COMPETITION STUDIES IN *RHIZOBIUM LEGUMINOSARUM*

by

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DEDICATION

This work is dedicated to my wife and children who persevered many hardships during my three years absence from them.

ABSTRACT

Competition among strains of Rhizobium for nodule formation is a complex phenomenon which involves multiple interactions. In this study an attempt has been made to examine the interactions of host genotype, bacterial genotype and environmental factors as they affect competition among strains of Rhizobium leguminosarum for nodulation of Pisum sativum, Vicia faba and Lens esculenta.

Competition among strains of R.leguminosarum for nodulation of P.sativum, V.faba and L.esculenta was studied in pairs as well as with a mixture of the six strains. Bacteria from randomly selected nodules were identified by "finger printing", using the intrinsic resistance of the strains to low levels of antibiotics. Wherever necessary this method was supplemented by phage typing, which was found to be more sensitive, but only contents of one nodule could be examined per Petri dish.

Three strains of R.leguminosarum, selected on their competitive properties in the previous experiment were used together and in pairs to study their competitiveness on five cultivars of P.sativum. The rhizobia within the nodules were identified with fluorescent antibodies.

The genotype differences of the three genera had greater effect on competition than did the genotype differences of the cultivars of P.sativum.

Symbiotic interactions among the three strains of R.leguminosarum and the six cultivars of P.sativum were studied. One of the strains was ineffective, forming many, small white nodules, which did not fix nitrogen. The other two strains were effective. The number of nodules formed by each strain on each cultivar appeared to be controlled by the cultivar, but was not found to correlate to the competitiveness of the strains in mixed inoculum.

Host strain interactions were also studied using varying mixtures of the strain in the inoculum. The effect of antagonism, probably through bacteriocin production, and synergism was apparent, although the host genotype effect was still predominant.

Two environmental factors, soil moisture and temperature, were studied in relation to the competitive hierachy of the strains; both affected the competitiveness of the strains, nodule number and nitrogen fixation.

The role that root exudates from P.sativum, V.faba and L.esculenta may have in influencing the attraction of strains of Rhizobium to roots of appropriate hosts was examined. The chemotactic response of the six strains tested varied with different exudates though it was not always related to attraction to a preferred host.

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GENERAL INTRODUCTION

Nitrogen is one of the most abundant elements in nature. Most of it is present in a fixed form in the earth's crust in rocks and sediments. According to Delwiche (1970) the atmosphere contains about 3.8×10^{15} tonnes of molecular N_2 and 18×10^{15} tonnes of N is found in the lithosphere. The soil accounts for only a minute fraction of lithosphere N and only a small proportion of this soil N is directly available to plants. This occurs in the form of NO_3^- or NH_4^+ ions.

Nitrogen plays a crucial role in primary productivity and it is one of the most important elements in animal and plant nutrition. Dry plant material contains about 2 to 4% N. In comparison with C content which is about 40% it appears rather low, but N is an indispensable element as it is a constituent of the numerous organic compounds of general importance (amino acids, proteins, nucleic acids). In the majority of plants the inorganic nitrogen requirement is met by uptake of ammonium and/or nitrate from the soil solution. Higher plants are major contributors to the large amounts of N which is continuously being converted from the inorganic to the organic form.

In many tropical agricultural soils nitrogen is (a nutrient) deficient. Correction of this deficiency is attempted by the use of fertilizer. The process by which the fertilizer is made, the Haber-Bosch process, requires high temperatures and pressures and hydrogen which is obtained from methane and other hydrogen feed-stocks like petroleum and other hydrocarbons. Nitrogen can also be added to the soil through rain water, lightning, industrial processes, mineralization and biological nitrogen fixation.

Biological nitrogen fixation is by far the most important non-industrial process by which N from the relatively inert inorganic molecular form in the atmosphere is fixed and converted to an organic form. So far only prokaryotes are capable of fixing (assimilating) molecular nitrogen. Nitrogen fixed by microorganisms accounts for 2 x 10^8 tonnes N per year (Quispel , 1974, Werner et al.,1980).

The enzyme responsible for this process is nitrogenase which is capable of reducing not only N_2 to NH_3 but also a variety of other triple bonded compounds such as acetylene ($HC \equiv CH$) to ethylene ($H_2C = CH_2$) and hydrogen cyanide ($HC \equiv N$) to methylamine ($H_3C - NH_2$) which can still break up to methane (CH_4) and ammonia (NH_3).

Its ability to reduce acetylene to ethylene is the basis of a rapid and sensitive assay used to measure nitrogen fixation (Dilworth, 1966, Hardy et al., 1973, 1977;Havelka and Hardy, 1976). The enzyme is produced and located in the bacteroid cell. Since the enzyme is oxygen-sensitive, all nitrogen fixing organisms have a mechanism for controlling oxygen or avoid it altogether by producing nitrogenase only under anaerobic conditions. The leghaemoglobin present in the host cell cytoplasm (Verma and Bal, 1976; Verma et al., 1978b, 1979) plays a role in transporting O_2 to the host cell cytoplasm at a sufficiently high rate for oxidative phosphorylation (Verma, 1981) while providing a sustained low pO_2 for the bacteroids.

The plant-bacteria collaboration that exists between Rhizobium and legumes is so intimate that one may conceive of the association as a new form of life. The complexities of nodule tissues and the high degree of specialization for gaseous and enzyme regulation and vascular transport indicate a long-standing co-evolution between the two partners.

The family Leguminosae has a worldwide distribution and is currently estimated to have 16,000 - 19,000 species in about 750 genera (Allen and Allen, 1981). In economic importance it is second only to the grasses, Gramineae; in size only to the Orchidaceae and the Compositae. Legumes

constitute more than 50 per cent of forest trees in the humid tropics. The often quoted estimates of 70 x 10^6 tonnes of nitrogen fixed per year probably do not include the fixed nitrogen pool of dominant tropical forests since no data exists on these nodulated giants. Many species of legumes particularly in the subfamilies Mimosoideae and Caesalpinioideae are valuable for their timber, dyes, tannins, resins, gums, insecticides, medicines and for fibres. Numerous members of the Papilionoideae are economically important as edible and highly nutritive crops for human and animal consumption, for forage fodder, ground cover, green manures, and erosion control, and as major sources of pollen and nectar for bees. They also act as pioneer crops in marginal and scrubland.

Rhizobium as described in the eighth edition of Bergey's Manual of Determinative Bacteriology (Jordan and Allen, 1974) is an aerobic non-spore forming Gram-negative short rod. They are distinguished physiologically by their characteristic fast or slow growth and production of acid or alkali, but the one characteristic that validates all of them is their ability to form nodules on a leguminous host (and on Parasponia (Trema)) (Trinick, 1973; Trinick, 1976; Trinick and Galbraith, 1976; Akkermans et al., 1978).

Early investigators regarded rhizobia as one species capable of nodulating all legumes, but now seven major

species are recognized based on the hosts they nodulate.

Table 1: Cross inoculation groups within the Leguminosae (Trinick, 1982 in Nitrogen fixation in plants edited by Vincent, J.M.).

Rhizobium species	Group	Representative genera
R.leguminosarum	Pea	*Pisum*, *Vicia*, *Lens*, *Lathyrus*
R.phaseoli	Bean	*Phaseolus*
R.meliloti	Medic	*Medicago*, *Melilotus* and *Trigonella*
R.japonicum	Soybean	*Glycine*
R.trifolii	Clover	*Trifolium*
R.lupini	Lupin	*Lupinus*, *Ornithopus*
R.spp.	Cowpea	A great number of genera from 3 sub-families
R.spp.	Others	eg. *Lotus*, *Cicer*, *Leucaena*, *Mimosa* *Sesbania*.

The assumption that each species of *Rhizobium* nodulates only plants from the specified cross-inoculation group and that all rhizobia within such a group nodulate all other members of the group (Fred *et al.*, 1932), has lost credibility (Allen and Allen, 1939; Wilson, 1944). Jordan and Allen (1974) writing in Bergey's Manual stated that

taxonomic position of Rhizobium based on the cross-inoculation (or plant affinity) concept is controversial and can be regarded as only tentative. Graham (1976) stated that among the limitations to the cross inoculation concept of classification was that (i) it had been shown that there was cross-infection from one affinity group to the other;(ii) there were insufficient data as only 8 - 9% of legume species had been examined for nodules and less than 1% had been studied with respect to their symbiotic relationship with nodule bacteria and (iii) there were scanty biochemical data.

Serological analysis of Rhizobium species (Graham, 1969) yielded 3 broad groups which were in agreement with the numerical taxonomic studies of Graham (1964a). De Ley and Rassel (1965) used DNA base composition and the position and the number of flagella as taxonomic criteria and found that the peritrichously flagellated fast growing rhizobia had a percentage GC composition ranging from 58.6 to 63.1% while the subpolarly flagellated, slow growing strains, ranged from 62.8 to 65.5%. Vincent (1977) found that fast-growers were differentiated from slow-growers by acid production while the latter were alkali producers.

Rhizobia can be introduced into the soil on the legume seed or in granules or liquid in a process known as inoculation. Soils supporting legumes develop Rhizobium

populations which may be effective or ineffective on a particular legume crop. In such a situation, the inoculum for the sown legume (often added in low numbers relative to soil populations) has to compete with the established rhizobia to become established in sufficient numbers to form reasonable proportions of nodules. The introduced strain has also to compete with other soil microorganisms for substrates and suitable niches in which to grow.

Competition is a complex phenomenon which involves multiple interactions between (i) host genotypes (ii) the symbionts and the environment and (iii) competing Rhizobium strains.

Preferential selection of a particular strain by the host legumes has been considered to be one of the mechanisms that confers competitive advantage to a strain (Russell and Jones, 1975a; Vincent and Waters, 1953). The events that lead to successful nodulation of one host plant by a particular strain are many and diverse.

There is evidence that plant lectins determine host range specificity through binding to characteristic carbohydrate groups on the cell wall of the bacteria (lipopolysaccharides (LPS) and extracellular polysaccharide (ECPS)). The polysaccharide composition of LPS varies in different strains of the same species (Carlson et al., 1978; Zevenhuizen et al., 1980). This

indicates that the binding of different strains would also be different. Bohlool and Schmidt (1974) showed that lectins from soybean bound to 22 out of 25 strains of R.japonicum, but not to any of the 23 strains of rhizobia from other cross inoculation groups. A glycoprotein from clover seeds (Trifolium repens) specifically bound to cells of R.trifolii, but not cells of other Rhizobium species (Dazzo et al.,1978). This protein, trifoliin - A, was also found on root hair surface of clover using an immunolocalization technique (Dazzo et al., 1978).

Antibody raised against purified LPS of one R. leguminosarum strain agglutinated some, but not all other R.leguminosarum strains as well as some R.phaseoli strains (Zevenhuizen et al.,1980). No straightforward correlation between LPS composition and host specificity was found.

Dazzo and Hubbell (1975a) suggested that the preferential adsorption of infective versus non-infective cells of R.trifolii occurs on the surface of T.repens roots by a cross-bridging of their common antigens with a multi-valent T.repens lectin. Later Dazzo et al. (1976) showed that this adsorption occurred through a 2-deoxy-glucose-sensitive receptor site. But Hrabak et al. (1981) did not find 2-deoxy-glucose in the R.trifolii LPS and its presence in extracellular polysaccharides was considered debatable.

Although the lectin-binding hypothesis is very attractive doubts have been raised against it. Chen and Phillips (1976), for example, observed the polar attachment of fluorescent-antibody coated R.leguminosarum cells to root hairs and root surfaces but noted the lack of specificity in attachment. Law and Strijdom (1977) also commented on this hypothesis after studying the binding of fluorescein isothiocyanate lectins from a number of legumes to a large collection of strains. They failed to find a correlation between the ability of various Rhizobium strains to nodulate soybean and to bind to fluorescein isothiocyanate-labelled soybean lectin (FITC-SBL). They also found that some heterologous strains of rhizobia did bind to FITC-SBL, although FITC-SBL did not bind to all the homologous R.japonicum.

These complexities suggest that recognition and infection may involve a multistep process rather than a single step mechanism (Bauer, 1981; Dazzo and Hubbell, 1982) and also that various Rhizobium species or strains may differ in the repertory of recognition mechanisms that they use.

In the soybean-R.japonicum symbiosis the suggestion is that a polysaccharide component of the Rhizobium cell wall is specifically recognized by lectin on the soybean root surface. In the clover-R.trifolii symbiosis however, the

suggestion is that a lectin from clover roots binds with a polysaccharide on the surface of both bacteria and root hairs (Dazzo and Hubbell, 1975; Dazzo *et al*, 1976). The lectin thus provides a bridge between root hairs and bacteria.

The difference between the two suggestions for the clover-*R.trifolii* symbiosis and that for the soybean-*R.japonicum* symbiosis is that the former requires a specific polysaccharide on the root hair surface.

Looking at the evidence as a whole one must echo the conclusion of Sequeira (1978) that the number of annoying exceptions is sufficient to question the validity of the lectin models. This, however, does not mean questioning the importance of attachment, but the attachment of bacteria to plant cell surfaces can only be regarded as one step in the infection process, just as the attachment of a rust fungus to a leaf surface by means of an appresorium is only one step in its infection process.

The first genetic evidence that plasmid genes in *Rhizobium* may be involved in the establishment of symbiosis and host range specificity was reported by Higashi (1967). He mixed together two *Rhizobium* species, *R.trifolii* and *R.phaseoli* in liquid culture and then eliminated the *R.trifolii* cells from the mixed culture by the use of phage. He checked other characteristics to show

that there were no phage-resistant R.trifolii remaining in the mixture before he added it to plants. He was thus able to demonstrate that the ability to infect clover could be transferred from R.trifolii to R.phaseoli.

Transfer of chromosomal DNA by conjugation was demonstrated in R.leguminosarum by Beringer and Hopwood (1976) and in R.meliloti by Mead and Signer (1977) and Kondorosi et al.,(1977a). Interspecific crosses were successfully carried out by Johnston and Beringer (1977) and Johnston, Setchell and Beringer (1978). The very first convincing evidence of the role of plasmids in controlling host range specificity and nodule formation was given by Johnston et al., (1978) using R.leguminosarum strain carrying a resident transmissible plasmid within which had been inserted a drug resistance gene. This strain acted as a donor in crosses with R.trifolii and R.phaseoli. Transconjugants arose at a frequency of about 10^{-2} and all of them were capable of forming nodules on peas as well as on their original hosts, Trifolium and Phaseolus. Moving a symbiotic R.trifolii plasmid into other Rhizobium species was found to alter host range (Beynon et al.,1980; Hoykaas et al.,1981). Transfer of an R.leguminosarum plasmid to a non-nodulating (Nod^-) R.leguminosarum mutant allowed the recipient cell to nodulate peas (Johnston et al.,1978; Brewin et al., 1980).

Recently Brewin et al.(1983) introduced symbiotic plasmids from R.leguminosarum into three different strains that lacked plasmid-linked symbiotic determinants. The recipient and the introduced symbiotic plasmid contributed about equally to competitiveness in forming nodules. Genes specific to the symbiosis have been identified by mutations. Several mutagenic techniques have been used to generate symbiotically-defective mutants (Maier and Brill, 1978; Beringer and Hopwood, 1976; Beringer et al.,1978; and Banflavi et al., 1981).

Legume plant breeders have been interested in producing new high yielding varieties through the incorporation of desirable traits into new genotypes. It is only until recently that attempts at breeding for increased N_2-fixing ability or compatibility with highly efficient N_2-fixing strains of Rhizobium (Chowdhury and Doto, 1982; Mytton, 1978; Rupela and Dart, 1982; Nambiar et al.,1982; Minchin et al., 1978) were begun. Selection for improved symbiosis is, unlike conventional breeding, however, because two genotypes have to be considered, the plant and the rhizobia as well as the effect of the environment and the interaction between all the three variables.

In search for plant genes for development of symbiosis with Rhizobium, Auger et al. (1979) determined the total complexity of mRNA from uninfected root and mature nodule

tissues by RNA-CDNA hybridization and concluded that a similar number of structural genes were active in root and nodule tissues. Using differential hybridizations to nodule-CDNA, a nodule-specific fraction was isolated (Auger and Verma, 1981). These sequences were found to be host specific and were found on host polysomes only after infection. These cloned sequences should make it possible to test the effects of mutations in Rhizobium and applications of phyto hormones, on the nodulation process (Verma et al., 1981).

Recent results with Vicia faba and a range of R.leguminosarum strains showed that 75% of the phenotypic variation in dry matter yield was due to host-strain interactions (Mytton, 1978, 1981; Mytton, Sherbeeny and Lawes, 1977). Thus when the genotype of the plant or Rhizobium alone is selected for only about half of the total pool of the genetically determined variation is considered.

It has also been observed that while breeding for some other traits it is possible to produce lines or cultivars which are no longer nodulated by rhizobia. Dart (1981), for example, reported that while breeding Arachis hypogea for rust resistance some plants in the F_2 population were no longer nodulated by strains that nodulated both the parents and F_1 plants. Preliminary genetic analysis

suggested that failure to nodulate was due to a pair of independent recessive genes. A pair of recessive genes rj1 rj1 were found by Devine and Weber, (1977) and Devine et al. (1979) to be responsible for specificity (restrictive nodulation) in soybean, but no clear explanation was given as to why it should happen in soybean and not in other legumes. The hypothesis was that a substance called rhizobitoxine conferred to R.japonicum the ability to nodulate rj1 rj1 soybeans. Among legumes, the pea is particularly amenable to genetic study being a self-pollinating diploid with a comparatively short generation time and a well marked linkage map (Blixt, 1974).

Another factor influencing competition has been found to be compatibility of the rhizobia and the host plants, sometimes expressed as effectiveness (Trinick, 1982). Effective symbionts can be, but not always, the most competitive in nodule formation. Robinson (1969a) found that the most effective strains of R.trifolii were the most successful in forming nodules. He concluded that the nitrogen fixing ability of the strain in association with a particular legume host somehow influenced the preferential infection of the strain into the particular host.

Marques Pinto et al.(1974) supported Robinson's proposition as they found that in both R.meliloti and

R.trifolii there were more cases of inferior strains forming less nodules on their hosts in paired competition with effective ones. Gaur and Lowther (1982) also found the *R.trifolii* strains which were highly competitive increased clover dry matter and N uptake as compared to the less competitive ones.

Other workers have found that effective strains were poor competitors with the ineffective ones. Van Rensburg and Strijdom (1969) using seven strains of *R.japonicum* on three cultivars of *Glycine max* found that three of the strains which produced effective nodules in pure culture on the three soybean cultivars practically failed to nodulate any of the cultivars when present in mixed culture with other strains. In this connection, the dominance of the different strains on different cultivars stresses the influence of the plant on the competitive ability of a strain; a process which may be considered as selective competition.Means *et al.*(1961) also demonstrated differential competition among strains of *R.japonicum* for nodulation of roots of soybean. They found that a strain of *R.japonicum* which amounted to 1% of the rhizobia in the rhizosphere of soybeans produced 85 % of the nodules.

The speed of nodule formation and the number of nodules formed by strains when inoculated singly on a particular host was not found to correlate with

competitiveness of the strain in mixed inoculum (Lahiri, 1974). Similarly Ruiz-Arguesso et al. (1977) found no correlation between effectiveness and competitiveness in R.japonicum and Skrdleta and Pelikan (1973) found that in R.japonicum nodule weight, nodule volume and effectiveness could not be correlated with competitiveness either. The nature of this competitiveness is, therefore, not apparent.

Johnston and Beringer (1976b) reported on pea root nodules containing more than one Rhizobium species. The results of inoculating peas with various strains of R.leguminosarum together with R.trifolii or R.phaseoli showed that at least one nodule on at least half of the plants contained the heterologous species. The frequency of such nodules was 2-27%.

Ineffective strains of R.leguminosarum mixed with R.trifolii yielded ineffective mixed nodules on peas showing the defects of the ineffective R.leguminosarum could not be overcome by R.trifolii strains i.e. there was no genetic complementation between R.trifolii and R.leguminosarum in a pea plant (Rolfe and Gresshoff, 1980). The ineffective strain of R.leguminosarum did not inhibit acetylene reduction when it occurred in the same pea nodule with the effective strain of R.leguminosarum (Johnston and Beringer, 1976a). When non-infective

strains of R.leguminosarum were inoculated with R.trifolii on pea no nodules were formed. When the non-infective strains of R.leguminosarum were mixed with effective strains, none of the non-nodulating strains were recovered from the nodules showing that although R.leguminosarum permitted entry of R.trifolii and R.phaseoli,it did not allow its non-nodulating mutant to do so. This may be due to severe competition that exists between very closely related strains but does not occur in species which are genotypically distant.

The basis of specificity in recognition mechanisms may reside in breaking a barrier or barriers, by the appropriate cross-inoculation group to host penetration. It appears that the heterologous Rhizobium species use the apparatus induced by R.leguminosarum so that there is a partial breakdown in specificity. The fact that R.trifolii was unable to fix nitrogen in pea nodules suggests that more than one barrier prevented Rhizobium of one cross-inoculation group forming effective symbiosis with a legume of a different group.

Rolfe and Gresshoff (1980) using a refined bacteroid isolation procedure were able to locate the inoculant strains within the nodule. They found that in a nodule containing two different strains of Rhizobium, only one of

the strains could be isolated from a single plant cell and never both together. Mixed nodules in which each strain was present accounted for 30%. Agrobacterium tumefaciens, however, was not "helped" into the nodules of Trifolium by a nodulating R.trifolii showing that there was some means of exclusion against accidental entry of the non-invasive bacterium into the nodule. This was in contrast with the findings of Imshenetskii et al. (1976) who found that A.tumefaciens was capable of entry into alfalfa root nodules. When a non-nodulating mutant of R.meliloti was mixed with a nodulating strain mixed nodules resulted and, in some cases, these mutants were found as bacteroids. The mutants on reisolation were still non-nodulating showing that no change had occurred to them due to passage through the nodule. Similarly they found that R.trifolii formed mixed nodules with R.leguminosarum on Trifolium repens at a frequency of 1-3%. The R.leguminosarum strain, however, did not acquire broadened host range, due to passage through Trifolium repens nodule, to nodulate both hosts, Pisum sativum and Trifolium repens nor did R.trifolii and R.leguminosarum strains lose their capacities to nodulate clover and pea respectively.

Van Rensburg and Strijdom (1972) also observed a bacterial contaminant in nearly 50% of the nodules of Vigna sinensis Endl that were inoculated with a mixture of slow-growing R.japonicum and fast-growing cells of WBK

which was a non-infective strain. The numbers of non-infective fast-growing and infective slow-growing cells within the soybean nodules were determined and they found that the ratio of non-infective to infective cells in the nodules was approximately $1:10^7$. Although the fast-growing contaminant was isolated from nodules produced by all Rhizobium spp.tested, it was found in larger percentage of the nodules formed by the slow-growing strains of R.japonicum, R.lupini and strains of cowpea group, than in nodules formed by the fast-growing strains of R.meliloti, R.trifolii and R.leguminosarum.

Tests carried out on this contaminant could not confirm whether it was Agrobacterium or Rhizobium since it did not form nodules on any legume in pure culture and it had no resemblance to Agrobacterium.

In some interactions Rolfe and Gresshoff (1980) observed a marked inhibition of the growth of the nodulated plants when non-nitrogen fixing mutants were mixed with effective strains. In the majority of cases though there was no observable difference to the nodulating response of the plant. These findings suggested that the ineffective mutants may have had a systemic plant response to regulate the effectiveness of

nodulation. Using a combination of two mutants defective in nodule persistence (Nop$^-$) one third of the plants appeared as partially effective may be as a result of large nodules which were formed by a mixture of these strains. The mutants retained the Fix$^-$phenotypes though. They concluded that this cooperation between symbiotically defective mutants of Rhizobium was a form of cross-feeding and not genetic complementation. Furthermore there were four possible steps where the inter-nodule interactions could occur between different strains of Rhizobium. These steps are: (i) infection (ii) nodule inititation (iii) bacteriod development and (iv) persistence of nodule function. Presumably, therefore, this particular combination of Nop$^-$mutants can interact in such a manner as to slow down the onset of early senescence within some plants and thus enable some nitrogen-fixing nodules to occur.

Broughton et al.(1980) observed that in mixed inoculum containing two strains of R.leguminosarum, a non-nodulating strain blocked the nodulating strain on P.sativum cv Afghanistan. Using ^{35}S-labelled rhizobia they were able to study the kinetics of their binding to seedling roots. In non-competitive situations both organisms bound to the root within 2 hours of exposure.

The non-nodulating mutant was found to bind onto the roots faster than the nodulating strain thus successfully competing for attachment on the root surface in mixed inoculation. Radioactive extracellular polysaccharides and lipopolysaccharides from either organism failed to bind to the seedling roots in measurable quantities. All the added radioactivity was recoverable in the seedling nutrient solution at the end of the experiment.

The fact that a non-nodulating strain was found to successfully compete with nodulating strains for binding on the root surface in mixed inoculation showed that the binding mechanism *per se* cannot explain the specific recognition mechanism for nodulation unless it can be demonstrated that there are specific sites. Broughton *et al.* (1982) further found that for this blockage of a nodulating strain by a non-nodulating strain to occur both cells must be alive and that cooperation of the plant was required.

The effect of relative growth rates of the competing strains on competitive success for nodule formation was studied by Nicol and Thornton (1941) and Purchase and Nutman (1957). Although relative nodulation response was found to be related to the size of the infective *Rhizobium* population in the rhizosphere or to the density of the inoculum no direct proportions were observed. Successful

nodulation response of a strain was found to be influenced by its competitive ability when used in mixed inocula (Vincent and Waters, 1953; Thornton and Kleckowski, 1950; Johnson et al., 1965). Again in this case competitiveness was generally found to be independent of the relative numbers of the bacterial strain used.

Franco and Vincent (1976) found that five out of the seven cases of nodulation success could be attributed to the number of cells of each strain on the root surface and the relative number of nodules formed by that strain. Inter-strain competition has been related not only to proportionate representation in the supplied inoculum, but also to the populations recovered from the seedlings at appropriate intervals after inoculation (Labandera and Vincent, 1975; Marques Pinto et al., 1974). Weaver and Fredrick (1974) found that for successful nodulation of the inoculum strain in a field which had indigenous strains of R.japonicum, large numbers ($3.3x10^6$ rhizobia per seed) were required.

There are a number of factors which can alter the proportions of the competing strains. One such factor was demonstrated by Schwinghammer (1975) and Marshall (1956) who found that the ability of some rhizobia to produce bacteriocins, temperate bacterio-phages and antibiotics can alter the proportions of the strains of rhizobia grown

in broth culture or in peat. Schwinghammer and Brockwell (1978) found that when strains which produced bacteriocins (and or lysogenic phages) were grown together with sensitive strains in broth culture the latter decreased proportionally to the former. In sterile peat the effect of bacteriocin production was found to be less severe, maybe because some of the bacteriocin was adsorbed to the peat. These results are relevant in the preparation of mixed strain inoculants if the depletion of one or more strains is to be avoided.

No information is available on the competitive advantage of bacteriocinogenic or lysogenic rhizobia in the complex field environment, although it is reasonable to assume that the production of those inhibitors could have greater adaptive survival value during bacterial multiplication in the nutritionally enriched plant rhizosphere.

Chemotactic behaviour, a familiar phenomenon in enteric and other bacteria, was also found in some Rhizobium strains (Vincent, 1970; Carlile, 1980). Beringer et al. (1979) reported on the fact that under controlled growth conditions, a single pea plant growing in 200ml of a mineral salts medium lacking fixed nitrogen and carbon compounds provided sufficient nutrients for auxotrophic mutants of R.leguminosarum to grow from a

population of about $10^2 ml^{-1}$ to $10^8 ml^{-1}$ within 2-3 weeks; all amino acid, purine, pyrimidine and vitamin auxotrophs tested responded in this way. The beneficial effect of the host on soil populations showed the selective advantage for rhizobia of an association with legume roots. Currier and Strobel (1976) showed that Rhizobium species were attracted to plant root exudates of both legumes and non-legumes. They also found that while chemotaxis was not absolutely specific, and therefore not mandatory to nodulation, most plants which are nodulated produce root exudates that attract Rhizobium.

Gitte et al.(1978) showed that a Rhizobium strain from Cicer arietinum (chick-pea) was attracted by the various amino acids and sugars they detected in the exudate. Napoli and Albersheim (1980) and Ames et al. (1980) observed that non-motile and non-chemotactic mutants could nodulate legumes, but were slower than the wild type. Hunter and Fahring (1980) and Gaworzewska and Carlile (1982) demonstrated that both non-chemotactic and non-motile mutants of R.leguminosarum were less competitive than wild types.

Bhaqwat and Thomas (1981) found that cowpea root exudates elicit faster nodulation response by Rhizobium species measured as the rate at which successful infection was initiated after inoculation with Rhizobium. Nutman

(1965), Mori and Bauer (1980) and Schmidt (1979) suggested that there were certain compounds or substances in legume exudates which stimulated the homologous Rhizobium strain for effective symbiosis. Those substances were, for example,found by Bhuvaneswari and Bauer (1978) to influence the formation of appropriate receptors for attachment to lectins on all 11 strains of R.japonicum which were cultured din root exudates of soybean (Glycine max). Only 5 out of 11 strains cultured under synthetic salt medium developed receptors. Heterologous species were also found to form receptors though to a lesser extent. These results demonstrated that the environment provided by plant roots is an important factor in the development of specific lectin receptors on the cell wall of R.japonicum but this does not mean that host specifity can be fully explained by this phenomenon.

The effect of root exudates on the formation of fibrillar appendages, pili or cellulose which may help in bacterium attachment to the root surface has been observed on R.meliloti and R.trifolii adsorbed on medic and clover roots respectively (Dart, 1971; Dart and Mercer, 1964). R.trifolii growing in clover root exudates was found to develop similar appendages (Dazzo, 1980). Root extracts from pea and clover were found to selectively degrade the capsular polysaccharide of the complementary rhizobia

(Bauer, 1980). It is apparent that, in addition to the lectin-binding mechanism, the recognition of the host plant by the complementary Rhizobium may be mediated by special signal compounds excreted by the host plant (Schmidt, 1979; Bauer, 1981). Van Egeraat (1975), for example, found that homoserine released from the roots of pea seedlings selectively stimulated the growth of R.leguminosarum when a mixture of Rhizobium strains, belonging to different cross-inoculation groups, was present in the medium surrounding the young pea roots.

The effect of the environmental factors on competition of various strains of rhizobia for nodulation of host legumes is very important. Successful inoculation of legumes in countries, like Tanzania, which has tropical and subtropical climates with wide variations in precipitation, even in one growing season, calls for selecting strains which are suitable for these environments. Strains which have been selected under temperate conditions may not be suitable as inoculants in the tropical countries, either because they might fail to become established in the rhizosphere or may be out-competed by indigenous rhizobia which are more adapted to tropical conditions.

Weber and Miller (1972) conducted experiments on the effect of varying soil temperatures on the symbiotic interaction between Rhizobium japonicum serogroups and

soybean. They found that plant development and nodulation were generally favoured by increasing temperature from10°C to 30°C. The optimum temperature for fresh weight of shoots and roots, plant height, nodule number and nodule weight was 30°C. Serogroup distribution of R.japonicum in the nodules was influenced by soil temperature. Serogroups which were infrequently recovered at lower temperatures were dominant at 30°C whereas serogroups forming the majority of nodules at 10 or 15°C formed fewer nodules at 30°C.

Caldwell and Weber (1970) also found that the distribution of different serogroups of Rhizobium japonicum in soybean nodules varied with the planting dates which corresponded to different temperatures. There were also variations in populations from year to year, since temperature patterns were different for each of the three years of study.

Masterson and Sherwood (1974) studied the nodulation pattern of R.trifolii on Trifolium repens (white clover) and Trifolium subterraneum (subterranean clover) as it is influenced by soil pH and soil moisture. They found that the proportion of the effective isolates that formed nodules on both hosts in Kilmuckridge soil (pH 5.3) was not consistently affected by soil pH but was affected by soil moisture. This was in agreement with the findings of

Singer et al. (1964),Singer (1964) and Masterson (1968). Osa-Afiana and Alexander (1979) found that survival of both R.trifolii and R.japonicum was affected by soil moisture. Flooding the soil greatly reduced the distribution of the inoculum in the soil.

The effect of pH was demonstrated by Russell and Jones (1975) who found that the proportion of nodules formed by an effective strain of R.trifolii was much higher in acid soil but was altered in favour of the ineffective strains at neutral and alkaline pH. Effectiveness of R.trifolii strains on commercial clover was found to correspond to the pH of the soil from which the isolates were made (Holding and King, 1963; Masterson, 1968; Jones and Burrows, 1969 and Russell and Jones, 1975).

When studying ecology, survival and competition between strains of Rhizobium one needs reliable, simple and rapid methods of recognizing different strains from nodules (Beringer, 1982). The aim of these methods should be to observe differences which can be measured and tested without difficulty. All differences observed are basically genetic and the more closely related the organisms are the fewer the gene differences and hence the more difficult it is to distinguish them. Several methods have been used to characterize strains in ecological studies: intrinsic antibiotic resistance (IAR) (Pinto et al., 1974; Johnston and Beringer, 1975; Diatloff, 1977;

Josey et al.,1979; Beynon and Josey, 1980; Kremer and Peterson, 1982;Bromfield et al.,1982; Stein et al.,1982), high level antibiotic resistance (Schwinghammer and Dudman, 1973; Johnston and Beringer, 1976b; Pankhurst, 1977; Jones and Bromfield, 1978; Bromfield and Jones, 1979; Pain, 1979), phage typing (Gerretsen et al., 1923, Laird, 1932; Dorosinskii, 1941; Conn et al., 1945; Demolon, 1951; Kleczkowska, 1957; Staniewski et al., 1962; Darrel and Wahba, 1964; Staniewski, 1970), bacteriocin production and sensitivity (Roslycky, 1967; Schwinghammer, 1971; Hirsch, 1979), serology (Vincent, 1941, 1970, 1974, 1982; Purchase et al.,1951; Koohty and Faber, 1961; Skrdleta, 1965; Means et al., 1964; Kishinevsky and Bar-Joseph, 1978; Read, 1953; Dudman and Brockwell, 1968; Dudman, 1977, Morley and Jones, 1980; Tchan, 1982), use of auxotrophic mutants (Higashi, 1967; Johnston and Beringer, 1976a; Beringer et al.,1980) and utilization of different substances as carbon and nitrogen sources (Graham, 1964; Graham and Parker, 1969).

The choice of the method to use depends on the types of experiment, facilities available and personal prejudice.

Intrinsic resistance to low levels of antibiotics has been used with varying levels of satisfaction. Josey et al. (1979) found that reliable identification of R.leguminosarum strains could be made with reference to

stock cultures or nodule isolates from host plants inoculated and grown under laboratory conditions. Throughout the experiment they found that resistance to low levels of antibiotics was a stable property of the strains examined and the results could be reproduced. Kremer and Peterson (1982) were able to screen 12 strains of R.leguminosarum, R.phaseoli, cowpea Rhizobium and peanut Rhizobium from different sources using this method. They found that crude suspension of nodule material or isolates of nodules of pea plants that were inoculated with specific, single strains exhibited the same patterns as the stock strains showing that it was possible to bypass the step of isolation from nodules. Inconsistencies, however, occurred on bush beans (Phaseolus), cowpea and peanut nodules which had been stored at - 10°C for 10 months. They attributed these inconsistencies to low numbers found in the nodules and concluded that inoculant size was critical in obtaining consistent growth of rhizobia from nodules on control and antibiotic supplemented media. Stein et al. (1982) found variations in the pattern of the intrinsic drug resistance of the Rhizobium phaseoli strains and attributed them to differences in inoculum concentration and the time of incubation. Bromfield et al. (1982) found that the

antibiotic concentration gradient method, using gradient plates, (Szybalski and Bryson, 1952) was suitable for identifying fast growing rhizobia, but unsuitable for slow growers. Antoum et al.(1982) successfully used low level antibiotic discs to identify 20 strains of R.meliloti. The results were amenable to computer cluster analysis.

Phage typing has been used extensively in epidemiological studies of bacteria of medical importance (Meynell, 1964, Stocker, 1955, Wentworth, 1963; Juhasz and Bonicke, 1965) and only limited use made on studies with Rhizobium particularly by Staniewski (1970). Although phage typing is very sensitive, the method is unsuitable for ecological studies where large numbers of nodules have to be screened. Only one nodule isolate can be typed per Petri dish.

Serological methods used judiciously and with the understanding of their underlying principle provide one of the most reliable methods for identifying strains in ecological studies. The use of serology is, however, restricted by the limited number of serotypes of Rhizobium which are found and also the raising of specific antisera is time consuming and expensive.

The fluorescent antibody technique (FAT) is one of the serological methods most suitable for the direct examination of nodules however small (Callao and Olivares,

1964) without the need to isolate bacteria. Trinick(1969) was able to examine even the smallest nodules from subterranean clover using this technique. Jones and Russell (1972) were also able to identify strains of R.trifolii in competition on white clover. Van der Merwe et al. (1972) used the method to detect a bacterial contaminant in nodule squashes of leguminous plants, Vigna sinensis, Arachis hypogea and Glycine max inoculated with either pure cultures of Rhizobium strains or with mixed suspensious of Rhizobium and a contaminant strain whose antiserum had been prepared. In some cases van de Merwe and Strijdom (1973)found the technique more sensitive than immunodiffusion. Lindermann et al. (1974) used it to detect doubly infected nodules of R.japonicum.

It is generally accepted that the serological characterisitcs of Rhizobium strains are stable and do not change with time, culture conditions or plant passage (Vincent 1954).

In this study an attempt has been made to examine the interactions of the genotypes of the symbionts and the environment and their effects on competition between strains of R.leguminosarum for nodulation of Pisum sativum, Vicia faba and Lens esculenta. Effect of the three genotypes of plants of different genera on competition were studied using six strains of R.leguminosarum.

Some cultivars of P.sativum were selected for studying the influence of closely related genotypes on competition.

The influence of soil moisture and temperature on competition was also examined using one cultivar of P.sativum as the two factors are very important. pH effects on both the plant and competition of introduced strain with indigenous rhizobia were observed.

GENERAL MATERIALS AND METHODS

Rhizobium and its legume host interact at many levels: as distinct organisms; as adjoining cell types within a complex developing organ; as inhabitants within a common cell wall boundary, and as producers of shared metabolites and compounds.

An understanding of the Rhizobium legume symbiosis requires examining interactions between the two organisms from several points of view and using various techniques and materials such as microscopic examinations,biochemical and genetic analysis and physiological studies of the two partners. Apart from these studies the environment in which the nodule is formed plays a crucial part in the interaction and has to be studied in relation to the genomic interactions of the symbionts.

BACTERIAL STRAINS AND MEDIA

Initially ten strains of Rhizobium leguminosarum from the Rothamsted Collection of Rhizobium (RCR) and four isolates from pea nodules received from Morogoro, Tanzania, were tested for their symbiotic performance, intrinsic resistance to antibiotics and sensitivity to phages. Thereafter six strains, five from the Rothamsted Collection and one from Tanzania, were selected for use in further work on competition.

The characteristics of these six strains are summarized in Table 2 below:

Table 2 : Characteristics of the six strains used in competition for nodulation of Pisum sativum, Vicia faba and Lens esculenta.

Strain	Synonym	Isolated from	Symbiotic response
RCR 1001	Dutch Pea	Pisum sp.in in Holland	Effective on P.sativum, V faba and L.esculenta.
RCR 1021	14	P.sativum in Delft Holland	Ineffective on P.sativum V.faba, V.hirsuta, but effective on L.esculenta
RCR 1031	V1	Vicia sativa in Dorset, England	Effective in P.sativum, V.hirsuta poorly effective on L.esculenta Ineffective on V.faba
RCR 1045	V19E	P.sativum in Ireland	Effective on P.sativum, poorly effective on V.faba,V.hirsuta and L.esculenta
RCR 1056	23C	V.faba in Wales	Effective on V.faba and L.esculenta, but ineffective on P.sativum
MR4	M_4	P.sativum in Morogoro,Tanzania	Effective on P.sativum, V.faba,V.hirsuta. Ineffective on L.esculenta.

Source: Rothamsted Rhizobium Collection Handbook (Dye 1980) and my personal observations.

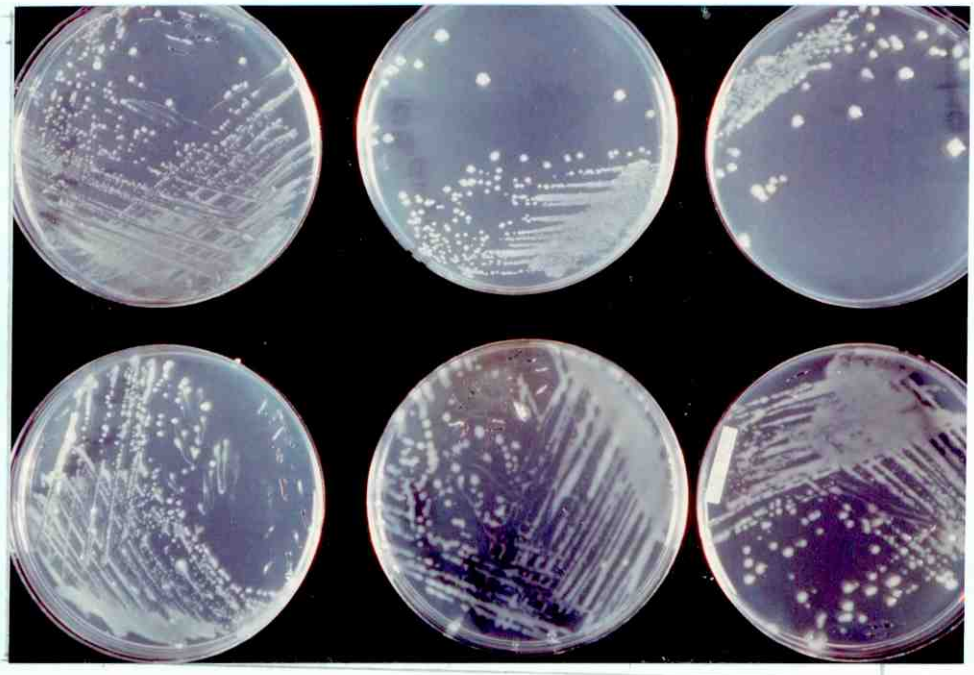


Plate 1: Appearance of the six strains of Rhizobium leguminosarum when streaked on Tryptone Yeast Extract (TY) agar

Front row, left to right: RCR 1001, RCR 1045, RCR 1056. Back row, left to right: RCR 1012, RCR 1013, MR4. The strains had been incubated for 3 days at 28°C.

The strains were grown on Tryptone Yeast Extract (TY) medium (Beringer, 1974) solidified with 1.5% agar for plates and slants. Exponentially growing cells from the slants were harvested in sterile distilled water centrifuged and stored in 20% glycerol at -20°C in a domestic freezer (Zelazna-Kowalska and Lorkiewz, 1971b). Yeast Extract Mannitol,(YEM) (Vincent, 1970) was used to grow and store the cultures on slants at 4°C. Minimal medium supplemented with sodium glutamate, sodium succinate and vitamins (Beringer, 1974) was used for making mutants and growing cultures for chemotaxis and motility experiments. In all cases, unless otherwise stated, all strains were incubated at 28°C and harvested after 3 days of incubation.

Details of the media generally used are given here below:

TY Complete medium (Beringer, 1974)

Bacto Tryptone (Difco)	5.0 g
Bacto Yeast extract (Difco)	3.0 g
$CaCI_2 \cdot 6H_2O$	1.3 g

The ingredients were dissolved in 1 litre of deionized water and dispensed into flasks containing agar before autoclaving if solid medium was required.

TY slants were made by dispensing the above medium into sterilized universal bottles when still hot (60°C)

and laid on a wooden block slanting at an appropriate angle.

Yeast Extract Mannitol (YEM) Agar (Vincent, 1970).

The following is the composition of medium per litre of distilled water:

K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.1 g
Mannitol (Difco)	10.0 g
Yeast extract (Difco)	1.0 g

The solution of the components (200ml) were dispensed in 250ml Erlenmeyer flasks with agar and autoclaved.

Minimum medium (Y) (Beringer, 1974).

The following is the compositioning of the minimum medium dissolved in 1 litre deionized water:

$MgSO_4 \cdot 7H_2O$	0.1 g
$CaCl_2 \cdot 6H_2O$	0.1 g
K_2HPO_4	0.22 g
$FeCl_3$	0.02 g

The components were usually made up in stock solution, 1000 times the final concentration, and kept at 4°C. The $FeCl_3$ stock was dissolved in 0.1 ml of IN HCL and made up with water. Then 1 ml of each was added to 1 litre of

deionized water and pH adjusted to 6.8 before dispensing into flasks containing 1.5% agar and autoclaved at 15 lb pressure for 15 minutes.

The medium was supplemented with the following ingredients which were prepared and autoclaved separately for 10 minutes only.

The following supplements were used:

Name of Supplement	Concentration of stock solution (gm/l)	Required final Concentration (gm/l)	Volume of stock taken to make up desired solution (ml)
Sodium glutamate	44 g	1.1 g	25 ml
Sodium succinate	27 g	135 mg	5 ml
Biotin	0.1 g	0.75 mg	7.5 ml
Thiamin	0.1 g	0.75 mg	
DL Panto-thenic acid	0.1 g	0.75 mg	

(Biotin, Thiamin, DL Panto-thenic acid: dissolve together)

GROWING OF RHIZOBIUM CULTURES

When agar culture media in the 250 ml Erlenmeyer flasks were required they were melted in a microwave oven or in a pressure cooker at 10 lb pressure. The melted medium was then dispensed in 9 cm vented plastic Petri dishes (Sterilin, Richmond, Surrey). Usually 20 ml were dispensed each time and left to set in a microflow cabinet for 15 minutes.

Liquid cultures were made by inoculating a loopfull of an expentially growing culture from a Petri dish or slant in 100 ml of TY in 250 ml Erlenmeyer flask and incubated in a shaker (Cooled Orbital Incubator from Gallenkamp) at 110-120 revolutions per minute.

Growth rates of the strains were determined at 12 hour intervals for 96 hours.

BACTERIOCIN PRODUCTION AND SENSITIVITY

To determine bacteriocin production and sensitivity the six strains of R.leguminosarum were tested on one another. The producers were grown on TY agar for 2-3 days until colonies were large enough.

They were then removed by inverting the Petri dish onto a sterile velvet cloth mounted on a replicating block. This procedure was repeated twice to try and remove as many cells as possible. The remaining cells were killed by exposing them to chloroform vapour for 45 minutes in a fume extractor chamber. The plates were then removed and put in a laminar flow cabinet to "air" for 30 minutes. Then 0.1 ml of the indicator strain was mixed with 3 ml of soft agar precooled to 50°C and immediately overlayed on the chloroform sterilized producer colonies. The plates were allowed to set for a few minutes and incubated in an inverted position for 2-3 days. Bacteriocin production and sensitivity was recorded where clear zones around the producer colonies were observed in a confluent growth of the indicator strain. Thus the indicator strain would be recorded as being sensitive to the bacteriocin produced by the producer strain.

The results of the test are given in Table 3 below.

Table 3: Bacteriocin production and sensitivity of the six strains of R.leguminosarum.

Producer Strains	Indicator strains					
	1001	1012	1031	1045	1056	MR4
1001	**	-	-	-	-	-
1012	-	**	-	-	-	-
1013	+++	-	**	-	+++	-
1045	++	-	-	**	-	-
1056	-	-	-	-	**	-
MR4	-	-	-	-	-	**

** = same strain (not done)

+++ = large zone of inhibition

++ = medium zone of inhibition

PLANT TYPES AND PLANT MEDIA

The legume host plants used were those found in the cross-inoculation group of *Rhizobium leguminosarum* which are:

Pisum sativum L (peas)
Vicia faba L (field beans)
Vicia hirsuta L (hairy vetch)
Lens esculenta L (lentiles)

Of the four host plants *Vicia hirsuta* was later dropped because it formed very few nodules *in vitro* and its nodulation patterns were too variable.

Pisum sativum and *Vicia faba* were successfully grown in 250 ml Erlenmeyer flasks containing 200 ml of Fahraeus (1957) medium whose composition per litre of deionized water is shown below:

$CaCl_2\ 2H_2O$	0.1 g
$MgSO_4 \cdot 7H_2O$	0.12 g
KH_2PO_4	0.1 g
$Na_2HPO_4.12H_2O$	0.15 g
Ferric citrate	0.005 g

The components of the medium were prepared in stock solution 100 times its final concentration. The medium was supplemented with 8 ml of Gibson's (1966) trace

elements whose composition per litre of water is given below:

$H_3 BO_3$	2.86 g
$Mg80.4H_20$	2.03 g
$Zn\$0_4.7H_20$	220 mg
$CuSO_4.7H_20$	80 mg
$H_2M_o0_4.H_20$	80 mg

The full plant medium (PF) (Cartwright,1967).

This medium was also used for growing plants particularly for exudation experiments. Its composition per litre of distilled water is given below:

$CaC0_3$	3.0 g
$CaCl_2H_20$	446 mg
KCl	65 mg
KH_2PO_4	200 mg
$Mg\$0_4.7H_20$	700 mg
$Na_2\$0_4$	200 mg

The components were supplemented with 8 ml of the following trace elements dissolved in 1 litre of water.

KCl	6.5 g
Kl	750 mg
$FeCl_3.6H_2O$	2.5 g
H_3BO_3	1.9 g
$MgSO_4.4H_2O$	2.2 g
$ZnSO_47H_2O$	290 g
$(NH_4)_6Mo_7O_{24}$	35 mg

GROWING PLANTS ON AGAR

Seeds were first dipped in 95% ethyl alcohol (ethanol) and washed with sterile distilled water before soaking them in sodium hypochlorite (13% available chlorine) for 30-40 minutes depending on the size and texture of the seeds. Thereafter they were washed seven times in sterile distilled water and left in the seventh wash to soak for 1 hour. They were then placed separately on TY agar to germinate. To facilitate faster germination the seeds were covered with black polythene sheet and incubated at 28°C. When the seeds had germinated properly they were transferred onto the Fahraeus agar in 250 ml Erlenmeyer flasks for inoculation.

INOCULATION OF SEEDS

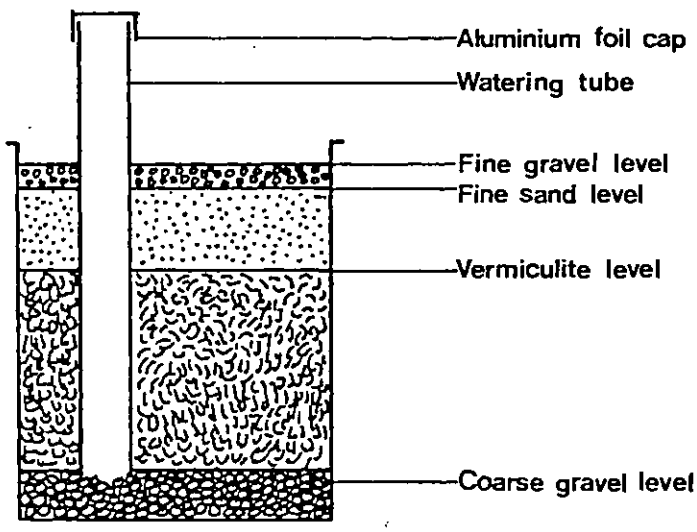
The pre-germinated seeds were inoculated with exponentially growing culture of *Rhizobium* cells either grown in TY liquid medium or on TY slant which was washed off with 10 ml of sterile water. In mixed strain inoculants, for example in paired strain mixtures, equal volumes of the strains containing about 10^8 cells/ml were mixed before use and shaken well.

The liquid inoculum (2 ml) was usually inoculated on the germinated seeds in each flask and swirled round to cover the surface of the agar. The flasks were then covered with black polythene bags and tied with elastic bands until the shoots, etiolated in the dark, were long enough to be pulled through the neck of the flask and foam bungs replaced.

PLASTIC CUP ASSEMBLY

Lentils (*L.esculenta*) were unable to nodulate on agar in the Erlenmeyer flasks. An alternative method was used and this was a modification of the glazed jar assembly (Burton *et al.*, 1972). Sand, gravel and vermiculite were sterilized separately at 15 lbs pressure in an autoclave for 30 minutes. The plastic cups were obtained commercially and were sterile before use. The assembly

Diagram 1; The plastic cup assembly used for growing Lens esculenta.



PLASTIC CUP ASSEMBLY

The above assembly is a modification of the Glazed Jar Assembly (Burton et al, 1972.)

was arranged as shown in Diagram 1.

The bottom of the cup was layered with one inch of coarse gravel, a sterilized glass tube inserted and horticultural grade vermiculite poured into the cup to keep the tube in position to about 3 inches from the top. A layer of mason's sand was then added on top of the vermiculite to about 1½ inches of the top. Nitrogen free nutrient solution (Fahraeus medium and Gibson's trace elements) was then added through the watering tube until the whole assembly was filled "per ascensum". A spatula which was sterilized by dipping it in 95% alcohol and flaming was used to dig two holes in the sand in each plastic cup. Seeds were placed in the holes by means of sterilized forceps and inoculated with 5 ml of expotentially growing broth culture. Fine gravel was finally layered on top to cover the seeds and sand as shown. Subsequent watering was done through the tube using 30 ml of Fahraeus nutrient solution and sterile distilled water alternatively twice a week.

CONDITIONS OF GROWTH OF PLANTS

Unless otherwise stated, all plants were grown in the glass house with temperatures and lighting controlled thermostatically to give 18/15°C, or 20/25°C day/night temperatures and 14/16 hour day length. Relative humidity

was kept above 60-70% by a simple humidifier.

Treatments were generally randomized so that spatial effects were nullified or at least minimized.

Controlled environment cabinets were used for specific experiments and these are describes in appropriate chapters.

HARVESTING OF PLANTS AND NODULES

Unless otherwise stated plants were harvested after six weeks of inoculation. Appropriate measurements of shoot weight were made immediately if fresh weights were desired. The plants were then dried in an 80°C oven for twenty four hours after which dry weights were obtained.

Nodules were harvested by individually excising them from the roots using forceps and scissors. The nodules were then placed in 20% glycerol and stored at -20°C in a domestic freezer.

ISOLATION OF RHIZOBIA FROM ROOT NODULES

When the nodules preserved as above were used they were first thawed and then washed twice with sterile distilled water to remove excess glycerol and or debris. They were then sterilized in sodium hypochlorite (13%

available chlorine) for 15-60 seconds depending on the size of the nodules. Hydrogen peroxide (20 volumes) was sometimes used particularly for small nodules. In this case nodules were kept in the hydrogen peroxide for 4-6 minutes. After sterilizing,either in sodium hypochlorite or hydrogen peroxide, the nodules were washed thoroughly six times in sterile distilled water. To isolate rhizobia from nodules each nodule was crushed in a drop of sterile distilled water in a Petri dish using a glass rod. It was possible to crush 10-15 nodules in one Petri dish at a time. To inoculate, a platinum wire loop was dipped into the crushed nodule and streaked on the surface of agar. The loop was then sterilized in the flame after each nodule. The inoculated Petri dishes were incubated at 28°C in an inverted position to avoid condensation of water vapour on the surface of the lid.

IDENTIFICATION OF STRAINS

The various methods used for identifying strains all aim at distinguishing closely related differences which are basically genetic. In this study 3 methods were used for studying strains that formed each nodule in mixed inoculum of up to six strains together.

(a) INTRINSIC ANTIBIOTIC RESISTANCE (IAR) METHOD

This method is also known as antibiotic "finger printing" method because of the different patterns of growth obtained for each strain on a range of antibiotics. It employs the use of a multiple inoculator which was made from aluminium and had 25 michrome prongs. The alignment and depth to which the prongs penetrate the matching wells was determined by the rods and collars in the well units (see Plate 2).

Medium:

All strains were grown on TY medium. Fresh solutions of antibiotics were added to melted TY medium which had cooled to 60°C to give the following final concentrations of mg/ml: neomycin sulphate (NEO) 5,10 (Sigma); kanamycin sulphate (KAN) 10,20 (Sigma; rifampicin (RIF) 2,4 ("Rimactane" CIBA); erythromycin (ERY) 5,10 (Cambrian Chemicals); streptomycin sulphate (STR) 5,10(Glaxo); nalidixic acid (NAL) 10,20 (Sigma) and tetracycline hydrochloride (TET) 0.5,1.0 (Sigma).

Procedure:

Antibiotic test plates were prepared as shown above by putting the appropriate volume of antibiotic from the concentrated stock solution in 200 ml of molten TY agar (60°C) and mixing thoroughly.

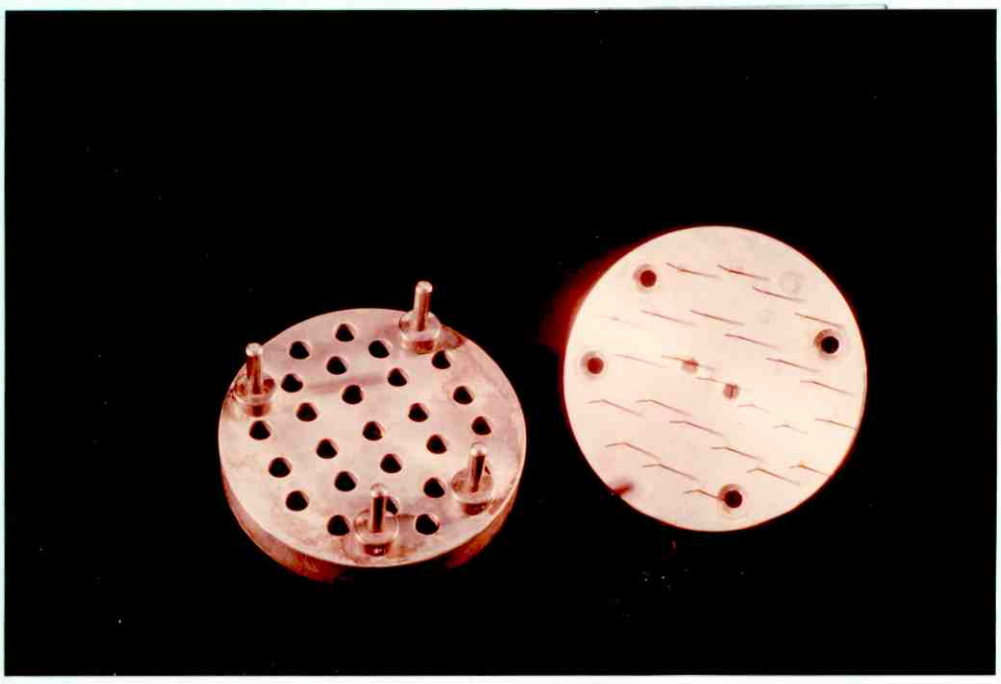


Plate 2: Multiple inoculator apparatus consisting of wells in the bottom part and prongs in the upper part. The collars ensure that the prongs can only reach a certain uniform depth and pick equal volume of the culture suspension.

Thereafter 20 ml of it was dispensed in each Petri dish and left to set in a microflow laminar flow cabinet for 15-20 minutes. Stock solutions were made up in sterile liquids and were not sterilized.

Below is the list of the antibiotics used giving their stock solution concentrations, solvents used, their final concentrations and the volumes used for 200 ml of TY agar.

Antibiotics concentrations used for "finger printing"

Anti-biotic	Stock solution conc.(mg/ml)	Solvent used	Final conc. (g/ml)	Volume used per 200ml (ml)
Rifampicin (RIF)	10	MeOH	2, 4	40, 80
Streptomycin (STR)	100	H_2O	10, 20	10,20
Kamamycin (KAN)	10	H_2O	10,20	200,400
Tetracycline (TET)	5	H_2O	0.5, 1.0	20, 40
Nalidixic acid (NAL)	10	NaOH	10,20	200,400
Erythromycin (ERY)	100	EtOH	5,10	10,20
Neomycin (NEO)	100	H_2O	5,10	10,20

Bacterial strain cultures that were stored at 20°C in glycerol were grown on TY agar for three days until single colonies were obtained, (see Plate 1). A loopful of the colony was picked and emulsified in 0.3 ml of sterile distilled water in marked wells of the multiple inoculator according to the plan drawn on paper. Each strain was replicated 4 times to give a total of 24 wells containing bacterial strains and 1 well with only sterile distilled water. The titre of the strain thus suspended in distilled water was found to be around 10^8 cells/ml estimated by serial dilutions of 0.1 ml of suspension. Each prong of the multiple inoculator transferred about 10^3 cells to the agar. The number of rhizobia transferred to the antibiotic medium by each prong was estimated from the agar removed from replicate inoculation site with a cork borer (6 mm diameter). The agar cores were mashed in sterile water containing beads and the number of rhizobia released was estimated from colony counts on TY agar.

To transfer suspensions from the wells to the antibiotic plates the part with prongs was matched with the bottom part with wells so that all the 25 prongs dipped into the wells at the same depth. The prongs were then stamped on the plates starting with one without antibiotics and ending with another one without antibiotics after going through all the antibiotics plates. This procedure of inoculating control plates was aimed at finding out if there was any carry over effect of the antibiotics on the growth of the control colonies.

Scoring growth on the test plates

The plates thus inoculated were incubated for 3 days at 28°C after which growth was scored using the control plates as standard which was designated number 4 (see Table 4 and Plates 3a & b and 4a & b) Thus 4 means that the colony on the antibiotic plate was the same as that on the TY control plate without antibiotics and 3 was ¾, 2 was a half, 1 was a quarter and - when there was no growth at all. Scores of 4 and 3 were generally regarded as resistant while 2, 1 and - were sensitive (to varying degrees).

This way of scoring was somewhat subjective, but if the same person did the scoring the pattern obtained was found to be reproducible. It was also possible to record minute differences in sensitivity. Stein et al.(1982) also stressed the importance of one person scoring all the plates for each experiment. If a second person was to score the same experiment it was recommended that he or she went over all the scores. The patterns obtained thus were found to be comparable.

Nodule isolates were first grown on TY agar before finger printing. Using nodule isolates for finger printing was attempted but the patterns obtained were so variable that it was not possible to reproduce the finger print of the strain. This was found to be due to variable numbers of cells from each nodule so that each time the

prongs transferred different titres of strains on the antibiotic plates. The time of incubation and the inclusion of reference strains was found to be important.

Double nodule occupancy was recorded when the finger print for one isolate was found to coincide with the pattern of two reference strains.

The table below shows the different finger prints of the six strains of Rhizobium leguminosarum used in this study.

Table 4: Patterns of intrinsic antibiotic resistance of the six strains of R. leguminosarum

	ANTIBIOTICS													
CONCEN-TRATION	RIF		KAN		STR		NEO		NAL		ERY		TET	
STRAINS	2	4	10	20	5	10	5	10	10	20	5	10	.5	1.0
RCR 1001	4	2	4	-	-	-	-	-	2	1	4	2	1	-
RCR 1012	3	3	-	-	-	-	-	-	-	-	2	2	4	2
RCR 1013	-	-	1	-	-	-	2	-	-	-	3	2	-	-
RCR 1045	-	-	-	-	-	-	4	-	2	-	4	4	-	-
RCR 1056	4	4	-	-	2	-	4	3	4	-	-	-	-	-
MR4	2	-	4	-	4	4	4	-	4	-	3	-	2	-

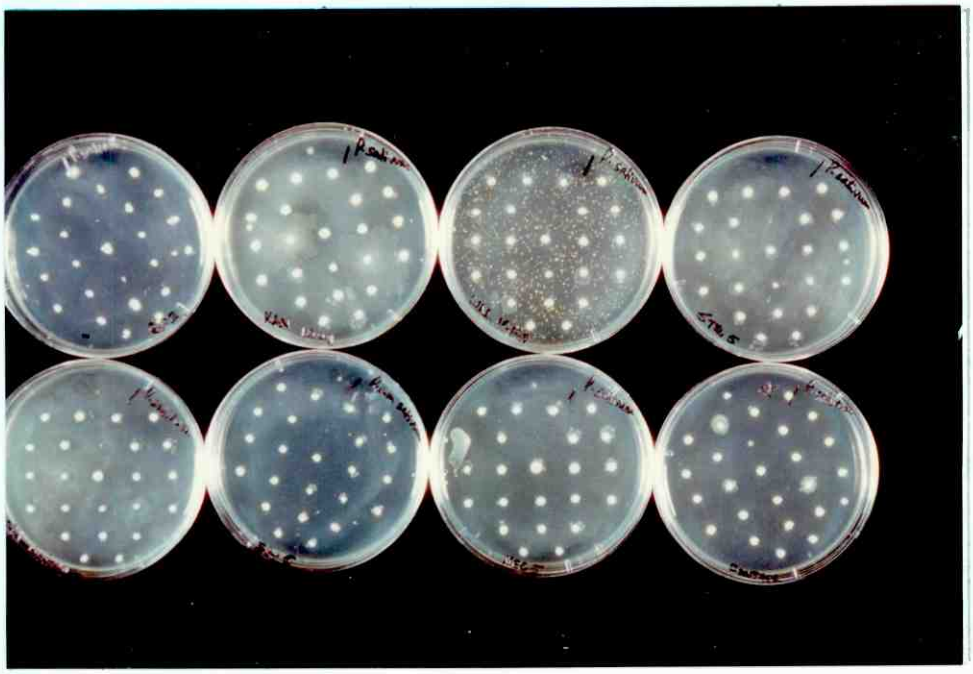


Plate 3a: Finger prints of the six strains of R.leguminosarum placed in wells 1-5 and 8 and isolates from P.sativum nodules in the other 19 wells at low concentration of seven antibiotics.

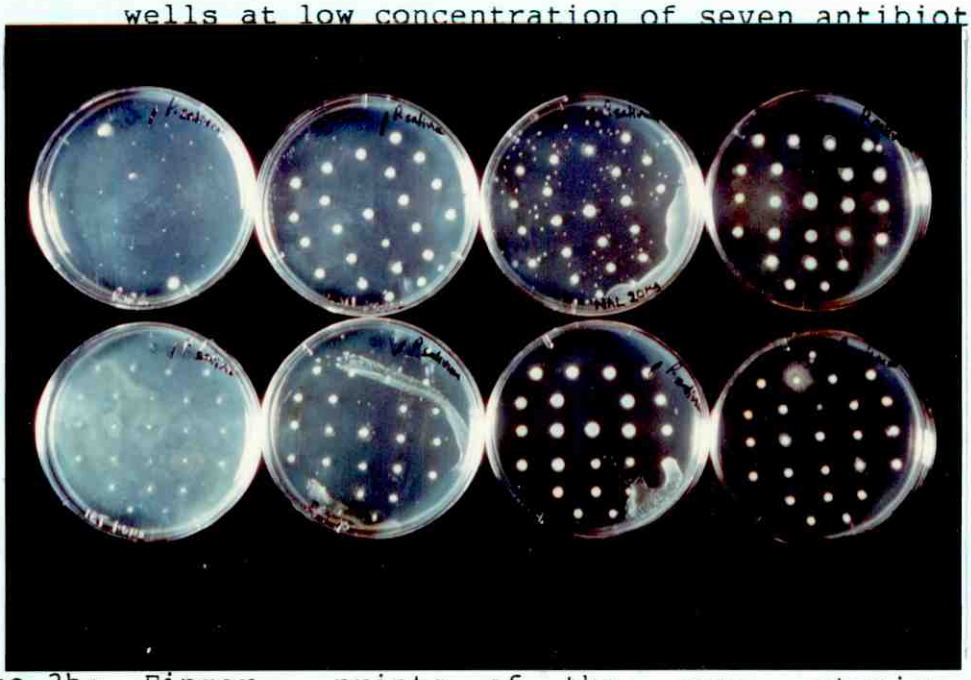


Plate 3b: Finger prints of the same strains and isolates at twice the concentration of antibiotics. The control plate in the bottom left hand corner was used for scoring the colony sizes on the test plates.

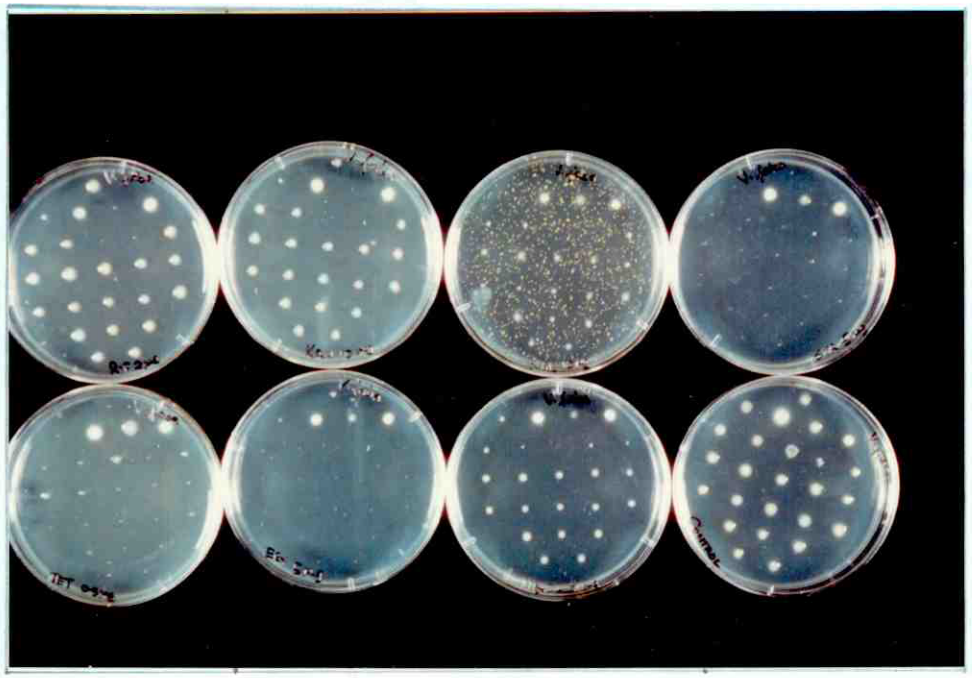


Plate 4a: The finger prints of the six strains of R.leguminosarum placed in wells 1-5 and 8 and isolates from V.faba nodules in the other 19 wells at low concentration of seven antibiotics.



Plate 4b: Finger prints of the same strains and isolates from V.faba nodules of twice the concentration of the antibiotics. Notice that the control plate was unchanged.



Plate 5: Motility and chemotaxis of the six strains of R. leguminosarum was determined on soft agar minimal medium supplemented with succinate and glutamate. The cells migrated in concentric circles from the centre where they were inoculated. From left to right;upper plates 1001, 1012, 1013. Lower plates 1045, 1056 and MR4.

(b) PHAGE TYPING

Bacteriophages were isolated from soil samples obtained from soils near and around Rothamsted. Some of the soils were obtained from Kenya (sent by Dr.S.O.Keya). The other phages (with Jl prefix) were obtained from John Innes Institute, Norwich.

To isolate phages from soil the following procedure was followed:

Soil samples were mixed with 100 ml of Vincent's yeast sucrose phage broth (VS) (Vincent, 1970) whose composition per litre is as follows:

Sucrose	2.5 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
$CaSO_4$	0.16 g
NaCl	0.1 g
$FeCl_3.6H_2O$	0.02 g
Bacto.yeast extract	0.5 g

The soil broth suspension was incubated overnight at 28°C in a rotary shaker (Gallenkamp). The suspension was then centrifuged and the supernatant filtered through a 0.45μ nitrocellulose filter membrane (Millipore or Amicon).

R.leguminosarum strain T3J1 (Beringer and Johnston, 1978) was used as bacterial indicator. To 3 ml of TY soft agar (which contains 0.6% instead of 1.5% agar) was added 0.1 ml of the filtrate and 0.1 ml of T3J1 freshly prepared from a TY slant. The mixture was overlayed on the solid TY agar and allowed to set in a microflow laminar cabinet and incubated inverted for two days at 28°C before observing plaques (clear zones surrounded by a lawn of bacterial growth). High titres of the phages were obtained by removing well formed plaques and dropping the agar portion into VS broth containing T3J1 and incubated overnight in an orbital shaker. Stocks were sterilized by filtering through a 0.25 μ or 0.45 μ nitrocellulose Millipore filter membrane and stored at 4°C in a domestic fridge.

To type different strains stock phages were placed in wells of the multiple inoculator according to a plan drawn on paper. Then 0.1 ml of the exponentially-growing cell suspension was pipetted in TY soft agar and overlayed on TY agar (as above). The soft agar, once set, was stabbed with 25 prongs of the multiple inoculator that had been dipped in the wells containing phage stocks. Between each inoculation the prongs were sterilized by dipping them in 95% alcohol in a Petri dish and flaming to avoid the carry-over of cells of different strains.

The soft agar thus inoculated was incubated for 2 days and the presence of plaques noted and scored by crosses

which denoted the size of the plaque and not necessarily the degree of sensitivity. The table below shows the pattern of phage sensitivity obtained by using 15 phages. Those with Jl (e.g.98Jl) were obtained from John Innes Institute, Norwich, England (Beringer's stock phages). The others were isolated from various soil samples as described earlier.

Table 5:Phage typing of the six strains of R.leguminosarum

Phages	R.leguminosarum strains						
	1001	1012	1013	1045	1056	MR4	T3J1
RL79	-	-	-	-	-	+	++
RL96	-	++	-	+++	-	-	++
RL84	-	+	+	-	++	-	+++
98JI	-	-	-	-	+	-	++
102JI	-	-	-	-	-	+	+
31JI	-	-	-	-	-	+	++
35JI	-	-	-	+++	-	-	++
4JI	-	-	-	+++	-	-	+++
RL104	-	-	-	-	+++	-	+++
RL24	-	+++	++	+++	-	-	+
RL23	-	+++	++	+++	++	-	++
RL29	-	++	-	-	++	-	+
6JI	-	-	-	+++	-	-	+++
RL18	-	-	-	++	+++	-	+++
107JI	-	-	-	-	++	-	+

- = Not lysed, no plaques observed.

(C) IMMUNOFLUORESCENT TECHNIQUE FOR IDENTIFICATION OF STRAINS OF R.LEGUMINOSARUM

There are two immunofluorescent techniques both of which use a fluorescent dye, usually fluorescein isothiocyanate (FITC), the direct and indirect ones. In the direct technique the antibody of the serum in question are labelled by the fluorescent dye, FITC. This fluorescent antibody preparation applied to a smear of cells becomes attached only to those which can react specifically.

The indirect technique avoids labelling of the anti-bacterial antiserum separately by using a second antiserum specific for the animal species that was used to prepare the antiserum, e.g. a goat antiserum specific for rabbit globulin. Only the cells that react with the primary specific antibodies will carry attached rabbit globulin molecules and so will be the only ones to retain the fluorescent antibodies. The indirect fluorescent antibody technique was used in this study.

DEVELOPMENT OF ANTISERUM

Preparation of antiserum against three strains RCR 1012, RCR 1056 and MR4 was done by Dr Govier, D.A. of the Plant Pathology Department, Rothamsted Experimental Station. The procedure according to Campbell et al. (1970)

involved injection of 1 ml of a cell suspension of about 10^9 bacteria per ml in sterile 0.85% NaCl into the thigh muscle of the rabbit's hind leg. The preparation was steamed for 30 minutes to inactivate the flagellar antigens. Some of the cell suspension was mixed with an equal volume of adjuvant (Freund's (Difco)).

After 4 weeks 1 ml of the bacterial suspension without adjuvant was inoculated into the rabbit intravenously. The animal was bled from the large marginal ear vein for the first time, six weeks after the initial inoculation. Other samples were taken every two weeks until the titre was adequate. In this study antisera obtained after the third bleeding was used and had adequate titre (see Tables 6 and 7 of agglutination and FAT tests below).

Blood samples were incubated for 1 hour at 37°C to facilitate clotting. The clot was cut and held at 4°C overnight to help extrusion of the serum which was then drawn off and centrifuged to free it from residual cells. The clear serum was frozen at -20°C in a domestic freezer.

TESTING OF A TITRE

The titre of the antiserum thus obtained was tested by agglutination and by the fluorescent antibody technique. The antisera for strains 1012, 1056 and MR4 were set up in tubes and serially diluted to 50, 100, 200, 400, 800, 1600, 3200 and 6400 cell suspensions (0.5 ml) of the six

strains were added to 0.5 ml of the diluted serum and incubated at 37°C or 52°C. Cross agglutinations are shown in the table below which shows the highest dilution at which the antisera agglutinated with the antigen.

Table 6: Highest dilution of the antisera where agglutintination with the antigens occurred.

ANTIGEN	Strains from where antisera were made		
Strains acting as antigens	1012	1056	MR4
1001	1:400	1:200	NONE
1012	1:400	NONE	NONE
1013	NONE	NONE	1:200
1045	1:50	1:100	1:50
1056	1:100	1:400	NONE
MR4	NONE	NONE	1:400

WORKING DILUTIONS OF THE ANTISERA AND FLUORESCENT ANTIBODY

It is important to determine the appropriate working dilutions of both the fluorescent antibody (FA) and the antisera before they can be used in identification of antigens (bacterial strains) in nodules. This ensures that cross-reactions which may occur at lower dilution of one of them is eliminated by using higher dilution of the other one.

PREPARATION OF SALINE BUFFER

Phosphate saline buffer (PBS) was used for diluting the antibodies, washing the slides as well as for preparation of the 90% glycerol which was used for mounting. The composition of the buffer per litre of water is as follows:

NaCl	8.5 g
Na_2HPO_4 (anhydrous)	12.8 g
NaH_2PO_4	1.56
pH 7.1	

After staining the slides were mounted in buffered glycerol which consists of 9 parts of glycerol and 1 part PBS.

DILUTION OF ANTISERA AND FLUORESCENT ANTIBODY

The antisera were serially diluted as above. FA was obtained from the Wellcome Foundation, was made up as a % (W/V) solution in PBS and was serially diluted to 1:20, 1:40 1:80. The cell suspensions from exponentially growing cultures on slants were dispensed into wells of special fluorescent microscope multitest slides (Flow Laboratories Ltd, Scotland) using a 20 µcapillary tube (Bilbate Precision Glass). The suspensions were air dried

and heat fixed by passing the slide through a flame twice. Antisera dilutions (30 µl) were dispensed on these cell suspensions onto the wells of the multitest slides using Eppendorf Varippette 4710 pipettes (10-100 µl). The slides on a small plastic tray were placed onto wet cotton wool in a larger plastic basin and wrapped well with aluminium foil. The humidity around the slides was thus maintained so that the antisera did not evaporate. The whole assembly was incubated at 37°C for 30 minutes. Thereafter the slides were placed in a rack and washed by gentle agitation in two changes of PBS for 10 minutes. The unconjugated γ- globulins of the antiserum were washed off by then. Slides were drained for 2-5 minutes.

FLUORESCENT STAINING

The fluorescent antibody, at different dilutions was then dispensed as for the antisera. The manner and duration of incubation was the same as for antisera. After incubation the slides were washed in two changes of PBS (ensuring minimum exposure to light) for 10 minutes and then in distilled water for 5 minutes. The slides were drained and mounted in glycerol buffered with phosphate-saline (pH 7.1) and covered with a microscope glass cover slip (Chance Propper Ltd). The specimens thus stained were examined under the fluorescent microscope (Ernst Leitz GmbH) with condenser and filters suitable for

fluorescein isothiocyanate (FITC). Positive cases fluoresce with apple green (pale green-yellow) colour in dark field with bright ground.

The results obtained for this exercise are given in the table below:

Table 7: Maximum dilution of antisera at a given concentration of Fluorescent Antibody which gave fluorescence with the antigens shown.

	ANTIBODY								
	1012			1056			MR4		
FA CONCENTRATION / ANTIGEN	1:20	1:40	1:80	1:20	1:40	1:80	1:20	1:40	1:80
1001	1:200	1:100	1:50	1:400	1:100	-	1:50	-	-
1012	1:800	1:400	1:200	-	-	-	1:100	-	-
1013	1:100	-	-	1:50	-	-	1:400	1:200	1:100
1045	1:200	1:50	-	1:100	-	-	1:100	-	-
RCR 1056	-	-	-	1:800	1:400	1:200	-	-	-
MR4	-	-	-	1:100	-	-	1:800	1:400	1:200

- No fluorescence i.e. no cross-reaction

From the above results titre combinations of the two antibodies appropriate for identifying the strains in various mixtures were worked out and these are shown in the table below:

Table 8: Appropriate FA concentration and corresponding antisera titre for identifying strains in nodules

Strain Combination in inoculum	No of slides	FA titre	Strain	Dilution of the antiserum	Result obtained
1001 + 1056	2	1:20	1012	1:200	+ 1001, - 1056
			1056	1:800	- 1001, + 1056
1001 + MR4	2	1:20	1012	1:200	+ 1001, - MR4
			MR4	1:200	- 1001, + MR4
1056 + MR4	2	1:20	1056	1:400	+ 1056, - MR4
			MR4	1:200	- 1056, + MR4
1001 + 1056 + MR4	3	1:20	1012	1:200	+ 1001,- 1056 - MR4
			1056	1:800	- 1001, + 1056 - MR4
			MR4	1:200	- 1001, - 1056 + MR4

+ = positive, fluorescence

- = negative, no fluorescence

TYPING NODULES

Nodules were washed thoroughly in sterile distilled water before crushing them separately in a drop of PBS and dispensed in multitest slide wells as described above. The number of slides used for each test depends on the number of strains used in the inoculum (see Table 8 above). The procedure of staining was, as described above. Double or even triple occupancy of one nodule can be recorded if the cells fluoresce in both or triple wells containing the same nodule isolate (antigen).

ACETYLENE REDUCTION METHOD

The acetylene reduction method has been used extensively in measuring the activity of nitrogenase, the enzyme responsible for reducing N_2 (Hardy et al., 1968).

Nodulated roots were excised from the shoots and immediately placed in 380 ml bottle and sealed with a suba-seal plug. Air (10% of the volume of the bottle) was withdrawn with a syringe and replaced with an equal volume of welding grade acetylene. The acetylene was collected in a football bladder from a gas cylinder. Incubation of the bottle was done under ambient conditions, but not in direct sunlight,for 30 or 60 minutes (but not longer). To sample the gases a syringe was inserted into the bottle and mixed by filling and emptying it several times. An

aliquot of the gas (about 1 ml) was withdrawn and stabbed into a rubber bung. Then 0.5 ml of the gas was injected into a gas chromatograph equipped with a flame ionization detector (FID) and a suitable column (in this case Porapak N). The areas under the ethylene and acetylene peaks were measured and the amount of ethylene produced was calculated by reference to the area under the peak of a standard of ethylene or a standard curve.

CHAPTER 1

GENOTYPE INTERACTIONS IN COMPETITION

INTRODUCTION

Introducing new strains of *Rhizobium* on a sown legume crop can only be of benefit if the inoculant strain can fix more nitrogen than the indigenous rhizobia. To ensure that this happens is not easy as the introduced strain has to compete with the indigenous rhizobia before it can successfully form nodules. The factors that control the success of any *Rhizobium* strain in forming nodules in the presence of other strains have been outlined in the General Introduction. In this chapter I shall examine how the host genotypes affect the success of different strains for nodule formation.

For all practical purposes *Rhizobium* has been classified on the basis of the host-plants they nodulate. The reason why this is so is because there are differences among the various species of host plants and the various races of root-nodule organism (Baldwin *et al.*,1927). Various workers have found evidence that both the bacteria and the plant contribute to the specificity (Nutman, 1952; Jones and Russell, 1972; Holl and LaRue 1976; Schwinghammer, 1977; Broughton, 1978; Graham, 1982,

Beringer, 1982; and Brewin et al., 1983).

Graham (1982) reported that while symbiotic nitrogen fixation in legumes is the culmination of a complex interaction between host, Rhizobium and the environment, it is the host which appears to play the more important role in controlling the symbiosis. Some of the host controlled traits regulating recognition of Rhizobium and initiation of infection have already been cited in the General Introduction.

The ability of legumes to select particular Rhizobium strains from the rhizosphere is also well documented but not well understood. Materon and Vincent (1980) compared the competitive ability of R.japonicum on lines of soybean with and without the rj2 gene and found that their competitiveness was significantly altered when different lines were used. In similar experiments with white clover Jones and Hardason (1979) identified some clover lines in which almost all nodules were formed by one strain. Inheritance of this trait was found to be addititive and without dominance (Hardason and Jones, 1979).

Devine and Weber (1977) found that desirable strains of R.japonicum could be established in soybean fields against the competition of aggressive but less productive indigenous strains by using a genetic system which substantially excluded the indigenous strains from

nodulating soybean cultivars which had a genotype rjl rjl. They observed that only strains which produced rhizobitoxine were able to infect rjlrjl soybeans. Previous reports had indicated that the rjl rjl genotype does not repress the development of rhizobia in the rhizosphere. Clark (1957) recovered equal numbers of rhizobia from roots of rjlrjl and Rjl Rjl plants grown in the field and green house. Elkan (1962) reported higher numbers of R.japonicum on rjl rjl than on Rjl Rjl isoline up to 40 days after seedling emergence. The effect of the rjl rjl genotype, then appears to be specific to infection event only. The mechanism and expression of the rjl rjl gene and host-symbiont interaction in the infection was further studied by Devine et al. (1979).

They found that inoculating plants carrying the rjl rjl gene with an inoculum of high cell density resulted in nodules containing both the infective and non-infective strain or only non-infective strains and infective strains separately showing that the plants were unable to distinguish between the rhizobitoxine producing strains (which are infective on rjl plants) and those that do not which are asymptomatic. Devine et al.(1979) suggested that the observation was due to some diffusible product produced by the compatible strains which was then absorbed by the cells of the compatible strain and transiently endowed them with infectivity, or that the compatible

strain acted on the root directly and rendered them receptive to infection by any Rhizobium in contact with an infective site. Their experimental results also established that once the asymptomatic strains had achieved infection it formed nodules of normal size containing abundant Rhizobium cells.

Nicol and Thornton (1941) were the first to note varying competitiveness of different Rhizobium strains in the root zone. Vincent and Waters (1953), however, could not find any marked host influence on the balance between R.trifoli strains colonizing the rhizosphere of various species of Trifolium.

Differences between cultivars of the same species in the ability to fix N_2 in symbiosis have now been demonstrated in a range of legumes including Trifolium (Gibson and Brockwell, 1968, Mytton, 1975), Glycine max (Hardy, Burns and Holsten, 1973), Phaseolus (Graham and Halliday 1977; Graham, 1981; Rennie and Kemp, 1981); Medicago (Gibson, 1962; Seetin and Barnes, 1977) Vigna (Zary et al.,(1978); Vicia (El-Sherbeeny, Lawes and Mytton, 1977) Pisum (Holl and LaRue, 1976) and Desmodium (Hutton and Coote, 1972; Imrie, 1975). The plant phenotypes are influenced by three independently measurable components of genetic variability. These are, additive effects of plant genotype, additive effects of Rhizobium genotype and non-additive effects of their joint action (Mytton, 1975).

It was the last component that was examined in this study since such interaction has important implications for improving nitrogen fixation through selection of superior plant and Rhizobium genotypes. If the non-additive component is large, both symbionts must be carefully matched to achieve maximum productivity.

The ability of strains to nodulate competitively is difficult to assess, but competition studies generally involve determining nodules attributable to each of the strains of Rhizobium that are simultaneously inoculated on a host. Methods for identifying the various strains have already been discussed in the General Materials and Methods.

The genotype interactions in this study were evaluated at four levels namely, genera-strain interactions; cultivar-strain interactions, using mixed inoculum where one of them was excluded; single strain-cultivar interaction to establish symbiotic compatibility and the interrelationships of the interacting genotypes.

GENOTYPE INTERACTIONS

M A T E R I A L S A N D M E T H O D S

MATERIALS AND METHODS

FIRST EXPERIMENT: Influence of hosts of different genera on the competitiveness of six strains of R.leguminosarum

Three host plants: Pisum sativum var Onward, Vicia faba var Throws. M.S. and Lens esculenta were used as representative of the three genera nodulated by Rhizobium leguminosarum. The other genus Lathyrus in the same inoculation group was not used.

Six strains of R.leguminosarum whose symbiotic characteristics have been described in the General Introduction were used in competition.

Seeds were pregerminated on agar and transferred aseptically to Fahraeus agar medium in 250 ml Erlenmeyer flasks and immediately inoculated with 2 ml of exponentially growing cultures of each strain. The seeds were etiolated and then the shoots were pulled through the neck of the flask using forceps sterilized by dipping in alcohol and flaming (Beringer, 1974) (see General Materials and Methods). Flasks were placed randomly on the bench in the green house where temperatures were between 15° and 18°C and about 20-25 (night/day) in winter and summer respectively.

Lentils were grown in plastic cups as described in the General Materials and Methods.

SECOND EXPERIMENT : Cultivar-Strain Interaction

Six cultivars of Pisum sativum were obtained from the John Innes Institute, Norwich, England. The characteristics of the cultivars and their place of origin are given below:

Cultivar No	Name	Origin
Jl 199	P.electius	Israel
Jl 305	Feltham First	Commercial cultivar
Jl 343	Onward	Commercial cultivar
Jl 502	Rondo	Commercial cultivar
Jl 1264	-	India
Jl 1326	Dark skinned Perfection	Commercial cultivar

Jl = John Innes Institute, Norwich, England.

Three strains of R.leguminosarum, RCR 1001 RCR 1056 and MR4, whose characterisitics can be found in the General Materials and Methods were used to inoculate those cultivars in pairs and in mixture of three.

Treatments were replicated three times and the experiment repeated to obtain a total of six independent replicates.



Plate 6a: The five cultivars of P.sativum as they are seen growing in the green house. From left to right the cultivars are Jl 199, Jl 1362, Jl 1264, Jl 502 and Jl 305. The flasks were covered with black polythene bags and the necks sealed with foam bungs which keep the plant shoot in position.



Plate 6b: The same plants as they are seen growing in Fahraeus agar with black polythene bags removed to show the cultivar numbers and the strain used to inoculate them (MR4).

THIRD EXPERIMENT : Determining the effect of absence of one strain on the competitive ability of a strain of Rhizobium leguminosarum

Pisum sativum var Onward and the six Rhizobium strains were used in this study which involved using six strains of R.leguminosarum in competition. In other treatments five strains were used as one strain was ommitted each time making a total of seven treatments as follows: all six, -1001, -1012, -1013, -1045, -1056, -MR4. The experiment was replicated four times. The number of nodules formed by each strain was determined by the intrinsic antibiotic resistance method described in General Methods.

FOURTH EXPERIMENT : Symbiotic performance of the strains with six cultivars

This experiment was conducted using the three strains of Rhizobium and the six cultivars of P.sativum described above. Each strain was inoculated singly on the pre-germinated seed of each cultivar on Fahraeus agar medium. The experiment was replicated four times. The plants were harvested after seven weeks, the time at which some of the cultivars had started to flower. The roots were cleared of agar and then immediately excised from shoots with a pair

of scissors. They were incubated in a jar containing 10% acetylene for one hour and the rate of reduction of acetylene to ethylene assayed using gas chromatography (see General Materials and Methods for details).

Nodules were counted and then removed from roots and weighed fresh.

RESULTS

GENERA - STRAIN INTERACTION

Effect of hosts from different genera on competitive ratios of six strains

Table 9 shows the overall picture of the effect of the different host plants on the number of nodules formed by each of the six strains in various inoculum combination. The number of nodules formed by each strain in paired combinations with different strains is significantly different showing that the strains used do not have the same competitive ability. Also the competitive performance of a strain was found to depend on the host plant on which the strains were inoculated in mixture.

From Table 9 the ratios of each pair of competing strains were tabulated and are presented separately in Table 10.

Percentage competitiveness of each strain was calculated using the percentages of the nodules it formed when competing in pairs against the other strains. The average of those percentages indicates the degree of competitiveness of the strain. The data are illustrated by Figure 1. The hierachy of the six strains was obtained by ranking them in positions as shown in the Table 11. This hierachy did not differ with that obtained when the six strains were used together (Table 12) although the general performance of each strain was different.

Table 9: Effect of hosts of different genera on the nodulation competitiveness of six strains of R.leguminosarum mixed in pairs

Hosts	Pisum sativum van sobat												Vicia faba van Throws M.S.												Lens esculenta											
Replicates	1			2			3			TOTAL			RATIO	1			2			3			TOTAL			RATIO	1			2			3			TOTAL			RATIO
Strains in the nodules Strain combination (A + B)*	A	B	Mix*	A	B	Mix	A	B	Mix	A	B	Mix	A:B	A	B	Mix	A	B	Mix	A	B	Mix	A	B	Mix	A:B	A	B	Mix	A	B	Mix	A	B	Mix	A	B	Mix	A:B
1001+1012	45	22	2	57	13	1	65	3	1	168	38	4	4:1	9	59	1	2	67	0	3	65	1	14	191	3	1:14	65	4	0	67	2	0	62	4	3	194	10	3	19:1
1001+1003	51	14	4	44	24	1	60	8	1	155	46	7	3:1	68	1	0	66	3	0	66	2	1	200	6	1	33:1	67	2	0	63	6	1	64	2	3	194	10	4	19:1
1001+1045	36	30	3	42	25	2	47	21	1	125	76	6	2:1	9	55	5	1	66	2	3	66	5	13	187	8	1:14	48	20	1	56	12	1	55	13	1	159	45	3	4:1
1001+1056	62	2	5	64	4	1	60	6	3	186	12	9	15:1	11	57	1	5	64	0	5	63	1	21	184	2	1:9	59	10	0	51	12	0	61	7	1	171	29	1	6:1
1001+MR4	1	57	0	3	63	3	6	63	0	10	183	3	1:18	2	67	0	2	66	1	9	58	2	13	191	3	1:15	59	8	2	61	6	2	68	1	0	188	15	5	12:1
1012+1013	45	12	0	51	18	0	54	15	0	150	45	0	3:1	3	66	0	6	61	2	2	61	6	11	188	8	1:17	8	59	2	3	66	0	2	66	1	13	191	3	1:15
1012+145	24	44	1	26	49	0	21	47	1	71	140	2	1:2	47	21	1	45	11	1	54	13	2	146	45	4	3:1	27	40	2	10	46	1	18	57	1	55	143	4	1:3
1012+1056	49	4	1	60	8	1	64	3	2	173	15	4	11:1	23	46	0	28	39	2	28	37	4	79	122	6	1:2	10	59	0	1	67	1	4	63	2	15	189	3	1:13
1012+MR4	1	63	5	2	63	4	5	63	1	8	189	10	1:12	1	68	0	1	63	5	5	62	2	7	193	7	1:27	57	12	0	51	16	2	67	2	0	175	30	2	6:1
1013+1045	2	68	0	15	54	0	3	65	1	20	187	1	1:9	3	65	1	15	53	1	12	55	2	30	173	4	1:6	40	15	2	58	11	0	54	14	1	152	40	3	4:1
1013+1056	66	2	1	57	4	0	61	8	0	184	14	1	13:1	2	67	0	1	56	0	1	65	3	4	188	3	1:47	67	2	0	51	6	0	68	1	0	186	9	0	21:1
1013+MR4	21	44	4	24	45	0	15	63	1	60	152	5	1:3	1	68	0	3	65	1	3	65	1	7	198	2	1:28	45	11	1	55	14	0	62	7	0	162	32	1	5:1
1045+1056	60	9	0	66	1	2	59	10	0	189	21	1	9:1	42	26	1	39	17	1	48	20	1	129	63	1	2:1	51	6	0	60	8	1	66	3	0	177	21	1	8:1
1045+MR4	6	63	0	3	57	6	8	60	1	17	180	4	1:10	30	37	2	25	38	6	30	34	5	85	109	13	1:1	41	15	1	58	11	0	58	9	2	157	39	3	4:1
1056+MR4	1	68	0	7	59	4	1	59	3	9	186	4	1:20	65	4	0	54	15	0	60	5	4	179	24	4	7:1	51	5	1	66	3	0	64	5	0	181	13	1	14:1

* Note: (1) A denotes nodules containing the first strain in the pair and B the competing partner.
(2) Mix denotes nodules containing both strains A and B in one nodule.

Table 10: Relative performance of the six strains of Rhizobium leguminosarum expressed in terms of ratios of the total number of nodules formed on the three host genera.

	HOST PLANTS		
Strain combination	P.sativum	V.faba	L.esculenta
1001:1012	4:1	1:14	19:1
1001:1013	3:1	33:1	19:1
1001:1045	2:1	1:14	4:1
1001:1056	15:1	1:9	6:1
1001:MR4	1:18	1:15	12:1
1012:1013	3:1	1:17	1:15
1012:1045	1:2	3:1	1:3
1012:1056	11:1	1:2	1:13
1012:MR4	1:12	1:27	6:1
1013:1045	1:9	1:6	4:1
1013:1056	13:1	1:47	21:1
1013:MR4	1:3	1:28	5:1
1045:1056	9:1	2:1	8:1
1045:MR4	1:10	1:1	4:1
1056:MR4	1:20	7:1	14:1

Table 11: Percentage of nodules formed by each of the strains when competing in pairs on P.sativum, V.faba and L.esculenta calculated from the ratios in tables 9 and 10.

Host Plants	P.sativum						V.faba						L.esculenta					
Strains competing against those below	1001	1012	1013	1045	1056	MR4	1001	1012	1013	1045	1056	MR4	1001	1012	1013	1045	1056	MR4
1001	**	20	25	33	7	95	**	93	3	93	90	94	**	5	5	20	14	8
1012	80	**	25	67	9	92	7	**	95	25	67	96	95	**	94	75	93	12
1013	75	75	**	90	8	75	97	5	**	86	98	97	95	6	**	20	5	17
1045	67	33	10	**	10	91	7	75	14	**	33	50	80	25	80	**	11	20
1056	93	91	92	90	**	95	10	33	2	67	**	12	86	7	95	89	*	7
MR4	5	8	25	9	5	**	6	4	3	50	88	**	92	88	83	80	93	*
Average % of competitiveness	64	45	35	57	8	90	25	42	23	64	75	70	90	26	71	56	43	13
RANK*	2	4	5	3	6	1	5	4	6	3	1	2	1	5	2	3	4	6

Rank = competition position of the strain; 1 is the highest rank and 6 is the lowest rank.

Competition of the six strains in mixed culture inoculant on the three host genera.

Table 12 shows the number of nodules formed by each of the strains competing in pairs and their percentages on the three host genotypes. The data were obtained from the experiments conducted under similar conditions by finger printing many nodules picked randomly. Ratios of the nodules formed by each strain were calculated from the above table and are shown separately in Table 13. Either the percentages or the ratios can be used to rank the strains in order of competitiveness. When compared with ranks obtained under paired competition a general agreement was found though in this case one strain demonstrated clear dominance over the other six than in pairs. These observations are discussed under strain - strain interactions.

Table 12: Number of nodules formed by each of the six strains used together to inoculate three plants of different genera

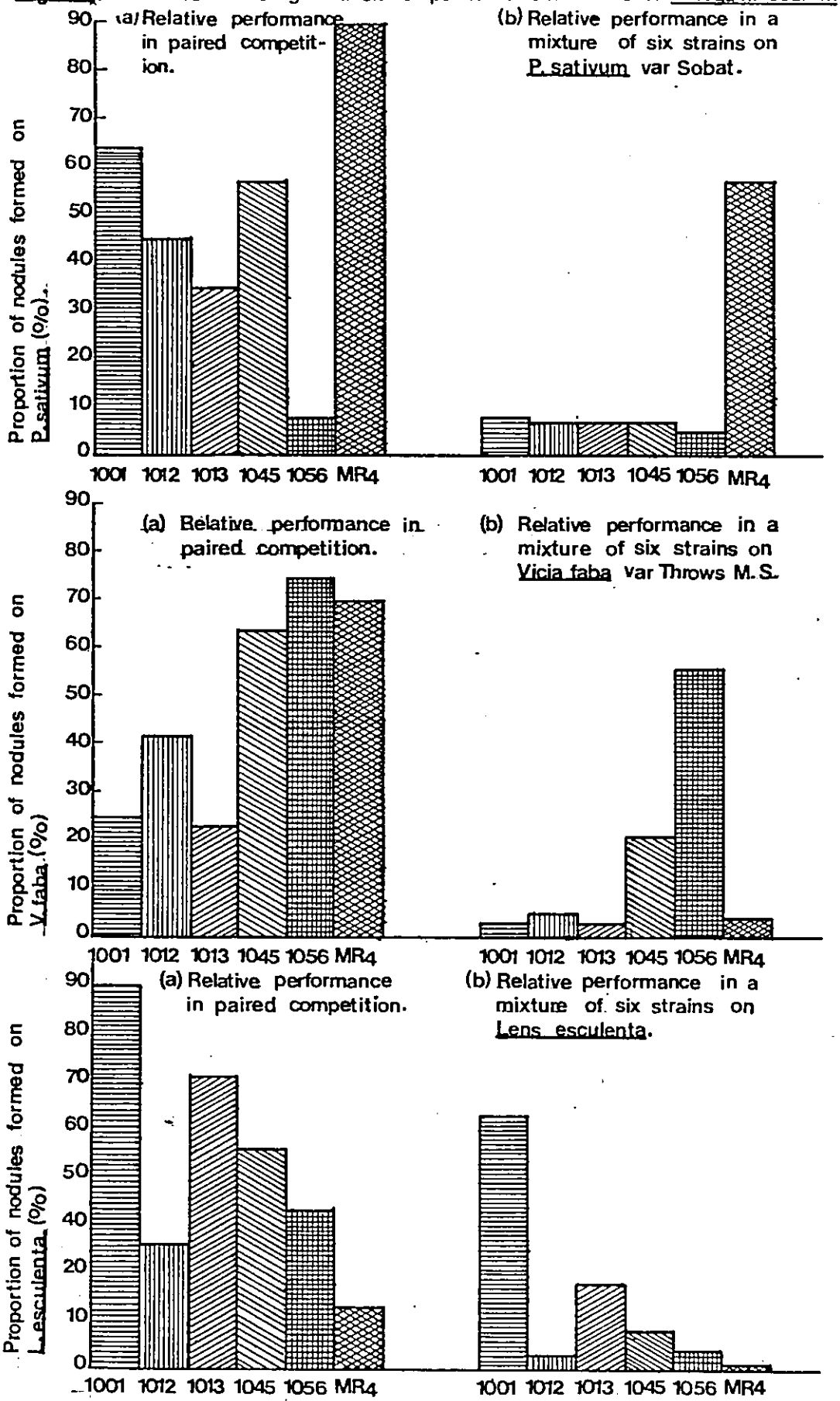
Host Plants	P.sativum					V.faba					L.esculenta				
Replicates / Strains	1	2	3	Total	%	1	2	3	Total	%	1	2	3	Total	%
1001	13	9	4	26	8	4	2	3	9	3	117	80	93	292	63
1012	6	7	11	24	7	7	6	4	17	5	2	6	4	12	3
1013	7	4	13	24	7	4	3	2	9	3	30	29	27	86	18
1045	6	7	11	24	7	27	23	20	70	21	8	14	13	35	8
1056	3	9	4	16	5	50	68	71	189	56	5	8	7	20	4
MR4	65	67	52	184	57	7	4	1	12	4	0	1	2	3	1
Mixed*	7	4	12	23	7	13	6	11	30	9	6	2	4	12	3
Totals	107	107	107	321		112	112	112	336		168	149	149	466	

* Mixed = Nodules contain two strains in one nodule.

Table 13: Ratios of the number of nodules formed by the six strains worked out from data in Table 12 above.

	Strains in the nodules						
Host Plants	1001	1012	1013	1045	1056	MR4	Mixed
P.sativum	2	2	2	2	1	13	1
V.faba	1	2	1	8	21	1	3
L.esculenta	97	4	29	12	7	1	4

Figure 1: Effect of host genera on competition of six strains of R. leguminosarum



CULTIVAR - STRAIN INTERACTIONS

Table 15 shows the effect of different cultivars on the relative number of nodules formed by each of the three strains of Rhizobium leguminosarum. Some of the nodules were formed by both strains and these are shown in a separate column. The percentage of nodules on each cultivar were calculated.

The data clearly show that different cultivars have different effects on the competitiveness of the three strains used. Strain 1001 was superior to 1056 on all cultivars. When the same strain was compared with strain MR4 it was less competitive on three cultivars out of five. The ratios which indicate the relative performance of the strains were reversed. Strain 1056 was found to be a very poor competitor against these two strains on all cultivars. The phenomenon of competition is, however, clearly an interaction one as both the host and the strains play a part. Table 17 gives the ratios of the number of nodules formed by the three strains competing in pairs to show the relative performance of each strain on each cultivar. The effect of genotype interaction is evident.

Table 14 gives the number of nodules formed by each of the three strains when equal volumes of exponentially growing cultures were mixed in pairs. The percentage

Table 14: Number of nodules formed by each of the three strains of R.leguminosarum when inoculated in pairs on five cultivars of P.sativum.

Host cultivars		J1 1499			J1 305			J1 502			J1 1264			J1 1362		
Strain in nodules		1001	1056	Mixed*	1001	1056	Mixed	1001	1056	Mixed	1001	1056	Mixed	1001	1056	Mixed
Strain con-	Repli-															
firmation	cates 1	34	10	1	23	20	2	30	12	3	30	15	0	22	23	0
	2	31	14	0	30	15	0	37	6	2	36	8	1	33	12	0
	3	33	12	0	24	15	6	28	15	2	34	11	0	34	11	0
	4	33	11	1	30	13	2	35	6	4	30	12	3	24	18	3
1001+1056	5	25	19	1	24	16	5	27	18	0	24	21	0	27	18	0
	6	23	19	3	31	14	0	30	14	1	30	13	2	30	13	2
	Total	179	85	6	162	93	15	187	71	12	184	80	6	170	95	5
	Aver.	29.8	14.1	1	27	15.5	2.5	31.1	11.8	2	30.6	13.3	1	28.3	15.8	0.8
		66	32	2	60	34	6	6g	26	5	68	30	2	63	35	2
	SD	4.7	3.9	1.1	3.7	2.4	2.5	3.9	4.9	1.4	4.1	4.4	1.2	4.8	4.6	1.3
Strains in nodules		1001	MR4	Mixed	1001	MR4	Mixed	1001	MR4	Mixed	1001	MR4	Mixed	1001	MR4	Mixed
Strain Com-	1	16	27	2	12	30	3	19	23	3	30	12	3	30	15	0
bination		13	32	0	10	29	6	18	23	4	26	17	2	26	15	4
	2	7	35	3	13	32	0	15	29	1	25	19	1	27	17	1
	3	9	34	2	9	34	2	9	33	3	27	18	0	39	6	0
1001+MR4	4	12	30	3	15	27	3	13	30	2	30	11	4	33	9	3
	5	19	26	0	14	30	1	14	31	0	27	18	0	29	16	0
	6	76	184	10	73	182	16	88	169	13	165	95	10	184	78	8
	Total	12.6	30.6	1.6	12.1	30.3	2.6	14.6	28.1	6.5	27.5	15.8	1.7	30.7	13	1.3
	Aver.	28	68	4	27	67	6	32	63	5	61	35	4	68	29	3
	%	4.4	3.7	1.3	2.3	2.4	2.07	3.6	4.2	1.4	2.1	3.4	1.6	4.7	4.4	1.7
	SD															
Strains in nodules		1056	MR4	Mixed	1056	MR4	Mixed	1056	MR4	Mixed	1056	MR4	Mixed	1056	MR4	Mixed
Strain Com-	1	18	26	1	10	35	0	16	29	0	13	32	0	15	30	0
bination		7	38	0	7	38	0	12	32	1	8	36	1	17	27	1
	2	6	39	0	3	39	3	5	39	1	2	42	1	5	39	1
	3	10	34	1	14	29	2	10	35	0	6	36	3	13	30	2
	4	11	31	3	8	36	1	11	31	3	4	40	1	9	36	0
	5	18	27	0	13	29	3	7	38	0	3	40	2	12	30	3
	6	70	195	5	55	206	9	61	204	5	36	226	8	71	192	7
	Total	11.7	32.5	0.8	9.1	34.3	1.5	10.1	34	0.8	6	37.6	1.3	11.8	32	1.16
	Aver.	26	72	2	20	76	4	22	76	2	13	84	3	26	71	3
	%	5.2	5.4	1.1	4.0	4.3	1,3	3.8	4	1.1	4.0	3.6	1.0	4.3	4.5	1.16

* Mixed = Nodules containing both strain together.

of the nodules formed was calculated and is presented in Figure 2. In this Figure the influence of the cultivar genotype, the performance of the three strains is evident. The Figure shows clearly the extent of the interaction between the cultivars and strains. The data indicated that cultivars Jl 199, Jl 305 and Jl 502 were best suited with strain MR4 as it formed most nodules in competition with the other two strains while strain 1001 was found to perform best on the other two cultivars.

When the strain-cultivar interaction was expressed as percentage of nodules each strain fromed in mixture of three it was evident that strain 1001 still performed best on cultivar Jl 1264 and Jl 1362; strain 1056 was the poorest performer on all cultivars while MR4 was the best on three cultivars out of the five. (Table 17 and Figure 3)

When the percentages in Table 15,obtained as averages of the paired competition, were compared with the percentages of the nodules formed by each strain in mixture of three (Table 17) the trend was found to be the same. The performance of strain 1001 was comparatively better in mixture of three together than its average performance from its paired competition. This indicates a possibility of interaction effect.

Table 15: Competitiveness of the three strains of R.leguminosarum on 5 cultivars of P.sativum measured in percentages * of nodules formed when mixed in pairs calculated from Table 14.

Host Cultivrs	J1 199	J1 305	J1 502	J1 1264	J1 1362
Strains					
1001	47	44	51	65	66
1056	29	27	24	22	31
MR4	70	72	70	60	50

* The figures above were obtained by calculating an average of the percentages of nodules formed by each strain in paired competition with each of the other two strains.

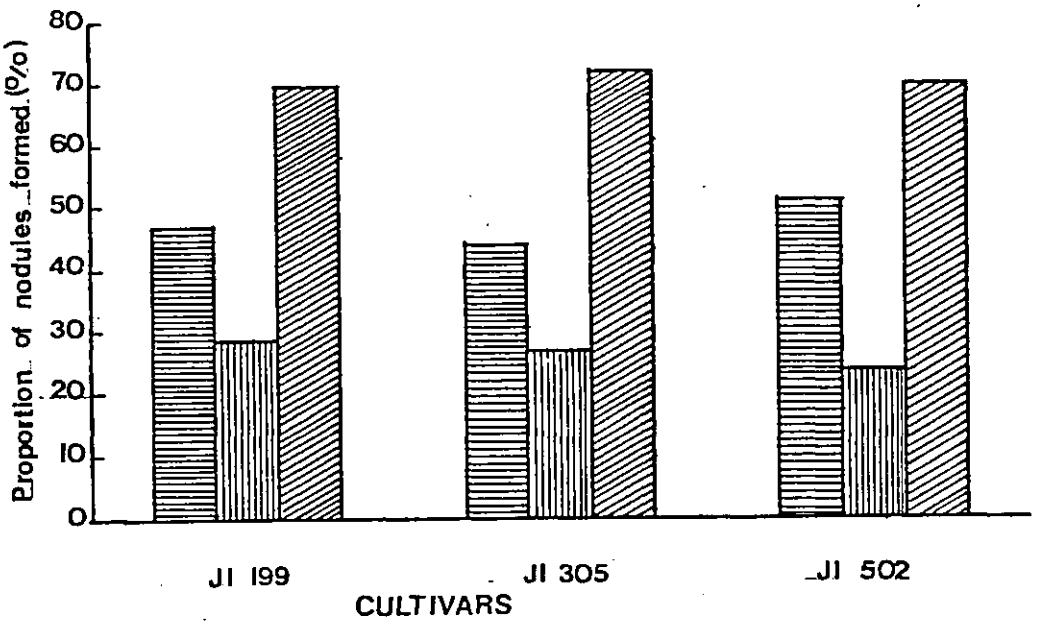
Table 16: The relative performance of the three strains competing in pairs on five pea cultivars expressed in ratios.

	Cultivars				
	J1 199	J1 305	J1 502	J1 1264	J1 1362
Strains combination					
1001:1056	2:1	2:1	3:1	3:1	2:1
1001:MR4	1:2	1:2	1:2	2:1	2:1
1056:Mr4	1:3	1:4	1:3	1:6	1:3

Table 17: Number of nodules formed by each of the three strains of R.leguminosarum when inoculated together on the five cultivars of P.sativum.

Host cultivars	J1 199				J1 305				J1 502				J1 1264							
Strains in nodules	100	1056	MR4	Mixed	100	1056	MR4	Mixed	1001	1056	MR4	Mixed	1001	1056	MR4	Mixed	1001	1056	MR4	Mixed
Replicates																				
1	9	6	29	1	10	11	22	3	12	9	23	1	19	7	14	5	24	12	6	3
2	10	5	27	3	11	15	17	1	14	3	22	6	21	3	18	3	20	6	15	4
3	9	1	32	3	13	4	24	4	20	2	18	5	17	6	18	4	19	3	20	3
4	11	9	24	1	15	5	21	4	14	4	23	4	31	2	10	2	21	6	15	3
5	13	3	27	2	11	5	24	5	15	9	19	2	26	4	12	3	26	8	10	1
6	10	2	29	4	14	4	22	5	13	10	19	3	30	3	10	2	31	2	10	2
Total	62	26	168	14	74	44	130	22	88	37	124	22	144	25	82	19	141	37	76	16
	10.3	4.3	28	2.3	12.3	7.3	21.6	3.7	14.7	6.2	20.7	3.5	24	4.2	13.6	3.2	23.5	6.2	12.7	2.
Percentages	23	10	62	5	27	16	48	8	32	14	46	8	53	9	30	7	52	14	28	6
Ratios	2	1	6		2	1	3		3	1	4		6	1	3		4	1	2	
SD	1.5	2.9	2.6	1.2	1.9	4.5	2.6	1.5	2.8	3.5	2.2	1.8	5.8	1.9	3.6	1.1	4.5	3.6	4.9	1.

Figure 2: Competitiveness of the three strains of R. leguminosarum on 5 cultivars of P. sativum expressed in percentage (overall) of nodules formed when strains were mixed in pairs



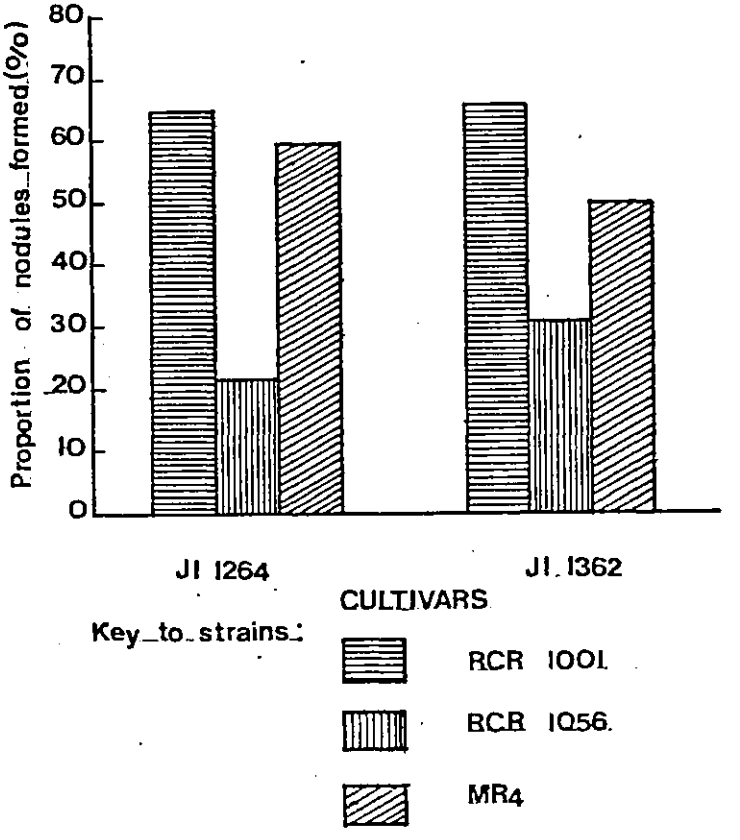
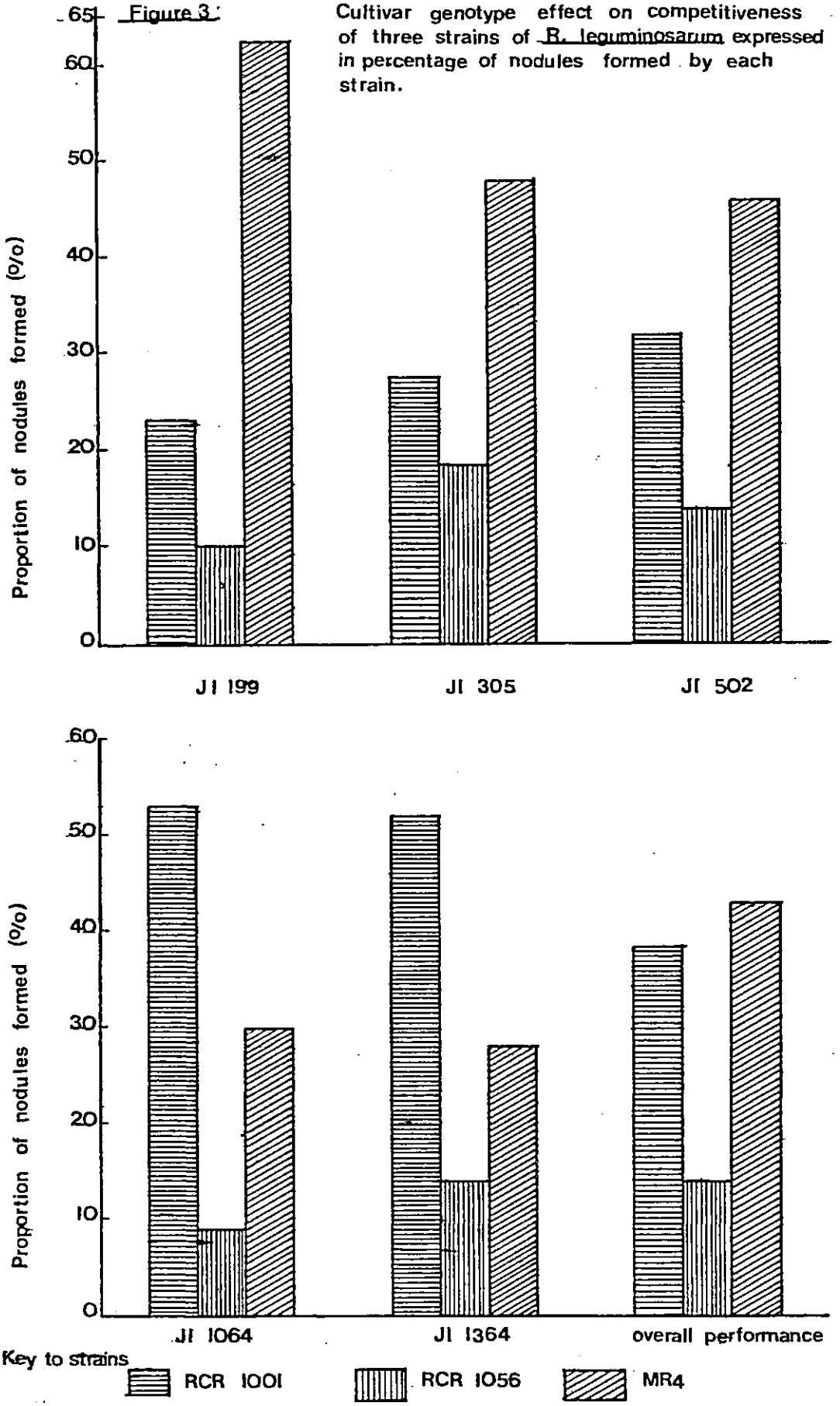


Figure 3

Cultivar genotype effect on competitiveness of three strains of R. leguminosarum expressed in percentage of nodules formed by each strain.



CULTIVAR - STRAIN INTERACTION

Single-strain inoculation of the six cultivars

Table 18 gives the interaction of cultivars with the three strains inoculated singly on each one of them. The number of nodules formed by each cultivar is related to:

(1) Cultivar differences in nodulation and,

(2) Strain differences in nodule formation.

All this information is found in the data in Table 18 and it is illustrated in Figures 4 and 5. Cultivar Jl 343 formed the biggest number nodules with all three strains. Strain 1056 formed most nodules on each of the six cultivars, but these were small and ineffective.

There is a significant variation even among plants inoculated with the same strains as is shown on the large Standard Deviation and variance ratios (see statistical Analysis).

Nodule fresh weights are also given. The weights of nodules did not always correspond to their number as some strains formed many but small nodules which weighed less, while others formed fewer, but bigger nodules which weighed more.

The average number of nodules formed by each cultivar indicates the degree of susceptibility to infection by the Rhizobium strains on the six cultivars. The average number of nodules formed by each cultivar is given in the brackets after the cultivar: Jl 199 (19), Jl 305 (61)

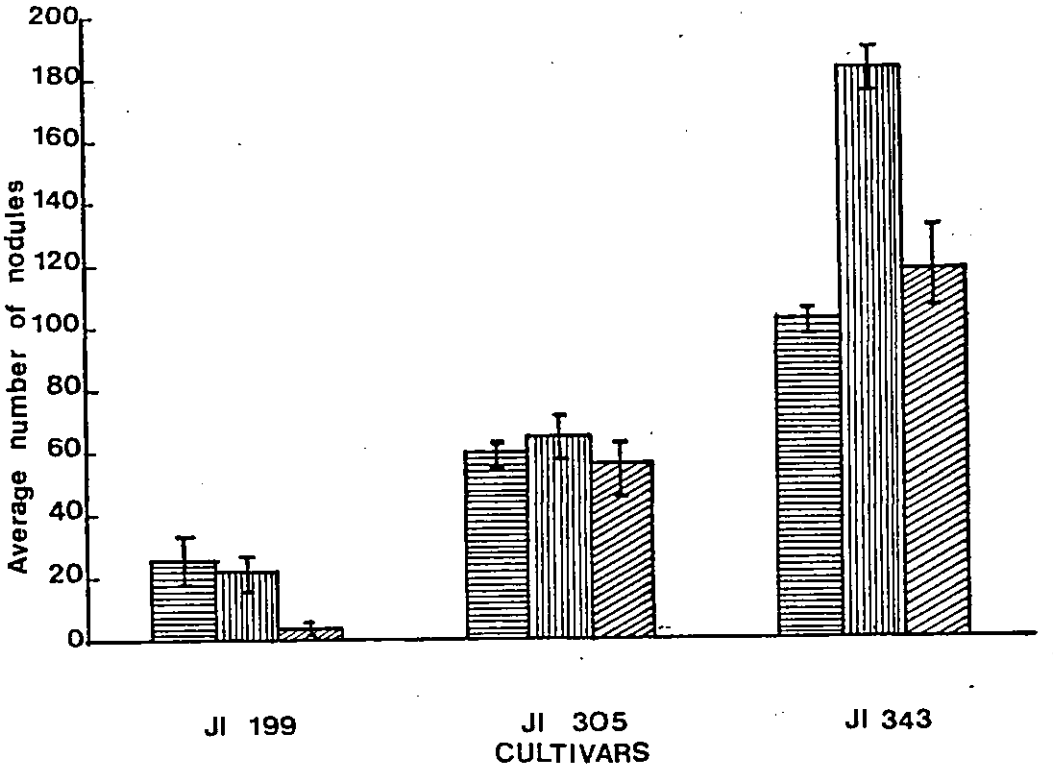
Table 18: Cultivar - strain interactions in nodule formation and nitrogen fixation

		Numbers of nodules formed by strains			Fresh weights of nodules (mg)			N_2 fixation rate *		
		1001	1056	MR4	1001	1056	MR4	1001	1056	MR
	1	46	18	1	93	22	2	29	0	2
J1 199	2	14	12	6	83	55	29	24	0	34
	3	18	39	10	83	72	37	12	0	18
	Aver.	26	23	8	86	50	23	22	0	18
	S/D	17.4	14.1	4.5	5.7	25.4	18.3	8.7		16
	1	60	59	45	153	104	123	6	0	32
	2	47	52	68	69	68	133	29	0	14
J1 305	3	65	76	38	122	121	92	15	0	21
	4	71	80	72	177	144	165	15	0	12
	Aver.	61	67	56	130	108	165	16	0	19
	S/D	10.2	13.4	16.7	46.6	32.0	30.0	9.5		9.0
	1	112	187	148	138	248	266	10	0	4
	2	106	194	107	174	258	266	2	0	5
J1 343	3	88	170	102	126	234	205	10	0	6
	Aver.	102	183	119	146	247	246	8	0	5
	S/D	12.4	12.3	25.2	24.9	12.0	35.2	4.6		0.6
	1	62	118	127	111	121	216	5	0	13
	2	70	139	114	176	162	136	20	0	15
J1 502	3	46	178	98	96	231	156	14	0	8
	4	63	74	109	163	106	142	8	0	13
	Aver.	60	127	112	137	155	163	12	0	12
	S/D	10.1	43.3	12.0	38.9	55.9	36.6	6.6		2.9
	1	58	172	32	125	186	95	17	0	5
	2	30	114	17	63	162	78	9	0	10
J1 1264	3	21	168	31	42	141	52	17	0	20
	4	53	73	39	94	53	115	7	0	3
	Aver.	41	137	30	81	136	85	13	0	9
	S/D	17.8	47.2	9.2	36.2	57.9	26.6	5.2		7.5
	1	63	137	51	114	167	79	9	0	19
J1 1362	2	43	42	41	36	55	102	7	0	6
	3	57	228	44	87	235	81	7	0	7
	Aver.	54	137	45	79	153	87	8	0	8
	S/D	10.2	93.0	5.1	39	90.8	12.7	1.1		2.1

* N_2 fixation rate was measured by acetylene reduction assay in μM/g/hr C_2H_4 and hence this indicates the rate of nitrogenase activity at t particular time of measurement.

S/D = Standard Deviation.

Figure 4: Average number of nodules formed by each host cultivar of P. sativum inoculated with each of the 3 strains of R. leguminosarum separately.



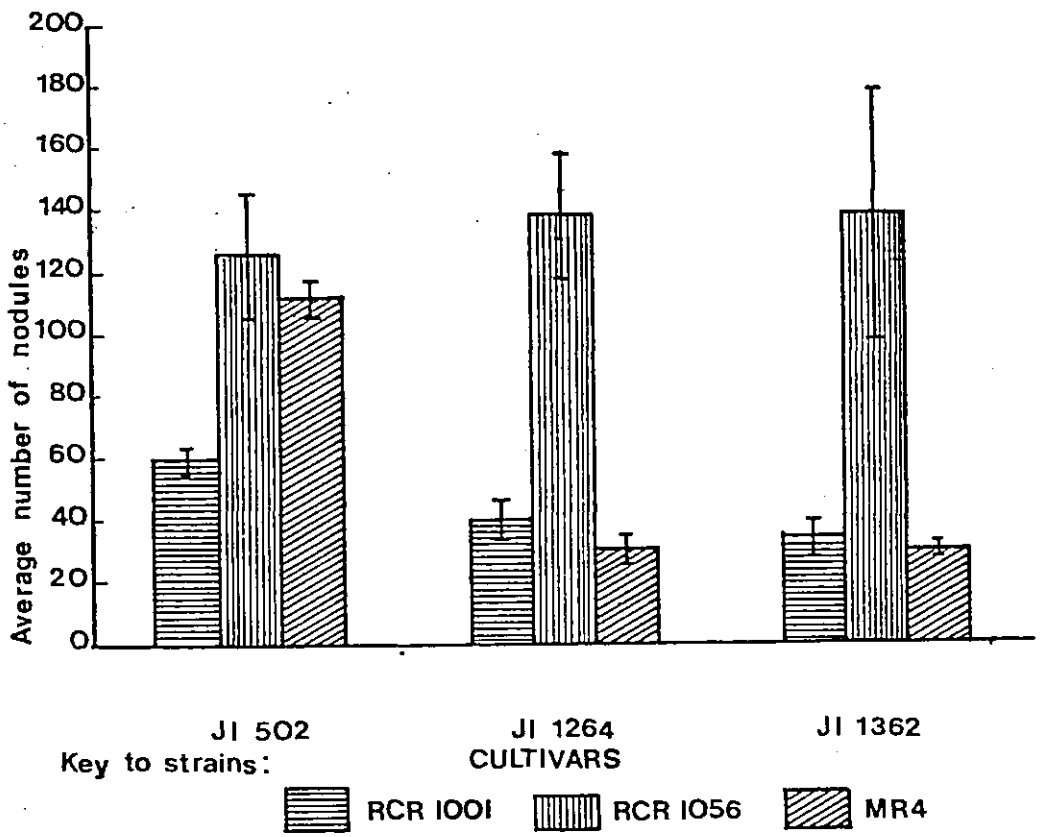


Table 19: The average nodule weight in mg formed by each strain-cultivar interaction

Cultivars	J1 199	J1 305	J1 343	J1 502	J1 1264	J1 1362
Strains						
1001	3.3	2.1	1.4	2.3	2.0	1.5
1056	2.2	1.6	1.3	1.2	1.0	1.1
MR4	2.8	2.9	2.1	1.5	2.8	1.9
SEM *	0.55	0.65	0.43	0.56	0.90	0.4

* SEM = Standard Error of mean = Standard Error

Table 20: Cultivar-strain interaction expressed in terms of rate of N_2 fixation (nM/nodule/hr)

Host Cultivar	J1 199	J1 305	J1 343	J1 502	J1 1264	J1 1362
Strains						
1001	71	35	10.8	27.0	24.8	11.3
1056	0	0	0	0	0	0
MR4	111	448	10.8	18.0	26.9	18.3
LSD *	54.3	292	0	6.3	1.48	4.9

* LSD - Least Significant Difference = SDX1.972 at 5% Confidence limits.

Figure 5.: Average nodule fresh weight per plant formed by each strain of R. leguminosarum on the six cultivars of P. sativum.

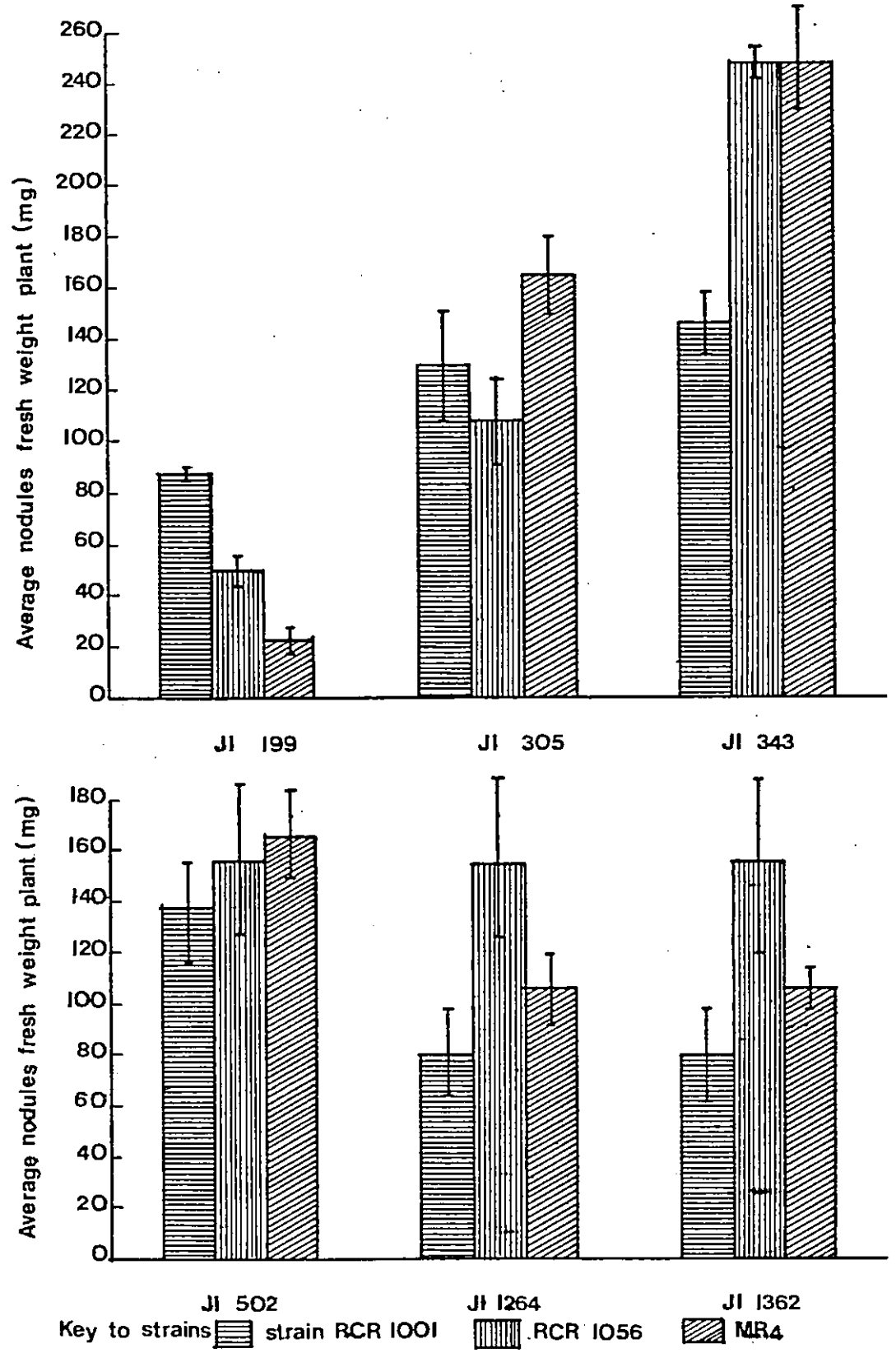


Figure 6.a: The rate of N_2 – fixation (measured by acetylene reduction assay) due to the two effective strains of R. leguminosarum.

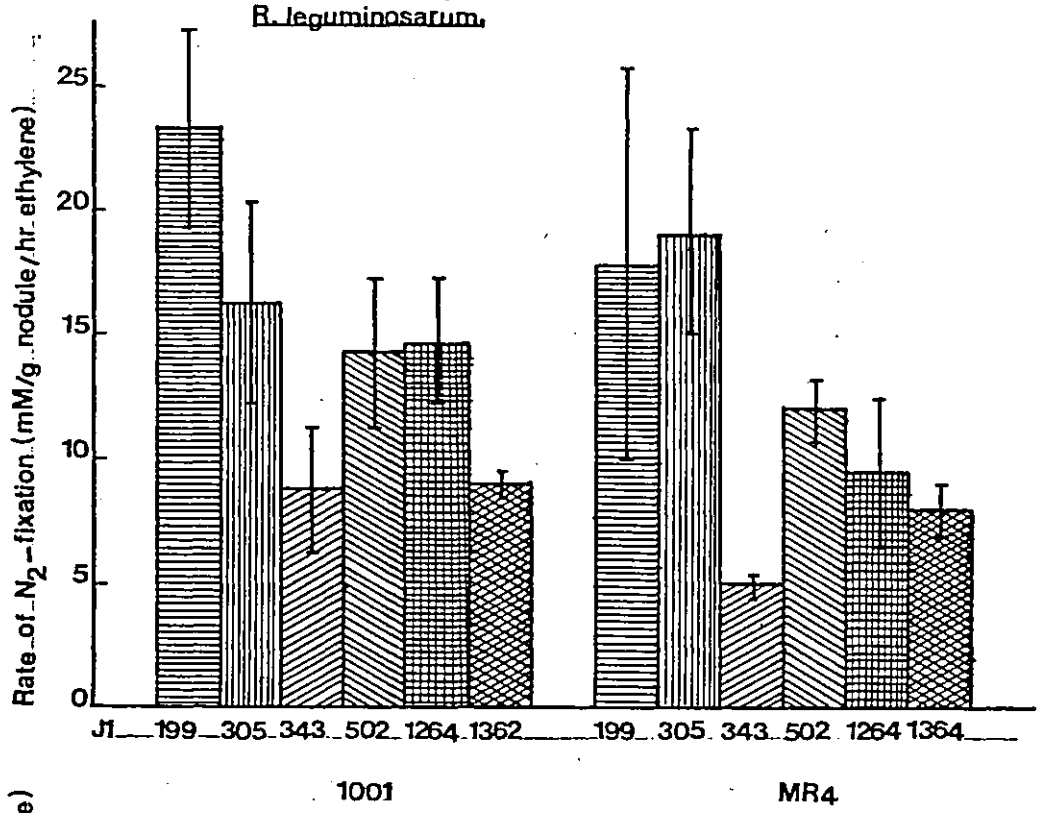
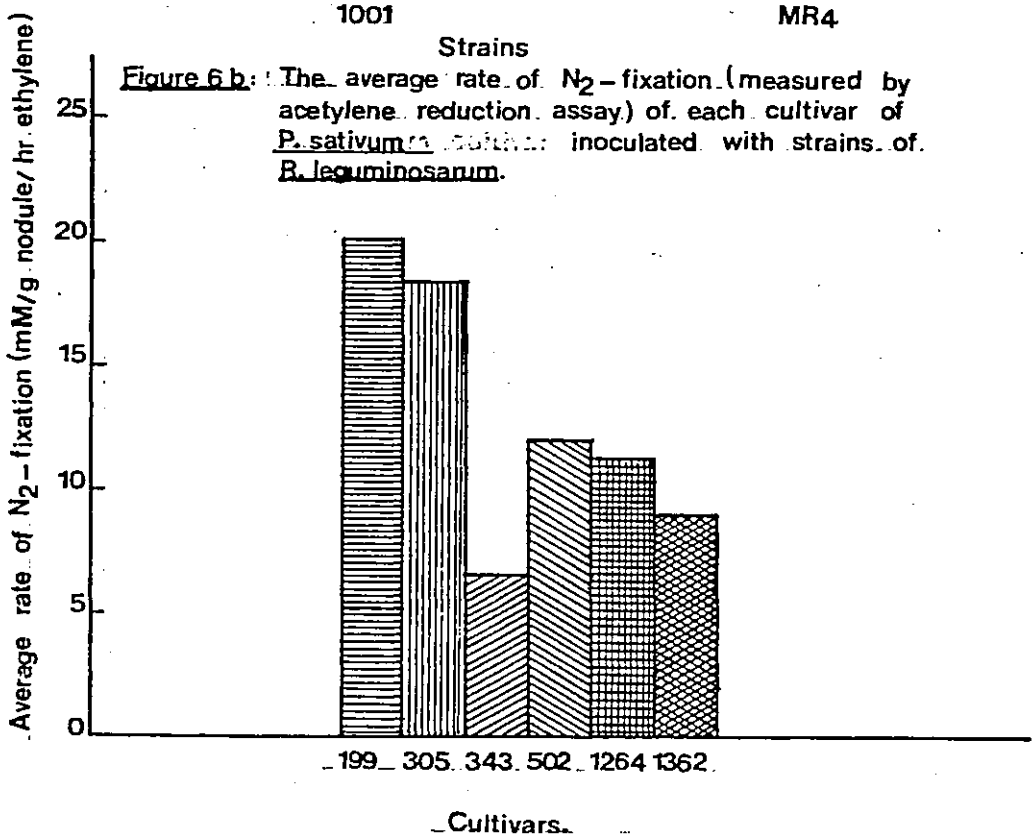


Figure 6.b: The average rate of N_2 – fixation (measured by acetylene reduction assay) of each cultivar of P. sativum [illegible] inoculated with strains of R. leguminosarum.



J1 343 (132) J1 502 (100), J1 1246 (69), and J1 1362 (79). The least significant difference of these averages at 5% significant limits was found to be 75. This indicates that J1 343 and J1 502 formed significantly more nodules than J1 199 but not the others.

The average fresh weight of each nodule formed by each of the three strains on the six cultivars was calculated from Table 18 and is presented in Table 19.

The rates of N_2-fixation (measured by acetylene reduction assay) are presented in Table 18 and 20 and illustrated in Figures 6a and 6b. The rates which were measured in nMoles of ethylene produced per hour per gram weight of nodule were found to be very variable (see the Standard Deviation figures in Table 18 and the large Least Significant Difference/(LSD) obtained at 5% confidence limits (Table 20).

STRAIN-STRAIN INTERACTIONS

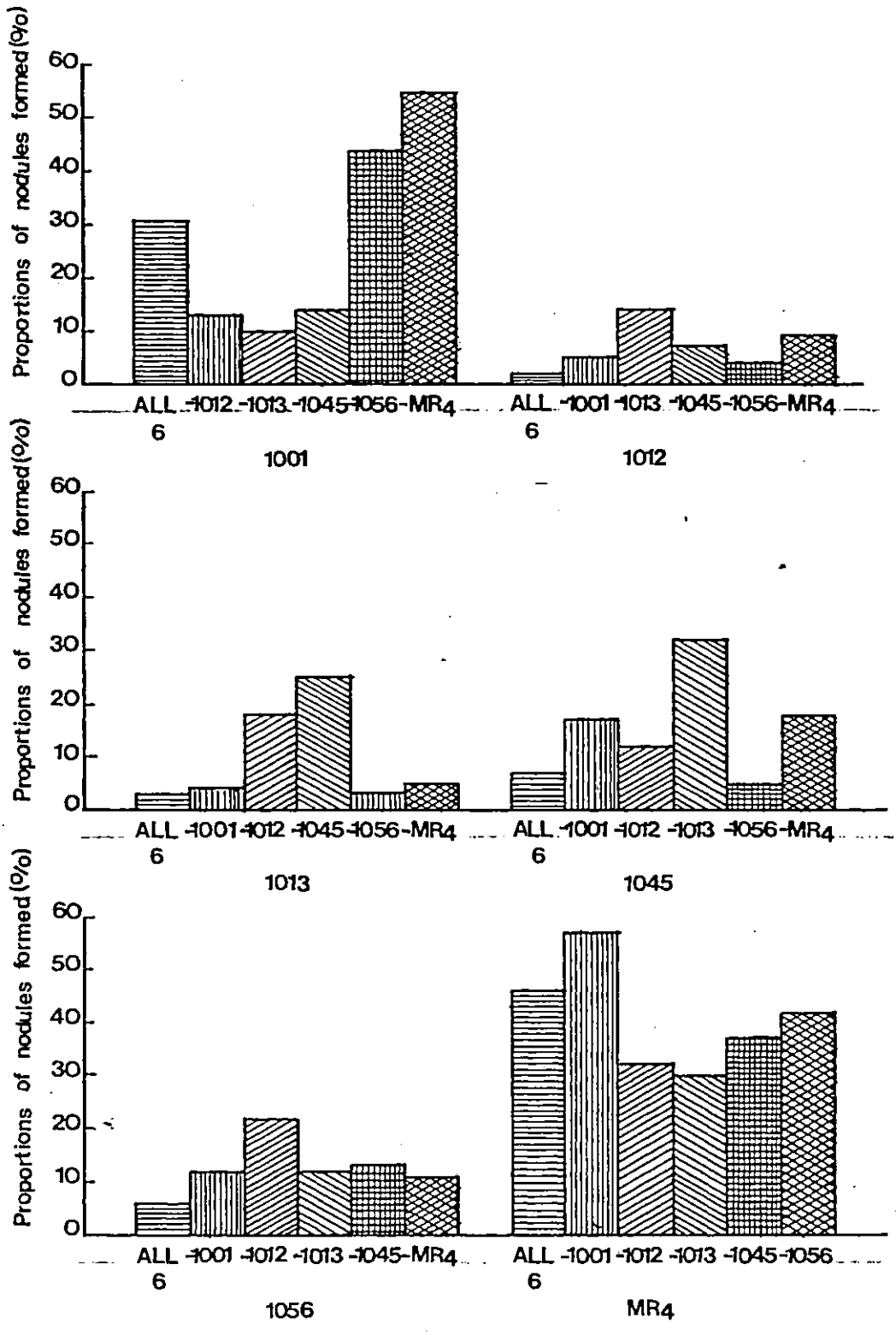
Rhizobium strains are said to interact at various levels before and after forming nodules on the host plants. One of such interactions is bacteriocin production and sensitivity. The results of the assay for these properties are given in Table 3 in the General Materials and Methods.

From Table 3 it can be seen that strain 1013 produced bacteriocins which formed large inhibition zones against strain 1001 and 1056 and also strain 1045 produced medium sized zone of inhibition against strain 1001.

Table 21: Number of nodules formed by each strain in mixtures of six and five (when one strain omitted on P.sativum var Onward

Strain found in nodules	Replicates	Composition of the inoculum (strains)						
		All six	-1001	-1012	-1013	-1045	-1056	-MR4
1001	1	26		10	4	10	35	65
	2	21		9	13	9	30	22
	3	23		12	6	12	40	45
	Total	70		31	23	31	105	132
	%	31		13	10	14	44	55
1012	1	3	3		15	4	3	6
	2	1	4		11	6	1	7
	3	1	5		6	5	5	8
	Total	5	12		32	15	9	21
	%	2	5		14	7	4	9
1013	1	1	3	17		13	3	4
	2	3	5	12		20	2	5
	3	3	2	13		24	3	4
	Total	7	10	42		57	8	13
	%	3	4	18		25	3	5
1045	1	9	12	10	41		6	9
	2	2	10	6	16		3	21
	3	4	15	12	18		3	12
	Total	15	37	28	75		12	42
	%	7	17	12	32		5	18
1056	1	2	11	15	5	11		7
	2	3	9	17	8	4		15
	3	8	6	19	15	15		5
	Total	13	26	51	28	30		27
	%	6	12	22	12	13		11
MR4	1	28	41	23	15	24	28	
	2	39	48	25	30	21	42	
	3	36	39	28	24	37	30	
	Total	103	128	76	69	82	100	
	%	46	57	32	30	37	42	
	Total	11	11	8	5	9	2	5
	%	5	5	3	2	4	1	2
	GRAND TOTAL	224	224	236	232	224	236	240

Figure 7: Relative number of nodules formed by three strains of R. leguminosarum competing in sixes and fives on P. sativum var Onward.



Competition studies were carried out using the six strains together and five strains together by removing one strain each time. The results of this study given in Table 21 show the number of nodules formed by each strain under these treatments and the various ratios calculated for each treatment. Further analysis is given in the Appendix (Statistical Analysis).

DISCUSSION

The results presented in this chapter have further confirmed that the phenomenon of competition among strains of Rhizobium for nodulation of host legumes is complex. The interaction between the host plant and the Rhizobium, introduced as inoculant strain,culminates in the formation of nodules. Nodule initiation, development and function entail intricate biochemical and biophysical processes many of which are not fully understood. Since the surest way of identifying Rhizobium from a host of other Gram-negative bacteria is its ability to form nodules on legume host, it is perfectly logical to study success or failure of a particular strain of Rhizobium by looking for its presence in the nodules.

The relative numbers of nodules containing a particular strain of Rhizobium used in competition with other strains clearly show the competitive ability of the strain. The ability of Rhizobium to fix nitrogen when in symbiosis with a compatible host plant is very important agronomically and seems to be under the control of both the host and the Rhizobium strain.

The introduction of incoculant strains that nodulate a range of legumes within one inoculation group has been taken for granted. For example, Van Rensburg and Strijdom (1969) used the relative nitrogen-fixing efficiencies of each of the seven strains to select suitable inoculants, but while this is a good attribute of a strain it is not necessarily a guarantee of success of the inoculant strain. As my data clearly show the host plant cultivar has also a vast influence on the amount of nitrogen fixed. It is also clear that in the presence of other strains which may be more or less equally effective, the interaction of the strain may result in depressed ability of nitrogen fixation. Rice and Olsen (1983) have shown that having two strains of R.meliloti which were equally competitive resulted in decreased nodulation and N_2 fixation when both were present. They found that plant weight was highest for the single strain inocula and was decreased 5-19% with dual incoulation.

Effectiveness of a strain of Rhizobium is not only a desirable characteristic agronomically, but the host legume also tends to select effective strains to form nodules in the presence of less effective or even ineffective ones.

In studying the genotype interactions between the six strains of R.leguminosarum and 3 genera of host plants, namely Pisum sativum, Vicia faba and Lens esculenta, it was possible to demonstrate that each host plant has specific preference for particular strains. This is shown

in Tables 9,10,11 and 12. From these Tables it is clear that P.sativum van Onward preferentially selected strain MR4. When strain MR4 was inoculated on Vicia faba and Lens esculenta in equal proportions with the other two strains however, it was,completely out-competed by 1056 and 1001 respectively. Strain 1056 was isolated from Vicia faba in Wales while MR4 was isolated from P.sativum from Tanzania. One would be tempted to conclude that since strain 1056 was isolated from effectively nodulated V.faba and forms highly effective symbiosis with the latter there was more symbiotic specialization between V.faba and 1056 than between V.faba and the other two Rhizobium strains isolated from P.sativum. This view is supported by the finding of Lie et al.(1983)of the existence of coevolution between local plant ecotypes and the indigenous Rhizobium populations, resulting in well nodulated and effectively nitrogen fixing plants. Also Dean and Clark (1978) found that faba beans (Vicia faba L.) inoculated with pea inoculum were significantly inferior to faba beans inoculated with rhizobia specific for faba beans.

Strain 1001 was by far the most competitive strain on Lens esculenta where it was also found to be very effective.

Cultivar-strain interactions have also demonstrated that the host plant has a strong influence on the competitiveness of the three strains (see Tables 14,15,16 and 17; also Figures 2 and 3). Lahiri (1974) in trying to explain this phenomenon used the numbers of nodules formed by each

strain on the host when inoculated singly as an indication of host preferentiality. The data obtained with these three strains and six cultivars did not indicate that the number of nodules formed by a strain on its own determined its relative success in forming nodules in the presence of other strains. Neither nodule fresh weight nor the amount of N_2 fixation correlated to the nodule number formed by these strains in competition. It can be concluded, at this juncture, that those individual symbiotic properties are independent of each other and cannot be used to explain the competitiveness of a particular strain. It is however useful to note that effectiveness coupled with the other characteristics did tend to influence the competitiveness of a particular strain. For example strain MR4 was found to be more competitive with host cultivars than strain 1001 on cultivars where it formed more nodules and fixed more nitrogen. The wide variations observed for these symbiotic properties are supported by the observations made by Volkel et al. (1983) who pointed out that the statistical evaluation of soybean response to Rhizobium and nitrate is limited because of high coefficients of variations. Indeed as one can see from the statistical analysis the variations are quite enormous in some cases even with Pisum sativum cultivars interacting with 3 strains. Graphical analysis permitted plant to plant comparisons and revealed several patterns of interactions between symbiotic properties and competitiveness. The data also show that different cultivars have different abilities for forming nodules but the degree of

susceptibility to infection by rhizobia was not reflected in the amount of N_2 fixed as well. In fact there appeared to be an inverse relationship. This observation supports the findings of Nutman (1969), Caldwell and Vest (1977) and Jones and Hardarson (1979) that host plants carry genetic variability to infection and can at some stage inherit resistance to invasion by specific rhizobia. Cultivar Jl 199 tended to be most resistant to infection of the six cultivars used.

While it has been possible to speculate from the limited data that competitiveness could be linked with effectiveness and invasiveness of the strain, care must however be taken in arriving at such a conclusion since there could be other hidden factors which have to be taken into account to explain the various discrepancies observed in the data. For example, strain MR4 forms fewer nodules and fixes less N_2 on cultivar Jl 199 than strain 1001 and yet it is more competitive in mixed inoculum. One interesting characteristic of P.elatius has been reported by Kneen and LaRue (1983) who found that the plant was relatively resistant host to nodulation. Indeed the present findings in this study agree with this report as this host formed the smallest number of nodules. It would appear, however,that in the presence of 1001, strain MR4 becomes more invasive to this host and forms more nodules.

In the study of the effect of competing strains on each other it has been possible to demonstrate that some strains are affected by the presence or absence of other

strains. One such effect may be expressed through bacteriocin production and sensitivity on strains inoculated on agar and sand and grit. While it was possible to show that the absence of strain 1013, which produces bacteriocin against 1001 and 1056, in the inoculum did not have any positive effects on 1001, strain 1056 performed better than when 1013 was present (see Table 21). At the same time strain 1045 produces bacteriocin against 1001, but there was no indication of increased competitiveness of strain 1001 in the absence of 1045. These two examples do indicate that bacteriocin production *per se* is not an important factor in determining competitiveness of a sensitive strain, particularly under the situation of high inoculum titre like the one used in the study.

It appears from the data, though, that strains which are clearly competitive tend to suppress each other and hence the absence of one greatly increases the competitiveness of the other. Strain 1001 and MR4, for example, were found to be almost equally competitive in the absence of 1045, and in the absence of any one of them, the other forms more than half of the total number of nodules (53% and 54%) were formed by MR4 and 1001 respectively. A similar situation occurs with the two least competitive strains 1012 and 1013. The absence of one greatly boosts the chances of the other one almost to the point of out competing the other strains which were better in the presence of the missing strain.

Stimulation of one strain by the other also exists, as was reported by Rolfe and Gresshoff (1980) and Devine et al. (1979). This is probably in the form of cross-feeding where some products produced by one strain may stimulate another to infect the host. The evidence of such stimulation can be found in Table 21 using strains 1012 and 1001 as examples. In the absence of one the relative performance of the other strain drops. Also the relative performances of some strains improved when all the six strains were competing together when their performance was compared with the average performance obtained from paired competitions (see Tables 12 and 13). It is apparent, however, that while some strains were stimulated by the presence of one or two strains, others were simultaneously suppressed. The knowledge of this level of interaction is important before deciding for some reason to mix certain strains in one inoculant.

Double occupancy of the nodules is a phenomenon which was considered to be a rare event (Purchase and Nutman, 1957) but thereafter it was shown to occur more frequently than had been considered (Lindemann et al., 1974; Skrdleta and Karimova, 1969; Bromfield and Jones, 1980). In this study double occupancy has been encountered with wide variations (see SD for mixed nodules in Tables 15 and 18). The phenomenon was found to be more frequent in plants that were grown in agar (P.sativum and Vicia faba) than that grown in sand and grit (Lens esculenta). This observation agrees with the findings of Hughes and Vincent

(1942),Vincent (1954) and Bromfield and Jones (1980).

Double occupancy of nodules tends to occur more frequently among strains that are not closely competitive. Rice et al (1983) found that it usually occurs more when the inoculum titre is higher and when the strains are mixed in a ratio close to 50:50. In this particular case double strain occupancy accounted for up to 12% of the nodules. In this study double occupancy accounted for up to 10% on agar, but less than 5% in sand and grit. The fact that double occupancy was found to be higher on agar than in sand and grit could have been due to the continuous fluid nature of the medium and hence the possibility of one strain "sneaking" into the root hair with its competing partner.

It can be concluded that the competitive advantage of one strain over the other depends more on the interactions of the symbionts involved rather than the individual excellence of the partners. Invasiveness of the bacteria and the susceptibility to infection of the host plants are certainly important in ensuring that nodulation does take place, but are not mandatory to the success of one strain over the other in forming nodules. The single strain-single host selection procedure currently in use for inoculant strains has the intrinsic weakness of not being able to predict the performance of that particular strain in competition with other strains which may even be symbiotically inferior than itself.

Effectiveness of a strain seems to confer some advantage to competitively nodulate the compatible host legume. It is important, however, that this symbiotic property should be developed in situ with the host.

A number of workers have approached the problem of improved symbiosis from one side, either from the Rhizobium side alone or from host. Legume breeders, for example, have been selecting for genes of increasing yield and other properties without taking into consideration their symbiotic compatibility with the homologous rhizobia.

It has to be borne in mind that Rhizobium legume interaction is a symbiotic system in which the two partners play an equal role in ensuring the success of the system,the fixation of gaseous nitrogen from the atmosphere.

CHAPTER 2

EFFECT OF SOIL MOISTURE ON COMPETITION BETWEEN STRAINS OF RHIZOBIUM LEGUMINOSARUM FOR NODULATION OF PISUM SATIVUM VAR ONWARD

INTRODUCTION

It is well known that plants wilt when the moisture content of the soil reaches a point known as the permanent wilting percentage (the soil moisture content at which soil cannot supply water at sufficient rate to maintain turgor in plant cells). Likewise it is well known that the addition of water to dry soils results in increased plant growth and greater yields. Water stress affects growth, yield and quality of various legume crops (Kilmer et al., 1960, Bourget and Carlson, 1962; Meriaux, 1972; Mack, 1973). Water is a major component of green plants, accounting for 70-90% of the fresh weight of most herbaceous species. It is contained in cell contents where it provides a suitable medium for many biochemical reactions.

Hamdi (1971) studied the movement of Rhizobium in the soil and found that it was governed by moisture and ceases when water film becomes discontinuous which occurs normally at water tensions still adequate for seed

germination. He found that at good moisture levels rhizobia move between 2 and 20 mm per day in sandy soils. Worrall and Roughley (1976), however, did not find any movement by R.trifolii over a 15-day period either through the soil or along the root system of Trifolium subterraneum roots even though the soils were kept at field capacity.

Brockwell and Whalley (1970) suggested that some of the variability in the nodulation of inoculated Medicago seed sown in dry soil may have been due to low soil moisture.

Sprent (1972a, 1972b and 1972c) found that nitrogen fixation (in Vicia faba and Glycine max) was sensitive to moisture; too much or too little decreased the nitrogen fixation. Worrall and Roughley (1976) showed that soil moisture and method of inoculation greatly affected the number and distribution of infected root hairs and nodules in young seedlings of Trifolium subterraneum. A reduction of soil moisture from 5.5 to 3.5% significantly decreased the number of infection threads and inhibited nodulation, although the number of rhizobia in the rhizosphere was unaffected. At low soil moisture levels the root hairs were abnormally short and swollen. Infection and nodulation were little affected between 5.5 and 9.5% moisture.

Osa-Afiana and Alexander (1979) showed that the survival of R.trifolii and R.japonicum was greater at 10% moisture content than at 22, 35 and 45% levels in both sterile and non-sterile soils. At higher levels, however, survival was better in sterile soils than in non-sterile soils.

Tu and Hietkamp (1977) tried to correlate moisture levels with acetylene reducing activity of soybean root nodules. He found that stressed plants showed no acetylene reducing activity and that too much moisture reduced it. The latter may have been due to defficiency of oxygen supply to root nodules (Sprent, 1971).

Skrdleta (1973) observed that competition between inoculum strains of R.japonicum was influenced by the planting season which corresponded to variation in soil moisture contents. Schwinghammer and Brockwell (1978) found that producing bacteriocin and phage, strains of R.trifolii, strongly suppressed growth of sensitive strains in broth culture. In peat culture their degree of suppression was much more marked in "wet" peat than in "damp" peat showing that the moisture content of peat was clearly an important variable. They also observed that there was hardly any difference in bacterial growth at the two moisture contents when the strains were grown separately. Since bacteriocin production was linked with bacterial growth in broth culture it can be assumed that bacteriocins were produced in comparable quantities in

both wet and damp peat but that the liquid in the wet peat facilitated mobility and the diffusion of inhibitor substances from the producer cells to the sensitive ones.

The role of the behaviour of microorganisms in ecological situations is an area where very little data exists. Motility and chemotaxis have been shown to provide competitive advantage for nodulation to strains possessing them (Napoli and Albesheim, 1980; Ames et al., 1980). Environmental factors such as pH, temperature and moisture have been found to influence growth and longevity of free-living rhizobia in the soil (Marshall, 1964; Munns, 1968; Vincent, 1952).

There is hardly any work on the effect of soil moisture on competition between different strains of Rhizobium for nodulation of host legumes. One reason why this is so may be because most studies have been done in the temperate regions where soil moisture is not as limiting as in the tropics. Another could be because soil moisture is a variable which is difficult to keep constant. There have been efforts to study moisture stress in the laboratory using various methods. One involves watering pots of plants with a certain amount of water so that weights of pots are kept constant for each moisture content desired. This is the most extensively used method for simulating the natural stress conditions of the field environment, but it has the drawback of creating moisture gradients between one watering exercise

and another ranging between levels causing water-logging and wilting. The other method uses the application of suction through a sintered glass funnel (Wallace,1958) to produce a moisture gradient in the soil but it does not permit very high moisture stresses to be obtained. A method of adjusting moisture with a pressure membrane apparatus (Richards, 1952b) submits organisms to undesirable pressure and if constant stress is to be obtained, the soil cannot subsequently be aerated during the experiment.

In this study an adaptation of the methods used by Painter (1966),Zur (1966) and de Guiran (1975) was used with the aim of avoiding the above drawbacks. The method employs the principle of osmosis where water moves from a region of low solute concentration to higher concentrations through a semipermeable membrane.

Zur (1966) and Painter (1966) used cellophane as a semipermeable membrane, but Guiran found fibran (material made of natural fibre reconstituted from leather refuse (split hide)) to be more suitable than cellophane in that it is not destroyed by cellulolytic microorganisms and hence can be used for longer periods than cellophane.

Soil moisture was maintained at fairly constant levels by the use of fibran membrane and varying concentrations of polyethylene glycol (PEG) of molecular weight 20,000 (see Materials and Methods).

Competition was measured by typing a number of nodules randomly selected from each plant using the Fluorescent Antibody technique (FAT).

MATERIALS AND METHODS

(a) Preparation of strains for inoculation

Three strains of Rhizobium leguminosarum RCR 1001, RCR 1056 and MR4 were grown in 200 ml of TY medium in 250 ml Erlenmeyer flasks and incubated in an orbital shaker for 3 days (see General Materials and Methods). The strains were then mixed in equal proportions by volume in pairs and all three together, shaken thoroughly, before aliquots of 5 ml were taken and inoculated onto the pregerminated seeds in irradiated Woburn soil from Woburn,Hertfordshire, UK.

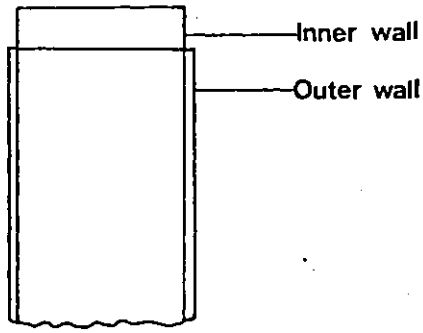
(b) Making soil moisture bags

The fibran material was supplied by F.Heinnemann Ltd., London as rolls of tubes (10 m long and 130 mm diameter). The tubes were cut into pieces, each 400 mm long and soaked in 40°C in a water bath for 1 hour. Each tube was then folded in two to form a double walled tube (see Diagram 2). A rubber band was coiled around the bottom of

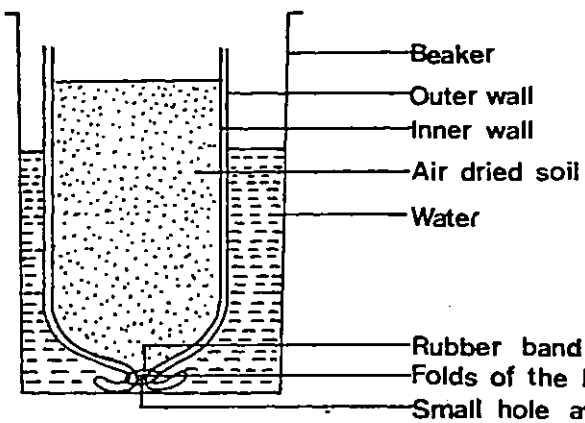
DIAGRAM 2:

HOW TO PREPARE AND USE "FIBRAN" TUBING FOR SOIL MOISTURE STUDIES

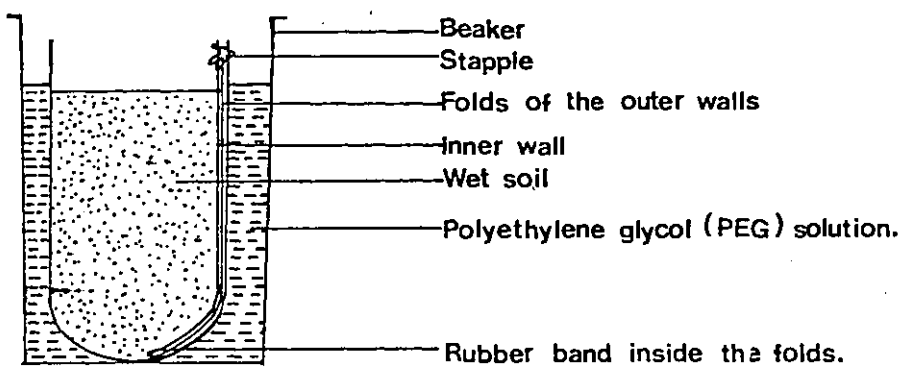
The tubing is cut in pieces twice the desired length and soaked in water bath at 40 ℃ for 1 hour. Then the following procedure is followed;



A. The "Fibran" tubing is folded into double-walled tube.



B. The folded end of the Fibran tube is firmly tied with rubber band so as to leave very small hole at the base. The bag is then filled with air dried crushed soil and placed in water and then soil allowed to wet "per ascensum".



C. When soil is wet unfold the outer walls of the bag and stapple them together on one side and immerse the bag in PEG solution.

the double-walled tube in order to obtain a bag with a very small hole at the bottom. The bag was then filled with air-dried soil (that had been γ-irradiated for two days - 2 megarads) and immersed in a beaker of sterile tap water against an illuminated background so that the water could be seen wetting the soil "per ascensum" (to the top). When the soil was fully wet the tube was unfolded on one side and the walls of the outer layer stripped over and strapped to the inner wall (see diagram 2 and Plates 8a and 8b).

The bag was then immersed in a known concentration of polyethylene glycol (20,000 MW) for 24 hours before planting the pregerminated seeds. The seeds were immediately inoculated with an exponentially growing mixed inoculum as described earlier (General Materials and Methods), treatments replicated 4 times and placed in controlled environment cabinets (see Plate 7).

Initially five concentrations of PEG were prepared to establish moisture stresses ranging between field capacity and wilting point (moisture at which the pregerminated seeds did not grow). PEG solutions of concentration 4,8,12,20 and 30% were used and had measured osmotic pressures of 0.5, 1,2,8 and 16 atmospheres respectively (de Guiran and Demeure, 1978). The water potential of the soil (PF) is equivalent to the osmotic pressure of the solution when the system is in equilibrium. In this case they found the PF to be equivalent to 2.7, 3, 3.3, 3.6 3.9 and 4.2 respectively. As there was no growth at 30% PEG only the 4 concentrations were subsequently used in this

study.

The concentration of PEG was kept constant by keeping the level of the solution in the beaker constant with sterile distilled water. To minimize drying of the soil from the top sterile black plastic beads were sprinkled on the surface of the soil up to 3 inches depth when the seedlings were 1 week old.

Plants were harvested six weeks after inoculation when some of the plants had started flowering and the bags were still intact. Shoots of the plants were weighed immediately they were cut off from the roots and then dried in 80°C oven in tin foil boats) for 24 hours. The soil samples were also taken simultaneously and 2 g weighed out and dried in the above oven for 24 hours.

Nodules were picked with forceps and scissors and counted before they were preserved in 20% glycerol at -20°C until they were typed using the fluorescent antibody technique. (see General Materials and Materials).

RESULTS

Table 22 and Figure 8 give the soil moisture content as a percentage of the soil dry weight at different concentration of polyethylene glycol (PEG). This was obtained by taking 2 g of the soil at the time of harvesting each treatment and drying it in an 80°C oven for 24 hours in tin foil cups. The soil moisture content ranged from 9.2% at 4% PEG to 4.3% at 20% PEG, with slight

Table 22: Moisture contents of soils at different PEG concentration and different combination of Rhizobium strains

Strain Combination	Reps	PEG Concentrations 4%	8%	12%	20%
	1	8.6	7.6	4.9	4.7
	2	8.2	5.4	5.2	4.6
1001+1056	3	9.6	6.5	4.7	4.3
	4	11.1	6.8	5.3	4.5
	Average	9.4	6.6	5.1	4.5
	1	8.8	5.6	4.1	4.1
1001+MR4	2	9.0	6.2	5.0	3.9
	3	8.7	5.6	4.9	4.3
	4	8.1	6.8	5.1	4.6
	Average	8.7	6.1	4.8	4.2
	1	10.4	5.8	5.4	5.3
1056xMR4	2	11.8	5.8	5.5	4.1
	3	7.4	6.2	5.0	3.6
	4	8.7	7.0	4.8	4.5
	Average	9.5	6.2	5.1	4.3
	1	9.3	5.3	5.3	4.1
All 3	2	7.8	6.2	4.2	5.2
together	3	8.5	6.9	5.2	4.4
	4	11.8	6.0	5.4	3.8
	Average	9.2	6.1	5.0	4.3
Experiment error(SD of mean)		0.355	0.238	0.141	0.135

Figure 8: Soil moisture content (o/o) at different PEG concentration (o/o).

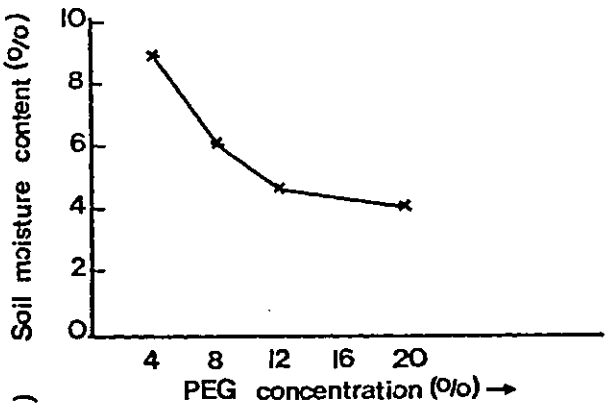


Figure 9: Effect of soil moisture on fresh and dry weights of shoots of P. sativum inoculated with strains of R. leguminosarum.

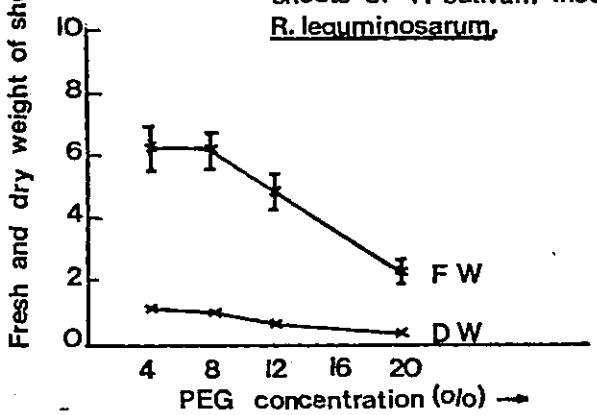
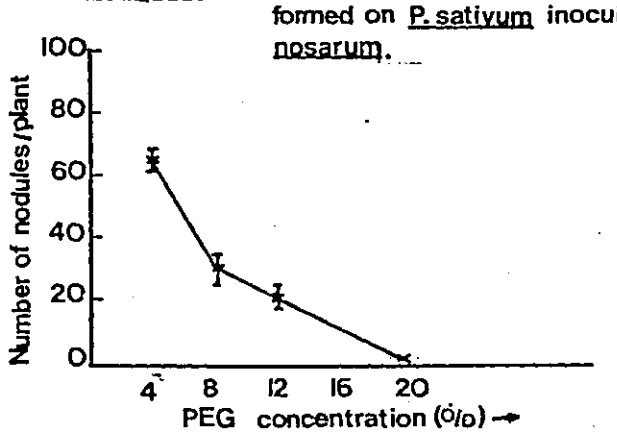


Figure 10: Effect of soil moisture on the number of nodules formed on P. sativum inoculated with strains of R. leguminosarum.



variations among the different treatments. Baver (1956) obtained considerable variation in moisture contents at given tension and he attributed this to hysteresis effect (an oscillator effect exhibited by elastic bodies operating at stresses below the proportional limit where in a given value of an operating parameter may result in multiple values probably due to deformation) and imperfections in the sampling method. The slight variations observed in this study were not significant.

Effect of soil moisture on the dry weight of shoots of plants nodulated by different combinations of inoculants

The effect of soil moisture on the host plant is shown in Table 23 which shows the percentage water content, fresh weight and dry weight of the shoots. Figure 9 gives the graphical presentation of the average dry weights (DW) and fresh weights (FW) of the shoots. The slight variations in different treatments shows the effect of different strain combinations at different moisture levels. From the data it is obvious that combinations involving strain 1056 yield lower values of the weights than those with the two effective strains 1001 and MR4. Also at 4% the predominance of 1056 was manifested by the low fresh and dry weight.

Table 23 Effect of soil moisture on the fresh and dry weights of host plants

Strain Combinations	Replicates	PEG CONCENTRATION 4% FW	4% DW	4% %H_2O	8% FW	8% DW	8% H_2O	12% FW	12% DW	12% H_2O	20% FW	20% DW	20% H_2O
	1	6.5	1.0	84	4.7	0.8	84	6.2	1.1	83	4.0	0.7	84
1001+1056	2	9.3	1.4	85	6.4	1.0	85	3.7	0.6	83	0.4	0.1	81
	3	6.7	1.1	83	3.1	0.5	84	4.2	0.7	82	1.2	0.2	81
	4	4.7	0.7	85	4.5	0.7	83	5.2	0.9	82	2.4	0.4	82
	Average	6.9	1.2	84	4.7	0.7	84	4.8	0.8	83	2.0	0.4	82
	SD	1.9	0.29		1.35	0.20		1.10	0.21		1.56	0.25	
	1	8.6	1.4	84	7.4	1.4	82	6.0	1.1	82	3.2	0.6	81
1001+MR4	2	8.4	1.3	85	5.9	1.0	83	1.9	0.3	82	1.1	0.2	82
	3	10.1	1.6	85	6.6	1.1	83	4.4	0.8	82	1.6	0.3	82
	4	6.1	1.1	85	7.9	1.3	84	2.7	0.5	83	4.7	0.9	82
	Average	8.3	1.3	85	6.9	1.2	83	3.8	0.7	82	2.6	0.5	82
	SD	1.6	0.18		0.9	0.14		1.8	0.32		1.6	0.29	
	1	7.5	1.1	86	3.8	0.7	82	4.2	0.7	83	3.4	0.6	83
1056+MR4	2	5.3	1.0	84	7.4	1.3	82	6.1	1.0	83	1.6	0.3	84
	3	6.2	1.0	84	11.1	1.9	83	6.9	1.1	84	0.6	0.1	78
	4	5.9	0.9	85	8.4	1.5	82	6.7	1.1	83	1.9	0.3	83
	Average	6.2	1.0	85	7.7	1.3	83	6.0	1.0	83	1.9	0.3	82
	SD	0.93	0.06		3.0	0.50		1.25	0.20		1.17	0.18	
	1	3.6	0.5	85	7.5	1.3	83	4.5	0.7	84	2.1	0.4	83
All 3	2	4.9	0.8	84	7.8	1.2	83	3.9	0.6	84	3.2	0.6	82
together	3	4.1	0.7	84	4.9	0.8	83	8.1	1.4	82	3.8	0.6	83
	4	5.7	0.9	84	9.4	1.5	84	5.4	0.9	84	2.8	0.5	82
	Average	4.6	0.7	84	7.2	1.2	84	5.5	0.9	84	2.9	0.5	83
	SD	0.88	0.16		1.8	0.27		1.86	0.36		0.71	0.11	
Exptal. Error = SD of mean		1.5	0.25	0.17	1.3	0.25	0.59	0.95	0.14	0.61	0.53	0.09	0.31

NB: FW = Fresh weight
DW = Dry weight
PEG = Polytheylene glycol (20,000 MW).

Table 24: The effect of soil moisture stress on the number of nodules formed by the plants inoculated by a mixture of strains.

Strain Combination	Replicates	% PEG CONCENTRATION			
		4%	8%	12%	20%
	1	63	37	15	5
1001+1056	2	72	36	16	0
	3	53	42	22	1
	4	86	39	13	0
	Average	69	39	17	2
	1	76	38	17	0
1001+MR4	2	68	40	15	0
	3	57	32	12	3
	4	72	31	23	2
	Average	68	35	17	1
	1	67	37	24	1
1056+MR4	2	61	29	29	0
	3	52	45	45	0
	4	70	33	33	2
	Average	63	36	36	1
	1	68	24	24	0
All 3	2	72	18	18	0
Together	3	49	33	33	2
	4	60	28	28	0
	Average	62	26	26	0.5
Exptal Error (SD of mean)		3.51	5.60	1.00	0.62



Plate 7: Plants, Pisum sativum, growing under different soil moisture stresses determined by the concentrations of PEG outside the Fibran bags containing soil. The beakers in which the bags were placed, were immersed in Wisconsin water tanks at 25°C.



Plate 8a: Plants growing in soil subjected to different moisture stresses by the different concentration of PEG solution surrounding the Fibran bag in the beaker.



Plate 8b: The Fibran bags with plants from different moisture stresses (as above) as they are seen outside the solution. Notice the level of soil in the bag.

Effect of soil moisture on the number of nodules formed by the strains in mixed inoculum

Soil moisture has a considerable effect on the number of nodules formed by the host plant (see Table 24). At 4% PEG (9.2% moisture) the plants formed nearly twice as many nodules as those at 8% PEG (6.3% moisture). More stress is, however, experienced at higher concentrations of PEG, 12% (5.0% moisture) and at 20% PEG (4.3% moisture). At the latter concentration there were so few nodulating plants that it was impossible to get any meaningful analysis.

Table 24 gives the number of nodules per plant at different soil moisture contents (due to different concentrations of polyethylene glycol,PEG) and is illustrated by Figure 10.

Irrespective of the strain combinations, the number of nodules significantly decreased with increasing soil moisture stress.

The effect of soil moisture on competition of 3 strains of R leguminosarum as measured by the number of nodules they formed on Pisum sativum var Onward

Table 25 shows the number of nodules formed by each strain in competition with the other two strains at different moisture tensions created by varying concentration of PEG (20,000 mw). The three strains manifested varying comptetitive abilities at different soil moisture. This phenomenon is clearly illustrated by the magnitudes of the different ratios. Strain MR4 is most competitive at all moisture tensions in paired combination generally, though strain 1001 is a slightly better competitor at higher moisture content (4% PEG, 9.2% moisture). Strain 1056 performed better at higher moisture but failed to compete at low soil moisture content, though the data also show that it was still surviving. Figures 11a&b show the performance of each strain at different moistures as determined by the percentage of nodules formed by each strain in competition of the 3 strains.

Table 25: Competition of the 3 strains measured by the number of nodules formed when inoculated in pairs

PEG CONCENTRATION (%)

Strain Combinations	REPS	4			8			12			20		
		A	B	Mix	A	B	Mix	A	B	Mix	A	B	Mix
A + B	1	8	6	1	14	1	0	11	4	0			
	2	10	5	0	9	5	1	11	3	1			
1001+1056	3	9	6	0	12	3	0	10	3	2			
	4	7	8	0	11	3	1	14	1	0	Too few nodules		
	Total	34	25	1	46	12	2	46	11	3	to analyse		
	%	57	42	2	77	20	3	77	18	5			
	Ratio	3	2		4	1		4	1				
A + C		A	C	Mix	A	C	Mix	A	C	Mix	A	C	Mix
	1	6	9	0	5	9	1	4	11	0			
	2	8	7	0	4	11	0	3	11	1			
1001+MR4	3	7	8	0	3	12	0	2	13	0			
	4	9	5	1	5	10	0	2	12	1	Too few nodules		
	Total	30	29	1	17	42	1	11	47	2	to analyse		
	%	50	48	2	28	70	2	18	78	3			
	Ratio	1	1		1	2.5		1	4				
		B	C	Mix	B	C	Mix	B	C	Mix	A	C	Mix
B + C	1	6	8	1	1	14	0	2	12	1			
	2	2	12	1	2	13	0	1	14	0			
1056+MR4	3	3	11	1	0	15	0	1	13	1			
	4	5	10	0	0	14	1	0	15	0	Too few nodules		
	Total	16	41	3	3	56	1	4	54	2	to analyse		
	%	27	68	5	5	93	2	7	90	3			
	Ratio	1	2.5		1	14	2	1	14				

Mix = Nodules containing both strains.

Table 26: Relative performance (in percentages) of the three strains in paired competition at different soil moisture contents.

	PEG CONCENTRATION (%)			
Strains	4	8	12	20
1001	53.5	53.5	47.5	
1056	34.5	12.5	12.5	
MR4	58.0	81.5	84.0	

* The percentages were obtained by calculating the average of the percentage of nodules formed by each strain in paired combination with the other two strains.

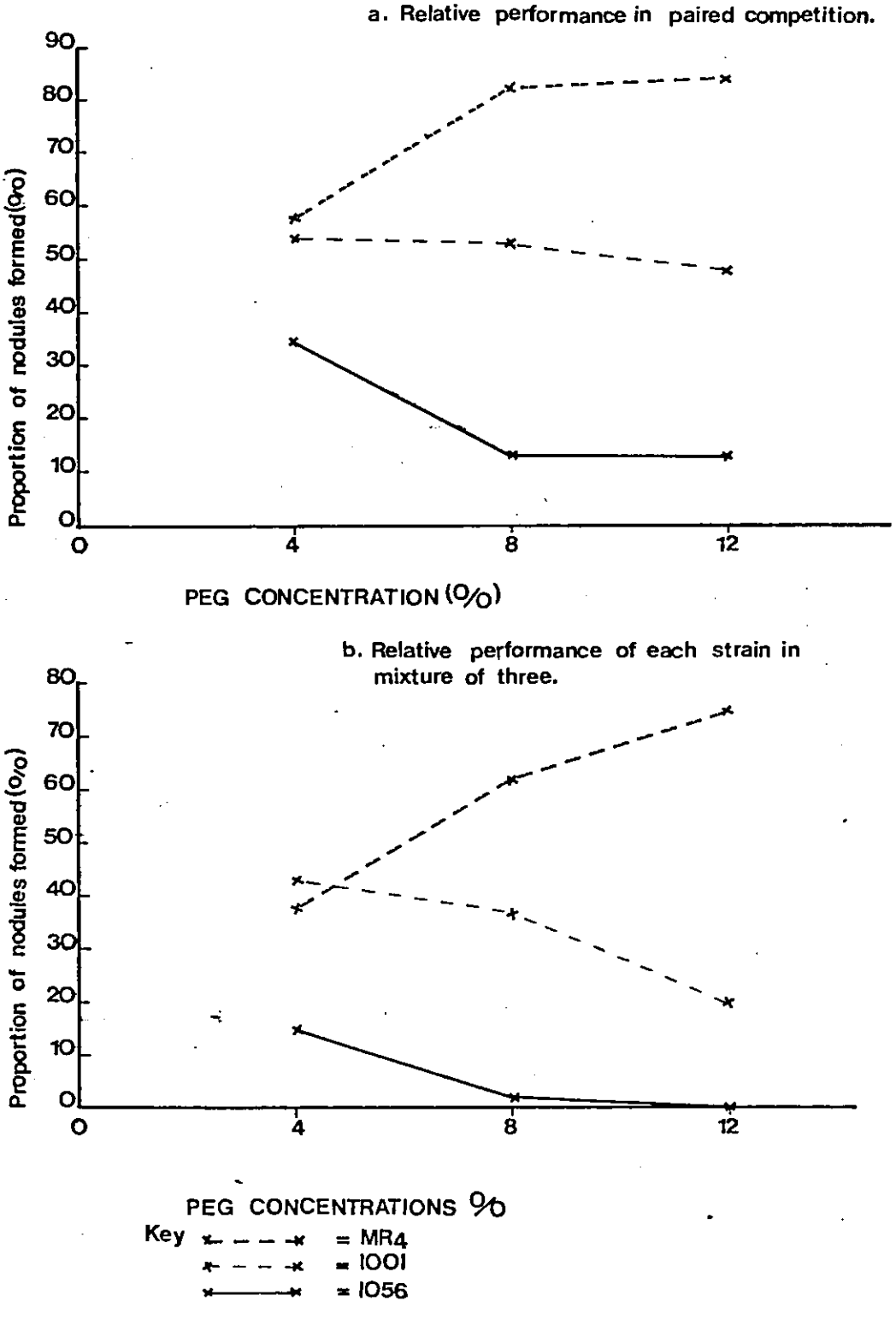
Table 27: Number of nodules formed by each of the three strains when competing with the other two strains at different soil moisture tensions.

	% PEG CONCENTRATION												
Replicates	4				8				12				20
	1001	1056	MR4	Mix	1001	1056	MR4	Mix	1001	1056	MR4	Mix	NA
1	9	2	4	0	7	0	8	0	4	0	10	1	
2	4	2	8	1	6	0	9	0	3	0	11	1	
3	7	3	5	0	4	0	11	0	3	0	12	0	
4	6	2	6	1	5	1	9	0	2	0	12	1	
Total	26	9	23	3	22	1	37	0	12	0	45	3	
%	43	15	38		37	2	61	0	20	0	75	5	
Ratio	3	1	2		18	1	30		1	0	4		

Mix = Nodules that contain more than one strain, usually two.

The competitive ratios obtained when the three strains were competing together correlate with their performance in paired competition.

Figure II : Effect of soil moisture on competitiveness of the 3 strains of R. leguminosarum inoculated on P. sativum var Onward.



DISCUSSION

Symbiotic response to physiological stress is a highly variable quantity in which tolerance or susceptibility seems to result from heritable factors in both the host and *Rhizobium*. The effect of genotype interaction has been discussed in Chapter 1.

In annual arable crops such as *Pisum sativum*, factors like sowing time, inoculation practice, soil texture, nutrient and water status are usually arranged close to optimum in order to get maximum yields. In countries where rainfall is unreliable, irrigation practices are used to avert drought. There are situations, however, when prolonged soil moisture stresses are inevitable and when this happens it is important for plants to be able to withstand the stress, at least for a short period, without loss of vitality.

The effect of moisture stress on the legume host is evident (see Table 23, Figure 9 and Plates 7,8a and 8b) in that fresh and dry weights and also the size of the plants are affected by the amount of soil moisture. Dry matter in the host legume used was highest at 9.2% moisture content which was near to field capacity. These findings are in agreement with those of Sprent (1972), Meriaux (1972) and Mack (1973).

Benefits from legume inoculation with Rhizobium are sometimes not evident even though the right host-strain combination selected under controlled environmental conditions, are used. One of the reasons for this failure is demonstrated by this study. At a moisture content of 4.3% (at 20% PEG) hardly any nodules were formed, showing failure in nodulation was due to moisture stress,since all the other conditions were kept constant. Also some strains were more competitive at good soil moisture levels, but failed to compete with other strains when soil moisture decreased, for example strain 1056 and even strain 1001 performed better at higher moisture levels than they did at lower ones. With decreasing moisture the latter strains were out-competed by strain MR4. It is tempting to relate the relative success of strain MR4 under those conditions with the prevailing conditions of its origin, Morogoro, which receives rain for seven months of the year and remains dry for the rest of the period. Also within the rainy season there is a short dry period of up to one month duration.

Worrall and Roughley (1976) showed that as the moisture level decreased, the number of root hairs decreased and they became abnormally short and thickened. This may account for the decrease in the number of nodules observed in this study. Probably thickened root hairs are less susceptible to infection for various reasons

including the suppression of pectolytic enzymes demonsrated by Fahraeus and Ljunggren (1959) and Martinez-Molina and Olivares (1982) to play apart in the infection process. The success of one strain to form nodules on the root under such conditions could mean that the bacteria is able to produce enzymes like pectinases which dissolve the thickened walls of root hair to allow for the formation of infection thread (Callahan, 1979).

It is also likely that the resistance to infection by legumes under stressed condition is one way of conserving energy as the plant struggles to acquire the necessary materials for survival. Under these conditions ineffective strains such as 1056, would tend to be unfavoured. A lot more data is, however, needed before one can explain the observed competitive advantage of one strain over the others at different moisture conditions.

CHAPTER 3

THE EFFECT OF TEMPERATURE ON COMPETITION

INTRODUCTION

Higher plants are unable to maintain their cells and tissues at a constant temperature and therefore their leaves,stems and roots are usually within a few degrees of ambient temperatures. The growth and metabolism of plants are therefore greatly affected by temperature.

Rhizobia are mesophilic bacteria which can survive at low temperatures. Various types of rhizobia differ markedly in their tolerance to elevated temperatures and are soon killed at temperatures above 50°C for more than a few hours (Diatloff, 1970). Survival of Rhizobium in soil and nodulation are known to be adversely affected by high soil temperatures (Bowen and Kennedy, 1959; Marshall, 1964).

The effect of temperature on the Rhizobium - legume symbiotic system has been reviewed by Lie (1974); Dart et al. (1976) and Lie (1981). The temperature effect is dramatic in that it appears to affect all stages of nodule formation and nodule functioning (Gibson, 1971, 1975; Dart and Day, 1971), Low temperatures retard infection and nodule initiation more than they affect nodule development and dinitrogen fixation (Gibson, 1967; Roughley, 1970; Roughley et al., 1970; Day et al., 1978). The threshold temperature for nodulation varies with plant species and bacterial strain.

The factors that affect both the bacteria and the host plant in this way are therefore of practical importance in selecting suitable strains for particular environments. Herridge and Roughley (1976) working on the influence of 3 temperatures on the symbiosis of 3 strains of Rhizobium and Macroptilium atropurpureum (Siratro) and Macroptilium axillare (axillaris) found that optimum host/strain temperature for siratro was 31/26°C (day/night) while axillaris grew best at 26/21°C which was lower than that reported for other tropical legumes; but higher than for temperate species. Growth of all host/strain combinations was inferior to their respective nitrate controls at 21/16°C and all control and inoculated plants of axillaris grew poorly at 31/26°C. Cadwell and Weber (1970) also observed that the occurrence of specific serogroups of an established population of Rhizobium japonicum in the nodules of Glycine max changed with planting date, which corresponded to different temperatures. Weber and Miller (1972) found that strains which formed most of their nodules at 30°C formed very few nodules at 10 or 15°C and vice versa.

The competitiveness of a strain used as an inoculant at a particular optimum temperature for plant growth is therefore of considerable practical significance. Bushby (1982) reported that Couper and Vincent had observed that a strain which was more competitive at optimal growth conditions (15-16°C) for Medicago truncatula was found to be less competitive on Medicago sativa at its optimal

growth temperature of 25-26°C. Hardarson and Jones (1979) found that a strain of R.trifolii which was competitive on clover at 12°C was completely outcompeted by another strain at 25°C.

Indications that strains of Rhizobium can undergo temperature adaptive advantage in competition was observed by Ek-Jander and Fahraeus (1971) using two strains of R.trifolii, one from the subarctic region of Scandinavia and the other from Southern Scandinavia. They found that the strain from Northern Scandinavia grew faster and nodulated clover faster than the strain from Southern Scandinavia when tested at 10°C. There was however, no difference between the two strains at 20°C. Hardarson and Jones (1979) however did not find any relationship between competitiveness at a particular temperature of one strain and its geographical origin.

Elevated temperature may delay nodule initiation (Gibson, 1967, Roughley and Dart, 1970; Munevar and Wollum, 1981) nodule development (Kumarasinghe and Nutman, 1979) and interfere with nodule structure (Roughley 1970) and nodule functioning in temperate legumes (Pankhurst and Gibson, 1973; Munevar and Wollum 1983) Date (1977) indicated that the optimum temperature for nodule formation and nitrogen fixation in tropical legumes was generally close to 30°C whilst Pankhurst and Gibson (1973) found 30°C to be the maximum temperature for dinitrogen fixation and at higher temperatures nodules showed abnormal senescence and accelerated senescence. For

tropical and subtropical species, including soybeans, the maximum temperature for dinitrogen fixation was 27-40°C (Gibson, 1976; Souto and Dobereiner, 1968; Dart *et al.*, 1975) with the range being due to differences between host plant species as well as strains of *Rhizobium* used. The optimum temperature for nodulated soybean has been reported as 20-30°C (Hardy *et al*, 1968),10-35°C (Dart and Day, 1971) while in another study, the response varied with the temperature conditions for growth prior to assay (Gibson, 1975). In the latter study with plants grown at 21/16°C (day/night) and incubated at 25°C the value was double that found for incubation at 20°C and 45% higher than those grown at 27/22°C (day/night) and incubated at 25 or 30°C. Such results indicate the need for caution in selecting the conditions used to determine overall levels of dinitrogen fixation from periodic C_2H_2 reduction assay.

MATERIALS AND METHODS

Plants were grown in plastic multipour beakers (Gallenkamp) containing soil, γ-irradiated with 2 Mrad. The beakers were immersed in Wisconsin tanks or "Wisconsin soil temperature tanks" which are essentially water baths designed in the 1920's at the University of Wisconsin, U.S.A. The water temperature in each tank is controlled by circulation of heated or chilled liquid depending on the temperature set on a thermostat(see Plate 9). The Zeal chart recorder indicates the water temperature in each unit.

Seeds were surface sterilized and pregerminated before planting them in the pots. Air temperature was maintained constant at 15°C with 16 hours of lighting, 24,000 lux at 0.54 m (from fluorescent tubes). There was no humidity control.

The plants were inoculated with mixed strains and the treatments placed in each Wisconsin tank in a randomized manner.They were watered daily with adequate amounts of sterile distilled water to keep the soil as near to field capacity as possible. Water levels in the tanks were kept constant above the soil level in the beakers, by adding adequate amounts of tap water daily. This ensured that the temperature of the surface soil was not significantly lower than the temperature of the bulk. Slight variations between the surface and the lower soils were observed, particularly at 35°C where there was up to 3°C difference in each pot. Topping-up of tanks with tap water also altered the temperature of water in the tanks, but this



Plate 9: Plants growing in Wisconsin tanks kept at different temperatures.

Far right-hand corner tank was at 15°C the one in the back-ground far left-hand corner was at 25°C and near left-hand corner tank was kept at 35°C.

Wisconsin tanks are essentially big "water baths".

The empty tank contained plants growing at 8°C but these had been removed to show the structure of the tank.

soon returned to normal (less than 10 minutes).

Plants were harvested after seven weeks growth under those conditions. The shoots were weighed fresh to obtain fresh weights and later dried in an 80°C oven for 24 hours to obtain dry weights. For acetylene reduction assays nodulated shoots were excised immediately after uprooting the plants from the soil and assayed as described in the General Materials and Methods.

After the acetylene reduction assay nodules were removed from the roots and weighed to obtain fresh weights for each temperature to calculate the rate of reduction per gram per hour.



Plate 10: The effect of temperature on plants, P.sativum, inoculated with various mixtures of R.leguminosarum strains was manifested in the plant vigour. Note the sizes of the plants at different temperature 8°C, 15°C, 25°C and 35°C (from left to right) were clearly different. The effect of temperature on competition of the three strains was equally dramatic.

RESULTS

Effect of temperature on dry weight of shoots of plants nodulated by a mixture of strains at different temperatures.

Plant shoots of the host plant, Pisum sativum var Onward show a distinct response to temperature, with the optimum at 25°C. The differences in weights between plants growing at 15°C and those growing at 35°C are very slight. No measurements were made on plants growing at 8°C as they had not nodulated up to the time of harvest.

The variation in dry weights between one treatment and another is noticeable, but not significant.

Table 28 shows the effect of temperature on dry weight of the shoots at different treatments. Figure 12 illustrates this effect on the dry weights of shoots of P.sativum nodulated by a mixture of different strain at different temperatures.

Effect of temperature on nodule weight

The effect of temperature on symbiosis expresses itself in the number, volume and weight of the nodules formed. In this study nodule weight was taken because it is one of the symbiotic characteristics found important in determining the interaction in terms of nodular material formed and dinitrogen fixation. The nodule weight was found to be highest at 25°C which was also optimal for shoot weight (see Table 28 and Figure 12). Figure 13

illustrates this relationship. It is evident that at all treatments the optimal temperature was 25°C. Performance at 15°C was better than at 35°C where hardly any nodules formed.

Effect of temperature on the rate of dinitrogen fixation (measured by acetylene reduction assay)

The rate of acetylene reduction shows the activity of the nitrogenase and is temperature dependent (Table 30). There is no evidence that the rate of fixation is significantly higher at 25°C than at 15°C. The wide variations in the rate of fixation among replicates makes the use of averages rather meaningless in certain cases. Clearly from these data, there was is hardly any enzymic activity at 35°C. Those nodules which were formed were elongated and white in colour showing that they were ineffective. Figure 14 illustrates the effect of temperature on N_2 fixation.

Effect of temperature on number of nodules formed by each strain in competition on P.sativum var Onward

The number of nodules formed by each of the three strains when in competition with the other two in pairs or together is shown in Table 31 and illustrated in Figures

Table 28: Effect of root temperature on dry weight of shoots of nodulated plants in (mg)

Strain Combinations	REPS	TEMPERATURES		
		15°C	25°C	35°C
	1	74	170	82
1001+1056	2	69	147	80
	3	53	127	40
	4	68	114	80
	5	76	160	60
	Average	68	143	68
	SD	9.0	23.0	18
	1	58	307	83
1001+MR4	2	64	106	58
	3	66	271	54
	4	72	240	84
	5	54	262	75
	Average	63	237	71
	SD	7.0	77.2	14
	1	93	257	20
1056+MR4	2	87	388	52
	3	109	440	54
	4	63	287	37
	5	57	285	94
	Average	82	331	51
	SD	21.5	78.5	27
	1	57	354	131
All three	2	89	224	82
together	3	88	356	152
	4	102	186	110
	5	62	425	96
	Average	80	310	115
	SD	19.2	100	27.8
SD of strains combinations		9.21	84.98	27.29

Table 29: Effect of temperature on nodule weight (in mg)

Strain Combinations	REPS	TEMPERATURES		
		15°C	25°C	35°C
	1	19	103	0
1001+1056	2	10	155	34
	3	10	115	0
	4	14	100	78
	5	12	103	0
	Average	13	115	22
	SD	3.7	22.9	
	1	59	100	12
1001+MR4	2	21	50	0
	3	17	108	0
	4	29	129	39
	5	22	150	0
	Average	30	107	10
	SD	16.9	37.5	
	1	16	104	0
1056+MR4	2	25	147	0
	3	17	112	0
	4	24	137	0
	5	16	109	13
	Average	20	122	3
	SD	4.5	18.9	
	1	12	130	63
All three	2	18	150	47
together	3	50	150	87
	4	117	128	41
	5	28	197	0
	Average	25	151	48
	SD	15.1	27.7	
SD of strain combination effect		6.89	19.17	19.90

Table 30: Rate of N_2 fixation (measured by acetylene reduction) (in ethylene µM/hr/g nodule).

Strain Combinations	REPS	TEMPERATURES		
		15°C	25°C	35°C
	1	12	22	0
1001+1056	2	27	15	6
	3	30	22	0
	4	14	20	1
	5	20	14	0
	Average	20	18	
	SD	7.8	3.8	
	1	32	29	6
1001+MR4	2	28	48	0
	3	21	19	0
	4	16	18	3
	5	19	50	0
	Average	23	33	
	SD	6.6	15.4	
	1	12	48	0
1056+MR4	2	14	45	0
	3	19	38	0
	4	16	29	0
	5	13	54	3
	Average	15	43	
	SD	2.7	9.6	
	1	32	26	2
All three	2	34	34	3
together	3	35	22	2
	4	28	26	5
	5	55	27	0
	Average	37	27	2
	SD	10.5	4.3	
Exptal Error (SEM)		9.42	10.5	0.57

Figure 12: Effect of root temperature on dry weight of shoots of P. sativum inoculated with R. leguminosarum (mg).

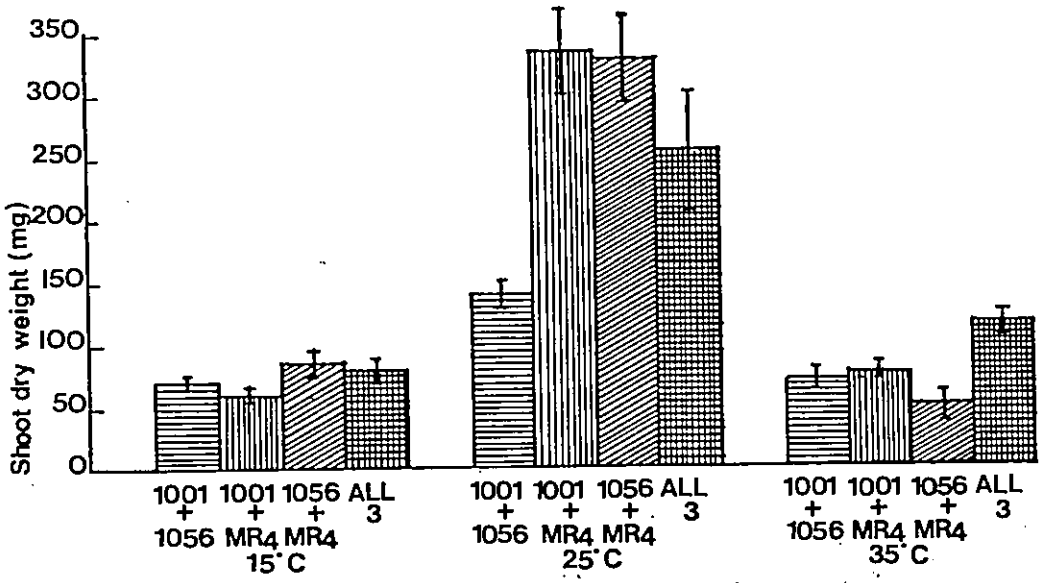


Figure 13: Effect of root temperature on nodule weight (mg)

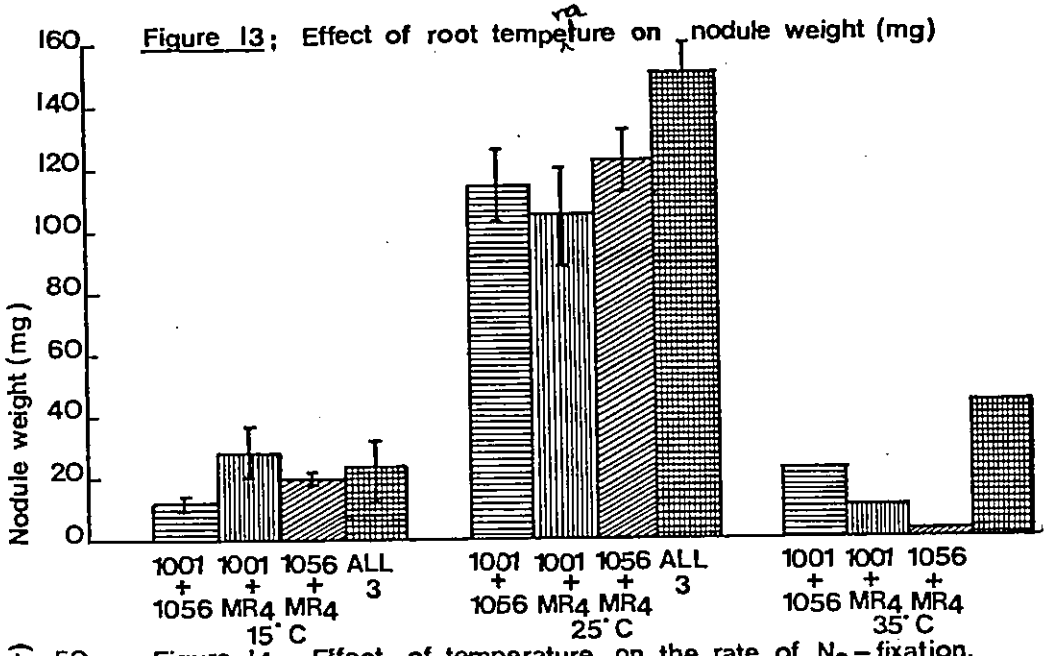


Figure 14: Effect of temperature on the rate of N_2-fixation.

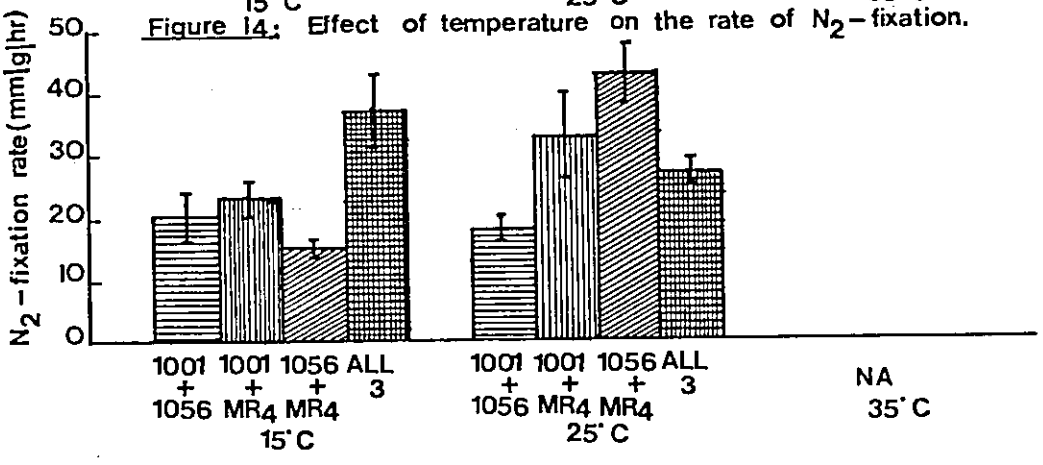


Table 31: Competition for nodule formation at different temperatures. Number of nodules formed by each strain in paired competition.

Strain Combinations	REPS	TEMPERATURES								
		15°C			25°C			35°C		
		1001	1056	Mixed	1001	1056	Mixed	1001	1056	Mixed
	1	3	12	0	6	9	0	0	0	0
	2	2	13	0	8	7	0	8	5	2
1001+1056	3	0	15	0	4	11	0	0	0	0
	4	1	13	1	8	7	0	11	3	1
	5	1	14	0	6	8	1	0	0	0
	Total	7	67	1	32	42	1	19	8	3
	%	9	90	1	43	56	1	63	27	10
	Ratio	1	10		3	4		3	1	
		1001	MR4	Mixed	1001	MR4	Mixed	1001	MR4	Mixed
	1	11	3	1	6	7	2	4	11	0
	2	11	3	1	4	9	2	0	0	0
1001+MR4	3	10	5	0	8	6	1	0	0	0
	4	10	5	1	5	9	1	1	13	1
	5	12	3	2	4	10	1	0	0	0
	Total	54	19	2	27	41	7	5	24	1
	%	72	25	3	36	55	9	17	80	3
	Ratio	3	1		2	3		1	5	
		1056	MR4	Mixed	1056	MR4	Mixed	1056	MR4	Mixed
	1	13	2	0	0	15	0	0	0	0
	2	12	2	1	3	12	0	0	0	0
1056+MR4	3	8	6	1	2	13	0	0	0	0
	4	9	6	0	3	11	1	0	0	0
	5	11	3	1	2	12	1	2	28	0
	Total	53	19	3	10	63	2	2	28	0
	%	71	25	4	13	84	3	7	93	0
	Ratio	3	1		1	6		1	14	

Table 32: Proportion of nodules formed by each strain (%) in paired competition calculated from above Tables (31)

Temperature	1001	1056	MR4	Ratio 1001 : 1056 : MR4 :
15°C	40.5	80.5	25	2 : 3 : 1
25°C	39.5	33	59	1 : 1 : 2
35°C	40	17	86.5	2 : 1 : 5

Table 33: Number of nodules formed by each strain when in mixture of three

Strain Combination	REPS	15°C				25°C				35°C			
		1001	1056	MR4	Mix	1001	1056	MR4	Mix	1001	1056	MR4	Mix
	1	6	18	4	2	8	4	16	2	4	0	10	1
	2	10	18	2	0	3	4	18	5	2	0	12	1
1001+1056+	3	7	15	4	4	4	5	17	4	1	1	11	2
MR4	4	5	17	7	1	5	5	17	3	2	0	12	1
	5	3	20	5	2	9	3	18	0	0	0	0	0
	Total	33	88	22	9	29	21	86	14	9	1	45	5
	%	22	58	14	6	19	14	57	10	15	2	75	8
	Ratio	1.5	4	1		1.5	1	4		9	1	45	

Mix = Nodules containing 2 strains each.

Figure 15 a: Effect of temperature on the competitiveness of the three strains of R. leguminosarum inoculated in pairs on P.sativum var Onward.

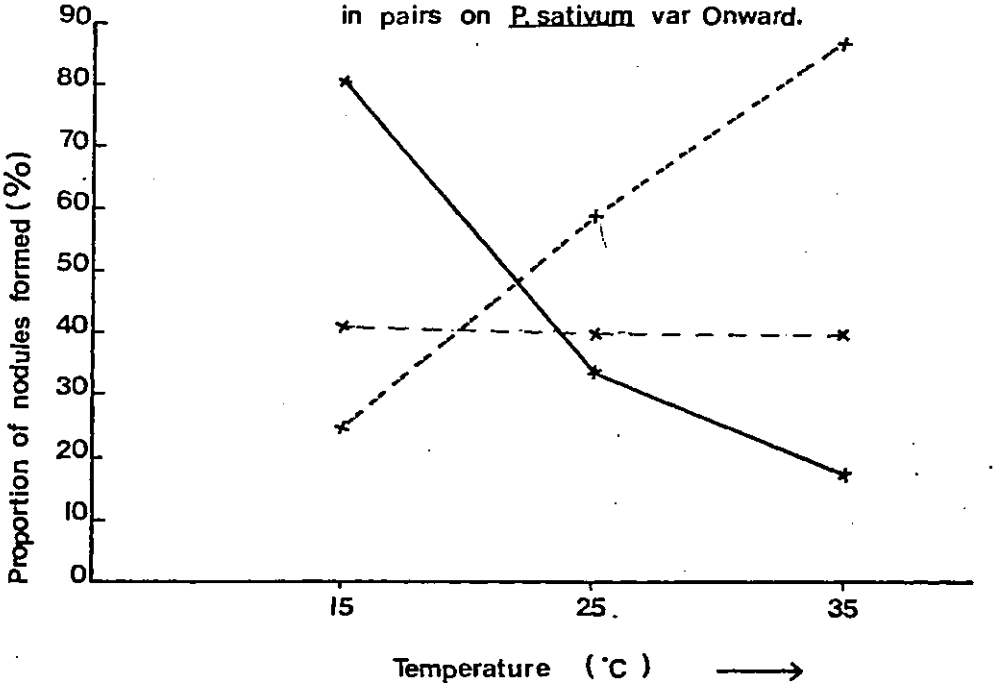
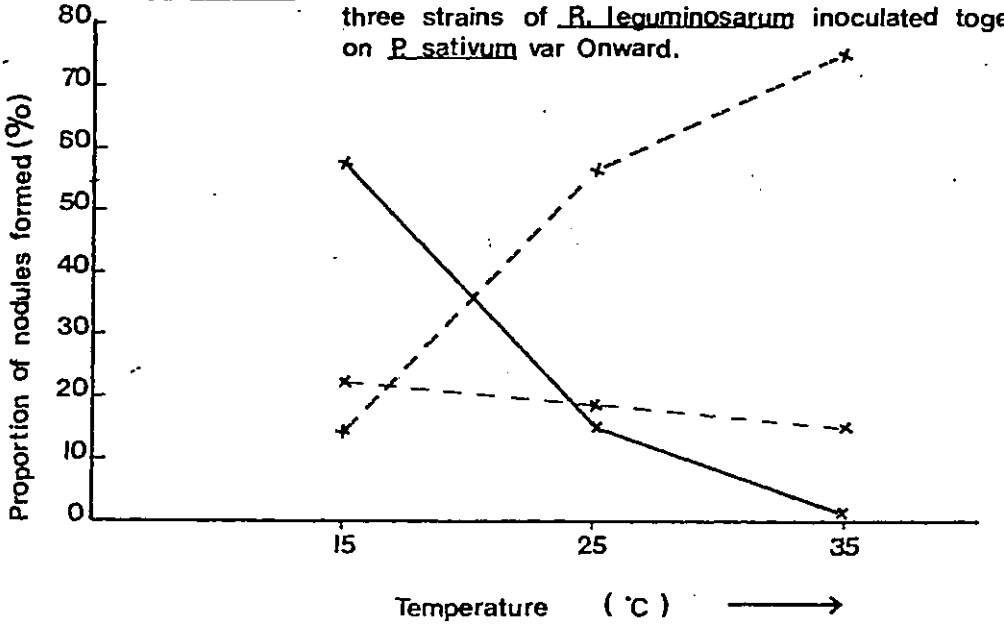


Figure 15 b: Effect of temperature on the competitiveness of three strains of R. leguminosarum inoculated together on P. sativum var Onward.



Key:

MR4

1001

1056

15a and 15b. The relative numbers of nodules formed by each strain were expressed in percentages and ratios (Table 32). The ratios obtained in pairs correspond to those obtained when all the three strains were inoculated together. The proportion of nodules formed as illustrated in Figure 15b are those from the mixture of all three strains. The proportions of the numbers of nodules formed by each strain in mixture of three strains are given in Table 33 while Table 32 gives the relative per-centages in paired competition.

DISCUSSION

In this study root temperature was varied from 8°C to 35°C. The plants at 8°C grew so slowly that by the time plants started flowering at the other temperatures no nodules had been formed on them, hence no analysis of nodulation at this temperature was made. The optimal temperature was found to be 25°C where plant vigor, expressed in dry weight was maximum. At this temperature dry weight was maximum when strains 1056 and MR4 were inoculated together. On analyzing the proportion of nodules formed by each of the two strains it was found that most of the nodules (84%) were formed by MR4. But when 1056 competed with 1001 at the same temperature 1056 formed a bigger proportion of nodules than 1001. The dry weight of the plants with this combination of strains was found to be low. This may be due to depressing effect of the ineffective strain, 1056, over the effective strain 1001.

These observations were further supported by fact that at lower temperature (15°C) where strain 1056 forms more nodules than the other two in paired combination, the dry weight of the plants was much less than at 25°C.

Comparison of rates of N_2 - fixation (Table 30) also revealed that where 1001 and 1056 were inoculated together and 1056 was the most competitive the rates of N_2 fixation were lower than where 1056 was outcompeted. There was however, no significant (hardly any) difference in N_2-fixation at two temperatures (15 and 25°C). At 15°C where 1056 was more competitive than MR4 the rate of N_2 fixation was lower than at 25°C where it was not. In mixtures of the 3 strains the combined effect of the two strains (1001 and MR4) tends to cancel out the ineffectiveness of 1056 and hence both the dry weight and rate of N_2 fixation were relatively higher than when the two strains competed separately with 1056. This observation agrees with the findings of Mytton (1975) and Hardason and Jones (1979) that Rhizobium genotypes can have additive effects.

It also agrees with the observations of Dart et al. (1976) that some strains were more effective at higher temperatures than others which were more effective at lower temperatures.

Nodule weight has been found to be affected by temperatures too (see Figure 13). At 15°C nodule weight was also found to be lower than at 25°C which was the optimal temperature for plant growth.

The relative performance of the three strains as shown in Tables 31, 32 and 33 and Figures 15a and 15b clearly show that temperature has a dramatic effect on the competitiveness of strains of Rhizobium. This finding is in agreement with those of Caldwell and Weber (1970), Weber and Miller (1972) and Hardason and Jones (1979). An explanation is not easy to find, but it may be due to the effect of temperature on both the host plant and the bacteria altering the preferentiality or susceptibility of host plants to one type of strain at different temperatures Lie et al.(1976), for example, found that one pea cultivar, Iran, was resistant to three strains of Rhizobium,which he called "avirulent", at 20°C, but was susceptible to all three at 26°C. The cultivar was susceptible to another strain at both temperatures. Kumarasinghe and Nutman,(1979) observed switching on "and switching off" of infection process depending on temperatures used. Lie et al.(1971;1978), Roughley and Dart (1969, 1970) and Dart and Day, (1971) observed deformations of root hairs at elevated temperatures. These changes can constitute nodulation barriers to some or all the strains as was observed at 35°C with strains 1001, 1056 and MR4 on P.sativum var Onward were hardly any nodules were formed. It appears though that some strains are better than others at breaking these barriers by producing special enzymes like pectinases as Martinez-

Molina and Olivares pointed out that the rhizobia legume interactions must be delicately balanced process and therefore the amount of hydrolytic enzymes and their activities must be well regulated at the site of infection and during infection.

It is also tempting to suppose that strain MR4 was less competitive at lower temperatures but more competitive at higher temperatures due to its geographical origin and apparent adaptation to higher temperatures which fluctuate between 35°and 24°C during the pea growing periods November to January and March to June respectively in this part of Tanzania.

As observed by Dart *et al.* (1976), the effect of the temperature on root nodule formation is strictly localized since shoot temperature was kept at 15°C in the controlled environmental chamber and only the root temperatures were altered. During the experiment, the soil in the pots which were placed in the water tank at 35°C, needed more watering than the others, due to faster evaporation and the effect of watering soils that are dry may also have had an effect in reducing nodulation. Pena-Gabriales and Alexander (1979) reported that there was a reduction in the population of *R.lupini* following successive wetting and drying. Salema *et al.*(1982) showed that rehydrating cells of *Rhizobium trifolii* on mung bean (*Vigna radiata*) seeds led to morphological deformation and eventual rupture. In this study, no morphological examinations

were undertaken, but it is possible that failure to nodulate at 35°C may be a combined effect of morphological and physiological changes in both the bacteria and the host plant.

It is clearly important to note that for success in inoculant production selection of the strains and hosts should be carried out under conditions that are similar to those where production is going to take place.



Plate 11: The effect of pH on plant growth was clearly noticeable. The plants grew poorly at lower pH. The relative performance of the mutant strain of R.leguminosarum inoculated on these plants in competition with indigenous rhizobia was, however, poor irrespective of the pH or titre of the inoculant.

CHAPTER 4

MOTILITY AND CHEMOTAXIS

INTRODUCTION

The mechanism and specificity of symbiosis association between *Rhizobium* and legumes have been studied from the point of view of both the bacteria and the plant. In the General Introduction the various theories of lectin polysaccharide recognition have been discussed but none of them seems to have been proved beyond doubt. The symbiotic process can be divided into five distinct stages namely, accumulation of bacterial cells in the rhizosphere, infection of the plant root by *Rhizobium*, growth of the nodules, functioning of the nodules and maintenance of the symbiosis.

Since rhizobia are motile bacteria (Carlile, 1980), it has been suggested that motility could play a part in symbiosis. Furthermore positive chemotaxis exerted by some substances exuded by plant roots could play a specific role in this association (Currier and Strobel, 1976;1977a).

Rhizosphere soil of a particular legume sustains more homologous rhizobia than in the non-rhizosphere soil nearby. Tuzimura and Watanabe (1961) found that there were 10^3-10^4 *Astralagus* rhizobia per gram of the *Astralagus* rhizosphere soil as compared to 10^1 per gram of

the other soil in the same pot. Van Egeraat (1972) observed interactions between free-living soil bacteria with the rhizosphere of various legumes,and, in general, that nodule bacteria were little affected by roots of non-legumes. Wilson (1930) observed that nodule bacteria were more strongly stimulated (attracted and multiplied) by those hosts that they were able to infect than by other legumes.

Robinson (1967) found that in a mixed pasture of clover (Trifolium subterraneum) and Lucerne (Medicago sativa), clover stimulated Rhizobium trifolii but not Rhizobium melitoti,while lucerne stimulated both species of Rhizobium. Gortz et al.(1982), after studying the response of Rhizobium to a range of amino acids suggested that the observed concentration in the rhizosphere of nodulating plants (Rovira, 1965) may be a consequence of a general chemotactic response to root exudates containing high proportions of amino acids. The subsequent specific interaction, which leads to invasion of the roots, the development of root nodules and the differentation of rhizobia into nitrogen fixing bacteroids (Vincent, 1974) is probably independent of the chemotaxis and motility (Ames et al., 1980; Napoli and Albersheim, 1980). The distinction between general and specific interactions has an obvious parallel in the genetic makeup of symbiotic Rhizobium species. Genes for motility and chemotaxis are

found,clustered next to at least one of the genes required for histidine biosynthesis, (Ames et al. 1980), while on the bacterial chromosome those for symbiosis in R.leguminosarum reside on large plasmids (Beynon et al., 1980).

Gaworzewska and Carlile (1982) demonstrated positive chemotaxis of Rhizobium leguminosarum towards root exudates of its host, Pisum sativum. Hunter and Fahring (1980) and Gaworzewska (1983) have shown that non-motile and non-chemotactic mutants are less competitive than the motile and chemotaxis wild strain types.

Plant exudation of organic components through their roots was found to be influenced by many factors (Rovira 1965). The age of the plant was also found to influence the nature of exudate.Rovira (1956) found that peas exuded more amino acids and sugars in the first ten days after germination than in the second ten days. Van Egeraat (1975) found that changes in free ninhydrim - positive compounds produced by pea seedlings were influenced by age, environmental conditions and nutrition. He found that homoserine was quantitatively more important after seven days, but this decreased rapidly, so that by the

eighteenth day homoserine was only present in minor quantities. Little is known about the role of homoserine in plants though it was found to be a precursor of threonine and methionine (Fowden, 1965).

Lie (1964) found that the accumulation of active substances (nodulation-stimulating substances) took place during the first two weeks after germination. He counted nodules formed on three aseptically grown pea seedlings that were inoculated at intervals of 7,14 and 25 days after germination and observed that with increasing age of the plant inhibiting substances were exuded by the plant leading to fewer nodules formed. More inhibition was obtained with roots of uninoculated plants of both ages. In continuous growth the inhibitory effect of the exudates of later stages was found to be partly counteracted by the active substance accumulated during the preceeding period.

In this study motility and chemotaxis of the six Rhizobium leguminosarum strains has been studied with the view of finding out if the two properties observed in these strains could be correlated with their competitive abilities.

MATERIALS AND METHODS

Bacterial strains

The same six strains used in the study of competition on three host plants (see General Materials and Methods) were used for motility and chemotaxis assays.

Media and Buffer:

All media and buffers were prepared with distilled water. The minimal medium used for growing the strains had the following composition per litre of water:

KH_2PO^4	0.11g
$CaCl_2.2H_2O$	0.11g
$MgSO_4.7H_2O$	0.1g
$FeCl_3.6H_2O$	3.3mg
Sodium succinate	6.gg
Sodium glutamate	1.1g
Na_2EDTA	0.372 mg
Thiamin hydrochloride	0.75 mg)dissolved
Biotin	0.75 mg)together
Calcium pantothanate	0.75 mg)

Sodium succinate, sodium glutamate and Na_2EDTA and vitamins were made in stock solutions 100 times the final concentration and sterilized separately at 15lb pressure for 10 minutes. They were then mixed with salts when the medium was being made, otherwise they were stored separately at 4°C in a domestic fridge.

The chemotaxis buffer consisted of the following components dissolved in 1 litre of distilled water:

K_2HPO_4	106 mg
KH_2PO_4	53 mg
EGTA	38 mg
$CaCl_2$	106 mg

PREPARATION OF ROOT EXUDATES

Seeds of Pisum sativum var Onward, Vicia faba var Throws M.S. and Lens esculenta were surface sterilized by soaking them in sodium hypochlorite for 20-30 minutes followed by seven washes in sterile distilled water (see General Materials and Methods). They were then transferred on TY agar aseptically and left in the dark to germinate. When the plumule was just visible (about 2 mm long), the seed was pinned to a sterile sharp stainless steel wire (10 in long) and placed in an Erlenmeyer flask

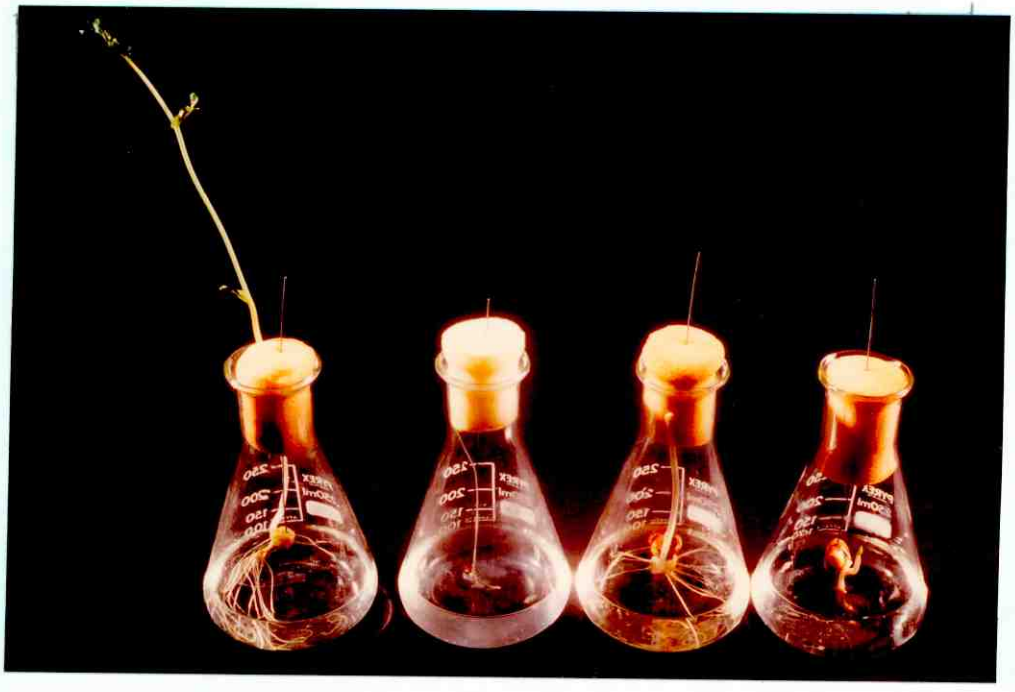


Plate 12: Method used to collect plant exudates. The germinated seed was held by a stainless steel wire and dipped into the nutrient solution so that the seed did not touch the liquid. The flask was then covered with black polythene bag until shoot was long enough to pull through the neck of the Erlenmeyer flask. The shoot was held in position by a foam bung which also ensured the solution was sterile throughout the collection of the exudate.

containing 100 ml of nutrient solution (see General Materials and Methods). The seed was then lowered in the flask until the radicle, but not the seed entered the medium (see Plate 12). The flasks were then covered with a black polythene bag for three to four days until the shoots were long enough to be pulled through the flask neck and the bung replaced. Seven days after immersing the root in the nutrient medium a 20 ml sample was taken with a sterile pipette and preserved in a Universal bottle at -20°C in a domestic freezer.

The plants were left to grow for another seven days and another 20 ml sample taken. The exercise was repeated after a further 14 days to obtain 28 d sample. All these samples were centrifuged for 10 minutes, at 8000g at 5°C, to remove cell debris, then decanted and freeze-dried. The samples were stored and redissolved in sterile chemotaxis buffer as required to obtain a protein content of 150 mg/ml as measured by the Folin-Lowry method (Herbert et al., 1977) described below.

Reagents used for the Folin-Lowry method were as follows:

(i) 5% $Na_2 CO_3$

(ii) 0.5% $CuSO_4.5H_2O$ 1% sodium tartarate

(iii) To 50ml of reagent (i) add 2ml reagent (ii) prepared immediately before use.

(iv) Diluted Folin - Ciocalteu (1927) reagent, which is essentially a phosphotungstic -

phosphomolybdic acid solution, is titrated with 1.0N NaDH using phenolphtalein as indicator. It is then diluted with distilled water to make the total acid exactly 1.0N. In this case 100ml was added to 87.6ml g stock solution.

(v) Protein-standard: bovine serum albumin (Armour), 200 g/ml.

Procedure:

Exudate (0.5ml) was measured into an ordinary test tube and 0.5 ml of 1.0N NaOH added. The mixture was placed in a boiling water bath for 5 minutes and cooled in cold water. 2.5ml of reagent (iii) (NaOH-Na_2CO_3-$CuSO_4$-tartarate reagent) was added to it and allowed to stand for ten minutes. 0.5ml Folin-Ciocalteu reagent was then added rapidly using a blow out pipette and mixed immediatley.

A reagent blank containing 0.5ml of distilled water instead of sample exudates, and a set of standard protein solutions (50-200mg protein) were treated in the same way including the heating stage. After about 30 minutes to allow full colour development optical densities were measured against a reagent blank in a Unican SP 600 spectophotometer using a Wavelength of 750mm and 1 cm cuvettes. The standard of optical density against

micrograms of standard protein was drawn and used to read off the amounts of the protein in the exudates.

MOTILITY MIGRATION EXPERIMENTS

Migration experiments were carried out on the minimal medium supplemented with 0.25% agar.

Bacteria from slant cultures (TY) were used to inoculate the centre of the Petri dish with a loopful of bacteria. The plates were incubated at 24°C (room temperature) covered in polythene bags to maintain moist conditions. The concentration of the agar was low enough to allow bacteria to swim in this medium. They migrated in rings whose diameters were measured at intervals of 24 hours.

CHEMOTAXIS MIGRATION

The chemotaxis assay was carried out using the Blindwell chamber method (Armitage et al., 1977), in which migration occurs through membrane into control or test solutions. Chemotaxis chambers and unipore polycarbonate membranes (5 μm pore size, 13 mm diameter) were obtained from Bio-Rad Laboratories. A bacterial suspension (0.15ml) was placed in the lower chamber and 0.3ml of chemotaxis buffer, in the upper chamber. The chambers



Plate 13: The blindwell apparatus consisting of two chambers, a lower chamber where the bacterial cells were placed and the upper chamber, which is screwed on, consisting of the test or buffer solution. The apparatus was incubated upside down when sealed with a parafilm.

were then sealed with Parafilm (Gallenkamp) and inverted and incubated at 28°C for 90 minutes before sampling.

Samples were taken from the upper chamber and serially diluted to obtain counts of bacteria. A chemotaxis ratio was obtained by comparing the numbers of bacteria that had passed into the test solution with the numbers that passed into the control solution. Each result was based on the averages of three replicates.

RESULTS

The data given in Table 22 show that all six Rhizobium leguminosarum strains were motile and chemotactic. The migration rates with bacteria are a fraction (not more than 1/10) of the speeds of individual cells. The rate of migration is an expression of the rates of metabolism of key attractants. Knowles and Carlile (1978) showed that migration rates increased with time and as biomass increased. It is probable, therefore, that the different rates of migration observed in the six strains indicate the different abilities of these strains to utilize the attractants. The soft agar assay clearly showed that the strains were motile and chemotactic and that they differed in their metabolic activities.

Chemotaxis of the Rhizobium cells towards root exudates was compared with those cells that had passed

Table 34: DISTANCES MOVED FROM CENTRE (mm) USED BY EACH STRAIN OF R.LEGUMINOSARUM

		TIME (HOURS)			
	Replicates	24	48	72	96
1001	1	5.5	12.0	29.0	41.0
	2	4.0	9.6	27.0	40.5
	3	6.0	13.0	33.0	41.5
	Average	5.2	11.5	29.0	41.0
	SD	1.0	1.7	3.0	0.7
1012	1	4.5	10.5	26.0	31.5
	2	4.0	8.0	22.0	29.0
	3	5.0	9.3	25.0	30.0
	Average	4.5	9.3	24.3	30.2
	SD	0.5	1.2	2.0	1
1013	1	1.5	3.7	8.5	17.0
	2	2.0	4.2	9.0	18.5
	3	2.4	5.3	10.0	20.0
	Average	2.0	4.4	9.2	18.5
	SD	0.45	0.8	0.7	1.5
1045	1	2.5	6.5	16.5	30.0
	2	2.0	5.0	15.0	29.5
	3	3.5	8.0	17.0	30.5
	Average	2.7	6.5	16.2	30.0
	SD	0.7	1.5	1.0	0.5
1056	1	5.0	10.0	23.0	30.5
	2	6.0	12.5	26.5	32.0
	3	6.0	12.0	26.0	33.0
	Average	5.7	11.5	25.2	31.8
	SD	0.5	1.3	1.8	1.2
MR4	1	6.5	11.0	30.5	32.5
	2	6.0	13.0	29.0	32.0
	3	7.0	14.0	28.5	33.5
	Average	6.5	12.7	29.3	32.7
	SD	0.5	1.5	1.0	0.7
SE (Exptal. Error of mean)		1.7	3.2	8.0	7.2

Table 35 Absorbance (O.D.) at 750 mm of the exudates obtained after 7,14 and 28 days.

PLANTS FROM WHERE EXUDATES CAME

	P.sativum			V faba			L esculenta		
Replicates	7	14	28	7	14	28	7	14	28
1	2.25	16.0	35.0	6.0	22.0	41.0	11.0	23.0	32.0
2	3.00	20.0	43.0	10.0	27.0	48.0	4.0	17.0	37.0
3	2.0	23.0	38.0	4.5	21.0	39.0	5.0	20.0	36.0
Average	2.4	19.7	38.7	6.8	23.3	42.7	6.7	20.0	35.0
SD	0.5	3.5	4.0	2.8	3.2	4.7	3.7	3.0	2.6

Table 37: Ratios of number of cells going through the membrane into the different exudates as compared with the control buffer.

STRAINS									
	Buffer	Pisum sativum			Vicia Faba			Lens esculenta		
		7	14	28	7	14	28	7	14	28
1001	1	11	148	7	55	7	5	10	107	48
1012	1	16	200	211	72	83	1	3	239	9
1013	1	1	112	26	94	14	1	2	218	10
1045	1	1	168	16	46	17	3	12	33	5
1056	1	2	24	4	7	9	2	16	13	1
MR4	1	3	210	14	108	14	14	6	78	4

Table 36: Number of cells attracted towards exudates obtained from plants at intervals of 7, 14 and 28 days and buffer

		BUFFER	PISUN SATIVUM			VICIA FABA			LENS ESCULENTA		
Strains			7	14	28	7	14	28	7	14	28
Strains	Reps 1	3×10^4	4×10^5	2.8×10^6	1.0×10^5	2.0×10^6	1.3×10^5	1.0×10^5	1.3×10^5	3×10^6	1.2×10^6
1001	2	4×10^4	2×10^5	7.9×10^6	1.3×10^5	1.8×10^6	3.4×10^5	2.5×10^5	$2x\ 10^5$	5×10^6	1.1×10^6
	3	1.8×10^4	3.4×10^5	2.1×10^6	3.6×10^5	1.0×10^6	1.0×10^5	9.0×10^4	5×10^5	1.25×10^6	2.0×10^6
Average		2.9×10^4	3.13×10^5	4.3×10^6	1.96×10^5	1.6×10^6	1.9×10^5	1.4×10^5	2.8×10^5	3.1×10^6	1.4×10^6
Ratio Exud: Buffer		7	11	148	7	55	7	5	10	107	48
	1	1.3×10^4	1.0×10^5	4.3×10^6	2.1×10^6	1.2×10^6	1.04×10^6	2.5×10^4	6.4×10^4	4.4×10^6	1.7×10^5
1012	2	2.4×10^4	3.5×10^5	5.0×10^6	7×10^6	1.4×10^6	1.4×10^6	1.3×10^4	4.5×10^4	3.7×10^6	1.2×10^5
	3	1.7×10^4	4.3×10^5	1.5×10^6	2.3×10^6	1.2×10^6	2.0×10^6	1.4×10^4	7.7×10^4	5.0×10^6	1.8×10^5
Average		1.8×10^4	2.9×10^5	3.6×10^6	3.8×10^6	1.3×10^6	1.5×10^6	1.7×10^4	6.2×10^4	4.3×10^6	1.6×10^5
Exud: Buffer		1	16	200	211	72	83	1	3	239	9
	1	1.9×10^4	2.0×10^4	2.5×10^6	3.8×10^5	1.2×10^6	3.8×10^5	1.7×10^4	1.5×10^4	5×10^6	1.0×10^5
1013	2	1.0×10^4	2.5×10^4	1.0×10^6	5.0×10^5	2×10^6	1.0×10^5	2.1×10^4	5×10^4	1.5×10^6	1.1×10^5
	3	3.0×10^4	1.0×10^4	2.0×10^6	3.5×10^5	1.0×10^6	2.2×10^5	2.5×10^4	1.0×10^4	3.9×10^6	2.7×10^5
Average		1.6×10^4	1.8×10^4	1.8×10^6	4.1×10^5	1.5×10^6	2.3×10^5	2.1×10^4	2.5×10^4	3.5×10^6	1.6×10^5
Exud: Buffer		1	1	112	26	94	14	1	2	218	10
	1	1.7×10^4	4.1×10^4	6.0×10^6	4.1×10^5	1.4×10^6	8.6×10^5	9.0×10^4	3.9×10^5	8.9×10^5	1.7×10^5
1045	2	3.7×10^4	2.4×10^4	4.5×10^6	6×10^5	1.7×10^6	2.6×10^5	8.5×10^4	2.6×10^5	9.1×10^5	1.1×10^5
	3	3.0×10^4	4.5×10^4	3.7×10^6	3.8×10^5	8.8×10^5	3.0×10^5	7.1×10^4	3.4×10^5	1.0×10^6	1.0×10^5
Average		2.8×10^4	3.7×10^4	4.7×10^6	4.6×10^5	1.3×10^6	4.7×10^5	8.2×10^4	3.3×10^5	9.3×10^5	1.26×10^5
Exud: Buffer		1	1	168	16	46	17	3	12	33	5
	1	2.9×10^5	3.4×10^5	7.5×10^6	7.9×10^5	1.0×10^6	2.0×10^6	2.7×10^5	4.1×10^6	1.12×10^6	1.0×10^5
1056	2	1.0×10^5	2.4×10^5	3.2×10^6	5.9×10^5	1.1×10^6	1.0×10^6	3.5×10^5	2.0×10^6	1.19×10^6	1.6×10^5
	3	2.0×10^5	2.9×10^5	2.7×10^6	7.0×10^5	2.2×106	2.3×10^6	6.2×10^5	3×10^6	5×10^6	2.3×10^5
Average		1.9×10^5	2.9×10^5	4.5×10^6	6.9×10^5	1.4×10^6	1.8×10^6	4.1×10^5	3.0×10^6	2.4×10^6	1.6×10^5
Exud: Buffer		1	1	24	4	7	10	2	16	17	1
	1	6.7×10^4	1.2×10^5	12×10^6	7.7×10^5	5.1×10^6	7.14×10^5	7.2×10^5	1.6×10^5	5.5×10^6	1.4×10^5
MR4	2	4.9×10^4	1.4×10^5	11×10^6	5.8×10^5	5.3×10^6	8.0×10^5	7.0×10^5	1.3×10^5	3.7×10^6	1.6×10^5
	3	3.6×10^4	1.3×10^5	9.0×10^6	8.1×10^5	6.0×10^6	7.0×10^5	6.5×10^5	7.0×10^5	2.9×10^6	2.8×10^5
Average		5.1×10^4	1.3×10^5	10.7×10^6	7.2×10^5	5.5×10^6	7.4×10^5	7.2×10^5	3.3×10^5	4.0×10^6	1.9×10^5

through the membrane into the buffer. Table 36 shows the number of bacteria obtained by plate counts of colonies on TY agar plates. They represent the number that have migrated through the membrane. The results clearly indicate that the exudates dissolved in buffer attract proportionally more bacteria than does the buffer alone. The 7 day exudate was generally less attractive than the subsequent ones obtained from the same plants. The concentrations of organic compounds (proteins) were assessed by measuring the absorbance of the exudates at 750mm.

DISCUSSION

In this study it was evident that exudates from different host legumes attracted Rhizobium leguminosarum strains. The exudates obtained after seven days contained lower amounts of organic compounds (see absorbance readings in Table 35) than those obtained after 14 and 28 days. There seemed to be no linear relationship, though, between the amounts of organic compounds in the exudates as measured by absorbance and the number of bacterial cells attracted to the exudates. The exudates obtained after 28 days seemed to attract fewer bacteria than those obtained at 14 days. This is in agreement with the findings of Lie (1964) who observed better nodulation

induced by pea exudates from 14 day old plants than from 25 day old plants. It appears that the exudates produced later contained inhibitory substances or it may be that they were too concentrated. Whatever the explanation, it has been observed that different strains accumulated differently in different exudates. This observation also agrees with the findings of Cleyet-Marel, Jean-Claude and Yves Crozat (1982) that in the rhizosphere only one host legume (Glycine max cv.Chipewa) supported high populations of strain G3. In another experiment with 4 cultivars of soybean, the populations of R.japonicum were 100-1000 times more numerous in the rhizoplane soil than in the normal soil.

Looking at chemotactic ratios of the six strains towards exudates obtained after 28 days, it is clear that there was generally less attraction to them than to those exudates from younger plants, except strain 1012 on P.sativum and MR4 on V.faba,where there appears to be unchanged attraction. There is, however, no evidence of preferential attraction of the strains that had been found to be more competitive on the respective host legume (see Table 9 and Figure 1) than the strains that were less competitive in nodulation.

Gaworzewska (1983) found that motility mutants failed to compete successfully with wild motile type strains and

accounted for only 18-26% of the total infections. She also found that chemotactic mutants were fairly competitive accounting for 34-40% of the total infections at high initial inoculum titre (10^7 cells/plant). At lower inoculum titre (10^3 cells/plant), however, the chemotactic mutants were less competitive. These findings are in some agreement with my observations.

The speed with which one strain is attracted to the host legume exudates may confer some additional advantage in the soil where the initial inoculum titre may be low and motility is guaranteed. The stimulating effects of root exudates observed by Peters and Alexander (1966) on Lotus corniculatus inoculated with a initial population of 10^4 per ml to 10^7-10^8 within two days implies that once a strain reaches the rooting zone it would increase in numbers in relation to those that do not get there as quickly.

GENERAL DISCUSSION AND CONCLUSIONS

When one refers to competition among strains of *Rhizobium* for nodulation of a specific host plant one is actually dealing with both the bacteria and the legume. The interaction between *Rhizobium* and legume is so intimate that the symbiotic success depends on both partners. Any factors that are introduced into the environment where both symbionts are present together will therefore affect their interaction.

In this thesis it has been shown that different strains of *Rhizobium leguminosarum* have different characteristics such as intrinsic antibiotic resistance, susceptibility to phages and production and or sensitivity to bacteriocins. Their symbiotic performances on the host legumes were also found to differ. Strain RCR 1056, for example, was found to nodulate *Vicia faba* and *Lens esculenta* effectively, but was ineffective on *Pisum sativum* (see Table 1). Various genes responsible for the different symbiotic responses have been mapped in various species of *Rhizobium* by various workers.

Rhizobium geneticists have looked at the possibility of increasing the competitive ability of an inoculant strain to form more nodules in the presence of other strains and hence enable the plant to fix more nitrogen. Recently, Brewin *et al.* (1983) have reported on the

introduction of different symbiotic plasmids into three different recipients that lacked the plasmid linked symbiotic determinants. When these constructed strains were tested for competitiveness against a reference strain the recipient strain and the introduced plasmid contributed equally to competitiveness in forming nodules. They further stated that both the sym plasmid and background genotype determined by the chromosome and by any other resident plasmids other that sym plasmid can exert a major influence on competition for nodulation.

While this work by Brewin et al. (1983) and other previous Rhizobium genetics studies have demonstrated the part played by bacterial genotype in symbiosis, competition determinants are still not clearly understood. The most important reason for this is that the interaction between the bacteria and the plant is not controlled by a single gene and it is a multistep one. It is further complicated by the fact that the factors that determine nitrogenase activities of strains are independent of those for competitiveness (Peterson et al., 1983).

From the data for competition of six strains of R.leguminosarum on hosts of different genera (Pisum, Vicia and Lens) and of three strains on five cultivars of P.sativum,it is obvious that host genotype plays a crucial role in the performance of each strain. It was also found that other symbiotic characteristics of the strain

like effectiveness (N_2-fixation), nodulation and others were not linked with the competitiveness of the strain. It has been also found that one strain can be the most competitive on one host and least so on another. Similarly it was found that one strain can be effective on one host but ineffective on another host within the same inoculation group. This phenomenon was, however, not found among cultivars of the same host species.

Some workers have offered evidence showing that effective strains were more competitive than those that were not effective on the host plants. My data on hosts of different genera, Pisum sativum, Vicia faba and Lens esculenta partly confirm these previous findings. Strain RCR 1056, for example, was effective on Vicia faba and not on Pisum sativum. The competitive performance of this strain showed that it was most competitive on V.faba but very poor on P.sativum. It is tempting to conclude that strain RCR 1056 was most competitive on Vicia faba because it was effective on this host and not so on the other two hosts where it was not so effective. This explanation does not, however, explain why those strains of R.leguminosarum which were also as effective on Vicia faba were not as competitive as strain 1056.

My data also show, as mentioned earlier, that effectiveness is not a common trait on all hosts of the cross inoculation group and therefore cannot be a stable

genetic factor either. It appears to me that both competitiveness and effectiveness are phenotypic characters expressed through the interaction of both the plant and the Rhizobium symbiotic genes. These characters, though they may be expressed together, could be independent of each other.

While studying competition among strains of Rhizobium leguminosarum the role of the host plants has been clearly demonstrated. Even at cultivar levels, the host plants still had remarkable effects on the competitiveness of the strains used in mixed inoculum. Another observation which is of ecological importance was the remarkable interaction among the strains themselves in mixed inoculum. It was evident that the presence or absence of one strain altered the competitiveness of one or two other strains. These observations,complemented by the findings of Peterson et al.(1983) that the expression of nitrogenase activities were influenced in a synergistic way by the presence of other inoculum strains, have great implications in multi-strain inoculation of legumes. In cases where the most competitive strain is also the most effective strain, this would lead to the best response to inoculation, but when the opposite is true then the interaction of some of the effective strains may mitigate the injurious effects of ineffective symbiosis.

The important role of the host has many facets and one of them which was investigated was the effect of root exudates on the numbers of the different strains attracted to them. The data in this study has clearly showed that root exudates profoundly attracted the six strains of Rhizobium. The data further showed that exudates from different plants and of different ages had different effects on the chemotactic responses of the strains. Furthermore the data indicated that all the strains were motile although they differed greatly in their rate of metabolic activities of the metabolites in the minimal soft agar medium.

The data in Tables 30 and 31 show that temperature had a considerable effect on the competitiveness of the three strains of Rhizobium leguminosarum as well as their nitrogenase activities. It is clear from this data that the effect of temperature on the competitive ability of a strain of Rhizobium for nodule formation was dramatic. The symbiotic performance in terms of nodule number, nodule weight and the nitrogenase activities were also affected in the same way. Clearly, therefore, the dry weight of the host plants were also affected (Table 28). It is evident therefore that strain selection of inoculant strains of Rhizobium should be done under optimal conditions in which the inoculated plants will be growing.

Soil moisture also was found to have profound effects on both the plant and the Rhizobium strains (Tables 23 and 24). The competitiveness of strains in mixed inoculum was generally found to change with moisture content. (Table 25 and 27). The significance of this is that inoculant strains should also be selected on the basis of their performance under the moisture regimes where they will be used. With considerable variations in soil moisture during one growing season, it is important to have a strain whose performance remains high under changing soil moisture contents.

The performance of strain 1001, for example, was found to be relatively unaffected by changing moisture contents although its competitiveness was throughout low. It showed a versatile response to both temperature and moisture stresses, but would be unsuitable when an ineffective strain, like RCR 1056, was present in the inoculum since strain 1001 was outcompeted by 1056 at higher moisture content and by MR4 at low moisture. It would not therefore be allowed to form enough nodules at any level when such kind of strains are present.

It was further observed that under stressed conditions of temperature and moisture the ineffective strain was least "preferred" by the host plant. This may be due to the fact that when the plant is struggling to survive it can ill afford the injurious effects of the non-effective strain. The barrier mechanisms to nodulation both

physical and physiological were not investigated although observations were made that nodulation ceased when plants were stressed.

Although the physical and chemical mechanisms leading to successful nodulation were not also investigated it is clear from this work that it is not possible to talk of a good strain without qualifying it. Even the much talked about "effectiveness" of a strain is relative and its expression dependent on the interaction with the host genotype. Competitiveness of a strain is another useful concept in strain selection but it is not a stable characteristic and one must always state the host and environmental conditions under which the strain was selected. It is, however, possible, with more understanding of the genotype interactions and the environmental interplay to select highly suitable strains in situ through genetic manipulation of both symbionts.

The scope for this kind of work is wide and deep and enables the researcher to gain a fuller understanding of the intimate relationship that exists between the symbionts as individual organisms as co-partners and how the different environmental factors affect them both as individuals and as partners.

The effect of pH was also investigated using a streptomycin labelled mutant strain of R.leguminosarum competing against indigenous (and hence naturalized)

strains in Rothamsted soils. The introduced mutant strain failed to compete with the indigenous rhizobia as it formed less than 10% of the total number of nodules even at the highest titre of 10^8 cells/ml at optimal pH 6.90.

It was possible, however, to observe the effect of pH on the plant growth (see Plate 11). It is possible to study the effect of pH and titre of inoculant strains using either agar or -irradiated soils. Although the effects of pH could not be analysed due to low numbers of nodules it is pertinent to draw from the finding of other workers like Jones and Morley (1981) who found that the preference of host plant for strains of Rhizobium trifolii was affected by pH. The pH findings together with finding for temperature and moisture presented in this thesis strongly underline the importance of selecting strains for inoculation in the environments where inoculation is to take place or simulating the environment as closely as possible in order to ensure the success of the inoculant utilization.

APPENDIX

STATISTICAL ANALYSIS

In addition to the mean, percentages, standard deviations and standard errors already indicated in the text in relevant chapters, the data were analysed graphically as shown in the various figures. The tables supplemented with figures give the true picture of the variations and interrelations among the different treatments and variables. Graphical analysis permitted and revealed several patterns and interactions.

The data was further analysed to give the standard errors, variance ratios, coefficients of variation and in some cases correlation coefficients, which are presented in this appendix. In all these cases sources of variation and degrees of freedom were given so that it was possible to use the various tables, like the F tables to find out if the variation was significant at 5% confidence limits or not.

Variance is a measure of dispersion from the mean. It is based on the idea that it is the size of the deviations from the mean which is important rather than their direction (above or below).

The Variance ratio (VR), that is the ratio of variance estimated from the samples, follows the distribution known as F. The variance ratio (VR) can be read off from

the F tables using the sample degrees of freedom as numerator and residual degrees of freedom as denominator.

The VR will be significant at the percentage confidence limits if it is more than the values in the F table.

Standard error of a treatment mean (SE) is the ratio of standard deviation (square root of variance,) divided by the sample size. With increasing sample size (r) the distribution of means becomes narrower and taller, that is, the standard deviation becomes smaller, but the mean remains the same. The standard error, is used repeatedly in ANOVA to estimate the variance per plot from a series of means assuming each mean is from a sample drawn from the same population.

Coefficient of variation is the ratio of the standard deviation /mean multiplied by 100 to obtain a percentage. This denotes the variability among experimental units of experiments involving different units of measurements and/or plot sizes.

Coefficient of correlation is a measure of the closeness of the relation between two variable and to find out if the relationship is real or accidental. Regression is the amount of change in one variable associated with a unit change in the other variable. Correlation refers to the closeness of the relationship between the nature of the relationship.

1. GENERA-STRAIN INTERACTION

NUMBER OF NODULES FORMED

A. ANALYSIS OF VARIANCE FOR EACH STRAIN

VARIANCE RATIOS (F-VALUE)

STRAIN	GENERA	STRAINS	GENERA-STRAINS	RESI-DUAL
DF	2	5	10	34
1001	73.109	14.932	7.407	
1012	12.573	12.795	18.598	
1013	49.747	23.242	13.308	
1045	9.659	80.804	42.569	
1056	67.441	4.204	5.823	
MR4	135.128	28.517	7.376	

B. STANDARD ERRORS (SE) AND COEFFICIENTS OF VARIATION (CV%).

BLOCK - GENERA - STRAINS

STRAIN	DF	SE	CV%
	34		
1001		0.5562	28.7
1012		0.5673	44.6
1013		0.5120	35.6
1045		0.2025	9.2
1056		0.6474	44.6
MR4		0.4047	21.1

C. (i) Table of correlation coefficients of the number of nodules formed by each strain when mixed with each one of the strain compared with numbers of nodules formed by the strain in mixture of six strains. Negative figures indicate that there is an inverse relationship between the two values. It indicates a possible suppression of the strain by the competing strain when mixed together.

Strain	1001	1012	1013	1045	1056	MR4
1001		0.6655	0.3241	0.6948	0.4640	0.9731
1012	0.1226		0.6771	0.0642	0.7347	-0.7273
1013	-0.2256	0.1718		0.7786	0.7053	0.8904
1045	0.8318	-0.8081	0.3922		-0.9039	-0.0071
1056	0.9793	0.0947	0.9812	0.8657		0.4486
MR4	0.5472	0.5476	0.2083	0.8694	0.9822	

(ii) Comparing numbers of nodules formed by strains in pairs with those formed when all six were mixed.

DF = 43

	1001	1012	1013	1045	1056	MR4
Correlation coefficient	0.5627	0.1574	0.4478	0.0539	0.5756	0.6050

E. STRAIN - STRAINS INTERACTIONS ON GENERA

(i) VARIANCE RATIOS (VR)

STRAIN - STRAIN 4

DF RESIDUAL 10

Strain	P.sativum	V.faba	L.esculenta
1001	52.498	153.645	2.636
1012	27.283	206.988	71.002
1013	48.677	200.724	37.365
1045	86.146	19.404	41.676
1056	0.395	26.818	197.186
MR4	5.198	151.549	4.469

(ii) STANDARD ERRORS (SE) AND COEFFICIENT OF VARIATION (CV%) FOR INTERACTION

BLOCK 2

DF BLOCK. UNITS 10

Strain	P.sativum		V.faba		L.esculenta	
	SE	CV%	SE	CV%	SE	CV%
1001	1.8902	15.4	1.3289	27.4	1.9734	10.1
1012	2.5681	31.4	1.1190	13.5	1.6647	35.9
1013	1.7889	26.6	0.9871	24.8	2.4178	17.6
1045	1.5271	13.2	2.1162	18.4	2.0193	18.8
1056	1.3617	113.1	2.1133	12.9	1.2987	16.0
MR4	1.7781	9.8	1.2108	9.2	1.1360	48.5

2. CULTIVAR - STRAIN INTERACTIONS

(a) Nodule number formed by 1001, 1056 and MR4

SUMMARY ANALYSIS OF VARIANCE

	RESIDUAL		CHANGE		MEAN	VR
	DF	SS	DF	SS	CHANGE	
INITIAL MODEL	62	170121.5	*	*		
MODIFICATIONS TO MODEL						
BLOCK	59	167009.4	3	3312.1	1037.4	1.31
CULTIVAR	54	95342.3	5	71667.1	14333.4	18.03

	RESIDUAL DF	RESIDUAL SS	CHANGE DF	CHANGE SS	MEAN CHANGE	VR
STRAIN	52	57800.8	2	37541.6	18770.8	23.61
CULTIVAR-STRAIN	42	33386.0	10	24414.8	2441.5	3.07
				R		

DENOMINATOR RATIO = $\frac{\text{RESIDUAL SS}}{\text{RESIDUAL DF}}$ = 794.9

(b) NODULE FRESH WEIGHT

SUMMARY OF ANALYSIS OF VARIANCE

	RESIDUAL DF	RESIDUAL SS	CHANGE DF	CHANGE SS	MEAN CHANGE	VR
INITIAL MODEL	62	253082	*	*		
MODIFICATION						
BLOCK	59	251111	3	1971	657	0.39
CULTIVAR	54	116228	5	134884	26977	16.11
STRAIN	52	107082	2	9145	4573	2.73
CULTIVAR-STRAIN	42	70311	10	36772	3677	2.20

DENOMINATOR RATIO $\frac{\text{RES SS}}{\text{RES DF}}$ = 1674

(c) RATE OF N_2 FIXATION

SUMMARY OF ANALYSIS OF VARIANCE

	RESIDUAL		CHANGE		MEAN	VR
	DF	SS	DF	SS	CHANGE	
INITIAL MODEL	62	4873.29	*	*		
MODIFICATION						
BLOCK	59	4798.22	3	75.07	25.02	0.68
CULTIVAR	54	4149.14	5	649.08	129.82	3.53
STRAIN	52	1906.06	2	2243.08	1121.54	30.59
CULTIVAR-STRAIN	42	1539.80	10	366.26	36.63	1.00

$$\text{DENOMINATOR RATIO} = \frac{\text{RES SS}}{\text{RES DF}} = 36.66$$

CORRELATION MATRIX

THOSE SYMBIOTIC PROPERTIES WHEN CORRELATED GAVE THE FOLLOWING CORRELATION COEFFICIENTS:

	ALL STRAINS	1001	1056	MR4
DF	61	19	19	19
CORRELATION COEFFICIENTS	-0.4386	-0.3823	-0.3857	-0.3344

In all cases there was a negative correlation showing that the rate of N_2 fixation decreases with increasing number of nodules.

(d) CULTIVAR-STRAIN INTERACTION IN COMPETITION FOR NODULE FORMATION

ANALYSIS OF VARIANE FOR CULTIVAR-STRAIN

	DF
CULTIVAR	4
STRAIN	2
CULTIVAR-STRAIN	8
RESIDUAL	70

VARIANCE RATIOS

STRAIN	CULTIVAR	STRAIN	CULTIVAR STRAIN
1001	5.068	10.291	4.745
1056	3.281	12.531	0.487
MR4	1.664	11.162	1.381

STANDARD ERRORS AND COEFFICIENTS OF VARIATION

	DF		
BLOCK	5		
BLOCK.CULT,STR.	70		
		SE	CV%
STRAIN			
1001		3.032	36.3
1056		1.624	47.8
MR4		3.089	32.0

3. INTER-STRAIN INTERACTION

(A) Average number of nodules

Tables of Means

STRAINS	GRAND MEAN	ALL	1001	-1012	-1013	-1045	-1056	-MR4
1001	1.756	2.135	x	1.337	0.643	1.337	2.493	2.59
1012	0.253	-0.327	0.576	x	1.009	-0.327	0.210	0.38
1013	0.363	0.039	-0.327	0.894	x	1.520	-0.327	0.38
1045	0.765	0.322	1.487	0.576	1.955	x	-0.693	0.9

STRAINS	GRAND MEAN	ALL	1001	-1012	-1013	-1045	-1056	-MR4
1056	1.121	0.322	1.141	1.760	1.121	1.205	x	1.179
MR4	2.338	2.489	2.636	2.206	1.999	2.253	2.448	x

(B) ANALYSIS OF VARIANCE

DF STRAINS 10

RESIDUAL 15

STRAINS	VARIANCE RATIOS	STANDARD ERRORS	COEFFICIENTS OF VARIATION
1001	4.743	0.6132	34.9
1012	1.797	0.6753	266.6
1013	1.945	0.9090	250.2
1045	2.857	0.9534	124.7
1056	1.887	0.5802	51.7
MR4	3.391	0.2160	9.2

4. EFFECTS OF MOISTURE

(A) SOIL MOISTURE (% DRY SOIL)

ANALYSIS OF VARIANCE

	DF	VR	SE	CV%
MOISTURE	3	98.413		
STRAIN	3	0.866		
MOISTURE-STRAIN	9	0.173		
RESIDUAL	45		0.871	14.0

(B) WATER CONTENT IN PLANTS (% DRY WEIGHT)

ANALYSIS OF VARIANCE

	DF	VR	SE	CV%
MOISTURE	3	17.516		
STRAIN	3	1.035		
MOISTURE-STRAIN	9	0.809		
RESIDUAL	45		0.90	1.1

(C) NUMBER OF NODULES

ANALYSIS OF VARIANCE

	DF	VR	SE	CV%
MOISTURE	3	308.352		
STRAIN	3	1.940		
MOISTURE-STRAIN	9	0.870		
RESIDUAL	45		6.27	21.4

(D) DRY WEIGHTS OF SHOOTS (G)

	DF	VR	SE	CV%
MOISTURE	3	21.250		
STRAIN	3	1.484		
MOISTURE-STRAIN	9	2.300		
RESIDUAL	45		0.271	31.6

(E) COMPETITION AMONG THREE STRAINS

NUMBER OF NODULES

VARIANCE RATIOS

	MOISTURE	STRAIN	STRAIN-MOISTURE	RESIDUAL
DF	2	2	4	24
1001	4.884	60.771	9.389	
1056	23.900	22.455	0.482	
MR4	44.634	32.082	1.360	

STANDARD ERRORS AND COEFFICIENTS OF VARIATION

	SE	CV%	DF
1001	1.433	21.1	45
1056	1.177	52.3	45
MR4	1.209	11.6	45

EFFECTS OF TEMPERATURE

(A) DRY WEIGHTS OF SHOOTS

ANALYSIS OF VARIANCE, STANDARD ERRORS AND COEFFICIENTS OF VARIATION

	DF	VR	SE	CV%
TEMPERATURE	2	102.110		
STRAIN	3	7.820		
TEMPERATURE-STRAIN	6	5.442		
RESIDUAL	44		0.4616	34.2

(B) NODULE FRESH WEIGHT (G)

ANALYSIS OF VARIANCE, STANDARD ERRORS AND COEFFICIENTS OF VARIATION

	DF	VR	SE	CV%
TEMPERATURE	2	147.36		
STRAIN	3	5.336		
TEMPERATURE-STRAIN	6	1.489		
RESIDUAL	44		0.2202	40.1

(C) RATE OE N_2 FIXATION

ANALYSIS OF VARIANCE, STANDARD ERRORS AND COEFFICIENTS OF VARIATION

	DF	VR	SE	CV%
TEMPERATURE	2	98.510		
STRAIN	3	4.490		
TEMPERATURE-STRAIN	6	8.168		
RESIDUAL	44		6.79	36.4

(D) COMPETITION FOR NODULE FORMATION

ANALYSIS OF VARIANCE

VARIANCE RATIOS

	TEMPERATURE	STRAIN	TEMPERATURE-STRAIN	RESIDUAL
DF	2	2	4	32
1001	17.485	3.602	10.508	
1056	277.507	21.780	13.274	
MR4	9.962	2.622	0.721	

STANDARD ERRORS AND COEFFICIENTS OF VARIATION

	SE	CV%	DF
1001	2.178	46.9	32
1056	1.561	24.1	32
MR4	5.387	70.3	32

5. CHEMOTAXIS OF RHIZOBIUM TO EXUDATES FROM THREE HOST LEGUMES

ANALYSIS OF VARIANCE, STANDARD ERRORS AND COEFFICIENTS OF VARIATION

	DF	VR	SE	CV%
CULTIVAR	2	5.013		
STRAIN	5	25.640		
TIME(DAYS)	2	162.335		
CULTIVAR-STRAIN	10	4.812		
CULTIVAR-TIME	4	92.176		
STRAIN-TIME	10	3.623		
CULT.STR.TIME	20	10.193		
RESIDUAL	106		0.6164	23.1

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