4. N_2-fixing tropical non-legumes

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1. Introduction

Non-leguminous plants with root nodules possessing N_2-fixing capacity are present in a large number of phylogenetically unrelated families and genera of dicotyledonous angiosperms. These plants occur in a wide variation of habitats and show a large range of morphological forms. Some of them are small prostrate herbs (e.g. *Dryas* spp.), others shrubs (e.g. *Ceanothus* spp. and *Colletia* spp.), while others are stout tree-like woody species (e.g. *Alnus* spp. and *Casuarina* spp.).

The only species of horticultural/agricultural significance is *Rubus ellipticus*, because it is a raspberry species producing a soft, edible fruit. All the other non-leguminous nodulated species are of no agricultural importance for crop production. The woody species are, however, important in forestry for reforestation and wood production and all of them play a prominent role in plant succession of natural ecosystems by covering bare soil of disturbed areas or sites. The section dealing with management and prospects for use in the tropics will discuss some of these properties in more detail.

2. Nodulated species

The older literature dealing with non-leguminous N_2-fixing dicotyledonous angiosperms can be found in the reviews of Becking [20, 21, 23, 26, 28] and Bond [40]. The present communication will cover more recent contributions in the field, with emphasis on the tropical species. Table 1 presents a complete enumeration of the non-leguminous dicotyledonous taxons possessing root nodules, including the more recent discoveries. As evident from this table, these nodulated plants comprise 8 orders, 9 families, 18 genera, and about 175 species of dicotyledonous plants.

With respect to root nodulation, two types of root-nodule symbiosis can be distinguished. The majority of these symbiosis are actinorhizal, i.e. caused by microorganisms belonging to the actinomycetes of the genus *Frankia* of the family Frankiaceae [22, 25]. In one genus of dicotyledons, however, i.e. the genus *Parasponia* of the Ulmaceae, a true bacterium (Eubacteriales) is involved in the symbiosis. This bacterium belongs to the genus *Rhizobium* of the Rhizobiaceae. It is to a certain degree promiscuous, as it can also produce root nodulation and effective N_2 fixation in a number of tropical legumes such as cowpea (*Vigna* spp.) and other leguminous species.

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Table 1. Classification of the non-leguminous dinitrogen-fixing Dicotyledons with *Frankia* symbioses

Order	Family	Tribe	Genus	Number of nodulated species (in parentheses total number of species)[a]		
Casuarinales	Casuarinaceae	–	*Casuarina*	25	(45)	
Myricales	Myricaceae	–	*Myrica*	26	(35)	
			Comptonia	1	(1)	
Fagales	Betulaceae	Betuleae	*Alnus*	33	(35)	
Rhamnales	Elaeagnaceae	–	*Elaeagnus*	17	(45)	
			Hippophaë	1	(3)	
			Shepherdia	3	(3)	
	Rhamnaceae	Rhamneae	*Ceanothus*	31	(55)	
		Colletieae	*Discaria*	6	(10)	
			Colletia	3	(17)	
			Trevoa	1	(6)	
Coriariales	Coriariaceae	–	*Coriaria*	14	(15)	
Rosales	Rosaceae	Rubieae	*Rubus*	1	(250)	(429)[b]
		Dryadeae	*Dryas*	3	(4)	
		Cercocarpeae	*Purshia*	2	(2)	
			Cercocarpus	4	(20)	
Cucurbitales	Datiscaceae		*Datisca*	2	(2)	

[a] Taxonomic estimates mainly based on Willis [136]
[b] According to Focke [58] 429 *Rubus* species occur worldwide, but Willis [136] gives as an estimate 250 *Rubus* species

2.1. Actinorhizal symbioses

The root nodulation in the genus *Casuarina* (single genus) of the Casuarinaceae is well known in the tropics. This species occurs spontaneously in Southeast Asia, including the Southwest Pacific and Australia. Representatives of this large genus (45 species) have, however, also been introduced in recent times in parts of Africa such as North Africa, Tunisia and Morocco, Dakar, and the Cape Verde Islands off the coast of Dakar; in tropical and subtropical American areas such as Florida [56, 109, 110]; and in some localities in Asia [7].

The genus *Alnus* of the Betulaceae, with 35 species, occurs mainly in temperate regions, but important representatives also occur in the tropics. For example, *Alnus jorullensis* occurs in South America and *A. nepalensis*, is found at the higher elevations in Southeast Asia (Nepal), while species like *A. japonica* and *A. maritima* are often introduced and thrive well at higher elevations in tropical Asia (Indonesia, Philippines).

The genus *Elaeagnus* of the Elaeagnaceae has many species present in natural ecosystems in Southeast Asia, including *Elaeagnus latifolia* and *E. conferta* on Java,

Indonesia [31], and *E. philippensis* in the Philippines [6]. Of the representatives of the genus *Myrica* (Myricaceae), several montane species such as *M. javanica* naturally inhabit the higher regions of Indonesia and the Philippines [8, 20, 23].

Of the order Coriariales, which has a single family and a single genus with 15 representatives, some species occur in Asia at the higher elevations, e.g. *Coriaria japonica* and *C. nepalenis*. For both species, nodulation and N_2 fixation has been established [45, 75].

In the Rosaceae, root nodulation has already been reported in three North-American species of the genus *Cercocarpus*, i.e. *C. betuloides*, *C. montanus* and *C. paucidentatus*. Recently, nodulation has also been observed in *Cercocarpus ledifolius* growing as pioneer species in *Pinus flexilis* stands in California in the United States [88]. The latter species probably also has a montane neotropic distribution. Of the tribe Dryadeae of the Rosaceae, root nodulation is reported in the genera *Dryas* (Fig. 1) and *Purshia*. Representatives of the genus *Dryas* have a northern temperate and subarctic distribution, but this plant genus occurs also at the higher elevations (montane zone) in North America, Europe (Alps), and some mountains in Asia such as the Himalayas. The genus *Purshia* of the tribe Dryadeae has a solely North-American distribution, like most members of the above-mentioned genus *Cercocarpus* (tribe Cercocarpeae of the Rosaceae). In the tribe Rubieae of Rosaceae, however, one member of this extensive group, i.e. *Rubus ellipticus* (Fig. 2), has been observed and confirmed to possess root nodulation and N_2 fixation [31, 40]. The observation of root nodulation in *Rubus* initially caused some surprise and even disbelief, because although nodulation was known in the tribes Dryadeae and Cercocarpeae of the Rosaceae, the genus *Rubus* is relatively unrelated to them. The nodulated species *Rubus ellipticus* occurs naturally on continental Asia, and on some islands: e.g., Sri Lanka and Luzon in the Philippines. So far nodulation and N_2 fixation (acetylene reduction) has only been reported for *Rubus ellipticus* specimens grown in Java, Indonesia. Root nodulation in one species of the large genus *Rubus* (tribe Rubieae) with about 250 species [136] or 429 species [58], is quite notable. Moreover in all non-leguminous nodulated plants investigated so far, nodulation is a generic character. Nodulation, however, is apparently far from a generic character in the genus *Rubus*, though perhaps other nodulating species will eventually be found in this very large genus.

In the Rhamnaceae, root nodulation in a representative of the genus *Colletia* of the order Rhamnales has been reported. The *Colletia* species has a principally neotropic distribution. Root nodulation in *Colletia* was first observed by Bond in a specimen of *C. paradoxa* (syn. *C. cruciata*) growing in the Glasgow Botanical Gardens, Scotland. This nodulation was reported at the final I.B.P. conference, Section PP-N at Edinburgh in 1973 [40]. Subsequently, Medan and Tortosa [89] mentioned nodulation in *Colletia paradoxa* and *C. spinosissima* plants growing in the Botanical Gardens of Buenos Aires, Argentina. They also reported nodulation occurring locally in natural vegetations in four species of the genus *Discaria* also belonging to the Rhamnaceae, i.e. *Discaria americana*, *D. serratifolia*, *D. trinervis* and *D. nana*. At present, nodulation in *Discaria* has only been reported in the

Fig. 1. Dryas sp. (tribe Dryadeae of Rosaceae). Although having a mainly temperate and subarctic distribution, some *Dryas* species occur at high elevations on Euro-Asiatic mountains, where they are prominent as pioneer plants on bare rocky soils. (a) *Dryas* sp., plant habitus in natural environment; (b) root nodules of *Dryas drummondii* Richardson; bar scale = 1 cm.

temperate species *D. toumatou* endemic to New Zealand [93] and in an unclassified species growing in the Botanical Gardens of Edinburgh, raised from seeds from neotropic origin, i.e. from wild plants in Chile [40]. *Colletia* species introduced in Asia also have been observed to bear root nodules, and N_2-fixing activity was established (acetylene reduction test), i.e. in *Colletia paradoxa* (syn. *C. cruciata*)

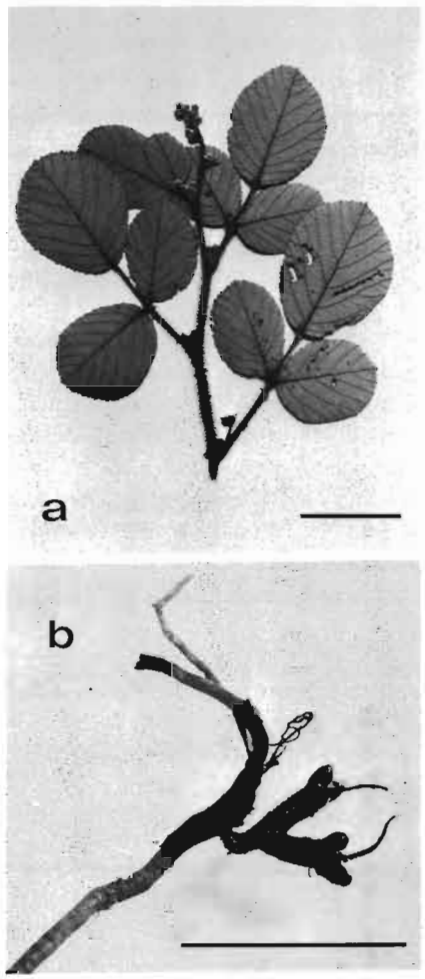

Fig. 2. Rubus ellipticus J.E. Smith. (a) Twig with leaves and flower buds; bar scale ≒ 4 cm; (b) root nodules; bar scale = 1 cm.

and in *C. armata* (syn. *C. spinosa*) growing in the Cibodas Mountain Gardens (altitude 1450 m) at Mt. Pangrango-Gedeh, W. Java, Indonesia [31]. Moreover, nodulation was observed in *C. paradoxa* and *C. armata* plants growing in various Botanical Gardens in Europe (Amsterdam, Cologne, Munich and Nantes)(Becking, unpublished).

Recently, also in another genus of the Rhamnaceae of neotropic and subtropic distribution, i.e. *Trevoa*, which is closely related to *Colletia*, root nodulation has been observed. The species *Trevoa trinervis* has been found to bear root nodules and subsequent acetylene reduction tests have revealed N_2 fixation in natural habitat [104] comparable to that of *Ceanothus* and *Cercocarpus* measured under similar conditions [55, 67, 77]. *Trevoa trinervis* is an important matorral shrub in Chile occurring primarily on disturbed sites, more or less in the same way *Ceanothus* and *Cercocarpus* species occur as xerophytic chaparral shrubs in California.

Unlike the above-mentioned Californian species, however, the leaves of *Trevoa trinervis* are drought deciduous. Therefore, with the onset of drought stress, although the *Trevoa* rapidly sheds its leaves, photosynthesis is maintained by chlorophyllous tissue of young stems and spines. With regard to the latter phenomenon, *Trevoa* closely resembles certain *Colletia* species (Fig. 3), which also shed their leaves during the unfavourable season, but in which phyllocladioid stems and spines continue with photosynthetic activity and serve the nodules with indispensable carbohydrates.

A recent discovery of this type of symbiosis has been the observation of root nodulation in the genus *Datisca* of the Datiscaceae of the order Cucurbitales. Up to now, this order was not considered to contain nodulated species as there is no affinity of this order to other nodulated taxons. Nodulation and N_2 fixation (acetylene reduction) was observed in *Datisca* in the two representatives of this genus, i.e. *Datisca cannabina* (see Fig. 4), with a Mediterranean distribution over the Indo-Arabian region to the Himalayas and Central Asia, and *D. glomerata*, found in southwestern, and northwestern Mexico. In both species, root nodulation was observed and N_2 fixation was confirmed by Chaudhary [47, 48, 137]. Root nodulation in *Datisca cannabina* had already been reported, however, by Severini [108], more than 55 years ago. Severini had in addition already conducted some growth experiments with nodulated plants in nitrogen-deficient medium and had demonstrated their N_2-fixing ability. In spite of the fact that this reference of root nodulation of *Datisca cannabina* had been cited in Metcalfe and Chalk's book [90], Severini's observation escaped notice until recently. This omission is probably due to the fact that the observed root nodulation was not connected with possible N_2 fixation.

2.2. Rhizobium symbioses

This type of symbiosis in non-leguminous plants has only recently been discovered in members of the genus *Parasponia* of the Ulmaceae of the Urticales. Evidence of root nodulation in the Ulmaceae by a *Rhizobium* species was first reported by Trinick [125], who mentioned the nodulation and N_2 fixation of *Trema aspera* occurring in the Pangia District of Papua (New Guinea). Later this species was classified as *Trema cannabina* var. *scabra* [126], but recently it has been reclassified as a *Parasponia* species [3, 4, 30]. The confusion between *Trema* and *Parasponia* is understandable, since representatives of both genera are morphologically very similar. In the past, specimens of both genera have been regularly confused as one can see from the many reidentifications and name changes found on labels of herbarium specimens present in the Herbarium Bogoriensis, Bogor, Indonesia and Rijksherbarium, Leiden University, The Netherlands. Members of both genera can be distinguished only by minor morphological characteristics. When these characters become known, however, it is relatively easy to discriminate between the two genera. *Parasponia* can be distinguished from *Trema* by its imbricate

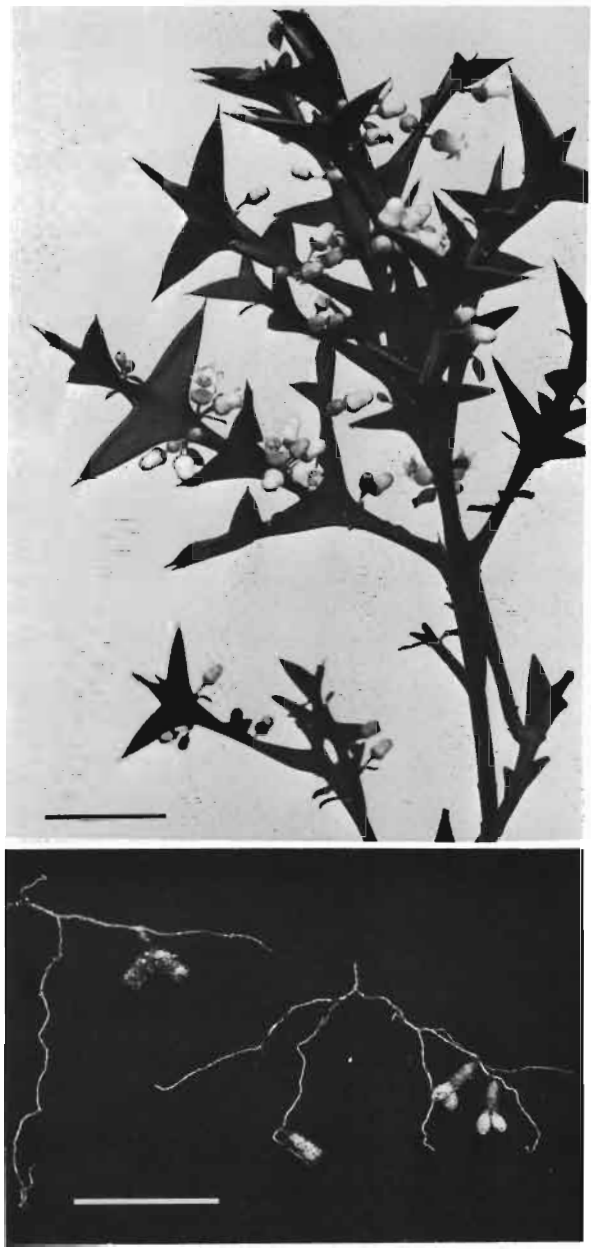

Fig. 3. Colletia paradoxa (Spreng.) Escalante (syn. *C. cruciata* Gillies ex Hook.). (a) Twig of a mature plant showing phyllocladioid stems with spines and flowers; (b) root nodules; bar scale = 2 cm.

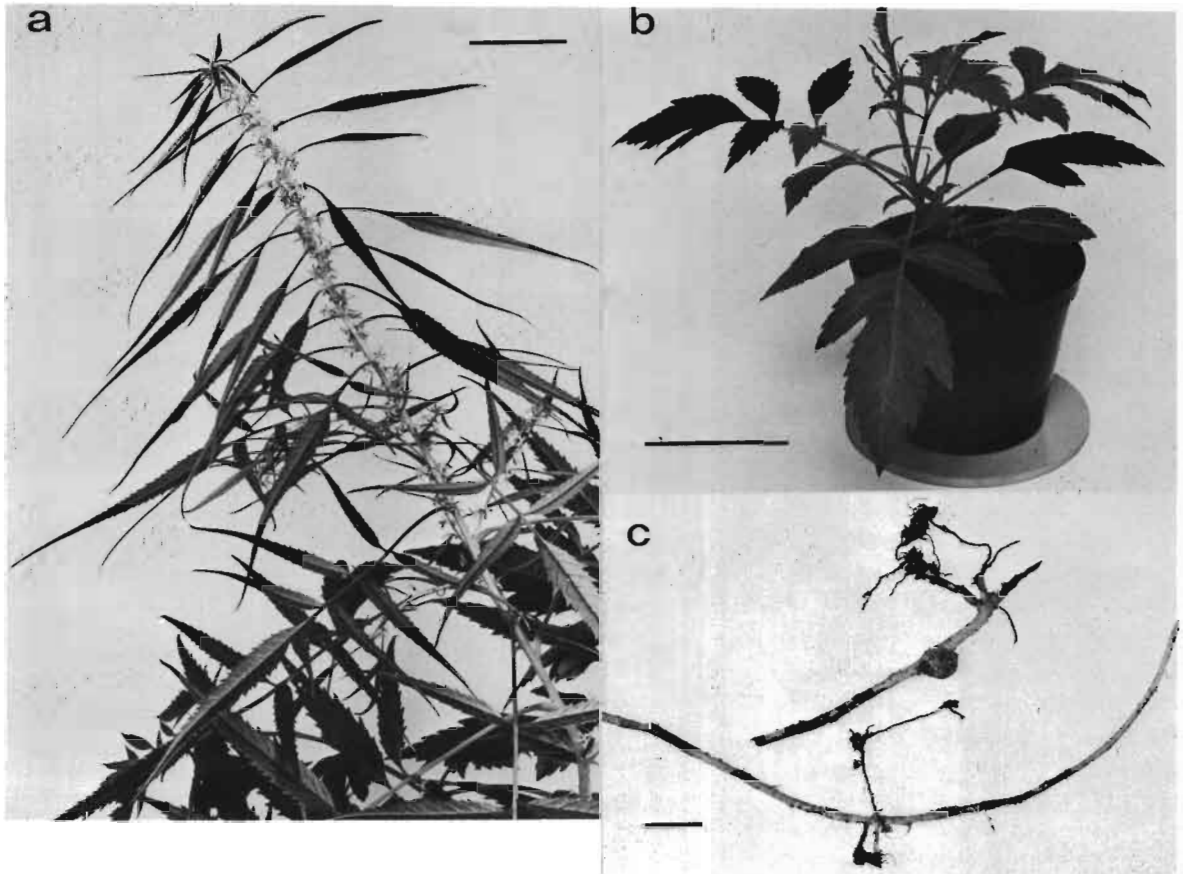

Fig. 4. Datisca cannabina L. (Datiscaceae, Cucurbitales). (a) Twig of mature plant with flowers; bar scale = 3 cm; (b) young plant; bar scale = 6 cm; (c) root nodules; bar scale = 1 cm. (Root nodules are abnormal because they showed no acetylene reduction activity.)

perianth lobes of the male flowers and by the presence of intrapetiolar connate stipules enclosing the terminal bud [11, 113].

Just as the presence of root nodules in *Datisca* was recorded much earlier in the literature, the presence of root nodules in *Parasponia* was recognized at a very early date. In a symposium on 'Green manure in Indonesia', Ham [65] in 1909 called attention to the fact that the non-leguminous tree called 'anggrung' in the Javanese language (central and eastern Java) or 'kuraj' in the Sundanese language (western Java) bore root nodules and that these root nodules probably had the capacity to 'collect nitrogen' as do so many leguminous plant species. The vernacular names 'anggrung' and 'kuraj' are sometimes also used indiscriminately for *Trema* and *Parasponia* species on Java.

Subsequently, Backer and Van Sloten [12] were able to confirm root nodulation in *Trema* in one case, but not in other cases. At that time, however, no rigid discrimination had been made between members of the genera *Trema* and *Parasponia*. It is therefore likely that the species were confused and that the tree bearing root nodules was in fact a *Parasponia* species. This situation was definitely resolved by Clason [49], who demonstrated clearly the pioneer habit of *Parasponia* on the volcanic ash soils of Mt. Kelut (eastern Java, Indonesia) in an extensive study on the regeneration of the flora in that region. He also observed the profuse root nodulation in this species. In addition, he stated very plainly that 'nitrogenous food is possibly obtained in this way' [49]. Dried herbarium specimens of root nodules of *Parasponia* collected at that time by Clason on Mt. Kelut are still present in the herbarium material of the Herbarium Bogoriensis at Bogor, Indonesia, but Clason was not the first who called attention to the pioneer properties of *Parasponia*. Junghuhn [74] had much earlier described *Parasponia* as a pioneer vegetation on Mt. Merapi (central Java, Indonesia) on places cleared by volcanic activity like solidified lava flows. He remarked that it was an active colonizer of bare, virgin soils on this mountain at altitudes between 1500 and 1800 m. Both Junghuhn and Clason attributed the plant species to *Parasponia parviflora* Miq., but herbarium material from these localities has recently been classified as *Parasponia rugosa* Bl [113]. This is the same species found in Papua by Trinick [125] and having a distribution from central Java over the Lesser Sunda Islands, some Greater Sunda Island (Sulawesi), and the Philippines as far as Papua.

Root nodulation in *Parasponia* has now been recorded in three of the five species within the genus, i.e. *P. rugosa* [49, 125], *P. parviflora* [3, 4, 30] (see Fig. 5a–c) and *P. andersonii* [127]. It is likely then that the two other unexamined *Parasponia* species confined to New Guinea are also nodulated.

It is noteworthy that the *Trema* species have a more western distribution than *Parasponia*. It is found in the western parts of the Malaysian archipelago (western Indonesia and Malay Peninsula), Africa, and the American continent, while the representatives of the genus *Parasponia* have a typical Asiatic distribution and are common in the eastern parts of the Malaysian archipelago (i.e. central and eastern Indonesia) as far as New Guinea (Papua).

Rhizobial root nodulation has also been reported in some xerophylic desert

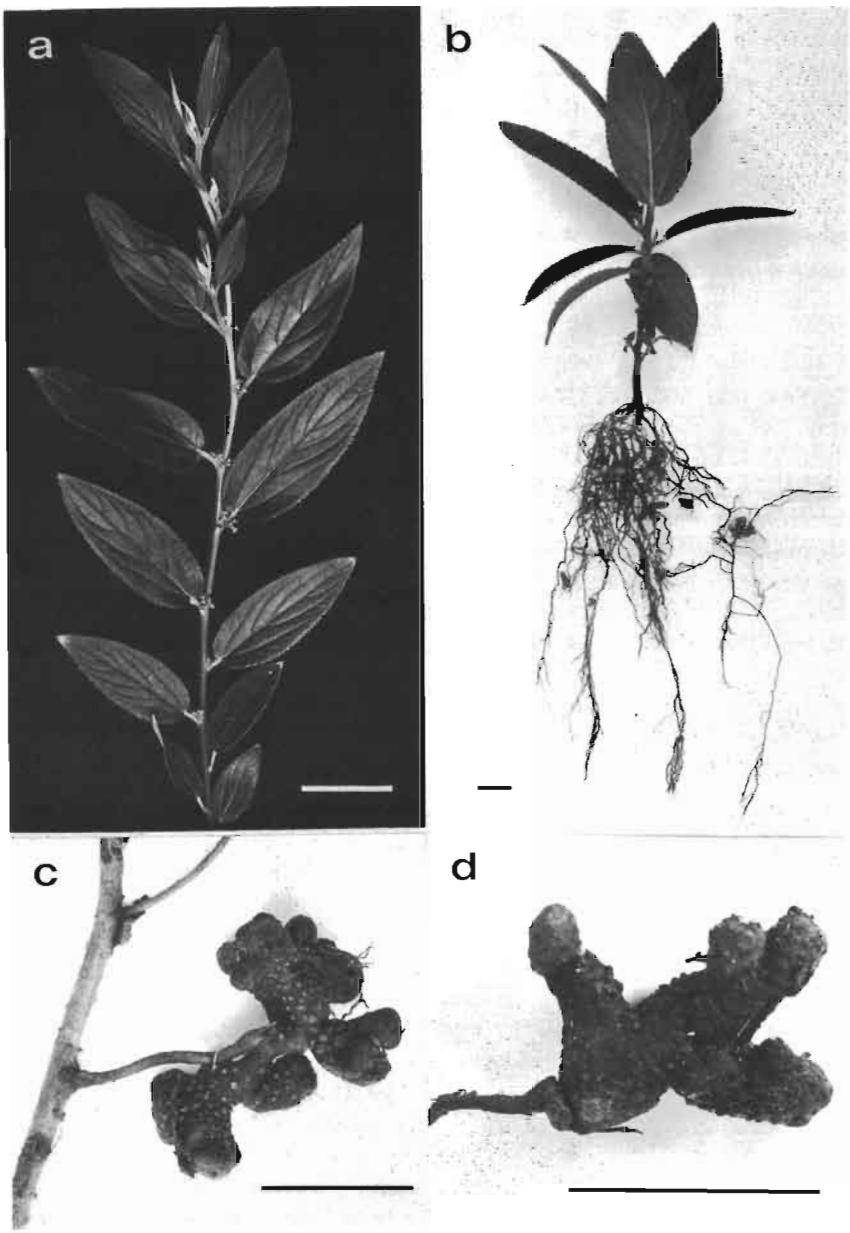

Fig. 5. Rhizobial root nodules of *Parasponia* (Ulmaceae, Urticales). (a) Twig of mature plant with flowers of *Parasponia parviflora* Miq.; bar scale = 3 cm; (b) young nodulated plant of *Parasponia parviflora* Miq.; bar scale = 1 cm; (c) and (d) root nodules; in both bar scale = 1 cm.

plants belonging to the Zygophyllaceae. Sabet [105] observed root nodulation occurring in a number of species of the genera *Zygophyllum* (i.e. *Z. coccineum*, *Z. album*, *Z. decumbens* and *Z. simplex*), *Fagonia* (*F. arabica*), and *Tribulus* (*T. alatus*) found in the poor sandy soils of the Egyptian deserts. Mostafa and Mahmoud [94] reported the isolation of *Rhizobium*-like strains from these root nodules. These strains could be cross-inoculated to produce effective root nodules with some legumes such as *Trifolium alexandrinum* and *Arachis hypogaea*. They reported in addition, however, that some of these zygophyllaceous species apparently had their own nodule bacterium. Both the Sabet and Mostafa and Mahmoud publications noted that when these zygophyllaceous plants were cultivated in sterilized and unsterilized soil, the nodulated plants in the unsterilized soil (or in the sterilized soil after innoculation) grew more vigorously compared to non-nodulated plants, which in due course showed nitrogen deficiency symptoms. The researchers concluded from these experiments that the root nodules supplied the host plant with nitrogen. Athar and Mahmood [9] confirmed the presence of root nodules on zygophyllaceous plants for *Zygophyllum simplex*, *Fagonia cretica*, and *Tribulus terrestris* growing in dry sandy soil low in nutritional elements near Karachi in South Pakistan. From morphological observations and staining techniques, they concluded that the endophyte within the root nodules was a *Rhizobium* species. The author of this chapter was able to examine *Zygophyllum coccineum* plants in Egyptian desert soil alongside the road between Cairo and Suez. Nodular structures were observed along the roots (Fig. 6b). Subsequent acetylene reduction tests carried out in the field *in situ* with this material, however, revealed no nitrogenase activity of these root nodules (Becking, unpublished).

3. Biology of the symbioses

3.1. Actinorhizal symbioses

About 175 plant species covering 17 genera, 8 families, and 7 orders of worldwide distribution have been reported to bear actinomycete-induced root nodules with N_2-fixing capacity (Table 1). The actinomycete involved has been classified to belong to the genus *Frankia* of the family Frankiaceae [22, 25].

Actinorhizal root nodules are morphologically and anatomically distinct from legume root nodules. Two main types can be distinguished (Fig. 7a–d). In the *Alnus* type, a coralloid root nodule, usually lacking nodule roots, is formed by dichotomous branching. The nodular lobes originate from lateral roots with inhibited or very slowly growing apical meristems. In the *Myrica/Casuarina* type of root nodule, the apex of each nodule lobe, in contrast, produces a normal but negative geotropic root. In this way the root nodule becomes clothed with upward growing rootlets. The distinction between these two root nodule types is, however, not clearcut. Thus, *Alnus rubra* inoculated with the *Alnus glutinosa* endophyte produces root nodules in which the nodular lobes give rise to negative-geotropic

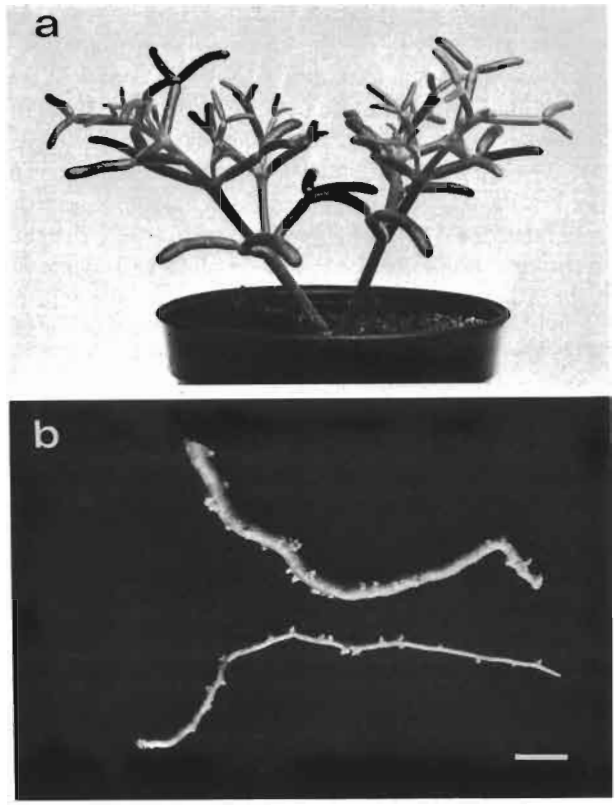

Fig. 6. Zygophyllum coccineum L. (Zygophyllaceae, Malpighiales). (a) Young plant; (b) root nodules; bar scale = 1 cm.

terminal rootlets (see Fig. 7d), and [20, 21, 23] and *Casuarina equisetifolia* growing in the field often exhibits coralloid root nodules reminiscent of the *Alnus* type.

As pointed out earlier by Becking [20, 21, 23, 26], the very initial root-nodule development of these actinomycetous root nodules is different from that of leguminous plants. In actinorhizal symbioses, a pre-nodule is first formed, with a morphological structure apparent only as a slight thickening of the main root. Pre-nodules appear in the longitudinal section to consist of only a few host-cell layers, and only a restricted number of cortical cells are invaded by the *Frankia* species coming from the root hair. From this pre-nodule stage, the true root nodule is formed by a lateral root primordium developing from meristematic proliferation of pericycle and mid-cortical cells of the main root. Not until a later stage of root-nodule development does the *Frankia* infection progress into the lateral root initiated within the pre-nodule. In the pre-nodule stage, the endophyte is already partially present in its vesicular form within a cortical parenchyma cell layer of only 3–4 cells thick[21, 26]. These pre-nodules, however, do not fix N_2. Glass-slide techniques have shown that pre-nodule initiation is accompanied by an extensive

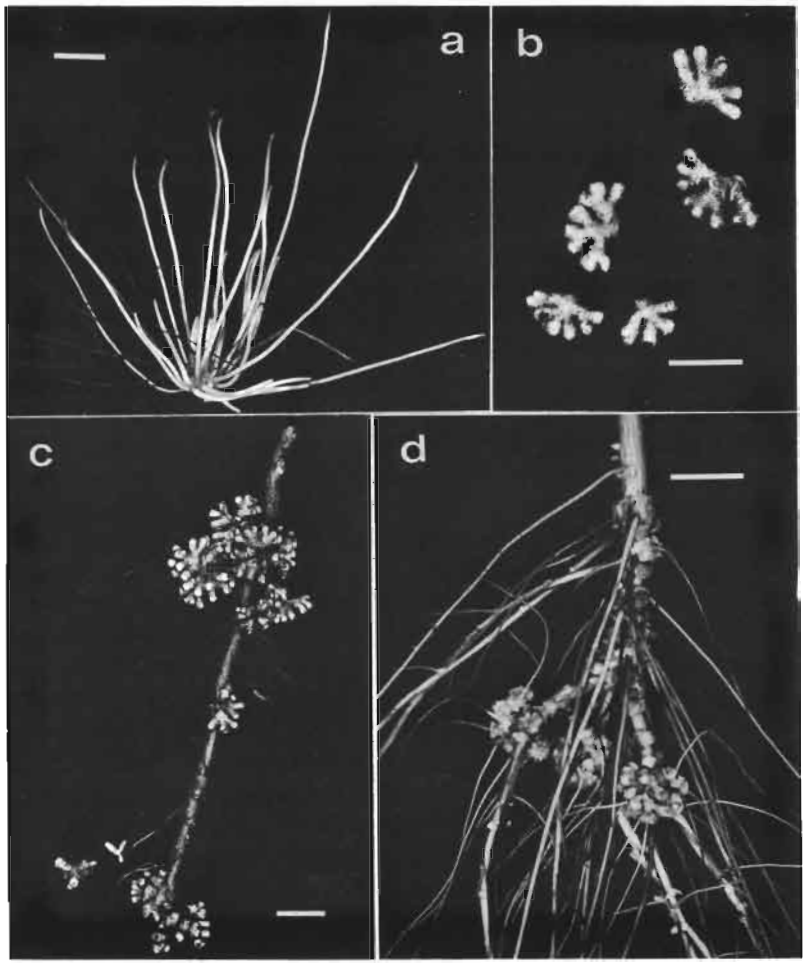

Fig. 7. Alnus and *Myrica/Casuarina* type of root nodules. (a) *Casuarina equisetifolia* L. root nodules showing that the apex of each nodule lobe gives rise to a negative geotropic root; (b) detached and divided root nodules of *Alnus glutinosa* L. (Gaertn.), showing dichotomous branching of nodule lobes; (c) coralloid root nodule of *Alnus glutinosa* L. (Gaertn.); (d) root nodules of *Alnus rubra* produced by an *Alnus glutinosa* inoculum. The nodular lobes produce some negatively geotropic root like those in *Casuarina* spp. and *Myrica* spp. In all figures bar scale = 1 cm.

curling of the initially straight root hairs at the infection site [21, 23, 26]. This stimulus is probably due to the production of auxin or related growth substances by the endophyte. At least one root hair becomes infected with the endophyte at the infection site. Torrey [122], Callaham and Torrey [43], and Torrey and Callaham [124] stated that only one or very few root-hair infections give rise to pre-nodule formation, a situation apparently different from that of leguminous

root nodules. In the case of the latter, root-hair infections and abortive infections are generally associated with the root-nodule formation. Penetration of the actinomycetes into the root hair comes at the root-hair tip, and subsequent events observed in the root hair are very reminiscent of those observed in leguminous root nodules. A pocket is formed at the root-hair tip by the invagination of the host-cell wall, and at this point an infection thread containing actinomycete cells develops inside the host cell. During its growth, the thread is accompanied by the host cell nucleus [21, 26]. When the infection thread reaches the main root, it is still sheathed by cell-wall material made up presumably of cellulose, pectins, and polysaccharides produced by the host. While this encapsulation material surrounds all the endophyte filaments throughout its entire life as endophyte in living host cells, it is absent when the host cell is dead or has been killed by the action of the endophyte. In the latter case, another form of the endophyte develops, characterized by the presence of spores (formerly called granulated bodies) liberated from sporangia [32, 130, 131]. When the *Frankia* endophyte invades new cortical parenchyma cells, penetration is probably the result of dissolution of the host-cell wall on its path from cell to cell as seen in micrographs (Fig. 8). Sometimes the endophyte does not pass the host cells intra-cellularly, but it continues its path for some distance intercellularly between host cells before re-entering a new host cell. When the endophyte proceeds from one cortical parenchyma cell to another, it apparently stimulates hypertrophy and cell division in the host-cell tissue. The enlarged host cells and the large nuclei they possess suggest that they are polyploids, but definite proof is still lacking. Moreover, it is worth noting that the endophyte does not penetrate a zone of cortical parenchyma cells adjacent to the epidermis nor those cells close to the endodermis surrounding the stele [21, 26]. This restriction in growth is probably the result of a physiological interaction, presumably hormonal, between the plant and the endophyte.

The study of the fine structure of the endophyte has revealed the presence of hyphae, vesicle structures, and spore-like particles seen through transmission and scanning electron microscopy mainly in the *Alnus* endophyte [24, 25, 26, 27, 32]. An *Alnus glutinosa* host cell containing vesicular endophyte structures is shown in the scanning electron micrograph of Fig. 9, while mature spore-like endophytic structures seen through transmission electron microscopy are presented in Fig. 10. Transmission of these spores to adjacent host cells is shown in transmission electron micrograph (Fig. 11). Gardner [61] studied the fine structure of other non-leguminous root nodules, such as those of *Hippophaë rhamnoides*, *Myrica gale* and *M. cerifera*, *Ceanothus velutinus*, and *Casuarina cunninghamiana*. Strand and Laetsch [115, 116] studied the cell and endophytic structures of the root nodules of *Ceanothus integerrimus* extensively. Finally, Lalonde and Knowles [81, 82], Lalonde and Devoe [80], and Lalonde *et al.* [84] contributed more detailed cytological studies on the sheathed material around the endophyte and on the origin of the membrane envelopes. They used cytological staining techniques in combination with transmission electron microscopy and freeze-etching microscopy, all in the *Alnus crispa* var. *mollis* root nodules.

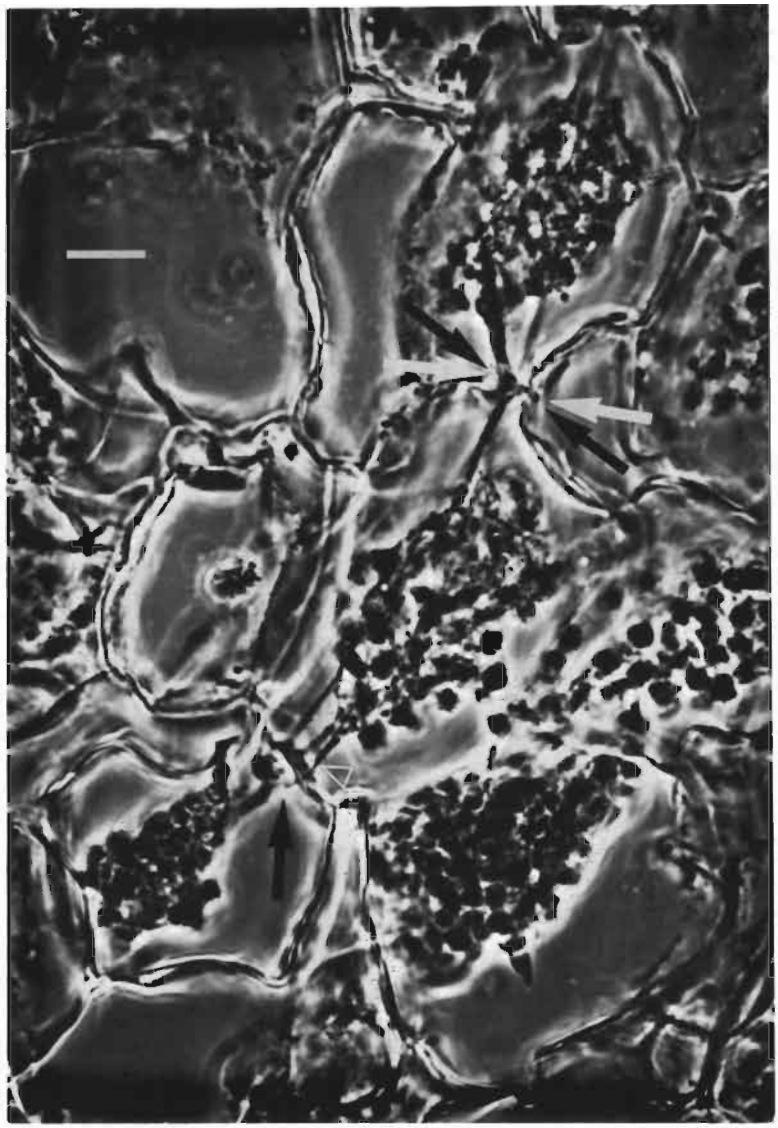

Fig. 8. Paraffin-wax section through a root nodule of *Alnus glutinosa* L. (Gaertn.) showing the perforation of the host-cell wall by the hyphae of the *Frankia* endophyte penetrating the successive host cells. In the center of the host cell, vesicular endophytic structures are visible; bar scale = 10 μm.

Root nodule initiation and root nodule growth of the *Myrica/Casuarina* root nodules have been studied extensively by Torrey and his group [42, 43, 95, 122, 123).

In the *Myrica/Comptonia* symbiosis, as in the *Alnus* symbiosis, a pre-nodule is

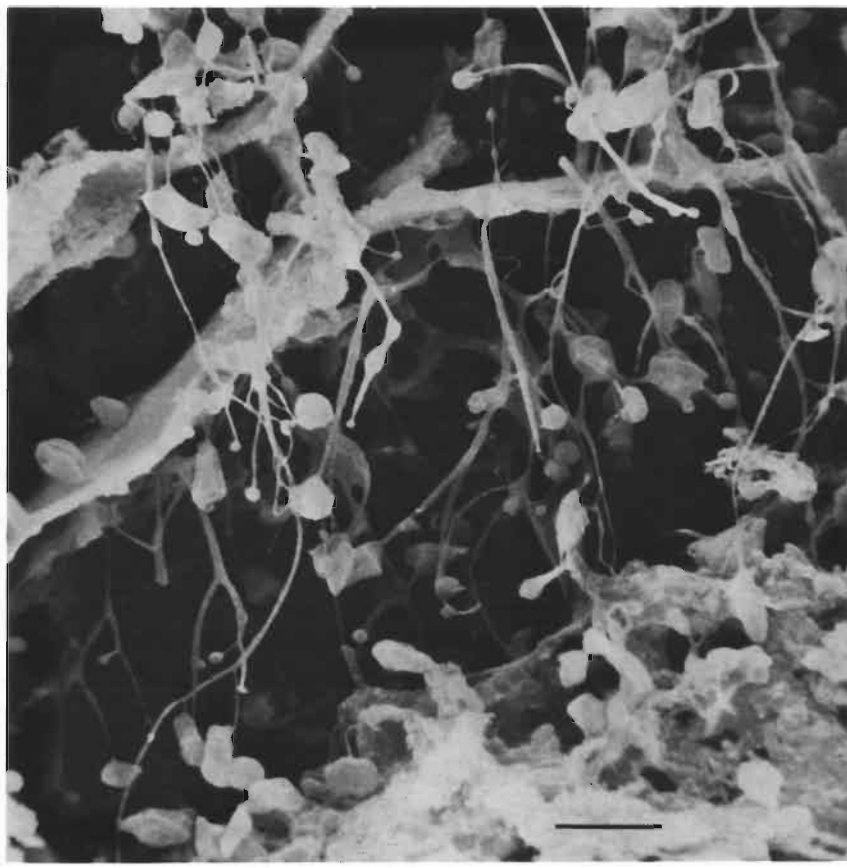

Fig. 9. Scanning electron micrograph of an opened host cell of *Alnus glutinosa* L. (Gaertn.) showing the vesicular structures of the *Frankia* endophyte at the tip of the hyphae; bar scale = 4 μm.

formed subsequent to root-hair infection. This stage is succeeded by three different phases of root-nodule development: nodule-lobe formation, a transitional or arrested stage of variable duration, and nodule-root primordium originating endogen-nodules, the primary nodule lobe is a lateral root primordium originating endogen-ously within the pre-nodule with a formation involving pericycle, endodermis, and cortical cell derivates. The nodule lobe develops slowly as the cortical parenchyma cells are invaded by the actinorhizal endophyte. After a period of arrest of variable duration, from a few days to several weeks, the nodule-lobe meristem begins altered developments, forming the elongate nodule root, which undergoes slow but continuous growth to about 3 to 4 cm in final length. New nodule-lobe primordia are initiated endogenously at the base of the existing nodule lobes, ultimately forming a cluster of nodule roots. Each nodule root has a terminal apical meristem with reduced root-cap formation and a modified root structure possessing an

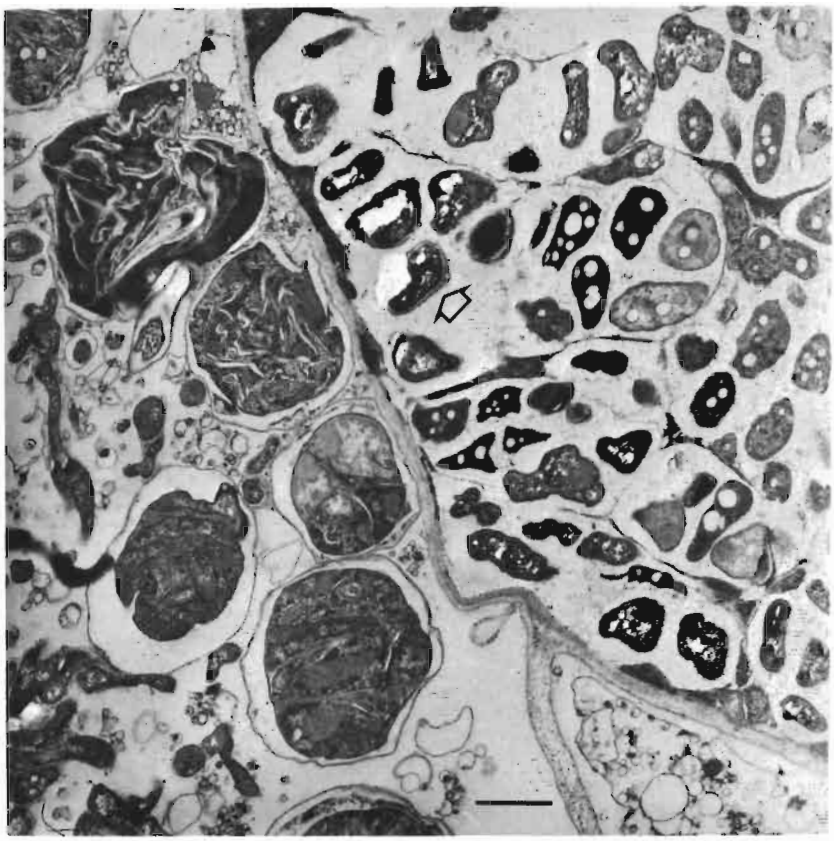

Fig. 10. Transmission electron micrograph of vesicles and hyphae (to the left) and mature spores (to the right) of the *Frankia* endophyte within the host cell. Note the dead host cell devoided of cytoplasmic content in the plant cell containing spores. The cell wall of the spore is very thick (see arrow) and these spore structures probably function as 'Dauerspores' for the survival and transmission of the endophyte; bar scale = 1 μm.

elaborate cortical intercellular space system and a reduced central cylinder. The endophyte is restricted to cortical cells of the nodule lobe and is totally absent from tissues of the nodule root. A probable function of the nodule roots in *Myrica/Comptonia* is to facilitate gas diffusion to the N_2-fixing endophyte site in the nodule lobe when nodules occur under conditions of low oxygen tension.

An anatomical study of nodule formation in *Casuarina* showed essentially the same sequence of events as the above-described observation for *Myrica/Comptonia* root nodules. Anatomical analysis of nodule formation in *Casuarina* revealed that the large number of nodule lobes formed are the result of repeated endogenous lateral root initiations, one placed upon another in a complexly branched and truncated root system. The endophyte-infected cortical tissues derived from successive root primordia form the swollen nodular mass [122].

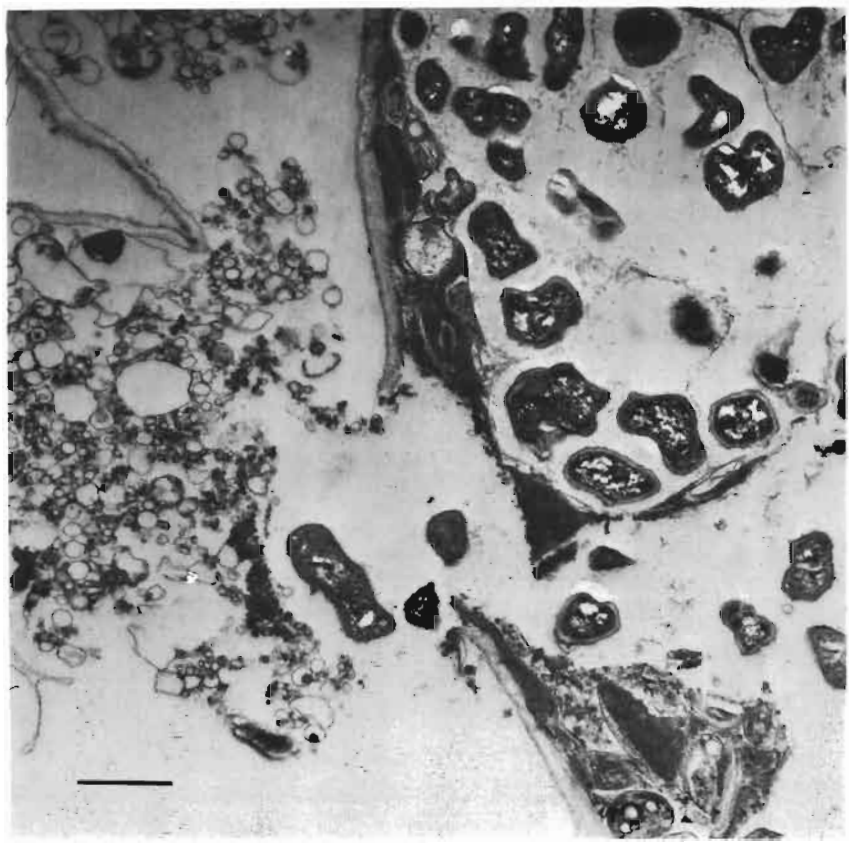

Fig. 11. Transmission electron micrograph of host cells of *Alnus glutinosa*, showing the transmission of spores of the *Frankia* endophyte from a dead host cell to an adjacent living host cell by the rupture of the host-cell wall; bar scale = 1 μm.

3.2. Rhizobium symbioses

The internal structure of the root nodule of *Parasponia rugosa* (formerly classified as *Trema aspera* or *T. cannabina* var. *scabra*) has been described by Trinick [125, 126] and Trinick and Galbraith [128]. Initially the nodule structure was described as bearing some resemblance to that of leguminous plants, but later, more affinities with non-leguminous root nodules were found. In transection the root nodules show a central vascular bundle with bacteroid infected host-cell tissue forming a horseshoe-shaped zone around it. The central vascular stele is a typical feature of non-leguminous root nodules. As in other non-leguminous root nodules, *Parasponia* nodules possess an apical meristematic zone which provides for the continuous elongation of the nodules. The infection thread has been observed to persist in this form and to penetrate the newly formed host cells immediately behind the apical

meristem. The infection thread can either pass directly through the host-cell wall to infect other cells, or it can enter young host cells from the intercellular spaces between the host cells. As in legumes and the actinorhizal non-legumes nodulated by the *Frankia* endophyte, the host cells invaded by the *Rhizobium* endophyte become hypertrophied. However, in contrast to the infection threads in leguminous root nodules, the *Rhizobium* cells in *Parasponia* species are very rarely released from the infection threads. Usually the infection thread continues to grow until it has completely filled the host cell. The wall of the infection thread was found by the above-mentioned investigators [126, 128] to be continuous with the host-cell wall, and therefore probably to consist of cellulose and pectins. The persistence of infection threads within host cells appears to be a peculiar feature of *Parasponia* root nodules. Because more than two thirds of the infected host cells throughout the nodule contained these structures, it was presumed that N_2 fixation occurred in the bacteria within the thread structures. Thus, bacteria inside the infection threads may have properties similar to bacteroids enclosed in a membrane envelope found in leguminous host cells.

A study of the internal structure of *Parasponia andersonii* root nodules showed that the infection-thread structures were even more dominant in this species than in the preceding species, since *Rhizobium* cells had never been found to be released from the infection threads into the host cytoplasm [127]. The persistence of the thread structures was confirmed by an examination of serial sections of host cells containing either little or very extensive infection. The absence of 'released' rhizobia in the light microscope sections may be due to either the improved resolution obtained in the Araldite-embedding material in comparison with wax used in the study with *Parasponia rugosa* root nodules [128] or to a difference between the species. Transmission electron micrographs showed that the walls of the threads varied greatly in thickness, and thread structures were often observed without a rigid wall and enclosed only by a cytoplasmic membrane. In view of the observed differences between the two *Parasponia* species, it was surmised that the two species probably represented different evolutionary stages of the *Rhizobium* infection [127].

A preliminary study of *Parasponia parviflora* root nodules from Java, Indonesia by Becking [30] showed that these nodules were of the coralloid type. They differ from the *Alnus* root-nodule type of *Frankia* symbioses, however, in that nodule branching is more irregular and not strictly dichotomous. Moreover, the larger root nodules of the *Alnus* type are usually sessile on the thicker roots, whereas the *Parasponia* root nodules often shown thin bases (Fig. 5c and d) at the point of attachment with the main root. Becking found bacteroids to be regularly released into the host cytoplasm. When released, however, these *Rhizobium* cells maintain their normal straight shape (Fig. 12) and do not become distorted as is usually the case in bacteroids of leguminous plants. Moreover, a large proportion of the rhizobial cells are found in thin-walled membrane envelopes consisting of a double-layered cytoplasmic membrane (Fig. 13). These membrane structures are long and thin, and within the host cell, the longitudinal axis of the envelopes is often orientated

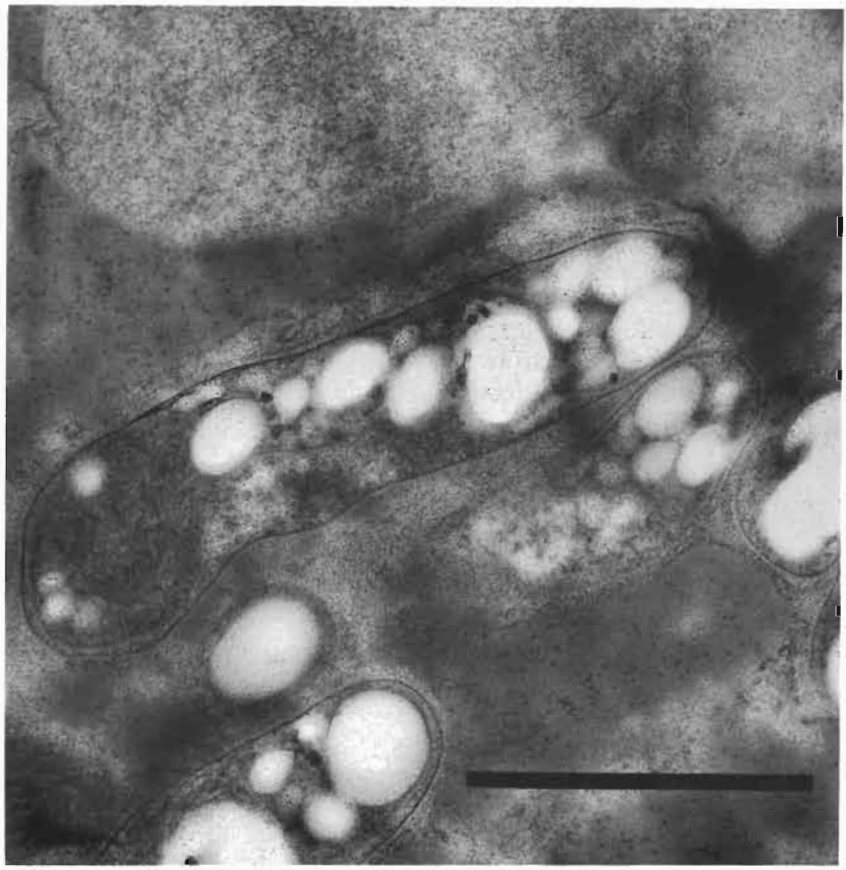

Fig. 12. Transmission electron micrograph of a free *Rhizobium* bacteroid in the cytoplasm of *Parasponia parviflora* Miq. root nodule cell. The reserve material inside the bacterium is poly-β-hydroxybutyrate; bar scale = 1 μm.

in the same direction as adjacent membrane envelopes. In extreme cases, thread-like envelope structures may develop (Fig. 14). In these elongated membrane structures, about 5–25 bacteria can be generally counted in cross-section [30], but sometimes there is only one. The total number of *Rhizobium* bacteroids within these membrane sacks or stretched envelopes in one direction is certainly a multiple of that observed in cross-section. In the membrane envelopes the individual *Rhizobium* cells are usually orientated with the long axis parallel to the long axis of the membrane structure or thread. The thread-like structures are usually very uneven and variable in length and width, so one has the impression that the thicker threads are either composed of multi-layered threads or that they become thicker through some internal growth process (Fig. 14).

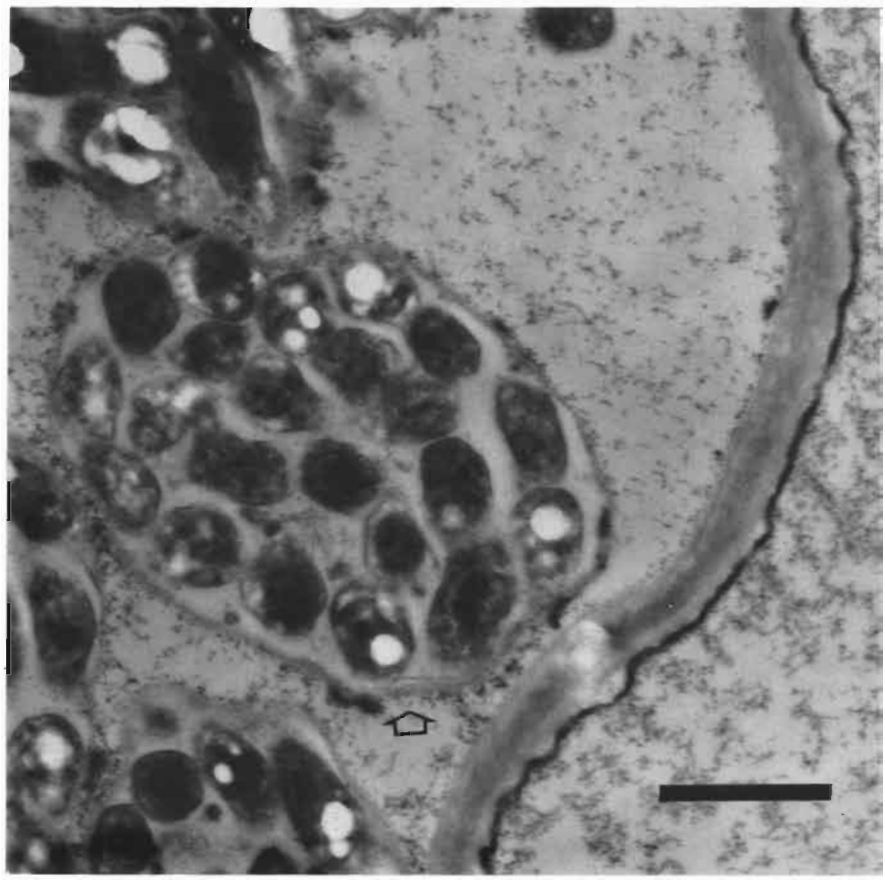

Fig. 13. Transmission electron micrograph. Transverse section through the membrane envelope enclosing *Rhizobium* cells in a *Parasponia parviflora* Miq. host cell. Note the living cytoplasm of the host cell and the double-layered cytoplasmic structure of the envelopes (see arrow); bar scale = 1 μm.

4. Isolation of the endophyte

4.1. Actinorhizal symbioses

Most of the earlier literature concerning isolation of the endophyte is summarized by Becking [21, 23, 26, 27, 28]. One study particularly worth mentioning was conducted by Pommer [97] who claimed that he had isolated the root-nodule endophyte of *Alnus glutinosa*. The isolate was in general morphologically very similar to the symbiotic endophyte (*Frankia* sp.) observed within the root-nodule cells, and moreover, it could induce root nodulation in aseptically grown *Alnus glutinosa* seedlings within 4–6 weeks. The isolate grows, although very slowly, on agar plates

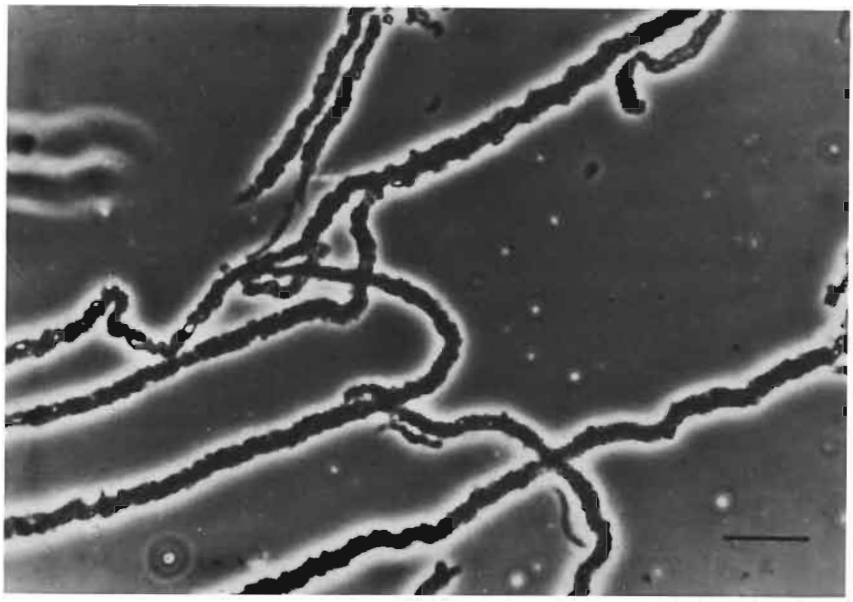

Fig. 14. Thread-like structures in *Parasponia parviflora* Miq. host cells enclosing *Rhizobium* bacteria. Note the variable thickness of the threads; phase contrast micrograph; bar scale = 1 μm.

containing a simple glucose-asparagin agar supplied with *Alnus* root-nodule extract and exposed to normal atmospheric conditions. Although it is likely that Pommer really isolated the endophyte (unfortunately the isolate was subsequently lost, Pommer personal communication), this experiment could not be repeated by others, e.g. Quispel [98] and Becking (personal observation).

By employing a complex nutrient medium used for the *in vitro* cultivation of plant tissue, Becking [19, 21, 23, 26] isolated the root-nodule endophyte of *Alnus glutinosa*. The actinomycetous endophyte thus obtained did not produce growth outside the plant tissue on this medium. The endophyte only invaded new-formed callus tissue very slowly, and because of this insufficient transmission, the endophyte was often lost in the subsequent callus explants. The isolated endophyte in this monoxenic culture produced root nodulation in axenic *Alnus glutinosa* seedlings, but the resulting root nodules contained only the hyphal form of the *Frankia* endophyte and no vesicles. Most likely as a result, these root nodules were ineffective with respect to nitrogenase activity [19].

Lalonde *et al.* [83] reported the isolation of the *Alnus crispa* var. *mollis* endophyte on a rather complex medium used for growth of plant tissues [66]. The isolate lost its resemblance to the nodular endophyte after several subcultures, and it failed to produce nodulation in aseptic *Alnus crispa* var. *mollis* seedlings. Extensive immuno-labelling tests were carried out in order to demonstrate that the isolate was an ineffective strain of the nodular endophyte. Since the substances involved in these immuno-labelling reaction are unknown and may be also present

in other actinomycete species (as previously shown by these authors in the same publication), definite proof of the strains identity with the nodular endophyte cannot be given. In fact, it is rather unlikely that this strain is the endophyte.

Using micro-dissection techniques and enzyme degradation, Callaham *et al.* [44] were able to isolate a slow growing actinomycete from the root nodules of *Comptonia peregrina* in a medium containing sucrose, mineral salts, amino acids, and vitamins, or in a yeast extract mannitol/sucrose medium supplemented with growth substances (thiamine-HCl, nicotine acid, pyridoxin-HCl, etc.) used in standing liquid medium in petri dishes. The isolate grew very slowly in axenic culture in the medium under these conditions, but somewhat better growth was obtained in yeast extract liquid medium (Difco Yeast Extract, 0.5% w/v) in unshaken test tubes filled only with 5–6 cm medium. The isolate appeared to be micro-aerophilic, since it grew best at the bottom of tubes of liquid medium, but it failed to grow under complete anaerobiosis. The isolate was able to produce root nodulation in *Comptonia* seedlings kept in sand culture in the greenhouse. Using the same isolation procedure as for the initial isolation, Callaham *et al.* were able to reisolate the endophyte from the nodules. According to the authors, the Koch's postulate was in this way fulfilled. It is reasonable to assume that the real endophyte was involved. However since the experiment was not conducted in a monoxenic culture with axenic *Comptonia* seedlings, the conclusion that Koch's postulate was fulfilled is disputable. Another factor which weakens that conclusion is the appearance in one of the experiments of a number of nodules in the uninoculated controls caused by contamination. The independent evaluation tests performed by Lalonde [78] suffer from the above-mentioned challengeable immuno-labelling reactions and therefore are inconclusive. Moreover, although surface-sterilized *Comptonia* seeds were used at the start of the experiment, no tests have been reported to verify how long the monoxenic conditions of the plant cultures were maintained.

New isolation techniques or modifications of the earlier method of Callaham *et al.* [44] have led to the isolation of several *Frankia* strains from root nodules of different non-leguminous plant species. Using a technique of sucrose-density sedimentation the researchers were able to separate the actinomycete from the root nodule and in this way isolate *Frankia* species from the root nodules of *Elaeagnus umbellata* (Elaeagnaceae) and *Alnus viridis* ssp. *crispa* [14, 15]. In these experiments, the sucrose-density fractions were poured on agar plates with a nutrient medium containing yeast extract, 0.5%; dextrose, 1.0%; casamino-acids, 0.5%; H_3BO_3, 1.5 mg l^{-1}; $ZnSO_4 \cdot 7H_2O$, 1.5 mg l^{-1}; $MnSO_4 \cdot 2H_2O$, 4.5 mg l^{-1}; $NaMoO_4 \cdot 2H_2O$, 0.25 mg l^{-1}; $CuSO_4 \cdot 5H_2O$, 0.04 mg l^{-1}; vitamin B_{12}, 1.6 mg l^{-1}; and Difco agar, 0.8%. The plates were sealed with Parafilm and incubated at 28 °C. Colonies of a finely filamentous actinomycete appeared on the plates after approximately 3–5 weeks.

Quispel and Tak [100, 101] stressed the use of an extract with petrol ether-soluble substances from Alder roots (or root nodules) to the nutrient medium for isolating and growing the *Frankia* species from *Alnus glutinosa* root nodules.

They succeeded in isolating what they called the spore$^-$ type of endophyte, but they failed to grow the *Alnus glutinosa* spore$^+$ type of endophyte under these conditions.

Gauthier *et al.* [62] were able to isolate two *Frankia* strains from the root nodules of *Casuarina equisetifolia* by plating 1 ml of a 10^{-3} suspension of young nodules on a so-called QMOD medium, i.e. a medium containing per liter deionized water: yeast extract (BBL), 500 mg; Bacto-Peptone (Difco), 5 mg; glucose, 10 g; KH_2PO_4, 300 mg; NaH_2PO_4, 200 mg; $MgSO_4 \cdot 7H_2O$, 200 mg, KCl 200 mg; ferric citrate (citric acid and ferric citrate, 1% sol.), 1 ml; some minor salts; lipid supplement, agar 15 g [79]. Small colonies (ca. 0.1 mm in diameter) appeared on this medium in 3–4 weeks and the microscope had to be used to observe and isolate the strains. The *Frankia* colonies could be easily discriminated from those of other bacteria (contaminants) by their typical 'starfish-like' appearance. These strains of *Frankia* were unable to nodulate *Casuarina equisetifolia* but effectively nodulated *Hoppohaë rhamnoides* (Gauthier, personal communication). The absence of nodulation with *Casuarina equisetifolia* is not yet understood.

5. Taxonomy and morphology

The endophyte of actinomycetous root nodules have been attributed to the genus *Frankia* of the family Frankiaceae by Becking [22, 25]. The taxonomic division in species was based on the morphology of the endophyte within the host tissue, such as the structure and dimensions of the hyphae, spherical vesicular bodies or club-shaped structures at the tip of the hyphae, and the presence or absence of spore-like endophytic cells, sometimes also called 'bacteria-like' cells, 'bacteroids', or 'granulae'. Moreover, analogous to the subdivision of the genus *Rhizobium*, use was made of cross-inoculation groups and an attempt was made to determine cell-wall constituents of the endophyte, because the classification of the actinomycetes is based on major cell-wall types [86].

Originally, the endophyte was described to be an obligate symbiont [22] because of the researcher's failure to isolate the species. Later, however, it was observed that there was considerable growth of the endophyte as a hyphae in monoxenic cultures in the rhizosphere of *Alnus glutinosa* seedlings [26]. In addition it was noted that there must be a free stage or viable form of the endophyte in soil, because it could induce root nodulation in axenic *Alnus* seedlings. For this reason, the term 'obligate' in respect to the association has purposefully been omitted in the description of this group in Bergey's Manual [37]. The above-mentioned description distinctly states that there is a free stage of the *Frankia* endophyte in soil and that the endophyte is moreover probably micro-aerophilic [25]. The latter property was proven to be the case after its final isolation [44].

The vesicular or club-shaped structures at the tip of the hyphae in *Frankia* species are probably degenerated sporangia modified under the influence of the host cytoplasm and very probably associated with N_2 fixation. This conclusion

is based on the observation that nodule slices containing the endophyte predominantly in vesicular form have more N_2 fixation (acetylene reduction) than similar slices containing the endophyte as hyphae or spore-like structures, but lacking vesicles [25, 29]. Such has been proven in *Frankia* sp. CpI1 strain cultured in pure culture in a defined nutrient medium, where the acetylene reduction activity of the culture appeared simultaneously with vesicle formation [121]. N_2 fixation (acetylene reduction) in pure culture was also established in *Frankia* sp. strain isolated from *Casuarina equisetifolia* root nodules. In these strains, the highest specific activity of acetylene reduction was observed when p_{O_2} was 10 kPa [62].

Cell-wall analyses performed in search for diaminopimilic acid (DAP) using paper chromatography and chemical methods revealed the presence of meso-DAP (but not LL-DAP), arabinose, galactose and glycine in the *Frankia* species of *Alnus glutinosa* root nodules. The cell-wall preparations were obtained by a filtration technique of nodule-tissue homogenates using filter cloth of different mesh and in which isolated hyphal and vesicular endophytic clusters were sedimented on cloth of 10 μm mesh. These endophyte clusters were carefully freed from adhering host-cell components by repeated washings with phosphate buffer. The endophytic cells were subsequently ruptured by ultra-sonic treatment and the sub-cellular fragments thoroughly washed with buffer in order to remove cytoplasmic constituents prior to hydrolysis and cell-wall analyses [25, 26, 27]. Although we could find DAP in the *Alnus* endophyte, we did not detect it in the *Casuarina* endophyte, and Quispel [99] reported its absence in the *Frankia* species of *Myrica gale* root nodules.

The division of the *Frankia* species in cross-inoculation groups has been subjected to criticism, since in a number of cases cross-inoculation was observed between representatives of different groups, e.g. the effective nodulation and N_2 fixation in the *Frankia* sp. CpI1 strain isolated from root nodules of *Comptonia peregrina* in *Alnus* species. Such compatibility is, however, not a reason to reject the general division, because incompatibility barriers are also not strict for certain *Rhizobium* strains and have apparently not devaluated the existing classification of this genus according to cross-inoculation groups. Rodriguez-Barrueco and Bond [103] observed that an *Alnus glutinosa* inoculum (crushed nodules) produced root nodulation in *Myrica gale*, but that the reciprocal combination of *Myrica gale* inoculum tested on *Alnus glutinosa* plants did not give nodulation. Recently, however, Miguel *et al.* [91] mentioned that they could produce root nodulation with the reverse combination. These authors also observed that a *Hippophaë rhamnoides* inoculum produced root nodulation in *Myrica gale*, *Coriaria myrtifolia* and *Elaeagnus angustifolia*, but that this inoculum did not nodulate *Alnus glutinosa.* Most of these experiments were, however, performed in water cultures under non-axenic conditions using an inoculum of crushed root nodules of water- or field-grown plants. Therefore an unambiguous proof cannot be presented because the involvement of other actinomycetes can not be excluded.

Further, a distinction between normal and abnormal combinations should probably be made, since in the combination of the *Alnus glutinosa* endophyte in

the *Myrica gale* host, the appearance of the endophyte within the host is abnormal and more like that of the *Alnus* endophyte (Rodriguez-Barrueco, personal communication). Moreover, the N_2-fixing capacity of this combination is much lower than usual. In addition, the normal endophytes of *Alnus* and *Myrica* cannot be identical, since the *Alnus* endophyte contains DAP and the *Myrica* endophyte does not, and this presence or absence of DAP in the cell walls of actinomycetes has diagnostic value [17, 18, 85, 87].

Analyses of the cell-wall components of the free-living actinomycete isolates from root nodules growing in axenic culture [14, 15, 16, 44, 62] will definitively give an insight into the taxonomy and interspecific relationships of *Frankia* species.

5.1. Rhizobium symbioses

The isolation of the rhizobial endophyte of non-leguminous root nodules (*Parasponia* spp.) does not offer any problems. Homogenates of surface-sterilized root nodules treated with ethanol 70% (v/v), and subsequently with hydrogen peroxide 6% (v/v) plated on agar medium containing mannite and yeast extract produced good rhizobial growth. This medium has the following composition: distilled water, 1000 ml; yeast extract, 1.0 g; mannite, 10 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NaCl, 0.1 g; $CaCO_3$, 3.0 g; trace-element solution of Allen and Arnon [5], 1 ml; agar, 12 g (pH 6.8–7.0)(Becking, unpublished). The *Rhizobium* strains often appear on these plates as a sole organism; in other cases (with less severe surface disinfection), they are at least the dominant organism.

6. Biochemical progress

6.1. Nitrogenase activity

N_2 fixation by nodule breis of non-leguminous plants using $^{15}N_2$ and C_2H_2 was first reported by Sloger and Silver [111, 112] and Sloger [110]. The nitrogenase activity of the nodule breis was found to be much lower than that of intact nodules. The presence of a reducing agent (sodium dithionite) and the absence of O_2 was essential during homogenization, but O_2 was required, presumably for the production of ATP, during exposure of the homogenate.

Van Straten *et al.* [132] and Akkermans *et al.* [2] reported reasonable nitrogenase activity in nodule homogenates with the acetylene reduction assay, as long as 0.3 *M* sucrose and 100 m*M* dithionite ($Na_2S_2O_4$) were present during anaerobic homogenization. The addition of Na-dithionite was essential in this experiment, because the nodule material contains large amounts of phenolic compounds which after oxidation inhibit nitrogenase activity. The reaction was found to be ATP-dependent, but strangely enough, the addition of an ATP-generating system (creatine phosphate (Cr ~ P)/creatine phosphokinase or phospho (enol) pyruvate

(PEP)/pyruvate kinase (PK)) decreased the nitrogenase activity during short-term experiments. Apparently, Cr ~ P and PEP inhibited acetylene reduction, but the nature of this inhibition could not be elucidated. The observed nitrogenase activity concerned mainly the vesicle clusters, as 60% of the homogenate activity was recovered by the resuspended residue from a 10 μm filter. The 10 μm filtrate consisting of hyphal fragments and disrupted vesicle clusters contained only 29% of the activity of the homogenate. The latter activity was all particle bound. The remaining 11% of the activity was lost during the procedure. This finding suggests again that the sites of N_2 fixation are the vesicles confirming earlier experiments of strong tetrazolium-reducing activity of the vesicles [1] and that tissue slices of nodules containing host cells filled with vesicles showed higher nitrogenase (C_2H_2 reduction) activity than nodule slices containing the other forms of the endophyte [25, 29].

Cell-free nitrogenase activity in actinorhizal root nodules was obtained by Benson *et al.* [36] with *Alnus glutinosa* root nodules by disruption of the root nodules in liquid nitrogen to release the actinomycetal endophyte. The powdered root nodules were suspended and centrifuged four times in 100 m*M* potassium phosphate buffer (pH 7.4) containing 10 m*M* dithonite and then repeatedly washed in tris-buffer (20 m*M* tris-HCl, pH 7.2) with 20 m*M* ascorbate and 2 m*M* dithionite to free the homogenate from most of the inhibitory phenolic compounds released during homogenization. Nitrogenase (C_2H_2 reduction) activity was stable even when degassed buffer alone was used in the later washings, indicating that dithionite is not needed in late washing to retain activity. Cell-free preparations were made by mixing the sedimented material with solid polyvinylpolypyrrolidone (PVP) and sonication for 1.5–2.0 min. Prolonged sonication rapidly inactivated the C_2H_2-reducing activity. The sonicated material was centrifuged down and the supernatant containing the cell-free extract had an initial activity of 0.5–1.0 μmole of C_2H_2 reduced per g of nodules per h, or about half the activity of the particulate homogenates. The cell-free enzyme is somewhat unstable after isolation as shown by the non-linear time course in 60 min. The cell-free nitrogenase required ATP, Mg^{2+}, and $Na_2S_2O_4$ for acetylene reduction.

Recently, N_2 fixation (acetylene reduction) has been established in *Frankia* species growing *in vitro* in pure culture in a defined medium [62, 121]. These free-living *Frankia* species were the *Frankia* sp. CpI1 strain isolated from *Comptonia peregrina* root nodules and two *Frankia* strains (str. D11 and G2) isolated from *Casuarina equisetifolia* root nodules. In *Frankia* sp. CpI1, inoculum size had an effect on growth (and vesicle formation) and acetylene reduction activity; both increased at higher inoculum density. In the *Frankia* sp. CpI1 strain, acetylene reduction was greater in stationary culture than in shaken culture. In the *Frankia* sp. strains isolated from *Casuarina* root nodules, the oxygen requirements for nitrogenase (C_2H_2) reduction) activity were studied in more detail.

6.2. Hydrogenase activity

Nitrogenase-dependent hydrogen evolution has been observed in detached legume and non-legume root nodules and in reaction mixtures containing cell-free nitrogenase.

An evaluation of the magnitude of hydrogen evolution seen as energy loss in terms of the efficiency of electron transfer to N_2 via nitrogenase, suggests that hydrogen production may severely reduce N_2 fixation in some plants. For most leguminous plants, it has been shown that 40–60% of the energy of the electron flow was lost through hydrogen evolution, but in some tropical legumes (i.e. *Vigna sinensis* and *Vigna radiata*) and in some non-leguminous plants (i.e. *Alnus rubra*, *Purshia tridentata*, *Elaeagnus angustifolia*, *Ceanothus velutinus* and *Myrica californica*), the relative efficiency is much higher, at 70–90%. The latter plants apparently have evolved a mechanism of minimizing net hydrogen production by recycling the produced hydrogen [107].

Regarding the above-mentioned observation, Benson *et al.* [36] studied the hydrogen production in crude homogenates and cell-free extracts of *Alnus glutinosa* root nodules. The H_2 evolution was ATP dependent and occurred at a rate comparable to the rate of C_2H_2 reduction. Since intact actinorhizal root nodules evolve little hydrogen, there must be a highly active or tightly coupled uptake hydrogenase in the system. As explained above, the relation between the uptake hydrogenase and ATP-dependent H_2 production by nitrogenase is of special interest, because part of the ATP expended for H_2 production can be recovered by coupled hydrogenase-catalyzed reoxidation of H_2. This process increases the efficiency of the system.

6.3. Other investigations

Using translocation studies with ^{14}C-labelled photosynthate to the root nodules of first-year *Alnus glutinosa* plants growing under natural illumination, but at constant temperature, Wheeler [135] showed that a maximum influx of new photosynthates occurred at the time of the midday peak in N_2 fixation. An analysis of fluctuations in the levels of the main free sugars present in the nodules suggested that a substantial part of the nodule carbohydrate was unavailable for N_2 fixation, and that maximal rates of fixation are attained only when new photosynthates are entering the nodules. Similar diurnal changes in the N_2 fixation rate were observed by Bond and Mackintosh [41] in *Casuarina cunninghamiana*, using the N-15 method.

Studies of hormones in non-leguminous root nodules have revealed the presence of cytokinin-like substances in *Alnus glutinosa* and *Myrica gale* root nodules [38, 102]. Analyses of the cytokinin extracts of different plant parts showed that a zeatin-9-glucoside-like substance was the prominent cytokinin in nodules and leaves of *Alnus*, but that a zeatin riboside-like substance was the major cytokinin present in the roots and root pressure sap [68, 69, 70, 71]. Cytokinin levels determined

by bioassay were also observed in other non-leguminous root nodules such as *Purshia tridentata*, *Myrica gale*, *Hippohaë rhamnoides* and *Colletia paradoxa* [72]. In addition to the above-mentioned hormones, gibberellin-like (GA-like) substances were detected in various parts of young nodulated plants as estimated by the lettuce hypocotyl bioassay [73].

7. Management and prospects for use in the tropics

Up till now, the only nodulated non-legume found to be of agricultural importance for food production is *Rubus ellipticus* (Rosaceae). Under normal conditions (in the field), this plant species can fix N_2 at a rate comparable with that achieved by other non-legume root nodules in similar conditions. This raspberry species is taxonomically a representative of the sub-genus *Idaeobatus* [58] along with the common raspberry *Rubus idaeus* and some other species. The fruit of *Rubus ellipticus* is similar in size to the common raspberry, is yellow in colour, and has good culinary quality. According to Bailey [13], the species, at least for some period, was grown as a crop plant in Florida and California under the name of Golden Evergreen Raspberry. It is possible that by crossing *R. ellipticus* with another raspberry or with soft fruits belonging to the genus *Rubus* (blackberries), hybrids can be obtained in which the nodulating habit is combined with other useful agricultural features of these *Rubus* species. Hybridization within the genus *Rubus* is common, including hybridization between raspberries and blackberries. Many natural hybrids of wild *Rubus* species have also been reported [34, 53, 64].

The economic importance of the other non-leguminous nodulated species is found mainly in their capacities to increase the nitrogen status of soils by their N_2-fixation ability and to reclaim land very poor in nitrogen. In certain soils deficient also in other elements, supplemental amelioration procedures are often necessary in addition to the use of N_2-fixing non-legumes. Thus, generally speaking, the cultivation of these nodulated plants will reduce the requirement for manufactured nitrogenous fertilizer and well increase the productivity of sites now deficient in nitrogen.

In temperate regions, extensive use has been made of Alder species (*Alnus glutinosa*, *A. viridis crispa*, etc.) to reforest distressed soils in the revegetation of mine spoils, rubbish heaps, and eroded land [46, 76].

For a long time both in Europe and in the United States, N_2-fixing non-leguminous plant species have been employed for nitrogen accretion in forest ecosystems. The species have been used either as the only or major component of the final harvest (Red Alder) or as a rotation crop, as in the case of alternating stands of Alder and needle-leaved trees (Black Alder – Spruce or Red Alder – Douglas Fir), or in unbroken continuity as in the case of Alder-Poplar mixtures [10, 51, 52, 106, 114, 117, 118, 120, 129, 133]. Nitrogen accretion and redistribution in the ecosystem by litter fall and organic matter decomposition is well known for Alder stands [39, 54, 60, 92, 96, 117, 119, 134, 139]. In silvicultural

systems, other combinations are sometimes employed, such as Autumn-Olive, *Elaeagnus umbellata*, and Black Walnut, *Juglans nigra* [59], Pitch Pine, *Pinus rigida*, or Black Pine, *P. thunbergii*, grown in combination with Bayberry, *Myrica pensylvanica* [120]. Many of these non-leguminous plants, furthermore, can grow on waste or mine-land soils under rather adverse conditions of heavy metal content and other toxic agents [46, 57]. Some woody N_2-fixing trees like *Alnus rubra* are in present-day forestry highly valued for wood production, as they produce a high biomass yield per unit of land area [39, 63, 117, 118, 138]. Alder wood is valued either for timber, fiber, or fuel, depending on its source and quality [33, 63].

Similar utilization of nodulated non-legumes is certainly possible in the humid and more arid tropics. As a counterpart of the commercially important European Black Alder, *Alnus glutinosa*, and the North-American Red Alder, *Alnus rubra*, *Alnus jorullensis* of South America is equally valuable. Up till now, relatively little has been done with this tree species in its native country Colombia. Selection and breeding of improved genotypes (as now have been obtained for *Alnus glutinosa* and *A. rubra*) will certainly increase the productivity and economic value of this species in the tropics. Moreover, certain temperate species of woody N_2 fixers may be used in the higher regions of tropical countries. Good growth and wood production by these non-legumes in these new areas may be possible. For instance the Green Alder, *Alnus viridis* has been imported in New Zealand for revegetation of eroded mountain slopes [35].

Moreover, some woody species typically found in the tropics are of considerable importance for reforestation and wood production in these areas. The littoral *Casuarina equisetifolia* is particularly valuable for the revegetation of dune sands and bare sandy soils near the coast, while the montane species *Casuarina junghuhniana* (syn. *C. montana*) is a very good colonizer of bare soil or eroded areas in mountainous regions of the tropics (e.g. Java, Sumatra in Indonesia). The Southeast Asiatic/Australian *Casuarina* species are widely imported in other tropical countries (Africa, South America and southern USA) for silvicultural purposes. *Casuarina* has a great silvicultural potential, and it would be profitable to make more extensive silvicultural investigations on the use of the various *Casuarina* species [c. 45 spp] in the tropics; these species are especially valuable because they exhibit relatively rapid growth combined with a good N_2 fixation capacity and show special potential to grow in hot, dry climates. In a quantitative estimate of the N increase in a sand-dune soil of the Cap Vert peninsula North of Dakar, West Africa, Dommerques [56] found that the initial bare soil contained 80 kg N ha^{-1}, and after afforestation for 13 years, the soil contained 309 kg N ha^{-1}, i.e. 137 kg N in the A_{00} horizon, 28 kg N in the A_0 horizon, and 144 kg N in the mineral horizon of 0–10 cm. Thus, the afforestation of this area produced an increase in soil N of 229 kg N ha^{-1}. In addition, the standing timber of the *Casuarina* forest was estimated to contain 531 kg N ha^{-1}, signifying an average net N_2 fixation of 58.5 kg N $annum^{-1}$, reminiscent of the N_2 fixation by temperate Alder species [1, 50, 96].

The potential for mixed plantations of commercial woody species and actinorhizal plants should also be studied in the tropics, such as in the case of the interplanting

of *Casuarina* species or of *Myrica* species (e.g. *M. pillulifera* or *M. javanica*) in *Pinus merkusii* plantations for tropical stands of conifers.

Finally, the value of a thorough investigation of natural ecosystems for finding economically valuable species should not be underestimated. In natural ecosystems, nodulated non-legumes often play a prominent role in plant succession covering bare soil of disturbed areas. Such sites may be caused by soil erosion, land-slides, fire, volcanic activity, or human interference in the form, for example, of road-building activities or savanah fires set for agricultural purposes or forest clearings. Many nodulated non-legumes are pioneer plants of forest denuded of timber or colonizers of bare soil forming a necessary chain in natural plant succession. Because of their pioneer character, nodulated non-legumes, especially the smaller species, are relatively short-lived. Through their nitrogen input they assist the revegetation of the area by other more aggressive plant species. After the latter have been established, the nodulated non-legumes are soon overgrown. For this reason, these non-leguminous nodulated plants are rarely found in climax vegetations, and when they do persist in such situations, it is merely due to the presence of extreme environmental conditions, e.g. *Myrica javanica* as a regular component of crater vegetation (fumarole gases) on Java and Sumatra (Indonesia) and *Parasponia rugosa* on recently erupted mud (lahar) streams produced by volcanic activity, e.g. at Mt. Kelut on Java, Indonesia. Besides the *Parasponia* species, *Myrica javanica* is also an active colonizer of bare, rocky soil produced by solidified lava streams. *Parasponia rugosa* is in addition a prominent colonizer of open soils after removal of the climax vegetation, such as in tea plantations, where it grows as weed in between the tea plant rows (Pangia district, Papua). The West-Javanese species *Parasponia parviflora* is a prominent pioneer plant of denuded primary forest and occurs therefore frequently in secondary forest. A study of non-leguminous nodulated plants in their natural habitat will certainly result in a selection of species applicable in current agricultural systems.

This book was in press when Diem *et al.* (C.R. Acad. Sc. Paris, Ser. D, 1982) reported the isolation of an infective and effective strain of *Frankia* from *Casuarina* nodules.

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