THE EFFECT OF ETHYLENE AND CARBON DIOXIDE ON ROOT-NODULE FORMATION AND NITROGEN FIXATION IN THREE GRAIN LEGUMES

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DECLARATION

I hereby declare that the work presented in this thesis was performed by me except where otherwise indicated, and that it has not been submitted in any previous application for a degree. All sources of information have been specifically acknowledged by reference to the authors.

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ABSTRACT

The involvement of ethylene, (C_2H_4) in the formation and persistence of legume root nodules, and its interaction with carbon dioxide, (CO_2) have been studied in three different legume species, namely, pea (*Pisum sativum* L.), bean (*Phaseolus vulgaris* L.) and lentil (*Lens culinaris Medik.*). This work was undertaken because of the possible connection between tolerance to ethylene in the root zone and tolerance to waterlogging.

Two different techniques for root aeration were employed: a 'constant flow-through' system and a 'closed-vessel' (recirculatory) system. In the constant flow-through system, legume roots were exposed to an air-stream containing 0, 0.11, 0.33, and 1 ppm of C_2H_4. In the closed-vessel system C_2H_4 and/or CO_2 produced by the roots were allowed to accumulate around them.

Exogenous C_2H_4 at 1 ppm significantly inhibited root-nodule formation in pea and bean. Nodule fresh weight (individual and total), nitrogenase activity and leghaemoglobin content per plant were significantly reduced. Total N accumulation in the shoots and shoot dry weight per plant were also significantly lower than in the other treatments. However, root dry weight and primary root length were not significantly affected in either species. Significant positive correlations were observed between leghaemoglobin content and nodule fresh weight per plant, nitrogenase activity and leghaemoglobin, total N accumulation in the shoots and nitrogenase activity, and root dry weight and total nodule fresh weight. Shoot N concentration, on the other hand, was negatively correlated with shoot dry weight and total nodule fresh weight.

No comparable effect of 1 ppm C_2H_4 on the lentil cultivar, which is known to be moderately waterlogging tolerant, was observed.

Similar results were observed in the closed-vessel experiments, where C_2H_4 (produced by the plant roots) reached concentrations of 0.6-0.8 ppm. Carbon dioxide (0.6-1%) in the root atmosphere of pea and bean plants caused an increase in individual and total nodule fresh weight and the nitrogenase activity of the nodules. However, increased CO_2 in the root atmosphere did not significantly increase total N accumulation and shoot dry weight in pea. Again, no significant effect was observed with lentil. No significant interactions between C_2H_4 and CO_2 were observed in either experimental system.

Comparisons of a range of bean cultivars in sealed vessels showed large differences in the amounts of C_2H_4 produced by the root systems. Abnormal shoot growth and defoliation occurred when the endogenous C_2H_4 was allowed to accumulate, suggesting that it was at least partly responsible for the effects.

The relationship between sensitivity to C_2H_4 of different species and cultivars, and tolerance to waterlogging is discussed.

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1. INTRODUCTION

Ethylene is well known as a gaseous plant hormone, that affects many plant growth processes, from seed germination to senescence. It is unique among the hormones in being a simple gaseous compound. One process which may be of considerable ecological and agricultural importance is the inhibitory effect of ethylene on nodulation of legumes (Grobbelaar et al., 1971; Goodlass and Smith, 1979). The gas is produced in the root zone both by the plants themselves (endogenous ethylene) and by soil microorganisms (exogenous ethylene).

It is possible that the well-documented suppression of legume nodulation under waterlogged conditions (Bishnoi and Krishnamoorthy, 1990) may involve ethylene, since waterlogging (and the consequent reduction in gaseous exchange between root environment and the atmosphere) favours the accumulation of both endogenous and exogenous ethylene around the roots. The increased endogenous ethylene production by higher plants subjected to such environmental stress conditions is a well known natural phenomenon (Beyer et al., 1984) and possibly acts as a trigger of growth responses. Crop species vary widely in their response to flooding conditions, and tolerance appears to be directly related to hormonal changes in the plant. Ethylene production may contribute to tolerance by inducing processes such as adventitious rooting in maize and tomato, and aerenchyma formation in maize, although not in rice (Jackson and Drew, 1984).

There is evidence of differences between varieties as well as species in the sensitivity of plant roots to exogenous ethylene (Smith and Robertson, 1971). El-Beltagy and Hall (1979) compared two varieties of broad bean (*Vicia faba*)

and found that the ethylene content of aerial parts and roots increased over nine days' waterlogging and that the variety with the greater concentration suffered considerably greater leaf abscission. They suggested that the measurement of endogenous ethylene production might provide a technique for screening plants for relative tolerance to water stress.

Differences in endogenous ethylene production due to stresses other than waterlogging may directly affect crop performance in the field. For example, the inhibition of hypocotyl elongation, which affected certain varieties of soyabean, was shown to be due to rapid endogenous production of ethylene at a temperature of 25°C as compared with a low rate of production at 20°C (Samimy, 1970).

Legume root nodulation and nitrogen fixation appear to be more sensitive to waterlogging than many other plant growth processes (Minchin and Pate, 1975; Sprent and Gallacher, 1976), and also more sensitive to the presence of low concentrations of ethylene (Grobbelaar et al., 1971). Therefore it seemed logical to explore the extent to which ethylene-induced effects were involved in the well known poor tolerance of some grain legumes to waterlogging, and also to examine the interaction of ethylene with the other physiologically important gases in the root zone, oxygen and carbon dioxide.

One of the aims of this project was to develop techniques that would allow the effects of changes in ethylene and/or oxygen and carbon dioxide concentrations to be observed with whole legume plants that were not subject to any physiological stress other than changes in the composition of the root atmosphere. Most of the previous experiments of this kind (see review of

literature, Section 2.8.3) were carried out by exposing excised legume roots, or legume seedlings cultured with *Rhizobium* in liquid medium in petri dishes or in test tubes, to exogenously applied ethylene or endogenous ethylene allowed to build up naturally within the test vessels. It is possible that symbiotic development under such conditions may not have accurately reflected processes under natural field conditions since the plants are probably suffering additional stress. Methods were therefore developed to allow studies to be made using whole plants up to about five weeks in age, as a step towards achievement of more realistic conditions (Section 3.2).

2. REVIEW OF LITERATURE

2.1 LEGUMES

The *Leguminosae* is a family of plants which is subdivided into three major subfamilies, the *Papilionoideae,* the *Mimosoideae* and the *Caesalpinioideae*. There is a detailed review of the legumes by Allen and Allen (1981). Recently updated and commonly accepted taxonomy is included in a report by Faria et al., (1989).

A major factor in the ecological success of members of the Leguminosae is their ability to enter into a beneficial relationship with soil bacteria of the genera *Rhizobium*, *Bradyrhizobium*, or *Azorhizobium*. In this association, the bacteria induce the plant to develop a new plant organ, the root nodule. Within this nodule, the ecological niche required for fixation of atmospheric nitrogen by the bacteria is created, thus rendering the plant independent of soil nitrogen (Bergersen, 1982).

The oldest agricultural records available indicate that legumes have been cultured for centuries and that they were valued for food and soil enrichment long before their ability to work symbiotically with *Rhizobium* was understood. It is estimated that food legumes play an important role in both the diet and health of close to one billion people in the world.

2.2 NUTRITIONAL CHARACTERISTICS

The importance of food legumes as components of traditional diets world-wide is based on nutritional characteristics given in Table 2.1; i.e. a relatively high

Table 2.1. Positive nutritional attributes of food legumes (Bressani and Elias, 1988).

1.	High protein concentration
2.	High lysine concentration
3.	Excellent supplementary protein to cereal grains
4.	Beneficial effects on blood cholesterol values
5.	Beneficial effects on blood glucose values

Table 2.2. Selected nutritional composition of milled polished rice, faba bean, pea, lentil, chickpea and wheat (composition per 100 g edible portion of dried mature whole seeds).

Component	Rice[1]	Faba bean[1]	Pea[1]	Lentil[1]	Chickpea[1]	Wheat[2]
Moisture (%)	11.8	13.8	13.6	12.0	11.0	13.0
Protein (g)	6.4	25.0	22.2	20.0	19.4	15.0
Oil (g)	0.8	1.2	1.4	0.6	5.6	2.1
Crude fibre(g)	0.3	5.1	6.0	NDa	2.5	2.4
Dietary fibre(g)	8.3	NDa	16.7	11.7	25.5	NDa
Starch (%)	81.1	51.6	54.1	59.1	54.9	82.0
Sugars (%)	NDa	5.0	8.1	6.1	13.1	2.5
Iron (mg)	1.9	4.2	4.4	7.0	2.2	5
Thiamin (mg)	0.10	0.45	0.77	0.46	0.46	0.29
Riboflavin (mg)	0.05	0.19	0.18	0.33	0.20	0.11
Niacin (mg)	2.1	2.4	3.1	1.3	1.2	5
Energy (kcal)	366	328	330	340	362	370

a. ND: Not determined.
[1]Aykroyd et al. (1982); [2]Kent (1975)

protein concentration with ample amounts of the essential amino acid lysine, which is deficient in cereal grain protein.

The protein-rich seeds of pea, bean, lentil, chickpea, mungbean, and pigeon pea are especially important where poverty, religion, or social circumstances either prevent or restrict the consumption of meat. It is well established that the proteins of food legumes and those of cereal grains are nutritionally complementary; the essential amino acids that are deficient in one may be provided by the other. Consequently, a balanced blend of amino acids from a food legume and cereal mixture may have a greater nutritional value than either ingredient alone (Newman et al., 1988) both for humans and for animals. Additionally, the fibre from legumes is beneficial in lowering serum cholesterol values and removing potentially toxic and/or carcinogenic compounds in the gastrointestinal tract of animals (Freeman, 1980; Chen and Anderson, 1984). Selected nutritional compositions of faba bean, pea, lentil and chickpea are given in Tables 2.2 and 2.3 (Aykroyd et al., 1982), where rice is included for comparison.

2.3 AGRONOMY

Grain legumes offer several advantages when grown in a rotational system with cereals and possibly with other crops, too (Papendick, 1982). In many cases the legumes, if properly inoculated and well nodulated, will fix a substantial part of the nitrogen needed for seed production and so will minimize depletion of soil nitrogen (Bezdicek et al., 1982). Over 1200 species of *Leguminosae* are recorded, of which about 10% have been examined for nodulation: the property is widespread among the *Papilionoideae* (some 85% of the species examined

Table 2.3. Protein as percentage of dry matter, and essential amino acids as percentage protein in milled polished rice, faba bean, pea, lentil, chickpea and wheat.

Component	Rice[1]	Faba bean[1]	Pea[1]	Lentil[1]	Chickpea[1]	Wheat[2]
Protein	7.3	29.0	25.7	23.0	21.8	16.3
Isoleucine	4.2	4.0	4.3	4.3	4.4	3.8
Leucine	8.2	7.1	6.8	7.6	7.5	6.7
Lysine	3.6	6.5	7.5	7.2	6.8	2.3
Methionine	2.1	0.7	0.9	0.8	1.0	1.7
Cystine	1.5	0.8	1.1	0.9	1.2	2.6
Phenylalanine	4.8	4.3	4.6	5.2	5.7	4.8
Tyrosine	3.2	3.2	2.7	3.3	2.9	2.7
Threonine	3.3	3.4	4.1	4.0	3.8	2.8
Valine	5.8	4.4	4.7	5.0	4.5	4.4

[1]Aykroyd et al. (1982); [2]Kent (1975)

Table 2.4. World production of the major food crop groups (FAO, 1984)

Commodity group	Average values of		
	Area harvested (Mha)	Yield (t ha^{-1})	Production (Mt)
Cereals	730	2.47	1802
Legumes	138	1.15	158
Roots or Tubers	471	2.75	93
Oil Seeds	41	0.95	39

nodulate), less common among the *Mimosoideae* (25%) and rare among the *Caesalpinioideae.* Thus most of the best-known nodulating *Leguminosae* belong to the *Papilionoideae* (e.g. peas, beans, clover, lucerne, and lupin in temperate areas; soyabeans, ground nuts, lentils, cowpeas, chickpeas, pigeon peas, and mung beans in tropical and subtropical areas). Nodules are almost always restricted to the roots, but two or three exceptions are known, such as the tropical marsh legume *Sesbania rostrata* which can develop a spectacular array of nodules down its stem (Dreyfus & Dommergues, 1981).

Legume crops normally contribute to the nitrogen requirements of succeeding non-legume crops. Inclusion of legumes in cropping systems is an old-established method to increase the productivity and fertility of soil by fixing atmospheric N and improving the physical, chemical and biological properties of the soil (Ghosh, 1981; Jain et al., 1985; Biswas et al., 1987). Available estimates indicate that legumes fix about 35 million tonnes of N per year in the world (Burris, 1977; Prasad, 1986). Intercropping legumes with non-legumes is the commonest cropping system in the tropics. Several reports have demonstrated that in an intercropped system, the presence of a grain legume increases the growth or nitrogen content of the non-legume (Agboola and Fayemi, 1972; Remison, 1978; Eaglesham et al., 1981; Reddy and Willey, 1981; Vasilas and Ham, 1985).

Legumes like chickpea, lentil and pea were reported in one Indian study to reduce the N requirement of the succeeding maize crop to the extent of 16-18 kg ha^{-1} as compared to wheat or fallow (Ahlawat et al., 1981). Sekhon (1983) reported that a mung bean-maize-wheat system could be sustained without fertilizer N. George and Prasad (1989) claimed similar results in multiple

cropping systems, namely: maize (fodder)-rice-lentil-wheat; maize (fodder)-rice-lentil; cowpea (fodder)-rice-lentil during the crop years (July-June) 1985-86 and 1986-87 in India. Interest has been shown in green manure to sustain lowland rice productivity, reduce farmers' dependence on mineral-N fertilizers, and lower their costs (Garrity and Flinn, 1988; Ladha et al., 1988). In tropical Asia, farmers plant green-manure legumes during the 40-60 day transition period between two rice crops (Garrity and Flinn, 1988). Biological nitrogen fixation (BNF) by leguminous green-manure crops has the potential to provide a substantial portion of the N required by rice (Bouldin, 1988; Ladha et al., 1988; Becker et al., 1990).

Legumes also contribute greatly to pasture production by providing high-protein forage, especially during the dry season when grass quality is poor (Willey, 1981). A nitrogen advantage for grass growing in a mixture with white clover (*Trifolium repens* L.) has been observed by many authors (Boller & Nosberger, 1988; Goodman, 1988).

Rotational cropping with legumes has long been used as a practical method of improving soil physical and chemical properties (Kurtz et al., 1984). The addition of legume residues containing large amounts of N obtained through symbiotic N_2 fixation resulted in improved soil fertility, soil structure and reduced erosion (Jansson and Peterson, 1982). Legumes in rotation with cereal crops can also be extremely effective for breaking disease cycles in both the cereal and the legume crop. A single year of pea or lentil is usually adequate to reduce the inoculum of pathogens to a safe level for a following wheat crop (Papendick et al., 1988).

Despite all the advantages, farmers often consider legumes as crops of secondary importance in comparison with cereals such as rice, wheat, and maize, and so they provide the latter with the greater share of the agronomic inputs and managerial attention. As a result legumes are often grown under marginal soil conditions. Yield figures for major food crops reveal that the legumes are poorly productive, although the total area devoted to them is relatively large, in keeping with their importance around the world (Table 2.4).

The poor yields and low overall production of many food legumes may be attributed to several major constraints (Al-Jibouri, 1977; Al-Jibouri and Bozzini, 1979). Legumes have poor tolerance and resistance to environmental factors such as low temperature, flooding, drought and salinity. In addition, disease and pest resistance is also poor. Lack of research into breeding higher yielding and more resistant or tolerant varieties has meant that there is strong competition for farmers to grow other food and cash crops which give better economic returns.

2.4 LEGUME-RHIZOBIUM SYMBIOSIS

In global terms, the production of crops is limited by the availability of fixed nitrogen, supplied to the soil by chemical or biological means. The major source of available nitrogen world-wide is biological (Fig. 2.1 contains estimates of the amount of atmospheric N_2 converted to combined form each year), introduced into the soil-plant system by the action of microorganisms living either under symbiotic conditions (e.g. *rhizobia*) or freely in the soil (e.g. *Azotobacter*) (Postgate, 1982). Leguminous root nodules are by far the largest single source of organic nitrogen in the global nitrogen cycle (Nap and Bisseling, 1990b). The microorganisms involved in these processes contain the

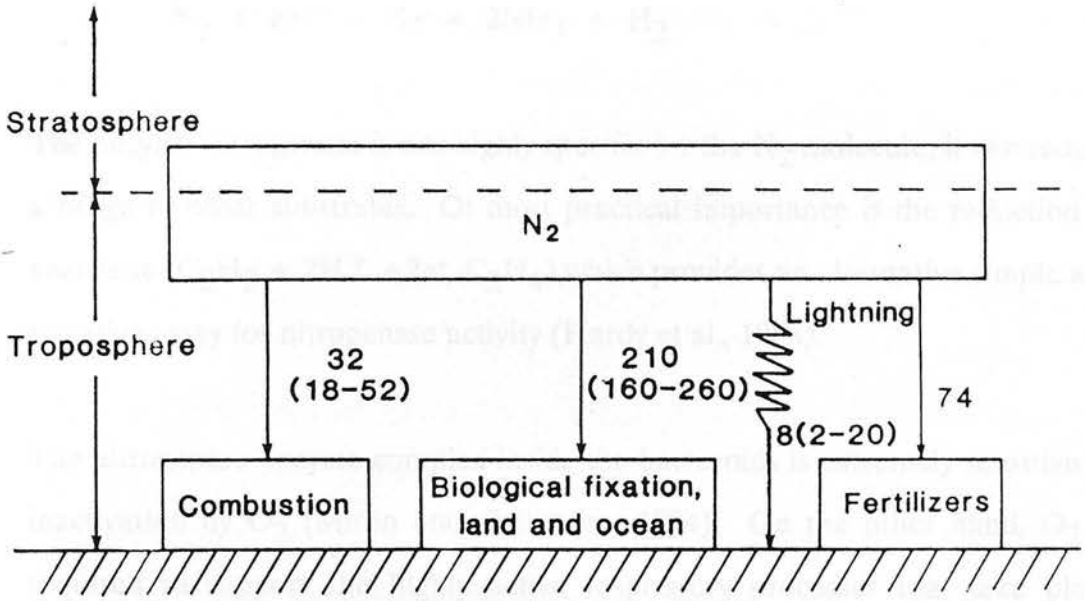


Fig. 2.1. Biological and chemical fixation of N_2 on the global scale. Units are millions of tonnes of nitrogen per year. The data are adapted from Jenkinson (1991), after Warneck (1988).

oxygen-sensitive metalloenzyme nitrogenase that converts the atmospheric N_2 gas to ammonia, according to the equation:

$$N_2 + 8H^+ + 8e^- = 2NH_3 + H_2$$

The enzyme nitrogenase is not highly specific for the N_2 molecule; it can reduce a range of other substrates. Of most practical importance is the reduction of acetylene ($C_2H_2 + 2H^+ + 2e^- \; C_2H_4$) which provides an alternative simple and sensitive assay for nitrogenase activity (Hardy et al., 1968).

The nitrogenase enzyme complex inside the bacteroids is extremely sensitive to inactivation by O_2 (Miflin and Cullimore, 1984). On the other hand, O_2 is required to support the highly active respiratory processes that take place aerobically in the plant and bacteroid compartments. Consequently, nodules have developed an efficient mechanism for delivering and dispersing O_2 in the central zone, while maintaining the free O_2 in this region at a concentration that ranges between 3 and 30 nM (Day and Copeland, 1991). The diffusion of O_2 into the central zone of nodules is regulated by a physical barrier which is probably located in a layer of cells in the subcortex (Day and Copeland, 1991). Under conditions of stress, the resistance to the diffusion of O_2 through this barrier increases, causing the concentration of O_2 in the central zone to fall to a very low level and nitrogen fixation to be inhibited (Day and Copeland, 1991).

During symbiosis, the host plant synthesises a certain number of proteins specific to nodule development and nitrogen fixation, called nodulins. The best known among these proteins is leghaemoglobin, which is found in all legumes and regulates oxygen tension in nodules. Leghaemoglobin constitutes a

buffering mechanism in legume root nodules, serving to minimize the O_2 gradient through the infected tissue and to provide sufficient O_2 for bacterial respiration, albeit at an extremely low free O_2 concentration (Appleby, 1984). By providing a sustained oxygen flux to bacteroids at low O_2 concentration, it protects the nitrogenase enzyme from excess O_2. Being located in the host cell cytoplasm, it may also provide a sufficiently high flux of O_2 to support the oxidative functions of the host cell and thus may serve a dual role in nodule tissue (Verma and Nadler, 1984).

Nitrogen-fixing microorganisms are found in most habitats, although for agricultural purposes the most significant ones in terms of the amounts of N_2 fixed per annum are those in nodules on plant roots (Beringer, 1984). One of the most beneficial interactions between bacteria and plants is symbiotic nitrogen fixation. Bacteria of the genus *Rhizobium* invade the root cells of different leguminous plants, resulting in nodule formation and eventually nitrogen fixation. The mutual advantages of the symbiosis are that the plants are supplied with fixed nitrogen, which is one of the most limiting nutrients, and on the other hand, bacteria are in a protected environment inside the nodule cells and are supplied with the product of photosynthesis (photosynthate) to satisfy their requirement for carbon and energy needs. Leguminous plants in symbiosis with *Rhizobium* can fix nitrogen at rates in the range 52-300 kg ha^{-1} y^{-1} (Phillips, 1980). The association between legumes and their appropriate rhizobia has been the focus of intensive investigation (Yates, 1980; Robertson and Farden, 1980; Pueppke, 1986).

2.5 NODULE INDUCTION ON PLANT ROOTS BY RHIZOBIUM

The process of symbiotic nitrogen fixation and the steps leading to it are highly complex, requiring interaction between the bacterium and the plant host (Fig. 2.2), and are influenced by genetic factors in both the partners (Fig. 2.3). Combined efforts of cytologists, plant physiologists, geneticists and molecular biologists have given insight into the processes of nodule formation and functioning (Brill, 1980; Beringer et al., 1980; Vincent, 1980; Kondorosi and Johnston, 1981; Postgate, 1982; Verma, 1982; Verma and Long, 1983; Verma and Nadler, 1984; Schubert, 1986; Rolfe and Gresshoff, 1988; Long 1989; Sprent, 1989; Nap and Bisseling, 1990a).

The first step in the establishment of symbiosis is the recognition and invasion of the appropriate legume by the bacteria. Rhizobia attached to the normally straight root hairs induce deformations, curling and branching. The curled root hairs resemble shepherds' crooks that can entrap bacteria in their fold. At this site, rhizobia penetrate the plant cell wall and a tubular infection thread forms which carries the bacteria into the root meristem. Meanwhile, the cortical cells are induced to divide and are then invaded by the bacteria released from the branching infection thread. Within the cortical cells the bacteria multiply and differentiate into the morphologically altered bacteroids. The enzyme nitrogenase synthesized in the bacteroids converts atmospheric molecular nitrogen to ammonia, which is then assimilated by the plant. Identification and analysis of plant and bacterial genes that direct nodule formation and function have progressed to a point where these processes can be described in considerable detail at the molecular level, showing that the signal-response pathways involved in the plant cell division and differentiation are quite unlike

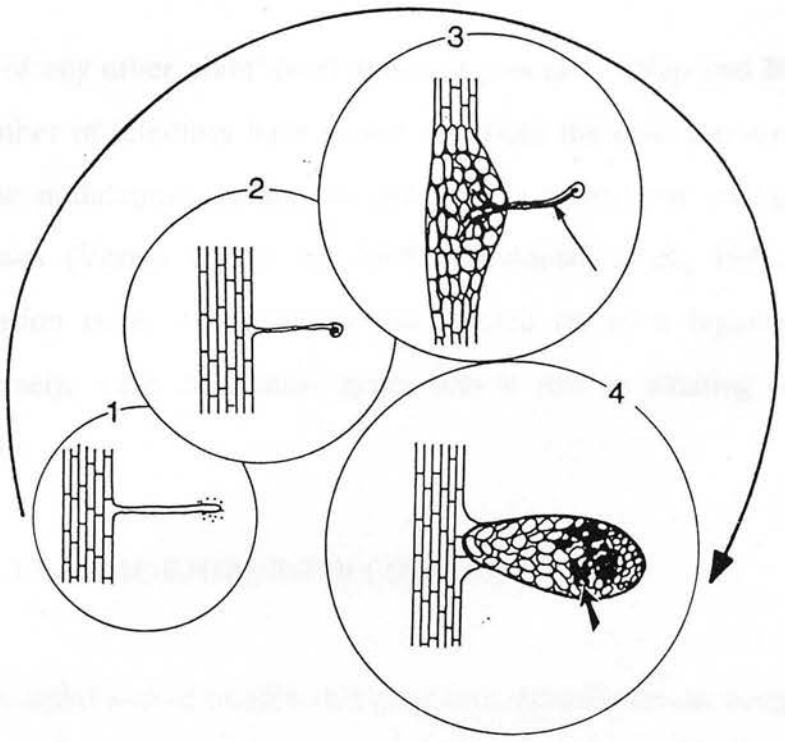


Fig. 2.2. Development of a symbiotic nodule. 1, attachment of bacteria to the root hair; 2, root hair curling and entrapment of bacteria ; 3, infection thread (arrow) growth; 4, mature nodule, the arrow points to plant cells packed with nitrogen-fixing bacteroids. (Kondorosi and Kondorosi, 1986).

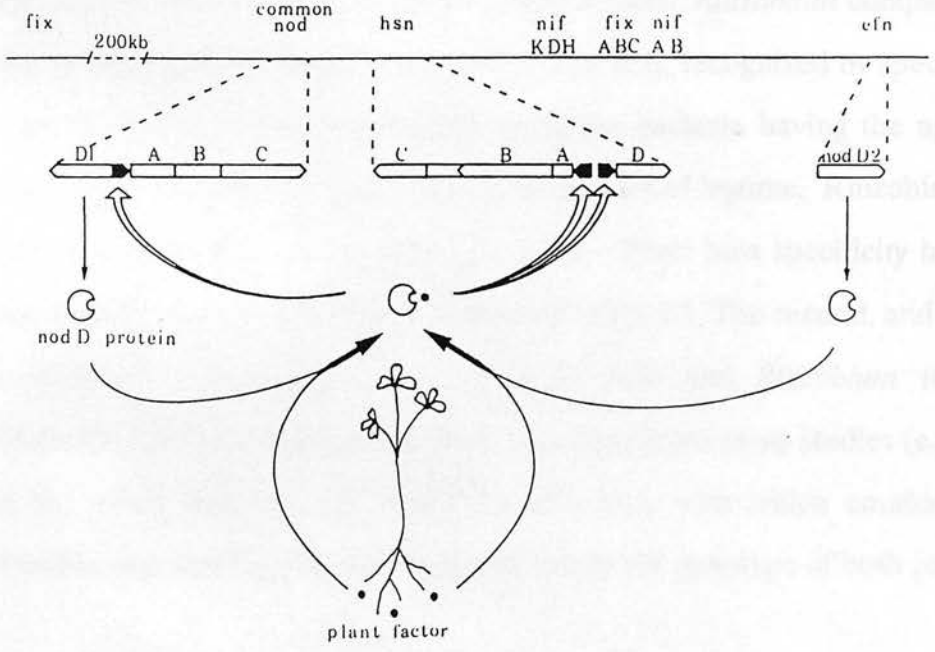


Fig. 2.3. Organization and regulation of nodulation genes in *R. meliloti* by the legume factor (Kondorosi and Kondorosi, 1986).

those of any other plant developmental processes (Nap and Bisseling, 1990b). A number of scientists have shown that both the common *nod* and *hsn* (Host specific nodulation) genes are specifically turned on during the symbiotic processes (Verma and Long, 1983; Kondorosi et al., 1984) and the early nodulation genes of *Rhizobium* are turned on by a legume-specific factor. Conversely, these nodulation genes play a role in eliciting cortical root cell division.

2.6 LEGUME-RHIZOBIUM COMPATIBILITY

A substantial part of an effective symbiosis depends on the compatibility factors between the host legume and the rhizobial strain (Gibson, 1980a), i.e. the symbiosis exhibits specificity, particular legumes being infected only by a limited range of rhizobial strains or species (Downie and Johnston, 1988). The formation of N_2-fixing root nodules depends upon interactions between compatible strains of *Rhizobium* and legume roots. *Rhizobium* compatibility has two components (Beringer et al., 1988). The first, recognized by speciation and cross-inoculation grouping, depends upon the bacteria having the appropriate genotype for nodule formation on a given species of legume. Rhizobia from pea plants, for example, do not colonize lupins. Their host specificity has formed the basis of their classification, outlined in Table 2.5. The second, and much less understood component, is the ability of host and *Rhizobium* to interact efficiently after nodules have formed. It is clear from many studies (e.g De Jong et al., 1982; Witty et al., 1983) the efficiency with which combinations of rhizobia and host legumes fix N_2 is affected by the genotype of both partners.

Table 2.5. Cross-inoculation groups of Rhizobium

Group	*Rhizobium* species	Representative hosts
Fast-growing, acid-forming types:		
Pea group	*R. leguminosarum*	Peas, broad beans, lentils, vetch
Bean group	*R. phaseoli*	Kidney beans, mung beans, runner beans
Clover group	*R. trifolii*	Clover
Alfalfa group	*R. meliloti*	Lucerne, melilot, fenugreek
Slow-growing types:		
Lupin group	*R. lupini*	Lupins, seradella
Soyabean group	*R. japonicum*	Soya beans
Cowpea group	'cowpea miscellany'	Cowpeas, peanuts etc.

Among other factors, field legume inoculation with *Rhizobium* and *Bradyrhizobium* species is also restricted by the presence in the soil of native strains capable of nodulating the host legumes (Moawad and Bohlool, 1984; Dowling and Broughton, 1986; Singleton and Tavares, 1986). It is generally difficult to displace indigenous rhizobia with inoculant strains, and most nodules on the host legume are formed by native rhizobia (Bohlool and Schmidt, 1973; Kvien et al., 1981; Moawad et al., 1984). Displacement is only likely to occur where the native *Rhizobium* population is low or absent (Materon and Hagedorn, 1982; May and Bohlool, 1983). The degree of establishment and the persistence of inoculant rhizobia generally decreases with increasing population density of the native rhizobia (Roughley et al., 1976; ICRISAT, 1981).

However, some inoculant strains have succeeded in forming the greater number of nodules even in the presence of active indigenous competing rhizobia, e.g., Viking 1 on French beans (*Phaseolus vulgaris* L.) (Robert and Schmidt, 1983), G 1067 on clover (*Trifolium*) (McLoughlin et al., 1984), and NC 92 on groundnuts (*Arachis hypogaea* L.) (Nambiar et al., 1984). The often poor ability of inoculant strains to compete with the native populations and the importance of identifying competitive strains have recently been reviewed by Schmidt (1988).

2.7 SOIL FACTORS AFFECTING THE SYMBIOSIS

Many reviews have been published dealing with the environmental factors controlling the establishment and the functioning of N_2-fixing symbioses (Vincent, 1980; Gibson et al., 1982a; Dommergues, 1982; Zahran, 1991). This literature review concentrates only on the soil factors affecting the system.

2.7.1 Moisture

Legumes in general are intolerant of shortage (water stress) and excess (waterlogging) of water. The functioning of the nodule is severely restricted by water stress. Intermittent stress can severely reduce nitrogen fixation (De Jong and Phillips, 1982; Becana et al., 1986; Davey and Simpson, 1989) and prolonged periods of stress may accelerate the rate of nodule senescence (Sprent et al., 1983; Sutton, 1983). Shedding of determinate and indeterminate nodules from the roots of both pasture and grain legumes may result from severe water-stress-induced senescence, and re-establishment of N_2 fixation is dependent upon the growth of new nodules (Sprent, 1981; Sinclair et al., 1988). The ability of rhizobia to survive really dry conditions varies with species, type of soil and other factors (Mary et al., 1986).

Several temperate legumes are reported to be sensitive to waterlogging (Sprent, 1972; Khan, 1974; Minchin and Pate, 1975; Sprent and Gallacher, 1976; Hong et al., 1977; Bisseling et al., 1980; Krishnamoorthy et al., 1981, 1987). Waterlogging inhibited growth of pigeon pea (*Cajanus cajan*) in deep vertisols in India (Reddy and Virmany, 1981), and cowpea (*Vigna unguiculata* L.) (Minchin and Summerfield, 1976) and significantly decreased the number of nodules and leghaemoglobin content at all stages of growth of groundnut (Bishnoi and Krishnamoorthy, 1990). Prolonged flooding of the root system of four-week-old soyabean (*Glycine max.* L.) plants inhibits nitrogenase activity and irreversibly alters the ultrastructure of the cells (Andreeva et al., 1987). However, there are reports indicating that several legumes, including soyabean (De Polli et al., 1973), have a substantial ability to adjust to waterlogged soils, particularly when the soil saturation occurs during early seedling growth, and

the water table is maintained at a constant level in the soil. One study showed that nodule mass was increased in high-watertable culture by up to 35 times that in control plants, and in some cases accounted for 5 percent of total plant dry matter (Hunter et al., 1980).

2.7.2 Temperature

Root temperature affects many processes in the *Rhizobium*-legume symbiosis (Lie, 1974; Frings, 1976; Gibson, 1977). The effect of root temperature is very complex, varying with the host species, as well as with the *Rhizobium* strain (Lie, 1971). Furthermore, different symbiotic processes may have different optimum and limiting temperatures (Dart and Day, 1971; Gibson, 1974; Frings, 1976; Gibson, 1977). Some aspects of *Rhizobium*-legume symbiosis affected by high root temperatures are (1) the growth and survival of rhizobia in the rhizosphere (Parker et al., 1977; Day et al., 1978; Somasegaran, 1984); (2) the formation and growth of root hairs (Lie, 1974; Frings, 1976; Fyson and Sprent, 1982); (3) the binding of the rhizobial cells to the root hair cells (Frings, 1976); (4) the formation of infection threads (Rao, 1977); (5) the structure, growth and development of root nodules (Pankhurst and Gibson, 1973; Lie, 1974); (6) the activity of the nitrogenase enzyme (Dart and Day, 1971; Munns et al., 1977); (7) the leghaemoglobin content (Frings, 1976); and consequently (8) the N content and dry matter production of the nodulated plants (Lie, 1971; Dart et al, 1975; Herridge and Roughley, 1976; Day et al., 1978; Munevar and Wollum, 1981).

A low temperature has been reported to affect the relative nodulating competitiveness (Rice and Olsen, 1988) and N_2-fixing efficiency of *Rhizobium* spp. (Layzell et al., 1983; Pankhurst and Layzell, 1984).

2.7.3 Salinity

Salt stress is one of the many environmental constraints that limit N_2 fixation in legumes. Legumes in general are known to be either sensitive or moderately resistant to salinity. Most legumes respond to moderate salinity with a decrease in growth (Helal and Mengel, 1981). This growth depression in legumes can be attributed to toxic ion accumulations (e.g., Na and Cl ions) in different plant tissues which may in turn induce changes in some enzyme activities and carbohydrate distribution pattern (Zahran, 1991). In contrast, rhizobia can survive in the presence of extremely high levels (0.5 kM m^{-3} NaCl) of salt, both in culture and in the soil, a limit which would kill any legume plant (Zahran, 1991). Generally fast-growing rhizobia are more salt-tolerant than slow-growing rhizobia (El Sheikh and Wood, 1990). However, the processes of nodulation in legumes are very sensitive to high salt concentration. El Sheikh and Wood (1990) observed an inhibition of nodulation in chickpea plants with 34.2 mol m^{-3} NaCl and complete inhibition with 61.6 mol m^{-3} NaCl. The concentration and nature of salts have been found to affect legume-rhizobium symbiosis differently in their growth, nodulation and N_2 fixing capacity (Imbaba, 1973; Balasubramanian and Sinha, 1976; Lauter et al., 1981; Winter and Lauchli, 1982; Nukaya et al., 1982a; Imamul Huq and Larher, 1983a; Yousef and Sprent, 1983; Rai and Prasad, 1983; Zahran and Sprent, 1986; El Sheikh and Wood, 1990; Subba Rao et al. 1990).

Unsuccessful symbiosis under salt stress may be due to failure in the infection process due to the effect of salinity on the establishment of rhizobia (Rai and Prasad, 1983; Singleton and Bohlool, 1984). Lakshmi-Kumari et al. (1974) showed a reduction in the number of root hairs of alfalfa (*Medicago sativa* L.)

plants by 70 to 100 mol m^{-3} NaCl and reduction in root hair infection to a minimum by 35 mol m^{-3} NaCl. These findings were supported by Yousef and Sprent (1983) and Zahran and Sprent (1986).

2.7.4 Nutrients and Acidity

The nutritional demands of legumes are often greater than those of non-legumes and although distinct differences are not always evident, N_2-fixing herbaceous legumes appear to have greater requirements for phosphorus, potassium and molybdenum than non-legumes (Sprent, 1983). N_2-fixing plants are often more sensitive to low pH than plants relying on mineral N (Sprent, 1983; Dixon and Wheeler, 1983) and therefore liming is likely to be of greater importance for N_2-fixing systems in the hills and uplands where soil pH is generally low (Newbould and Floate, 1979). Legumes, which are frequently cultivated on infertile acid soils in tropical and temperate zones, can be severely limited, in terms of establishment and production, by the individual or combined influence of soil acidity factors (Munns, 1978). The early stages in the development of symbiosis between a legume and its endosymbiont *Rhizobium* or *Bradyrhizobium* are particularly sensitive to low pH and high aluminium concentrations. For example, the nodulation of Common stylo (*Stylosanthes*) is depressed by aluminium (De Carvalho, 1981, 1982) and the multiplication of slow-growing rhizobia is more strongly inhibited by low pH and high aluminium concentrations (Alexander, 1985) than by low calcium or high manganese concentrations (Keyser and Munns, 1979).

Leguminous plants grow less luxuriantly in acid media than in neutral or slightly alkaline conditions, which could indirectly be due to lower colonization by

Rhizobium of the soil and rhizosphere, leading to inadequate nodulation (Subba Rao, 1980). Inhibition of soil and rhizosphere colonization by low pH and calcium has been reported for many fast-growing temperate rhizobia (Rovira, 1961; Jones, 1966; Munns, 1968; Lie, 1969; Lowther and Loneragan, 1968; Robson and Loneragan, 1970a,b). Soils of high acidity frequently have low levels of phosphorus, calcium, and molybdenum and high concentrations of aluminium and manganese. The effects on nodule formation and host plant growth of soil acidity and of the related factors of toxic concentrations of aluminium and manganese and inadequate supply of phosphorus, calcium, and molybdenum have been studied for a wide range of legumes, and the literature has been adequately reviewed by Munns (1977, 1978).

One of the factors that may limit N_2-fixation is Mo deficiency (Franco et al., 1978), aggravated by Mo immobilization at low soil pH (Siqueira and Velloso, 1978). In several acid soils of Brazil, a positive response of beans to Mo application was only observed when the soil pH was raised above 5.5. Reports of soil-acidity-induced aluminium toxicity on the nodulation process are available. The nodulation process is more sensitive to aluminium toxicity than is the growth of the host legume. De Carvalho (1981) observed reduction in nodule production, without any significant change in the growth pattern of the host legume, at an aluminium concentration of 100 mmol m^{-3}. However, there are reports that both host and bacterium show variable adaptation to environmental factors, such as acidity, aluminium and manganese concentrations (Vargas and Graham, 1988).

2.7.5 Combined Nitrogen

The inhibitory effect of combined nitrogen (i.e. NH_4^+ and NO_3^-) on rhizobial infection, nodule development and nitrogen fixation in legume-*Rhizobium* symbiosis has been well documented (Munns, 1977; Rigaud, 1981; Gibson and Jordan, 1983; Nelson, 1987). Differences in the extent of inhibition by nitrate of nodulation and C_2H_2 reduction activity (see Section 3.4) have been reported to occur among legume species (Manhart and Wong, 1980; Harper and Gibson, 1984) including soyabean cultivars (Gibson and Harper, 1985). Mutants of pea (*Pisum sativum* L.) (Jacobsen and Feenstra, 1984) and soyabean (Carroll et al., 1985a,b) which nodulate in the presence of high concentrations of nitrate have been isolated. Rhizobial strains vary in their ability to nodulate and reduce C_2H_2 when combined nitrogen is present at low concentration (Heichel and Vance 1979; McNeil, 1982; Nelson, 1983; Gibson and Harper, 1985).

2.7.6 Phytohormones

Since nodule development involves meristematic activity, plant hormones are believed to be integral to the process. Legume nodules contain high concentrations of all three major groups of plant growth-promoting hormones (auxins, cytokinins and gibberellins) and it is likely that they play important roles in early nodule development (Libbenga and Bogers, 1974). Involvement of hormones in nodule initiation has been suggested by several authors (Phillips and Torrey 1972; Libbenga et al., 1973; Libbenga and Bogers, 1974; Syono et al., 1976).

A number of microorganisms have been reported to produce phytohormones that cause deformation of root hairs (Fahraeus and Ljunggren, 1968). A culture filtrate of *Rhizobium* can induce curling or branching of host root hairs, but the complete curling characteristic of infection is only produced by the presence of live bacteria (Yao and Vincent, 1976). This may be due to specific attachment and a localized hormone action (Verma and Nadler, 1984).

Following the invasion of root hair cells by rhizobia an early response of the host is the elicitation of cell division in the cortex some distance away from the site of infection (Newcomb et al., 1979a). A similar observation has been made in the early formation of nodules on non-legumes by symbiotic association with actinomycete (*Frankia*) spp. (Callaham and Torrey, 1977; Newcomb et al., 1978). This suggests that a diffusible substance stimulating cell division is produced by the endosymbiont. Extensive morphological evidence exists that diffusible factors play a role in nodule development (Libbenga et al., 1973; Newcomb et al., 1979a,b).

Involvement of auxins and cytokinins in nodule initiation has been suggested by several authors (Phillips and Torey, 1972; Libbenga et al., 1973; Libbenga and Bogers, 1974; Syono et al., 1976; Nap and Bisseling, 1990b; Upadhyaya et al., 1991a,b). Before the bacteria enter the roots, they have to multiply in the rhizosphere, at the expense of root exudates (Van Egeraat, 1975). A selective stimulation of *R. leguminosarum* strains by homoserine, a major component of root exudate, was observed by Van Egeraat (1975). No growth was obtained with *R. trifolii* and *R. phaseoli* when homoserine was used as a source of nitrogen, whereas an inhibitory effect on *R. meliloti* was found. Among the amino acids exuded by the roots, tryptophan has received particular attention,

because it is easily converted by *Rhizobium* to the plant hormone indole-acetic acid (IAA) (Kaneshiro and Kowlek, 1985; Hunter, 1989). It is assumed to play a role in the infection mechanism, but it is more likely that IAA stimulates the formation and elongation of the root hairs (Fahraeus and Ljunggren, 1968). Since infection is closely linked with active growth of the root hair, it is presumably favoured by IAA. Microbial production of plant growth hormones is a feature of several plant-microbe interactions (Kado, 1984). It is now generally accepted that both *Rhizobium* and *Agrobacterium*, closely related members of the *Rhizobiaceae*, produce IAA and cytokinins (Verma and Nadler, 1984). The generation of phytohormones like auxins, gibberellins and cytokinins by many other organisms has been reported in soil (Vancura and Macura, 1960; Gonzalez-Lopez et al., 1986; Nieto and Frankenberger, 1989). Interestingly, the physiological responses to and regulation of many of the phytohormones thought to be involved in the nodulation processes are largely influenced by the soil conditions in situ. Under flooded conditions, supply of auxins to the roots (Phillips , 1964), synthesis of cytokinins (Burrows and Carr, 1969) and gibberellin (Reid and Crozier, 1971) is inhibited, while the synthesis of ethylene (Kawase, 1972) and abscisic acid (Hiron and Wright, 1973) is increased. Such hormonal changes may be partly responsible for poor nodulation under flooded conditions (Bishnoi and Krishnamoorthy, 1990).

2.8 THE GASEOUS ENVIRONMENT OF THE NODULATED ROOT

2.8.1 Oxygen

Rhizobia are normally considered to be aerobic organisms, and therefore anoxic conditions in soils would be expected to have a detrimental effect on their survival (Eaglesham and Ayanaba, 1984). However, many rhizobia, particularly among the slow growers, can utilize nitrate as an electron acceptor under anaerobiosis (Zablotowicz et al., 1978; Daniel et al., 1982), and may therefore be described as facultative anaerobes. Some of these rhizobia not only survive anaerobic conditions but can even increase in numbers (Daniel et al., 1982). It is also well known that root-hair formation is reduced at low oxygen concentrations, and as the infection is closely linked with the active growth of root hairs low oxygen may lead to the complete failure of symbiotic association. Gallacher and Sprent, (1978) reported retarded nodule development at low oxygen concentrations. However, a marked effect of O_2 on the morphological features of cowpea nodules grown in different O_2 concentrations from an early seedling stage was noted by Dakora and Atkins (1990a); the percentage of the surface area of the nodule occupied by lenticels was greatest at 1 and 2.5% O_2 and least at the highest O_2 concentration of 80%. In nodules cultured in 1% O_2, either the bacteroids contained a greater level of nitrogenase or the supply of reductant and ATP per bacteroid was enhanced. Carbon dioxide evolution per unit N_2 fixed was also greater (Dakora and Atkins, 1990b).

Although nitrogen fixation in legume nodules requires that the bacteroids receive a steady supply of O_2 sufficient to support oxidative phosphorylation, the O_2 concentration must be low enough not to destroy nitrogenase (Dakora

and Atkins, 1991). In other words, the oxygen requirement for oxidative phosphorylation to supply energy is balanced against the sensitivity to oxygen of nitrogenase (Parsons and Day, 1990). Recent studies suggest that many nodules achieve this by a combination of leghaemoglobin and a variable resistance to oxygen diffusion (Sheehy et al., 1985). Direct measurements of oxygen concentration within tissues of soyabean nodules, using O_2-specific microelectrodes (Witty et al., 1987), have shown a sharp fall in O_2 between the cortex and the central infected tissue. They indicated that a layer of cells in the inner cortex provides a resistance to diffusion of oxygen; oxygen concentrations on the outer side of this layer are close to atmospheric, but the concentration falls to almost zero on the inner side as a result of the respiratory activity within the infected zone and the resistance to gas diffusion. Recent research has demonstrated the dependence of legume-nodule functioning on mechanisms which effectively regulate endogenous O_2 supply so as to match closely the demand for O_2 within the N_2-fixing tissue (Witty et al., 1986; Dakora and Atkins, 1989). As a consequence of these mechanisms the free O_2 concentration close to the bacteroids is maintained around 10 μmol m^{-3} (10 nM) (Appleby, 1984) and the inactivation of nitrogenase by O_2 is prevented. The major O_2-consuming reaction in nodules is that involving the terminal oxidase of bacteroid respiration, which functions to provide ATP for N_2 fixation (Atkins et al., 1990). The particular oxidase involved is uniquely adapted, through having a K_m (O_2) close to 5 μmol m^{-3} (Appleby, 1984), to function effectively in the microaerobic conditions within the infected tissue of the nodules. However, a number of other important reactions of nodule metabolism also require a ready supply of O_2 (Atkins et al., 1990). These include the terminal oxidase of mitochondrial electron transport (both in infected and uninfected

cells) and, in those symbioses forming ureids as products of N_2 fixation, ureate oxidase.

In soils, the gas phase oxygen concentration is usually well below 20%, suboptimal for both nodule formation and N_2 fixation. It is commonly observed that legumes are generally intolerant of poorly-aerated or waterlogged soils. Many authors have suggested that oxygen deficiency is the major soil atmospheric constraint on symbiotic efficiency under such conditions. Huang et al. (1975a,b), Tu and Hietkamp, (1977) and Hopmans et al. (1982) have observed in a number of legumes that nitrogen fixation was affected by different soil water content, and suggested that gas exchange in the soil pore space may control the activity of the nodules. Periods of waterlogging lead to decreased production of nodule tissue and decreased nitrogenase activity (Minchin and Pate, 1975), presumably since a thin layer of water surrounding the nodule reduces the oxygen supply (Sprent, 1969; Schwinghamer et al., 1970). Huang et al. (1975a,b) used intact soyabean plants in special chambers to show that flooding inhibited N_2-fixation by interfering with gas exchange between soil and nodules, thus restricting oxygen supply. Sprent and Gallacher (1976) suggested that the effect of excess water on nitrogen fixation could be overcome by increasing oxygen supply. Mundy et al. (1988) reported low rates of N_2-fixation of white clover grown with flood irrigation and suggested that they were probably due to low oxygen supply resulting from reduced oxygen diffusion in the soil with its low air-filled porosity. A reduction in oxygen diffusion-resistance of the nodules in response to low oxygen concentrations outside the nodules (Sheehy, et al., 1983; Witty et al., 1984; Minchin et al., 1985) under such conditions may not be sufficient to compensate for the low oxygen supply. Respiration and N_2-fixation studies on detached nodules exposed to different

oxygen tensions show that the efficiency of carbohydrate consumption during N fixation is highest at near-atmospheric levels of oxygen (Bergersen, 1971).

2.8.2 Carbon dioxide

The process of nitrogen fixation is energetically very expensive. Estimates vary widely, between 1.1 and 19 g of carbon required per gramme of nitrogen fixed, but several studies suggest intermediate values of 6-8 g per gramme of N fixed (Phillips, 1980). The nitrogen reaction alone utilizes as many as 16 molecules of ATP and reductant equivalent to $8e^-$ per molecule of N_2 reduced. In addition, carbon substrates are required for the assimilation of fixed ammonia into organic compounds and for nodule growth and maintenance (Day and Copeland, 1991). In many cases, these costs may represent 15 to 35% of the total photosynthetic capacity of the host plant (Coker and Schubert, 1981). For this reason, any factors which affect the rate of photosynthetic carbon reduction have a direct effect on nitrogen fixation.

CO_2 is important in legume nodulation studies for two reasons. First, there is a widely-accepted close relationship between photosynthesis, N_2-fixation, and plant growth (Jones et al., 1984; Jones et al., 1985; Acock et al., 1985; Baker et al., 1989; Campbell et al., 1990). Second, enrichment of the rhizosphere with CO_2 has long been known to be beneficial in symbiotic N_2-fixation, especially in soils of low pH (<5.5) (Mulder and Van Veen, 1960). They also observed an increase in N_2-fixation by *Trifolium pratense*, *Pisum sativum* and *Phaseolus vulgaris* grown in hydroponic culture purged with air containing 4% CO_2. Their interpretation, that the effect of CO_2 was direct on the root rather than through photosynthetic reduction of CO_2 by the shoot, was supported by Bergersen

(1971) who found that CO_2 stimulated N_2 reduction by detached soyabean nodules exposed to O_2 no greater than 30%. Phillips (1976), showed that a CO_2 enrichment of up to 0.12% in the growth chambers for four weeks increased plant dry weight, N content, root nodule mass, number of nodules and mean nodule dry weight in Pea (*Pisum sativum* L. cv. Alaska) significantly compared to control plants grown at 0.032% CO_2. Many authors have suggested that the major effect of increased CO_2 concentration is an increased rate of CO_2 fixation in nodules (Lawrie and Wheeler, 1975; Christeller et al. 1977; Coker and Schubert, 1979; Layzell et al. 1979; Rawsthorne et al. 1980). Coker and Schubert (1981), showed that legume root nodules can recycle 9 to 30% of nodule respiratory carbon in soyabean. It has been suggested that reassimilation of respired CO_2 may increase the apparent energy use efficiency of legume-*Rhizobium* symbioses, and that selection for increased CO_2 fixation may be a feasible means of increasing legume productivity (King et al., 1986).

Several CO_2-fixing enzymes are known to occur in legume nodules, but PEP carboxylase has been suggested to be the most important (Coker and Schubert, 1981; King et al., 1986). These authors have shown that, during the vegetative stages of growth, CO_2 fixation in soyabean nodules increased at the onset of N_2 fixation and continued at the higher rate until shortly before there was a decrease in the rate of N_2 fixation. They provided evidence that this CO_2 fixation is required both for energy-yielding metabolism and for supplying carbon skeletons for NH_4^+ assimilation and amino acid biosynthesis. Their results also confirmed that at least 66% of dark CO_2 fixation in soyabean nodules might be involved in the production of organic acids, which when oxidized would be capable of providing at least 48% of the requirement for ATP equivalents to support nitrogenase activity.

Other, earlier studies showed that pure cultures of *Rhizobium* require CO_2 for growth (Lowe and Evans, 1962). Jackson and Coleman (1959), provided evidence for CO_2 fixation in plant roots. However, CO_2 at concentrations of 3% has been reported to be completely inhibitory towards nodulation and nitrogen fixation in pea plants (Grobbelaar et al., 1971).

2.8.3 Ethylene

The first observation (Small et al., 1968) that nodulation of cultured bean roots with *Rhizobium* was inhibited if culture vessels were tightly sealed led Grobbelaar et al., (1970, 1971) to test the influence of ethylene. They found that absorbing accumulated olefins with mercuric perchlorate increased nodulation from 0 to 42 nodules per cultured root system, while 0.4 ppm (40 mPa) of ethylene supplied to the roots in a flow-through system reduced the number of nodules from 35 to 4 per root system over 18 days. Furthermore, when ethylene treatment (10 ppm) was delayed until after nodulation, the ability of the nodules to incorporate ^{15}N was suppressed by 90%. These findings have been supported by work by Day et al. (1975) using subterranean clover (*Trifolium subterraneum*), and Goodlass and Smith (1979), using pea (*Pisum sativum*) and white clover (*Trifolium repens*), and also by Drennan and Norton (1972) using pea. These workers used Ethrel, an ethylene-releasing compound, rather than C_2H_4 gas.

More recently, Peters and Crist-Estes (1989) showed that nodule formation by *Rhizobium meliloti* on alfalfa roots was doubled when the inhibitor of C_2H_4 biosynthesis aminoethoxyvinylglycine (AVG) was added in the inoculum.

However, the average nitrogen fixation by the existing nodules remained unchanged by AVG treatment and was independent of nodule numbers in their experiment. Earlier, Zaat et al. (1989) reported a more obscure role for C_2H_4 in the nodulation of the roots of common vetch (*Vicia sativum* L.) induced by one particular strain of *Rhizobium* that caused stunting, swelling, copious root hair development, and abnormal nodulation that was restricted to emerging lateral roots. These symptoms of aberrant morphology induced by the bacteria, or by extracts from bacterial cultures, could be reversed with aminoethoxyvinylglycine (AVG), suggesting that C_2H_4 was involved in the process.

It has also been shown that the biosynthesis of C_2H_4 from methionine, and C_2H_4 action in plant tissues, can be inhibited by a number of other agents such as Ag^+, Co^+, norbornadiene (NBD), diiodohydroxybenzoic acid (DIHB), EDTA and CO_2 (Adams and Yang, 1981; Yang and Sisler, 1990). Ethylene biosynthesis is prevented by inhibition of any of the enzyme systems in the biosynthetic pathway from methionine to C_2H_4 (Methionine SAM ACC C_2H_4) (Adams and Yang, 1981).

The action of C_2H_4 on plant tissues is counteracted by competitive inhibitors of C_2H_4, by their occupation of the C_2H_4 binding sites. For example, when 2,5-norbornadiene was applied to green-mature tomato, it inhibited the onset of ripening which is an ethylene-induced process (Sisler and Yang, 1984). The formation of root aerenchyma in non-wetland and wetland plant species such as maize (*Zea mays* L.) and rice (*Oryza sativa* L.), and the possible causal relationship with C_2H_4 have been extensively studied by Drew et al. (1981) and Jackson et al. (1985a,b), by using AVG, $AgNO_3$ and $CoCl_2$ as inhibitors. They

have shown that when ethylene production by roots exposed to 5% oxygen was inhibited by AVG, dissolved in the nutrient solution, aerenchyma formation in maize was retarded. Similar studies with $AgNO_3$, AVG and/or $CoCl_2$ on rice shows that the aerenchyma formation in this species is probably not C_2H_4-controlled. Liu et al. (1990) studied the involvement of C_2H_4 in the adventitious rooting in hypocotyls of sunflower (*Helianthus annuus*) seedlings by using two different forms of silver (silver nitrate and silver thiosulphate, STS). This showed that $AgNO_3$ promoted rooting, but at the same time it produced necrotic lesions and greatly promoted C_2H_4 production, while STS inhibited rooting and only slightly promoted C_2H_4 production. They suggested that the difference in effects of the two forms of silver might have been because the nitrate form more quickly became immobile by binding to some components inside the cells, and as a consequence the immobile silver could have some toxic effect which stimulated C_2H_4 production. Arshad and Frankenberger (1988) studied the classical "triple response" of etiolated pea seedlings to microbially produced C_2H_4, by treating them with various concentrations of foliarly applied Ag as $AgNO_3$ (0, 60, 120, 180 and 240 mg litre^{-1}). They showed that Ag^+ blocked the ability of the C_2H_4 to produce the response (reduction in elongation, swelling of the hypocotyl, and a change in the direction of growth).

Ligero et al. (1986) reported that ethylene is an important factor in the control of nodule development, maintenance, and senescence and showed that the functional development of nodules is associated with a peak of ethylene production in alfalfa (*Medicago sativa* L.) plants inoculated with *Rhizobium meliloti*. According to Pierce and Bauer (1983), this ethylene production by plants upon infection is autoregulated and might be directed to control possible overnodulation.

It is possible that the well-known depressing effect of nitrate fertilizer on the extent of nodulation by *Rhizobium* may involve ethylene, since in alfalfa, ethylene production increases with the concentration of nitrate (Ligero et al., 1987).

The mechanism by which ethylene influences the symbiotic development is not quite clear. However, ethylene production, like many other processes, inhibits absorption, transportation and metabolism of auxins (Mallorch and Osborne, 1976; Lieberman, 1979) and DNA synthesis (Burg, 1973; Sato et al.,1976); thus according to Coleman et al. (1980) the presence of C_2H_4 could satisfactorily explain the inhibition of a complex morphogenic event such as the initiation of a root primordium, and therefore the inhibition of nodulation.

There is also a role of ethylene in cellular reorganisation in the plants. Exposing intact pea plants to exogenous C_2H_4 causes swelling of the elongating sub-apical region by the re-orientation of growing cells predominantly in the lateral rather than the longitudinal direction (Sargent et al., 1973). Moreover, C_2H_4 causes an increase in the levels of cytoplasmic and cell wall-bound peroxidases and an increase in the hydroxyproline content of the cell wall proteins. It was proposed (Ridge and Osborne, 1971) that C_2H_4 is directly responsible for enhancing the hydroxylation of proline-peptides in the cytoplasm and the subsequent transfer of this hydroxyproline-rich protein to the wall where it decreases wall plasticity and elasticity (Sargent et al., 1973) and the orientation of the growth.

2.9 FACTORS AFFECTING THE COMPOSITION OF THE SOIL ATMOSPHERE

2.9.1 O_2 Supply and Demand

Aerobic soil respiration consumes O_2 and the consumption rate (C) varies from 4.7 to 25.1 g O_2 m^{-2} d^{-1} for soil profiles at 17 to 25°C (Currie, 1970, 1975). In comparison, soil to a depth of 0.3 m contains 9.4 g O_2 m^{-2} in the soil air when the air-filled porosity e = 0.1 m^3 m^{-3} and the gaseous O_2 content is 21% at 17°C. Saturated soil to a depth of 0.3 m may contain up to 1.2 g O_2 m^{-2} dissolved in the water (Kemper and Amemiya, 1957). These values suggest that stored O_2 in soil can supply the respiratory demand for only a short period and is therefore a minor contributor to total O_2 consumption. Despite this, O_2 content is commonly used as an index of aeration.

In well aerated soil the O_2 content of the air is close to that of the atmosphere (Kramer, 1969; Russell, 1973). The O_2 removed is readily replaced by diffusion, and the replacement keeps pace with the requirement of roots and soil microorganisms for aerobic respiration. However, the net movement of O_2 into the soil in response to that respiratory demand is considerable: as much as 0.017 m^3 per m^2 of land area per day was recorded with row crops and a moderately cool (17°C) temperature (Brown et al., 1965). As fluxes of molecular O_2 of such magnitude can only take place by movement in the gaseous phase, it is inevitable that when soils become increasingly wet (and a greater proportion of the pore space becomes water filled) or compacted, an increasing fraction of the volume of the soil becomes poorly aerated (Currie, 1970; Radford and Greenwood, 1970; Fluehler, 1976; Smith, 1980; Currie, 1984;

Currie and Rose, 1985; Hodgson and McLeod, 1989). Following heavy rainfall, or with excessive irrigation on slowly draining or poorly structured soil (Meyers et al., 1987), much of the soil pore space becomes water filled. Dissolved O_2 is soon consumed by respiring organisms, while the presence of water in most of the soil pores effectively blocks further movement of O_2 from the atmosphere, and the soil is described as 'flooded' or 'waterlogged'. The optimal environment is thus a compromise between the need to store water in the soil pores for supply to microbes and roots and the requirements for gas exchange (Drew, 1990).

When the soil becomes excessively wet, root growth and function are likely to be inhibited long before O_2 is exhausted in the soil water. Oxidative phosphorylation depends on molecular O_2 as the terminal electron acceptor at the end of the cytochrome chain. Root growth itself, comprising cell division, cell expansion and differentiation, is highly energy-dependent, particularly in the apical meristem where protein and nucleic acid synthesis are most rapid. It is a common observation that root extension is slowed by hypoxia and stopped by anoxia. The turnover of ATP in aerobic root tips of maize, for example, is extremely rapid (Roberts et al., 1985) and any slowing of ATP synthesis in O_2-deficient cells will immediately be reflected in the rates of ATP-consuming processes such as nucleic acid and protein synthesis.

Radial ion transport by roots to the xylem, and hence to the shoot, is an energy-dependent process and is strongly inhibited by O_2 deficiency (Trought and Drew, 1980, 1981; Drew, 1988). Root O_2-deficiency affects the water relations of plants (Bradford and Hsiao, 1982; Everad and Drew, 1987, 1989). Synthesis and supply to the shoot of IAA, gibberellins and cytokinins are all diminished by

root O_2-deficiency (Reid and Bradford, 1984; Kuiper et al., 1988, 1989) and could conceivably play a role in shoot responses to flooding (Drew, 1990). By contrast, there are large increases in the production of abscisic acid in leaves (Zhang and Davies, 1987). Abscisic acid has also been closely associated with growth responses and changes in gene expression in response to a wide range of other stresses, including high salinity (Singh et al., 1987), freezing (Mohapatra et al., 1988) and water deficit (Davies, 1990). Another hormonal message to the shoots originating in the roots is in the form of ethylene or its precursor ACC. Root O_2-deficiency was shown by Bradford and Yang, (1980b) to stimulate synthesis of the C_2H_4 precursor ACC, which travels in the xylem to the shoots where, in contact with air, it is converted enzymatically to C_2H_4, and may induce epinasty (Drew, 1990).

Anaerobic metabolism may also lead to the production of ethanol, but the phytotoxicity to plant cells of ethanol in water is relatively low (Jackson et al., 1982; Alpi and Beevers, 1983; Perata et al., 1986).

The major factor detrimental to root and microbial (aerobic) propagation and function attributed to waterlogging is low availability of O_2. This was confirmed by experiments in hydroponic systems which clearly showed that many of the physiological responses of intact plants to soil flooding were caused simply by de-oxygenation of the nutrient solution (Trought and Drew, 1980). Such observations provide a rationale for using oxygen deficiency alone as an environmental stress factor, but it is important to emphasize that it is unlikely to be the only factor of significance (Mendelssohn et al., 1981; Waldren et al., 1987; Koch and Mendelssohn, 1989; Moore and Patrick, 1989).

2.9.2 Processes in Anaerobic Soil

During the transition from aerobic to anaerobic (oxygen-free) conditions, the physico-chemical properties of the soil undergo a sequence of changes that is reflected in more reducing conditions (Grambel and Patrick, 1978; Ponnamperuma, 1984). Once molecular O_2 has been consumed in respiration by aerobic organisms, various populations of anaerobic microorganisms utilize other terminal electron acceptors to respire. The sequence of reductions takes place at specific redox potentials. Typically, NO_3^- is reduced to NO_2^-, followed at a lower redox by reduction of Mn^{4+} to Mn^{2+}, then Fe^{3+} to Fe^{2+}, the reduced forms of these metal ions having a relatively high solubility in the soil water. Under very low redox conditions SO_4^{2-} can be reduced to S^{2-} and released as H_2S. Furthermore, products of microbial metabolism such as acetic and butyric acid can accumulate (Drew and Lynch, 1980; Harper and Lynch, 1982). Each of these products has been found in flooded soils, on some occasions at concentrations sufficiently high to be potentially phytotoxic (Grambel and Patrick, 1978). Also, O_2 deficiency under waterlogged conditions can lead to the production of metabolically active gases, the accumulation of which at substantial concentrations can induce specific physiological changes in plants. C_2H_4 is one of the most important of these gases (Smith and Restall, 1971; Jackson, 1982; Jackson et al., 1984; Van Cleemput and El Sebaay, 1985). The accumulation of CO_2 in waterlogged soils does not seem to be particularly damaging (Jackson, 1979).

2.9.3 Movement of Gases

Movement of gases into and out of the soil is a natural phenomenon that allows O_2 to get in for the respiration of living roots and microorganisms, and gaseous byproducts formed as a result of various metabolic activities (aerobic/anaerobic) to get out. Accumulation of the byproducts could be deleterious to root growth. Gas movement is a continuous process until and unless a barrier is set up somewhere in the exchange mechanism, and it is largely determined by the air-filled porosity (Currie, 1984; Hodgson and MacLeod, 1989), pore size distribution and continuity (Douglas, 1986), connectivity and tortuosity of the pores (Ball, 1981), texture, structure, density and moisture content of the soils, and temperature. The net concentration and distribution of any gas at any depth in a given soil is determined by the balance between diffusion (inward/outward) and production/consumption.

Relatively large pores provide a low-resistance pathway for the movement of gases into and out of the bulk soil by gaseous diffusion, and also by mass flow in response to temperature gradients and pressure surges (Hillel, 1980). Although the contribution of mass flow in the mechanism of gas exchange has not been estimated, it is likely to be small. Diffusion is therefore the major mechanism for gas exchange in soil. As O_2 is consumed, partial pressure gradients cause O_2 to diffuse towards the area of consumption. If diffusion is in one direction and the concentration gradient is constant in space and time, a steady state exists, and the flux can be expressed by Fick's law:

$$F = 1/A\ dq/dt = -D\ dc/dx$$

where F is the oxygen flux density in the x direction (g m^{-2} s^{-1}), A is the area of cross section (m^2), dq/dt is the amount of gas per unit time crossing any plane normal to the flow direction (g s^{-1}), D is the diffusion coefficient (m^2 s^{-1}), and dc/dx is the oxygen concentration gradient in the medium (g m^{-3} m^{-1}) (Hodgson and MacLeod, 1989). The concentration gradient is negative since it increases in the direction opposite to that of the diffusive flow.

Diffusion through water is extremely slow (10^4 times slower than air). Thus the water content is frequently the dominant factor determining the aeration status of a soil, and therefore the O_2 supply to the soil largely depends on the air-filled porosity (e). The minimum e at which gaseous diffusion first occurs (e_0) varies from 0.08 to 0.15 m^3 m^{-3}, though 0.10 m^3 m^{-3} is the value most commonly found (Wesseling, 1974). Many attempts have been made by different authors to relate the relative diffusivity D/D_0 (where D_0 is the diffusivity in free air) and air-filled porosity e, since the relative diffusivity is a crucial factor in determining the aeration status of a soil and because e is an easily-measured variable (Fig. 2.4).

2.9.4 Ethylene in the Soil Atmosphere

Since C_2H_4 is physiologically active at concentrations as low as 0.04 ppm, with most plant growth responses becoming saturated at or below 1 ppm (Smith, 1976a,b), its production in soil has been of considerable interest in agricultural research. Microbially-produced C_2H_4 in soil can influence the growth of both plants and microorganisms (Smith and Cook, 1974; Arshad and Frankenberger, 1988; 1990a).

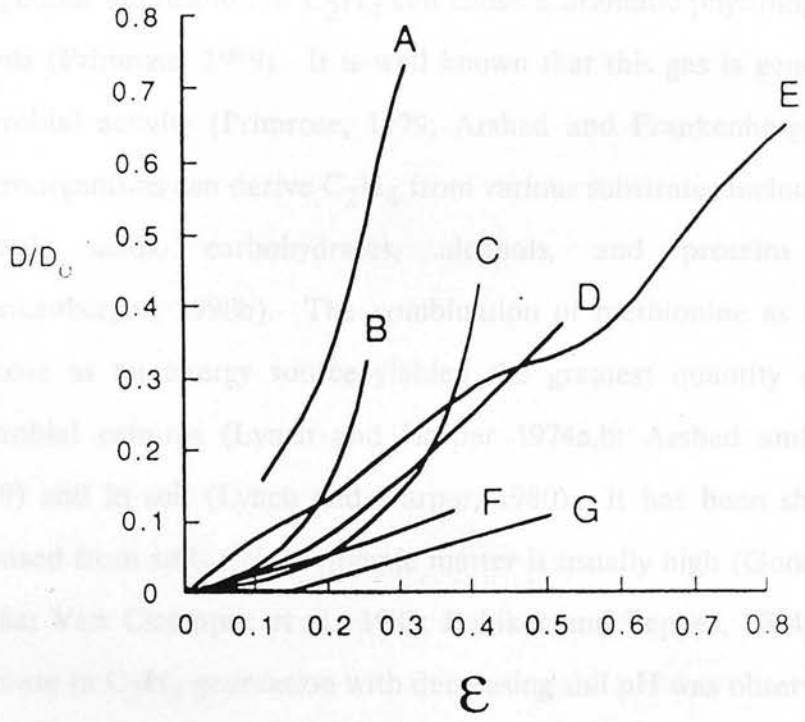


Fig. 2.4. Relationship between relative diffusivity (D/D_0) and air-filled porosity (e) for soils and porous solids determined by several authors using different methods: A (Raney, 1949); B (Blake and Page, 1948); C (Millington, 1957); D (Taylor, 1949); E (Penman, 1940); F (Gradwell, 1962); G (Hodgson and MacLeod, 1989). Redrawn and modified by Hodgson and MacLeod, 1989 after McIntyre, 1962.

Field studies have revealed that C_2H_4 concentrations in the soil atmosphere can typically range from <0.1 to 75 ppm (10 mPa- 7.5 Pa) (Dowdell et al., 1972; Smith and Dowdell, 1974), with accumulation in the upper range usually occurring under waterlogged conditions. As little as 0.01 ppm (1 mPa) of an exogenous application of C_2H_4 can cause a dramatic physiological response in plants (Primrose, 1979). It is well known that this gas is generated in soil by microbial activity (Primrose, 1979; Arshad and Frankenberger 1990a). Soil microorganisms can derive C_2H_4 from various substrates including amino acids, organic acids, carbohydrates, alcohols, and proteins (Arshad and Frankenberger, 1990b). The combination of methionine as a precursor and glucose as an energy source yielded the greatest quantity of C_2H_4 gas in microbial cultures (Lynch and Harper 1974a,b; Arshad and Frankenberger 1989) and in soil (Lynch and Harper, 1980). It has been shown that C_2H_4 released from soils rich in organic matter is usually high (Goodlass and Smith, 1978a; Van Cleemput et al., 1983; Babiker and Pepper, 1984). A significant increase in C_2H_4 generation with decreasing soil pH was observed by Goodlass and Smith (1978a).

Many species of C_2H_4-producing microorganisms have been isolated from soil. Mucoraceous fungi (Dasilva et al., 1974; Lynch and Harper, 1980) as well as facultative anaerobic spore-forming bacteria (Smith and Restall, 1971) have been proposed to be the major producers of C_2H_4 in soil.

It is important to understand the behaviour of waterlogged soils in C_2H_4 formation because of the close similarity between the physiological responses of plants growing in flooded soils and those exposed to C_2H_4. This subject has been extensively reviewed by Jackson (1985). There is ample evidence that

ethylene can accumulate in waterlogged soils at concentrations in excess of those known to affect plant growth (Smith and Russell, 1969; Smith and Dowdell, 1974). The accumulation of C_2H_4 in waterlogged soils is a result of immobilization in water and enhanced stability (Arshad and Frankenberger, 1990b). Under well aerated conditions, C_2H_4 is metabolized by soil microorganisms, but not under anoxia (deBont, 1976). Jackson (1985) suggested that during the early stages of waterlogging, aerobic microorganisms synthesize C_2H_4 while O_2 is still available, but with time, C_2H_4 becomes trapped in the water into a subsequent anoxic phase and is preserved by slow rates of degradation.

There are conflicting reports regarding C_2H_4 synthesis in aerobic vs. anaerobic conditions in soil. Lynch (1975) suggested that an interactive oxidative-reductive process may be involved, where anaerobic microsites allow the synthesis of C_2H_4-forming intermediates while aerobic conditions enhance C_2H_4 production. Hunt et al. (1982) proposed that C_2H_4 can accumulate under both oxidative and reduced conditions as a result of production and consumption, while Smith (1976a) postulated that C_2H_4 production is regulated by the O_2 supply in soil.

Methionine (MET) was first suggested as a possible precursor of ethylene by Lieberman and Mapson (1964) as it was rapidly converted into ethylene in a model system consisting of Cu^{2+} and ascorbic acid. Since then it has been believed that MET serves as the biological precursor of ethylene in all higher plant tissues. It is now recognised that microbial utilization of MET in synthesizing C_2H_4 may follow the same pathway as demonstrated in plant

tissues (Adams and Yang, 1977; Adams and Yang, 1979). The biosynthetic pathway for C_2H_4 production in plants is as shown in Fig. 2.5.

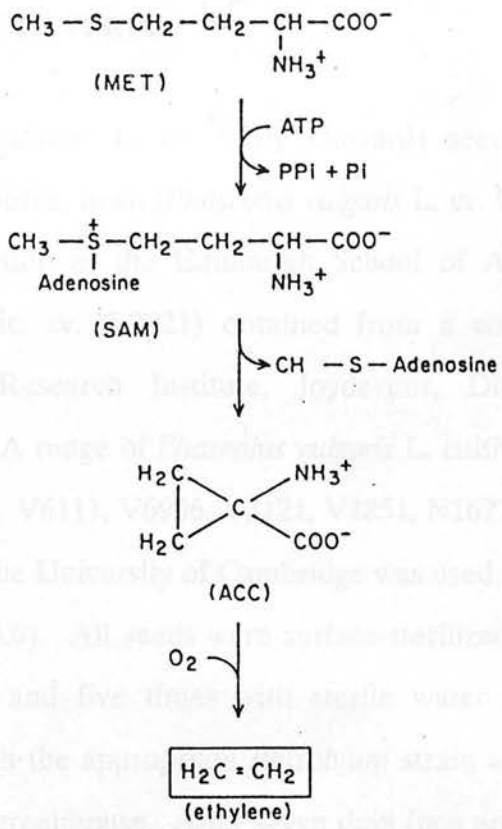


Fig. 2.5. Ethylene biosynthesis in higher plants (Frankenberger and Phelan, 1985, after Adams and Yang, 1977, 1979).

3. MATERIALS AND METHODS

3.1 PLANT MATERIAL

Pea (*Pisum sativum* L. cv. Early Onward) seeds purchased from a local commercial source, bean (*Phaseolus vulgaris* L. cv. Canadian Wonder) obtained from a collection at the Edinburgh School of Agriculture and lentil (*Lens culinaris* Medic. cv. P-2021) obtained from a collection at the Bangladesh Agricultural Research Institute, Joydevpur, Dhaka were used in most experiments. A range of *Phaseolus vulgaris* L. cultivars (V6905, V4407, V6033, V6754, V6896, V6111, V6906, V2121, V2851, N1622, N1 1085, N1 1098) from a collection at the University of Cambridge was used in one series of experiments (see Section 3.9). All seeds were surface-sterilized by rinsing them once with 95% ethanol and five times with sterile water (Gibson, 1980b) and then inoculated with the appropriate *Rhizobium* strain and allowed to germinate on perlite in the greenhouse. After seven days (pea and lentil) and 10 days (bean) the seedling roots were re-inoculated (in order to ensure higher infection) and then transplanted into perlite (see Section 3.2) or hydroponic growth medium (see Section 3.8) containing a further 2.0 ml of the appropriate *Rhizobium* inoculum suspension. Transplantation was necessary to ensure that the plant shoots emerged through the holes in the lids of the growing vessels. When seeds were germinated directly in the vessels it was found that significant numbers of the shoots failed to emerge through the holes in the lids, becoming trapped in the body of the vessel.

3.2 PLANT GROWTH CONDITIONS (MAIN EXPERIMENTS)

The major requirement in the project was that the legume plants be grown in a system which allowed the roots to be exposed to differing concentrations of C_2H_4 and CO_2 and the shoots to remain in a normal atmospheric environment.

The use of soil as the root-growing medium was rejected because soil acts as a natural source of C_2H_4 which could interfere with the imposed treatments, and because uniform diffusion and distribution of gases was likely to be more difficult to achieve.

Attempts were made to allow the legume root systems to grow in Kilner jars (volume approx. 1700 ml) with three 8 mm holes drilled in their lids (one for the plant, one for the gas inlet and one for the outlet) as in the method described by Goodlass and Smith (1979). Seedling roots were carefully passed through one of the holes into the jars and were allowed to grow on a perspex slope covered with moist filter paper. The plants were fed via the filter paper with 200 ml of nutrient solution kept in the bottom of the jars (Plate 3.1).

A slightly different technique was also tried out, using the same principle, but employing a purpose-built flat perspex chamber (borrowed from Dr. M.B. Jackson at the Long Ashton Research station, Bristol) with gas inlet and outlet and a nutrient reservoir at the bottom (Plate 3.2).

Neither technique proved satisfactory. The high temperatures and long day-lengths characteristic of the greenhouse resulted in high rates of transpiration, and the filter paper system was unable to supply enough water to the plants, and plant growth was poor. There were also problems with substantial fungal

Plate 3.1 Root growth system using Kilner jar with perspex slope covered with filter paper "wick", based on system of Goodlass and Smith (1979).

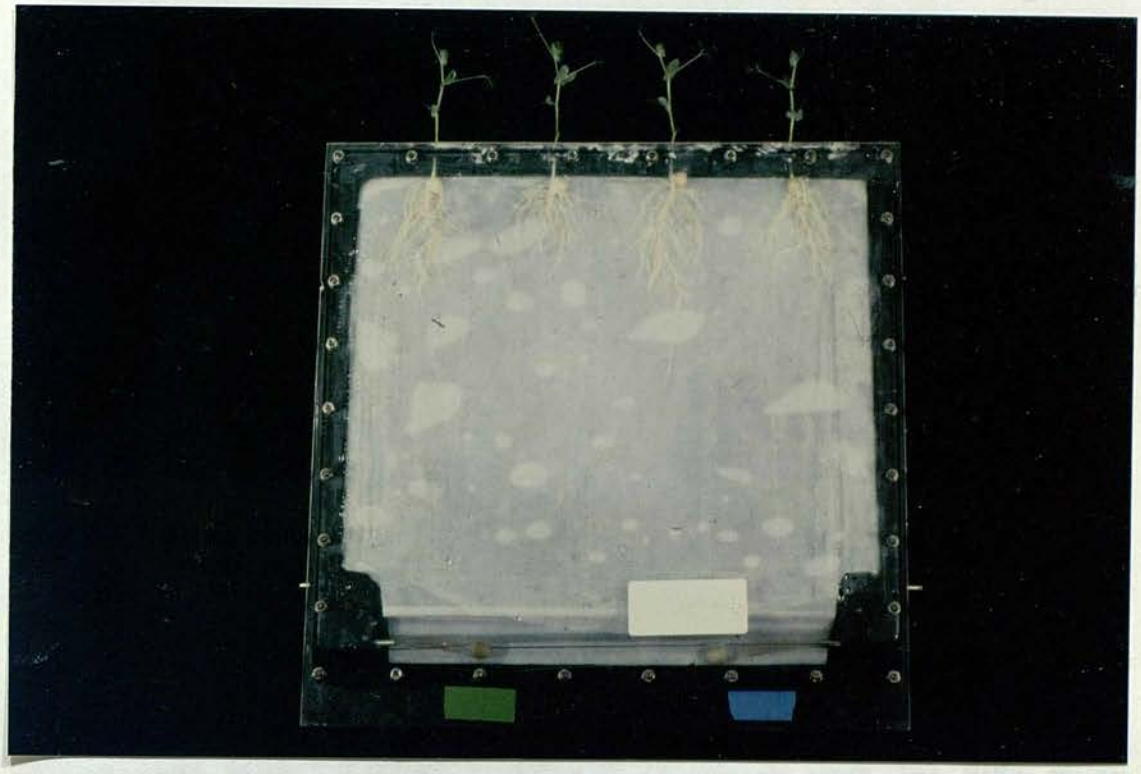


Plate 3.2 Root growth system using flat perspex chamber with filter paper "wick" (obtained from Dr M B Jackson, Long Ashton Research Station).

growth on the moist filter paper, and it was considered that attempting to conduct the experiments in sterile conditions would introduce a new level of complexity.

The plants were grown in a greenhouse and fed with virtually nitrogen-free nutrient solution [($CaSO_4.2H_2O$, 0.25 g; $Ca_3(PO_4)_2$, 0.25 g; $MgSO_4.3H_2O$, 0.25 g; NaCl, 0.08 g; KCl, 0.52 g; $FeCl_3$, 0.005 g)/l mixed with 1.0 mg/l Mo as $(NH_4)_6Mo_7O_{24}.4H_2O$]. The temperature in the greenhouse ranged from 19 to 28°C during the photoperiod of 16 h and from 14 to 18°C during the dark period. During winter months the photoperiod was maintained at 16 h with the aid of high pressure sodium lamps.

The technique using Kilner jars was then modified, by replacing the filter paper 'wick' by perlite. Perlite is an alumino-silicate, volcanic glass containing small quantities (2 to 5% v/v) of water trapped inside the mineral during rapid cooling of the lava. When crushed and heated rapidly to high temperature (c. 800°C), the superheated water explodes the softened particles into a white, lightweight foam. Expanded perlite is chemically inert, has little cation exchange capacity (c. 2 meq/100 g), a neutral reaction and practically no extractable plant nutrients; and above all is sterile. Being highly porous (air-filled porosities range from c. 12% to 60%, depending on particle size), perlite had the advantage of facilitating gas diffusion and thus of allowing gases to become fairly uniformly distributed, as long as the moisture content was carefully controlled.

The jars were filled with 140 g of perlite (sieved to discard fine powder), moistened with 280 ml of nutrient solution. Plants grew well in perlite and all the legume species studied nodulated well when inoculated with specific

rhizobial strains. As the aim was to look at the effects of C_2H_4 and CO_2 on nodulation it was necessary to identify the most significant period of nodule development and change in nitrogenase activity. To determine the time of maximum nodule development and activity, small harvests of three plants each were taken at four-day, intervals starting seven days after root inoculation. From these harvested samples it was found that nodule development and activity were maximal between 20 and 35 days after transplanting. This therefore was the time period over which harvests were made in subsequent experiments.

The Kilner jar ("Closed-vessel") system was used for experiments in which endogenously produced C_2H_4 and/or CO_2 were allowed to accumulate around the roots. Ten sealed jars were connected to each other in series by PVC tubing (Fig. 3.1 and Plate 3.3). The gases inside the jars were circulated through the respective trapping agents by a peristaltic pump running on a one-hour-on and one-hour-off basis. There were four treatments: C_2H_4 trapped out, CO_2 trapped out, both C_2H_4 and CO_2 trapped out and both gases present. Mercuric perchlorate (0.25 kmol m^{-3} (0.25 M)) in 2 kmol m^{-3} (2.0 M) perchloric acid solution (Young et al., 1952) and sodium hydroxide solution (5 kmol m^{-3} (20% w/v)) were used as C_2H_4 and CO_2 traps, respectively. Accumulated C_2H_4, oxygen and CO_2 concentrations were estimated by the methods described in Sections 3.8 and 3.9. The concentration of O_2 in the jars was restored to 21% by the addition of pure O_2, using a large plastic syringe. The jars were covered with black plastic sheeting and placed in boxes to exclude all light from the root medium.

Another technique (the "Constant Flow-through System") was devised in which the plants were grown in 60 x 7 cm pvc pipes filled with perlite and the root

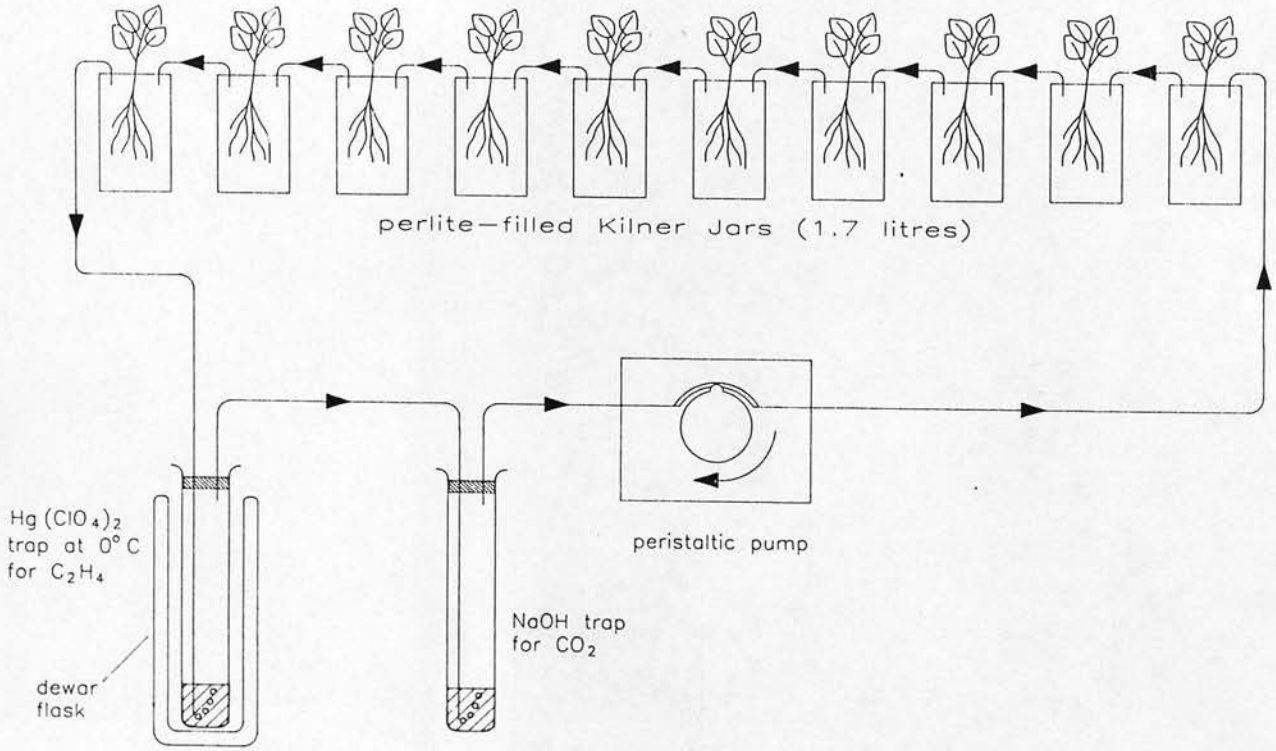


Fig. 3.1. Closed-vessel system for comparisons of root growth and nodulation in presence and absence of endogenous ethylene and carbon dioxide. Four treatments per experiment: that illustrated is "-C_2H_4/-CO_2", i.e. with both C_2H_4 and CO_2 traps present; in other treatments, one or other or both traps removed. Jars were thermally incubated in boxes filled with perlite (see Plate 3.1).

Plate 3.3 "Closed-vessel" system for comparisons of root growth in presence and absence of endogenous ethylene and carbon dioxide.

systems were exposed to a continuous flow of air containing 0, 0.11, 0.33 and 1.0 ppm C_2H_4 (Fig. 3.2 and Plate 3.4). The C_2H_4 concentrations were achieved by diluting 1000 ppm C_2H_4 in N_2 from a cylinder with moist air, using an electric pump. The air and the C_2H_4/N_2 mixture were mixed in a pipe containing internal vanes. The mixture was passed through a manifold from where, via parallel tubes, it passed into the bottom of the pvc pipes, and finally to the atmosphere via outlet tubes near the top. Regular gas chromatographic measurements were made to ensure the desired C_2H_4 concentrations were achieved, fine control of the supply of C_2H_4 being achieved by means of needle valves at the cylinder outlet. Flow rates through the individual pipes were maintained at c. 300 ml min^{-1}, by adjustment of screw clamps on the plastic tubes.

To allow complete control of the rooting environment in the Kilner jars and pvc pipes, it was necessary to seal the plant stems into the holes in the lids with a suitable material that was not damaging to the growing plants. Several different silicone adhesives/sealing agents were tried: Silastics 732, 734 and 738 (Dow Corning), and plaster of Paris ($CaSO_4$). Silastic 738 proved to be most suitable and was used in all the experiments.

3.3 BACTERIAL CULTURES

Rhizobium leguminosarum bv. *viciae*, isolate RH-17, was obtained from Professor J. Sprent, Department of Microbiology, University of Dundee, and bv. *viciae* isolate L-30B and bv. *phaseoli* isolate RCR-3622 from Dr. J.M. Day,Rothamsted Experimental Station. They were grown in a liquid yeast extract/mannitol (YEM) medium [(mannitol, 10 g; yeast extract, 0.5 g; $CaCO_3$, 3 g; K_2HPO_4, 0.5 g; $MgSO_4.3H_2O$, 0.2 g; NaCl, 0.1 g; Fe-EDTA, 0.2 g)/l; pH

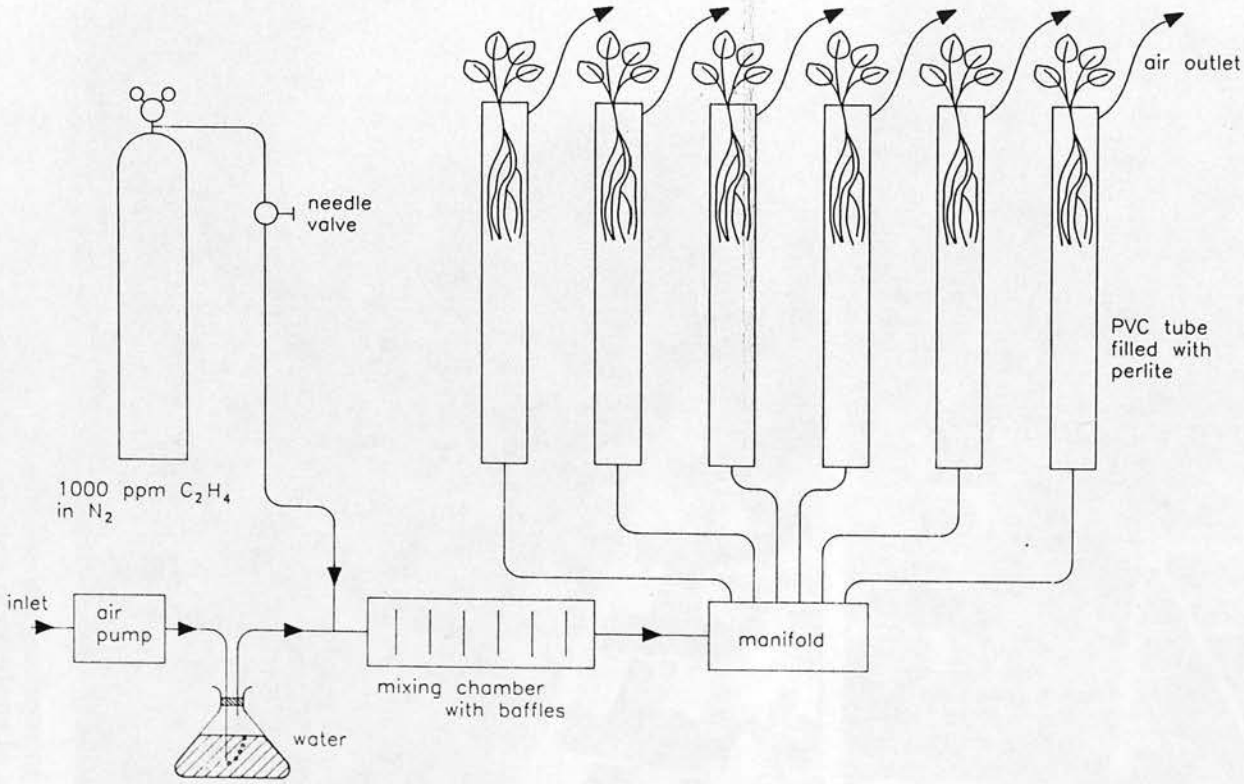


Fig. 3.2. "Constant flow-through" system for comparisons of root growth and nodulation in presence of different concentrations of ethylene in air.

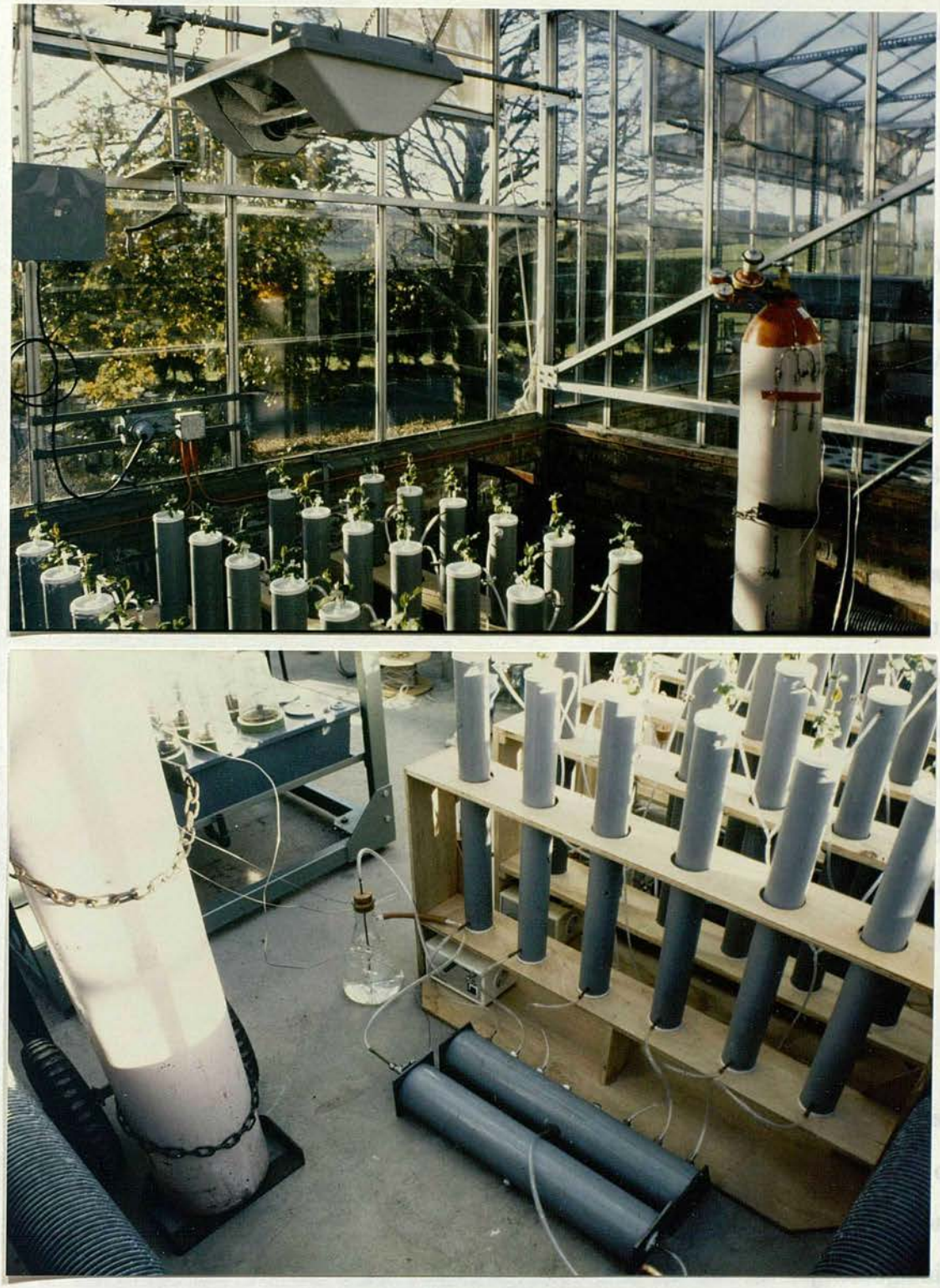


Plate 3.4 "Constant flow-through" system for comparisons of root growth in presence of different concentrations of ethylene in air. (a) View of tops of plant tubes and needle valve system attached to cylinder containing 1000 ppm C_2H_4 in N_2. (b) View of lower part of plant tubes, air-pump, mixing chamber for diluting and mixing the 1000 ppm C_2H_4 with air, and distribution manifold.

adjusted to 6.8] at 26°C as shaken culture (250 r min^{-1} orbital) for two days before being used for inoculation.

3.4 ACETYLENE REDUCTION ASSAY FOR NITROGENASE

Acetylene reduction assays were carried out by a method similar to that described by Hardy et al. (1968). Excised nodulated roots were placed in 70-ml test tubes sealed with a Subaseal stopper. Incubation with acetylene (10% v/v) was carried out in the laboratory at ambient temperatures ranging from 20 to 25°C. Ethylene was determined using a Pye Unicam Series 104 gas chromatograph equipped with a flame ionisation detector. One ml gas samples were taken from the incubation tubes 30 minutes after the addition of acetylene. Gases were separated on an alumina column partially desensitized with sodium iodide, with N_2 as the carrier gas flowing at 40 ml min^{-1} and oven and detector temperatures of 110 and 120°C, respectively (Smith and Arah, 1991). Separation of the C_2H_4 peak from C_2H_6 and C_2H_2 was satisfactory. Output signals from the detector were recorded by a Hewlett Packard 3390 integrator. The chromatograph response to ethylene was calibrated using standard gas mixtures of known C_2H_4 concentration, and found to be linear.

3.5 HARVESTING AND SAMPLE PREPARATION

Plants were harvested at 20, 27 and 34 days after transplantation. At harvest, root nodules were counted and assayed for nitrogenase by determination of C_2H_2 reduction rate. After the assay the nodules were carefully removed from the roots with a sharp scalpel, weighed fresh and assayed for leghemoglobin content (Section 3.6). Root lengths (main axis) of were measured and all plant samples were oven-dried at 100°C to determine dry matter yields. Plant tops

were finely chopped (lentil) or finely ground (to a flour-like consistency) in an agate ball mill (bean, pea) to produce homogeneous samples from which small replicate subsamples could accurately be taken for N analysis (Robinson and Smith, 1991). Subsamples were analysed for total nitrogen content (Section 3.7).

3.6 DETERMINATION OF LEGHAEMOGLOBIN

The leghaemoglobin content of the nodules was assayed fluorometrically according to the method described by LaRue and Child (1979). Fresh nodules were ground in a Waring blender in 50 ml of solution containing 0.02% (w/v) potassium ferricyanide and 0.1% (w/v) sodium bicarbonate. The samples were centrifuged and 0.2 ml of clear supernatant and 2.0 ml saturated aqueous solution of oxalic acid (recrystallized once from dioxane and once from water to remove fluorescent impurities) were added to each of three screw-capped tubes. Two tubes were sealed and heated for 30 minutes at 120^{o}C in an autoclave, then cooled to room temperature. The fluorescence of the solutions was measured using a Luminescence Spectrometer (Perkin Elmer LS30). The excitation wavelength was 405 nm and the emission monochromator setting was 650 nm. The difference in fluorescence between heated and unheated samples was determined and taken as being proportional to the haemprotein concentration. Reference values were obtained from standards of known haemprotein content made using bovine haemoglobin (Haemoglobin Crystalline; BDH Chemicals Ltd., Poole, England).

3.7 DETERMINATION OF TOTAL N

Samples were analysed for total N content using a VG Isogas MM 622 mass spectrometer linked to a Carlo-Erba 1400 automatic N analyser which converts

nitrogen compounds to N_2 by the Dumas oxidation-reduction system. This system is normally used for simultaneous analysis of total nitrogen and $^{15}N/^{14}N$ ratio. However, in this work only the total nitrogen information was required. Subsamples (8-10 mg) of the prepared plant material were accurately weighed into small tin cups and sealed for analysis. In the Dumas system (Fig. 3.3) the sample is combusted at a very high temperature in a stream of O_2 in an oxidation column packed with NiO. The tin cup acts as an oxidation catalyst, raising the temperature to ca. 1700°C to ensure complete combustion. The N in the sample is converted to nitrogen oxides and then to N_2 by reduction by metallic copper. The helium acts as an inert carrier gas to move the N_2 and other combustion products through the system. Water, CO_2 and other contaminants are removed as the N_2 is passed over absorbent columns. A small fraction of the gas stream passes to the mass spectrometer where the N_2 is ionised, and after passing through a magnetic field the ion beam currents are measured, and the total N and $^{15}N/^{14}N$ ratio calculated by computer. Reference values are obtained from standards of known N content which are analysed in the same batch.

3.8 MEASUREMENT OF C_2H_4 RELEASED BY ROOT SYSTEMS

Ethylene trapped in the mercuric perchlorate solution in the 'closed vessel' experiments was estimated at different times by releasing it from the ethylene-mercury complex using the method described by Young et al. (1952). Two ml of ethylene-mercury complex solution was transferred by pipette to a 70 ml test tube and sealed with a Subaseal. Three ml of 2 kmol m^{-3} (2 M) HCl solution was added and the tube shaken by mechanical shaker for 15 min at room temperature. A 1.0 ml gas sample was then taken from the tube head space with a hypodermic syringe and analysed in the gas chromatograph as described

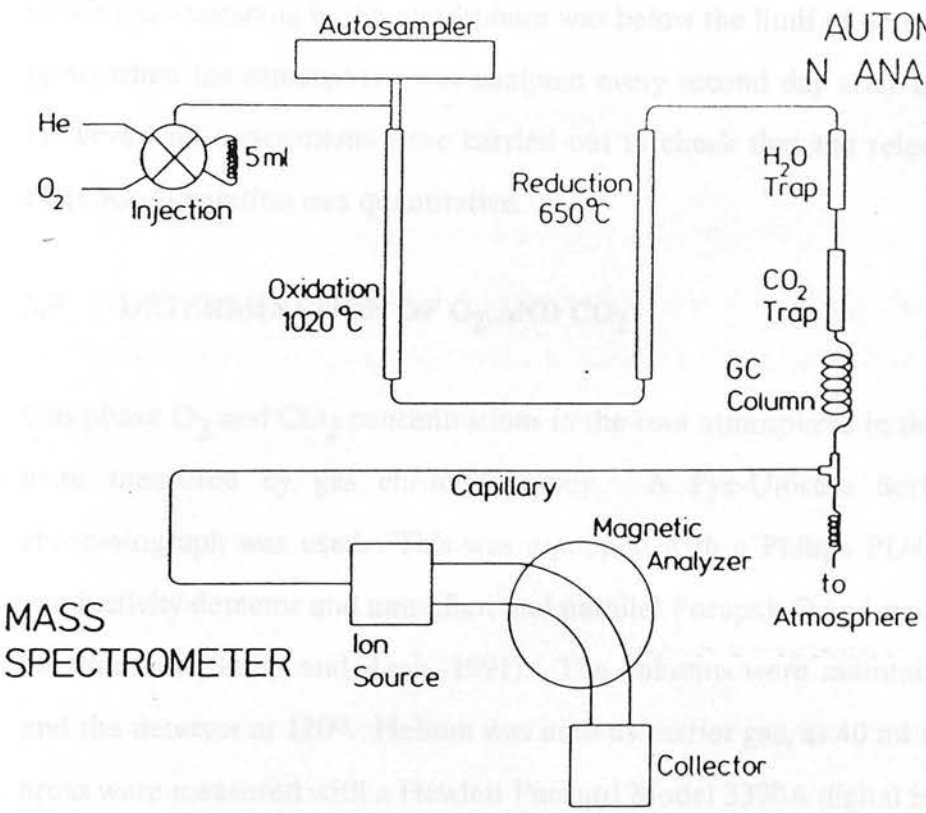


Fig. 3.3. Schematic diagram of automatic nitrogen analyser with direct combustion system, interfaced with isotope mass spectrometer (Robinson and Smith, 1991).

in Section 3.4. The C_2H_4 concentration obtained was then used for estimating total C_2H_4 accumulated in the mercuric perchlorate trapping solution. The trapping of C_2H_4 from the root atmosphere was quantitative; the concentration of the gas remaining in the atmosphere was below the limit of detection (< 0.01 ppm) when the atmosphere was analysed every second day after transplanting. However, no experiments were carried out to check that the release from the $Hg(ClO_4)_2$ solution was quantitative.

3.9 DETERMINATION OF O_2 AND CO_2

Gas phase O_2 and CO_2 concentrations in the root atmosphere in the Kilner jars were measured by gas chromatography. A Pye-Unicam Series 104 gas chromatograph was used. This was equipped with a Philips PU4500 thermal conductivity detector and amplifier, and parallel Porapak Q and molecular sieve 5A columns (Smith and Arah, 1991). The columns were maintained at 110^o and the detector at 120^o. Helium was used as carrier gas, at 40 ml min^{-1}. Peak areas were measured with a Hewlett Packard Model 3390A digital integrator.

3.10 HYDROPONIC CULTURE

For experiments to investigate the effects of Ag^+ and Co^{++} ions, plants were grown hydroponically in Kilner jars plus/minus Ag (Ag_2SO_4, 0.063 mol m^{-3}) or Co ($CoCl_2$, 0.5 mol m^{-3}). Eight treatments ($+O_2+C_2H_4+Ag/Co$, $+O_2+C_2H_4$-Ag/Co, $+O_2$-C_2H_4+Ag/Co, $+O_2$-C_2H_4-Ag/Co, $-O_2+C_2H_4+Ag/Co$, $-O_2+C_2H_4$-Ag/Co, $-O_2$-C_2H_4+Ag/Co, $-O_2$-C_2H_4-Ag/Co) were employed in full strength nutrient solution, and one ($-O_2$-C_2H_4-Ag/Co) in distilled water. With the C_2H_4-treated plants, roots were exposed to a continuous stream of 1.0 ppm C_2H_4. Laboratory-grade air from a cylinder was used as a carrier gas; for

the minus-O_2 treatments O_2-free N_2 was used instead. The C_2H_4 concentration was achieved by diluting 1000 ppm C_2H_4 in N_2 in a vaned mixing pipe as described in Section 3.2. Gases were passed into the solution via sintered-glass gas bubblers.

3.11 COMPARISON OF BEAN CULTIVARS

The rates of ethylene production by a range of bean cultivars, and the effects of that production and subsequent build up of the gas around the root systems on the plant shoots, was examined first by growing plants in individual sealed kilner jars with a gas sampling port. Gas samples were extracted at various times by syringe and were analysed in the gas chromatograph, and the growth symptoms that developed were scored by visual observation. Follow-up experiments were performed by trapping out the gas from the root zone as described in Section 3.2 to see if there was any causal relationship between the rate of C_2H_4 production and the severity of the symptoms developed by the plant leaves.

3.12 LAYOUT OF THE EXPERIMENTS AND STATISTICAL ANALYSES OF THE DATA

The experiments were all laid out in a completely randomized design containing between 2 to 4 replicates, depending on the experiment carried out. Statistical analyses were carried out using the Minitab computer package (Release 7.1). The "closed-vessel" experiments were analysed by an incomplete 3-way analysis of variance. In the "flow-through" experiments a 2-way incomplete analysis of variance was carried out. Standard errors of the differences between means were calculated from the analysis of variance and these were used to compare treatment means. Correlation coefficients were also calculated to study the relationships between different parameters.

4. RESULTS AND DISCUSSION

4.1 RESULTS OF EXPERIMENTS USING THE "CONSTANT FLOW-THROUGH" SYSTEM

The results reported in this section are from experiments carried out using the system described in Section 3.2, in which plant root systems were exposed to continuous streams of air containing different concentrations of C_2H_4, ranging from 0 to 1.0 ppm of C_2H_4.

4.1.1 Pea (*Pisum sativum* L.)

4.1.1.1 Nodule numbers per plant

Nodulation in roots was very significantly ($p<0.01$) inhibited by 1.0 ppm C_2H_4 throughout the period of observation (Fig. 4.1). At day 20, nodule numbers on pea roots treated with 0, 0.11 and 0.33 ppm of C_2H_4 ranged between 72 and 80 per plant, with no significant difference between these treatments. However, only 39 nodules per plant were found on the pea roots treated with 1.0 ppm of C_2H_4, i.e. about a 50% reduction compared with the values obtained in the 0, 0.11 and 0.33 ppm treatments. Similar results were obtained at the second and the last harvest (days 27 and 34, respectively).

Nodule numbers per plant increased significantly between days 20 and 34 in all treatments - by 62, 56, 54 and 100% in the C_2H_4-free control, 0.11, 0.33 and 1 ppm treatments, respectively, but the numbers in the 1 ppm treatment were substantially lower throughout.

Nodules on roots exposed to 0, 0.11 and 0.33 ppm C_2H_4 were largely distributed on the secondary laterals, with a few on the primary root (plate 4.1a). The individual nodule sizes ranged from 1 to 2 mm. In contrast, nodules on roots exposed to 1 ppm C_2H_4 were mostly located on the primary root, were mostly clustered, and were mainly located on the basal half of the root (plate 4.1b). The few nodules that were formed on the secondary laterals were scattered, larger in size (2.5-3.5 mm), and were mainly located near the root tips.

4.1.1.2 Nodule fresh weight

Nodules formed in the presence of 1 ppm C_2H_4 had significantly ($p<0.01$) lower fresh weights at 27 and 34 days than those formed in all the other treatments (Fig. 4.2). However, at day 20 the differences were statistically not significant. Individual nodule fresh weights increased rapidly in all the treatments except 1 ppm C_2H_4, to about 4.25 mg by day 27 compared with an average value of 3.35 mg at 1 ppm C_2H_4. No significant changes in individual nodule fresh weight occurred between days 27 and 34 in any of the treatments (Fig. 4.2).

The inhibitory effect of C_2H_4 at a concentration of 1 ppm was found to be even more significant ($p<0.001$) when total nodule fresh weight per plant was taken into consideration (Fig. 4.3). At day 20, pea roots treated with 0, 0.11 and 0.33 ppm had total nodule fresh weights of about 270 mg plant^{-1}. In contrast, roots exposed to 1 ppm yielded nodules weighing 125 mg plant^{-1}. Total nodule fresh weight in the control and the two lower C_2H_4 concentrations increased significantly up to the final harvest to about 500 mg plant^{-1} (about a 2-fold increase over the two week period of observation). At 1 ppm C_2H_4,

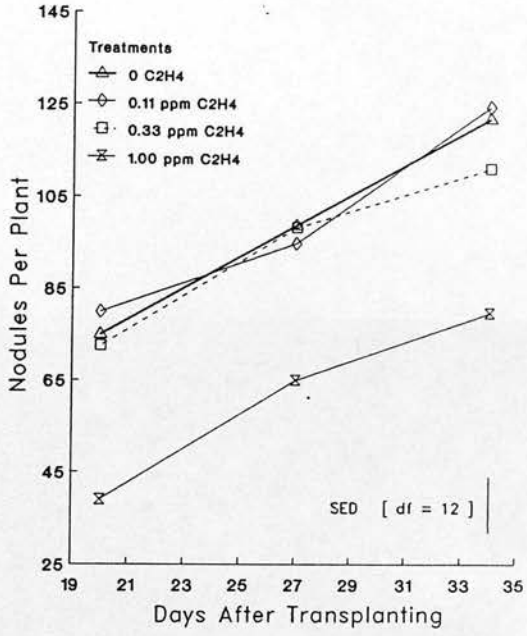


Fig. 4.1 Effect of different concentrations of C_2H_4 on nodulation of pea roots

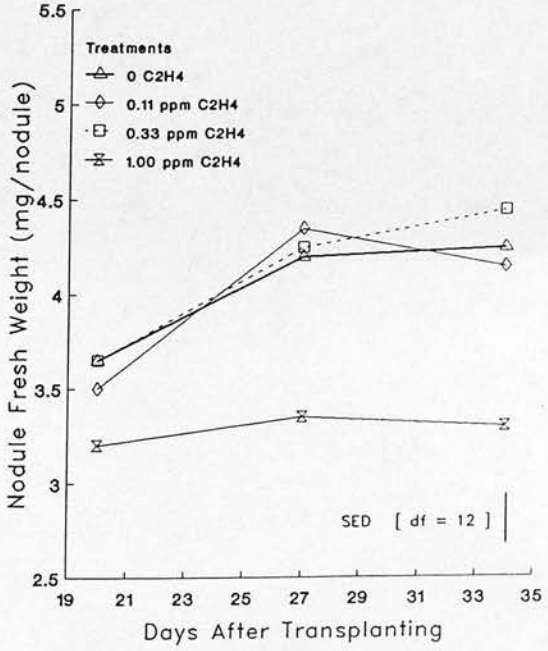


Fig. 4.2 Effect of different concentrations of C_2H_4 on individual nodule fresh weight (pea)

Plate 4.1a Nodules on pea roots exposed to air in the absence of ethylene (flow-through system).

Plate 4.1b Nodules on pea roots exposed to 1 ppm of C_2H_4 in air (flow-through system).

there was a significant increase in total nodule fresh weight between days 20 and 27 but thereafter the increase was very slow and was statistically not significant. At the final harvest the nodule fresh weight at 1 ppm C_2H_4 was about 250 mg plant^{-1}, just half of the corresponding values in the control and the lower C_2H_4 treatments.

4.1.1.3 Nodule nitrogenase activity

A very significant ($p<0.001$) inhibition of nodule nitrogenase activity by 1 ppm C_2H_4 was observed at day 20 when the first harvest was made, and the difference from the other treatments became more pronounced with time (Fig. 4.4). At day 20 the nitrogenase activity in nodules formed at 1 ppm C_2H_4 was about 0.50 μmol C_2H_4 plant^{-1} hr^{-1}, compared with 1.0 μmol C_2H_4 plant^{-1} hr^{-1} in the 0, 0.11 and 0.33 ppm treatments. Nodule nitrogenase activity increased significantly in these latter treatments up to the last harvest (day 34) reaching 3.5 μmol C_2H_4 plant^{-1} hr^{-1} (a 3½-fold increase). Compared with this, only a 1½-fold increase in activity was observed in the 1 ppm C_2H_4 treatment and this increase in activity was significant only between days 20 and 27.

4.1.1.4 Nodule leghaemoglobin content

Pea root nodules in the 1 ppm C_2H_4 treatment had a significantly ($p<0.01$) lower leghaemoglobin content (0.02 mg plant^{-1}) than those in the treatments with lower concentrations of ethylene and in the control (0.05 mg plant^{-1}), at day 20. In the 0, 0.11 and 0.33 ppm treatments the leghaemoglobin content increased by about 300% over the period from 20 to 34 days, reaching a maximum value of about 0.2 mg plant^{-1}, whereas the

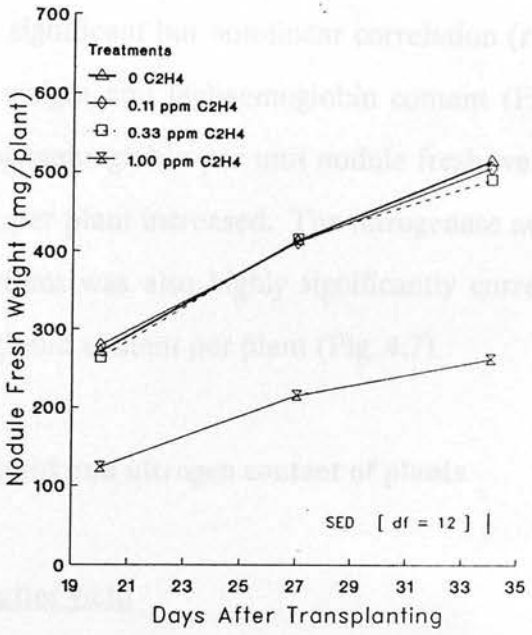


Fig. 4.3 Effect of different concentrations of C_2H_4 on total nodule fresh weight (pea)

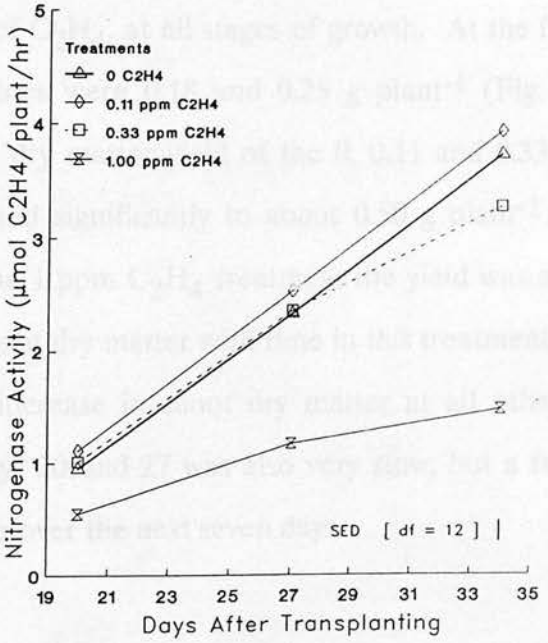


Fig. 4.4 Effect of different concentrations of C_2H_4 on nodule nitrogenase activity (pea)

corresponding value obtained in the 1 ppm C_2H_4 treatment was about 0.05 mg plant^{-1} (Fig. 4.5).

There was a highly significant but non-linear correlation ($r = 0.97^{***}$) between total nodule fresh weight and leghaemoglobin content (Fig. 4.6), because the concentration of leghaemoglobin per unit nodule fresh weight increased as the total nodule weight per plant increased. The nitrogenase activity per plant at all ethylene concentrations was also highly significantly correlated ($r = 0.94^{***}$) with the leghaemoglobin content per plant (Fig. 4.7).

4.1.1.5 Dry matter yield and nitrogen content of plants

(a) Shoot dry matter yield

The pea plants whose roots had been treated with 1 ppm ethylene gave significantly ($p < 0.01$) lower shoot dry matter yields than those exposed to 0, 0.11 or 0.33 ppm of C_2H_4, at all stages of growth. At the first harvest (day 20) the respective values were 0.18 and 0.25 g plant^{-1} (Fig. 4.8). By the final harvest, the shoot dry matter yield of the 0, 0.11 and 0.33 ppm C_2H_4-treated plants had increased significantly to about 0.50 g plant^{-1} (i.e. about a 2-fold increase) but in the 1 ppm C_2H_4 treatment the yield was about 0.25 g plant^{-1}. The increase in shoot dry matter with time in this treatment was statistically not significant. The increase in shoot dry matter at all other concentrations of C_2H_4 between days 20 and 27 was also very slow, but a faster and significant increase took place over the next seven days.

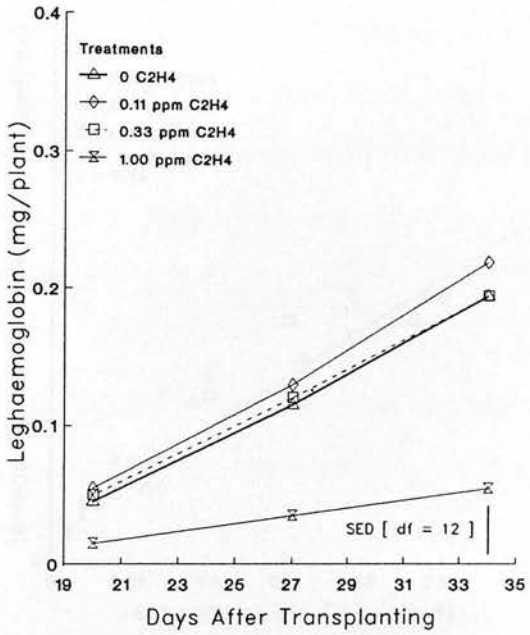


Fig. 4.5 Effect of different concentrations of C_2H_4 on nodule leghaemoglobin content (pea)

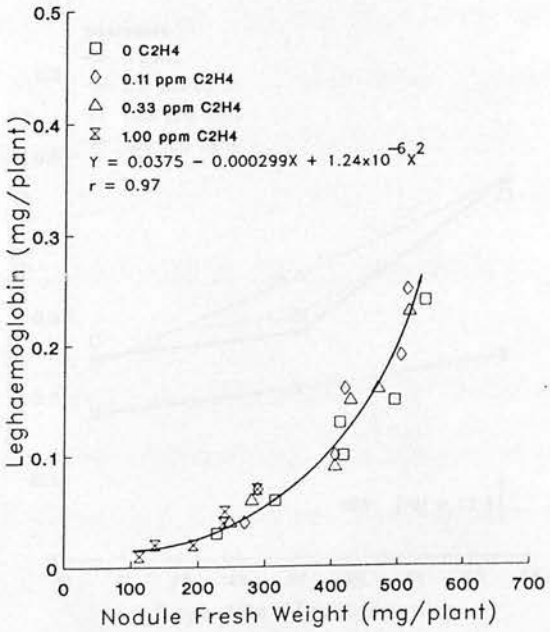


Fig. 4.6 Relationship between leghaemoglobin content of pea root nodules and nodule fresh weight

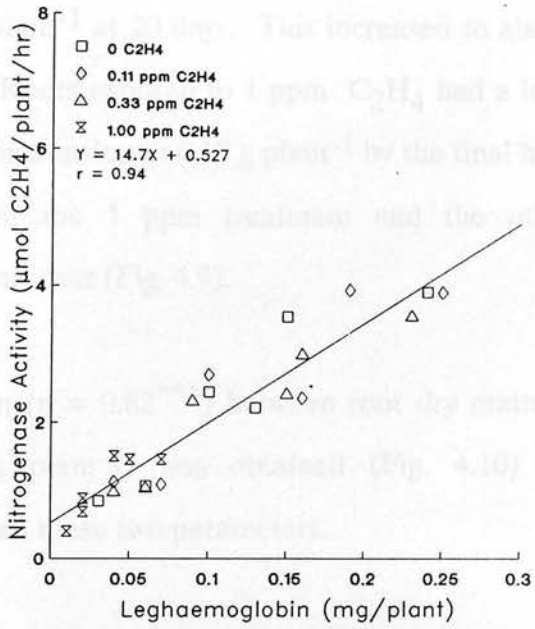


Fig. 4.7 Relationship between nitrogenase activity and leghaemoglobin content of pea root nodules

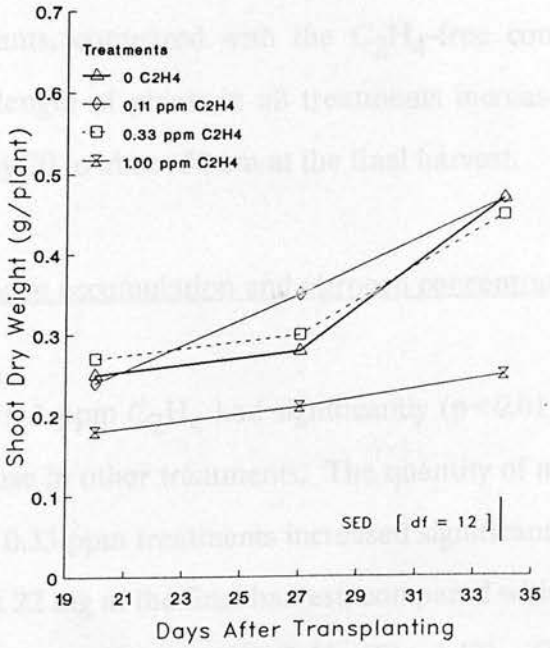


Fig. 4.8 Effect of different concentrations of C_2H_4 on shoot dry weight (pea)

(b) Root dry matter yield

The dry matter content of the roots exposed to 0, 0.11 and 0.33 ppm of C_2H_4 was about 0.18 g plant^{-1} at 20 days. This increased to about 0.26 g plant^{-1} by the final harvest. Roots exposed to 1 ppm C_2H_4 had a lower root dry matter (0.13 g) at day 20, increasing to 0.19 g plant^{-1} by the final harvest. However the difference between the 1 ppm treatment and the other treatments was statistically not significant (Fig. 4.9).

A good correlation ($r = 0.82^{***}$) between root dry matter yield and nodule fresh weight (mg plant^{-1}) was obtained (Fig. 4.10) indicating a direct relationship between these two parameters.

(c) Primary root length

None of the C_2H_4 treatments had any significant effect on the primary root length of pea plants, compared with the C_2H_4-free control treatment (Fig. 4.11). The root length of plants in all treatments increased significantly from about 30 cm at day 20 to about 50 cm at the final harvest.

(d) Total nitrogen accumulation and nitrogen concentration in shoots

Plants treated with 1 ppm C_2H_4 had significantly ($p<0.01$) lower N content in the shoot than those in other treatments. The quantity of nitrogen in the shoots in the 0, 0.11 and 0.33 ppm treatments increased significantly from about 12 mg at day 20 to about 22 mg at the final harvest, compared with an increase from 10 to 13 mg in roots treated with 1 ppm C_2H_4 (Fig. 4.12). The change in total N

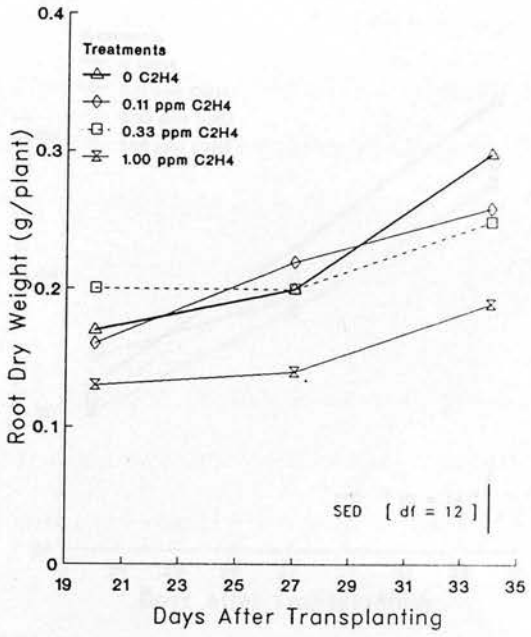


Fig. 4.9 Effect of different concentrations of C_2H_4 on root dry weight (pea)

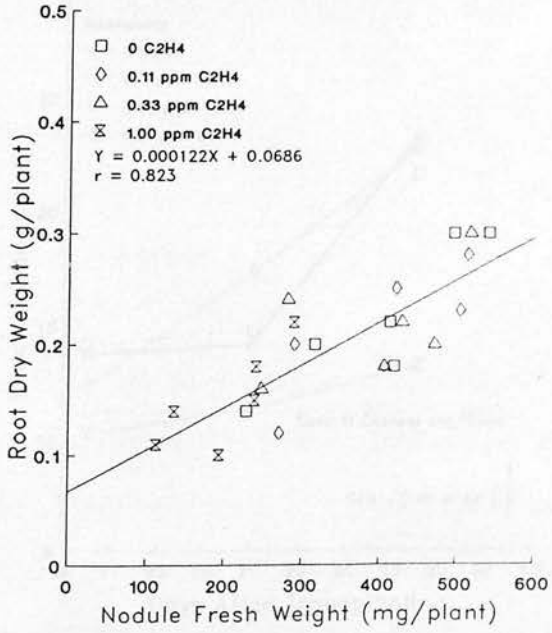


Fig. 4.10 Relationship between root dry weight and nodule fresh weight (pea)

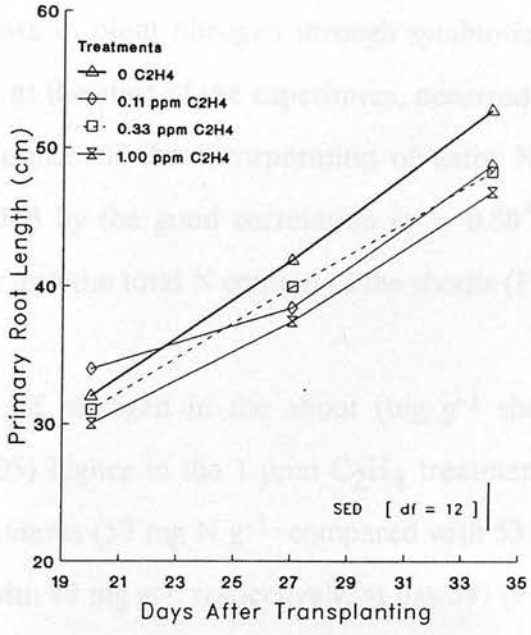


Fig. 4.11 Effect of different concentrations of C_2H_4 on primary root length of pea plants

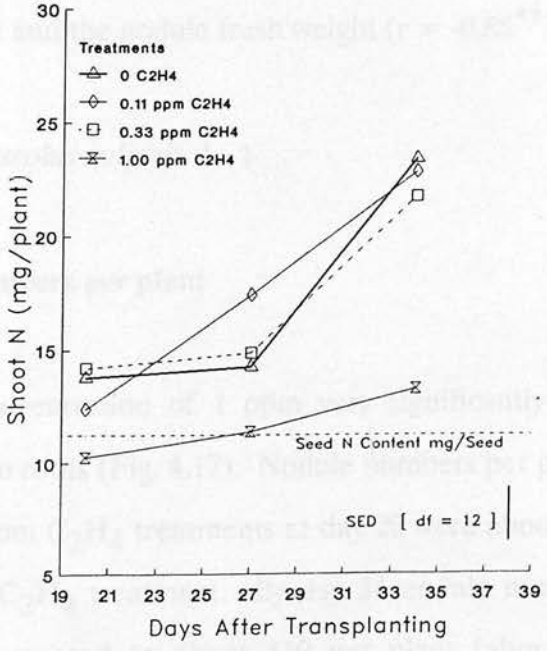


Fig. 4.12 Effect of different concentrations of C_2H_4 on total nitrogen content of pea shoots

content in roots exposed to 0, 0.11 and 0.33 ppm C_2H_4 was very rapid between day 27 and day 34 compared with that between days 20 and 27.

A significant increase in plant nitrogen through symbiotic fixation, above that present in the seed at the start of the experiment, occurred within the last seven days. Further evidence of the incorporation of extra N into the shoots by fixation was provided by the good correlation ($r = 0.88^{***}$) between nodule nitrogenase activity and the total N content of the shoots (Fig. 4.13).

The concentration of nitrogen in the shoot (mg g^{-1} shoot dry weight) was significantly ($p<0.05$) higher in the 1 ppm C_2H_4 treatment than in the 0, 0.11 and 0.33 ppm treatments (57 mg N g^{-1} compared with 53 mg N g^{-1}, at day 20, and 53 compared with 49 mg g^{-1}, respectively, at day 34) (Fig. 4.14).

There were good negative correlations between mg N g^{-1} shoot dry weight and the shoot dry weight (g $plant^{-1}$) ($r = -0.84^{***}$) (Fig. 4.15), and between mg N g^{-1} shoot dry weight and the nodule fresh weight ($r = -0.85^{***}$) (Fig. 4.16).

4.1.2 Bean (*Phaseolus vulgaris* L.)

4.1.2.1 Nodule numbers per plant

Ethylene at a concentration of 1 ppm very significantly ($p<0.01$) inhibited nodulation of bean roots (Fig. 4.17). Nodule numbers per plant recorded in the 0, 0.11 and 0.33 ppm C_2H_4 treatments at day 20 were about 75, compared with 59 in the 1 ppm C_2H_4 treatment. By day 34 nodule numbers in the former treatments had increased to about 110 per plant (about a 45% increase),

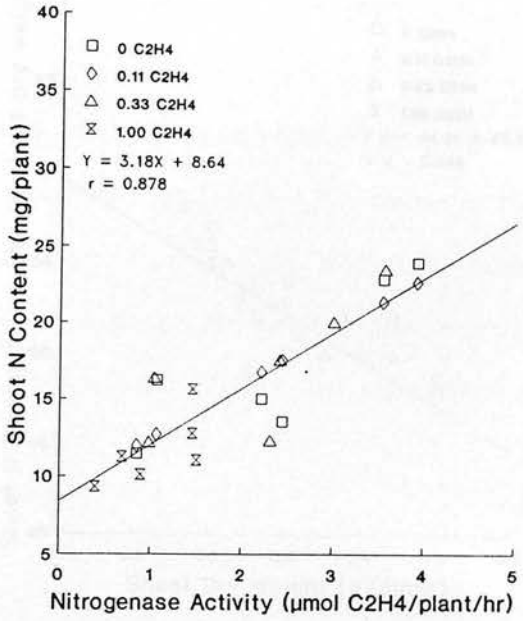


Fig. 4.13 Relationship between shoot nitrogen content and nodule nitrogenase activity (pea)

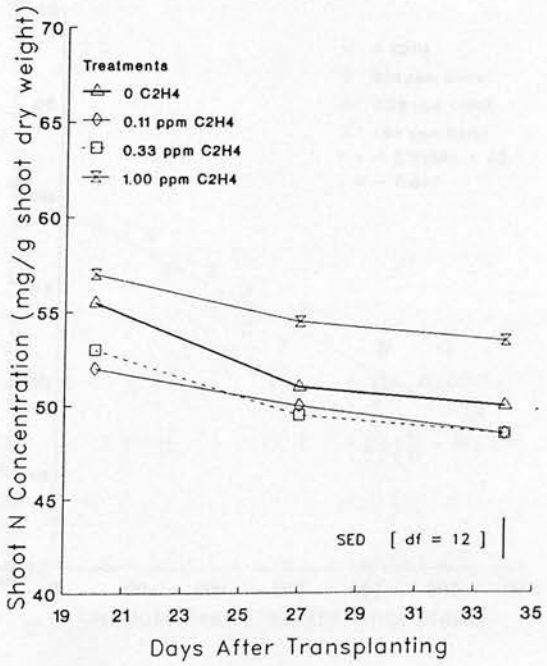


Fig. 4.14 Effect of different concentrations of C_2H_4 on nitrogen concentrations of pea shoots

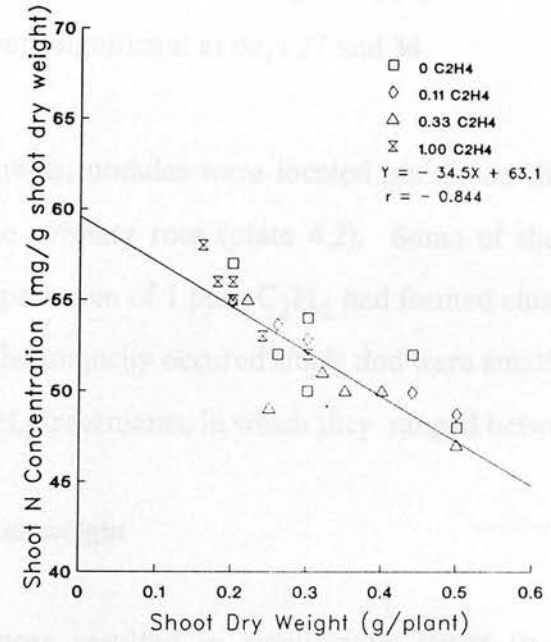


Fig. 4.15 Relationship between shoot nitrogen concentration and shoot dry weight (pea)

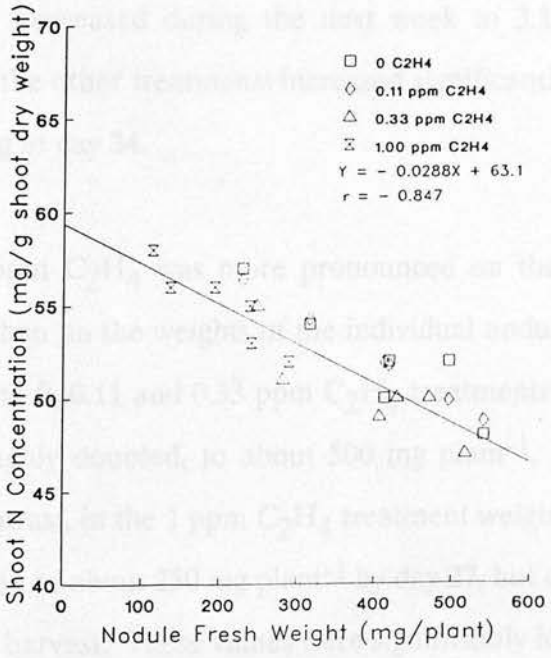


Fig. 4.16 Relationship between shoot nitrogen concentration and nodule fresh weight (pea)

whereas nodule numbers on roots exposed to 1 ppm C_2H_4 showed no significant change over the 14-day period. The differences in nodule number between the treatment with the highest ethylene concentration and the remainder were very significant at days 27 and 34.

In all four treatments, nodules were located mainly on the secondary laterals, with a few on the primary root (plate 4.2). Some of the nodules which had developed in the presence of 1 ppm C_2H_4 had formed clusters in size 3-3.5 mm in diameter, but the majority occured singly and were smaller than in the 0, 0.11 and 0.33 ppm C_2H_4 treatments, in which they ranged between 1.5 and 2.5 mm.

4.1.2.2 Nodule fresh weight

The size differences resulted in significantly lower ($p<0.01$) nodule fresh weights being obtained in the 1 ppm C_2H_4 treatment than in the other treatments (Fig. 4.18). The values increased from 2.75 mg at day 20 to 3.5 at day 27, but then decreased during the next week to 3.15 mg nodule^{-1}. In contrast, those in the other treatments increased significantly from about 3.5 mg at day 20 to 4.5 mg at day 34.

The effect of 1 ppm C_2H_4 was more pronounced on the total nodule fresh weight per plant than on the weights of the individual nodules. At day 20, total fresh weights in the 0, 0.11 and 0.33 ppm C_2H_4 treatments were about 250 mg plant^{-1}. This roughly doubled, to about 500 mg plant^{-1}, by the final harvest (Fig. 4.19). In contrast, in the 1 ppm C_2H_4 treatment weights of 150 mg plant^{-1} at 20 days increased to about 250 mg plant^{-1} by day 27, but decreased to 200 mg plant^{-1} by the last harvest. These values were significantly lower than the values obtained in the other treatments.

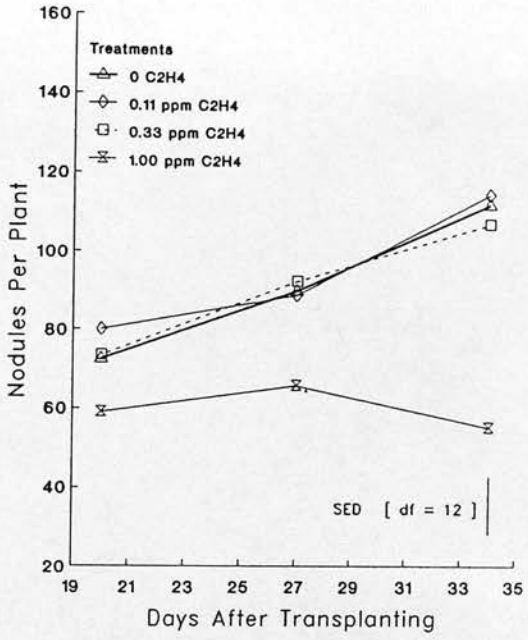


Fig. 4.17 Effect of different concentrations of C_2H_4 on nodulation of bean roots

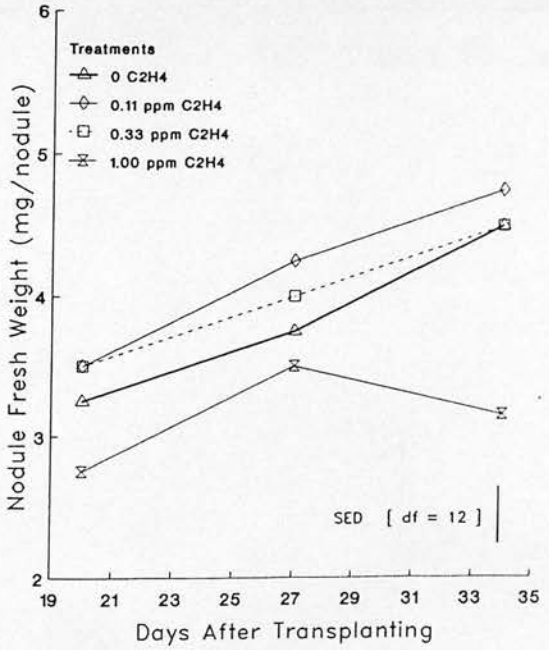


Fig. 4.18 Effect of different concentrations of C_2H_4 on individual nodule fresh weight (bean)

Plate 4.2 Nodules on bean roots exposed to air in the absence of ethylene (flow-through system).

4.1.2.3 Nodule nitrogenase activity

A very significant inhibition of bean nodule nitrogenase activity ($p < 0.001$) by 1 ppm C_2H_4, compared with all other treatments, was observed (Fig. 4.20). However, nitrogenase activity was significantly ($p < 0.05$) higher in the 0.33 ppm C_2H_4 treatment at all stages of growth, and in the 0.11 ppm C_2H_4 treatment at days 27 and 34, than in the control. The activity increased from about 1 to 2.5 μmol C_2H_4 plant^{-1} hr^{-1} by the final harvest in the 0.11 and 0.33 ppm C_2H_4 treatments, and from 0.75 to just over 2 μmol C_2H_4 plant^{-1} hr^{-1} in the control. In the 1 ppm treatment the activity increased significantly from about 0.5 to 1 μmol C_2H_4 plant^{-1} hr^{-1} by day 27 but then remained constant up to day 34.

4.1.2.4 Nodule leghaemoglobin content

Over the period of the experiment, the nodules that formed on roots treated with 1 ppm of C_2H_4 had a significantly lower ($p < 0.01$) leghaemoglobin content than did nodules treated with 0, 0.11 or 0.33 ppm (Fig. 4.21), although the differences were not significant at the first harvest (day 20). By day 34 the leghaemoglobin content per plant at 0, 0.11 and 0.33 ppm C_2H_4 had increased to 0.14-0.18 mg from about 0.04 mg at day 20. In contrast, the leghaemoglobin content of those exposed to 1 ppm C_2H_4 increased only from about 0.02 mg plant^{-1} to 0.03 mg plant^{-1}.

As found previously for pea, leghaemoglobin content per plant increased curvilinearly with increasing nodule fresh weight. Again, there was a highly significant correlation ($r = 0.94^{***}$) between these two

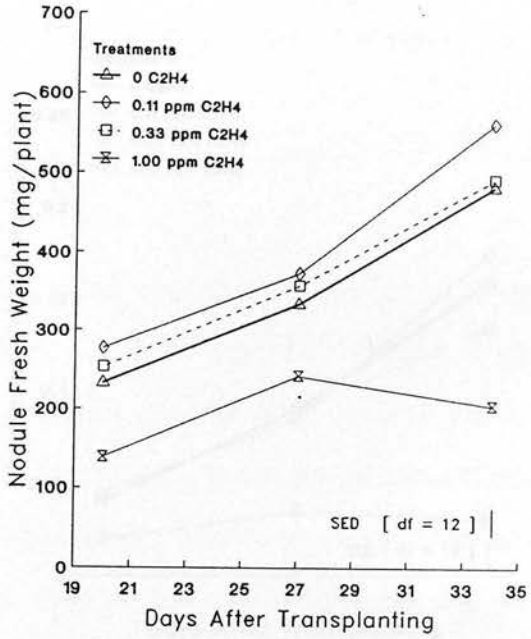


Fig. 4.19 Effect of different concentrations of C_2H_4 on total nodule fresh weight (bean)

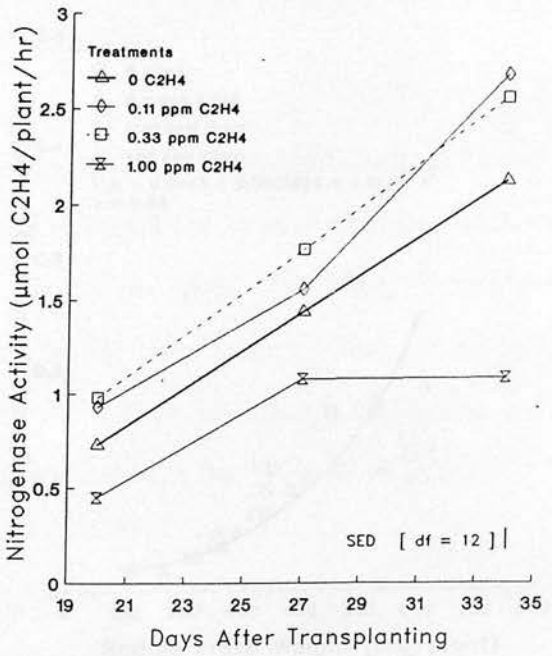


Fig. 4.20 Effect of different concentrations of C_2H_4 on nodule nitrogenase activity (bean)

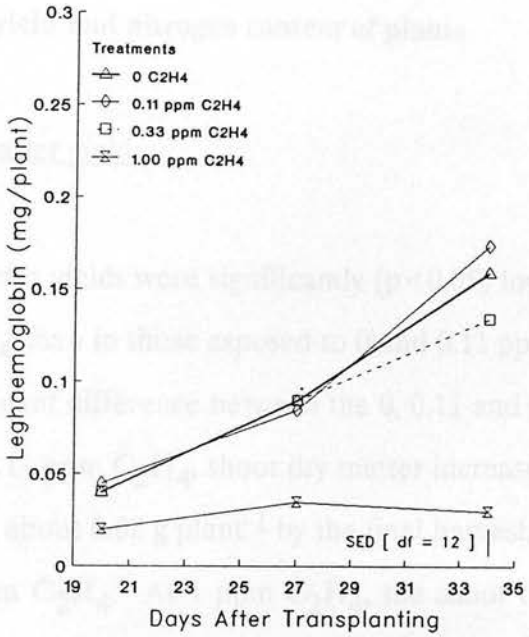


Fig. 4.21 Effect of different concentrations of C_2H_4 on nodule leghaemoglobin content (bean)

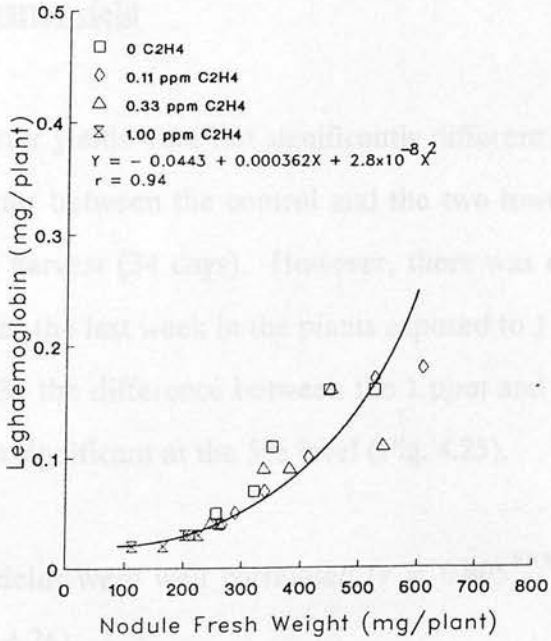


Fig. 4.22 Relationship between leghaemoglobin content of bean root nodules and nodule fresh weight

quantities (Fig. 4.22). Nodule nitrogenase activity was also highly significantly correlated ($r = 0.93^{***}$) with the leghaemoglobin content per plant (Fig. 4.23).

4.1.2.5 Dry matter yield and nitrogen content of plants

(a) Shoot dry matter yield

Bean shoot dry matter yields were significantly ($p < 0.05$) lower in plants treated with 1 ppm of C_2H_4 than in those exposed to 0 and 0.11 ppm at days 27 and 34. However, no significant difference between the 0, 0.11 and 0.33 ppm treatments was observed. In 0.11 ppm C_2H_4, shoot dry matter increased from about 0.35 g plant^{-1} at day 20 to about 0.62 g plant^{-1} by the final harvest, and to 0.56 g plant^{-1} in 0 and 0.33 ppm C_2H_4. At 1 ppm C_2H_4, the shoot dry matter increased much more slowly, from 0.25 g plant^{-1} to only 0.40 g plant^{-1} by the final harvest (Fig. 4.24).

(b) Root dry matter yield

Bean root dry matter yields were not significantly different between treatments at 20 or 27 days, nor between the control and the two lower concentrations of C_2H_4 at the final harvest (34 days). However, there was no increase at all in root dry matter over the last week in the plants exposed to 1 ppm C_2H_4 with the result that by day 34 the difference between the 1 ppm and the 0 and 0.11 ppm treatments was just significant at the 5% level (Fig. 4.25).

Root dry matter yields were well correlated ($r = 0.805^{***}$) with total nodule fresh weights (Fig. 4.26).

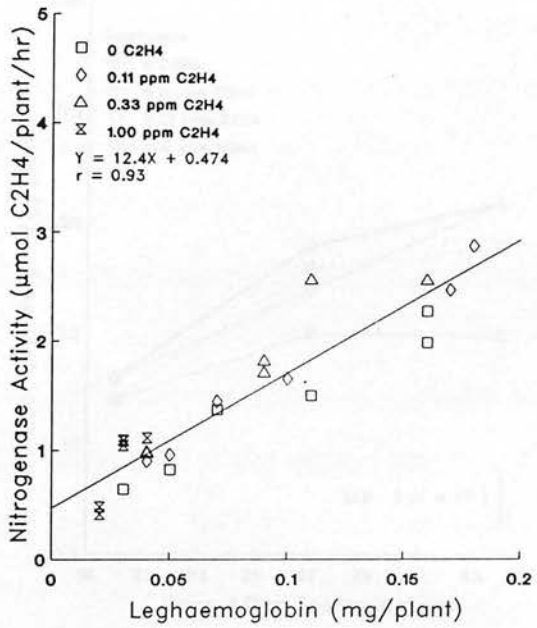


Fig. 4.23 Relationship between nitrogenase activity and leghaemoglobin content of bean root nodules

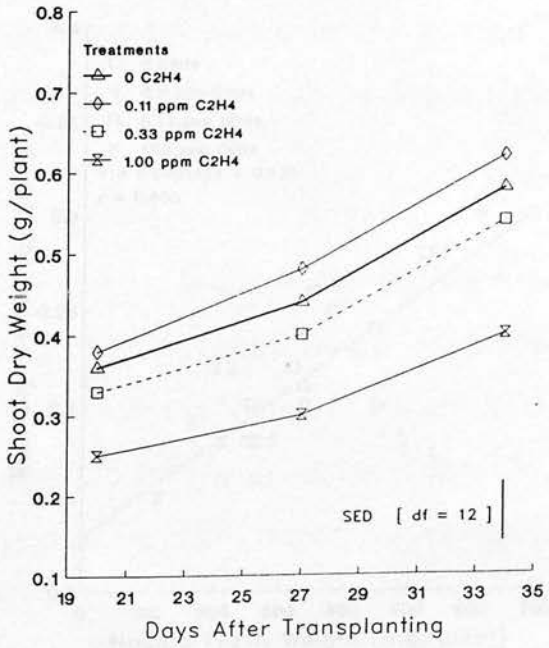


Fig. 4.24 Effect of different concentrations of C_2H_4 on shoot dry weight (bean)

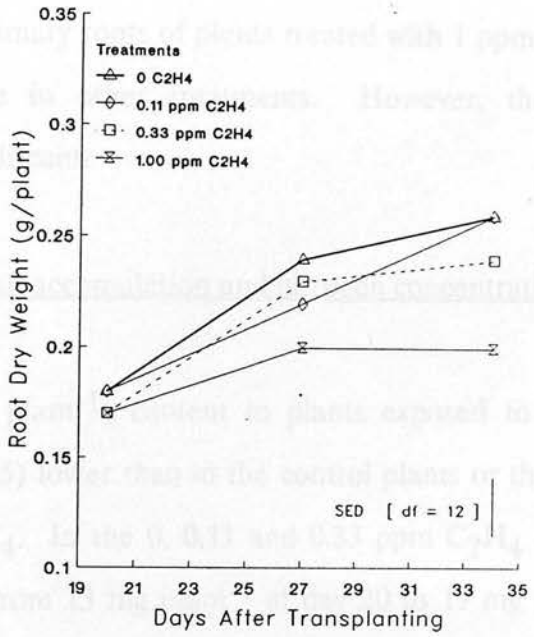


Fig. 4.25 Effect of different concentrations of C_2H_4 on root dry weight (bean)

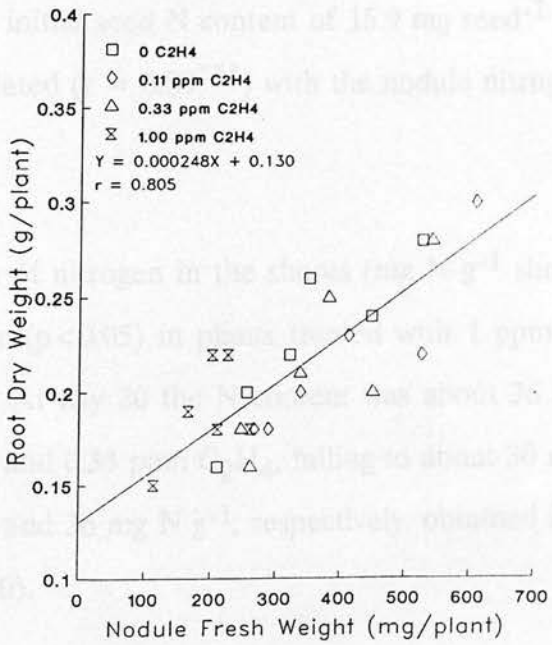


Fig. 4.26 Relationship between root dry weight and nodule fresh weight (bean)

(c) Primary root length

Primary root length increased steadily in all treatments between days 20 and 34 (Fig. 4.27). The primary roots of plants treated with 1 ppm C_2H_4 were slightly shorter than those in other treatments. However, the differences were statistically not significant.

(d) Total nitrogen accumulation and nitrogen concentration in shoots

The shoot N (mg plant^{-1}) content in plants exposed to 1 ppm C_2H_4 was significantly ($p<0.05$) lower than in the control plants or those exposed to 0.11 and 0.33 ppm C_2H_4. In the 0, 0.11 and 0.33 ppm C_2H_4 treatments shoot N content increased from 13 mg plant^{-1} at day 20 to 17 mg plant^{-1} by the final harvest, compared with 10 mg plant^{-1} increasing to 14 mg plant^{-1} at 1 ppm C_2H_4. The differences were just significant at the 5% level. In all treatments the increase in shoot N content over the period of observation was equivalent to about 25% of the initial seed N content of 15.9 mg seed^{-1} (Fig. 4.28). The N content was correlated ($r = 0.80^{***}$) with the nodule nitrogenase activity (Fig. 4.29).

The concentration of nitrogen in the shoots (mg N g^{-1} shoot dry weight) was significantly higher ($p<0.05$) in plants treated with 1 ppm C_2H_4 than in the other treatments. At day 20 the N content was about 36 mg N g^{-1} in plants exposed to 0, 0.11 and 0.33 ppm C_2H_4, falling to about 30 mg N g^{-1} at day 34, compared with 41 and 36 mg N g^{-1}, respectively, obtained in the 1 ppm C_2H_4 treatment (Fig. 4.30).

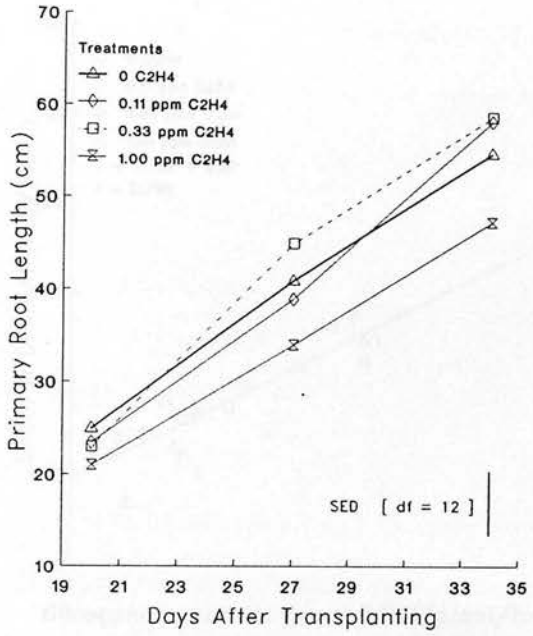


Fig. 4.27 Effect of different concentrations of C_2H_4 on primary root length of bean plants

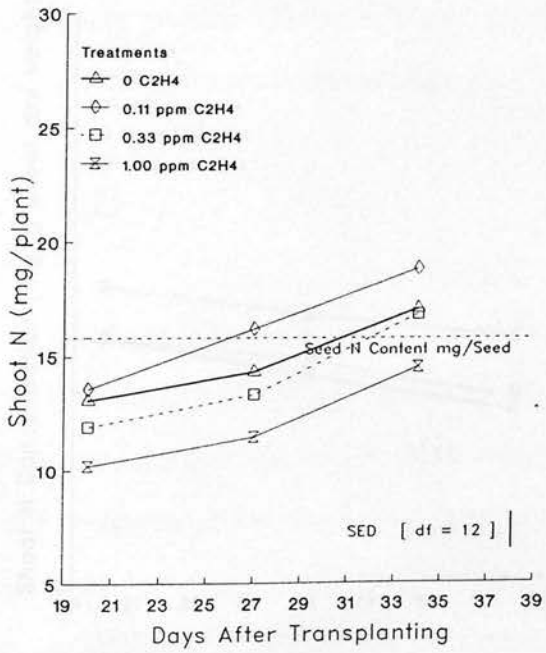


Fig. 4.28 Effect of different concentrations of C_2H_4 on total nitrogen content of bean shoots

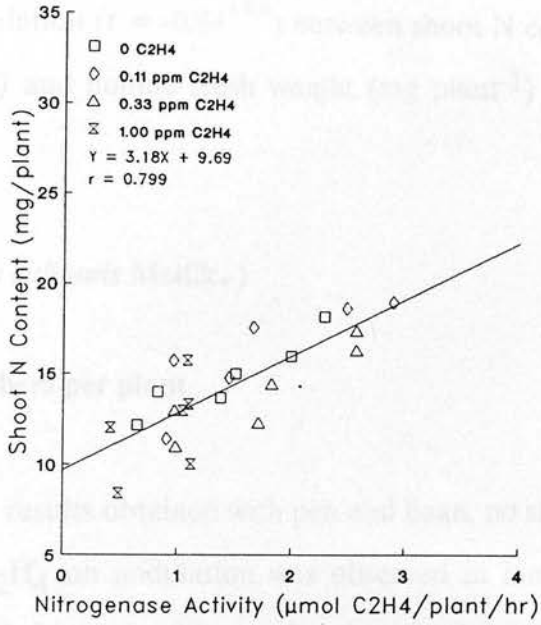


Fig. 4.29 Relationship between shoot nitrogen content and nodule nitrogenase activity (bean)

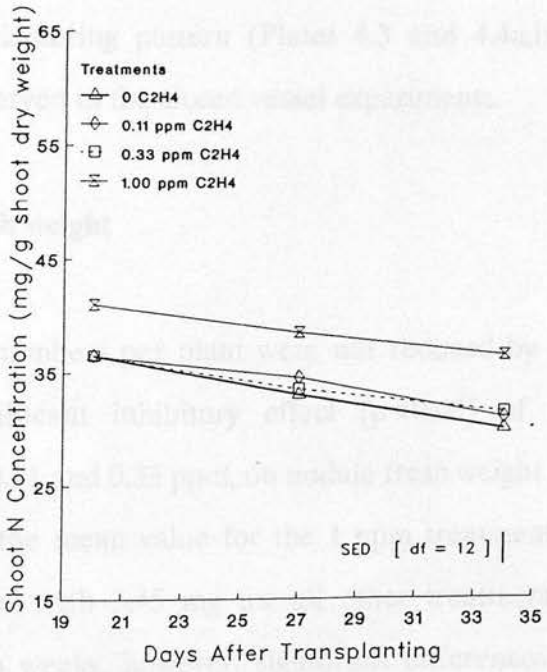


Fig. 4.30 Effect of different concentrations of C_2H_4 on nitrogen concentrations of bean shoots

The shoot N concentration (mg g^{-1} shoot dry weight) decreased significantly with time. A high negative correlation ($r = -0.94^{***}$) was obtained between mg N g^{-1} shoot dry weight and shoot dry matter yield (g $plant^{-1}$) (Fig. 4.31). Also a good negative correlation ($r = -0.84^{***}$) between shoot N concentration (mg g^{-1} shoot dry weight) and nodule fresh weight (mg $plant^{-1}$) was obtained (Fig. 4.32).

4.1.3 Lentil (*Lens culinaris* Medik.)

4.1.3.1 Nodule numbers per plant

In contrast with the results obtained with pea and bean, no significant inhibitory effect of 1 ppm C_2H_4 on nodulation was observed in lentil plants. Nodule numbers were much lower in this species than in the other two, but at all concentrations of C_2H_4 the numbers per plant increased significantly with time, from about 24 per plant at day 20, to about 35 at day 34 (Fig. 4.33). The nodule distribution and clustering pattern (Plates 4.3 and 4.4a,b) was found to be similar to that observed in the closed vessel experiments.

4.1.3.2 Nodule fresh weight

Although nodule numbers per plant were not reduced by 1 ppm of ethylene, there was a significant inhibitory effect ($p<0.05$) of this concentration, compared with 0, 0.11 and 0.33 ppm, on nodule fresh weight (mg $nodule^{-1}$) (Fig. 4.34). At day 20 the mean value for the 1 ppm treatment was about 3.1 mg $nodule^{-1}$, compared with 3.45 mg for all other treatments (not significant). Over the next two weeks, however, significant differences did emerge. The nodule fresh weights increased to an average of about 4.1 mg at day 34 in the 0,

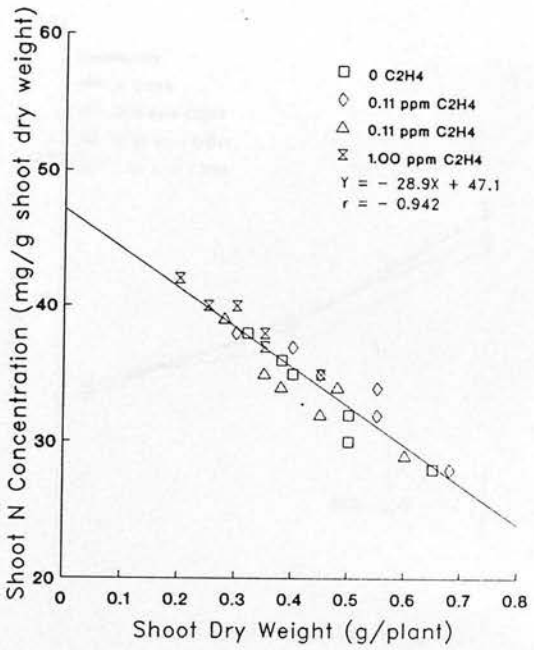


Fig. 4.31 Relationship between shoot nitrogen concentration and shoot dry weight (bean)

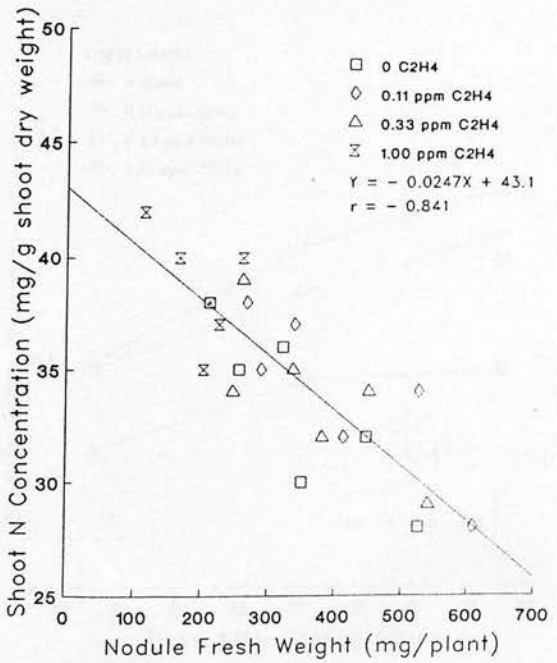


Fig. 4.32 Relationship between shoot nitrogen concentration and nodule fresh weight (bean)

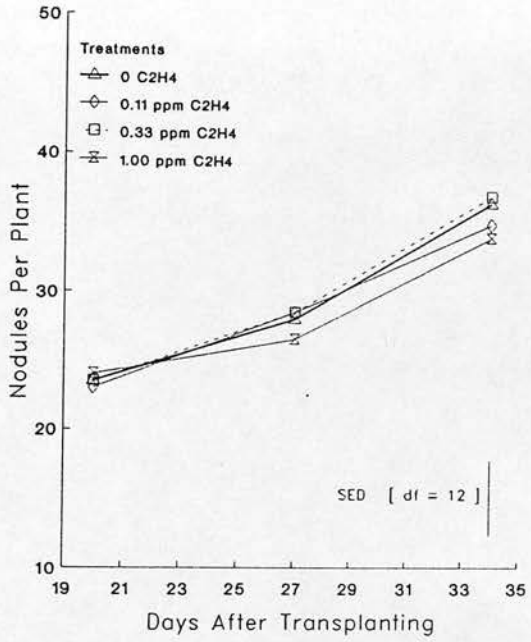


Fig. 4.33 Effect of different concentrations of C_2H_4 on nodulation of lentil roots

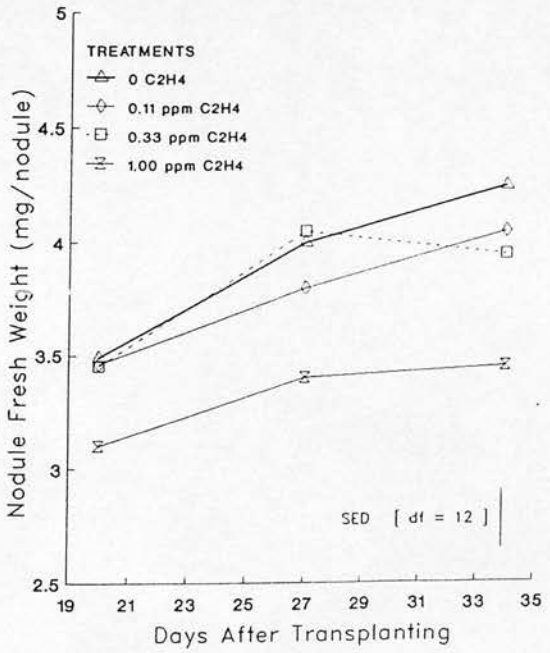


Fig. 4.34 Effect of different concentrations of C_2H_4 on individual nodule fresh weight (lentil)

Plate 4.3 Nodules on lentil roots exposed to air in the presence of different concentrations of ethylene (flow-through system). Left to right: 0 ppm; 0.11 ppm; 0.33 ppm; 1 ppm.

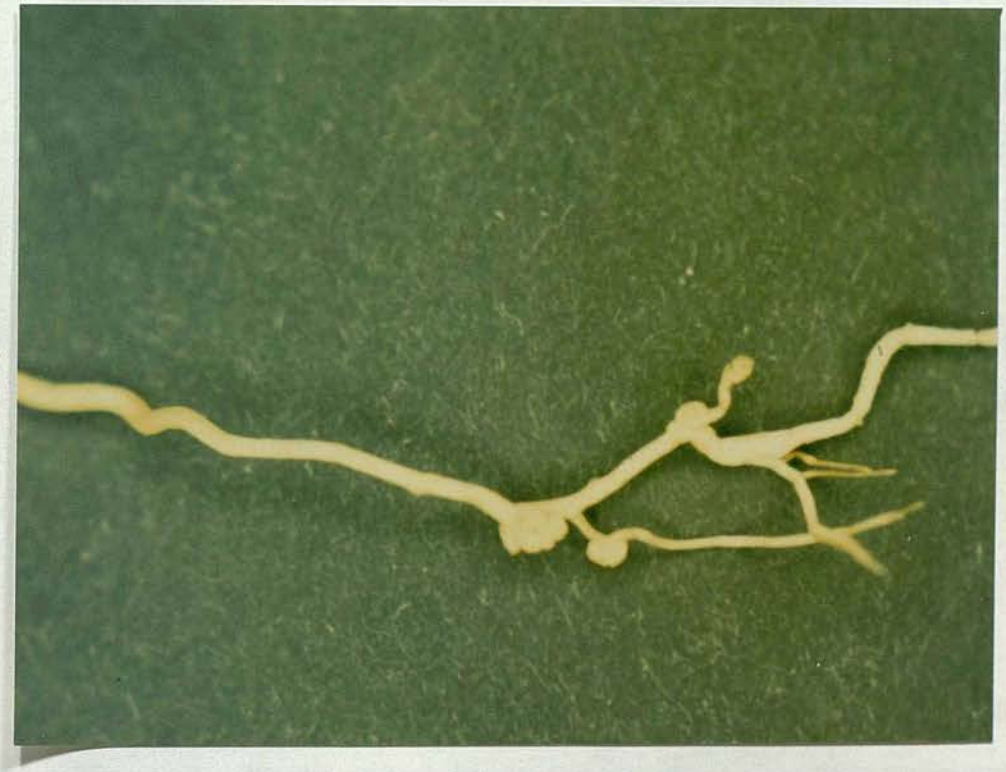


Plate 4.4a,b Nodules on lentil roots exposed to 1 ppm of C2H4 in air (flow-through system)

0.11 and 0.33ppm C_2H_4 treatments, whereas at 1 ppm an increase to about 3.4 mg at 27 days was followed by almost no increase in the following week.

Total nodule fresh weight (mg plant^{-1}) increased from about 80 at day 20 to 155 mg plant^{-1} by the final harvest in control plants. The corresponding value obtained at day 34 for the 0.11 and 0.33 ppm C_2H_4 treatments, 140 mg plant^{-1}, was not significantly lower (Fig. 4.35). An average total weight of 116 mg plant^{-1} was recorded for nodules developed in 1 ppm C_2H_4. This was just significantly lower than the control treatment, but the differences from the 0.11 and 0.33 ppm treatments were not significant.

4.1.3.3 Nodule nitrogenase activity

There were no significant differences in nodule nitrogenase activity between treatments. The activity increased significantly in all the treatments over the period of observation (Fig. 4.36), from 0.35 μmol C_2H_4 plant^{-1} hr^{-1} at day 20 to an average of about 0.86 μmol C_2H_4 plant^{-1} hr^{-1} at day 34, in all treatments except the 1 ppm C_2H_4, and to about 0.80 μmol C_2H_4 plant^{-1} hr^{-1} in the latter. Nodule nitrogenase activity at the highest concentration of C_2H_4 was always lower than in the other treatments, but the differences were statistically not significant.

4.1.3.4 Nodule leghaemoglobin content

The leghaemoglobin content at day 20 was about 0.02 mg plant^{-1} in all treatments. By the end of the experiment the content had increased to about 0.06 mg plant^{-1} in the 0, 0.11 and 0.33 ppm C_2H_4 treatments, and to

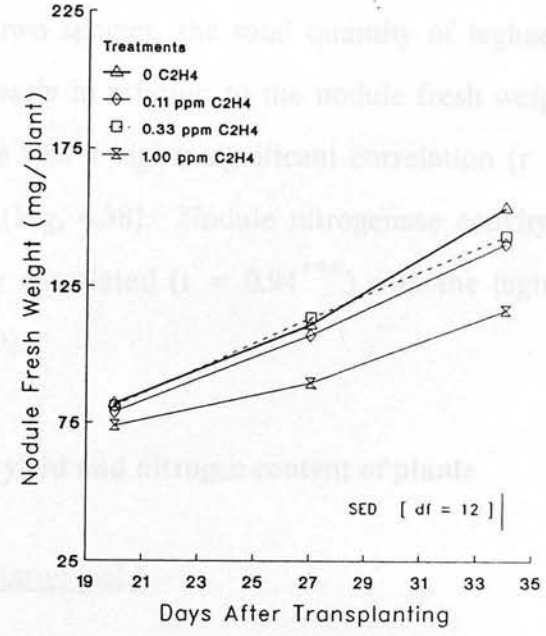


Fig. 4.35 Effect of different concentrations of C_2H_4 on total nodule fresh weight (lentil)

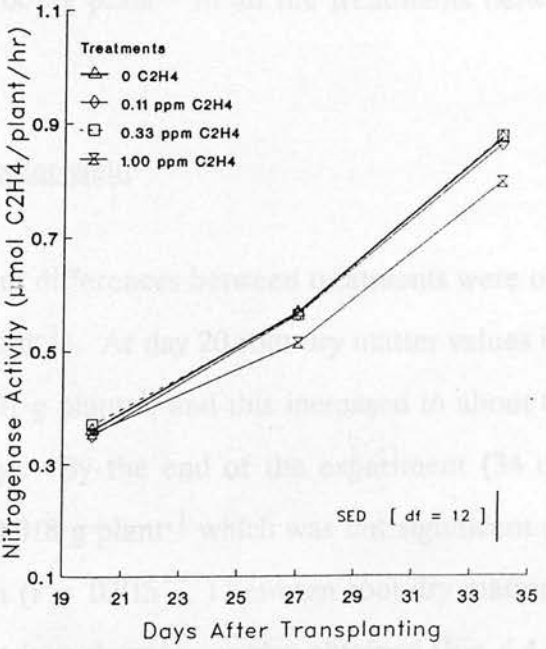


Fig. 4.36 Effect of different concentrations of C_2H_4 on nodule nitrogenase activity (lentil)

about 0.45 mg in the 1 ppm C_2H_4 treatment (Fig. 4.37). However, the difference was not significant.

As for the other two species, the total quantity of leghaemoglobin per plant increased curvilinearly in relation to the nodule fresh weights per plant, in all treatments. There was a highly significant correlation ($r = 0.97^{***}$) between these parameters (Fig. 4.38). Nodule nitrogenase activity per plant was also highly significantly correlated ($r = 0.94^{***}$) with the leghaemoglobin content per plant (Fig. 4.39).

4.1.3.5 Dry matter yield and nitrogen content of plants

(a) Shoot dry matter yield

None of the C_2H_4 treatments had any significant effect on lentil shoot dry matter production. Shoot dry matter increased steadily with time, from about 0.02 to 0.042 ± 0.002 g plant^{-1} in all the treatments between days 20 and 34 (Fig. 4.40).

(b) Root dry matter yield

Again, no significant differences between treatments were observed for root dry matter yields (g plant^{-1}). At day 20 root dry matter values in all the treatments averaged about 0.01 g plant^{-1}, and this increased to about 0.016 g plant^{-1} over the next seven days. By the end of the experiment (34 days) the value had increased only to 0.018 g plant^{-1} which was not significant (Fig. 4.41). A good positive correlation ($r = 0.815^{***}$) between root dry matter yield and the total nodule fresh weight (mg plant^{-1}) was also obtained (Fig. 4.42).

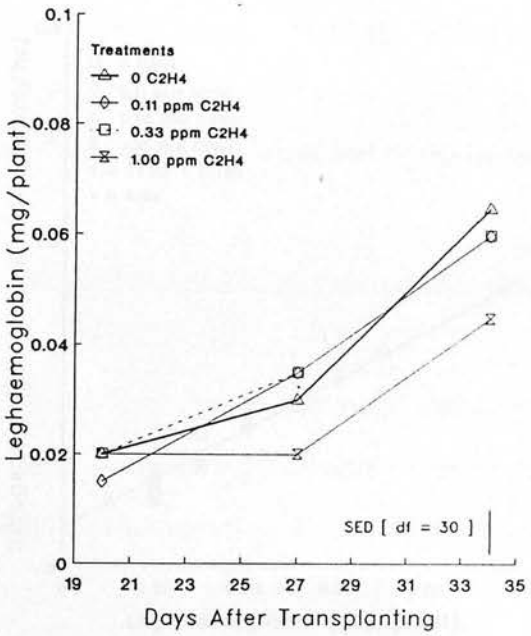


Fig. 4.37 Effect of different concentrations of C2H4 on nodule leghaemoglobin content (lentil)

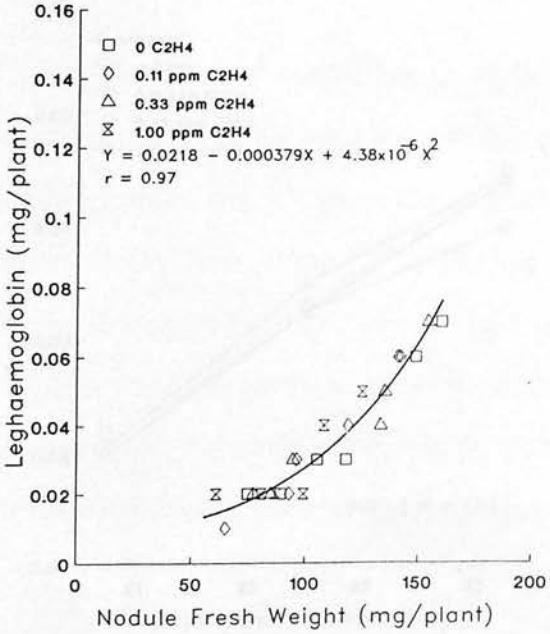


Fig. 4.38 Relationship between leghaemoglobin content of lentil root nodules and nodule fresh weight

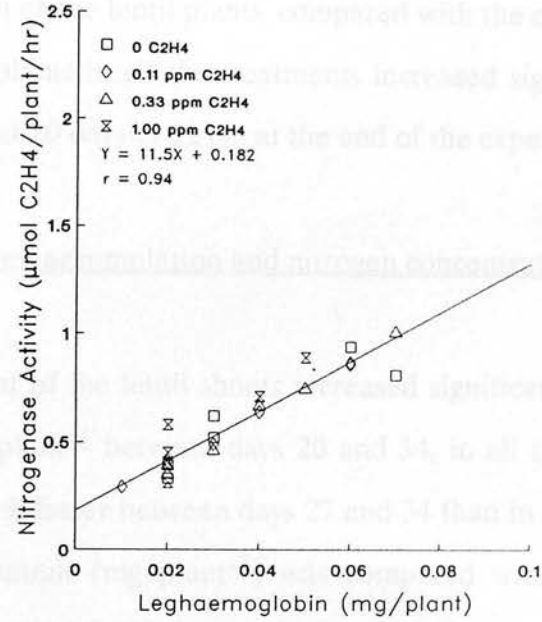


Fig. 4.39 Relationship between nitrogenase activity and leghaemoglobin content of lentil root nodules

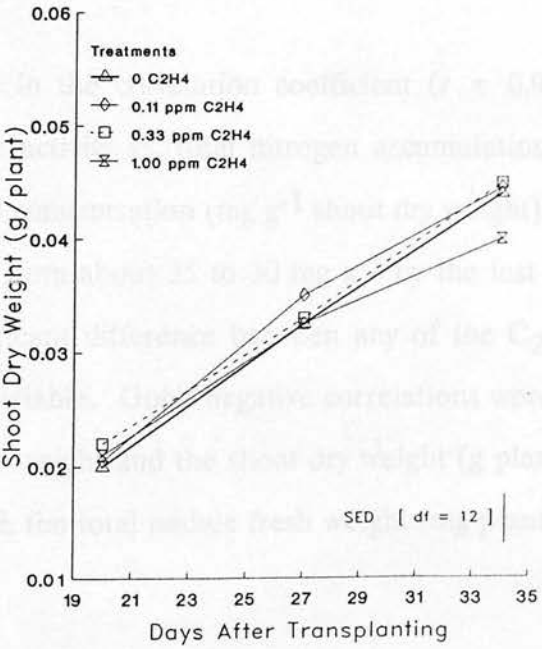


Fig. 4.40 Effect of different concentrations of C_2H_4 on shoot dry weight (lentil)

(c) Primary root length

No significant effect of any of the C_2H_4 treatments was observed on the primary root length of the lentil plants, compared with the control. The primary roots of all lentil plants in all the treatments increased significantly with time, from about 12 cm at 20 days to 22 cm at the end of the experiment (Fig. 4.43).

(d) Total nitrogen accumulation and nitrogen concentration in shoots

The total N content of the lentil shoots increased significantly from about 0.75 to just over 1 mg plant^{-1} between days 20 and 34, in all the treatments. The increases were much faster between days 27 and 34 than in the previous period. When shoot N content (mg plant^{-1}) was compared with the amount of N initially present in the seed, the results showed that a substantial input of atmospheric nitrogen into the shoot dry matter had occurred in lentil, as was found with pea and bean (Fig. 4.44).

This was reflected in the correlation coefficient ($r = 0.92^{***}$) obtained for nodule nitrogenase activity vs. total nitrogen accumulation in the shoot (Fig. 4.45). The shoot N concentration (mg g^{-1} shoot dry weight), on the other hand, decreased steadily from about 35 to 30 mg g^{-1} by the last harvest (Fig. 4.46). However, no significant difference between any of the C_2H_4 treatments was observed for this variable. Good negative correlations were obtained between mg N g^{-1} shoot dry weight and the shoot dry weight (g plant^{-1}) ($r = -0.89^{***}$) (Fig. 4.47), and with the total nodule fresh weight (mg plant^{-1}) ($r = -0.775^{***}$) (Fig. 4.48).

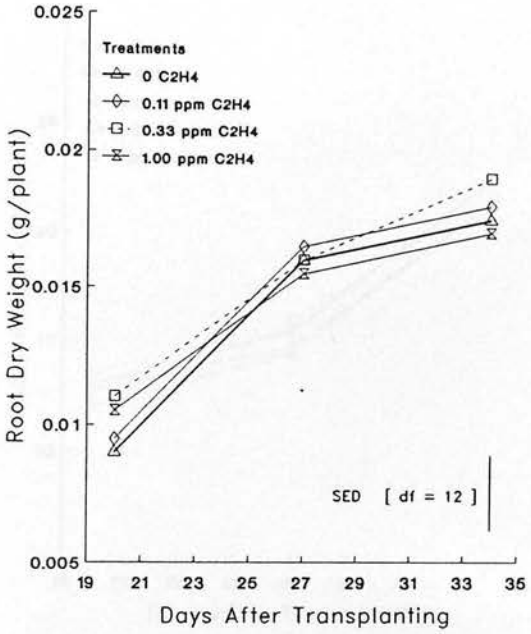


Fig. 4.41 Effect of different concentrations of C_2H_4 on root dry weight (lentil)

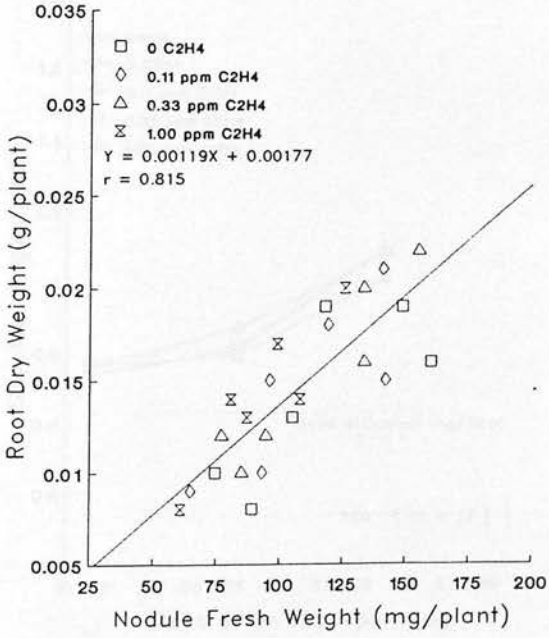


Fig. 4.42 Relationship between root dry weight and nodule fresh weight (lentil)

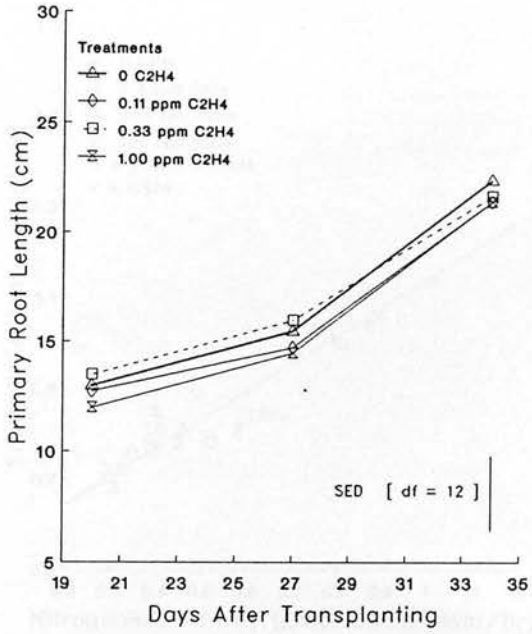


Fig. 4.43 Effect of different concentrations of C_2H_4 on primary root length of lentil plants

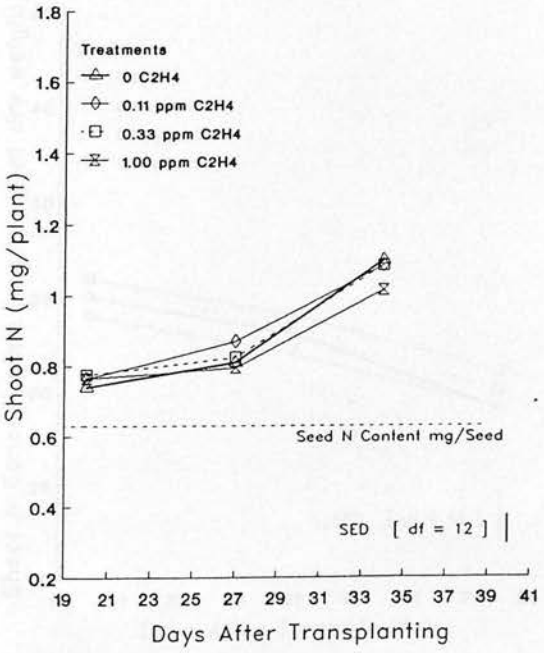


Fig. 4.44 Effect of different concentrations of C_2H_4 on total nitrogen content of lentil shoots

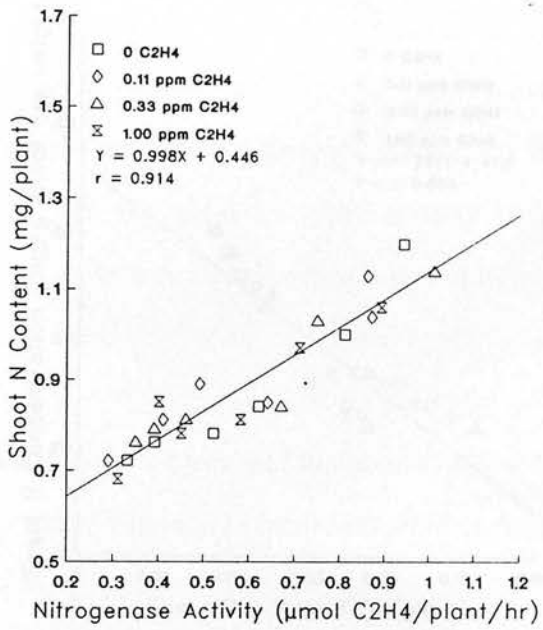


Fig. 4.45 Relationship between shoot nitrogen content and nodule nitrogenase activity (lentil)

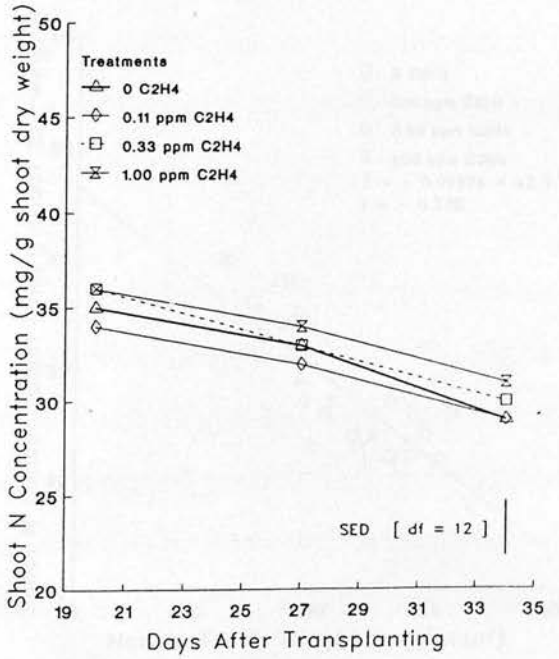


Fig. 4.46 Effect of different concentrations of C_2H_4 on nitrogen concentrations of lentil shoots

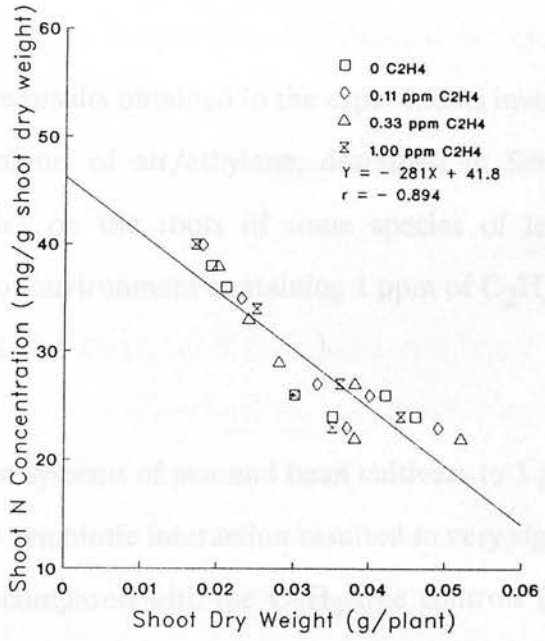


Fig. 4.47 Relationship between shoot nitrogen concentration and shoot dry weight (lentil)

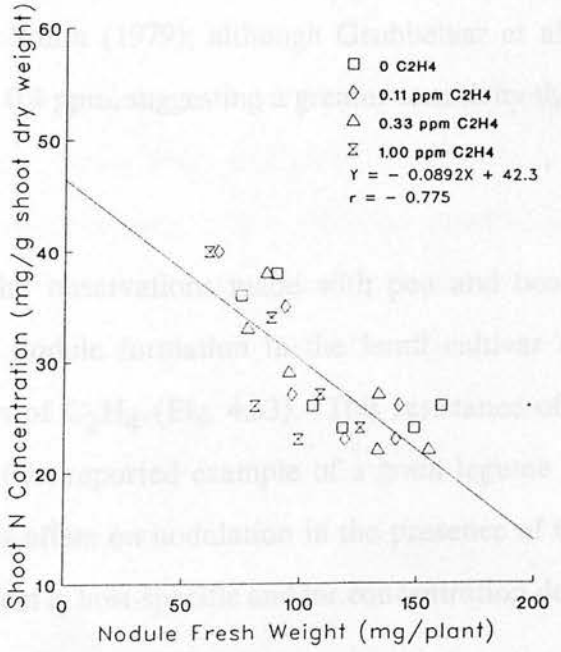


Fig. 4.48 Relationship between shoot nitrogen concentration and nodule fresh weight (lentil)

4.2 DISCUSSION OF THE RESULTS CONTAINED IN SECTION 4.1

4.2.1 Effects of C_2H_4 on nodulation

It is evident from the results obtained in the experiments involving root aeration with continuous streams of air/ethylene, described in Section 4.1, that the formation of nodules on the roots of some species of legume is inhibited significantly by a root environment containing 1 ppm of C_2H_4, whereas another species is unaffected.

The exposure of root systems of pea and bean cultivars to 1 ppm of C_2H_4 from the beginning of the symbiotic interaction resulted in very significant reductions in nodule numbers compared with the C_2H_4-free controls (Figs. 4.1 and 4.17, respectively). However, the two lower concentrations of C_2H_4 applied (0.11 and 0.33 ppm) had no discernible effect. Inhibition of nodulation by C_2H_4 was also reported by Grobbelaar et al. (1970, 1971), Drennan and Norton (1972), and Goodlass and Smith (1979); although Grobbelaar et al. observed a large effect with bean at 0.4 ppm, suggesting a greater sensitivity than observed in this work.

In contrast with the observations made with pea and bean cultivars in this investigation, root nodule formation in the lentil cultivar examined was not inhibited by 1 ppm of C_2H_4 (Fig. 4.33). This resistance of the lentil cultivar appears to be the first reported example of a grain legume species or cultivar showing no obvious effect on nodulation in the presence of C_2H_4. The results suggest that the effect is host-specific and/or concentration dependent.

Generally, lentil species are more tolerant than some other grain legumes of extreme environmental conditions (Muehlbauer et al., 1985). In preliminary trials at the start of this project, this particular cultivar was found to be waterlogging-tolerant, and independent confirmation of this has been provided by other work in Edinburgh (Young, 1991). It is also tolerant of other stresses such as pest damage (BARI Annual Report, 1979-1980, Pulses Improvement Project).

The mechanism by which C_2H_4 inhibits nodulation in legumes is not very clear. However, it may be a part of a feedback autoregulatory mechanism that inhibits cortical cell division and/or the supply of photosynthates to the actively respiring bacteroids (Pierce and Bauer, 1983; Caetano-Anolles and Bauer, 1988; Hacin et al., 1990; Caetano-Anolles and Gresshoff, 1991). The results obtained with pea and bean suggest that the inhibition might have occurred during the early stages of the symbiosis, possibly during infection and/or nodule development.

Although the inhibitory effects were similar in pea and bean, the nature of the inhibition appears to have been different. Inhibition in pea root nodule formation might have occurred in the infection process, whereas in bean it might be that the nodule developmental process, or both infection and development, were inhibited. It can be seen from the data on nodules per plant (Section 4.1.1.1) that the nodule numbers in pea exposed to 1 ppm of C_2H_4 were almost 50% lower than in the other three treatments (0, 0.11 and 0.33 ppm 19 days after treatment began). However, from then on, nodule numbers in the 1 ppm treatment increased significantly and at about the same rate as in the other treatments up to the final harvest at day 34 (Fig. 4.1). The nodules

formed in the 1 ppm treatment were mostly restricted to the primary roots. This distribution pattern has been observed to be characteristic of legumes grown from inoculated seed (Hardarson et al., 1989) so may not be a result of the C_2H_4 treatment. On the other hand, the similarity between the effects of seed inoculation and 1 ppm of C_2H_4 suggests that C_2H_4 could be involved in determining the outcome of early symbiotic events.

The nodules on the primary roots were formed exclusively at the sites of lateral emergence (Plate 4.1). This is an observation which differs from that made earlier by Zaat et al. (1989), namely that in a thick short root phenotype of common vetch (*Vicia sativa*), nodules were induced by C_2H_4 produced in response to the *Rhizobium* bacteria, and were formed exclusively on the laterals, at the sites where they emerged from the primary root. In the presence of the inhibitor of ethylene action AVG, nodulation on the primary root was restored to normal.

The relative variation with time in the numbers of nodules formed on bean roots exposed to the different concentrations of C_2H_4 was significantly different from that observed with pea. Bean nodule numbers were significantly lower in the 1 ppm treatment throughout the experiment (see Section 4.1.2.1), but the difference between the numbers formed in the presence of 1 ppm and the numbers in the other treatments was less than in pea at day 20, and the nodules were not restricted to the primary root. However, unlike pea, there were no significant changes in bean nodule numbers in the 1 ppm C_2H_4 treatment after day 20 (Fig. 4.17), suggesting the possibility of the inhibition of nodule development by some mechanism (possibly autoregulatory) that suppresses the supply of photosynthates to the late infections. Developing nodules act as

strong sinks for photosynthates (Hacin et al., 1990) and it is possible that infections deprived of essential photosynthates might have been aborted at the early stage of development. It is also possible that the increased C_2H_4 concentration might have had a stimulatory effect on the pea plant's autoregulatory mechanism (ie the capacity of existing nodules to inhibit further nodulation)(Pierce and Bauer, 1983; Caetano-Anolles and Gresshoff, 1991), that was exerted at the level of nodule initiation. In contrast with the situation for pea, nodulation in bean roots exposed to 1 ppm of C_2H_4 was not restricted to primary roots; rather, the nodules were scattered throughout the root system. The reason for this is not obvious, but it may have been due to the slow suppression response of bean plants' autoregulatory mechanism (George and Robert, 1991).

4.2.2 Nodule fresh weight

Nodules that were formed in the roots of pea and bean exposed to 1 ppm of C_2H_4 had significantly lower nodule fresh weights (Figs. 4.2 and 4.18, respectively). The level of significance was much higher when total nodule fresh weight per plant was taken into consideration (Figs. 4.3, 4.19). This reflects the formation of significantly fewer nodules in this treatment as well as a direct effect on individual nodule size.

Although the number of nodules per bean plant was not changed significantly by 1 ppm of C_2H_4 during the experiment, individual nodule fresh weights were found to increase up to day 27 (although this was not statistically significant) and then declined at day 34. The differences between pea and bean in the effect of C_2H_4 on nodule numbers, and the suggestion of a difference in the

effect on nodule weight, partly supports the hypothesis that the mechanism of inhibition of root nodule formation by C_2H_4 might not be the same for these two species, although the extent of inhibition appears to be of a similar order. It is possible that, in pea, available photosynthate was utilised in forming new nodules (Fig. 4.1), while in bean available photosynthate was utilised in the growth of existing nodules because, in the latter species, autoinhibition of nodulation is stronger. Recently, Caetano-Anolles and Gresshoff (1991) showed that the autoregulation in bean is exerted at the level of nodule initiation, rather than during infection development.

The individual nodule fresh weights in pea remained unchanged throughout, but increased in bean up to day 27 and then declined. The effect with bean was not statistically significant; it may have been merely the consequence of sampling variation. However, possibly there may have been an induction of early nodule senescence by 1 ppm of ethylene. The experiment would need to be repeated, with more replication, to find out if there was a real effect.

Although there were no significant effects of 1 ppm of C_2H_4 on root nodule numbers in the lentil cultivar examined, the individual fresh weights, and therefore the total weight per plant of the nodules formed in this treatment were lower than in the other three treatments (Figs. 4.34, 4.35), although the differences were only just significant at the 5% level. It is not clear why 1 ppm of C_2H_4 should have had no effect on the process of nodulation but a negative effect (although to a lesser extent than with the other species) on the nodule fresh weight.

4.2.3 Relationships between shoot N concentration, shoot dry weight, and nodule fresh weight

Correlations between shoot N concentration and shoot dry weight were examined, in an attempt to understand the nitrogen dynamics in the legumes when they were fixing nitrogen. In general, the N concentration in the shoots of all three species went down as the shoots increased in weight (Figs. 4.15, 4.31, 4.47). This is presumably because of the normal dilution of N as growth proceeds, observed in legumes and non-legumes alike. However, the greater N concentration at any particular time in pea and bean treated with 1 ppm of ethylene, as compared with the other treatments (Figs. 4.14 and 4.30) may have been due in part to a reduction in the N sink in the nodules, resulting from reduced nodulation in the 1 ppm C_2H_4 treatment.

The decrease in N concentration in shoots with increasing nodule fresh weight (Figs. 4.16, 4.32, 4.48) is not to be regarded as a direct effect, but rather as a result of the general positive correlation between plant size and nodule weight per plant.

4.2.4 Nitrogenase activity, leghaemoglobin content and shoot N content

The leghaemoglobin content per plant, in all three species, increased rapidly with increasing total nodule fresh weight, as a result of an increase in both nodule weight and leghaemoglobin concentration per mg of nodule, with time (Figs. 4.6, 4.22, 4.38). This interaction of the two factors was responsible for the non-linearity of the relationships shown in the figures. There was very little scatter in the data, and the correlation coefficients were very high.

The overall relationship for each species between nitrogenase activity and leghaemoglobin content was linear and showed very high correlations (Figs. 4.7, 4.23, 4.39). For pea, the nodule fresh weight per plant at 34 days was reduced by 1 ppm of C_2H_4 to 52% of the mean of the other three treatments (Fig. 4.3), whereas the corresponding mean reduction in leghaemoglobin content and nitrogenase activity was greater, to about one-third (Fig. 4.7). The nitrogenase activity also decreased on a per-nodule basis (Figs. 4.1, 4.7). For bean, the corresponding reduction in nodule fresh weight per plant at 34 days was to only 40% of the mean of the other treatments (Fig. 4.19). The reduction in nitrogenase activity per plant was similar (Fig. 4.23). However, the effect on leghaemoglobin was greater still; its net production at 1 ppm of C_2H_4 had ceased after 27 days (Fig. 4.21), and this resulted in a calculated reduction to only 20% of the value for the other treatments, at 34 days. There was no reduction in nitrogenase activity per nodule. For lentil, the reduction in nodule fresh weight in the presence of 1 ppm of C_2H_4 was to 76% of the mean of the other treatments; the corresponding reduction in leghaemoglobin was similar; the effect on nitrogenase activity was rather less. Here also, there was no reduction in nitrogenase activity per nodule.

These results indicate different effects on the three species: in pea only, there was a reduction in nitrogenase activity per nodule as well as per plant; in bean only, there was a complete cessation in leghaemoglobin production after 27 days; in lentil, unlike the other two species, there was no reduction in leghaemoglobin content beyond that pro rata with the reduction in total nodule fresh weight. Further work is required to establish the significance of the changes in leghaemoglobin content for the capacity of the different species to fix nitrogen.

The results for pea agree with previous observations, in which Goodlass and Smith (1979), working with the same species, and also Day et al. (1975) working with *Trifolium*, found a decline in nitrogenase activity on a per-nodule basis. The absence of an effect with bean may have been due to the fact that C_2H_4 concentrations above 1 ppm were not used in the present study. Work by Grobelaar et al. (1971) showed that higher concentrations of C_2H_4 did inhibit the N-fixation activity of bean explants. They showed that the $\%^{15}N$ values of bean explants supplied with $^{15}N_2$ were significantly higher when C_2H_4 was continuously removed than when the explants were treated with 10 or 100 ppm of C_2H_4 or when no C_2H_4 was added or removed. There was no significant difference between $\%^{15}N$ values in these three treatments, suggesting that the concentration of C_2H_4 produced naturally was high enough to have an effect comparable with that of the 10 or 100 ppm applied artificially. The much smaller air volume/root volume ratios used by Grobelaar et al., compared with those used in the present work, could be expected to have given rise to concentrations of several ppm from endogenous ethylene alone (based on the results described below in Section 4.3.2).

Correlations between shoot N content and nitrogenase activity were studied to assess whether atmospheric N incorporation into the legumes through symbiotic association with *Rhizobium* was occurring to a significant extent. The significant positive correlations obtained for these two variables (Figs. 4.13, 4.29, 4.45) suggest that fixation did occur, and furthermore that the acetylene reduction assay is suitable for measuring nitrogenase activity *in vitro* for short-term assessments as in the present investigation.

4.2.5 Shoot dry weight

Exposure of the root systems to 1 ppm of C_2H_4 resulted in a significant reduction in shoot biomass production in pea plants (Fig. 4.8). This, however, represents an effect of C_2H_4 which is independent of its effect on nitrogen supply, through reduction of nodulation and N-fixation activity. The shoots of the plants treated with 1 ppm of C_2H_4 had a higher N concentration than those in the other treatments (Fig. 4.14), and therefore limitation of N supply to the shoot was clearly not the mechanism involved. Further work with non-nodulated plants would be useful in clarifying this aspect.

Shoot dry matter yields for bean (Fig. 4.24) were reduced by 1 ppm of C_2H_4. Similarly to what had been found for pea, the shoot N concentrations were highest in this treatment (Fig. 4.30) and so here, also, N supply limitation did not appear to be the cause of lower shoot weight.

4.2.6 Root dry weight

The effect of 1 ppm of C_2H_4 on root biomass production in all the species examined (Figs. 4.9, 4.15, 4.41) was not significant except at day 34 in bean, when the root dry weight (excluding nodules) was significantly lower (at the 5% level) than in all the other treatments (Fig. 4.25). In general, however, pea and bean roots exposed to 1 ppm of C_2H_4 always had lower dry weights. Inhibition of nodule formation and nitrogenase activity might explain this, although it would be difficult to differentiate between an indirect effect of that sort and a direct effect of C_2H_4.

4.2.7 Relationship between nodule weight and root weight

The relationships between nodule weight and the root weight were examined to identify whether one is dependent on the other. It was not possible from the data to correlate root dry weight and nodule dry weight, because nodules were sacrificed for the determination of leghaemoglobin. However, when nodule fresh weight was compared with root dry weight, significant positive correlations were obtained for all three legume species (Figs. 4.10, 4.26, 4.42). It is possible that the increased root mass provided the nodules with more photosynthates to cope with the demand for their own development. It is also possible that the increased root weight and the associated formation of more laterals, and therefore more surface area exposed for the bacteria to infect, was a factor in increasing the number of nodules per plent. The root dry weights of all the species were unaffected by C_2H_4 to any significant extent.

4.2.8 Primary root length

Primary root lengths of all three species were not affected to a statistically significant extent by 1 ppm C_2H_4 (Figs. 4.11, 4.27, 4.43). However, the root tips of pea and bean were swollen, and there was induction of root hair formation on the root tips of pea (characteristic of C_2H_4 action) but not on those of bean. In general, the number of lateral roots formed by pea and bean at 1 ppm C_2H_4 (from visual observation) was lower than 0, 0.11 and 0.33 ppm C_2H_4, and this reduction was responsible for the reduction in root weight (Fig. 4.9).

4.2.9 Shoot N concentration

Shoot N concentration in all three species decreased with time, irrespective of C_2H_4 treatment (Figs. 4.14, 4.30, 4.46). However, in pea and bean the 1 ppm C_2H_4 treatment always had a significantly higher shoot N concentration than all other treatments. Generally, shoot N concentration decreased as the plant biomass increased over time. For pea, a rapid decrease in shoot N concentration over the 20-27 day period coincided with a rapid increase in nodule weight, suggesting that retention of nitrogen in the nodules and/or translocation of nitrogen from the shoots down to the nodules (Verma and Nadler, 1984) had occurred during the nodule developmental phase.

4.3 EXPERIMENTS IN THE "CLOSED VESSEL" SYSTEM (WITH RECIRCULATED ATMOSPHERES)

4.3.1 Composition of root atmospheres

The results reported in this section are from experiments carried out using the system described above in Section 3.2. In this system, plant root systems were sealed into Kilner jars and the atmosphere in the jars was circulated in a closed loop. Absorbent traps removed C_2H_4 and/or CO_2, and there was also a control treatment without absorbent.

The C_2H_4 and CO_2 concentrations present in the atmosphere around pea, bean and lentil roots are shown in Figs. 4.49-4.5l, respectively. The ethylene reached a concentration of 0.6-0.7 ppm with pea, around 0.6 ppm with bean and around 0.5 ppm with lentil by day 20, when recording of nodule numbers commenced. In the experiment with pea, the concentration of C_2H_4 in the treatment where neither trap was used (i.e. $+C_2H_4/+CO_2$) remained fairly constant up to day 34, but in the treatment where CO_2 was trapped out (i.e. $+C_2H_4/-CO_2$) a decrease in C_2H_4 concentration was observed, to 0.4 ppm by day 34.

The concentration of ethylene in the experiment with bean increased steadily up to day 26 and then slightly decreased in both the $+C_2H_4/-CO_2$ and $+C_2H_4/+CO_2$ treatments. However, the concentration in the vessels never dropped below 0.6 ppm within the period between 20 and 34 days after closure.

In the corresponding experiment with lentil, the concentration of C_2H_4 increased to about 0.6 ppm by day 24; however, it decreased slightly after that, to between 0.4 and 0.5 ppm by day 34 in both the treatments.

The carbon dioxide in the untrapped systems reached 0.6% in the pea experiment, 0.8% in that with bean, and 0.4% in that with lentil by day 20. The concentration reached a maximum of 1.1% with bean, 0.7-0.8% with pea, and around 0.6% with lentil by day 34.

For both the gases, the most rapid increase in concentration was observed between days 10 and 20 with pea and bean, but between days 14 and 24 with lentil.

As can be seen in Figs. 4.49-4.51, neither ethylene nor CO_2 was detectable in treatments in which the appropriate absorbent trap for the gas was included in the circulatory system.

The amounts of C_2H_4 absorbed by the $Hg(ClO_4)_2$ traps in the $-C_2H_4$ treatments were compared with the amounts accumulated in the Kilner jars around the roots in the $+C_2H_4$ treatments (calculated from the measured concentrations and volume of the system). The results are presented in Table 4.1.

The amount of ethylene collected by $Hg(ClO_4)_2$ traps over the first 20 days was significantly higher than that accumulated in the untrapped systems, for all three species. The rate of increase of gas concentrations slowed dramatically in the later stages, or even fell to zero or turned into a decline. The generally good

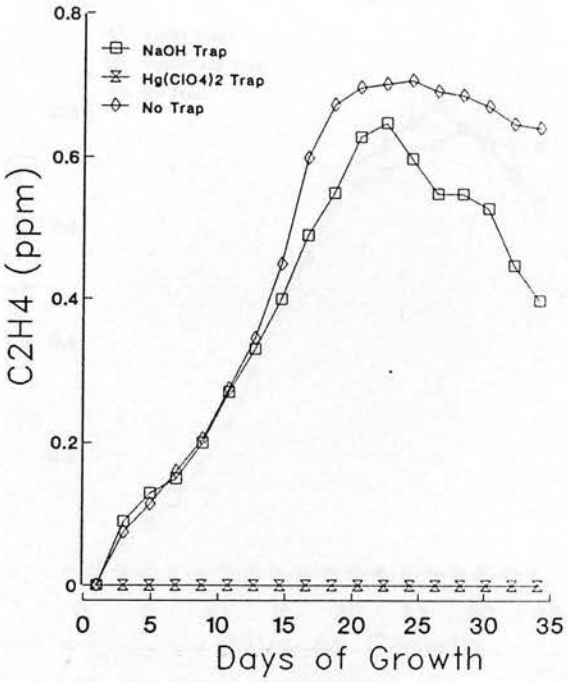


Fig. 4.49(a) Accumulation of endogenous ethylene in atmosphere around pea roots, in presence and absence of $Hg(ClO_4)_2$ traps

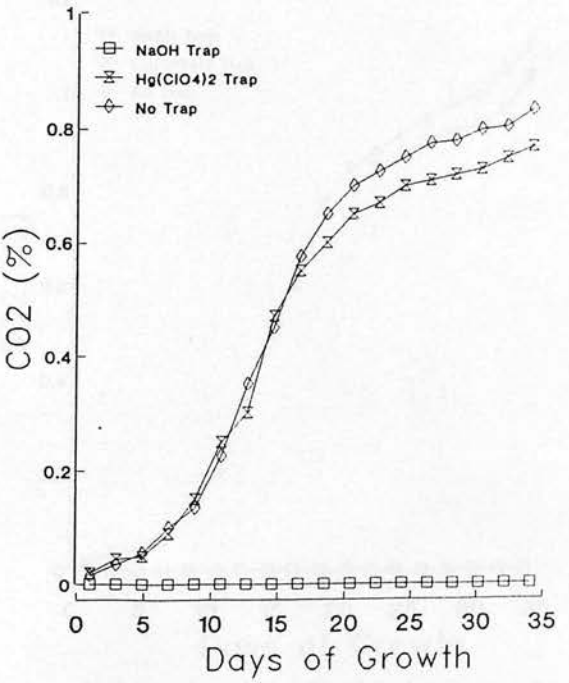


Fig. 4.49(b) Accumulation of carbon dioxide in atmosphere around pea roots, in presence and absence of NaOH traps

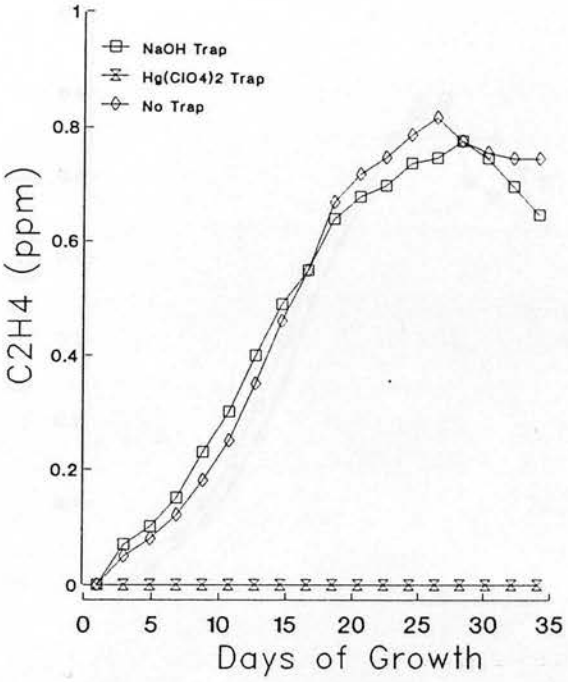


Fig. 4.50(a) Accumulation of endogenous ethylene in atmosphere around bean roots, in presence and absence of $Hg(ClO_4)_2$ traps

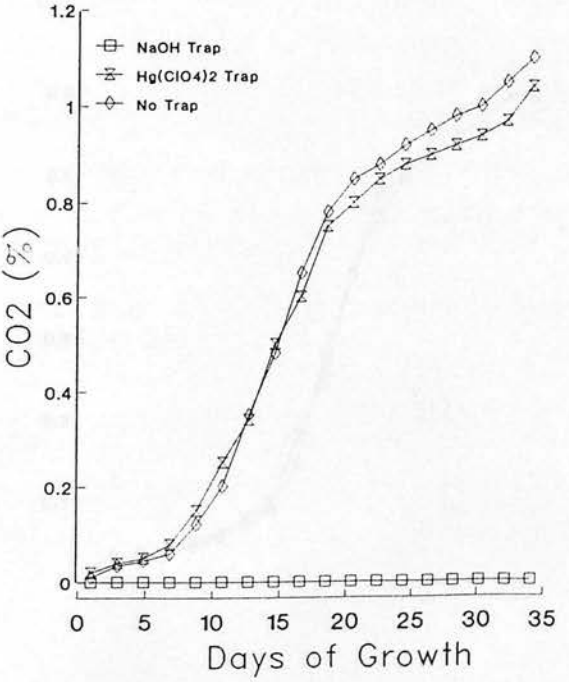


Fig. 4.50(b) Accumulation of carbon dioxide in atmosphere around bean roots, in presence and absence of NaOH traps

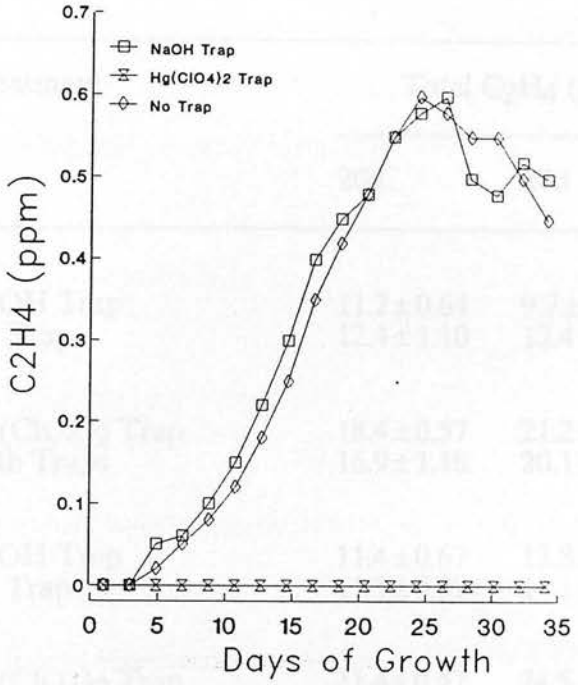


Fig. 4.51(a) Accumulation of ethylene in atmosphere around lentil roots, in presence and absence of $Hg(ClO_4)_2$ traps

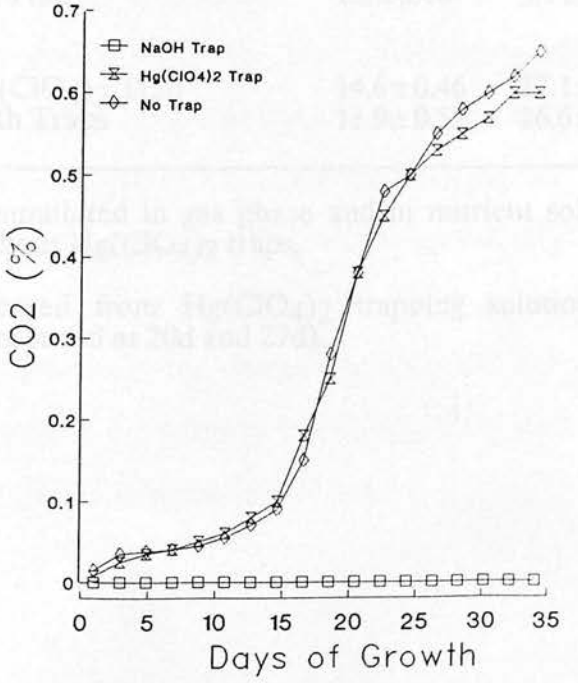


Fig. 4.51(b) Accumulation of carbon dioxide in atmosphere around lentil roots, in presence and absence of NaOH traps

Table 4.1. Comparison of total C_2H_4 production by enclosed root systems, in presence and absence of $Hg(ClO_4)_2$ traps, at three stages of growth (values are means of three replicate measurements with standard error of the mean)

Species	Treatment	Total C_2H_4 (μg) in system		
		20d	27d	34d
Pea	NaOH Trap	11.2±0.64	9.7±0.99	7.1±0.52
	No Trap	12.4±1.10	12.4±1.21	11.5±0.51
	$Hg(ClO_4)_2$ Trap	18.4±0.57	21.2±0.87	24.2±0.72
	Both Traps	16.9±1.18	20.1±1.02	23.5±0.36
Bean	NaOH Trap	11.4±0.67	13.8±0.53	11.7±0.62
	No Trap	11.5±0.29	14.2±0.64	13.3±0.92
	$Hg(ClO_4)_2$ Trap	21.4±0.42	24.5±0.40	27.9±0.64
	Both Traps	20.7±0.76	23.6±0.46	26.9±0.51
Lentil	NaOH Trap	8.1±0.71	8.7±0.38	8.9±0.59
	No Trap	7.8±0.40	9.7±0.40	8.0±0.29
	$Hg(ClO_4)_2$ Trap	14.6±0.46	17.1±0.45	19.8±0.53
	Both Traps	13.9±0.52	16.6±0.35	19.2±0.44

*either accumulated in gas phase and in nutrient solution in treatments without $Hg(ClO_4)_2$ traps,

or released from $Hg(ClO_4)_2$ trapping solutions (fresh solutions introduced at 20d and 27d).

agreement between the pairs of treatments where the gas in question was not being trapped (Figs. 49-51) strongly suggests that leakage was not the major cause of these differences. In the presence of the traps for ethylene, the daily production rate of the gas showed a decline between 20 and 27 days, compared with the previous period, but then an increase to a higher rate. This pattern was consistent for all three species (Table 4.2).

4.3.2 Pea *(Pisum sativum* L.*)*

4.3.2.1 Nodule numbers per plant

The presence of endogenously-produced ethylene around the pea roots significantly ($p<0.05$) reduced the number of nodules per plant at day 27 and reduced them very significantly ($p<0.01$) at day 34, compared with the numbers found in the treatments in which ethylene was removed (Fig. 4.52). Carbon dioxide in the absence of C_2H_4 also had a significant effect on the nodule numbers at day 34. The number of nodules per plant in the $-C_2H_4/+CO_2$ treatment almost doubled over the period during which measurements were made (20-34 days), whereas there was hardly any increase in the nodule numbers in the $+C_2H_4/-CO_2$ treatment after day 20. The difference between the nodule numbers in the $-C_2H_4/+CO_2$ and $-C_2H_4/-CO_2$ treatments at day 34 was significant, confirming that CO_2 had a stimulatory effect on nodule production. However, the absence of CO_2 did not prevent nodulation; the number of nodules per plant in the $-C_2H_4/-CO_2$ treatment increased from 75 to about 120 (60%) between days 20 and 34.

Table 4.2. Daily production rate of C_2H_4 by enclosed root system, in presence of $Hg(ClO_4)_2$ traps (produced using data presented in Table 4.1)

Species	Mean C_2H_4 Production Rate (μg/plant/day)		
	0-20d[a]	20-27d[b]	27-34d[c]
Pea	0.088	0.061	0.114
Bean	0.105	0.061	0.120
Lentil	0.071	0.053	0.095

(a) 10 plants/treatment
(b) 7 plants/treatment; 3 plants harvested at 20d
(c) 4 plants/treatment; 3 plants harvested at 27d

Although CO_2 in the absence of C_2H_4 resulted in a significant increase in the number of nodules per plant, this did not occur in the presence of C_2H_4.

In the $+C_2H_4/-CO_2$ and $+C_2H_4/+CO_2$ treatments, the nodules were located mainly on the primary root and were mostly clustered (multiple heads containing up to seven individual nodules were recorded). However, there were also nodules on the secondary laterals, and these were bigger in size (2.5-3.5 mm) than those on the primary roots (2 mm). These larger nodules looked superficially like clusters, but this was very difficult to confirm and they were therefore recorded as single nodules.

In contrast with the nodulation pattern observed in the treatments in which C_2H_4 was present, the nodules formed in the $-C_2H_4/+CO_2$ and $-C_2H_4/-CO_2$ treatments were scattered throughout the secondary laterals, with a few on the primary root. There were some clusters, but these were mainly located on the basal half of the primary root. The sizes of the individual nodules ranged between 1 and 2 mm in these treatments.

4.3.2.2 Nodule fresh weight

Individual nodule fresh weights at the final harvest (day 34) were very significantly ($p<0.01$) affected by C_2H_4 and CO_2 (Fig. 4.53). When nodule fresh weights were compared on a per-plant basis, there were no significant effects at day 20, but C_2H_4 caused significant ($p<0.001$) inhibition and CO_2 significant ($p<0.05$) stimulation at day 27 and 34; the stimulation by CO_2 was more significant at day 34 than at day 27 (Fig. 4.54).

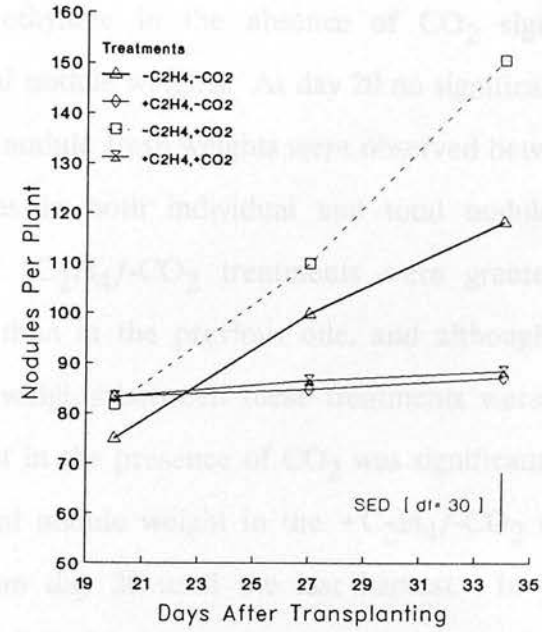


Fig. 4.52 Effect of C_2H_4 and CO_2 on nodulation of pea (closed system)

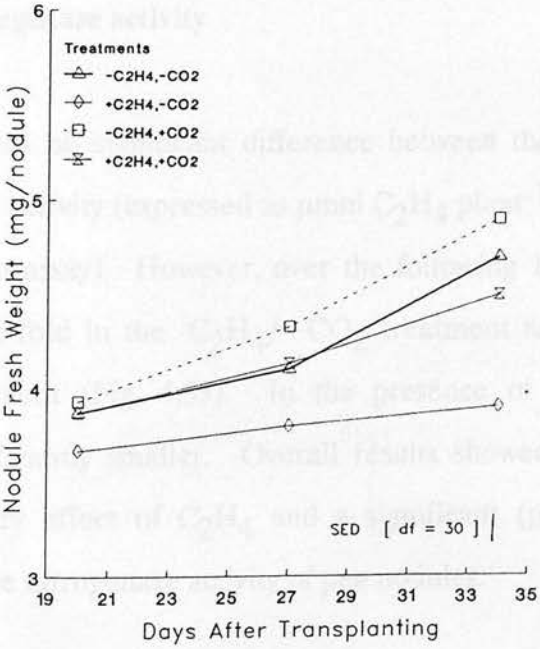


Fig. 4.53 Effect of C_2H_4 and CO_2 on fresh weight of pea nodules (closed system)

In the absence of ethylene, CO_2 increased the mean individual nodule fresh weight from just under 4 to nearly 5 mg (Fig. 4.53) and the total nodule fresh weight per plant from about 325 to 750 mg, an increase of 130% (Fig. 4.54). On the other hand, ethylene in the absence of CO_2 significantly decreased individual and total nodule weights. At day 20 no significant differences in the individual or total nodule fresh weights were observed between the treatments. However, increases in both individual and total nodule weights in the -C_2H_4/+CO_2 and -C_2H_4/-CO_2 treatments were greater during the final sampling interval than in the previous one, and although differences in the individual nodule weights between these treatments were not significant the total nodule weight in the presence of CO_2 was significantly higher at day 34. Individual and total nodule weight in the +C_2H_4/-CO_2 treatment remained fairly constant from day 20 until the last harvest. In the +C_2H_4/+CO_2 treatment individual nodule weight was significantly increased at day 34 but total nodule weight did not change significantly.

4.3.2.3 Nodule nitrogenase activity

At day 20 there was no significant difference between the treatments in the nodule nitrogenase activity (expressed as μmol C_2H_4 plant^{-1} hr^{-1} evolved in the acetylene reduction assay). However, over the following 14 days, nitrogenase activity increased 5-fold in the -C_2H_4/+CO_2 treatment and 3½-fold in the -C_2H_4/-CO_2 treatment (Fig. 4.55). In the presence of C_2H_4 the activity increase was significantly smaller. Overall results showed a very significant ($p<0.001$) inhibitory effect of C_2H_4 and a significant ($p<0.05$) stimulatory effect of CO_2 on the nitrogenase activity of pea nodules.

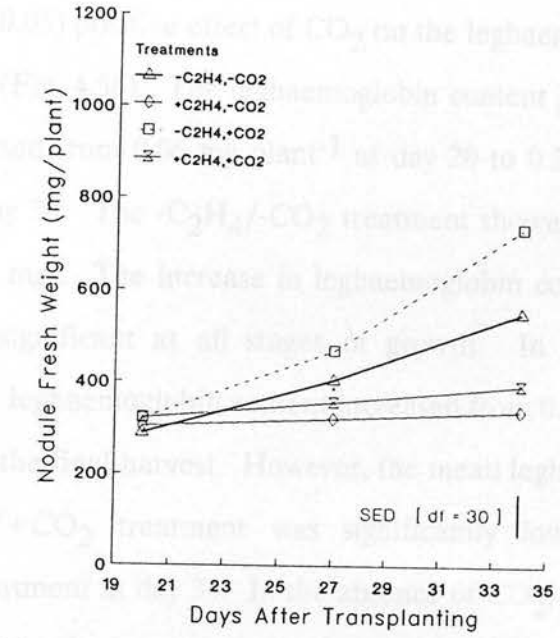


Fig. 4.54 Effect of C_2H_4 and CO_2 on total fresh weight of pea nodules (closed system)

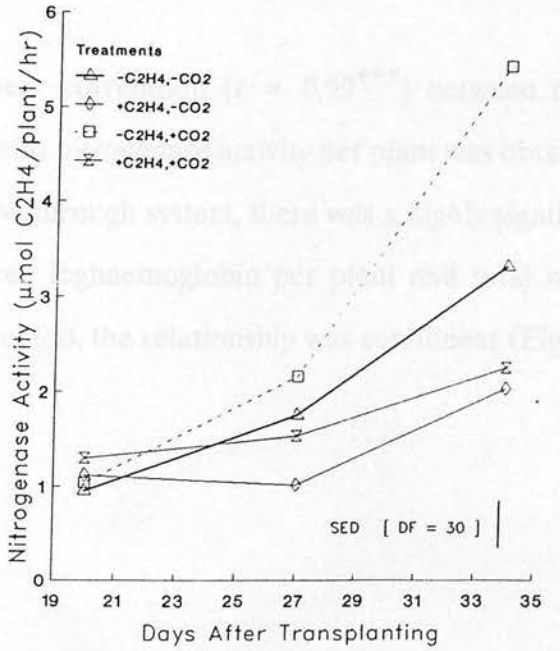


Fig. 4.55 Effect of C_2H_4 and CO_2 on nitrogenase activity of pea nodules (closed system)

4.3.2.4 Nodule leghaemoglobin content

Overall results showed a very significant ($p<0.01$) negative effect of C_2H_4 and a significant ($p<0.05$) positive effect of CO_2 on the leghaemoglobin content per plant at 34 days (Fig. 4.56). The leghaemoglobin content in the $-C_2H_4/+CO_2$ treatment increased from 0.06 mg plant^{-1} at day 20 to 0.28 mg plant^{-1} by the last harvest at day 34. The $-C_2H_4/-CO_2$ treatment showed a smaller increase (0.05 mg to 0.19 mg). The increase in leghaemoglobin content in this pair of treatments was significant at all stages of growth. In the $+C_2H_4/+CO_2$ treatment nodule leghaemoglobin content increased from 0.06 mg plant^{-1} at day 20 to 0.12 mg by the final harvest. However, the mean leghaemoglobin content in the $+C_2H_4/+CO_2$ treatment was significantly lower than in the $-C_2H_4/+CO_2$ treatment at day 34. In the absence of CO_2, ethylene ($+C_2H_4/-CO_2$ treatment) resulted in a significant decrease in the leghaemoglobin content by day 27, compared with the $-C_2H_4/-CO_2$ treatment. The difference between these treatments was still significant at day 34.

A remarkable linear correlation ($r = 0.99^{***}$) between the leghaemoglobin content per plant and nitrogenase activity per plant was obtained (Fig. 4.57). As in the constant flow-through system, there was a highly significant correlation ($r = 0.95^{***}$) between leghaemoglobin per plant and total nodule fresh weight (mg plant^{-1}). Here, too, the relationship was curvilinear (Fig. 4.58).

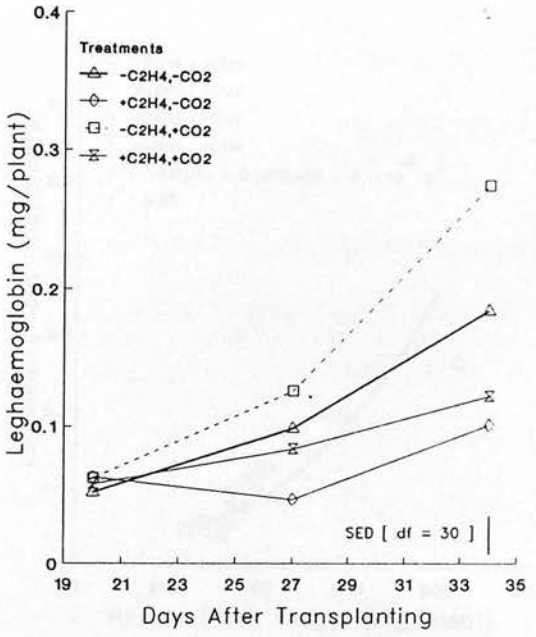


Fig. 4.56 Effect of C_2H_4 and CO_2 on leghaemoglobin content of pea nodules (closed system)

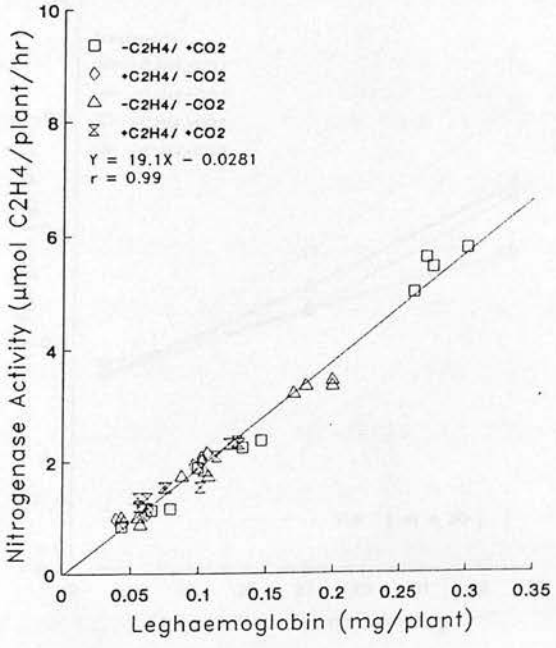


Fig. 4.57 Relationship between nitrogenase activity and leghaemoglobin content of pea nodules (closed system)

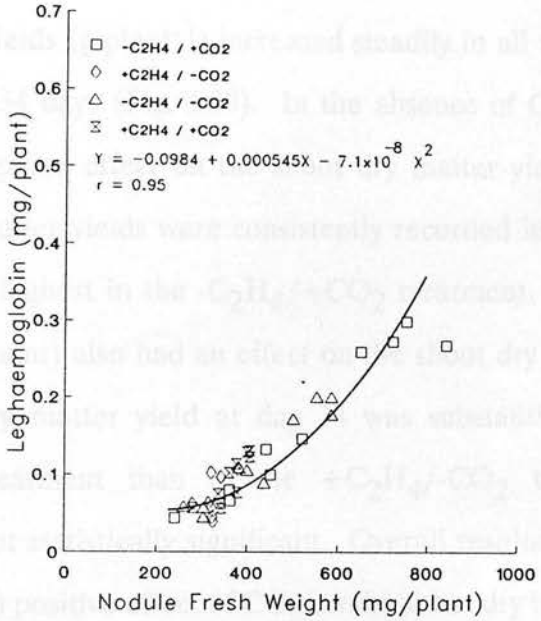


Fig. 4.58 Relationship between leghaemoglobin content and nodule fresh weight (pea, closed system)

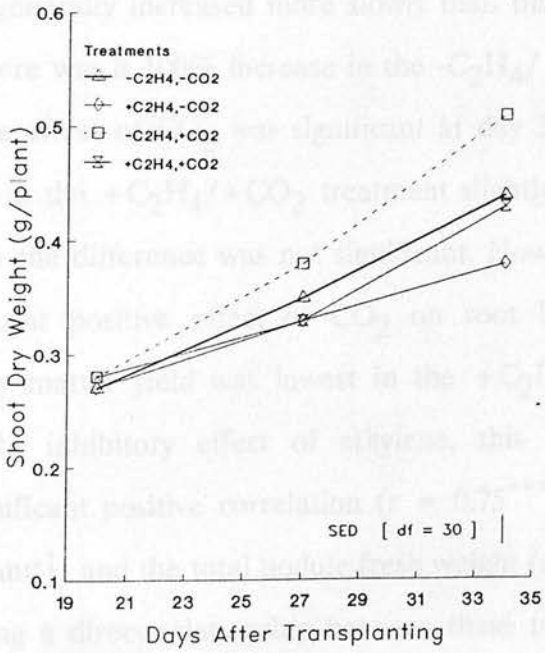


Fig. 4.59 Effect of C_2H_4 and CO_2 on shoot dry weight (pea, closed system)

4.3.2.5 Dry matter yield and nitrogen content of plants

(a) Shoot dry matter yield

Shoot dry matter yields (g plant^{-1}) increased steadily in all treatments over the period from 20 to 34 days (Fig. 4.59). In the absence of CO_2 ethylene had a very significant negative effect on the shoot dry matter yield at day 34. The lowest shoot dry matter yields were consistently recorded in the $+C_2H_4/-CO_2$ treatment and the highest in the $-C_2H_4/+CO_2$ treatment. Removing CO_2 ($-C_2H_4/-CO_2$ treatment) also had an effect on the shoot dry matter yield at day 34. The shoot dry matter yield at day 34 was substantially greater in the $+C_2H_4/+CO_2$ treatment than in the $+C_2H_4/-CO_2$ treatment, but the differences were not statistically significant. Overall results however showed a significant ($p<0.05$) positive effect of CO_2 on the shoot dry matter yield.

(b) Root dry matter yield

Root dry matter generally increased more slowly than that of the shoot (Fig. 4.60), although there was a 100% increase in the $-C_2H_4/+CO_2$ treatment. In that treatment the effect of CO_2 was significant at day 34, but although the presence of CO_2 in the $+C_2H_4/+CO_2$ treatment slightly increased root dry matter production the difference was not significant. However, overall results showed a significant positive effect of CO_2 on root biomass production. Although root dry matter yield was lowest in the $+C_2H_4/-CO_2$ treatment, suggesting a slight inhibitory effect of ethylene, this difference was not significant. A significant positive correlation ($r = 0.75^{***}$) between root dry matter yield (g plant^{-1}) and the total nodule fresh weight (mg plant^{-1}) was also obtained, indicating a direct relationship between these two parameters (Fig. 4.61).

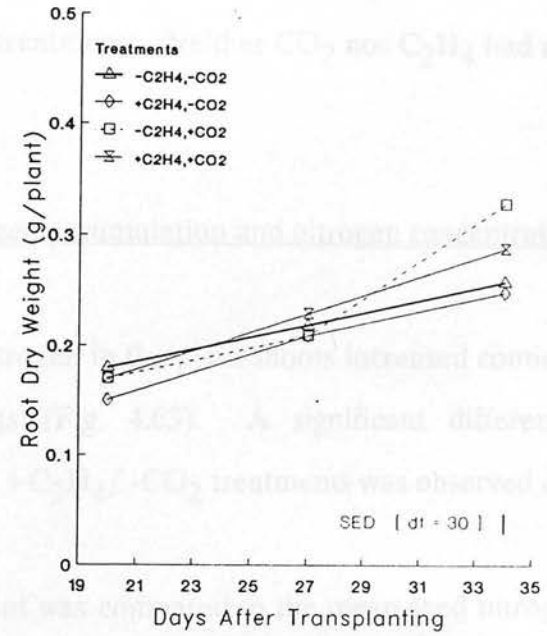


Fig. 4.60 Effect of C_2H_4 and CO_2 on root dry weight (pea, closed system)

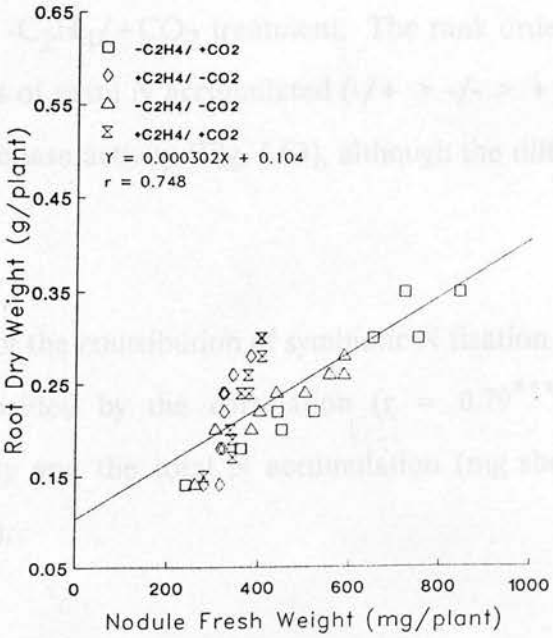


Fig. 4.61 Relationship between root dry weight and nodule fresh weight (pea, closed system)

(c) Primary root length

The length of the primary roots increased by about 50% over the period of observation in all treatments. Neither CO_2 nor C_2H_4 had any significant effect (Fig. 4.62).

(d) Total nitrogen accumulation and nitrogen concentration in shoots

The quantity of nitrogen in the plant shoots increased continuously over time in all the treatments (Fig. 4.63). A significant difference between the $-C_2H_4/+CO_2$ and $+C_2H_4/-CO_2$ treatments was observed at day 34.

When the N content was compared to the mean seed nitrogen content of 11.25 mg (the only source of available nitrogen at the initial stage of growth in these experiments) it was clear that a substantial input of atmospheric N must have occurred as a result of the pea-Rhizobium symbiosis in all treatments, but particularly in the $-C_2H_4/+CO_2$ treatment. The rank order of the treatments at 34 days in terms of extra N accumulated (-/+ > -/- > +/+ > +/-) was the same as for nitrogenase activity (Fig. 4.63), although the differences were much smaller.

Further evidence of the contribution of symbiotic N fixation to the N content of the shoots is provided by the correlation ($r = 0.79^{***}$) between nodule nitrogenase activity and the total N accumulation (mg $shoot^{-1}$) in the shoot biomass (Fig. 4.64).

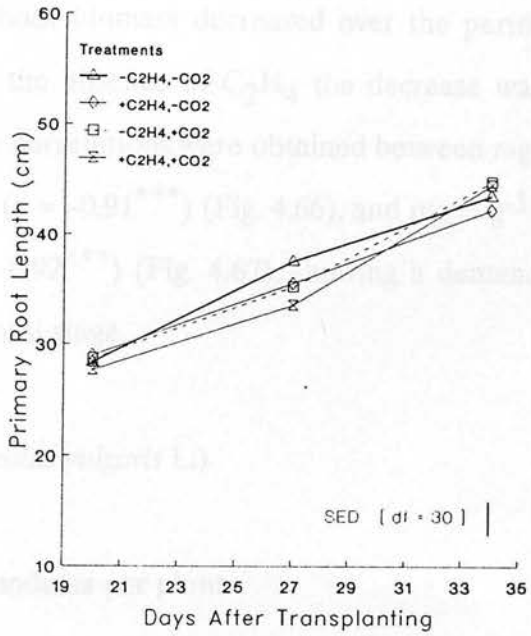


Fig. 4.62 Effect of C_2H_4 and CO_2 on length of primary roots of pea (closed system)

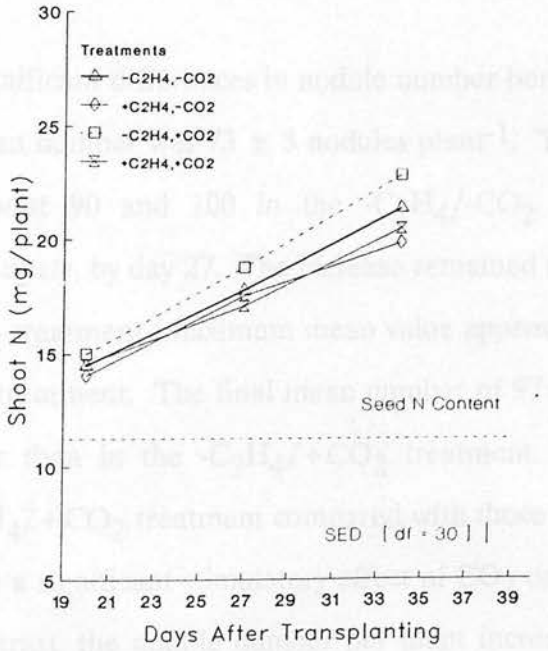


Fig. 4.63 Effect of C_2H_4 and CO_2 on total nitrogen content of pea shoots (closed system)

The concentration of nitrogen in the shoot (mg N g^{-1} shoot dry weight) was significantly affected by ethylene at day 20 ($p = 0.048$) and at day 34 ($p<0.001$) and by CO_2 ($p<0.01$) at day 34. In all treatments except $+C_2H_4/-CO_2$, the N concentration in shoot biomass decreased over the period of the experiment (Fig. 4.65), but in the absence of C_2H_4 the decrease was more rapid. Very significant negative correlations were obtained between mg N g^{-1} shoot biomass and shoot biomass ($r = -0.91^{***}$) (Fig. 4.66), and mg N g^{-1} and the total nodule fresh weight ($r = -0.92^{***}$) (Fig. 4.67), showing a demand for nitrogen at the nodule developmental stage.

4.3.3 Bean (*Phaseolus vulgaris* L.)

4.3.3.1 Number of nodules per plant

The effects of C_2H_4 and CO_2 on the nodulation of bean roots were similar to those observed with pea. The results obtained are presented in Fig. 4.68.

There were no significant differences in nodule number between the treatments at day 20; the mean number was 73 ± 3 nodules $plant^{-1}$. The values increased rapidly up to about 90 and 100 in the $-C_2H_4/-CO_2$ and $-C_2H_4/+CO_2$ treatments, respectively, by day 27. The increase remained rapid up to day 34 in the $-C_2H_4/+CO_2$ treatment (maximum mean value approx. 126) but slowed in the $-C_2H_4/-CO_2$ treatment. The final mean number of 97 nodules $plant^{-1}$ was significantly lower than in the $-C_2H_4/+CO_2$ treatment. The higher mean values in the $-C_2H_4/+CO_2$ treatment compared with those in the $-C_2H_4/-CO_2$ treatment indicate a significant stimulatory effect of CO_2 on nodule production at day 34. In contrast, the nodule number per plant increased only slightly in

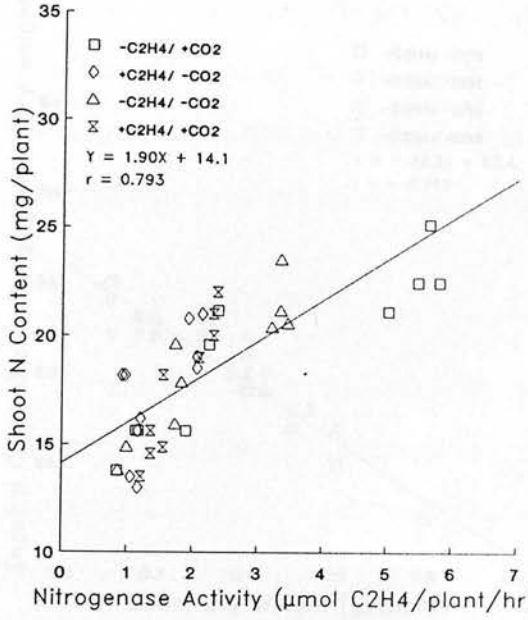


Fig. 4.64 Relationship between shoot nitrogen content and nitrogenase activity (pea, closed system)

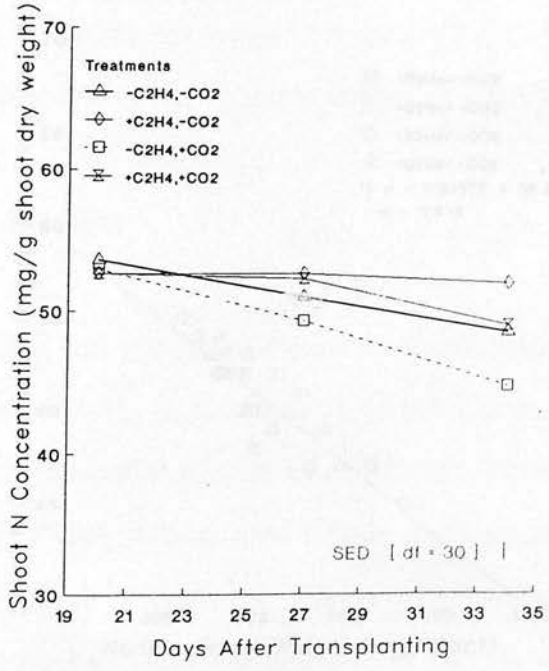


Fig. 4.65 Effect of C_2H_4 and CO_2 on nitrogen concentration of pea shoots (closed system)

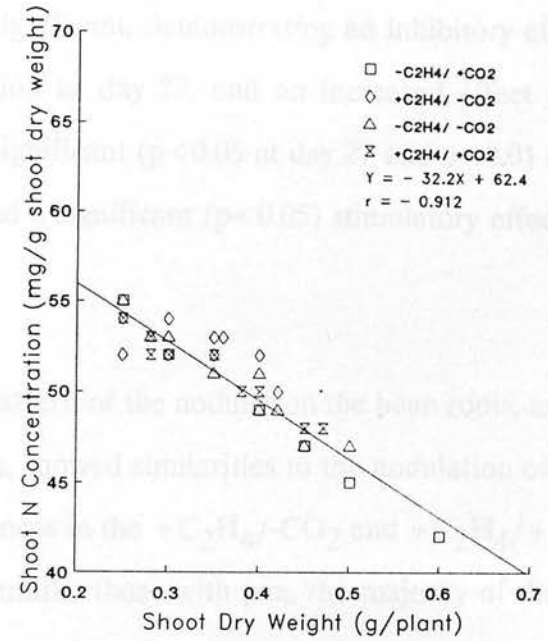


Fig. 4.66 Relationship between shoot nitrogen concentration and shoot dry weight (pea, closed system)

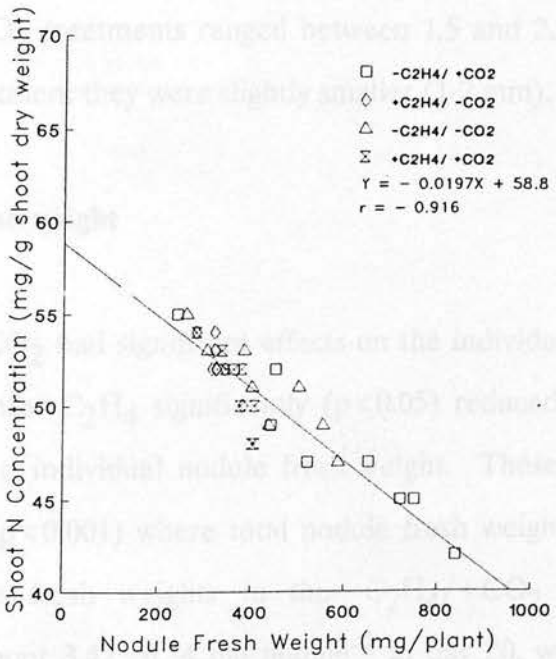


Fig. 4.67 Relationship between shoot nitrogen concentration and nodule fresh weight (pea, closed system)

the $+C_2H_4/+CO_2$ treatment, and remained fairly constant in the $+C_2H_4/-CO_2$ treatment up to day 27, decreasing thereafter to the lowest observed values of 82 and 72, respectively, at the final harvest. Differences between treatments were significant, demonstrating an inhibitory effect of C_2H_4 on the extent of nodulation at day 27, and an increased effect at day 34. Overall, results showed a significant ($p<0.05$ at day 27 and $p<0.01$ at day 34) inhibitory effect of C_2H_4 and a significant ($p<0.05$) stimulatory effect of CO_2 on nodule production.

The distribution pattern of the nodules on the bean roots, and the occurrence of clusters of nodules, showed similarities to the nodulation of pea, although there were some differences in the $+C_2H_4/-CO_2$ and $+C_2H_4/+CO_2$ treatments. In these treatments, unlike those with pea, the majority of the nodules (clustered or single) were located on the secondary laterals rather than on the primary roots. The greatest individual nodule sizes were recorded in the $+C_2H_4/-CO_2$ treatment (2-3.5 mm diameter). Individual nodule sizes in the $-C_2H_4/+CO_2$ and $+C_2H_4/+CO_2$ treatments ranged between 1.5 and 2.5 mm, while in the $-C_2H_4/-CO_2$ treatment they were slightly smaller (1-2 mm).

4.3.3.2 Nodule fresh weight

Both C_2H_4 and CO_2 had significant effects on the individual and total nodule fresh weight. Whilst C_2H_4 significantly ($p<0.05$) reduced, CO_2 significantly ($p<0.05$) increased individual nodule fresh weight. These effects were even more significant ($p<0.001$) where total nodule fresh weights were concerned. Individual nodule fresh weights in the $-C_2H_4/+CO_2$ treatment rapidly increased from about 3.42 ± 0.24 mg nodule^{-1} at day 20, when there were no

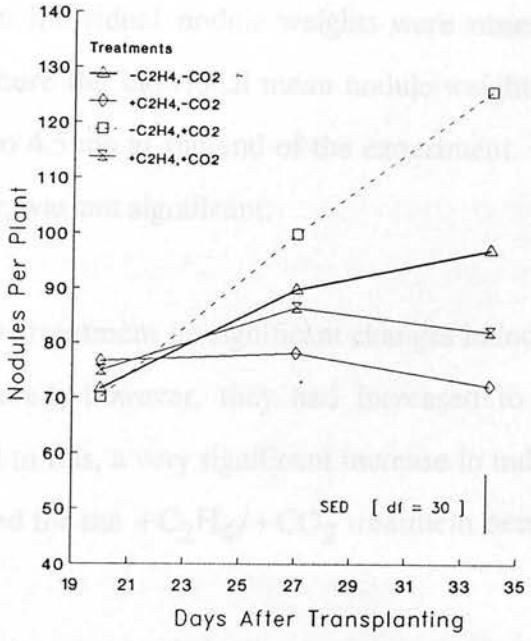


Fig. 4.68 Effect of C_2H_4 and CO_2 on nodulation of bean (closed system)

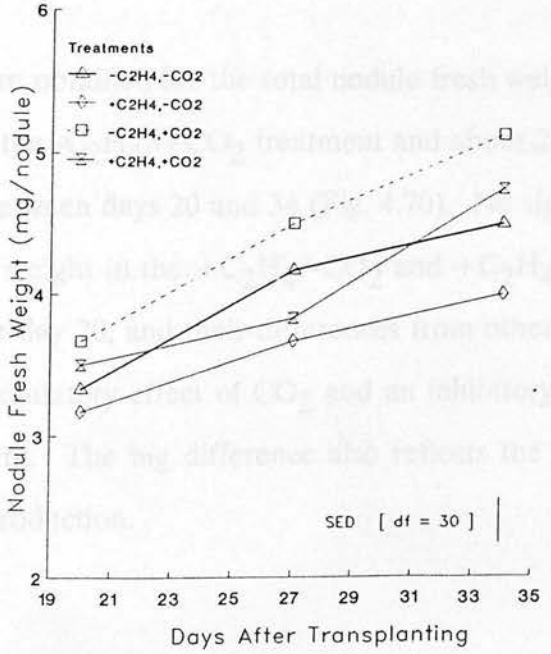


Fig. 4.69 Effect of C_2H_4 and CO_2 on fresh weight of bean nodules (closed system)

significant differences between the treatments, to about 4.5 mg by day 27, and a maximum of 5.13 mg at the end of the experiment (day 34) (Fig. 4.69).

Similar increases in individual nodule weights were observed in the $-C_2H_4/-CO_2$ treatment, where the individual mean nodule weight rose rapidly to 4.17 mg by day 27 and to 4.5 mg at the end of the experiment. The increase in the last 7 days, however, was not significant.

In the $+C_2H_4/-CO_2$ treatment no significant changes in individual nodule fresh weights were observed; however, they had increased to 4 mg by the final harvest. Compared to this, a very significant increase in individual nodule fresh weights was recorded for the $+C_2H_4/+CO_2$ treatment between 27 and 34 days of growth.

These observations clearly demonstrate that C_2H_4 had a significant inhibitory effect on nodule development, whereas carbon dioxide had a stimulatory effect.

Similar results were obtained for the total nodule fresh weight which increased almost 2½-fold in the $-C_2H_4/+CO_2$ treatment and about 2-fold in the $-C_2H_4/-CO_2$ treatment between days 20 and 34 (Fig. 4.70). No significant increase in total nodule fresh weight in the $+C_2H_4/-CO_2$ and $+C_2H_4/+CO_2$ treatments was observed after day 20, and their differences from other treatments show a very significant stimulatory effect of CO_2 and an inhibitory effect of C_2H_4 on nodule development. The big difference also reflects the inhibitory effect of C_2H_4 on nodule production.

4.3.3.3 Nodule nitrogenase activity

Nodule nitrogenase activity was measured by acetylene reduction assay, as described in Section 3.5. The results obtained, presented in Fig. 4.71, show an almost 250% increase in activity in the $-C_2H_4/+CO_2$ treatment, from 0.84 to 2.91 μmol C_2H_4 plant^{-1} hr^{-1}, between days 20 and 34. Compared to this, a 170% increase in nitrogenase activity was observed in the $-C_2H_4/-CO_2$ treatment, from 0.73 to 1.98 μmol C_2H_4 plant^{-1} hr^{-1} over the same time period. The activity in the nodules in the $+C_2H_4/-CO_2$ treatment, although slightly greater at day 20 compared to that in the $-C_2H_4/+CO_2$ and $-C_2H_4/-CO_2$ treatments, remained constant up to day 27 and then decreased to 0.84 μmol C_2H_4 plant^{-1} hr^{-1} by the final harvest (day 34). In the $+C_2H_4/+CO_2$ treatment, however, there was a significant increase in the nitrogenase activity up to day 27 but it had decreased by day 34. Although there were no significant differences between the values obtained with these two treatments, 50% greater activity in the nodules in the $+C_2H_4/+CO_2$ treatment was observed, showing that the presence of CO_2 could partly counteract the inhibitory effect of C_2H_4 on nodule nitrogenase activity. This was reflected in the statistical analysis, where a significant interaction ($p<0.05$) between these two gases was observed. Results as a whole show that C_2H_4 very significantly suppressed ($p<0.001$) the nodule nitrogenase activity, while CO_2 stimulated the activity to the same extent at days 27 and 34.

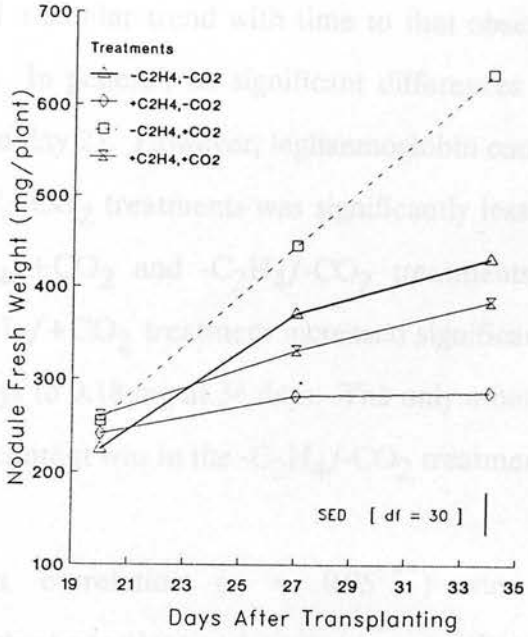


Fig. 4.70 Effect of C_2H_4 and CO_2 on total fresh weight of bean nodules (closed system)

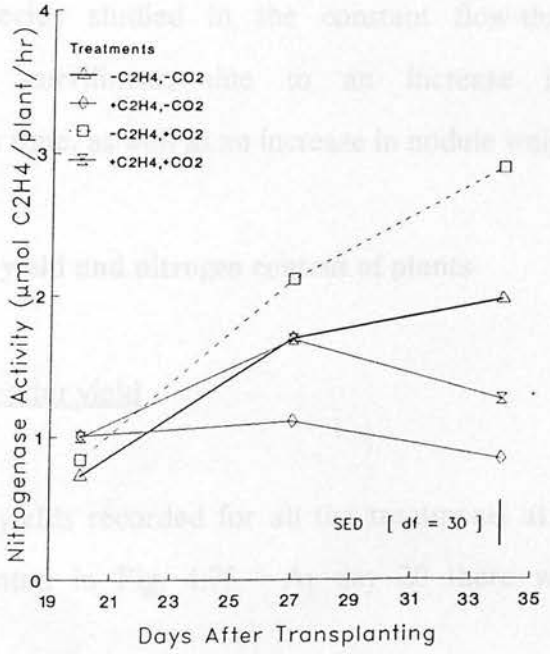


Fig. 4.71 Effect of C_2H_4 and CO_2 on nitrogenase activity of bean nodules (closed system)

4.3.3.4 Nodule leghaemoglobin content

Leghaemoglobin content per plant measured by the method described in Section 3.3 showed a similar trend with time to that observed for nitrogenase activity (Fig. 4.72). In general, no significant differences between treatments were observed up to day 27. However, leghaemoglobin content in the $+C_2H_4/-CO_2$ and $+C_2H_4/+CO_2$ treatments was significantly less ($p<0.05$) by day 34 than in the $-C_2H_4/+CO_2$ and $-C_2H_4/-CO_2$ treatments. Leghaemoglobin content in the $-C_2H_4/+CO_2$ treatment increased significantly from about 0.04 mg plant^{-1} at 20 days to 0.18 mg at 34 days. The only other significant increase in leghaemoglobin content was in the $-C_2H_4/-CO_2$ treatment.

A very significant correlation ($r = 0.95^{***}$) was obtained between leghaemoglobin content per plant and nitrogenase activity per plant (Fig. 4.73). A very similar correlation ($r = 0.94^{***}$) between leghaemoglobin content and the total nodule fresh weight was also obtained (Fig. 4.74). As for pea, and as for all three species studied in the constant flow-through system, this relationship was curvilinear, due to an increase in leghaemoglobin concentration with time, as well as an increase in nodule weight.

4.3.3.5 Dry matter yield and nitrogen content of plants

(a) Shoot dry matter yield

Shoot dry matter yields recorded for all the treatments at different stages of growth are presented in Fig. 4.75. At day 20 there were no significant

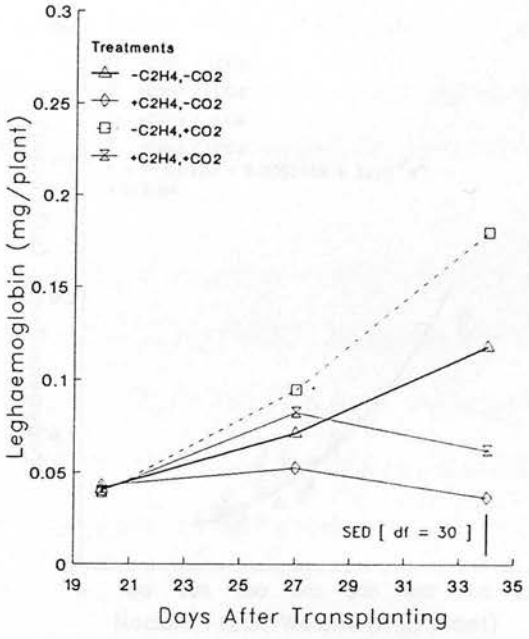


Fig. 4.72 Effect of C_2H_4 and CO_2 on leghaemoglobin content of bean nodules (closed system)

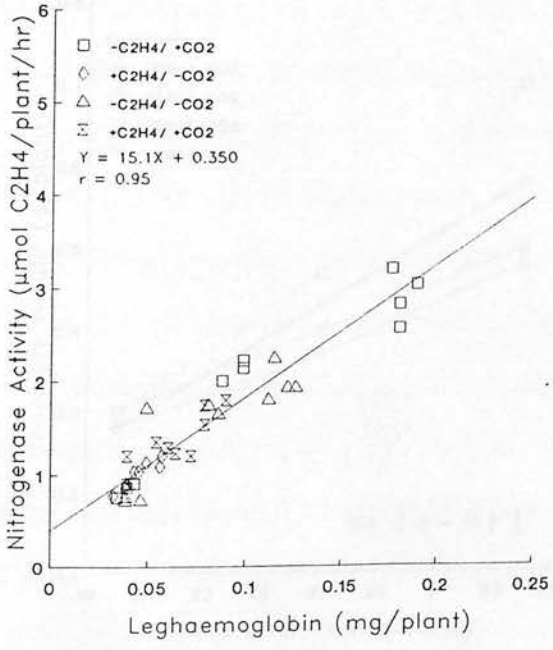


Fig. 4.73 Relationship between nitrogenase activity and leghaemoglobin content of bean nodules (closed system)

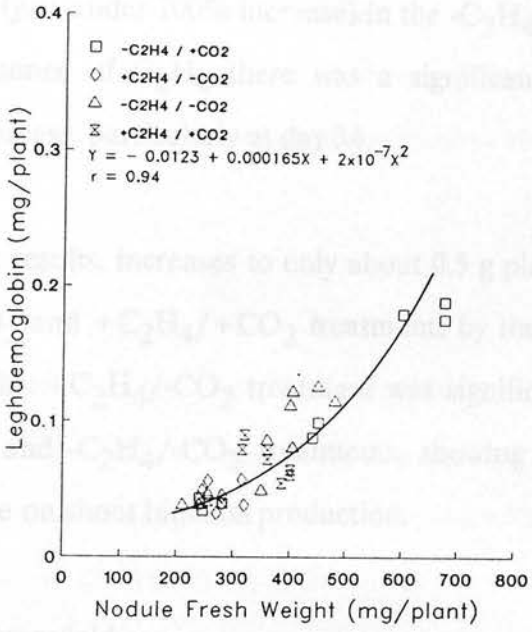


Fig. 4.74 Relationship between leghaemoglobin content and nodule fresh weight (bean, closed system)

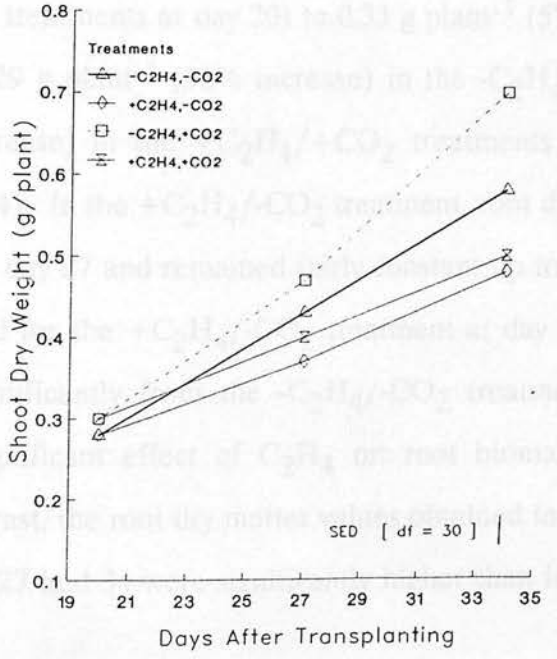


Fig. 4.75 Effect of C_2H_4 and CO_2 on shoot dry weight (bean, closed system)

differences in shoot dry matter yields between treatments when a mean weight of 0.29 ± 0.01 g plant^{-1} shoot dry matter was found for all the treatments. This increased very rapidly to 0.70 g plant^{-1} (a 140% increase) in the $-C_2H_4/+CO_2$ treatment and 0.57 (just under 100% increase) in the $-C_2H_4/-CO_2$ treatment by day 34. In the absence of C_2H_4 there was a significant effect of CO_2 in increasing shoot biomass, particularly at day 34.

In contrast to these results, increases to only about 0.5 g plant^{-1} were recorded in the $+C_2H_4/-CO_2$ and $+C_2H_4/+CO_2$ treatments by the final harvest. The value obtained in the $+C_2H_4/-CO_2$ treatment was significantly lower than in the $-C_2H_4/+CO_2$ and $-C_2H_4/-CO_2$ treatments, showing a significant effect ($p < 0.01$) of ethylene on shoot biomass production.

(b) Root dry matter yield

Root dry matter increased significantly from about 0.21 ± 0.01 g plant^{-1} (the average of all the treatments at day 20) to 0.33 g plant^{-1} (57% increase) in the $-C_2H_4/+CO_2$, 0.29 g plant^{-1} (38% increase) in the $-C_2H_4/-CO_2$, and 0.30 g plant^{-1} (43% increase) in the $+C_2H_4/+CO_2$ treatments at the end of the experiment (day 34). In the $+C_2H_4/-CO_2$ treatment, root dry matter increased significantly up to day 27 and remained fairly constant up to day 34. However, the value obtained for the $+C_2H_4/-CO_2$ treatment at day 34 (0.28 g plant^{-1}) did not differ significantly from the $-C_2H_4/-CO_2$ treatment, and therefore statistically no significant effect of C_2H_4 on root biomass production was observed. In contrast, the root dry matter values obtained in the $-C_2H_4/+CO_2$ treatment at days 27 and 34 were significantly higher than in the $-C_2H_4/-CO_2$

treatment demonstrating a stimulatory effect of CO_2 on root dry matter production (Fig. 4.76).

A good correlation ($r = 0.77^{***}$) between root dry matter yield and the total nodule fresh weight was also obtained, which shows that the increase in nodule biomass somehow related to the root biomass production (Fig. 4.77).

(c) Primary root length

Neither CO_2 nor C_2H_4 had any statistically significant effect on the primary root length of bean (Fig. 4.78), although a slightly higher mean root length (44 cm) was observed in the $-C_2H_4/+CO_2$ treatment than in the $+C_2H_4/-CO_2$ treatment (38 cm) at the end of the experiment. Primary root lengths in all the treatments significantly increased from day 20 to day 34, when the final harvest was made.

(d) Total nitrogen accumulation and nitrogen concentration in shoots

There was a significant effect ($p<0.01$) of C_2H_4, and of CO_2 ($p<0.05$), on the shoot N concentration (mg g^{-1} shoot dry weight). The presence of carbon dioxide caused a rapid decrease in shoot N concentration concurrent with increasing nodule mass and shoot dry matter, whereas in the presence of C_2H_4 this decrease was much slower (Fig. 4.79). The decrease in mg N g^{-1} shoot dry weight was significant in the $-C_2H_4/+CO_2$ treatment, where it decreased from about 35 to 29 mg g^{-1} between days 20 and 34; the decrease in the last 7 days was more rapid than in the previous period. In the $-C_2H_4/-CO_2$ treatment the shoot N concentration of 38 mg g^{-1} decreased to 32 mg g^{-1} by the last harvest.

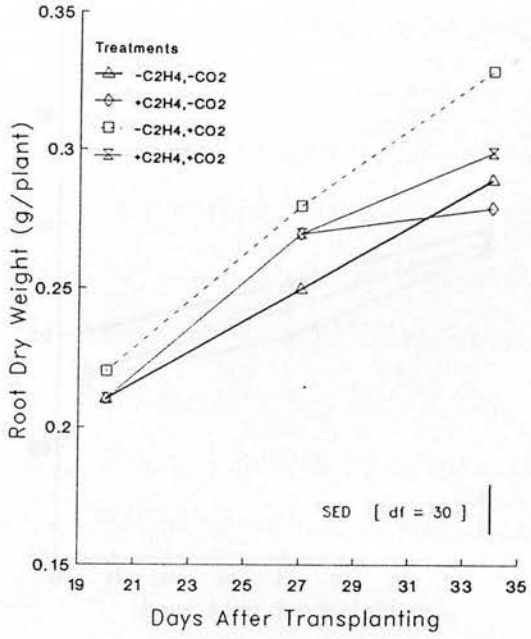


Fig. 4.76 Effect of C_2H_4 and CO_2 on root dry weight (bean, closed system)

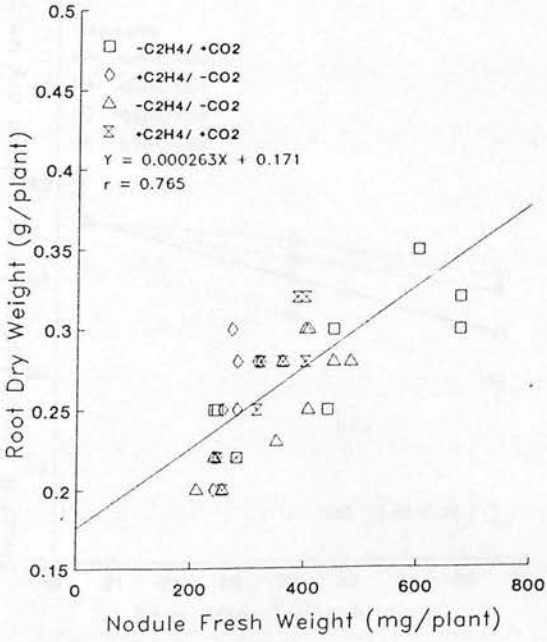


Fig. 4.77 Relationship between root dry weight and nodule fresh weight (bean, closed system)

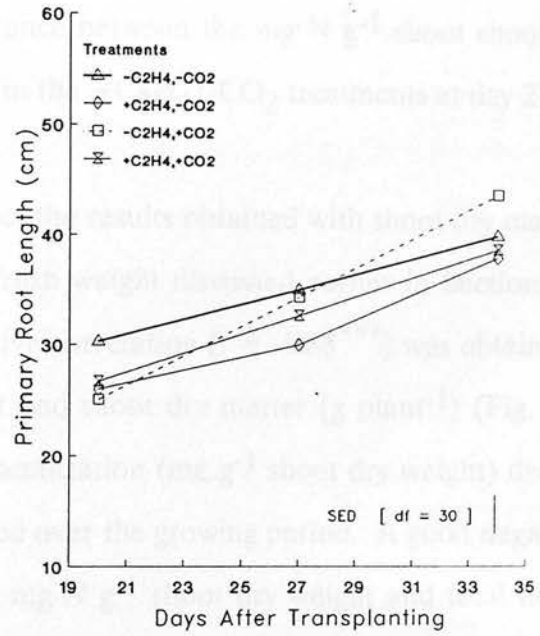


Fig. 4.78 Effect of C_2H_4 and CO_2 on length of primary roots of bean (closed system)

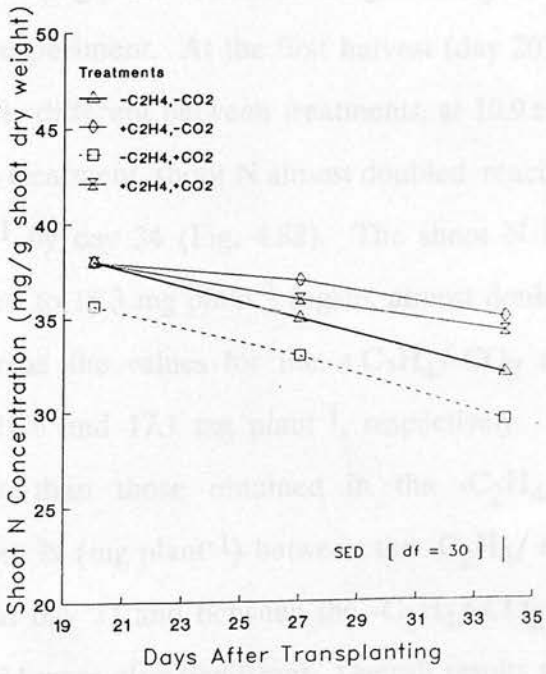


Fig. 4.79 Effect of C_2H_4 and CO_2 on nitrogen concentration of bean shoots (closed system)

In comparison, the concentration in the $+C_2H_4/-CO_2$ and $+C_2H_4/+CO_2$ treatments decreased from 38 to 35 and 34 mg g^{-1}, being always significantly higher than in the $-C_2H_4/+CO_2$ and $-C_2H_4/-CO_2$ treatments. There was also a significant difference between the mg N g^{-1} shoot shoot dry weight in the $-C_2H_4/+CO_2$ and in the $+C_2H_4/-CO_2$ treatments at day 27.

These results reflect the results obtained with shoot dry matter yield (g $plant^{-1}$) and total nodule fresh weight discussed earlier in Sections 4.3.3.5 and 4.3.3.2. Also, a good negative correlation ($r = -0.88^{***}$) was obtained between mg N g^{-1} shoot dry weight and shoot dry matter (g $plant^{-1}$) (Fig. 4.80), reflecting the fact that the N concentration (mg g^{-1} shoot dry weight) decreased as the shoot dry matter increased over the growing period. A good negative correlation ($r = -0.74^{***}$) between mg N g^{-1} shoot dry weight and total nodule mass was also obtained (Fig. 4.81).

The shoot N content (mg $plant^{-1}$) increased significantly in all the treatments up to the end of the experiment. At the first harvest (day 20), the shoot N value was not significantly different between treatments, at 10.9 ± 0.60 mg $plant^{-1}$. In the $-C_2H_4/+CO_2$ treatment, shoot N almost doubled, reaching its highest value of 20.5 mg $plant^{-1}$ by day 34 (Fig. 4.82). The shoot N in the $-C_2H_4/-CO_2$ treatment increased to 18.3 mg $plant^{-1}$ (again, almost doubling) over the same time period, whereas the values for the $+C_2H_4/-CO_2$ and $+C_2H_4/+CO_2$ treatments were 16.6 and 17.1 mg $plant^{-1}$, respectively. These values were significantly lower than those obtained in the $-C_2H_4/+CO_2$ treatment. Differences in shoot N (mg $plant^{-1}$) between the $-C_2H_4/+CO_2$ and $+C_2H_4/-CO_2$ treatments at day 27 and between the $-C_2H_4/-CO_2$ and $-C_2H_4/+CO_2$ treatments at day 34 were also significant. Overall results show that both CO_2

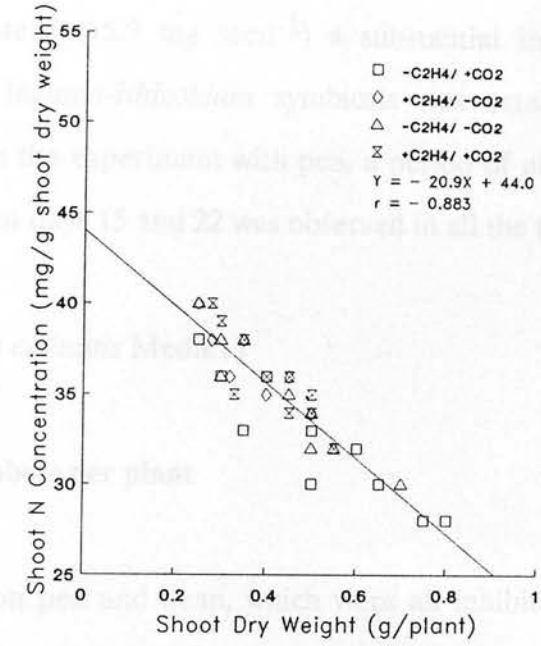


Fig. 4.80 Relationship between shoot nitrogen concentration and shoot dry weight (bean, closed system)

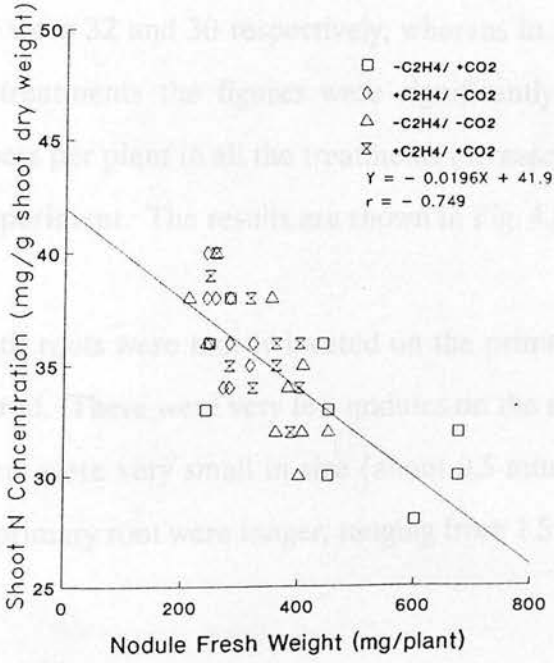


Fig. 4.81 Relationship between shoot nitrogen concentration and nodule fresh weight (bean, closed system)

and C_2H_4 had a significant ($p<0.05$) effect on the shoot N (mg plant^{-1}). A good positive correlation ($r = 0.70^{***}$) between nodule nitrogenase activity and the shoot N (mg plant^{-1}) was also obtained (Fig. 4.83). When compared to seed nitrogen content (15.9 mg seed^{-1}) a substantial input of atmospheric nitrogen through legume-*Rhizobium* symbiosis was established (Fig. 4.82). However, unlike in the experiment with pea, a period of nitrogen starvation in bean plants between days 15 and 22 was observed in all the treatments.

4.3.4 Lentil (*Lens culinaris* Medik.)

4.3.4.1 Nodule numbers per plant

Unlike its effects on pea and bean, which were all inhibitory, ethylene had a significant ($p<0.05$) stimulatory effect on nodule production in lentil at day 20; however this did not persist for the remainder of the experimental period. Nodule numbers per plant in the $+C_2H_4/-CO_2$ and $+C_2H_4/+CO_2$ treatments recorded at day 20 were 32 and 30 respectively, whereas in the $-C_2H_4/+CO_2$ and $-C_2H_4/-CO_2$ treatments the figures were significantly lower, about 24. Mean nodule numbers per plant in all the treatments increased to about 40 ± 2.5 by the end of the experiment. The results are shown in Fig. 4.84.

The nodules on lentil roots were mostly located on the primary root and many of them were clustered. There were very few nodules on the secondary laterals; the few that did form were very small in size (about 0.5 mm). Single nodules that formed on the primary root were longer, ranging from 1.5 to 2.5 mm.

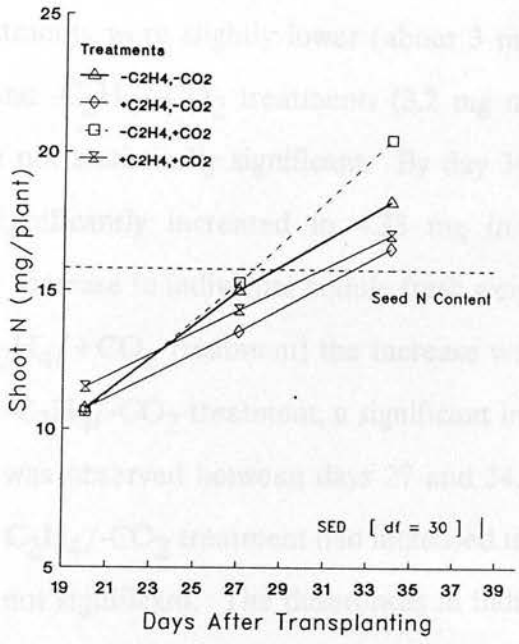


Fig. 4.82 Effect of C_2H_4 and CO_2 on total nitrogen content of bean shoots (closed system)

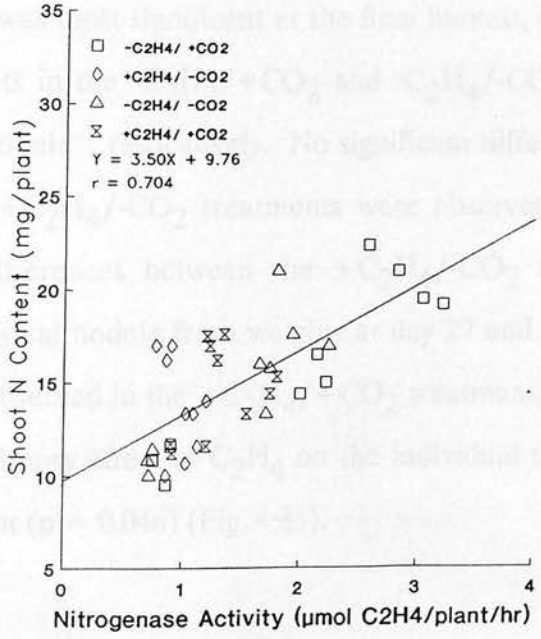


Fig. 4.83 Relationship between shoot nitrogen content and nitrogenase activity (bean, closed system)

4.3.4.2 Nodule fresh weight

Individual nodule fresh weights at day 20 in the $+C_2H_4/-CO_2$ and $+C_2H_4/+CO_2$ treatments were slightly lower (about 3 mg nodule^{-1}) than in the $-C_2H_4/+CO_2$ and $-C_2H_4/-CO_2$ treatments (3.2 mg nodule^{-1}). However the differences were not statistically significant. By day 34, individual nodule fresh weight had significantly increased to 4.33 mg in the $-C_2H_4/+CO_2$ treatment. A similar increase in individual nodule fresh weight (to 4.05 mg) was observed in the $+C_2H_4/+CO_2$ treatment; the increase was particularly rapid up to day 27. In the $-C_2H_4/-CO_2$ treatment, a significant increase in individual nodule fresh weight was observed between days 27 and 34. Individual nodule fresh weight in the $+C_2H_4/-CO_2$ treatment had increased to 3.65 mg by the last harvest but this was not significant. The differences in individual nodule fresh weights between treatments show that CO_2 had a significant ($p<0.05$) stimulatory effect.

The effect of CO_2 was most significant at the final harvest, when the individual nodule fresh weights in the $-C_2H_4/+CO_2$ and $-C_2H_4/-CO_2$ treatments were 4.33 and 3.85 mg nodule^{-1} respectively. No significant differences between the $-C_2H_4/-CO_2$ and $+C_2H_4/-CO_2$ treatments were observed. However, there were significant differences between the $+C_2H_4/-CO_2$ and $-C_2H_4/+CO_2$ treatments in individual nodule fresh weights at day 27 and 34. Higher nodule weights were also observed in the $+C_2H_4/+CO_2$ treatment. These results as a whole show an inhibitory effect of C_2H_4 on the individual nodule fresh weight that is just significant ($p = 0.046$) (Fig. 4.85).

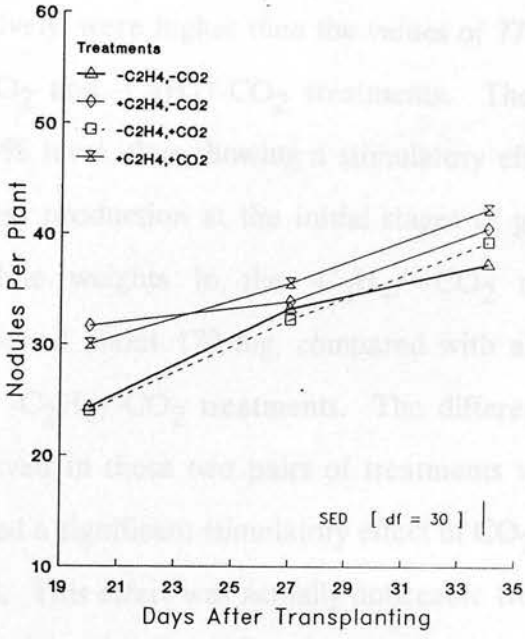


Fig. 4.84 Effect of C_2H_4 and CO_2 on nodulation of lentil (closed system)

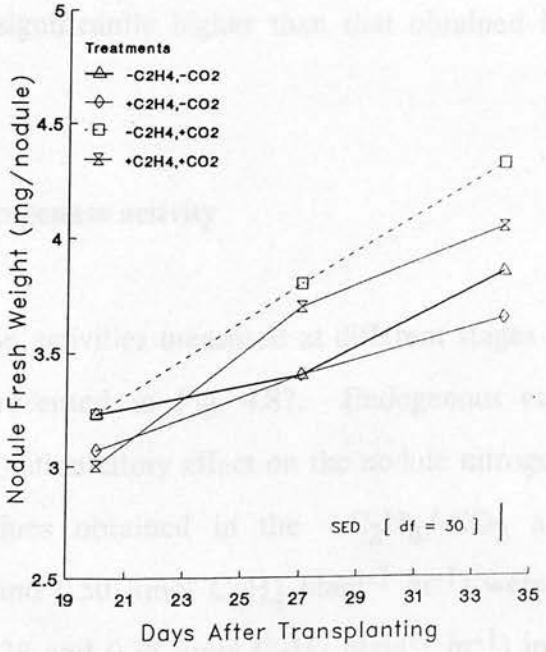


Fig. 4.85 Effect of C_2H_4 and CO_2 on fresh weight of lentil nodules (closed system)

Total nodule fresh weight values in all the treatments increased significantly up to the final harvest (Fig. 4.86). At day 20 the mean total nodule fresh weight values of 96 and 89 mg, obtained in the $+C_2H_4/-CO_2$ and $+C_2H_4/+CO_2$ treatments, respectively, were higher than the values of 77 and 78 mg obtained in the $-C_2H_4/+CO_2$ and $-C_2H_4/-CO_2$ treatments. These differences were significant at the 5% level, thus showing a stimulatory effect of C_2H_4 on the total nodule biomass production at the initial stages of growth. At the final harvest, total nodule weights in the $-C_2H_4/+CO_2$ and $+C_2H_4/+CO_2$ treatments had reached about 170 mg, compared with about 145 mg in the $+C_2H_4/-CO_2$ and $-C_2H_4/-CO_2$ treatments. The differences in total nodule fresh weights observed in these two pairs of treatments were very significant ($p<0.01$) and showed a significant stimulatory effect of CO_2 on the total nodule biomass production. This effect was actually noticeable from day 27, when the total nodule fresh weight values obtained in the $-C_2H_4/+CO_2$, $+C_2H_4/-CO_2$, $C_2H_4/-CO_2$ and $+C_2H_4/+CO_2$ treatments were 121, 115, 112 and 131 mg, respectively, and the values obtained in the $-C_2H_4/+CO_2$ and $+C_2H_4/+CO_2$ treatments were significantly higher than that obtained in the $-C_2H_4/-CO_2$ treatment.

4.3.4.3 Nodule nitrogenase activity

Nodule nitrogenase activities measured at different stages of growth in all the treatments are presented in Fig. 4.87. Endogenous ethylene had a very signifcant ($p<0.01$) stimulatory effect on the nodule nitrogenase activity at day 20, when the values obtained in the $+C_2H_4/-CO_2$ and $+C_2H_4/+CO_2$ treatments (0.55 and 0.50 μmol C_2H_4 plant^{-1} hr^{-1}) were much higher than those obtained (0.38 and 0.36 μmol C_2H_4 plant^{-1} hr^{-1}) in the $-C_2H_4/+CO_2$

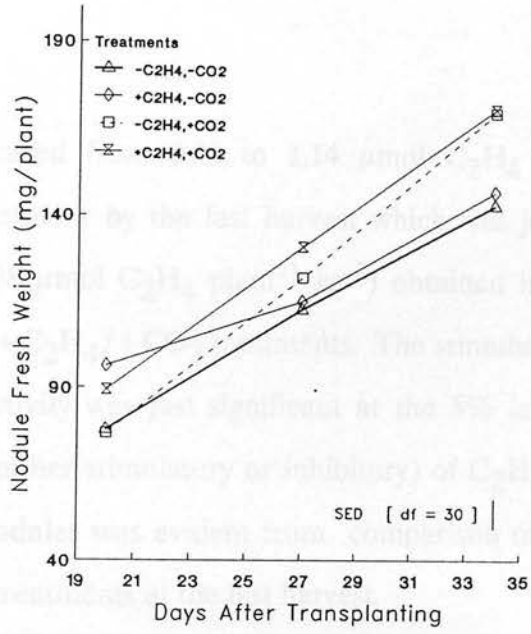


Fig. 4.86 Effect of C_2H_4 and CO_2 on total fresh weight of lentil nodules (closed system)

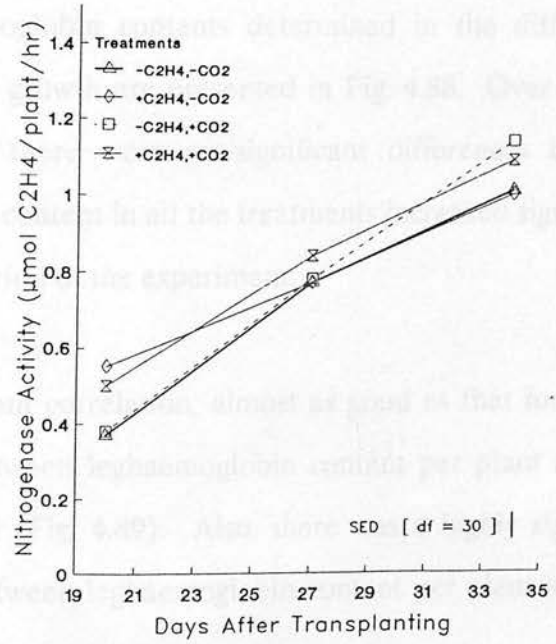


Fig. 4.87 Effect of C_2H_4 and CO_2 on nitrogenase activity of lentil nodules (closed system)

and -C_2H_4/-CO_2 treatments. Nodule nitrogenase activity in all the treatments increased significantly up to the last harvest, although no significant differences in the nodule nitrogenase activity between treatments were observed at days 27 and 34.

The activity increased from 0.38 to 1.14 μmol C_2H_4 plant^{-1} hr^{-1} in the -C_2H_4/+CO_2 treatment by the last harvest which was just above the values (1.00, 1.01 and 1.08 μmol C_2H_4 plant^{-1} hr^{-1}) obtained in the +C_2H_4/-CO_2, -C_2H_4/-CO_2 and +C_2H_4/+CO_2 treatments. The stimulatory effect of CO_2 on the nitrogenase activity was just significant at the 5% level. In contrast, no significant effect (either stimulatory or inhibitory) of C_2H_4 on the nitrogenase activity of lentil nodules was evident from comparison of the +C_2H_4/-CO_2 and -C_2H_4/-CO_2 treatments at the last harvest.

4.3.4.4 Nodule leghaemoglobin content

Nodule leghaemoglobin contents determined in the different treatments at various stages of growth are presented in Fig. 4.88. Over the whole period of the experiment, there were no significant differences between treatments. Leghaemoglobin content in all the treatments increased significantly (about 2½-fold) over the period of the experiment.

A highly significant correlation, almost as good as that for pea ($r = 0.98^{***}$), was obtained between leghaemoglobin content per plant and the nitrogenase activity per plant (Fig. 4.89). Also, there was a highly significant correlation ($r = 0.97^{***}$) between leghaemoglobin content per plant and the nodule fresh

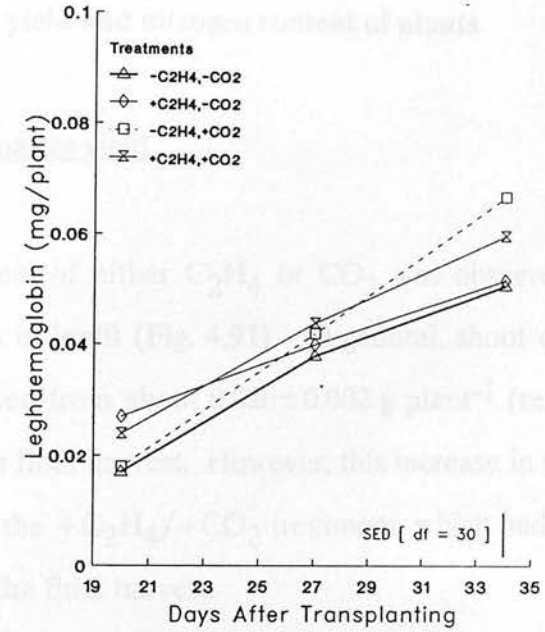


Fig. 4.88 Effect of C_2H_4 and CO_2 on leghaemoglobin content of lentil nodules (closed system)

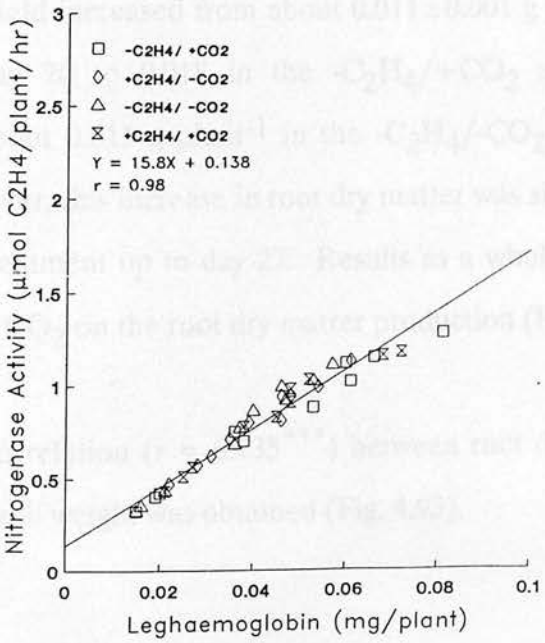


Fig. 4.89 Relationship between nitrogenase activity and leghaemoglobin content of lentil nodules (closed system)

weight (mg plant^{-1}) (Fig. 4.90). As in the other experiments, this relationship was curvilinear.

4.3.4.5 Dry matter yield and nitrogen content of plants

(a) Shoot dry matter yield

No significant effect of either C_2H_4 or CO_2 was observed on the shoot dry matter production in lentil (Fig. 4.91). In general, shoot dry matter in all the treatments increased from about 0.026 ± 0.002 g plant^{-1} (recorded at day 20) to 0.043 ± 0.003 at the final harvest. However, this increase in shoot dry matter was significant only in the $+C_2H_4/+CO_2$ treatment, which had the highest value of 0.047 g plant^{-1} at the final harvest.

(b) Root dry matter yield

Root dry matter yield increased from about 0.011 ± 0.001 g plant^{-1} (mean of all treatments) at day 20 to 0.018 in the $-C_2H_4/+CO_2$ and $+C_2H_4/+CO_2$ treatments and about 0.015 g plant^{-1} in the $-C_2H_4/-CO_2$ and $+C_2H_4/-CO_2$ treatments. However, this increase in root dry matter was significant only in the $+C_2H_4/+CO_2$ treatment up to day 27. Results as a whole show a significant ($p < 0.05$) effect of CO_2 on the root dry matter production (Fig. 4.92).

A good positive correlation ($r = 0.735^{***}$) between root dry matter yield and the total nodule fresh weight was obtained (Fig. 4.93).

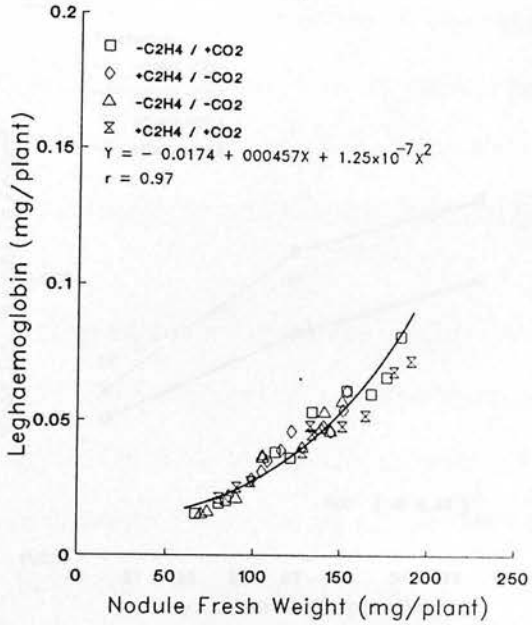


Fig. 4.90 Relationship between leghaemoglobin content and nodule fresh weight (lentil, closed system)

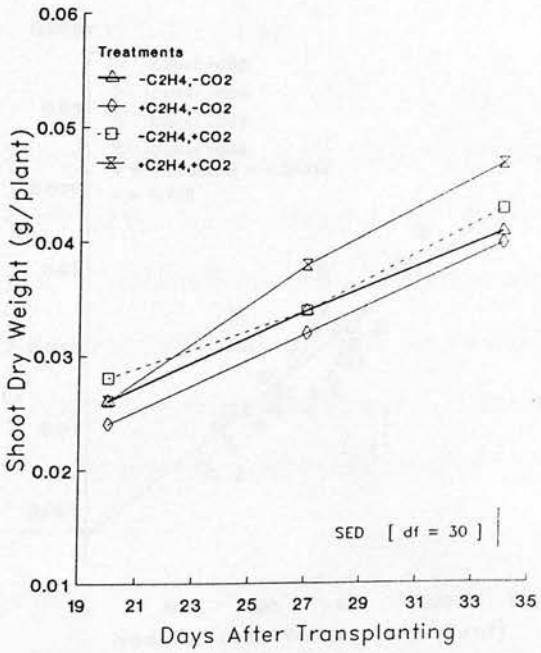


Fig. 4.91 Effect of C_2H_4 and CO_2 on shoot dry weight (lentil, closed system)

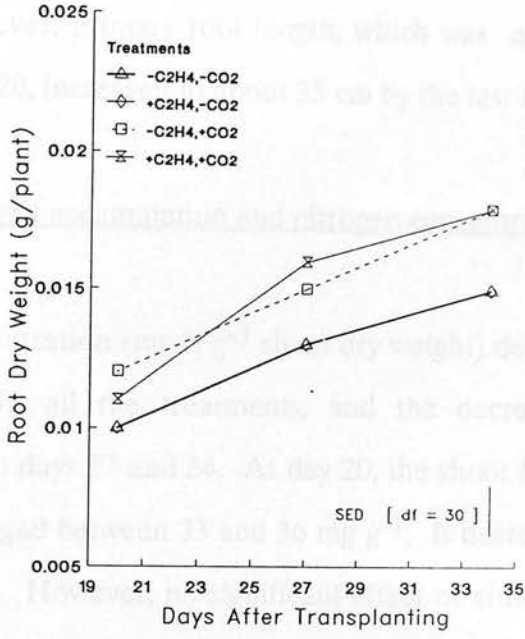


Fig. 4.92 Effect of C_2H_4 and CO_2 on root dry weight (lentil, closed system)

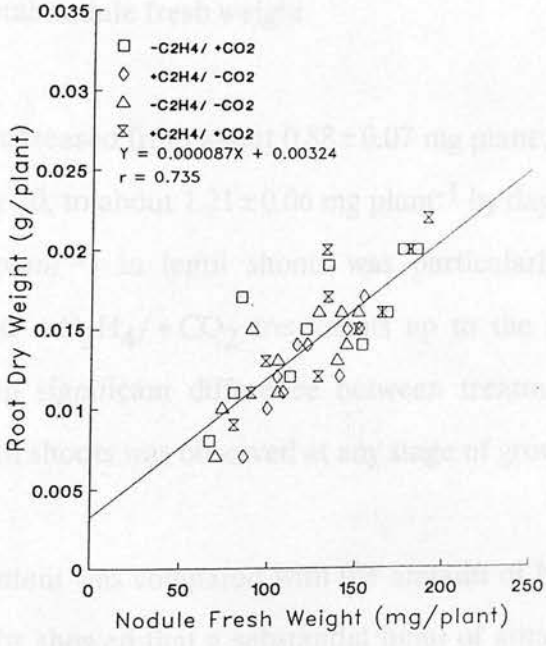


Fig. 4.93 Relationship between root dry weight and nodule fresh weight (lentil, closed system)

(c) Primary root length

No significant effect of either CO_2 or C_2H_4 was observed in any of the treatments. However, primary root length, which was about 15 cm in all the treatments at day 20, increased to about 35 cm by the last harvest (Fig. 4.94).

(d) Total nitrogen accumulation and nitrogen concentration in shoots

The shoot N concentration (mg N g^{-1} shoot dry weight) decreased steadily up to the last harvest in all the treatments, and the decrease was particularly significant between days 27 and 34. At day 20, the shoot N concentration in all the treatments ranged between 33 and 36 mg g^{-1}. It decreased to 27-30 mg g^{-1} by the last harvest. However, no significant effect of either C_2H_4 or CO_2 was observed (Fig. 4.95). There was a strong negative correlation ($r = -0.88^{***}$) between mg N g^{-1} shoot dry weight and shoot biomass production (Fig. 4.96). The mg N g^{-1} shoot dry weight was also negatively correlated ($r = -0.845^{***}$) (Fig. 4.97) with total nodule fresh weight.

Shoot N content increased from about 0.88 ± 0.07 mg $plant^{-1}$, (average of all the treatments) at day 20, to about 1.21 ± 0.06 mg $plant^{-1}$ by day 34. The increase in N content (mg $plant^{-1}$) in lentil shoots was particularly significant in the $+C_2H_4/-CO_2$ and $+C_2H_4/+CO_2$ treatments up to the second harvest (day 27). However, no significant difference between treatments in the total N content of the lentil shoots was observed at any stage of growth (Fig. 4.98).

When shoot N content was compared with the amount of N initially present in the seed, the results showed that a substantial input of atmospheric nitrogen to

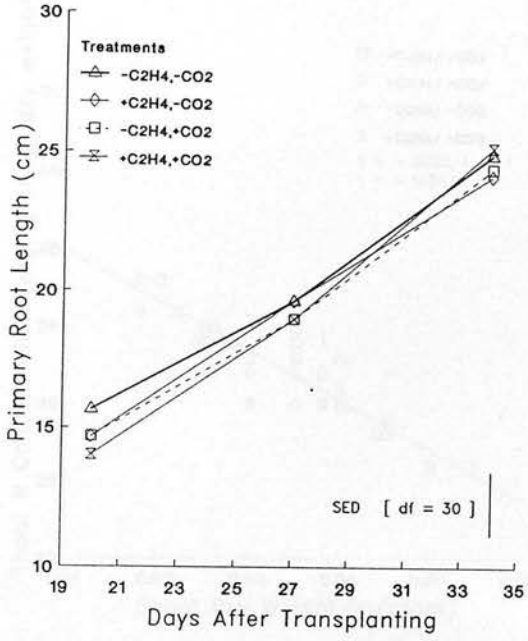


Fig. 4.94 Effect of C_2H_4 and CO_2 on length of primary roots of lentil (closed system)

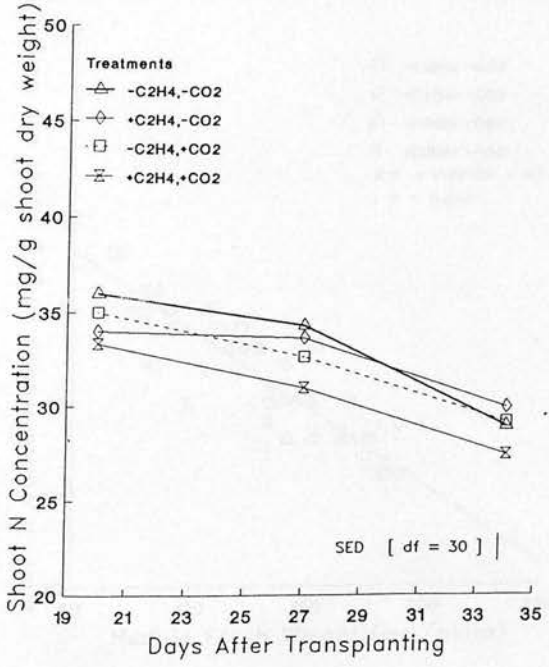


Fig. 4.95 Effect of C_2H_4 and CO_2 on nitrogen concentration of lentil shoots (closed system)

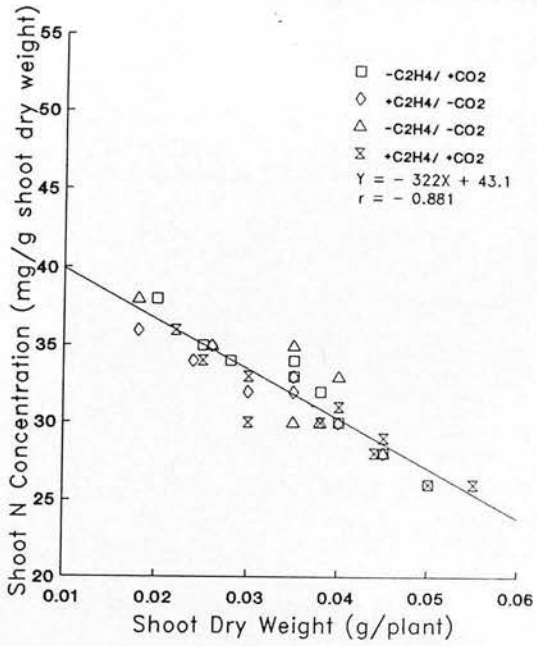


Fig. 4.96 Relationship between shoot nitrogen concentration and shoot dry weight (lentil, closed system)

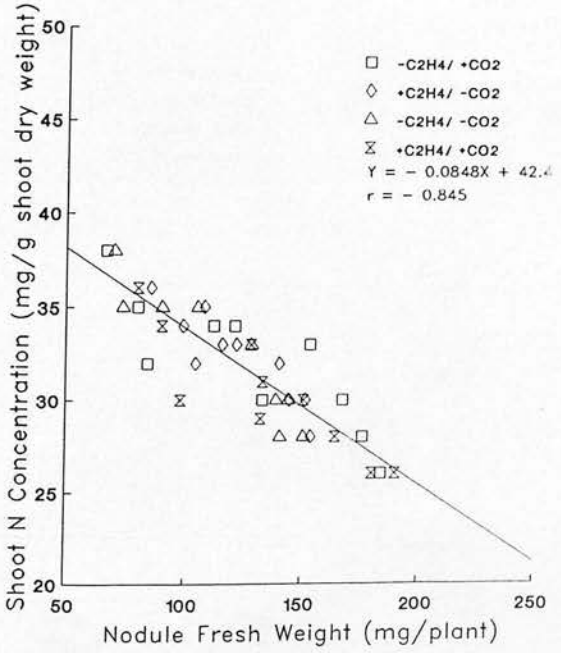


Fig. 4.97 Relationship between shoot nitrogen concentration and nodule fresh weight (lentil, closed system)

the shoot biomass had occurred in lentil, as in pea and bean (Fig. 4.98). This was reflected in the correlation coefficient ($r = 0.76^{***}$) obtained for the nodule nitrogenase activity vs. total nitrogen accumulation in the shoot (Fig. 4.99).

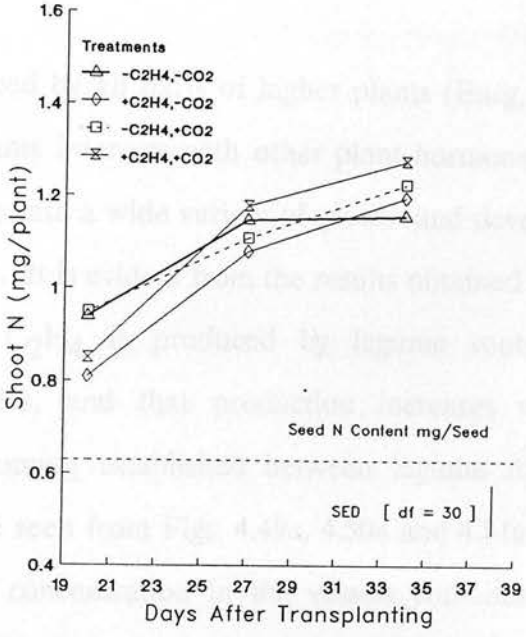


Fig. 4.98 Effect of C_2H_4 and CO_2 on total nitrogen content of lentil shoots (closed system)

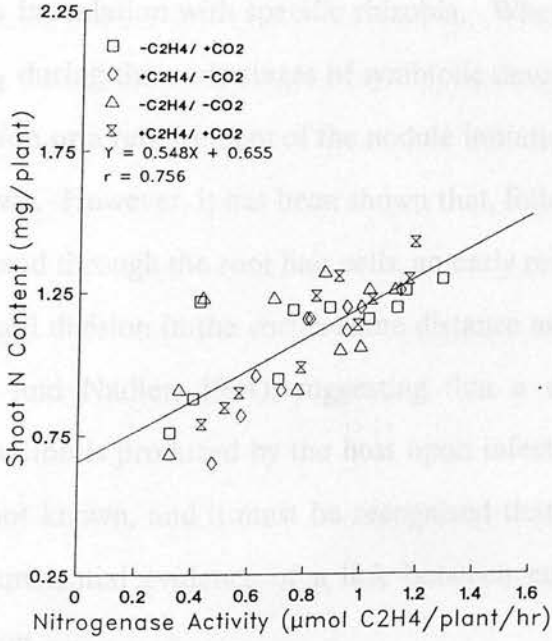


Fig. 4.99 Relationship between shoot nitrogen content and nitrogenase activity (lentil, closed experiment)

4.4 DISCUSSION OF THE RESULTS CONTAINED IN SECTION 4.3

4.4.1 Endogenous ethylene production, and its role in nodulation

Ethylene is produced by all parts of higher plants (Burg, 1962; Abeles, 1973) and in trace amounts interacts with other plant hormones, notably auxins, to coordinate and regulate a wide variety of growth and developmental processes (Beyer et al., 1984). It is evident from the results obtained in the "closed vessel" experiments that C_2H_4 is produced by legume roots in physiologically significant quantities, and that production increases when the symbiotic association is becoming established between legume roots and *Rhizobium* bacteria. It can be seen from Figs. 4.49a, 4.50a and 4.51a that the most rapid increase in C_2H_4 concentration in the vessels containing the root systems occurred between days 10 and 20 with pea and bean and between days 14 and 24 with lentil. The results are consistent with the findings of Ligero et al. (1986) who observed a similar increase in endogenous C_2H_4 in the roots of *Medicago sativa* plants upon inoculation with specific rhizobia. Whether this increase in endogenous C_2H_4 during the early stages of symbiotic development is merely a symptom of infection or a requirement of the nodule initiation and development process is not known. However, it has been shown that, following the formation of an infection thread through the root hair cells, an early response of the host is the elicitation of cell division in the cortex some distance away from the site of infection (Verma and Nadler, 1984), suggesting that a diffusible substance stimulating cell division is produced by the host upon infection. The nature of this substance is not known, and it must be recognised that these observations provide only circumstantial evidence of a link between endogenous ethylene and nodule initiation.

Considering the ability of C_2H_4 to diffuse freely from cell to cell, a concentration-dependent role of C_2H_4 cannot be ruled out. Various enzymes, such as cellulase, peroxidase, phenylalanine ammonia lyase and phosphatase, have been found to increase in activity following treatment with C_2H_4, and it is possible that C_2H_4 may exert its effect by regulating protein synthesis (Wareing and Phillips, 1978).

During the symbiosis, the host plant expresses a certain number of proteins specific to nodule development and nitrogen fixation, called nodulins, eg leghaemoglobin. Ethylene may stimulate the transcription of nodulin genes.

The development and persistence of a functioning nodule requires a high degree of regulation, and it is generally believed that hormonal balance is an important factor not only in the control of nodule initiation and development, but also in its maintenance and senescence (Badenoch-Jones et al., 1983). In particular, the auxin/cytokinin ratio in the host plant during the symbiotic development may be an important factor in determining the success of the association (Verma and Nadler, 1984). It is possible that C_2H_4 may have a role in host plants through its interaction with auxins. For example, it has been suggested that the promotive action of C_2H_4 in abscission involves the destruction of auxin and inhibition of its synthesis and transport, since auxin counteracts the action of C_2H_4 (Beyer et al., 1984).

Evidence presented recently by Smulder et al. (1991) suggests that the products of genes on the T-DNA of *A. rhizogenes*, a rhizosphere bacterium, affect auxin

action indirectly by preventing C_2H_4 from reducing the sensitivity of the tissue to auxin.

Although low concentrations of C_2H_4 may play a role in promoting symbiotic development, higher concentrations seem to have a deleterious effect, possibly determined by the host phenotype. The accumulation of endogenous C_2H_4 around the legume root systems in the closed vessels resulted in a significant inhibition of nodule production in pea and bean but not in lentil (Figs. 4.52, 4.68 and 4.84).

Similar observations were made in the "constant-flow" experiments discussed in Section 4.2. However, the effects of endogenous C_2H_4 accumulation in the closed systems became apparent only a week later (day 27) than those observed in the constant-flow system, and the effects were more significant at day 34 than at day 27 when the final harvest was made.

There were no significant differences between treatments in the number of nodules per pea or bean plant at day 20, but the number remained almost unchanged in the $+C_2H_4/-CO_2$ treatment compared with a significant increase in the $-C_2H_4/+CO_2$ and $-C_2H_4/-CO_2$ treatments up to the final harvest. It thus appears that the effects of endogenous C_2H_4 on root nodule formation were exerted at a time (between days 20 and 27) when the concentrations in the vessels had increased to above the minimum concentration that is presumably inhibitory to nodulation. It can be seen from Figs. 4.49a to 4.51a that the concentration of C_2H_4 in the vessels in the absence of $Hg(ClO_4)_2$ traps was about 0.6-0.7 ppm with pea and bean and about 0.5 ppm with lentil at day 20

and that it never fell below 0.4 ppm with any of the species, although some decrease was observed in the later stages of growth.

Although the effect on nodulation appears to be concentration-dependent, the results suggest that it is also time-dependent; ie the expression of responses to the accumulated C_2H_4 in the closed vessels experiments occurred some time after the increase in C_2H_4 concentration, and occurred later than in open systems (Figs. 4.1, 4.17, 4.33). The inhibition may have occurred during nodule development and was possibly coupled with induction of nodule senescence.

The failure of nodule development and possible shedding of senescent nodules between days 20 and 34 would account for the lack of an increase in nodule numbers in the treatments where C_2H_4 was allowed to accumulate.

In contrast to the inhibitory effects of C_2H_4 on nodulation in pea and bean at the later stages of growth, nodule formation in lentil was significantly stimulated at day 20 in the $+C_2H_4$ treatments compared with the $-C_2H_4/+CO_2$ and $-C_2H_4/-CO_2$ treatments. Later, there was no effect. The number of nodules increased up to the final harvest much as in the $-C_2H_4/+CO_2$ and $-C_2H_4/-CO_2$ treatment, the increase being particularly significant between days 27 and 34 (Fig. 4.84).

Ethylene might also have a secondary effect on the clustering and positioning of nodules. Pierce and Bauer (1983) have pointed out that the rapid regulatory response may be an important factor contributing to the clustering of nodules in the crown region of soyabean roots in field-grown plants and suggested the existence of another regulatory phenomenon affecting nodulation in soyabean

plants. Nodules in the C_2H_4 treatments in the present investigation were distributed and clustered on the primary roots in pea and lentil; but in bean, although the nodules were clustered, they were not restricted to the primary roots. These observations were similar to those observed in the constant-flow experiments discussed above in Section 4.2.

4.4.2 Interactions of CO_2 with C_2H_4, and effects on nodulation

Unlike C_2H_4, CO_2 stimulated root nodule formation in pea and bean. However, the effect was significant only at the final harvest (day 34).

The mechanism by which CO_2 stimulates nodule formation is not clear, but since the effects were more evident at the later stages of growth they were probably exerted at the level of nodule development rather than at infection or nodule initiation. Mulder and Van Veen (1960) and Phillips et al. (1976) reported similar stimulatory effects of CO_2 on root nodulation formation in *Trifolium pratense*, *Phaseolus vulgaris* and *Pisum sativum*. In another study, Lowe and Evans (1962) showed that pure cultures of *Rhizobium* require CO_2 for growth. Recently, Rosendahl et al. (1990) showed that the products of dark CO_2 fixation in pea root nodules support bacterial metabolism.

Although CO_2 stimulated nodule numbers in pea and bean in the absence of C_2H_4, nodule numbers did not increase when endogenous C_2H_4 was allowed to build up in the same vessels.

In the treatments where both the gases were present, the inhibitory effect of C_2H_4 dominated throughout. This suggests that CO_2, although known to be an

inhibitor of C_2H_4 action at concentrations of the order of 10% (Abeles, 1973), was not acting in this way to a very great extent at the concentrations prevailing in the present investigation: up to about 0.8-1.0% with pea and bean (Figs. 4.49b, 4.50b). No stimulatory effect of CO_2 on root nodule formation either in the presence or absence of C_2H_4 was observed with the lentil cultivar. Again, this suggests that it is plant genotype that determines the effect of this environmental stress.

4.4.3 Effect of endogenous C_2H_4 and CO_2 on nodule fresh weights

Individual nodule fresh weights in all three species were stimulated by CO_2 and inhibited by C_2H_4. The extent of inhibition was less in the lentil cultivar (Fig. 4.85) than in pea and bean (Figs. 4.53, 4.69), but the effects were most apparent at the final harvest. Similar results were obtained for the total nodule fresh weights (Figs. 4.54, 4.70 and 4.86), but the differences were more significant. The stimulation by CO_2 was not pronounced in bean. These greater differences were mainly because of the differences in nodule number between treatments.

The inhibitory effect of C_2H_4 on total nodule fresh weight in bean was similar to that in pea, but CO_2 was stimulatory both in the presence and in the absence of C_2H_4. With lentil, C_2H_4 was stimulatory only in the absence of CO_2 but, as with bean, CO_2 was stimulatory whether C_2H_4 was present or not.

It was not possible to correlate root dry weight and nodule dry weight because nodules were sacrificed for the determination of leghaemoglobin. However, total nodule fresh weight in all three species correlated reasonably with root dry weight (Figs. 4.61, 4.77 and 4.93). As in the constant flow-through

experiments, the explanation may lie in increased root area resulting in increased bacterial infection and thus in increased nodule numbers.

Significant positive effects of CO_2 on nodule fresh weights may be attributed to increased accumulation of starch. Phillips et al. (1976) observed similar effects of CO_2 enrichment (0.12%) and showed that the greater nodule mass in CO_2-enriched plants was correlated with an increase in material giving a positive response in the periodic acid-Schiff reaction, in the cells containing bacteroids. They suggested that the PAS-positive material might be starch granules rather than cell wall materials. There is no obvious explanation for the negative effects of C_2H_4 on the nodule fresh weights as observed in all three species in the present investigation. However, it may have resulted from selective destruction of nodule proteins or the inhibition of the supply of photosynthates to the actively respiring bacteroids.

4.4.4 Nitrogenase activity, leghaemoglobin content and shoot N content

The relationships between leghaemoglobin content per plant and nodule fresh weight per plant, and between nitrogenase activity and leghaemoglobin content, were remarkably similar to those found in the experiments with the constant flow-through system.

As in the earlier experiments, the leghaemoglobin content per plant, in all three species, increased rapidly with increasing total nodule fresh weight, as a result of an increase in both nodule weight and leghaemoglobin concentration per mg of nodule, with time (Figs. 4.58, 4.74, 4.90). This interaction of the two factors was, once again, responsible for the non-linearity of the relationships shown in

the figures. There was very little scatter in the data, and the correlation coefficients were very high.

The overall relationship for each species between nitrogenase activity and leghaemoglobin content was linear and, as in the "flow-through" experiments, showed remarkably high correlations (Figs. 4.57, 4.73, 4.89). For pea and bean, the greatest differences were between the $+C_2H_4/-CO_2$ and $-C_2H_4/+CO_2$ treatments. For pea, comparing these two treatments, the former resulted in a reduction of nodule fresh weight per plant to 46% of that observed with the latter. The reductions in leghaemoglobin content and nitrogenase activity per plant were both to 37%. Nitrogenase activity in the $+C_2H_4/-CO_2$ treatment was also reduced on a per nodule basis, compared with the $-C_2H_4/+CO_2$ treatment. For bean, the corresponding reductions were: nodule fresh weight per plant, to 45%; leghaemoglobin, to 20%; nitrogenase activity, to 29%. This greater reduction in leghaemoglobin in bean is analogous to the results of the constant flow-through experiments with different concentrations of C_2H_4. However, the fact that the nitrogenase activity of bean also fell on a per nodule basis, contrasts with the results from the other series of experiments. For lentil, the reductions were: nodule fresh weight per plant, to 87%; leghaemoglobin, to 58%, nitrogenase activity, to 88%. For this species there was no change in nitrogenase activity on a per nodule basis.

The results are generally compatible with those obtained with the constant flow-through system. Bean was again the species showing the most drastic reduction in leghaemoglobin content. It was found that, as in the earlier experiment, leghaemoglobin did not increase after 27 days in the presence of 0.7-0.8 ppm of C_2H_4 (Figs. 4.50(a), 4.72). Lentil again showed only a relatively small effect on

nodule fresh weight and nitrogenase activity. However, the leghaemoglobin content fell rather more in the closed system (comparing the $+C_2H_4/-CO_2$ and $-C_2H_4/+CO_2$ treatments), and this suggests the absence of CO_2 is more damaging than the presence of C_2H_4 for this species. Further work is required to establish the significance of the changes of leghaemoglobin content for the capacity of the different species to fix nitrogen, and to identify the mechanisms of the interactions between CO_2 and C_2H_4 with respect to their influence on leghaemoglobin content and nitrogenase activity.

Correlations between shoot N content and nitrogenase activity were studied to assess whether atmospheric N incorporation into the legumes through symbiotic association with *Rhizobium* was occurring to a significant extent. The significant positive correlations obtained for these two variables (Figs. 4.64, 4.83, 4.99) suggest that, as in the flow-through system, fixation did occur. In this series of experiments also, the acetylene reduction assay appears to have been suitable for measuring nitrogenase activity *in vitro* for short-term assessments.

The presence of CO_2 and absence of ethylene increased the shoot N content, as compared with the reverse treatment, for pea and bean (Figs. 4.63, 4.82), but there was no corresponding effect with lentil (Fig. 4.98). These results simply reflect the effects of the gases on nitrogenase activity, as discussed above.

The effects of C_2H_4 on pea and bean observed in these experiments may have involved reduced synthesis of leghaemoglobin and nitrogenase, and (for bean) possibly enhanced leghaemoglobin breakdown. It is not known whether C_2H_4 can induce proteolytic activity leading to the destruction of nodule specific proteins. Significant declines in leghaemoglobin contents and in all the enzymes

involved in carbon and nitrogen metabolism in nodules, ie invertase, sucrose synthase, UDP glucose pyrophosphorylase, aspartate amino transferase, glutamine synthase, phosphoenolpyruvate carboxylase, and malate dehydrogenase, following stress-induced defoliation, have been reported by Gordon and Kessler, 1990). Two thiol proteases have also been purified from French bean senescent nodules and they appear to be active only during senescence (Manen et al., 1991). It is not known whether C_2H_4 can induce the expression of these proteases.

The stimulatory effects of CO_2 on nodule nitrogenase activity in all three legume species studied are consistent with the findings of Mulder and Van Veen (1960). who showed that hydroponically cultured roots of *Trifolium pratense*, *Pisum sativum* and *Phaseolus vulgaris*, when flushed with air containing 4% CO_2, exhibited increased N_2 fixation. They suggested that the effect of CO_2 was directly on the root rather than through photosynthetic reduction of CO_2 by the shoot. This was partly supported by Bergersen (1971), who observed that CO_2 stimulated N_2 reduction by detached soyabean nodules exposed to O_2 levels no greater than 30%. Presumably the higher oxygen concentrations tested promoted respiration and relieved any CO_2 limitation. Phillips et al. (1976) also showed a very significant increase in C_2H_2 reduction activity by *Pisum sativum* L. grown in the light for 6 hr at a CO_2 enrichment of 0.12%.

It is possible that the increase in the nitrogenase activity of the nodules exposed to the elevated CO_2 concentration in the vessels in the present study resulted from the dark fixation of CO_2 by the roots and nodules, involving the recycling of substantial amounts of respiratory carbon and thus the provision of additional

photosynthate throughout the growing period (Christeller et al., 1977; Coker and Schubert, 1979; Lawrie and Wheeler, 1975; Layzell et al., 1979; Rawsthore et al., 1980).

4.4.5 Effects on shoot N concentration

Significant positive effects of C_2H_4 on the N concentrations in pea and bean shoots were observed at day 34 (Figs. 4.65 and 4.79). That was true whether CO_2 was present or not. The increased N concentrations were not due to enhanced rates of N-fixation; this parameter decreased with increasing C_2H_4. Rather, they were due to a reduction in shoot dry matter production which was proportionately greater than the decrease in N-fixation.

Carbon dioxide had a significant negative effect at day 34 in pea and bean, although in the latter the inhibition by CO_2 occurred only in the absence of C_2H_4. This reflects the proportionately greater dry matter production in the presence of CO_2. No significant effects of either C_2H_4 or CO_2 on shoot N concentration were observed with lentil (Fig. 4.95).

4.4.6 Effects on shoot dry weight

Significantly lower shoot dry weights were observed in pea at day 34 in the $+C_2H_4/-CO_2$ treatment than in the other treatments (Fig. 4.59). On the other hand, CO_2, in the absence of C_2H_4, increased shoot dry weight. In beans, C_2H_4 had an inhibitory effect on shoot dry weight production at day 34, but only when CO_2 was present (Fig. 4.75). No significant effects of CO_2, either in the presence or absence of C_2H_4, were observed. As was concluded in the

discussion of the constant flow-through experiments, the reduction in shoot growth may well have been due to ethylene-induced effects unconnected with the role of the gas in nodulation and/or N-fixation. Work with nodulating and non-nodulating plants would help to confirm this.

Neither C_2H_4 nor CO_2 had any significant effect on shoot dry weight production in lentil, despite significant stimulation of nodule production by C_2H_4 at the initial stages of growth (Fig. 4.91).

4.4.7 Effect on root dry weight

Ethylene had no significant effect on the root dry weights of any of the plant species studied (Figs. 4.60, 4.76 and 4.92). However, CO_2 resulted in a significant increase in root biomass production in all three species in the absence of C_2H_4. This increase in the root biomass production correlated significantly with the total nodule biomass production, suggesting that the stimulatory effect of CO_2 on nodule numbers was mediated through the increase in root biomass production rather than by affecting infection development. These results are consistent with the findings of Phillips et al. (1976), who observed significantly higher plant dry weight, N content, root nodule mass, number of nodules and mean nodule dry weight in *Pisum sativum* L. grown for four weeks at 0.12% CO_2 than in control plants grown at 0.032% CO_2.

4.4.8 Effects on root length

Primary root lengths of the species studied were not affected by C_2H_4 and/or CO_2 (Figs. 4.62, 4.78 and 4.94). Thus, it appears that the concentration required for the inhibition of the main root system is possibly much higher than that attained in the root growth vessels during the present study.

4.4.9 Summary of effects

The effects of endogenous C_2H_4 and/or CO_2 on nodulation, subsequent N_2-fixation and overall plant growth in pea, bean and lentil cultivars as observed in the closed vessels experiments, have been summarised in Tables 4.4.1, 4.4.2 and 4.4.3.

Table 4.4.1 *Effect of endogenous C_2H_4 and/or CO_2 in pea root nodulation and related plant growth*

Measured variables	Effect of C_2H_4 in presence of CO_2			Effect of C_2H_4 in absence of CO_2			Overall effect of C_2H_4 +/- CO_2	Effect of CO_2 in presence of C_2H_4			Effect of CO_2 in absence of C_2H_4			Effect of CO_2 +/-C_2H_4
	20d	27d	34d	20d	27d	34d		20d	27d	34d	20d	27d	34d	
Nodules plant^{-1}	NE	-	-	NE	-	-	**	NE	NE	NE	NE	NE	+	NS
Nodule fresh weight (mg nodule^{-1})	NE	NE	NE	NE	NE	-	**	NE	NE	+	NE	NE	NE	**
Nodule fresh weight (mg plant^{-1})	NE	-	-	NE	-	-	***	NE	NE	NE	NE	+	+	*
Nitrogenase activity (µmol C_2H_4 plant^{-1} hr^{-1})	NE	-	-	NE	-	-	***	NE	NE	NE	NE	+	+	*
Leghaemoglobin content (mg plant^{-1})	NE	NE	-	NE	-	NE	**	NE	NE	+	NE	NE	+	*
Shoot N concentration (mg g^{-1} shoot dry weight)	NE	NE	+	NE	NE	+	**	NE	NE	-	NE	NE	-	*
Shoot N content (mg plant^{-1})	NE	NE	NE	NE	NE	NE	*	NE	NE	NE	NE	NE	NE	NS
Shoot dry weight (g plant^{-1})	NE	NE	NE	NE	NE	-	**	NE	NE	NE	NE	NE	+	NS
Root dry weight (g plant^{-1})	NE	NE	NE	NE	NE	NE	NS	NE	NE	NE	NE	NE	+	*
Primary root length (cm)	NE	NE	NE	NE	NE	NE		NE	NE	NE	NE	NE	NE	NS

NE --no effect

Table 4.4.2 Effect of endogenous C_2H_4 and/or CO_2 in bean root nodulation and related plant growth

Measured variables	Effect of C_2H_4 in presence of CO_2			Effect of C_2H_4 in absence of CO_2			Overall effect of C_2H_4 +/- CO_2	Effect of CO_2 in presence of C_2H_4			Effect of CO_2 in absence of C_2H_4			Effect of CO_2 +/-C_2H_4
	20d	27d	34d	20d	27d	34d		20d	27d	34d	20d	27d	34d	
Nodules plant^{-1}	NE	-	-	NE	-	-	**	NE	NE	NE	NE	NE	+	NS
Nodule fresh weight (mg nodule^{-1})	NE	NE	-	NE	NE	-	*	NE	NE	NE	NE	NE	NE	*
Nodule fresh weight (mg plant^{-1})	NE	-	-	NE	-	-	***	NE	+	+	NE	+	+	**
Nitrogenase activity (µmol C_2H_4 plant^{-1} hr^{-1})	NE	-	-	-	-	-	***	NE	+	+	NE	+	+	**
Leghaemoglobin content (mg plant^{-1})	NE	NE	-	NE	NE	-	**	NE	NE	+	NE	NE	+	*
Shoot N concentration (mg g^{-1} shoot dry weight)	NE	NE	+	NE	NE	+	*	NE	NE	NE	NE	NE	-	*
Shoot N content (mg plant^{-1})	NE	NE	-	NE	NE	NE	*	NE	NE	NE	NE	NE	+	*
Shoot dry weight (g plant^{-1})	NE	NE	-	NE	NE	NE	**	NE	NE	NE	NE	NE	NE	*
Root dry weight (g plant^{-1})	NE	NE	NE	NE	NE	NE	NS	NE	NE	NE	NE	NE	NE	*
Primary root length (cm)	NE	NE	NE	NE	NE	NE	NS	NE	NE	NE	NE	NE	NE	NS

NE - no effect

Table 4.4.3 *Effect of endogenous C_2H_4 and/or CO_2 in lentil root nodule formation and related plant growth*

Measured variables	Effect of C_2H_4 in presence of CO_2			Effect of C_2H_4 in absence of CO_2			Overall effect of C_2H_4 +/- CO_2	Effect of CO_2 in presence of C_2H_4			Effect of CO_2 in absence of C_2H_4			Effect of CO_2 +/-C_2H_4
	20d	27d	34d	20d	27d	34d		20d	27d	34d	20d	27d	34d	
Nodules plant^{-1}	NE	NE	NE	+	NE	NE	*	NE	NE	NE	NE	NE	NE	NS
Nodule fresh weight (mg nodule^{-1})	NE	NE	NE	NE	NE	NE	*	NE	NE	+	NE	+	+	**
Nodule fresh weight (mg plant^{-1})	NE	NE	NE	+	NE	NE	*	NE	NE	+	NE	NE	+	**
Nitrogenase activity (µmol C_2H_4 plant^{-1}hr^{-1})	NE	NE	NE	+	NE	NE	NS	NE	NE	NE	NE	NE	+	NS
Leghaemoglobin content (mg plant^{-1})	NE	NE	NE	NE	NE	NE	NS	NE	NE	NE	NE	NE	NE	NS
Shoot N concentration (mg g^{-1} shoot dry weight)	NE	NE	NE	NE	NE	NE	NS	NE	NE	NE	NE	NE	NE	NS
Shoot N content (mg plant^{-1})	NE	NE	NE	NE	NE	NE	NS	NE	NE	NE	NE	NE	NE	NS
Shoot dry weight (g plant^{-1})	NE	NE	NE	NE	NE	NE	NS	NE	NE	NE	NE	NE	NE	NS
Root dry weight (g plant^{-1})	NE	NE	NE	NE	NE	NE	NS	NE	NE	NE	NE	NE	+	*
Primary root length (cm)	NE	NE	NE	NE	NE	NE	NS	NE	NE	NE	NE	NE	NE	NS

NE - no effect

4.5 GENERAL DISCUSSION

4.5.1 Comparisons between species and experimental systems

Ethylene was evolved by the roots of all three legume species studied in the present investigation. The concentration of C_2H_4 reached in the closed vessels (Figs. 4.49a, 4.50a and 4.51a) was higher with bean and pea than in lentil, but on a root-dry-weight basis lentil roots evolved the most C_2H_4.

Nodules were formed on the roots of all three legume species, after inoculation with host-specific *Rhizobium*. However, the number of nodules formed on the roots of the lentil cultivar was much lower than on those of pea and bean. The reason for this is not obvious but it might result in part from differences in the efficacy of the nodulating strains. When subcultured on agar plates, the strain used with lentil did not produce as many colonies as did those used with pea and bean. Another possible reason is that the lentil plants had fewer lateral roots and therefore less exposed surface for the bacteria to interact with.

Bean nodules had slightly greater nodule fresh weights than did those of pea and lentil, but a lower nitrogenase activity per nodule. In all three species, however, nitrogenase activity correlated well with total shoot-N accumulation. This does not rule out the possibility that some of the accumulated N could have come from the seeds.

Natural abscission of leaves was observed during the early stages of growth in bean, presumably resulting in a decrease in photosynthate supply to the roots

and perhaps partly explaining the lower than expected nitrogenase activity per nodule.

For pea and bean, exposure to 1 ppm of C_2H_4 in the constant flow-through system and to 0.6-0.8 ppm in the closed system had a generally greater inhibitory effect on leghaemoglobin than on nodule fresh weight or nitrogenase activity. For the most resistant species, lentil, the inhibition was more pronounced in the closed system in the absence of CO_2. The relationship between the effect of gas concentrations on leghaemoglobin and the consequences for N fixation is a complex one, and more work is required to provide an adequate understanding of it.

At day 20, the N content of the shoots of pea and lentil was only as high as the original seed-N content, and that in bean was substantially less. Even at the final harvest the total N content in bean shoots was only just above the original seed-N content and this was reflected in the symptoms of nitrogen starvation observed in bean but not pea and lentil at the initial stages of nodule growth and development.

Early nodule development and seedling growth in general is dependent upon carbon and nitrogen present in the cotyledonous reserves, and it is possible that the formation of new nodules on the roots causes substantial competition between nodules and the rest of the plants for those reserves at that stage (Hansen et al., 1990; Hungria et al., 1991). For beans there is an additional problem; unlike in pea and lentil the cotyledons come above ground and are abscised by an autoregulatory mechanism within 7-10 days after germination. The plant is thus deprived of the cotyledonous reserves including the supply of

nitrogen, for which the demand is greatest during the early stages of nodule growth and development.

The difference in sensitivity to C_2H_4 of the species studied may reflect differences in tolerance to environmental stress conditions. Pea and bean are well known as being intolerant of waterlogging, ie being intolerant of anaerobic conditions (even of short duration) in their root zones. Lentil, on the other hand, is generally accepted in many countries, including the author's own country of Bangladesh, as being a particularly waterlogging-tolerant grain legume. Also, the particular cultivar used in this study, brought from Bangladesh, was shown in unpublished work of Young (1991) in this department to have this tolerance. It is very evident from the experiments described in this thesis that the reputed variation in tolerance to aeration correlates well with the observed tolerance to ethylene, at least as far as phenomena associated with nodulation and N-fixation are concerned.

Comparable correlations between tolerance to ethylene and to poor aeration have been well documented for growth parameters such as root extension, in the case of non-legumes (Smith and Robertson, 1971; Konings and Jackson, 1979). Of course, the fact that the two tolerances are correlated does not by itself prove cause and effect. However, it is becoming clear that the quantity of circumstantial evidence of a link has grown over the years, and the body of data reported here add considerably to it. The apparent paradox that ethylene is neither formed nor demonstrates physiological activity in the absence of oxygen is resolved for at least some growth phenomena, in that ACC produced in oxygen-deficient roots is converted to ethylene in the presence of atmospheric O_2 in the shoots (see Section 2.9.1) and it is this ethylene which produces visible

effects on the shoots. It is possible that ethylene produced in this way in turn induce processes which reduce nodulation and N-fixation.

The flow-through and the closed-vessel techniques allowed investigation of different aspects of the involvement of C_2H_4 and CO_2 in the *Rhizobium* legume symbiosis. The plants grew well and appeared healthy in both systems throughout the experiments despite the unconventional growth conditions.

The flow-through technique was designed to determine a threshold C_2H_4 concentration capable of having effects on the symbiosis. As discussed in Section 3.2, the different C_2H_4 concentrations were obtained by diluting 1000 ppm of C_2H_4 in N_2 with air, so the results obtained do not show the effects of C_2H_4 in the complete absence of CO_2. The concentration of CO_2 in the C_2H_4-containing air-stream was always close to the ambient atmospheric CO_2 level.

The concentrations of C_2H_4 used in the flow-through technique were fairly constant throughout the experiments, except in the control treatment where some C_2H_4 (not exceeding 0.1 ppm) was occasionally detected in the air emerging from the vessels, particularly during the first few days after transplanting the plants into the PVC pipes.

In the closed vessels the concentration of neither gas was constant; the concentrations of both CO_2 and C_2H_4 increased steadily over the first three weeks or so (Figs. 4.49-4.51) and it is therefore not possible to say exactly what concentrations began to have an effect. However, the good agreement between the observations in the two types of experiment suggests that the threshold

concentration of C_2H_4 capable of inducing inhibitory effects lies between 0.5 and 1 ppm.

This concentration range is well below the concentrations found in the soil atmosphere in the field in several studies (eg Dowdell et al., 1972; Smith and Dowdell, 1974; Arshad and Frankenberger, 1990b,c). Therefore, if no other factors were involved, it could be inferred that field conditions potentially inhibitory to nodulation are relatively common. In much of the published work referred to here, it was concluded that the ethylene in the soil atmosphere was produced by soil microorganisms. One outcome of the present study is the demonstration that the root system itself is capable of generating self-inhibitory concentrations of ethylene, even in a medium with an air-filled porosity many times that of normal soils. Thus it can be concluded that even more inhibitory concentrations could result from growth in an environment with a reduced air space and thus a diminished dilution of the evolved ethylene.

That said, it must also be pointed out that the inhibitory effects of ethylene in the root environment under field conditions may be moderated considerably by the presence of enhanced CO_2 concentrations - in view of the results described here - and of reduced O_2 concentrations. Complex interactions between ethylene and O_2 concentration in the root environment are likely, and deserve to be the subject of further study. In this work the O_2 concentration in the flow-through technique was always close to the ambient atmospheric level, and in the closed system the O_2 was replenished by the addition of pure O_2 every two days. As a result, even in the latter system the O_2 concentration in the vessels never fell below 17%. The results obtained in this model system are only likely to relate to the field in circumstances such as the very transient formation of a

perched watertable after heavy rain, when gas exchange with the atmosphere is prevented but the soil at rooting depth retains a reasonably high air-filled porosity and a reserve of O_2, at least for a time.

The extent and pattern of nodule formation were quite similar in the two techniques. However, relatively higher nodule nitrogenase activity was observed in pea and lentil nodules in the control (no C_2H_4) treatment of the flow-through experiments than in the -C_2H_4/-CO_2 treatment of the closed-vessel experiment. This higher nodule nitrogenase activity might be accounted for by the presence of CO_2 in the flow-through system. The results obtained in this work were either in the presence of atmospheric CO_2 concentrations (in the flow-through system) or at concentrations approaching 1% (in the closed system). It would be useful, in future work, to study the impact of more elevated CO_2 concentrations in excess of 1%, as these commonly occur in the field, and also interactions involving high CO_2/low O_2 combinations, on processes such as nodulation.

4.5.2 Relationship between this work and other studies of ethylene-root interactions

The importance of biological nitrogen fixation, particularly through the legume-*Rhizobium* symbiotic association, has long been recognised. The process is principally responsible for the input of nitrogen into natural soil-plant ecosystems, and is also the main source of N in many agricultural ecosystems, especially in the developing world.

The development of an effective symbiotic association is vulnerable to many ecological constraints including the presence of inhibitory concentrations of ethylene in the root environment. The gaseous hormone is involved in many processes of plant growth and development, possibly including the root nodule formation of legumes. However, the gas has been shown to be inhibitory to the nodulation process (Grobelaar et al., 1970, 1971; Goodlass and Smith, 1979) at concentrations which are known to occur in soils and/or which can be generated by the emission of endogenous ethylene from the plant root system.

The present investigation was initiated to study the effect of C_2H_4 on the nodulation and N-fixation of legumes growing without any additional physiological stresses. The results obtained confirm previous reports that C_2H_4 inhibits the processes of root nodule formation in some legumes. However, the results also show a degree of variation in sensitivity between different species that is comparable with the differences in effects on root extension reported elsewhere (eg Smith and Robertson, 1971). In the present investigation C_2H_4 appeared to inhibit nodule production and other processes involved in the symbiosis in pea and bean only above a certain threshold concentration that lies between 0.5 and 1 ppm. No significant effects of C_2H_4 in this range on the lentil cultivar studied were observed.

Although the higher concentrations of C_2H_4 used resulted in a significant inhibition of nodulation and N-fixation, lower concentrations may have had a positive role in the nodulation process. There was some evidence of a stimulation of lentil nodulation at the lower C_2H_4 concentrations used. It may well be that increasing C_2H_4 concentration first results in a stimulation of the process, but further increases ultimately result in inhibition, producing a

response curve similar to that observed for other phenomena such as root extension (Smith and Robertson, 1971). If this is the case, then as with root extension it is likely that a given concentration of ethylene will be stimulatory for one species, relatively neutral for another, but inhibitory for a third species.

In contrast to the inhibitory effects of C_2H_4 on root nodule formation of some legumes, a significant positive effect of CO_2, particularly in increasing root and nodule dry weight and the nitrogenase activity of the nodules, was observed for the pea and bean cultivars used. In the lentil cultivar the only effect observed was an increase in nodule dry weight.

4.5.3 Suggestions for future research

4.5.3.1 Root atmosphere-nodulation-N fixation interactions

It would be worth investigating the effects of C_2H_4 on the nodulation and N-fixation of plants growing in soil systems where C_2H_4 production (endogenous and exogenous) and accumulation are influenced by the soil type, texture, structure, porosity, and above all the temperature and moisture content of the soil. The concentrations of endogenously produced C_2H_4 produced in confined air-filled pore space around the roots could well become much higher than the concentration in the loosely packed and very porous perlite medium. However, interactions with O_2 concentration, which would be likely to fall to lower values than in the work described here, might offset any resulting effects. The role of soil physical conditions on these interactions should be explored.

4.5.3.2 Mechanism of C_2H_4 action in nodulation and related processes

There is scope for considerable further work on the mechanism of ethylene action in nodulation and related processes. The inhibition of root nodule formation in the roots of the pea cultivar used in the present study may have occurred during infection development. It is possible that high concentrations of C_2H_4 may eventually induce host-plant defence responses. The first step towards the development of an effective symbiotic association must be the removal or suppression of host defence mechanisms. This is made possible by a mechanism involving controlled invasion of host tissue by bacteria and strict compartmentalisation within the nodule. The invasion (or internalisation) is mediated by a tubular structure known as the infection thread, which grows inwards towards the region of cells that becomes the nodule meristem and restricts the invading microbe to an extracytoplasmic location. The *Rhizobium* is finally released from the infection thread but remains enclosed in a membrane envelope (peribacteroid membrane). Thus, the plant and bacteria remain separated from each other throughout the symbiosis. Rae and Brewin (1991) characterised the presence of a 95 kD glycoprotein in the lumen of the infection thread and suggested that the glycoprotein is synthesised by plant cells associated with the bacteria at all stages of internalisation. Recently, the accumulation of a basic glycoprotein induced by C_2H_4 in the leaves of Azuki bean (*Vigna angularis*) has been confirmed by Fumiharu et al. (1991). It is possible that high concentrations of C_2H_4 in the legume rhizosphere may eventually induce host-plant defence responses including the synthesis of such glycoproteins to such an extent that invading bacteria are confined in the lumen of the infection thread and nodule development is aborted. This would be an intriguing area to investigate.

A number of specific *Rhizobium* mutants affecting various stages of nodule development and thereby preventing an effective association have been isolated (Verma and Nadler, 1984). It might be possible to use these to investigate the developmental stages of the symbiosis and examine how C_2H_4 exerts its inhibitory effect.

It would be worth investigating whether changes in phytohormone levels occur during the infection process, from the first stage (root-hair curling) to the production of a fully-functioning nodule and whether C_2H_4 at relatively high concentrations can regulate the auxin/cytokinin ratio in the host plant.

Symbiotic root nodule formation is the legumes response to rhizobial infection and it is very likely that changes in phytohormone ratios may be involved (Nap and Bisseling, 1990a), much as the differential gene expression involved in normal plant development appears to be regulated by phytohormones (Horgan, 1984). Auxins (Verma and Nadler, 1984), cytokinins (Upadhyaya et al., 1991a,b) and ethylene (Ligero et al., 1986) may all play a role in controlling nodule development. Rhizobia are themselves capable of producing auxins and cytokinins (Verma and Nadler, 1984 and Upadhyaya et al., 1991b) but it is not clear whether bacterial production of the hormones is involved in nodulation. The introduction of a gene involved in cytokinin synthesis can partly complement Nod^- mutants of *R. meliloti* (Sprent, 1989), suggesting that cytokinin production may be involved, but non-nodulating and nodulating strains of rhizobia apparently produce similar amounts of auxins, which suggests that the bacterial production of auxins is not involved (Verma and Nadler, 1984). Inhibition of auxin transport in alfalfa leads to the formation of nodule-like structures in which early nodulin genes are expressed (Nap and Bisseling,

1990a). The implication is that an alternative bacterial signal leads to changes in the plants own hormone levels. It is possible that C_2H_4 may have a role in regulating the auxin/cytokin ratio in host-plant through its interactions with auxins. The promotive action of C_2H_4 in abscission has been suggested to involve the destruction of auxin and inhibition of its synthesis and transport, since auxin counteracts the action of C_2H_4 (Beyer et al., 1984).

In spite of the linear relationships found in this work between leghaemoglobin content per plant and nitrogenase activity, detailed examination of the results indicated significant differences between the species in the effects of C_2H_4 (and CO_2) on these parameters and their relationships with nodule mass and nodule number. It would be useful to investigate fully the nature of the relationships between changes in gas concentrations, leghaemoglobin synthesis and disappearance, and the capacity of the plants to fix nitrogen, and to identify the mechanisms involved. Studies should, perhaps, include measurements from earlier stages of growth; it is possible that the use of constant time periods for the experiments with all three species in this work did not reflect equivalent stages in physiological development, and thus may have observed a more comparable pattern of response to gas concentrations.

Another worthwhile area for further research would be to look at whether C_2H_4 at high concentrations can induce the production of proteases involved in nodule senescence. Recently, Manen et al. (1991) have purified two thiol proteases from French bean senescent nodules and they appear to be active only during senescence. The benefits of the legume-*Rhizobium* symbiosis could be prolonged if senescence can be delayed; from an agronomic point of view this would be very advantageous.

5. OTHER EXPERIMENTS: RESULTS AND DISCUSSION

5.1 Investigation of C_2H_4 production by *Rhizobium* in culture

5.1.1 Introduction

Many species of C_2H_4-producing microorganisms have been isolated from soil, and these microorganisms have been shown to derive C_2H_4 from various substrates including amino acids, organic acids, carbohydrates, alcohols, and proteins (Arshad and Frankenberger, 1990b). A combination of methionine as a precursor and glucose as an energy source yielded the greatest quantity of C_2H_4 in microbial cultures and incubated soil samples (Arshad and Frankenberger, 1989; Lynch and Harper, 1980). However, there is no direct evidence of C_2H_4 production by *Rhizobium* despite its possible importance in the interactions between the bacteria and legumes.

An attempt was made to investigate whether C_2H_4 is produced by *Rhizobium* in normal culture. A suspension culture amended with methionine (0.5 g l^{-1}), DL-homoserine (0.5 g l^{-1}) or five times the normal yeast extract content (ie 2.5 g l^{-1}) was used. Treatments were as follows:

(a) Control (culture solution only)("CS")

(b) Culture solution + *Rhizobium* ("CS + Rh")

(c) Culture solution + *Rhizobium* + methionine ("CS + Rh + ME")

(d) Culture solution + *Rhizobium* + homoserine ("CS + Rh + HS")

(e) Culture solution + *Rhizobium* + yeast extract ("CS + Rh + Y5")

The cultures (containing *Rhizobium leguminosarum* bv. *viceae*) were shaken (250 r min^{-1}) in 250 ml conical flasks fitted with Subaseal stoppers, at 26-29°C for 60 hr. Gas samples were collected every 12 hours using hypodermic syringes and analysed for C_2H_4 by gas chromatography as described in Section 3.4.

The results of the analyses are presented in Fig. 5.1. Ethylene was detected in the CS + Rh, CS + Rh + HS and CS + Rh + Y5 treatments 12 hours after the start of the incubation. The total amount produced increased significantly in all three treatments up to 36 hours and then remained unchanged for the next 24 hours. There were no significant differences in C_2H_4 production between the treatments at 12 hours. However, by 36 hours the C_2H_4 concentration in the CS + Rh + Y5 treatment was much higher than in the other two treatments, ie 12 ppm, compared with 6.5 ppm in the CS + Rh + HS treatment, and 1.8 ppm in the CS + Rh treatment. No C_2H_4 was detected in the CS + Rh + ME or CS (control) treatments.

These results indicate that C_2H_4 is produced in significant amounts by *Rhizobium* in culture and that the production increases very substantially in the presence of yeast extract. Yeast extract is rich in many amino acids and vitamins. It is possible that *Rhizobium* uses them as substrates for C_2H_4 production. Homoserine is a major component of root exudate (Van Egeraat, 1975) and may also serve as a substrate for C_2H_4 production.

The fact that C_2H_4 was produced in the CS + Rh treatment but not in the CS + Rh + ME treatment, suggests that methionine may suppress C_2H_4 production. This is in contrast to the observation reported earlier that it might

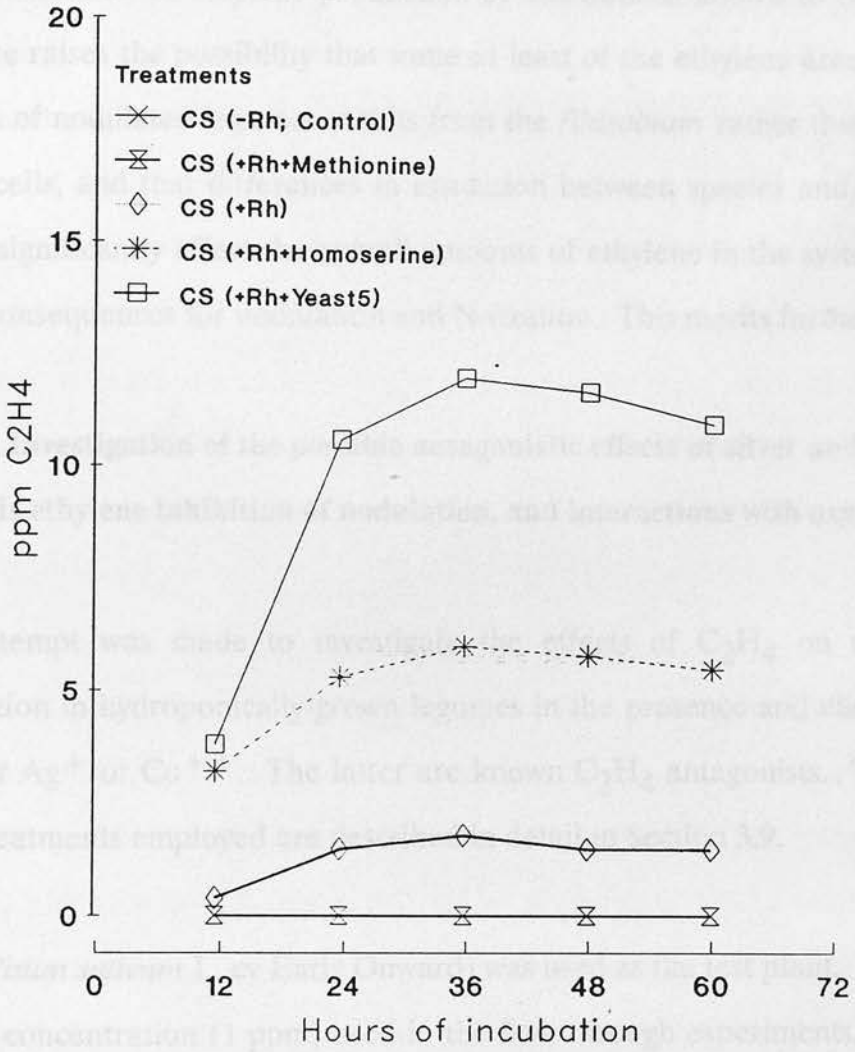


Fig. 5.1. Production of C_2H_4 over time, in flasks containing *Rhizobium* culture incubated with different amendments.

serve a precursor of C_2H_4 (Lynch, 1972). There is no obvious reason for the inconsistency.

The stimulation of ethylene production by compounds known to occur in root exudate raises the possibility that some at least of the ethylene around the root system of nodulated legumes results from the *Rhizobium* rather than the higher plant cells, and that differences in exudation between species and/or cultivars could significantly affect the overall amounts of ethylene in the system and thus have consequences for nodulation and N-fixation. This merits further study.

5.2 Investigation of the possible antagonistic effects of silver and cobalt ions towards ethylene inhibition of nodulation, and interactions with oxygen

An attempt was made to investigate the effects of C_2H_4 on root nodule formation in hydroponically-grown legumes in the presence and absence of O_2 and/or Ag^+ or Co^{++}. The latter are known C_2H_4 antagonists. The method and treatments employed are described in detail in Section 3.9.

Pea (*Pisum sativum* L. cv Early Onward) was used as the test plant. The highest C_2H_4 concentration (1 ppm) used in the flow-through experiments, and shown to inhibit root nodule formation in pea and bean in those experiments, was employed.

Seven-day-old seedlings were transplanted into Kilner jars containing full-strength nutrient solution (as described in Section 3.2) or distilled water mixed with 5 ml of *Rhizobium* culture solution. The experiment was continued for 35 days.

In none of the treatments except the $+O_2/-C_2H_4/-Ag^+$ or Co^{++} and the distilled water/$-O_2/-C_2H_4/-Ag^+$ or Co^{++} treatments did nodulation occur. These results suggest that Ag^+, Co^{++} and C_2H_4 all inhibit nodule formation, and that in nutrient solution, though not in distilled water, an absence of O_2 may also be inhibitory.

All the plants in the Co^{++} treatment died within 14 days of transplantation. Plants treated with Ag^+, on the other hand, looked very unhealthy from about 7 days after transplantation until the end of the experiment, but did not die. The Ag^+ ions promoted the growth of secondary laterals (Plate 5.1a,b) whether O_2 and/or C_2H_4 were present or not. Co^{++} was obviously more toxic to the plants than was Ag^+.

In the $-O_2/-C_2H_4/-Ag^+$ or Co^{++} treatment with distilled water, stress became evident soon after transplanting but the plants later recovered. The main stem of the plants died within 14 days (Plate 5.2), but the roots still looked healthy although growth was rather slow. A few days later a secondary shoot emerged just above the upper root region and within another few days nodules appeared on the roots of those plants (Plate 5.3). By this time the plants were flowering. This delayed nodulation deserves further investigation. It is interesting that the ability of plants to withstand the physiological stress induced by the absence of aeration corresponds with their ability to nodulate.

The concentration of Ag^+ used in the nutrient solution was the same as that used in work by Arshad and Frankenberger (1988) as a foliar spray, in earlier work on the antagonistic effects of this element towards C_2H_4 action in plants.

Plate 5.1a. Effect of Ag^+ on lateral root growth in peas grown in hydroponic culture ($+O_2/+C_2H_4$ treatment).

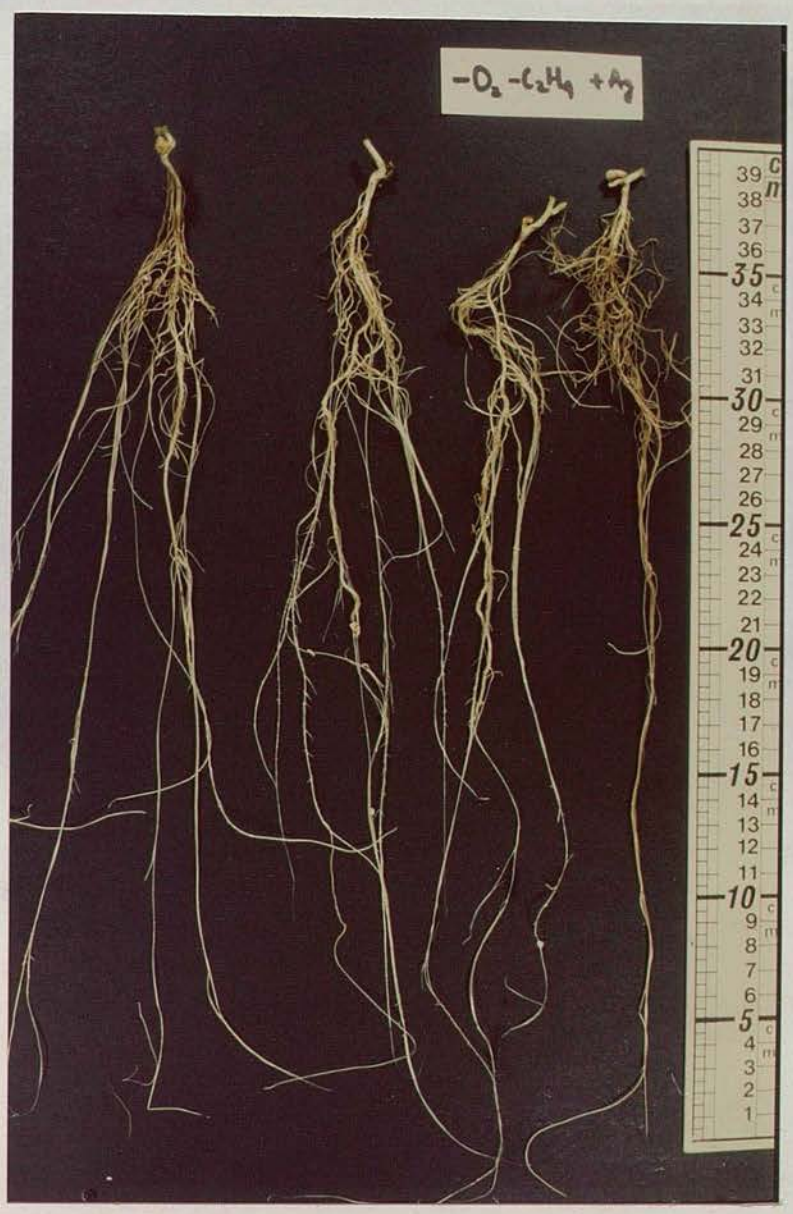


Plate 5.1b. Effect of Ag^+ on lateral root growth in peas grown in hydroponic culture ($-O_2/-C_2H_4$ treatment).

Plate 5.2. Shoot development of peas grown in hydroponic culture without the addition of nutrients, O_2, C_2H_4, Ag^+ or Co^{++}.

Plate 5.3. Root and nodule development of peas grown in hydroponic culture without the addition of nutrients, O_2, C_2H_4, Ag^+ or Co^{++}.

It is clear that a much lower concentration should have been used around the root system, to avoid toxic effects, which inevitably made interpretation of effects as C_2H_4 difficult. However, there was insufficient time available to repeat this work at a lower Ag^+ concentration.

5.3 Comparison of C_2H_4 production by roots of a range of bean cultivars, and effects on the shoots

The production of ethylene by the roots of a range of bean cultivars (Section 3.1) was measured. Ten-day-old seedlings were transplanted into replicate Kilner jars, then the root atmospheres were sampled at 3-day intervals over a 15-day period and analysed for C_2H_4 (Section 3.4).

Ethylene was produced by all 12 cultivars (Table 5.1). Generally, the greatest rate of accumulation in the root atmosphere was during the first 3 days, after which the rate slowed down. The rates over the initial period varied about 5-fold between the lowest and the highest. Seven out of the 12 achieved concentrations of 5 ppm or more. The concentrations generally remained constant between days 9 and 12 before falling significantly by day 15.

The plants growing under the influence of the higher C_2H_4 concentrations showed signs of abnormal leaf growth (Plate 5.4a,b) and necrosis and ultimately defoliation between days 10 and 15. Those cultivars which were low producers of C_2H_4 (N1 1085, V6754, N1 l622, N1 1098) did not exhibit the abnormal leaf morphology exhibited by the high producers.

Table 5.1. Endogenous C_2H_4 production by a number of bean cultivars

Bean cultivar	Concentration of C_2H_4 (ppm) at different times after transplantation				
	3d	6d	9d	12d	15d
V2121	8.35	11.20	11.51	11.35	4.48
V2851	5.81	8.08	10.30	10.32	6.00
V6111	4.02	7.23	10.16	11.02	7.58
V6905	6.93	9.59	9.89	8.44	5.68
V4407	8.35	10.98	10.03	12.14	7.53
V6906	7.27	11.43	11.18	11.62	5.91
V6033	8.06	9.95	10.66	11.20	7.95
V6896	5.0	8.55	10.66	11.39	6.77
N1 1085	4.23	8.26	9.00	9.01	5.04
V6754	1.68	2.75	4.29	4.45	3.25
N1 1622	2.33	3.38	4.18	4.23	3.43
N1 1098	2.83	4.23	4.63	4.75	3.45

Plate 5.4a. Leaf development of bean grown in perlite medium in the presence of endogenous C_2H_4 (cultivars V2121 and V6896).

Plate 5.4b. Leaf development of bean grown in perlite medium in the presence of endogenous C_2H_4 (cultivars V6033 and V4407).

Ethylene is known to be a natural regulator of leaf abscission (Jackson and Osborne, 1970). The decline in C_2H_4 concentrations around the root systems between days 12 and 15 suggested that some C_2H_4 may have diffused via the roots to the shoots, causing abnormal leaf growth and abscission.

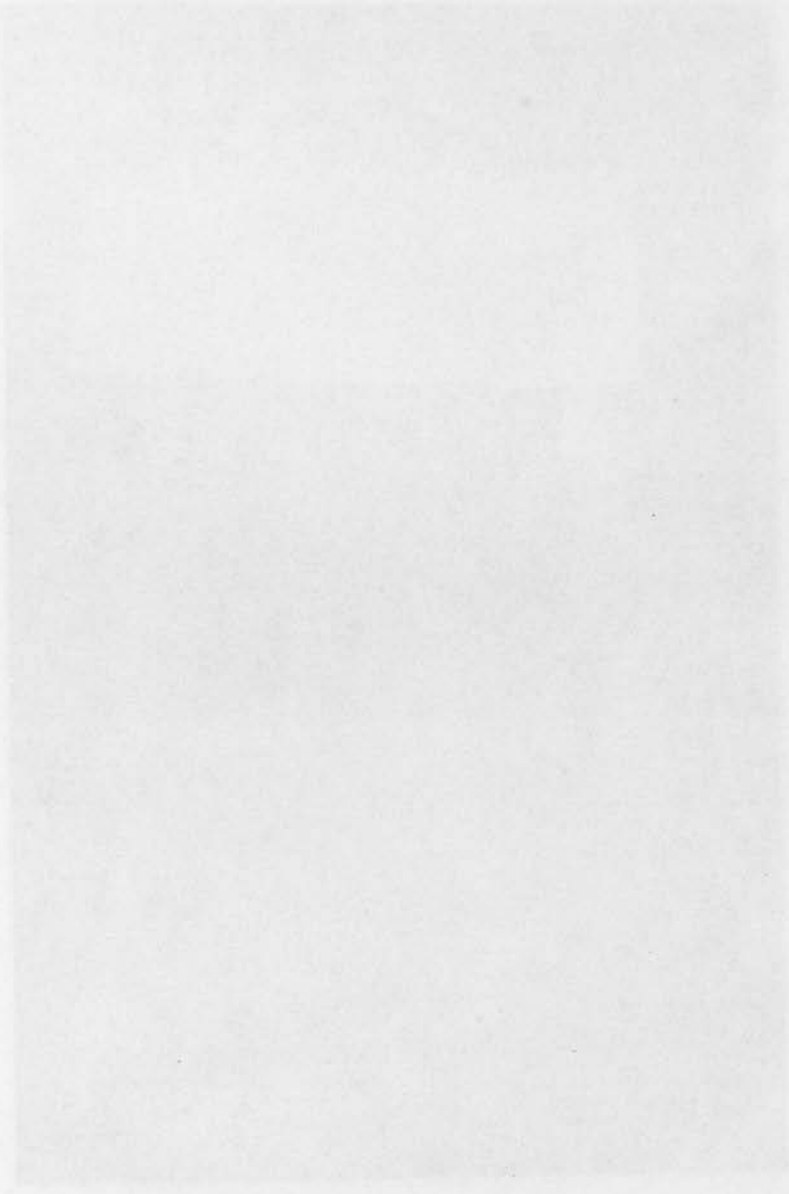
To investigate this a small experiment was carried out with two treatments. In one treatment, C_2H_4 was continuously scrubbed out of the root atmosphere by means of $Hg(ClO_4)_2$ traps, and in the other treatment C_2H_4 was allowed to build up naturally. In both treatments symptoms of abnormal leaf growth did occur (Plate 5.5). However, the severity of the effect was greater in those plants which were growing with their roots continuously exposed to C_2H_4, and also the symptoms appeared 2-3 days earlier than in the other treatment.

Leaf defoliation was observed in both treatments, which suggested that a build-up in concentration of endogenous C_2H_4 around the root systems was not wholly responsible for the aberrant leaf morphology; some other factors might have been involved. For example, the plants were all grown in nitrogen-free nutrient solution and it was possible that lack of nitrogen might have had a role.

To test this hypothesis, another small experiment was carried out along the same lines as the one described above, but with the inclusion of treatments with and without nitrogen. Inclusion of nitrogen did not bring about any significant overall reduction in leaf damage. However, the symptoms in the +N treatment appeared 2-3 days later than in the -N treatment, in the absence of C_2H_4, indicating an effect of N on the rate of damage of development.

Plate 5.5. Leaf development of three cultivars of bean grown in perlite in the presence or absence of endogenous C_2H_4 (treatments grown in the absence of C_2H_4 are situated to the right of the variety label on each basket).

In general, the roots of all the plants looked very healthy and there were no obvious abnormalities in their growth (Plate 5.6).



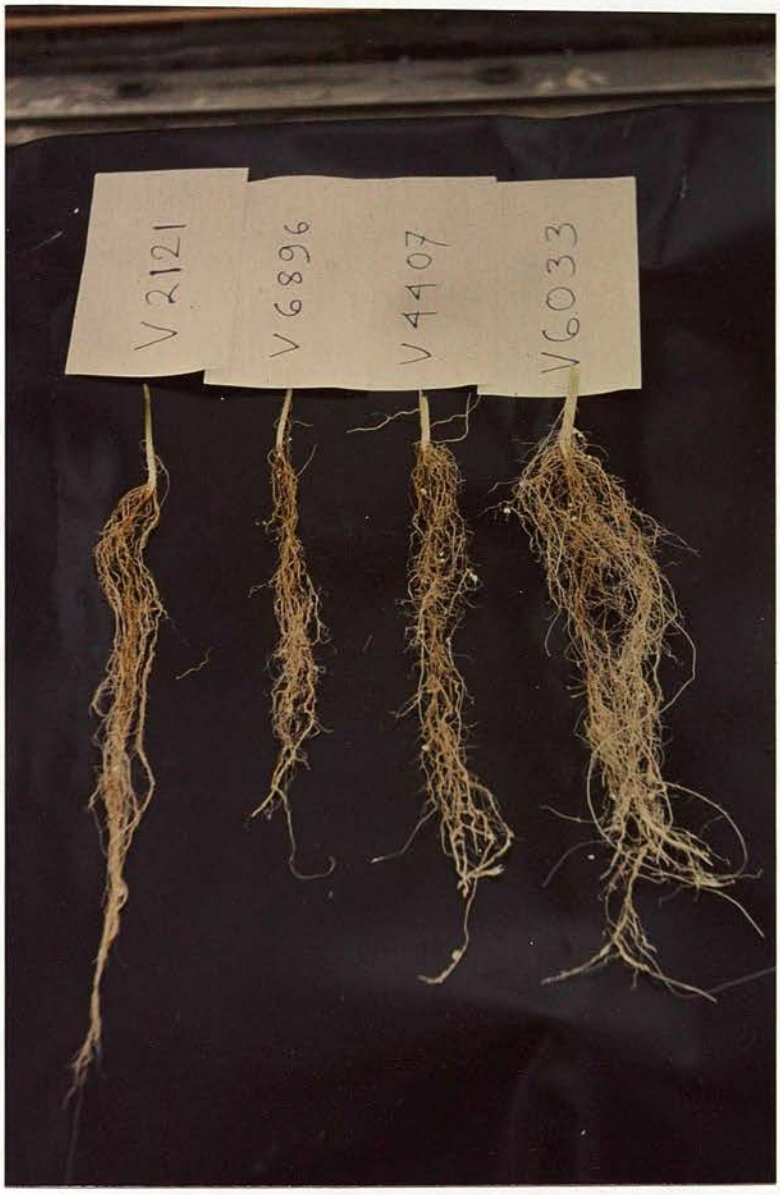


Plate 5.6. Root development of bean grown in perlite medium in the presence of endogenous C_2H_4.

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