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Nitrogen Fixation by Actinomycete-Nodulated Angiosperms

John G. Torrey

Nitrogen fixation by symbiotic associations between soil bacteria belonging to the actinomycetes and root systems of a diversified group of woody dicotyledonous plants is less generally well known than that by the legume-*Rhizobium* symbiosis. The fixation of dinitrogen by nodulated legumes is a major mechanism for entry of reduced nitrogen into agricultural lands and, to a much lesser extent, to wooded ecosystems. For forested areas, woodlands, wetlands, and fields, nodulated plants like the alders (*Alnus*), bog plants like sweet gale (*Myrica gale*), and roadside and disturbed area invaders such as sweet fern (*Comptonia*), bayberry (*M. pensylvanicum*), and various species of *Ceanothus* represent the major mode of fixing atmospheric nitrogen. The actinomycete-induced nodulation of these woody species and their role in N_2 fixation have begun to be recognized as comprising one of the largest sources for biological fixation of atmospheric dinitrogen, at least comparable to the legume symbioses.

Lags in appreciation of the importance of this group are understandable in retrospect. Knowledge of the presence of root nodules in natural populations requires excavation of root systems, sometimes in difficult terrain. Only deliberate and sustained efforts at sampling, as under the recent stimulus of the International Biological Programme (Bond 1976), have demonstrated the extent of the occurrence of this type of symbiosis. At present, about 160 species in 15 genera among 7 families (Table 1) have been reported worldwide to have actinomycete-induced nodulated nitrogen fixation. The

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TABLE 1. Plants that form root nodules following actinomycete infection, their relationships, and distribution. (Updated from Rodriguez-Barrueco 1968 and Bond 1976; systematic estimates based on Willis 1966.)

Genus	No. of species nodulated/total no. of species in genus	Family	Geographical distribution	Ecological sites
Alnus	33/35	Betulaceae	Europe, Siberia, N. America, Japan, Andes	poor soils, sand hills/dunes, glacial till, wet bogs, mine dumps, gravel, wasteland, volcanic ash
Casuarina	24/45	Casuarinaceae	Australia, Tropical Asia, Pacific Islands, widely introduced elsewhere	sand dunes, salt marshes, tropical forests, desert areas
Ceanothus	31/55	Rhamnaceae	N. America, esp. Western USA	dry forest & chaparral, subalpine zones
Cercocarpus	4/20	Rosaceae	Western USA & Mexico	high altitudes, poor soils
Colletia	1/17	Rhamnaceae	S. America (cf. Bond 1976)	
Comptonia	1/1	Myricaceae	N. America	disturbed sandy or gravely areas
Coriaria	13/15	Coriariaceae	Mediterranean to Japan, New Zealand, Chile to Mexico	lowlands & subalpine, sandy or gravely soils or clay
Discaria	2/10	Rhamnaceae	Andes, Brazil, New Zealand, Australia	gravely soils, arid zones
Dryas	3/4	Rosaceae	Alaska, Canada, circumpolar	postglacial areas, sandy, gravely soils
Elaeagnus	16/45	Elaeagnaceae	Asia, Europe, N. America	disturbed areas, sand dunes, poor soils
Hippophaë	1/3	Elaeagnaceae	Asia, Europe, from Himalayas to Arctic Circle	sand dunes, coastal areas
Myrica	26/35	Myricaceae	many tropical, subtropical, & temperate regions, extending nearly to Arctic Circle	acidic bogs, sand dunes, mine wastes
Purshia	2/2	Rosaceae	Western N. America	dry sand soils, as understory plant in *Pinus* forests
Rubus	1/250	Rosaceae	Indonesia (cf. Bond 1976); otherwise cosmopolitan n. temperate	
Shepherdia	3/3	Elaeagnaceae	N. America	sand soils, disturbed areas

very diversity of these nodulated plants has blocked the recognition of the extent and importance of their role in the nitrogen cycle in nature.

Furthermore, few of these plants have been of direct agricultural importance in the commerce of man. Some among the alders are good-sized trees and produce timber; others, such as *Casuarina*, are sizeable, handsome, and useful trees in semi-tropical climates. The alders have been used experimentally for interplantings or as alternate crops in forested timber plantations as biological sources of fixed nitrogen. Many of the others are shrubby species abundant in some habitats yet relatively inconspicuous in the vegetation as a whole. The fact that the microbial agent had not been identified except from structural studies has further inhibited development of our understanding of the relationship and its importance.

Over the past 25 years, largely through the efforts and persistence of a few research laboratories, our knowledge and understanding of the group have grown. Recently, the pace of research has accelerated, and the immediate future promises a rapid exploitation of this area, both in research and in practical application. The subject of symbiotic nitrogen fixation has received much recent attention by reviewers. The reader will find useful discussions of actinomycete-nodulated plants by Becking (1970a, 1975, 1977a), Bond (1963, 1967, 1976), Silvester (1976, 1977), and Quispel (1974).

Root nodules induced by soil actinomycetes are morphologically and anatomically distinct from legume nodules. Typically these nodules result from a localized stimulus at the infection site of multiple, modified lateral roots whose condensed and repeated branching produces a more or less spherical mass, sometimes up to several centimeters in diameter. Two general types of nodules occur: the *Alnus*-type in which knobby, coralloid structures are formed and the *Myrica*-type in which each terminal lobe of the nodule produces a determinate nodule root, which grows more or less vertically upward (Figs. 1, 2). Within the basal swollen lobes, the actinomycete occupies specific cortical cell layers where the enzyme nitrogenase develops, which catalyzes the fixation of dinitrogen from the atmosphere and converts it to ammonia through a series of reductions.

Until quite recently, cultivation of these plants in the greenhouse or laboratory depended on growing seedlings with their roots in water culture or in nutrient mist and inoculating the root systems with nodule suspensions prepared by grinding up older nodules taken from field-collected or greenhouse-grown plants. Under favorable conditions, nodules develop within about three weeks, and their structure and function can be readily studied. The infection process appears to be remarkably similar in a number of genera. Recent structural studies of nodulation in *Alnus*, *Myrica*, *Comptonia*, and *Casuarina* (Angulo-Carmona 1974, Bowes et al. 1977, Callaham and Torrey 1977, Lalonde 1977, Torrey 1976) allow one to begin to formulate some generalizations, which should foster further research.

THE INFECTION PROCESS

Within a day or two of applying a nodule suspension to a young seedling root system of the appropriate host, one can observe extensive root hair deformation, i.e., curling and branching among the still-developing root hairs. In this region of the seedling roots, nodule initiation occurs. In all of the four genera mentioned, there now exists evidence based on light and electron microscopy that infection occurs by root hair invasion. One can clearly demonstrate the presence of actinomycetous filaments, usually as multiple strands, in deformed hairs (Figs. 4, 5). Many hairs are deformed; typically only one is invaded at each nodule site.

Evidence of the nature of the microsymbiont outside the root and the exact process of endophyte infection has been difficult to obtain. Until the process has been analyzed, using a cell preparation from a pure culture, it will continue to be difficult to sort out the structural form of the microorganism in its soil environment. A number of related problems including the persistence of the bacterium in the soil, the question of its multiplication in the soil, and its mode of attack on the root surface are important and should become accessible to study in axenic systems into which the cultured actinomycete can be introduced.

The endophyte observed within the root hair is sheathed in a polysaccharide layer produced by the host. This encapsulation, which appears to be pectic in nature (Lalonde and Knowles 1975), surrounds all of the endophyte filaments throughout its entire life as an endophyte, and a substantial portion of the activities and energies of the host cortical cells is involved in the ongoing process of encapsulation (Newcomb et al. 1978b).

Once within the root hair, the branching filamentous actinomycete grows through the cell walls of the outer cortical cell, dissolving the various cell layers in its invasion path. It passes from one cortical cell to the next, stimulating hypertrophy and cell division of the host cortex (Fig. 3), forming a pre-nodule or invasion site (Callaham and Torrey 1977).

FIG. 1. Nodules on the root system of *Ceanothus americanus*, typical of the coralloid *Alnus*-type. (× 0.5.) FIG. 2. Root nodules of *Casuarina cunninghamiana*, showing upward-growing nodule roots, typifying the *Myrica*-type. (× 1.2.)

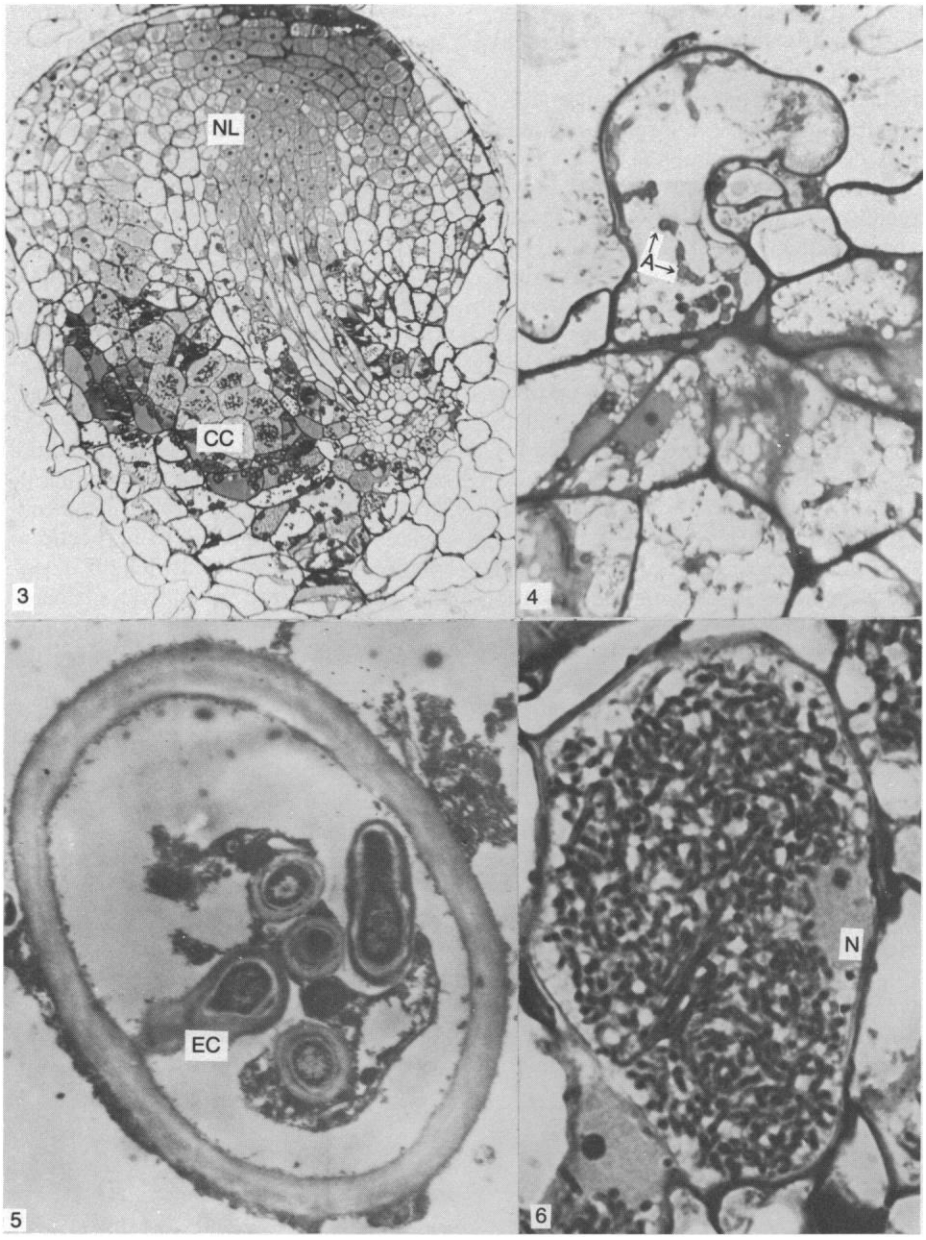

FIG. 3. Transection of lateral root of *Comptonia peregrina*, showing a young nodule lobe (NL) in longitudinal section with actinomycete-infected cortical cells (CC). (× 150.) FIG. 4. Longitudinal section of an infected root hair in the epidermis of a root of *Comptonia*, showing actinomycete filaments (A). (× 1,210.) FIG. 5. Electron micrograph of transection of root hair of *Casuarina* with actinomycete filaments cut in section. Note capsule (EC) surrounding each filament. (× 9,880.) FIG. 6. Enlarged light-microscopic section of a cortical cell in a nodule lobe of *Casuarina cunninghamiana*, filled with actinomycete filaments. N is the nucleus of the cortical cell. (× 1,500.)

Associated with this invasion is the stimulation of multiple, modified lateral-root-like primordia within a few hundred microns of each other. In *Comptonia*, the number of primary nodule lobe primordia may be as high as 12–15 within a couple of millimeters of the root hair infection site. In *Myrica gale* only 2–3 nodule lobes are usually initiated at the outset. In *Casuarina*, usually one nodule lobe is initiated at the outset, followed sequentially by further branchings. Thereafter, new primordia are stimulated to form endogenously within the first-formed nodule lobes, until a condensed, highly branched system is formed.

ULTRASTRUCTURE OF THE HOST-ENDOPHYTE RELATIONSHIP

Since the first report of Becking et al. (1964) on *Alnus*, the ultrastructure of the actinomycete endophyte in nodules has been studied in detail (cf. reviews by Becking 1975, Gardner 1976). The actinomycetous character of the endophyte is generally apparent. The organism is a filamentous, branched, and septate prokaryote. Three distinctive phases in its symbiotic development in situ have been described. Its invasive stage involves a filamentous hyphal-like structure, 0.5–1.0 μm in diameter, which branches profusely, ramifying within cells (Figs. 6–9), causing perforations in cell walls, and passing from one cortical cell to another from the pre-nodule into the cortical cell layers of successive nodule lobes, always encapsulated by a polysaccharide layer of host origin. Within the cytoplasm of the host cell, extensive organelle activity devoted to the encapsulation process occurs (Newcomb et al. 1978b).

Vesicle formation by the endophyte marks the second stage in the development of the symbiosis. In nodules of many different genera, terminal swellings or vesicles develop at the ends of the filaments after a cortical cell becomes filled with the endophyte. These vesicles may be club-shaped or spherical, enlarged (3.0–5.0 μm) and septate or subdivided. The function of the vesicles remains unclear, although Akkermans (1971), Angulo et al. (1976), and others have suggested that nitrogenase activity is associated with these terminal vesicles. Van Straten et al. (1977) reported, in fact, that isolated clusters of vesicles from *Alnus* showed nitrogen-fixing activity under appropriate incubation conditions. However, vesicles have not been observed at all in some genera (e.g., *Casuarina*), although nitrogen fixation occurs.

A third phase in the structural development of the endophyte in situ is the formation of structures referred to in published accounts as bacteroids, granules, or, most recently, spores. Van Dijk and Merkus (1976) described in detail the origin of spore formation in *Alnus glutinosa*, showing that spores were derived from local transverse enlargements of thick endophytic hyphae, which formed "spindles" or "granulated bodies." Spores are produced from septation of the swollen regions of the hyphae; the segmented components round up, undergo cell wall thickening and internal ultrastructural changes, including deposit of electron-opaque material (probably storage matter), and are finally released upon the death of the host cell. The spore-like appearance of these structures, which are about 1.0–1.5 μm in diameter and usually round or oval in shape, is reminiscent of sporangiospores in known actinomycetes. The spindles are similar to structures formed in actinomycetes in

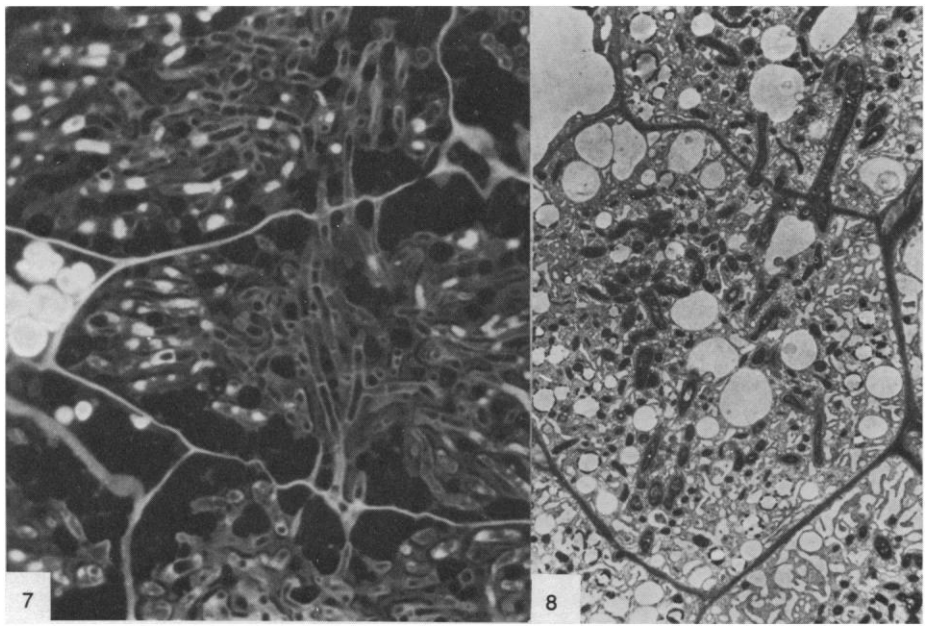

FIG. 7. Infected cortical cells of a nodule lobe of *Myrica gale*, stained with a fluorescent stain specific for polysaccharide and photographed with fluorescent light. Starch grains stain brightly in uninfected cells. The encapsulation of the endophyte is fluorescent, as is storage material within the filament. (× 1,400.) FIG. 8. Low-power electron micrograph of cortical cell of *Myrica gale*, showing actinomycete filaments within the cell and penetrating across cell walls. (× 2,100.)

culture (see below), which can properly be termed sporangia. Such sporangia have been observed in nodules of species of *Alnus*, *Myrica*, *Hippophaë*, and *Casuarina*, among others.

ISOLATION AND CULTURE OF THE ENDOPHYTE

Progress in working with the symbiotic relationship between angiospermous host plants and root-nodulating actinomycetes has been hampered seriously by the lack of understanding of the microbial symbiont. Until the infective agent is isolated and grown in pure culture separate from the host, one is unable to develop an understanding of the life cycle of the microorganism, its state and longevity in the soil, the nature of the infection process, and the whole range of relationships between the host and the microbial symbiont controlled by the genetic constitutions of the partners. Practical field application and manipulations would flow naturally from an understanding of these considerations.

Attempts to culture the endophyte have been frequent and can be traced back at least to the studies of Peklo in 1910, who first claimed success in growing the actinomycete isolated from roots of *Alnus*. Successes and more frequent failures have been reviewed over the years (Bond 1963, 1967, Fletcher and Gardner 1974, Uemura 1964). Although many actinomycetes have been readily isolated and grown in culture, they seldom were able to cause nodulation when applied to test plants (cf. Lalonde et al. 1975). Researchers in the field generally accept that successful isolation and routine culture of actinomycete endophytes from root nodules have yet to be achieved.

We have recently reported (Callaham et al. 1978) the isolation and culture of the actinomycete from root nodules of *Comptonia peregrina*, a close relative of *Myrica* species. Further, we have achieved the reinfection of seedling roots of *Comptonia*, demonstrated the production of effective nodule formation (based on acetylene-reduction tests), and the successful reisolation of the same organism from the nodules of the reinfected roots. Repeated successful inoculations of roots of *Comptonia* seedlings grown in sand culture or aeroponics have been made and high levels of acetylene-reduction observed. Other host plants in which the *Comptonia* isolate will induce nodulation include *Myrica gale*, *M. cerifera*, *Alnus rubra* (our laboratory), *A. glutinosa*, and *A. crispa* (Lalonde 1978). Other tests of cross-inoculation capacity are in progress.

Like many other actinomycetes (Sykes and Skinner 1973), the organism isolated from nodules of *Comptonia* grows slowly as an unpigmented, filamentous mat in a yeast-extract liquid medium in a range of oxygen levels, but not anaerobically (Figs. 10, 11). In liquid culture, the organism forms numerous sporangia (Figs. 10, 12); in plates of agar medium, these sporogenous structures are submerged, not aerial.

The development of sporangia can occur in two ways: either by enlargement and septation in the filament itself (Fig. 12 inset) or by swelling and segmentation of a short branch filament. Numerous spores are formed within the swollen filament; they are surrounded only by a thin membranous covering, possibly from the filament itself. The spores when released are thick-walled and oval-shaped, varying in size from 1.5–3.5 μm. In the electron microscope (Newcomb et al. 1978a), they appear to be filled with electron-opaque material, probably storage matter. Spore suspensions plated in agar nutrient medium show spore germination in a few days, with the formation of a swollen germ tube reminiscent of spore germination in *Streptomyces* (Attwell and Cross 1973). Filamentous colonies develop, which enlarge and later form new sporangia.

Comparison of the structures observed in the cultured endophyte and in the organism within the host plant shows striking similarities and some sharp differences. The filamentous nature of the actinomycete is seen in both conditions—a filamentous, septate, and branched vegetative body of 0.5–1.0 μm diameter. Internal structure is the same (cf. Fig. 9 and Fig. 11). In vitro, the cultured actinomycete shows no encapsulation, confirming that the capsule is probably a host-produced structure. The vesicle stage observed in nodules has yet to be demonstrated conclusively in the cultured actinomycete.

Spore formation within sporangia has been described in *Alnus* nodules by Van Dijk and Merkus (1976), and the structures they observed are strikingly similar to sporangial development in the cultured *Comptonia* isolate. One must conclude that both spores and sporangia are normal components in the life cycle of this complex prokaryote, either in infected cells of root nodules or in culture.

Successful isolation of the organism that nodulates *Comptonia* encourages one to believe that systematic attempts to isolate the effective organism in other symbioses will be possible. Nutrient requirements for the *Comptonia* isolate, although not fully defined, are not complex. However, growth in culture is slow, and patience is a requirement for successful isolation.

Isolations must be made on as many genera as possible so studies can be made of cross-inoculations, e.g., as tested by Rodriguez-Barrueco and Bond (1976). One hopes to study the genetic nature of the effective microbial agents and ways of increasing the effectiveness of inoculation, perhaps by selection of strains of high effectiveness and applying them to seeds, seedlings, or plantations, thereby increasing the nitrogen-fixing capacity of the host-microbial symbioses. Identifying the organisms and placing them in their proper systematic position (cf. Becking 1970b) will be much facilitated.

SIGNIFICANCE OF ACTINOMYCETE-NODULATED PLANTS

In making an assessment of the importance of nitrogen fixation by symbiotic actinomycete-infected plants, several features should be appreciated. It has only recently begun to be generally accepted that N_2-fixation rates in these plants are of the same order of magnitude as those observed in legumes. Rates reported among rhizobial-induced nodulated species (legumes) and actinomycete-induced nodulated plants (several families) are presented in Table 2. In herbaceous legumes and seedling stages of the nonlegumes, rates are strikingly similar. Direct comparisons of acetylene-reduction by root nodules, calculated on a fresh weight basis, can be made, or one can compare calculations based on field samples extrapolated to area per unit time. Using either measure, rates of fixation by actinomycete-nodulated plants fall in the same range of values as for legumes.

Actinomycete nodulation is known to occur only among woody dicotyledonous species. Restriction of this type of symbiosis to woody dicots is of interest; Bond (1963) speculated that the actinomycete-woody dicot type of symbiosis arose at a geological time when only woody species were extant. At present, we have no good structural or physiological hypothesis to explain this limitation of host range, any more than we understand why *Rhizobium* infection is restricted to the legumes, be they herbaceous or woody species. There is one recently described exception, the unusual association reported between the tree species *Trema cannabina*[1] of the family Ulmaceae and a rhizobial-like endophyte (Trinick 1976). The diversity of host plants susceptible to infection by soil actinomycetes suggests that still broader host ranges will be found in nature, and other species, genera, or families will be added as the search is extended. The possibility also exists that the host range can be usefully extended by man's intervention and ingenuity.

Because of the perennial nature of the hosts, nitrogen fixed by actinomycete-nodulated plants is made available to the soil only on a long-term basis. Nitrogen fixed in the nodules is rapidly transported to the shoot; leaves of many of these species show well above average levels of nitrogen on a fresh weight basis. The fixed nitrogen is returned to the soil on an annual basis largely as leaf litter, and only when the nodules are sloughed off or the plant dies is the nitrogen in the root system made available to other

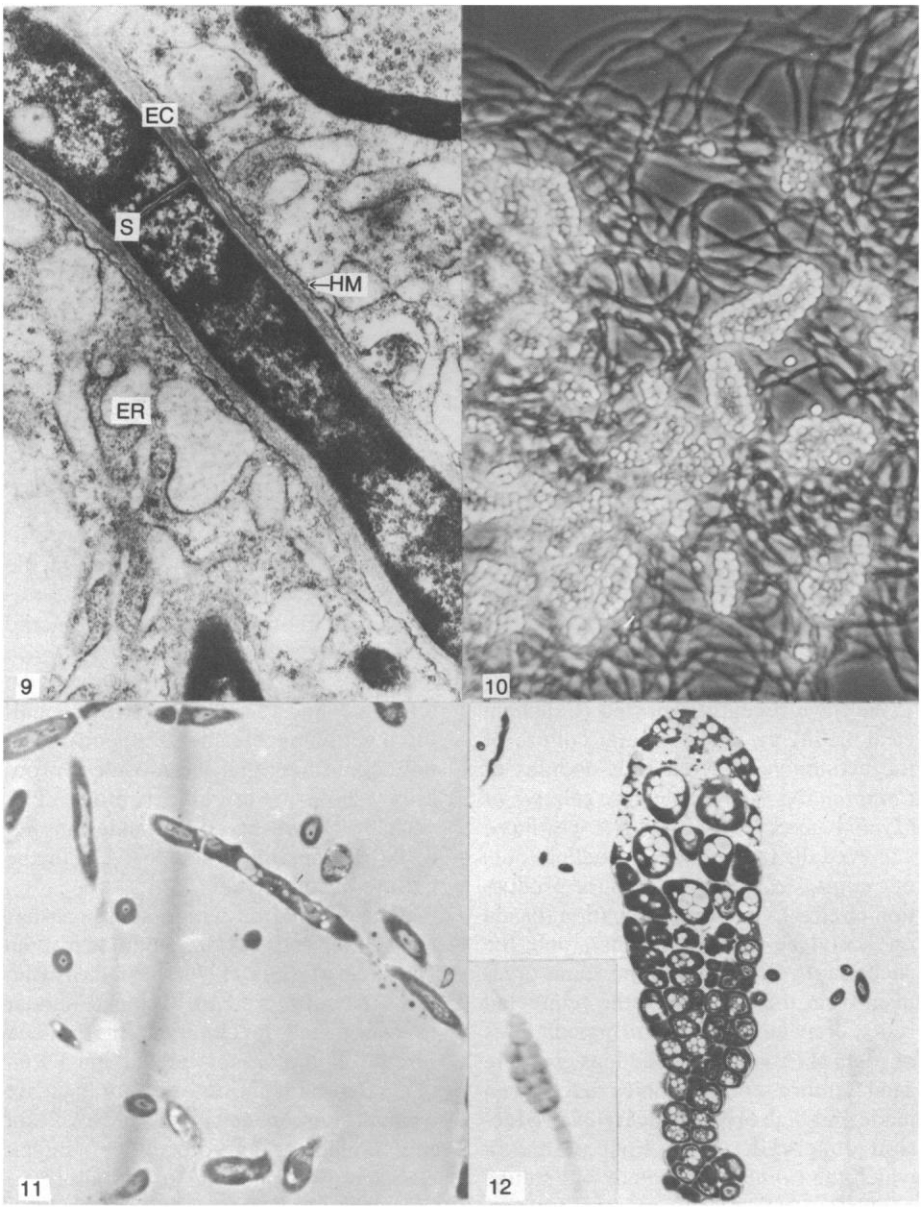

FIG. 9. High-power electron micrograph of actinomycete filament within the cortical cell of a nodule lobe of *Myrica gale*, showing the septa within the filament (S), the encapsulating material (EC), expanded endoplasmic reticulum (ER), and the host cell membrane (HM). (× 16,100). FIG. 10. Phase-contrast micrograph of a colony of the *Comptonia* isolate cultured in yeast-extract medium. Dark filaments and brightly refractile sporangia containing spores are evident. (× 750.) FIG. 11. Low-power electron micrograph of the actinomycete isolated from *Comptonia* nodules and grown in axenic culture. Note the absence of encapsulation, the presence of septa and storage material. (× 8,270.) FIG. 12. Electron micrograph of a sporangium formed in a culture of the *Comptonia* isolate. (× 3,970.) Inset: Light-microscope photograph of a stained filament of the *Comptonia* isolate, showing intrafilament septation leading to sporangium formation. (× 1,200.)

[1]The correct identification of this plant has been established by A. D. L. Akkermans as *Parasponia parviflora* Miq. of the Ulmaceae (*Plant and Soil*, in press).

TABLE 2. Comparison of rates of nitrogen fixation by rhizobial-induced nodulation (legumes) and actinomycete-induced nodulation.

Plant	Acetylene-reduction (μ mol/hr/g fr. wt. of nodule)	Plant	Total N fixed kg/ha/yr
ACTINOMYCETE-INDUCED		ACTINOMYCETE-INDUCED*	
Comptonia peregrina seedlings†	9.6–22.2	*Alnus crispa* trees	40–362
Elaeagnus angustifolia in H_2O culture‡	14.8	*Alnus rubra* trees	140–300
Alnus rubra‡	6.9	*Hippophaë rhamnoides* trees	15–179
Alnus glutinosa seedlings§	9–92.0	*Casuarina equisetifolia*	58–200
RHIZOBIAL-INDUCED‡		RHIZOBIAL INDUCED‖	
Glycine max	7.3	*Glycine max*	103 (av.)
Medicago sativa	15.1	*Medicago* sp.	54–463
Pisum sativum	17.4	*Pisum sativum*	52–77
Trifolium subterraneum	2.1	*Vicia faba*	45–552

*Cf. Silvester 1976.
†Callaham et al. 1978.
‡Schubert and Evans 1976.
§Akkermans 1971.
‖Cf. Nutman 1976, ch. 19.

plants. If one takes into account the long-term contribution made by the symbiotic nonlegumes, it is clear that these diverse genera add a very significant amount of fixed nitrogen to the total ecosystem in which they are found. Some attempt has been made by Silvester (1976, 1977) to assess the magnitude of this contribution on a genus-by-genus basis. Global estimates need to be recalculated to place the contribution of these plants in true perspective. Surely the actinomycete-induced symbioses are at least on an equal par with the legumes on a worldwide basis.

While the legumes are primarily tropical or semi-tropical in their natural occurrence or have been adapted by man to a range of agricultural situations, the actinomycete-nodulated plants are primarily north and south temperate in their distribution, with only *Casuarina* extending into tropical areas. They occupy a range of ecological sites varying from wet habitats as pioneers along rivers, ponds, and bogs, and in postglacial sites, to dry sandy dunes and hills, disturbed or devastated areas, and even dry chaparral and desert environments (cf. Table 1). Thus, in their diversity, these plants are found adapted to a wide variety of sites and are widespread in their distribution.

Although actinomycete-nodulated symbiotic nitrogen-fixing plants occur predominantly in natural stands and contribute to total nitrogen availability in ecosystems largely unmanaged by man, increasing recognition has been given to the potential value of these genera in providing nitrogen through mixed plantations, succession plantings, and other management practices, especially in the context of forestry. As Silvester (1977) has pointed out, the use of *Alnus* in crop rotation with millet has been long practiced in Formosa, and *Casuarina* is used for soil improvement in highland New Guinea. Uemura (1971) reported that over wide areas of Japan, species of *Alnus, Myrica,* and *Elaeagnus* have been planted for many decades, mixed with pines, cedar, cypress, and other trees for erosion control and soil improvement. Similarly, in Europe, appreciation of the benefit of alders to soil improvement has been common among foresters.

Since about 1955, increasing interest and attention have been focused on the possibilities of using actinomycete-nodulated plants in forestry management. Silvester (1976) has reviewed some of these practices, including land reclamation, soil improvement by adding to soil nitrogen, and interplanting actinomycete-nodulated plants with economically important tree crops.

In the Pacific Northwest, experimental plantations have included mixed planting of black cottonwood (*Populus trichocarpa*) and red alder (*A. rubra*) for short-term fiber production and soil improvement or Douglas fir (*Pseudotsuga menziesii*) with red alder as nurse crop. Elsewhere, experiments on mixed plantings have involved black walnut (*Juglans nigra*) with autumn olive (*Elaeagnus umbellata*) as nurse crop or poplar-alder mixtures for pulp and total biomass production. In all of these, significant contributions to the soil nitrogen have been recorded and reflected in increased growth of the crop plantation.[2] Still other experimentation has focused on the use of actinomycete-nodulated plants for reclaiming strip-mine areas, revegetating roadsides, and maintaining and improving forested ecosystems.

[2]These and related aspects of symbiotic nitrogen fixation in actinomycete-nodulated plants were discussed at a conference held in April 1978 at the Harvard Forest in Petersham, Massachusetts. The papers presented at that meeting will be published shortly as a special issue of the *Botanical Gazette*.

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