Plant and Soil 118, 43–50, 1989.

PLSO FA-08

Is birch a suitable reference in estimating dinitrogen fixation in alders by isotope dilution?

L. CHATARPAUL and D. LACHANCE
Petawawa National Forestry Institute, Canadian Forestry Service, Chalk River, Ontario, Canada KOJ 1JO

Key words: *Alnus incana*, *Betula papyrifera*, N-fixation, isotope dilution, ^{15}N, reference plant

Abstract

Seedlings of *Alnus incana* (nodulated and non-nodulated) and *Betula papyrifera* were fertilized with varying amounts (0, 10, 25, 50, 100, 250 and 500 $\mu g\,N\,g^{-1}$ soil) of labelled ammonium-N or nitrate-N ($\simeq$ 5.2 A% excess ^{15}N as ammonium sulphate or potassium nitrate). After 4 months in the greenhouse, ^{15}N excess in the plants were determined and an isotope dilution equation was applied to determine the percent of biomass N fixed by the *A. incana*/*Frankia* system. When ammonium was used as the sole N source and birch as the non-fixing reference, N-fixation accounted for 95%, 87% and 60% of the plant nitrogen yields with 10, 25 and 50 $\mu g\,N\,g^{-1}$ rates, additions respectively. At the 100 $\mu g\,N\,g^{-1}$ fertilization and above N-fixation accounted for less than 10% of the N yield. Similar results were obtained when non-nodulated *A. incana* was used as non-fixing reference. With nitrate as the sole N source, N-fixation accounted for 98%, 97%, 97%, 86%, 56% and 12% of N yield with 10, 25, 50, 100, 250 and 500 $\mu g\,N\,g^{-1}$ additions respectively. These values were similar for both types of reference plants. The direct isotope dilution method was compared to that of the total nitrogen difference method. There was good agreement between the two methods up to 50 $\mu g\,N\,g^{-1}$ for ammonium and up to 100 $\mu g\,N\,g^{-1}$ for nitrate. The difference method produced negative values at high concentrations of nitrogen fertilization. Again similar results were obtained by the two reference plants. The results indicate that birch can be used as a non-fixing control in isotope dilution studies but that care must be exercised in selecting the type and quantity of labelled nitrogen fertilizer.

Introduction

Alders are among the actinorhizal group of plants which show great potential for reforestation of extreme environments and impoverished soils (Chatarpaul and Carlisle, 1983; Dawson, 1986). As a result, recent interest in the ecological significance of nitrogen fixed by actinorhizal plants has been intensified. Reported quantities of N fixed by these plants varied widely. These estimates were based on an equally varied number of methods. A complete review of the methods used for estimating N-fixation is given by Knowles (1981). The classical approach to estimating N-fixation is by the difference method using total nitrogen (Kjeldahl) analysis or indirectly by measuring nitrogenase activity (acetylene-reduction). Both these methods present enormous difficulties when applied in the field.

More recently, the use of ^{15}N labelled fertilizers have been applied to quantify N-fixation in the field. These studies however have largely been confined to the legume-Rhizobium system. The principles, pros and cons of ^{15}N methodology have been much discussed in recent reviews by Witty (1983), Chalk (1985), Danso (1986) and Rennie (1986) among others. One of the few studies using ^{15}N methodology on actinorhizal plants was done by Gauthier *et al.* (1986).

It is now generally accepted that the ^{15}N-isotope dilution technique, which was first described by McAuliffe *et al.* (1958) and subsequently tested by others (see Goh *et al.*, 1978; Rennie *et al.*, 1978;

Phillips 1980), could be a useful tool for quantifying N-fixation if sufficient care is taken. One of the major considerations is selecting an appropriate reference plant (non-fixing control) because a fundamental assumption is that the reference plant should be able to absorb available N from soil and fertilizer in the same proportion (though not in the same quantities) (Wagner and Zapata, 1982). Reference plants used in legume/Rhizobium studies have included non-legumes, grasses, and non-nodulated, and ineffective nodulated legumes. In the field, non-inoculated or those inoculated with ineffective strains can become nodulated by wild strains (Rennie, 1982). For actinorhizal plants, the absence of non-nodulating isolines and the ubiquitous nature of *Frankia* have made the selection of the non-fixing reference difficult. Other problems in applying the isotope dilution technique to quantify N-fixation are related to type and quantity of the labelled fertilizer used in the studies (Chalk, 1985).

In this study the purpose was to compare the isotope dilution technique to the classical difference approach to evaluate N-fixation by *Alnus incana* using either non-inoculated *A. incana* or a close relative, white birch (*Betula papyrifera*) as reference plants. A secondary objective was to determine the optimum level and type of ^{15}N label for such studies. *Alnus incana* was chosen as the test plant because of its importance to the nitrogen economy of temperate forest ecosystems (Johnsrud, 1978) and its potential for use in Canada (Burgess *et al.*, 1986).

Methods

Seedling production and maintenance

Seeds of *Alnus incana* and *Betula papyrifera* were surfaced sterilized with 30% hydrogen peroxide for 15 min, rinsed several times with sterile distilled water before they were planted in Hilson's type Spencer-Lemaire containers (32 cavities/box, 175 ml capacity). Each cavity contained 205 g (air dry weight) sterilized sand. Two weeks after germination seedlings were thinned to one per cavity leaving only those which were uniform in height. Later (6 weeks after inoculation) plants in alternate cavities were removed in order to reduce shading effects. Seedlings were kept in the greenhouse under 24 h lighting at 25°C ± 2°C. Watering was done manually once a day to achieve no more than 95% water holding capacity in order to prevent leaching. Plants were fertilized weekly with $\frac{1}{4}$ strength N-free Crone's solution supplemented with minor elements. Regular misting with deionized water prevented excessive drying.

Inoculation

Four weeks after germination three groups of *A. incana* seedlings were inoculated with pure cultures of *Frankia*. Each group was inoculated with one of 3 isolates obtained from field nodulated 2-year-old *A. japonica*, *A. incana* and *A. glutinosa*. A fourth group, which was not inoculated, served as non-fixing control as did one group of *B. papyrifera*. The *Frankia* isolates were cultivated in liquid Q-mod following the isolation technique of using osmium tetroxide (Lalonde and Calvert, 1979). Four week old cultures were washed twice in sterile distilled water through repeated centrifugation (2,000 rpm, 5 min) and resuspension. Following determination of the packed cell volumes (PCV), the mycellial mats were homogenized in $\frac{1}{4}$ strength Crone's solution by repeated passages through a 23 g needle attached to a 10 ml syringe. The homogenate was then diluted in $\frac{1}{4}$ strength Crone's solution such that upon inoculation with five ml of the mixture, each seedling received 0.3 μl PCV.

Labelled nitrogen treatment

To each group of seedlings of inoculated *A. incana*, non-inoculated *A. incana* and *B. papyrifera* labelled ammonium-N ($\simeq$ 5.2 atom percent excess as ammonium sulphate) or nitrate-N ($\simeq$ 5.2 atom percent excess as potassium nitrate) in varying concentrations of 10, 25, 50, 100, 250 and 500 $\mu g\ N\ g^{-1}$ soil was applied. The nitrogen was applied weekly in twelve equal amounts beginning at the time of *Frankia* inoculation. Deionized water served as zero nitrogen control for one set of each group of seedlings. Thirteen treatments per group with eight plants per treatment resulted from the design.

Analyses

Thirteen weeks after inoculation seventeen week old seedlings were harvested and individual biomass determined (ODW, 80°C). The eight plants per treatment were pooled and a subsample was used to determine their total nitrogen content by Kjeldahl procedures. The ^{15}N excess of the pooled samples were determined on a SIRA-9 mass-spectrometer following conversion procedures with Lithium hypobromite outlined by (IAEA, 1976). The percentage of N in the nodulated *A. incana* derived from biological nitrogen fixation (BNF) was calculated using Equation 1 isotope dilution method or Equation 2 for difference method:

$$\% \text{ N fixed} = 1 - \left(\frac{^{15}\text{N excess (fs)}}{^{15}\text{N excess (nfs)}}\right) \times 100 \quad (1)$$

$$\% \text{ N fixed} = \left(\frac{\text{yield of N(fs)} - \text{yield of N (nfs)}}{\text{yield of N(fs)}}\right) \times 100 \quad (2)$$

where fs = fixing system (test plant), nfs = non-fixing system (reference plant).

Results

Biomass accumulations

Frankia isolates from the three *Alnus* hosts made no difference in the growth of *A. incana* seedlings, so they were treated as replicates of the inoculation treatment. The results are presented in Figs. 1A and B. Nodulated unfertilized (no nitrogen) *A. incana* seedlings accumulated the most biomass. The growth of these seedlings were reduced by increasing additions of both ammonium-N and nitrate-N, with the former showing slightly greater toxicity. At 500 $\mu g\,N\,g^{-1}$ fertilization level, the seedlings were severely stunted and only a few survived. Non-inoculated *A. incana* and *B. papyrifera* seedlings which received no nitrogen fertilizer were also severely stunted. However these seedlings responded sharply to ammonium-N up to the 50 $\mu g\,N\,g^{-1}$ level in the case of *A. incana* and up to the 100 $\mu g\,N\,g^{-1}$ in the case of *B. papyrifera*. Growth peaked at 250 μg N g^{-1} then dropped sharply at 500 $\mu g\,N\,g^{-1}$. At these higher ammonium-N levels, biomass of the non-inoculated *A. incana* and *B. papyrifera* was higher than that of the inoculated *A. incana*. Birch appeared to be less sensitive to ammonium toxicity. The response of the reference plants to nitrate-N was less dramatic than that of ammonium-N. The plants fertilized with nitrate-N peaked at 50% of those fertilized with ammonium-N. With nitrate-N, growth of both *A. incana* (non-inoculated) and *B. papyrifera* were identical up to the 100 $\mu g\,N\,g^{-1}$. As with ammonium-N fertilization, the biomass of the reference plants were higher than the inoculated *A. incana* when fertilized with 500 $\mu g\,N\,g^{-1}$ of nitrate-N.

N data and N yield

The atom percent ^{15}N excess of the inoculated *A. incana* increased gradually with ammonium fertilization up to the 50 $\mu g\,N\,g^{-1}$ (Table 1) then sharply at the 100 $\mu g\,N\,g^{-1}$ rate and above to a level approximating the labelled source. In contrast, the labelled ammonium-N in the control plants rose sharply even with 10 $\mu g\,N\,g^{-1}$ addition with almost identical values for non-inoculated *A. incana* and *B. papyrifera*. When nitrate-N was used (Table 2), the uptake of the labelled N was more gradual in response to increasing N levels but the overall pattern was similar to that of ammonium-N fertilization.

The nitrogen yield of the inoculated *A. incana* was highest in the plants which received no nitrogen (Tables 1 and 2). N yield declined gradually with increasing levels of N regardless of which source. N-yield of the control plants were identical to the 250 $\mu g\,N\,g^{-1}$ fertilization level for both N sources. However the plants fertilized with ammonium-N yielded roughly five times the amount of N than those fertilized with nitrate-N. The N yield of the control plants exceeded that of the test plant fertilized with ammonium-N over the 100 $\mu g\,N\,g^{-1}$ level whereas this occurred at the 500 $\mu g\,N\,g^{-1}$ level with nitrate-N fertilization.

Dinitrogen fixation

The two equations mentioned above were used

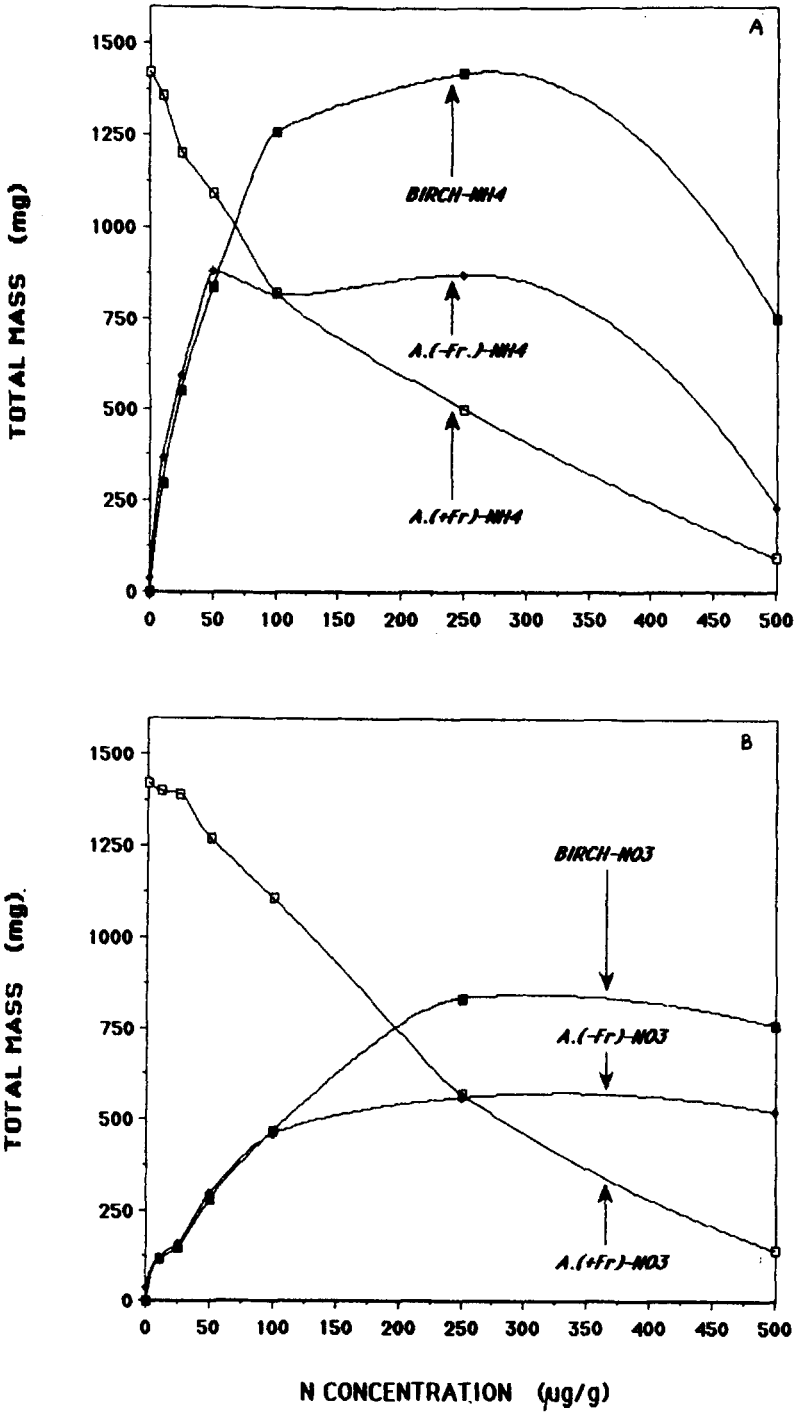

Fig. 1. **A** Changes in total biomass of nodulated (+ Fr), and non-nodulated (− Fr) *Alnus incana* (A.) and white birch seedlings fertilized with varying amounts of ammonium-N. **B.** Changes in total biomass of nodulated (+ Fr), non-nodulated (− Fr) *Alnus incana* and white birch seedlings fertilized with varying amounts of nitrate-N.

Table 1. Ammonium-N effects on ^{15}N excess (%) and nitrogen yield (mg) in test and control plants

N Conc ($\mu g\,g^{-1}$)	^{15}N Excess (%)			Nitrogen yield (mg)		
	Test plant Inoc Ai	Control 1 Non-Inoc Ai	Control 2 Bp	Test plant Inoc-Ai	Control 1 Non-Inoc Ai	Control 2 Bp
0	0.00	0.00	0.00	25.05	0.28	0.01
				±0.20	±0.00	±0.00
10	0.153	3.112	2.856	24.36	2.15	2.02
	±0.014	±0.000	±0.000	±2.90	±0.00	±0.00
25	0.548	4.145	4.219	21.04	3.83	3.52
	±0.048	±0.000	±0.000	±0.41	±0.00	±0.00
50	1.856	4.636	4.632	19.57	9.07	6.78
	±0.403	±0.000	±0.000	±2.58	±0.00	±0.00
100	4.734	5.005	5.132	16.23	18.64	17.65
	±0.137	±0.000	±0.000	±2.02	±0.00	±0.00
250	5.111	5.211	5.199	15.06	29.99	30.70
	±0.021	±0.000	±0.000	±5.75	±0.00	±0.00
500	5.081	5.058	5.218	4.09	6.66	23.65
	±0.093	±0.000	±0.000	±1.82	±0.00	±0.00

Ai = *Alnus incana*, Bp = *Betula papyrifera*.

to calculate the percentage of N in the test plant which was derived from biological nitrogen fixation (BNF). When ammonium-N is the source of nitrogen (Table 3), the isotope dilution and difference methods yielded similar BNF values regardless of which control plants were used. Increasing rates of ammonium-N, however, gradually reduced BNF values to about 60% at $50\,\mu g\,N\,g^{-1}$ rate. At $100\,\mu g\,N\,g^{-1}$ and over, the difference method gave negative values because of greater N yield of control plants. With nitrate-N fertilization good agreement between the two methods was observed up to the $100\,\mu g\,N\,g^{-1}$ level (Table 4). The difference method yielded lower BNF values at $250\,\mu g\,N\,g^{-1}$ and negative values at $500\,\mu g\,N\,g^{-1}$. Increasing nitrate-N also gradually reduced BNF values to about 55% for the isotope dilution method and to about 30% for the difference method at the

Table 2. Nitrate-N effects on ^{15}N excess (%) and nitrogen yield (mg) in test and control plants

N Conc ($\mu g\,g^{-1}$)	^{15}N Excess (%)			Nitrogen yield (mg)		
	Test plant Inoc Ai	Control 1 Non-Inoc Ai	Control 2 Bp	Test plant Inoc-Ai	Control 1 Non-Inoc Ai	Control 2 Bp
0	0.00	0.00	0.00	25.05	0.28	0.01
				±0.19	±0.00	±0.00
10	0.034	1.842	1.695	25.27	0.73	0.79
	±0.006	±0.000	±0.000	±3.70	±0.00	±0.00
25	0.075	3.186	2.675	26.83	1.13	1.06
	±0.015	±0.000	±0.000	±4.33	±0.00	±0.00
50	0.118	3.749	3.974	22.94	1.87	1.85
	±0.019	±0.000	±0.000	±0.81	±0.00	±0.00
100	0.584	4.335	4.106	20.30	2.96	3.33
	±0.189	±0.000	±0.000	±3.13	±0.00	±0.00
250	1.936	4.333	4.400	9.57	6.79	6.42
	±0.532	±0.000	±0.000	±1.49	±0.00	±0.00
500	4.370	4.751	4.981	2.58	9.31	13.23
	±0.015	±0.000	±0.000	±0.35	±0.00	±0.00

Ai = *Alnus incana*, Bp = *Betula papyrifera*.

Table 3. Comparison of % N fixed by isotope dilution and difference methods with ammonium N and two control plants

N Conc ($\mu g\,g^{-1}$)	Isotope dilution		Difference method	
	Control = Ai	Control = Bp	Control = Ai	Control = Bp
0	100.00	100.00	98.88	99.96
	±0.00	±0.00	±0.01	±0.00
10	95.09	94.65	91.09	91.63
	±0.16	±0.50	±1.09	±1.02
25	86.79	87.02	81.79	83.27
	±1.15	±1.13	±0.35	±0.33
50	59.96	59.92	53.15	64.98
	±8.69	±8.70	±5.80	±4.34
100	5.47	7.76	−15.99	−9.83
	±2.74	±2.76	±13.69	±12.97
250	1.92	1.70	−127.06	−132.59
	±0.41	±0.41	±110.29	±112.98
500	−0.46	2.63	−97.48	−601.25
	±1.83	±1.78	±117.46	±417.06

Ai = Non-nodulated *Alnus incana*, Bp = *Betula papyrifera.*

250 $\mu g\,N\,g^{-1}$ level. Again, the two reference plants gave similar BNF values at all levels of fertilization.

Discussion

In a carefully controlled greenhouse study, we have demonstrated that seedlings of white birch were comparable to non-inoculated *Alnus incana* seedlings as reference plants in estimating biological nitrogen fixation by nodulated *A. incana* using the isotope dilution technique. Further, there was very good agreement between this method and the difference method based on total Kjeldahl nitrogen, especially when low to moderate levels of inorganic nitrogen were applied. It was also shown that nitrogen fixation based on both calculations was lowered by increasing levels of ammonium and nitrate sources of N.

The question of reference plant in assessing nitrogen fixation in actinorhizal plants has rarely been addressed. Gauthier *et al.* (1985) used non-inoculated *Casuarina equisetifolia* in methyl bromide fumigated soil to study N-fixation of that species with ^{15}N labelled ammonium. They found that although isotope dilution gave the highest esti-

Table 4. Comparison of % N fixed by isotope dilution and difference methods with nitrate N and two control plants

N Conc ($\mu g\,g^{-1}$)	Isotope dilution		Difference method	
	Control = Ai	Control = Bp	Control = Ai	Control = Bp
0	100.00	100.00	98.88	99.96
	±0.00	±0.00	±0.01	±0.00
10	98.32	98.18	97.07	96.83
	±0.35	±0.38	±0.40	±0.43
25	97.65	97.20	95.72	95.98
	±0.46	±0.55	±0.68	±0.64
50	96.84	97.02	91.84	91.93
	±0.50	±0.47	±0.29	±0.29
100	86.31	85.54	85.21	83.35
	±4.37	±4.61	±2.10	±2.36
250	55.31	55.99	27.97	31.90
	±12.28	±12.09	±10.37	±9.80
500	8.01	12.26	−265.30	−419.11
	±1.58	±1.51	±49.10	±69.77

Ai = Non-nodulated *Alnus incana*, Bp = *Betula papyrifera.*

mation of N-fixation, it was not significantly different from those obtained by the difference and A-value methods, because of highly variable data. In related legume/Rhizobium studies, Rennie (1979) reported that the percent of total plant N in navy beans derived from N-fixation was best determined by the isotope dilution method using non-inoculated plants as reference.

It was not our intention to define the effects of inorganic-N on nodulated *A. incana*. However, we observed a decline in biomass and nitrogen yield as well as calculated biological N-fixation with increasing rates of ammonium and nitrate. Such effects of N fertilization on N-fixation in actinorhizal plants are known (Rodriguez-Barrueco *et al.*, 1970) but stimulation of growth of nodulated alders by inorganic nitrogen has also been described (Mackay *et al.*, 1970). Many factors are implicated in nitrogen nutrition of woody plants. Growth and chemical composition of plants differ considerably when subjected to different forms of nitrogen nutrition. These differences are largely attributable to the differential mode of uptake and energy requirement for the assimilation of different forms of nitrogen and the necessity for the maintenance of an ionic equilibrium within the plant. These in turn are largely influenced by the regulation of pH within the cells (Raven and Smith, 1976).

As opposed to the reduction and assimilation of nitrate-N in both above-ground and below-ground parts of the plant, ammonium-N is largely assimilated in the roots. This could probably explain the toxicity attributed to ammonium-N nutrition (Lea and Miflin, 1980; Raven and Smith, 1976). Differences among species have been reported for actinorhizal plants with regard to the effect of the form of combined nitrogen on the symbiotic system. *Alnus glutinosa* is less sensitive to NH_4-nutrition as compared to *Ceanothus velutinus* and *Hippophae rhamnoides*, which are highly sensitive (Rodriguez-Barrueco *et al.*, 1970; Stewart and Bond, 1961; Wheeler and McLaughlin, 1978).

We conclude from our greenhouse study that white birch (*Betula papyrifera*) could be used as a non-fixing reference plant for estimating nitrogen-fixation by *Alnus incana* in ^{15}N aided studies. We also caution that care should be taken in selecting the form and the amount of the labelled source of nitrogen. Because of the potential for field application, further evaluation of *B. papyrifera* reference plant is underway.

References

Burgess D, Chatarpaul L and Hendrickson O 1986. Field performance of Frankia-inoculated and non-inoculated alders (*Alnus* spp.): Preliminary results. Inf. Rep. PI-X-67, Canadian Forestry Service, Chalk River.

Chalk P M 1985. Estimation of N_2-fixation by isotope dilution: An appraisal of techniques involving ^{15}N enrichment and their application. Soil Biol. Biochem. 17, 389–410.

Chatarpaul L and Carlisle A 1983 Nitrogen fixation – a biotechnological opportunity for Canada. For. Chron. 59, 249–259.

Danso S K A 1986 Review: Estimation of N_2-fixation by isotope dilution: An appraisal of techniques involving ^{15}N enrichment and their applications – Comments. Soil Biol. Biochem. 18, 243–244.

Dawson J D 1986 Actinorhizal plants: Their use in forestry and agriculture. Outl. Agric. 15, 202–208.

Gauthier D, Diem H G, Dommergues Y R and Ganry F 1985 Assessment of N_2-fixation by *Casuarina equisetifolia* inoculated with Frankia ORSO21001 using ^{15}N methods. Soil Biol. Biochem. 17, 375–379.

Goh K M, Edmeades D C and Robinson B W 1978 Field measurements of symbiotic nitrogen fixation in an established pasture using acetylene reduction and a ^{15}N method. Soil Biol. Biochem. 10, 13–20.

IAEA 1976 Tracer Manual on Crops and Soils. Tech. Rep. No. 171. IAEA, Vienna. 277 p.

Johnsrud S C 1978 Nitrogen fixation by root nodules of *Alnus incana* in a Norwegian forest ecosystem. OIKOS 30, 475–479.

Knowles R 1981 The measurement of nitrogen fixation. *In* Current Perspectives in Nitrogen Fixation. Eds. A H Gibson and W E Newton. pp 327–333. Australian Academy of Science, Canberra.

Lalonde M and Calvert H E 1979 Production of *Frankia* hyphae and spores as an infective inoculant for *Alnus* species. *In* Symbiotic Nitrogen Fixation in the Management of Temperate Forests. Eds. J C Gordon, C T Wheeler and D A Perry. pp 95–110. Forest Research Laboratory, Corvallis.

Lea P J and Miflin B J 1980 Transport and metabolism of asparagine and other nitrogen compounds within the plant. *In* The Biochemistry of Plants: A comprehensive treatise. Vol. 5: Aminoacids and Derivatives. Ed. B J Miflin. Acad. Pres. New York. pp 569–607.

Mackay J, Simon L and Lalonde M 1987 Effect of substrate nitrogen on the performance of *in vitro* propagated *Alnus glutinosa* clones inoculated with sp^+ and sp^- *Frankia* strains. Plant and Soil 103, 21–31.

McAuliffe C, Chamblee D S, Hribe-Arango H and Woodhouse W W Jr. 1958 Influence of inorganic nitrogen fixation by legumes as revealed by ^{15}N. Agron. J. 50, 334–337.

Phillips D A 1980 Efficiency of symbiotic nitrogen fixation in legumes. Annu. Rev. Plant Physiol. 31, 29–49.

Raven J A and Smith F A 1976 Nitrogen assimilation and transport in vascular land plants in relation to intracellular pH regulation. New Phytol. 76, 415–431.

Rennie R J 1979 Comparison of ^{15}N-aided methods for determining symbiotic dinitrogen fixation. Rev. Ecol. Biol. Sol. 16, 455–463.

Rennie R J 1982 Quantifying dinitrogen (N_2) fixation in soybeans by ^{15}N isotope dilution: The question of non-fixing control plant. Can. J. Bot. 60, 856–861.

Rennie R J 1986 Comparison of methods of enriching a soil with nitrogen-15 to estimate dinitrogen fixation by isotope dilution. Agron. J. 78, 158–163.

Rennie R J, Rennie D A and Fried M 1978 Concepts of ^{15}N usage in dinitrogen fixation studies. *In* Isotopes in Biological Dinitrogen Fixation. IAEA, Vienna, pp 107–133.

Rodriguez-Barrueco C, Mackintosh A H and Bond G 1970 Some effects of combined nitrogen on the nodule symbioses of *Casuarina* and *Ceanothus*. Plant and Soil 33, 129–139.

Stewart W D P and Bond G 1961 The effect of ammonium-nitrogen on fixation of elemental nitrogen in *Alnus* and *Myrica*. Plant and Soil 14, 347–359.

Wagner G H and Zapata F 1982 Field evaluation of reference crops in the study of nitrogen fixation by legumes using isotope techniques. Agron. J. 74, 607–612.

Witty J F 1983 Estimating N_2-fixation in the field using ^{15}N-labelled fertilizer: Some problems and solutions. Soil Biol. Biochem. 15, 631–639.