THE NATURE AND DISTRIBUTION WITHIN THE PLANT OF THE BACTERIA ASSOCIATED WITH CERTAIN LEAF-NODULATED SPECIES OF THE FAMILIES MYRSINACEAE AND RUBIACEAE.

A THESIS

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ABSTRACT

An investigation has been undertaken into the nature and distribution within the plant of the bacteria associated with one Myrsinaceae species (*Ardisia crispa*) and with three hitherto uninvestigated Rubiaceae species (*Psychotria emetica*; *Psychotria nairobiensis* and *Pavetta grandiflora*).

A cyclic symbiosis has been confirmed in *A. crispa*, demonstrated in *Psy. nairobiensis* and partially demonstrated in *Psy. emetica* and *P. grandiflora*. Evidence that infection of the plant occurs in the germinating seed stage has been presented in the case of *A. crispa* and *Psy. nairobiensis*. The method of transmission of the symbiont from one generation of plant to the next has been confirmed in the case of *A. crispa*, and the findings reported herein suggest that a similar method is involved in the three Rubiaceae species. Evidence has been presented that, in general, the bacteria present within the tissues are specifically identical from location to location and from plant species to plant species.

A number of different bacterial species, some of which are capable of nitrogen-fixation when grown in pure culture, have been isolated from these plants, and the cultural characteristics of these have been studied. Fluorescent antibody tracing studies, along with morphological and isolation studies, indicate that only one of these species is the symbiont of *A. crispa* and *Psy. nairobiensis*, and this has been tentatively identified as a *Rhizobium* species.

The possible relationship between the bacterial species isolated and the host plant species have been discussed, and an attempt has been made to assess the results in the light of previous knowledge.

CONTENTS

1. INTRODUCTION

(a) General remarks.

It is well known that certain plants (e.g. members of the Leguminosae) have irregular knob-like growths on their roots. Although these nodules (as they are called) were at one time thought to be pathological, they have been conclusively shown to be indispensable for the healthy growth of the particular plants with which they are associated. This was proved by Hellriegel and Wilfarth (1886-1888), who succeeded in demonstrating that root nodules were the means of providing the host plant with combined nitrogen, obtained from the atmosphere through the activity of bacteria inhabiting the nodules. Once symbiotic nitrogen fixation by Leguminosae was accepted as an established fact, work was undertaken to discover whether plants of other orders might, under certain conditions, "fix" atmospheric nitrogen.

A large number of plant orders were examined and investigations were also directed to parts of the plants other than roots, namely leaves, leaf-margins,

and seeds. The rapidity of vegetative growth in the tropics led some investigators to direct their attention to the flora of those regions for testing their theories. An even more intimate bacterial symbiosis has been claimed to exist in the leaves of certain tropical and semi-tropical Myrsinaceae and Rubiaceae, than exists in Leguminous nodules. Over the past half-century, upwards of 400 plant species of these two families have been reported in the literature as possessing leaf-nodules, in some cases with bacteria.

While this may suggest that a symbiotic mechanism is present in these species, only a few of the plants have received any serious attention from the microbiological standpoint; and indeed only three, namely, Ardisia crispa A.DC. in the Myrsinaceae, and Pavetta zimmermanniana and Psychotria bacteriophila in the Rubiaceae, have been the subjects of extensive research in respect of the bacteria contained within their nodules; a number of workers have considered the possession of nodules as being an important taxonomic feature, but have not concerned themselves with the bacterial content of the nodules.

In this field of research, the names of three workers stand out. F. C. von Faber (1912, 1914) demonstrated that bacteria are present in the leaf nodules, terminal buds, and seeds of Pavetta zimmermanniana and Psychotria bacteriophila Val. (since re-named Psychotria punctata Vatke.), and has further claimed that bacteria isolated from the leaf nodules are capable of nitrogen fixation. Hugo Miehe (1911, 1914) demonstrated the presence of bacteria in the leaf nodules, terminal buds, and seeds of Ardisia crispa A.DC. More recently Ph. de Jongh (1938) has shown that bacteria are also present in floral buds of the same plant. Both Miehe and de Jongh have rejected von Faber's suggestion that the nodular bacteria serve as nitrogen fixing symbionts, but suggest that a growth factor which serves the plant in some unknown way is possibly produced by the bacteria. The work of these three authors is described in some detail, and discussed in the light of subsequent investigations, in the present thesis.

(b) Literature survey

(i) Geographical distribution of species

Plant species exhibiting bacterial leaf nodules have a wide distribution in tropical and semi-tropical regions of the world, but so far as is known are confined to the Families Rubiaceae and Myrsinaceae.

1. The family Rubiaceae, in which approximately 400 species have been reported in the literature as being leaf-nodulated, contains, by far, the largest number of bacteriophilous species. The latter are members of 3 genera, namely Pavetta, Psychotria and Coprosma.

Pavetta species are distributed over Africa, Arabia, India, Indo-China, South China, Formosa, the Malayan Archipelago, tropical Australia, Melanesia.

Africa has the largest number of species of Pavetta and here tropical West Africa, Tangyanika, S. Rhodesia, Portuguese E. Africa, Kenya and South Africa are particularly well provided. Nodulated species also occur in Ceylon and the Philippines in large numbers.

Psychotria species are tropical and semi-tropical in habit.

Bremekamp (1933) described 42 leaf-nodulated species of this genus which are found in tropical and semi-tropical regions of Africa, e.g. West Africa, East Africa, the Congo, Angola, Rhodesia and South Africa. More recently (1960) he has described a further 7, hitherto undiscovered, leaf-nodulated Psychotria species which are native to Madagascar.

A number of taxonomists have described further Psychotria species but have made no reference to a possible presence of nodules. Thus Standley and Steyermark (1943) noted and named Psychotria species that formed part of the natural flora of Guatemala, British Honduras, Costa Rica and Mexico. Merrill (1950) drew attention to 5 Psychotria species which he found to occur in Borneo. Bacigalupo (1952) described Psychotria species as native to the Argentine. Cuban species of the genus have recently been described by Acuna and Roig (1962). These workers list some 67 species of Psychotria as naturally occurring in Cuba, 9 of which were considered to be new and hitherto unnamed species.

At least one leaf-nodulated Psychotria species (Psychotria emetica) is known by the author to be native to the Costa del Caribe region of Columbia, South America.

Coprosma is a genus with about 60 species, approximately half of which have been reported by Stevenson (1953) as possessing leaf nodules. The genus has a fairly wide distribution, but is chiefly found in New Zealand. Species of the genus also occur in the South Temperate and Antarctic areas.

2. Myrsinaceae. The largest genus, Ardisia, has some 240 species in the warmer parts of both hemispheres, and of these Mez (1902) has listed 30 species as bacteriophilous.

They are mainly tropical in habitat, and Mez reported them as being distributed in Indo-China, Malacca, the Malayan Archipelago, and the Philippines.

Mez also reported 5 species of the genera Amblyanthopsis and Amblyanthus as possessing leaf nodules; these genera are also tropical in habitat.

In the last twenty years or so several new species of Ardisia, many of which are nodulated, have been described.

Smith (1940) recorded that he had found Ardisia species in Peru, Venezuela and Columbia, while Lundell (1941) reported Ardisia species to be present in Mexico, British Honduras and Costa Rica. Neither Smith nor Lundell made any reference to the possible presence of bacterial nodules on the leaves of those newly discovered Ardisia species; but both workers, in their taxonomic descriptions of these plants, noted that the leaves of some species had crenated margins, indicating that nodules were possibly present.

New species of Ardisia, which exhibit conspicuous orange "glands" on their sepals and petals, were reported to occur in Panama by Woodson and Schery (1941). Examples given of Panamanian Ardisia species were A. rigidifolia and A. Woodsoni. Lundell sp., both of which exhibit these "glands". Walker (1942) described four new species of Ardisia from Indo-China, one of which

Ardisia prionata, Walker sp. nov., had nodules arranged around the margins of the leaves.

Guatemalan species of Ardisia have been described by Standley and Steyermark (1947) and, more recently, Steyermark (1955) has noted the presence of Ardisia species in El Slavador and Mexico.

Leaf-nodulated species of Ardisia indigeneous to Japan have been described by Hanada (1954)

(ii) Survey of earlier investigations of bacteriophilous species.

1. The bacterial leaf nodules of the RUBIACEAE.

In 1894, Trimen, in his "Handbook to the Flora of Ceylon", drew attention to the small "warts" on the leaves of certain Rubiaceae; the plants he mentioned as possessing these "warts" were Pavetta indica L., Pavetta augustifolia Th.w., Pavetta involucrata Th.w. and Pavetta Gleniei Th.w..

Other than noting them as taxonomic features, Trimen did not attempt to define any possible function of the "warts".

The first reference to a bacterial symbiosis in the Rubiaceae is found in a paper by A. Zimmermann (1902), in which he recorded the presence of "bacterial nodules" on the leaves of three Pavetta species housed in the Botanic Gardens at Buitenzorg, Java. Another plant labelled Grumilia micrantha Hiern. was also noted by this worker to be nodulated; but as this plant had neither flowers nor fruits, it was impossible at the time to identify it. However, shortly after Zimmermann's departure from Java, it flowered and proved to be a new species of Pavetta, which Valeton (1908) subsequently named Pavetta zimmermanniana. An important feature of these four Pavetta species, observed by Zimmermann, was the constant presence of bacteria in their leaf nodules. Stomatal penetration of the bacteria into the leaves in the terminal bud stage, resulting in local swellings, was suggested by Zimmermann as the means of infection. In addition to drawing attention to these four Pavetta species, Zimmermann also pointed out that pustules on

the shoots and leaves of Heterophila pustulata Hook. and Lecanosperma lycoides Rusby., had a structure similar to the bacterial leaf nodules of the four Pavetta species.

That bacterial nodules were present in a genus very closely related to Pavetta was shown a few years later, when Valeton (1908) described a species of Psychotria, also cultivated in the Buitenzorg Gardens, as leaf nodulated. This plant was thought to have come from the Comores, and as it appeared to be distinct from all other Mascarene species, Valeton regarded it as new and named it Psychotria bacteriophila. Comparison of this plant with the bacteriophilous species of Psychotria in the Kew Herbarium has shown, however, that it is identical with Psychotria punctata Vatke., from the coast of tropical East Africa, where it is apparently quite common. Bremekamp (1933), who did this work, considered its occurrence in the Comores doubtful, though not quite impossible, as no other bacteriophilons species of Psychotria was known to occur in those islands.

Boas (1911) showed that bacterial nodules were not confined solely to this one species of Psychotria.

He gave an anatomical description of bacterial nodules in the leaves of two Psychotria species collected from the Cameroons, namely Psychotria alsophila K.Sch. and Psychotria umbellata Thon.. In the former species the nodules resembled those present on the leaves of Psychotria punctata, being more or less circular in outline and irregularly scattered over the surface of the whole leaf; whereas in Psychotria umbellata. Thon, they were elongated and arranged in a double file along the midrib of the leaf.

A major contribution in the study of the Rubiaceous leaf nodules was made by von Faber, who published an account of his researches in two papers (1912, 1914). In a field of research that has been virtually untouched in the past fifty years, and indeed was only briefly mentioned before the turn of the century, von Faber's contribution is outstanding. He claimed to have separated the causative bacterium from the plant, to have demonstrated its capacity for nitrogen fixation, and to have successfully re-infected bacteria-free plants with his isolates.

Von Faber noted the following species as showing leaf nodulation:-

Pavetta indica. L.

Pavetta indica. fol. var.

Pavetta indica. var polycantha

Pavetta indica. var. tomentosa.

all species being found in the Indian monsoon region.

Pavetta lanceolata. Ek. L. From South Africa

Pavetta augustifolia.Th.w. From Ceylon. (This plant has subsequently been renamed Pavetta agrostiphylla Brem. nom. nov. by Bremekamp in 1934.).

Pavetta zimmermanniana Val. From West Africa

Pavetta gardaeniaefolia. Hochst.

Pavetta oligantha From Borneo

Psychotria bacteriophila Val. From East Africa and the Comore Islands. (This plant was later found by Bremekamp in 1933 to be identical with Psychotria punctata Vatke.).

Psychotria alsophila K.Sch.

Psychotria umbellata. Thon.

both from the Cameroons

A microscopic examination of leaf nodules of these plants showed the presence of bacteria in all.

The bacterial nodules were present in the "chlorophyll tissue", where assimilation takes place, and the starch granules present in these regions disappeared upon complete formation of the nodules; it was suggested that a part of the starch, in the form of reduced sugar, was utilised by the bacteria. As well as the bacteria which inhabited the nodules, von Faber noted the presence of "well-formed dark green chlorophyll granules". From a study of the different stages of leaf development, it appeared that the nodular bacteria were digested by the plant, the bacterial membranes being dissolved away, until a complete lysis of the bacteria was noted; and, in very old leaves just prior to leaf-fall, only remnants of the bacteria remained.

Having demonstrated the presence of bacteria in foliar nodules, he then turned his attention to ascertaining how the nodules originated. On examination of the youngest leaves of Pavetta zimmermanniana. Val., namely those in the terminal bud, he noticed that bacteria were present in the cavities formed between the stipules and the young leaves. These bacteria penetrated through "stipular holes" into the leaves, claimed von Faber;

these "holes" appeared on both surfaces of the leaves, and penetration took place before stomata were open. Thus, von Faber was opposed to Zimmermann's theory (page 9) of stomatal penetration as the means of bacterial infection. Having penetrated the leaf surface, the bacteria collected in the intercellular spaces of the mesophyll, where they disturbed the surrounding cells, resulting in the formation of a cavity. A self-defence mechanism caused the cells immediately surrounding the cavity to modify cytologically. Von Faber envisaged an equilibrium being set up between those cells and the invading bacteria, so that eventually the mesophyll cells became circumscribed and enlarged around the bacteria, and hence nodules were formed. The nodule consisted of very small cells, between which lived the bacteria. Meanwhile, the pore through which the bacteria had originally penetrated, had closed, thus imprisoning the bacteria, and a membrane separated the nodular cells from the bacteria. With age, the nodules turned into "secretion containers", in which the secretion was identical with that sealing off the bacteria in young nodules.

Having shown the presence of bacteria in the foliar buds, von Faber attempted to discover how they

arrived at this stage. Extraneous infection - as occurs in the root nodules of the Leguminosae - he ruled out; for, when he grew externally sterilised seeds of Pavetta zimmermanniana under aseptic conditions (sterile soil, etc.), they germinated to produce nodulated plants. Microscopic examination showed bacteria to be present inside the seeds, where they were situated in a layer between embryo and endosperm. In order to discover how bacteria arrived in the seed, blossoms of various ages were studied. The results of this study were not published by von Faber. He considered that the bacteria present between the young leaves of a terminal bud had initially been present in the seed and that on germination of the latter, they were mechanically carried with the growing tip of the stem. On the basis of these results, he has postulated that a "hereditary symbiosis" existed between the bacterium and the nodulated members of the Rubiaceae, the phenomenon apparently being widespread in the family.

With two plant species, namely Psychotria bacteriophila. Val. and Pavetta zimmermanniana. Val., von Faber carried out a survey on bacterial distribution within the plant body. Bacteria were found to be present

in the seeds, terminal buds, and nodules of both plants.

In Psychotria bacteriophila. Val., the bacteria were observed in the seeds by skimming off the embryos in a drop of water; they proved to be non-motile, slightly curved, single rods, one end of which was slightly swollen; their dimensions he recorded as 1μ - 3μ long x 0.5μ wide. They were Gram-negative, and stained evenly with carbol fuchsin, but were embedded in a slimy mass which made staining difficult.

As the bacteria were present in the terminal bud in much larger amounts, he found it was much easier to work with them. He noted that the bacteria here were similar to those present in the seed, but a few longer forms were also present. In addition to the latter type of bacterium, which was common to both seed and terminal but, he noted that a low percentage of "branched" forms were also present (about 1%). These bacteria were slightly longer and thinner than the other bud bacteria, having dimensions of 1μ - 4μ x 0.5 - 0.7μ. Unlike the former type of bacterium present in the bud, the "branched" rods stained unevenly with carbol fuchsin.

Quite characteristic, however, was the appearance of the bacteria present in the nodules. By microscopic examination of squashed nodule tissue, two types of bacterial rod were observed. The first type was similar to seed and terminal bud bacteria, but possibly were slightly shorter and thicker; they were non-motile rods, which were slightly curved, and which stained evenly with carbol fuchsin. They were Gram-negative, and although no chains were observed (i.e. they were single cells), they tended to bunch together in a sticky material within the nodule. The second type (c.f. terminal bud bacteria) was present in large amounts, and consisted of long, thin rod forms, slightly swollen at one end. These rods were "branched", and von Faber reported that "X", "Y", and "T" shapes were present in large proportions in this type; von Faber compared these irregularly shaped cells to the "bacteroids" of Leguminous nodules. This type of bacterial rod, which accounted for the majority of organisms in the nodules, stained unevenly with carbol fuchsin, only small round grains in the cells staining intensively, but they were always stained at the poles. The "branched" forms, he noted, still persisted in old nodules.

For Pavetta zimmermanniana. Val., von Faber claimed that only minor differences existed between the microorganisms as found in this plant compared with those in Psychotria bacteriophila. Val.. Their distribution in the plant was similar, the bacteria being found in seeds, buds, and nodules; as with Psychotria bacteriophila. Val., bacteria were found in the seed between embryo and endosperm, and had similar staining and morphological properties to bacteria found in that plant. The bacteria found at the terminal bud were similar to those in the seed (c.f. Psychotria bacteriophila), but now only one type of bacterium was found, and unlike Psychotria bacteriophila, no "branched" forms were present. In nodules, he once again found two types of bacterial rod, in similar proportion to those found in Psychotria bacteriophila; the majority of rods were long, thin forms, slightly swollen at one end, and having a high proportion of branched "bacteroid" types which stained unevenly with carbol fuchsin; the second type, which closely resembled the seed and terminal bud bacteria, consisted of short, single rods, the protoplasm of which stained evenly with carbol fuchsin.

A further section of von Faber's work dealt with the isolation of the nodular bacterium from the plant. In his preliminary experiments, he encountered numerous difficulties; all attempts at isolation of the bacterium from mature nodules were without success, and this, he considered, was due to the nodular bacteria "losing their capacity for adaptation with age".

However, he claimed successful isolation of the nodular bacterium from the very young nodules of leaf buds. Leaf buds, which had been surface-sterilised by washing in sterile water and absolute alcohol, were sliced and planted out onto a leaf-extract medium, to which gum arabic and a small amount of asparagine had been added as carbon and nitrogen sources respectively. Using this method, he obtained isolates from Psychotria bacteriophila, Pavetta indica and Pavetta zimmermanniana, but he was unable to obtain isolates from any other Pavetta species; he could not explain the reason for this failure.

From under nodule sections lying on the surface of plant extract medium, grey-white colonies emerged in 4 days; colony growth, slow at first, was quite good

after 10 days, the improvement in growth being due, according to von Faber, to an "adaption to environment" on the part of the bacterium. He claimed to have isolated the same microorganism from the aforementioned 3 plants, because to the naked eye the colonies on leaf-extract medium appeared identical.

Von Faber made but brief mention of "occasional infections" which grew up on his isolation plates; for instance, yellow colonies which appeared to be a type of Sarcina, the possible origin of which von Faber considered might be due to a colonisation of the young nodules by foreign microorganisms before the pore was completely closed.

Taking the microorganism isolated from Pavetta zimmermanniana as his example (all others being similar), von Faber described its properties on artificial media; 6- to 7-day old cultures, on microscopic examination, showed non-motile rods, of dimensions 1μ - 4μ x 0.5μ, and which sometimes occurred in short chains. The individual cells were slightly curved and showed irregular edges. Coccal and weakly branched forms also appeared (c.f. nodular bacteria), all shapes stained

evenly with carbol fuchsin. With age the differences in length of rods increased (1μ - 8μ), the longer rods staining unevenly but their swollen ends always staining: these rods had a "branched" appearance and, for this reason, von Faber considered he was dealing with the same microorganism as was present in the plant. Although in artificial culture, these rods were far slimmer than their counterparts in the nodules, he considered this to be due to differences in environmental conditions. The short rods still stained evenly.

In older cultures he found mainly "branched" forms, while the small regular rods hardly ever appeared. Eventually, it appeared that the culture consisted only of "cocci", the strongly staining grains in the old rods giving this effect.

The cultural characteristics of the isolates were as follows: starch was hydrolysed, and strong acid-formation was noted in sugar media; arabinose and starch were seemingly the best carbon sources, sucrose, glucose and mannose being not very good in this respect; neither lactose nor cellulose could be used as carbon sources.

Von Faber named his isolate Mycobacterium rubiacearum, placing it in the Mycobacteriaceae because it was acid-fast and bore a strong morphological resemblance to tubercule bacilli.

Re-infection of healthy plants with his isolate was von Faber's next aim, and for one plant, Pavetta zimmermanniana, he claimed success in this respect. On examination of a large number of seeds of this plant, von Faber found bacteria to be present in all. This he confirmed by growing a number of seeds of this plant, all of which germinated to produce nodulated plants; so, before he could perform re-infection experiments, von Faber had to produce healthy, bacteria-free (i.e. non-nodulated), plants. After many failures, using such techniques as tissue culture of the extracted seed embryos, and attempts at producing bacteria-free plants from cuttings, he finally reported successful results. (1914).

Bacteria-free plants could be obtained by suitable heat-treatment of the seeds of Pavetta zimmermanniana. Internal sterilisation of the seed was effected by hot-water treatment (50°C x 25 minutes), 65% of the seeds

germinating to produce non-nodulated (i.e. bacteria-free) plants. The same temperature for a longer time killed the seeds, but when applied for shorter periods, the resultant plants were nodulated to the usual degree.

Seeds in which the bacteria had been killed germinated much more slowly than those in which the bacteria were still alive, and von Faber was of the opinion that the symbiotic microorganism might play a role in germination, though the nature of this role, if any, was unknown to him. Bacteria-free plants also grew more slowly and had smaller leaves. Sand culture experiments (using acid-washed, nitrogen free sand), showed that bacteria-free plants when deprived of nitrogen developed very poorly and showed all the symptoms of nitrogen deficiency, whereas bacteria-containing plants grew well under similar conditions.

At first, all attempts at re-infection of bacteria-free plants failed, and this, he considered, might be due to the condition of the inoculum; maintaining cultures on an artificial medium might have caused them to lose their capacity for infection, and thus to become unable to function as symbionts. But, he argued,

successful re-inoculation experiments had been performed with root-nodule isolates on Leguminous plants, and these isolates had been maintained on artificial culture media; thus, Rhizobium had not lost its ability to re-infect the host plant. Why then, should the Pavetta organism behave in this way? Was it that the stage of the plant development at which the inoculations were made was the governing factor as to whether a successful re-infection could be attained? Working along these lines, von Faber carried out a thorough anatomical study of the embryo development in order to discover which stage of germination was suitable for infection experiments. Using thoroughly surface-sterilised, and heat-treated (i.e. theoretically bacteria-free) seeds of Pavetta zimmermanniana, sowed individually in pots and maintained under aseptic conditions, he followed their germination. Control pots were also set up under the same conditions; in this case, the heat-treatment for the seeds was omitted. Again the heat-treated seeds germinated much more slowly than untreated ones, and a certain number of heat-treated seeds still contained live bacteria. Several days after the emergence of the plumule from the seeds, and observing aseptic precautions, he introduced a small amount of a pure culture of his isolate onto the terminal bud of both

heat-treated and control plants. From the original batch of heat-treated seeds, 65% were bacteria-free, and, of these, approximately 50% were successfully re-infected, as shown by the presence of nodules. On this evidence, von Faber concluded that his isolate was the true hereditary companion of the Pavetta plant in nature.

Although heat-treated seeds gave rise to slower-growing plants than untreated ones, and the plants had a different habit, treated seeds which were not bacteria-free showed the same phenomena, and therefore he assumed that heating had a harmful effect on the plasma. They recovered, however, after two months, after which time they were indistinguishable from the controls. Bacteria-free plants which had not been reinfected never recovered and remained always slow growing. Their appearance was characteristic: short, thin, yellow leaves, and the decussate arrangement, typical of normal plants, had become irregular, the small leaves being twisted to one side, as if they had been exposed to uneven illumination.

Bacteria-free seedlings successfully re-infected

showed, at first, the same features as heat-treated plants, i.e. slower growth and irregular leaf formation; however, with time, this difference disappeared and the re-infected plants appeared identical with the controls.

A major section of von Faber's work concerned the possible function served by the nodular bacterium. He envisaged that a kind of "hereditary symbiosis" was involved, and argued that the close association between the nodule microorganism and the plant must make large amounts of carbohydrate available to the bacteria, and that bacteria and plant must benefit. He compared the association with that existing in Leguminosae root nodules, and postulated that nitrogen-fixation was occurring. This suggestion he considered strengthened when he found that the natives of India used leaves of Pavetta indica as a green manure. The benefit of these, reasoned von Faber, lay in their high nutrient value, due to the "fixed" nitrogen they contained.

In attempts to confirm the nitrogen-fixation theory, von Faber grew the organism isolated from Pavetta zimmermanniana buds in aerated, nitrogen-free, liquid media. He claimed values for nitrogen fixation as high

as 20.4 mg. nitrogen fixed per 100 ml. solution over a period of twenty days. Further experiments led him to conclude that there was a gain in the amount of nitrogen fixed if a small amount of bound nitrogen (asparagine 0.1 mg/ml) was included in the synthetic medium. Growing the organism isolated from Psy. bacteriophila (which organism von Faber considered to be identical with that isolated from Pavetta zimmermanniana), under similar conditions he obtained much lower values - 8.5 mg/100 ml being the highest value. He envisaged that nitrogen-fixation in the intact plant was probably far more efficient, as the plant ensured that the assimilation products were led away.

From the results of his experiments, von Faber concluded that Mycobacterium rubiacearum could fix atmospheric nitrogen when grown in pure culture; and as his other Rubiaceae isolates appeared to be identical, he assumed that they also would fix nitrogen under similar conditions. The "granules" present in cells (which von Faber concluded to be of a nucleoprotein nature), he considered to be part of the products of nitrogen fixation, as other known nitrogen-fixing organisms had similar protoplasmic inclusions.

Working at the Royal Botanic Gardens, Kew, Georgevitch (1916) described bacterial nodules on the leaves of a plant named Kraussia floribunda. Harv.. This plant possessed leaf nodules of similar anatomical structure to those on Pavetta species; the nodules were elliptical and were separated from the leaf tissue by two or three layers of modified cells. From the nodules he claimed to have isolated a spore-forming Bacillus species on potato agar. No further results were reported by him, and he made no mention of any possible function of the microorganism in the plant, although he considered the association to be symbiotic in nature.

In 1923, Adinarayan Rao, working in Ceylon, took up von Faber's work on the symbiosis of Pavetta, particularly in relation to the common usage of Pavetta leaves in that country as a green manure. Rao described two leaf-nodulated species which he had investigated, namely, Chomelia asiatica and Pavetta indica. He obtained bacterial isolates from young leaf nodules and leaf buds, thus confirming von Faber's findings that isolations could be obtained from these sources. The isolates on solid media were very slow growing, and the irregular shapes, first noticed by von Faber, were noticed

also by Rao, when he examined 6-7 days old cultures microscopically. Young colonies consisted of typical rods which stained Gram-positively.

Rao found bacteria to be present at all the locations in the plant noted by von Faber (i.e. terminal bud, seeds, nodules) in both Pavetta and Chomelia; and he also claimed high values for nitrogen fixation by the Pavetta and Chomelia isolates, when these were grown in nitrogen-free liquid media. Rao's conclusions were similar to von Faber's, namely, that the symbiosis was of a hereditary character, and that the symbiotic microorganism functioned as a nitrogen fixer.

Korinek (1928) studied the bacterial population of isolated Psychotria leaves and concluded that the bacteria brought about neither physiological nor morphological changes. According to him, it would seem hardly possible to consider this symbiosis as a form of "balanced parasitism" (an idea first propounded by von Faber in 1912), as in this case the bacteria should attack the tissue of the isolated leaves.

Boodle (1923) has written a short review on the bacterial leaf nodules of Psychotria and Pavetta; he quotes von Faber's work freely and, although he did not perform any further experimental work, accepted von Faber's nitrogen fixation theory as being the correct interpretation as to the basis of the symbiosis.

Over the last 30 years Bremekamp has published a series of monographs dealing with the Psychotria and Pavetta genera. He considered the possession of leaf nodules to be an important taxonomic feature - when taken alongside other characters - for the differentiation of species within these two genera. His work has been taxonomic in character and not primarily concerned with the role of the bacteria within the nodules. Nevertheless, his contribution is of great signficance for he has discovered and classified a great number of hitherto unknown leaf nodulated species of those two genera.

When Bremekamp first approached the problem of bacterial symbiosis in Psychotria, the bacteriophilous condition had only been described in 3 species of the genus, and was thought to be a comparatively rare

phenomenon. However, when he published his first monograph on the subject (1933), he described some 42 species of this genus as possessing leaf nodules.

According to Bremekamp, the bacteriophilous species of Psychotria fall naturally into 3 groups; in the first, the bacterial nodules follow the midrib, whereas in the second and third groups the nodules are irregularly scattered over the leaf surface; the species of the second group have a ring of hairs at the insertion of the stamens, whereas in those of the third group the inside of the corolla tube is glabrous.

Psychotria species which never bear bacterial nodules were also listed by Bremekamp; examples were Psychotria abrupta. Hiern. and Psychotria amboniana.K.Sch., both members of the section Inundatae (sub-genus Eupsychotria).

Originally, Bremekamp had considered that Psychotria species were restricted to the African Continent. However, in a second paper (1960) he has recorded that this view was incorrect. In this paper he

described and classified a further 7 leaf nodulated species, all of which were indigenous to Madagascar.

Bremekamp has also produced monographs dealing with the Pavetta genus (1934, 1939a, 1939b, 1948, 1953, 1956). In those papers he has described and named many new species; in all he has listed, up to date, 427 species of Pavetta, of which 339 are nodulated.

As with Psychotria, the presence of leaf nodules, and their form and distribution of nodules over the leaf surface, he considered to be of taxonomic importance for the differentiation of species within the genus.

H. J. Humm (1944) carried out a taxonomic survey of the plant species exhibiting leaf nodules, and reported that in the genus Pavetta 294 species, and in the genus Psychotria 42 species, were bacteriophilous. For these results he quotes Bremekamp as the authority. Similarly for the Myrsinaceae, he reported 30 species of the genus Ardisia, 5 species of the genus Amblyanthus, and 5 species of the genus Amblyanthopsis as being

nodulated. In the family Dioscoreaceae, a single species of the genus, namely Dioscorea macroura. Harms., was said to have bacteria in glands located in the attenuated leaf-tips. Orr had discovered this in 1923.

More recently, Stevenson (1953), working in New Zealand, has drawn attention to another Rubiaceae genus in which bacterial leaf nodules occur. In the genus, Coprosma, she found some 28 species exhibited stipular nodules. A microscopic examination of cross-sections of stipular nodules showed that they consisted of a palisade-like layer of cells, which contained masses of bacteria. One point, immediately noticeable in her descriptions, and which differed from previous workers' findings, was that she considered the bacterial infection to be intracellular in nature (as opposed to intercellular). Throughout the whole genus, domatia were found on the under-surface of the leaves and these were surrounded by bacteria-containing hairs, a similar condition being noted on the stipules. Young buds were also covered with a gum which contained bacteria.

Bacterial isolates were obtained by Stevenson from the stipular glands of Coprosma australis, Coprosma foetidissima, Coprosma lucida, Coprosma repens, and Coprosma robusta; and, in each case, a bacterium which appeared to be the infective organism was isolated on a leaf-extract-glucose medium.

The properties of the isolates in cultivation were not examined, and no experiments on the production of bacteria-free plants and subsequent re-inoculations were performed.

Stevenson did not investigate the relationship between these plants and their stipular nodular bacteria, but several facts led her to conclude that the association was symbiotic in nature, and that the bacterial symbionts might possibly function in the fixation of atmospheric nitrogen. Three observations led her to this conclusion:-

(a). In all plants examined, a red pigment was seen to be present at some stage either in the cells which contained bacteria or in neighbouring ones. This pigment, she compared to the red pigment found in Leguminous root nodules.

(b). Many Coprosma species (e.g. C. repens, C. robusta) grow well on gravel or stony ground, both of which are highly deficient in nitrogeneous matter.

(c). Calcium oxalate crystals were present in cells immediately beneath the stipular tissue containing the bacteria. As the bacterial tissue aged these crystals largely disappeared, and Stevenson suggested that this was due to the oxalic acid being used up in bacterial activity; possibly as a starting point for amino acid synthesis in nitrogen-fixation.

2. The bacterial leaf nodules of the MYRSINACEAE.

The leaf nodules on the crenated leaf margins of Ardisia crispa A.DC. were studied by von Höhnel as early as 1882. He referred to them as "proteinaceous glands" and claimed to have observed "proteinaceous bodies" in the intercellular spaces of such structures.

In later years, Solereder (1899), and Grosse (1908), followed up von Höhnel's work on "proteinaceous glands", but neither was able to add anything further to the latter worker's findings.

In a series of four papers (1911 (a), 1911 (b), 1914, 1919) published under the general heading "Javanische Studien", Miehe, working at the Botanic Gardens in Buitenzorg, Java, gave a detailed account of this curious association of bacteria-with-plant. Miehe's contribution to our knowledge concerning the leaf nodules of the Myrsinaceae is analogous to von Faber's work with Rubiaceae; he was the pioneer in the study of Myrsinaceae nodules, and for this reason his work has been considered in some detail.

He was chiefly interested in Ardisia crispa A.DC., and all his results quoted in this survey apply to this species; but he also observed nodules in other species of the genus, e.g. Ardisia Cumingiana. A.DC..

In his first paper (1911(a)), Miehe reported that he first looked at the nodules of fully-developed

leaves of Ardisia crispa A.DC., i.e. those leaves well down the stem away from the terminal bud. In these older stages, a cross-section of the nodule showed a cavity filled with a foamy mucus, which contained rod-shaped bacteria; a high proportion of these bacteria were of "bacteroid" appearance.

Having shown bacteria to be present in the leaf nodules (which correspond respectively to von Höhnel's "proteinaceous bodies" and "glands"), Miehe then attacked the problem of how bacterial infection of the leaves arose. Starting with the very youngest leaves of the plant, namely those of the terminal bud, he found a great many bacteria were present between the very young leaves of the bud. These bacteria were embedded in a "mucilaginous membrane" which lay between the leaf primordmordia and leaves in all stages of development in the bud. Between the oldest leaves in the bud, the membrane seemed stretched and broken by the growth of the leaves.

In the axils of every leaf, dormant buds were present, and Miehe found that if these buds were stimulated (by removal of the terminal bud) then bacteria

were found to be present at the same places as in the terminal bud. In dormant buds which had not been stimulated in this way, he never found bacteria; he concluded that bacteria were probably present in the dormant axillary buds all the time, but that their small numbers made detection impossible. Removal of the terminal bud had, in some way, stimulated the bacteria to increase in number, thereby making it possible to detect them.

The leaf bases in buds (terminal and dormant) were found to cover each other alternately with the oldest bud-leaves rolled in to such an extent that the margins nearly touched the morphological upper surface of the leaf, and, according to Miehe, the nodules first appeared at this stage. Hydathodes had been found by von Höhnel (1882) at the margin on the dorsal surface of the leaf, and Miehe found that the entrace pore of the hydathodes contained bacteria, while the deeper intercellular cavities were still free from bacteria. Miehe considered that the hydathodes were the point of infection of the leaves.

He continued to trace the development of

nodules, noting that, even whilst the bud leaves were little more than primordial tissue, the intercellular cavity was closed by growth of the cells near the pore of the hydathode, thus isolating the bacteria from the outside world. Looking at older leaves in the bud, he noted that further development of the outer cells of the hydathode pushed the bacterial mass inwards, the mass being extended in such a way as to cause the cells lining the cavity to be pushed in, so that they assumed a concave outline. At a still later stage, the bacteria occupied a position exclusively in the intercellular spaces of the tubular cells in the nodule; these tubular cells were elongated, and were arranged radially into the nodular cavity; at a slightly later stage, an extension of the bacterial mass took place into them.

Miehe noted that in young nodules rod-shaped bacteria were present, embedded in a foamy mucus, whilst in older leaves the nodules carried only "bacteroid" types; in very old leaves the number of bacteria was reduced considerably, often almost to none.

The origin of the bacteria in the terminal bud was Miehe's next problem. He was convinced that extraneous bacterial infection of the plant from the soil, as for instance occurred in Leguminous root nodules, was not involved; for, when he grew seeds under aseptic conditions, nodulated plants invariably resulted. On microscopic examination of mature seeds, he discovered that bacteria were present in these seeds, where they were situated between the embryo and the endosperm, at the radical pole. Although the bacteria in the nodules and seeds were morphologically different, Miehe considered that they were one and the same. How the seed became infected in the first place, and how the bacteria arrived next to the radical pole when once inside the seed, puzzled Miehe. On one occasion he observed bacteria to be present between the carpels of a very young flower; but he did not follow up this observation. He assumed, in fact, that the bacteria could be brought inside the embryo-sac by means of the pollen tube. The fate of the bacterium in the intermediate stages (flower and fruit formation) was not investigated by Miehe, and he was not able to account for their presence in the seed on the basis of ontogeny. He concluded that

the association between bacterium and plant represented a type of "cyclic symbioses" (c.f. von Faber's "hereditary symbiosis" in Rubiaceae); but the benefits derived by the respective partners in this association were unknown to Miehe.

In a later paper (1914), Miehe dealt with the isolation of the nodular bacteria from the Myrsinaceae. He described numerous unsuccessful attempts to isolate the symbiont from nodules. As the bacteria were present in large quantities in mature nodules, he concluded that failure to isolate the organism was due to the fact that a symbiotic bacterium would be very selective in its nutrient requirements, and hence might require a special type of substrate for its isolation. He therefore tried isolations on a plant extract media, namely pea agar, but this also failed to produce isolates from mature nodules. Similarly, he was unable to isolate the bacterium from ungerminated seeds. Because sterilisation of terminal buds proved difficult and infections always occurred on his plates, he abandoned attempts at isolation of the nodular bacterium from this source.

Eventually he obtained definite bacterial isolates from germinating seeds of Ardisia crispa A.DC., the isolations being carried out as follows:- germinating seeds were externally sterilised and the young developing plants kept under aseptic conditions; they were then plated out onto pea agar and incubated at 25°C for a few days, after which time slimy white bacterial colonies appeared around the majority of plant embryos.

At first, Miehe was not certain whether this bacterial isolate was the same organism as that present in the plant; for his isolate was a motile rod, whereas the symbiont had never been observed to show motile stages in the plant. Nevertheless, when examined in old culture, his isolate showed non-motile swollen types of rods, which he likened to the "bacteroids" of Leguminous root nodules. The irregular shaped rods were also similar in morphological and staining properties to the bacteria which appeared in old leaf nodules of Ardisia; and for this reason Miehe claimed to have isolated the true bacterial symbiont of Ardisia crispa A.DC.. He studied the morphological and physiological

properties of the isolate and named it Bacillus foliicola n.sp.. He also found an incidental "fellow-traveller", a long rod, forming yellow colonies on pea agar, which he named Bacterium repens. n.sp.. Although the latter organism only occurred occasionally in his isolations, Miehe paid much attention to it.

Miehe recorded the following properties for Bacillus foliicola n.sp. :- One-day old cultures in liquid or on solid media consisted of motile rods, having dimensions 1 - 2μ x 0.5μ. They were mainly single, no chains being observed, but were sometimes stuck together in irregular masses by a mucilaginous sheath. They were flagellated, the number of flagella per cell ranging from 1 to 4, arranged around the cell in non-polar fashion. The non-motile stage was reached on glucose-peptone-agar after 3 days incubation at 25°C. Four-week old cultures showed non-motile, curved rods and also "involution forms"; the latter were swollen, occasionally branched, and stained irregularly with carbol fuchsin. Miehe noted their similarity on culture media to the "bacteroids" of old leaf nodules of Ardisia crispa. He was fully aware that morphological

properties taken by themselves were not a sufficient criterion to say that one organism was the same as another, and hence he was reluctant, at first, to conclude that his isolate (Bacillus foliicola) and the nodular organism were one and the same organism. Only successful re-infection of healthy, non-nodulated, Ardisia crispa plants, with the subsequent production of nodulated plants, would prove that his isolate was the natural symbiont, and therefore, at first he only assumed that his isolate was the same as the nodular bacterium. On solid media Bacillus foliicola n.sp. gave well-developed smooth, white, shiny, round colonies in 2 days, after incubation at 25°C. A membrane was formed on liquid media in a similar time, indicating the aerobic nature of the organism. It appeared that the bacterium preferred a neutral medium for growth, and that plant-extracts (e.g. pea, malt, etc.) were more suitable for growth of the organism than were, for example, peptone media. On plant-extract media the organism brought about a slight shift in the pH of the media to the alkaline side of neutrality, whereas peptone media were rendered slightly acid. Gelatin was not liquefied by the isolate, indicating

that proteolytic enzymes were not secreted into the medium. Asparagine was seemingly the best nitrogen source for growth, but the isolate failed to grow in the complete absence of combinednitrogen. No fermentation of glucose, lactose, or sucrose occurred when the isolate was grown in media incorporating these sugars. 25 - 30°C was the optimum temperature for growth of the isolate.

A most important feature of Miehe's work was that he was unable to obtain evidence of nitrogen fixation by the organism when growing in vitro. This result was the basis of a long-standing scientific controversy between Miehe and von Faber, who at the same time was researching on leaf nodules of the Rubiaceae. (This will be discussed more fully on page 76).

Miehe's last paper (1919) dealt with bacteria-free plants ("cripples"), the crippled appearance of which he considered to be a living demonstration of a truly symbiotic component deprived of its partner. Without bacteria, the plant developed but poorly, remained dwarfed, and never flowered.

In order to prove that his isolates were identical with the nodule-inhabiting bacterium, Miehe tried to produce bacteria-free plants; he had already shown bacteria to be present in the seeds of Ardisia crispa A.DC., and claimed that these followed the growth of the plant, being found in the terminal bud, whence they infected the bud-leaves through the hydathodes. Hence Miehe was faced with the same problem with Ardisia crispa as was von Faber with his Rubiaceae species; namely, the production of bacteria-free (and hence, nodule-free) plants from seeds that already housed the nodule-inhabiting microorganism. In theory, at least, he could then carry out reinfection experiments with his isolates on the bacteria-free plants.

Three methods were used by Miehe in an attempt to produce bacteria-free plants. His first method involved keeping young plants in the dark so that etiolation would take place. He thought it was possible that the vigorous growth of the terminal bud of a plant in an etiolated condition might cause it to pierce the bacterial film, leaving it behind, and hence producing a terminal bud devoid of bacteria. When he brought

the etiolated plants back into the light, however, normal development of the plant took place, i.e. subsequent leaves were nodulated, and he abandoned the use of this method.

The second method Miehe tried was also found unsuitable for the production of bacteria-free plants. He thought that wound-callus might form bacteria-free buds, so accordingly he cut the thin bark of Ardisia crispa A.DC. in various places. But bud formation was never observed on would surfaces, or on cuttings free of dormant-buds.

Eventually Miehe claimed success in the production of bacteria-free plants by use of a method analogous to that used by von Faber with Rubiaceae species, namely heat-treatment of the seeds. Heating Ardisia crispa seeds at 40°C for 2 days was sufficient to kill the bacterial symbiont, whilst still retaining viability of the plant embryo. Seeds thus treated always developed into crippled plants on germination; the latter Miehe designated as "experimental cripples". Heating to temperatures below 40°C for a similar duration of time yielded only a very low percentage of "cripples".

He also found that most batches of untreated seeds gave rise to a certain percentage of "cripples" on germination. These "spontaneous cripples", as Miehe called them, sometimes accounted for about 48% of all plants arising from untreated seeds. Miehe explained this high figure as being due to a variable frequency of bacteria in the seed, even to the extent that there might be some naturally occurring bacteria-free seeds, which would yield "spontaneous cripples". The "experimental" and "spontaneous crippes" appeared to be morphologically identical.

Initially there appeared to be no difference in the seedlings originating from heated or unheated seeds; they germinated at the same rate and development of the first leaves took approximately the same time. But, after this point was reached, further development of the two sets of plants was markedly different. When the plants reached a height of about 4 cm. differences in their external morphology were evident. Only about 3 foliage leaves were present on all plants at this stage, there being nothing unusual about this; but, whereas the terminal bud was

typically pointed in the normal untreated plant, it was stunted in the "cripple". On following the subsequent development of the two sets of plants, the differences between them became even more marked. The young "cripples" showed multiple bud formation in the foliage leaf and cotyledonary axils, these buds apparently developing from the dormant buds already present in these axils. These unusual buds were roundish, swollen, and thickened, and seemed unable to form leaves. Eventually the stunted buds developed into irregular protruberances, reaching appreciable dimensions. No further longitudinal growth of the stem took place and flowering did not occur. The root-system of the "cripples" was normal (as compared with normal untreated plants) but, after about 3 years, the few leaves possessed by the "cripple" withered and died; simultaneously, the swollen bud masses rotted and the plant finally died.

Over a similar period of time, the normal plants had grown into well-developed, healthy plants, approximately ten times the height of the "cripples"; fruit had been produced, and the numerous leaves developed by the normal plant were all nodulated.

Miehe also noted the presence of stages intermediate between normal and crippled plants. Unlike the "cripples" described above, these plants could revert spontaneously to normal plants, even after exhibiting a crippled appearance for 6 weeks, a normal shoot developing from a cotyledonary bud to form a normal plant.

Microscopic examination of thin sections of the enlarged tissue of the terminal and cotyledonary buds of "cripples" showed that bacteria were no longer present in these regions. Similarly the enlarged meristematic regions were devoid of bacteria. Miehe noticed, however, that nodules were still present on the leaves of crippled plants (both "experimental" and "spontaneous") and bacteria were occasionally present therein.

Miehe made numerous attempts at reinfection of the "experimental cripples" with the two isolates he had obtained from germinating Ardisia crispa A.DC. seeds; on no occasion, however, was he able to produce normal plants from the crippled forms.

Miehe was opposed to the idea that leaf nodular bacteria functioned in symbiotic nitrogen fixation. He considered that the association between bacterium and plant was symbiotic in nature, but the precise nature of the symbiosis was unknown to him. Eventually he concluded that a "stimulative effect" emanated from the symbiotic bacteria.

In 1932 a Czechoslovakian worker, Nemec, turned his attention to the function of leaf nodular bacteria of the Myrsinaceae. Little of outstanding interest was added to Miehe's results by this worker. Nemec favoured Miehe's theory that the symbiotic bacteria did not serve the plants in the fixation of atmospheric nitrogen, and added further that the bacterium, even when living in symbiosis in Ardisia leaf nodules, was not capable of nitrogen fixation. Nemec considered that a hormonal substance, excreted by the nodular bacterium, induced normal development of the host.

In an attempt to repeat and extend Miehe's work, de Jongh (1938), working at the University of Leiden, took up the problem of the Ardisia symbiosis.

De Jongh reported that the bacterial symbiosis of Ardisia, as conceived by Miehe, could be confirmed by him in almost all aspects. He isolated a bacterium from Ardisia crispa A.DC. which he concluded was the true bacterial symbiont in nature. With this bacterium, which in pure culture exhibited identical properties to Bacillus foliicola Miehe n.sp., he claimed (unlike Miehe) to have successfully re-infected bacteria-free ("crippled") Ardisia plants. Like Miehe and Nemec, de Jongh was not able to arrive at any definite conclusion as to the nature of the symbiosis, but deemed it most likely that a growth-promoting substance was being produced and excreted by the bacterium within the plant, thus serving the plant in some unknown capacity. His bacterial isolates were incapable of nitrogen fixation when grown in artificial culture.

As Miehe had done before him, de Jongh investigated the distribution of the bacterium in the plant tissues, and similarly concluded that the symbiosis was "cyclic" in nature. Closely following the methods adopted by Miehe, he confirmed that the bacterium

occurred in the plant in foliar nodules, seeds, and active and dormant meristems (the root and anther meristems being excepted). The bacteria found in the various locations exhibited a different morphology according to their habitat in the plant; thus, while rods were found in the neighbourhood of the terminal bud, the foliar nodules contained "involution forms". The Ardisia bacterium was always intercellular.

Although Miehe (1911) had given a very detailed description of the Ardisia bacterium in the mature seed, where it occurred between the embryo and the endosperm at the radical pole, he was not able to show how it arrived at this location. De Jongh particularly concentrated on this gap in Miehe's work, examining in detail the flower and fruit formation of Ardisia crispa A.DC., for he considered that these stages represented the most interesting part of the "cycle", as the presence of bacteria in the seed had to be accounted for ontogenetically. He claimed success in demonstrating the method of seed infection. De Jongh described typical rod-shaped bacteria, as occurring in successive stages of fruit formation, ranging from young developing flowers, through mature flowers, and finally up to mature fruits.

De Jongh reasoned that the method of fruit infection would be closely connected with the embryology of the plant, and considered that there were at least three ways by which the Ardisia embryo could become infected in nature:-

(a) As only pollinated flowers yielded fruit, it was possible that the bacteria could enter the embryo-sac via the pollen tube, and in this way develop between the endosperm and the embryo. Both von Faber (with Pavetta) and Miehe (with Ardisia) had favoured this idea as a possible mode of entry of the bacterium into the seeds. De Jongh rejected the idea, for he never found a pollen-tube occurring in the flower. In addition to this, for von Faber and Miehe's hypothesis to be correct, the bacteria would have to be intracellular at one stage in their life-cycle, whereas they had never been found in this condition.

(b) It was also possible that the bacteria might conceivably enter the embryo-sac through a special channel in the ovule wall. But de Jongh never observed any such channels and hence he also discarded this idea.

(c) After microscopical investigation of Ardisia flowers and seeds in all stages of development, de Jongh postulated that infection of the seeds arose in the following way:- The bacteria, enclosed between the floral parts at flower-formation, were locked within the carpels and finally surrounded the ovules. During development of the integuments, the bacteria, already present near the apex of the ovules, were enclosed between the nucellus and the interior integument, the bacterial membrane being activated by the developing meristem and forming a film under the micropylar region. The micropyle was not situated at the apex of the ovule, being pushed by the fast-growing dorsal flank of the interior integument to the ventral side (tendency to kampylotropy). De Jongh attached much importance to the peculiar wedge-shaped part of the ventral flank of the interior integument; for, in addition to serving as a method of closing the micropyle, it had cellular contents which were much denser than those of the surrounding cells. De Jongh claimed, as Jaensch had done before him (1905), that this wedge-shaped cell mass was embryogenic tissue, the embryo being formed adventitiously from this

particular region of the interior integument. Confirming Jaensch's observations, a true egg-apparatus was only seen in about 3% of all flowers examined; this egg-apparatus consisted of 4 cells which de Jongh interpreted as being 2 synergids, the egg-cell, and a vegetative cell. This condition, occurring in the flower before pollination, was most unusual, as it was absent from the large majority of flowers examined; no such egg-apparatus was ever seen in pollinated flowers, indicating the apogamous nature of embryo formation. Only one ovule per flower (out of a total 4 or 5) developed further, the others degenerating in the very young fruit stage. Following the process a stage further, the bacteria adhered to the radical pole of the adventitiously-formed embryo, where they remained in a resting, but reversible, state until the seed germinated. On germination of the seed the bacteria were pushed over the cotyledons, where they infected the axillary bud, while a bacterial mass was also deposited on the terminal bud.

The relationship between number, form and activity of the bacterium in the various locations within the plant (foliar buds, floral buds, seeds, nodules) was studied

by microscopical observations and experiments. The bacteria were always present in large numbers as rods near meristematic tissues. De Jongh found that within the plant the bacteria were always non-motile; they seemed to be most active at active stem-meristems, and inactive (though reversible to the active state) in dormant buds, where they could renew activity after several years of rest. De Jongh was in full agreement with Miehe's "cyclic symbiosis" theory, for he considered that his own findings with young developing fruits of Ardisia represented the missing links in the bacterial life cycle as postulated by Miehe.

All attempts to isolate the bacterial symbiont in pure culture from the leaf nodules failed; and numerous "infections" occurred when foliar buds were used for the same purpose. Eventually however, a successful isolation of the bacterial symbiont was achieved from germinating seeds, by using a slight modification of Miehe's method. Plating out crushed plantule suspensions (as opposed to intact plantules as used by Miehe) onto pea agar resulted in the development of bacterial colonies of two types - white slimy colonies and yellow colonies, the former predominating.

In pure culture the organism which produced white slimy colonies exhibited similar morphological and physiological properties to Bacillus foliicola n.sp. and de Jongh concluded that this and his new isolate were identical. Notwithstanding the fact that only 3 colonies of B.foliicola n.sp. had grown up on his original isolation plates, de Jongh considered that this species represented the true bacterial symbiont in nature; for, when he subsequently re-infected crippled seedlings with this bacterium, approximately 20% of such seedlings developed into normal plants. As further proof that this bacterial species and the nodular bacterium were identical, he drew attention to the high proportion of swollen, distorted rod-forms which were found to occur in 3-5 week old pure cultures of the isolate, and which he likened to the "bacteroids" present in old foliar nodules of A. crisp A.DC..

The bacterium which gave rise to yellow colonies was morphologically different from Miehe's second isolate (Bacterium repens n.sp.).

De Jongh carried out an extensive study of "experimental" and "spontaneous cripples". For the

production of "experimental cripples", a wider range of temperature and time than used by Miehe was employed. De Jongh considered that maintaining seeds at 52°C for only 7 minutes - as advocated by Miehe - was not a sufficient length of time for allowing temperature equilibrium to be reached within the seed. Accordingly, for experiments where only short period heat-treatments were involved (10 minutes x 55°C, 10 minutes x 50°C and 7 minutes x 52°C), he pierced the seeds with thermoneedles before immersing them in hot water. In this way, he reasoned, an equal temperature both inside and outside the seed could be attained within two minutes. For experiments where the exposure time was of the order of hours (e.g. 24 hr. x 40°C, 48 hr. x 40°C), he dispensed with the use of thermoneedles, placing the seeds in moist sand, the temperature of which was thermostatically controlled. As controls, unheated seeds, grown under the same conditions, were employed.

Exposure to 55°C for 10 minutes was fatal to seeds. However, all the other batches of heated seeds yielded "cripples" and, in most cases, the percentage of such plants was within the region of 90-100%. In the control batch all seeds germinated to produce normal

plants. De Jongh commented that this result was somewhat unusual, especially in view of the fact that seeds from the same plant had, in another experiment, yielded 18% "spontaneous cripples", when grown under identical conditions.

The germination time of seeds in all batches was approximately the same, being of the order of 50-55 days. Seedlings originating from heated- and control-seeds were morphologically identical in their very early stages of development. However, after about four months, "cripples" developed from the heated seeds. Such plants remained dwarfed, longitudinal growth ceasing over the entire plant (with the exception of the root). At the same time the axillary cotyledonary buds started to proliferate, resulting in the formation of a "wart-like mass"; this continued over a number of years.

De Jongh considered that temperature was probably the most important factor governing the development of "cripples"; and, as the temperature range for heat-treatment of the seeds (as described above) seemed relatively wide compared to the temperature

most likely to be encountered by the plant in its natural milieu, he carried out a similar investigation on the effect of lower-range heat-treatments. Temperatures between 30°C - 40°C inclusive were employed, batches of seeds being exposed to these temperatures for times ranging from 15 minutes up to 32 hours. These seeds were germinated and grown under optimal temperature conditions. Non-heated seeds, grown under similar conditions, served as controls.

The percentage of seeds germinating in all batches was high, in most cases being 100%; in no batch was a value lower than 97% recorded. The germination time of both heated and control seeds was approximately the same (50 days). Thus, in both high (40°C - 50°C) and low (30°C - 40°C) temperature range experiments, the rate of germination did not appear to be affected by heating to those temperatures.

In contrast to the results obtained from seeds which had been exposed to high temperatures, the seeds exposed to the lower temperature range gave rise to only very few "cripples"; thus 3% "cripples" were noted in plants developing from seeds maintained at

30°C, 32.5°C or 35°C for 32 hours; and 6% resulted in plants developed from seeds which had been held at 40°C for 8 hours; all the remaining series, including controls, showed normal plants exclusively.

On the basis of these results, de Jongh concluded that the high temperatures employed in his first experiment were probably responsible for such a mass occurrence of "cripples"; growing plants from seeds which had been subjected to temperatures roughly approximating to that of their natural habitat, greatly reduced the number of "cripples".

De Jongh confirmed Miehe's finding that most batches of untreated seeds gave rise to a certain percentage of "spontaneous cripples", but the proportion of such "cripples" was markedly less (about 20% as compared with 48%). "Spontaneous" and "experimental cripples" were morphologically identical.

The percentage of "spontaneous cripples" developing from seeds harvested from different individual A. crispa plants was extremely variable, even when the

seeds were grown under constant temperature conditions. With batches of seeds collected from 7 different plants, he observed values ranging from 0% - 21% for "spontaneous cripples".

Not only did the source of seeds affect the proportion of such "cripples"; for when he cultivated seeds which had been collected over 3 successive years from the same plant, "spontaneous cripples" accounted for 18%, 0% and 0% of the plant populations respectively.

Miehe had assumed that the occurrence of "spontaneous cripples" could be due to a variable frequency of bacteria within the seeds, some of which might be entirely free from bacteria. De. Jongh's microscopical investigations revealed that flowers and fruits of A. crispa were always infected with the bacterial symbiont and hence he rejected Miehe's assumption. He considered that the external conditions prevailing during maturation or germination of the seed were more likely to govern the appearance of "cripples" and, of these conditions, temperature was probably important.

Microscopical investigations showed that the stem meristems of normal and crippled plants were markedly different in anatomical structure, size and number. The swollen terminal and axillary buds of "cripples" contained a number of quite distinct meristematic cell-groups, sunk deeply below the surface. This number was very variable and seemed to increase with age. It was difficult to assess the number of meristems in the normal plant, as the vegetation point proper merged into the foliar primordia; but the meristems were always in "free contact with the outer world and with the bacterial film". It appeared that the number of nuclei per unit area in a "cripple"-meristem was smaller, and the average cells larger, than in a normal plant meristem.

"Cripples", like normal plants, were diploid and hence the nanism of the former did not result from a change in chromosome number.

The swollen meristems of "cripples" were always devoid of bacteria and this factor was thought to be probably responsible for the abnormal appearance of "cripples".

Microchemical tests showed that catalase and peroxidase were present in the terminal bud of "cripples", whereas, in normal plants, only catalase was present. Because of this, de Jongh tentatively suggested that the bacterial film present in normal plants might influence the oxido-reduction potential of the terminal bud.

In order to prove that the nodular bacteria were essential for the normal development of A. crispa, de Jongh attempted to re-infect crippled plants with his two bacterial isolates. Of the two bacterial isolates, only one - Bacillus foliicola n.sp. - seemed capable of converting crippled plants into normal plants; and, using this bacterium, he claimed successful re-infection of crippled seedlings.

Ten-month old "cripples" seemed no longer susceptible to re-infection, whilst similar experiments on 4 month old "cripples" yielded inconclusive results. However, by treating the terminal buds of very young crippled seedlings (i.e. seedlings from which the plumule has just emerged) with an aqueous suspension of

B. foliicola n.sp., he claimed that 20% of them subsequently developed as normal plants. He concluded that, whilst these results were far from complete, they could be considered as positive evidence for successful re-infection; thus demonstrating that his bacterial isolate and the *in vivo* bacterial symbiont were identical.

Turning his attention to the function of the symbiosis, de Jongh considered a number of possibilities. He rejected nitrogen fixation as being the basis of the symbiosis, for neither of his bacterial isolates exhibted this capacity when grown on artificial media. Another possible explanation of the symbiosis was that the bacterial film covering the vegetation point might act as an "oxygen-trap", thereby lowering the oxygen-tension in the meristematic region of the stem. As bacteria-free *Ardisia* plants showed stunted development, the resulting "cripple" might therefore be a product of an oxygen overdosage at the meristematic region of the stem apex.

A consideration of the morphological and physiological abnormalities of "cripples" led de Jongh to conclude that the most likely explanation of the symbiosis was that a growth-promoting substance was being produced by the symbiotic bacterium; this, in turn, was utilised by the plant in some unknown manner.

In order to test the validity of de Jongh's suggestion that crippled plants might conceivably arise as a result of oxygen overdosages, Bok (1941) raised Ardisia crispa plants from seed in a closed system where the oxygen concentration of the atmosphere could be maintained at controlled levels.

Heat-treated (i.e. internally sterilised) surface-sterilised Ardisia crispa seeds were divided into batches prior to planting on a sterilised mineral salts - agar growth medium, the latter being contained in sterile flasks, which in their turn formed part of the closed system. Germination and subsequent development thus took place in a sterile environment.

An oxygen-nitrogen gas mixture of known

composition was fed continuously into each flask over a 5 month period, the relative proportions of the two gases varying from flask to flask. Thus each plant was grown in an atmosphere of known oxygen tension.

Surface-sterilised, unheated seeds, grown under exactly the same conditions were used as controls.

She found that the percentage of "cripples" originating from heat and surface-sterilised seeds could be considerably reduced by raising the plants at oxygen tensions lower than that of the normal atmosphere.

At an increased oxygen tension compared to normal air, all the sterilised seeds - as well as most of the control seeds - developed into "cripples".

Bok concluded that her results would seem to corroborate de Jongh's suggestion that dwarfing is due to an oxygen overdosage of the vegetation point, such a phenomenon normally being prevented by the covering bacterial film.

The leaf nodule symbiosis of Ardisia hortorum Maxim has been investigated by Hanada (1954). His investigations, whilst being similar in many respects to those carried out by Miehe (1911 - 1919) and de Jongh (1938) with Ardisia crispa A.DC., have, however, led him to a different conclusion as regards the function of the symbiosis. A bacterium isolated from the nodules was shown to be capable of fixing atmospheric nitrogen; and this fact, taken alongside his discovery that addition of nitrate to nodulated plants grown in nitrogen-free sand produced no noticeable change in the appearance of these plants, led him to conclude that nitrogen-fixation formed the basis of the symbiotic association. Hanada rejected Miehe's hypothesis that the bacterial symbiont supplied the host with a "stimulatory substance".

The nodules of A. hortorum. Maxim, arranged as in A. crispa along the crenated periphery of the leaf, were found to occur in much larger number than in A. crispa. Hanada considered that the variegation of A. hortorum Maxim leaves, which in summer extends along the whole leaf edge but in winter develops at only a

few regions, is, in some unknown way, connected with the "seasonal vitality" ("Lebenskraft") of the nodular bacteria.

An anatomical investigation revealed that the leaf nodules always developed below pore-type structures which he termed "elongated apertures" ("spaltöffnung") and where there were no such structures no nodules developed; but the converse did not hold. Hanada considered that bacterial infection took place through these structures. The leaf nodules exhibited a similar anatomical structure to those of A. crispa, having loosely packed cells interspersed with a proteinaceous slimy material. In A. hortorum. Maxim. nodules, however, the symbiotic bacteria, in addition to being present in the intercellular slime, were also found to occur inside the nodular cells. The closely packed host cells of developing nodules contained, initially, only a small number of bacteria. Hanada suggested that these bacteria multiplied within the host cells; when the host cells divided the bacteria were passively transferred to the daughter cells.

By plating out surface-sterilised leaf nodules of A. hortorum. Maxim. on Bouillon's agar, he isolated a bacterium which, he claimed, was the nodular symbiont; for, when he subsequently re-inoculated this bacterium into the leaves of bacteria-free plants (i.e. those plants which developed from seeds which had been maintained at 50°C for 30 minutes in order to kill the bacterial symbiont), structures thought to be nodules were produced in a few cases.

In pure culture the bacterium exhibited morphological and physiological properties different from Bacillus foliicola n.sp. and Mycobacterium rubiacearum. It was a Gram negative, non-sporing, non-capsulated, rod-shaped organism having cellular dimensions of 2 - 3μ x 0.4 - 0.5μ. It was motile, possessing one (and sometimes two) polar flagella. On leaf extract agar it grew rapidly, forming moist, translucent, cream colonies. Gelatin was entirely liquefied and a strong hydrolysis of starch was noted. Acid (but no gas) was produced in sucrose, glucose and lactose broths. Nitrate broth was reduced to nitrite, litmus milk was turned acid and no indol was produced in tryptone broth. It was a facultative anaerobe with an

optimum temperature for growth of 27°C. Hanada considered it to be a new species and named it Xanthomonas hortoricola n.sp..

Hanada concluded that Xanthomas hortoricola n.sp. was a nitrogen-fixing organism for when he cultivated it in a mineral salts liquid medium, containing mannitol (1%) as carbon source and potassium nitrate (0.01%) as a "starter", he noticed that the nitrogen content of the medium increased. Thus, over a 5 day period, a nitrogen gain of 0.07 mg/10 c.c. solution was found. With time, the amount of nitrogen fixed increased (e.g. over a 7 - 12 day period the gain was 0.13 mg/ 10 c.c. solution). The isolate would not grow in a medium that was totally devoid of nitrogen and Hanada noted that a similar effect had been recorded by McBeth in 1913 for Azotobacter. The addition of small quantities of nitrate (from 0.005% - 0.1%) resulted in an increased fixation of nitrogen, but further addition above 0.1% reduced the amount fixed.

In order to investigate the nature of the symbiotic association between host plant and bacterium,

Hanada cultivated A. hortorum Maxim. from seed under various conditions. Seeds, surface sterilised and then heat-treated at 50°C for 30 minutes, were germinated and grown under sterile conditions in nitrogen-free sand.

As a control a further batch of surface-sterilised non-heated seeds were sown under the same conditions.

During the first two months after sowing there was no noticeable morphological difference between experimental (i.e. bacteria-free) and control plants, both sets being of approximately the same size and bearing a similar number of leaves. After 2 months, however, differences could be observed. Thus, whilst the control plants appeared healthy, green and nodulated, the bacteria-free plants showed signs of starvation and were non-nodulated.

After a further 5 - 6 months the differences were more pronounced, the bacteria-free plants bearing pale green leaves, whilst the control plants bore dark green, healthy-looking leaves. Growth ceased in the

bacteria-free plants after about one year, when they had reached a height of 25 cm; no new leaves developed, the stem was of peculiar form and the terminal bud was swollen. The control plants continued to develop normally. The bacteria-free plants eventually died, whereas the control plants continued normal development.

Addition of nitrate to the control plants produced no change in their appearance, and a similar nitrate addition to bacteria-free plants produced very little change in their appearance, their development still remaining very poor. Hanada concluded that the plants depended, not so much on a supply of nitrate from the soil, but rather upon a supply of nitrogenous compounds arising from the fixation of atmospheric nitrogen by the symbiont. He considered that the proteinaceous material found in the leaf nodules represented the products of bacterial assimilation, the atmospheric nitrogen having been incorporated into "carbohydrate" material derived from the plant. These nitrogenous materials were utilised by the plant.

The bacterial leaf nodules of a third Ardisia species have been described by Bose (1955), who drew

attention to the "schizogenous glands" (i.e. nodules) which he found occurring on the leaves, stems, roots, flowers and seed endosperm of Ardisia humilis. Vahl.. Such "glands", which were particularly numerous on the edges of the leaves and also over the whole surface of the sepals and petals, were pinkish in colour; this pink pigmentation was very noticeable in young leaves (especially at the margins) and in the sepals, petals, anthers, carpels and seeds.

A microscopic examination showed bacteria to be present in large numbers close to these "glands" and likewise the conspicuous irregular nodular swellings on the roots were heavily infected with similar bacteria. These bacteria were irregularly shaped and had a granulated cytoplasm and Bose concluded that they represented B. type L. forms of bacteria. The pink pigment was present in large amounts around the "glands" and Bose considered that it possibly represented a metabolic product of the bacteria. Notwithstanding their high degree of bacterial contamination, the Ardisia humilis Vahl. plants appeared perfectly healthy and bore abundant flowers and fruits.

The bacterial symbiont was aseptically isolated from the leaves and roots and was grown in pure culture on nutrient agar, malt agar and in liquid malt, when a large number of small granulated bodies - which Bose interpreted as L. forms of bacteria - were found along with the bacterial colonies. Bose's work is still continuing.

3. Comparative studies on MYRSINACEAE and RUBIACEAE.

In 1914 a controversy arose between Hugo Miehe and F. C. von Faber concerning the interpretation of the bacteriophilous condition as existing in the Myrsinaceae and Rubiaceae. On the basis of his findings with A. crispa (Myrsinaceae), Miehe (1914) raised a number of objections to von Faber's findings (1912) with Rubiaceae species.

On comparing Bacillus foliicola n.sp. (the bacterium which he had isolated from germinating seeds of A. crispa) with Mycobacterium rubiacearum (the bacterium isolated by von Faber from terminal buds of P. zimmermanniana, P. indica and Psy. bacteriophila) Miehe noted that it differed from the latter in a number of characters, among which were motility, non

acid-fast staining, and the absence of elemental nitrogen fixation. As regards nitrogen fixation Miehe found that when Bacillus foliicola n.sp. was inoculated into nitrogen-free media and incubated for a number of days there was an increase in the amount of total nitrogen, but the increase was so small as to be within the limits of experimental error. He concluded that Bacillus foliicola n.sp. was not a nitrogen-fixing bacterium. Miehe considered that the two bacteria represented distinctly different types and further criticized the identity of von Faber's isolate with the true symbiont, on the basis of his own experience with A. crispa, where he had isolated a variety of bacteria from terminal buds. He suggested that von Faber's isolate was probably a water-borne aerial contaminant of Rubiaceae terminal buds, and subsequently confined his attention to isolation from surface-sterilised seeds.

Miehe also disagreed with von Faber's systematic placing of the Rubiaceae isolate in the Mycobacteriaceae; for, when he examined a number of Pavetta species, he noted that not only did the acid-fast property of the nodular bacterium vary from one

plant to another, but it was not constant, depended upon the time of application of the reagents, and in most cases was negative.

In Miehe's opinion the nature of the association was a symbiosis based, not upon nitrogen-nutrition, but upon a "stimulatory effect" due to the bacteria.

A major portion of von Faber's second paper (1914) was devoted to refuting Miehe's criticisms.

At the outset von Faber pointed out that as they both worked with different plants from different families and under different conditions, then there was little basis for comparison.

Concerning their respective isolates he agreed with Miehe that the two bacteria exhibited unmistakable differences and were probably different bacterial species, but, in spite of this, they were basically similar, and the close relationship of the two symbioses could hardly be questioned. Notwithstanding the non-motile character of Mycobacterium rubiacearum in pure culture, von Faber

did not exclude the possibility that it might be motile at some stage in its intimate partnership with the host plant, though he could not obtain evidence for this.

Turning his attention to Miehe's assertion that foliar buds, on account of the fact that they might harbour water-borne aerial contaminants, were unsuitable as sources for the isolation of leaf nodular bacteria, von Faber recognized that whilst this could conceivably be the case with A. crispa, it would certainly not apply to Rubiaceae foliar buds; whereas the more open structure of the Ardisia foliar bud could lend itself to the immigration of bacteria, the stipular coverage and gum-filled interfoliar spaces of Rubiaceae species would render the penetration of aerial bacteria impossible.

As regards the systematics of the Rubiaceae bacterium, von Faber repeated his claim that it should be placed in the Mycobacteriaceae. He considered that the acid-fast character of the bacterium was variable, being more pronounced for the Psy. bacteriophila isolate than for the P. zimmermanniana isolate,

but on no occasion did he find that this character was completely absent. The acid-fast nature of the bacterium, taken alongside a number of other taxonomic characters, led him to re-assert that it was a member of the Mycobacteriaceae.

When he repeated his nitrogen-fixation experiments with Mycobacterium rubiacearum, von Faber obtained almost identical results to those he first reported in 1912. The nitrogen-fixing capacity of Mycobacterium rubiacearum varied considerably from one experiment to another, but always, nitrogen-enrichment values high enough to be outside the limits of experimental error were obtained. He concluded that Mycobacterium rubiacearum was a nitrogen-fixing bacterium and that its function in the nodular symbiosis was one of nitrogen-fixation.

During the course of a research on nitrogen-fixing bacteria, de Vries and Derx (1950) attempted to isolate the leaf nodular bacteria from a number of Rubiaceae and Myrsinaceae species.

Closely following the methods of von Faber (1912, 1914) and Rao (1923) they isolated a pink-pigmented, rod-shaped bacterium from the leaf nodules and foliar buds of several Pavetta species (including P. zimmermanniana), Psychotria bacteriophila, and A. crispa. Like Mycobacterium rubiacearum, it was Gram negative, non-sporing and bacteroid-forming, but it differed from the former in dimensions, in being motile and in forming pink gelatinous colonies. They named it Mycoplana rubra.

They found that, although Mycoplana rubra would grow well on media of low nitrogen-content, it failed to grow on a medium that was completely nitrogen-free, and hence concluded that it was not a nitrogen-fixing organism.

In further experiments it proved possible to isolate Mycoplana rubra from the leaves of non-nodulated Pavetta species, from river water and from soil; but, from each of these sources, the number of Mycoplana rubra colonies which developed on isolation plates was relatively low as compared with that obtained from

leaf-nodulated species. De Vries and Derx concluded that Mycoplana rubra was not the nodule-causative organism. They considered it to be a regular, but secondary, companion of the organisms which initiate such structures.

Ziegler (1958) has investigated the plant-bacteria relationship in certain members of both Rubiaceae and Myrsinaceae, namely Pavetta zimmermanniana Val. and Ardisia crispa A.DC.. From each species he obtained bacteria by preparing smears of aqueous suspensions of crushed seed embryos, terminal buds and leaf nodules. Comparison of the morphological features of the bacteria, using the electron microscope, convinced him that the symbiotic bacteria present in the two plants were of different species. Those found in seed embryo suspension of Pavetta zimmermanniana. Val. were non-flagellated rods, measuring 2.8μ - 3.1μ in length by 0.9μ - 1.2μ in breadth. The majority of the bacteria in the terminal bud suspension were morphologically similar to the seed microorganism, but, in addition, there were some much longer rod-forms present; in some cases the latter measured up to 12μ in length. Irregular forms of bacterial rods, often branched and

swollen, were found in suspensions of fully developed leaf nodules; only in very young nodules could unchanged rods be observed. In contrast, the seed and terminal bud bacteria of Ardisia crispa. A.DC., whilst similarly being a non-flagellated rod-shaped organism, exhibited a wider variation in length (2μ - 3.5μ) than the bacterial symbiont as found in the corresponding locations in Pavetta zimmermanniana Val.; the width of the bacterial cells (1μ) was approximately the same. The bacterial population of the leaf nodules consisted almost exclusively of bacteroids, but these were much less bizarre than those found in Pavetta species.

Ziegler isolated the bacterial symbiont, in pure culture, from the two plants by dissecting out the seed embryos and plating them out on 2% sweet wort agar; from each plant a different kind of bacterium was isolated. Electron microscopic examination showed that the pure-cultured bacterial isolate of Pavetta zimmermanniana. Val. was exactly similar in size and form to that present in the seed embryo suspensions of this plant. The pure-cultured Ardisia crispa bacterium,

however, was a bi-flagellated rod. Notwithstanding the fact that the bacteria in Ardisia seeds were non-flagellated, Ziegler concluded that his bacterial isolate was identical with that present in vivo, as they were morphologically very similar in all other respects e.g. size, shape and granulated appearance of their cytoplasm.

Electron-microscopic observation of thin sections of Pavetta zimmermanniana Val. leaf nodules confirmed the findings of earlier workers with regard to the position of the bacteria within the nodules. They were intercellularly arranged, being present in large numbers in the slime-containing regions of the nodule. But, under the high resolving power of the electron-microscope, he was able to see structures which, owing to their small size, were undiscernible by the ordinary light microscope. Thus, he found the intercellular spaces of the nodule were interlaced with fine, thread-like structures, which he termed "plasmodesma". The latter seemed to connect the bacteria and nodular slime to the modified mesophyll cells of the nodule, whose cell-membranes were apparently

completely permeated by such structures.

Ziegler noted that his findings seemed to confirm von Faber's view (1914) that the leaf nodule symbiont of Pavetta zimmermanniana Val. was, in fact, a different bacterium from that present in Ardisia crispa A.DC. leaf nodules. The morphological differences between the two, especially when observed in vivo, were only very slight and for this reason he considered that only a species difference was involved. Accordingly, he suggested that the Pavetta and Ardisia symbionts should be renamed Bacterium rubiacearum (von Faber) Miehe (originally designated in 1912 by von Faber as Mycobacterium rubiacearum) and Bacterium foliicola. Miehe (originally named Bacillus foliicola n.sp. by Miehe in 1914) respectively.

(c) Scope of the thesis.

The literature on leaf-nodule bacteria shows, in particular, that the identitiy of the symbiont, or symbionts, is in doubt and that the biological significance of the association is equally controversial.

In this thesis the problem has been re-approached through a comparative study of the distribution, identity and biological nature of the organisms associated with leaf-nodule plants. The question of nitrogen fixation by some of these organisms, has been re-investigated.

The availability of material has made it necessary to restrict the investigations to Ardisia crispa of the Myrsinaceae and to Psychotria emetica, Psychotria nairobiensis and Pavetta grandiflora of the Rubiaceae.

2. MATERIALS AND METHODS

I. Plant species investigated

Ardisia crispa A.DC. plant material was provided by the Royal Botanic Gardens, Kew and by the Chelsea Physic Garden.

Psychotria emetica and *Pavetta grandiflora* plant materials were supplied by the Chelsea Physic Garden.

Psychotria nairobiensis. Brem. plants were grown from seed donated by the East African Agricultural and Forestry Research Organisation, Nairobi, Kenya and the Scott Agricultural Laboratories, also of Nairobi.

II. Preparation of plant material for microscopical examination.

For the rapid preliminary examination of plant tissues for the presence of bacteria, slides were prepared as follows:-

Aqueous suspensions of leaf and bud macerates, smeared onto slides and stained by the Kopeloff and Beerman modification (1922) of Gram's method, were examined microscopically under oil immersion.

Because of their woody nature, seeds did not lend themselves readily to maceration. Hence fresh seeds were sectioned by using a freezing microtome. Both transverse and longitudinal sections (10μ in thickness) were stained by Stoughton's carbol thionin - Orange G method (1930).

For the preparation of permanent mounts the following methods were adopted:-

Fresh plant materials were fixed in a mixture of Formaldehyde, (10% v/v), Glacial acetic acid, (5% v/v), 95% Ethyl alcohol, (50% v/v), distilled water, (35% v/v) ('F.A.A."). Leaves, foliar and floral buds were soaked in F.A.A. for 3 to 7 days prior to dehydration. Because of their woody nature, seeds were given 3 to 4 weeks in the fixative.

For dehydration and wax-infiltration of the fixed plant tissues, Johansen's Tertiary Butyl Alcohol (T.B.A.) method (1940) was used. As fixation had been effected in F.A.A. containing 50% ethyl alcohol, partial

dehydration had already been accomplished. Hence the fixed materials were transferred directly to the appropriate T.B.A. solution, which similarly contained 50% alcohol by volume. Plant material was immersed in ten times its own volume of the T.B.A. solutions and for all plant materials, with the exception of seeds, the immersion times specified by Johansen were used; double the specified time was used for seeds.

The plant material was embedded in paraffin wax of M. pt. 56°C.

5 - 10μ thick serial sections (T.S. and L.S.) of the plant tissues were prepared using a Cambridge rocker microtome.

Haupt's gelatin method (1930) was employed for affixing the serial sections to microscope slides.

De-waxing of the sections was accomplished by immersing the slides for 5 mins. in each of the following solutions:- xylol; xylol - absolute ethyl alcohol (1 : 1); absolute ethyl alcohol - ether (1 : 1);

95%, 70%, 35%, 15%, 10%, 5% ethyl alcohol; distilled water. Where alcoholic-based stains were used, the process was terminated at the 35% alcohol stage.

A variety of staining techniques were tried in an attempt to find the most suitable procedure for demonstrating the presence of bacteria in the tissues. The criterion, by which a particular staining technique was judged, was its ability to give a good differentiation of the bacteria within the tissues. In each case, the same staining technique was applied to a smear from the nodular tissue of all plants under investigation.

The following staining methods were investigated:-

1) Carbol-thionin - Orange G (Stoughton, 1930). This method differed from Stoughton's former method (see above) in that the sections were stained in carbol thionin for 1 hour (instead of 15 mins.). Differentiation was effected by staining with Orange G for 1½ mins.

2) Safranin - Fast Green method (Modification of Conant's Quadruple stain - Johansen (1940)).

3) Methyl Green - Pyronin (after Conn and Darrow (1943)).

4) The carbol fuchsin method:-

The sections were

(a) stained in 1% carbol fuchsin for 1 min.

(b) washed in water

(c) washed briefly in 95% ethyl alcohol

(d) immersed in a 1:1 solution of 95% ethyl alcohol - xylol, prior to clearing in xylol.

5) Basic Fuchsin - Gentian Violet - Iodine method (MacCallum, 1919).

All permanent preparations were mounted in "Euparal".

Of the above staining methods Stoughton's modified carbol thionin - Orange G method was found to give very good results. By this method, the bacteria in the plant tissues were stained a red-purple colour, which contrasted well against the blue and yellow-stained host background. One important advantage this method possesses over the others is its ability to distinguish intracellular bacteria in plant tissues. The carbol fuchsin method had the advantage of being quick and easy to apply; it was found to be a particularly good method for demonstrating the presence of bacteria in the "mucilaginous membranes" of the

terminal buds and flowers of the plant species under study. The Basic Fuchsin-Gentian Violet-Iodine method was particularly good for staining "bacteroids". The Safranin-Fast Green method, in addition to giving a brilliant differentiation of nodular tissue from the surrounding mesophyll cells (staining it red as opposed to the green colour of the latter), showed up the nodular bacteria as bright red refractive bodies in the nodules. The Methyl Green - Pyronin method did not give a good differentiation of bacteria in the plant tissues. Methods 1), 2), 4) and 5) were adopted.

III. The isolation of bacteria from leaf nodulated plants.

(1) Methods employed for the surface sterilisation of plant materials.

The following methods were used:-

Mechanical method

Plant materials were washed, with shaking, in several changes (approx. 15) of sterile distilled water. The volume of water employed for each wash was roughly 100 times as great as that of the plant materials under investigation.

Chemical methods

The plant materials were either (a) mechanically shaken in 0.1% $HgCl_2$ for 20 minutes, followed by several washes (approx. 10) in sterile distilled water, or (b) mechanically shaken in 1% Chloramine T for 30 minutes followed by approximately 10 washes in sterile distilled water.

(2) Culture media

Various culture media were tried in an attempt to find which was most suitable for the isolation of the leaf nodular bacterium. Details of the composition and preparation of those culture media are as follows:-

Nutrient agar

"Oxoid" Nutrient broth No. 2	25 g.
"Oxoid" agar No. 3	10 g.

Made up to 1 l. with distilled water. pH 7.

Malt agar

Malt extract	30 g.
"Oxoid" agar No. 3	20 g.

Made up to 1 l. with distilled water. pH. 6.4.

Potato dextrose agar.

Peeled, chopped potatoes (200g.) were

immersed in 400 ml. tap water and steamed for 30 mins. The resulting suspension was filtered through muslin, and the volume of the filtrate was then made up to 1 litre with tap water. Dextrose (10 g.), "Oxoid" agar No. 3 (20 g.) and calcium carbonate (1 g.) were added to this solution prior to sterilisation.

Pea extract agar.

Dried peas (150 g.) were soaked overnight in 500 ml. phosphate buffer (pH 7.0) and were then finely ground in a blender. The minced pea tissue suspension was steamed for 1 hour and then filtered and pressed through muslin. Any fine grains remaining in the filtrate were removed by centrifugation (3,000 r.p.m. for 20 mins), decanting off the supernatant and filtering same through filter paper (Whatman No. 42 grade) on a Buchner pump. To the filtrate, sucrose (20 g.) and "Oxoid" agar No. 3 (15 g.) were added, the volume being made up to 1 litre by addition of phosphate buffer. After sterilisation the pH of this medium was 6.8.

Leaf-extract agar.

Approximately 100 leaves* were minced in

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* All 4 leaf nodulated plant spp. were used for making leaf extract media.

250ml. phosphate buffer (pH 7) in a blender. After filtering and pressing the leaf suspension through muslin and then centrifuging (3,000 r.p.m. x 30 mins) to bring down fine sediment, the supernatant was decanted off and filtered through Whatman filter paper (grade 41) to remove further traces of sediment. The volume of the filtrate was re-adjusted to 250 ml. by addition of further phosphate buffer, the resulting solution being filter-sterilised through an "Oxoid" membrane filter in a Seitz filtration apparatus.

A solution of "Oxoid" agar No. 3 (5 g.) and sucrose (10 g.) in phosphate buffer (250 ml. at pH 7) was sterilised by autoclaving (15 p.s.i. x 15 mins). The leaf extract solution was added to this hot solution prior to pouring plates.

"Nitrogen-free" synthetic medium*

All chemicals used in the preparation of this medium were "Analar" grade; it consisted of -

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* This medium was also employed for certain nitrogen-fixation experiments.

Na_2HPO_4	0.25g.
KH_2PO_4	0.10g.
$Na_2MoO_4.2H_2O$	0.01g.
$MgSO_4.7H_2O$	0.10g.
$FeSO_4.7H_2O$	0.10g.
$MnSO_4.3H_2O$	0.01g.
$ZnSO_4.7H_2O$	0.002g.
$CuSO_4.5H_2O$	0.001g.
$CaCO_3$	0.10g.
Glucose	10g.
Ion Agar (acid washed)	20g.
Glass distilled water	1 litre.

The medium was adjusted to pH 7 prior to sterilisation.

Yeast + glucose + all amino acids + all sugars.

Since glucose is partly decomposed when autoclaved in the presence of phosphate, the medium was prepared and sterilised without the glucose; a 20% (v/v) solution of glucose in glass distilled water was autoclaved separately and 50 ml. of this solution were added per litre of medium just before dispensing for use.

Except where otherwise stated, all culture

media used for bacterial isolation were sterilised by autoclaving at 15 p.s.i. for 15 mins.

(3) Maintenance of cultures

All bacterial isolates were maintained on nutrient agar, being incubated at 25°C and transferred weekly

IV. Methods used in the characterisation of the bacteria isolated from leaf nodulated plants.

For taxonomic purposes a number of morphological and biochemical tests were performed. Observations were made at frequent intervals during incubation of the cultures and each character was investigated at least twice yearly in order to assess its stability.

Except where specifically mentioned, young (24 hrs. old) nutrient agar slope cultures were used for the inoculation of characterisation media, the latter then being incubated at 25°C (with the exceptions of nutrient gelatin stab cultures which were incubated at

22°C, and of cultures used in experiments concerning the effect of temperature on bacterial growth).

For sterilisation of characterisation media 15 p.s.i. for 15 mins. was used unless otherwise stated.

[A] Morphology and staining reactions

(i) Morphology, Gram-reaction and size.

Air dried, heat-fixed films of young (24 hrs. old) nutrient broth and nutrient agar cultures were stained by Kopeloff and Beerman's modification (1922) of the Gram stain.

Measurement of the size of the stained organisms were made by using a calibrated eye piece graticule.

(ii) Motility and flagella staining.

The bacterial isolates were examined for motility by the hanging drop method.

Gray's method (1926) was adopted for flagella staining.

(iii) Slime and capsule formation.

The bacterial isolates were grown in slope culture on a medium of the follcwing composition:- peptone, 0.5% w/v; glycerol, 1% w/v; agar, 2% w/v; distilled water.

For the detection of capsular material Duguid's Indian Ink method (1951) was used.

In addition, Anthony's capsule staining method (1931) was employed.

Aerobacter aerogenes was used as a control.

(iv) Acid-fast staining.

The Ziehl-Neelsen method was adopted for determination of the acid-fastness of the bacterial isolates.

Mycobacterium phlei was used as a control.

In addition, the bacterial isolates were examined by von Faber's (1914) method:- The heat-fixed bacterial smear was treated with hot Ziehl's carbol

fuchsin for 2 - 3 minutes. The preparation was then washed in changes of 5% H_2SO_4 (15 seconds), 70% alcohol (30 seconds) and finally in water.

(v) Spore formation.

3 - 7 day old nutrient agar slope cultures of the bacterial isolates were examined for the presence of spores by:-

(a) The heat test. A turbid suspension of bacteria in sterile distilled water was maintained at 80°C for 10 minutes, after which a loopful was sub-cultured onto a nutrient agar slope. The latter was incubated at 25°C and examined daily over a 7-day period for the presence of bacterial growth.

(b) Schaeffer and Fulton's malachite green - safranin spore staining method (1933).

Bacillus subtilis was used as a control for both methods.

[B] Growth characters.

(i) Growth characteris in and on meat-extract media.

The growth characters were determined in

nutrient broth ("Oxoid" nutrient broth No. 2, 2.5% w/v; in distilled water; pH 7) and on slope and streak-plate cultures of nutrient agar.

(ii) The ability to grow on a "nitrogen-free" synthetic medium.

Streak plates of the bacterial isolates were prepared on the "nitrogen-free" synthetic medium and were incubated at 25°C. Where growth occurred, its characters were recorded.

(iii) Chromogenesis.

The bacterial isolates were inoculated in Georgia and Poe's medium ($MgSO_4.7H_2O$, 0.1%; K_2HPO_4, 0.05%; asparagine 0.3%; distilled water) and then incubated. The cultures were examined (in daylight and under u.v.) at intervals over a 7 day period.

[C] Physiological characters.

(i) Anaerobic conditions:-

Nutrient agar streak plates of the bacterial isolates were incubated at 25°C in an atmosphere of hydrogen in a McIntosh and Filde's jar.

(ii) Temperature:-

Ability to grow at 4 - 5°C, 15 - 17°C, 20°C, 25°C, 30°C, 37°C on nutrient agar slopes was determined. The cultures were examined after 2, 4 and 7 days.

(iii) The influence of pH of the medium on growth.

10 ml. quantities of nutrient broth buffered to pH 2.8, 4.4, 5.2, 6.0, 7.0, 7.6, 8.4 and 9.2 were inoculated and incubated. Growth, assessed in terms of turbidity, was examined visually.

(iv) The influence of salt concentration on growth.

10 ml. quantities of peptone water (peptone, 1%; distilled water) containing 0.0, 0.5, 1.0, 2.0, 5.0 and 10% (w/v) NaCl were inoculated and incubated and growth assessed at intervals in terms of turbidity.

[D] Biochemical characters.

(i) Action on carbohydrates.

The ability of the bacterial isolates to ferment a number of different carbohydrates was investigated.

The following carbohydrates were employed:-

Monosaccharides (hexose):- glucose, mannose.

Disaccharides:- sucrose, maltose, lactose.

Alcohols (trihydric):- glycerol.

" (hexahydric):- mannitol, sorbitol.

These substances were incorporated as 1% solutions in a peptone water basal medium (peptone, 1%; NaCl, 0.5%; tap water; pH 7) along with an indicator (bromcresol purple) and a Durham fermentation tube. With the exception of glucose (which was autoclaved at 10 p.s.i. for 10 mins. as a 20% aqueous solution and then added aseptically to tubes of sterilised peptone water-indicator solutions), all carbohydrate media were sterilised at 10 p.si. for 10 mins. with the carbohydrate *in situ* in the tubes.

In addition to employing peptone water as the basal medium for carbohydrate broths, a basal mineral salts (peptone -free) solution (S.A.B. Manual of Methods, II, 14, 1944) was also used. Lactose and salicin were incorporated (as 1% solutions) in this basal medium. Bromcresol purple was the indicator.

Carbohydrate cultures were examined daily over a 21 day period.

(ii) Nitrate reduction.-

5 ml. quantities of nitrate broth (KNO_3, 1% w/v in peptone water) were inoculated with the bacterial isolates and then incubated. 7 days after inoculation the cultures were tested for the presence of nitrite by the Griess-Ilosvay reagent. Production of any gas was noted.

(iii) Action on litmus milk.

10 ml. quantities of litmus milk ("Oxoid" litmus milk powder 10% w/v in distilled water; tubed and sterilised at 10 p.s.i. for 5 mins. on 2 successive days) were inoculated and incubated. Cultures were examined at intervals over a 21 day period for any changes in the appearance of the medium.

(iv) Gelatin hydrolysis.

10 ml. quantities of nutrient gelatin ("Oxoid" nutrient gelatin 13% w/v in distilled water) were stab inoculated with the bacterial isolates. All cultures were incubated at 22°C, and examined at intervals over a 21 day period.

(v) Indole production

10 ml. quantities of tryptone broth (1% w/v "Oxoid" tryptone in distilled water) were inoculated and incubated for 7 days. The cultures were then tested for indole.

(vi) Catalase production

A loopful of bacterial growth from a 24 hr. old nutrient agar slope culture was added to 1 drop of "10 vol" H_2O_2 solution. The formation of gas bubbles is a positive indication that the isolated bacterium has produced catalase.

(vii) Starch hydrolysis.

Plates of starch agar (0.2% w/v soluble starch added to nutrient agar before sterilisation) were streak-inoculated with the bacterial isolates. After 7 days incubation, the plates were tested for hydrolysis of starch by the usual iodine stain.

(viii) Voges-Proskauer (V.P.) and Methyl red (M.R.) tests.

5 ml. quantities of glucose-phosphate-peptone broth (peptone, 0.5% w/v; K_2HPO_4, 0.5% w/v; glucose

0.5% w/v; distilled water; pH 7.5) were inoculated (2 tubes/bacterium) and incubated.

After 5 days the V.P. and M.R. tests were performed.

The V.P. test was carried out according to O'Meara's method (1931).

The M.R. tests was performed by the addition of 5 drops of 0.04% methyl red solution to the cultures. The development of a magenta-red colour is regarded as a positive result.

(ix) Oxidase test.

The oxidase test was carried out according to Kovac's method (1956).

(x) Aerobic versus anaerobic utilisation of glucose.

The method of Hugh and Liefson (1953) was used.

(xi) Liquefaction of pectate gel.

Dowson's modification (1957) of Wieringa's method (1949) was used.

After inoculation the plate-cultures were incubated at 25°C, being examined for liquefaction at 3, 7, 14 and 21 days.

Bacillus polymyxa was used as a control.

V Fluorescent antibody techniques

(1) Preparation of antisera.

As no facilities for the preparation of antisera were available in this Department, these were kindly prepared by Dr. A. M. Paton of the University of Aberdeen and by Mr. K. Bettelheim of the Central Public Health Laboratories, Colindale, N.W.9.

Washed suspensions (4×10^8 cells/ml) of 24 hour old nutrient agar -, or, where appropriate, "N_2-free" synthetic agar -, slope cultures of the bacteria were made up in physiological saline and were then either -

(a) ultrasonically disintegrated and heat treated at 63°C for 30 mins. (the method used by Paton) or

(b) formolised for two days by the addition of formalin (0.3% v/v) to the suspension. (the method adopted by Bettelheim).

- prior to intravenous injection of rabbits.

After drawing off blood samples (for use in the preparation of non-immune sera), one of the following immunization courses was given:-

Course "a"

Time (days)	Amount of ultrasonically disintegrated Inoculum (ml)
1	4.0
8	4.0
15	4.0
21	BLEED

Course "b"

Time (days)	Amount of Formolised Inoculum (ml)
1	0.5
4	1.0
9	2.0
14	4.0
21	BLEED

Non-immune and immune antisera were prepared (from pre- and post-immunization blood samples respectively) by allowing the blood samples to clot, decanting off and serum and then centrifuging to remove the remaining red blood cells. Dreyer's method (1906) was employed for investigating the antibody activities of the antisera.

(2) Subsequent treatment of the antisera.

Each antiserum prepared by course "a" was adjusted to pH 7.1 by dialysis against 0.02M phosphate buffer. Such a solution, henceforth referred to as an "Unlabelled specific antiserum", was employed only in the Indirect ("Sandwich") Method of staining.

One antiserum was prepared by course"b" and this was used, after further treatment, in both the Direct and Indirect Methods of Staining. The crude γ-globulin fraction (which contains the antibody active proteins) was precipitated from the antiserum by overnight dialysis against a large volume of half-saturated ammonium sulphate solution (Cohn et al, 1940). By using this method sudden changes in the composition of the protein solution are avoided, with a consequent reduction in the co-precipitation of other protein fractions. After centrifugation, further purification of the γ-globulin precipitate was effected by washing in three changes of half-saturated ammonium sulphate solution. The precipitate was then dissolved in physiological saline and dialysed against several changes of physiological saline until all the ammonium sulphate had been removed.

At this point, the γ-globulin solution was divided into two equal portions, one of which was then adjusted to pH 7.1 by dialysis against 0.02M phosphate buffer; such a solution, henceforth designated as an "unlabelled specific antibody", was set aside for subsequent use in both the Direct and Indirect Methods of Staining.

The remaining portion was conjugated with fluorescein isothiocyanate* (F.I.T.C.) prior to use in the Direct Method of Staining. Conjugation was carried out as follows:-

The γ-globulin solution, adjusted to pH 9 by dialysis for one hour against 0.5M carbonate-bicarbonate buffer ($NaHCO_3$, 3.7g; Na_2CO_3 anhydrous, 0.6g; distilled H_2O, 100 ml; pH 9), was filtered to remove insoluble matter, and then the volume of solution was re-adjusted to the original serum volume by addition of carbonate-bicarbonate buffer.

An approximate estimation of the protein concentration of the solution was obtained by measuring the optical density at 280 mμ in a 1cm. cell and then dividing the reading obtained by the factor 1.24 (Goldstein et al., 1961; 1mg protein/ml ≡ O.D. of 1.24). Rinderknecht's conjugation method (1960) was chosen for antibody-labelling, a quantity of F.I.T.C. dispersed (4% w/w) on diatomaceous earth being added to the solution, which was then stirred for 30 mins. at room temperature. The quantity of dye required for optimum

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* Obtained from B.D.H., Ltd., Poole, Dorset.

fluorescent labelling (i.e. that quantity which gives the correct number of F.I.T.C. molecules/molecule antibody) will vary from one serum to another, depending on the antibody concentration of the serum.

When conjugation is complete, the reaction mixture contains several unwanted components (e.g. buffer salts, hydrolysed F.I.T.C. and other fluorescent substances which may have been present in the original dyestuff or formed during the course of the reaction) which, if not removed, could cause non-specific staining of microscopical preparations; these substances, referred to collectively as unreacted fluorescent material (U.F.M.), were removed by gel filtration through "Sephadex" (Porath and Flodin, 1959); the mixture was centrifuged and the supernatant allowed to soak into a column (2.8 x 18cm) of "Sephadex G 100" previously equilibrated to pH 7.5 with 0.02M phosphate buffer. The column was then developed with phosphate buffer and, as the solution descended, two bands separated, the faster of which was the fluorescein-conjugated antiserum. After collection of the conjugate, U.F.M. was removed from the column by elution with more buffer. The

conjugate was rendered free of "Sephadex" by filtration through an "Oxoid" membrane filter. Such a conjugated γ-globulin solution is henceforth referred to as a "labelled specific antibody".

An average value for the degree of conjugation was obtained by measuring the dye and protein concentrations of the labelled specific antibody - in terms of optical densities - in a spectrophotometer (see Fothergill, 1962). When carrying out the optical density measurements, in order to minimise the risk of fluorescence interfering with optical density, the sample under investigation was diluted with 0.02M phosphate buffer to $\frac{1}{30}$ of the original serum concentration. Dye concentration was determined from the optical density at the wavelength of maximum absorption (λ_{max}) of the dye component; for F.I.T.C. conjugates this is 495mμ. Protein concentration was determined after measuring the optical density at an absorption peak of 280mμ. From the readings obtained, the ratio of dye:protein in the purified labelled antibody was calculated, and for the bacteria under investigation a ratio of approximately 0.5:1 was found to be satisfactory.

(3) Staining methods

(i) Direct Method (cf. Coons and Kaplan, 1950)

Microscopical preparations were methanol-fixed, dried, and then covered with two drops of labelled specific antibody. The slide was placed on a moistened filter paper in a petri dish (to prevent drying) and staining allowed to proceed for 30 minutes at room temperature. The antibody solution was then poured off and the preparation washed in two or three changes (3 to 5 minutes each) of phosphate buffered saline (pH 7.1) prior to microscopical examination.

The "blocking test" (Mellors, 1959), which was employed as a control, was carried out in an almost identical manner to the above. It differed in that the control microscopical preparations were treated with unlabelled specific antibody prior to the application of labelled specific antibody.

(ii) Indirect ("Sandwich") Method

Paton's modification (personal communication; method awaiting publication) of Weller and Coons (1954) method was used.

Acetone fixed microscopical preparations were treated for 10 minutes with dimethylformamide solution and then washed for 10 minutes in phosphate buffered saline (pH 7.1). Unlabelled specific antiserum (or, in one case, an unlabelled specific antibody) was then applied, being allowed to act for 15 minutes, before the preparations were washed for 30 minutes in phosphate buffered saline (pH 7.1). Labelled anti-globulin serum ("Difco" freeze-dried goat anti-rabbit serum conjugated with fluorescein) was applied and allowed to act for 15 minutes. After washing for 15 minutes in further buffer, the preparations were counterstained for 5 minutes with Aluminium-chelated Eriochrome Black T.* Excess counterstain was removed from the preparations by rinsing in phosphate buffered saline for 10 minutes.

In the control microscopical preparations an unlabelled non-specific antiserum (i.e. an antiserum prepared against a different bacterial species from

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* Chelated with aluminium by the method of Hall and Hansen (1962).

the one under investigation and not conjugated with F.I.T.C.) was included as the middle layer of the "sandwich".

(4) Examination of microscopical preparations.

The microscopical preparations were mounted in glycerol-saline (90% v/v glycerol; 10% v/v saline) and examined by UV/Blue light with a Reichert Zetopan Fluorescence Microscope, using a glycerol immersion 100 x UV stopping lens and 8 x eyepiece.

VI Materials and methods employed in the nitrogen fixation studies.

(1) Culture media

Chemically defined culture media, prepared exclusively from "AnalaR" grade chemicals, were employed.

The following culture media were used:-

(a) "Nitrogen-free" solid synthetic medium

The composition and preparation of this medium have been described under section III (2) of "Materials and Methods".

(b) "Nitrogen-free" liquid synthetic medium

Contents and method of preparation as for (a), but omitting the ion agar.

(c) Casein-supplemented synthetic medium

Contents and method of preparation as for (a), but with the addition of 0.2% w/v vitamin-free casein hydrolysate prior to sterilisation. This medium was employed in certain preliminary nitrogen-fixation investigations.

Strict precautions were taken to ensure that all the glassware used in those investigations was nitrogen-free.

(2) Analytical methods

(a) The micro-Kjeldahl method

In preliminary experiments on nitrogen fixation, all nitrogen analyses were carried out by the micro-Kjeldahl method. The catalyst employed in the digestion was a copper sulphate-selenium dioxide mixture. 1% (w/v) boric acid was used as absorbent in the final distillation

and $^{N}/_{50}$ hydrochloric acid was employed as titrant. Because it gives a very distinct end-point, the Bromocresol Green-Methyl Red mixed indicator method of Ma and Zuazaga (1942) was adopted.

(b) The use of isotopic nitrogen.

1 Preparation of atmospheres

Isotopically labelled atomospheres of the following composition were prepared and stored in bulbs of 1 litre capacity:-

	Atmosphere I.	Atmosphere II.
Nitrogen (containing 31.3 atom % of excess N^{15})	79.6% (v/v)	58% (v/v)
Oxygen	20.4% "	20% "
Argon	0.0% "	22% "

The apparatus used (see Fig. I) was of pyrex glass construction throughout, all movable connections, such as those between the gas generation unit and the vacuum line, being made by the use of greased standard ground-glass joints.

For the sake of simplicity, the preparation of Atmosphere I will be described; the preparation

FIGURE I — DIAGRAMMATIC REPRESENTATION OF THE APPARATUS USED IN THE PREPARATION OF LABELLED ATMOSPHERES

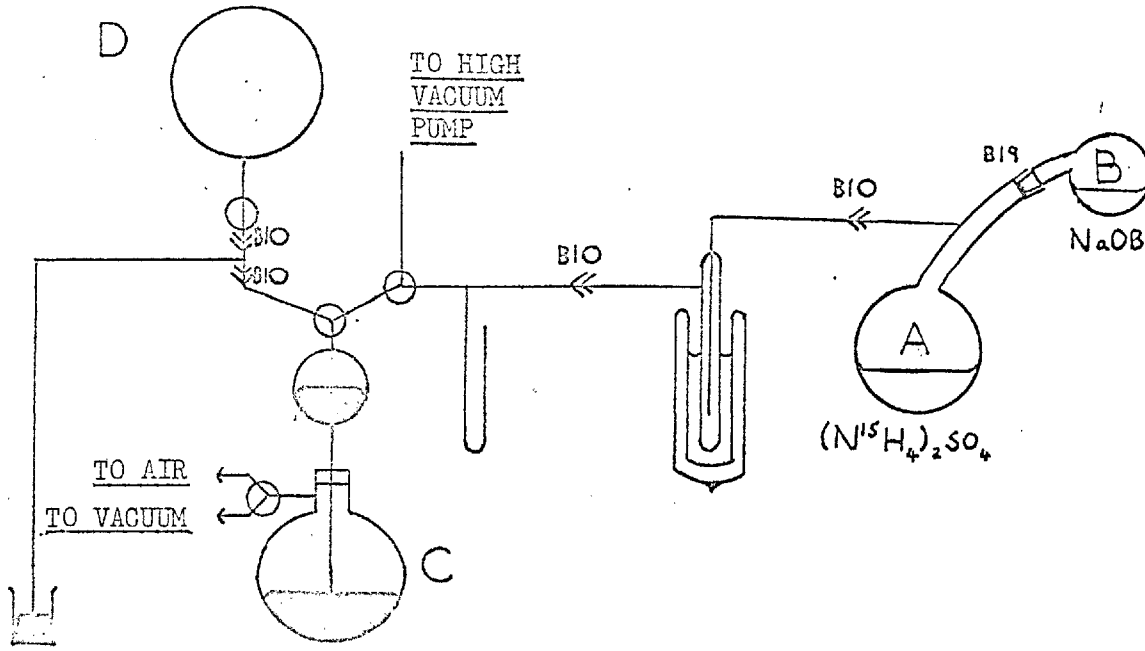
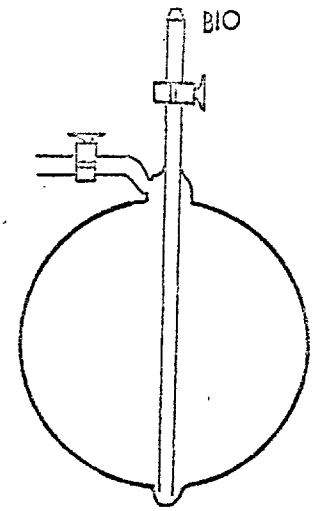


FIGURE II — DIAGRAM OF FLASK USED FOR EXPOSING BACTERIAL SUSPENSIONS TO LABELLED ATMOSPHERES



of Atmosphere II follows on a completely analogous basis.

Pure nitrogen gas was prepared by the action of sodium hypobromite on N^{15}-enriched ammonium sulphate. The resulting oxidation takes place quantitatively according to the equation:-

$$2NH_3 + 3NaOBr = N_2 + 3NaBr + 3H_2O$$

The appropriate quantity of labelled ammonium sulphate (1.15g.) was weighed out, dissolved in water, and transferred quantitatively to flask 'A' of the nitrogen generation unit. Into flask 'B'was poured a calculated volume (25 ml) of freshly prepared alkaline hypobromite (80 ml air-free distilled water containing 20g. sodium hydroxide, cooled, and shaken with 8 ml. liquid bromine; 1 ml. of this solution is sufficient to oxidise 25 mg. of ammonia to nitrogen.). Both component flasks were cooled in solid carbon dioxide/methanol mixture until their contents were frozen solid. The gas generation unit was then assembled and fitted to the vacuum line. The cold trap was surrounded with liquid nitrogen and flasks and contents completely evacuated. After approximately 15 minutes the contents

of both flasks had melted (and by now were adjudged to have degassed sufficiently), when they were shut off from the main vacuum line. The hypobromite solution was then gradually added to the ammonium solution by rotating 'B' around the ground-glass joint.

At this stage the liquid nitrogen surrounding the trap was replaced by liquid oxygen, which served to condense impurities such as nitrous oxide, ammonia, bromine and water, without condensing any of the nitrogen being generated. The nitrogen produced was completely transferred by means of a Toepler pump C to a previously evacuated storage bulb D, the pressure being read off on the attached manometer.

Pressure of nitrogen (cm. Hg.) = Initial reading - final reading.

Assuming Charles Law to operate over the volume of the storage bulb and the associated dead space, it is now a relatively simple procedure to make up the gas mixture according to the desired proportions. If the tap at the top of the Toepler pump is used as a reference, gases may be added through it in amounts according

to the ratio of the pressures required (this ratio being the same as that of the ratio of volumes of gas at N.T.P.) provided a constant total volume is maintained (as "defined" by the Toepler pump tap).

Thus, in the case of Atmosphere I, the following manometer readings were obtained:-

Pressure of Nitrogen = 76.2 - 20.3 = 55.9 cm. Hg.

" " Oxygen = 20.3 - 6.0 = 14.3 cm. Hg.

From comparison of these relative pressures at constant volume, it follows that the composition of the mixture is:-

Nitrogen	79.6% (v/v)
Oxygen	20.4% "

Oxygen and Argon gases used in the preparation of Atmospheres were obtained directly from commercially supplied cylinders.

The main difficulty in this method was in making sure that a gas, once in the storage bulb, did not diffuse back into the Toepler pump and thus be discarded along with any excess gas. This was avoided by

ensuring that all the gas in the Toepler pump was added to the storage bulb. Care was also taken to ensure that the amount of gas in the Toepler pump was not more than that required to increase the total pressure above the desired figure. This was avoided, as far as possible, by always adding less gas than the expected amount and by making the additions progressively smaller as the final pressure figure was approached on the manometer.

2 Method of exposing bacterial suspensions to isotopically labelled atmospheres.

The type of flask used for exposing bacterial suspensions to isotopically labelled atmospheres was similar to that reported by Stevenson (1958). It was of 300 c.c. capacity and is illustrated in Figure II.

Labelled atmospheres were introduced into the exposure flask by using the apparatus as illustrated in Figure III. The system consisted of the storage bulb A (containing a labelled atmosphere), which was connected to the exposure flasks B by an adaptor and to a Toepler pump C by a three-way tap. The Toepler pump was further connected by a three-way tap to the main

FIGURE VII — DIAGRAM OF THE APPARATUS USED FOR INTRODUCING LABELLED ATMOSPHERES INTO THE EXPOSURE FLASKS

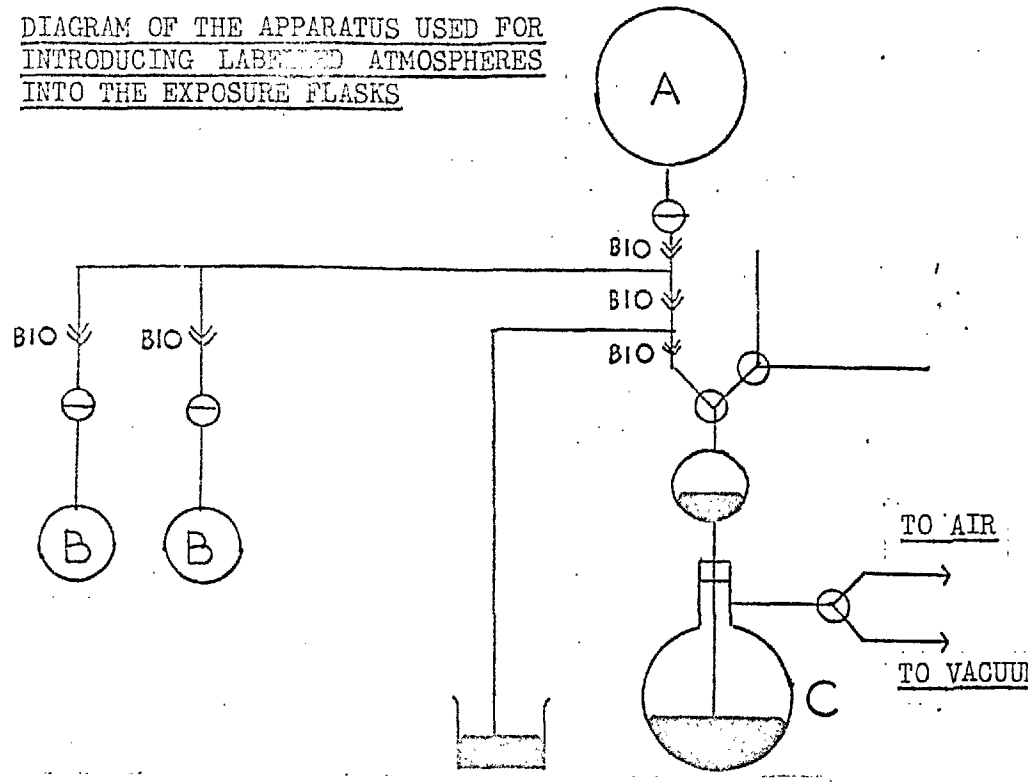
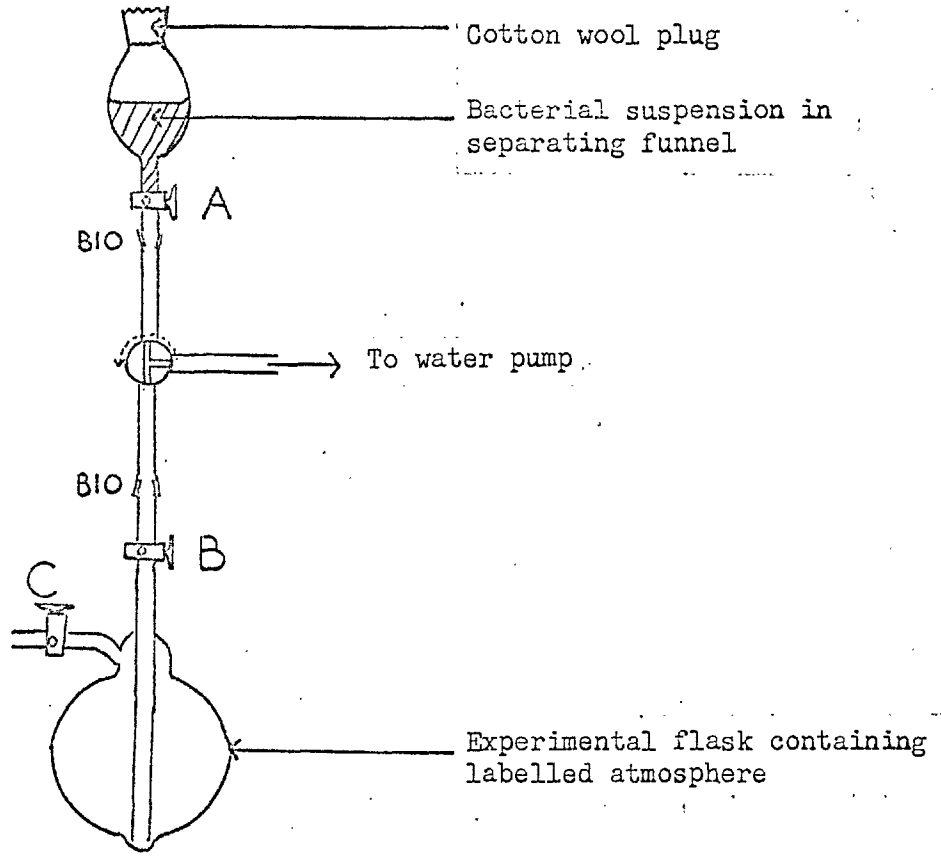


FIGURE IV — METHOD OF INTRODUCING BACTERIAL SUSPENSION INTO FLASKS CONTAINING LABELLED ATMOSPHERES



vacuum line and to air. Gas pressures were measured by a mercury manometer.

After the exposure flask had been completely evacuated, the labelled atmosphere from the storage bulb was transferred to each flask in turn via the Toepler pump.

Figure IV shows the type of apparatus which was devised and employed for introducing a bacterial suspension into an exposure flask containing a labelled atmosphere. By using such a system the bacterial suspension can be delivered into the exposure flask under aseptic conditions, whilst, at the same time, leakage of labelled atmosphere from the flask is prevented. The separating funnel and T- piece, first plugged and sterilised (10p.s.i. x 10 mins.) were connected up aseptically to the exposure flask, taps A, B and C all being in the "closed" position. The bacterial suspension was then poured into the separating funnel under aseptic conditions. With the T- piece three-way tap in the position as indicated (Fig. IV), the region between taps A and B and the water pump was evacuated. The T- piece three-way tap was then turned through 180°, thereby isolating the system from the

water pump. On opening tap A, the bacterial suspension flowed down and filled the tube as far as tap B. The latter was then opened, thus allowing the suspension to flow into the exposure flask. Suspension flow ceased when approximately 100 c.c. had entered the flask, due to a pressure-equilibrium being set up between the exposure flask and the head of suspension. Enough suspension (150 c.c.) was poured into the separating funnel beforehand to ensure that sufficient remained in the funnel and in the tube at equilibrium condition. This safeguarded against leakage of labelled atmosphere into the surrounding atmosphere and vice versa. Due to the head of liquid, the gas pressure in the exposure flask was slightly in excess of atmospheric pressure ($\simeq$ 1% higher). Tap B was reclosed, the exposure flask removed and the culture mechanically shaken at 25°C for a fixed amount of time.

3 Method of detecting nitrogen enrichment in samples.

A small quantity of the labelled atmosphere was drawn off from the experimental flask by using the same method and apparatus as was previously employed for introducing the atmosphere into the experimental flask.

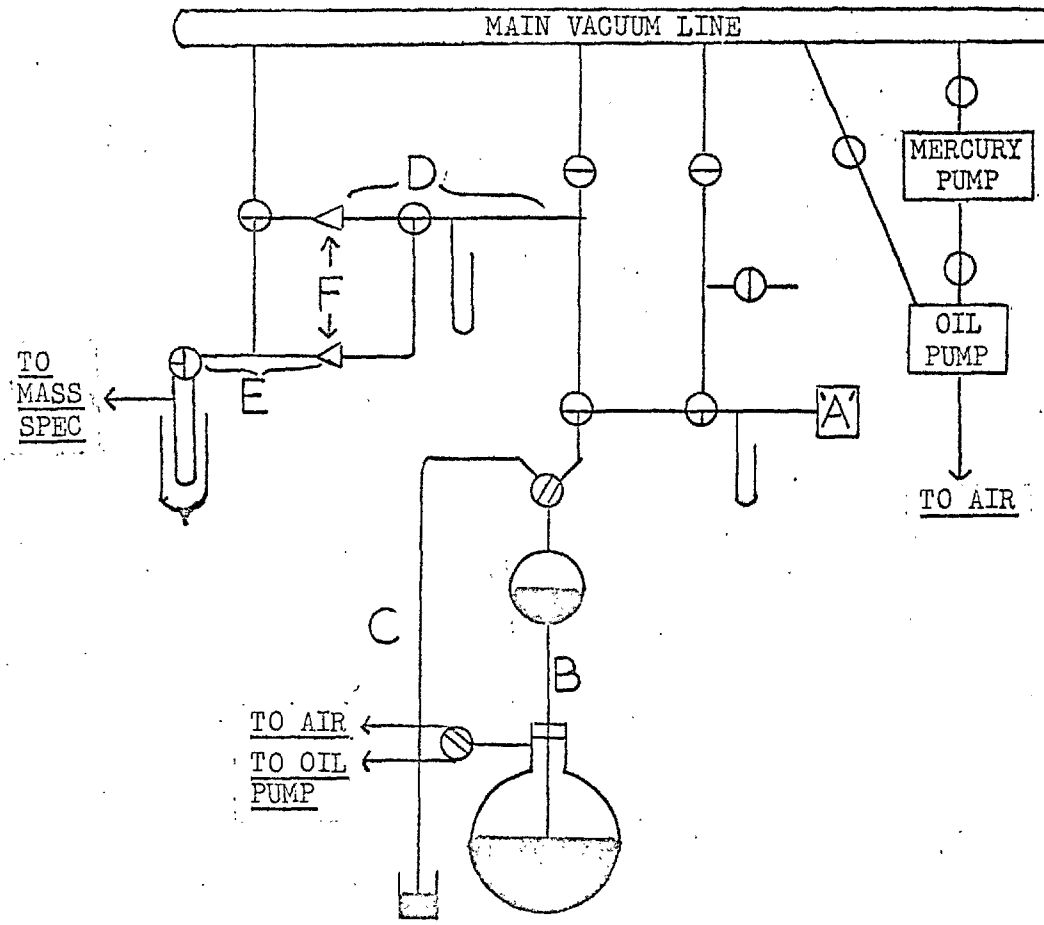
The gas sample, collected in a Thunberg tube attached to the apparatus (see Fig. III) at the location previously occupied by the atmosphere storage flask (A), was then analysed in the mass spectrometer.

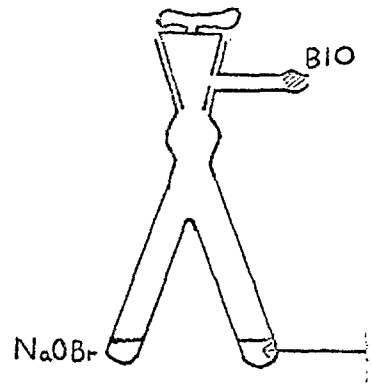
When carrying out a micro-Kjeldahl total nitrogen analysis of a bacterial suspension which had been exposed to an isotopically-labelled atmosphere, a slightly modified procedure from the one reported earlier (see Section VII (2) (a)) was adopted. In Kjeldahl digestions it is normally sufficient to heat the acid-digest for only two hours after the contents become colourless. Incomplete digestion, however, may lead to the formation of methylamine (CH_3NH_2), which can be ionised in the mass spectrometer to CH_3N^+, of mass number 29. This would interfere with the determination of ^{15}N abundance, since the 28 and 29 peaks are measured for this purpose. To safeguard against this, the samples were digested for eight hours after the contents had become colourless. Ammonia, liberated from the samples by steam distillation under alkaline conditions, was collected in 5 ml. of nitrogen-free water. Two drops of 0.2% Bromocresol Green indicator (chosen since it does not contain nitrogen) were added

to the latter, which was then back-titrated against N/50 hydrochloric acid. Upon reaching the end-point, 2 ml. excess acid were added to the titrated sample, which was then concentrated down to 3 ml.

Nitrogen was regenerated from this solution by the action of sodium hypobromite; the apparatus used for this purpose, and also for admitting gas samples into the mass spectrometer, is illustrated in Figure V. A Rittenburg tube, containing sodium hypobromite (3 ml.) in one arm and the concentrated sample (3 ml.) in the other, was connected (by a greased ground-glass joint) to the mass spectrometer line at inlet 'A'. When the two solutions had been outgassed under low vacuum (10^{-2} mm. Hg produced by the oil pump), the Rittenburg tube tap was closed and the solutions were then frozen by immersion in a slurry of solid carbon dioxide in methanol. The mercury diffusion pump (backed by the oil pump) was then used to produce a hard vacuum (10^{-6}mm. Hg) in the system. On re-opening the Rittenburg tube tap, the two frozen solutions were thus subjected to a high vacuum, which completed the outgassing process. After reclosing the Rittenburg tube tap, the frozen solutions were thawed out and then mixed by rotating the tube

FIGURE V — VACUUM LINE AND APPARATUS USED FOR ADMISSION OF ISOTOPIC SAMPLES INTO MASS SPECTROMETER





RITTENBURG TUBE ATTACHED TO APPARATUS AT POSITION - 'A'

around the ground-glass joint at 'A'. The nitrogen thus liberated was transferred by the Toepler pump B into the portion D of the vacuum line behind the leaks (F). When the pressure in this section was at a suitable level (1 to 3 cm. of mercury), that portion E between the leak and the mass spectrometer tube was isolated. The leak was then opened and the gas sample was released slowly into E and thence into the mass spectrometer tube. Once a steady flow of gas was visible through the leak - as observed by a Tesla discharge - the peaks corresponding to ions of mass number 28 and 29 were tracked and their heights measured. After corrections for zero and peak residuals the percentage of ^{15}N in the sample was readily determined by application of the equation:

$$\%^{15}N = \frac{100}{\left(2x\frac{\text{Peak Ht 28}}{\text{Peak Ht 29}} \times S\right) + 1}$$

S is the shunt ratio required to bring the peak heights to the same order of magnitude. Percentage enrichment was obtained by deducting natural abundance of $^{15}N(0.38\%)$ from each value.

3. EXPERIMENTAL AND RESULTS.

PART 1.

THE DISTRIBUTION OF BACTERIA IN LEAF NODULATED PLANTS

Description of leaf nodules

The leaf nodules of *A. crispa* (Fig. VI) are arranged in a regular fashion along the crenated margin of the leaf. They are present on every leaf (including those of the terminal bud) and are most readily observed on the ventral surface of the leaf edge, where their dark green colour contrasts well against the paler green undersurface of the leaf. In length they range from 0.5 to 3mm., in breadth from 0.5 to 1.5 mm. A large number of pink-pigmented areas are also noticeable scattered over the leaf surfaces; these are particularly numerous in young leaves, a high proportion being found both in the region of the nodules and in the petiolar region.

On leaves of *Psy. emetica* (Fig. VII), *Psy. nairobiensis* (Fig. VIII) and *P. grandiflora* (Fig. IX)

FIG.VI *Ardisia crispa*. Ventral surface of leaf showing the distribution of nodules.

FIG.VII *Psychotria emetica*. Ventral surface of leaf showing the distribution of nodules.

FIG.VIII Psychotria nairobiensis. Ventral surface of leaf showing the distribution of nodules.

FIG. IX Pavetta grandiflora. Ventral surface of leaf showing the distribution of nodules.

the nodules are very numerous and on all leaves are scattered irregularly over the surface. Many nodules are found to occur on small veins, but an approximately equal number show no visible attachment to the vascular system. A remarkable feature of nodule distribution on leaves of these species is that the number of nodules on one side of the leaf midrib closely approximates (± 1%) to the number on the other side. Each nodule extends from the dorsal to the ventral surface of the leaf and, in dimension, ranges from 0.5 to 5 mm. in length by 0.5 to 2 mm. in breadth. As with A. crispa, each nodule of these species is more readily observed on the ventral than on the dorsal surface of the leaf.

Serial sections of the following plant tissues, prepared and stained using the methods described under section II of "Materials and Methods", were examined microscopically both under low (x 30 objective) and high (x 95 oil immersion) power:-

MYRSINACEAE

Ardisia crispa

(a) Leaf nodules of various ages.

(b) Non-nodulated regions of nodulated leaves of various ages.

(c) Terminal Buds.

(d) Inflorescences.

(e) Fruits.

(f) Mature Seeds.

(g) Germinating Seeds.

RUBIACEAE

Psychotria emetica

As for A. crispa, with the exceptions that, due to lack of plant material, inflorescences, mature seeds and germinating seeds were not studied.

Psychotria nairobiensis

As for A. crispa.

Pavetta grandiflora.

As for A. crispa, with the exceptions that, due to lack of plant material, mature seeds and germinating seeds were not investigated.

For the purposes of convenience and simplicity each plant has been considered separately, and the results obtained were as follows:-

MYRSINACEAE.

Ardisia crispa

(a) Leaf nodules of various ages

Old leaf nodules (i.e. those found on well-established leaves), when viewed under low power (L.P.) were found to be set in the spongy mesophyll. A layer of long, brick-shaped cells of about three to four cells depth was found to be surrounding the nodule. The inner layers of this "sheath" contained a number of pigmented cells, and similar cells were found to occur in abundance in the palisade and spongy mesophyll. Cells comprising the sheath appeared to be stretched to surround the nodule. Each nodule was supplied with a vascular bundle which terminated in the "sheath".

The nodular tissue itself consisted of loosely packed "mucilaginous packets" (Fig. X). Remnants of broken cell walls could be seen amongst these "packets"

and, from this, it was concluded that the "packets" represented the products of broken down cells. No plant cellular structure was evident within the nodule and the "packets" were not arranged in any definite pattern.

Under high power (H.P.) the mucilaginous packets were seen to contain very large numbers of highly-refractile bacterial cells. These were difficult to stain effectively, for they took up the stain much more strongly in some regions of the cell than in others. Whilst areas within each cell remained unstained, the poles of the cell were always stained. Carbol thionin-Orange G was found to be the most suitable stain for demonstrating the presence of bacteria within the nodules. Nodular bacteria were of most bizarre shapes, all of which were variations of rods. They bore a strong morphological resemblance to the "bacteroids" of Leguminosae root nodules and in the leaf tissues were confined to the mucilaginous packets of the nodule (Fig. XI).

"Middle-aged" leaf nodules (i.e. those present on leaves arising at the 3rd or 4th node below the

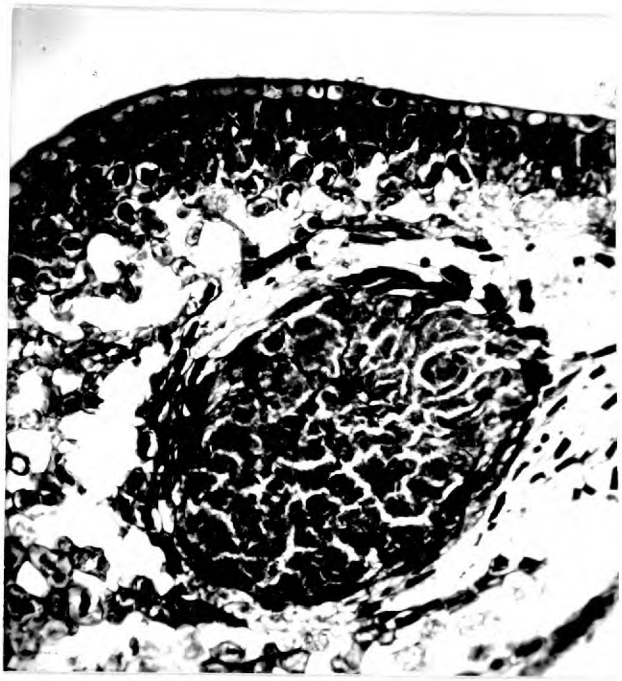


FIG. X Ardisia crispa. T.S. old leaf nodule
[Carbol thionin - Orange G. x 120]

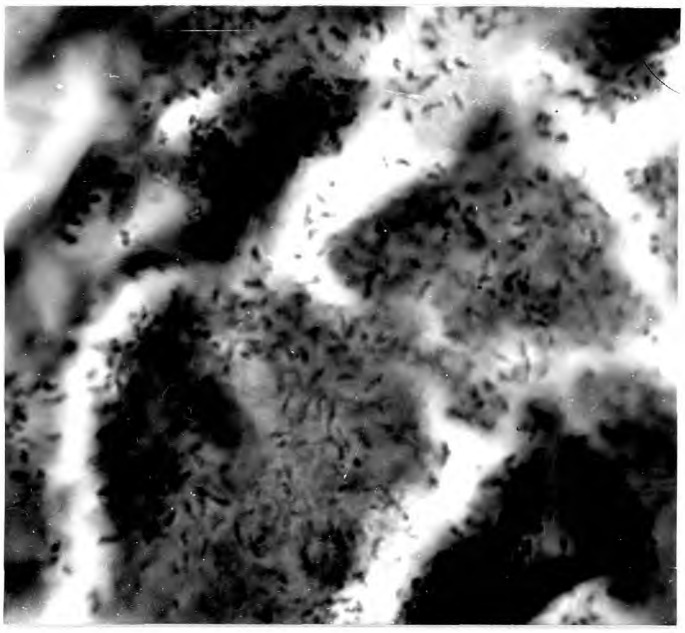


FIG. XI Ardisia crispa. Bacteroids present in the mucilaginous packets of an old leaf nodule.
[Carbol thionin - Orange G. x 1520]

terminal bud) were seen, under L.P., to be surrounded by a sheath (three to four cells deep) of brick-shaped cells, some of which were heavily pigmented. Once again (cf. old nodule) a vascular bundle was always found to be attached to the nodule via the sheath.

Unlike old nodules, middle-aged nodules contained a large number of loosely packed plant cells. These differed markedly from the surrounding mesophyll cells, being a good deal smaller than the latter. Nodular plant cells were relatively thick-walled (i.e. as compared with mesophyll cells) and were very tightly packed at the nodule periphery. They increased in size towards the centre of the nodule, where they were more loosely packed and interspersed with mucilaginous packets. The nodular cells were quite irregular in shape and arrangement (Fig. XII).

Of the stains employed, Carbol thionin-Orange G and Safranin-Fast Green - Orange G were found to be the most suitable for demonstrating the presence of bacteria within middle-aged nodules. H.P. examination of nodular tissues revealed the presence of very many

"bacteroid" - type cells. The latter were embedded in a mucilaginous matrix which penetrated between the plant cells present in the nodules; they were irregular shaped rods, which occurred singly and were difficult to keep in focus (Fig. XIII). Some of these bacterial cells also appeared to be present inside the plant cells of the nodule. There seemed to be a few, more typical, rod-shaped bacteria between the mesophyll cells outside the nodule. Unlike the "bacteroids" characteristic of the leaf nodules, these bacteria were slightly longer and thinner (1 - 3.5μ x 0.5μ as opposed to 1.5 - 2μ x 1.2 - 1.5μ for "bacteroids") and stained evenly. Within the tissues similar staining "bodies" were seen to be surrounding the numerous chloroplasts of the palisade mesophyll cells; but whether or not these "bodies" were bacteria could not be ascertained.

Young leaf nodules (i.e. those present on leaves arising immediately below the terminal bud) were almost entirely enclosed in the spongy mesophyll, and comprised much smaller, thicker-walled, and more closely packed cells than the surrounding mesophyll tissues (Fig. XIV). The outer layers of the nodule consisted of small plant cells, the cell size increasing towards the centre of

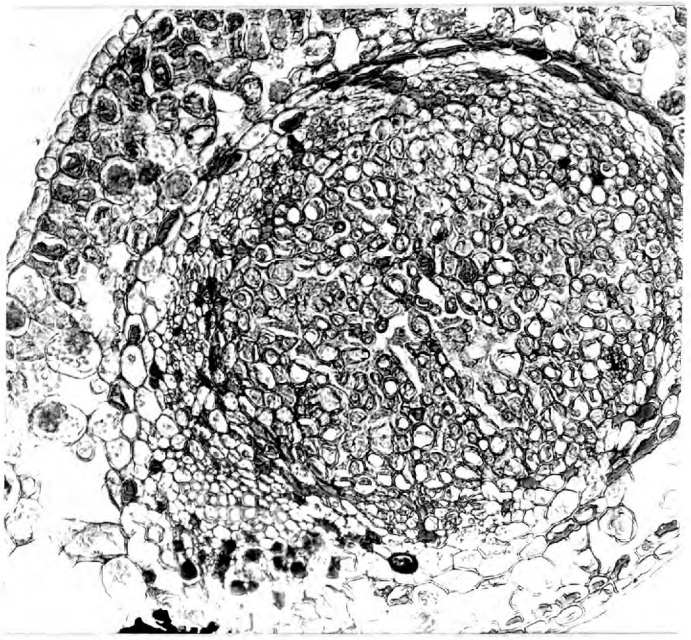


FIG. XII Ardisia crispa. Middle-aged leaf nodule.
[Unstained x 160]

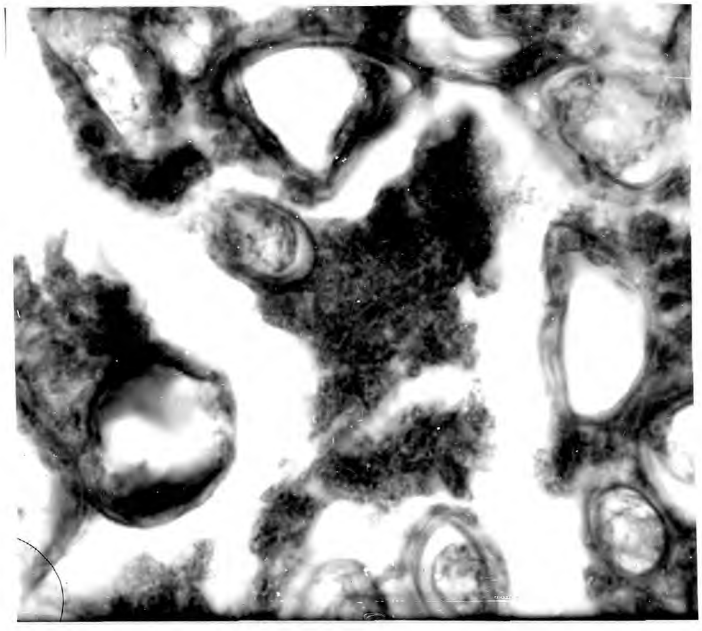


FIG. XIII Ardisia crispa. Bacteria present in the mucilaginous masses between the plant cells of a middle-aged leaf nodule.
[Safranin - Fast Green - Orange G. x 1520]

the nodule. Pigmented plant cells were numerous around the nodule, but only few appeared to be inside the nodule.

A definite layer of cells was in the process of formation at the periphery of the nodule, and a vascular bundle served the nodule via this developing sheath tissue.

Staining with Carbol thionin - Orange G, failed to reveal any bacteria in the nodules, but there were indications that bacteria were washing out of the sections onto the slide, for large numbers were observed in the vicinity of the sections under examination. The combination Basic fuchsin - Gentian Violet - Iodine was found to be very suitable for staining these bacteria, and the following observations were made when the sections were examined under H.P. after staining by this method:-

In the vicinity of every section examined a large number of rod-shaped bacterial cells had been washed out of the sections onto the slides. Such bacteria were not confined solely to the area on the

slide immediately surrounding a nodule, but appeared to have been washed out from the entire section (i.e. leaf mesophyll tissues included); they.fell off in concentration with increasing distance from the section. Two distinct types of bacteria, both of which were rod-shaped, were present in this bacterial film (Fig. XV). Type 1 consisted of large (1.5 - 2μ x 1.0 - 1.5μ), slightly curved, swollen, irregularly shaped cells; they occurred mainly in pairs, but a few single forms were also present. These rods stained unevenly, had a striated appearance, and were seemingly surrounded by a mucilaginous film; they corresponded, both in dimensions and in staining characteristics, to the bacteria as seen in old and middle-aged nodules. The cells of type 2 were more variable in length and much thinner than those of type 1 (1 - 3μ x 0.5μ). Whilst they were not as intensely stained as the cells of type 1, they likewise stained irregularly, being more strongly stained at the poles than elsewhere in the cell. They were mainly arranged singly, but a certain number occurred as pairs of cells joined end to end. Cells of type 2 were slightly curved.

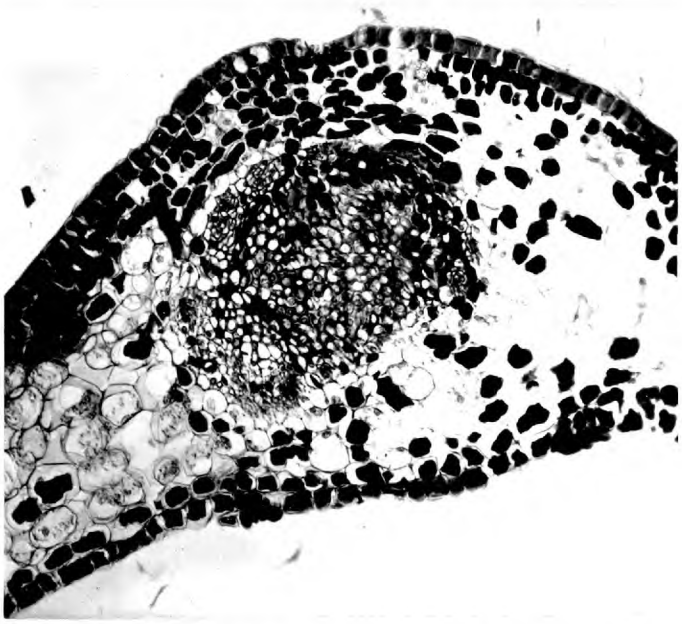


FIG. XIV Ardisia crispa. Young leaf nodule.
[Carbol thionin - Orange G. x 160]

FIG. XV Ardisia crispa. Showing the two types of bacteria which were washed out of sections of young leaf tissues (containing a nodule) onto the surrounding areas of the slide.
[Basic fuchsin - Gentian Violet - Iodine x 1520]

Under H.P., the plant cells of the nodule appeared to contain large numbers of the "bacteroid" - type cells (type 1), which were also present in abundance in the small spaces between such cells. It was not easy to ascertain whether rods of type 2 were present in the nodules, since they were much more refractile and thus, if present, would tend to be masked by the cytoplasm of the nodular cells, which, when stained, was a similar colour. Both types of bacterial cell appeared to be present in profilic numbers both inside and outside nearly all the mesophyll cells of the leaf (Fig. XVI). Since many bacteria of both types had washed out of the sections during manipulation, it is not possible to affirm definitely whether those seen within the plant cells are, in fact, truly intracellular in nature.

Very young leaf nodules (i.e. those present in the outer leaves of the terminal bud) were made up of numerous, minute, thin-walled, closely packed cells. The latter were set in the spongy mesophyll. No "sheath" layer had as yet been formed. A few pigmented plant cells, rather haphazardously arranged, occurred both within and without the developing nodule.

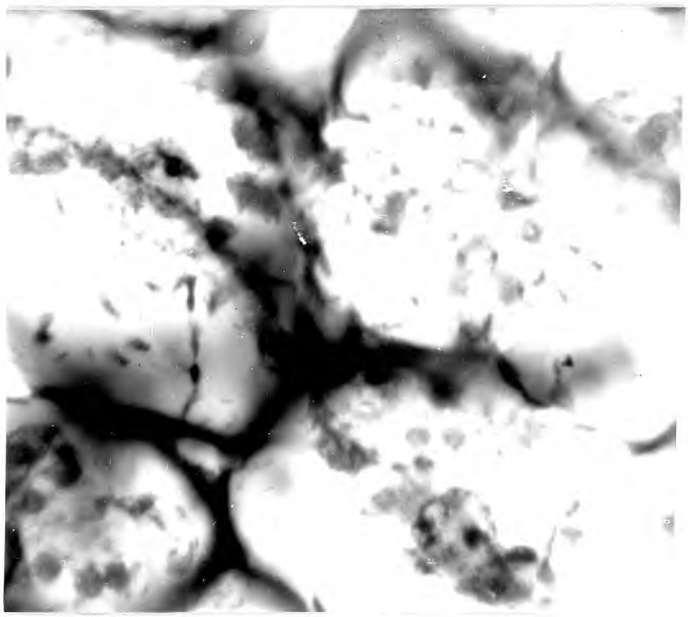


FIG. XVI Ardisia crispa. Showing the two types of bacteria present inside cells of the mesophyll tissues.
[Basic fuchsin - Gentian Violet - Iodine x 1520]

Preparations stained by the Carbol thionin - Orange G method, when viewed under H.P., were found to harbour highly-refractile bacterial rod-shaped cells; these were mostly concentrated between the spongy mesophyll cells in the region of the developing nodule. The whole tissue was seemingly permeated by these bacteria, which resembled the type 2 - rods seen in young nodule preparations.

(b) Non-nodulated regions of nodulated leaves.

Microscopical examination of leaf tissue sections which contained young leaf nodules had revealed the presence of many bacterial cells within the non-nodulated regions of the mesophyll tissue. But, as many similar bacteria had been found in the area on the slide immediately surrounding such preparations, it was not possible to reach a definite conclusion as to whether these bacteria did, in fact, occur naturally in the mesophyll tissues or had merely been washed out from the nodule into such tissues during the handling of the pre - paration. Accordingly, leaves in various stages of development were taken and from each a segment was cut which did not contain any nodules. Each segment, sectioned and stained by the same methods as had been adopted

for nodule-containing tissues, was examined microscopically, when bacteria were observed only in the mesophyll tissues of young leaves; these bacteria were of type 2, were very few in number and, once again, some had been washed out of the sections onto the areas of the slide surrounding the sections.

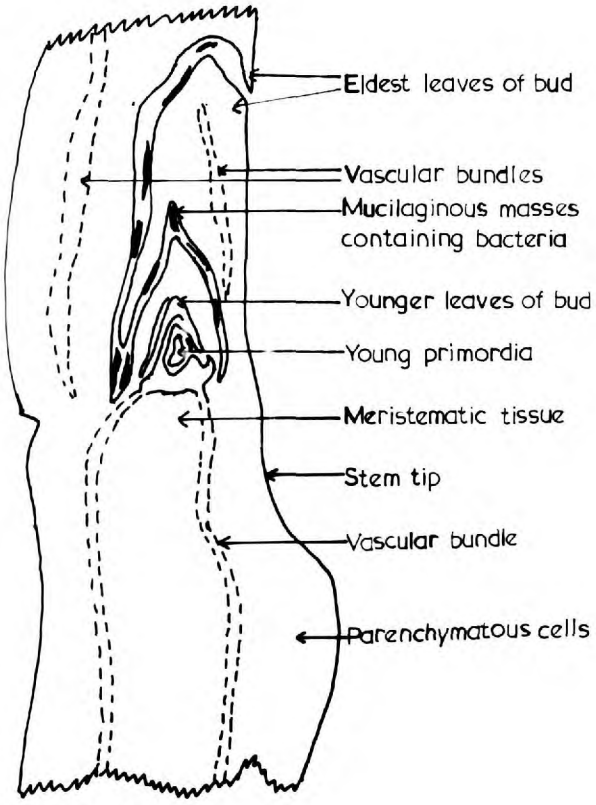
(c) Terminal buds.

In examining the terminal buds, use was made of two staining methods, each having its respective advantages. The general picture, as seen when either of these methods was employed, was as shown in Figs. XVII and XVIII.

Longitudinal sections of buds, stained in 1% Carbol fuchsin, were found to contain a mottled, pink-staining mucilaginous film, which enveloped all the primordial leaves on the stem apex. Fragments of this film were also found on the surfaces of the more mature leaves of the bud.

Under H.P. the mucilaginous film was seen to be well supplied with closely-bunched bacteria and rarely

ARDISIA CRISPA terminal bud



Bacterial film in black

FIG. XVII
Ardisia crispa.
L.S. terminal bud
(diagrammatic representation)

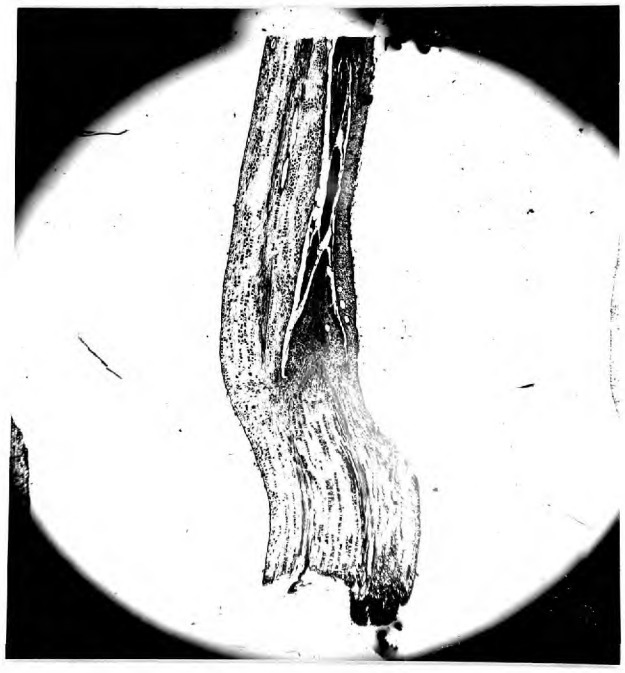


FIG. XVIII
Ardisia crispa.
L.S. terminal bud
[1% Carbol fuchsin
x 30]

were bacteria observed to occur outside this film. Only one type of bacterial rod was found and this appeared to be an intermediate form of types 1 and 2 (Fig XIX). Thus, like the cells of types 1 and 2, the cells of this form stained in an irregular fashion and were slightly curved. They possessed dimensions of 1 - 2.5μ x 0.5 - 1μ. The cytoplasmic content of the cells appeared to be granular and was mostly concentrated at the poles. Staining with Carbol fuchsin did not reveal the presence of bacteria within the plant cells of the apical meristem.

When longitudinal sections of the terminal buds were stained by the Carbol thionin - Orange G method, a picture similar to that shown in Fig. XX was obtained. Epidermal cells were stained light blue, cell walls and the mucilaginous film were stained yellow and nuclei were stained green (save in the innermost primordial areas where they were stained red). Certain areas in the primordial leaf mesophyll tissue, namely those destined to occur near the periphery of the fully developed leaf, were stained purple-red; two such areas (marked by arrows) are shown in Fig. XX.

FIG. XIX Ardisia crispa. Bacteria present in the mucilage of the terminal bud.
[1% Carbol fuchsin x 1520]

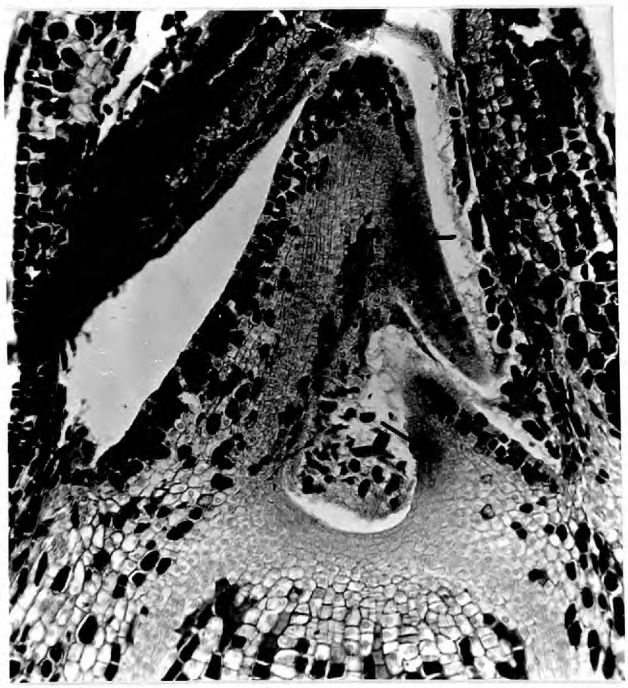


FIG. XX Ardisia crispa. Apical meristem of the terminal bud.
[Carbol thionin - Orange G. x 120]

Under H.P. the mucilaginous film was found to contain very many red-stained, refractile bacterial rods, which were similar to those seen in the Carbol fuchsin-stained preparations. Due to their uneven staining, the rods had a "beaded" appearance (Fig. XXI).

Bacterial cells, similar to those present in the mucilage, were found to occur in large numbers in the purple-red staining mesophyll regions (Fig. XXII). Many of these appeared to be inside the mesophyll cells, where they stained fairly evenly and were single (no chains observed). Examination of the meristematic cells within the stem apex failed to reveal the presence of any bacteria. The outer leaves of the terminal bud contained developing nodules. The latter consisted of numerous, closely packed cells which were set in the spongy mesophyll; the nodular cells were much smaller than those of the mesophyll, and bacteria, similar to those noted in primordial leaves, were found to occur between the nodular cells. Large numbers of bacteria were also found between the mesophyll cells in the region of the developing nodule.

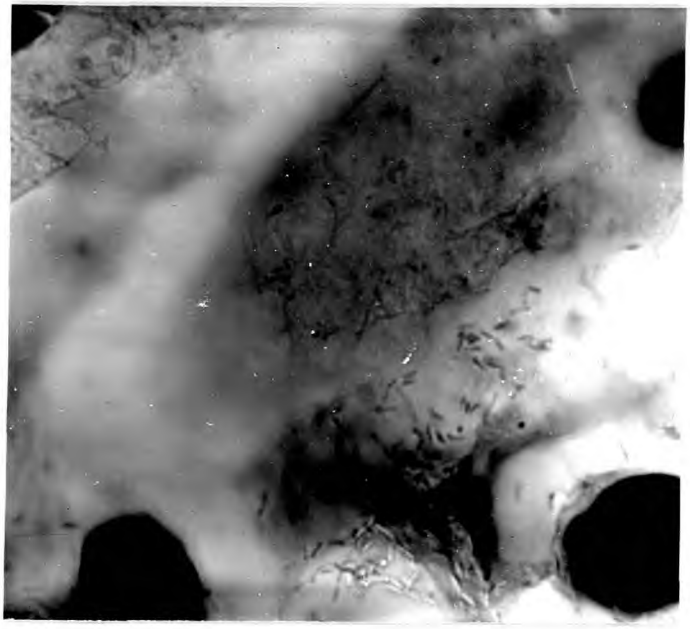


FIG. XXI Ardisia crispa. Showing the bacteria present in the mucilaginous film of the terminal bud
[Carbol thionin - Orange G. x 1520]

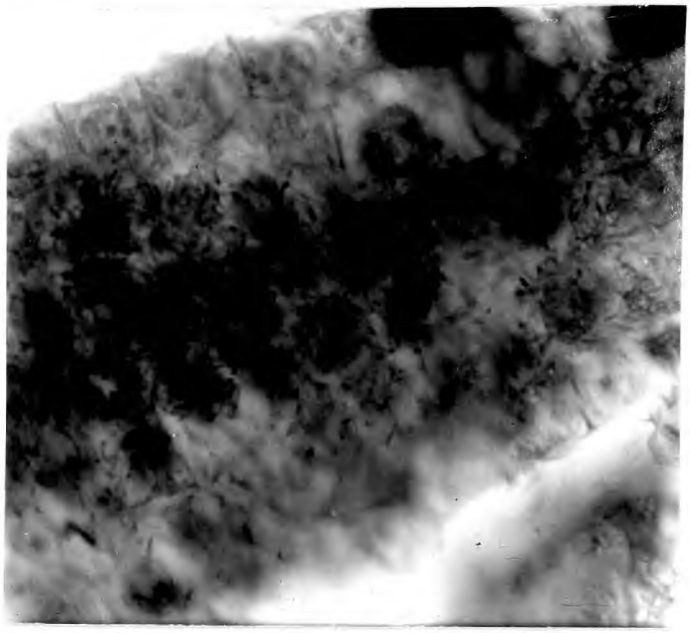
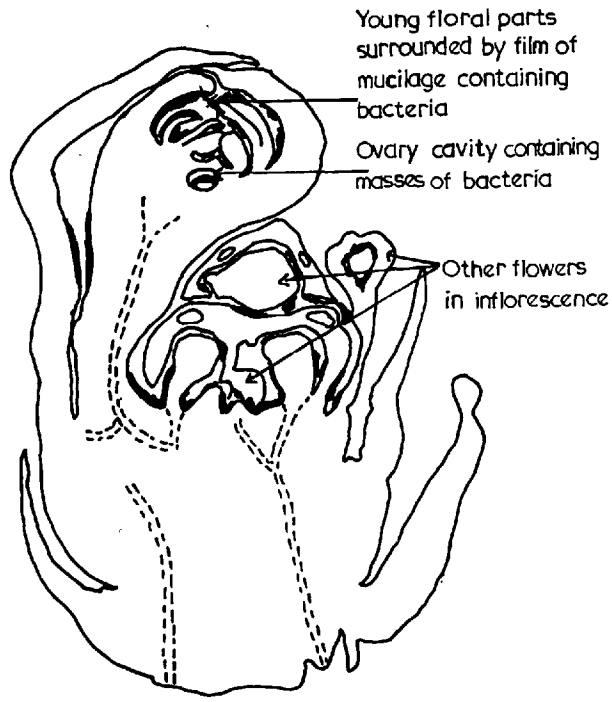


FIG. XXII Ardisia crispa. Showing the bacteria present in the mesophyll tissues of a young, terminal bud, leaf.
[Carbol thionin - Orange G. x 1520]

(d) Microtome sections of young floral buds (i.e. those buds which were still in the primordial stages) were prepared and stained in 1% Carbol fuchsin. Figures XXIII and XXIV illustrate the general picture observed when sections (L.S. and T.S. respectively) of such inflorescences were examined under L.P.. A pink-staining mucilaginous film permeated the spaces between each separate flower in the inflorescence. All the developing floral parts of each individual flower were enveloped by this film (Figs. XXV and XXVI), which also lined the cavities formed between the bract leaves and sepals.

Examination of the mucilaginous film under H.P. showed the constant presence of large numbers of rod-shaped bacteria. Apart from containing a low percentage (approximately 1%) of "bacteroid"-type rods (which in dimension and staining characters resembled type 1 bacteria of leaf nodules), the majority of cells making up the mucilaginous film's bacterial population were similar in all respects to the type 2 leaf nodular bacterium. These were the most "typical" rods observed and were particularly concentrated around and between the developing anthers and carpels (Fig. XXVII). They were also observed inside the

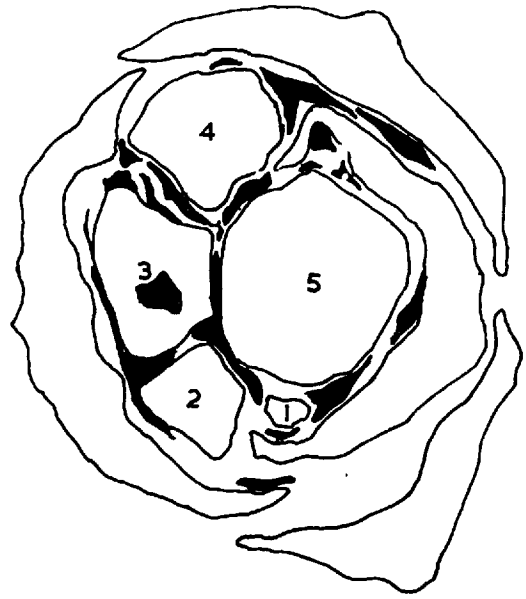
ARDISIA CRISPA inflorescence



Bacterial film in black

FIG. XXIII
Ardisia crispa.
L.S. inflorescence (containing four developing flowers cut at different levels), showing the distribution of the bacterial film between the individual flowers (diagrammatic representation)

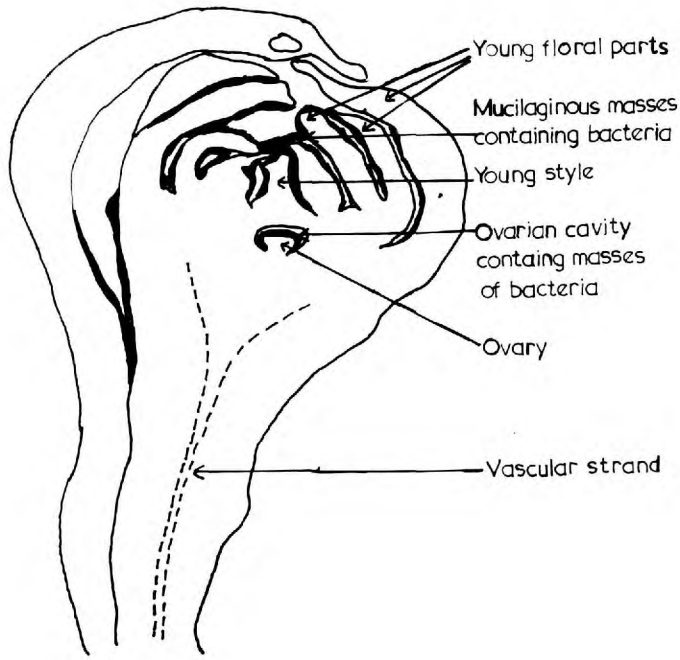
ARDISIA CRISPA inflorescence



Bacterial film in black

FIG. XXIV
Ardisia crispa.
T.S. inflorescence (containing five flowers), showing the distribution of the bacterial film between the individual flowers (diagrammatic representation)

ARDISIA CRISPA single young flower



Bacterial film in black

FIG. XXV
Ardisia crispa.
L.S. single young flower, showing the distribution of the bacterial film (diagrammatic representation).

FIG. XXVI
Ardisia crispa.
L.S. single young flower.
[1% Carbol fuchsin x 120]

ovarian cavity, where they were concentrated between the peak of the placental tissue and the roof of the cavity (Fig. XXVIII). The meristematic tissues of the carpels and anthers also appeared to contain similar bacteria; however, this fact was difficult to judge, as the cells comprising such tissues were very small, closely packed and had dense cytoplasmic contents - but rod-like "objects" did appear in such meristematic tissues.

Only slight traces of the bacteria-containing film remained on the sepals, petals, anthers and carpels of fully developed (i.e. open) flowers. Inside the ovarian cavity, however, there appeared to be no reduction in the number of bacteria.

Microtome section of A. crispa inflorescences, stained in Carbol thionin-Orange G, exhibited similar features to those recorded above for 1% Carbol fuchsin - stained tissues.

(e) Fruits

The plant materials studied under this heading

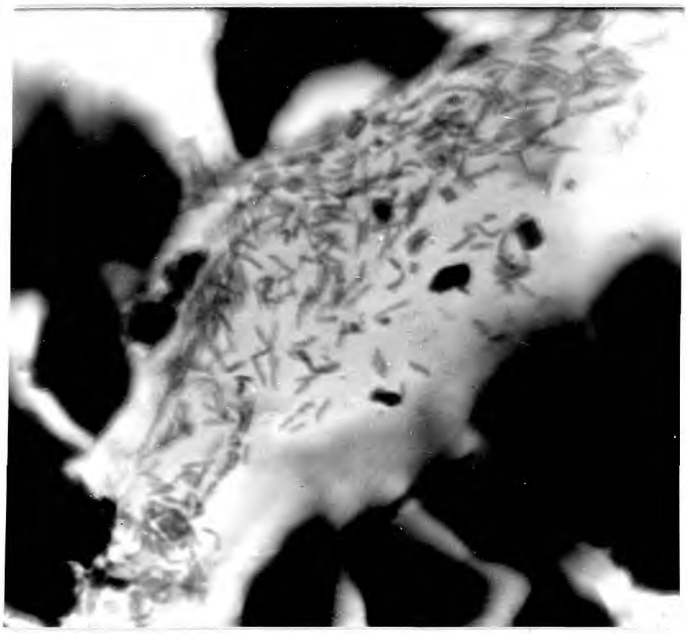


FIG. XXVII Ardisia crispa. Showing the bacteria present in the spaces between anthers and carpels of a young flower.
[1% Carbol fuchsin x 1520]

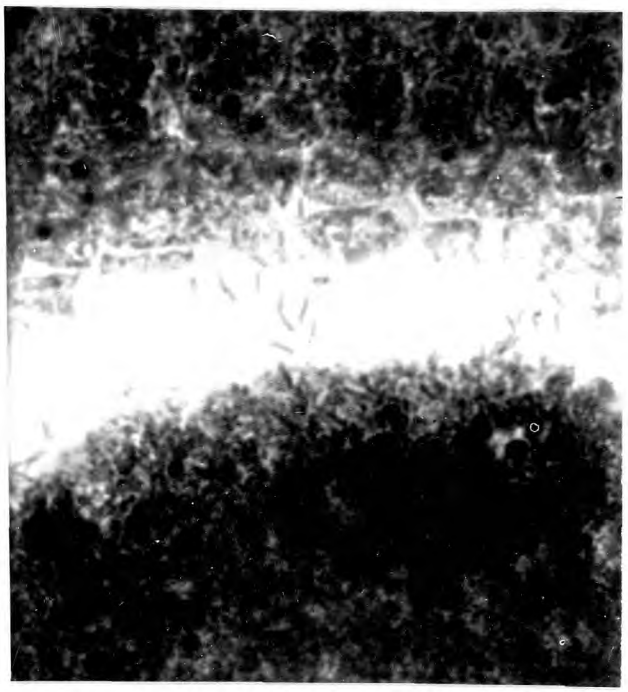


FIG. XXVIII Ardisia crispa. Showing the bacteria present in the ovarian cavity of a young flower.
[1% Carbol fuchsin x 1520]

ranged, in age, from flowers just after pollination* to fruits of 3mm. diameter; the results of these investigations confirmed de Jongh's findings (1938) as to the mode of bacterial entry into the seeds. Figures XXIX and XXX show the distribution of bacteria within a flower just after pollination. Large numbers of bacteria were found in the ovarian cavity. These were all of the same type, and in shape, dimension and staining characteristics, appeared to be intermediate in form between types 1 and 2. Many of the bacteria in the ovarian cavity were situated in the region between the apex of the placental tissue and the entrance of the style-canal (Fig. XXXI). A bacterial film (broken in parts) lined the outer wall of the ovarian cavity and bacteria were also found to be concentrated at the micropylar end of the developing seed (this region is marked with an 'X' in Fig. XXX). It is possible that bacteria were present inside the micropyle, since

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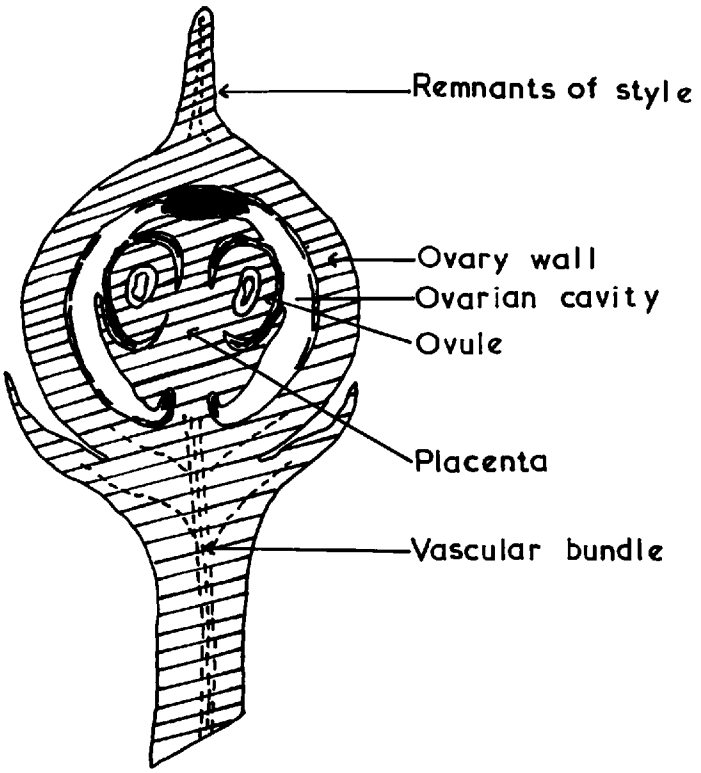
* According to de Jongh (1938), A crispa reproduces asexually, the seed embryo being formed in an apogamous fashion from the integumentary tissues of the ovule.

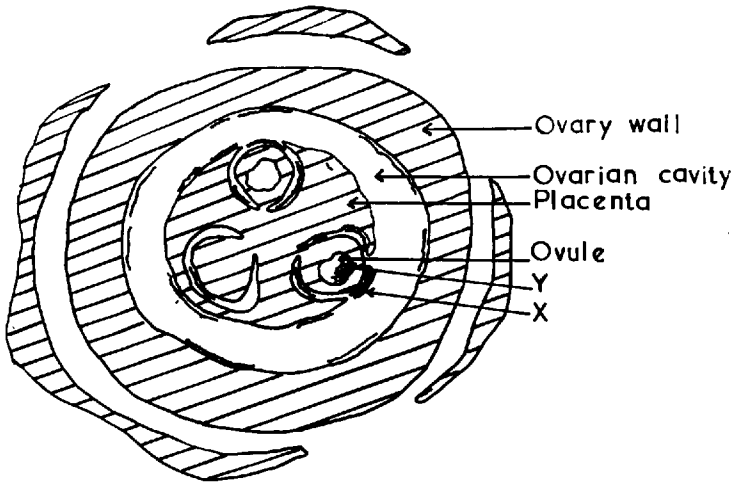
ARDISIA CRISPA flower just after pollination



Bacterial film in black

FIG. XXIX
Ardisia crispa.
L.S. very young fruit, showing the distribution of the bacterial film (diagrammatic representation).

ARDISIA CRISPA flower just after pollination



Bacterial film in black

FIG. XXX
Ardisia crispa.
T.S. very young fruit, showing the distribution of the bacterial film (diagrammatic representation).

they were also found to occur in a space between the nucellus and the integuments (at the region marked with a 'Y' in Fig. XXX, and also shown under H.P. in Fig. XXXII), but, due to the closely packed nature of the cells in the integumentary tissues, this could not be ascertained. No pollen tubes were observed.

Fig. XXXIII shows the general picture presented by a fruit of 3mm. diameter. Two of the ovules were degenerating, whilst the seed was developing within the tissues of the third. The embryo sac had enlarged and the inner integument had been almost entirely resorbed, save for a small region at the micropylar side of the developing seed. Confirming de Jongh's observation (1938), the developing embryo, which was situated near the micropyle, appeared to have arisen adventitiously from the tissues of the inner integument. It was still enclosed within some of these cells and was totally surrounded by a bacterial film which also permeated between the few remaining cells of the inner integument.

(f) Mature seeds.

All initial attempts at sectioning wax-embedded seeds on the rocker microtome failed, owing

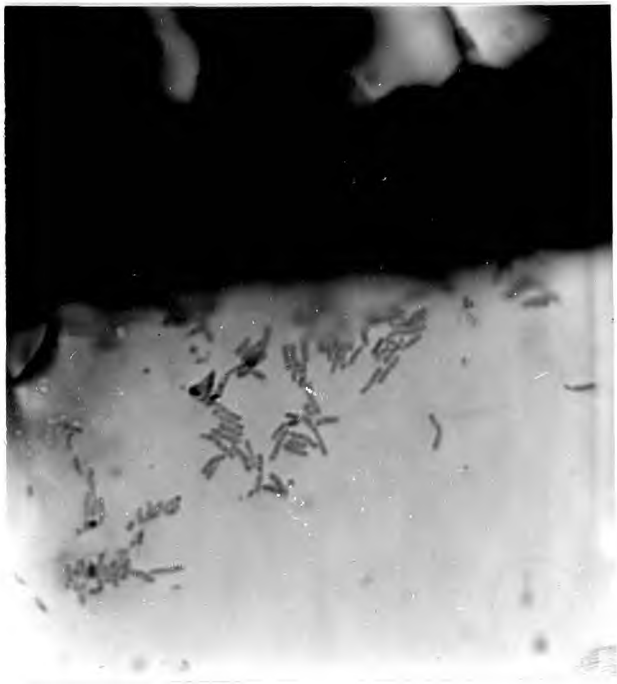


FIG. XXXI Ardisia crispa. Showing the bacteria present in the ovarian cavity of a very young fruit.
[1% Carbol fuchsin x 1520]

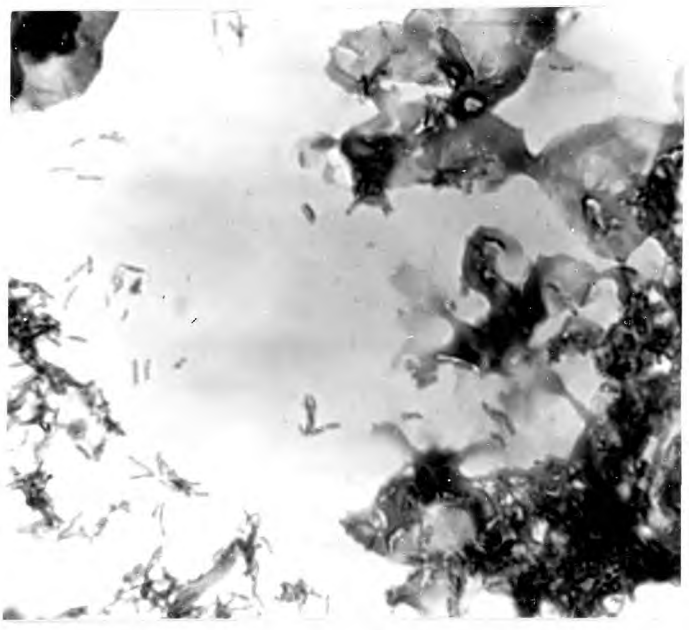
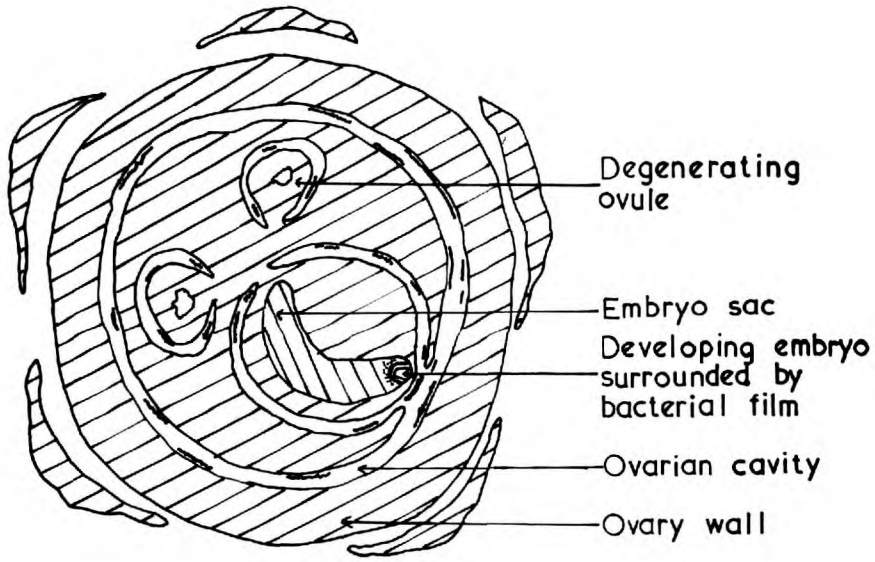


FIG. XXXII Ardisia crispa. Bacteria present in the space between the integuments and the nucellus in an ovule of a very young fruit.
[1% Carbol fuchsin x 1520]

to the fact that the seeds were so hard and woody that they continually sliced out of the wax blocks. Eventually, sections were obtained (of thicknes 5 - 8μ) by using a freezing microtome. These were stained in 1% Carbol fuchsin and examined microscopically, when the following results were obtained:-

Confirming Miehe's observations (1914), a mucilaginous bacteria-containing layer was present between the embryo and the endosperm of the seed. Remnants of this layer can be seen near the micropyle of the seed shown in Fig. XXXIV. Under H.P. the mucilaginous layer was found to contain very many bacterial rods (Fig. XXXV), which were of quite characteristic shape and were fairly uniform in width; they were mainly arranged singly (a few pairs were observed), were slightly curved, showed bipolar staining and some appeared to be capsulated. They bore a strong morphological resemblance to the bacteria found elsewhere in the plant, but were possibly slightly shorter and fatter (1.5 - 2μ x 1μ) than those observed in early stages of seed formation. Uneven staining of the cell thus appears to be characteristic of the Ardisia bacterial symbiont.

ARDISIA CRISPA developing fruit



Bacterial film in black

FIG. XXXIII Ardisia crispa. T.S. fruit at a late stage in development, showing the distribution of the bacterial film around the developing embryo (diagrammatic representation).

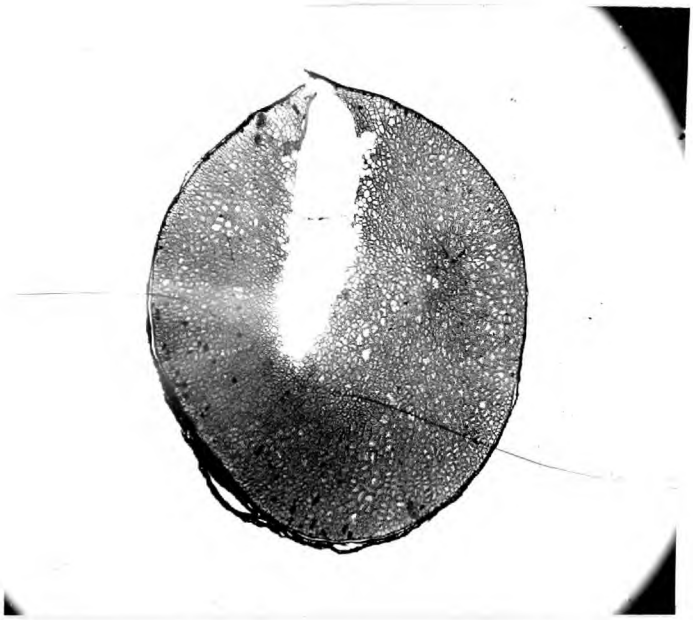


FIG. XXXIV Ardisia crispa. T.S. mature seed, showing remnants of the mucilaginous film at the micropyle region (in this preparation the embryo has been displaced during section-cutting).
[1% Carbol fuchsin x 30]

Contrary to reports by Miehe (1914) and de Jongh (1938), which state that the bacterial film is restricted to the micropyle region, the embryo was found to be totally enveloped by the film, and a large number of similar bacteria - free from the film - were found to occur both along the walls of the invaginated endosperm cells and within such cells (Fig. XXXVI). This was particularly the case for endosperm cells which were in the immediate vicinity of the embryo; no bacteria were seen inside the endosperm cells at any further distance away from the embryo, but a number were still present on the cell walls.

Examination of seed tissues stained in Carbol thionin - Orange G confirmed the findings recorded above for Carbol fuchsin-stained tissues.

(g) Germinating seeds.

Fresh seeds were germinated on moistened blotting paper at 25°C. After 12 days the radical had emerged from most seeds, which were then fixed, wax-embedded and sectioned on the rocker microtome.

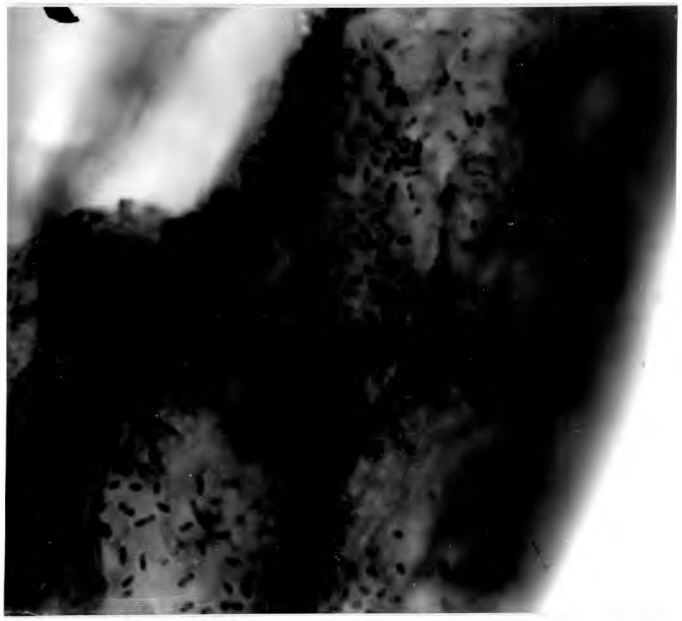


FIG. XXXV Ardisia crispa. Showing the bacteria present in the mucilaginous film which occurs between embryo and endosperm in mature seeds.
[1% Carbol fuchsin x 1520]

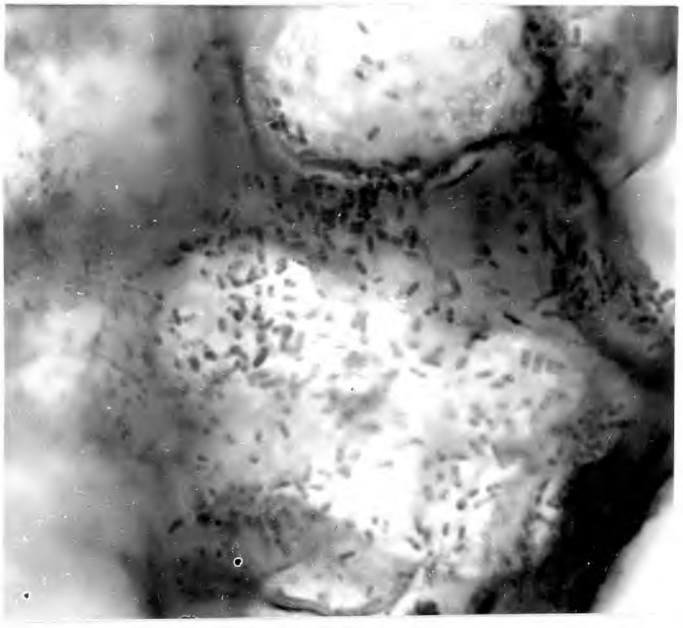


FIG. XXXVI Ardisia crispa. Showing the bacteria present within the cells and lining the walls of the seed endosperm.
[1% Carbol fuchsin x 1520]

Whilst only slight traces of the mucilaginous film remained on the radical, the cotyledons, stem apex and stem were still enveloped by the film. It also permeated between the partially digested cells of the endosperm. One such preparation, stained with 1% Carbol fuchsin, is shown in Fig. XXXVII. When examined under H.P., the film was found to contain many bacteria, the majority of which closely resembled (in shape, dimensions and staining characteristics) the type 2 bacteria present in flowers, developing seeds and young leaf nodules; the remainder (approximately 5%) were similar to those found in mature resting seeds and likewise appeared to be capsulated. Examples of both types of bacteria are illustrated in Fig. XXXVIII.

Attempts to demonstrate the presence of bacteria within tissue-sections of the plantules of germinating seeds (by staining with either Carbol fuchsin or Carbol thionin - Orange G) were unsuccessful. However, the presence of bacteria in the plantule tissue was demonstrated as follows:- After removal from a germinating seed, the plantule was washed free of mucilaginous film by shaking in several

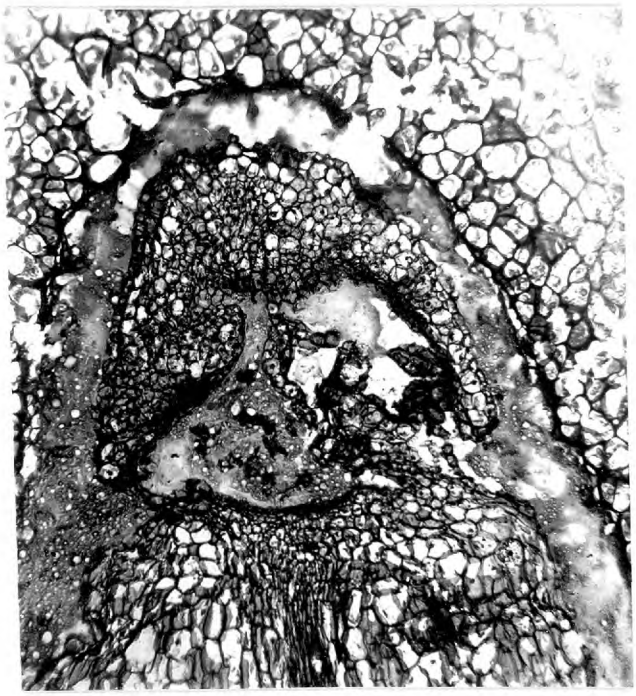


FIG. XXXVII Ardisia crispa. T.S. germinating seed, showing the cotyledons and stem apex enwrapped in the mucilaginous film.
[1% Carbol fuchsin x 60]

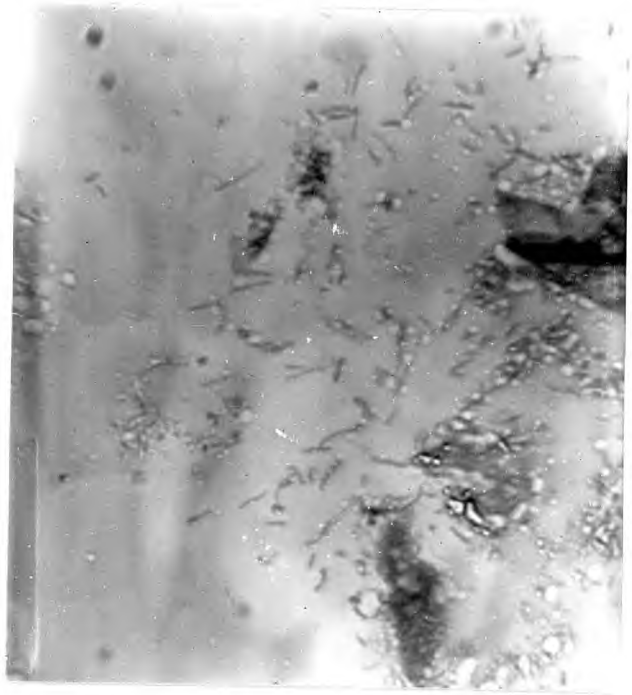


FIG. XXXVIII Ardisia crispa. Bacteria present in the mucilaginous film of a germinating seed.
[1% Carbol fuchsin x 1520]

changes of 95% alcohol. It was then squashed onto a slide and stained with Carbol fuchsin. Many bacteria were found in such preparations and these all resembled type 2 bacteria (Fig. XXXIX).

RUBIACEAE.

Psychotria emetica

(a) Leaf nodules of various ages.

Under L.P., old leaf nodules showed up as well-differentiated structures which were set mainly in the spongy mesophyll layers. The nodule, oval in outline, swelled the leaf on both dorsal and ventral surfaces (Fig. XL).

No plant cellular structure was seen in the nodule, but there was evidence of some organisation of the tissues. Thus, the nodule contained what appeared to be packets of "mucilage" (cf. A. crispa nodules). These packets were largest at the periphery of the nodule and were separated by channels, which originated in the central portions of the nodule and ran outwards towards the periphery (Fig. XLI). A vascular bundle which terminated in a distinct 'sheath'

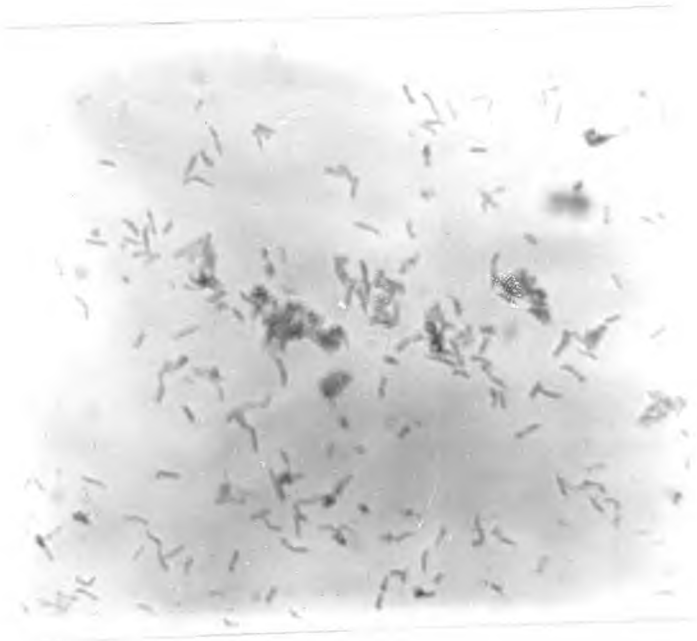


FIG. XXXIX *Ardisia crispa*. Bacteria as observed in suspensions of squashed plantules.
[1% Carbol fuchsin x 1520]

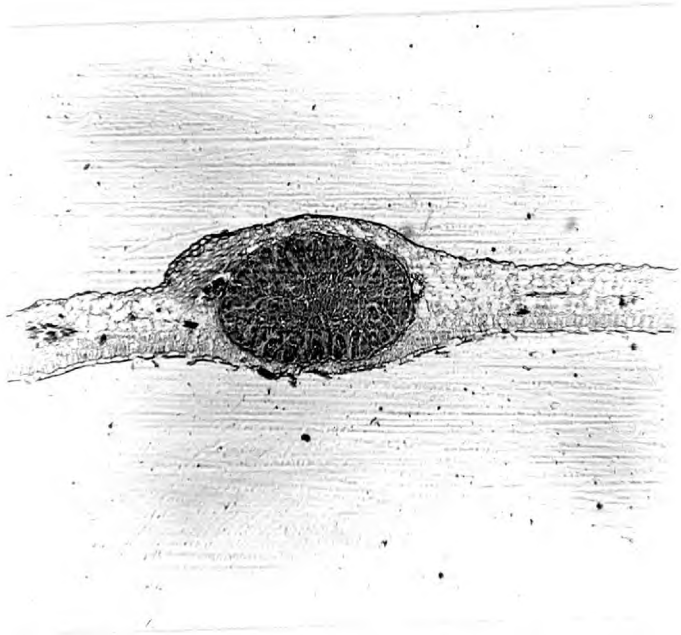


FIG. XL Psychotria emetica. T.S. old leaf nodule.
[Carbol fuchsin - Fast Green x 30]

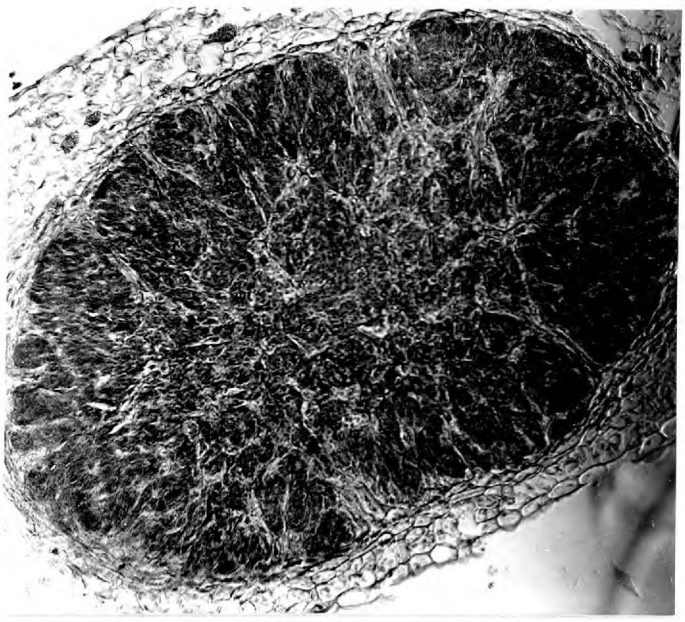


FIG. XLI Psychotria emetica. T.S. old leaf nodule, showing mucilaginous packets separated by channels.
[Carbol fuchsin - Fast Green x 120]

layer (two to three cells deep) of modified mesophyll cells, was present in the immediate vicinity of all nodules examined. The cells of the 'sheath' were spindle-shaped. They overlapped lengthwise and appeared to form a wall, which demarked the nodule from the surrounding mesophyll tissue (cf. A. crispa nodules).

The nodular mucilage was mottled with thousands of dark red-staining granules.

Under H.P., the mottled appearance of the nodular mucilage was seen to be due to masses of bacteria. The bacterial population consisted almost wholly of "bacteroid" type organisms. The latter were very refractile, for each cell was stained only at the poles. They were aggregated in large masses within the mucilaginous packets and this type of arrangement, together with the uneven staining of the cells, accounted for their "coccoid" appearance. These "bacteroids", which were stubby, swollen and had dimensions of (approximately) 0.5 - 1.5μ x 1.5 - 2μ, resembled the "bacteroids" present in A. crispa nodules (Fig. XLII).

In addition a number of larger bacterial rods (0.5μ x 3 - 4μ), which stained more evenly than the "bacteroids", were found within the nodular mucilage.

Examination of the surrounding leaf tissues showed that the palisade mesophyll cells contained (in addition to a number of chloroplasts) rod-shaped "objects" which were stained in a similar manner to the nodular "bacteroids". But, since the cytoplasmic contents of the palisade layer cells were so dense, it was not possible to discern whether or not such "objects" were, in fact, bacteria. A few similar "objects" were found inside the spongy mesophyll cells, but bacteria were not found to occur either in the intercellular spaces of the mesophyll tissue or in the vascular bundle.

In most preparations of old nodules, the mucilaginous contents of the nodule were in direct contact with the outer atmosphere via a pore, which terminated in the upper epidermis of the leaf. The entrance to the pore was surrounded by modified epidermal cells (not resembling stomata) and the pore

was lined with layers of cells which were continuous with the nodule "sheath".

Serial sections of old nodules, stained with either Carbol thionin - Orange G or Basic fuchsin - Gentian Violet - Iodine, presented a similar picture to that recorded above for Carbol Fuchsin - Fast Green stained tissues.

Middle-aged nodules differed only in minor detail from old nodules. The channels which, in old nodules, separated the mucilaginous packets, were found, in middle-aged nodules, to contain multicellular hairs; the latter originated from cells in the sheath layer.

As in the case of old nodules, most middle-aged nodules were found to contain a pore and the bacterial content was predominantly "bacteroid" in nature. The multicellular hairs of the nodule also appeared to contain intracellularly arranged "bacteroids". Bacteria were not found in the intercellular spaces of the mesophyll.

In structure, young leaf nodules were very similar to middle-aged nodules; other than containing multicellular hairs, no organised plant cellular structure was visible within the nodule (c.f. A. crispa). The sheath layer was in the process of being formed and a vascular bundle was in the vicinity of each nodule. In this young stage, whilst as yet no swelling of the epidermal surfaces had occurred, most nodules showed the presence of a pore.

When stained with Carbol thionin - Orange G, the majority of the nodular bacteria were found (c.f. old and middle-aged nodules) to be "bacteroid" in nature.

However, as in the case of A. crispa, a second type of bacterium was found to be present in the tissues of young leaves. Great numbers of this type had washed out of every section onto the slide. These cells were rod-shaped and mainly occurred singly, although a number of pairs were also present. They had a characteristic "banded" appearance, due

to the fact that their cytoplasm stained irregularly and was always more intensely stained at the poles than elsewhere in the cell. These bacteria closely resembled the type 2 organisms found in young leaf tissues of A. crispa, for, in addition to being irregularly stained, they possessed similar dimensions (1 - 2.5μ x 0.5μ) and were slightly curved.

Initially it was thought that these bacteria were being washed out of the nodules both onto the slide and into the rest of the tissues, since a few had been found to be present within the nodules. However, they were very evenly distributed around the sections, indicating that they had probably washed out from the whole section of leaf tissue - and not just from the nodules. Examination of mesophyll cells under H.P. would suggest that this was the case, for similar bacteria appeared to be present in large numbers inside these cells. In order to confirm this, some nodule-free leaf tissues were examined:-

(b) Non nodulated regions of nodulated leaves.

Whilst no bacteria were found to be present in the non-nodulated tissues of old and middle-aged

leaves, many bacteria were found in the corresponding regions of young leaf tissues (cf. A. crispa). The bacteria were all of type 2 and were situated both in the intercellular spaces of the mesophyll and inside the cells of the mesophyll and epidermal layers (Fig. XLIII). However, since many bacteria of similar type had been washed out of each preparation onto the slide (c.f. young leaf nodule sections), it is not possible to pinpoint their original location (i.e. whether they were arranged inter- or intracellularly).

(c) Terminal buds.

The general picture observed when longitudinal sections of terminal buds were stained with 1% Carbol fuchsin is as shown in Figures XLIV and XLV. The bud consists of an alternate system of foliage leaves and stipules, the dorsal and ventral surfaces of which carry numerous multicellular hairs. In the preparations examined many developing nodules were found in the foliage leaf tissues. A mucilaginous, finely granulated material partially filled and permeated all the bud spaces and was in intimate

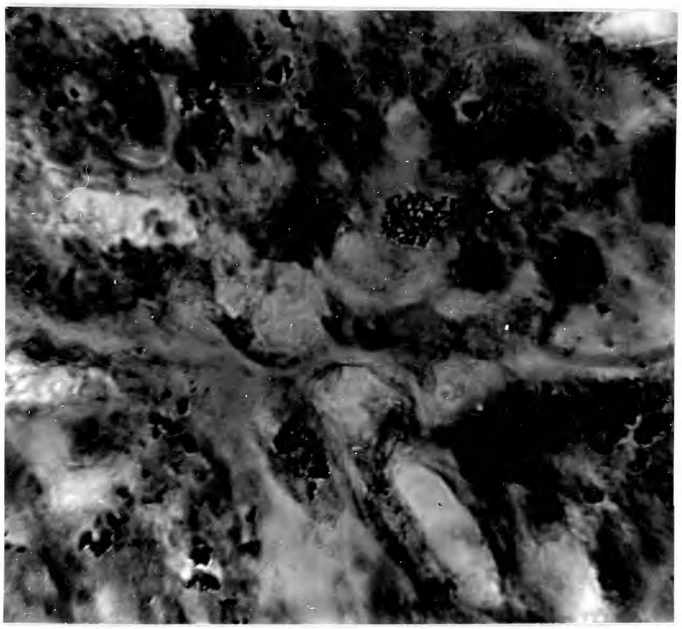


FIG. XLII Psychotria emetica. Aggregates of bacteroids present in an old leaf nodule.
[Basic fuchsin - Gentian Violet - Iodine x 1140]

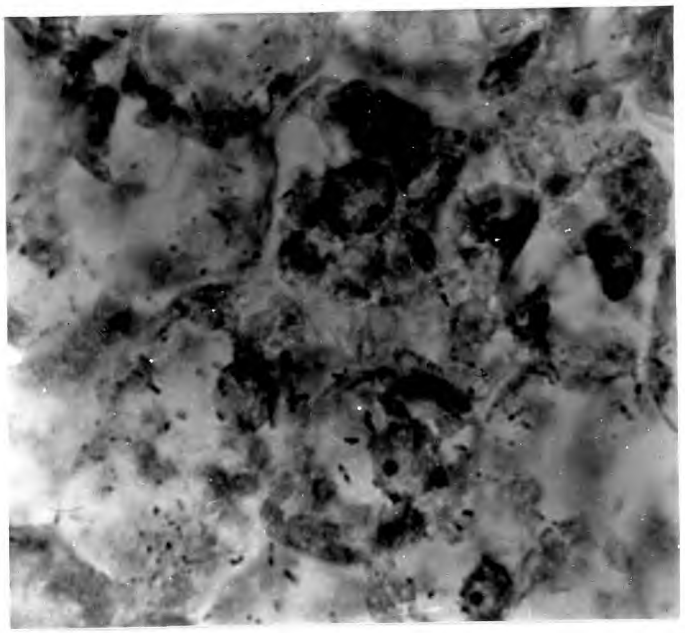


FIG. XLIII Psychotria emetica. Bacteria present in non-nodulated regions of nodulated young leaves.
[Carbol thionin - Orange G. x 1520]

PSYCHOTRIA EMETICA terminal bud

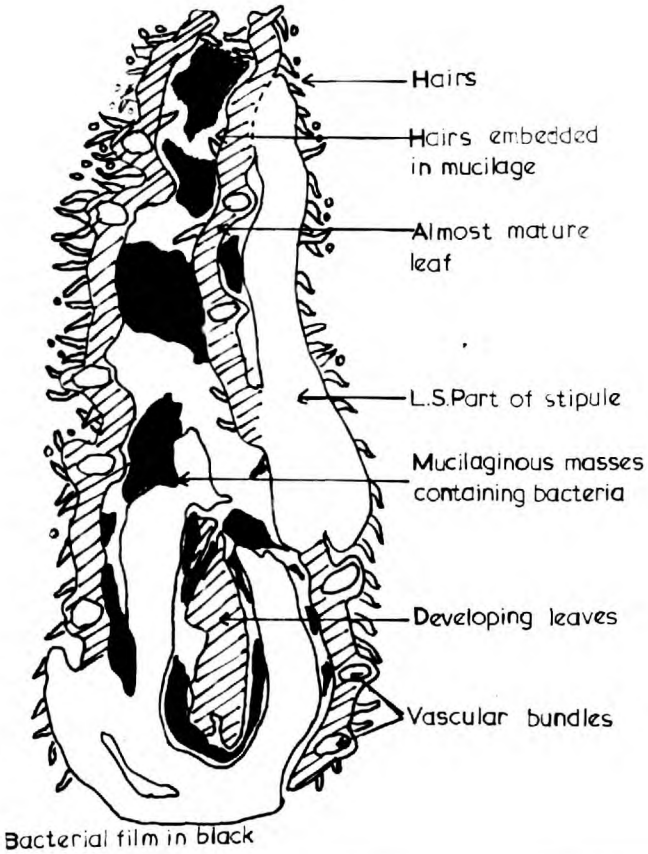


FIG. XLIV
Psychotria emetica.
L.S. terminal bud
(diagrammatic
representation).

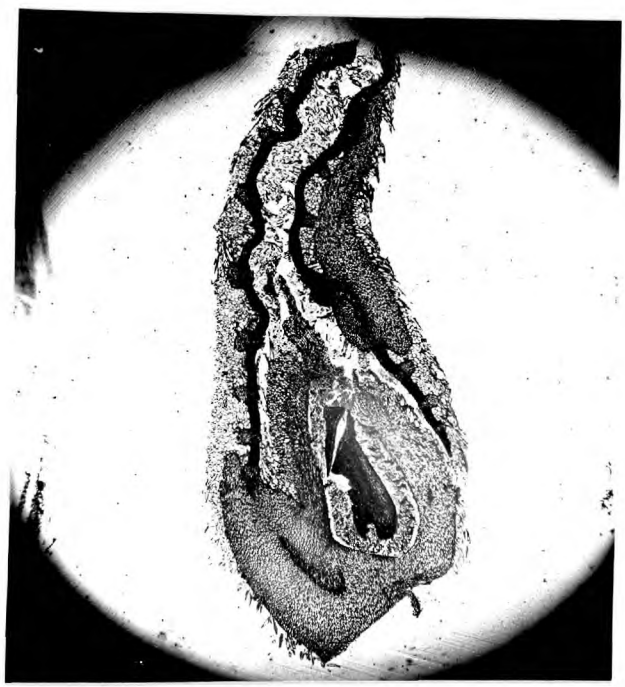


FIG. XLV
Psychotria emetica.
L.S. terminal bud.
[1% Carbol fuchsin
x 30]

contact with the hairs and epidermal tissues of the developing bud leaves.

When examined under H.P. the bud mucilage was found to contain very many bacteria (Fig. XLVI), the majority of which appeared to be intermediate in form between the two types of bacteria found in young leaf nodulated tissues. In shape, dimensions and staining characteristics, these bacteria were indistinguishable from the bacterial population of A. crispa terminal buds.

However, unlike the interfoliar mucilage of A. crispa terminal buds, the mucilage of Psy. emetica terminal buds housed several types of morphologically distinguishable bacterial cells, some of which were probably representatives of different bacterial species. Thus approximately 10% of the total bacterial population was "bacteroid" in appearance and closely resembled the type 1 nodular bacteria. A number (approximately 5%) of longer, thinner rods (2 - 5μ x 0.3 - 0.5μ), arranged in chains, were also seen, as were several (approximately 5%) large (2 - 5μ x 2μ), oval-shaped, bipolar-staining rods.

The bacterial content of the developing nodules was predominantly "bacteroid" in nature and consisted almost exclusively of type 1 bacteria. A few representatives of type 2 were also found.

In Carbol fuchsin-stained preparations it was not possible to ascertain whether bacteria were present between or within the cells of the apical meristem and foliage leaves, but the multicellular leaf hairs appeared to contain bacteria of type 2.

When terminal buds were stained by the Carbol thionin-Orange G method, a picture similar to the one shown in Figure XLVII was obtained. The mesophyll tissues of the foliage leaves were stained an intense red-purple colour, as were certain regions of the apical meristem, portions of the multicellular hairs and scattered areas of the mucilaginous film. All other tissues were stained yellow.

This method of staining gave a very good differentiation of developing leaf nodules, which appeared as oval, purple-stained regions in the spongy mesophyll

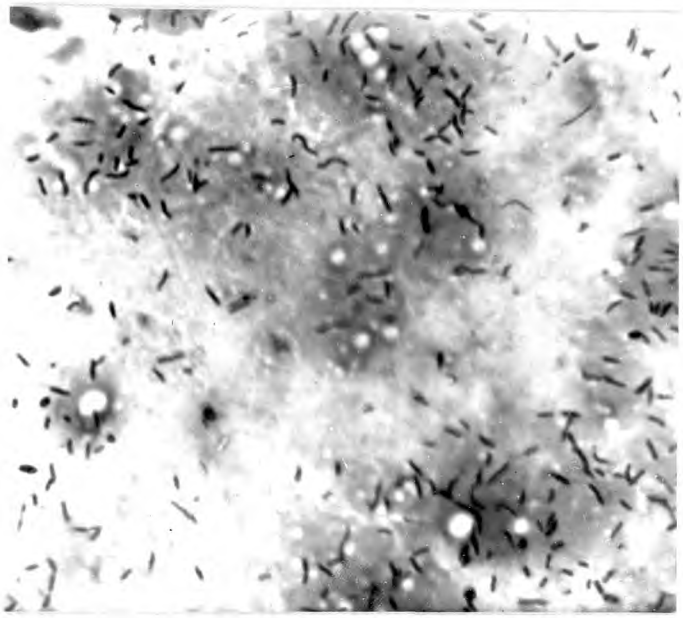


FIG. XLVI Psychotria emetica. Bacteria present in the terminal bud mucilage.
[1% Carbol fuchsin x 1140]

FIG. XLVII Psychotria emetica. Staining pattern observed in terminal bud tissues.
[Carbol thionin - Orange G. x 40]

tissue of the foliage leaves. Even primordial foliage leaves possessed nodules. Several nodules can be seen in Figure XLVII and one such structure, more highly magnified, is shown in Figure XLVIII. No organised plant cellular structure (i.e. excluding hairs) was visible within the nodules and, even at such an early stage in their development, most nodules possessed a pore.

Examination of Carbol thionin-Orange G-stained preparations under H.P. showed that bacteria were present in various locations within the bud. Irregularly stained "bacteroids" of type 1 and quite a large number of cells of type 2 were found in the mucilaginous packets of the developing nodules. Several different types of bacteria were found in the interfoliar mucilage, thus confirming the results obtained with 1% Carbol fuchsin. The purple staining regions of foliage leaves and apical meristems contained a very large number of bacteria of type 2. Along with the plant cell nuclei, which likewise stained purple, these bacteria imparted the purple colour to the tissues. Literally hundreds of these bacteria lined the walls between the cells in the

mesophyll tissues and many were found inside the mesophyll cells (Fig. XLIX).

(d) Fruits

Only two or three fruits of this species, all of which were at the same stage of development, were available. The following results were obtained when serial sections of one such fruit (of 3mm. diameter) were stained with Carbol thionin-Orange G and subsequently examined microscopically:-

The overall morphology of the fruit, as seen in longitudinal section, is as shown in Fig. L. Floral leaf nodules resembled foliage leaf nodules in structure.

Within the fruit, bacteria were found to occur at various locations, each of which is indicated in Figure LI. Under H.P. the floral nodules were found to contain type 1 "bacteroids". Unevenly stained bacteria of type 2 were present in the immediate vicinity (marked with an 'X' in Fig. LI) of the developing embryo and these are illustrated in

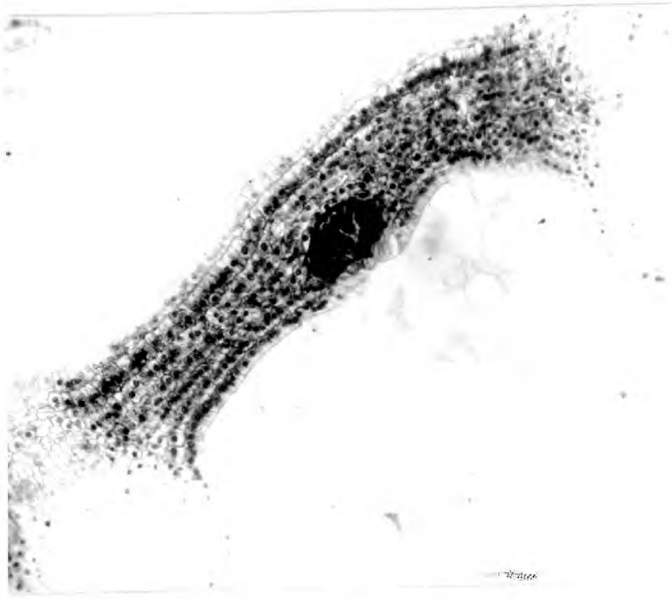


FIG. XLVIII Psychotria emetica. Young nodule present on a terminal bud foliage leaf.
[Carbol thionin - Orange G. x 160]

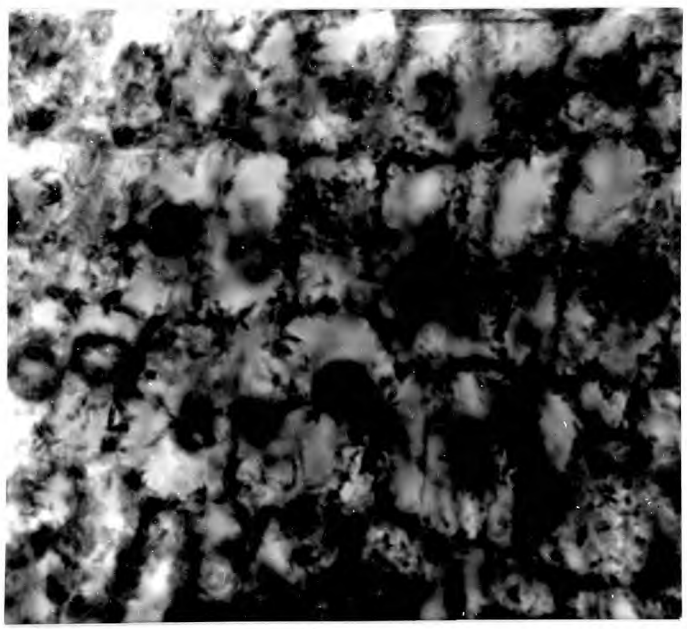


FIG. XLIX Psychotria emetica. L.S. terminal bud leaf showing intracellular bacteria of type 2 in the mesophyll tissues.
[Carbol thionin - Orange G. x 1520]

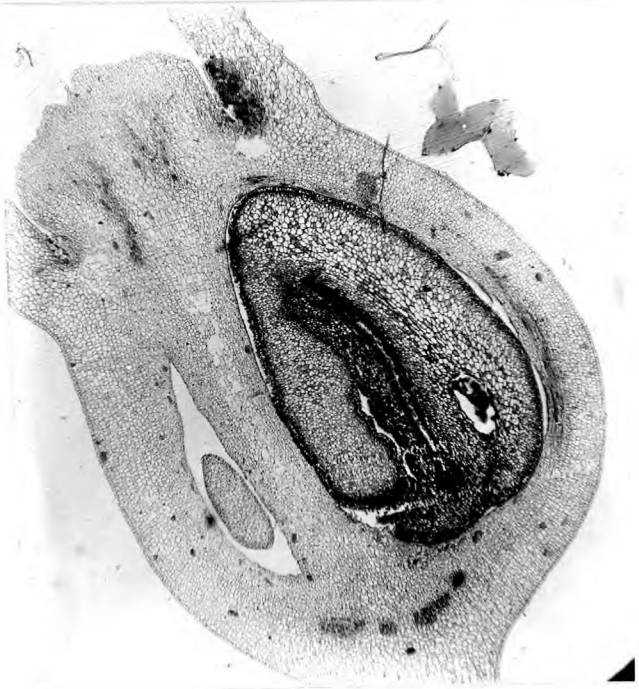


FIG. L
Psychotria emetica.
L.S. developing fruit.
[Carbol thionin - Orange G. x 40]

PSYCHOTRIA EMETICA
developing fruit

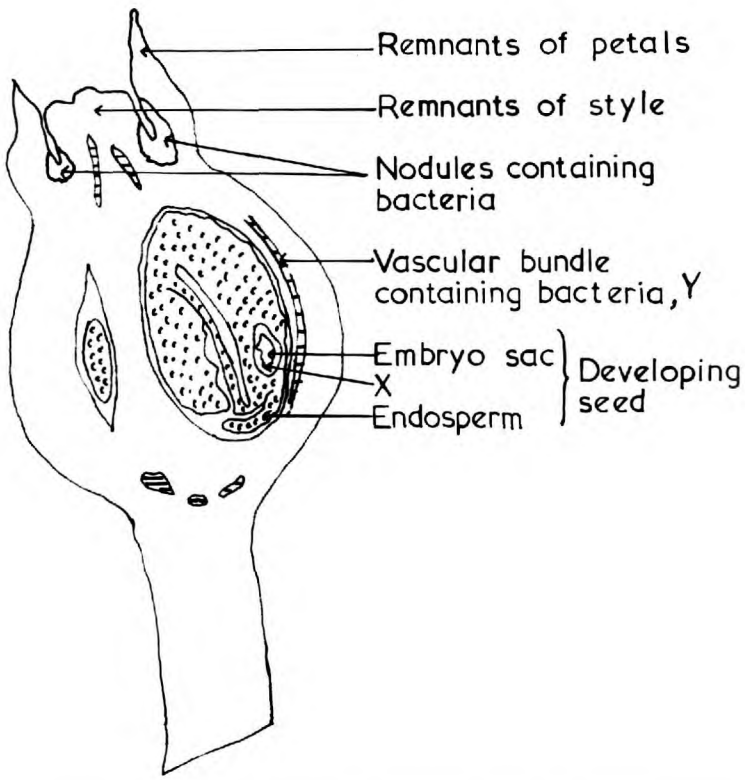


FIG. LI
Psychotria emetica.
Showing the distribution of bacteria within a developing fruit (diagrammatic representation).

Figure LII. Similar bacteria, whose location is marked with a 'Y' in Figure LI, were found within the tissues of a nearby vascular bundle.

Psychotria nairobiensis

(a) Leaf nodules of various ages.

At all stages in their development, the leaf nodules of Psy. nairobiensis bore a close morphological resemblance to the leaf nodules of Psy. emetica; they were likewise set in the spongy mesophyll and were bounded at the periphery by a sheath of modified mesophyll cells. Examples of old- and middle-aged nodules are shown in Figures LIII and LIV respectively.

In the mucilaginous packets of nodules of all ages, only one type of bacterium was seen and in its staining and morphological characteristics this appeared to be identical with the bacteria of type 1 (bacteroids) of A. crispa and Psy. emetica (Fig. LV). In old and middle-aged leaves, the bacterial population was restricted to the nodules. However, just as in the case of A. crispa and Psy. emetica, the

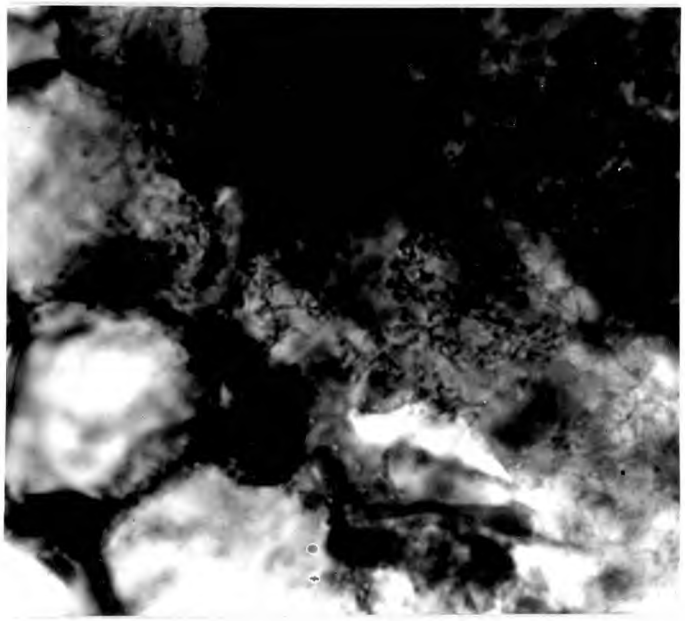


FIG. LII Psychotria emetica. Showing the bacteria present in the vicinity of the developing embryo.
[Carbol thionin - Orange G. x 1520]

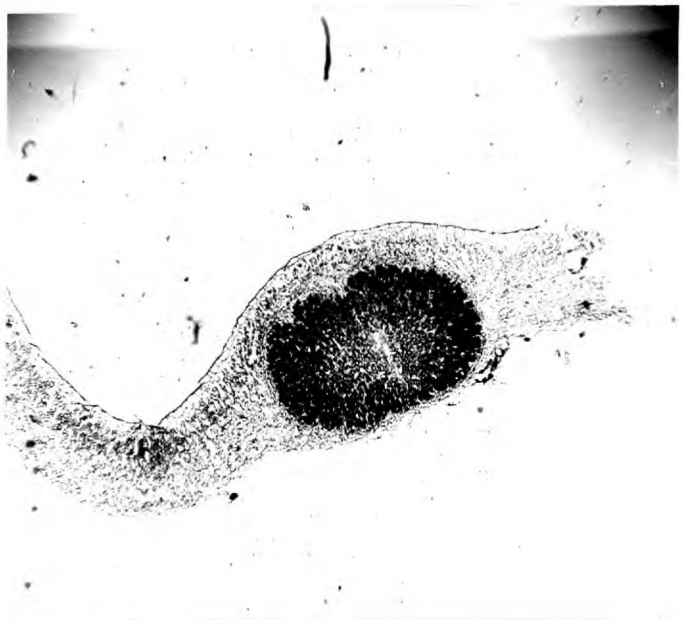


FIG. LIII Psychotria nairobiensis. T.S. old leaf nodule.
[1% Carbol fuchsin x 60]

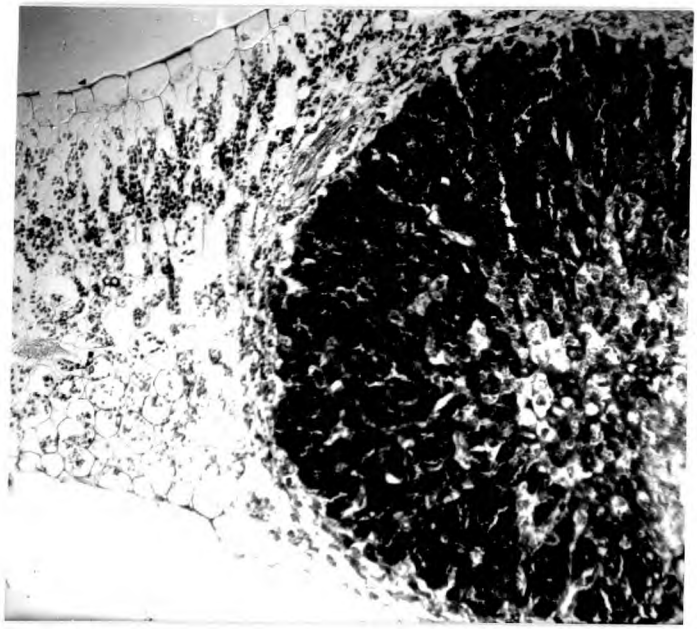


FIG. LIV Psychotria nairobiensis. T.S. middle-aged leaf nodule.
[1% Carbol fuchsin x 120]

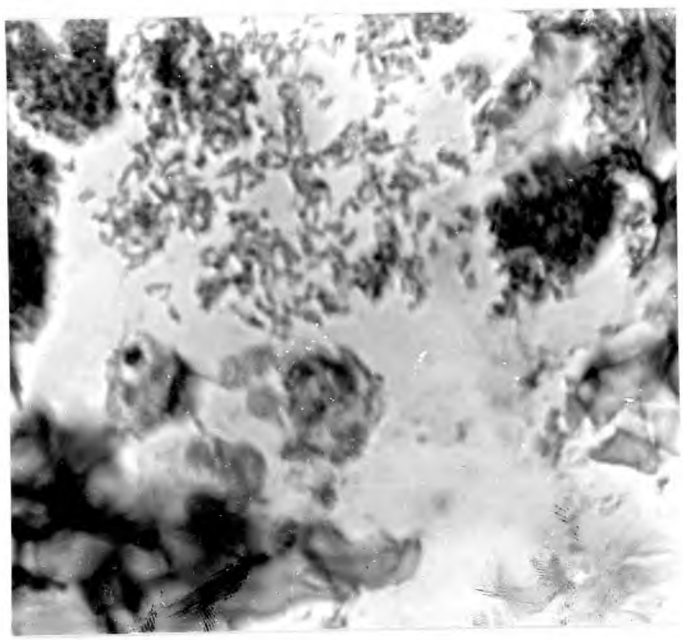


FIG. LV Psychotria nairobiensis. Bacteroids situated in the pore of a middle-aged leaf nodule.
[Safranin - Orange G. x 1520]

mesophyll tissues of young leaves were found to contain a second type of bacterium; in staining and morphological characteristics, this type resembled bacteria of type 2. Only a few cells of this type were observed (cf. A. crispa and Psy. emetica) and, since most of these had been washed out from the sections onto the slide, it was not possible to assess whether they had been displaced from the nodules or from within or between the cells of the mesophyll.

(b) Non-nodulated regions of nodulated leaves.

All preparations of young leaf tissues were found to contain bacteria of type 2. These were more or less uniformly distributed throughout the sections and were also found in the areas on the slide immediately surrounding such sections. Compared to the corresponding tissues of A. crispa and Psy. emetica, young leaves of Psy. nairobiensis contained only very few bacteria of this type. No bacteria of this type were found within the tissues of older leaves.

(c) Terminal buds.

Psy. nairobiensis buds were found to be anatomically very similar to those of Psy. emetica (Figs. LVI and LVII). The spaces between the bud hairs were lined with mucilage, though this was not so abundant as in Psy. emetica. A number of developing leaf nodules were present in the tissues of the foliage leaves.

Serial sections of the bud, stained with 1% Carbol fuchsin, were found to contain only one type of bacterium (cf. A. crispa and Psy. emetica); this type (Fig. LVIII) closely resembled the bacteria of type 1 and was present in both the interfoliar mucilage and interfoliar spaces of the bud.

Whilst staining with Carbol fuchsin did not reveal the presence of bacteria inside the foliar and apical meristem tissues, their presence in these tissues was demonstrated by staining with Carbol thionin-Orange G. The result obtained was similar to that as already recorded for Psy. emetica, bacteria of type 2 being present in large amounts in the mesophyll tissues of the developing leaves and "bacteroids" being found in the developing leaf nodules.

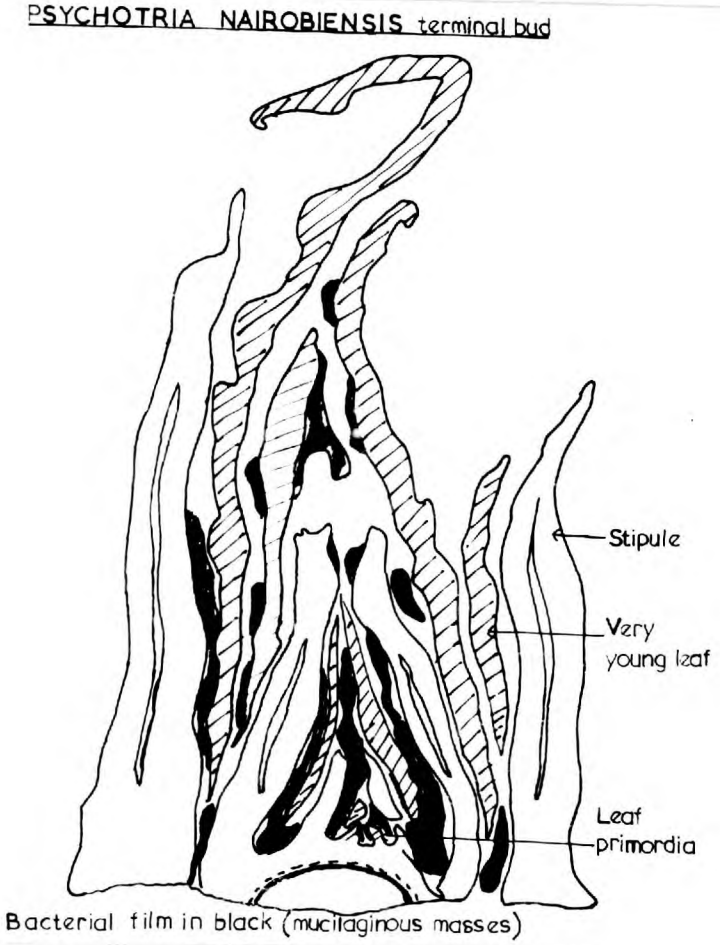


FIG. LV
Psychotria nairobiensis.
L.S. terminal bud showing the distribution of the bacteria-containing mucilaginous film (diagrammatic representation).

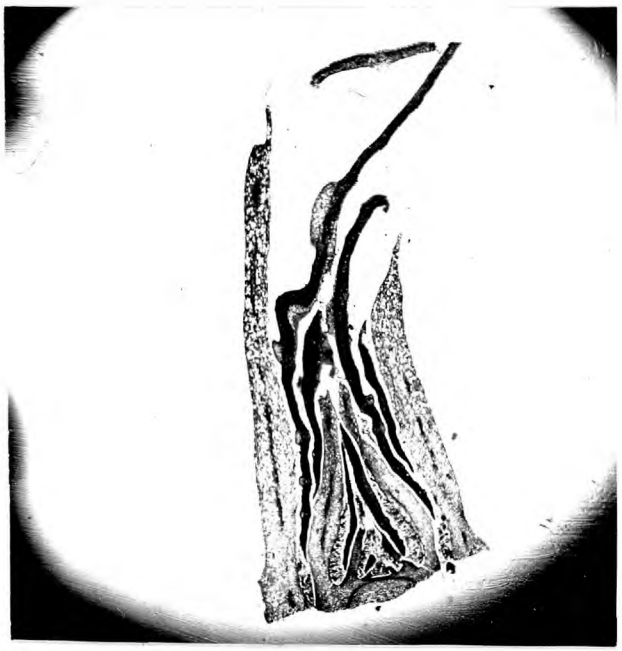


FIG. LVII
Psychotria nairobiensis.
[1% Carbol fuchsin x 30]

(d) Inflorescences.

Microscopical examination of Carbol fuchsin-stained serial sections of inflorescences of all ages yielded similar results to those obtained with A. crispa. In young closed floral buds a mucilaginous film, which contained numerous type 2 bacteria, enveloped all the primordial floral parts. Type 2 bacteria were also present in the ovarian cavity.

In open flowers, whilst only remnants of the bacterial film remained on the surfaces of the sepals, petals and style, no trace of the film could be found on the stamens. A few bacteria of type 2 were again found in the ovarian cavity.

(e) Fruits

Fruits, which were similar both in size and in developmental stage to those described for Psy. emetica, were sectioned and subsequently stained by the same methods.

Both the distribution of bacteria within the fruit and the morphological characteristics of these bacteria were similar to those previously recorded for fruits of Psy. emetica.

(f) Mature seeds.

Just as in A. crispa, a mucilaginous, bacteria-containing, layer completely enveloped the embryo. This layer was best seen in Carbol fuchsin-stained preparations and under H.P. the cells comprising its bacterial population appeared to be identical (in size, shape and staining characteristics) to the bacteria present in mature seeds of A. crispa. A number of those bacteria were capsulated. Bacteria were not found within the embryo tissues.

With the staining methods employed it was not possible to demonstrate the presence of bacteria within or between the cells of the endosperm. (cf. A. crispa)

(g) Germinating seeds.

When seeds of Psy. nairobiensis were germinated under conditions similar to those recorded previously for A. crispa, it was found that the mucilaginous layer contained a large number of bacteria (Fig. LIX). The majority of these bacteria, which were longer and thinner than those found in mature seeds, exhibited similar morphological and staining characteristics to the type 2 bacteria found elsewhere in the

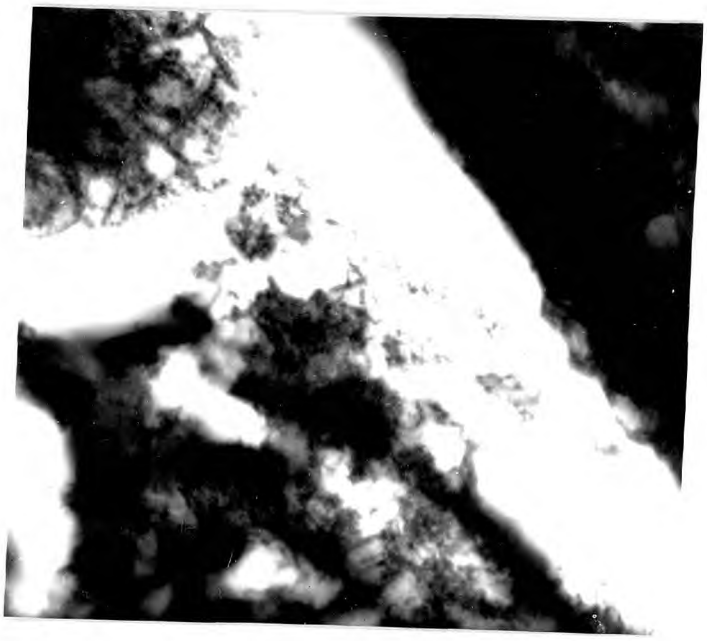


FIG. LVIII Psychotria nairobiensis. Bacteria present in the interfoliar spaces of the terminal bud.

[1% Carbol fuchsin x 1140]

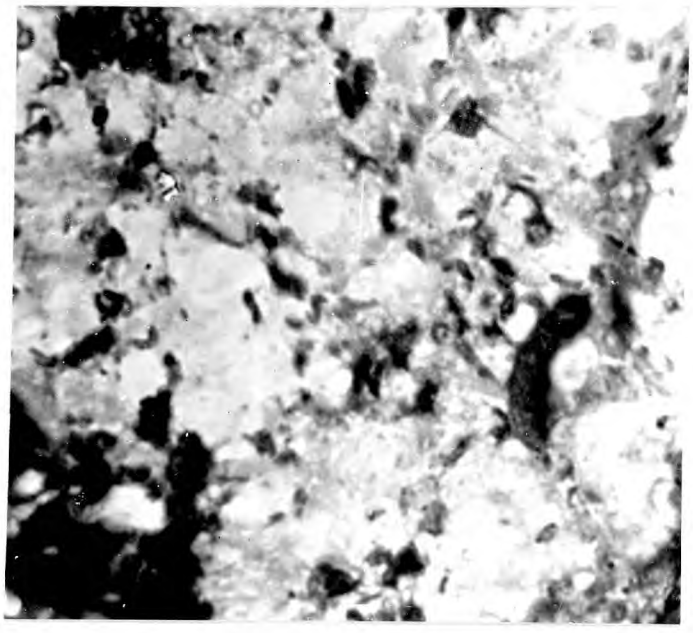


FIG. LIX Psychotria nairobiensis. Bacteria present in the vicinity of the embryo in a germinating seed.

[1% Carbol fuchsin x 1520]

plant; in addition they closely resembled the type 2 bacteria of A. crispa germinating seeds. Unlike germinating seeds of A. crispa and mature seeds of Psy. nairobiensis, the germinating seeds of Psy. nairobiensis were not found to contain any capsulated bacterial rods. The remainder of the seed mucilage bacterial population consisted of bipolar staining, rod-shaped cells, whose width was similar to that of the type 2 cells; their length (1.5 - 2.5μ) was intermediate between that of the cells found in mature seeds and that of the type 2 cells of germinating seeds. They appeared to be intermediate morphological forms of these two types.

Both of these types were also found in the partially digested cells of the endosperm.

Pavetta grandiflora.

(a) Leaf nodules of various ages.

At all stages in their development, the leaf nodules of P. grandiflora, as seen in serial sections, were anatomically very similar to those of Psy. emetica and Psy. nairobiensis. They were likewise set in the spongy mesophyll, being walled off from the parenchymatous

cells of the latter by a distinct sheath of brick-shaped cells. Examples of old and young leaves nodules are illustrated in Figures LX and LXI respectively.

Whilst the only type of bacterium present in old and middle-aged nodules were those of type 1 (bacteroids), young nodules, in addition, contained a large number of cells of type 2 (Fig. LXII).

(b) Non-nodulated regions of nodulated leaves.

Young leaf tissues were found to contain large numbers of type 2 bacteria, which were uniformly distributed throughout the serial sections and were also found in the areas on the slide immediately surrounding such sections.

(c) Terminal buds.

Anatomically, the terminal bud of P. grandiflora is similar to that of Psy. nairobiensis. As in the other three plant species, developing nodules were observed in the leaves of P. grandiflora terminal bud. A mucilaginous film capped the apical meristem and lined the interfoliar spaces of the bud.

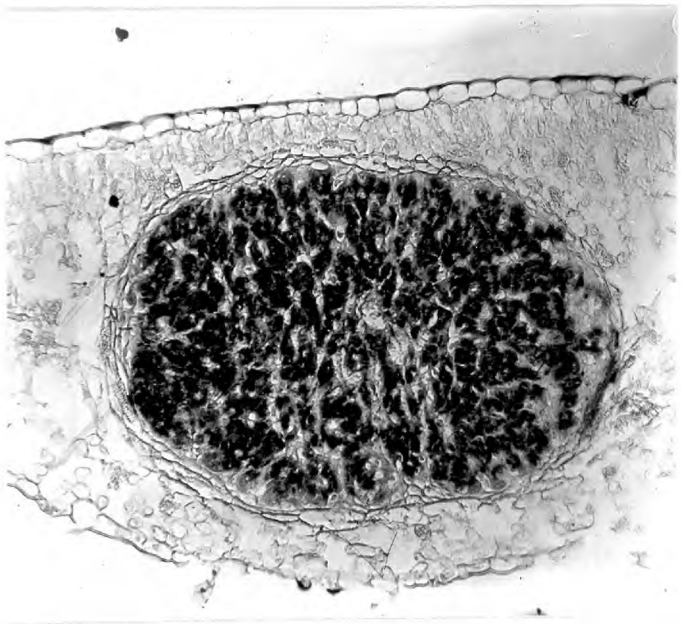


FIG. LX Pavetta grandiflora. T.S. old leaf nodule.
[Carbol thionin - Orange G. x 120]

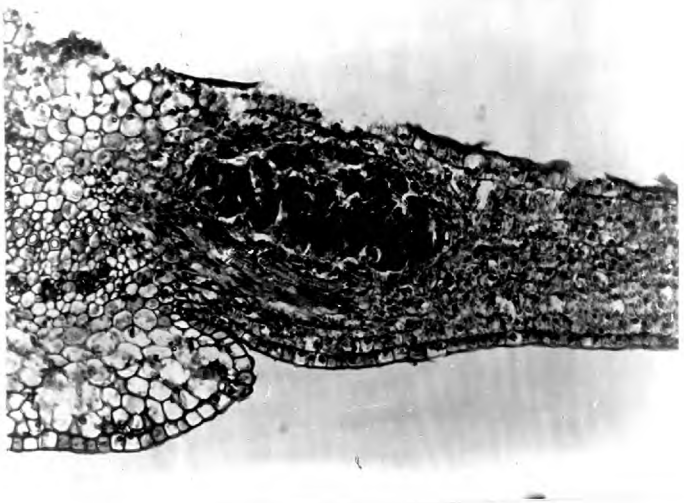


FIG. LXI Pavetta grandiflora. T.S. very young nodule present in a terminal bud leaf.
[1% Carbol fuchsin x 160]

When examined under H.P., the carbol fuchsin-stained bud mucilage was found to contain a large number of bacteria, the majority of which appeared to be intermediate in form between the two types of bacteria found in young leaf nodulated tissues (cf. A. crispa and Psy. emetica). The remainder of the terminal bud bacterial population consisted of a few bacteria of type 1 (bacteroids), of several large (2 - 5μ x 2μ), oval shaped bipolar-staining rods and of a few cocci.

In the very young nodules of the terminal bud leaves, bacteria of types 1 and 2 were found to occur in approximately equal numbers.

(d) Inflorescences.

Figure LXIII shows the general picture observed when longitudinal sections of young, developing floral buds were stained by the Carbol thionin-orange G method. A mucilaginous film, which contained bacteria of type 2, enveloped all the floral components and was also found in the ovarian cavity.

Whilst the major portion of the bud tissues were stained yellow, a number of regions within the

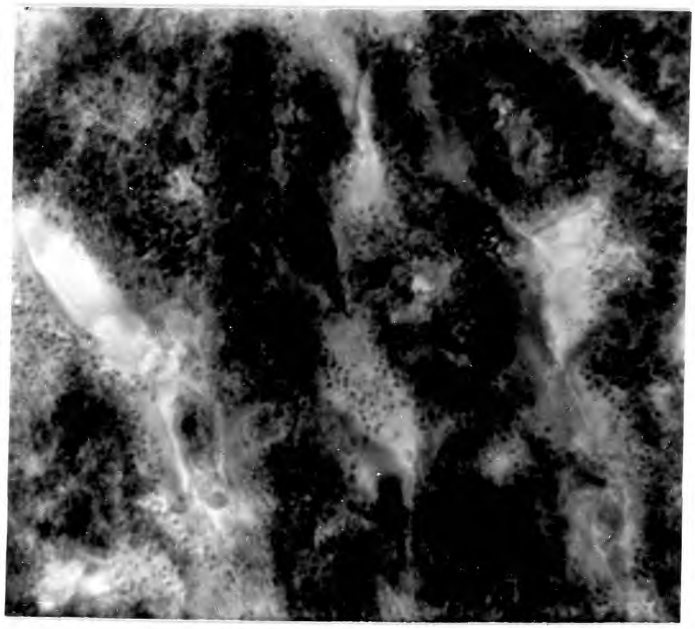


FIG. LXII Pavetta grandiflora. Showing the bacteria present in very young leaf nodules.
[Carbol thionin - Orange G. x 1520]

FIG. LXIII Pavetta grandiflora. L.S. developing floral buds.
[Carbol thionin - Orange G. x 14]

tissues (e.g. the vascular tissues and the peripheral regions of the placenta and stamens) were stained an intense purple colour. These regions, which can be seen in Figure LXIV as the darker areas, were found to contain many purple-stained, rod shaped "objects"; the latter were more evenly stained and more angular in appearance than the bacteria found elsewhere in the plant. Initially, it was considered that these "objects" represented either over-stained bacteria of type 1 or crystals of stain. Accordingly, similar serial sections were stained with Carbol thionin-Orange G, but, in these preparations, the Carbol thionin was applied for 5 minutes only. A similar result was obtained and, since no crystals of stain were seen in the preparation, it was concluded that these "objects" were bacteria. In size they closely approximated to cells of type 1.

In open flowers, the mucilaginous film, which was found only within the ovarian cavity, contained a few bacteria of type 2.

(e) Fruits.

At all stages in their development fruits of

P. grandiflora are morphologically quite similar to those of Psy. nairobiensis and Psy. emetica.

In longitudinal section (Fig. LXV), a developing fruit of P. grandiflora showed a similar appearance to the fruits of the other Rubiaceae species investigated.

The bacterial distribution within the fruit, as also the morphological characteristics of these bacteria, were similar to the results previously obtained with fruits of Psy. emetica and Psy. nairobiensis.

FIG. LXIV Pavetta grandiflora. Showing the bacteria present in the vascular tissue of developing floral buds.
[Carbol thionin - Orange G. x 1520]

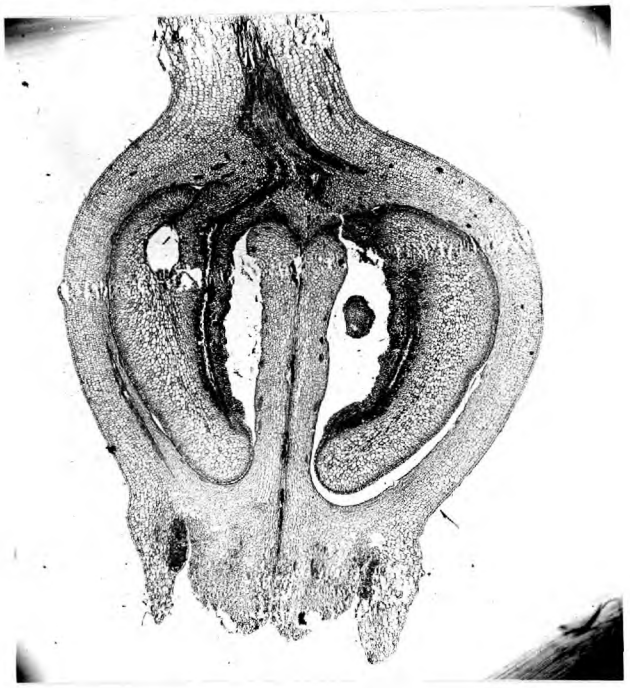


FIG. LXV Pavetta grandiflora. L.S. developing fruit.
[Carbol thionin - Orange G. x 14]

PART 2

THE ISOLATION AND CHARACTERISATION OF THE BACTERIA ASSOCIATED WITH LEAF NODULATED PLANT SPECIES.

I. THE ISOLATION OF BACTERIA.

The literature on leaf nodule-bearing plant species shows that a controversy still exists as to the identity of the bacterial symbiont, or symbionts, within these species. Accordingly, this section of the work was undertaken in an attempt to resolve this question.

Bacterial isolations were made from the four plant species as listed previously (p. 87). In each case, isolation was attempted from leaf nodules, from non-nodulated regions of nodulated leaves and from foliar buds. Additionally, in the case of A. crispa and Psy. nairobiensis, ungerminated and germinated seeds were investigated; seeds of the other two plant species could not be obtained.

During the course of this investigation, a large number of different bacterial species were isolated and, from these, several were selected for further study. The selected species were those which:-

(i) occurred consistently and abundantly on the isolation plates in replicate isolation experiments.

(ii) were morphologically similar to any of the bacterial types observed in serial sections of plant tissues

and (iii) were Gram-negative or Gram-variable.

It was considered that the leaf nodule symbiont was most likely to be found from amongst these species.

All these bacterial species were obtained in pure culture by the application of standard techniques and from a consideration of their morphological, cultural and biochemical characteristics, an attempt was made to establish their identity.

For convenience' sake, the results obtained from each plant species are treated separately.

Ardisia crispa

(a) Leaf nodules of all ages.

In initial attempts to isolate the bacterial symbiont from old leaf nodules, the following technique was adopted:- Approximately 50 nodules were cut out intact from old leaves (i.e. fully developed leaves which were attached to the stem at a position several nodes distant from the terminal bud). After surface-sterilising the nodules by using the mercuric chloride method (p. 93), the nodules were suspended and squashed, under aseptic conditions, in 4 ml. sterile water. The suspension was examined microscopically, when non-motile, Gram-negative, bacteroids of type 1 were observed. This suspension was then plated out onto nutrient agar-, onto malt agar- and onto "nitrogen-free" synthetic agar media, all of which were of pH 7. The plates were incubated at 25°C and examined daily over a 7 day period for bacterial growth.

In all experiments conducted in this way, at the end of the incubation period no bacterial colonies had developed on any of the plates.

Initially it was considered that this failure to isolate the bacterial symbiont from old leaf nodules was possibly due to the employment of incorrect cultural conditions and, of these, the type of culture media used was probably the most critical. Accordingly, the experiment was repeated but in this instance, in addition to employing the same three media, potato dextrose agar and A. crispa leaf extract agar were employed. As before, no colonies developed on the plates.

It seemed unlikely that the physical conditions employed ($25^{o}C$ and pH 7) could be responsible for the inability to isolate the leaf nodule symbiont, since these conditions must be approximately similar to those encountered by the bacterium in its natural, tropical milieu.

A number of other factors were considered as the cause of these negative results:- (i) the sterilising agent ($HgCl_2$) killed not only the bacterial population on the leaf surface, but also penetrated the plant tissues and killed the nodule bacteria; (ii) $HgCl_2$ was adsorbed by the plant tissues

and, even after washing, a concentration of Hg^{++}, sufficient to inhibit bacterial growth, remained in the aqueous suspension and plating medium; or (iii) the bacterial suspension (inoculum) was too dilute.

With the object of testing these possibilities, a number of other experiments were performed. In these experiments, surface-sterilisation was effected by using the chloramine-T method and instead of crushing the surface-sterilised nodules in 4 ml. sterile water, only 1 ml. was employed. The cultural conditions were similar to those employed above and the results obtained, at least insofar as bacterial colonies were concerned, were again negative. Thus, whilst in one or two experiments fungal colonies developed on the isolation plates, in other, similar, experiments the plates remained free of growth of any type of microorganism.

Accordingly, insofar as old nodules were concerned, the use of chloramine T was abandoned and instead it was decided to resort to simple, mechanical washing of the leaf nodule surfaces in several

changes of sterile water. Once again, the results were negative, yeast colonies appearing on all isolation plates in three experiments, whilst no growth of any description was obtained on any of the isolation plates in two similar experiments.

The histological investigations, reported elsewhere in this Thesis, had shown that the bacterial population of old leaf nodules was exclusively "bacteroid" in nature and possibly this factor was the governing factor accounting for the inability to isolate the bacterial symbiont from old leaf nodules. In view of this, it was decided to attempt bacterial isolation from very young leaf nodules (i.e. those present on the outer leaves of the terminal bud). In all these experiments, the leaf surface microflora was removed by mechanical washing in several changes of sterile distilled water and cultural conditions, similar to those employed in isolation attempts from old nodules, were used. In four replicate experiments no bacterial colonies developed on the isolation plates.

(b) Non-nodulated regions of nodulated leaves of all ages.

When attempting bacterial isolation from this source, the technique adopted throughout this work was as follows:- An intact leaf was mechanically rinsed in several changes of sterile water. Whilst employing aseptic conditions, several non-nodulated segments (each of approximately 1 mm. sq.) were cut out from the leaf and introduced into 1 ml. sterile water. After macerating the leaf segments with a heat-sterilised glass rod, the resulting suspension was examined microscopically prior to plating out onto the same media as employed for leaf nodule-bearing tissues. All plates were incubated at 25°C for 7 days and then examined for the presence of bacterial colonies.

Four replicate experiments, employing old leaves, were performed in this way. In each case, the leaf-macerate inoculum was found to contain non-motile cells of type 2. The latter were present in low concentration (approximately 10^2 cells/ml) and were Gram-negative. In one of these experiments,

bacterial colonies appeared on all culture media with the exception of "nitrogen-free" synthetic medium; the colonies were identical in colour (pink) and in form, but an average of only 2 colonies/plate was recorded. When examined microscopically, they were found to consist of rod-shaped cells, whose dimensions (2 - 5μ x 1μ) were at variance with those of the bacteria seen in the original inoculum. In the other replicate experiments, all plates remained free of bacterial growth.

Similar experiments were performed, in replicate, on non-nodulated regions of young leaves, when very similar results to those recorded for old leaf tissues were obtained. In some of these experiments, yeast colonies developed.

(c) Terminal buds.

In initial attempts to isolate the leaf nodule symbiont from this source, the following method was employed:- Excised shoot tips (containing the terminal bud) were washed, with shaking,

in several changes of sterile water. They were then introduced into test tubes (one bud/tube), each of which contained 1 ml. sterile water, and macerated with a heat-sterilised glass rod. Each suspension was examined microscopically for the presence of bacteria, prior to plating out onto nutrient agar, malt agar, potato dextrose agar, A. crispa leaf-extract agar and "nitrogen-free" synthetic medium.

Microscopic examination showed that the inoculum contained a large number of bacteria, the majority of which were of types similar to those noted in serial sections of A. crispa terminal buds. All types were non-motile and Gram-negative and, as in serial sections, the "bacteroids" were unevenly stained. In addition, a few yeast-like cells were present.

However, in spite of the fact that the bud suspension contained numerous bacteria, when plates were inoculated with this suspension and incubated for 7 days, only very few bacterial colonies developed; plates of nutrient agar, malt agar and potato dextrose agar contained, on average, two or three bacterial colonies, whilst none developed on plates of the other

two media. A number of cream-coloured, yeast colonies (an average of 10/plate) developed on the plates of all media with the exception of the "nitrogen-free" medium. The bacterial colonies were of two types, coloured pink and yellow respectively. In Gram-staining (Gram-negative) and morphological properties, the organism producing pink colonies was identical to the pink colony-forming bacterium isolated from non-nodulated tissues of old leaves; the yellow colonies were made up of large bacterial cocci, whose arrangement in tetrads caused them to resemble microorganisms of the genus Sarcina. These two bacterial species failed to conform to criteria (i) and (ii) (page 204) and hence were not studied further in this context.

This experiment was repeated on two further occasions, when similar results were obtained.

It is not easy to find an explanation as to why the leaf nodule bacterium had not developed on the isolation plates, for it appeared to be present in high concentration in the inoculum. One possible explanation could be that the culture media employed were not suitable for supporting the growth of this bacterium,

although this would seem to be unlikely, since one of the media used was made up from tissue extracts of A. crispa leaves.

It was decided to investigate another method of isolation and this was done as follows:- Terminal buds were sterilised by the chloramine T method and then introduced (one bud/tube) into test tubes containing either 0.5 ml. sterilised phosphate buffer (KH_2PO_4 - Na_2HPO_4; 0.1 M; pH 7) or 0.5 ml. sterilised phosphate buffer supplemented with 1% (w/v) glucose. The buds were then aseptically crushed and the resulting leaf extract suspension incubated at 25°C. This initial incubation procedure was adopted with a view to giving the terminal bud bacteria a chance to multiply under conditions which simulated those existant in their natural environment.

After 4 days, the contents of the tubes were visually examined, when a light turbidity was noted in all. Microscopical examination showed that little or no increase in the number of bacteria had occurred; a large proportion of the bacteria were "bacteroid" in

nature. The suspensions were plated out onto media similar to those used in the initial isolation attempts and also onto pea-extract agar. The plates were incubated at 25°C and examined daily for 7 days, after which time no bacterial colonies had developed.

Further modifications to this method were introduced, but they, likewise, failed to bring about isolation of the terminal bud bacteria. Such modifications included:- (i) experiments in which water-sterilised, crushed buds were incubated at 25°C for periods of 3 to 7 days in either phosphate buffer (0.5 ml) or in similar buffer supplemented with sugars (either 1% w/v glucose or 1% w/v sucrose), prior to plating out onto the same six media as used previously; (ii) a similar experiment to the above in which water-sterilised, crushed buds (suspended in sugar-supplemented phosphate buffers) were incubated aerobically or anaerobically, prior to plating out onto the same six media, which were then incubated aerobically or anaerobically; or (iii) incubating water-sterilised, crushed buds in sugar-supplemented phosphate buffer of varying pH (6.0, 6.5, 7.0) prior to plating onto the same six media.

(d) Seeds

Aqueous suspensions of macerated, ungerminated seeds were found to contain large numbers of non-motile, rod-shaped bacteria. These bacteria, all of which exhibited Gram-negative staining characteristics, were similar to those seen in serial sections of ungerminated seeds.

At the outset of this series of experiments, it was decided to revert to the use of the mercuric chloride method for effecting surface-sterilisation. This decision was based mainly on the fact that the surface of A. crispa seeds is woody and lightly-ridged and hence simple mechanical washing in water might fail to remove that portion of the seed surface microflora which was buried at the base of the ridges. It was considered that the mercuric chloride could exert no effect on the bacterial symbiont, since the latter is buried deeply within the tissues of the seed.

Initially, the following isolation technique was adopted:- Whilst observing aseptic precautions, a number of ungerminated seeds, which had been surface-sterilised by the mercuric chloride method, were sliced

so as to expose the embryo. These half-seeds were then placed (cut-face downwards) onto plates of nutrient agar and malt agar. Examination of the isolation plates after 4 days incubation at 25°C, showed that, whilst 5 out of the 48 half-seeds plated on nutrient agar were surrounded by cream-coloured bacterial colonies, no bacterial colonies had developed on the malt agar plates. On repeating this experiment, a similar result was obtained. It is difficult to interpret the reason for the low frequency of bacterial isolation, since the seed contains a large number of cells of the bacterial symbiont. The experiment was repeated with other culture media, namely pea-extract agar, A. crispa leaf-extract agar and "nitrogen-free" solid synthetic agar, when again only very few bacterial colonies developed. Another factor, which could possibly have accounted for the low frequency of isolation, is the retention of mercury by the seeds. However, this interpretation would seem to be erroneous, since, when the chloramine-T method was adopted for surface sterilisation, a similar result was obtained.

All the seeds employed in the aforementioned

experiments were taken from a batch which had been stored for at least one year, and hence, in order to test the possibility that age might influence the viability of the bacterial symbiont, another experiment, in which fresh (unstored) seeds were used, was performed. In this experiment, surface sterilisation was effected by the mercuric chloride method, after which the seeds were sliced and then plated onto nutrient agar and malt agar. A number of fresh, unsliced, surface-sterilised seeds, placed onto similar media, served as a control. All plates were incubated at 25°C and after 4 days were examined for the presence of bacterial colonies. On both media, all half-seeds were surrounded by a large number of confluent bacterial colonies. The latter appeared to be a mixed culture, since numerous white, pinpoint colonies were dotted on a more confluent, cream, mucoid type of colony. Microscopical examination confirmed that two types of bacteria were present. Both of these exhibited Gram-negative staining characteristics and were of approximately similar dimensions to the bacteria seen in seed tissues. The white pinpoint - and cream mucoid colonies, separated and purified by

"plating out", were set aside for further study, being designated as Ac.1 (A. crispa bacterial isolate No. 1) and Ac. 2 repsectively. In the control experiment 3 out of 40 seeds on nutrient agar were surrounded by a bacillus type of growth, while 1 out of 40 on malt agar was contaminated by a fungal growth; all other unsliced seeds remained sterile.

This experiment was repeated on several occasions, each time with variable degrees of success. This, whilst approximately 95% of the control seeds, in all experiments, remained free of bacterial growth, the frequency of appearance of Ac. 1 and Ac. 2 colonies around the sliced seeds was very variable and ranged, in some experiments, from 20% of the sliced seeds showing both types of colony, up to 100% showing both types. Fresh seeds were employed in all subsequent isolation experiments.

The possibility exists that Ac. 1 and Ac.2 were not originating from within the immediate vicinity of the embryo; thus they could be present just below the surface of the testa and, if this be the case, they

would be exposed as a result of the cutting process. In order to test this possibility, a number of seeds, from which the testa had been removed, were surface-sterilised and then plated onto nutrient agar. After incubation for several days at 25°C, no bacterial colonies had developed around any of the seeds.

Histological studies (see p 164) had indicated that bacteria were present, not only in the immediate vicinity of the embryo, but also within the endosperm tissues. An attempt was made to isolate the bacteria from the endosperm tissues and for this experiment the following technique was adopted:- A number of seeds, from which the testa had been removed, were surface sterilised by the mercuric chloride method and were then sliced in such a way that the embryo was not exposed; the sliced seeds were then placed (cut-face downwards) on nutrient agar and incubated under the same conditions as in the previous experiments. After 3 days incubation, colonies of organisms Ac. 1 and Ac. 2 had developed from under the majority of sliced seeds.

Large numbers of Ac. 1 and Ac. 2 colonies were also obtained when the embryos, aseptically

removed from surface-sterilised, ungerminated seeds, were placed on nutrient agar plates and incubated for 3 days.

Bacterial isolation was also attempted from germinated seeds and for these experiments the following technique was adopted:- Fresh (unstored) seeds were rinsed in water and placed on moist blotting paper in a petri dish. The latter was then placed in an incubator at 25°C and germination allowed to proceed over a 20 day period; throughout this period the seeds were kept moist by the addition of small quantities of water at 4 day intervals. The germinated seeds were surface sterilised by the mercuric chloride method and then introduced and squashed into tubes containing 2 ml. sterile distilled water (1 seed/tube). On microscopic examination, all suspensions were found to contain a very large number of bacteria, which in staining and morphological characteristics were identical to these bacteria seen in serial sections of germinating seeds. Each suspension was plated out onto nutrient agar and onto "nitrogen-free" synthetic medium, being then incubated at 25°C.

A large number of experiments were carried out according to this technique, all of which yielded similar results. Examination of the nutrient agar plates after 2 days incubation showed that, on all plates, hundreds of pale yellow, mucoid, spreading colonies had developed. Microscopical investigation showed that these colonies consisted of slowly motile (many non-motile similar types were also observed), single, rod-shaped bacteria, which in dimensions (1.5 - 2.5μ x 0.5μ), staining characteristics (Gram-negative), shape and arrangement were very similar to those bacteria seen in the original inoculum. This bacterium, which was obtained in pure culture by employing the streak-plate method, was set aside for further study, being designated as Ac. 3. Additionally, nutrient agar plates supported the growth of a few, white, pinpoint colonies (which were macroscopically identical to Ac. 1) and of even fewer, cream, mucoid colonies (macroscopically identical to Ac. 2). The colonies of Ac. 3 appeared to be repressing the growth of colonies of Ac. 1 and Ac. 2.

After 2 days incubation, the "nitrogen-free" isolation medium remained clear of immediately visible

bacterial growth, but, when examined with a hand lens, micro-colonies were seen to be developing. After a further 3 days, all plates of this medium showed the presence of a vast number of grey, mucoid, spreading colonies, these being the sole type of colony which had developed. Microscopical examination showed them to consist of slime-producing, rod-shaped bacteria, many of which appeared to be capsulated. In Gram-stained preparations, these bacteria (Gram-negative) exhibited a remarkable resemblance to the bacteria seen in serial sections of ungerminated seeds; thus, in addition to being of similar dimensions to the bacterial symbiont seen in ungerminated seeds, they were, likewise, stained in a bi-polar fashion. Since colonies of this bacterium were repeatedly isolated in large numbers on "nitrogen-free" synthetic medium, a colony was retained, after purification, for further study; this bacterium was designated as Ac. 4.

In order to check whether those bacteria were coming from the environs of the embryo and <u>not</u> from the seed coat surface, the experiment was repeated in a slightly modified form. After surface-sterilisation

of the germinated seeds, the young plantule was aseptically removed from the seed. A number of plantules were plated, without further treatment, onto nutrient agar and "nitrogen-free" synthetic medium; other plantules (controls) were surface-sterilised by the mercuric chloride method and then plated under identical conditions. After 5 days incubation at 25°C, the plantules on nutrient agar were found to be surrounded by very many colonies of organism Ac. 3; no other organisms had developed. 50% of the plantules on the "control" nutrient agar plates were similarly surrounded by Ac.3 colonies, whilst the remaining plantules were bacteria-free. Colonies of Ac. 4 were present around all plantules on "nitrogen-free" synthetic medium; 35% of the plantules on the "control" "nitrogen-free" plates were likewise surrounded by Ac. 4 colonies.

When this experiment was repeated on further occasions, these results were reproduced. The percentage of plantules in the "controls" which remained sterile varied from one experiment to another, but, without exception, in all of these experiments a number of "control" plantules were surrounded by colonies of the aforementioned bacteria. One possible explanation of this phenomenon

is that mercuric chloride had failed to penetrate between the two cotyledons; if this be the case, then the bacteria which occur in this region (p166) would not be affected by the mercuric chloride treatment.

Psychotria emetica

(a) Leaf nodules of all ages

Examples of both Gram-positive and Gram-negative, rod-shaped bacteria were found in macerated leaf nodules of all ages.

As in the case of A. crispa, in initial attempts to isolate the bacterial symbiont from Psy. emetica nodules, either mercuric chloride or chloramine-T was employed for the surface sterilisation of the excised nodules. Regardless of the age of the nodules or of the use of an extended range of culture media (including Psy. emetica leaf extract medium), the results obtained were similar to those recorded for A. crispa, insofar as no bacterial colonies developed repeatedly from one experiment to another.

When the mechanical (repeated washing) method was employed for removal of the surface microflora from leaf nodules, the results obtained were very different from those recorded for A. crispa. In all of many replicate experiments, a bacterium which produced pink, pinpoint, butyrous colonies was always present in large numbers on all types of media investigated (including "nitrogen-free" synthetic medium). This bacterium was rod-shaped and was larger (1.5 - 4μ x 1 - 1.2μ) than any of the types of bacteria present in serial sections of leaf nodules (p168). In certain respects, however, it did resemble the "bacteroids" of leaf nodules; thus, it was Gram-negative and exhibited bi-polar staining characteristics. This organism, which was repeatedly isolated from old-, middle-aged and young-leaf nodules, was retained for further study; it was designated as Pe. 1 (Psy. emetica bacterial isolate No. 1)

However, Pe. 1 was rarely the sole bacterium which developed on the isolation plates, Two other organisms, which produced bright yellow coloured and fawn coloured colonies respectively, were repeatedly isolated (in large or small numbers, dependant upon the culture medium employed) from nodules of all ages. Microscopical examination showed that both of these

organisms were non-motile, Gram-positive, coryneform bacteria. Additionally, in some similar experiments, yeast and bacillus colonies developed.

When very young leaf nodules were investigated by similar methods, another organism, in addition to Pe. 1, was repeatedly isolated in large numbers. This organism, which gave rise to cream coloured, flat, spreading colonies, was Gram-negative and of similar dimensions to the nodule bacteria of type 2. It differed from the latter, however, in showing no curvature and in being evenly stained. Since it was consistently isolated in large numbers on nutrient agar, malt agar, potato dextrose agar and Psy. emetica leaf extract agar, it was retained for further study, being designated as Pe. 2.

The number of different bacterial species isolated from water-washed nodules was much in excess of the number of different types of bacteria, which histological investigations (p168) had shown to be present in this material. Accordingly, with a view to investigating the possibility that some of these

organisms were coming from the leaf surface, some further experiments were conducted.

In these experiments, young leaf nodules were mechanically washed in approximately 15 changes of sterile water. After each wash, the washings were plated out onto nutrient agar. The washed nodules, after maceration and suspension in 1 ml. water, were similarly plated out. The general pattern of results was similar in each of these experiments. On all isolation plates which had been inoculated with suspensions of leaf nodule tissue, colonies of organisms similar to those described above were obtained in abundance. Plates which had been inoculated with the first washing presented a similar appearance, large number of Pe. 1 and Pe. 2 colonies, amongst others, being present. Similar colonies appeared (but now much less abundantly) on those plates which had been inoculated with the 2nd, 3rd, 4th, 5th or 6th washings respectively. On the remaining plates, no bacterial colonies developed.

These results indicate that the majority of the leaf nodule bacterial isolates (including Pe. 1 and Pe. 2) are probably surface inhabiting microorganisms. However, since bacteria (Pe. 1 and

Pe. 2) were also obtained from nodules which had been washed 15 times (the later washings yielding no bacteria), it may be concluded that these bacteria are, in fact, present within the nodular tissue. (The possibility that bacteria remain undisturbed in the "furrows" on the leaf surface must not be ignored).

(b) Non-nodulated regions of nodulated leaves.

A Gram-stain, performed on an aqueous suspension of macerated, non-nodulated leaf tissue, yielded a similar result to that recorded above for nodulated leaf tissue.

When isolation was attempted from this source, results similar to those recorded for water-washed leaf nodules were obtained.

(c) Terminal buds

A Gram-stain, performed on macerated terminal bud tissues, showed that, in all probability, more than one bacterial species is present in this location. Whilst the majority of terminal bud bacteria exhibited Gram-negative staining characteristics, a number of

Gram-positive forms were also present. Included in the former group were types 1 and 2 (see p 176), as also were several, chain-forming bacterial cells.

Isolation experiments conducted with water-washed buds confirmed these findings. Thus, in addition to bearing innumerable colonies of organisms Pe. 1 and Pe. 2, the isolation plates (of nutrient agar, malt agar, potato dextrose agar and Psy. emetica leaf extract agar) showed the presence of several other organisms. Included amongst these were the two Gram +ve coryneform bacteria (previously isolated from leaf nodulated tissues), several yeast colonies and a very large number of colonies of a Gram-negative, rod-shaped bacterium which produced lemon-yellow colonies. This latter organism was retained for further study and was designated as Pe. 3.

Histological studies (p 176) had shown that, whilst a mixed bacterial population was present in the mucilage-filled interfoliar spaces of the bud, the primordial foliage leaves appeared to contain only one type of bacterium. An attempt was made to isolate

the bacteria from primordial foliage leaves. After being stripped of stipules and outermost foliage leaves, the bud was rinsed in 95% alcohol (to remove the mucilage from the stem apex and primordial leaves) and then treated by the mechanical washing method, prior to maceration and plating. Colonies of Pe. 1, Pe. 2 and Pe. 3 again developed in large numbers.

Psychotria nairobiensis

(a) Leaf nodules of all ages.

Gram-negative bacteroids were the sole type of organism observed in smears of macerated leaf nodules.

Irrespective of the method of sterilisation employed, of the culture media investigated and of the age of the leaf nodules, no bacterial culture was ever isolated (i.e. in abundant amount and repeatedly) from this source.

(b) Non-nodulated regions of nodulated leaves.

Bacteria were not observed in tissue macerates of non-nodulated regions of old leaves; a few

Gram-positive bacteria of type 2 (p 189) were present in smear preparations of squashed young leaves.

All attempts to isolate the bacteria from non-nodulated regions of young leaves were unsuccessful.

(c) Terminal buds

Only one type of bacterium, namely an unevenly-stained, Gram-negative rod, was found in smears of macerated terminal bud tissue.

Notwithstanding the fact that the terminal bud contained large numbers of these bacteria, all initial attempts to isolate bacteria from this source failed.

When water-washed buds were squashed into tubes of phosphate buffer (0.5 ml; pH 7; one bud/tube) and then incubated for 2 days prior to plating out onto a range of plant extract media (Psy. nairobiensis leaf extract agar; malt agar; potato dextrose agar), numerous pale-cream, fluorescent colonies grew up on all media. These colonies were isolated in 3 out of 5

replicate experiments and microscopical examination showed that they were produced by a Gram-negative, straight-sided, rod-shaped bacterium of approximately similar dimensions to the bacterium seen in terminal bud macerates. This organism was retained for further study, being designated as Pn. 1 (Psy. nairobiensis bacterial isolate No. 1).

(d) Seeds.

In all attempts to isolate the bacterial symbiont from seeds, fresh (unstored) seeds were employed and surface sterilisation was effected by the mercuric chloride method.

As with A. crispa, isolation of the bacterial symbiont was attempted both from ungerminated and from germinated seeds; a series of isolation experiments, similar to that described previously for A. crispa, was undertaken.

When bacterial isolation was attempted from ungerminated seeds, the following results were obtained:-

After 3 days' incubation at 25°C, the majority of seeds which had been sliced through the embryo prior to plating onto nutrient agar and malt agar, were surrounded by two different types of bacterial colonies. These were coloured cream-white and lemon-yellow respectively and after purification by the streak-plate technique, each was examined microscopically. The cream-white colonies consisted of small (1-1.5μ x 0.5μ), motile, Gram-negative rods, whilst the lemon-yellow coloured colonies were produced by slightly larger (1.5-2μ x 1μ), motile, Gram-negative to Gram-variable rods. Since these organisms were obtained in large numbers in each of several similar experiments, they were retained for further study, being labelled as Pn. 2 and Pn. 3 respectively. Neither of these organisms was obtained on the control (unsliced seeds) plates. In most of these experiments, however, not all of the control seeds remained sterile. Thus, in some instances, as many as 20% of the control seeds were surrounded by a bacillus type of growth. Colonies of this bacterium were not present around the sliced seeds; it would thus appear that the development of

this organism was being prevented, in some unknown way, by organisms Pn. 2 and/or Pn. 3.

This bacillus type of colony was also obtained when, in an experiment similar to that recorded previously for A. crispa, seeds, which had had their testa removed prior to surface-sterilization, were plated onto nutrient agar. However, the majority of seeds (95%) remained bacteria-free. In all probability this bacillus was a seed surface micro-organism.

Organisms Pn. 2 and Pn. 3 were also obtained when surface-sterilised seeds, which had been sliced in such a way that the embryo was not exposed, were plated and incubated on nutrient agar.

Microscopically-examined, Gram-stained preparations of aqueous suspensions of macerated, germinated seeds were found to contain large numbers of Gram-negative, rod-shaped bacteria, whose staining and morphological characteristics were similar to those bacteria as seen in serial sections of germinated seeds.

Isolation attempts from germinated seeds were carried out under exactly similar conditions to those described previously for A. crispa. Examination of the nutrient agar isolation plates after 2 days incubation showed that hundreds of pale-yellow, mucoid, spreading colonies, of very similar appearance to colonies of Ac. 3 (the bacterium isolated from germinated seeds of A. crispa), had developed. Additionally (cf. A. crispa), a few colonies of bacteria Pn. 2 and Pn. 3 were obtained. Microscopical examination confirmed that the pale-yellow, mucoid colonies consisted of bacteria, which in size, shape and staining characteristics, were very similar to bacterial species Ac. 3. Since this bacterium was repeatedly obtained in many replicate experiments, it was retained for further study, being designated as Pn. 4.

Large numbers of grey, mucoid, spreading colonies were the sole type of colonies present on plates of the "nitrogen-free" synthetic medium (cf. A. crispa). To the naked eye these colonies resembled the colonies obtained as a result of plating macerated,

germinating seeds of A. crispa onto the same medium (p 222). Microscopical examination confirmed that these bacteria were morphologically very similar both to organism Ac. 4 and to the bacteria seen in ungerminated seeds of Psy. nairobiensis. This bacterium appeared consistently and in large number in all such isolation experiments; hence it was retained for further study and was labelled Pn. 5.

Pavetta grandiflora

(a) Leaf nodules of all ages.

In many respects the results of attempted isolations from leaf nodules of P. grandiflora were similar to those recorded previously in this Section for Psy. emetica.

The mechanical (repeated washing) method was adopted for the removal of surface microflora from nodules. Using identical isolation procedures one particular type of bacterial species was isolated in the majority of experiments; this was a rod-shaped

bacterium which exhibited very similar morphological and staining characteristics to bacterial isolate Pe. 1. It was retained for further study being designated as Pg. 1 (P. grandiflora bacterial isolate No. 1). Several other species of microorganisms were isolated during the course of these investigations, but, since they did not conform to the criteria laid down at the beginning of this section (p 204), they were not studied further.

(b) Non-nodulated regions of nodulated leaves.

Bacterium Pg. 1 was isolated in large numbers from water-washed non-nodulated leaf tissues. Several, coryneform, Gram-positive bacterial species - similar to those isolated from nodulated tissues - were also obtained. However, as in the case of isolation attempts from leaf nodules, these species were not obtained consistently from one experiment to another. In an experiment similar to that described for Psy. emetica (p 227), both Pg. 1 and the coryneform bacteria developed on plates which had been inoculated with a sample from each of the first few washings. The vast majority of plates which had been inoculated either from the later washings or with the

washed, macerated, non-nodulated leaf tissues remained sterile. It would thus appear that the bacteria isolated in these experiments are leaf surface-inhabiting organisms.

(c) Terminal buds

A Gram-stain, performed on macerated terminal bud tissues, showed that, possibly, more than one bacterial species is present at this location. Thus, whilst all of the bacteria observed in terminal buds were Gram-negative, a number of morphologically distinct types were present. The majority of bacteria exhibited morphological characteristics similar to those of the bacteria seen in serial sections of terminal buds. A few, larger (1.5-4μ x 1.5-2μ), unevenly stained, rods were also present.

When isolation was attempted from water-washed buds, a large number of different bacterial species developed on the isolation plates. In all probability, many of these species were surface-inhabiting microorganisms and hence, in an attempt to cut down the number of contaminants, resort was made to

the use of a sterilising agent. Buds were surface sterilised in mercuric chloride, prior to washing, macerating and plating out. Again, a large number of different bacterial species developed on the isolation plates (cf. A. crispa, Psy. emetica and Psy. nairobiensis). It would appear that, in the case of P. grandiflora, the mercuric chloride had failed to penetrate the stipular coverage; if this be the case, then this fact could possibly explain why so many bacteria appeared on the isolation plates. It would also seem probable that the interfoliar spaces of the terminal bud housed a mixed bacterial population.

An attempt was made to isolate those bacteria which are present within the primordial foliage leaves of the bud (see p 197). After a bud had been stripped of stipules and outermost foliage leaves, it was rinsed in 95% alcohol and then treated by the mechanical (repeated washing) method. The bud was then macerated in 2 ml. sterile water and plated onto nutrient agar, malt agar and potato dextrose agar. After 3 days incubation, the plates showed the presence of two types of colonies, both of which occurred abundantly. One type consisted of pink colonies of bacterium Pg. 1,

whilst the second type consisted of pale yellow, circular, colonies, which were produced by a motile, Gram-negative rod-shaped bacterium. Since the latter organism was obtained in 4 out of 5 replicate isolation experiments, it was set aside for further study, being designated as Pg. 2.

II. CHARACTERISATION OF THE BACTERIAL ISOLATES.

Using the methods previously described (see pp. 97-107), an attempt was made to establish the identity of each of the selected bacterial isolates. The morphological and growth characteristics of each isolate are summarized below, and the physiological and biochemical characteristics are set out in Table I.

(a) Morphological and Growth characters.

Some features were common to all the selected isolates; thus, each was a Gram-negative, non-sporing, rod-shaped organism, which was not acid fast when stained by the Ziehl-Neelsen method but was acid-fast when stained by von Faber's method.*

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* The bacteria present in plant tissues likewise were not acid-fast when stained by the Ziehl-Neelsen method, but were all found to be acid-fast when von Faber's method was employed. The latter method was considered to be unsatisfactory for investigating the acid-fastness of bacteria, since even a known non-acid-fast bacterium (*Escherichia coli*) was positive when stained by this method. Only the control bacterium (*Mycobacterium phlei*) was found to be acid-fast by the Ziehl-Neelsen method and hence it was concluded that neither the bacterial isolates nor the *in vivo* bacteria were acid-fast.

Bacterial isolate Ac. 1.

[A] Morphology and staining characters.

Cells grown in nutrient broth or on nutrient agar were straight, evenly-staining, non-capsulated, motile (1 - 4 polar flagella) rods, arranged singly or in pairs, with rounded ends and measuring 1 - 2μ x 0.3μ.

[B] Growth characters.

In nutrient broth, after 2 days incubation, there was an abundant growth, as evidenced by a dense uniform turbidity, a moderate viscid deposit and a thin smooth surface pellicle. There was little change after 7 days.

Growth on nutrient agar (in slope or plate culture), after 2 days incubation, was abundant. Individual colonies were circular, 2 mm. in diameter, convex, amorphous, white and translucent, with a smooth glistening surface and an entire edge. They were of butyrous consistency and were easily emulsifiable in water. There was little change after 7 days.

No growth occurred on "nitrogen-free" media. When grown in Georgia and Poe's medium, after 2 days, the culture exhibited a duck-egg blue fluorescence when examined under U.V. light. In visible light the medium fluoresced to a pale green colour after 7 days.

Bacterial isolate Ac. 2.

[A] Morphology and staining characters.

In dimensions, cell arrangement, motility (1 - 4 polar flagella) and staining characters, organism Ac. 2 resembled organism Ac. 1. It differed from the latter, however, in a number of respects; thus the cells of Ac. 2. were pointed at the poles, were capsulated and produced slime.

[B] Growth characters.

In nutrient broth, after 2 days incubation, there was an abundant growth, as shown by a dense, uniform turbidity, an abundant, fine, flocculent deposit and a thick, granular, friable pellicle. A similar result was recorded after 7 days.

Growth on nutrient agar (in slope or plate culture), after 2 days, was abundant and largely confluent. Individual colonies were circular, 2 - 6 mm. in diameter, convex, amorphous, cream coloured and opaque, with a smooth, moist surface and an entire edge. They were butyrous and easily emulsifiable in water. After 7 days there was little change in the appearance

of the bacterial growth, but the medium was turned dark brown in colour by the diffusion of some unknown substance from the culture.

This bacterium grew extremely well on "nitrogen-free" synthetic medium, producing grey, mucoid circular, raised colonies. No fluorescent pigment was produced in Georgia and Poe's medium.

Taxonomic investigations showed that bacterial isolates Ac. 3 and Ac. 4 were specifically identical; the designation Ac. 4 is, therefore, abandoned.

Bacterial isolate Ac. 3.

[A] Morphology and staining characters.

Cells from nutrient agar were slightly curved, irregularly stained, capsulated rods, occurring either singly or in pairs or in long, thin, wavy chains (up to 8 cells long); the wavy nature of the chains was due both to the slight curvature of the individual cells and to a type of "snapping-fission" which these cells exhibited. The individual cells had irregular sides, pointed ends and were of dimensions 1.5 - 3.5μ x 0.3 - 0.5μ. They were non-motile, produced a profuse amount of slime and bore a strong morphological resemblance to the bacterium seen in germinating seeds.

When cultured on "nitrogen-free" synthetic medium, this isolate showed a pronounced tendency to pleomorphism. Cells from 2-day-old cultures were slightly curved, bipolar-stained, capsulated, slime-producing, non-motile rods, occurring singly or in pairs,

with rounded ends, slightly bulging sides and measuring 1.5 - 2.5μ x 0.5μ; these cells were morphologically very similar to the bacteria seen in ungerminated seeds. After 7 days, all cells were swollen and ellipsoidal in shape; cultures examined after a further 14 days consisted exclusively of bipolar-stained, Azotobacter-type cells, each enclosed in a greatly enlarged capsule (Fig. LXVI).

[B] Growth characters

This isolate produced a very poor growth in nutrient broth; no growth was evident after 2 days, whilst after 7 days the cultures showed a slight friable ring, a faint turbidity and a moderate amount of a pale yellow viscid deposit.

Growth on nutrient agar, after 2 days, was abundant and, even in plate culture, was largely confluent. Individual colonies were irregular, up to 10 mm. across at their widest point, raised, amorphous, pale yellow and opaque, with a smooth moist surface and an abrupt edge. They were mucoid and easily emulsifiable in water. After 7 days the colonies were covered

by a dull, contoured skin; at this stage they were of viscid consistency and were firmly attached to the surface of the medium. Notwithstanding the fact that it grew rapidly and abundantly on nutrient agar, Ac. 3 was unable to survive on this medium for longer than 7 days.

Ac. 3. grew extremely well on the "nitrogen-free" synthetic medium, producing irregular, raised, amorphous, smooth, moist, spreading, grey translucent colonies. Continued culture on this medium resulted in the appearance of a "rough" variant, which was observed when such cultures were subsequently plated on nutrient agar; this was designated as Ac. 3 "R" to distinguish it from the normal "smooth" type Ac. 3"S" (isolate Ac. 3 as described above). In morphological and growth characters, Ac. 3"R" differed from Ac. 3"S" in the following respects:- Rods from nutrient agar (see Fig. LXVI) were more evenly stained, occurred mainly singly (no chains observed) and measured 1.5 - 2.5μ x 0.5μ; they were motile in young cultures (up to 36 hr. old) by a single polar flagellum. In nutrient

broth a flocculent deposit was formed, whilst, on nutrient agar, discrete, circular, 2 mm. diameter, convex, coarsely granular colonies, with an entire edge, were produced.

Bacterial isolate Pe. 1.

[A] Morphology and staining characters.

Cells grown in nutrient broth or on nutrient agar were straight, bipolar-staining, non-capsulated, rods, occurring mainly singly but also in pairs. They were motile (single polar flagellum) in young culture (up to 24 hr.), of dimensions 1.5 - 4μ x 1.0 - 1.2μ and their rounded ends and bulging sides gave them an ellipsoidal appearance.

[B] Growth characters.

In nutrient broth, after 2 days, there was only scanty growth; no surface growth occurred, the medium remained clear and there was a slight, viscid, pink deposit. There was a slight turbidity and a moderate viscid deposit after 7 days incubation.

Growth on nutrient agar, after 2 days, was scant. Individual colonies were circular, pinpoint, convex, amorphous, pink and transparent, with a smooth, glistening surface and an entire edge. After 7 days,

the colonies were of 2 mm. diameter and were convex, opaque, viscid, finely granular and of a deeper shade of pink; they adhered strongly to the agar and were difficult to emulsify in water.

A scant amount of growth was produced on "nitrogen-free" synthetic medium. No fluorescent pigments were detected when this organism was cultured in Georgia and Poe's medium.

Bacterial isolate Pe. 2.

[A] Morphology and staining characters.

Cells grown in nutrient broth or on nutrient agar were slightly curved, evenly-stained, non-capsulated, motile (1 - 4 polar flagella) rods, arranged singly or in pairs, with rounded ends, parallel sides and measuring 1 - 2μ x 0.3μ.

[B] Growth characters.

In nutrient broth, after 2 days incubation, there was a moderate growth, as evidenced by a moderate, uniform turbidity, a slight flocculent deposit and a surface ring around the wall of the tube. After 7 days, the medium was densely turbid and a thin, friable pellicle covered the medium.

Growth on nutrient agar, after 2 days, was moderate. Individual colonies were circular, up to 2 mm. in diameter, convex, amorphous, cream coloured and translucent, with a smooth moist surface and an entire edge. A yellow-green fluorescent pigment had diffused from the culture into the medium. The colonies

were butyrous and easily emulsifiable in water. There was little change after 7 days.

No growth occurred on "nitrogen-free" medium. In Georgia and Poe's medium a duck-egg blue fluorescence was noted under U.V. after 2 days.

Bacterial isolate Pe. 3.

[A] Morphology and staining characters.

Cells grown in nutrient broth or on nutrient agar were straight, evenly stained, motile (single polar flagellum) rods, occurring singly or in pairs, with rounded ends, bulging sides and measuring 0.4 - 1μ x 0.3μ. Slime was produced, but it is doubtful whether a capsule was present.

[B] Growth characters.

A rapid and abundant growth was produced in nutrient broth; after 2 days, a dense, uniform turbidity and a moderate, flocculent deposit were present. After 7 days a ring was present at the surface of the medium.

Growth on nutrient agar (in slope and plate culture), after 2 days incubation, was moderate. Individual colonies were circular, of 2 - 4 mm. diameter, convex, amorphous, pale yellow and translucent, with a smooth glistening surface and an entire edge. They were of butyrous consistency and easily emulsifiable in

water. After 7 days, an abundant number of colonies, the colour of which had intensified to lemon yellow, was present.

Pe. 3 was unable to survive more than one transfer on "nitrogen-free" medium. No fluorescent pigments were produced in Georgia and Poe's medium.

Bacterial isolate Pn. 1.

[A] Morphology and staining characters.

Cells from nutrient broth and nutrient agar were straight, evenly-stained, non-capsulated, motile (1 - 4 polar flagella) rods, arranged either singly (the majority of cells showed this arrangement) or in pairs or in short chains (3 - 4 cells long), with rounded ends and measuring 0.5 - 2μ x 0.4μ.

[B] Growth characters.

In nutrient broth, after 2 days incubation, there was an abundant growth, the cultures showing a dense, uniform turbidity, a moderate, viscid deposit and a thin, friable pellicle. The deposit was abundant after 7 days.

Growth on nutrient agar (in slope and plate culture), after 2 days incubation, was abundant. Individual colonies were circular, up to 3mm. in diameter, low convex, amorphous, cream coloured and translucent, with a smooth moist surface and an entire edge.

They were butyrous and easily emulsifiable in water. After 7 days, a yellow-green, fluorescent pigmentation of the medium was observed.

No growth occurred on "nitrogen-free" media. In Georgia and Poe's medium a duck-egg blue fluorescence of the medium was noted under U.V. after 2 days.

Bacterial isolate Pn. 2.

[A] Morphology and staining characters.

Cells from nutrient broth or nutrient agar were straight, evenly stained, non-capsulated, motile (peritrichate flagellation) rods, occurring mainly in pairs, with rounded ends and measuring 0.8 - 1.5μ x 0.5μ. Small amounts of slime were produced by the cells.

[B] Growth characters.

In nutrient broth, after 2 days, there was a copious growth, the cultures showing a dense, uniform turbidity, an abundant, flocculent-viscid deposit and a surface ring around the wall of the tube. After 7 days the deposit was viscid.

Growth on nutrient agar (in slope or plate culture) was moderate, after 2 days incubation. Individual colonies were circular, 1 - 3 mm. in diameter, low convex, amorphous, cream coloured and opaque, with a smooth, glistening surface and an entire edge.

Such colonies were butyrous and easily emulsifiable in water. A large number of colonies (4mm. in diameter) were observed after 7 days.

No growth occurred on "nitrogen-free" media and no fluorescent pigment was produced in Georgia and Poe's medium.

Bacterial isolate Pn. 3.

[A] Morphology and staining characters.

Cells grown in nutrient broth or on nutrient agar were straight, evenly stained, capsulated, motile (3 - 8 peritrichate flagella) rods, arranged singly or in pairs, with rounded ends and of dimensions 1 - 1.5μ x 0.5μ.

[B] Growth characters.

In nutrient broth, after 2 days, there was a dense, uniform turbidity, an abundant, fine flocculent deposit and a slight, friable surface ring. After 7 days, no surface growth was evident, the medium had clarified and there was an abundant amount of deposit.

Growth on nutrient agar, after 2 days, was moderate, greatly increasing after 7 days. Individual colonies were circular, 1 - 2 mm. in diameter, convex, amorphous, lemon-yellow coloured and translucent, with a smooth, glistening surface and an entire edge. They were of butyrous consistency and easily emulsifiable in water.

No growth occurred on "nitrogen-free" media and no fluorescent pigmentation was produced in Georgia and Poe's medium.

Taxonomic investigations showed that bacterial isolates Pn. 4 and Pn. 5 were specifically identical; the designation Pn. 5 is, therefore, abandoned.

Bacterial isolate Pn. 4.

[A] Morphology and staining characters.

Cells grown on nutrient agar exhibited identical characters to those observed when Ac. 3"S" was cultured on the same medium.

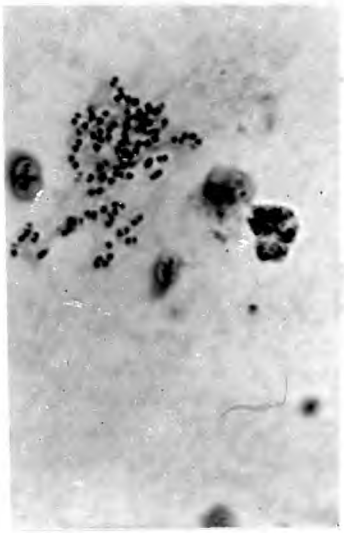
Just as in the case of Ac. 3"S" and Ac. 3"R", when Pn. 4 was cultured on "nitrogen-free"synthetic medium it exhibited pleomorphism. With the exception that they were slightly shorter (1 - 2μ), cells from 2-day old cultures of Pn. 4, in morphological and staining characters, were similar to correspondingly aged cells of Ac. 3"S" and Ac. 3"R"; in older cultures (21 day old) Pn. 4 was morphologically identical to this isolate (Fig. LXVII).

[B] Growth characters.

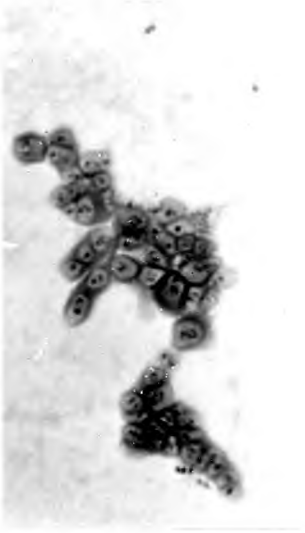
The growth characters of Pn. 4, in nutrient broth and on nutrient agar and "nitrogen-free" synthetic

Nutrient agar (2 days)

"N_2-free" agar (2 days)

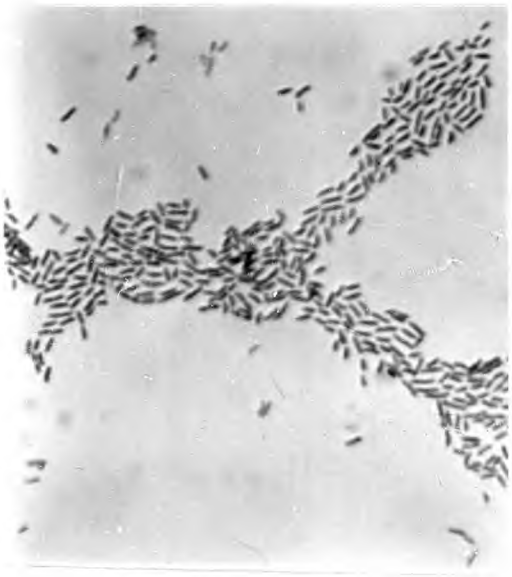


"N_2-free" agar (7 days)

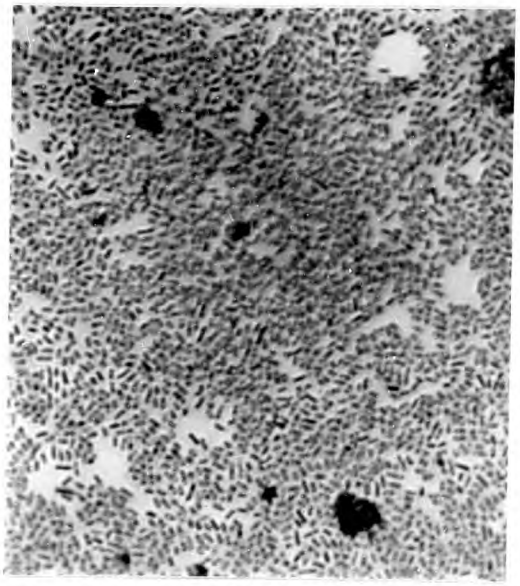


"N_2-free" agar (21 days)

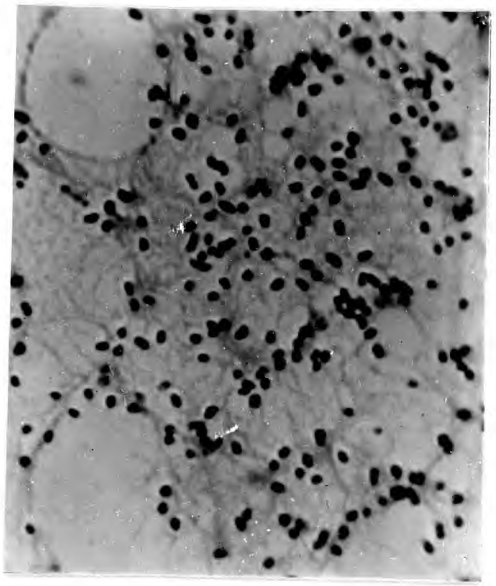
FIG. LXVI Isolate Ac.3. Showing the effect of media and of age of culture on cell morphology.



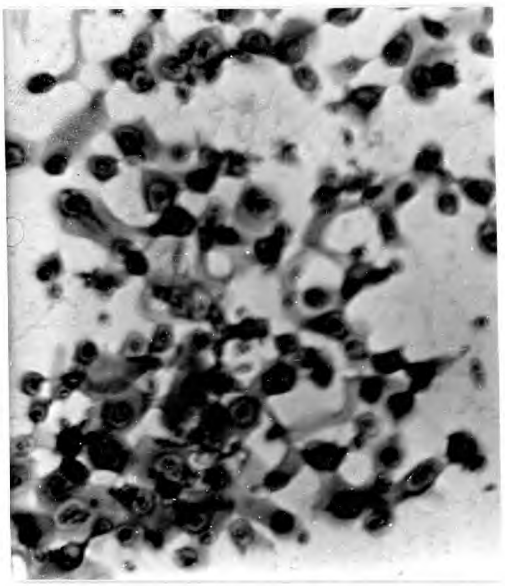
Nutrient agar (2 days)



"N_2-free" agar (2 days)



"N_2-free" agar (7 days)



"N_2-free" agar (21 days)

FIG. LXVII Isolate Pn.4. Showing the effect of media and of age of culture on cell morphology.

medium, were identical to those recorded previously for Ac. 3"S". Continued culture of Pn. 4 on the "nitrogen-free" medium resulted in the appearance of a "rough" variant, which was observed when such cultures were subsequently plated on nutrient agar; this was designated as Pn. 4"R" to distinguish it from the normal smooth type Pn. 4"S" (isolate Pn. 4 as described above). In cell morphology, staining reactions and growth characters, Pn. 4"R" was identical to Ac. 3"R".

Bacterial isolate Pg. 1.

In morphology, staining reactions and growth characters this isolate was identical to isolate Pe. 1.

Bacterial isolate Pg. 2.

[A] Morphology and staining characters.

Cells from nutrient broth and nutrient agar were straight, evenly stained, non-capsulated, motile (single polar flagellum) rods, arranged singly or in pairs, with rounded ends and measuring 0.8 - 1.5μ x 0.5μ.

[B] Growth characters.

In nutrient broth, after 2 days incubation, there was a moderate growth, the culture showing a moderate flocculent turbidity and a slight flocculent deposit. No surface growth was evident. The medium had clarified after 7 days, but a friable surface ring and an abundant flocculent deposit were present.

Growth on nutrient agar, after 2 days, was abundant. Individual colonies were circular, 1 - 5mm.

in diameter, low convex, amorphous, pale yellow and transparent, with a smooth glistening surface and an entire edge. A yellow-green fluorescent pigment had diffused from the culture into the medium. After 7 days, the colonies were butyrous and easily emulsifiable in water.

No growth occurred on "nitrogen-free" media. In Georgia and Poe's medium a duck-egg blue fluorescence was noted under U.V. after 2 days.

(b) Physiological and Biochemical characters.

See Table I.

All isolates were found to be catalase positive. None of the isolates either hydrolysed starch or liquefied pectate gel. These characters are omitted from Table I.

Explanations of abbreviations used in Table I

Ability to grow under strict anaerobic conditions

\+ = growth - = no growth

Effect of temperature

For each isolate the optimum temperature for growth was approximately 25°C.

The range of temperature over which growth was observed is given as A - B, where A is the minimum temperature, and B the maximum temperature of the range. The range tested was from 5°C to 37°C and where either figure is quoted, growth may be possible beyond this range.

Effect of pH

The range of pH over which growth was observed is given as A - B, where A is the minimum pH, and B the maximum pH of the range. The range tested was from 4.4 to 8.4 and where either figure is quoted, growth may be possible beyond this range.

Effect of salt concentration

The range of salt concentration over which growth was observed is given as A - B, where A is the minimum concentration, and B the maximum concentration of the range. The range tested was from 0 to 10% and where 10% is quoted, growth may be possible at a higher concentration.

Fermentation characters

A = acid produced
a = slight amount of acid produced
G = gas produced
g = slight amount of acid produced
- = neither acid nor gas produced

Nitrate

\+ = nitrate reduced to nitrite
- = no nitrite detected
g = gas produced

Litmus milk

A = medium became acid
Alk = medium became alkaline
pep = medium was peptonized
cl = medium was clotted
- = no change in medium

Gelatin

CL = crateriform liquefaction
SL = saccate liquefaction
IL = infundibuliform liquefaction
St.L = stratiform liquefaction
- = no liquefaction

Indol

\+ = Indol produced
- = no Indol produced

V.P.; M.R.

\+ = positive reaction
- = negative reaction

Oxidase

++ = positive, i.e. development of a purple colour within 10 sec.
\+ = delayed positive, i.e. development of a purple colour within 10 - 60 sec.
- = negative, i.e. development of a purple colour after 60 sec. or no development of purple colour.

The superscript figures refer to the number of days incubation required for the particular property to become evident. The properties recorded in Table I refer to the final state of the test.

PHYSIOLOGICAL CHARACTERS	Ac1	Ac2	Ac3"S"	Ac3"R"	Pe1	Pe2	Pe3	Pn1	Pn2	Pn3	Pn4"S"	Pn4"R"	Pg1	Pg2
Anaerobic conditions	-	+	+	+	-	-	-	-	-	-	+	+	-	
Temperature range	17-30	17-37	17-37	17-37	17-30	17-37	17-37	5-30	17-37	17-37	17-37	17-37	17-30	
pH range	5.2-7	5.2-8.4	5.2-7	5.2-7	5.2-7	6-8.4	5.2-8.4	6-8.4	5.2-8.4	5.2-7	5.2-7	5.2-7	5.2-7	
[salt]	0-5	0-5	0-10	0-10	0-1	0-5	0-5	0-5	0-5	0.5-5	0-10	0-10	0-1	
BIOCHEMICAL CHARACTERS														
Fermentation characters: Glucose	A^3	A^1G^1	a^{21}	-	-	A^7	A^1	A^5	A^1G^1	A^1	a^{21}	-	-	A^1
Mannose	a^{14}	A^1G^1	-	-	a^5	A^5	A^1	A^5	A^1G^1	A^1	-	-	a^{21}	A^1
Sucrose	-	A^1G^1	a^{21}	-	-	A^{14}	A^1g^5	-	A^1G^1	A^1	a^{21}	-	-	A^1
Maltose	-	A^1G^1	a^{21}	-	a^{14}	-	A^1	-	A^1G^1	A^1	a^{21}	-	a^{14}	A^1
Lactose	-	A^1G^1	-	-	-	-	A^1	-	A^5g^4	-	-	-	-	A^1
Glycerol	-	A^1G^1	-	-	a^{14}	-	A^4	-	-	a^{21}	-	-	a^{14}	A^3
Mannitol	-	A^1G^1	-	-	a^{14}	-	A^1	-	A^1G^1	A^1	-	-	a^{14}	A^1
Sorbitol	-	A^1G^1	-	-	a^{14}	-	A^1	-	A^1G^1	-	-	-	a^{14}	A^1
Lactose (inorganic medium)	-	A^1G^1	A^{21}	A^{21}	a^4	-	a^4	-	A^4G^4	-	A^{21}	A^{21}	a^4	A^5
Salicin (inorganic medium)	-	A^1G^1	-	-	-	-	a^4	-	A^1G^1	-	-	-	-	A^4
Nitrate	-	$+^4g^4$	-	-	-	$+^4g^4$	-	$+^3g^3$	$+^3g^3$	$+^3$	-	-	-	-
Litmus milk	Alk^2 pep^2	A^1	Alk^2 cl^7	Alk^7	Alk^7	Alk^1 pep^3	A^2 cl^{14}	Alk^5 pep^{14}	A^7 cl^{14}	Alk^5 pep^{21}	Alk^7 cl^7	Alk^7	Alk^5	A^4 cl^7
Gelatin	$St.L^3$	$St.L^4$	CL^{21}	CL^{21}	-	$St.L^3$	SL^{14}	$St.L^7$	-	SL^3	CL^{21}	CL^{21}	-	SL^{21}
Indol	-	-	-	-	-	-	$+^7$	-	-	-	-	-	-	$+^7$
V.P.	-	-	-	-	-	-	$+^5$	-	$+^5$	$+^5$	-	-	-	$+^5$
M.R.	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	$+^1$	$++^1$	$++^1$	$++^1$	$++^1$	$+^1$	$++^1$	$+^1$	$+^1$	$++^1$	$++^1$	$++^1$	$+^1$

TABLE I

PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERS

PART 3

THE IDENTIFICATION OF THE BACTERIAL SYMBIONT.

A number of criteria which must be fulfilled in order to establish that a particular pathological condition is caused by a particular parasite were laid down by Koch in 1891. These state that a disease is caused by a specific bacterium if it can be shown that:-

(i) the bacterium is present in the lesions;

(ii) the bacterium can be isolated and grown in pure culture;

(iii) the isolated bacterium reproduces the disease when inoculated in a healthy host; and

(iv) the bacterium re-isolated from the artificially inoculated host exactly corresponds in all its characters with the bacterium isolated from the lesions.

The work so far described fulfils the first criterion only. However, since a number of different bacterial species were isolated from the four leaf-nodulated plant species, it is uncertain which of

these, if any, is the bacterial symbiont. Normally this question would be resolved by the inoculation of a disease-free host to see if the same symptoms of disease were produced. In the case of the four plants under investigation, however, histological studies have shown that all naturally occurring plants contain bacteria within their tissues; hence, before re-infection experiments could be undertaken, it was necessary to produce bacteria-free plants. Since a reasonable supply of Psy. nairobiensis seeds was available, it was decided to restrict these investigations to this species.

When 30 surface-sterilised (0.1% $HgCl_2$) seeds were raised in sand under aseptic conditions, all 6 plants thus produced showed the presence of leaf nodules. From this result it was concluded that the bacteria present within the seed are responsible for the production of leaf nodules.

von Faber (1912), Miehe (1914), de Jongh (1938) and Hanada (1954) have reported that effective internal sterilisation of seeds can be achieved by the application of suitable heat treatments; in the

present study, all attempts to produce bacteria-free plants involved the use of such methods.

A number of seeds were divided into batches prior to immersion in water, the temperature of which was thermostatically controlled. The combinations of temperature and time to which separate batches of seeds were subjected were 55°C x 10 min., 52°C x 10 min. and 50°C x 10 min. All treated seeds were potted in John Innes soil and raised under greenhouse conditions (23°C; 70 - 90% Relative Humidity). As a control, a number of untreated seeds were sown under the same conditions. Four months after sowing, the results shown in Table II were obtained.

SERIES	TEMPERATURE AND DURATION OF HEATING.	NO. OF SEEDS SOWN.	NO. OF PLANTS PRODUCED.
I	55°C x 10 min.	25	0
II	52°C x 10 min.	25	0
III	50°C x 10 min.	25	2
IV	Control.	25	5

Table II. Psy. nairobiensis. The results obtained after heat treatment of seeds.

All the plants obtained in this experiment appeared to be perfectly normal at the end of 4 months; they bore healthy-looking, green, nodulated leaves. Initially it was thought that heating had exerted a deleterious effect on the seeds of series I, II and III; however, since only 5 out of 25 control seeds had germinated, it would appear that this was not the sole factor accounting for these unsatisfactory results. On repeating the experiment, a similar result was obtained (no seeds in series I germinated; 2, 4 and 7 seeds germinated in series II, III and IV, respectively).

It seemed unlikely that the temperature and humidity conditions employed were at fault, since these approximated closely to the conditions existing in the natural habitat of this species. All seeds employed in the above experiments had been in storage for at least one year, and in order to test the possibility that storage might have caused loss of germination, a number of fresh, unstored seeds were procured. These were employed in a similar experiment to the one described above, and in this experiment another temperature/time combination

(43°C x 24 hrs.) was investigated. The potted seeds were examined periodically and after 4 months the results shown in Table III were recorded.

SERIES	TEMPERATURE AND DURATION OF HEATING.	NO. OF SEEDS SOWN	NO. OF PLANTS PRODUCED.	TYPES OF PLANTS.		
				"normal"	Nodulated "cripples"	Non-nodulated "cripples"
I	55°C x 10 min.	10	0	0	0	0
II	52°C x 10 min.	14	5	0	0	5
III	50°C x 10 min.	34	21	4	13	4
IV	43°C x 24 hrs.	34	23	12	11	0
V	Control	28	25	19	6	0

Table III Psy. nairobiensis. Examination of heat-treated and control seeds 4 months after sowing.

The percentage of germinated seeds in the control series (89%) was significantly higher than that found in the treated series (0%, 36%, 62% and 68%, for series I, II, III and IV respectively) and it was concluded that for seeds of series I to IV, the various heat treatments had affected their ability to germinate.

The seedlings obtained both in the control and treated series emerged above ground level approximately 60 days after sowing, thereby showing that the heat treatments did not affect the germination rate. At this stage there appeared to be no morphological difference between the control and treated seedlings.

Four-month-old "normal" plants (Table III) were tall (average stem length 6 cm) and healthy-looking; they bore green, distinctly nodulated leaves (average n° of 6 leaves/plant), which were arranged on the stem in a decussate fashion. Both the nodules and the terminal buds of "normal" plants contained bacteria. These plants have continued to grow normally and were in every respect normal after 2 years.

Four-month-old "cripples" were characterised by showing short stems (average height 2 cm), which bore small, thin, twisted, pale yellow leaves (average of 3 leaves/plant) and a swollen terminal bud. On the leaves of the majority of "cripples", minute (less than 0.1 mm diameter), bacteria-con-

taining nodules were present. Whilst bacteria were found in the mucilage-filled interfoliar spaces of the terminal buds of nodulated "cripples", none were detected at this location in non-nodulated "cripples".

Nine months after sowing, approximately half of the nodulated "cripples" of series III, IV and V had recovered, and after one year were identical with the "normal" plants. The average height of the stems of these plants was 20 cm. Over a similar period of time, the remaining nodulated "cripples" and all the non-nodulated "cripples" had reached an average height of 5 cm. Very few leaves had developed on these "cripples" and in all such plants the normal terminal bud was replaced by several smaller buds. Eventually the latter developed into irregular protruberances and after one year, when these plants had reached an average height of 8 cm, no further longitudinal growth of the stem took place. (Fig. LXVIII) Two years after sowing the few leaves possessed by these "cripples" withered and died. Simultaneously the swollen terminal bud masses rotted and the plants died.

FIG. LXVIII Psychotria nairobiensis. Showing non-nodulated crippled plant, A, and normal plant, B, after 14 months.

In one respect the results of this experiment were not entirely satisfactory; thus, only 9 out of an initial total of 120 seeds had germinated to produce bacteria-free plants. On the other hand, the experiment had yielded a number of useful results; thus, it had shown that unstored seeds were more suitable for use in heat-treatment experiments than were stored seeds, and also that a temperature/time combination of 52°C x 10 min. was possibly the most suitable combination to employ in subsequent heat-treatment experiments.

Another similar experiment, in which 50 unstored seeds were subjected to 52°C for 10 min. prior to sowing, was undertaken. The percentage germination (40%) in this experiment was similar to that found in the previous experiment, and after four months only one plant showed "normal" features; 11 out of 19 "cripples" were non-nodulated and, at this stage, no bacteria were detected in the tissues of such plants. However, after a further five months, 7 of these non-nodulated "cripples" showed bacteria-containing leaf nodules. They were morphologically similar to the remaining nodulated "cripples"; with

time (14 months), 2 of the newly-nodulated "cripples" assumed a normal appearance.

In any reinfection experiment, a comparison must be made between the effect of reinfection and non-reinfection on the non-infected host. Since, in the two experiments described above, initially non-nodulated plants had become nodulated in none out of 9 cases in one experiment and in 7 out of 11 cases in the other, it is evident that, so as to take account of normal reversion, a reinfection experiment must

(i) be based on a sufficient number of non-nodulated "cripples", infected at random, and

(ii) be designed on a statistical basis.

Furthermore, the proportion of seeds germinated was low and reversion of non-nodulated "cripples" was evident only after nine months.

Thus, to resolve the identity of the supposed symbiont (or symbionts) by the method of Koch's postulates, many seeds and plants and a long period of time are necessary. In this event, it

was considered advisable to approach the problem of identification of the bacterial symbiont(s) by the use of the fluorescent antibody tracing technique.

The underlying principle of the fluorescent antibody method, introduced by Coons et al (1942), is based upon the observation that proteins, including serum antibodies, can be labelled by chemical combination with fluorescent dyes, without material effect on the biological or immunological properties of the proteins. When layered over a histological section or cytological preparation, fluorescent antibody is deposited from solution at sites of specific combination with antigen. The reaction sites are perceived in characteristic colour by fluorescence microscopy. For this the preparation is illuminated by UV/Blue light, which is stopped above the object by a filter designed to transmit only the visible fluorescence emission.

In the last twenty years numerous papers dealing with the biological and medical applications of the fluorescent antibody method have been published, and adequate reviews have been written by

Mellors (1959) and by Nairn et al (1962). Paton (1960) has demonstrated its potentiality as a bacterial antigen-tracing technique in the study of plant-bacteria relationships.

(1) Selection of material for fluorescent antibody studies, and methods adopted for the preparation of antisera.

For reasons put forward in a later section (p[323]), leaf nodules and terminal buds were considered to be unreliable sources for the isolation of the bacterial symbiont. Consequently, fluorescent antibody investigations were restricted to bacterial cultures isolated from seeds of A. crispa (Myrsinaceae) and Psy. nairobiensis (Rubiaceae). A number of bacterial cultures, originally isolated from these sources, were selected for fluorescent antibody study on the basis of:-

(a) frequency of isolation and

(b) morphological similarity to the bacteria seen in plant tissue sections.

The selected isolates, along with the methods adopted for the preparation of antisera, are listed in Table IV.

BACTERIAL CULTURES SELECTED FOR FLUORESCENT ANTIBODY STUDIES.	METHOD OF ANTISERA PREPARATION	
	Culture medium	Immunization Course
Ac. 1	Nutrient agar	Course "a"
Ac. 2	Nutrient agar	Course "a"
Ac. 3 "S"	"N_2-free" agar	Course "a"
Pn. 2	Nutrient agar	Course "a"
Pn. 4 "S"	"N_2-free" agar	Course "b"

Table IV Showing the bacterial isolates selected for fluorescent antibody studies.

(2) Assessment of antibody activity of the antisera; subsequent treatment of antisera.

All antisera, prepared against these bacteria by using the methods described previously (pp 107 - 109), were examined for antibody activity by Dreyer's method. The antiserum prepared against culture Pn. 2 showed strong agglutinating activity when tested (against Pn. 2) by this method; only

weak agglutinating activity was shown when antisera prepared against Ac. 1, Ac. 2, Ac. 3"S" and Pn. 4"S" were examined by the same method. The presence of specific antibodies in these antisera was detected, however, when each bacterial culture was tested against its corresponding antiserum by Elek's gel diffusion technique (1950). It is interesting to note that cultures Ac. 1 and Ac. 2 gave strong, almost identical lines of precipitation by this method, thereby indicating their close relationship. Culture Ac. 3 "S" yielded similar results to culture Pn. 4 "S" in the gel diffusion method.

Unlabelled specific antisera were obtained from each of the four antisera prepared by Course "a" by using the method previously described (p. 109); from the antiserum prepared against isolate Pn. 4 "S", both the corresponding unlabelled specific antibody solution and the corresponding labelled specific antibody solution were obtained (pp 110 - 113).

(3) Fluorescent antibody tracing experiments.

(a) Experiments involving the use of the Direct Staining Method.

Labelled specific antibody solution prepared against isolate Pn. 4 "S" was employed in the Direct Method.

An essential requirement in the employment of the fluorescent antibody method is a decisive demonstration of the immunospecificity of the staining reaction. Accordingly, before any attempt was made to stain the bacteria present in the tissues of A. crispa and Psy. nairobiensis, the labelled Pn. 4 "S" specific antibody was examined for specificity of staining as follows:-

Smears of each of the bacterial isolates obtained from the four leaf-nodulated plant species were prepared. Using the Direct Staining Method, all preparations were treated with labelled Pn. 4 "S" specific antibody. The results of this investigation are shown in Table V. The bacterial cells showing specific staining were strongly fluorescent and

apple-green in colour, whereas those showing non-specific staining did not fluoresce and were dull green in colour.

BACTERIAL ISOLATES TREATED WITH LABELLED Pn. 4"S" SPECIFIC ANTIBODY.	STAINING PATTERN OBSERVED.
Ac. 1	Non-specific
Ac. 2	Non-specific
Ac. 3 "S"	Specific
Ac. 3 "R"	Specific
Pe. 1	Non-specific
Pe. 2	Non-specific
Pe. 3	Non-specific
Pn. 1	Non-specific
Pn. 2	Non-specific
Pn. 3	Non-specific
Pn. 4 "S"	Specific
Pn. 4 "R"	Specific
Pg. 1	Non-specific
Pg. 2	Non-specific

Table V Results of staining bacterial isolates with labelled Pn. 4 "S" antibody.

Those bacterial cultures which stained specifically with this labelled antibody were further checked for specificity of staining by the "blocking test" (p.114). The results, shown in Table VI, confirmed the findings of the previous test.

BACTERIAL ISOLATES EXAMINED	SERUM USED FOR TREATMENT OF SMEARS PRIOR TO STAINING	STAIN USED	STAINING PATTERN OBSERVED
Pn. 4"S"	A	Labelled Pn. 4"S" specific antibody	Non-specific
Pn. 4"S"	B	"	Specific
Pn. 4"R"	A	"	Non-specific
Pn. 4"R"	B	"	Specific
Ac. 3"S"	A	"	Non-specific
Ac. 3"S"	B	"	Specific
Ac. 3"R"	A	"	Non-specific
Ac. 3"R"	B	"	Specific

A = Unlabelled Pn. 4"S" specific antibody (i.e. "blocked" preparation).

B = Non-immune serum.

Table VI Results of the "blocking test".

Having thus established its staining specificity, the labelled Pn. 4"S" specific antibody was then employed in experiments to investigate the presence of bacterium Pn. 4"S" in nodulated plant species; in all such experiments, the "blocking test" was employed as a control. The following results were obtained when Psy. nairobiensis tissues were examined by this method:-

Approximately 95% of the many bacteria present in smears of macerated leaf nodules were found to be stained specifically by labelled Pn. 4"S" specific antibody.

The bacteria present in smears of macerated non-nodulated regions of nodulated leaves, whilst being much fewer in number, were likewise predominantly (95%) stained specifically by the labelled antibody.

Of the numerous bacteria present in terminal bud macerates, only 30% were stained specifically. However, a considerable quantity of mucilage was present in these preparations, and in its staining

and fluorescent characteristics this resembled those bacteria which were stained specifically by labelled Pn. 4"S" specific antibody. Since in the control preparation the mucilage did not fluoresce, the fluorescence noted above must be due to specifically-stained material contained in the mucilage. As the latter was known to contain a large number of bacteria (see Part 1), the fluorescence could be due to some of these bacteria staining specifically and hence imparting their fluorescence to the mucilage as a whole; an alternative explanation could be that some of these bacteria (30%) were identical to Pn. 4"S" and were producing a mucilage-soluble antigen which, in turn, was stained by the labelled Pn. 4"S" specific antibody.

Whilst smears of squashed germinating seeds contained only very few bacteria, almost 100% of those present exhibited specific staining. The paucity of bacteria in seed macerates was possibly due to the fact that, in this case, smears had been prepared from aqueous suspensions of crushed seeds; this maceration method, employed in order to facilitate crushing of the woody-textured seeds, would result in some dilution of the seed bacterial population.

In all cases the control experiments confirmed these findings. From these results it was concluded that bacterium Pn. 4"S" is present in the leaf nodules, non-nodulated regions of nodulated leaves, terminal buds and germinating seeds of Psy. nairobiensis.

In view of the fact that bacterial cultures Ac. 3"S" and Ac. 3"R" had stained specifically with labelled Pn. 4"S" specific antibody, and had each shown very similar taxonomic characteristics to Pn. 4"S" and Pn. 4"R", it was decided to examine A. crispa tissues with the aid of the labelled Pn. 4"S" specific antibody. As far as smears of leaf nodules, of non-nodulated regions of nodulated leaves and of germinating seeds were concerned, the results obtained with A. crispa compared favourably - both with regards to specific staining and to the percentage of specifically-stained bacteria - with those recorded above for the corresponding tissues of Psy. nairobiensis. Macerated terminal bud tissue of A. crispa, whilst likewise containing many bacterial cells, differed from its Psy. nairobiensis counterpart in containing a much higher percentage (almost 100%) of specifically-stained cells.

These results would indicate that bacterium Pn. 4"S" is also present in A. crispa tissues.

(b) Experiments involving the use of the Indirect Staining Method.

The undermentioned antisera were employed in the Indirect Method:-

(i) Unlabelled Ac. 1 specific antiserum.

(ii) Unlabelled Ac. 2 specific antiserum.

(iii) Unlabelled Ac. 3"S" specific antiserum.

(iv) Unlabelled Pn. 2 specific antiserum.

(v) Unlabelled Pn. 4"S" specific antibody.

Before attempting to stain the bacteria present in plant tissues, each antiserum was checked for specificity of staining. For this, duplicate smears of the bacterial cultures Ac. 1, Ac. 2, Ac. 3"S", Pn. 2 and Pn. 4"S" were prepared. One smear of each culture was treated with its corresponding unlabelled specific antiserum (or antibody in the case of Pn. 4"S") and stained according to the method previously described (p. 115); in the other (control) smear, unlabelled non-specific antiserum replaced the unlabelled specific antiserum as the

middle layer of the "sandwich". Fluorescent staining obtained in the first set of smears and lack of similar staining in the controls indicates specificity of staining. All the unlabelled specific antisera (and the unlabelled Pn. 4"S" specific antibody) were found to exhibit staining specificity when examined in this way. A similar control procedure was adopted when plant tissues were examined by the Indirect Method.

When the Indirect Method of Staining was applied to Psy. nairobiensis and A. crispa tissues, the following results (set out below in the order of unlabelled specific antisera employed as the middle layer of the "sandwich") were obtained:-

(i) Unlabelled Ac. 1 specific antiserum.

Bacterium Ac. 1 could not be detected in macerates of Psy. nairobiensis nodular tissue. Only very few (approximately 1%) of the many bacterial cells present in smears of A. crispa leaf nodules were stained specifically with this antiserum.

(ii) Unlabelled Ac. 2 specific antiserum.

Results similar to those recorded for unlabelled Ac. 1 specific antiserum were obtained.

(iii) Unlabelled Ac. 3"S" specific antiserum.

The vast majority of the bacterial cells present in Psy. nairobiensis leaf nodule macerates exhibited a brilliant apple-green fluorescence, indicating specific staining. Macerates of non-nodulated regions of nodulated leaves of the same plant, whilst containing fewer bacterial cells, were similarly found to contain a high proportion of specifically-stained cells. Terminal bud- and germinating seed tissue macerates yielded results similar to those obtained when corresponding tissues were stained by the Direct Method.

When unlabelled Ac. 3"S" specific antiserum was applied to A. crispa tissues, a pattern of results, similar to that obtained when corresponding tissues were stained with labelled Pn. 4"S" specific antibody in the Direct Method, was found.

(iv) Unlabelled Pn. 2 specific antiserum.

Bacterium Pn. 2 was detected in macerates of Psy. nairobiensis leaf nodule tissue, about 1% of the total number of bacterial cells being stained specifically. This bacterium was not detected in A. crispa tissues.

(v) Unlabelled Pn. 4"S" specific antibody.

Tissue macerates of Psy. nairobiensis and A. crispa, treated with this antibody, yielded results analogous to those obtained when corresponding tissues of these two plants were treated with unlabelled Ac. 3"S" specific antiserum.

Unlabelled Pn. 4"S" specific antibody was also used for the detection of bacteria in serial sections of Psy. nairobiensis and A. crispa germinating seeds. For staining these sections, Paton's modification of the Indirect Method (which employs a background counterstain and thereby enhances the contrast between stained bacteria and stained plant tissue) was found to be particularly suitable. Germinating seeds of both plant species were found to contain (almost exclusively) specifically-stained bacteria, which were localized in the embryo and in its surrounding slime.

PART 4

INVESTIGATIONS INTO THE NITROGEN-FIXING ABILITY OF THE BACTERIA ISOLATED FROM MYRSINACEAE AND RUBIACEAE SPECIES.

A number of workers have concluded that the function served by the bacterium in the leaf nodule symbiosis is one of nitrogen-fixation; reference has already been made, in this connection, to the work of von Faber (1912, 1914) and Rao (1923) with Rubiaceae species and of Hanada (1954) with Myrsinaceae species. Since the bacteria isolated by these workers were each of different species, it would appear that a number of different bacterial species, all of which are capable of fixing atmospheric nitrogen when grown in pure culture, are associated with leaf nodulated plants.

Accordingly, the nitrogen-fixing abilities of all the bacterial cultures which had been isolated from the four leaf nodulated plant species under investigation, were examined.

(1) Preliminary investigations.

A number of preliminary "screening" experiments were performed:-

Experiment 1

In this experiment, in addition to examining all the bacterial cultures which had been isolated from the four leaf nodulated plants, several classified bacterial species were included as a control; of this latter group, only Azotabacter chroococcum was known to be a nitrogen-fixing organism.

Saline-washed suspensions (1 x 10^8 cells/ml) of each of the organisms in nitrogen-free physiological saline were streaked out onto plates of both "nitrogen-free" solid synthetic medium and casein-supplemented synthetic medium (p. 116 - 117). All plates were incubated for 5 days at 25°C and then examined for the presence of bacterial colonies. The results of this experiment are set out in Table VII and a selection of these is illustrated in Figs. LXIX and LXX.

ORGANISM EXAMINED	AMOUNT OF GROWTH* ON "N_2-FREE" MEDIUM AS COMPARED WITH AMOUNT OF GROWTH OF SAME ORGANISM ON CASEIN-SUPPLEMENTED MEDIUM.
Experimental organisms.	
Ac. 1	-
Ac. 2	++
Ac. 3"S"	++
Ac. 3"R"	++
Pe. 1	+
Pe. 2	-
Pe. 3	+
Pn. 1	-
Pn. 2	-
Pn. 3	-
Pn. 4"S"	++
Pn. 4"R"	++
Pg. 1	+
Pg. 2	-
Control organisms.	
Escherichia coli	-
Pseudomonas fluorescens	+
Erwinia aroideae	+
Bacillus subtilis	-
Azotobacter chroococcum	++

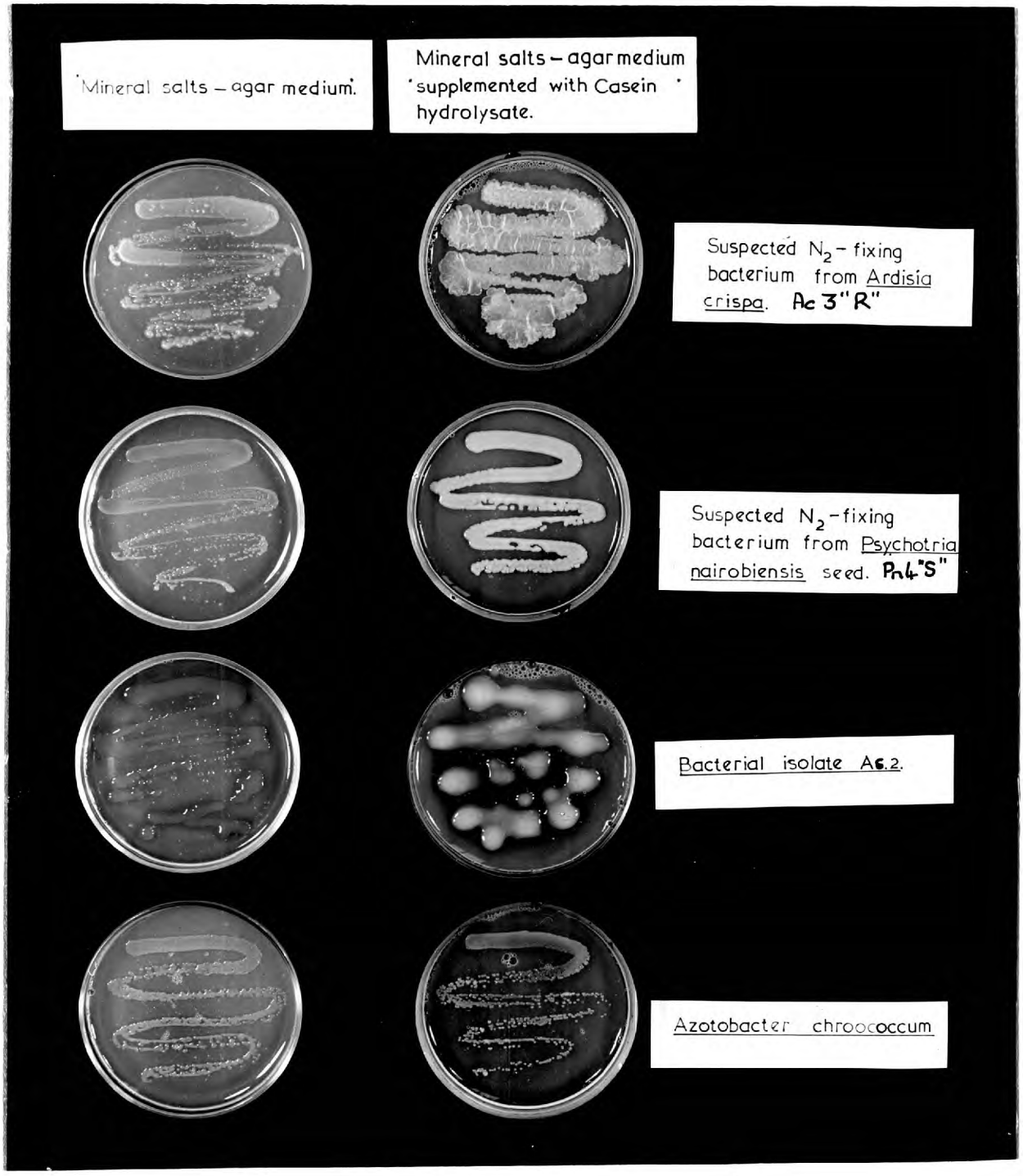
* assessed visually

++ = amount of growth on "N_2-free" medium approximately equal to that produced by same organism on casein-supplemented medium.

\+ = amount of growth much less on "N_2-free" medium.

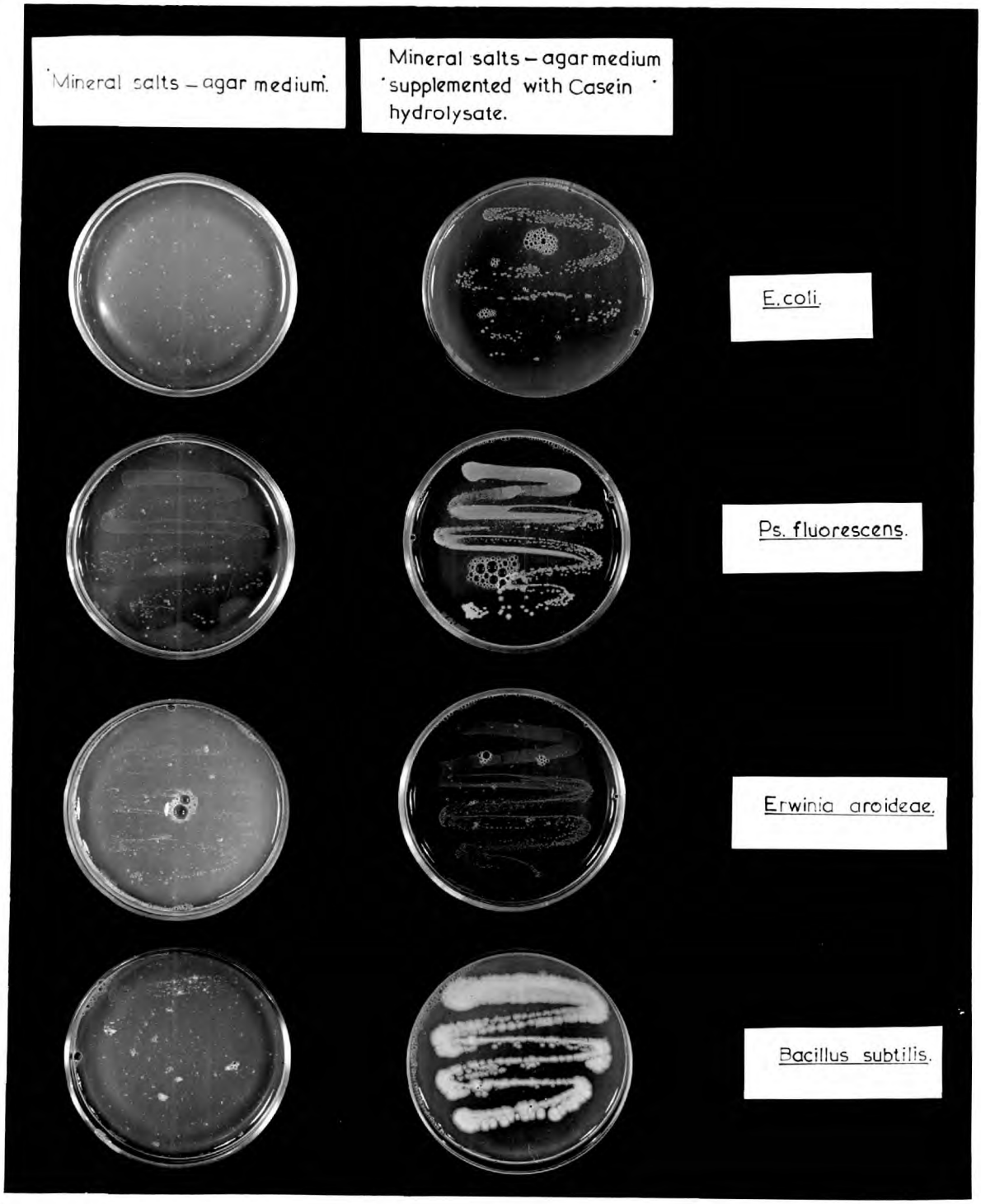
\- = no growth on "N_2-free" medium and hence unable to make a comparison.

Table VII Showing the results of Experiment No. 1.



Comparative growth on N_2-free and Casein supplemented media.

FIG. LXIX



Comparative growth on N_2-free and Casein supplemented media.

FIG. LXX

Whereas all the organisms examined grew on the casein-supplemented medium, the majority were unable to grow on the "nitrogen-free" medium. Those organisms which did not grow on the "nitrogen-free" medium were considered, under the conditions of the experiment, to be incapable of fixing atmospheric nitrogen and were rejected for the purposes of the present study.

Of the remaining organisms some grew vigorously and equally well on both media, whilst others gave very poor growth on the "nitrogen-free" medium as compared with their growth on casein-supplemented medium. As a known nitrogen-fixing organism (Azotobacter chroococcum) was a member of the group of organisms which gave growth on both media, it was considered that the other organisms included in this group were also possible nitrogen-fixers.

Experiment 2

All those organisms, which in Experiment 1 had grown on "nitrogen-free" solid synthetic medium, were subcultured onto plates of the same medium and incubated for 5 days at 25°C.

On examination of the plates, organisms Ac. 2, Ac. 3"S", Ac. 3"R", Pn. 4"S", Pn. 4"R" and Azotobacter chroococcum were found to have produced an abundant amount of growth, whilst organisms Pe. 1 and Pg. 1 had produced only trace amounts of growth. No growth had occurred in the case of the remaining organisms, and accordingly these were struck out from the group of possible nitrogen-fixers and not employed in subsequent nitrogen-fixation experiments.

One possible explanation as to why the latter group of organisms were able to grow under the cultural conditions of Experiment 1, yet under the apparently identical conditions of Experiment 2 did not survive, could be that, in Experiment 1, the inocula used in the case of these organisms were not absolutely free of nutrient agar (the medium on which all isolates were stock-cultured).

Repeated subculture of organisms Ac. 2, Ac. 3"S", Ac. 3"R", Pn. 4"S" and Pn. 4"R" did not result in any diminution in the amount of growth produced, which result would again indicate that these organisms are capable of nitrogen-fixation.

Organisms Pe. 1 and Pg. 1 survived several subcultures on "nitrogen-free" solid synthetic medium; however, since the amounts ofgrowth produced by each of these isolates was small, they were not investigated further in this Section.

Since there is always the possibility that solid "nitrogen-free"media contain traces of nitrogen (e.g. from the ion agar), the suspected nitrogen-fixing organisms were tested for their ability to grow in a nitrogen-free liquid medium:-

Experiment 3

For each of the organisms Ac. 2, Ac. 3"S", Ac. 3"R", Pn. 4"S" and Pn. 4"R", a physiological saline suspension (containing 1 x 10^6cells/ml) was prepared. One ml. of each of these suspensions was inoculated (one suspension/flask) into 100 ml. quantities of nitrogen-free liquid synthetic medium (p. 117)

contained in 150 ml. Erlenmeyer flasks. All flasks were then incubated at 25°C and examined for the presence of bacterial growth after 3, 7 and 14 days.

A good bacterial growth was evident in all flasks after 3 days. The medium inoculated with Ac. 2 showed little change in turbidity after 7 days, but that inoculated with Ac. 3"S", Ac. 3"R", Pn. 4"S" or Pn. 4"R" respectively was strongly turbid and contained a pale yellow film which rested on the bottom of the flask. After 14 days, the medium in the Ac. 2 flask had cleared and a cream, granular bacterial deposit had formed. In the flasks containing the remaining cultures the turbidity had further increased, the deposited film had thickened and the supernatant was extremely viscous.

(2) Analytical investigations.

(a) MicroKjeldahl studies.

The preliminary investigations had yielded results which indicated that bacterial isolated Ac. 2, Ac. 3"S", Ac. 3"R", Pn. 4"S" and Pn. 4"R" were possibly capable of fixing atmospheric nitrogen. In view of

this, and so as to verify these results, some quantitative investigations were undertaken. Only organisms Ac. 2, Ac. 3"S" and Pn. 4"S" were employed in these experiments; Ac. 3"R" and Pn. 4"R" were not studied further in this connection, since taxonomic studies, completed at about this time, had indicated that they were probably mutant forms of Ac. 3"S" and Pn. 4"S" respectively.

Experiment 4

To each of three Erlenmeyer flasks (250 ml), each containing nitrogen-free liquid synthetic medium (100 ml; 1% w/v glucose; pH7), was added 1 ml. of a physiological saline suspension (1 x 10^6 cells/ml) of organism Ac. 2; for subsequent use as a control, the contents of one flask were boiled for 5 minutes immediately after inoculation. All flasks were then incubated at 25°C for 11 days, after which time a 50 ml. sample was withdrawn from each and analysed for nitrogen content by the microKjeldahl method.

Organisms Ac. 3"S" and Pn. 4"S" were similarly investigated.

The results of the micro-Kjeldahl analyses are set out in Table VIII. From a consideration of these results, it will be seen that, for each organism, the nitrogen content in the experimental flasks had increased approximately twofold (i.e. as compared with the control flask) during the course of the experiment - thereby indicating that nitrogen fixation had taken place.

ORGANISM	SAMPLE UNDER INVESTIGATION	CALCULATED QUANTITY OF NITROGEN IN EACH SAMPLE (μg)	GAIN OF NITROGEN IN EXPERIMENTAL FLASKS (μg)
Ac. 2	Experimental Flask No. 1	89.6	33.6
	Experimental Flask No. 2	108.4	52.4
	Control Flask	56.0	-
	Blank*	-	-
Ac. 3"S"	Experimental Flask No. 1	98.0	56
	Experimental Flask No. 2	98.0	56
	Control Flask	42.0	-
	Blank*	-	-

Table VIII (continued on page 304).

ORGANISM	SAMPLE UNDER INVESTIGATION	CALCULATED QUANTITY OF NITROGEN IN EACH SAMPLE (μg)	GAIN OF NITROGEN IN EXPERIMENTAL FLASKS (μg)
Pn. 4"S"	Experimental Flask No. 1	112.0	56
	Experimental Flask No. 2	98.0	42
	Control Flask	56.0	-
	Blank*	-	-

* A "blank" determination, included so as to check that the apparatus and analytical reagents were nitrogen-free, was performed in a similar manner, the reagents in this case being glass-distilled water (10 ml) and 40% NaOH (2 ml). For each of the three organisms, the blank was performed; as the highest concentration of nitrogen found in the analytical reagents and apparatus only amounted to 0.6μg, contamination from these sources was not significant and was therefore neglected.

Table VIII The results of the micro-Kjeldahl analyses performed in Experiment 4.

Experiment 5

Organisms Ac. 3"S" and Pn. 4"S" were studied in this experiment, which was designed to investigate whether the initial concentration of nitrogen in the medium had any effect on the nitrogen-fixing capacity of the organisms.

This experiment repeated Experiment 4 with the exceptions that in Experiment 5 :-

(a) for each organism, experimental flask No. 1 contained 50 ml. of the liquid medium, as opposed to the 100 ml. contained both in experimental flask No. 2 and in the control flask; and

(b) the <u>total</u> content of each flask was analysed at the end of the experiment.

A one ml. inoculum was again used for each flask; in this way, the initial nitrogen content of flask No. 1 would be twice that of flask No. 2 and twice that of the control flask (since flask No. 1 initially contained 2n bacterial cells/ml, whereas the other two flasks only contained n bacterial cells/ml).

The results of this experiment are set out in Table IX.

ORGANISM	SAMPLE UNDER INVESTIGATION	CALCULATED QUANTITY OF NITROGEN IN EACH SAMPLE (μg)	GAIN OF NITROGEN IN EXPERIMENTAL FLASKS (μg)
Ac.3"S"	Experimental Flask No. 1	360	224.0
	Experimental Flask No. 2	252	112.0
	Control Flask	140	-
	Blank*	-	-
Pn.4"S"	Experimental Flask No. 1	336	257.6
	Experimental Flask No. 2	168	89.6
	Control Flask	78.4	-
	Blank*	-	-

* Values of 0.6μg N_2 and 0.6μg N_2 for the two blanks; insignificant and therefore neglected.

Table IX The results of the micro-Kjeldahl analyses performed in Experiment 5.

From the results of this experiment, it is evident that the concentration of bacteria initially present in the medium (which, in turn, governs the initial nitrogen content of the medium) has a marked

effect upon the amount of nitrogen subsequently fixed. Thus, at the end of the experiment, in the case of both organisms, whilst the nitrogen content in flask No. 2 was approximately double that in the control flask, the nitrogen content in flask No. 1 (which at the beginning of the experiment contained twice as many bacterial cells/ml as was contained in flask No. 2) had increased threefold in the case of organism Ac. 3 "S", and fourfold in the case of organism Pn. 4 "S".

(b) N^{15} studies.

The micro-Kjeldahl analyses had yielded results which indicated that three bacterial isolates are capable of effecting nitrogen fixation when grown in pure culture. In order to confirm this, some isotopic nitrogen investigations were undertaken for two of these isolates.

Experiment 6

The methods involved in the preparation of an isotopically-labelled atmosphere, in exposing a bacterial culture to such an atmosphere, and in the subsequent analysis of an exposed culture, have

previously been described (pp 118 - 130); hence, only the details relevant for a particular experiment are given below:-

Into each of two exposure flasks, one of which contained Atmosphere I (79.6% v/v N_2 : 20.4% v/v O_2) and the other which contained Atmosphere II (58% v/v N_2 : 20% v/v O_2 : 22% v/v Ar), was introduced 100 ml. of a heavy suspension (1 x 10^8 cells/ml) of a 24-hour-old "nitrogen-free solid synthetic medium" culture of Ac. 3 "S" in nitrogen-free liquid synthetic medium. Into a further two flasks, containing Atmosphere I and Atmosphere II respectively, non-inoculated nitrogen-free liquid medium (100 ml/flask) was introduced; each of these flasks served as a control.

Organism Pn. 4 "S" was treated similarly.

The cultures and controls were then mechanically shaken at 25°C for 16 hours, after which time a sample of labelled Atmosphere was withdrawn from each exposure flask and set aside for mass-spectrometric analysis. The contents of the

exposure flasks were centrifuged and micro-Kjeldahl total nitrogen analyses were then performed on the separated bacterial and supernatant fractions. After acidifying and evaporating each titrated sample down to 3 ml., the atom % of N^{15} excess (i.e. N^{15} abundance) in each sample was determined in the mass spectrometer. The percentage enrichments in N^{15} of each bacterial and supernatant fraction were obtained by deducting the natural abundance of N^{15} in the air (0.38%) from the values recorded for each of these samples.

The results obtained for Ac. 3 "S" and Pn. 4 "S" are listed in Tables X and XI respectively; in no case was N^{15} detected in the uninoculated (control) flasks.

The minimum value required for proof of nitrogen-fixation in experiments of this type, as established by Burris and Wilson (1957) and Roberts (private communication), is a percentage enrichment in N^{15} of 0.015. Thus the results presented in Tables X and XI amply confirm that organisms Ac. 3"S" and Pn. 4"S" are capable of fixing nitrogen, both in

the presence of a high (Atmosphere I) and of a low (Atmosphere II) partial pressure of nitrogen.

Since the controls did not show the presence of N^{15}, it can only be concluded that the high values recorded for N^{15} abundance in the supernatant fractions from all experimental flasks are due either to autolysis of the bacterial cells or to excretion of nitrogenous substances from the bacterial cells. The latter explanation is the more probable, since the experiment was conducted with young cells and was run for only 16 hours, which is an insufficient period for an appreciable degree of autolysis to have occurred.

ATMOSPHERE	SAMPLE UNDER INVESTIGATION	TOTAL NITROGEN IN SAMPLE (ASSESSED BY MICRO-KJELDAHL METHOD) (µg)	ISOTOPE ABUNDANCE (ATOM % N^{15} EXCESS) OF EACH SAMPLE (%)	PERCENTAGE ENRICHMENT IN N^{15}
I	Storage Flask Atmosphere	-	28.3 (±0.6)	-
	Exposure Flask atmosphere (sampled at the end of the experiment)	-	5.32*1 "	-
	Bacterial cells	546	24.8 "	24.42(±0.6)
	Supernatant	1596	28.6 "	28.22(±0.6)
II	Storage Flask Atmosphere	-	7.9*2 (±0.6)	-
	Exposure Flask atmosphere (sampled at the end of the experiment)	-	7.25 "	-
	Bacterial cells	182	5.5 "	5.12(±0.6
	Supernatant	112	2.5 "	2.12(±0.6)

*1 The low value recorded for N^{15} abundance in the exposure flask indicates that a leakage of atmosphere had occurred at some stage during the experiment. When the N^{15} abundance in the storage flask Atmosphere was re-determined, it was again found to be (approx.) 28-29%. Hence, the leakage must have occurred at some stage during exposure of the culture. Since the bacterial- and supernatant fractions were each found to exhibit N^{15} abundances approximately equal to that of the storage flask Atmosphere, it can only be assumed that the leakage occurred either near or at the end of the exposure time, and thus did not appreciably affect the amount of N^{15} fixed by Ac. 3"S".

*2 The low value recorded in this case, indicates that air leakage has occurred into the storage flask prior to conducting the experiment.

Table X The results obtained after exposing isolate Ac. 3"S" to isotopically-labelled Atmospheres.

ATMOSPHERE	SAMPLE UNDER INVESTIGATION	TOTAL NITROGEN IN SAMPLE (ASSESSED BY MICRO-KJELDAHL METHOD) (µg)	ISOTOPE ABUNDANCE (ATOM % N^{15} EXCESS) OF EACH SAMPLE (%)	PERCENTAGE ENRICHMENT IN N^{15}
I	Storage Flask Atmosphere	-	28.3 (±0.6)	-
	Exposure Flask atmosphere (sampled at the end of the experiment)	-	5.25*1 "	-
	Bacterial cells	700	26.3 "	25.92(±0.6)
	Supernatant	1610	29.5 "	29.12(±0.6)
II	Storage Flask Atmosphere	-	7.9*2 (±0.6)	-
	Exposure Flask atmosphere (sampled at the end of the experiment)	-	7.1 "	-
	Bacterial cells	154	3.4 "	3.02(±0.6)
	Supernatant	98	2.22 "	1.84(±0.6)

*1 The low value recorded for N^{15} abundances in the exposure flask indicates that a leakage of Atmosphere had occurred at some stage during the experiment. (cf. result obtained with Ac. 3"S"). Since the exposure flask atmospheres both in the case of Ac. 3"S" and Pn. 4"S" had shown low values for N^{15} abundance, it is evident that (since both exposure flasks were sampled simultaneously on the same apparatus) the leakage occurred into both at the same time. Since high values for N^{15} abundance in bacterial- and supernatant fractions were recorded, in all probability the leakage occurred at the sampling stage, i.e. at the end of the experiment.

*2 cf. Ac. 3"S"; an air leakage had occurred into the storage flask prior to conducting the experiment.

Table XI The results obtained after exposing isolate Pn. 4"S" to isotopically-labelled Atmospheres.

4. DISCUSSION

In many symbioses involving microorganisms the mutual benefits and the means by which they are secured are obscure. One such example is the leaf nodule symbiosis of semi-tropical and tropical Rubiaceae and Myrsinaceae species. Although reference was first made to this symbiosis as long as eighty years ago, not much practical attention has since been paid to it. The small amount of work that has been performed has been unsystematic and has yielded inconclusive results. The literature on leaf-nodule bearing plants shows that not only is the identity of the bacterial symbiont in doubt, but also that the biological significance of the association is equally controversial.

Leaving aside for the moment the question of the mechanism and significance of the symbiosis, the cytological distribution and the nature of the bacterial populations which have been reported by various workers as being present in the tissues of plant species in these two families may first be considered.

All workers who have carried out a survey of the bacterial distribution in these families are in agreement on a number of issues. Thus, they have shown that bacteria are always present at certain locations within the plant, namely the seeds, terminal buds and leaf nodules. All agree that the bacterial population of fully developed leaf nodules is almost exclusively bacteroid, whilst the terminal buds house a variety of forms, mainly rods of varying dimensions. Von Faber (1912) found that the seeds of P. zimmermanniana, P. indica and Psy. bacteriophila contained evenly-staining rods which were embedded in a slimy matrix, the latter being situated between the embryo and the endosperm, whilst Miehe (1911a) reported a similar finding with A. crispa. Additionally, each worker has concluded that the bacteria found in each of these locations within the plants he has investigated are one and the same bacterial species.

On other issues, however, discrepancies are apparent. Thus, a number of workers, studying the same plant species, each claimsto have isolated a different bacterial species, basing their claims on the cultural characteristics of the microorganisms concerned. For instance, von Faber (1912) isolated an acid-fast rod, to which he gave the name Mycobacterium rubiacearum, from the terminal buds of P. indica, P. zimmermanniana and

Psy. bacteriophila. Rao (1923) claimed to have isolated the same microorganism from the same source in P. indica, but a comparison of the staining properties and cultural characteristics given by Rao strongly suggests that his bacterial isolate was not the same as that of von Faber. (Particularly open to criticism is the technique used both by von Faber and Rao for establishing the acid-fastness of their organisms). More recently, Silver (private communication to the author) has isolated a Klebsiella species from germinating seeds of Psy. bacteriophila; each of these workers, on the basis of re-infection experiments, has subsequently concluded that his isolate is the leaf-nodule symbiont of the particular plant from which isolation was obtained.

Other investigators, each studying different plant species, have isolated different bacterial species. For example, Georgevitch (1916) isolated a Bacillus species from the leaf nodules of Kraussia floribunda. Harv, whilst Hanada (1954) obtained Xanthomonas hortoricola from the same source in A. hortorum Maxim., and Bose (1955) isolated a B. type L-form bacterium from the leaves and roots of A. humilis Vahl; with the exception of Georgevitch, these workers have claimed identity

between their respective isolates and the bacterium present *in vivo*. On the other hand, both Miehe (1914) and de Jongh (1938) have isolated *Bacillus foliicola* from germinating seeds of *A. crispa* - the only indisputable isolation of the same bacterium from nodule plant tissues by two independent workers.

With the exception of von Faber, earlier workers have shown perhaps insufficient appreciation of the fact that the bacteria associated with members of the Rubiaceae may well be quite distinct from those of the Myrsinaceae, and this fact has become reflected in general texts dealing with leaf-nodule symbiosis. Von Faber did in fact point out (1914), in a reply to Miehe's criticisms (1914), that this distinction between bacterial populations in different plant families was important.

In the present work, realising that much of the previous work did not take into account the possible, or probable, differences between the bacterial populations in different plant families, an attempt has been made to resolve some of these outstanding problems. The plants chosen for study included one representative (*A. crispa*) of the Myrsinaceae and three (*Psy. emetica*,

Psy. nairobiensis and P. grandiflora) of the Rubiaceae. In its simplest form the problem may be stated thus:-

(i) What is (are) the bacterial symbiont(s)?

(ii) Is (are) the bacterial symbiont(s) of the Rubiaceae identical to that (those) of the Myrsinaceae?

(iii) What is the mechanism of transmission of bacteria from one generation of the plant to the next?

(iv) What is (are) the function(s) of the symbiosis?

Most attention has been paid in past work to A. crispa, and the present work has confirmed many of the findings reported by Miehe and de Jongh. Thus, in agreement with de Jongh, the presence of bacteria was demonstrated in foliar and floral buds and in seeds; and old leaf nodules were found to contain exclusively "bacteroid" (type 1) forms. Young nodules contained two morphologically distinct types of bacterial rod (types 1 and 2), an observation which is implied, but not definitely stated in the account given by Miehe (1911). However, in contrast to the findings of Miehe and de Jongh, only one type (type 2) of bacterium was found in very young leaf nodules, and in

addition bacteria of type 2 were also found to occur in non-nodulated regions of young (but not of older) nodulated leaves. Both Miehe and de Jongh made unsuccessful attempts to isolate the bacterial symbiont from leaf nodules, and this finding was confirmed in the present study. Whereas Miehe and de Jongh abandoned attempts to isolate the symbiont from terminal buds, because of the high incidence of "secondary contaminants" on their isolation plates, in the present work, no such "secondary contaminants" were obtained; in fact, despite the introduction of many modifications to the isolation technique, the terminal buds never consistently yielded an isolate.

Both Miehe and de Jongh isolated B. foliicola from germinated, but not from ungerminated, seeds. Miehe also isolated "Bacterium repens", whilst de Jongh isolated a different, but unnamed organism, which resembled Bacterium repens in forming yellow colonies. The present writer, using similar isolation techniques, isolated two bacterial species (Ac. 1 and Ac. 2) from ungerminated seeds, while germinated seeds readily yielded another organism (Ac. 3), although a small population of Ac. 1 and Ac. 2 persisted. Another isolate (Ac. 4), which

accompanied Ac. 3, was initially distinguished by growing on a "nitrogen-free" medium, but taxonomic investigations subsequently showed that Ac. 4 was in fact identical to organism Ac. 3. The latter was found to occur in two forms - smooth and rough - the former type predominating. None of these isolates was specifically identical with B. foliicola or Bacterium repens.

As to the location of the bacteria in the seeds, both Miehe and de Jongh concluded that they were present in a mucilaginous film which was situated between the embryo and the endosperm at the radical region of the seed. The results obtained in the present study indicate that this conclusion is only true in part; thus, in ungerminated seeds, bacteria were found to occur in a mucilaginous film which totally envelops the embryo, and additionally, bacteria similar to those found in the film were present both inside the endosperm cells and lining the walls between such cells. On germination, it appeared that the bacteria seen in ungerminated seeds underwent a transformation from being a short, bipolar-staining rod into one resembling the thin, more evenly stained rods of type 2.

With the three Rubiaceae species now studied, bacteria were again found at similar locations to those recorded by von Faber (1912) for P. zimmermanniana, P. indica and Psy. bacteriophila. Histological studies showed that in fully developed nodules of Psy. nairobiensis and P. grandiflora, the bacterial population was exclusively "bacteroid" (type 1), whilst in Psy. emetica nodules, in addition to the "bacteroids", a few, larger bacterial rods were present. These observations mainly agree with those recorded by von Faber, so far as his account allows comparison. Young nodules of both the Psychotria species and of the Pavetta species resembled those of A. crispa in containing two types of bacteria, one of which (type 1) was more abundant. Confirming the observations of both von Faber and Ziegler (1958), the "bacteroids" of Rubiaceae species appear to be more distorted than those of the Myrsinaceae species. The second type of nodule bacterium (type 2) was also present in large numbers throughout the mesophyll tissues in non-nodulated regions of young, nodulated leaves of Psy. emetica and P. grandiflora, an observation not recorded by previous workers.

Parallel studies, in which macerates of nodules of Psy. emetica and P. grandiflora were examined, revealed

the presence in all instances of a mixed bacterial population, which included both Gram-positive and Gram-negative types; this finding has not been recorded by previous workers, and since Gram-positive bacteria were never observed within the tissues of these species, it is concluded that these types are present as a result of secondary, external infection of the nodule. The bacterial population of Psy. emetica nodules, as established by isolation studies, included a Gram-negative, bi-polar staining rod (Pe. 1), which gave rise to pink colonies, a shorter Gram-negative rod (Pe. 2) and two Gram-positive coryneform types, which produced yellow and fawn coloured colonies respectively. Taxonomic investigations (see Part 2) showed that organism Pe. 1 is probably identical with Mycoplana rubra, described by de Vries and Derx (1950); it was again isolated (as "Pg. 1") from nodules of P. grandiflora. This organism, while undoubtedly present within the nodules, is also to be found on the external surface of the nodules and the leaf, and de Vries and Derx have identified it in non-nodulated plant species and even in river water and soil. It is perhaps significant that no bacteria could be isolated from nodules of Psy. nairobiensis.

Terminal buds of Psy. nairobiensis were found to contain "bacteroid" forms, morphologically similar to those found in the nodules; in the terminal buds of Psy. emetica and P. grandiflora, there were very many bacteria which were intermediate in form between the "bacteroids" (type 1) and the type 2 rods. However, several other types of bacteria, including some coccal forms and Gram-positive rods, were observed at this location in these species (see Part 1), and it is concluded that a number of different bacterial species are housed in the terminal bud mucilage. This conclusion is borne out by the results of the isolation investigations (see Part 2) conducted with these tissues; thus, in addition to obtaining isolates Pe. 1, Pe. 2 and Pe. 3 from Psy. emetica buds, and isolates Pg. 1 and Pg. 2 from P. grandiflora buds, a large number of colonies of different organisms developed on the isolation plates when isolation was attempted from these sources. Both the histological and bacterial isolation studies undertaken with these two species have thus yielded results which support Miehe's contention (and refute von Faber's contention; see p. 79) that the interfoliar mucilage of Rubiaceous terminal buds harbours a mixed bacterial population.

The bacteria present in ungerminated seeds of Psy. nairobiensis were found to be morphologically identical with those found in ungerminated seeds of A. crispa, although their distribution, as far as could be ascertained, was restricted to a region between the endosperm and the embryo. From these seeds two isolates (Pn. 2 and Pn. 3) were obtained, while on germination yet another type (Pn. 4) appeared consistently and in large numbers. The latter resembled isolate Ac. 3, and likewise gave rise to rough and smooth forms after culturing on nitrogen-deficient media.

It is evident from the investigations, summarized above, that all the plant species examined carried in their tissues a very mixed bacterial population, and it would seem unlikely that all of these bacterial species are functional symbionts in the sense that they contribute, in some manner or other, to the maintenance of a healthy condition in the host; and indeed some of them might, under certain conditions, even become harmful. The findings with terminal buds and old nodules raise the question as to whether these two sources are suitable for the isolation of the bacterial symbiont.

As regards terminal buds, the histological and isolation results obtained with Psy. emetica and P. grandiflora indicate that the stipular coverage (which according to von Faber (1914) prevents the entrance of aerial bacteria into the interfoliar spaces of the bud) does not exclude the invasion of the bud spaces by secondary contaminants; and although no evidence was obtained in the present study to support the view that the same situation can arise in Psy. nairobiensis buds, it is conceivable that the buds of this species - which are anatomically similar to those of Psy. emetica and P. grandiflora - can also become infected in the same way. It is also possible that A. crispa buds, with their more open structure, can be infected in a similar manner. Whilst the results reported herein do not support the latter conclusion, the isolation results obtained by Miehe and de Jongh suggest that the bud spaces of A. crispa house several species of bacteria. For these reasons, terminal buds are considered to be unsuitable sources for the isolation of the bacterial symbiont.

With regard to the suitability of old leaf nodules as isolation sources, the evidence obtained in the present study would indicate that they are not entirely

suitable. Many of the "bacteroids" in old leaf nodules appear to be in the process of degenerating, and since no isolations were obtained from this source in A. crispa and Psy. nairobiensis (both of these species house "bacteroids" almost exclusively in their old nodules) it is conceivable that many, if not all, of the bacteria found in old nodules are no longer viable (cf. Rhizobium see Jordan (1962)). Additionally, the nodules of Psy. emetica and P. grandiflora were found to contain a mixed bacterial population.

From this superficial study of the bacterial populations it is not possible to decide which of the numerous bacterial species is (are) the functional symbiont(s). There are, however, certain criteria that can be applied in this context for selecting isolated organisms for further study (i.e. for tracking down the true bacterial symbiont(s)), and these (p. 204) have been followed in this investigation. However, by following these criteria, some twelve different bacterial isolates (Ac. 3"R" and Pn. 4"R" are rough variants of Ac. 3"S" and Pn. 4"S" respectively), representative of ten different bacterial species, were isolated from the four plant species investigated. It was assumed that a number of these isolates are surface contaminants

and this assumption is supported by the fact that they did not appear in preparations made from tissues which had been surface-sterilised by $HgCl_2$, but only from those which had been washed with water; the washings themselves usually carried a mixture of these same isolates, namely Pe. 1, Pe. 2, Pe. 3, Pn. 1, Pg. 1 and Pg. 2. Accordingly, these isolates were considered to be unlikely candidates for the role of bacterial symbiont and hence were not studied further in this context. A list of the species which remained for consideration is given in Table IV; it will be seen that all of these were obtained from seeds which had been surface-sterilised by treatment with $HgCl_2$.

The procedure normally adopted for establishing that a particular pathological condition is caused by a particular microorganism is as stated in Koch's postulates (p. 269). However, in the present study, when surface-sterilised ($HgCl_2$) Psy. nairobiensis seeds were raised under aseptic conditions, all plants obtained possessed leaf nodules, thereby showing that bacteria present within the seed were responsible for the production of leaf nodules. This result confirms the findings of von Faber (with P. zimmermanniana), of Miehe and de Jongh (with A. crispa), of Hanada (with A. hortorum. Maxim) and of Silver (with Psy. bacteriophila; private communication to the author). Hence, before any re-infection experiments could be undertaken, it would be necessary to kill the bacteria present within the seed.

Using similar techniques to those employed by previous workers, only partial success was achieved, and for reasons discussed more fully below no re-infection experiments were undertaken. Stored seeds were difficult to germinate, and this can probably be explained as being due to the fact that the seeds had become partially dehydrated during storage. Fresh (unstored) seeds were found to germinate more readily, but in a series of

heat-treatment experiments, they yielded results that - at least in so far as producing a sufficient number of bacteria-free plants was concerned - were somewhat unsatisfactory. Thus, in the first experiment (p. 272 et seq) with fresh seeds, whilst a large number of plants exhibiting "crippled" features were obtained, from an initial total of 120 seeds (92 of which were heat-treated) only nine non-nodulated, bacteria-free "cripples" were obtained. Nodulated, bacteria-containing "cripples", in addition to being obtained from heat-treated seeds, were likewise obtained in the control series, a result previously noted by Miehe (1919) with A. crispa; these "spontaneous cripples" (i.e. "cripples" arising from untreated seeds) were morphologically identical with the nodulated, bacteria-containing "cripples" obtained in the heated series. Many of the nodulated "cripples", including a number of the "spontaneous cripples", recovered and after nine months were identical with "normal" plants. Since, in this experiment, all of the non-nodulated, bacteria-free "cripples" died, whilst all the "normal" plants and approximately half of the nodulated, bacteria-containing "cripples" survived, it can only be concluded that the nodule bacteria are essential for the survival of Psy. nairobiensis. It is difficult to explain the death of the remaining nodulated, bacteria-containing "cripples", but this could conceivably be due to

the fact that heating had brought about a reduction in the number of viable bacteria present in the seed, and that this number was insufficient to support the continued development of these plants. In contrast to von Faber's findings (with P. zimmermanniana), but in agreement with the findings of Miehe and of de Jongh (with A. crispa) and of Hanada (with A. hortorum. Maxim.), none of the heat-treatments employed significantly affected the rate of germination of those seeds which survived heating.

In the second experiment employing fresh seeds (p. 277), the results obtained, as assessed on the basis of the production of a sufficient number of bacteria-free plants, were again unsatisfactory. Thus, in this experiment of eleven plants which were initially (after four months) non-nodulated (and at this stage adjudged to be bacteria-free), seven were found to exhibit bacteria-containing nodules after nine months growth. A similar "spontaneous reversion" of non-nodulated, bacteria-free plants was recorded by Miehe in 1919. It is quite conceivable that four month-old plants did in fact contain viable bacteria, but that so few bacteria were present that they remained undetected;

the bacteria had probably multiplied at some stage between the fourth and ninth month of growth. However, whatever the cause of these somewhat unexpected results, the fact remains that in the first experiment none of the non-nodulated, bacteria-free "cripples" survived for a significant period of time, whilst in the second, seven out of eleven non-nodulated "cripples" later became nodulated (and bacteria-containing).

It seemed to be of no avail to attempt re-infection of crippled plants when one considered that:-

(a) only a few bacteria-free, non-nodulated "cripples", insufficient in number for the performance of an adequately controlled re-infection experiment, were obtained.

(b) the percentage of "spontaneously reverting cripples" was not known with any degree of accuracy.

and (c) "spontaneously reverted cripples" could only be recognised after growing for nine months.

It is possible that the problem of killing the seed bacteria could be solved more readily by treating the seeds or seedlings with antibiotics - i.e. instead of using the heat-treatment method. However,

if the latter method is to be used, then further research is necessary (a), to determine the percentage of seeds in any batch which "spontaneously" revert, and (b), to be able to recognise a true "cripple" (i.e. a plant which is non-nodulated, dwarfed and bacteria-free) in its very early developmental stages.

The only way to solve the first requirement, (a), satisfactorily would be to perform replicate experiments (employing a large number of batches of seeds) which are designed on a statistical basis. In this way the "spontaneous" reversion rate could be determined with some accuracy. Then and only then (assuming question (b) to have been solved) could one safely conduct re-infection experiments of significance. If in these experiments, likewise designed on a statistical basis, one could show that the percentage of leaf nodulated, bacteria-containing plants which developed from re-infected "cripples" was significantly higher than the percentage of similar plants developed as a result of "spontaneous reversion" in the controls (i.e. non-nodulated, bacteria-free "cripples" which had not been re-infected), then one could safely conclude that the bacterium isolated was the cause of

nodulation. To solve these problems, a large number of seeds and a long period of time would be required, and hence as time for the completion of this research was limited, it was decided to employ an alternative method. The one chosen was the "fluorescent antibody" method; this technique, which combines the sensitivity and specificity of immunology with the precision of microscopy, was considered to be particularly suitable for employment in this case.

From the results obtained when plant tissues were investigated by the fluorescent antibody technique, a number of conclusions can be drawn. The low incidence of bacterial isolates Ac. 1 and Ac. 2 in macerates of A. crispa leaf nodules, and likewise the low incidence of isolate Pn. 2 in Psy. nairobiensis tissue macerates, would suggest that these bacterial species are present, in or on their respective hosts, as secondary infections. On the other hand, the high incidence of isolates Ac. 3"S" and Pn. 4"S" (which taxonomic investigations had shown to be specifically identical; see page333) in leaf nodules, non-nodulated regions of nodulated leaves, terminal buds and germinating seeds of both A. crispa and Psy. nairobiensis would make it more probable that here we are dealing with the true functional symbiont.

It must be recognised that the fluorescent antibody method is not infallible, but all foreseeable pitfalls were guarded against when conducting these experiments (pp 279-292). Since re-infection experiments were not performed, it cannot be stated categorically that organism Ac. 3"S" (Pn. 4"S") is the functional symbiont of A. crispa and Psy. nairobiensis, but the contention that it is the symbiont may be supported by the following arguments:-

(a) A high incidence of this species (i.e. Ac. 3"S" or Pn. 4"S") was recorded within the tissues of A. crispa and Psy. nairobiensis, when such tissues were investigated by the fluorescent antibody method.

(b) When grown on "nitrogen-free" media, this species exhibits very similar morphological and staining characters to those of the bacteria present in resting seeds; likewise, when grown on nutrient agar, it shows a very close morphological resemblance to the bacteria present in germinated seeds.

(c) This species was consistently isolated from germinating seeds of both A. crispa and Psy. nairobiensis.

Brief mention was made above to the fact that bacterial isolates Ac. 3"S" and Pn. 4"S" are

identical, and in support of this statement the following facts may be noted:-

(a) They are indistinguishable in physiological and biochemical characters.

(b) They exhibit identical morphological and growth characters, with the possible exception that Pn. 4"S" is slightly smaller in its early stages of growth on a "nitrogen-free" medium. On the latter medium they both show pleomorphism, each occurring initially (after 2 days) as a small, bi-polar staining rod, which eventually (after 21 days) develops into a large irregularly shaped, capsulated, azotobacter-like cell (see Figs. LXVI and LXVII). After prolonged cultivation on a "nitrogen-free" medium, both give rise to a proportion of "rough" colonies (Ac. 3"R" and Pn. 4"R"), which are likewise indistinguishable, one from the other, in their cultural characteristics.

(c) Labelled antisera prepared against Ac. 3"S" were found to stain Pn. 4"S" and Pn. 4"R" specifically, whilst labelled antisera prepared against Pn. 4"S" stained Ac. 3"S" and Ac. 3"R" specifically.

(d) They showed similar precipitation patterns when examined by Elek's gel diffusion technique.

Ac. 3"S" (Pn. 4"S") exhibits cultural characteristics distinctly different from those recorded by von Faber (1912), Miehe (1914), Rao (1923), de Jongh (1938) and Hanada (1954) for their respective bacterial isolates, and in all probability it is generically distinct from any of the isolates described in the literature. An attempt to identify this organism by reference to Bergey (1957) and Skerman (1959) has presented considerable difficulty, for its cultural characteristics are such that they do not allow it to be placed in any of the known genera with any degree of certainty. For instance, on the basis both of its ability to fix atmospheric nitrogen and of the fact that it is a Gram-negative, unicellular, asporogenous, straight to slightly curved, motile, heterotrophic rod, it could conceivably be placed in any one of the genera Azotomonas, Aerobacter, Azotobacter, Rhizobium or Pseudomonas. However, it differs from each of these genera in several fundamental respects and is quite probably not a member of any. Thus, its carbohydrate metabolism rules out its placing in the genus Azotomonas, whilst its carbohydrate metabolism plus the fact that it is oxidase-positive eliminate the possibility that it is a member of the genus Aerobacter.

The fact that it is a facultative anaerobe and polar flagellated probably excludes its placing in the genus Azotobacter. Likewise, the fact that it fixes atmospheric nitrogen when growing non-symbiotically suggests that it is not a member of the genus Rhizobium, whilst its highly pleomorphic nature taken alongside the fact that it does not produce water-soluble pigments probably rule out its placing in the genus Pseudomonas.

If the facts that it fixes nitrogen when grown in pure culture and that it was not isolated from root nodules are ignored, Rhizobium is the most likely genus to house this organism, for, in addition to the cultural characters referred to above, Ac. 3"S" (Pn. 4"S") exhibits several other properties which are characteristic to Rhizobium species, and a number of these can be cited:-

(a) It is very similar in size to Rhizobium species.

(b) It is motile when young, commonly changing to bacteroidal forms when cultured on nitrogen-deficient media (cf. "bacteroids" of leaf nodules).

(c) Acid production from carbohydrates is very poor and very slow. Glucose is degraded oxidatively

(as assessed by the method of Hugh and Leifson, 1953).

(d) It is able to induce nodulation in plant tissues.

In all probability, however, Ac. 3"S" (Pn. 4"S") belongs to none of the genera described in the Manual, but, in the absence of a completely satisfactory genus, it is provisionally placed in the genus, Rhizobium. Further study of this organism is required before its identification can be made more precise.

The remaining bacterial isolates were identified by reference to Bergey (1957), Dowson (1959) and Steel (1961). Isolates Ac. 1, Pe. 2, Pn. 1 and Pg. 2 are all readily placed as members of the genus Pseudomonas. Isolates Pe. 1 and Pg. 1 are culturally identical (except for the fact that Pe. 1 produces acid in mannose more rapidly than does Pg. 1) and are considered to be specifically identical; they are also identical with Mycoplana rubra (de Vries and Derx, 1950), so far as could be judged by the tests performed in the present study. Isolate Pe. 3 is considered to be a Xanthomonas species whilst Pn. 2 probably belongs

to the genus Aerobacter. Isolate Pn. 3 could well be placed in either of the closely related genera Erwinia or Pectobacterium; it does not liquefy pectate gel, however, and is therefore placed under Erwinia. Isolate Ac. 2 is of doubtful classification since, although it exhibits many features characteristic of the genus Aeromonas, it nevertheless differs from this genus in its apparent ability to fix atmospheric nitrogen (p. 303). In the absence of a completely satisfactory genus it is provisionally classified as an Aeromonas species.

Reference has already been made (pp. 79,80) to the controversy which arose between Miehe and von Faber concerning the acid-fastness of the nodule bacteria of Myrsinaceae and Rubiaceae species. The results obtained in the present study clearly show that, when the Ziehl-Neelsen staining method is employed, none of the bacteria present in A. crispa, Psy. emetica, Psy. nairobiensis or P. grandiflora is acid-fast. On the other hand, when von Faber's staining technique is used, all bacteria present in these species are

"acid-fast". Since Escherichia coli (a known non-acid-fast organism) was found to exhibit "acid-fastness" when stained by the latter method, it can only be concluded that von Faber's method is inadequate for assessing this character. The fault of this method probably lies in the fact that the sulphuric acid employed is not strong enough to dissolve out the carbol fuchsin from the stained bacteria. It is concluded that none of the bacteria associated with the four plants now studied are acid-fast, and hence that they should not be placed under the genus Mycobacterium. Silver (private communication) has reported a similar finding with one of the species investigated by von Faber, namely Psy. bacteriophila.

Evidence which supports the view advanced by Miehe (1914) that the symbiosis of A. crispa is cyclic in nature has been obtained in the present study; and additionally, on the basis of the results reported herein, it is concluded that in the three Rubiaceae species investigated this is also the case.

Thus, in all of these species, bacteria were found to occur in the leaf nodules, in the terminal buds, in the floral buds and in the fruits, whilst, in the case of A. crispa and Psy. nairobiensis, bacteria were also found to occur in the seeds*.

The possibility always exists that the bacteria found in each of these locations in a particular plant are not specifically identical from one location to another. For instance, four morphologically distinct rods, namely the type 1 bacteroids of old nodules, the type 2 cells of young nodules, of non-nodulated regions of nodulated young leaves, of mesophyll tissues of primordial bud leaves and of germinating seeds, the bacteria of the bud mucilage which are intermediate in form between types 1 and 2, and the short bipolar-staining cells of the resting seed,

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* Seeds of Psy. emetica and P. grandiflora were not investigated, but in all probability, had this been done, bacteria would be found within the seeds, since they were found to occur in the flowers and fruits of both these species.

were found to occur in A. crispa. However, notwithstanding this diversity in form, it is concluded that all of these bacterial types in A. crispa are specifically identical pleomorphic forms. Similarly, with the three Rubiaceae species investigated, if we exclude those locations (old leaf nodules of Psy. emetica; terminal bud mucilage of Psy. emetica and P. grandiflora) which are known to house secondary contaminants, the same conclusion can be reached.

A number of findings obtained in the present study can be advanced in support of the conclusions reached above:-

(a) With a very few exceptions, there is a pattern of distribution of different types of bacteria common to each of the four plant species investigated:- (i) Bacteroids (type 1 cells) are the only type of cells present in old nodules; (ii) types 1 and 2 are found in middle aged and young nodules; (iii) type 2 cells are the sole type of cell present in very young nodules; (iv) type 2 cells occur in the mesophyll tissues of young leaves and in the tissues of the leaf primordia of the bud; (v) type 2 cells are present in flowers and fruits; (vi) short bipolar-staining rods are the sole type of bacterium present

in resting seeds. A feature common to all these rods is the property of bipolar staining.

(b) Only one type of bacterium, namely the short bipolar-staining rod, is present in ungerminated seeds of A. crispa and Psy. nairobiensis, whereas, in germinated seeds of these species, the majority (95%) of bacteria are of type 2. Observations suggest that the appearance of type 2 cells is simultaneous with germination of the seeds, and it is considered that they are derived from the shorter, bipolar-staining rods mentioned above. It is of interest to recall that, in germinated seeds of Psy. nairobiensis, types intermediate between the short, bipolar-stained rod and the type 2 cells were present. Organism Ac. 3"S" (Pn. 4"S") undergoes a similar transition when transferred from "nitrogen-free" media to nutrient agar.

(c) In the fluorescent protein tracing studies conducted with A. crispa and Psy. nairobiensis (p. 279 - 292), the vast majority of bacteria present at the various locations within these plants were stained specifically with the aid of Ac. 3"S" or Pn. 4"S" antisera.

On the basis of previous workers' results and in agreement with the results now obtained, it is suggested that transmission of the symbiont from one generation of plants to the next takes place in the following manner:-

Seeds invariably carry a bacterial population (short bipolar-staining rods); on germination, these bacteria simultaneously multiply and undergo morphological modification from the short bipolar-staining form into one which is longer and thinner (type 2). It is probable that it is at this stage (i.e. whilst the embryo is still within the germinating seed) that infection of plantule takes place (see pp. 166, 168 and 292); infection conceivably takes place into the plumule, but the exact mode of entry of the type 2 bacteria was not ascertained in the present study. The bacteria will thus reach the primordial leaves and as these develop will continue to multiply, so that they will be found (as "type 2") in all parts of the very young leaf tissues.

Possibly the bacteria arrive in the flowers by a similar method of transmission (i.e. they are passively carried in the meristematic tissues of the

floral bud), since all meristematic tissues examined appeared to carry a bacterial population. (The possibility that bacteria are carried to the flowers via the vascular system is not supported by any experimental evidence obtained in the present study).

Both von Faber (1912) and Miehe (1914) tentatively suggested that infection of the seed arose as a result of bacterial penetration through the pollen tube of open flowers. But the present study shows that, in all plants investigated, bacteria are present in the ovary cavity of the flowers a long time before any pollen tubes are formed (and, in fact, in A. crispa no pollen tubes were ever observed); thus, bacteria were found in the ovary cavity of flowers of A. crispa, Psy. nairobiensis and P. grandiflora, even whilst these flowers were still in the primordial stages. These bacteria - all of type 2 - are conceivably passively enclosed in the ovary when the walls of the latter fuse, since many bacteria of similar type were found in the mucilaginous film which completely envelops the primordial components of the floral bud. Within the ovary cavity of open flowers of each species investigated, the bacteria were mostly concentrated at the apices of the placenta and of the ovules.

Confirming de Jongh's (1938) observation, in A. crispa fruits, the bacteria appear to be passively enclosed between the nucellus and the inner integument of the developing seed. The exact mode of entry of the bacteria into the seeds of the Rubiaceae species investigated could not be ascertained. However, in all probability, infection of the seeds takes place through the micropyle of the developing ovule; this suggestion is based on two findings, namely, (i) in the open-flower stage, bacteria of type 2 were found to be mainly concentrated at the apex of the ovules (i.e. at the site of formation of the micropyle), and (ii) in the fruit, similar bacteria were found to occur in the immediate vicinity of the developing embryo (which is situated near the micropyle) in the developing seed.

As to the formation of nodules in the leaves, several interesting points may be considered. The striking regularity in the distribution of nodules in any one leaf may be a reflection of some structural regularity in the leaf, for example the symmetry of distribution of stomata (cf. the method of leaf infection

postulated by Zimmermann, 1902) in Rubiaceae species or the regular distribution of hydathodes in Myrsinaceae species, or it may be due to the fact that the nodule bacteria require some critical concentration of food supply, which is brought to them via the vascular system.

Earlier workers are in disagreement with regard to the mechanism of infection of the leaves. For instance, Zimmermann (1902) considered that stomatal penetration occurred; von Faber (1912) suggested that bacteria entered the leaves via "stipular holes"; Hanada (1954) stated that penetration took place through "spaltöffnung", whilst both Miehe (1911(a)) and de Jongh (1938) favoured hydathodes as the means of infection; all of these workers are agreed that infection takes place in the terminal bud stage. It is difficult to reconcile some of these suggestions with the findings obtained in the present study, and attention is particularly drawn to the fact that bacteria (type 2) were found <u>throughout</u> the primordial leaf tissues in the foliage buds of <u>all</u> plants investigated, whereas by the method of infection proposed by the above-named workers a localised concentration of

bacteria would be expected to occur in the leaf tissues.

Although there are differences in the precise detail of the pathway of nodule development in the Myrsinaceae and Rubiaceae (which are interpreted as being due to different reactions on the part of the host), the development of the nodules in both families follows a similar course. The type 2 bacteria, typical of primordial leaf tissues, migrate, either passively or as a result of some stimulus provided by the host environment, to particular foci in the leaf. Having collected at these foci, two developments take place; (1) type 2 cells undergo modification leading to the continued appearance of type 1 ("bacteroids") until, in old nodules, the whole population is of type 1; and (2) there is simultaneous development of a "sheath" of modified mesophyll cells, which demarkates the boundary of the nodules. One explanation of this phenomenon may be that we have here a protective mechanism whereby the plant removes a proportion of the bacterial population that has already ceased to serve any useful function. This suggestion is not without foundation since the bacteroid type

appears to be no longer viable, as evidenced by the impossibility of obtaining the bacterial symbiont from this source.

In the past, several theories have been advanced to explain the function of the bacterial component in the leaf nodule symbiosis of Myrsinaceae and Rubiaceae species. Of these, the two that have found most favour with later workers are the nitrogen fixation theory, postulated by von Faber (1912), and the "stimulative effect" theory, postulated by Miehe (1914). Evidence in support of the former theory has since been put forward by Rao (1923) and Silver (private communication), on the basis of their findings with P. indica and Psy. bacteriophila respectively, and by Hanada (1954), who investigated A. hortorum. Maxim.; Miehe's theory has been supported by Nemec (1932), who postulated (although without experimental evidence) that the "stimulative effect" was due to a growth hormone, and by de Jongh (1938); both of these workers investigated A. crispa.

Evidence in support of the nitrogen fixation theory was obtained in the present study. An investigation into the nitrogen-fixing ability of each of the bacterial isolates revealed that two of these (Ac. 3"S" and Pn. 4"S") are undoubtedly capable of fixing atmospheric nitrogen when grown in pure culture (as established by N^{15} studies), whilst another (Ac.2) is probably a nitrogen fixing organism (as indicated by microKjeldahl experiments). In the isotopic nitrogen investigations isolates Ac. 3"S" and Pn. 4"S" - the single bacterial species which is considered to be the functional symbiont of A. crispa and Psy. nairobiensis - excreted large amounts of nitrogen into the medium, and it is conceivable that this organism, when growing in symbiosis with these plants, can fix atmospheric nitrogen more efficiently and can also pass on a proportion of the nitrogenous products of fixation to the host. In the present study, however, no investigations were undertaken into the nitrogen-fixing ability of the intact symbiotic system (i.e. bacterium plus plant), and hence it is not possible, on the sole basis of the results reported herein, to state categorically that this is the case. However, certain results obtained by independent investigators

provide evidence that the intact symbiotic system is capable of effecting atmospheric nitrogen fixation, and these results can be summarized as follows:-

(a) Cone (private communication) has reported that when young shoots of Psy. nairobiensis were exposed for a period of seven days to an isotopically labelled atmosphere (O_2, 15.1%; CO_2, 6.9%; N^{14}, 54.8%; N^{15}, 23.2%), the nitrogenous content of the nodulated leaves was enriched (by a value of 0.08% N^{15} excess) in N^{15}.

(b) Von Faber (1912), working with P. zimmermanniana in a series of sand-culture experiments, found that whilst bacteria-containing plants grew normally when deprived of bound nitrogen, bacteria-free plants developed very poorly and showed all the symptoms of nitrogen deficiency; similar investigations, recently conducted by Silver and Centifanto (private communication) with Psy. bacteriophila yielded similar results.

The results obtained in the present study, taken alongside the evidence of the independent investigations cited above, strongly indicate that at least one function of the bacterial symbiont in leaf-ndoulated Rubiaceae and Myrsinaceae species is the fixation of atmospheric nitrogen.

Although the suspected bacterial symbiont (isolates Ac. 3"S" and Pn. 4"S") and several associated bacterial species (e.g. isolate Ac. 2 obtained in the present study; Mycobacterium rubiacearum obtained by von Faber; X. hortoricola obtained by Hanada) from nodulated plants can effect the fixation of atmospheric nitrogen, one cannot conclude that this is the only function of the bacteria beneficial to the plant. If this were so and the bacteria simply passed certain amounts of nitrogenous compounds into the plants, it might be expected that by supplying the plants with suitable amounts of "bound" nitrogen the dwarfism of nodule-free plants would be overcome. Direct attempts by Hanada (1954) and Silver (private communication) to show such beneficial effects of bound nitrogen gave entirely negative results, although it must be pointed out that the chemical nature of the nitrogenous compounds necessary might be somewhat specific. Since a vast number, if not the majority of green plants, are capable of obtaining their nitrogenous nutrients directly from the soil without the intervention of nitrogen fixing-organisms, then if nitrogen fixing organisms are essential to leaf-nodulated plant species, it argues that there must be some abnormalities in the root absorption

system of these plants. Unless there is this abnormality, some other beneficial activity of the bacteria must be sought, and the possibility cannot be discounted that they are concerned, in some undefined way, with the production of one or more substances of the "growth factor" type. Whilst no direct evidence was obtained in support of this possibility, a number of findings obtained in the present study can be advanced as indirect evidence in its favour:-

(a) Normal, healthy plants of all four species investigated were found to contain a large number of bacteria (type 2) within the tissues of the stem meristems, i.e. in regions where hormone production takes place.

(b) Plants which contained no (or few) viable bacteria exhibited crippled features; the dwarfed nature of such plants can be interpreted as being due to an upset in hormonal balance.

With the exception of some details concerning nodule development, present evidence suggests that the symbiotic association is the same in the two families studied - as has been assumed in the past from somewhat superficial evidence.

SUMMARY

1. A review of the literature appurtenant to leaf-nodulated Myrsinaceae and Rubiaceae species has been presented. From a consideration of the literature it is evident that there is much controversy as to the identity of the bacterial symbiont or symbionts and also as to the biological function of the symbiosis.

2. A histological survey of the bacterial distribution within the tissues of one Myrsinaceae species (A. crispa) and of three hitherto uninvestigated Rubiaceae species (Psy. emetica; Psy. nairobiensis and P. grandiflora) has been undertaken.

3. In all species, bacteria were found to occur in leaf nodules of all ages, in non-nodulated regions of nodulated young leaves, in foliage and floral buds and in developing fruits. Seeds of A. crispa and Psy. nairobiensis only were examined and were found to contain bacteria.

4. In spite of the diversity in their morphology, evidence has been presented that, in general, the bacteria present are specifically identical from

location to location and from plant species to plant species.

5. A cyclic symbiosis has been demonstrated in A. crispa and Psy. nairobiensis, and partially demonstrated in Psy. emetica and P. grandiflora.

6. Several bacterial species were isolated from these sources and the cultural characteristics of these have been investigated.

7. The results of fluorescent antibody tracing studies, and of morphological and isolation studies, indicate that only one of these species is the symbiont of A. crispa and Psy. nairobiensis, and this has been tentatively identified as a Rhizobium species.

8. Nitrogen fixation in pure culture has been demonstrated in two of the bacterial species, including the proposed symbiont.

9. The possible relationship between the bacterial isolates and the host plant species have been discussed, and an attempt has been made to assess the results in the light of previous knowledge.

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