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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 $620 \text{ Kg N ha}^{-1} \text{ yr}^{-1}$ (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 (Bohloul & 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 num-

ber 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).

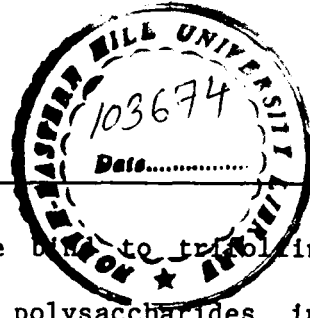
The initial phase of attachment is followed by firm anchorage of

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 to trkA in *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 (Badenoch-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 deficiency 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 N₂ fixation including size and number of nodules, time of appearance, nodule morphogenesis and N₂ 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 inhibitory 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 alleviated 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 N₂ 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 N₂ 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 CaCO₃ 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 rhizo-

sphere 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 edaphic-

ally 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 N₂ 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 N₂ 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 N₂ to absorbed CO₂ 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 N₂ 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 N₂ 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 ($\text{cm}^2 \text{g}^{-1}$ 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 photo-period 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 N₂ 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 ¹⁴C, 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 N₂ 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 N₂ 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 $\text{ha}^{-1} \text{yr}^{-1}$ 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 recommended 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 $295 \text{ Kg ha}^{-1} \text{yr}^{-1}$ and N transfer of $76 \text{ Kg ha}^{-1} \text{yr}^{-1}$ 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 N₂ 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.