SOME ASPECTS OF SYMBIOTIC NITROGEN FIXATION IN VICIA CRACCA L. (LEGUMINOSAE)

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Some Aspects of Symbiotic Nitrogen Fixation in *Vicia cracca* L. (Leguminosae)

by

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ABSTRACT

Studies were undertaken to characterize the symbiosis between an uncultivated legume, Vicia cracca L., and a strain of Rhizobium bacteria isolated from its root nodules.

Root nodules were found to be elongate and often branched at maturity. Tritiated-thymidine incorporation studies, monitored by autoradiography, indicated that nodules develop from an apically-located meristem. Nitrogen fixation, measured by acetylene reduction, occurred at rates commensurate with values reported for other legumes. Vicia cracca seeds and plants are small and can be conveniently cultured in vitro but a rigorous scarification procedure, immersion in concentrated sulfuric acid, was required to produce appreciable germination rates. Sulfuric acid simultaneously scarified and surface-sterilized Vicia cracca seeds. This permitted nodulation of V. cracca, by selected Rhizobium strains, to be conveniently studied.

The Rhizobium isolate, referred to as R. sp. VC 2, was identified as a wild strain of Rhizobium leguminosarum. Studies were undertaken to characterize the bacterium with respect to carbon and nitrogen nutrition and with respect to the effects of temperature and pH on growth. A wide variety of carbon and nitrogen sources was used by R. sp. VC 2. Total growth of shake cultures was significantly affected by carbon and nitrogen source and markedly dependent on the

initial pH of the growth medium. Total growth of bacteria in shake culture, at pH values below 7.0, was found to be greater at 24 C than at 30 C. The cell surface of R. sp. VC 2 was shown to possess receptor sites for a lectin from V. cracca seeds. The lectin was extracted from V. cracca seeds by affinity chromatography and labelled with fluorescent isothiocyanate (FITC). Nitrogen fixing activity was induced in free-living cultures of Rhizobium sp. VC 2. A number of carbohydrates, alone and in combination, supported nitrogen fixation. R. sp. VC 2 nodulated V. cracca as efficiently as two commercial strains of Rhizobium leguminosarum.

V. cracca seedlings, being small, could be easily cultured in vitro and nodulated by Rhizobium leguminosarum. These attributes permit the V. cracca - Rhizobium leguminosarum symbiosis to be used as a convenient model for the Pisum sativum (pea) - Rhizobium leguminosarum symbiosis. Uncultivated V. cracca nodules could be used as a source of new genetic material for commercial producers of Rhizobium inocula. Also, the Vicia cracca - Rhizobium leguminosarum symbiosis has potential to be used in land reclamation and land maintenance projects.

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INTRODUCTION

1.1 General Introduction

Bacteria of the genus Rhizobium frequently form nodules on the roots of legume plants. Mature nodules are characteristically capable of reducing atmospheric nitrogen (N_2) into ammonia (NH_3) through the catalytic action of a bacterial enzyme, nitrogenase (N'ase). The chemical process is termed 'nitrogen fixation' and its stoichiometry, in biological systems, can be expressed as follows:

$$N_2 + 8e^- + 8H^+ \xrightarrow{N'ase} 2NH_3 + 2H_2$$

(Schrauzer, 1977)

Other organisms are capable of fixing nitrogen (Table 1) and non-biological means also exist (Table 2) although associations of legumes and rhizobia are the dominant source of fixed nitrogen on a worldwide basis (Rao, 1980).

The process of nitrogen fixation is biologically important because nitrogen, being an integral part of proteins and nucleic acids, is required by all organisms but can only be used by most when it is present in its fixed or reduced state. Much of the usable, i.e., reduced, nitrogen is lost by the action of denitrifying bacteria, fires and various other means (Payne et al., 1980; Wigley and

Table 1 - Some Biological Nitrogen-Fixing Organisms

Category	Organim(s)
asymbiotic prokaryotes	
aerobic	Aquaspirillum, Azomonas Azotobacter, Azotococcus Beijerinckia, Derxia, Gleocapsa
microaerobic	Azospirillum, Plectonema Rhizobium, Thiobacillus, Xanthobacter
facultative	Bacillus, Citrobacter
anaerobic	Chlorobium, Chromatium, Clostridium, Desulfotomaculum, Desulfovibrio, Ectothiospira, Proprionibacterium, Rhodopseudomonas, Rhodospirillum, Thiopedia
symbiotic associations	legumes x Rhizobium, Parasponis x Rhizobium, woody plants x Frankia, Paspalum x Azotobacter, various associations of grasses and weeds x prokaryotes, various algae x fungi, Azolla x Anabena, Cycads x Nostoc, ruminant animals and termites and Homo sapiens x enterobacteria

(from Postgate, 1980)

Table 2 - Methods of Nitrogen Fixation and the Relative Contribution of Each to the Worldwide Nitrogen Economy

Method	Rate *	Reference
Biological Fixation	175-101	Rao, 1980
Industrial Fixation	40	Rao, 1980
Lightening	10	Burns and Hardy, 1975
TiO_2 Fixation	68-7	Henderson-Sellars and Schwartz, 1980

(*) million metric tons per annum

Brimblecombe, 1981) so processes which maintain pools of fixed nitrogen are important.

Plants generally have a "high requirement" for nitrogen (Bidwell, 1974, p.238) so agricultural crops constantly reduce pools of fixed nitrogen from the soil. As a result, supplemental nitrogen must be applied to maintain the fertility of the soil. The usual methods include application of commercial fertilizer or manure and cultivation of 'green' crops which are plowed under at maturity. The vetches, Vicia spp. and clovers, Trifolium spp., are common green crops. Nitrogen fertilizers are used extensively in North American agriculture to supplement soil nitrogen levels and current agricultural practices are heavily dependent on them.

The preferred method to increase levels of usable soil nitrogen is a contentious issue (Rao, 1980) since problems of economics, biology and technological appropriateness must all be accounted for. Associations of legumes and rhizobia are, however, currently important in supplying fixed nitrogen to the soil and in other aspects of agriculture (Isely, 1982). Moreover, their importance will probably continue into the immediate future since there will be an estimated three-fold increase in demand for fixed nitrogen over the years 1980-2000 (Verghese, 1977). Industrially produced fertilizers may not meet this demand for reasons of economics and resource management (Postgate, 1980; Rao,

1980). New strategies that link biological fixation with abiological fixation may evolve or genetic improvement of existing plant and bacterial genomes may occur (Ausubel, 1981; Brill and Ela, 1981). Legume-*Rhizobium* symbioses, or genetically-reconstituted associations of them, will undoubtably have an important role in supplying future demands for fixed nitrogen.

In addition to their agricultural importance, legume-*Rhizobium* symbioses are biologically interesting because they involve elaborate interactions between two very disparate taxa. For example, Verma *et al.* (1978) have shown that during the 'infection' of soybean roots, the plant cell wall is degraded by a coordinated enzymatic process in which the plant releases cellulase and *R. japonicum* releases pectinase. These activities permit inward movement of the 'infective' bacterium through the plant root.

Despite the importance of legume-*Rhizobium* symbioses to agriculture and their interest to biology, only 16% of the legume species have been examined for root nodules (Allen and Allen, 1981); this indicates that many new classes of associations may yet exist. Of the legumes that have been examined for root nodules, the biology of only 10 or 20 of the most agriculturally important species has been studied extensively. These data indicate that the biology of legume-*Rhizobium* symbioses is not well understood. This

lack of knowledge must be eliminated if associations of legumes and rhizobia are to be manipulated, to their fullest degree, in the service of man.

Preliminary studies of an isolate from Vicia cracca nodules by this author (A.N.) suggested that the isolate was a wild strain of R. leguminosarum, the endosymbiont of Garden Pea (Pisum sativum L.), Faba Bean (Vicia faba L.) and Lentil (Lens culinaris Medic.). The rhizobia from Vicia cracca nodules might, therefore, be of benefit to local agriculture since the strains have undergone some selection for fitness to the local soil environment. Moreover, the V. cracca symbiosis could be useful in studies of pea-type symbioses since the respective plants and bacteria appear quite closely related.

Characterization of the symbiosis of Vicia cracca and its endosymbiotic Rhizobium partner was therefore seen as an interesting biological study that had considerable application to agriculture in Canada.

1.2 Statement of Research Aims

The primary aim of this research project is to characterize some aspects of the Vicia cracca - Rhizobium sp. symbiosis by examining important features of the plant and the bacterium as well as some salient aspects of their interaction.

Selected aspects of the symbiosis will be compared with those from symbioses of commercially important legumes, particularly from faba bean (Vicia faba L.) and garden pea (Pisum sativum L.). These plants are related to V. cracca and their symbioses have been extensively studied.

The usefulness of the Vicia cracca - Rhizobium symbiosis, with regard to agriculture and land management, will also be discussed in this thesis.

1.3 Terminology Notes

Throughout this thesis the convention of the literature has been followed and the term 'infection' has been used to refer to processes that occur prior to the development of a mature legume nodule. The term has not been used in its normal pathological sense in this case. Similarly, the word 'host' refers to the legume partner of the symbiosis. Rhizobia which are internalized within the host are said to be 'endosymbionts'.

2. Literature Review

2.1 References that Pertain to the Vicia cracca Symbiosis

An annotated list of the literature that pertains to the Vicia cracca symbiosis is given in Appendix A. In summary, there are early references to nodulation of V.

cracca, a description of the seasonal pattern of nodulation in Vicia cracca and biochemical studies of the Vicia cracca seed lectins.

2.2 Taxonomy, Distribution and Description of Vicia cracca

The cow vetch, Vicia cracca L., is a member of the tribe Viceae, from the sub-family Lotoidea (Papilionatae; Faboidea;) of the pea family, Leguminosae (Fabaceae). The ten tribes of the Lotoidea represent some 375 genera and include most of the temperate climate representatives of the pea family (Lawrence, 1951). Members of this family usually possess a gamosepalous calyx and a papilionaceous corolla (Lawrence, 1951). Some of the more well-known representatives of the tribe Viceae include peas (Pisum sativum), lentils (Lens culinaris Medic.), sweet peas (Lathyrus spp.) and road beans or aba beans (Vicia faba L.).

Vicia cracca has been collected from a large number of sites throughout the world such as Eurasia, Greenland, North America, South Africa, the Falkland Islands, New Zealand and Tasmania (Hulten, 1968). In Canada the plant has been reported from all ten provinces and the Yukon but not from the Northwest Territories (Scoggan, 1978; Porsild and Cody, 1980). Collections of V. cracca from insular Newfoundland in the Ayre Herbarium of this university indicate that it is

widely distributed on this island.

V. cracca is generally held to be an introduction from Europe (Frankton, 1955; Fernald, 1970; Scoggan, 1978) although Gleason (1963, p. 443) writes,

> "(it is) native to the n. part of our range (n.e. United States and adjacent Canada) but apparently only introduced from Mass. to Ind. and southward."

The other representatives of the genus Vicia in insular Newfoundland, V. sativa L. and V. faba L. (Rouleau, 1978), are also believed to be European introductions (Scoggan, 1978).

The synonomy of V. cracca L., as recognized by Scoggan (1978) for Canada, is as follows:

var. tenuifolia (Roth)Beck
  f. tenuifolia (=V. tenuifolia Roth)
  f. albiflora (Aschers and Graebn.) Gams

var. cracca
  f. cracca (includes var. linearis (Pet.) Gams and its f. etiamelba Boivin
  f. sericea (Peterm.) Beck
  f. albida (Peterm.) Gams (includes f.albiflora Kitt)

All specimens sampled by this author (A.N.) were typical representatives of the species .

V. cracca is known throughout North America by various colloquial names including Cow vetch (Scott, 1977), tufted vetch, wild vetch, purple tufted vetch (Frankton, 1955),

bluebirds (Scoggan, 1950), Canada pea, or, in the French language, jargeau and vesque craque (Fernald, 1970). Throughout the remainder of this thesis I shall use only the name cow vetch as a colloquial synonym for Vicia cracca L.

Vicia cracca is a perennial herb with bluish-purple or (rarely) white flowers that are borne on one side of a long-peduncled raceme. The flowers become flattened pods containing several reddish-brown, oval to round (2.5-3.5 mm long) seeds. The thin, weak stem bears opposite leaves that consist of 8-12 leaflets. Leaves end in branched tendrils which allow the plant to climb adjacent objects such as erect plants or fenceposts. This twining habit probably accounts for the plant's frequent designation as a 'weed' (Frankton, 1955; Alex and Switzer, 1976). The cow vetch can be found in,

> "meadows, pastures, gardens, wasteplaces, grain fields, [and other] cultivated fields" (Frankton, 1955, p.100) as well as on "sea cliffs; river gravels; [and] beaches " on the Gaspe Penninsula (Scoggan, 1950, p. 253).

2.3 Seasonal Aspects of Growth and Nodulation in Vicia cracca

Pate (1958) studied seasonal aspects of growth and nodulation in field-grown Vicia cracca from Northern

Ireland. In V. cracca,

> "A short-lived, fibrous root system is developed and nodulated in the first few months of seedling growth. This is replaced by an extensive system of starch-storing rhizomes, each with its own adventitious roots. Portions of the rhizome system may become separated from the parent plant. Nodules predominate on adventitious roots from the current season's rhizomes".
>
> (Pate, 1958, p.510-11)

Wedderburn and Gwynne (1981) studied the seasonal nature of growth and nitrogen fixation in another rhizomatous perennial legume, Lotus uliginosus Schkuhr. (the Marsh Birdsfoot Trefoil) in southwest Scotland. Production of aerial shoots and rhizomes (dry weight of tissue) reached a peak in September and October while nitrogen fixation began in June and rose to a midsummer peak in July. The commencement of nitrogen fixation, "coincided with increasing soil temperature" while the "decline in fixation was associated with increasing rainfall, decreasing soil temperature, flowering and onset of rhizome production" (Wedderburn and Gwynne, 1981, p. 5).

In Lotus uliginosus, "Renewed aerial shoot growth in spring was initiated mainly from nodes on rhizomes formed in the previous autumn." Pate (1958, p. 511) suggested a similar role for overwintered V. cracca nodules in Northern Ireland:

> "Nodules may remain on a root system for 12-14 months and hence two sets of nodules may coexist for a short period in the early spring, the older set still haemoglobin-pigmented and presumably active in fixation. It is possible

that one set of aging nodules may provide the necessary growth materials for juvenile members of the same root system. A large proportion of each season's nodule set survives the normal Irish winter".

2.4 Lectins and the Initiation of Legume-*Rhizobium* Symbioses

Many legumes contain one or more proteins known as lectins which are believed (Bohool and Schmidt, 1974; Albersheim and Anderson-Prouty, 1975) to mediate 'recognition' between strains of *Rhizobium* and their appropriate legume hosts. Some comprehensive treatments of relevant aspects of the lectin literature are provided by Gold and Balding (1975), Sharon (1977), Roth (1978), Bauer and Bhuvaneswari (1980) and Pistole (1981).

The hypothesis that legume lectins recognize specific strains of *Rhizobium* (Bohool and Schmidt, 1974) was based on observations that lectin from soybean seed bound to 22 out of 25 strains of the soybean endosymbiont, *Rhizobium japonicum*, but not to 23 strains of *Rhizobium* which do not nodulate soybean. A model for the biological role of legume lectins, which arose from these and other experiments, stated that lectin on the surface of the root could 'recognize' bacteria on the basis of sugar moieties on the bacterial cell surface. This hypothesis is especially appealing since lectins are renowned for their ability to

bind specifically to certain sugars but not to others. The term 'lectin' is derived from the Latin verb legere, to "pick or to choose" (Sharon, 1977).

Lectins have been isolated from a great number of legumes including soybean seeds (Lotan, 1974) and roots (Gade et al., 1981), from pea seeds (Trowbridge, 1974; Kinje et al., 1980) and roots (Gatehouse and Boulter, 1980; Kinje et al., 1980) and from seeds and roots of Lotononis bainesii (Law and Strijdom, 1977). Lectins have also been isolated from seeds of various other legumes such as lentil (Howard et al., 1972), clover (Dazzo et al., 1978) and alfalfa (Paau et al., 1981). Among the vetch species, lectins have been isolated from seeds of Vicia villosa (Braciale et al., 1981; Grubhoffer et al., 1981), V. sativa (Van Driessche et al., 1980) and V. faba (Wang et al., 1974; DeClerque et al., 1976). In the cow vetch, Vicia cracca, lectins have been reported from the seeds (Baumann et al., 1979; Horejsi and Kocourek, 1978) as well as from the leaves, stems and tops of this plant (Cazal and Lalaurie, 1952).

The seeds of V. cracca possess two distinct lectin fractions (Baumann et al., 1979). One fraction binds to N-acetyl-galactosamine (Gal-N-Ac) and galactose and specifically agglutinates Type A1 erythrocytes (Ruediger, 1977) The other fraction binds to glucose (Glu) but does not display any hemagglutination specificity (Aspberg et al.,

1968). The Gal-N-Ac binding fraction is a tetramer composed of four identical subunits while the Glu binding fraction is composed of a smaller polypeptide chain, the alpha chain, and a larger chain, the beta chain. Analysis of N-terminal amino acid sequences and immunochemical properties reveal that the Glu binding fraction is far more homologous with the lectins of pea and lentil than with the Gal-N-Ac fraction of V. cracca (Baumann et al., 1979). Those authors suggest (p. 217) that the two V. cracca lectins are coded for by separate genes, "which have a common origin, but which diverged a number of years ago".

The Gal-N-Ac binding fraction can be further resolved into two sub-fractions on the basis of pH-dependent desorbtion from an affinity chromatography column with a galactose ligand (Ruediger, 1977). One sub-fraction desorbs at pH 6.8 and the other desorbs at pH 4.5; both contain several components that have similar ionic charges, electrophoretic mobility and hemagglutination capacity.

The precise role of lectins in initiating legume-Rhizobium symbioses is unknown and quite controversial since some rhizobia bind lectin from plants that they do not nodulate (Dazzo and Hubbell, 1975; Chen and Phillips, 1976; Law and Strijdom, 1977; Wong, 1980). In addition, the existence of lectins on legume roots, not seeds, has only been demonstrated in four species to date (Law and Strijdom, 1977; Horejsi et al., 1978; Gatehouse

and Boulter, 1980; Kijne et al., 1980; Gade et al., 1981). Moreover, the suspected lectin binding sites on the Rhizobium cell surface are lipopolysaccharides and/or acid polysaccharides and these groups show sugar compositions and immunodominant structures that vary as much between Rhizobium species as within Rhizobium species (Carlson et al., 1978; Kamberger, 1979; Robertson et al., 1981). It can be concluded that lectins on the surface of legume roots may play a role in initiating legume-Rhizobium symbioses but the extent and nature of that role is not presently understood.

Initiation of legume-Rhizobium symbioses may also involve the exudation of 'priming' compounds from the host root to prepare the rhizobia for the nodulation process. Egeraat (1975a, 1975b) claims that the dominant amino acid exuded by pea (Pisum sativum L.) seedlings, homoserine, selectively stimulates growth of the pea endosymbiont, Rhizobium leguminosarum, but not several other species of Rhizobium. By contrast, Gaworzewska and Carlile (1982) have shown that low molecular weight pea root exudates attract R. leguminosarum, other Rhizobium species and Escherichia coli. The exudates from other plants, including non-legumes, also attract R. leguminosarum and the other bacteria. On the basis of this data, Gaworzewska and Carlile (1982) concluded that these exudates facilitate infection of legumes but probably play no direct role in selection of the appropriate

bacterium for nodulation. Bhagwat and Thomas (1982) have isolated an exudate from roots of Cowpea (Vigna sinensis) which enables cowpea rhizobia, Rhizobium sp., to infect the host plant more quickly.

2.5 Nodule Morphogenesis and Histology

Legume root nodules develop after cells of a suitable strain of Rhizobium attach to the root surface and become internalized within the root cortex. Bacteria are internalized by means of a tube-like structure called the infection thread which is present in the developing nodules of most, but not all, legumes.

The plant meristem is initiated, following internalization of the Rhizobium cells, by a process that is still poorly understood (Dart, 1977; Newcomb, 1981) but believed to involve exchange of chemical 'messages' from the bacterium to the the plant (Truchet, 1978). The meristem produces the greatly enlarged or hypertrophied host cells which form the mature nodule. Within the hypertrophied cells the highly differentiated rhizobia, called bacteroids, are found. These bacteroids produce the enzyme nitrogenase that catalyzes the nitrogen fixation reaction.

The histology of the mature leguminous nodule has been extensively described by Dangeard (1926), Bieberdorf (1938), Allen and Allen (1954), Libbenga and Bogers (1974), Dart

(1977) and Newcomb (1981). The nodule consists of "four zones of tissue differentiation" (Allen and Allen, 1954, p. 218) or tissues which include, from outside to inside, an area of parenchymatous cells (the nodule cortex), with elements of the vascular system interspersed as distinct vascular bundles. A meristematic region and a zone of bacteroid-containing cells (the bacteroid zone) also exists in mature nodules. Developing nodules may have a fifth zone present (the root cortex) which consists of stretched and broken cells that are smaller than the nodule cortical cells. Root cortical cells are derived from the root meristem while the other cells which constitute the nodule are derived from nodule meristem.

Pate et al. (1969, p. 13) describe the vascular system of legume nodules as being, "sparse and peripheral" (in the cortex) and comprised of vascular bundles that contain an endodermis plus xylem and phloem. In most bundles the xylem is external to the phloem although in some nodules the situation is reversed. Occasionally an additional endodermis is present in the cortex in a position that is external to the regular endodermis (Allen and Allen, 1954).

The meristematic zone is internal to the nodule cortex and, in nodules of Pisum, Trifolium, Medicago and Vicia, displaced to one end of the nodule. The meristem produces vascular plus cortical cells to the outside and cells

destined for the bacteroid zone to the inside. In Pisum, Trifolium, Medicago and Vicia, the nodules are elongate-cylindrical and mature nodules may branch into two or more lobes. In plants with round nodules such as soybean, Glycine max, the meristem is found in pockets throughout much of the nodule.

The bacteroid-containing zone includes hypertrophied cells that are often filled with bacteria. Infected cells are probably sites of nitrogen fixation since there is ready access to nitrogenase (from the bacteria) and components of a nitrogenase protection system as well as ATP (from the plant). Uninfected cells in the bacteroid zone may play a role in synthesis of the nitrogenous compounds that are exported from the nodule (Newcomb and Tandon, 1981). The bacteroid zone can be distinguished from other tissues of the nodule in free-hand, unfixed sections because it has a reddish hue. The colour is due to the presence of a heme-containing pigment called leghemoglobin.

2.6 Localization of the Nodule Meristem

The nodule meristem determines the shape, size and longevity of nodules so it is important to their development and function. The location and extent of the meristem within the nodule has, by convention, been determined solely from observations of light- and electron-micrographs (eg.

Dart, 1977); these data indicate that meristem cells are densely-stained and smaller than adjacent cortical cells.

It is possible to demonstrate meristematic activity in cells by experimental means using thymidine incorporation by the insoluble fraction of the nucleus as a criterion of meristematic activity. Meristem cells actively synthesize DNA in S-phase of the cell cycle and incorporate thymidine in the replicating DNA. Thymidine incorporation can be demonstrated by autoradiographic procedures with tritium-labelled thymidine (Jensen, 1962). Sites that incorporate tritiated thymidine are indicated by dense deposits of silver grains.

2.7 *Rhizobium* Taxonomy and Biology

2.7.1 General

The family Rhizobiaceae, as recognized in Bergey's Manual of Determinative Bacteriology (1974), includes 6 species of *Rhizobium* and 4 species of *Agrobacterium*. Members of the Rhizobiacea are aerobic, Gram-negative, non spore-forming, motile bacilli.

The *Rhizobium* bacteria are named for their ability to invade roots of legume plants and cause organized, non-pathological cortical hypertrophies (nodules) to form : *rhiza*, Greek noun meaning 'a root'; *bios*, G.n. meaning 'life' hence *Rhizobium*, 'that which lives in a root' (Jordan and Allen, 1974). Species of *Agrobacterium* cause

disorganized, pathogenic growths called galls in more than 40 families of plants.

The current classification of the genus Rhizobium is generally considered "tentative" (Jordan and Allen, 1974) because it relies heavily on a character with disputed taxonomic value; the ability to form root nodules on legumes. The legumes that a particular Rhizobium strain will nodulate are usually, but not always, closely related. Use of this character to classify or identify rhizobia is problematic because some strains are 'promiscuous' and can nodulate more than one group of legumes. Also, the ability to nodulate a specific host can be lost or reduced if strains are cultured for extended periods so host nodulation ability is not a stable character. More comprehensive accounts of the the Rhizobium taxonomy are provided by Jordan and Allen (1974), Graham (1976), Vincent (1977) and Elkan (1981).

Most rhizobia can be categorized into one of two groups that have fairly broad boundaries. The first includes the 'slow-growing' rhizobia (generation time approximately 6-8 h) which prefer pentose carbon sources, form small colonies(=or< 1 mm dia.) and do not produce acid on yeast extract-mannitol-mineral salts (YEM) media after 5-7 days (Vincent, 1977). Usually the bacteria are isolated from tropical legumes or tropical soils and display polar or subpolar flagella. The second Rhizobium grouping includes

the 'fast-growers' (generation time approximately 2-4 h) which can use a wide variety of carbon sources and produce large colonies 2-4 mm dia.) after 3-5 days on YEM media. Fast-growers are characteristic of temperate legumes and temperate soils and possess 2-6 peritrichous flagella. The slow-growing group, with the major hosts indicated parenthetically, includes R. japonicum (soybean) and R. lupini (lupin). The fast-growing group includes R. leguminosarum (pea, lentil and vetch), R. phaseoli (bean), R. trifoli (clover) and R. meliloti (sweet clover, alfalfa and fenugreek) (Jordan and Allen, 1974). Not all rhizobia conform to this convenient dichotomy of fast or slow growth rate, as evidenced by the rhizobia from Lotus nodules which may be either fast growers or slow growers (Vincent, 1977). Moreover, generation time values are not diagnostic characters of Rhizobium species since tremendous variations can be observed between strains of a single species. For example, the generation times of R. japonicum vary from 3 to 10 h (Elkan, 1971) or 2.9 to 13.0 h (Keyser et al., 1982).

2.7.2 Carbohydrate Use

Fast- and slow-growing rhizobia display large differences in the number and kind of carbohydrates that they can use (Vincent, 1977) so tests of carbohydrate use

were begun to aid in the characterization of a Rhizobium isolate from Vicia cracca.

The effect of carbon source on total growth of the bacterium was also studied.

2.7.3 Nitrogenase Activity

Rhizobia do not normally fix nitrogen unless they are within the legume nodule and have undergone the tremendous morphological and metabolic changes that are required of functioning endosymbiotic rhizobia. The transformed rhizobia are referred to as 'bacteroids'; more complete accounts of their biology are given by Jordan (1962) and Bergersen (1974).

Free-living rhizobia are capable of nitrogen fixation if cultured under the appropriate conditions (Pagan et al., 1975; Kurz and LaRue, 1975; McComb et al., 1975; Keister, 1975; Bednarsky and Reporter, 1978; Manhart and Wong, 1979; Skotnicki et al., 1979; Egeraat and Timmermans, 1980). Induction of N'ase activity in free-living cultures demonstrates that the genes for nitrogen fixation ('Nif') reside in the bacterial genome and that they are normally repressed in the free-living state.

Induction of N'ase activity (i.e., de-repression of the Nif genes) also provides a model with which the physiology and biochemistry of the endosymbiotic Rhizobium state can be

studied. For example, the identity of the molecules that are supplied to the bacteroids by the plant is unknown and the subject of some recent speculation (Bergersen, 1977; Trinchant, et al., 1981). To provide some insight on the effect of carbon source on nitrogenase activity, free-living cultures of R. sp. VC 2 were grown on various carbon sources and N'ase activity was measured. Appreciable differences in nitrogenase activity due to carbon source have been reported in free-living, slow-growing rhizobia (Kurz and LaRue, 1975), in fast-growing antibiotic-resistant mutants (Skotnicki et al., 1979) and in bacteroid suspensions from pea and other legumes (Trinchant et al., 1981).

2.7.4 Temperature and pH Effects on Growth

The influences of temperature on rhizobial growth and rhizobial growth mechanisms are not well known. Optimal rhizobial growth generally occurs in the range of 20-28 C but fast-growing rhizobia can grow at 4 C and Rhizobium leguminosarum is tolerant of maximal temperatures ranging from 31 C or higher (Vincent, 1977).

The combined results of some preliminary experiments suggested that R. sp. VC 2 grew faster at 30 C than at 20 C but final cell density was greater at the lower temperature than at the higher one. Since this novel

temperature-dependant growth response has ecological implications regarding strategies for soil colonization and plant nodulation, studies were begun to confirm the preliminary results.

Studies of the effect of pH on growth were also begun to understand optimal growth conditions.

MATERIALS and METHODS

Collection of Plant Specimens

Seeds and root nodules of V. cracca were collected on or near the St. John's campus of Memorial University of Newfoundland, Newfoundland, Canada. Seeds were collected from field-collected V. cracca plants then cleaned of extraneous material and stored in 125 ml glass flasks at 4 C. All specimens were typical representatives of the species.

Nodules used to illustrate external nodule morphology were periodically immersed in water to prevent dessication and photographed within one half hour of collection. No fixatives were used to preserve these specimens.

Localization of the Nodule Meristem

The nodulated root system from an entire V. cracca plant was carefully uprooted, washed in distilled water then incubated for 1 h at 18 C with tritiated thymidine (New England Nuclear Co.) in Sorenson's phosphate buffer at pH 7.2. The initial specific activity of the tritiated thymidine was 104.0 mCi/mmole and the concentration of the radiolabel was 0.01 mCi/ml.

Nodules were fixed in FAA, dehydrated in an

ethanol-tertiary butyl alcohol series then embedded in paraffin (Jensen, 1962). Sections made on a rotary microtome were de-paraffinized and prepared for liquid emulsion autoradiography by immersion in Kodak NTB-2 autoradiographic emulsion. After 7 days exposure at 4 C the sections were developed in Kodak D-19 Developer and fixed for 6 min. in Kodak FS Fixer. The fixed sections were then washed 20 min. in running tap water, allowed to dry and observed. The autoradiographed sections were photographed using a Zeiss Photomicroscope.

The FAA fixative contained 5 ml of commercial formalin, 5 ml of glacial acetic acid plus 90 ml of 50% ethanol (Jensen, 1962). Sorenson's phosphate buffer (pH 7.2) is made by adding 70 ml of solution A (7.1 g of Na_2HPO_4 in 500 ml H_2O) with 30 ml of solution B (6.8 g KH_2PO_4 in 500 ml of H_2O).

Assay for Nitrogenase Activity

Nitrogenase activity was assayed by the acetylene-reduction method of Hardy et al. (1968) which measures the rate that acetylene (C_2H_2) is reduced to ethylene (C_2H_4). Nodulated roots of Vicia cracca were collected from uncultivated plants and from cultivated V. cracca nodules. Vincent's (1970) method was used to produce V. cracca nodules in vitro. Seeds were surface sterilized

(this procedure described in 'Seed Germination'), inoculated with a Rhizobium isolate from V. cracca nodules (described in 'Sources of Bacteria and Method of Collection'), and grown in sterile flats containing sterile Vermiculite. Nitrogenase activity was assayed 26 days after inoculation with the bacterium.

Nodulated root sections were incubated in 'Vacutainer' glass serum tubes (13 ml capacity; Becton, Dickerson and Co., Mississauga, Ontario) while bacterial cultures were incubated in 1/4 oz. glass Bijou bottles (6.8 ml capacity; Johns Scientific Co., Toronto, Ontario). Roots were incubated at 24 C for 2 h while bacteria were incubated at 30 C for 4 h. The incubation gas mixture consisted of 0.1 atm. acetylene and 0.9 atm. air.

One (1.0) ml volumes of the gas mixture were chromatographed on a previously described system (Boyle and Patriquin, 1980) that consisted of a model GC 9700 'Basic' gas chromatograph (Carle Instruments, Anaheim, California) with a 'Poropak T' column and a flame ionization detector. The detector gave output to an 'Omniscribe' recorder model B 5118 from Houston Instruments of Austin, Texas. Column temperature was 50 C and the carrier gas flow rate was 20 ml/min. Helium, hydrogen and medical air were of the highest grades obtainable from the Canadian Liquid Air Company, St. John's, Newfoundland. Acetylene was generated by reacting calcium carbide with distilled water. Gases

were held in glass serum tubes and transferred with gas-tight syringes (Hamilton Co., Reno, Nevada) after extensive flushing with the appropriate gases.

Ethylene peak heights were measured directly from the paper recorder and compared to a standard curve which related the peak heights to known molar quantities of ethylene gas. A sample calculation is given in Appendix F (b).

Isolation and FITC-Labelling of V. cracca Lectin

Lectin from V. cracca seed was isolated by a modification of Ruediger's (1977) technique. Ten g of cleaned seed were ground to a fine powder by mortar and pestle and extracted with a 0.05 M Tris/HCl buffer at pH 8.0. The seed slurry was acidified to pH 5.0 and centrifuged for 30 min. at approximately 27,000g. The supernatant was saved and brought to pH 8.0 with 1 N NaOH then dialysed extensively against the extraction buffer.

An affinity chromatography column was prepared using a modified 10 ml glass pipette tube (7 x 1 cm). Slurried column material was swollen, poured into the column and equilibrated with the extraction buffer. The column material ('Selectin 5' from the Pierce Chemical Co. of Rockford, Ill. USA) consisted of an N-acetyl-D-galactosamine ligand bound to a matrix of

cross-linked "Beaded Agarose".

The dialysed product was applied to the column and flushed with 50 ml of a high ionic strength buffer, EB-1, at pH 8.0. To elute the lectin a continuously-decreasing pH gradient was developed by mixing the first buffer (EB-1) with another high ionic strength buffer (EB-2) at pH 3.0. The eluate was collected in 1 ml volumes and the absorbance at 280 nm was read on a double beam spectrophotometer (Shimadzu Seisakushu Ltd./Bausch and Lomb model 'Spectronic 210 UV'). Successive pairs of 1 ml fractions were combined and pH was monitored on a Fisher 'Model 7' pH-meter.

An absorption peak was noted and the fractions which constituted it were pooled, brought to pH 8.0 and re-applied to the column. The 280 nm-absorbing material was eluted with another continuous pH gradient that was developed using the EB-1 at pH 8.0 and at pH 6.0.

The re-chromatographed material also produced an absorption peak at 280 nm so the high-absorption fractions were again pooled, dialysed extensively against distilled water and then lyophilized and stored at 4 C.

The procedure to conjugate fluorescent isothiocyanate (FITC) to seed lectin and subsequently react it with Rhizobium strains followed the protocol set out by Bohool and Schmidt (1974). This procedure involved dissolving the lyophilizate in saline to make a 1 % solution then adding 4 ml of 0.1M sodium phosphate buffer (pH 7.0) to 10 ml of this

solution. 4 ml of 0.1M sodium phosphate buffer (pH 8.0) containing 5 mg of fluorescent isothiocyanate (isomer 1; British Drug House) was added. The mixture was adjusted to pH 9.0, merthiolate was added to make a concentration equal to (1mg/10L) and the mixture allowed to react for 24 h. Unbound dye was removed by dialysis against phosphate-buffered saline (0.02 M sodium phosphate; pH 7.2) until the dialysate was completely colourless. The FITC-Lectin conjugate was dispensed in 1 ml aliquots and stored at -20 C.

The lectin extraction buffer included 7.90 g of 'Tris' [= 2-amino-2(hydroxy-methyl)propane-1:3-diol] HCl and 0.20 g of sodium azide in 1000 ml of water. The pH was adjusted to 8.0.

The first seed lectin elution buffer (EB-1) consisted of 3.03 g of 'Tris', 1.70 g of imidazole, 116.88 g NaCl and 1000 ml H_2O. The pH of this buffer was 8.0 except where noted. The second elution buffer (EB-2) consisted of 1.44 g of glacial acetic acid, 1.06 ml of 90% formic acid, 116.88 g NaCl and 997.5 ml of H_2O. The pH of this buffer was adjusted to 3.0.

Sodium phosphate buffer was used at 3 pH values and made by adding different amounts of solution A (27.8 g monosodium phosphate in 1000 ml H_2O) to solution B (53.65 g $Na_2HPO_4.7H_2O$ or 71.7 g $Na_2HPO_4.12H_2O$ in 1000 ml H_2O). For solutions at pH 7.0, 39 ml of A and 61 ml of B were mixed;

for pH 8.0, 5.3 ml of A were added to 94.7 ml of B; for pH 7.2, 28 ml of A and 72 ml of B were used. In the last buffer the solution was diluted ten times to obtain a working solution at 0.02M.

Seed Germination

Seeds of V. cracca were surface-sterilized by one of several methods:

1) a brief dip in ethanol then a 10-20 minute immersion in either acidic mercuric chloride, $HgCl_2$, or in 5.3-6.0% (commercial strength) sodium hypochlorite, NaOCl.
2) a brief dip in ethanol then either a 10, 20, or 30 minute exposure to concentrated hydrochloric acid, HCl, or a 20 or 30 minute exposure to concentrated sulfuric acid, H_2SO_4.

Seeds were washed extensively in distilled water and germinated in Petri dishes containing water-moistened filter paper. The Petri dishes were held in darkness at room temperature for one week. Throughout the procedure, sterile materials were used and aseptic conditions were maintained.

The 'percentage germination' values were calculated as follows:

$$\frac{\text{Number of seeds that produced a radicle}}{\text{Total number of seeds}} \quad (x100)$$

Sources of Bacteria and Method of Collection

A strain of Rhizobium was isolated from nodules of uncultivated V. cracca by Vincent's (1970) method. The isolate is referred to as 'Rhizobium sp. VC 2' throughout the remainder of the thesis. A second Rhizobium isolate was obtained from nodules produced in vitro by inoculation of sterile Vicia cracca seedlings with a pure culture of Rhizobium sp. VC 2. The second isolate is referred to as 'Rhizobium sp. VC 2 re-isolate'.

To surface-sterilize nodules for isolation of bacteria, the nodules were dipped briefly in 95% ethanol, immersed in acidic mercuric chloride ($HgCl_2$) and washed extensively in sterile distilled water. The nodules were crushed in a sterile tissue homogenizer with 1 ml of sterile distilled water and the slurry was diluted 100 times with sterile distilled water. The liquid was plated on YEM medium (Vincent, 1970), grown at 29 C for 4-5 days and single colonies were isolated.

Dr. J. Burton of the Nitragin Company of Madison,

Wisconsin kindly provided the following material: R. leguminosarum 128C56 and 97H3, R. trifolii 10328; R. phaseoli 137K14; R. japonicum 61A76 and 61A89; R. lupini and R. sp. 127E15. Dr. Lucien Bordeleau of Agriculture Canada (Ste-Foy, Quebec) supplied R. japonicum Jc-7. The bacteria Streptococcus faecalis, Staphylococcus aureus UWO-231 and Escherichia coli E11775 are from the Bacterial Collection of the Biology Department, M.U.N., and were obtained from the American Type Culture Collection in Rockville, Maryland.

Single colonies of each strain were used to inoculate YEM broth and these were lyophilized in either 7.5% glucose (in 50% horse serum) or in YEM broth. The lyophilized cultures were stored at 4 C and new cultures were periodically started to ensure that cultures from successive experiments were not appreciably different from one another.

Electron Microscopy

A log-phase culture of R. sp VC 2 was centrifuged and re-suspended in sterile distilled water then fixed in an equal volume of Karnovsky's (1965) fixative. Karnoysky's fixative was made by adding 2 g paraformaldehyde to 25 ml of water and heating the solution to 60-70 C. Three drops of 1N NaOH were added and the mixture was allowed to cool to room temperature. Five ml of 50% glutaraldehyde was added

and the total volume made up to 50 ml with the addition of Sorenson's buffer at pH 7.2.

The bacterial suspension was dried on copper grids (200 mesh) that were pre-coated with Formvar and carbon. Observations were made using a Zeiss EM-9 transmission electron microscope.

Catalase, Litmus Milk and Gram Reactions

One loopful of YEM-plated culture was transferred onto a cleaned glass slide and reacted with 1-2 drops of 3% hydrogen peroxide (Blair et al., 1970). Positive results were recorded when gas bubbles formed and negative results were recorded when gas bubbles did not form. The positive controls were Staphylococcus aureus and Escherichia coli while the negative control was Streptococcus faecalis.

A litmus milk broth (BBL Litmus Milk; Becton, Dickensen and Co., Cockeysville, Maryland) was prepared, inoculated and then observed after 14 days. Cultures with a distinctly clear fluid were said to have formed a 'serum zone'. The positive control was Rhizobium leguminosarum and the negative control was R. japonicum.

The Gram stain was done according to Blair et al. (1970). Young (1-2 day old), YEM-grown cultures were air-dried and heat-fixed on clean glass slides. These were stained with Hucker's Crystal Violet for 1 minute, rinsed

with tap water and stained with iodine for 1 minute. The smears were then rinsed with tap water, flooded with 95% ethanol, stained with Safranin-O for 0.5 minutes and rinsed with tap water. Observations were made on a light microscope. The positive control was Streptococcus faecalis and the negative control was Escherichia coli.

Measurement of Bacterial Growth

Bacterial growth rates can be measured by calculation of the generation time (G) which indicates the time required for a doubling of the bacterial number over the logarithmic or exponential phase of growth. The optical density of bacterial cultures is directly proportional to the cell number at low cell densities or when the appropriate corrections are made (Lawrence and Maier, 1977). Generation times were calculated as follows:

$$G = \frac{T \ (\log 2)}{(\log OD\text{-}2) - (\log OD\text{-}1)}$$

where :

G = the generation time (hours)
T = time 2 - time 1
log 2 = log (base 10) of 2 = 0.301
OD-2 = optical density of the bacterial culture at time 2
OD-1 = optical density of the bacterial culture at time 1

(Gow, J.A., 1973, Ph.D. Thesis; McGill University; Montreal)

Bacterial Growth Media

Most of the media described below were autoclaved but heat-labile compounds were filter-sterilized with 'Nalgene' filtering devices (pore size = 0.45 micrometers). Batches of media that contained filter-sterilized compounds were left uninoculated, overnight or longer, to detect microbial contamination. The bacterial growth media used in this thesis included:

a) Yeast Extract-Mannitol (YEM) Medium (Vincent, 1970):

K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
mannitol	10.0 g
yeast extract	0.4 g
H_2O	1000. ml

The pH was adjusted to 7.0. When a solid medium was required 15 g of agar (Difco) was added. In studies of carbohydrate use, glucose and xylose were substituted for mannitol.

b) Defined Medium (Manhart and Wong, 1979).

K_2HPO_4	0.76 g
KH_2PO_4	1.00 g
KNO_3	0.80 g
$MgSO_4$	0.18 g
$CaSO_4.2H_2O$	0.13 g
mannitol	3.00 g
arabinose	3.00 g
H_3BO_3	1.45 mg
$CuSO_4.7H_2O$	0.05 mg
$MnCl_2.4H_2O$	0.04 mg
$ZnSO_4.7H_2O$	1.08 mg
$Na_2MoO_4.2H_2O$	2.50 mg
$CoCl_2.6H_2O$	0.10 mg
$FeCl_3.6H_2O$	4.00 mg
$Na_2EDTA.2H_2O$	5.50 mg
riboflavin	0.10 mg
p-aminobenzoic acid	0.10 mg
nicotinic acid	0.10 mg
biotin	0.12 mg
thiamine HCl	0.40 mg
pyridoxine HCl	0.10 mg
Ca pantothenate	0.50 mg
inositol	0.50 mg
cyanocobalum	0.10 mg
H_2O	1000. ml

The pH of the medium was adjusted to 7.0.

c) LNB5 Medium for Nitrogenase Induction (Kurz and LaRue, 1975):

KNO_3	1.00 g
$(NH_4)_2SO_4$	0.50 g
$MgSO_4 \cdot 7H_2O$	0.25 g
$NaH_2PO_4 \cdot H_2O$	0.15 g
$CaCl_2 \cdot 2H_2O$	0.15 g
sucrose	20.00 g
ferric EDTA	28.00 mg
myo-inositol	100.00 mg
$MnSO_4$	10.00 mg
thiamine HCl	10.00 mg
H_3BO_3	3.00 mg
$ZnSO_4 \cdot 7H_2O$	2.00 mg
nicotinic acid	1.00 mg
pyridoxine HCl	1.00 mg
$Na_2MoO_4 \cdot 2H_2O$	0.25 mg
KI	0.78 mg
$CoCl_2 \cdot 6H_2O$	0.025 mg
$CuSO_4$	0.025 mg
H_2O	1000. ml

The pH of the medium was adjusted to 5.5 and 12 g of agar was added. Ferric EDTA was substituted for "Fe (as Sequestrene 330)" (Kurz and LaRue, 1975, p.408) since the latter was not available. 'Sequestrene' is the tetrasodium salt of EDTA (The Merck Index 9th Ed., The Merck Co., Rahway, New York).

Carbohydrate Use

Carbohydrate use in R. VC 2 and R. leguminosarum 128C56 was studied by a slight modification of Vincent's (1970) method. Test tubes containing 5.0 ml of yeast extract-mineral salts solution (Vincent, 1970) with various carbohydrates substituted at a concentration of 1% (w/v), were inoculated with 0.10 ml suspensions (0.040 O.D.U.) of the inocula. The inocula had been streaked on YEM plates, grown for 7 days at 30 C then suspended in distilled water. The carbohydrates included: arabinose, dulcitol, fructose, galactose, glucose, maltose, mannitol, raffinose, rhamnose, sucrose, trehalose and xylose. Uninoculated media were also used to demonstrate the sterility of media. After 10 days at 24 C the presence or absence of turbidity was noted. Citrate use was studied in the same manner but in an independent experiment. Citrate use was also studied using test tube slants of Simmon's Citrate Agar (Difco Co.). Cultures were observed after 10 days growth at 29 C.

The effect of mannitol, glucose or xylose on total growth of R. sp. VC 2 was also studied using yeast extract-mineral salts solution (Vincent, 1970) with either mannitol, glucose, or xylose present at a concentration of 1% (w/v). Side-arm flasks (125 ml capacity) containing 30 ml of medium were inoculated with 1.0 ml of mid log-phase, YEM-grown broth cultures and held at 30 C with

shaking (125 rpm). Turbidity was measured after incubation for 53 h and corrected for inherent optical error by the method of Lawrence and Maier (1977).

Nitrogen Use and Effect of Nitrogen Source on Total Growth

The effect of various nitrogen sources on the total growth of R. sp. VC 2 was studied using the defined medium of Manhart and Wong (1979). The nitrogen sources were: potassium nitrate (KNO_3), aspartic acid, glutamic acid, homoserine, and threonine. All nitrogen sources were supplied at a concentration of 8.0 mM which is approximately equal to the molar concentration (8.0 vs. 8.1 mM) of the regular nitrogen source, potassium nitrate, in the Manhart and Wong (1979) medium. All amino acids used were in the L-isomer form.

Temperature and pH Studies

The effect of incubation temperature on growth of R. sp. VC 2 was studied turbimetrically. Side-arm flasks (125 ml capacity) containing 30 ml of YEM were inoculated with 1 ml of log-phase culture (= .200 mg dry wt./ml). The inoculum was grown for 3 days at 25 C. Cultures were shaken

at 125 rpm in darkness at either 20 C or 30 C. Temperature fluctuations were always less than 1 degree C. Turbidities were measured using a Klett-Somerson instrument (The Klett Manufacturing Co., New York) using nine replicates at each temperature.

In studies on the effect of media pH on total bacterial growth, YEM broth was adjusted to pH 3, 4, 5, 6, 7, or 8 and dispensed in 30 ml aliquots to 125 ml flasks. The media were inoculated with 1.0 ml of log-phase culture of R. sp. VC 2 and held at 24 C, with shaking (125 rpm). Turbidity was read after 68 h as described above.

The interactive effects of temperature and pH on total growth of R. sp. VC 2 were also studied. The pH of YEM was adjusted to 4.5, 5.5 or 6.5 and cultures were maintained at either 24 C or 30 C. Three replicates of each combination of temperature and pH were used and turbidities were measured after 96 h as described above.

Assay for Lectin-Binding by Rhizobia

6-10 day old cultures, grown on Vincent's (1970) YEM medium, were air-dried and then heat-fixed. The smears reacted for 20 min. with FITC-labelled lectin that was pre-diluted (1:4) in phosphate-buffered saline (PBS). The smears were washed 15 min in PBS and allowed to dry.

The smears were mounted in a glycerol-based medium that

reduces fluorescence fading (Johnson and C. de Nogueira-Araujo, 1981) then observed on a Zeiss Photomicroscope equipped with epifluorescent and bright-field illumination. The value '% Lectin-Bound Cells' (Table 9) was calculated as follows:

$$\frac{\text{No. of fluorescing cells}}{\text{Total number of cells}} \quad (\times 100)$$

Five fields of view were observed per strain of Rhizobium.

The microscope mounting medium of Johnson and C. de Nogueira-Araujo (1981) consisted of 10 ml of phosphate-buffered saline (0.15M NaCl in 0.01 M Sorenson's phosphate buffer at pH 7.4) containing 100 mg p-phenylenediamine added to 90 ml of glycerol. The pH was adjusted to 8.0 with 0.5 M carbonate-bicarbonate buffer at pH 9.0. The carbonate-bicarbonate buffer consisted of 2.5 ml of solution A (6.30 g of anhydrous sodium carbonate in 100 ml H_2O) and 47.5 ml of solution B (42.4 g sodium bicarbonate in 100 ml H_2O).

Nitrogenase Activity in Free-Living Rhizobium Cultures

The protocol used to study the effect of carbohydrate source on nitrogenase activity essentially duplicated that described by Kurz and LaRue (1975), using LNB5 medium. LNB5 basal medium, excluding carbohydrates and heat-labile

vitamins, was prepared then membrane-filtered solutions of vitamins and the appropriate sugars were added. The carbohydrates included sucrose, arabinose, arabinose plus sucrose, galactose, galactose plus sucrose, mannitol, mannitol plus sucrose, xylose and xylose plus sucrose. Each carbohydrate source was present at a final concentration of 1% (w/v). When two carbohydrates were used, the concentration of each was 5 g/litre; when one was used, the concentration was 10 g/litre. For each medium, 3 ml volumes were dispensed into 5 Bijou bottles (6.8 ml capacity) then the bottles were capped and left for 7 days to detect contamination.

The media were inoculated with 0.10 ml of a suspension of R. sp. VC 2 in sterile distilled water. The inoculum had a turbidity of 0.08 optical density units and was a suspension of 5 day-old colonies grown on YEM agar plates. After 10 days growth at 30 C, cultures were capped with sterile rubber caps and the atmosphere adjusted to 10% (v/v) acetylene. After incubation at 30 C for approximately 4 h, 1.0 ml gas samples were withdrawn and chromatographed on the system described above. Controls did not have acetylene in the incubation atmosphere.

Nodulation Studies

The ability of Rhizobium cultures to nodulate V.

cracca was determined by Vincent's (1970) method in which sterile seedlings, prepared as described above (Seed Germination), were inoculated with R. sp. VC 2 and R. leguminosarum strains 97H3 and 128C56. Cultures were shaken (120 rpm) in YEM broth at room temperature and the turbidity was adjusted to 0.128 OD units (= 0.20 mg dry weight per ml) (Lawrence and Maier, 1977).

Inoculated seedlings were transferred to test tube slants of Jensen's Seedling Agar (Vincent, 1970) and grown at a daytime temperature of 20 C and a nighttime temperature of 18 C. Plants were maintained on a 16 h photoperiod. After 30 days the number of nodules on each plant was counted:

Jensen's Seedling Agar (Vincent, 1970) contained:

$CaHPO_4$	1.0 g
K_2HPO_4	0.2 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.2 g
$FeCl_3$	0.1 g
H_2O	1000. ml

One ml of Trace Element Solution was added to 1 liter of the first solution. Trace Element Solution contained:

H_3BO_3	.05 mg
$MnCl_2.4H_2O$	.05 mg
$ZnSO_4.7H_2O$	.005 mg
Na_2MoO_4	.005 mg
$CuSO_4.7H_2O$	.002 mg
H_2O	1000. ml

The pH was adjusted to 6.5 and 10 g agar (Difco) was added.

Statistical Considerations

For consistency and to avoid redundant explanations, the word 'significant' has only been used to describe results where the probability of obtaining a Type I, or alpha, error is equal to or less than 5%. The 5% alpha level was chosen because it is the most commonly-used alpha level in statistical methods, although other alpha values are sometimes used (Zar, 1974).

Analysis of variance (ANOVA) procedures, outlined in Sokal and Rohlf (1969) and Zar (1974), were used to analyze the effects of carbon source, incubation temperature, pH and interactions of temperature and pH on bacterial growth. ANOVA was also used to determine if there were significant differences in the nitrogenase activity of free-living bacterial cultures. To analyze the effect of inoculum on nodule formation, raw data were transformed by the square root transformation:

$X' =$ the square root of $(X + 0.5)$

This transformation was used to normalize the data and permit analysis by ANOVA procedures. All data analyzed by ANOVA showed equality of variances, as determined by Bartlett's test.

The effect of nitrogen source on growth of *R.* sp. VC 2 was analysed non-parametrically using the Kruskal-Wallis procedure outlined in Zar (1974). This

analysis was used because the data showed significant inequality of variances when Bartlett's test for homogeneity of variances was applied so there was some question as to whether the parametric analysis (ANOVA) could be validly used. ANOVA procedures require that the variances be equal or approximately so.

In most experiments where significant factor effects were found the Student-Newman-Keuls multiple range test (Zar, 1974) was used to locate significantly different means. For the experiment in which growth on 5 nitrogen sources was compared, a non-parametric multiple comparison test (Zar, 1974, p. 156) was used to locate the significantly different means.

Calculations for ANOVA's were done on Minitab, a pre-programmed library of statistical procedures from the Statistics Department of Pennsylvania State University.

RESULTS

1. The Plant
   1.1 Nodule Shape

Vicia cracca L. root nodules are initially hemispherically-shaped but become elongate-cylindrical with age (Figure 1). Older nodules sometimes branch into 2 or more lobes and reach 1.5 cm in length (Figure 1). Twenty-six days after inoculation, cultivated root nodules were elongate and cylindrically-shaped but unbranched (Figure 2).

The lobes of older, branched nodules do not lie in a single plane, creating a "fan-shaped, palmate" nodule as reported by Allen and Allen (1981) for *Acacia* and *Crotalaria* nodules. Instead, older *Vicia cracca* nodules have lobes that lie in more than one plane. The lobes are of equal or approximately equal size.

Nodules collected from the field tend to occur individually or, less frequently, in pairs. Extensive nodulation of *Vicia cracca*, such as that observed in many commercial legumes, rarely occurs in roots collected from the field.

Figure 1 - Nodulated Root System of Vicia cracca Collected from the Field.
Lobed (|) and unlobed (U) nodules are present.
Major scale divisions of ruler are centimeters.

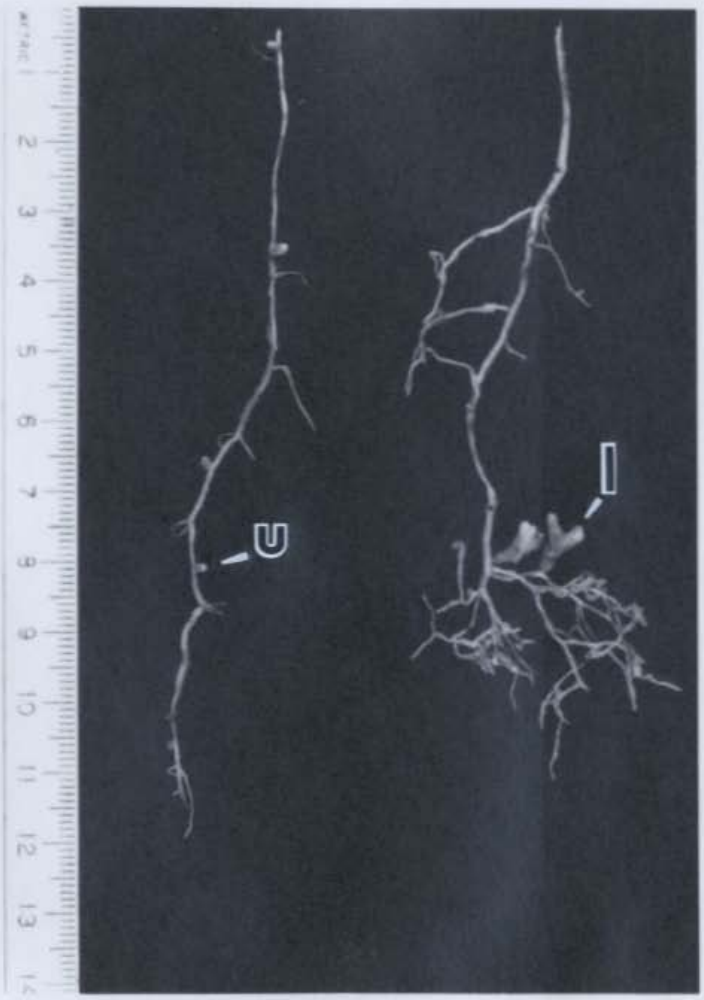
S
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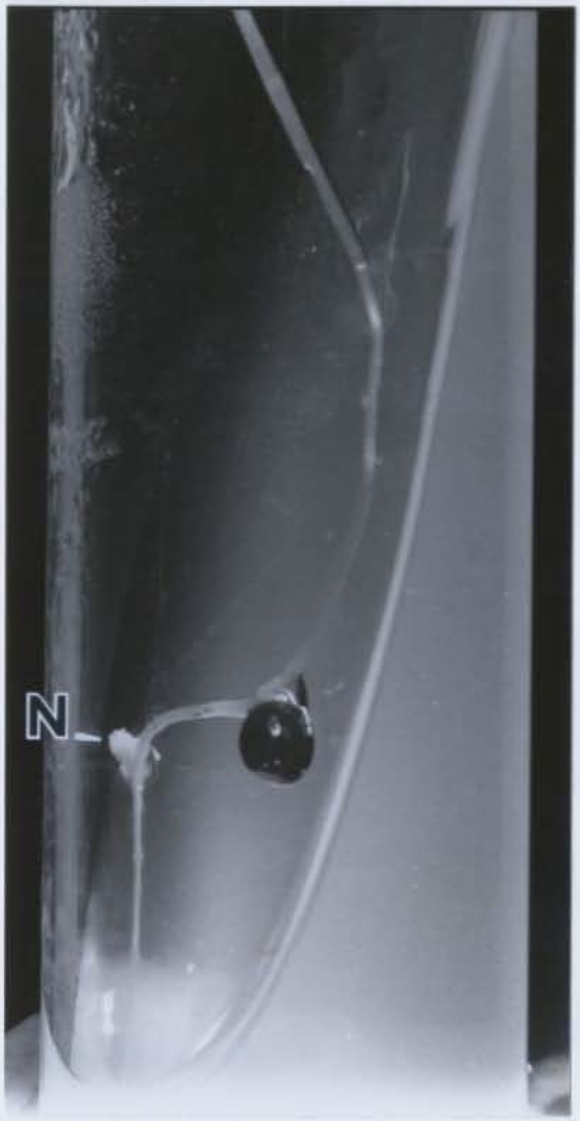
Figure 2 - Nodule of Vicia cracca x Rhizobium Species VC 2 Produced in vitro. Nodule indicated by (**N**). Magnification = 3.25.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1.2 Meristem Localization

Nodules incorporated radiolabelled thymidine at the nodule apex (Figures 3 and 4) in the nuclei of small cells within 5-10 cell layers of the nodule surface. These data indicate that the tissue is a zone of DNA synthesis and meristematic activity.

Some thymidine incorporation was also observed in the nuclei of enlarged, infected cells of the bacteroid zone (Figure 4).

1.3 Nitrogenase Activity

Nitrogenase activity (acetylene reduction) was demonstrated in field-collected nodules and in nodules produced under _in vitro_ conditions (Table 3).

1.4 Lectin Isolation

Seeds of _V. cracca_ possessed a lectin fraction that bound to an affinity chromatography column with an N-acetyl-galactosamine ligand. The lectin fraction could be eluted by decreasing the pH along a continuous gradient.

Two ultraviolet (280 nm) absorption peaks occurred in the elution profile (Figure 5a); one peak eluted at pH 6.8

and the other at approximately pH 4.7. The first peak was re-chromatographed and eluted at pH 6.8 again. (Figure 5B).

1.5 Germination

A 30 minute immersion in concentrated sulfuric acid provided 94% germination, the highest germination rate of the various regimes that were tested (Table 4). Seeds treated in this way were simultaneously scarified and surface-sterilized.

2. The Bacterium
2.1 Morphology

Using electron microscopy, R. sp. VC 2 was observed to possess 2 flagella (Figure 6).

2.2 Physiological/ Biochemical Features
2.2.1 Aspects of Growth
2.2.1.1 Growth Rate

A generation time of 6.8 h was calculated for R. sp. VC 2 grown at 24 C with 125 rpm shaking (Table 5). Under these conditions turbidity was visible 24-48 h after inoculation. The growth rate of R. sp. VC 2 is faster than the growth rates of Rhizobium leguminosarum, Rhizobium japonicum and Rhizobium sp. 127E15.

Figure 3 - Localization of the Vicia cracca Nodule Meristem. An autoradiograph of a longitudinal section of a nodule treated with ^{3}H-thymidine for 1 hour. Note the dense groups of silver grains (arrow) over the nuclei of the apical meristem. Magnification = 60x.

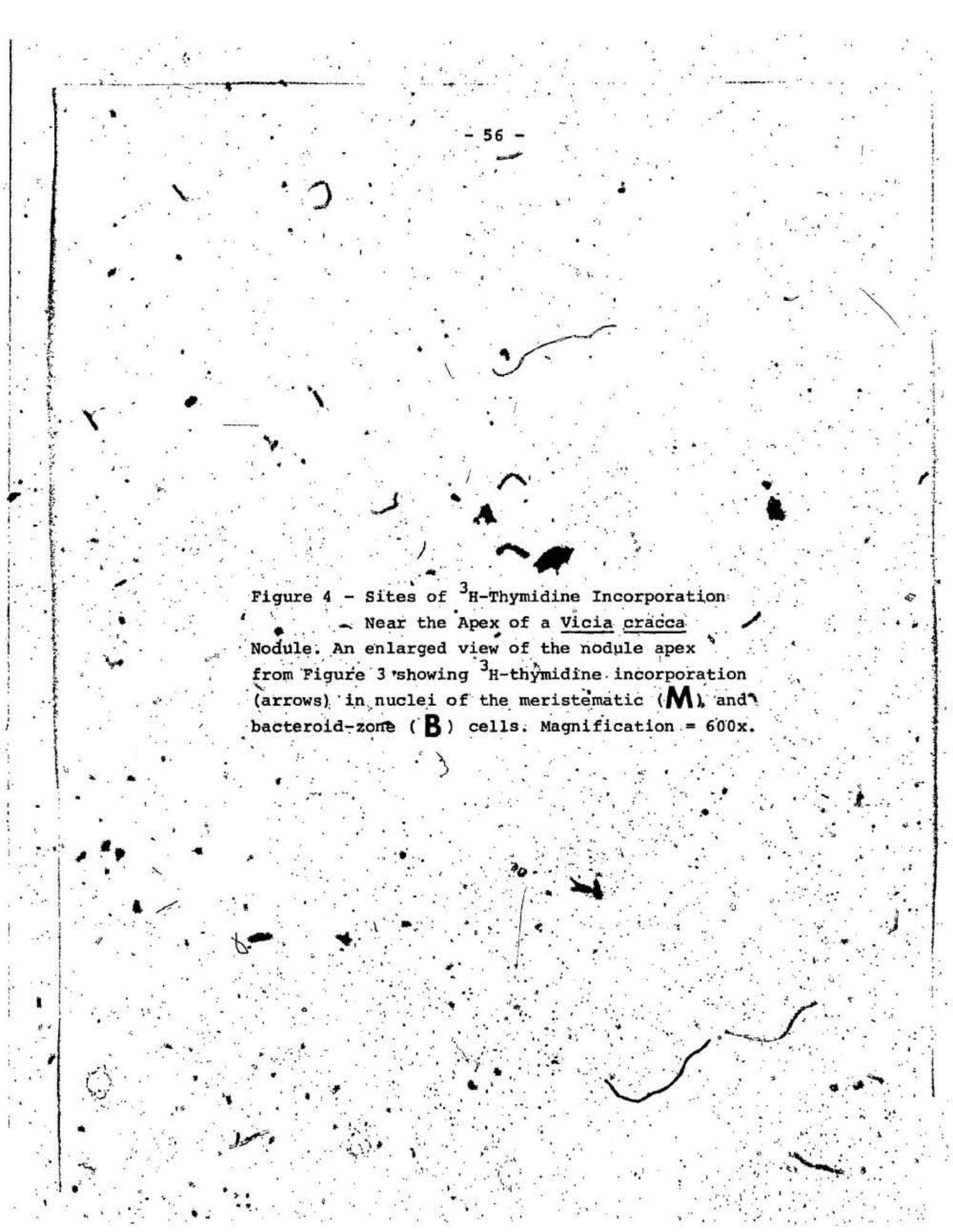


Figure 4 - Sites of ^{3}H-Thymidine Incorporation Near the Apex of a Vicia cracca Nodule. An enlarged view of the nodule apex from Figure 3 showing ^{3}H-thymidine incorporation (arrows) in nuclei of the meristematic (M) and bacteroid-zone (B) cells. Magnification = 600x.

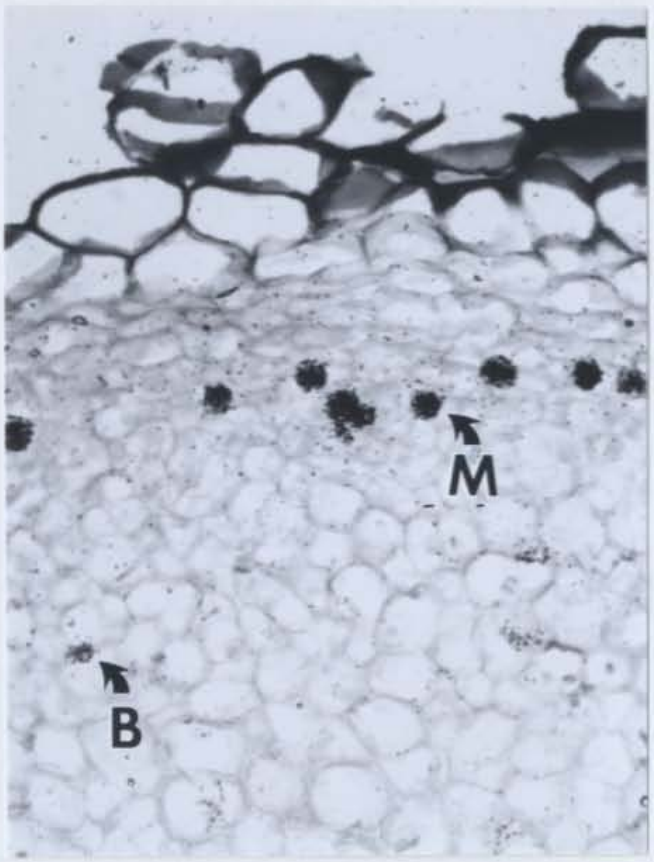
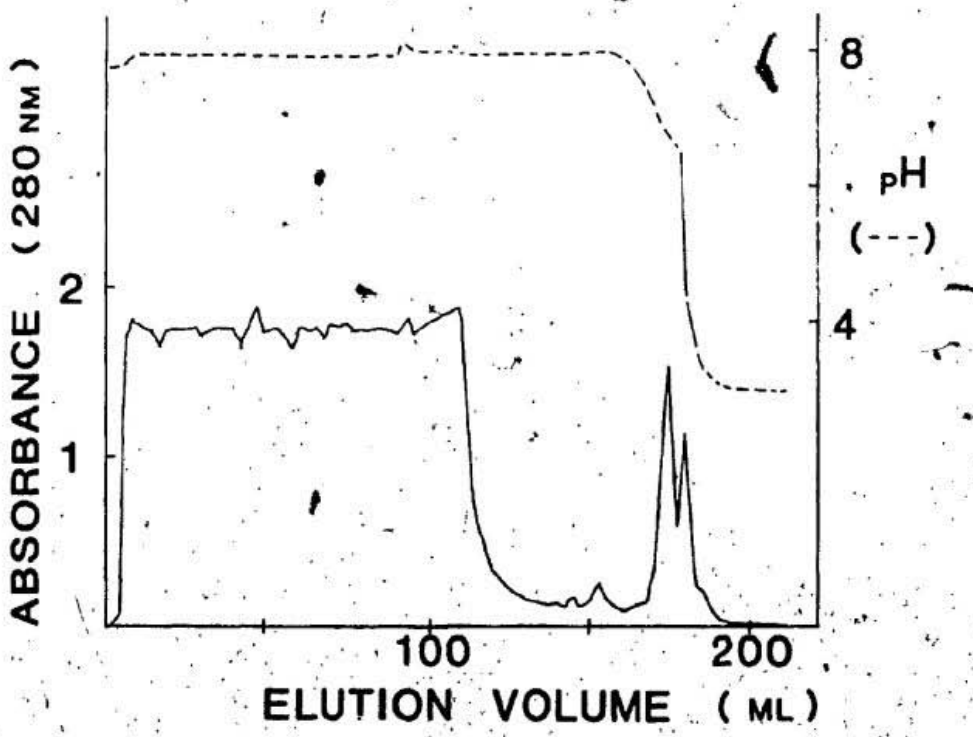

M
B

Table 3 - Nitrogenase Activity of Vicia cracca Nodules

Growth Condition	Nitrogenase Activity* (mean ± s.d.) per mg Nodule Fresh Weight	per Plant
Field-Collected Nodules	6.7 ± 4.5	———
Cultivated Nodules	12.0 ± 7.1	36.5 ± 23.3

(*) measured as nmoles ethylene produced per hour

Figure 5 - Elution Profiles for the Isolation of a Vicia cracca Seed Lectin by Affinity Chromatography. The top panel, Figure 5a, indicates the initial isolation and purification. Two 280nm-absorbing peaks (solid line) eluted along a continuously decreasing pH gradient (hatched line) at pH 6.8 and 4.7. The bottom panel, Figure 5b, indicates the secondary purification. The fraction that eluted at pH 6.8 in Figure 5a was re-applied to the column at pH 8.0 and found to elute again at pH 6.8 when the pH was lowered. The ligand used was N-acetyl-galactosamine.


ABSORBANCE (280 NM)
2
1
8
pH
(---)
4
100
200
ELUTION VOLUME (ML)

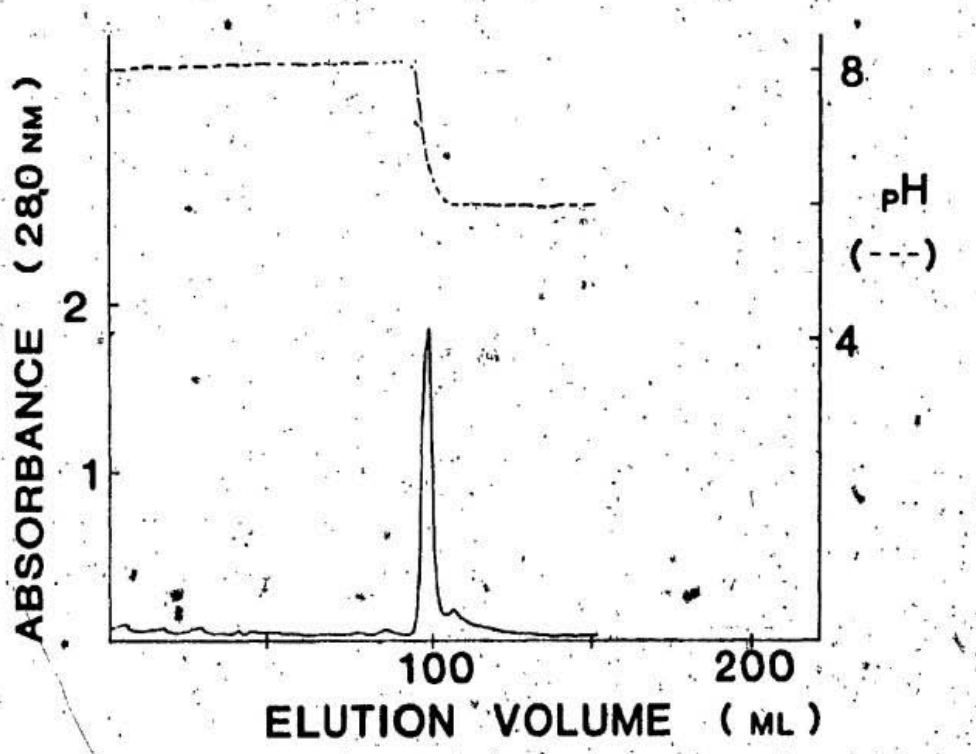

ABSORBANCE (280 NM)
2
1
8
pH
(---)
4
100
200
ELUTION VOLUME (ML)

Table 4 - Germination Rates for Various Treatments of *Vicia cracca* Seeds

Treatment	Percentage Germination (mean ± s.d.)
HCl -10 min	1.9 2.6
HCl -20 min	2.7 0.6
$NaClO_2$-20 min	2.7 3.9
H_2SO_4-10 min	48.0
H_2SO_4-20 min	58.3
H_2SO_4-20 min (soaked 2h in H_2O)	74.0
H_2SO_4-30 min	94.3

$31 < n < 59$ seeds for each replicate

Figure 6 - Whole Mount of Rhizobium species VC 2.
Arrows indicate the flagella.
Magnification = 22,100.

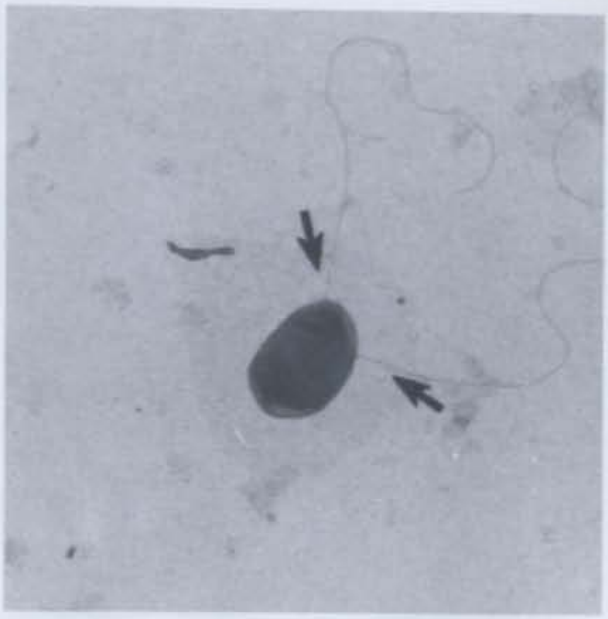


Table 5 - Generation Times of Some Rhizobia Grown on YEM Medium at 24 C

Rhizobium	Generation Time (h) mean ± s.d.
R. sp. VC 2	6.8 1.0
R. leguminosarum 128C56	9.4 0.7
R. japonicum 61A76	17.3 2.3
R. sp. 127E15	28.9 1.3

2.2.1.2 Carbohydrate Use

R. sp. VC 2 and R. leguminosarum used all of the carbohydrates that were tested (Table 6). This group includes arabinose, dulcitol, fructose, galactose, glucose, maltose, mannitol, raffinose, rhamnose, sucrose, and xylose.

2.2.1.3 Effect of Carbohydrate Source on Growth

The total growth of R. sp. VC 2 was significantly affected ($.005 < P < .01$) by carbohydrate source (Table 7). There were no significant differences in total growth of R. sp. VC 2 between xylose and mannitol treatments although there were significant differences between glucose and xylose treatments and glucose and mannitol treatments ($P < .05$).

2.2.1.4 Nitrogen Use

R. sp VC 2 was able to use all five compounds as sources of fixed N but significant differences ($.01 < P < .025$) in total growth were observed (Table 8). Glutamic acid produced the greatest total growth while threonine provided the least.

The pH of the media containing potassium nitrate and

threonine fell to 5.43 and 6.02, respectively, although media containing aspartic acid and glutamic acid hardly fell at all (6.93 and 6.98, respectively, versus 7.0). The pH of the homoserine-containing medium rose from 7.00 to 7.13. There was no apparent correlation between total bacterial growth and final pH of the nitrogen-containing media so pH effects were probably not marked.

2.2.1.5' Effects of Temperature and pH

R. sp. VC 2 grew faster at 30 C than at 20 C (generation times = 5.1 ± .3 and 8.1 ± 1.3 hours, respectively) but final bacterial density was greater at the lower temperature (Figure 7). Approximately 63 h after inoculation, the final cell densities were nearly equal but by 72 h post-inoculation the total growth of 20 C cultures was significantly greater ($P < .0005$) than that of 30 C cultures (Figure 7). This trend could still be observed 51 hours later, at 123 h post-inoculation, when sampling ceased.

The optimal pH of the isolate for total growth on YEM medium appeared to be near pH 8 (Figure 8). Studies of pH and temperature interaction indicated that total growth of R. sp. VC 2 on YEM media was greater at 24 C than at 30 C over the pH range of 4.5, 5.5 and 6.5 inclusive (Figure 9). There were significant differences in the total growth of R.

sp. VC 2 due to incubation temperature ($P < .0005$) and initial media pH ($P < .0005$). There was no significant interaction of temperature and pH on bacterial growth ($.10 < P < .25$).

2.2.2 Lectin Binding Property

Cells of R. sp. VC 2 more frequently bound a lectin (from seeds of the host plant) than did R. leguminosarum, R. trifolii or R. lupini (Table 9). Cells of R. japonicum and R. phaseoli did not bind the lectin and N-acetyl-galactosamine inhibited binding of the lectin to R. sp. VC 2.

2.2.3 Catalase, Litmus Milk and Gram Reactions

Rhizobium sp. VC 2 was catalase positive and, on litmus milk medium, formed a serum zone (Table 10). Young cultures of the isolate were Gram negative (Table 10). All 'known' strains of rhizobia gave the expected results (Jordan and Allen, 1974) with regard to the catalase, litmus milk and Gram reactions. An isolate from V. cracca nodules infected with R. sp. VC 2, designated 'R. sp. VC 2 re-isolate', gave results that were identical to those of the 'original' isolate.

Table 6 - Carbohydrate Use by *Rhizobium* sp. VC 2 and *R. leguminosarum*

Carbohydrate	*R.* sp. VC 2	*R. leguminosarum* 128C56	Percentage of 14 Strains of *R. leguminosarum* *
arabinose	+	+	100
dulcitol	+	+	86
fructose	+	+	100
galactose	+	+	100
glucose	+	+	100
maltose	+	+	100
mannitol	+	+	86
raffinose	+	+	93
rhamnose	+	+	NT
sucrose	+	+	100
trehalose	+	+	100
xylose	+	+	100

\+ = observable turbidity
NT = not tested
(*) = from Graham, 1964

Table 7 - Effect of Carbohydrate Source on Total Growth of Rhizobium sp. VC 2 at 30 C

Carbohydrate	Turbidity* (mean ± s.d.)	Significantly Different From
Glucose	185.0 30.7	Xylose, Mannitol
Xylose	253.7 12.4	Glucose
Mannitol	294.2 24.7	Glucose

(*) measured in Klett Units

Table 8 - Effect of Nitrogen Source on Total Growth of Rhizobium sp. VC 2

Nitrogen Source	Turbidity* (mean ± s.d.)	Final pH
Threonine	35.7 1.2	6.02
Homoserine	73.7 2.1	7.19
Aspartic Acid	76.7 7.8	6.93
Potassium Nitrate	76.7 1.2	5.43
Glutamic Acid	96.3 3.5	6.98

(*) measured as Klett Units

Figure 7 - Growth Curves of Rhizobium sp. VC 2
Incubated at 20 C and 30 C on YEM Medium.
Average total growth indicated by (●) and
standard deviation by the vertical bars.

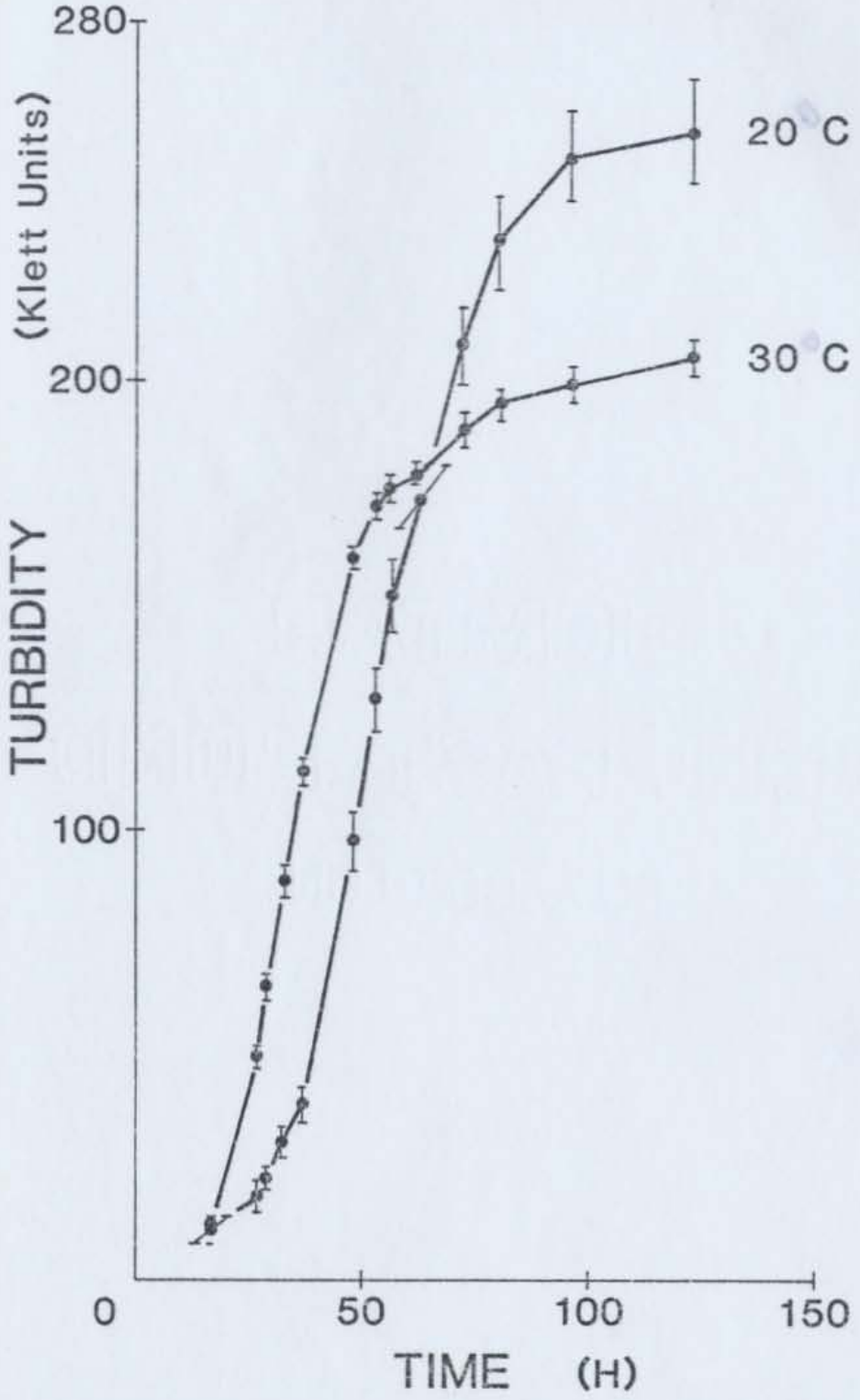
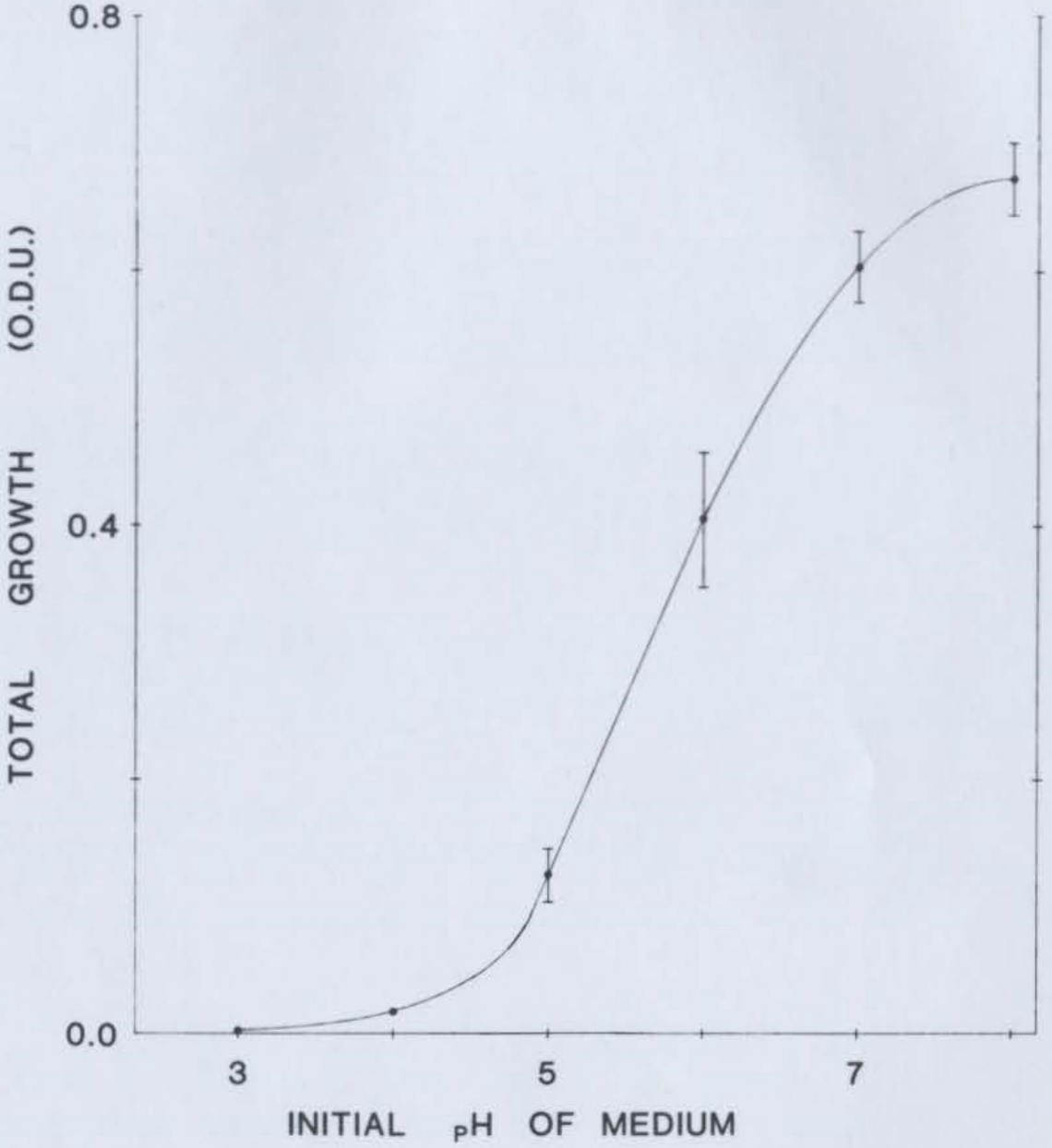

280
200
100
0
50
100
150
TURBIDITY
(Klett Units)
TIME (H)
20 °C
30 °C

Figure 8 - Growth of Rhizobium sp. VC 2 at Various Initial pH Values. Average total growth indicated by (●) and standard deviation by the vertical bars.


0.8
0.4
0.0
TOTAL GROWTH (O.D.U.)
3
5
7
INITIAL pH OF MEDIUM

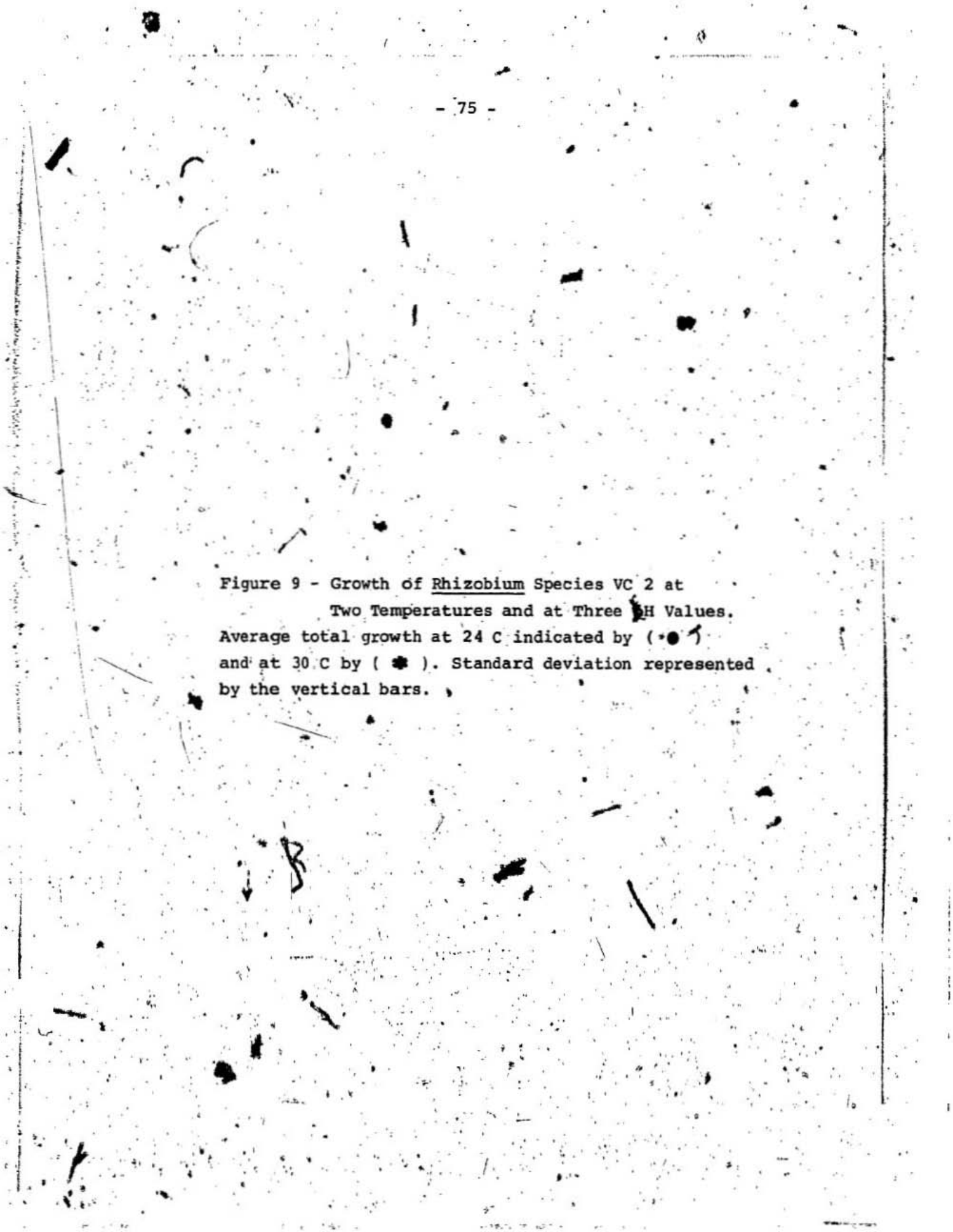


Figure 9 - Growth of Rhizobium Species VC 2 at Two Temperatures and at Three pH Values. Average total growth at 24 C indicated by (●) and at 30 C by (✱). Standard deviation represented by the vertical bars.

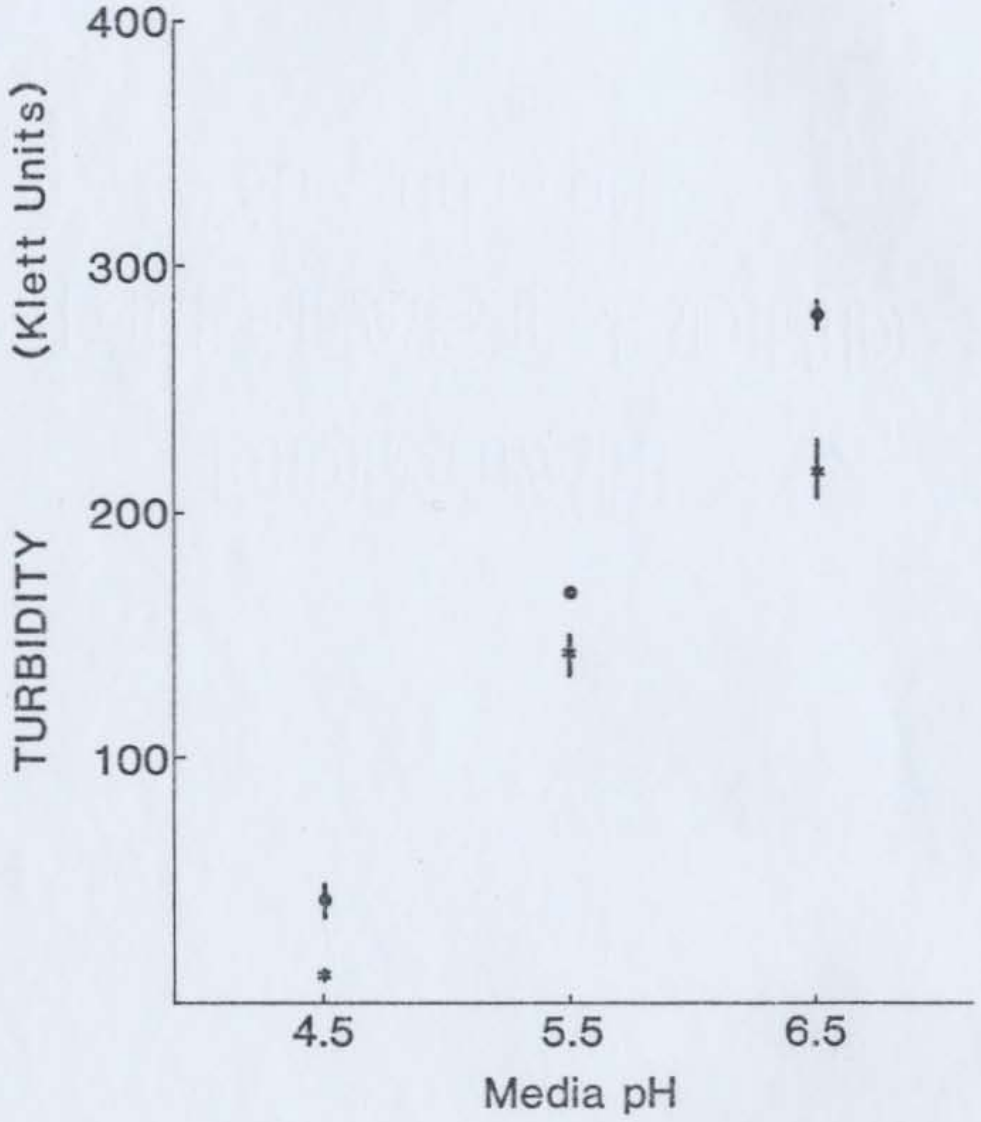

TURBIDITY (Klett Units)
400
300
200
100
4.5
5.5
6.5
Media pH

Table 9 - Lectin Binding Properties of *Rhizobium* sp VC 2

Rhizobium	Percentage of Lectin-Bound Cells (Mean ± s.d.)	
R. sp. VC 2	24.1	11.7
R. sp. VC 2 plus hapten (hapten = Gal-N-AC)	0.0	0.0
R. leguminosarum		
128C56	15.1	12.4
97H3	10.6	9.7
R. trifolii	9.3	7.9
R. lupini	4.3	7.4
R. phaseoli	0.0	0.0
R. japonicum Jc-7	0.0	0.0

Table 10 - Results of Catalase and Litmus Milk Tests and the Gram Reactions

Bacterium	Catalase Activity	Litmus Milk Serum Zone	Gram Reaction
Rhizobium sp. VC 2			
original	+	+	-
re-isolate	+	+	-
R. leguminosarum			
97H3	+	+	-
128C56	+	+	-
R. trifolii 10328	+	+	-
R. phaseoli 137K14	+	+	-
R. japonicum			
61A76	+	-	-
61A89	+	-	-
Jc-7	+	-	-
R. lupini 10318	+	-	-
Positive Control	+	+	+
Negative Control	-	-	-

+ = positive result

- = negative result

2.2.4 Nitrogenase Activity in Free-Living Cultures

R. sp. VC 2 was able to reduce acetylene on LNB5 medium when any of the carbon sources was present (Table 11). When galactose was the carbon source the N'ase rates were maximal and when sucrose-xylose was the carbon source the rates were minimal.

There were significant differences ($.0025 < P < .005$) in the nitrogenase activity supported by the various carbon sources but it was not possible, using the Newman-Keuls test, to determine which carbon sources supported significantly different rates of nitrogenase activity.

2.3 Nodulation Ability

R. sp. VC 2 and two strains of R. leguminosarum nodulated Vicia cracca (Table 12). Ninety percent (90%) of the inoculated plants formed nodules but control plants, which were uninoculated, did not form nodules. There were no significant differences ($.10 < P < .25$), due to the strain of Rhizobium, in the transformed number of nodules formed per plant.

Table 11 - Effect of Carbon Source on Nitrogenase Activity of Free-Living Cultures of *Rhizobium* sp. VC 2

Carbon Source	N'ase Activity * mean ± s.d.
sucrose + xylose	2.03 .19
sucrose + mannitol	2.09 .49
xylose	2.14 .30
mannitol	2.19 .27
sucrose	2.74 .71
sucrose + arabinose	2.79 .56
sucrose + galactose	2.87 .56
arabinose	3.04 .39
galactose	3.20 .33

(*) decimoles ethylene/ culture/ h

Table 12 - Effects of Three Inocula on Nodule Formation in *Vicia cracca*

Inoculum	Percentage of Plants Nodulated	Nodules Formed per Plant (mean ± s.d.)	
Rhizobium sp. VC 2	92	3.4	2.2
R. leguminosarum 97H3	94	3.0	2.4
128C56	81	2.1	1.9
None (control)	0	0.0	0.0
Overall Average (excluding control)	90	2.9	2.2

2.4 Identity of the Isolate

Information relevant to the identification of R. sp. VC 2 is summarized, from the above data, in Table 13.

Table 13 - Comparison of *Rhizobium* sp. VC 2 and *Rhizobium leguminosarum*

Character	*R.* sp VC 2	*R. leguminosarum**
Gram (-)ve rods	+	+
Endospores Formed	-	-
Citrate Used	-	-
Flagella Number	2	2-6
Litmus Milk Serum Zone	+	+
Growth Rate	fast	fast
Nodulates One or More of *Vicia*, *Pisum* or *Lens*	+	+

(*) as cited in *Bergey's Manual of Determinative Bacteriology* (8th Ed.; 1974)

DISCUSSION

1. The Plant

1.1 Nodule Shape and Meristem Localization

The shapes that Vicia cracca nodules assume during organogenesis are similar to those reported by Libbenga and Bogers (1974) and Dart (1977) for other species of Vicia and for Pisum and Medicago nodules. Nodules of Vicia cracca appear to result from the activity of an apically-located meristem which, according to Dart (1977, p. 416), "produces files of cells mainly towards the bacteroid zone".

The extent and location of the Vicia cracca nodule meristem, as indicated by tritiated-thymidine incorporation, is consistent with earlier interpretations of the meristem in stained Vicia nodule sections (Pate, 1977). Thymidine incorporation in the nuclei of cells in the bacteroid zone has been observed in pea and alfalfa nodules and is believed to occur because infected host cells become polyploid (Truchet, 1978).

In summary, tritiated-thymidine autoradiographic procedures indicate that the meristem of V. cracca nodules is an apically-located, cap-shaped band of small cells.

1.2 Nitrogenase Activity

Vicia cracca nodules fix nitrogen at rates comparable to those reported by Stripf and Werner (1978) for Glycine max nodule tissue (7-12 vs. 2-18 nmoles C_2H_4/h/mg nodule fresh wt). The rates of N fixation for entire plants are, however, much different (37 vs 1700 nmoles /h/plant). These differences reflect the fact that Vicia cracca is a smaller plant and that it tends to form fewer nodules than Glycine max does (<10 versus 26 nodules at 26 days post-inoculation). V. cracca also forms smaller nodules than Glycine max does; the mean weight of nodule tissue in 26 day-old Vicia cracca is 3.5 mg whereas comparably-aged Glycine max nodules are approximately 260 mg (Stripf and Werner, 1978).

Van Brussel et al. (1982) report that entire 24 day-old Vicia hirsuta plants fix nitrogen at rates between 10-600 nmoles C_2H_4/h which is similar to the rate reported here for V. cracca nodules (37 nmoles C_2H_4/h/plant).

In conclusion, field-collected and cultivated Vicia cracca nodules fix appreciable amounts of atmospheric nitrogen. The rates of N fixation are commensurate with values reported from soybean nodules (Stripf and Werner, 1978) and entire Vicia hirsuta plants (Van Brussel et al., 1982).

1.3 Isolation of Seed Lectin

The lectin fraction that was isolated from Vicia cracca seed and subsequently labelled with FITC appears identical to the fraction reported by Ruediger (1977). The fraction strongly absorbs ultraviolet (280 nm) light, binds to N-acetyl-galactosamine and eluted as a single major peak at pH 6.8. These data agree with Ruediger's (1977) description of the fraction and indicate that the lectin fraction reported here is very similar to the one described by Ruediger (1977).

The protein peak which was eluted from the affinity column at pH 4.7 may correspond to the peak which Ruediger (1977) reported to have eluted at pH 4.5. The differences in elution pH may reflect differences in the ligand used. In the extraction process this author (A.N.) used N-acetyl-galactosamine as the lectin ligand while Ruediger (1977) used galactose. It is possible that the acylamino moiety of N-acetyl-galactosamine interacted with the lectin and that this accounted for the discrepancy in the elution pH of the second lectin fraction.

1.4 Germination

Seeds of V. cracca are small and possess a hard seed coat which must be partially digested for appreciable rates

of germination to occur. For in vitro nodulation studies of V. cracca, a simple method of surface sterilizing the seeds was also required. Scarification and surface-sterilization were simultaneously achieved by immersion of seeds in sulphuric acid. This method of preparing sterile legume seedlings has been mentioned by Vincent (1970). The method is simple and conveniently permits in vitro studies of V. cracca nodulation by Rhizobium leguminosarum. The V. cracca - Rhizobium leguminosarum. symbiosis can be used as a model system to study the Pisum sativum (pea) - Rhizobium leguminosarum symbiosis because V. cracca seeds are quite small (2.5-3.5 mm long) and they lend themselves to culture in standard size test tubes. The seeds and seedlings of Pisum sativum are much larger and are not easily grown in standard test tubes. Van Brussel et al. (1982) evaluated a number of plants from the pea cross-inoculation group, including V. cracca, for suitability to in vitro culture and nodulation by R. leguminosarum. They reported that plants from the genus Vicia were most suitable because they can be grown most easily in test tubes, owing to their small size. Nodules were first visible in V. hirsuta 4-6 days after infection but plants were medium-sized. In V. cracca nodules were first visible 13 days after infection but plants were small-sized.

Vicia cracca is a rhizomatous plant so it may use vegetative growth to spread and colonize available sites.

Conversely, there is some evidence that V. cracca could spread by means of animals which ingest the seeds and scarify them during passage through the intestinal tract. Seeds require a rigorous acid scarification treatment for appreciable germination to occur and acidic secretions of the stomach are used by many vertebrate animals (Schmidt-Nielson, 1975, p.176-8), including birds (Welty, 1975, p.42), to degrade foods. Also, V. cracca seeds possess an inhibitor of the intestinal digestive protein, trypsin (Sundberg, 1970). There is, however, no evidence to judge whether V. cracca seeds are eaten by any animals so this interaction of plant and animal is purely speculative.

2. The Bacterium

2.1 Aspects of Growth

2.1.1 Growth Rates

A comparison of growth rates indicated that R. sp. VC 2 is a fast-growing strain since its mean generation time was markedly less than that of a known fast-grower, R. leguminosarum, and less than one-half that of a known slow-grower, R. japonicum. Fast-growing rhizobia are reported to "grow much faster (less than one-half the doubling time)" than the slow-growers (Elkan, 1981, p. 7).

2.1.2 Carbohydrate Use

R. sp. VC 2 and RA leguminosarum 128C56 were capable of using a wide range of carbohydrates; this property is characteristic of the fast-growing rhizobia and of R. leguminosarum (Vincent, 1977).

2.1.3 Effect of Carbohydrate Source on Total Growth

It is possible that the by-products of glucose, xylose and mannitol catabolism, such as acetic acid (Vincent, 1977), are different in form or quantity and these compounds are responsible for the differences in growth that were noted. The by-products of glucose and mannitol are not likely to be different in form since glucose and mannitol are quite similar in structure and, in R. meliloti, metabolized by the same pathway (Ronson and Primrose, 1979). Both carbohydrates were present in nearly equimolar concentrations (glucose at 55.5 mM and mannitol at 54.9 mM) so the amounts of inhibitory, or stimulatory, by-products of hexose metabolism are essentially identical. It seems more likely that the greater total growth realized by mannitol-containing cultures over glucose-containing cultures reflects greater energetical efficiency in securing and oxidizing mannitol. If Rhizobium sp. VC 2 uses the same oxidative pathways for glucose and mannitol catabolism

then the differences in energy efficiency probably result from reactions that preceed entry of glucose-1-phosphate into the tricarboxylic acid cycle. An example of an appropriate reaction is transport of mannitol across the cell membrane by mannitol permease versus transport of glucose by glucose permease.

2.1.4 Effect of Nitrogen Source on Total Growth

The five nitrogen sources supported significantly different amounts of bacterial growth although the final pHs of the media were not appreciably different. The differences in growth of R. sp. VC 2 supported by the various nitrogen sources may reflect differences in efficiency of nitrogen assimilation or differences in the amount and type of metabolic by-products that are produced when the different nitrogen sources were used. The experiment indicated that the isolate was able to assimilate all the nitrogen sources that were studied; the nitrogen sources that supported the most total growth should not be interpreted as the 'preferred' nitrogen sources of the isolate.

2.1.5 Effects of Temperature and pH

Soil temperature and pH probably exert great influences on the density and composition of the Rhizobium soil community since there are appreciable differences in the optimal and tolerable limits of these values among rhizobia.

Soil temperature and pH are important to biological nitrogen fixation in legumes since the number of successful infections, i.e. nodules formed per plant, is roughly proportional to the density of bacterial cells (Purchase and Nutman, 1957; Bhuvaneswari et al., 1977). Rhizobium sp. VC 2 produces greater bacterial densities at moderate temperatures (20 C or 24 C versus 30 C) and at neutral or alkaline pH values rather than at acidic ones.

The growth responce of R. sp. VC 2 to higher incubation temperatures, i.e. decreased total growth and increased growth rate, has not previously been reported for any strain of R. leguminosarum or any other Rhizobium species. However, a similar pattern of growth has been reported for a psychrophilic Bacillus isolate (Stokes, 1968). In the latter, total growth increased and generation times decreased with increased incubation temperatures over the range of 0, 5, 10, 15, 20 and 25 C.

Stokes (1968) notes that the reduction in total growth of psychrophiles at higher temperatures is probably due to heat-labile enzyme systems. The decrease in total growth of

R. sp. VC 2 at higher temperatures is probably also due to heat-labile enzyme systems.

Total growth of R. sp. VC 2 in insular Newfoundland soils is probably limited by soil pH since soils are typically acidic, having pH values at or below 5.0 (Page, 1971; Niles, A.M., 1980, B.Sc. thesis, M.U.N.) and since the bacterium shows little growth over this pH range. The acidic conditions might also be an indirect constraint to nodulation of suitable legumes if growth of R. sp. VC 2 is restricted since fewer bacteria would be present to cause nodulation. Lie (1969) reports that R. leguminosarum strain PRE forms appreciably fewer nodules on Pisum sativum cv. 'Rondo' at pH 4.5 than at pH values between 5.0 and 8.0.

2.2 Lectin Binding Property

The V. cracca lectin did not bind exclusively to rhizobia of the R. leguminosarum cross-inoculation group so the data do not support Bohool and Schmidt's (1974) recognition theory. Results similar to these have been reported by a number of other workers including Dazzo and Hubbell (1975), Chen and Phillips (1976), Law and Strijdom (1977) and Wong (1980).

Vicia cracca seeds possess two lectin fractions with distinct sugar-specific binding affinities. The two lectins

are known to interact in vitro (Baumann et al., 1980) although the product has not been characterized with regard to its biochemical properties or physiological role in nature. This lectin complex may be the basis of the Rhizobium recognition phenomenon in the Vicia cracca-Rhizobium leguminosarum symbiosis.

2.3 The Catalase and Litmus Milk Tests and the Gram Reaction

The catalase and litmus milk tests and the Gram reaction were useful aids in preliminary identification of the isolate.

2.4 Nitrogenase Induction in Free-Living Cultures and Carbohydrate Effect

A number of carbon sources supported acetylene reduction in free-living cultures of R. sp. VC 2. These results were not unexpected since R. leguminosarum and other fast-growing rhizobia can use a variety of carbon sources for growth on artificial media (Vincent, 1977) and for reduction of acetylene in pure culture (Skotnicki et al., 1979). The slow-growing rhizobia are more fastidious with regard to the carbon sources on which they can grow

(Vincent, 1977) or reduce acetylene (Kurz and LaRue, 1975). The latter authors (K. and L.) reported that a cowpea strain R. sp. 32H1, only reduced acetylene in pure culture when more than one carbon source was available. The reasons for the multiple carbon source requirement are unknown.

Kurz and LaRue (1975) found N'ase activity in R. leguminosarum TA101 on LNB5 medium with xylose and sucrose added but they did not state what other carbon sources, or combinations of carbon sources, also supported acetylene reduction in free-living cultures of R. leguminosarum. Skotnicki et al. (1979) reported that a spectinomycin-resistant strain of R. leguminosarum reduced acetylene if grown on a mannitol-containing medium and then transferred to a medium that contained succinate as the added carbon source.

Studies of substrates that supported nitrogen fixation in bacteroid preparations (Bergersen, 1977; Trinchant, 1981) indicated that hexoses may be the dominant substrates supplied to the bacterium from the plant. This viewpoint is based on several observations: glucose and sucrose are the main photosynthetic products translocated to root nodules (Bach et al., 1958; Streeter and Bosler, 1976; Singh et al., 1980) and several enzymes required for hexose use have been isolated from bacteroids of assorted rhizobia (Wong et al., 1971). Also, glucose and sucrose support acetylene reduction in bacteroids (Trinchant et al., 1981) at oxygen

tensions that are commensurate with values recorded from nodules (Wittenberg et al., 1974) The organic acids succinate, fumarate and pyruvate also support acetylene reduction in pure cultures of rhizobial bacteroids and some authors (Ronson and Primrose, 1979) believe that they are the dominant substrates supplied by the plant to the bacteria. However, succinate does not occur in the host nodule cells (Antoniw and Sprent, 1978) and it requires much higher oxygen tensions to support comparable rates of acetylene reduction than do glucose or sucrose (Trinchant et al., 1981).

2.5 Identity of the Isolate

Rhizobium sp. VC 2, an isolate from nodules of Vicia cracca, displayed all the characteristics cited by Bergey's Manual (1974) for R. leguminosarum. The isolate therefore appears to be a wild strain of that species.

3. Implications for Agriculture and Land Management

The Vicia cracca-Rhizobium leguminosarum symbiosis can be used as a convenient model for studying the Pisum sativum (pea) - R. leguminosarum symbiosis because the former can be more easily cultivated than the latter. The attributes

of the Vicia cracca symbiosis are discussed above in the 'Germination' section of the Discussion.

The second major agricultural application of the Vicia cracca-R. leguminosarum symbiosis would be as a source of new genetic material for the commercial producers of legume inocula. In this respect, cow vetch nodules can be thought of as 'libraries' of bacterial genotypes that have been selected by nature. This role is especially important since recent technological developments have made the recognition and transfer of gene sequences, which code for attributes such as cold tolerance, a fairly straightforward process.

The Vicia cracca-Rhizobium leguminosarum symbiosis also has considerable potential as a means to retard erosion and encourage colonization of unvegetated mineral soils in temperate climates since the plants grow readily and do not require maintenance. Cow vetch is especially suited for land reclamation or land maintenance projects because it is a perennial plant and can provide permenant support to eroding soils. Also, the plants fix atmospheric nitrogen so soil fertility is likely to be increased wherever they grow.

CONCLUSIONS

1) The Vicia cracca- Rhizobium sp. VC 2 symbiosis fixes atmospheric nitrogen at rates commensurate with other temperate legume species.

2) The Rhizobium isolated from Vicia cracca root nodules is a wild type of R. leguminosarum.

3) The symbiosis is similar to that of the garden pea (Pisum sativum L.) x Rhizobium leguminosarum with regard to shape and development of the nodule, and with regard to the the general characteristics of the bacterial partner. For these reasons, and because Vicia cracca seedlings can be grown more easily in vitro than pea seedlings, the Vicia cracca- Rhizobium leguminosarum symbiosis holds promise as a tool to facilitate investigation of the pea symbiosis.

4) The Vicia cracca- Rhizobium leguminosarum symbiosis is potentially useful in land reclamation and land maintenance projects and as a source of new genetic material for commercial producers of Rhizobium inocula.

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APPENDICES

Appendix A- References that Pertain to Symbiotic Nitrogen Fixation in Vicia cracca

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- An isolation procedure for, and a biochemical characterization of, Gal-N-Ac-specific V. cracca seed lectin

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Pate, 1958. The influence of various environmental factors on symbiotic expression in the vetch (Vicia sativa L.) and other legumes.
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Appendix A (cont.)- References that Pertain to Symbiotic Nitrogen Fixation in Vicia cracca

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- Report of tyrosinase activity in a Rhizobium strain isolated from V. cracca nodules
- data added after defense of this thesis

Sundberg, 1970. Simultaneous isolation of trypsin inhibitor and anti-A phyto-hemagglutinin from Vicia cracca seed by means of biospecific absorption.
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Tschirch, 1887. Beitraege zur kenntiss der wurzelknoellchen der leguminosen, Bericht der Deutschen Botanischen Gesellschaft 5: 58-98
- Mention of V. cracca nodule shape
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Van Brussel et al., 1982. Small Leguminosae as test plants for nodulation of Rhizobium leguminosarum and other rhizobia and agrobacteria harbouring a sym-plasmid.
- Mention of V. cracca and other small legumes nodulated by R. leguminosarum.

Wilson, 1939a. Leguminous plants and their associated organisms.
- V. cracca was nodulated by rhizobia isolated from Lens esculenta, Vicia villosa and Vicia villosa var. Gore.

Appendix A (cont.)- References that Pertain to Symbiotic Nitrogen Fixation in Vicia cracca

Wilson, J.K., 1939b. A relationship between pollination and nodulation of the Leguminosae.
- Vicia cracca is relatively self-pollinating but only formed nodules when inoculated with 3 (unspecified) strains from a diverse collection of 32 Rhizobium strains

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- this paper not read
- data added after defense of this thesis

Appendix B - Effect of Carbohydrate Source on Total Growth of R. sp. VC 2

	Growth *			
	Replicate			
Carbohydrate	1	2	3	mean
Glucose	172.5	220.0	162.5	185.0
	(28.0)	(31.9)	(23.5)	(30.0)
Xylose	262.5	245.0	-----	253.7
	(42.6	(39.7)		(41.1)
Mannitol	282.5	277.5	322.5	294.2
	(45.8)	(45.0)	(52.3)	(47.7)

(*) Values not in parentheses are turbidity readings given in Klett Units; values in parentheses are mg dry weight per ml equivalents

(--) missing datum

Appendix C (a) Total Growth of R. sp. VC 2 on Manhart and Wong's (1979) Defined Medium with Various Nitrogen Sources

	Turbidity (Klett Units)				
N source	1	2	3	Mean	s.d.
threonine	35	35	37	35.7	1.2
homoserine	76	73	72	73.7	2.1
KNO_3	78	76	76	76.7	1.2
aspartate	68	82	80	76.7	2.1
glutamate	99	95	92	95.3	3.5

Appendix C (b) Non-parametric Multiple Comparison of Total Growth of R. sp. VC 2 on Manhart and Wong's Defined Medium with Various Nitrogen Sources

Treatments Compared	Calculated q values	Table q values	Conclusion
5 vs 1	4.6476	3.858	Reject Ho: The turbidities are the same
5 vs 2	3.6824	3.633	Reject Ho: The turbidities are the same
5 vs 3	3.3731	3.314	Reject Ho: The turbidities are the same
5 vs 4	4.629	2.772	Reject Ho: The turbidities are the same
4 vs 1	3.3627	3.633	Accept Ho: The turbidities are the same

where Treatment 1 represents threonine (and similarly); 2, homoserine; 3, KNO_3; 4, aspartic acid and 5, glutamic acid.

Appendix D - Total Growth of R. sp. VC 2 at 20 C and 30 C

	Turbidity (Klett Units)			
Temperature	20 C		30 C	
Hours Post-Inoculation	96	123	96	123
Mean	250.0	255.3	199.4	204.9
S.D.	10.4	11.4	4.1	4.2

Appendix E - Effects of Temperature and pH on Total Growth of *R.* sp. VC 2.

Temperature	20 C			30 C		
pH	4.5	5.5	6.5	4.5	5.5	6.5
	32	170	264	8	150	204
	46	164	262	12	132	218
	46	166	252	12	140	228
mean	41.3	166.7	259.3	10.7	140.7	216.7
s.d.	8.1	3.1	6.4	2.3	9.0	12.1

Values are Klett units

Appendix F (a) - Nitrogenase Activity of Free-Living R. sp VC 2 Grown on LNB5 Medium with Various Carbon Sources

Carbon Source	N'ase Activity	Carbon Source	N'ase Activity
arabinose	3.56	mannitol	2.50
	2.90		2.24
	3.06		1.84
	2.63		2.19
sucrose	1.81	sucrose-mannitol	1.59
	2.90		1.88
	2.74		2.45
	3.52		2.53
sucrose-arabinose	2.31	xylose	2.39
	3.41		2.21
	3.15		1.81
	2.31		
galactose	2.96	sucrose-xylose	1.95
	3.67		1.95
	2.96		2.32
	3.19		1.90
sucrose-galactose	2.15		
	3.31		
	2.70		
	3.31		

* nitrogenase activity was measured in decimoles of ethylene/culture/hr

Appendix F (b) - Sample Calculation of Nitrogenase Activity in Free-Living R. sp VC 2 with Arabinose as the Carbohydrate Source

Step	Calculation	Value
1	peak height	1.08 inches/culture
2	equivalent amount of ethylene calculated from a standard curve (1.08 x 2.67)	2.88 decimoles/culture
3	volume correction (2.88 x 3.70 ml)	10.7 decimoles/culture
4	time correction	3.56 decimoles/culture/h

Appendix G - Number of Nodules Formed on *V. cracca* by Three Strains of *Rhizobium*

Plant	*R.* sp. VC 2	*R. leguminosarum* 97H3	*R. leguminosarum* 128C56
1	2	1	1
2	0	3	2
3	1	8	1
4	3	1	0
5	0	7	2
6	2	6	3
7	1	6	0
8	7	2	1
9	5	2	0
10	2	2	2
11	5	3	2
12	3	2	4
13	3	3	5
14	2	1	2
15	7	1	7
16	2	0	1
17	1	-	-
18	6	-	-
19	1	-	-
20	3	-	-
21	6	-	-
22	6	-	-
23	5	-	-
24	6	-	-
25	3	-	-
26	5	-	-
Mean	3.4	3.0	2.1
SD	2.2	2.4	1.9