Nitrogen-Fixing Nodule Characterization and Morphology of Four Species in the Northern Intermountain Region

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Abstract—*Purshia tridentata* (antelope bitterbrush), *Ceanothus velutinus* (snowbrush), *Ceanothus sanguenius* (redstem ceanothus), and *Shepherdia canadensis* (buffaloberry) are native shrubs of the Northern Intermountain Region that are generally characterized as nitrogen-fixing species. These species occupy a range of habitats from steppe to alpine environments. Nodulation of these species is initiated through root infection by *Frankia* species and the resulting nodules are described as coralloid. Nodulation is not necessarily confirmation that nitrogen-fixation is taking place in the nodular and root tissue of the shrub. Nodule formation, abundance, and functionality on individual plants is strongly influenced by soil moisture, soil nutrient balance, and age and health of the individual shrub, thus the character of nitrogen-fixation as an ecological process across a shrub population, and through time, may be tremendously varied. We determined the degree of nodulation and the nodule morphological characteristics on plants between stands and sites to be consistent.

Introduction

Non-legume nitrogen-fixing shrubs are frequently among the first colonizers on disturbed forest and range sites especially when nitrogen availability is one of the principal limiting factors. Non-leguminous shrubs and trees that fix atmospheric nitrogen for plant use are known as actinorhizal plants and the roots of these plants form a symbiotic relationship with an actinomycete in the genus *Frankia*. Actinorhizal plants are found almost everywhere in the world and most species, as far as is presently known, are found in temperate climate regimes.

The potential to incorporate actinorhizal plants in the design of vegetative covers for severely disturbed areas is great and often a significant means of stimulating and stabilizing nutrient cycling on these sites. In this paper we have documented the nitrogen-fixing nodular structures on each of four native woody shrub species found in the Northern Intermountain Region. This study is part of a large research effort examining soil nutrient regimes that will promote native vegetative development on severely disturbed sites. Initially, we assumed that each species has distinct nodule morphology. Our study focused on characterization of nodule morphology but included nodule occurrence within a stand, nodule placement on individual roots and root types, and the mode of nodule attachment to roots. The character of nodules that occur on these wild plants will be used as a means for comparison to nodule development on experimentally grown plants being researched for reclamation of severely disturbed landscapes.

Nodule Form and Function in Wild Nitrogen-Fixing Plants—Knowledge of Northern Intermountain Region actinorhizal plants in the wild is limited. The majority of research has been made on agricultural and horticultural nitrogen fixation and nitrogen fixation under controlled conditions. Relatively few research efforts to this point have addressed native nitrogen-fixing plants in the wild and the role they can play in reclamation vegetation cover in severely disturbed landscapes in the Northern Intermountain Region. Genera receiving the most research are *Alnus*, *Ceanothus*, and *Purshia* in that order. In the United States there are over a 100 actinorhizal native species and several naturalized species considered to be noxious weeds (Paschke 1997). The genus with the greatest number of actinorhizal species is *Ceanothus*. This genus has more than 30 actinorhizal species. *Ceanothus* and *Purshia* are the most prevalent actinorhizal shrub genera in the Northern Intermountain Region. In the Northern Intermountain Region of North America there are several nitrogen-fixing woody species (table 1) that are important as wildlife cover, livestock and wildlife browse, and considered pioneers in ecological succession.

The site of nitrogen fixation is in nodules on plant roots (fig. 1). Morphologically, nodules in actinorhizal species are modified lateral roots. The nodulation originates in the pericycle of the root (Strand and Laetsch 1977; Berry and Sunell 1990). *Frankia* infections are known to initially take place by intracellular penetration in which the bacteria enters a deformed host root hair and by an intercellular host cell penetration that is capable of infecting multiple sites in mature root cortical tissues. The type(s) of infection depends on the host plant species (Berry and Sunell 1990). Vascular tissue is central in the nodule and the bacterial organisms are found in the cells of the nodule cortex. This is different from the structure of legume nodules that are more tumor-like in which vascular tissue surrounds the nitrogen-fixing organism (Baker and Schwintzer 1990). The stele in nodules is not invaded by the *Frankia* bacteria and there are no root hairs, root cap (Nelson and Schuttler 1984), or root epidermis (Strand and Laetsch 1977).

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Table 1—Pacific Northwest native nitrogen-fixing shrubs and trees of forests and rangelands.

			Environments			
Family	Genus	Species	Moist forest	Dry forest	Subalpine	Steppe
Elaeagnaceae	*Shepherdia*	*canadensis*	+			
		argentia	+			
	Elaeagnus	*commutata*				+
Myricaceae	*Myrica*	*californica*	+			
		gale	+			
Betulaceae	*Alnus*	*rubra*	+			
		incana	+			
		rhombifolia	+			
		sitchensis	+			
Rhamnaceae	*Ceanothus*	*velutinus*	+	+	+	
		sanguineus	+	+		
		cuneatus		+		
		integerrimus	+			
		prostratus		+		
Rosaceae	*Cercocarpus*	*ledifolius*		+		+
		montanus		+		+
	Dryas	*drummondii*			+	
		integrifolia			+	
					+	
	Purshia	*tridentata*		+		+

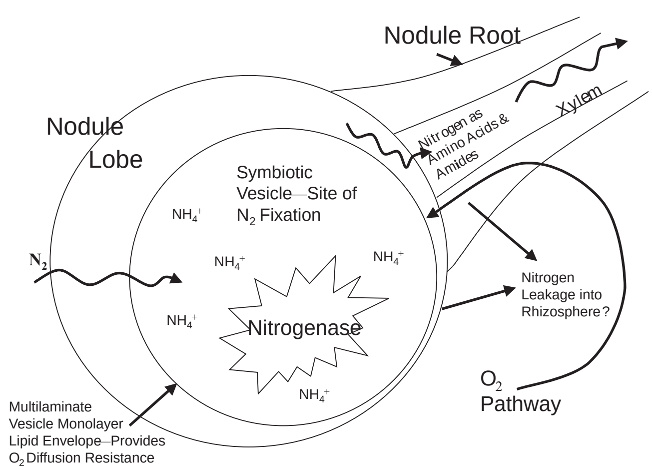

Figure 1—Generalized process of nitrogen-fixation (adapted from Huss-Danell 1990; Silvester and others 1990; Smith and others 1999).

Atmospheric nitrogen is reduced by a bacterial nitrogenase enzyme system provided by the *Frankia* species. Nitrogenase catalyzes the dinitrogen reduction reaction to form the ammonium ion (Huss-Danell 1990). Ammonium is a form of nitrogen able to be used by plants (Sprent and Sprent 1990) and can give them a survival advantage in nitrogen limited environments (Wollum and Youngberg 1969; Postgate 1982; Righetti and others 1986; Sprent 1993; Walker 1993; Kohls and others 1994; Paschke 1997).

The enzymatic reduction of atmospheric nitrogen is relatively slow and very expensive in terms of ATP provided by the host plant (Huss-Danell 1990; Sprent 1993). Because large amounts of ATP energy are consumed for metabolic activities and nitrogen fixation, the nodules require increased amounts of oxygen energy derived from plant respiration. At the same time the need for oxygen is increased, nitrogenase has to be protected from oxygen exposure because the enzyme is lethally sensitive to it (fig. 1). The host cell wall and plasmalemma surround the bacteria and are thought to afford it the needed protection (Silvester and others 1990).

Frankia induced nodules have been described in almost all actinorhizal species as coralloid clusters and consist of multiple dichotomously branching lobes (Krebill and Muir 1974; Wood and others 1989). Their colors range from light tan to dark brown (Krebill and Muir 1974) and almost whitish (Nelson 1983).

Various authors have determined that *Frankia* nodules are perennial (Strand and Laetsch 1977; Nelson and Schuttler 1984). A nodule cluster may arise from a single infection site and may be effective for several years in the field (Schwintzer and Tjepkema 1996). The nodules, as does the whole plant in temperate regions, become dormant in the winter (Huss-Danell 1990). Kummerow and others (1978) determined the age of some *Ceanothus* species nodules to be 6 to 8 years old. Nelson (1983) observed *Cowania stansburiana* nodules to be on roots that were distinctly larger than roots of a comparable age. He postulated that this root enlargement was perhaps hormonally mediated and contributed to longevity of nodules as well as more secure nodule attachment and provided for the larger volume of needed vascular translocation.

Only the distal portion of nodules (about 10 to 20 percent of live weight) is known to be fixing nitrogen (Kummerow and others1978). The tips of nodules are much lighter than the rest of the nodule. The number of nodules clusters on each plant is inversely correlated to the mass of the nodules (Sangina and others 1996). There appear to be large differences in the numbers of nodules between *Cowania* and *Purshia* species and the nodule clusters are often larger on field plants that have fewer numbers of nodules (Nelson 1983).

Nodulation and nodule activity generally thrive when the whole plant thrives and are affected by pH, temperature, and available moisture. Optimal pH range is 6.5 to 8.0 and below 5 nodules become inactive (Alexander 1977). Burggraaf and Shipton (1982) found some *Frankia* isolates to grow well on media at pH 6.5 to 7.0 but can extend their range from 4.6 to 8.0. Generally, actinomycetes temperature range for optimal growth is 29 to 37 °C. There is little growth below 5 °C and none at 39 °C (Alexander 1977). When soil temperature at 20 cm is increased above 10 °C (Dalton and Zobel 1977) nodule growth and activity begins. Under strongly aerobic conditions, *Frankia* require temperatures of around 25 to 30 °C (Stowers 1987). Youngberg and Wollum (1976) feel the optimal temperature for nodule activity is 23 °C.

Nitrogenase is severely depressed under even slight soil/water deficit (Abdel Wahab and Abd-Alla 1995). Righetti and others (1986) found evidence that nodules slough off in dry years. In excessive water they fail to thrive because when O_2 is lacking they cannot metabolize (Alexander 1977).

Righetti and Munns (1981) thought nitrogen additions inhibited nodulation and nodule activity. However, nitrogen levels in the soil were not thought to be the reason for lack of nodules or nodule nitrogen fixation activity (Righetti and others 1986). When nitrogen is supplied to plants the roots form more callus than plant roots grown in nitrogen deficient soil. Callus is incapable of fixing nitrogen.

Nodulation does not necessarily mean nitrogen fixation is taking place and some nodules exist but never fix nitrogen (Kohls and others 1994). Nitrogenase activity is one means by which researchers have been able to measure nitrogen fixation. Several methods of measuring nitrogenase activity have been available for the past 40 years and of these methods, acetylene reduction assay has been determined to be the most sensitive and the least expensive (Sprent 1979). Estimates of nitrogen addition by *Ceanothus* species systems are more a function of the *Frankia* species than the host species (Nelson and Lopez 1989). Nitrogen fixation rates are also thought to vary with plant species and *Frankia* combination, plant and nodule age, growth conditions in the field, time of day, season of the year, method of measurement, and how the rates are expressed (Huss-Danell 1990).

Seasonal patterns of nodule lobe growth and nitrogenase activity have been studied to some extent and are found to vary throughout the growing season. In central Oregon, nodule growth and activity in *Purshia tridentata* begins mid-May at the time the soil temperature at 20 cm below the surface rises above 10 °C. The greatest nodule activity appears to be in late June, and in July. In late July and August, activity declines with higher air temperatures and increased water stress (Dalton and Zobel 1977). The nitrogenase enzyme system is greatly affected by water stress and depletion of carbohydrates derived from photosynthesis is often effected by stomatal closure (Dalton and Zobel 1977).

Dalton and Zobel (1977) also found diurnal and seasonal diurnal patterns in nitrogen-fixation activity. Rates of acetylene reduction were low at predawn and late in the afternoon. Activity was greatest in the mid day. The declines in nodule activity were greater from June to August than were the mid day nitrogenase reduction rates.

The Role of Nitrogen-Fixing Woody Plants in Revegetation—*Purshia tridentata* is a dominant in several distinct climax forest and steppe vegetation types of the Northern Intermountain Region. Because of its prevalence in some areas, it may also occur as an early seral species on xeric soils. *Purshia tridentata* will commonly colonize recently burned *Pseudotsuga menziesii* sites and dominate the shrub layer until shaded out by other shrubs or development of an overstory tree layer. *Purshia* species can pioneer on semiarid foothills, plains, and mountain slopes and nodulation within the first 20 to 30 cm of root depth might be important for vegetation establishment in nitrogen deficient pioneer situations (Righetti and others 1986). Field observations show moisture is the limiting factor in nodule formation (Kummerow and others 1978). When nitrogen in the system is limiting and other factors are plentiful, nitrogen-fixing symbioses can have a significant role. Once nitrogen is no longer limiting, nitrogen fixers are not as competitive and other species take over in succession (Sprent 1993). Sprent (1993) postulated removal of nitrogen limitations could encourage more rapid cycling of all nutrients and lead to possible losses due to leaching and addition of nitrates to groundwater (Sprent 1993).

Both *Ceanothus velutinus and Ceanothus sanguineus* are most prevalent during the early vegetation stages of disturbed forest sites where the overstory layer has been removed and the site subjected to fire. *Ceanothus* species are also very often present following stand-replacing wildfire events. Because of surface and soil depletion by fire and other soil disturbances, these sites are generally considered deficient if not devoid of available soil nitrogen. Youngberg and Wollum (1976) estimated that *Ceanothus velutinus* brings nitrogen concentrations up to pre-burn levels in just 7 years.

Shepherdia canadensis is most prevalent in more advanced successional stages of disturbed forest vegetation and is present, but not dominant, in old-growth forests. In early succession, *Shepherdia* will maintain itself as part of the seral vegetation.

Nitrogen-fixing plants are able to survive in environments unfavorable to other plants and, therefore, build up organic matter. Woody nitrogen fixers, in addition to adding to nitrogen accumulation in the soil in the form of dead leaf and root tissue, can affect other species' successful colonization by providing shade, moist microsites, seed capture, and soil stabilization. In fact, nitrogen fixation may not be the most important contribution. Actinorhizal species such as *Alnus rubra* when planted with *Pseudotsuga menziesii* tend to suppress fungal root diseases and increase wood production in the conifer (Dawson 1990). Nitrogen fixers can sustain themselves in otherwise limiting environments and by the mere fact of their presence, pave the way for later successional species (Walker 1993). Pioneer succession plants add to soil organic matter. *Ceanothus velutinus* is one of the species that increases soil organic matter. Organic matter is the primary storage medium for soil N and the relatively decay resistant nature of humidified organic matter means that chemically bound organic nitrogen will be released fairly slowly (Hibbs and Cromack 1990). Soil pH can be reduced, which will minimize nitrogen and ammonia losses due to volatization (Alexander 1977) but can increase the loss of cations by encouraging their release from soil and subsequent loss by leaching (Paschke and others 1989).

The role of nitrogen fixers in revegetation has only been postulated based on inference that nitrogen fixers put nitrogen back into their environments (Munshower 1993). Nitrogen-fixing plants can also become a trapping mechanism for nitrogen and in turn, release it slowly into its soil environment. Dawson (1993) estimated tropical systems to be some of the most important nitrogen-fixing systems on the earth but estimates of their contribution to global nitrogen fixation may be underestimated because accurate data from these areas are lacking. Burns and Hardy (1975) estimate $4{,}100 \times 10^6$ ha in forests and woodlands to be responsible for fixing 10 kg nitrogen per hectare per year. This amounts to 28 percent of terrestrial ecosystems' contribution of nitrogen fixed every year.

Others working with actinorhizal nitrogen fixers have obtained specific results that vary from 0.057 kg N ha^{-1} yr^{-1} in *Purshia tridentata* (Dalton and Zobel 1977), 80 kg N ha^{-1} yr^{-1} for *Ceanothus velutinus* (Cromack and others 1979), 24–50 kg N ha^{-1} yr^{-1} in *Ceanothus sanguineus* (Binkley and Husted 1983), 42–100 kg N ha^{-1} yr^{-1} for Ceanothus in the Pacific Northwest (Binkley and others 1982), 101 kg N ha^{-1} yr^{-1} (McNabb and Cromack 1983), and 50–200 kg N ha^{-1} yr^{-1} for *Alnus rubra* (Hart and others 1997).

Most woody tree and shrub actinorhizal plants are shade intolerant early colonizers of disturbed sites. *Ceanothus velutinus* is considered to compete negatively with *Pseudotsuga menziesii* in regeneration of severely disturbed sites (Dawson 1990). On the other hand, Binkley and others (1982) argue that *Ceanothus velutinus* competes for water, light, and nutrients early on in the stand regeneration but over time, nitrogen accretion is enough to compensate for nitrogen losses generated by the disturbance. They feel the species is critical in regeneration of secondary successional sites.

Actinorhizal plants can also benefit from associations with other plant species. In *Alnus rubra* stands, for instance, associated species such as *Thuja plicata* offer wind protection and controlled spacing, resulting in increased tree diameter and straighter stems (Dawson 1990).

Materials and Methods

The incidence and morphology of nitrogen-fixing nodules were examined on the shrubs *Purshia tridentata, Ceanothus velutinus, Ceanothus sanguineus,* and *Shepherdia canadensis.* These species are native to the Northern Intermountain Region and are being studied for their utility in reclamation vegetation covers.

Sample Populations—Three areas in Washington and Idaho were chosen for collection of plant materials for all four species.

Two populations of *Purshia tridentata* were sampled on the Spokane Indian Reservation, Stevens County, east-central Washington. Both sites are near the abandoned uranium Midnite Mine for which native vegetation covers are being studied. The soil of both sites is sandy gravel glacial till and both belong to the *Pinus ponderosa/Purshia tridentata* habitat type.

Three populations of *Shepherdia canadensis* plants were sampled. Two of the sites are on the Spokane Indian Reservation. Both sites had been logged and prescribed burned within the last 5 years and represent the *Pseudotsuga menziesii/Calamagrostis rubescens* and the *Pseudotsuga menziesii/Symphoricarpos albus* habitat types. The third population is from a gravely soil, *Pseudotsuga menziesii/Symphoricarpos albus* habitat type that was also recently logged and burned. This site is south of Thrapp Mountain, Twisp Ranger District, Wenatchee National Forest, Okanogan County in north-central Washington.

Ceanothus velutinus was taken from the same Okanogan site. A second population of *Ceanothus velutinus* was sampled from disturbed sites in a *Tsuga heterophylla/Clintonia uniflora* habitat type of the St. Joe National Forest, Potlatch Ranger District, Idaho. All soils of these sites were a gravely sand.

Ceanothus sanguineus was located the same area as the first *Shepherdia* and second *Shepherdia* sites.

Sample Collection and Analysis—Fifteen cubic decimeters soil (65 cm diameter by 45 cm deep) with root material were carefully removed from around the root crown of each shrub. On every plant, all nodule distances from the crown were noted. The number of nodules and their root attachments were recorded. Crown height and dry weights, and root length and dry weights were determined. Nodules were carefully examined, photographed, and dry weights taken. Averages of data from plants in each species were computed for comparison between species.

Results and Discussion

Species Characteristics in the Northern Intermountain Region—*Purshia tridentata* in the Northern Intermountain Region is often a species with winter persistent leaves and found in low elevations with coarse textured, well-drained soils. It is considered a late successional species but can be an early colonizer on disturbed sites of more moist habitat types. It is found on warm, dry sites in steppe and forest zones. *Purshia* quickly develops a large, single, deep taproot that appears to be an adaptation to survive in water stressed habitats.

Ceanothus velutinus is an early seral evergreen species found on *Pseudotsuga menziesii* to warm *Abies lasiocarpa* habitat types. This species is best adapted to dry forest conditions and grows on hillsides and is commonly found on coarse textured soils. This forest shrub is not shade tolerant and is limited in its successional role to early states of vegetation development following fire and other kinds of severe soil disturbances on cold and drought-prone sites. This species is considered to have a deep taproot; however, in our samples, none of the plants formed a distinct taproot. The upper portion of the root beneath the caudex was a single large diameter root that divided within a depth of 15 to 20 cm into small diameter, lateral, spreading roots.

Ceanothus sanguineus in the Northern Intermountain Region is a deciduous forest shrub in the *Pseudotsuga menziesii* to warm *Abies lasiocarpa* sites. It is a seral species with low-moderate shade tolerance, grows on dry rocky hillsides, and is fire adapted. The caudex develops into a large structure that resembles the start of a taproot but no distinct taproot was evident in any of the plants we sampled. Primary branching produces fairly large lateral branches. These lateral branches became a diffuse root system within a short distance from the plant. It is more drought and cold tolerant than *Ceanothus velutinus* and overlaps *Ceanothus velutinus* in range and habitat characteristics (Hibbs and Cromack 1990).

Shepherdia canadensis is a deciduous forest shrub found on moist, cool sites with fine to sandy, gravely, rocky soils. It is found on *Pseudotsuga menziesii* to warm *Abies lasiocarpa* sites. It is shade and fire tolerant. This species has no distinct taproot and branching of the main lateral roots is evident immediately below the crown.

Species Nodule Characteristics—In our *Purshia* study areas, only 50 percent of the *Purshia* plants examined were nodulated. The nodules were predominantly small masses of 8 mm in diameter with an average weight of.0.081 grams (table 2). Single lobed nodule development was scarce and the nodules were usually found on fine lateral roots at least 15 cm but no deeper than 25 cm below the crown of the plant. No nodules were observed directly attached to the primary root. The nodules were irregularly lobed globular clusters and were very fragile, easily breaking apart from their attachments to one another and associated roots to which they were attached (fig. 2). There was a strong tendency for the clusters to form a spherical shape.

Ceanothus velutinus nodules were irregularly shaped lobed masses. Seventy percent of the plants excavated had nodules at one site and 60 percent at the other site had nodules. The nodules were large (33 mm diameter and dry weight of 1.191 g), and relatively sturdy. Most of the nodules were found at an average depth of 20 cm below the crown and attached directly to the main root. A distinguishing characteristic of these nodule clusters was the long lobe roots. The average nodule lobe root length was 5 mm (fig. 3) compared to 3.5 mm and less in the other species we examined.

Ceanothus sanguineus nodules were generally large and irregularly shaped masses. In comparison to *Purshia* and *Shepherdia*, the nodules were large in size, averaging 24 mm diameter, and heaviest in dry weight. Nodules in this *Ceanothus* species were the least fragile of the four plant species examined. The nodule lobes were thick, short, and firmly attached to one another (fig. 4). Most of these nodules were found on fine roots within 5 to 30 cm of the caudex.

Shepherdia nodules, in contrast to *Purshia*, were 80 percent attached immediately below the root crown and nodule masses varied substantially in size from small to large. Clusters of nodules in this species were also attached to young, fine roots. *Shepherdia* nodules had a tendency to branch in one plane only and produce fan-shaped masses (fig. 5) even when nodules' growth were not restricted by rocks in close proximity. This species often had numerous strings of single lobed nodules along roots.

General Observations—There was a greater tendency for larger nodule masses to form rather than single lobed structures. With each species, we found that as the root

Table 2—Characteristics of plants and attached nodule clusters in field-collected samples.

Character		*Purshia tridentata*	*Ceanothus velutinus*	*Ceanothus sanguineus*	*Shepherdia canadensis*
Plant and stem dry weight (g)	mean	111	209	125	187
	range	114–259	14–612	62–180	63–416
Plant age (yr)	mean	7	11	5	6
	range	2–5	7–18	3–8	3–9
Percentage of plants with nodules		50	65	100	100
Sites of nodule attachment		10% lateral off secondary root, 85% lateral off primary, 5% tap root	100% primary root, some lateral	100% lateral root off primary root	100% lateral root off primary root
Number of nodule clusters in sample	mean	8	many	19	13
	range	1–26	3 to many	4–59	3–37
Diameter of clusters (mm)	mean	8	33	24	19
	range	1–14	10–55	10–47	3–27
Tendency to form clusters nodules		90% intermediate clusters form	100% very large clusters form	90% small clusters form	20% intermediate, 80% small single lobed
Nodule dry weight (g)	mean	0.081	1.191	0.142	0.096
	range	0.012–0.218	0.212–2.547	0.017–0.644	0.01–0.0259

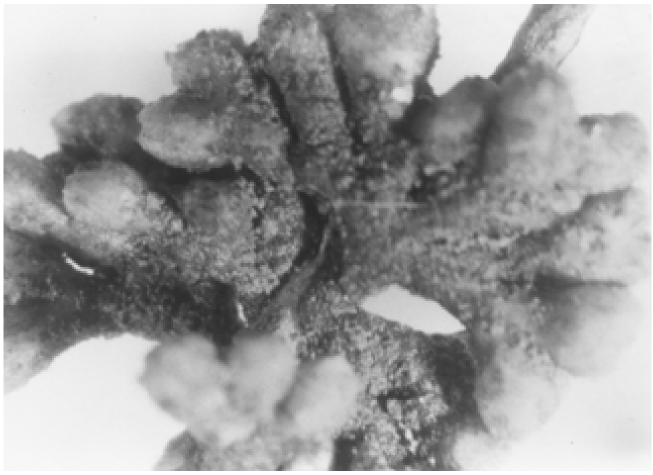

Figure 2—*Purshia tridentata*—Irregular globular clusters of fragile individual lobes.

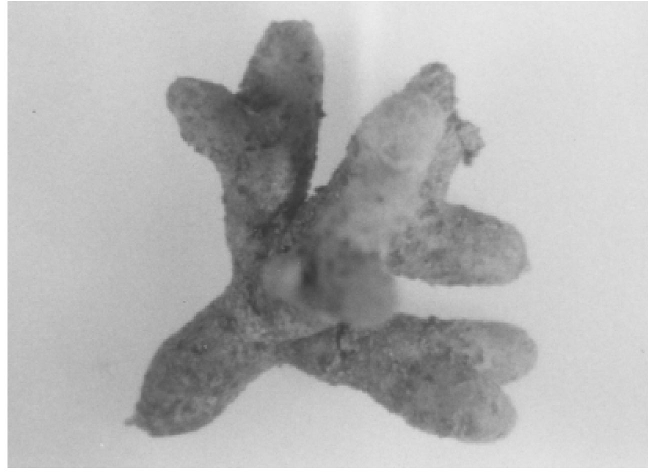

Figure 4—*Ceanothus sanguineus*—Plump, strong nodule lobes.

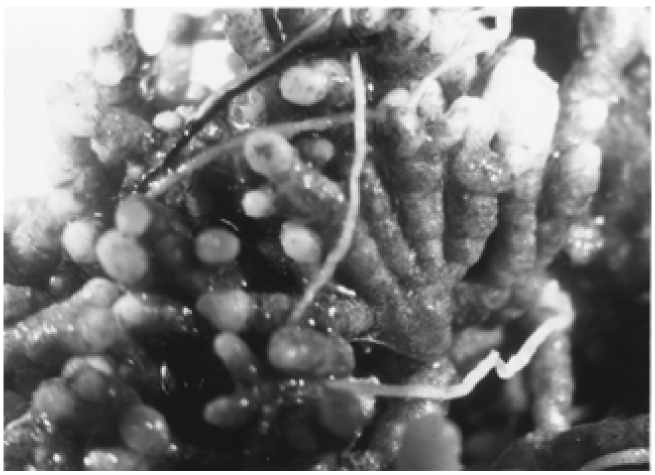

Figure 3—*Ceanothus velutinus*—Long lobe roots averaging 5 mm.

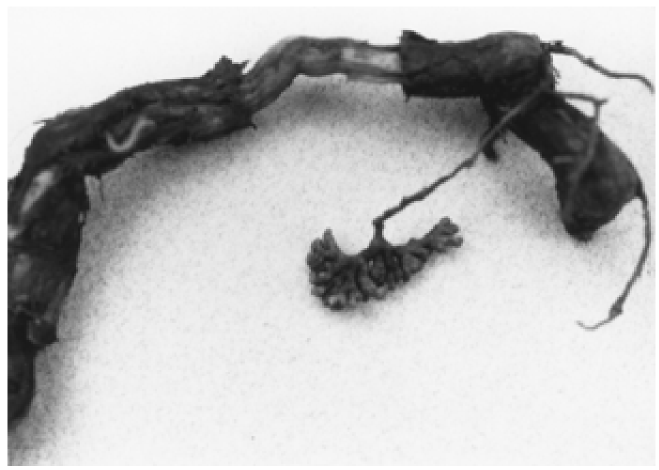

Figure 5—*Shepherdia canadensis*—Nodule clusters form in one plane and result in fan shaped clusters.

diameter increased, there was a greater prevalence of senesced nodule masses. The most viable nodular masses were strongly associated with younger and smaller roots.

Nodules were generally found within the first 10 to 30 cm below the root crown of the plants examined. Nodules also tended to be present on lateral roots off secondary roots.

Each of the actinorhizal species examined in this study had nodules with physical features that were distinct for the host plant. Distinguishing external morphological features set them apart from one another based on size and form of nodule mass, cluster weight, fragility of nodule tissue, and nodule root characteristics.

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