Occurrence, Structure, and Nitrogen-Fixation of Root Nodules of Actinorhizal Arizona Alder

J. O. Dawson
Department of Natural Resources and Environmental Sciences, University of Illinois at Urbana-Champaign, Urbana, IL

G. J. Gottfried
Rocky Mountain Research Station, USDA Forest Service, Phoenix, AZ

D. Hahn
Department of Chemical Engineering, New Jersey Institute of Technology, Newark, NJ

Abstract—*Actinorhizal plants are nodulated by the symbiotic, nitrogen-fixing actinomycete* Frankia. *The genus* Alnus *in the family Betulaceae is one of the 24 genera in 8 families of angiospermous plants that are actinorhizal. Arizona alder (*Alnus oblongifolia *Torr.) occurs in isolated populations associated with the watersheds of Madrean Sky Islands in the Southwestern United States between 1,370 and 2,285 m in elevation. We have found root nodules on alder trees from Oak Creek Canyon in central Coconino County, Arizona, and from the Santa Catalina Mountains in southeastern and central Pima County, Arizona. We describe the occurrence of nodules at two locations at or near opposite latitudinal limits of Arizona alder's main range. Capacity of Arizona alder nodules to fix atmospheric nitrogen is confirmed by the acetylene reduction assay and the occurrence of vesicles in infected cortical cells of nodule lobes. Nodule location on roots, nodule morphology, and cellular anatomy confirm symbiotic structure similar to that of other alder species.*

Introduction

Arizona alder (*Alnus oblongifolia* Torr.) is an important riparian tree species that occurs in mountain canyons of the Southwestern United States and Northern Mexico, including many areas within the Madrean Archipelago or "Sky Islands." The genus *Alnus* is actinorhizal and is associated with the symbiotic, nitrogen-fixing actinomycete *Frankia*. Nitrogen is one of the major nutrients that limit productivity of naturally occurring ecosystems throughout the world (Maars and others 1983), and nitrogen fixation by actinorhizal plants is a major source of nitrogen replenishment in many natural terrestrial ecosystems (Dawson 1986). Atmospheric nitrogen also is fixed in the symbiotic microorganism-plant relationship with nodules containing *Rhizobium* bacteria, found in legumes and the elm-family genus *Parasponia*.

A specific relationship between Arizona alder and Frankia, although suspected, has not been previously confirmed. It is not possible to assume that geographically isolated populations of Arizona alder are nodulated by Frankia capable of fixing nitrogen symbiotically because of the complexity of exact dispersal mechanisms, host specificity mechanisms, and isolation factors for Frankia populations. This relative isolation of actinomycete and host populations presents the possibility that the characteristics of its symbiotic biology are unique. Moreover, not even the most rudimentary symbiotic features of this large, riparian tree species have been carefully examined. A symbiotic relationship would be an important source of nitrogen for the ecologically critical riparian communities of the Madrean Archipelago.

Arizona alder is found in riparian habitats along canyons and perennial and intermittent streams within oak woodlands and ponderosa pine (*Pinus ponderosa*) forests generally at 1,370 and 2,285 m in elevation. Arizona alder is a medium to large tree with a straight trunk that can grow to 18 to 24 m in height and to 60 to 80 cm in diameter (Little 1950). The largest Arizona alder tree is found in New Mexico and has a height of 39 m, a circumference of 505 cm, and a spread of 15 m (American Forests 2004). The species grows along many stream channels in southeastern and central Arizona, from Pima County in the south to Oak Creek Canyon in Coconino County in the north. In the Sierra Ancha Mountains of Gila County in central Arizona, alder is associated with big-toothed maple (*Acer grandidentatum*), narrow-leaf cottonwood (*Populus angustifolia*), box elder (*A. negundo*), Arizona walnut (*Juglans major*), several coniferous forest species and Gambel oak (*Quercus gambelii*) (Minkley and Brown 1982; Reynolds and Johnson 1964). Common herbaceous species include fowl mannagrass (*Glyceria striata*), false-Solomonseal (*Smilacina racemosa*), and wondering violet (*Viola nephrophylla*) (Pase and Johnson 1968).

Actinorhizal plants including Arizona alder are nodulated by the symbiotic, nitrogen-fixing actinomycete *Frankia* and include 24 genera in 8 families of angiospermous plants (table 1). Actinorhizal plant families, together with all legumes and the rhizobially nodulated genus Parasponia, have been placed

in the rosid clade containing plants with a predisposition to nodular symbiosis with diazotrophs (Soltis and others 1995). The symbiotic organ is a multi-lobed coralloid or compact spherical root nodule formed upon primary roots infected by the actinomycete *Frankia*. Nitrogen fixation by actinorhizal plants is a major source of nitrogen in diverse and widespread terrestrial ecosystems including forests, bogs, swamps, coastal dunes, landslides, glacial deposits, riparian zones, shrub lands, prairies, and deserts (Dawson 1986). Actinorhizal plants play important roles in wildland ecosystem function and have been used in land reclamation, range management, forestry, agroforestry, and horticulture. Dixon and Wheeler (1986) estimated that the contribution of actinorhizal plants to terrestrial global nitrogen fixation could be as great as 25% of the total. Estimated rates of actinorhizal nitrogen fixation are comparable to those of legumes. Nitrogen fixation rates vary widely within and among actinorhizal species and according to assay methodology used as well as ecological and genetic factors. It follows that the ranges of values reported for taxa that have been studied tend to be wide. Estimates of N_2 fixation by *Alnus rubra*, a similarly large tree in the same subgenus as Arizona alder, range from 22 to over 300 kg $ha^{-1}y^{-1}$ (summarized in Hibbs and Cromack 1990). Riparian alders, such as red alder and Arizona alder, may be keystone species in stream corridors, influencing productivity and diversity by increasing nitrogen quantity and availability in soil through primary input via N-enriched litter and root decomposition.

The purpose of the present research was to describe some aspects of the occurrence, morphology, and nitrogen fixation capacity of Arizona alder. Observations of the occurrence and morphology of root nodules on Arizona at distant locales near the northern and southern extremes of its main range that lies within the United States are preliminary to further studies of the genetics and ecology of this prominent tree species. Specific objectives of our study were to determine whether or not Arizona alders were nodulated at our two study sites and to describe in detail for any alder nodules: their location on roots, size and distribution on roots, depth of occurrence in soil, gross morphology, and microscopic features of cells.

Table 1—Currently known actinorhizal plant families and genera (adapted from Bond 1983 and Baker and Schwintzer 1990).

Family	Genus	Number of species
Betulaceae	Alnus	35
Casuarinaceae	Allocasuarina	54
	Casuarina	16
	Ceuthostoma	2
	Gymnostoma	18
Coriariaceae	Coriaria	16
Datiscaceae	Datisca	2
Elaeagnaceae	Elaeagnus	38
	Hippophae	2
	Shepherdia	2
Myricaceae	Comptonia	1
	Myrica	28
Rhamnaceae	Ceanothus	31
	Colletia	4
	Discaria	5
	Kentrothamnus	1
	Retanilla	2
	Talguenea	1
	Trevoa	2
Rosaceae	Cercocarpus	4
	Chamaebatia	1
	Cowania	1
	Dryas	3
	Purshia	2

Study Areas, Materials, and Methods

Nodule samples were collected from three trees along Oak Creek south of the Pine Flat Campground in the Coconino National Forest at approximately 1,705 m in elevation. Ponderosa pine forests occupy the surrounding slopes. Nodule samples were also collected from trees on the headwaters of Sabino Creek in the Santa Catalina Mountains within the Coronado National Forest, south of Summerhaven and north of the Marshall Gulch Campground. Elevations were about 2,315 m. Trees sampled were at least 15 cm in stem diameter at breast height and were located on the stream margins. Samples were collected in July and September of 2003.

Two indicators of nitrogen fixation capacity were examined: acetylene-reduction ability of nodules and the presence of vesicles in infected cortical cells of nodule lobes.

Nodules with attached root pieces were removed and washed in water. The average diameter of each nodule was determined using calipers. The source tree, position with respect to the root collar, color, shape, and the configuration of nodule lobes were observed and recorded for the nodules. Microtome sections from the base of peripheral nodule lobes were examined microscopically for the presence in cortical cells of Frankia hyphae, sporangia, and vesicles. The nitrogenase enzyme responsible for nitrogen fixation resides within the protective layers of vesicles in alder nodule cortical cells, and their presence is diagnostic for the capacity to fix dinitrogen.

A subsample of 6 small (~ 5 mm in diameter) alder nodules with attached root pieces, two from each tree at the Oak Creek site, were placed according to source tree on moist filter paper in loosely sealed plastic bags. Small nodules were chosen because of their relatively high ratio of active, infected cortical cells in relation to functionally inert woody tissue compared to older, larger perennial nodules. Nodule samples were stored in an insulated container cooled by plastic-encased frozen gel refrigerants. The temperature was maintained between 2 and 12 °C as determined by a min-max thermometer for 20 hours prior to gradual warming to 25 °C in a laboratory. Two nodules with attached root pieces from each tree were incubated at 25 °C in a 10% acetylene in air gas mixture in 10 cc glass tubes sealed with a serum stopper. Incubation time was 1 h. Duplicate gas samples were taken from each tube through the serum stopper after incubation using 1-cc gas syringes. Root segments without attached nodules were used to control for low levels of ethylene contaminants in the

acetylene source and any plant-derived ethylene hormone in the incubation medium.

In the 10% acetylene in air gas mixture, acetylene is reduced to ethylene by the nitrogenase enzyme while the acetylene blocks dinitrogen fixation, allowing ethylene gas evolution to serve as an indirect assay for nitrogen fixation capacity (for details see Winship and Tjepkema 1990). The acetylene-reduction assay employed in this study was intended only to detect the presence or absence of nitrogenase activity and not to quantitatively represent the actual acetylene reduction rate of the intact system measurable for a brief instant immediately after exposure to acetylene. Rates of acetylene reduction were undoubtedly reduced by excision, chilling, a rapid, acetylene-induced decline in nitrogenase activity, and the time delay between removal from the roots system and the assay. Chilling was intended to slow respirational loss of energy substrates in nodules and nitrogenase turnover during transport.

Ethylene evolution indicated by the amount of ethylene in the gas samples from the nodule incubation vessels was measured by a gas chromatograph fitted with a flame ionization detector.

Results

All trees sampled at both sites were nodulated. The nodules collected were reddish brown, 0.3 to 3 cm in diameter, with compact lobes producing a solid, spherical structure. In some cases nodules were irregular in shape where rocks or other obstructions interfered with growth. The largest nodules were found near the root collar and most nodules at the two Arizona sites were found near the soil surface. None were found at depths exceeding 5 cm. Some were located at the surface of the humic soil under rocks. Soils were moist owing to the proximity to the creeks. Cortical cells of all nodule lobes examined from each site contained *Frankia* hyphae and vesicles. Vesicles in alder nodules contain the nitrogenase enzyme. They only develop in pure culture in nitrogen limited growth media necessary to induce nitrogen fixation. Vesicles are indicative of the capacity of Arizona alder nodules to fix nitrogen.

The cellular anatomy of infected cells from Arizona alder nodules was generally similar to that of other alder species. However, Arizona alder nodules sampled differed in one respect from alder nodules developed on disturbed sites. This difference was the presence in many but not all of the Arizona alder nodule lobes examined of sporogeneous bodies. Such spore nodules produce intracellular sacs containing spores, and are characteristic of alder nodules developed in natural areas long occupied by the actinorhizal host species (Schwintzer 1990).

Nodules from all three trees sampled at Oak Creek Canyon reduced acetylene to ethylene at an average rate of 6 µmole per g dry nodule weight per h (± 3 units standard deviation). This value is clearly indicative of functional nitrogenase activity. The level is equal to low nodule rates that occur early in the spring and late in the fall in field nodules of European black alder (Zitzer and Dawson 1989). Experimental limitations allowed us to determine only that there were biologically significant rates of nitrogenase activity in Arizona alder nodules from two sites, but not to provide rates representative of the actual value or rates that can be compared quantitatively with those of assays performed under conditions of highest stringency (Winship and Tjepkema 1990).

Discussion

Many important ecological interactions, patterns, and functions can be strongly regulated by nitrogen fixation carried out by actinorhizal (*Frankia*-nodulated) plant symbiotic associations. Much information on the patterns and functions of the *Frankia* symbiosis has appeared in the literature (for review see Huss-Danell 1997), and some of this information has important implications concerning its presence and role in riparian ecosystems of the Madrean Archipelago. Of specific concern is the ability of *Frankia* in soils to form nodules on suitable host plants, such as alder, to fix nitrogen symbiotically, to disperse, and to survive.

Actinorhizal Plant Symbiosis and Its Importance

Infectious *Frankia* is known to be widespread throughout the world. This symbiosis occurs in plants on all continents (except Antarctica) and on many islands. However, its occurrence varies widely both spatially (Paschke and others 1994; Simonet and others 1999) and temporally (Wollum and others 1968). Nodulation of an actinorhizal plant may not occur because *Frankia* is absent or, more likely, because specific strains able to nodulate a given host are not present in a soil, or are unable to nodulate a host under existing soil conditions (e.g., soil oxygen limitations). Within their native ranges, most actinorhizal plant species nodulate with *Frankia* strains capable of symbiotic nitrogen fixation, although nodulation with ineffective strains can also occur in nature (Wolters and others 1997). Soil near actinorhizal hosts generally has greater nodulation capacity then surrounding soils (Jeong and Myrold 2001; Smolander 1990; Zimpfer and others 1999).

However, infective *Frankia* can be found in a variety of soils both within and outside the immediate influence of actinorhizal hosts as well as outside the range of actinorhizal plant species (Burleigh and Dawson 1993; Lawrence and others 1967; Maunuksela and others 1999, 2000; Paschke and Dawson 1992a; Zimpfer and others 1997). *Frankia* strains that are able to nodulate *Alnus*, *Myrica*, *Dryas*, and *Elaeagnus* are widespread in occurrence outside the native range of their host plants, suggesting the capacity to persist as a sporophyte (Kohls and others 1994; Maunuksela and others 1999, 2000; Nickel 2000; Nickel and others 1999).

The presence of plant species such as birches that are not actinorhizal, but that are closely related to actinorhizal genera, can increase the overall nodulation capacity of the soil for the actual host species (Gauthier and others 2000; Paschke and Dawson 1992b; Smolander 1990). Increased rhizosphere soil nodulation capacity of actinorhizal hosts and some other closely related plant species suggest the release of compounds that stimulate *Frankia* growth, infectious capacity, or both (Zimpfer and others 2002, 2003). A compound or compounds

from the roots of an actinorhizal plant can stimulate *Frankia* spore germination (Krumholz and others 2003). This stimulation could be due to flavonoids, which have been found to stimulate nodulation of actinorhizal plants (Benoit and Berry 1997; Laplaze et al. 1999).

A spatial pattern probably resulting from soil variables was evident on the Oak Creek Canyon site, where nodules were limited to soil depths less than 5 cm. In this location, the soil was saturated near the surface resulting in a high streamside water table, which may have restricted nodule development under hypoxic conditions at deeper soil depths. Low soil oxygen reduces the occurrence of infective *Frankia* in soil and can also inhibit nodule development and function. Thus, the hypoxic conditions in soil could have caused a reduction in respiration and decrease the amount of available energy substrates that would be required in large quantities for nodule development and subsequent nitrogen fixation.

Spore Dispersal Mechanisms

It cannot be assumed that a little-studied species such as Arizona alder is nodulated, or nodulated by *Frankia* populations capable of fixing nitrogen symbiotically because the exact dispersal mechanisms, host specificity mechanisms, and isolating factors for soil *Frankia* populations are not known. However, the dispersal of propagules, such as spores, is an important characteristic that would enable *Frankia* to become widely distributed among host plants of different terrestrial plant communities, including riparian areas. The mechanisms of dispersal of *Frankia* have not been empirically established in nature. However, it is known that infective *Frankia* is present in newly deposited glacial till and on sand dunes prior to colonization by host plants (Kohls and others 2003; Young and others 1992). Common mechanisms for *Frankia* dispersal are wind (anemochoric dispersal), water (hydrochoric dispersal), and biological vectors (zoochoric dispersal). All three of these mechanisms operate, to some extent, in riparian environments of the Madrean Archipelago where the two most important mechanisms are water and biological.

An important mechanism enhancing the dispersal of *Frankia* throughout riparian areas probably involves an earthworm-bird interrelationship. It has been reported that *Casuarina*-infective *Frankia* can pass through the digestive tracts of earthworms, which probably disperse *Frankia* vertically together with large volumes of soil (Reddell and Spain 1991). Birds in turn consume earthworms and other soil invertebrates and also ingest large soil particles that function as grit for grinding food in their gizzards. Some bird species also transport mud containing infective Frankia for nest construction as well as soil invertebrates as a food source for nestlings (Paschke and Dawson 1993). Furthermore, it has been demonstrated that *Frankia* spores can survive and maintain the ability to infect host plants after passage through the digestive tracts of birds (Burleigh and Dawson 1995).

Birds provide a possible aggressive vector for the importation of *Frankia* spores over long distances. For example, it has been reported that soils of tropical lowland forests of Costa Rica contain *Frankia* spores although these extensive lowland forests lack any known actinorhizal hosts (Paschke and Dawson 1992a). This is believed to occur because the migratory routes of many bird species funnel though Costa Rica as they move annually between North and South America along the Central American isthmus. Many of these migrating birds eventually visit and concentrate in the moister environments provided by the riparian corridors found along the streams of the Sky Islands of Northern Mexico and Southwestern United States. Thus it is possible that isolated riparian corridors may not lose soil microbial diversity or develop distinct ecotypes of soil microorganisms owing to frequent genetic mixing of *Frankia* and other soil microorganisms transported long distances by birds.

Summary

Arizona alders from two geographically isolated populations were found to bear actinorhizal nodules typical of species of the genus *Alnus*. Many nodules were spore+, which is a characteristic of alder nodules developed in natural areas long occupied by the actinorhizal host species. The presence of *Frankia* vesicles in infected cells and a positive result from the acetylene-reduction assay for nitrogenase activity indicates that Arizona alder has the capacity to fix atmospheric nitrogen. These findings indicate the possibility that Arizona alder may be a keystone species important for primary nitrogen inputs into biotically diverse and ecologically critical communities of riparian corridors.

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