DUAL MUTUALISTIC ASSOCIATIONS IN SAINFOIN

(Onobrychis viciifolia Scop.)

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ABSTRACT

Recent studies established that many legumes, when infected with the appropriate *Rhizobium* spp. and arbuscular fungi, nodulated better and exhibited greater dinitrogen fixation than plants infected with only the rhizobia.

A similar study, therefore, was carried out in a glasshouse using sainfoin *(Onobrychis viciifolia* Scop.*)*, a legume that is rapidly gaining recognition as a potential forage crop in New Zealand and other parts of the world. Pre-germinated seeds (cv. Fakir) were planted in sterilized soils and incubated with an effective *Rhizobium* spp. (strain NZP 5301), a mixture of endophytes *(Gigaspora magarita* Becker & Hall, *Glomus fasciculata* (Thax. sensu Gerd.) Gerdemann & Trappe and *Glomus tenuis* (Greenall) Hall), or both eht rhizobia and endophytes. The experiment also included a control, without any inoculation. Endophyte infection, nodulation and dinitrogen fixation, total nitrogen and phosphorus concentrations, and plant growth and development were determined on eleven sequential samplings over about twenty weeks, up to the stage of green inflorescence.

Arbuscular mycorrhiza formation did not occur with the first endophyte inoculation, containing *Gigaspora magarita* Becker & Hall, even after 93 days of growth. This is probably because the inoculum used consisted of a low quantity of viable spores and mycelia. The second inoculation, containing the three endophyte species, produced only a low degree of infection between day 115 and 137, possibly because the extensive root lignification and relatively higher root phosphorus concentration (0.50%) restricted fungal invasion and establishment within the root cortex. Mycorrhiza formation did not increase phosphate uptake, improve nodulation and dinitrogen fixation, or increase plant growth. This is due probably to the already well-developed root systems that were efficiently exploiting the small soil volume within the bags.

Rhizobia-inoculated plants produced more nodules, larger nodules and consequently, a greater nodule dry weight than the uninoculated plants. The nodules produced in the inoculated plants were red

instead of green as in the uninoculated plants, and exhibited a greater dinitrogen fixation. As a result, these inoculated plants contained a higher concentration of shoot, root and nodule nitrogen, and a greater dry weight accumulation in the shoots and nodules. The shoot and nodule phosphorus concentrations, however, were lower in the rhizobia-inoculated than in the uninoculated plants due to the greater amount of shoot and nodule tissues which caused a dilution effect. These rhizobia effects on nodulation and dinitrogen fixation, nitrogen and phosphorus concentrations, and plant growth and development became more prominent with time.

The relatively higher nodule phosphorus concentration when compared with the shoot and root phosphorus concentrations suggests that phosphorus was presumably required in large quantities by the dinitrogen-fixing system.

PREFACE

Coexistence of organisms has long been recognised as an axism of life. In 1952, Paul R. Bulkholder formally and objectively interpreted coexistence as different biological interactions. Based on his coaction theory, these interactions were classified into and named as nine separate categories of which the most studied in agricultural ecology are competition and mutualism.

In this thesis, two examples, of mutualism, involving a forage legume *(Onobrychis viciifolia* Scop.*)*, a nitrogen-fixing bacterium *(Rhizobium* spp.*)* and three species of arbuscular fungi *(Gigaspora magarita* Becker & Hall, *Glomus fasciculatus* (Thax. sensu Gerd.) Gerdemann & Trappe and *Glomus tenuis* (Greenall) Hall), are examined. The intention of this study was to investigate the real value of coexistence of these organisms from an agricultural standpoint and, therefore, emphasis is placed on the effects of the bacterium and fungi on the nutrition, and growth and development of sainfoin. While the bulk of chapters 4, 5, 6 and 7 is devoted to these topics, the relevant background information of the research is also included in the first three chapters.

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CHAPTER 1

INTRODUCTION

Nitrogen is an essential element for plant growth and reproduction as it is required in the synthesis of proteins, enzymes, deoxyribonucleic acids and many intermediate metabolic compounds. It is, therefore, a key constituent of all plant cells. Dry matter of vascular plants contains about 15 000 μg N g^{-1}DM (1.5% nitrogen), making it the most abundant soil element in plant tissue (Table 1.1).

The supply of nitrogen in current and future agricultural systems is a major determinent of adequate food production to meet the ever-expanding human population. During the green revolution, many agricultural plants were selected and bred for responsiveness to fertilizer supply, resulting in the necessity for intensive application of fertilizers particularly nitrogen (Cummings and Gleason, 1971; Engibous, 1975; Jackson *et al*, 1975; Cox and Atkins, 1979). Scrimshaw and Taylor (1980) have worked out that the primary factor responsible for increases in crop production between 1961 and 1976 was the increasing use of fertilizers. Between the same period, the world's annual consumption of nitrogen fertilizers rose sharply from 11 to 45 Gkg, a dramatic 309% increase (*Fertilizers - Annual Review*, 1968; *FAO Fertilizer Yearbook*, 1980). In 1980, it reached a record of 57 Gkg $year^{-1}$ (*FAO Fertilizer Yearbook*, 1981). With this increasing trend in nitrogen fertilizer usage, it is perhaps not an exaggeration to reaffirm the statement of Viets (1965) that more crops are deficient of nitrogen than of any other element.

The primary supplement of nitrogen to crop plants is from industrial nitrogen fertilizers. However, the existing method of industrial synthesis of nitrogen fertilizers requires a high input of expensive fossil energy. For instance, in the manufacture of ammonia, the precursor for various types of nitrogen fertilizers, a temperature of 400 to 500°C and a pressure of 15 to 35 MPa must be created to drive the Haber-Bosch process in a modern plant, with a production capacity of about 900 Mkg day^{-1} (Bridger *et al*, 1979). In this process alone, the natural gas feed and fuel cost contributes 25% of the total manufacturing cost (Finneran and Czuppon, 1979). With the addition of other fuel expenditure as in the conversion of ammonia into nitrogen fertilizers, in transport and in application, the final nitrogen fertilizer applied to

TABLE 1.1

CONCENTRATION OF SIXTEEN ELEMENTS IN COMPLEX PLANTS (AFTER STOUT, 1961)

Element	Concentration ($\mu g\ g^{-1}$ DM)
From the atmosphere and water,	
carbon	450 000
oxygen	450 000
hydrogen	60 000
From the soil,	
nitrogen	15 000
potassium	10 000
calcium	5 000
magnesium	2 000
phosphorus	2 000
sulphur	1 000
chlorine	100
iron	100
manganese	50
boron	20
zinc	20
copper	6
molybdenum	0.1

TABLE 1.2

EFFICIENCY AND CONTRIBUTION FROM VARIOUS DINITROGEN-FIXING SYSTEMS (AFTER BURNS AND HARDY, 1975, WITH ALTERATIONS IN PARENTHESIS FROM PAUL, 1978)

Dinitrogen-fixing system	Land use (Mha)		Rate of fixation (kg $N_2 ha^{-1}$ $year^{-1}$)	Total contribution (Gkg $N_2 year^{-1}$)
Legume-*Rhizobium*	legume	250	140 (80)	35 (20)
Legume-*Rhizobium*	permanent grassland	3 000	15 (8)	45 (24)
Blue-green algae	rice	135	30	4
Free-living and "loose" associations	other crops	5	5	5

the field is an expensive item for many farmers.

In conjunction with this high cost, tracer studies reveal that the use of applied nitrogen fertilizers in the field by crop plants is inefficient. Depending on the type of crop, agricultural practices, fertilizer, climate and soils (Winteringham, 1980), between 20 to 60% of the total applied nitrogen is absorbed by crop plants (Allison, 1965, 1966; Bartholomew, 1971; Gutschick, 1980; Hauck, 1981). The work of Myers and Paul (1971) showed that wheat (*Triticum aestivum* L.) grown in a sandy loam and a clay soil, recovered about 25% and 50% of the applied ammonium nitrate respectively in the shoots. In two other studies, a first year maize (*Zea mays* L.) crop utilized only about 22% of the labelled urea, this being from grain and straw (Arora *et al*, 1980), while a first year dwarf bean (*Phaseolus vulgaris* L.) crop removed about 30% of the labelled ammonium sulphate (Cervellini *et al*, 1980). The unrecovered nitrogen is "lost" through immobilization, leaching, erosion, denitrification and volatilization of which leaching and erosion, if excessive, pose a serious threat to environmental pollution and public health (Mulder *et al*, 1977; Wild and Cameron, 1980).

From the foregoing discussion, it is apparent that the continual heavy reliance on nitrogen fertilizers in the future is becoming a questionable proposition. The emphasis of current nitrogen and crop research is, therefore, strongly orientated towards improving biological dinitrogen fixation (Evans, 1975; Hardy *et al*, 1975; Brill, 1980; Hardy, 1980a, b; Lambourg, 1980; Subba Roa, 1980). Several biological dinitrogen fixing systems are available for incorporation into agricultural production as shown in Table 1.2. The most important and efficient of which, in relative terms, is the legume-*Rhizobium* mutualistic association. The data in Table 1.2 indicate that this type of association fixes an average of between 80 to 88 kg N_2 $ha^{-1}year^{-1}$ and, thus, contributes between 20 to 44 Gkg N_2 $year^{-1}$ to cultivated land under legumes and permanent grassland. However, a fixation as high as 171 kg N_2 $ha^{-1}year^{-1}$ has been obtained in the developed New Zealand pastures in which the principal legume component is white clover (*Trifolium repens* L.) (Hoglund *et al*, 1979).

Although the legume-*Rhizobium* association is the most efficient by comparison, it is widely recognised that its dinitrogen-fixing activity seldom attains the optimal rate. For example, the clovers in the New Zealand pastures are capable of fixing a potential of 215 to 336 kg

N_2 $ha^{-1}year^{-1}$ (Sears *et al*,1965; Levy, 1970). Improvement on the rate of dinitrogen fixation is, therefore, an imperative research endeavour in order to sustain the necessary agricultural production levels.

The physical and biological factors that directly and indirectly influence the legume-*Rhizobium* relationship have been identified and comprehensively reviewed by various authors (Lie, 1974; Gibson, 1977; Munns, 1977; Pate, 1977; Parker *et al*, 1977; Dommergues, 1978; Vincent, 1980; Grandhall, 1981). One of these factors is soil phosphorus, an essential element for the growth and nodulation of legumes (van Schreven, 1958; Andrew, 1977; Andrew and Jones, 1978). Many legumes, when infected with arbuscular fungi, show an enhanced phosphate absorption and, subsequently, an associated increase in growth, nodulation and dinitrogen fixation (Crush, 1974; Daft and El-Giahmi, 1974, 1975,1976; Powell, 1976; Mosse *et al*, 1976; Mosse, 1977; Abbott and Robson, 1977; Smith and Daft, 1977; Carling *et al*, 1978; Azcon-G. de Aguilar *et al*, 1979; Smith *et al*, 1979). Similar studies on sainfoin (*Onobrychis viciifolia* Scop.) have yet to be carried out and since it is a legume which is gradually gaining world-wide recognition as a potential forage crop, the purpose of this study is to examine the endophyte-phosphate interaction, and its effects on the nodulation and dinitrogen fixation in sainfoin.

CHAPTER 2

TERMINOLOGY

2.1 INTRODUCTION

Over the years, the interdisciplinary interest in plant science has resulted in some confusing terminology in the literature on soil-plant studies. This chapter, therefore, proposes to define the meanings of commonly used terms so as to ensure a greater precision of meaning. For convenience, each term is discussed in alphabetical order under two sections. Section 2.2 is concerned with the nomenclature used in dinitrogen fixation, while section 2.3 deals with the terms used in mycorrhizae.

Similarly, to reduce ambiguity in units of expression (Incoll *et al*, 1981), it is proposed to use the Système International d'Unités (SI) endorsed by the International Organisation for Standardization in 1960. A list of the units (and other common abbreviations) used, with their symbols, names and definitions are presented in Appendix A.

2.2 TERMS USED IN DINITROGEN FIXATION

2.21 Infection, Invasion and Infectivity (Fåhraeus and Ljunggren, 1968)

The term *infection* is used to describe the establishment of a parasitic and not a pathogenic relationship within the host. The term *invasion* is sometimes used as a synonym of infection, irrespective of whether the process is pathogenic or non-pathogenic. Since virulence or pathogenicity expresses the capacity of an organism to produce a disease, it is substituted by another term, *infectivity*.

2.22 Mutualism and Symbiosis (Starr, 1975)

Symbiosis is a general term which means the living together of two dissimilar organisms and, thus, includes all types of interactions. However, *mutualism* refers specifically to the association in which the two organisms involved, benefit from each other.

Both terms as defined are also used to describe mycorrhizae.

2.23 Rhizobia, *Rhizobium* and *Rhizobium* spp. (Jordan and Allen, 1974; Skerman *et al*, 1980)

Rhizobium Frank 1889 is the generic name for a genus of bacteria that form a mutualism with legumes and fix dinitrogen. *Rhizobia* is sometimes used as a common name when referring to this group of bacteria, but other terms such as *"Rhizobia"*, and "rhizobial" are regarded as unsatisfactory. *Rhizobium* spp. refers to certain unclassified dinitrogen-fixing bacteria. On the basis of cross-inoculation studies, these bacteria can be described either as "promiscuous", that is, capable of forming mutualism with legumes of a wide range of genera from different tribes, or as specific.

2.3 TERMS USED IN MYCORRHIZAE

2.31 Endophyte (Braun-Blanquet , 1928)

The term *endophyte* (plural *endophytes*) means a plant that invades and lives in its host. It is commonly used to refer to fungi that form mycorrhizae (see Kelley, 1950). Fungi are not classified as plants of course, but the use of endophyte to define mycorrhiza-forming fungi is continued here. However, it is not employed when referring to bacteria such as the *Rhizobium* spp. that reside in legumes.

2.32 Mycorrhiza Types (Lewis, 1975)

The term *mycorrhiza* (plural *mycorrhizae* in American usage and mycorrhizas in English usage) (Nicholson, 1967) means fungus-root and is strictly used to describe the mutualism between a fungus and plant root. Terms such as "mycorrhizal fungus" (fungus-root fungus) and "mycorrhizal root" (fungus-root root) are, therefore, best avoided.

If the fungus exists only in the intercellular spaces in the root and forms a fungal sheath around the root, the association is known as an *ectomycorrhiza*. However, if the fungus is both intercellular and intracellular, and does not form a sheath, the association is called *arbuscular, orchidaceous*, or *ericoid mycorrhiza* depending on the host type. Orchidaceous and ericoid mycorrhiza occur specifically in the family Orchidaceae and the order Ericales respectively, while arbuscular mycorrhiza occurs generally in many families. A fifth group, named as *arbutoid mycorrhiza*, is also specific to the order Ericales, but the fungus involved is intercellular and intracellular, as well as sheath-producing.

CHAPTER 3

REVIEW OF LITERATURE

3.1 INTRODUCTION

The Fabaceae (or Leguminosae), comprising 650 genera and 18 000 species, is the third largest family of flowering plants after the Asteraceae and Orchidaceae (Polhill *et al*, 1981). Within the Fabaceae, three subfamilies -- the Caesalpinioideae, the Mimosoideae and the Papilionoideae -- are generally recognised. In the Caesalpinioideae and the Mimosoideae, the species included are mainly trees and shrubs of the tropics and subtropics (Clapham *et al*, 1952; Hutchinson, 1964). In contrast, the Papilionoideae consists mostly of annual and perennial herbs which are more widely distributed, extending from the tropical to the temperate regions (Hutchinson, 1969). Almost all the important agricultural legumes are also included in this subfamily (Okigbo, 1978).

Economically, legumes are considered to be the second most important group of plants after grasses (Sinha,1977; National Academy of Sciences, 1979). The findings of Harlan (1976) suggest that legumes have been cultivated since the beginning of ancient civilization and agriculture. Throughout history, therefore, man appeared to have highly regarded the value of legumes. Today, legumes maintain their key role in modern agriculture, covering a wide range of purposes including agricultural uses (Skerman, 1977; Dobereiner and Campelo, 1977; Mulder *et al*, 1977), forestry (National Academy of Sciences, 1979), horticulture (Heywood, 1971; National Academy of Sciences, 1979), and others (Heywood, 1971; Willis, 1973).

Although legumes are cultivated for various reasons, their primary economic importance is attributed to the ability of their root nodules to convert dinitrogen into ammonia which is readily utilized by the plants. This dinitrogen-fixing ability is the result of a successful mutualistic association between legume roots and a genus of soil bacteria, *Rhizobium*. Not all legumes exhibit this mutualistic relationship, but the majority of species in the Mimosoideae and the Papilionoideae are known to form root nodules (Allen and Allen, 1961), including all the agricultural legumes except for certain lines of red clover (*Trifolium*

pratense L.) (Nutman, 1949) and soybean (*Glycine max* (L.) Merr.) (Williams and Lynch, 1954; Clark, 1957).

In recent years, the potential dinitrogen-fixing capacity of legumes is recognised to be of immense economic and ecological value. This is reflected in the release of several exclusive monographs by the National Academy of Sciences (1975, 1977, 1979), Skerman (1977), Duke (1980), and Summerfield and Bunting (1980) on the potential and the utilization of both food and forage legumes. In addition to the major agricultural legumes, the generated research interest is extended also to many under-utilized species with desirable characteristics.

In this chapter, a review is presented of the botany and agronomy of one such under-utilized legume called sainfoin (*Onobrychis viciifolia* Scop.), with special emphasis on the supply of phosphates through mycorrhiza formation, and its effects on nodulation and dinitrogen fixation. In the section on dinitrogen fixation (section 3.3) and mycorrhizae (section 3.4), where information is scanty on sainfoin, an endeavour is made to broaden the review to include other legume species and, whenever necessary, other non-legume species.

3.2 SAINFOIN AS A FORAGE CROP

3.21 General Distribution and History

3.211 Distribution. The genus *Onobrychis* Mill., belonging to the tribe Hedysareae, is comprised of some 130 species (Clapham *et al*, 1952; Polhill, 1981) herbaceous, or sometimes shrubby and spiny perennials (Bailey, 1944) and annuals (Ball, 1968). The origin of *Onobrychis* spp. is in the Near Eastern Centre which includes the regions of Turkey, Iraq, Iran, and the Caucasus and the east of the Caspian Sea, belonging to the Soviet Union (Vavilov, 1951). However, their present distribution is wide, covering the Mediterranean, central Europe, western Asia, and central Asia from the Caspian Sea to Lake Baykal (Kernick, 1978). A number of species such as *O. arenaria* (Kit.) D.C., *O. transcaucasica* (Shain, 1959), *O. caput-galli* (L.) Lam., *O. crista-galli* (L.) Lam., (Smith, 1976), *O. vaginalis* C.A. Mey., *O. tanaitica* Sprengel, *O. inermis* Stev. and *O. altissima* Grossh. (Duke, 1980) have been cultivated in their native areas. However, the most important and widely grown species is *O. viciifolia* Scop. (sainfoin) which can be found commonly today in the Mediterranean nations, west Asian countries,

the United States and Canada (Kernick, 1978).

3.212 History. The writings of Vavilov (1951) suggest that sainfoin is indigenous to the mountains of the Near Eastern Centre, especially in the Caucasus, existing in many forms as land races and ecotypes. Sainfoin cultivation probably commenced in the tenth century in Azerbadjan, Armenia and Georgia, all of which are situated just south of the Caucasus (Shain, 1959). In the fifteenth century, its cultivation spread to France from which it was extended to Switzerland, Germany and England two centuries later (Stebler and Schroter, 1889). In the eighteenth century, some seeds were also carried to Italy (Stebler and Schroter, 1889) and Wales (Rees, 1928), and in the early nineteenth century to the United States (Clark and Malte, 1913).

From the time of its introduction to the nineteenth century, sainfoin was highly esteemed as a forage or pasture plant in both continental Europe and England (Stebler and Schroter, 1889; Whyte *et al*, 1953). This popularity was because of its successful adaptation to the then barren, dry calcareous soils, where it grew well for as long as 3 to 20 years without manuring (Stebler and Schroter, 1889; Davies, 1960). Owing to such extraordinary qualities, the French named it as sainfoin (formerly written incorrectly as Saint Foin), meaning wholesome hay (Clark and Malte, 1913).

However, after the 1920's, the popularity of sainfoin steadily declined, with sainfoin acreages being considerably reduced in the continent (Smith, 1976) as well as in England (Hutchinson, 1965; Bland, 1971). This decline was due to the introduction of improved lucerne and clover cultivars that are higher-yielding and more adaptable to a wider range of soils and climate (Rogers, 1975; Smith, 1976), and due to the changing agricultural ststem that requires intensive application of nitrogen fertilizers instead of lower-yielding legumes (Hutchinson, 1965; Aldrich, 1974). In Europe today, therefore, the chief sainfoin-growing areas are restricted to France, Italy, the Soviet Union and Romania (Fortune and Withers, 1980). However, as a result of the escalating cost of nitrogen fertilizers in the last decade, interest in sainfoin has been renewed in recent years (Sheehy and Popple, 1981).

In North America, sainfoin cultivation and evaluation were carried out on many farms and experimental stations in the nineteenth century, but it never attained agricultural importance (Piper, 1924; Jensen and Sharpe, 1968). In the 1960s, however, there was renewed

interest in this species in both the United States (Eslick, 1968) and Canada (Hanna, 1968). This is because of its potential in dry calcareous soils (Dubbs, 1968; Roath, 1968; Ryerson and Taylor, 1968), its high palatability (Hanna and Smoliak, 1968; Jensen *et al*, 1968), its non-bloating characteristics (Krall, 1968; Cooper *et al*, 1968a), its resistance to alfalfa (lucerne) weevil (*Hypera postica* Gyll.) (Carleton *et al*, 1968b; Wallace, 1968) and most of all, the availability of genotypes well-adapted to the adverse winters (Eslick, 1965; Carleton, 1968; Hanna, 1968; Cooper *et al*, 1968b). With the release of improved cultivars such as Eski in 1964 (Eslick *et al*, 1967), Melrose in 1969 (Hanna *et al*, 1970), Remont in 1971 (Carleton and Delaney, 1972) and Nova in 1980 (Hanna, 1980), sainfoin cultivation is becoming profitable and important in North America.

3.22 Botanical Description

3.221 Morphology. Sainfoin is a deep-rooted perennial, with a soil-level crown which produces a number of hollow stems that grow up to about 80 cm high. The leaves are borne on long petioles and are imparipinnately compound, with 7 to 35 oblong-obvate and entire leaflets (Thomson, 1951a; Smith, 1972). The inflorescences are conspicuous, 5 to 13 cm long racemes (Piper, 1924), terminating on long, slender peduncles (Percival, 1921). Each raceme consists of 5 to 80 rose-pink florets (Carleton and Weisner, 1968). The pods formed are single-seeded, indehiscent, slightly compressed, with reticulate ridges (Stebler and Schroter, 1889). Hulled seeds are kidney-shaped, yellowish-green to brown or black, and weigh about 15g per 1 000 seeds (Percival, 1921; Thomson, 1951b).

The root system is extensive with a strong woody tap root which has several large branches and numerous fine laterals (Masaudilov, 1958). The tap root usually penetrates 1 to 2 m down the soil profile, but occasionally reaches to 10 m deep (Andreev, 1963). The nodules formed are white-orange and are found mainly on the fine lateral roots (Smith, 1972). They are large and characteristically branched, but when under-developed may be merely prolate, ovoid, elongate, or lobed, with apical meristems and, therefore, are classified as astragaloid nodules (Corby, 1981).

3.222 Growth pattern. Broadly, two types of sainfoin are

recognised (Rees, 1928; Thomson, 1938). One is a single-cut group, with greater stem production and late flowering, and with a relatively slow regrowth (e.g. variety *communis* Ahlef., cultivar Eski, Melrose and Nova). The other is a double-cut (or multi-cut) group, with lower stem production and early flowering, and with a fairly quick regrowth (e.g. variety *bifera* Hort., cultivar Remont, Fakir and Othello).

Both types of sainfoin exhibit three similar phases of growth and development in their seeding year. The first phase is germination which is described as phaneroepigeal (Duke and Polhill, 1981), with the thick, fleshy cotyledons turning green and opening above the soil surface (Thomson, 1938). Delayed germination of unhulled sainfoin seeds has been indicated in several early tests (Rees, 1931, 1933; Thomson, 1938). In 1952, Thomson explained that it was probably due to mechanical resistance of seed pod to radicle emergence and not due to a water-soluble inhibitor in the seed pod. However, this explanation was refuted by Cavazza (1952), Carleton *et al*, (1968a), and Smith (1979), who all found that at least one type of water-soluble inhibitor was present.

The second phase is associated with the formation of a rosette of about 6 leaves close to the ground (Percival, 1921; Thomson, 1938). The first leaf may be unifoliate, bifoliate, trifoliate, or even pentafoliate, with the unifoliate form being predominant (Thomson, 1938). Cooper (1974) has noted differences in leaf area among the various types of first leaf, but no associated long-term effect upon photosynthesis and growth. The cotyledon may remain green to yellowish-green throughout this phase of growth. However, the major photosynthetic contribution from the cotyledons occurs during the unfolding and expansion of the first leaf, with a 100% contribution when the cotyledons are just unfolded, 54% when the first leaf is unfolded, 39% when the first leaf is fully expanded, and only 18% when the second leaf is unfolded (Cooper and Fransen, 1974). The same authors showed also that cotyledon food reserves do not contribute significantly to sainfoin growth during this phase of development as compared to germination. The positive correlation obtained, therefore, between seed size (in the range of 13.8 to 19.5 mg seed $^{-1}$) and seedling performance (Carleton and Cooper, 1972) is due possibly to the greater contribution of cotyledon food reserves from larger seeds during germination, and the greater photosynthesis from larger cotyledon area during the early second phase

of growth. In addition to seed size, this phase of growth depends also on the inherent vigour of the sainfoin seedling (Cooper, 1977).

The third phase of growth begins soon after the formation of the sixth leaf, that is, when lateral buds start to develop (Thomson, 1938). These buds produce numerous leaves, with their internodes normally short initially, but which elongate later and climax in the development of inflorescences. According to Rees (1931), the amount of flowering in the seeding year is variable and depends on the type of sainfoin and time of sowing. Double-but sainfoin commonly produces elongated stems and flowers two to five times after each cutting in its first growth season. However, single-cut sainfoin, when sown in spring, does not produce elongated stems and inflorences until the second season (Thomson, 1951a; Badoux, 1965) when vernalization and photoperiodic requirements have been satisfied (Sheely, 1977).

3.23 Some Agricultural Characteristics

3.231 Adaptive characteristics. Sainfoin is well-known to perform favourably in marginal agricultural lands having a dry climate and poor, calcareous soils. Besides being tolerant to drought, limestone and low fertility, it is reported also to exhibit tolerance to frost, steep country, salt, high pH and grazing (Duke, 1980). Space here permits only the discussion of its adaptation to drought and high pH including limestone.

Although areas with an annual precipitation of 400 to 800 mm are recommended for sainfoin growing, it also shows tolerance to an annual rainfall of up to 1 200 mm (Duke, 1980), provided that the soil is well-drained (Mansfield, 1945). Two species, *Onobrychis echidna* Lips. and *O. cornita* (L.) Desv. from central Asia, have been described as xerophytes (Kul'tiasov, 1961, 1962), but not sainfoin per se. However, it might well be classified as a xerophyte because of its semi-arid habitat, deep extensive root system, and typically small, thick and hairy leaflets. A cross-section of each leaflet reveals the characteristic thick article, thickened epidermal cell walls, and water-bearing cell layer under the epidermis (Dalenvoa, 1961). Such xeromorphic features intimate that sainfoin seems to possess both avoidance and tolerance mechanisms like many arid and semi-arid plants (Chabot and Bunce, 1979), but these mechanisms are yet to be

elucidated. Avoidance is possibly more important than tolerance because of its ability to maintain a relatively high and constant leaf water potential at greater than -400 kPa throughout its growth period (Sheehy *et al*, 1978; Sheehy and Popple, 1981).

Sainfoin is a calcicole, but it is tolerant to a relatively wide pH range of 5.3 to 8.2 (Duke, 1980). Information on pH effects and the physiology of this species is limited. Crop failure at pH below 5.3 is due probably to the high concentration of injurious hydrogen ions (Moore, 1974), toxic aluminium ions (Ronson, 1958, 1965) and toxic manganese ions (Murray, 1968), low availability of calcium and magnesium ions, phosphates and molybdates (Donahue *et al*, 1977), or a combination of these factors. At pH greater than 8.2, other ions such as those of iron, manganese, zinc, copper and boron exist in low concentrations in the soil solution and, thus, the related deficiencies may develop (Donahue *et al*, 1977).

3.232 Herbage quality. The nutritional qualities of sainfoin herbage include its non-bloating property, and relatively high palatability, digestibility, and protein and mineral content.

The non-bloating characteristic of sainfoin forage when grazed by or fed to ruminants is an exceedingly important quality. Bloat is caused by the generation of a stable foam which traps the fermented gaseous products in the reticulo-rumen, leading to a rise in pressure in the rumen (Reid and Johns, 1957; Reid, 1960). Although a number of factors contribute to formation of this foam (Clarke and Reid, 1974), soluble plant protein appears to be a major foaming agent (Jones *et al*, 1970). The anti-bloating characteristics of sainfoin herbage are attributed to the presence of certain compounds in the leaves (Cooper *et al*, 1966), later identified as condensed tannins (Kendall, 1966; Jones *et al*, 1973) which inhibit the production of stable foam in the rumen (Reid *et al*, 1974). Gutek *et al* (1974) found large quantities of condensed tannins in sainfoin in all seasons and at all stages of growth and thus established the persistence of this non-bloating property of sainfoin.

In addition to the prevention of foam generation, condensed tannins in sainfoin can slow down deamination of proteins in the rumen, thereby increasing nitrogen assimilation (Reid *et al*, 1974). Plant tannins have been shown in many experiments to be toxic to

animals, but no evidence has been collected for sainfoin tannins (Krall, 1968) or other forage tannins (McLeod, 1974).

There is evidence that tannins are secondary metabolic compounds that are "distasteful" and do inhibit the action of proteases (Harborne, 1977). Such properties have been found to deter herbivores. For instance, the presence of condensed tannins, possibly procyanidin, (Sakar *et al*, 1976), in sericea (*Lespedeza sericea* Benth.) was suggested as the cause of its low palatability (Donelly and Anthony, 1969) and poor digestibility (Cope and Burns, 1971). However, such problems are not found in sainfoin due probably to the absence of procyanidin in the flavolan composition (Sarkar *et al*, 1976). The intake of sainfoin herbage by sheep (Raymond, 1966; Osbourn *et al*, 1966; Hanna and Smoliak, 1968) and cattle (Jensen *et al*, 1968; Ulyatt *et al*, 1977) respecitvely was higher or at least similar to that of lucerne (*Medicago sativa* L.), a recognised nutritious forage legume, indicating that the palatability of sainfoin herbage is better or equivalent to that of lucerne herbage. In another study involving Hereford cattle, the digestible dry matter percentage of sainfoin was as high as that of lucerne (Jensen *et al*, 1968). In addition, greater liveweight gains were obtained with steers grazed on sainfoin than on a mixture of white clover (*Trifolium repens* L.) and tall fescue (*Festuca arundinacea* Schreb.) or a mixture of bromegrass (*Bromus inermis* Leyss.) and cocksfoot (*Dactylis glomerata* L.) (Krall, 1968).

2.233 Herbage yield. Dry matter yield of sainfoin is highly variable, depending on the climate, soils, management, age of stand and cultivar. In its indigenous mountains of the Caucasus in Azerbadjan, the Soviet Union, Mamedov and Aliev (1972) recorded an annual yield of 6 000 kg DM ha^{-1} on level plots, 4 050 kg DM ha^{-1} on northern slopes, and 3 600 kg DM ha^{-1} on southern slopes. On a sloping eroded land in Romania, sainfoin yielded 5 400 kg DM ha^{-1} $year^{-1}$ (Popa, 1976). In Italy, a dry matter yield of 9 380 kg ha^{-1} $year^{-1}$ was reported (Covarelli, 1975). In England, Cotswold Common yielded 11 000 kg DM ha^{-1} $year^{-1}$ in three cuttings in both the first and second harvest year (Smith, 1972). In a trial using monocultures or mixtures of Eski and Remont in the United States, Eski and Remont respectively contributed 9 450 and 9 590 kg DM ha^{-1} $year^{-1}$ in two cuts in the second year (Cooper, 1972). In Canada, however, Nova, Melrose, Remont, and Eski respectively produced up to 14 600, 13 600, 13 500, and 13 500 kg DM

ha^{-1} $year^{-1}$ in 3 cuts in the second season (Hanna, 1980).

When comparing with two other important forage legumes, lucerne and red clover, the herbage yield of sainfoin is generally lower (Cooper and Roath, 1965; Hanna and Smoliak, 1968; Smith, 1972; Rogers, 1975). However, in conditions suitable to sainfoin, it can outyield lucerne or red clover (Roath, 1968; Carleton *et al*, 1968; Smoliak and Hanna, 1975). Since the improved cultivars such as Eski, Melrose, Remont and Nova have shown potential in dry matter yeild, the selection and breeding for suitable high-yielding cultivars is, therefore, a major priority in many countries including New Zealand.

3.24 Suitability in New Zealand Conditions

3.241 Early introduction and evaluation. In New Zealand, a series of studies on the causes and control of bloat in cattle that graze on clovers and lucerne (Johns, 1954; Reid and Johns, 1957; Mangan and Johns, 1957; Reid, 1958; Mangan *et al*, 1959; Reid *et al*, 1961) has generated considerable interest in non-bloating legume species including sainfoin (Jones *et al*, 1970; Jones and Lyttleton, 1971; Jones *et al*, 1973; Ross and Jones, 1974; Wright and Reid, 1974). Introduced into New Zealand in the early 1970s, it was evaluated for its non-bloating property and the associated nutritional qualities (Special Correspondent, 1974). Excellent results were obtained with both sheep and cattle and, thus, it was recommended as a potential nutritive forage legume in New Zealand (Reid *et al*, 1974; Derrick, 1977; Ulyatt *et al*, 1977).

In the following years, the agronomic characteristics of sainfoin have been studied at the Ministry of Agriculture and Fisheries in Rotorua, and at the Grassland Division of the Department of Scientific and Industrial Research (DSIR), Palmerston North, involving the Agronomy Department at Massey University. Currently in DSIR, a breeding programme, aiming to select a cultivar better suited to New Zealand is also underway (W. Rumball, personal communication).

3.242 Diversity of genetic potential. The indigenous habitats of sainfoin are related to the semi-arid and sub-humid Mediterranean climatic zones (between 30 to 40° latitude), with an average rainfall of between 400 and 600 mm, and 600 and 800 mm respectively (Le Houeroa, 1977). The winter is wet, particularly in December and January, and

cool (-5 to 15°C), while the summer is dry and hot (30 to 40°C). In these climatic regions, sainfoin is known to exist in a range of habitats from the lowland to the substeppe zone, up to an elevation of about 2 000 m (Kernick, 1978). It is dominant in calcareous pasture lands (Le Houeroa, 1977) and in marls (Kernick, 1978).

Since its spread into the Soviet Union and Europe, sainfoin, like many other legumes, displays a wider adaptation to diversity in environmental conditions (Adams and Pipoly III, 1980). A number of different ecotypes of sainfoin have been collected from many geographical locations especially from its indigenous habitats (Llovet, 1963-1965; Sinskaya, 1958; Hanna, 1968, 1980). This wide biological variation suggests that it is possible to select and fit the specific requirements and desired performance of certain ecotypes to the specific resources of the New Zealand environment.

3.243 Sainfoin and the New Zealand environment. Much of the cultivated land in the North Island and east of the South Island lies between 37 and 45° latitudes, and experiences a Mediterranean-like climate, characterised by a wet and cool winter, and a dry and warm summer (Mitchell, 1963). The mean annual rainfall in these regions is generally between 380 to 1 520 mm with maximum precipitation occurring in the winter (New Zealand Meteorological Service, 1959). The average annual maximum and minimum temperature is -5 and 30°C respectively. These regions include areas in Gisborne, Hawke's Bay, Central Plateau, Manawatu, Wairarapa, Canterbury and Central Otago.

The period which limits pasture production most in the above-mentioned regions is during the summer and autumn, when a serious water deficit is compounded by the shallow, less vigorous root systems of pasture species and high surface soil temperatures (Mitchell, 1963). As much as 40% reduction in potential pasture production between October and April in Palmerston North was estimated (Brougham, 1966). Consequently, certain perennial Mediterranean plant species, having the characteristics of a vigorous, deep root system, low growing meristems, greater stem production and regrowth capacity, smaller leaves, tall vegetative structure of 1 to 2 m high, and good digestability and nutritional value were proposed as desirable forage plants for these regions (Mitchell, 1960,1963,1966). Some cultivars and ecotypes of sainfoin seem to satisfy most of these requirements.

Considering this information, sainfoin cultivation may be profitable in these regions especially the well-drained soils, but liming will be necessary also in many areas.

At present, there is limited quantitative data available for critical assessment of the suitability of sainfoin in these regions. The only published research so far was conducted by Percival and McQueen (1980), between 1976 and 1980, on the pumice soils in the Central Plateau. The report reveals that in the overall 5 years, the annual dry matter yield of the cultivar Melrose was 8 500 kg ha^{-1}, similar to the dry matter yield of pasture, but much lower than the 12 700 kg ha^{-1} of lucerne. Future research endeavour should be directed to investigating more cultivars and ecotypes at various places in both North and South Island, and the nature of their growth responses.

At this moment, therefore, sainfoin appears to have a specific role in the New Zealand agriculture as a specialised forage for certain drier and well-drained areas due mainly to its drought resistance, its nutritive merits, and its resistance to pests that seriously affect lucerne such as the blue-green lucerne aphid (*Acyrthosiphon kondoi* Shinji), pea aphid (*Acyrthosiphon pisum* (Harris)), and spotted alfalfa aphid (*Therioaphis trifolii* fm maculata) (Lance, 1980). The spotted alfalfa aphid is recently reported to have arrived in New Zealand. Scott (1979) calculated that sainfoin growing for bloat control is less economical than lucerne growing plus the existing bloat control methods, but this financial analysis must be reserved for a later reconsideration, together with further yield information and other findings, of the economics of sainfoin cultivation.

3.3 DINITROGEN-FIXING SYSTEM OF LEGUMES

3.31 Development of the Legume-*Rhizobium* System

A number of authors, including the more recent ones (Dart, 1975, 1977; Pate, 1977a; Bergersen, 1978; Schmidt, 1978,1979; Dazzo, 1980; Vincent, 1980; Bauer, 1981), have reviewed comprehensively the sequence of interactive events from recognition to dinitrogen fixation between legumes and their respective *Rhizobium* spp. In these reviews, the information is derived from work done mainly on the major agricultural

legumes such as pea, soybean, dwarf bean, lucerne, clovers and lupins, but similar observations are expected to occur in other legumes. Here, a short discussion on the three generally recognised phases of interaction is presented in relation, as much as possible, to sainfoin.

3.311 Recognition. In this first phase of interaction, the appropriate strain or species of bacterium, specifically (Rovira, 1961; Robinson, 1967; Munns, 1968; van Egeraat, 1975) or non-specifically (Peters and Alexander, 1966) stimulated by root exudates, attach to the surface of root hairs perpendicularly (Sahlman and Fåhraeus, 1963; Marshall *et al*, 1975; Dazzo and Hubbell, 1975). Using pea seedlings, van Egeraat (1978) detected a large quantity of a root-tip exudate, γ-L-glutamyl-D-alanine, which inhibited the growth of *Rhizobium leguminosanum* strain PRE before the lateral roots emerged. However, when the lateral roots were emerging, they exudated also substantial amounts of homoserine which selectively stimulated rhizobia growth, while the exudation of another compound called 2-alanyl-isoxazolin-5-one appeared to diminish the inhibiting action of γ-L-glutamyl-D-alanine. The mechanism or mechanisms of recognition is still under much speculation although lately, certain legume proteins, called lectins (carbohydrate-binding glycoproteins) (Hamblin and Kent, 1973; Bohlool and Schmidt, 1974; Dazzo and Hubbell, 1975), are postulated to bind with the polysaccharide produced by the bateria (Wolpert and Albersheim, 1976; Dazzo and Brill, 1977; Calvert *et al*, 1978; Zevenhuizen *et al*, 1980). Firm adherence to the root hair surface at later stages of recognition is provided by the production of extracellular cellulose microfibrils (Napoli *et al*, 1975).

The first visible effect of recognition is root hair deformation, characterized by swelling, curling and branching of the hairs often leading to the formation of shepherd's crooks (Fåhraeus and Ljuggren, 1968). Root hair deformation is caused by a partially dialyzable, heat-stable, undefined substance (Li and Hubbell, 1969; Hubbell, 1970; Yao and Vincent, 1969,1976), containing a nucleic acid and a protein or polysaccharide (Solheim and Raa, 1973), produced by the bacteria.

No study has been done on sainfoin in the recognition phase at the cellular level but sainfoin rhizobia have been found to secrete polysaccharides (Nalbandyan and Sayadyan, 1977), while a lectin has been isolated from sainfoin seeds and young roots (Hapner and Robbins,

1979). Early inoculation studies suggested that sainfoin bacteria were strain-specific (Allen and Allen, 1981). However, in more recent experiments, bacterial isolates from sweetvetch (*Hedysarum* spp.) and crownvetch (*Coronilla* spp.) can also infect and nodulate sainfoin (Burton and Curley, 1968; van Schreven, 1972).

3.312 Infection. Infection begins when infection threads are initiated in deformed root hairs by way of invagination of the primary walls (Dart, 1977). Three mechanisms of threat initiation have been proposed. The first hypothesis suggests that thread initiation may be caused by inward cell wall synthesis induced by impediments to normal wall growth in curled root hairs (Nutman, 1956). According to the second hypothesis, bacterial penetration is probably enhanced by cell wall softening, induced by host polygalacturonase (Ljuggren and Fåhraeus, 1961). The third hypothesis deduces that thread initiation may be due to direct infection by small, multi-flagellated coccoid rhizobia, called swarmers, through gaps in the cellulose microfibrils of the root hair cell wall (Dart and Mercer, 1964).

The infection thread, once initiated, progresses towards the inner root cortex by a coordinated bacterial division and cell wall deposition along the thread (Dart, 1974,1977; Sprent, 1979). While passing through the cortex, it also branches and ramifies. The nuclei of cortical cells enlarge and cells adjacent to the threads may divide. Cortical cell division is probably stimulated by indole-acetic acid (IAA) and cytokinin, secreted by the bacteria enclosed in the threads (Libbenga *et al*, 1973; Libbenga and Torrey, 1973; Liggenga and Bogers, 1974).

Dangeard (1926) has observed some infection threads in sainfoin nodules, while Hume (1981) has related the greater number of nodules to the abundance of root hairs. These results indicate that, as in most legumes (Dart, 1974,1977), infection by rhizobia in sainfoin is probably via root hairs.

3.313 Nodulation. After the infection thread passes through the outer six layers of cortical cells, the inner cortical cells show increased RNA contents and subsequently divide to form a nodule primordium which later become infected by the advancing infection thread (Sprent, 1979). This young primordium then loses its meristematic activity, but the adjacent cells become meristematic and form the nodule meristem which divides repeatedly and emerges from the root (Libbenga and Bogers, 1974). Cell division is probably stimulated by the cyto-

kinins in the meristems (Syōno *et al*, 1976). Meanwhile, back in the non-meristematic zone, bacteria are released from the infection thread tips as membrane droplets (Kijne, 1975; Newcomb, 1975; Bassett *et al*, 1977), later freed and dispersed into the host cells (Robertson *et al*, 1978). They multiply and enlarge progressively, and differentiate into dinitrogen-fixing bacteroids, enclosed in membrane envelopes of the host origin (Bergersen, 1974; Bassett *et al*, 1977). Nodulation is completed when leghaemoglobin, vascular tissues and nodule cortex are developed (Appleby, 1974; Bergersen, 1974,1978).

With an effective inoculum, sainfoin nodule swellings can be detected between 14 to 30 days after inoculation (Dangeard, 1926; Karpov, 1957; Hume, 1981). At this stage, cell divisions and nodule primordia had occurred (Dangeard, 1926). Within 3 to 4 days, the swellings mature into nodules (Hume, 1981), containing bacteroids that are spherical, 1 to 5 μm in diameter, and which sometimes occur in pairs (Dangeard, 1926). Koter (1965) reported that a small amount of combined nitrogen stimulated nodulation and dinitrogen fixation, but a large quantity decreased nodule number in sainfoin. Recently, Hume (1981) showed that in the presence of 210 μg N cm^{-3} solution as sodium nitrate, the number and dry weight of nodules, as well as leghaemoglobin production were significantly suppressed. Other nutritional disorders such as massive accumulations of calcium carbonate in the roots (Ross and Delaney, 1977) may also depress nodulation and dinitrogen fixation. In general, however, sainfoin (Burton and Curley, 1968) and other *Onobrychis* spp. (Hely and Ofer, 1972) are reasonably nodulated in the presence of an effective inoculum, with the nodules formed being larger than those in lucerne (Karpov, 1957).

3.32 Dinitrogen Fixation

3.321 Physiology and biochemistry. The present concept of the physiology and biochemistry of dinitrogen fixation in legume nodules is a synthesis of research results mainly from soybean, lupins, pea and seradella (*Ornithopus sativus* Brot.), but applicable to other legume species (Bergersen, 1980a).

Dintrogen fixation in legume nodules is under rigid genetic control (Whiting and Dilworth, 1974; Page, 1978) and catalysed by the nitrogenase enzyme system (Kennedy *et al*, 1966; Bergersen and Turner, 1967; Koch *et al*, 1967 in the bacteroids. The nitrogenase enzyme

system, consisting of two protein components, a heavier molybdenum-iron protein called component I and a lighter iron protein called component II (Brill, 1977; Eady *et al*, 1980), catalyses a series of complex reductive reactions in which dinitrogen is ultimately reduced to ammonium ions (Evans and Russell, 1971; Eady and Postgate, 1974; Stiefel, 1977; Chatt, 1980) and protons are reduced to hydrogen gas (Bulen *et al*, 1965; Schubert and Evans, 1976,1977; Evans *et al*, 1980).

Energy, required in large quantity in the fixation process, is derived from the oxidation of photosynthetic products, whose forms are yet to be established (Bergersen, 1978,1980a). The oxidation of these products generates reducing power which is used for the production of ATP in oxidative pathways (Appleby *et al*, 1975) and for the generation of reductants for the nitrogenase system (Evans and Philips, 1975). In the bacteroids of certain rhizobia strains, another enzyme called hydrogenase is present and oxidizes the generated hydrogen gas to protons and hence, recovering these reductants and possibly synthesizing ATP as well (Dixon, 1972; Schubert and Evans, 1976,1977; Schubert *et al*, 1977; Schubert and Ryle, 1980). Oxygen, also required in the fixation process (Bergersen, 1974,1977), is transported to active sites through facilitated-diffusion by leghaemoglobin, thereby preventing the inactivation of the nitrogenase system (Bergersen, 1980b).

The ammonium ions reduced are transported to and assimilated in the nodule host cells (Bergersen, 1980a). The assimilatory pathways and, thus, assimilatory products vary among species. In general, the final assimilatory products for xylem transport later in temperate legumes are primarily amides (Pate, 1977b, 1980), while in tropical legumes these are mainly ureides (Pate *et al*, 1980).

Dinitrogen fixation in sainfoin, as in all other legumes, follows an exponential increase with time during the vegetative growth phase (Hume, 1981). There is as yet no published report on its rate of fixation during the reproductive growth phase, but dinitrogen fixation is expected to decline rapidly from anthesis. At similar growth stages, the fixation in sainfoin is generally lower compared with that in the more improved legumes such as pea (Phillips *et al*, 1976), subterranean clover (Eskart and Raguse, 1980), soybean (Nelson and Weaver, 1980; Cassman *et al*, 1980), lucerne (Sheehy *et al*, 1980; Craig *et al*, 1981; Hersman *et al*, 1981), red clover (Craig *et al*, 1981), and cowpea

(Zablotowicz *et al*, 1981).

3.322 Energetics. From various experiments involving legumes, the evidence obtained strongly indicates that the major limitation in dinitrogen fixation of all the factors investigated is the energy source, photosynthate, available to the nodules (Hardy and Barelka, 1977; Hardy, 1977). However, other factors must also be considered because of their potential adverse effects on plant growth and photosynthesis and, thus, on dinitrogen fixation (Hardy, 1977, 1980a; Andersen and Shanmugam, 1977; Andersen *et al*, 1981).

Measurements on the time course of dinitrogen fixation for field grown soybean (Hardy *et al*, 1968; Weber *et al*, 1971; Ham *et al*, 1976), pea (LaRue and Kurz, 1973), dwarf bean (Bethlenfalvay and Phillips, 1977; Graham and Halliday, 1977), cowpea (*Vigna unguiculata* (L.) Walp.) (Herridge and Pate, 1977) and white lupin (*Lupinus albus* L.) (Pate and Herridge, 1978) show a general exponential increase until pod-filling after which there is a rapid decrease. These results demonstrate that photosynthate is a limiting factor in dinitrogen fixation, when the demand for photosynthate by reproductive organ becomes large. Indirectly, enrichment of carbon dioxide to the canopy of field-grown legumes from pod-filling to senescence also increases dinitrogen fixation (Hardy and Havelka, 1975,1977; Havelka and Hardy, 1976) by reducing photorespiration and increasing photosynthate available to the nodules. Similarly, photosynthesis and dinitrogen fixation are increased by supplementing light intensity (Lawn and Brun, 1974) or by grafting a second shoot on a rootstock (Streeter, 1974), while they are decreased by reducing light intensity or shading (Hardy *et al*, 1968; Chu and Robertson, 1974; Sloger *et al*, 1975) and defoliation (Hardy *et al*, 1968; Moustafa *et al*, 1969; Chu and Robertson, 1974). In a more direct study involving the measurement of soluble carbohydrate, Graham and Halliday (1977) found a strong positive correlation between acetylene reduction and soluble nodule carbohydrate in *Phaseolus* spp. Phillips (1981), in a more recent experiment, showed that nitrogen was the chief limiting factor during the early growth stages in soybean. It appears, therefore, that carbon is a limiting factor at the later growth stages when the dinitrogen-fixing system has greatly developed and is more active in fixation.

Various workers have estimated the energy cost of dinitrogen

fixation in whole plant studies using different techniques which produced striking differences in results (Phillips, 1980; Schubert and Ryle, 1980). However, measurements based on intact root systems can be taken with reasonable confidence. Three such experiments involving intact root systems are summarised by Pate and Minchin (1980) (Table 3.1). In the table, both the carbon consumed-nitrogen fixed ratio and percentage of net photosynthate utilized by nodules vary considerably probably due mainly to differences in plant species, rhizobia strain and length of growth period. The net photosynthate utilized by nodules ranges from 10 to 32% and, thus, suggests that dinitrogen fixation is an energy-intensive process (Schubert and Ryle, 1980). Although the three species show large differences in the quantities of carbon used in the export of fixed nitrogen, in nodule respiration and in the accumulation of nodule dry matter, the proportion of consumed carbon used in these three activities are similar. Between 48 to 52%, 36 to 40% and 9 to 16% of the consumed carbon are used in the export of fixed nitrogen from the nodules, in nodule respiration lost and in accumulation of nodule dry matter respectively. These variations among species may be explained by the differences in nitrogen-metabolic pathways, and the complex relationships in dinitrogen fixation, hydrogen gas evolution and hydrogenase activity (Pate and Minchin, 1980).

Cellular estimates of the energetics of the fixation process *in vivo* are based on the stoichiometry of ATP hydrolysis using a reductant and purified nitrogenase system. The overall reaction (equation 3) is given by the reduction of dinitrogen (equation 1) and reduction of protons (equation 2) as shown below (Phillips, 1980):-

1	$N_2 + 12ATP + 6\bar{e} + 8H^+ \longrightarrow 2NH_4^+ + 12ADP + 12P_i$	
2	$+ 4ATP + 2\bar{e} + 2H^+ \longrightarrow H_2 + 4ADP + 4P_i$	
3	$N_2 + 16ATP + 8\bar{e} + 10H^+ \longrightarrow 2NH_4^+ + H_2 + 16ADP + 16P_i$	

Although 16 mole ATP mole $^{-1}$ N_2 fixed is the total energy involved in the above stoichiometry, between 12 to 24 mole ATP mole^{-1} N_2 fixed is the commonly cited range (Brill, 1977,1980), depending on the reaction conditions (Schubert and Ryle, 1980). The ATP-ADP ratio in nodules has been shown to relate directly to dinitrogen fixation (Appleby *et al*, 1975; Ching *et al*, 1975).

TABLE 3.1

CARBON AND NITROGEN IN NODULES OF THREE LEGUMES
(AFTER PATE AND MINCHIN, 1980)

	Cowpea	White lupin	Garden pea
Period of growth (days)	0 - 78	0 - 94	21 - 30
N_2 fixed (mg plant^{-1})	726	788	27
C used in export of fixed N from nodules (mg plant^{-1})	969 (48%)	1789 (52%)	53 (48%)
C lost as CO_2 in nodule respiration (mg plant^{-1})	789 (39%)	1372 (40%)	40 (36%)
C incorporated into nodule dry matter (mg plant^{-1})	253 (13%)	298 (9%)	18 (16%)
Total C consumed by nodules (mg plant^{-1})	2011 (100%)	3459 (100%)	122 (100%)
Plant net photosynthate utilized by nodules (%)	2.8 10.2	4.4 13.0	4.1 32.0

Sainfoin, growing in a controlled environment (Burton and Curley, 1968) and in the field (Sims *et al*, 1968), has been reported to develop symptoms of nitrogen deficiency although the plants were well-nodulated. The deficiency was readily corrected with supplementary combined nitrogen (Sims *et al*, 1968). In a more recent study, Hume (1981) found that sainfoin plants depending solely on the dinitrogen fixed in the nodules were capable of fixing only approximately 43% of the amount of nitrogen required. When comparing these plants with those that were supplied with combined nitrogen, their relative growth rates were lower than the latter. It is, therefore, evident that dinitrogen fixation in sainfoin is relatively inefficient due probably to inefficient strains of rhizobia (Burton and Curley, 1968) and inadequate supply of energy from photosynthesis (Hume, 1981).

3.33 Methods of Improving Dinitrogen Fixation

The many possibilities of enhancing dinitrogen fixation in legumes have been examined by Evans (1975), Brown *et al*, (1975), Postgate (1977,1978), Hardy and Havelka (1977), and Subba Rao (1980). The principal, short-term method is by ameliorating the efficiency of energy consumption by the nodules. Theoretically and to a certain extent, practically, this is achieved when the energy supply to the nodules is increased, and the energy wastage in hydrogen evolution is reduced (Figure 3.1).

3.331 Photosynthesis. As discussed earlier, dinitrogen fixation is strongly dependent on photosynthesis. This was undoubtedly demonstrated in experiments in which dinitrogen fixation was greatly enhanced by light supplement, carbon dioxide enrichment and shoot grafting on a rootstock. Although these methods are not practical in extensive agricultural production systems, the underlying principle is the same, that is, to create an efficient photosynthetic system (Hardy, 1977). This can be achieved through plant breeding involving three major approaches -(1) optimization of the canopy structure, primarily to attain an optimal leaf area index and better canopy architecture to increase light interception and absorption (Nasyrov, 1978; Gifford and Evans, 1981), (2) improvement of efficiency of the photosynthetic process, with minimal photorespiration and dark respiration to reduce energy lost (Zelitch, 1971; Postgate, 1977; Tolbert,

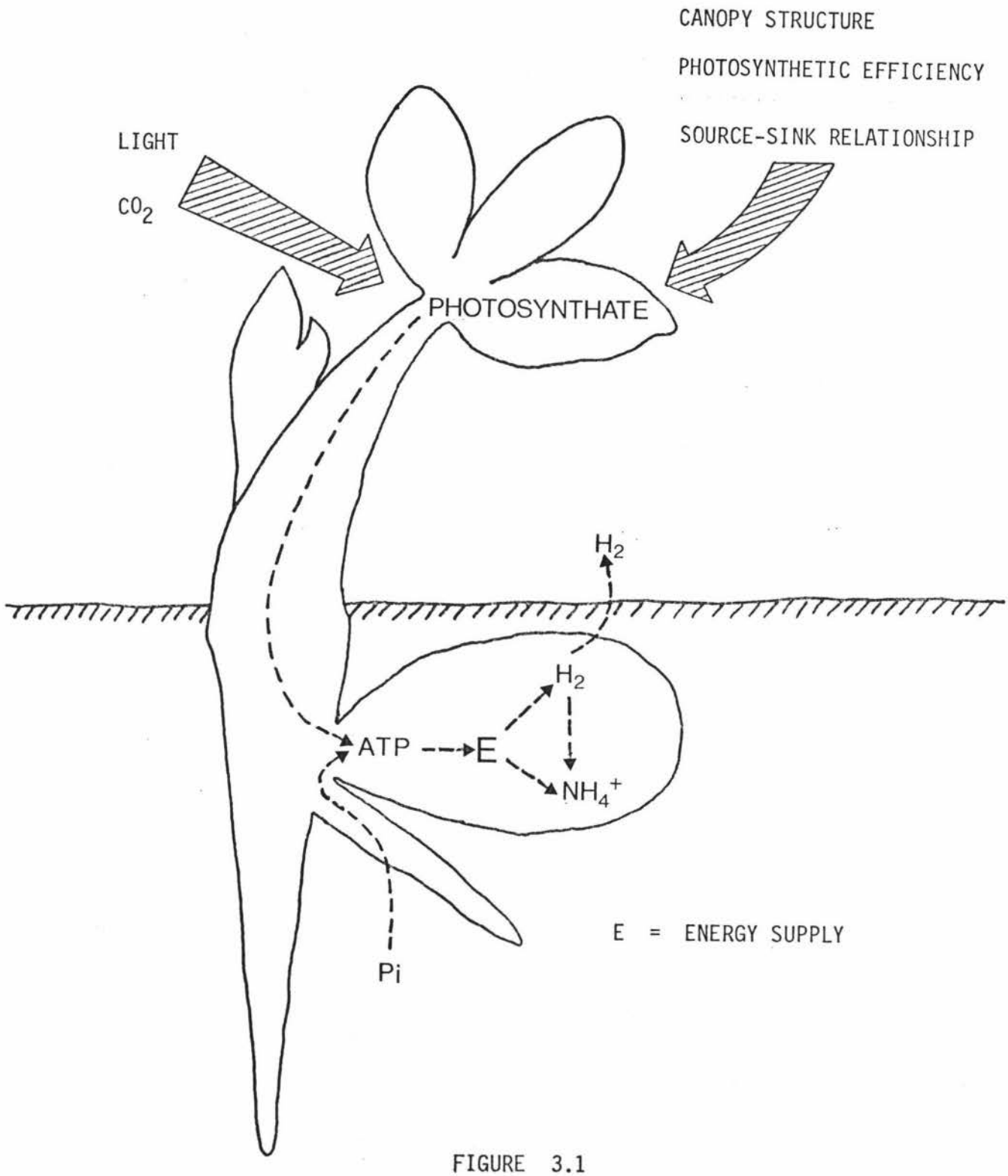


FIGURE 3.1

FACTORS AFFECTING THE ENERGY SUPPLY FOR DINITROGEN FIXATION IN A LEGUME NODULE

1977), and (3) rational partition and usage of assimilates for maximum expression of yield (Nasyrov, 1978; Gifford and Evans, 1981) and, thus, in the case of forage legumes the shoot dry weight.

3.332 Phosphate supply. In many experiments, nodulation and dinitrogen fixation in legumes are closely related to phosphate fertilization (van Schreven, 1958; Vincent, 1965; Gukova and Arbuzova, 1969; Gates, 1970,1974; Fillery, 1974; Andrew, 1977; Munns, 1977; Robson, 1978; Andrew and Jones, 1978; Hanson, 1978-1979). This is because of the common limiting phosphate supply from the soil solution in many soils (Dudal, 1976). Since phosphorus is a constituent of many metabolic compounds associated with dinitrogen fixation (Martin and Bushman, eds, 1978), it is very likely that phosphorus is inadequate for the synthesis of these metabolites especially ATP, the energy-yielding compound required in large quantity (Mortenson, 1966; Bullen and LeComte, 1966). Moustafa *et al*, (1971) have positively correlated the nodule ATP, ADP and AMP with the foliar-applied labelled orthophosphate in lotus major, but the relationships between inorganic soil phosphate supply and ATP and dinitrogen fixation are still to be investigated.

3.333 Hydrogenase activity. Part of the dinitrogen-fixing process in legumes is associated with the energy-wateful reduction of protons to hydrogen gas. In this reaction, up to 12 mole ATP $mole^{-1}$ N_2 fixed or an equivalent of up to 50% of the total energy used in fixation is lost (Schubert and Evans, 1976,1977). However, the bacteroids of certain rhizobia strains possess another enzyme, hydrogenase, which is capable of oxidizing the hydrogen gas into protons, thereby also synthesizing ATP. Additional evidence also shows that different legumes inoculated with the same strain of bacteria produced nodules with different levels of hydrogenase activity, indicating that host plants play a role in the exppression of nitrogenase (Dixon, 1972; Schubert and Evans, 1976,1977).

In the range of legumes surveyed, the hydrogenase activity in each legume species varies widely and, in general, this activity is greater in tropical legumes such as soybean and cowpea than in temperate legumes (Evans *et al*, 1977). In certain tests, nodules of soybean and cowpea are also capable of taking up hydrogen gas from their external surroundings in the presence of oxygen (Schubert and Evans,

1976,1977). When determining the fate of recycled energy in the soybean cultivar Anoka-*R. japonicum* strain USDA 110 system, part of the energy was channelled to dinitrogen fixation which showed a 7% increase, and the balance to dry matter accumulation which recorded a 24% increase (Schubert and Ryle, 1980). These results, therefore, show that more research ought to be conducted to identify and develop more energy-efficient systems of legume cultivars and rhizobia strains, capable of high hydrogenase activity.

3.334 Improving the dinitrogen-fixing efficiency of sainfoin. Sainfoin, being a relatively new forage crop, has not been well-selected for its dinitrogen-fixing capacity. Burton and Curley (1968) have studied the efficiency of the dinitrogen-fixing systems of various sainfoin cultivars using different rhizobia isolates, but no attempt was made to select or breed for efficient fixation. It is, therefore, apparent that such research endeavour is a requisite for further level of sainfoin yield.

The prospective areas of research in plant breeding include the screening and selection for a larger leaf area index, greater stem production, low hydrogen evolution, and more efficient rhizobia strains. Compared with lucerne, Sheehy and Popple (1981) have, in fact, demonstrated a lower rate of canopy photosynthesis in sainfoin (var. *bifera* Hort.) because of its considerably lower leaf area index. This lower leaf index as compared to lucerne was also reported in the cultivars Remont, Melrose and Fakir (J.A. Fortune, unpublished data), and Hume (1981) has found that the leaf area of Fakir was positively correlated with dinitrogen fixation ($R = 0.74$, $P < 0.001$). Currently at Massey University, an initial screening programme for cultivars with a larger leaf area index is in progress (N.J. Withers, personal communication).

3.4 MYCORRHIZA SYSTEM OF LEGUMES

3.41 Occurrence

Some 200 families and 1 000 genera of plants (Trappe and Fogel, 1977) have been identified as developing arbuscular, orchidaceous, ericoid and arbutoid mycorrhizae (Lewis, 1975). This thesis concerns only with the arbuscular type.

The phenomenon of arbuscular mycorrhizae is ubiquitous, occurring in numerous terrestrial species of the Bryophyta (liverworts, mosses and hornworts), Polypodiophyta (ferns), Pinophyta (gymnosperms),

and Magnoliophyta (angiosperms) (Gerdemann, 1968,1974,1975). They are formed by some 90 distinct taxa of non-specialized fungi (Hall and Fish, 1979; Hall, in preparation) of the Endogonaceae, a family of the Phycomycetes (Mosse, 1956; Nicolson and Gerdemann, 1968; Gerdemann, 1971).

Arbuscular mycorrhizae are of particular interest because of their widespread occurrence in numerous agricultural (Gerdemann, 1968; Mosse, 1973; Safir, 1980) and horticultural (Maronek *et al*, 1980; Hayman, 1981) crops. In the Fabaceae, they are found in most species of the two subfamilies, the Mimosoideae and the Papilionoideae (Malloch *et al*, 1980), including those in the agriculturally important genera *Arachis*, *Glycine*, *Lens*, *Lotus*, *Medicago*, *Onobrychis*, *Phaseolus*, *Pisum*, *Trifolium*, *Trigonella* and *Vicia* (Jones, 1923,1924; Peyronel, 1924; Samuel, 1926; Asai, 1944; Strzemska, 1969a,b, 1970,1973,1975b; Crush, 1975). Of the *Onobrychis* spp., sainfoin is the only one investigated (Strzemska, 1975b). Members of the third subfamily, the Caesalpinioideae, also develop arbuscular mycorrhizae except those in the tribes, Amherstieae and Detarieae; those regularly form ecto-mycorrhizae (Malloch *et al*, 1980).

3.42 Development of Arbuscular Mycorrhizae

On the basis of growth and development of the endophytes, arbuscular mycorrhiza formation is described in four phases -- recognition, infection, arbuscle and vesicle production, and extra-matrical development. All these four phases are found typically in different plant species. In the following account, therefore, data from non-legumes are also used wherever necessary.

3.421 Recognition. In the rhizosphere, spore germination and subsequent growth are stimulated by adjacent root exudates (Bevege and Bowen, 1975; Hepper and Mosse, 1975), decayed organic litter (Nicolson, 1958,1960,1963; Guttenberg, 1963; Went, 1974) particularly disintegrated roots, and the presence of other soil microorganisms (Mosse, 1956,1959,1962). Certain unknown compounds are particularly crucial as indicated in experiments involving artificial cultures, in which spore germination and subsequent fungal growth are extremely limited (Godfrey, 1957b; Mosse, 1963). The germ tubes produced from spores become the first hyphae which grow and branch, resulting in the

pre-infection mycelia (Bevege and Bowen, 1975; Hepper and Mosse, 1975).

The endophytes generally are known to infect a wide range of hosts (Gerdemann, 1955; Koch, 1961; Mosse, 1962,1963,1973,1975), but certain species are reported to exhibit some degree of specificity (Tolle, 1958). However, zero infection is less common than restricted infection, that is, "preferential" association (Hawker, 1962).

Specificity in the recognition phase is the result of root-endophyte interaction involving root exudates and possibly other physiological properties as shown in the study of Bevege and Bowen (1975). Using clover, onion (*Allium cepa* L.), common wall cress (*Arabidopsis thaliana* (L.) Heynh.), and pine (*Pinus radiata* Don.), inoculated with three arbuscular fungal species (white-reticulate spore type, *Endogone araucareae* Bevege, *Glomus mosseae* (Nicol. & Gerd.) Gerdemann & Trappe), they observed that all these endophytes developed pre-infection growth and later infected the clover and onion roots in varying degrees, depending on the endophyte species. However, these endophytes failed to infect the roots of common wall cress and pine although pre-infection mycelia were present except for the white-reticulate type which even failed to germinate in the rhizosphere of common wall cress. Despite the absence of infection in pine, *E. araucarea* formed external vesicles besides hyphae, while *G. mosseae* also developed occasional appressoria in addition to hyphae and external vesicles around the roots. These cross-inoculation results suggest that the selection of highly susceptible host cultivars and highly infective fungal species is likely to effect a more intensive infection.

3.422 Infection. Infection begins when hyphae, produced from spores (Manjunath and Bagyaraj, 1981) or infected roots (Hirrel and Gerdemann, 1979; Heap and Newman, 1980; Manjunath and Bagyaraj, 1981), attach to the epidermal or root-hair surface (Nicolson, 1959; Davidson and Christensen, 1977) and produce appressoria, from which projections penetrate the wall and form the invading hyphae (Hepper and Mosse, 1975) of 6 to 12 µm across (Jones, 1924). The penetration process is probably aided by enzymes and mechanical pressure (Kaspari, 1975). These aseptate, yellow hyphae become coarser, measuring 12 to 13 µm in diameter (Jones, 1924), and subsequently traverse and ramify the root cortex intercellularly and intracellularly (Jones, 1923, 1924; Samuel, 1925; Nicolson, 1959), developing an infection unit

(Cox and Sanders, 1974) often with coiled hyphae (Jones, 1924; Nicolson, 1959; Kinden and Brown, 1975a). The host cells react to invasion with an increase in cytoplasm and an accumulation of all organelles such as mitochondria, dictyosomes, and endoplasmic recticulum cisternae in the vicinity of the hyphae (Kaspari, 1975). Ultrastructural studies showed a continual digestion of the intracellular hyphae by the host cells (Scannerini *et al*, 1975; Kinden and Brown, 1975b), but the host plasmalemma remained morphologically and cytochemically unchanged in the interaction (Bonfante-Fasolo *et al*, 1981; Marx *et al*, 1982) in contrast to host-pathogen interactions (Littlefield and Bracker, 1972; Gil and Gay, 1977), indicating a mutualistic symbiosis instead of a true host-pathogen association.

The degree of infection spread in the cortex depends on the interaction between the host species and fungal species. Bevege and Bowen (1975) observed a very rapid infection spread in clover but a localised spread in onion for three different fungal species. Khan (1980) obtained a general infection spread in onions with *Glomus fasciculatus* (Thax. sensu Gerd.) Gerdemann & Trappe, *Glomus mosseae*, and *Glomus macrocarpus* Tul. & Tul. var. *macrocarpus*, but a localised one with *Sclerocystic rubifosmis* Gerdemann & Trappe. Generally, the spread is greater in the outer cortex than in the middle or inner cortex (Jones, 1924; Nicolson, 1959).

3.423 Arbuscle and vesicle production. In the middle and inner cortex, and to a certain extent in the outer cortex, the intercellular hyphae parallel to the root axis produce lateral branches that penetrate further cortical cells and form arbuscles (Peyronel, 1923; Jones, 1924; Nicolson, 1959; Kinden and Brown, 1975a). Each arbuscle develops within a host cell by repeated dichotomous branching of the invading hypha to produce a cluster of fine filaments (Kinden and Brown, 1975c). The host nucleus is also significantly enlarged with a prominent nucleolus (Mosse, 1963). At a later stage, the arbuscles are digested within the host cells, forming a granular mass (Kaspari, 1975; Scannerini *et al*, 1975). Arbuscular digestion is initiated at the tips of the finest branches and progresses basipetally (Kinden and Brown, 1976). These greenish-yellow arbuscles (Jones, 1923) are structurally analogous to haustoria of the fungi in the family, Peronosporaceae (Peyronel, 1923), and are probably the sites of photosynthate and nutrient exchange, and especially phosphate (Cox and Tinker, 1976; Tinker, 1978).

At the later stage of mycorrhiza formation, vesicles may develop in the middle or from the tips of hyphae, mainly in the intercellular spaces of the outer cortex where few arbuscles are present (Jones, 1924; Kinden and Brown, 1975a,b). They vary in shape but typically are ovate and vary in length from 25 to 65 µm (Jones, 1924). Their functions are associated with possibly the temporary storage of certain compounds as indicated by the presence of oil droplets within (Kinden and Brown, 1975b), and in reproduction as shown by the germination and growth of single excised vesicles (Koch, 1961).

Both the number of arbuscles and vesicles produced varies considerably in different roots of the same plant (Jones, 1924) and also in different species (Jones, 1924; Samuel, 1926; Bevege and Bowen, 1975). Such variations are clearly due to host and endophyte interactions (Bevege and Bowen, 1975; Khan, 1981).

3.424 Extramatrical development. While the internal fungal structures are being formed in the cortex, hyphae grow out into the soil to form a loose network of mycelium. These extramatrical hyphae are dimorphic with a system of coarser hyphae, 20 to 30 µm across, thick-walled and aseptate, and a system of finer hyphae, 2 to 7 µm across, thin-walled and septate with time (Mosse, 1959; Nicolson, 1959,1963). These hyphae extend several centimetres into the soil and contribute to the entire nutrient absorptive system of the plant (Hattingh *et al*, 1973; Pearson and Tinker, 1975; Rhodes and Gerdemann, 1975; Cooper and Tinker, 1978). The extent of extramatrical mycelium development differs enormously among different endophytes (Bevege and Bowen, 1975; Khan, 1981).

The coarser hyphae also produce vesicles, single chlamydospores (resting spores) and sporocarps (fruiting bodies) (Nicolson, 1967). The sporocarps contain chlamydospores, zygospores or sporangia, but sometimes chlamydospores and zygospores together (Godfrey, 1957; Gerdemann, 1965). These chlamydospores, zygospores and sporangiospores of the sporacarps, and the single chlamydospores are able to produce new infections in adjacent roots (Gerdemann, 1955,1961; Gerdemann and Nicolson, 1963; Mosse and Bowen, 1968).

The morphological features of these spores such as size, shape, wall structure, origin, as well as the appearance of the hyphae from which they arise have been employed to distinguish different spore groups (Gerdemann and Nicolson, 1962,1963; Mosse and Bowen, 1968)

which became the foundation for later endophyte classification into various genera and species (Gerdemann and Trappe, 1974,1975; Hall and Fish, 1979; Hall, 1982; Hall, in preparation). Besides these keys, a special slide collection has been prepared also for the identification and classification of arbuscular endophytes (Hall and Abbott, in preparation).

3.43 Arbuscular Mycorrhizae and Phosphorus Nutrition

3.431 Soil phosphates and organic phosphorus. Soil phosphates (inorganic phosphorus compounds) are recognised to exist in three defined pools in equilibrium relationships (Sutton and Gunary, 1969) as shown in Figure 3.2. The soil solution pool also links to the organic pool existing as organic matter (Dalal, 1977).

The non-labile pool is by far the largest inorganic fraction (Larsen, 1967,1976), in which phosphates are held mainly as calcium, fluorine, aluminium and iron compounds in mineral lattices such that they are not immediately exchangeable with ions in the soil solution (Larsen, 1967). These compounds are only sparingly soluble and, thus, their solubility in the soil solution is strongly influenced by the soil pH and the action of microorganisms (Larsen, 1967). The fertilizer known as rock phosphate, being sparingly soluble, is also considered in this category (Tinker, 1975a).

The labile pool describes the phosphate component that can be released into the soil solution (Sutton and Gunary, 1968). The size of this pool may be determined by isotopic dilution of labelled phosphorus such as ^{32}P, with the pool sampled either physically via the soil solution, in which case the result is called E value, or biologically by growing a test plant in the soil, in which case the result is called L value (Larsen, 1967). If the E and L values are closely similar, then the test plant is absorbing only from the soil solution or the labile pool in equilibrium with it (Sanders and Tinker, 1971).

The soil solution is the pool in which plants obtain their phosphate supply as orthophosphates mainly in the forms $H_2PO_4^-$ and HPO_4^{2-} (Larsen, 1967). The concentration of total phosphorus in the soil solution is extremely low, usually between 0.1 and 10.0 μM, equivalent to less than 60 $\mu g\ P\ kg^{-1}$ moist soil or about 0.005% of the soil phosphorus (Pierre and Parker, 1927; Fitter and Hay, 1981).

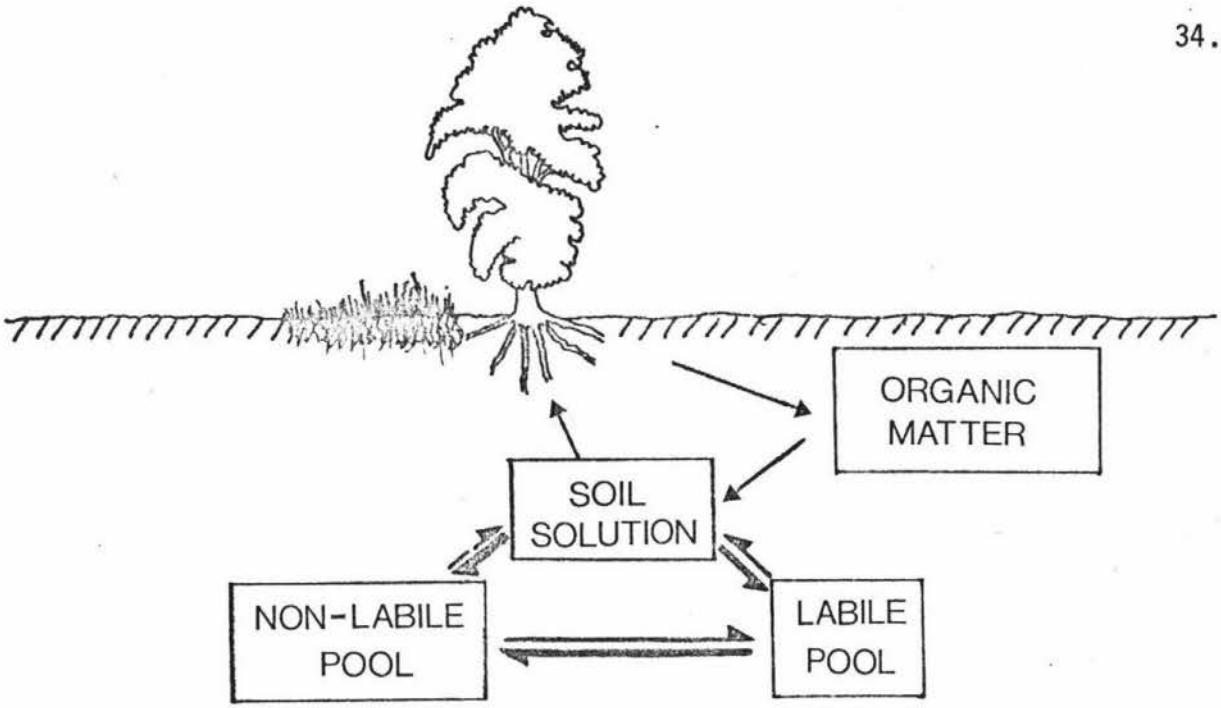


FIGURE 3.2

MODEL OF SOIL PHOSPHATES AND INORGANIC PHOSPHORUS IN DIFFERENT POOLS (THE THICKNESS OF THE ARROWS INDICATES THE BALANCE OF THE EQUILIBRIUM) (MODIFIED AFTER FITTER AND HAY, 1981)

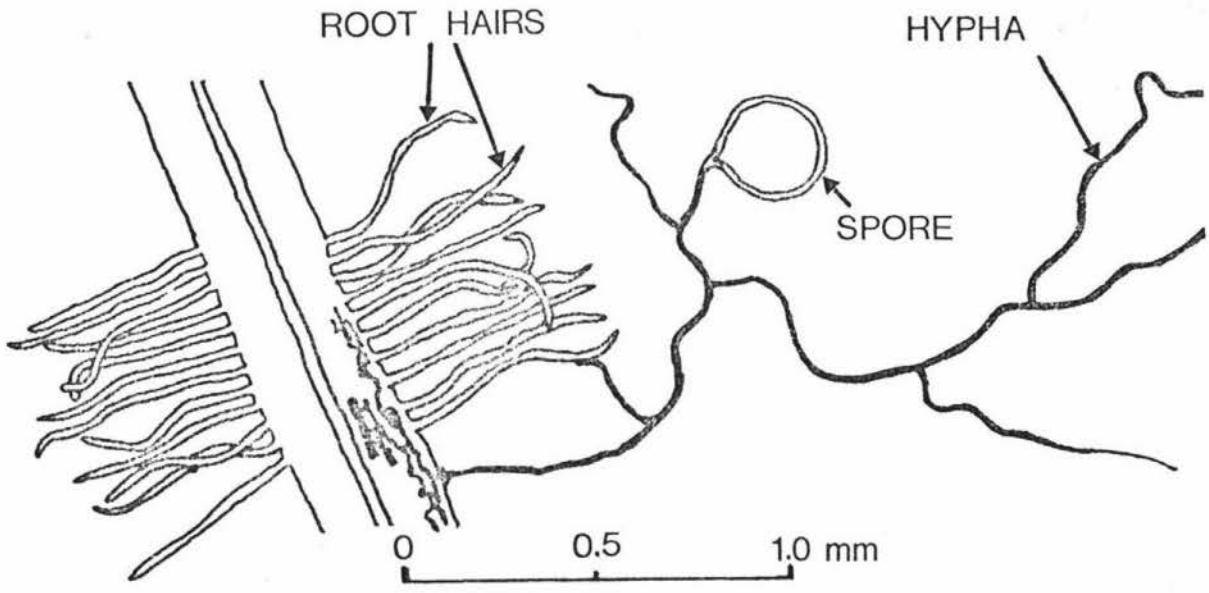


FIGURE 3.3

ROOT HAIRS AND EXTRAMATRICAL MYCELIUM IN THE SOIL (MODIFIED AFTER POWELL, 1975b)

Phosphates, the inorganic fraction, constitutes less than 0.1 μM (Pierre and Parker, 1927). As shown in Table 3.2, the availability of phosphates for plant uptake depends on the replenishment from the labile and, to a limited extent, the non-labile pool.

The organic phosphorus pool may comprise between 20 and 80% of the total soil phosphorus (Wells and Saunders, 1960), and consists of organic as well as organic-inorganic fractions (Larsen, 1967; Dalal, 1976,1977). The compounds so far identified are inositol phosphates (0.4 to 83.0%), phospholipids (0.5 to 7.0%), and nucleic acids (0.2 to 0.4%) (Andersen, 1967; Dalal, 1976,1977). About half the soil organic phosphorus is still in an unknown form (Andersen and Malcolm, 1974).

3.432 Absorption by uninfected roots. Phosphate ions and complexes, like all other mineral nutrients, move through the soil to the root surface by convection (mass flow) and diffusion (Barber, 1962; Olsen and Kemper, 1968). Although the behaviour of the soil-root interface is still ambiguous (Oades, 1978; Greenland, 1979), actively absorbing plant roots are known to quickly deplete phosphate in the solution in their immediate vicinity (Barber *et al*, 1963). For instance, the amount of phosphorus needed by a crop of corn for a yield of 9 500 kg ha^{-1} is 38 kg ha^{-1}, but the approximate amount supplied by convection is only 2 kg ha^{-1} (Barber and Olson, 1968). The remaining phosphate supply, therefore, depends on the release of phosphates from the labile pool and diffusion of these released phosphates to the root surface. However, because phosphates are extremely immobile, the rate of phosphate diffusion to the roots is the slowest (Nye, 1966), with a diffusion coefficient of 0.3 to 3.3 x 10^{-9} cm^2 s^{-1} (Rowell *et al*, 1967) compared to that of nitrate (1.0 x 10^{-6} cm^2 s^{-1}) (Nye, 1966) and potassium (1.0 to 28.0 x $10^{-8}$$cm^2$ s^{-1}) (Drew *et al*, 1969).

In such circumstances, root hairs play a supplementary role in phosphate absorption to meet the plant's demand (Bouldin, 1961; Nye, 1966,1969; Barley, 1970; Drew and Nye, 1970; Newman and Andrews, 1973; Phat and Nye, 1974a,b). The greater zone of phosphate depletion around root hairs as shown by autoradiographs (Lewis and Quirk, 1967a, b; Bhat and Nye, 1973) is due primarily to the greater length of root hairs that penetrate further into adjacent soils, generating a more effective soil-root interface, thereby decreasing the distance for

phosphate diffusion to the root surface (Nye, 1966; Brewster *et al*, 1976b). However, other reasons such as the larger total root absorbing surface area per unit radius (Bouldin, 1961; Bhat and Nye, 1974b; Brewster *et al*, 1976a), greater total volume of soil exploited (Nye, 1966; Bhat and Nye, 1974b) and possibly also greater surface root exudations that aid in solubilizing non-labile phosphates (Cormack, 1962; Brewster *et al*, 1976b) are also regarded as important contributing factors.

In studies of phosphate uptake, rape (*Brassica napus* L.), a species with abundant root hairs, absorbed 10.50×10^{-7} mole P in 5 days (Bhat and Nye, 1974a), while onion, a species with few root hairs, absorbed only 0.25×10^{-7} mole P in 12 days, about 40 times less phosphate, in a low-phosphate soil with comparable amount of root development (Bhat and Nye, 1974b).

3.433 Absorption by infected roots. Beginning in the late 1950's, various workers compared the elemental composition between plants infected and uninfected by arbuscular fungi. A higher level of phosphorus and an associated greater plant growth were found in many species including forest trees (Baylis, 1959,1967; Hall, 1975), horticultural crops (Mosse, 1957; Holevas, 1966; Possingham and Obbink, 1971; Kleinschmidt and Gerdemann, 1972), and agricultural species such as cereals (Gerdemann, 1964; Khan, 1972,1975; Saif and Khan, 1977; Bagyaraj and Manjunath, 1980), grasses (Powell, 1977d; Powell and Sithamparanathan, 1977) and legumes (Ross and Harper, 1970; Ross, 1971; Crush, 1974,1976; Mosse *et al*, 1977; Bagyaraj and Manjunath, 1980) when they were grown in low-phosphate soils. Baylis (1959), Gerdemann (1964) and Holevas (1966) suggested that this consistent, higher concentration of phosphorus and greater plant growth in infected than in uninfected plants is due to the enhanced phosphate absorption by the mycorrhiza systems. Besides greater phosphate absorption, the uptake of other elements such as potassium (Powell, 1975d; Lambert *et al*, 1979; Powell *et al*, 1980a), sulphur (Rhodes and Gerdemann, 1978), and certain micronutrients including zinc, copper, iron and manganese (Swaminathan and Verma, 1979; Lambert *et al*, 1979,1980; Bagyaraj and Manjunath, 1980) were occasionally reported to be higher in mycorrhiza than in non-mycorrhiza systems.

Over the years, five hypotheses have been put forward to explain the increased phosphate uptake by infected plants (Tinker, 1975b).

Tinker (1975b) examined their validity and concluded that the enhanced phosphate absorption is due to the provision either of additional or more efficient, absorption in greater hypha length with subsequent transfer to the host. This hypothesis is strongly supported by the evidence that in infected onions, the mean phosphate inflow is 15.3 x 10^{-14} mole P cm^{-1} s^{-1}, but in non-infected onions, it is only 3.6 x 10^{-14} mole P cm^{-1} s^{-1} (Sanders and Tinker, 1971;1973), indicating that the fungal hyphae contributed a substantial increase in phosphate influx. Although these values were calculated on indirect evidence, the actual inflow of phosphorus in hyphae of mycorrhiza systems measured in split-plate culture techniques is still high, ranging between 1 x 10^{-10} and 2 x 10^{-9} mole cm^{-2} s^{-1} (Pearson and Tinker, 1975; Cooper and Tinker, 1978). A similarly high phsophate inflow value of 2.7 x 10^{-8} mole P cm^{-2} s^{-1} has been recently calculated based on the transport of polyphosphate granules in the hyphae (Cox *et al*, 1980).

The significant contribution of hypha length in phosphate absorption was also supported by the evidence that infected onions absorbed more labelled phosphorus, when placed 2.7 cm away from the root surface, than uninfected onions (Hattingh *et al*, 1973; Hattingh, 1975). Similar data were also obtained using white clover with labelled phosphorus at about 1.0 to 2.0 cm away from the root surface (Pearson and Tinker, 1975), and using onions with the labelled phosphorus as far as 7.0 cm away from the root surface (Rhodes and Gerdemann, 1975). This information, therefore, confirms that the extramatrical mycelia of infected roots extend several centimeters into the adjacent soils, thereby decreasing the distance for phosphate diffusion through the formation of a more effective soil-root interface, similar to the role played by root hairs (Baylis, 1972,1975; Clarkson and Hansen, 1980; Clarkson, 1981).

The available data based on isotopic dilution to date srongly suggest that the external hyphae of arbuscular mycorrhizae absorb phosphates from only the soil solution pool or the labile pool in equilibrium with it (Sanders and Tinker, 1971; Hayman and Mosse, 1972; Tinker, 1975a, Powell, 1975c) and not, as indicated in ectomycorrhizae, from the non-labile pool (Bowen, 1973; Barlett and Lewis, 1973) or from the organic matter pool (Bowen, 1973). Nevertheless, greater phosphorus concentration and dry weight increases have been demonstrated in infected plants than in uninfected plants when grown

in soils treated with sparingly soluble phosphates such as rock phosphate and tricalcium phosphate. Such differences have not been shown when the less soluble superphosphate and monocalcium phosphate were applied (Daft and Nicolson, 1966; Murdoch *et al*, 1967; Mosse *et al*, 1976; Hall, 1975; Powell and Daniel, 1978; Powell, 1979). This is probably because the soils used were highly phosphate retentive, thereby rendering the soluble superphosphate and monocalcium phosphate unavailable to the plants. However, the less soluble rock phosphate and tricalcium phosphate, with a better residual effect, were not significantly affected by the retention capacity of the soils and, thus, the greater phosphate absorption in infected plants than in uninfected plants was still evident (Powell and Daniel, 1978; Powell, 1979,1980a; Powell *et al*, 1980b).

The exudation of hydrogen ions and organic acids such as citrate and 2-ketogluconic acid that solubilize non-labile phosphate compounds have been detected from plant roots (Moghimi *et al*, 1978; Nye, 1979; Kepert *et al*, 1979), but not from extramatrical hyphae. However, phosphate enzymes that can hydrolyse organic phosphorus compounds have been demonstrated on both plant roots (Ridge and Rovira, 1971; Martin, 1973; Boero and Thien, 1979) and intercellular and intracellular hyphae (Gianinazzi *et al*, 1979). Recently, Allen *et al*, (1981) detected alkaline phosphatase activity in mycorrhiza system, but the exact localization within the system is not known (M.F. Allen, personal communication). It seems, however, that the hydrolysis and utilization of organic phosphorus compounds by extramatrical hyphae, if any, is of little importance (Sanders and Tinker, 1971,1973).

3.434 Mechanism of uptake by endophyte. The process of phosphate supply to the host in arbuscular mycorrhizae involves the absorption of phosphates from the soil by external hyphae, the translocation of phosphates in the hyphae, and the release of phosphates from fungal structures to the root cells (Rhodes and Gerdemann, 1980).

Relatively little is known about phosphate absorption by the external hyphae. Absorption is inhibited by 10^{-3}M potassium cyanide or low temperature at 2°C, indicating an active mechanism (Bowen *et al*, 1975). Woolhouse (1975) has suggested the involvement of a carrier such as a phosphate-binding protein in the membranes of hyphae. Cress *et al* (1979) has, in fact, demonstrated a greater phosphate-binding affinity in hyphae than in roots of infected plants.

The mechanism of phosphate translocation in the hyphae is imperfectly understood. Phosphates are probably translocated as polyphosphate granules in hyphal vacuoles (Cox *et al*, 1975,1980; Ling-Lee *et al*, 1975; Callow *et al*, 1978; White and Brown, 1979) by rapid cytoplasmic streaming (Cox *et al*, 1980), possibly bidirectionally (Rhodes and Gerdemann, 1980). This transport mechanism may be associated with the intense alkaline phosphatase activity in the hyphal vacuoles (Gianinazzi *et al*, 1979).

The release of phosphates into host cells occurs in the structurally adapted host-endophyte interface in the arbuscles (Cox and Sanders,1974; Cox *et al*, 1975; Dexheimer *et al*, 1979) by a transfer process across living membranes (Harley, 1969; Cox and Tinker, 1976; Tinker, 1978), involving the expenditure of energy (Marx *et al*, 1982), rather than by the earlier suggestion of arbuscular digestion (Kelly, 1950; Kinden and Brown, 1975c,1976) or hypha digestion (Kinden and Brown, 1975b). In ultrastructural studies, polyphosphate granules have been detected in the arbuscular branches (Cox *et al*, 1975; Strullu *et al*, 1981) and in these regions, intense ATPase activity has been located in the host plasmalemma (Marx *et al*, 1982).

3.435 Efficiency of root hairs and external hyphae. Plant species differ enormously in the root hair abundance (Cormack, 1962) which generally shows an inverse relationship with mycotrophy (Baylis, 1970,1972,1974,1975). On the basis of the length and frequency of root hairs, as well as the thickness of the ultimate rootlet, Baylis (1975) has broadly classified plant species into three groups -- (1) magnolioid root type with rootlets normally greater than 0.5 mm in diameter, a larger cortical section and few or no root hairs of less than 0.1 mm in length (e.g. the Magnoliaceae), (2) intermediate root type with rootlets generally 0.1 to 0.5 mm in diameter, a smaller cortical section and moderate amount of root hairs of 0.1 to 1.0 mm in length, and (3) graminoid root type with rootlets usually less than 0.1 mm in diameter, a small cortical section and abundant root hairs greater than 1.0 mm in length (e.g, Poaceae, Cyperaceae and Juncaceae). The degree of mycotrophy, and hence the dependence on mycorrhizae to absorb phosphate in low-phosphate soils is in the descending order from the magnolioid root type to the graminoid root type (Baylis, 1975; Cooper, 1975) with the Cyperaceae and Juncaceae basically non-mycotrophic (Powell, 1975a). Baylis's hypothesis was confirmed by St John

(1980) who examined the roots of 89 Brazilian forest species and established a significant association between magnolioid root characteristics and mycotrophy.

In the light of the preceding argument on the greater hypha than root length, it appears that endophytes provide access to soil phosphates that root hairs cannot exploit. Assuming equal specific gravity, 1 mg of hyphae of 10 μm in diameter has an equivalent length as 600 mg of root of 400 μm in diameter or 1 to 4 mg of root hairs (Bowen *et al*, 1975). Compared to root hairs and in particular roots, hyphae are, therefore, highly efficient absorptive systems that require possibly less energy. Consequently, the exploitation of efficient endophytes in crop plants in low-phosphate soils could be important in reducing the cost of phosphate fertilizer input in both agricultural and horticultural production systems.

3.44 ARBUSCULAR MYCORRHIZAE AND NITROGEN NUTRITION

3.441 Absorption of organic and inorganic nitrogen. Few studies have been carried out in relation to the absorption of organic nitrogen, nitrate and ammonium ions by arbuscular fungi (Smith, 1980; Bowen and Smith, 1981). The hypothesis that arbuscular fungi can take up organic and inorganic nitrogen is, therefore, based largely on circumstantial evidence.

The greater nitrogen concentration present in infected non-legumes such as strawberries (*Fragaria* spp.) (Holevas, 1966) and grape vine (*Vitis vinifera* L.) (Possingham and Obbink, 1971) than in uninfected plants may be due to the greater absorption of inorganic nitrogen by the roots after the correction of phosphate deficency rather than to the direct uptake of inorganic nitrogen by the external hyphae. Since nitrate is a very mobile ion (Nye, 1969), its supply to the root by diffusion is not expected to be limiting and, thus, the contribution of extramatrical hyphae in nitrate absorption, if any, appears to be insignificant.

As most microorganisms can utilize organic matter and assimilate a certain amount of organic nitrogen (Atlas and Bartha, 1981), it is possible that arbuscular fungi can use, perhaps in small quantities, simple organic nitrogen compounds such as amides, amino acids, urea and nucleic acids. However, the significance of this contribution to

the host plants is yet to be established.

3.442 Enhancement of dinitrogen fixation. There is no conclusive data to show that arbuscular fungi or indeed any other eucaryotic organisms are capable of dinitrogen fixation (Bowen and Smith, 1981), although such ability is suggested to exist in arbuscular fungi in semi-arid and arid ecosystems (Farnsworth *et al*, 1978). However, both nodulation and dinitrogen fixation have been repeatedly demonstrated to occur at greater level in infected than uninfected legumes.

In 1944, Asai observed that several legumes failed to nodulate and developed stunted growth in autoclaved soils unless they formed mycorrhizae. Thirty years later, many workers subsequently confirmed that inoculation with various arbuscular-fungal species usually promotes phosphorus absorption, plant growth, nodulation and dinitrogen fixation in many tropical and temperate legumes (Appendix B). In some cases, although dinitrogen fixation has not been measured, significant increases in phosphate uptake, plant growth and nodulation have been demonstrated (Abbott and Robson, 1977; Powell and Sithamparanathan, 1977; Azcon-G. de Aguilar, 1978; Azcon-G. de Aguilar *et al*, 1979; Bagyaraj *et al*, 1979; Barea *et al*, 1980; Azcon-Aguilar and Barea, 1981). In the dwarf beans, Daft and El-Giahmi (1974,1975) reported also a greater concentration of leghaemoglobin in nodule tissues. However, such beneficial effects vary enormously depending on the soil phosphate concentration, soil pH, indigenous arbuscular fungi, efficiency of introduced endophytes and the type of phosphate fertilizer added (Mosse *et al*, 1976; Mosse, 1977; Abbott and Robson, 1977; Waidyanatha *et al*, 1979).

The increase in nodulation and dinitrogen fixation in infected legumes is due primarily to the improved phosphorus nutrition of the plants. This is evident in the above mentioned experiments in which nodulated plants, when given adequate phosphate fertilizers, have been shown to perform better than or as well as nodulated and infected plants not given any phosphate fertilizer in phosphate uptake, plant growth, nodulation and dinitrogen fixation (Crush, 1974; Daft and El-Giahmi, 1974,1975; Abbott and Robson, 1977; Carling, 1978). Phosphate availability is well-recognised to be low or even deficient in many soils especially when their phosphate retention is also high,

and thus a greater supply of phosphate tends to promote legume growth and subsequent nodulation and dinitrogen fixation (van Schreven, 1958; Gates, 1970,1974; Fillery, 1974).

Phosphorus, being the fifth most abundant soil element in plants (Figure 1.1), is an essential element required in large amounts, particularly in legumes (van Schreven, 1958), for the manufacture of various metabolites to participate in biochemical and physiological processes (Arnon, 1953; Williams, 1978; Clarkson and Hanson, 1980). One of such important metabolites is the energy-yielding molecule, ATP, which is consumed in great quantities in the nodules and often cited as an important limiting factor in dinitrogen fixation (Bergersen, 1971; Franco, 1977; Munns and Mosse, 1980; Grandhall, 1980). Moustafa *et al*, (1971), after applying labelled phosphorus to the leaves of lotus major (*Lotus pedunculatus* Cav.), found that 52% of the radioactivity was in ATP, ADP and AMP of the nodules. In this view, it is possible that the greater phosphate uptake in legumes infected by arbuscular endophyte results also in a greater phosphate translocation to the nodules where it is used to synthesize ATP, thereby enhancing dinitrogen fixation.

3.5 AGRICULTURAL SIGNIFICANCE OF DUAL MUTUALISTIC ASSOCIATIONS IN LEGUMES

With the skyrocketing prices of nitrogen and phosphorus fertilizers due to the energy crisis, the heavy dependence on these high-cost inputs in existing farming systems is becoming untenable. In order to sustain the necessary agricultural production levels for the rapidly growing population, the other alternative is the exploitation of dual mutualistic associations in legumes involving both the rhizobia and arbuscular endophytes. As reviewed earlier in this chapter, these bacteria and fungi play an indispensable role in the cycling of nutrients and more importantly, in the potential improvement of plant nutrition and production. In addition to the reduction in fertilizer usage, the use of legumes can also minimize environmental pollution and increase the protein component in human diet.

The thorough exploitation of land by civilization for various purposes including agriculture means that in the future, any new land for additional cropping programmes has to come from the less productive

environment in which nutrient deficiencies are most probably widespread. To amend these marginal soils with heavy fertilization is not economically feasible (Devine, 1982) and ecologically unsound (Cox and Atkins, 1979) and, therefore, the use of a dual mutualistic system by legume growing appears to be a rational approach (Ruehle and Marx, 1979).

With this in mind, future research in dual mutualistic associations ought to orientate towards the selection of highly effective and efficient combinations of rhizobia and endophyte strains, and the breeding of host cultivars that are highly susceptible to rhizobia and endophytes and which can tolerate low soil fertility (Clark, 1982; Devine, 1982). Progress has been achieved to a certain degree in selecting efficient legume-*Rhizobium* systems (Caldwell and Vest, 1977; Gibson, 1980; Nutman, 1981; Nutman and Riley, 1981), but the selection for desirable legume-endophyte systems is only at the initial stage. So far, there is evidence suggesting that efficient legume-endophyte combinations can be produced. Under controlled conditions, host genetic variation to various endophyte species has been obtained in silver birch (*Betula pendula* Roth.) (Mason, 1975), corn (Hall, 1978), white clover (Hall *et al*, 1977), soybean (Skipper and Smith, 1979; Carling and Brown, 1980), lucerne (Lambert *et al*, 1980), wheat (Azcon and Ocampo, 1981), barley (Jensen, 1982), and cassava (*Manihot esculenta* Crantz) (Howeler *et al*, 1982). For the future, further research on the dual mutualistic association in legumes in the natural field conditions (Carling and Brown, 1980; Crush and Caradus, 1980; Lambert *et al*, 1980) should offer new challenges to both plant physiologists and plant breeders.

CHAPTER 4

EXPERIMENTAL MATERIALS AND METHODS

4.1 INTRODUCTION

In order to confirm that arbuscular fungi are found naturally in the field and may be introduced experimentally into the test plants, two preliminary studies were performed between March 28 and April 28, 1981. The results obtained, as shown in section 5.2, confirm the occurrence of arbuscular fungi in sainfoin roots. On the basis of these findings, the principal experiment was planned.

This was conducted between May 29 and October 15, 1981 in glasshouse No 3 at the Plant Growth Unit, Massey University. The overall growth period of the plants lasted 137 days from radicle emergence to green inflorescence development.

The preliminary and the principal experiments are fully described in the following sections, while a programme summary for these experiments is presented in Appendix C.

4.2 PRELIMINARY EXPERIMENTS

4.21 Glasshouse Test

Pre-germinated sainfoin seeds (variety *bifera* Hort.) were planted in 2 000 cm^3 planter bags at four per bag, on March 28. The bags were potted with Manawatu very fine sandy loam and sterilized with methyl bromide. The experiment consisted of 12 bags and 3 treatments, giving 4 bags per treatment, but without a control and replications. At planting, each treatment was inoculated with 4 to 6 g of one of the three fungal cultures -- R4, NP15 and Gm which are described in Appendix D.

On day 5, 10 and 31 from planting, one bag per treatment was sampled and examined for endophyte infection. The root systems were sectioned into about 1.5 cm segments which were cleared and stained according to the procedure described by Hayman and Phillips (1970) (see section 4.64). The roots were examined under a 100x magnification, and the absence (0) or presence (1) of infection was recorded as shown

in section 5.2.

4.22 Field Test

Eighteen-month old sainfoin plants (cvs. Fakir and Remont) were sampled at random from two of Mr J.A. Fortune's experimental blocks, on April 27. The blocks were situated on Manawatu sandy loam, with a pH of about seven. Three plants from the Fakir block and five plants from the Remont block were considered respectively as three and five replications in this test.

The roots were washed, cut into approximately 1.5 cm segments, and cleared and stained as described in section 4.64. The roots were examined under a 40x magnification and the degree of infection was recorded, this time, based on a quantitative scoring system which had been developed (see Appendix I). The results are tabulated and discussed in section 5.2.

4.3 THE PRINCIPAL EXPERIMENT

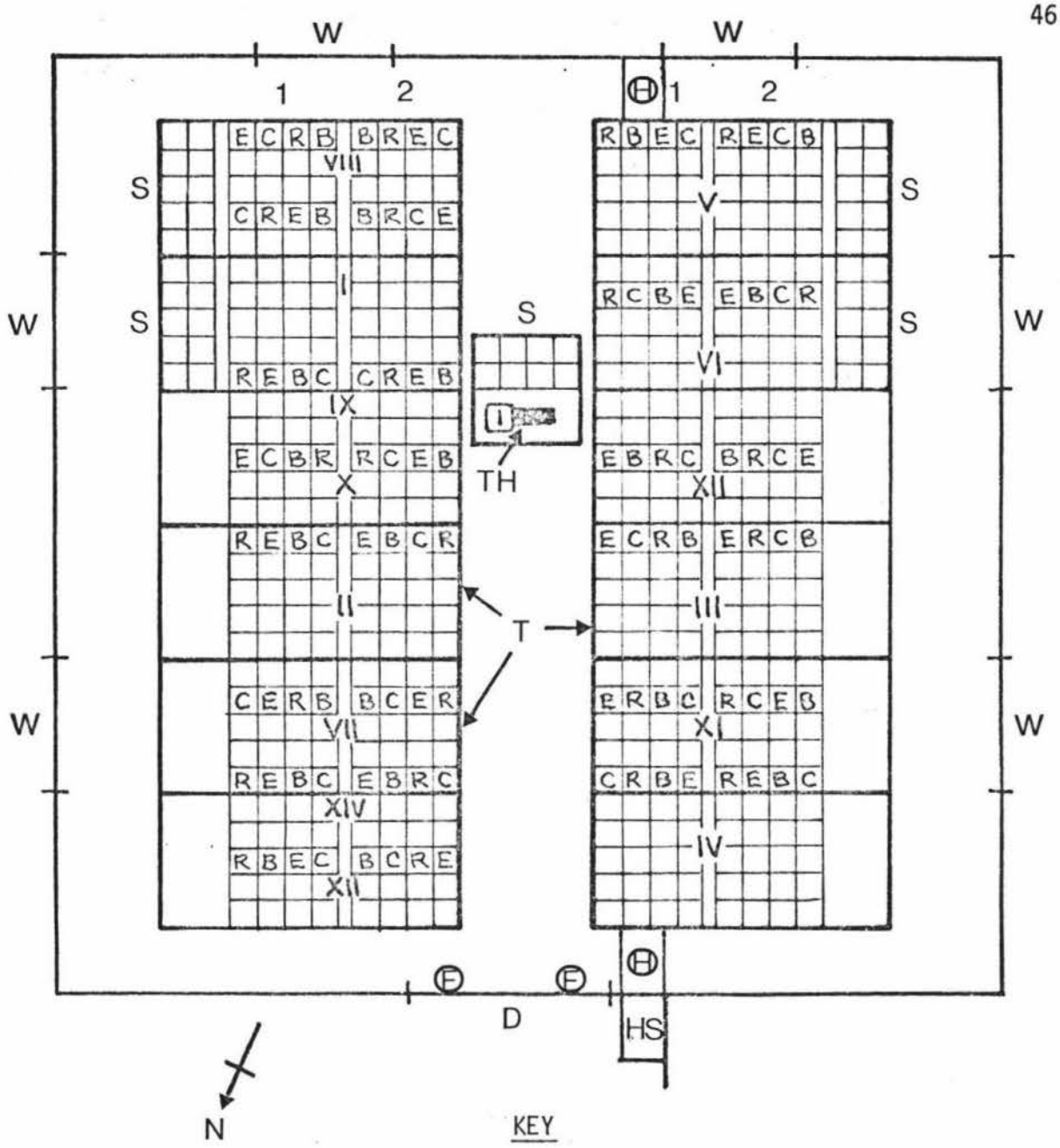
4.31 Experimental Design and Layout

The purpose of this experiment was to investigate the interaction between two mutualistic systems, namely the sainfoin-*Rhizobium* and sainfoin-endophyte associations, over a period of time. Four treatments, therefore, were chosen as follows:

C control or no inoculation
E inoculated with endophytes only
R inoculated with rhizobia only
B inoculated with both rhizobia and endophytes

Owing to extensive sample preparation and handling, a total of some 500 planter bags was chosen for a series of randomised complete-block designs pooled across time, with 14 samplings and 2 replicates in each treatment, giving 8 experimental units per sampling. There were thus a total of 486 experimental bags and 48 spare ones as replacements.

The 486 bags were placed on trolleys in a glasshouse and arranged randomly to form four 4-row blocks (replicates), labelled 1 and 2 (Figure 4.1). Two levels of randomisation were performed. Firstly, the 14 individual designs in the series were randomised to form 14 sequential sampling times, designated by capital roman numerals



KEY

Sampling time	I,II,III,....XIV	Overhead fan	⊕
Block	1,2	Thermohygrograph	TH
Treatment	C,E,R,B	Trolley	T
Spare bags	S	Window	W
Overhead heater	⊕——⊕	Door	D
Heat source	HS		

FIGURE 4.1

EXPERIMENTAL LAYOUT IN THE GLASSHOUSE

in Figure 4.1. Subdivisions according to the size of each individual design were, therefore, made across the blocks. Secondly, randomisation was carried out within each block to form the four treatments, designated by the appropriate letters.

The 48 spare bags were placed and completely randomised in the five sites marked "S" as shown.

4.32 Schedule of Destructive Sampling

Initially, the plants were sampled when 90% of the population developed a fully expanded new leaf. However, as the plants matured, variability of leaf growth and development was significant and thus, sampling on the basis of the above criterion was not possible. A nine-day sampling interval was, therefore, decided on.

After 45 days of growth, endophyte infection was yet to occur. In order to allow time for the development of this mutualistic system, the sampling interval was lengthened from 9 to 16 days. This longer interval was later reduced to 11 days due to the restriction of time available. For the same reason, the experiment was terminated at the eleventh sampling. Table 4.1 presents the schedule of the eleven destructive samplings.

TABLE 4.1

SCHEDULE OF DESTRUCTIVE SAMPLING

Sampling No	Date	Days from germination	Interval
	May 31	Radicle emergence	0
I	June 18	18 (1 leaf)	18
II	June 27	27 (2 leaves)	9
III	July 6	36 (3 leaves)	9
IV	July 15	45	9
V	July 31	61	16
VI	August 16	77	16
VII	September 1	93	16
VIII	September 12	104	11
IX	September 23	115	11
X	October 4	126	11
XI	October 15	137	11

4.33 Experimental Measurements

In order to reduce the extensive laboratory work, the plants from each experimental bag were bulked and the measurements, except for the nitrogen and phosphorus concentration in tissue, were expressed as the appropriate units per bag. These measurements were computed and re-expressed, whenever necessary, as the appropriate units per plant to form a list of new variables for later analyses and discussion (Table 4.2).

4.4 PREPARATION OF MATERIALS

4.41 Growth Containers

Polythene planter bags, of 2 000 cm^3, were used.

4.42 Growth Medium

A top 15 cm sample of Manawatu very fine sandy loam was extracted, sieved through a 0.5 cm screen and homogenised to obtain minimal physical and chemical variations. Later, methyl bromide was applied to kill the indigenous microbiota and seeds. The prepared growth medium was stored in new brown paper bags until potting after adequate aeration.

A subsample of the medium was taken for pH, organic matter, phosphate retention, total soluble salts and macronutrients determination. The results, as shown in Appendix E, indicate that the pH at 6.2 was satisfactory, phosphorus and calcium concentrations were medium, but organic matter and potassium, magnesium and sodium concentrations were low.

4.43 Sainfoin Seedlings

Encased sainfoin seeds (cv. Fakir), with a germination of 84% on day 6, was supplied by Mr J.A. Fortune (Agronomy Department, Massey University). In order to achieve a greater and more even germination, seed pods were cracked by rubbing seeds between two pieces of wood to facilitate water entry to the testa, and the cracked pods were pre-soaked for 12 hours in water to remove the water-soluble germination inhibitors (Carleton *et al*, 1968; Smith, 1979).

The pre-soaked seeds were rinsed and placed on wetted perlite in perforated plastic trays, which were fed with water from below and

TABLE 4.2

COMPUTED VARIABLES USED IN STATISTICAL ANALYSES

Variable No.	Description	Unit
1	Total plant dry weight	g plant^{-1}
2	Cotyledon dry weight	g plant^{-1}
3	Shoot dry weight	g plant^{-1}
4	Root dry weight	g plant^{-1}
5	Nodule dry weight	g plant^{-1}
6	Root-shoot ratio	ratio
7	Secondary stem number	unit plant^{-1}
8	Nodule number	unit plant^{-1}
9	Nodule dry weight per nodule	g nodule^{-1}
10	Acetylene reduction per plant	µmoles hr^{-1} plant^{-1}
11	Acetylene reduction per nodule DW	µmoles hr^{-1} g^{-1}
12	Total plant nitrogen	µg g^{-1} tissue
13	Total cotyledon nitrogen	µg g^{-1} tissue
14	Total shoot nitrogen	µg g^{-1} tissue
15	Total root nitrogen	µg g^{-1} tissue
16	Total nodule nitrogen	µg g^{-1} tissue
17	Total plant phosphorus	µg g^{-1} tissue
18	Total shoot phosphorus	µg g^{-1} tissue
19	Total root phosphorus	µg g^{-1} tissue
20	Total nodule phosphorus	µg g^{-1} tissue
21	Endophyte infection	score view^{-1}
22	Plant age	days from germination

placed in the glasshouse. The maximum and minimum glasshouse temperatures were 25 and 15°C respectively. Some radicles appeared in about a day but most of the seeds had germinated with their cotyledons opened and turned green by the third day.

4.44 Rhizobia Inoculum

Sainfoin rhizobia, *Rhizobium* spp. strain 3157 (NZP 5301), was obtained and cultured on to yeast-mannitol agar slopes from which subcultures were made once every four weeks to maintain viability. The yeast-mannitol medium (Vincent, 1970) is presented in Appendix F.

About 10 days before the inoculations, 5 000 cm^3 each of rhizobia liquid culture was prepared using the same medium but without the agar. The prepared cultures were incubated at 21°C for eight days after which they were stored at 6°C in a cool room until inoculation.

4.45 Endophyte Inoculum

Initial inocula of R4, NP15, Gm (*Gigaspora magarita* Becker & Hall), and MX (a mixture of *Gigaspora magarita* Becker & Hall, *Glomus tenuis* (Greenall) Hall, and *Glomus fasciculatus* (Thax. sensu Gerd.) Gerdemann & Trappe) were supplied by Dr C.Ll. Powell (Ruakura Soil and Plant Research Station, MAF, Hamilton). The fungal populations were multiplied in open-pot culture by mixing the inocula with sterilized Manawatu very fine sandy loam in a ratio of 1:10 or 1:13 (C.Ll. Powell, personal communication) (Appendix D). About 30 to 40 corn seeds were planted in each pot as the host plants, and allowed to grow until senescence.

During the growth period, corn roots were periodically sampled and examined for endophyte infection. Towards the later growth stages, a reasonable infection, with hyphae, arbuscles and vesicles, was observed (Plate 5.1, p.68a).

At harvesting, plant tops were discarded, while the soil-root components were retained, mixed and packed in plastic bags which were stored at 6°C in a cool room until needed. Since Gm was a monospecies culture and (earlier) known to infect sainfoin, it was chosen as the inoculum for the principal experiment.

4.46 Nutrient Solutions

Two types of nutrient solution, a low-nitrogen and high-nitrogen,

were prepared. The nutrient composition of the low-nitrogen solution was similar to that of Small and Leonard (1969), and contained various essential macronutrients and micronutrients at adequate levels except nitrogen (Appendix G). This composition was used when experimenting with peas (*Pisum sativum* L.) and subterranean clover (*Trifolium subterraneum* L.) (Small and Leonard, 1969), and sainfoin (*Onobrychis viciifolia* Scop. cv. Fakir) (Hume, 1981), but the low nitrogen present (4 μg cm^{-3}) was not reported to depress nodulation.

The high-nitrogen solution included 0.01% ammonium nitrate and, thus, contained 35 μg N cm^{-3} which was used to supply a maintenance requirement of the plants.

4.5 EXPERIMENTAL PROCEDURES

4.51 Control of Glasshouse Conditions

Initially, the glasshouse was funigated by heating 6 cm^3 of Vapona® (containing 1.08 g cm^{-3} dichlorvos as active ingredient) at 65°C.

A temperature of between 15 and 25°C was maintained throughout the experiment. The maximum, minimum and average weekly temperatures recorded are shown in Appendix H. During the first 3 weeks and between week 10 to 15, the heating device was not operating. From week 14 onwards, it was increasingly difficult to maintain the day temperature at 25°C.

The plants were grown under natural day length and light intensity.

4.52 Potting

Five hundred and twenty-eight planter bags were potted with equal weights of sterilized Manwatu very fine sandy loam soil. Of this total, 264 bags contained 2 500 g soil, while in the other 264 bags, there was 2 485 g soil plus 15 g Gm fungal inoculum added later which was a Manawatu very fine sandy loam soil-maize root mixture.

4.53 Inoculation of Endophyte

Four days before planting, all the bags containing 2 485 g soil were given 15 g fungal inoculum to form treatments E and B. Inoculation

was carried out by mixing the soil-root mixture into the top 2 to 3 cm soil of each bag.

4.54 Potting

Healthy, two-day old sainfoin seedlings were selected and planted on June 2, 1981 by placing seedlings in previously bored holes of equal distance apart. Immediately after placement, the holes were filled with adjacent soil particles, and the pots watered carefully.

To allow for selection during thinning and to ensure an adequate quantity of plant tissue for nutrient analysis in the earlier harvests, a larger plant population per bag was established. The initial 6 samplings consisted of 14 seedlings per bag, while the later 8 consisted of 6 per bag.

4.55 Inoculation of Rhizobia

On day 5 and 16, inoculation with rhizobia cultures was completed after these had reached room temperature, diluted with 0.1% sterile peptone water (1:40 v/v for the first inoculation and 1:3 v/v for the second inoculation). A subsample of each inoculation was kept and estimated for the number of viable bacteria by the plate count method (Vincent, 1970). The number of viable cells in the first and second diluted cultures was respectively 2 150 x 10^6 and 1 180 x 10^6 cm^{-3}.

At each inoculation, 20 cm^3 of the culture was evenly injected into each bag of treatments R and B using a Cornwall syringe. The number of applied viable bacteria in each bag was, therefore, 43 000 x 10^6 and 23 600 x 10^6. It was assumed that these bacterial populations would give satisfactory levels of infection and nodulation. After inoculation, all the bags in the four treatments were watered to pot capacity, assessed by weight differences.

4.56 Thinning and Replacement of Seedlings

Two thinnings were performed on day 9 and 13. In the first thinning, the seedling number was reduced from 14 to 12 for the first 6 samplings, and from 6 to 4 for the remaining harvests. In the second thinning, the plant number for the initial 6 samplings was further reduced from 12 to 10, while the number for the later 8

samplings was maintained the same. Each time, those seedlings with the smallest cotyledons and first developing leaves were pulled out using a pair of forceps.

The death of some seedlings warranted their replacement. To prevent the development of disease organisms, removal of dead and dying seedlings was done every alternate day, and fresh seedlings transplanted into new sites in the bags. Dead and dying seedlings were placed in 95% ethanol to reduce aerial spore dispersal.

4.57 Watering

Pot capacity was determined by the method of weight differences every day, and used as a guide to derive the amount of water needed in each watering. During the early growth stages, watering was less frequent, being done every two to three days. This watering frequency was increased to once a day, and in late September and October, twice a day in order to keep up with the increased evapotranspiration.

On day 91 and 98, all the bags were flushed with 300 cm^3 water in case of large accumulation of sales in the soils.

4.58 Nutrient Application

The prepared low-nitrogen and high-nitrogen solutions were applied at various stages of plant growth (Appendix G). Except for treatments C and E in which plants developed nitrogen-deficient symptoms due to low level of dinitrogen fixation, other nutritional deficiencies were not observed.

After the flushing of all bags on day 91 and 98, nutrients were reapplied to replace those that were flushed out. In each case, 20 cm^3 low-nitrogen solution was added to all bags, while 10 cm^3 high-nitrogen solution was applied to bags of treatments C and E only.

4.6 ANALYTICAL TECHNIQUES

4.61 Sampling Method

At each destructive sampling, plants of the first replicate were transferred to the laboratory, washed free of soil, and prepared for various experimental measurements. For harvests 6 to 11, acetylene reduction was first determined before the bags were taken into

the laboratory.

In the laboratory, the washed plants were divided into two lots, the first lot consisting of two plants per bag in each treatment, for the estimation of endophyte infection and the second lot for dry weight, nodule number, secondary stem number and total nitrogen and phosphorus measurements. The entire sampling method was repeated for the second replicate. Between the sampling of the two replicates, there was an interval of about three to four days and, therefore, the median was taken as the sampling date.

4.62 Removal and Washing of Plants

Bags were inverted on sieves and immersed in water in buckets for about 20-30 minutes. The planter bags were removed from the soil-plant components which were later broken down by a water jet. The plants with their best possible intact root systems were extracted from the soil and repeatedly washed to remove the adhered soil particles. Detached roots were collected on sieves and washed. The estimated root recovery was about 95% of the total root dry weight.

Washed plants from each bag were placed between wetted paper towels to prevent tissue drying. They were put on trays and transferred to a cool room for temporary storage at 6°C.

4.63 Determination of Plant Dry Weight

Plants were dissected into cotyledons, shoot (which included leaves, petioles, apex and epicotyl stem), root and nodules. Nodule and secondary stem numbers for each plant were noted. The dissected plant components were placed seperately in glass jars and dried in an oven at 80°C for 24 hours.

Desiccators were used to cool and store the dried plant components. When ready, they were weighed on a Mettler A30 digital balance which read up to four decimal places.

After weighing, all the plant parts of the same component and treatment were bulked for grinding. Ground tissues were stored in labelled plastic tubes.

4.64 Estimation of Endophyte Infection

The assessment of root infection by arbuscular fungi included

the preparation of root materials, and the examination and quantification of infected cortical tissues. In the initial preparation, washed root systems were sectioned into approximately 1.5 cm segments with 30 to 40 segments from each plant being randomly sampled for cleaning and staining by the method of Phillips and Hayman (1970). The segments were heated in 10% potassium hydroxide solution in McCartney bottles at 90°C for 25 minutes, acidified in 1M hydrochloric acid for 5 minutes, and then simmered in 0.1% trypan blue in lactophenol in a fume cupboard for 5 minutes. To increase the contrast between root tissue and fungal structures, the roots were destained by soaking in lactic acid on spotting porcelain trays. Lactic acid was preferred instead of lactophenol because the latter is health hazardous (Hayman, 1981).

Twenty root segments were selected from the spotting trays and examined under a compound microscope at the 40x magnification. For each segment, two random fields of view were examined. The degree of endophyte infection in each field was quantified by assigning a score with the aid of an assessment key developed (Appendix I).

4.65 Determination of Dinitrogen Fixation

Dinitrogen fixation, a measure of the instantaneous nitrogenase activity, was determined by the modified acetylene-ethylene assay of Hardy *et al* (1968), involving two seperate sequential steps, namely incubation and gas chromatography.

In each incubation run, one replicate, consisting two bags per treatment plus duplicate soil blanks, plant blanks and acetylene blanks, was assayed *in situ*, that is, with the soil-plant system undisturbed. The soil blanks, plant blanks and acetylene blanks provided assessments of the ethylene produced by free-living organisms in the soil, ethylene in the background and ethylene impurity in the acetylene gas respectively.

After being watered to a similar soil moisture content of about 11% of the total bag weight (pot capacity at 17%), intact plants in bags were placed in 4 500 cm^3 opaque white containers with gas-tight lids which were fitted with rubber septa to allow for the introduction and withdrawal of gases (Plate 4.1). From each container, 150 cm^3 of air was removed by a 50 cm^3 syringe. Immediately, an equal volume of industrial (commercial) grade acetylene was injected to give an

acetylene partial pressure of 5 270 to 5 670 Pa (0.052 to 0.056 atm) which was adequate to saturate the nitrogenase in the nodules (Hardy and Holsten, 1977; Crush and Tough, 1981). To reduce diurnal variation of nitrogenase activity, all the containers were incubated, for one hour, within the 1030 and 1230 hour peroid.

At the end of each incubation, three 0.6 cm^3 gas samples were taken using 1.0 cm^3 disposable plastic syringes. The samples were plunged in rubber bungs and stored for about two hours prior to gas chromatography using a Pye series 104 chromatograph (Plate 4.2). The preparation of ethylene standards, gas chromatography and the method of calculation are detailed in Appendix J.

4.66 Determination of Total Nitrogen and Phosphorus

Total herbage nitrogen and phosphorus were simultaneously determined by a modified technique of autoanalysis as described by Blakemore *et al* (1981), involving micro-Kjeldahl digestion, dilution and colorimetric automated analysis. In micro-Kjeldahl digestion, 4 cm^3 of the digest reagent was added to 0.1 g of weighed tissue in 24 x 200 mm pyrex test-tubes which were placed in an aluminium digestion block in which they were heated to about 400°C for 8 hours. One replicate of samples, represented by duplicate samples of every plant component for each treatment and two standards of known nitrogen and phosphorus concentrations previously determined, was digested each time to control any variation.

After digestion, the samples were washed, diluted into 50 cm^3 stop flasks and stored in ordinary test-tubes which were covered with tin foil.

The automated analyses were carried out using the Technicon Industrial Automated Analyser (Autoanalyser) (Plate 4.3 and 4.4). The samples were loaded into 2 cm^3 cups from which they were pumped to a nitrogen and a phosphorus colorimeter where they reacted with other reagents to produce their distinctive colours that were signalled to the recorders. Two series of standards, one each for nitrogen and phosphorus, were also analysed to produce their respective standard graphs from which the total nitrogen and phosphorus concentrations of the samples were read. The method of calculation and preparation of various reagents and standards are described in Appendix K.

PLATE 4.1

INCUBATION AND GAS SAMPLING IN THE FIRST STEP
OF ACETYLENE REDUCTION ASSAY

PLATE 4.2

GAS CHROMATOGRAPHY IN THE SECOND STEP OF
ACETYLENE REDUCTION ASSAY

PLATE 4.3

AUTO-ANALYSER USED IN THE ANALYSIS OF TOTAL NITROGEN AND PHOSPHORUS

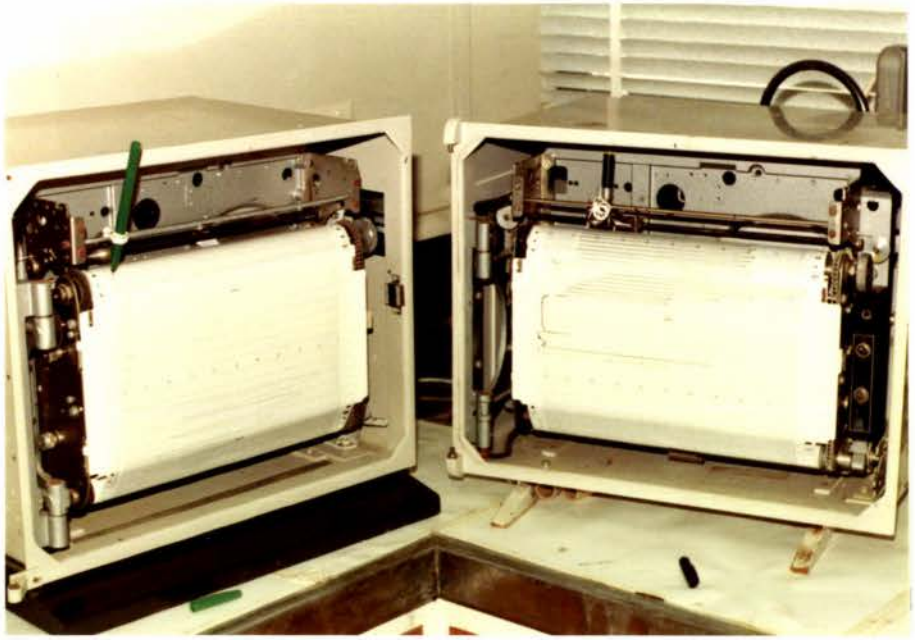


PLATE 4.4

AUTO-ANALYSER RECORDERS

4.7 PROBLEM WITH ENDOPHYTE INFECTION

4.71 Reisolation of Gm

Although sainfoin roots were expected to develop arbuscular mycorrhizae with *Gigaspora magarita* Becker & Hall after 30 to 40 days of growth, there was no infection even at day 91 (Sampling V) and, therefore, a reisolation experiment to investigate its presence or absence in the inoculated bags was performed. Eight spare bags, two from each of the four existing treatments, were randomly selected and planted with surface-sterilized corn seeds at six per bag. At day 26, 34 and 40 from planting of the seeds, two corn plants from each bag were harvested and stained as before for endophyte infection.

In addition, fresh sainfoin plants were grown from seeds in a 100% Gm inoculum to investigate its infectivity as a high inoculum.

4.72 Reinoculation with MX

At day 97, a mixture of highly infective and efficient fungal species (MX), including *Glomus fasciculatus* (Thax. sensu Gerd.) Gerdemann & Trappe, *Glomus tenuis* (Greenall) Hall as well as *Gigaspora magarita* Becker & Hall (C. Ll. Powell, personal communication) was inoculated to treatments E and B in order to continue the principal experiment. To ensure adequate viable fungi in the inoculum, the quantity used was increased to 30 g per bag, twice the amount applied in the first inoculation.

Meanwhile, eight spare bags, two from each of the four existing treatments, were also randomly selected, inoculated with MX at 30 g per bag and planted with surface-sterilized corn seeds at six per bag to act as controls. At day 18 and 61 from planting of the seeds, two corn plants from each bag were harvested and stained as before for endophyte infection.

4.73 Detection of Condensed Tannins

With the overwhelming evidence of tannins providing host resistance to fungal infection (McLeod, 1974; Harbourne, 1977) and tannins occurring in sainfoin (Jones and Lyttleton, 1971; Sarkar *et al*, 1976; Goplen *et al*, 1980), plant materials remaining after the estimation of endophyte infection in Sampling VIII (day 104) were

collected for the detection of condensed tannins using the vanillin-hydrochloric acid (HCl) technique described by Jones *et al* (1973). A younger leaf and older leaf, the crown, the main and a few secondary roots, several younger roots and a few nodules were crushed between two Watman No. 3 filter papers and one imprint was applied with the vanillin-HCl reagent, while the other imprint was applied with only the HCl reagent to act as a control. The colour reaction was scored as follows:

SCORE	GRADE	COLOUR
0	negative	blue-green
1	trace	a few pink spots within imprint
2	slight	light pink over imprint
3	medium	light red over imprint
4	intense	intense red-violet over imprint

4.74 Determination of Root Morphology

This exercise aimed to provide an additional information on the degree of mycotrophy in sainfoin based on the hypothesis of Baylis (1975). Eight sainfoin (cv. Fakir) and four white clover plants were grown in the same seterilized Manawatu very fine sandy loam soil and harvested when they reached the fourth or fifth leaf stage. Each plant was treated as a replication and the diameter of ten ultimate rootlets and length of ten straight root hairs, after staining for three minutes with 0.005% aqueous Nile blue, were randomly selected for measurement using a compound microscope. Relative root hair abundance was also visually compared.

4.8 STATISTICAL METHODS

4.81 Data Transformation

Except for cotyledon dry weight, acetylene reduction per nodule dry weight, total plant nitrogen, total plant phosphorus, total shoot phosphorus and total root phosphorus, all other variables did not approximate a normal distribution and were, therefore, transformed to other scales using the statistical programme, Minitab (Ryan *et al*, 1981). All transformed data resembled more or less a bell-shaped

distribution as indicated in their histograms and approached a near straight line as shown in their normal probability plots (Daniel, 1959; Bliss, 1967). For many variables in which increases in numbers were proportional to the numbers already present (e.g. dry weights), natural logarithmic transformations were performed, while for variables in which data consisted of integers (e.g. secondary stem number), square root type of transformations were carried out (Barlett, 1947). Since the variable, fungal infection score, was constructed from a percentage scale, it was transformed by a standard arcsine equation (Snedecor and Cochran, 1978).

Table 4.3 presents the different variables with their equations of transformation.

4.82 Analysis of Variance

The pooled analysis of variance (ANOVA) used was a split-plot model (Steel and Torrie, 1980) given by

$$Yijk = \mu + \rho i + \alpha j + \gamma ij + \beta k + (\alpha\beta)jk + \xi ijk$$

where
- μ = grand mean for the whole experiment
- ρ = effect of block i
- α = effect of treatment j
- γij = error (a) for a plot totalled one time
- βk = effect of time k
- $(\alpha\beta)jk$ = interaction of treatment j and time k
- ξijk = error (b) for a plot at a particular time

In order to examine the different combinations of rhizobia and endophyte, the analysis of variance was extended to include the comparison between without rhizobia and with rhizobia, and between without endophyte and with endophyte in the treatment effect. The pooled ANOVA was performed on all variables except plant age (Table 4.3) by the general statistical programme, GENSTAT (Alvey *et al*, 1980). As environmental effects were much controlled, a fixed model was assumed (Eisenhart, 1947; Fisher, 1951; Plackett, 1960).

Although the variable, endophyte infection, consisted of data expressed as scores, it was based on an ordinal and interval percentage scale and, therefore, it can be argued that the use of the ANOVA is reasonable (Abelson and Turkey, 1959; Labovitz, 1970,1972).

TABLE 4.3

EQUATIONS OF TRANSFORMATION OF VARIOUS VARIABLES

Variable	Equation of transformation
Total plant dry weight	$[(\ln x) + 1.5]^3 + 100$
Cotyledon dry weight	not necessary
Shoot dry weight	$\ln (235\ x)$
Root dry weight	$[(\ln x) + 2.7]^3 + 100$
Nodule dry weight	$\sqrt{(\sqrt{x})}$
Root-shoot ratio	$\ln (50\ x)$
Secondary stem number	$\sqrt{(x)}$
Nodule number	$\sqrt{\ [(x + 1)]}$
Nodule dry weight per nodule	$\sqrt{(x)}$
Acetylene reduction per plant	$\sqrt{[\sqrt{(x + 0.01)}]}$
Acetylene reduction per nodule DW	not necessary
Total plant nitrogen	not necessary
Total cotyledon nitrogen	$\ln (x + 0.001)$
Total shoot nitrogen	$(10^{-5}x - 0.7)^3 + 1$
Total root nitrogen	$\ln (x)$
Total nodule nitrogen	$\ln (x^{5 \cdot 8} + 0.001)$
Total cotyledon phosphorus	$\ln (x^{2 \cdot 0} + 0.001)$
Total shoot phosphorus	not necessary
Total root phosphorus	not necessary
Total nodule phosphorus	$\ln (x^{5 \cdot 5} + 0.001)$
Endophyte infection	arcsin (x)
Plant age	not necessary

x is the original value

For samplings with the entire data set being zero, they were not included in the analysis as they would have produced unmeaningful results.

4.83 Coefficient of Variation and Standard Error

The formula used for calculating the coefficient of variation (CV) (Steel and Torrie, 1980) is given by

$$CV = \frac{\sqrt{EMS}}{\bar{x}} \times 100\%$$

where EMS = error mean square,

$\bar{x}$ = grand mean.

The standard error (SE) (Steel and Torrie, 1980) is calculated by

$$SE = \sqrt{\frac{EMS}{n}}$$

where EMS = error mean square,

n = number of replications.

4.84 Duncan's New Multiple Range Test

Tests of significance between all means at each time were carried out for all variables using Duncan's new multiple range test (Duncan, 1955) as this is more appropriate than the least significant difference test in comparing control and non-control means (LeClerg *et al*, 1962; Gomez and Gomez, 1976). The final results are presented in tabular form in which means significantly different from each other at $P < 0.05$ are denoted with different letters.

4.85 Regression Analysis

A simple regression analysis was used to examine the relationship between nodule dry weight per plant and nodule number, nodule dry weight per plant and nodule dry weight per nodule, shoot dry weight and root dry weight, and shoot dry weight and nodule dry weight per plant. The dependent variables are predicted from a linear function of the form (Draper and Smith, 1981),

$$\hat{Y} = a + bX,$$

where $\hat{Y}$ = estimated value of the dependent variable Y,

a = intercept,

b = regression coefficient which is the slope,

X = independent variable.

The analysis was performed by SPSS (Nie *et al*, 1975) which also determined the coefficient of multiple determination (R^2) using the formula

$$R^2 = \frac{SSyy - SSres}{SSyy}$$

where SSyy = sum of squares in Y,

SSres = residual sum of squares.

The square root of this ratio is the correlation coefficient (R) between variables X and Y. The correlation coefficient was used to measure the strength of the linear relationship between dry weights of various plant components and their nitrogen and phosphorus concentrations.

In addition, a multiple regression analysis was performed on the dependent variable, nodule dry weight, with the independent variables, nodule number and nodule dry weight per nodule, and on acetylene reduction per plant with nodule dry weight per plant and shoot dry weight. The general form of the regression (Draper and Smith, 1981) is

$$\hat{Y} = a + b_1X_1 + b_2X_2$$

where the subscripts, 1 and 2, denote variables 1 and 2 respectively. To determine the respective contribution of each independent variable to explained variance, a forward (stepwise) inclusion in the analysis was done using SPSS (Nie *et al*, 1975).

As for ANOVA, samplings with the entire data set as zero were dropped from the analysis to avoid unmeaningful results.

The homogeneity of the two regression coefficients of the regression lines, $\hat{Y}_1 = a_1 + b_1 X_1$ and $\hat{Y}_2 = a_2 + b_2 X_2$, were analysed using the t-test formula (Gomez and Gomez, 1976).

$$t = \frac{b_1 - b_2}{\sqrt{\left[s^2 \left(\frac{1}{\Sigma X_1^2} + \frac{1}{\Sigma X_2^2} \right) \right]}}$$

where $Sp^2 = \dfrac{(n_1 - 2)\, Sxy_1{}^2 + (n_2 - 2)\, Sxy_2{}^2}{n_1 + n_2 - 4}$

= pooled residual mean square,

$Sxy_1{}^2$ = residual mean square,

$\Sigma xi^2 = \dfrac{Sxyi^2}{(SEbi)^2}$

This is equivalent to testing that $b_1 = b_2$, that is, the slopes of the regression lines are the same.

CHAPTER 5

EXPERIMENTAL RESULTS

5.1 INTRODUCTION

In this chapter, the experimental results are presented in five major sections. Section 5.2 deals with the information obtained from the two preliminary experiments, while in other sections, data on endophyte infection, rhizobia infection, nodulation and dinitrogen fixation, total plant nitrogen and phosphorus, and plant growth and development from the principal experiment is sequentially considered.

Unless otherwise stated, the conventional statistical notations of the abbreviation ns, one star (*) and two stars (**) are used to represent non-significance ($P>0.05$), a significance at the 5% probability ($P<0.05$) and a significance at the 1% probability ($P<0.01$) respectively. The use of the capital letters C, E, R and B (as previously introduced in Chapter 4) to denote the four treatments control, endophyte, rhizobia, and both endophyte and rhizobia respectively is continued here and throughout the rest of the thesis.

5.2 PRELIMINARY RESULTS

5.21 Glasshouse Test

In this experiment, endophyte infection was observed in all the three endophyte treatments on day 31 (Table 5.1), demonstrating that these arbuscular fungi can be introduced into sainfoin plants. At this growth stage, the infection was probably new because the fungal structures occurred in isolated patches in the root cortex with few entry points, some hyphae and a limited number of arbuscles.

5.22 Field Test

In the field-grown sainfoin plants (cvs. Fakir and Remont), the mean infection score was respectively 1.3 and 1.5 (Table 5.2) which was about 18 and 22% of the infected cortical area per microscope field of view, on the basis of the percentage scale. The fungal structures

TABLE 5.1

MEANS OF ENDOPHYTE-INFECTED ROOT CORTEX
OF SAINFOIN (VAR. *bifera* Hort.)
IN THE GLASSHOUSE

Days after planting	Means of infected root segments (%)			
	Control	Culture R4	Culture NP15	Gm (*Gigaspora magarita* Becker & Hall)
5	0	0	0	0
10	0	0	0	0
31	0	40	20	10

TABLE 5.2

MEANS OF ENDOPHYTE-INFECTED ROOT CORTEX
OF SAINFOIN (CVS. FAKIR AND REMONT)
IN THE FIELD

Sainfoin cultivar	Infection score of each plant (replicate)					Mean
	1	2	3	4	5	
Fakir	1.5	1.9	0.6	-	-	1.3 ± 0.7
Remont	1.3	1.9	1.4	1.4	1.6	1.5 ± 0.2

observed included entry points, extensive hypha network, arbuscles and vesicles. Sainfoin is, therefore, basically mycotrophic.

5.3 DEVELOPMENT OF ARBUSCULAR MYCORRHIZAE

5.31 First Inoculation with Gm

The fungus, *Gigaspora magarita* Becker & Hall, did not infect sainfoin plants in treatments E and B even after 93 days of growth, although infection was expected to have occurred around day 30. Even in the roots of the later introduced corn plants, infection was extremely low in both treatments E and B regardless of the days of growth (Table 5.3).

On the contrary, in another test, when sainfoin plants were grown in a high inoculum (100% soil inoculum of Gm), about 47% of the forty 1.5 cm root segments examined was infected after 34 days of growth.

5.32 Condensed Tannins and Root Morphology of Sainfoin

As shown in Table 5.4, condensed tannins were absent in both the roots and nodules of 104 day-old sainfoin plants, but a substantial quantity was detected in their aerial organs.

The mean tertiary rootlet diameter and root hair length of sainfoin were respectively greater than 0.35 mm and less than 0.18 mm (Table 5.5). When compared with the root morphology of white clover, the tertiary rootlets of sainfoin were markedly thicker ($P<0.01$) and produced less root hairs which were often stumpy and much shorter ($P<0.01$). These data suggest that sainfoin belongs to the intermediate root type of Baylis (1975).

5.33 Second Inoculation with MX

At day 115, that is, 16 days after the second inoculation, the fungi had just begun invasion in the sainfoin roots of treatment B. In the subsequent samplings at day 126 and 137, the plants in both treatments E and B were infected with many entry points, hyphae and arbuscles, but not vesicles (Plates 5.2, 5.3 and 5.4). The endophytes did not invade nodules confirming the observations of Crush (1974), Carling *et al* (1978) and Smith *et al* (1979) in other legumes. The results are plotted against time in Figure 5.1 (p.72). Treatment

TABLE 5.3

MEANS OF INFECTED ROOT CORTEX OF CORN WITH *Gigaspora magarita* Becker & Hall

Days after planting	Mean infection score per view			
	C	E	R	B
26	0.00	0.00	0.00	0.05
34	0.00	0.05	0.00	0.00
40	0.00	0.05	0.00	0.05

TABLE 5.4

MEANS OF CONDENSED TANNIN SCORE OF DIFFERENT SAINFOIN PLANT COMPONENTS

Plant component	C	E	R	B
Older leaf	2.08	2.16	1.58	1.08
Younger leaf	3.58	3.75	2.75	2.72
Crown	3.58	3.67	3.50	3.25
Older roots	0.00	0.00	0.00	0.00
Younger roots	0.00	0.00	0.00	0.00
Nodules	0.00	0.00	0.00	0.00

TABLE 5.5

MEAN ROOT DIAMETER, MEAN EXTENSION OF THE ROOT HAIR CYLINDER FROM THE EPIDERMIS AND ROOT HAIR ABUNDANCE FOR TERTIARY ROOTLETS OF FOUR LEGUMES

Species	Root diameter (mm)	Root-hair length (mm)	Roots with root hair (%)
Centrosema pubescens Benth.†	0.288 ± 0.074	0.106 ± 0.031	17
Stylosanthes guianensis (Aubl.) Sw. †	0.285 ± 0.049	0.108 ± 0.045	6
Onobrychis viciifolia Scop.††	0.379 ± 0.030	0.155 ± 0.026	less†††
Trifolium repens L.††	0.204 ± 0.025	0.225 ± 0.009	more†††
Trifolium repens L.†	0.221 ± 0.087	0.213 ± 0.087	79
Lotus pedunculatus Cav.†	0.229 ± 0.042	0.809 ± 0.242	99

† Investigated by Crush (1974).

†† Investigated in this experiment. Using the two-tailed *t*-test formula, (Steel and Torrie, 1980),

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\left[s^2\left(\frac{n_1 + n_2}{n_1 n_2}\right)\right]}}$$

both the means of root diameter and root-hair length between sainfoin and white clover were significantly different from each other at $P<0.01$.

††† Comparison between sainfoin and white clover by visual estimation of their root-hair densities.

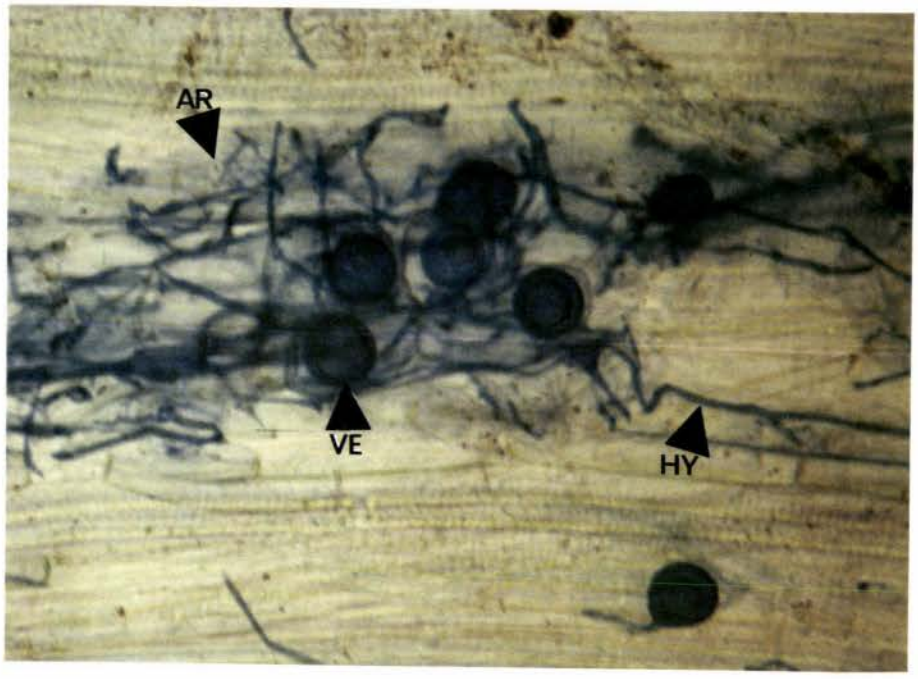


PLATE 5.1

GENERAL VIEW OF INFECTION WITH *Gigaspora magarita* Becker & Hall IN CORN (x1 220)(HY, HYPHA; AR, ARBUSCLE; VE VESICLE)

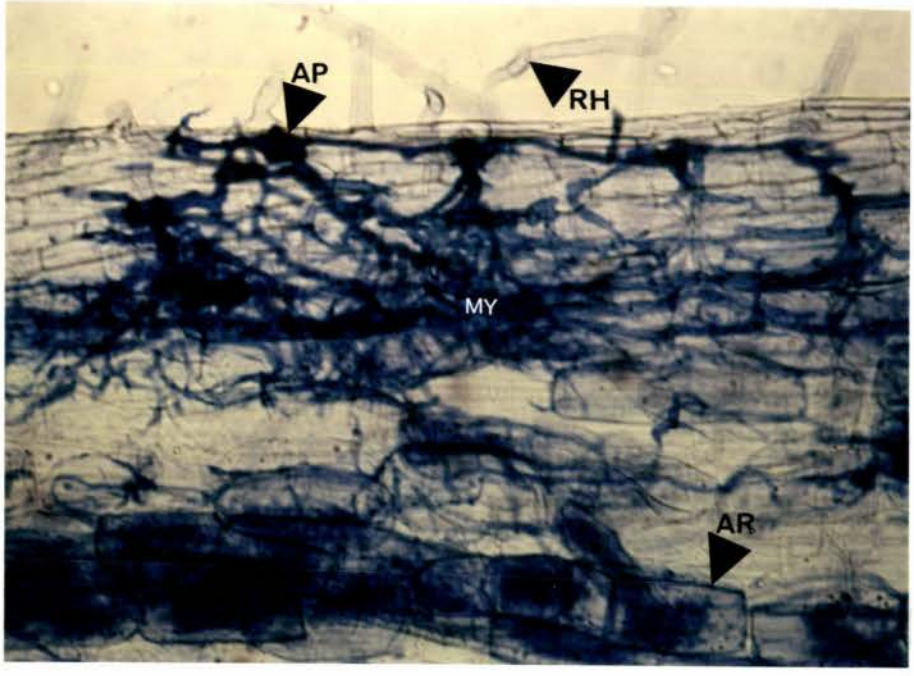


PLATE 5.2

GENERAL VIEW OF INFECTION WITH MX 1N SAINFOIN AT DAY 137 (x380) (AP, APPRESSORIUM; MY, MYCELIUM; AR, ARBUSCLE; RH, ROOT HAIR)

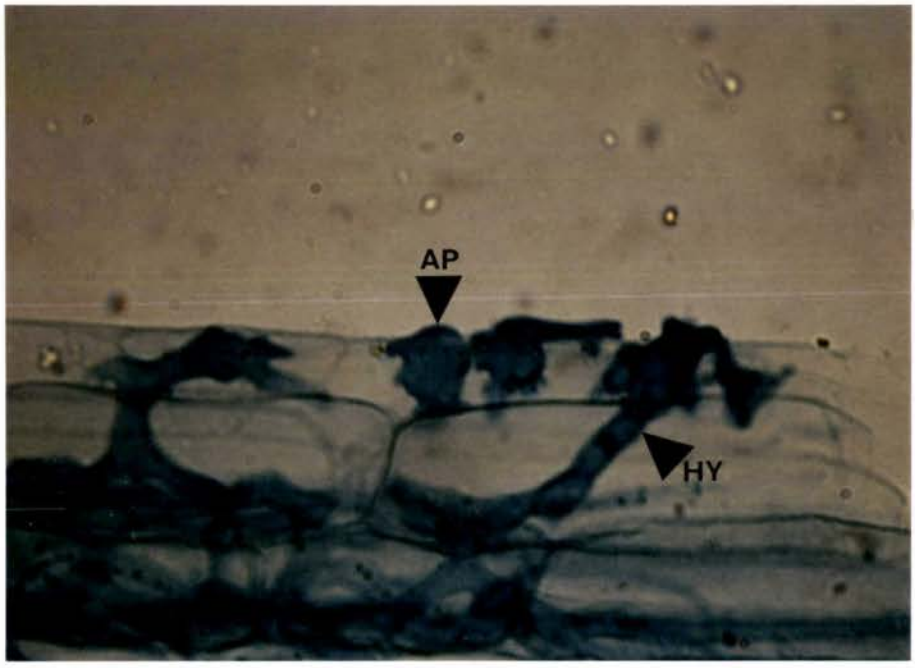


PLATE 5.3

ENDOPHYTE ENTRY POINTS IN SAINFOIN ROOTS AT DAY 137 (x1 220)(AP, APPRESSORIUM; HY, HYPHA)

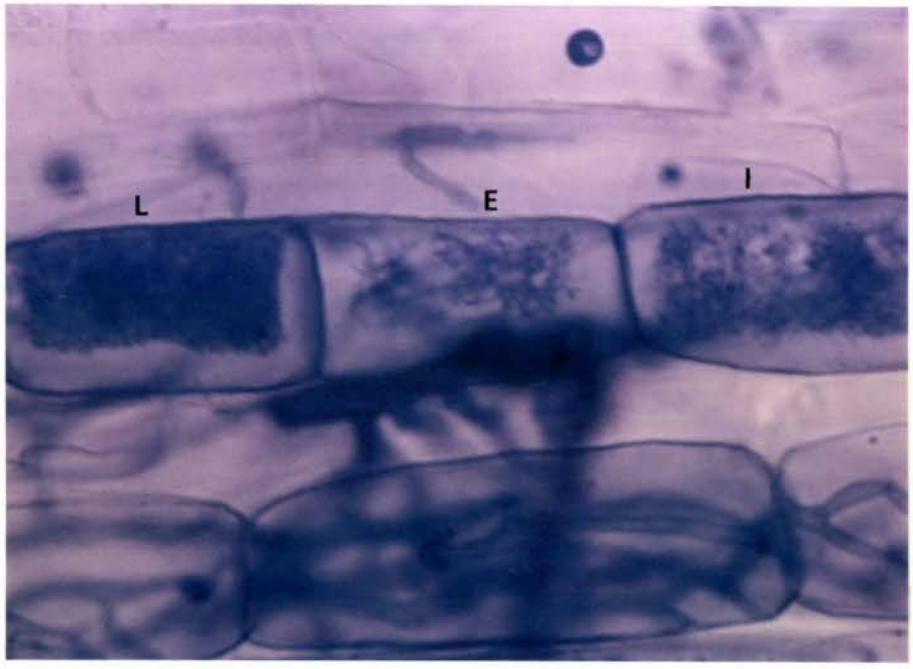


PLATE 5.4

ARBUSCLES AT DIFFERING STAGES OF DEVELOPMENT IN SAINFOIN AT DAY 137 (x1 220)(E, EARLY; I, INTERMEDIATE; L, LATE)

differences in the degree of infected cortical area were significant ($P<0.05$), as an average across time, according to the pooled ANOVA (Appendix L). However, Table 5.5 shows only two significant comparisons in infection score at day 137, that is, treatment B was greater than treatments C and R.

The high coefficient of variation for both error (a) (202%) and error (b) (221%) (see Appendix L) indicates a high variability in the data, presumably due to the small root sample (20 segments) taken for each plant.

At day 162, examination of the sainfoin plants left over from the principal experiment showed that the mean infection in treatments E and B were respectively 0.13 and 0.48, or 7.47 and 28.68 after arcsine transformation (Table 5.6). These results, although they cannot be statistically tested, appear to indicate an increase in infected root tissue since the last sampling at day 137 (Table 5.7).

5.4 DEVELOPMENT OF DINITROGEN-FIXING SYSTEM

5.41 Infection

The first nodule swelling was observed on day 27 when the plants were having a second expanded leaf and, thus, infection first occurred around this time. This was 22 days after the first rhizobia inoculation, similar to the result of Dangeard (1926), Karpov (1957) and Hume (1981) who all reported nodulation within 14 to 30 days of inoculation when the plants had a developed second or third leaf.

Microscope examination of root segments confirmed that rhizobia infection in sainfoin is via root hairs, with the characteristic deformation and shepherd's crook formation (Plate 5.5) (Fåhraeus and Ljuggren, 1968). Commonly, numerous hairs within the proximity of each other were infected at the same time (Plate 5.6) and, thus, gave rise to two or more nodules adjacent to each other. These nodules could also merge together at a later stage to form a larger nodule (Kidby and Goodchild, 1966).

5.42 Nodulation

The nodules formed were of various shapes, sizes and colour (Plate 5.7). Basically, they were branched, as large as 2.5 cm in

TABLE 5.6

MEANS OF INFECTED ROOT CORTEX OF CORN AND SAINFOIN WITH A MIXTURE OF ENDOPHYTES (MX)

Days after planting		Mean infection score per view					
		C	E (30 g inoculum)	R	B	Without sainfoin (30 g inoculum)	Without sainfoin (high inoculum)
Corn	18	0.00	0.25	0.00	0.06	0.00	0.25
	61	0.00	0.93	0.00	1.98	0.90	1.93
Sainfoin	162	0.00	0.13	0.00	0.48	-	-

TABLE 5.7

TRANSFORMED† TREATMENT MEANS†† ††† OF ENDOPHYTE INFECTION PER FIELD OF VIEW ($x10^{-2}$)

Days from germination	C	E	R	B
115	0.00 a	0.00 a	0.00 a	2.72 a
126	0.00 a	3.55 a	0.00 a	3.55 a
137	0.00 a	2.51 ab	0.00 a	8.62 b

† Equation for transformation is arcsine x.

†† Within each time, means not sharing a common letter differ significantly at $P<0.05$.

††† For all means, SE = 1.45×10^{-2}.

length and orange-white, similar to the descriptions of Smith (1972) and Corby (1981). When some nodules were sliced, the internal tissues of the inoculated treatments (R and B) were red, but those from the uninoculated treatments (C and E) were green (Plate 5.8), indicating that the former were effective and the latter ineffective in dinitrogen fixation (Vance and Johnson, 1981).

5.421 Nodule number per plant. The pooled ANOVA for this variable is shown in Table M.1 (Appendix M). As indicated in the analysis, there were significantly more nodules at certain times but not at other times ($P<0.01$), and in the rhizobia treatments (R and B) but not in the uninoculated treatments (C and E) ($P<0.01$). This is evident in Figure 5.2. The strong interaction between time and rhizobia ($P<0.01$) also implies that the differences in nodule numbers, as an average of the two endophyte levels, between rhizobia and uninoculated treatments were not the same for the various times; and the differences in nodule numbers at various times differed between the rhizobia and uninoculated treatments. Figure 5.2 and Table 5.8 show that the rhizobia effect was particularly prominent at the later stages of growth.

Similarly, the significance of the endophyte effect and time-endophyte interaction (both at $P<0.01$) indicate that the differences in nodule number, as an average of the two rhizobia levels, between the endophyte (E and B) and uninoculated (C and R) treatments were different for the various growth stages; and the differences in nodule numbers in various growth stages were not the same between the endophyte and uninoculated treatments. As shown in Table 5.8, nodule number was generally greater in treatments C and R than in treatments E and B respectively.

5.422 Nodule dry weight per plant. Table M.2 (Appendix M) presents the pooled ANOVA for nodule dry weight per plant. Unlike the previous variable, only the time, rhizobia and time-rhizobia interaction were highly significant at $P<0.01$. Contrasting differences in nodule dry weight were, therefore, indicated at different growth stages and in different treatments, inoculated and uninoculated with rhizobia. The rhizobia effect also changed with time such that nodule dry weight increased rapidly at the later stages of plant growth beginning from day 45 (Figure 5.3 and Table 5.9).

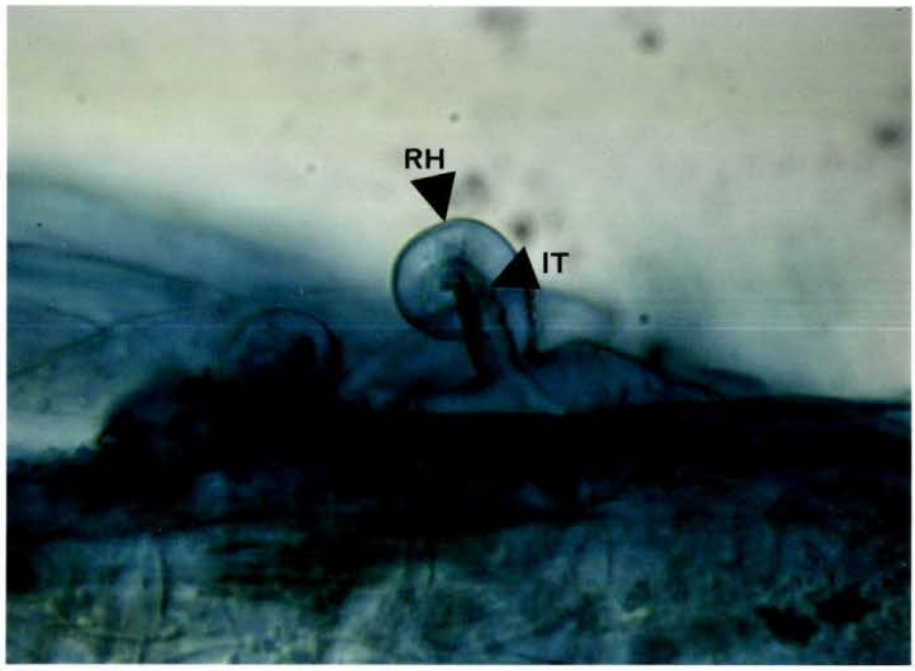


PLATE 5.5

A SAINFOIN ROOT HAIR INFECTED WITH *Rhizobium spp.* (x1 910)(RH, ROOT HAIR; IT, INFECTION THREAD)

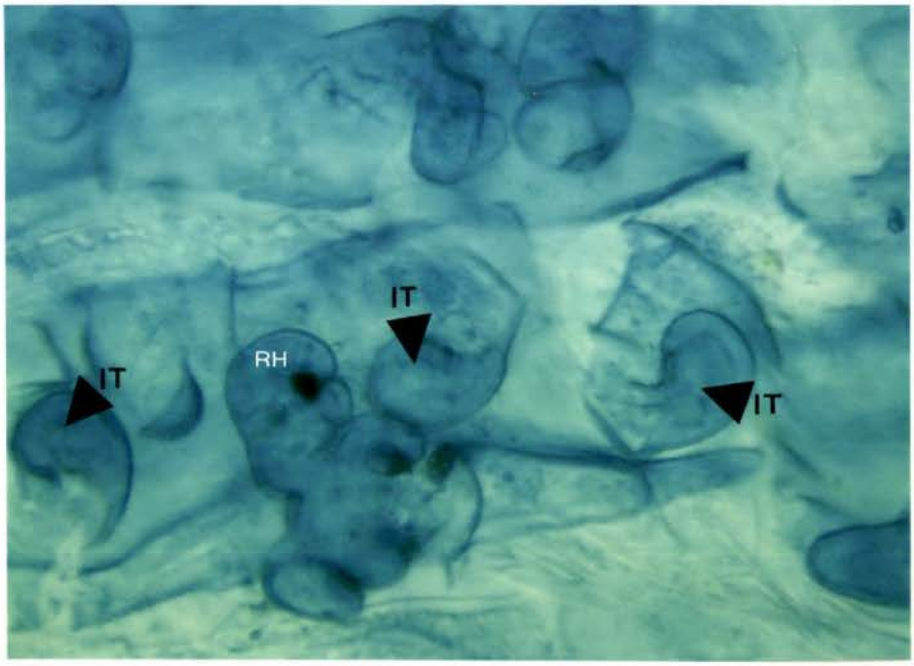


PLATE 5.6

GENERAL VIEW OF A REGION WITH MANY INFECTED ROOT HAIRS (x1 910)(RH, ROOT HAIR; IT, INFECTION THREAD)

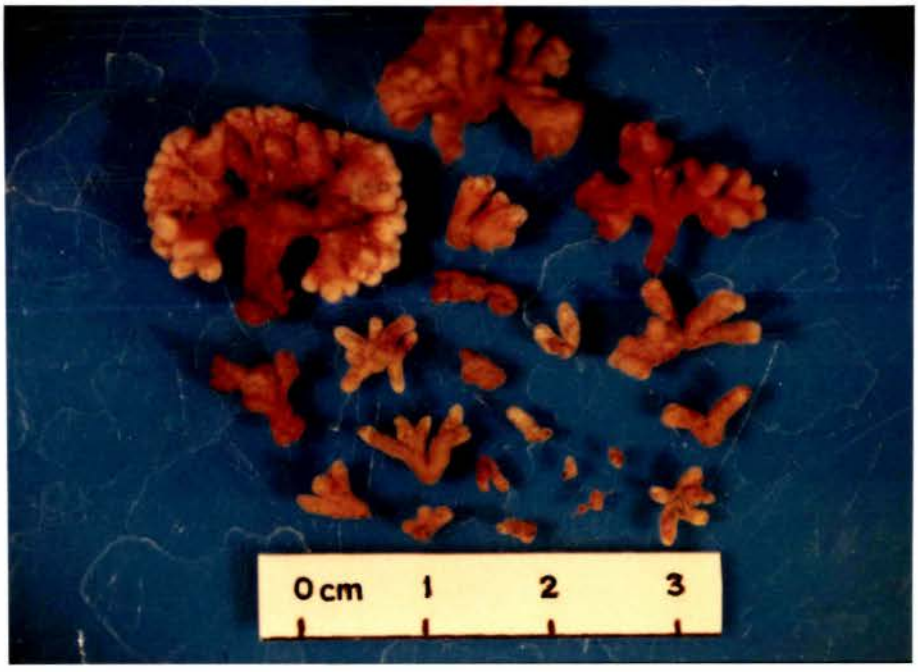


PLATE 5.7

SAINFOIN NODULES OF VARIOUS SIZES, SHAPES AND COLOUR

PLATE 5.8

GREEN (LEFT) AND RED (RIGHT) NODULES FROM THE UNINOCULATED (C AND E) AND RHIZOBIA-INOCULATED (R AND B) TREATMENTS

For Figure 5.1 to 5.4, original data are plotted:

(treatment C, ○——○; treatment E, ●——●,
treatment R, △——△; treatment B, ▲——▲).

FIGURE 5.1

MYCORRHIZA INFECTION OF SAINFOIN MEASURED AS SCORE OF INFECTED CORTEX PER MICROSCOPE FIELD OF VIEW OVER TIME (ZERO INFECTION OBTAINED FOR ALL TREATMENTS BEFORE DAY 115)

FIGURE 5.2

NODULE NUMBER PER SAINFOIN PLANT OVER TIME (ZERO DATA SET FOR ALL TREATMENTS BEFORE DAY 36)

FIGURE 5.3

NODULE DRY WEIGHT PER SAINFOIN PLANT OVER TIME (ZERO DATA SET FOR ALL TREATMENTS BEFORE DAY 36)

FIGURE 5.4

NODULE DRY WEIGHT PER SAINFOIN NODULE OVER TIME (ZERO DATA SET FOR ALL TREATMENTS BEFORE DAY 36)

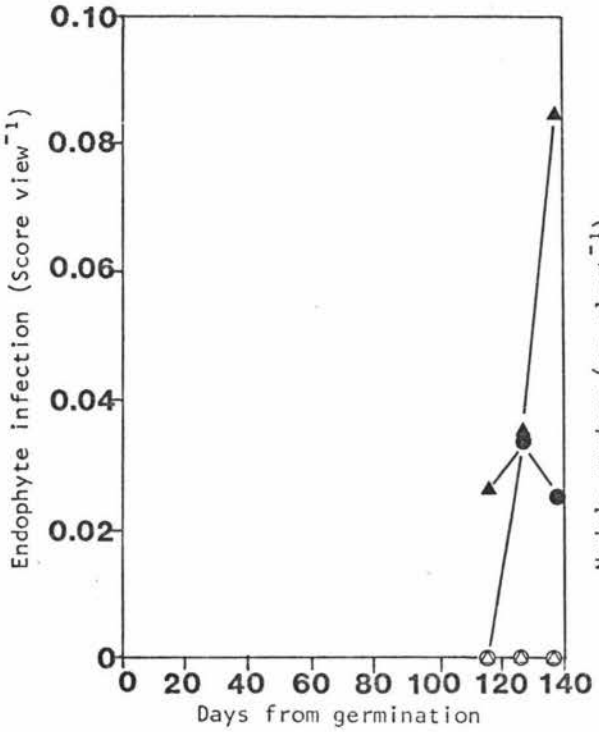


FIGURE 5.1

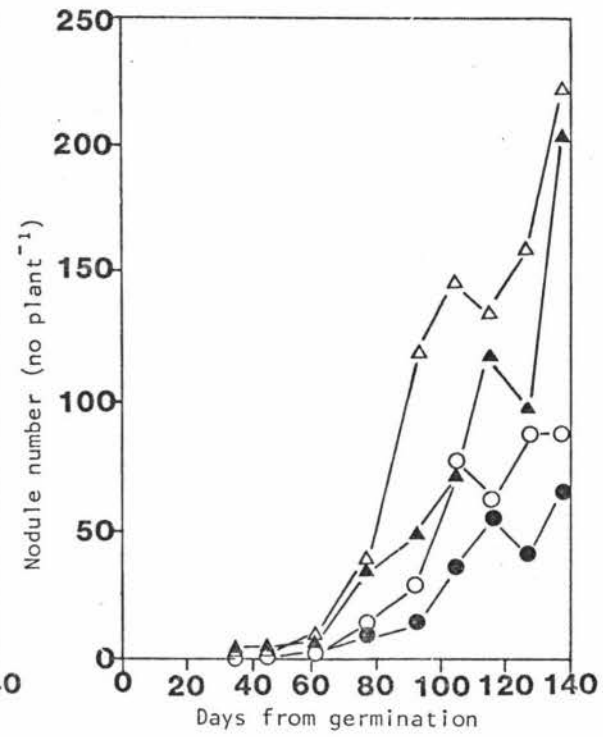


FIGURE 5.2

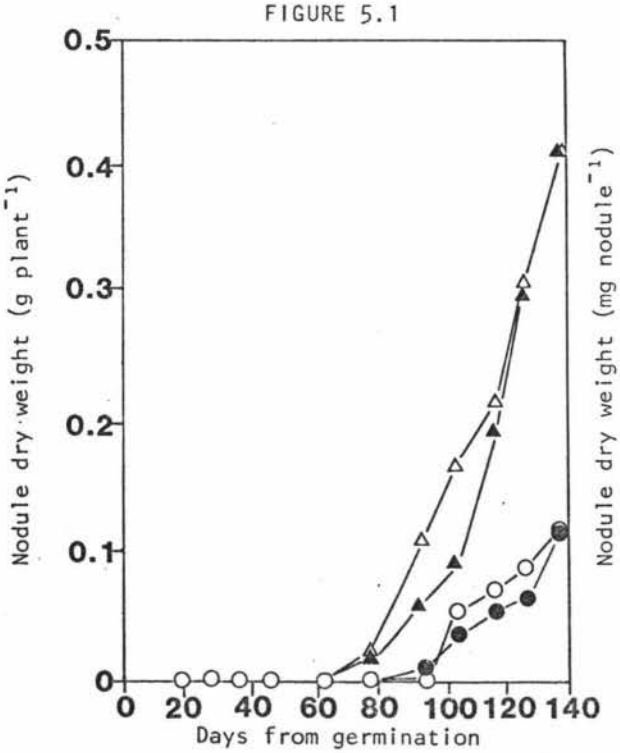


FIGURE 5.3

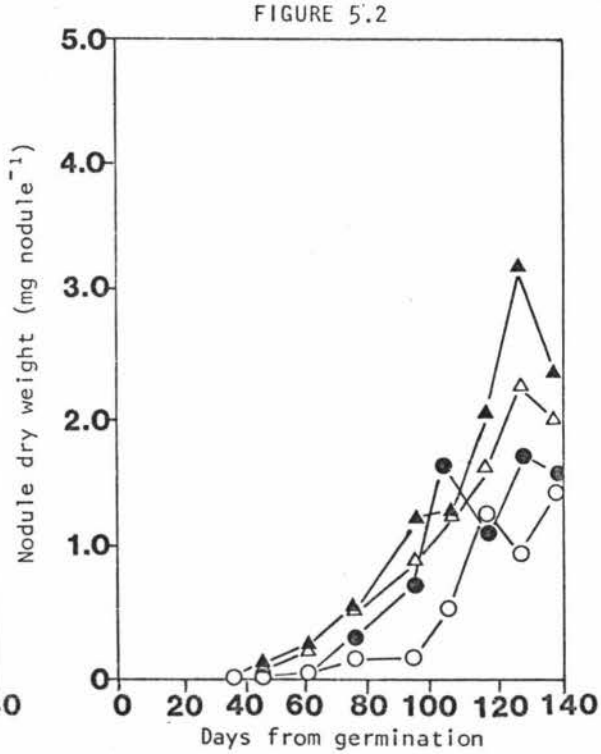


FIGURE 5.4

TABLE 5.8

TRANSFORMED† TREATMENT MEANS†† ††† OF NODULE NUMBER PER PLANT

Days from germination	C	E	R	B
36	1.00 a	1.00 a	1.24 a	1.33 a
45	1.00 a	1.00 a	1.38 b	1.41 b
61	1.13 a	1.14 a	1.78 b	1.74 b
77	1.91 a	1.72 a	2.48 b	2.39 b
93	2.21 a	1.93 a	3.27 c	2.64 b
104	2.96 b	2.35 a	3.47 c	2.90 b
115	2.74 a	2.59 a	3.40 b	3.25 b
126	3.06 b	2.43 a	3.50 c	3.12 b
137	3.06 a	2.82 a	3.82 b	3.78 b

† Equation of transformation is $\sqrt{[\sqrt{(x + 1)}]}$.

†† Within each time, means not sharing a common letter differ significantly at $P<0.05$.

††† For means from 36 to 77, SE = 0.08 while from time 93 to 137, SE = 0.11.

TABLE 5.9

TRANSFORMED† TREATMENT MEANS†† ††† OF NODULE DRY WEIGHT PER PLANT

Days from germination	C	E	R	B
36	0.00 a	0.00 a	0.04 ab	0.05 b
45	0.00 a	0.00 a	0.10 b	0.11 b
61	0.05 a	0.05 a	0.21 b	0.21 b
77	0.21 a	0.19 a	0.37 b	0.36 b
93	0.24 a	0.26 a	0.57 c	0.49 b
104	0.45 a	0.41 a	0.64 c	0.55 b
115	0.51 a	0.46 a	0.68 b	0.66 b
126	0.54 a	0.48 a	0.75 b	0.74 b
137	0.59 a	0.56 a	0.80 b	0.80 b

† Equation of transformation is $\sqrt{(\sqrt{x})}$.

†† Within each time, means not sharing a common letter differ significantly at $P<0.05$.

††† For means from time 36 to 77, SE = 0.97×10^{-2} while from time 93 to 137, SE = 1.37×10^{-2}.

Besides the rhizobia effect, there is also a significant endophyte effect ($P<0.05$). This is evident in Table 5.9 in which nodule dry weight was generally lower in treatments E and B than in treatments C and R respectively, with the results being significant ($P<0.05$) between R and B at day 93 and 104.

5.423 Nodule dry weight per nodule. The pooled ANOVA is shown in Table M.3 (Appendix M). For each nodule, the dry weight increased with time for all the treatments ($P<0.01$) (Figure 5.4). In the rhizobia inoculated treatments, the nodule size was much larger than that in the uninoculated treatments (C and E) ($P<0.01$), and these differences were more prominent at the later plant growth stages as indicated by the time-rhizobia interaction ($P<0.05$). These trends are illustrated in Figure 5.4 and Table 5.10.

The endophyte effect was also significant ($P<0.05$), with the endophyte treatments (E and B) showing a smaller nodule size than the uninoculated treatments (C and R) (Table 5.10).

5.43 Dinitrogen Fixation

5.431 Acetylene reduction per plant. Table M.4 (Appendix M) provides the pooled ANOVA for this variable. As expected, the time, rhizobia and time-rhizobia interaction were all highly significant at $P<0.01$. Marked differences were, therefore, indicated in the amount of acetylene reduced by a plant in each hour at various growth stages across the treatments and between the rhizobia (R and B) and uninoculated (C and E) treatments. With the significant time-rhizobia interaction, the rhizobia effect on acetylene reduction in each plant also changed with time, and as shown in Figure 5.5 and Table 5.11, acetylene reduction per plant sharply increased with plant maturity in the rhizobia treatments.

Since the endophyte effect is non-significant, there was neither actual acetylene reduction nor direct stimulation of acetylene reduction by the arbuscular fungi.

5.432 Acetylene reduction per gram nodule dry weight. Table M.5 (Appendix M) shows the pooled ANOVA. Differences in acetylene reduction were obtained between the rhizobia (R and B) and uninoculated (C and E) treatments ($P<0.01$), and at various growth stages ($P<0.05$).

TABLE 5.10

TRANSFORMED† TREATMENT MEANS†† ††† OF NODULE DRY WEIGHT PER NODULE ($x10^{-2}$)

Days from germination	C	E	R	B
36	0.00 a	0.00 a	0.23 a	0.29 a
45	0.00 a	0.00 a	0.66 ab	0.77 b
61	0.51 a	0.52 a	1.51 b	1.59 b
77	1.32 a	1.53 a	2.32 b	2.34 b
93	1.31 a	2.20 ab	3.06 bc	3.50 c
104	2.39 a	3.59 b	3.43 b	3.58 b
115	3.56 ab	3.29 a	4.04 ab	4.41 b
126	3.10 a	4.01 ab	4.72 bc	5.69 c
137	3.78 a	3.91 a	4.42 a	4.49 a

† Equation of transformation is $\sqrt{x}$.

†† Within each time, means not sharing a common letter differ significantly at $P<0.05$.

††† For means from time 36 to 77, SE = 0.16×10^{-2} while from 93 to 137, SE = 0.22×10^{-2}.

TABLE 5.11

TRANSFORMED† TREATMENT MEANS†† ††† OF ACETYLENE REDUCED PER HOUR PER PLANT

Days from germination	C	E	R	B
77	0.32 a	0.32 a	0.71 b	0.73 b
93	0.42 a	0.34 a	1.24 b	1.11 b
104	0.46 a	0.44 a	1.35 b	1.29 b
115	0.44 a	0.56 a	1.34 b	1.34 b
126	0.32 a	0.43 a	1.59 b	1.46 b
137	0.61 a	0.41 a	1.85 b	1.78 b

† Equation of transformation is $\sqrt{[(\sqrt{x} + 0.01)]}$.

†† Within each time, means not sharing a common letter differ significantly at $P<0.05$.

††† For means at time 77, SE = 0.08 while from time 93 to 137, SE = 0.12.

TABLE 5.12

ORIGINAL TREATMENT MEANS† †† OF ACETYLENE REDUCED PER HOUR PER GRAM NODULE DRY WEIGHT

Days from germination	C	E	R	B
77	1.57 a	0.06 a	16.60 a	16.03 a
93	16.80 a	0.18 a	23.98 a	23.37 a
104	6.63 a	2.02 a	20.14 ab	33.02 b
115	1.79 a	6.55 a	17.26 a	17.52 a
126	0.00 a	0.84 a	21.64 a	15.93 a
137	4.49 ab	1.47 a	32.11 c	28.66 b

† Within each time, means not sharing a common letter differ significantly at $P<0.05$.

†† For means at time 77, SE = 4.03 while from time 93 to 137, SE = 5.70.

For Figure 5.5 to 5.8, original data are plotted:

(treatment C, ○——○; treatment E, ●——●;
treatment R, △——△; treatment B, ▲——▲).

FIGURE 5.5

ACETYLENE REDUCED PER HOUR PER SAINFOIN PLANT OVER TIME (NO ASSAY MADE BEFORE DAY 77)

FIGURE 5.6

ACETYLENE REDUCTION PER HOUR PER GRAM NODULE DRY WEIGHT OF SAINFOIN OVER TIME (NO ASSAY MADE BEFORE DAY 77)

FIGURE 5.7

SIMPLE REGRESSION PLOTS OF NODULE DRY WEIGHT AGAINST NODULE NUMBER PER SAINFOIN PLANT FOR TREATMENTS C, E, R AND B

FIGURE 5.8

SIMPLE REGRESSION PLOTS OF NODULE DRY WEIGHT PER SAINFOIN PLANT AGAINST NODULE DRY WEIGHT PER NODULE FOR TREATMENTS C, E, R AND B

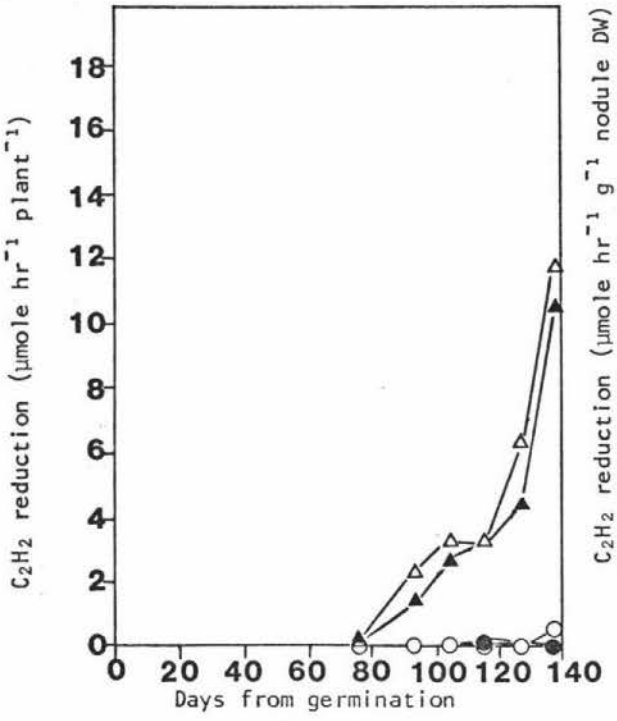


FIGURE 5.5

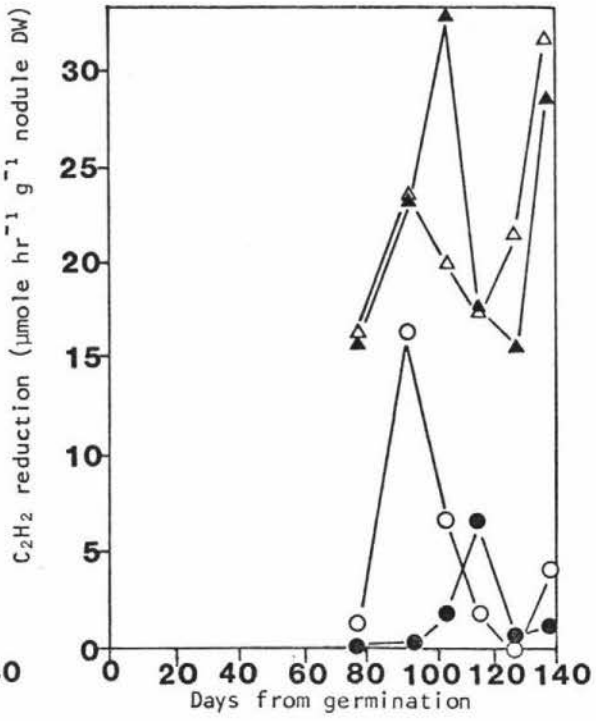


FIGURE 5.6

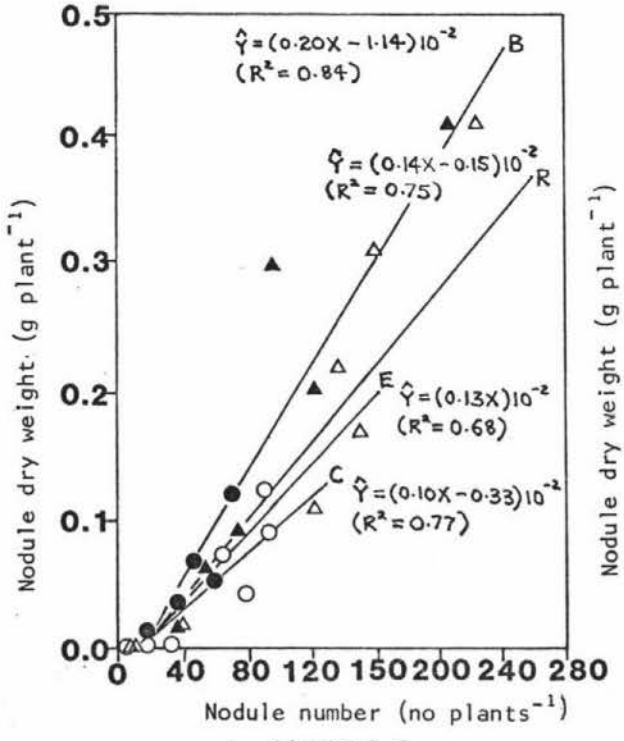


FIGURE 5.7

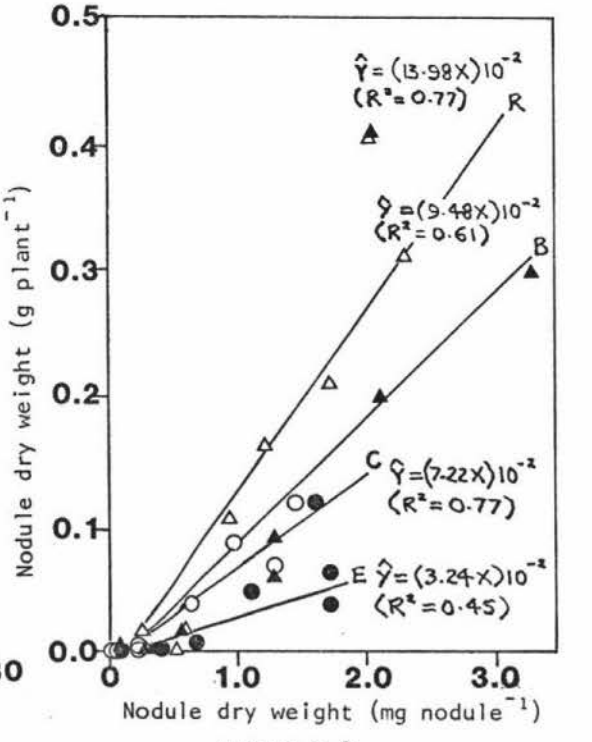


FIGURE 5.8

While nodule activity was definitely superior in the rhizobia inoculated treatments than in the uninoculated treatments across all times, the pattern of activity with time showed some periodic fluctuations (Figure 5.6 and Table 5.12).

The high coefficient of variation calculated for error (a) (114%) and error (b) (94%) is possibly due to both the inherent variation in nodule activity of the plant and the environmental variation, especially temperature, between assays.

5.44 Regression Analysis

Simple regressions of nodule dry weight per plant and nodule number and nodule dry weight per plant and nodule size are respectively given in Figures 5.7 and 5.8. Their slopes were tested for homogeneity and significant differences were obtained between the rhizobia (R and B) and uninoculated (C and E) treatments, as well as within the rhizobia and uninoculated treatments (Table 5.13).

Table 5.14 provides a summary of the stepwise multiple regression between the nodule dry weight per plant (dependent variable) and nodule number and nodule size (independent variables). The summary shows that for treatments C, E and B, nodule number was the principal determinant of nodule dry weight per plant, but for treatment R, nodule size was the principal determinant of nodule dry weight per plant.

5.5 TOTAL NITROGEN AND PHOSPHORUS IN PLANT TISSUE

5.51 Total Nitrogen

5.511 Cotyledon nitrogen concentration. The pooled ANOVA is presented in Table N.1 (Appendix N). Marked differenced in the cotyledon nitrogen occurred across time ($P<0.01$) for all treatments. Figure 5.9 shows that these differences were due to the steady decline in tissue nitrogen from day 18 to 77.

Endophyte inoculation appeared to have significant effect on the cotyledon nitrogen concentration among treatments ($P<0.05$). From Table 5.15 however, only one comparison exhibited contrasting levels of nitrogen; that is, treatment C was greater than treatment B at day 77. In general, plants inoculated with endophytes had a slightly lower level of cotyledon nitrogen.

TABLE 5.13

T-TEST VALUES FOR HOMOGENEITY OF REGRESSION SLOPES BETWEEN NODULE DRY WEIGHT AND NODULE NUMBER, AND NODULE DRY WEIGHT AND NODULE SIZE

Regression	Comparisons	DF	t-test
$\hat{Y}$ = nodule dry weight per plant X = nodule number per plant (from Figure 5.7)	b_C v b_E	152	2.029 *
	b_R v b_B	152	3.689 **
	b_C v b_R	152	2.660 **
	b_E v b_B	152	2.366 *
$\hat{Y}$ = nodule dry weight per plant X = nodule dry weight per nodule (from Figure 5.8)	b_C v b_E	152	6.633 **
	b_R v b_B	152	3.537 **
	b_C v b_R	152	5.505 **
	b_E v b_B	152	6.214 **

ns non-significant with P>0.05
* significant at P<0.05
** significant at P<0.01

TABLE 5.14

SUMMARY OF STEPWISE MULTIPLE REGRESSION ANALYSIS FOR DIFFERENT TREATMENTS

Treatment	Independent variable	R^2	R^2 change	b	a
C	nodule number	0.77	0.77	0.60×10^{-3}	-0.74×10^{-2}
	nodule size	0.90	0.13	42.07	
E	nodule number	0.68	0.68	1.00×10^{-3}	-0.70×10^{-2}
	nodule size	0.81	0.13	19.38	
R	nodule size	0.77	0.77	86.40	-0.28×10^{-1}
	nodule number	0.93	0.16	0.86×10^{-3}	
B	nodule number	0.84	0.84	0.15×10^{-2}	-0.29×10^{-1}
	nodule size	0.92	0.08	43.96	

For Figure 5.9 to 5.12, original values are plotted:

(treatment C, o——o ; treatment E, ●——● ;
treatment R, Δ——Δ ; treatment B, ▲——▲).

FIGURE 5.9

TOTAL COTYLEDON NITROGEN CONCENTRATION OF SAINFOIN OVER TIME (INADEQUATE TISSUE FOR ANALYSIS AT DAY 93, WHILE ZERO DATA SETS AFTER DAY 104)

FIGURE 5.10

TOTAL SHOOT NITROGEN CONCENTRATION OF SAINFOIN OVER TIME

FIGURE 5.11

TOTAL ROOT NITROGEN CONCENTRATION OF SAINFOIN OVER TIME

FIGURE 5.12

TOTAL NODULE NITROGEN CONCENTRATION OF SAINFOIN OVER TIME (INADEQUATE TISSUE FOR ANALYSIS AT DAY 36, 45 AND 61, WHILE ZERO DATA SET BEFORE DAY 27)

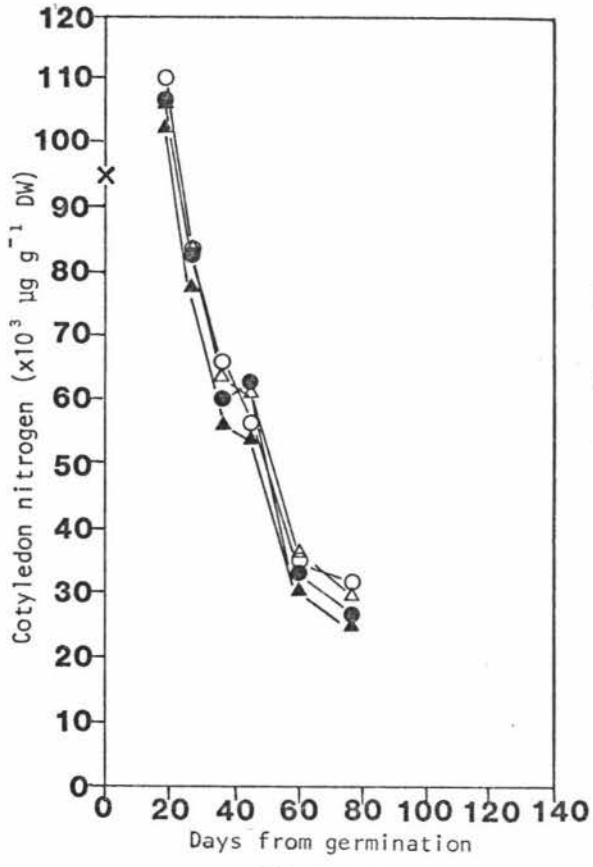


FIGURE 5.9

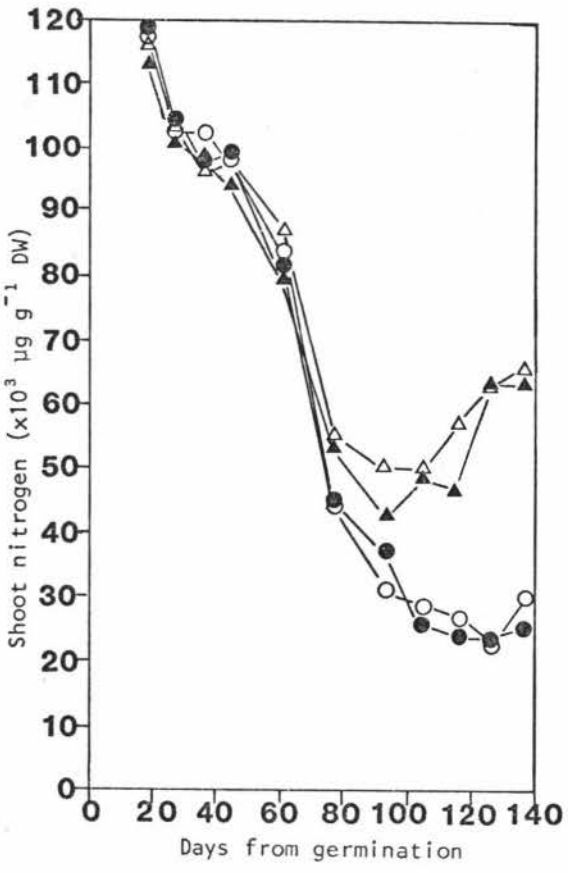


FIGURE 5.10

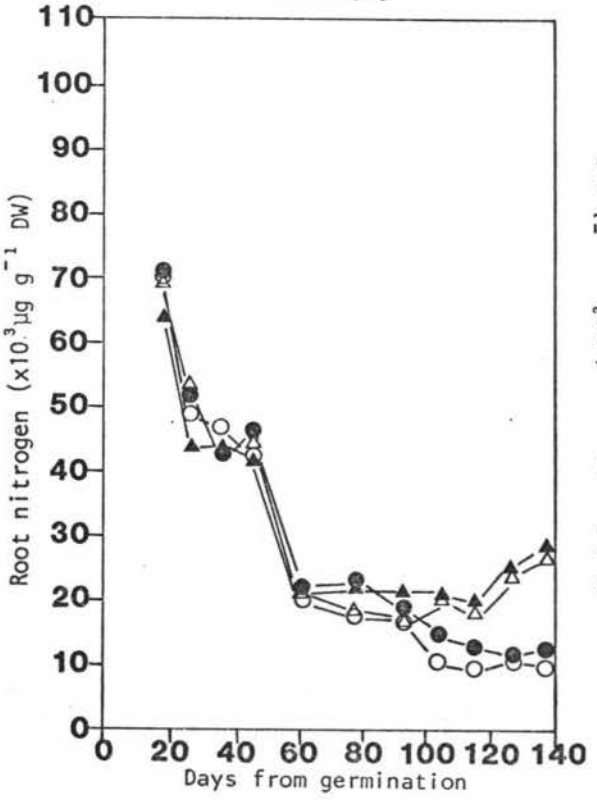


FIGURE 5.11

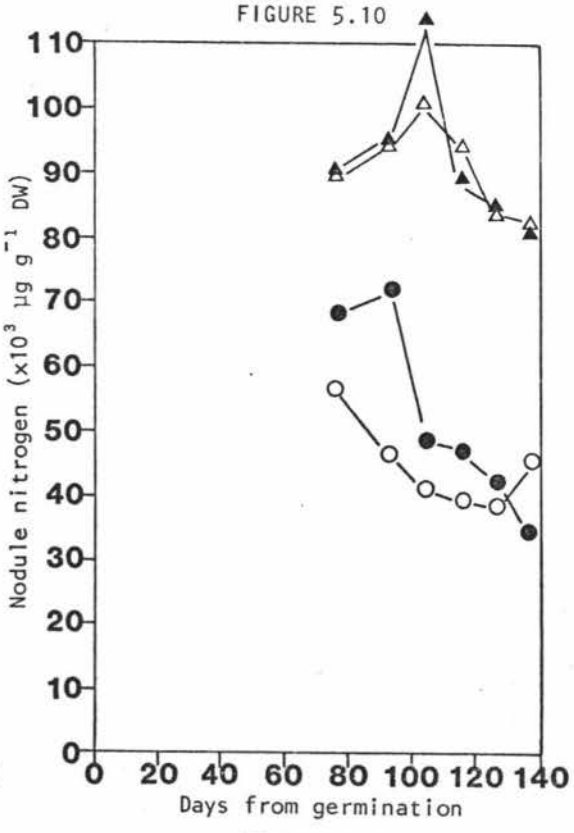


FIGURE 5.12

TABLE 5.15

TRANSFORMED† TREATMENT MEANS†† ††† OF TOTAL COTYLEDON NITROGEN

Days from germination	C	E	R	B
18	11.61 a	11.57 a	11.58 a	11.52 a
27	11.32 a	11.32 a	11.34 a	11.25 a
36	11.09 a	11.00 a	11.06 a	10.93 a
45	10.95 a	11.04 a	11.04 a	10.90 a
61	10.48 a	10.40 a	10.49 a	10.32 a
77	10.39 b	10.22 ab	10.32 ab	10.17 a

† Equation for transformation is $ln\ (x + 0.001)$.

†† Within each time, means not sharing a common letter differ significantly at $P<0.05$.

††† For all means, SE = 0.04.

TABLE 5.16

TRANSFORMED† TREATMENT MEANS†† ††† OF TOTAL SHOOT NITROGEN

Days from germination	C	E	R	B
18	1.11 a	1.12 a	1.10 a	1.08 a
27	1.03 a	1.04 a	1.04 a	1.03 a
36	1.03 a	1.02 a	1.02 a	1.02 a
45	1.02 a	1.02 a	1.02 a	1.02 a
61	1.00 a	1.00 a	1.01 a	1.00 a
77	0.98 a	0.98 a	1.00 a	1.00 a
93	0.94 a	0.96 a	0.99 a	0.98 a
104	0.93 a	0.92 a	0.99 b	0.99 b
115	0.92 a	0.91 a	1.00 b	0.99 b
126	0.89 a	0.90 a	1.00 b	1.00 b
137	0.94 a	0.91 a	1.00 b	1.00 b

† Equation of transformation is $(10^{-5}x-0.7)^3+1$.

†† Within each time, means not sharing a common letter differ significantly at $P<0.05$.

††† For all means from time 18 to 77, SE = 9.38×10^{-3} while from time 93 to 137, SE = 1.32×10^{-2}.

TABLE 5.17

TRANSFORMED† TREATMENT MEANS†† ††† OF TOTAL ROOT NITROGEN

Days from germination	C	E	R	B
18	11.17 a	11.17 a	11.14 a	11.06 a
27	10.78 a	10.86 a	10.90 a	10.69 a
36	10.75 a	10.66 a	10.67 a	10.69 a
45	10.66 a	10.77 a	10.72 a	10.64 a
61	9.92 a	10.05 a	9.98 a	10.00 a
77	9.82 a	10.05 a	9.84 a	9.99 a
93	9.77 a	9.84 a	9.72 a	9.98 a
104	9.30 a	9.59 ab	9.96 b	9.97 b
115	9.21 a	9.51 ab	9.78 bc	9.92 c
126	9.34 a	9.38 a	10.10 b	10.12 b
137	9.30 a	9.51 a	10.21 b	10.28 b

† Equation of transformation is $ln\ (x)$.

†† Within each time, means not sharing a common letter differ significantly at $P<0.05$.

††† For all means from time 18 to 77, SE = 0.06 while from time 93 to 137, SE = 0.09.

TABLE 5.18

TRANSFORMED† TREATMENT MEANS†† ††† OF TOTAL NODULE NITROGEN

Days from germination	C	E	R	B
77	63.43 a	64.18 a	66.10 b	66.11 b
93	62.20 a	64.88 b	66.44 b	66.48 b
104	61.95 a	62.45 a	66.88 b	67.54 b
115	61.32 a	62.34 a	66.35 b	66.05 b
126	61.15 a	61.69 a	65.78 b	65.92 b
137	62.10 a	60.45 a	65.72 b	65.56 b

† Equation of transformation is $ln\ (x^{5.8} + 0.001)$.

†† Within each time, means not sharing a common letter differ significantly at $P<0.05$.

††† For means at time 77, SE = 0.41 while from time 93 to 137, SE = 0.59.

TABLE 5.19

ORIGINAL TREATMENT MEANS† †† OF TOTAL PLANT NITROGEN

Days from germination	C	E	R	B
18	99 371.90a	98 991.90a	97 567.90a	92 972.90a
27	78 104.90a	79 891.40a	80 447.40a	73 688.90a
36	71 735.40a	66 846.90a	nd	nd
45	65 936.40a	69 737.40a	nd	nd
61	nd	nd	59 185.90a	59 487.30a
77	37 922.90a	40 781.90a	48 612.40b	47 984.40b
93	nd	nd	nd	nd
104	26 911.40a	29 950.40a	57 826.40b	61 329.90b
115	25 342.90a	28 246.40a	56 449.90b	57 069.40b
126	24 038.90a	25 566.90a	57 342.90b	58 081.90b
137	28 564.40a	23 986.40a	58 946.40b	57 974.40b

† Within each time, means not sharing a common letter differ significantly at $P<0.05$.

†† For means from time 18 to 77, SE = 2233.64 while from time 104 to 137, SE = 3158.85.

nd Not determined due to missing data in one or more plant organs.

5.512 Shoot nitrogen concentration. Table N.2 (Appendix N) gives the pooled ANOVA for this variable. The nitrogen concentrations in the shoot again differed at various growth stages ($P<0.01$). The trend of nitrogen concentration for each treatment is shown in Figure 5.10.

The pooled ANOVA also demonstrates a significant rhizobia ($P<0.05$) and time-rhizobia interaction ($P<0.01$). As shown in Figure 5.10 and Table 5.16, rhizobia inoculation resulted in greater shoot nitrogen in the rhizobia treatments (R and B) than in the uninoculated ones (C and E) between day 93 and 137. Such differences became more prominent with time.

5.513 Root nitrogen concentration. Table N.3 (Appendix N) shows the pooled ANOVA. As indicated in the analysis, nitrogen in the root occurred in different concentrations at certain times ($P<0.01$) with a general decline in the four treatments (Figure 5.11).

The highly significant time-rhizobia interaction and rhizobia effect (both at $P<0.01$) demonstrate that the levels of root nitrogen between the rhizobia (R and B) and uninoculated (C and E) treatments were different at certain times. Table 5.17 shows that this occurred between day 104 and 137.

5.514 Nodule nitrogen concentration. Table N.4 (Appendix N) shows the pooled ANOVA. The rhizobia and uninoculated treatments differed enormously in the nodule nitrogen concentration ($P<0.01$). These differences are evident in Figure 5.12 and Table 5.18 in which the differences between the rhizobia (R and B) and uninoculated (C and E) treatments were significant at all times.

Nitrogen concentration in the nodules of treatments R and B increased to a peak at day 104 after which it steadily decreased, but the concentration in the nodules of treatments C and E decreased with time (Figure 5.12). These variations were significant at $P<0.05$.

5.515 Plant nitrogen concentration. In Table N.5 (Appendix N), the pooled ANOVA indicates significant differences in the total plant nitrogen between times and between the rhizobia (R and B) and uninoculated (C and E) treatments (both at $P<0.01$), and a strong time-rhizobia interaction ($P<0.01$). Figure 5.17 and Table 5.19 reveals that the differences in plant nitrogen between treatments R and B and treatments

C and E were significant from day 77 and became more distinctive in the last few samplings.

5.52 Total Phosphorus

5.521 Cotyledon phosphorus concentration. The pooled ANOVA of cotyledon phosphorus is shown in Table N.6 (Appendix N). Marked differences in cotyledon phosphorus were obtained between times ($P<0.01$) and between the endophyte (E and B) and uninoculated (C and R) treatments. Cotyledon phosphorus steadily declined with plant maturity for all treatments (Figure 5.13), but this decline was generally lesser in treatments C and R than in treatments E and B with the differences in means being significant at day 36, 61 and 77 (Table 5.20).

5.522 Shoot phosphorus concentration. Table N.7 (Appendix N) presents the pooled ANOVA for this variable. Shoot phosphorus concentrations were different between times ($P<0.01$) and between the rhizobia (R and B) and uninoculated (C and E) treatments ($P<0.01$). A strong time-rhizobia interaction was also evident ($P<0.01$). In general, the phosphorus concentrations of treatments R and B decreased with plant maturity, but those of treatments C and E initially declined and after day 77, increased (Figure 5.14). In Table 5.21, the multiple range tests showed that these differences in phosphorus concentrations between treatments R and B and treatments C and E were significant at day 126 and 137.

5.523 Root phosphorus concentration. Table N.8 (Appendix N) shows the pooled ANOVA for this variable. Unlike the shoot phosphorus concentrations, the only differences in root phosphorus concentrations obtained were between times ($P<0.01$) and between the endophyte (E and B) and uninoculated (C and R) treatments ($P<0.05$). The effect due to time and endophytes were also independent of each other. The trends of treatment phosphorus levels in the roots were illustrated in Figure 5.15. In the multiple range test, there was only one significant comparison, that is, between treatments C and B at day 137 and, therefore, indicate that generally, the treatment means within each time were similar (Table 5.22).

5.524 Nodule phosphorus concentration. In Table N.9 (Appendix N), the pooled ANOVA shows significant effects due to time and rhizobia (both at $P<0.01$). Phosphorus concentrations in the

For Figures 5.13 to 5.16, original values are plotted
(treatment C, o——o ; treatment E, ●——● ;
treatment R, △——△ ; treatment B, ▲——▲ .

FIGURE 5.13

TOTAL COTYLEDON PHOSPHORUS CONCENTRATION OF SAINFOIN OVER TIME (INADEQUATE TISSUE FOR ANALYSIS AT DAY 93, WHILE ZERO DATA SETS AFTER DAY 104)

FIGURE 5.14

TOTAL SHOOT PHOSPHORUS CONCENTRATION OF SAINFOIN OVER TIME

FIGURE 5.15

TOTAL ROOT PHOSPHORUS CONCENTRATION OF SAINFOIN OVER TIME

FIGURE 5.16

TOTAL NODULE PHOSPHORUS CONCENTRATION OF SAINFOIN OVER TIME (INADEQUATE TISSUE FOR ANALYSIS AT DAY 36, 45 AND 61, WHILE ZERO DATA SET BEFORE DAY 27)

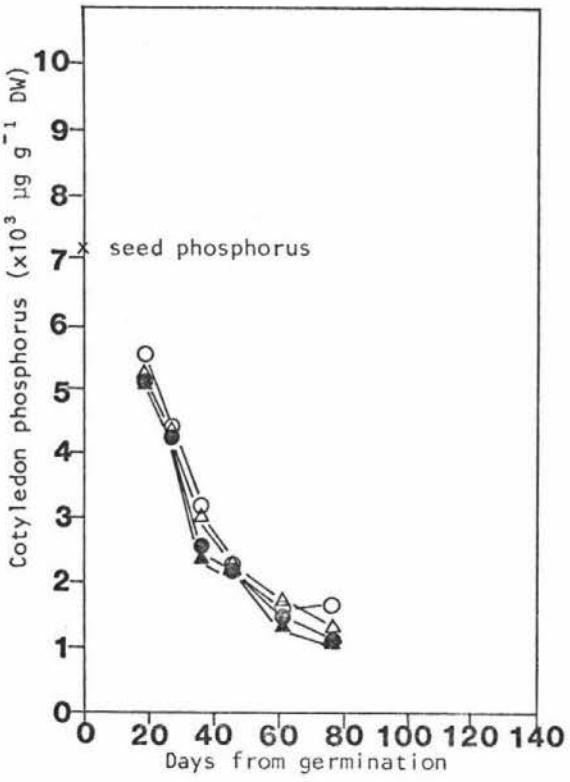


FIGURE 5.13

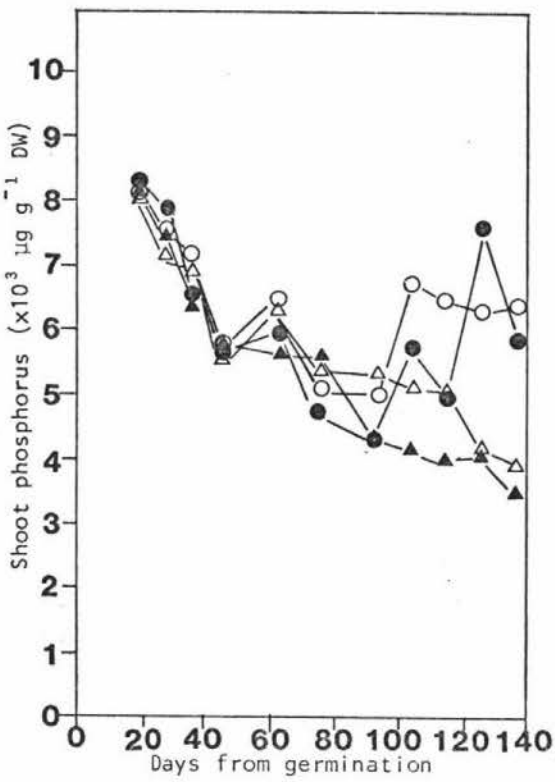


FIGURE 5.14

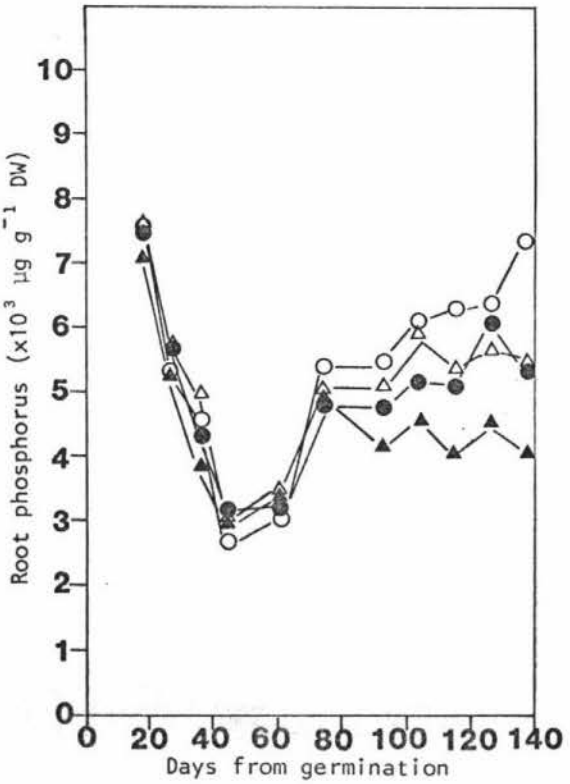


FIGURE 5.15

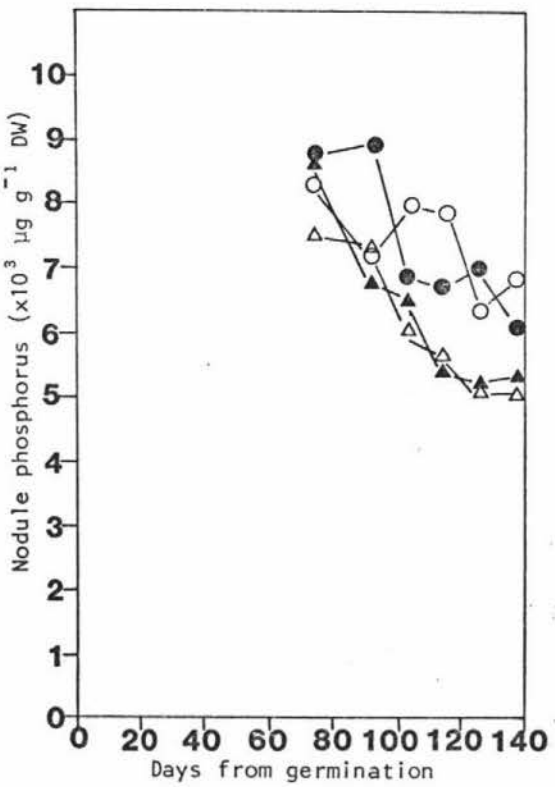


FIGURE 5.16

For Figures 5.17 to 5.18, original values are plotted. Broken lines denote missing values within due to inadequate tissue for analysis (treatment C, o——o ; treatment E, ●——● ; treatment R, △——△ ; treatment B, ▲——▲).

FIGURE 5.17

TOTAL PLANT NITROGEN CONCENTRATION OF SAINFOIN OVER TIME

FIGURE 5.18

TOTAL PLANT PHOSPHORUS CONCENTRATION OF SAINFOIN OVER TIME

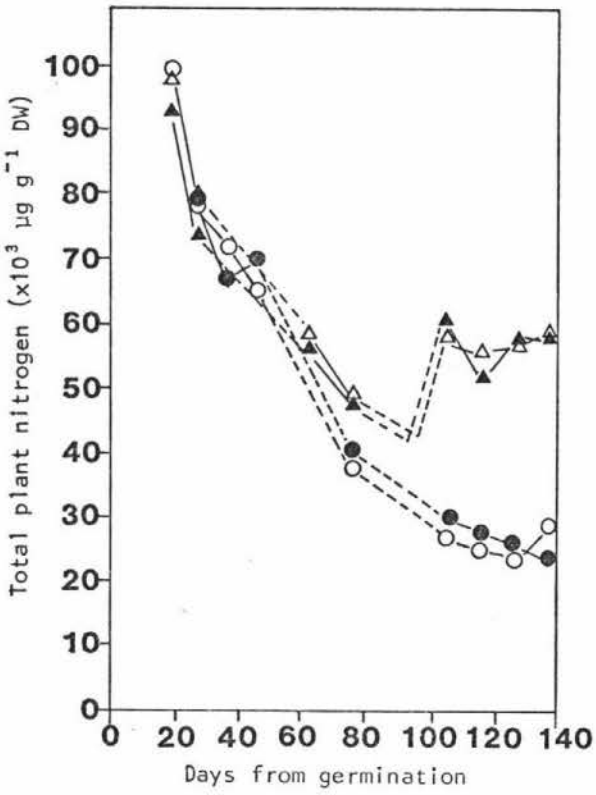


FIGURE 5.17

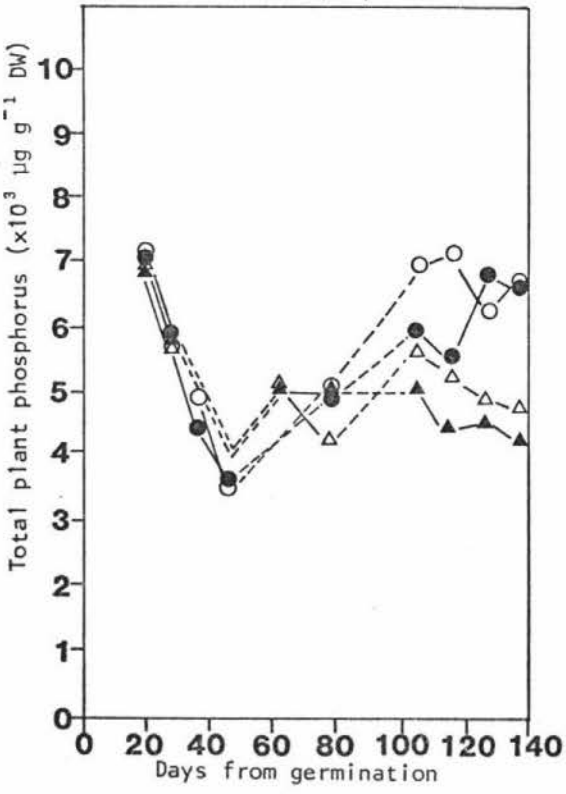


FIGURE 5.18

TABLE 5.20

TRANSFORMED† TREATMENT MEANS†† ††† OF TOTAL COTYLEDON PHOSPHORUS

Days from germination	C	E	R	B
18	17.23 a	17.09 a	17.10 a	17.09 a
27	16.74 a	16.67 a	16.70 a	16.64 a
36	16.07 b	15.61 ab	15.90 ab	15.47 a
45	15.38 a	15.37 a	15.45 a	15.37 a
61	14.77 ab	14.47 a	14.99 b	14.28 a
77	14.83 b	14.06 a	14.33 ab	14.23 a

† Equation for transformation is $ln(x^{2.0} + 0.001)$.

†† Within each time, means not sharing a common letter differ significantly at $P<0.05$.

††† For all means, SE = 0.11.

TABLE 5.21

ORIGINAL TREATMENT MEANS† †† OF TOTAL SHOOT PHOSPHORUS

Days from germination	C	E	R	B
18	8 093.50a	8 266.50a	7 910.50a	8 062.50a
27	7 625.00a	7 787.50a	7 125.00a	7 489.00a
36	7 144.00a	6 640.50a	6 915.00a	6 445.50a
45	5 716.50a	5 750.50a	5 480.00a	5 812.50a
61	6 375.00a	5 887.50a	6 281.50a	5 687.50a
77	5 098.50a	4 726.10a	5 375.00a	5 666.50a
93	4 982.00a	4 285.50a	5 354.00a	4 321.00a
104	6 725.00c	5 850.50bc	5 010.00ab	4 099.50a
115	6 426.50b	4 904.00a	4 922.00ab	3 971.00a
126	6 257.00b	7 625.00b	4 184.50a	4 009.50a
137	6 387.00b	5 781.50b	3 875.00a	3 412.00a

† Within each time, means not sharing a common letter differ significantly at $P<0.05$.

†† For all means from time 18 to 77, SE = 237.35 while from time 93 to 137, SE = 335.66

TABLE 5.22

ORIGINAL TREATMENT MEANS† ††OF TOTAL ROOT PHOSPHORUS

Days from germination	C	E	R	B
18	7 577.50a	7 517.50a	7 561.50a	7 112.00a
27	5 312.50a	5 815.50a	5 762.00a	5 665.50a
36	4 574.50a	4 299.00a	5 005.50a	3 820.50a
45	2 730.00a	3 220.50a	3 070.50a	3 000.00a
61	3 130.00a	3 221.00a	3 488.00a	3 443.00a
77	5 534.00a	4 854.91a	5 133.00a	4 877.50a
93	5 477.00a	4 801.50a	5 058.50a	4 231.00a
104	6 199.50a	5 252.50a	6 031.50a	4 736.50a
115	6 315.50a	5 071.50a	5 375.50a	4 106.00a
126	6 374.50a	6 072.00a	5 726.50a	4 656.00a
137	7 209.50b	5 448.50ab	5 502.50ab	4 168.00a

† Within each time, means not sharing a common letter differ significantly at $P<0.05$.

†† For all means from 18 to 77, SE = 379.14 while from 93 to 137, SE = 536.19.

TABLE 5.23

TRANSFORMED† TREATMENT MEANS†† ††† OF TOTAL NODULE PHOSPHORUS

Days from germination	C	E	R	B
77	49.64 ab	49.90 b	49.11 a	49.92 b
93	48.83 a	49.91 b	49.02 a	48.51 a
104	49.40 b	48.62 ab	47.82 a	48.23 a
115	50.01 c	48.48 b	47.45 a	47.29 a
126	48.17 bc	48.70 c	46.83 a	47.03 a
137	48.64 b	47.85 ab	46.98 a	47.28 a

† Equation of transformations is $ln(x^{5.5} + 0.001)$.

†† Within each time, means not sharing a common letter differ significantly at $P<0.05$.

††† For means at time 77, SE = 0.14 while from time 93 to 137, SE = 0.20.

TABLE 5.24

ORIGINAL TREATMENT MEANS† †† OF TOTAL PLANT PHOSPHORUS

Days from germination	C	E	R	B
18	7 068.99b	6 979.99b	6 881.99ab	6 776.99a
27	5 754.00a	5 952.50b	5 703.00a	5 784.50ab
36	4 989.50b	4 481.49a	nd	nd
45	3 545.50a	3 714.00a	nd	nd
61	nd	nd	5 193.50b	5 226.32a
77	5 161.00b	4 873.58a	4 838.00a	5 129.16b
93	nd	nd	nd	nd
104	6 964.50d	6 007.99c	5 669.50b	5 097.99a
115	7 212.99d	5 602.99c	5 323.50b	4 500.50a
126	6 330.99c	6 912.99d	4 972.49b	4 613.99a
137	6 845.50d	5 759.50c	4 835.00b	4 332.00a

† Within each time, means now sharing a common letter differ significantly at $P<0.05$.

†† For means from time 18 to 77, SE = 42.19 while from time 104 to 137, SE = 59.67.

nd Not determined due to missing values in one or more plant organs.

nodules of all treatments decreased with time (Figure 5.16). The rhizobia treatments showed a lower concentration of nodule phosphorus than the uninoculated treatments (C and E), with the significant differences occurring from day 93 onwards (Table 5.23).

5.525 Plant phosphorus concentration. In the pooled ANOVA for total plant phosphorus concentration (Table N.10 in Appendix N), the effect of time, rhizobia and endophyte were all highly significant ($P<0.01$). Significant time-rhizobia interaction ($P<0.01$) and rhizobia-endophyte interaction ($P<0.05$) were also obtained. The analysis indicates that differences in the plant phosphorus concentration due to rhizobia changed with time and influenced by the inoculation of endophytes. In addition, the endophyte effect was also dependent on the rhizobia inoculation. Such complex interactions are revealed in both Figure 5.18 and Table 5.24 in which consistent trends were apparent only from day 104 onwards. During this period of growth, the presence of either rhizobia or endophyte generally resulted in a lower phosphorus level when comparing the means of treatments C and R, E and B, C and E, and R and B.

5.53 Correlation Studies

As shown in Table 5.25, a consistent positive correlation was obtained in both treatments R and B between acetylene reduction per plant and total shoot nitrogen, and between acetylene reduction per plant and total root nitrogen (both correlations at $P<0.01$). In the same treatments, however, acetylene reduction per plant was negatively correlated with the total nodule nitrogen ($P<0.05$). There was no consistent result obtained in treatments R and B between acetylene reduction per gram nodule dry weight and any of the tissue nitrogen concentration.

On examining the correlation coefficients of acetylene reduction and tissue phosphorus (Table 5.26), a consistent negative correlation was derived in treatments R and B between acetylene reduction per plant and total shoot phosphorus, and between acetylene reduction per plant and total nodule phosphorus, (both correlations at $P<0.01$). However, the acetylene reduction per gram nodule dry weight was not correlated with any of the tissue phosphorus concentration.

TABLE 5.25

CORRELATIONS OF TISSUE NITROGEN AND ACETYLENE REDUCTION

Acetylene reduction	Treatment†	Total plant nitrogen	Total shoot nitrogen	Total root nitrogen	Total nodule nitrogen
µmole C_2H_2 plant^{-1} hr^{-1}	C	0.01 ns	0.05 ns	-0.21 ns	0.00 ns
	E	-0.13 ns	-0.36 *	-0.24 ns	-0.15 ns
	R	0.66 **	0.62 **	0.81 **	-0.40 *
	B	0.36 ns	0.59 **	0.68 **	-0.39 *
µmole C_2H_2 g^{-1} nodule dry weight hr^{-1}	C	0.05 ns	0.08 ns	0.30 ns	0.29 ns
	E	-0.12 ns	-0.30 ns	-0.18 ns	-0.13 ns
	R	0.32 ns	0.17 ns	0.53 **	-0.48 **
	B	0.48 *	-0.04 ns	0.03 ns	-0.33 ns

† C, control; E, endophyte; R, rhizobia; B, both

ns non-significant with P >0.05

* significant at P <0.05

** significant at P <0.01

TABLE 5.26

CORRELATIONS OF TISSUE PHOSPHORUS AND ACETYLENE REDUCTION

Acetylene reduction	Treatment†	Total plant phos-phorus	Total shoot phos-phorus	Total root phos-phorus	Total nodule phos-phorus
	C	0.17 ns	0.09 ns	0.41 *	-0.12 ns
µmole C_2H_2	E	0.23 ns	0.23 ns	0.13 ns	-0.18 ns
$plant^{-1}$ hr^{-1}	R	-0.15 ns	-0.80 **	0.13 ns	-0.64 **
	B	-0.60 **	-0.67 **	-0.34 ns	-0.57 **
	C	0.21 ns	-0.24 ns	-0.29 ns	0.02 ns
µmole C_2H_2	E	0.10 ns	0.04 ns	0.00 ns	-0.10 ns
g^{-1} nodule dry	R	-0.01 ns	0.29 ns	0.02 ns	-0.12 ns
weight hr^{-1}	B	0.09 ns	-0.28 ns	-0.05 ns	-0.13 ns

† C, control; E, endophyte; R, rhizobia; B, both

ns -on-significant

* significant at P <0.05

** significant at P <0.01

5.6 GROWTH AND DEVELOPMENT OF SAINFOIN

5.61 Plant Dry Weight

Plate 5.9 and 5.10 respectively show the growth differences among the four treamtents at day 93 and 137. Nitrogen-deficient symptoms were beginning to develop in the plants of treatments C and E at day 104 and after day 115, the symptoms were more pronounced even with a low maintenance supply of nitrogen from the high-nitrogen solution (Plate 5.11).

5.611 Cotyledon dry weight per plant. The pooled ANOVA for this variable is given in Table 0.1 (Appendix 0). Cotyledon dry weights for all the treatments were significantly different at various growth stages ($P<0.01$). Figure 5.19 shows that the dry weights declined in a more or less constant rate with time. Dry weight variation between treatments was absent (Table 0.1 and Table 5.27).

5.612 Shoot dry weight per plant. Table 0.2 (Appendix 0) presents the pooled ANOVA for this variable. Statistical significance was recorded in the time and rhizobia effects, as well as the time-rhizobia interaction (all at $P<0.01$). Owing to dry weight increases with plant maturity, the dry weights between times were different, particularly after day 77 (Figure 5.20). Dry weight increases due to rhizobia inoculation (treatments R and B) were much greater than that without rhizobia (treatments C and E) (Figure 5.20). This rhizobia effect was dependent on time, with greater dry weight differences between the rhizobia and uninoculated treatments at the later growth stages. Specifically, such significant differences occurred after day 93 (Table 5.28).

5.613 Root dry weight per plant. Table 0.3 (Appendix 0) shows the pooled ANOVA for this variable. Unlike the shoot dry weight per plant, the analysis demonstrates only one significant effect which was time ($P<0.01$). Root dry weights for all treatments increased with plant maturity, especially after day 77 (Figure 5.21). Since there was no treatment effect, the multiple range tests in Table 5.29 are not considered in this discussion.

5.614 Nodule dry weight per plant. This was discussed earlier in section 5.422.

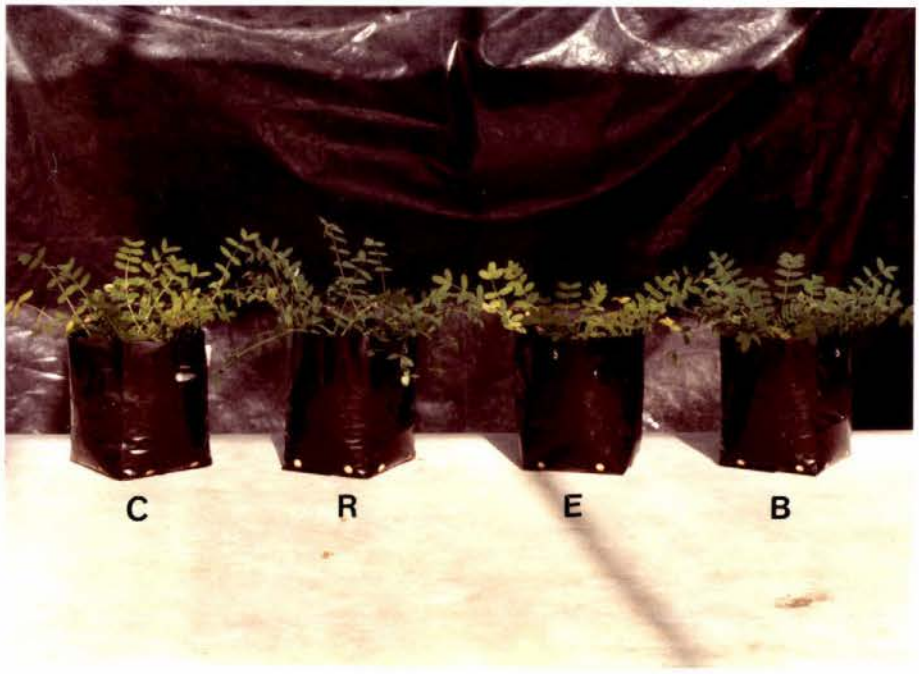


PLATE 5.9

SAINFOIN PLANTS AT DAY 93 (C, CONTROL; E, ENDOPHYTE; R, RHIZOBIA; B, BOTH INOCULATIONS)

PLATE 5.10

SAINFOIN PLANTS AT DAY 137 (C, CONTROL; E, ENDOPHYTE; R, RHIZOBIA; B, BOTH INOCULATIONS)

PLATE 5.11

NITROGEN DEFICIENCY SYMPTOMS OF SAINFOIN PLANTS
IN TREATMENT C AND E AT DAY 115

PLATE 5.12

SECONDARY STEM INITIATION AT THE END OF ROSETTE
LEAF STAGE AT DAY 77

For Figures 5.19 to 5.22, original values are plotted

(treatment C, ○——○ ; treatment E, ●——● ;
treatment R, △——△ ; treatment B, ▲——▲).

FIGURE 5.19

COTYLEDON DRY WEIGHT PER SAINFOIN PLANT OVER TIME (ZERO DATA SETS AFTER DAY 104)

FIGURE 5.20

SHOOT DRY WEIGHT PER SAINFOIN PLANT OVER TIME

FIGURE 5.21

ROOT DRY WEIGHT PER SAINFOIN PLANT OVER TIME

FIGURE 5.22

TOTAL PLANT DRY WEIGHT PER SAINFOIN PLANT OVER TIME

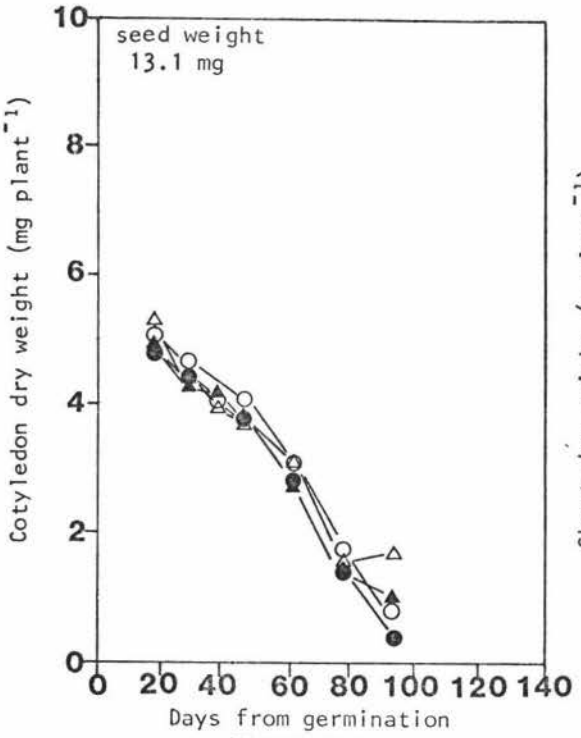


FIGURE 5.19

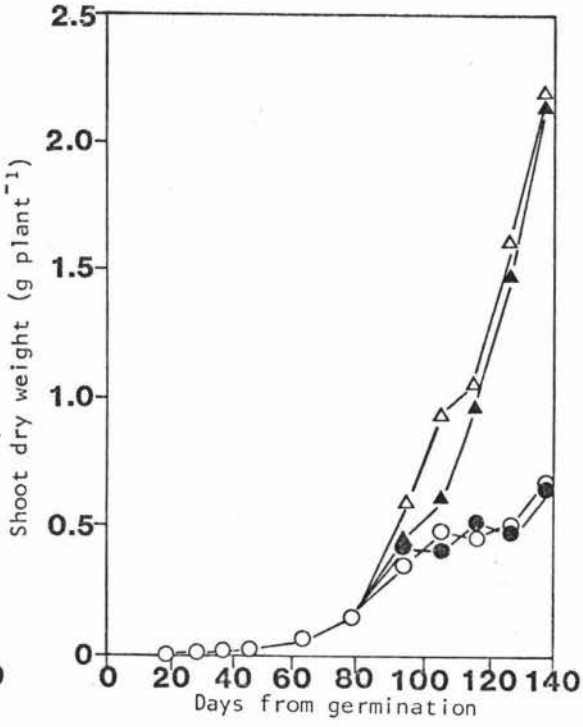


FIGURE 5.20

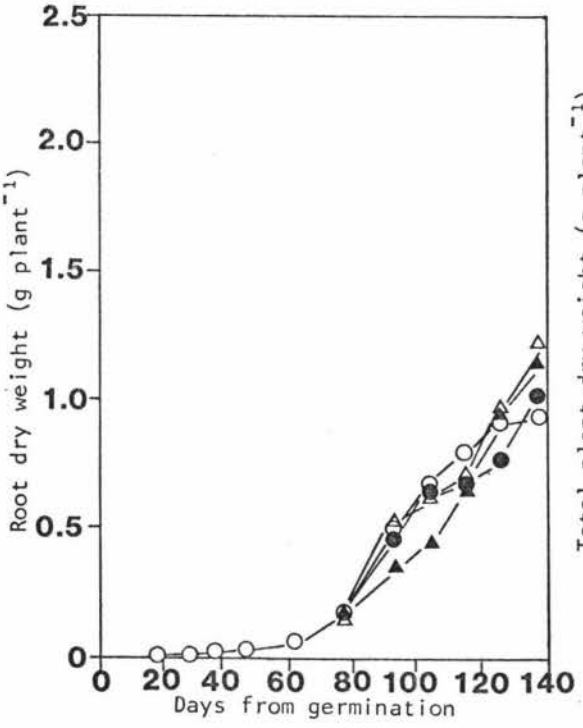


FIGURE 5.21

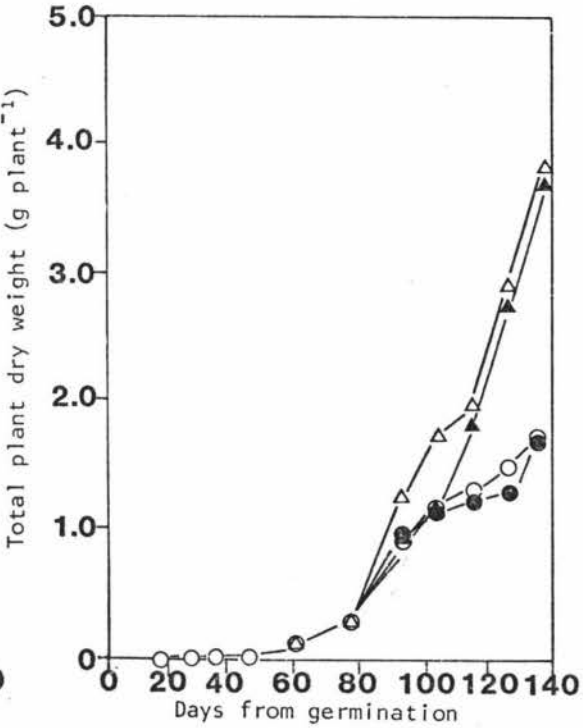


FIGURE 5.22

TABLE 5.27

ORIGINAL TREATMENT MEANS† †† OF COTYLEDON DRY WEIGHT PER PLANT ($x10^{-3}$)

Days from germination	C	E	R	B
18	5.124 a	4.819 a	5.284 a	4.936 a
27	4.717 a	4.379 a	4.429 a	4.333 a
36	4.049 a	3.970 a	3.939 a	4.143 a
45	4.135 a	3.794 a	3.762 a	2.838 a
61	2.994 a	2.810 a	3.132 a	2.757 a
77	1.790 a	1.415 a	1.611 a	1.415 a
93	0.692 a	0.383 a	1.758 a	1.094 a

† Within each time, means not sharing a common letter differ significantly at $P<0.05$.

†† For all means from time 18 to 77, SE = 0.232×10^{-3} while for time 93, SE = 0.328×10^{-3}.

TABLE 5.28

TRANSFORMED† TREATMENT MEANS†† ††† OF SHOOT DRY WEIGHT PER PLANT

Days from germination	C	E	R	B
18	0.30 a	0.23 a	0.37 a	0.31 a
27	0.99 a	0.88 a	0.84 a	0.90 a
36	1.38 a	1.39 a	1.42 a	1.50 a
45	1.76 a	1.65 a	1.62 a	1.66 a
61	2.79 a	2.76 a	2.73 a	2.82 a
77	3.54 a	3.59 a	3.51 a	3.51 a
93	4.44 a	4.61 ab	4.88 b	4.64 ab
104	4.72 a	4.61 a	5.41 b	4.92 a
115	4.71 a	4.81 a	5.48 b	5.41 b
126	4.76 a	4.72 a	5.95 b	5.83 b
137	5.07 a	5.01 a	6.25 b	6.21 b

† Equation for transformation is $ln\ (235x)$.

†† Within each time, means not sharing a common letter differ significantly at $P<0.05$.

††† For all means from time 18 to 77, SE = 0.06 while from time 93 to 137, SE = 0.09.

TABLE 5.29

TRANSFORMED† TREATMENT MEANS†† ††† OF ROOT DRY WEIGHT PER PLANT

Days from germination	C	E	R	B
18	76.03 a	73.97 a	77.47 a	77.36 a
27	92.82 b	89.11 ab	88.66 a	91.06 ab
36	94.36 a	94.39 a	94.21 a	94.50 a
45	94.31 a	91.62 a	92.82 a	92.60 a
61	99.92 a	99.72 a	99.87 a	99.95 a
77	100.75 a	100.67 a	100.31 a	100.27 a
93	108.40 a	107.03 a	108.92 a	104.92 a
104	112.31 a	111.22 a	110.88 a	106.83 a
115	115.23 a	111.59 a	113.10 a	111.50 a
126	117.54 a	114.61 a	119.35 a	118.25 a
137	118.53 a	120.30 ab	124.66 b	122.77 ab

† Equation of transformation is $[(\ln x)+2.7]^3 + 100$.

†† Within each time, means not sharing a common letter differ significantly at $P<0.05$, but the pooled ANOVA (Appendix 0.3) does not indicate a signficant treatment effect.

††† For all means from time 18 to 77, SE = 0.89 while from time 93 to 137, SE = 1.26.

TABLE 5.30

TRANSFORMED† TREATMENT MEANS†† ††† OF TOTAL PLANT DRY WEIGHT PER PLANT

Days from germination	C	E	R	B
18	79.65 a	78.08 a	80.09 a	79.76 a
27	90.07 a	87.49 a	87.11 a	88.48 a
36	92.73 a	92.82 a	92.85 a	93.58 a
45	94.90 a	98.53 a	93.59 a	93.81 a
61	99.71 a	99.51 a	99.66 a	99.79 a
77	100.06 a	100.07 a	100.05 a	100.03 a
93	102.63 a	102.74 a	105.26 a	102.71 a
104	104.88 ab	104.04 a	108.74 b	104.55 ab
115	105.90 ab	105.04 a	110.54 c	109.35 bc
126	106.99 a	105.68 a	117.13 b	115.56 b
137	108.87 a	109.16 a	123.18 b	122.18 b

† Equation for transformation is $[(\ln x) + 1.5]^3 + 100$.

†† Within each time, means not sharing a common letter differ significantly at $P<0.05$.

††† For all means from time 18 to 77, SE = 0.66 while from time 93 to 137, SE = 0.93.

5.615 Total plant dry weight per plant. In Table 0.4 (Appendix 0), the pooled ANOVA shows that the effects of time, rhizobia and time-rhizobia interaction were all significant at $P<0.01$. Treatment differences in the total plant dry weight, therefore, differed between times, and between the rhizobia (R and B) and uninoculated (C and E) treatments. The presence of interaction, however, indicates that the rhizobia effect varied at various growth stages, with the effect increasingly prominent in the later period of growth (Figure 5.22). Table 5.30 demonstrates that such differences were apparent after day 115.

5.62 Root-Shoot Ratio

Table 0.5 (Appendix 0) gives the pooled ANOVA for the root-shoot ratio. As shown in the analysis, the effects of time, rhizobia and time-rhizobia interaction were all highly significant ($P<0.01$). These results are clearly demonstrated in Figure 5.23 in which the root-shoot ratio for all treatments changed as the plants matured. Between day 18 and 77, the ratio was similar for all treatments at each time, but beginning from day 77, this ratio was becoming smaller in the rhizobia treatments (R and B), while it was increasingly larger in the uninoculated treatments (C and E) (Table 5.31).

The pooled ANOVA also indicated an endophyte effect and rhizobia-endophyte interaction (both at $P<0.05$). On examining Table 5.31, the difference in root-shoot ratio was shown at day 27, 45, 93 and 115 between treatment C and E in which the former had a greater ratio than the later. Such difference, however, was not significant between treatment R and B.

5.63 Secondary Stem Production

Towards the end of the rosette leaf stage, that is, around day 77, secondary stem buds were initiated (Plate 5.10) and later elongated to form secondary stems. More stems were developed with time and, thus, a significant difference in stem number due to time for all treatments was obtained ($P<0.01$), as shown in the pooled ANOVA (Table 0.6 in Appendix 0). There was also a rhizobia effect ($P<0.01$) and a time-rhizobia interaction ($P<0.05$). Differences in secondary stem number between the rhizobia (R and B) and uninoculated (C and E) treatments occurred after day 115 (Figure 5.24 and Table 5.32).

For Figure 5.23 to 5.26, original values are plotted

(treatment C, ○——○ ; treatment E, ●——● ;
treatment R, △——△ ; treatment B, ▲——▲).

FIGURE 5.23

ROOT-SHOOT RATIO OF SAINFOIN OVER TIME

FIGURE 5.24

SECONDARY STEM NUMBER PER SAINFOIN PLANT OVER TIME (ZERO DATA SET BEFORE DAY 77)

FIGURE 5.25

REGRESSION PLOTS BETWEEN SHOOT DRY WEIGHT AND ROOT DRY WEIGHT OF SAINFOIN (R_1 AND B_1 ARE PLOTTED FROM DAY 18 TO 77, WHILE R_2 AND B_2 ARE PLOTTED FROM DAY 93 TO 137)

FIGURE 5.26

REGRESSION PLOTS BETWEEN SHOOT DRY WEIGHT AND NODULE DRY WEIGHT OF SAINFOIN (C_1 AND E_1 ARE PLOTTED FROM DAY 36 TO 93; C_2 AND E_2 ARE PLOTTED FROM DAY 93 TO 137; R AND B ARE PLOTTED FROM DAY 77 TO 137)

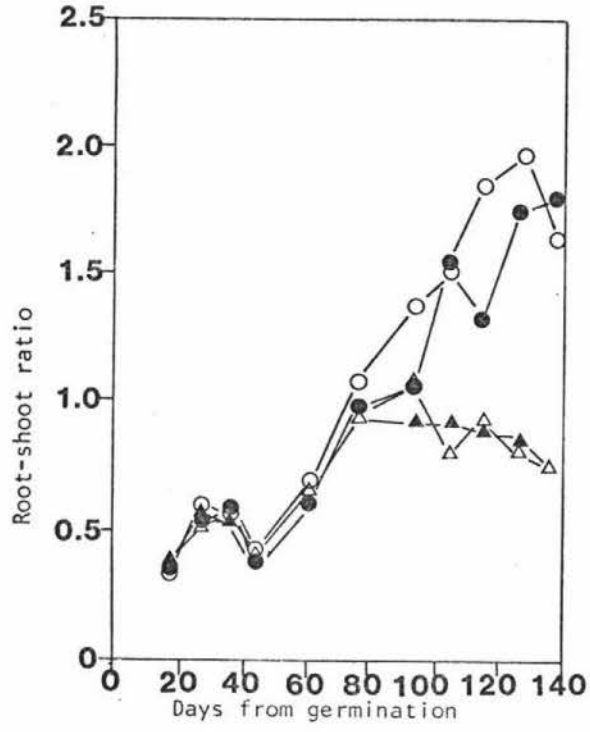


FIGURE 5.23

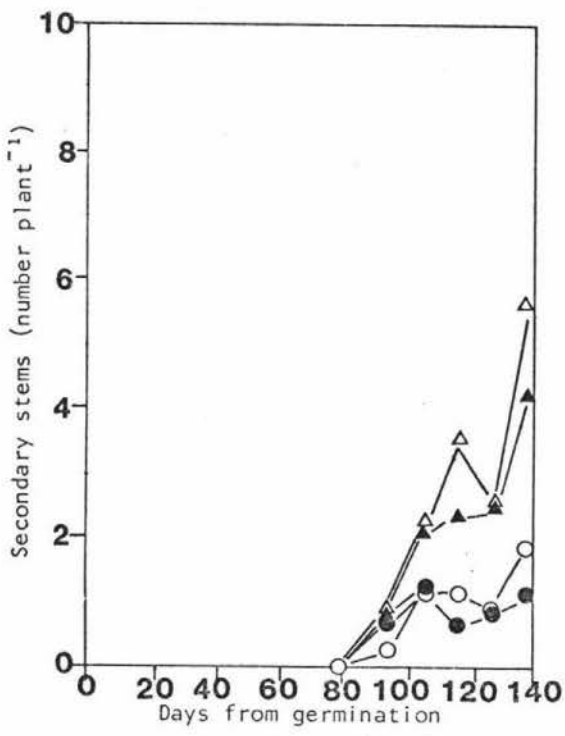


FIGURE 5.24

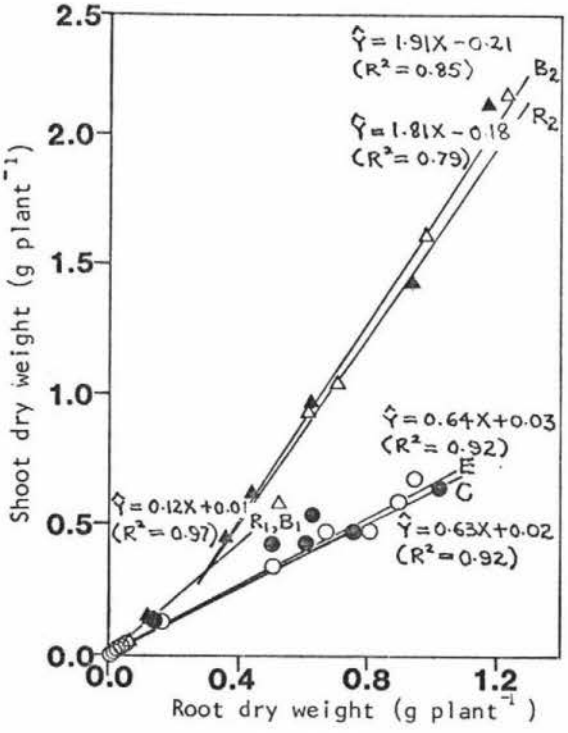


FIGURE 5.25

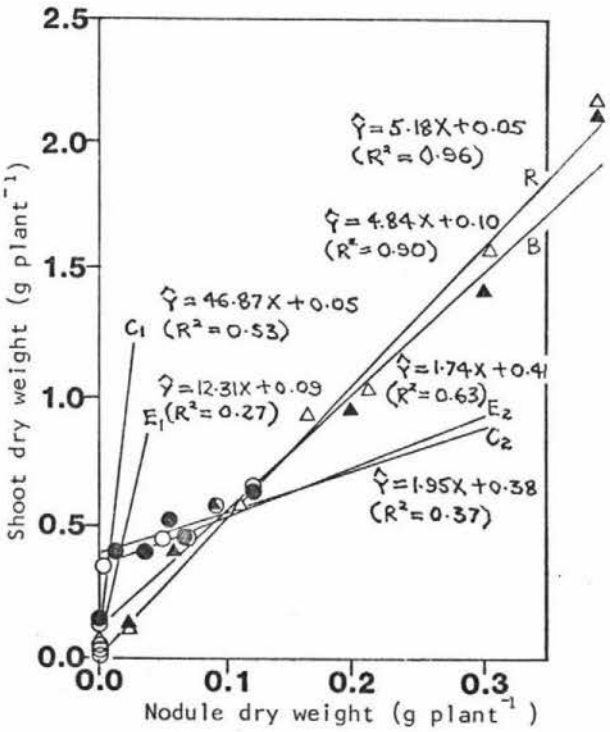


FIGURE 5.26

TABLE 5.31

TRANSFORMED† TREATMENT MEANS†† ††† OF ROOT-SHOOT RATIO

Days from germination	C	E	R	B
18	2.86 a	2.84 a	2.87 a	2.95 a
27	3.42 b	3.25 a	3.23 a	3.36 ab
36	3.32 a	3.33 a	3.27 a	3.24 a
45	3.03 b	2.85 a	2.99 b	2.95 ab
61	3.52 a	3.39 a	3.51 a	3.53 a
77	3.97 a	3.90 a	3.87 a	3.85 a
93	4.25 b	3.97 a	3.97 a	3.83 a
104	4.31 b	4.35 b	3.69 a	3.81 a
115	4.52 c	4.18 b	3.81 a	3.78 a
126	4.59 b	4.45 b	3.68 a	3.74 a
137	4.37 b	4.48 b	3.61 a	3.60 a

† Equation of transformation is $ln\ (50x)$.

†† Within each time, means not sharing a common letter differ significantly at $P<0.05$.

††† For all means from time 18 to 77, SE = 0.04 while from time 93 to 137, SE = 0.05.

TABLE 5.32

TRANSFORMED† TREATMENT MEANS†† ††† OF SECONDARY STEM NUMBER PER PLANT

Days from germination	C	E	R	B
93	0.33 a	0.70 a	0.87 a	0.86 a
104	0.98 a	1.14 a	1.51 a	1.26 a
115	0.98 ab	0.65 a	1.85 c	1.50 b
126	0.86 a	0.72 a	1.56 b	1.55 b
137	1.24 a	1.04 a	2.33 b	1.97 b

† Equation for transformation is $\sqrt{x}$.

†† Within each time, means not sharing a common letter differ significantly at $P<0.05$.

††† For all means, SE = 0.21.

TABLE 5.33

T-TEST VALUES FOR HOMOGENEITY OF REGRESSION SLOPES BETWEEN SHOOT DRY WEIGHT AND ROOT DRY WEIGHT AND BETWEEN SHOOT DRY WEIGHT AND NODULE DRY WEIGHT

Regression	Comparisons	DF	t-test
$\hat{y}$ = shoot dry weight per plant x = root dry weight per plant (from Figure 5.25)	b_{R_1} v b_{R_2}	98	1.652 ns
	b_{B_1} v b_{B_2}	98	1.929 ns
	b_C v b_E	200	0.497 ns
	b_{R_2} v b_{B_2}	56	0.413 ns
	b_C v b_{R_2}	128	11.861 **
	b_E v b_{B_2}	128	14.254 **
$\hat{y}$ = shoot dry weight per plant x = root dry weight per plant (from Figure 5.26)	b_{C_2} v b_{E_2}	56	0.400 ns
	b_R v b_B	80	1.098 ns
	b_{C_2} v b_R	68	5.822 **
	b_{E_2} v b_E	68	5.046 **

ns non-significant with $P>0.05$

** significant at $P<0.01$

For Figures 5.27 and 5.28, original values are plotted (treatment C, ○ ; treatment E, ● ; treatment R, △ ; treatment B, ▲).

FIGURE 5.27

SCATTERPLOTS OF ACETYLENE REDUCTION AGAINST NODULE DRY WEIGHT FROM DAY 77 TO 137

FIGURE 5.28

SCATTERPLOTS OF ACETYLENE REDUCTION AGAINST SHOOT DRY WEIGHT FROM DAY 77 TO 137

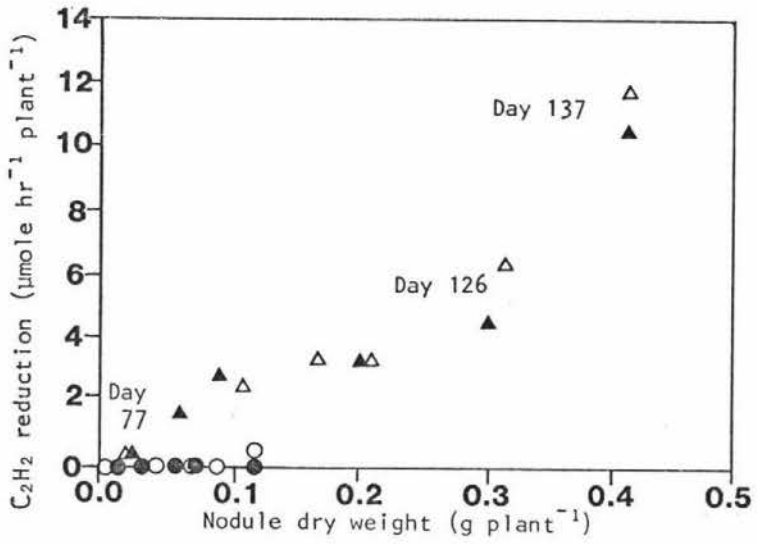


FIGURE 5.27

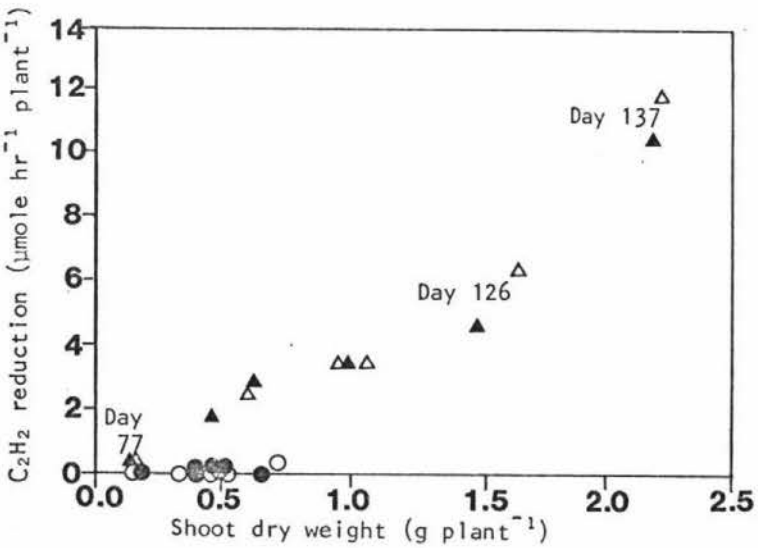


FIGURE 5.28

TABLE 5.34

SUMMARY OF STEPWISE MULTIPLE REGRESSION ANALYSIS FOR DIFFERENT TREATMENTS

Treatment	Growth Period	Independent variable	R^2	R^2-change	b	a
C	77-137	shoot dry weight per plant	0.27	0.27	1.83	-0.13
		nodule dry weight per plant	0.32	0.05	-2.71	
E	77-137	shoot dry weight per plant	0.03	0.03	0.24	-0.13×10^{-1}
		nodule dry weight per plant	0.04	0.01	-0.44	
R	77-126	nodule dry weight per plant	0.85	0.85	15.10	-0.54×10^{-1}
		shoot dry weight per plant	0.85	0.00	0.96	
	126-137	shoot dry weight per plant	0.70	0.70	7.09	-11.31
		nodule dry weight per plant	0.72	0.02	21.43	
B	77-126	nodule dry weight per plant	0.74	0.74	12.35	0.63
		shoot dry weight per plant	0.74	0.00	0.37	
	126-137	shoot dry weight per plant	0.72	0.72	6.70	-4.08
		nodule dry weight per plant	(not computed by SPSS)			

The slightly larger coefficient of variations of both error (a) (43%) and error (b) (34%) (Appendix 0.6) are due probably to inherent variation in stem production between plants within the Fakir cultivar.

5.64 Regression Analysis

The simple regression results between shoot dry weight and root dry weight, and shoot dry weight and nodule dry weight are shown in Figures 5.25 and 5.26 respectively. The high R^2 (between 0.79 and 0.97) for all treatments in Figure 5.25 indicates that the partitioning of shoot dry weight was strongly related to the the root dry weight. In Figure 5.26, the R^2 of the rhizobia-inoculated treatments (0.90 and 0.96) was higher than that of the uninoculated treatments (0.37 and 0.63) and, therefore, suggests that the shoot dry weight was closely associated with the nodule dry weight when the treatments contained effective rhizobia.

Table 5.33 presents the t-test values for comparisons of the regression slopes between treatments. The slopes of treatments R and B were significantly different from those of treatments C and E respectively ($P<0.01$). However, the slopes of R_1 and R_1, and B_1 and B_2 (see Figure 5.25) were not different from each other and, thus, suggest that each of the treatments R and B can be fitted with a single regression line.

Scatterplots of acetylene reduction per plant with nodule dry weight per plant and acetylene reduction per plant with shoot dry weight per plant are presented in Figures 5.27 and 5.28 respectively. The stepwise inclusion multiple regression results provide evidence that nodule dry weight was the principal determinant of thd dependent variable acetylene reduction between day 77 and 127 for both treatments R and B, but between day 126 and 137 it was shoot dry weight (Table 5.34). For treatments C and E, the major determinant of acetylene reduction was the shoot dry weight, but the proportion of variance that accounted for acetylene reduction was low.

CHAPTER 6

DISCUSSION

6.1 INTRODUCTION

For convenience of reference, the discussions of experimental results are presented in four main sections in this chapter. In these four sections, the development of arbuscular mycorrhizae, the development of dinitrogen-fixing system, the total plant nitrogen and phosphorus, and the growth and development of sainfoin are considered in their order.

6.2 DEVELOPMENT OF ARBUSCULAR MYCORRHIZAE

6.21. First Inoculation with Gm

Sainfoin seedlings (var. *bifera* Hort.), inoculated at planting, were infected with a range of New Zealand endophytes and the monoculture of *Gigaspora magarita* Becker & Hall after about 30 days of growth. In the principal experiment, however, such results were not repeated with the cultivar Fakir when inoculated with *Gigaspora magarita* Becker & Hall even after 93 days of growth. The absence of infection in Fakir with the first inoculation may be due to host specificity involving the production of inhibitory substances in the roots, high concentration of soil and/or plant phosphates, or a low viable spore number and limited living mycelia in the inoculum used.

Although host specificity is rare in arbuscular mycorrhiza formation (Mosse, 1973,1975), the involvement of toxic compounds from the seed coats and possibly also the root exudates of lupin (*Lupinus cosentinii* Guss.) in preventing proper mycorrhiza establishment was noted (Morley and Mosse, 1976). If inhibitory substances were produced in Fakir roots, then they were very likely to be condensed tannins. Pankhurst *et al* (1979) and Pankhurst and Jones (1979) have found the presence of condensed tannins in the nodules of lotus major (*Lotus pedunculatus* Cav.). However, the vanillin-hydrochloris acid spot

test showed a negative result in both the roots and nodules of sainfoin although the leaves, petioles and crowns gave a positive red-violet colour response (Table 5.4). These results confirm the earlier findings obtained by W.T. Jones (unpublished data) and J.A. Fortune (personal communication). The presence of other phenolic compounds or phytoallexins (McLeod, 1974; Mann, 1978) other than condensed tannins cannot be totally ruled out. However, their involvement in a complete resistance to fungal invasion in Fakir roots is not expected because Ling-Lee *et al* (1977) have found ectomycorrhiza development in brown barrel (*Eucalyptus fastigata* Deane & Maiden) even though localized phenolic substances were detected in its root tissues. Furthermore, later-grown Fakir plants in a 100% inoculum soil, in fact, established arbuscular mycorrhizae with *Gigaspora magarita* Becker & Hall at day 34.

The total amount of phosphorus applied to each bag as KH_2PO_4 from day 0 to 93 was 0.62 mg P, that is, 0.25 mg P kg^{-1} soil (or equivalently 0.30 mg P 1000 cm^{-3} soil) when expressed as per unit of soil in the bag. Prior to the start of the experiment, the soil contained 21.90 mg P kg^{-1} soil (or equivalently 26.50 mg P 1000 cm^{-3} soil) according to Olsen's test (Appendix E). Regardless of the small losses out of each bag through drainage during watering, therefore, the maximum quantity of phosphorus in the soil per bag during the growth period between planting and day 93 was 22.15 mg P kg^{-1} soil or 26.80 mg P 1000 cm^{-3} soil. This is a comparatively low figure when considering the application of 64.00 mg P 1000 cm^{-3} soil as $Na_2H_2PO_4.2H_2O$ to pot-grown white clover and perennial ryegrass (Powell and Daniel, 1978), and as high as 167.20 mg P 1000 cm^{-3} agar medium as $Na_2H_2PO_4.2H_2O$ to cane blue stem (*Bouteloua gracilis* H.B.K. Lag ex. Steud.) (Allen *et al*, 1981). In both these experiments, mycorrhiza infection in the plants was reported, suggesting that the seemingly high phosphorus quantities applied did not inhibit fungal invasion. In another study, Powell (1977a) showed that in a range of soils with Truog test values between 44 to 120 µg P cm^{-3}, which are considered as high in the Olsen scale, mycorrhiza infection in white clover was between 5 to 82%. This information indicates that the phosphorus level in the principal experiment did not appear to be a major inhibitory effect in mycorrhiza formation by *Gigaspora magarita* Becker & Hall.

Recently, Sanders (1975) established that foliar application of phosphate to onion plants reduced the rate of spread and intensity

of mycorrhiza infection and, therefore, suggested that the degree of mycorrhiza infection was related to internal host phosphorus rather than soil phosphates. On examining the root phosphorus of sainfoin, the concentration between day 36 and 93 was below 4 500 μg P g^{-1} tissue, (0.45%), but the concentration between day 93 and 140 was slightly higher, about 5 000 μg P g^{-1} tissue (0.50%). Since mycorrhiza infection was eventually recorded after day 104, it appeared that the internal phosphorus concentration of sainfoin roots, although relatively high, was not a possible reason for zero infection for the first inoculation. Furthermore, total resistance to fungal infection was not reported by Sanders (1975) and this was not expected to occur in sainfoin.

Consequently, the most likely reason for the failure of fungal infection in Fakir roots with the first endophyte inoculum is the low spore number and mycelia in the inoculum used. This is supported by the evidence that Fakir plants growing in the same but 100% inoculum soil formed mycorrhizae with 47% of the root segments examined infected.

6.22 Second Inoculation with MX

With the second inoculation using a mixture of highly efficient endophytes, mycorrhiza infection occurred 16 days after inoculation. The infection was, however, diffuse, occurring in isolated patches even at day 137, that is, 36 days after inoculation. There are two possible reasons for this observation. One is probably because the root system had undergone much structural charges especially lignification which prevented fungal invasion into more root tissues (Vance *et al*, 1980). Infection was, therefore, limited to the smaller number of newly formed roots which were yet to lignify. Two is probably because the relatively higher root phosphorus concentration (about 0.50% between day 115 and 137) reduced the rate of spread and intensity of infection within the root cortex (Sanders, 1975).

Based on an infection rating technique involving cross-sections (Strzemska, 1975a), Strzemska (1975b) noted a moderate infection in sainfoin roots when compared with other legumes. In the preliminary field test, infection in field-grown sainfoin plants was 1.4 out of a maximum score of 5. These observed mycorrhiza occurrences and the characteristic intermediate root type of sainfoin (Table 5.5) based on Baylis's root classification (1975) strongly suggest that sainfoin is mycotrophic. In addition, the presence of arbuscular mycorrhizae in

Fakir, Remont, var. *bifera* Hort., and the cultivar of Strzemska (1975b) established that sainfoin is basically a non-specific host of a range of arbuscular fungal species including those in the New Zealand soils.

6.3 DEVELOPMENT OF DINITROGEN-FIXING SYSTEM

6.31 Infection

In this experiment, rhizobia infection via root hairs was formally established (Plate 5,5 and 5.6), confirming the suggestion made by Hume (1981) based on his observation of a positive correlation between root hair abundance and nodule number, and Dangeard's observation (1926) of infection threads in sainfoin nodules.

The large number of nodules produced (up to a mean of 214 nodules per plant) in the rhizobia treatments (R and B) suggests that numerous root hair infections actually developed into a successful association. Since plants in the uninoculated treatments (C and E) also produced a number of nodules (up to a mean of 86 nodules per plant), the rhizobia contaminants, possibly the survivals left in the soil after sterilization, from the air and/or from the adjacent rhizobia-inoculated bags, were also infective and capable of forming an association with the host plants. Nevertheless, like many other legumes studied (Dart 1974,1977), not all root hair infections in sainfoin gave rise to nodules. This is particularly evident in root regions with numerous root hair infections adjacent to each other (Plate 5.6), but only a few of these developed into nodules. Similar results on several clover species and lucerne were also reported by Purchase (1958). The rate of successful root hair infection leading to normal nodulation between day 61 and 137 was estimated at about 2.6 new infections per day in the rhizobia treatments, and about 1.0 new infection per day in the uninoculated treatments (Figure 5.2).

6.32 Nodulation

Nodule dry weight of each plant increased exponentially with time in all treatments, with the exponent being larger in the rhizobia (R and B) than in the uninoculated (C and E) treatments (Figure 5.3).

This greater increase in treatments R and B was the result of a large, infective population of rhizobia present in the soils. These rhizobia produced a larger number of root hair infections and subsequently, a greater number of nodules which also increased in size at a greater rate that the nodules of treatments C and E. Of the two variables that contributed to the nodule dry weight gain, nodule number and nodule size, nodule number was the major determinant in treatment B, but nodule size was more important in treatment R (Table 5.14).

The effect of endophyte on nodulation was more complex. Although a generally lower nodule number and nodule dry weight per plant occurred in treatment E than in treatment C and in treatment B than in treatment R between day 93 and 115, a larger nodule size was recorded in treatment E than in treatment C and in treatment B than in treatment R between the same period with several multiple range tests being significant at $P<0.05$ (Tables 5.8, 5.9 and 5.10). Endophyte inoculation, therefore, appeared to have a depressive effect on nodule number, specifically on root hair infections by the rhizobia. Owing to the reduced nodule number, each nodule probably received a greater share of photosynthesis from the shoot, resulting in the larger nodule size. Since endophyte infection did not occur until day 115, both infection and nodulation were not affected by mycorrhizae. The depressive effect was more likely due to other soil microflora, associated with the Gm inoculum, that could be antagonistic towards the rhizobia activity in the rhizosphere (Vincent, 1977), thereby reducing root hair infections. In addition, the free-living endophytes in the soils were not suspected to have a depressive effect on nodulation because endophyte inoculation was consistently reported to increase the number of nodules in many other legumes such as red clover (Mosse *et al*, 1976), lucerne (Smith and Daft, 1979) and subterranean clover (Smith *et al*, 1979).

On examining the growth period between day 126 and 137 when mycorrhizae had developed in sainfoin, Figure 5.2 seems to show a "catching-up" in nodule number in the endophyte treatments (C and E) than in the uninoculated treatments (C and R). Consequently the final nodule dry weights of each plant between treatments C and E and between treatments R and B were similar. This feature might be due to the stimulating effect of mycorrhiza formation although this effect did

not attain statistical significance. Unfortunately, the limited time available did not permit any further samplings for a more thorough investigation after day 137.

Major *et al*, (1979) reported a 36 mg nodule dry weight per sainfoin plant (an average for 34 cultivars and ecotypes) at day 70, while Hume (1981) showed a 34 mg nodule dry weight for each Fakir plant at day 73. In this experiment, a much lower figure of 21 mg for treatment R and 18 mg for treatment B at day 77 were obtained. These differences are primarily because of the differing growth conditions in these three experiments. Major *et al*, (1979) conducted their experiment in spring with the seedlings planted on April 1 in a 18 to 23°C glasshouse, while Hume (1981) planted his seedlings after mid-winter on July 24 and maintained a glasshouse temperature of between 13 to 23°C. In contrast, the principal experiment was carried out at the onset of winter, with planting on June 2 in a 15 to 25°C glasshouse and, thus, a slower growth rate probably occurred.

6.33 Dinitrogen Fixation

Acetylene reduction per plant, the measure of dinitrogen fixation, in treatments R and B increased exponentially from day 77 to 137, but the reduction activity in treatments C and E remained at a low level within the same growth period (Figure 5.5). In terms of each unit weight of nodule, the mean specific acetylene reduction was about 5-8 times greater in the rhizobia (R and B) than in the uninoculated (C and E) treatments (Figure 5.6). The rhizobia inoculum used, therefore, was a more effective strain in establishing a proper mutualistic system that was more efficient in dinitrogen fixation than the system derived from rhizobia contaminants in Treatments C and E. In fact, when some nodules from both the rhizobia-inoculated and uninoculated treatments were cross-sectioned, the nodules from the former contained a red to deep red central tissue, while most nodules from the latter contained a green central tissue (Plate 5.8). The red tissue indicated the presence of leghaemoglobin which is necessary for dinitrogen fixation (Bergersen, 1974), and the green tissue demonstrated the lack of such essential compound as well as a premature nodule senescence (Vance and Johnson, 1981). This kind of rhizobia-induced ineffectiveness has also been shown in lucerne nodules using certain ineffective

rhizobia strains (Vance and Johnson, 1981). Host-induced ineffectiveness was not observed in this experiment, but Hume (1981) has found that certain Fakir plants were possibly non-nodulating.

Although arbuscular fungi could be involved in stimulating the production of nodule number in sainfoin as discussed in the previous section, their effect on dinitrogen fixation was not evident even at day 137 (Figures 5.5 and 5.6). The likely reason for this is that there was no enhancement in phosphate absorption by the endophyte-infected plants (Figures 5.14, 5.15 and 5.16). In many other legumes investigated, the increase in nodulation and dinitrogen fixation in plants infected with arbuscular endophytes were due to an improvement in phosphate nutrition of the hosts (Crush, 1974; Mosse *et al*, 1976; Mosse, 1977; Daft and El-Giahmi, 1974,1975,1976; Carling *et al*, 1978; Bagyaraj *et al*, 1979; Smith *et al*, 1981) and/or due to a direct availability of phosphate to the nodules (Smith and Daft, 1977) for nodule growth and leghaemoglobin development (Gates, 1974), and possibly ATP synthesis (Bergersen, 1971; Moustafa *et al*, 1971). In addition, other indirect effects suggested are the greater photosynthate supply to the nodules as a result of a general improvement in mineral nutrition of the hosts (Smith and Daft, 1977; Smith *et al*, 1979), and hormonal stimulation (Powell and Sithamparanathan, 1977; Bagyaraj *et al*, 1979). However, these endophyte effects were obviously absent in this experiment.

The similar acetylene reduction activity between treatments C and E, and treatments R and B indicates that the sainfoin mycorrhiza system was not capable of fixing dinitrogen, confirming the findings of Schenk and Hinson (1973).

Between day 77 and 126, acetylene reduction was primarily explained by the nodule dry weight in treatments R and B, indicating that the dinitrogen-fixing system was probably the primary determinant of dinitrogen fixation and not the photosynthetic system. On the contrary, acetylene reduction was explained by the shoot dry weight after about day 126 and, therefore, the photosynthetic system was the primary determinant of dinitrogen fixation (Table 5.34). This appears to agree with Hume (1981) that sainfoin, dependent on dinitrogen fixation for its nitrogen supply, was limited by an inefficient dinitrogen-fixing system and a poor photosynthetic system. In another recent

experiment, Phillips (1981) found that during the early growth stages of soybean (from germination to day 22), nitrogen was the chief growth-limiting factor and not the photosynthate supply.

Major *et al*, (1979) showed that sainfoin (70 day old), lucerne (49 day old) and cicer milkvetch (*Astragalus cicer* L.) (49 day old) respectively reduced 0.44, 0.18 and 0.18 μmole C_2H_2 $plant^{-1}$ hr^{-1}. In Hume's experiment (1981), sainfoin reduced 2.33 and 4.33 μmole C_2H_2 $plant^{-1}$ hr^{-1} at day 84 and 94 respectively. In this experiment, however, the acetylene reduction was 0.25 and 0.28 μmole C_2H_2 $plant^{-1}$ hr^{-1} in treatments R and B respectively at day 77, and 2.46 and 1.61 μmole C_2H_2 $plant^{-1}$ hr^{-1} in treatments R and B respectively at day 93. These lower results obtained in the principal experiment are probably attributed to the contrasting experimental conditions and rhizobia strains used.

6.4 TOTAL NITROGEN AND PHOSPHORUS IN PLANT TISSUE

6.41 Total Nitrogen

The concentrations of cotyledon, shoot and root nitrogen in treatments C and E all declined with plant maturity (Figures 5.9 to 5.11). However, in the rhizobia treatments (R and B), the nitrogen concentration in the tissues initially decreased from day 18 to 93 after which the shoot and root nitrogen gradually increased. This increase between day 93 and 137 was due to the effective nodulation and the subsequent more efficient dinitrogen fixation. This rhizobia effect was particularly distinctive as shown in Figure 5.12 in which the nodule nitrogen concentration was higher in the rhizobia (R and B) than in the uninoculated (C and E) treatments throughout the period between day 77 and 137.

There was no overall significant effect of endophytes on the nitrogen concentrations in various organs since mycorrhiza development did not improve dinitrogen fixation in the rhizobia-inoculated plants.

Between germination and day 18, the increase in cotyledon nitrogen concentration was because of a greater decrease in cotyledon dry weight than in total cotyledon nitrogen (Figure 5.9). The total cotyledon nitrogen actually decreased from 1235 μg $seed^{-1}$ at germination to 525 μg at day 18 to 360 μg at day 27. Nitrogen was, therefore,

exported out of the cotyledons to other parts of the plants at a faster rate during the unfolding of the first leaf (between day 0 to 18) than during the unfolding of the second leaf (between day 18 and 27). This agrees with the result of Cooper and Fransen (1974). The utilization of cotyledon nitrogen by other plant parts between day 0 and 77 (throughout the rosette growth stage) was not affected by endophyte inoculation, or nodulation and dinitrogen fixation. During this growth period, therefore, reserve nitrogen in the cotyledons played an important role in the nitrogen nutrition of sainfoin as the supply of nitrogen from dinitrogen fixation was probably inadequate before day 77 (Figure 5.5).

Unlike cotyledon nitrogen concentration, the declining nitrogen concentrations in the shoot and root tissues between day 18 to 93 were due to a dilution effect as plant dry weight increased more than the accumulation of nitrogen. In treatments C and E, typical chlorotic symptoms of nitrogen deficiency developed on the leaves around day 104. At this growth stage, the critical concentration of nitrogen in the shoot was 27 000 $\mu g\ g^{-1}$ tissue which was nearly twice the average crtical value of 15 000 $\mu g\ g^{-1}$ tissue for most plants (Stout, 1961). In the field, Sims *et al*, (1968) observed chlorotic symptoms on sainfoin plants with a nitrogen concentration of 34 000 $\mu g\ g^{-1}$ shoot tissue, but not on plants with a concentration of 18 000 $\mu g\ g^{-1}$ shoot tissue. Sainfoin, thus, requires a substantial amount of nitrogen for normal growth and development. Consequently, to ensure a good, healthy sainfoin crop, an infective and effective inoculum should be used such that dinitrogen fixation can contribute greatly to its nitrogen requirement (Burton and Curley, 1968; Sims *et al*, 1968).

Since nodule nitrogen concentration was consistently greater in the rhizobia-inoculated treatments than in the uninoculated treatments, the strain of rhizobia used was much more efficient than the contaminants (Figure 5.12). In treatments C and E, the nitrogen concentration generally decreased with time, while in treatments R and B, it increased to a peak at day 104 after which it decreased with time. The decrease was probably because of a dilution effect and a steady export of nitrogen to the shoot for growth. Nitrogen was lower in the root than in the shoot, implying that nitrogen was not accumulated but used for shoot growth. Major *et al* (1979) have, in fact, demonstrated that shoot growth in sainfoin was proportional to the amount of dinigrogen fixed in the nodules.

In the experiment of Major *et al* (1979), the shoot nitrogen concentration of sainfoin (70 day old), lucerne (49 day old) and cicer milkvetch (49 day old) were respectively 34 100, 29 900 and 27 200 μg g^{-1} tissue. In Hume's study (1981), the shoot nitrogen concentration at day 84 was 29 300 μg g^{-1} tissue. However, the concentration in the principal experiment was 56 000 and 53 000 μg g^{-1} tissue at day 77 for treatments R and B respectively. These higher nitrogen concentrations were probably because of the lower dry weight accumulation due to the slower growth rate in winter.

6.42 Total Phosphorus

Similar to the total nitrogen concentration, the total phosphorus concentration in cotyledons declined with plant maturity from germination to day 77 (Figure 5.13). When considering the total phosphorus instead of its concentration, there was a rapid drop from 96 μg $seed^{-1}$ at germination to 27 μg at day 18. At day 27, the total phosphorus declined to 19 μg in the cotyledons. Phosphorus was, therefore, exported out of the cotyledons to the shoot and root at a faster rate during the unfolding of the first leaf (between day 0 and 18) than during the unfolding of the second leaf (between day 18 and 27). This agrees with the suggestion of Cooper and Fransen (1974). Like the reserve nitrogen in the cotyledons, cotyledon phosphorus also played an important part in the phosphorus nutrition of sainfoin between day 0 and 77 (during the rosette growth stage).

The concentrations of shoot phosphorus in treatments R and B steadily declined with plant maturity from day 18 to 137 (Figure 5.14). However, in the uninoculated treatments (C and E), the phosphorus concentration initially decreased until day 93, after which it increased. This greater concentration of phosphorus in the shoot of treatments C and E was due to the lower shoot dry weight that was restricted by the limited available nitrogen. On the contrary, the lower concentrations of phosphorus in the shoot of treatments R and B were because of a greater shoot dry weight that was stimulated by the efficient dinitrogen fixation.

Like shoot phosphorus concentration, the concentrations of root phosphorus of all treatments declined between day 18 and 45, but after which it increased with the differences between the rhizobia

(R and B) and uninoculated (C and E) treatments being non-significant (Figure 5.15). This is because the root growth between the rhizobia and uninoculated treatments were similar.

Nodule phosphorus concentrations for all treatments steadily decreased for all treatments, but generally the concentrations in the rhizobia treatments (R and B) were greater than in the uninoculated treatments (C and E) (Figure 5.16). Again, the reason was due to the greater dry weights of nodules in the rhizobia than in the uninoculated treatments, thereby producing a diluting effect on phosphorus. In general, nodules contained a higher concentration of phosphorus than the associated root systems, similar to those reported by Mosse *et al* (1978) for centro (*Centrosema pubescens* Benth.), Mosse (1977) for stylo (*Stylosanthes guianensis* (Aubl.) Sw.), and Smith *et al* (1979) for subterranean clover (*Trifolium subterraneum* L.). This concentrated level of phosphorus in the nodules suggest that phosphorus could well be an important element for dinitrogen fixation, very probably involved in the synthesis of large quantities of ATP that was necessary for the fixation process (Bergersen, 1971; Moustafa *et al*, 1971). Besides this particular direct role of initiation, development of leghamoglobin, and establishment of an effective mutualistic association between rhizobia and sainfoin as remarked by Gates (1974). This high nodule phosphorus concentration could be a deterrent to any fungal invasion into the nodule tissues (Crush, 1975).

The total plant phosphorus concentration, as shown in Figure 5.18, decreased between day 18 and 45 after which it rose gradually over the rest of the growth period. For treatments C and E, and treatments R and B respectively, the final phosphorus concentrations were 6 800 and 4 600 $\mu g\ g^{-1}$ tissue, both of which were higher than the critical concentration of 2 000 $\mu g\ g^{-1}$ tissue for most plants (Stout, 1961). Since the phosphorus concentrations were higher in the root than in the shoot, it seems that the phosphorus absorbed was partly accumulated in the root and partly translocated to the shoot.

There was no distinctive effect due to endophyte infection on the phosphorus concentrations in all tissues even at day 137. This is probably due to the low infection degree in sainfoin roots and due to the already well-developed, large root system that was efficiently exploiting the soils in each bag. The total plant phosphorus concentrations were well over the critical mark of 2 000 $\mu g\ g^{-1}$ tissue and

thus suggests an efficient phosphorus absorption by the root system. Hudson (1982), who experimented with pot-grown blueberry (*Vaccinum corymbosum* L.), also found that the phosphorus concentrations between plants infected and uninfected with ericaceous fungi were similar due to their well-developed root systems that efficiently exploited the soils in the pots.

6.5 GROWTH AND DEVELOPMENT OF SAINFOIN

6.51 Plant Dry Weight

The growth and development of Fakir were similar to the descriptions given by Percival (1921) and Thomson (1938). From germination to about day 77, this is called the rosette growth phase with the formation of about 6 to 10 rosette leaves, and after day 77, it is called the stem production phase with the initiation and elongation of the primary and numerous secondary stems.

Generally, cotyledon dry weight decreased with plant maturity with no marked effect due to rhizobia or endophyte (Figure 5.19). During this rosette growth stage, the carbohydrate reserves in the cotyledons were particularly important to the growth and development of the sainfoin seedling. The greatest contribution of carbohydrates from the cotyledons to the shoot and root occurred between germination and day 18 during which the first leaf was developing and, therefore, confirms the findings of Cooper and Fransen (1974). Within this period, the cotyledon dry weight dropped rapidly from about 13 mg to about 5 mg in all treatments, while the decline in the subsequent 18 days, between day 18 and 36, was only 1 mg.

The shoot, root and nodule dry weight all increased exponentially with time (Figure 5.20, 5.21 and 5.3). With the infective inoculum and effective nodulation, nodule dry weights increased at a greater rate than that of the uninoculated treatments beginning from day 45. Subsequently, an associated greater increase in dinitrogen fixation also occurred. With this larger supply of nitrogen to the shoots of the rhizobia-inoculated plants, they grew better than the uninoculated plants, displaying a sharp exponential growth. However, there was no such clear effect due to rhizobia on the growth of the roots, demonstrating that the dinitrogen fixed in the nodules was mainly transported to the shoot for growth (Major *et al*, 1979).

The common pattern of dry weight accumulation for all treatments indicates also that root growth was predominantly under the genetic control of the plant and not the amount of dinitrogen fixed, or the amount of nitrogen absorbed. Such a direct genetic control in root growth and development has the advantage of maintaining an extensive root system irrespective of the environmental influence and hence, is probably related to the drought resistance mechanism.

There was no distinctive effect due to endophyte on shoot, root and nodule growth. As discussed earlier in section 6.42 the most likely reason is that the supply of phosphorus was not limiting as a result of the well-developed root systems which were efficient in absorbing phosphates from the soils.

The pattern of whole plant dry weight accumulation followed an exponential growth curve, similar to that in shoot and nodule growth (Figure 5.22). These results are in agreement with those of Hume (1981) who also used the same sainfoin cultivar.

6.52 Root-Shoot Ratio

Figure 5.23 shows that the root-shoot ratio generally increased from day 18 to 77 for all treatments. After day 93, this ratio for treatments C and E continue to increase, but the ratio for treatments R and B decline slightly. The initial increase in root-shoot ratio suggests that the early root growth and development was possibly more important than shoot growth. This is possibly related to the mechanism of drought resistance (Chabot and Bunce, 1979). When the shoot dry weight was actually plotted against the root dry weight, the slope was less than unity at the early stages, indicating a greater photosynthate partitioning into root growth (Figure 5.26). Unfortunately, the differences between the two slopes of treatment R, as well as the two slopes of treatment B were not large enough to be statistically significant.

From day 77 onwards, shoot growth became dominant in treatments R and B due to the onset of another phase of growth associated with stem bud initiation and secondary stem development, and the availability of nitrogen through an efficient dinitrogen-fixing system. In contrast, the continual increase in root-shoot ratio in treatments C and E was due to the limited nitrogen available for growth, leading to a stunted shoot

growth. Reduced shoot growth resulting in a greater root-shoot ratio are due to the deficiency of mineral nutrients as was demonstrated in perennial ryegrass and white clover (Davidson, 1969).

6.53 Secondary Stem Production

Although buds were formed at around day 77, these buds did not elongate and develop into secondary stems until after day 77 (Figure 5.24). After day 77, secondary stem production increased rapidly due to the increasingly favourable growth conditions including a longer photoperiod, higher temperatures and due to the increase in dinitrogen fixation. Since a more efficient fixing system has developed in treatments R and B than in treatments C and E, more stems were produced in the rhizobia-inoculated treatments than in the uninoculated ones.

CHAPTER 7

CONCLUSIONS

Sainfoin is mycotrophic and a non-specific host to a range of cultured and indigenous endophytes of New Zealand. *Gigaspora magarita* Becker & Hall, inoculated into bags 4 days before planting, did not infect Fakir roots between day 18 and 93 probably because the inoculum used contained a very low number of viable fungal spores and a limited amount of living mycelia. However, 16 days after the second inoculation (day 115) with a mixture of *Gigaspora magarita* Becker & Hall, *Glomus fasciculatus* (Thax. sensu Gerd.) Gerdemann & Trappe and *Glomus tenuis* (Greenall) Hall, arbuscular mycorrhizae were established in Fakir roots, but the infection remained sparse from day 115 to 137. This low infection is presumably due to an extensive root lignification which deterred fungal invasion and establishment, and due to the relatively high root phosphorus concentration (0.50%) which reduced the rate of spread and intensity of infection within the root cortex.

Arbuscular mycorrhiza formation was not associated with any increase in phosphorus concentration in the shoots, roots or nodules probably because the well-devleoped root systems, between day 115 and 137, were efficiently exploiting the comparatively small soil volume. Possibly as a consequence, nodulation and dinitrogen fixation, plant nitrogen concentration, and plant growth and development between the endophyte-inoculated and uninoculated treatments were basically similar.

Like most legumes, rhizobia infection in sainfoin roots was via root hairs. With the inoculation of a known infective and effective rhizobia strain, sainfoin plants produced more nodules, longer nodules and consequently, a greater total nodule dry weight than plants that were infected with rhizobia contaminants. The increase in nodule dry weight in treatment R was due mainly to an increase in nodule size while in treatment B, it was due primarily to an increase in nodule number. These nodules contained a red to deep-red central tissue and were efficient in dinitrogen fixation. In contrast, those

nodules produced from the rhizobia contaminants were green and inefficient in dinitrogen fixation. Both the nodule dry weight and dinitrogen fixation were lower than that obtained in other sainfoin experiments due probably to the less favourable winter growth conditions and the differences in rhizobia strains used.

Cotyledons appeared to play a significant role in supplying carbohydrate, nitrogen and phosphorus reserves to the shoot and root during the rosette growth stage between germination and day 77. After day 77, dinitrogen fixation became more important in meeting the nitrogen requirement of the plants which responded with a significant increase in root, nodule and especially shoot growth. The increase in shoot growth was due to the initiation and production of many secondary stems. There is evidence that dinitrogen fixation in sainfoin was determined by the amount of dinitrogen-fixing tissues between day 36 and 126, but when the dinitrogen-fixing system was well-developed after day 126, the fixation was strongly influenced by the photosynthetic system.

Owing to the greater dinitrogen fixation particularly after day 77, the shoot, root and nodule nitrogen concentrations were considerably higher in the rhizobia-inoculated than in the uninoculated treatments. On the contrary, the shoot and nodule phosphorus concentrations were lower in the rhizobia-inoculated than in the uninoculated treatments due to the significant increase in dry weights which caused a dilution effect. The phosphorus concentrations in the nodules of all treatments, between 0.50 and 0.90%, were higher than those in the shoot and root tissues and, therefore, phosphorus was presumably required in large quantity by the dinitrogen-fixing system.

APPENDICES

APPENDIX A. SI AND SI-DERIVED UNITS AND OTHER COMMON ABBREVIATIONS

TABLE A.1

SYMBOLS, NAMES AND DEFINITIONS OF SI AND SI-DERIVED UNITS

Symbol	Name	Definition
m	metre	unit of length
g	gram	unit of mass
s	second	unit of time
Pa	pascal	unit of pressure
C	celsius	unit of temperature (0°C ≈ 273K)
mol	mole	amount of substance
n	nano	10^{-9}
μ	micro	10^{-6}
m	milli	10^{-3}
c	centi	10^{-2}
k	kilo	10^{3}
M	mega	10^{6}
G	giga	10^{9}
ha	hectare	$10^{4}m^{2}$ (not a SI unit)
M	molality	mole 1000 cm^{-3}

TABLE A.2

NAME OF COMMON ABBREVIATIONS

Abbreviation	Name
ADP	adenosinediphosphate
AMP	adenosinemonophosphate
ANOVA	analysis of variance
A.R.	analytical reagent
ATP	adenosinetriphosphate
cv.	cultivar
DF	degree of freedom
DM	dry matter
EDTA	ethylenediaminetetraacetic acid ferric monosodium
EMS	error mean square
hr	hour
me	milliequivalent
MS	mean square
n	number of replicates
N	nitrogen
N_2	dinitrogen
P	phosphorus
R	correlation coefficient
R^2	coefficient of multiple determination
RNA	ribonucleic acid
SE	standard error
$\bar{x}$	grand mean
var.	variety
VR	variance ratio (F-test)
v/v	volume per volume

APPENDIX B. EFFECTS OF ENDOPHYTES ON NODULATION AND DINITROGEN FIXATION

Species	Reference	Plant growth	Phosphorus concentration	Nodulation	Dinitrogen fixation
TROPICAL LEGUMES					
peanut (*Arachis hypogaea* L.)	Daft and El-Giahmi, 1976	+	+	+	+
	Daft, 1978	+	+	+	+
centro (*Centrosema pubescens* Benth.)	Crush, 1974	+	+	+	+
	Mosse *et al*, 1976	+	+	+	+
soybean (*Glycine max* (L.) Merr.)	Carling *et al*, 1978	+	+	+	+
	Bagyaraj *et al*, 1979	+	+	+	ND
dwarf bean (*Phaseolus vulgaris* L.)	Daft and El-Giahmi, 1974	ND	+	+	+
	Daft, 1978	+	+	+	+
puero (*Pueraria phaseoloides* (Roxb.) Benth.)	Waidyanatha *et al*, 1979	+	+	+	+
stylo (*Stylosanthes guianensis* (Aubl.) Sw.)	Crush, 1974	+	+	+	+
	Mosse *et al*, 1976	+	+	+	+
	Mosse, 1977	+	+	+	+
	Waidyanatha *et al*, 1979	+	+	+	+

APPENDIX B. Contd.

TEMPERATE LEGUMES

(*Lotus pedunculatus* cav.)	Crush, 1974	0	0	0	0
	Powell and Sithamparanathan, 1977	+	0	+	ND
lucerne (*Medicago sativa* L.)	Smith and Daft, 1977	+	+	+	+
	Daft, 1978	+	+	+	+
	Azcon-G. de Aguilar and Barea, 1978	+	ND	+	ND
	Smith and Daft, 1978	+	+	+	+
	Azcon-G. de Aguilar *et al*, 1979	+	+	+	ND
	Barea *et al*, 1980	+	+	+	ND
	Azcon-G. de Aguilar and Barea, 1981	+	+	+	ND
red clover (*Trifolium pratense* L.)	Mosse *et al*, 1976	+	+	+	+
	Powell and Sithamparanathan, 1977	+	ND	+	ND
white clover (*Trifolium repens* L.)	Crush, 1974	+	0	+	+
	Powell and Sithamparanathan, 1977	+	+	+	ND
subterranean clover (*Trifolium subterraneum* L.)	Abbott and Robson, 1977	+	+	+	ND
	Smith *et al*, 1979	+	+	+	+
	Smith *et al*, 1981	ND	ND	+	+

+ = improvement 0 = no change ND = not determined

APPENDIX C. PROGRAMME SUMMARY

Date		Event
		Beginning of Preliminary Experiments
March	28	Planting
April	2	Sampling I
April	7	Sampling II
April	27	Field test
April	28	Sampling III
		End of Preliminary Experiments
May	1	Soil extraction
May	4-10	Soil sieving and mixing
May	11-12	Soil sterilization
May	13	Soil packing for temporary storage
May	20-22	Glasshouse fumigation
May	23-26	Potting
May	27-28	Arrangement of potted bags
		Beginning of Principal Experiment
May	29	Endophyte (Gm) inoculation
May	30-31	Seed germination
June	2	Planting
June	4	Nutrient application (both low-N and high-N)
June	5	First rhizobia inoculation
June	9	First thinning and replacement
June	11	Second replacement
June	13	Second thinning and third replacement
June	15	Fourth replacement
June	16	Second rhizobia inoculation
June	18	Sampling I
June	27	Sampling II
July	1	Nutrient application (only low-N)
July	6	Sampling III

APPENDIX C. Contd

July	15	Sampling IV
July	20	Nutrient application (only low-N)
July	31	Sampling V
August	4	Planting of corn seeds for "trapping" Gm
August	16	Sampling VI
August	30	First corn sampling for Gm
		Bag flushing
September	1	Sampling VII
		Nutrient application (both low-N and high-N)
September	6	Bag flushing
September	7	Second corn sampling for Gm
September	8	Nutrient application (both low-N and high-N)
September	9	Endophyte (MX) reinoculation
		planting of corn seeds for "trapping" MX
September	12	Sampling VIII
September	13	Third corn sampling for Gm
September	23	Sampling IX
September	27	First corn sampling for MX
October	4	Sampling X
October	15	Sampling XI
		End of Principal Experiment
November	9	Second corn sampling for MX

APPENDIX D. ENDOPHYTE CULTURE

TABLE D.1

DESCRIPTION OF FOUR ENDOPHYTE INOCULA SUPPLIED BY DR C. Ll. POWELL

Name	Description
R4	A sample (#4), collected in Raglan county, containing a mixture of New Zealand endophyte species.
NP15	A sample (#15), collected in New Plymouth, containing a mixture of New Zealand endophyte species.
Gm	A sample containing *Gigaspora magarita* Becker & Hall.
MX	A sample containing *Gigaspora magarita* Becker & Hall, *Glomus tenuis* (Greenall) Hall, and *Glomus fasciculatus* (Thax. sensu Gerd.) Gerdemann & Trappe.

TABLE D.2

CULTURING OF FOUR ENDOPHYTE INOCULA

Bag no.	Planting date	Harvesting date	Endophyte type	Weight of soil (g)	Weight of inoculum (g)	Ratio
1	24/3	12/5	Gm	2 500	194	13:1
2	24/3	12/5	Gm	2 500	194	13:1
3	24/3	12/5	NP15	3 000	295	10:1
4	24/3	12/5	NP15	3 000	295	10:1
5	24/3	12/5	R4	2 500	265	10:1
6	24/3	12/5	R4	2 500	265	10:1
7	24/4	8/9	MX	2 500	244	10:1
8	24/4	12/6	MX	2 500	244	10:1
9	24/4	12/6	NP15	2 500	250	10:1
10	24/4	12/6	NP15	2 500	250	10:1
11	24/4	12/6	R4	3 100	317	10:1

APPENDIX E. SOIL-TEST RESULTS

TABLE E.1

BASIC SOIL TESTS

Type of test	Value	Inference
Bulk density (g cm^{-3})	1.21	-
pH	6.20	satisfactory
Phosphorus ($\mu g\ cm^{-3}$)	26.50	medium
Extractable cations (me 100 g^{-1})		
Potassium	0.40	low
Calcium	7.10	medium
Magnesium	0.85	low
Sodium	0.13	very low
Cation exchange capacity (me $100g^{-1}$)	10.50	low
Percentage saturation (%)		
Potassium	3.70	satisfactory
Calcium	65.50	satisfactory
Magnesium	7.85	satisfactory
Sodium	1.25	satisfactory

TABLE E.2

SPECIAL TESTS

Type of test	Value	Inference
Organic matter (%)	2.00	low
Total soluble salts (mm ho cm^{-1})	0.69	medium
Phosphate retention (%)	5.00	low
Reserve magnesium (me 100 g^{-1})	23.50	high

APPENDIX F. YEAST-MANNITOL MEDIUM

TABLE F.1

COMPOSITION OF YEAST-MANNITOL NUTRIENT MEDIUM
(VINCENT, 1970)

Chemical compound	Quantity (g)
K_2HPO_4	0.5
$MgSO_4$	0.2
NaCl	0.1
Mannitol	10.0
Yeast	0.4
Agar†	15.0
Tap water	1 000 cm^3

† For liquid culture, agar is omitted

APPENDIX G. NUTRIENT SOLUTIONS

TABLE G.1

CHEMICAL COMPOSITION OF LOW-NITROGEN SOLUTION
(pH = 7.8) (AFTER SMALL AND LEONARD, 1969)

Concentration of chemical compounds in stock solution	Volume of stock solution used per 1000cm^3 nutrient solution (cm^3)	Concentrations of elements in nutrient solution (μg cm^{-3})
Compounds for macroelements		
1.0 M $MgSO_4.7H_2O$	1.0	Mg - 24.31 ; S = 32.06
1.0 M KH_2PO_4	0.5	K = 19.95 ; P = 15.48
0.5 M K_2SO_4	5.0	K =195.50 ; S = 80.15
0.5 M $CaCl_2$	5.0	Ca =100.20 ; Cl = 177.25
Compounds for microelements		
$(NH_4)_6Mo_7O_{24}.4H_2O$(0.090 per 1000cm^3)	1.0	N = 0.01 ; M_o = 0.05
$ZnSO_4$ (0.220 g per 1000 cm^3)	1.0	Zn = 0.09 ; S = 0.04
$CuSO_4.5H_2O$ (0.790 g per 1000 cm^3)	1.0	Cu = 0.20 ; S = 0.10
H_3BO_3 (2.860 g per 1000 cm^3)	1.0	B = 0.50
$MnSO_4.H_2O$ (1.540 g per 1000 cm^3)	1.0	Mn = 0.50 ; S = 0.29
$C_o(NH_3)_2.6H_2O$ (0.025 g per 1000 cm^3)	1.0	N = 0.00 ; C_o = 0.01
10% EDTA - Ferric monosodium (27.500 g per 1000 cm^3)	2.0	Fe = 8.37 ; Na = 3.44 N = 4.20

TABLE G.2

CONCENTRATION OF ELEMENTS IN LOW-NITROGEN SOLUTION

Element	Concentration ($\mu g\ cm^{-3}$)
N	4.21
P	15.48
K	215.05
S	112.64
Ca	100.20
Mg	24.31
Na	3.44
Cl	177.25
Fe	8.37
Mn	0.50
B	0.50
Cu	0.20
M_o	0.05
Zn	0.09
Co	0.01

TABLE G.3

QUANTITY OF NUTRIENT SOLUTIONS APPLIED AT VARIOUS TIMES

Days from germination	Volume of low-nitrogen solution applied (cm^3)	Volume of high-nitrogen (0.01% NH_4NO_3) solution applied (cm^3)
4	10	10
31	10	-
50	20	-
91	F L U S H I N G	
93	20	10 (to treatment C & E)
98	F L U S H I N G	
100	20	10 (to treatment C & E)
137	Termination of Experiment	

APPENDIX H. GLASSHOUSE TEMPERATURES

TABLE H.1

MINIMUM, MAXIMUM AND MEAN WEEKLY TEMPERATURES IN THE GLASSHOUSE

Week	Minimum	Maximum	Mean
1	7	27	14
2	5	25	17
3	13	25	18
4 (July)	13	26	18
5	13	27	18
6	13	23	17
7	13	25	15
8	13	26	19
9 (August)	13	25	18
10	13	25	18
11	4	27	15
12	5	28	18
13 (September)	10	28	20
14	9	30	21
15	14	29	21
16	13	31	20
17	14	31	20
18 (November)	14	30	21
19	13	32	22
20	14	33	23

APPENDIX I. DEVELOPMENT OF ENDOPHYTE INFECTION ASSESSMENT KEY

While many methods have been used to assess quantitatively, endophyte infection, Giovannetti and Mosse (1980) reviewed that the percentage of cortex occupied by the endophyte appears to be the most common means. This approach was adopted in the experiment described. A scoring system was invented, using a series of standard area diagrams which is a commonly used technique in plant pathology to assess fungal infection of plant organs.

The assessment key was made up of six classes of endophyte infection ranging from 0 to 89% of the infected cortical tissue. A 90 to 100% infection is virtually impossible because the endophyte do not occupy the epidermis and vascular tissues. Scores from 1 to 6 were assigned ordinally to the classes (Table I.1).

From the maximum infection percentage of every class, standard area diagrams were drawn to resemble as accurately and as closely as possible to the observed infected roots of field-grown sainfoin. As shown in Figure I.1, each standard area diagram represents a microscopic view with a stained, flattened root lying on the diameter of the field at a 40X magnification.

TABLE I.1

ENDOPHYTE INFECTION ASSESSMENT KEY

Infected cortical area (%)	Score
0 - 14	1
15 - 29	2
30 - 44	3
45 - 59	4
60 - 74	5
75 - 89	6

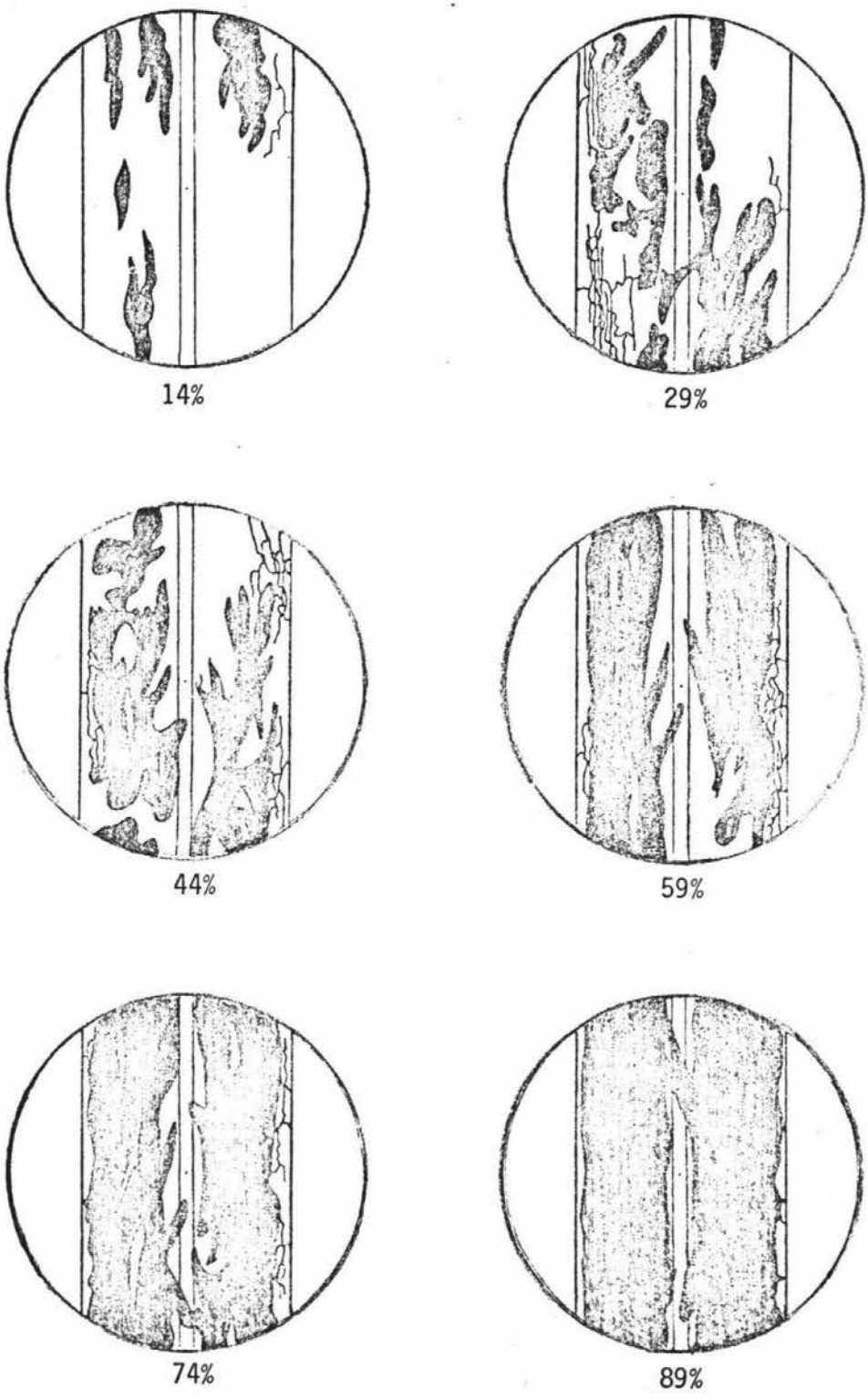


FIGURE I.1

STANDARD AREA DIAGRAMS DRAWN FROM FIELD SAMPLES OF INFECTED SAINFOIN ROOTS

APPENDIX J. GAS CHROMOTOGRAPHY

Preparation of Ethylene Standards

Stock ethylene (C_2H_4). Using a 10 cm^3 disposable syringe, 10.00 cm^3 of pure C_2H_4 was withdrawn from the ethylene gas cylinder and introduced, through a suba seal, into a 190 cm^3 cornical flask from which 10.00 cm^3 of air was previously withdrawn using another 10 cm^3 syringe. The amount of C_2H_4 in the vessel was 450 000 nmoles.

From this vessel, 2.50 cm^3 of the diluted ethylene was withdrawn and introduced into a 190 cm^3 cornical flask in a similar way to produce the second stock ethylene which contained 5.921 nmoles in 190 cm^3.

Working standards. A higher and a lower dilution series were produced by withdrawing the appropriate amounts from stocks 1 and 2 respectively and introducing into the vessels as shown in Figure J.1. The working standards, therefore, consisted of 10.000, 4.000, 2.000, 1.000, 0.500, 0.100, 0.050, 0.010, 0.005 and 0.001 nmoles C_2H_4 in a standard 0.2 cm^3 delivery. In case of any leakage from these vessels, fresh stocks and standards were reproduced in each new run.

Gas Chromatography and Calculation

The Pye series 104 chromatograph, fitted with a heated dual flame ionisation detector, was attached with a Servoscribe potentiometric recorder which printed out signals in graphical form. In the oven, the column, made of glass tubing and packed with Parapak T 80-100 mesh, was 0.5 m long. During the analysis of gas samples, the following conditions were maintained.

Oven (column) temperature	100°C
Injector port temperature	100°C
Detector oven temperature	125°C
Carrier gas (N_2) flow rate	45 $cm^3\ min^{-1}$
Hydrogen gas flow rate	45 $cm^3\ min^{-1}$
Air flow rate	700 $cm^3\ min^{-1}$

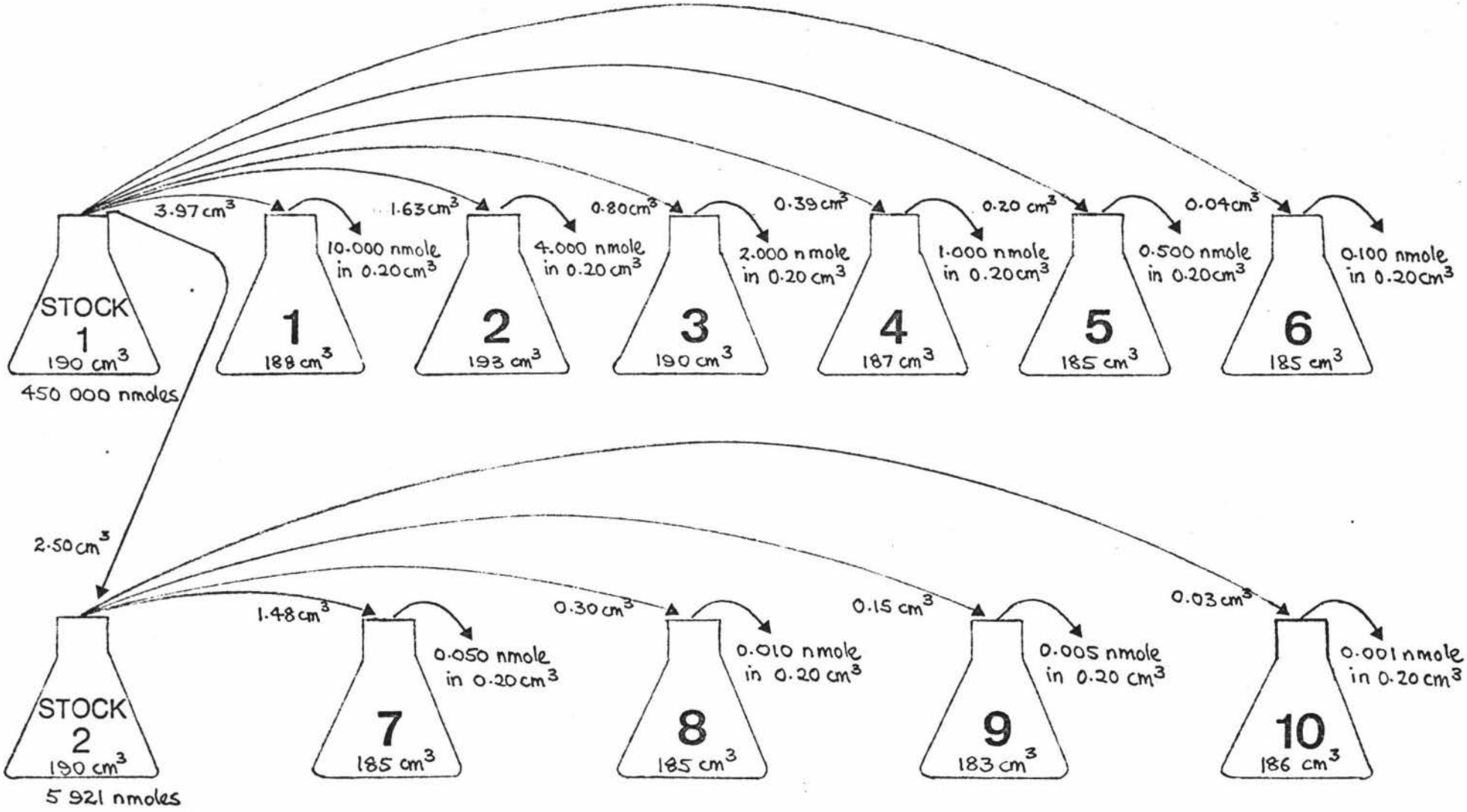


FIGURE J.1

DILUTION SERIES FOR THE PREPARATION OF ETHYLENE STANDARDS

In each run, a series of ehtylene standards was analysed. This was followed by the gas samples collected. A standard volume of 0.2 cm gas was injected for each standard and sample. From the results of ethylene standards on the recorder chart, a regression equation was derived and used to obtain a final equation for calculating the ethylene production in each container. This final equation is

$$Z = (a + b\ X)\left(\frac{V_1}{V_2}\right)\left(\frac{I}{T}\right)$$

where Z = ethylene production in μmoles hr^{-1} bag^{-1},

X = ethylene peak height in units,

a = intercept of the regression equation,

b = slope of the regression equation in μmoles $unit^{-1}$,

V = incubation volume in cm^3,

V = injection volume in cm^3,

T = incubation times in hour.

Using the same equation, the correction factors for ethylene in the soil blanks (S), plant blanks (P) and acetylene blanks (A) were also deduced and, therefore, the ethylene production attributed to the nitrogenase activity in the sainfoin nodules (N) is

$$N = Z - (S + P + A)$$

APPENDIX K. COLORIMETRIC AUTOMATED ANALYSIS (BLAKEMORE *et al*, 1981)

Preparation of Reagents

Kjeldahl digest reagent. To 1 000 cm^3 concentrated sulphuric acid, 100 g potassium sulphate and 1 g selenium powder were added. The mixture was heated for approximately 3 hours at 280 to 300°C until it cleared.

Wash solution. This was prepared by diluting 715 cm^3 Kjeldahl digest reagent to 10 000 cm^3 with distilled water.

Alkali phenol solution. To 1 400 cm^3 distilled water, 160 g phenol crystals, 132 g sodium hydroxide pellets and 228 g potassium-sodium tartrate were added. The solution was filtered to remove black solid impurities and then made up to 2 000 cm^3 with distilled water.

Acid ammonium molybdate. In about 800 cm^3 distilled water, 5 g ammonium molybdate was dissolved. Then, 56 cm^3 concentrated sulphuric acid was added and the solution was made up to 1 000 cm^3.

Hypochloride. Janola at household concentration was used.

Water. About 6 drops of Brij-35 wetting agent were added to 1 000 cm^3 distilled water.

Preparation of Standards

Stock solution (1 cm^3 = 500 µg N and 100 µg P). In approximately 800 cm^3 distilled water, 2.358 g ammonium sulphate (A.R grade and dried at 105°C) and 0.440 g potassium dihydrogen phosphate (A.R grade) were dissolved. Then, 50 cm^3 concentrated sulphuric acid was added and the solution was made up to 1000 cm^3 with distilled water in a volumetric flask.

Working standards. To 250 cm^3 volumetric flasks, 0.0, 12.5, 25.0, 37.5 and 50.0 cm^3 of stock solution were pipetted and each made up to volume with previously prepared wash solution. These standards contained 0, 25, 50, 75 and 100 µg cm^{-3} N and 0, 5, 10, 15 and 20 µg cm^{-3} P respectively.

Colorimetric Automated Analysis

During the operation of the Autoanalyser, the following conditions were maintained.

Heating bath	40°C
N colorimeter	660 nm
P colorimeter	630 nm; aperture = 4
Sample - wash ratio	2:1

Calculation

Standard curves of μg cm^{-3} N against peak height and μg cm^{-3} P against peak height were prepared. From these curves, the sample N and P concentrations were read and used to calculate the plant tissue N and P concentrations with the formula

$$T = \frac{SXV}{W}$$

where T = plant tissue N or P concentration in μg g^{-3},

S = sample N or P concentration in μg cm^{-3},

V = dilution volume which was 50 cm^3,

W = dry weight of plant tissue digested which is o.1 g.

APPENDIX L. POOLED ANOVA FOR ENDOPHYTE INFECTION

TABLE L.1

POOLED ANOVA FOR ENDOPHYTE INFECTION PER FIELD OF VIEW

Source of variation	DF	MS	VR	
Block	1	1.986×10^{-3}	0.833	ns
Rhizobia (R)	1	3.904×10^{-3}	3.111	ns
Endophyte (E)	1	2.193×10^{-2}	17.478	*
RXE	1	3.904×10^{-3}	3.111	ns
Error (a)	3	1.255×10^{-3}	0.526	
Time (T)	2	2.653×10^{-3}	1.770	ns
TXR	2	1.411×10^{-3}	0.942	ns
TXE	2	2.653×10^{-3}	1.770	ns
TXRXE	2	1.411×10^{-3}	0.942	ns
Error (b)	8	1.499×10^{-3}	0.628	
Error (c)	48	2.385×10^{-3}		

ns P>0.05

* P<0.05

** P<0.01

$CV(a) = \frac{\sqrt{(1.255 \times 10^{-3})}}{0.0175} \times 100 = 202.43\%$, $CV(b) = \frac{\sqrt{(1.499 \times 10^{-3})} \times 100}{0.0175}$

$= 221.24\%$

APPENDIX M. POOLED ANOVA FOR NODULATION AND DINITROGEN FIXATION VARIABLES

TABLE M.1

POOLED ANOVA FOR NODULE NUMBER PER PLANT

Source of variation	DF	MS	VR	
Block	1	0.225	3.698	ns
Rhizobia (R)	1	25.017	363.579	**
Endophyte (E)	1	1.880	27.329	*
RXE	1	0.027	0.393	ns
Error (b)	3	0.069	1.133	ns
Time (T)	8	25.206	266.081	**
TXR	8	0.322	3.402	**
TXE	8	0.432	4.569	**
TXRXE	8	0.049	0.514	ns
Error (b)	32	0.095	1.560	
Error (c)	240	0.061		

ns P>0.05

* P<0.05

** P<0.01

$CV(a) = \frac{\sqrt{0.06881}}{2.05} \times 100 = 12.80\%$, $CV(b) = \frac{\sqrt{0.09473}}{2.05} \times 100 = 15.01\%$

TABLE M.2

POOLED ANOVA FOR NODULE DRY WEIGHT PER PLANT

Source of variation	DF	MS	VR	
Block	1	0.018	7.449	**
Rhizobia (R)	1	1.934	1714.288	**
Endophyte (E)	1	0.015	13.215	*
RXE	1	0.000	0.044	ns
Error (a)	3	0.001	0.474	
Time (T)	8	2.143	372.389	**
TXR	8	0.041	7.204	**
TXE	8	0.004	0.714	ns
TXRXE	8	0.003	0.606	ns
Error (b)	32	0.006	2.418	
Error (c)	240	0.277		

ns P>0.05

* P<0.05

** P<0.01

$CV(a) = \frac{\sqrt{0.00128}}{0.29} \times 100 = 11.58\%$, $CV(b) = \frac{\sqrt{0.005756}}{0.29} \times 100$

$= 26.16\%$

TABLE M.3

POOLED ANOVA FOR NODULE DRY WEIGHT PER NODULE

Source of variation	DF	MS	VR	
Block	1	4.868×10^{-5}	1.123	ns
Rhizobia (R)	1	5.520×10^{-3}	182.050	**
Endophyte (E)	1	3.966×10^{-4}	13.077	*
RXE	1	6.180×10^{-6}	0.204	ns
Error (a)	3	3.033×10^{-5}	0.700	
Time (T)	8	8.893×10^{-3}	133.948	**
TXR	8	1.548×10^{-4}	2.332	*
TXE	8	8.823×10^{-5}	1.329	ns
TXRZE	8	3.367×10^{-5}	0.507	ns
Error (b)	32	6.639×10^{-5}	1.532	
Error (c)	240	4.335×10^{-5}		

ns $P>0.05$

* $P<0.05$

** $P<0.01$

$CV(a) = \frac{\sqrt{(3.033 \times 10^{-5})}}{0.01917} \times 100 = 28.73\%$, $CV(b) = \frac{\sqrt{(6.639 \times 10^{-5})}}{0.01917} \times 100$

$= 42.50\%$

TABLE M.4

POOLED ANOVA FOR ACETYLENE REDUCED PER HOUR PER PLANT

Source of variation	DF	MS	VR	
Block	1	0.035	1.307	ns
Rhizobia (R)	1	28.551	341.402	**
Endophyte (E)	1	0.041	0.496	ns
RXE	1	0.014	0.172	ns
Error (a)	3	0.084	3.111	
Time (T)	5	1.584	30.374	**
TXR	5	0.851	16.334	**
TXE	5	0.035	0.677	ns
TXRXE	5	0.025	0.478	ns
Error (b)	20	0.052	1.940	
Error (c)	48	0.027		

ns P>0.05

* P<0.05

** P<0.01

$CV(a) = \frac{\sqrt{0.08363}}{0.82} \times 100 = 35.27\%$, $CV(b) = \frac{\sqrt{0.05215}}{0.82} \times 100 = 27.85\%$

TABLE M.5

POOLED ANOVA FOR ACETYLENE REDUCED PER HOUR PER GRAM NODULE DRY WEIGHT

Source of variation	DF	MS	VR	
Block	1	1 168.980	34.138	**
Rhizobia (R)	1	13 920.390	71.507	**
Endophyte (E)	1	81.050	0.416	ns
RXE	1	122.620	0.630	ns
Error (a)	3	194.670	5.685	
Time (T)	5	388.310	2.927	*
TXR	5	179.610	1.354	ns
TXE	5	123.220	0.929	ns
TXRXE	5	163.690	1.234	ns
Error (b)	20	132.650	3.874	
Error (c)	48	34.240		

ns $P>0.05$

* $P<0.05$

** $P<0.01$

$CV(a) = \frac{\sqrt{194.67}}{12.25} \times 100 = 113.90\%$, $CV(b) = \frac{\sqrt{132.65}}{12.25} \times 100 = 94.02\%$

APPENDIX N. POOLED ANOVA FOR TOTAL NITROGEN AND PHOSPHORUS CONCENTRATIONS

TABLE N.1

POOLED ANOVA FOR TOTAL COTYLEDON NITROGEN

Source of variation	DF	MS	VR	
Block	1	3.339×10^{-1}		
Rhizobia (R)	1	1.075×10^{-1}	5.070	ns
Endophyte (E)	1	5.349×10^{-1}	25.233	*
RXE	1	1.097×10^{-1}	5.173	ns
Error (a)	3	2.120×10^{-2}		
Time (T)	5	1.204×10^{1}	188.456	**
TXR	5	1.487×10^{-3}	0.023	ns
TXE	5	3.648×10^{-2}	0.571	ns
TXRXE	5	2.327×10^{-2}	0.364	ns
Error (b)	20	6.390×10^{-2}		
Error (c)	240	0.000		

ns P>0.05

* P<0.05

** P<0.01

$CV(a) = \frac{\sqrt{(2.120 \times 10^{-2})}}{10.93} \times 100 = 1.33\%$, $CV(b) = \frac{\sqrt{(6.390 \times 10^{-2})}}{10.93} \times 100$

$= 2.31\ \%$

TABLE N.2

POOLED ANOVA FOR TOTAL SHOOT NITROGEN

Source of variation	DF	MS	VR	
Block	1	1.186×10^{-2}		
Rhizobia (R)	1	3.242×10^{-2}	30.720	*
Endophyte (E)	1	6.250×10^{-4}	0.592	ns
RXE	1	8.050×10^{-4}	0.763	ns
Error (a)	3	1.055×10^{-3}		
Time (T)	10	8.246×10^{-2}	115.653	**
TXR	10	1.499×10^{-2}	21.024	**
TXE	10	2.230×10^{-4}	0.313	ns
TXRXE	10	6.631×10^{-4}	0.930	ns
Error (b)	40	7.130×10^{-4}		
Error (c)	320	0.000		

ns P>0.05

* P<0.05

** P<0.01

$CV(a) = \frac{\sqrt{(1.055 \times 10^{-3})}}{1.01} \times 100 = 3.22\%$, $CV(b) = \frac{\sqrt{(7.130 \times 10^{-4})}}{1.01} \times 100$

$= 2.64\%$

TABLE N.3

POOLED ANOVA FOR TOTAL ROOT NITROGEN

Source of variation	DF	MS	VR	
Block	1	1.052×10^{-2}		
Rhizobia (R)	1	1.892	40.905	**
Endophyte (E)	1	3.407×10^{-1}	7.367	ns
RXE	1	2.488×10^{-1}	5.380	ns
Error (a)	3	4.624×10^{-2}		
Time (T)	10	1.109×10^{1}	220.712	**
TXR	10	8.891×10^{-1}	17.698	**
TXE	10	9.886×10^{-2}	1.968	ns
TXRXE	10	4.600×10^{-2}	0.916	ns
Error (b)	40	5.024×10^{-2}		
Error (c)	320	0.000		

ns P>0.05

* P<0.05

** P<0.01

$CV(a) = \frac{\sqrt{(4.624 \times 10^{-2})}}{10.30} \times 100 = 2.09\%$, $CV(b) = \frac{\sqrt{(5.024 \times 10^{-2})}}{10.30} \times 100$

$= 2.18\%$

TABLE N.4

POOLED ANOVA FOR TOTAL NODULE NITROGEN

Source of variation	DF	MS	VR	
Block	1	1.028×10^1		
Rhizobia (R)	1	5.748×10^2	278.904	**
Endophyte (E)	1	6.202	3.009	ns
RXE	1	4.504	2.185	ns
Error (a)	3	2.061		
Time (T)	5	1.262×10^1	3.257	*
TXR	5	9.869	2.548	ns
RXE	5	3.309	0.854	ns
TXRXE	5	2.787	0.719	ns
Error (b)	20	3.873		
Error (c)	120	0.000		

ns P>0.05

* P<0.05

** P<0.01

$CV(a) = \frac{\sqrt{2.061}}{64.38} \times 100 = 2.23\%$, $CV(b) = \frac{\sqrt{3.873}}{64.38} \times 100 = 3.06\%$

TABLE N.5

POOLED ANOVA FOR TOTAL PLANT NITROGEN

Source of variation	DF	MS	VR	
Block	1	9.762×10^{8}		
Rhizobia (R)	1	1.620×10^{10}	95.064	**
Endophyte (E)	1	1.963×10^{8}	1.152	ns
RXE	1	3.197×10^{8}	1.877	ns
Error (a)	3	1.704×10^{8}		
Time (T)	9	1.656×10^{10}	304.785	**
TXR	9	2.354×10^{9}	43.328	**
TXE	9	8.953×10^{7}	1.648	ns
TXRXE	9	3.490×10^{7}	0.642	ns
Error (b)	36	5.432×10^{7}		
Error (c)	304	0.000		

ns P>0.05

* P<0.05

** P 0.01

$CV(a) = \frac{\sqrt{(1.704 \times 10^{8})}}{62926.70} \times 100 = 20.74\%$, $CV(b) = \frac{\sqrt{(5.432 \times 10^{7})}}{62926.70} \times 100$

$= 11.71\%$

TABLE N.6

POOLED ANOVA FOR TOTAL COTYLEDON PHOSPHORUS

Source of variation	DF	MS	VR	
Block	1	4.217	797 636.500	**
Rhizobia (R)	1	2.721×10^{-1}	1.761	ns
Endophyte (E)	1	4.945	32.004	*
RXE	1	6.031×10^{-2}	0.390	ns
Error (a)	3	1.545×10^{-1}	29 231.598	
Time (T)	5	5.806×10^{1}	69.417	**
TXR	5	8.800×10^{-2}	0.105	ns
TXE	5	5.851×10^{-1}	0.700	ns
TXRXE	5	3.737×10^{-1}	0.447	ns
Error (b)	20	8.363×10^{-1}	158 210.844	
Error (c)	240	5.286×10^{-6}		

ns P>0.05

* P<0.05

** P<0.01

$CV(a) = \frac{\sqrt{(1.545 \times 10^{-1})}}{15.66} \times 100 = 2.51\%$, $CV(b) = \frac{\sqrt{(8.363 \times 10^{-1})}}{15.66} \times 100$

TABLE N.7

POOLED ANOVA FOR TOTAL SHOOT PHOSPHORUS

Source of variation	DF	MS	VR	
Block	1	4.130×10^6	7 593.163	**
Rhizobia (R)	1	2.791×10^7	41.295	**
Endophyte (E)	1	5.229×10^6	7.736	ns
RXE	1	7.785×10^4	0.115	ns
Error (a)	3	6.760×10^5	1 242.879	
Time (T)	10	4.782×10^7	19.288	**
TXR	10	9.123×10^6	3.680	**
TXE	10	2.498×10^6	1.008	ns
RXRXE	10	5.936×10^5	0.239	ns
Error (b)	10	2.479 c 10^6	4 558.453	
Error (c)	320	5.439×10^2		

ns P>0.05

* P<0.05

** P<0.01

$CV(a) = \frac{\sqrt{(6.760 \times 10^5)}}{6133.90} \times 100 = 13.40\%$, $CV(b) = \frac{\sqrt{(2.479 \times 10^6)}}{6133.90} \times 100$

$= 25.65\%$

TABLE N.8

POOLED ANOVA FOR TOTAL ROOT PHOSPHORUS

Source of variation	DF	MS	VR	
Block	1	9.020×10^6	34 469.000	**
Rhizobia (R)	1	6.116×10^6	3.546	ns
Endophyte (E)	1	1.930×10^7	11.193	*
RXE	1	2.396×10^6	1.398	ns
Error (a)	3	1.725×10^6	6 590.489	
Time (T)	10	6.775×10^7	23.830	**
TXR	10	2.357×10^6	0.829	ns
TXE	10	2.913×10^6	1.025	ns
TXRXE	10	4.532×10^5	0.159	ns
Error (b)	40	2.843×10^6	10 864.150	
Error (c)	320	2.617×10^2		

ns $P>0.05$

* $P<0.05$

** $P<0.01$

$CV(a) = \frac{\sqrt{(1.725 \times 10^6)}}{4989.20} \times 100 = 26.32\%$, $CV(b) = \frac{\sqrt{(2.843 \times 10^6)}}{4989.20} \times 100$

$= 33.80\%$

TABLE N.9

POOLED ANOVA FOR TOTAL NODULE PHOSPHORUS

Source of variation	DF	MS	VR	
Block	1	5.938	1 693.011	**
Rhizobia (R)	1	37.416	161.428	**
Endophyte (E)	1	0.154	.0.644	ns
RXE	1	1.709	7.373	ns
Error (a)	3	0.232	66.089	
Time (T)	5	19.957	12.922	**
TXR	5	2.727	1.766	ns
TXE	5	1.878	1.216	ns
TXRXE	5	1.972	1.277	ns
Error (b)	20	1.544	440.365	
Error (c)	120	0.004		

ns P>0.05

* P<0.05

** P<0.01

$CV(a) = \frac{\sqrt{0.231781}}{48.65} \times 100 = 0.99\%$, $CV(b) = \frac{\sqrt{1.544418}}{48.65} \times 100$

$= 2.55\%$

TABLE N.10

TOTAL ANOVA FOR TOTAL PLANT PHOSPHORUS

Source of variation	DF	MS	VR	
Block	1	3.347×10^5	9 405.004	**
Rhizobia (R)	1	4.074×10^7	1 907.206	**
Endophyte (E)	1	4.565×10^6	213.690	**
RXE	1	6.463×10^5	30.254	*
Error (a)	3	2.136×10^4	600.358	
Time (T)	9	4.253×10^7	37.871	**
TXR	9	5.226×10^6	4.653	**
TXE	9	1.765×10^6	1.571	ns
TXRXE	9	6.000×10^5	0.534	ns
Error (b)	36	1.123×10^6	31 564.281	
Error (c)	304	3.558×10^1		

ns $P>0.05$

* $P<0.05$

** $P<0.01$

$CV(a) = \frac{\sqrt{(2.136 \times 10^4)}}{5282.52} \times 100 = 2.77\%$, $CV(b) = \frac{\sqrt{(1.123 \times 10^6)}}{5282.52} \times 100$

$= 20.06\%$

APPENDIX O. POOLED ANOVA FOR PLANT GROWTH VARIABLES

TABLE 0.1

POOLED ANOVA FOR COTYLEDON DRY WEIGHT PER PLANT

Source of variation	DF	MS	VR	
Block	1	5.923×10^{-6}	19.682	**
Rhizobia (R)	1	1.025×10^{-7}	0.159	ns
Endophyte (E)	1	3.720×10^{-6}	5.755	ns
RXE	1	2.353×10^{-7}	0.364	ns
Error (a)	3	6.464×10^{-7}	2.148	
Time (T)	6	8.784×10^{-5}	118.705	**
TXR	6	9.424×10^{-7}	1.274	ns
TXE	6	2.863×10^{-7}	0.387	ns
TXRXE	6	1.849×10^{-7}	0.250	ns
Error (b)	24	7.400×10^{-7}	2.459	
Error (c)	256	3.009×10^{-7}		

ns $P>0.05$

* $P<0.05$

** $P<0.01$

$$CV(a) = \frac{\sqrt{(6.464 \times 10^{-7})}}{0.003444} \times 100 = 23.34\%, \quad CV(b) = \frac{\sqrt{(7.400 \times 10^{-7})}}{0.003444} \times 100 = 24.98\%$$

TABLE 0.2

POOLED ANOVA FOR SHOOT DRY WEIGHT PER PLANT

Source of variation	DF	MS	VR	
Block	1	0.721	19.915	**
Rhizobia (R)	1	4.835	100.744	**
Endophyte (E)	1	0.096	1.993	ns
RXE	1	0.000	0.001	ns
Error (a)	3	0.048	1.327	
Time (T)	10	131.069	2 824.393	**
TXR	10	1.655	35.673	**
TXE	10	0.063	1.348	ns
TXRXE	10	0.074	1.591	ns
Error (b)	40	0.046	1.283	
Error (c)	320	0.036		

ns P>0.05

* P<0.05

** P<0.01

$CV(a) = \frac{\sqrt{0.048}}{2.7549} \times 100 = 7.95\%$, $CV(b) = \frac{\sqrt{0.04641}}{2.7549} \times 100 = 7.82\%$

TABLE 0.3

POOLED ANOVA FOR ROOT DRY WEIGHT PER PLANT

Source of variation	DF	MS	VR	
Block	1	30.032	4.018	*
Rhizobia (R)	1	4.748	0.501	ns
Endophyte (E)	1	92.929	9.812	ns
RXE	1	25.368	2.678	ns
Error (a)	3	9.471	1.267	
Time (T)	10	6 115.515	270.219	**
TXR	10	30.020	1.326	ns
TXE	10	10.010	0.442	ns
TXRXE	10	17.247	0.762	ns
Error (b)	40	22.632	3.028	ns
Error (c)	320	7.474		

ns P>0.05

* P<0.05

** P<0.01

$CV(a) = \frac{\sqrt{9.471}}{98.70} \times 100 = 3.12\%$, $CV(b) = \frac{\sqrt{22.632}}{98.70} \times 100 = 4.82\%$

TABLE 0.4

POOLED ANOVA FOR TOTAL DRY WEIGHT PER PLANT

Source of variation	DF	MS	VR	
Block	1	21.060	5.067	*
Rhizobia (R)	1	374.994	72.427	**
Endophyte (E)	1	41.940	8.100	ns
RXE	1	3.109	0.600	ns
Error (a)	3	5.178	1.246	
Time (T)	10	3 910.228	435.502	**
TXR	10	154.811	17.242	**
TXE	10	5.707	0.636	ns
TXRXE	10	8.333	0.928	ns
Error (b)	40	8.979	2.160	
Error (c)	320	4.156		

ns P>0.05

* P<0.05

** P<0.01

$CV(a) = \frac{\sqrt{5.178}}{97.23} \times 100 = 2.34\%$, $CV(b) = \frac{\sqrt{8.979}}{97.23} \times 100 = 3.08\%$

TABLE 0.5

POOLED ANOVA FOR ROOT-SHOOT RATIO

Source of variation	DF	MS	VR	
Block	1	0.056	2.663	ns
Rhizobia (R)	1	3.295	214.210	**
Endophyte (E)	1	0.187	12.166	*
RXE	1	0.345	22.444	*
Error (a)	3	0.015	0.736	
Time (T)	10	8.019	128.687	**
TXR	10	0.912	14.637	**
TXE	10	0.057	0.915	ns
TXRXE	10	0.035	0.563	ns
Error (b)	40	0.062	2.981	
Error (c)	320	0.021		

ns P>0.05

* P<0.05

** P<0.01

$CV(a) = \frac{\sqrt{0.01538}}{3.5234} \times 100 = 3.52\%$, $CV(b) = \frac{\sqrt{0.06232}}{3.5234} \times 100 = 7.09\%$

TABLE 0.6

POOLED ANOVA FOR SECONDARY STEM NUMBER PER PLANT

Source of variation	DF	MS	VR	
Block	1	0.098	0.358	ns
Rhizobia (R)	1	13.109	50.610	**
Endophyte (E)	1	0.360	1.392	ns
RXE	1	0.221	0.853	ns
Error (a)	3	0.259	0.949	
Time (T)	4	2.744	16.563	**
TXR	4	0.576	3.477	*
TXE	4	0.254	1.535	ns
TXRXE	4	0.080	0.483	ns
Error (b)	16	0.166	0.607	
Error (c)	80	0.273		

ns P>0.05

* P<0.05

** P<0.01

$CV(a) = \frac{\sqrt{0.259}}{1.195} \times 100 = 42.59\%$, $CV(b) = \frac{\sqrt{0.1657}}{1.195} \times 100 = 34.06\%$

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