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Effects of substrate nitrate concentration on symbiotic nodule formation in actinorhizal plants

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Abstract

The response of *Alnus glutinosa*, *Casuarina cunninghamiana*, *Elaeagnus angustifolia* and *Myrica cerifera* to a range of substrate nitrogen levels in solution, in relation to plant growth, infection, nodulation and root fine structure was studied. Nine concentrations of potassium nitrate ranging from 0.05 to 3.0 m*M*, were tested on each of the species. Plants were inoculated with *Frankia* pure cultures after a two week exposure to one of the nine levels of added nitrate. After six more weeks with constant exposure to nitrate, plants were harvested and assayed. With the exception of Myrica, regression analyses of whole plant dry weights as a function of added nitrate were highly significant. There was a tendency for nodulated plants grown at intermediate levels of added nitrate to exhibit higher relative growth rates, probably due to the additive effect of substrate nitrogen and fixation of atmospheric nitrogen. The mean numbers of nodules per plant were, with the exception of Alnus, significantly higher at intermediate levels of added nitrate, as were mean nodule dry weights. A highly significant inverse relationship between nodule weight as a percentage of whole plant weight was found in Elaeagnus and Myrica. The observed response of Elaeagnus to added nitrate compared to other actinorhizal plants appears to demonstrate that root hair infected plants are much more sensitive to the inhibitory effects of added nitrate than plants infected by intercellular penetration. A sharp reduction in the presence of root hairs at high concentrations of nitrate was observed. This indicates that the inhibition of nodulation in some actinorhizal plant species results from nitrate induced root hair suppression.

Introduction

Substrate nitrogen has been shown to have an inhibitory effect upon infection and nodule development in leguminous plants (Streeter, 1988). Relatively few studies have been undertaken to examine similar phenomena in actinorhizal species, and the majority of these employed nutrient solutions supplemented with ammonium nitrogen (*e.g.* Bond *et al.*, 1954). Unfortunately, data are sparse for studies in which nitrate was used as the form of substrate nitrogen to determine influence on nodulation. Pizelle (1966) studied the effect of nitrate on previously nodulated *Alnus glutinosa* seedlings using a split-root technique. He observed that nitrate inhibited the development of existing nodules, and that the response was systemic and not a localized phenomenon. Benecke (1970) utilized mature nodulated *Alnus viridis* plants in sand culture and determined that nodule growth was related inversely to the amount of nitrate supplied. He also observed a reduction in plant growth at low concentrations of nitrate. Bond and Mackintosh (1975) investigated the effect of nitrate on one year old nodulated *Coriaria arborea* and *Hippophae rhamnoides* plants and demonstrated that by supplying as little as 25 mg l^{-1} nitrate, nodule dry weight was significantly reduced.

It is generally agreed that legume infection and nodule development are more sensitive to nitrate rather than ammonium inhibition (Rys and Phung, 1984). Bond and Mackintosh (1975) suggested that nitrate is also a more inhibitory compound than ammonium in their study of Coriaria and Hippophae.

The purpose of this investigation was to determine the extent of and possible mechanisms for nitrate inhibition of infection and nodule development in several actinorhizal plants. We chose to supply the young seedlings with nitrate previous to inoculation so that we could ascertain its effects on the earliest stages of infection.

Materials and methods

Plant material

Seeds of *Alnus glutinosa* and *Elaeagnus angustifolia* were obtained from F.W. Schumacher, Inc. and Native Plants, Inc., respectively. Excised Elaeagnus embryos and Alnus seeds were germinated on moist sand. Young shoot cuttings of *Casuarina cunninghamiana* and *Myrica cerifera* from stock plants originally obtained from collections held at Harvard Forest, were rooted in nutrient solution as described by Lundquist and Torrey (1984). Shoots of Myrica were pulse treated with 50 μM IBA for one hour before transferring into the nutrient solution. Cuttings of Casuarina required no hormone treatment for rooting. Seedlings or rooted cuttings that were 3–4 weeks old were subsequently transferred to 400 ml hydroponic solution jars with three plants per jar. One-quarter strength nitrogen free Hoaglands nutrient solution, pH 5.5 (Hoagland and Arnon, 1950) was utilized as substrate. Immediately upon transfer, young plants were subjected to one of nine different levels of combined nitrogen, 0.05, 0.1, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5 or 3.0 mM, in the form of potassium nitrate. Two jars (six plants) were established for each concentration of nitrate. Due to lack of observable inhibition of nodulation in Elaeagnus, a second study on this species was initiated at higher concentrations of nitrate, 0.5, 3.0, 5.0, 7.5 and 10.0 mM. Plants were grown for a total of eight weeks within an environmental chamber. Nutrient solutions were completely changed on a weekly basis during the first four weeks and on a twice weekly basis during the last four weeks of the study. Photon flux density (PAR) was determined to be 500–600 μM with a light dark cycle of 16:8 h and a day:night temperature cycle of 28:25°C. Relative humidity was kept constant at 50%. Plants were randomized within the growth chamber in regard to nitrate concentration.

After two weeks of exposure to the nitrate-containing solutions, the plants were inoculated with an appropriate pure cultured *Frankia* strain (Table 1). Each plant received one ml of a 50:1 dilution (packed cell volume) of washed and homogenized *Frankia* cells.

Light microscopy

Samples of two to three cm long sections of terminal roots were collected from plants in each treatment level for light microscopy on the day prior to inoculation. Tissues were fixed in 2.5% glutaraldehyde in 0.2 M potassium phosphate buffer, pH 6.8, washed in an 8:1 dilution of buffer and dehydrated in a graded acetone series. The specimens were embedded in a low viscosity resin as described previously (Spurr, 1969), and sectioned at 1 μm. Thin sections were stained with 0.5% toluidine blue in 1.0% sodium tetraborate buffer.

Physical analyses

At eight weeks, plants were harvested and the following characteristics of each plant were measured: shoot length, root length, fresh shoot weight, fresh root weight, fresh nodule weight and numbers of nodules per plant. Dry weights were determined after drying at 80°C for 12 h.

Statistical analyses

Raw data were analyzed using Statview 2 + soft-

Table 1. Strains of *Frankia* used in this study

Strain Registry No.	Trivial designation	Host species inoculated
DDB 01310210	ArI4	*Alnus glutinosa*
HFP 020203	CcI3	*Casuarina cunninghamiana*
LLR 02022	R43	*Elaeagnus angustifolia* *Myrica cerifera*

ware. Means for each parameter were determined for each concentration of nitrate and examined inter- and intra-specifically.

Results

Recognizable nodules appeared one to two weeks following inoculation. Of those plants that did nodulate, nodules were observed sooner on Elaeagnus seedlings (7–8 days), than on other genera (10–20 days). Timing of nodulation in Elaeagnus was independent of nitrate concentration, while in other genera it was highly dependent. The appearance of root nodules on Casuarina was delayed significantly longer than for the other genera tested, often as much as three or more weeks. Without exception, nodules on plants other than Elaeagnus were first observed on plants at the lower concentrations of nitrate. Additionally, a distinct trend was observed in the position of nodules or nodule clusters on plants held at the higher concentrations of nitrate. Nodules were routinely confined to the upper portions of the tap root in the highest concentrations. No such nodule distribution pattern was observed with Elaeagnus.

Statistical analyses of the primary data are presented in Figs. 1–3. Unless otherwise noted, analyses of variance in regression analysis (F-test) were significant at $P \leqslant 0.05$; all means (T-test) were significantly different at $P \leqslant 0.0001$.

Discussion

Plant growth

Growth of the majority of plants was substantially increased when exposed to increasingly higher concentrations of nitrate. With the exception of Myrica, linear regressions of whole plant dry weight, shoot dry weight and root dry weight were significant (Fig. 1). At the highest concentrations of nitrate tested, plant biomass was the greatest even though the root systems failed to nodulate. This is consistent with the results of Ingestad (1980) and Sellstedt (1986) in studies of Alnus provided with ammonium nitrogen.

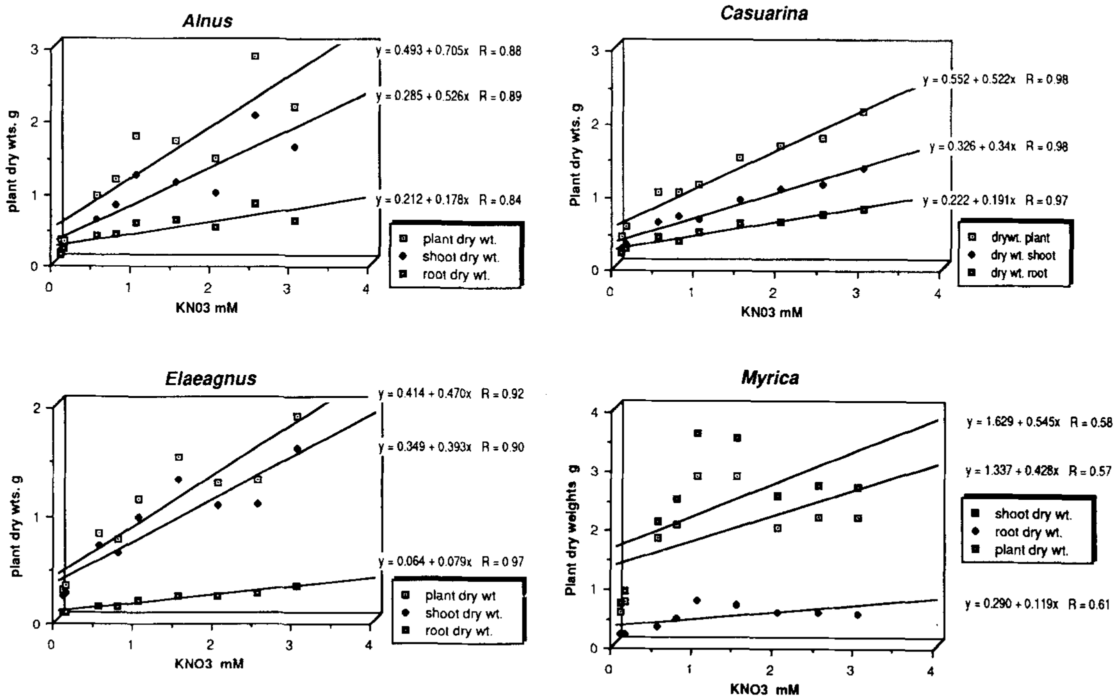

Fig. 1. Relationships of whole plant, shoot and root mean dry weights to nitrate concentrations. With the exception of Myrica, $P \leqslant 0.05$.

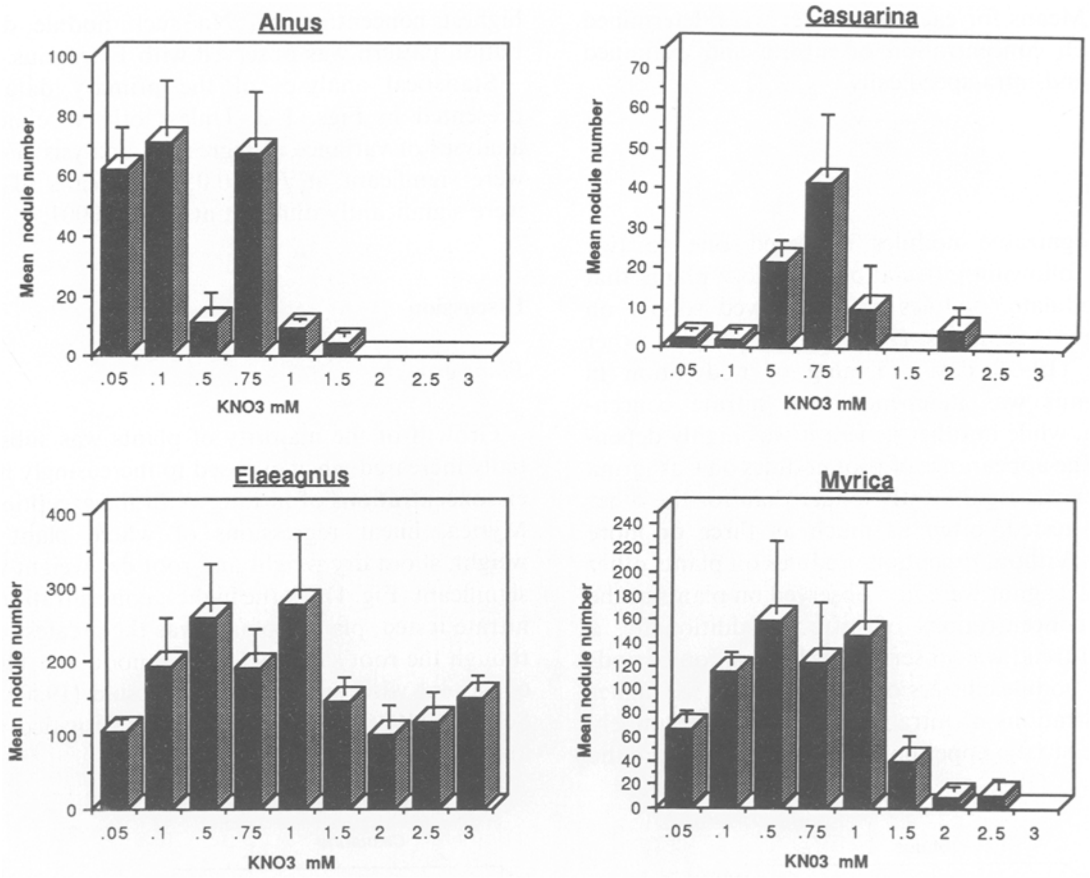

Fig. 2. Mean nodule numbers for plants cultivated at different nitrate concentrations, $P \leqslant 0.0001$.

Of particular interest are the results obtained from Myrica. Significantly greater biomass was produced at the intermediate levels of nitrate (Table 2, Fig. 1). This suggests that in the early stages of the symbiosis the combination of nitrogen fixation and assimilation of nitrate nitrogen supports optimum growth. A similar trend was observed in Alnus and Elaeagnus, but the effect was not significant.

Nodule number

A distinct tendency for the mean number of nodules per plant to vary as a function of added nitrate occurred. As shown in Table 3 and Fig. 2, a pattern where nodule number gradually increases and then abruptly falls over an increasing range of nitrate concentrations was observed. This trend was most pronounced in Casuarina, with a 49% increase in mean nodule number observed between 0.5 and 0.75 m*M* nitrate and a 77% decline from 0.75 to 1.0 m*M*. A very similar pattern was observed with Myrica. Over the entire range of nitrate concentrations, mean nodule number increased 49% between 0.05 and 0.5 m*M*, but fell dramatically between 0.5 and 2.5 m*M*. Alnus demonstrated a slightly different pattern. Nodule numbers were relatively high at lower concentrations. An abrupt decrease (93%) in nodule number was observed between 0.75 and 1.5 m*M* nitrate. It is not clear why such a pattern was observed.

The results obtained with Elaeagnus are in sharp contrast to the other three species. The mean number of nodules, even at the highest concentrations, did not vary significantly from those at lower concentrations.

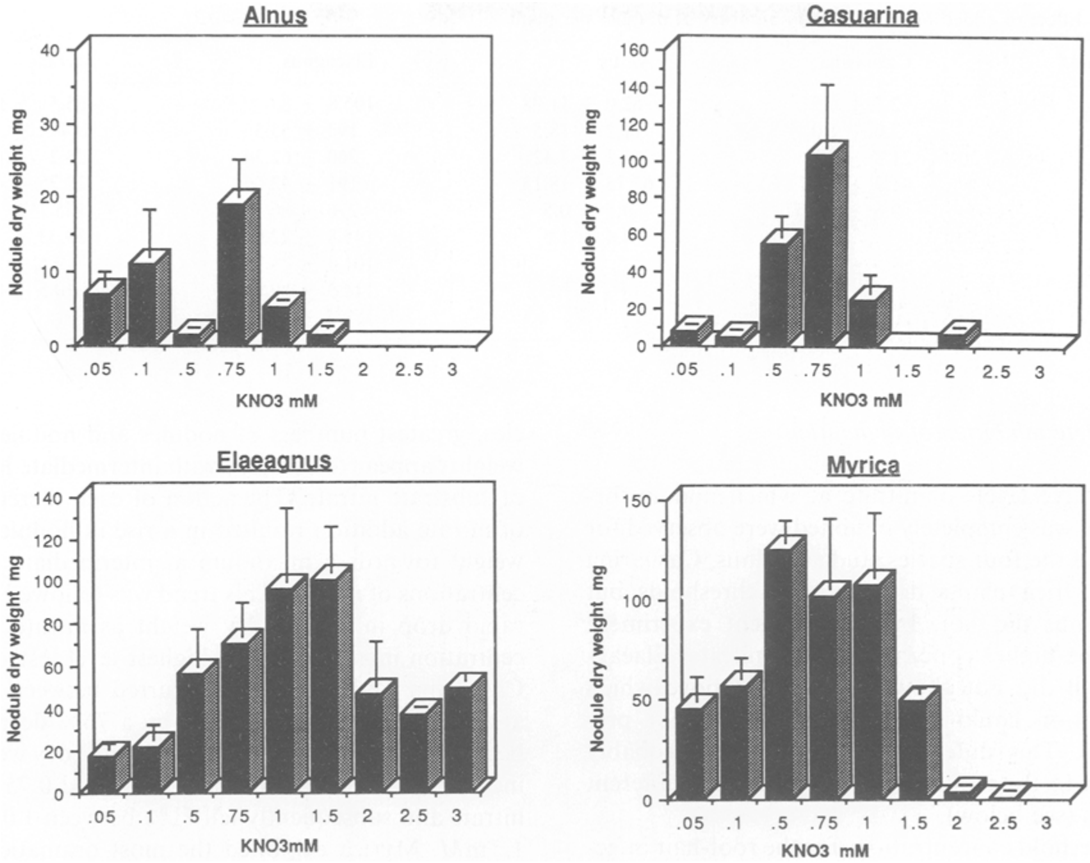

Fig. 3. Mean dry weights of nodule biomass of plants cultivated at different nitrate concentrations, $P \leq 0.0001$.

Table 2. Effect of added nitrate on shoot, root, and whole plant dry weights

Added KNO_3 mM	Shoot	Root	Whole plant	Shoot	Root	Whole plant
	Casuarina			**Alnus**		
0.05	0.266 ± 0.067	0.164 ± 0.04	0.39 ± 0.105	0.145 ± 0.028	0.083 ± 0.013	0.288 ± 0.041
0.1	0.293 ± 0.11	0.23 ± 0.08	0.523 ± 0.19	0.172 ± 0.066	0.166 ± 0.069	0.266 ± 0.097
0.5	0.602 ± 0.15	0.397 ± 0.1	0.998 ± 0.25	0.577 ± 0.21	0.342 ± 0.115	0.92 ± 0.32
0.75	0.68 ± 0.18	0.32 ± 0.07	1.0 ± 0.25	0.778 ± 0.15	0.37 ± 0.04	1.14 ± 0.189
1.0	0.64 ± 0.19	0.45 ± 0.15	1.1 ± 0.34	1.21 ± 0.14	0.537 ± 0.06	1.75 ± 0.19
1.5	0.9 ± 0.16	0.56 ± 0.09	1.47 ± 0.24	1.1 ± 0.29	0.58 ± 0.14	1.68 ± 0.42
2.0	1.04 ± 0.2	0.59 ± 0.12	1.62 ± 0.32	0.95 ± 0.29	0.48 ± 0.174	1.43 ± 0.46
2.5	1.11 ± 0.25	0.69 ± 0.14	1.7 ± 0.4	2.03 ± 0.56	0.81 ± 0.14	2.84 ± 0.71
3.0	1.32 ± 0.15	0.775 ± 0.07	2.1 ± 0.21	1.6 ± 0.49	0.57 ± 0.145	2.16 ± 0.64
	Elaeagnus			**Myrica**		
0.05	0.202 ± 0.02	0.055 ± 0.009	0.257 ± 0.03	0.54 ± 0.14	0.145 ± 0.05	0.685 ± 0.19
0.1	0.24 ± 0.05	0.058 ± 0.015	0.298 ± 0.07	0.71 ± 0.09	0.16 ± 0.05	0.875 ± 0.11
0.5	0.67 ± 0.16	0.11 ± 0.25	0.782 ± 0.19	1.8 ± 0.41	0.287 ± 0.09	2.07 ± 0.507
0.75	0.61 ± 0.13	0.115 ± 0.026	0.727 ± 0.16	2.02 ± 0.66	0.43 ± 0.14	2.45 ± 0.8
1.0	0.94 ± 0.14	0.172 ± 0.031	1.11 ± 0.167	2.8 ± 0.77	0.74 ± 0.17	3.55 ± 0.94
1.5	1.3 ± 0.34	0.212 ± 0.05	1.49 ± 0.39	2.8 ± 0.99	0.65 ± 0.22	3.46 ± 1.2
2.0	1.05 ± 0.29	0.213 ± 0.06	1.26 ± 0.35	1.95 ± 0.78	0.153 ± 0.24	2.48 ± 1.0
2.5	1.05 ± 0.3	0.233 ± 0.07	1.28 ± 0.47	2.14 ± 0.16	0.525 ± 0.25	2.66 ± 0.13
3.0	1.57 ± 0.15	0.305 ± 0.032	1.87 ± 0.17	2.15 ± 0.26	0.505 ± 0.26	2.65 ± 0.13

Table 3. Effect of added nitrate on mean numbers of nodules per plant

KNO_3 m*M*	Casuarina	Alnus	Elaeagnus	Myrica
0.05	2.5 ± 0.5	62.0 ± 11.98	105.8 ± 8.6	65.5 ± 9.8
0.1	2.0 ± 1.0	71.2 ± 18.5	193 ± 55.3	114 ± 11.8
0.5	21.3 ± 4.0	11.3 ± 7.42	260 ± 62.34	158.2 ± 62.35
0.75	41.3 ± 15.5	67.75 ± 18.15	191 ± 42.56	122.75 ± 45.2
1.0	9.6 ± 5.9	9.5 ± 0.5	276 ± 86	145.7 ± 39.1
1.5	—	4.4 ± 1.5	145.8 ± 22.9	39.33 ± 16.3
2.0	4.5 ± 2.5		101.3 ± 28.7	9.5 ± 2.5
2.5			118.5 ± 29.5	10.5 ± 8.5
3.0			150.5 ± 19.46	

Complete inhibition of nodulation

Relative levels of nitrate at which nodule formation was completely inhibited were observed for three of the four species studied. Alnus, Casuarina and Myrica plants demonstrated thresholds but Elaeagnus did not. In a subsequent experiment with the higher concentrations of nitrate, Elaeagnus still did not show a threshold above which nodulation could be suppressed (data not presented). This difference in response is probably related to the mode of infection of these different species (see below).

Threshold concentrations for the root-hair infected species were observed to be between 1.5 and 2.5 m*M* nitrate. The actual threshold values may be slightly below these concentrations since it is difficult to maintain absolute nitrate concentrations in standing water cultures in the presence of rapidly assimilating plants. These values are significantly lower than those observed for leguminous plants infected by Rhizobium. Truchet and Dazzo (1982) noted that nitrate levels as high as 18 m*M* may be required to suppress nodulation in legumes. It is not clear why there should be such a large difference in threshold concentrations for such similar symbiotic systems. The data in Table 3 suggest that Alnus is the most sensitive of the three genera. Myrica was the least sensitive and Casuarina demonstrated an intermediate sensitivity.

Nodule weight

The effect of added nitrate on mean nodule dry weight was similar to the results observed in nodule number (Fig. 4). Among the root-hair infected species, greatest numbers of nodules and nodule dry weights appear to coincide with intermediate levels of substrate nitrate. The action of each increment of nitrate addition resulted in a rise in nodule dry weight towards a maximum at intermediate concentrations of nitrate. This trend was followed by a rapid drop in nodule dry weight as nitrate concentration increased to the highest level tested. In Casuarina a 91% increase occurred between 0.05 and 0.75 m*M* nitrate followed by a 76% decrease between 0.75 and 1.0 m*M*. Alnus nodule dry weight increased by 65% between 0.05 m*M* and 0.75 m*M* nitrate and subsequently fell 71% between 1.0 and 1.5 m*M*. Myrica exhibited the most dramatic decrease with a 93% decline in nodule dry weight between 1.5 and 2.0 m*M* nitrate. An increase of 63% in nodule dry weight was observed between 0.05 and 0.5 m*M* nitrate.

Although the response of Elaeagnus to increased nitrate is not as pronounced as in the other species, a definite trend was observed. Over the range of concentrations from 0.05 to 1.0 m*M* nitrate, an 82% increase in nodule dry weight was observed. From 1.5 to 3.0 m*M* nitrate a 51% decrease was

Table 4. Effect of added nitrate on nodule dry weight (mg)

KNO_3 m*M*	Casuarina	Alnus	Elaeagnus	Myrica
0.05	8 ± 0	7 ± 2	18 ± 2.6	46 ± 12
0.1	5 ± 0.01	11 ± 6.4	22 ± 7	57 ± 10
0.5	55 ± 11	1.6 ± 0.01	56 ± 18	125 ± 11
0.75	104 ± 34	19 ± 5.2	70 ± 16	102 ± 35
1.0	25 ± 10	5.4 ± 0	96 ± 35	107 ± 33
1.5	0	1.6 ± 0.04	100 ± 22	49 ± 3
2.0	7 ± 0.01		47 ± 21	3.6 ± 0
2.5			37 ± 2.4	0.6 ± 0
3.0			49 ± 3.6	

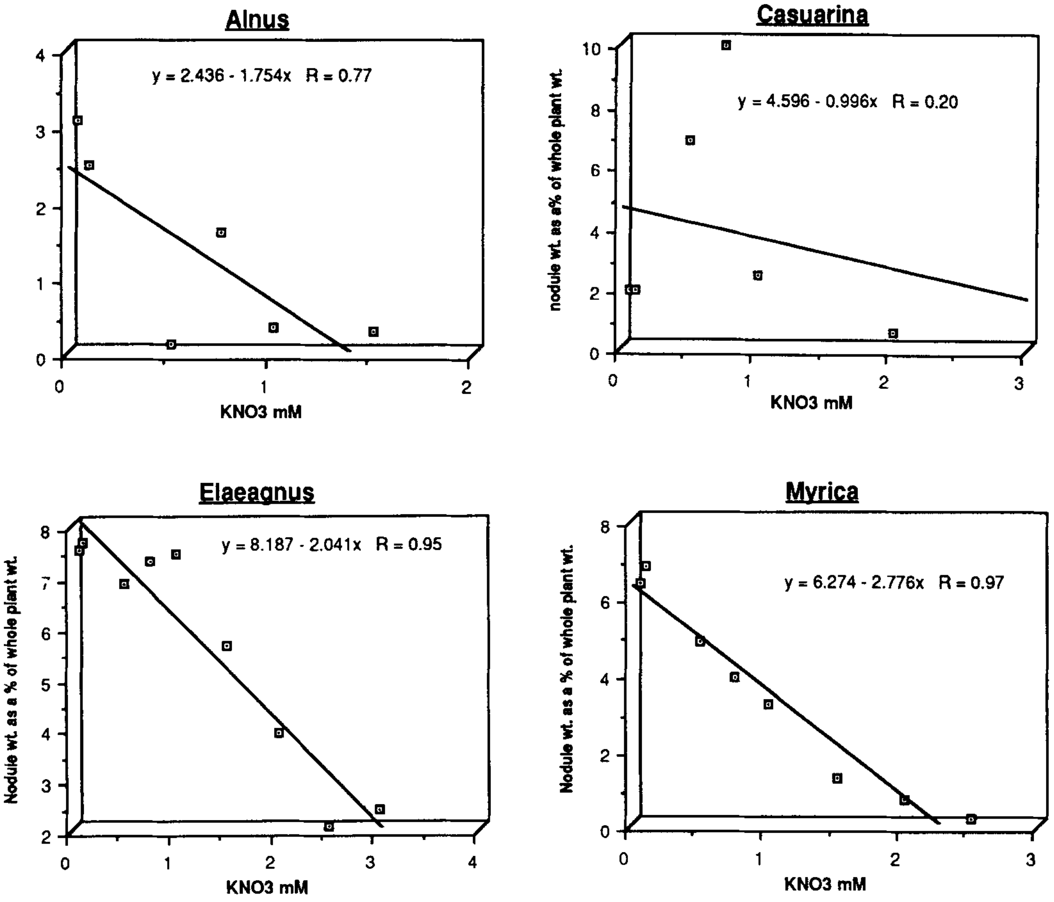

Fig. 4. Relationships between mean dry weights of nodule biomass as a percentage of whole plant dry weight. $P \leqslant 0.05$, with the exception of Casuarina.

Table 5. Nodule dry weight as a percentage of whole plant dry weight

KNO_3 m*M*	Casuarina	Alnus	Elaeagnus	Myrica
0.05	1.86 ± nd	3.07 ± 0.6	7.46 ± 0.55	6.33 ± 0.64
0.1	1.85 ± nd	7.3 ± 0.57	7.62 ± 0.75	6.6 ± 0.23
0.5	6.78 ± 1.2	0.11 ± 0.052	6.81 ± 1.25	4.7 ± 1.1
0.75	9.8 ± 0.72	1.56 ± 0.241	7.24 ± 0.65	3.8 ± 0.29
1.0	7.3 ± 0.19	0.315 ± nd	7.41 ± 0.86	3.1 ± 0.84
1.5	—	0.278 ± 0.186		
			5.5 ± 0.49	1.9 ± 0.63
2.0	0.51 ± nd			
			3.88 ± 0.67	0.67 ± nd
2.5				
			2.03 ± nd	0.16 ± 0.017
3.0				
			2.38 ± 0.28	

nd = not determined.

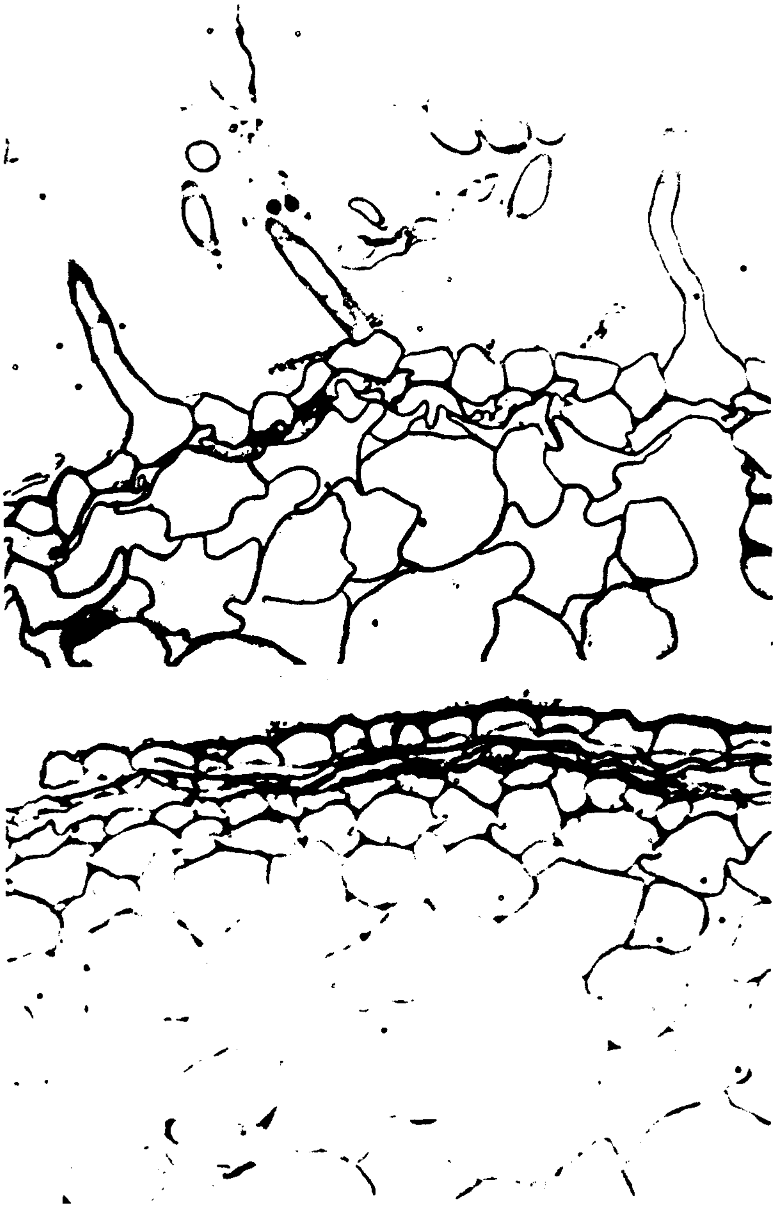

Fig. 5. Representative photomicrograph of transverse sections of Casuarina root. The plant in the upper micrograph was cultivated at 0.05 m*M* nitrate; the plant in the lower micrograph was cultivated at 3 m*M* nitrate. 400 ×.

observed. Even so, the relatively high biomass of nodules found at high levels of nitrate demonstrate that Elaeagnus is less affected in regard to both infection and nodule development than the other species.

Light microscopic observation of those plants which did not nodulate showed an absolute lack of root hairs (Fig. 5). Since infection of Alnus, Casuarina and Myrica occurs via the root hair (Callaham *et al.*, 1979, Berry *et al.*, 1986), the absence of root hairs would preclude the infection of the root system. We feel that this simple cause and effect phenomenon is the mechanism by which nodulation was inhibited. Thornton (1936) investigated the action of sodium nitrate on root hair development and morphology in Medicago. He concluded that the presence of nitrate had a definite inhibitory effect on absolute numbers of root hairs. More recently, Munns (1968) observed the same results. Truchet and Dazzo (1982) failed to observe reduction in root hairs of Medicago, but did observe absence of root hair curling and nodule initiation at 18 m*M* nitrate. These results are related to the fact that nitrate exposure was coincident with inoculation. In our experiment nitrate exposure preceded inoculation by two weeks, enough time to allow existing functional root hairs to mature and senesce. Therefore, any root hairs actively growing at the time of inoculation were formed in the presence of the imposed nitrate concentration.

In Elaeagnus, a species which is not infected via root hairs (Miller and Baker, 1985), no suppression of infection and nodulation occurred, thus strengthening the conclusion that nitrate's role is at the root hair level. Both Myrica and Elaeagnus were inoculated with the same Frankia strain, LLR 02022. This strain, being 'flexible' as defined by Miller and Baker (1986), infects host plants by either intercellular penetration or root hair mediated processes. Only nodulation of Myrica was inhibited at high nitrate levels with this strain, supporting the hypothesis that nitrate affects not the microsymbiont, but the host plant.

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