Nitrogen fixation
Volume 2: Rhizobium

Edited by

W. J. Broughton

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Preface

Proceedings of several conferences that have included rhizobial aspects of nitrogen fixation have recently been published. Good soil microbiology texts are also available, but not single volume has been entirely devoted to *Rhizobium*. This is an important omission since the legume–*Rhizobium* symbiosis is assuming ever increasing importance in parallel with better management of our natural resources. Furthermore, *Rhizobiums* close relationship to *Agrobacterium* (they are the only two genera in the bacterial family Rhizobizceae) is exciting molecular biologists. Along with many others, pioneers of plant-microorganism research hope that the plant transformation systems now being developed with *Agrobacterium* will also be applicable to *Rhizobium*. Thus, this volume should be a timely and extremely useful addition to the literature on nitrogen fixation.

Functionally, the book is organized so that it forms both an introduction to the field and a critical review of knowledge in the main disciplines. In Chapter 1 Gloria Lim and Joe Burton answer the question 'Which legumes are nodulated?', 'Van' Bushby discusses rhizobial distribution in the soil and factors affecting it, while Mike Trinick has provided a comprehensive account of rhizobial biology. As the biggest difference between rhizobia—that between fast- and slow-growing organisms—basically concerns varying abilities to metabolize carbohydrates, Gerry Elkan and David Kuykendall have closely examined metabolism. John Beringer, Nick Brewin, and Andy Johnston reviewed the most rapidly expanding field—the genetics of the microsymbiont, and Tsien Hsien-Chyang described the cells as seen under the microscope. Three chapters deal directly or indirectly with the outer structure of the cells—Russel Carlson with their chemistry, Jim Vincent with their immunological reactions, and Frank Dazzo and David Hubbell with the implications of various rhizobial and legume components in regulation of the interaction. Finally, Eltjo Meijer correlated observations on nodule development with known biochemical events. In short, this treatise is a thorough examination of both the freeliving *Rhizobium* and its relationship to legumes.

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Preparation of this volume was largely dependent of the fifteen authors and I am exceedingly grateful to them for their expert contributions. Others whose help was essential included Fräulein Antonia Maria Schafer and Professor Fritz Lenz of the University of Bonn and Fräulein Elisabeth Schölzel and Professor Jeff Schell of the Max-Planck-Institut für Züchtungsforschung in Köln. It has been my privilege to co-ordinate the efforts of so many knowledgable and pleasant people.

Cologne   WJB

Contents

1 Nodulation status of the Leguminosae

G. LIM AND J. C. BURTON

1.1 Introduction

The legumes form one of the largest families of flowering plants, ranking third in terms of world-wide occurrence, with about 600 genera and 18 000 species, although estimates of actual numbers vary considerably (from 590 to 700 genera and 12 000 to 18 000 species according to different authors; Hutchinson 1964; Airy Shaw 1966; Keng 1970; Heywood 1971; Anonymous 1979; Allen and Allen 1981).

The oldest agricultural records available indicate that leguminous crops have been cultured for centuries and that they were valued for food and soil enrichment long before their ability to work symbiotically with bacteria was understood.

Fossils of Leguminosae have been traced back to the Cretaceous or last division of the Secondary or Mesozoic era, 95–120 million years ago (Fred, Baldwin, and McCoy 1932). However, no reference was made to the presence of nodules on these ancient legumes. It has been suggested by Fred *et al.* (1932) that a re-examination of these fossils for the occurrence of nodules should be made to determine the antiquity of the *Rhizobium*–leguminous plant association.

Our knowledge of the beneficial association of rhizobia with leguminous plants in utilizing atmospheric nitrogen (N_2) is very recent when compared to the very long period that leguminous crops have been cultured and valued for food and soil enrichment. According to Fred *et al.* (1932), credit for the first published picture of nodules should be given to Fuchs who portrayed nodules on the roots of *Aphaca, Vicia faba,* and *Trigonella foenum-graecum* in the first edition of *Historia stirpium commentarii insignes* in 1542. No mention was made in this book of the nodules, which were apparently considered to be normal plant structures rather than hypertrophies induced by bacteria. It was not until 1886 that Hellriegel (1886) revealed the true origin and function of the

nodule which enabled the leguminous host plant to utilize atmospheric nitrogen. Two years later the bacteria (rhizobia) were isolated from the nodule and cultured on laboratory media by a Dutch microbiologist (Beijerinck 1888).

In the tropics, leguminous plants are prevalent and constitute one of the largest groups of flora. Norris (1956) suggested that the legumes originated in the tropics; in fact tropical trees comprise a large part of the family which is said to be basically a tropical arborescent family (Tutin 1958).

In the humid tropical Malaysian peninsula and Singapore, the legumes form the fourth largest family with 66 genera and 266 species (Keng 1970), most of them woody species. According to Whitmore (1972) however, there are 70 genera and 270 species in the lowland and mountain forests of Malaya of which 53 species are trees reaching at least 1 m girth. He also considered the legumes to be amongst the loftiest trees in Malayan forests, especially the species of *Koompassia* and *Intsia*, and he regarded the family as only second to the Dipterocarpaceae in abundance among the emergent trees of the lowland rain forest. Among this emergent layer, appreciable numbers of legumes occur, mainly of the genera *Dialium, Koompassia*, and *Sindora* (Whitmore 1975). In fact, in the Malayan peninsula, legumes form the second most important timber-producing family after the Dipterocarps, *Intsia palembanica* being the most important individual species, while *Pterocarpus indicus* is said to outshine teak in natural beauty (Whitmore 1972).

In the monsoon forests of the Far East, in the Malesian region, the major dominant trees include a large number of legumes, ten species out of 26 typical species belong to the Leguminosae family, while the rest belong to various other families (Whitmore 1975). These ten species are two each of *Acacia* and *Albizia*, and one species of each of *Butea, Caesalpinia, Cassia, Dalbergia, Dichrostachys*, and *Tamarindus*.

Among the common cultivated plants in Malaysia and Singapore are legumes such as the various beans, ornamentals such as *Cassia, Caesalpinia*, and *Mimosa* spp, and park, roadside, and garden trees and shrubs such as *Acacia* spp, Flame of the forest (*Delonix regia*), raintree (*Samanea saman*), Angsana (*Pterocarpus indicus*), Bauhinias, and tamarind.

In India, the Leguminosae are considered to be the second most dominant family in order of abundance. Some genera occurring in the forests are *Ougeinia, Mastersia*, and *Wagatea*. In the delta forests of the gangetic plains, *Cynometra ramiflora* are found, and in the sandal forests of south India legumes occur including *Dalbergia latifolia, Albizia* 'spp, *Pterocarpus marsupium*, and *Pongamia glabra*. Other species found in various parts of India include *Indigofera* spp, *Sesbania aculatea, Aeschynomene* spp, *Alysicarpus vaginalis, Acacia* spp, and *Rhynchosia minima* (Puri 1960).

Many legumes are also cultivated for food and feed, for example, *Cicer, Crotalaria, Phaseolus, Tephrosia,* and *Vigna* spp, as well as many other grain and pulse legumes.

A large number of indigenous legume species occur in Africa and many more have been introduced and cultivated. As a result, legumes are fairly well-represented among the flora, although some areas are richer in legume species than others. In West Africa for instance, legumes are well-represented in forests with species of *Piptadeniastrum, Albizia,* and *Tetrapleura* occurring, and some rainforests are dominated by *Cynometra ananta* (Lawson 1966). *Pericopsis* is abundant in western Ashanti but rare elsewhere; *Afzelia africana* and *Parkia clappertoniana* are said to be the most common trees in the Guinea savannas (Lawson 1966); and in the subtropical regions, *Acacia* spp abound.

In the East African region, *Cynometra* spp are abundant in Tanzania and Western Uganda, and may constitute a dominant part of the lowland forests, forming 70–80 per cent of single layered canopy (Lind and Morrison 1974). Genera such as *Brachystegia, Isoberlinia,* and *Julbernardia* are dominant over wide areas of woodland in tropical Africa. In northern Nigeria, *Parkia filicoidea* is a very common tree of park savanna (Purseglove 1968).

The neotropics are also rich in legume flora with a variety of growth forms. Over 50 per cent of the trees in some forest associations in British Guiana were found to consist of legume species (Davies and Richards 1934). Jenny (1950) reported on an estimate of 50 per cent legume trees on forest soils examined in Colombia. In fact central and south America are said to be promising areas for finding species useful for improvement of soil nitrogen and for grazing (Williams 1967).

In temperate regions legumes are less predominant among the flora, and in terms of number of genera and species, fewer legumes occur there than in tropical areas. A good account of the distribution of legumes has been given by Norris (1956). Examples of legumes which are found in temperate regions are those of tribe Vicieae such as species of *Vicia,* e.g. *V. faba, Pisum* spp, and *Lathyrus* spp such as *L. odoratus* (sweet pea); members of tribe Loteae such as *Lotus* spp and *Anthyllis* spp; and Trifolieae genera such as *Trifolium, Medicago,* and *Melilotus.*

The Leguminosae is generally subdivided into four subfamilies— Mimosoideae, Caesalpinioideae, Swartzioideae, and Papilionoideae— the latter being the largest and Swartzioideae the smallest. Tutin (1958) and Allen and Allen (1961) have given excellent accounts of the descriptions, classification, size, and distribution of members of this family, and the latter reference additionally reviewed the scope of nodulation in the Leguminosae up to 1959.

Tropical legumes represent all four subfamilies, members comprising very few herbaceous species, mainly being shrubs, woody climbers, and

trees, some of which are very large trees in rainforest areas. Although the humid tropics are regarded as the ancestral home of legumes, and indigenous species abound, the leguminous flora of many tropical countries contains a fairly large proportion of introduced species. These latter species were brought into a country for agricultural, economic, or ornamental purposes. Whatever the reasons, many such plants thrive well and have become established as part of the country's present-day flora.

The subfamily Mimosoideae is normally regarded as the most primitive of the four subfamilies, in terms of evolutionary development, although Hutchinson (1964) considered the Caesalpinioideae to be the most primitive. Members of the subfamily with the exception of *Acacia*, are said to occur exclusively in the tropics and most abundantly in rainforests and semi-arid subtropics (Allen and Allen 1961). Many species of *Acacia* occur in temperate and alpine regions of Australia.

The subfamily Caesalpinioideae is also regarded as being confined to the warmer parts of the world. Tropical species are largely woody shrubs, trees, and climbers. Temperate ones consist of only three genera, *Gleditsia*, *Cercis*, and *Ceratonia*.

Swartzioideae, with only 9–10 genera and about 100–150 tropical species, is native to South America and Africa, and is regarded as intermediate between Papilionoideae and Caesalpinioideae in taxonomic position (Corner 1951). All species are woody.

Papilionoideae is the only subfamily with equal distribution in tropical and temperate countries, though many more genera are tropical. The tropical species are predominantly woody, large trees, or big, woody climbers; the temperate ones are predominantly shrubs or herbs.

1.2 Rhizobiaceae

The Rhizobiaceae family of bacteria consists of only two genera: *Rhizobium* and *Agrobacterium*. In discussing the genus *Rhizobium*, it is appropriate to begin with nodulation, because the ability to incite cortical hypertrophies or nodules on leguminous roots is the one criterion currently accepted in distinguishing rhizobia from other bacteria (Buchanan and Gibbons 1974). As Vincent (1977) points out, however, the genus *Rhizobium* also includes bacteria which have lost their invasive properties, as long as there is authentic proof of clonal descent from a culture capable of inducing nodules to form on leguminous plants.

In contrast to *Rhizobium* the other genus, *Agrobacterium*, has the ability to incite hypertrophies (galls) on the roots and stems of diverse plant species. These hypertrophies may be mistaken for nodules, but they are tumorous galls consisting of unorganized tissues or structures and are

usually harmful to their host. Only one species, *Agrobacterium radiobacter*, does not produce galls. The latter, because of similarity in morphology, is sometimes mistaken for fast-growing rhizobia which have lost their ability to produce nodules. However, these bacteria can be differentiated easily from rhizobia by cultural methods (Graham and Parker 1964; Vincent 1977).

Rhizobium *species*

Early studies of nodulation were concerned primarily with readily accessible leguminous plants, cultured in temperate climates mainly in the United States and Europe, as sources of food. Observations that all leguminous crops did not respond similarly to inoculation with *Rhizobium* cultures led to a grouping of plant hosts according to their nodulation responses. Leguminous plants nodulated by the same nodule bacteria constituted a group. Rhizobia isolated from nodules on a plant in the group usually produced nodules on other plants in the same group. These plant groups were called 'cross-inoculation' groups. Rhizobia able to nodulate plants in one of these groups were considered a *Rhizobium* species. Only six species of *Rhizobium* have been named, but other groups of plant genera are mutually susceptible to nodulation by a common *Rhizobium* (Table 1.1). The rhizobia without species names were identified with their parent host as cowpea, lotus, crownvetch, and other kinds of rhizobia.

TABLE 1.1

Groups of leguminous plants (genera)
nodulated by a single species or kind of Rhizobium

Rhizobium species or kind	Legume genera
1. *Rhizobium meliloti*	*Medicago, Melilotus, Trigonella* spp
2. *Rhizobium trifolii*	*Trifolium* spp
3. *Rhizobium leguminosarum*	*Lathyrus, Lens, Pisum, Vicia* spp
4. *Rhizobium phaseoli*	*Phaseolus coccineus, P. augustimus, P. vulgaris*
5. *Rhizobium japonicum*	*Glycine* spp
6. *Rhizobium lupini*	*Lupinus* and *Ornithopus* spp, *Lotus* (certain species)
7. *Rhizobium* spp (cowpea)	*Acacia, Alysicarpus, Andira, Apios, Arachis, Baptisia, Cajanus, Cassia, Canavalia, Crotalaria, Cyamopsis, Cytisus, Desmodium, Dolichos, Erythrina, Indigofera, Lespedeza, Phaseolus, Macroptilium, Pueraria, Stylosanthes, Vigna,* and others
8. *Rhizobium* spp (lotus)	*Lotus, Anthyllis, Dorycnium*
9. *Rhizobium* spp (crownvetch)	*Coronilla, Onobrychis, Petalostemum, Leucaena, Dalea* spp

The rhizobia which induce nodules on certain genera of leguminous plants are currently considered to differ from any of those listed in Table 1.1. These are *Amorpha, Amphicarpaea, Astragalus, Caragana, Cicer, Laburnum, Robinia, Sesbania, Strophostyles,* and *Wistaria.* In some genera, not all plant species are nodulated by the same cultures of rhizobia. *Vicia floridana* and *Vicia acutifolia* are examples of this (Carroll 1934); they are not nodulated by *R. leguminosarum.* Several species of *Phaseolus: P. lunatus, P. acutifolius, P. wrightii, P. ritensis, P. adenanthus,* and *P. heterophyllus* are nodulated by cowpea rhizobia whereas *P. vulgaris, P. coccineus,* and *P. augustifolia* are nodulated by *Rhizobium phaseoli.* There is a similar situation in the genus *Lupinus.* New groups will undoubtedly be formed and new additions to old groups will be made as our knowledge of the nodulating characteristics of leguminous species expands. So far, studies have involved only about 10 per cent of the total leguminous species.

The cross-inoculation concept, based on mutual susceptibility of plants within a group to nodulation by a common *Rhizobium,* has not proven acceptable because of the numerous irregularities. None the less, the grouping has been very useful in searching for good nitrogen-fixing *Rhizobium* strains. Leguminous plants can derive benefit only from rhizobia capable of nodulating their roots. Groupings based on nodulation permits focusing on good prospects when searching for highly effective *Rhizobium* strains, regardless of bacterial taxonomy.

The grouping of leguminous plants which tend to give effective nitrogen-fixing response to the same strains of rhizobia, appears to be far more useful than groupings based entirely on susceptibility to nodulation (Burton 1979). *Lotus* species are all nodulated by the same strains of rhizobia, but *Lotus* species fall into two groups when nitrogen fixation is considered (Fig. 1.1). Strains of rhizobia effective on birdsfoot trefoil, *Lotus corniculatus,* are not effective on deervetch, *Lotus pedunculatus,* and vice versa. All of the *Lotus* species studied so far can be classed either as the birdsfoot trefoil or big trefoil types.

Certain strains of cowpea rhizobia work effectively on many genera, species, and varieties of legumes. In contrast, the clover cross-inoculation group which embodies only *Trifolium* species, requires different strains of rhizobia for many of its species (Burton 1979).

Numerical taxonomy of rhizobia based on the Adasonian principle, DNA-base composition, and homology, is being studied in an attempt to clarify classification of *Rhizobiaceae.* This is discussed later in this volume (Chapter 3).

Nodules

The nodule is the focal point of reaction between the *Rhizobium* and its host plant. When a susceptible, leguminous plant and a compatible
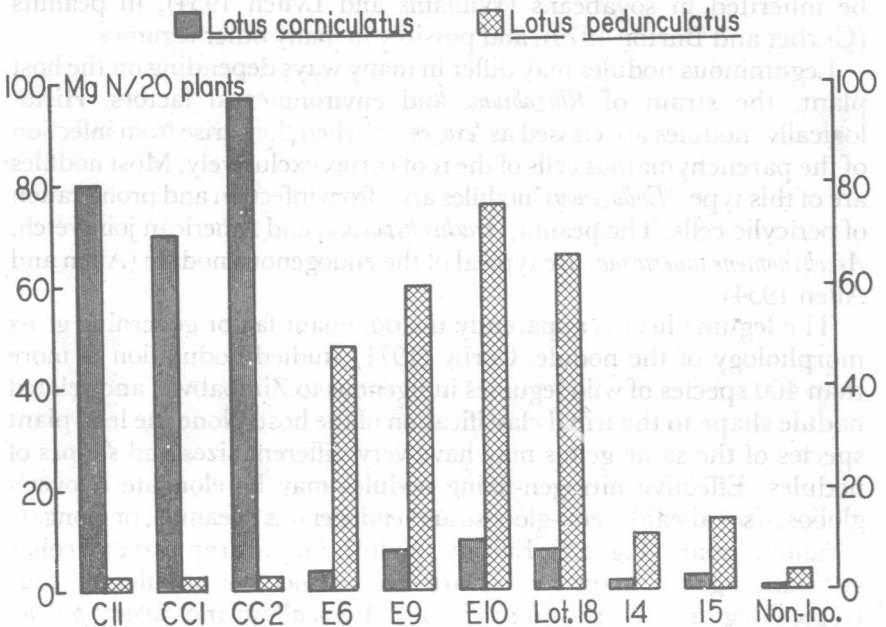
FIG. 1.1. Nitrogen fixation by two *Lotus* species. *Rhizobium* strains C11, CC1, and CC2 were isolated from and are effective on *Lotus corniculatus*. Strains E6, E9, E10, and Lot. 18 were isolated from and are effective on *Lotus pedunculatus*. All *Rhizobium* strains produced nodules on their parent host and on the other test species of *Lotus*.

strain of rhizobia are brought together under conditions favourable for growth and infection, a nodule will form. The surface chemistry of the *Rhizobium* and of the root hair, partly determines the compatibility of the two symbionts and whether or not a nodule will form. The intricate chemistry and mechanics of the rhizobia and of the root hair, are covered in Chapters 7 and 9 of this volume.

Not all leguminous plants form nodules. Up to 1981, it was thought that 90 per cent of 388 plants examined in the subfamily Mimosoideae were nodulated; 98 per cent of 2462 species in the subfamily Papilionoideae bore nodules; and only 28 per cent of 258 species examined in the subfamily Caesalpinioideae were nodulated (Allen and Allen 1961). For nodulation to occur, the host plant must be susceptible and a compatible infective strain of *Rhizobium*, capable of multiplying, must be present on the root. It is not known whether lack of nodule development in some leguminous species is due to lack of appropriate rhizobia, or to structural problems in the legume root system. The possibilities are discussed in detail by Funk (1956). Susceptibility to nodulation may

be inherited in soyabeans (Williams and Lynch 1954), in peanuts (Gorbet and Burton 1979), and possibly in many other legumes.

Leguminous nodules may differ in many ways depending on the host plant, the strain of *Rhizobium*, and environmental factors. Histologically, nodules are classed as *'exogenous'* when they arise from infection of the parenchymatous cells of the root cortex exclusively. Most nodules are of this type. *'Endogenous'* nodules arise from infection and proliferation of pericylic cells. The peanut, *Arachis hypogaea*, and American jointvetch, *Aeschynomene americana*, are typical of the endogenous nodule (Allen and Allen 1954).

The legume host is apparently the dominant factor governing gross morphology of the nodule. Corby (1971) studied nodulation of more than 400 species of wild legumes indigenous to Zimbabwe, and related nodule shape to the tribal classification of the host. None the less, plant species of the same genus may have very different sizes and shapes of nodules. Effective nitrogen-fixing nodules may be elongate (clover), globose (soyabean), semi-globose and endogenous (peanut), or elongate coralloid (guar) (Fig. 1.2). Effective nodules may also appear as a collar surrounding the main root (Dart 1977). Effective nodules on four tropical legumes are shown in Fig. 1.4: *Acacia albida* and *Robinia pseudoacacia* are trees, and bear nodules perenially.

The leguminous plant with nodules on its roots induced by an effective strain of *Rhizobium*, is considered to be in symbiosis with the bacteria. The symbionts are considered to be mutually beneficial, but this relationship may shift with age. Allen and Allen (1954) suggest that in the early stages of nodule development, the bacteria are the dominant factor. As the nodule matures structurally, there is a period of balance when each symbiont is supplying and being supplied by the other. This is a period of mutual benefit; nitrogen is being supplied to the plant by the bacteria, and carbohydrates are being supplied to the bacteria for growth and nitrogen fixation. When the plant matures and begins fruiting, it becomes the dominant controlling factor and eventually initiates senescence in the nodule. The length of these stages will vary with different leguminous plants and environmental conditions (see Volume 3, Chapters 3 and 4).

This type of nodulation which results in nitrogen fixation is called 'effective' nodulation. The nodules tend to be large, concentrated on the upper root system, and usually have reddish interiors from the haemoglobin, which characterizes an effective nodule (Fig. 1.3). When nodules develop, but fix little or no nitrogen they are called 'ineffective'. These nodules are usually small, numerous, and widely scattered throughout the root system. Ineffective nodules may sometimes appear to be moderately large (Viands, Vance, Heichel, and Barnes 1979).

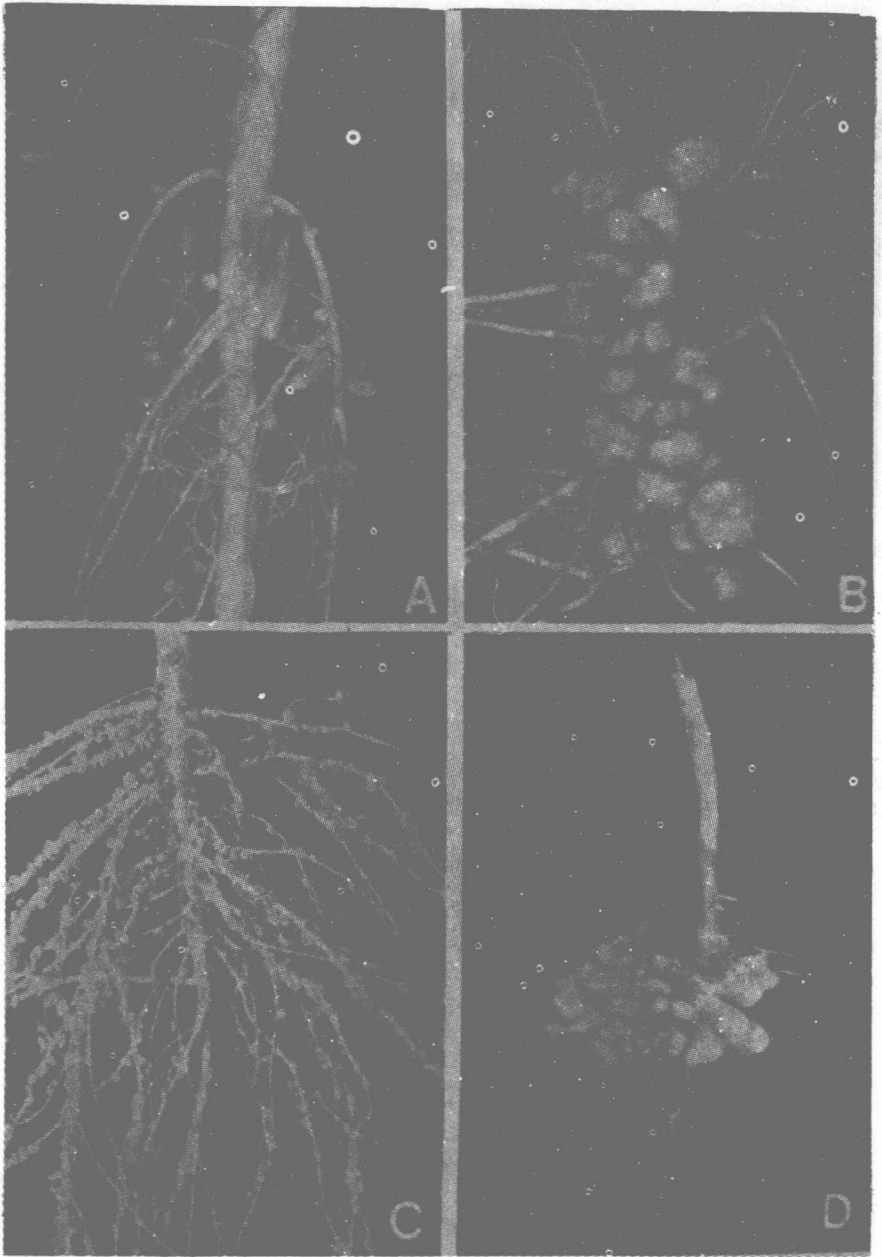In describing nodule function, the terms effective and efficient are

FIG. 1.2. Nitrogen-fixing nodules. (A) Elongate nodules on *Trifolium amabile*. (B) Globose nodules on *Glycine max* L. Merr. (C) Endogenous semi-globose nodules on *Arachis hypogaea*. (D) Elongate coralloid nodules on *Cyamopsis tetragonoloba*.