NITROGEN FIXATION IN RIVERINE WETLAND PLANT COMMUNITIES

A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy.

by Caroline Elizabeth Allan

DECLARATION

I hereby declare that this thesis is composed of work carried out by myself unless otherwise acknowledged and cited and the thesis is of my own composition. The research was carried out in the period October 1991 to September 1995. This dissertation has not in whole or in part been previously presented for any other degree.

I dedicate this work to my Dad,
who sadly is not here to see this thesis completed.

ABSTRACT

The occurrence of biological nitrogen fixation was examined in wetland plant communities of three riverine wetland catchments of differing geography, climate and land use in the British Isles. Two of the three were also targeted by an EC project 'Functional Analysis of European Wetland Ecosystems'. Within the three catchment areas, a total of 17 sites were surveyed.

Repeated visits were made to the sites during the period 1993-1995. Species abundance (frequency), legume density and cover and a suite of environmental parameters were measured. Indirect measurements of legume and soil (free-living) nitrogen fixation (acetylene reduction assays) were conducted at three sites in Ireland and Scotland. Further detailed studies using both acetylene reduction and ^{15}N methodology were focused on one site at Ring Bog, Loch Lomond, Scotland.

By using TWINSPAN to classify sites by their species composition and successfully comparing these groups to NVC and CORINE categories, it was confirmed that several different wetland plant communities had been surveyed. Nine species of legumes were found at the riverine wetlands investigated in this study. Canonical correspondence analysis revealed that the most influential of the measured environmental factors on species distribution were flooding regime, soil wetness class, soil pH and land management. Legume species were ranked along these environmental gradients. Legume cover was negatively correlated with the flooding regime gradient.

Decreases in both growth and nodulation in *Trifolium pratense* and *Trifolium dubium* in 1994 were attributed to early winter (1993) and late spring (1994) floods. Mowing for hay in 1993 however, was not detrimental to subsequent growth and nodulation in *Lathyrus pratensis* and *Vicia cracca* when compared to 1994, when mowing took place after assessments were completed. All the species of legumes examined for nitrogenase activity reduced acetylene to ethylene, illustrating their potential to fix nitrogen. Although no accurate value of nitrogen fixation was assigned to the Irish species (*L. pratensis*, *V. cracca*, *T. dubium* and *T. pratense*) all had the potential to fix nitrogen and therefore to contribute to the nitrogen economy of the riverine wetlands in which they were found.

The cyclic nature of growth and nodulation in *Lotus pedunculatus* was revealed (1993-1995). Die back and decay occurred in the winter months (thus providing input of biologically fixed nitrogen into Ring Bog), with regrowth the following

spring. Root and nodule growth occurred mainly in the above-ground vegetation and litter layer in *L. pedunculatus* at Ring Bog, a phenomenon not previously recorded.

L. pedunculatus at Ring Bog also reduced acetylene to ethylene and more detailed investigations revealed its ability to incorporate $^{15}N_2$. The rate of nitrogen fixation for *L. pedunculatus* was estimated conservatively at 11.2 kg N ha^{-1} yr^{-1}. An isotope dilution experiment conducted in the greenhouse on material collected from the field gave the percent contribution of atmospheric nitrogen to the tops of *L. pedunculatus* as 73-76%. Natural abundance of ^{15}N in *L. pedunculatus* and non-legume species of similar growth form in Ring Bog illustrated the unsuitability of using the two source model of Shearer & Kohl (1986) in an ecosystem far removed from the homogenous agricultural situation.

In the more detailed study at Ring Bog, the acetylene reduction assay showed the nitrogen fixing potential of surface peat soil and bryophytes. This was reinforced by direct measurements of nitrogen fixation by incorporation of $^{15}N_2$. Nitrogen fixation by the peat soil was attributed to free-living bacteria and the annual rate of nitrogen fixation in the surface peat soil was estimated conservatively to be 55.8 kg N ha^{-1}. The fixation occurring in the bryophytes may be attributed to an association between the mosses (*Sphagnum palustre*, *Sphagnum recurvum* and *Polytrichum commune*) and cyanobacteria and/or heterotrophic bacteria. Rates of nitrogen fixation in the mosses were estimated conservatively at 0.3-1.4 kg N ha^{-1} yr^{-1}. $^{15}N_2$ incorporation showed the occurrence of N_2 fixation in association with the monocotyledonous species (*Agrostis stolonifera*, *Carex curta*, *Juncus acutiflorus* and *Molinia caerulea*). Rates of nitrogen fixation for these monocotyledonous plants were estimated conservatively at 0.04- 9.7 kg N ha^{-1} yr^{-1}.

Following the exploratory findings of the vegetation analysis and the environmental influences on legume distribution, a number of greenhouse experiments examining flooding (at different plant ages and flooding duration), limited water supply and defoliation were conducted on six selected legume species. Of these species, *T. dubium* and *T. pratense* were most susceptible to short term flooding and limited water supply, both biomass accumulation and nitrogen fixation were reduced by these treatments. Short term flooding and limited water supply had no significant effect on biomass accumulation and nitrogen fixation in *L. pedunculatus*, *T. repens* and *V. cracca*. Nitrogen fixation in *L. pratensis* however, was reduced by limiting water supply but short term flooding had no significant effect on this species.

T. pratense was the only species to suffer detrimental effects from short term flooding (with recovery period) at the seedling stage of development. *L. pedunculatus* was not affected by flooding until the treatment was imposed for a prolonged period of time, and even then only biomass was reduced, nitrogen fixing activity remaining unaffected. The distribution of these legumes at field sites with varying flooding regimes (as shown by CCA analysis), corresponds well to their ability to tolerate flooding shown in the greenhouse experiments.

Out of the six species, greenhouse experiments showed that, *L. pedunculatus* and *T. repens* have the greater ability to recover from defoliation/disturbance (as imposed by managerial practices such as cutting for hay or grazing) as they recovered from defoliation and also developed adventitious roots. *V. cracca*, recovered from defoliation but did not develop adventitious roots. Both *T. pratense* and *T. dubium*, formed adventitious roots but did not recover well from defoliation. The species apparently least likely to recover from defoliation/disturbance was *L. pratensis*, which did not recover from defoliation or form adventitious roots. These greenhouse findings however, do not correspond well to the inferred rankings from CCA analysis (in order of likeliness to be found at a managed (cut or grazed site): *T. dubium*> *T. pratense*> *V. cracca*> *L. pratensis*> *T. repens*> *L. pedunculatus*). This difference is probably due to difficulties of matching in the greenhouse, the severity of cutting/grazing pressures to which legumes were exposed in the field.

ACKNOWLEDGEMENTS

This project was carried out with funding from the Biotechnology and Biological Sciences Research Council and I would like to thank Professor R. Cogdell (formerly of the Botany Department) and Professor F. Huntingford of the Division of Environmental and Evolutionary Biology for allowing me to use their facilities. I would like to thank Dr Chris Wheeler and Dr Kevin Murphy for their supervision and for giving me the opportunity to make numerous visits to the Republic of Ireland.

I am very grateful to the many people who helped me with field work, often having to endure less than pleasant weather conditions; Vicky Abernethy, Aileen Adam, Irene Durant, Jeremy Hills, Anna Milligan, Paula Rattray, Mark Tebbitt, Nigel Willby and to Gordon Allison for getting me there.

Thanks must also go to everyone at the Garscube Research Laboratory; Don Clarke, Bob Cuthbertson, Margaret Gallacher, Jamie Graham, Rita McCormick and particularly Aileen Adam whose help I would have been lost without. Other technical assistance was most gratefully provided by Eoin Robertson for carrying out nodule sections, Jim Muckersie for creating my magnificent wheelbarrow amongst other things and Alison Sutcliffe for all her help, in particular for teaching me the joys of kjeldahl digestions. I would particularly like to thank Vicky and Paula for helping me pick and count nodules well into the night on more than one occasion.

A number of organisations outside the University must also be acknowledged; the Irish Wildlife Service, in particular Debbie Hacket and Jim Ryan; Scottish Natural Heritage and Macleans Farm for allowing me access to Ring Bog, Linda Handley and Charlie Scrimgeour of the Scottish Crop Research Institute for their great amount of help in my $\delta^{15}N$ analysis and the Beatson Cancer Research Institute for use of their five place balance. Thanks also to Andy MacMullen for helping with moss identification, Clyde River Purification Board for providing rainfall data and all the FAEWE team for their help and provision of environmental data and Nigel Willby for providing Plate 5. I am grateful to Vicky Abernethy, Nigel Willby and Linda Handley for comments on the text and to Linda Morrison (the qualified proofreader!) for proof reading the final version. Fieldwork in Ireland was made all the more easier with the kind help of Séamus and Céline Grennan who always made us feel very welcome and Killeens Public House for their free-flowing hospitality.

I am indebted to my entire family for their love and support through my numerous years at University and to all my friends who made doing this PhD more bearable. A special thanks must go to Paula who was always there to take my mind off work and made sure my drinking arm was regularly exercised and last but not least, I would like to thank Mark Tebbitt, cheers!

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LIST OF ABBREVIATIONS

The following abbreviations were used in this thesis:

ARA:	Acetylene Reduction Assay
TWINSPAN:	Two-Way Species Indicator Analysis
CA:	Correspondence Analysis
DCA:	Detrended Correspondence Analysis
CCA:	Canonical Correspondence Analysis
NVC:	National Vegetation Classification

Chapter 1
INTRODUCTION

1. INTRODUCTION

1.1 AIMS

The study had four main aims:

1) To establish the contribution of legumes to the species composition of riverine wetland plant communities in the British Isles.

2) To investigate the role of legumes as a source of biologically fixed nitrogen within riverine wetlands.

3) To identify sources of biologically fixed nitrogen other than legumes that may contribute to the nitrogen economy of riverine wetlands.

4) To determine the effects of the wetland environment on legume growth and nitrogen fixation.

This study ran in conjunction with a previously established interdisciplinary research programme "Functional Analysis of European Wetland Ecosystems" (FAEWE) (Maltby *et al.*, 1994). The FAEWE project concentrated on river marginal wetlands, its objective being to develop "science based procedures for evaluating the functional characteristics of European wetland systems". The lack of information on the importance of biological nitrogen fixation to wetland ecosystems makes this investigation pertinent for the understanding of the processes operating within them. All the sites used in this study were FAEWE selected sites with the exception of one (Ring Bog, see Chapter 2) and data was used and discussed collaboratively within the project. However, this study was sufficiently detailed and comprehensive to stand alone. A combination of field and greenhouse studies was used to achieve the above aims.

1.2 WETLAND ECOSYSTEMS

1.2.1 Nitrogen cycle

Following the classification scheme of Cowardin *et al.* (1979) a wetland is defined as "an area where the water table, is at, near, or above the land surface long enough to promote hydric soils, where the predominant vegetation is hydrophytes." Thus wetlands have properties of both terrestrial and aquatic ecosystems, and are distinguished by their potentially changing water levels and waterlogged soil. The cycling of nutrients in wetlands is therefore different to that found in other ecosystems.

Nitrogen cycling in wetland soils has been described by many authors, including: Howard Williams (1985); Bowden (1987); Reddy & Graetz (1988). The cycling of nitrogen in any wetland is governed by various interacting factors (Gosselink & Turner 1978) which can be categorised as follows:

- Climatic factors - If precipitation does not exceed evapotranspiration, nitrogen transformations are impaired in the soil by the reduced water supply and temperature affects the processing of nitrogen by microbes and plants.
- Geological and hydrological factors - The source and flux of water controls nitrogen input into wetlands. "Riverine wetlands occur along the shores of rivers or other types of situations where water is primarily restricted to a stream channel", (Whigham & Bayley, 1978) and most freshwater riverine wetlands receive a continual input and output of nutrients and organic matter as a result of hydrological fluctuations.
- Biological factors - Nitrogen is utilized in plant production, thus reducing losses from the soil and internal nitrogen dynamics can be controlled by plant litter and its associated microbes.

In submerged soils the principle nitrogen source for higher plants is ammonium. Such waterlogged soils have characteristically low oxygen concentrations. The penetration of oxygen is restricted, and that reaching the soil surface is used rapidly, either by microbial action or by the chemical oxidation of reduced iron and manganese. Two distinct layers form: an oxidized (aerobic) layer and an underlying reduced (anaerobic) layer (Patrick & Tusneem, 1972). If the consumption of oxygen is very high and the oxygen renewal rate from floodwater is low then the soil may be completely anaerobic. A deeper aerobic layer will occur if oxygen consumption rates are low (Buresh *et al.*, 1980; Reddy & Patrick, 1984).

The full cycle of nitrogen in wetland soils is illustrated in Fig. 1.1. The decomposition of dead plant material (organic nitrogen) to inorganic nitrogen (ammonification) is the major process supplying nitrogen in a form available to wetland plants (Reddy & Graetz, 1988). Ammonification rates are usually highest in the aerobic zone, but this is usually less than 1 cm in depth, so aerobic ammonification contributes little to overall mineralization compared to anaerobic ammonification at greater depths. Some of this ammonium is lost from the soil via ammonia volatilization, (particularly when the overlying water is alkaline). The formation of two layers (aerobic and anaerobic) promotes simultaneous nitrification and denitrification in wetland soils (Patrick & Reddy, 1976). The aerobic layer favours the oxidation of ammonium to nitrite and then to nitrate (nitrification). After nitrate diffusion to the anaerobic layer, the reducing conditions

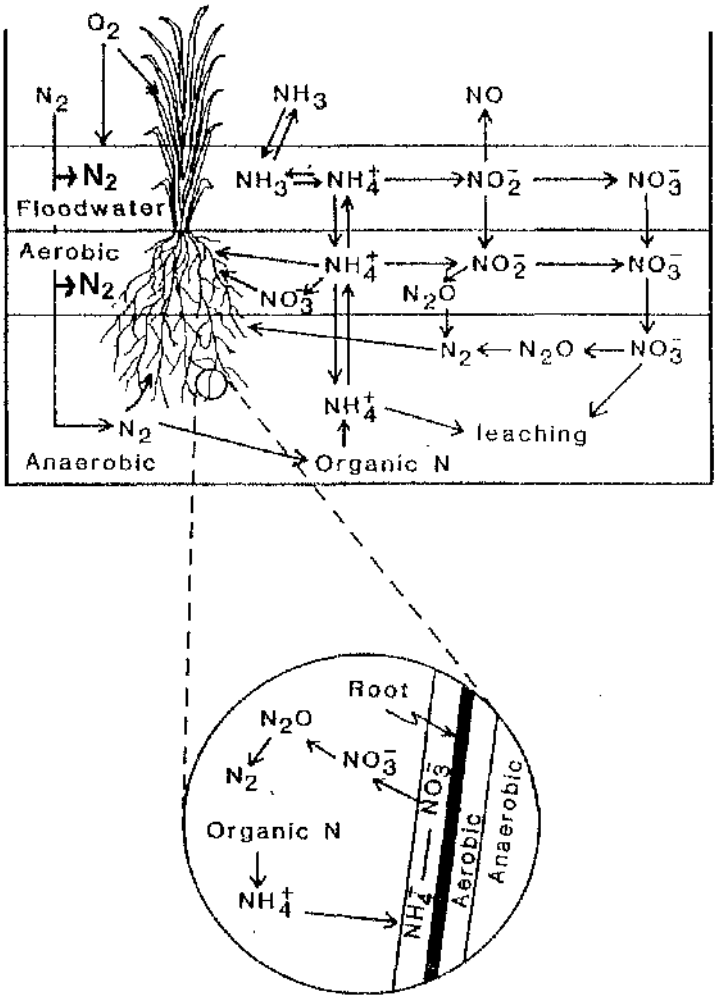


Fig. 1.1 Nitrogen transformations in wetland soils. Reproduced from Reddy & Graetz (1988).

promote the denitrification of nitrate to molecular nitrogen and nitrous oxide. Low nitrate levels in waterlogged soil can be explained by this nitrification/denitrification process. The majority of nitrogen lost from wetland soils is lost via denitrification (Patrick & Tusneem, 1972).

1.2.2 Nitrogen fixation

Four fifths of the world`s atmosphere consists of nitrogen in its gaseous form, N_2. Nitrogen is essential for protein formation and all organisms need it in some form. Most plants obtain nitrogen in the form of nitrate (NO_3^-) or ammonium (NH_4^+) ions, but some are able to utilise reduced molecular nitrogen (N_2) from association with microbial complexes that fix (reduce) N_2 to ammonia making it available to the plant. Nitrogen is usually the limiting nutrient to plant growth in wetlands and that fixed biologically can be an important nitrogen input (Bowden, 1987). In his review, rates of N_2 fixation in wetlands (measured by acetylene reduction assay) have been recorded from "a few hundredths... to about 10g N m^{-2} yr^{-1} with most values about 1 g N m^{-2} yr^{-1}" (compared to rates of legumes in agriculture estimated between 10-60 g N m^{-2} yr^{-1} (Sprent & Sprent, 1990)). Nitrogen fixation may therefore be an important source of nitrogen in some riverine wetland plant communities.

1.3 LEGUMES IN WETLANDS

Legumes are the most widely studied of all plants that form nitrogen fixing symbioses, but their role in semi-natural ecosytems has been studied less than their role in agriculture. This study examines the legumes found within riverine wetlands of the British Isles and assesses their importance to these ecosystems.

1.3.1 Species studied

Six main species were investigated: all are members of the Fabaceae, within the subfamily Papilionoideae (Stace, 1991). Nodulation of all these species has been widely reported by many investigators (listed in Allen & Allen, 1981). Clapham *et al.* (1987) and Stace (1991) were the main texts consulted to compose the descriptions and for the habitat information the main source was Grime *et al.* (1988). Additional information from other sources is cited as such. Nomenclature is according to Stace (1991).

a) *Lathyrus pratensis* L. Meadow Vetchling

Description (Fig. 1.2.a)

Climbing perennial, height 30-120 cm, finely pubescent. Stem angled. Leaves with one pair of leaflets and simple or branched tendrils, leaflets lanceolate with parallel

veins, stipules leaf like, arrow shaped. Racemes axillary, peduncle longer than leaves, flowers 5-12, hermaphrodite, yellow, 10-18 mm, narrowly triangular lower calyx teeth. Pod flattened, glabrous to finely hairy, 25-35 mm, dehiscing explosively, seeds usually 3-6 (Brunsberg, 1977), mean seed size is 3.3 x 2.8 mm and mean weight is 12.85 mg (Grime *et al.*, 1988). Flowering season: May to August. 2n – 14, 28 (Grime *et al.*, 1988).

Reproduction

Vegetative reproduction is by horizontal rhizomes; some up to 7 m long have been recorded (in cultivation) after one vegetative period (Brunsberg, 1977). Flowers are pollinated by insects, self fertilisation has been shown to occur but it is not known to what extent selfing and cross fertilisation occur in the wild (Brunsberg, 1977). As the seeds are large they are not dispersed far by the plant itself but may be spread over long distances by man, in hay for example or by herbivores (Brunsberg, 1977). Seeds germinate in spring (Grime *et al.*, 1988). There are no seed bank data on *L. pratensis*.

Habitat

L. pratensis is found mainly in grasslands, especially meadows, waysides and wasteland, occasionally in spoil habitats and riverbanks, absent from woodland, aquatic and most skeletal habitats (with the exception of scree). Although infrequent in mires, it has been recorded on moist soil at the edges of both topogenous and soligenous mires. It is supported by its tendrils on surrounding vegetation on fertile soils. *L. pratensis* is usually found in soils of pH range 6.36 - 7.68, but not in soils of pH <5.2 or >7.78 (unpublished data, Wheeler & Shaw, 1992). It has been recorded at a wide range of altitudes, usually to 380 m (maximum recorded = 457 m (Wilson, 1956)).

Distribution

It is native to Britain where it is found in 100% of vice-counties (Perring & Walters, 1990), it is common but is declining from farmland (Grime *et al.*, 1988). It is also native in North Africa, nearly all of Europe, Asia and introduced in North America (Clapham *et al.*, 1987).

Agricultural Importance

None.

b) *Lotus pedunculatus* Cav. (*Lotus uliginosus* Schkukr) Greater Birdsfoot Trefoil

Description (Fig. 1.2.b)

Erect or scrambling perennial, height 15-100 cm, glabrous to sparsely pubescent. Stem hollow. Leaves with 5 leaflets, 2 of which arise from the base of stem like stipules, leaflets ovate to obovate. Stipules minute, falling early. Cymes axillary, peduncles to 15 cm, flowers 5-12, hermaphrodite, yellow, 10-18 mm, calyx teeth recurved in bud, the 2 upper with an acute sinus. Pod elongate, not ridged or angled, 15-35 mm, dehiscing explosively. Seeds approximately 14 per pod, mean seed size is 1.1 x 1.1 mm and mean weight is 4 mg (Grime *et al.*, 1988). Flowering season: June to August. 2n =12.

Reproduction

L. pedunculatus regenerates vegetatively by means of rhizomes (Wedderburn & Gwynne, 1981). Once well developed, rhizomes were found to be very important for lateral spread and shoot production of *L. pedunculatus* in the uplands of Scotland (Wedderburn & Gwynne, 1981). Reproduction is also by seed, which germinate in spring (Muller, 1978). Turner (1933) reported a persistent seed bank. Flowers are pollinated by insects and are self-incompatible (Duke, 1981).

Habitat

L. pedunculatus is a mire or wet grassland plant, found most commonly in unshaded mire and wasteland, occasionally in pasture, waysides and aquatic habitats, absent from skeletal, arable, woodland and spoil habitats. It is the only British legume commonly found in wetlands; it is most abundant in the drier parts of topogenous and soligenous mires (but not in permanently submerged areas). *L. pedunculatus* is usually found in soils of pH range 5.04 - 7.04, but not in soils of pH <2.2 or >7.78 (unpublished data, Wheeler & Shaw, 1992). It has been recorded at a wide range of altitudes usually to 395 m (maximum recorded = 490 m (Wilson, 1956)).

Distribution

It is native to Britain and is present in nearly 100% of vice-counties, rarer in the extreme North of Scotland and Central Ireland (Perring & Walters, 1990), it is decreasing as a result of wetland destruction (Grime *et al.*, 1988). It is also native to West, Central and Southern Europe, Asia and North Africa (Clapham *et al.*, 1987), introduced to Australasia, North and South America (Duke, 1981).

Agricultural Importance

Is widely used as a pasture plant, particularly in North America and New Zealand, several cultivars have been developed (Duke, 1981). It is noted for its feed qualities, such as non-bloating characteristics, increased amino acid absorption in the rumen, insect resistance and ability to grow on acid and waterlogged soils (Harris *et al.*, 1993).

c) Vicia cracca **L. Tufted Vetch**

Description (Fig. 1.2.c)

Scrambling perennial, height 60-200 cm, pubescent. Leaves with 5-15 pairs of leaflets and branched tendrils. Leaflets oblong-lanceolate to linear-lanceolate, acute or mucronate. Stipules half arrow shaped, lower lobe entire. Racemes axillary, peduncle 2-10 cm, flowers 10-30, hermaphrodite, blue-purple, 8-12 mm, calyx teeth very unequal, upper minute. Pod ovate, obliquely truncate, glabrous, 10-25 mm, longitudinally dehiscent, 2-6 seeds. Mean seed size is 2.8 x 2.6 mm and mean weight is 14.29 mg (Grime *et al.*, 1988). Flowering season: June to August. 2n = 14, 27, 28, 30.

Reproduction

V. cracca can regenerate vegetatively, but has limited capacity to do so by means of spreading underground roots, 1-25 stems may be produced from a small rhizome (Chrtkova-Zertova, 1973). It produces mainly by seed, which germinate in the autumn (Muller,1978). There are no seed bank data for *V.cracca.* Flowers are insect pollinated (Aarsen *et al.*, 1986).

Habitat

V. cracca is found mainly on rough grassland, e.g. wasteland, riverbanks, unimproved pasture and hedgerows, occasionally in mires, meadows and woodland margins, absent from aquatic and skeletal habitats (except scree). It is found on relatively fertile and moist soils, and is sometimes found at the edges of topogenous mires. It is found most frequently in soils of pH 5.5-6.0, not in soils with pH <4.5 or >7.5 (Zarzycki, 1976). It is observed most frequently between altitudes of 100-200 m but has been recorded up to 448 m (Wilson, 1956)).

Distribution

It is native to Britain and is found in 100% of vice-counties (Perring & Walters, 1990), it is probably decreasing, not being efficient in colonising artificial habitats

(Grime *et al.*, 1988). It is native in nearly all of Europe, Asia and Greenland and introduced in North America (Clapham *et al.*,1987).

Agricultural Importance
None.

d) *Trifolium dubium* Sibth. Lesser Yellow Trefoil

Description (Fig. 1.2.d)
Procumbent or ascending annual, height to 25 cm, very sparsely hairy. Leaves palmately trifoliate, leaflets to 11 mm, obcordate or obovate, base cuneate. Stipules ovate, acuminate. Racemes globose, terminal and axillary, peduncles exceed petioles, flowers 3-15, hermaphrodite, yellow (turning dark brown), 3-4 mm. Pedicels approximately 1 mm, shorter than calyx tube. Standard narrow, folded longitudinally over pod. Pod ovoid, 2.5-3 mm, 1 seeded, dehiscent. Mean seed size is 1.5 x 0.9 mm and mean weight is 0.32 mg (Grime *et al.*, 1988). Flowering season: May to October, though if the site is droughted only May to June (Grime *et al.*, 1988). 2n = 28, 32.

Reproduction
T. dubium regenerates entirely by seed which germinate in the autumn (Grime *et al.*, 1988). Charlton (1977) reported the occurrence of a persistent seed bank. Flowers are self pollinated (Fryxell, 1957).

Habitat
T. dubium is found in two different types of moderately infertile habitats; open habitats (especially rocky outcrops) and grassland sites such as meadows, wasteland, waysides and lawns. It has not been recorded in wetlands (but has been found on one of the floodplains studied here). There is no detailed information on the pH range of *T. dubium*, but Grime *et al.*, (1988) only recorded it on sites with pH >5.0. It is only abundant at altitudes less than 300 m (maximum recorded = 487 m (Wilson, 1956)).

Distribution
It is native to Britain, found in 100% of vice-counties, though it is rarer in Northern Scotland (Perring & Walters, 1990) it is increasing, colonising lawns and sown grasslands (Grime *et al.*, 1988). It is also native throughout most of Europe (except the extreme North) and North Africa (Clapham *et al.*, 1987).

Agricultural Importance

It is much less productive and persistent than white clover and its use in pasture seed mixtures is not recommended (Langer & Hill, 1982).

e) *Trifolium pratense* L. Red Clover

Description (Fig. 1.2.e)

Decumbent to erect perennial, height 5-100 cm, pubescent. Leaves palmately trifoliate, leaflets 10-30 mm, elliptical to obovate, hairy underneath, glabrescent above with whitish crescentric spot. Stipules ovate to oblong, free part of stipule triangular with a setaceous point, usually pressed to petiole. Petioles erect to 20 cm. Racemes globose to ellipsoid, terminal, sessile, approximately 100 flowers (Grime *et al.*, 1988), hermaphrodite, pink-purple (sometimes whitish), 12-18 mm. Pod obovoid, 2.2 mm, 2 distinct parts, lower membranous, upper thickened, dehiscent. Mean seed size is 2.1 x 1.5 mm and mean weight is 1.35 mg (Grime *et al.*, 1988). Flowering season: May to September. 2n = 14.

The agricultural variant var. *sativum* Schreber is more robust with hollow stems and entire leaflets unlike var. *pratense* which has solid stems and toothed leaflets.

Reproduction

T. pratense regenerates entirely by seed which germinate in autumn (Grime *et al.*, 1988). Chippendale & Milton (1934) reported the presence of a persistent seed bank. Flowers are insect pollinated (Knuth, 1906).

Habitat

T. pratense is a common agricultural plant, found mainly in meadows and pastures and also in other artificial habitats e.g. waysides and spoil heaps. It is absent from most skeletal habitats (rocky outcrops, the exception) and wetland habitats (except unshaded mires). It is a plant of continuously moist habitats, able to survive waterlogged soils, but only transiently. *T. pratense* occurs in infertile and fertile soils (Fitter, 1978), though on the more fertile (and disturbed) habitats it is suspected that only var. *sativum* is present. It is most frequently found in soils of the pH range 5.5-6.0, but not in soils with pH < 5.0 or >7.0 (Zarzycki, 1976). It is widely distributed up to altitudes of 360 m (maximum recorded = 850 m (Wilson, 1956)).

Distribution

It is native to Britain and is found in 100% of vice-counties (Perring & Walters, 1990), it is thought that var. *pratense* is decreasing, but var. *sativum* is increasing in

agricultural use (Grime *et al.*, 1988). It is also native in Europe and introduced in Australasia and South and North America (Clapham *et al.*, 1987).

Agricultural Importance

Is widely grown for pasture, hay and green manure, many cultivars have been developed (Duke, 1981).

f) *Trifolium repens* L. White Clover, Dutch Clover

Description (Fig. 1.2.f)

Creeping perennial, height to 50 cm, glabrous, rooting at the nodes. Leaves palmately trifoliate, leaflets 10-30 mm, obovate or obcordate, usually with light or dark markings, veins translucent. Stipules ovate to oblong with short narrow points. Petioles erect and glabrous, to 14 cm. Racemes globose, axillary, peduncles up to 20 cm, flowers 40-80, hermaphrodite, white or pink, rarely purple or red, scented, 7-10 mm. Pedicels to 6 mm, calyx tube white with green veins, calyx teeth triangular-lanceolate, about half as long as the calyx tube. Standard folded over pod, rounded at the base. Pod oblong, 4-5 mm, indehiscent, 3-4 seeded. Mean seed size is 1 x 1 mm and mean weight is 0.56 mg (Grime *et al.*, 1988). Flowering season: June to September. $2n = 32$.

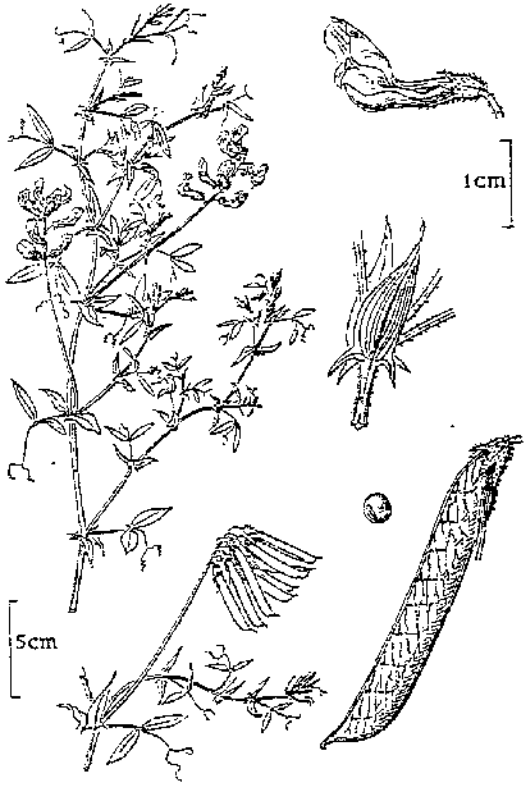
Reproduction

T. repens reproduces mainly by vegetative stolons but also by seed (Burden, 1983). The lateral extension of stolons is very important and shows marked seasonal variation, depending on genotype and its interaction with the environment (Burden, 1983). Flowers are mainly pollinated by insects. Seed reproduction is more important for colonising new habitats (Burden, 1983) and persistent seed banks have been recorded (Odum, 1978). Flowers are mainly pollinated by insects, seed germinates in the spring (Grime *et al.*, 1988).

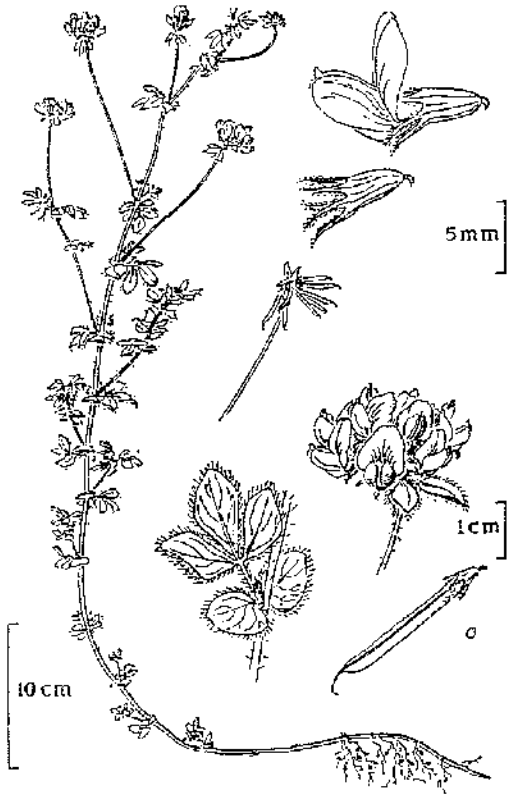
Habitat

T. repens is found in a wide range of habitats, most commonly in meadows, pastures, road verges and paths, also on arable land, soil heaps, mine spoil and demolition sites. It occurs to a lesser extent on wasteland, skeletal habitats and wetlands, and is practically absent from woodland. It is most frequent on fertile and moist habitats and is occasionally found in soligenous mires. It is most frequently found in soils of pH 5.0-6.5 and is rarely found at pH <4.0 (Burden, 1983). It is mainly found at altitudes below 400 m (maximum recorded = 900 m (Burden, 1983).

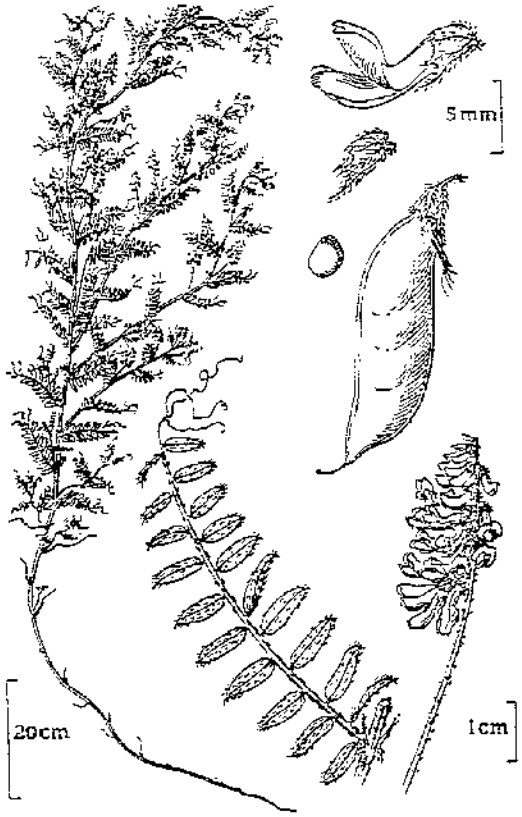
Fig. 1.2 Illustrations of the 6 legume species studied (a.- f.) Reproduced from Clapham *et al.*(1957).



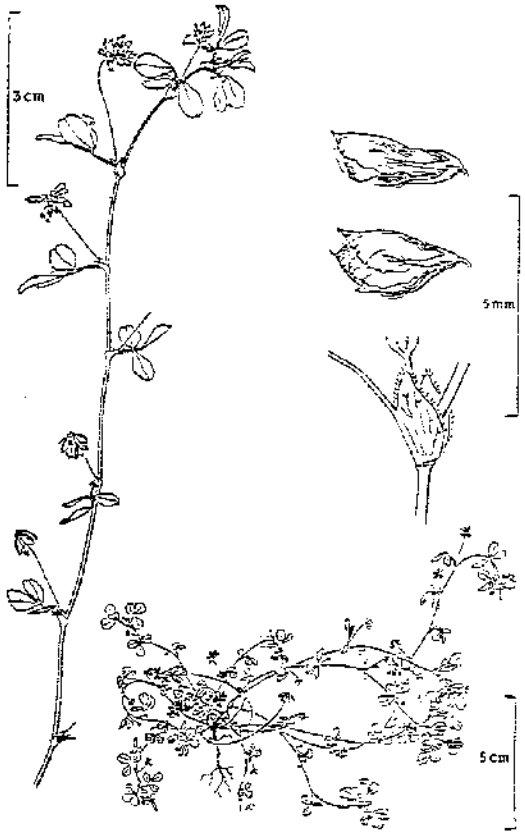
a. *Lathyrus pratensis* L.



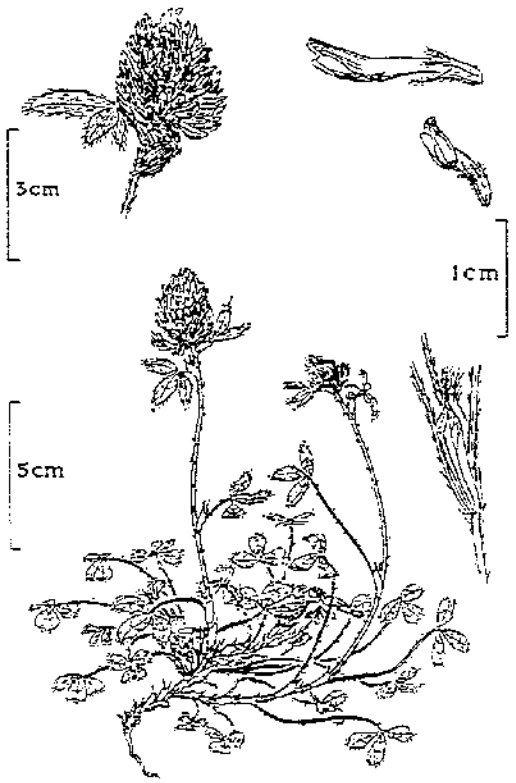
b. *Lotus pedunculatus* Cav.



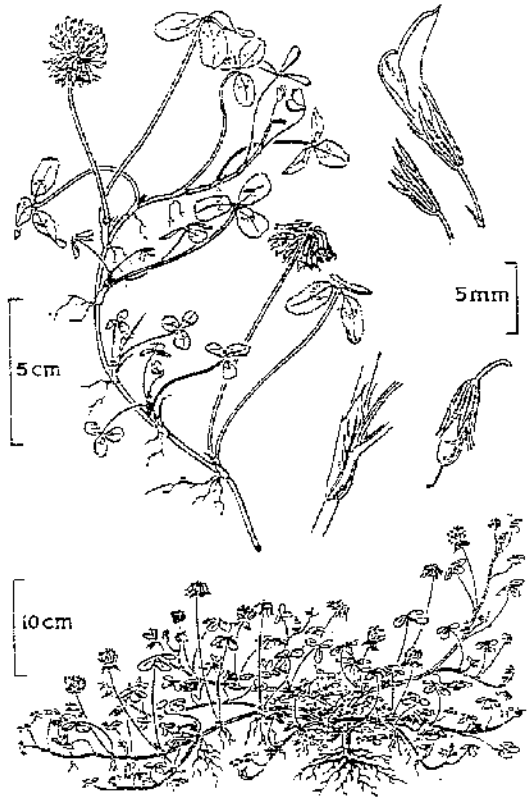
c. *Vicia cracca* L.



d. *Trifolium dubium* Sibth.



e. *Trifolium pratense* L.



f. *Trifolium repens* L.

Distribution

It is native to Britain, and is present in 100% of vice-counties (Perring & Walters, 1990), the abundance of *T. repens* is maintained at an artificially high level as a result of agriculture (Grime *et al.*, 1988). It is also native in nearly all of Europe, North and West Asia, North Africa and introduced in Southern Africa, North and South America and East Asia (Clapham *et al.*, 1987).

Agricultural Importance

T. repens is one of the most important forage legumes in the world, used extensively (in a variety of cultivars) for its high nutritional value and persistence (Duke, 1981).

1.3.2 Legume-rhizobium symbiosis

Many leguminous plants form a symbiosis with the soil bacterium rhizobium, in this work, the term "rhizobia/rhizobium" embraces those genera of the Rhizobiaceae, species of which elicit the formation of hypertrophic growths on roots (nodules). These provide the locus for symbiotic N_2 fixation by the bacterium (Sprent & Sprent, 1990). Vincent (1980) outlines the different stages of nodule development. This framework has been modified and recent advances are summarised in Sprent & Sprent (1990) and Franssen *et al.* (1992). Nodule development can be divided into the following stages (Franssen *et al.*, 1992).

1) Pre-infection

There are three methods of infection: via the epidermis, via wounds (cracks) or by root hair infection as is the case with the species used in this study. Rhizobia interact with root hairs to induce root hair curling, which traps the bacteria.

2) Infection and nodule formation

The bacteria invade the root hair cell and root cortex through an infection thread. Cortical cells are stimulated to divide and the infection threads carry rhizobia to these cells and usually release them into the cortical cell cytoplasm. The bacteria lose most of their own cell wall and are surrounded by a membrane derived from the host cell plasma membrane. These altered bacteria divide to fill the cytoplasm.

3) Nodule function and maintenance

The bacteria differentiate into bacteroids, which fix nitrogen.

Both *Vicia* and *Lathyrus* belong to the tribe *Fabeae* and are usually associated with *Rhizobium leguminosarum* biovar *viciae* (Young, 1992). *Trifolium* belongs to the tribe *Trifolieae* and is usually associated with *Rhizobium leguminosarum* biovar

trifolii (Holt *et al.*, 1994). In both the tribes *Fabeae* and *Trifolieae* the infection thread penetrates cells near the root stele, following which rhizobia are released within membrane bound vacuoles. The nodules have an apical meristem and are of indeterminate growth. The nodule vascular system extends and branches as the nodule grows, the nodule becoming cylindrical and elongate and branching when reaching maturity (Bergersen, 1982) (Fig. 1.3.a). The infection threads persist and sequentially infect newly formed cells (Sprent & Sprent, 1990). Gaseous exchange takes place via intercellular spaces over the surface of the nodule; there are no lenticels (Sprent, 1983).

Lotus belongs to the tribe *Loteae* and is associated with *Rhizobium loti* and *Bradyrhizobium* sp. (*Lotus*) (Holt *et al.*, 1994). Nodules are of determinate growth and it has been suggested that the vascular system becomes 'closed' when growth ceases (Bergersen, 1982). After cells have been infected with rhizobia via the infection thread, they divide until the onset of nitrogen fixation. Following this, nodule size increases by cell expansion (Franssen *et al.*, 1992), giving rise to almost spherical (globose) nodules (Fig.1.3.b). Gaseous exchange is assisted by lenticels on the nodule surface (Sprent, 1983).

1.3.3 Legume nitrogen fixation

The legume-rhizobia symbiosis is one that has been studied extensively since its discovery last century, and is thus the best known of all nitrogen fixing systems. Nitrogen fixation by grain and forage legumes is estimated to contribute from less than 100 to 600 kg N ha^{-1} yr^{-1}(Sprent & Sprent, 1990). Legumes are very important in agriculture, the most economically important legumes in the world are listed by Duke (1981). Most research therefore has been conducted in agriculture rather than examining the role they play in their natural habitats. This study aimed to fill some of the gaps in our knowledge of nitrogen fixation in riverine wetlands.

Fig. 1.3 Nodule shape a) Elongate, cylindrical: *Lathyrus*, *Vicia*, *Trifolium* and b) Globose: *Lotus* (not to scale). Reproduced from Allen & Allen (1981).

1.4 NON-LEGUME NITROGEN FIXERS IN WETLANDS

In addition to the legume-rhizobia symbiosis there are many other biological nitrogen fixing systems, be they symbiotic, associative or free-living. A brief introduction to each of those investigated in this study follows and further details are presented in Chapter 4B. This subject is extensive and the discussion will focus on organisms that are possible important sources of biologically fixed nitrogen in riverine wetlands. Non-legume nitrogen fixation studies were restricted to one field site only (Chapter 4B).

1.4.1 Free-living nitrogen fixing bacteria

A vast variety of free-living nitrogen fixing bacteria has been recorded. Most are heterotrophic, with the exception of cyanobacteria and nitrogen fixing photosynthetic bacteria, thus the former are limited to habitats where sufficient carbon is available. Free-living cyanobacteria have been estimated to fix up to 20-25 kg N ha^{-1} yr^{-1}(Mishustin & Shil'nikova, 1971) as compared to heterotrophic azotobacters and clostridia which have been estimated to fix less than 0.5 kg N ha^{-1} yr^{-1} (Stewart, 1977).

1.4.2 Bryophyte-cyanobacteria associations

Cyanobacteria form an association with members of the Hepaticae (liverworts), Anthocerotae (hornworts) and Musci (mosses) (Sprent & Sprent, 1990). Only the latter will be considered here, the other two being absent or rare components of the sites studied. Generally, cyanobacteria form epiphytic associations with mosses, though there are endophytic exceptions (Meeks, 1990). One of the best known cyanobacteria/bryophyte association is that found in *Sphagnum* bogs. If 100% cover of *Sphagnum* is assumed, *Sphagnum*-cyanobacteria fixation is estimated to be up to 94 kg N ha^{-1} yr^{-1}(Basilier *et al.*, 1978).

1.4.3 Bacteria-angiosperm root associations.

An association has been described between nitrogen fixing bacteria in the rhizosphere of some angiosperms and the plant itself. The rhizosphere is considered to be the soil near to plant roots where microbes are affected by the root's presence. In addition to the rhizoplane (root surface) outwards (the ectorhizosphere), the root interior also provides a niche for microbes (the endorhizosphere) (Killham, 1994). Nitrogen fixing bacteria have been found in both (Döbereiner & Day, 1976; Patriquin & Döbereiner, 1978). Associative nitrogen fixation has been noted in tropical grasses (Döbereiner & Day, 1976), temperate grasses (e.g. Bristow, 1974) and dicotyledons (Harris & Dart, 1973). Associative fixation in both dicotyledonous

and monocotyledonous plants was estimated to contribute 11 kg N ha^{-1} yr^{-1} to a wetland community (Eckardt & Biesboer, 1988).

1.5 MEASURING NITROGEN FIXATION: A REVIEW OF TECHNIQUES USED IN THE PRESENT STUDY

1.5.1 Acetylene reduction assay (ARA)

Nitrogenase is the enzyme complex by which nitrogen is reduced to ammonia. The enzyme consists of two proteins, one known as the Fe protein or dinitrogenase reductase (containing iron and labile sulphur) and the other the Mo-Fe protein or dinitrogenase (containing molybdenum-iron and labile sulphur). Both Mg^{2+} and adenosine triphosphate (ATP) are needed for nitrogen reduction. An electron is donated to dinitrogenase reductase by a reduced electron donor (flavodoxins and ferredoxins are primary electron donors). The dinitrogenase reductase reacts with $MgATP^{2-}$ while the dinitrogen molecule is reduced by dinitrogenase at an active centre that usually incorporates Mo. When the two components join, two ATP molecules are hydrolysed and electrons flow from the dinitrogenase reductase to dinitrogenase. Dinitrogen gas and protons are then simultaneously reduced.

$$8H^+ + N_2 + 8e^- \rightarrow 2NH_3 + H_2$$

There is a high demand for oxygen in the infected (central) region of the nodules to meet the respiratory requirements of nitrogen fixation (oxygen is needed to support oxidative phosphorylation to provide ATP). The concentration of free oxygen however, must remain low as nitrogenase is inactivated by oxygen. Oxygen diffusion, therefore, has to be controlled. A barrier exists in the nodule cortex (Tjepkema & Yocum, 1974; Witty *et al.*, 1986) that helps regulate oxygen flux from the atmosphere to the infected region. Leghaemoglobin facilitates the transport of oxygen in bound form within the infected cells (Bergersen, 1980, 1982). The diffusion barrier is variable, the resistance to oxygen diffusion varying depending on the demand for oxygen (Hunt & Layzell, 1993). The actual mode of operation of the barrier is yet to be determined though a number of hypotheses have been put forward (e.g. Hunt *et al.*, 1988; Sheehy *et al.*, 1987).

In addition to nitrogen, the nitrogenase complex reduces other substrates. The first to be discovered, the reduction of acetylene to ethylene (Dilworth, 1966; Schöllborn & Burris, 1966) has been used widely to assay nitrogenase activity in nitrogen fixing systems. Early methodology is reviewed by Hardy *et al.* (1968), who recommended

the use of a closed assay vessel with detopped, substrate free nodulated root systems, as the 'standard' assay system.

At least 8 electrons are used for every N_2 reduced and 2 are used to reduce acetylene to ethylene. Theoretically therefore, for every 4 C_2H_2 reduced 1 N_2 is reduced, suggesting that an estimate of nitrogen fixation can be made. However, a large number of drawbacks to the use of acetylene reduction as a quantitative technique have been reported, and will be considered later in Chapters 4A and 4B.

1.5.2 $^{15}N_2$ incorporation assays

$^{15}N_2$ incorporation methods have been used since Burris (1942) used it to show *Azotobacter vinelandii* fixed $^{15}N_2$. In the same year, Burris *et al.* showed that $^{15}N_2$ was fixed in the nodules of legumes. The sample under investigation is exposed to a ^{15}N labelled N_2 enriched atmosphere for a short period of time and then analysed for ^{15}N enrichment using a mass spectrometer. ^{15}N labelled N_2 is expensive and is rarely used in the field.

1.5.3 ^{15}N natural abundance

This method is based on the fact that the natural abundance of ^{15}N in atmospheric N_2 (0.3663 atom % ^{15}N (Junk & Svec, 1958)) is either lower or higher than that found in soil sources of N (Shearer & Kohl, 1986). However, in most soils the natural abundance of ^{15}N is higher than that of atmospheric N_2 (Shearer *et al.*, 1978) as a result of discrimination between the ^{14}N and ^{15}N isotopes during N transformations (Delwiche & Steyn, 1970). Differences in the ratio of $^{15}N:^{14}N$ between samples are small, so measurements are compared to a standard using the following equation (Griffiths, 1991)

$$\delta\ (‰) = \frac{\text{R-sample - R-standard}}{\text{R-standard}} \times 1000$$

where R = molar abundance ratio

δ = natural abundance, expressed as per mil (‰)

There is very little variation in ^{15}N abundance of atmospheric N_2 (Mariotti *et al.*, 1981). This is thus a 'reliable standard' and is set at zero, 1 δ ^{15}N unit (‰) = 0.3663 x 10^{-3} atom % excess ^{15}N. It is assumed therefore, that ^{15}N is more abundant in non-nitrogen fixing plants than nitrogen fixing plants as the latter utilises both soil and atmospheric N_2. This difference has been used in attempts to estimate the amount of N_2 fixed. The non-fixing plants are used as references and ideally their protoplasm

should have a ^{15}N concentration similar to that available to the plant in the soil N. The nitrogen fixing plants reduce atmospheric N_2 and have proportionately lower ^{15}N concentrations. For estimating the fractional contribution of biologically fixed nitrogen to plant N uptake (following the methods of Shearer & Kohl, 1986), three values of $\delta^{15}N$ abundance are needed; that of nitrogen fixing and reference plants growing at the same site, and that of the nitrogen fixing plant grown in N free medium.

$$\text{Fraction of N derived from atm } N_2 = \frac{\delta^{15}N \text{ reference} - \delta^{15}N \text{ fixer in field}}{\delta^{15}N \text{ reference} - \delta^{15}N \text{ fixer grown - N}}$$

This method has been applied more successfully to agricultural settings (Bergersen, *et al.*, 1990) where plants and soils are more homogenous than in natural ecosystems. However, there are a number of problems when applying this method to uncultivated natural ecosystems. These are reviewed by Handley & Scrimgeour (1996) and are discussed briefly here.

Soil N is not a single resource. Plants largely require nitrogen in its mineral form, mostly NH_4^+ and NO_3^-. However, a wide range of organic nitrogen compounds can also be utilised by some plant species (Raven *et al.*, 1993), usually assisted by ecto-mycorrhizal associations (Read, 1991). For example, amino acids are taken up directly by some mycorrhiza infected Arctic plant species (Kielland, 1994). Atmospheric N deposition can also add an extra source of N to soils (Pearson & Stewart, 1993; Department of the Environment, 1994). In the natural environment, N does not occur homogeneously as in 'artificial' agricultural systems. Plants therefore exploit different reserves of N in this heterogeneous environment (Caldwell & Pearcy, 1994). Plants use different soil horizons and $\delta^{15}N$ of total soil N often increases with soil depth therefore plants may use soil with different levels of $\delta^{15}N$ (e.g. Schulze *et al.*, 1994). However, Ledgard *et al.* (1984) found that in an agricultural situation, the plant available fraction of soil N did not vary with depth. Therefore this should be investigated for each particular situation. Non-fixing reference plants are not always a proxy for $\delta^{15}N$ of the typical soil N used by both the reference and fixing plant. Several authors (Ledgard *et al.*, 1985; Pate *et al.*, 1993; Handley *et al.*, 1994) have been unable to find suitable reference plants. It is unlikely that in a mixed stand (as in natural ecosystems) at any one time, that two taxa are using the same soil N. The origin of fixed N can be confounded by free-living microbes contributing fixed N_2 to legumes and reference plants (Hansen & Pate, 1987; Piccolo *et al.*, 1994). Although soil sources provide an enriched or

depleted $\delta^{15}N$ value compared to that of atmospheric N ($\cong$ 0 ‰) on a global scale, it is not necessarily true for any particular site, for example Herman & Rundel (1989) found that $\delta^{15}N$ for NH_4^+ and NO_3^- N could be 0 ‰ (the same as atmospheric N_2). The $\delta^{15}N$ value for nitrogen fixing species grown without N has been found to change with rhizobia strain (Steele *et al.*, 1983), nutrient supply and watering frequency (Ledgard, 1989). Therefore these values should be measured for the situation in question. Variation of the $\delta^{15}N$ within a plant varies as a result of fractionation during transport of N from one tissue to another, therefore the $\delta^{15}N$ plant parts sampled should reflect that of the whole plant or the whole plant itself should be sampled (Shearer & Kohl, 1986).

1.5.4 ^{15}N isotope dilution

In this method, the soil on which nitrogen fixing plants are to be grown, is labelled by applying low levels of ^{15}N enriched fertiliser (McAuliffe *et al.* 1958). It has been reviewed by several authors (e.g. Rennie & Rennie, 1983; Chalk, 1985; Hauck & Weaver, 1986). Again a non-fixing plant is used as a reference and it is assumed that the nitrogen fixing and reference plants take up the same proportion of soil N. The difference between the ^{15}N concentrations of the nitrogen fixing and reference plants is directly proportional to the amount of atmospheric N_2 fixed. This equation (Fried & Middleboe, 1977) is used to calculate the percentage of nitrogen derived from the atmosphere:

$$\% \text{ N fixed} = 1 - \frac{\text{atom percent excess } ^{15}\text{N in N fixer}}{\text{atom percent excess } ^{15}\text{N in non N fixer}} \times 100$$

Phillips *et al.* (1986) consider the advantages of the ^{15}N dilution method; it is possible to estimate soil N utilisation and nitrogen fixation in a single sample and to integrate nitrogen fixation over time. They also state that a single application at the beginning of the first season has been sufficient to conduct a ^{15}N dilution assay for several years at the same site, although, according to Rennie (1986), this may lead to incorrect estimates. Shearer *et al.* (1980) found that the ^{15}N concentration of seeds in soybean most closely resembled the ^{15}N of the whole plant but the distribution of ^{15}N within the plants varied with experimental conditions. As in natural abundance studies it is essential that the plant part sampled reflects the ^{15}N of the whole plant.

A disadvantage of this method is that when studying natural systems the addition of N fertiliser disturbs the system. The root activity of the reference plant, under N deficient conditions may be lowered and soil N uptake can be effected. The

assumption that the isotope incorporated into the soil N is equally available to both the N fixing plant and the reference plant is not always the case. Soil spatial variability can effect the distribution of ^{15}N label in the plant and so effect nitrogen fixation estimates. Special care must also be taken when choosing reference plants (Reichardt, 1990; Reichardt et al., 1987) and errors arise if there is a mismatch between reference plants and nitrogen fixers. They should be of similar growth form and phenology, with a similar rooting pattern (Doughton *et al.*, 1995).

It can be seen therefore, that there is no truly accurate method for measuring nitrogen fixation rates. Although this is true for both the laboratory and the field, "...the further one moves from *in vitro* studies towards the field situation, the greater are the operational and interpretative problems" (Turner & Gibson, 1980). Even exposure to $^{15}N_2$ gas has problems of field application due to the impossibilty of desiguing containment vessels for incubation that do not perturb the environment. When estimating nitrogen fixation it is necessary to use a number of different methods, to build up a picture of the nitrogen fixing situation.

Chapter 2
STUDY SITES

2. STUDY SITES

2.1 RING BOG, ENDRICK MARSHES, LOCH LOMOND NATIONAL NATURE RESERVE, SCOTLAND.

Ring Bog is one of three bogs comprising the Aber Bogs (Aber and Gartocharn being the other two), situated west of the River Endrick (56°0'N, 4°25'W) in the Loch Lomond National Nature Reserve (L.L.N.N.R.) (Fig.2.1 and Plate 1). It covers 16 ha and lies at an altitude of approximately 10 m; the climate is cool and wet. The mean annual rainfall is thought to have increased by 25% over the last 20 years and is now approximately 1400 mm (Doughty & Maitland, 1994). The mean annual temperature is 11.3°C with a mean annual minimum and maximum of 7.8°C and 11.9°C respectively (Jones, 1991). The bedrock is Old Red Sandstone with overlying glacial and alluvial deposits (Maitland, 1966) and more recent peat deposits on Ring Bog itself.

The Aber Bogs are reported to have been cut regularly for bog hay into the 1930's with the exception of Ring Bog, which has not been cut in living memory (Mitchell, 1989). Instead, Ring Bog was used for rough grazing, the Ross Drain preventing cattle from invading the bog hay meadows (Fig. 2.1). The last harvest was recorded in 1952, up until when the other bogs were cut intermittently. As this practice declined, so did necessary maintenance, fences and ditches were left to fall into disrepair and the bogs gradually dried out. In 1963 the whole area was used for rough grazing, but in 1968 the bogs were refenced due to the loss of animals in the over-grown ditches (Mitchell, 1989). Most of Ring Bog was fenced from cattle, but it was not until 1982 that the area where this study was conducted was completely fenced off.

The first official recognition of the Aber Bogs conservation value came in 1959 when they were notified as part of the Endrick Water Mouth SSSI. Ring Bog became part of L.L.N.N.R. in 1962 by which time the state of the bogs was very poor. The lack of regular cutting resulted in the invasion of *Phalaris* into the *Juncus/Carex* stands and *Salix* colonisation was also evident. The quality of water feeding into the bogs had also declined due to increasing amounts of agricultural and domestic pollutants within the catchment area. A comprehensive management of the whole area commenced in 1986 after Aber and Gartocharn Bogs were bought by the Nature Conservancy Council in 1984 (Mitchell, 1989). Aber Burn which receives outflow from Gartocharn Sewage Works plus agricultural pollutants

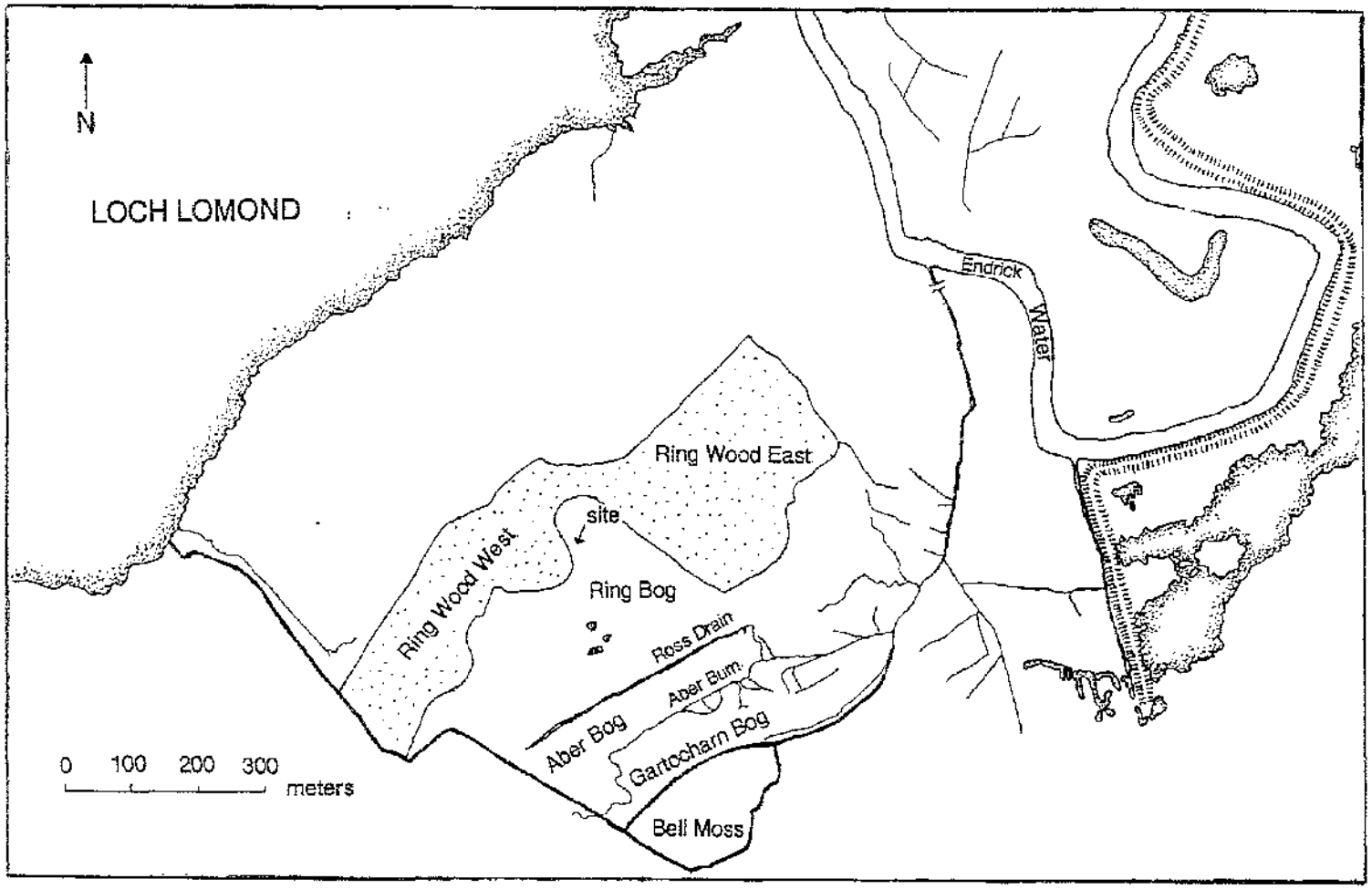


Fig. 2.1 Map showing location of Ring Bog (marked by arrow) within Loch Lomond National Nature Reserve.

Plate 1. Ring Bog, viewed from the boundary of Ring Wood West.

from the surrounding area and Ross Drain which receives domestic waste from a caravan site, were both diverted south away from the Bogs. Plans to control *Phalaris* by raising the water table have been set back by leaks in the embankments (Mitchell, pers. comm., 1993). *Phalaris* has not encroached into the study site location and much of northern Ring Bog. This may be a result of the fall in ground level from Ross Drain northwards, leading to water retention and thus discouraging *Phalaris* advancement (Eccles, 1989).

The study site, (Plate 1) is within an "enclave" area between Ring Wood East and Ring Wood West, the trees making it relatively sheltered. *Juncus acutiflorus* is dominant, *Filipendula ulmaria* and *Molinia caerulea* are also common. *Lotus pedunculatus*, is the only legume found at this site. The presence of *Sphagnum* gives the vegetation a hummock/hollow aspect (Plate 1). This tussocky nature of the vegetation may also have been increased by the poaching effect of cattle on wet ground (Scott, 1993). The site remains waterlogged throughout the year.

2.2 CLONMACNOISE AND LITTLE BROSNA, MIDDLE SHANNON, REPUBLIC OF IRELAND.

Clonmacnoise and Little Brosna are situated within the middle catchment area of the River Shannon (Fig. 2.2). They lie at an altitude of less than 60 m and the average annual rainfall is 850 mm. The mean annual temperature is 10°C, dropping to a mean 4.4°C in January and rising to a mean 15°C in July (Maltby *et al.*, 1994). The bedrock at the study site is Carboniferous limestone, overlain by peat and silty alluvium (Hooijer, 1996).

Both sites are known in Ireland as Callows, semi-natural floodplain grasslands beside the River Shannon in Central Ireland. The Irish word "*caladh*" means river meadow and it is from this that callow is derived (Heery, 1993). These fields flood each year in winter and spring and occasionally in summer. The floodplains are used both for pasture and meadow. The annual floods make the practice of ploughing and reseeding land impossible, hence the grassland is composed of native species, though some grasses may have been introduced by the old (pre 1920's) practice of spreading seed over fields once spring floods receded (Heery, 1993). Spring and winter floods on the callows provide ideal habitats for wading birds and wildfowl, and the callows are also home to the now endangered corncrake (*Crex crex*). It is therefore not surprising that the Shannon Callows are listed in the 'Manual Of Most Important Bird Areas' (Heery, 1993). Little Brosna is important for waders, as it

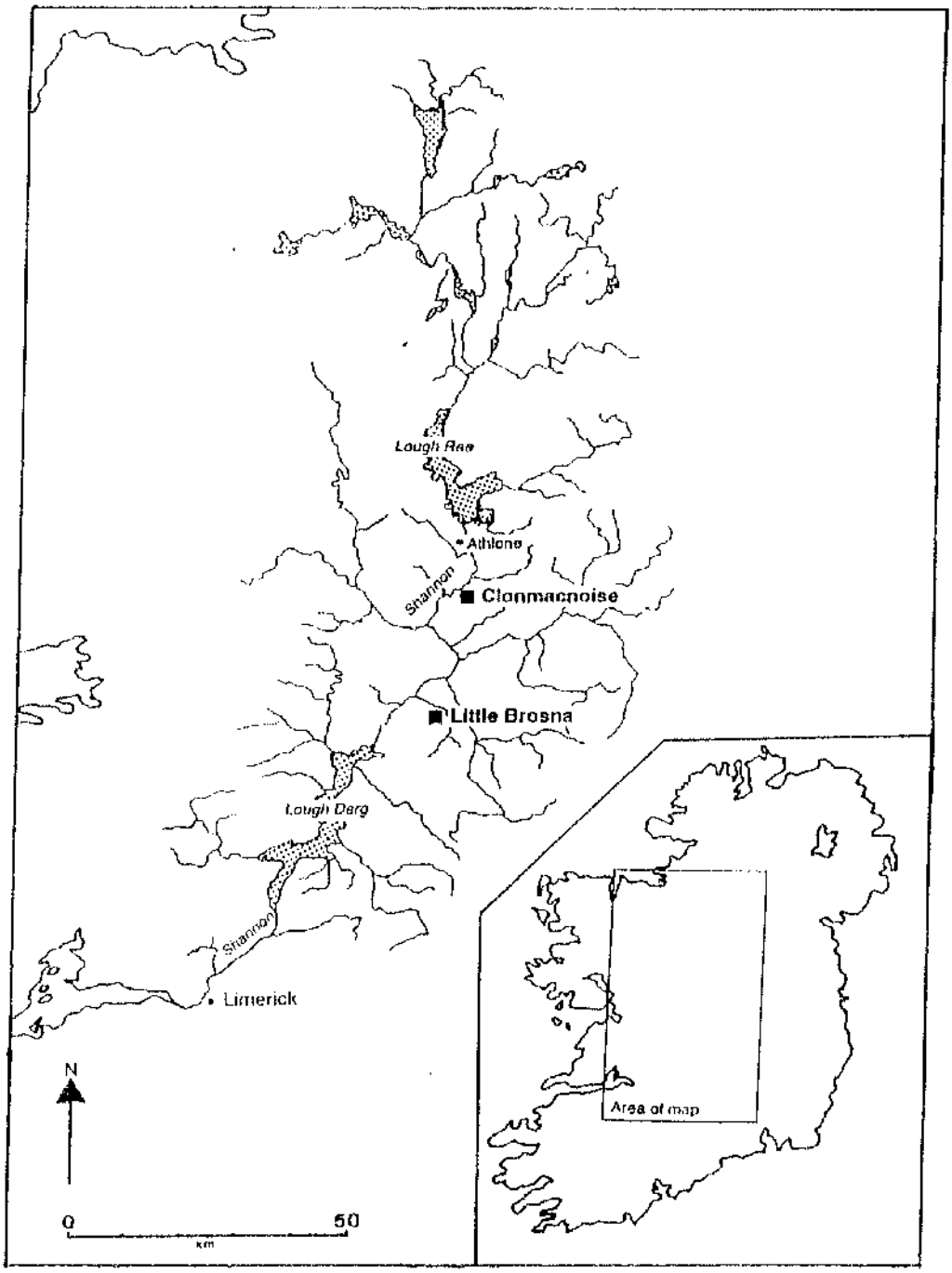


Fig. 2.2 Map showing location of Irish sites in relation to the River Shannon catchment area. Reproduced from Maltby *et al.* (1994).

floods throughout winter and spring (Plate 2a) whereas Clonmacnoise is flooded only for a few weeks in winter/spring (Maltby *et al.*, 1994).

The Little Brosna site (Plate 2a and b) is a floodplain off the Little Brosna, a downstream tributary of the Shannon (53°8'N, 7°55'W), impacted by sedimentation from commercial peat extraction within the area. The Clonmacnoise site (Plate 3) is a floodplain directly off the Shannon (53°20'N, 7°58'W) and is less affected by human activities than the Little Brosna.

Transects mapped out by the FAEWE project are shown in Fig. 2.3a and b, illustrating the position of the sites used in this study relative to the river and their underlying geology and vegetation types. At Little Brosna, work was mainly carried out at a site between station 1 and 2 (marked by an m) (Fig. 2.3a). At this site grasses are dominant; *Agrostis stolonifera*, *Festuca rubra*, *Phleum pratense* and *Lolium perenne* being the most frequent. *Ranunculus repens* and *Carex disticha* are also common. *Trifolium dubium* and *T. pratense* are the most abundant legumes at the Little Brosna site, together with *Vicia cracca* and *T. repens*. This study concentrated mainly on station 3 at Clonmacnoise (Fig. 2.3b), where several grasses; *Agrostis stolonifera*, *Festuca rubra* and *Anthoxanthum odoratum* are abundant, as is *Carex nigra* and *Filipendula ulmaria*. *Lathyrus pratensis* is the dominant legume, but *Vicia cracca*, *Lathyrus palustris*, *Trifolium pratense* and *T. repens* also occur.

Plate 2. Little Brosna, looking towards the river,
a) In flood.

b) During summer months

Plate 3. The floodplain at Clonmacnoise
(photographed by Nigel Willby)

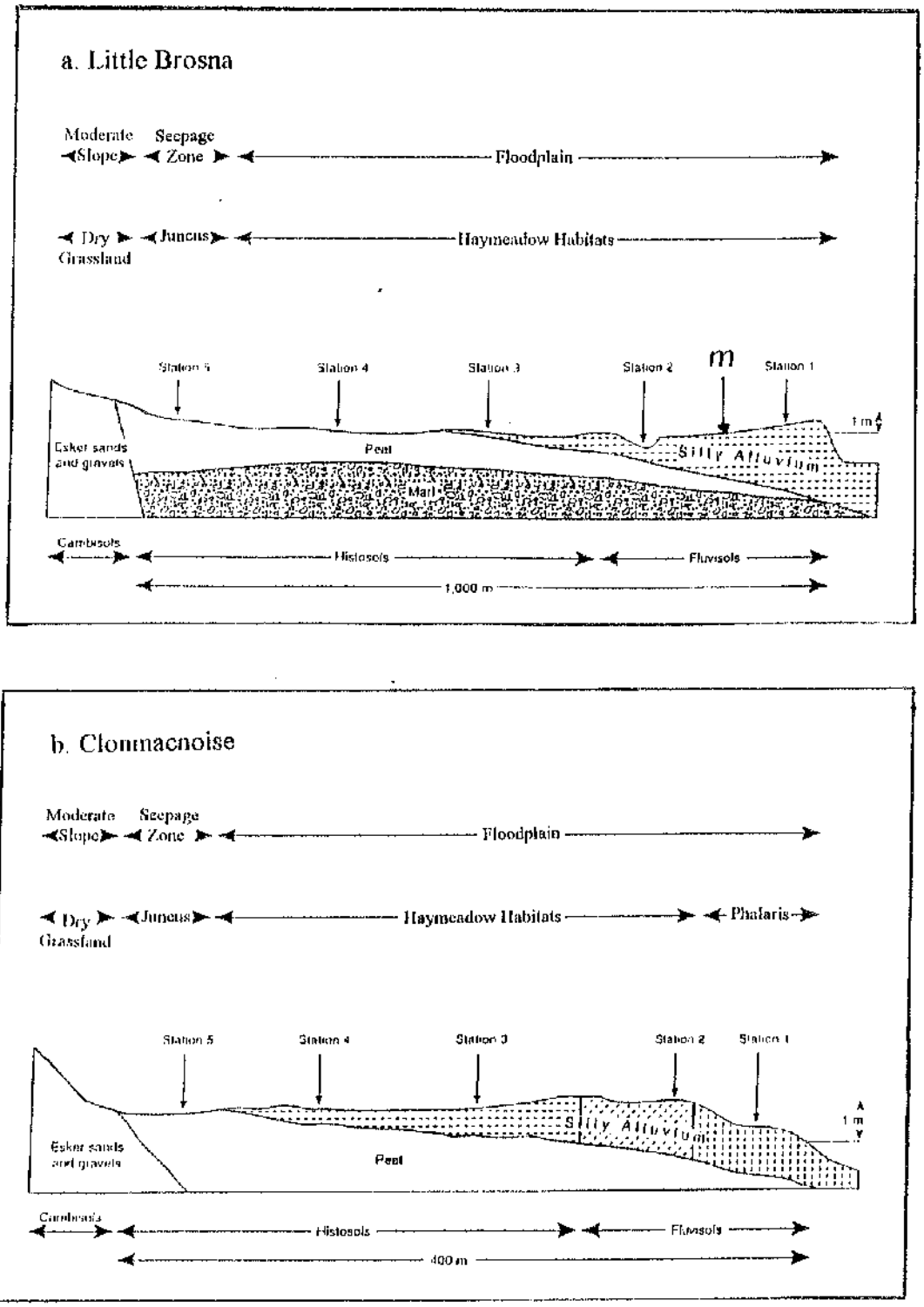


Fig. 2.3 Schematic cross-sections of the Irish study site transects ; a. Little Brosna; b. Clonmacnoise. Reproduced from Maltby *et al.*(1994).

2.3 KISMELDON AND BRADFORD MILL, DEVON, ENGLAND

Two extra field sites in England on the River Torridge were included in the vegetation survey only, details are from Maltby *et al.* (1994). Kismeldon and Bradford Mill are situated within the River Torridge catchment (Fig. 2.4). They lie at an altitude of about 55 m. The annual average rainfall at Kismeldon is 1353 mm, and at Bradford Mill 1133 mm. The mean annual temperature is in the range 7.5-10.0°C, the mean annual minimum and maximum ranges being 2.5-5.0°C and 15-17.5°C respectively. The bedrock is Carboniferous rock (comprising sandstones and shales). Kismeldon (50°55'N, 4°20'W) is situated 7 km from the sea on the River Torridge, Bradford Mill (50°55'N, 4°15'W) is further inland, at the confluence of the Rivers Torridge and Waldon. Transects mapped out by FAEWE are shown in Fig. 2.5, the underlying geology and vegetation types are shown.

The codes for all these sites are shown below in Table 2.1.

Table 2.1 Site Codes

SITE	CODE
Clonmacnoise 1-5	CL1 CL2 CL3 CL4 CL5
Little Brosna 1-4, & extra site	LB1 LB2 LB3 LB4 LBM
Ring Bog	RB
Kismeldon 1-3	KM1 KM2 KM3
Bradford Mill 1-3	BM1 BM2 BM3

2.4 SELECTION CRITERIA

The study sites were selected on the grounds of their differing geography, climate and landuse. Climatic conditions at the study sites ranged from oceanic in Central Ireland to eu-oceanic in West Scotland and South West England. Ring Bog (West Scotland) is relatively undisturbed, though there may be light grazing by wild herbivores such as deer or rabbits. The Irish sites however are either grazed by cattle (Little Brosna) or cut for hay (Clonmacnoise). The Devon sites are both grazed, of the two sites Kismeldon has the lowest nutrient availability as it is less affected by agricultural improvements than Bradford Mill which is impacted by fertiliser applications.

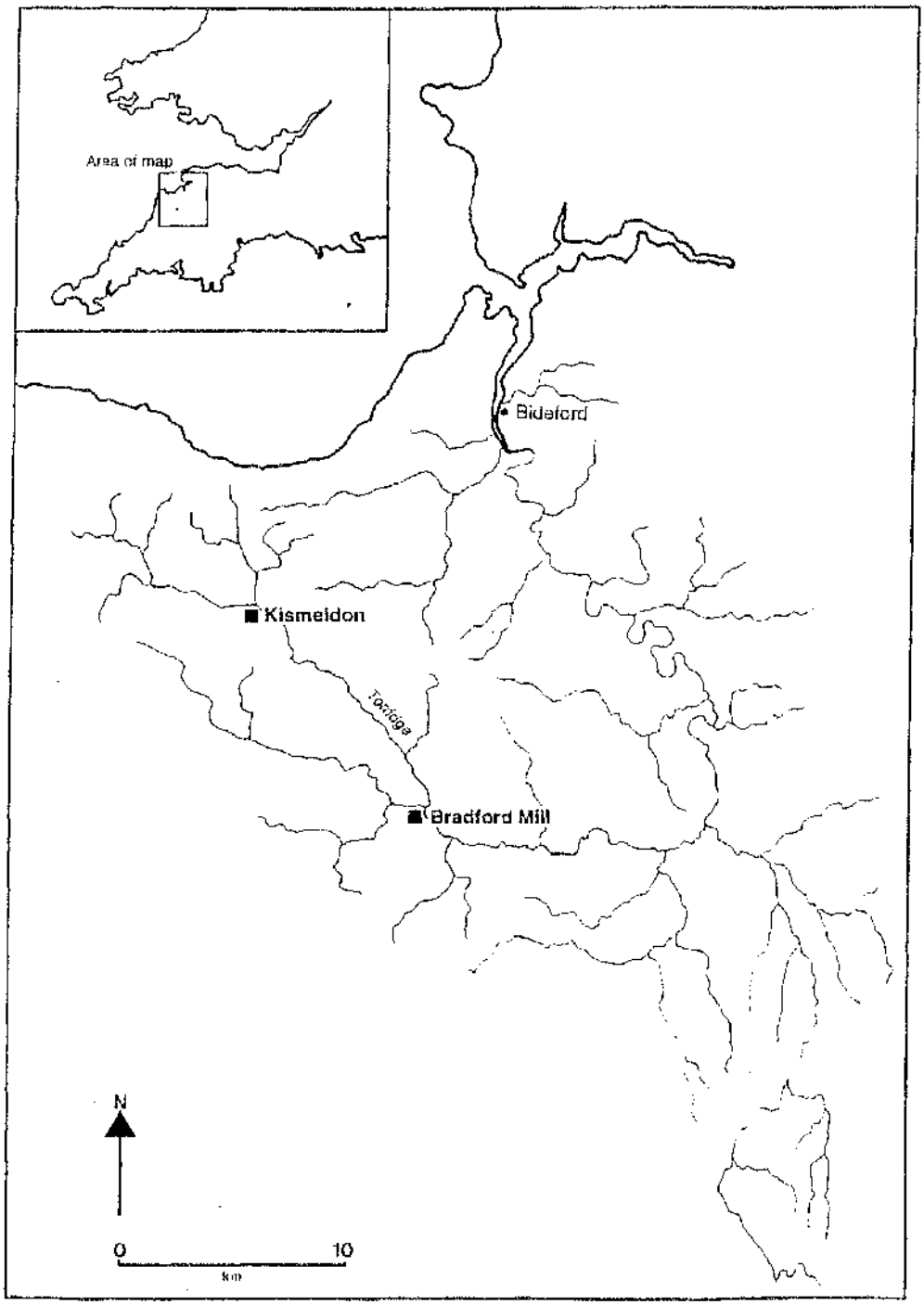


Fig. 2.4 Map showing location of English study sites in relation to the River Torridge catchment area. Reproduced from Maltby *et al.* (1994).

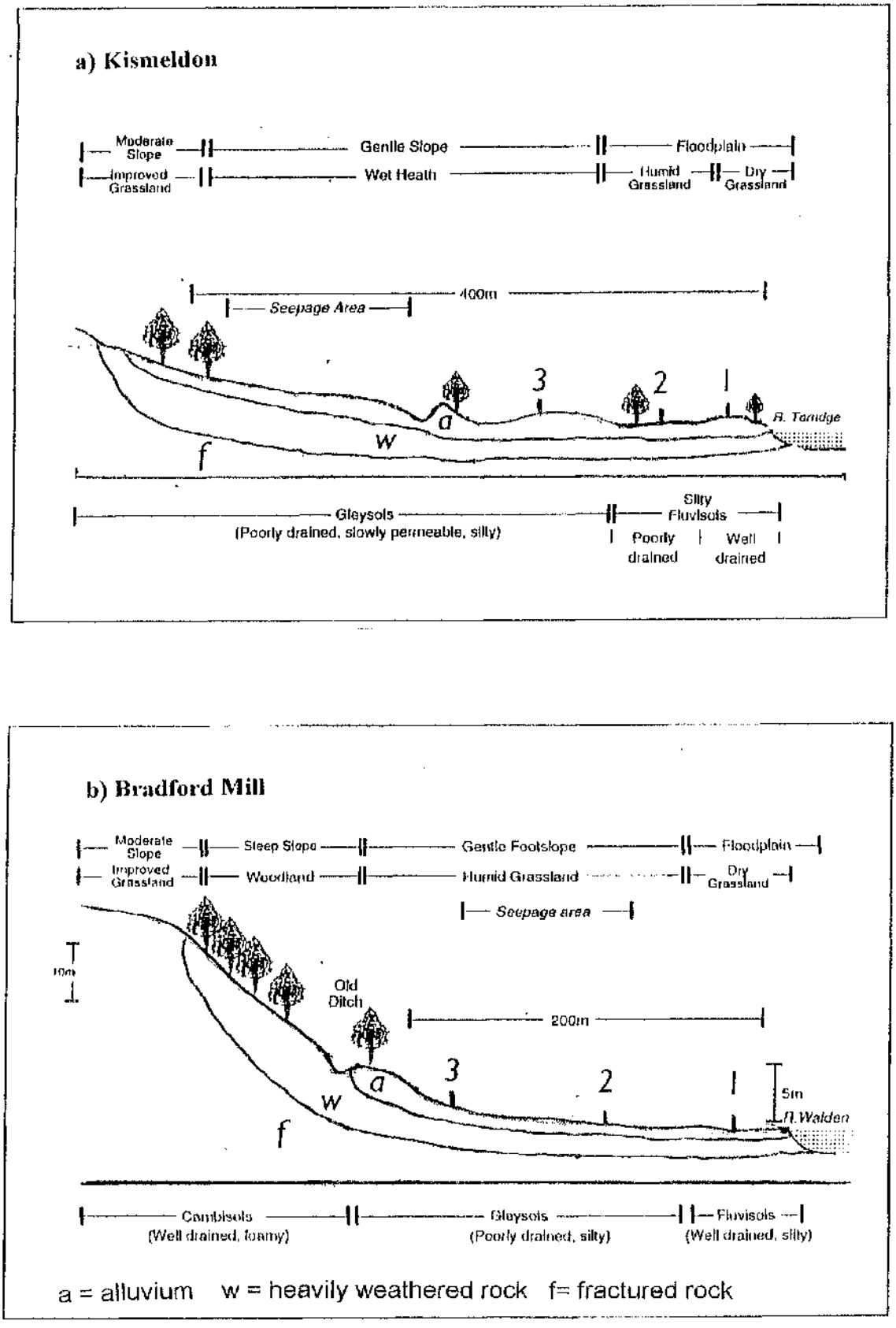


Fig. 2.5 Schematic cross-sections of the English study site transects; a) Kismeldon and b) Bradford Mill. Reproduced from Maltby *et al.* (1994).

Chapter 3
SPECIES DISTRIBUTION AND ABUNDANCE

3. SPECIES DISTRIBUTION AND ABUNDANCE

3.1 INTRODUCTION

The aims of this chapter are to

- Investigate the variation between different wetland sites in terms of species assemblage.
- Examine the sites in relation to measured environmental variables.
- Examine legume distribution within the wetland sites, in relation to the measured environmental variables.

Vegetation surveys were conducted at the sites listed in Table 2.1, spanning a range of different riverine wetland habitats. The abundance of all plant species was measured as percentage frequency (estimated using a subdivided 0.5 m^2 quadrat). The percentage cover and shoot density (m^2) were also measured for legumes. Environmental data was collected to investigate plant-environment relationships.

Ordination analysis was carried out to formulate hypotheses as to which environmental factors are important in influencing the distribution of legume species. These were then tested by experimental work (Chapter 5).

3.2 MATERIALS AND METHODS

3.2.1 Vegetation

At Ring Bog (Endrick marshes, Scotland) a fixed section of vegetation was studied (5 x 10 m strip) where *L. pedunculatus* was found in abundance. At the Irish sites the transects of the FAEWE team were followed. The sites in Devon were included in the vegetation survey only. At each of these sites a 10 x 10 m fixed quadrat was used for vegetation measurements at each unit along the transects. Destructive sampling was carried out outside the quadrats. The mean values of five visits to Ring Bog, three visits to Ireland in 1994, and one visit to Devon in July 1993 were used for data analysis. The scientific names, authorities and common names of all plant species encountered in this study are given in Appendix A.

The following measurements of legume abundance were also made. Codes are given in bold type after each variable.

i) Species frequency

The presence or absence of each plant species within the 25 subdivisions of each 5 randomly placed 0.5 x 0.5 m quadrats were recorded at each site throughout the field season. The species frequency data and legume codes are shown in Appendix B.

ii) Legume cover **(COVER)**

A pin frame (Philip Harris Education) was used to estimate the cover of legume species. The pins were lowered one at a time and each legume species touched by each pin was recorded. The pinframe (0.5 m in height, with ten pins at 5 cm intervals) was placed randomly (10 times) within each study site (quadrat) throughout the field season. The % cover of Legume A is given in the equation:

$$\% \text{ cover Legume A} = \frac{\text{Number of pins which hit Legume A at least once}}{\text{Total number of pins}} \times 100$$

iii) Legume density **(DENS)**

In the case of the legumes, it is difficult to distinguish individual plants, therefore the number of erect shoots within a 0.5 x 0.5 m quadrat was recorded. Ten random quadrats were assessed at each study site throughout the field season.

3.2.2 Environment

The following environmental parameters were determined for each site (mean values collected over several field visits), codes are given in bold type. The environmental data are given in Table 3.1.

i) Soil pH (**pH**)

Soil and 0.01N calcium carbonate (1:1) were mixed in a plastic beaker to a paste. A pH probe (Jenway model 3070 meter) was placed in this mixture and allowed to equilibrate for 10 minutes before a reading was taken. This was done for 5 replicate soil samples. The pH values were then placed in the following categories:

1 -< 4.5
2 - 4.6 - 5.5
3 - 5.6 - 6.5
4 - 6.6 - 7.5
5 - >7.5

ii) Soil wetness class (Maltby *et al.*, 1994) **(WET)**

Soil moisture was placed in one of the following categories (the number of days specified is not necessarily a continuous period and 'in most years' is defined as more than 10 out of 20 years):

1 The soil profile is not wet within 70 cm depth for more than 30 days in most years.
2 The soil profile is wet within 70 cm depth for 30-90 days in most years.
3 The soil profile is wet within 70 cm depth for 90-180 days in most years.
4 The soil profile is wet within 70 cm depth for more than 180 days, but is not wet within 40 cm depth for more than 180 days in most years.
5 The soil profile is wet within 40 cm depth for more than 180 days and is usually wet within 70 cm depth for more than 335 days in most years.
6 The soil profile is wet within 40 cm depth for more than 335 days in most years.

iii) Soil organic carbon status (Maltby *et al.*, 1994) **(CARBON)**

Soil for each site was placed in one of the following organic carbon status categories:

- Non-humous mineral soil (1). **NOHUM**
- Humous mineral soil (2). **HUM**
- Organic soil (peat) (3). **ORG**

iv) Water regimes (Maltby *et al.*, 1994) **(WATER)**

One of the following water regimes were selected for each site (based on Cowardin *et al.*, 1979):

- Not flooded (1). **NOFL**
- Intermittently flooded (2). The substrate is usually exposed, but surface water is present for variable periods, without detectable seasonal periodicity. Long periods, sometimes years may intervene between periods of inundation. **INTFL**
- Saturated (3). The substrate is saturated to the surface for extended periods during the growing season, surface water is seldom present. **SAT**
- Seasonally flooded (4). Surface water is present for extended periods early in the growing season but usually absent by the end of the season in most years. When surface water is absent, the water table is often near the surface. **SEFL**
- Semipermanently flooded (5). Surface water is present throughout the growing season, if surface water is absent, the water table is usually at or very near to the land surface. **SEPEFL**

v) Soil total nitrogen **(TOTN)**

The total nitrogen (mg N g^{-1} dry weight) of soil samples was measured using the Kjeldahl method (Appendix C). Data for the Irish and English sites were provided by Dr David Hogan (University of Exeter, FAEWE).

1 - 0-5
2 - 6-10
3 - 11-15
4 - 16-20
5 - 21-25
6 - 26-30

vi) Management **(MAN)**

Land management for each site was placed in one of the following categories:

- Grazed (1) **GRAZE**
- Mown (2) **MOWN**
- Unmanaged (3) **UNMAN**

Table 3.1 Environmental data for each site (site codes are given in Table 2.1).

SITE	pH	WET	CARBON	WATER	TOTN	MAN
BM1	2	1	1	2	1	1
BM2	2	5	2	1	2	1
BM3	2	5	2	1	2	1
CL1	3	6	3	5	3	2
CL2	3	4	2	2	2	2
CL3	2	6	3	4	3	2
CL4	2	6	3	4	3	2
CL5	2	5	3	4	5	2
LB1	4	5	2	2	2	1
LB2	4	6	3	4	4	1
LB3	3	5	3	4	3	1
LB4	2	5	3	4	6	1
LBM	4	5	2	2	2	1
KM1	2	1	2	2	1	1
KM2	2	5	2	3	2	1
KM3	2	5	2	1	2	1
RB	2	6	3	3	2	3

3.3 RESULTS

3.3.1 Analysis : background

Classification is useful in that it breaks down large data sets into more manageable groups, though it should be remembered that it is not strictly applicable to a situation of continuous change (Causton, 1988). Two-Way Indicator Species Analysis (TWINSPAN) was used to classify the sites (Hill, 1979). TWINSPAN characterises groups of sites by a group of differential species (this idea originating from phytosociology), using quantitative and qualitative data (Jongman *et al.*, 1987). It works on the basis of correspondence analysis (CA) (Hill, 1973). CA is an indirect ordination technique which works on the principle that species abundance data show a unimodal response to environmental variables (as opposed to a linear response as assumed, for example, by Principle Components Analysis (PCA) (Gauch, 1982)). A dichotomy is made at the centre of gravity of the first axis of an ordination of the sites, creating two groups which are then divided, and those groups created further are ordered and divided until an arbitrary end point is reached. The groups formed at each side of the dichotomy are known as negative (left hand) and positive (right hand) indicators and preferential species are given for each division. The program utilizes quantitative data by using the concept of the pseudospecies (Hill *et al.*, 1975). Each species abundance is replaced by at least one pseudospecies, the number of pseudospecies needed for any one species is proportional to the total range of abundances of that species. A limited number of pseudospecies can be assigned, and the species abundance range for each must be set, this is known as the 'cut level'.

Each site was also classified according to both the CORINE Biotopes and National Vegetation Classification (NVC). The TABLEFIT program (Hill, 1993) was used to help determine the NVC class for each site as described by Rodwell (1991, 1992, 1995). TABLEFIT determines a measure of goodness-of-fit between vegetation samples and the expected species composition of each vegetation type. Classes were only assigned when the goodness-of-fit was above 60%. CORINE biotopes is "an inventory of sites of major importance for nature conservation in the European Community" (Commission of the European Community, 1991) which classifies vegetation on a more habitat oriented, Europe wide basis.

Two ordination techniques were used to analyse the relationship between species composition and environment. The first, Detrended Correspondence Analysis (DCA), is based on CA and uses the species composition to order sites in multidimensional space. DCA 'flattens' the arching effect that can occur in CA by

'detrending' (Hill, 1979; Hill & Gauch, 1980). Usually the first two or three axes are sufficient to explain much of the variance in the dataset. Sometimes one or two environmental variables are strongly correlated with the main (or first) axis of variation in the species composition data. This can easily be investigated by regression of the DCA axis 1 scores and the environmental variables. The second technique, Canonical Correspondence (CCA) is a canonical ordination technique used if one or two of the environmental variables cannot obviously explain the main variation in the species composition. It is used to detect patterns in variations of species data and explain them by reference to measured environmental variables. This is a direct gradient ordination technique, the axes are constrained by the environmental variables. An arching effect which occurs if several environmental variables are highly intercorrelated can be rectified by detrending as in DCA but can also be 'flattened' without detrending by removing one of each pair of intercorrelated variables. Arching is usually found in the second axis and variables highly correlated with this axis are more likely to be superfluous (ter Braak, 1987). Intra-set correlations must be considered in CCA interpretation: these are the correlation coefficients between the ordination axes and environmental variables (ter Braak, 1986, 1987). The computer program CANOCO (ter Braak, 1991) was used for both DCA and CCA analysis.

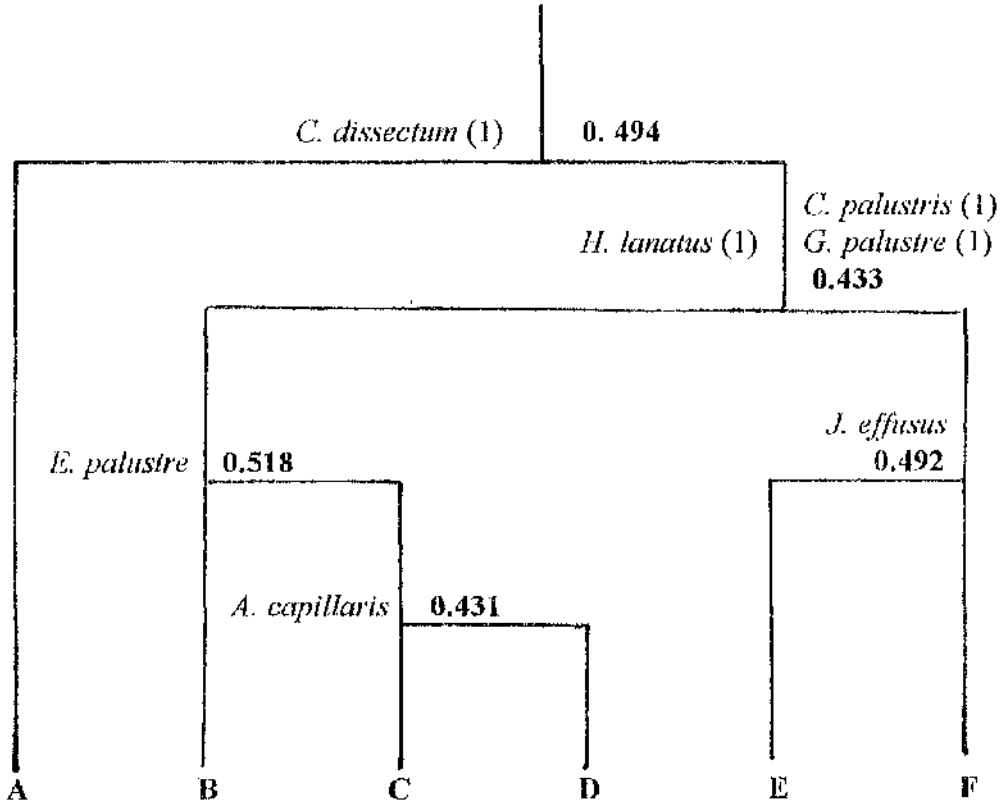
3.3.2 Analysis : Interpretation

TWINSPAN classification (with four cut levels) was carried out on the species frequency data for all sites. Subdivisions were stopped when the eigenvalue (which is a measure of the variance along the axis) fell below 0.400 and the sites were split into six groups, illustrated in Fig. 3.1. The NVC classifications and assigned CORINE biotopes for each site, together with their TWINSPAN group are shown in Table 3.2. The codes for each site are given in Chapter 2.

Group A contains the Torridge sites, KM2 and KM3, both the NVC classification and CORINE biotope define this group as acid purple moor grass meadows (C37.312), the main community present was *Molinia caerulea-Cirsium dissectum* with the subcommunity defined as *Juncus acutiflorus-Erica tetralix* (M24c).

Ring Bog and the Devon sites BM2, BM3 comprise Group B. It possesses two mire communities (*Filipendula ulmaria-Angelica sylvestris* (NVC category M27 and CORINE C37.1) and *Juncus acutiflorus-Galium palustre* (NVC category M23a and CORINE C27.217). The grassland community is *Holcus lanatus-Deschampsia cespitosa* (NVC category MG9 and CORINE C37.213).

Fig. 3.1 Dendogram of sites classified by species (using TWINSPAN). At each division the indicator pseudospecies and eigenvalue are given.



A	B	C	D	E	F
KM2	BM2	KM1	BM1	CL5	CL1
KM3	BM3		CL2	LB4	LB2
	RB		CL3		LB3
			CL4		
			LB1		
			LBM		

Table 3.2 TWINSPAN groups with NVC classifications and CORINE biotopes.

GROUP	SITE	NVC CLASSIFICATION	CORINE
A	KM2	M24c *Molinia caerulea-Cirsium dissectum* subcommunity *Juncus acutiflorus-Erica tetralix*	C37.312 Acid purple moor grass meadows
A	KM3	M24c *Molinia caerulea-Cirsium dissectum* subcommunity *Juncus acutiflorus-Erica tetralix*	C37.312 Acid purple moor grass meadows
B	RB	M23a *Juncus effusus/acutiflorus-Galium palustre*	C37.217 Soft rush meadows
B	BM2	MG9 *Holcus lanatus-Deschampsia cespitosa*	C37.213 Tufted hairgrass meadows
B	BM3	M27 *Filipendula ulmaria-Angelica sylvestris*	C37.1 Meadow sweet stands
C	KM1	M27 *Filipendula ulmaria-Angelica sylvestris*	C37.1 Meadow sweet stands
D	LBM	MG11a *Festuca rubra-Agrostis stolonifera Potentilla anserina* subcommunity *Lolium perenne*	C37.242 Creeping bent and tall fescue swards
D	LB1	MG11a *Festuca rubra- Agrostis stolonifera- Potentilla anserina* subcommunity *Lolium perenne*	C37.242 Creeping bent and tall fescue swards
D	BM1		38.1 Mesophile pastures
D	CL2	M27b *Filipendula ulmaria-Angelica sylvestris* subcommunity *Urtica dioca-Vicia cracca*	C37.1 Meadow sweet stands
D	CL3	M27 *Filipendula ulmaria-Angelica sylvestris*	C37.1 Meadow sweet stands
D	CL4	M27 *Filipendula ulmaria-Angelica sylvestris*	C37.1 Meadow sweet stands
E	CL5	M27a *Filipendula ulmaria- Angelica sylvestris* subcommunity *Valeriana officinalis-Rumex acetosa*	C37.1 Meadow sweet stands
E	LB4	SD17 *Potentilla anserina- Carex nigra*	C16.34 Dune slack grasslands
F	CL1	S19c *Eleocharis palustris* swamp subcommunity *Eleocharis palustris*	C15.339 *Eleocharis palustris* beds
F	LB2	S19c *Eleocharis palustris* swamp subcommunity *Eleocharis palustris*	C15.339 *Eleocharis palustris* beds
F	LB3	S19c *Eleocharis palustris* swamp subcommunity *Eleocharis palustris*	C15.339 *Eleocharis palustris* beds

Group C consists of one site only, KM1 a mire community *Filipendula ulmaria-Angelica sylvestris* (NVC category M27 and CORINE C37.1).

Group D is the largest group, it too has both mire and mesotrophic grassland communitites represented. The main mire community present in this group is defined as *Filipendula ulmaria-Angelica sylvestris* (NVC category M27 and CORINE C37.1). The mesotrophic grassland communities present are *Holcus lanatus-Juncus effusus* (NVC category MG9 and CORINE C37.213), *Festuca rubra-Agrostis stolonifera-Potentilla anserina* (NVC categories MG11 and CORINE C37.242). It was not possible to assign BM1 a sensible NVC class, but the CORINE biotope of mesophile grassland fitted well (C38.1).

Group E consists of two Irish sites, CL5 defined as *Filipendula ulmaria-Angelica sylvestris* (NVC category M27 and CORINE C37.1) and LB4. Site LB4 was assigned to a dune grassland community (NVC category SD17 and CORINE C16.34) this may be due to the strong influence of marl in the overlying peat (Nigel Willby, pers. comm., 1996).

The Irish sites, CL1, LB2 and LB3 comprise Group F. This group is defined as *Eleocharis palustris* swamp (S19a) by NVC and (C15.339) by CORINE.

A DCA was carried out in order to determine the relationship between sites and display this graphically. A summary of the DCA using species composition followed by a multiple regression of the species scores on the first DCA axis against the measured environmental variables is shown in Table 3.3. This indicates that the environmental variables show significance ($p<0.05$) in explaining to some extent the species scores along the gradient explained by DCA axis 1. The long gradients (greater than 2 standard deviations) show that DCA was the appropriate method to use as opposed to PCA, for example, which is more suitable for shorter gradients (ter Braak, 1986). A DCA ordination of the sites using species composition with TWINSPAN groups displayed is shown in Fig. 3.2. Axes 1 and 2 only are displayed as the eigenvalue of axis 3 falls to 0.227 and it explains little more of the species variance than the first two axes.

Table 3.3 DCA Summary

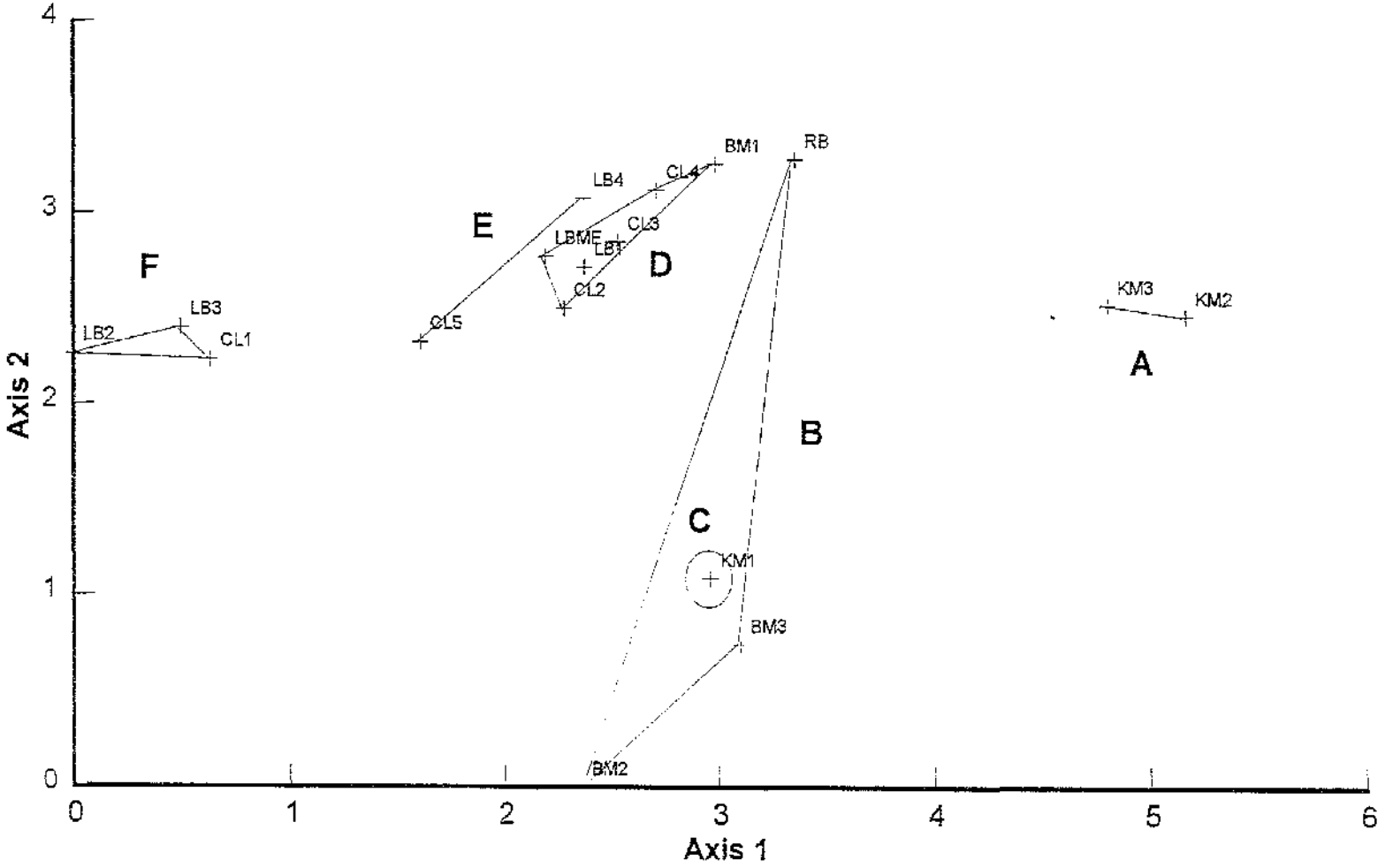
Axes	1	2	3	4	Total Inertia
Eigenvalues	.656	.466	.227	.139	4.202
Lengths of gradient	5.162	3.286	2.811	1.859	
Cumulative percentage variance of species data	15.6	26.7	32.1	35.4	
Sum of all unconstrained eigenvalues					4.202

Regression equation

DCA axis 1 = 7.07 - 1.06 pH + 0.424 WET - 1.40 ORG C - 0.062 WATER - 0.159 TOTN + 0.014 MAN

R-sq (adj) = 48.0% ANOVA p:0.041

Fig. 3.2 DCA ordination of all sites (using species composition).
TWINSPAN groups overlain (see Table 3.2).



To investigate further the findings put forward by the DCA, a CCA was carried out. As a method of standardisation the environmental variables were ranked. To avoid any arching effects the data were examined for any superfluous environmental variables. The last category within a group of nominal variables was classed as redundant, and therefore not included in the final analysis. CANOCO allows the forward selection of environmental variables which enables redundant variables to be identified. Each variable was added sequentially in order of ability to explain the variation in the species data. A Monte-Carlo permutation test was run on each variable to determine its significance in explaining the remaining variation. The saturated, not flooded and intermittently flooded water regimes, soil wetness class and pH all explained significant variation independently of other variables ($p<0.05$). At a lower significance level ($p<0.1$) unmanaged land management explained a significant amount of variation. An overall Monte-Carlo permutation test using the selected variables showed that the species data were significantly related to the environmental variables ($p<0.05$).

Two extra variables, legume cover and density, (in addition to the redundant variables) were run as passive variables, enabling them to be placed on the ordination diagram without including them in the actual analysis. A summary of the CCA is shown in Table 3.4. The eigenvalue for each axis is relatively high, indicating that species separation is satisfactory and 35.8% of the species-environment relation is contained in the first two axes. The environmental variables measured were found to explain a large amount of the species variation, shown by the high ratio between the sum of canonical and unconstrained eigenvalues. The CCA ordination of site scores (site codes not shown, TWINSPAN groups are overlaid) and legume species scores with environmental biplots/centroids is shown in Fig. 3.3. Only axes 1 and 2 are plotted. Much variation was explained by axes 3 and 4 (see Table 3.4) but further examination revealed that they did not correlate strongly with any of the environmental variables and were therefore not plotted. Nominal variables are plotted as centroids (the weighted average of sites/species belonging to that category) and are represented by filled circles, continuous variables are plotted as arrows (which correspond approximately to the ranking of the weighted average of the sites/species according to an amount of the environmental variable). The length of an arrow give an indication of its importance compared to other variables. Species and sites are represented by a cross.

Table 3.4 CCA Summary

Axes	1	2	3	4	Total Inertia
Eigenvalues	.623	.487	.427	.336	4.202
Species-environment correlations	.991	.978	.997	.868	
Cumulative percentage variance					
of species data	14.8	26.4	36.6	44.6	
of species-environment relation	20.1	35.8	49.5	60.4	
Sum of all unconstrained eigenvalues					4.202
Sum of all canonical eigenvalues					3.101

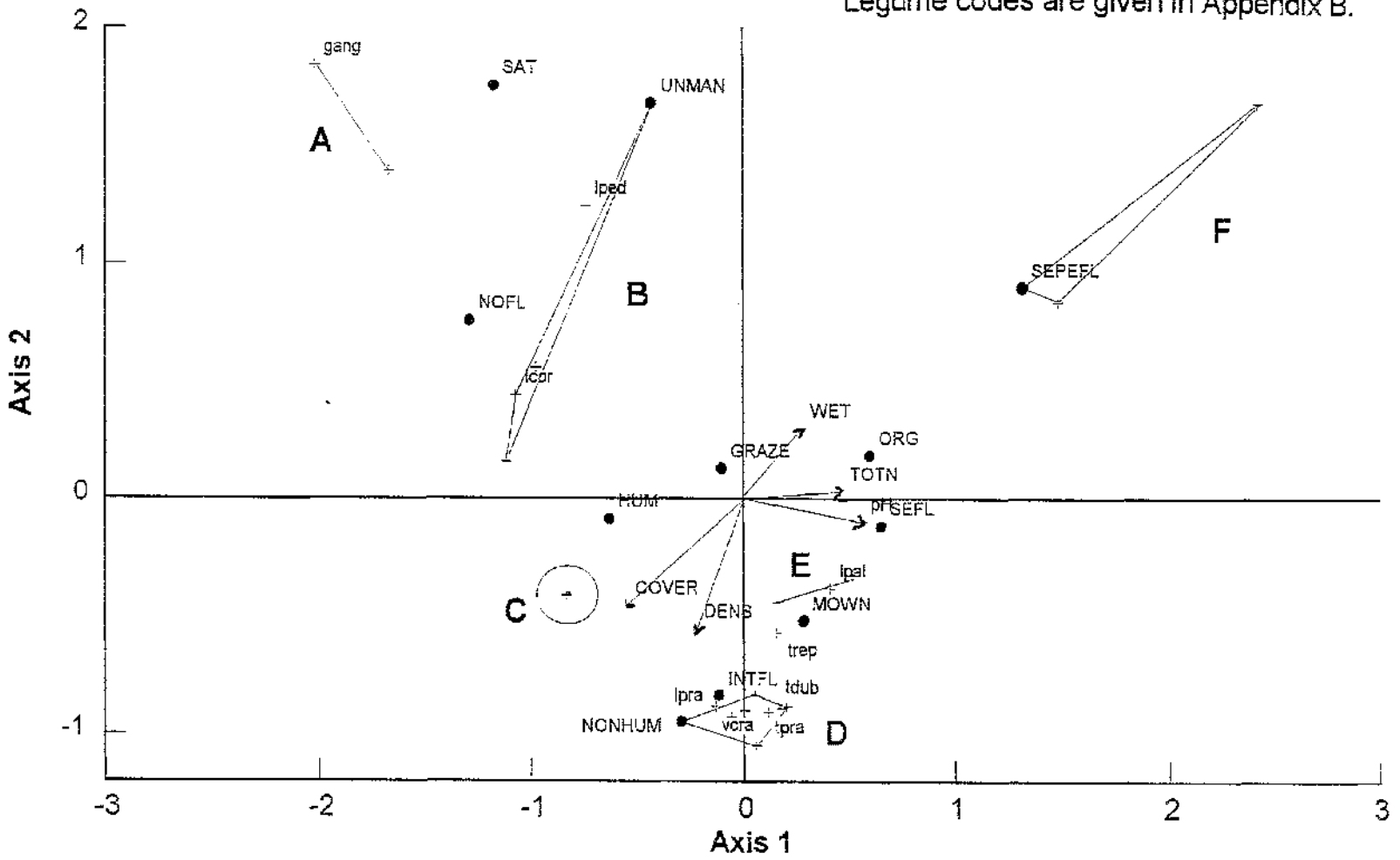
The intra-set correlations are shown in Table 3.5. Organic (peat) soil organic status, soil pH and total nitrogen, seasonally flooded and semi-permanently flooded water regimes correlated positively with Axis 1, humous soil organic carbon status, legume cover and saturated and not flooded water regimes were negatively correlated. Unmanaged management type was positively correlated with the second axis, intermittently flooded water regime and mown management type were negatively correlated.

Table 3.5 Intra-set Correlations

	AXIS 1	AXIS 2
Organic (peat) *	.5940	.1801
Legume cover *	-.5515	.4473
Humous	-.5641	-.0756
pH	.5570	-.0946
Seasonally flooded	.5215	-.0914
Not flooded	-.4835	.2795
Total Nitrogen	.4569	.0297
Saturated	-.3967	.5831
Semi-permanently flooded *	.3011	.2030
Legume density *	-.2773	-.2577
Wetness class	.2712	.2928
Mown	.1943	-.3579
Grazed *	-.1390	.1601
Unmanaged	-.1038	.3955
Intermittently flooded	-.0847	-.5752
Nonhumous	-.0721	-.2270

* = passive variables

Fig. 3.3 CCA biplot of site scores (labels not shown), legume scores and environmental biplots/centroids.
TWINSPAN groups overlain (see Table 3.2) environmental variable codes given in Table 3.1.
Legume codes are given in Appendix B.



The TWINSPAN groups on the left of the CCA biplot of site scores are mainly those which are not regularly flooded (though some are saturated). The negative correlation of legume cover (and density to a lesser extent) with axis 1 implies that as flooding regularity increase these decrease.

The legume species were ranked along the more influential environmental parameters by dropping perpendicular lines from the species co-ordinates to the environmental arrows or by measuring distance to centroids. Rankings were given for soil wetness class, soil pH (arrows) water regime and management type (centroids) and are presented in Table 3.6. The species at the top of the list are found at high values of the particular environmental parameter (arrows). For the nominal variables, the species at the top of the list is the one most likely to be found at a site of that environmental category.

Table 3.6 Ranking of legume species along influential environmental parameters (from CCA). Environmental codes are given in section 3.2.2.

WATER REGIME	WATER REGIME	MANAGEMENT
SAT	**INTFL**	**UNMAN**
L. pedunculatus	*L. pratensis*	*L. pedunculatus*
G. anglica	*V.cracca*	*L. corniculatus*
L. corniculatus	*T. pratense*	*G. anglica*
L. palustris	*T. dubium*	*L. palustris*
T. repens	*T. repens*	*T. repens*
L. pratensis	*L. palustris*	*L. pratensis*
T. dubium	*L. corniculatus*	*V. cracca*
V. cracca	*L. pedunculatus*	*T. pratense*
T. pratense	*G. anglica*	*T. dubium*

SOIL WETNESS CLASS	SOIL pH
L. pedunculatus	*L. palustris*
L. palustris	*T. dubium*
G. anglica	*T. pratense*
T. repens	*T. repens*
L. corniculatus	*V. cracca*
T. dubium	*L. pratensis*
T. pratense	*L. pedunculatus*
V. cracca	*L. corniculatus*
L. pratensis	*G. anglica*

3.4 DISCUSSION

3.4.1 Classification of sites

Comparison of the TWINSPAN groups with other classifications (NVC and CORINE) (Table 3.2) showed that several different wetland plant communities occurred at the wetland sites, although this does not imply that variation between sites is discontinuous. The majority of TWINSPAN groups corresponded well with both NVC and CORINE classifications. With the exception of Group D, the TWINSPAN groups represented one or two NVC and CORINE classifications, which is not surprising as NVC is also based on TWINSPAN analysis. However, it was valuable to perform my own separate TWINSPAN analysis as this highlighted site differences in species composition which were too subtle for broad scale classification systems such as NVC or the even less detailed CORINE, for example, the NVC category M27 occurred in four TWINSPAN groups.

3.4.2 Influential environmental parameters on species composition

A number of environmental parameters influenced both community and legume distribution. From the CCA ordination (Fig. 3.3) it was found that water regime, soil wetness class, soil pH and management type, were all influential in defining site and species scores and will be discussed further.

3.4.2.1 Influence of water regime and soil wetness class on species composition

Flooding was recorded by two different measures: soil wetness class and water regime. Each represent a different aspect of flooding; soil wetness class relates to soil moisture whereas water regime denotes flooding frequency and duration.

a) Water regime

This (flooding frequency and duration) influences the site scores along CCA axis 1 (Fig. 3.3). For example, at the negative end of the axis, TWINSPAN groups A and B are found, comprising not flooded and saturated sites. Characteristic species of group A are *Molinia caerulea* and *Cirsium dissectum*, while *Carex panicea*, *Festuca rubra* and *Potentilla erecta* are also present. These are species characteristic of well drained conditions (Grime et al., 1988) with the exception of *C. panicea* which is often found on damp soils (Grime *et al.*, 1988). Group B however has species tolerant of moister soils, such as *Deschampsia cespitosa*, *Filipendula ulmaria* and *Epilobium palustre* and various *Juncus* species: *acutiflorus*, *effusus* and *inflexus* (Grime *et al.*, 1988). At the other extreme, group F is found at the positive end of the axis, containing sites which are semi-permanently flooded. Species characteristic of much wetter conditions are found here: *Eleocharis*

palustris, *Phalaris arundinacea*, *Caltha palustris* and *Mentha aquatica* (Grime *et al.*, 1988).

Groups in between these extremes are not regularly flooded and are characterised by a mixture of species with varying degrees of flood tolerance. Species characteristic of well-drained soils such as *Festuca rubra* and *Plantago lanceolata* are found alongside species capable of withstanding impeded drainage, such as *Agrostis stolonifera*, *Cardamine pratensis*, *Carex disticha*, *Rumex acetosa* and *Filipendula ulmaria* (Grime *et al.*, 1988). It would seem that group C consisting of one site is separated from the large group D mainly by the presence of *Agrostis capillaris*, a species characteristic of free-draining soils (Grime *et al.*, 1988). This group is placed to the left of the diagram at the negative end of the flooding regime gradient associated with axis 1. Group E is found to the right of group D on the CCA, further towards the positive end of the flooding regime gradient. The TWINSPAN output gave *Juncus effusus* as the indicator species important in separating this group from the semi-permanently flooded group (F). This species, although capable of surviving wet conditions is generally not found in habitats which are submerged for long periods of time (Grime *et al.*, 1988). Water regime was also found to be an important influence on species composition on the Irish callows by Heery (1991) and Hooijer (1996).

It is well known that differences in flooding tolerance occur between species e.g. Etherington (1984) found differences in adaptations between two morphologically similar species: *Epilobium hirsutum* (wetland) and *Chamerion angustifolium* (aerated soil). In addition differences arise within genera and within species. Voesenek *et al.* (1988) found that *Rumex acetosa*, *R. crispa* and *R. palustris* differed in ability to tolerate flooding. Different populations of the same species have also been found to vary in flooding tolerance, for example: *Festuca rubra* and *Agrostis stolonifera* (Davies & Singh, 1983); *Carex flacca* (Heathcote *et al.*, 1987) and *Dactylis glomerata* (Etherington & Thomas, 1986).

b) Soil wetness class

Soil moisture in addition to flooding frequency and duration was also influential as indicated by the importance of soil wetness class. Waterlogging of the soil by flooding cannot be regarded as a single stress to plants as it has profound effects on the soil environment (Davy *et al.*, 1990). Waterlogging eliminates nearly all gas-filled pores and gas exchange is severely restricted since oxygen and carbon dioxide diffuse 10,000 times slower in water than in air (Grable, 1966). Thus the soil

becomes oxygen deficient. The electrochemistry of the soil solution is also affected greatly, the solution is diluted immediately and redox potential (a measure of the tendency of the soil to act as an oxidizing or reducing agent (Lincoln *et al.*, 1982)) is greatly reduced (Armstrong, 1982). Reduced soil is characterised by the absence of NO_3^-, and the presence of Mn^{2+}, Fe^{2+}, NH_4^+ and S^{2-} (Ponnamperuma, 1984). Lack of oxygen prevents the conversion of ammonium to inorganic nitrogen, thus ammonium accumulates in flooded soils. Nitrate is converted (denitrified) to nitrous oxide (N_2O) or nitrogen (N_2) and is lost from the system. In addition to the chemical processes involved, micro-organisms also contribute to the reduction of mineral ions in flooded soil, a detailed account of this subject is given in Laanbroek (1990). The reduction of Mn (IV) to the more soluble form Mn (II) precedes that of ferric ion Fe (III) to ferrous ion Fe (II) which again is more soluble and therefore more available for plant uptake. At very low redox potentials associated with prolonged inundation, sulphate SO_4^{2-} is reduced to S^{2-}.

The consequences of soil waterlogging for plant growth are numerous since not only does the plant face lack of oxygen, but also the potentially toxic effects of high concentrations of reduced metal ions. The response of plants to waterlogging is varied (Jackson & Drew, 1984). Crawford (1989) uses the approach of Levitt (1980), classifying plants which can withstand waterlogging as either 'avoiders' or 'tolerators'. Although higher plants are dependent on oxygen for growth and survival, plants do differ in their ability to tolerate anoxia, although lack of oxygen is not the only stress (as outlined previously). Barclay & Crawford (1982) found that the underground organs of some wetland species could tolerate a completely anaerobic environment for several months.

It can be seen therefore how soil wetness class may influence plant species composition of a particular site. Wetland plant species have different adaptations allowing them to inhabit waterlogged habitats. The adaptations of plants to flooding have been reviewed extensively by many authors, including Hook *et al.* (1984), Crawford (1989), Blom *et al.* (1990), Ernst (1990) and Armstrong (1994). Several species develop aerenchyma which facilitate transportation of oxygen from the shoots to waterlogged roots, allowing oxidation of the root tip, by reduced resistance to O_2 movement and development of lignified and suberised cell walls on the upper parts of the roots prevents radial diffusion of oxygen into the soil (Armstrong, 1979; Armstrong & Beckett, 1987). The origins of aerenchyma tissue differs between species; it can be formed either by the enlargement of intercellular spaces (schizogeny) e.g. *Filipendula ulmaria*, *Caltha palustris* or by cell

disintegration (lysigeny) to form large air filled spaces (lacunae) e.g. *Juncus effusus*, *Mentha aquatica* (Smirnoff & Crawford, 1983). On examination of 91 plant species from different habitats, Justin & Armstrong (1987) found that the species more tolerant of flooding were more likely to have a large amount of aerenchyma than those that were intolerant. In addition to oxygen supply, aerenchyma also detoxify reduced mineral ions by oxidation of the rhizosphere.

Some species are adapted to high manganese and iron concentrations. Avoidance of uptake is facilitated by aerenchyma, since the oxidation of the rhizosphere results in the formation of oxides. The commonly observed reddish-brown deposits in waterlogged soils is ferric hydroxide. This renders the iron unavailable for plant uptake, but it is thought to decrease uptake of phosphate and other nutrient ions (Ernst, 1990). This iron 'plaque' can also prevent uptake of sulphide by the precipitation of iron sulphide (FeS). Resistance can also be due to the ability to tolerate high cellular concentrations of toxic ions, high concentrations of manganese and iron occur in plants growing in flooded soils (Ernst, 1990). The accumulation of various organic acids by plants growing in waterlogged conditions is an area of plant flooding tolerance which is unresolved (Ernst, 1990) and there is generally a lack of agreement on the adaptive value of metabolic responses to flooding (Smith *et al.*, 1986; Crawford, 1989, Davy *et al.*, 1990).

3.4.2.2 Influence of water regime and soil wetness class on legume distribution

With the exception of *L. pedunculatus*, *L. corniculatus* and *G. anglica* most legume species are found at the intermittently flooded sites. These three species were not found at flooded sites, however *G. anglica* and *L. pedunculatus* inhabited saturated sites. This distribution is reflected by their inferred wetness class ranking. *G. anglica* and *L. pedunculatus* are within the top section of this list (Table 3.6), along with *L. palustris*, a rare legume (Heery, 1993) which is locally abundant in patches at Clonmacnoise. This species is recorded as being found in marshes and fens (Stace, 1991) which is supported by the findings inferred from the CCA. Both *L. pedunculatus* and *L. pratensis* are featured in Newbold and Wheeler's (1996) review of water requirements of wetland plants (the only legumes to be included). *L. pedunculatus* had a preferred water table depth higher than that of *L. pratensis*. Somewhat surprising in this analysis is the ranking of *T. dubium* in terms of wetness class and water regime, Grime *et al.* (1988) states that this species is not found in wetlands, so it would be expected to have the lowest wetness class ranking. However, although it is in the bottom section of the list (Table 3.6) another three species follow which have all been recorded in wetlands (Grime *et al.*, 1988). It has

long been established that prolonged flooding has a detrimental effect on legume growth (Bolton & McKenzie, 1946) both at the mature and seedling stage of plant growth (McKenzie, 1951). This supports the inverse relationship between soil wetness class and legume cover revealed by the CCA. It is speculated that legumes are generally absent from wetlands, probably as a result of the conflict between the need for oxygen to support the respiratory demands for nitrogen fixation and the oxygen deficient conditions associated with wetland soils (Grime *et al.*, 1988). However this study indicates that some legumes can grow successfully in flooded sites albeit irregularly, and in the case of *L. pedunculatus* and *G. anglica* in saturated soils. Chapter 5 will explore these findings further and determine individual legume response to waterlogging.

3.4.2.3 Influence of management on species composition

Management of sites was classified as either mown, grazed (by cattle) or left unmanaged. In this study it is difficult to separate the effects of mowing and grazing, therefore sites which are grazed may be situated closer to the mown centroid and vice versa, but it is apparent that no management at all contributes significantly to species composition (i.e. no defoliation versus defoliation is an important influence).

It has long been established that defoliation (mowing or grazing) can have great effect on plant communities. It is well known that mowing (or grazing) can maintain plant species diversity because if it ceases a few individuals increase in size leading to a reduction in plant number and thus plant species (Darwin, 1859). Many studies have been carried out on individual plant response to defoliation and these are often confused with arguments at the community and ecosystem level (Belsky, 1987). It has been widely proposed (reviewed independently by Belsky (1986) and McNaughton (1986)) that defoliation by herbivory may have a positive effect on individual plant growth and survival. Belsky (1986, 1987) suggests that the reports of overcompensation (increased growth compared to non-grazed plants) by grazed plants lack definitive evidence, whereas evidence of the negative effects of herbivory is well-documented (Crawley, 1983). Whatever arguments exist pertaining to individual plant response to defoliation, it is accepted that by removing competitors or adding additional nutrients to the system (from faeces and urine or nutrients leached from damaged leaves), grazing or mowing can drastically alter plant communities (Belsky, 1987).

Theoretical models have been put forward suggesting that changes in both species diversity and composition in grasslands are to some extent related to grazing

(Milchunas *et al.*, 1988). Grazing results in a loss of individual plant organs therefore altering canopy structure. The plant community will alter according to individual plant recovery and the impact of defoliation on the competitive relationships in the canopy.

The only site studied which is not managed is Ring Bog. Species richness was no different to that of managed sites, however Ring Bog differs in terms of its species composition. *L. pedunculatus* is a palatable herb which is now widely naturalised in New Zealand where it is grown as a pasture legume on account of its numerous qualities (Harris *et al.*, 1993). Its abundance at Ring Bog may be a result of the cessation of grazing but this can only remain speculation as records of species composition prior to this were not available. However if one examines the grazed sites where *L. pedunculatus* is present, its frequency is much lower. Other species found at Ring Bog are also 'susceptible' to grazing pressure, such as *Valeriana officinalis* and *Angelica sylvestris* (Grime *et al.*, 1988); these species are either absent or only found in very small numbers at other (grazed) sites. Conversely, *Molinia caerulea* and *Deschampsia cespitosa* are less abundant at Ring Bog than at the other sites. Both species are tolerant of grazing and higher abundance at the grazed sites may be a result of the management regime in conjunction with drier soil conditions when compared to Ring Bog. It should be noted that lack of grazing/mowing is not the only influence on the plant species found in Ring Bog. Also there was likely to have been a very light grazing pressure from wild herbivores such as deer and rabbits at Ring Bog.

3.4.2.4 Influence of management on legume distribution

The effects of defoliation on nodulation and nitrogenase activity of legumes is complicated. Nodules are either shed or undergo bacteroid degeneration and regrow as in *Medicago sativa* for example (Vance *et al.*, 1979). Either way nitrogen fixation is reduced and recovery depends on the amount of stored carbohydrate that is available for new shoot growth (Sprent & Minchin, 1983). It is interesting that the ranking of the legume species according to their likelihood to inhabit an unmanaged site corresponds well to the findings of a previous study investigating the effects of defoliation on root and nodule growth. Three species were examined: *T. repens*, *T. pratense* and *L. uliginosus* (= *L. pedunculatus*), the latter species was the least tolerant to defoliation, with *T. pratense* the more tolerant of the two clovers (Butler *et al.*, 1959). In this study *L. pedunculatus* was found to be most likely to inhabit an unmanaged site while *T. pratense* was one of the least likely. The effects of defoliation on individual legume species are examined further in Chapter 5.

3.4.2.5 Influence of soil pH on species composition

As discussed earlier, flooding cannot be regarded as a single factor. From the CCA it can be seen that soil pH is also correlated with axis 1, in addition to water regime. The pH of an acid soil increases on prolonged flooding, the opposite occurs in alkaline soils. Most flooded mineral soils have pH values between 6.7 and 7.2 (unless a soil has a low reducible ion content, in which case the pH may not rise above 5.0). If a flooded alkaline soil has a low organic matter content, the pH may not fall below 8.0 (Ponnamperuma, 1984). At the negative end of axis 1 the sites on the more acid soils are found. Groups A and B for example are found on the more acidic soils of the sites studied. Species such as *Potentilla erecta*, *Molinia caerulea* and *Lotus pedunculatus* are common to both these groups; these are indicative of acid soils (though *Molinia* is also found at the other extreme on calcareous soils) (Grime *et al.*, 1988). At the positive end of the axis, species usually found in the less acidic pH range of 6-7 are found associated with sites in group F such as *Eleocharis palustris*, *Phalaris arundinacea*, *Caltha palustris*, *Mentha aquatica* and *Ranunculus repens* (Grime *et al.*, 1988). In general, high pH is associated with deficiencies in nutrients such as iron, manganese, calcium, potassium or phosphorus, whereas low pH is associated with high levels of phytotoxic ions such as aluminium and iron (Ponnamperuma, 1984). Some plant species have a very narrow range of pH tolerance (characteristic of acid or alkaline soils) but most species are found in-between these extremes and grow within a wide range of soil pH (Crawley, 1986).

3.4.2.6 Influence of soil pH on legume distribution

Most legumes grow better at a neutral or slightly acidic pH for growth (Bordeleau & Prévost, 1994). In addition to effects on the host plant *per se*, the legume-rhizobium symbiosis is affected (Munns, 1986; Graham, 1992). Extremes of pH and accompanying nutrient disorders can effect the survival of rhizobia in the soil, the infection process and also nodule function. Inhibition of nodulation generally occurs at pH values <4.5-5.0 (Edmeades *et al.*, 1991). The inferred ranking of legumes along the soil pH gradient, generally follows the findings of previous studies, with species such as *G. anglica* and *L. pedunculatus* at the low pH end of the list, along with *L. corniculatus* which inhabits a wide range of soil pH (Grime *et al.*, 1988). The other legumes were all found at the higher soil pH's recorded in this study. Literature on the soil pH requirements of *L. palustris* is lacking, but in this study, of all the legumes, it is found at the highest pH along with *T. dubium* and *T. pratense*. The pH range of these sites is small compared to the known range in which these legumes can occur, therefore extrapolation of the rankings found here to other situations may be unrealistic.

3.5 SUMMARY

- Species composition was used successfully to classify the sites into groups which related well to already existing classification systems (NVC and CORINE) (Table 3.2).

- Multivariate approaches for exploratory data analysis revealed that species composition of the sites was influenced mainly by flooding regime, soil wetness class, land management and soil pH (Canonical Correspondence Analysis including Fig. 3.3).

- Nine species of legumes were found in the wetland sites surveyed. Legume cover was negatively correlated with the flooding regime gradient reflected by CCA axis 1 (Table 5.3). The legume species were ranked according to the influential environmental variables listed above (Table 3.6). These preliminary findings are explored further on selected legume species and environmental parameters in greenhouse studies in Chapter 5.

Chapter 4
NITROGEN FIXATION
SECTION A: LEGUMES

4. NITROGEN FIXATION
SECTION A : LEGUMES

4A.1 INTRODUCTION

The overall aim of the work detailed in this chapter was to identify and to compare the major sources of biological nitrogen fixation in the wetland study sites. Specifically, the experiments were designed to:

- assess plant growth and nodulation of legume species of riverine wetlands.
- determine whether the legume species found in riverine wetlands are active in nitrogen fixation.
- determine the amounts of nitrogen fixed by *Lotus pedunculatus* using ^{15}N techniques.

Nodulation studies and acetylene reduction assays were carried out at the Irish sites on 4 legume species: *L. pratensis* and *V. cracca* at Clonmacnoise; *T. dubium* and *T. pratense* at Little Brosna. The nitrogen fixing capacity of *L. pedunculatus* was studied in greater detail at Ring Bog, using ^{15}N methodology in addition to nodulation and acetylene reduction assays.

4A.2 MATERIALS AND METHODS

4A.2.1 Legume growth and nodulation

All the legumes at the study sites were found to be nodulated. Isolates of rhizobium were obtained from *Lathyrus pratensis*, *Lotus pedunculatus*, *Trifolium dubium*, *Trifolium pratense*, *Trifolium repens* and *Vicia cracca* for use in the experiments detailed in Chapter 5. Details of the methods used for isolation are described in that chapter (5.2.1.)

i) Harvest of plant material

Steel cylinders (8 cm in diameter, 20 cm in length) were used to isolate whole plants and associated soil and then extracted from the ground. This coring technique was used at Clonmacnoise and Little Brosna on seven visits to these sites from May 1993 to September 1994. The number of replicate cores collected for each species on each occasion was three or four in Ireland. At Ring Bog the coring technique was used in 1993 only, and the number of replicates for *L. pedunculatus* was five. Turves 20 x 20 x 5(deep) cm were dug at Ring Bog in 1994 and 1995. Because of the strictures on minimising change to the Nature Reserve, use of this destructive sampling technique was limited to only one turf per visit (a total of eight, collected from June 1994 to September 1995).

ii) Measurements

a) Nodules were carefully removed from the cores/turves and number, colour and dry weight (g) of the nodules were recorded.

b) For core samples, above-ground biomass was also measured for each plant and presence/absence of flowers noted. All plant tissue above-ground level was removed and dried at 70°C for 48 hours.

c) For turf samples, above and below-ground biomass was measured for each turf (as described above) and the total nitrogen content of the plant tissue was measured as described in Appendix C.

4A.2.2 Nitrogen fixation

4A.2.2.1 Nitrogenase activity - acetylene reduction assays (ARA)

The four principle methods used to measure acetylene reduction were:-

a) Assay of cores : *in situ*

A coring cylinder, consisting of a steel tube (internal diameter of 8 cm, length 20 cm) was inserted into the ground around a legume, leaving 5 cm of the tube above the ground. A thick polyethylene bag of low permeability (pre-tested for ethylene/acetylene diffusion) was then secured to the top of the cylinder using a

jubilee clip and rubber 'O' ring. The bag was fitted with an inlet port, constructed using the middle section of a 20 ml syringe barrel, Blu-tac and a vaccine stopper ("Suba Seal") (Plate 4).

The assay vessel was then inflated with air and 500 ml of the volume of air removed through the rubber septum using a hypodermic syringe (Becton & Dickinson Ltd). The volumes of the assay vessels were 4.5 l (= 0.04 (s.e)) and were measured accurately prior to commencing the experiment. 450 ml of acetylene and 50 ml of propane were then introduced to the vessel, again through the septum. To facilitate the mixing of the added gases with the gas-phase within the assay vessel, the syringe was pumped several times.

The gas phase was sampled at 0, 0.5, 1.5 and 2.5 hours using a hypodermic syringe to transfer gas to a pre-evacuated (13 ml) blood sampling tube, 'Vacutainer' (Becton & Dickson Ltd). Duplicate samples were taken from each assay vessel at each sampling time. Ten separate soil temperatures were recorded at midday using a temperature probe (Jenway model 3070 meter), which measured temperature within the first 8 cm of soil.

b) Detached nodulated roots

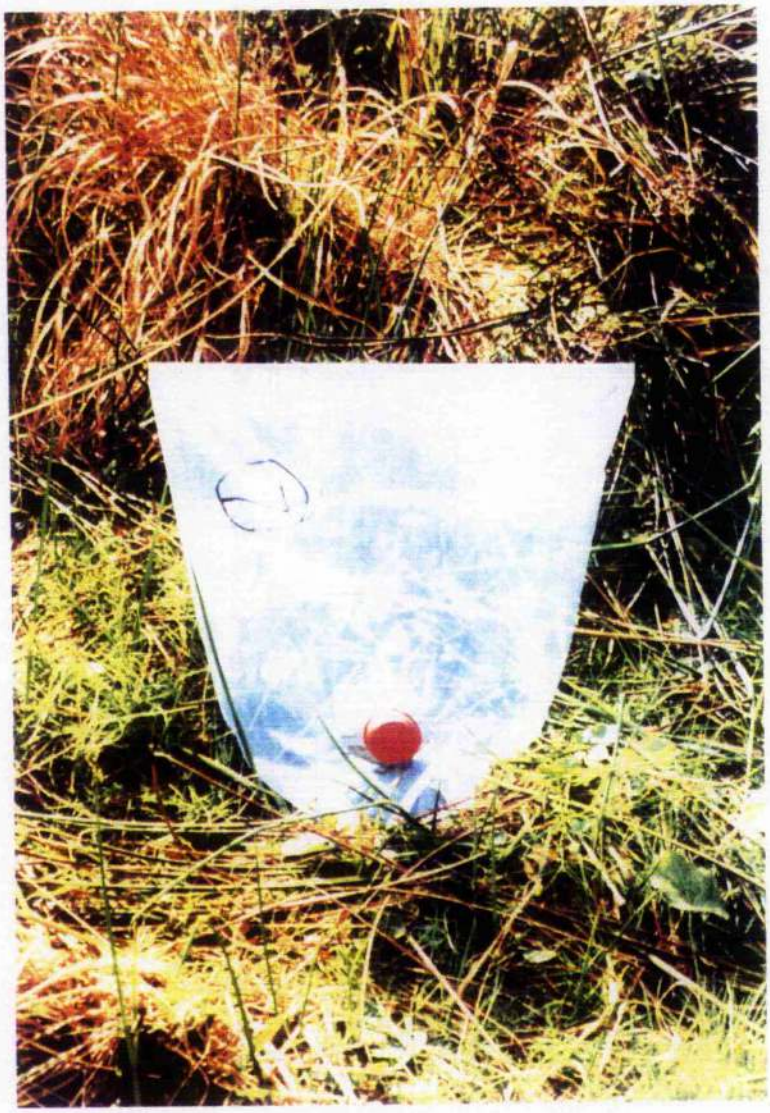
A closed assay system was utilised in Ring Bog during 1994. Detached nodulated roots of *L. pedunculatus* were placed in round bottomed glass flasks (0.5 l) which were sealed using "Suba Seals". The assay was conducted as in (a).

All assays in the field were carried out at the same time each day, the starting time being between 10.30 and 11.00 am.

c) Growth room assays

Methods were as for b) with the exception of the omission of propane, the use of different gas sampling techniques and larger glass assay vessels (1 l). Unlike gas samples collected in the field, in these experiments samples were analysed within a short time of collection and were not stored in 'Vacutainers'. The gas samples were removed using a hypodermic syringe (as before), the needle of the syringe was stabbed into a large rubber bung to stop leakage and the gas analysed within two hours of sampling. Tests showed that leakage from these syringes was negligible over this period of storage.

Plate 4. Acetylene reduction assay vessel : cores (*in situ*).



d) Gas analysis

Gas samples were analysed by gas chromatography (flame ionization detector) using a Pye series 104 gas chromatograph (GC), with a 3 ft long x 1/4" diameter column packed with Poropak N 100/120 mesh. The column operating temperature was 65°C with a N_2 carrier 13.5 seconds/10 ml gas flow rate. Gas samples (0.5 ml) were injected into the column. The peaks of ethylene, acetylene and propane were recorded respectively on a potentiometric recorder. The peak heights correspond to the concentration of each of the three gases (the linear relationship between peak height and gas concentration for each gas was tested). During gas analysis 0.5 ml samples of standard gas (ethylene in argon) was injected periodically into the GC (for calibration purposes).

Data analysis

The amounts of ethylene produced were calculated as follows:

For methods a) and b) above the following procedure (adapted from Knowles, 1980) was used.

Assume that:

V_1 and V_2 = the volumes (ml) occupied by the gases at t_1 and t_2

P – the amount (mol) of propane injected into the assay vessel

t_1 and t_2 = the 1st and 2nd sampling times (hrs)

p_1 and p_2 = the propane concentrations (mol ml^{-1}) of samples taken at t_1 and t_2

e_1 and e_2 = the ethylene concentrations (mol ml^{-1}) of samples taken at t_1 and t_2

a = the area of the surface being assayed (m^2)

w – the nodule weight (g)

The nitrogenase activity A (rate of ethylene production, either mol m^{-2} or g^{-1} nodule weight) hr^{-1} was calculated as follows :

$$A = \frac{e_2V_2 - e_1V_1}{a(t_2 - t_1)} \quad \text{or} \quad \frac{e_2V_2 - e_1V_1}{w(t_2 - t_1)}$$

where $V_1 = P/p_1$ and $V_2 = P/p_2$

therefore

$$A = \frac{e_2P/p_2 - e_1P/p_1}{a(t_2 - t_1)} \quad \text{or} \quad \frac{e_2P/p_2 - e_1P/p_1}{w(t_2 - t_1)}$$

$$A = \left(\frac{P}{a\,(t_2 - t_1)}\right)\left(\frac{e_2}{p_2} - \frac{e_1}{p_1}\right) \quad \text{or} \quad \left(\frac{P}{w(t_2 - t_1)}\right)\left(\frac{e_2}{p_2} - \frac{e_1}{p_1}\right)$$

This can be further simplified, viz:

e'/p' = the ratio of ethylene to propane peak heights obtained when equimolar quantities of the two gases are analysed on the GC (over the range usually covered by acetylene reduction sample analysis).

h_{e1}/h_{p1} and h_{e2}/h_{p2} = the ratios of ethylene to propane peak heights obtained in the samples taken at t_1 and t_2. Then:

$$A = \left(\frac{Pp'}{ae'(t_2 - t_1)}\right)\left(\frac{h_{e2}}{h_{p2}} - \frac{h_{e1}}{h_{p1}}\right) \quad \text{or} \quad A = \left(\frac{Pp'}{we'(t_2 - t_1)}\right)\left(\frac{h_{e2}}{h_{p2}} - \frac{h_{e1}}{h_{p1}}\right)$$

Therefore if the amount of propane injected into the assay vessel and the GC sensitivity (e'/p') remain constant, only peak heights of ethylene and propane are needed (Knowles, 1980).

Calculations for the growth room assays differ from those described above as propane was not used and the gas sample was analysed immediately. Firstly we need to establish the concentration (mol) of ethylene in one 0.5 ml injection into the GC. Ethylene (in argon) gas (used as a standard in all GC analysis) contained 103.6 ppm ethylene (0.1036 µl ethylene/ml) at a laboratory temperature of 20°C and pressure 760 mm, therefore every 0.5 ml ethylene injected contained 2.15 nmole.

Then A, the nitrogenase activity (the rate of ethylene production, nmoles g^{-1} nodule weight hr^{-1}) can be calculated when:

Ep = peak height (cm) (including attenuation) of 0.5 ml calibration ethylene gas

Sp = peak height (cm) (including attenuation) of 0.5 ml sample gas

V = volume assay vessel (ml)

W = nodule dry weight (g)

t_1 and t_2 = the 1st and 2nd sampling times (hrs)

E = amount of ethylene (nmoles) injected into GC (2.15)

I = volume (ml) gas sample injected into gas chromatograph (0.5)

$$A = \left(\frac{Ep \times E}{Sp}\right)\left(\frac{V}{I\,W\,(t_2 - t_1)}\right)$$

4A.2.2.2 Incorporation of $^{15}N_2$

Experiments to assess N_2 fixation from incorporation of $^{15}N_2$ gas were conducted initially in a growth room (using material from turves removed from Ring Bog (28/6/94) and established in the greenhouse for four weeks) and subsequently in the field on two occasions at Ring Bog only (5/6/95 and 2/8/95). Intact plants (5 in growth room, 4 *in situ*) of *L. pedunculatus* were removed from the turf/ground, placed in glass incubation vessels (1 l) and sealed with bull dog clips, Vaseline and "Suba Seals". 25 or 30 ml of 98.8 atom % ^{15}N labelled N_2 was added using a hypodermic syringe (after the equivalent amount of air was removed). The assay vessels were then either returned to a growth room or, when in the field, placed in a pit to keep the samples as near pre-assay temperature as possible (Plate 5).

The samples were incubated for 3 hours and duplicate samples of the gas phase within each vessel were taken using a two-way needle and pre-evacuated gas sampling tubes (Europa Scientific Ltd., Crewe, England). Following the $^{15}N_2$ incubations, ARA's were conducted over the subsequent 3 hour period as described in 4A.2.2.1 c). Plants were not harvested for 48 hours after the assay, to allow translocation of ^{15}N to different plant parts. This period of time is thought to be sufficient for distribution of ^{15}N between plant parts to reach stable equilibrium (Warembourg, 1993). The distribution of ^{15}N between above and below-ground parts was examined.

Plate 5 $^{15}N_2$ incubation vessel situated in the ground, Ring Bog.

Plant samples were separated into shoots, roots and nodules and prepared for ^{15}N analysis. Samples were dried at 40°C to a constant weight, ground coarsely in a mill with blades, then transferred to a ball mill and ground to a very fine powder. Each sample was mixed thoroughly by repeated quartering. The ground sample (1 mg) was then weighed into a tin cup (Europa Scientific Ltd.) using a 5 place balance. The tin cup was crimped into a tight ball and placed in the well of a disposable titre plate. Both the weight and position of the sample in the titre plate were recorded.

The weighed samples were sent to the Scottish Crop Research Institute (SCRI), Invergowrie, Scotland for analysis. The 1 mg samples were analysed to calculate the nitrogen content (% dry weight) and therefore how much sample was needed for 100 µg (± 10 µg) of nitrogen in order to obtain accurate measurements of ^{15}N. Once this had been calculated the samples were reweighed in duplicate so that each contained 100 µg of nitrogen and resent to SCRI.

A continuous flow isotope ratio mass spectrometer (CF-IRMS) coupled to ANCA combustion unit model 20-20 (Europa Scientific Ltd) was used for analysis (Handley *et al.*, 1993). The samples in the tin cups were loaded into an automated sampler, combusted in an automated combustion unit. The resulting gases were then purified by gas chromatography and the residual N_2 transferred to a mass spectrometer.

Both gas and plant samples were analysed for ^{15}N enrichment. Plants were separated into above and below-ground parts. Nodules were grouped with roots as their weights were too low for separate analysis by CF-IRMS. Non-legume assays were conducted simultaneously with the *L. pedunculatus* assays on each occasion (Chapter 4B). The $\delta^{15}N$ (as defined in Chapter 1, section 1.5.3) of *Lotus* (five replicates) not exposed to $^{15}N_2$ was calculated, and subtracted from the $^{15}N_2$ atom % of the exposed plant to calculate ^{15}N atom % excess.
The amount of N_2 fixed was calculated using the equation:

$$N_2 \text{ fixed} = \frac{\text{atom \% } ^{15}\text{N exposed plant - atom \% } ^{15}\text{N non exposed plant}}{\text{atom \% } ^{15}\text{N of gas phase}} \times \text{Total N}$$

4A.2.2.3 ^{15}N Natural Abundance

Analysis of $\delta^{15}N$ was carried out on the following plant samples:

The tops of five *L. pedunculatus* plants cut from Ring Bog on 7/9/93 and separated into 'old' and 'young' tissue and into leaves and stem.

Three intact *L. pedunculatus* plants were collected from Ring Bog on 22/6/94 and again on 28/7/94 and separated into above and below-ground parts.

Turves (20 x 20 x 5 cm deep) were cut on 5/6/95 and 2/8/95 and separated into above and below-ground parts. Plant material was then prepared for $\delta^{15}N$ analysis (as described above).

4A.2.2.4 Contribution of *L. pedunculatus* to the nitrogen economy of Ring Bog

i) Vegetation survey of Ring Bog 'enclave' area (2.2).

An overall vegetation survey of the enclave area of Ring Bog was conducted on 30/6/94. Three transects were investigated, the presence or absence of each species was recorded from a 1 x 1 m quadrat every 20 m. Twelve quadrats were completed in total over the three transects.

ii) Associated species : Nearest neighbour.

Species associated with *L. pedunculatus* were determined by throwing a pointed marker at random within the designated strip and locating the nearest *Lotus* stem to it. The stem nearest to that of *Lotus* was located and the species recorded. This was repeated 50 times.

iii) Total nitrogen of associated species

The plant tops of *Lotus* and the two species most commonly associated (*Agrostis stolonifera* and *Juncus acutiflorus*) with *Lotus* were removed within a randomly placed 0.2 x 0.2 m quadrat. Material was harvested from 10 quadrats containing *Lotus* and 10 quadrats from which *Lotus* was absent. The plant material was dried as described previously and the total nitrogen content determined following the methods outlined in Appendix C.

4A.2.2.5 ^{15}N isotope dilution (uptake and fractionation of ^{15}N within plant material from Ring Bog maintained in the greenhouse).

Five turves containing *L. pedunculatus* were cut from Ring Bog (21/9/93) to fit in plastic containers measuring 34 x 20 x 12 (deep) cm. The turves were maintained in the greenhouse over a two month period. Plant species in each were recorded and four out of the five turves were selected at random for further experimentation. Five shoots (no below-ground material) of the dicotyledonous species in each turf were removed and prepared for $\delta^{15}N$ analysis.

Following this initial harvest 0.58 atom % ^{15}N ($\delta^{15}N$ 583.4 ‰) - labelled potassium nitrate was added to the turves at a rate of 50 mg N m^{-2} (0.5 kg N ha^{-1}). The turves were then left for a further four weeks before harvesting above-ground material (of the same species analysed initially) and analysing as before.

4A.2.3 Environmental parameters

i) Soil moisture

In addition to temperature and the environmental data collected in Chapter 3, soil moisture was measured for Ring Bog and the Irish sites. The fresh weight (g) of 5 separate soil samples was recorded in the laboratory before they were oven dried at 70 °C, placed in a desiccator to cool and re-weighed. The soil moisture was then calculated as % dry weight :

$$\% \text{ dry weight} = \frac{\text{fresh weight - dry weight}}{\text{fresh weight}} \times 100$$

At Ring Bog, the following environmental parameters were also measured :

ii) Water level

Plastic cylinders (6) with a 7.5 cm internal diameter and 40 cm in length, perforated with holes (2.5 cm in diameter) were inserted into the ground (13/7/93). The rim of each was as level with the ground as possible. On subsequent visits the water level in each was measured using a meter rule.

iii) Daily Rainfall

Daily rainfall values for the Ring Bog area were kindly provided by the Clyde River Purification Board for 1993-1995.

4A.2.4 Data analysis

In order to determine if there was any relationship between the environmental variables (described in this chapter and in Chapter 3) and the acetylene reduction assays and legume growth and nodulation, stepwise multiple regressions were conducted using the UNISTAT statistical program (version 4). The following independent variables were used in the analysis: soil pH, soil wetness class, soil organic carbon status, water regime, soil total nitrogen, management regime, soil moisture and in the case of Ring Bog, water level and rainfall. The month in which sampling was carried out, flowering and location (site) were also included in the analysis as independent variables. The dependent variables used were; acetylene reduction (μmoles ethylene evolved/g nodule dry weight), above-ground biomass, nodule biomass, nodule number and individual nodule biomass. Data were normalised using $\log_{10}$ transformations where necessary.

4A.3 RESULTS

4A3.1 Legume growth and nodulation

The results are presented in the form of bar charts, values are means per 100 cm^2, standard error bars are shown. An asterisk denotes flowering at time of sampling.

4A.3.1.1 Legumes at Clonmacnoise

Vegetation was assessed in the growing period of 1993 and 1994. However, the conditions under which plants grew were very different in the two years since the area was cut for hay in July 1993 and in September 1994 (after measurements had been taken).

Lathyrus pratensis

Above-ground biomass was higher in June 1993 and July 1994 than in late spring (May) or autumn (October 1993 and September 1994) (Fig. 4A.1 a)). The autumn decrease in biomass was more marked in 1994 than in 1993, where there was considerable regrowth following cutting.

Patterns of nodulation were also affected by cutting. Thus in 1993, there was an apparent increase in plant total nodule biomass and number in October following the July cut (Figs. 4A.1 (b) and (c)). In 1994, however, total nodule biomass and number were highest in July and decreased by September. Individual nodule biomass was lower in October 1993, reflecting the presence of a large number of smaller nodules, while there was relatively little difference in individual nodule biomass in July and September 1994 (Fig. 4A.1 (d)).

The nodule colour changed throughout the growing season from white (May) to pink (June/July) to brown in September 1994, but pale pink nodules were found in October 1993.

Vicia cracca

Despite cutting for hay in July 1993, the pattern of change in above-ground biomass followed a similar pattern in both 1993 and 1994 with a peak in June/July and a substantial decrease in the autumn (September/October) (Fig 4A.2 (a)). Total nodule biomass and number both decreased from peaks in the spring (May) (Figs. 4A.2 (b) and (c)). While individual nodule biomass tended to increase throughout the year (Fig. 4A.2 (d)). The nodule colour changed throughout the growing season from white (May) to pink (June/July) to brown in September/October.

4A.3.1.2 Legumes at Little Brosna

Vegetation was assessed in the growing period of 1993 and 1994. However, the conditions under which plants grew were very different in the two years since the area was flooded in October 1993 and early spring 1994 (Séamus Grennan, pers. comm., 1994).

Trifolium dubium

Above-ground biomass (Fig. 4A.3 (a)) increased from May to June but declined in July 1993 (no October value is available as Little Brosna was flooded). However in 1994 above-ground biomass increased from May to July, but by September no plants of *T. dubium* could be. Nodule biomass (Fig. 4A.3 (b)) and number (Fig. 4A.3 (c)) follow the pattern of change in above-ground biomass. Individual nodule biomass (Fig. 4A.3 (d)) continued to increase from May to July in 1993 and 1994. Nodule and above-ground growth in 1994 was much lower than that of the previous year. Nodule colour changed from white in May to pink in June to dark pink in July. *T. dubium* plants were in flower on each sampling occasion with the exception of May 1994.

Trifolium pratense

There was little change in above-ground biomass in 1993. In 1994, however, it increased from May to July and as for *T. dubium*, no plants of *T. pratense* could be found by September (Fig. 4A.4 (a)). Total nodule biomass (Fig. 4A.4 (b)) changed little throughout 1993 and 1994. Nodule number (Fig. 4A.4 (c)) increased from May to July in 1993 but changed little in 1994, however the mean was much higher in May 1994 than in the previous year. Individual nodule biomass (Fig. 4A.4 (d)) decreased from May in 1993 but changed little in 1994 (the values for 1994 were much lower than for 1993). Nodule colour was as for *T. dubium*. Plants were in flower in May and June 1993 but only in July 1994.

Fig. 4A.1 Growth and nodulation of *Lathyrus pratensis* at Clonmacnoise,1993-1994. Data are means (n=4) per 100 cm^2 with standard error bars and * denotes flowering.

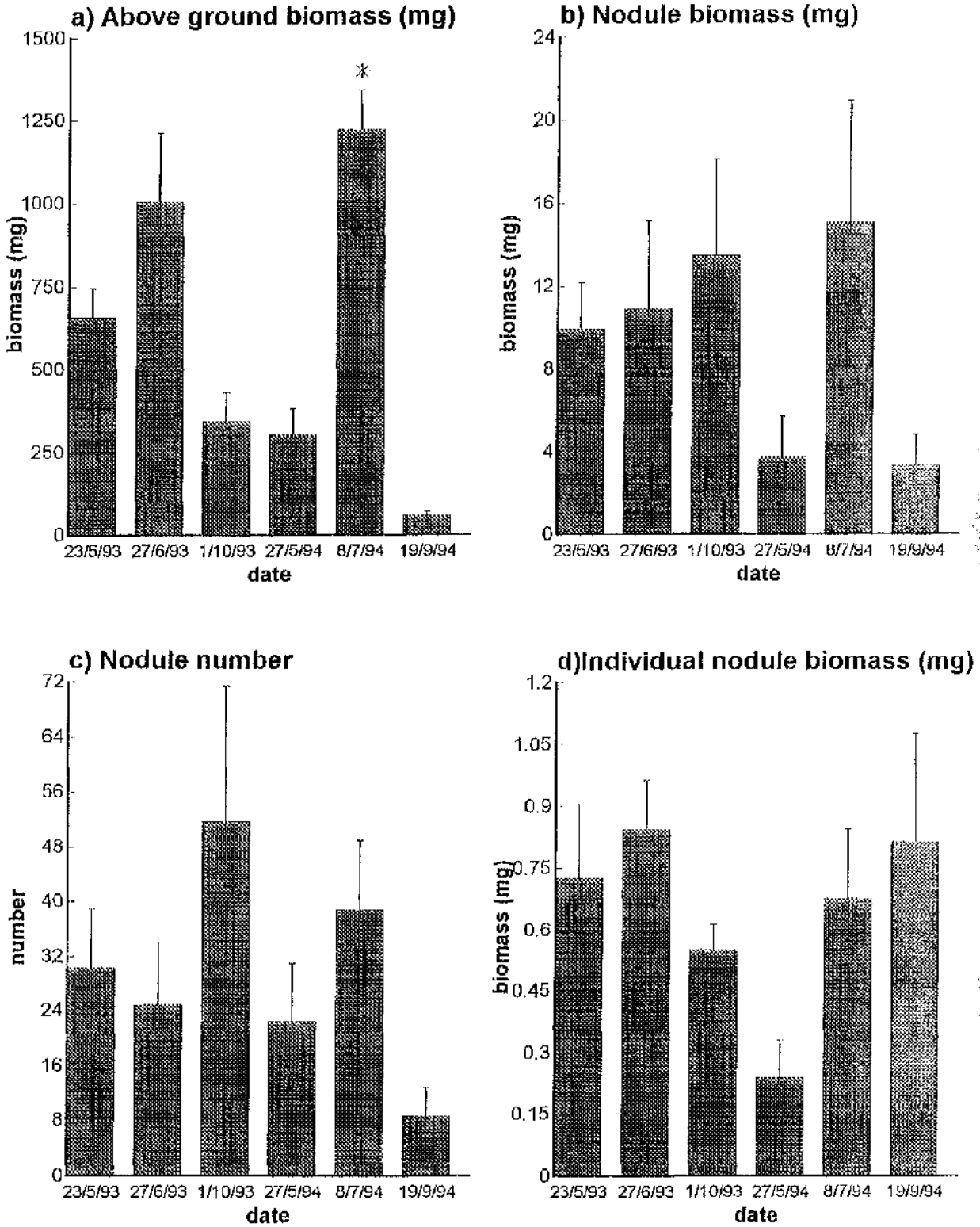


Fig. 4A.2 Growth and nodulation of *Vicia cracca* at Clonmacnoise, 1993-1994. Data are means (n=3) per 100 cm^2 with standard error bars and * denotes flowering.

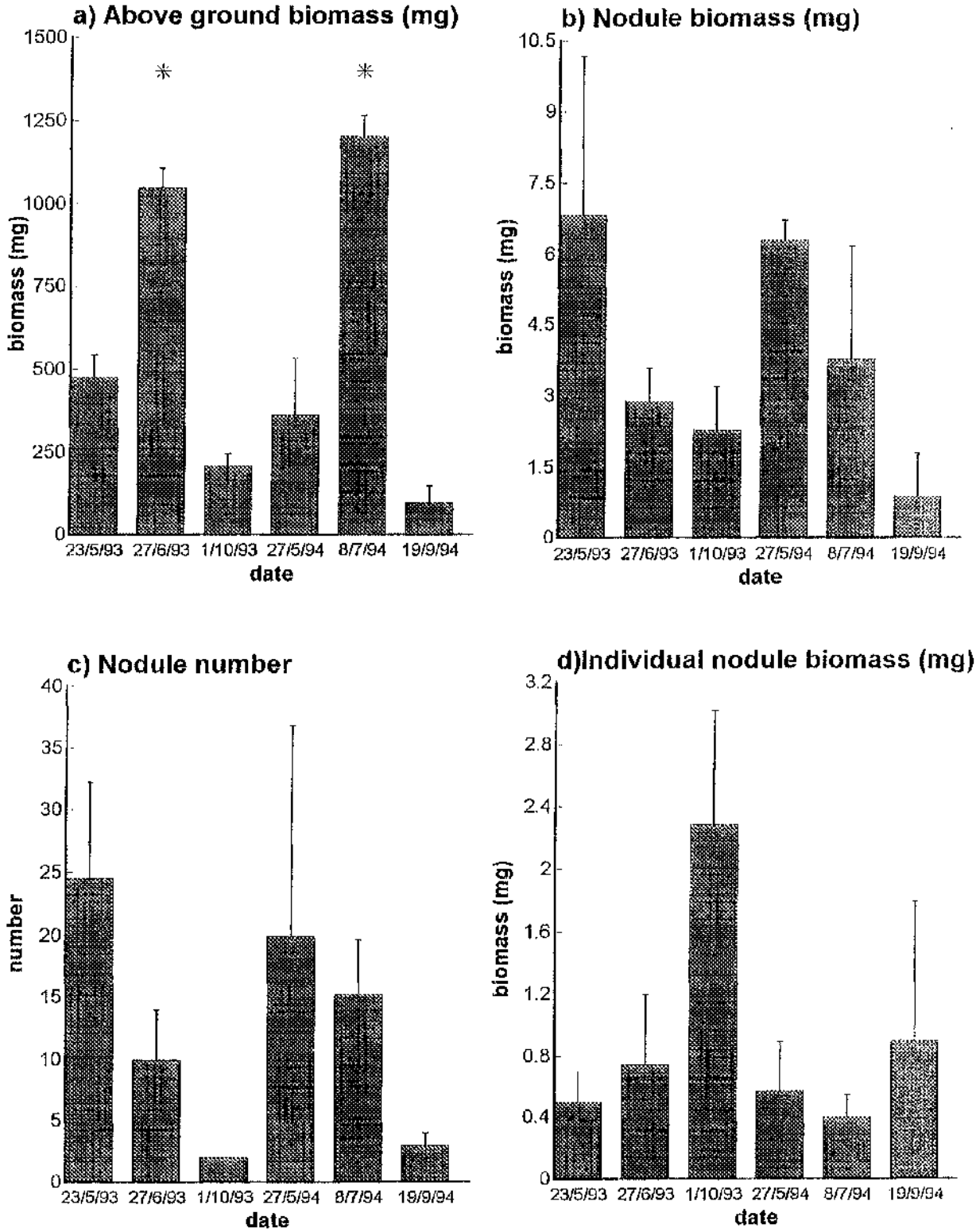


Fig. 4A.3 Growth and nodulation of *Trifolium dubium* at Little Brosna, 1993-1994. Data are means (n=3) per 100 cm^2 with standard error bars and * denotes flowering.

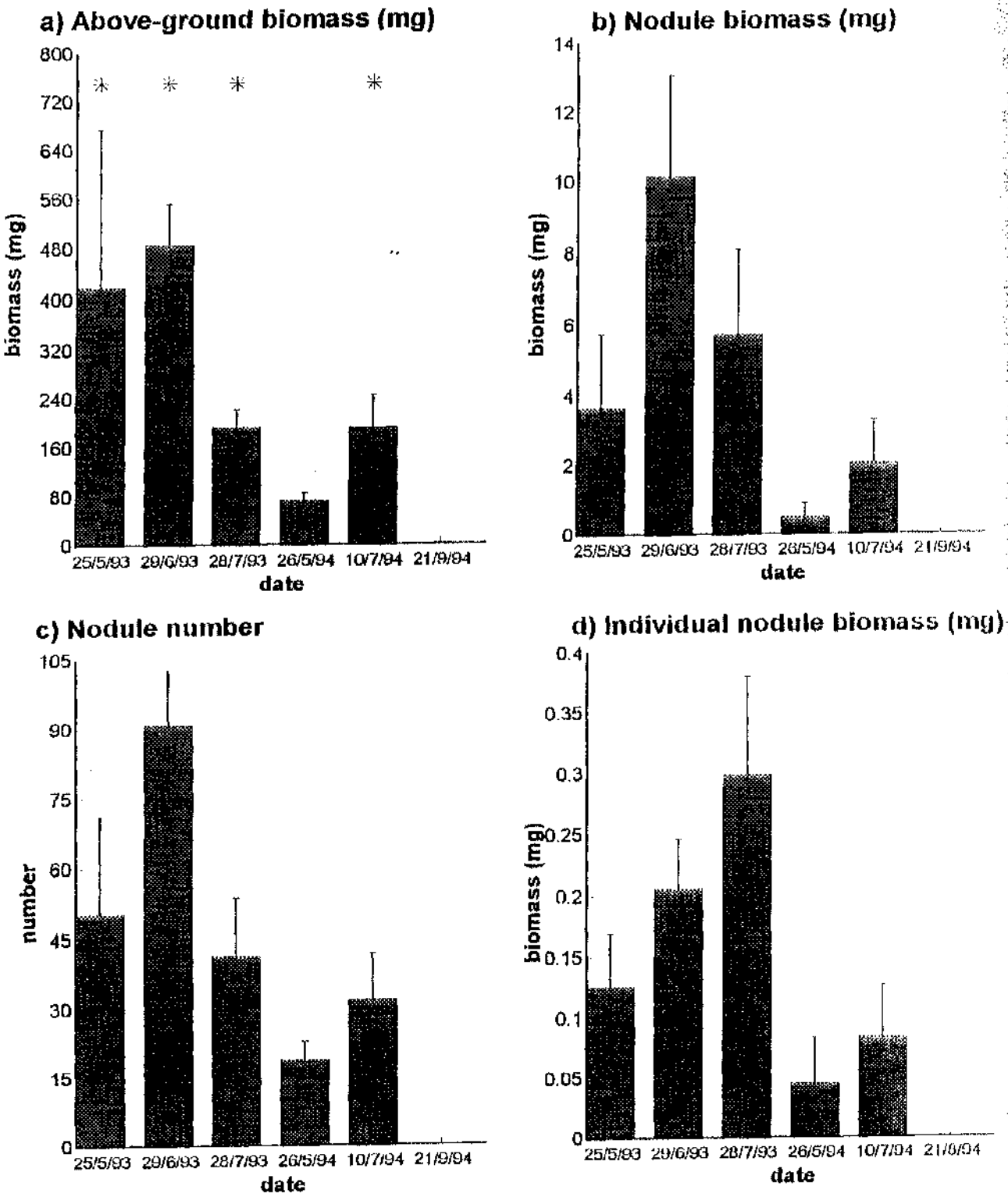
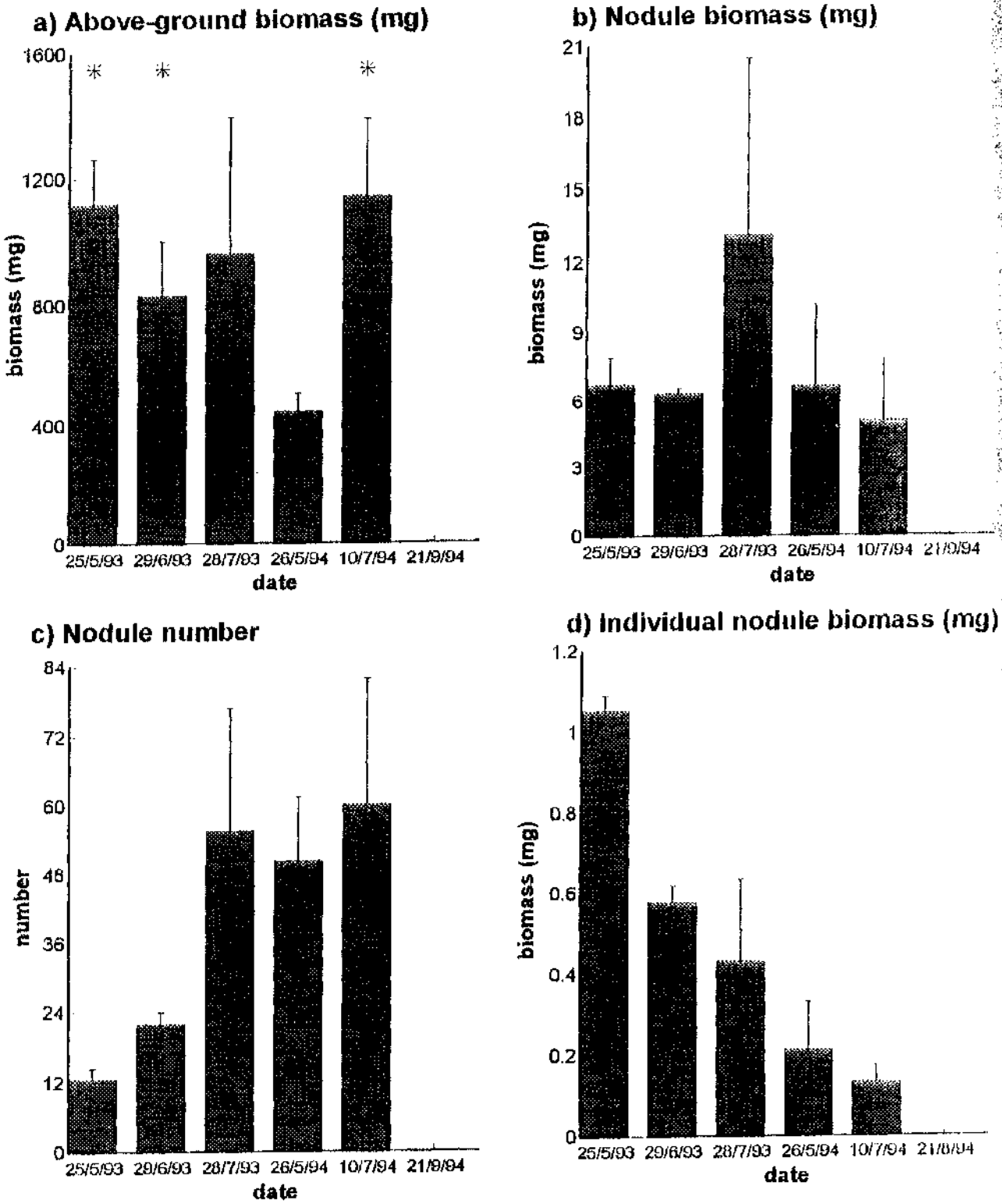


Fig. 4A.4 Growth and nodulation of *Trifolium pratense* at Little Brosna, 1993-1994. Data are means (n=4) per 100 cm^2 with standard error bars and * denotes flowering.



4A.3.1.3 *Lotus pedunculatus* at Ring Bog

1993: Above-ground biomass (Fig. 4A.5 (a)), nodule biomass (Fig. 4A.5 (b)), the number of nodules (Fig. 4A.5 (c)) all changed little throughout 1993. Individual nodule biomass (Fig. 4A.5 (d)) reached a peak in September and declined in October. The plants were in flower in July.

1994 & 1995: Sampling frequency on these occasions was reduced to minimise damage to this N.N.R. Changes in the parameters measured broadly followed the same pattern as shown by the replicated measurements of 1993 (Fig. 4A.5). Thus, above-ground biomass was maximum in July/August and declined in September/October (Fig. 4A.6 (a)). Below-ground biomass (roots and nodules) was greatest in the autumn (October 1994) and spring (May 1995) (Fig. 4A.6 (a) and (e)). Nodule biomass was maximum in July/August (Fig. 4A.6 (b)), the peak in nodule numbers occurred in June (Fig. 4A.6 (c)). Individual nodule biomass was greatest in the period July-September (Fig. 4A.6 (d)). Plant total nitrogen (Fig. 4A.6 (f)) was highest in the below-ground biomass in autumn (October 1994) and spring (May and early June 1995). For the above-ground biomass, plant total nitrogen was greatest in the summer months (late June 1995, July 1994 and 1995, late August 1994/early September 1995).

In all years nodule colour changed throughout the growing season, generally from pale pink to pink with a white lenticel (July) to darker pink with white lenticel (August) to brown in September/October.

Root and nodule growth in the litter layer

In all the study years, a notable feature of growth in *Lotus* was the production of roots in the above-ground vegetation and litter layer (which formed a 2 cm layer above the soil) and this was investigated in detail during 1994 and 1995. For brevity, the term 'litter layer' will be used on the graphs to describe this, but in all cases roots and nodules were found intertwined in the vegetation matt above the litter layer (including the first 1 cm section). This growth feature was evident at all times of survey, except in June 1994 and May and July 1995 when roots were also found at greater depths in the soil (Fig. 4A.6 (c)). Nodules were found in the 2 cm litter layer only, with the exception of June 1994 and May and July 1995 when nodules were also found in the deeper 3 cm layer but this number was small relative to the total number of nodules found (Fig. 4A.6 (e)).

Fig. 4A.5 Growth and nodulation of *Lotus pedunculatus* at Ring Bog, 1993. Data are means (n=5) per 100 cm^2 with standard error bars and * denotes flowering.

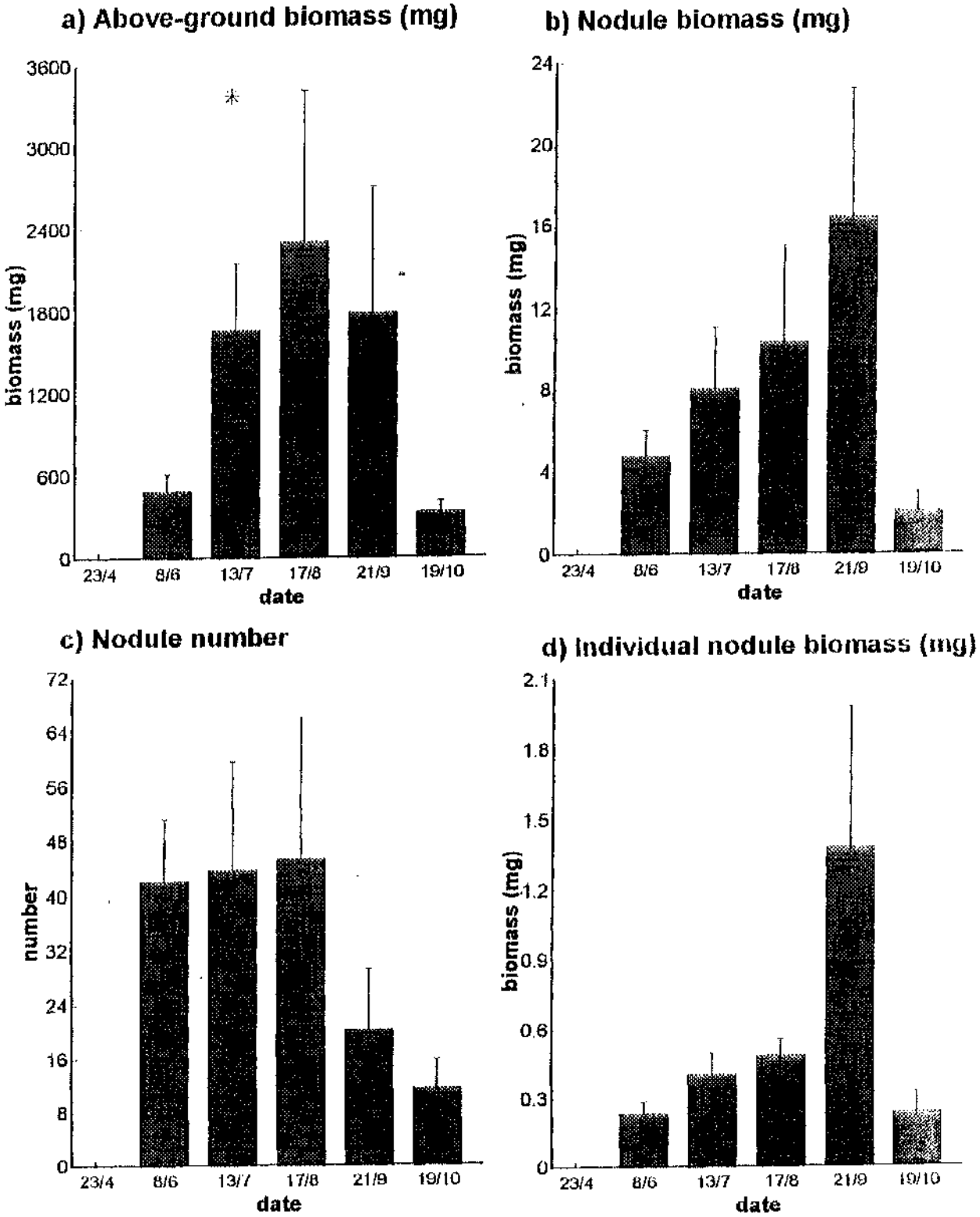
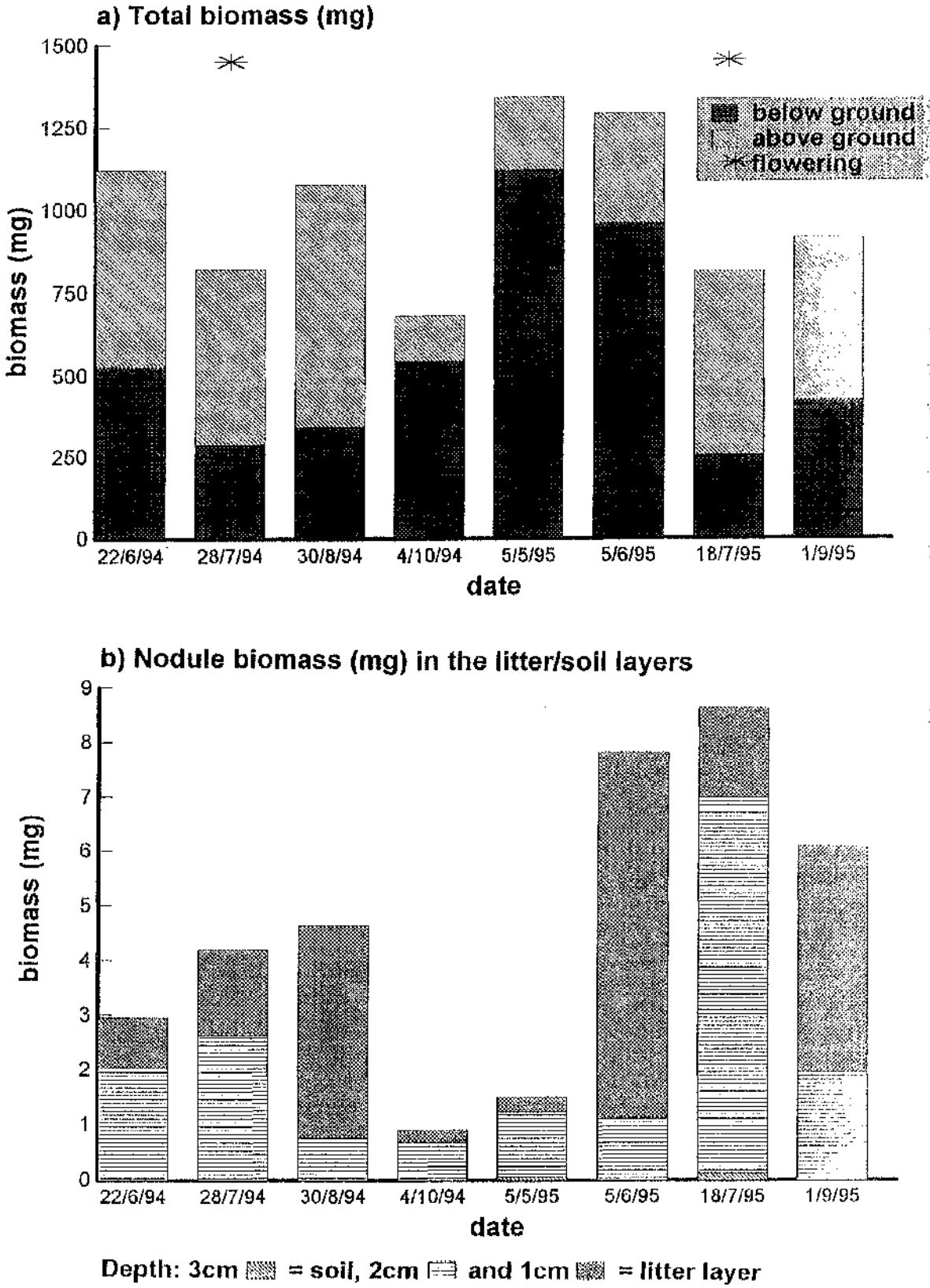
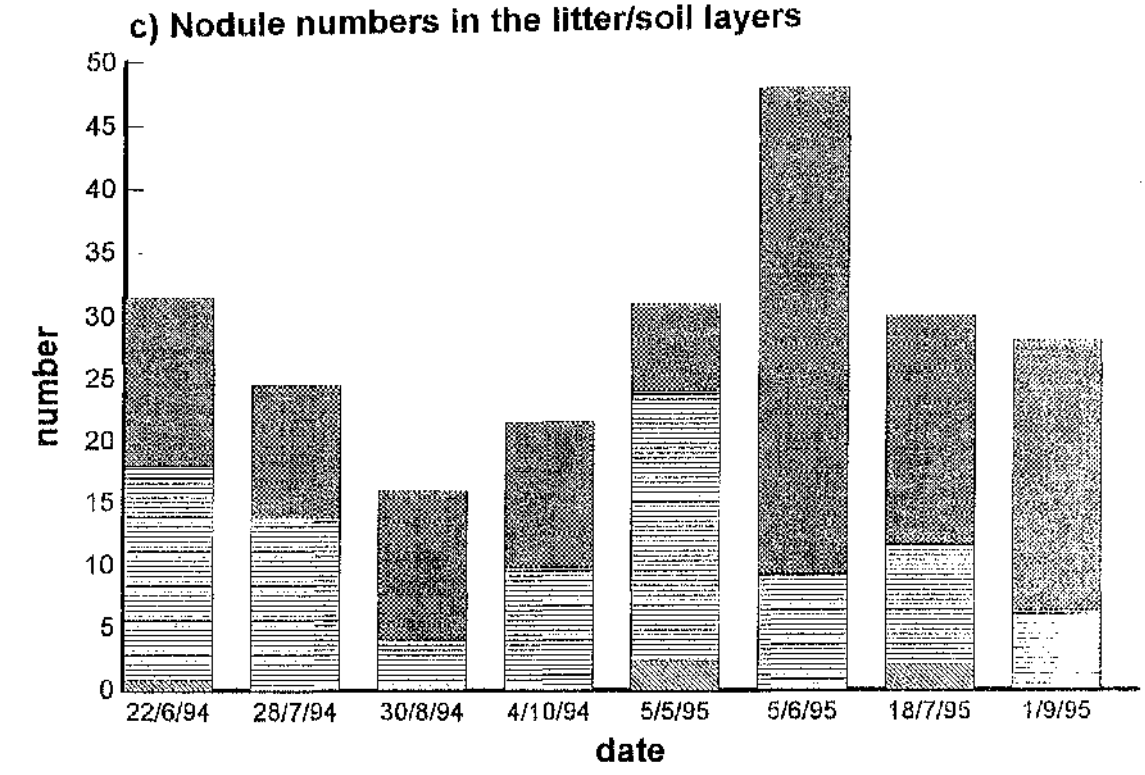
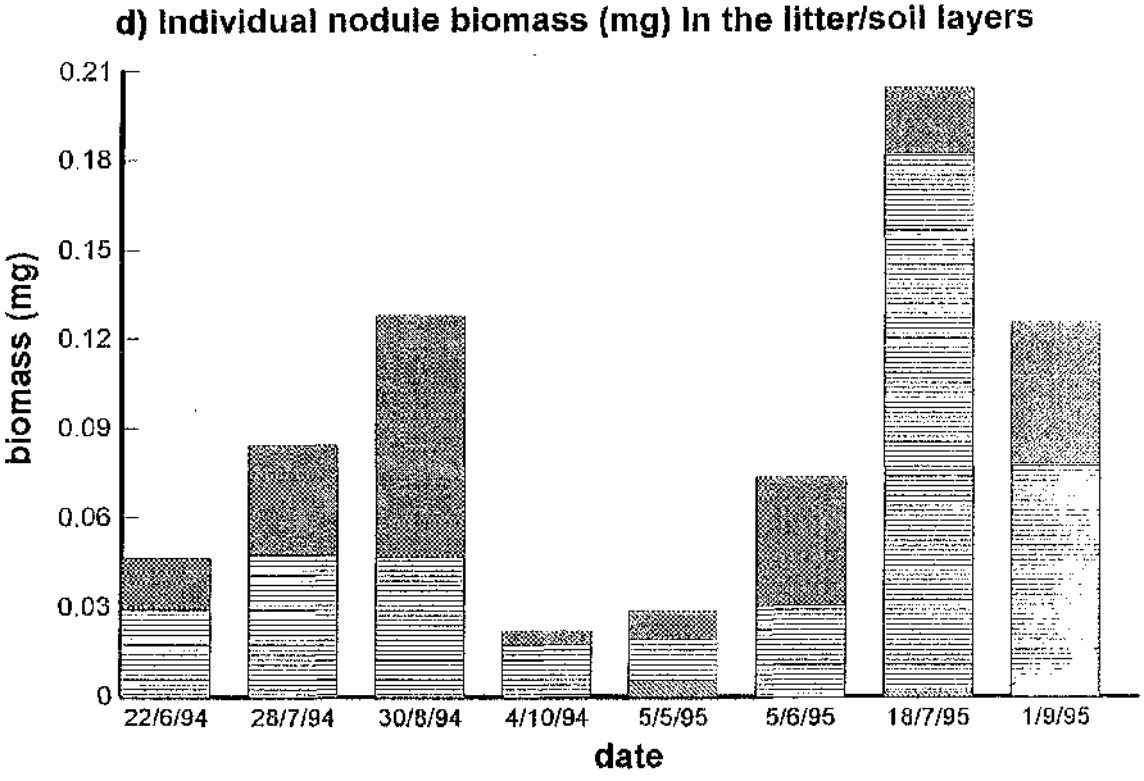


Fig. 4A.6 Growth and nodulation of *Lotus pedunculatus* at Ring Bog, 1994-1995. Data are per 100 cm^2 (one turf only).



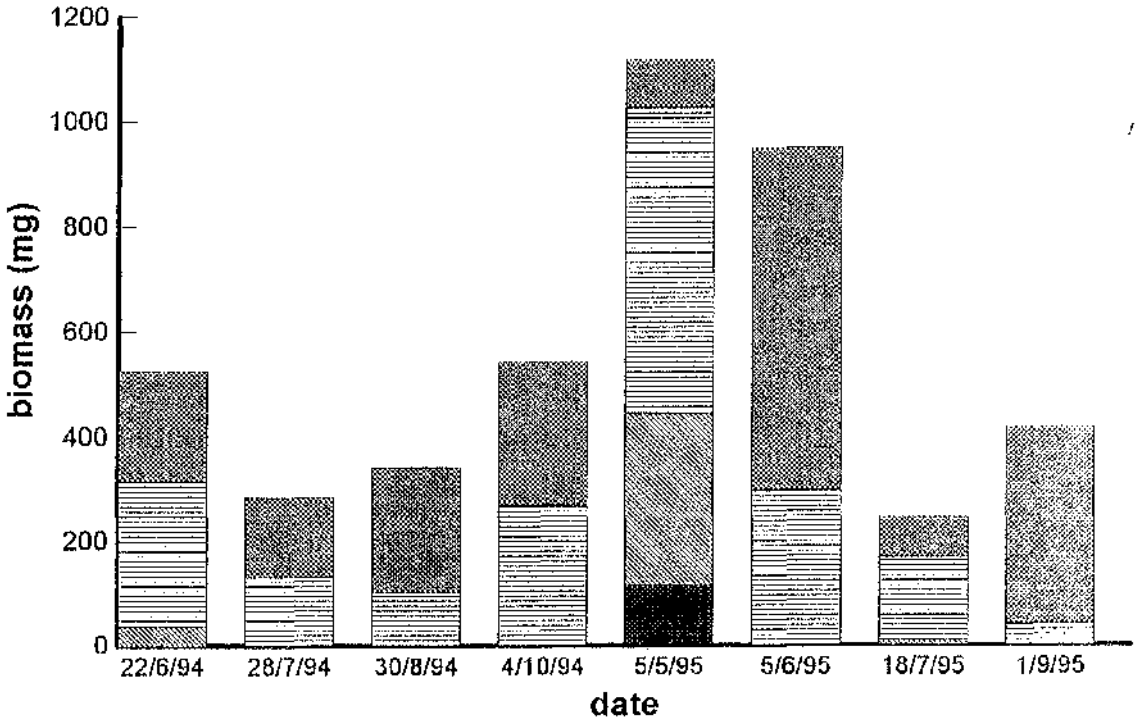




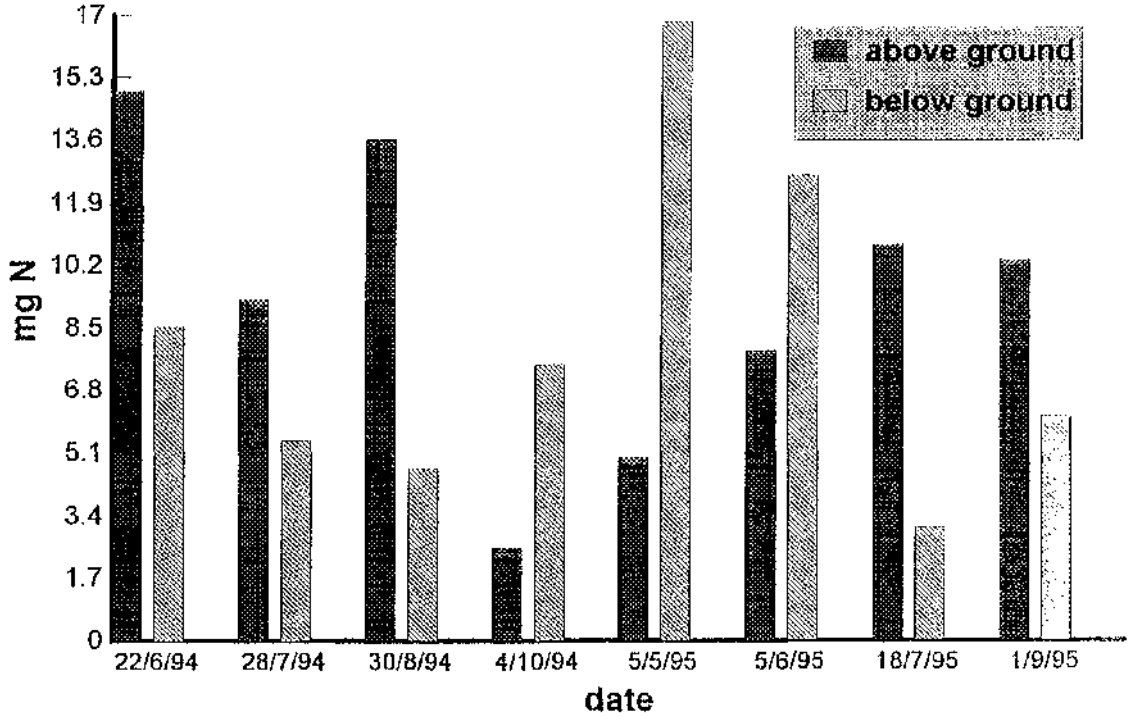
Depth: 3cm = soil, 2cm and 1cm = litter layer

Depth: 4cm and 3cm = soil, 2cm and 1cm = litter layer

e) Root biomass (mg) in the litter/soil layers



f) Plant total nitrogen (mg total N)



4A.3.1.4 Environmental factors effecting legume growth and nodulation

When each species was analysed separately, none of the independent variables were selected by stepwise linear regression to explain the variation in legume growth and nodulation, but when analysed as one 'legume' group, some interesting relationships were found. Analysing the species as one group was justified as there was no significant variation between species ($p>0.05$) in growth and nodulation. No independent variables were selected to explain the variation in above-ground biomass and nodule biomass. The environmental data are given in Chapter 3 (Table 3.1). However, 25% of the variation in nodule number was explained by water regime (R-squared (adjusted) = 24.8, $p<0.01$) (negative relationship). The water regime categories are defined in Chapter 3 (section 3.2.2). Where flooding was only intermittent (i.e. not every year) nodule number was highest (Little Brosna) and where flooding occurred every year (seasonal flooding) nodule number is lowest (Cloumacnoise). The category in between was saturated. This was represented by Ring Bog which is not flooded but the soil is saturated. Soil total nitrogen explained 32% of the variation in individual nodule biomass (R-squared (adjusted) = 31.6, $p<0.005$) (positive relationship). As soil total N (Table 3.1) increased so did individual nodule biomass. No other independent variables were selected. The monthly rainfall values and water levels for Ring Bog are shown in Appendix D.

4A.3.2 Nitrogen Fixation

4A.3.2.1 Nitrogenase activity

Nitrogenase activities measured by the acetylene reduction assays are summarised in Table 4A.1. The hourly rate is for μmoles of ethylene evolved between 0.5 and 1.5 hours g^{-1} nodule weight (accumulative data for 2.5 hours is given in Appendix E). It is evident from the large standard errors of the mean that there was a great amount of variation amongst the samples assayed. The amount of ethylene evolved per square meter is also given in Table 4A.1, this is to allow comparisons to be made with soil assays and these will be discussed in Chapter 4B. The data suggest that nitrogenase activity in *L. pratensis* was at its maximum in May, and in June for *V. cracca*, the latter value was very high but from one plant only. *T. dubium* and *T. pratense* show similar patterns in activity, increasing from May through June to a maximum in July. Potential nitrogenase activity in *L. pedunculatus* in both 1993 and 1994 was maximum in June, decreasing in July, increasing slightly in August before declining in September/October. Values for *L. pedunculatus* in 1994 (cores) were higher than those for the following year (detached nodulated root systems).

Stepwise multiple linear regression was carried out on the species as one 'legume' group. There was no significant variation between species ($p>0.05$) in acetylene reduction rates. Soil moisture accounted for 49% (R-squared (adjusted) = 49.24%, $p<0.01$) (negative relationship) of the variation in acetylene reduction rates and soil total N explained 70% (R-squared (adjusted) = 70.48%, $p<0.005$) (positive relationship). No other independent variables were selected. Mean measurements of soil moisture (% dry weight) over 1993-94 (Ireland) and 1993-95 (Ring Bog) revealed that it was greatest in Ring Bog (83.56 ± 0.98) and that Clonmacnoise was slightly greater (69.85 ± 0.54) than Little Brosna (61.04 ± 1.09). The monthly rainfall values and water levels for Ring Bog are shown in Appendix D.

Table 4A.1 Nitrogenase activity of legumes, measured in the field (± standard error).

Species & Date	(n=)	Soil Temp. (^{0}C)	Nitrogenase activity µmol ethylene evolved g^{-1} dry wt. nodules h^{-1}	$m^{-2} h^{-1}$
L. pratensis **(CL)**				
23/5/93	(3)	12.3 ± 0.1	226 ± 215	97 ± 83
27/6/93	(4)	20.2 ± 0.3	116 ± 80	159 ± 97
1/10/93	(3)	11.3 ± 0.1	172 ± 96	65 ± 20
V. cracca **(CL)**				
23/5/93	(3)	12.3 ± 0.1	162 ± 107	44 ± 8
27/6/93*	(1)	20.2 ± 0.3	1219 ± NA	269 ± NA
1/10/93	(2)	11.3 ± 0.1	135 ± 72	24 ± 4
T. dubium **(LB)**				
25/5/93*	(3)	12.4 ± 0.2	15 ± 9	18 ± 9
29/6/93*	(3)	17.2 ± 1.1	98 ± 83	82 ± 59
28/7/93*	(4)	15.8 ± 0.1	269 ± 155	105 ± 45
T. pratense **(LB)**				
25/5/93*	(3)	12.4 ± 0.2	47 ± 15	35 ± 16
29/6/93*	(1)	17.2 ± 1.1	81 ± NA	54 ± NA
28/7/93	(3)	15.8 ± 0.1	105 ± 58	71 ± 17
L. pedunculatus **(RB)**				
8/6/93	(5)	13.1 ± 0.1	85 ± 58	42 ± 27
13/7/93*	(5)	12.4 ± 0.2	20 ± 13	21 ± 11
17/8/93	(5)	11.8 ± 0.2	76 ± 24	60 ± 33
21/9/93	(5)	11.1 ± 0.1	53 ± 33	33 ± 7
22/6/94†	(4)	12.8 ± 0.26	32 ± 12	
28/7/94†*	(4)	17.9 ± 0.35	24 ± 3	
30/8/94†	(5)	12.9 ± 0.1	27 ± 12	
4/10/94†	(5)	7.5 ± 0.3	16 ± 9	

Data are mean nitrogenase activity for 2-5 samples (except for *V. cracca* and *T. pratense* 6/93 which had only 1 sample), over the period 0.5-1.5 hours from initiation of incubation. See Appendix E for accumulative values over 2.5 hours.

n= number of samples † Detached nodulated root systems

* in flower NA= not applicable

4A.3.2.2 Incorporation of $^{15}N_2$

The results of the $^{15}N_2$ incorporation experiment are shown in Table 4A.2. The highest rate of fixation was found in the assay conducted under controlled conditions (elevated temperature and lighting). Of the two assays carried out *in situ*, the one conducted in June showed the highest rate of $^{15}N_2$ uptake. Distribution of ^{15}N varied between plants, giving high standard errors. Approximately half of the fixed nitrogen was translocated to the above-ground parts in the lab experiment and *in situ* 5/6/95 but was considerably less *in situ* 2/8/95. It should be noted that at the time of sampling on 2/8/95, when data showed lower levels of $^{15}N_2$ fixation, *Lotus* was in flower.

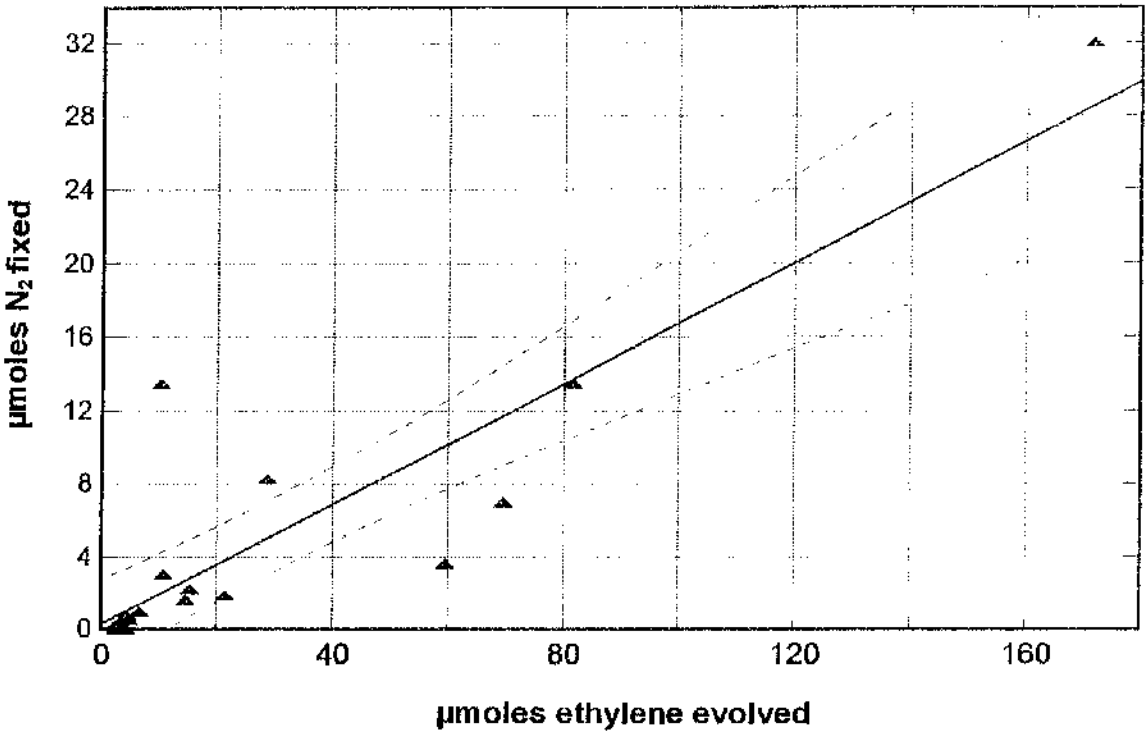
Table 4A.2 Incorporation of $^{15}N_2$ by *L. pedunculatus* (µmoles N_2 fixed g^{-1} nodule dry weight) over a 3 hour assay period (± standard error)

	growth room 28/7/94 (n=5)	***in situ* 5/6/95 (n=4)**	***in situ* 2/8/95 (n=4)**
N_2 fixed (µmoles)	354.64 ± 99.99	136.09 ± 50.72	61.97 ± 27.29
% ^{15}N above-ground	46.13 ± 12.12	58.85 ± 0.75	34.45 ± 20.24
temperature/°C	22.5 ± 0.28	12.12 ± 0.26	18.53 ± 0.16

Figure 4A.7 shows the linear relationship between the amount of nitrogen fixed by direct means ($^{15}N_2$ incorporation) compared to the amount of ethylene evolved (acetylene reduction) for each assay vessel (including non legumes) over consecutive 3 hour periods in the field (R-squared (adjusted)=78.8% and $p<0.0001$). The ratio between the amounts of ethylene evolved and N_2 fixed was close to 5:1.

The assays carried out in the growth room did not produce a significant relationship between the amount of nitrogen fixed by the $^{15}N_2$ method and the amount of ethylene evolved (acetylene reduction).

Fig. 4A.7 Relationship between µmoles ethylene evolved and µmoles N_2 fixed ($^{15}N_2$ incorporation) for each assay vessel over 3 hours (*in situ* 5/6/95 and 2/8/95). The best fit line (linear least squares) and the confidence interval (0.95) are shown. R-squared (adjusted)=78.8% and p<0.0001 (n=18).



4A.3.3.3 ^{15}N natural abundance

The natural abundance values for different plant parts of *L. pedunculatus* are shown in Table 4A.3. Old and new stems had similar values, old and new leaves however were quite different.

Table 4A. 3 $\delta^{15}N$ (‰) of *L. pedunculatus* (five plants bulked together) sampled from Ring Bog (7/9/93).

PLANT PART	%N	$\delta^{15}N$ (‰)
Old Leaves	2.45	-2.34
New Leaves	2.21	-1.09
Old Stem	1.25	-2.42
New Stem	1.39	-2.30

On examining the data for the natural abundance of ^{15}N in *Lotus* in 1994 and 1995 (Table 4A.4) it is apparent that there are differences in $\delta^{15}N$ between the above and below-ground parts and also not unexpectedly from one month to another. These differences are consistent in both 1994 and 1995.

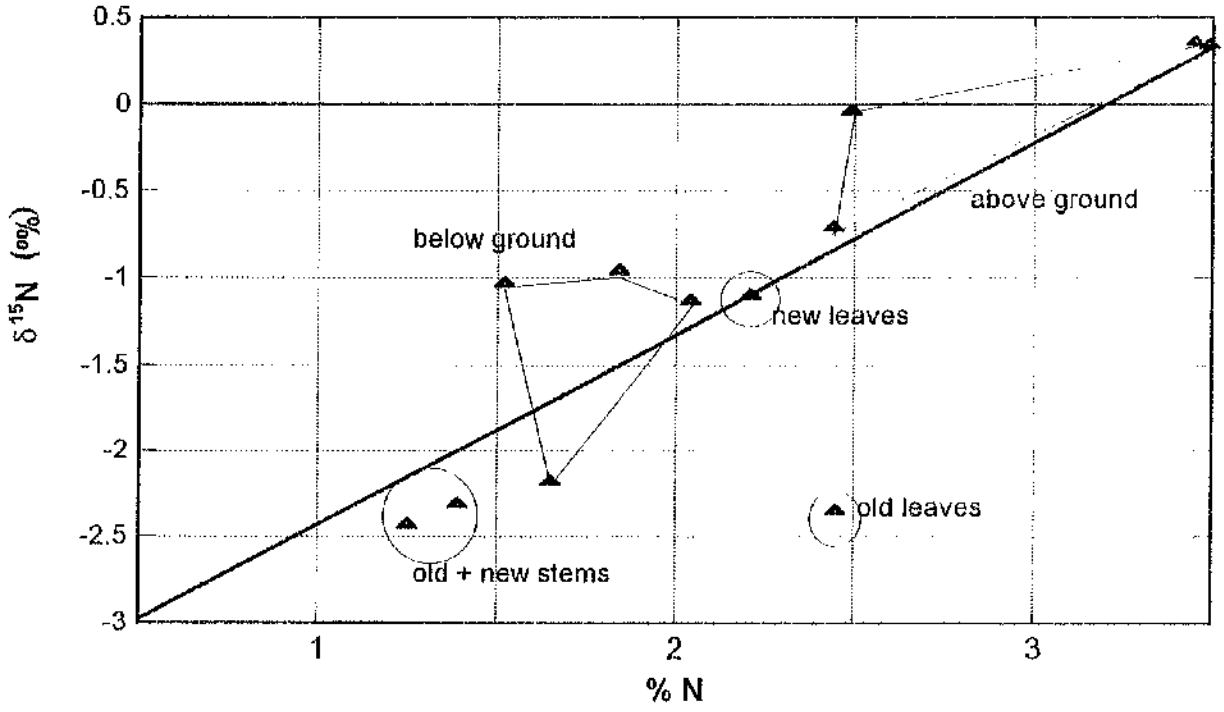
Table 4A. 4 $\delta^{15}N$ (‰) of *L. pedunculatus*, Ring Bog 1994-1995.

	ABOVE		BELOW	
	$\delta^{15}N$ (‰)	%N	$\delta^{15}N$ (‰)	%N
22/6/94 (3)	0.35 ± 0.04	3.49 ± 0.27	-1.12 ± 0.21	2.04 ± 0.22
28/7/94 (3)	-0.70 ± 0.12	2.44 ± 0.07	-2.17 ± 0.51	1.65 ± 0.21
5/6/95 (1)	0.37	3.45	-0.95	1.84
2/8/95 (1)	-0.03	2.49	-1.02	1.52

(sample numbers are given in brackets)

A regression (UNISTAT version 4) of the data in Table 4A.3 and 4A.4 ($\delta^{15}N$ (‰) against % N (dry weight)) gave a significant linear relationship (R-sq(adj) = 58.9 % and $p<0.005$). No data transformations were necessary. This relationship is shown in Fig. 4A.8, $\delta^{15}N$ (‰) increased as % N increased.

Fig. 4A. 8 Relationship between $\delta^{15}N$ (‰) and %N (dry weight) in *Lotus pedunculatus*. R-squared (adjusted) =58.9% and p<0.005 (n=12).



4A.3.3.4 Contribution of *L. pedunculatus* to the nitrogen economy of Ring Bog

An overall survey of the enclave area gave an estimate of *Lotus* frequency at 23.2 % (± 1.69). Results of the vegetation survey including all the species recorded is given in Appendix F. By using the nearest neighbour technique it was found that the plants nearest to *Lotus* were usually *A. stolonifera* (36% of plants closest to *Lotus* were of this species) and *J. acutiflorus* (28%). The results of the total nitrogen contents of associated species, 'with' and 'without' *Lotus* in the quadrats are given in table 4A.5.

Table 4A.5 Mean total nitrogen (% dry weight) content of *Agrostis* and *Juncus* 'tops' growing in close proximity to *Lotus* at Ring Bog (n=10).

	with *Lotus*	without *Lotus*	ANOVA
J. acutiflorus	1.108 ± 0.061	0.995 ± 0.068	F=1.51 p=0.236 (NS)
A. stolonifera	1.220 ± 0.100	1.094 ± 0.032	F=1.34 p=0.269 (NS)

A oneway ANOVA (MINITAB version 9.2) revealed that concentrations of total nitrogen were not significantly affected in *Agrostis* and *Juncus* by growth in close proximity to *Lotus*.

4A.3.3.5 ^{15}N isotope dilution (uptake and fractionation of ^{15}N within plant material from Ring Bog maintained in the greenhouse).

Table 4A.6 shows the results of the ^{15}N fractionation experiment. *L. pedunculatus* had the smallest increase in ^{15}N (10.01 ‰) followed by *A. sylvestris* which was double that of *Lotus* (20.80 ‰). Enrichment of ^{15}N in other species increased to a much greater extent (33-59%). The application of the isotope dilution calculation as outlined in Chapter 1 using the mean ^{15}N (‰) of the eight non-fixing plants and *Lotus* after the $K^{15}NO_3$ application, gave the % contribution of atmospheric nitrogen to the nitrogen content in the above-ground biomass of *Lotus* as 75.81% (over the four weeks). If the $\delta^{15}N$ of the plants before $K^{15}NO_3$ application is taken into account and the difference (after minus before) used in the calculation then the value is 72.87%.

Table 4A.6 $\delta^{15}N$ (‰) of 9 plant species (including *Lotus*) from Ring Bog turves, maintained in the greenhouse. (± standard errors where applicable) (supplied NO_3=583.4 (‰))

Species	No.*	Shoot % N	Initial $\delta^{15}N$ (‰)	Increase in $\delta^{15}N$ (‰) After $K^{15}NO_3$ addition
Lotus pedunculatus	4(5)	2.70 (±0.22)	-1.54 (±0.24)	10.01 (±2.23)
Angelica sylvestris	1(2)	1.4	-1.37	20.80
Carum verticillatum	1(3)	2.55	-1.50	33.32
Hydrocotyle vulgaris	1(5)	1.47	-1.36	36.70
Potentilla erecta	1(3)	1.42	-1.75	28.67
Rumex acetosa	2(5)	2.44 (±0.59)	-0.53 (±1.58)	47.44 (±11.20)
Scutellaria galericulata	1(3)	2.53	-0.85	37.77
Valeriana officinalis	1(2)	2.55	-0.93	26.52
Viola palustris	2(5)	2.64 (±0.78)	-1.08 (±0.63)	58.64 (±5.36)

* Number of samples analysed (values in brackets = number of shoots bulked together for analysis)

4A.4 DISCUSSION

4A.4.1 Legume growth and nodulation

There were differences between the legume species studied in their pattern of above-ground and nodule growth (section 4A.3.1).

4A.4.1.1 Legumes at Clonmacnoise

The pattern of nodule growth in *L. pratensis* was different in 1993 and 1994 (Fig. 4A.1). The large nodule number in October 1993 suggested growth of new nodules. However, the individual nodule biomass was low, suggesting the formation of young small nodules. The white colour of the nodules also suggested new growth. Nodule number and biomass declined in September in 1994 and the individual nodule biomass increased, suggesting the persistence of a few large (and therefore mature) nodules. The nodule colour (brown) supports this. The difference in nodule growth in *L. pratensis* in these two years might be attributable to the effects of mowing, which seems to have resulted in the formation of new nodules after the cut. It is thought that regrowth after cutting can improve the yield of certain species (e.g. McNaughton, 1983) and this may explain why above-ground biomass is slightly higher in October 1993 (cut) than in September 1994 (cut after measurements taken). This hypothesis will be considered further in Chapter 5.

The general pattern of growth was similar in *V. cracca* in 1993 and 1994 (Fig. 4A.2). The peak of nodule growth in *V. cracca* at the start of the growing season suggests that this species put more effort into producing nodules early in the season and less as time went on, with individual nodule size increasing over time, again suggesting an increase in number of larger (older) and decrease in small (young) nodules. *V. cracca* responded differently to the mowing, no re-growth of nodules was noted and the above-ground biomass was no different after cutting to that at the equivalent sampling time the following year (cut after measurements taken).

4A.4.1.2 Legumes at Little Brosna

There were large differences in growth in *T. dubium* (Fig 4A.3) and *T. pratense* (Fig. 4A.4) between 1993 and 1994. These distinct differences at Little Brosna between 1993 and 1994 could be attributed to the early flooding of the site in October 1993 and the unusually long spring flood of 1994 (Séamus Grennan, pers. comm., 1994). In *T. dubium* both above-ground and nodule growth (number, biomass and individual biomass) was reduced. In *T. pratense* nodule number was higher and individual nodule biomass was lower in 1994 compared to 1993, indicating smaller nodules but in greater numbers. Flowering was delayed and both

species died back earlier in 1994, no plants were found in mid September. As discussed in Chapter 3, flooding can have profound effects on legume growth, in this case the species recovery after a prolonged flood was negatively affected. This hypothesis will be considered further in Chapter 5.

4A.4.1.3 *Lotus pedunculatus* at Ring Bog

The more detailed study of *Lotus* (Fig 4A.6) reveals that below-ground biomass comprises a substantial proportion of its total biomass, a finding similar to those of Sheath (1977) and Wedderburn & Gwynne (1981). The roots of *Lotus* (and therefore nodules) were located in the top 2 cm of the turves in the above-ground vegetation and litter layer. This may explain how these plants are able to fix nitrogen (see further) in such adverse conditions (high soil moisture and low pH). These conditions are 'avoided' to a certain extent by elevation of the N_2 fixing tissues above the soil itself.

4A.4.1.4 Environmental factors effecting legume growth and nodulation

As individual species did not explain a significant amount of variation in any of the four measured aspects of legume growth and nodulation, (when stepwise linear regressions were carried out on the five species as one 'legume' group) (section 4A.3.1) there is no evidence that the species differed significantly from each other in these respects. The environmental parameters measured did not predict any of the variation in above-ground and nodule biomass. The water regime category was negatively associated with nodule number, nodule biomass however was not, therefore only nodule initiation was associated. This is unusual as it is usually nodule development and/or activity that is affected by waterlogging (Sprent & Sprent, 1990). The positive association of soil total nitrogen and individual nodule biomass is not surprising. Combined nitrogen is known to both reduce nodule development and nitrogen fixation (e.g. Becana & Sprent, 1987) and stimulate nodule development and nitrogenase activity (e.g. Wedderburn, 1983; Pankhurst, 1981). The levels of soil nitrogen at the sites were low (Table 3.1) and were therefore not at inhibitory levels, which might explain the positive relationship found in this case. Gibson & Jordan (1983) reported that responses of legume nitrogen fixation to combined nitrogen vary from significant levels of inhibition to significant levels of stimulation. Of course, the concentration of nitrogen determines a particular legume-rhizobia response and the response of each species to different soil N levels requires further, detailed greenhouse studies for resolution.

4A.4.2. Nitrogen fixation

4A.4.2.1 Nitrogenase activity

Assays were carried out throughout the growing season and at a set time each day as data for the acetylene reduction assay are dependent on both the time of day and the time of year the assay is conducted. Root nodules are reported to function diurnally, both in legumes (e.g. Bergersen, 1970; Mague & Burris, 1972; Minchin & Pate, 1974) and non-legumes (Wheeler *et al.*, 1969, 1971). This diurnal cycle is related to daily environment changes, particularly temperature and light which also affect nitrogen fixation season to season in addition to day to day (e.g. Sloger *et al.*, 1975).

It is probable that the rates obtained from these assays are under-estimates and should therefore be regarded as minimal potential rates. Minchin *et al.* (1983), using continuous gas flow techniques found that nitrogenase activity and respiration in many legume species declined rapidly in the presence of acetylene (within 8 minutes). This decline was thought to be linked to the cessation of ammonia production and this was later confirmed by Sheehy *et al.* (1983) and Witty *et al.* (1984). They found also that nodules become more diffusion resistant in the presence of acetylene, causing a decrease in oxygen flow to the bacteroids. The method for quantitative measurement of nitrogenase activity now recommended employs a continuous gas flow through system to measure the maximum rates of activity in the first few minutes of the assay before the decline occurs. The decline is not detectable in closed assays and therefore the rates of activity can be under-estimated, sometimes up to 50% (Minchin & Witty, 1989).

However, the gas flow through technique is not without problems. Gordon *et al.* (1989) found that even when using a gas flow through technique, nitrogen fixation was under-estimated by 50% in white clover (when compared to N accumulation data). The technique requires special equipment which can be expensive and is not available to everyone as opposed to that used in closed assays which is relatively cheap and simple. To use this technique in the field, highly sophisticated equipment is needed (e.g. Sheehy *et al.*, 1991) and as a result most assays are carried out in the laboratory. It is argued (Vessey, 1994) that with proper calibrations, the closed acetylene reduction technique is still a useful tool in comparative nitrogenase assays of legume nodules (e.g. Hansen & Atkins, 1987). This is disputed by Minchin *et al.* (1994), who consider that only trends can be detected by this method, not absolute values. It is in this context that the latter authors have used a closed assay in a later paper to measure nitrogenase activity (Pugh *et al.*, 1995).

The use of the acetylene reduction technique to measure N_2 fixation is therefore contentious. In view of its limitations it was used in this study as an indicator of nitrogenase activity only, not to provide quantitative measurements. For this purpose a closed system was acceptable.

The results obtained provide an indication of potential nitrogenase activity and do not reflect the specific rates for each species at any time of the year. The great variability between plants of the same species is shown by the large standard errors. In essence the results illustrate that all the species assayed actively reduced acetylene to ethylene and therefore showed potential to fix N_2. The amounts of ethylene evolved were not converted to amounts of nitrogen reduced as the ratio of 4 C_2H_2:1 N_2 is a theoretical one only. It has been shown that in the nodules of many legume species 30-60% of the electron flux through nitrogenase goes towards H_2 production (Schubert & Evans, 1976), therefore not all the electrons flowing through nitrogenase reduce N_2 to NH_3.

The core sampling technique using intact plants was not always successful in enclosing the root system of any particular plant and was not apparent until after the assay and therefore occasionally resulted in loss of replicates. It was felt, however that this problem outweighed the problems encountered when using disturbed plants as disturbance of the nodulated root system is known to reduce nitrogenase activity (Mague & Burris, 1972; Wheeler *et al.*, 1978; Wych & Rains, 1978). Using a continuous gas flow through system, Minchin *et al.* (1986) found that using detopped and substrate free (by shaking) nodulated root systems resulted in a 75% reduction of nitrogenase activity in white clover and soybean.

Another cause of loss of replicates was the problem of the safe transportation of the 'vacutainers': despite careful packaging, some were lost. This happened more frequently on the Irish field visits. As the initial number of replicates had to be kept to a minimum due to the destructive nature of the technique used, a combination of both of the above problems sometimes resulted in a large loss of replication. For example, in the case of *V. cracca* and *T. pratense* in June, the final values obtained were from one plant (of each species) only.

Disruptive events such as the cutting of Clonmacnoise and the flooding at Little Brosna, could perhaps have been accommodated if it had been possible to make more frequent visits to Ireland, but distance and cost prevented this. It was for this

reason and those mentioned previously that acetylene reduction assays were carried out in Ireland in 1993 only.

The nitrogenase activity for all species was highly variable, masking any possible seasonal changes. However it was notable that in *Lotus* (Ring Bog) at the time of flowering in both 1993 and 1994 that the mean nitrogenase activity was apparently lower than either before or after this event (Table 4A.1). The slight apparent decrease associated with flowering each year was due to a decrease in specific nitrogenase activity (g^{-1} nodule dry weight) and not a decrease in nodule biomass. A reduction in nitrogenase activity on flowering has been reported previously in white clover (Masterson & Murphy, 1976) and *Lotus uliginosus* (= *L. pedunculatus*) (Wedderburn & Gwynne, 1981). In some grain legumes however, a marked decline is more commonly reported on pod-filling (Vikman & Vessey, 1992). This is perhaps a reflection of the greater amount of dry matter that grain legumes put into reproductive growth. The apparently slightly lower rates recorded in Ring Bog in 1994 may be a result of the more 'disturbed' methods used (detached nodulated roots) when compared to the less disturbed method of whole plants used in 1993. As mentioned previously, disturbance is known to cause a decrease in nitrogenase activity. The overall seasonal pattern of nitrogenase activity found in *Lotus* was similar to that found previously for this species (Wedderburn & Gwynne, 1981). They associated the decline with increased rainfall, decreasing soil temperature, flowering (flowering occurred much later than in this study) and the start of rhizome production.

With the loss of replicates and the flooding and mowing at the Irish sites, it was not possible to see a seasonal pattern with certainty (Table. 4A.1).

Although it was not possible to detect patterns for each species separately, the nitrogenase activity of the five species as a whole was seen to be influenced by both soil moisture and soil total nitrogen (section 4A.3.2.1). The negative relationship between nitrogenase activity and soil moisture is well known (e.g. Minchin & Pate, 1975). This suggests that although *Lotus* nodules were found in the litter layer, they were still affected by the moisture of the substrate in which they were found. Similarly to individual nodule biomass, the relationship between nitrogenase activity and soil total nitrogen was positive and was discussed earlier. Both nodule development and function were positively related to soil total nitrogen, however further work would be needed to explain this relationship.

4A.4.2.2 Incorporation of $^{15}N_2$

The incorporation of $^{15}N_2$ by *Lotus* gave amounts of nitrogen fixed over the three hour assay period (Table 4A.2). The value obtained under controlled conditions in the growth room gave the highest amount of nitrogen fixed (355 ± 100 µmoles N_2 fixed g^{-1} nodule dry weight over a 3 hour assay period), which is not surprising as both light and temperature were at an optimum and both are known to affect nitrogen fixation (Sloger *et al.*, 1975). Although only two $^{15}N_2$ assays were carried out in the field, both appear consistent with the findings of the acetylene reduction assays, the highest of the two values being that for June (136 ± 51 µmoles N_2 fixed g^{-1} nodule dry weight over a 3 hour assay period). Again a decrease in nitrogen fixing activity was noted when the plants were in flower (62 + 27 µmoles N_2 fixed g^{-1} nodule dry weight over a 3 hour assay period). High standard errors were obtained in the assays, again reflecting the high variability between plants. The proportion of ^{15}N found in the above-ground material was higher earlier in the growing season (June = 59 %) than later when the plants were in flower (August = 34 %), therefore this has important implications for what may be interpreted from plant parts versus whole plants in ^{15}N studies.

The significant linear relationship (Fig. 4A.7) between N_2 fixed ($^{15}N_2$ incorporation) and ethylene evolved (ARA) conducted in the field, supports the use of the ARA's carried out previously to indicate nitrogenase activity. The amount of ethylene evolved to N_2 fixed ratio was approximately 5:1 (compared to the theoretical 4:1 ratio), highlighting the problem of using the theoretical ratio to convert acetylene reduction rates to amounts of N_2 fixed. No relationship however, was found between N_2 fixed and ethylene evolved in the assays carried out under controlled conditions (4A.3.3.2). This can be explained by the fact that not every putative nitrogen fixer was actually analysed for ^{15}N, therefore the N_2 values would not correspond to ARA values which were the total amounts for each assay vessel. This problem was rectified later in the subsequent assays conducted in the field.

The annual amount of nitrogen fixed by *Lotus pedunculatus* can be estimated by using the mean values obtained from the $^{15}N_2$ incorporation assays in the field (Table 4B.3) and converting the g^{-1} dry weight values to m^{-2} by using the mean total biomass of *Lotus* (1994-1995) 100 cm^{-2} (Fig. 4A.6 (a)). If one assumes 8 hours of activity a day for a period of 24 weeks a year, then the amount of nitrogen fixed by *Lotus pedunculatus* in Ring Bog is estimated at 11.2 kg N ha^{-1} yr^{-1}. This is low compared to the rates obtained in agricultural situations, for example the annual nitrogen fixation rate for a *T. repens* sward (high density) in Northern Ireland was

estimated to be 270 kg N ha^{-1} (Halliday & Pate, 1976). However, this rate was predicted using the acetylene reduction technique and the problems incurred when converting the amount of acetylene reduced to nitrogen fixed have been discussed previously. Also, it is often assumed that the legume is in monoculture, no such assumption is made in this study, the calculations are based on a mean biomass value (random areas of vegetation sampled over 2 years). In Chapter 6 this estimate of nitrogen fixation by *Lotus* will be compared to the estimates obtained in Chapter 4B for the non-legumes and soil.

4A.4.2.3 ^{15}N natural abundance

As discussed in Chapter 1 (1.5.3) many problems arise when the Shearer and Kohl (1986) two-source natural abundance model is used in natural uncultivated situations (Handley & Scrimgeour, 1996). The application of the two source natural abundance model to the particular case of *Lotus* in Ring Bog proved to be unsuitable. The following were used in the equation outlined in Chapter 1 (1.5.3) to obtain the fraction of N derived from N_2: the mean initial $\delta^{15}N$ of the non-fixers in the turf (above-ground material) in Table 4A.6 (-1.17 ‰); $\delta^{15}N$ for *Lotus* in the turf (above-ground material) in Table 4A.6 (-1.54 ‰) and the $\delta^{15}N$ for *Lotus* (above-ground material) grown in N-free medium (-0.13 ‰, mean for several different *Rhizobium* strains) reported by Steele *et al.* (1983). A negative value that is biologically impossible was calculated.

The $\delta^{15}N$ values of the stems of *Lotus* were more negative than values for the leaves (Table 4A.3). A similar result was found by Yoneyama (1987) in forage legumes growing in a nitrogen free medium. He suggested that this was due to isotopic discrimination during the metabolism and translocation of N. He also found that the magnitude of isotopic fractionation at nitrogen fixation was small in legumes (-0.2 to -2 %), which suggests that the old leaves and old stems were slightly too negative for all the depletion to be due to nitrogen fixation.

The below-ground material was more depleted in $\delta^{15}N$ than the above-ground material on the four sampling occasions over the two years, whereas the nitrogen content of below-ground material was 50-70% of above-ground on these occasions (Table 4A.4). These $\delta^{15}N$ values suggest that transfer of stored N from the roots to the above-ground plant parts did not occur, if this was the case the N in the above-ground material would be less enriched than the below-ground material. The significant linear relationship between $\delta^{15}N$ and % N (Fig. 4A.8) shows that there was little net enzymatic discrimination between source and sink N of this species

within a particular plant part (Linda Handley, pers. comm., 1996). The decrease in $\delta^{15}N$ of the shoots throughout the growing season, noted in this study was also found by Bergersen *et al.* (1988) for soybean grown in an N-free medium. However, unlike in this study, they found that the $\delta^{15}N$ of the roots invariably remained positive and that of nodules increased (to about 10‰) a phenomenon widely reported in legume nodules (Yoneyama (1988) and authors cited therein). Roots and nodules were not analysed separately in this study, but it is apparent that the two combined declined in $\delta^{15}N$ during the growing season. *Lotus* $\delta^{15}N$ was much lower (by at least 3‰) than that of the total soil N (Table 4B.2).

Although the mean of the non-fixing species (-1.17 ‰) was lower than that of *Lotus* (-1.54 ‰) all had a negative $\delta^{15}N$ and several had values close to *Lotus* and that of *Potentilla erecta* (-1.75 ‰) was even lower. The % N of *Lotus* was only slightly higher than the non-fixers. This does not conform with the characteristics (nitrogen fixing plants with higher %N and lower $\delta^{15}N$ than non-fixers) of reference plants commonly reported (Virginia & Delwiche, 1982). Similar problems in finding suitable reference plants have been found by several authors (Ledgard *et al.*, 1985; Pate *et al.*, 1993; Handley *et al.*, 1994).

Handley & Raven (1992) suggested that low $\delta^{15}N$ of reference plants could be due to their use of unmineralised (therefore unenriched) N fixed by another organism or to the reference plants fixing nitrogen themselves by symbiotic or associative means. It is unlikely that the latter is the true explanation as all four of the dicotyledon species examined for $^{15}N_2$ incorporation (Table 4B.3) in Chapter 4B did not take up $^{15}N_2$. However, the former suggestion may have some relevance to Ring Bog. The peat soil in Ring Bog was found to fix nitrogen (Tables 4B.1 and 4B.3) therefore free-living nitrogen fixers may have contributed unenriched N to the non-fixers (and *Lotus*), thus confounding the $\delta^{15}N$ measurements. It should also be noted that *Lotus* and the non-fixers have all (with the exception of *Carum verticillatum*, for which there is no information available) been reported to possess vesicular-arbuscular mycorrhiza (Harley & Harley 1987, 1990). Although some reports have also found mycorrhiza to be absent (Harley & Harley, 1987, 1990) there is a possibility that mycorrhiza may affect the $\delta^{15}N$ signatures in the plant soil system (Handley & Scrimgeour, 1996) at Ring Bog. However, this is speculation without confirmation from further work. In some cases where the $\delta^{15}N$ of non-fixing plants are close to the $\delta^{15}N$ of nitrogen fixers, it has been concluded that the fixers are not actually fixing nitrogen (e.g. in the case of tropical tree legumes (Yoneyama *et al.*, 1993a)). This can be ruled out in this study as the ARA's indicated and the $^{15}N_2$ incorporation

assays proved that *Lotus* actively fixes nitrogen in Ring Bog. Another possible explanation for the negative values of $\delta^{15}N$ values of both non-fixers and *Lotus* is that these low values were due to ammonia assimilation by glutamine synthetase (Yoneyama *et al.*, 1991, 1993b).

4A.4.2.4 Contribution of *L. pedunculatus* to the nitrogen economy of Ring Bog

The proximity of the *Lotus* associated species *Juncus acutiflorus* and *Agrostis stolonifera* to *Lotus* did not significantly affect the species' total nitrogen content. However, the sample size used (ten samples with and ten samples without *Lotus*) may have been too small for significant differences to be detected. Another factor which may confound results is the associative nitrogen fixation of these two monocotyledonous species. Both were found to incorporate $^{15}N_2$ in Chapter 4B.

4A.4.2.5 Isotope dilution

Doughton *et al.* (1995) in their comparison of ^{15}N isotope dilution and natural abundance methods reinforce the fact that the choice of reference plant is important. All the non-fixing species in the current study had the same growth form as *Lotus* (perennial, hemicryptophyte) but as mentioned previously (4A.4.1), *Lotus* has very shallow roots in Ring Bog, and no other species was found to exhibit a similar rooting pattern and for the reasons mentioned earlier (possibility of effects of mycorrhiza and contribution of unenriched ^{15}N by other nitrogen fixing organisms), none of the non-fixers examined (Table 4A.6) were obvious reference plants for *Lotus*. However, as the percent of nitrogen contributed by fixation to the above-ground material was high (76 %), the mismatch may have only resulted in a small deviation from the actual value (Danso *et al.*, 1993; Doughton *et al*, 1995). This value is only an estimate and must be treated with caution due to the problems with the methodology. Problems would arise if this was then used to calculate the amount fixed per plant (Danso *et al.*, 1993).

4A.5 SUMMARY

- Nodulation patterns were determined for five species of legumes at three different sites (Fig. 4A.1-4A.6). Detailed studies of *Lotus pedunculatus* at Ring Bog (frequently water saturated) revealed that root and nodule growth was restricted to the above-ground vegetation and litter layer, thus 'avoiding' the more hostile soil environment.

- All five legume species examined for nitrogenase activity using the acetylene reduction technique reduced acetylene to ethylene (Table 4A.1) and thus showed the potential to fix nitrogen.

- In a more detailed study on *Lotus pedunculatus* at Ring Bog, the nitrogen fixing potential of this species was confirmed by the direct uptake of $^{15}N_2$ (Table 4A.2). The annual rate of nitrogen fixation for *Lotus pedunculatus* in Ring Bog was estimated conservatively at 11.2 kg N ha^{-1}.

- Studies on the natural abundance of ^{15}N of *Lotus pedunculatus* and non-fixing plants growing in Ring Bog revealed that the two source natural abundance model was not suitable for the semi-natural situation found at Ring Bog (4A.4.2.3).

- Further confirmation of the nitrogen fixing capacity of *Lotus pedunculatus* was given by using the ^{15}N isotope dilution technique in a greenhouse experiment (Table 4A.6). The contribution of atmospheric N_2 to the above-ground biomass of *Lotus* was estimated at 76% (over 4 weeks), illustrating the importance of N_2 fixation as a source of nitrogen for new growth in this species.

Chapter 4
NITROGEN FIXATION
SECTION B: NON-LEGUMES AND SOIL

4. NITROGEN FIXATION
SECTION B : NON-LEGUME AND SOIL

4B.1 Introduction

The aim of this chapter is to:

- determine which, if any, of the following fix nitrogen within riverine wetlands:
  soil (due to free-living nitrogen fixers)
  bryophytes (cyanobacteria)
  non-legume angiosperms (associative fixation)

Peat soils are known to reduce acetylene (Waughman & Bellamy, 1972; Waughman, 1976) and are therefore potential sources of biologically fixed nitrogen. The acetylene reduction technique was used at the Irish sites and Ring Bog to determine nitrogenase activity in the soil. Ring Bog was selected for more detailed investigation of non-legume sources of biologically fixed nitrogen.

As mentioned in Chapter 1, some moss-algal associations have nitrogen fixing potential (Sprent & Sprent, 1990). Ring Bog has several different mosses in abundance, and the three most frequent were selected for examination: *Polytrichum commune* Hedw.; *Sphagnum palustre* L. and *Sphagnum recurvum* P. Beauv.

Nitrogen fixation associated with angiosperm roots that lack root nodules is well documented (e.g. Boddey & Döbereiner, 1988; Chalk, 1991). It has been suggested that the generally low levels of combined nitrogen in wetlands may result in associative fixing plants being favoured in these habitats (Tjepkema & Evans, 1976). Bowden (1987) proposed that 'imported' nitrogen is not important to marsh communities because of internal cycling. However, Eckardt & Biesboer (1988) found associative fixation to be an important source of nitrogen to a wetland community. Associative fixation in angiosperms (monocotyledons and dicotyledons) was studied in Ring Bog.

4B.2 MATERIAL AND METHODS

4B.2.1 Soil

i) Nitrogenase activity (ARA)

Nitrogenase activity of soil at all three sites was assayed throughout 1993. The methods used are described in 4A.2.2.1 (a). Five to seven replicate soil cores were assayed on each occasion (simultaneously with the legume cores).

In order to determine if there was any relationship between the environmental variables measured in Chapters 3 and 4A and the nitrogenase activity of soil (the three sites analysed collectively), a stepwise linear regression was carried out using UNISTAT (version 4).

ii) Nitrogenase activity (ARA) v ethylene accumulation

Ten soil cores (7 cm in diameter and 12 cm in length) were collected from Ring Bog (28/10/94) and assayed under growth room conditions (4A.2.2.1 (c)) with the exception that acetylene was not added to half the samples to allow the detection of endogenous ethylene accumulation. Gas samples were taken at 0, 1, 2 and 3 hours.

iii) ^{15}N natural abundance

Two soil samples were collected from Ring Bog (7/9/93) and prepared for $\delta^{15}N$ analysis (4A.2.3). This was repeated on 5/6/95 and 2/8/95 using five replicate soil samples on each occasion.

iv) Incorporation of $^{15}N_2$

This was carried out in the field at Ring Bog on two occasions (5/6/95 and 2/8/95). Three replicate soil samples (approximately 600 ml in volume) were assayed on each visit (4A.2.2.2).

4B.2.2 Bryophytes

i) Nitrogenase activity (ARA)

a) In the field: Nitrogenase activity of *P. commune* and *S. palustre* was measured at Ring Bog (7/9/94). Three replicate samples of each species were assayed as described previously (4A.2.2.1 (b)).

b) In the greenhouse: Eight turves were collected from Ring Bog (30/8/94) and established in plastic containers (19.5 x 13.5 x 15 (deep) cm) over a period of three and a half months. Four samples each of *P. commune*, *S. palustre* and *S. recurvum* were split into above and below-ground material and assayed as in 4A.2.2.1 (c) with

the exception that 500 ml round bottomed flasks were used. Gas samples were taken at 0, 1, and 2 hours.

ii) Incorporation of $^{15}N_2$

Two samples of *P. commune* and *S. palustre* were included in the $^{15}N_2$ incorporation assay conducted on turves established in the greenhouse (4A.2.2.2). In addition to the latter two bryophyte species, *S. recurvum* was also included in the $^{15}N_2$ incorporation assays carried out in the field (5/6/95 and 2/8/95).

4B.2.3 Associative fixation

Incorporation of $^{15}N_2$

Two samples of *Juncus acutiflorus* L. were initially examined for $^{15}N_2$ uptake as described in 4A.2.2.2. This examination was subsequently extended to include a further 7 plant species (excluding *L. pedunculatus*) in the field (4A.2.2.2). Due to the variability in vegetation composition, the number of replicates for each species varied.

4B.3 RESULTS

4B.3.1 Soil

Nitrogenase activity values for soil cores are given in Table 4B.1. The hourly rate is given for μmoles of ethylene evolved between 0.5 and 1.5 hours m^{-2} (accumulative data for throughout the 2.5 hours is shown in Appendix G). As with the legume ARA's, the standard errors are large, illustrating the large variation between the soil samples. It should be stated again that these are only indications of the potential amounts of nitrogenase activity. If one refers back to the legume ARA's (Table 4A.1) it can be seen that there is apparently more fixation in the soil at Ring Bog than can be ascribed to *Lotus*, with the exception of June when no activity was detected in the soil. Nitrogenase activity appears to be much higher in Ring Bog than the two Irish sites, but not at comparable dates. Activity of soils for all sites is lowest in June. A stepwise linear regression found that none of the measured environmental variables explained a significant amount of variation in soil nitrogenase activity.

The results of the comparison of nitrogenase activity and endogenous ethylene evolution of Ring Bog soil under controlled conditions are shown in Fig. 4B.1. There was some ethylene evolution in the vessels without added acetylene, but the amounts were very small (10.87% of ethylene evolved as a result of putative nitrogenase activity after 3 hours).

The natural abundance of nitrogen in Ring Bog soil is given in Table 4B.2. The relatively high standard errors indicate the variability of the soil.

Confirmation of the nitrogen fixation in the soil is given in Table 4B.3, it can be seen that Ring Bog soil reduced $^{15}N_2$. The amounts incorporated by fixation were greater in June than in August.

Table 4B.1 Nitrogenase activity of soil, measured in the field (± standard error)

Site & Date	(n=)	Soil Temp. (^{o}C)	Nitrogenase activity (μmol ethylene evolved $m^{-2}h^{-1}$) ± standard error
Clonmacnoise			
23/5/93	(5)	12.3 ± 0.1	28 ± 15
27/6/93	(5)	20.2 ± 0.3	5 ± 3
1/10/93	(7)	11.3 ± 0.1	37 ± 19
Little Brosna			
25/5/93	(5)	12.4 ± 0.2	14 ± 8
29/6/93	(3)	17.2 ± 1.1	0 ± 0
28/7/93	(7)	15.8 ± 0.2	14 ± 7
Ring Bog			
8/6/93	(3)	13.1± 0.1	0 ± 0
13/7/93	(4)	12± 0.2	20 ± 16
17/8/93	(3)	12 ± 0.2	94 ± 75
21/9/93	(4)	11 ± 0.1	81 ± 23

* Mean nitrogenase activity for 5-7 samples, over the period of 0.5-1.5 hours from initiation of incubation. See Appendix G for accumulative values over 2.5 hours. (n= number of samples)

Table 4B.2 $\delta^{15}N$ (‰) of total soil N at Ring Bog ± standard error.

DATE (No. samples)	%N	$\delta^{15}N$ (‰)
7/9/93 (2)	1.75 ± 0.38	3.59 ± 0.50
5/6/95 (5)	2.21 ± 0.28	2.85 ± 0.40
2/8/95 (5)	2.33 ± 0.28	3.31 ± 0.30

Fig. 4B.1 Nitrogenase activity (with acetylene) and endogenous ethylene evolution (without acetylene) of Ring Bog soil (μmoles ethylene evolved m^{-2} h^{-1}) with standard error bars (n=5)

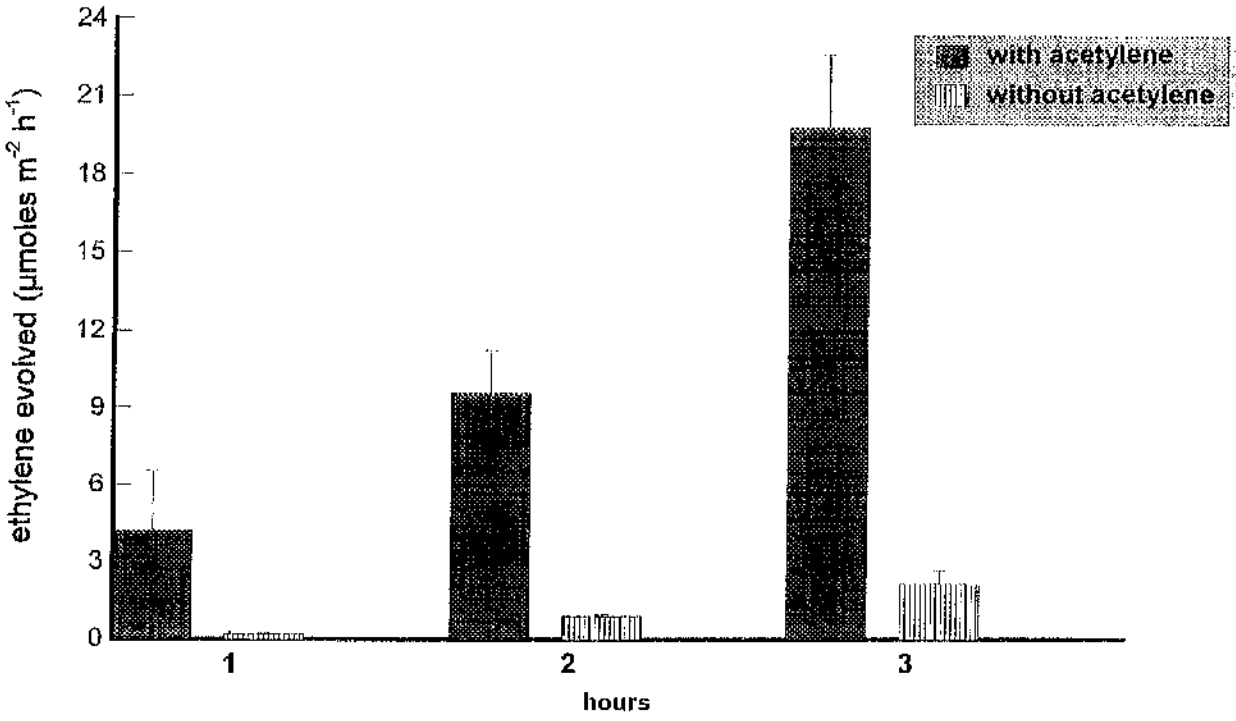


Table 4B.3 Fixation of N_2 by Ring Bog soil (µmoles N_2 fixed g^{-1} dry weight), eight non-legume plant species (plus *Lotus*) and three bryophytes (µmoles N_2 fixed g^{-1} dry weight) (± standard errors) over three hours

	GROWTH ROOM 28/7/94	*in situ* 5/6/95	*in situ* 2/8/95
SOIL		0.21 ± 0.08 (3)	0.01 ± 0.01 (3)
DICOTYLEDONS			
Lotus pedunculatus	3.02 ± 0.23 (5)	3.06 ± 2.03 (4)	0.46 ± 0.20 (4)
Hydrocotyle vulgaris		0.00 ± 0.00 (3)	0.00 ± 0.00 (3)
Potentilla erecta		0.00 ± 0.00 (3)	0.00 ± NA (1)
Rumex acetosa		0.00 ± 0.00 (4)	0.00 ± NA (1)
Viola palustris			0.00 ± 0.00 (2)
MONOCOTYLEDONS			
Agrostis stolonifera		0.00 ± 0.00 (3)	0.02 ± 0.02 (5)
Molinia caerulea			0.07 ± 0.06 (2)
Carex curta		0.08 ± NA (1)	0.26 ± 0.22 (2)
Juncus acutiflorus	0.26 ± 0.03 (2)	0.17 ± 0.06 (5)	0.29 ± 0.22 (5)
BRYOPHYTES			
Polytrichum commune	0.19 ± 0.01 (2)	0.01 ± 0.01 (2)	
Sphagnum palustre	1.32 ± 0.39 (2)	0.04 ± 0.02 (3)	0.19 ± 0.13 (3)
Sphagnum recurvum		0.06 ± NA (1)	0.16 ± 0.08 (3)
TEMPERATURE/°C	22.5 ± 0.28	12.12 ± 0.26	18.53 ± 0.16

Sample numbers are given in brackets NA= not applicable

4B.2.2 Bryophytes

The nitrogenase activity (μmol ethylene evolved g^{-1} dry weight h^{-1}) of *Sphagnum palustre* and *Polytrichum commune* measured in the field (7/9/94) was 0.05 ± 0.03 and 0.02 ± 0.004 respectively. The values were taken between 0.5-1.5 hours (see Appendix G for values over the 2.5 hour assay period). Mean soil temperature was 11.18°C (± 0.14).

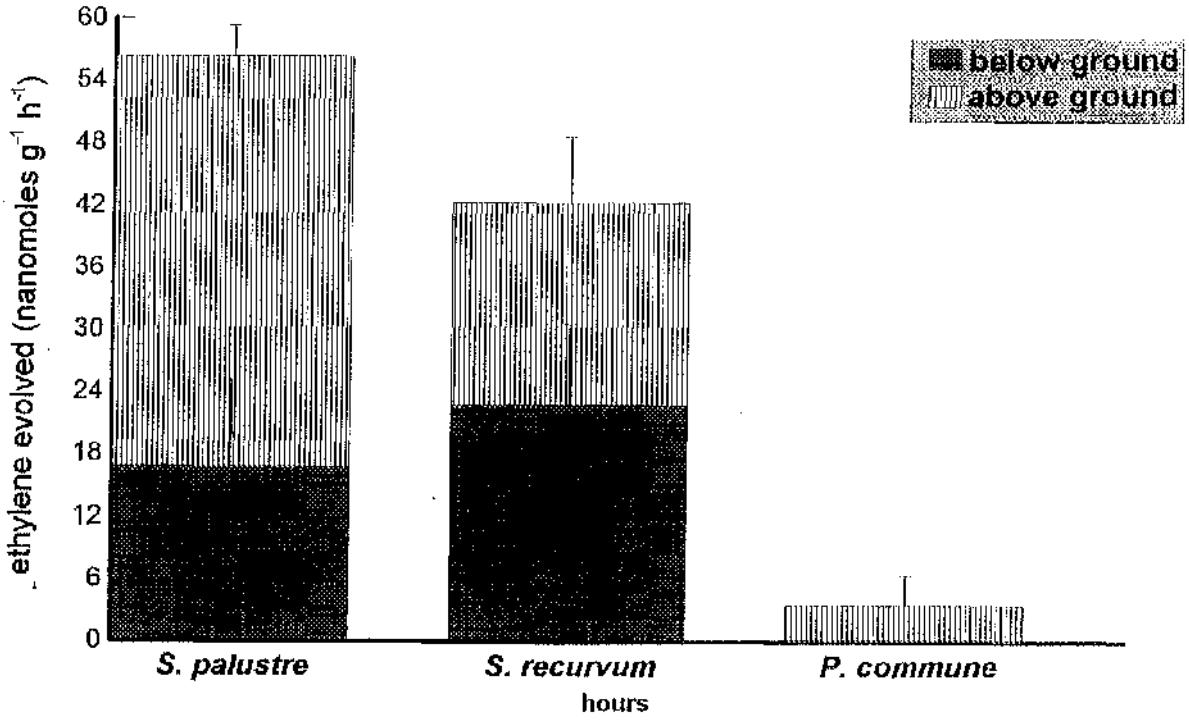
The results of the acetylene reduction assays carried out in the growth room are given in Fig. 4B.2. The mean soil temperature was 25 °C (± 0.58). Both above and below-ground parts of the *Sphagnum* species were active, while *Polytrichum* was only slightly active in the below-ground section and was the least active moss overall. Of the two *Sphagnum* species, *S. palustre* had the highest rate of nitrogenase activity.

All three mosses reduced $^{15}N_2$ (Table 4B.3), *Polytrichum* incorporated the least. The amounts and pattern of $^{15}N_2$ incorporation was similar in the two *Sphagnum* species. The improved conditions for nitrogen fixation provided by the growth room resulted in higher rates of incorporation when compared to the *in situ* assays.

4B.2.3 Associative fixation

From Table 4B.3 it can be seen that *Lotus* was the only dicotyledonous plant sampled to reduce $^{15}N_2$. All the monocotyledonous species incorporated $^{15}N_2$, *A. stolonifera* to a lesser extent than the other 3 species. *A. stolonifera*, *C. curta* and *J. acutiflorus* incorporated a greater amount of $^{15}N_2$ in August than in June.

Fig. 4B.2 Nitrogenase activity of three bryophyte species under controlled conditions (nanomoles ethylene evolved g^{-1} dry weight h^{-1}) with standard error bars (n=4)



4B.4 DISCUSSION

4B.4.1 Soil

The ARA's conducted on soil from all three sites (Table 4B.1) reveal that they reduce acetylene to ethylene and thus have the potential to fix nitrogen. This activity is presumed to be due to free-living prokaryotes. Acetylene reduction by peat soils has been observed by several workers (e.g. Waughman & Bellamy, 1972; Granhall & Selander, 1973; Dooley & Houghton, 1973). It was beyond the scope of this project to identify the free-living nitrogen fixers. Waughman (1976) found the dominant form of nitrogenase activity in peat soils to be heterotrophic, but cyanobacteria may also contribute (Granhall & Selander, 1973). Cyanobacteria have only local importance in peat soils, but can be important in other soil systems (Sprent & Sprent, 1990). It has been suggested that the nitrogen fixing bacteria could be in either the aerobic or anaerobic zone of the soil (Waughman & Bellamy, 1972) and regular fluctuations in water table levels would create periods of anaerobicity and aerobicity in the upper peat layers (Waughman *et al.*, 1981).

Problems encountered with assaying soil and associative fixation were among the first to be reported and are reviewed by Giller (1987). Acetylene has been found to inhibit the natural oxidation of ethylene in soils and over-estimates of nitrogenase activity can therefore occur due to ethylene accumulation (de Bont, 1976; Witty, 1979). It was for this reason that ethylene accumulation was examined in Ring Bog soil, where rates of nitrogenase activity were rather high (higher than that of the *Lotus*). The rates of endogenous ethylene evolution were low (Fig. 4B.1), but it must be noted that Ring Bog soil has the potential to evolve endogenous ethylene and this may contribute to the high rates of acetylene reduction that were recorded.

Although the acetylene assay method has many problems, the values obtained for soil in this study fall within those previously found for a number of European peatlands (Waughman & Bellamy, 1980). None of the environmental variables measured could explain the variation in soil nitrogenase activity. It is interesting that nitrogenase activity was detected in Ring Bog, where the pH of the soil was below 5.5 since any fixation below this pH is considered unusual (Mishustin & Shil'nikova, 1971). However, Waughman & Bellamy (1980) also found nitrogenase activity in peat soils with a pH below 5.5.

The occurrence of nitrogenase activity in soil was further supported by the $^{15}N_2$ assays on Ring Bog soil (Table 4B.3). The annual nitrogen fixation rate for the free-living bacteria in the peat soil within Ring Bog can be calculated using the mean

values obtained from the $^{15}N_2$ incorporation assays in the field (Table 4B.3). If one assumes that the activity occurred eight hours a day for 24 weeks of the year then the rate of nitrogen fixation is estimated at 55.8 kg N ha^{-1} yr^{-1}.

The $\delta^{15}N$ value of the total soil N of Ring Bog soil increased during the growing season (Table 4B.2). A similar finding was reported by Handley & Scrimgeour (1996) studying an abandoned agricultural field. The % total N of the soil was high in this study, but the amounts in different soil N fractions were not determined.

Studies have shown that the $\delta^{15}N$ of total soil N often correlates with soil moisture content, due to the loss of ^{15}N depleted N to the atmosphere by nitrification, denitrification and ammonia volatilization and to groundwater from leaching (Handley & Raven, 1992). As the soil moisture content of Ring Bog is high (84% dry weight) it might be expected that the $\delta^{15}N$ of total soil N would be more enriched than it is. This discrepancy may be explained by the apparent input of ^{15}N depleted N by *Lotus* (discussed in the previous chapter), free-living microbes in the soil and monocots (see further). Confirmation of this hypothesis would, of course, need a considerable amount of work involving detailed examination of soil processes. Only nitrogen fixation was considered in this study.

4B.4.2 Bryophytes

The ARA's carried out on mosses revealed that *Sphagnum palustre*, *Sphagnum recurvum* and *Polytrichum commune* all reduced acetylene to ethylene (section 4B.3.2), therefore implying a potential to fix nitrogen. Fixation in bryophytes, with cyanobacteria as the active organism, has been reported widely (Meeks, 1990). Although the *Sphagnum*-cyanobacteria association is one of the best known, an extensive literature search failed to locate any published information relating to the nitrogen fixing ability of the two *Sphagnum* species studied here. The acetylene reduction rates recorded for the *Sphagnum* sp. under controlled conditions (Fig. 4B.2) and *Sphagnum palustre* (section 4B.3.2) are within the rates previously recorded by Granhall & Selander (1973) for the *Sphagnum lindbergii*-algal relationship at two sites within a Swedish mire (4-90 nmol ethylene evolved g^{-1} dry weight h^{-1}). However, fixation was attributed solely to algae in the upper leaves, whereas in this study, non photosynthetic bacteria were also active (Fig. 4B.2), below-ground nitrogenase activity was detected. The nitrogen fixing capacity of *Polytrichum commune* has been reported previously (Alexander & Schell, 1973; Bowden, 1991) with conflicting views as to which micro-organisms are important in nitrogen fixation. Bowden (1991) suggested that fixation by cyanobacteria was

unlikely to be important as rates of fixation were not reduced by periods of total darkness (a conclusion that ignores the possibility that cyanobacteria may utilise carbon from the host plant in addition to their own photosynthetic carbon), whereas Alexander & Schell (1973) attributed the fixation to cyanobacteria (*Nostoc*).

The cyanobacteria most often reported in association with *Sphagnum* is *Nostoc*, but others such as *Hapalosiphon* and *Anabaena* have also been recorded (reviewed by Grilli Caiola, 1992). Basilier (1980) demonstrated the transfer of nitrogen fixed by epiphytic *Nostoc* to the *Sphagnum*'s growing apex using $^{15}N_2$, thus proving metabolic interaction, the mechanism of which has not been studied to date. The algae are either intracellular or extracellular, the latter is the most commonly found (Gibson & Jordan, 1983). In the former case, the cyanobacteria enter through pores in the hyaline cells of the moss (Granhall & Hofsten, 1976). The hyaline cells, it is assumed, provide a more favourable habitat, their buffering capacity allowing the cyanobacteria to avoid the inhibitory effects of low pH outside the cells.

The potential nitrogen fixing capacity of the mosses indicated by the ARA's were confirmed by the $^{15}N_2$ incorporation assays. *Polytrichum* was the least active species in both the ARA and $^{15}N_2$ assays. The results of the ARA where gametophytes and rhizomes were analysed separately (Fig. 4B.2) show that rhizome activity was negligible in *Polytrichum*, suggesting that activity was due to cyanobacteria only. However, in both the *Sphagnum* species the below-ground parts also reduced acetylene, suggesting that other organisms were actively fixing nitrogen in addition to the assumed cyanobacteria in the above-ground parts. This is not necessarily a clear reflection of the situation in the field as both temperature and light were elevated in this particular experiment. From the $^{15}N_2$ incorporation assays it would seem that rates of $^{15}N_2$ incorporation in the mosses increase as temperature increases, though other factors may also be important.

The annual nitrogen fixation rate for each of the mosses within Ring Bog can be calculated using the mean values obtained from the $^{15}N_2$ incorporation assays in the field (Table 4B.3) if one assumes that the turfs collected for $^{15}N_2$ incorporation were representative of the vegetation in Ring Bog and that activity occurred eight hours a day for 24 weeks of the year. The following values are based on the mean biomass of the vegetation included in the assays, whereas the estimate for *L. pedunculatus* (4A.4.2.2) was based on a mean biomass value calculated from data (turves) collected over 2 years.

The estimate of nitrogen fixation for *Sphagnum palustre* = 1.5 kg N ha^{-1} yr^{-1}, *Sphagnum recurvum* = 0.3 kg N ha^{-1} yr^{-1} and *Polytrichum commune* = 0.55 kg N ha^{-1} yr^{-1}.

Lack of time prevented further study of the presumed association between the mosses studied and cyanobacteria. However, taking into account current literature and the results obtained, it appears that a moss-cyanobacterial nitrogen fixing association is present in Ring Bog.

4B.4.3 Associative fixation

None of the non-leguminous dicotyledonous plant species fixed nitrogen (Table 4B.3). This is perhaps not surprising as most associative nitrogen fixation in plant rhizospheres has been reported in monocotyledons (Sprent & Sprent, 1990). The study by Harris & Dart (1973) is a notable exception. Amongst the species in which they detected nitrogenase activity (acetylene reduction) was *Rumex acetosa*. This species was not found to reduce $^{15}N_2$ in the current study.

All the monocotyledonous species examined (*Agrostis stolonifera*, *Carex curta*, *Juncus acutiflorus* and *Molinia caerulea*) in this study however, incorporated $^{15}N_2$ (Table 4B.3), a direct indication of their nitrogen fixing association with rhizosphere bacteria. This is not the first time British wetland monocots have been examined for associative nitrogen fixing ability. The acetylene reduction assay showed activity in the following species: *Typha latifolia* L. (Biesboer, 1984), *Phalaris arundinaceae* L., *Phragmites australis* (Cav.) Trin, *Juncus effusus* L. (Ogan, 1982a, b). However, an extensive literature search failed to locate any published information on nitrogen fixation by the monocots found to fix nitrogen in this study. The organisms in the rhizosphere associated with the monocots were not identified.

The annual nitrogen fixation rate for each of the monocots within Ring Bog can be calculated using the mean values obtained from the $^{15}N_2$ incorporation assays in the field (Table 4B.3) if one assumes that the turfs collected for $^{15}N_2$ incorporation were representative of the vegetation in Ring Bog as a whole and that activity occurred eight hours a day for 24 weeks of the year. These values are based on the mean biomass of the vegetation included in the assays, whereas the estimate for *L. pedunculatus* (4A.4.2.2) was based on a mean biomass value calculated from data (turves) collected over 2 years. The estimate of nitrogen fixation associated with *Agrostis stolonifera* = 0.04 kg N ha^{-1} yr^{-1}, *Carex curta* = 11.2 kg N ha^{-1} yr^{-1}, *Juncus acutiflorus* = 9.7 kg N ha^{-1} yr^{-1} and *Molinia caerulea* = 1.2 kg N ha^{-1} yr^{-1}.

4B. SUMMARY

- Soil samples from three sites (Clonmacnoise, Little Brosna and Ring Bog) were found to reduce acetylene to ethylene (Table 4B.1) and thus exhibited the potential to fix nitrogen. This nitrogenase activity was attributed to free-living micro-organisms in the soil. This potential was confirmed in Ring Bog which incorporated $^{15}N_2$ (Table 4B.3). The annual rate of nitrogen fixation by free-living organisms in the (surface) soil at Ring Bog was estimated at 55.8 kg N ha^{-1} yr^{-1}.

- Three bryophytes species were found to reduce acetylene to ethylene at Ring Bog (Fig. 4B.2) (*Sphagnum palustre, Sphagnum recurvum and Polytrichum commune*). This nitrogen fixing potential was confirmed by direct uptake of $^{15}N_2$ by all three species (Table 4B.3). Nitrogen fixation may have been due to moss-cyanobacteria associations although some heterotrophic bacteria may also have been active in the underground parts of the *Sphagnum* species. The annual rate of nitrogen fixation by mosses at Ring Bog was estimated to be between 0.3 and 1.4 kg N ha^{-1} yr^{-1}.

- Four monocotyledonous species (*Agrostis stolonifera, Carex curta, Juncus acutiflorus* and *Molinia caerulea*) were found to incorporate $^{15}N_2$ (Table 4B.3). Nitrogen fixation was attributed to bacteria living in the rhizosphere of these species. None of the dicotyledonous plant species examined fixed nitrogen. The annual rate of nitrogen fixation by monocots at Ring Bog was estimated to be between 0.04 and 11.2 kg N ha^{-1} yr^{-1}.

Chapter 5
ENVIRONMENTAL EFFECTS ON LEGUME GROWTH AND NITROGEN FIXATION: GREENHOUSE STUDIES

5. ENVIRONMENTAL EFFECTS ON LEGUME GROWTH AND NITROGEN FIXATION: GREENHOUSE STUDIES

5.1 INTRODUCTION

The aims of this chapter are to:

- Investigate in greenhouse studies, the hypothesis put forward in Chapter 3 that flooding is a major influence on legume distribution in wetland sites.

- Examine the additional influence of defoliation on legume distribution, as discussed in Chapter 3, again by carrying out studies under greenhouse conditions.

The detrimental effects of waterlogging on growth of land plants are well documented (e.g. Jackson & Drew, 1984) and are discussed in Chapter 3. The majority of studies which have examined the response of legumes to waterlogging focused on legumes of agricultural importance, for example: *Glycine max* (L.) Merrill (Stanley *et al.*, 1980); *Medicago sativa* L. (Rogers, 1974); *Phaseolus aureus* Roxb. (Musgrave & Vanhoy, 1989); *Pisum sativum* L. cv. Sprite (Cannell *et al.*, 1979; Jackson, 1979); *Vigna unguiculata* (L.) Walp. (Hong *et al.*, 1977; Minchin *et al.*, 1978). It was generally found that waterlogging was detrimental to nodule production, formation and function and host plant growth. The effects were noted after only four days of waterlogging in *Pisum sativum* (Jackson, 1979) and none of the experiments imposed a waterlogging treatment for more than eight days. Waterlogging in older plants (flowering-podset) was reported to have the most deleterious effects while younger plants (vegetative growth) were more tolerant (Cannell *et al.*, 1979; Jackson, 1979; Stanley *et al.*, 1980). However, Minchin *et al.* (1978) found the adverse effects of waterlogging were more acute, the earlier the stage of development in *Vigna unguiculata*.

This chapter will examine the effects of long term flooding on legumes native to the riverine wetlands visited in this study. The age of a legume, in addition to flooding duration affects its ability to recover from waterlogging, therefore effects of flooding on different aged plants were examined.

The extremes of drought and flooding imposed experimentally occur naturally within riverine wetlands where water levels can fluctuate considerably. The

response of one of the more flood tolerant legumes from the first experiment (*L. pedunculatus*) to more severe waterlogging was examined.

Major additional influences on the wetland sites studied were mowing and grazing by cattle. The effect of defoliation and the ability of legumes to develop adventitious roots from cuttings were examined in the greenhouse.

In Chapter 6, the findings of this Chapter will be discussed relative to the field situation (as discussed in Chapter 3).

5.2 MATERIALS AND METHODS

A number of greenhouse experiments were carried out on six legume species *L. pratensis*, *L. pedunculatus*, *T. dubium*, *T. pratense*, *T. repens* and *V. cracca*.

5.2.1 Isolation of rhizobia

Rhizobia from the above six legume species were isolated for use in the experiments. Nodules were mostly collected for isolation of rhizobia during preliminary visits to the field sites in October 1992 (Table 5.1).

Table 5.1 Species collected for rhizobia isolation.

Species	Site	Date Collected
Lathyrus pratensis	Clonmacnoise	26/10/92
Lotus pedunculatus	Ring Bog	23/10/92
Trifolium dubium	Little Brosna	27/5/93
Trifolium pratense	Little Brosna	26/10/92
Trifolium repens	Clonmacnoise	26/10/92
Vicia cracca	Clonmacnoise	26/10/92

Individuals of each legume species were selected at random in the field. Rhizobium isolation methods followed those outlined by Vincent (1970) and Carpenter and Robertson (1983). After washing off the loose dirt with tap water, older and smaller nodules were briefly sterilised in ethanol for 10 seconds, younger nodules were surface sterilised with 0.1% acidified mercuric chloride for three-four minutes (Vincent, 1970). After sterilisation, the nodules were crushed aseptically in approximately 10 ml sterile water, using a homogeniser. This was then diluted 7 fold with sterile water. Two drops from each stage of the series dilution were placed onto yeast-mannitol agar plates (Appendix H) using sterile pipettes. The drops were spread out on the plates which were then inverted (Date, 1982) and incubated at 26°C. Possible contaminants were identified by using congo red dye (which also has fungicidal properties). An aqueous solution of congo red (10 ml of 0.25%) was added by sterile filtration to 1L of melted medium prior to use. Rhizobia generally do not absorb this dye strongly, permitting recognition and elimination of possible contaminants which do. Cultures were replicated until well isolated colonies could be picked out. Bacteria from well separated, single colonies were repeatedly sub-cultured and finally transferred to yeast mannitol agar for storage at 3°C until required.

By using bromothymol blue any pH change as a result of bacterial growth could be observed. The pH indicator (5 ml of 0.5% alcoholic solution per litre) was

added to melted agar prior to use, the agar turning yellow in acid conditions, blue in alkali. Isolate growth on glucose peptone agar (Appendix H) for two days at 30°C was examined as rhizobia do not grow well on this substrate.

Once isolated, the rhizobia were authenticated by an infection test with a suitable legume host. The post-emergence inoculation technique of water-inoculum suspension was used. Rogers *et al.* (1982), found that this method gave satisfactory nodulation and plant growth when plants were inoculated two and four weeks after plant emergence. Seed trays were cleaned with methanol and filled with sterile perlite and horticultural sand (ratio of 1:1). This was moistened with Crone's solution (nitrogen free formula) and minor element concentrate (A-Z) (Appendix H). Seeds were surface sterilised (rinsed in 95% ethanol followed by 3 minutes with 0.2% $HgCl_2$ and washed with five changes of sterile water) and spread out on the surface and covered lightly with the sand/perlite mixture and a transparent cover was placed over the tray. The moisture of the substrate was maintained until germination. Two weeks after emergence, the seedlings were transplanted into sterile perlite/sand filled pots (disposable plastic growth pouches) moistened with Crone's solution and A-Z as before. A suspension of rhizobia was made in sterile water and adjusted to give 10^7 rhizobia ml^{-1} of sterile water (counted using a haemocytometer). This suspension (5 ml) was applied to each seedling of the homologous plant species. A second inoculation was carried out 12 days after the first using the same procedure. Ten seedlings of a given plant species were inoculated and 10 left un-inoculated as controls. Controls (separated from inoculated seedlings by transparent plastic dividing sheets) were used to monitor the occurrence of cross contamination.

With the exception of experiment 5.2.4, plants were grown from seed (Emorsgate Seeds, Terrington St. Clement, Norfolk). Seeds were germinated and seedlings transferred to individual pots (one plant/pot) containing sterile sand and perlite (1:1) and inoculated with rhizobium as described above. Plants were supplied weekly with Crone's nutrient solution and minor elements (A-Z) (Appendix H). Those plants under a 'standard' watering regime were free-draining and watered daily with distilled water.

Plants were grown in a heated greenhouse (20°C ± 5) under a 16 hr/day light regime. Natural daylight was supplemented with Navilux 400W sodium lamps. The mean PAR above the plant canopy was 234 µmol m^{-2} s^{-1} in the

winter period from September to March (when most of the experiments were conducted).

In order to determine plant biomass, harvested plant material was oven dried at 70°C for 48 hours.

5.2.2 Effects of short term flooding and limited water supply

This experiment ran from January-March 1994. After transferral to 10.5 cm diameter pots, the plants (arranged using randomly generated numbers) received the standard watering regime. After ten weeks the plants were arranged into eight blocks, each containing 18 pots in a random block design. One complete set of species treatment combinations was represented in each block. The following treatments were applied:

i) standard control: free-draining
ii) flooded: water level maintained 1 cm above substrate level
iii) dry: watered every four days only

All plants were placed in larger 1 litre pots (14 cm in diameter), the flooded pots did not have holes for drainage. The watering regimes were maintained for 20 days before harvesting.

The following variables were measured: number of nodules; total nodule dry weight; shoot dry weight; root dry weight; plant total nitrogen content (Appendix C). Yellowing of shoots, nodule colour, presence or absence of adventitious roots and hypertrophy were also noted. It was also intended to measure nodule cortex area, but due to an accident involving the loss of most of the nodules preserved for sectioning, (whilst being transported from one building to another when not in my care) this was not possible.

5.2.3 Effects of short term flooding with recovery period on seedlings

This experiment ran from October-December 1994. The effects of flooding and a subsequent recovery period on seedlings of *L. pratensis*, *L. pedunculatus*, *T. pratense*, and *V. cracca* were examined (it was intended that *T. repens* and *T. dubium* should be included in this experiment but their seedlings were destroyed by an infestation of mice in the greenhouse).

After transferral to plastic growth pouches (6 cm in diameter, 4.5 cm deep), the plants were arranged into 10 blocks, each containing four plants (one from each species) placed in a tray (35.5 x 21.5 x 6 (deep) cm). A flooding treatment was

applied (water level 1 cm above substrate) to five of these trays (without drainage holes) and the remainder were left as a control. After three weeks the water in the flooded blocks was poured away and the plants were treated as for the control. The plants were harvested after a further three weeks.

The following variables were measured: number of leaves, leaf area, number of nodules, total nodule dry weight, shoot dry weight, root dry weight. Yellowing of shoots, nodule colour, presence or absence of adventitious roots and hypertrophy were also noted.

5.2.4 Effects of long term flooding on *Lotus pedunculatus*

The effects of flooding on *L. pedunculatus* stock from Ring Bog (maintained in the greenhouse) were examined in an experiment which ran from November 1993-February 1994.

Equal lengths of stem (15 cm) and root (6 cm) were taken from 20 plants and planted out in garden soil and perlite (3:1) in 1 litre pots. The plants were watered daily and maintained in the greenhouse for seven weeks before the flooding treatment was applied. The plants (18) were placed into individual plastic containers (19.5 x 13.5 x 15 (deep) cm). Half of these containers had drainage holes. The containers were arranged in a random design. The water level was maintained at 1 cm above substrate level on half the plants, the remainder received the standard watering regime. The watering regimes were applied for five weeks before harvesting.

The following variables were measured: number of nodules, total nodule dry weight, shoot dry weight, root dry weight, nitrogenase activity, nodule cortex area, plant total nitrogen content (Appendix C). Yellowing of shoots, nodule colour, presence or absence of adventitious roots and hypertrophy were also noted.

Nitrogenase activity was measured on five randomly selected plants from each treatment (4A.2.2.1 (c)). The nodule cortex area was measured on seven nodules from each treatment, sectioned by Eoin Robertson (Electron Microscopy Centre, University of Glasgow) using the following method: samples were fixed in Formalin Acetic acid Alcohol (FAA) (90 ml 70% ethanol, 5 ml formalin and 5 ml glacial acetic acid) for 24 hours, then rinsed in distilled water and dehydrated in an ethanol series 30%, 50%, 70%, 90%, 100% for a minimum of 4 hours at each stage. After three changes in 100% ethanol they were transferred to xylene for clearing for a

minimum of 24 hours. The xylene was then removed, leaving just enough to cover the specimen and paraffin wax pastels were added. The specimens were then placed in an oven at 60°C for 24 hours, three changes of molten paraffin wax were made. The specimens were embedded in moulds with fresh wax and sectioned at 7 μ onto glass slides smeared with adhesive (glycerine albumin). The slides were stained with Heidenhein's haematoxylin and Fast Green and mounted in DPX.

5.2.5 Recovery from defoliation

This experiment ran from October 1993-January 1994. Methods follow those outlined by Bossard & Hillier (1993). After transferral to 13 cm diameter pots, the plants were arranged into five blocks, 12 pots in each (two from each species) in a random block design. Plants were watered as standard. After 31 days, half of the plants (one of each species in each block) were cut with scissors, so that approximately 3/4 of the shoot was removed. The plants were left to recover for 35 days before the final harvest.

The following variables were measured; number of leaves, stem length (mean of five), shoot dry weight, root and nodule dry weight (combined).

5.2.6 Adventitious rooting of cuttings

The ability of the six legume species to develop adventitious roots after cutting was examined, following methods outlined by Hodgson *et al.* (1993). Mature shoots (seven to ten of each species) were collected from the field on two occasions at the Irish sites (19/7/94 n=7 and 4/5/95 n=10) and at Ring Bog (28/7/94 n=7, 7/6/95 n=10). Shoots from greenhouse grown plants (after 12 weeks growth) were also harvested (6/1/95 n=10).

Shoot apices with three leaf nodes and well developed leaves were removed from each shoot (with the exception of *T. pratense* and *T. repens* in which removal of an apical cutting was impossible and a whole leaf rosette was therefore used with the root removed). The cuttings were then floated in individual shallow dishes containing 200 ml tap water. The water level was topped up daily and changed every three days. The cuttings were examined for root growth every two days for up to 40 days. Once rooting was observed the cutting was discarded.

All the treatments described above are summarised below in Table 5.2.

Table 5.2 Summary of treatments applied

TREATMENT NAME (AND EXPERIMENT No.)	TREATMENT (brief description)	APPLIED TO:	LENGTH OF TIME APPLIED
SHORT TERM FLOODING (5.2.2)	water level 1 cm above substrate level	6 legume species (12 week old plants)	20 days
LIMITED WATER SUPPLY (5.2.2)	watered every 4 days	6 legume species (12 week old plants)	20 days
SHORT TERM FLOODING WITH RECOVERY PERIOD (5.2.3)	water level 1 cm above substrate level followed by recovery period	4 legume species (2 week old seedlings)	3 weeks of flooded followed by 3 weeks of recovery
LONG TERM FLOODING (5.2.4)	water level 1 cm above substrate level	*Lotus pedunculatus* plants from the field and grown on for 7 weeks	5 weeks
RECOVERY FROM DEFOLIATION (5.2.5)	removal of 3/4 of shoot followed by recovery period	6 legume species (46 day old plants)	recovery period of 35 days
ADVENTITIOUS ROOTING OF CUTTINGS (5.2.6)	shoot apices with three leaf nodes removed and floated in tap water	6 legume species (field and greenhouse plants)	40 days

5.2.7 Data Analysis

Two way analysis of variance (ANOVA) was conducted on experiments 5.2.2 and 5.3.5 using the UNISTAT statistical program (version 4). Each species was analysed separately with treatments and blocks as factors. The blocks were not a significant source of variation in either of these experiments. Where the ANOVA showed significance, Tukey's Honestly Significant Difference (HSD) values were calculated for experiment 5.2.2 using the standard error of difference of the mean, $p=0.05$. A one way ANOVA was conducted on 5.3.3 and 5.3.4 using MINITAB (version 9.2). The data are presented in the form of bar charts, with standard error bars. It should be noted that in Fig. 5.1 (a-i) between treatment HSD bars are positioned above the species to which they apply.

5.3 RESULTS

5.3.1 Rhizobia isolation

a) Morphology and colonal characteristics

All isolates were motile rods, between 1-3 μm long. None took up congo red. Very little growth was noted for all six isolates on peptone glucose agar after two days at 30°C.

The isolates from *Trifolium*, *Vicia* and *Lathyrus* all produced colonies approximately 2-4 mm in diameter after five days (on yeast mannitol agar at 26°C). Colonies were circular, raised and white. A large amount of white watery slime was produced. The isolates from *Trifolium*, *Vicia* and *Lathyrus* all acidified the medium bromothymol blue.

The isolate from *Lotus* produced little growth after this time. After 10 days, colonies were approximately 2-3 mm in diameter, they too were circular and white, but slightly more raised than the other isolates. A dense sticky gum was produced. The isolate from *Lotus* did not change the colour of bromothymol blue.

b) Infection test

All isolates successfully nodulated seedlings of the host plant species in several trials (the controls remained non-nodulated).

5.3.2 Effects of short term flooding and limited water supply

The effects of short term flooding and limited water supply are shown in Fig. 5.1. ('dry' denotes the limited water supply treatment, 'standard' is the control and 'flooded' is the short term flooding treatment).

Whole plant

The total biomass response to short term flooding and limited water supply is shown in Fig. 5.1 (a). Both the treatments significantly reduced the total biomass of *T. pratense* and *T. dubium* (Table 5.3).

The effect of short term flooding and limited water supply on the above:below ground biomass ratio is shown in (Fig. 5.1 (b)). The limited water supply significantly reduced the ratio for *T. dubium* and *V. cracca* (Table 5.3). The flooding treatment significantly increased the ratio for *T. dubium* and *T. pratense* (Table 5.3).

Significant differences between limited water supply and flooding treatments were noted in *L. pedunculatus* and *T. repens* (Table 5.3), but not with respect to the control.

Shoot

Yellowing of the shoots occurred in flooded plants of *T. dubium* and *V. cracca*, and in flooded and plants with limited water supply of *T. repens* and *T. pratense*. Shoots of *L. pedunculatus* and *L. pratensis* remained green in each watering regime. Both species exhibited hypertrophy of the stem base following the flood treatment. The effect of short term flooding and limited water supply on shoot biomass is shown in Fig. 5.1 (c). The limited water and flooded conditions suppressed shoot biomass accumulation in *T. dubium* while in *T. pratense* the limited water supply reduced shoot biomass (Table 5. 3).

Root

The effect of short term flooding and limited water supply on root biomass is shown in Fig. 5.1 (d). The root biomass of *T. dubium*, *T. pratense* and *T. repens* was significantly reduced by the flooding treatment (Table 5.3). Flooded plants of *T. repens and L. pedunculatus* developed adventitious roots. There was a significant difference between the flooded and limited water treatment in root biomass in *V. cracca* (Table 5.3).

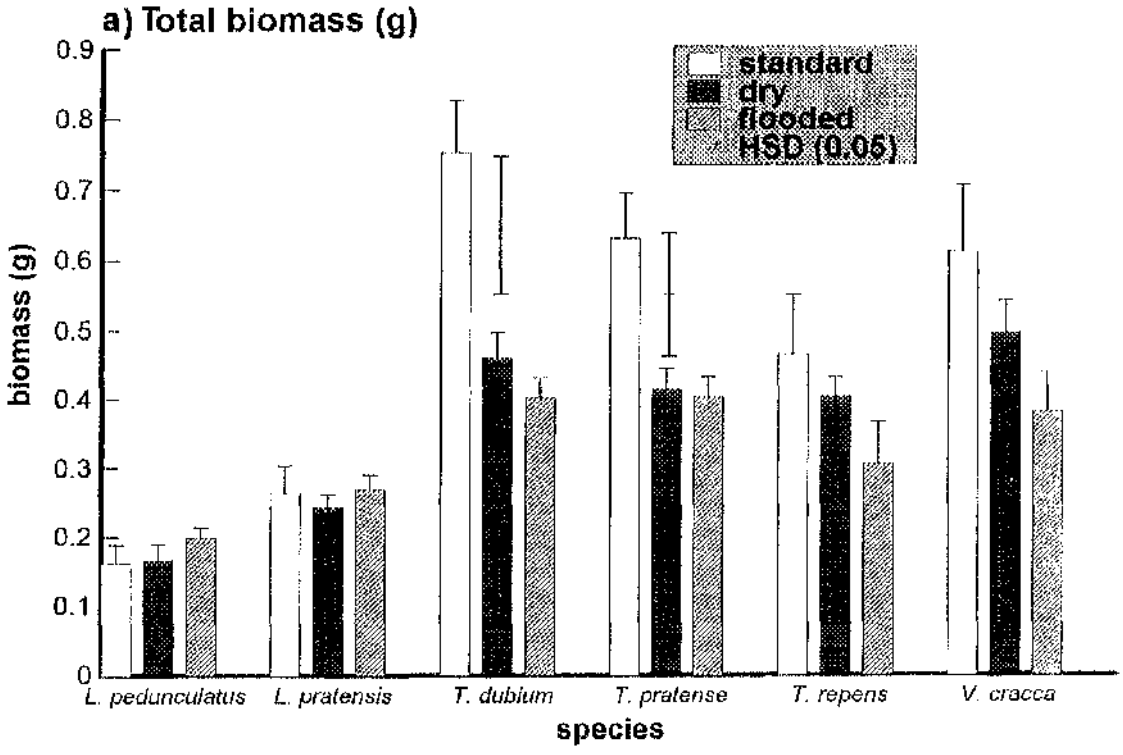
Nodule

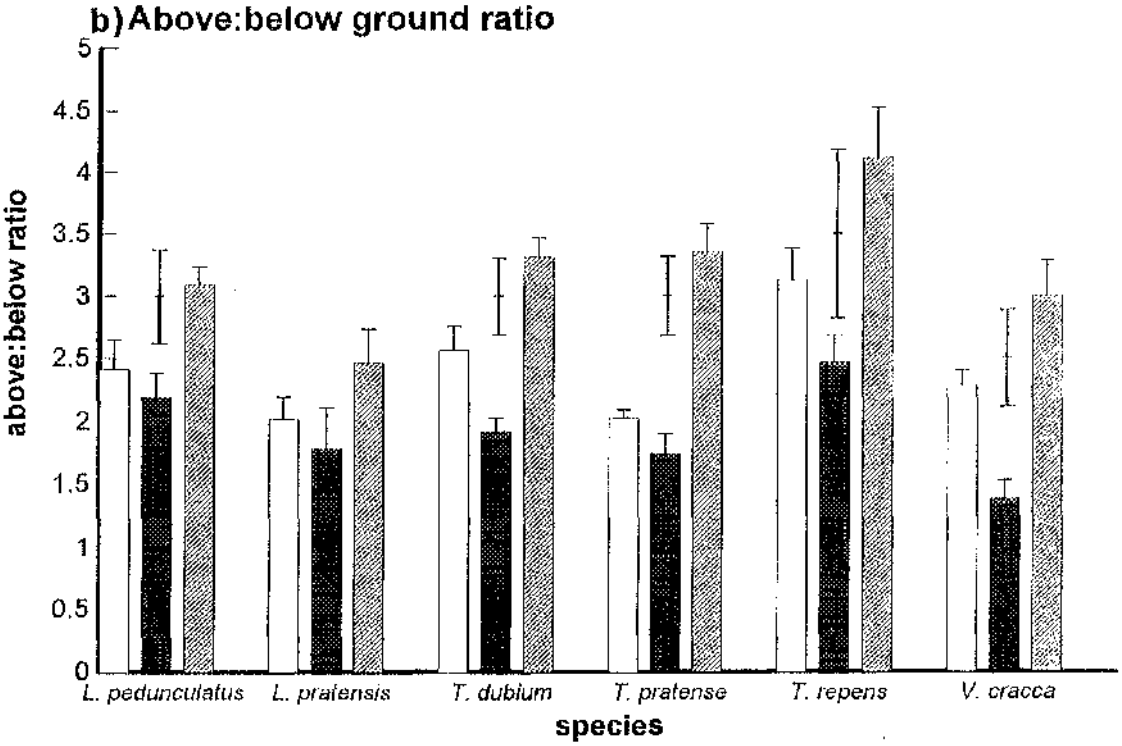
Nodule colour in all species, with the exception of *L. pedunculatus* was affected by the watering treatments. The nodules in *L. pedunculatus* remained pink with a white lenticel outgrowth, although the lenticel outgrowth was more pronounced in flooded nodules. In the other species the nodules of the standard watered plants were pink, those receiving the dry treatment were grey/green and those flooded were green, although in *T. repens* and *L. pratensis* the tips of the nodules were pink. The effect of short term flooding and limited water supply on nodule biomass is shown on Fig. 5.1 (e). Nodule biomass in *T. pratense*, *T. repens* and *V. cracca* was suppressed by the flooding treatment (Table 5.3). The limited water treatment reduced nodule biomass in *T. dubium*, *T. pratense* and *V. cracca* (Table 5.3). The effect of short term flooding and limited water supply on nodule number is shown on Fig. 5.1 (f). The limited water treatment reduced the number of nodules of *T. dubium*, the flooding treatment had a similar reducing effect on nodule number in *V. cracca* (Table 5.3).

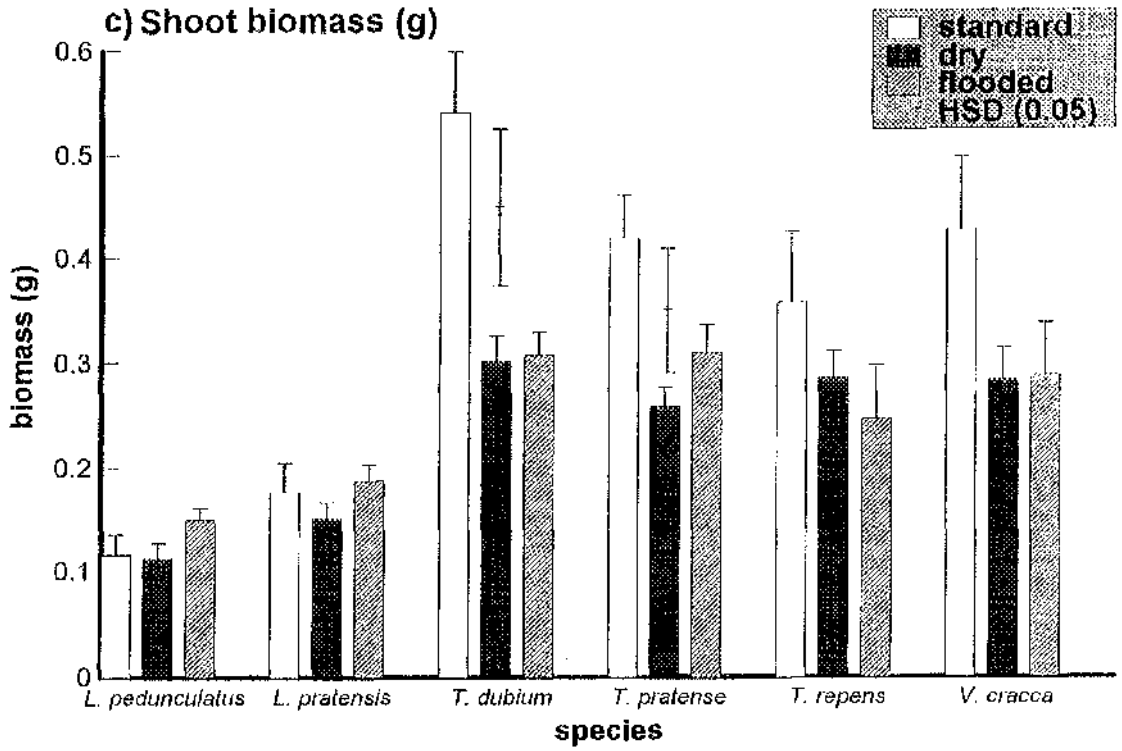
Nitrogen Fixation

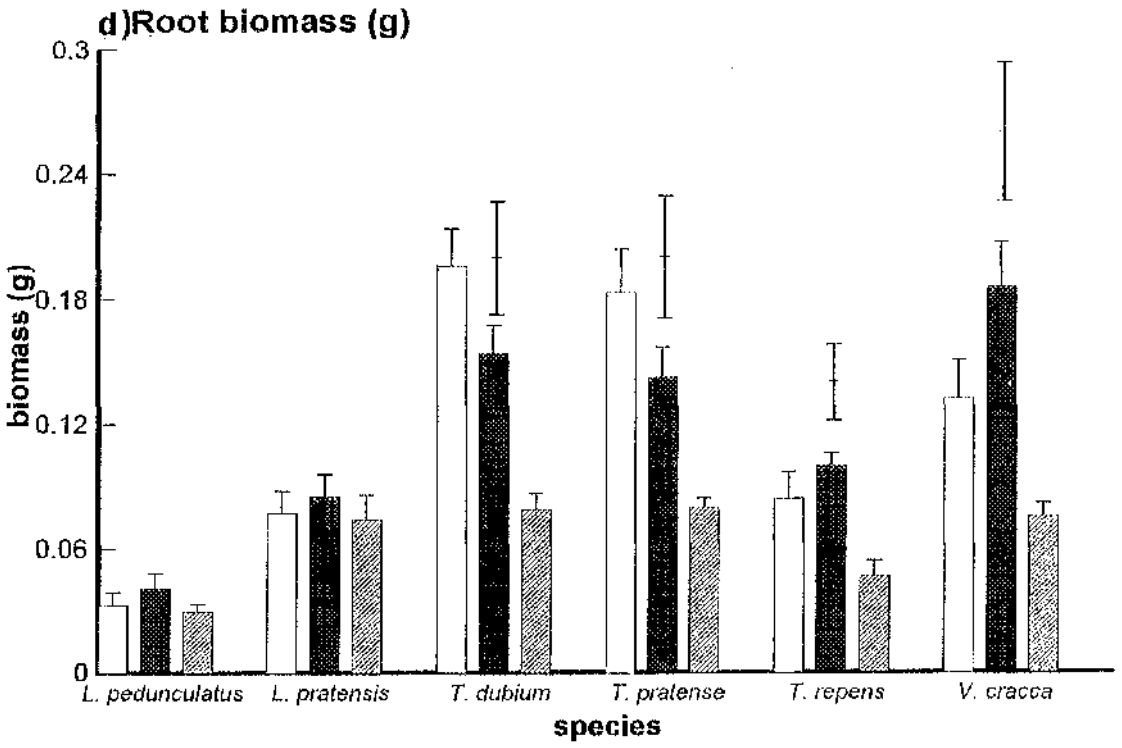
The effect of short term flooding and limited water supply on % nitrogen (% dry weight) is shown in Fig. 5.1 (g). Both the limited water and flooding treatments reduced the % nitrogen of *T. dubium* and *T. pratense* (Table 5.3). The % nitrogen of *L. pratensis* was reduced by the limited water treatment only (Table 5.3). The effect of short term flooding and limited water supply on nodule effectivity (g nitrogen fixed g dry weight nodule^{-1}) is shown in Fig. 5.1 (h). This was increased by the limited water treatment in both *T. dubium* and *V. cracca* and reduced by the flooding treatment in *T. dubium* (Table 5.3). The effect of short term flooding and limited water supply on plant nitrogen content (g) is shown in Fig. 5.1 (i). This was reduced by both the limited water and flooding treatments in *T. dubium*, *T. pratense* and *V. cracca* (Table 5.3).

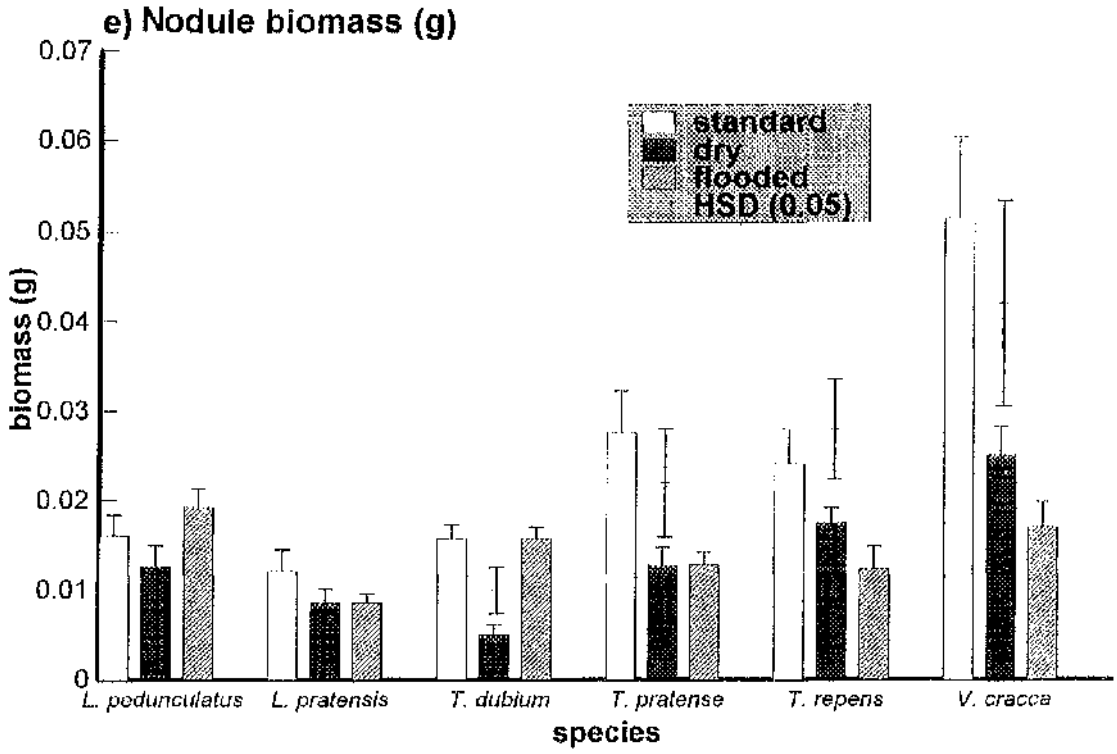
Fig. 5.1 The effects of short term flooding and limited water supply (mean values/plant) (n=8) with standard error bars and HSD bars (calculated where ANOVA outcomes of $p<0.05$ were obtained)

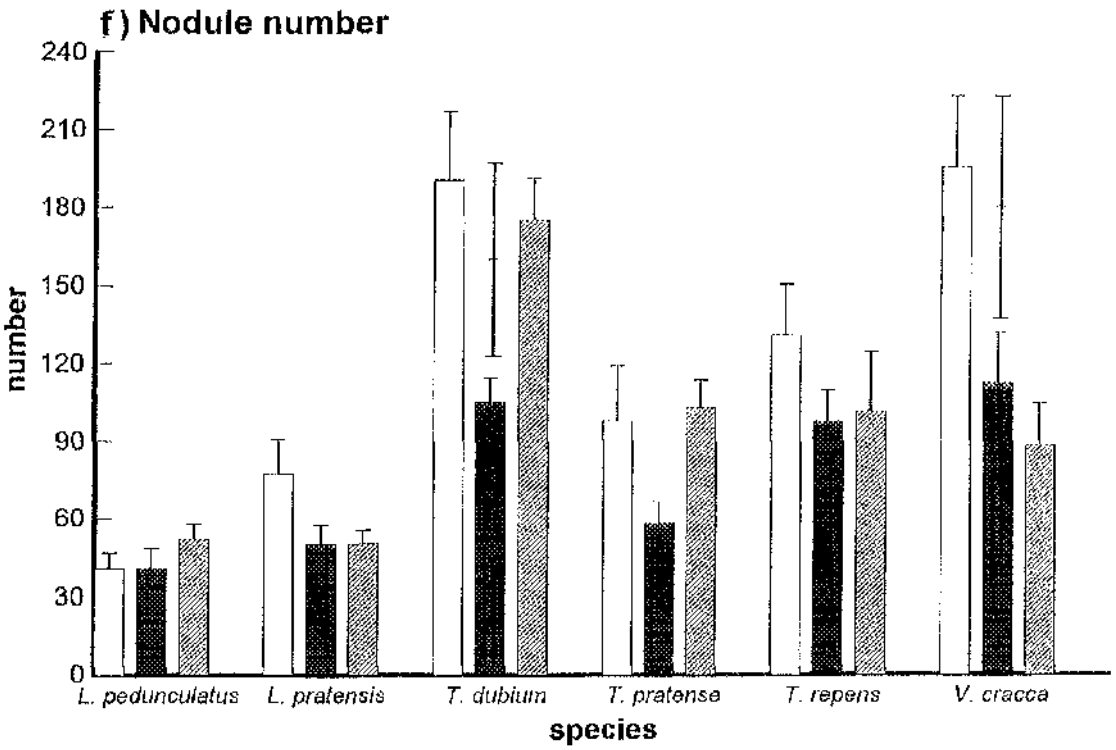


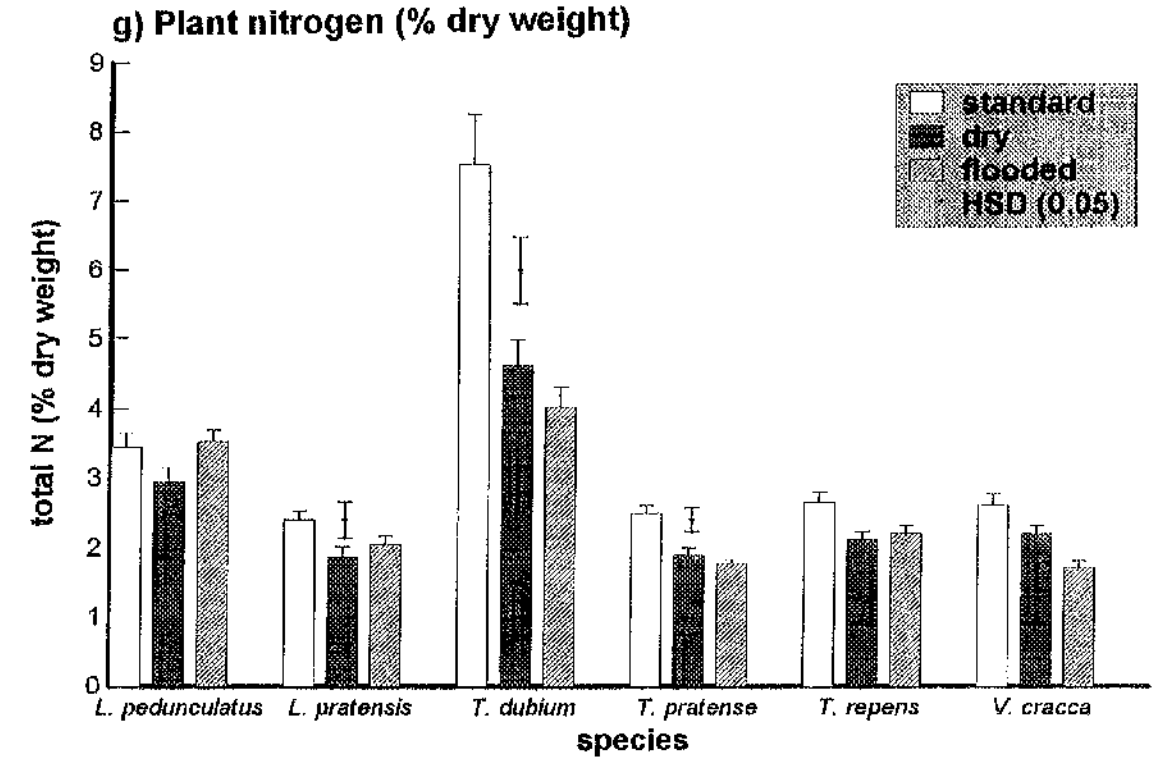


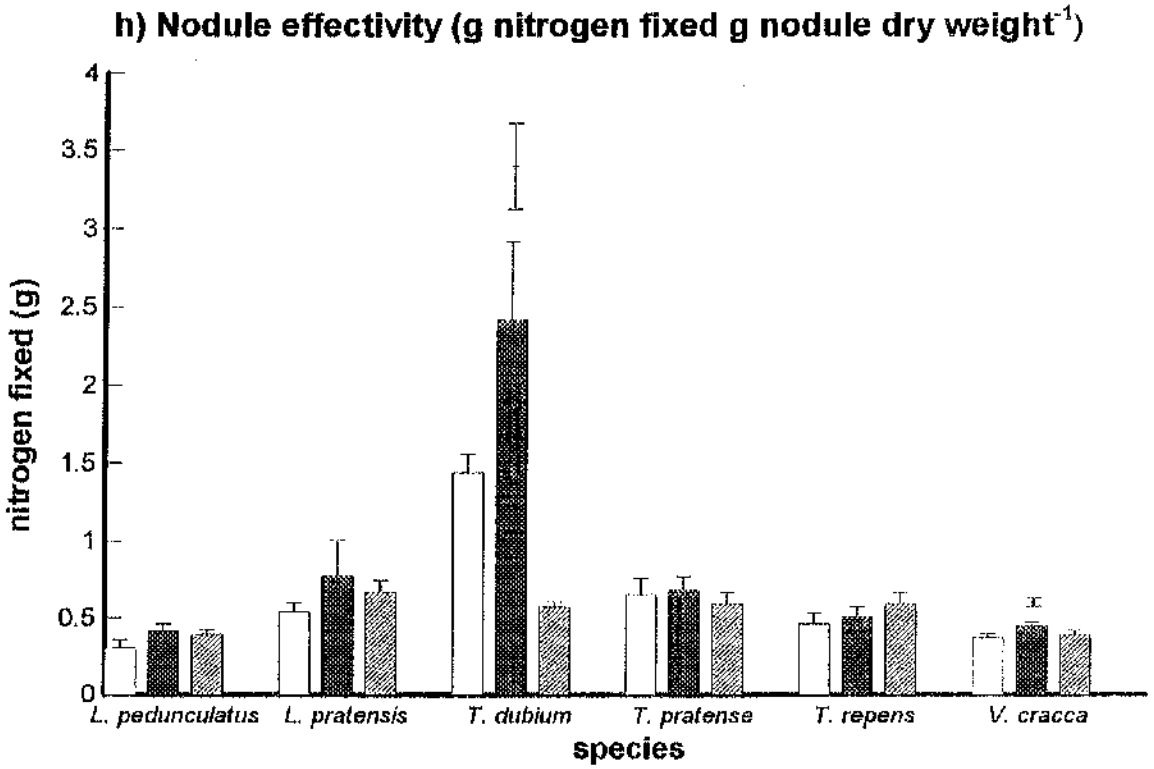

c) Shoot biomass (g)
standard
dry
flooded
HSD (0.05)
biomass (g)
0.6
0.5
0.4
0.3
0.2
0.1
0
L. pedunculatus
L. pratensis
T. dubium
T. pratense
T. repens
V. cracca
species


d)Root biomass (g)
biomass (g)
0.3
0.24
0.18
0.12
0.06
0
L. pedunculatus
L. pratensis
T. dubium
T. pratense
T. repens
V. cracca
species


e) Nodule biomass (g)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
biomass (g)
standard
dry
flooded
HSD (0.05)
L. pedunculatus
L. pratensis
T. dubium
T. pratense
T. repens
V. cracca
species


f) Nodule number
240
210
180
150
120
90
60
30
0
number
L. pedunculatus
L. pratensis
T. dubium
T. pratense
T. repens
V. cracca
species


g) Plant nitrogen (% dry weight)
standard
dry
flooded
HSD (0.05)
total N (% dry weight)
0
1
2
3
4
5
6
7
8
9
L. pedunculatus
L. pratensis
T. dubium
T. pratense
T. repens
V. cracca
species


h) Nodule effectivity (g nitrogen fixed g nodule dry weight-1)
nitrogen fixed (g)
0
0.5
1
1.5
2
2.5
3
3.5
4
L. pedunculatus
L. pratensis
T. dubium
T. pratense
T. repens
V. cracca
species

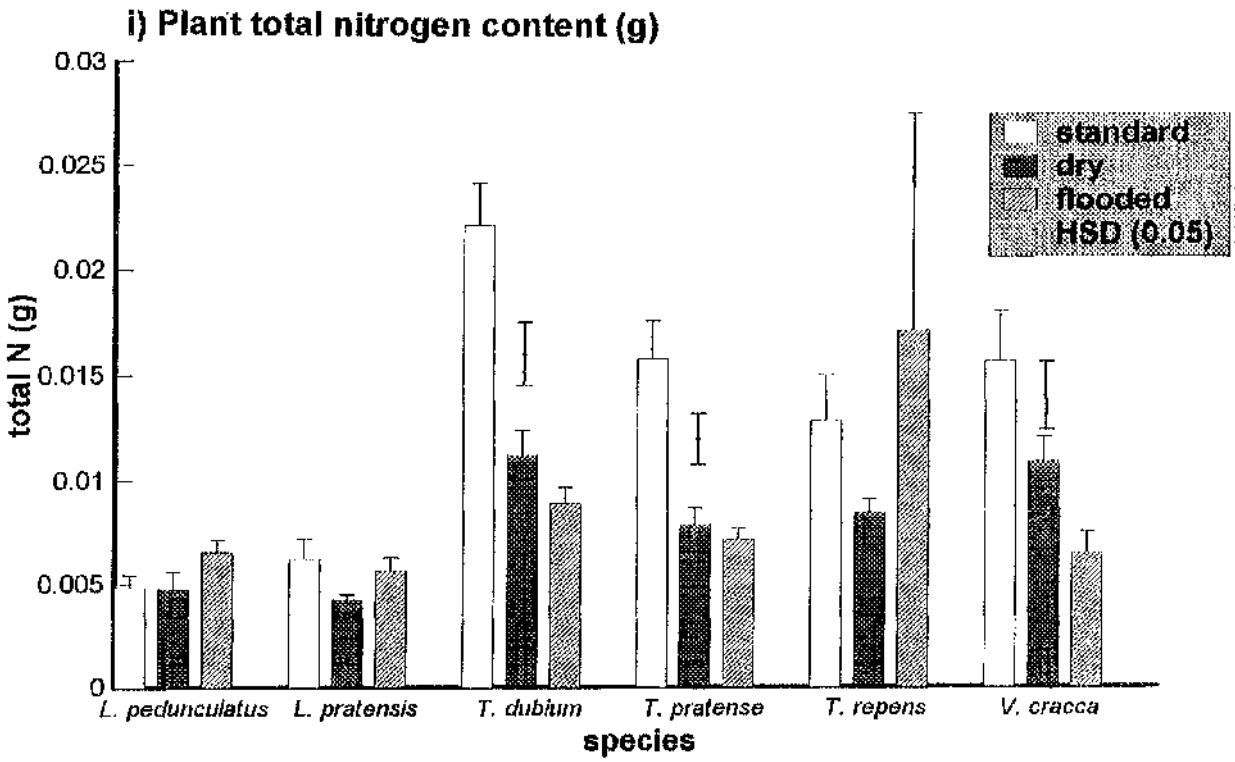

i) Plant total nitrogen content (g)
0.03
0.025
0.02
0.015
0.01
0.005
0
total N (g)
standard
dry
flooded
HSD (0.05)
L. pedunculatus
L. pratensis
T. dubium
T. pratense
T. repens
V. cracca
species

Table 5.3 Summary of the effects of water stress (short term flooding and limited water supply) (ANOVA).

Levels of significance are as follows NS >0.05, * p < 0.05, ** p < 0.01, *** p <0.005

Species	Shoot biomass	Root biomass	Nodule biomass	Total biomass	Number of nodules	Above : below	Nitrogen (%)	Nodule effectivity	Nitrogen content (g)
L. pedunculatus	NS	NS	NS	NS	NS	** B (f)	NS	NS	NS
L. pratensis	NS	NS	NS	NS	NS	NS	* d-	NS	NS
T. dubium	** f- d-	*** f-	*** d-	*** f- d-	* d-	*** f+ d-	*** f- d-	*** f- d+	*** f- d-
T. pratense	** d-	*** f-	** f- d-	** f- d-	NS	*** f+	*** f- d-	NS	*** f- d-
T. repens	NS	** f-	* f-	NS	NS	** B (f)	NS	NS	NS
V. cracca	NS	*** B(d)	** f- d-	NS	* f-	*** d-	NS	** d-	*** f- d-

biomass = g

nodule effectvity = nitrogen fixed (g)/nodule dry weight (g)

Nitrogen (%)= N % dry weight

f = flooding treatment d = dry treatment

- = negative effect of treatment compared to the control (standard)

+ = positive effect of treatment compared to the control (standard)

B = differences between dry and flooding treatment only (not significantly different from the control (standard))

(d = dry greater than flooded f= flooded greater than dry)

5.3.4 Effects of short term flooding with recovery period on seedlings

Whole Plant

The effect of the flood/recovery treatment on seedling total biomass is shown in Fig. 5.2 (a). The treatment resulted in a significant decrease in the total biomass of *T. pratense* seedlings (Table 5.4). The above: below ground ratio is shown in Fig. 5.2 (b). The treatment did not affect the above:below ground ratio in any species (Table 5.4).

Shoot, root and leaf

Yellowing of shoots, production of adventitious roots and hypertrophy were not evident at the end of the experimental period. The effect of the flood/recovery treatment on seedling shoot and root biomass is shown in Fig. 5.2 (c) and (d) respectively. Only the root and shoot biomass of *T. pratense* was significantly reduced (Table 5.4). Seedling leaf number (Fig. 5.2 (e)) was unaffected by the flood/recovery treatment. The effect of the flood/recovery treatment on seedling leaf area is shown in Fig. 5.2 (f). This was significantly reduced by the treatment in *T. pratense* seedlings, but unaffected in the other species (Table 5.4).

Nodule

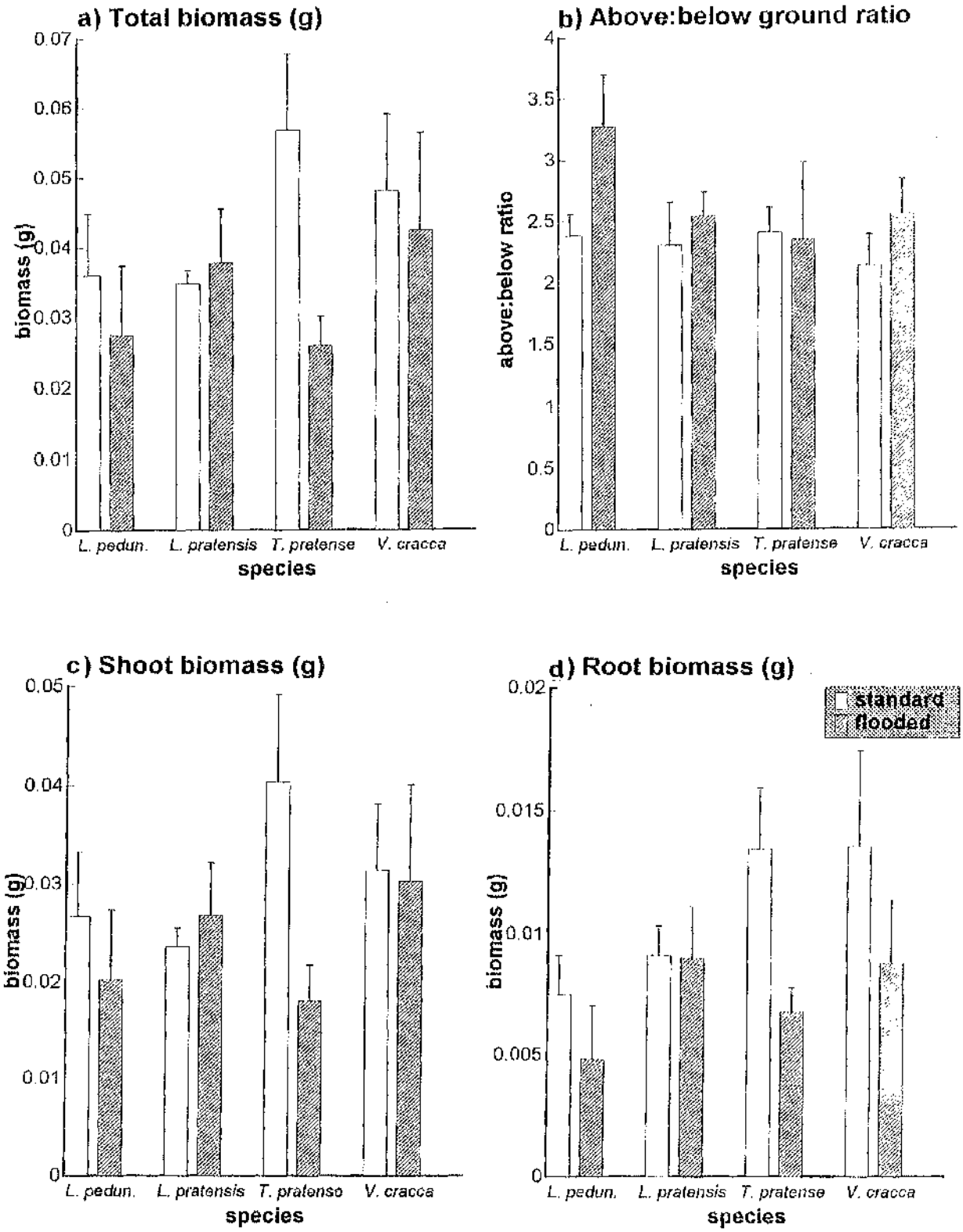
Nodules were pink for standard and flooded plants. The effect of the flood/recovery treatment on seedling nodule biomass and number is shown in Fig. 5.2 (g) and (h) respectively. Only the nodule biomass of *T. pratense* was significantly reduced (Table 5.4).

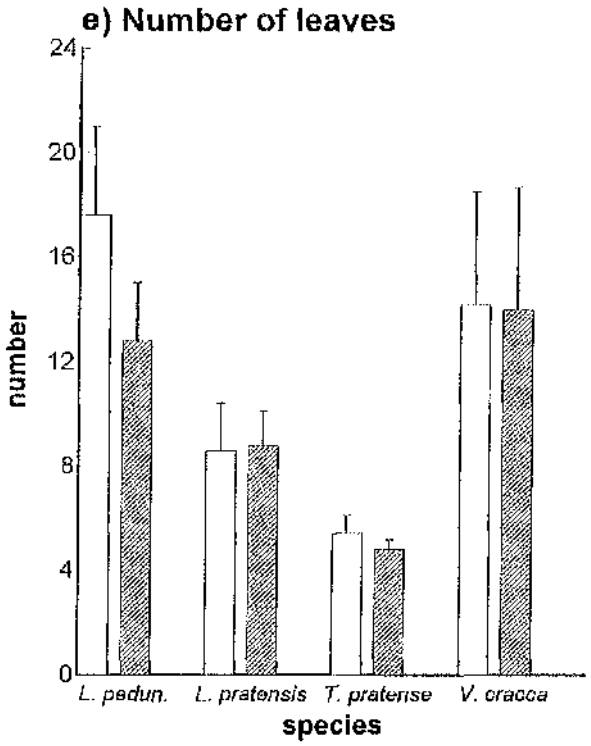
Table 5.4 Summary of the effects of flooding with recovery period on seedlings (ANOVA). Levels of significance are as follows NS >0.05, * p < 0.05.

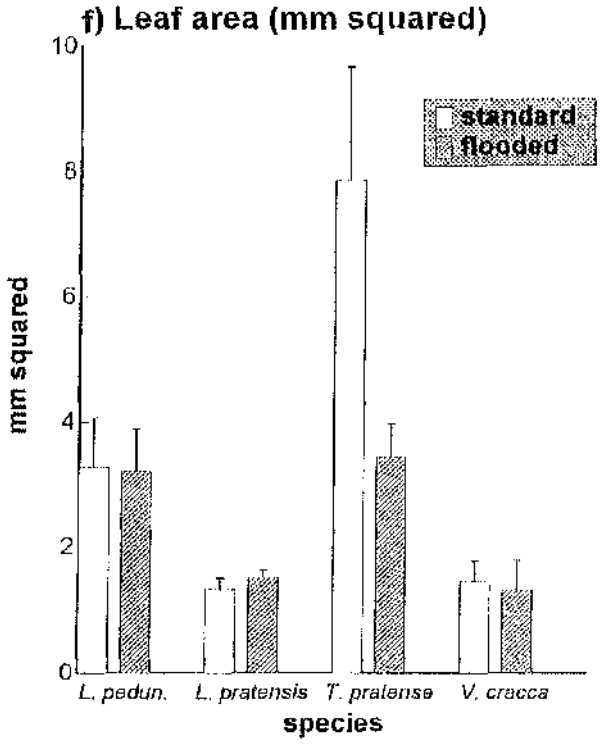
	L. pedunculatus	*L. pratensis*	*T. pratense*	*V. cracca*
Total biomass (g)	NS	NS	* -	NS
Above:below ratio	NS	NS	NS	NS
Shoot biomass (g)	NS	NS	* -	NS
Root biomass (g)	NS	NS	* -	NS
Number of leaves	NS	NS	NS	NS
Leaf area (mm^2)	NS	NS	* -	NS
Nodule biomass (g)	NS	NS	* -	NS
Number of nodules	NS	NS	NS	NS

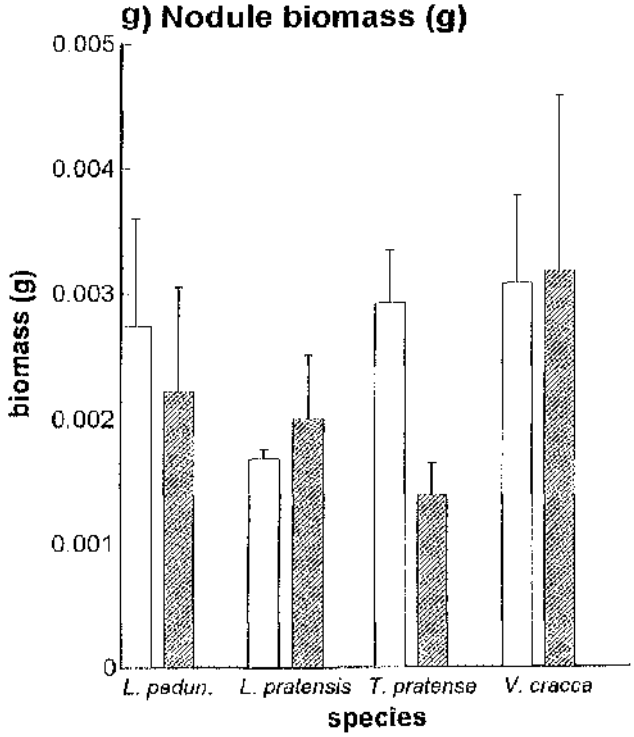
(- = negative effect)

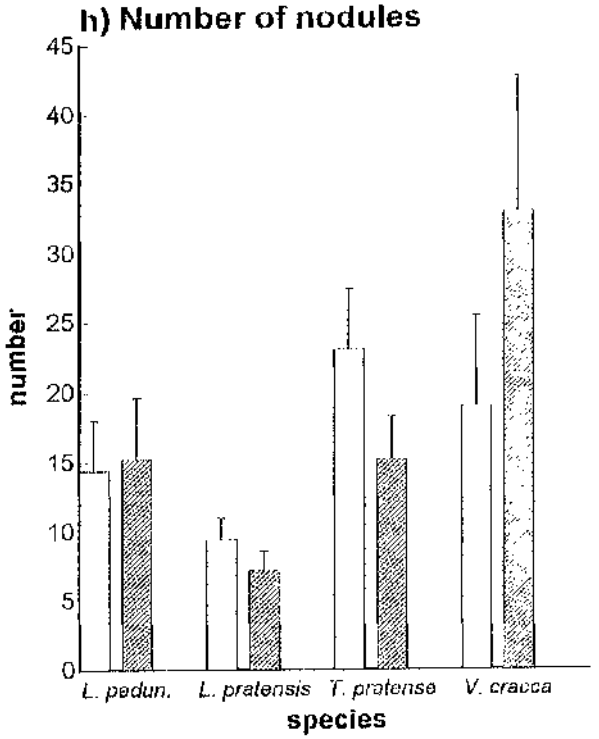
Fig. 5.2 The effects of short term flooding with recovery period on seedlings (mean values/plant) (n=5) with standard error bars




e) Number of leaves
number
24
20
16
12
8
4
0
L. pedun.
L. pratensis
T. pratense
V. cracca
species


f) Leaf area (mm squared)
mm squared
10
8
6
4
2
0
standard
flooded
L. pedun.
L. pratensis
T. pratense
V. cracca
species


g) Nodule biomass (g)
biomass (g)
0.005
0.004
0.003
0.002
0.001
0
L. pedun.
L. pratensis
T. pratense
V. cracca
species


h) Number of nodules
number
45
40
35
30
25
20
15
10
5
0
L. pedun.
L. pratensis
T. pratense
V. cracca
species

5.3.5 Effects of long term flooding on *Lotus pedunculatus*.

Whole plant

The effect of long term flooding on total biomass of *L. pedunculatus* is shown in Fig. 5.3 (a). The long term flooding had a significant reducing effect (Table 5.5) on shoot (and hence total) biomass. The above:below ground ratio (Fig. 5.3 (b)) was unaffected by prolonged flooding (Table 5.5). Flowering was adversely affected by long term flooding (Fig 5.3 (c)), since only the standard watered plants produced flowers.

Shoot and root

The long term flooded plants showed yellowing of the shoots, hypertrophy of the stem and adventitious rooting. The effect of long term flooding on the root and shoot biomass of *L. pedunculatus* is shown in Fig. 5.3 (a). Only the shoot biomass was significantly reduced (Table 5.5).

Nodule

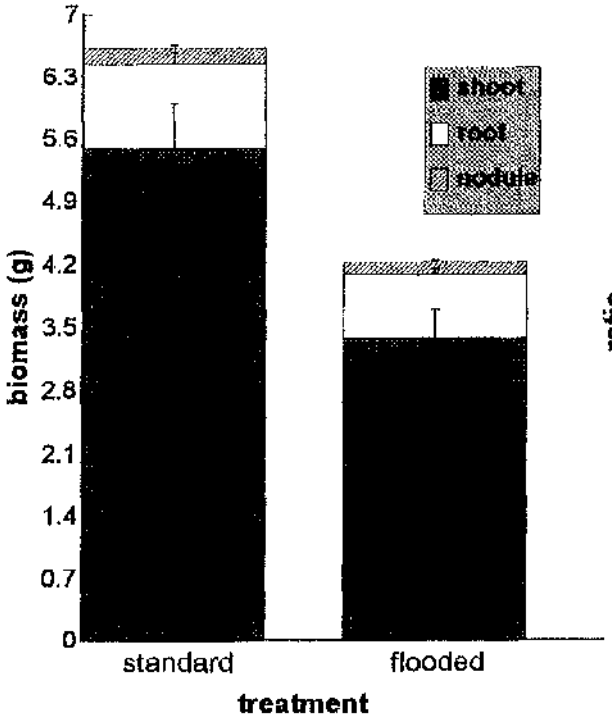
Nodule colour was unaffected by long term flooding of *L. pedunculatus*, (pink with white lenticel) but the lenticel outgrowths increased in the flooded nodules. The effect of long term flooding on nodule biomass and number is shown in Figs. 5.3 (a) and (d). Only nodule number was reduced significantly (Table 5.5). The cortex area as percentage of nodule transverse area was not significantly affected by the prolonged flooding treatment (Fig. 5.3 (e) and Table 5.5).

Nitrogen fixation

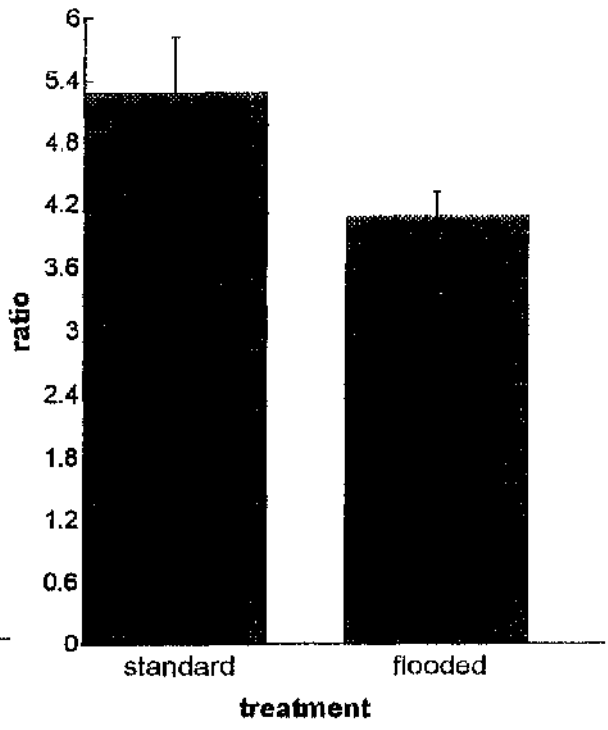
There was no significant effect of long term flooding on nitrogenase activity in *L. pedunculatus* (Fig. 5.3 (f), Table 5.5). The % nitrogen (Fig. 5.3 (g)) and nodule effectivity (g nitrogen fixed g nodule dry weight^{-1}) (Fig. 5.3 (h)) were also not significantly affected by long term flooding (Table 5.5). However, the total nitrogen content (g) (Fig. 5.3 (i)) was reduced by long term flooding (Table 5.5).

Fig. 5.3 The effects of long term flooding on *Lotus pedunculatus* (mean values/plant) (n=9) with standard error bars

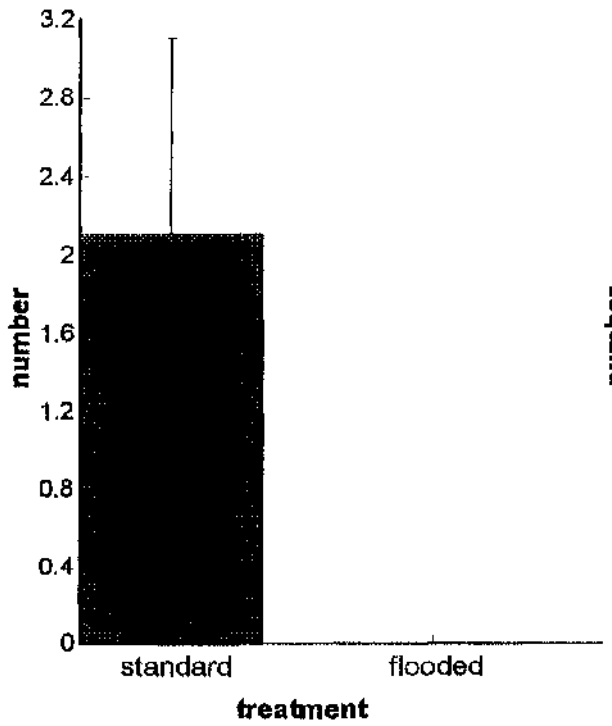
a) Total biomass of plant parts (g)



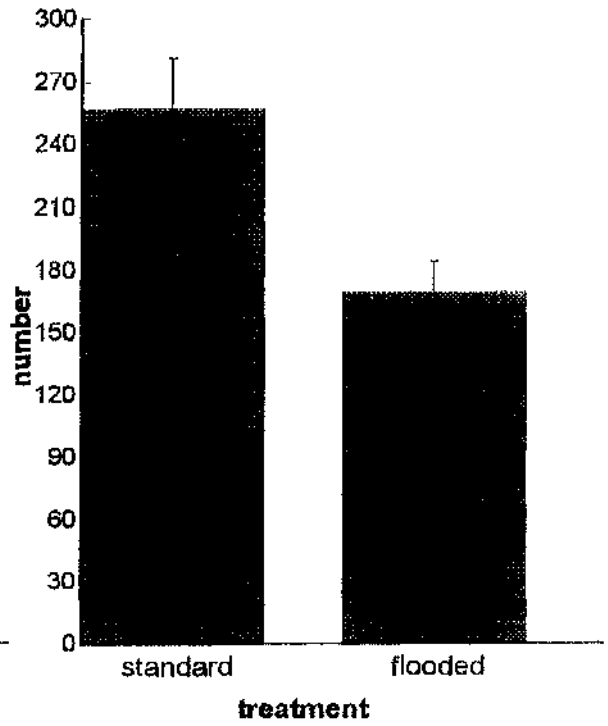
b) Above:below ground ratio



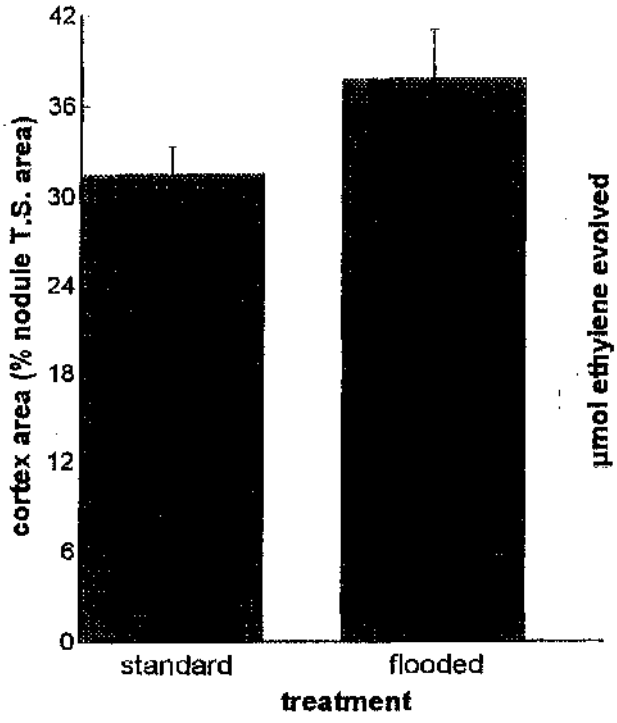
c) Number of flowers



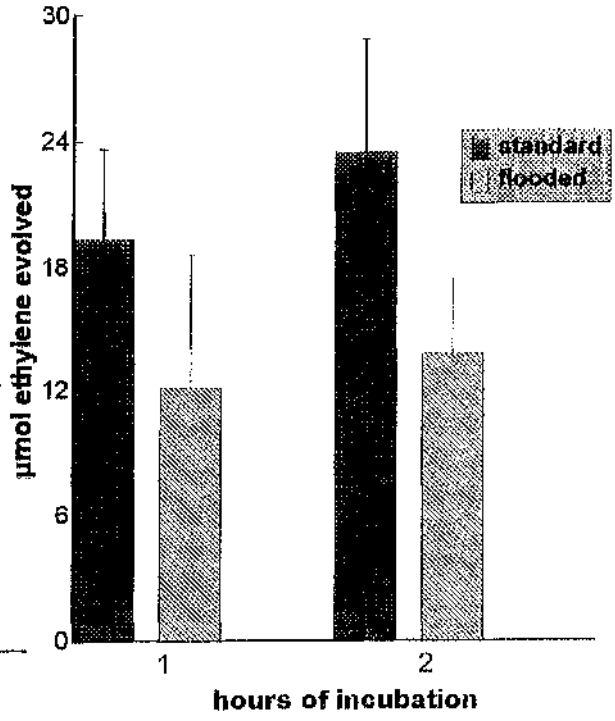
d) Number of nodules



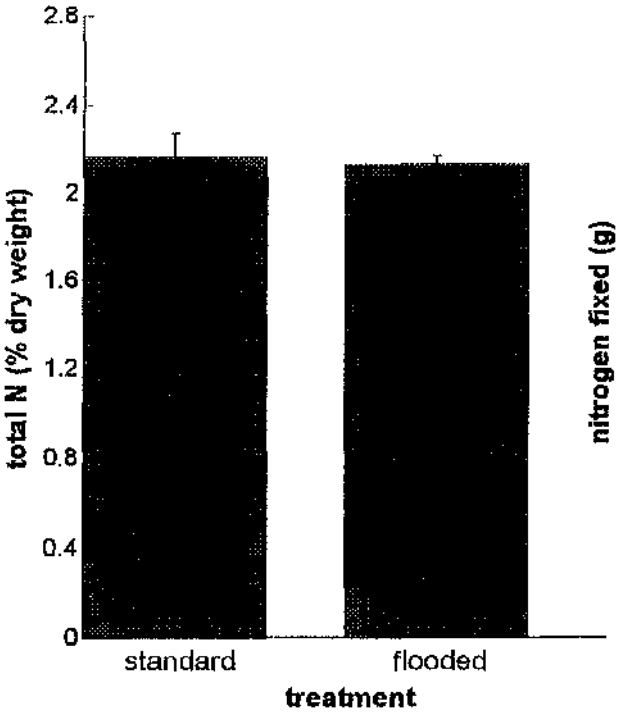
e) Cortex area (% nodule transverse sectional area)



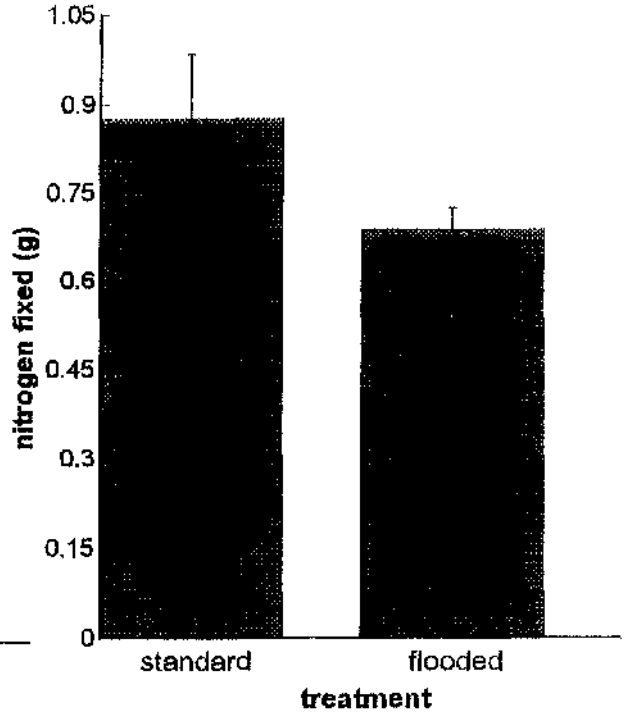
f) Nitrogenase activity (µmol ethylene evolved g^{-1} nodule dry weight h^{-1}) (n=5)



g) Plant nitrogen (% dry weight)



h) Nodule effectivity (g nitrogen fixed g nodule dry weight^{-1})



i) Plant total nitrogen content (g)

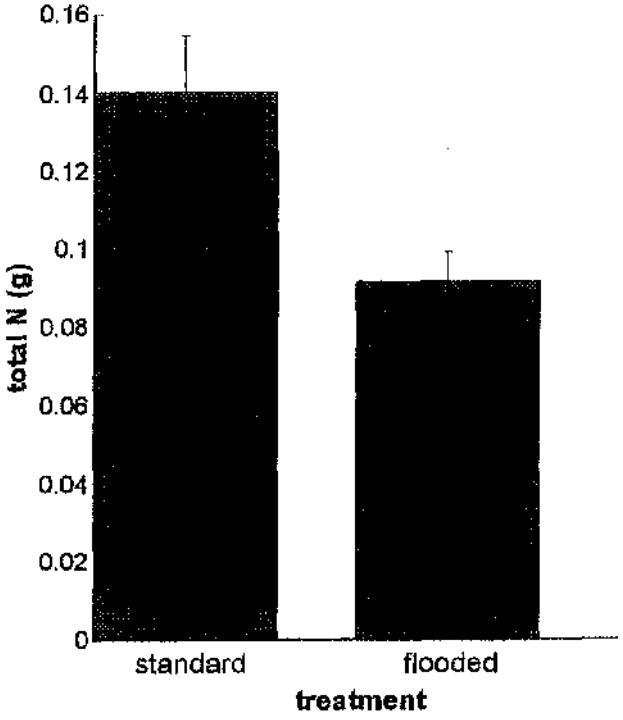


Table 5.5 Summary of the effects of long term flooding on *L. pedunculatus* (ANOVA).

Levels of significance are as follows NS >0.05, * p <0.05, ** p < 0.01.

Variable	Significance
Total biomass (g)	** -
Shoot biomass (g)	** -
Root biomass (g)	NS
Nodule biomass (g)	NS
Above:below ground ratio	NS
Number of flowers	* -
Number of nodules	** -
Cortex area	NS
ARA 1 hour	NS
ARA 2 hour	NS
Plant nitrogen (%)	NS
Nodule effectivity	NS
Total nitrogen content (g)	** -

ARA 1 hour = µmol ethylene evolved g nodule dry weight^{-1} hour^{-1} after 1 hour of incubation

ARA 2 hour = µmol ethylene evolved g nodule dry weight^{-1} hour^{-1} after 2 hours of incubation

Cortex area = cortex area as a percentage of nodule transverse sectional area

Plant nitrogen (%) = % dry weight

Nodule effectivity = g nitrogen fixed g nodule dry weight^{-1}

- = negative effect

5.3.5 Recovery from defoliation

Plants were harvested 35 days after defoliation.

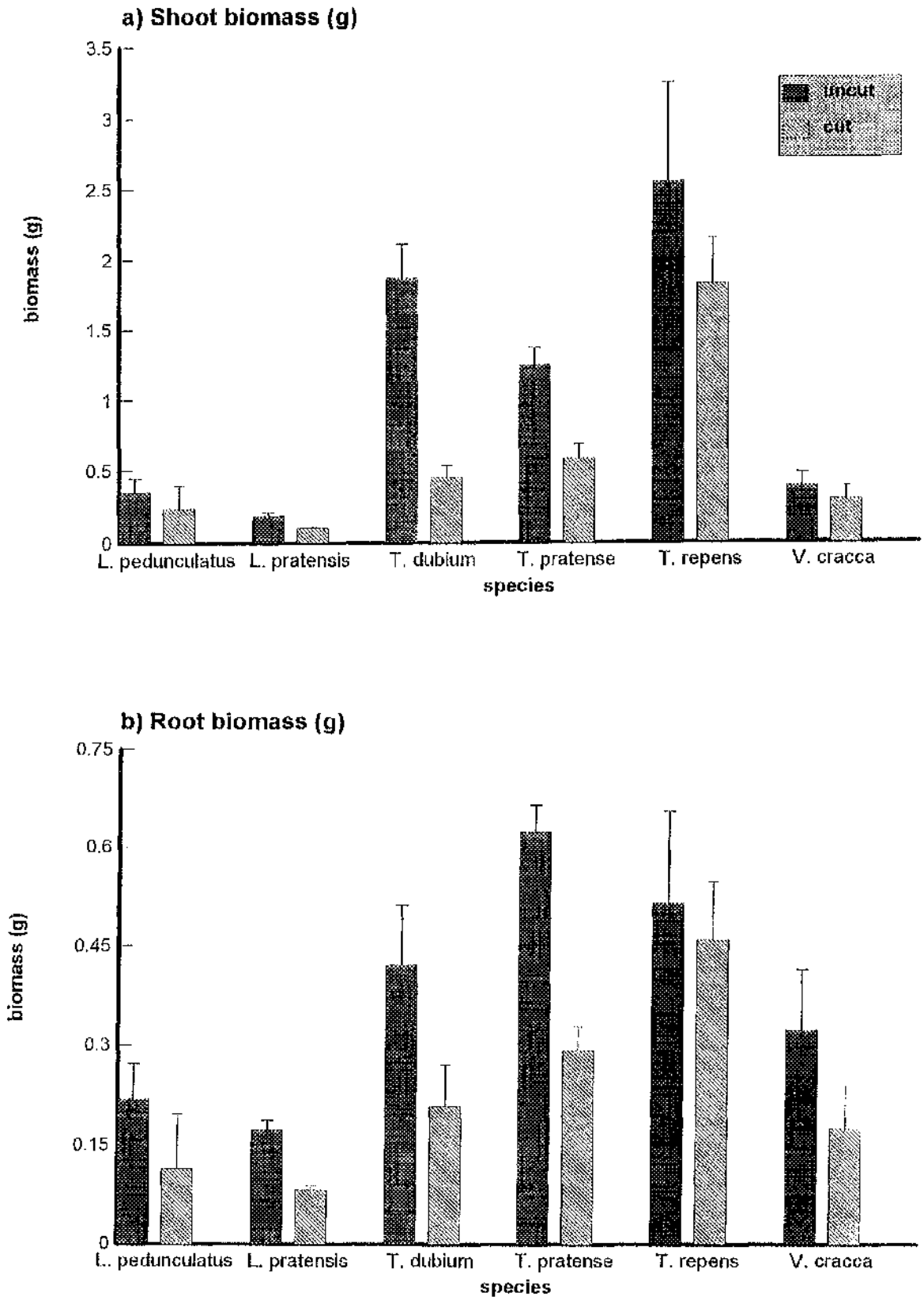
Biomass accumulation

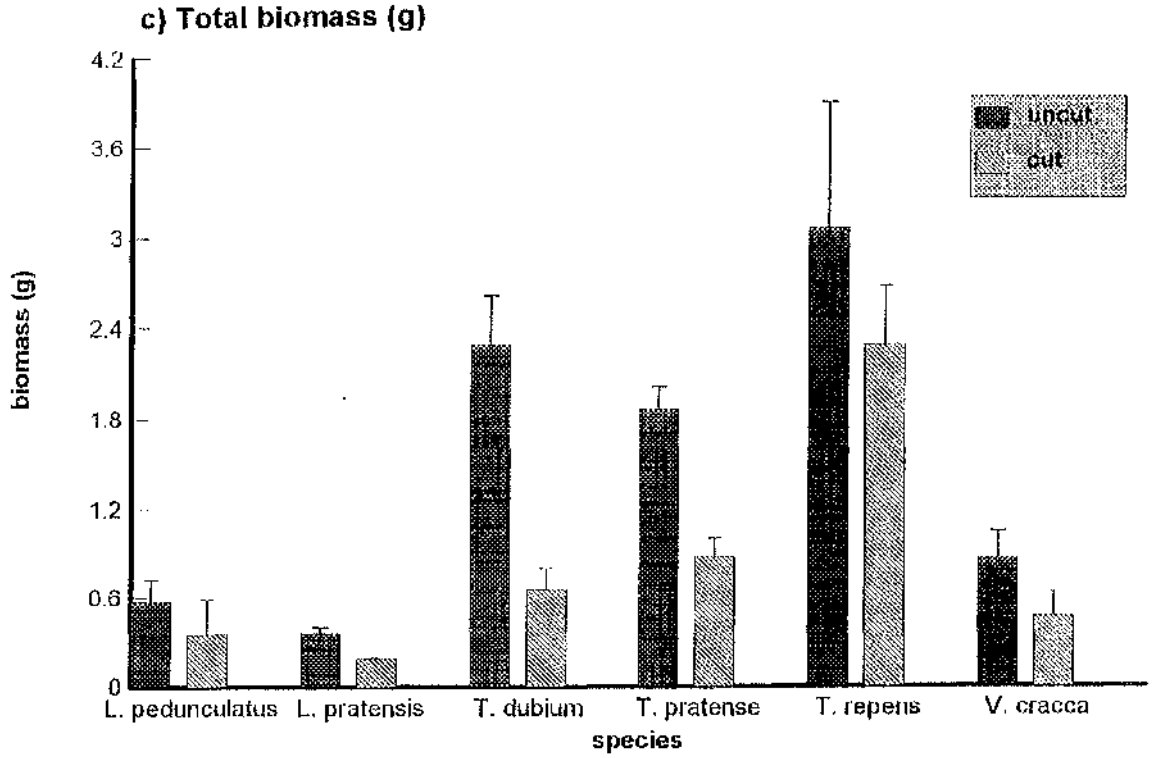
The effect of defoliation on shoot biomass is shown in Fig. 5.4 (a). The shoot biomass of defoliated plants had not recovered to that of the controls in half the species; *L. pratensis*, *T. dubium* and *T. pratense* (Table 5.6). Root (and nodule) biomass of defoliated *L. pratensis* and *T. pratense* (Fig. 5.4 (b)) had not recovered to that of the controls (Table 5.6). The total biomass (Fig. 5.4 (c)) of defoliated *L. pratensis*, *T. dubium* and *T. pratense* did not recover to that of the controls (Table 5.6).

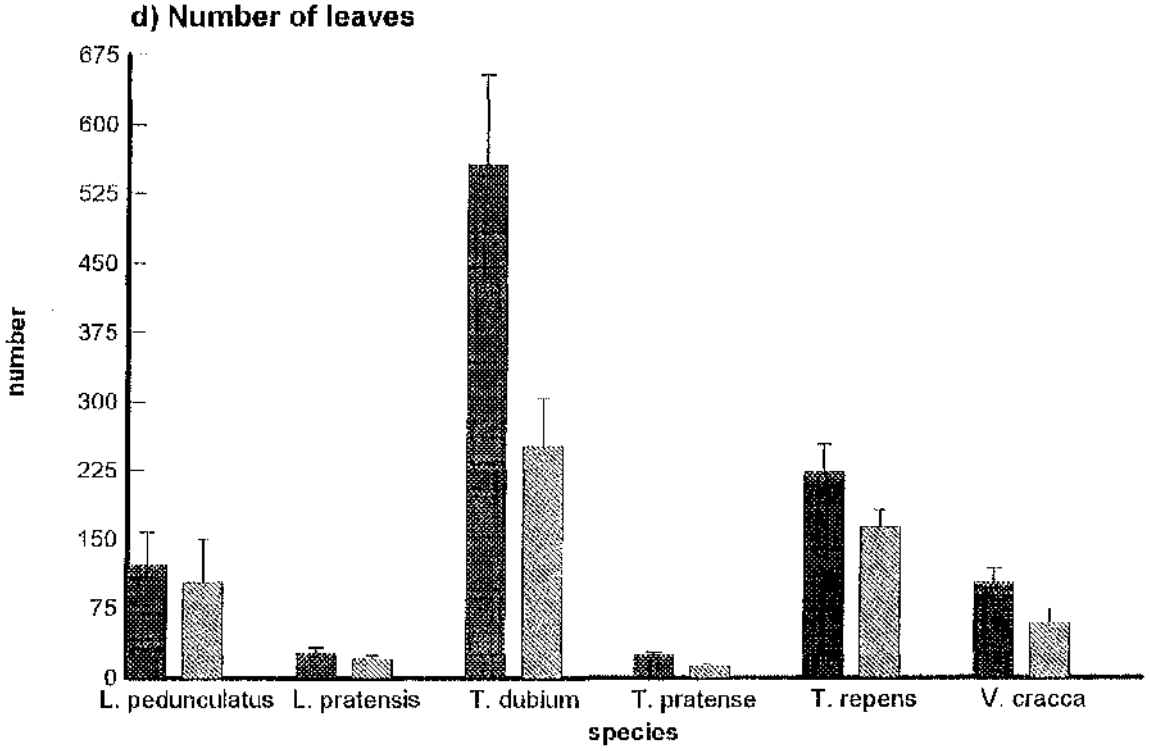
Leaves and stem

The recovery of leaf number from defoliation is shown in Fig. 5.4 (d). The leaf number did not recover from defoliation in *T. dubium* and *T. pratense* (Table 5.6). Stem length did not recover from defoliation in *L. pratensis*, *T. dubium* and *T. pratense* (Fig. 5.4 (e) and Table 5.6).

Fig. 5.4 Recovery from defoliation (mean values/plant) (n=5) with standard error bars




c) Total biomass (g)
biomass (g)
4.2
3.6
3
2.4
1.8
1.2
0.6
0
uncut
cut
L. pedunculatus
L. pratensis
T. dubium
T. pratense
T. repens
V. cracca
species


d) Number of leaves
number
675
600
525
450
375
300
225
150
75
0
L. pedunculatus
L. pratensis
T. dubium
T. pratense
T. repens
V. cracca
species

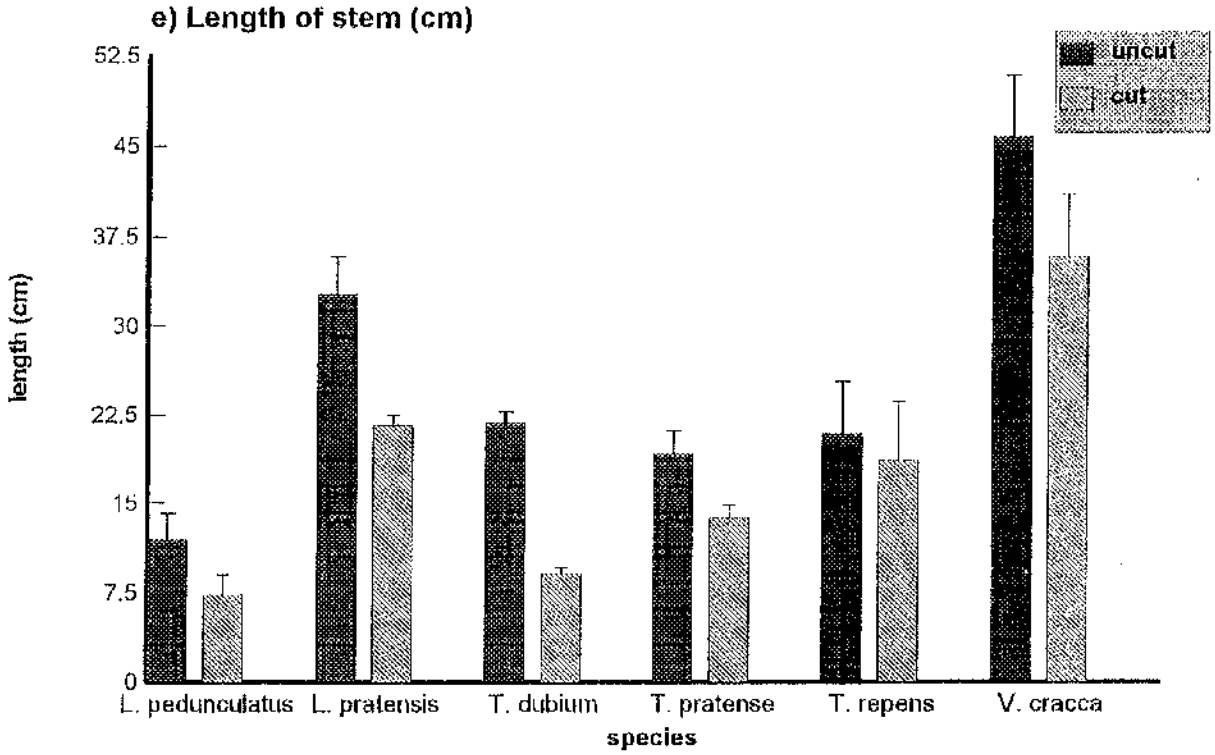


Table 5.6 Summary of the recovery from defoliation (ANOVA)

Levels of significance are as follows NS >0.05, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Species	Shoot Biomass (g)	Root Biomass (g)	Total Biomass (g)	Number of Leaves	Length of Stem (cm)
L. pedunculatus	NS	NS	NS	NS	NS
L. pratensis	***	***	***	NS	**
T. dubium	***	NS	***	*	***
T. pratense	**	***	***	**	*
T. repens	NS	NS	NS	NS	NS
V. cracca	NS	NS	NS	NS	NS

length of stem = mean of 5 stems per plant

All the significant differences are negative (defoliation has reducing effect in all cases)

5.3.6 Adventitious rooting of cuttings

From Table 5.7 it can be seen that a high percentage of cuttings of: *T. dubium*, *T. pratense* and *T. repens* and *L. pedunculatus* formed adventitious roots, both from plants growing in the field and grown in the greenhouse. Cuttings of *L. pratensis* and *V. cracca* had a lower percentage of cuttings taking root, this was increased in *L. pratensis* when greenhouse grown plants were used. In general, those species from which cuttings formed a higher percentage of roots, produced roots sooner than those that formed a lower percentage of roots.

Table 5.7 Summary of the ability of selected legumes to form adventitious roots

	Field		**Field**		**Greenhouse**	
	July 1994		**May-June 1995**		**January 1995**	
Species	**% root**	**T_{50}**	**% root**	**T_{50}**	**% root**	**T_{50}**
L. pedunculatus	85.7	17	100.0	10	90.0	5
L. pratensis	0.0	*	40.0	*	70.0	28
T. dubium	85.7	16	NA	NA	100.0	10
T. pratense	85.7	7	NA	NA	100.0	10
T. repens	100.0	5	100.0	3	100.0	5
V. cracca	0.0	*	10.0	*	0.0	*

% root = Percent of cuttings forming roots

T_{50} = number of days taken for 50% of cuttings to form roots (* = not applicable)

NA = not available

5.4 DISCUSSION

The morphology and colony characteristics of the bacteria isolates from each of the plant species agree with Jordan's (1984) description of the Rhizobiaceae. They did not take up congo red or grow on peptone glucose agar and nodulated the species of legume from which they had been isolated. It is not possible to determine which strains have been isolated without serological or molecular analysis, which was out with the scope of this study. However, differences were noted in their growth rates and ability to produce acid. The isolate from *Lotus* was a 'slow grower' and did not produce acid in yeast mannitol agar. The others were 'fast growers', producing acid on yeast mannitol agar. The isolates were not speciated but it is probable that the isolates from *Trifolium*, *Lathyrus* and *Vicia* belong to the genus *Rhizobium,* being fast growers and acid producers (Holt *et al.*, 1994). Although *Lotus* was a slow grower, no change in pH was noted, therefore the isolate could either be a slow growing strain of *Rhizobium loti* or *Bradyrhizobium* (meaning: the slow growing rhizobium) but the latter usually produces alkali (Holt *et al.*, 1994). The isolates were used for inoculation of plants in the greenhouse experiments so that results would reflect more closely the situation in the field (though of course greenhouse experiments cannot replicate the field environment).

The overall effects of water excess (and deficiency) on plant growth and nitrogen fixation will be discussed, followed by an examination of individual species response. The effects of defoliation on legume growth will then be considered.

5.4.1 Response of legumes to water stress (short term flooding and limited water supply)

Biomass accumulation

Biomass accumulation in *T. dubium* and *T. pratense* was depressed by both short term flooding and limited water supply (Table 5.3). It is assumed that the range of changes brought about by the flooding of roots (transport of minerals, water, hormones, toxins and assimilates (Jackson & Drew, 1984)) ultimately resulted in a reduction of photosynthesis and thus a cessation of growth (and therefore biomass accumulation). Heinrichs (1970) showed that white clover could tolerate a period of 20 days flooding (the duration used in this study). A water deficit can effect most plant functions, including carbon dioxide assimilation and root nutrient uptake (Schulze, 1991). Even a small decrease in soil water can affect photosynthesis and growth (Etherington, 1982). Heinrichs (1970, 1972) found yellowing to be a good visual indicator of flooding damage, several legume species in this study exhibited yellowing on flooding.

Shoot and Root

In the plants of *T. dubium* and *V. cracca* supplied with limited water, a reduced above:below ground ratio (Table 5.3) may be interpreted functionally as a reduced ratio of transpiring to absorbing surface (Etherington, 1982). This is seen as a beneficial strategy during dry periods, plants which experience predictable drought often have a low shoot:root ratio (Etherington, 1982). The organic nutrition of the root is not greatly affected by drought whereas shoot growth is reduced (Crawford, 1989). This is shown in *T. dubium* and *T. pratense*. The shoot biomass of both species was significantly reduced whereas root biomass was unaffected (although the reduction in shoot biomass was significant in *T. pratense*, it was not sufficient to change the above:below ground ratio). In *V. cracca*, although shoot and root biomass were not significantly effected, a combination of the two resulted in a decrease in above:below ground ratio from receiving the limited water treatment.

Shoot and root growth are normally reduced by flooding (Levitt, 1980). However, reduction in shoot biomass was seen in *T. dubium* only and reduction of root biomass was seen in *T. dubium*, *T. pratense* and *T. repens*. The increased above:below ground ratio of the flooded plants of *T. dubium* and *T. pratense* is commonly found in waterlogged plants (Sena Gomes & Kozlowski, 1980; Kozlowski, 1984; Voesenek *et al.*, 1988).

Adventitious rooting was noted in two species (*L. pedunculatus* and *T. repens*). The development of adventitious rooting in flooded plants is generally thought to aid survival (Jackson & Drew, 1984). However, the mechanisms by which they are beneficial have yet to be established. Etherington (1984) found that the pruning of adventitious roots limited growth in flooded *Epilobium hirsutum* L. but Wample & Reid (1978) did not find adventitious roots contributed to the survival of flooded sunflowers.

Two species (*L. pedunculatus* and *L. pratensis*) exhibited stem hypertrophy on flooding. This is the swelling of the base of the stem on flooding, resulting from corticular cell enlargement, and accompanied usually by the collapse of some cells to form gas-filled spaces (Kawase, 1981). This is considered to increase tolerance of waterlogging by increasing the surface area of the stem available for oxygen diffusion, thus facilitating internal aeration of the roots. It is appropriate therefore that the species which exhibited hypertrophy in this study were those least affected by flooding (Table 5.3).

Nodules and nitrogen fixation

Nodule colour

This is of subjective value as an indicator of the activity of a nodule in nitrogen fixation. Active nodules have a distinct pink colour due to the pigment haemoglobin produced by the host plant, which maintains oxygen transport at a low concentration (Appleby, 1984). It was thought that change of nodule colour from pink to green due to leghaemoglobin degradation, was an indication of senescence, but it has now been established that this is reversible to some degree (e.g. Pfeiffer *et al.*, 1983). However, the colour of nodules still provides some indication of possible nitrogen fixing activity.

Nodule development

In the short term flooding and limited water supply experiment, nodule initiation would have been established before the watering treatments were applied, therefore any decrease in number or biomass is likely to have been due to inhibition of nodule development. Such a decrease in nodule development was brought about by flooding in *T. pratense*, *T. repens* and *V. cracca* and by limited water availability in *T. dubium*, *T. pratense* and *V. cracca* (Table 5.3). In waterlogged conditions, a build up of carbon dioxide may occur which may inhibit nodulation at high concentrations (Bordeleau & Prévost, 1994). A major factor implicated in decreased nodulation is low oxygen concentration (Loveday, 1963). In field conditions, ethylene is evolved from anaerobic soils and can depress nodulation at low concentrations (Grobbelaar *et al.*, 1970; Goodlass & Smith, 1979). Water deficiency has been shown to inhibit root hair infection and nodule development, as reported by Worrall & Roughley (1976) for *Trifolium subterraneum*. On rewatering however, after the period of water deficiency, normal nodulation was resumed. In *Vicia faba* water deficiency restricted nodule expansion, but enlargement occurred on rewatering (Gallacher & Sprent, 1978).

Nitrogen fixation

The nitrogen content of the plants in this study provided a measure of nitrogen fixation. The plant nutrient source was free of combined N and therefore it can be assumed that nitrogen accumulation by the plants relates to nitrogen fixation (Table 5.3). Both the nodule effectivity (g nitrogen fixed g dry weight nodule^{-1}) and overall nitrogen fixed (% nitrogen and nitrogen content (g)) were reduced by flooding in *T. dubium*. Although the % nitrogen and nitrogen content was reduced in *T. pratense*, the nodule effectivity was unaffected by flooding. This suggests that the nodule tissue present remained active and the overall reduction was a result of a reduction

in nodule biomass. The reduction in nitrogen content in flooded *V. cracca* plants is simply a reflection of the reduction in root and nodule biomass, as neither % nitrogen or nodule effectivity were affected by flooding (i.e. as root and nodule biomass were reduced, the nitrogen content was also reduced).

Nitrogen fixation as measured by % nitrogen in *L. pratensis*, *T. dubium* and *T. pratense* was also reduced by limited water availability as was the nitrogen content in limited water supplied plants of *T. dubium*, *T. pratense* and *V. cracca* (Table 5.3). In *T. dubium*, this reduction was a reflection of the reduced total biomass as neither % nitrogen nor nodule effectivity were effected. The increase in nodule effectivity in *T. dubium* and *V. cracca* plants which received a limited water supply (Table 5.3) suggests that a higher proportion of nodule tissue was active in the plants with limited water supply than the controls. However, the nodule biomass was so reduced by the limited water treatment that a reduction in overall nitrogen fixed (% nitrogen) still occurred (Table 5.3). In *L. pratensis* however, the reduction in % nitrogen did not correspond to a change in nodule effectivity, suggesting that this reduction resulted from reduced nodule biomass.

Nitrogen fixation is known to be affected by water supply. Sprent (1972) found that there was a strong positive correlation between soil water content and nitrogen fixing activity (ARA) in field grown *Vicia faba* and, once past the field capacity, waterlogging reduced activity. Decreased nodule activity caused by waterlogging is presumably a result of oxygen deficiency. Minchin & Pate (1975) found that nitrogenase activity of *Pisum sativum* (harvested roots) was reduced by waterlogging and once removed from the excess water, activity progressively increased. This was attributed to increasing oxygen availability as the water evaporated from the nodule surface. Bisseling *et al.* (1980) found that a decrease in nitrogenase activity in waterlogged *Pisum sativum* was paralleled by a decline in the amount of active nitrogenase found in the bacteroids. The synthesis and therefore the amount of nitrogenase component II (Fe protein) was decreased by waterlogging (component I (Mo-Fe protein) was unaffected). Therefore, in addition to reduced oxygen supply to the nodules, decreased amounts of active nitrogenase also contributes to reduced nitrogen fixation.

Reduction in nitrogen fixing activity as a result of water deficit may be a result of direct effects on nodules. Export of fixed nitrogen out of the nodules may be depressed as a result of lower rates of water movement, therefore inhibiting nitrogen fixation by means of a feedback mechanism (Pate *et al.*, 1969). Nitrogen fixing

activity and respiration in nodules has been found to relate directly to nodule water potential (Pankhurst & Sprent, 1975a). This reduction in activity may also be an indirect result of lack of photosynthate supply from the stressed shoot (Huang *et al.*, 1975; Guerin *et al.*, 1990). However, Durand *et al.* (1987) found no direct correlation between nitrogenase activity and carbohydrate availability. More recently, Sridhara *et al.* (1995) reported that in soybean, nodule water status and nitrogenase activity (but not photosynthesis) returned to control levels on rewatering after a dry period. This would support the hypothesis that nitrogenase activity is directly related to nodule water status.

5.4.2 Response of seedlings to short term flooding with recovery period

Out of the four species studied, only *T. pratense* was affected by the flood/recovery treatment. Biomass of all plant parts' (and thus total biomass) and leaf area were reduced (Table 5.4), therefore the subsequent three week period of free-drainage was not sufficient for recovery. Reduction of leaf area is common in plants affected by flooding, as growth (and therefore leaf initiation and expansion) is inhibited. The leaf area of wheat was reduced by 83% after 25 days of root anoxia (Sojka *et al.*, 1975). Nutrient deficiencies are a principal cause, but ethylene is also thought to contribute (Jackson & Drew, 1984).

The other three species (*L. pratensis*, *L. pedunculatus* and *V. cracca*) were either unaffected by the flooding or recovered sufficiently in the three weeks of free drainage that followed. It has been found that nodules of flooded cowpea (*Vigna unguiculata*) resumed growth and activity following subsequent drainage (Minchin & Summerfield, 1976), although this compensatory nodule growth resulted in a decrease in overall growth. A reduction in overall biomass was not shown by the three tolerant species in this study, suggesting that no compensatory nodule growth occurred (at the expense of overall growth) and nodule growth may have continued through the flood and recovery period. Further studies are required to confirm this.

5.4.3 Response of *Lotus pedunculatus* to long term flooding

Biomass accumulation

The prolonged flooding in *L. pedunculatus* resulted in a reduction of shoot (and thus total biomass (Table 5.5)), whereas the less severe flooding treatment had no affect (Table 5.5). This illustrates the importance of flood duration in terms of plant response.

Reproduction

The experiments 5.2.1 and 5.2.2 were not run until plants flowered, however, the *L. pedunculatus* plants in experiment 5.2.3 did reach flowering stage. Flowering occurred in the non-flooded plants but did not occur in prolonged flooded plants of *L. pedunculatus* (Fig. 5.3 g)). It has been shown that flooding can delay flowering (van der Sman *et al.*, 1988) and this often leads to reduced seed production.

Nodules and nitrogen fixation

Long term flooding of *L. pedunculatus* brought about a reduction in nodule number and a reduction in overall nitrogen content (g/plant) (Table 5.5). However, this reduction in nitrogen content was probably a reflection of the reduction of the total biomass since nitrogenase activity and % nitrogen and nodule effectivity were not affected. It is apparent that *L. pedunculatus* is particularly tolerant of flooding as neither short nor long term flooding reduced nitrogen fixation (% nitrogen or nitrogenase activity) (Table 5.5). This tolerance may be a result of this species' ability to develop adventitious roots and stem hypertrophy (as discussed previously) and enlarged lenticels. These enlarged lenticels are regarded as an adaptation which enables continued nitrogen fixation and growth (Minchin & Summerfield, 1976). They are thought to be of importance in the transport of oxygen to the central bacteroid region (Pankhurst & Sprent, 1975b). In waterlogged conditions where oxygen supply is limited, increased lenticel development may therefore facilitate oxygen transport for continued nitrogen fixation. Several authors have examined nodule anatomy at different rhizosphere O_2 partial pressures (e.g. Dakora & Atkins, 1989, 1991; Parsons & Day, 1990) and it would seem that nodule cortical anatomy changes to suit environmental growth conditions. However where cortex area (as a percentage of nodule transverse sectional area) was examined in this study (in *L. pedunculatus* plants receiving prolonged flooding treatment) no significant increase in nodule cortication was noted (Table 5.5). It is apparent that more detailed studies would be necessary to elucidate the exact mechanisms of flood tolerance in *L. pedunculatus*, but the continuation of nitrogenase activity implies the development of an internal route between the submerged nodules and the atmosphere for gaseous exchange (Pugh, *et al.*, 1995).

5.4.4 Recovery from defoliation

As discussed previously in Chapter 3, it has been hypothesised that herbivory (under certain conditions) may lead to improved plant growth and fitness (e.g. McNaughton, 1983, 1986) although this is also widely disputed (Belsky, 1986, 1987; Verkaar, 1988). The latter authors state that very few reports showing

overcompensation actually contain evidence to support this claim. Shoot growth may be increased by defoliation, but it might be at the roots expense, thus increasing the risk of mortality (Bentley & Whittaker, 1979). It has been reported that root biomass is usually reduced by grazing (Crawley, 1983). It is for this reason that both root and shoot biomass were measured in this study, to gain an insight into whole plant response rather than above ground biomass alone, as is often the case (Verkaar, 1988). All six species received the same defoliation treatment, a procedure not usually adopted in earlier studies where growth form/rate determined the defoliation treatment applied (Bossard & Hillier, 1993). Thus the effects of defoliation on each species are comparable in this study.

L. pedunculatus, *T. repens* and *V. cracca* recovered sufficiently from defoliation (Table 5.6) but overcompensation did not take place. However plants of *L. pratensis*, *T. dubium* and *T. pratense* did not recover well from defoliation (Table 5.6). Total biomass accumulation of each species was significantly reduced by defoliation, as was stem length. Unlike in *L. pratensis* and *T. pratense*, root growth was not depressed significantly in *T. dubium*. Leaf number was not significantly reduced in *L. pratensis*, but was in *T. dubium* and *T. pratense*. Defoliation thus affected the growth of each species differently.

The ability of the legumes to develop adventitious roots is an indicator of regenerative capacity from detached shoots (e.g. from flooding or mowing) (Hodgson *et al.*, 1993). The two species lacking this ability: *L. pratensis* and *V. cracca* were the most abundant legumes found at the only site where mowing occurs (Clonmacnoise). It can be assumed therefore that regrowth after mowing is probably a result of the regeneration of the damaged rooted shoot. The clovers and *Lotus* however, all exhibited the ability to form adventitious roots, suggesting that if their habitats were disturbed (e.g. by mowing, grazing), regeneration would be possible from detached shoots.

If we consider the results of the defoliation and adventitious rooting together, of all the legume species examined, *L. pedunculatus* and *T. repens* would have greater ability to recover from defoliation/disturbance as they have been shown to recover from defoliation and develop adventitious roots. *V. cracca*, although able to recover from defoliation did not develop adventitious roots. Both *T. pratense* and *T. dubium*, were able to form adventitious roots but did not recover well from defoliation. The species least likely to recover from defoliation/disturbance was *L. pratensis*, which did not recover from defoliation or form adventitious roots.

5.5 SUMMARY

Water stress (both flooding and drought) and disturbance by defoliation were applied to legumes in the greenhouse. The response of each legume species follows and is summarised in Table 5.8.

Lathyrus pratensis

Short term flooding and limited water supply had no significant effect on overall biomass accumulation of *L. pratensis* plants, however % nitrogen was significantly reduced by limited water supply, i.e. nitrogen fixation was reduced by limited water availability.

L. pratensis seedlings recovered well from the flood/recovery treatment. No detrimental effects were noted.

L. pratensis did not recover from defoliation and did not form adventitious roots from cuttings.

Lotus pedunculatus

Total biomass accumulation and nitrogen fixation were unaffected by short term flooding and limited water supply. However, long term flooding on *L. pedunculatus* significantly reduced total biomass accumulation. Reproduction was also significantly reduced, none of the long term flooded plants produced flowers. However, it would appear that nitrogen fixation was not significantly affected, since % nitrogen (dry weight) and nitrogenase activity were not altered by prolonged flooding.

L. pedunculatus seedlings recovered well from the flood/recovery treatment. No detrimental affects were noted.

L. pedunculatus tolerated defoliation and showed the capacity to form adventitious roots from cuttings.

Trifolium dubium

Short term flooding and limited water supply treatments resulted in significant reductions of total biomass accumulation and nitrogen fixation, only limited water supply decreased nodule growth.

T. dubium did not recover well from defoliation, but was able to form adventitious roots from cuttings.

Trifolium pratense

Short term flooding and limited water supply treatments resulted in significant reductions of total biomass accumulation, nodule growth and nitrogen fixation.

T. pratense seedlings were susceptible to flooding. The flood/recovery treatment reduced shoot, root and nodule (and thus total) biomass and leaf area.

T. pratense did not recover well from defoliation, but was able to form adventitious roots from cuttings.

Trifolium repens

Only root and nodule biomass were reduced significantly by short term flooding, overall nitrogen fixation and total biomass accumulation were not affected.

T. repens recovered well from defoliation and showed a large capacity to form adventitious roots from cuttings.

Vicia cracca

Similarly to *T. repens* nitrogen fixation and total biomass accumulation were not affected by short term flooding and limited water supply, but nodule growth was reduced by both treatments.

V. cracca seedlings recovered well from the flood/recovery treatment. No detrimental affects were noted.

V. cracca plants tolerated defoliation, but were unable to form adventitious roots from cuttings.

Table 5.8 Summary of the response of selected legumes to water stress and defoliation

SPECIES	RESPONSE TO STRESS										
	LIMITED WATER SUPPLY			SHORT TERM FLOODING			FLOOD/ RECOVERY	LONG TERM FLOODING		RECOVERY FROM DEFOLIATION	ADV. ROOTING ? (with rank)
	YIELD	N	nod	YIELD	N	nod	YIELD	YIELD	N*	YIELD	YES/NO
L. pratensis	NS	-	NS	NS	NS	NS	NS	*	*	NS	YES (5)
l. pedunculatus	NS	NS	NS	NS	NS	NS	NS	-	NS	NS	YES (3)
T. dubium	-	-	+	-	-	-	*	*	*	-	YES (4)
T. pratense	-	-	NS	-	-	NS	-	*	*	-	YES (2)
T. repens	NS	NS	NS	NS	NS	NS	*	*	*	-	YES (1)
V. cracca	NS	NS	+	NS	NS	NS	NS	*	*	NS	NO

YIELD = Total biomass (g) N = Nitrogen fixation (% nitrogen)

N* = Nitrogen fixation (% nitrogen, nodule effectivity and nitrogenase activity)

nod = nodule effectivity (g nitrogen fixation g nodule dry weight^{-1}

- = Negative effect + = positive effect NS = no significant effect

* = Not available

(Rank = in decreasing order of ability to form adventitious roots)

Chapter 6
CONCLUSIONS

6. CONCLUSIONS

6.1 Did the study achieve its aims?

To answer this question, each of the aims outlined in Chapter 1 will be addressed separately.

6.1.1 What is the contribution of legumes to the species composition of riverine wetland plant communities in the British Isles?

Of the 114 plant species recorded at the 17 riverine sites, nine (8%) were members of the Fabaceae (*Genista anglica*, *Lathyrus palustris*, *Lathyrus pratensis*, *Lotus corniculatus*, *Lotus pedunculatus*, *Trifolium dubium*, *Trifolium pratense*, *Trifolium repens* and *Vicia cracca*). This percentage may seem small, but in fact it is the highest percentage of any dicotyledonous family (along with Asteraceae which also comprised 8%). Only the monocotyledonous families Poaceae (18%) and Cyperaceae (9%) were represented to a greater extent. The Fabaceae (along with Asteraceae and Poaceae) is one of the largest families of flowering plants (Stace, 1991) therefore this is not surprising. However, this does not detract from the fact that legumes were well represented in the plant species list from the 17 riverine wetland sites. Of course this is only a reflection of the species present and not their abundance at the wetland sites.

Trifolium repens was found at a total of 7 sites, the most widely spread species of legume. None of the legumes were dominant at any of the sites. The greatest percent frequency of any one legume was 33% for *V. cracca* at Clonmacnoise (site 2), closely followed by 32% for *L. pedunculatus* at Ring Bog and 30% for both *L. pratensis* and *L. corniculatus* at Clonmacnoise (site 3) and Bradford Mill (site 2) respectively. Canonical correspondence analysis suggested that site species composition was influenced by certain environmental factors, this will be discussed further in section 6.1.4.

6.1.2 What is the role of legumes as a source of biologically fixed nitrogen within riverine wetlands?

Limitations in the methodology used to estimate the rates of nitrogen fixation allowed comparison of the order of magnitude of N_2 fixation by different sources at one site (Ring Bog) only. All legumes tested reduced acetylene to ethylene, all had the potential to fix nitrogen and therefore were possible sources of nitrogen to the riverine wetland environment. The conservative estimate of the annual rate of nitrogen fixation for one species of legume, *Lotus pedunculatus* at Ring Bog was

11.2 kg N ha^{-1}. This value will be discussed in relation to the magnitude of nitrogen fixation determined for other sources in the following section.

6.1.3 What are the sources of biologically fixed nitrogen other than legumes that may contribute to the nitrogen economy of riverine wetlands?

A number of sources other than legumes were found to fix nitrogen in Ring Bog: free-living bacteria in the peat soil, mosses (undetermined association, possibly cyanobacteria) and monocotyledon-rhizosphere bacteria associations. The mosses had the lowest annual input of fixed nitrogen in Ring Bog, with estimates ranging from 0.3 and 1.4 kg N fixed ha^{-1} followed by the monocotyledon-rhizosphere bacteria association with estimates between 0.04 and 11.2 kg N fixed ha^{-1}. By far the highest rate of nitrogen fixation was estimated for the free-living bacteria in the surface peat soil at 55.8 kg N ha^{-1} yr^{-1}. This compares to the rate of 11.2 kg N ha^{-1} yr^{-1} estimated for *Lotus pedunculatus*. It is obvious therefore, that the free-living bacteria in the peat soil are the single most important source of fixed nitrogen in Ring Bog. Mosses fix relatively small amounts of nitrogen as do two of the monocotyledons, *Agrostis stolonifera* (0.04 kg N ha^{-1} yr^{-1}) and *Molinia caerulea* (1.4 kg N ha^{-1} yr^{-1}). However, the two other species of monocotyledon examined both had rates close to that of *Lotus* (*Juncus acutiflorus* (9.7 kg N ha^{-1} yr^{-1}) and *Carex curta* (11.2 kg N ha^{-1} yr^{-1})). It can be concluded that *Lotus pedunculatus*, *Carex curta* and *Juncus acutiflorus* together provide an important input of nitrogen at Ring Bog, second only to that contributed by the free-living bacteria in the surface peat soil. However, it should be noted that not all the plant species at Ring Bog were examined for nitrogen fixing ability, therefore other sources may also be present.

Between all the sources, nitrogen fixation contributes a substantial amount of nitrogen to Ring Bog; the total annual rate is estimated to be at least 91.4 kg N ha^{-1}. It is likely that peaks in input from the different sources occur at different times of the year. However, nitrogen fixation is only one source of nitrogen input to riverine wetlands. Atmospheric deposition can be an important input (Morris, 1991); precipitation inputs are well known, but dry deposition can exceed wet deposition (Boring *et al.*, 1988). Total deposition of nitrogen (wet and dry) for the area in which Ring Bog lies was estimated at 15-20 kg N ha^{-1} yr^{-1} (1989-1992) (Department of the Environment, 1994). The total input of nitrogen from biological fixation therefore exceeds that from atmospheric deposition in Ring Bog. Atmospheric deposition therefore exceeds the input of *Lotus pedunculatus*, the only legume

present in Ring Bog. The only single biological nitrogen fixing source with a higher input is free-living bacteria in the peat soil.

6.4 What are the effects of the wetland environment on legume growth and nitrogen fixation?

Water regime (flooding) was the most important environmental influence on riverine wetland vegetation composition, more so than soil wetness class, soil pH and land management which were also found to be of importance. It is recognised that wetland hydrology is more complicated than the descriptive categories assigned to each site in this study, but it is interesting that a detailed study also found flooding regime (although measured differently) to be an important influence on species composition in riverine wetlands (Irish callows) (Hooijer, 1995). The rankings of the legume species along the environmental gradients compare well to the findings of the greenhouse studies (flooding and land management studied only).

The vegetation analysis inferred that *L. pedunculatus* was likely to be found in soil of high wetness class (Table 3.6). This was further supported by the greenhouse experiments (Table 5.8). It was found to be one of the most tolerant legumes of flooding. Twelve week old *L. pedunculatus* plants not only withstood short term flooding but also recovered well from short term flooding at the seedling stage (flooded for three weeks after germination). Only long term flooding (5 weeks) reduced the yield of *L. pedunculatus* but nitrogen fixation remained unaffected. Detailed studies of *L. pedunculatus* at Ring Bog revealed that root and nodule growth was restricted to the above-ground vegetation and litter layer. This was thought to be an avoidance mechanism of this species as the nitrogen fixing tissues remained above the saturated soil. However, the greenhouse experiments revealed that nitrogen fixation in *L. pedunculatus* was unaffected by long term flooding. Given its ability to survive flooding, it is somewhat surprising to find that *L. pedunculatus* was one of the least likely legume species to be found at flooded sites, suggesting that within the range of sites examined in the current study an unmeasured factor may have influenced the distribution of *L. pedunculatus* (due to time limits it was not possible to investigate all environmental parameters, for example other nutrient concentrations, such as phosphorus may have been influential).

L. pratensis and *V. cracca* were ranked as the legumes most likely be found in intermediate flooded sites (Table 3.6) and this was supported by the greenhouse studies which revealed them to be flood tolerant (Table 5.8). Short term flooding at

twelve weeks old had no affect, neither did flooding for three weeks following germination, implying that these species could withstand continuous flooding at an early stage of growth and sudden flooding as applied at twelve weeks old. The intermediate flooding rank given to both these species therefore seems reasonable, what does seem surprising is that they were ranked as least likely to be found in soil of high wetness class. This suggests that, although tolerant of periods of flooding, *L. pratensis* and *V. cracca* were more likely to grow in soils which were not waterlogged for substantial parts of the year.

The two species which were most severely effected by short term flooding and limited water supply (at an age of twelve weeks) were *T. dubium* and *T. pratense* (Table 5.8), suggesting a narrower ecological amplitude than the other species. This applies to *T. pratense* in particular since this species was the only one affected at the seedling stage of development by short term flooding followed by a recovery period. The effects of flooding in the field were revealed by the legume growth and nodulation studies (Figs 4A.3 and 4A.4) where reductions in growth and nodulation were related to long periods of inundation. It is perhaps not surprising, therefore, that these two species are found in the middle section of both the soil wetness class and intermediate flooding rankings (Table 3.6).

Although *T. repens* was tolerant of both short term flooding and limited water supply (Table 5.8), it was ranked in the middle of both soil wetness class and intermediate flooding (Table 3.6). The greenhouse experiments suggest that this species has a wide ecological amplitude, being able to survive both flooding and limited water supply). Because it is found in-between the extremes of soil wetness class and intermediate flooding, it can be concluded that within the sites studied that *T. repens* is not at the limits of its ecological ability in the sites studied.

Land management practices (cutting for hay and grazing) also influenced species distribution. However, the greenhouse experiments which investigated different aspects of defoliation (Table 5.8) produced data which did not relate well to the inferred legume rankings from the vegetation analysis (Table 3.6). Thus, two of the three legume species found to be intolerant of defoliation (*T. dubium* and *T. pratense*) in the greenhouse were ranked as the two species most likely to be found at managed (i.e. defoliated) sites. The other species intolerant of defoliation was *L. pratensis*, in which an apparent increase in growth following cutting at Clonmacnoise (compared to growth at a similar time the following year) was attributed to cutting of this species. In all three species it was expected therefore

that defoliation would not have a negative effect on plant growth. However, this was not the case. In *T. dubium* and *T. pratense* this conflict may be explained either by the severity of the defoliation treatment applied or because their ability to develop adventitious roots from cut stems plays an important part in regrowth after defoliation (*L. pratensis* did not exhibit this ability). In the greenhouse experiments, three-quarters of the photosynthetic material was removed and therefore may not have truly reflected the defoliation pressures due to cutting for hay and grazing in the field.

6.2 Future work

This study has given clear indication of the occurrence of nitrogen fixation in riverine wetlands, but due to the restrictions of time and money, questions have been raised that cannot be answered without further work:

1) Which free-living bacteria fix nitrogen in the soil at Ring Bog?

The free-living bacteria which fix nitrogen in Ring Bog (and reduce acetylene to ethylene at the Irish sites) could be aerobic and/or anaerobic. *Bacillus polymyxa* may be present; this has been isolated from peat soils and is known to fix nitrogen in both anaerobic and aerobic conditions (Waughman *et al.*, 1981). A great deal of work would be needed to isolate and identify the nitrogen fixing bacteria in the soils of the study sites.

2) Do cyanobacteria and/or heterotrophic bacteria form a nitrogen fixing association with *Sphagnum palustre*, *Sphagnum recurvum* and *Polytrichum commune* in Ring bog and what is the nature of this association?

Although epiphytic cyanobacteria associations with mosses are more common than endophytic, there is a possibility that either and/or both may be present at Ring Bog. Heterotrophic bacteria may also be associated with *Sphagnum* and this also needs further investigation.

3) Which bacteria form a nitrogen fixing association with the monocotyledons found to fix nitrogen at Ring bog and what is the nature of the association?

The most widely reported angiosperm rhizosphere bacteria is *Azospirillum* and has been isolated from the roots of many species in many regions (Gibson & Jordan, 1983). However, species belonging to this genus are not the only diazotrophs associated with roots and it would therefore be interesting to isolate and identify those present and their location (root surface or within the roots inner cortex) in the rhizospheres of the nitrogen fixing monocotyledons in Ring Bog.

This study did not attempt to explore the nitrogen cycle as a whole within riverine wetlands, only one aspect of it: nitrogen fixation and detailed investigations were restricted to one site (Ring Bog) only. It has suggested that input of nitrogen by free-living soil bacteria is the major source of biologically fixed nitrogen in the ecosystem studied. Other wetland sites need to be studied in order to permit detailed comparison with the study presented. In the long term, more extensive studies to determine the relationship of biological nitrogen fixation to the overall cycling of nitrogen in these ecosystems are required.

6.3 Summary

The following can be concluded:

- Legumes are present in riverine wetlands, nine species were recorded at the sites visited, legume frequency of any one species reached approximately 30% (m^{-2}) at any one site. Plant species composition was influenced by flooding regime, soil wetness class, soil pH and land management. Greenhouse studies on six selected legume species revealed that responses to flooding treatments and defoliation varied between legume species.

- All of the five legume species examined using the acetylene reduction technique reduced acetylene to ethylene, showing their potential to fix nitrogen. Table 6.1 shows the four sources of nitrogen fixation identified at Ring Bog (in order of magnitude based on conservative estimates of their annual rates of nitrogen fixation, calculated from $^{15}N_2$ assays in the field).

Table 6.1 Nitrogen fixing sources at Ring Bog

NAME	NATURE OF SOURCE	Estimated rate (kg N ha^{-1} yr^{-1})
peat soil	free-living bacteria	55.8
Lotus pedunculatus	legume	11.2
Carex curta	monocotyledon-rhizosphere bacteria	11.2
Juncus acutiflorus	monocotyledon-rhizosphere bacteria association	9.7
Sphagnum palustre	bryophyte (cyanobacteria association?)	1.5
Molinia caerulea	monocotyledon-rhizosphere bacteria association	1.2
Polytrichum commune	bryophyte (cyanobacteria association?)	0.55
Sphagnum recurvum	bryophyte (cyanobacteria association?)	0.3
Agrostis stolonifera	monocotyledon-rhizosphere bacteria association	0.04

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APPENDICES

APPENDIX A: List of plant species: scientific names, authorities and common names.

Scientific Name & Authority	Common name
Achillea ptarmica L.	Sneezewort
Agrostis canina L.	Velvet Bent
Agrostis capillaris L.	Common Bent
Agrostis stolonifera L.	Creeping Bent
Alopecurus geniculatus L.	Marsh Foxtail
Angelica sylvestris L.	Wild Angelica
Anthoxanthum odoratum L.	Sweet Vernal-grass
Arrhenatherum elatius (L.) J.S. & C. Presl.	False Oat-grass
Barbarea stricta Andrz.	Small-flowered Winter-cress
Bellis perennis L.	Daisy
Briza media L.	Quaking-grass
Bromus commutatus Schrader	Meadow Brome
Calluna vulgaris (L.) Hull	Heather
Caltha palustris L.	Marsh-marigold
Cardamine pratensis L.	Cuckooflower
Cardamine hirsuta L.	Hairy Bitter-cress
Carex acutiformis Ehrh.	Lesser Pond-sedge
Carex curta Gooden	White Sedge
Carex demissa Hornem.	Common Yellow-sedge
Carex disticha Hudson	Brown Sedge
Carex echinata Murray	Star Sedge
Carex hirta L.	Hairy Sedge
Carex nigra (L.) Reichard	Common Sedge
Carex panicea L.	Carnation sedge
Carum verticillatum (L.) Koch	Whorled Caraway
Centaurea nigra L.	Common Knapweed
Cerastium glomeratum Thuill.	Sticky Mouse-ear
Cerastium fontanum Baumg.	Common Mouse-ear
Cirsium dissectum (L.) Hill	Meadow Thistle
Cirsium palustre (L.) Scop.	Marsh Thistle
Crepis paludosa (L.) Moench	Marsh Hawk's-beard
Cynosurus cristatus L.	Crested Dog`s-tail
Dactylorhiza fuchsii (Druce) Soó	Common Spotted-orchid
Deschampsia cespitosa (L.) P. Beauv.	Tufted Hair-grass
Eleocharis palustris (L.) Roemer & Schultes	Common Spike-rush
Eleocharis palustris ssp. *vulgaris*	Common Spike-rush
Epilobium obscurum Schreber	Short-fruited Willowherb
Epilobium palustre L.	Marsh Willowherb
Equisetum fluviatile L.	Water Horsetail
Equisetum palustre L.	Marsh Horsetail
Erica tetralix L.	Cross-leaved Heath
Eriophorum angustifolium Honck.	Common Cottongrass
Festuca arundinacea Schreber	Tall Fescue
Festuca rubra L.	Red Fescue
Filipendula ulmaria (L.) Maxim.	Meadowsweet
Galium palustre L.	Common Marsh-bedstraw
Galium uliginosum L.	Fen Bedstraw
Galium saxatile L.	Heath Bedstraw

Scientific Name & Authority	Common name
Galium verum L.	Lady`s Bedstraw
Genista anglica L.	Petty Whin
Glyceria fluitans (L.) R.Br.	Floating Sweet-grass
Heracleum mantegazzianum Sommier & Levier	Giant Hogweed
Hippuris vulgaris L.	Mare`s -tail
Holcus lanatus L.	Yorkshire-fog
Hydrocotyle vulgaris L.	Marsh Pennywort
Iris pseudacorus L.	Yellow Iris
Juncus acutiflorus Ehrh. ex Hoffm.	Sharp-flowered Rush
Juncus articulatus L.	Jointed Rush
Juncus effusus L.	Soft Rush
Juncus inflexus L.	Hard Rush
Lathyrus palustris (L.) L.	Marsh Pea
Lathyrus pratensis L.	Meadow Vetchling
Leontodon autumnalis L.	Autumn Hawkbit
Lolium perenne L.	Perennial Rye-grass
Lotus corniculatus L.	Common Bird`s-foot-trefoil
Lotus pedunculatus Cav.	Greater Bird`s-foot-trefoil
Luzula campestris (L.) DC.	Field Wood-rush
Lychnis flos-cuculi L.	Ragged-Robin
Lycopus europaeus L.	Gipsywort
Lysimachia nummularia L.	Creeping-Jenny
Lythrum salicaria L.	Purple-loosestrife
Mentha aquatica L.	Water Mint
Molinia caerulea (L.) Moench	Purple Moor-grass
Myosotis laxa Lehm.	Tufted Forget-me-not
Myosotis scorpioides L.	Water Forget-me-not
Narthecium ossifragum (L.) Hudson	Bog Asphodel
Odontites vernus (Bellardi) Dumort.	Red Bartsia
Oenanthe fistulosa L.	Tubular Water-dropwort
Pedicularis palustris L.	Marsh Lousewort
Persicaria amphibia (L.) Gray	Amphibious Bistort
Persicaria bistorta (L.) Samp.	Common Bistort
Phalaris arundinacea L.	Reed Canary-grass
Phleum pratense L.	Timothy
Plantago lanceolata L.	Ribwort Plantain
Poa pratensis L.	Smooth Meadow-grass
Poa trivialis L.	Rough meadow-grass
Potentilla anserina L.	Silverweed
Potentilla erecta (L.) Raeusch	Tormentil
Potentilla palustris L. Scop.	Marsh Cinquefoil
Prunella vulgaris L.	Selfheal
Ranunculus acris L.	Meadow buttercup
Ranunculus flammula L.	Lesser Spearwort
Ranunculus lingua L.	Greater Spearwort
Ranunculus repens L.	Creeping Buttercup
Rhinanthus minor L.	Yellow-rattle
Rorippa nasturtium-aquaticum (L.) Hayek	Water-cress

Scientific Name & Authority	Common name
Rumex acetosa L.	Common Sorrel
Rumex crispus L.	Curled Dock
Rumex obtusifolius L.	Broad-leaved Dock
Scutellaria galericulata L.	Skullcap
Scutellaria minor Hudson	Lesser Skullcap
Senecio aquaticus Hill	Marsh Ragwort
Sonchus palustris L.	Marsh Sow-thistle
Stachys officinalis (L.) Trev.	Betony
Stellaria palustris Retz.	Marsh Stichwort
Succisa pratensis Moench	Devil's-bit Scabious
Taraxacum sect. *Ruderalia* Kirscher, Oellgaard & Stepanek	Dandelion
Thalictrum flavum L.	Common Meadow-rue
Triglochin palustre L.	Marsh Arrowgrass
Trifolium dubium Sibth..	Lesser Trefoil
Trifolium pratense L.	Red Clover
Trifolium repens L.	White Clover
Valeriana officinalis L.	Common Valerian
Veronica catenata Pennell	Pink Water-speedwell
Vicia cracca L.	Tufted Vetch
Viola palustris L.	Marsh Violet

BRYOPHYTES

Polytrichum commune Hedw.
Rhytidiadelphus squarrosus (Hedw.) Warnst
Sphagnum palustre L.
Sphagnum recurvum P. Beauv.

APPENDIX B: Plant species frequency (%) for all sites

Species Names & Authorities	LB1	LB2	LB3	LB4	LBM	CL1	CL2	CL3	CL4	CL5	RB	KM1	KM2	KM3	BM1	BM2	BM3
Achillea ptarmica	0	0	0	0	0	0	4	0	0	0	1	4	0	2	0	0	0
Agrostis canina	0	0	0	0	0	2	6	0	0	1	0	0	0	0	0	0	0
Agrostis capillaris	0	0	0	0	0	0	0	0	0	1	0	43	6	0	0	0	0
Agrostis stolonifera	38	41	84	54	76	73	85	68	37	44	91	0	0	0	97	0	0
Alopecurus geniculatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
Angelica sylvestris	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0
Anthoxanthum odoratum	1	0	0	0	13	0	1	24	20	0	0	0	2	0	6	0	0
Arrhenatherum elatius	0	0	0	0	0	0	0	0	0	0	0	9	0	0	0	0	0
Barbarea stricta	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Bellis perennis	4	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Briza media	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0
Bromus commutatus	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Calluna vulgaris	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0
Caltha palustris	1	9	27	1	3	27	0	0	0	1	0	0	0	0	0	0	0
Cardamine pratensis	15	7	25	7	16	6	29	28	25	25	0	0	0	0	0	0	0
Carex acutiformis	0	0	59	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Carex curta	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0
Carex demissa	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Carex disticha	9	30	52	43	43	19	12	27	32	80	0	0	0	0	0	0	0
Carex echinata	0	0	0	0	0	0	0	1	45	0	1	0	0	0	0	0	0
Carex hirta	9	0	0	1	5	0	1	0	0	0	0	0	0	0	0	0	0
Carex nigra	0	8	10	27	5	0	17	58	55	26	17	0	0	0	0	0	0

Species Names & Authorities	LB1	LB2	LB3	LB4	LBM	CL1	CL2	CL3	CL4	CL5	RB	KM1	KM2	KM3	BM1	BM2	BM3
Carex panicea	0	0	0	58	0	6	0	4	10	0	0	0	40	30	0	0	0
Carum verticillatum	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0
Centaurea nigra	0	0	0	0	0	0	1	0	1	0	0	2	0	0	0	0	0
Cerastium glomeratum	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Cerastium fontanum	2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Cirsium dissectum	0	0	0	0	0	0	0	0	0	0	0	0	46	29	0	0	0
Cirsium palustre	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Cynosurus cristatus	2	0	0	0	2	0	0	0	6	0	0	0	0	0	0	0	0
Dactylis glomerata	0	0	0	0	0	0	0	3	0	0	0	0	0	0	43	0	0
Dactylorhiza fuchsii	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Deschampsia cespitosa	0	0	0	0	0	0	18	0	0	0	1	62	0	0	0	66	10
Eleocharis palustris	0	88	78	5	2	43	0	0	0	0	0	0	0	0	0	0	0
Epilobium obscurum	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Epilobium palustre	0	5	1	0	0	0	0	0	0	0	2	0	0	0	0	1	2
Equisetum fluviatile	0	0	1	0	0	28	0	0	0	2	3	0	0	0	0	2	0
Equisetum palustre	3	5	10	1	2	0	0	0	0	0	0	0	0	0	0	0	0
Erica tetralix	0	0	0	0	0	0	0	0	0	0	0	0	52	6	0	0	0
Eriophorum angustifolium	0	0	0	0	0	0	0	1	5	0	0	0	0	0	0	0	0
Festuca arundinacea	15	0	0	0	0	0	9	8	9	14	0	0	0	0	0	56	30
Festuca rubra	76	0	0	23	65	0	47	98	68	0	2	20	59	84	100	0	20
Filipendula ulmaria	25	0	10	26	38	4	60	76	57	81	10	74	0	1	0	21	42
Galium palustre	2	21	29	4	5	49	4	0	0	37	16	9	0	2	0	0	2
Galium uliginosum	0	0	0	0	0	0	0	32	0	0	0	0	0	0	0	0	0
Galium saxatile	0	0	0	1	0	0	0	0	36	0	0	0	0	0	0	0	0

Code	Species Names & Authorities	LB1	LB2	LB3	LB4	LBM	CL1	CL2	CL3	CL4	CL5	RB	KM1	KM2	KM3	BM1	BM2	BM3
	Galium verum	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
gang	*Genista anglica*	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0
	Glyceria fluitans	0	46	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Heracleum mantegazzianum	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0
	Hippuris vulgaris	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Holcus lanatus	11	0	0	0	4	0	0	1	15	0	0	23	1	0	92	0	12
	Hydrocotyle vulgaris	0	0	0	49	0	0	0	0	0	0	4	0	0	0	0	0	0
	Iris pseudacorus	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Juncus acutiflorus	0	0	0	0	0	0	0	0	0	0	86	0	0	0	0	0	0
	Juncus articulatus	2	1	8	12	16	0	0	0	0	1	1	0	0	0	0	0	0
	Juncus effusus	0	0	0	1	18	0	0	1	0	1	0	30	0	0	0	16	14
	Juncus inflexus	18	0	0	0	1	0	0	0	0	0	0	34	6	22	0	18	96
lpal	*Lathyrus palustris*	0	0	0	0	0	0	3	1	0	7	0	0	0	0	0	0	0
lpra	*Lathyrus pratensis*	1	0	0	0	7	0	2	30	0	0	0	6	0	0	14	0	0
	Leontodon autumnalis	6	0	0	29	1	0	5	2	1	0	0	0	0	0	0	0	0
	Lolium perenne	51	1	0	0	43	0	0	0	0	0	0	0	0	0	0	0	0
lcor	*Lotus corniculatus*	0	0	0	4	0	0	1	0	0	0	0	0	0	0	9	30	27
lped	*Lotus pedunculatus*	0	0	0	0	0	0	0	0	0	0	32	3	0	18	0	0	0
	Lychnis flos-cuculi	0	0	0	0	0	0	0	1	3	0	0	0	0	0	0	2	2
	Lysimachia vulgaris	0	0	0	0	0	0	9	0	0	0	0	0	0	0	0	0	0
	Lysimachia nummularia	1	0	3	0	0	5	0	1	0	0	0	0	0	0	0	0	1
	Lythrum salicaria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	Luzula campestris	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
	Mentha aquatica	0	22	44	0	0	12	0	0	0	1	0	0	0	0	0	1	0
	Molinia caerulea	0	0	0	0	0	0	0	0	15	0	34	0	100	100	0	0	0

Species Names & Authorities	LB1	LB2	LB3	LB4	LBM	CL1	CL2	CL3	CL4	CL5	RB	KM1	KM2	KM3	BM1	BM2	BM3
Myosotis laxa	0	6	10	1	0	0	1	0	0	0	0	0	0	0	0	0	0
Myosotis scorpiodes	0	0	0	0	0	8	3	0	1	0	0	0	0	0	0	0	0
Narthecium ossifragum	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
Odontites vernus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
Oenanthe fistulosa	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Pedicularis palustris	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0
Persicaria amphibia	7	5	3	1	0	7	6	0	1	0	0	0	0	0	0	0	0
Persicaria bistorta	2	0	1	0	9	0	0	0	0	0	0	0	0	0	0	0	0
Phalaris arundinacea	0	14	38	0	0	36	0	0	0	0	0	0	0	0	0	28	0
Phleum pratense	64	0	0	0	39	0	32	0	0	4	0	0	0	0	0	0	0
Plantago lanceolata	33	0	0	17	19	0	38	39	31	0	0	26	0	0	6	0	0
Poa pratensis	0	0	0	1	0	0	0	0	3	0	0	0	0	0	0	0	0
Poa trivialis	24	0	0	0	18	0	4	2	0	0	0	0	0	0	0	0	0
Potentilla anserina	0	1	0	15	0	0	0	0	0	0	0	0	0	0	0	0	0
Potentilla erecta	1	0	0	0	0	0	1	5	3	0	13	0	28	49	0	0	0
Potentilla palustris	0	0	0	3	0	0	0	1	1	2	9	0	0	0	0	6	0
Prunella vulgaris	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
Ranunculus acris	12	0	1	5	3	0	7	10	10	1	0	0	0	1	0	0	0
Ranunculus flammula	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0
Ranunculus lingua	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	1
Ranunculus repens	39	11	37	35	36	25	18	31	14	13	0	0	0	1	4	0	2
Rhinenthus minor	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0
Rorippa nasturtium-aquaticum	0	1	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0
Rumex acetosa	3	0	0	1	20	0	26	14	7	0	16	21	0	0	25	0	26
Rumex crispus	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0

Code	Species Names & Authorities	LB1	LB2	LB3	LB4	LBM	CL1	CL2	CL3	CL4	CL5	RB	KM1	KM2	KM3	BM1	BM2	BM3
	Rumex obtusifolius	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
	Scutellaria galericulata	0	0	0	0	0	0	0	0	0	0	7	0	0	0	0	0	0
	Scutellaria minor	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
	Senecio aquaticus	1	1	9	3	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sonchus palustris	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	Stachys officinalis	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0
	Stellaria palustris	0	0	0	0	0	3	0	0	0	0	0	7	0	0	0	0	1
	Succisa pratensis	1	0	0	1	5	0	1	4	9	1	0	1	0	0	2	0	0
	Taraxacum sp.	9	0	1	6	3	0	9	3	0	0	0	0	0	0	4	0	0
	Thalictrum flavum	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
	Triglochin palustre	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
tdub	*Trifolium dubium*	1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
tpra	*Trifolium pratense*	2	0	0	0	2	0	0	1	1	0	0	0	0	0	0	0	0
trep	*Trifolium repens*	20	0	0	6	8	0	1	0	2	16	0	9	0	0	0	0	0
	Valeriana officinalis	0	0	0	0	0	0	0	1	0	0	13	2	0	0	0	0	0
	Veronica catenata	0	6	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
vcra	*Vicia cracca*	5	0	0	0	1	0	33	11	7	4	0	0	0	0	0	0	0
	Viola palustris	0	0	0	0	0	0	0	0	0	0	20	0	0	0	0	0	0

APPENDIX C: Kjeldahl digestion method for determining total N

The Kjeldahl method is most often used to determine total nitrogen (N) in soils, and plant material (Nelson & Sommers, 1973), the alternative being the Dumas combustion technique. The Kjeldahl procedure involves the digestion of the dried sample by boiling in sulphuric acid, with a salt to raise the boiling point (usually potassium sulphate) and a catalyst to promote the oxidation of organic matter (e.g. mercury, selenium or copper) until oxidation of organic material is complete and organic nitrogen has been converted to ammonia. The concentration of ammonium-N (and thus total N) can then be determined from the digest via colorimetry or titration.

Before the 1960's, Kjeldahl analysis was carried out using macro methods (e.g. Bremner, 1965), where large digestion flasks (350-800 ml) were used. After this time, semi-micro methods (e.g. Goh, 1972) were developed using much smaller samples and flasks (30-50 ml). Semi-micro-methods are those commonly used today (Bremner & Mulvaney, 1982).

Methods used were:

Preparation

Dried samples were ground coarsely using a mill with blades and then to a fine powder using a ball mill.

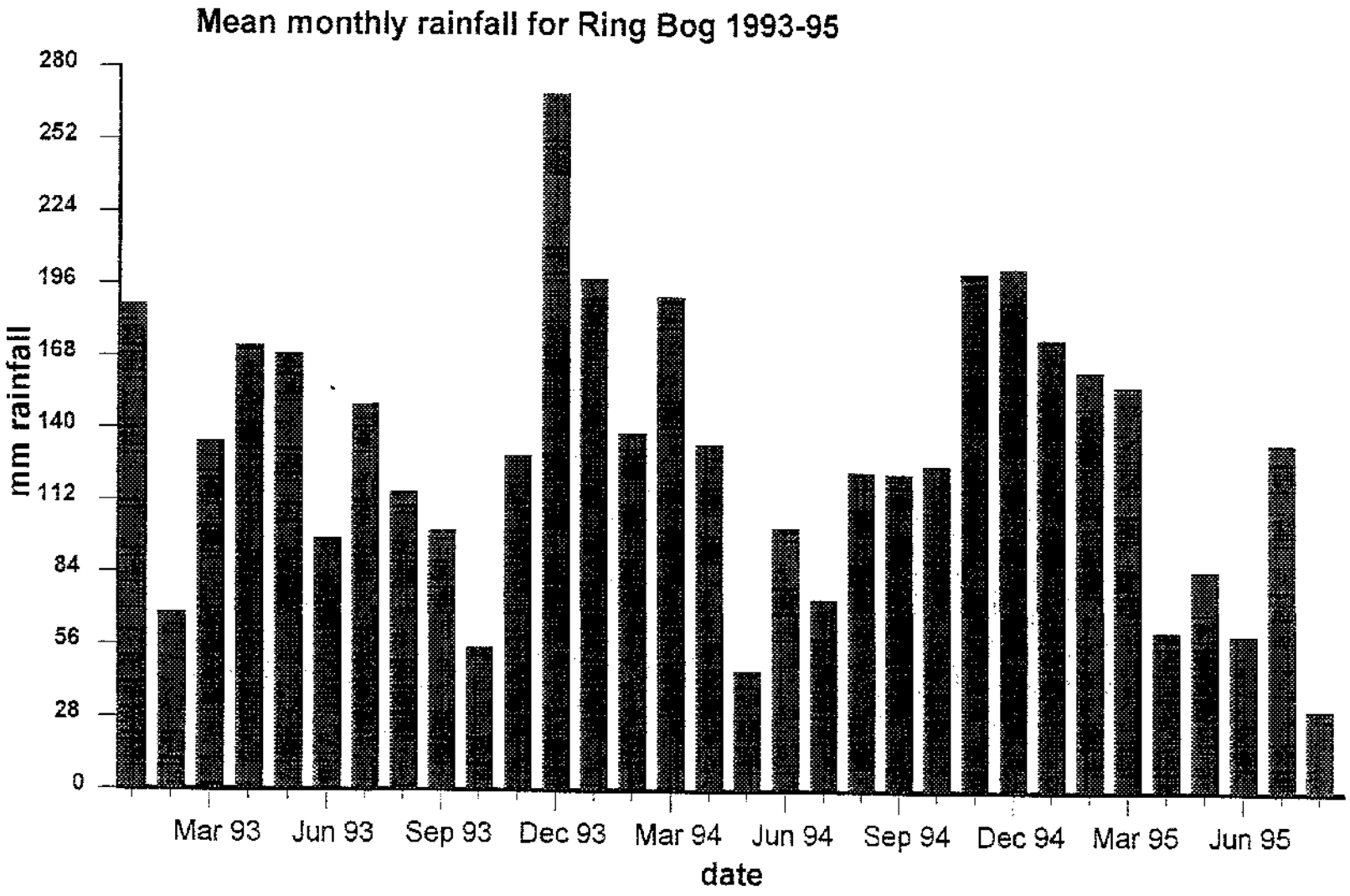
Digestion

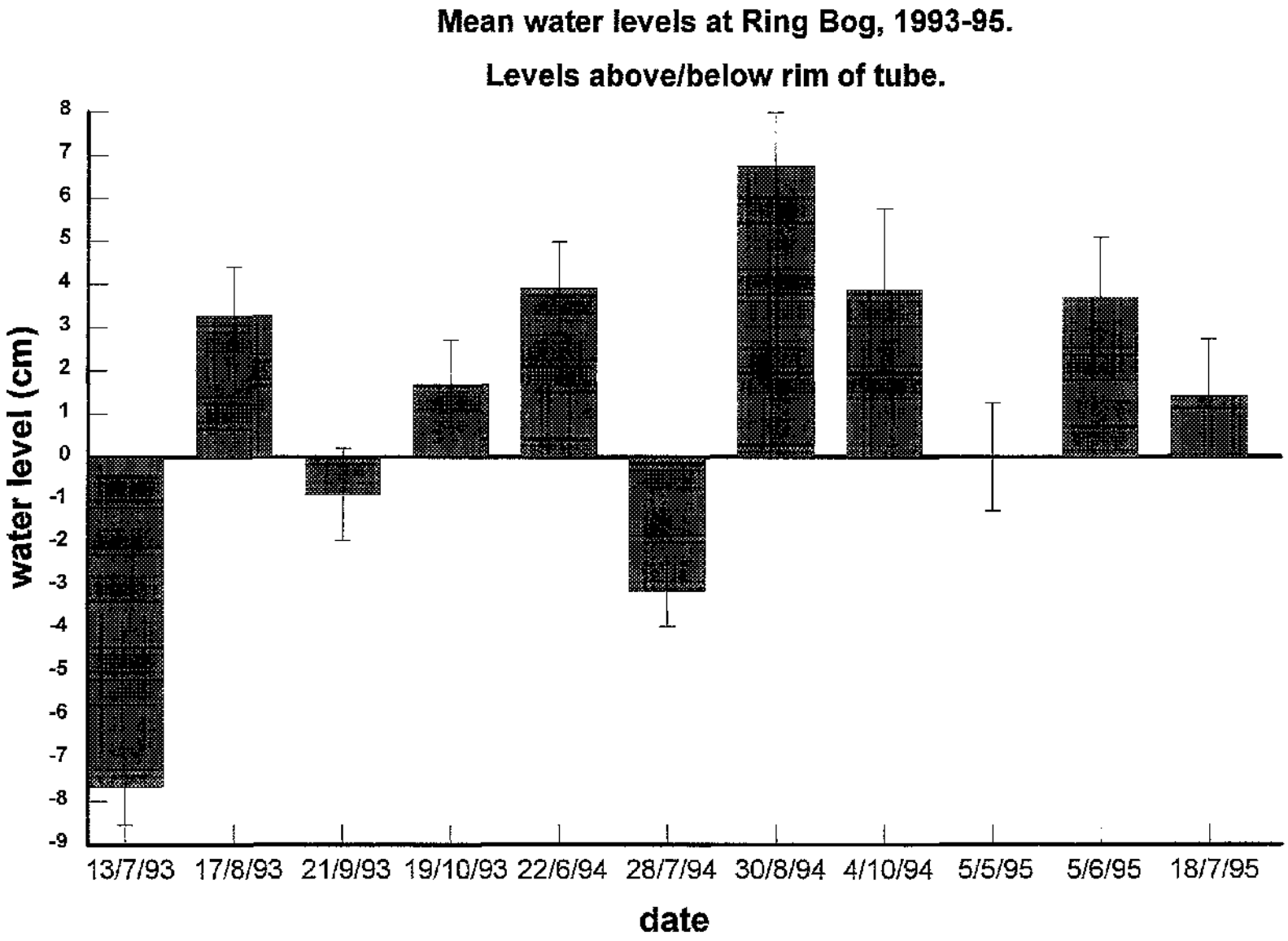
0.1 - 0.3 g ground sample was placed into a digestion tube. 2 ml of conc. H_2SO_4 and a small spatula tip of catalyst (8g potassium sulphate, 1g copper sulphate, 1g mercuric oxide) were added. If the sample was a field grown plant that might contain nitrate then salicylic acid was also added (30 g salicylic acid to 1 l of H_2SO_4). A blank tube of acid and catalyst only was used as a control. The digestion block was heated gradually up to 330^{o}C once the acid fumes had evolved. The flasks were turned frequently throughout the digestion, until the digest turned green - colourless. After a further 30 minutes the tubes were removed and allowed to cool, the sides of the tube were washed with distilled water and the digest made up to 25 ml with distilled water in a volumetric flask.

Steam-distillation and Titration

5 ml of digested sample was steam distilled for 3 minutes with 40% NaOH and collected in 5 ml of 2% boric acid plus 3 drops of mixed indicator. The distillate was then titrated with 0.01N HCl until the indicator turned from green to pink.

APPENDIX D: Rainfall and water level data for Ring Bog


Mean monthly rainfall for Ring Bog 1993-95
mm rainfall
280
252
224
196
168
140
112
84
56
28
0
Mar 93
Jun 93
Sep 93
Dec 93
Mar 94
Jun 94
Sep 94
Dec 94
Mar 95
Jun 95
date


Mean water levels at Ring Bog, 1993-95.
Levels above/below rim of tube.
water level (cm)
8
7
6
5
4
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
13/7/93
17/8/93
21/9/93
19/10/93
22/6/94
28/7/94
30/8/94
4/10/94
5/5/95
5/6/95
18/7/95
date

APPENDIX E: Nitrogenase activity of legumes measured *in situ* (μmoles ethylene evolved g^{-1} nodule dry weight)

L. pratensis and *V. cracca* (Clonmacnoise) 1993

	L. pratensis				*V. cracca*		
23/05/93	0.5 HR	1.5 HR	2.5 HR	23/05/93	0.5 HR	1.5 HR	2.5 HR
	145.8	802.4	865.9		177.4	549.5	614.4
	20.2	41.0	40.7		10.1	99.0	168.9
	0.0	0.0	0.0		27.5	51.1	54.3
MEAN	55.3	281.1	302.2		71.7	233.2	279.2
STDEV	78.9	451.9	488.6		92.0	275.0	295.9
STERR	45.5	260.9	282.1		53.1	158.7	170.8
	L. pratensis				*V. cracca*		
27/06/93	0.5 HR	1.5 HR	2.5 HR	27/06/93	0.5 HR	1.5 HR	2.5 HR
	6.0	98.9	156.3				
	0.0	23.2	479.6				
	0.0	0.0	0.0		0.0	1219.4	1410.2
	0.0	349.3	461.7		8.9		95.5
MEAN	1.5	117.9	274.4		4.4	1219.4	752.9
STDEV	3.0	160.0	235.5		6.3		929.6
STERR	1.5	80.0	117.8		4.4		657.3
	L. pratensis				*V. cracca*		
01/10/93	0.5 HR	1.5 HR	2.5 HR	01/10/93	0.5 HR	1.5 HR	2.5 HR
	31.1	189.7	329.7				
	0.0	344.0	371.5		372.2	578.5	1029.5
	57.7	69.8	69.5		253.4	316.6	443.8
MEAN	29.6	201.2	256.9		312.8	447.6	736.6
STDEV	28.9	137.5	163.7		84.0	185.2	414.1
STERR	16.7	79.4	94.5		59.4	130.9	292.8

T. dubium and *T. pratense* (Little Brosna) 1993

	T.dubium				***T. pratense***		
25/05/93	0.5 HR	1.5 HR	2.5 HR	25/05/93	0.5 HR	1.5 HR	2.5 HR
	17.1	30.6	34.3		27.0	99.4	111.0
	45.1	77.1	114.2		41.5	90.3	113.5
	0.0	0.0	0.0		77.6	97.6	123.6
MEAN	20.7	35.9	49.5		48.7	95.8	116.0
STDEV	22.8	38.8	58.6		26.0	4.8	6.7
STERR	13.1	22.4	33.8		15.0	2.8	3.9
	T.dubium				***T. pratense***		
29/06/93	0.5 HR	1.5 HR	2.5 HR	29/06/93	0.5 HR	1.5 HR	2.5 HR
	0.0	30.9	115.7				
	0.0	263.7	518.4		0.0	81.3	613.6
	0.0	0.0	0.0			195.3	
MEAN	0.0	98.2	211.4		0.0	138.3	613.6
STDEV	0.0	144.1	272.1			80.6	
STERR	0.0	83.2	157.1			57.0	
	T.dubium				***T. pratense***		
28/07/93	0.5 HR	1.5 HR	2.5 HR	28/07/93	0.5 HR	1.5 HR	2.5 HR
	83.7	293.4					
	156.5	217.5	304.8		155.3	242.3	356.6
	1026.3	1750.4	1896.8		191.5	404.8	1672.5
	75.6	155.7	785.4		16.4	32.0	68.5
MEAN	335.5	604.3	995.6		121.1	226.3	699.2
STDEV	461.9	766.1	816.6		92.4	186.9	855.2
STERR	231.0	383.1	471.5		53.4	107.9	493.7

L. pedunculatus (Ring Bog) 1993-94

08/06/93	0.5 HR	1.5 HR	2.5 HR	08/06/93	0.5 HR	1.5 HR	2.5 HR
	6.2	296.7	320.5		0.0	10.4	146.8
	105.2	238.3	237.5		2.1	0.0	0.0
	0.0	0.0	0.0		9.5	23.1	26.2
	0.0	0.0	0.0		65.0	122.2	170.1
	0.0	0.0	0.0				
MEAN	**22.3**	**107.0**	**111.6**		**19.2**	**38.9**	**85.8**
STDEV	**46.4**	**148.0**	**155.6**		**30.8**	**56.3**	**85.1**
STERR	**20.8**	**66.2**	**69.6**		**15.4**	**28.2**	**42.6**
17/08/93	**0.5 HR**	**1.5 HR**	**2.5 HR**	**17/08/93**	**0.5 HR**	**1.5 HR**	**2.5 HR**
	293.0	336.2	567.1		129.1	144.4	160.8
	49.5	58.8	72.4		39.6	46.3	75.8
	68.8	193.9	227.8		328.3	479.5	570.4
	784.4	849.6	1766.2		79.0	117.4	135.0
	43.0	181.2	330.7				
MEAN	**247.7**	**324.0**	**592.8**		**144.0**	**196.9**	**235.4**
STDEV	**317.5**	**309.9**	**680.1**		**128.2**	**192.9**	**226.1**
STERR	**142.0**	**138.6**	**304.2**		**64.1**	**96.4**	**113.1**
22/06/94	**0.5 HR**	**1.5 HR**	**2.5 HR**	**22/06/94**	**0.5 HR**	**1.5 HR**	**2.5 HR**
	45.0	113.9	230.5		0.0	16.5	20.1
	0.0	3.8	47.7		26.4	55.9	65.2
	68.1	115.1	282.9		33.2	64.4	111.7
	27.6	54.9	90.2		23.8	42.3	76.5
	31.3	45.0	114.3		16.2	41.0	67.0
MEAN	**34.4**	**66.5**	**153.1**		**19.9**	**44.0**	**68.1**
STDEV	**24.9**	**47.8**	**99.3**		**12.7**	**18.2**	**32.7**
STERR	**11.2**	**21.4**	**44.4**		**5.7**	**8.1**	**14.6**
30/08/94	**0.5 HR**	**1.5 HR**	**2.5 HR**	**30/08/94**	**0.5 HR**	**1.5 HR**	**2.5 HR**
	88.7	160.8	209.5		0.0	0.6	6.9
	32.8	53.9	96.5		0.0	12.1	22.0
	178.7	199.7	204.0		16.9	31.1	46.2
	116.4	125.4	196.8		6.1	10.8	31.7
	224.2	235.6	248.5		46.3	95.2	95.8
MEAN	**128.2**	**155.1**	**191.0**		**13.8**	**30.0**	**40.5**
STDEV	**76.1**	**70.0**	**56.6**		**19.4**	**38.1**	**34.1**
STERR	**33.6**	**31.3**	**25.3**		**8.7**	**17.0**	**15.2**

APPENDIX F: Plant species frequency (%) for Ring Bog 'enclave' area (30/6/93)

SPECIES	MEAN	STERR
Agrostis stolonifera	**68.80**	**2.03**
Angelica sylvestris	17.33	0.84
Caltha palustris	2.13	0.43
Cardamine hirsuta	1.07	0.21
Carex curta	**12.00**	**1.58**
Carex echinata	1.33	0.22
Carex nigra	25.33	1.65
Carex panicea	5.87	1.17
Carum verticullatum	0.80	0.16
Crepis palludosa	2.67	0.53
Dactylorhiza fuchsii	0.53	0.07
Deschampsia cespitosa	6.93	1.33
Epilobium palustre	1.60	0.23
Equisetum fluviatile	2.93	0.26
Eriphorum angustifolium	2.67	0.38
Festuca rubra	13.33	1.43
Filipendula ulmaria	16.80	1.48
Galium palustre	6.40	0.48
Galium saxatile	7.20	0.86
Holcus lanatus	29.33	2.20
Hydrocotyle vulgaris	8.80	1.25
Juncus acutiflorus	**94.40**	**0.76**
Juncus effusus	1.87	0.23
Lotus pedunculatus	**23.20**	**1.69**
Lychnis flos-cucculi	0.80	0.12
Lycopus europaeus	1.33	0.27
Luzula campestris	1.07	0.17
Molinia caerulea	**54.67**	**2.27**
Narthecium ossifragum	2.13	0.43
Polytrichum commune	**6.67**	**0.91**
Potentilla erecta	36.53	1.91
Potentilla palustris	2.67	0.24
Ranunculus repens	6.93	1.33
Rhytidiadelphus squarrosus	72.27	2.21
Rumex acetosa	48.00	1.99
Scuttelaria galericulata	6.40	0.79
Sonchus palustris	0.53	0.07
***Sphagnum* spcs.**	**33.33**	**2.22**
Stellaria palustris	10.93	0.52
Succisa pratensis	9.07	1.08
Valeriana officinalis	28.00	1.43
Viola palustris	13.07	0.86

Those in bold were shown to incorporate $^{15}N_2$ in Chapter 4 (A & B).

STERR = standard error

NVC CLASSIFICATION: M23a *Juncus effusus/acutiflorus-Galium palustre*

CORINE BIOTOPE: C37.217 Soft rush meadows

APPENDIX G: Nitrogenase activity of soil measured *in situ* (μmoles ethylene evolved m^{-2}) and moss (μmoles ethylene evolved g^{-1} dry weight)

Clonmacnoise and Little Brosna (1993)

	Clonmacnoise						
23/05/93	0.5 HR	1.5 HR	2.5 HR	27/06/93	0.5 HR	1.5 HR	2.5 HR
	63.1	97.4	168.3		0.0	0.0	0.0
	3.8	14.9	17.2		0.0	11.3	102.8
	20.6	29.2	158.7		0.0	0.0	25.7
	105.6	190.9	203.7		0.0	0.0	0.0
	0.0	0.0	0.0		0.0	12.6	141.5
MEAN	38.6	66.5	109.6		0.0	4.8	54.0
STDEV	45.0	78.9	93.9		0.0	6.5	64.6
STERR	20.1	35.3	42.0		0.0	2.9	28.9
01/10/93	0.5 HR	1.5 HR	2.5 HR				
	7.0	8.0	18.1				
	0.0	16.1	54.0				
	0.0	0.0	29.0				
	35.5	41.3	46.9				
	34.0	66.7	100.9				
	0.0	68.8	72.1				
	56.7	191.2	206.6				
MEAN	19.0	56.0	75.4				
STDEV	22.9	65.6	64.0				
STERR	8.7	24.8	24.2				
	Little Brosna						
25/05/93	0.5 HR	1.5 HR	2.5 HR	34149.0	0.5 HR	1.5 HR	2.5 HR
	0.0	0.0	78.9		0.0		591.2
	0.0	0.0	0.0		0.0		827.3
	0.0	41.3	63.1		0.0	0.0	95.5
	32.9	55.2	60.2		0.0	0.0	192.9
	59.7	65.8	67.2		0.0	0.0	158.2
MEAN	18.5	32.5	53.9		0.0	0.0	373.0
STDEV	27.1	30.9	30.9		0.0	0.0	320.0
STERR	12.1	13.8	13.8		0.0	0.0	143.1
28/07/93	0.5 HR	1.5 HR	2.5 HR				
	12.2	52.9	284.5				
	185.3	190.3	373.2				
	12.2	51.5	94.4				
	0.0	0.0	93.9				
	0.0	5.6	98.0				
	0.0	4.9	52.9				
	0.0	0.0	0.0				
MEAN	29.9	43.6	142.4				
STDEV	68.7	68.8	134.4				
STERR	26.0	26.0	50.8				

Ring Bog (1993) and moss (1994)

08/06/93	0.5 HR	1.5 HR	2.5 HR	13/07/93	0.5 HR	1.5 HR	2.5 HR
	0.0	0.0	0.0		46.3	51.4	62.0
	0.0	0.0	0.0		75.6	142.4	156.7
	0.0	0.0	64.3		26.8	31.5	51.7
					0.0	4.6	61.9
MEAN	**0.0**	**0.0**	**21.4**		**37.2**	**57.5**	**83.1**
STDEV	**0.0**	**0.0**	**37.1**		**31.9**	**59.7**	**49.3**
STERR	**0.0**	**0.0**	**21.4**		**15.9**	**29.9**	**24.7**
17/08/93	0.5 HR	1.5 HR	2.5 HR	17/08/93	0.5 HR	1.5 HR	2.5 HR
	161.5	194.7	205.7		145.7	170.5	278.2
	33.0	276.0	388.4		39.9	105.9	139.5
	152.1	158.3	177.1		184.8	315.6	390.3
					587.6	691.5	815.3
MEAN	**115.6**	**209.7**	**257.1**		**239.6**	**320.9**	**405.8**
STDEV	**71.6**	**60.3**	**114.6**		**240.1**	**262.2**	**291.6**
STERR	**41.4**	**34.8**	**66.2**		**120.1**	**131.1**	**145.8**
	Polytrichum commune				***Sphagnum palustre***		
07/09/94	0.5 HR	1.5 HR	2.5 HR	07/09/94	0.5 HR	1.5 HR	2.5 HR
	0.0	0.0	0.1		0.1	0.1	0.2
	0.1	0.1	0.2		0.1	0.1	0.1
	0.1	0.1	0.1		0.0	0.1	0.1
MEAN	0.1	8.8	16.6		0.1	0.1	0.1
STDEV	0.0	17.4	33.0		0.1	0.0	0.0
STERR	0.0	0.0	0.0		0.0	0.0	0.0

APPENDIX H: Growth media (rhizobia) and nutrient solutions

Yeast - mannitol agar

Mannitol	10.0g
diPotassium phosphate	0.5g
Magnesium sulphate	0.2g
Sodium chloride	0.2g
Calcium chloride	0.2g
Ferric chloride	0.01g
Yeast extract	0.4g
Agar	15.0g

(pH adjusted to 6.8) per litre distilled water

Peptone glucose agar

glucose	5g
peptone	10g
agar	15g

per litre distilled water

Crone's nutrient solution

Potassium chloride	375g	
Calcium sulphate	182g	(dihydrate =250g)
Magnesium sulphate	250g	
Calcium phosphate	125g	
Iron orthophosphate	125g	

All salts were first finely powdered, then mixed in a mill with blades. 0.5g of this mixture was added to each 1 l of distilled water, in the case of seedlings and young plants half this strength was used.

A- Z (Minor Elements)

Boric acid	0.62g	
Sodium silicate	0.43g	
Manganese chloride	0.40g	
Potassium permanganate	0.40g	
Copper sulphate	0.055g	
Zinc sulphate	0.055g	
Aluminium sulphate	0.055g	
Nickel sulphate	0.055g	per litre distilled water
Cobalt chloride	0.055g	
Titanium oxide	0.055g	
Lithium sulphate	0.035g	
Stannous chloride	0.035g	
Potassium iodide	0.035g	
Potassium bromide	0.035g	
Sodium molybdate	0.03g	

0.2 ml of A-Z was added to every 1 l of Crone's mixture, half this strength was used with seedlings and young plants.