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Title: NITROGEN FIXATION BY PURSHIA TRIDENTATA: SOME ECOLOGICAL ASPECTS AND ROOT NODULE ANATOMY

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This study examines several aspects of nitrogen fixation by Purshia tridentata (Pursh) D. C., a rosaceous shrub widespread in the central Oregon pumice region, especially as an understory species in Pinus ponderosa and Pinus contorta forests. Anatomical studies of root nodules under the light microscope revealed strong similarities to other non-legume nodules. The endophyte, apparently an actinomycete, was visible as a mass of hyphae with peripheral spherical vesicles in swollen infected cells in the central cortex of the nodules. Seasonal variations in morphology and anatomy are described.

Acetylene reduction was used to assay nodule activity in both field and greenhouse plants. The maximum rates were observed at 20°C., although summer soil temperatures were frequently around 15°C., at which a much lower rate was observed. Acetylene reduction by excised nodules was linear for 5 hours and then slowly declined until finally ceasing after 19 hours. Nodule activity was found to decline in

water stressed plants, essentially ceasing in plants with xylem pressure potentials below -25 bars.

Field studies at 5 different sites revealed that nodule activity began in mid-May or early June when soil temperature at 20 cm. increased to above 10°C. Activity began later and remained lower until July 20 in plants located under Pinus contorta probably because of the cooler temperatures at this site. Nodule activity at all sites was maximum in June and July. In late July, nodule activity declined sharply, corresponding with moisture stress readings in the -25 bar range. Daily acetylene reduction rates declined sharply each night; this decline was even more severe late in the season.

Only 46% of Purshia plants were found to be nodulated. Several possible explanations for this low percent are discussed, but the primary reason appears to be low soil temperature and unfavorable moisture conditions. Previous speculations that Purshia may contribute significant amounts of nitrogen to the ecosystems in which it occurs are disputed using estimates based on seasonal acetylene reduction rates and a determination of nodule biomass/ha. The estimated nitrogen accretion rate was only 0.057 kg N/ha · yr.

Nitrogen Fixation by *Purshia tridentata*: Some Ecological Aspects and Root Nodule Anatomy

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NITROGEN FIXATION BY PURSHIA TRIDENTATA: SOME ECOLOGICAL ASPECTS AND ROOT NODULE ANATOMY

INTRODUCTION

The significance of nitrogen fixation by non-legumes has received increasing attention in recent years. The interest centers in part on the ability of these plants, in some instances, to contribute large amounts of nitrogen to other members of the biotic community and the subsequent potential for increased productivity of these other species. The most thoroughly examined non-legume is Alnus. Studies have indicated nitrogen accretion rates in Alnus stands as high as 320 kg N/ha· yr (Newton et al. 1968) and as much as a 22 fold stimulation of growth of associated trees such as Populus (Lawrence 1958). These are extreme examples, but numerous reports have appeared describing nitrogen accretion rates from 50-150 kg N/ha· yr and some beneficial effect on the growth of Picea (Virtanen 1957), Pinus (Wollum and Youngberg 1964) and Pseudotsuga (Tarrant 1961). Other non-legumes have also been implicated in the improved growth of associated trees. For example, Uemura (1971) reported that under-planting with Myrica rubra probably improves the growth of Pinus thunbergii on poor soils in Japan. Wollum and Youngberg (1964) found higher foliage nitrogen levels in Pinus ponderosa seedlings when growing under Ceanothus.

Purshia tridentata (Pursh) D. C. (bitterbrush or antelope brush)

is in one of the three rosaceous shrub genera that have been reported to bear nodules. The other nitrogen fixers in this family are some species of Dryas and Cercocarpus. Nodulation of Purshia was not reported until 1958 when Wagle noted the presence of nodules on both P. tridentata and P. glandulosa, the only two species in this genus. Wagle and Vlamis (1961) reported that nodulated P. tridentata plants grew well in low nitrogen soil while Purshia plants without nodules showed signs of nitrogen deficiency. Nitrogen fixation ability of P. tridentata was confirmed using N-15 by Webster et al. (1967).

Purshia tridentata is widespread in open range and as an understory in Pinus ponderosa and Pinus contorta forests. The species has a distribution range of about 340 million acres in 11 western states and southern British Columbia (Hormay 1943). The general autecology of Purshia has received attention in several reports (Stanton 1959, Nord 1965).

Although the potential ecological significance of nitrogen fixation by Purshia is occasionally mentioned (Krebill and Muir 1974, McArthur et al. 1974), the subject has remained unexplored. This is especially surprising because Purshia is one of the few non-leguminous nitrogen-fixing plants with any direct economic potential. It is highly desirable as a browse species and is used heavily by both deer and domestic livestock (Cook 1954, Dixon 1943, and Julander 1955). Purshia has a fairly high nutritional value, even in winter months, and, of several

species tested by Bissell et al. (1955) was found to be the only natural food capable by itself of sustaining caged deer. It is possible that some of Purshia's nutritional value is due to its nitrogen fixation capabilities.

Purshia's widespread occurrence in pine forests suggests that it may be important in the nitrogen budget of these areas. Dickson and Crocker (1953) observed nitrogen accretion rates of 53 kg N/ha· yr in young stands of Purshia tridentata and Pinus ponderosa. At the time, the nitrogen fixing ability of Purshia was not known. The authors were puzzled by this high accretion rate and by the apparently healthy appearance of the pines and suggested that free-living nitrogen-fixing bacteria were involved. Now that Purshia's nitrogen fixation capability is known, Silvester (1975) has speculated that Purshia was responsible.

Based largely on the production of a true mycelium and the lack of a nuclear membrane, as verified by electron microscopy (Gardner 1965, Becking et al. 1964), the nitrogen-fixing endophytes of non-legumes are considered to be actinomycetes. Becking (1970 a & b) has suggested that the endophytes of non-legumes be considered as belonging to a new monogeneric family, Frankiaceae, based on their common morphological and physiological characteristics. Although there is considerable variability in the gross morphology of root nodules from the 14 genera of nodule bearing non-legumes, the

appearance of the endophyte is similar. Becking has proposed that all non-legume endophytes be placed in the genus Frankia, with 10 species distinguished on the basis of specificity of host plant interaction. In most cases the endophyte is named after the host plant, e.g. Frankia alni for Alnus and F. purshiae for Purshia. F. purshiae was the last of Becking's ten species to receive research attention when Krebill and Muir (1974) provided a brief account of internal structure in Purshia nodules. They observed infected cortical cells occupied by mycelial masses with swollen obovoid terminal vesicles similar in many respects to other non-legumes.

The present study is an attempt to evaluate nitrogen fixation by Purshia in the field and some of the environmental conditions (temperature and moisture) that control activity of the nitrogen-fixing enzyme system. Variations due to season and time of day are also examined. In addition, due to the almost complete lack of information on this species, a brief description of root nodule anatomy is provided.

ROOT NODULE ANATOMY

Materials and Methods

Nodules were collected for examination in both winter and summer, since Dalton and Naylor (1975) have shown considerable seasonal variation in nodule anatomy and morphology of Alnus. All nodule material for microscopic examination was fixed in formalin-acetic acid-alcohol (FAA) for at least 24 hours and then thoroughly washed with a fine water jet to remove soil particles. Standard paraffin embedding procedures were employed except that much better results were obtained when the final stages of infiltration were carried out under vacuum. A number of different staining procedures were used including safranin-fast green and a modification of the technic reported by Krebill and Muir (1974). This second method yielded the best general results and was used most often. Sections were de-paraffinized, brought to water, and stained 30-40 min. in 1% orseillin BB in 3% acetic acid. After transfer through a graded alcohol series, the sections were counter-stained in .25% aniline blue in 90% ethanol for 2 min.

Nuclei were studied using the Feulgen method of Gomori (1952) in which sections were hydrolyzed in 1 N HCl at 60°C for 15 min. or in 5 N HCl at 20°C for 5 min. and then stained with Schiff's reagent.

The Azure method of Flax and Himes (1952) was also used. This involved staining tissue sections in a solution containing .25 mg/ml of Azure B in citrate buffer at pH 4.0 for 2 hours at 50°C. Differential extraction of DNA and RNA was attempted using two technics. Following the perchloric acid method of Erickson et al. (1949), sections were placed in 1 N perchloric acid for 24 hours at 4°C to remove RNA and/or .5 N perchloric acid at 70°C for 40 min. to remove DNA. The second method involved enzyme extraction as described by Brachet (1953). RNA was removed by placing sections in a 0.1% ribonuclease solution at pH 6.8 for 1 hour at 40°C. DNA was removed by placing sections in a .2 mg/ml solution of deoxyribonuclease in 0.003 M $MgSO_4$ at pH 6.5 for 1 hour at 25°C.

Results and Discussion

Apical portions of nodules were characterized by a brown color in the winter; in the summer they were slightly swollen and distinctly lighter colored, sometimes almost white. Nodule branching was highly variable, some nodules branching only once or twice, with others forming a ramiform coralloid mass never greater than 2 cm in diameter (Figure 1 and 2). Branching was dichotomous, each branch from 1 to 5 mm in length and 0.5 to 1 mm in diameter. Active nodule clusters frequently contained senescent branches easily distinguished by a dark brown-black appearance and the lack of terminal

swelling. Microscopic examination revealed the complete breakdown of cellular structure throughout suggesting that these nodule branches were being shed. Nodules were located on small lateral roots mostly at depths of 9-30 cm. The occurrence of large clusters on small thread-like roots was common, making excavation of nodules difficult and probably incomplete in many cases.

The orseillin BB - aniline blue staining sequence was found to produce excellent results with clear distinction between endophyte and host tissue. This staining sequence has proven useful in the study of fungal infections of higher plants because the host cell walls stain red and are easily distinguished from infecting hyphae, which stain blue. The technic was described by Strassburger (1923) who correctly attributed the difference in staining reaction to the callose content of the fungal cell walls. Under proper conditions, callose is specifically stained by aniline blue. More recently, the procedure has been used successfully by Jewell (1958) and Peterson and Shurtleff (1965). However, in Purshia, active endophyte tissue stained red and host plant cells blue. The chemistry of this reaction is unclear and Krebill and Muir (1974), who also used this technic with Purshia nodules, did not comment on the reversed color scheme. Cohen and Doak (1935) reported a similar response with the same stains in which mycorrhizal mycelia appeared red and host cell tissue blue, but provided no explanation for the basis of the reaction.

The color distinction between host and endophyte was lost in inactive winter nodules where the endophyte also stained blue. In the tips of growing nodules, newly forming endophyte stained light red, followed by a region of dark staining endophyte and then, further back from the nodule apex, old senescent endophyte which did not stain (Figure 5 & 6). The older endophytes appeared to be disintegrating and were not visible in more proximal host tissue.

Infected cells consisted of a central mycelial mass. The details of hyphal structure were not discernible with light microscopy, but studies with other non-legumes using the electron microscope (Becking et al. 1964, Gardner 1965) have revealed that the mycelium is septate and branched. In the present study, the mycelium appeared as a granular and amorphous clump (Figure 3). Conspicuous spherical vesicles are formed on the periphery of the central mycelium just inside the host cell wall. Although the exact function of these vesicles is not clear, Akkermans' (1971) work with tetrazolium dyes has suggested that they are the actual sites of nitrogen fixation in Alnus. These vesicles were absent in winter nodules, but the central mycelium remained (Figure 4). The area previously occupied by the vesicles became empty, resulting in the mycelium appearing separated from the host cell wall by a clear ring (Figures 4 & 7).

Infected cells retained the nucleus and nucleolus, both of which became enlarged and misshapen when compared to those in surrounding

uninfected cells (Figure 4). Becking (1970 a & b) believes the infected cells of nonlegumes are probably polyploid and in the material examined here, two or three nucleoli were often seen in the same infected cell. The increased size of the nucleolus made it very conspicuous and was probably an indication of increased protein synthesizing activity. The enlarged nucleolus stained well with safranin and was readily apparent in both summer and winter nodules. It was frequently highly visible while the rest of the nucleus was not discernible. Special technics employed for nucleic acid staining and for differential DNA and RNA extraction revealed more clearly the presence of enlarged nuclei and established the identity of the nucleolus. However, the nucleus responded poorly to most treatments, staining very weakly by the Feulgen method and only slightly better with Azure B. Nucleic acid extraction was only partially complete, with the perchloric acid technic more effective than enzymes.

It is interesting to note that at least some of these changes in nuclear size and structure in infected cells are surprisingly similar to abnormalities found in tumorous animal cells (Busch and Smetana 1970) and in plant cells infected by pathogenic fungi (Gaumann 1950). Even more noteworthy are the findings of Bahadur (1969) that root cells of Nardus infected by vesicular-arbuscular mycorrhizae developed much enlarged nuclei and nucleoli. In addition, staining responses were analogous to those mentioned above in infected cells

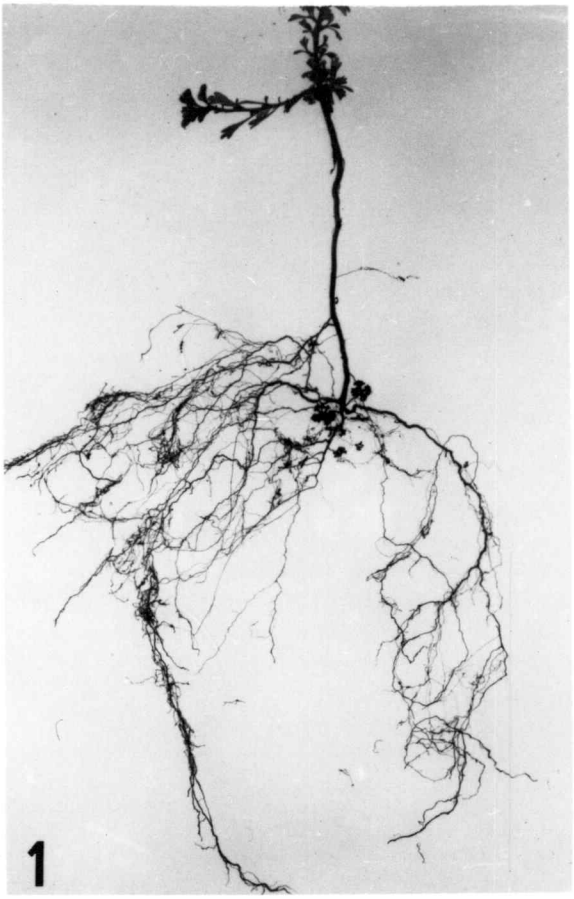
of Purshia. Bahadur reported the nucleolus stained much darker in infected cells than in non-infected cells, but the rest of the nucleus stained only lightly with both Feulgen and Azure B methods. The increase in nuclear size accompanied by less intense Feulgen staining was interpreted as an indication that the total DNA content was unchanged.

Unlike those of legumes, root nodules of Purshia and other non-legumes consist basically of modified lateral roots. The central region was occupied by a normal stelar arrangement (Figure 7 and 8). Growth and branching proceeded from a terminal meristem complete with the typical Angiosperm tunica-corpus configuration (Figure 7). Winter nodules were completely encased in a corky periderm. In summer nodules, the active growing nodule tips lacked this cork covering, but it persisted further back from the apex (compare Figures 6 and 7).

In some non-legumes, small polyhedral bacteria-like particles called bacteroids or granulae have been observed in infected cells. Their role is unclear but they may represent a resting stage involved in propagating the endophyte. Granulae are not universal and have been reported for only about half of the species of Frankia. The situation is complicated by the fact that this phase of the endophyte life cycle is generally not observed even in material from species in which

it is known to occur. No granulae were found on any material observed in this study.

Figure 1 and 2. Root nodules of a 7 month old Purshia seedling grown in the greenhouse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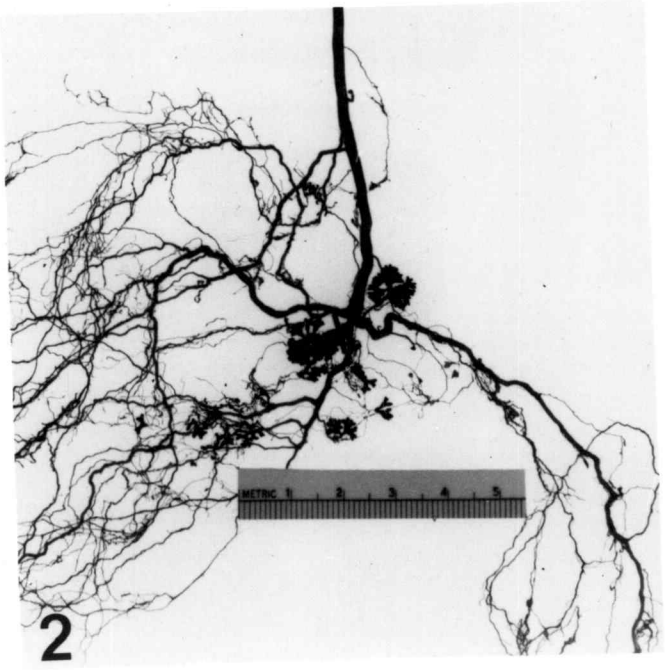
METRIC
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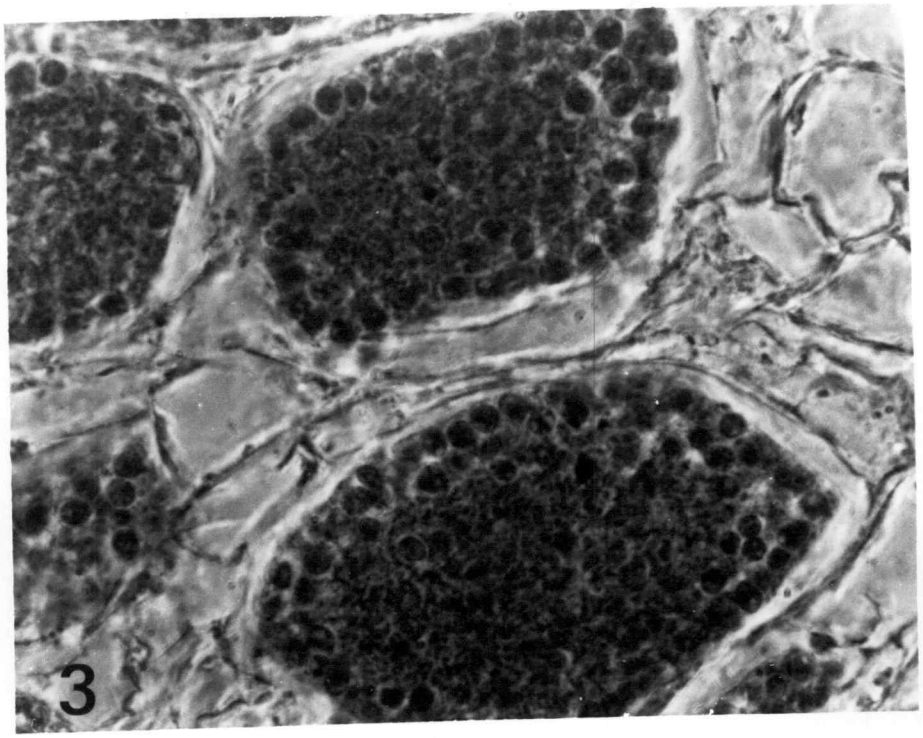
Figure 3. Infected cortical cells of an active nodule showing central mycelium and peripheral spherical vesicles. Safranin and fast green, 1320 X, oil.

Figure 4. Infected cortical cells of an inactive winter nodule showing the enlarged nuclei and nucleoli. Safranin and fast green, 210 X. Inset: 1040 X, oil.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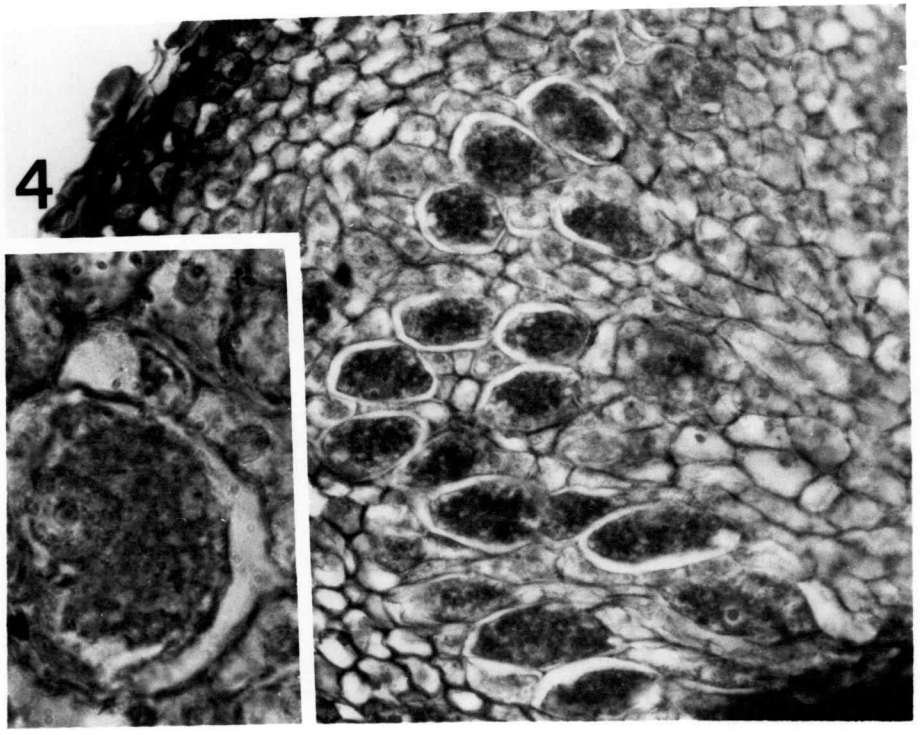
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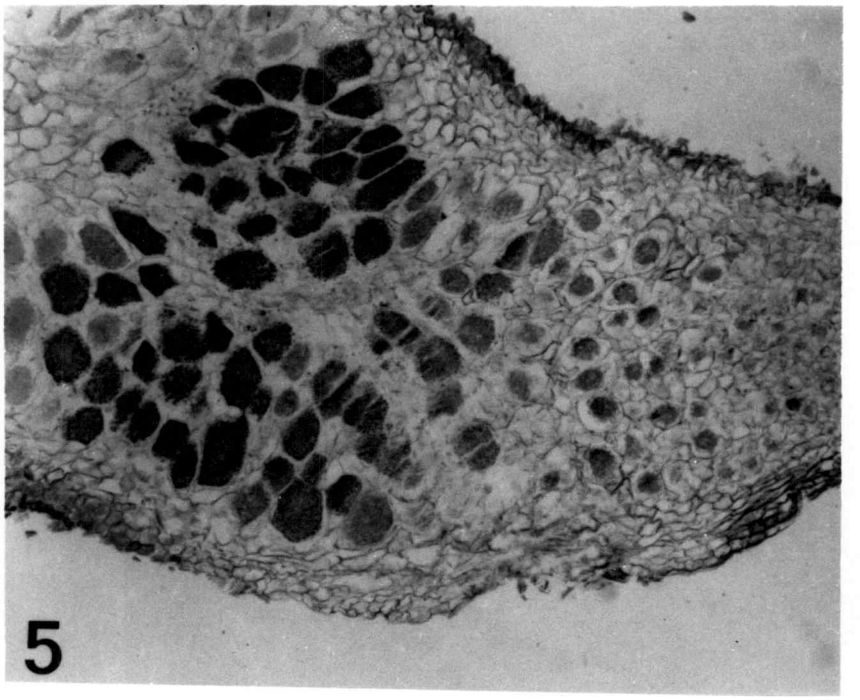
Figure 5 and 6. Tangential sections through an active nodule showing regions of newly forming endophyte, established regions, and senescent regions. Orseillin and aniline blue, 135 X.



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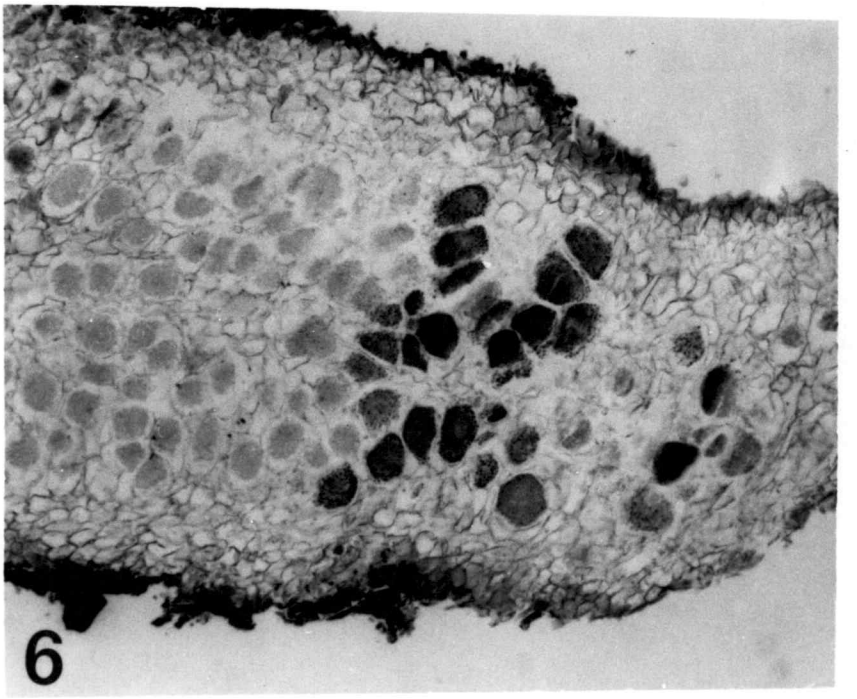

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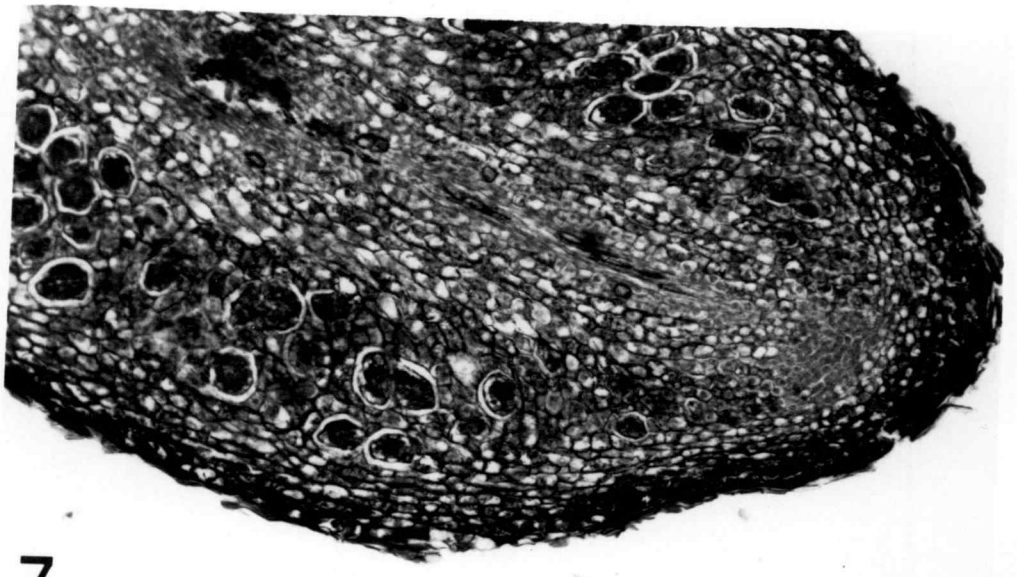
Figure 7. Median longitudinal section of an inactive nodule showing the stele and meristem. Safranin and fast green, 135 X.

Figure 8. Cross section of an active nodule. Safranin and fast green, 215 X.



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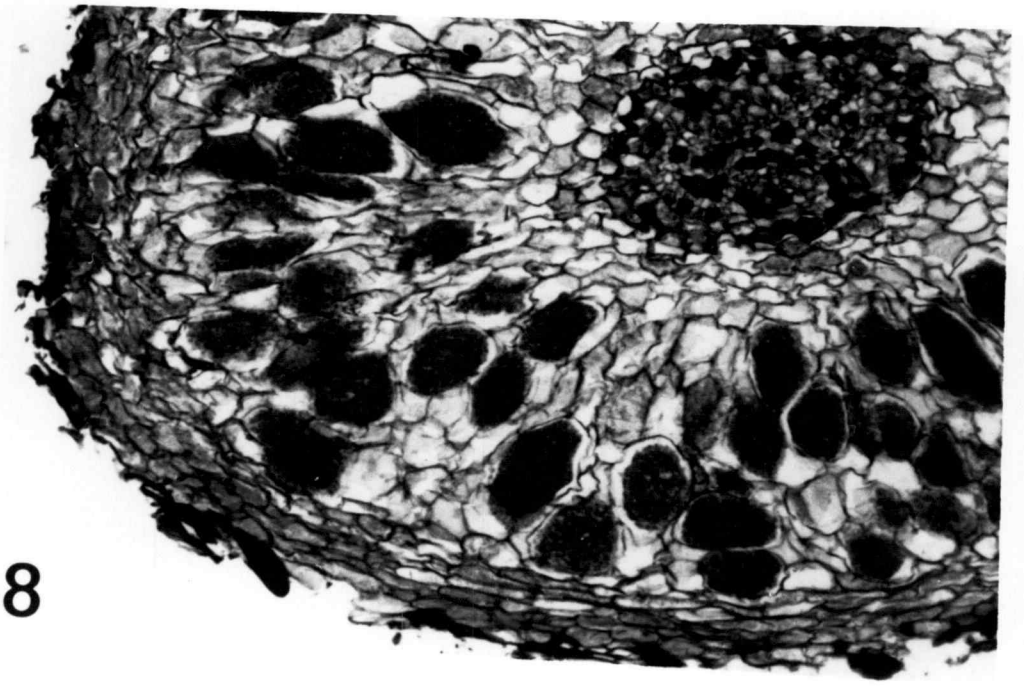

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DESCRIPTION OF STUDY SITES

Five different study sites were selected in the central Oregon pumice region. Soils in this region are a fairly recent mixture of aeolian pumice and glacial outwash. Dyrness and Youngberg (1966) have described the properties of some Oregon pumice soils. Total nitrogen levels are modest (.105 - .160%) under Purshia communities and carbon-nitrogen ratios are surprisingly high. Due to the abundance of small pores, these soils have favorable moisture retaining properties.

The high Cascades immediately to the west remove much of the moisture from air masses moving inland from the Pacific. As a result precipitation in the area is slight. Most precipitation occurs as snow. Summer rainfall accounts for only about 12% of the yearly total, and occurs mostly as light, scattered showers (Wells 1941) which are ineffective for most plant growth. Generally air temperatures show strong diurnal variation with cool nights and warm days in the summer. Summer frosts are not uncommon. Of the five study sites, three were located in the Pinus ponderosa zone, one in the Pinus contorta zone, and one in an open stand dominated by Artemesia tridentata.

Sites in the Pinus ponderosa zone lay within the Deschutes National Forests along a 24 km. transect near the route of US 20 from

Suttle Lake to the town of Sisters. The elevation in this area drops gently from 1030 m to 980 m from west to east. Robinson (1967) presents extrapolations of data from the US Army Corps of Engineers that indicate an annual precipitation range of from 1270 mm at Suttle Lake to 460 mm at Sisters. Precipitation values for the individual study sites have been estimated from Robinson's data and are presented in the study site descriptions that follow. Air and soil temperature were measured with recording thermographs at two stations within this area, air temperature at 60 cm. under an A-frame shelter, and soil temperature at a depth of 20 cm. (Table 1). According to West (1964), data collected at Sisters indicate an average January temperature of -0.5°C. and an average July temperature of 17.5°C. Soils are generally frozen in winter months and in 1975 remained so in spots until mid-April. Soil temperature as measured at 20 cm. was found to never exceed 17°C. in the summer during the course of this study.

Community descriptions of the Pinus ponderosa zone in central Oregon have been provided by Dyrness and Youngberg (1966). In order of increasing moisture, the communities are Pinus ponderosa/Purshia tridentata, Pinus ponderosa/Purshia tridentata/Festuca idahoensis, Pinus ponderosa/Purshia tridentata - Arctostaphylos patula, Pinus ponderosa/Ceanothus velutinus - Purshia tridentata, and Pinus ponderosa/Ceanothus velutinus.

Table 1. Temperature (°C.) at study sites 1, 3, and 4.

Date	Site	Air Temperature: Average daily high	Average daily low	High	Low	Soil Temperature: Average at 20 cm.
5/17 to	1	na*	na	37.0	- 6.5	13.0
6/2	3	23.0	-1.0	33.0	-13.0	10.0
	4	21.0	0.5	34.5	- 8.0	9.5
6/8 to	1	na	na	35.0	- 3.5	14.0
6/18	3	27.0	0.0	34.0	- 7.0	13.0
	4	25.0	3.0	33.0	0.0	9.5
6/19 to	1	19.0	3.0	24.5	- 3.5	13.0
7/2	3	20.0	3.0	24.5	- 3.0	12.0
	4	18.0	0.5	24.5	- 3.5	9.5
7/3 to	1	na	na	37.0	- 0.5	14.5
7/20	3	31.0	8.5	38.0	0.0	13.0
	4	30.0	8.5	37.0	0.5	13.0
7/21 to	1	31.5	5.0	37.0	3.0	16.0
7/30	3	32.0	5.0	39.5	1.5	15.0
	4	31.0	4.0	38.0	- 3.0	15.0
7/31 to	1	na	na	35.0	na	16.5
8/13	3	30.0	-0.5	34.5	- 5.0	14.5
	4	30.5	2.0	33.5	- 0.5	15.0
8/14 to	1	24.0	3.5	33.0	- 1.5	14.5
8/31	3	23.5	1.1	33.0	- 6.1	12.0
	4	21.5	3.0	31.5	- 3.0	13.5

* Values not available due to thermograph malfunction.

The fourth study site was chosen in the Pinus contorta zone. This tree is considered to be a topoedaphic climax species in wide areas of the south central Oregon pumice region (Berntsen 1967). The study area was located approximately 40 km. southwest of Bend. Annual precipitation is approximately 510 mm. (Soil Conservation Service 1964). Temperature measurements were made as described previously and are presented in Table 1. Ranging mostly from 1200 to 1525 m. in elevation, P. contorta occurs in many broad level depressions known as "lodgepole flats." Cold air drains off upper slopes and creates significantly lower night temperatures in these basins. As a result, P. contorta, which is highly cold tolerant, occupies the bottom of these depressions, while the dominant changes to P. ponderosa just a few meters higher on lava ridges and cinder cones (Berntsen 1967).

Youngberg and Dahms (1970) have described ten P. contorta communities in this area. The most widespread type is Pinus contorta/Purshia tridentata. Other important types include the Pinus contorta/Purshia tridentata/Ribes cereum and Pinus contorta/Purshia tridentata/Festuca idahoensis communities.

The fifth study area was located on the western edge of the high desert of eastern Oregon, near the base of Pine Mt. The vegetation is dominated by Artemesia tridentata. Stanton (1959) also worked in this area and indicated an annual precipitation of about 310 mm. No

temperature records are available; however, the open nature of the stand and the higher elevation suggest cooler night and warmer day temperatures than those recorded under the ponderosa pine. Anthesis of Purshia at this site was reached about 7-10 days later than in the ponderosa pine and about the same time as in the lodgepole pine stand. Only limited sampling was conducted on this site because Purshia was not well represented, and nodules were very difficult to locate.

Artemesia and Purshia occur together only in limited areas in the central Oregon pumice region. Purshia's presence on this particular site may be due to the presence of a layer of "popcorn" pumice which favors its taproot habit, and allows it to compete successfully with Artemesia.[1]

Vegetation analysis of all study sites was undertaken using the approach of Daubenmire and Daubenmire (1968). A 15 x 25 m. rectangle was marked off and divided into three "macroplots" of 5 x 25 m. Understory vegetation was sampled in 25 2 x 5 dm. "microplots" placed at 1 m. intervals along the inner side of the edge of the center macroplot. In contrast to Daubenmire's procedure, microplot readings were made along only one side of the macroplot. In each microplot placement, coverage for each species of shrub and herb was estimated and recorded. Overall percent coverage was determined by

[1] Dr. W. W. Chilcote, OSU Dept. of Botany, personal communication.

averaging values from all microplots. Frequency values indicate the percent of microplots in which a particular species occurred. The results of this sampling are presented in Table 2. Species present in the stand, but not encountered in the microplots are indicated by a "+" in the table.

The d.b.h. of all trees greater than 1 m. tall within the 15 x 25 m. plot was tallied by decimeter class. Trees less than 1 m. tall were sampled in two 1 m. wide strips along the inner sides of the central macroplot and the numbers adjusted to a 375 m^2 basis to correspond to the data collected for larger trees. Basal area (m^2/ha) was calculated using the basal area of the midpoint of each diameter class interval. These figures are presented in Table 3.

Brief descriptions of the individual study sites follow:

Site 1. Southwest 1/4 Sec 31 T13S R9E, 6 km. southeast of Suttle Lake. Elevation: 1025 m. precipitation: 1200 mm. See Figure 9.

Only light to moderate grazing, but heavy logging disturbance was evident. The occurrence of Abies grandis and even Pseudotsuga menziesii suggested the pine may be seral. Pine regeneration, as seen in Table 3, was probably the result of logging disturbance. Purshia's presence here may have also been due to this disturbance or to the greater degree to which the soil has been reworked by glacial outwash and the resulting poor soil moisture relations. The glacial

Table 2. Percent frequency and percent coverage for higher plant species at each study site.

Species	Study Site 1	2	3	4	5
	% frequency/% coverage				
Shrubs:					
Arctostaphylos patula	+*	4/2.5			
Castanopsis chrysophylla	+				
Ceanothus velutinus	8/.7				
Chrysothamnus viscidiflorus	4/.1	+	28/.7		24/3.5
Purshia tridentata	60/19.1	60/25.2	44/15.7	76/20.4	4/1.5
Ribes cereum			+	+	+
Chrysothamnus nauseosus					+
Artemesia tridentata					32/11.8
Graminoids:					
Bromus sp.	48/1.7	4/.1		12/.3	
Carex sp.	44/1.1	4/.1	24/.6	20/.5	12/.3
Festuca idahoensis	44/5.5	76/10.5	96/25.3	20/.5	32/4.7
Stipa sp.	+	12/.3	+	+	+
Sitanion sp.	+	+	+	+	+
Agropyron sp.			+		44/1.1
Bromus tectorum					4/.1
Forbs:					
Agoseris glauca	24/.6				
Balsamorhiza sagittata	+				
Campanula sp.	16/.9				
Castilleja sp.	+				
Chimaphila umbellata	+				
Cirsium sp.	+				
Epilobium angustifolium	20/.5				
Hieracium cynoglossoides	36/.9				
Horkelia fusca	+				
Pteridium aquilinum	28/3.6				
Lathyrus lanszwertii	24/1.1	68/3.7			
Lomatium triternatum	16/.4		8/.2		
Fragaria virginiana	64/1.6			4/.1	
Phacelia heterophylla	+				+
Viola purpurea	+	+		28/.7	
Achillea millefolium	40/1.5		36/.9		+
Gayophytum humile	+	+	+		
Lupinus caudatus	8/.2		20/1.5	8/.2	4/.1
Collinsia parviflora		24/.6	36/.9		
Madia minima		20/.5	12/.3		
Microsteris gracilis		12/.3	4/.1		40/1.0
Montia spathula		4/.1			

Table 2. Continued.

Species	Study Site 1	2	3	4	5
	% frequency/% coverage				
Penstemon humilis		4/.6	+		
Pterospora andromedea		+		+	
Collomia linearis			8/.2		80/2.0
Eriogonum umbellatum			4/.1		+
Eriophyllum lanatum			4/.1		4/.1
Lithospermum ruderale			+		
Gilia aggregata			+		
Frittilaria atropurpurea				+	
Spraguea umbellata				+	
Agoseris heterophylla					20/.5
Arabis sp.					+
Senecio integerrimus					+

* + Indicates a species was present on the site, but not encountered in any microplots.

Table 3. Tree population analyses at study sites.

Species	d.b.h. in dm. 0-.5	.5-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	Basal area m^2/ha
	no. in 375 m^2									
Site 1										
Pinus ponderosa	219	18	3			1		1		19.5
Abies grandis	11		3	1						2.93
also present:										
Pseudotsuga menziesii										
Site 2										
Pinus ponderosa	8		2		3	2	1			23.55
also present:										
Juniperus occidentalis										
Site 3										
Pinus ponderosa	41	16	2				1			9.68
Juniperus occidentalis	11	3	1							.97
Site 4										
Pinus contorta	83	23	11	4	3	1				20.97

outwash might account for the scarcity of Ceanothus velutinus, which would be the normal dominant shrub in ponderosa pine forests with this high amount of precipitation.

Site 2. Northeast 1/4 Sec 26 T14S R9E, 1 km. north of Cold Springs Campground. Elevation: 1010 m. precipitation: 690 mm. See Figure 10.

Grazing and logging disturbance were minimal. Purshia regeneration was excellent, and nodules were abundant and easily located. This community belongs in the Pinus ponderosa/Purshia tridentata/Festuca idahoensis association of Dyrness and Youngberg (1966), which is widely represented in this general area.

Site 3. Northwest 1/4 Sec 17 T15S R10E, 2 km. south of Sisters. Elevation: 1005 m. precipitation: 510 mm. See Figure 11.

Grazing pressure was moderate. Large ponderosa pine were logged a number of years ago. Pine regeneration was prolific but clumped, with open areas and fairly dense stands interspersed. Temperature extremes (both high and low) on this site frequently exceeded the values from other sites (Table 1), due to the open nature of the canopy. This community is intermediate between the Pinus ponderosa/Purshia tridentata association and the drier Juniperus occidentalis zone.

Site 4. Southeast 1/4 Sec 19 T20S R10E, 1 km. northwest of Pistol Butte. Elevation: 1315 m. precipitation: 510 mm. See

Figures 12 and 13.

Grazing and logging disturbance at this site were minimal. The presence of Ribes and Fragaria placed this community in the Pinus contorta/Purshia tridentata/Ribes cereum type of Youngberg and Dahms (1970).

Site 5. Northeast 1/4 Sec 29 T20S R16E, 11 km. southeast of Millican. Elevation: 1450 m. precipitation: 310 mm. See Figure 14.

Livestock utilization of this area was very heavy. This appeared to be leading to the decrease of Purshia, since no young seedlings were present. Artemesia regeneration was good. Established Purshia plants showed signs of heavy graving. Stanton (1959) placed this site in the Artemesia tridentata/Festuca idahoensis association of Eckert (1957).

Figure 9. Study site 1.

Figure 10. Study site 2. The range pole is marked in 1 ft increments.


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Figure 11. Study site 3.

Figure 12. Study site 4.


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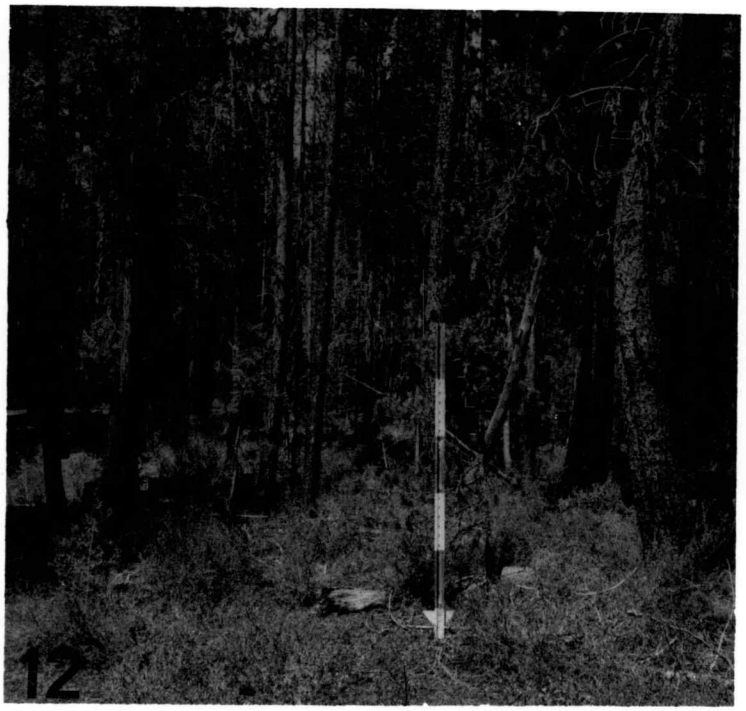
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Figure 13. View from Pistol Butte, near study site 4, showing the distribution of Pinus ponderosa on elevated sites and Pinus contorta in the depressions. Purshia occurs under both species.

Figure 14. Study site 5.

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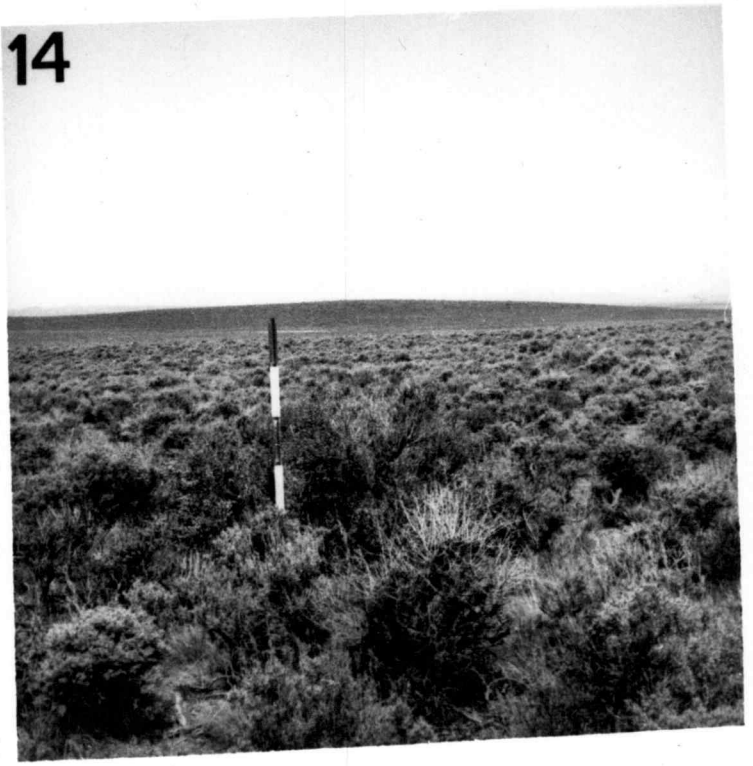

ACETYLENE REDUCTION

General Materials and Methods

The discovery by Schollhorn and Burris (1966) and Dilworth (1966) that acetylene is reduced to ethylene by nitrogenase has greatly facilitated research on nitrogen fixation. This assay procedure is becoming increasingly more popular and is especially applicable to field work. In this study, acetylene reduction was used to measure nitrogenase activity as related to temperature, plant moisture stress, and time after excision of nodules from the roots. Diurnal and seasonal variation were also examined.

Due to the small size of Purshia nodules, small (8.8 ml.) incubation vials were used. Vials containing approximately 8 - 60 mg. fresh weight of nodules were sealed with a rubber serum stopper, .88 ml. of the atmosphere withdrawn with a syringe, and an equal amount of acetylene injected to give a final concentration of 10%. To prevent contamination from gas absorbed by rubber stoppers, these were discarded after only one use. Acetylene was generated immediately before use by reacting CaC_2 with water.

Incubation times varied generally from 40 - 65 min., but all acetylene reduction values were converted to a 60 min. basis on a linear scale. Incubation temperature was controlled in the labratory by submerging the vials in a water bath and in the field by burying the

vials in the soil at a depth of 20 cm. The exact conditions under which acetylene reduction was measured in different experiments are described in later sections.

Gas samples from laboratory work were analyzed immediately on the gas chromatograph. Gas samples from field work were collected in evacuated blood sample tubes ("vacutainers") as described by Schell and Alexander (1970). The entire gas mixture in the incubation vials was forced into the vacutainer tube by injecting water through the vial serum stopper. The vacutainer tubes were then brought back to the laboratory where their contents were analyzed within a week. Water was injected into the vacutainers immediately before removing samples for gas chromatography so that pressure inside the vacutainers was increased to above 1 atm. When samples were then withdrawn for injection into the gas chromatograph, this slightly increased pressure resulted in a portion of the gas mixture escaping from the syringe. The portion remaining in the syringe was uniformly an undiluted sample at room atmospheric pressure. The only pressure calculations that were then required were those resulting from elevation differences between the study sites and the laboratory.

The use of water to displace gas samples has also been reported by Steyn and Delwiche (1970). Ethylene is essentially insoluble in water. Control samples with known amounts of ethylene were treated

as described for test samples to verify that no ethylene was lost in solution in the water. On a short term basis, ethylene amounts of the controls remained unchanged. Storage of the vacutainers for more than a few hours after injection of the water sometimes resulted in a decrease in ethylene content but it is uncertain whether this decline was due to some ethylene dissolving in the water or leaking from the serum stopper, which by then had been pierced 5 - 6 times by syringe needles.

Other controls with known ethylene concentrations revealed that the vacuum inside the vacutainers was incomplete. A slight correction factor was determined and applied to all samples. Unused vacutainers contained small traces of ethylene. If pure 10% acetylene in air was introduced into the tubes, this level of background ethylene approximately tripled, perhaps due to the release of bound ethylene from the rubber serum stopper. The amount of endogenous ethylene was still small and became significant only when very low nodule activity was being measured (less than 0.1 μmoles/g·hr). In such cases an average value of background ethylene was subtracted from the sample value.

Gas samples were analyzed on a Varian Aerograph model 600-D gas chromatograph equipped with a hydrogen flame ionization detector and a Honeywell recorder. Chromatograms were run at 50^{o}C. in a 6 ft. x 1/8 in. column packed with Poropak R. Nitrogen was used as

the carrier gas at a flow rate of 25 ml/min. Sample injection size was 0.5 ml.

All nodule weights were determined on a fresh weight basis. Nodules from field samples were placed in formalin-acetic acid-alcohol (FAA) until they could be weighed. In weighing only active nodule material was considered. All senescent nodule branches and woody tissue proximal to active branches were removed.

Time Course

Materials and Methods

A time course study was carried out on a set of greenhouse plants that had been treated as described for the plants used in the temperature studies (see next section). Nodules were excised at approximately 9:00 am PST and incubated in a vial with acetylene at 22°C. Gas samples were withdrawn for analysis at time intervals of 1 - 4 hours up to 24 hours. At each sampling time two .55 ml. samples were removed from the vial and 1.1 ml. of gas mixture containing 10% acetylene re-injected to maintain normal atmospheric pressure. Values for ethylene production were calculated to include the amount of ethylene present in the vial at the sampling time as well as the total amount of ethylene that had been removed in all previous samples.

Results and Discussion

The results of the time course study are shown in Figure 15. Acetylene reduction was linear through the first 5 hours, and then declined slowly, essentially ceasing after 19 hours, presumably due to the exhaustion of carbohydrate reserves. The average rate during the first 5 hours was 3.5 μmoles C_2H_4/g·hr. Varying incubation times were used throughout this study and the initial linearity of acetylene reduction allowed for the easy conversion of all results to a standard 60 min. basis.

Similar time course studies have been reported for Myrica by Morris et al. (1974). Acetylene reduction was linear up to 8 hours, the longest time tested. In contrast, activity of excised Alnus nodules has been found to decline rapidly after only 1 - 2 hours (Dalton and Naylor 1975).

Effects of Temperature

Materials and Methods

To examine the effect of incubation temperature on acetylene reduction, approximately 30 nodulated plants, 12 to 40 cm. tall, were removed from study site 2 in February. The plants were transplanted into 20 cm. plastic pots containing the pumice soil from this site and placed in the greenhouse. All plants were watered twice weekly and

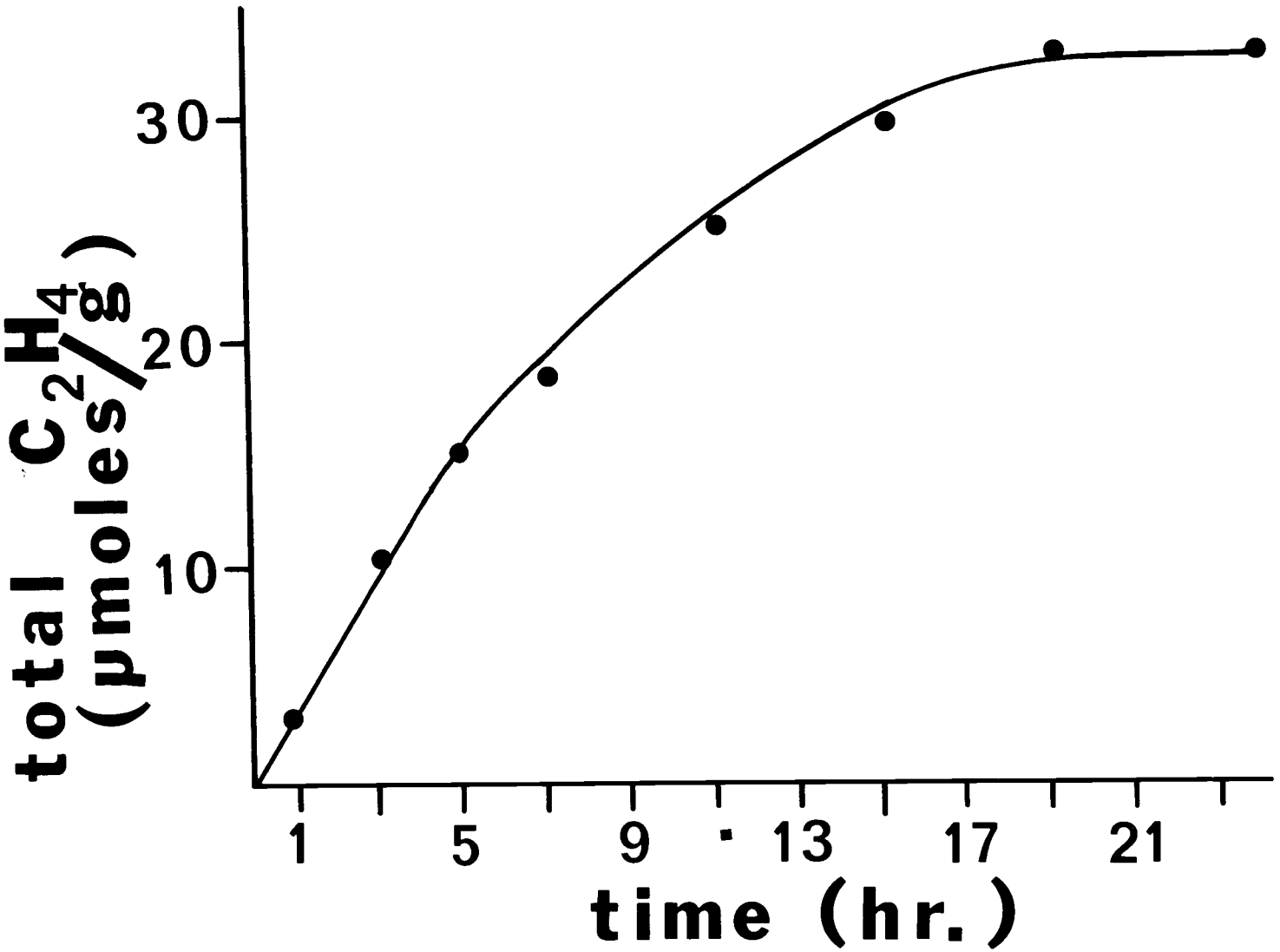

Figure 15. Time course of acetylene reduction by excised Purshia nodules. Mean of 3 trials.

allowed to grow 2 - 3 months before acetylene reduction was measured. To determine the response of nodule activity to different temperatures, unsealed vials containing nodules were partially submerged in a water bath at 20°C for 10 min. to equilibrate. The vials were then sealed and acetylene introduced as previously described. After 30 min., gas samples were withdrawn for gas chromatography. Serum stoppers were removed and the vials containing the same nodules placed in another water bath at one of the test temperatures: 5, 10, 15, 20, 25, 30, 35, or 40°C. Vials and nodules were allowed to equilibrate to the new temperature for 10 min. with frequent flushing with air to remove traces of the previous gas mixture. The vials were again sealed, acetylene injected and after 30 min. ethylene production measured. Ethylene production was then expressed on a basis relative to that during the initial incubation at 20°C. Recovery was examined by returning the vials and nodules to 20°C., equilibrating, flushing, and measuring acetylene reduction as before.

A brief comparison of relative acetylene reduction rates of field plants was undertaken by incubating 6 samples each of freshly collected nodules in water baths at 15° and 20°C. and collecting gas samples in vacutainers.

Results and Discussion

The results of comparative acetylene reduction measurements

made on the same nodules at two different temperatures are presented in Figure 16. This procedure was adopted because of the limited number of greenhouse plants available and the high variability in acetylene reduction activity between nodules from different plants.

The rate for nodules held at 20°C. for all three stages was found to actually increase after each 30 min. period. The rate during the second stage (the test stage) at 20°C. was 119% of the initial rate. The rate during the third (recovery) stage increased to 151%. Nodules treated identically in the first stage at 20°C. and then incubated in the second stage without the addition of acetylene showed no release of ethylene. These increases in ethylene production are puzzling and may be due to the release of bound ethylene in the presence of acetylene or a time delay in temperature equilibration.

The results indicate a sharp maximum of acetylene reduction around 20°C. The rate at 15°C. was only 12.1% of the initial rate of 20°C. Data shown in Table 4 indicate that a similar drop is evident in field grown samples. Reversing the treatments for greenhouse plants (15°C. first followed by 20°C.) also resulted in a much higher rate at 20°C. This sudden drop cannot be explained by enzyme kinetics. The apparent Q_{10} from 10° to 20°C. is too large - greater than 8.2. Cold injury may account for this drastic decrease. Although 15°C. is in the normal range of soil temperature, the sudden 5°C. shift may have some harmful effect. Perhaps a more plausible

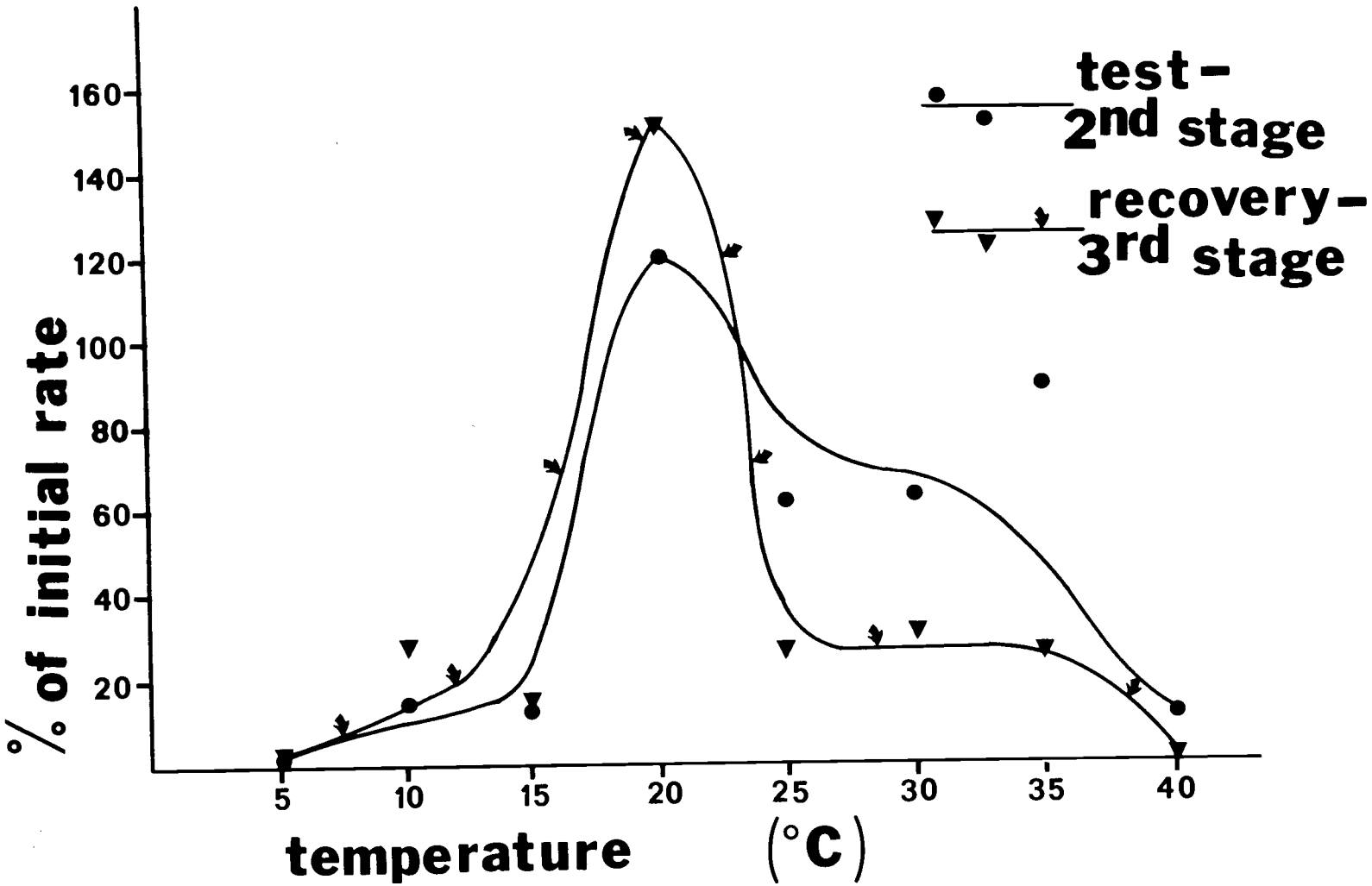

Figure 16. Response of acetylene reduction rates to temperature treatments. Arrows distinguish the recovery response line. See text for explanation. Mean of 3 trials.

explanation involves reduced gas diffusion in and out of the nodule brought about by cooler temperatures and condensation of water vapor on the nodule surface. Hardy et al. (1968) have reported that a slight film of water around nodules greatly reduces acetylene reduction rates.

The effects of temperature on Ceanothus nodule activity have been examined by Webster (1968) using nitrogen 15. The optimum temperature was found to be around 23°C. The rate at 15°C. was 37.5% of that at 23°C. Unfortunately, no assays were made between 15° and 23°C. Akkermans (1971) reported optimum temperatures for acetylene reduction by Alnus nodules near 20°C. Wheeler (1971) reported a value near 25°C. also for Alnus. The decline in rates at 15°C. was much less severe in both cases than that observed here for Purshia.

Table 4. The effects of incubation temperature on acetylene reduction by nodules from plants at study site 2 on June 21.

Temperature	Acetylene reduction (μmoles/g · hr)*		
	mean	high	low
20°C.	1.32	3.17	0.27
15°C.	0.04	0.09	0.01

* 6 replicates at each temperature

It seems likely that the rates measured at 15°C. are artificially low. Soil temperature at the study sites was generally around 15°C. throughout the summer, but substantial acetylene reduction was measured in vials buried in the soil at this temperature. Nevertheless, soil temperature appears to be below the optimum for nitrogen fixation. Cool soil temperature may be responsible for the delay in spring until late May before nodule activity begins (see later sections). This is especially true for study site 4 which is located in a cold air drainage basin. Soil temperatures were 2 to 6°C. cooler in the spring and early summer than those at the other study sites (Table 1). Nodule activity began later and was generally lower throughout the summer (Figure 24).

Effects of Plant Moisture Stress

Materials and Methods

The effects of plant moisture stress on acetylene reduction were examined on both greenhouse plants and field plants. Approximately 50 nodulated plants were removed from study site 2 and treated as described for the plants used in the temperature experiments. All plants were watered twice a week for 3 weeks. After this time new leaves were fully emerged and most plants were growing rapidly. The plants were then divided into two groups with approximately equal size

distributions. One group was watered as before but the other received no water. After 1, 6, and 14 days acetylene reduction measurements were made at 22°C. on 3 to 12 plants in both groups. Water stress was determined in each case with a pressure chamber (Scholander et al. 1965). To examine recovery from water stress, the xylem pressure potential of 4 additional plants from the unwatered group was measured on day 14 but nodule activity was not examined. These plants were then returned to the greenhouse and watered regularly. Their xylem pressure potential and acetylene reduction activity were measured after an additional 12 days.

Field moisture stress measurements were made throughout the growing season on most plants sampled for acetylene reduction. These readings were taken mostly from 9:00 am to 2:00 pm PST, but predawn measurements also were made at study site 2. All pressure chamber readings were taken on plants that had been uprooted to sample nodules, immediately after the nodules were removed.

On July 30, approximately 60 plants on study site 2 were selected for irrigation. These plants were 5 - 12 years old and in groups of 5 - 10 individuals resulting from old seed caches. A trench was dug around the base of each group of plants and filled with water 5 - 6 times allowing time in between for each dose to soak in. Each group received a total of about 8 liters. Predawn and midday acetylene reduction and water stress measurements were made on these plants 2

days later and compared to nearby non-watered plants. Similar measurements were made on August 13.

Results and Discussion

Greenhouse plants under low to moderate water stress had highly variable rates of acetylene reduction that were not correlated well with water stress (Figure 17). Extremes ranged from 0 to 10.5 μmoles ethylene/g·hr with the average being 3.69 for all plants above -20 bars, 2.25 for plants between -20 and -25 bars, and 0.11 for all plants below -25 bars. Recovery from this low activity of highly stressed plants was good after 12 days with regular watering, provided the initial stress was not too severe (Table 5). One plant with a very low xylem pressure potential (below -68 bars) lost many of its leaves and was the only plant in which nitrogenase activity did not recover, although it might have eventually.

Table 5. Recovery of nodule activity by greenhouse plants subjected to water stress and then provided with water for 12 days.

Xylem pressure potential (-bars)		Acetylene reduction (μmoles/g·hr)
initial	final	
45.5	10.9	2.96
44.4	11.3	3.05
57.6	15.4	2.29
>68	19.7	0

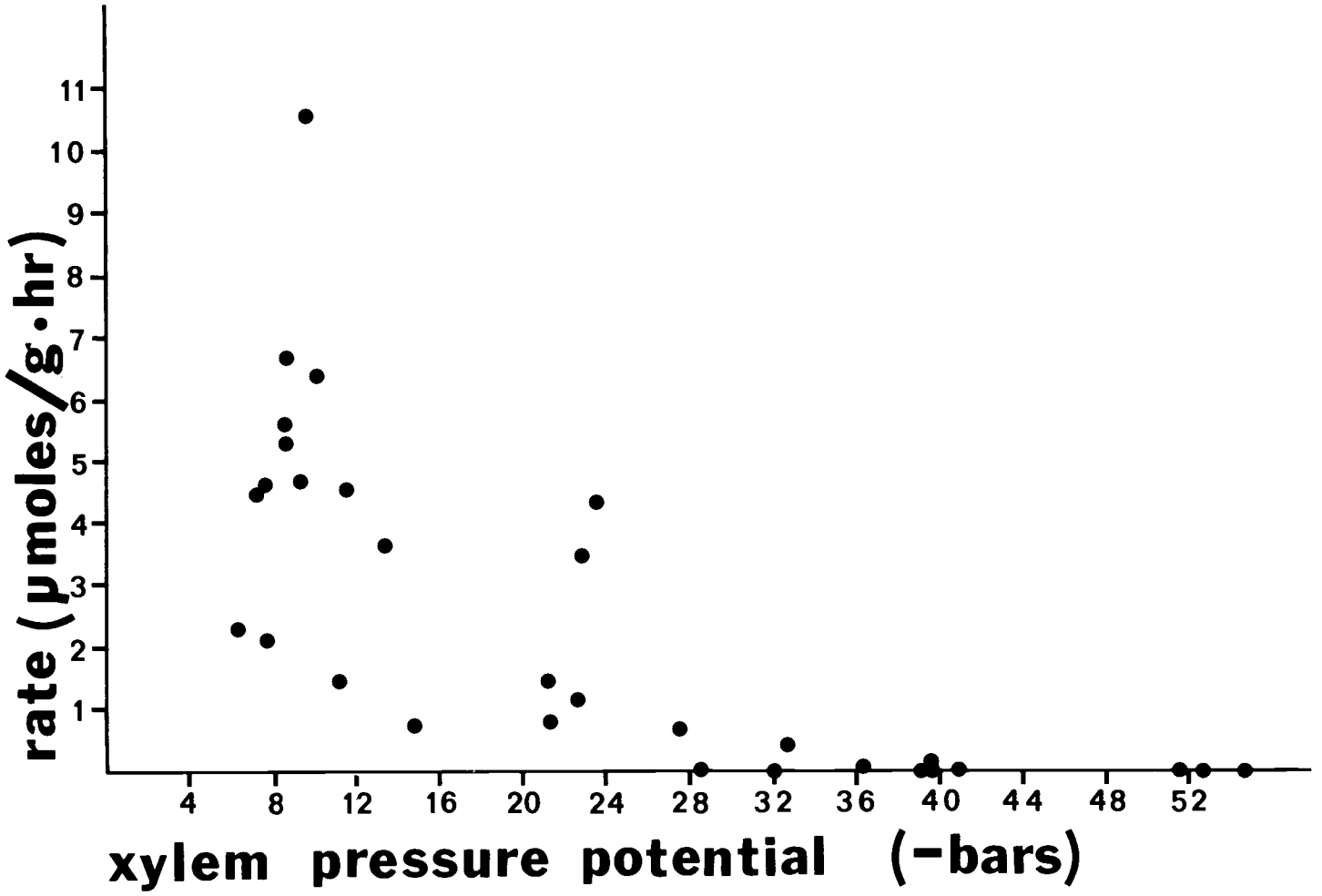

Figure 17. Acetylene reduction by nodules from plants at different moisture stress levels. Field plants were transplanted into the greenhouse for 3-6 weeks.

Midday field moisture measurements from all sites and predawn readings from site 2 are presented in Table 6. Water stress corresponded somewhat with differences in annual precipitation between the sites. Moisture stress variation due to different sampling times during the day undoubtedly accounts for some irregularities, but the general drying trend can be seen throughout the growing season. The drop in acetylene reduction rates that was observed in late July (see next section) corresponds roughly with the water stress in the -25 bar range which was observed to reduce nodule activity in greenhouse plants. The continuation of some nodule activity into September may have been due to the unusually wet summer in 1975. August precipitation probably exceeded 2.0 cm. on all sites except possibly no. 5.

Irrigated field plants showed a significant increase in both midday and predawn acetylene reduction only 2 days after watering (Table 7). At this time, predawn xylem pressure potentials were slightly higher, but midday readings were actually slightly lower on the watered plants. After an additional 2 weeks, the same water stress patterns were repeated with higher predawn and lower midday readings on the irrigated plants. Nodule activity of watered plants had declined sharply after this 2 week period. Predawn acetylene reduction rates were very low, and midday rates were even lower than those of the unwatered plants. The lower midday xylem pressure potentials and eventually the lower acetylene reduction rates of the watered

Table 6. Xylem pressure potential (- bars) at each study site. Data for sites 1, 3, 4 and 5 are for midday (900-1400 PST). Means of 6-8 samples.

Date	Study Site 1	2-predawn	2-midday (- bars)	3	4	5
5/17-18	10.9	nd*	7.2	8.9	6.7	12.6
6/7-8	13.5	nd	12.8	9.7	11.3	15.4
6/18-19	12.5	nd	12.5	8.4	rain	nd
7/1-2	14.5	6.6	16.0	13.1	5.9	15.9
7/20-21	13.6	6.0	23.7	20.0	24.8	nd
7/30-31	13.7	11.3	26.5	24.6	22.4	22.2
8/13-14	17.1	15.2	24.1	26.0	19.4	nd
9/4-5	20.7	14.4	23.7	24.2	19.5	nd

*Not determined

Table 7. Comparison of water stress and nodule activity of irrigated and non-irrigated plants in the field. Water was applied on only one day (7/30) using the method described in the text.

Date	Plant treatment	Xylem pressure potential (- bars)		Acetylene reduction (μmoles/g·hr)					
		predawn	midday	predawn			midday		
				high	low	average	high	low	average
8/1	irrigated	9.2	29.1	0.40	0.07	0.22	1.59	0.28	0.67
8/1	not irrigated	11.3	26.5	0.17	0	0.05	0.67	0	0.28
8/13	irrigated	13.4	30.3	0.11	0	0.03	0.32	0.07	0.22
8/13	not irrigated	15.4	24.1	0.14	0	0.04	1.03	0.18	0.44

plants were unexpected. The addition of a large supply of water may have interrupted the normal processes of water conservation, such as stomatal closure, to the point that more severe water stress conditions resulted. The plants appeared to suffer uncontrolled water loss during the day with good recovery at night due to the extra soil moisture. At first, perhaps there was enough water to compensate for the loss, but eventually the high water stress resulted in a decrease in acetylene reduction rates below those of unwatered plants, in which normal water conservation had not been disrupted.

Caution must be used in interpreting the low xylem pressure potentials reported here. Kaufmann (1968) reported considerable discrepancies between pressure chamber and psychrometer technics for some plants. Pressure chamber readings as much as 16 bars lower were obtained for 2 species of oaks. This difference was interpreted as resulting from water moving into air spaces in the large vessel members during pressure measurements. Like oaks, Purshia has a ring-porous xylem structure with fairly large vessels, and it is possible that this caused pressure potential readings to be artificially low. Another problem arises from the fact that pressure chamber readings were taken on plants that had been uprooted to remove nodules. Generally about 2 min. elapsed before pressure measurements were made, during which time the roots were exposed. A comparison of uprooted and undisturbed plants revealed about a 2 to 3 bar difference resulting

from root damage and drying through the exposed roots. Despite these complications the readings are valuable when considered on a relative basis.

The reduced nitrogen-fixing activity of nodules brought about by water stress is probably related to reduced carbohydrate supply. Wheeler (1971) has shown that the reserve of carbohydrate, primarily sucrose, in the nodules drops rapidly in Alnus plants placed in the dark. This drop in sucrose levels corresponds to a sharp decrease in acetylene reduction rates. The same effects might be achieved by drought induced stomatal closure which would halt photosynthesis and the flow of carbohydrates to the nodules.

Water stress leads to structural changes within legume nodules, and if severe enough, will result in the nodule being shed (Sprent 1972). Sprent (1971) and Minchin and Pate (1975) have reported reduced acetylene reduction rates resulting from water stress. Pankhurst and Sprent (1975) attributed these lower rates to reduced respiration in the nodule due to structural changes that reduce O_2 diffusion. Rates in moderately stressed nodules could be completely restored by increasing the pO_2, but severely stressed nodules recovered only partially. There are considerable structural differences between legume and non-legume nodules but it is possible that water stress may also reduce O_2 diffusion in non-legume nodules as well.

The critical region appears to be the peripheral uninfected cortex (Sprent 1972), which is similar in both types of nodules.

Seasonal and Diurnal Variation

Materials and Methods

The variation in acetylene reduction rates throughout a growing season was examined by making repeated trips to study sites at 2 - 4 weeks intervals from April to September. During each visit, acetylene reduction trials were conducted on 6 - 8 plants. Mostly small plants, 10 - 30 cm. tall, were selected. Ring counts were not made, but most plants were probably 3 - 12 years old. No more than 2 plants were used from the group resulting from any one seed cache. Air temperature at a height of 60 cm. and soil temperature at a depth of 20 cm. were recorded. Acetylene vials were buried in the soil at a depth of 20 cm. as a temperature control. Initially acetylene reduction measurements were taken any time from 8:00 to 6:00 pm PST, but after mid-June, when the extent of diurnal variation was discovered, all measurements were confined to between 9:00 am and 2:00 pm.

Diurnal variation in acetylene reduction rates was determined by comparing rates on nodules removed at different times of the day. Usually this involved a series of measurements made shortly before dawn (3:00 - 4:00 am) and another series at midday (11:00 am - 2:00 pm). In one case additional measurements were taken at 7:30 am and

7:30 pm. To observe seasonal changes in diurnal patterns, predawn and midday rates were determined at intervals from mid-June to mid-August. All plants used in this part of the study were from study site 2 because of the abundance of easily located nodules there.

Results and Discussion

Air and soil temperatures recorded at each sampling time are presented in Table 8. Winter nodules sampled from early February to mid-April failed to produce any detectable amounts of ethylene. During this period soil incubation temperatures generally ranged from 0 to 4°C. Nodules placed in vials and incubated at room temperature for up to 48 hours also remained totally inactive.

Seasonal patterns of midday acetylene reduction rates at all sites are presented in Figures 18-22. The first signs of nodule activity appeared on May 18 when small amounts of ethylene where produced by some nodules from sites 2, 3, and 5. At this time leaves were fully emerged, but anthesis was not reached for another 7 - 10 days. By June 7, nodules from site 1 showed moderate activity, but those from site 4 produced only traces of ethylene.

Throughout June some nodules from each of the 5 sites retained very low or no activity, but average values increased over those in May. Generally, the individual nodules with the highest activity were also measured in June, making this month the period of greatest

Table 8. Temperature (°C.) at acetylene reduction sampling times.

Date	Site	Air	Soil	Date	Site	Air	Soil
3/1	2	9.0	1.0	8/13-14	1	24.5	17.0
	4	8.0	0.0		2	9.0	16.0
4/19	2	13.0	5.0		3	27.0	15.0
5/17-18	1	20.0	8.0		4	22.0	15.0
	2	16.0	11.5	9/4-5	1	20.0	12.0
	3	22.0	12.0		2	20.5	13.0
	4	12.0	6.5		3	22.0	9.5
	5	11.0	11.0		4	20.0	11.0
6/7-8	1	23.5	15.0				
	2	18.5	15.5				
	3	17.0	12.0				
	4	14.0	10.0				
	5	22.0	16.0				
6/18-19	1	17.0	14.0				
	2	20.0	15.0				
	3	14.5	13.5				
	4	9.5	9.5				
7/1-2	1	19.0	13.0				
	2	21.5	14.0				
	3	18.5	11.0				
	4	5.0	10.0				
	5	23.5	13.0				
7/19-20	1	23.5	16.0				
	2	31.0	16.5				
	3	23.5	15.0				
	4	18.5	15.5				
7/30-31	1	16.0	16.5				
	2	18.0	16.5				
	3	16.0	14.0				
	4	22.0	15.0				
	5	23.5	13.0				

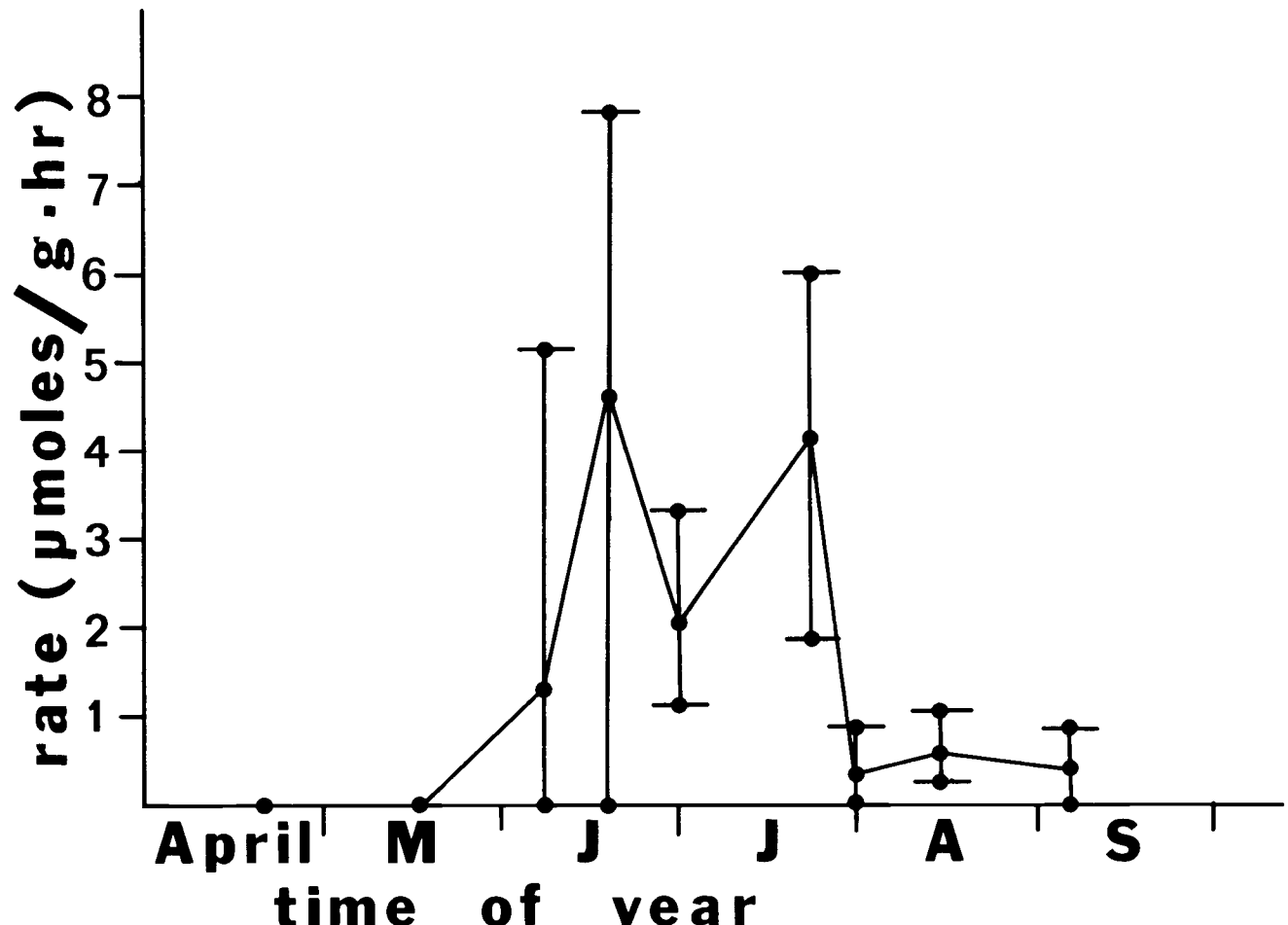

Figure 18. Seasonal pattern of midday acetylene reduction rates at study site 1. Vertical lines indicate the extremes observed at each determination. Means of nodules from 6-8 plants.

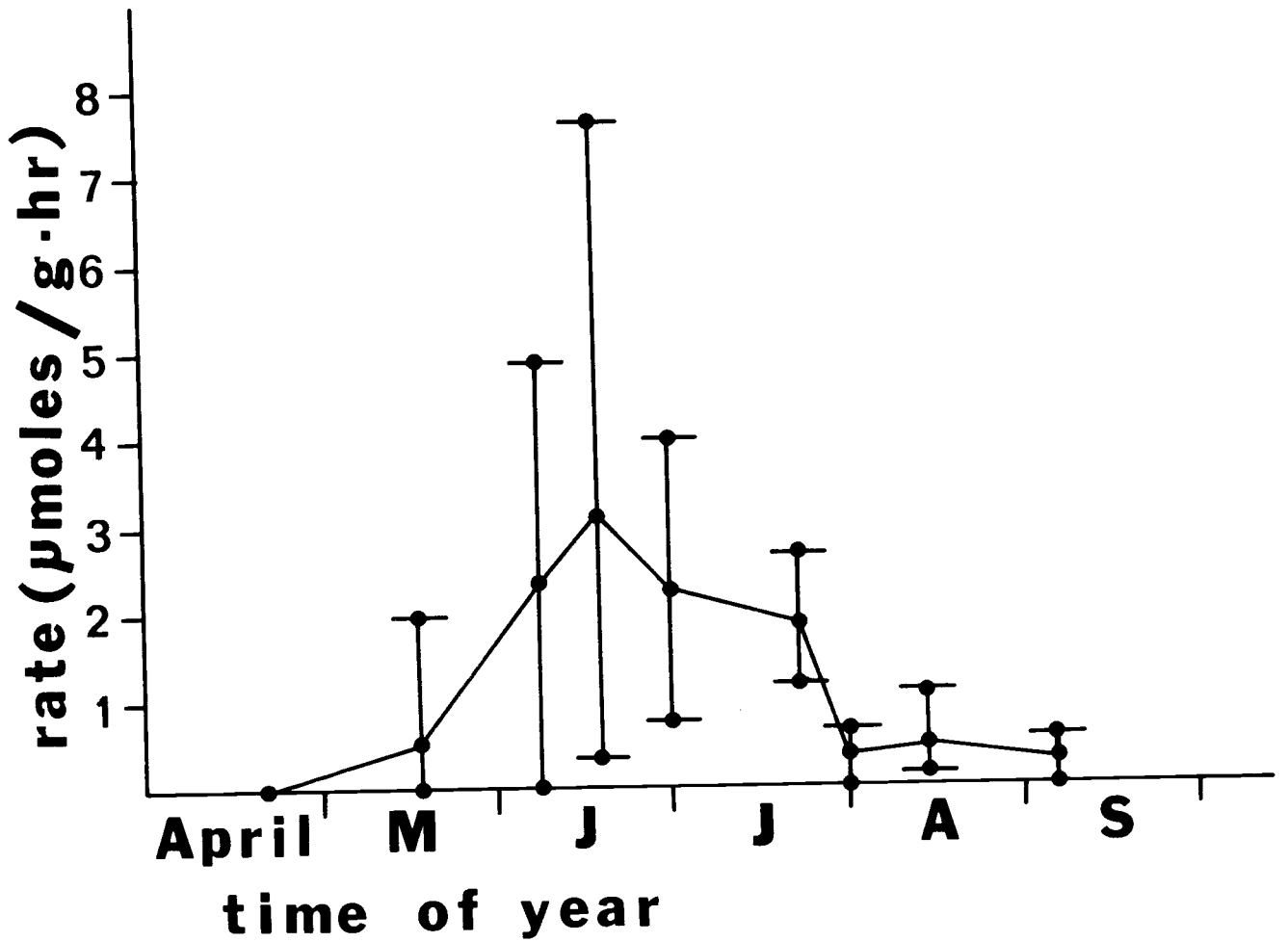

Figure 19. Seasonal patterns of midday acetylene reduction rates at study site 2. Vertical lines indicate the extremes observed at each determination. Means of nodules from 6-8 plants.

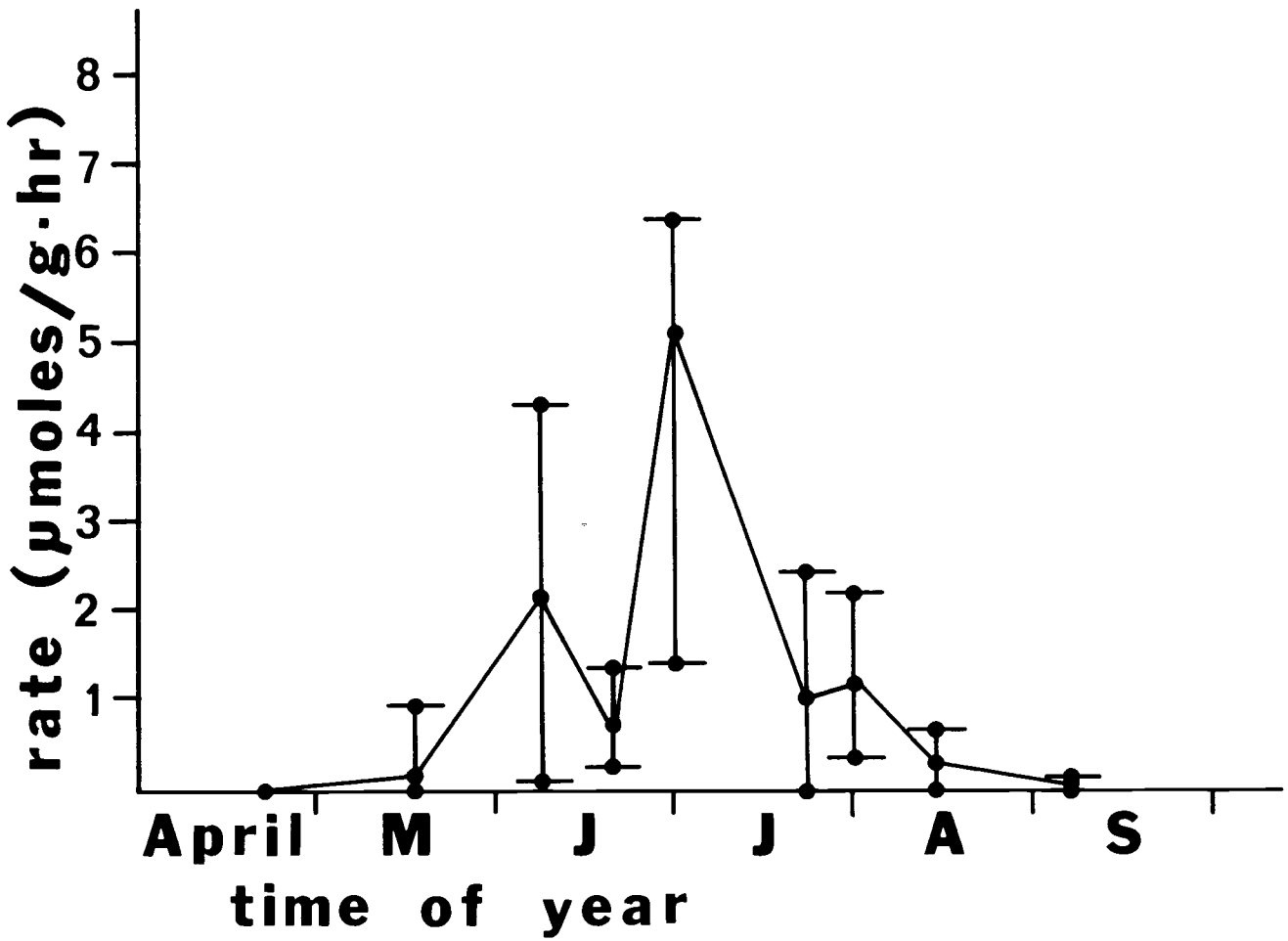

Figure 20. Seasonal patterns of midday acetylene reduction rates at study site 3. Vertical lines indicate the extremes observed at each determination. Means of nodules from 6-8 plants.

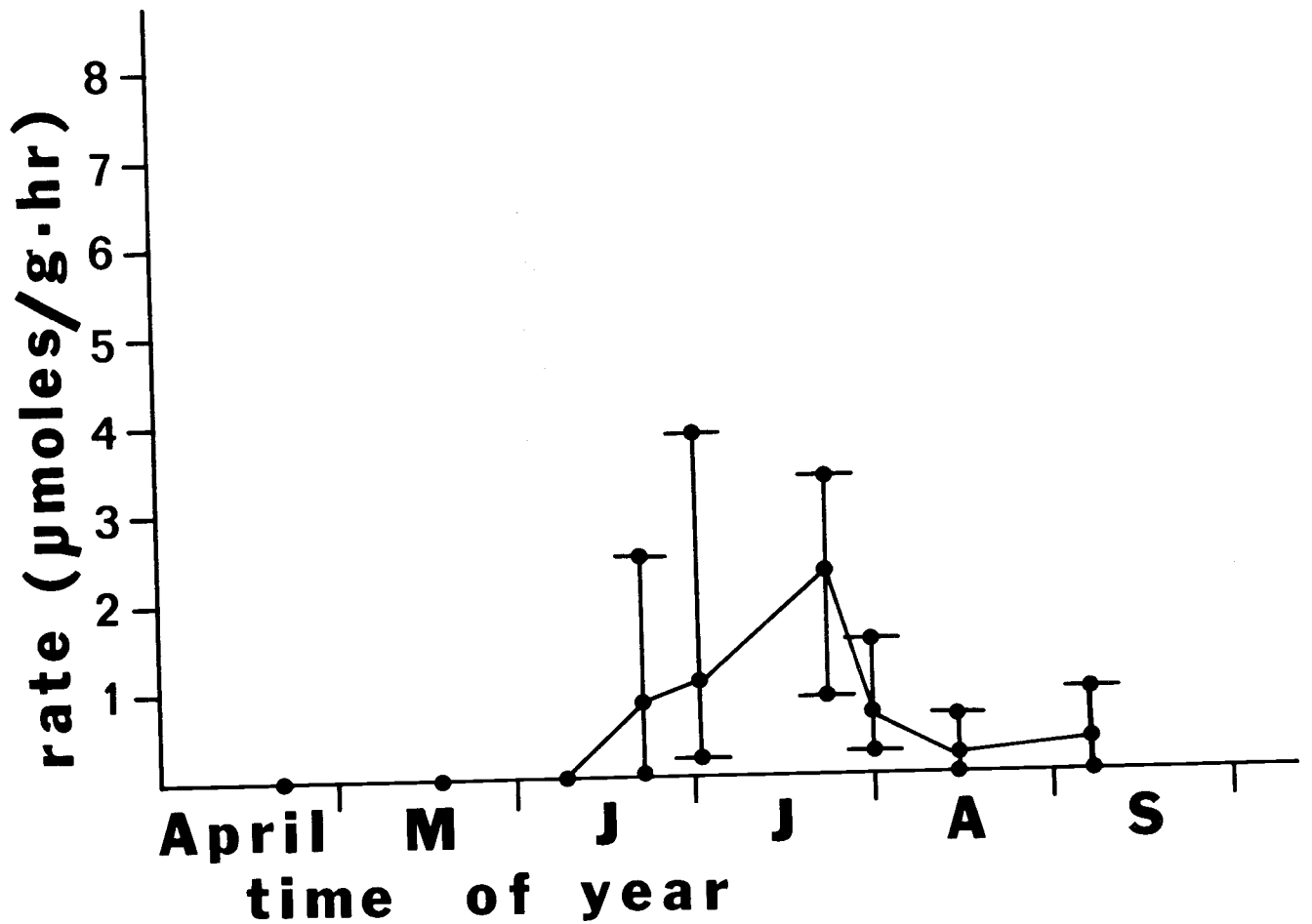

Figure 21. Seasonal patterns of midday acetylene reduction rates at study site 4. Vertical lines indicate the extremes observed at each determination. Means of nodules from 6-8 plants.

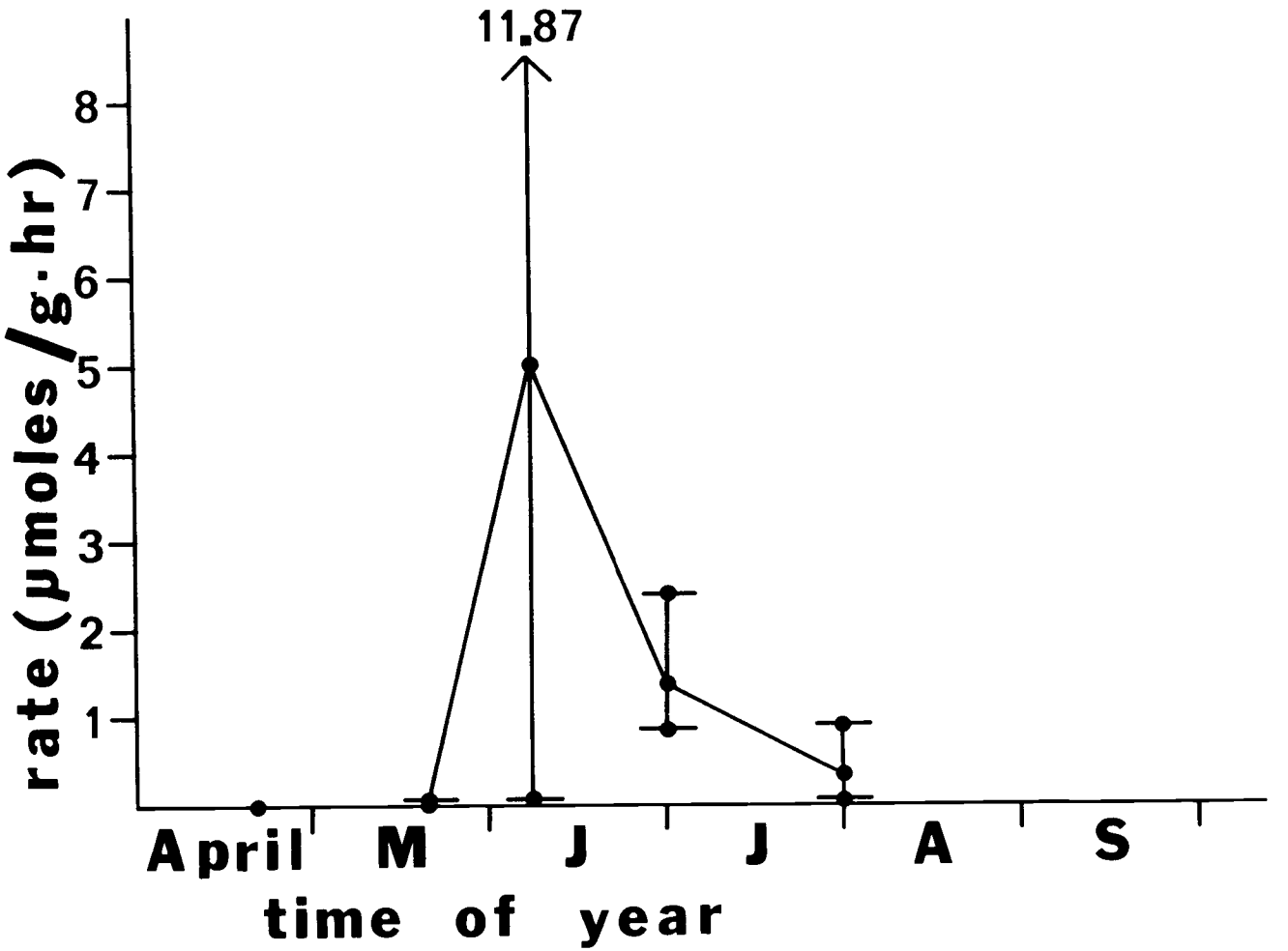

Figure 22. Seasonal patterns of midday acetylene reduction rates at study site 5. Vertical lines indicate the extremes observed at each determination. Means of nodules from 6-8 plants.

variability between nodules. By the end of July, nodule activity at all sites decreased sharply. This low rate continued throughout August and early September.

The late starting date for nodule activity is apparently due to the cool temperature encountered in this region. Substantial activity was detected only after soil temperatures exceeded 10°C. The delay in initiation of nodule activity at site 1 and especially site 4 is tied closely to the lower soil temperatures in May and June at these sites (Tables 1 and 8). The cool temperatures at site 4 and to some extent site 1 were due to cold air drainage as described earlier. Due to the open nature of the canopy on site 3, both high and low temperature extremes frequently exceeded those of other sites, but moderately warm soil temperatures were maintained and nodule activity began by mid-May.

Soil temperatures at site 4 remained substantially cooler until late July. Consequently acetylene reduction rates were lower and never reached the high levels measured on other sites (Figure 23). After July 20, when soil temperature at this site did finally reach levels comparable to those of other sites, nodule activity activity was short-lived due to moisture conditions and by July 30 activity had rapidly declined at this site as well as the others.

This decline in rates at the end on July at all sites corresponds to xylem pressure potential readings in the range of -25 bars (Table 6) which were observed to greatly reduce rates in greenhouse plants

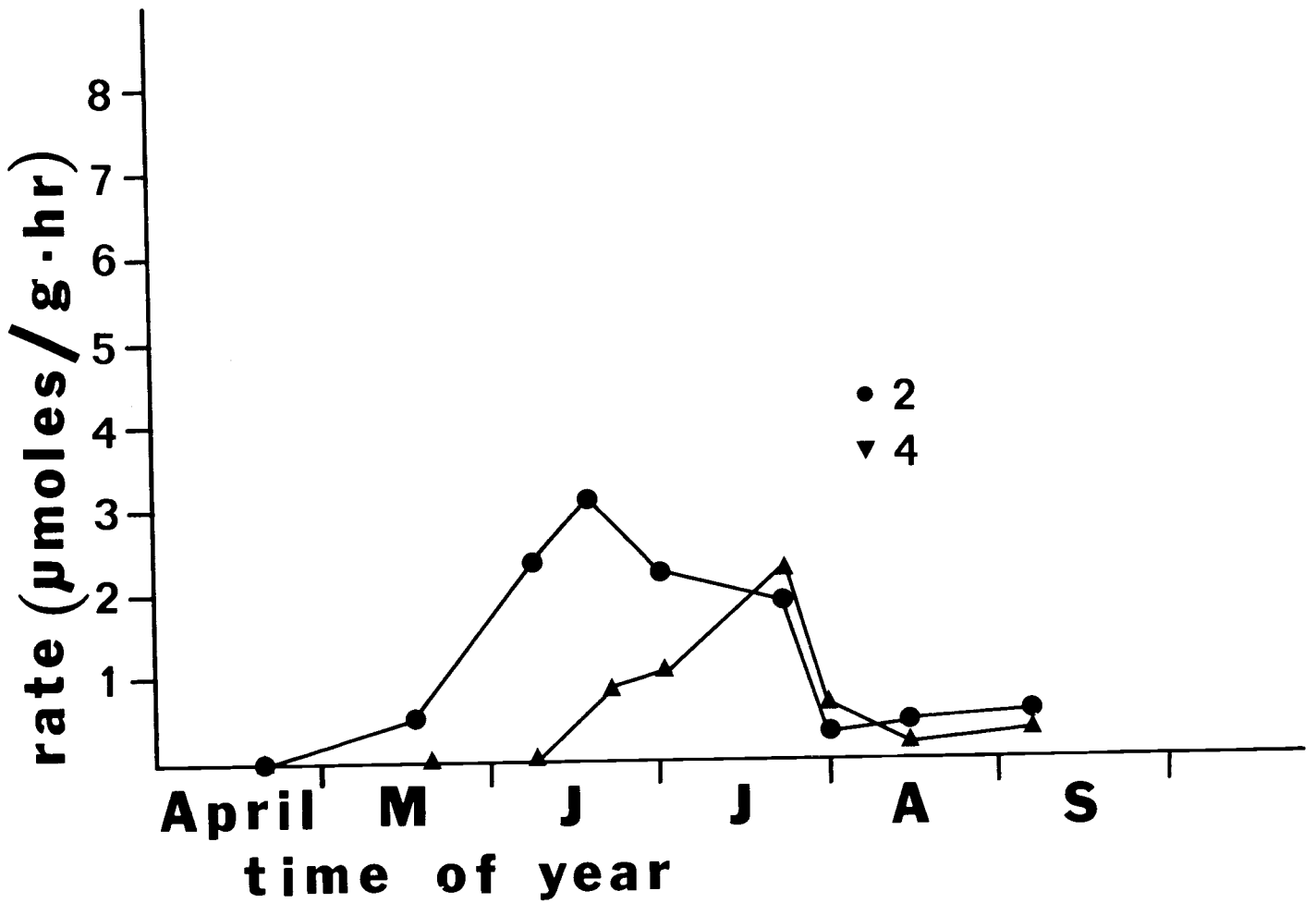

Figure 23. Comparison of seasonal pattern of midday acetylene reduction rates at study sites 2 and 4.

(Figure 17). August 1975 was unusually wet. The continuation of small amounts of nodule activity into early September may have been the result of this extra moisture. Some signs of leaf drop were observed in late July, but this was temporarily arrested by the August rains.

Previous information of seasonal rates of nitrogen fixation by non-legumes is limited. Akkermans (1971) reported that acetylene reduction activity of Alnus nodules from plants growing in the Netherlands was first detected at low levels on April 16. Soil temperature at this time was 9°C. and leaves were unfolded and up to 3 cm. long. Activity increased rapidly and did not decline to a low value until early November.

Strong diurnal patterns occurred in nodule activity. The variation through the course of one day (July 20 - 21) is shown in Figure 24. Following a midday maximum in acetylene reduction, activity declined in the late afternoon reaching a low in predawn hours. Rates began to climb again shortly after sunrise. In addition, diurnal patterns showed a seasonal change. Predawn rates declined much more rapidly from June to August than did midday rates when the two rates are compared on a percentage basis (Figure 25).

Diurnal variation in nodule activity has been reported for soybeans (Bergersen 1970) and Alnus and Myrica (Wheeler 1969). Wheeler (1971) has shown that these fluctuations, at least in Alnus, are due to the depletion of carbohydrate levels in the nodules in the dark. The

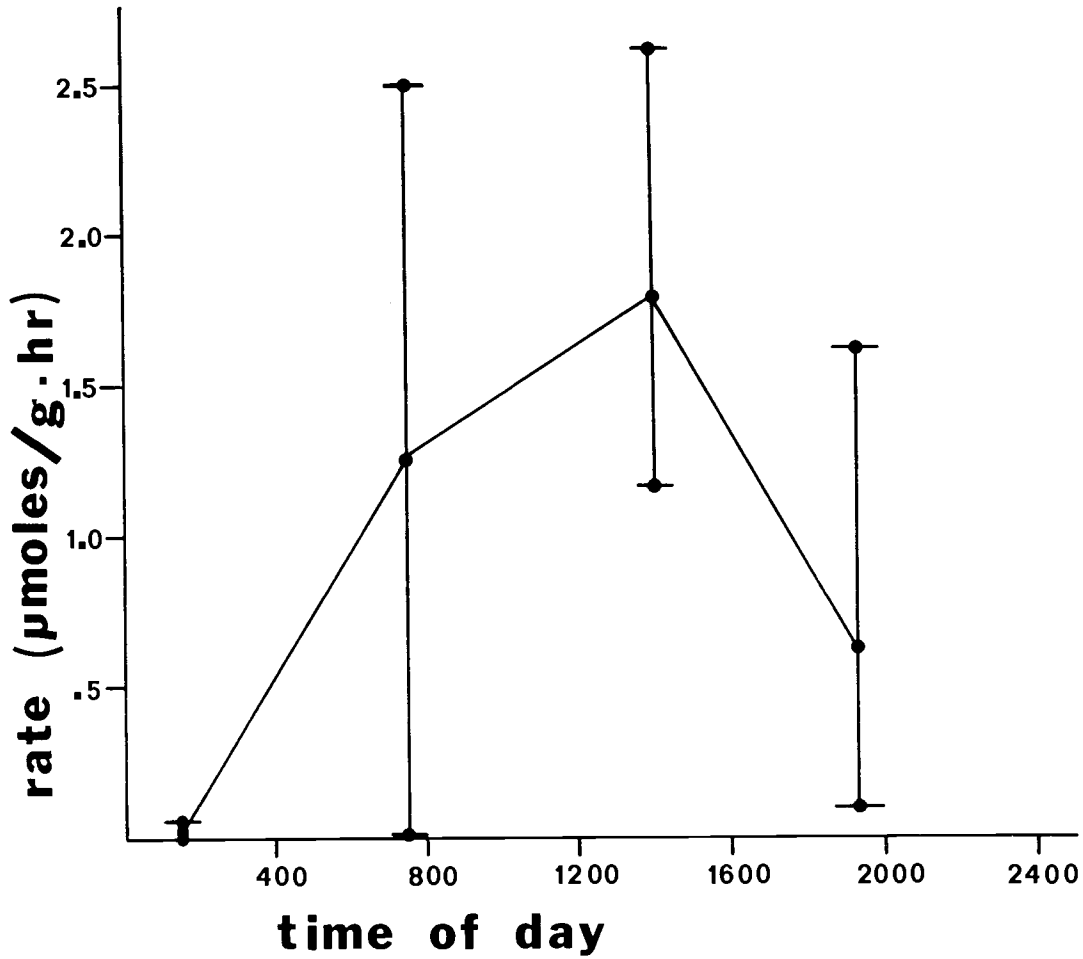

Figure 24. Variation in acetylene reduction rates of nodules sampled at several times during 24 hours. All determinations were made on June 20-21 at study site 2. Vertical lines indicate the extremes observed at each determination. Means of nodules from 6-8 plants.

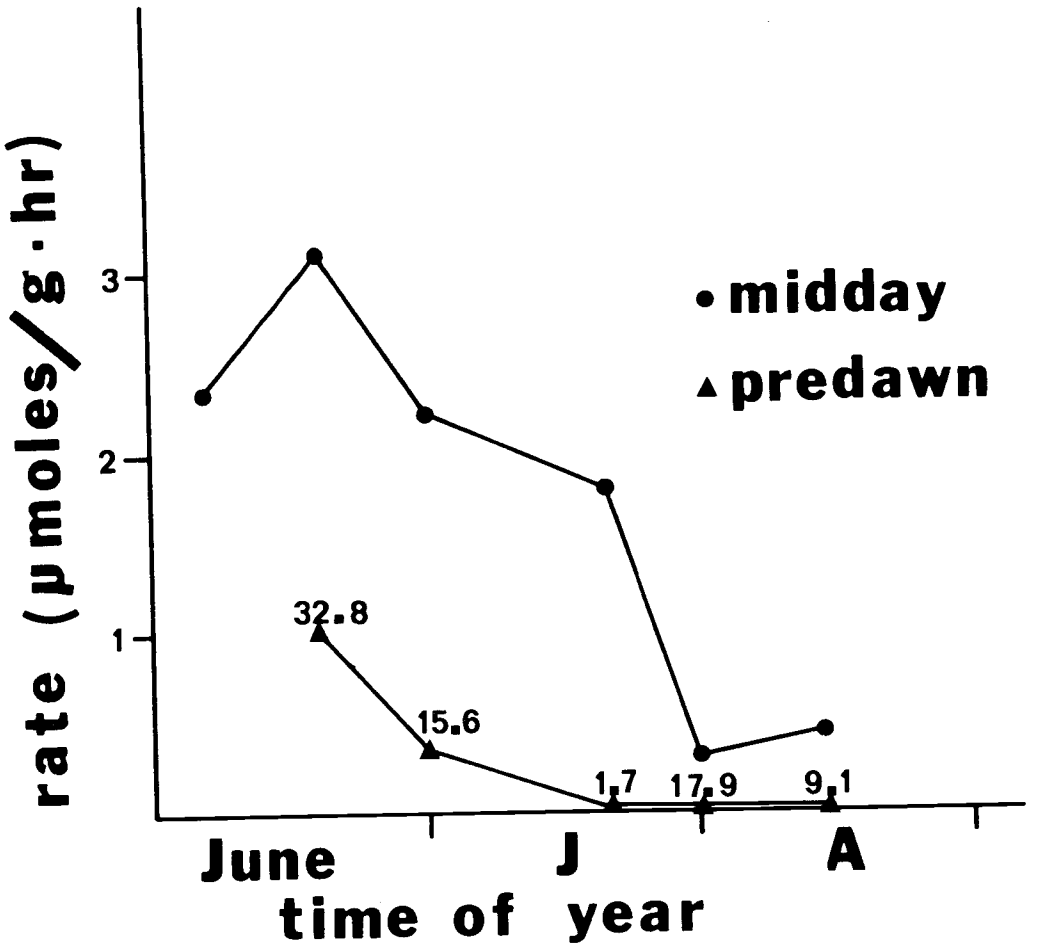

Figure 25. Seasonal pattern of midday and predawn acetylene reduction rates at study site 2. Numbers above each predawn point are the percent of predawn rates relative to midday rates on the same date. Means of nodules from 6-8 plants.

percent of predawn acetylene reduction rates relative to midday rates (32.8%) of Purshia nodules in early June compares well with the values provided by these other reports. The substantial decline in predawn rates later in the season suggests that carbohydrate levels were also decreasing throughout the season, becoming more completely exhausted each night. This decline of carbohydrate levels is probably also due to reduced photosynthesis brought about by moisture stress.

The high variability in average acetylene reduction rates between sampling times from June to mid-July such as observed at sites 1 and 3, might also be due to fluctuation in carbohydrate levels. Bergersen (1970) reported that soybean nodule activity declined on a cloudy day. Light showers and changes in temperature also might effect the daily carbohydrate level and nodule activity of Purshia although a direct comparison of thermograph data and nodule activity failed to show a strong relationship. Other factors which account for part of this variability are random sampling variability and the fact that all acetylene reduction measurements were not made at the same time of day.

EVALUATION OF PURSHIA NODULATION

Materials and Methods

The percent of Purshia plants with nodules was determined by carefully excavating 364 plants from sites 1 - 4. Any plant whose roots appeared badly damaged or severed was discarded. Each acceptable plant was numbered and the presence or absence of nodules recorded. Plants with only senescent nodules were not considered to be nodulated. A short segment of stem near the base of each plant was clipped off and returned to the laboratory for age determination. Thin sections of each segment were cut with a razor blade and the annual rings counted under a dissecting microscope.

An evaluation of nodule biomass per hectare was conducted on study site 2. A small plot (8.12 m^2) was selected at random in an area with high Purshia coverage. All Purshia plants in the plot were carefully excavated, making every effort to remove the roots intact. All nodues were excised and placed in FAA and returned to the laboratory for weighing.

Greenhouse seedlings were grown from seed provided by Ms. Jean Alderfa from 12 different sources scattered across several Western states. Germination was promoted by soaking seeds in a 10% thiourea solution for 5 min. following the procedure of Hubbard and Bennett (1958). After drying, the seeds were planted in 20 cm. plastic

pots containing a mixture of wet pumice soil collected from two areas in central Oregon where nodulated Purshia occurs. Soil in all pots was watered to saturation once weekly. After one month, the seedlings were thinned to 6 or fewer per pot and a -N fertilizer solution (Evans et al. 1972) was applied once. After 3 months the seedlings were harvested, acetylene reduction measured on intact root systems, and % N of foliage determined. At least 5 seedlings were examined from each seed source. Acetylene reduction measurements were made at 22°C. following the methods previously described except that complete root systems with nodules intact were measured.

Kjeldahl analysis of foliage nitrogen of the greenhouse seedlings and some field plants was conducted with a modification of the method described by Block and Bolling (1951). Approximately 50 mg. of oven dried leaves was weighed and then digested in a mixture of 2.5 ml. concentrated sulfuric acid, 2 Hengar selenized granules, and 2 g. K_2SO_4 - $CuSO_4$ mixture (20:1 by weight). After boiling 90 min., an additional selenized granule was added and the mixture boiled for another hour. The solution was then made alkaline with NaOH and distilled into a saturated boric acid solution. A methyl red-methylene blue indicator was added to the distillate and the ammonia titrated with 0.02 N HCl.

Results and Discussion

Percent Nodulation

All seedlings grown from seed in the greenhouse were found to have nodules by the age of 3 months. Two month old seedlings often had not formed nodules. All of these nodules reduced acetylene at varying rates with the exception of one group of plants. These plants were from seeds collected from a source at Bryce Canyon, Utah. Nodules from 4 2-month-old, 12 3-month-old and 4 4-month-old seedlings from this seed source were examined and no trace of ethylene was produced by any. There was no visible distinction between the nodules of these plants and the active nodules of plants from other sources. These plants were, however, distinguished by a low growth habit of older plants. Apparently Purshia plants from this source are genetically different from other Purshia plants and are not adapted to the endophyte found in central Oregon soils.

The percent nodulation of plants from sites 1 - 4 is shown in Table 9. There is no consistent trend in age and nodulation, but all sites except no. 2 have a much lower percentage of young plants (2 - 5 years old) with nodules. Plants younger than 2 years almost never had nodules. Although no precise data were collected it was apparent that even though plants greater than 15 years old were just as likely to have nodules as plants 5 - 15 years old, the nodules on older plants were

Table 9. Percentage of plants with nodules. The numbers in parenthesis are the total number of individuals examined in each case.

Age (years)	Study Site 1	2	3	4	all sites
2-5	21 (28)	60 (84)	29 (24)	23 (26)	43 (162)
6-10	40 (43)	57 (14)	35 (31)	62 (34)	47 (122)
11-15	63 (16)	50 (2)	50 (4)	38 (8)	53 (30)
> 15	75 (12)	48 (21)	67 (3)	36 (14)	50 (50)
all ages	42 (99)	57 (121)	35 (62)	43 (82)	46 (364)

generally small and only a few occurred on any one plant. Even with the much smaller root system of younger plants the average nodule biomass per plant was much greater. This corresponds partially with the description of Hippophae nodules by Akkermans (1971) who reported less nodule biomass/m^2 in older stands. Stewart and Pearson (1967) indicated similar findings for Hippophae but noted that 100% of the plants examined had nodules.

Under normal conditions, nodulation percentages of other nitrogen-fixing non-legumes are generally high, close to 100% (Bond 1974). The occurrence of lower nodulation percentages in Purshia has several possible explanations. The chance of nodules forming even under ideal conditions might be small if the symbiotic relationship is poorly developed. The degree to which host and endophyte have adapted to each other is a well known factor in controlling the success of nitrogen fixation in legumes. Poorly adapted symbionts may form few nodules or nodules with low activity. It should be noted that nodulation of other genera in the Rosaceae appears to be marginal. Of 20 species of Cercocarpus, only 3 are known to be nodulated (Vlamis et al. 1964, Hoeppel and Wollum 1971) although more may eventually be discovered. Dryas species have been found to be nodulated only in Alaska and Canada despite careful searching elsewhere (Bond 1971). It may be that nitrogen fixation by actinomycetes is poorly suited to members of the Rosaceae or that the evolutionary introduction of nodules into this

family was a fairly recent event and that sufficient time has not elapsed for "fine tuning" of the symbiotic relationship.

Another explanation for low percent nodulation involves the effect of unfavorable environmental conditions such as moisture stress or low soil temperature. This theory is supported by the observation that all seedlings planted from seed and grown in the greenhouse with ample moisture and warm temperatures developed nodules. Also, a few of the plants removed from the field and placed in the greenhouse developed a profusion of new nodules such as never observed in the field.

The effects of moisture stress on the process of nodulation has not been thoroughly investigated. According to Sprent (1971), moisture availability has been generally accepted as affecting nodule initiation and longevity since Wilson's (1931) observations in which up to 57% of nodules on bean plants were shed under moderate water stress. Purshia nodule clusters generally become senescent and are shed before they reach a large size. Although there is no direct evidence, moisture probably is important in controlling the loss of old Purshia nodules as well as the formation of new ones.

Summer soil temperature at 20 cm. in Purshia communities in this study never exceeded 17°C. and was frequently lower. Soil temperatures were probably slightly below normal due to the cool, wet summer of 1975; however even in normal years, soil temperature

throughout this region would still be quite cool. Nodule initiation of legumes is reduced by low temperature (Lie 1974). While infection, growth of the nodule and nodule activity were also slowed by low temperature, the process of initiation was more sensitive. Wollum and Youngberg (1969) studied the effect of soil temperature on nodulation of Ceanothus velutinus which, like Purshia, grows in the central Oregon pumice region. Only 10% of their plants formed nodules when grown at a soil temperature of 15°C. and no nodules were formed at 10°C. However, all plants grown at 22°C. developed nodules. The nodules that did form at 15°C. required 70 days to develop, compared to less than 45 days for those at 22°C. If Purshia nodulation behaves similarly, then nodule initiation might not begin until spring soil temperatures exceeded 10°C. - approximately mid-May or early June. Since summer soil temperature is generally around 15°C., few nodules would form and those that did might require much of the summer for full development. This would mean that the final stages of nodule development would occur late in the growing season when moisture conditions are unfavorable.

There are other environmental factors that might account for the failure of some plants to nodulate. Dyrness and Youngberg (1966) found pH in the range of 6.0 - 6.4 and exchangeable Ca at 2.35 - 3.60 meq/100 g. in central Oregon pumice soils, so it is unlikely that these factors limit the formation of nodules. Total nitrogen was reported

at .105 - .160%, so the levels of available nitrogen are probably not high enough to inhibit nodulation. Pumice soils are well aerated and the supply of O_2 in the soil atmosphere should be adequate. Lie (1974) has suggested that nodulation of forest understory legumes may be affected by shading patterns of the canopy. Sunlight filtered through green leaves contains more far-red than red light. Since the nodulation of legumes has been shown to be partially controlled by phytochromes, with far-red light inhibitory, plants in the shade might form fewer nodules. Although Purshia is one of very few species that grows well under ponderosa pine without regard to light and shade patterns (Robinson 1967), it may be that nodulation is partially dependent on this factor.

Another factor which affects nodule initiation is the distribution of the endophyte in the soil. If a nitrogen-fixing plant invades an area where it has not previously grown, nodule formation may be scarce until the soil population of the endophyte is built up. Wollum et al. (1968) have shown that the endophyte for Ceanothus persists in Oregon soils up to 200 years after the host plant disappears from an area and that symbiotic populations are increased rapidly when the host plant returns. However, all of the Purshia communities in this study are climax and Purshia has been present on each site for a number of years, as proven by the occurrence of some individuals over 25 years old at each sampling area. Consequently, soil populations of the

endophyte should have been built up to sufficient levels to insure universal nodulation. Even in case of a fire, Purshia should become reestablished before soil endophyte populations decline seriously. Several other observations suggest that it is not endophyte distribution that is limiting nodulation. Frequently, 1 or 2 plants in an old seed cache group will be nodulated, while other plants in the same group will have roots intertwined with the nodulated roots, but no nodules of their own. Finally, as noted earlier, nodulation was complete in plants grown in identical soil brought into the greenhouse.

In review, it seems likely that nodulation of Purshia is limited primarily by low soil temperatures and moisture stress. Several other factors may also be involved and the low nodulation percentages which were observed may be the result of the combined interaction of several of these factors.

Foliage Nitrogen and Nitrogen Accretion

Kjeldahl analysis of leaves from 14 field plants, 7 with and 7 without nodules, revealed an average nitrogen content of $1.5 \pm 0.3\%$ for non-nodulated and $1.7 \pm 0.3\%$ for nodulated plants. Foliage levels of 3 month old greenhouse seedlings (all nodulated) ranged from 1.3 to 2.5% with the average being 1.8%. Nitrogen levels were higher in seedlings with nodules that were more active as shown in Figure 26. The group of plants in which only inactive nodules formed is not included in this

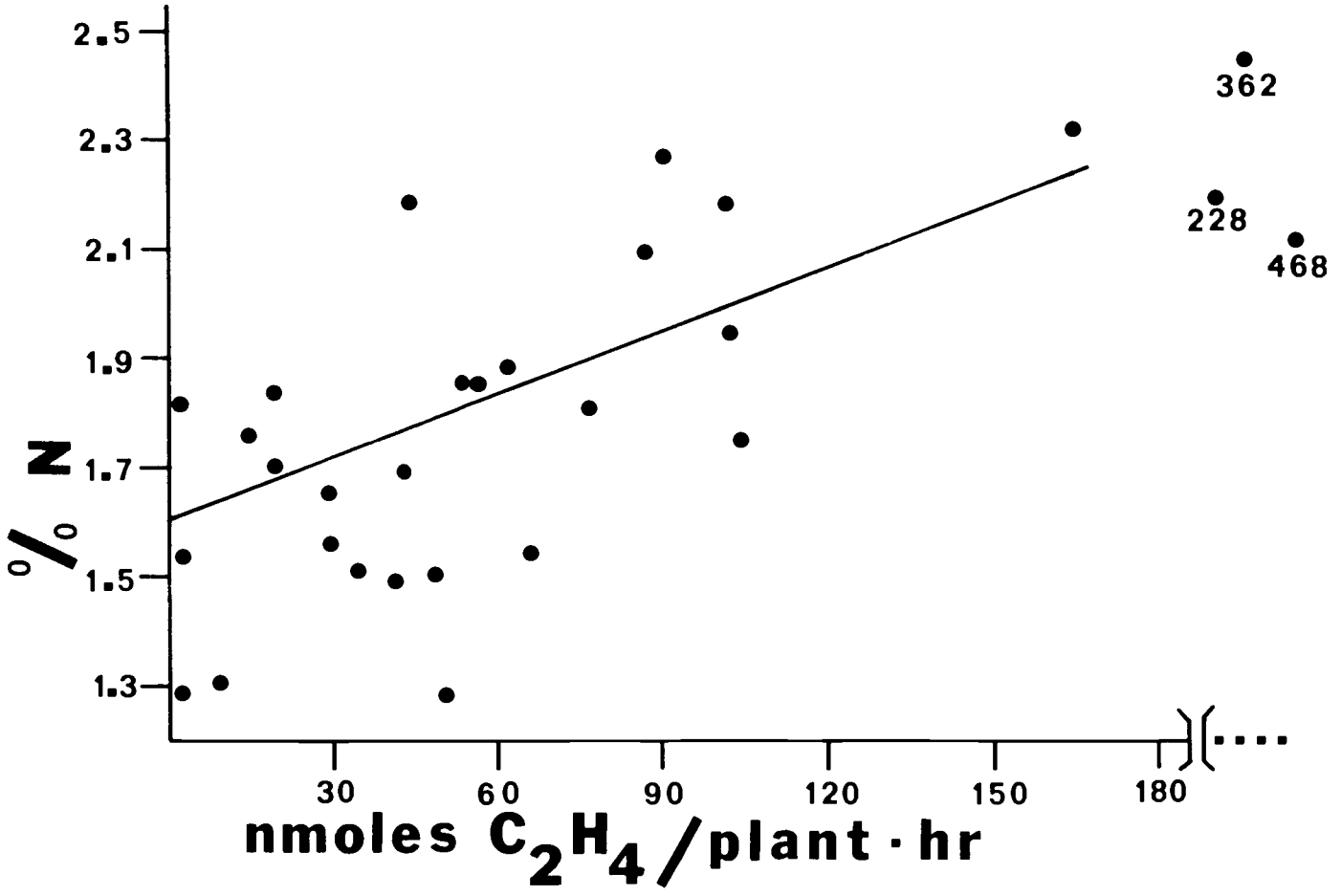

Figure 26. Relationship of foliage nitrogen levels to nodule activity of 3 month old greenhouse seedlings. Regression analysis revealed a correlation coefficient of .64.

diagram. Regression analysis showed a correlation coefficient of .64.

These nitrogen levels are low when compared to several other plants including legumes (soybeans 5.8%, cowpeas 5.6%; Miller 1929) and alder (3.34%; Hughes et al. 1968). Silvester (1975) has suggested that low foliage nitrogen in some nitrogen-fixing plants may be an indication that nitrogen fixation by these plants is of little ecological significance and it appears that his argument may be extended to include Purshia.

An effort was made at site 2 to estimate the total contribution of nitrogen by Purshia to the ecosystem. This site was chosen because of the long season of nodule activity and the clear abundance of nodules relative to the other sites. Consequently, this estimate should represent an upper limit of accretion rates within the sites studied. The procedure for estimating the total nitrogen accretion was based on a knowledge of nodule biomass per unit ground surface area and seasonal acetylene reduction rates. Akkermans (1971) used a similar method in describing nitrogen rates of 56 - 130 kg N/ha·yr by Alnus glutinosa with a dry weight root nodule biomass of 444 kg/ha. The Purshia root nodule biomass at site 2 was calculated to be only 1.91 kg/ha, on a fresh weight basis.

Several assumptions were made to arrive at an estimate of how much nitrogen this amount of nodules could fix in one year. Nodules were assumed to function at the observed midday rates for 12 hours

and the predawn levels for 12 hours each day. Predawn rates previous to June 19 (the earliest date predawn readings were taken) were considered to identical to the relative activity observed on this day (32.8% of the midday rate). Similarly, predawn rates after August 13 were taken as 9.1% of the midday rate. Acetylene reduction values from each sampling date were treated as a constant over a time span extending from halfway to the previous sampling date to halfway to the next sampling date. Nodule activity after September 4 was considered negligible since acetylene reduction rates were very low and in years with normal precipitation, would have probably have approached zero by this time. The conversion factor for C_2H_2 reduced to N_2 fixed of 2.4 was used, based on reports for Alnus, which is the only non-legume for which this factor has been calculated (Hardy et al. 1973 based on data from Akkermans 1971 and Russell and Evans 1970).

This results in an accretion estimate of only 0.057 kg N/ha·yr. This extremely low value arose from several factors. Nodules were active for only a short time each year, with substantial acetylene reduction rates occurring only from early June to late July. Even during this period, nodule activity was slight during night hours. Soil temperatures were consistently below the level required for maximum nodule activity. Most important was the very small nodule biomass for a given ground surface area that arose from the small size of nodules and the low percentage of plants with nodules. The biomass

estimate was probably below the actual value, due to loss of nodules in the process of excavation, despite careful digging. The percentage of nodules lost in this way was probably low; however, even assuming a substantial loss, the estimated nitrogen accretion rate would still be low.

Such a small nitrogen accretion rate has negligible ecological significance. Based on Youngberg and Dyrness (1964) data on pumice soil nitrogen (.105 - .160%) and bulk density (0.7 g/cc) and assuming all nitrogen is in the top 15 cm. of soil, Purshia would be increasing soil nitrogen by only 0.005% or less each year. This accretion rate is also small when compared to probable input from nitrogen in precipitation. Fredriksen (1975) reported yearly inputs of dissolved nitrogen of from 0.36 to 1.12 kg N/ha/100 cm of precipitation in the Western Oregon Cascades. Extrapolated to study site 2, this would suggest a probable yearly input of 0.25 to 0.77 kg/ha.

The occurrence of nodules may have a direct impact on Purshia plants. Survival of plants, especially those less than 15 years old, may be improved by the presence of nodules, considering the low soil nitrogen levels and the intense competition from other plants in the same seed cache. As noted earlier, the younger plants generally have larger nodules.

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