this equation, small changes in the diameter profoundly affect flow rate. Therefore, we used the equation to select the capillary tube diameters that best met our needs and resulted in easily managed tube lengths.

2.4. Example

We used our system to determine the optimal pO_2 for N_2 fixation in actinorhizal plants. Eight O_2 tensions (0, 2, 4, 8, 12, 16, 20, and 32 kPa) were delivered to 80 individual plant root systems. Nitrogen gas was maintained at a tension of 68 kPa across all treatments. We adjusted the O_2 tension for each treatment by varying the proportions of O_2 and Ar (Fig. 3). A MiniOX® Responder remote O_2 detector (Mine Safety Appliances, Pittsburgh, Penn.) was used to measure ambient O_2 concentrations within individual Mason jars before plants were installed and after plants were removed from treatments.

Although we used a continuous gas-exchange system to maintain plants at constant pO_2, our N_2-fixation assay was performed under closed gas-exchange conditions. At the time of assay, plants were sealed within their containers and subjected to 10% acetylene for 1 h before measurement of ethylene production. Because we used the acetylene-reduction assay as a one-time comparison of N_2-fixation rates across treatments, a closed assay system was adequate. An open-exchange assay may be desirable for measuring short-term changes in N_2-fixation rates over time. Our system can be modified easily to accommodate an open-exchange assay by adding a cylinder of acetylene into the gas-delivery system, adding sampling ports to the inflow and outflow tubing, and measuring the difference in ethylene content between outgoing and incoming gases. The flow rate of gases would need to be adjusted to match the rate of the phenomenon being measured (Winship and Tjepkema, 1990).

3. Results and Discussion

All plants survived in our aeroponic system. Plants exhibited no signs of water stress and developed vigorous root systems with many root hairs. Further, a large number of

nodules developed on root systems exposed to pO_2 greater than those we found to be required for nodulation. We often documented nodule counts that were one order of magnitude greater than the counts typical of plants of similar age grown in a soil-based medium. Oxygen tensions within individual jars were within ± 1.0% of prescribed values, and pO_2 values after plants were removed were not different from pO_2 data collected before the treatments began ($P < 0.0001$). These data demonstrate consistency in rhizospheric gas concentrations over time.

Several aeroponic systems have been devised and used successfully for propagation and maintenance of a variety of woody and herbaceous plants. Reported benefits of such systems include the ability to access roots with minimal disturbance, decreased plant water stress, enhanced plant growth rates, and optimal aeration of the root zone. Zobel et al. (1976) designed a system that used a spinner and rotor to atomize water droplets and provide water and nutrients to plant roots continuously. They attributed enhanced plant growth and good root hair development in diverse taxa grown aeroponically to the greater access of roots to an aerobic environment. Hubick et al. (1982) modified the system of Zobel et al. (1976) to manipulate the water available to plants of *Helianthus annuus* L. cv. Russian. Plants in aeroponics grew larger than plants grown with conventional irrigation.

Martin-Laurent et al. (1997) compared three methods for culture of the woody legume *Acacia mangium* Willd. and judged aeroponics superior to either liquid or sand culture. After 17 weeks, aeroponically cultured plants had grown faster and matured earlier than plants cultured in either liquid or sand. Further, aeroponically grown plants developed more nodules than did liquid-cultured plants, and rhizobial inoculation increased the N content of plants grown aeroponically, but not in liquid, over that of uninoculated controls. Researchers studying N_2 fixation often maintain nodulated plants within water- or nutrient-

filled cuvettes (Silvester et al., 1989; Winship and Tjepkema, 1990), with the nodulated portion of the root system suspended above the water. This is a modification of a liquid culture system and is not considered truly aeroponic (Weathers and Zobel, 1992) because gases must diffuse through water before they reach the nodules.

Our system is similar to others in which roots are misted intermittently with dilute nutrient solution (Zobel et al. 1976; Hubick et al. 1982; Nir 1982; Peterson and Krueger 1988; Burgess et al. 1998). Intermittent misting with nutrient solution can lead to precipitation of nutrient salts within the fog nozzles, which may limit the widespread use of aeroponics (Weathers and Zobel, 1992). Nutrient salts precipitated within our nozzles after four weeks but did not interfere with nozzle function or lead to obvious plant stress. After four weeks, precipitate was removed easily from inside fog nozzles by using a dissecting tool, and nozzles could be cleaned periodically, if necessary, during treatments exceeding four weeks. The timer in our system was programmed to activate for 2 s every 20 min (every 30 min on cloudy days and every 60 min after dark). This schedule may need to be adjusted for other plants and different environmental conditions (Peterson and Krueger, 1988).

Aeroponic systems described previously, and those available commercially, use a common container for delivery of liquid to plant roots. Generally, plant roots are suspended in a darkened box, with the shoots emerging from a horizontal surface above the water-delivery system. A finite number of plants can be accommodated by one system, and multiple systems may be necessary for large-scale experiments. No other system of which we are aware allows for the use of an individual plant as an experimental unit. This is an advantage when the number of treatment replications is important and randomization is necessary.

4. Conclusion

To our knowledge, this is the first report of a true aeroponic system used in combination with a gas-delivery apparatus for control of individual root-zone atmospheres of intact plants. When this system is used for N_2-fixation assays, abiotic stress caused by transfer of plants to assay conditions is avoided, and the gas phase around plant roots and nodules can be controlled accurately and precisely. This system is flexible and can be modified to accommodate a wide range of sample sizes and gas treatments. It is simple to construct from readily available, economical materials, yet it provides accurate and reproducible results.

Acknowledgements

This project was supported by the Iowa Agriculture and Home Economics Experiment Station and State of Iowa funds through the Hatch Act. We would like to express our appreciation to Arlen Patrick, Greenhouse Agriculture Specialist of the Department of Horticulture at Iowa State University, for his assistance with construction of the aeroponic system.

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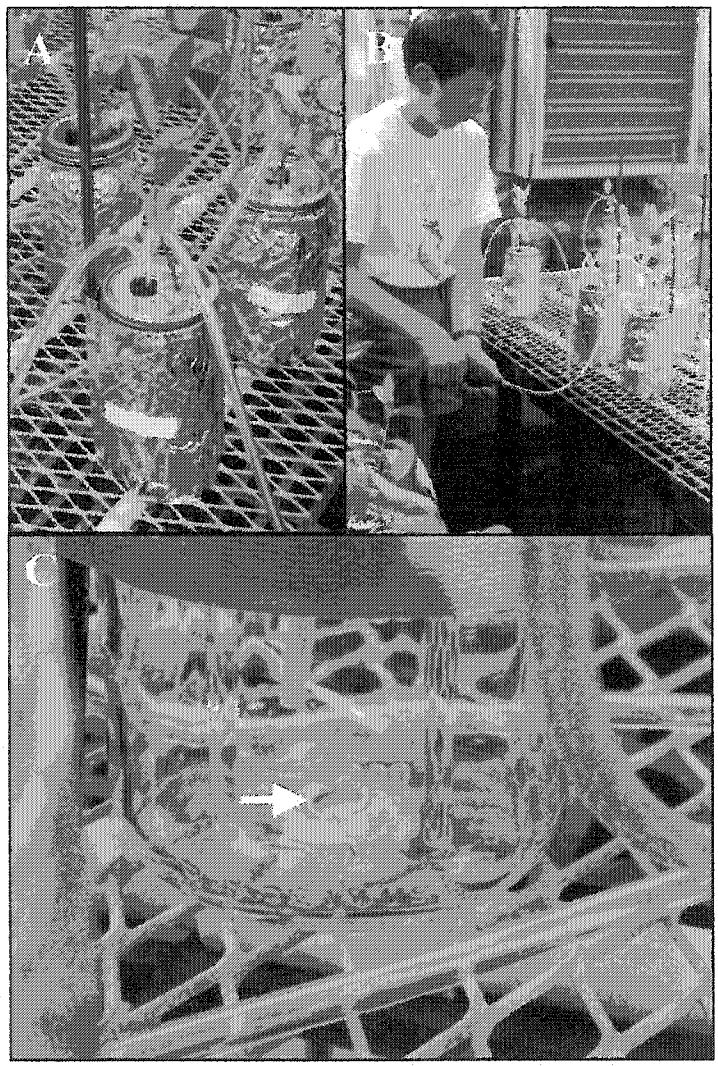


Fig. 1. Roots of intact plants were held in 1-L Mason jars. (A) Each plant was held in place by using a one-hole stopper inserted into a hole in the dome lid. Gas inlet and outlet tubes were held within a two-hole stopper inserted into a second hole in the dome lid. (B) A pipette bulb was used to aspirate excess nutrient solution that collected in the bottom of the jars. (C) Each jar was positioned over a fog nozzle, which emerged through a hole drilled in the jar bottom (arrow). Rope caulk was used to seal the opening between the fog nozzle and the perimeter of the hole.

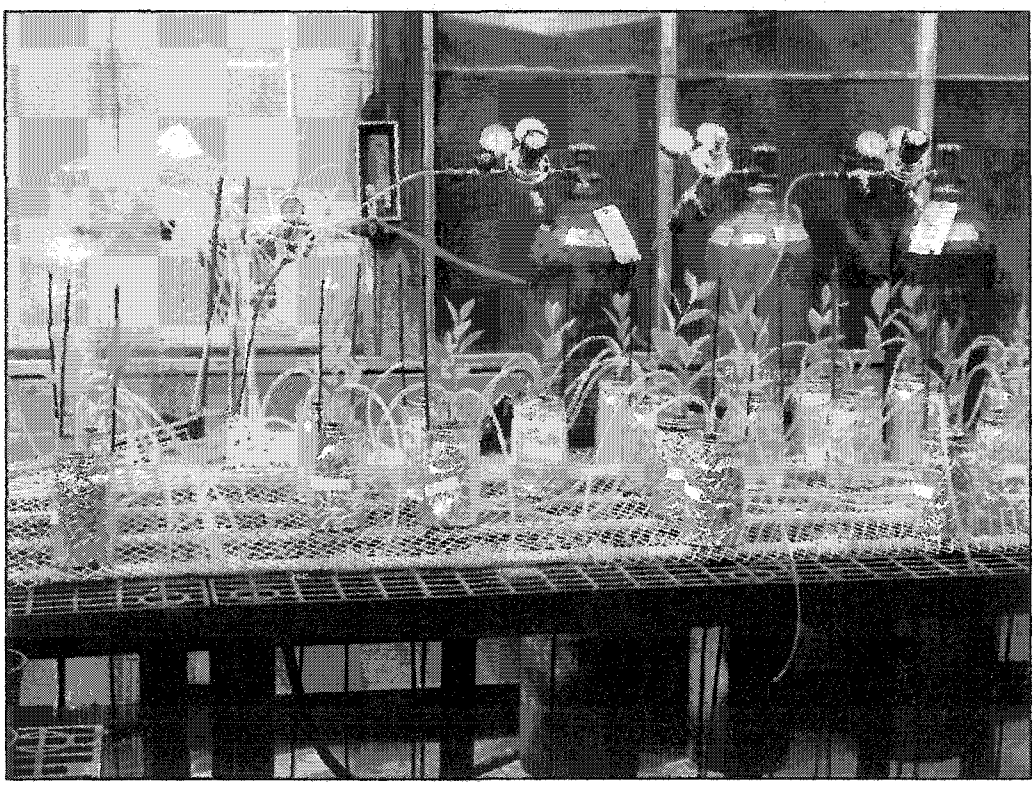
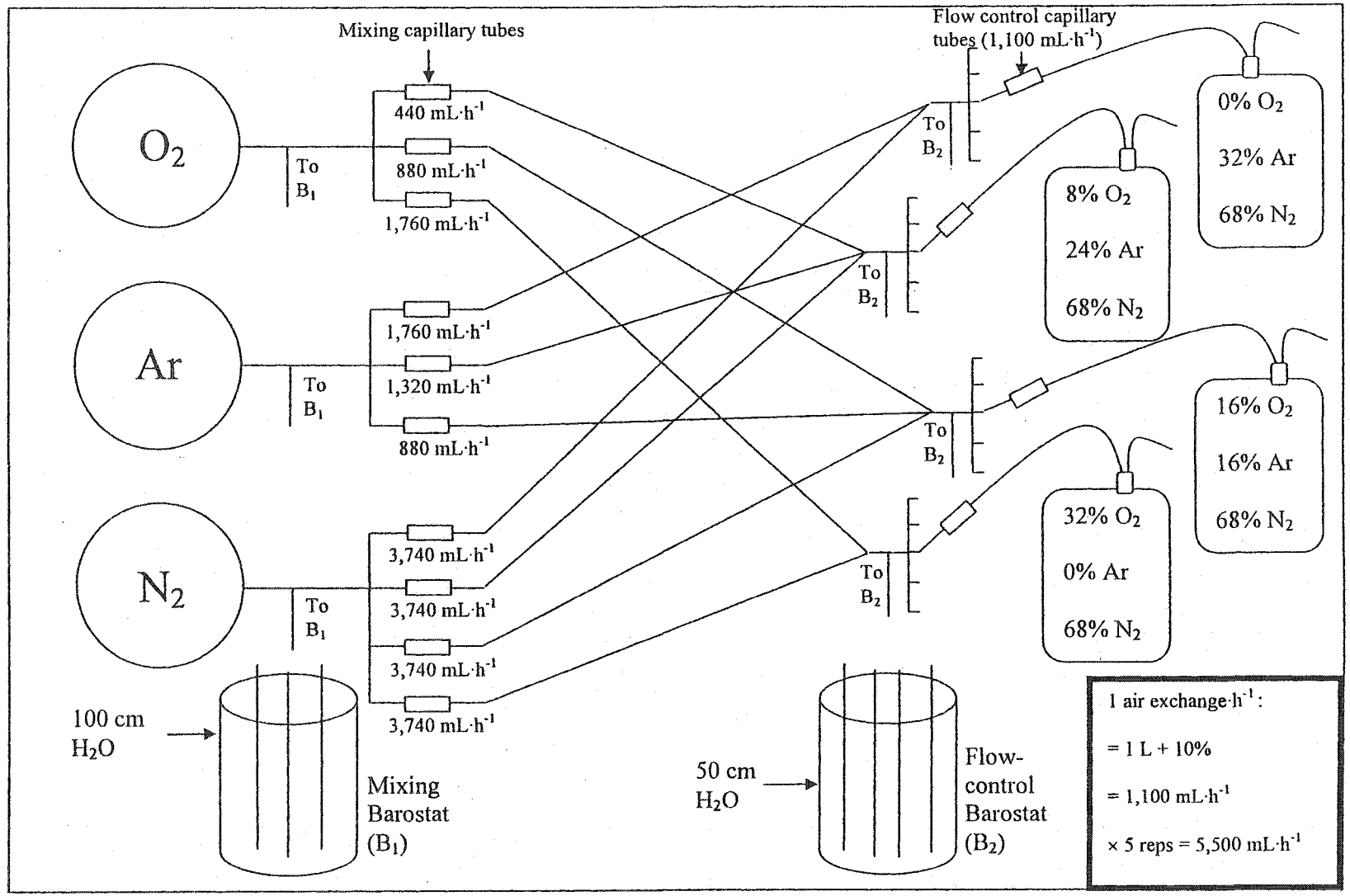


Fig. 2. The aeroponic system was constructed of PVC pipe. Plant containers rested on a sheet of expanded metal. Fog nozzles emerged through openings in the expanded metal sheet. Containers were stabilized by using duct tape wrapped around the jar and a 1.5-m-long stake. Aluminum foil was used to prevent algal growth within jars and was removed during periodic observation of nodule development.

Fig. 3. Schematic of gas-delivery system depicting four gas-mixture treatments and five replications per treatment. Gases were mixed and delivered by using a system of barostat towers and capillary tubes. The pressure across the system was indicated by the difference in water height between the mixing and flow-control barostats. Capillary tubes of defined length and inner diameter controlled the rate of gas flow through the system. When determining desired flow rates, a factor of 10% was added to the volume of each jar to ensure adequate pressure across the system was achieved. Excess gas escaped through the tubes within each barostat tower. Note that the cumulative flow rate of each gas mixture is equal to the overall rate of gas flow to a set of plants in a treatment (5,500 $mL \cdot h^{-1}$).


Mixing capillary tubes
Flow control capillary tubes (1,100 mL·h-1)
O2
Ar
N2
To B1
440 mL·h-1
880 mL·h-1
1,760 mL·h-1
1,760 mL·h-1
1,320 mL·h-1
880 mL·h-1
3,740 mL·h-1
3,740 mL·h-1
3,740 mL·h-1
3,740 mL·h-1
To B2
0% O2
32% Ar
68% N2
8% O2
24% Ar
68% N2
16% O2
16% Ar
68% N2
32% O2
0% Ar
68% N2
100 cm H2O
Mixing Barostat (B1)
50 cm H2O
Flow-control Barostat (B2)
1 air exchange·h-1:
= 1 L + 10%
= 1,100 mL·h-1
× 5 reps = 5,500 mL·h-1

CHAPTER 6. OXYGEN CONCENTRATION AFFECTS NITROGENASE ACTIVITY AND NODULE ANATOMY OF *ALNUS MARITIMA*

A paper to be submitted to *Plant, Cell, and Environment*

Heidi A. Kratsch, William R. Graves

KEYWORD INDEX. Seaside alder, *Frankia*, symbiosis, hemoglobin, actinorhizal, acetylene-reduction assay

Abstract

Alnus maritima is a shrub that associates with N_2-fixing *Frankia* in the wetlands in which it is native. Despite low concentrations in waterlogged soils, O_2 is critical to maintenance of this symbiosis, and *Frankia*-infected nodules exist on plants in native stands. Our objective was to determine how root-zone O_2 concentration influences N_2 fixation and the anatomy of *A. maritima* nodules. Root zones of plants inoculated with soil from native stands were exposed to eight O_2 concentrations. Nitrogenase activity increased with increasing O_2 concentration. Photosynthetic rate, plant dry mass, leaf N content, and nodule fresh mass were maximal in plants maintained with 15 to 25% O_2 in the root zone. Nodule counts peaked at 10 and above 25% O_2, and nodules that developed at $\leq$ 2% O_2 were < 2 mm in diameter and single-lobed. Mean total area of air spaces within nodules decreased, and mean area per space increased, with increasing O_2 concentration. Seasonal and O_2-dependent nodule pigmentation was observed. Our data suggest that the capacity to gain access to soil O_2 is critical to the development of functional symbioses, and that nodules of this species possess mechanisms for responding to the root-zone O_2 environment.

Introduction

Alnus maritima (Marsh.) Muhl. ex Nutt. is a rare shrub that is unique among alders in its degree of restriction to low-O_2 soils of wetlands (Schrader & Graves 2002). An actinorhizal (nonlegume) species with promise for use in low-maintenance landscapes and wetland regeneration, *A. maritima* develops a root-nodule symbiosis with the nitrogen-fixing bacterium *Frankia* Brunchorst (Stibolt 1978). *A. maritima* is indigenous to only three disjunct locations in North America, and distinct subspecies were recognized recently (Schrader & Graves 2002). *Alnus maritima* subsp. *maritima* is found in and along streams on the Delmarva Peninsula, and nodulation occurs on plants in native stands (Kratsch & Graves 2004a). Despite its confinement to waterlogged soils, development and function of nodules of this subspecies are sensitive to flooding (Kratsch & Graves 2004b). Inundation changed nodule structure, but whether low O_2 directly led to altered anatomy has not been confirmed.

Although symbiotic N_2 fixation is sensitive to O_2, *Frankia* within actinorhizal nodules fix N_2 over a wide range of O_2 concentrations due largely to the capacity of nodules to adjust morphologically, anatomically, and physiologically to root-zone O_2 conditions. Examples of morphological adjustments include negatively geotropic growth of roots from the apex of nodule lobes of *Myrica* L. (Silvester et al. 1988b), thickening of the periderm of *Coriaria* L. (Silvester & Harris 1989), and development of lenticels along the periderm of *Alnus* Miller (Bond, Fletcher & Ferguson 1954). Anatomical adjustments, like increased number and distribution of air spaces of *Myrica* (Silvester et al. 1988b), changes in nodule diffusion resistance of *Coriaria* (Silvester & Harris 1989), and changes in thickness of the lipid-rich vesicle surrounding *Frankia* cells in *Alnus* (Silvester, Silvester & Torrey 1988a; Kleeman et

al. 1994), modulate the O_2 environment within the nodule. Nodules of other actinorhizal species that are obligate wetland natives, like *Myrica* spp., also are physiologically adapted to low O_2 and express the O_2-transporting protein hemoglobin (Pathirana & Tjepkema 1995).

Because we have measured hypoxia in the soils of this obligate wetland shrub, we hypothesized that *A. maritima* subsp. *maritima* has adapted to low root-zone O_2 concentrations by way of physiological and structural adjustments within its nodules. Our objectives were to determine the range of O_2 concentrations under which *A. maritima* subsp. *maritima* fixes N_2 and to describe the effects of O_2 concentration on nodule development and anatomy.

Materials and Methods

Plant materials

Alnus maritima subsp. *maritima* was propagated from seed collected in Dorchester Co., Md. Seeds were rinsed in distilled, deionized water and cold-stratified at 4 °C for five weeks. Seeds were germinated at room temperature between two pieces of moist filter paper in plastic petri dishes. Germinated seedlings were sown communally in round plastic pots (height = 13.5 cm, volume = 3154 cm^3) filled with germination mix (Conrad Fafard, Agawam, MA). The pots were held on a greenhouse bench where 16-h photoperiods were provided by using 400-W, high-pressure sodium lamps. Seedlings were irrigated with tap water once daily and fertilized three times per week with Peters Excel All-Purpose (25% of stock solution) and Cal-Mag® (75% of stock solution) (16.4N–2.2P–16.6K) (Scotts Sierra Horticultural Products, Marysville, OH), providing N at 8.6 mM. After three weeks, seedlings were transplanted into individual plastic pots (height = 9.5 cm, volume = 857 cm^3)

filled with soilless substrate (Sun Gro® Horticulture, Seba Beach, Alberta, Canada). Ten days before treatments began, each seedling was inoculated at the base of the stem with 30 mL soil harvested from beneath indigenous plants of *A. maritima* subsp. *maritima* plants in Dorchester Co. Md.

Treatments and experimental design

Forty uniform seedlings that were sixteen weeks old were considered experimental units and assigned randomly to one of eight treatments (n = 5): 0, 2, 4, 8, 12, 16, 20, and 32% O_2. An additional five seedlings were harvested destructively the day treatments began to provide a baseline for plant dry mass and leaf N content. Experimental plants were removed from their pots, and the roots were washed free of substrate and inspected for premature nodule development. Plants were installed in a randomized complete block design in an aeroponic gas delivery system (Kratsch, Graves & Gladon 2004). Briefly, plants were maintained in closed 1-L wide-mouth Ball® Mason jars (Alltrista Zinc Products, Greeneville, TN). Each jar held one root system, and the shoot emerged from a hole drilled in the dome lid. A hole drilled in the center of the bottom of the Mason jar fit over a Baumac® fog nozzle (Extra Fine) (Hummert International, St. Louis, M.), which was controlled by a programmable timer (Phytotronics, Earth City, MO). The timer was adjusted so that roots were sprayed periodically with quarter-strength, N-free Hoagland's solution (Hoagland & Arnon 1950).

Defined atmospheres within each jar were maintained by mixing the gases from cylinders of O_2, N_2, and Ar. This was accomplished by using a system of capillary tubes (Ace Glass, Vineland, NJ) of fixed diameter and length to control flow rates. N_2 gas concentration was maintained at 68% over all eight treatments. We adjusted the O_2

concentration for each treatment by varying the proportions of O_2 and Ar. The flow rate of gas mixture through each jar was 1,100 $mL \cdot h^{-1}$.

Plant growth and physiology

Treatments were initiated 2 August 2003, and plants were harvested four weeks later. We used a data logger equipped with appropriate sensors to monitor the environment during treatments and found air averaged 24 °C (range 22 to 30 °C), mean relative humidity was 85% (range 40 to 90%), and mean photosynthetically active radiation was 530 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (average over 24 h). Weights of all leaf blades, stems, petioles, and roots were determined after tissues dried for 3 d at 67 °C. Root:shoot ratios were calculated by dividing the total mass of each root by the total mass of each shoot (leaves, petioles, and stems). Dried leaf blades were ground in a tissue homogenizer (Thomas-Wiley Laboratory Mill, Philadelphia, PA) and analyzed for N content by using the Kjehldahl method.

Photosynthetic rate was measured for each plant the day before harvest by using an LI-6400 portable photosynthesis system (Li-Cor, Lincoln, NE). Nitrogenase activity was measured on the day of harvest by using the acetylene-reduction assay. Plants were sealed within their containers and subjected to 10% C_2H_2 for 1 h before measurement of C_2H_4 production. Ethylene production was measured by injecting 0.1-ml gas samples directly into a Varian Star 3600 CX gas chromatograph (Varian, Walnut Creek, CA) fitted with an alumina column, flame ionization detector, and sampling loop.

Nodule formation and anatomy

NODULE COUNTS. Nodules on each root system were counted as plants were harvested. Each nodulation event, whether it resulted in a single nodule lobe or a cluster of lobes, was counted as one nodule. Nodule fresh mass was recorded for each plant. Dry mass

of nodules was not included in calculations of plant dry mass because nodules from selected plants were harvested, weighed, and fixed for microscopy. Six of the 40 experimental plants had a single small nodule when treatments began; these were noted and not included in analysis of nodule counts.

COMPOUND LIGHT MICROSCOPY AND IMAGE ANALYSIS. All nodules from one randomly chosen plant from each O_2 treatment were harvested for chemical fixation. Nodules were fixed in a solution of 2% glutaraldehyde and 2% paraformaldehyde in a 0.1 M sodium cacodylate buffer at pH 7.2. Nodule clusters were separated under fixative into individual lobes and stored at 4 °C overnight in fresh fixative. After rinsing with buffer, nodule lobes were postfixed in 1% aqueous osmium tetroxide, dehydrated through a graded series of ethanols, and embedded in Spurr's resin (Spurr 1969). Serial thick sections (1 μm) through the N_2-fixation zone of at least two nodule lobes from each treatment were stained with toluidine blue and viewed with an Olympus Bx40 (Olympus Optical, Tokyo) compound light microscope. Images were captured by using a Zeiss Axiocam HRc digital camera (Carl Zeiss MicroImaging, Thornwood, NY). Five images from various locations within the N_2-fixation zone of each lobe were randomly selected for analysis. Air spaces in each image were hand-drawn on transparencies, scanned, and the area analyzed by using AnalySIS® software (Soft Imaging System, Lakewood, CO). The resulting data were burned as an overlay onto the image of origin.

STEREO LIGHT MICROSCOPY. Nodule lobes were harvested from plants grown in a greenhouse or from plants grown in a field in Ames, IA. Some nodule lobes were fixed overnight in a solution of 2% glutaraldehyde and 2% paraformaldehyde in a 0.1 M sodium cacodylate buffer at pH 7.2 before capturing images. All nodule lobes were hand-sectioned,

and images captured, while the lobes were submersed in water. Images were viewed by using an Olympus SZH10 (Leeds Precision Instruments, Minneapolis, MN) stereo light microscope and captured by using a digital camera as for compound light microscopy. For root-zone O_2 effects, nodules were harvested from plants treated in the aeroponic gas-delivery system described herein. For root-zone moisture effects, nodules were harvested from plants subjected to three treatments: daily watered and drained, partially flooded, and totally flooded (Kratsch & Graves 2004b).

Data analysis

Data from all experiments were analyzed with the general linear model (GLM) procedure of SAS/STAT®, Version 8.2 (1999-2001) to test for overall treatment differences, and Fisher's least significant difference (LSD) test was used at $P \leq 0.05$ to separate treatment means. Levene's test was used to evaluate data for homogeneity of variances. Based on these results, percentage leaf N and percentage nodule air space were transformed to log base 10 to achieve homogeneity of variances for further analyses. Regression analyses were used to test linear and polynomial effects of O_2 concentration on the dependent variables. Pearson's correlation coefficient was used to test for association of photosynthetic rate, plant dry mass, and leaf N content with nitrogenase activity.

Results

Plant materials

All plants in the eight O_2 treatments survived. Plants treated with $\leq 4\%$ O_2 showed stunted growth, and leaves were mottled and chlorotic (Fig. 1). Root systems remained less than 8 cm and did not extend below the top half of the Mason jar. Adventitious roots formed

from the stem above the root system, and hypertrophied lenticels developed at the base of the stems. The mean height of plants treated with 8 to 16% was 46 cm, whereas the mean height of plants treated at ≤ 4% O2 was 28 cm. The leaves of plants at 8 to 16% O_2 were dark green. The root systems appeared fibrous due to the formation of many secondary and tertiary roots, and many of the roots extended to the bottom of the Mason jars. Plants treated with 20 or 32% O_2 also were dark green, but plants were not taller than those treated with ≤ 4% O_2. Plants with roots at 32% O_2 had extremely short (≤ 2 cm) internodes.

Plant growth and physiology

Quadratic regression functions best described the influence of O_2 concentration on plant dry mass and total leaf N content, with maximal dry mass among plants with roots at ≥ 16% O_2 and maximal N concentration among plants with roots at > 20% O_2 (Fig. 2A). Mean dry mass of plants with roots treated with 8 to 16% O_2 was almost twice that of plants with roots at ≤ 4% O_2. Mean dry mass of plants with roots at 16% O_2 was double that of plants harvested the day treatments began, whereas the mean dry mass of plants at ≤ 4% O_2 was not different from plants harvested the day treatments began. Root:shoot ratios were not affected by O_2 concentration, although the overall mean root:shoot ratio of all treated plants at harvest was only 46% of the root:shoot ratio of plants harvested the day treatments began. Mean leaf N content of plants with roots at ≥ 16% O_2 was twice that of plants with roots at ≤ 4% O_2. Mean leaf N content of plants with roots treated with O_2 ≤ 4% was similar to that of plants harvested the day treatments began.

Quadratic regression functions also described the influence of O_2 concentration on photosynthetic rate (Fig. 2B). Photosynthetic rate was maximal among plants with roots at > 16% O_2. Linear regression functions described the influence of O_2 on nitrogenase activity,

which increased as O_2 concentration increased. Photosynthetic rate ($r = 0.665$), plant dry mass ($r = 0.624$), and leaf N content ($r = 0.703$) were correlated positively with nitrogenase activity ($P < 0.0001$).

Nodule formation and anatomy

Cubic regression functions best described the effect of O_2 concentration on nodule count, with peaks occurring at 10 and above 25% O_2 (Fig. 3A). Regression analysis predicted nodule count per plant to be greatest at ~ 10% O_2. (Fig. 3A). Nodule count was reduced between 10 and 25% O_2, and these nodules appeared comparatively large and multilobed. Few nodules formed on roots at 0% O_2, whereas roots held at 2 to 8% O_2 formed many small, mostly single-lobed nodules. Nodules on roots of plants at $\leq$ 12% O_2 often developed adjacent to one another, resembling beads on a string (Fig. 3A inset). A quadratic regression function described the influence of O_2 concentration on nodule fresh mass, with maximal nodule fresh mass occurring at > 16% O_2. Nodule fresh mass ($r = 0.747$) was correlated positively with nitrogenase activity ($P < 0.0001$), although nodule count was not.

Linear regression functions best described the influence of O_2 concentration on percentage of nodule area occupied by air space and mean area per air space (Fig. 3B). Nodules that formed on roots at 0 and 2% O_2 were < 2 mm in diameter, and air spaces developed in hypertrophied lenticels and infiltrated between *Frankia*-infected cortical cells (Fig. 4A) (mean air space area = 7.3% of total area). Nodules that formed at 12% O_2 were 2 to 4 mm in diameter and multilobed. Air spaces in these nodules occupied only 5% of the total area (Fig. 3B); but comparatively more spaces were distributed between cortical cells, and lenticels were not hypertrophied (Fig. 4B). Nodules that developed on roots at $\geq$ 16% O_2 were 2 to 8 mm in diameter and often multilobed; the *Frankia*-infected cortical cells were

tightly packed with no discernible air spaces between them (Fig. 4C). Air spaces occurred only between the uninfected cortical cells close to the nodule periderm and were especially prominent between cells near nodule lenticels. Mean air-space area ranged from 2.4% at 16% O_2 to < 1% at 32% O_2 (Fig. 3B). Mean area per air space at 32% O_2 was five-fold greater than it was at 0% O_2, but the spaces were limited to the nodule periphery. Nodules from all treatments contained phenolic compounds, although these cells were limited to the outer nodule cortex at 0 and 12% O_2. At 16, 20, and 32% O_2, phenolic-containing cells developed in tightly packed rows, up to four cell-layers thick, throughout the nodule (Fig 4D); at 4 and 8% O_2, they occurred throughout the area of *Frankia*-infected cortical cells, in no discernible pattern.

We observed brownish-pink pigmentation in *Frankia*-infected areas of the nodule cortex, the intensity of which was dependent upon O_2 concentration in the root zone during nodule development (Fig. 5A-C), moisture content of the root zone (Fig. 5D-F), and stage of plant dormancy (Fig. 5G-I). Nodules that were submerged for two weeks lacked pigmentation in *Frankia*-infected areas when first cut (Fig. 5J), but they developed pigmentation within 15 s after cutting that intensified over time (Fig. 5K-L).

Discussion

Despite hypoxia in root zones within its native habitat, *A. maritima* subsp. *maritima* fixes N_2 optimally at root-zone O_2 concentrations above those of the atmosphere, and as N_2-fixation rates increase, so do accumulation of leaf N and plant dry mass. Nodule fresh mass and nodule counts are influenced differently by O_2 concentration, and nodules adjust to different O_2 conditions by altering the position and extent of air-space development between

Frankia-infected cortical cells. Further, seasonal and O_2-dependent changes in nodule pigmentation were observed in areas where *Frankia* infection occurs, and a preliminary spectrophotometric assay confirmed the presence of CO-reactive heme within nodules (data not shown). Taken together, these data suggest that the capacity to gain access to soil O_2 is critical to the establishment and function of this subspecies in native stands, and that hemoglobin may play a role in sequestering O_2 in the vicinity of *Frankia*-infected cells.

Our data showing the influence of low O_2 concentration on plant dry mass, leaf N accumulation, and nodule anatomy confirm that effects of flooding reported in previous work (Kratsch & Graves 2004b) resulted from low root-zone O_2 induced by flooding. In that work, plants of *A. maritima* subsp. *maritima* were chlorotic and stunted in their growth when roots were submerged continuously and entirely, nodules were ≤ 3 mm in diameter, and large air spaces developed in the area of hypertrophied lenticels and between *Frankia*-infected cortical cells. Oxygen concentrations in the root zone during flooding treatments ranged from 0.5 to 1.5%. Also in accordance with the results of the present work, optimal plant growth and accumulation of leaf N occurred in partially flooded and in drained soils, in which O_2 concentrations were 13 to 18%, and nodule numbers were greatest at the moisture treatment corresponding to 12% O_2. Nodule counts overall were as much as one order of magnitude greater in this study compared with nodule counts made earlier (Kratsch and Graves 2004b). This may be related to the growth of plants in an aeroponic system because Martin-Laurent et al. (1997) found enhanced nodulation in plants of *Acacia mangium* Willd. grown aeroponically.

We observed that nodule fresh mass increased with O_2 concentration and was greatest between 20 and 25% O_2, whereas the number of nodules peaked at 10% O_2 and then again

above 25% O_2 (Fig. 3A). Others have observed that nodule mass of actinorhizal plants, but not number of nodules, remains relatively constant (Nelson 1983; Tjepkema et al. 1986). Consistent with this pattern, the decrease in number of nodules between 15 and 25% O_2 likely was associated with an increase in the average size of nodules within this range of O_2 concentration. As nodule numbers increased again at O_2 > 25% O_2 (Fig. 3A), nodule mass showed a corresponding decrease (Fig. 3A). This suggests that there exists a maximum carrying capacity for nodule mass in a nodulated plant, possibly related to the efficiency of N_2 fixation (Valverde, Wall & Huss-Danell 2000) or to the photosynthetic capacity of the plant (Huss-Danell 1990).

Our data demonstrate N_2 fixation in *A. maritima* subsp. *maritima* across a broad range of O_2 concentrations, with optimal rates above 21% O_2. Similarly, in work with plants of *Alnus incana* (L.) Moench (Kleeman et al. 1994) and *Myrica gale* L. (Silvester et al. 1988b) grown for 14 days and 28 days, respectively, at 5, 21, or 40% O_2, optimal nitrogenase activity occurred in plants grown at 21% O_2. In experiments with detached nodules held for $\leq$ 90 min at 5 to 75% O_2, optimal nitrogenase activity occurred between 15 and 30% O_2 among plants of *Alnus rubra* Bong. (Wheeler, Gordon & Ching 1979) and between 20 and 40 kPa O_2 in plants of *Coriaria arborea* Lindsay (Silvester & Harris 1989). Consequently, it is apparent that *Frankia* within actinorhizal plants studied to date are capable of N_2 fixation over a range of O_2 concentrations. The major differences between taxa are revealed in the various adaptations nodules have developed for controlling O_2, which are likely a result of the environmental conditions under which they evolved. Our study challenged this pattern by testing an obligate wetland plant, and results still fit the pattern set previously by other plants that are less restricted to wetlands.

Wheeler et al. (1979) described the structure of nodules of field-grown *A. rubra* and characterized the distribution of intercellular spaces based on the shape and location of spaces within the cortex. They described a gradation of intercellular spaces within nodules, from large, elongated spaces in the outer cortex to small, triangular spaces in the inner cortex between *Frankia*-infected cells, which progressively restricts the diffusion of O_2 into the nodule. They did not identify the O_2 concentration of the soil from which nodules were sampled, nor did they test for changes in air-space area or distribution with changes in O_2 concentration. Nodules of *A. maritima* subsp. *maritima* exhibited a similar gradation in nodule air-space size and distribution that was altered in response to changes in O_2 concentration (Fig. 3B). We also observed progressively greater organization of phenolic-containing cells as O_2 concentration increased. Phenolic-containing cells occurred in tightly packed rows, one or two cell layers wide starting at 16% O_2 (Fig. 4C), and up to four cell layers wide at 32% (Fig. 4D). These tightly packed cells might also have provided a barrier to O_2 diffusion within the nodule.

The presence of hemoglobin in symbiont-infected areas is proposed as another mechanism by which legumes and certain actinorhizal taxa facilitate the transport of O_2 within N_2-fixing root nodules. The O_2 concentration within the symbiont-infected zone must be low relative to the concentration of hemoglobin, or zones of low O_2 must exist, if the protein is to function in O_2 transport (Silvester, Harris & Tjepkema 1990). This condition is met in legumes by virtue of a layer of tightly packed cells surrounding the central bacteroid that establishes an O_2 gradient within the nodule (Denison & Layzell 1991). High concentrations of heme have been found in nodules of *Casuarina* L. (Tjepkema & Asa 1987) and *M. gale* (Pathirana & Tjepkema 1995), where it occurs in amounts similar to those found

in nodules of legumes. Oxygen-diffusion barriers exist in these nodules, creating areas of low O_2 concentration near *Frankia*-infected cells. Cut nodules from these plants appear pink due to the presence of the O_2-binding protein.

We consistently observed brownish-pink pigmentation in *Frankia*-infected areas of nodules, which could be due to the presence of heme. Our data suggest that expression of the putative protein is seasonal because dormant nodules did not become pigmented, even after they were cut and exposed to air for ≥ 5 min. The increased intensity of pigmentation with increases in O_2 concentration or decreases in soil moisture could be due to increased expression of the putative protein, or it may be due to heme binding greater amounts of O_2 as it becomes available. The latter is feasible, because flooded nodules became pigmented after 30 s in air, and the intensity increased over time (Fig. 5J-L).

The presence of true hemoglobin has been confirmed in nodules of *Alnus glutinosa* L. (Suharjo & Tjepkema 1995), but its role within these nodules is unclear because it is present in small amounts. But *A. maritima* subsp. *maritima* is an obligate wetland inhabitant, where its roots are under water, and nodules with viable *Frankia* can form in flooded root zones (Kratsch & Graves 2004b). In some areas, this subspecies is subject to tidal changes in water depth (Graves & Gallagher 2003). Zones of low O_2, which could facilitate the function of hemoglobin, may be created within nodules of this subspecies by a combination of flood-induced hypoxia and resulting alterations in intercellular space distribution and organization of phenolic-containing cells. It is plausible that hemoglobin might sequester O_2 when tidal water levels are low for later use when soils become waterlogged and O_2-deficient.

We have provided the first evidence of plant-regulated, O_2-dependent anatomical changes in *Alnus* nodules, and we have postulated a role for hemoglobin in modulating

effects of O_2 concentration during changes in root-zone O_2. Future studies should focus on identifying the cause of the pigmentation in nodules of *A. maritima* subsp. *maritima*. If hemoglobin is present, it would be valuable to know where the protein is localized within the nodule, how expression levels are affected by changes in root-zone O_2, and how concentrations in nodules of this subspecies compare to those in nodules of other taxa in the genus *Alnus*.

Acknowledgments

This project was supported by the Iowa Agriculture and Home Economics Experiment Station and State of Iowa funds through the Hatch Act. We would like to express our appreciation to Richard Gladon for his assistance with design of the gas-delivery system, and to Harry Horner and Tracey Pepper for technical advice regarding microscopy and image analysis.

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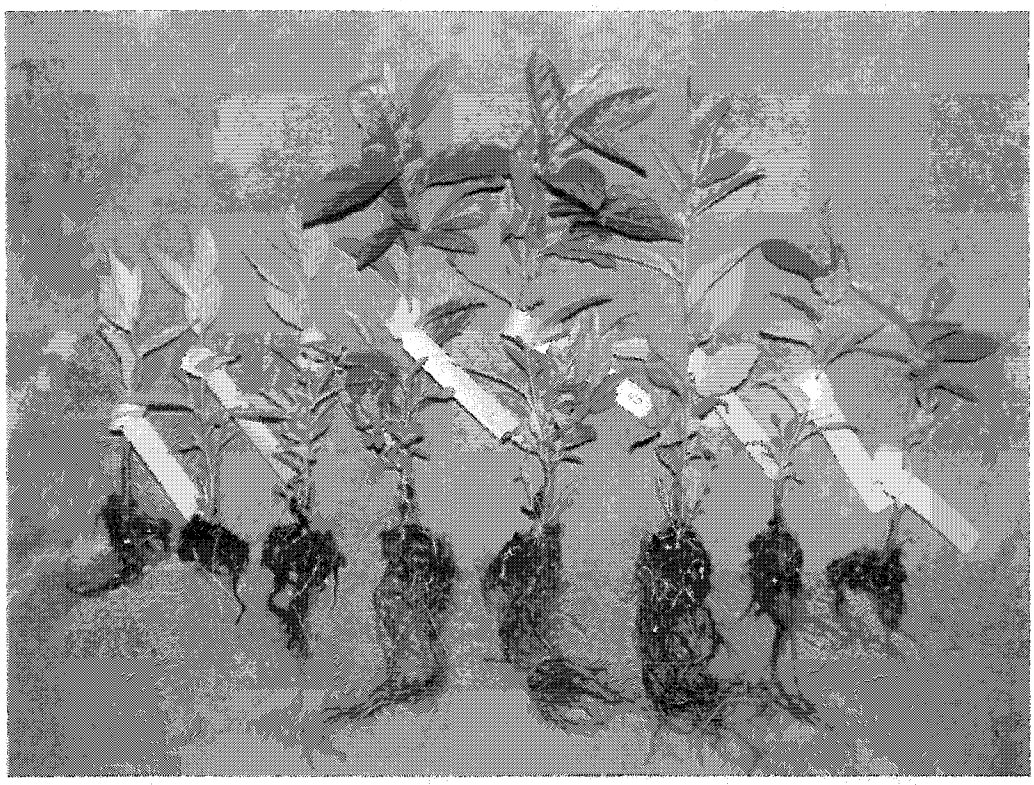


Fig. 1. Plants of *Alnus maritima* subsp. *maritima* were treated for 4 weeks with 0, 2, 4, 8, 12, 16, 20, and 32% O_2 (from left to right) in the root zone. Plants were assayed for nitrogenase activity and subsequently harvested in blocks over 5 consecutive days. A representative block was selected for the photograph. Bar = 20 cm.

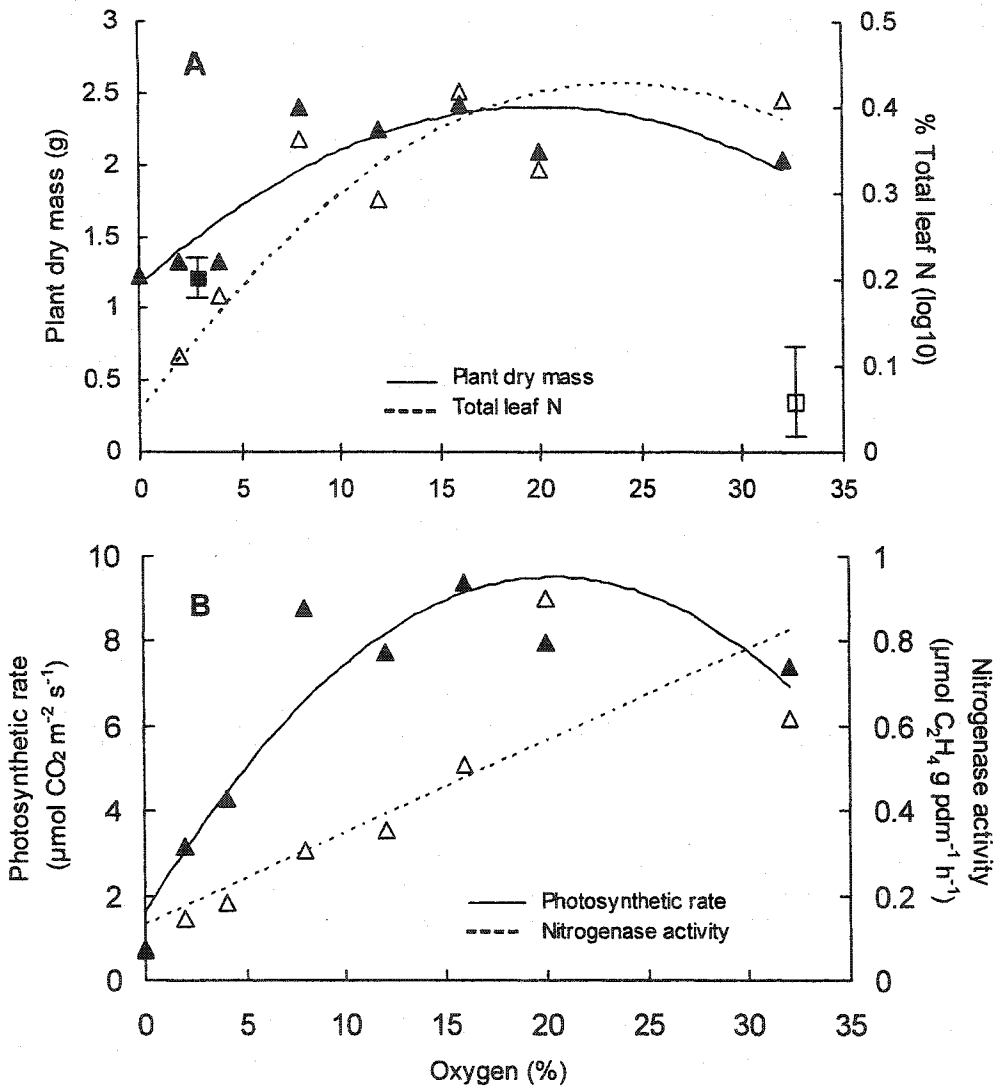


Fig. 2. (A) Plant dry mass = $-0.003[O_2]^2 + 0.124[O_2] + 1.166$, $r^2 = 0.783$, $P = 0.03$; % total leaf N (log10) = $-0.001[O_2]^2 + 0.032[O_2] + 0.045$, $r^2 = 0.844$, $P = 0.05$. Closed and open boxes with error bars indicate the range of plant dry mass and total leaf N concentration (log10), respectively, of plants harvested on the day treatments began. (B) Photosynthetic rate = $-0.019[O_2]^2 + 0.774[O_2] + 1.62$, $r^2 = 0.876$, $P = 0.008$; nitrogenase activity = $0.022[O_2] + 0.133$, $r^2 = 0.698$, $P = 0.01$ (pdm = plant dry mass).

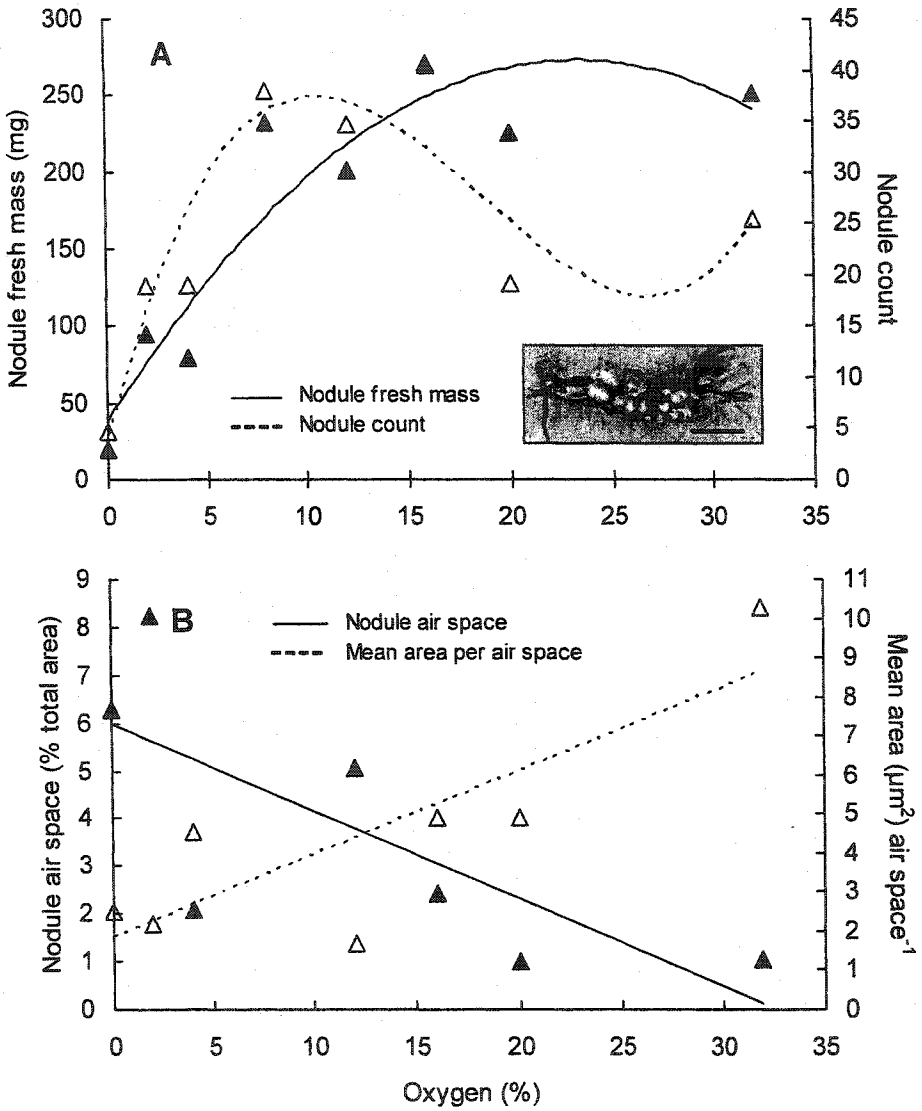


Fig. 3. (A) Nodule fresh mass = $-0.43[O_2]^2 + 20.1[O_2] + 38.287$, $r^2 = 0.868$, $P = 0.03$; nodule count = $0.009[O_2]^3 - 0.496[O_2]^2 + 7.464[O_2] + 3.327$, $r^2 = 0.832$, $P = 0.06$. Inset image shows closely arranged discrete nodules on a root at 8% O_2. Bar = 5 mm. (B) Nodule air space data was log-transformed for statistical analysis to achieve homogeneity of variance. Means of raw data are presented in the figure for clarity. Nodule air space = $-0.002[O_2] + 0.06$; $r^2 = 0.656$, $P = 0.03$; mean area per air space = $0.013[O_2] + 0.114$, $r^2 = 0.7$, $P = 0.02$.

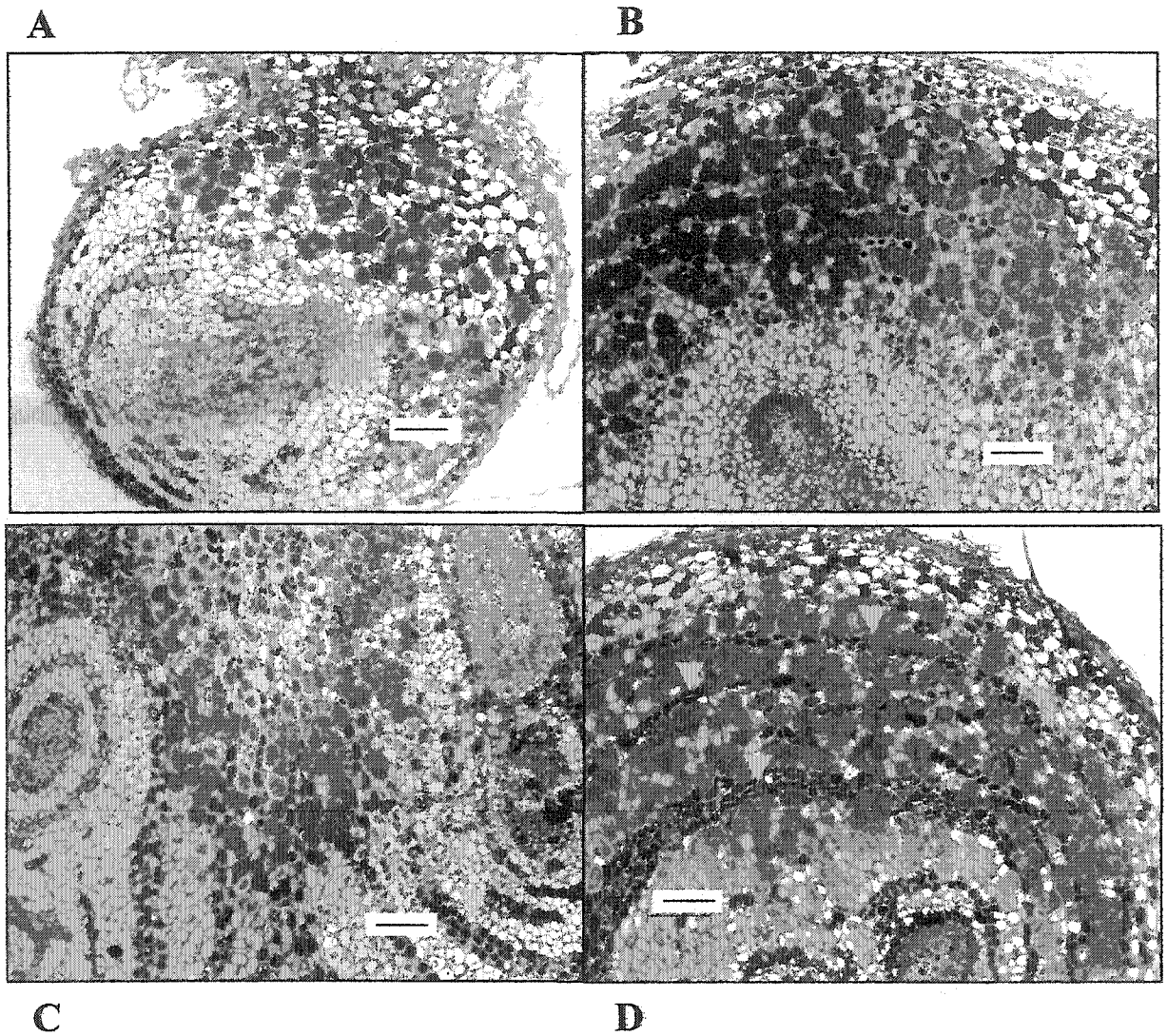
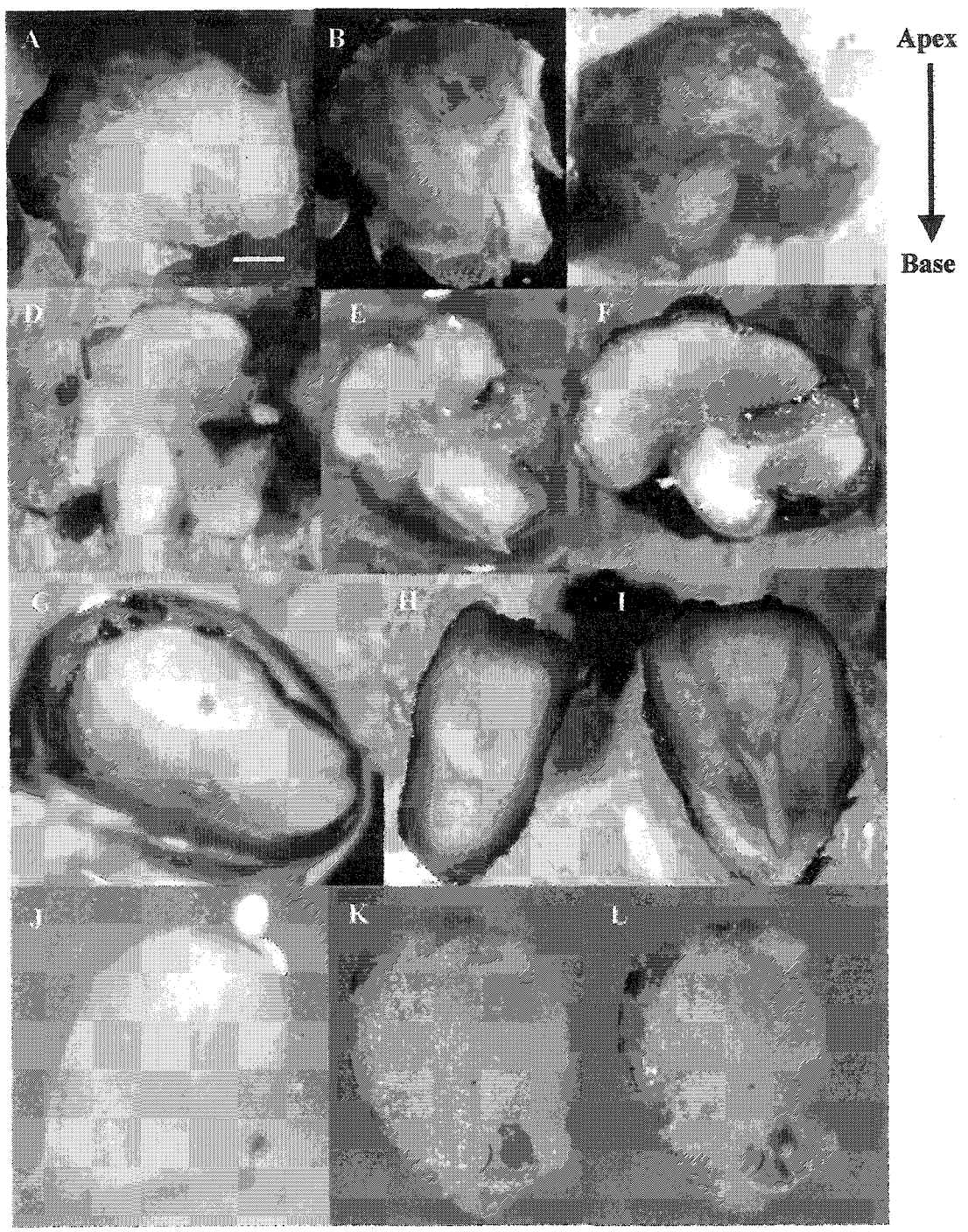


Fig. 4. Cross sections of nodules from plants treated with (A) 0%, (B) 12%, (C) 16%, and (D) 32% O_2. Air spaces developed well into the zone of infected cortical cells in nodules at 0 and 12% O_2. Air spaces in nodules at 16 and 32% O_2 occurred primarily in the periphery of the nodule cortex between uninfected cells. Yellow arrows point to rows of phenolic-containing cells. Bar = 100μm for A-D.

Fig. 5. Nodule lobes, dissected apex to base, exhibited seasonal and O_2-dependent changes in pigmentation. Nodules grown at 0% (A), 2% (B), and 8% (C) O_2 were harvested, stored frozen at −20 °C, and chemically fixed for 24 h before dissection and viewing. Nodules from totally flooded (~1% O_2) (D), partially flooded (~13% O_2) (E), and watered and drained (~17% O_2) (F) greenhouse-grown plants were harvested and dissected, and images were captured within 60 s of dissection. A dormant nodule from a greenhouse-grown plant was harvested and dissected, and the image was captured within 60 s of dissection (G). Nodules from field-grown plants that were dormant (H) and had expanding leaves after winter dormancy (I) were harvested and chemically fixed before dissection and viewing. Nodules from totally flooded greenhouse-grown plants (~1% O_2) were harvested and chemically fixed before dissection and viewing (J) or harvested fresh and images captured 30 s (K) and 3 min (L) after dissection. Bar = 1 mm for A-L.


A
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Apex
Base

CHAPTER 7. NITROGENASE ACTIVITY AND NODULATION OF SYMPATRIC *ALNUS MARITIMA* SUBSP. *MARITIMA* AND *ALNUS SERRULATA*

A paper to be submitted to *Symbiosis*

Heidi A. Kratsch, William R. Graves

Keywords: seaside alder, hazel alder, symbiosis, hemoglobin, acetylene-reduction assay, *Frankia*

Abstract

Alnus maritima and *Alnus serrulata* are sympatric in their occurrence on the Delmarva Peninsula, but *A. maritima* is unique in its occurrence in waterlogged soils. Both species are actinorhizal and develop root-nodule symbioses with *Frankia* bacteria, however the hypoxic condition of its root zone may be a challenge to the symbiosis between *Frankia* and *Alnus maritima*. Our objective was to compare nitrogenase activity and nodulation of the two species under similar root-zone O_2 concentrations. Plants of *Alnus maritima* subsp. *maritima* and *A. serrulata* were inoculated with soil from native stands and individual root zones exposed to O_2 concentrations of 0, 2, 4, 8, 16, and 20%. Nitrogenase activity and nodule fresh mass increased in *A. serrulata* with increases in O_2 concentration, but they were maximal in *A. maritima* at 16% O_2. Nodule fresh mass was similar in both species at 2% O_2, but only *Frankia* within *A. maritima* fixed N_2 at that O_2 concentration. Nodule count was sensitive to O_2 concentration in *A. serrulata*, but not in *A. maritima*. Nodules of *A. maritima*, but not *A. serrulata*, exhibited O_2-dependent brownish-pink pigmentation that future work may confirm is hemoglobin. Our results show interspecific differences in efficiency of N_2

fixation and nodulation between the two species that suggest adaptation of the *Frankia-A. maritima* symbiosis to its wetland habitat.

1. Introduction

Alnus maritima (Marsh.) Muhl. ex Nutt. is a rare shrub that is unique in its disjunct distribution. The species is found in only three widely separated populations in North America. *Alnus maritima* subsp. *maritima* (hereafter referred to as *A. maritima*) is native to the Eastern Shore of Maryland and to southern Delaware, where it is sympatric with *Alnus serrulata* (Aiton) Willd. Both *A. maritima* and *A. serrulata* are actinorhizal species that form root-nodule symbioses with the N_2-fixing soil bacterium *Frankia* Brunchorst (Stibolt, 1978). Although both species grow in wet soils, only *A. maritima* grows with its roots partially or totally submerged (Stibolt, 1981), and nodules form on plants in native stands (Kratsch and Graves, 2004a). This creates a challenge for *Frankia* associated with this subspecies because O_2 is essential for the establishment and function of the symbiosis. Unlike *Alnus glutinosa* (L.) Gaertner., which has adapted to growth in wet soils by forming nodules only above the water table (Akkermans and Van Dijk, 1976), *A. maritima* can form nodules on roots that are submerged (Kratsch and Graves, 2004b), and *Frankia* within these nodules fix N_2 in a hypoxic root-zone environment (Kratsch and Graves, unpublished data). Given the different soil-moisture conditions of their native stands, we hypothesized that nodulation and N_2 fixation in *A. serrulata* are more sensitive to low O_2 than are those traits of *A. maritima*. The objective of this work was to compare nodulation and N_2 fixation in these two species at physiologically relevant O_2 concentrations.

2. Materials and Methods

Plant materials

Alnus maritima and *Alnus serrulata* were propagated from seeds collected in Dorchester Co., MD. Seeds were rinsed in distilled, deionized water and cold-stratified at 4 °C for five weeks. Seeds were germinated at room temperature between two pieces of moist filter paper in plastic petri dishes. Germinated seedlings were sown in round plastic pots (height = 13.5 cm, volume = 3154 cm^3) filled with germination mix (Conrad Fafard, Agawam, MA). The pots were held on a greenhouse bench where 16-h photoperiods were provided by using 400-W, high-pressure sodium lamps (P.L. Light Systems, Canada). Seedlings were irrigated with tap water daily and fertilized three times per week with Peters Excel All-Purpose (25% of stock solution) and Cal-Mag® (75% of stock solution) (16.4N–2.2P–16.6K) (Scotts Sierra Horticultural Products, Marysville, OH), providing N at 8.6 mM. After three weeks, seedlings were transplanted into individual plastic pots (height = 9.5 cm, volume = 857 cm^3) filled with soilless substrate (Sun Gro® Horticulture, Seba Beach, Alberta, Canada). Ten days before treatments began, each seedling was inoculated at the base of the stem with 30 mL soil harvested from beneath native *A. maritima* plants in Dorchester Co. MD.

Experimental design and methods

Experiments were conducted over two three-week periods that began 10 Sept 2003 and 15 Oct 2003. Twenty-four uniform seedlings of each species were considered experimental units and assigned randomly to one of six treatments (n = 4): 0, 2, 4, 8, 16, and 20% O_2. An additional four seedlings of each species were destructively harvested at the time treatments began to provide a baseline for plant dry mass and leaf N content.

Experimental plants were removed from their pots, and the roots were washed free of substrate and inspected for premature nodule development. Plants were installed in a randomized complete block design in an aeroponic gas-delivery system (Kratsch et al., 2004). Plants were maintained in closed 1-L Ball® Mason jars (Alltrista Zinc Products, Greeneville, TN). Each jar held one root system, and the shoot emerged from a hole drilled in the dome lid. A hole drilled in the center of the bottom of the Mason jar fit over a Baumac® fog nozzle (Extra Fine) (Hummert International, St. Louis, Mo.), which was controlled by a programmable timer (Phytotronics, Earth City, Mo.). The timer was adjusted so that roots were sprayed periodically with quarter-strength, N-free Hoagland's solution (Hoagland and Arnon, 1950).

Defined atmospheres within each jar were maintained by mixing the gases from cylinders of O_2, N_2, and Ar. This was accomplished by using a system of capillary tubes (Ace Glass, Vineland, NJ) of fixed diameter and length to control flow rates. Nitrogen gas concentration was maintained at 68% over all treatments. We adjusted the O_2 concentration for each treatment by varying the proportions of O_2 and Ar. The flow rate of gas mixture through each jar was 1,100 $mL \cdot h^{-1}$.

We used a data logger equipped with appropriate sensors to monitor the environment during treatments and found air temperature averaged 24 °C (range 22 to 27 °C), mean relative humidity was 65% (range 30 to 80%), and the maximum photosynthetically active radiation on cloudless days was 690 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Weights of all leaf blades, stems, petioles, and roots were determined after tissues dried for 3 d at 67 °C. Dried leaf blades were ground in a tissue homogenizer (Thomas-Wiley Laboratory Mill, Philadelphia, PA) and analyzed for nitrogen content by using the Kjehldahl method.

Photosynthetic rate was measured for each plant the day of harvest by using a LI-6400 portable photosynthesis system (Li-Cor, Lincoln, NE). Nitrogenase activity was measured on the day of harvest by using the acetylene-reduction assay. Plants were sealed within their containers and subjected to 10% C_2H_2 for 1 h before measurement of C_2H_4 production. Ethylene production was measured by injecting 0.1-ml gas samples directly into a Varian Star 3600 CX gas chromatograph (Varian, Walnut Creek, CA) fitted with an alumina column, flame ionization detector, and sampling loop.

Nodulation

We recorded the number of nodules on each root system as plants were harvested. Each nodulation event, whether it resulted in a single nodule lobe or a cluster of lobes, was counted as one nodule. Nodule fresh mass was recorded for each plant. Nodule lobes were harvested from experimental plants, frozen at −20 °C, and later fixed for 24 h in a solution of 2% glutaraldehyde and 2% paraformaldehyde in a 0.1-M sodium cacodylate buffer at pH 7.2 before capturing images. All nodule lobes were hand-sectioned, and images captured, while the lobes were immersed in water. Images were viewed by using an Olympus SZH10 (Leeds Precision Instruments, Minneapolis, MN) stereo light microscope and captured using a Zeiss Axiocam HRc digital camera (Carl Zeiss MicroImaging, Thornwood, NY).

Data analysis

Plants in each experiment were analyzed over 2 d, and the resulting four data sets were treated as blocks. Data were analyzed by using the general linear model (GLM) procedure of SAS/STAT®, Version 8.2 (1999-2001) to test for overall treatment differences among species, and Fisher's least significant difference (LSD) test was used at $P < 0.05$ to separate treatment means. Regression analyses were used to test linear effects of O_2

concentration on the dependent variables. The mixed procedure was used to test for fixed effects between species, and to test for equality of the regression curves.

3. Results

Plant growth and development

Plants did not accumulate dry mass during the brief experiments, and dry mass of plants from the two experiments was similar (mean = 6.08 g). There was an overall species-related difference in plant dry mass ($P = 0.0005$); however, treatment-related differences in plant dry mass were not observed. Plants did not accumulate N in their leaves during the experiments, but plants treated with $\leq 4\%$ O_2 lost N from their leaves, and this was not species-dependent. Photosynthetic rate was similar, regardless of species and treatment, and averaged 3.43 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Nitrogen fixation and nodulation

Quadratic and linear regression functions best described the influence of O_2 concentration on nitrogenase activity of *A. maritima* and *A. serrulata*, respectively (Fig. 1A). Nitrogenase activity of *A. maritima* peaked at 16% O_2 and increased in *A. serrulata* with increases in O_2. Quadratic and linear regression functions also described the influence of O_2 concentration on nodule fresh mass of *A. maritima* and *A. serrulata*, respectively (Fig 1B). Nodule fresh mass at 2% O_2 was similar in both species, but only *Frankia* within nodules of *Alnus maritima* actively fixed N_2 at that O_2 concentration.

The surface of nodules that formed on roots of *A. maritima* was brown, and many nodules were multilobed and as large as 4 mm in diameter at $\geq 8\%$ O_2. Nodules that formed on roots of *A. serrulata* were rose-pink on the exterior, mostly single-lobed, and < 2 mm in diameter (Fig. 2). Nodule counts on roots of *A. maritima* were higher than those of on roots

of *A. serrulata*. They averaged 73 at 4% O_2 and 134 at 16% O_2, but only 9 and 69 at the same O_2 concentrations in *A. serrulata*. Nodule count of *A. serrulata* was strongly dependent upon O_2 concentration ($P = 0.0018$), and a linear regression function best described the relationship (nodule count = $5.18[O_2] - 0.389$, $r^2 = 0.93$); nodule count was greater as O_2 concentration increased. No nodules occurred on either species at 0% O_2, although two plants assigned to this treatment had nodules before treatments began. Dissected nodules showed O_2-dependent changes in pigmentation in *A. maritima*, but not in *A. serrulata* (Fig. 2). Nodules grown at 0% O_2 were creamy-white inside, except for the vascular cylinder and the periderm. At 2% O_2, pigmentation changes were observed, and at $\geq$ 4% O_2, the nodule cortex was brownish-pink. In contrast, dissected nodules of *A. serrulata* were rosy-pink throughout the cortex, consistent with the color of their exterior.

4. Discussion

Our results demonstrate that *Frankia* within nodules of *A. maritima* fix N_2 more efficiently within a wide range of O_2 concentrations than does *Frankia* within nodules of *A. serrulata*, primarily because nodulation in *A. maritima* is less sensitive to O_2 concentration than is nodulation in *A. serrulata*. Further, dissected nodules of *A. maritima* exhibit O_2-dependent alterations in nodule cortical pigmentation that are not apparent in *A. serrulata*. Some plants of *A. maritima* grow in and along streams that are subject to tidal changes in water depth (Graves and Gallagher, 2003). Together, these data suggest that nodules of this species, or the *Frankia* within, have developed mechanisms for adjusting to changes in the root-zone O_2 environment.

Our conclusions are in conflict with those of Stibolt (1978), who compared the nitrogenase activities of nodules on detached roots from native stands of *A. maritima* and *A. serrulata* and concluded that nodules of *A. serrulata* fixed N_2 at higher rates than nodules of *A. maritima*. In that study, nodulated roots were collected and assayed in serum bottles. One set of roots was tested 1 h after harvest, and another set was tested 24 h after harvest. After 1 h, *A. serrulata* exhibited higher N_2-fixation rates than did *A. maritima*. Twenty-four hours after harvest, N_2-fixation rates of *A. serrulata* decreased, while those of *A. maritima* increased. The significance of these results is unclear because assays that use detached roots or nodules are no longer recommended due to exposure of nodules to changing conditions (Silvester and Harris, 1989).

Our findings are consistent with previous work that demonstrated the capacity of *A. maritima* to nodulate and fix N_2 across a broad range of O_2 concentrations from 0 to 32% (see Chapter 6 of this dissertation). In that work, linear regression functions best described the influence of O_2 concentration on nitrogenase activity; and quadratic regression functions described the influence of O_2 concentration on photosynthetic rate and plant dry mass, with maximal rates occurring at O_2 concentrations > 20%. The present study did not show treatment-related differences in plant dry mass or photosynthetic rate. This may have been a result of imposing O_2 conditions for only three weeks, or to the time of the year when treatments were imposed.

We consistently have observed brownish-pink pigmentation in the nodule cortex of *A. maritima* in known areas of *Frankia* infection, and the current study suggests that this pigmentation is dependent upon the presence of O_2. Similar pigmentation was not observed in *A. serrulata*. Young seedlings of *A. serrulata* have bright pink stems and petioles, and

some mature leaves, roots, and nodules also contain pink pigmentation, presumably due to the presence of anthocyanins. It is likely that the rose-pink pigmentation observed in dissected nodules is also due to anthocyanins because the color consistently is observed throughout the entire nodule, rather than only in the infection zone as in *A. maritima*, and is not dependent upon O_2 concentration. The interior of nodules of legumes are bright pink, as are nodules of *Myrica* L., due to the O_2-transporting protein hemoglobin, and the presence of hemoglobin in nodules of *Alnus glutinosa* L. has been confirmed (Suharjo and Tjepkema, 1995). Like *A. maritima, Myrica* spp. are obligate wetland inhabitants and develop nodules on submerged roots (Schwintzer and Lancelle, 1983). It is feasible that the two species have evolved similar mechanisms for control of the O_2 environment within nodules.

We have provided the first evidence of interspecific differences in efficiency of N_2 fixation between *A. maritima* and *A. serrulata* related to differences in root-zone O_2 concentration of native stands. Given the sympatry of these two alders and their competition for resources near water sources, each may have developed unique strategies for survival within their habitat. Future work should focus on confirming the presence of hemoglobin in *A. maritima* nodules, and on assaying for differences in expression of the hemoglobin gene between nodules of *A. maritima* and *A. serrulata.*

Acknowledgments

This project was supported by the Iowa Agriculture and Home Economics Experiment Station and State of Iowa funds through the Hatch Act. We would like to express our appreciation to Philip Dixon for his advice regarding statistical analysis.

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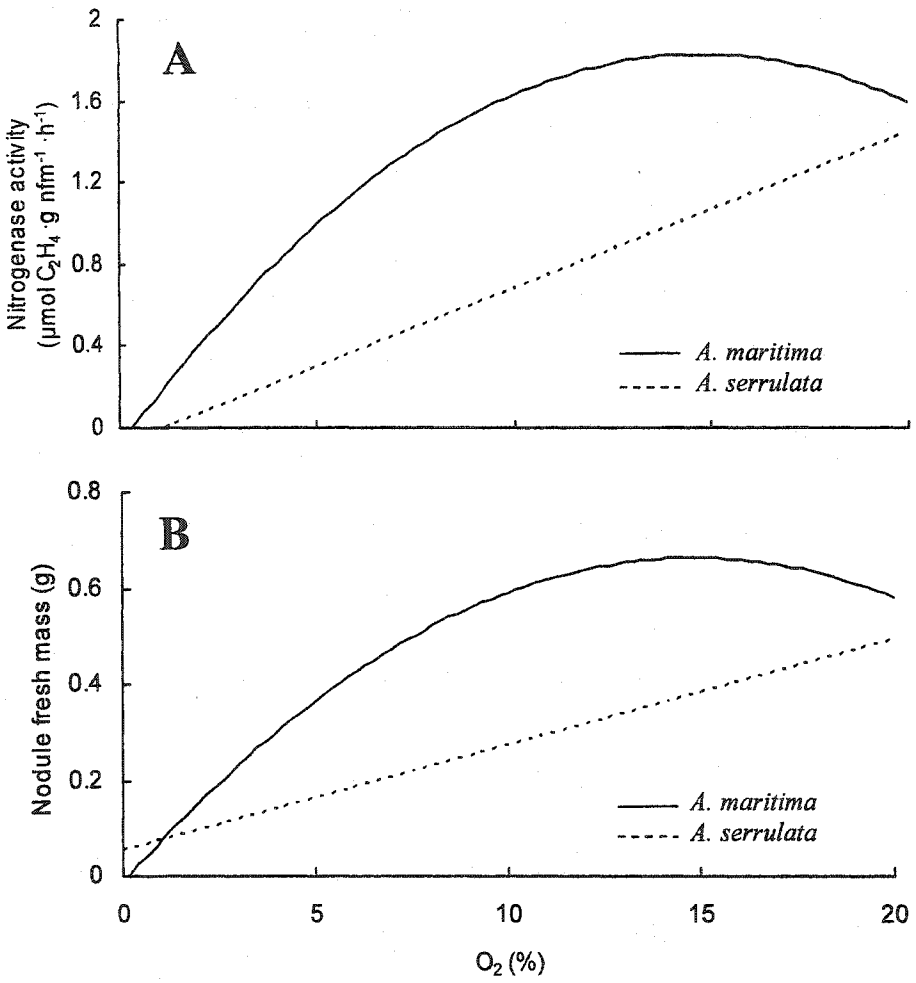

Fig. 1. Regression curves for dependent variables as a function of O_2 concentration. (A) Nitrogenase activity (*A. maritima*) = $-0.009[O_2]^2 + 0.259[O_2] - 0.085$, $r^2 = 0.95$, $P = 0.04$; nitrogenase activity (*A. serrulata*) = $0.077[O_2] - 0.085$, $r^2 = 0.99$, $P = 0.0001$. (B) Nodule fresh mass (*A. maritima*) = $-0.003[O_2]^2 + 0.091[O_2] - 0.014$, $r^2 = 0.97$, $P = 0.03$; nodule fresh mass (*A. serrulata*) = $0.022[O_2] + 0.052$, $r^2 = 0.90$, $P = 0.004$.

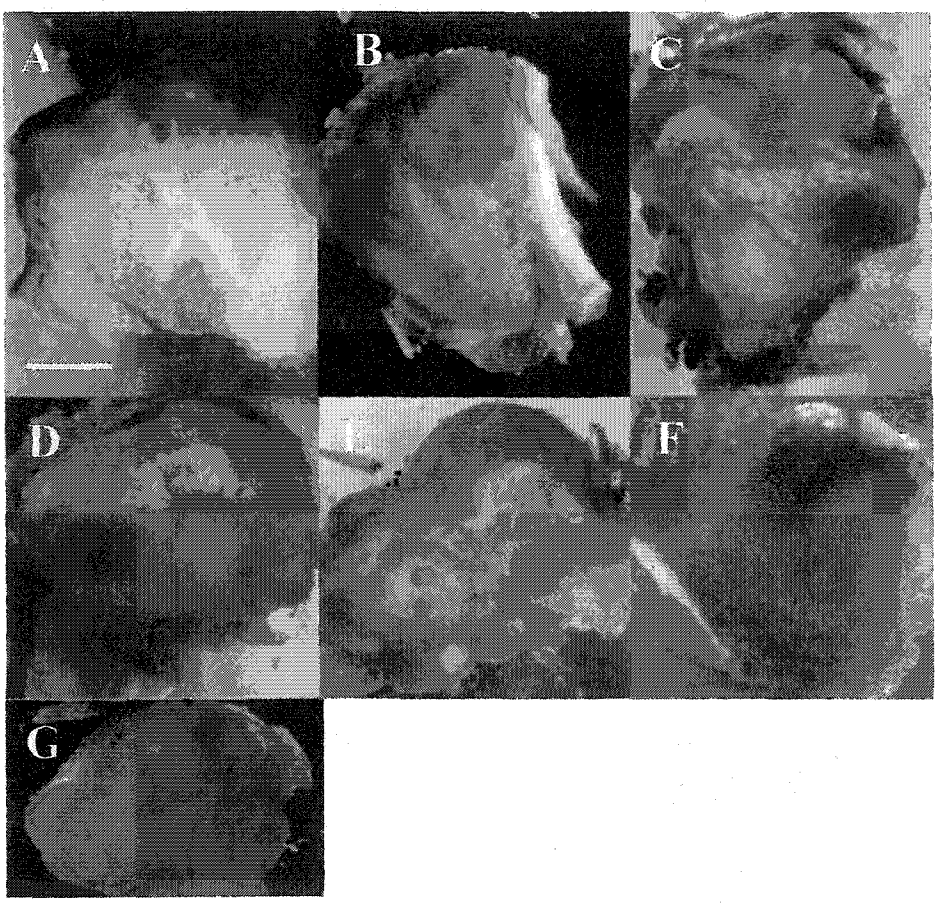

Fig. 2. Nodule lobes of *A. maritima*, but not *A. serrulata*, exhibit O_2-dependent changes in pigmentation. Nodule lobe of *A. maritima* grown at 0% (A), 2% (B), 4% (C), 8% (D), 16% (E), and 20% O_2 (F) and nodule lobe of *A. serrulata* (G) grown at 8% O_2 were harvested, stored frozen at −20 °C, and chemically fixed for 24 h before dissection and viewing. Bar = 1 mm for A-G.

CHAPTER 8. GENERAL CONCLUSIONS

Nodules occur on plants of *Alnus maritima* subsp. *maritima* in their native habitat, and these nodules are organized in a way that reflects their perennial nature. *Frankia* invades nodule cortical cells acropetally, and tightly packed layers of phenolic-containing cells restrict the bacteria to specific areas within the nodule. Although the presence of oxygen is essential to establishment and maintenance of an effective symbiosis, nodules of *A. maritima* subsp. *maritima* can develop on roots in flooded soils. *Frankia* within the flooded nodules is intact, despite the fact that flooding limited the extent of its infection of nodule cells. Further, nitrogen fixation within nodules of *A. maritima* subsp. *maritima* is oxygen-limited below atmospheric oxygen concentrations, and nodules adjust anatomically to different concentrations of oxygen in the root zone. Oxygen-dependent pigmentation, suggestive of the presence of heme, was observed within nodules. Finally, a comparison of *A. maritima* subsp. *maritima* with the sympatrically occurring *A. serrulata* provided important clues regarding the ecology of these two species in their native habitat. Nodulation and nitrogen fixation in *A. maritima* subsp. *maritima* were less sensitive to low oxygen than were those traits of *A. serrulata*, and this may help to explain the native occurrence of the two species. *Alnus maritima* is found with its roots partially or totally submerged, and *A. serrulata* occurs upland in drier soils.

These data suggest that *Alnus maritima* subsp. *maritima* has found a niche in low-oxygen, waterlogged soils. This has led to environmentally induced adaptations that have allowed this subspecies to persist in soils that are uninhabitable by other taxa. Despite the fact that it flourishes in these soils, it also survives in drained, higher-oxygen soils typical of horticultural landscapes. Because of its capacity to adjust to changes in soil oxygen, *A. maritima* subsp. *maritima* shows promise for use in low-input, managed landscapes.

APPENDIX A. ADDITIONAL DATA FROM CHAPTER 6

Table 1. Means from raw data with standard deviations.

Root-zone $[O_2]$ (%)[z]	Plant dry mass (g)		Total leaf [N] ($mg \cdot g^{-1}$)		Photosynthetic rate (µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$)		Nitrogenase activity (µmol $C_2H_4 \cdot g\ pdm^{-1} \cdot h^{-1}$)[y]		Nodule fresh mass (mg)		Nodule count (nod. $plant^{-1}$)		Nodule air space (%)		Mean area (μm^2) $airspace^{-1}$	
	mean	st dev	mean	st dev	mean	st dev	mean	st dev	mean	st dev	mean	st dev	mean	st dev	mean	st dev
0	1.2	0.3	9.7	2.6	0.7	1.3	0.07	0.05	19.9	34.1	4.6	8.6	6.3	0.9	2.5	1.1
2	1.3	0.2	12.3	3.5	3.2	1.0	0.15	0.09	95.3	66.2	19.0	15.0	8.2	3.7	2.2	0.9
4	1.3	0.3	15.2	5.2	4.3	2.6	0.18	0.10	79.5	56.2	19.0	18.4	2.1	0.6	4.5	2.6
8	2.4	0.7	23.0	2.7	8.8	0.9	0.31	0.08	232.8	103.4	38.0	19.2	x	x	x	x
12	2.3	1.0	19.6	6.5	7.8	4.4	0.36	0.21	201.0	181.6	34.6	43.9	5.1	1.0	1.7	0.7
16	2.4	0.7	26.2	3.7	9.4	1.9	0.51	0.08	269.4	59.2	40.6	38.1	2.4	3.6	4.9	2.9
20	2.1	1.1	21.3	5.0	8.0	3.1	0.90	1.07	225.7	135.5	19.2	23.4	1.0	0.5	4.9	2.0
32	2.0	1.0	25.7	1.8	7.4	2.8	0.62	0.03	252.3	163.6	25.4	31.3	1.0	0.5	10.3	7.7

[z]n = 5 plants per treatment.

[y]pdm = plant dry mass.

[x]data not available (phenolic compounds masked air spaces).

APPENDIX B. PRELIMINARY UNPUBLISHED DATA

Introduction and Background

I consistently have observed the presence of brownish-pink pigmentation on the cut surface of nodules of *Alnus maritima* subsp. *maritima* that intensifies upon exposure to air and occurs only in areas of the cortex known to be infected by *Frankia*. No other alder species shows similar pigmentation (Alison Berry, personal communication). Hemoglobin is an oxygen-transporting protein found in nodules of all symbiotic legumes and is thought to function in the transport of oxygen across the peribacteroid membrane for nitrogen fixation by *Rhizobium*. Tjepkema and Asa (1987) have assayed several actinorhizal species for the presence of CO-reactive heme and have found significant amounts in nodules of *Alnus*, *Comptonia*, and *Ceanothus*. Nodules of other taxa, such as *Casuarina* and *Myrica*, contain CO-reactive heme in amounts similar to those found in legume nodules. Since then, hemoglobin has been purified from nodules of *Myrica gale* (Pathirana and Tjepkema, 1995) and *Casuarina glauca* (Fleming et al., 1987). Based on physiological properties and similarity of amino acid and gene sequences to other symbiotic hemoglobins, it was concluded that these are true hemoglobins. More recently, a true hemoglobin (based on optical absorption spectra, molecular mass, and formation of a stable complex with oxygen) has been purified from nodules of *Alnus glutinosa* (Suharjo and Tjepkema, 1995). The average concentration, however, was found to be much lower than that of legume nodules, and its function remains unclear. It has been suggested, however, that if the protein were localized to a limited area within the nodule (like *Frankia* vesicles, for example), a gradient could be established such that it could function in the transport of oxygen.

Hypothesis

Given my visual observations of nodule pigmentation, recent discovery of a true hemoglobin in nodules of *Alnus*, and the low oxygen concentration of soils in which they occur, I hypothesize that nodules of *Alnus maritima* subsp. *maritima* contain hemoglobin that functions to modulate the effects of oxygen within the nodules.

My objective was to confirm the presence of CO-reactive heme in nodules of *A. maritima* subsp. *maritima.*

Results and Discussion

Using the protocol of Suharjo and Tjepkema (1995), I was able to extract CO-reactive heme from nodules of *A. maritima* subsp. *maritima.* Hemoglobin shows an optical absorption peak that shifts at a wavelength of 420 nm when it converts from the carboxy state to an oxygenated form, and it has a molecular mass of around 18 kDa. Based on the optical absorption spectrum obtained from a crude nodule extraction, I concluded that CO-reactive heme was present in *A. maritima* subsp. *maritima* nodules (Fig. 1). I also separated the proteins in the crude extract by SDS-PAGE and discovered a faint band just below the 20 kDa molecular weight marker (Fig. 2). Unfortunately, the amount of protein obtained from the crude extract was not sufficient to make a definitive claim. Further attempts at extracting protein from the nodules were unsuccessful. Strict anaerobic conditions and the use of a detergent are necessary to remove CO-reactive heme from actinorhizal nodules (Tjepkema and Asa, 1987). The assay is difficult, and to date I have not perfected the methodology.

Conclusions and Future Work

Although initial attempts at protein extraction proved difficult, the preliminary evidence is promising, and further efforts should be undertaken. An alternate approach might be to create a cDNA library from extracted mRNA and use primers designed from known areas of homology in symbiotic hemoglobins to find the gene (or genes) and sequence it. I attempted this using genomic DNA extracted from nodules (Fig. 3) but was not successful in amplifying any regions. Once a suitable primer is found, it would be useful to use *in situ* hybridization to localize the protein. If it is discovered to be associated with *Frankia* vesicles, these could be isolated from nodules and further attempts made at obtaining the protein.

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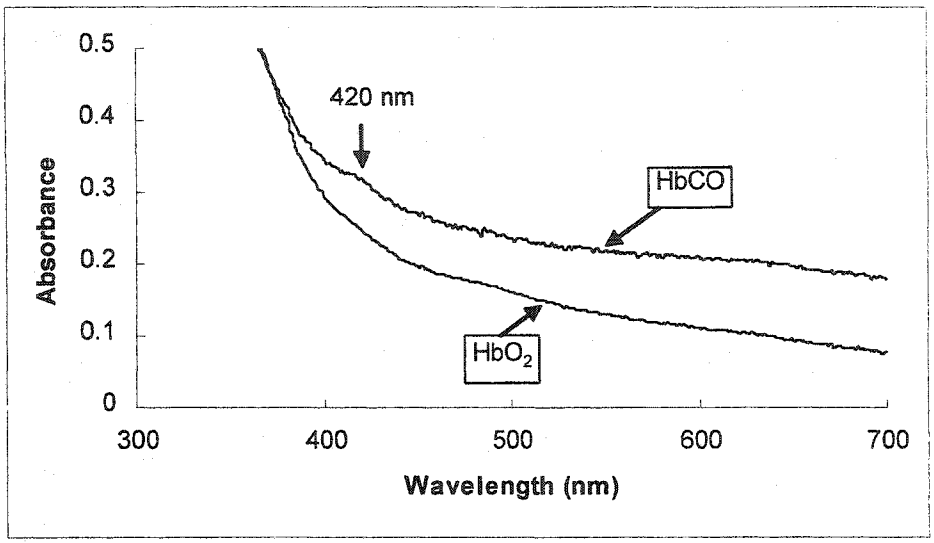

Fig. 1. Spectragraph of crude nodule extract. Proteins from fresh nodules of *A. maritima* subsp. *maritima* were extracted under CO-enriched, reducing conditions. HbO_2 was formed by exposing the extract to air for 30 min. HbCO was formed by bubbling the extract with CO for 5 s. Spectra were recorded from 700 to 385 nm by using a spectrophotometer.

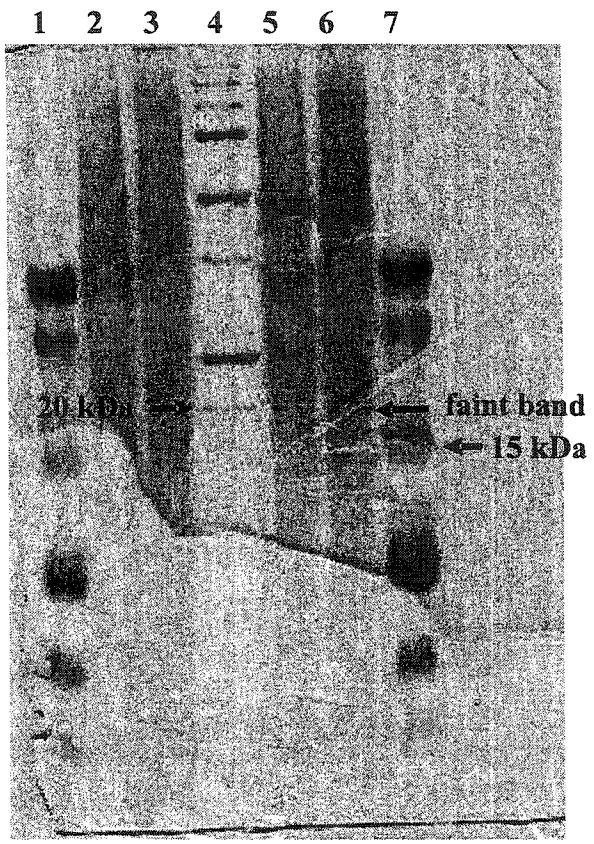

Fig. 2. SDS-PAGE of proteins precipitated from nodule extract. Lanes 2, 3, 5, and 6 are filtered, concentrated protein extracts. Lanes 1, 2, and 7 are molecular weight markers. Molecular weights of hemoglobin from other plant sources, as determined by SDS-PAGE, range from ≈ 15 to 20 kDa.

Primers:

Primer 1 (upper): 5′ ACWGARAARCARGARGCTTT 3′
(lower): 5′ AGYTTRGGRTTRTTYTCHGG 3′
Primer 2 (upper): 5′ GTGAGTTTGTGAMCTTGTGA 3′
(lower): 5′ TGGGCTTATTCTTTCATCTC 3′
Primer 3 (upper): 5′ GTTTGTGAMCTTGTGAGAGA 3′
(lower): 5′ CTTCAGTCCATGCACAACCC 3′

Fig. 3. Primer pairs used to probe genomic DNA. Primer pair 1 was used as reported by Gherbi et al., 1997. Primer pairs 2 and 3 were designed based on regions of homology in conserved regions of other plant hemoglobin genes.

ACKNOWLEDGMENTS

I would like to acknowledge the support and encouragement of my major professor, Dr. Bill Graves. Thank you for providing excellent opportunities for my professional growth and, most importantly, for giving me the confidence to take advantage of them. There have been many times during my graduate career that I questioned the sanity of my decision to pursue a doctoral degree, but I have never regretted my decision to study with you. I also would like to thank the rest of my committee: To Dr. Gwyn Beattie, who gently challenged me to think more deeply; to Dr. Dick Gladon for being not only a great mentor but a friend; to Dr. Rick Hall for fostering my willingness to engage in academic debate; to Dr. Harry Horner for his wonderful patience and attention to detail; and to Dr. Coralie Lashbrook, who is a gifted teacher, careful researcher, and a great listener.

I am grateful for the support and encouragement provided by the staff of the Preparing Future Faculty program. I am particularly indebted to PFF Program Director, Dr. Donna Kienzler, who was generous with her time and praise. I also appreciate the guidance and friendship provided by my PFF mentor, Dr. Cheryll Reitmeier, who reminded me to slow down and keep my perspective. I am also indebted to the advisors, fellow board members, and senators of the Graduate and Professional Student Senate, who gave me an opportunity to see the administrative side of university life. I am particularly indebted to Dee Egdorf, who encouraged me to get involved; and to Dr. John Mayfield, by whose example I learned patience and persistence.

I wish to acknowledge my lab mates, both past and present: Mark Kroggel, the "go-to" guy who could fashion practically anything from nothing; Dr. Jim Schrader, fellow *Alnus-maritima* nerd and IPPM-soul mate; Ryan Stewart, who inspires me to keep my

priorities straight; Tiffany Laws, whose boundless energy and enthusiasm are contagious; and Carol Foster, who was technically no longer in the lab when I arrived, but was a source of guidance and encouragement nonetheless. I will miss you all, and I sincerely hope you will keep in touch.

I gratefully acknowledge the financial support I received from the Plant Sciences Institute in the form of a Fellowship, the Graduate College, the Interdepartmental Plant Physiology Program, the Department of Horticulture, the Men's Garden Club of Des Moines, and my family.