Plant and Soil XXVI, no. 2 April 1967

NODULATION AND NITROGEN-FIXATION BY *HIPPOPHAË RHAMNOIDES* L. IN THE FIELD

by W. D. P. STEWART and M. C. PEARSON

Westfield College, University of London, and University of Nottingham

It is generally accepted that the bulk of the biologically fixed nitrogen on the surface of the Earth is leguminous in origin. There is good evidence, however, that other nitrogen-fixing organisms, such as the nodulated non-leguminous angiosperms, for example Alnus, Myrica and Casuarina, and free-living nitrogen-fixing micro-organisms, such as the blue-green algae and the bacteria, contribute considerable quantities of fixed nitrogen to the soil [23]. The latter groups are probably more important in natural ecosystems than in the cultivated soils of which the legumes are so typical.

A natural population which has been studied, particularly from the point of view of nitrogen-fixation by blue-green algae, is that associated with salt marshes [24] and areas of sand dune slack [22]. Evidence to date indicates that in these habitats nitrogen-fixation by free-living organisms may be appreciable. At one of the sites studied – Gibraltar Point Nature Reserve, Lincolnshire (British National Grid Reference TF 555576), a large area of the sand dune system is characterised by the abundance of the nodulated non-leguminous angiosperm *Hippophaë rhamnoides* L., a species which in the laboratory has been shown conclusively to fix nitrogen, both on the basis of long-term growth analyses [6] and by use of the sensitive N^{15} technique.[3] The biology of this plant has recently been reviewed by Pearson and Rogers [18].

Because of the abundance and vigour of Hippophaë at Gibraltar point and thus its large potential contribution to the nitrogen status of the habitat, the experiments to be described in this paper were carried out. These involved measuring the degree of nodulation and nitrogen-fixing capacity of field material and relating this to the nitrogen status of the soil. It is hoped that such studies will help in our understanding of the sources of combined nitrogen in coastal soils.

MATERIALS AND METHODS

The stands of Hippophaë under investigation

In the area studied, *Hippophaë* comprised the dominant vegetation of the dune system and four stands (I to IV) were selected for detailed study. These represented various stages of plant succession, ranging from embryo to mature dunes. The whole area was exposed to rather uniform conditions and there was nothing to suggest a major factor other than the abundance of Hippophaë which would differentially affect the nitrogen status of any of these sites.

Growth and nodulation studies

These were carried out in July, 1963 on selected one square metre areas at each of the four sites. These areas were chosen so that within each there was present what appeared to be a typical sample of the standing crop. The entire Hippophaë plant material, i.e. shoots, roots and nodules within each square metre quadrat, was then extracted carefully, and the nodules counted and separated from the remaining plant material. The term 'nodule' in this paper refers to the branched clustered structure which appears to arise from a single infection point. Dry weight data on root and shoot material were obtained by chopping the plant tissue into small pieces and drying to constant weight at 95° C. Nodule dry weights were also obtained by drying at 95° C. These materials were then milled separately and triplicate samples taken from each were analysed for total nitrogen by the Kjeldahl method. The age of the standing crop was determined by counting the number of growth rings present at ground level on each leader shoot within the sampling area. The mean value obtained gave a measure of the age of the stand.

Measurement of nitrogen-fixation

Two independent methods were employed:

(a) Comparison of total nitrogen yield at stands of increasing age. By this method the total soil nitrogen present (including leaf litter) was determined by taking triplicate cores of soil 0–16 cm deep and 6.4 cm in diameter from beneath the *Hippophaë* stands. Intact root systems present in the cores were not included in the total soil nitrogen estimations. Each sample was then sieved to separate leaf litter from soil, sand, and small organic material, and the dry weight of each fraction determined as described above. Triplicate samples were then assayed for total nitrogen by Kjeldahl analyses and the total soil nitrogen present per site to a depth of 16 cm calculated. This, together with the total plant nitrogen already estimated, was expressed as total nitrogen per site. By difference in total nitrogen and in age of the various stands, total nitrogen accretion in the presence of Hippophaë plants of known age range was calculated. The method of calculation is perhaps best illustrated by taking a specific example, thus: the data in Table 3 (column 5) show that at Site II the plants were 11 years old and that a total of 65.5 g nitrogen per square metre was present, while at Site III the plants were 13 years old and 92.2 g nitrogen per square metre were present. Thus in the presence of plants 11 to 13 years old (92.2–65.5)/2 g nitrogen per square metre per annum, *i.e.* 13.3 g per square metre per annum, were accreted. As will be discussed later not all this nitrogen is necessarily due to nitrogen fixation.

(b) Use of the isotope N^{15}. This method involves exposing the test material to an atmosphere containing N^{15} and after a suitable exposure period determining the quantities of nitrogen assimilated. In studies on free-living nitrogen-fixing organisms it has proved satisfactory [10 12 22] for with these, it is possible because of their small size to enclose intact nitrogen-fixing units. This generally is not feasible with large nodulated angiosperms and the other possibility – that of exposing the actual nitrogen-fixing structures – the root nodules to N^{15} has proved unsatisfactory in the case of legumes, for when detached from the plants the nodules rapidly lose their nitrogen-fixing capacity [2 16]. Despite this, the N^{15} method suggested itself in this particular instance for the two following reasons: Firstly, it would show conclusively whether or not field material of Hippophaë was fixing nitrogen and it would also give an indication of its quantitative significance. It would furthermore eliminate the possibility of measuring in addition gains in total nitrogen due to non-symbiotic nitrogen-fixing organisms, or other sources.

Secondly, Bond [3] showed that detached non-legume root nodules retain their nitrogen-fixing activity for considerably longer than do those of legumes, and that *Hippophaë rhamnoides* is the most active non-legume in this respect. He furthermore showed in studies with legume nodules [5] that by keeping portions of the root systems attached to the nodules, the latter fixed nitrogen more vigorously than did excised nodules. It thus seemed feasible that exposure of nodulated portions of Hippophaë roots to N^{15} for a short period would give an indication of the rate of fixation and this procedure was subsequently adopted. It was continuously borne in mind, however, that the data so obtained would have to be considered as *minimal* values and that the fixation rates by nodules still attached to the plant may be considerably higher. The following are the details of the method: At each of the four sites Hippophaë root systems were excavated carefully and nodulated portions, approximately 12 cm long and bearing at least eight typical nodules clusters, were detached and exposed immediately to N^{15}. The sample containers were specially constructed 150 ml capacity specimen tubes. After insertion of the plant material, the entire system was evacuated and flushed several times with argon, prior to introducing the gas mixture which initially contained 20 per cent nitrogen, enriched with 18.016 atom per cent N^{15}, 20 per cent oxygen, 0.03 per cent carbon dioxide, the remainder being argon. In all instances the nodulated roots were exposed to the N^{15} mixture within 12 minutes of detachment. The containers were then placed on the soil surface in the shade and covered with a thin layer of soil during a 2-hour exposure period. The nodules were then excised from the root material and the attached soil brushed off. They were acidified and subsequently taken back to the laboratory where they were estimated for N^{15}-enrichment by mass spectrometer assay after Kjeldahl digestion and distillation, using methods and instruments reported previously [21].

Nitrate and ammonium-nitrogen in the soil

Every two months, over a 12-month period (January, 1963 to December, 1963) triplicate 200-g fresh-weight samples of the top 16 cm of soil were taken for nitrate-nitrogen, and for ammonium-nitrogen analyses. Nitrate-nitrogen was estimated colorimetrically using a nitrophenol–disulphonic acid method [15] after extraction with copper sulphate and removal of chlorides with silver sulphate. Ammonia was obtained in solution from which it was then released by treatment with a water saturated solution of sodium bicarbonate [19] and distilled into $N/10$ HCl. The ammonia present was then determined colorimetrically by Nesslerization [7].

DATA OBTAINED

Plant growth and dry weight data

These are presented in Table 1. As is to be expected the Hippophaë plants increased from Site I to Site IV, both in age and in total dry weight (columns 2 and 3). Increase in the latter was due not only to increase in height of the bushes but also to increase in density of the stands due to the extensive vegetative propagation of the underground system. Indeed at Site IV it was almost impossible to penetrate the Hippophaë thicket.

TABLE 1

Growth and dry-weight data for a field population of *Hippophaë rhamnoides* (plant material per square metre of soil surface)

(1) Site	(2) Mean age of plants (years)	(3) Total plant weight (g)	(4) Total nodule weight (g)	(5) Nodule weight as % of total plant weight	(6) Number of nodules	(7) Mean weight per nodule (mg)
I	3	1053	2.5	0.24	98	26
II	11	1997	39.0	1.95	370	105
III	13	4317	64.2	1.49	313	205
IV	16	13929	2.7	0.02	22	123

Nodule weight (column 4), in contrast to total plant weight, was greatest at mid-dune levels (Sites II and III) and decreased sharply at Site IV to a level comparable with that at Site I. Thus nodule weight as a percentage of total plant weight (column 5) was twelve times less at Site IV than at Site I, with highest values being obtained at mid-dune levels. The increase in percentage nodule weight to a maximum at Site II indicates that the bushes at Site I were not fully nodulated; the decrease at Sites III and IV may be related to the higher levels of combined nitrogen which occur on moving up the dune system (see next paragraph). Nodule numbers (column 6) and nodule size (column 7) were greatest at Sites II and III respectively; in the embryo dune a large number of small nodules was present in contrast to Site IV where the opposite was the case. Thus nodulation in terms of total dry weight, percentage dry weight, nodule number and dry weight per nodule was always greatest at mid-dune levels.

Plant-nitrogen data

The data obtained are shown in Table 2. It is clear (column 3) that the percentage nitrogen content of the root plus shoot material is lower at Site IV than at the lower dune levels. The percentage nitrogen contents of the nodules (column 5) are approximately twice as great as that of the root plus shoot material, suggesting that the nodules are fixing nitrogen. In general, total plant nitrogen and total nodule nitrogen values are proportional to total dry matter values.

TABLE 2

Nitrogen data for a field population of *Hippophaë rhamnoides*

(1) Site	(2) Mean age of plants (years)	(3) Mean percentage N-content of roots plus shoots	(4) Total N in roots plus shoots per m² of soil surface (g)	(5) Mean percentage N-content of nodules	(6) Total N in nodules per m² of soil surface (g)	(7) Total plant N per m² of soil surface (g)
I	3	0.61	6.41	1.5	0.04	6.45
II	11	0.64	12.53	1.2	0.47	13.00
III	13	0.66	28.07	1.3	0.84	28.91
IV	16	0.54	75.20	1.0	0.03	75.23

Nitrogen fixation data

a) Total nitrogen analyses. The data on total plant plus soil nitrogen at successive sites are shown in Table 3. Data on total nitrogen present in a typical embryo dune system where Hippophaë plants had not yet become established and where *Ammophila arenaria* was

TABLE 3

Total nitrogen present in *Hippophaë rhamnoides* stands of different ages

(1) Site	(2) Mean age of plants (years)	(3) Total soil N per m² of soil surface (g)*	(4) Total plant N per m² of soil surface (g)	(5) Total soil plus plant N per m² of soil surface (g)	(6) N accretion per m² per annum (g)
0	–	1.96	–	1.96	–
I	3	3.68	6.45	10.13	2.72
II	11	52.54	13.00	65.54	6.93
III	13	63.28	28.91	92.19	13.33
IV	16	70.62	75.23	145.85	17.89

* Any non-Hippophaë plant nitrogen is included in this value.

the only angiosperm present, are included to provide a measure of the total nitrogen initially present (Site 0). Plant nitrogen and soil nitrogen both increased from site I to site IV, largest gains in soil nitrogen being associated with plants 3 to 11 years old (column 3) and largest gains in plant nitrogen with 13 to 16 years old plants (column 4). The large relative increase in plant: soil nitrogen at Site IV perhaps implies that assimilation of soil nitrogen by the plant is occurring on an appreciable scale. The increases in total nitrogen on passing from Sites I to IV are large and if extrapolated into kg per hectare per annum give the values summarised in Table 4, that is increases range from 27 kg per hectare per annum in the presence of plants 0 to 3 years old to 179 kg per hectare per annum in the presence of plants 13 to 16 years old. As large expanses of the dune system at Gibraltar Point are rather uniformly covered with Hippophaë this extrapolation into kilograms per hectare seems justified.

TABLE 4

Increases in total nitrogen (kg per hectare per annum) in the presence of Hippophaë bushes of known age range

Age range	0–3 years	3–11 years	11–13 years	13–16 years
Increase in N	27.2	69.3	133.3	178.9

b) N^{15}-analyses. The data (Table 5) show convincingly that the nodules at all sites fix atmospheric nitrogen and that fixation was on a considerable scale considering the short exposure period (two hours). They furthermore confirm the assumption made on the basis of laboratory data that field nodules should fix nitrogen [3 6] and extend the range of nodulated non-legumes for which nitrogen fixation by field material has been demonstrated using N^{15}, [4 13 17 28].

The N^{15}-labelling of the nodules (column 3) show that those on the youngest plants are most active in fixation and that efficiency decreases with age. This is probably due to the nodules being perennial structures and thus the older ones contain a higher proportion of

TABLE 5

Assimilation of N^{15} by field nodules of *Hippophaë rhamnoides*

(1) Site	(2) Mean age of plants (years)	(3) Mean atom % N^{15} in exposed nodules*	(4) N fixed per m^2 of soil surface during exposure period (mg)**	(5) N fixed per m^2 per annum (g)
I	3	0.509	0.30	0.44
II	11	0.478	2.86	4.19
III	13	0.453	3.93	5.75
IV	16	0.432	0.10	0.15

* Atom % N^{15} in gas mixture = 18.016; atom % N^{15} in air sample = 0.368; atom % N^{15} in unexposed nodule material = 0.370.

** The exact quantities of nodule nitrogen present per site were: Site I = 38 mg; Site II = 468 mg; Site III = 835 mg; Site IV = 27 mg.

wood and cork which contributes nothing to fixation. The total amount of nitrogen fixed per site however is not dependent solely on the efficiency of the nodules in fixation. The total nodule nitrogen present must also be taken into account and when this is done it is seen that increase in total nodule nitrogen at Site III (Table 2, column 6) offsets the decrease in nodule efficiency so that plants 11 to 13 years old fix the largest quantities of nitrogen (Table 5, column 4). By extrapolation of these N^{15} data it is possible to obtain an estimate of the quantities of nitrogen fixed, assuming that as in Alnus [20] fixation occurs during a 4-month summer period. These data (Table 5, column 5) show a rather similar trend to the total nitrogen data (Table 3 column 6) except at Site IV where the N^{15}-data and total nitrogen data are in complete contrast. The estimates obtained using the N^{15}-method are considerably lower than those achieved using the total nitrogen method, and because of the various assumptions which have to be made in extrapolation, are best considered as orders of magnitude rather than as specific values. Possible reasons for the difference in values obtained using both methods will be discussed later.

Seasonal variation in inorganic soil nitrogen

The data on ammonium-nitrogen, nitrate-nitrogen and average monthly rainfall during 1963 are shown in Table 6. As with total nitrogen both forms of inorganic nitrogen increase up the dune system. They also show a marked seasonal variation with maximum values being obtained in the winter months and minimum values in the summer. In general there is an inverse correlation between the seasonal variation in rainfall and inorganic nitrogen levels.

TABLE 6

Levels of ammonium- and nitrate-nitrogen present (ppm oven-dry soil) under *Hippophaë rhamnoides* and average monthly rainfall (inches) during the sampling period

Nitrogen type	Site	Month					
		February	April	June	August	October	December
Ammonium nitrogen*, ppm	I	2.0	0.7	< 0.1	< 0.1	2.4	2.9
	II	5.2	5.8	< 0.1	< 0.1	1.6	6.0
	III	6.1	4.7	0.1	0.1	2.4	7.8
	IV	9.9	9.8	0.1	0.1	2.8	11.2
Nitrate-nitrogen*, ppm	I	0.2	1.2	< 0.1	< 0.1	< 0.1	1.5
	II	1.8	4.7	0.1	3.0	< 0.1	4.7
	III	5.1	2.5	0.1	1.5	0.2	10.0
	IV	9.7	6.2	1.0	8.0	0.1	29.0
Mean monthly rainfall (inches)		0.1	1.4	1.1	3.8	0.8	0.5

* Each value is the mean of triplicate determinations.

DISCUSSION

At Gibraltar Point, Lincolnshire, like various other coastal habitats in Britain, *Hippophaë rhamnoides* is an important early coloniser of areas initially low in combined nitrogen. Laboratory data [3 6] provide strong evidence that in such a situation this plant should fix nitrogen providing: 1) effective root nodules are present, and 2) the levels of combined nitrogen in the soil are not sufficiently high to inhibit the process completely.

The presence of root nodules on plants from all sites (Table 1) satisfies the first requirement. The increased nodulation at Sites II and III compared with Site I signifies an increase in the number of infection points, or of infection points which ultimately develop into nodules. The sharp fall off at Site IV is attributable in the main to shedding of the old nodules with few fresh nodules being formed.

As can be seen (Table 3) the total quantities of nitrogen present increase with increase in age of the dune system and, except at Site I, soil nitrogen is higher or of the same order as total plant nitrogen. The low level of soil nitrogen at the latter site is probably due to the presence of large amounts of freshly blown sand in the embryo dune system. The high soil nitrogen:plant nitrogen ratios obtained are in contrast with the findings of Dommergues [11], who observed in his particular studies on Casuarina that on the whole plant nitrogen was considerably greater than soil nitrogen. The levels of inorganic nitrogen in the soil are inversely correlated to the level of monthly rainfall but assimilation of inorganic nitrogen by the plants undoubtedly also contributes to the low levels during the summer (Table 6). These variations are of particular interest in relation to nitrogen fixation for they imply that during the summer months (the time of the year when nitrogen fixation occurs) the levels of inorganic nitrogen in the soil are so low that they are unlikely to inhibit fixation. The N^{15}-data in Table 5 do in fact confirm that nitrogen fixation occurs.

While it is reasonable to attribute much of the total nitrogen gains to nitrogen fixation by Hippophaë, the exact amounts fixed by this angiosperm cannot be determined with certainty using the total nitrogen method. As mentioned in the Introduction asymbiotic nitrogen-fixing organisms may be abundant and important in natural habitats. Also, nitrogen fixation by such organisms has been demonstrated, using N^{15}, in a region of sand-dune system not very different from the one at present under consideration [22]. (It should be mentioned however that from superficial examination there was no evidence of a rich flora of potential nitrogen fixing algae at any of the sites studied in this investigation). It has also been reported [14] that nitrogen fixation is associated with the rhizosphere bacteria of the non-nodulated monocotyledon *Ammophila arenaria*, and in fact almost all the combined nitrogen recorded in the fore-dune sand not yet colonised by Hippophaë (Table 3, Site 0) was present in the Ammophila plants in the samples. This of course does not necessarily mean that the nitrogen present on the fore-dune sand was due to nitrogen fixation. A possible source of combined nitrogen is the sea.

It is known that the thin surface layer of the ocean is rich in organic material [26], and that ocean foam and spray when blown on to the land contributes appreciable quantities of combined nitrogen [27]. In studies on coastal soils therefore this may lead to an overestimation, when using the total nitrogen method, of the quantities of nitrogen actually fixed. On the debit side some fixed nitrogen must be lost by leaching particularly during the early stages in succession when the plants are growing on almost pure sand. Secondly, denitrification may be occurring but its magnitude, like that of leaching, is unknown. However, Allison [1] on reviewing some of the lysimeter data for American agricultural soils, concluded that on average approximately 40 per cent of the total nitrogen added to soils cannot be accounted for even when losses by leaching are taken into account. Despite these limitations there is no doubt that large increases in total nitrogen do occur in the presence of Hippophaë bushes and that these are comparable with gains obtained in arable regions in the presence of a good leguminous crop.

The data obtained using N^{15} on the other hand give values for fixation of approximately sixty-four to forty-three per cent of that obtained at Sites II and III using the total nitrogen method, while those at Sites I and IV are considerably lower. It is impossible to say with certainty whether this is due to the total nitrogen method overestimating fixation, the N^{15} method underestimating fixation, or both. As stressed earlier, because of the possible loss of activity on detaching the roots from the plants, the values obtained using the isotopic method must be regarded as minimum values, but at the same time they do show conclusively that the plants are actually fixing nitrogen and at Sites II and III at least a considerable proportion of the total nitrogen present is due to fixation by Hippophaë.

The very large discrepancy in the results obtained at site IV using both methods requires closer scrutiny. It is clearly impossible for bushes 16 years old to fix approximately 18 kg per square metre per annum, as the total nitrogen data suggest (Table 3), for as can be seen from the nodulation data (Table 1), few nodules are present and supposing these fixed nitrogen many times more efficiently than the most efficient nitrogen fixing nodules (those at Site I) the total amount of nitrogen fixed would still be very small. It appears likely therefore that fixation increases to a maximum in bushes more than

13 but less than 16 years old when the mean fixation per square metre would in fact be greater than 18 kg and that this establishes a climax vegetation in which large-scale nodule shedding without fresh nodulation occurs. In this connection it should be noted that nodule shedding in legumes is reported [8] to be stimulated by shading and defoliation; that is, by rather similar conditions to those which exist at Site IV of the present study.

Finally, two points may be considered. First, the data presented here on growth, nodulation and nitrogen fixation by Hippophaë in the field do, on the whole, confirm and extend those obtained by Bond and his co-workers on the basis of laboratory studies [3 6] and thus emphasise the important contributions which the latter make to our understanding of the biology of this particular group of nodulated non-legumes which are difficult to examine in the field. Secondly, the data now available for Hippophaë support the contention that these non-legumes markedly increase the nitrogen status of the environments in which they occur. If one compares the Hippophaë data obtained using the total nitrogen method with values obtained by other workers for other non-legumes, it is seen that those for Hippophaë compare favourably. For example Dommergues [11] demonstrated that 58 kg per hectare per annum were fixed over a 13-year period in the presence of *Casuarina equisetifolia* in a tropical sand dune system. It has also been calculated that *Alnus crispa* trees contribute to the soil 49 kg nitrogen per hectare per annum over a 50 year period [9] and that in an *Alnus glutinosa* grove of plants up to 8 years old about four times this amount may be fixed per annum [25]. It seems likely on the basis of data such as the above that the quantities of nitrogen fixed by nodulated non-legume plants in the field have not always been fully appreciated.

SUMMARY

1. The extent of nodulation and nitrogen fixation by a population of *Hippophaë rhamnoides* bushes occurring on a sand dune system on the east coast of England has been studied.

2. Nodules were found on all plants (the latter ranged from 3 to 16 years in age) and using the isotope N^{15} it was confirmed that these nodules fixed nitrogen. The nodules on the youngest plants were most active in fixation but because of the greater total nodule mass older plants fixed the largest quantities of nitrogen.

3. Large increases in total nitrogen (*i.e.* soil plus plant nitrogen) occurred with increase in age of the plants, such nitrogen increases ranging from 27 kg per hectare per annum in the presence of bushes 0 to 3 years old to 179 kg per hectare per annum in the presence of bushes 13 to 16 years old.

4. The N^{15}-technique indicated that a proportion of the nitrogen accumulated was due to fixation by *Hippophaë* but nitrogen contributions from other sources could not be ruled out.

5. The levels of nitrate-nitrogen and ammonium-nitrogen in the soil under *Hippophaë* showed a marked seasonal variation, increasing to a maximum in the winter and to a minimum in the summer. These levels were in general inversely related to the level of average monthly rainfall. Because of the low levels of combined nitrogen in the soil during the summer months it seems unlikely that combined nitrogen should markedly inhibit fixation in this situation.

ACKNOWLEDGEMENTS

The authors are grateful to Professor G. E. Fogg, F. R. S. and Professor G. Bond for their interest in this work and for their suggestions on improving the manuscript. Thanks are also due to Professor C. G. C. Chesters in whose department part of the work was carried out. The mass spectrometer used was made available through a grant from the Department of Scientific and Industrial Research to the Department of Botany, Westfield College. We wish to thank Mr. T. Lymn for technical assistance and the Lincolnshire Naturalists' Trust for allowing us to do the work at Gibraltar Point and for supplying the rainfall data.

Received January 11, 1965.

REFERENCES

1 Allison, F. E., The enigma of soil nitrogen balance sheets. Advances Agron. **7**, 213–250 (1955).

2 Aprison, M. H. and Burris, R. H., Time course of fixation of N_2 by excised soybean nodules. Science **115**, 264–265 (1952).

3 Bond, G., An isotopic study of the fixation of nitrogen associated with nodulated plants of Alnus, Myrica and Hippophaë. J. Exptl. Botany **6**, 303–311 (1956)

4 Bond, G., Evidence for fixation of nitrogen by root nodules of alder (Alnus) under field conditions. New Phytol. **55**, 147–153 (1956).

5 Bond, G., Inhibition of nitrogen fixation in non-legume root nodules by hydrogen and carbon monoxide. J. Exptl. Botany **11**, 91–97 (1960).

6 Bond, G., Fletcher, W. W., and Ferguson, T. P., The development and function of the root nodules of Alnus, Myrica and Hippophaë. Plant and Soil **5**, 309–323 (1954).

7 Burris, R. H., and Wilson, P. W., Methods for measurement of nitrogen fixation. *In* Methods in Enzymology **4**, 355–365 (1957).

8 Butler, G. W. and Bathurst, N. O., The underground transferance of nitrogen from clover to associated grass. 7th Intern. Grassl. Congr. Palmerston North, New Zealand, Paper No. **14**, (1956).

9 Crocker, R. L. and Major, J., Soil development in relation to vegetation and surface age at Glacier Bay, Alaska. J. Ecol. **43**, 427–448 (1955).
10 Delwiche, C. C. and Wijler, J., Non-symbiotic nitrogen fixation in soil. Plant and Soil **7**, 113–129 (1956).
11 Dommergues, Y., Evaluation du taux de fixation de l'azote dans un sol dunaire reboise en filao (*Casuarina equisetifolia*). Agrochim. **7**, 335–340 (1963).
12 Dugdale, V. A. and Dugdale, R. C., Nitrogen metabolism in lakes, II. Role of nitrogen fixation in Sanctuary Lake, Pennsylvania. Limnol. Oceanogr. **7**, 170–177 (1962).
13 Harris, G. P. and Morrison, T. M., Fixation of nitrogen by excised nodules of *Coriaria arborea* Lindsay. Nature (London) **182**, 1812 (1958).
14 Hassouna, M. G. and Wareing, P. F., Possible role of rhizosphere bacteria in the nitrogen nutrition of *Ammophila arenaria*. Nature (London) **202**, 467–469 (1964).
15 Jackson, M. L., Soil Chemical Analysis. London, Constable (1958).
16 Magee, W. E. and Burris, R. H., Fixation of N_2^{15} by excised nodules. Plant Physiol. **29**, 199–200 (1954).
17 Morrison, T. M., Fixation of nitrogen-15 by excised nodules of *Discaria toumatou*. Nature (London) **189**, 945 (1961).
18 Pearson, M. C. and Rogers, J. A., *Hippophaë rhamnoides* L. Biological flora of the British Isles. J. Ecol. **50**, 501–513 (1962).
19 Piper, C. S., Soil and Plant Analysis, Adelaide, University of Adelaide (1942).
20 Stewart, W. D. P., A quantitative study of fixation and transfer of nitrogen in Alnus. J. Exptl. Botany **13**, 250–256 (1962).
21 Stewart, W. D. P., Nitrogen fixation by Myxophyceae from marine environments. J. Gen. Microbiol. **36**, 415–422 (1964).
22 Stewart, W. D. P., Nitrogen turnover in marine and brackish habitats, I: Nitrogen fixation. Ann. Botany (London) N.S. **29**, 229–239 (1965).
23 Stewart, W. D. P., Nitrogen Fixation in Plants. London, Athlone Press, University of London (1966).
24 Stewart, W. D. P. and Pugh, G. J. F., Blue-green algae of a developing salt marsh. J. Mar. Biol. Assoc. U.K. **43**, 309–317 (1963).
25 Virtanen, A. I. and Miettinen, J. K., Biological nitrogen fixation. *In* Plant Physiology, ed. F. C. Steward, Vol. **3**, p. 539–668. New York; Academic Press (1963).
26 Wangersky, P. J., The organic chemistry of sea water. Am. Sci. **53**, 358–374 (1965).
27 Wilson, A. T., Surface of the ocean as a source of air-borne nitrogenous material and other plant nutrients. Nature (London) **184**, 99–101 (1959).
28 Ziegler, H. and Hüser, R., Fixation of atmospheric nitrogen by root nodules of Comptonia peregrina. Nature (London) **199**, 508 (1963).