Isolation and Properties of Microorganisms from Root Nodules of Non-leguminous Plants. A review with extensive bibliography.

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I. Introduction

ALLEN and ALLEN (1958) summarized the literature on non-leguminous root nodule bearing plants of the Dicotyledoneae and stated that 65 species of trees and shrubs within 8 families are reported as non-leguminous species with root nodules. Since then, *Dryas drummondii* (family Rosaceae) and *Discaria toumatou* (family Rhamnaceae) were added to this category.

In addition, it is known that within the families Cycadaceae, Taxaceae and Pinaceae of the Gymnospermae, some species also have nodules or nodule-like swellings on their roots, although there is some question whether those of the latter two families are typical nodules or mycorrhizal hypertrophies.

In succession, SCHWARTZ (1959), NORRIS (1962), McKEE (1962) and BOND (1963) reviewed the literature concerning nodulated non-leguminous plants. At present, the following 17 genera within 12 families (Table 1) contain nodulating species of trees and shrubs. Not all of the species belonging to these genera have been examined for nodule formation, nor have all of those reported been confirmed.

Probably, different types of nodules exist on the non-leguminous species of these families; however, the nodules of Betulaceae, Elaeagnaceae, Myricaceae, Casuarinaceae, Coriariaceae and Rhamnaceae appear to be somewhat interrelated to each other. Reference is made to these in the literature citation appended to this paper.

The nodule endophytes of the above mentioned families have been variouly interpreted as bacteria, actinomycetes, filamentous fungi or members of the Plasmodiophorales. Some authors claim to have isolated successfully microorganisms from nodules of these non-leguminous species, but most of these isolates have failed to incite nodule formation upon attempted re-inoculation of their host plants. However, PLOTHO (1941) and POMMER (1959) reported to have succeeded in the nodule formation on alders in sterile culture with their isolated nodule endophytes of an actinomycete or actinomycete-like organism respectively, but as yet their results have not been confirmed.

However, it does resolve in the general sense that the weight of evidence favors actinomycetes as the causal agents of the root nodules of these families.

The reports of root nodules on species within the family Zygophyllaceae are meagre. Whereas their nodules and causative organisms were reported to be similar to those of leguminous plants by SABET (1946), MOSTAFA and MAHMOUD (1951) and MONTASIR and SIDRAK(1952), ALLEN and ALLEN (1949) did not confirm this view about the nodular hypertrophies on roots of *Tribulus cistoides*.

In reporting the occurrence of root nodules on two *Coffea* species (Rubiaceae), STEYAERT

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Table 1. Non-leguminous families and genera which have root-nodule bearing species.

Family	Genus	Family	Genus
Dicotyledoneae		7. Rosaceae	*Dryas*[2]
1. Betulaceae	*Alnus*	8. Zygophyllaceae	*Fagonia*
2. Elaeagnaceae	*Elaeagnus*		*Tribulus*
	Hippophaë		*Zygophyllum*
	Shepherdia	9. Rubiaceae	*Coffea*
3. Myricaceae	*Myrica* (*Comptonia*)	Gymnospermae	
4. Casuarinaceae	*Casuarina*	10. Cycadaceae	*Cycas*[3]
5. Coriariaceae	*Coriaria*	11. Taxaceae	*Podocarpus*[4]
6. Rhamnaceae	*Ceanothus*	12. Pinaceae	*Sciadopytis*[5]
	Discaria[1]		

Note 1 : Morrison and Harris (1958a, b) reported nodule formation on *Discaria toumatou* Raoul Choix in New Zealand.

Note 2 : Lawrence (1953), Crocker and Major(1955), Crocker and Dickson(1957), Cooke and Lawrence (1959) and Sprague and Lawrence (1960) reported that *Dryas drummondii* in Alaska has root nodules and is considered to be capable of nitrogen fixation from field observations (cf. Photo. 8).

Note 3 : All the genera of Cycadaceae except *Microzamia* are found possessing root nodules by Spratt (1915) and Kellerman (1911).

Note 4 : Other genera closely related to *Podocarpus* are reported to have root nodules by Hooker (1854) and Spratt (1912b).

Note 5 : The genus *Sciadopytis* comprise only one species, namely, *S. verticillata*, distributed in restricted areas of Japan.

(1932) interpreted the causal agents of the nodules as bacteria, *Bacillus coffeicola* ; on the contrary Rayner (1948) classified them in the mycorrhiza. Therefore, further studies are needed to confirm the nature of the nodule organism. Some species of this family produce leaf nodules.

As to the nodule endophytes of *Podocarpus* species, Nobbe and Hiltner (1899), Shibata (1902), Hiltner (1903), Petri (1903), Yeates (1924), Saxton (1930), Schaede (1943) and Ferreira dos Santos (1947) maintained the fungal theory : on the contrary Bottomley (1912a), Spratt (1912b), McLuckie (1923a), Phillips (1932) asserted the bacterial one ; as yet no one has successfully produced nodules with the isolated microorganisms.

The nodules of *Sciadopytis verticillata* were reported to be mycorrhizal by Noellê (1910) and by Laing (1923), but no one has confirmed the nodule endophytes of them.

Investigators have observed several kinds of microorganisms in the root nodules of *Cycas* (Cycadaceae), such as algae (Reinke 1879, Hariot 1892, Prantl 1889, Spratt 1911, Watanabe 1924, Winter 1935, Takeshige 1937, Guttenberg 1941, Schaede 1944 and Douin 1953), bacteria (Prantl 1889, Schneider 1894, Pampaloni 1901, Life 1901, Kellerman 1911, Burrill and Hansen 1917, and McLuckie 1922), and fungi (Brunchorst 1886a, Zach 1910 and Schaede 1944) ; however, confirmation of the nodule-producing organism remains to be proved.

On the other hand, in this decade many workers, especially Bond and his co-workers have studied the nitrogen fixation of the nodules of these non-leguminous plants (*Alnus*, *Myrica*, *Elaeagnus*, *Hippophaë*, *Shepherdia*, *Casuarina*, *Coriaria* and *Ceanothus*) by the methods with or without ^{15}N. Presumably, these nodules are analogous in function to those of legumes. These findings were reviewed in the papers of Bond (1958a, 1959, 1963).

The author has studied for more than twenty years the root nodules of non-leguminous

plants, especially those on *Alnus* species, and has described some new methods for isolation of actinomycetes. Some parts of the experimental results on *Alnus* species have already been reported (1952c, d ; 1961).

By these methods, chiefly the one using the nodule stele, it was possible to isolate microbes from the root nodules of species of *Alnus*, *Elaeagnus*, *Myrica*, *Casuarina*, *Ceanothus*, *Coriaria*, *Podocarpus* and *Sciadopytis*. Actinomycetes apparently definite to each species have been commonly isolated.

This report deals with the isolation of actinomycetes from the root nodules of these non-leguminous plants, and with some of the cultural properties of the major actinomycetal strains isolated.

The discussion concerns the problems encountered in proving that these endophytes cause nodulation.

II. Isolation experiments of actinomycetes

A. Methods and materials

1. Materials.

The plant species used for these experiments were the following :

Genus *Alnus* : *Alnus tinctoria* SARG. var. *glabra* CALL., *A. japonica* SIEB. et ZUCC., *A. Sieboldiana* MATSUMURA, *A. firma* SIEB. et ZUCC., *A. glutinosa* GAERTNER, *A. incana* WILLD., *A. cordata* DESF. Among these, the first four species were used principally.

Genus *Elaeagnus* : *Elaeagnus umbellata* THUNB.

Genus *Myrica* : *Myrica rubra* SIEB. et ZUCC.

Genus *Casuarina* : *Casuarina equisetifolia* MIQ.

Genus *Coriaria* : *Coriaria japonica* A. GRAY.

Genus *Ceanothus* : *Ceanothus americanus* L.

Genus *Podocarpus* : *Podocarpus macrophylla* D. DON

Genus *Sciadopytis* : *Sciadopytis verticillata* SIEB. et ZUCC.

Fresh and complete nodules with uninjured epidermis of the one- or two-year-old seedlings were selected as materials for the isolation experiments, from the above mentioned plant species. All plants were grown in the nursery of the Government Forest Experiment Station, Meguro, Tokyo, Japan.

Note : In the nursery of Government Forest Experiment Station in Tokyo, *Ceanothus americanus*, *Hippophaë rhamnoides* and *Shepherdia argentea* were sown in the early spring of 1961 from seeds received from Dr. C. O. OSTROM (Director of the Division of Forest Management Research, United State Department of Agriculture). Seedlings of *Ceanothus americanus* bore nodules, but the other two species failed to produce them and died gradually.

2. Methods of isolation technics.

Two methods A and B, devised by the author, were used. But, as method B was used only for reference, the result of it will be omitted in this report.

Method A, using the top of nodule stele :

(1) Nodulated roots of seedlings were gently cleaned superficially with a writing brush in running tap water. Then, the small root pieces, about 1.5 cm length with attached nodules were cut off and, after washing with soap and sterilized water, were transferred into a PETRI dish.

(2) Next, a small root piece was picked from the PETRI dish with sterilized forceps and blotted with sterilized blotting paper. Holding the root at the nodule attachment point with the sterilized forceps in one hand, the nodule cortex was stripped from the root piece by means of sterile forceps held in the other hand to expose the nodule stele attached to the root piece.

When this operation was done favourably, the entire nodule stele remained on the base of the nodule which was attached to the root. However, such steles were not obtained easily from the nodules of all species ; young nodules were more satisfactory than older ones.

(3) A cooled, sterile, platinum needle was stroked against the tip of the nodule stele two or three times, and the trace of nodule stele on the platinum needle was transferred to the surface of an agar slope of a test tube.

The isolation of the trace was tried usually twice from the same nodule stele. The isolated traces were transferred to the upper and lower places of the same agar slope in a test tube.

(4) For isolation, yeast-glucose-peptone agar medium, which is the modified yeast-mannitol agar medium mentioned by PLOTHO in 1941, was used exclusively. This medium seemed to be one of the most favourable for the isolation of actinomycetes from alder nodules in the former studies (1952c, d).

Formula of yeast-glucose-peptone agar medium.

Glucose	20.0 g
Peptone (Poly peptone, TAKEDA Co.)	10.0 g
K_2HPO_4	0.2 g
KH_2PO_4	0.3 g
NaCl	0.1 g
$CaSO_4 \cdot 7H_2O$	0.005 g
Yeast Water	100.0 cc
Water, distilled	900.0 cc
Agar	15～18 g

The yeast water was prepared by adding 100 g of brewer's yeast (Ebiosu powder) to one liter of tap water followed by sterilization for 1 hour in a KOCH's steam pot and a 24-hour settling period at room temperature ; the clear supernatant was then decanted. The medium was adjusted to pH 6.8 with NaOH.

Methcd B, using some disinfectants :

(1) The fresh and complete nodules from 1- to 2-year-old seedlings were washed repeatedly with soap and sterilized water, then immersed in 0.2 per cent Uspulun solution (Semesan : BAYER E. Co.) for 2 to 5 minutes (or 0.1 per cent mercuric chloride solution for about 5 minutes) for the purpose of sterilizing the nodule surfaces. The nodules were transferred to yeast-glucose-peptone agar medium in PETRI dishes. About five nodules were used per plate.

Note : Uspulun in one of the mercury compounds, hydroxymercurichlorophenol ; $C_6H_5 \cdot ClHgO_2$, as a seed disinfectant.

(2) In order to confirm the effectiveness of surface sterilization, the dishes of nodules were incubated for about 72 hours at 28°C ; at intervals of about 24 hours, each nodule was rotated on the medium to insure exposure of the entire surface to test against contamination.

(3) Only those nodules whose surfaces proved to be completely disinfected were crushed and used for isolation by the plate culture method.

The above-mentioned isolation methods, A and B, especially method A were carried out on the root nodules of each species of these non-leguminous plants as follows :

B. Experiments and results

1. *Alnus* species

The root nodules of *Alnus* species, like those of *Elaeagnus*, *Hippophaë*, *Shepherdia*, *Myrica*, *Casuarina*, *Coriaria* and *Ceanothus* species are regarded as modified lateral roots (cf. Photos. 1～8).

In the nursery, the nodules of *Alnus* species were usually apparent on two-month-old seedlings.

Four alder species, *Alnus tinctoria* var. *grabra*, *A. japonica*, *A. Sieboldiana* and *A. firma*, were used in the isolation experiments. These species were classified into two groups; one, *Alnus* group, the former two species, and the other, *Alnaster* group, the latter two species.

From July 1950 to March 1956, isolation tests by method A was repeated 18 times with 229 culture attempts for the *Alnus* group; on the *Alnaster* group, 21 times with 372 test tubes (Table 2).

Table 2. Results of the isolation experiments by method A on *Alnus* and *Alnaster* groups.

Host plant	Number of tubes which yielded mono- or mixed cultures of microorganisms			
	Actinomycetes	Bacteria	Fungi	None
Alnus group	30	5	4	194
Alnaster group	32	9	6	327
Total: number (607)	62	14	10	521
%	10.21	2.31	1.65	85.83

The detailed data of the major part of this experiment were presented in a previous report (1952d), hence only the general result of the whole experiment, including isolation tests which were made since then, are reported here.

Throughout the entire isolation trials on these non-leguminous root nodules including *Alnus* species, one to three colonies of the same actinomycete usually developed on the agar slope of a test tube, but in rare cases, two actinomycete-types developed. Therefore, the total number of isolated actinomycetes exceeded in some cases those of test tubes which yielded the cultures.

Thirty-two strains of actinomycete were isolated from the *Alnus* group and 36 strains from the *Alnaster* group. The majority of the former strains belonged to the first type actinomycetes mentioned in the next section (cf. Table 5); and most of the later strains to the second type.

A limited number of isolation tests of the above-mentioned plant species showed that method B might yield similar types of actynomycete as method A, but less frequently.

The nodule steles were derived more easily from the young nodules of the *Alnaster* group (*A. Sieboldiana* and *A. firma*) than from those of the *Alnus* group (*A. tinctoria* var. *glabra* and *A. japonica*). Among some exotic alder species, the nodule steles were easily separated from the nodules of *A. cordata*, *A. maritima*, *A. nepalensis*, but it was almost impossible to expose and streak the steles of the nodules of *A. glutinosa* and *A. incana*, because the nodules together with their steles were easily separated from the root, even in the three- to six-month-old seedlings. On these species, isolation method B was applied in parallel and yielded frequently the same kinds of actinomycete with the common isolates from the *Alnus* group.

2. Other species

Besides *Alnus* species, the author has carried out several isolation trials by methods A and B in this decade on the other non-leguminous species.

The detailed data of isolation trials on them are omitted here, but total results of isolations

of microorganisms by method A on each of them are shown in Table 3.

Table 3. Results of isolation experiments by method A on other species.

Host plant	Number of tubes which yielded mono- or mixed cultures of microorganisms			
	Actinomycetes	Bacteria	Fungi	None
Elaeagnus umbellata	27	42	3	53
Myrica rubra	17	23	2	129
Casuarina equisetifolia	12	14	5	53
Coriaria japonica	15	35	6	42
Ceanothus americanus	9	9	0	22
Podocarpus macrophylla	29	36	4	83
Sciadopytis verticillata	11	18	0	23
Total : number (722)	120	177	20	405
%	16.6	24.51	2.77	56.09

(1) *Elaeagnus umbellata*

Mature root nodules of this species resemble those of *Alnus* species in their external appearance. Nodule formation on the seedlings of this species was noticed in about four months after sowing (cf. Photo. 2).

Elaeagnus umbellata yielded perfect nodule steles in about 20 to 30 per cent of the attempts (cf. Photo. 12). Total result of 10 separate trials with 116 test tubes is shown in Table 3.

Among the test tubes used in this experiment, two tubes yielded two different types of actinomycetes on the same agar slant respectively, therefore 29 actinomycetal strains were isolated. The types of them will be described in the next section (cf. Table 5).

(2) *Myrica gale*

The nodules of this species closely resembled those of *Casuarina* species in their external appearance and usually had fibrous roots growing from the nodule tips or lobes (cf. Photo. 4). In the nursery, nodules first appeared on 5-month-old seedlings. Nodule formation on this species was considerably slower than that of *Alnus* and *Elaeagnus* species.

Entire nodule steles were obtained comparatively easily from about 30 per cent of the younger nodules (cf. Photo. 13), but with difficulty and rarely from the older ones. Total result of 10 separate isolation trials with 168 test tubes is shown in Table 3.

In this experiment, all but one of the 17 strains of actinomycete isolated belonged to the Type II group of *Streptomyces* described in the next section (cf. Table 5).

As previously stated, isolations by methods A and B frequently yielded similar actinomycetes.

(3) *Casuarina equisetifolia*

The nodules of this species resembled externally those of *Myrica* species. They have considerably well-developed fibrous roots growing from the tips or nodule lobes (cf. Photo. 5).

In Japan, the nodule endophytes of *C. equisetifolia*, *C. cunninghamiana*, *C. stricta*, *C. glauca* and *C. Huegeliana* are not widely distributed or prevalent. But in the nursery, nodule formation was accomplished within two or three months after sowing seeds inoculated with crushed nodules of *C. equisetifolia* or *C. cunninghamiana*. Perfect nodule steles were evidenced in about 30 to 50 per cent from the young nodules (cf. Photo. 14). Table 3 shows the total result of 6 separate isolation trials with 83 test tubes.

In this experiment, one of the test tubes used yielded two different type actinomycetes on

the same agar slant and 13 strains of actinomycetes were isolated. Most of them belonged to the Type I group described in the next section (cf. Table 5).

（4） *Coriaria japonica*

The nodules of this species closely resembled those of *Elaeagnus* species in external appearance ; they lacked the fibrous nodule roots of *Casuarina* and *Myrica* species. The nodules appeared on nursery seedlings about 5 months after sowing (cf. Photo. 6).

Perfect nodule steles were easily collected from about 30 per cent of the young nodule samples. Total result of 3 separate isolation trials with 88 test tubes are shown in Table 5.

In this experiment, 15 strains of actinomycete were isolated. Most of them belonged to the Type I group referred to in the next section (cf. Table 5).

In confirmation of their isolation by method A, actinomycetal strains were also isolated occasionally by method B.

（5） *Ceanothus americanus*

Species of the genus *Ceanothus* are not distributed in Japan, but in the nursery of the Government Forest Experiment Station in Tokyo, nodule formation was accomplished on the 5-month-old seedlings of *C. americanus* after sowing without any inoculation treatment. The nodules resembled those of *Coriaria japonica* in outer appearance (cf. Photo. 7).

The number of seedlings obtained were limited. The tests by method A using 40 agar culture tubes for attempted isolations were repeated only 3 times (Table 3). Perfect nodule steles were collected comparatively easily.

In this experiment, 9 strains of actinomycete were isolated and most of them belonged to the Type I group as will be shown in the next section (cf. Table 5).

（6） *Podocarpus macrophylla*

The nodules of this species were remarkably different in outer appearance from those of other non-leguminous plants mentioned above, and in some cases they are termed thickening strip, pearl necklace or mamillate (cf. Photo. 9).

The nodules of *P. macrophylla* were noticed on four-month-old nursery seedlings.

The young fresh nodules of two-year-old seedlings of *Podocarpus macrophylla* were used for the isolation tests. Perfect nodule steles were collected rarely, and with difficulty, from both young and old nodules, because the whole nodules including nodule steles were easily detached from the root. Only from early mature nodules were the nodule steles developed to proper thickness to allow isolations (cf. Photo. 15).

In this experiment, one of the test tubes used yielded two different type actinomycetes on the same agar slant, therefore 30 strains of actinomycete were isolated ; most of them belonged to the Type I group as will be explained in the next section (cf. Table 5).

Similar actinomycetes were isolated with comparative ease by both methods A and B. Some of them developed in pure condition in plate culture.

（7） *Sciadopytis verticillata*

Nodules of this species closely resembled those of *Podocarpus macrophylla*, but they were smaller and appeared as narrow ellipsoides (cf. Photo. 10).

In the nursery, the nodules were first apparent on one-year-old seedlings. The seeds of this species were sown in early spring and sprouted in autumn.

The fresh nodules of two-year-old seedlings were used for the isolation. Although perfect nodule steles were difficult to secure, a sharp nodule stele (cf. Photo. 16) was obtained at the rate of one in thirty trials from the fresh nodules ; from these the actinomycetes were isolated

comparatively easily.

The isolation trials by method A were repeated 4 times using 50 transfers for agar slopes in test tubes. Total result is shown in Table 3.

Of 11 strains of actinomycete isolated, all but one belonged to the same *Streptomyces* (Type III), as will be seen in the next section (cf. Table 10).

The isolation tests by method B using PETRI dishes provisionally, confirmed the presence of similar actinomycetal strains.

Among the non-leguminous plants used in this experiment, the ease or difficulty in obtaining the nodule steles from the nodules was considerably different when the species were different or when the nodules were young or old, even in the same species. The isolation percentages of actinomycetal strains from these species were higher when the perfect nodule steles were used.

By methods A and B, bacteria and fungi were also isolated in some degree from these non-leguminous plants. But in most cases, they seemed to be different species, even though they were isolated from one and the same plant species ; they have not yet been confirmed.

Judging from the results of the above mentioned experiments, it seems that when the isolation trials are carried out in favourable conditions, the averaged isolation percentages of microbes by methods A and B were fairly consistent and comparable as shown in Table 4.

Table 4. Averaged isolation percentages of microbes by methods A and B from non-leguminous root nodules.

Isolation method	Isolation percentages of each kind of microbes			
	Actinomycetes	Bacteria	Fungi	None
Method A	10—30	10—30	0—10	30—70
Method B	10—20	30—50	0—20	20—40

It is of great interest to note that no microbial growth occurred on 30 to 70 per cent of the agar slants used for the isolation experiment by method A, nor on about 20 to 40 per cent of the PETRI dishes by method B.

III. Classification of the actinomycetes

A. Types of actinomycetal isolates and their properties

The actinomycetal isolates numbered 32 from the root nodules of the *Alnus* group, 36 from the *Alnaster* group, 29 from *Elaeagnus umbellata*, 17 from *Myrica rubra*, 13 from *Casuarina equisetifolia*, 15 from *Coriaria japonica*, 9 from *Ceanothus americanus*, 30 from *Podocarpus macrophylla*, and 11 from *Sciadopytis verticillata*.

Three types (groups) of actinomycetes were frequently isolated ; however, four types were provisionally defined on the basis of cultural characters.

Type I : These were characterized by comparatively rapid growth on yeast-glucose-peptone agar medium visible to the naked eye in 48 hours ; they attained 0.5 cm in diameter in one to two weeks.

The colonies were cartilaginous in appearance, pale yellow or pale yellowish brown in color that adhered tenaciously to the medium and usually lacked aerial hyphae but occasionally produced them in a later stage. Frequently, some of the colonies changed the surrounding substrate to a medium dark brown color even at the beginning of their development. In general, the

actinomycetes belonging to this type seem to resemble those which are commonly isolated from soil.

Type II : Colonies of this type developed slowly on yeast-glucose-peptone agar medium and usually attained a diameter of 2 to 3 mm in 2 to 3 weeks.

The surfaces of these colonies were covered with white powdery aerial hyphae, the vegetative mycelia were orange or reddish purple in color, and tenaciously adhered to the medium. These colonies did not usually produce pigment on the medium except on old cultures ; the reddish purple pigment was observed on the closely surrounding medium.

Type III : Colonies of this type developed fairly rapidly on the medium and reached a diameter of 1 to 2 mm in a week. The surfaces of these colonies bore white, powdery hyphae. The vegetative mycelia were reddish orange in color and brittle, but did not adhere to the medium tenaciously.

Type IV : The isolates which did not belong to the former three types were included in this type provisionally. So, this type included different kinds of actinomycetal strains, but they were very rarely isolated.

The numbers of actinomycetal isolates from each plant species are shown in Table 5.

Table 5. Type classification of the actinomycetal isolates from non-leguminous plants.

Host plant	Number of actinomycetal isolates	Number of actinomycetal type isolates			
		I	II	III	IV
Alnus group	32	23	5	3	1
Alnaster group	36	8	28	0	0
Elaeagnus umbellata	29	15	11	2	1
Myrica rubra	17	1	16	0	0
Casuarina equisetifolia	13	9	2	0	2
Coriaria japonica	15	13	0	1	1
Ceanothus americanus	9	8	1	0	0
Podocarpus macrophylla	30	27	1	1	1
Sciadopytis verticillata	11	1	0	10	0

B. Cultural properties of actinomycetal type isolates I, II and III

The cultural properties of about 40 representative actinomycetal isolates from these non-leguminous species were compared with strain v. PLOTHO, CBS of *Actinomyces alni* PEKLO. These data appeared in a previous report (1961). This strain of *Actinomyces alni* seemed to be a *Streptomyces* belonging to the Type I actinomycetes. Recently, WAKSMAN (1961, p. 197) identified it as *Streptomyces coelicolor* (MÜLLER) WAKSMAN and HENRICI. The distinctive characteristics between these types are tabulated in Table 6.

The following new interesting facts were noted in the cultural studies :

(a) A purplish soluble pigment produced by the Type II actinomycetes (*Streptomyces*) in CZAPEK's solution.

In culturing the strains on the many organic and synthetic media used, all strains belonging to the Type II actinomycetes produced a purplish soluble pigment in CZAPEK's solution. The amounts of pigment production by these strains varied considerably, but seemed to be more pronounced by freshly isolated strains. Some of them lost pigment-producing abilities upon repeated culture on artificial media, especially on organic media.

Table 6. Distinctive characteristics between nodule actinomycetal types I, II and III.

Type / Characteristics	Actinomycetal strain types		
	I	II	III
Color of colony on the isolation medium	yellow to yellowish brown usually	orange or purplish orange	reddish orange
Form of aerial hyphae	not definite, spiral, wave, straight and no aerial hyphae	compact spiral usually	straight usually
CZAPEK's agar	medium growth	slow/poor growth	no growth
Starch agar	good growth	poor growth	no growth
Ca-malate glycerine agar	good growth	no growth	medium growth
CZAPEK's solution	no soluble pigment usually	purplish soluble pigment such as phenazine	no soluble pigment
Bouillon solution	acid	alkaline	very alkaline
Hydrolysis of starch	+	−	−
Liquefaction of gelatin	+	−	−
Peptonization and coagulation of milk	+	−	−

This pigment seemed to be a specific one compared with those produced by other actinomycetal strains belonging to the Types I and III. Ether extractions of CZAPEK's solution were analysed chemically after growth for one month at 28°C. This pigment proved to be one of the phenazine derivatives and closely resembled the iodinin which is produced by *Pseudomonas iodinum* (DAVIS) TOBIE (Syn. *Chromobacterium iodinum* DAVIS).

(b) Vesicular swellings of vegetative hyphae produced by some strains characterized the Type I actinomycetes.

Most strains belonging to the Type I actinomycetes from *Alder* (including *Actinomyces alni* Peklo isolated by v. PLOTHO) produced round swellings, resembling the vesicles within alder nodules, when they were cultured for more than one month on calcium-tartarate glycerine agar medium consisting of calcium tartarate 10 g, NH_4Cl 0.5 g, K_2HPO_4 0.5 g, Glycerine 10 g, dist. water 1000 cc, pH 7.0. These round swellings were usually on the terminal tips of hyphae, but some were located at the sides or within the hyphae (cf. Photos. 25～26). They were not observed on all parts of vegetative mycelium, but usually occurred in submerged mycelium in ager media. The stages of their development varied considerable acccrding to their position. They consisted of a thin cell membrane containing two or three vacuolated areas as shown by FEULGEN's staining method (cf. Photo. 26). The diameters of the mature swellings were three to four microns and were slightly smaller than those within mature alder nodules. They were stained intensely with carbol fuchsin, carbol gentian violet and were gram positive. These methods confirmed their close resemblance to vesicles of alder nodules. Drop cultures of these swellings in the ordinary

media, i.e., yeast-glucose-peptone and glucose bouillon liquid media, did not germinate or develop. Judging from the above mentioned facts, it seems that these swellings were involution forms of actinomycetes which had lost their developing power. But it is not certain that they were one and the same as the alder nodule vesicles.

IV. Discussion and Conclusion

1. Isolation methods :

For the purpose of isolating the actinomycetes from the root nodules of non-leguminous plants, especially from *Alnus*, *Elaeagnus* and *Myrica* species, the author performed several other experiments by different methods, including the one used by PLOTHO (1941). But all experiments failed to find a definite isolation method of actinomycetes or other microorganisms from these root nodules, although there were some rare cases in which the actinomycetal strains or other microorganisms were isolated perhaps by chance. Even in the isolation experiments by PLOTHO's method, the actinomycetal strains were only rarely isolated, and the percentages of isolation of actinomycetes were very low (1952c).

Satisfactory isolation methods were achieved by using nodule steles in method A, or using disinfectants for nodule surface sterilization in method B. Method A was preferable for the isolation of the actinomycetes from root nodules of *Alnus tinctoria* var. *glabra*, *A. japonica*, *A. Sieboldiana*, *A. firma*, *A. cordata*, *A. maritima*, *A. nepalensis*, *A. orientalis* ; *Elaeagnus umbellata* ; *Myrica rubra* ; *Casuarina equisetifolia*, *C. cunninghamiana*, *C. stricta*, *C. glauca*, *C. Huegeliana* ; *Coriaria japonica* ; *Ceanothus americanus* and *Podocarpus macrophylla* from which the perfect nodule steles were collected comparatively easily. Isolation method B was more favourable for root nodules of *Alnus glutinosa*, *A. incana* and *Sciadopytis verticillata*, from which the perfect nodule steles were obtained only rarely and with great difficulty.

2. Types and kinds of isolated actinomycetes :

The results of isolation experiments by methods A and B on these non-leguminous root nodules have made clear that restricted actinomycetal strains occur for each host species.

These isolated actinomycetal strains were divided into four types on the basis of their developing state. The first and second type actinomycetes were frequently isolated from the root nodules of most of these non-leguminous plants ; the third type actinomycetes were isolated usually from only the *Sciadopytis species*, and rarely from *Alnus*, *Elaeagnus*, *Coriaria*, *Podocarpus* species. The fourth type actinomycetes included the isolates that did not belong to the other three types ; these actinomycetes were very rarely isolated.

From consideration of the isolation percentages of the first, second and third type actinomycetes, the non-leguminous root-nodule bearing plants used in this experiment seem to be divided into the following three plant groups.

（1） The first plant group : The plant species having root nodules from which the Type I actinomycetes were isolated most frequently, although the Type II actinomycetes were sometimes also present.

The following species : *Alnus* species (especially subgenus *Alnus*), *Elaeagnus umbellata*, *Casuarina equisetifolia*, *Coriaria japonica*, *Ceanothus americanus* and *Podocarpus macrophylla* seem to be in this group at the present time.

（2） The second plant group : The plant species having root nodules from which the Type II actinomycetes are isolated principally or most frequently, but not exclusively, were *Alnus*

species (especially subgenus *Alnaster*) and *Myrica rubra*.

(3) The third plant group : This contains only one species, *Sciadopytis verticillata*, having root nodules from which the Type III actinomycetes are isolated principally.

It is of interest to note that the Type I actinomycetes were usually isolated from the root nodules of *Podocarpus macrophylla* and the Type III actinomycetes from *Sciadopytis verticillata*, although both nodules are classified by many authorities as mycorrhizal.

The following facts were made clear from the cultural investigations :

(1) The Type I actinomycetal strains were not always the same kind of actinomycete, even when they were isolated from the same host plant. Several different kinds of actinomycetes are involved. Most of them belonged to the genus *Streptomyces*, but certain isolates appeared to be in the genus *Nocardia*.

Although these isolates differed in detailed properties, they had something in common with each other in some important characteristics, especially in their physiology.

(2) The Type II actinomycetal strains were one and the same species belonging to the genus *Streptomyces*, despite their isolation from the root nodules of different kinds of species or genera. A distinctive characteristic was the production of a purplish soluble pigment, a phenazine derivative, closely resembling iodinin in CZAPEK's solution.

(3) All of the Type III actinomycetal strains seemingly belonged to the genus *Streptomyces* and appeared to be almost one and the same species, although there were a few differences in their detailed properties.

(4) The actinomycetal strains belonging to the Type VI were rarely isolated from some of these non-leguminous root nodules. They seemed not dominant but chance isolations, and among them different kinds of actinomycetes belonging to *Streptomyces*, *Nocardia* were involved.

3. Attempts to produce nodules by re-inoculation with the isolates.

The author conducted re-inoculation tests with those organisms which were most frequently isolated from the root nodules of *Alnus*, *Elaeagnus*, *Myrica*, etc. Primarily on the alder species, several inoculation tests with each type of actinomycetal strain, the bacterial strains and the fungal strains, individually and in combination, have been done repeatedly throughout twenty years in a variety of ways and manner. These techniques employed the use of sterile seedlings grown in liquid, in sand and agar media in large test tubes, or in semi-sterile condition with the sterile seedlings grown in sterile sand in small pots. But none of these inoculation tests led to the development of nodules on the *Alnus* species or other non-leguminous species.

In general, whenever a species was re-inoculated with the particular actinomycetal strains which were isolated originally from that species, growth was superior to that of the control series ; especially when seedlings were grown in nitrogen-free culture media, although the author has not yet obtained nitrogen analysis data about them to provide evidence (cf. Photo. 27).

In 1941 PLOTHO reported success in obtaining nodule formation on seedlings of *Alnus glutinosa* in water culture by inoculation with *Actinomyces alni* PEKLO which she had isolated from the root nodule of *Alnus incana*. Because PLOTHO did not mention seed sterilization, used unsterilized tap water for culture, and moreover, in so far as some of the culture vessels were excluded from the inoculation test by reason of contamination by algae, it is doubtful that she kept the seedlings in a sufficient sterile condition. On the other hand, her experiment suggests that *Actinomyces alni* PEKLO is one of the indispensable agents in nodule formation on alder in as much as only those alder seedlings inoculated with *Actinomyces alni* produced nodules.

Under sterile conditions with sand and water cultures the author sought supplementary proof

of PLOTHO's inoculation experiment, using seedlings of the same alder species (*Alnus glutinosa*) and the *Actinomyces alni* PEKLO obtained from the "Centraalbureau voor Schimmelcultures, Baarn, Holland". Satisfactory nodule formation did not result. However, in most cases the seedlings inoculated with *Actinomyces alni* showed a superior growth compared with the non-inoculated seedlings.

Casuarina seedlings observed growing in the unsterilized nursery soil in Tokyo lacked nodules. Apparently their natural endophyte was lacking in this soil. However, seedlings produced root nodules when they were inoculated with some of the actinomycetal strains isolated from *Casuarina* nodules. But if the soil was first sterilized and the seedlings were then inoculated, no nodule formation on the seedlings occurred.

Judging from the above-mentioned facts, it seems that the actinomycetal strains isolated from the alder and *Casuarina* nodules can not produce nodules on their host plants by themselves, but they might play an important part in the nodule formation. The author assumes that on *Elaeagnus, Myrica, Casuarina,* and *Podocarpus* species, the same presumption may also apply.

In the author's experience with methods A and B, the isolation percentages of actinomycetes from the root nodules of these non-leguminous plants were in most cases only 10 to 30 per cent among the test tubes or PETRI dishes used, 20 to 70 per cent were negative. Therefore, further investigations are needed to decide the extent and importance of these actinomycetes in the nodule formation on their host plants.

The inoculation tests using aseptically grown alder seedlings showed that a considerable quantity of nodule crushes or soil were needed to assure production of nodules on the seedlings. As one of the main causes of these phenomena, the author supposed at first that the growth-promoting substances such as vitamins, amino-acids etc., which are involved within the inoculants (the nodule crushes or the soil), may in addition to nodule endophytes be needed to produce the nodules. To test this idea, sterile alder seedlings were inoculated with the various actinomycetal isolates and cultivated in sterile sand in test tubes or in pots containing a considerable quantity of the water extract of nodules or soil that had been filtered through a bacterial membrane filter. However, all attempts failed to bring about the nodule formation.

Curious forms of the nodule endophytes observed by most of the earlier investigators are the so-called vesicles in the root nodules of *Alnus, Elaeagnus, Hippophaë, Myrica, Casuarina, Coriaria, Podocarpus* etc. (cf. Photo. 18). These vesicles have been interpreted as spores, sporangia, bacteroids and involution forms of the endophytes. Although opinions still differ, most investigators interpret them to be involution forms. From cytological studies of nodules of *Alnus, Myrica* and *Hippophaë* species, HAWKER and FRAYMOUTH (1951) regarded the vesicles formed in older cells as endophytic sporangia and concluded that the nodule organisms are members of the Plasmodiophorales.

However, some differences prevail in the staining properties between the spores of *Plasmodiophora brassicae* obtained from club-root of turnip and alder nodule vesicles. The former, stained with carbol gentian violet, showed unstained peripheries which seemed to be thick cell walls (cf. Photo. 23); whereas all parts of the latter were stained uniformly by the same staining method (cf. Photos. 19, 21). When these were stained with 1 to 20 dilute carbol gentian violet, most of the spores of *P. brassicae* contained a deeply stained spot (Photo. 24), which alder nodule vesicles did not contain. Furthermore, the author has observed germination of the resting spores of *P. brassicae* in filtrated soil extract solution, and yet similar attempts to germinate alder nodule vesicles failed.

Cytological investigations on crushed alder nodules (*Alnus Sieboldiana*) by the electronmicroscope and by the bacterial stainings (cf. Photos. 19～22) by the author have shown that the vesicles of nodules are produced on the ends of hyphae and remain attached for a certain period of time. Furthermore, in the older culture on calcium tartarate agar medium, some actinomycetal strains (especially those of Type I isolated from alder nodules) produced often the vesicle-like swellings on the tips or laterals of vegetative hyphae (cf. Photos. 25, 26). They were slightly smaller in diameter compared with the matured vesicles in nodules and seemed to have lost their viability.

Therefore, it seems that the vesicles of alder nodules are more likely involution forms of actinomycetes than sporangia of the members of Plasmodiophorales.

In addition to nodule vesicles, PEKLO (1910), SHIBATA and TAHARA (1917), ARCULARIUS (1928), SCHAEDE (1933, 1939), HAWKER and FRAYMOUTH (1951), TAUBERT (1956), and KÄPPEL and WARTENBERG (1958) reported the existence of bacteroidal bodies in some of the root nodules of non-leguminous plants, especially in *Alnus* species. The nature of these bacteroids is not sufficiently clear, but most of the investigators have intepreted them as the special form in one of the developing stages of nodule endophytes.

From a cytological investigation of *Alnus* and *Myrica* root nodules, SCHAEDE (1933, 1939) concluded that the bacteroids are the resistant form of nodule endophytes (*Actinomyces*). HAWKER and FRAYMOUTH (1951) regarded them as the minute granules, or spores, developed from the sporangium (vesicles) of *Plasmodiophora* in the nodules.

KÄPPEL and WARTENBERG (1958) reported a detailed study on the morphological development of endophytes in the root nodules of *Alnus glutinosa* GAERTN. They asserted that the endophytes of alder nodules are actinomycetes (*A. alni* PEKLO), that the nodule vesicles are the involution forms of *A. alni*, and that the bacteroids develop not from the vesicles but from the main hyphae of endophytes in the nodules, and are the only forms with the ability of infecting the roots and producing the root nodules. They also stated that they succeeded in producing nodules on alders by inoculating them with bacteroids collected from the nodules of alder seedlings previously grown in nitrogen-free CRONE's solution.

The author grew seedlings of *Alnus Sieboldiana* and *A. tinctoria* var. *glabra* in nitrogen-free culture solution for two years and examined the hand-sectioned nodules to collect the masses of bacteroids as KÄPPEL and WARTENBERG (1958) stated, but failed to find them in the materials.

Although POMMER (1956) asserted that nodules of alder are caused by actinomycetes, he failed to reproduce nodules with his twenty-three actinomycetal isolates. He also stated that he succeeded in obtaining the fungal nodule formations on alder by inoculating with his fungal isolates, *Penicillium albidum* SOPP. and *Cylindrocarpon radicicola* WR. respectively. But these fungal root nodules were small and short lived, and he never found the vesicular stage of endophytes in them. Therefore, we may assume that these were not true alder nodules. From the cytological point of view, TAUBERT (1956) reported the detailed processes of infection and development of nodule endophyte of *Alnus glutinosa*; but he did not clarify the nature of endophyte, although he denied its actinomycetal nature.

More recently, POMMER (1959) reported that from the root nodules of *Alnus glutinosa*, he isolated an actinomycete-like microorganism different from *Actinomyces alni* PEKLO which produced nodules on the same *Alnus* species in sterile plant-growth conditions. This organism developed well in ordinary glucose-asparagine medium and produced the vesicles, or bacteroids, *in vitro* similar to those *in vivo* on alder. He also succeeded in isolating similar endophytes from the

nodules of *Elaeagnus*, *Hippophaë*, and *Shepherdia* species, but not from those of *Casuarina*, *Ceanothus*, and *Myrica* species. QUISPEL (1960) failed to confirm POMMER's findings, but suggested that the latter's success may be attributed to the existence of physiological variants of the endophyte. The author also has tried but could not confirm POMMER's results.

On the other hand, QUISPEL (1954a, b ; 1955) reported that all attempts to grow the endophytes of alder nodules *in vitro* have ended in failure in ordinary methods, but succeeded in the growth *in vitro* of the endophyte of *Alnus glutinosa* by using the alcoholic extracts of usuable peat or nitrogen-poor alder root, prepared after a previous water extraction. Later he (1960) reported that an alcoholic substance of alder nodule is absolutely necessary for the growth of nodule endophyte, and this substance can be purified by extracting with petroleum ether. Furthermore, he asserted that the growth of alder nodule endophyte is inhibited by substances formed during autoclaving of glucose and yeast autolysate, and peptone is indispensable for the growth of endophyte. Although he did not identify clearly the nodule endophyte, his work is invaluable for future research on the possibility of artificial culture of the alder nodule endophyte.

In preliminary experiments adopting the culture method of QUISPEL, the author occasionally obtained growth of the endophyte in a combined yeast-soil extract glucose solution upon the addition of an alcoholic extract derived from alder nodules that was prepared after a previous water extraction. When the nodule steles of alders were cultured in this medium, one could observe in hanging drop culture in a humid chamber that some of the cell contents were transformed into well-defined square bodies, 2μ in length in a few days at 28°C. Most of these square bodies developed into two bacteroidal bodies in one to two months (cf. Photos. 28～31).

It is not clear whether these curious bodies are one of the developing stages of *Actinomyces alni* or a different kind of alder nodule endophyte. In preliminary tests using stelar tissue containing these bacteroidal bodies to inoculate alder seedlings in sterile culture, some of the seedlings produced nodule-like swellings on their roots in four to five weeks after inoculation. Though these swellings are not yet confirmed to be true alder nodules, it is the first instance whereby small quantities of the pure nodule tissue, i. e., nodule stele which seemed to be free from the microbes developing in ordinary culture media, produced nodule-like swellings on the alder seedlings in sterile culture.

Although no one has yet confirmed the causative endophytes of these non-leguminous nodules, the following conclusions seem warranted :

The actinomycetes commonly isolated from these non-leguminous root nodules seem to be one of several nodule endophytes, though none of these *per se* has the ability to produce root nodules.

The failure of these actinomycetal isolates to cause nodulation can be attributed either to the possibility that they have lost their nodule-producing ability during growth *in vitro*, or that they require other co-operative nodule endophytes which do not develop on the usual media.

Summary

1. By the isolation methods A and B, the author succeeded in the isolation of actinomycetes which were characteristic of nodules of *Alnus*, *Elaeagnus*, *Myrica*, *Casuarina*, *Ceanothus*, *Podocarpus* and *Sciadopytis* species respectively.

As materials for isolation, only the nodule steles of these plants were used in method A ; in method B, the entire nodules were disinfected with weak Uspulun (semesan) or mercuric solutions. Yeast-extract glucose peptone agar medium was used principally as the culture medium

for isolation.

2. The actinomycetal strains isolated were divided into four types on the basis of their cultural characteristics.

3. Type I actinomycetal strains were isolated from the root nodules of *Alnus*, (especially from the subgenus *Alnus*), *Casuarina*, *Coriaria*, *Ceanothus* and *Podocarpus* species principally, often from *Alnus* (especially from the subgenus *Alnaster*), *Elaeagnus* species and very rarely from *Myrica* and *Sciadopytis* species.

Among them, many kinds of actinomycetes were involved, but all had something in common with each other in their physiological characteristics. Most of them clearly belonged to the genus *Streptomyces*; others suggested inclusion in the genus *Nocardia*.

4. Type II actinomycetal strains were isolated from the nodules of *Alnus* (especially from subgenus *Alnaster*, but seldom from *Alnus*) and *Myrica* species principally, often from *Elaeagnus* species and rarely from *Casuarina*, *Ceanothus* and *Podocarpus* species.

All of them are one and the same species belonging to the genus *Streptomyces* with the distinctive characteristic of producing purplish soluble pigment in Czapek's solution, which is one of the derivatives of phenazine closely resembling iodinin.

5. Type III actinomycetal strains were isolated from the nodules of *Sciadopytis* species principally and rarely from *Alnus* (subgenus *Alnus*), *Elaeagnus*, *Coriaria* and *Podocarpus* species to date. All of them were closely related species belonging to the genus *Streptomyces*.

6. Type IV actinomycetal strains were isolated very rarely from some of these root nodules; these different ones did not belong to any of the former three types.

7. None of the inoculation tests with the principal isolated actinomycetal strains on their host plants has resulted in the development of nodules; but, in most cases, the host plants inoculated showed a superior growth compared with ones uninoculated.

8. Judging from the results of the author's investigations, especially in alder root nodules to data, the actinomycetal strains isolated usually from each of these plant species seemed to be one of the nodule endopytes of their host plants, although none of them had the ability of producing the nodules by itself.

It seems appropriate to conclude that the failure of nodule formation on the host plants inoculated with the isolated actinomycetal strains can be attributed either to the fact that the isolated strains have lost their nodule-producing power during the isolation and following growth *in vitro*, or the fact that to produce nodules the actinomycetes require other co-operative nodule endophytes which do not develop on the usual media.

9. The nature of nodule vesicles and bacteroids observed in some of these non-leguminous nodules and the results of attempts to produce nodulation with them are discussed.

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* Symbols of host plants: A=*Alnus*, CA=*Casuarina*, CE=*Ceanothus*, CO=*Coriaria*, COF=*Coffea*, CY=*Cycas*, DIS=*Discaria*, DRY=*Dryas*, E=*Elaeagnus*, FAG=*Fagonia*, G=*General*, H=*Hippophaë*, M=*Myrica*, P=*Podocarpus*, S=*Shepherdia*, SC=*Sciadopytis*, TRI=*Tribulus*, Z=*Zygophyllum*.

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Explanation of plates

Plate 1

Root nodules of non-leguminous plants.

Photo. 1. *Alnus Sieboldiana*.

Photo. 2. *Elaeagnus umbellata*.

Photo. 3. *Shepherdia argentea.* (From Alaska)

Photo. 4. *Myrica rubra.*

Photo. 5. *Casuarina equisetifolia.*

Photo. 6. *Coriaria japonica.*

Photo. 7. *Ceanothus americanus.*

Photo. 8. *Dryas drummondii.* (From Alaska)

Plate 2

Root nodules of non-leguminous plants and nodule steles of non-leguminous plants.

Photo. 9. Root nodules of *Podocarpus macrophylla.*

Photo. 10. Root nodules of *Sciadopytis verticillata.*

Photo. 11. Nodule stele of *Alnus Sieboldiana.*

Photo. 12. Nodule stele of *Elaeagnus umbellata.*

Photo. 13. Nodule stele of *Myrica rubra.*

Photo. 14. Nodule stele of *Casuarina equisetifolia.*

Photo. 15. Nodule stele of *Podocarpus macrophylla.*

Photo. 16. Nodule stele of *Sciadopytis verticillata.*

Plate 3

Types of actinomycetal strains isolated from non-leguminous root nodules.

Photo. 17. Three types of actinomycetal strains which were usually isolated from non-leguminous root nodules. Cultured for two weeks at 28°C on yeast-glucose-peptone agar slopes. ×1.7

a : Type I actinomycetal strain (Strain A-136)

b : Type II actinomycetal strain (Strain A-1020)

c : Type III actinomycetal strain (Strain A-144b)

Plate 4

Cross section of the root nodule of alder.

Photo. 18. Cross-section of the root nodule of one-year old alder seedling (*Alnus japonica*). Note many vesicles in the nodule cells. Fixed with chromo-osmo-acetic sol. Stained with HEIDENHAIN's iron haematoxylin. ×1,200

Plate 5

Nodule vesicles of alder, resting spores of *Plasmodiophora brassicae* in the club-roots of turnip and vesicular swellings of actinomycetal mycelium.

Photo. 19. Vesicles in the crushed alder root nodules (*Alnus Sieboldiana*). Stained with carbol gentian violet. ×800

Photo. 20. Electro-micrograph of a vesicle (*A. Sieboldiana*). ×12,000

Photo. 21. Nodule vesicles stained with carbol gentian violet (*A. Sieboldiana*), Note the hyphae connected with the vesicle (left one). ×1,250

Photo. 22. Vesicles stained by the method of NISHIZAWA-SUGAWARA's flagella stain. ×4,000

Photo. 23. Resting spores of *Plasmodiophora brassicae* in the club-roots of turnip (crushed materials). Stained with carbol gentian violet. ×900

Photo. 24. Same organisms as in Photo. 23. Stained with 1/20 dil. carbol gentian violet. ×1,800

Photo. 25. Vesicular swelling produced on the mycelium of strain A-1017a (Type I actinomycete). Six months culture on calcium-tartarate glycerol agar. Stained with carbol gentian violet. ×1,800

Photo. 26. Vesicular swellings stained by FEUGEN's method. ×4,000

Plate 6

Inoculation effects of the isolated nodule actinomycetes.

Photo. 27. Inoculation effects of the isolated actinomycetes on their host plants (*Alnus tinctoria* var. *glabra*).
A : Strain *Act. alni* (Type I), B : Strain A-136 (Type I), C : Strain A-141 (Type I), D : Strain A-1041 (Type II), E : Control. 150 days culture in nitrogen-free culture solution.

Plate 7

Hanging drop cultures of the alder nodule steles (*A. Sieboldiana*) in the mixed culture solution of yeast-glucose solution and soil-extract solution plus alcoholic extract of alder root nodule.

Photo. 28. Development of the well-defined square bodies in the drop culture solution. 1 week culture at 28°C. ×700

Photo. 29. Same as Photo. 28 in two-months culture at 28°C. Note that some of the square bodies changed into the two bacteroidal bodies respectively. ×700

Photo. 30. Development of the square bodies beneath a cover glass in the culture drop. 1 week culture at 28°C. ×2,000

Photo. 31. Same as Photo. 30 in two-months culture at 28°C. Note that most of the square bodies changed into the two bacteroidal bodies respectively. ×2,000

非マメ科根粒からの，微生物の分離方法ならびに分離菌の類別

（非マメ科植物の根粒に関する文献）

植　村　誠　次[1]

摘　　要

現在までに報告されているマメ科以外の根粒植物を科，属にまとめてみると，第1表に示すようになる。しかしながら，これらの属のすべての種類について，根粒の形成が調べられているわけではなく，また一部の学者によっては根粒として取り扱うのに異論をもたれているものも含まれている。

なお以上のうち，チョウノスケソウ，*Discaria* の2属は最近加えられたものであり，調査が進むにつれてさらに新しい種類が発見されるものと思われる。

林業上肥料木として重要な位置を占めているハンノキ，グミ，ヤマモモ，モクマオウ属の植物は，古くから非マメ科根粒植物に属する代表的なものとされている。

第1表　非マメ科根粒植物の属する科および属

双子葉植物		
1.	カバノキ（Betulaceae）科	ハンノキ（*Alnus*）属
2.	グミ（Elaeagnaceae）科	グミ（*Elaeagnus*）属
		Hippophaë 属：外国産
		Shepherdia 属：外国産
3.	ヤマモモ（Myricaceae）科	ヤマモモ（*Myrica*）属
4.	モクマオウ（Casuarinaceae）科	モクマオウ（*Casuarina*）属
5.	ドクウツギ（Coriariaceae）科	ドクウツギ（*Coriaria*）属
6.	クロウメモドキ（Rhamnaceae）科	*Ceanothus* 属：外国産
		Discaria 属：外国産
7.	バラ（Rosaceae）科	チョウノスケソウ（*Dryas*）属
8.	ハマビシ（Zygophyllaceae）科	*Fagonia* 属：外国産
		ハマビシ（*Tribulus*）属
		Zygophyllum 属：外国産
9.	アカネ（Rubiaceae）科	*Coffea* 属：外国産
裸子植物		
10.	ソテツ（Cycadaceae）科	ソテツ（*Cycas*）属
11.	イチイ（Taxaceae）科	マキ（*Podocarpus*）属
12.	マツ（Pinaceae）科	コウヤマキ（*Sciadopytis*）属

第1表記載の根粒植物のうち，カバノキ，グミ，ヤマモモ，モクマオウ，ドクウツギ，クロウメモドキ，バラ科の根粒は，いずれもほぼ類似した外部形態ならびに内部構造をもつものと考えられ，とくにハンノキ属，グミ科，ヤマモモ属，モクマオウ属の根粒については，これまでに多数の研究（文献参照）が行なわれてきたが，内生菌の分離がきわめて困難なため，その本体については，細菌，糸状菌，放射状菌，粘

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菌説など，種々異説が主張されてきた。しかし，最近の研究では一部に異説（Hawker および Fraymouth 1951）も主張されているが，放射状菌（Actinomycetes）説が有力視されている（Krebbr 1932, Schaede 1933, 1939; Plotho 1941, Pommer 1956, 1959；Taubert 1956, Käppel および Wartenberg 1958, Niewiarowska 1959, 1961）。しかし，いずれの根粒についても，異論のない内生菌の実証は残された問題となっている。

ハマビシ科およびアカネ科のうち，後者は葉粒植物を含むものであるが，これらの根粒についての研究例は少なく，多くはマメ科根粒菌類似の細菌説（Sabet 1946, Mostafa および Mahmoud 1951, Montasir および Sidrak 1952, Steyaert 1932）が主張されているが，異説（Allen および Allen 1949, Rayner 1948）も見られ，根粒そのものについては，なお検討が要望されている。

裸子植物のうち，ソテツ属の根粒内には，観察者により藍藻類（Reinke 1879, Hariot 1892, Prantl 1889, Spratt 1911, Watanabe 1924, Winter 1935, Takeshige 1937, Guttengerg 1941, Schaede 1944, Douin 1953），細菌類（Prantl 1889, Schneider 1894, Pampaloni 1901, Life 1901, Kellerman 1911, Burrill および Hansen 1917, McLuckie 1922），および糸状菌類（Brunchorst 1886a, Zach 1910, Schaede 1944）の存在が報告されているが，根粒形成菌の実体は明らかにされていない。

イヌマキ属の根粒については糸状菌説（Nobbe および Hiltner 1899, Shibata 1902, Hiltner 1903, Petri 1903, Yeates 1924, Saxton 1930, Schaede 1943, Ferreira dos Santos 1947）および細菌説（Bottomley 1912a, Spratt 1912b, McLuckie 1923a, Phillips 1932）が主張されており，コウヤマキの根粒についてはわずかに Noellê（1910）および Laing（1923）によって菌根として報告されているにすぎなく，これらの内生菌の本体は，今後の研究により開拓されねばならない分野と考えられる。

以上のように，非マメ科根粒植物の根粒菌の分類的位置づけについては，いずれも確実に実証されたものはなく，未解決にとどまっている現状である。

筆者はこれまで主としてハンノキ属樹種の根粒を対象として，多年にわたってその内生菌の分離に関する実験を行ない，根粒の中心柱を用いる方法（分離方法A）と根粒の表面消毒による方法（分離方法B）の2方法により，これらの根粒から樹種に応じてほぼ一定の放射状菌が分離されることを見いだし，またおもな分離放射状菌の細菌学的諸性質を調査した結果，その多くは第Ⅰ，第Ⅱ，第Ⅲの3つの群に分けられることを実証し，その一部を取とまとめて発表した（1952c, d；1961）。

その後アキグミ，ヤマモモ，モクマオウ，ドクウツギ，*Ceanothus americanus*（北米産），イヌマキ，コウヤマキの根粒についても，引きつづき分離方法A，B，とくにA法によって多数の分離試験を実施した結果，これらからもハンノキ属の場合と同様，樹種に応じてほぼ一定の放射状菌を分離することに成功した。

本報告は，以上の非マメ科植物の根粒について，最近10か年間にわたって実施した分離試験の結果と，分離されたおもな放射状菌の特性ならびに類別を取りまとめたものである。また同時に実施してきた寄主植物に対する分離放射状菌の接種試験の結果とあわせて最近報告された諸外国の研究動向を紹介して，これらの非マメ科植物の根粒菌の実体について論議ならびに考察を行なった。なお文献欄には，1963年までのおもな非マメ科植物の根粒についての広汎な報告を収録した。

なお，本分離試験の結果得られた成果のおもなものは次のようである。

1. 酵母水・ブドウ糖・ペプトン寒天培地を分離用培地として，分離方法A（根粒の中心柱利用），B（根粒の表面消毒）の2方法により，ハンノキ属のほか，アキグミ，ヤマモモ，モクマオウ，ドクウツギ，

Ceanothus americanus，イヌマキ，コウヤマキの根粒からも比較的容易に，それぞれの樹種に一定した放射状菌（Actinomycetes）が分離された。

とくにこれまで菌根説が有力視されているイヌマキ，コウヤマキの根粒からも，放射状菌が容易に分離されることを明らかにした。

2. これらの分離放射状菌の大部分は，その細菌学的諸性質から，便宜上第Ⅰ，第Ⅱ，第Ⅲ，第Ⅳの4つの群に分類されたが，その大部分は前3者の型に属した。

3. 第Ⅰ型の放射状菌は，ハンノキ属のうちおもにハンノキ類，モクマオウ，ドクウツギ，*Ceanothus*，イヌマキの根粒から最も頻繁に分離され，ヤシャブシ類，アキグミからは時おり，ヤマモモ，コウヤマキからはきわめてまれに分離された。

これらの放射状菌の多くは，*Streptomyces* に属しており，かなり異なった種類のものも含まれているが，重要な生理学上の諸性質では共通した特性を示した。

4. 第Ⅱ型の放射状菌株はヤシャブシ類，ヤマモモの根粒から最も頻繁に分離され，ハンノキ類，アキグミからは時おり，モクマオウ，*Ceanothus*，イヌマキからまれに分離された。

いずれも同一種類の *Streptomyces* 属の放射状菌で，フェナチン（Phenazine）誘導体の色素を形成する点で特色ある種類と思われた。

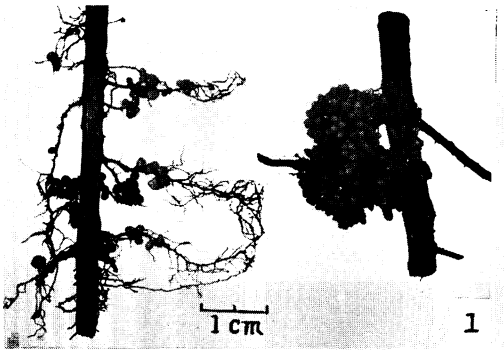
5. 第Ⅲ型の放射状菌株は，コウヤマキの根粒から最も多く分離され，これまでのところハンノキ類，アキグミ，ドクウツギ，イヌマキの根粒からまれに分離された。

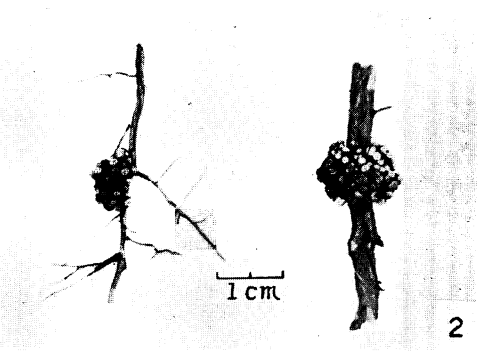
これらの菌株は，いずれも生理上かなりの特色がある，*Streptomyces* 属の同一放射状菌と思われた。

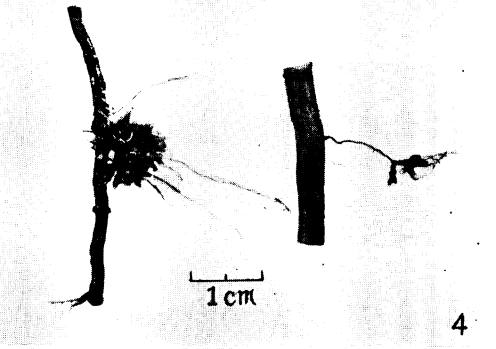
6. これらの根粒を通じてきわめてまれに分離された第Ⅰ，第Ⅱ，第Ⅲ型以外の数種の放射状菌株は，第Ⅳ型として類別した。

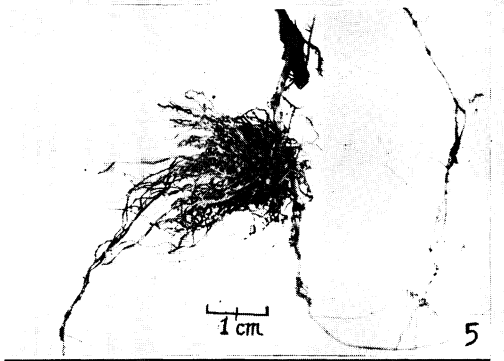
なお，これまでの研究結果から判断すると，これらの根粒から主として分離される放射状菌は，根粒内生菌のひとつとして重要な位置を占めるものと思われるが，いずれも接種試験において，寄主植物の根粒形成を実証し得なかった理由としては，未知の培養接種の条件が関与するか，あるいは普通の培地では発育しえない他の内生菌の協力を必要とするものと考察された。

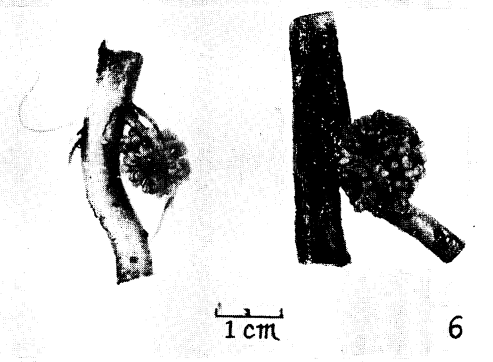
—Plate 1—

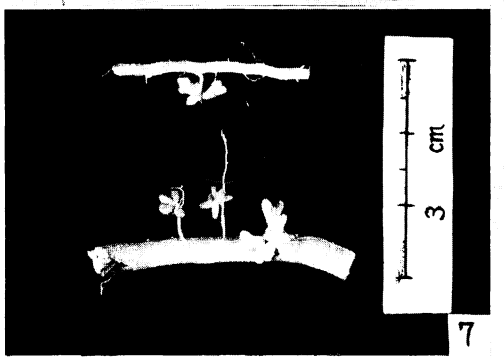


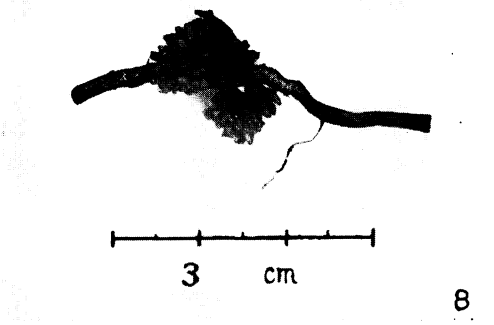




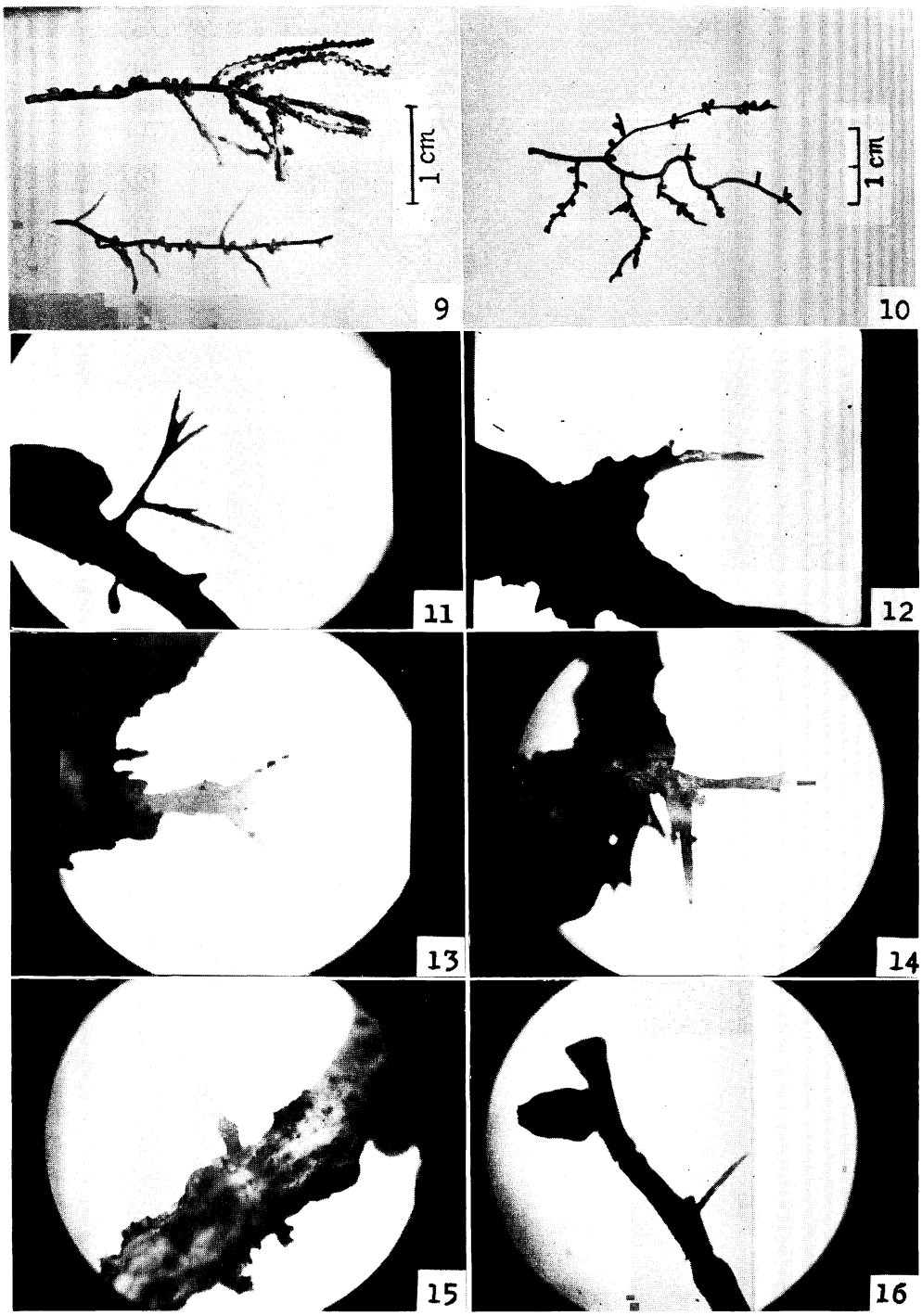






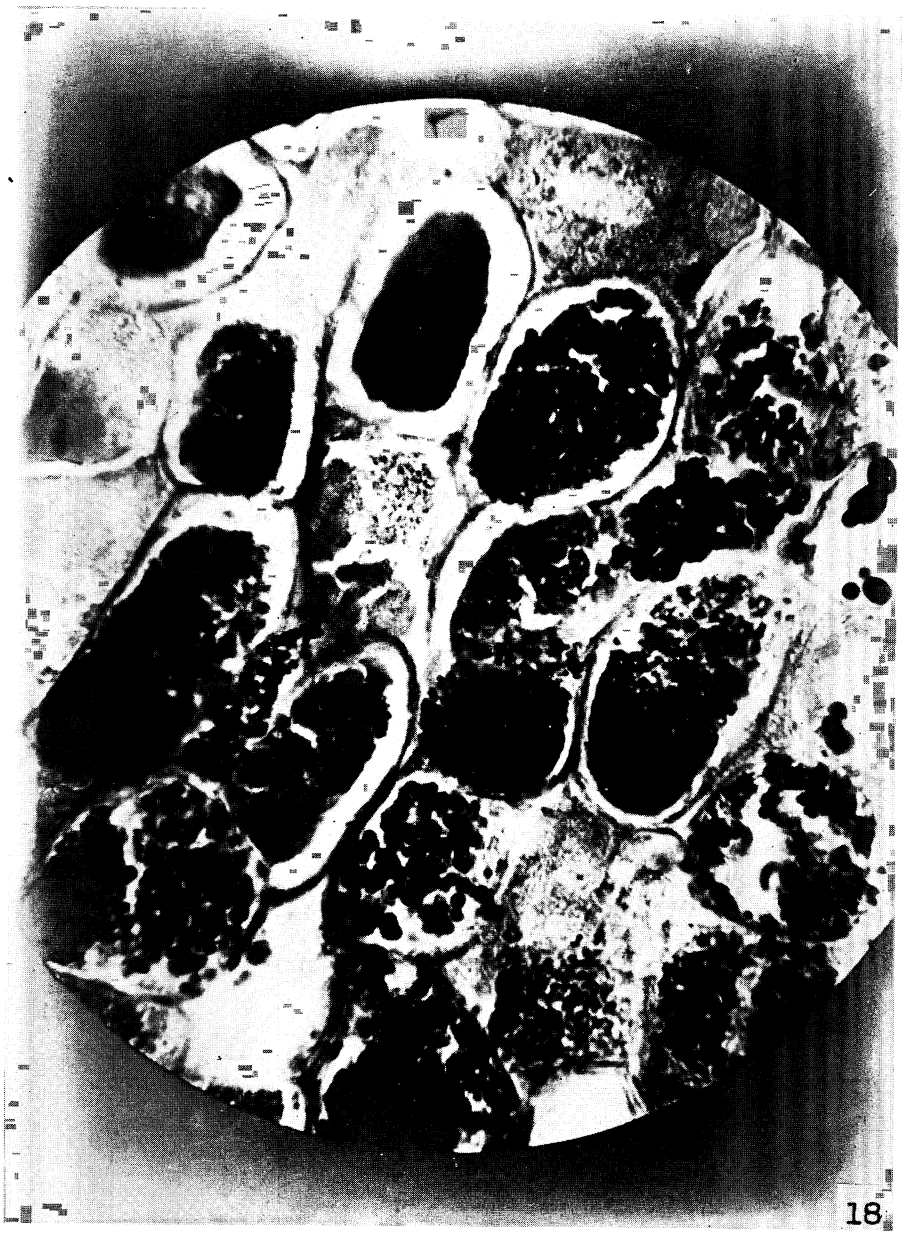


—Plate 2—



—Plate 3—

—Plate 4—



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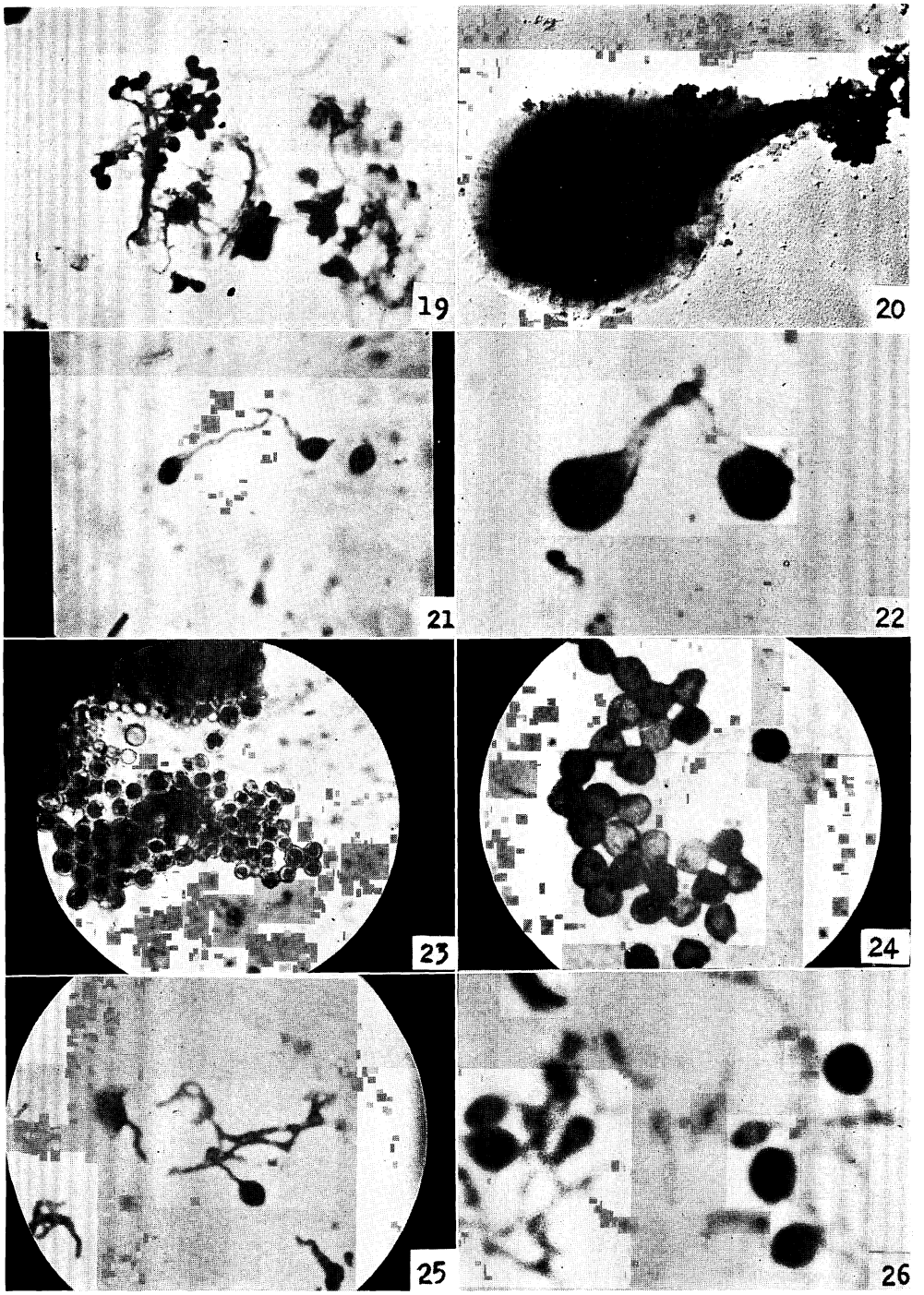
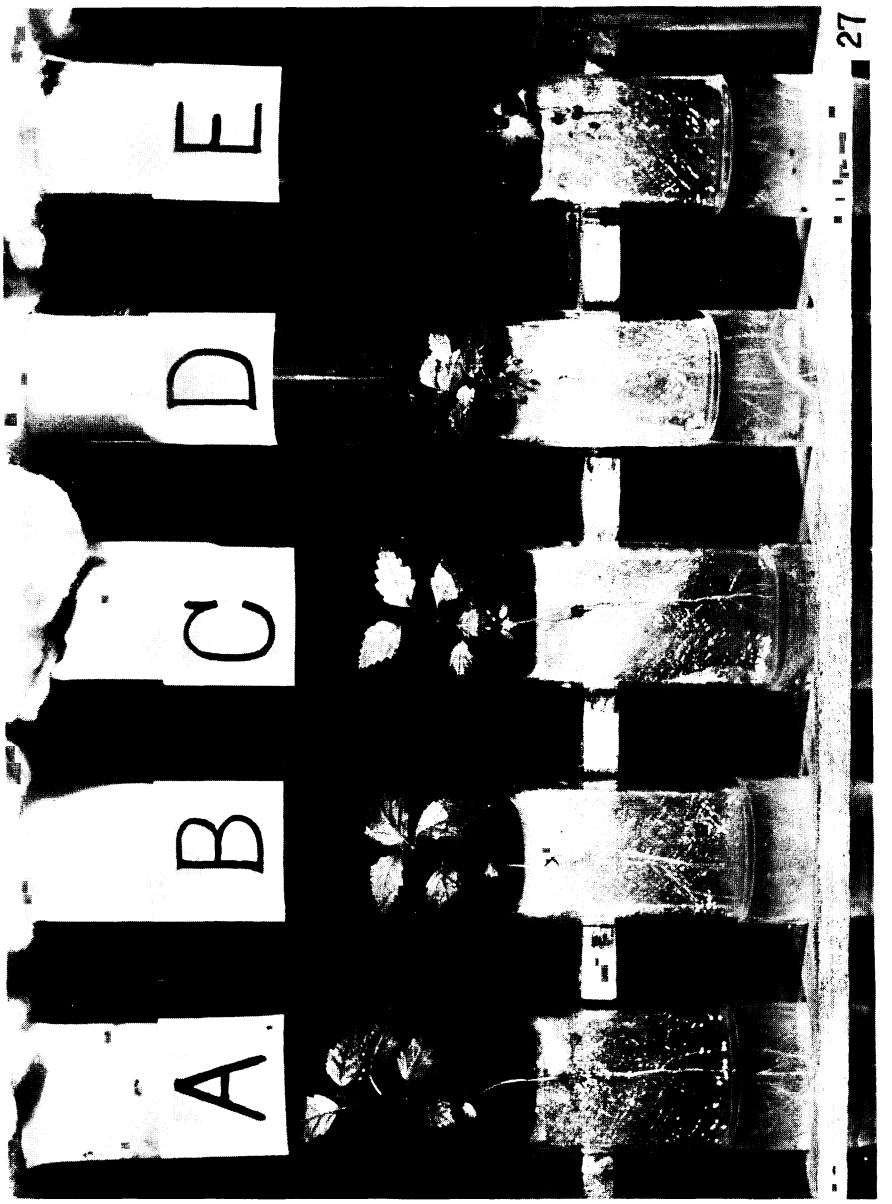


—Plate 6—



—Plate 7—