AN ANALYSIS OF NODULE POPULATION DYNAMICS IN WHITE CLOVER (*TRIFOLIUM REPENS* L.) UNDER DIFFERENT ECOLOGICAL CONDITIONS

by

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THESIS SUBMITTED IN FULFILMENT OF THE DEGREE OF
DOCTOR OF PHILOSOPHY IN BOTANY

NORTH-EASTERN HILL UNIVERSITY
SHILLONG
1994

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I certify that the thesis entitled "**An analysis of nodule population dynamics in white clover** (*Trifolium repens* L.) **under different ecological conditions**" submitted by Debashis Dutta, for the degree of **Doctor of Philosophy** of the North-Eastern Hill University, Shillong, embodies the record of original investigation by him under my supervision. He has been duly registered and the thesis presented is worthy of being considered for the award of the **Ph.D. Degree**. The work has not been submitted for any degree of any other University.

Shillong
The 12th December 1994.

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ACKNOWLEDGMENTS

This doctoral dissertation is the culmination of the study that started in 1990.

My supervisor and teacher Professor R.S. Tripathi has been most supportive and encouraging during the whole process. His enthusiasm, knowledgeable guidance and criticism were most profitable during the course of this study. To him I express my heartfelt gratitude and sincere thanks. I am also grateful to Dr. H.N. Pandey for his valuable suggestions.

I am thankful to the Head, Department of Botany, North-Eastern Hill University, Shillong, for providing necessary laboratory facilities. I would also like to thank Dr. G. Gopal for help with the isolation of rhizobia, and Dr. Y. Kumar for field photography.

The completion of this study is largely due to the help and support received from my lab mates: Dr. A.K. Das, Dr. L. Boral, Dr. P. Rao, Ms. T. Lyngdoh, Dr. J. Misra, Dr. S.K. Barik, Dr. G. Pradhan, S. Dasgupta, Dr. U.K. Sahoo, Ms. S. Rynjah, Arunachalam and Ms. K. Maithani.

I would also like to thank my friends Suman, Virginia, Nora, Santa, Mahendra, Don, Mayashree, Nitul and Banita, and my cousins Ashish and Raja for their help in a number of ways.

I am most grateful to my parents for their encouragement and support.

My senior colleagues at the Botany Department, St. Edmund's College, deserve special thanks for their co-operation and valuable suggestions. I also thank the Principal of the college for allowing me to pursue the Ph.D. research.

Shillong
The 12 December 1994.

Debashis Dutta

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1 Introduction

Nitrogen is indispensable to plant life as it is an essential constituent of chlorophyll, proteins and protoplasm. This element is thus required in considerable amounts by the plants for growth, especially of the shoot system. Among the major elements, nitrogen is also the one which is readily lost from the ecosystem (Jefferies *et al*. 1981) as the rate of its uptake by the growing plants generally exceeds the rate at which it becomes available by decomposition of complex organic molecules into simpler inorganic forms. Consequently, soils rarely contain enough nitrogen for maximal plant growth. This deficit is usually compensated in forest ecosystems by nitrogen input through litter and in agro-ecosystems by the addition of nitrogenous fertilizers. However, in grasslands this compensation is rarely brought about as the rate of litter accumulation or input is minimal owing to the low above-ground biomass and its frequent partial removal due to grazing. This may result in the sward becoming moribund due to poor growth and decreasing ground cover thereby enhancing leaching and erosion losses of soil (Skeffington & Bradshaw 1980, Jefferies et al. 1981).

Of the large number of living organisms on the earth, only a few prokaryotic microorganisms, the diazotrophs, have the capacity to utilize atmospheric nitrogen for their growth by a process called biological nitrogen fixation. They are responsible for more than 60% of the earth's newly fixed nitrogen (Postgate 1982). Nitrogen fixation contributes substantially to the nitrogen status of arable lands and productivity of agroecosystems. In future agriculture, nitrogen fixation is expected to be of still greater importance since it offers an alternative to the energy-demanding production of nitrogenous fertilizers. Biological nitrogen fixation is only matched by industrial processes like the Haber-Bosch process where the nitrogen is reduced in the presence of powerful catalysts at very high temperature and pressure, for the production of ammonia. The microorganisms which fix nitrogen may be either free living or those which enter into symbiotic association with higher plants.

Symbiotic nitrogen fixation is one of the most important biological processes affecting plant production and soil fertility due to the large amounts of elementary nitrogen fixed by this system. These amounts, when calculated per unit of nitrogen-fixing cell material, are 100-200 times higher than those calculated for free living nitrogen fixers.

Among higher plants, two groups of nitrogen-fixing systems are known: (i) bacteria belonging to the genus *Rhizobium* living in symbiosis with leguminous plants like *Trifolium*, and (ii) micro-organisms belonging to the Actinomycetes (Frankia) which enter into symbiotic associations with non leguminous plants like *Alnus*, *Myrica* and *Hippophae* (Burns & Hardy 1975). These symbiotic associations are expressed morphologically by the formation of specialized galls called nodules on the host plant. Nodulation is, thus, a symptom of colonization by an endophyte and the formation of a diazotrophic symbiosis. These nodules are usually formed on the roots of the host, as in the above-mentioned symbiosis, but may be formed on the leaf (as in *Psychotria*) or on the stem (as in *Sesbania*). This symbiotic association between the two organisms involved, results in the development of an elaborate system which leads to the host plants becoming independent of soil nitrogen.

Leguminosae (Fabaceae) is one of the largest families of the higher plants comprising 3 sub-families (Papilionaceae, Mimosaceae and Caesalpinaceae) (Allen & Allen 1981). The family comprises about 640 genera with 12,000 species. The family is probably of tropical origin but now members of this family can be found in the tropical, sub-tropical, temperate and even in arctic regions (Allen *et al*. 1964). They range in habit from small forbs like *Trifolium* to trees like *Acacia*. Of the large number of legume species recorded, only *ca*. 10% of them have been examined for nodulation. Nodulation is widespread in Papilionaceae (85% of the examined species nodulate), less common among Mimosaceae (25%) and rare among Caesalpinaceae.

The nodule bacterium, Rhizobium belonging to the family Rhizobiaceae, includes a number of species which are soil saprophytes (Bergey 1984). They are capable of nodulating certain legumes and symbiotically fixing atmospheric nitrogen. Rhizobia are microaerobic and were, for long, thought to be obligate symbiotic diazotrophs, unable to fix nitrogen in the absence of the host plant. However, later some strains were shown to be capable of microaerobic diazotrophy *ex planta* (Gibson *et al*. 1977).

Each Rhizobium species comprises a number of different strains which may differ in their nodulating and nitrogen fixing ability. However, effectiveness of the symbiosis depends not only on the bacterium but also on the host legume and prevailing environmental conditions.

Symbiosis is attained after a series of successful events for which a synchronized co-operation between the legume and the endophyte is required. To begin with, a well established population of the bacteria in the rhizosphere of the potential host is a prerequisite for nodulation. The bacteria are attached to the root hair surfaces by linkages between specific polysaccharides secreted by the rhizobia and lectins present on the plant roots (Dazzo & Hubbell 1975). Adhesion of bacteria to root hairs leads to curling or deformation of the root hairs (Martensson 1980). This curling, commonly referred to as the shepherd's crook, entraps some of the bacteria. At this site an infection thread - an invaginated tube of root hair cell wall within which the entrapped rhizobia multiply and are confined in a chain along the lumen of the tube - develops (Callaham & Torrey 1981, Bhuvaneshwari 1984). As it grows, the 'thread' penetrates the basal cell of the root hair, grows between the cortical cells and ramifies, initiating some cortical cells to divide (Newcomb 1980) and finally a young nodule is formed containing the bacteria which are initially rod shaped but gradually become irregular bacteroids (Bergersen 1974). Leghemoglobin, an oxygen buffering protein, is synthesized within the nodules and serves as an oxygen barrier for the nitrogenase enzyme (which is sensitive to oxygen and is damaged by it). As the host plant supplies energy to the nodules in the form of photosynthates, nitrogenase is activated and begins the function of nitrogen fixation.

Nodulation is thus of immense ecological significance as the nodulated legumes contribute substantially to soil fertility by virtue of their remarkable nitrogen fixing capability. The nitrogen fixation rates associated with legumes like T. repens are at least ten times greater than those of non leguminous species (Skeffington & Bradshaw 1980) and have been reported to be as high as 620 Kg N $ha^{-1} yr^{-1}$ (Nutman 1974). This makes nodulated legumes the largest single contributors to fixed nitrogen in the biosphere. When grown in association with grasses, a considerably large part of this fixed nitrogen is released into the soil during decomposition of legume litter. Also, there is conclusive evidence to show that in a mixed population of Trifolium repens and Paspalum dilatatum grown at nil soil nitrogen level, some amount of nitrogen fixed by the legume is passed on to the grass (Pradhan & Tripathi 1985) even much before the decomposition of the legume litter takes place. Introduction of legumes into pastures or to degraded ecosystems is of much advantage as (a) the legumes use elementary atmospheric dinitrogen and thus an unending reserve of the element can be utilised, (b) the legume residues accumulating in the soil contain larger amounts of decomposable nitrogenous compounds than the grass residues, and (c) the use of expensive nitrogenous fertilizers can be done away with as the legume growth contributes nitrogen to the soil system and the energy cost of using legumes to maintain a sward has been estimated to be considerably less than using fertilizers (Laidlaw & Wright 1980). The use of legumes as a source of nitrogen supply in derelict ecosystems is an attractive proposition and an actively fixing legume can supply nitrogen to the root zone of a sward continuously in a way no fertilizer can (William & Cooper 1976).

T. repens forms an important pasture component of the grasslands in and around Shillong where it plays a significant role in enriching the nitrogen status of the swards. The clover is composed of two distinct leaf morph populations - one characterised by the presence of distinct 'V'-shaped white markings on the leaflets (marked population) and the other devoid of such leaf markings (unmarked population) (Plate 1). Two linked groups of genes control the leaf marking in _T. repens_ (Corkill 1971), which is due to the presence of air spaces within the palisade tissue, and is controlled by multiple alleles at a locus in one of these groups (Carnahan

Plate 1. A view of the study site showing profuse growth of <u>Trifolium repens</u>

et al. 1955). Views on the adaptive significance of the leaf markings in *T. repens* are contradictory. Charles (1968) is of the view that the marked leaves of clover might help grazing animals like sheep to form an image, so as to select it from the grass mixture because of its palatability, while Cahn & Harper (1976) have demonstrated that sheep avoid *T. repens* with marked leaves (Plate 2).

Though considerable work has been carried out on the *T. repens* - *R.trifolii* symbiosis, nodulation patterns in the two leaf morph populations have not been studied. A detailed analysis of the various ecological factors on nodulation of this legume has also not been carried out in spite of the fact that white clover is an important component of the Indian grasslands at higher elevations. The high annual rainfall received in the north-eastern states causes severe leaching of nutrients from the top soil, leaving it porous and infertile and therefore, a luxuriant growth of *T. repens* in the local grasslands assumes special significance in the maintenance of soil fertility. Even though white clover plays a pivotal role in the ecology of these swards, its importance has been grossly undermined. Consequently, this is a neglected field of research, except for studies by Pradhan (1984) and Pradhan & Tripathi (1985) on its competitive ability.

The present study was conducted in swards at Shillong as well as in the green house at the North-Eastern Hill University during 1990-1993. The major objectives of the study were as follows: (i) To determine the variations in the nodule population of *T. repens*

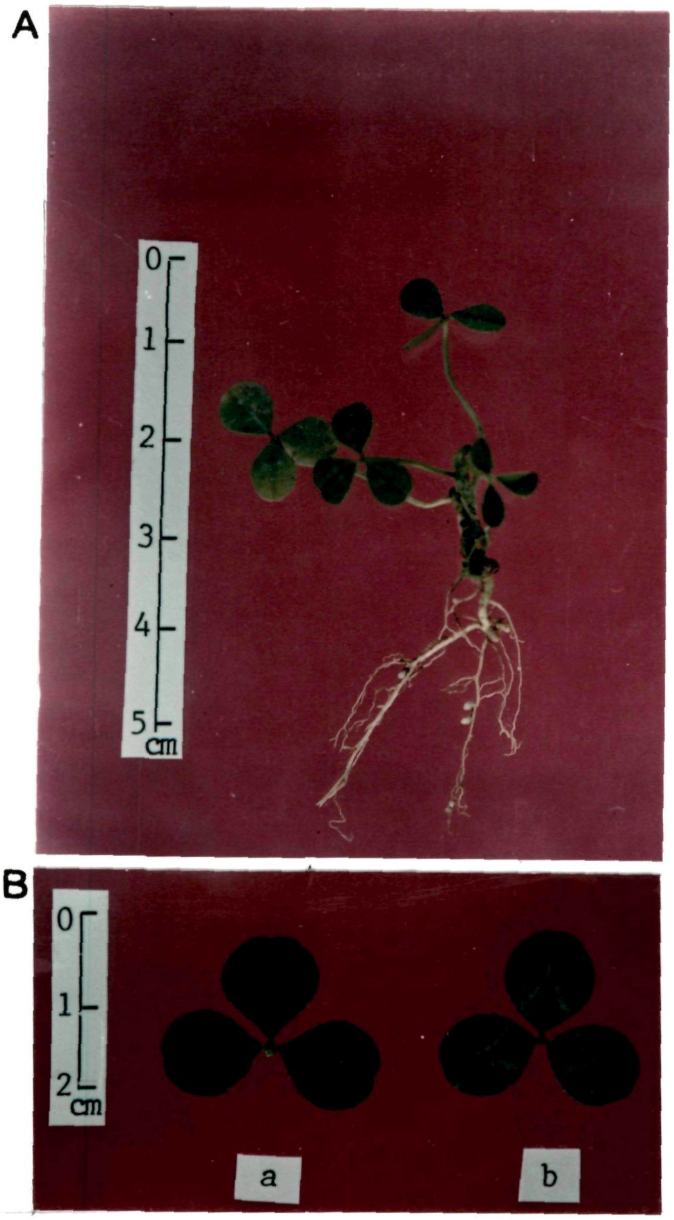

Plate 2. (A) *T. repens* plant bearing root nodules.
(B) Leaves of the (a) unmarked and (b) marked leaf morph populations.

under different ecological conditions, (ii) To determine the differences, if any, in the nodulation pattern in the marked and unmarked and unmarked leaf morph populations of T. repens, (iii) To determine whether nitrogen accumulation rates varied in the two leaf morph populations, and (iv) To suggest ecological conditions and management practices that could augment nodulation and consequent nitrogen fixation.

The thesis has been divided into ten chapters. The subject matter of the thesis has been introduced in Chapter 1 (the present chapter). A brief outline of the legume-rhizobium symbiosis and the main objectives of the study are also included in this chapter. Chapter 2 (Review of Literature) presents a review of the selected and important studies carried out on the subject of the thesis and related areas. Chapter 3 dealing with 'Materials and Methods' gives a brief description of the protocols followed for the different experiments and the materials used. Seasonal changes in nodule population and corresponding changes in soil nitrogen levels in field conditions have been presented in Chapter 4. The results of the greenhouse experiments are dealt with in Chapters 5-8. Chapter 5 embodies the results pertaining to the effects of substrate nitrogen on the nodule population. Chapter 6 deals with the nodulation pattern in relation to varying soil pH, and the effects of soil moisture content on nodule population have been presented in the following chapter (Chapter 7). Chapter 8 contains the results on the nodulation pattern in relation to defoliation and NPK levels. The results presented in Chapters 4-8 have been discussed in detail in each of these chapters. However, the findings of the studies on the various aspects have been briefly discussed in an integrated manner in Chapter 9 (General Discussion) and a summary of the entire study has been presented in Chapter 10.

2

Review of Literature

The atmosphere contains about 10^5 tonnes of N occurring as nitrogen gas and nearly ten times as much dissolved in oceans or occluded in rocks. However, the enormous quantity of atmospheric N is largely unavailable as a biological resource because the N_2 molecule is relatively inert. The soil bacteria classified as *Rhizobium* are characterised by their ability to successfully infect the root system of legumes (Bergey 1984) which results in the formation of nodules within which the bacteria fix atmospheric nitrogen into a form which can be assimilated by the host. This symbiosis between *Rhizobium and legumes has been recognised and exploited* in agriculture for long (cited by Fred *et al*. 1932). Among the legumes, *T. repens* occupies an important position due to its immense N_2 fixing capabilities which have been reported to be as high as 620 Kg N $ha^{-1} yr^{-1}$ (Nutman 1974). Besides, due to its stoloniferous habit, this legume can rapidly cover a large area, rooting at the nodes and bearing a profusion of N_2 fixing root nodules. This has resulted in legumes like T. repens being widely adopted for increasing the N status of degraded grasslands and other ecosystems. However, the efficient development and functioning of this symbiosis depends on conducive environmental conditions too. Considerable work has also been done over the years on various aspects of this symbiosis though the clues to details of the mechanism are being revealed only lately.

The scanning of literature reveals that studies on nodule formation in legumes and related aspects have engaged considerable attention. The studies made so far cover the following aspects:

(a) Host lectins and their role in symbiosis
(b) Bacterial polysaccharides and symbiosis
(c) Host response
(d) Role of phytohormones
(e) Nodule types
(f) Nutritional factors of the host affecting symbiosis
(g) Genes involved in symbiosis
(h) Ecological factors affecting symbiosis
(i) Reclamation studies

The significant studies relating to the above aspects have been reviewed in the following pages.

The host lectin

Hamblin & Kent (1973) were one of the first to report on the role of lectin in the legume - *Rhizobium* symbiosis, followed by another report that soybean-nodulating rhizobia interact specifically with soybean lectin (Bohlool & Schmidt 1974). In the following years numerous reports covering different legume - *Rhizobium* symbioses followed (Dazzo & Hubbell 1975, Kamberger 1979, Kato *et al*. 1979, Paau *et al*. 1981). This led to the proposal that recognition between rhizobia and legume roots involves a binding of the plant lectins to carbohydrates of the bacterial symbiont.

In the clover - *Rhizobium* symbiosis, a new lectin was discovered, which was named trifoliin A (after the host legume *Trifolium*) (Dazzo *et al*. 1978). Trifoliin A was found to have the unique property to be able to bind specifically to, and agglutinate *R. trifolii* even at very low concentrations. Interaction of this lectin with bacterial cells and their polysaccharides was also studied by several workers (Dazzo & Hubbell 1975, Dazzo & Brill 1977, Dazzo *et al*. 1978, Hrabak *et al*. 1981). Trifoliin A was found to be present in greater concentration at the growing tip of the root hairs than towards the base of the root hairs. The lectins of pea, alfalfa and soybean also show a similar trend (Gatehouse & Boulter 1980, Gade *et al*. 1981).

The mode of bacterial attachment to host root hairs was also studied by several workers. Microscopic studies of the clover - *Rhizobium* symbiosis by Napoli & Hubbell (1975) and Kumarasinghe & Nutman (1977), have shown multiple mechanisms (specific and non-specific) of bacterial attachment to root hairs. Dazzo *et al*. (1978) reported that in the case of specific mechanism a much larger number of bacterial cells of the selective species are attached per unit length of root hair. Similar host-specific diazotroph attachment has also been reported in soybean - *R. japonicum* (Stacey *et al.* 1980) and pea - *R. leguminosarum* (Kato *et al.* 1979) root systems. However, Chen & Phillips (1976) observed that specificity was not maintained when the number of rhizobia in the rhizosphere was very high. In the clover - *R. trifolii* symbiosis, the attachment was found to be strong in case the specificity was maintained, and loose if the same was non specific (Van Resenberg & Strijdom 1982). This probably results in successful infections following specific attachments.

Dazzo *et al.* (1976) and later Zurkowski (1980) suggested the involvement of trifoliin A in rhizobial attachment to clover root hairs. Involvement of respective host lectins of pea and soybean in attachment of diazotrophs was also reported (Stacey *et al.* 1980). Dazzo & Brill (1977) studied the interaction between clover root surfaces and trifoliin A - binding polysaccharides of *R. trifolii* and found that the bacterial capsule polysaccharide bound specifically to root hairs according to distribution of trifoliin A. Similar trends were reported from diazotrophic symbioses with other plants (Kato *et al.* 1979). Further, it was noticed that nitrate supplemented media resulted in reduction in trifoliin A on root hair surfaces of clover and corresponding decrease in attachment of rhizobia (Dazzo & Brill 1979).

The initial phase of attachment is followed by firm anchorage of the diazotroph to root hair surface. This triggers deformation and curling of the root hairs to different degrees (Napoli *et al.* 1975) resulting in the formation of a "shepherd's crook" and subsequent penetration of root hair cell wall. Dazzo & Gardiol (1985) have by scanning electron microscopy, recognised the presence of fibrillar material associated with attached bacteria. These fibrillar structures were reported to be produced by many rhizobia (Dienema & Zevenhuizen 1971) and though their true nature is not confirmed, Dazzo & Gardiol (1985) feel that they may play an important role in the post attachment adhesion process.

Polysaccharides

Considerable work has also been done on bacterial polysaccharides with an aim to identify which ones of these bind to the host lectin and help initiate the symbiosis. In the clover - *R. trifolii* symbiosis it has been reported that the diazotroph specifically binds to clover lectin (trifoliin A) through their capsular polysaccharide and their lipopolysaccharide (Kamberger 1979, Dazzo & Brill 1979). Exopolysaccharide (EPS) secretion was also shown to be dependent on a large molecular weight plasmid and related to ex-planta nitrogenase activity in *Rhizobium* (Udupa *et al.* 1991). Those strains that produce large quantities of EPS showed ex-planta activity of the nitrogenase enzyme.

Age-dependent changes in rhizobial polysaccharides have also received considerable attention. Hrabak *et al.* (1981) showed that in the early stationary phase of growth of *R. trifolii*, both the capsular polysaccharide and lipopolysaccharide bind to trifoliin A. They also worked out subsequent changes in polysaccharides in an ageing culture. In T. repens they reported that plants had more infected root hairs when inoculated with rhizobia in the early stationary phase than with cells in the mid-exponential phase.

Another substance that affects the infection process is the root exudate. The pretreatment of R. trifolii with exudates of clover causes an increase in the rate of infection as reported by Solheim (1975) and Napoli (1976).

Host response

An early structural response of the host is expressed as curling of root hairs which is probably due to inhibition of cell growth at the attachment site (Bauer 1981). This is common to both legume - rhizobium as well as majority of actinorrhizal associations (Callaham & Torrey 1977). The curling occurs to different degrees and forms the characteristic shepherd's crooks which entrap the rhizobia. Nutrients are probably concentrated within the crooks and a micro-environment is created which is conducive to invasion of the host (Callaham 1979). In their electron microscopic study, Callaham & Torrey (1981) postulated that rhizobia dissolve the host cell wall at a specific point, following which the plasma membrane of the root hair invaginates forming the infection thread with the rhizobia within. The endosymbiont is thus transported to host cells via the infection thread (Kijne 1992). According to Hirsch (1992), of the many root hairs that deform, only a small percentage form bona fide shepherd's crooks. Even the next step, i.e. infection thread formation and rhizobial penetration, are often unsuccessful as shown by Wood & Newcomb (1989). From their studies with alfalfa seedlings, they found that in a set of 10 seedlings with ca. 80,000 root hairs, only 27 root nodules were formed. These originated from a total of 52 infection threads, of which 2 were initiated in branched hairs, 17 in intertwined hairs and 33 in shepherd's crooks. This low rate of successful nodulation is probably due to a high incidence of abortion of the threads (Libbenga & Harkes 1973) even though they may penetrate up to the cortical cells (Dart 1977). Sprent & de Faria (1988) have compared the modes of infection in both leguminous and non-leguminous diazotrophic systems.

Phytohormones

Thimann (1936) was the first to suggest the involvement of auxin in pea root nodulation and since then plant hormones have been presumed to play a major role in nodulation. Following invagination of root hair cells and development of the infection thread, an early response of the host is the elicitation of cortical cell division (Newcomb et al. 1979). This is assumed to be initiated by a diffusible growth promoting substance produced by the endophyte. Besides implicating growth of infection threads and nodule morphogenesis this substance is probably responsible for endoreduplication and increase in ploidy levels of the infected cells (Libbenga et al. 1973).

The presence of a rhizobial 'nodule organogenesis-inducing principle' was postulated by Truchet _et al_. (1980). Although the exact nature of this 'inducer' is unknown, indole acetic acid (IAA) and cytokinin are probable contenders. These compounds have been identified unambiguously in several species of fast-growing rhizobia like _R. trifolii_ (Badnenoch-Jones _et al_. 1982).

Mitotic division of the cortical cells ultimately results in the formation of the nodule. Spatial dispositions adopted by dividing cells of the meristem cause the nodules of various legumes to differ in shape and gross anatomy with associated differences in fine structure.

Rhizobia, which have reached the nodule primordium via the infection thread, are released into cells from wall-less branches of the threads, the infection droplets (Newcomb 1976). According to Kijne (1992) this is brought about probably by a phagocytosis-like process. Bacteria from the infection droplets eventually become enclosed by a plant-derived membrane - the peribacteroid membrane (Newcomb 1976, Robertson _et al_. 1978) or the symbiosome membrane (Streeter 1991).

Nodule types

The type of nodule that ultimately develops depends on the host and not on the rhizobial strain (Dart 1977, Newcomb 1981). Sprent (1989) divided root nodules into 2 broad classes - determinate and indeterminate. The two main classes of nodules can be distinguished from one another in a number of ways. **Determinate nodules** are usually spherical with no persistent meristem. The infection threads are relatively short and thus have little or no involvement in distribution of bacteria to the nodule cells. Bacteroids are only slightly enlarged and enclosed in groups of two or more within membrane-enclosed vesicles which occupy a large proportion of the host cell volume. **Indeterminate nodules** are elongated, with a persistent meristem which gives rise to a nodule whose tissues are of graded age from the meristem to the point of attachment and consequently, the vascular system is open. The bacteroids are almost always grossly enlarged (10-20 times the original size) with a single bacteroid almost completely filling its membrane-enclosed vesicle. Cytoplasm occupies a small portion of host cell volume. This type of nodule is common among temperate legumes as in clover infected with rhizobia. In _T. repens_, the nodules are of the indeterminate, crotalarioid type.

Although shapes of nodules vary, there are certain significant common features amongst them. All contain centrally a relatively large proportion of tissue composed of enlarged parenchymatous cells. The cytoplasm is packed with the enlarged bacteria, the bacteroids. The volume of the central tissue and the duration for which it remains in an intact state are the major determinants of the extent of N_2 fixation (Chen & Phillips 1976). With age and/or stress, the bacteroid containing tissue breaks down - centrally in determinate and from the base in indeterminate nodules. All nodules have a spongy cortex, divided into inner and outer portions by an endodermal layer. The central tissue, which is derived from cells of the nodule meristem, contain both infected and uninfected cells. Studies by Dart (1977) and Newcomb (1981) have revealed that a maximum of about 50% of the total cells are infected by Rhizobium, interspersed with uninfected cells. In soybean, these interstitial cells outnumber the infected cells by ca. 3:2 (Vanden Bosch & Newcomb 1988). The DNA content of the infected cells also increases several fold (Libbenga & Bogers 1974). Further details on cellular organisation have been dealt with by Verma & Nadler (1985) and Hirsch (1992).

Nutritional role of the host

In the legume - rhizobium symbiosis, the endosymbiont is known to depend on the host for its carbon requirement and in turn supplies the legume with fixed nitrogen. However, Verma et al. (1979) have established that N_2 fixation commences at least ten days after infection. Since the process of N_2 fixation is very energy intensive and is dependent on the carbohydrate supply from the host, the onset of fixation cannot be delayed as a N-deficient situation can prove deleterious to the host.

Carbohydrate supply: Hardy & Havelka (1976) have shown the non-availability of photosynthates as a major limiting factor in N_2 fixation of legumes. The photosynthates are continuously required both as a respiratory substrate (for host and endosymbiont) as well as as carbon skeletons for assimilation of nascent ammonia. Thus factors that tend to increase photosynthate supply, like high carbon dioxide levels and increased illumination, also tend to increase N_2 fixation. Therefore the N_2 fixation rates decrease due to defoliation or dark phases which adversely affect photosynthesis. The length of time that a species can continue fixing nitrogen without simultaneous photosynthesis depends on the amount of carbohydrates stored.

The respiratory substrates required by the host and the endosymbiont vary and a number of reports are available on the varying alternatives (Bergersen 1974, Glenn & Brewin 1981, Ronson _et al._ 1981). Studies by Ronson & Primrose (1979) on _R. trifolii_ have shown that the endophyte utilizes dicarboxylic acids to support N_2 fixation. Mutant forms of rhizobia defective in dicarboxylic acid transport failed to grow on other alternative carbon sources like fumarate, succinate and malate, and formed ineffective nodules on clover (Ronson _et al._ 1981). However, the ineffective nodules contained morphologically normal bacteroids and this prompted the suggestion that though organic acids were the substrates for N_2 fixation, other carbon sources could be utilised up to the nodule formation stage. These organic acids may also be essential as substrates for heme synthesis in rhizobia (Ronson & Primrose 1979, Ronson _et al._ 1981).

Nitrogen metabolism: In the legume - rhizobium symbiosis, the endophyte is responsible for 'fixing' atmospheric N_2 and transporting the same to the host cells in a stable form (O'Gara & Shanmugam 1976). The host plays an apparently secondary role in further assimilation of the supplied nitrogen, functioning more as a sink for the fixed nitrogen. However, studies have revealed that the host too provides a range of nitrogenous compounds like amino acids, besides other nutrients, to the endophyte during symbiosis (Schwinghamer 1970). Thus mutant strains of Rhizobium requiring amino acids, may still induce effective nodules indicating that the defeciency of the endosymbiont is compensated by the host.

According to Bergersen (1974), apart from carbon and nitrogen compounds, the symbionts are also involved in the exchange of iron, sulphur, heme, cobalt and molybdenum.

Genes involved

Considerable work has also been done on the genetics of the legume - Rhizobium symbiosis. This can be grouped under two broad heads - studies on host genes involved in symbiosis, and studies on genetics of Rhizobium. The genetics of the host seems to play a more important role as several characters of the bacteroids are affected by the given host (Sutton et al. 1981). Inheritable characteristics in the host plant are also known to affect practically all aspects of symbiotic N_2 fixation including size and number of nodules, time of appearance, nodule morphogenesis and N_2 fixing activity (Vincent 1980, Verma & Long 1983). Also, Brewin (1991) has shown that hydrogenase activity is host-controlled. Verma & Nadler (1985) among others, have dealt with this aspect in considerable detail.

The genetics of the endosymbiont has also been the focus of a number of studies. Among them, the work of Rolfe and his co-workers is particularly noteworthy (Rolfe *et al*. 1980, 1981, Chakravorty *et al*. 1982). The importance of extrachromosomal genetic matter or plasmids in nodulation and in host specificity has also been established by Zurkowski (1980), Zurkowski & Lorkiewicz (1979) and Morrison *et al*. (1983). Udupa *et al*. (1991) have shown that ex planta nitrogenase activity by rhizobia is often related to the presence of a large molecular weight plasmid, especially in the fast growers.

Ecological factors affecting symbiosis

Legume species and cultivars and their associated rhizobial strains differ in their response to different ecological/edaphic conditions. The important studies carried out on the effects of ecological factors on nodulation and related aspects having some bearing on the present work have been briefly reviewed below.

Substrate nitrogen: Fred & Graul (1916) were among the first to report on the negative effects of substrate N on nodulation. Since then, extensive work has been done on the subject and the detrimental effects of combined N, particularly NO_3^--N, on root hair infection, nodule initiation, nodule development and N_2 fixation have been demonstrated by several researchers (Dart 1977, Munns 1977, Gibson & Pagan 1977, Streeter 1988). The extent of these effects is influenced by the host plant, the bacterial strain involved, and the form and concentration of the combined N.

Contrary to the above generalization, legumes often require a small amount of combined N as a 'starter' to initiate nodulation and subsequent nitrogenase activity. In some legumes, as in Phaseolus vulgaris, in the absence of a 'starter', the initiation of the symbiosis is considerably delayed (Streeter 1988).

NO_3^--N affects a broad range of infection events including a decrease in root hair deformation (Thornton 1936, Truchet & Dazzo 1982), a decrease in the binding capacity of rhizobia to root hairs (Dazzo & Brill 1979), a decrease in the number of infection threads (Munns 1977) and an increase in the number of aborted infections (Munns 1977). Truchet & Dazzo (1982) found that although high levels of nitrate completely inhibit nodule formation, a delay of just five days in the commencement of nitrate treatment after inoculation, allows some infection to generate functional nodules.

Indole acetic acid (IAA), synthesized from tryptophan by rhizobia, is supposed to be involved in infection, and this growth promoter seems to be destroyed by nitrite, formed from supplied nitrate (Dixon 1969). Sherwood et al. (1984) found that high levels of nitrate reduce the rhizobia-binding capacity of the lectin on the root surface.

Growth and activity of nodules are depressed at nitrate concentrations greater than 2 mM (Streeter 1981, Eardly et al. 1984). With long-term exposure of plants to 4-8 mM nitrate, the detrimental effects are greater on nodule growth than on acetylene reduction assay (Streeter 1981, Ursino et al. 1982). Miller et al. (1982) reported similar trends with field-grown legumes like cowpea.

Allos & Bartholomew (1955, 1959) were among the first to establish the inhibitory effects of combined nitrogen on N_2 fixation. They reported that the effects were proportional to the amount of nitrogen supplied and independent of the legume species or the rooting medium. The inhibitory effects of high levels of nitrate (10-20 mM) on acetylene reduction activity is very rapid, apparently after day one, whereas inhibition of nodule growth is not evident before day two or three after initiation of treatment (Skrdleta et al. 1980).

This inihibitory effect may be due to two possible reasons. According to the first view, reduction and assimilation of supplied nitrate requires energy such that carbohydrates, otherwise available to the nodules, are channellized for this purpose, leading to decreased nodule growth and activity. The results of the experiment with ^{14}C-labelled photosynthate in pea and subterranean clover (Small & Leonard 1969) support the above view. The above trends have been confirmed by Kahn & Kahn (1981) in cowpea and by Latimore et al. (1977) Rabie et al. (1980), Kouchi & Yoneyama (1984) and Kouchi et al. (1986) in soybean.

The second alternative explanation provided is that the inhibitory effects are due to nitrite, formed in or transported to the nodules. Nitrite has long been known to be toxic to living organisms and has been demonstrated to be a potent inhibitor of nitrogenase from a wide range of diazotrophs, including Rhizobium species (Kennedy et al. 1975). Besides, nitrite has been shown to oxidise leghemoglobin to the inactive ferric form (Rigaud & Puppo 1977) thereby impairing the protective nature of the enzyme.

There seems to be contradictory reports as to which form of combined nitrogen (NO_3^--N or NH_4^+-N) is a stronger inhibitor. According to Dazzo & Brill (1978), the infection process as studied in *T. repens* seedlings, is much more sensitive to nitrate addition than to ammonium, and urea is least detrimental (Imsande 1986). However, according to an earlier report, nodule formation is delayed to a greater extent by nitrate than by either ammonium or urea (Darbyshire 1966).

Wedderburn (1983) studied the effects of source, concentration and time of application of nitrogen on the growth, nodulation and N_2 fixation in *T. repens*. Potassium nitrate, sodium nitrate and ammonium nitrate in varying concentrations were applied to transplanted legumes at different intervals. Root weight was found to increase with increasing concentrations of potassium nitrate and ammonium nitrate only. Weight and number of effective nodules increased at low N levels, though N_2 fixation was unaffected. At higher levels of N, however, both number and weight of nodules decreased sharply.

Similar results were reported by Rys & Mytton (1985) in *T. repens* supplied with sodium nitrate ranging in concentrations from 0.36-22.84 mM. They found that though plants supplied with a low starter dose initially showed depressed symbiotic activity, this was only transitory and these treatments were subsequently associated with enhanced rates of nodule formation and N_2 fixation. In a similar experiment they reported that when supplied with abundant NO_3-N, nodule number per plant ranged from 0-20 with corresponding low acetylene reduction assay rates. With nil N, number of nodules per plant averaged 50, with comparatively higher acetylene reduction rates. Skeffington & Bradshaw (1980) studying N_2 fixation by plants grown on reclaimed china clay waste found that up to 50 Kg N ha^{-1} (as ammonium nitrate) could be applied to _T. repens_ without affecting its N_2 fixing potentiality.

Depressed nodulation and nitrogenase activity with increased nitrate concentrations was also reported in pea and faba bean (Buttery & Gibson 1990). Houwaard (1980) found that detached pea nodules supplied with succinate, fumarate and malate were most effective in stimulating nitrogenase activity, but this was inhibited when the same received ammonium chloride, although nitrogenase activity of isolated bacteroids was not affected. The negative effects of combined nitrogen on nodulation could be aleviated to some extent in wet soils.

Dazzo & Brill (1978) and Truchet & Dazzo (1982) studied the effect of combined nitrogen on the attachment of rhizobia to legume roots. They reported that levels of trifoliin A on the root surface decreases sharply with a corresponding increase in substrate nitrate. It was also revealed that substrate nitrate concentrations do not hamper the synthesis of rhizobium-binding lectin, but the synthesized end product (lectin) does not accumulate on the legume root cell wall at required concentrations. In clover, NO_3^--N affects the root cell wall composition (Dazzo et al. 1981, Diaz et al. 1981). The infection process could thus be impaired as changes in the chemistry of the cell wall could inhibit successful penetration by the diazotroph.

pH and related factors: Acidic soils can limit plant growth in many locations (Foy 1984) and poor productivity of pastures has occasionally been attributed to restricted nodulation or total nodulation failure (Coventry et al. 1985) of the associated legumes, in acid soils. The pH of the soil is thus an important factor affecting N_2 fixation (Mulder et al. 1966, Andrew 1976). The effect of soil acidity and pH-related factors have been reviewed by Munns (1977) and Flis et al. (1993). The legume host as well as the associated rhizobial strain exhibit different degrees of tolerance to acid soils (Graham et al. 1982). Munns et al. (1981) studied the effect of liming on yield and N_2 fixation of a number of legumes at pH 4.8. They reported that the legumes could be broadly divided into groups - acid tolerant ones, whose yield increased less than 30% with liming and maximum yield was obtained with 1 ton $CaCO_3$ ha^{-1}, and the acid sensitive ones, whose yield increased more than five times with liming, and a large quantity of lime is required for maximum yield.

The effects of factors associated with soil acidity like low pH, low calcium, high aluminium and high manganese, on the clover - rhizobium symbiosis were investigated by Wood et al. (1984). The pH range for survival of R. trifolii was reported to be 4.5-4.7 (Bryan 1923). Wood et al. (1983) observed that at pH 5.5, there was growth characteristic of non-stressed conditions, but at pH 4.3 the rhizosphere population of the endosymbiont perished. Consequently, root hair formation was also unaffected at this pH.

Effects of acidic soil factors on the soybean - rhizobium symbiosis were examined in acidified soils by Cline & Kaul (1990). They reported that resultant reduced plant growth was caused by the effects of acidified soil on nodulation, probably brought about by H^+ ion concentration, the effects of aluminium, manganese or molybdenum appearing less likely.

Pijnenborg _et_ _al_. (1990a) showed the effect of lime pelleting seeds in neutralizing the detrimental effects of an acid soil. It was found that a lime pellet around seeds significantly increased crown nodulation. This was attributed largely to the neutralizing effect of lime and only partly to increased calcium availability. However, the study carried out to establish the effect of calcium on nodulation of the legume, using EGTA, a specific calcium chelator, indicated that depletion of soil calcium did indeed depress nodule formation (Pijnenborg _et_ _al_. 1990b). This is in accordance with Snaydon (1962) who reported that distribution of _T_. _repens_ on some soils is correlated with calcium concentrations and not with soil pH.

Grauer & Horst (1990) compared the effect of nitrogen source (NO_3^--N and NH_4^+-N) on the aluminium tolerance of lupin, in acid soils. The aluminium toxicity was found to be less severe in presence of NH_4^+-N than NO_3^--N. Thus soluble aluminium is a major factor limiting plant growth in acid mineral soils. (Downes & Beckwith (1951) investigated the variations in soil pH over short distances in an edaphically heterogeneous area and concluded that differences as great as 1 pH unit occur over a distance of only 0.3 m. This apparently determines the distribution of several species.

Wolff et al. (1993) dealt with the influence of acid soil on nodulation in common bean. In both field and pot experiments, nodule numbers during the initial stages of plant growth were reduced by 30-50% in acid soils. The effect of moisture stress on growth and nodulation of *T. subterraneum* in acid and limed soils was studied by Davey et al. (1989). This was further extended to study the N_2 fixing activity of the legume under similar stress (Davey & Simpson 1989).

Soil moisture: Soil moisture and soil aeration play an important role in nodulation and N_2 fixation of legumes (Sprent 1976). Clay soils, which have high moisture content, have been often associated with poor nodulation, due to poor aeration. Thus soil texture is important as this determines both moisture content and aeration.

Kuo & Boersman (1971) reported that the ratio of fixed N_2 to absorbed CO_2 decreases with increasing soil water tensions. Sprent (1971) showed that nodules from slightly wilted plants exhibited a reduced rate of acetylene reduction assay. Holter (1978) studied nodulation and N_2 fixation in four legumes, *Trifolium pratense*, *T. arvense*, *Vicia augustifolia* and *Medicago lupulina*, in relation to soil moisture. Plants of *T. pratense* showed almost no acetylene reduction assay when the soil had less than 8.0% of moisture, but *T. arvense* was more resistant to moisture stress and showed high N_2 fixation rates even at 2.2% soil moisture, ultimately ceasing at a moisture level of 1.2%. However, he found that N_2 fixation increased with increasing soil moisture content until a maximum value above which it gradually began to decrease, probably due to oxygen deficiencies. The detrimental effects of moisture stress on nodulation and growth were confirmed by Davey _et al._ (1989) in _T. subterraneum_. Thomas (1984) studied the effect of drought on growth of two _T. repens_ cultivars - a small leaved and a large leaved one. Water stress imposed from day 28-56 after sowing, reduced the shoot growth and stress imposed from month 4-5 after sowing reduced the cut herbage weight, but not the total shoot weight. The remaining stubble weights were much greater than control plants. The inhibitory effects of moisture stress on N_2 fixation was also observed in non-legumes like _Purshia tridentata_ by Dalton & Zobel (1977). Engin & Sprent (1973) studied the effects of water stress on growth and N_2 fixing activity of _T. repens_. The water stress depressed both growth and acetylene reduction activity rates of the legume. Ability of plants to recover and the time taken to do so were both related to the duration of the stress period. On resuming watering, meristematic activity of the nodules also resumed proving that plants with indeterminate nodules were more adapted to stress conditions compared to those bearing nodules of the determinate type. N_2 fixation estimated by the acetylene reduction assay was comparable to total Kjeldahl nitrogen of the plants.

Defoliation: The effect of defoliation of _T. repens_ plants on its nodulating ability, under set stocking and rotational grazing with sheep on hill pastures receiving low and high levels of fertilizers, was studied by Clark et al. (1984). They found that in swards, clover is exposed to higher defoliation risk as compared to grasses because in clover the leaves were nearly always completely removed as opposed to the grasses where the leaves were only partially removed. Chapman (1986) examined the development and fate of white clover leaves in swards under three grazing managements. Longer defoliation intervals led to development of a greater number of leaves per stolon, but the differences in defoliation pattern between managements were not large enough to cause substantial differences in legume performance. The effect of defoliation on the source/sink ratio of white clover plants was the aim of the study by Chapman et al. (1990). When two out of three mature leaves were removed from a stolon, there was a 10% increase in net photosynthesis of the residual mature leaf and a greater export of carbon from this leaf to the apex. Another response to defoliation was a rapid increase in the specific leaf area (cm^2 g^{-1} dry weight) of new leaves. Ohyama & Harper (1991) studied the effect of shoot removal on N_2 fixation and assimilation in soybean and observed a rapid decline following decapitation. They suggested that this was primarily due to the interruption of carbohydrate supply from the shoot.

Ecological and reclamation studies

Chapman (1983) studied the growth and demography of T. repens stolons in pastures grazed by sheep and cattle. Similar reports are available on growth of clover in five hill swards grazed by sheep (Wilman & Simpson 1988). The latter workers reported that ground cover of the clover increased between February and June due to a four fold increase in leaflet size which attained a peak in summer. Seasonal changes in leaflet size with peak values during June-July was confirmed by Briseno (1980) and Hollington & Wilman (1985), and an increase in clover ground cover in the British Isle between late winter and July was also confirmed by Burdon (1983). Leaflet size attaining a peak in summer suggests that day length probably had more effect on leaflet size, than temperature which peaked around August. However, Arnott & Ryle (1982) have shown that both photoperiod and temperature affect leaflet size in clover.

The growth of the parent stolon and branches in clover was reported to be greatest where its ground cover was increasing from relatively low levels (Wilman & Simpson 1988). White clover appears to be inhibited in some way from growing into a part of the sward which it already occupies, whereas it may readily invade grassy areas (Turkington 1983). The stolon extension rate appeared to be severely restricted by soil moisture deficits and low light intensities (Wilman & Simpson 1988) and to a lesser extent by soil acidity and competition from grasses (Turkington 1983). Snaydon (1962) identified contrasting natural populations of white clover with a wide edaphic tolerance, capable of growing on soils ranging from highly calcareous to markedly acidic and such 'edaphic ecotypes', when analysed, had a marked difference in calcium content.

Competition from grasses can also substantially retard the seasonal flush of white clover growth (Spedding & Diekmahns 1972, Rhodes & Mee 1984). During grazing, sheep have been reported to select white clover in preference to grasses (Milne et al. 1982, Clark & Harris 1985) which may further restrict clover growth. However, faster growing companion grasses often lower soil N levels when the clover again takes the upper hand due to its N_2 fixing ability.

A typical white clover plant is a collection of physically connected modules formed due to repeated branching of the parent stolons over which the main stolon exerts some control (Newton 1986, Thomas 1987). Chapman et al. (1991) using ^{14}C, studied translocation of carbohydrates between the parent stolon and branches, and amongst the branches of T. repens plants. They reported that of the total amount of carbon exported by the leaves (source tissue), 22% moved to the stolon apex, 16% to the stolon tissue of the parent stolon, 29% to the branches and as much as 34% to the adventitious roots on the main stolon, mostly utilized by older nodulated roots during N_2 fixation.

Degraded ecosystems are of worldwide occurrence and their revegetation is inhibited due to deficiency of vital nutrients among which the deficiency of N is an important factor limiting plant growth. In such cases, it is uneconomical to provide N in the form of fertilizers every year because it is quickly leached away. Legumes, by virtue of their N_2 fixing ability are essential in land restoration practices and perennial legumes like T. repens are particularly suitable for derelict lands (Bradshaw & Chadwick 1980). Several reports are available on the exploitation of the legume - Rhizobium symbiosis in the reclamation of degraded lands. In such cases, T. repens provides the crucial N for the companion grasses while itself yielding herbage rich in protein and minerals and of high digestibility. The resulting grassland can yield up to 5000 Kg dry matter $ha^{-1} yr^{-1}$ and the number of sheep that can be carried will increase five or ten fold. The soil develops different characters and gradually changes into a brown earth (Bradshaw & Chadwick 1988).

Legumes are also vital in restoration of mine spoils and swards established on colliery spoils were found to deteriorate quickly without a legume component (Bradshaw & Chadwick 1988). The use of legumes for N accumulation into china clay waste was emphasized by Dancer et al. (1977) and Lanning & Williams (1980). Skeffington & Bradshaw (1980) have reported on the effectiveness of various legume and non-legume associations in fixing nitrogen. Fixation rates with legumes, including T. repens were at least ten times greater than those of non-legume and thus legumes could be recomended as the most valuable means of promoting N accumulation. Legume growth, N accumulation and N transfer to companion species were compared amongst different legume species established on colliery spoils and on sand wastes (Jefferies et al. 1981a) and N accumulation of 295 Kg $ha^{-1} yr^{-1}$ and N transfer of 76 Kg $ha^{-1} yr^{-1}$ from the legume to companion grasses was apparent within 2 years from sowing. Among different legumes, Trifolium was found to be the most effective source of N with N_2 fixation rates estimated to be 70% higher than the maximum rates estimated for other legumes like *Ulex* (Dancer *et al*. 1977).

Bradshaw & Chadwick (1988) found degraded areas in Minnesota colonised by two legumes - *T. repens* and *Melilotus alba*, both successful under such stressed conditions due to their N_2 fixing ability. In another spoil, they found *T. repens* to be the pioneer species, appearing within 2 years and by a 13 year period had a frequency of 95% from the initial 5%. Under such conditions, the rate of N accumulation was reported to increase from 45 Kg ha^{-1} to 258 Kg ha^{-1} within a short span of one year (Bradshaw & Chadwick 1988).

Pulverised fuel ash spoils provide another example of a poor substrate on which only certain species can grow. *T. repens* was found to colonize this substrate at seral stage 3. Once established on such barren areas, *T. repens* exhibits excellent growth and fixation, steadily building up the N status of the substratum and making it more hospitable to colonization by other seral species.

There are a number of reports on the factors affecting establishment and growth of these legumes during reclamation (Szabo *et al*. 1974, Fail & Wochok 1977, Bennet *et al*. 1978, Palmer *et al*. 1979). Palmer (1982) reported a greater accumulation of mineralizable and total N under swards dominated by *T. repens*, compared to swards without the clover. Jefferies *et al*. (1981b) reported similar findings from naturally occurring patches of white clover. *Agrostis castellana* was found to have a higher N concentration when grown with white clover on colliery spoils and china clay waste compared to those of the grass in monoculture (Jefferies *et al*. 1981b). Palmer & Iverson (1983) have attempted to elucidate the factors affecting N_2 fixation by *T. repens* on colliery spoils. Residual soil phosphate levels affected fixation, the rates being higher under high residual phosphate levels. Sunshine hours, spoil temperature and substrate moisture level affected N_2 fixation most.

Over the years, white clover has emerged as the legume of choice for the purpose of reclamation of degraded ecosystems on account of its remarkable N_2 fixing ability and its capability to reproduce both from stolons as well as by seeds; furthermore, due to its prostrate habit, it provides good ground cover as it radially extends outwards by rapid stolon extension and rooting at the nodes.

3 Materials and Methods

Study site

Location: The present study was conducted at Shillong (latitude 25° 34'N, longitude 91° 56'E; altitude 1500 m aMSL), the capital of Meghalaya in north-east India. The field study was conducted in a sward having a profuse growth of *T. repens* (Plate 1). The pot experiments were conducted in net houses at the North-Eastern Hill University campus. In the chosen sward, two plots of around 0.25 ha each, seperated by a distance of about 100 m, were selected. The swards were protected from grazing and human interference by a fencing which had been erected for this purpose. Both the plots had a level topography. The first plot (Plot 1) received sunlight for the major part of the day, whereas the second plot (Plot 2) was partially shaded (light intensity 58% less than that at Plot 1) due to the growth of a row of *Prunus* trees which existed towards the southern periphery of the site. Prior to fencing the sward was not supplied with any fertilizers, but was exposed to mild grazing and human interference and occasional mowing. Sampling was done monthly over a two-year period during June 1991-May 1993.

Climate: The climate of Shillong is subtropical. The year can be divided into four seasons - spring, rainy, autumn and winter. The spring commencing from early March and extending till April, experiences mild to warm temperatures at times and occasional light showers. The rainy season is of long duration (May-September), warm and very wet, the rainfall being frequent and heavy. Autumn extending from October to November, represents a transitional period between rainy and winter seasons. The temperatures start declining during this period. It is followed by the winter season (December-February). The winter is cold and dry except the occasional light showers, and the nights are frosty. There is a sharp dip in the mercury levels (mean minimum 4.7°C, mean maximum 16.1°C) and at times even sub-zero temperatures are recorded. Mean annual minimum and maximum temperatures are 12.1°C and 22.5°C, respectively (Fig. 3.1). Mean annual rainfall during the study period is 1735.7 mm, about 80% of which occurs during June-October. Humidity ranges from 91% in June 1991 to 66% in November 1992.

Geology: The Shillong plateau is situated at an elevation of ca. 1500 m aMSL overlooking the alluvial plains of the Brahmaputra valley in the north and is made up largely of Precambrian rocks acutely folded and steeply dipping with an overturned fringe of Mesozoic and Tertiary sedimants. The southern slopes are abrupt scarps following fault zones rejuvenated in late Tertiary (Gansser 1964). The largest area of the plateau is formed by Archaean gneisses and granites. The gneisses are finely banded, grey to pink in colour, and contains microcline, biotite, subordinate quartz and plagioclase.

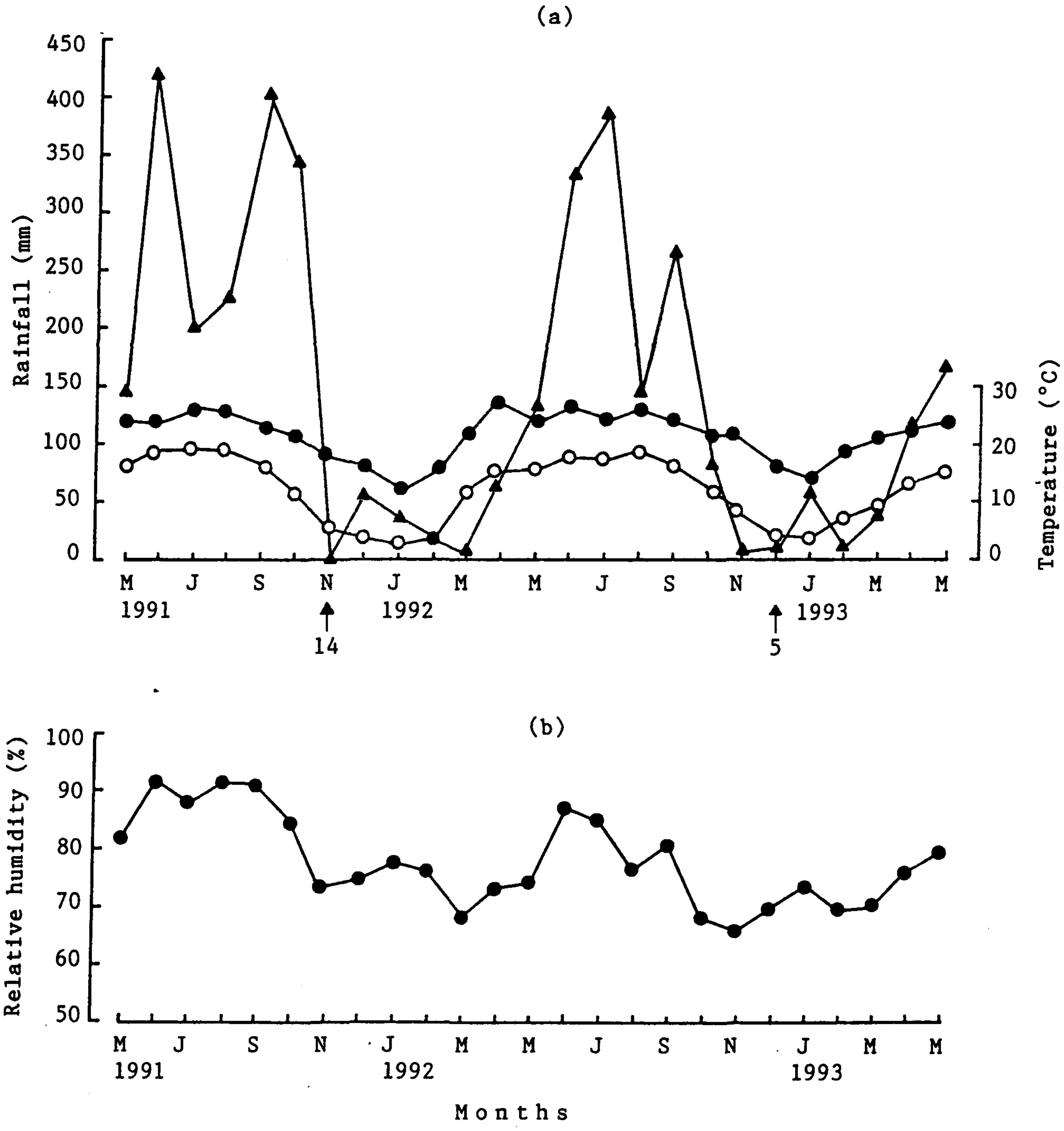

Fig.3.1. (a) Total monthly rainfall (▲), mean maximum (•) and mean minimum (o) temperatures, and (b) mean humidity of the study site during the study period. Arrow (↑) indicates date on which first frost of the season was recorded.

The intrusive granites are mostly porphyritic with large flesh coloured microclines, some acid plagioclase, orthoclase and biotite. These granites also intrude the Shillong schists, but are less frequent in the Shillong quartzites. Sandstones, limestones and conglomerates with subordinate clays superimposed over these rocks also occur in the Shillong plateau (Zimba 1977).

The soils are derived from the underlying gneisses, schists and granites of Archaen age (Gnasser 1964). They have been grouped under latosol (oxisol) type (Pascoe 1950). The top layer (0-10 cm) of soil at the study site is a sandy loam and shows acidic reaction (pH 5.6) and has an organic matter content of 3.6%. Details of the physico-chemical properties of the soil at the two plots are given in Table 3.1.

Vegetation: The sward contains Anemone rivularis Ham., Arundinella spp., Axonopus compressus (Sw.) Beauv., Centella asiatica L., Fimbristylis dichotoma Vahl., Hypocharis radicata L., Pennisetum clandestinum Hochst.ex.Chiov., Plantago major L., Trifolium repens L., moss and other minor constituents (Table 3.2).

T. repens comprises two common leaf morph populations - one with conspicuous 'V' shaped white markings on the leaflets (marked population) and the other with no such markings (unmarked population) (Pradhan & Tripathi 1984). The leaf markings in T. repens are very distinct and can be used to identify individual clones (Harberd 1963). The markings are controlled by the multiple alleles present in one of the two linked groups of genes (Corkill 1971).

Table 3.1. Physico-chemical characteristics of soil samples from the 'open' (P_1) and 'shaded' (P_2) plots. (n = 5).

Soil characteristics	Plots	
	'open' (P_1)	'shaded' (P_2)
% of soil particles		
Sand (%)	68.7	52.1
Silt (%)	19.1	26.9
Clay (%)	12.2	21.1
Textural class	Sandy loam	Sandy clay loam
Water Holding Capacity(%)	58.7	66.4
pH (1:2.5 0.01M $CaCl_2$)	5.2	5.7
Organic Matter (%)	2.7	3.9
CEC (meq 100 mg^{-1})	10.2	11.4

Table 3.2. Floristic composition of the plant communities in the two plots and life forms of the component species.

Species	Habit	Life form	Plot 1		Plot 2	
			1991-92	1992-93	1991-92	1992-93
GRASSES AND SEDGES:						
Arundinella bengalensis (Spreng) Druce	P	H	+	+	+	+
Arundinella nepalensis Trin.	P	H	+	+	-	-
Arthroxon lancifolius Hochst.	A	Th	+	+	-	-
Axonopus compressus (Sw.) Beauv.	P	H	+	+	+	+
Bulbostylis densa (Wall.ex Rox.) Handmazz	A	Th	+	-	+	-
Cyperus compressus Linn.	A	Th	+	+	-	-
Cyperus tuberosus Rottb.	P	H	+	+	+	-
Eragrostis nigra Nees & Steud.	P	H	+	+	-	-
Eulalia pallense (Hack.) Kuntze	P	H	-	+	+	-
Fimbristylis dichotoma Vahl.	A	Th	+	+	+	+
Imperata cylindrica Beauv.	P	H	+	+	-	+
Kyllinga triceps Rottb.	P	G	+	-	-	-
Lolium rigidum Gaud.	A	Th	-	+	-	-
Mariseus sieberianus Nees	P	H	-	+	-	-
Microchloa setacea R.Br.	P	H	+	+	-	-
Paspalum longifolium Roxb.	P	H	+	+	-	+
Pennisetum clandestinum Hochst.ex Chiov.	P	H	+	+	+	+
Poa annua Linn.	A	Th	-	-	-	+
Pycerus latespicatus Cl.	A	Th	-	-	+	-
Setaria palmifolia (Koen) Stapf.	A	Th	+	-	-	-
Sporobolus indicus R. Br.	P	H	+	+	-	+

Table 3.2.(contd.)

Species	Habit	Life form	Plot 1		Plot 2	
			1991-92	1992-93	1991-92	92-93
LEGUMES:						
Cassia mimosoides Linn.	P	Ch	+	-	-	-
Desmodium heterophyllum D.C.	P	Ch	-	-	-	+
Desmodium floribundum Don	P	Th	-	-	+	-
Smithia ciliata Royle	P	Ch	-	+	-	-
Trifolium repens Linn.	P	Ch	+	+	+	+
FORBS:						
Anemone rivularis Ham.	P	H	+	+	+	+
Brunella vulgaris Linn.	P	G	-	+	+	+
Centella asiatica (Linn.) Urb.	P	H	+	+	+	+
Commelina benghalensis Linn.	A	Th	+	-	-	+
Cyanotis vaga (Lour) J.A.et J.H. Schult	A	Th	-	-	-	+
Drymaria cordata (Linn.)Willd.ex Roem.	A	Th	-	+	+	-
Duchesnia indica (Andr.) Focke	P	Ch	+	-	+	+
Eriocaulon cristatum Mart.	A	Th	+	-	-	-
Hypericum japonicum Thunb.ex Murr	P	H	-	+	+	-
Hypocharis radicata Linn.	P	H	+	+	+	+
Plantago major Linn.	P	H	+	+	+	+
Scutellaria discolor Wall.ex Benth.	A	Th	+	+	-	+
Sida rhombifolia Linn.	A	Th	-	-	-	+
Sonchus asper (Linn.) Hill	A	Th	-	-	+	-
Spiranthes lanceae (Thunb.ex Sw.)B.B.&V.S.	P	G	+	-	-	-

\+ = Present
\- = Absent
A = Annual
P = Perennial

Ph = Phanerophyte
Ch = Chamaephyte
H = Hemicryptophyte
G = Geophyte
Th = Therophyte

Community analysis: The phytosociological analysis of vegetation was done annualy during peak vegetative growth which occurred in early September. The sampling was done at both plots by randomly laying 10 quadrats of 50 x 50 cm size. In case of grasses, each tiller and in case of creeping plants, any unit with shoot and roots was considered as an individual. Species were identified with the help of floras (Bor 1940, Hooker 1872-97).

Sampling of the legume

T. repens plants, from both marked and unmarked populations, were sampled at monthly intervals, over a period of two years from both the plots. During sampling, 5 randomly chosen plants from each of the two leaf morph populations were carefully excavated, taking special care of the root system. In order to excavate the roots intact, the soil monoliths comprising the roots were taken out from the sward. The sampled plants were sealed in polythene bags and transferred to the laboratory. The soil monoliths containing the root system were placed in 2 l buckets and soaked in water overnight. The roots were then washed with a fine jet of water over 2.0 mm and 0.5 mm mesh seives successively. This ensured minimum damage to the fine roots and maximum retrieval of the root nodules. The shed nodules, if present in the soil monolith, were found floating on the surface of the water in the bucket and were subsequently betrieved. The harvested plants were sorted out into live leaves, senescent leaves, inflorescence (if any), roots and nodules. The fresh, healthy nodules were seperated from the senescent ones.

The leaf area was computed by taking the mean of the areas of the largest and smallest unfolded compound leaves in each plant. Photosynthetic area was expressed as the mean leaf area multiplied by the number of unfolded live leaves. The nodule size was measured using a calibrated ocular micrometer. As the nodules are of the indeterminate type and continue to grow by meristematic activity, the size (length) and number of lobes were taken as the criteria to arbitrarily divide them into 5 categories (Plate 3) as follows:

(i) category 'a' - Unbranched and 0-3 mm in length

(ii) category 'b' - Those with 2 lobes or unbranched and 3-4.5 mm in length

(iii) category 'c' - Those with 3 lobes and up to 4.5 mm in length

(iv) category 'd' - Branched and 4.5-6.0 mm in length

(v) category 'e' - Those > 6.0 mm in length

Larger nodules (categories c, d and e) showed progressively more lobes. Data regarding stolon length, number of nodes, leaves, inflorescence and rooting nodes, and petiole lengths were recorded and the different plant parts were oven-dried at 60°C. Nodules were dried for 2 h, whereas other plant parts were dried for 12 h. Dry weights were recorded and samples were ground and stored for the analysis of Kjeldahl nitrogen, which was done following the method described by Allen *et al.* (1974).

Physico-chemical properties of soil

Soil samples representing 0-20 cm soil depth were collected

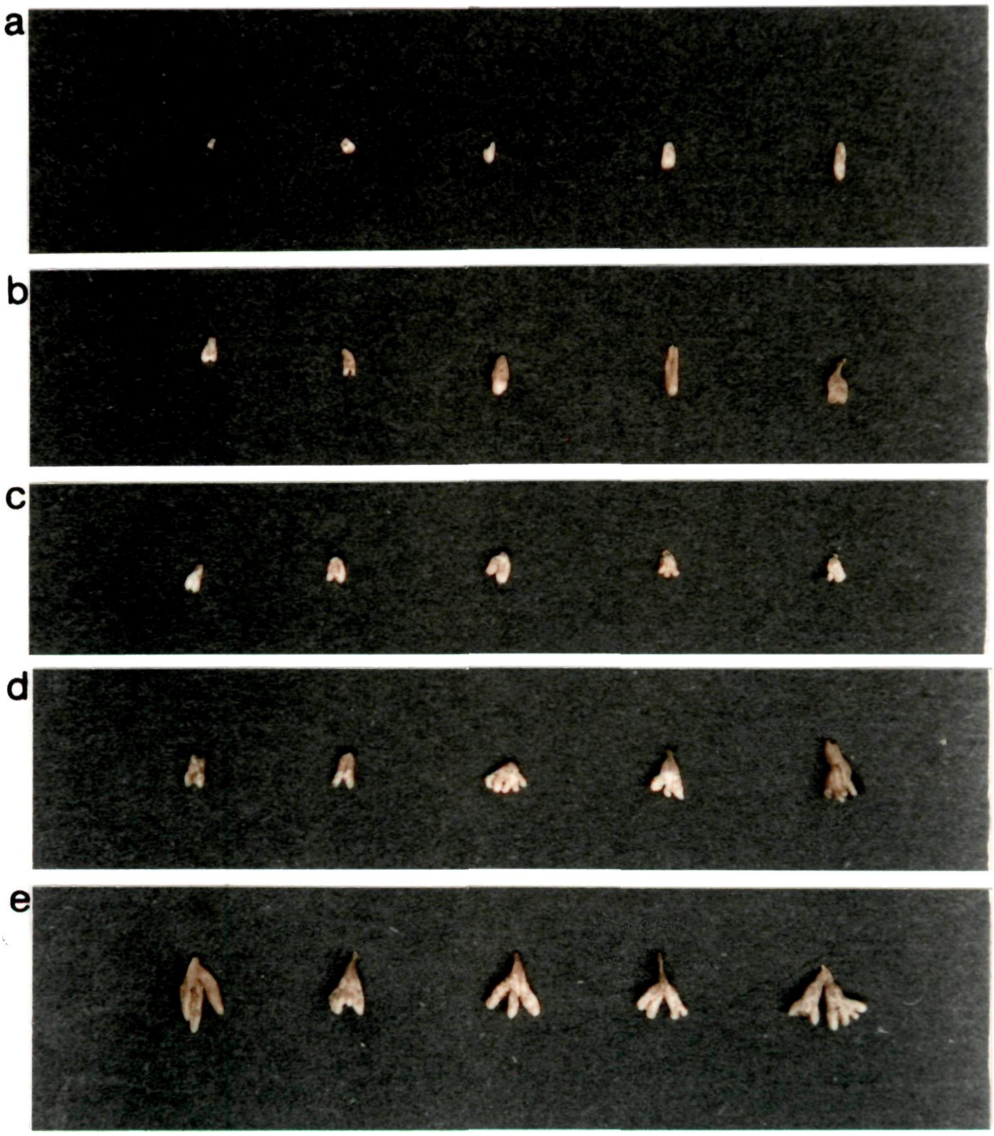

Plate 3. Morphology of nodules of the 5 categories (a to e) (x 1.5)

randomly from five different places at each plot for the determination of soil properties. Composite soil samples were made by mixing the soil samples collected from a plot. The samples were air-dried (or dried at 30-35°C in an oven during rainy season), seived through a 2 mm mesh seive followed by 80 mesh screen, and the seived samples were used for analysis. Soil samples were analysed once for water holding capacity, texture and bulk density following the standard methods outlined in Piper (1942) and Allen _et al_. (1974). The water holding capacity was determined using a Keen-Raczkowski box holding metallic perforated dishes (Piper 1942), texture by hydrometer method and bulk density by using a metallic cylinder (diam. 6.5 cm, length 11.5 cm).

The temperature, moisture content, pH and nitrogen content of soil (Fig. 3.2) were determined at monthly intervals. Soil temperature was recorded by inserting a soil thermometer down to 20 cm depth. Recordings were taken at midday. Soil moisture content was determined gravimetrically and results expressed on oven dry weight basis (Allen _et al_. 1974). pH of the soil (soil : 0.01 M calcium chloride suspension in the ratio of 1:2.5) was determined using a digital pH meter (Systronics 335). NO_3^--N and NH_4^+-N levels in soil were determined by the Phenoldisulphonic acid and Indophenol blue methods, respectively, as outlined by Allen _et al_. (1974). Kjeldahl N was determined by digesting the air dried soil samples with concentrated H_2SO_4 using K_2SO_4+HgO mixture as catalyst. The digest was distilled in a micro-Kjeldahl distillation apparatus and titrated against 1/140 N HCl (Allen _et al_. 1974). Soil organic matter was determined

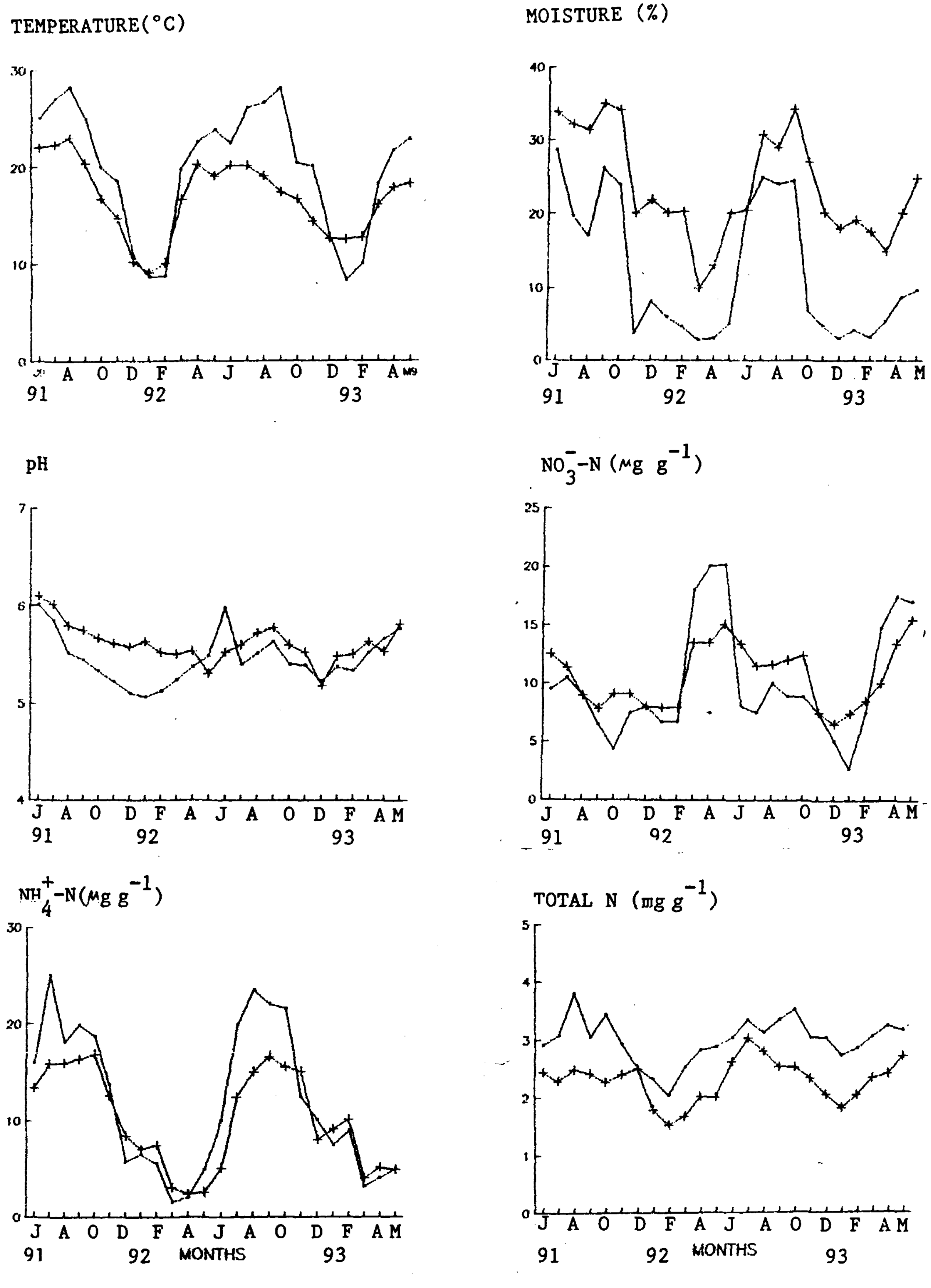

Fig. 3.2. Monthly variation in microenvironmental factors in the 'open'(P_1) (---) and 'shaded' (P_2) (-+-) plots during the study period.

by the rapid titration method of Walkley & Black (1934), and Cation Exchange Capacity (CEC) by extracting the soil with 1 M ammonium acetate (pH 7) and by following the methods outlined by Allen *et al.* (1974).

Isolation of rhizobia

Fresh, healthy nodules were collected from the field and excised from the roots in such a manner that a 1-2 mm segment of the root remained attached to a nodule. The nodules were thoroughly washed under running tap water and then with a mild detergent solution to remove any adhering soil particles. After rinsing, the nodules were surftce-sterilized by momentarily exposing them to 95% (v/v) ethanol and then in 0.01% (w/v) $HgCl_2$ for 4-10 min, according to size, and finally washing thoroughly in sterilized water. A nodule was then taken on a sterilized slide with a drop of sterile water and aseptically crushed with forceps until the water appeared milky and nodule tissue disintegrated. The suspension was streaked on to Yeast Extract Mannitol (YEM) agar medium (Table 3.3). The Petri plates were inverted and incubated at 28°C for 3 days and colony formation observed. Colonies were circular, convex, semi-translucent, raised and mucilaginous, secreting abundant amount of polysaccharides; approximately 2-4 mm in diameter. When sub-cultured in YEM broth, turbidity was observed in 2-3 days (Bergey 1984).

Confirmation of identity: To confirm that the isolate from the root nodules is indeed *Rhizobium*, the following tests were conducted:

Table 3.3. Composition of Yeast Extract Mannitol (YEM) medium.

Constituent	Weight (g)
Mannitol	10.0
Yeast extract	0.5
NaCl	0.1
K_2HPO_4	1.0
KH_2PO_4	1.0
$MgSO_4.7H_2O$	0.18
Distilled water	1.0 litre
Agar (if required)	15.0 g

pH adjusted to 6.8 and autoclaved

(a) Microscopic observations: When observed under a phase contrast microscope (Leitz Dialux 20 EB), the isolated organisms were seen to be minute, unicellular and rod-shaped. When processed and observed under a Scanning Electron Microscope (JEOL 35 CF), their unicellular, rod-shaped nature was clearly revealed; some of the bacilli were seen to be dividing by fission (Plate 4).

(b) Acetylene Reduction Assay (ARA): To test for nitrogenase activity, the isolate was subjected to the ARA. A loopful of an isolate was suspended in a few drops of sterilized water in rubber-stoppered vials. Intact nodules, collected from the field, were also placed in similar vials over pieces of moist filter paper. All vials were injected with a known volume of acetylene gas and incubated at room temperature. The reduced ethylene gas was assayed in a Gas Chromatograph (Tracor 540) fitted with a Porapak T column and a flame ionization detector. Nitrogenase activity was positive in all the samples assayed.

(c) Nodulation tests: Since the isolate is known to form symbiotic associations with white clover resulting in the formation of N_2 fixing root nodules, tests were performed to assess the capability of the isolate to nodulate the host plant. For this purpose, stolons of white clover were collected from the sward and cut into pieces 2 cm long, each with a node in the middle to serve as propagules (Pradhan & Tripathi 1984). The stolon pieces were thoroughly washed under running tap water to remove all adhering foreign particles, and then surface-sterilized in 80% ethanol for 5 s followed by

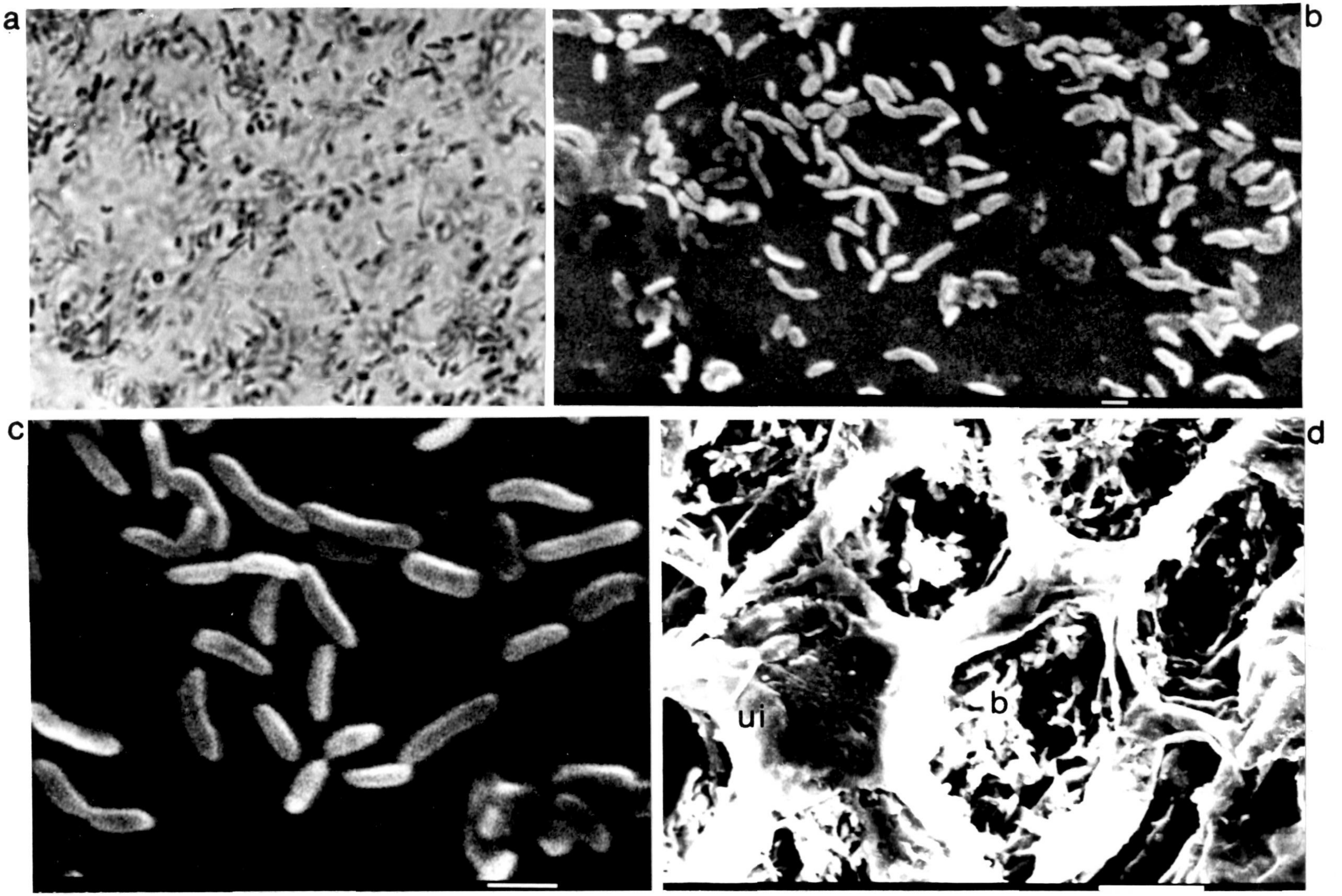

Plate 4. (a) Light micrograph of isolated rhizobia, (b) Scanning electron micrograph (SEM) of isolated rhizobia showing distinct rod-shaped cells. Bar=1.0μ, (c) Portion of 'b' magnified. Some cells can be seen undergoing fission. Bar=1.0μ, (d) SEM of a section of a root nodule of clover formed by the isolate. Bacteroids are clearly seen within the infected cells. b=bacteroids, ui= uninfected cell. Bar=10.0μ.

0.01% $HgCl_2$ for 3 min. Thereafter, they were thoroughly washed with at least three changes in sterilized distilled water. The propagules were placed in plastic cups (7.5 cm diam.) containing fine sterilized acid-washed sand and were supplied with a sterilized N-free nutrient solution (Table 3.4) diluted 1:1 with distilled water. The pots were covered with a transparent plastic film to prevent aerial contamination and watered with sterilized distilled water as and when required. The axillary buds of the propagules began to sprout from day 7 onwards. The ramets were thinned to give sprouts of uniform size (with one trifoliate leaf) and one ramet per cup. After 2 days, the ramets were inoculated with a suspension of the isolate containing $10^7 - 10^8$ cells ml^{-1} by injecting 1 ml of the suspension _ca_. 1 cm below the surface of the substratum in the rhizosphere region. A few uninoculated cups served as negative controls.

Similar tests were carried out in Petri plates with sprouts placed on moistened filter papers, as well as on seedlings grown on nutrient agar media in enclosed tubes (Vincent 1970). Inoculum was same as described earlier. The cups were kept in a growth chamber with 12 h photoperiod and 70% relative humidity. Root systems of the ramets from Petri plates were examined periodically for root hair deformation and subsequent development (Plate 5). The negative controls did not show any nodule formation; chlorosis of leaves was observed in some indicating symptoms of N deficiency.

Morphological observations, ARA, and nodulation tests all indi-

Table 3.4. Nitrogen-free plant nutrient solution

Compound	Molecular weight	Concentration of stock solution		Volume of stock solution l^{-1} of final solution (ml)
		(mM)	(g l^{-1})	
$CaHPO_4$	136.07	1×10^3	136.07	1.0
K_2HPO_4	174.18	1×10^3	174.18	0.2
$MgSO_4 \cdot 7H_2O$	246.49	1×10^3	246.49	1.0
KCl	74.55	50.0	3.728	1.0*
H_3BO_3	61.84	25.0	1.546	
$MnSO_4 \cdot H_2O$	169.01	2.0	0.338	
$ZnSO_4 \cdot 7H_2O$	287.55	2.0	0.575	
$CuSO_4 \cdot 5H_2O$	249.71	0.5	0.125	
H_2MoO_4 (85% MoO_3)	161.97	0.5	0.081	
Fe-EDTA**	346.08	20.0	6.922	1.0
Distilled water	1.0 litre			

* A combined stock solution is made up containing all micronutrients except iron.

** Ferrous ethylenediamine tetraacetic acid.

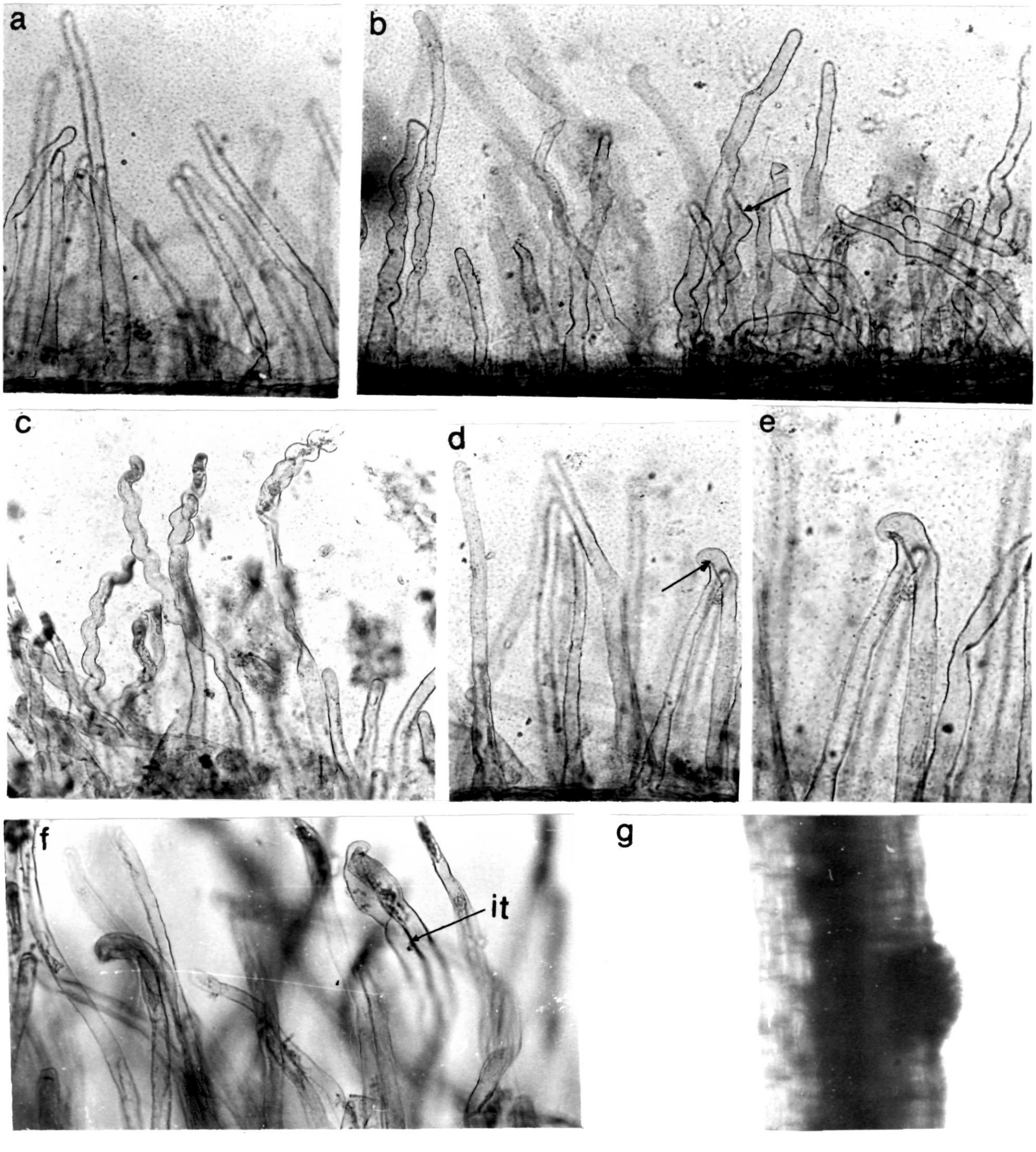

Plate 5. Phase contrast micrographs of root hair deformations and nodulation in *Trifolium repens*. (a) Root hairs of clover seedlings inoculated with *R. trifolii* and grown in 6 mM NO_3. Note the straight root hairs. 250x. (b) & (c) The same when grown in N-free medium shows marked deformations. Arrow indicates root hair tip showing branching tendency. 300x. (d) The tip of a root hair shows tight curling resulting in a 'shepherd's crook'. 300x. (e) The same, magnified. 450x. (f) Invagination of the root hair results in the formation of an infection thread (it) through which the bacteria are transported. 600x. (g) A pre-nodule. 100x.

cated that the isolate was indeed Rhizobium trifolii.

Nethouse experiments

Plastic pots (15 cm diam., 16 cm depth) with a basal hole for drainage, were thoroughly cleaned and the inside of each was surface-sterilized with absolute alcohol prior to filling them with the sterilized potting mixture (acid-washed sand / soil:sand mixture). The experiments were conducted in a nethouse, the roof of which was covered with a polythene sheet for protection against rain, but otherwise exposed to ambient conditions. The maximum temperature of 30.5°C was recorded in August and minimum of 16.0°C in September 1992. PFD (PAR) at foliage level was 500 μmols $m^{-2}s^{-1}$. A polythene sheet was spread on the floor of the nethouse to facilitate regular hosing for cleanliness. Each pot was raised ¼" above the floor by placing it over two longitudinal strips of wood placed parallel to each other. This was to prevent contamination from the excess water/nutrient solution that may drain through the bottom.

Stolons of the two leaf morph populations of T. repens were collected from the previously-tagged mother plants (one marked and one unmarked) from the natural swards, whose apices had been repeatedly clipped to encourage profuse branching by growth of latent axillary buds. Stolons of uniform diameter were cut into 2 cm pieces, each with a node in the middle, to serve as propagules. The stolon pieces were washed with a mild detergent solution followed by a rinse in tap water and surface-sterilized as described earlier. Thereafter, they were washed in at least three changes of sterilized distilled water and sown.

Substrate nitrogen: The stolon pieces of a particular leaf morph population were sown on 16 June 1992 in pots (15 cm diam., 16 cm depth) filled with sterilized acid-washed sand giving a depth of 15 cm. The concentrations of NO_3^--N and NH_4^+-N in the acid-washed sand, prior to filling were 1.0 μg g^{-1} and 0.6 μg g^{-1}, respectively. After sowing the stolon pieces, the pots were irrigated with deionized water to encourage sprouting of the axillary buds and after a week, ca. 70% of them had sprouted. The propagules were thinned on 23 June 1992 to give sprouts of uniform size (with one trifoliate leaf) with an overall density of 3 plants pot^{-1}. This date was taken as the date of commencement of the experiment. The substrate N was supplied in either of two forms - nitrate nitrogen (NO_3-N) or ammonium nitrogen (NH_4-N). The first harvest (H1) was taken on 21 July 1992 after a 4 week period and the second and final harvest (H2) on 15 September 1992, i.e. 12 weeks after commencing the experiment.

Experiment 1: Each pot received 100 ml of N-free nutrient solution (Table 3.4) weekly, diluted 1:1 with deionized water and amended with required amounts of KNO_3 to give any of the 7 different levels of NO_3-N: 0, 0.1, 0.5, 0.75, 1.5, 3 and 6 mM. The treatments were replicated three times involving a total of 18 plants per treatment. After 3 days, each plant was inoculated with a 1 ml suspension (10^7 10^8 cells ml^{-1}) of a 3 day old culture of the previously-isolated R. trifolii in YEM broth (Vincent 1970). Plants were watered with sterilized deionized water as and when required.

Experiment 2: Each pot received 100 ml of the N-free nutrient solution weekly, diluted 1:1 with deionized water and amended with required amounts of NH_4Cl to give NH_4-N levels of 0, 0.1, 0.5, 0.75, 1.5, 3 and 6 mM. Other conditions were similar to those in Experiment 1.

pH: To determine the effects of varying pH levels on nodulation in T. repens, the pots were filled with a soil:sand mixture in the ratio of 2:1 (v/v), the pH of which measured prior to filling, was 5.5 and the CEC 10.2 meq 100 mg sample^{-1}. The pH of the medium was raised or lowered by using finely ground calcium carbonate or 3% sulphuric acid respectively, in calculated amounts (Thompson & Troeh 1975) to give pH values of 4.5, 5.0, 5.5, 6.0 and 6.5. The reagent was thoroughly mixed with the potting mixture which was then kept for a one month period to allow for the pH to stabilize. The pots were covered with polythene sheeting to prevent contamination and the medium watered occasionally. After a fallow period, the media were tested to verify the pH and stolons were planted as described earlier.

Moisture content: Oven-dried samples of the soil:sand mixture were filled into sterilized plastic cups (7.5 cm diam.) with a basal hole for drainage. 170 g of the potting mixture was filled into each cup to give a depth of 9.0 cm. Three stolon pieces of either of the leaf morph populations were placed in each cup on 16 June 1992 and watered with calculated amounts of deionized water to give soil moisture contents (on dry weight basis) of 10, 20, 30 and 40%. The pots were weighed regularly and the weight loss due to evapo-transpiration made up by adding requisite amounts of deionized water. Most of the stolons sprouted after a week when they were thinned to one plantlet pot^{-1} of uniform size. This day (23 June 1992) was taken as the date of commencement of the experiment. Inoculation with rhizobia was done as described previously. Moisture content in the pots was maintained at the desired levels by taring regularly and adding requisite amounts of deionized water. The first harvest (H1) was taken on 21 July 1992 and the second and final harvest (H2) on 15 September 1992.

Defoliation and NPK treatments: Stolon pieces from the two leaf morph populations were sown in the soil:sand mixture, as described earlier, on 7 June 1993. On 14 June 1993 they were thinned to 3 ramets pot^{-1} of either leaf morph population. The potting mixture without any amendments represented low NPK level. The potting mixture with amendments (50 mg NH_4NO_3, 20 mg of KH_2PO_4 and 30 mg of KCl added per pot prior to sowing) represented high NPK level.

Two defoliation treatments were imposed - high and low - to try and mimic the effects of grazing. In the local swards, observations revealed that during spring, when associated grasses show slow growth, T. repens dominates. At this stage, the clover leaves together with the major part of the petiole are consumed due to herbivory. However, during peak vegetative growth around September, luxuriantly growing associated species, especially grasses and sedges, overshadow the leaflets of T. repens. Grazing during this period mostly causes removal of the greater part of the monocot foliage although the apical parts of the clover leaf and petioles (>6 cm height) are also consumed to some extent. The severed petioles were in the height range of 1-6 cm. In view of this, the plants were subjected to the following defoliation treatments: (i) Defoliation treatment I - It was imposed by clipping all the foliage above 1 cm from the soil surface. (ii) Defoliation treatment 2 - It was imposed by clipping all foliage above 6 cm from the soil surface. The pots were irrigated with deionized water as and when necessary. The first harvest (H1) was taken on 12 July 1993 and the final harvest (H2) on 6 September 1993.

For all the experiments, pots were arranged in a randomized block design. A number of pots were also used as uninoculated controls, well spread amongst the experimental pots, to check for contamination by aerial load of rhizobia, if any.

During harvesting, the pots were soaked in water to loosen the roots which were carefully excavated. The roots were washed over 0.5 mm seive with a fine jet of water and the plants sorted into leaves (live and senescent), stolons, roots and nodules. Harvested plant parts were oven-dried, weighed, ground and analysed as described earlier.

Computational procedures

Relative growth rate (R_W) and relative nitrogen accumulation rate (R_N) were calculated using the following formulae proposed by Vincent (1970):

$$R_W = \frac{\log W_2 - \log W_1}{t_2 - t_1}$$

where W_2, W_1 are the dry weights of plants at times t_2 and t_1, respectively, and

$$R_N = \frac{\log N_2 - \log N_1}{t_2 - t_1}$$

where N_2, N_1 are total plant Kjeldahl N at times t_2 and t_1, respectively.

Statistical treatments

The data was statistically analysed using 3-way ANOVA to study the variations between harvests, leaf morph populations and different treatments on the growth parameters of the legume. Linear regression models were proposed to estimate the relationship between nodule number per plant and mean nodule weight, wherever applicable. Pearson's correlation coefficients (r) between different growth parameters of the legume and edaphic factors with the two leaf morph populations of the clover from the two plots were calculated in order to study their effects on the nodule population. Standard error was calculated wherever necessary (Zar 1984).

4

Temporal variation in nodule population under field conditions

The development of effective N_2 fixing nodules on the root system of leguminous plants depends not only on the presence of a compatible rhizobial strain in the rhizosphere of the host, but also on conducive microenvironmental conditions. The prevailing ecological conditions play a key role in the initiation of this symbiosis and also in its effectiveness over time. This is significant considering the fact that in Trifolium repens the nodules are of the indeterminate type and can continue to grow and function for a considerable period of time as a result of activity of the nodule meristem.

Under favourable environmental conditions, not only does the number of nodules per plant increase as a result of successful infection, but their longevity also increases. This results in a temporal and spatial enhancement in the diazotrophic process leading to larger amounts of N fixed per unit area. On the contrary, unfavourable conditions often lead to arrested development of nodules, even if the process is initiated, and under severe stressed conditions like drought, previously functional and healthy nodules may be shed from the host root system to prevent channeling of valuable photosynthates towards the nodules.

Nodule formation and subsequent N_2 fixation are usually encouraged under N deficient edaphic conditions, but as the status of the soil improves following N_2 fixation and subsequent mineralization, the rates of nodule formation and growth are drastically reduced almost as if a self regulating system is in operation.

This chapter attempts to analyse the nodule population dynamics over a two year period at the study site. An attempt has also been made to correlate the effect of various environmental factors on the nodule population dynamics of T. repens growing in the two microhabitats viz., open and shaded plots, at the study site.

MATERIALS AND METHODS

These have been described in Chapter 3

RESULTS

Rooting nodes

The number of rooting nodes per stolon exhibited a significant ($P<0.01$) variation both between the plots and between the two leaf morph populations. The number of rooting nodes per stolon in the unmarked population was maximum in the month of September 1991 in the shaded plot (Fig. 4.1) and minimum during February 1993 in the open plot. Both marked and unmarked populations in the shaded plot had greater number of rooting nodes per stolon than in the open plot.

Photosynthetic area

The photosynthetic area (PSA) per plant did not vary significantly either between plots or between the two leaf morph populations during the study period. PSA was maximum in August 1992 (Fig. 4.2) in the marked leaf morph population in the open and in the unmarked population in the shade respectively. This corresponded with the period of peak vegetative growth in both leaf morph populations. Minimum values for PSA were obtained during winter - in January 1993 and February 1992 in the marked population from the open and shaded plots respectively. In general, PSA per plant was greater in both leaf morph populations from the shaded plot.

Dry weights

The shoot dry weights varied significantly ($P < 0.01$) between the two leaf morph populations although there was no significant difference between the open and shaded plots. In general, the shoot dry weight was maximum during summer when the stolon length was also maximum due to conducive environmental conditions (Table 4.1), whilst the minimum values were recorded during winter.

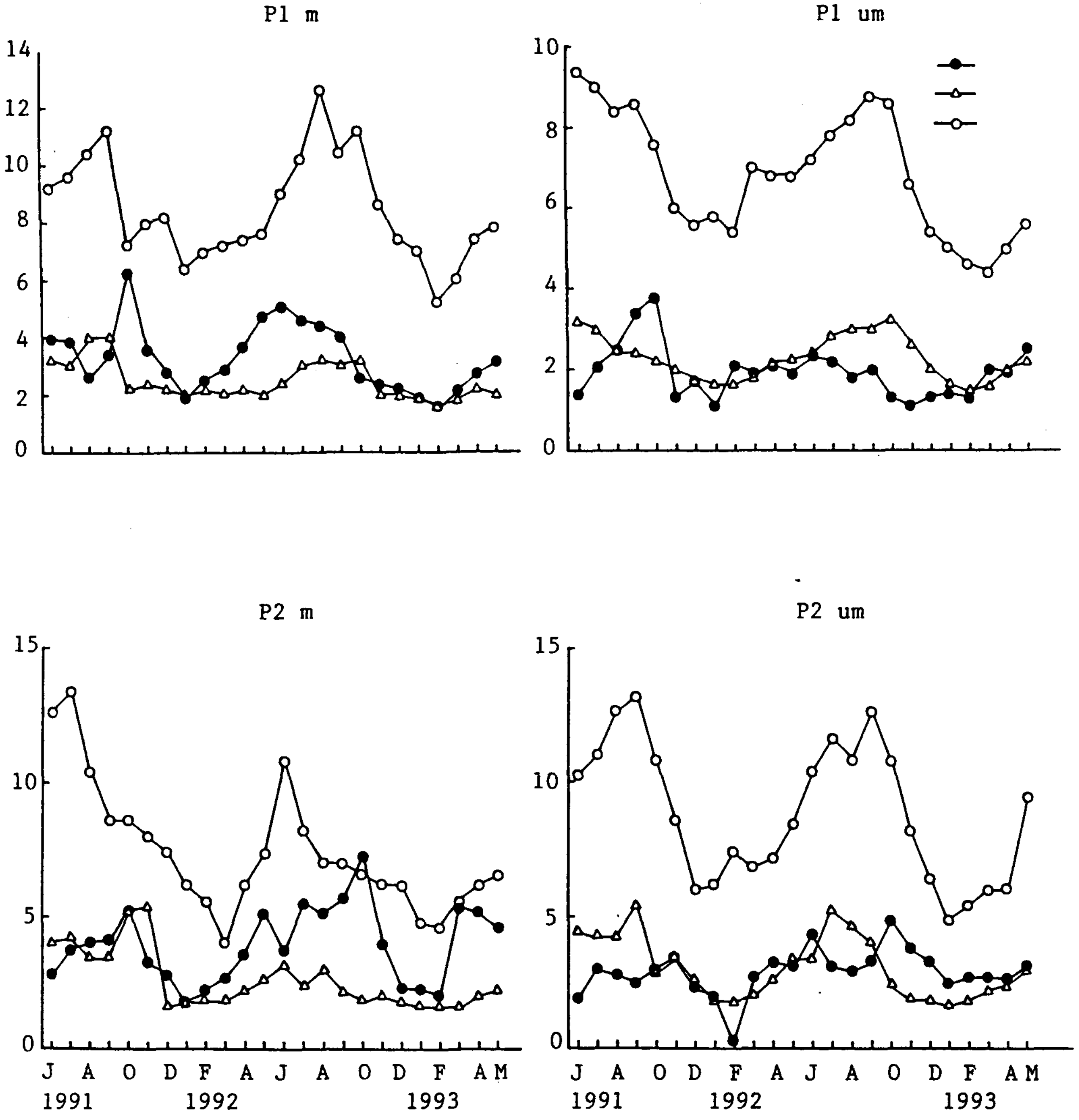

Fig. 4.1. Monthly variation in the number of nodes per stolon (o—o), rooting nodes per stolon (Δ—Δ) and nodules per rooting node (●—●) in the two leaf morph populations growing in 'open' and 'shade'. P1=Plot 1, P2=Plot 2, m=marked population, um=unmarked population.

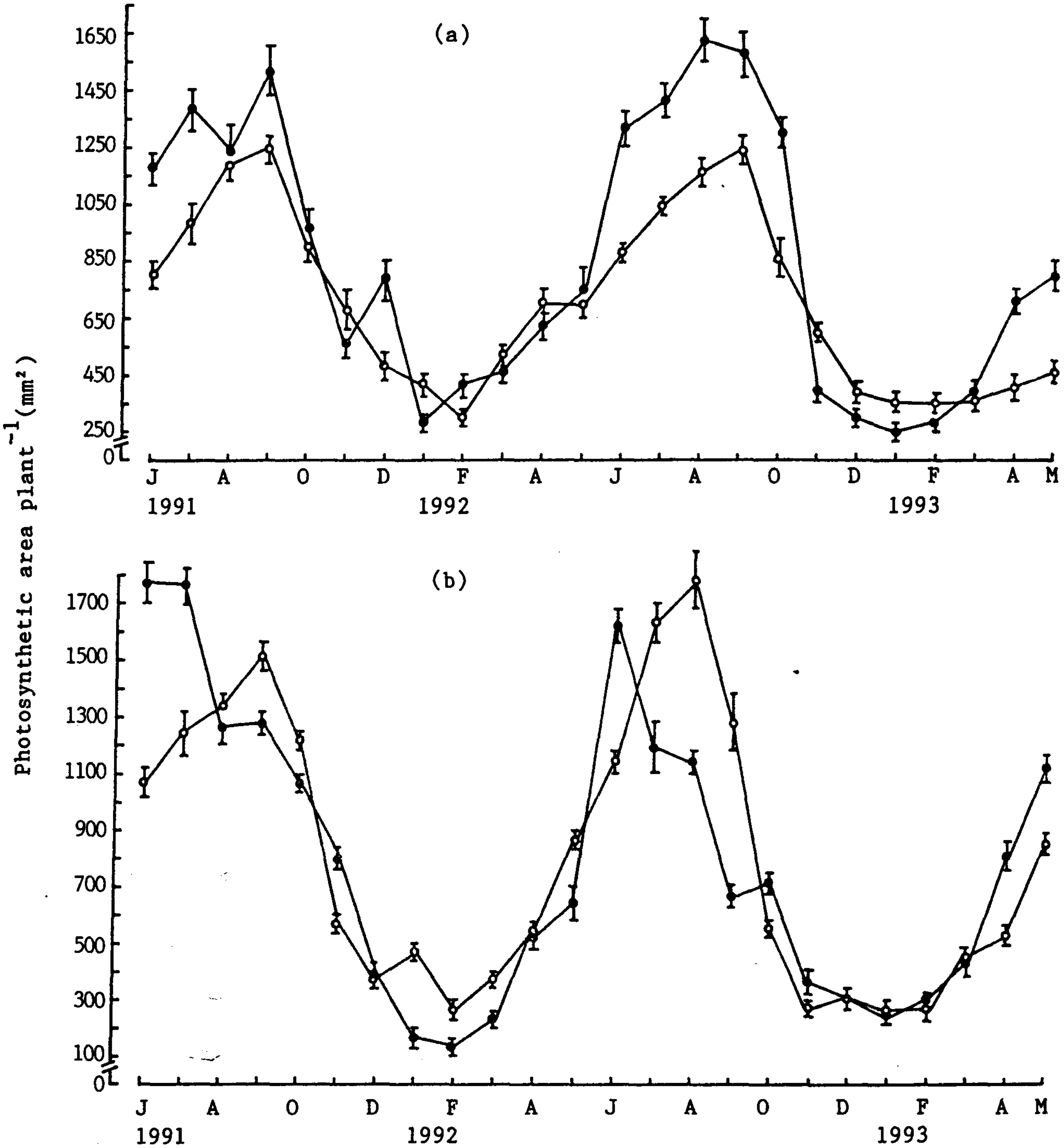

Fig.4.2. Monthly variation in photosynthetic area plant^{-1} of the marked (●—●) and unmarked (○—○) leaf morph populations growing in (a) 'open' and (b) 'shade'.

Table 4.1. Mean dry weights (mg) of shoot, root and inflorescence per stolon of the marked and unmarked leaf morph populations in 'open' and 'shade' during the study period. (n = 5)

Months	Open						Shade					
	marked population			unmarked population			marked population			unmarked population		
	Shoot	Root	Inflorescence	Shoot	Root	Inflorescence	Shoot	Root	Inflorescence	Shoot	Root	Inflorescence
Jun '91	33.96	4.03	2.78	32.03	3.04	-	31.60	3.78	17.48	29.76	3.42	7.62
Jul	33.89	4.17	-	33.71	3.27	-	33.50	4.10	4.72	33.04	4.21	-
Aug	34.05	4.09	-	29.62	4.13	-	33.86	3.84	-	33.21	4.07	-
Sep	46.13	4.52	-	34.06	4.79	-	37.64	3.52	-	39.58	3.98	-
Oct	29.14	3.98	-	23.14	4.92	-	27.58	3.16	-	23.10	4.53	-
Nov	20.73	3.70	-	18.67	4.13	-	22.14	2.52	-	21.72	3.03	-
Dec	19.64	3.91	-	17.05	3.07	-	20.72	2.96	-	19.64	2.96	-
Jan '92	21.37	2.96	-	16.92	3.10	-	18.64	2.72	-	18.22	2.08	-
Feb	38.01	3.03	4.72	17.00	2.98	9.07	18.32	3.00	2.62	17.04	2.14	7.31
Mar	39.37	3.79	8.53	18.63	3.32	9.34	37.46	2.96	4.62	18.13	3.90	16.50
Apr	47.46	4.56	16.01	23.04	4.04	14.00	46.14	4.62	16.94	29.32	4.73	17.29

Table 4.1. (contd.)

Months	Open						Shade					
	marked population			unmarked population			marked population			unmarked population		
	Shoot	Root	Inflorescence	Shoot	Root	Inflorescence	Shoot	Root	Inflorescence	Shoot	Root	Inflorescence
May	57.89	4.37	12.50	37.01	4.26	13.62	67.36	5.42	6.52	40.71	5.69	8.05
Jun	70.34	6.07	4.03	37.13	6.73	3.41	86.24	7.22	2.72	39.86	6.04	5.52
Jul	74.61	6.38	-	43.06	7.98	3.02	62.14	9.04	-	48.90	8.57	-
Aug	82.02	7.34	-	53.90	9.04	-	92.20	8.02	5.62	67.32	7.20	4.01
Sep	63.00	7.51	-	73.20	9.37	-	77.52	7.62	-	59.81	7.31	-
Oct	39.20	6.43	-	69.62	7.21	-	70.02	7.84	-	59.07	6.04	-
Nov	23.31	6.78	-	38.01	7.03	-	57.90	6.42	2.40	32.22	3.62	-
Dec	22.47	4.08	-	21.31	4.24	-	20.34	2.62	-	23.04	2.97	-
Jan '93	19.07	4.31	-	19.76	3.96	-	20.32	2.56	-	16.38	3.34	-
Feb	17.34	4.01	2.84	17.30	3.17	5.63	20.40	2.72	4.02	16.79	5.71	9.71
Mar	18.51	3.07	3.97	17.63	3.24	9.70	22.14	3.04	8.42	14.51	5.09	13.27
Apr	20.31	4.78	6.03	20.41	5.17	12.62	29.64	3.46	16.72	27.31	7.22	14.50
May	23.40	4.53	9.17	23.60	6.93	12.01	34.72	3.84	14.02	36.50	7.63	13.02

\- absence

Root dry weights did not show any significant variation either between the two plots or between the two leaf morph populations.

Inflorescence dry weights showed significant variation ($P < 0.01$) between the plots but the variation between the two leaf morph populations was not significant. Mean inflorescence dry weight was maximum in April 1992 (in the case of unmarked population in the shade) and minimum in November 1992 (in the marked population in the shade) (Table 4.1).

There was a significant ($P < 0.01$) difference in the mean nodule dry weights both between the open and shaded plots as well as between the two leaf morph populations. In the marked population, the mean nodule dry weight was maximum in September 1992 in the shaded plot and minimum in November 1991 in the same plot (Fig. 4.3). In the unmarked population, mean nodule dry weight was maximum in October 1992 in the shaded plot and minimum in February 1993 in the open plot.

Nodule number

The number of nodules per plant showed a significant ($P < 0.01$) variation both between the two plots and between the two leaf morph populations. Table 4.2 gives the percentage distribution of the total nodule number belonging to the five categories from the two leaf morph populations occurring in the two plots during the study period. The nodule number was greater in the marked population compared to the unmarked population in both open and shaded plots.

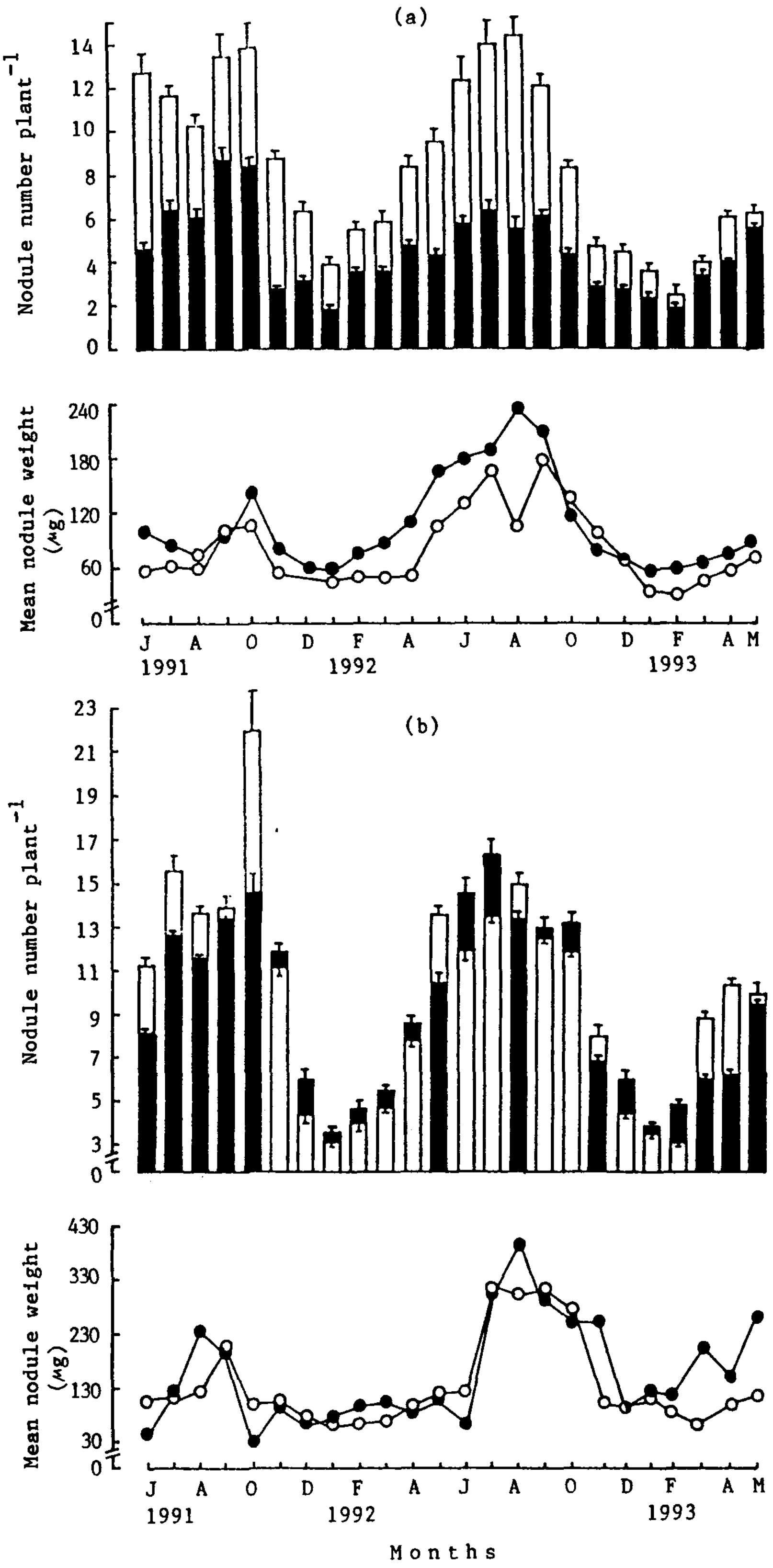

Fig. 4.3. Monthly variation in nodule number per plant and mean nodule dry weight of the marked and unmarked leaf morph populations growing in (a) 'open' and (b) 'shade'. Symbols: Nodule number per plant= □, marked population; ■ ,unmarked population; Mean nodule dry weight=-•-, marked population; -o-, unmarked population.

Table 4.2. Percentage distribution (%) of the total number of the 5 nodule categories from the two leaf morph populations occurring in the two plots during the study period. Values in parantheses indicate actual numbers.

	Plot 1 (open)		Plot 2 (shaded)	
	Marked	Unmarked	Marked	Unmarked
a	47.92 (565)	60.15 (317)	46.57 (496)	47.64 (466)
b	21.63 (255)	19.92 (105)	21.97 (234)	22.59 (221)
c	14.59 (172)	13.09 (69)	14.36 (153)	14.11 (138)
d	9.25 (109)	3.79 (20)	8.45 (90)	7.66 (75)
e	6.61 (78)	3.03 (16)	8.63 (92)	7.97 (78)
Total	31.75 (1241)	13.61 (527)	28.78 (1117)	25.86 (1001)

The unmarked population produced far larger number of nodules in shade than in open, whilst the marked population produced more nodules in open (Table 4.3).

Figure 4.3 represents the mean nodule number per plant in the two leaf morph populations from the two plots under consideration. The data reveal that the nodule number per plant increases during the spring and rainy seasons, decreasing sharply with the onset of the dry winter season. The marked population showed higher nodule number per plant than the unmarked population in the open during the entire study period. However, in the shade the nodule number per plant was marginally higher in the unmarked population than in the marked population (higher values were recorded at 13 observation dates out of the 24). In general the marked leaf morph population had greater number of nodules per plant than the unmarked population during the major part of the year.

In the marked population, the number of nodules per plant was maximum (22.0) during October 1991 in the shaded plot and minimum (2.4) during February 1993 in the open plot. Corresponding values for the unmarked population were 16.2 and 7.8 during July 1992 and January 1993, respectively.

Nodule types

The number of nodules in each of the five nodule categories varied significantly ($P < 0.01$) both between plots and between leaf morph populations. Of the five nodule categories, the maximum

Table 4.3. Analysis of variance of (a) morphological parameters and (b) dry weights of the clover as influenced by the plots at the study site and the leaf morph populations.

(a)

Parameters	Source of variation	df	F value	Level of significance
PSA	Plots	1	0.02	ns
	Leaf morphs	1	0.55	ns
	Plot x l.morph	1	0.66	ns
Rooting nodes	Plots	1	4.07	$P < 0.01$
	Leaf morphs	1	1.44	$P < 0.01$
	Plotxl.morph	1	6.13	$P < 0.01$
Nodule number	Plots	1	17.30	$P < 0.01$
	Leaf morphs	1	10.45	$P < 0.01$
	Plot l.morph	1	3.60	$P < 0.01$

(b)

Parameters	Source of variation	df	F value	Level of significance
Shoot dry weight	Plots	1	0.45	ns
	Leaf morphs	1	4.40	$P < 0.01$
	Plot x l.morph	1	0.12	ns
Root dry weight	Plots	1	0.25	ns
	Leaf morphs	1	0.73	ns
	Plot x l.morph	1	0.01	ns
Inflorescence dry weight	Plots	1	1.25	$P < 0.01$
	Leaf morphs	1	0.35	ns
	Plot x l.morph	1	0.05	ns
Nodule dry weight	Plots	1	8.04	$P < 0.01$
	Leaf morphs	1	4.78	$P < 0.01$
	Plot x l.morph	1	2.06	$P < 0.01$

PSA = Photosynthetic area

ns = not significant

number of nodules were of category "a" in both marked and unmarked leaf morph populations from the open and shade. Table 4.2 shows that 60.1% of the nodules were of this category in the unmarked leaf morph population from the open, while the unmarked population from the shade, marked population from the open and marked population from the shade respectively had 47.6%, 47.9% and 46.5% of their nodule population belonging to category "a".

Conversely, the unmarked leaf morph population from the open which had the highest percentage of its nodules in category "a", had the lowest percentage (3.03%) of the nodules in category "e". This trend was also exhibited for the marked population from the shade which had the lowest percentage of its nodules belonging to category "a", but had the highest proportion (8.6%) of nodules in category "e".

In general, there was a significant ($P < 0.01$) variation in the nodule number belonging to each of the five nodule categories between the plots and between the leaf morphs (Table 4.4).

Figure 4.4 shows the monthly variation in nodule numbers of the five categories from the two leaf morph populations in the open and shade. The nodules belonging to category "e" were absent during the major part of the study period in the case of the unmarked population, especially when growing in the open, whereas the small nodules (belonging to category "a") were best represented throughout the study period, comprising up to 77.8% of the nodule population in January 1992. The percentage of smaller nodules

Table 4.4. Analysis of variance of the five nodule categories of the clover as influenced by the plots at the study site and the leaf morph populations.

Nodule categories	Source of variation	df	F value	Level of significance
'a'	Plots	1	8.10	$P < 0.01$
	Leaf morphs	1	5.32	$P < 0.01$
	Plot x l.morph	1	0.98	ns
'b'	Plots	1	18.64	$P < 0.01$
	Leaf morphs	1	13.60	$P < 0.01$
	Plot x l.morph	1	3.77	$P < 0.01$
'c'	Plots	1	12.01	$P < 0.01$
	Leaf morphs	1	6.97	$P < 0.01$
	Plot x l.morph	1	1.33	$P < 0.01$
'd'	Plots	1	9.76	$P < 0.01$
	Leaf morphs	1	4.94	$P < 0.01$
	Plot x l.morph	1	1.17	$P < 0.01$
'e'	Plots	1	3.94	$P < 0.01$
	Leaf morphs	1	1.55	$P < 0.01$
	Plot x l.morph	1	4.11	$P < 0.01$

ns = not significant

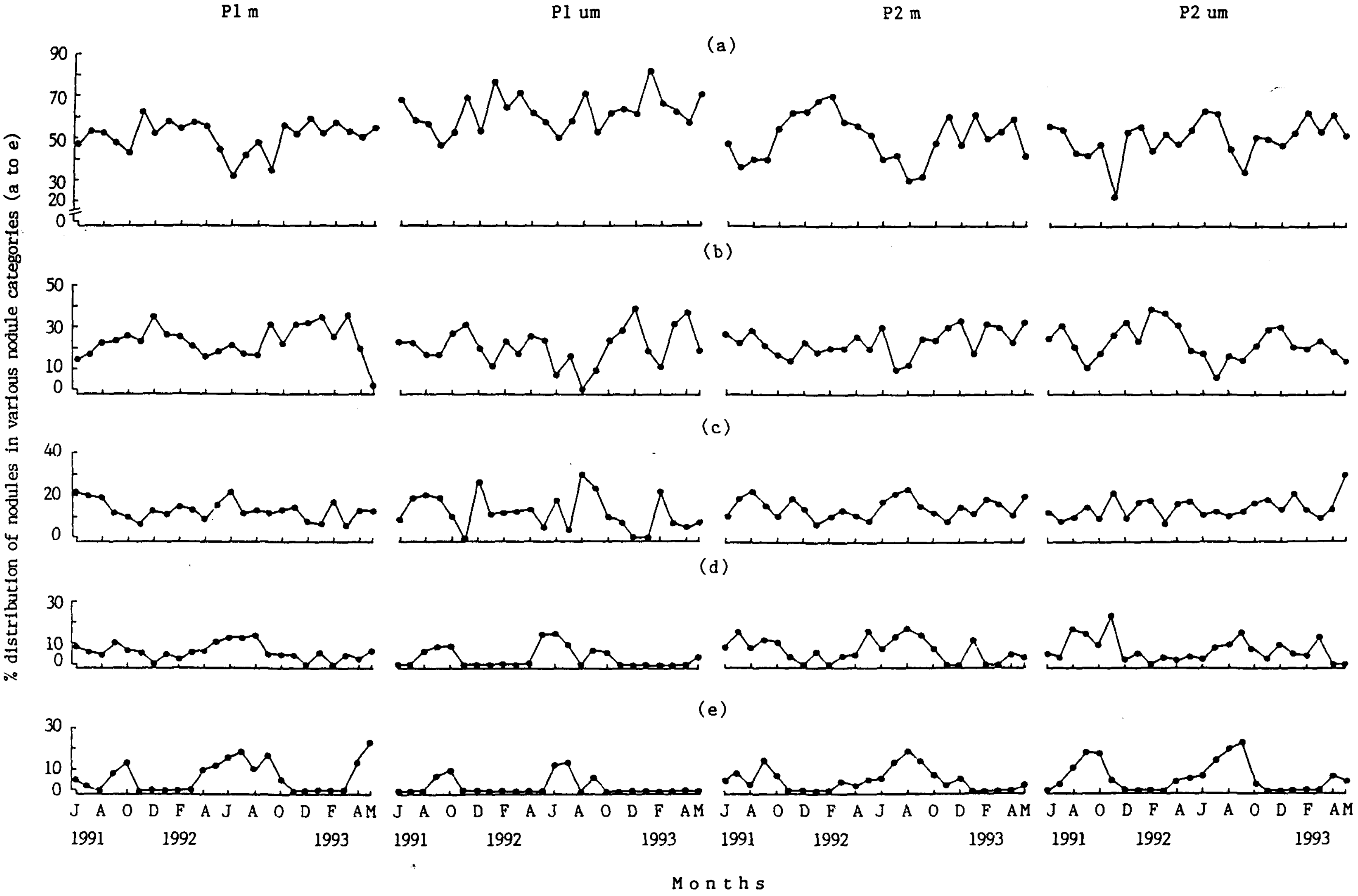

Fig.4.4. Percentage distribution (%) of the 5 nodule categories in the marked (m) and unmarked (um) leaf morph populations growing in 'open' (P1) and 'shade' (P2).

(categories "a" & "b") increased abruptly in relative proportions during the early part of the rainy season, declined during the latter part of the rainy season and the early part of the following autumn and increased again during dry winter months. The increase in the relative proportion of the smaller nodules is accompanied by a simultaneous decrease in the proportion of the largest nodules (category "e") and vise-versa.

Effect of edaphic factors on nodule population

Table 4.5a gives the correlation coefficients between important edaphic variables (soil temperature, soil moisture, pH, NO_3^--N, NH_4^+-N and total-N) and nodule number in the two leaf morph populations growing in open and shade. Soil temperature and soil moisture showed a significant positive correlation with nodule number in both marked and unmarked populations, the correlation being stronger for the open plot than for the shaded one.

The nodule number was also correlated with soil pH in all cases except for the unmarked population in shade.

NO_3^--N did not show any significant correlation with nodule number for either leaf morph populations in either plot, whereas NH_4^+-N and total N were significantly correlated with nodule number for both populations in open as well as in shade (Table 4.5).

Effect of legume parameters on nodule population

The correlation coefficients between important growth para-

Table 4.5. Correlation coefficient 'r' between (a) edaphic factors and nodule number, and (b) growth parameters of the legume and nodule number in the two leaf morph populations of *T. repens* growing in 'open' (P1) and 'shade' (P2).

(a)

Edaphic factors	Pearson's Correlation Coefficient 'r'				n
	Open		Shade		
	marked	unmarked	marked	unmarked	
Soil Temperature	0.773*	0.733*	0.620*	0.708*	24
Soil Moisture	0.886*	0.803*	0.694*	0.654*	24
pH	0.464*	0.433*	0.405*	0.349	24
NO_3^--N	-0.074	-0.004	0.129	0.346	24
NH_4^+-N	0.721*	0.598**	0.532**	0.584**	24
Total N	0.495	0.557**	0.519**	0.754*	24

(b)

Growth parameters of the legume	Pearson's Correlation Coefficient 'r'				n
	Open		Shade		
	marked	unmarked	marked	unmarked	
Rooting nodes	0.7303*	0.5627**	0.8241*	0.8493*	24
P.S. area	0.8964*	0.8028*	0.6659*	0.8474*	24
Shoot dry wt.	0.712*	0.412**	0.363	0.704*	24
Root dry wt.	0.482**	0.403**	0.372	0.553**	24

* Significant at $P<0.001$

** Significant at $P<0.01$

Table 4.6. Analysis of variance of edaphic factors at the study site as influenced by plots and year of study.

Edaphic factor	Source of variation	df	F value	Level of significance
Soil temperature	Plots	1	3.78	$P < 0.01$
	Years	1	0.05	ns
	Plot x Year	1	0.02	ns
Soil moisture	Plots	1	22.26	$P < 0.01$
	Years	1	0.18	ns
	Plot x Year	1	0.01	ns
pH	Plots	1	6.09	$P < 0.01$
	Years	1	0.02	ns
	Plot x Year	1	2.63	$P < 0.01$
NO_3^--N	Plots	1	0.19	ns
	Years	1	0.05	ns
	Plot x Year	1	0.35	ns
NH_4^+-N	Plots	1	0.88	ns
	Years	1	0.04	ns
	Plot x Year	1	0.06	ns
Total N	Plots	1	0.01	ns
	Years	1	1.86	$P < 0.01$
	Plot x Year	1	1.06	$P < 0.01$

ns = not significant

meters (number of rooting nodes per stolon, PSA, shoot dry weight and root dry weight) and nodule number of the two leaf morph populations are presented in Table 4.5b. Number of rooting nodes per stolon and PSA showed a significant correlation with the number of nodules in both leaf morph populations. Nodule number is most strongly correlated with number of rooting nodes per stolon in the unmarked population in the shade whereas in case of PSA, the correlation with nodule number was strongest for the marked population in the open. The nodule number was correlated to shoot and root dry weights in both leaf morph populations in open situation whereas in the case of the marked population growing in shade there was no such correlation. The correlation between nodule number and shoot dry weight was strongest in the marked population in the open. The correlation between root dry weight and nodule number, though significant (except for the marked population in shade), was not strong.

DISCUSSION

T. repens has a stoloniferous growth habit with leaves and adventitious roots being produced at the nodes and the species exists principally as stolons growing forward at the apex and decaying at the base (Beinhart *et al.* 1963, Harvey 1970). Increase in the number of rooting nodes per stolon is directly proportional to the nodule number probably due to an increase in possible sites of infection of legume roots by rhizobia. Between the plots, mean number of rooting nodes was significantly ($P<0.01$) higher in shade than in open during 1991-92 and 1992-93. This could be attributed to the significantly higher levels of soil moisture content in the shaded plot (Fig. 3.2). Similarly, a decline in the mean number of rooting nodes per stolon during winter and a decrease in mean root dry weights could be caused by a sharp decrease in soil moisture content during this season. Variations in the PSA were related to changes in vegetative growth and the peak values attained during July-September are in accordance with the findings of Mitchell (1956) that peak vegetative growth is attained when temperatures approach 20-25°C. Minimal values obtained in winter correspond to minimal vegetative growth of white clover at temperatures below 9°C (Pascoe 1973) and 5°C (Munro & Hughes 1966). In general, both leaf morph populations tended to show greater PSA per plant in shade than in open which could be considered as a mean of compensating for a decrease in photosynthetic rate under lower light intensities.

Since PSA did not show any significant variation between leaf morph populations, the observed significant difference in the shoot dry weights of the two populations could be attributed to differences in dry weights of the stolon tissues. Higher shoot dry weight values in case of the marked population is probably due to higher rates of channelling of photosynthates from the leaves, which is the 'source', to the stolon which acts as the 'sink', leading to rapid stolon growth (Chapman _et_ _al_. 1991). These 'sinks' play a crucial role in transporting of photosynthates to the nodule tissues in times of need.

The mean nodule dry weights peaked during late rainy season in both leaf morph populations which is in tune with the pattern of vegetative growth of the host plant. Chapman et al. (1991) reported that the largest part (up to 34%) of the photosynthates manufactured in the leaves, were channellized to adventitious roots bearing nodules. This provides a possible explanation for the similarity in the patterns of nodule growth and shoot growth.

As the young indeterminate nodules mature, they increase in size due to continuing activity of their apical meristems and thus during the latter part of the rainy season, a major proportion of the nodules belonged to categories "d" and "e" (Fig. 4.4). Though the increase in mean nodule number per plant may be only marginal, if at all, presence of a greater proportion of older and larger nodules contributes to the steep rise in nodule dry weights during September-October.

Soil moisture is one of the most important factors determining not only nodule formation and growth, but also the fate of functional nodules (Sprent 1976). A drastic reduction in soil moisture contents during winter was associated with a corresponding fall in mean nodule dry weights. This could be attributed to the high water content of white clover, reported to vary between 85.8% - 89.3% for Kent and New Zealand cultivars, respectively (Hayward 1953), which makes it particularly susceptible to drought. Unlike xerophytic legumes like Acacia which shed their leaves during transitory dry phases but retain their functional root nodules, temperate legumes like white clover are unable to react in a similar fashion owing to their persistent leafiness throughout the season. Shedding of root nodules, especially larger ones, is a result of reaction of the legume to such situations, probably to check the channelling of valuable photosynthates to nodule tissue. This also aids to overcome the decreasing ratio of fixed N_2/absorbed CO_2 with increasing soil water tensions and low temperatures (Kuo & Boersma 1971). The open plot is particularly subjected to dessicated conditions (Fig. 3.2) due to the bright sunshine it receives for the better part of the day coupled with the sandy substratum. A sharp decrease in the number of larger nodules (belonging to categories "d" and "e") during dry spells in this plot lends support to this view (Fig. 4.4).

A comparison of nodule number between the populations from the open and shaded plots shows that the marked population accounted for the maximum number of nodules in both open as well as shade (Table 4.2) as compared to the unmarked population. This is even more significant considering the fact that the marked population exhibited better nodulation in the open plot which has a sandy substratum with soil moisture levels well below those in the shaded plot. As reported by Pradhan & Tripathi (1985), the marked population has a higher N requirement than the unmarked population. The better nodulation in the marked population is probably a means of fulfilling its higher N requirement via the symbiotic association in the root nodules. Between the plots, the substratum of the open plot, due to its sandy texture, enhances leaching losses which could result in the sudden decrease in NO_3^--N levels (Fig. 3.2) following rainy spells, due to higher solubility of NO_3^- ions. This contributes to a lowered level of soil N (especially NO_3^--N) in the open plot, even if for brief periods, which triggered the host plant to respond by forming more root nodules as a compensatory reaction.

Though soil moisture is vital for nodule function, as discussed earlier, excessive levels are inhibitory for successful diazotrophy as waterlogged conditions limit aeration and restrict oxygen supply to the nodules (Bergersen 1971). Development of microaerobic conditions, even temporarily, in the shade during the rainy season could possibly be another reason for lower nodule population in this plot as compared to the open.

The population of small nodules showed a sharp increase during the early part of the rainy season in both leaf morph populations. Enhanced soil moisture conditions are reported to increase movement of rhizobia in soils (Hamdi 1971), rate of infection of root hairs (Worrall & Roughley 1976) and nodule growth and activity. The forementioned effects of soil moisture coupled with the increased availability of photosynthates from the new flush of leaves following rains, explain the sudden increase in these small young nodules. Though only a small fraction of these nodules persists and grows (to sizes comparable to those in category "d" and "e"), the occurrence of these 'juvenile' nodules in large numbers is an important factor in maintaining the optimal nodule population of the two leaf morph populations within a microenvironment.

The unmarked population from both open and shade had a higher proportion of their nodules belonging to category "a" as compared to the marked population whilst the reverse was true with respect to the largest nodules (category "e"). This suggests that longevity of nodules is more in the marked population whereby this population has a greater proportion of larger nodules which are more efficient in N_2 fixation compared to the juveniles. The unmarked population, on the other hand, tends to lose more nodules which results in a greater reduction in the number of mature nodules as compared to the marked population. This may be due to the lower N requirement of the unmarked population compared to the marked population (Pradhan & Tripathi 1984).

The prominent drop in nodule number in winter associated with an increase in the proportion of smaller nodules is of debatable significance. Whereas a decrease in nodule number and size is thought to be associated with a sharp decline in N_2 fixation rates, prevailing environmental stresses - namely moisture stress and low temperature - are in themselves inhibitory to N_2 fixation as has been confirmed by several workers (Sprent 1971, Kuo & Boersma 1971, Holter 1978). Moreover, N_2 fixation in temperate legumes like white clover has been reported to drop sharply below 9.8°C (Martin 1967) which suggests that N_2 fixation could be severely inhibited during winter months even though occasional showers may have temporarily raised soil moisture levels, as temperatures during this period remained well below the above mentioned limit. This is in conformity with the findings of Dalton & Zobel (1977).

Overwintering nodules would, however, be of special significance in the ecosystem as they resume function during the ensuing spring following a rise in temperature, soil moisture and increased supply of photosynthates from the flush of new leaves which arise from the dormant axillary buds present on the parent stolon.

5 Effect of nitrogen source on nodule population

The early development of a legume - Rhizobium endosymbiosis, manifested by the formation of root nodules, is vital for successful establishment and growth of legumes in nitrogen deficient soils. The symbiosis results in a nutritional complementation where the legume can be considered as a carbon-rich, nitrogen-deficient phototroph and the rhizobia as a carbon-deficient, nitrogen-fixing heterotroph (Verma & Nadler 1985). The resulting symbiosis makes the plant autotrophic with respect to the availability of reduced N, a limiting factor in plant nutrition. However, increased N input via fixation is self-limiting as high soil N levels inhibit nodulation. This is because legumes use combined N in preference to fixing atmospheric dinitrogen (N_2) (Allos & Bartholomew 1955). Substrate N has been shown to have an inhibitory effect on (i) the infection of legume root hairs by the endosymbiont,

(ii) the nodule mass per plant, although the reduction in nodule mass may sometimes be compensated for by nodule number per plant, which may not be seriously affected, and (iii) nitrogenase activity of the nodules, expressed as N_2 fixation rate of nodulated plants (Vincent 1965, Gibson 1974, Vallis 1978, Streeter 1988). On the contrary, nil or very low amounts of combined nitrogen may produce N deficiency symptoms and minimize nodulation and N_2 fixing capabilities due to reduced growth and vigour of the host plants. The detrimental effects of combined nitrogen also vary depending upon the form upon the form of N to which the root system is exposed i.e. NO_3^--N or NH_4^+-N.

The two leaf morph populations of T. repens seem to differ in their N requirement (Pradhan & Tripathi 1984). This study was therefore, undertaken to investigate the nodulation pattern and N_2 fixing abilities of the two clover populations as affected by varying levels of combined N, and to obtain data which would indicate the source and level of N that may result in maximum yield, nodulation and subsequent N_2 fixation. In order to achieve these objectives, two experiments were performed. Experiment 1 was carried out using NO_3^--N (supplied as KNO_3) and experiment 2 was performed using NH_4^+-N (supplied as NH_4Cl).

MATERIALS AND METHODS

These have been described in Chapter 3

RESULTS

Experiment 1

Plant growth

Increasing additions of KNO_3 resulted in an increase in shoot weight in both the marked and unmarked leaf morph populations of white clover (Fig. 5.1a). The difference in shoot weights between the two leaf morph populations and among the different levels of KNO_3 was not significant at the first harvest (H1). However, at the second and final harvest (H2), there was a significant variation in shoot dry weights both between the leaf morph populations and amongst KNO_3 levels ($P < 0.01$), the unmarked population exhibiting higher values. The large increase in shoot dry weights at H2 was also highly significant ($P < 0.01$) as compared to weights at H1 (Tables 5.1, 5.2).

There was a corresponding increase in root dry weight with increasing levels of KNO_3 in both the clover populations (Fig. 5.1b). The root dry weights were significantly higher ($P < 0.05$) in the marked population as compared to the unmarked at H2. This is contradictory to that of shoot dry weights which were significantly higher in the unmarked population.

Though there was an increase ($P < 0.01$) in plant weights with addition of KNO_3 at both harvests, there was no significant difference in weights between the two leaf morph populations (Tables 5.1, 5.2).

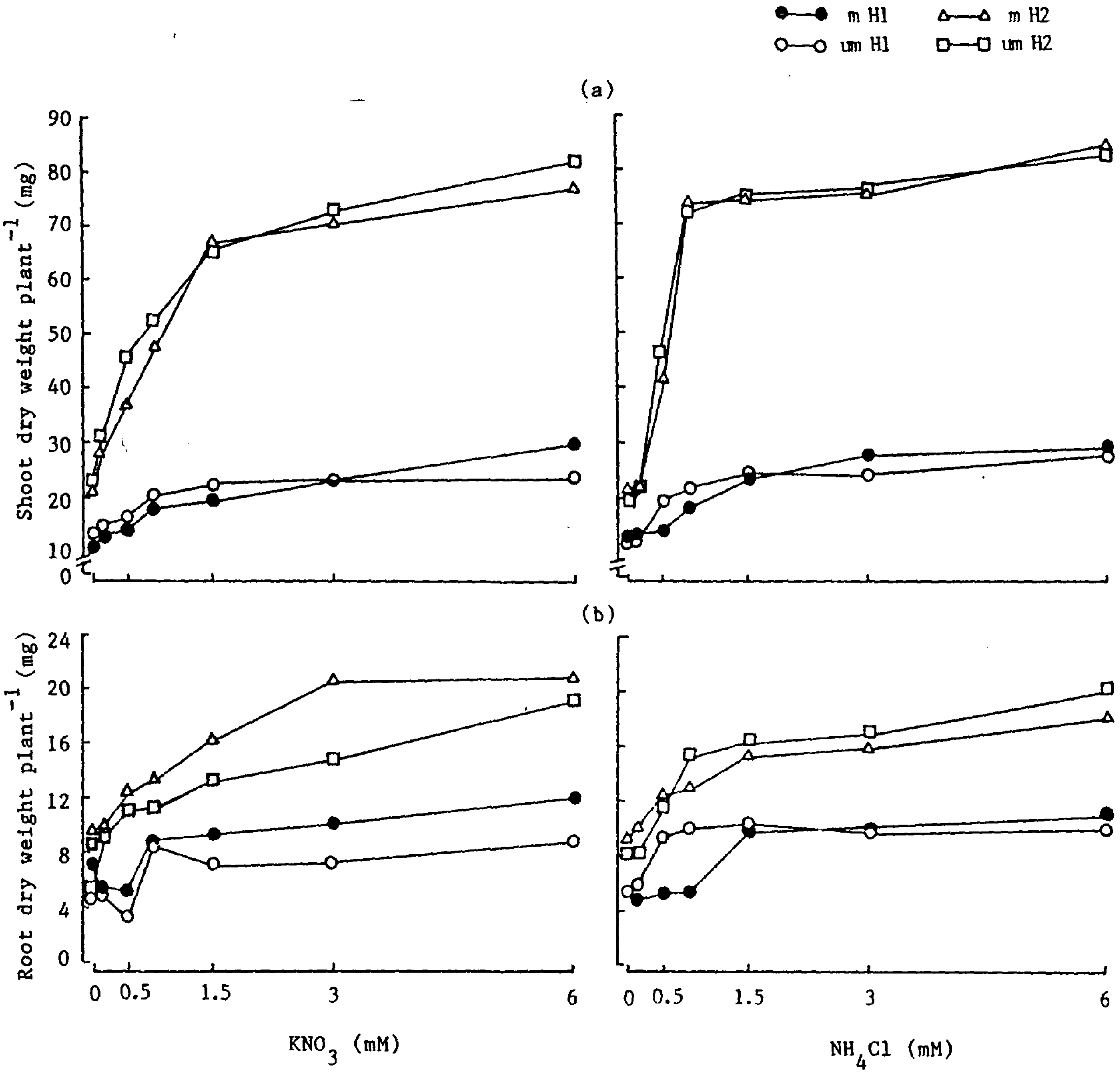

Fig.5.1. Effect of increasing concentrations of NO_3^- and NH_4^+ on (a) shoot dry weight and (b) root dry weight per plant in the two leaf morph populations of the clover at the two harvests. H1, harvest 1; H2, harvest 2; m, marked population um, unmarked population.

Table 5.1. Analysis of variance of legume parameters as influenced by the two leaf morph populations, different N levels and the two forms of N at harvest 1.

Plant parameters	Source of variation	df	F value	Level of significance
Shoot dry weight	Leaf morphs	1	0.32	ns
	N levels	6	0.21	ns
	N forms	1	2.75	ns
	L.morph x form	1	0.30	ns
	L.morph x level	6	0.78	ns
	Form x level	6	2.20	ns
Root dry weight	Leaf morphs	1	2.57	ns
	N levels	6	31.89	$P< 0.01$
	N forms	1	7.81	$P< 0.05$
	L.morph x form	1	32.59	$P< 0.01$
	L.morph x level	6	4.27	ns
	Form x level	6	4.52	$P< 0.05$
Plant dry weight	Leaf morphs	1	0.07	ns
	N levels	6	66.56	$P< 0.01$
	N forms	1	6.78	$P< 0.05$
	L.morph x form	1	3.29	ns
	L.morph x level	6	1.78	ns
	Form x level	6	6.41	$P< 0.05$
PSA	Leaf morphs	1	7.49	$P< 0.05$
	N levels	6	260.62	$P< 0.01$
	N forms	1	32.50	$P< 0.01$
	L.morph x form	1	4.88	ns
	L.morph x level	6	2.13	ns
	Form x level	6	6.41	$P< 0.05$

ns = not significant

Table 5.2. Analysis of variance of legume parameters as influenced by two leaf morph populations, different N levels and the two forms of N at harvest 2.

Plant parameters	Source of variation	df	F value	Level of significance
Shoot dry weight	Leaf morphs	1	17.76	$P < 0.01$
	N levels	6	1169.58	$P < 0.01$
	N forms	1	83.34	$P < 0.01$
	L.morph x form	1	7.96	$P < 0.05$
	L.morph x level	6	48.23	$P < 0.01$
	Form x level	6	3.30	ns
Root dry weight	Leaf morphs	1	7.96	$P < 0.05$
	N levels	6	47.35	$P < 0.01$
	N forms	1	0.56	ns
	L.morph x form	1	11.64	$P < 0.05$
	L.morph x level	6	1.26	ns
	Form x level	6	1.19	ns
Plant dry weight	Leaf morphs	1	1.05	ns
	N levels	6	971.99	$P < 0.01$
	N forms	1	60.07	$P < 0.01$
	L.morph x form	1	0.03	ns
	L.morph x level	6	32.15	$P < 0.01$
	Form x level	6	3.64	ns
PSA	Leaf morphs	1	11.29	$P < 0.05$
	N levels	6	162.27	$P < 0.01$
	N forms	1	7.19	$P < 0.05$
	L.morph x form	1	0.59	ns
	L.morph x level	6	0.62	ns
	Form x level	6	1.69	ns

ns = not significant

Photosynthetic area

Increase in plant with addition of KNO_3 was associated with significant ($P < 0.01$) increase in the photosynthetic area (PSA) per plant (Table 5.3), which was significantly ($P < 0.05$) greater in the unmarked leaf morph population as compared to the marked one (Tables 5.1, 5.2).

Nodule number

The mean nodule number per plant did not show any definite correlation with addition of KNO_3 in either of the leaf morph populations. At H1, the marked population exhibited an increase in the nodule number with increasing KNO_3 levels whereas the unmarked population showed an initial increase after which the number decreased sharply with no nodules being formed at higher exposures to KNO_3. There was a significant ($P < 0.01$) difference in the nodule number per plant between the two leaf morph populations, the marked population exhibiting higher values at both harvests. Nodule number per plant was significantly higher at H2 as compared to that at H1. At H2, the nodule number showed an initial increase with additions of KNO_3 after which the number sharply decreased with no nodules being formed at higher concentrations of KNO_3. The marked population, which nodulated even at higher concentrations of KNO_3 at H1, showed a suppression in nodulation at similar concentrations at H2. The nodule number per plant varied considerably amongst KNO_3 concentrations and the variations were significant (Tables 5.4, 5.5) at both harvests (Fig. 5.2).

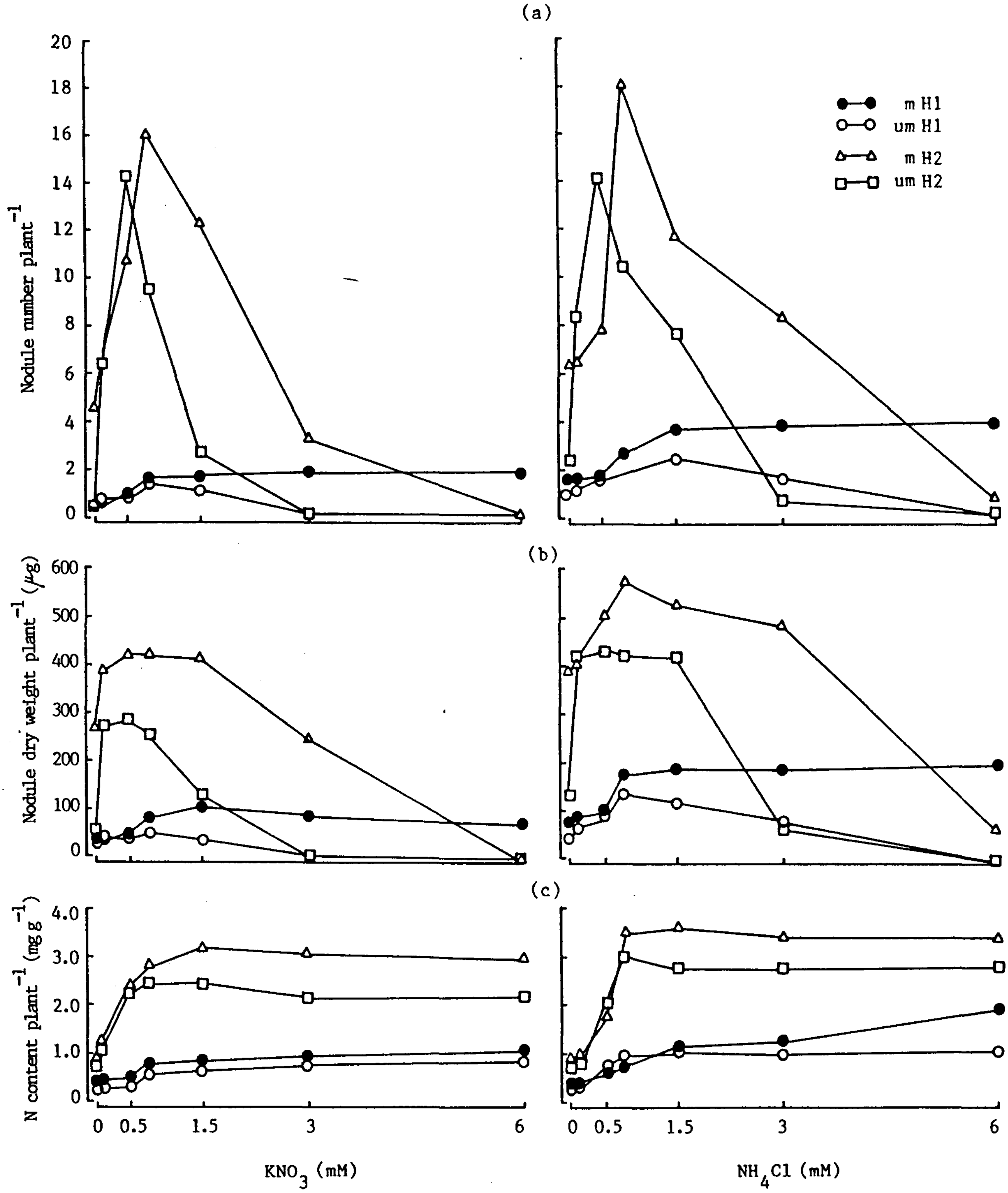

Fig.5.2. Effect of increasing concentrations of NO_3^- and NH_4^+ on (a) nodule number (b) nodule dry weight, and (c) N content per plant in the two leaf morph populations of the clover at the two harvests.

Table 5.3. Effect of increasing concentrations of KNO_3 on the nodule dry weight, plant N concentration and photosynthetic area (PSA) per plant in the two leaf morph populations of the clover at the two harvests.

	Harvest 1						Harvest 2					
	Marked population			Unmarked population			Marked population			Unmarked population		
KNO_3 concentration (mM)	Nodule dry weight (μg)	Plant N concentration (mg g^{-1})	PSA per plant (mm^2)	Nodule dry weight (μg)	Plant N concentration (mg g^{-1})	PSA per plant (mm^2)	Nodule dry weight (μg)	Plant N concentration (mg g^{-1})	PSA per plant (mm^2)	Nodule dry weight (μg)	Plant N concentration (mg g^{-1})	PSA per plant (mm^2)
0	48.6	23.85	143.64	47.3	16.73	140.40	58.7	28.79	129.32	70.9	26.51	131.93
0.1	52.2	25.01	148.96	49.7	18.08	191.10	59.3	33.90	160.93	43.2	27.09	459.00
0.5	51.0	25.13	198.72	50.3	18.63	229.50	39.6	35.46	291.18	20.1	30.12	507.00
0.75	49.3	27.98	588.00	33.6	18.79	576.00	26.3	40.09	491.30	26.7	31.19	553.84
1.5	60.3	28.03	585.01	34.9	21.01	625.11	34.7	38.67	814.46	49.3	30.74	910.65
3.0	47.3	28.76	615.78	-	17.31	782.46	78.9	33.51	1186.18	-	26.03	1349.64
6.0	45.0	26.00	666.54	-	18.63	809.19	-	30.06	1418.10	-	22.14	1378.98

\- absence

Table 5.4. Analysis of variance of nodule characteristics of the clover as influenced by two leaf morph populations, different N levels and the two forms of N at harvest 1.

Nodule characteristics	Source of variation	df	F value	Level of significance
Nodule number plant-1	Leaf morphs	1	50.32	$P < 0.01$
	N levels	6	7.02	$P < 0.05$
	N forms	1	68.62	$P < 0.01$
	L.morph x form	1	4.74	ns
	L.morph x level	6	1.48	ns
	Form x level	6	6.52	$P < 0.05$
Nodule weight per plant	Leaf morphs	1	29.02	$P < 0.01$
	N levels	6	6.94	$P < 0.05$
	N forms	1	3.22	ns
	L.morph x form	1	1.61	ns
	L.morph x level	6	1.40	ns
	Form x level	6	4.69	$P < 0.05$

ns = not significant

Table 5.5. Analysis of variance of nodule characteristics of the clover as influenced by two leaf morph populations, different N levels and the two forms of N at harvest 2.

Nodule characteristics	Source of variation	df	F value	Level of significance
Nodule number per plant	Leaf morphs	1	52.59	P < 0.01
	N levels	6	25.35	P < 0.01
	N forms	1	27.75	P < 0.01
	L.morph x form	1	0.09	ns
	L.morph x level	6	0.88	ns
	Form x level	6	3.40	P < 0.05
Nodule weight per plant	Leaf morphs	1	1.71	ns
	N levels	6	1.24	ns
	N forms	1	2.33	ns
	L.morph x form	1	0.01	ns
	L.morph x level	6	0.34	ns
	Form x level	6	0.59	ns

ns = not significant

Mean nodule weight

Although the number of nodules per plant showed a significant ($P<0.05$) increase with the addition of KNO_3 in both the leaf morph populations at H1, there was no correlation between nodule number and mean nodule weight in either population. At H2, however, there was a strong negative correlation between nodule number per plant and mean nodule weight (Fig. 5.3).

Nodule weight per plant

The total nodule mass per plant showed a distinct trend, being proportional to that of nodule number per plant (Fig. 5.2b). The difference in the nodule weights was significant between the two leaf morph populations ($P<0.01$) and NO_3^- levels ($P<0.05$) at H1 with the marked population exhibiting greater nodule weight per plant. Between harvests, the variation in nodule mass per plant was highly significant ($P<0.01$) with the weights being much higher at H2 than at H1.

Total N per plant

Figure 5.2c gives the corresponding N content per plant with increasing NO_3 levels. The N content showed an increase following NO_3 additions. The N content varied significantly between NO_3 levels ($P<0.01$) at both H1 and H2. The marked population showed a higher N content ($P<0.01$) as compared to the unmarked population at both harvests (Tables 5.6, 5.7).

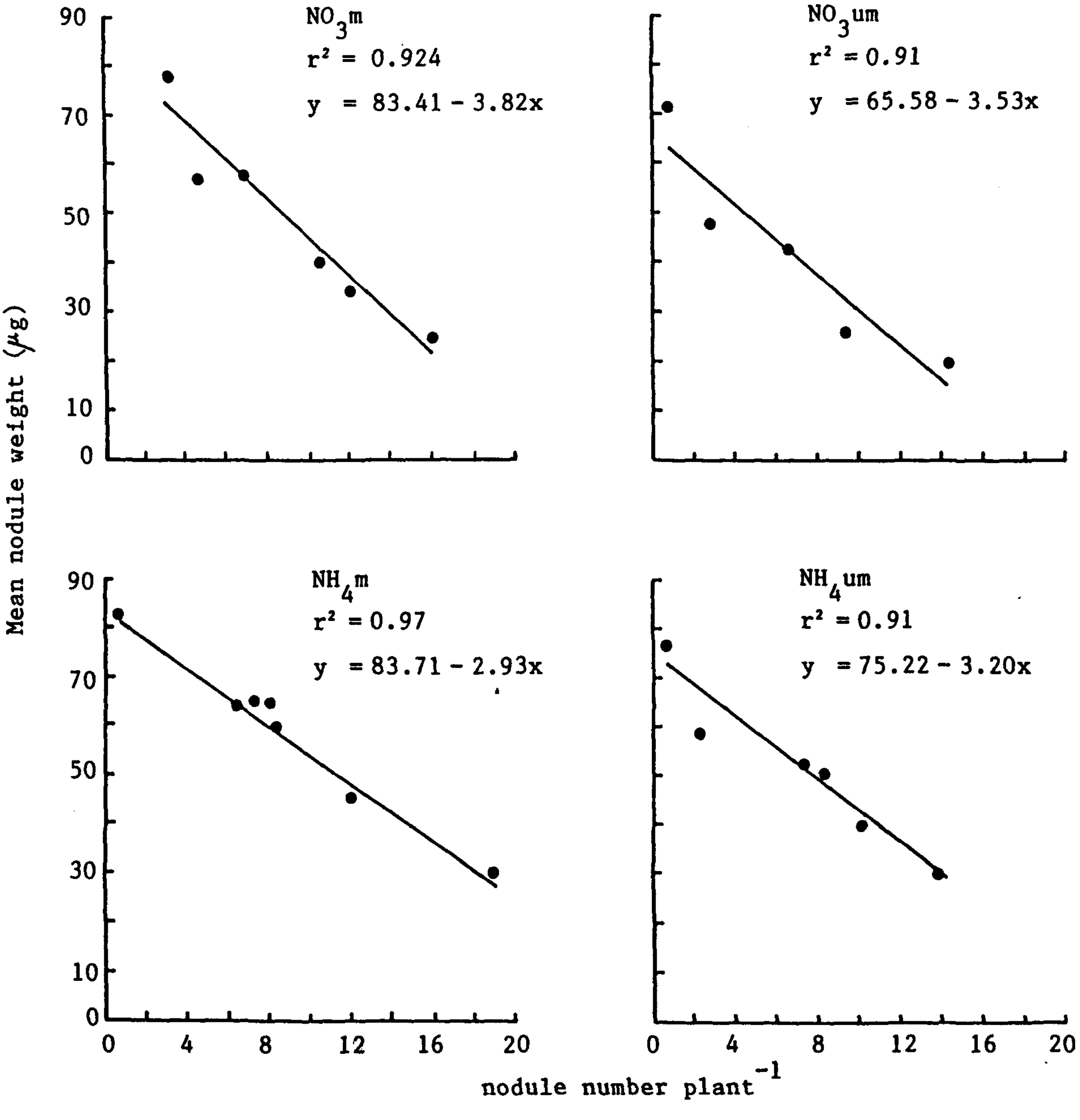

Fig.5.3. Regression showing the relationship between nodule number $plant^{-1}$ and mean nodule weight in the marked (m) and unmarked (um) leaf morph populations of clover supplied with NO_3^- and NH_4^+.

Table 5.6. Analysis of variance of growth parameters of the legume as influenced by two leaf morph populations, different N levels and the two forms of N at harvest 1.

Growth parameters	Source of varaition	df	F value	Level of significance
R_W	Leaf morphs	1	0.07	ns
	N levels	6	65.39	$P < 0.01$
	N forms	1	4.75	ns
	L.morph x form	1	2.68	ns
	L.morph x level	6	3.01	ns
	Form x level	6	1.99	ns
R_N	Leaf morphs	1	7.14	$P < 0.05$
	N levels	6	13.42	$P < 0.01$
	N forms	1	11.75	$P < 0.05$
	L.morph x form	1	0.71	ns
	L.morph x level	6	0.41	ns
	Form x level	6	0.53	ns
Total N per plant	Leaf morphs	1	19.71	$P < 0.01$
	N levels	6	52.59	$P < 0.01$
	N forms	1	49.52	$P < 0.01$
	L.morph x form	1	5.55	ns
	L.morph x level	6	2.33	ns
	Form x level	6	3.26	ns

ns = not significant

Table 5.7. Analysis of variance of growth parameters of the legume as influenced by two leaf morph populations, different N levels and the two forms of N at harvest 2.

Growth parameters	Source of variation	df	F value	Level of significance
R_W	Leaf morphs	1	0.08	ns
	N levels	6	22.96	$P < 0.01$
	N forms	1	2.77	ns
	L.morph x form	1	1.24	ns
	L.morph x level	6	0.80	ns
	Form x level	1	4.49	$P < 0.05$
R_N	Leaf morphs	1	0.92	ns
	N levels	6	7.42	$P < 0.05$
	N forms	1	4.66	ns
	L.morphs x form	1	0.40	ns
	L.morph x level	6	1.03	ns
	Form x level	1	1.71	ns
Total N per plant	Leaf morphs	1	199.78	$P < 0.01$
	N levels	6	521.20	$P < 0.01$
	N forms	1	34.66	$P < 0.01$
	L.morph x form	1	1.81	ns
	L.morph x level	6	18.58	$P < 0.01$
	Form x level	1	28.25	$P < 0.01$

ns = not significant

Relative growth rate and Relative N accumulation rate

The relative growth rates (R_W) (Table 5.8) showed a significant ($P< 0.01$) positive relationship with increasing KNO_3 levels at H1 for both leaf morph populations. At H2, the R_W values seemed to follow the trend exhibited by nodule number, nodule mass and N content of the clover populations, showing a sharp initial rise after which the R_W values either decreased (as in the marked population) or did not show any particular trend (as in the unmarked population). The variations in the R_W values were, however, significant at H2 ($P<0.01$) as in H1.

The relative N accumulation rates (R_N) showed a similar trend to that of R_W and increased significantly ($P <0.01$) with the addition of KNO_3 at H1 for both the leaf morph populations. Values were significantly ($P< 0.05$) higher for the marked population as compared to the unmarked one. At H2, the R_N values varied significantly ($P< 0.05$) showing a trend similar to that of R_W, reaching a maximum at 0.5 mM KNO_3 in both leaf morph populations, following which there was a gradual decline at higher concentrations (Tables 5.6, 5.7).

Experiment 2

Plant growth

Figure 5.1a shows the relationship between NH_4^+ levels and corresponding shoot weights in the two clover populations. The shoot

Table 5.8. Effect of increasing concentrations of KNO_3 on relative growth rate (R_W)(μg d^{-1}) and relative N accumulation rate (R_N)(μg d^{-1}) in the two leaf morph populations of the clover at the two harvests.

KNO_3 concentration (mM)	Harvest 1				Harvest 2			
	Marked population		Unmarked population		Marked population		Unmarked population	
	R_W	R_N	R_W	R_N	R_W	R_N	R_W	R_N
0	3.96	10.10	3.34	4.01	3.80	5.27	3.02	6.58
0.1	4.08	10.98	4.11	5.98	5.46	7.82	5.57	8.70
0.5	4.23	11.21	4.43	6.72	7.35	12.38	10.80	14.55
0.75	9.67	18.31	10.68	13.14	7.39	10.18	7.58	11.51
1.5	10.61	19.27	10.78	14.96	8.18	10.68	7.70	10.23
3.0	12.72	21.78	11.39	16.59	7.78	8.97	8.14	8.85
6.0	16.17	18.79	12.64	18.71	6.70	6.62	8.68	7.46

weights increased ($P< 0.01$) with NH_4 increments at both harvests. At H1, there was no significant difference in shoot dry weight either between the two leaf morph populations or between the two N sources (NO_3 and NH_4). At H2, not only did the shoot weights show a significant ($P< 0.01$) increase with additions of NH_4Cl but there was also a significant ($P < 0.01$) variation between the two leaf morph populations, the unmarked plants exhibiting greater shoot weights. The plants exposed to NH_4 too showed better shoot growth as compared to the NO_3 treated plants in both leaf morph populations. There was a marked increment in the shoot weights at H2 as compared to H1 ($P< 0.01$) for both the leaf morph populations in the NH_4 treated plants.

A corresponding increase in root biomass with additions of NH_4Cl was observed ($P< 0.01$) for both leaf morph populations at H1, though there was no significant difference in the root weights between the populations. Plants supplied with NH_4 also exhibited greater root weights ($P<0.05$) than those supplied with NO_3. At H2, the root weight in the unmarked population was significantly ($P <0.01$) higher compared to the marked population. Root growth was enhanced with the addition of NH_4 and there was a significant variation between NH_4 levels. Between harvests, the root weight showed a significant ($P <0.01$) increase at H2 compared to H1 for both leaf morph populations.

The total plant weights did not show any significant variation between the two leaf morph populations at either harvest although the increase in weight associated with NH_4 additions was significant at both harvests. The NH_4 treated plants also showed better growth, expressed as greater plant weight, as compared to NO_3 treated plants, both at H1 ($P<0.05$) and at H2 ($P<0.01$).

Photosynthetic area

Table 5.9 gives the PSA per plant corresponding to increasing levels of NH_4. PSA showed a significant ($P<0.01$) increase between NH_4 levels for both leaf morph populations at both harvests. Of the two leaf morph populations, PSA was greater in the unmarked population ($P<0.05$) both at H1 and H2. Plants supplied with NH_4 also showed greater PSA as compared to NO_3 treated plants, both at H1 ($P<0.01$) and H2 ($P<0.05$).

Nodule number

As in experiment 1, the nodule number per plant (Fig. 5.2a) did not show any definite relation with the addition of NH_4Cl. At H1, the increase in NH_4 levels caused an increase in nodule number per plant in the marked population, while in the unmarked population, there was an initial increase followed by a decline with no nodules being formed at 6 mM NH_4. The marked population showed a significantly ($P<0.01$) better nodulation with a larger number of nodules being formed per plant as compared to the unmarked population at both H1 and H2. The variation in nodule number per plant with increasing NH_4 levels was significant, both at H1 ($P<0.05$) and at H2 ($P<0.01$). In general, the clover populations had a significantly ($P<0.01$) larger number of nodules per plant at H2 as compared

Table 5.9. Effect of increasing concentrations of NH_4Cl on the nodule dry weight, plant N concentration and photosynthetic area (PSA) per plant in the two leaf morph populations of the clover at the two harvests.

NH_4Cl concentrations (mM)	Harvest 1						Harvest 2					
	Marked population			Unmarked population			Marked population			Unmarked population		
	Nodule dry weight (μg)	Plant N concentration ($mg\ g^{-1}$)	PSA per plant (mm^2)	Nodule dry weight (μg)	Plant N concentration ($mg\ g^{-1}$)	PSA per plant (mm^2)	Nodule dry weight (μg)	Plant N concentration ($mg\ g^{-1}$)	PSA per plant (mm^2)	Nodule dry weight (μg)	Plant N concentration ($mg\ g^{-1}$)	PSA per plant (mm^2)
0	50.3	25.11	129.01	46.3	21.36	138.04	63.8	29.31	143.64	59.3	26.03	165.36
0.1	50.9	25.07	144.97	46.7	22.01	146.83	64.0	30.01	163.59	50.9	26.91	188.71
0.5	52.7	28.03	153.44	53.1	23.71	181.76	64.1	33.13	294.68	30.1	34.18	401.71
0.75	62.0	30.18	353.06	50.0	30.11	329.31	30.3	41.01	499.97	40.2	34.16	566.72
1.5	48.9	33.07	582.01	42.1	31.70	543.00	45.1	40.03	735.21	53.7	30.03	880.08
3.0	50.0	33.16	631.12	41.0	30.80	629.01	59.8	37.01	1008.72	78.6	29.85	1016.89
6.0	49.8	35.01	665.60	-	28.17	734.16	83.4	33.16	1274.91	-	26.51	1403.43

\- absence

to numbers at H1.

Plants supplied with NH_4^+ developed a larger number of nodules per plant as compared to NO_3^- treated plants. There was a complete inhibition of nodulation in the unmarked population occurring at the highest level of NH_4-N i.e. 6 mM at both harvests (Table 5.10).

Mean nodule weight

At H1, there was no correlation between nodule number and mass per nodule in either leaf morph population. Figure 5.3 gives the regression model of nodule number per plant plotted against mean nodule weight at H2. Of the two leaf morph populations, the correlation was stronger in the marked ($r^2 = 0.97$) than in the unmarked ($r^2 = 0.91$) population. Between the harvests, there was no significant difference in mean nodule weight in either leaf morph population.

Nodule weight per plant

The total nodule mass per plant (Fig. 5.2b) showed an initial increase with addition of NH_4 for both leaf morph populations. At higher levels of NH_4, the nodule weight per plant did not increase much in the marked population, and in the unmarked population there was a gradual decline associated with increased levels of NH_4 from 1.5 mM onwards. At H2, the nodule mass per plant was significantly ($P < 0.01$) greater than at H1 for both leaf morph populations. Between the two leaf morph populations, there was a significant ($P < 0.01$) difference in the nodule mass per plant

Table 5.10. Effect of increasing concentrations of KNO3 and NH4Cl on the nodule number per unit (100 mg) plant dry weight.

Nitrogen levels (mM)	KNO_3				NH_4Cl			
	Harvest 1		Harvest 2		Harvest 1		Harvest 2	
	marked	unmarked	marked	unmarked	marked	unmarked	marked	unmarked
0	2.97	3.09	14.81	2.09	8.21	5.57	19.66	8.03
0.1	2.94	3.46	17.34	16.52	8.62	6.77	19.77	26.91
0.5	4.64	4.01	21.55	24.19	8.71	5.40	14.66	24.01
0.75	5.73	4.95	26.38	14.92	11.62	9.06	21.76	11.74
1.5	5.77	3.62	14.57	3.38	10.98	8.10	12.95	8.40
3.0	5.21	-	3.49	-	10.04	4.61	9.02	0.84
6.0	4.17	-	-	-	7.93	-	0.66	-

- absence

with the marked population exhibiting higher values (Tables 5.4, 5.5).

Total N per plant

Addition of NH_4Cl resulted in a steady increase in the total N content per plant (Fig. 5.2c) in both marked and unmarked leaf morph populations at H1. Total plant N at H2 showed a steep rise with increasing NH_4^+ concentration up to 0.5 mM level but at higher concentrations, the values did not increase. Plants of the marked population had significantly ($P < 0.01$) greater amounts of total N than those of the unmarked population, at both harvests. Total N increments associated with additions of NH_4Cl were significant ($P < 0.01$) at both harvests.

Relative growth rate and Relative N accumulation rate

Table 5.11 gives the corresponding values of R_W with the additions of NH_4Cl. The R_W values increased steadily up to 0.75 mM NH_4 following which there was either no definite trend (marked population) or only a marginal increase (unmarked population) on further additions of NH_4Cl. Variations in corresponding values of R_W was significant ($P < 0.01$) among NH_4 levels for both leaf morph populations. The R_W values at H1 showed a rapid increase with additions of NH_4Cl, especially in the marked population, with the 6 mM NH_4 treatment recording about 2.5 times the value in the case of the N free control. The unmarked population showed a sharper increase in the R_N values with a three fold increase as compared to the N free control At H1, the R_N values were generally higher for the marked population

Table 5.11. Effect of increasing concentrations of NH_4Cl on relative growth rate (R_W)(μg d^{-1}) and relative N accumulation rate (R_N)(μg d^{-1}) in the two leaf morph populations of the clover at the two harvests.

NH_4Cl concentration (mM)	Harvest 1				Harvest 2			
	Marked population		Unmarked population		Marked population		Unmarked population	
	R_W	R_N	R_W	R_N	R_W	R_N	R_W	R_N
0	4.07	11.01	3.18	7.60	3.95	5.16	3.73	5.48
0.1	3.31	10.24	3.25	8.16	4.43	5.82	3.99	11.09
0.5	4.76	13.42	10.56	16.63	7.73	9.03	5.51	8.35
0.75	7.65	17.25	11.23	21.01	10.04	12.52	8.31	9.29
1.5	12.87	24.10	12.97	23.54	7.67	9.15	7.73	7.31
3.0	14.72	25.98	13.03	23.15	6.84	7.69	7.75	7.52
6.0	15.65	27.76	14.45	16.88	7.19	6.78	7.98	8.35

($P < 0.05$) than the unmarked, and varied significantly ($P < 0.01$) between the NH_4 levels.

At H2, R_N values in both leaf morph populations increased up to 0.75 mM NH_4 level after which the values gradually decreased with further increments of NH_4Cl. Plants exposed to NH_4 (experiment 2) recorded higher values ($P<0.05$) for R_N than plants exposed to NO_3 (experiment 1) at H1. At H2, however, this trend was reversed with the NO_3 treated plants recording R_N values marginally higher than NH_4 treated plants (Tables 5.6, 5.7).

DISCUSSION

Addition of KNO_3 or NH_4Cl led to a steady increase in the weights of both shoot and root of the two clover populations at H1. Though shoot weights were greater than corresponding root weights at respective N levels, this difference was markedly pronounced at H2 when shoot weights were up to four times that of the root, especially at higher N levels (Fig. 5.1a). This trend suggests that most of the photosynthates produced as a result of augmented plant growth at higher N levels was used for shoot development which also functions as a sink for assimilates (Batra 1978, Chapman 1986).

Although the total plant weights did not differ significantly between the two leaf morph populations, the allocation pattern showed a conspicuous variation with the marked plants exhibiting better root development as compared to the shoot. This trend, though insignificant in NH_4 treated plants, was obvious in the plants exposed to NO_3. On the contrary, shoot development appears to be better in the unmarked population. The development of nodules at nil N in both the leaf morph populations, indicates that nodule formation is not N dependent but the initial increase in nodule number per plant following N addition, clearly indicates that low levels of N in soil enhance nodule formation and growth. There is a time lag between nodule formation and active N_2 fixation by the newly formed nodules and during this lag period the host plant is dependent on N present in the soil to tide over the temporary N crisis. Therefore, such plants show signs of N deficiency, even if only temporarily, when soil N levels were low (Streeter 1988). Plants of the N free controls, though nodulated, faced initial N deprivation and exhibited poor growth as indicated by low plant biomass (Figs. 5.1a, b). The nodule numbers per plant at H1 at 0.1 mM and 0.5 mM nitrogen were only marginally higher than at nil N but at H2 there was almost a four fold increase in nodule number and a sharp corresponding increase in weights of nodules and host plants.

Plants from the N free controls at H2 exhibited stunted growth and had low plant and nodule biomass. They were pale and occasionally had chlorotic leaves thereby exhibiting the classical symptoms of N deficiency. This indicates that initially some N is required by the legume to tide over the temporary period of crisis prior to commencement of N_2 fixation. Thus, the plants grown at nil N exhibited poor growth in spite of nodulating considerably. This is in conformity with the findings of Harper (1974), Pankhurst & Jones (1979), Das (1982) and Rawsthorne *et al.* (1985).

In both experiments, the sudden drop in nodule number following the sharp initial increase at H2, ultimately culminating in the total inhibition of nodulation, is interesting. Of the two forms of N available to a nodulated legume - combined N and molecular dinitrogen (N_2) - the first form is usually the preferred form (Wery *et al.* 1978). Nodule development and function are energy demanding processes requiring a continuous supply of photosynthates from the aboveground sinks (Bergersen 1974, Chapman 1983) and the initial development of nodules is dependent on the growth of the host plant which is enhanced with the addition of combined N. After the plants have grown considerably as was the case at H2, they require larger quantities of N which is mostly provided by the increased number of root nodules. However, the nodule number per plant which showed a sharp increase at lower N levels, starts decreasing rapidly with N additions in both leaf morph populations, indicating that the legume probably switches over to the more preferred source of N i.e. combined N (Stone & Buttery 1986). The combined N inhibits nodulation by affecting a broad range of infection events which include a decrease in root hair deformations (Truchet & Dazzo 1982), a decrease in the binding of rhizobia to root hairs (Dazzo & Brill 1979), a decrease in the number of infection threads (Munns 1977) and an increase in the number of aborted infections (Munns 1977). According to Dazzo & Brill (1979) higher levels of combined N hamper nodulation which is mediated via inhibition of infection of root hairs by the rhizobia. They have reported that with an increase in N levels, the accumulation of the host lectin on root hair surfaces is drastically reduced even though there may not be any inhibition of its synthesis by the host. This in turn prevents R. trifolii from accumulating on and adhering to root hair surfaces, thereby reducing infection and subsequent nodule formation.

Between harvests, the marked population actually showed a reduction in nodule number at H2 as compared to H1 at 6 mM NO_3 or NH_4 (Fig. 5.2). The plants harvested at H1 were exposed to the high N level only for a short duration and therefore, the detrimental effects of increased levels of soil N on nodulation were not obvious. However, after prolonged exposure to the high nitrogen, the plants substituted combined N for atmospheric dinitrogen (N_2) and do not need to develop any more nodules afresh. Existing nodules are also shed as they are temporarily denominated as non-functional and probably become expensive for the host to maintain due to channeling of photosynthates (Wedderburn 1983). In both experiments the marked population showed an increased root growth which contributes to an increase in infection sites resulting in greater number of nodules being formed. Greater nodule number per plant in the marked population was also exhibited under field conditions (Chapter 4). According to Pradhan & Tripathi (1984) the marked population has a greater N requirement than the unmarked population and the better nodulating ability of the former is probably a means of fulfilling its higher N requirement. The newly formed nodules nodules also act as sinks for photosynthates from the shoot and therefore, any increase in number of nodules would probably contribute to increase in nodule weight (Wedderburn 1983). This seems to be true in the present study. Though nodule number per plant drops sharply with increasing N levels, there is an increase in mean nodule weight (Fig. 5.3) indicating that higher levels of N may be detrimental to nodule initiation and formation but not to nodule growth. Thus the host plant compensates for the fewer successful infections by an increase in mass per nodule - an adaptation to sparse nodulation. The strong negative correlation between the two parameters exhibited by the marked population in both the experiments, indicates the greater tendency of the N demanding marked population to compensate for the curtailed number of nodules by augmented nodule mass. Between the two experiments, the coefficient of regression (r) was lower in the NO_3 treated plants indicating that in comparison to NH_4, not only was NO_3 a stronger inhibitor of nodulation but also of nodule growth. The probable causes for this are discussed after the following section.

Though mean nodule weight did not vary significantly between the two leaf morph populations in either experiment, the values tended to be higher in the marked plants which could be considered an important strategy to meet the higher N requirement of the marked plants, as N_2 fixation is a function of nodule weight.

The growth response of the nodules, measured as total nodule mass per plant, shows a sharp rise with low N increments which can be attributed both to a steep increase in nodule number and a simultaneous rapid growth of newly formed nodules triggered by a flush in shoot growth stimulated by N additions. The decrease in the total nodule mass following a drop in nodule number is lessened due to compensation for fewer successful infections with an increase in mass per nodule. Though the drop in nodule number is associated with an increase in mean nodule weight, the number of nodules was too low to maintain the total nodule mass per plant. Compensation thus, does not preclude the importance of nodule number and in case of sparse nodulation, increased numbers will be required to bring about an increase in nodule biomass.

Results of the present investigation indicate that of the two forms of N supplied, NO_3 is a more potent inhibitor of nodulation in clover. This holds good for inhibition of infection as well as inhibition of nodule growth and activity. According to Dazzo & Brill (1978), the infection process is more sensitive to NH_4 than to NO_3, but the findings of the present study are in conformity with those of the majority of workers on a wide range of legumes, who hold the opposite view (Darbyshire 1966, Dixon 1969, Streeter 1981, Ursino _et al_. 1982, Eardly _et al_. 1984, Sawhney _et al_. 1985). Wong (pers. commn.) also holds the latter view. Nodule growth has also been reported to be more sensitive to NO_3 than NH_4 (Imsande 1986). Our findings are in conformity with those of Streeter (1981), Ursino _et al_. (1982) and Sawhney _et al_. (1985) who also reported that several weeks exposure to 4-8 mM NO_3 leads to a reduction in nodule growth. In soybean, long term NO_3 supply was, however, found not to influence the number of infected, uninfected or cortical cells (Streeter 1988). Thus the decline in nodule size in response to N supply could probably be ascribed to a decrease in cell size.

When NO_3^- is supplied, its reduction and assimilation via nitrate reductase requires considerable reducing power and as such the amount of photosynthates, that could have been otherwise available for distribution to nodules is reduced. Studies on clover (Small & Leonard 1969), cowpea (Kahn & Kahn 1981) and soybean (Truchet & Dazzo 1982, Kouchi & Yoneyama 1984) showing a decrease in ^{14}C-labelled photosynthates to nodules in the presence of NO_3^-, lends support to this view. Nitrate is also partially reduced to nitrite which, besides being toxic to all living organisms including Rhizobium, is known to destroy IAA which is required for infection. This view has been confirmed by Dixon (1969) and Munns (1977) who reported that IAA supplied with NO_3^- could mitigate the inhibitory effects to some extent. Nitrite also oxidises leghaemoglobin to its inactive ferric form (Rigaud & Puppo 1977) which could affect the nitrogenase enzyme. Temperate legumes reduce a greater proportion of their nitrate in roots than tropical legumes (Andrews 1986). The inhibitory effects of NO_3^- on nodulation in white clover, a temperate legume, are probably mediated via the above-mentioned mechanisms.

Latimore et al. (1977) and Rabie et al. (1980) suggest that NH_4-N also acts in a similar way, but the inhibitory effects are less severe due to its reduced state. NH_4-treated plants also exhibited increased root growth leading to a greater number of infection sites which probably contributes to the increase in nodule number and weight (Wedderburn 1983) as discussed earlier.

Results indicated that NO_3^--treated plants failed to nodulate at higher N levels (3 mM for unmarked population and 6 mM for marked population) vis-a-vis to NH_4^+-treated plants where nodulation was totally inhibited only at 6 mM for the unmarked population (the marked population still forming some nodules). This confirms that not only is NO_3 a stronger inhibitor of nodulation but also the marked population is more resistant to the inhibitory effects of combined N as indicated by its greater nodule number as compared to the other population.

Between the two leaf morph populations, the marked population exhibited a marginally higher N content per plant in both the experiments. This could be attributed to the greater nodule number per plant of the marked population coupled with its higher N requirement which could be fulfilled by the uptake of combined N by the root system if the amount of symbiotically fixed N_2 proved inadequate. The higher N contents per plant in the NH_4 treated ones (experiment 2) compared to the NO_3 treated ones is probably the outcome of the greater number of nodules which develop in response to NH_4 addition. Nodule mass per plant was also higher in NH_4 treatments and thus the greater amount of potential N_2 fixing tissue could be responsible for the increased levels of total plant N in experiment 2.

At nil N lesel, the corresponding increment in total N of the legume is a measure of the amount of N_2 fixed by the root nodules. The total N content of the unmarked plants is comparable to that of the marked plants in spite of their lower nodule number and nodule mass per plant. This implies that the nitrogenase activity and subsequent fixation rates are considerably higher in the unmarked population probably fuelled by larger amounts of photosynthates from significantly greater aboveground biomass and photosynthetic area. However, the marked population has an edge over the unmarked population in total N content per plant, which is probably indicative of its higher N requirement, fulfilled by a much greater nodule number and nodule mass per plant (Fig. 5.2). With the addition of N to the substratum, corresponding estimates of fixation are, however, difficult to assess as the legume prefers to take up the readily available combined N in lieu of fixing atmospheric N_2. According to Ladha _et_ _al_. (1988) contribution of N by fixation varies from 50-80% of the total N content of a legume, the relative proportion of fixed N_2 increasing with a corresponding decrease in the amounts of combined N in the substratum. A more conservative estimate of contribution to the total N content in legumes by the symbiotic process has been put at 50% (Pate _et_ _al_. 1979). In the present study, as the amount of added N was increased, corresponding contribution of N via fixation decreased along with nodule number and biomass. Since this is not obvious due to total plant N remaining somewhat constant, the process may more specifically be referred to as N_2 assimilation in lieu of N_2 fixation. This symbiosis is thus of immense ecological significance in the N economy of the habitat, especially in N deficient environments.

Whether growth and nodulation of the two leaf morph populations of white clover was stimulated or depressed, was dependent on the form and concentration of the applied N. In this study, both NO_3^- and NH_4^+ supplied at 0.75 mM had the greatest beneficial effect on nodule number and nodule mass per plant in both leaf morph populations. The better-nodulating marked population developed nodules at 3 mM and 6 mM NO_3^- and NH_4^+ respectively, even after the 12 week period of exposure to N. However, it is possible that N levels not inhibitory in adequately watered small container experiments could be inhibitory in the field where lower soil moisture levels might make N nonlimiting and also restrict the development of an adequate soil population of the appropriate microsymbiont. The marked leaf morph population of T. repens thus tended to overcome the self limitation imposed due to high soil N levels and can be expected to nodulate and continue to fix N_2 under such stress. The shallow rooting local clover populations with nodulation and N_2 fixation occurring within a 20 cm depth, is therefore vital in the N economy of grasslands and for vegetation establishment in N-deficient situations.

6

Effect of pH on nodule population

Soils exhibiting acidic reactions are of common occurrence worldwide as also in this part of our country. This is mainly caused due to leaching of bases by percolating water which becomes more pronounced following heavy rainfall resulting in lowered pH of the soil leaving it porous and eroded, with subsequent low levels of organic matter and reduced fertility. Low pH ($=$ 5) is associated with aluminium and manganese toxicities (Munns 1977). Low pH also results in limited availability of calcium, molybdenum and phosphate (Coventry & Evans 1989). A shift in pH also reduces or increases the availability of many other elements and nutrients in the soil. These effects may occur either by biological or chemical mechanisms or by both.

Soil pH and the associated factors affect the growth and activity of the root nodule bacteria, the host legume and the symbiosis as such. Availability of calcium, which is closely linked to pH, affects the nodulation process, besides the metabolic processes of the endophyte. The local populations of T. repens and the associated strains of symbiotic R. trifolii, found in this part of the country are also strongly influenced by the abovementioned factors as far as colonization and nodulation of the legume by the endophyte is concerned. Both leaf morph populations of T. repens *seem to prefer slightly acidic soils whereas still lower pH* values though apparently not a severe stress, limit the nodulation process resulting in low N_2 fixation. Poor productivity of pastures has occasionally been attributed to nodulation failure in acid soils (Foy 1984, Coventry et al. 1985). This chapter attempts to analyse the nodulation pattern over an acidic pH range in the two legume populations.

MATERIALS AND METHODS

These have been described in Chapter 3

RESULTS

Plant growth

There was a significant ($P < 0.05$) increase in shoot weights of the clover populations with increasing pH levels (Fig. 6.1a). Between the two leaf morph populations, there was a significant ($P < 0.01$) variation in the shoot weights with the unmarked population exhibiting higher values for shoot dry weights at both harvests. The increase in shoot biomass from H1 to H2 was also highly significant ($P < 0.01$) (Table 6.1).

Along with increasing values of shoot dry weight, there was a corresponding increase ($P < 0.05$) in root dry weight in both marked and unmarked clover populations (Fig. 6.1b). The root biomass of the marked population showed a distinct edge over the unmarked at H1, though at H2 the difference in dry weights between the two populations disappeared. Unlike the shoot dry weight, the root dry weight of the marked population was generally greater than the unmarked population, though the difference was not significant. The root dry weights at H2 were significantly ($P < 0.01$) greater.

Increasing above- and below-ground biomass values, associated with increasing pH levels, led to simultaneous increments ($P < 0.01$) in the total plant dry weights. However, the difference between the two leaf morph populations was not significant. Total plant biomass exhibited a near three fold increase at H2 as compared to H1 (Table 6.1).

Photosynthetic area

The PSA showed a significant ($P < 0.01$) increase with increasing pH showing almost a four fold increase at pH 6.5 as compared to pH 4.5. Between the two leaf morph populations there was no significant variation in PSA although values were marginally higher for the marked population at both the harvests. There was a more than two fold increase in the PSA from H1 to H2 (Tables 6.1, 6.2).

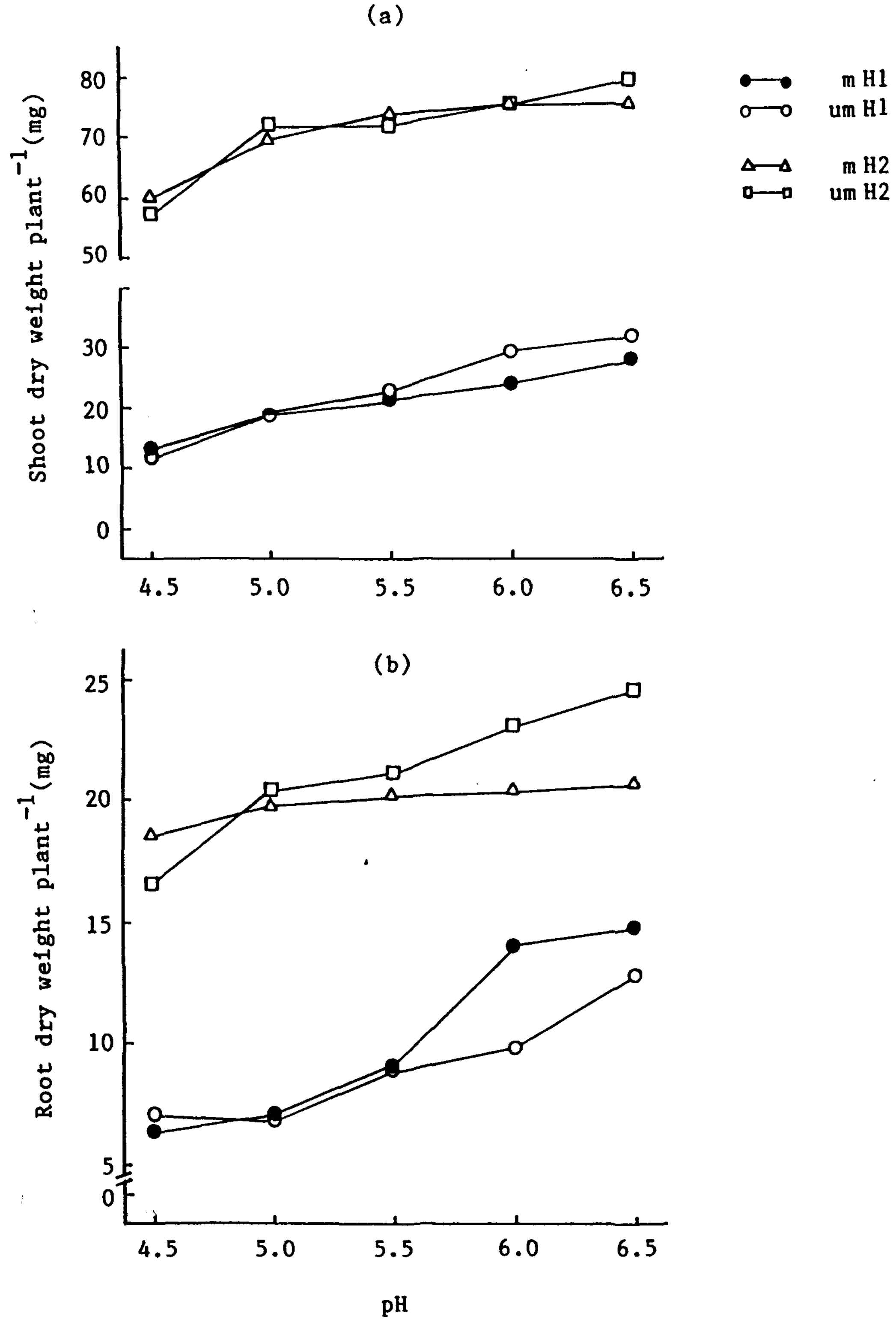

Fig.6.1. Effect of pH on (a) shoot dry weight and (b) root dry weight per plant in the two leaf morph populations of the clover at the two harvests.

Table 6.1. Analysis of variance of the legume growth parameters as influenced by harvests, leaf morph populations and varying pH.

Plant parameters	Source of variation	df	F value	Level of significance
Shoot dry weight	Harvests	1	2171.50	$P < 0.01$
	Leaf morphs	1	15.82	$P < 0.05$
	pH levels	4	12.01	$P < 0.05$
	Harv x morph	1	18.89	$P < 0.05$
	Harv x pH	4	0.79	ns
	Morph x pH	4	0.74	ns
Root dry weight	Harvests	1	147.28	$P < 0.01$
	Leaf morphs	1	0.00	ns
	pH levels	4	6.67	$P < 0.05$
	Harv x morph	1	1.42	ns
	Harv x pH	4	0.50	ns
	Morph x pH	4	0.06	ns
Plant dry weight	Harvests	1	6937.90	$P < 0.01$
	Leaf morphs	1	2.14	ns
	pH levels	4	139.00	$P < 0.01$
	Harv x morph	1	0.12	ns
	Harv x pH	4	4.75	ns
	Morph x pH	4	1.32	ns
PSA	Harvests	1	205.02	$P < 0.01$
	Leaf morphs	1	0.09	ns
	pH levels	4	87.52	$P < 0.01$
	Harv x morph	1	0.99	ns
	Harv x pH	4	1.58	ns
	Morph x pH	4	0.99	ns

ns = not significant

Table 6.2. Effect of pH on the nodule dry weight, plant N concentration and photosynthetic area (PSA) per plant in the two leaf morph populations of the clover at the two harvests.

pH levels	Harvest 1						Harvest 2					
	Marked population			Unmarked population			Marked population			Unmarked population		
	Nodule dry weight (μg)	Plant N concentration ($mg\ g^{-1}$)	PSA per plant (mm^2)	Nodule dry weight (μg)	Plant N concentration ($mg\ g^{-1}$)	PSA per plant (mm^2)	Nodule dry weight (μg)	Plant N concentration ($mg\ g^{-1}$)	PSA per plant (mm^2)	Nodule dry weight (μg)	Plant N concentration ($mg\ g^{-1}$)	PSA per plant (mm^2)
4.5	-	18.02	142.31	-	17.61	140.61	-	18.75	383.76	-	18.48	357.00
5.0	47.0	24.16	264.32	43.7	23.99	270.40	78.1	31.12	456.21	76.3	26.79	447.58
5.5	45.3	28.64	490.00	40.1	24.87	501.31	39.2	37.00	952.60	40.7	28.29	1078.77
6.0	40.8	31.49	578.24	39.7	27.08	518.63	40.1	40.79	1080.72	38.9	30.08	1128.08
6.5	40.1	33.00	646.28	37.0	24.97	654.31	64.6	39.00	1281.36	45.3	29.53	1092.78

- absence

Nodule number

The mean nodule number per plant increased ($P < 0.01$) with increasing pH (up to pH 6.0) in both leaf morph populations. However there was a drop in nodule number at pH 6.5. At pH 4.5, plants of either leaf morph population failed to nodulate even after 12 weeks of growth. Plants of the marked population nodulated better and exhibited a significantly ($P < 0.01$) higher nodule number per plant as compared to unmarked plants at both harvests. Except at pH 4.5 when the legume failed to nodulate, at all other pH levels, nodule number per plant showed almost a four fold increase ($P < 0.01$) at H2 as compared with numbers recorded at H1 (Tables 6.3, 6.4).

Mean nodule weight

The mean nodule weight showed an inverse relationship with nodule number and with increasing pH levels (Table 6.2) at both harvests. Variation in mean nodule weight with pH was significant ($P < 0.01$). At both harvests, the mean weight per nodule was greater in the marked population than in the unmarked, though the difference was not significant. The mean nodule weights were significantly ($P < 0.01$) greater at H2 than at H1 (Table 6.3).

Nodule weight per plant

Figure 6.2b shows the variation in total nodule weight per plant with pH. This parameter showed a trend similar to that of nodule number per plant (Fig. 6.2 a). Except at pH 4.5, the total nodule weight per plant showed a gradual increase up to pH 6.0 at H1. At

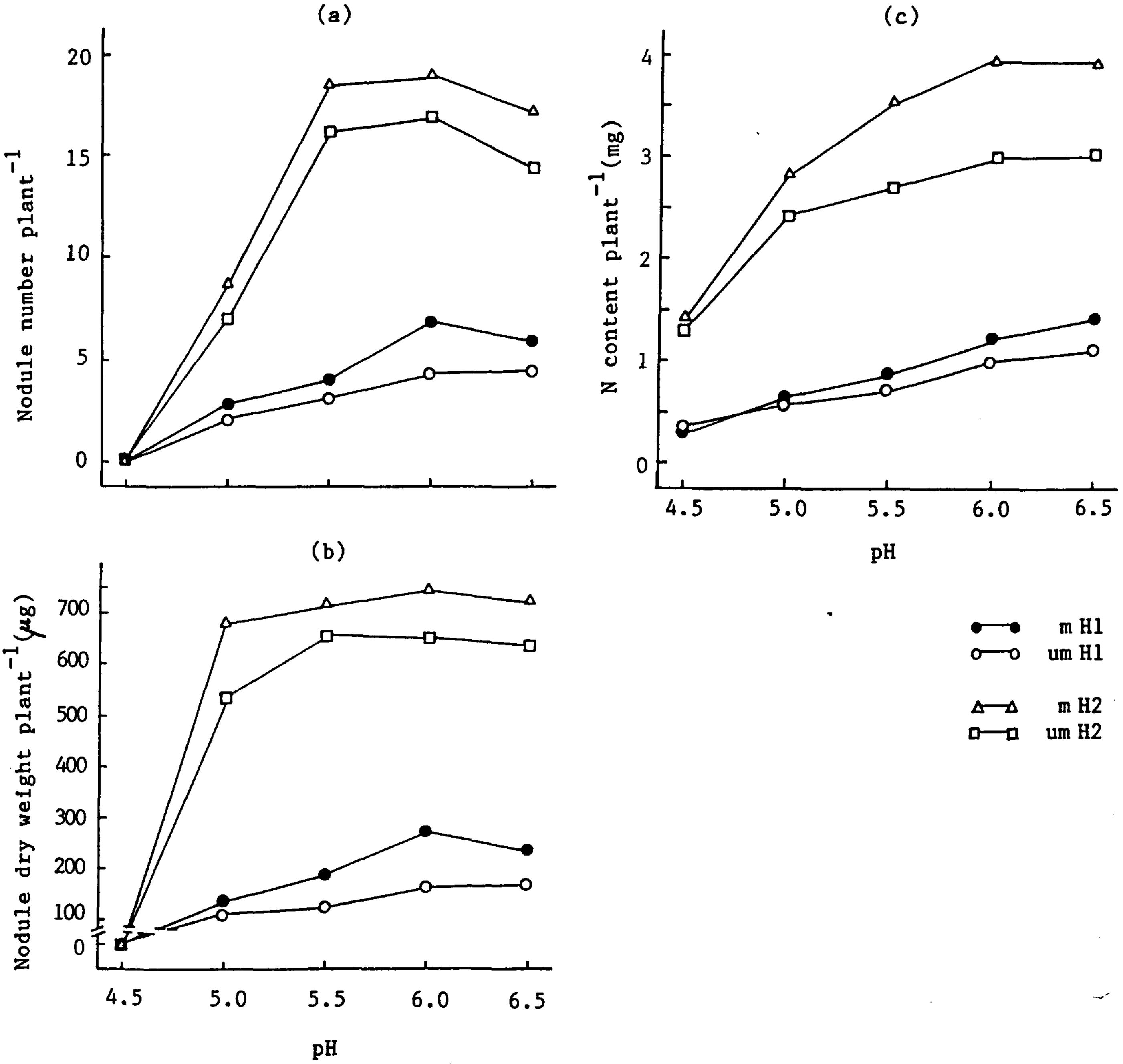

Fig.6.2. Effect of pH on (a) nodule number (b) nodule dry weight, and (c) N content per plant in the two leaf morph populations of the clover at the two harvests.

Table 6.3. Analysis of variance of nodule characteristics of the clover as influenced by harvests, leaf morph populations and varying pH.

Nodule characteristics	Source of variation	df	F value	Level of significance
Nodule number	Harvests	1	1667.34	P< 0.01
	Leaf morphs	1	43.11	P< 0.01
	pH levels	4	457.50	P< 0.01
	Harv. x morph	1	2.46	ns
	Harv. x pH	4	154.47	P< 0.01
	Morph x pH	4	3.54	ns
Mean nodule weight	Harvests	1	21.42	P< 0.01
	Leaf morphs	1	2.99	ns
	pH levels	4	111.22	P< 0.01
	Harv. x morph	1	0.18	ns
	Harv. x pH	4	11.92	P< 0.05
	Morph x pH	4	1.07	ns
Nodule weight per plant	Harvests	1	1158.12	P< 0.01
	Leaf morphs	1	29.39	P< 0.01
	pH levels	4	217.02	P< 0.01
	Harv. x morph	1	1.07	ns
	Harv. x pH	4	73.07	P< 0.01
	Morph x pH	4	2.18	ns

ns = not significant

Table 6.4. Effect of soil pH on the nodule number per unit (100 mg) plant dry weight.

Soil pH	Harvest 1		Harvest 2	
	marked population	unmarked population	marked population	unmarked population
4.5	-	-	-	-
5.0	11.04	9.38	9.69	7.60
5.5	13.23	9.92	19.24	16.99
6.0	17.36	10.80	19.15	16.76
6.5	13.59	10.10	17.12	13.72

- absence

H2, there was a steep rise at pH 5.0 from the initial nil value at pH 4.5, thereafter a gradual increase was observed up to pH 6.0 after which the values either increased marginally or decreased at pH 6.5. Variations in total nodule mass per plant were significant ($P < 0.01$) between pH levels. Of the two leaf morph populations, the marked one consistently exhibited greater nodule mass per plant ($P < 0.01$) at both harvests. At H2 the total nodule mass per plant recorded a three to four fold increase ($P < 0.01$) as compared to the values obtained at H1 (Table 6.3).

Total N per plant

The total N content per plant showed a steady significant ($P < 0.01$) increase with increasing pH at both harvests. Between the two leaf morph populations, the marked population exhibited higher ($P < 0.01$) N content per plant as compared to the unmarked population at both harvests (Table 6.5).

Relative growth rate and relative N accumulation rate

Table 6.6 presents the relative growth rates (R_W) and relative N accumulation rates (R_N) corresponding to the different pH levels for the two leaf morph populations at H1 and H2. There was a significant ($P < 0.01$) positive correlation between R_W and pH levels. However, at H2, the R_W values exhibited a reverse trend, decreasing gradually with increasing pH levels in both leaf morph populations. The difference in R_W values was significant ($P < 0.01$) amongst pH levels though the variation in R_W between the two leaf morph

Table 6.5. Analysis of variance of growth parameters of the clover as influenced by harvests, leaf morph populations and varying pH.

Growth parameters	Source of varaition	df	F value	Level of significance
Total N $plant^{-1}$	Harvests	1	948.03	$P < 0.01$
	Leaf morphs	1	29.95	$P < 0.01$
	pH levels	4	70.99	$P < 0.01$
	Harv. x morph	1	14.31	$P < 0.05$
	Harv. x pH	4	13.18	$P < 0.05$
	Morph x pH	4	2.59	ns
R_W	Harvests	1	472.88	$P < 0.01$
	Leaf morph	1	1.09	ns
	pH levels	4	124.37	$P < 0.01$
	Harv. x morph	1	3.39	ns
	Harv. x pH	4	499.53	$P < 0.01$
	Morph x pH	4	0.27	ns
R_N	Harvests	1	447.48	$P < 0.01$
	Leaf morph	1	7.61	ns
	pH levels	4	124.37	$P < 0.01$
	Harv.x morph	1	0.86	ns
	Harv.x pH	4	105.76	$P < 0.01$
	Morph x pH	4	0.60	ns

ns = not significant

populations was not significant.

The relative N accumulation rates (R_N) showed a trend similar to that of R_W (Table 6.6).

DISCUSSION

Soil acidity at pH 5.0 and below results in toxicity due to increased uptake of Al and Mn and limited availability of Ca in particular (Foy 1984, Coventry & Evans 1989). There was a steady increase in shoot dry weight in both leaf morph populations with increasing pH at both harvests. In legumes, shoot dry weights are known to decrease sharply with increased H^+ ion concentration (Cline & Kaul 1990). Thus the low shoot dry weights recorded at lower levels of pH could be attributed to toxicity resulting primarily from increased accumulation of H^+ besides Al and Mn. As the pH increases, there is an enhancement of shoot weights following partial alleviation of the prevailing toxic condition. Results of this study indicate that toxicities due to H^+ accumulation and related factors also affect root development which is reflected in reduced dry weight. This is in conformity with the findings of Kehoe & Curnow (1963) who reported that in acid soils, root development in subterranean clover was restricted to such an extent that the plants could not utilize subsoil moisture as the soil dried. Al toxicity similarly results in inhibition of root growth and proliferation (Foy 1984) which restricts ability of plants to fully exploit soil moisture (Bromfield et al. 1983).

Table 6.6. Effect of pH on relative growth rate (R_W)(μg d^{-1}) and relative N accumulation rate (R_N)(μg d^{-1}) in the two leaf morph populations of the clover at the two harvests.

pH	Harvest 1				Harvest 2			
	Marked population		Unmarked population		Marked population		Unmarked population	
	R_W	R_N	R_W	R_N	R_W	R_N	R_W	R_N
4.5	4.24	6.06	4.78	6.24	10.96	11.27	10.28	10.66
5.0	9.04	15.41	8.95	15.21	9.56	11.52	9.79	10.65
5.5	11.29	20.35	11.83	18.61	8.88	10.84	8.63	9.63
6.0	15.00	25.48	15.26	23.37	7.23	9.23	7.35	8.18
6.5	16.81	28.02	17.50	24.38	6.45	7.75	6.48	7.79

Both nodule number and nodule mass per plant showed a steady increase at pH >4.5 up to pH 6.0 following which there was a slight decrease in both. Low soil pH, especially below pH 5.0, primarily results in toxicity due to Al (Foy 1984, Coventry & Evans 1989). Al is an abundant element in the soil occurring in a wide variety of mineral forms which are largely inert at neutral pH (Flis *et al.* 1993). As the pH decreases, Al is mobilized into the soil solution and may become toxic to plants and soil organisms. Distribution of various ionic species of Al is pH-dependent (Martin 1991) and slight changes in pH may significantly affect the relative concentration of various charged Al species and hence, the toxicity of Al. Small changes in pH can therefore significantly affect the growth of rhizobia in soil (Thornton & Davey 1983, Richardson & Simpson 1989). Cultures of root nodule bacteria have been reported to grow after a large initial decline in viability following exposure to Al (Keyser & Munns 1979) and it has been suggested that this probably involves some physiological adaptation of the rhizobia to the presence of Al. This trend has also been confirmed for *R. trifolii* (Whelan & Alexander 1986). Such an adaptation may require some time to develop. This may explain the initial time lag between inoculation by *Rhizobium* and nodule formation, and perhaps the wide variation in the nodule number per plant between the two harvests might have been caused on account of this. Though the local populations of *T. repens* and the associated *R. trifolii* usually grow in slightly acidic conditions which prevail over most of the areas in the hill region of north-east India, tolerance to acidity does not necessarily confer on plants the tolerance to Al (Thornton & Davey 1983).

In the pH range of 4.3 - 5.0, toxicity due to Al and related factors severely affects both the legume and the endosymbiont. Recent studies have revealed the adverse effects of Al on the legume-Rhizobium symbiosis at pH < 5.0 (de Carvalho et al. 1981, 1982, Franco & Munns 1982) when the Al remains in solution. Al was reported to affect growth and survival and hence the number of root nodule bacteria in soil (Cooper et al. 1983, Coventry & Evans 1989), nodule initiation (de Carvalho et al. 1982, Murphy et al. 1984, Brady et al. 1990) and root hair formation of the host (Hecht-Buchholz et al. 1990). At pH 4.3-4.7, prevailing Al concentrations are high enough to be severely toxic to T. repens (Wood et al. 1983) leading to stunted growth of roots and total inhibition of root hair development. Results of the present investigation reveal that nodule formation in T. repens was totally inhibited at pH 4.5 for both leaf morph populations. It is not clear whether the lack of nodulation was directly due to inhibition of Rhizobium multiplication in the rhizosphere or due to the inhibition of root hair development of the legume, but it is probable that both the endosymbiont as well as the legume were affected. The critical pH for R. trifolii survival in soil was reported to be 4.5- 4.7 (Bryan 1923) and for growth in laboratory media pH 4.5 -5.0 (Graham & Parker 1964). Thus at pH 4.5, the presence of a considerably large rhizospheric population of R. trifolii is doubtful. The increase in nodule number and nodule mass per plant at pH 5.0 and 5.5 in both leaf morph populations could be attributed to the decrease in H^+ ion concentration and reduced toxicity levels of Al and Mn. At pH 5.0 the nodule number had still not increased appreciably although the nodule mass showed a marked increase, especially at H2. This could be because Al suppresses nodule initiation at concentrations which do not affect nodule growth and function (Robson & Bottomley 1991). At pH 5.5 Al is no longer toxic as it precipitates out of solution (Flis et al. 1993) and this is probably expressed by the large increase in nodule number at H2 in both the leaf morph populations.

Mn toxicity resulting from increased solubility at low pH is also known to inhibit nodulation. Dobereiner (1966) has suggested that the sensitivity of legumes to acid soils is due to the specific effect of Mn on the legume - Rhizobium symbiosis. Transitory waterlogged conditions are known to increase the levels of exchangeable divalent Mn. It has been reported that at low pH, Mn toxicity retards the growth of R. trifolii (Holding & Lowe 1971) and reduces the number of nodules formed on several varieties of white clover (Vose & Jones 1963). In the present study, though development of waterlogged conditions conditions in the pots even transitorily, is improbable, Mn toxicity resulting from low pH could well have played a part in restricting nodulation. Besides, acidic conditions are usually associated with Ca deficiency (Munns 1977) and Ca being a macronutrient, its deficiency could be a critical factor in determining growth of the legume.

Temperate legumes like T. repens, generally require higher levels of Ca, especially when depending on symbiotic N_2 fixation. However, more Ca is needed for nodule formation than for either N_2 fixation or plant growth (Lowther & Loneragan 1968, Lie 1974, Andrews 1976) and thus the time of nodule initiation is the most Ca demanding and acid sensitive step (Munns 1968). According to Smit _et_ _al_. (1987), a Ca-binding protein Rhicadhesin, identified in _Rhizobium_, is involved in the initial attachment of the diazotroph to the host root hairs and thus Ca is required for successful initiation of the symbiosis. The infection threads which develop are also composed of Ca-rich material (Sethi & Reporter 1981) and the non availability of Ca could lead to an increase in the number of aborted infections thereby drastically reducing the nodule number.

Ca plays a variety of roles in root nodule bacteria (O'Hara _et_ _al_. 1988, Robson & Bottomley 1991), many of them apparently involved with the stabilization of the bacterial lipopolysaccharide. Though Ca can slightly alleviate the Al-mediated inhibitions in the legume - _Rhizobium_ symbiosis, this is only partial (Richardson _et_ _al_. 1988) and Keyser & Munns (1979) conclude that Ca offers too little protection against Al to be biologically significant. The results of the present investigation indicated by the positive response of the symbiosis to the addition of Ca is in conformity with those of Coventry _et_ _al_. (1985) and Richardson _et_ _al_. (1988). It is probable that the beneficial effects of Ca are mainly mediated via its neutralizing effect and not due to the presence of the element itself as suggested by Richardson _et_ _al_. (1988) and Pijnenborg _et_ _al_. (1990).

The marginal drop in nodule number and nodule mass per plant at pH 6.5 is interesting. The soils exhibit slightly acidic reaction in this part of the country and it may be assumed that the local population of white clover and associated R. trifolii are adapted to such conditions. The root nodule bacteria are known to be extremely sensitive to small changes in pH which can significantly affect its growth (Thornton & Davey 1983, Richardson & Simpson 1989) and it is probable that at pH 6.5, when the soil reaction tends towards neutrality, the growth of the rhizospheric population of rhizobia is affected leading to reduced numbers and, subsequently, low infectivity of host roots. This could explain the marginal drop in nodule number at pH 6.5. It is also likely that senescence and abscission of nodules may have increased under these conditions (Davey et al. 1989) as a reaction of the host plant to pH conditions approaching neutrality.

Studies by Evans et al. (1980) and Wolff et al. (1993) have revealed that nodule growth (expressed as nodule weight) is more sensitive to changes in soil pH than nodule number. This marginal drop in nodule mass per plant following a decrease in nodule number could have been further caused by the reduction in nodule growth under prevailing conditions. Between the two leaf morph populations, the marked one exhibited significantly ($P < 0.01$) higher nodule number per plant as compared to the unmarked population at both harvests (Fig. 6.2a). This could be attributed to the higher N requirement of the marked population (Pradhan & Tripathi 1984) which forms more root nodules as a means of fulfilling this requirement by symbiotic N_2 fixation as discussed in Chapter 5.

There was no significant variation in mean nodule weight between the two leaf morph populations and the higher nodule mass per plant exhibited by the marked population could be attributed to a greater number of nodules that develop per plant in the marked population.

However, it is interesting to note that a reduction in nodule number per plant was not compensated by an increase in mean nodule weight. This indicates that low pH is not only detrimental to nodule initiation (infection response) but also to nodule growth (growth response). This is in conformity with the findings of Wolff _et al_. (1993) who observed that the nodule growth was more sensitive to changes in soil pH than nodule number.

H^+ ion toxicity arising at low pH, besides hampering nodulation, is also deleterious to N_2 fixation of the nodulated legumes. Cline & Kaul (1990) reported that such conditions affect N_2 fixation more than plant growth since legumes grew well if supplied with N but showed deficiency if dependent on N_2 fixation. Thus at low pH, reduced nodulation coupled with inhibited N_2 fixation (expressed by R_N) would lead to N deficiency and could be responsible for the suppressed growth of the inoculated plants (Mengel & Kamprath 1978, Alva _et al_. 1987) as the present results suggest.

The recommended pH for the growth of white clover in hill pastures is 5.2-5.8 (HFRO 1979). The results of the present investigation suggest that nodule formation and symbiotic function can be considerably reduced at the lower end of this pH range. Though soils in and around Shillong have a pH range of 5.5-6.0, it is probable that this value may drop due to leaching of soluble bases following heavy rains. Transitory waterlogged conditions may also lead to Mn toxicity and both these factors would severely hamper nodulation and N_2 fixation under natural conditions.

Of the two leaf morph populations, the marked one exhibits better nodulation over the acidic pH range in terms of production of greater number and mass of nodules per plant, and thus its role in the N economy of the swards would be more important under prevailing conditions of low pH.

7 Effect of soil moisture on nodule population

Soil moisture plays a pivotal role in the growth and functioning of all plants and soil microorganisms with the growth response being proportional to the moisture level, up to a certain extent. Soil moisture levels also determine the availability of nutrients which are concentrated predominantly in the upper layers of the soil profile (Pinkerton & Simpson 1986). According to Bartels (1966) and Stiles (1966), the clover content in mixed swards is sensitive to soil moisture and could be increased by irrigation. However, Reid & Castle (1965) reported that irrigation had no significant effect on the proportion of clover. Data obtained from the field observations (Chapter 4) indicate that increased soil moisture prevailing during the rainy season may have beneficial effects on the growth of the clover.

Observations on the effects of soil moisture on the nodulated root system of legumes dates back to Wilson (1931) who first reported that drought adversely affected the root nodules of legumes. Later, Mansfield (1961) and Doku (1970) confirmed the beneficial effects of irrigation on nodule number and size reported earlier. Soil moisture conditions are known to affect (i) the survival and movement of rhizobia in soil (Hamdi 1971), (ii) the rate of infection of legume root hairs (Worrall & Roughley 1976), and (iii) nodule growth and function (Davey & Simpson 1989).

However, most of the work on this aspect has been confined to legumes like soybean which bear spherical, determinate nodules of limited growth and thus the observed effects of soil moisture are immediate on pre-formed nodules. T. repens on the contrary, bears elongated indeterminate nodules which are scattered all over the fibrous root system. Each nodule has an apical meristem which allows it to continue to grow and function over a considerable period of time.

The Shillong plateau is characterised by dry winter and spring whereas heavy rainfall is recorded during the rainy season with almost 85% of the total annual rainfall occurring during the months of June-September. Corresponding soil moisture levels also vary widely ranging from 8% in winter to 35% during the rainy season (Fig. 3.2). Thus the white clover populations are exposed to a wide range of soil moisture levels and, therefore, an analysis of the effects of soil moisture on the nodule population dynamics in the two leaf morph populations of the clover could be quite revealing.

MATERIALS AND METHODS

These have been described in Chapter 3

RESULTS

Plant growth

Figure 7.1a represents the variation in shoot dry weight with soil moisture levels in the two leaf morph populations of white clover. At nil soil moisture level, all the plants died within 12 days. The increasing soil moisture levels were associated with a significant ($P< 0.01$) corresponding increase in shoot weights in both the leaf morph populations. Though there was no significant difference in shoot weights between the two leaf morph populations, variations in shoot weights between the two harvests were highly significant (Table 7.1) with weights at H2 exhibiting a near three fold increase over those at H1.

Increasing soil moisture levels were associated with a corresponding significant ($P <0.01$) increase in the root dry weights (Fig. 7.1b). At both harvests, the marked population exhibited a significantly ($P<0.01$) greater root biomass. At H2, the biomass allocation pattern in the above- and below-ground portions are diametrically opposite in the two leaf morph populations; the marked population allocated significantly greater biomass to root, whereas the unmarked population allocated more biomass to shoot.

The total plant weights also showed a significant ($P < 0.01$)

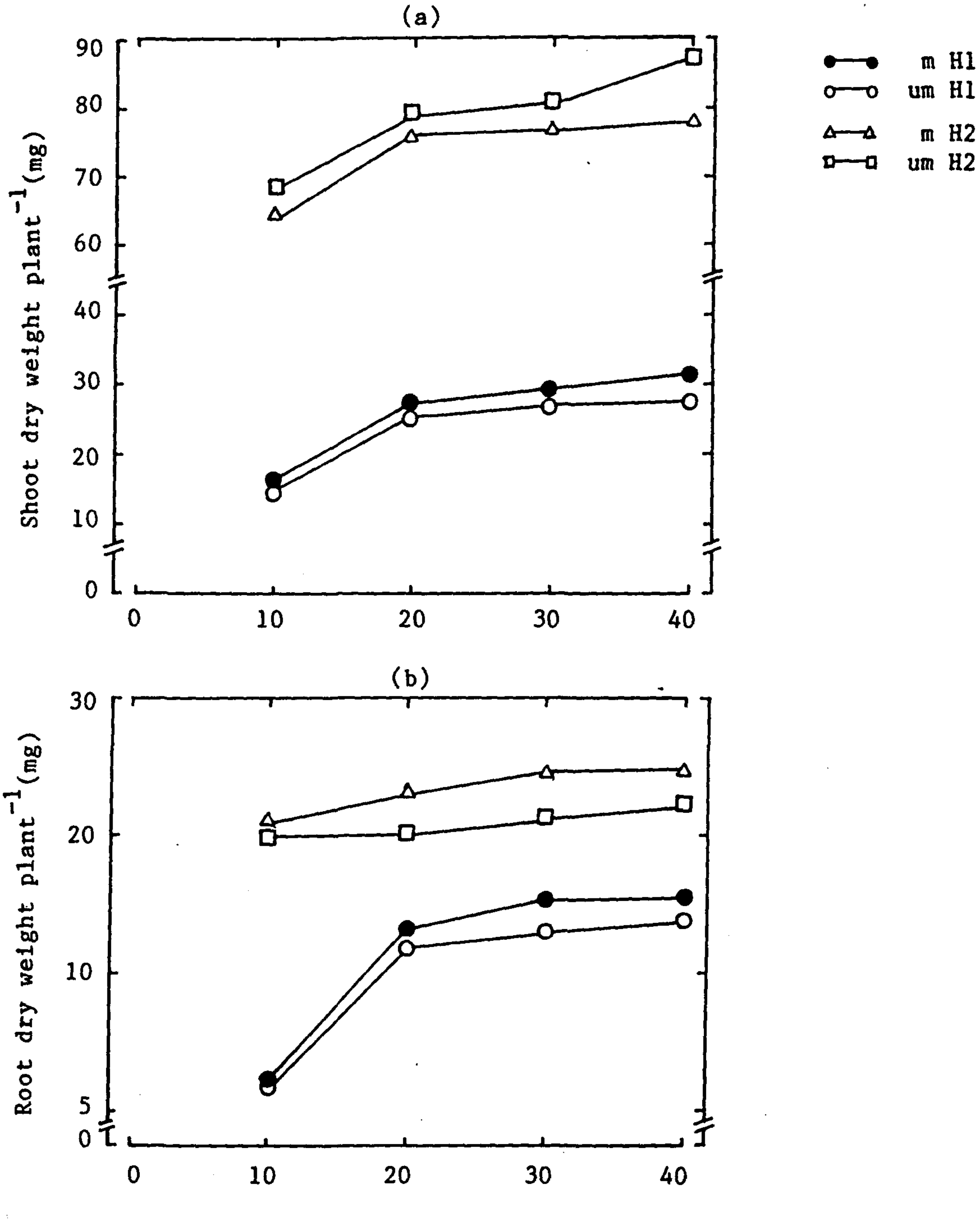

Fig.7.1. Effect of soil moisture on (a) shoot dry weight and (b) root dry weight per plant in the two leaf morph populations of the clover at the two harvests.

Table 7.1. Analysis of variance of the legume growth parameters as influenced by the harvests, leaf morph populations and different moisture regimes.

Plant parameters	Source of variation	df	F value	Level of significance
Shoot dry weight	Harvests	1	2985.46	$P < 0.01$
	Leaf morphs	1	3.92	ns
	Moisture %	3	52.80	$P < 0.01$
	Harv x Morph	1	11.83	$P < 0.05$
	Harv x Moist	3	0.49	ns
	Morph x Moist	3	0.30	ns
Root dry weight	Harvests	1	3760.38	$P < 0.01$
	Leaf morphs	1	86.47	$P < 0.01$
	Moisture %	3	223.49	$P < 0.01$
	Harv x Morph	1	8.19	ns
	Marv x Moist	3	63.61	$P < 0.01$
	Morph x Moist	3	3.77	ns
Plant dry weight	Harvests	1	2817.00	$P < 0.01$
	Leaf morphs	1	0.12	ns
	Moisture %	3	62.91	$P < 0.01$
	Harv x Morph	1	5.87	ns
	Harv x Moist	3	1.25	ns
	Morph x Moist	3	0.20	ns
PSA	Harvests	1	1571.10	$P < 0.01$
	Leaf morphs	1	5.00	ns
	Moisture %	3	316.56	$P < 0.01$
	Harv x Morph	1	0.50	ns
	Harv x Moist	3	64.50	$P < 0.01$
	Morph x Moist	3	0.33	ns

PSA = Photosynthetic area

ns = not significant

increase with increasing soil moisture levels. At H2 the dry weights showed a more than three fold increase ($P < 0.01$) although there was no significant difference in dry weights between the two leaf morph populations (Table 7.1).

Photosynthetic area

Increasing soil moisture levels were also associated with a significant ($P < 0.01$) increase in the PSA per plant and at H2 the values were more than twice that at H1. However, there was no significant difference in the PSA between the two leaf morph populations (Tables 7.1, 7.2).

Nodule number

Figure 7.2a shows the variation in mean nodule number per plant with increasing soil moisture content. At H1, the nodule number in both the leaf morph populations increased with increase in soil moisture up to 30% above which there was a conspicuous drop in the nodule number. At H2, the nodule number per plant increased only up to 20% soil moisture level above which the number dropped steeply. The marked population exhibited a sharp increase in nodule number over the unmarked population at H2 (Tables 7.3, 7.4).

Mean nodule weight

Though increasing soil moisture levels at H1 were associated with an increase in the mean weight per nodule, this trend was reversed at H2 (Table 7.3). There was no significant variation in the weight per nodule either amongst the moisture levels or between

Table 7.2. Effect of soil moisture on the nodule dry weight, plant N concentration and photosynthetic area (PSA) per plant in the two leaf morph populations of the clover at the two harvests.

	Harvest 1						Harvest 2					
	Marked population			Unmarked population			Marked population			Unmarked population		
Soil moisture (%)	Nodule dry weight (μg)	Plant N concentration (mg g^{-1})	PSA per plant (mm^2)	Nodule dry weight (μg)	Plant N concentration (mg g^{-1})	PSA per plant (mm^2)	Nodule dry weight (μg)	Plant N concentration (mg g^{-1})	PSA per plant (mm^2)	Nodule dry weight (μg)	Plant N concentration (mg g^{-1})	PSA per plant (mm^2)
10	37.6	20.13	131.92	36.9	18.60	130.54	70.3	40.80	904.00	72.0	35.90	928.00
20	43.3	23.60	320.96	40.3	24.00	339.09	61.0	38.20	1028.00	70.2	35.07	1092.67
30	45.0	32.11	628.16	43.2	27.30	701.96	40.2	33.04	1125.80	56.4	30.10	1143.12
40	48.1	33.07	973.00	47.0	27.61	965.21	37.9	29.18	1228.20	48.8	27.62	1289.92

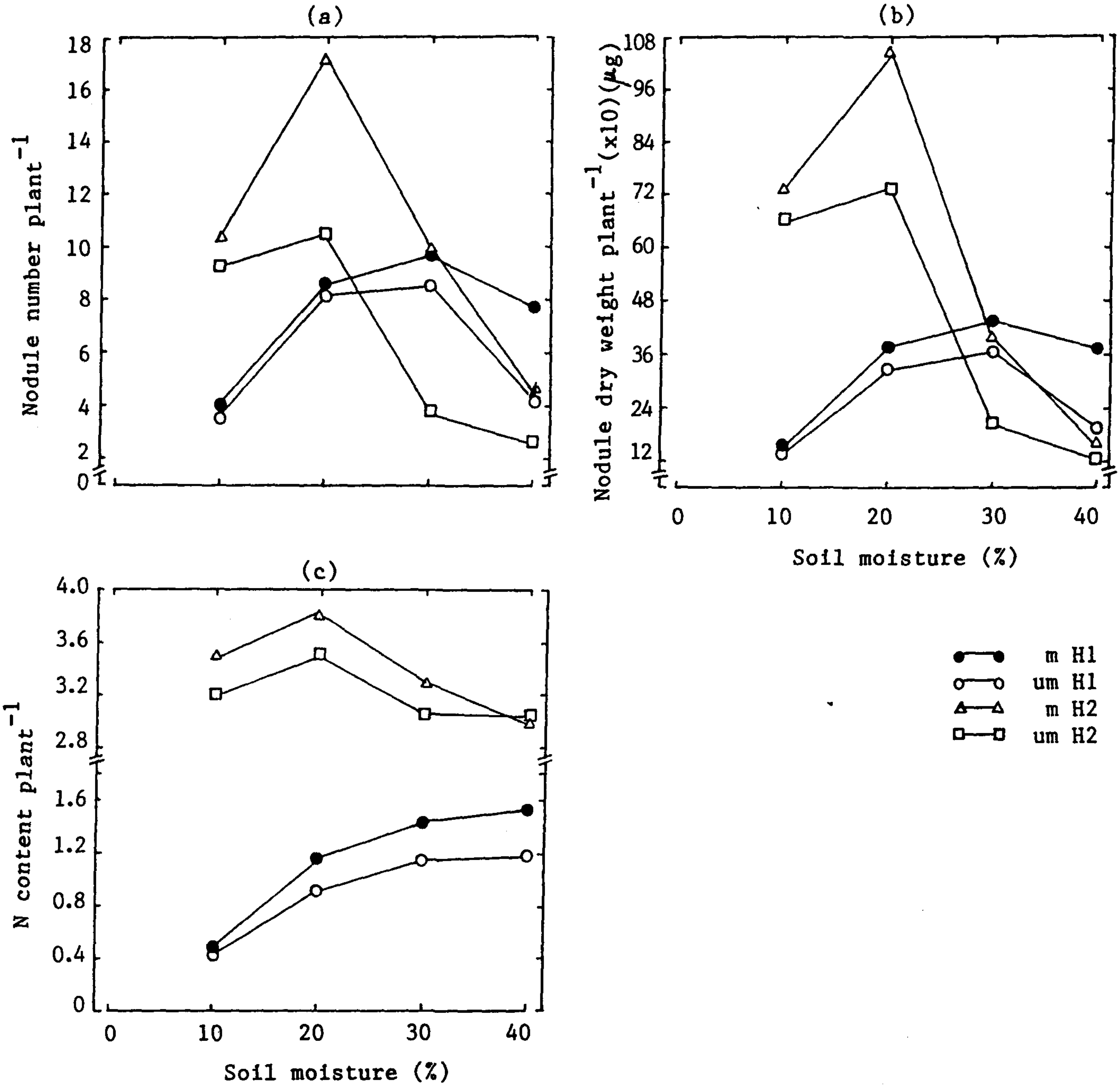

Fig.7.2. Effect of soil moisture on (a) nodule number (b) nodule dry weight and (c) N content per plant in the two leaf morph populations of the clover at the two harvests.

Table 7.3. Analysis of variance of nodule characteristics of the clover as influenced by the harvests, leaf morph populations and different moisture regimes.

Nodule characteristics	Source of variation	df	F value	Level of significance
Nodule number	Harvests	1	3.02	ns
	Leaf morphs	1	7.88	ns
	Moisture %	3	7.89	ns
	Harv. x Morph	1	1.98	ns
	Harv. x Moist	3	6.01	ns
	Morph x Moist	3	0.52	ns
Mean nodule weight	Harvest	1	79.74	$P < 0.01$
	Leaf morphs	1	5.90	ns
	Moisture %	3	8.48	ns
	Harv. x morph	1	11.91	$P < 0.05$
	Harv. x Moist	3	29.34	$P < 0.05$
	Moist x Morph	3	0.77	ns
Nodule weight per plant	Harvests	1	23.34	$P < 0.05$
	Leaf morphs	1	6.91	ns
	Moisture %	3	15.27	$P < 0.05$
	Harv. x Morph	1	0.87	ns
	Harv. x Moist	3	19.53	$P < 0.05$
	Moist x Morph	3	0.44	ns
Total N $plant^{-1}$	Harvests	1	951.60	$P < 0.01$
	Leaf morphs	1	9.12	ns
	Moisture %	3	7.41	ns
	Harv. x Morph	1	0.03	ns
	Harv. x Moist	3	16.27	$P < 0.05$
	Morph x Moist	3	0.44	ns

Table 7.4. Effect of soil moisture on the nodule number per unit (100 mg) plant dry weight.

Soil moisture (%)	Harvest 1		Harvest 2	
	marked population	unmarked population	marked population	unmarked population
10	17.14	16.98	12.08	10.34
20	21.03	21.01	17.14	10.32
30	21.36	19.91	9.64	3.52
40	16.31	9.60	4.28	2.36

the two leaf morph populations. However, at H2 the mean weight per nodule was significantly ($P < 0.01$) higher than that at H1 (Table 7.3).

Nodule weight per plant

The nodule weight per plant (Fig. 7.2b) showed a trend similar to that of nodule number per plant, and varied significantly ($P < 0.01$) between the soil moisture levels. Between the two leaf morph populations, there was no significant variation, although the marked population showed consistently higher values at both harvests. At H2, the nodule mass per plant had increased considerably and was significantly ($P < 0.01$) greater than that at H1.

Total N per plant

At H1, the total N content per plant showed a steady increment with increasing soil moisture levels in both the leaf morph populations. However at H2, the total N content increased up to 20% soil moisture after which the values decreased with a corresponding further increase in soil moisture levels. Although there was no significant variation in total N per plant either amongst the soil moisture levels or between the two leaf morph populations, values at H2 showed a three fold increase ($P < 0.01$) as compared to values at H1 (Table 7.3).

Relative growth rate and relative N accumulation rate

The relative growth rates (R_W) of the two leaf morph populations at varying soil moisture levels are given in Table 7.5. At H1, R_W

Table 7.5. Effect of soil moisture on relative growth rate (R_W)(μg d^{-1}) and relative N accumulation rate (R_N)(μg d^{-1}) in the two leaf morph populations of the clover at the two harvests.

Soil moisture (%)	Harvest 1				Harvest 2			
	Marked population		Unmarked population		Marked population		Unmarked population	
	R_W	R_N	R_W	R_N	R_W	R_N	R_W	R_N
10	7.27	10.78	10.12	15.60	6.54	8.84	10.74	15.71
20	15.90	24.88	6.99	9.24	15.29	21.55	7.33	10.27
30	17.36	28.14	6.23	6.55	16.53	24.78	6.82	7.58
40	18.26	29.49	5.98	5.01	16.67	25.10	7.33	7.33

showed a positive correlation with increasing soil moisture levels for both leaf morph populations. However, at H2 this trend was reversed and increasing soil moisture levels were associated with decreasing R_W values. There was no significant ($P < 0.01$) variation in R_W amongst the moisture levels though it did not vary significantly between the two leaf morph populations. At H2, the R_W values decreased considerably ($P < 0.01$) as compared to values at H1.

The relative N accumulation rates (R_N) in both the leaf morph populations showed a trend similar to that of R_W (Table 7.6).

DISCUSSION

White clover plants have a high water content and thus plant growth is extremely sensitive to moisture stress, resulting in plants becoming dwarf (Johns & Lazenby 1973, Thomas 1984). This view finds support from the findings of Bartels (1966) and Stiles (1966) that the white clover content in mixed swards can be considerably increased by irrigation. The plants in the unwatered treatments failed to survive beyond a 12 day period, but those exposed to 10% soil moisture could grow and nodulate normally. Increased soil moisture contents (up to 30%) brought about a sharp increase in the plant biomass accompanied by an increment in the nodule number per plant of both the leaf morph populations at H1 following which the nodule number dropped at higher soil moisture levels although plant biomass continued to increase. However, at H2 the nodule number per plant increased considerably and maximum

Table 7.6. Analysis of variance of growth parameters of the legume as influenced by harvests, leaf morph populations and different moisture regimes.

Growth parameters	Source of varaition	df	F value	Level of significance
R_W	Harvests	1	881.99	$P < 0.01$
	Morphs	1	0.30	ns
	Moisture %	3	50.40	$P < 0.01$
	Harv X Morphs	1	13.92	$P < 0.05$
	Harv X Moist	3	238.02	$P < 0.01$
	Morph x moist	3	0.01	ns
R_N	Harvets	1	637.31	$P < 0.01$
	Morphs	1	4.99	ns
	Moisture %	3	17.02	$P < 0.05$
	Harv x Morphs	1	21.02	$P < 0.05$
	Harv x Moist	3	168.13	$P < 0.01$
	Morph x moist	3	0.01	ns

ns = not significant

nodulation was observed at 20% soil moisture, but with further increase in soil moisture content, nodulation declined (Fig. 7.1).

Rhizobia are small to medium sized organisms - 0.5-0.9 x 1.2-3.0 μm - and it has been reported that they are unable to move actively over significant distances (Hamdi 1971). Passive transport via soil water to lower depths is rare as the rhizobial cells are usually retained either due to adsorption (Tan et al. 1991) or as a result of sieving effect (Bitton et al. 1974). As a result, T. repens exposed to higher soil moisture at H2 may find inadequate rhizobial population in the root hair region thereby resulting in low nodule number.

Though the increase in mean nodule weight at H2 as compared to H1 is significant ($P < 0.01$), a reduction in the number of nodules per plant is not compensated for by an increase in the size of the nodules which indicates that extremes of soil moisture are detrimental to both root hair infection and nodule growth. Since stressed conditions imposed due to extremes of soil moisture result in nodules being shed from the roots, it is difficult to determine whether such stress is actually detrimental to nodule growth. At lower soil moisture levels, this fall in nodule number is partially compensated for by the development of juvenile nodules but this compensatory mechanism probably fails at high moisture levels due to the absence of a substantial rhizobial population in the rhizosphere as discussed above. Therefore, the failure to compensate reduced numbers by enhanced growth of surviving nodules results in the nodule mass per plant being proportional to the nodule number (Figs. 7.1a,b).

Studies on *Rhizobium* have indicated that these bacteria are carried passively in water flowing through saturated soils (Roughley & Worrall 1984) and according to Hamdi (1974) and Madsen & Alexander (1982), percolating water increases the vertical movement of rhizobia through soils. Water flow due to water uptake by plant roots may enhance the movement of bacteria towards root surfaces (Breitenbeck *et al*. 1988) and the bacteria may also move along or with the roots which may act as a vector in transferring them to greater depths (Bashan *et al*. 1986; Madsen & Alexander 1982). The development of a small number of nodules per plant at 10% soil moisture, especially at H1, could be attributed to the susceptibility of the clover as well as *R. trifolii* to relatively drier conditions. *T. repens* is particularly sensitive to soil moisture as highlighted above. The root nodule bacteria are also severely affected by this stress factor and their susceptibility is known to be proportional to the amount of water retained by cells during drying (Bushby & Marshall 1977) and to the organic matter content of soils. According to Davey *et al*. (1989), drying of soils, particularly of the uppermost layer of the soil profile, caused nodule number in clover to decrease by 50-90%. Worrall & Roughley (1976) reported that this resulted from a sharp drop in the number of infection threads and nodulation by *R. trifolii* was consequently inhibited even if the rhizobial population was not severely affected. According to Vincent *et al*. (1962) and Bromfield *et al*. (1983), a considerable decrease in the bacterial population due to low soil moisture could also be expected. At low soil moisture, rhizobia survive within water 'lenses' formed within soil pores but movement of the bacteria is severely restricted as such 'lenses' do not form an extensive network of continuous pathways (Griffin & Quail 1968). This view has been supported by Marshall (1971) and Hamdi (1971). Low soil moisture also retards the rate of infection of root hairs (Worrall & Roughley 1976), nodule growth and activity (Issa et al. 1993) and leads to the shedding of pre-formed nodules (Nelson 1983). Such restrictions could explain the reduced nodule numbers at 10% soil moisture.

The above-mentioned findings could help explain the increase in nodule number per plant associated with higher soil moisture levels which would result in enhanced accumulation of rhizobia in the rhizospheric region following passive transport by water (Hamdi 1971). Higher levels of soil moisture, besides triggering off a flush of shoot growth, also makes nutrients such as P, available to the legume which results in significantly ($P < 0.01$) increased plant biomass at successively higher moisture levels (Table 7.1). This would not only enhance growth of belowground root systems thereby increasing possible sites of infection, but also make more photosynthates available to newly formed nodules. Soil moisture is also related to the rate of infection of legume root hairs (Worrall & Roughley 1976). However, at 40% soil moisture, edaphic conditions conditions are exposed to transitory inundation. Excessive soil moisture limits aeration and drastically reduces rhizobial population (Bergersen 1971). Thus in spite of significantly ($P < 0.01$) increased plant biomass, which would have ensured both an enhanced supply of photosynthates and an increase in possible sites of infection, the nodule number drops sharply. This could be attributed to the presumed fall in rhizospheric rhizobial population at this high soil moisture level. According to Graham (1982), excessive soil water results in shedding of existing nodules after only 2 days of imposition of the stress. Recovery from stress may also take considerable time if inundation occurred soon after initial nodulation. Presumably, a percentage of pre-formed nodules were shed leading to reduced numbers. Rapid renodulation under such stress does not occur as it poses a severe stress on carbohydrate reserves of the host.

Prolonged exposure to the above-mentioned soil moisture levels leads to a highly significant ($P < 0.01$) increase in plant biomass (Fig. 7.1a) as well as a sharp rise in the nodule number per plant up to 20% soil moisture in both leaf morph populations (Fig. 7.2a). However, the steep drop in nodule number with further increase in soil moisture could be attributed to the view that moisture levels which prove conducive for maximum nodulation at H1 are detrimental over a prolonged period probably bacause long term exposure leads to excessive accumulation of water thereby limiting soil aeration. Temporary inundation could also lead to pre-formed nodules being shed and the reduction in nodule numbers at H2 as compared to H1 at 40% soil moisture (Fig. 7.2a) lends support to this view.

Comparatively lower values for total N content per plant (Fig. 7.2c) corresponding to low soil moisture could be attributed primarily to poor nodulation exhibited by the legume. Water stress is also known to cause irreversible damage to the symplastic connections between cells of existing nodules, destroying nodule function (Sprent 1976) and their shedding from host plants (Dalton & Zobel 1977). Reduced soil moisture also leads to a sharp decrease in the acetylene reduction assay rates (Sprent 1971, Minchin & Pate 1975) which may ultimately come to a halt (Davey & Simpson 1989). This is probably brought about due to reduced respiration rates resulting in lowered N_2 fixing efficiency of stressed nodules. This view finds support from the studies of Engin & Sprent (1973) and DeJong & Phillips (1982). On the contrary, reduced N accumulation at high soil moisture levels could be attributed primarily to the steep drop in nodule number and nodule mass per plant leading to reduced N_2 fixing tissue.

There was no significant difference between the marked and unmarked leaf morph populations with respect to any of the parameters studied except for nodule number and root biomass, both of which were greater in the marked plants. Since both the leaf morph populations were exposed to similar soil moisture levels and watering frequencies, soluble NO_3 ions would have been equally leached leading to marked plants becoming comparatively N-starved owing to their higher N requirement. These plants therefore develop a larger number of N_2 fixing root nodules as a means of overcoming this crisis.

Results of the present study indicate that nodulation is best achieved at intermediate soil moisture levels which appear to be between 10-20% on dry weight basis. This amount of soil moisture appears to prevent prolonged desiccation, provides conducive conditions for growth of both the legume and the bacteria and, at the same time, allows adequate aeration by preventing waterlogged conditions to be manifested. Though this range of soil moisture is recorded at the swards during the major part of the year, the dry winter spells lowered soil moisture to levels well below the above-mentioned range. Drying of soils coupled with low temperatures during these months could act as a major stress. However, rhizobia are known to withstand such stressed spells by surviving within water 'lenses' (Issa et al. 1993) and can multiply and re-establish their population the following spring. Clover nodules which manage to overwinter are also known to respond effectively to re-watering, due to their indeterminate nature, and can thereby continue to grow and function in the following spring with the return of favourable conditions.

8 Effect of defoliation and NPK treatments on nodule population

White clover is an important pasture component of the grasslands in and around the Shillong plateau. Besides being a significant contributor of fixed nitrogen to these ecosystems via its root nodules, it serves as a forage legume for cattle. This results in clover leaves being frequently defoliated, along with companion grasses, during grazing. During periods of peak vegetative growth, the clover leaves are less accessible due to greater grass growth. However, during the drier periods of the year the growth of grasses is less vigorous and clover leaves are more accessible to grazers (Clark _et_ _al_. 1984). This would lead to mild and severe defoliation of clover in the former and latter situations, respectively. Moreover, the high defoliation risk for clover arises because leaves are nearly always completely removed, whereas in grasses leaves are only partially grazed (Chapman _et_ _al_. 1990).

In a prostrate, stoloniferous plant like *T. repens*, the trifoliate compound leaves thus serve as the primary photosynthesizing tissue from where the photosynthates are channelized to different plant parts. Partial removal of the leaves would, therefore, disturb the primary carbon 'source' and affect partitioning of photosynthates to the different 'sink' tissues and affect the growth of the clover population. According to Suckling (1976) and Lambert *et al.* (1982, 1986), growth of *T. repens* is also affected if deficiencies of nutrients, particularly of P, exist.

Several studies have been made to investigate the effects of defoliation in white clover by Chapman and his co-workers (Chapman 1983, Chapman *et al.* 1984, Clark *et al.* 1984, Chapman *et al.* 1990, Chapman *et al.* 1991, Chapman *et al.* 1992). However, these studies deal with the pathways of carbon following removal of the source tissue. N_2 fixation being an energy-intensive, root nodules of legumes consequently have a high energy demand (Ryle *et al.* 1985, Gordon *et al.* 1985). It would therefore be interesting to determine how the number, growth and activity of nodules in the two leaf morph populations of white clover were affected following removal of the leaf (source tissue) and addition of NPK, to alleviate nutrient deficiencies, if any.

MATERIALS AND METHODS

These have been described in Chapter 3

RESULTS

Plant growth

Figure 8.1a represents the mean shoot dry weights of the two leaf morph populations exposed to the two defoliation and NPK levels, compared to the controls at the two harvests. The shoot dry weights varied significantly ($P < 0.01$) between treatments with the weights being highest in the plants of both populations exposed to 'low defoliation - high NPK (LH)' treatments at H1. At H2, however, the control plants of both populations had the highest shoot dry weights. At both harvests, 'high defoliation - low NPK (HL)' treatments resulted in minimum shoot dry weights. Between harvests, there was a significant difference in the shoot dry weights with values recorded at H2 being almost twice that at H1 for all treatments including the 'control' plants. However, there was no significant difference in the shoot dry weights between the marked and unmarked plants (Table 8.1).

Root dry weights also varied significantly ($P < 0.01$) between the treatments. The highest values were recorded under the LH treatments and lowest under the H1 treatment for both the leaf morph populations. At H2, root dry weights were significantly ($P < 0.01$) higher compared to the values recorded at H1. Of the two leaf morph populations, the marked one exhibited better root growth and had significantly ($P < 0.01$) greater dry weights at both harvests (Fig. 8.1b).

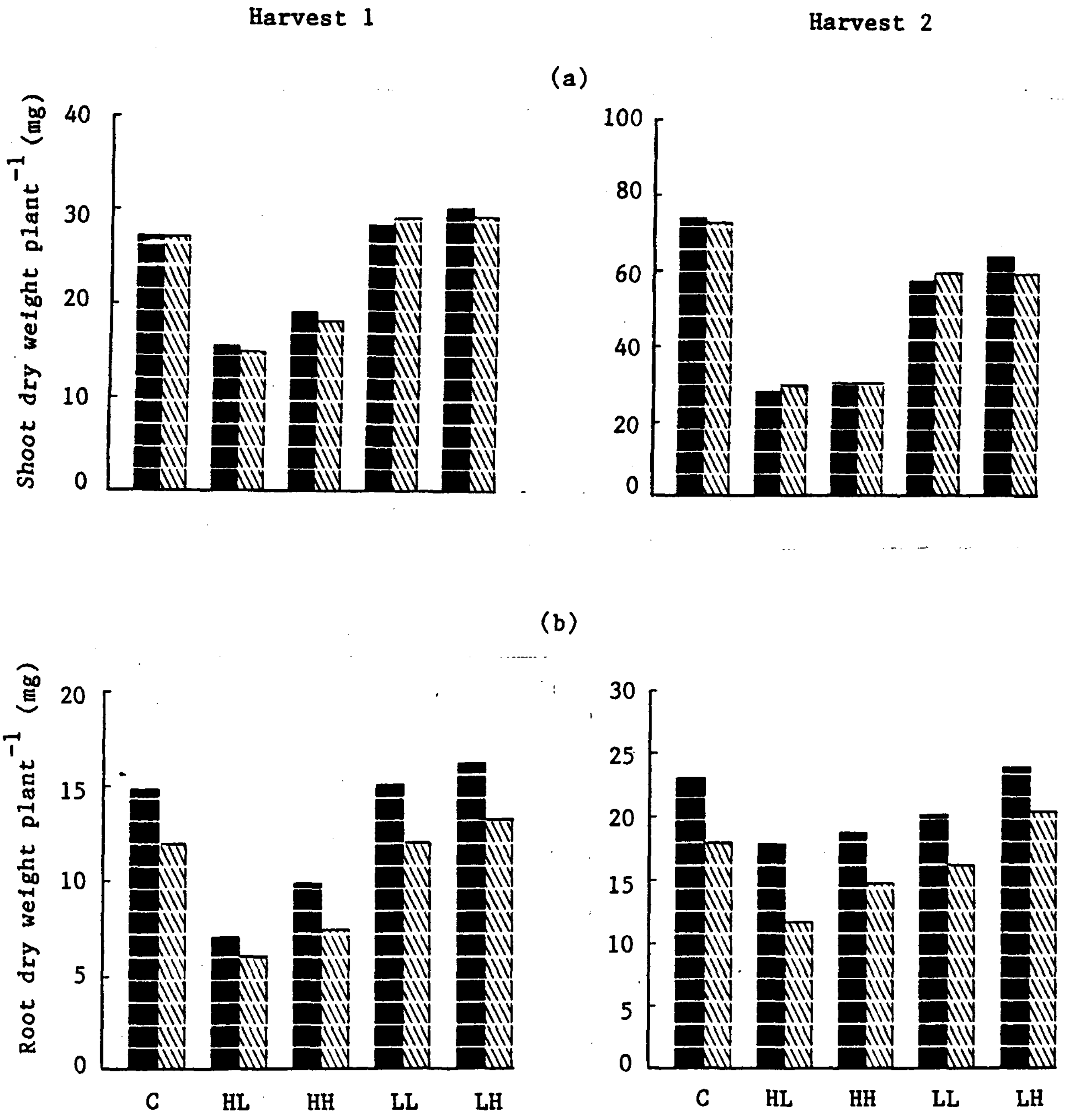

Fig.8.1. Effect of defoliation and NPK treatments on (a) shoot dry weight and (b) root dry weight per plant in the marked (■) and unmarked (▨) leaf morph populations of the clover at the two harvests. C = control, HL = high defoliation-low NPK, HH = high defoliation-high NPK, LL = low defoliation-low NPK, LH = low defoliation-high NPK.

Table 8.1. Analysis of variance of the legume growth parameters as influenced by harvests, leaf morph populations and defoliation and NPK treatments.

Plant parameters	Source of variation	df	F value	Level of significance
Shoot dry weight	Harvests	1	2164.99	$P < 0.01$
	Leaf morphs	1	0.28	ns
	Defoln. treat	4	403.29	$P < 0.01$
	Harv x Morph	1	0.01	ns
	Harv x defoln	4	120.71	$P < 0.01$
	Morphs x defol	4	1.74	ns
Root dry weight	Harvests	1	75.01	$P < 0.01$
	Leaf morphs	1	28.75	$P < 0.01$
	Defoln. treat	4	109.13	$P < 0.01$
	Harv x Morph	1	2.04	ns
	Harv x defoln	4	22.24	$P < 0.01$
	Morphs x defol	4	4.45	ns
Plant dry weight	Harvests	1	5724.39	$P < 0.01$
	Leaf morphs	1	77.38	$P < 0.01$
	Defoln. treat	4	1037.00	$P < 0.01$
	Harv x Morph	1	5.59	ns
	Harv x defoln	4	188.75	$P < 0.01$
	Morphs x defol	4	2.62	ns
PSA	Harvests	1	272.06	$P < 0.01$
	Leaf morphs	1	15.18	$P < 0.05$
	Defoln. treat	4	1105.13	$P < 0.01$
	Harv x Morph	1	3.76	ns
	Harv x defoln	4	0.98	ns
	Morphs x defol	4	7.60	ns

ns = not significant

Table 8.2. Effect of defoliation treatments on the nodule dry weight, plant N concentration and photosynthetic area (PSA) per plant in the two leaf morph populations of the clover at the two harvests.

Defoliation treatments	Harvest 1						Harvest 2					
	Marked population			Unmarked population			Marked population			Unmarked population		
	Nodule dry weight (μg)	Plant N concentration (mg g^{-1})	PSA per plant (mm^2)	Nodule dry weight (μg)	Plant N concentration (mg g^{-1})	PSA per plant (mm^2)	Nodule dry weight (μg)	Plant N concentration (mg g^{-1})	PSA per plant (mm^2)	Nodule dry weight (μg)	Plant N concentration (mg g^{-1})	PSA per plant (mm^2)
Control	47.8	31.07	763.00	40.8	27.63	818.77	70.1	39.7	1291.08	66.3	32.6	1380.50
HL	30.4	20.79	105.46	30.7	20.00	145.12	30.1	26.3	150.90	29.7	23.1	160.51
HH	31.7	22.86	113.16	32.9	21.36	164.05	30.7	27.0	169.54	29.8	24.6	166.51
LL	39.3	30.36	681.00	28.7	27.89	690.30	56.2	33.5	1040.01	48.1	30.0	1138.63
LH	42.6	32.11	1065.03	39.0	29.01	1129.65	57.9	34.7	1118.26	52.0	30.9	1228.36

Total plant dry weights also varied significantly ($P < 0.01$) between the treatments with the largest weights recorded for the Lh treatments at H1 and for the control plants at H2 in both leaf morph populations. Minimum weights were recorded for the HL treatments at both harvests. The increment in plant weights at H2 was highly significant ($P < 0.01$). Of the two leaf morph populations, the marked one invariably recorded significantly ($P < 0.01$) greater values for plant dry weights at both harvests (Table 8.1).

Photosynthetic area

The mean PSA per plant showed wide variation ($P < 0.01$) between the treatments. At H1, maximum PSA was recorded for the plants exposed to LH treatments and at H2 for the control plants. The minimum PSA was recorded under H1 treatments at both harvests. Between harvests, the PSA varied significantly ($P < 0.01$) especially with respect to the controls where values at H2 were almost twice that at H1. Between the two leaf morph populations, the unmarked plants exhibited significantly ($P < 0.01$) greater PSA at both harvests (Tables 8.1, 8.2).

Nodule number

The nodule number per plant of the two leaf morph populations exposed to different treatments at the two harvests are presented in Figure 8.2a. The nodule number varied significantly ($P < 0.01$) between the treatments with the maximum number of nodules per plant formed for the plants exposed to LH treatment at H1 and for the 'controls' at H2. Minimum number of nodules was formed under

HL treatment in both leaf morph populations at either harvest. Plants of both populations at H2 developed significantly ($P < 0.01$) greater number of nodules; of the two leaf morph populations, the marked plants had significantly ($P < 0.01$) greater number of nodules at both harvests (Table 8.3).

Mean nodule weight

The mean weight per nodule showed significant ($P < 0.05$) variation between the treatments. The 'controls' of both the leaf morph populations formed the largest nodules and the plants under H1 treatment formed the smallest nodules. However, there was no significant difference in nodule weight either between the marked and unmarked plants or between the harvests (Table 8.2).

Nodule weight per plant

The nodule weight per plant showed a significant ($P < 0.01$) variation between the treatments. At H1 the maximum values were obtained either under LH treatment (marked population) or under 'control' (unmarked population) whereas at H2, the maximum values were recorded for the 'controls' in both leaf morph populations. Lowest nodule mass per plant was recorded for the plants under HL treatment at both H1 and H2. At H2, both leaf morph populations exhibited a significant ($P < 0.01$) increase in nodule mass per plant. At both harvests, nodule mass per plant was significantly ($P < 0.01$) more in the plants of the marked population than in the unmarked ones (Fig. 8.2b)(Table 8.4).

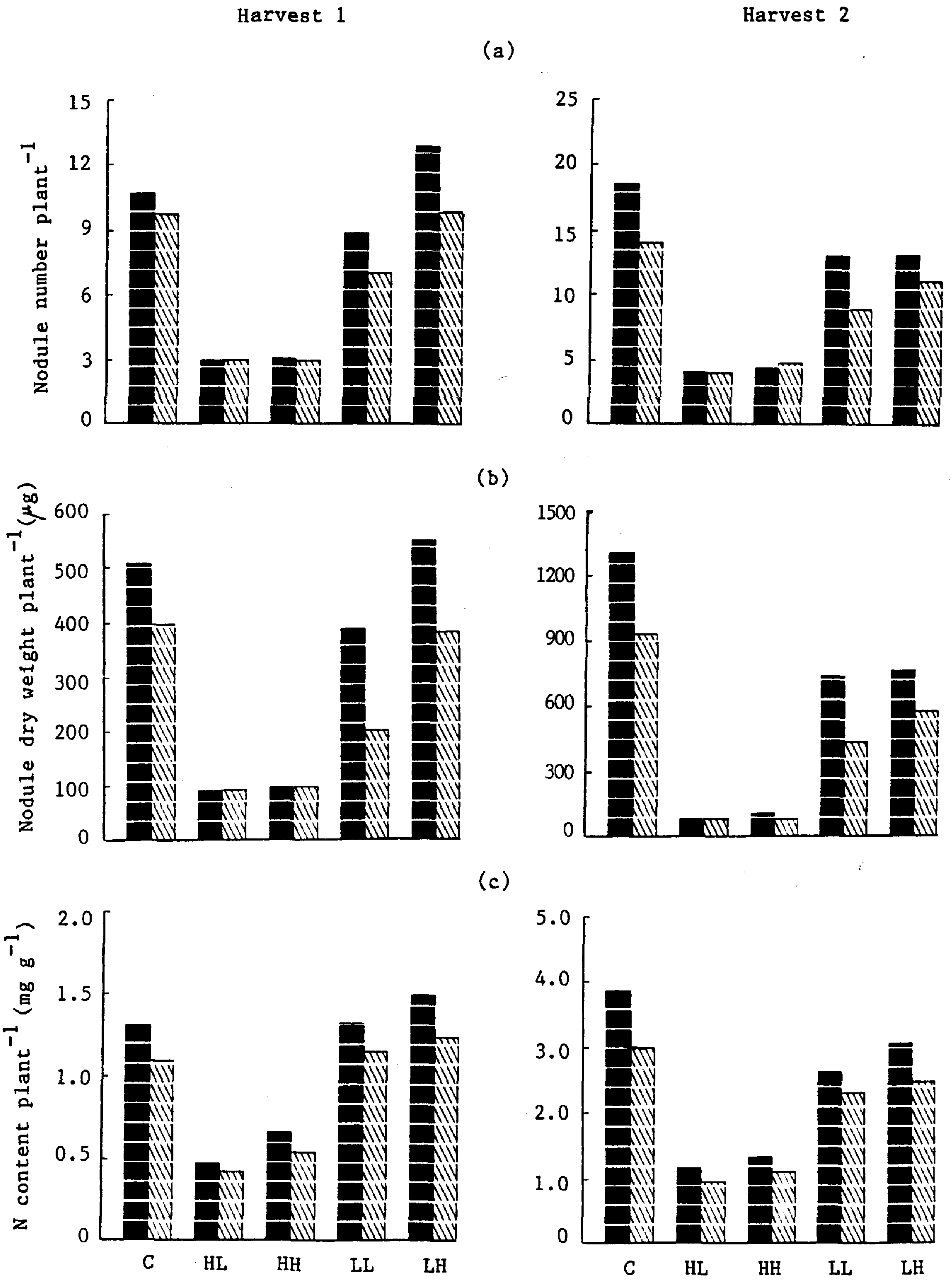

Fig.8.2. Effect of defoliation treatments on (a) nodule number (b) nodule dry weight and (c) N content per plant in the marked (■) and unmarked (▨) leaf morph populations of the clover at the two harvests.

Table 8.3. Effect of defoliation treatments on the nodule number per unit (100 mg) plant dry weight.

Defoliation treatments	Harvest 1		Harvest 2	
	marked population	unmarked population	marked population	unmarked population
Control	25.14	24.69	18.90	15.28
HL	13.31	14.37	8.84	9.58
HH	10.59	11.78	9.14	10.64
LL	20.37	17.01	16.66	11.80
LH	27.58	23.01	14.80	13.86

Table 8.4. Analysis of variance of nodule characteristics of the clover as influenced by harvests, leaf morph populations and defoliation and NPK treatments.

Nodule characteristics	Source of variation	df	F value	Level of significance
Nodule number	Harvests	1	24.05	$P < 0.05$
	Leaf morphs	1	22.03	$P < 0.01$
	Defoln. treat	4	122.93	$P < 0.01$
	Harv x Morph	1	1.29	ns
	Harv x defoln	4	8.62	$P < 0.05$
	Morph x defol	4	3.05	ns
Mean nodule weight	Harvests	1	4.83	ns
	Leaf morphs	1	1.07	ns
	Defoln. treat	4	20.13	$P < 0.05$
	Harv x Morph	1	2.79	ns
	Harv x defoln	4	4.03	ns
	Morph x defoln	4	0.95	ns
Nodule weight per plant	Harvests	1	91.15	$P < 0.01$
	Leaf morph	1	31.22	$P < 0.01$
	Defoln. treat	4	126.10	$P < 0.01$
	Harv x Morph	1	0.65	ns
	Harv x defoln	4	3.21	ns
	Morph x defoln	4	2.80	ns

ns = not significant

Total N per plant

Total N per plant varied widely between treatments with values varying significantly ($P<0.01$). At H1, maximum values were obtained under LH treatment, whereas at H2, peak values were recorded for the 'controls' for both leaf morph populations. The minimum values were recorded under HL treatment at both harvests. Although there was no significant variation in the total N per plant between the two leaf morph populations, values were significantly ($P < 0.01$) higher at H2 as compared to H1 (Fig. 8.2c).

Relative growth rate and Relative N accumulation rate

Relative growth rates (R_W) varied significantly ($P< 0.01$) between treatments with R_W being highest for the LH plants at H1 and for the 'controls' at H2. Lowest R_W was recorded under HL treatments at H1 and under HL treatments at H2. Variation in R_W was also significant ($P<0.01$) between the two leaf morph populations with the marked one showing higher values for R_W, and between harvests ($P<0.01$) with R_W being much greater at H1 as compared to H2.

Relative N accumulation rates (R_N) too differed widely between the treatments, populations and harvests (all $P< 0.01$) and generally followed the same trend as R_W (Tables 8.5, 8.6).

DISCUSSION

In a wide range of plant forms including T. repens, it has been well established that the leaves by virtue of being the most

Table 8.5. Effect of defoliation treatments on relative growth rate (R_W) (μg d^{-1}) and relative N accumulation rate (R_N)(μg d^{-1}) in the two leaf morph populations of the clover at the two harvests.

Defoliation treatment	Harvest 1				Harvest 2			
	Marked population		Unmarked population		Marked population		Unmarked population	
	R_W	R_N	R_W	R_N	R_W	R_N	R_W	R_N
Control	16.53	26.79	15.39	23.83	6.51	8.41	6.54	7.83
HL	6.72	10.75	5.59	8.99	5.53	7.36	5.37	6.49
HH	10.63	16.13	8.62	13.05	4.12	5.41	4.43	5.53
LL	16.95	26.85	16.09	24.68	4.53	5.29	4.77	5.37
LH	18.04	28.81	16.63	25.82	4.93	5.53	4.86	5.35

Table 8.6. Analysis of variance of growth parameters of the clover as influenced by harvests, leaf morph populations and defoliation and NPK treatments.

Growth parameters	Source of variation	df	F value	Level of significance
Total N $plant^{-1}$	Harvests	1	194.97	$P < 0.01$
	Leaf morphs	1	5.82	ns
	Defoln. treat	4	27.99	$P < 0.01$
	Harv x Morph	1	5.80	ns
	Harv x defoln	4	27.90	$P < 0.01$
	Morph x defoln	4	1.00	ns
R_W	Harvests	1	4268.64	$P < 0.01$
	Leaf morphs	1	25.82	$P < 0.01$
	Defoln. treat	4	354.72	$P < 0.01$
	Harv x Morph	1	32.07	$P < 0.01$
	Harv x defoln	4	326.43	$P < 0.01$
	Morph x defoln	4	0.59	ns
R_N	Harvests	1	5344.91	$P < 0.01$
	Leaf morphs	1	53.92	$P < 0.01$
	Defoln. treat	4	317.57	$P < 0.01$
	Harv x Morph	1	0.54	ns
	Harv x defoln	4	3.75	ns
	Morph x defoln	4	0.54	ns

ns = not significant

important photosynthetic tissue, are the major exporters of fixed carbon (C) in the form of carbohydrates, to the other plant parts on a priority basis (Hoshino *et al.* 1964, Chapman 1983, Robin *et al.* 1987, Chapman *et al.* 1991). Thus in *T. repens* the leaves normally act as the 'source' from where C is transported into compartments of shoot apices, stolons, branches, adventitious roots and nodules (which function as 'sinks') depending on prevailing nutritional requirements of these compartments. Conversely, grazing by herbivores, which causes partial defoliation, may lead to carbohydrates being translocated from previous sinks like stolon tissue towards the damaged part of the plant to compensate for the lack of photosynthate production in previously existing sources (Chapman *et al.* 1991). The marked reduced growth of *T. repens*, exhibited by both leaf morph populations following severe defoliation could therefore be attributed to a restriction in the size of the C source which determines growth of the defoliated plants (Chapman *et al.* 1991). Though the absence of mature leaves in the severely defoliated plants could partially explain their greatly reduced weights, this was more strongly related to the restricted development of the stolon tissue which accounts for the major part of the biomass of this legume. Addition of NPK nutrients could hardly help alleviate the detrimental effects of severe defoliation (Fig. 8.1a).

Though defoliation in *T. repens* has been reported to result in a marginal increase in the size of the remaining leaves within a 3-4 day period (Chapman *et al.* 1990), severe defoliation can have more serious and far-reaching consequences. Compared to companion grasses, white clover is known to be more sensitive to defoliation (Clark et al. 1984) and up to 30% of all stolon death and 30% of leaf loss have been reported to be related to the occurrence of severe defoliation (Chapman et al. 1984).

The nodulation pattern of the defoliated and 'control' T. repens plants is interesting. During the initial period of growth, light defoliation coupled with the addition of NPK (LH treatment) resulted in a flush of new leaves developing to compensate for the partial loss of source tissue. This might have led the plants under 'LH' treatments to produce a large number of leaves and consequently higher PSA as compared to the controls, in both the leaf morph populations at H1. This could explain the development of the largest number of nodules per plant in this treatment for both leaf morph populations at the end of the 4 week period (H1). Conversely, better root growth in this treatment (Fig. 8.1b), probably fuelled by the added NPK, would per se result in increased sites of infection by Rhizobium leading to the enhanced number of symbiotic root nodules formed. However, at the end of the 12 week period (H2), the nodule number in the 'controls' superseded that of the LH treatments. The formation of a greater number of nodules in plants exposed to LH treatments probably leads to the imposition of severe demands of the nodulated root system for carbohydrates from the source resulting in periods of crisis since the young leaves are not able to cope up with such demands (King 1978). The control plants not being subjected to any such imposed stress show better growth producing a larger number of leaves and greater PSA, and are thus able to form and sustain a larger number of nodules.

Nodulated roots of legumes require a continuous supply of C drawn from the source tissue for their growth and development. In addition, roots often act as reserves for deposition of carbohydrates (Danckwerts & Gordon 1989) and it has been estimated in T. repens, using ^{14}C, that of the total amount of C moving from the source to different sinks, the major portion (37-47%) is channelized to the root system. N_2 fixation is an energy-intensive process and nodules and nodules, consequently, have high energy demands (Ryle et al. 1985, Gordon et al. 1985). Respiratory losses associated with N_2 fixation are also high at ca. 9 moles of CO_2 / mole N_2 fixed as measured by the acetylene reduction assay (Ryle et al. 1989), accounting for nearly half of the C respired by whole plants (Gordon et al. 1987). The low number of nodules that develop in the severely defoliated plants could thus be attributed to the removal of the source tissue in these plants resulting in their becoming deficient in carbon. Starvation of sinks results in their poor growth (as indicated by the low root biomass), which not only drastically reduces sites of infection by Rhizobium but probably also leads to aborted infections in the absence of C for sustenance.

According to Chapman et al. (1990), the leaves of white clover attain an autotrophic state with respect to C supply when they have expanded to about 35% of their maximum surface area and reached about 50% of their final dry weight. Thereafter, they export an increasing percentage of the C they fix to the rest of the plant as they develop further. However, prior to attaining autotrophy young leaves also act as significant C sinks importing C from stolons. In the present study, severe defoliation resulted in removal of all mature leaves and the newly emerging leaves were possibly acting as sinks instead of a source, and thereby the flow of carbon to roots may decrease. Thus severe defoliation, which restricted the development of nodules (via its detrimental effects on root growth) also affected nodule growth as C would be channelized in the opposite direction towards young emerging sink leaves. This explains the low nodule weights associated with reduced nodule number per plant. As a result, the nodule mass per plant was proportional to the nodule number (Fig. 8.2c).

N_2 fixation being an energy demanding process, the 'low defoliation' and 'control' plants, which developed both the highest nodule number as well as nodule mass per plant, were the most efficient N_2 fixers fuelled by a continuous supply of C from the aboveground source tissue. This explains the high concentration of Kjeldahl N and total plant N in the plants of these treatments (Tables 8.2, 8.4).

Severe defoliation of T. repens plants, which would retard the transition of emerging leaves from C sink to C source, would therefore be the major attribute restricting nodule number as well as nodule growth.

Of the two leaf morph populations, the marked one consistently developed more nodules per plant (significant at P 0.01). This was true for the defoliation treatments as well as the 'control' at both harvests. The marked population is known to have a greater requirement for N (Pradhan & Tripathi 1984) and the larger number of nodules borne by this population is a probable attempt to fulfil its higher N requirement via symbiotic N_2 fixation. Results indicate that the root dry weights of the marked plants are significantly (P 0.01) greater as compared to that of the unmarked plants at both harvests. This indicates that the marked plants have a larger zone of their root system accessible to infection by the Rhizobium and this would aid increasing the number of nodules formed, as discussed earlier. Though there was no significant difference in mean nodule weights between the two populations, the marked plants by virtue of their greater nodule number, had a significantly (P 0.01) greater nodule mass per plant. However, the concentrations of Kjeldahl N were significantly higher (P 0.01) in the marked population although their PSA was considerably (P 0.01) lower. This reveals that the marked population, despite a significantly smaller C source, is a more efficient N_2 fixer.

The results of this experiment indicate that the stolon tissue plays an important role in facilitating a high degree of physiological plasticity with respect to C distribution and utilization in T. repens. The shoot meristems also took priority over sites of C utilization or storage, such as the nodulated roots and stolons, in response to defoliation and appear to act as the major sinks of carbon. Stolons being a rich source of stored carbohydrates (Baur-Hoch et al. 1990) are used in restoration of leaf tissue of defoliated plants (Danckwerts & Gordon 1989) at the expense of other sinks like nodulated roots. Though alleviation of nutrient deficiencies, particularly of P, is known to enhance the clover growth (Suckling 1976, Lambert et al. 1982, 1986), the present results indicate that under defoliation stress, alleviation of nutrient deficiencies does little to remedy the detrimental effects of drastic reductions in the C source of nodulated legumes.

9 General Discussion

The growth and establishment of a legume plays a pivotal role in the N economy of the habitat due to the immense N_2 fixing capability within its root nodules. However, the development and functioning of the nodules depend not only on the presence of a compatible rhizobial strain in the rhizosphere of the legume roots, but also on conducive micro-environmental condiions. The nodules in Trifolium being of the indeterminate type are further important as they can continue to grow and fix N_2 over prolonged periods of time.

As the young nodules belonging to category 'a' mature, they increase both in size as well as in the number of lobes. Thus they become larger and heavier so much so that they could be put under categories 'd' or 'e'. Though the nodule number in Trifolium during the later part of the year (September-October) increases only marginally, there is a sharp increase in the nodule dry weights during this period, which may be attributed to the greater proportion of older and larger nodules in the nodule population.

White clover has a high water content (Hayward 1953) and is susceptible to drought. It therefore reacts to the dry winter spells by shedding its larger root nodules which help it overcome the decreasing ratio of fixed N_2/absorbed CO_2 with increasing soil water tensions and low temperatures (Kuo & Boersma 1971). Field studies revealed that the nodule number per plant increased sharply following rains. Loss of soil nutrients and drop in NO_3 levels caused by heavy rains could trigger the formation of a greater number of nodules as a compensatory mechanism. The increased soil moisture levels would result in greater movement of rhizobia in soil (Hamdi 1971), increase the rate of infection of root hairs (Worrall & Roughley 1976) and enhance the supply of photosynthates from the flush of newly formed leaves - which would all lead to development of a greater number of 'juvenile' nodules.

The unmarked population had a greater proportion of their nodules belonging to the younger category whilst the reverse was true for the marked population, which indicates that the longevity of nodules is greater in the latter population. The marked population can therefore be expected to be more efficient in N_2 fixation compared to the unmarked population which bears a greater percentage of 'juvenile' nodules. Under stress, the unmarked population probably sheds a greater percentage of its larger nodules which results in an increase in the proportion of 'juvenile' nodules.

Trifolium repens exhibited a decrease in the nodule number per plant when supplied with increasing levels of NO_3 or NH_4, but the reduction in nodule number was compensated by an increase in the weight of the remaining nodules. This indicates that increased levels of N are detrimental to nodule initiation but not to nodule growth, and increased nodule mass may be seen as an adaptation to sparse nodulation under such conditions. Of the two leaf morph populations, the marked one shows stronger negative correlation between nodule number and mean nodule weight, which indicates that the marked population is better adapted to compensation of reduced number by increased weight of nodules.

Of the two forms of N, NO_3 was a stronger inhibitor to both nodule initiation and nodule growth compared to NH_4. The reduction and assimilation of NO_3 requires considerable energy which leads to a reduction in photosynthates otherwise available to the nodules. This has been confirmed using ^{14}C on a wide range of legumes including clover, by several workers (Small & Leonard 1969, Kahn & Kahn 1981, Truchet & Dazzo 1982). Also, nitrite reduced from nitrate besides being toxic to living organisms, destroys IAA which is required for infection (Dixon 1969, Munns 1977). The inhibitory effects of NH_4 are less severe due to its reduced state (Rabie et al. 1980). The increase in nodule number in the NH_4 treated plants may be attributed to the increased root growth of these plants which in turn, causes an increase in the number of infection sites (Wedderburn 1983) as discussed in Chapter 4. Between the two forms of nitrogen, the N content per plant was consistently higher in the NH_4^+ treated plants than in the NO_3^- treated ones, which is the outcome of a greater number of nodules which develop in response to NH_4^+ addition. Increased nodule mass per plant in the NH_4^+ treatments also indicated that NH_4^+ is not as severe an inhibitor to nodulation as NO_3^-.

As the clover plants grow, their stoloniferous habit leads to the plants encountering a wide mosaic of edaphic variables including variations in the soil pH. The pH of the microenvironment may be reduced following heavy rains which would result in leaching of soluble bases. When edaphic conditions are strongly acidic (pH 4.5), survival of the R. trifolii population in the soil is doubtful which is reflected in the failure of nodulation of the clover, whereas at pH 5.0-6.0 there was a steady increase in both nodule number per plant as well as in the nodule mass. Nodule growth was more sensitive to low pH than the nodule number. At reduced pH levels, decrease in nodule population coupled with low N_2 fixation rates would lead to N deficiency and suppressed growth. Strongly acidic conditions also severely affect root growth due to which the clover may fail to utilize sub-soil moisture leading to apparent moisture stress.

R. trifolii is also susceptible to dryness (Davey et al. 1989) and the reduced nodule number exhibited by both the leaf morph populations at 10% soil moisture could be attributed to the sharp decrease in the number of infection threads (Worrall & Roughley 1976). Moisture stress may also result in shedding of pre-formed nodules from the host root system leading to a further decrease in the nodule population. Results of the present investigation reveal that intermediate levels of soil moisture (10-20%) are most conducive for nodulation as this range also ensures adequate aeration.

In the grasslands, *T. repens* is commonly subjected to defoliation along with companion grasses during grazing by cattle. Removal of the leaves would result in the loss of the photosynthesizing tissue of the legume which acts as the 'source' of fixed carbon for the other plant parts. Defoliation may result in the export of stored photosynthates from 'sinks' like stolons, adventitious roots and nodules to the defoliated shoot apices. This causes a decrease in the number of nodules in the severely defoliated plants. Low root biomass resulting in such situations leads to a decrease in the number of infection sites (Wedderburn 1983) and an increase in the number of aborted infections in the absence of a carbon source.

Nodules are 'sheltered places' (Bergersen 1971) for the endophytes within, which pay back the host by providing nitrogenous compounds. For efficient N_2 fixation, homeostasis in terms of a steady supply of energy, constant removal of fixed products and the maintenence of a precisely poised oxygen concentration, are all important for sustained activity. Senescing nodules may also provide a survival mechanism for the endophyte. As they are shed, the husk provides first, a source of nutrients, then a shelter, thereby minimizing the effects of desiccation and other adverse soil conditions.

Long term exposures to higher concentrations (>1.5 mM) of N were severely inhibitory to nodule initiation. Though low concentrations (0.1 0.75 mM) proved greatly beneficial, nil N strongly inhibited nodule initiation. Compared to NH_4^+, NO_3^- was a stronger inhibitor - both to nodule initiation and nodule growth. Nodulation was totally inhibited at pH 4.5, thereafter there was a sharp rise in the nodule population up to pH 5.5-6.0, following which the nodule population dropped as conditions approached neutrality. Although short-term exposures to high soil moisture levels (up to 30%) led to an increase in nodule population, in the long term, 10-20% soil moisture proved to be optimum. High defoliation severely affected nodule initiation and low defoliation proved less detrimental. Incorporation of NPK did little to alleviate the negative effects due to the imposed stress. Only N-supplied plants exhibited a significant negative correlation between nodule number per plant and mean nodule weight, which indicates that higher levels of N was the only stress that was inhibitory to nodule initiation but not to nodule growth.

Management practices to ensure the growth of a well-established clover population bearing an appreciably high number of nodules, would include minimizing grazing during the dry winter and spring when the clover is particularly susceptible to defoliation. Soil N levels, prevailing in the swards, do not seem to be inhibitory to nodulation and growth of the clover as is evident from Chapter 4. Liming of the swards would not be beneficial as results of the present study indicate that the growth and nodulation of the clover is best at pH 5.5-6.0, which is the usual range of soil pH in the swards around Shillong.

Of the two leaf morph populations, the marked one consistently exhibited greater number of nodules per plant as well as greater nodule mass. Increased number of potential sites of N_2 fixation coupled with greater amount of N_2 fixing tissue in this population indicates that this population is of greater significance in determining the N status of the habitat. Prevailing environmental stresses in winter - low soil moisture and temperature - are inhibitory to N_2 fixation and therefore, the clover nodules in winter serve more as an overwintering population (than as potential sites of N_2 fixation) which can resume growth and function the following spring with the return of favourable conditions.

10

Summary

The present study on nodule population dynamics in *Trifolium repens* conducted at Shillong in north-east India during June 1991 - May 1993, aimed at determining how the nodule population in the two distinct populations of this legume, viz., the marked and unmarked leaf morph populations, were affected under different ecological conditions. The study was carried out in two parts - field study under natural conditions and net house experiments where conditions could be manipulated.

The field study was conducted in a sward having a profuse growth of *Trifolium repens*. The sward also contains *Anemone rivularis* Ham., *Arundinella* spp., *Axonopus compressus* (Sw.) Beauv., *Centella asiatica* L., *Fimbristylis dichotoma* Vahl., *Hypocharis radicata* L., *Pennisetum clandestinum* Hochst.ex.Chiov., *Plantago major* L., moss and other minor constituents. *T. repens* comprises two distinct leaf morph populations - one with conspicuous 'V' shaped white markings on the leaflets (marked population) and the other with no such markings (unmarked population).

In the sward, two plots of around 0.25 ha each were selected. The first plot received sunlight for the major part of the day whereas the second plot was partially shaded due to an existing row of trees towards the southern periphery of the site. *T. repens* plants of both leaf morph populations were sampled at monthly intervals and the shape, size and number of root nodules and various other growth parameters were studied. The edaphic variables of the study plots were also studied.

In the net house experiments, white clover plants were raised in plastic pots (15 cm diam., 16 cm depth) filled with acid washed sand or with a mixture of sand and soil, inoculated with previously-isolated rhizobia, and the effects of different controlled conditions on the nodulation pattern studied. The plants were raised in the pots using the uniform pieces of ramets obtained from the field populations.

The field observations revealed that nodule number per plant varied seasonally with the maximum number of nodules borne during the late rainy season, corresponding to peak vegetative growth, and the minimum number developing during the winter. Of the two leaf morph populations, the marked population consistently exhibited greater nodule number per plant. In the marked population, the mean number of nodules per plant was maximum (22.0) during October 1991 and minimum (2.4) during February 1993. Corresponding values for the unmarked population were 16.2 and 1.8 during July 1992 and January 1993, respectively. The nodules were grouped into five categories based on their size and the number of lobes: category 'a' - unbranched and 0-3 mm in length, category 'b' - those with 2 lobes or unbranched and 3-4.5 mm in length; category 'c' - with 3 lobes and up to 4.5 mm in length; category 'd' - 2 or more lobes and 4.5-6.0 mm in length, and category 'e' - 3 or more lobes and >6 mm in length.

The maximum number of nodules recorded from both the leaf morph populations were of category 'a'. Up to 60.1% of the nodules belonged to this category in the unmarked population from the 'open'. The unmarked population from 'shade', marked population from 'open' and marked population from 'shade' had lower percentage of nodules in category 'a', the respective values being 47.6%, 47.9% and 46.5%. The unmarked population from 'open', which had the highest percentage of its nodules in category 'a', had the lowest percentage (3.0%) of the nodules in category 'e'. Among the various populations sampled, the marked population from the 'shade' had the highest proportion (8.6%) of nodules in category 'e'.

Among the major edaphic variables, soil temperature and soil moisture showed a significant positive correlation with nodule number in both leaf morph populations. Nodule number was also correlated with soil pH. Among important growth parameters, the number of rooting nodes per stolon and photosynthetic area per plant showed a significant positive correlation with nodule number in both leaf morph populations.

The effect of varying levels of soil N, soil pH, soil moisture and defoliation on the nodule population of T. repens were studied by performing several nethouse experiments. To study the effect of varying levels of soil N on the nodule population of white clover, the plants were exposed to 0, 0.1, 0.5, 0.75, 1.5, 3 and 6 mM NO_3 or NH_4 (supplied as KNO_3 and NH_4Cl respectively). With increasing levels of NO_3 or NH_4, the nodule number per plant showed an initial increase and then sharply declined with further increase in N levels showing a total inhibition of nodulation at the highest N level. In both NO_3 and NH_4 treatments, the plants of the marked population developed a greater number of nodules as compared to the unmarked population. Of the two treatments, nodule number per plant was greater in the NH_4 treated plants. The mean nodule weight of plants from both leaf morph populations showed a strong negative correlation with nodule number per plant. This was true for both NO_3-treated as well as NH_4-treated plants. Of the two populations, the marked one also exhibited greater nodule mass per plant. The total N content per plant varied significantly between the different N levels. In both NO_3 and NH_4 treatments, the N content per plant was significantly greater in the marked population than in the unmarked population.

The clover was grown in sand+soil mixtures adjusted to pH 4.5, 5.0, 5.5, 6.0 or 6.5 to study the effect of varying pH levels on the nodule population dynamics. At pH 4.5, plants of both leaf morph populations failed to nodulate, but with increasing pH the nodule number increased sharply up to pH 6.0 beyond which level, the nodule number dropped considerably. In general, the marked population exhibited significantly greater number of nodules as well as nodule mass per plant compared to the unmarked population. The total N content per plant showed a steady increase with increasing pH in both the leaf morph populations. The total N content per plant was significantly greater in the marked population compared to the unmarked population.

The effect of varying levels of soil moisture on nodulation was determined by growing the two leaf morph populations in sand+ soil mixture at 10%, 20%, 30% and 40% moisture levels on dry weight basis. The nodule number per plant showed a gradual increment with an increase in soil moisture levels up to 20%, beyond which there was a drastic reduction in the nodule population. The marked population consistently exhibited greater nodule number per plant compared to the unmarked population. The total nodule mass per plant also varied significantly amongst moisture levels. Total N per plant increased up to 20% soil moisture level and thereafter decreased with increasing soil moisture levels.

To study the effect of defoliation and NPK levels on the nodule population in T. repens, the plants were exposed to two defoliation and two NPK levels - high and low. The sand+soil mixture represented low NPK level, to which amendments were made to maintain a high NPK level. The plants of both leaf morph populations were thus subjected to one of the following treatment combinations: (i) 'low defoliation-high NPK (LH), (ii) 'high defoliation-high NPK' (HH),

(iii) 'low defoliation-low NPK' (LL), and (iv) 'high defoliation-low NPK' (HL). Besides, respective controls were also maintained for the two leaf morph populations. The nodule number as well as nodule mass per plant varied significantly between treatments and the greatest number of nodules per plant was recorded in LH and the smallest number in HL treatments for both the leaf morph populations. The marked population consistently exhibited greater nodule number and nodule mass per plant compared to the unmarked population. The nodule mass per plant as well as total N per plant were highest in the 'controls' and lowest in the HL treatments in both populations.

Results of the study indicate that nodulation in *T. repens* is affected by a wide range of ecological conditions, directly or via their effects on the growth of the host legume. Not only is there a seasonality in the growth and functioning of the root nodules as obvious from the field data, but short-term localised changes in the edaphic conditions or external stress factors also affect their development, longevity and functioning. Of the two leaf morph populations, plants of the marked population consistently exhibited greater nodule number and nodule mass per plant under various ecological conditions. This results in plants of this population having a larger quantity of N_2 fixing nodule tissue which makes this population, under identical conditions, more efficient in so far as N_2 fixation is concerned. Thus the marked population of *T. repens* could be expected to play a more significant role in the nitrogen economy of grassland ecosystems compared to the unmarked population.

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