ON THE SYMBIOSIS OF ARDISIA CRISPA (THUNB.) A. DC.

BY

PH. DE JONGH

FROM THE BOTANICAL INSTITUTE OF THE GOVERNMENT UNIVERSITY, LEYDEN

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CHAPTER I.

Review and discussion of the literature.

§ 1. *Introductory remarks.*

Amongst the various types of bacterial symbioses of higher plants our knowledge of the most favoured case — the classical root-symbiosis of the Leguminosae — is far greater than that of the foliar symbionts. Only a few investigations have given us anatomical and experimental data on the foliar symbioses and it is no exaggeration to state that after these few papers on this subject little work has been done. It is true that taxonomic literature yields more data on the presence of "leaf-nodules" in various plants, but these statements have not materially furthered our subject.

As far as known to us bacterial nodules only occur in the leaves of tropical plants and this occurrence is restricted to three families; the Rubiaceae, the Myrsinaceae and the Dioscoreaceae.

With the exceptions of the bacterial symbiosis of Ardisia (Myrsinaceae) the available literature will be reviewed in this Chapter. My own experimental work is so closely related to that of HUGO MIEHE that comprehensive references to his observations and experiments will be scattered throughout this paper.

The sequence in which the families are discussed is historical. Therefore we shall start with the Myrsinaceae, in which cyclic symbiosis was established first.

§ 2. *Anatomical and experimental studies.*

a. *Myrsinaceae.*

The nodules on the crenulated leaf-margins of Ardisia crispa A. DC. were investigated by VON HÖHNEL (22) as early as 1882.

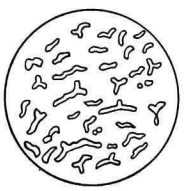

Fig. 1. Bacteroids from foliar nodules, "highly magnified", called by VON HÖHNEL "proteinaceous bodies" (after VON HÖHNEL 1882, Fig. 18).

According to him they are "proteinaceous glands". He correctly observes the "proteinaceous bodies" in the intercellulary spaces of the nodules; "Im fertigen Zustande erscheinen die grossen Intercellularräume des Markes ganz mit bakterienartigen feinen, die Molekularbewegung aufweisenden Körperchen erfüllt, die das Aussehen eines Niederschlages besitzen, aber schon im lebenden Blatt vorhanden und kein Kunstproduct sind." It is remarkable that VON HÖHNEL uses the term "bacteria". A reproduction of his figure is given as Figure 1. The

particles show great resemblance to the bacterial involution-forms later described by MIEHE (31). Neither SOLEREDER (42) 1899 and GROSSE (18) 1908, who both studied these curious organs were able to add anything to VON HÖHNEL's account.

Without exaggeration it may be said that we possess one classic in the literature of foliar symbiosis, and this classic is the work of HUGO MIEHE, who as a result of his work at the Botanic Gardens at Buitenzorg in 1910 published a detailed account of the Ardisia-symbiosis in his "Javanische Studien" (31). In this publication the problem is already clearly outlined.

The bacteria are present on the vegetation-point of the growing plant. Infection of the leaf margins occurs in the hydathodes of the young leaves. Constrictions occur at the margins and in the centre of these constrictions the bacterial nodules occur. The bacteria are always inter- never intracellular. In the seed MIEHE found bacteria between the embryo and the endospern; he could not account for their presence in the seed on the base of ontogeny. With this study MIEHE gave the first example of a cyclic symbiosis between a higher plant and a bacterium; extraneous infection, as in the Leguminosae, does not occur. The relation between the components in this symbiosis could not be defined in this first paper.

MIEHE is certain, however, that the case represents a new and peculiar instance of symbiosis.

MIEHE studied chiefly Ardisia crispa A. DC., but also observed the nodules in other species of this genus (table 1).

His second study (32) is concerned with the bacterium; Bacillus foliicola MIEHE n. sp. MIEHE describes its morphological and physiological characters and its mode of isolation. The constant companion ("Ständige Begleiter") of foliicola, Bacterium repens MIEHE n. sp., is also described. Neither of the forms seems able to develop on nitrogen-free media.

MIEHE's third study (33) deals with bacteria-free plant ("Krüppel") the crippled appearance of which is a living demonstration of a truly symbiontic component without its counterpart. Without bacteria the plant develops but poorly, it remains dwarfed and never flowers. According to MIEHE the very existence of the plant, therefore, depends upon its bacterial symbiosis. Bacteria-free plants are obtained by him by means of heat treatment of the seed.

Artificial infection of the "cripple" did not succeed. Nutrition-experiments showed that nothing pointed towards a possible N-fixation of the bacteria.

MIEHE assumes a stimulative effect emanating from the bacteria ("Reizwirkung"); "Die Ardisia ist auf ein gewisses Stimulans oder mehrere eingestellt, die von der Anwesigkeit der Bakterien abhängt, und hat sich phylogenetisch ähnlich daran gewöhnt, wie der Opiumraucher sich ontogenetisch an sein Opium gewöhnt hat."

The comparison is quite apt, it would be perfect if opium-eating depended upon a heriditary lesion!

ERWIN F. SMITH (41) considers, as a phytopathologist, the Ardisia- and

Pavetta bacteria. According to him they are to be classed as comparatively harmless parasites, producing "weaker toxins and enzymes" as the harmful parasites. They are able to attack only actively growing plants and may supply these plants with nitrogen.

The complete set of data as given by MIEHE did not come to SMITH's attention, maybe because of the war. If he had known MIEHE's 1919 paper he would not have said: "The plant may be dwarfed by the bacteria, because Ardisia grows very slowly, if deprived of them would it grow at all."

One of SMITH's assistants, Dr JODIDI has grown Ardisia's from surface-sterile seeds in sterile environment on media poor in nitrogen (the substrate proved to contain nitrogen at the end of the experiment). The plants grew slower than the controls. Conclusions may hardly be drawn from his experiments.

NĚMEC's (36) point of view on the Ardisia-symbiosis is exactly that of MIEHE. The bacterium, even in contact with the host, cannot fix atmospheric nitrogen, as is shown by the fact that the bacterium is not necessary for root-formation, which formation requires a large amount of nitrogen.

NĚMEC is of the opinion that a hormonal substance, excreted by the bacterium induces normal development in the host. In a few words he confirms all of MIEHE's results, the experimental data, however, are too few to warrant these conclusions.

b. *Rubiaceae.*

TRIMEN (44) 1894 was the first to call attention to the small knob-like excrescences which he observed to occur on the leaves of certain Ceylon Rubiaceae. This characteristic proved to be of taxonomic value, but the true nature of the excrescences remained hidden to this author.

ZIMMERMANN (47), during his sojourn at the Botanic Gardens at Buitenzorg in 1902 observed the constant presence in the foliar nodules of at least 4 species of Javanese Pavetta's (table 1).

He refers to "bacterial nodules" and showed the presence of bacteria between the very young leaves on the vegetation point of these plants. The bacteria enter the young leaves through the stomata and cause local swelling near the midrib and, in other species, over the entire leaf-surface. As no experimental work was performed, no evidence as to the symbiontic nature of these bacteria could be obtained by him.

BOAS (4) 1911 followed by giving an anatomical description of bacterial nodules in the leaves of two Psychotria-species, collected in the Kamerun (table 1).

VON FABER (12) made a comprehensive study of the above-mentioned symbiosis at the Buitenzorg Gardens in 1912. He investigated both the relationship of the bacteria to Pavetta and to Psychotria (table 1). VALETON (45) previous to VON FABER's work, gave a description of the

bacterial nodules in Psychotria (table 1). VON FABER was able to confirm ZIMMERMANN's results as to the mode of entry of bacteria in the leaves of Pavetta. He found, however, that the bacteria are inclined to parasitize upon the host, and that therefore, the bacterial invasion is not without its danger.

VON FABER found the bacteria in the seed, between embryo and endosperm. According to him the bacteria are passively included in the flower after the carpels originate. They enter through the micropyle and gather above the egg-cell. They are thought to enter the embryosac during fertilization, with this entry the bacterial cycle is closed – a cycle – characterized by VON FABER as "heriditary symbiosis" ("erbliche Symbiose"). It seems to us that this term is not very luckily chosen, especially when we refer the mechanism of inheritance to the gametes. The bacterium was isolated and named Mycobacterium rubiacearum VON FABER n.sp. The organism proved able to fix atmospheric nitrogen on nitrogen-free media. To this result VON FABER attaches much interest, as he was able to obtain bacteria-free plants by heating seeds to a temperature near the thermal death-point of the bacteria. These bacteria-free plants developed very badly on nitrogen-free media (both sand- and watercultures) while the normal, bacterial, plants showed copious growth on these substrates.

From these data the conclusion seems justified that the bacteria provide the plant with nitrogen.

A second paper of VON FABER (13) deals with the infection of bacteria-free plants with the bacterium. This result showed that the form isolated actually functions as symbiont. The work of VON FABER shall be further discussed later in this paper.

GEORGEVITCH (16) described bacterial nodules in the leaves of a plant named Kraussia (table 1) in cultivation at Kew Gardens. He isolated from the leaves a spore-forming bacterium on potato-agar. No further results were reported by him. Both the identity of the plant and of the bacterium seem exceedingly doubtful to us.

ADINARAYAN RAO (1) continued VON FABER's work on the symbiosis of Pavetta at Ceylon, particularly in relation to the suitability of Pavetta-leaves as green-manure. He also described bacterial nodules from the leaves of Chomelia asiatica (table 1).

The bacteria isolated by him were able to fix atmospheric nitrogen on N-free mannitol media and he concludes, that both for Pavetta and Chomelia, there is no doubt that VON FABER's interpretation is correct.

As RAO's work is rather fragmentary it may hardly be considered as an important confirmation.

KOŘINEK (26), studying isolated Psychotria-leaves observed that the bacteria bring about neither physiological, nor morphological changes. According to this author, it seems hardly possible, therefore, to consider this symbiosis as a form of "balanced parasitism", as in this case the bacteria should attack the tissue of the isolated leaves.

c. *Dioscoreaceae.*

The attenuated leaf-apices ("Vorläuferspitze") of this family have been investigated in detail by GENTNER (15) in 1905.

In such a leaf-apex a number of longitudinal intercellular canals are present, the intercellular spaces being filled with slime. GENTNER mentions Dioscorea macroura HARMS as an example. The canals should play a role not only in the water economy of the plant, but also in the assimilation and the respiration. Experimental data to prove this assertion are, however, lacking.

ORR (37) observed the bacteria in the intercellular mucus of Dioscorea macroura HARMS (table 1). Similar bacteria were found by him on the vegetation point. The isolated bacterium was able to fix nitrogen on N-free substrate. ORR concludes from this fact that the symbiosis is of the N-fixing (Leguminous) type.

§ 3. *Studies of a taxonomical or phytographical nature.*

a. *Myrsinaceae.*

According to MEZ (29) the "glandulae albuminiferae" in the Myrsinaceae are restricted to the genus Ardisia, subgenus Crispardisia, comprising 30 species and to 5 species belonging to the genera Amblyanthus and Amblyanthopsis. The distribution of the species mentioned includes Indo-China, Formosa, Malacca, the Malayan Archipelago and the Philippines.

b. *Rubiaceae.*

Apart from the older work of TRIMEN (44) and VALETON (45), BREMEKAMP has recently published a monograph on the genus Pavetta (7) in which the bacterial nodules are considered as a, taxonomically, useful and important character. Useful, because African and Asiatic Rubiaceae may be classified in some cases on leaf-characteristics only. Asiatic Pavetta's proved to possess the character throughout. In both subgenera of Pavetta we find both bacteriophilous and bacteria-free species. BREMEKAMP mentions in total 343 species of the genus Pavetta, of which 294 species (under which there are a great number of new species) are bacteriophilous. The latter group is distributed over Africa, Arabia, India, Indo-China, South China and Formosa, the Malayan Archipelago, Tropical Australia and Melanesia. The geographical distribution of the section Pavettaster is given in BREMEKAMP's monograph in a table on page 24 of his paper, to which special attention is called.

A second publication of this author (6) deals with the genus Psychotria. 42 Species, all bacteriophilous, are described, amongst which there are many new species. Psychotria's are only found on the African continent.

c. *Dioscoreaceae.*

Attenuated apices occur in a great number of representatives of this

family, according to KNUTH's monograph (25). It is obvious that, without further study, no conclusions may be drawn from the shape of the leaf alone, while in the Rubiaceae and Myrsinaceae, like in the Leguminosae, the bacteria cause visible swellings, which are quite characteristic and may be recognized as such. It is to be regretted that KNUTH seems unacquainted with ORR's paper, otherwise he might have been on the lookout. Thusfar Dioscorea macroura HARMS is the only example of this family. It seems to be restricted to Tropical Africa.

Summarizing we may say that we find foliar bacterial symbiosis in three unrelated families in the genera:

Ardisia	30	species
Amblyanthopsis and Amblyanthus	5	,,
Pavetta	294	,,
Psychotria	42	,,
Dioscorea	1	,,
In total	372	species

§ 4. *Comments upon the results obtained by other workers.*

a. *The nature of foliar symbioses.*

All investigators, with the exception of MIEHE and NĚMEC, compare the symbioses with the root-symbioses of Leguminous plants and they describe the relationship as if its chief factor, or even only factor, were nitrogen fixation.

Due to a dearth of facts it will be impossible in the near future to compare foliar- and root-symbiosis in detail. Still it seems possible to point out a few facts that seem to weaken the proofs given by VON FABER, RAO and ORR. These authors claim to have isolated, from the leaves of bacteriophilous plants, bacteria capable of nitrogen-fixation. At the time of their investigations it was still doubtful whether Rhizobium radicicola BEY. itself was capable of N-fixation when grown in pure culture.

In 1929 various investigators, under whom we mention LÖHNIS (28) succeeded in showing that *the root-symbiont of the Leguminosae is incapable of fixing free nitrogen in pure culture.*

Opinions to the contrary are accounted for by erroneous analyses, faulty procedure or lack of pure cultures.

A fair amount of scepticism is warranted, therefore, when considering the data on the N-fixation of foliar bacteria.

Until now N-fixation has only been put beyond doubt for various species of the genus Azotobacter, and for one species of the genus Clostridium.

It seems premature to view all forms of symbiosis from the same angle. To illustrate this two types of symbiosis may be, arbitrarily chosen from the known instances. From the comprehensive work of BURGEFF (10) on

the endotrophic mycorrhiza of the Orchids it follows that neither the symbiontic components, nor the complete symbiosis is able to fix atmospheric nitrogen.

The relationship of algal- and fungous components in the Lichens may be mentioned as a second example. Here the functional connection between the autotrophic and the heterotrophic elements seems again to be quite different from that met with the Leguminous root symbionts (BURGEFF 9).

MIEHE (33) and NĚMEC (36), however, ascribe the foliar symbiosis to a stimulative effect emanating from the bacterium or to a hormonal substance secreted by the bacterium. This, at least, seems a new principle worth investigating.

VON FABER, who started with his work on Pavetta after MIEHE's visit to Java, has reached results different from those of MIEHE. His polemic with MIEHE, questioning his results (with Ardisia) and comparing these results with his own (on Pavetta) seems rather pointless, the more so because comparatively few data were available in each case. In this paper we shall consider both symbioses separately.

As already mentioned, VON FABER's term "heriditary symbiosis" may better be avoided. We shall use "cyclic symbiosis" instead, reserving the epithet "heriditary" for the lesion in the bacteria-free plant which might be caused by mutation, parallel cases of this "semi lethal" loss-mutation being found in totally unrelated families!

b. *Enumeration of bacteriophilous plants.*

As mentioned above, foliar symbiosis, while restricted to few families and genera, is really of wide occurrence. A little less than four hundred species are mentioned.

However trustworthy these data and however probable the bacteriophilous nature of these species we may still doubt any diagnosis based upon macroscopic examination alone. For this reason we have restricted ourselves to these few plants on which there exist anatomical or experimental data. Table 1 gives an enumeration of these plants. The nomenclature of the various investigators is given in the first column while the second column gives modern nomenclature (for the Rubiaceae we follow BREMEKAMP), and, where necessary, the author's name mentioned.

On the case of Chomelia asiatica and Kraussia floribunda it seems probable that the authors mean something else.

Chomelia may have been a Pavetta and Kraussia a Psychotria, but this is no more than a guess.

Chomelia's and Kraussia's present in the Government Herbarium of the University, Leiden, did not contain nodules [1]).

Systematic anatomical investigation of the leaf-apices of the genus Dioscorea might lead to extension of the number of bacteriophilous species.

[1]) At this place I want to express my thanks to Dr S. J. VAN OOSTSTROOM, head-assistant at the Herbarium, for his expert advice.

TABLE 1.

Name used by the investigators	Modern nomenclature	Author
Myrsinaceae		
Ardisia crispa A. DC.	Ardisia crispa (THUNB.) A. DC.	MIEHE (1911—1919)
incl. var. compacta		SMITH (1920)
		NĚMEC (1932)
Ardisia Cumingiana A. DC.	id.	MIEHE (1911)
Ardisia spec. from Gunung Tjisalak	?	MIEHE (1911)
Rubiaceae		
Pavetta lanceolata EKL.	id.	ZIMMERMANN (1902)
		VON FABER (1912)
Pavetta indica L.	id.	ZIMMERMANN (1902)
incl. several subspecies		VON FABER (1912)
		ADINARAYAN RAO (1923)
		HEUBEL (1933)
Pavetta angustifolia THW.	Pavetta agrostiphylla BREM. nom. nov.	ZIMMERMANN (1902)
		VON FABER (1912)
Grumilea mikrantha HIRN	Pavetta Zimmermanniana VAL.	ZIMMERMANN (1902)
		VON FABER (1912)
Pavetta gardaeniaefolia HOCHST.	id.	VON FABER (1912)
Chomelia asiatica (author?)	Pavetta species?	ADINARAYAN RAO (1923)
Psychotria bacteriophila VAL.	Psychotria punctata VATKE	VALETON (1908)
		VON FABER (1912)
		KORINEK (1928)
Psychotria alsophila K. SCH.	id.	BOAS (1911)
Psychotria umbellata THON.	id.	BOAS (1911)
Kraussia floribunda HARV.	Psychotria spec.?	GEORGEVITCH (1916)
Dioscoreaceae		
Dioscorea macroura	Dioscorea macroura HARMS	ORR (1926)

All the Rubiaceae mentioned in table 1 (the Psychotria's of BOAS and Pavetta lanceolata excepted) were examined and showed the presence of bacteria. The material consisted either of living leaves from plants grown in the Botanic Gardens of the University of Leiden, or alcohol material collected by Prof. Dr L. G. M. BAAS BECKING at Buitenzorg in 1936.

On the Myrsinaceae, apart from Ardisia crispa A. DC. the presence of bacteria could be demonstrated in Ardisia crispa var. fructu albo (HORT) and in Ardisia vestita WALL.

A living plant of Dioscorea macroura HARMS showed copious numbers of bacteria in the leaves.

§ 5. *Application of bacteriophilous plants as green manure.*

The fact that the Ceylon natives use Pavetta-leaves as green manure is mentioned by various authors, VON FABER (10) and ADINARAYAN RAO (1).

HEUBEL, in a recent article (21) again points to the advisability to use Pavetta's as ground cover in Hevea plantations. HEUBEL seems convinced of the fact that the Rubiaceae of this type are capable of nitrogen-fixation. This idea seems premature, to say the least, as long as we are still waiting for extensive and careful research on this matter.

CHAPTER II.

Statement of the problem.

From a survey of the literature it follows that only for the Pavetta- and for the Ardisia-symbiosis detailed investigations are available. We feel very much attracted to the work of HUGO MIEHE on Ardisia, not only because of the lucid exposition of the problems, but also because of the careful and unbiased way in which he analysed the various factors. More than VON FABER he aims at an objective treatment and this objectivity has kept him from looking for analogies.

Dearth of material has undoubtedly been the cause of the fact that MIEHE's work is incomplete. But the reading of his three papers on this subject is so stimulating that it urged us to try to follow his trail and repeat his experiments with a larger amount of material and extend them with the aid of modern botanical theory. This paper, therefore, aims to be no more, and is probably less, than what MIEHE himself would have done if he had tackled the problem again. As Ardisia is cultivated at almost all Botanic Gardens it proved comparatively easy to obtain a large amount of material.

MIEHE's results are enumerated below and the gaps in these results constitute therefore our problem.

1. The foliar-symbiosis of Ardisia is a cyclic symbiosis. In this cycle, however, there is one missing link, in so far as MIEHE does not know how the developing fruit obtains its bacteria. As spontaneous "cripples" (i.e. bacteria-free plants) develop from seeds, the assumption follows that not every seed becomes infected. Or, on the other hand, that the external conditions are responsible for the appearance of those cripples.

2. The bacterium is isolated and, as far as possible, characterized morphologically as well as physiologically.

Some uncertainty, however, remains. For Bacillus foliicola MIEHE should, only on the base of its morphological characteristics, be considered as the symbiont, as it forms involution-forms on certain substrates, analogous to the forms found in the marginal nodules of the leaf.

Bacterium repens MIEHE, the "constant companion", is considered by MIEHE as a secondary and accessory form. Further reasons for this opinion are not given by him.

3. MIEHE obtained, experimentally, bacteria-free plants by means of heat-treatment. Spontaneous bacteria-free plants were, moreover raised by him, from $\pm$ 50 % of the seeds. The cause of this phenomenon remains obscure. The percentage of "spontaneous cripples" remains to be explained. To a geneticist the number might look significant!

The "experimental" and the "spontaneous cripple" are identical in every respect. A detailed anatomical investigation was not performed by MIEHE.

As the cripple does not flower and does not seem to propagate vegetatively, the bacterium seems necessary to the very existence of the plant. From a phylogenetic point of view this is indeed remarkable, as many species of Ardisia are known without bacteria.

4. Nitrogen-fixation probably cannot account for the symbiosis. Normal plants react markedly on the application of additional nitrogen to the substrate. Moreover, root-formation takes place copiously in the bacteria-free plants. As long as it remains impossible to raise normal (non-crippled) bacteria-free plants, the problem of N-fixation remains insoluble and should be approached by other means.

5. MIEHE did not succeed to perform the synthesis of the symbionts, either by means of pure cultures or by bacterial tissue of normal plants. In the former case the proof should have been given of the identity of the isolation and in both cases the necessity of the bacterium for the normal development of the plant should have been demonstrated.

The above facts and speculations served as a guide for further research. Due to the artificial conditions of the plants in many experiments we want to state that the symbiosis has not been studied under natural conditions. A brief summary of the following chapters is given below.

a. Material from various places was collected and tested as to its specific homogeneity. The conditions of its cultivation are defined (Chapter III).

b. Following MIEHE, the distribution of the bacterium in the plant was investigated. The infection of the fruit was especially studied. The relation between number, form and activity of the bacterium was studied by observation and experiment (Chapter IV).

c. Isolation of the bacterium was repeated on a more extensive scale. The properties of the isolated strains are given (Chapter V).

d. The method to obtain crippled plants (experimental cripples) and the spontaneous occurrence of cripples (spontaneous cripples) was studied. The external appearance of both types of cripples is given. Anatomical details are given in order to compare normal and bacteria-free plants, chiefly in order to see what changes are caused by the absence of bacteria. Especially the condition of the meristematic tissues in the cripple compared with that of the normal plant was studied. Cytological observations were carried out in order to see if the "chromosome portrait" is changed in cripples. The state of the oxido-reduction system in homologous tissues in cripples and normal plants was studied. Quantitative data of the occurrence of spontaneous cripples are given. Those cripples were studied in their early stage of development. The temperature as a causative factor to obtain experimental cripples is finally discussed (Chapter VI).

e. It was tried by various means to incite cripples to normal growth. The influence of hetero-auxin on cripples and on normal plants was studied

in relation to other cases of nanism, mentioned in the literature. (SCHLENKER and MITTMANN). Various grafts were prepared. Normal was grafted on cripple, cripple on normal to show eventually the influence of the normal scion on the crippled stock and of the normal stock on the crippled scion. Infection experiments with the isolated strains were performed in order to obtain proof both of the specific nature of the strains isolated and of the nature of the symbiosis. Normal plants were mutilated by removal of the terminal bud and the leaf-nodules in order to localize the role of the bacteria. Experiments with intermediates were carried out to obtain eventual normal shoot formation on the "opposite side". (Chapter VII).

f. The data obtained in the previous chapters are used to form an idea about the nature and the origin of the foliar symbiosis in Ardisia crispa A. DC. (Chapter VIII).

CHAPTER III.

Material. Method of culture.

As it seemed advisable to investigate a large number of plants it was necessary to obtain as much material as possible.

Apart from the plants present in the Hortus Academicus of Leyden seed was obtained from the Botanic Gardens; Baarn (Cantonspark), Buitenzorg (Hortus Bogoriensis), Liège (Hortus), Rotterdam (Zoological Gardens) and Wageningen (Arboretum).

The Rotterdam Zoological Gardens kindly put at our disposal ten three-year old, fruiting plants. We purchased, moreover, a 10—15 year old plant, which bore fruit copiously, from a private garden at Hilversum. Several hundred seedlings were obtained from a commercial establishment at Aalsmeer [1]).

With the kind help of Dr J. TH. HENRARD, conservator at the Government Herbarium, the plant material was classified and proved to belong to the species Ardisia crispa (THUNB.) A. DC. Usual synonyms, especially in Botanic Gardens, are Ardisia crenulata LODD. and Ardisia crenata ROXB.

Nearly all of the seeds yielded Ardisia crispa (THUNB.) A. DC. The seeds from Buitenzorg, however, yielded another bacteriophilous species of the genus Ardisia which, as it remained sterile, could not be classified as yet.

Ardisia crispa var. fructu albo (HORT) also possesses nodules. The seed of this species I also obtained from Liège.

The Pavetta seeds I used in the temperature experiments (Chapter VI) were sent from Buitenzorg. They all yielded Pavetta Zimmermanniana VAL.

The Ardisia's were sown and raised in glass-houses in the Hortus. The method of sowing will be given in Chapter VI. At this place we shall mention the fluctuations in temperature in the two greenhouses used, as temperature is an important factor in the sterilization of the seeds (Chapter VI). The temperatures are obtained from corrected thermograph data.

a. *Nursery.* Most of the material was sown and raised in this tropical house. Grafting and infection-experiments were also carried out in this place. It is a low building, the main axis of which is in the N—S direction.

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[1]) At this place it is a pleasure to thank several persons who helped me to gather the material especially Dr K. KUIPER, Director of the Rotterdam Zoological Gardens, Mr. G. TH. ODIJK, orchid-grower at Voorschoten, Professor Dr M. J. SIRKS, Dr D. F. VAN SLOOTEN, chief of the Buitenzorg Herbarium and Mr. H. VEENDORP, Hortulanus at Leiden.

The plants were raised at the West-side. Seed pans or pots were dug into peat on heated planting tables. Larger plants were treated similarly [1]).

The temperature-fluctations run as follows:

	Max.	Min.	Av. Max.	Av. Min.
June 1936	36° C	17	30.5	20.1
July 1936	35	20	34.2	22.2
Nov. 1936	25	15	22.2	18.1
Dec. 1936	20	14	18.1	15.6

The humidity in this house is very high, the air being practically always saturated with water-vapour.

b. *Orchid-house.* A number of experiments with seeds were carried out in this house, which is also low, main axis N—S. The seeds germinated on the E-side in a similar way as in the nursery-house. Due to a deficiency in the construction of the tables, the high water level in peat caused trouble, because of bad drainage. The house is not heated in summer.

Temperature-fluctuations:

	Max.	Min.	Av. Max.	Av. Min.
June 1936	30° C	15	24.4	17.8
July 1936	30	16	24.4	18.2
Oct. 1936	27	19	25.4	19.6

The fluctuations in this house, during the growing season, were therefore less than in the nursery-house [2]).

c. *Constant temperature room.*

Here seeds were germinated in the dark, at a temperature of 25° C. ± 0.1°. No humidity control was used, the saturation percentage varying from 70—80.

[1]) Mr. A. LAGENDIJK, gardener, has spared no efforts to assist us in this work. For four years he has raised and cared for countless Ardisia plants. Without his constant help and his wise council the work could not have been brought to a successful end.

[2]) Mr. A. J. TAFFIJN, gardener, has been of great help to me in these experiments

CHAPTER IV.

The occurrence of bacteria in the plant.

In order to obtain a clear idea on the nature of the symbiosis it is necessary to know the distribution of the bacteria on or within the plant-body. Only in this way it is possible to reconstruct the ontogenetic side of the symbiosis.

But not only the localization of the bacteria is important in this connection. For already MIEHE pointed to the fact that the bacteria in the plant occur in different forms. While he found rods in the neighbourhood of the vegetation point, the foliar nodules showed bacterial involution-forms. Moreover the frequency of the bacteria is also variable. The three points mentioned are considered in this chapter in their mutual relation.

§ 1. *Material and methods.*

The sections were cut from material originating from our own cultures. Picroformol (Regaud I) was used as a fixative. The objects were sectioned in the usual way in series of 6 μ in thickness. Heavier sections were less useful especially when the bacterial frequency was high, as the individuals could not be distinguished in this case. The sections were either stained with HEIDEHAIN's haematoxylin or with FLEMMING's triple stain. Haematoxylin stains the bacteria blue, while the Gentian Violet in the triple stain colours the entire bacterial mass. The former method is, therefore, preferable. Difficulties in interpretation were encountered when the bacteria are situated very close to, or on top of living cells, as mitochondria and other plasmatic inclusions might be taken for them. As, however, the bacteria are always intercellular only in rare cases this difficulty led to uncertain diagnosis. Later it proved unnecessary to apply MILOVIDOV (34) beautiful, but complicated and tedious method by which bacteria are differentiated from chondriosomes. The haematoxylin often formed rod-shaped precipitates which also gave a misleading picture. Soon, however, one obtains the routine necessary for trustworthy interpretations. Moreover, if absolute certainty about the presence of bacteria was not obtained, their absence was stated in the protocols [1]).

§ 2. *The occurrence of the bacteria on the vegetation-point.*

MIEHE demonstrated the presence of large numbers of bacteria between

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[1]) Mr. R. BOOM, voluntary technician at the Botanical Laboratory prepared the numerous slides used in this work with great accuracy. I want to thank him for this valuable assistance.

the developing leaves in a terminal bud. The bacteria are, as it were, imbedded in a mucilaginous membrane situated between the leaf-primordia and leaves in all stages of development. They are also present near the vegetation point proper.

On the places indicated by MIEHE we always found bacteria, also on the meristematic vegetation point, which is almost flat, the leaf primordia showing as protuberances. (Photograph 1). The bacteria appear as, often feebly curved, rods in a mucilaginous layer (Photograph 2). In this membrane we found, in confirmation of MIEHE's observation, also the multicellular hairs, which are inserted on the various elements of the foliar bud.

On the ventral side of older leaves and especially a greater distance from the vegetation point proper, the number of bacteria decreases materially and their clear rod-shape is lost. The membrane seems stretched by the growth of the leaf.

The same image is observed in the primordia of the inflorescence. The development of this lateral shoot is restricted to an axis with 2—3 normal leaves. The terminal bud of this axis remains dormant for a year, in the next season the umbel is formed. The bacteria remain in a latent condition for over a year and resume their activity when the flower develops.

A similar resting- or latent condition of the bacteria occurs in the dormant buds. In the axils of every leaf we find endogenous buds, which buds only develop after the terminal bud has been removed, which is a well known phenomenon in horticultural practice. If a dormant bud becomes active by such manipulation, we find bacteria here at the same places as in the terminal buds. These bacteria should have been present there before, but their small numbers made their localization impossible.

Experiments showed that all dormant buds may become active and yield normal shoots. MIEHE found that two year old buds could be raised to development. My own observations show that 3—4 year old plants, when cut back sufficiently developed shoots from dormant buds just above the soil. The bacterium should have been in its latent, but reversible stage, for over three years!

§ 3. *The development of the bacterial nodules.*

As our observations on this point confirmed those of MIEHE, it will suffice to briefly mention his results. As shown in § 2 the bacteria are present almost everywhere in the foliar bud. The leaf-bases in such a bud cover each other alternatingly (Photograph 6). The oldest stages are rolled-in to such an extent that the margins nearly touch the morphological upper-surface of the leaf. In this stage the nodules appear. At the dorsal side of the leaf, at the margin, hydathodes occur at regular intervals. They are formed earlier than the stomata at the ventral surface. In this stage, the atrium of the hydathode contains bacteria, while the deeper, intercellular, cavity and the subsidiary cavities still are free from bacteria.

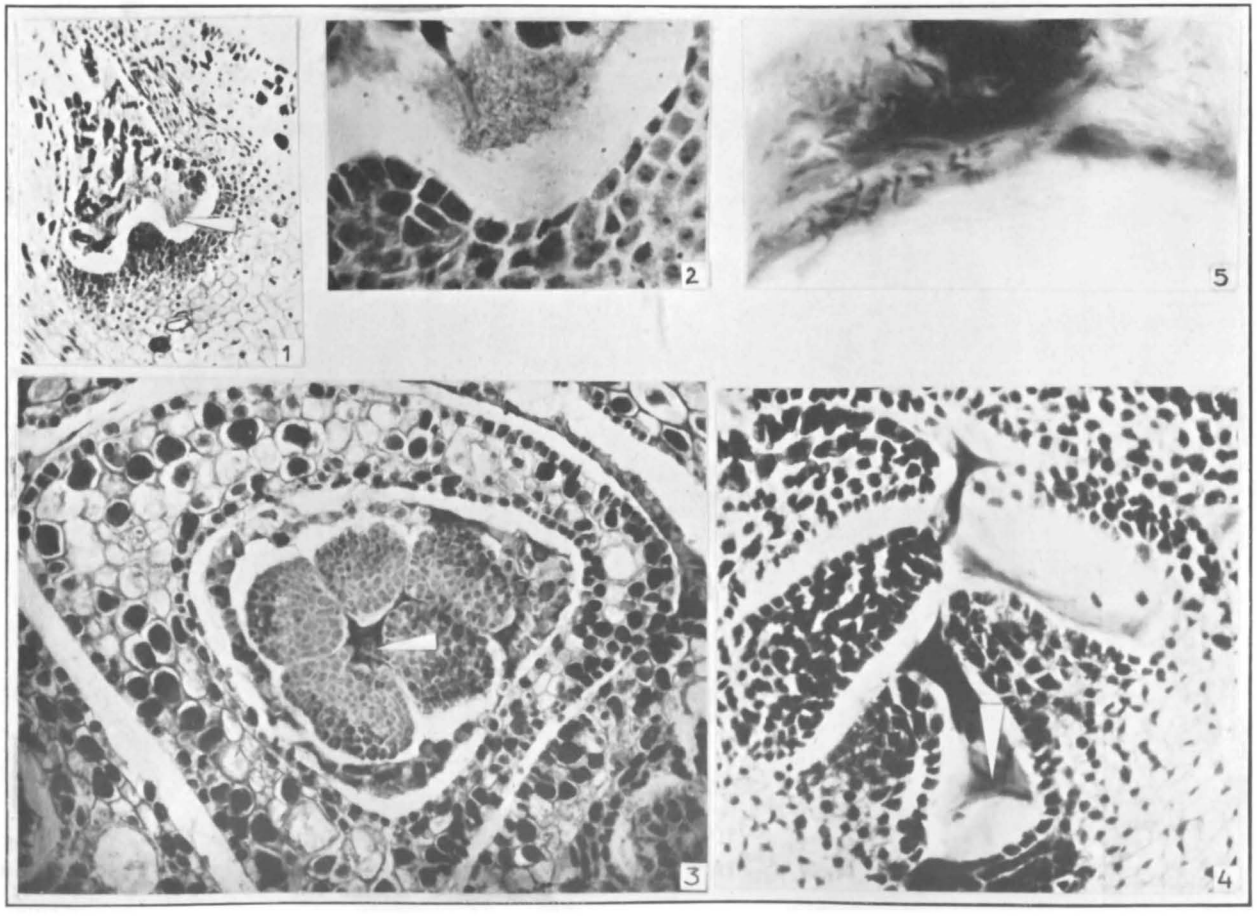
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PLATE I.

The bacteria in foliar buds and in floral buds.

Photograph 1. Transverse section through a terminal bud. Young leaf-primordium visible. The arrow points to the bacterial mass.
Haematoxylin. 480 ×.

Photograph 2. Detail of Ph. 1. 1080 ×.

Photograph 3. Cross section through young flower within its bract. Bacterial mass in the centre, surrounded by calyx-primordia.
FLEMMING's triple stain. 170 ×.

Photograph 4. Longitudinal section through young, developing flower. Bacterial film within the primordia, a large mass being enclosed by the carpels (near the arrow).
Haematoxylin. 340 ×.

Photograph 5. Detail of Ph. 4. Bacteria in mucilaginous membrane. 2000 ×.

In older stages we find a foamy mucus in this cavity, which mucus contains the rod-shaped bacteria (Fig. 2, after MIEHE). The intercellular cavity is closed by the growth of the cells near the pore, and the bacteria are isolated from the outer world.

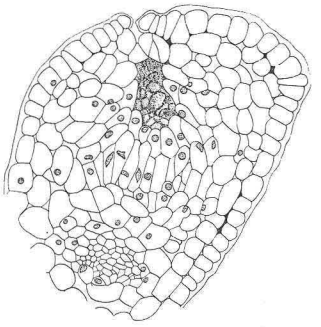

Fig. 2. Section through a young foliar nodule, 348 ×. (after MIEHE, "Javanische Studien", Fig. 20).

The leaves which originate from the terminal bud show marked changes. The development of the outer cells of the hydathode pushes the bacterial mass inward, which mass is extended in such a way as to cause the cells lining the cavity to be pushed in, so that they assume a concave outline. In a further stage the bacteria are present exclusively in the intercellular spaces of the tubular cells in the growing nodule.

The bacterial mass increases in size only after a procambial strand is given off by a lateral rib of the leaf. The tubular cells are placed radially into the cavity, in which later the extension of the bacterial mass takes place.

The mature nodule shows a spherical outline in cross section. The bacteria are massed together. I found, however, a few isolated irregular tubular cells in the nodule (Photograph 7).

On tangential section the contour of the nodule is bean-shaped. Although MIEHE applied many microchemical tests, no "special" substance seemed to be present which might give a clue as to the nature of the symbiosis.

In young nodules we find, in the foamy mucus, rod-shaped bacteria, while in the older leaves we only find bacterioids. In very old leaves the number of bacteria is materially reduced, often to almost none. A bacteria-free nodule seems to cause accumulation of starch in the surrounding tissue.

§ 4. *The infection of the fruit.*

Probably because of the lack of material, MIEHE did not study this part of the cycle. In one case he states the presence of bacteria inside the carpels of a very young flower. A very detailed description is given by him, however, of the bacteria in the mature seed. Here they are localized between the plantule and the endosperm, at the radical pole. The fate of the bacterium in the intermediate stages, flower and fruit formation, were not investigated by him. These stages represent the most interesting part of the cycle, as the presence of the bacteria between plantule and endosperm has to be accounted for ontogenetically. It is clear that these stages should be closely linked with the embryology of the plant.

The embryology of Ardisia is partly described in a detailed study (unfortunately not illustrated) by JAENSCH (23), who demonstrates the occurrence of apogamy. This is all we could find in the literature, a few observations of DAHLGREN (11), confirmatory of JAENSCH, excepted.

There are at least three ways in which the embryo of Ardisia may become infected.

1. Notwithstanding the observations of JAENSCH, according to which apogamy occurs as a rule, he also observed, in one case, a dividing egg-cell. In our own experiments only pollinated flowers yielded fruit. One might, therefore, accept the possibility that bacteria, once present inside the carpels, could be brought inside the embryo sac by means of the pollentube. in this way the bacteria could develop between the endosperm and the embryo. MIEHE is inclined towards this assumption, without giving further arguments in favour of it. VON FABER (12) accepts a similar mechanism for the infection of the seeds of Pavetta, because he observed, in two cases, bacteria on the micropyle.

2. Apart from sexual or asexual reproduction, it is conceivable that the bacterium might enter the ovulum by some special or incidental cleft or channel and in this way either enter the embryo sac or, at a later stage arrive between embryo and endosperm. In this case the bacterium should be already present within the carpels.

3. During the development of the integuments the bacteria, already present near the apex of the ovule, are enclosed between nucellus and interior integument. The bacterial membrane is, as is usual, activated by the developing meristem and forms a film under the micropylar region. The apogamous embryo finds the bacteria at its radical pole.

In view of the extracellular character of the bacterium, other hypotheses in which the microbes should penetrate the cell, are excluded. Only in the first hypothesis the organism is, at one stage, intracellular. This hypothesis should be excluded, however, as neither by JAENSCH nor by myself pollen-tubes are found within the flower. The influence of pollination is, as we shall see later, an indirect one.

The second hypothesis is also improbable. Neither in the ovule or in any of the structures derived from it clefts or channels were observed.

The probability of the last-mentioned hypothesis (3) is great, when we take into account the detailed embryological description of JAENSCH, which could be confirmed on all points.

Inasmuch as embryological details were to me only means to an end, reference is made to JAENSCH' results, and at this place only the points necessary to elucidate the life cycle of the symbiosis are mentioned.

The infection of the fruit will be considered in four successive stages; the young, developing flower, closed flower, fruit 2 mm in diameter, fruit 4 mm in diameter.

a. *Young, developing flower.*

The floral bud, reminding us in its structure of a foliar bud, it might be expected that the distribution of the bacteria should also be similar in both organs. A transverse section through a young floral bud shows that this is indeed the case (Figure 3). The entire inflorescence is still

enclosed by bracts and successive stages of floral development may be observed within those bracts. The individual flowers are each surrounded by a bract, within which we invariably find a bacterial membrane, shrunk away from its original position by fixation of the material. This membrane surrounds all developing floral elements (Photograph 3). We also find here the trichomes previously observed in the foliar buds. The great similarity between assimilating leaves and floral leaves is strikingly shown in longitudinal sections. Here not only the leaf-primordia are conspicuous, but also it may be seen how the carpels approach one another, enclosing a quantity of bacteria (Fig. 4 and Photograph 4). The observations give the impression that the bacteria are enclosed passively, and purely mechanically. At this stage all parts of the flower are meristematic and the bacteria rod-shaped (Photograph 5). The other floral parts, anthers, petals and sepals are also surrounded by a bacterial film, but nodules could in no case be detected. In all flowers investigated in this stage bacteria were found to be present and we might therefore conclude that every flower is supplied with the symbiont — which conclusion is important in view of the occurrence of so-called "spontaneous cripples" (Chapter VI). It appears that, generally, the bacteria are more numerous in the neighbourhood of meristematic tissues, while the film is stretched by the growth of the various floral elements and therefore contains, at later stages, a decreasing number of bacteria.

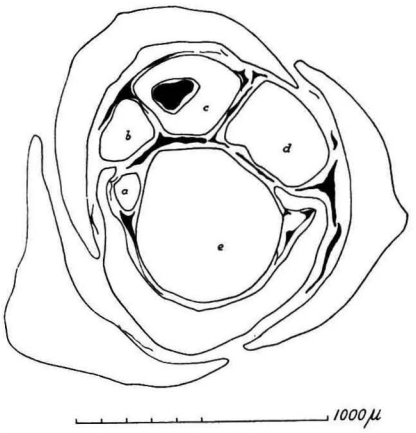

Fig. 3. Cross section through a young inflorescence, containing five flowers *a*, *b*, *c*, *d* and *e* cut at different levels, only outlines of structures are given. Bacterial film in black.

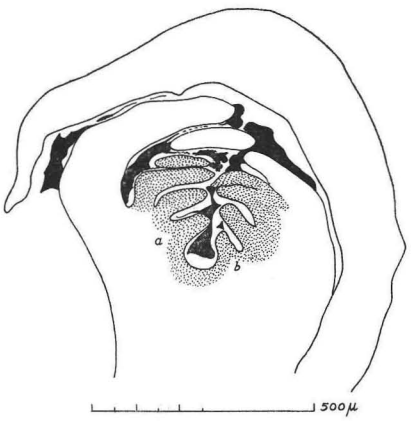

Fig. 4. Longitudinal section through a very young flower. Bacterial film in black. The film is being surrounded by the carpels *a* and *b*.

b. *Closed flowers* [1]).

Young flower-buds show an entirely meristematic placenta with develop-

[1]) At this place I should like to thank Miss ADA P. VERMEULEN, B. Sc., for her keen interest in the work and the thorough examination of the numberless slides.

ing ovules (3—5), the bacteria appear as a cap-shaped mass on top of the placenta (Photograph 8). Because from now on interpretation becomes difficult, it seems well to follow JAENSCH' description rather closely.

According to this author the integuments and the nucellus become differentiated simultaneously at an early stage. The ovules develop as small protuberances on the placenta; (the picture that DAHLGREN gives of this stage seems misleading to me) in these protuberances the development of the integuments and the nucellus may be observed. Both integuments originate from definite initial-cells of the placentar dermatogen, which initials flank the nucellar initials. The interior integument develops first, it surrounds the nucellus before the exterior integument arrives at the micropyle. This micropyle is not situated at the apex of the ovule, but is pushed, by the fast-growing dorsal flank of the interior integument, to the ventral side (tendency to kampylotropy). The peculiar wedge-shaped part of the ventral flank of the interior integument which helps to close the micropyle was observed by JAENSCH and deserves special mention. The cellular contents of this wedge are much denser than those of the surrounding cells.

The nucellus forms simultaneously with the integuments, at first its position is central, later the mass is pushed, by many divisions at the ventral side of the ovule, towards the apex. According to JAENSCH the embryo sac apparatus is but rarely present and reduced to four cells. DAHLGREN gives a picture of the equatorial plate of dividing macrospore showing the haploid number of chromosomes viz. 23.

JAENSCH does not attach much importance to the one dividing egg-cell found by him. Because he rarely found germinating pollen and also because he observed embryo's originating from integumentary tissue, he concludes that Ardisia crispa is apogamous (according to WINKLER's nomenclature we should call this adventitious embryo-formation rather than apogamy). My own observations warrant the conclusion that, with great probability, in the young closed flower bacteria are enclosed in the micropylar region between nucellus and integument, in the neighbourhood of the wedge-shaped cell-mass. The bacteria are close to the nucellus because of the fact that the interior integuments arrive at the micropylar pole before the exterior integuments. In the neighbourhood of the micropyle small numbers of bacteria could be observed.

Longitudinal section of a closed flower shows a central, almost sessile placenta, with 3—5 ovula. The ovula are almost entirely surrounded by placental outgrowths (Photograph 9). The above conclusion according to which bacteria are enclosed between the interior integument and the nucellus, is founded on the fact that in very young flowers the apex of the placenta carries large numbers of bacteria (Photograph 8). While transverse sections of the same development stage showed bacteria in the neighbourhood of the ovula, but not with absolute certainty. The number of bacteria is quite small at the moment when the interior integument

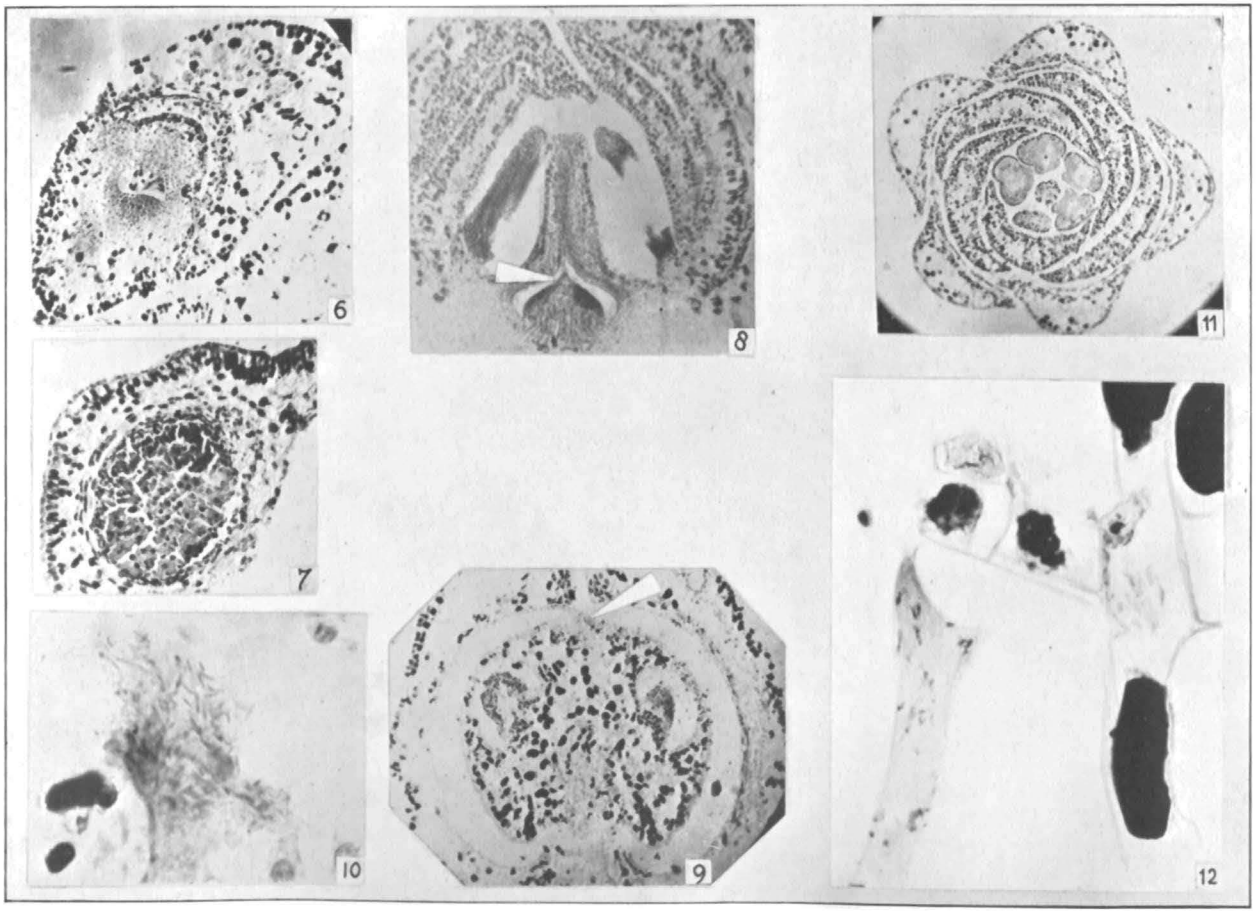
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PLATE II.

The bacteria in the leaf nodule and in the flower.

Photograph 6. Cross section through a terminal bud. Older stage in foliar development at upper-right. Vegetation point and bacterial mass at the centre. Haematoxylin. 120 ×.

Photograph 7. Cross section through a mature foliar nodule. The black dots near the central part are cross sections of the tubular cells. The greyish mass surrounding these cells are bacteria. FLEMMING's triple stain. 120 ×.

Photograph 8. Longitudinal section through very young flower. Calyx, corolla, anthers, carpels and meristematic central placenta. On top of placenta the bacterial mass. Haematoxylin. 120 ×.

Photograph 9. Longitudinal section through the ovary of a closed flower. Two ovula visible, surrounded by placentary outgrowths. Centre top; style-canal, bacterial mass just below its entrance. Haematoxylin. 70 ×.

Photograph 10. Bacterial mass from Ph. 9. 1000 ×.

Photograph 11. Cross section through a closed flower. Calyx, corolla, anthers, style. Bacterial film within the calyx, visible as a thin line. Haematoxylin. 70 ×.

Photograph 12. Detail of photograph 11. Calyx with multicellular hair. Bacterial film disrupted, probably by the fixative. Haematoxylin. 1000 ×.

covers the nucellus. At this stage the number of the bacteria on the placental apex is also very small (Photograph 10).

In thirty flowers investigated the egg-apparatus was only seen once. Confirming JAENSCH' observations it consisted of four cells. (Figure 5). The wedge-shaped protrusion of the ventral flank of the interior integument is clearly differentiated from the rest of the tissue by its dense cell-contents.

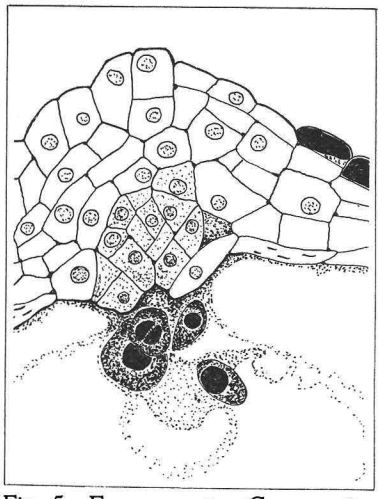


Fig. 5. Egg-apparatus. Cross section through a still closed flower. Two synergids (?), an egg cell and a "vegetative" cell are visible below the wedge-shaped part of the ventral interior integument. Series 41. 4. 2 mm Immersion, eyepiece K 7 Zeiss.

Just below this wedge we see two cells, which may be interpreted as being synergids. The other two cells are probably the egg-cell and the vegetative cell. This egg-apparatus is similar as that described by JAENSCH, but the position of the synergids is important because of the development of the young embryo from the wedge-shaped cell mass. As the embryo's are diploid, and as DAHLGREN demonstrated reduction-division in the embryo sac mother-cell, a direct development from the synergid seems excluded. The nucellar tissue, at this stage, is completely resorbed by the developing embryo sac, which also begins to attack the interior integument. The inner epidermis of this integument shows, in its cells, peculiar, spindle-shaped bodies, which take haematoxylin and which bodies may be indicative of an incipient resorption.

Outside the carpels the bacterial film is much reduced at this stage. We only find it back at the interior side of the calyx, the trichomes of its inner epidermis protruding into this film (Photograph 11 and 12). Neither on the interior flank of the corolla, nor on the anthers a reduced bacterial film could be observed. Probably the active longitudinal growth of the latter elements has stretched the film and disrupted it.

c. *Pollinated flowers.*

In all cases investigated the situation seemed similar to that found in closed flowers. No bacteria were found on the apex of the placenta, however. A longitudinal section showed that the exterior integument has not yet closed the micropyle, while the inner integument appears fully developed (Photograph 13). The resorption of the inner integument proceeds farther. No egg-apparatus was found and no pollen tubes, either in the style-canal or near the placental region. The observations again confirm those of JAENSCH.

d. *Young fruit.*

Only one of the ovules is conspicuous in this stage, the others seem to degenerate. A marked extension of the embryo sac is observed with proceeding resorption of the integuments. The inner integument almost entirely disappears, apart from the wedge-shaped cell-mass. The kampylotropy is more pronounced. In young fruits of a diameter of 2 mm many endosperm nuclei, situated in irregular strands of protoplasm and without intervening walls, could be observed. The protoplasm appears as a curious strand, winding to and fro in the embryo sac. Between the cells of the wedge-shaped mass at the micropylar end numerous rod-shaped bacteria are observed in a film or membrane (Photograph 14).

e. *Fruit 4 mm diameter.*

Many important changes occur here. In all cases investigated I observed curious growths within the growing embryo sac. The growths originated from the wedge-shaped mass near the micropyle and they may protrude more than 240 μ into the embryosac. At the micropylar end of these growths, which I shall call embryogenic tissue, a bacterial film is invariably present. The first case examined showed a proembryo surrounded by the cell-wedge and, at its radical pole, by bacteria (Photograph 15). This embryo could be followed through three sections. In another developing fruit a similar proembryo was observed, and in still another case two embryo's were seen, surrounded by embryogenic tissue. One of these embryo's was in an advanced stage of development (Photograph 16) probably just before the differentiation of the cotyledonary primordia. The type of this embryo (SOUAGÈS) could not be ascertained, however, because it was sectioned obliquely. The proembryo's mentioned all carried bacteria at the suspensor-side, which is also the micropylar side. The large embryo depicted in Photograph 16 could be followed for several sections and also showed bacteria at the suspensor-side (Photograph 17). In this stage of development the embryo sac is cucumber-shaped and shows, at its ventral flank, a ridge, which ridge ends into a blind channel at its apical side. A schematical longitudinal section of the embryo sac is given in Figure 6. The embryo sac is entirely surrounded by placental tissue, which also protrudes into the channel. The chalaza is placed almost central and dorsal of the embryo sac. The endosperm consists, at this stage, of thin

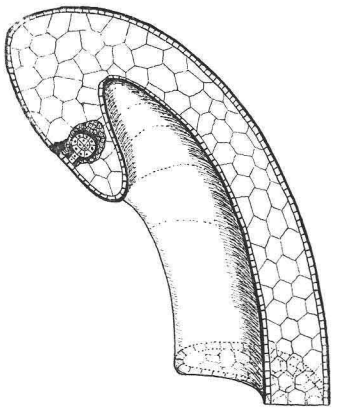
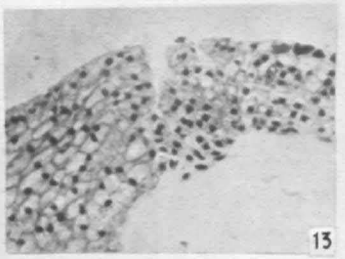


Fig. 6. Schematical reconstruction of a median section through a mature embryo sac from a 4 mm fruit. Size of cells arbitrary, only exterior integument shown. Adventitious embryo within embryogenic tissue, at the basal pole a bacterial mass. Just above the embryo; the micropyle.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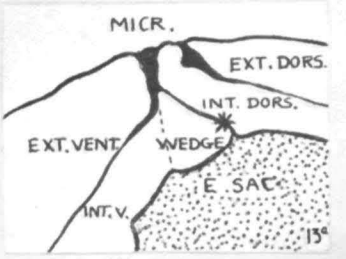

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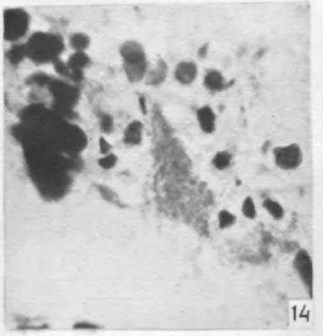
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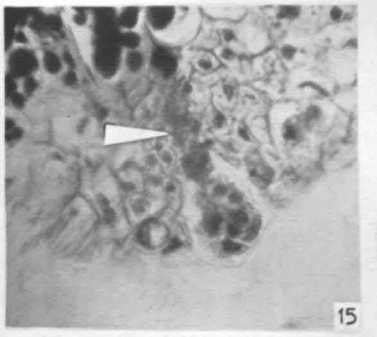
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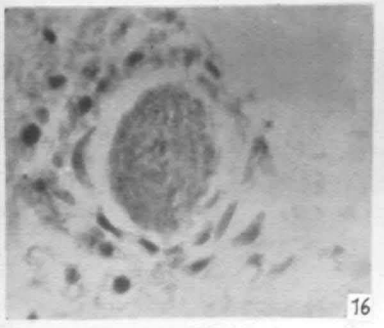
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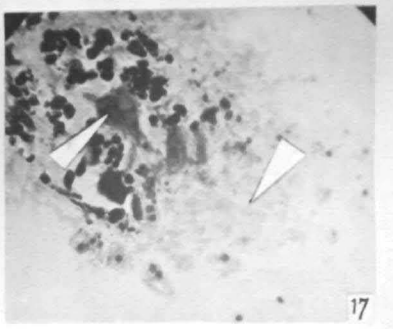
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PLATE III.

The bacteria in the development of the embryo.

Photograph 13. Longitudinal section through the micropylar region of the ovule of an open flower. Haematoxylin. 480 ×.

Photograph 13*a*. Interpretation of Ph. 13. micr. = micropyle. Ext. vent. = ventral flank of outer integument. Ext. dors. = dorsal flank of outer integument. int. v. = ventral flank of inner integument with its enlarged part, the wedge. int. dors. = dorsal flank of inner integument. E. sac. = embryo sac. The asterisk shows the place where the bacteria are entrapped.

Photograph 14. Cross section through young fruit, 2 mm diameter. Wedge of inner integument with bacterial mass. Haematoxylin. 1080 ×.

Photograph 15. Cross section through a fruit, 4 mm diameter. Bacterial mass near the wedge (arrow) just below the suspensor side of a developing proembryo. Haematoxylin. 800 ×.

Photograph 16. Cross section through the fruit, 4 mm diameter. Embryo in advanced state of development, surrounded by embryogenic tissue. Haematoxylin. 800 ×.

Photograph 17. Same series as Ph. 16. about 60 μ lower. The left arrow indicates bacterial mass, the right arrow the suspensor-side of the embryo, surrounded by embryogenic tissue. 400 ×.

wallel pentagonal or hexagonal parenchymatous cells, which are formed from the plasmatic skein of the previous stage. These cells fill the entire embryo sac apart from the embryogenic tissue. These parenchyma cells subsequently show heavy, ridged walls in the ripe seed (see textfigure 38 of DAHLGREN's paper) which make section of these seeds very difficult.

It is to be deplored that JAENSCH' excellent description appeared in 1905, long before MIEHE's discoveries. The embryological work of JAENSCH, however, has proved to be a competent guide. This author also found polyembryony, but states that only one plantule develops from an embryo sac. I found amongst 500 seeds of one plant one twin embryo and one year later amongst 800 seeds of the same plant two twins. In all three cases the seed consisted of two hemispherical structures, closely pressed together. Every half contained one embryo as was clearly seen at the radical pole. It seems more probable that these twins originate from two differentiated ovules, but as no further anatomical analysis was made, no certainty exists on this point. As said before the embryo-formation should be called adventitious rather than apogamous, as the embryo develops from the vegetative parts of the ovule. The origin from the synergids seems very improbable as the egg-apparatus could only be observed in one early stage and apparently almost always degenerates later. When we exclude the possibility of synergid-embryo's we are able to localize the embryogenic cells as the wedge-shaped protrusion of the ventral flank of the interior integument.

f. *The seed.*

Large fruits cannot be sectioned because of their horny endosperm. It seemed, moreover, superfluous to give a detailed description of the localisation of the bacteria in this stage as MIEHE has given us already such an account.

According to him the symbionts occur in the mature seed between endosperm and embryo at the radical side. This situation may now be accounted for by the developmental stages described by us in this chapter.

The plantule is situated transversely in relation to the longitudinal axis of the fruit in the berry, the radical pole pointing towards the periphery, as characteristic for the Myrsinaceae.

The brown seed coat apparently develops from the rests of the exterior integument, while the outer fleshy part is formed from the carpels.

g. *The germinating seed.*

The bacterial film at the radical pole seems to remain at their original location when the root pushes through. This root reaches an appreciable length before it is followed by the hypocotyledon. Later the vegetation point follows, while the cotyledons remain inside the seed, as a kind of haustorial organs. The bacterial film now covers the vegetation point and also the axial buds of the cotyledons. The bacterial cycle of Ardisia is closed.

§ 5. *Experiments in relation to the development of the fruit.*

In the spring and summer of 1937 the anthers were removed from a number of full grown closed flowers (103) of different plants. The plants treated in this way were isolated in order to prevent cross-pollination. All castrated flowers dropped after 2—3 weeks, without any trace of fruit formation.

As a control 30 flowers were pollinated, they all formed normal fruits.

Cleistogamic pollination, as observed by VON FABER on Psychotria seems excluded. In Ardisia the pollengrains are still enclosed in the thecae in this stage and no grains appeared after touching or shaking of the anthers.

MEZ (29) supposes that the flower structure, in analogy with Solanum species, is adapted to pollination by bees or bumble-bees.

Apogamic and allied phenomena seemed excluded on the basis of these experiments, while our anatomical evidence drove us to assume such phenomena. For the presence of pollengrains on the stigma seemed necessary for development of the fruit, while no pollen tubes were ever observed. Possibly the pollengrains secrete substances which induce parthenocarpy, and even pseudogamy.

Contrary to the experiece of JAENSCH' I found the pollen germinable, especially on 0.5 and 0.6 molar saccharose solutions, where the percentage of germinated grains amounted to 82.3 resp. 78.1 %!

From the literature, FITTING (14), LAIBACH (27) and GUSTAFSON (19) it appears that either pollen-paste or specific substances such as indole-3 n-propionic acid, phenyl-acetic acid and β-indolyl-acetic acid may cause experimental parthenocarpy with various plants. It seemed logical, therefore, to see whether hetero-auxin should induce experimental parthenocarpy or pseudogamy; the formation of adventituous embryo's. The experiments were only started recently but the castrated flowers treated with 1 : 500 hetero-auxin lanolin paste are forming fruits. Most of the castrated flowers treated with water lanolin paste are already shed. In one or two instances a much retarded fruit formation also seems to take place in this instance. It may be that in our original experiments the unprotected gynaceum had lost too much water to yield a fair result.

The objects are still too small to investigate on the presence of embryo-formation.

§ 6. *Discussion and summary.*

The supposition given at the beginning of this chapter, according to which the number and the form of the bacteria in relation to their localization in the plant might give us an idea as to the role of this symbiont, has been fruitful.

In the first place it is evident that the bacteria are present in large numbers, always as rods, near meristematic tissues, e.g. of the terminal bud, of the floral bud, the proembryo-formation. This stage I propose to

call, the active stage (Stage II), it occurs whereever the plant performs important functions, such as leaf- or flower-formation or embryo-formation. Here the bacterium seems to play an active part.

In the dormant buds, in the bud of the floral axis which will develop next year, near the micropyle in the closed flower the bacterium is in a resting stage for a longer or shorter period. The number is small or very small and the identification difficult. After a longer or shorter period the bacterium may resume its activity, but only when the neighbouring plant-tissue reassumes its development, and becomes again meristematic!

The intermission may take as long as 4 years (in the case of dormant buds), it may take one year (terminal bud of floral axis) or may be a few weeks (micropylar region). The bacteria in this stage are resting but may be raised to activity; Stage III is a reversible stage.

In very young foliar nodules according to MIEHE the bacterium occurs initially in Stage I, but during later development it shows involution-forms. This stage may be considered to be irreversible, the bacterium is "played out" and the symbiont cannot be isolated and be brought into Stage I, the motile stage, which probably only occurs on artificial media. This Stage IV, the irreversible stage, also occurs on the ventral side of full-grown leaves where remains of the bacterial film are still found. According to MIEHE the motile stage occurs only on the plant when the bacteria penetrate the hydathodes. We also mentioned his idea about their invasion of the micropyle. I never found motile bacteria in living foliar buds, but always the bacterial film could be observed. Figure 7 gives a schematical representation of the different stages of the bacterium observed in the plant.

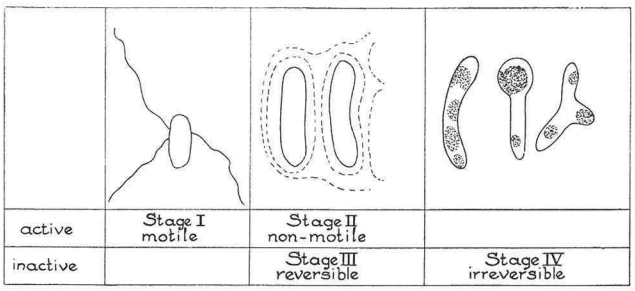

Fig. 7. Schematical representation of the various stages of Bacterium foliicola MIEHE as observed in cultures and in the plant.

For completeness' sake we also mention here the motile stage, only known from artificial substrates (Chapter V).

In confirmation of JAENSCH embryo-formation is asexual, but rather adventitious than apogamous. The pollengrains apparently secrete an auxin-like compound which induces parthenocarpy and, maybe, pseudo-gamous embryo-formation.

A schematical representation of the bacterial cycle of Ardisia is given in

Figure 8. Roman numerals indicate the ontogenetic sequence in the bacterial cycle. Stage II is always present near meristematic tissue, the root-meristem

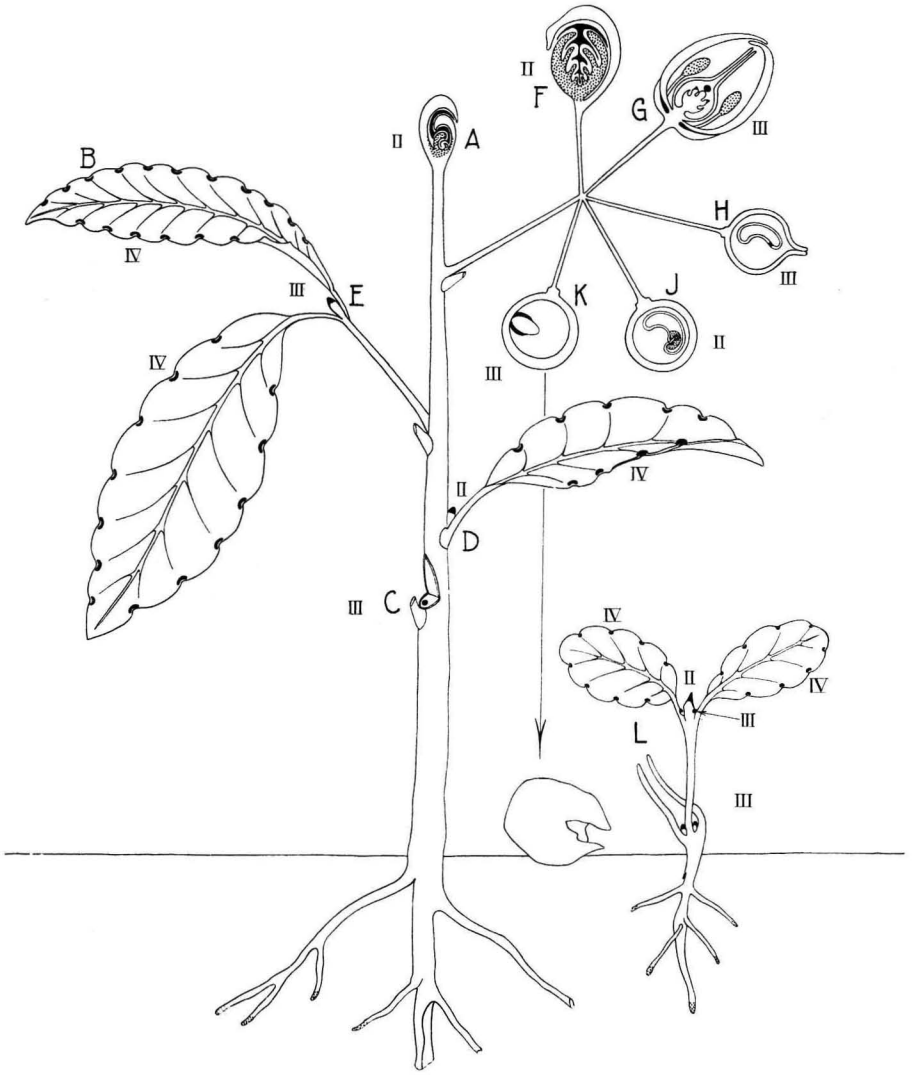

Fig. 8. Schematical representation of the bacterial cycle. The various parts are not drawn to scale. Bacteria shown in black, meristems dotted. The Roman numerals refer to the stages given in Fig. 7. Note the absence of bacteria on the roots and on the anthers, in all other cases the bacteria accompanying meristems.

A. terminal bud
B. foliar nodules
C. endogenous (dormant) bud
D. axillary bud
E. inflorescence-bud
F. young developing flower
G. closed flower
H. fruit, 2 mm diameter
I. fruit, 4 mm diameter
K. mature fruit
L. seedling

and the growing anther excepted. At the latter two localities no bacteria were observed by me.

CHAPTER V.

The bacterial strains isolated and their properties.

In this chapter I intend to describe the isolation of the bacterial symbiont, and to describe its properties, while aware of the fact that no far-reaching conclusions may be drawn from the behaviour of the bacteria on various culture media in respect to their symbiontic behaviour. For artificial media are very different from the natural environment in the host-plant.

§ 1. *Isolation of the bacteria.*

a. MIEHE's *attempts.*

MIEHE describes many attempts to isolate the microbe, for a long time without much success. This failure he contributed to the fact that the bacteria should be very selective in their milieu and should require a special substrate. It also appeared, however, that the locality in the plant from which the inoculum was taken, is of great importance. Neither from the leaf nodules, nor from the ungerminated seeds MIEHE could isolate the bacterium. Terminal buds proved unsuitable because sterilization is difficult. Remained as an infection-source the germinating seed, from which MIEHE was finally able to obtain definite bacterial strains. His method was the following; germinating seeds are sterilized externally and the plantule is isolated under rigorously sterile conditions. MIEHE was well aware of the danger of misinterpretation resulting from extraneous infection. Still it is remarkable that he obtained the bacterium in this way and it gives another tribute to his manipulatory skill. The isolated plantule was placed on a neutral medium, preferably pea agar, and incubated. Around the plantule he observed, after a few days incubation, a slimy white zone in which zone he observed very motile rods.

As MIEHE never observed motile stages of the microbe within the plant, he doubted at first the identity of the symbiont with the form isolated. But in older cultures he found also non-motile, swollen rods and, moreover, branched bacteroids. These involution-forms seemed microscopically identical with those observed in older leaf nodules and for this reason MIEHE claimes to have isolated the symbiont.

The bacterium, isolated and obtained in pure culture in the above-mentioned way he called Bacillus foliicola n.sp. As an incidental constant companion to this form he found an accessory symbiont, a long rod, forming yellow colonies and which he called Bacterium repens n.sp.

Notwithstanding the fact that B. repens only occurred sporadically in his isolation, MIEHE pays much attention to this form. Only infection-

experiments on crippled plants might prove its actual nature. Possibly it is an incidental infection, which has entered the flower through the open style-canal.

MIEHE's experiments are purposedly mentioned rather in detail in order to show that the findings of this expert authority together with our own results warrant the conclusion that B. foliicola Miehe is actually the symbiont of Ardisia crispa.

b. *Own results.*

On the basis of MIEHE's data extensive isolation-experiments were started and the nature of the isolated forms was ascertained. Preliminary experiments showed that no bacteria could be isolated from the leaf nodules, while many infections occurred when it was endeavoured to isolate the form from foliar buds. Later it was tried to isolate plantules from germinating seeds according to MIEHE's method. This method had to be abandoned because of great technical difficulties encountered. Even in a sterile chamber infections occurred and we sorely lacked the master's hand of MIEHE. Moreover, many isolated plantules became inactive and the plates remained sterile, possibly because of a too rigorous flaming of the seed, as follows from other experience. Possibly also the lack of contact between bacteria in the plantule (enclosed between the cotyledons) and the substrate might have played a role.

After comparison with the strains isolated by other means, the above method yielded only one form (strain 48).

A modification of MIEHE's method yielded more reliable results. The plates were inoculated by a smear of a suspension of crushed plantules in sterile water. In order to eliminate infections, several dilutions of this suspension were also used. A description of the method follows:

Germinating seeds are shaken in a 1 : 1000 solution of sublimate in alcohol for 15 minutes, and rinsed afterwards 5—6 times in sterile water. The plantules are then extracted from the seeds by means of sterile forceps and needles and crushed in an agate mortar in 3 cc sterile phosphatebuffer solution (pH = 7.0). Flaming was avoided because of the low thermotolerance of the bacterium, which idiosyncrasy appeared from other experiments. As the form proved to be sensitive to acid, crushing of the seeds without buffer, the cell sap (which proved to be acid) undoubtedly harms the bacteria.

From the 3 cc suspension obtained, dilutions ranging from 1 : 10 — — 1 : 10000 were prepared. Smears from original suspension as wel as from the dilutions were made on the same nutrient medium and incubated at 25° C.

All manipulations were carried out in a sterile chamber. The result of the first experiment is given in table 2. Only the plates inoculated with the undiluted suspension yielded white, irregular, slimy colonies and a few yellow colonies. Inoculations from the dilutions, however, remained sterile.

TABLE 2.

Result of second isolation-attempt after 5 days culture at 25° C of plates inoculated with undiluted suspension. Inoculum 10 plantules, 3 weeks old. Controls remained sterile.

Agar-medium (pH = 7.0)	Plate	Number colonies white	Number colonies yellow	Number of strains isolated
malt	51	4	3	1 white 1 yellow
pea	52	3	2	1
pea	53	4	0	1
peptone	54	2	0	1

Microscopic investigations showed that the bacteria stick together in the mucilaginous membrane, while isolated individuals appear very scarce. The latter fact accounts for the lack of success of the dilution-experiments.

The irregular form of the colonies is probably due to the development of many bacteria. All white colonies raised on pea- or malt agar showed the white- and slimy appearance as given by MIEHE for B. foliicola. On peptone agar the colonies were much smaller, but on all media the bacteria showed motility. Arbitrarily selected cultures were transferred from the various media to pea-agar. On this medium they all showed the same type of colonies, the effect on peptone-agar being therefore, most probably, due to the medium. Transfers were repeated three times, without any variation in the type of colony. The yellow colonies, which also proved constant on various media, however, yielded bacteria certainly not identical with MIEHE's Bacterium repens, our form possessing different dimensions. A pure culture, which proved constant after transfers, was isolated of this form (strain 51 yellow). Table 3 gives the result of a duplicate experiment. The result seems identical with that given in table 2. No results were obtained from diluted suspensions, and both yellow and white colonies occurred. The white and yellow colonies as well as the bacteria possessed the same characteristics as those isolated in the former experiment. After careful control five strains were isolated in pure culture from this series.

TABLE 3.

Result of second isolation-attempt after 5 days culture at 25° C of plates inoculated with undiluted suspension. Inoculum 15 plantules, 3 weeks old. Controls remained sterile.

Agar-medium (pH = 7)	Plate	Number colonies white	Number colonies yellow	Number of strains isolated
peptone	88	4	0	1
malt	89	0	0	
pea	90	4	2	1
do	91	0	0	
do	92	3	2	1
do	93	2	0	
do	94	2	0	1
do	95	2	0	1

As no air infection of fungi or other microbes occurred the isolation seems reliable.

Strain 51 yellow was not thoroughly investigated. It may have been an infection of the opened flower, penetrating through the style-canal and, once within the carpels, remaining within the developing fruit. The low frequency of this form is also indicative of its subordinate role. The strain was also used later for infection-experiments (Chapter VII, § 4).

The "white" strains all agreed almost completely in their morphological and physiological characteristics. It may suffice, therefore, to give a general description of this form. The morphological characteristics agree entirely with those given by MIEHE. The behaviour of the form on various culture-media was not investigated by us as thoroughly as was done by this author, however. A number of MIEHE's findings, however, could be duplicated. The next paragraphs, therefore, only give but little additional information.

§ 2. *Morphology.*

The most striking property of the isolated bacterium is its motility, chiefly because of the fact that motile stages are never observed within the plant. While MIEHE assumes (Chapter IV) an active penetration of the microbe into the style-canal no proof is given of this assumption. It seems more warranted to assume that the bacterium on the plant, in its mucous membrane, never shows any motility.

In 3—4 days old cultures (25° C) we saw both in liquid and on solid media, these motile rods, performing, as MIEHE says, a veritable "Mückentanz". The length of these rods is 1—2 μ, their thickness 0.5 μ.

Motility is caused, according to MIEHE, by 1—4 cilia (mostly 3), the implantation of which is irregular, but never polar. This stage we mentioned already in chapter IV as Stage I (Fig. 7).

In a week-old culture a change in form occurs, we saw non-motile, weakly curved rods. These forms are somewhat larger than the motile stages, their dimensions being 2—5 $\mu \times 1$ μ. The non-motile rods are often united to irregular bundles or packages. This adhesion, which is always irregular (no chains or other structured masses being observed) is caused by a mucilaginous sheath, 1 μ thick, which surrounds the rods. This sheath is easily observed by the BURRI - "ink-method". A common sheath surrounds each bacterial mass. The non-motile stage is reached on peptone-glucose agar already after 2—3 days of incubation. Three to five weeks-old cultures yield a third stage, together with the non-motile curved rod, which shows the characteristics of an involution-form. The rods are usually swollen, with a differentiated contents (irregular staining with carbolfuchsin). Branched forms occur also, but they are decidedly in the minority. These involution forms occurring on artificial media are morphologically identical with the bacteroids in older foliar nodules of Ardisia crispa (see VON HÖHNEL's figure in Chapter I). As morphological characteristics are dangerous taxonomic criteria in Bacteriology, MIEHE

only concludes as to the probable identity of the isolated form and the symbiont. Only infection-experiments, only the "curing" of cripples by means of the isolated strain will yield the proof.

We could moreover confirm that the bacterium is Gram-negative, non spore-forming and non acid-fast.

§ 3. *Physiology.*

The characteristics of the colony and the tendency to membrane-formation will be discussed first, as both the nature of colonies and of the membrane formation varies on various media.

Colonies: well developed after 2 days incubation at 25° C. They are round, spherical, smooth, with smooth contour, slimy and white. An opaque margin occurs around the colony after 4—5 days incubation.

Membrane: only on liquid so-called "natural extracts", pellicle formation takes place (after 2 days on pea- or malt-extract). This membrane starts as a ring at the rim of the culture-vessel, which ring increases in area until a regular membrane is formed. The zoogloea is wet, slimy and of a rather tough consistency. Finally the membrane caves in at the centre.

Table 4 shows the characteristical development in various media. In general it appears that the bacteria prefer a neutral medium and that plant extracts (pea or malt) are better suited than e.g. peptone medium.

The nature of the nitrogen supply seems very important. If various N-compounds are administered together with carbohydrate (glucose) the development seems to go parallel with the nature of the nitrogen source. Asparagin seems very well suited. Without combined nitrogen no development took place. Glucose, starch and gum arabic proved suitable as carbon source. No fermentation of either saccharose, glucose or lactose took place, only membrane formation occurred on these media, indicative of the aerobic nature of the bacterium, which shows, moreover, a marked catalase-reaction.

On plant extracts the bacterium forms alkali. Artificial media, like peptone-glucose-mineral salts, are rendered slightly acid.

No proteolytic enzymes are secreted into the medium.

The above data all confirm MIEHE's results. This author, moreover, states that experiments on possible nitrogen fixation yielded negative results. MIEHE gives, moreover, as temperature characteristics: minimum 7° C, optimum 25—30° C, maximum 35° C. The cells die, according to him after 48 hours sojourn at 40° C. This fact is of importance for obtaining experimental "cripples" of Ardisia (Chapter VI).

The strains isolated by me were much inhibited in their development after a heat treatment of 24 hours at 40° C. Membrane formation only took place after 8 days, while the controls showed membranes already after 2 days incubation. Newly inoculated malt-extract cultures were placed

TABLE 4.
Behaviour of the isolated strain in various media at 25° C.

Media at pH 7	Incubation-time in days	Development [1]	Remarks
Solid:			
pea agar	2	+++	starch corrosion after 7 days
malt agar	2	+++	
yeast agar, glucose, chalk	2	+++	no detectible acid-formation
peptone glucose agar	2	++	forms non motile, as involution forms
peptone agar	5	+	small colonies
glucose agar, no N-comp.	21	0	
pea gelatin	5—6	++	no liquefaction of gelatin
Liquid:			
pea-extract	2	+++	membrane, liquid opaque, after 7 days pH average 8.3
malt-extract	2	+++	membrane, liquid opaque and later alkaline
gum arabic-peptone	3	++	opaque, later cloudy sediment pH after 2 weeks average 8.2
malt-extract pH 4.2	21	0	
Glucose + salts + various N-compounds:			
asparagin	9	+++	membrane
peptone	9	++	opaque, slimy flocculent precipitate, pH 5.0—5.2 after 2 weeks
NH_4Cl	9	+	moderate precipitate
KNO_3	9	+	moderate precipitate
Fermentation media in Einhorn-tubes:			
yeast-extract saccharose	14	+	membrane formation in open arm of tube
yeast-extract glucose	14	+++	
yeast-extract lactose	14	+	no fermentation

[1] +++ copious, ++ medium, + moderate, 0 no development

for 24 hours in an incubator at 40° C and afterwards brought back to 25° C. The controls were incubated at 25° C. A temperature of 40° C seems decidedly harmful. The heavy inoculations used in my experiments, however, allowed a few extreme variants to develop.

§ 4. *Discussion and summary.*

The bacterium isolated by me proved to be identical in all respects with the form isolated by MIEHE, Bacillus foliicola. Definite proof, however, of the identity of this form with the symbiont will be given by the infection-

experiments described in Chapter VII, § 4. The yellow strain was also used in these experiments.

Nitrogen fixation on artificial media seems excluded. The relatively low temperature rangs of the bacterium is remarkable, also in view of the natural tropical occurrence of Ardisia crispa, and the subgenus Crispardisia in general, which plants therefore live very close to the limits of their potential milieu (Chapter VI). Except for the involution-forms, the curved-rod stage and the membrane-formation, the morphological work did not yield any important indications as to the role of this organism as a symbiont.

Comparing the various stages within the plant with those observed in artificial media, the following relations both in natural and artificial milieu seem to exist:

Stage I	Stage II. Stage III.	Stage IV
motile on artificial culture media	weakly curved rods, non-motile, with mucilaginous sheath. In plant (meristeme) and on artificial media	bacterioids not capable of reproduction. In nodules and on old culture media

The old name Bacillus foliicola MIEHE is no longer tenable, as according to international agreement (BERGEY, 3) the generic name Bacillus refers to aerobic sporeforming rods. Perhaps the form might be placed under the genus Alcaligenes cf. KLUYVER and VAN NIEL (24), but this would require a comparative study of all species listed under this rather badly known genus. For this reason I prefer as a new name Bacterium foliicola MIEHE, keeping in mind that the generic name has to be considered as a neutral name, and that the form is only provisionally placed under this genus.

CHAPTER VI.

The plant without bacteria; the cripple.

The purpose of this chapter is to describe the other symbiontic component: the plant without bacteria.

In the preceding chapter it was shown that the bacterial component grows vigorously on artificial media, without the help of the host plant.

On the other hand, the lack of bacteria in the plant causes, as will be shown in this Chapter, so many profound changes in its entire being, that we cannot consider the bacteria-free plant as an independent component. The phenomena caused in the absence of the bacteria yield the proofs for the symbiosis.

The behaviour and the condition of the "cripple", together with the description of the bacterial component might give a clue as to the nature of this symbiosis. It seems, therefore, necessary to compare the appearance and the behaviour of normal and "crippled" Ardisia at all stages of development.

As MIEHE (33) devoted much time to an analysis of the "cripple", we found the trail cleared here also and the directions for further work indicated.

In the first place the experimental procedure necessary to obtain cripples and the spontaneous occurrence of such stunted dwarfs will be considered. The habit, or macroscopic appearance of both forms will be described. Intermediate forms between normal- and crippled forms are considered next.

A microscopic examination of the cripple both as to the absence of bacteria and the condition of its meristematic tissue logically follows.

In relation to the problem of dwarfing both cytological and physiological evidence had to be obtained which evidence in the latter case, was concerned with the oxido-reduction system.

After the comparative analysis of cripple and normal plant the so-called "spontaneous cripple" is investigated further. The influence of the external milieu upon the incidence of these dwarfs is studied.

The influence of temperature upon the formation of cripples is of special interest, as it appears that bacteriophilous and tropical Ardisia's live near the limits of their potential milieu. A quantitative study of the germination of normal- and crippled plants completes this Chapter.

§ 1. *The methods to obtain crippled plants (experimental cripples) and the spontaneous occurrence of cripples (spontaneous cripples).*

MIEHE has shown that heating is a mean to deprive Ardisia of its bacteria. He heated seeds or apical parts of potted plants and cuttings

PLATE IV.

Habit of cripple and normal plants.

Photograph 18. Three spontaneous cripples and three normal plants, all from the same mother. Series IV, sown June 1936. One year old.

Photograph 19. Three experimental cripples and three normal plants, all from the same batch of seeds, sown January 1935. 2½ years old.

for 48 hours at 40° C. These temperatures are lethal to the bacteria (a statement which could be confirmed by us) while the plant remains unharmed.

In our experiment seeds were used exclusively. The experimental procedure will be treated under § 7.

Other means than heat treatment do not yield bacteria-free plants. Wound-callus might form bacteria-free buds, but MIEHE never observed bud formation on wound-surfaces or cuttings free of sleeping buds. Etiolation might cause the vegetation-point, by its vigorous growth, to pierce the bacterial film, but MIEHE observed that plants, etiolated in the dark, showed normal development when brought back into the light. We were able to confirm this observation.

The remarkable fact remains, however, that bacteria-free plants appear spontaneously. Inasmuch as the spontaneous- and the experimental cripple are apparently identical, we may conclude that the heating most probably only harms the bacterium and not the plant.

The occurrence of spontaneous cripples is, therefore, helpful to obtain a diagnosis. MIEHE also uses this argument, but starts from the idea that the spontaneous cripple develops from bacteria-free seeds. As we will show the evidence may lead to a different interpretation.

For the time being, however, the experimental- and the spontaneous cripple shall be described separately in detail in order to prove the identity of these forms.

§ 2. *The external appearance of the cripple.*

Initially there appears no difference in the seedlings originating from heated or unheated seeds. Germination takes the same time in both cases (see § 7) and this also pertains to the development of the first leaves. After this stage the difference between the crippled and the normal condition becomes demonstrable. After the formation of 3—5 leaves (which are in both cases obtuse) the terminal bud, which is always pointed in the normal plant, becomes blunted in the cripple. Sometimes a minute leaf is formed, but with this last gasp growth ceases completely. The cripple is, in that case, 3—5 cm high. In the leaf axils spherical buds appear later, and still later similar thickened buds appear in the cotyledonary axils.

Ageing of the cripple is concomitant with the formation of subsidiary buds next to the terminal bud. These subsidiary buds are equally unable to form leaves.

Both thickened axilary cotyledonary buds develop, on the long run, into irregular excrescences — undoubtedly consisting of masses of newly formed buds. These bud-masses may finally reach appreciable dimensions; up to 1 cm wide and 1 cm high.

Photographs 18 and 19 show the differences in external appearance between the crippled and the normal plant. The difference is very marked after one year. More striking is the picture of $2\frac{1}{2}$ year's development,

where the normal plants are already in fruit. The cripple never reaches the flowering stage, but continues only to form more cotyledonary buds. (Photographs 20 and 21). Attempts at shoot-formation were sometimes observed, but they always proved abortive. According to our observations the span of life of the cripple is ± 3 years. After that time the leaves decolorize, they become yellow and are finally shed. The end comes by the rotting of the cotyledonary bud-mass.

The root system of the cripple shows, strikingly enough, a normal development. This root system easily regenerates when the root of the potted plant has grown into the peat of the planting-bed, and has to be cut back.

The above-described cripple remains a dwarf, we never observed spontaneous reversion to the normal conditions in a three-year period. At this place we should mention, however, intermediate stages which were also known to MIEHE.

These plants initially behave as cripples, but at the period of the development of the cotyledonary buds, after the (irreversible) thickening of the terminal bud, a normal shoot develops from a cotyledonary bud, from which shoot a normal plant is generated (Photograph 22).

This shoot formation occurs 10 weeks after the seedlings appear above the ground. The plant has been a cripple in its appearance for over 6 weeks.

Anticipating the discussion of the quantitative data, where these intermediates are also dealt with, it should be stated that intermediates are nearly only formed from unheated seeds, from which seeds also spontaneous cripples may develop.

§ 3. *Anatomical investigations.*

a. *Absence of the bacteria in the cripple.*

In both spontaneous and experimental dwarfs we have seen that growth has come to a standstill and we have therefore called such a plant a "cripple". No sufficient stress has been laid upon the fact, however, that these plants are devoid of bacteria.

Microscopic investigations of the thickened cotyledonary- and in terminal buds never revealed the presence of these bacteria in these organs. The large number of stained sections used warrant the conclusion that the bacteria were actually absent. The same pertains to the investigation of the meristematic tissues. The leaf-nodules also appeared free from bacteria. In contrast to the occasional presence of bacteria in the nodules of the cripple, as stated by MIEHE, we never saw anything that could be interpreted as a bacterium. However, in the tissues around the bacteria-free nodules we found an accumulation of starch, which could be also observed in all thickened buds. The presence of starch is indicative of active photosynthesis, but may also indicate deficient translocation and (or) dissimilation.

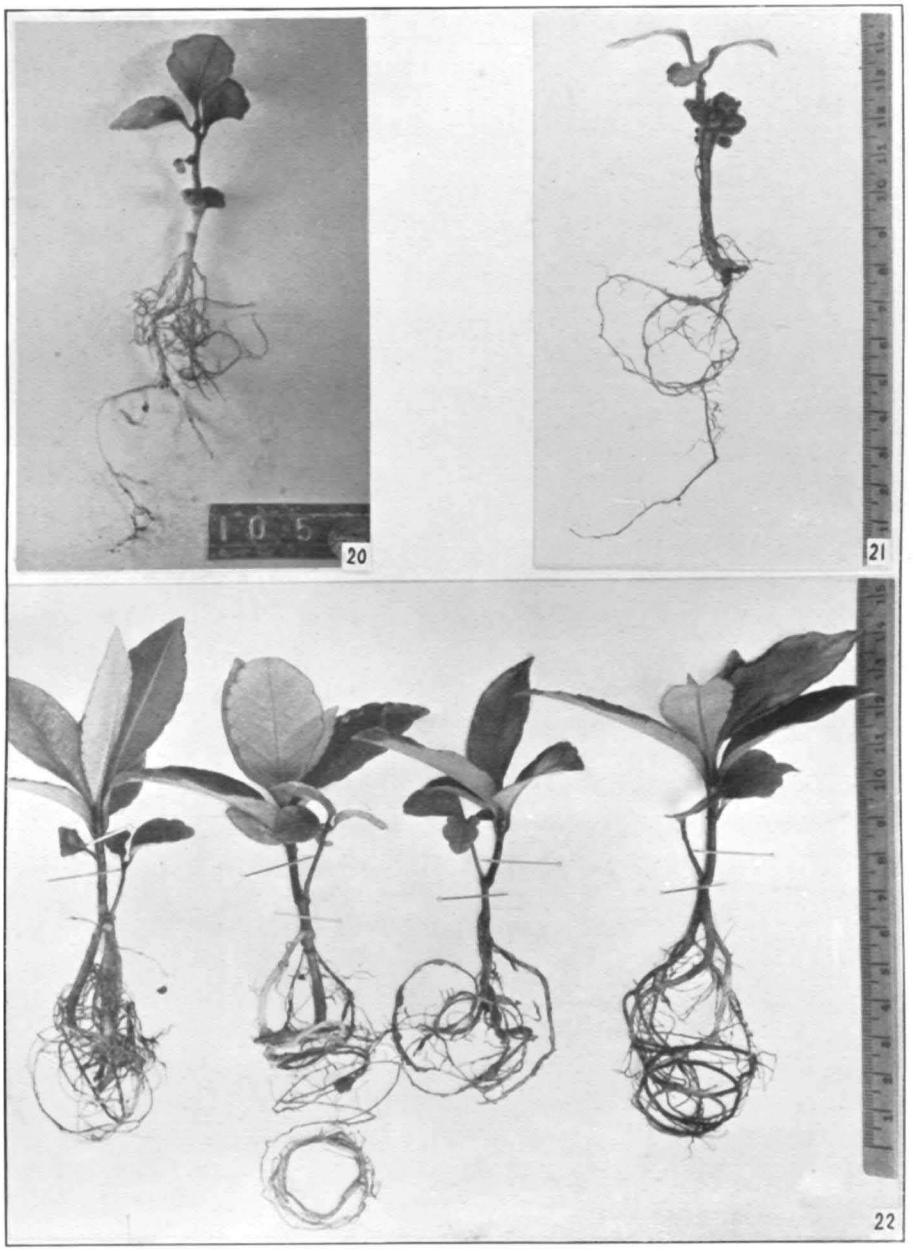

PLATE V.

Cripples and intermediates.

Photograph 20. Experimental cripple, one year old. Enlarged axillary cotyledonary buds, root system well developed.

Photograph 21. The same plant as shown in Ph. 20, 2½ years old. Cotyledonary buds further enlarged, leaves and stem stagnated. The photo is taken from the opposite side as in Ph. 20.

Photograph 22. Intermediates, one year old. The oldest crippled part is shown emerging from the hypocotyledon as a very slender shoot, the heavy shoot is developed from a cotyledonary bud.

b. *The condition of the meristematic tissue in the cripple, compared with that of normal plants* [1]).

Consideration of the cyclic symbiosis has shown that, in normal plants, the bacterium is present in large numbers and in active state (Stage II) near meristematic tissues. The possibility exists that the bacterium exerts a great influence on the development of the meristems. The nature of this influence we still leave undefined.

We have to state, however, that apart from the absence of bacteria, the meristems of the cripple show many abnormalities, both in topography, in size, in number and in their nature.

These abnormalities might be caused by the absence of bacteria.

A terminal- or an axillary bud of a cripple appears to consist chiefly of a large-celled "Dauergewebe" in which leaf structure is hardly differentiated. Sunk deeply below the surface we find "lumps" of meristematic tissue (Photograph 23). In certain cases an intercellular channel connects the meristem with the outer world (Photographs 24 and 25). The number of meristematic cell-groups within a single bud is variable and seems to increase with age (table 5).

The topography of the cripple-meristem is, therefore, totally different from that of the normal plants. In the latter case the meristem is in free contact with the outer world and with the bacterial film (Photograph 26).

The activity of the cripple-meristem appears from the topography of its surrounding parenchyma the orientation of which is in concentric circles around the meristematic tissue. Cell size decreases in the centripetal direction, i.e. towards the meristematic tissue. (Photograph 23).

After finding meristems in the buds comparative measurements suggest themselves. The nature of the meristem may follow from the measurement of cell-size. For in the normal plant cell-size decreases regularly from inactive parenchyma towards meristem, while the most active parts of the meristem show the smallest cells.

Chiefly due to the transparancy of the cell walls of the cripple, cell size is almost impossible to measure. The number of nuclei in a given volume of meristem was counted instead. This was performed in the following way:

A square corresponding to an area of $180\ \mu \times 180\ \mu$ was etched in a round coverslip and used in a measuring-eyepiece. The number of nuclei in a successive set of sections of both crippled and normal plants was counted in each section. The thickness of the sections was $6\ \mu$. The meristem was therefore divided into slices, measuring $180 \times 180 \times 6\ \mu$. The number of nuclei was ascertained in each slice. The number of sections counted also gives a dimension of the meristem.

The results are brought together in table 5. From this table it appears that the number of nuclei per unit area in a cripple-meristem is smaller

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[1]) I am much indebted to Mr. R. J. VAN DER LINDE B. Sc. for assistance in this part of the work.

TABLE 5.

Result of the countings of nuclei in meristems of crippled- and of normal plants.

Series	Type	Age	Direction of section	Meristems	Sections counted	Total number of nuclei	Average number of nuclei per unit of volume
I	terminal bud exp. cripple	4 months	longitudinal	4	34	496	14.6
II	do	4 „	„	4	13	161	12.4
III	terminal bud normal	4 „	„	—	6	158	26.3
IV	terminal bud spont. cripple	8 „	transverse	11	142	1983	15.0
V	do	8 „	„	12	—	—	—
VI	axill. cotyl. bud plant S. V	8 „	„	4	50	763	15.3
VII	axill. cotyl. bud plant S. IV	8 „	„	many	—	—	—
VIII	terminal bud normal	18 „	„	—	130	2622	20.2
IX	do	18 „	„	—	19	443	23.3
X	do	18 „	longitudinal	—	44	898	21.4
XI	do	18 „	„	—	26	533	20.6
XII	dormant bud, developing	4 weeks	„	—	30	651	21.7

than that in a normal meristem; the cells in the cripple meristem are larger. If the most active meristem cells are also the smallest, it would appear that the cripple meristem contained only few actively-dividing cells, or it may be that the active cells are larger in the cripple than in the normal plant. The first supposition seems more probable, for microscopic examination shows very few division-stages in the cripple meristem. Photograph 25 and 26 show the differences between a cripple and a normal meristem as to the appearance of the quantity of meristematic cells.

The direction of the sections is unimportant, as we always consider averages pertaining to number of nuclei per unit volume. The number of meristems seems to increase with age, confirming the observation of the formation of accessory buds with advancing age. The terminal- or axillary bud in column 2 of table 5 represents in reality a mass of buds to which are continuously added accessory ones, all represented in the sections by meristematic zones.

The number of meristems in a normal plant can hardly be ascertained, as the vegetation-point proper merges into the foliar primordia.

Detailed characteristics of the individual meristems of crippled- and of normal plants are given in tables 6 and 7.

Series IV table 5 (cripple) and series VIII table 5 (normal) are comparable as the total number of meristems is present in 142, respectively 130 sections. In the latter case, there is, strictly speaking, only one

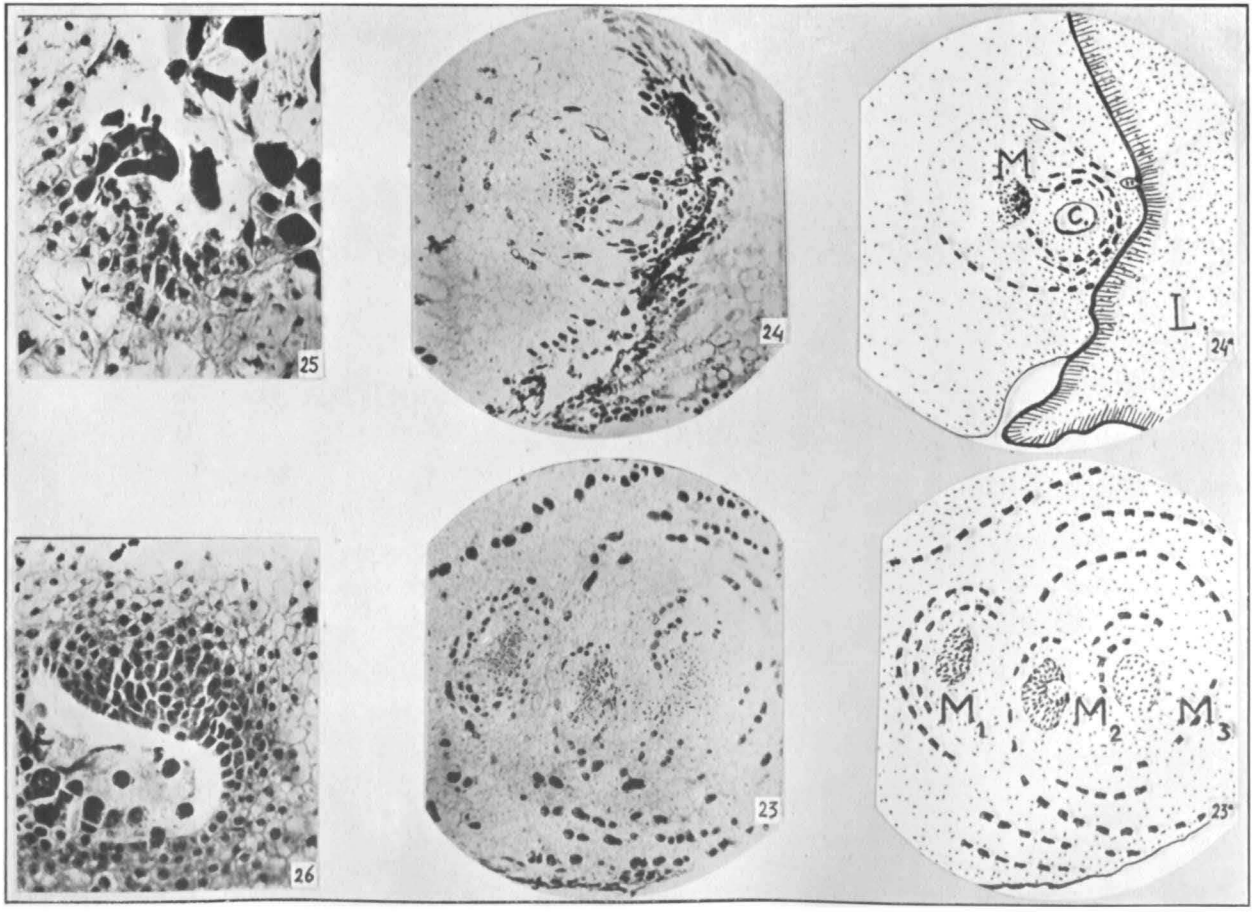

25
24
M
C
L
24'
26
23
M1
M2
M3
23'

PLATE VI.

Anatomy of the terminal bud of the cripple.

Photograph 23. Transverse section through a terminal bud of a cripple.
Haematoxylin. 120 ×.

Photograph 23*a*. Interpretation of Ph. 23. The dotted lines represent the direction in which the meristem showed activity. Individual leaf primordia undeveloped. At M_1, M_2 and M_3 meristematic regions; M_2 partly surrounds a channel.

Photograph 24. Transverse section through a terminal bud of a cripple.
Haematoxylin. 120 ×.

Photograph 24*a*. Interpretation of Ph. 24. The hatched part on the right, marked L represents leaf-tissue. At M meristematic region, at C the channel. Near C, on the leaf-epidermis, a multicellular hair.

Photograph 25. The meristematic tissue, consisting of a small number of cells, described in drawing 24*a* near channel C.
Haematoxylin. 480 ×.

Photograph 26. Transverse section through the terminal bud of a normal plant. Large number of meristematic cells. Note the multicellular hairs and the bacterial film.
Haematoxylin. 480 ×.

TABLE 6.

(Cripple) Details Series IV, Table 5.

Meristems	Sections	Total nuclei	Av. nuclei per unit of volume
1	8	96	12.0
2	13	162	12 5
3	18	102	12.8
4	cannot be counted		
5	15	234	15.6
6	27	431	15.9
7	14	220	15.7
8	12	198	16.5
9	12	207	17.3
10	10	133	13.3
11	13	200	15.4
Total	142	1983	15.0

meristem, the vegetation-point, the other figures refer to leaf-primordia. The size of the individual cripple meristems (table 6) seems to vary from 48 (8×6) to 162 (27×6) μ. A large meristem does not need to be more active (as measured by cell size) than a small one; meristem 6, while larger than meristem 9 has a smaller "nuclear density". In the normal plant (table 7) the vegetation point is the largest, 258 (43×6) μ, while the leaf-primordia show large differences in nuclear density.

TABLE 7.

(Normal) Details Series VIII, Table 5.

Meristems	Sections	Total nuclei	Av. nuclei per unit of volume
1	12	249	20.8
2	43	896	20.8
3	17	339	19.9
4	14	279	19.9
5	11	214	19.5
6	12	235	19.6
7	21	410	19.5
N. B. 2 veg. point all others are leaf-primordia			
Total	130	2622	20.2

The average nuclear volume in the cripple is $\frac{3}{4}$ of the normal nuclear volume. For the overage obtained from all series (table 5) the value is smaller (0.64).

From the above analysis it appears that topography, size and nature of the meristematic tissues in the cripples differ strongly from those in normal plants.

The meristems in the thickened buds of the cripple are still active, but much less active as compared to normal buds. It is difficult to prove whether this meristem is less active because of the absence of bacteria or that the following cell-elongation, and consequently the elongation of shoot and development of the leaves is hampered by this absence. This may, in its time, inhibit the division-rhythm. Some evidence for the latter opinion may be derived from the fact that the meristems of the cripple are sunk, and that the peripheral tissue is formed by them, but might refuse to elongate.

In both cases the bacteria might act as donors of growth-substance, as these substances seem to promote both division and elongation and as it is known that many microorganisms produce auxin.

§ 4. *Cytological observations.*

SCHWARNIKOV (36) observed that a temperature treatment at 40° C of seeds of Crepis-species caused chromosome-translocations and the occurrence of polyploidy (tetra-octoploidy). GERASSIMOVA (14) obtained haploid Crepis plants by irradiating the gametes with X-rays. These haplonts were dwarfs. These two observations illustrate the fact that external physical circumstances might influence the nuclear division and may cause changes in the chromosomes, both in number and in form. It is striking that dwarf-plants, combined with haploidy, were obtained in one instance.

It is possible that the chromosome-portrait of the heat-treated Ardisia is changed in a similar way, so that the dwarf plants should possess a reduced chromosome number. This possibility had to be investigated, although it would not account for the spontaneous cripple, unless Ardisia possessed an inordinately high mutability in response to external factors.

According to DAHLGREN (9) and to SUGUIRA (39) Ardisia crispa. A. DC. shows a diploid set of 46 chromosomes.

In our own work root-tips were used. It was soon found that, due to the extreme smallness of the chromosomes, translocations in the sense of SCHWARNIKOV could not be observed. The number of chromosomes could be ascertained in cross sections through metaphase-equatorial plates in a few cases.

An experimental cripple showed	47	Fig. 9
a spontaneous cripple	53	Fig. 10
and a normal root-tip	55 chromosomes.	Fig. 11.

These results are far from satisfactory. It is difficult, however, to obtain the right stage, as the two last-mentioned numbers probably refer to an advanced stage of the metaphase and several chromosomes were counted double. It seems excluded, however, that the cripples should be haploids.

According te BREMER (6), the size of the guard-cells of the stomates in the leaves of Saccharum-hybrids correlates positively with the number of chromosomes in these hybrids. If significant differences in the size of these

cells occurred in cripples and in normal plants, we might consider this as indicative of a possible changed chromosome-constellation.

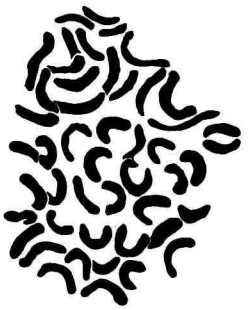


Fig. 9. Equatorial plate, metaphase, root tip of experimental cripple, showing 47 chromosomes. Immersion 2 mm, eyepiece 20 ×.

Fig. 10. Equatorial plate, metaphase, root tip of spontaneous cripple, showing 53 chromosomes. Immersion 2 mm, eyepiece 20 ×.

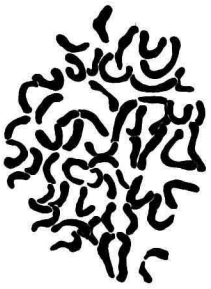


Fig. 11. Equatorial plate, metaphase, root tip of normal plant, showing 55 chromosomes. Immersion 2 mm, eyepiece 20 ×.

However, measurements of the stomatal apparatus in both cripples and normal plants did not indicate any difference.

The results of the measurements of the stomatal apparatus are as follows:

	Cripples	Normal
number of stomates measured	97	110
av. length	17.9	17.4
av. width	9.8	9.3

There is great possibility that there are no cytological differences between crippled and normal plants.

§ 5. *The oxido-reduction system in crippels and in normal plants.*

The necessity to investigate the enzymatic activity of various tissues became apparent after perusal of an interesting study by VAN OVERBEEK (38) on a dwarf maize. This dwarf differed only in one gene from plants with normal growth. Its nanism is apparently caused by the continuous destruction of its growth-substances. For the dwarf form showed, in its extracts, a much higher oxidation-power than the normal plant. Both catalase and peroxidase seemed more active. Especially the increased peroxidase-content was held responsible for the changed mode of growth by VAN OVERBEEK.

By heating normal plants to 45—50° C VAN OVERBEEK obtained smaller plants with an increased catalase-activity and with less growth-substance.

DE HAAN and GORTER (20) were able to demonstrate an analogous difference between a race of low growing- and trailing peas. Tips of the small "white raisin" strain contained more catalase than tips of the high "slender pea". Presumably, in the "white raisin" pea more growth-substance was destroyed with a concomitant decrease in growth-intensity, which decrease reflected itself also in the respective cell-sizes of the two strains.

TABLE 8.

Peroxidase and catalase-reactions in cripples and in normal plants [1]).

Series	Material investigated	Nadi + H_2O_2	Guaiac + H_2O_2	Benzidin + H_2O_2	H_2O_2
I	term. bud. norm. 7 weeks	—	0	0	+
II	do 1 year old	—	—	—	+
III	do spont. cripp. 1 year	+	0	0	+
IV	do exp. cripp. 1 year	+	+	+	+
V	ax. cot. spont. cripp. 1 year old	+	+	+	+
VI	do exp. cripple 1 year old	+	+	+	+
VII	stem, normal 1 year old	±	—	—	+
VIII	stem, exp. cripp. 1 year old	±	—	—	+
IX	nodules, normal 1 month	—	0	0	+
X	do one year	—	0	0	+
XI	do two years	—	0	0	+
XII	central part leaf, normal	—	0	0	+
XIII	terminal bud cripp., heated	—	0	0	—
XIV	terminal bud normal, heated	—	0	0	—

N.B. No oxidase reactions.

[1]) + positive — no reaction
± doubtful 0 not tried

Our own work is only concerned with qualitative tests for oxidase, peroxidase and catalase, chiefly in the terminal buds of both types of plants. Quantitative work, or tests for growth-substances were not carried out, our program being overburdened as it was.

We confined ourselves to the application of the wellknown reagents; gum-guaiac, benzidin, α-naphtol and "Nadi-reagent" (equal parts of the following three solutions, freshly prepared; 0.01 mol. dimethyl-p-phenylene-diamin hydrochloride in 50 % ethanol, 0.01 mol. α-naphtol in 50 % ethanol, 0.25 % aqueous solution of sodium carbonate) applied with (peroxidase test) or without (oxidase test) H_2O_2.

The terminal buds were washed and crushed in a phosphate buffer of pH 7. Drop--reactions were carried out on a white porcelain colour-mixing plate.

The results are summarized in table 8.

Together with H_2O_2, Nadi-reagent always yielded a prompt and definite reaction, α-naphtol was in no case oxidized, while benzidin + H_2O_2 and

guaiac + H_2O_2 also showed marked reactions. Probably due to the less intense colouration in the case of α-naphtol, the reaction could not be observed.

It appears therefore that both terminal- and axillary cotyledonary buds of both experimental and spontaneous cripples show the presence of peroxidase, while the presence of this enzyme could not be demonstrated in normal plants.

In series VII and VIII, table 8 parts of the stem are used because of the fact that the "Dauergewebe" of the terminal buds of the cripple corresponds in its character to the stem-parenchyma. A faint Nadi-reaction was obtained in both cases. This reaction is doubtful, the more so when we take into consideration the absence of a reaction with H_2O_2 + benzidin or guaiac in those cases.

It seemed possible that peroxidase content might decrease with the decrease of the number and nature of the bacteria during the ageing of the foliar nodule. In none of the nodules investigated, however, peroxidase seemed to be present.

All objects investigated were able to decompose hydrogen peroxide.

It would be premature to draw conclusions from the facts mentioned in this paragraph as no determination of growth-substances have been carried out. As a tentative opinion we may state that, because of the fact that enzymatic differences occur between normal- and crippled plants near the places where the bacterium is present normally, the nanism is caused by the absence of the bacteria, because these bacteria might influence the oxido-reduction state of the vegetation-point. In this respect the dwarfed Ardisia resembles the dwarf-maize of VAN OVERBEEK.

§ 6. *The occurrence of spontaneous cripples under various conditions.*

MIEHE states that from batches of resp. 44 and 31 untreated seeds 48 % of the seedlings appeared to be crippled (resp. 22 and 14). He accounts for this fact by the assumption of a variable frequency of bacteria in the seed. According to him, there should be naturally-occurring, bacteria-free seeds, which should yield spontaneous cripples. The mechanism of this process is not discussed by him. He also mentions intermediary forms, but, probably due to the limited material, he does not give quantitative data.

From Chapter IV, § 4, it follows that every flower examined contained bacteria and that, therefore, probably all fruits examined should be infected. Every seed is provided with bacteria by the mother plant and it seems plausible that external circumstances during maturation of the seed or during germination might influence the occurrence of spontaneous cripples. Under these external factors temperature should play an important role, considering the low thermotolerance of the bacteria.

Extensive sowing-experiments might, therefore, yield a clue.

The material for these experiments was obtained from various Botanical Gardens. Only fresh material, i.e. berries, were used. The pulp was

removed from the seeds and every seed was planted in a separate flower pot. The pots were always dug into a peat-bed.

As a measure of the velocity of development we used the lapse of time between sowing and the date on which the plant made its appearance (period of elongation of the hypocotyledon). The seed coat is carried on the tip, the cotyledons, which act as haustoria, remain within the seed for a long time.

A cripple may be recognized with certainty about 4 months after appearance of the seedling.

The results are summarized in table 9. Series IV and V excepted, in which case the seeds were harvested from one plant, the material is very heterogenous.

In all series cripples occurred, their percentage is extremely variable, but was never as high as MIEHE observed. Both under constant conditions (25° C) as well as in both plant houses (under variable conditions, see Chapter III) with different temperature-conditions, we always met with a number of dwarfs.

TABLE 9.

Spontaneous cripples and intermediate forms from various lots of seed under various conditions.

Series	Seed obtained from	Place where sown	Date	Number of seeds	% Germination	% Normal	% Intermediates	% Cripples	Remarks
I	Liège Botanical Gardens	nursery	February 1936	126	96.0	85.1	—	14.9	intermediates not counted
II	Leyden Botanical Garden	orchid house	February 1936	98	65.3	88.9	—	11.1	intermediates not counted
III	Wageningen Botanical Gardens	orchid house	February 1936	60	90.0	77.8	14.8	7.4	
IV	Hilversum private collection	nursery	June 1936	441	79.1	71.6	10.6	17.8	seeds all from one plant. All seeds weighed
V	Leyden Botanical Garden	constant temperature room	March 1936	61	72.3	81.8	2.3	15.9	seeds all from one plant
VI	Baarn Botanical Gardens	constant temperature room	March 1936	66	90.9	38.4	40.0	21.6	cultivated in the dark for four months. All seeds weighed.

After germination in the (dark) constant temperature-room, the seedlings were brought into the nursery. In series VI, however, the plants were kept in the dark for over 4 months. The normal plants showed all characteristics

of etiolation and reached a height of 8—9 cm in this period. In the nursery they subsequently developed into normal plants, in confirmation of MIEHE's observations.

The cripples in this series did not show abnormal longitudinal growth. They reached a height of 2—3 cm, smaller than the cripples in the light. The leaves remained small and were soon shed.

In series IV and V the seeds were weighed separately, in order to correlate this weight with the nature of its development. For a small, badly developed seed might yield a cripple, while a well developed seed might yield a normal plant. However, no correlation whatsoever could be found between the weight of the seed and its subsequent behaviour. Both heavy and light seeds yielded cripples while both types of plants germinated equally well.

The percentage of "intermediates" is equally variable. It is remarkable that they occur in great numbers in series VI, the dark series. It may be that in this case the increased longitudinal growth has disrupted the bacterial film of the vegetation point, while the axillary cotyledonary buds, from which buds the "intermediates" always form shoots still contain bacteria. In view of the enormous variability in behaviour it seems dangerous to draw conclusions from this one series.

Apart from the series mentioned in table 9 a batch of 150 seedlings, bought at a nursery at Aalsmeer contained 23 cripples and 127 normals.

A second batch of seedlings from Aalsmeer contained also a large percentage of cripples. Thirteen seedlings of A. crispa var. fructu albo (HORT) obtained from the Botanical Gardens at Liège showed the presence of one spontaneous cripple.

These data, incomplete as they are, still demonstrate the widespread occurrence of spontaneous cripples.

The rate of development of the two types of plants is illustrated in Fig. 12.

The season influences this rate markedly, the development in February being much retarded, while our constant temperature-series agrees closely with series IV (in the nursery) sown in June. The cripples develop generally at the same rate as normal plants, or a little slower. The difference, if real, disappears under constant or favourable conditions. Their first germination-stages up to the appearance of the first true leaves are therefore not hampered by the absence of the bacteria.

We mention here the occurrence of three twins (see Chapter IV). In two cases they yielded normal plants, while in the third case one cripple and one normal plant appeared.

Spontaneous cripples seem to occur both after germination under constant as under variable conditions, after germination both in the dark as in the light. The percentages, however, are lower as given by MIEHE. Six series, representing 844 plants, yielded an average of about 15 % spontaneous cripples while MIEHE found nearly 50 %!

In two cases, with seeds obtained from one plant (table 10, control) we found no or almost no cripples. A recent experiment (table 12, control),

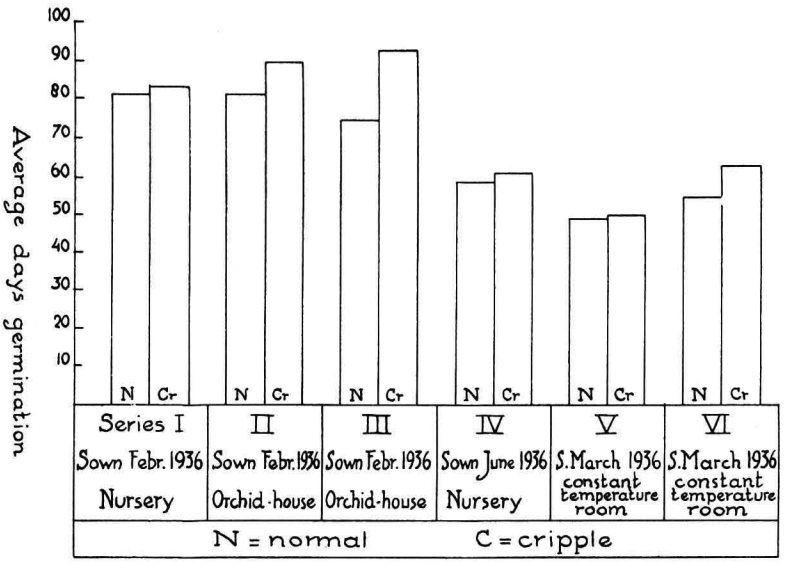


Fig. 12. Rate of germination of normal plants and spontaneous cripples under various conditions.

where almost 800 plants were raised, yielded similar results. The conditions necessary for the occurrence of the cripple seem to be finely balanced.

§ 7. *The temperature as a causative factor of crippling.*

As already mentioned in § 1 of this Chapter, cripples may be obtained by temperature-sterilization of the seeds. We obtain, in this way, experimental cripples which appear, in all respects, identical with spontaneous cripples. MIEHE used 48 hours heating at 40° C or 7 minutes at 52° C.

A large number of seeds, obtained from one plant, (series IV, § 6) allowed us to carry out more extensive observations on the temperature factor. As 7 minutes heating at 52° C, as advocated by MIEHE, seems a very short period to allow temperature-equilibrium to be reached within the seed, thermo needles were stuck into the seeds, which were placed in water of 50° C. Within 1—2 minutes equilibrium was reached and these 2 minutes were henceforward deduced from the heating-periods. The short-period treatment was carried out in water-baths, the long-period treatment in thermostats, the seeds being placed in petri-dishes in moist sand.

The results are summarized in table 10.

The heated seeds were raised both in the constant temperature room (C) or in the nursery (N). The rate of development was determined for every individual.

Nearly all heated seeds yielded cripples. Some series are mutilated by damage by snails, but in spite of the irregularities caused by this factor, we

TABLE 10.

Results of heating seeds obtained from one plant sown May 1937.

Series	Temperature and duration of heating		Number of seeds	% Germinated	% Normal	% Cripple	% Damage
I	10 min. 55° C	C[1]	25	0	—	—	—
II	do	N[2]	25	0	—	—	—
III	10 min. 50° C	C	25	92	0	96	4
IV	do	N	25	92	4	91	5
V	7 min. 52° C	C	30	93	0	96	4
VI	do	N	25	92	0	100	0
VII	24 h. 40° C	C	30	100	3	83	14
VIII	do	N	25	100	0	96	4
IX	48 h. 40° C	C	25	100	0	92	8
X	do	N	25	96	0	100	0
XI	do	C	25	92	0	78	22
XII	do	N	27	96	0	100	0
XIII	control	C	30	100	97	0	3
XIV	do	N	25	80	100	0	0

[1]) C = constant temperature room.
[2]) N = nursery.

may assume nearly 100 % cripples occurred. Series I and II apparently exceed the thermal deathpoint, which should be situated in the neighbourhood of 52° C. All dwarfs retained their character, no intermediate stages being observed.

It is remarkable that from series XIII and XIV, the controls, only or practically only normal plants developed, especially when we keep in mind that seeds from the same plant yielded in another experiment 18 % cripples (series IV, § 6).

The germination rate of the seeds is represented in Fig. 13. No

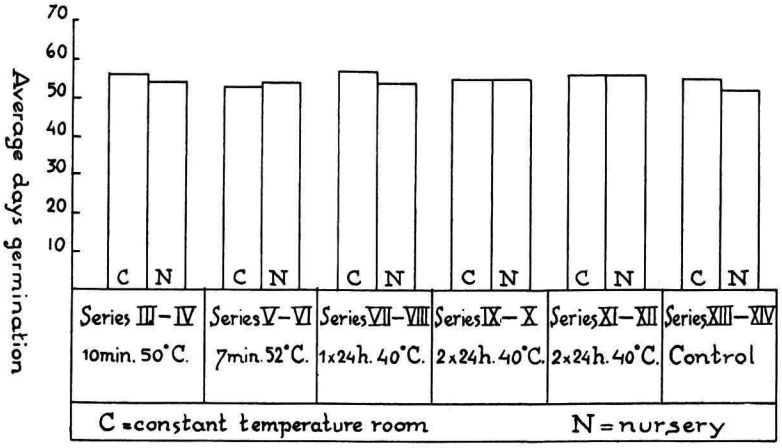


Fig. 13. Rate of germination of normal plants and experimental cripples under different conditions.

significant differences, even between heat-treated seeds and the controls, could be observed. Again the cripples develop at the same rate as the normal plants.

A large number of seeds obtained from Buitenzorg under the name Ardisia crispa A. DC. was also heated. A small percentage of those seeds germinated and we obtained a different bacteriophilous Ardisia, a species unknown to us. No cripples occurred, however. We should have wished to see cripples originating from seeds harvested in the tropics also. Also from Buitenzorg we obtained seeds of Pavetta Zimmermanniana VAL. in order to compare two unrelated bacteriophilous plants.

According to VON FABER bacteria-free Pavetta plants may also be obtained by heating. The heated seeds yield, after the development of the 3rd or 4th pair of leaves, dwarf plants. These cripples may be recognized only 6—8 months after germination.

The result of the experiments is given in table 11.

The Pavetta shows a behaviour analogous to that of Ardisia. The percentage germination does not seem to be influenced by temperature. Spontaneous cripples also occurred in the controls. These spontaneous cripples are not mentioned in VON FABER's work. Due to our absence from the institute at the time of germination no data on the comparative rate of development were obtained.

TABLE 11.

Pavetta Zimmermanniana VAL. Heat treatment of seeds. Sown July 1936, in the Orchid-house.

Series	Temperature and duration of heating	Number sown	% Germinated	% Normal	% Cripple	% Damage
I	2 min. 50° C	80	61	2	82	16
II	24 h. 40° C	80	66	2	92	6
III	48 h. 40° C	80	70	2	96	2
IV	control	80	64	76	21	3

Seeds of various non-bacteriophilous Ardisia-species were submitted to heat treatment, in order to see whether this treatment might induce other changes in development. Unfortunately none of these seeds, neither in the heated- nor in the control series, showed any sign of germination.

As in the foregoing experiments the temperature used seemed relatively high, a systematic survey of the influence of lower temperatures in an extended range was indicated.

As shown in Chapter V, § 3, the bacterium isolated showed a low thermotolerance, which seems remarkable for a symbiont of a tropical plant. The assumption seems therefore justified that Ardisia crispa lives near the limit of its potential milieu. This limit is determined by the presence or absence of the bacterium, for without the bacterium normal development is excluded.

Temperature is probably the most important factor for the origin of cripples, but also the time factor may come into play. As the study of both factors required a large amount of material, the experimental temperatures were chosen so as to approach those present in the natural, tropical, milieu.

Climatological data are to be found in the extensive report by BRAAK (5). When we confine ourselves to air- and soil-temperatures at Buitenzorg, BRAAK's findings may be summarized as follows: The air temperatures show average-maxima of 28°—31° C while the absolute maxima vary from 31° to 34° C (duration 2—4 hours). Soil-temperatures (in the shade) do not show much influence of depth or of season. At a depth of 3—5 cm the soil-temperature averages is about 28° C.

The temperatures used in my experiments were 30° C, 32°.5 C, 35° C and 40° C. The first two fall certainly within the range of maxima which may occur for two to four hours daily, while the temperatures of 35° C and 40° C form a link with the temperature treatments as described in a previous experiment (table 10). The times of heating varied between 0.25 and 36 hours for the three lowest temperatures. Lack of material caused me to restrict the 40° C treatment to a series where the seeds were treated for 8 hours.

As a lengthy sojourn at 40° C yields cripples exclusively (as previous experiments showed) the 8-hour treatment would form a necessary addition. Apart from soils exposed to direct sunlight, it seems improbable that a temperature of 40° C could be reached in the tropics.

The seed was procured from the same plant which yielded the seeds used in the experiment described in table 9 Series IV and which also yielded the seeds used in all series represented in table 10. The seeds of this plant were therefore used in three consecutive harvests. Heating of the seeds was performed as described before in this Chapter.

Due to the early season (February 1938) the seeds were planted under optimal conditions of temperature in seed pans, which pans were dug into peat on a separate planting table in the nursery, where the temperature fluctuated between 16° and 33° C. Four months after sowing the results became apparent (see table 12). Percentage germination was very high in all series and approach 100 %.

The number of cripples is extremely small, only prolonged heating (36 hours) of the seeds at one of the temperatures used caused a very small percentage of cripples to occur (series VII, XIV and XXI). Heating for 8 hours at 40° C also caused some cripples to develop (series XXVI). In series I a, probably, spontaneous cripple, made its appearance, while the control, series XXVII, yielded only normal plants. This result is in agreement with the data given in table 10, series XIII and XIV.

Successive harvests of the same plant yielded, respectively 18 % (series IV, table 9), 0 % (series XIII and XIV, table 10) and 0 % (series XXVII, table 12) of cripples. While an explanation of these facts seems

TABLE 12.

Results of heating seeds obtained from one plant sown February 1938.

Series	Temperature and duration of heating	Number of seeds	% Germinated	% Normal	% Cripple	% Intermediate	% Damage
I	¼ h. 30° C	30	100	97	3	0	0
II	1 h. 30° C	30	100	100	0	0	0
III	2 h. 30° C	30	97	97	0	0	3
IV	4 h. 30° C	30	97	100	0	0	0
V	8 h. 30° C	30	100	100	0	0	0
VI	16 h. 30° C	30	100	100	0	0	0
VII	32 h. 30° C	30	100	97	3	0	0
VIII	¼ h. 32°.5 C	30	100	100	0	0	0
IX	1 h. 32°.5 C	30	97	100	0	0	0
X	2 h. 32°.5 C	30	100	100	0	0	0
XI	4 h. 32°.5 C	30	97	97	0	3	0
XII	8 h. 32°.5 C	30	100	97	3	0	0
XIII	16 h. 32°.5 C	30	100	97	0	0	3
XIV	32 h. 32°.5 C	30	100	86	7	7	0
XV	¼ h. 35° C	30	97	100	0	0	0
XVI	1 h. 35° C	30	97	100	0	0	0
XVII	2 h. 35° C	30	100	100	0	0	0
XVIII	4 h. 35° C	30	100	100	0	0	0
XIX	8 h. 35° C	30	100	97	0	3	0
XX	16 h. 35° C	30	100	100	0	0	0
XXI	32 h. 35° C	30	97	97	3	0	0
XXII	¼ h. 40° C	30	100	100	0	0	0
XXIII	1 h. 40° C	30	100	94	3	0	3
XXIV	2 h. 40° C	30	93	100	0	0	0
XXV	4 h. 40° C	30	97	100	0	0	0
XXVI	8 h. 40° C	30	97	91	6	3	0
XXVII	control	45	98	98	0	0	2

still far, it may be that spontaneous cripples do not occur when the conditions for germination are optimal.

"Intermediates" were equally scare in these experiments (series XI, XIV, XIX and XXVI). At higher temperatures those forms never occurred (table 10). Less intensive temperature-treatment might possibly be the cause of the development of these intermediate forms, but the divergent results do not warrant definite conclusions.

The velocity of germination of the normal plants fluctuated between narrow limits, between 49.4—51.8 days. The average rate of germination of the cripples and the "intermediates" was viz. 49.8 and 51.3 days. These experiments did not yield an accurate limit of the potential milieu. It seems probable, however, that this limit is determined by both temperature and time, as a prolonged sojourn at a medium temperature (32°.5 C) already may give rise to crippled plants, while a short stay at a higher temperature (40° C) may also damage some plants.

The mass- occurrence of cripples as described in previous experiments

(table 10) should therefore be ascribed to the more extreme temperatures to which the seeds were subjected — temperatures not to be expected to occur in the natural milieu. As all the results were obtained under artificial conditions it seems interesting to investigate the external conditions (especially of temperature) under which Ardisia crispa occurs in its natural habitat. Especially the presence of spontaneous cripples should be looked for.

§ 8. *Discussion and summary.*

The plant component in the symbiosis is an individual entirely different from the normal plant. This difference is apparently caused by the absence of bacteria. Not only its habit, but also its inability to flower characterizes the cripple, which proved in all cases to be free of bacteria.

The remarkable condition of its meristematic tissues made us suspect that the extracellular bacterium might form substances in the external milieu which substances would influence growth.

Cytological investigation did not reveal any important difference between normal and dwarfed plants, so that genetical differences seem improbable. The presence of peroxidase in the crippled plants does not give us the right to directly compare our results with those obtained with maize and with peas.

If we might consider the cripple as a dwarf, the nanism is caused by the absence of the bacterium which might, therefore, form substances which penetrate into the growth-meristem and possibly modify the peroxidase activity.

We are unable to give a complete explanation for the origin of the spontaneous cripples. Our results differ markedly from those of MIEHE, as the percentage of spontaneous cripples found by us varied from 0—21 %. In order to interpret these variable results we are driven to consider the external circumstances as the causative agent.

As from the same plant, in subsequent harvests, totally different results were obtained it seems that the external circumstances during ripening of the seed might play an important role. The conditions during germination may also contribute.

Of these conditions the temperature is amongst the most important, as shown by heat-treatment of the seed under various conditions.

The experimental cripple appears identical in all respects with the spontaneous cripple.

CHAPTER VII.

Experiments with cripples, normal plants and intermediates.

Although this Chapter deals with very heterogeneous experiments performed on all three types of plants, it was deemed advisable to bring together these uncorrelated experiments into one Chapter of their contributory value for a final interpretation of the symbiosis.

In the first place we applied the methods used in horticultural practice for stimulating longitudinal growth.

The influence of hetero-auxin-treatment on the cripple was considered next.

Reciprocal graftings of cripples on normal plants were carried out, in order to obtain a clue as to possible transport of growth-promoting substances to the cripple. Furthermore, various strains of bacteria, isolated from normal plants were used to inoculate crippled Ardisia in order to obtain the proof of the symbiosis.

Mutilation of normal plants adduced evidence for the localization of the bacteria, as already hinted at in Chapter IV. Removal of one axillary bud of the intermediates was also tried, in order to see whether the dormant "partner-bud" was still capable of development.

§ 1. *The "forcing" of cripples.*

In practical horticulture methods know as "forcing" induce dormant plants to renewed longitudinal growth and concomitant flower-formation. The methods used are described by MOLISCH (35). Smoke, ether vapour or chloroform vapour were all tried but without any success. An eight-hour treatment in either ether- or chloroform vapour proved to be lethal for the crippled plants. An eight-hour treatment with smoke caused damaged leaves, the leaves became yellow, as after nicotin treatment, but the plants remained alive, although no trace of stimulation could be observed. Cripples kept at 2° C for 12 hours did not show any subsequent change in behaviour.

A method used by gardeners to stimulate the formation of shoots by removal of the terminal bud did not yield any result. In this case the cripples were cut back, so that only the cotyledonary buds remained.

§ 2. *The influence of hetero-auxin on cripples and on normal plants.*

The experiments of SCHLENKER and MITTMANN (39) with dwarf-hybrids of Epilobium hirsutum L. showed that these plants (in which longitudinal growth is inhibited both in the leaves and in the internodes) could be stimulated in growth by the application of hetero-auxin (see especially

strain 11 and Fig. 2 of their publication). The increased longitudinal growth showed itself in the increased growth in the stem-epidermis. The number of leaves did not increase after the application of hetero-auxin.

The nanism of Ardisia is almost certainly not caused by genetic differences, but is only due to the absence of (specific?) bacteria in the plant. Still, the paper of SCHLENKER and MITTMANN contained a valuable suggestion. It seemed therefore logical to investigate the influence of hetero-auxin on the crippled and on normal plants.

The anatomical work (see Chapter VI, § 3) showed that in the cripple the elongation of both internodes and leaves in the terminal bud was almost totally inhibited. At the other hand, the formation of hetero-auxin by various bacteria and fungi is an established fact (see WENT (46) Chapter IV) and it seems possible that the bacterium, present in the buds of the normal plant forms hetero-auxin which permeates into the surrounding cells. NĚMEC (36) seems to hold a similar opinion, although his experimental evidence is scanty. Unfortunately the same epithet applies to our own work. Due to the lack of material, hetero-auxin could be only applied by one method, while neither auxin *a* or *b* or vitamin B_2 were used.

The hetero-auxin (β-indolyl-acetic acid) was obtained from HOFFMANN and LA ROCHE, Basle.

The terminal buds of the plants were treated several times a day either by placing a drop of hetero-auxin solution on the bud or by painting this solution on the bud by means of a fine brush.

Controls were treated with tapwater in an identical way.

Concentrations varying from 1 : 500—1 : 5.000.000 were used. Every 3—4 days fresh solutions were prepared, which were kept in the ice-chest. The experiments were carried out in the nursery-house February–March 1938. In connection with the unfavourable season part of the plants were electrically illuminated during the night with an intensity of 500—600 Lux.

The differences between the dark and the light-series are very slight, the untreated normal plants showed perhaps a slightly better leaf-development. Consequently the results of both series seem comparable and are treated together.

Series I.

Hetero-auxin concentration 1 : 500.

Material: 25 experimental cripples and 10 normal plants originated from the same batch of seeds (Chapter VI, T. 10), age 8 months.

The hetero-auxin was applied twice daily for twenty days.

1. *Cripples.* Sixteen plants showed development of rot, due to the treatment. A strong indole-odour occurred in all cases. In the hothouse the hetero-auxin is probably soon decomposed; micro-organisms might play a role in this process. The plants which showed rot, all perished.

The nine remaining plants all behaved in the same way. Three weeks after the beginning of the experiment on all cripple-stems small root-tips

appeared. These tips pierced the epidermis, causing small, slit-like, openings. The number of roots per plant is not large, from 2—6. The roots reached a length of 2—3 cm, 5 weeks after their appearance, and penetrated into the soil.

This root-formation seemed to occur over the entire length of the epicotyledonary axis, either immediately above the mass of cotyledonary buds or below the bud. One plant was removed from its pot after five weeks and showed increased root-formation (Photograph 27). Three months after the inception of the experiment the other plants were removed from their pots. The roots showed a material increase in length as compared with the controls. As a rule the roots originated from the hypocotyledon (Photograph 28).

The only other change observed in the nine surviving plants was the vertical position of the petioles, probably caused by a response of the dorsal flank. The leaves assumed a more upright position.

After 4 months neither the terminal buds nor the cotyledonary bud-mass showed any visible change. Increase in longitudinal growth as compared with the controls could not be demonstrated.

2. *Normal plants*, treated with 1 : 500 hetero-auxin twice daily for twenty days. None of the ten plants died by rot, only the terminal bud was destroyed. After 2—3 weeks root tips appeared on the stems and even on the petioles. After 5 weeks a great many (often more than twenty) root tips appeared on the stems (see Photograph 29). Root-formation in this case, occurs almost exlusively above the hypocotyledon.

Remarkable torsions in the stem, especially near the terminal bud and change in the position of the petiole may be caused by the uneven distribution of the hetero-auxin applied. Longitudinal growth, however, appeared unstimulated. About 2 months after the cessation of the experiment the normal plants (with damaged terminal buds) formed new shoots from their dormant buds.

Series II.

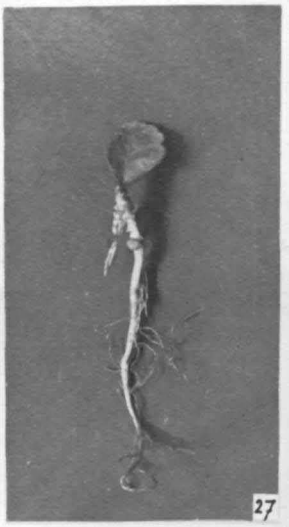
Hetero-auxin-concentration 1 : 5000.

Material: 12 experimental cripples and 10 normal plants from the same batch of seeds (Chapter VI, T. 10), age 8 months.

Hetero-auxin applied 4 times a day for 28 days.

1. *Cripples.* During the treatment no changes in the parts above ground could be observed. No losses occurred. After termination of the treatment the plants were taken from their pots. In nine out of twelve cases the plants appeared to have formed 1—6 new roots below the cotyledonary buds. These roots were already 1—2 cm long. After 3 months these roots had reached a length of 5—6 cm. The cripples of these series as compared with the controls, showed a marked increase in root formation (Photograph 28).

2. *Normal plants.* After 28 days' treatment the plants showed copious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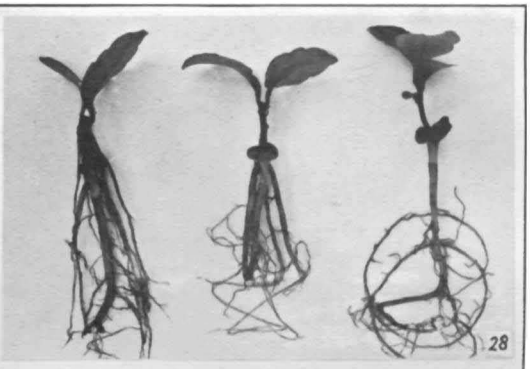
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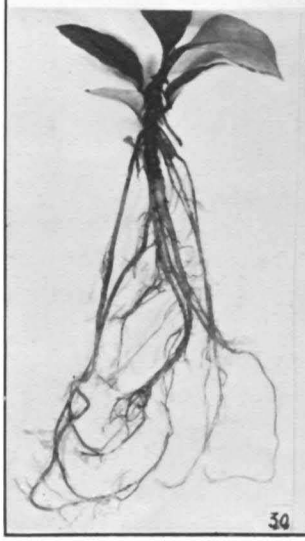
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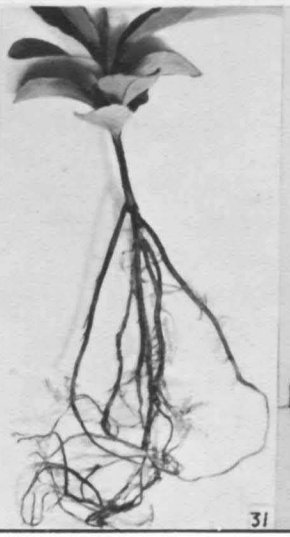
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PLATE VII.

The influence of hetero-auxin.

Photograph 27. Cripple of series I (hetero-auxin 1 : 500). Five weeks after beginning of treatment. Root-development in epicotyledonary region.

Photograph 28. From left to right; cripple of series I (hetero-auxin 1 : 500); cripple of series II (hetero-auxin 1 : 5000); cripple control, untreated. Three months after beginning of treatment. The cripple of series I shows root-formation both in epicotyledonary- and hypocotyledonary region. The cripple of series II only shows hypocotyledonary roots.

Photograph 29. Normal plants of series I (hetero-auxin 1 : 500), five weeks after beginning of treatment. Large number of rootlets on epicotyledon. Torsion of stem-tip and petiole.

Photograph 30. Normal plant of Series I (hetero-auxin 1 : 500). 3 months after beginning of treatment. Roots emerging from hypocotyledon, epicotyledon, petiole and midrib of leaf.

Photograph 31. Normal plant of series II (hetero-auxin 1 : 5000) 3 months after beginning of treatment. Roots emerging only from hypocotyledon.

Photograph 32. Normal plant, control, untreatment, of the same age as the plants depicted in Ph. 30 and Ph. 31.

root formation in the hypocotyledonary region, 3—7 roots being formed on a single plant. The length of these roots varied from 3—4 cm. After 3 months the hypocotyledonary roots had reached the same length as the original main-root. In comparison with the control (Photograph 32) which shows only lateral root-formation from the main root, the treated plants show a much better developed root-system (Photograph 31).

Series IV, V and VI.

Application of hetero-auxin in concentrations 1 : 50.000 resp. 1 : 500.000 and 1 : 5.000.000, 4 times a day for 60 days caused no changes either in cripples or in normal plants. Two months after the termination of the experiment all normal plants from the different series showed a slightly better developed root-system as compared to the controls. Up to this date (the end of May 1938) the cripples have remained unchanged.

Apart from the torsion effects in the stem and in the petioles *the application of hetero-auxin only seems to stimulate root-formation in both crippled- and normal plants,* application of low concentrations of hetero-auxin causes root formation only in the hypocotyledon, while higher concentrations moreover induce the epicotyledon and even the petiole to form roots. The effect is much less pronounced in the cripple, but essentially identical with the effect in normal plants. It may be that either the lower assimilatory surface of the cripple or its disturbed metabolism or both are the cause of this difference.

For root-formation the presence of bacteria seems unnecessary. Inasmuch as nitrogen is required to build up new organs it appears that the cripple itself is able to furnish the nitrogen-compounds necessary for this root-formation. The bacterium, itself unable to grow on nitrogen-free substrates seems, therefore, also incapable to fix nitrogen in a symbiontic condition. A similar conclusion was drawn by NEMEC from his experiments on root-formation from bacteria-free leaves of Ardisia.

§ 3. *The influence of grafting.*

If a normal plant be used as scion and a cripple as stock or vice versa the possibility may be considered that either *A.* the bacterium is transported from the normal to the cripple, where it may cause normal development or *B.* substances secreted by the bacterium enter into the normal plant, and these substances may pass into the cripple and induce normal development. The first assumption, transport of the bacteria, seems untenable, because an intracellular occurrence, as occurring in the Leguminosae, was never observed by us. In order to test the second assumption a great many grafts were prepared.

Horticulture knows many forms of transplants; such as the bud-grafting, the veneer graft and the cleft graft. Bud grafting did not yield any results. This is probably due to the fact that, after insertion of the thickened cripple-bud under the normal bark, I omitted to cut off the terminal bud

of the normal plant, which is common practice. Hence the nutrition-stream was diverted to this terminal bud and the graft did not "take". This seems to be deplored because only with the bud-graft a crippled meristem is brought into immediate contact with normal tissue.

The thin stem of the cripple made the reverse experiment impossible. Veneer grafts do not take for the same reason. The crippled stem is too thin to stand a firm application of twine. The so-called cleft-graft remained as a possibility. Preliminary trials showed that, for a successful graft, scion and stock should be of approximately the same diameter. This limited us in the choice of our material. Normal plants and both spontaneous- and experimental cripples were used. The joints were wound with cotton yarn. The grafts were placed under glass jars, until the joints were properly healed.

a. *Normal scion, normal stock.*

This control was prepared in order to see whether Ardisia would be susceptible to this form of grafting. The scion consisted of a terminal shoot, while as stock one year-old plants were chosen. In four of these grafts the callus-formation showed through the twine already after 10 days. After 3—4 weeks the yarn was removed and the scion appeared completely joined to the stock. This scion seemed to have stagnated in growth, but developed normally after the junction was completed. The stock showed no shoot-formation from dormant buds.

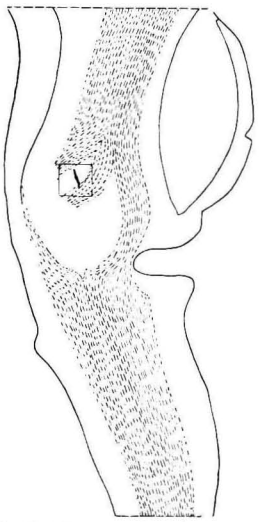
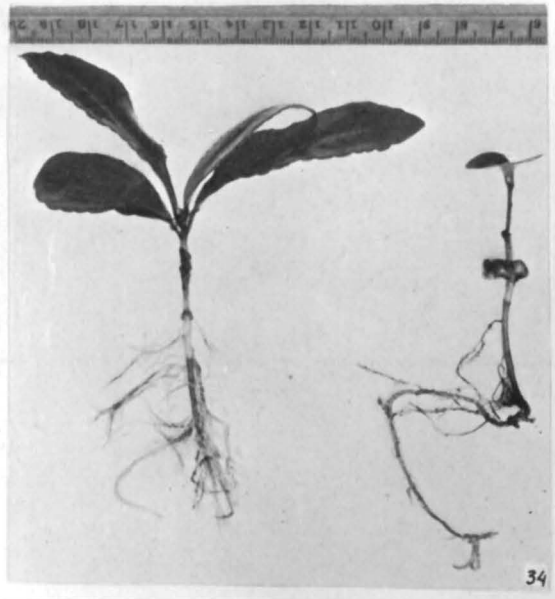


Fig. 14. Longitudinal section of a graft; normal scion on cripple stock 4 ×. The square is the area covered by the detail given in Fig. 15.

b. *Normal scion, cripple stock.*

The cripple stock was incised just above the cotyledonary bud-mass. The wedge-shaped scion (a young terminal, normal, shoot with 3—5 leaves) was inserted into this incision. The junction was wound with yarn. The stock consisted in this case of spontaneous cripples of Series IV, § 6. Eight grafts of this type were prepared, two of which did not take, because of insufficient tautness of the yarn. The six grafts that did "take" behaved as follows: one to two weeks after the preparation of the graft there was copious callus-formation, after 1—2 months the normal terminal shoot showed new leaf-development which development had hitherto stagnated. In this stage the yarn was removed (Photograph 33). The joint seemed complete. Longitudinal sections show a complete contact between the tissues of both scion and stock. The course of a connecting vascular bundle is shown in Fig. 15 which was taken from the part of the



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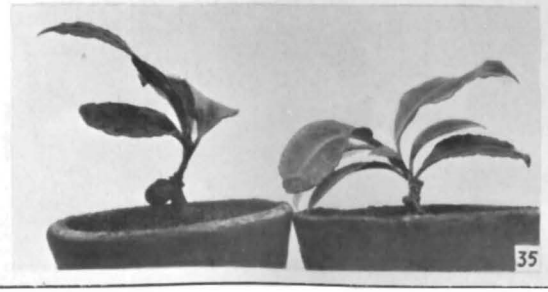

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PLATE VIII.

Cleft-grafts; normal scion, cripple stock.

Photograph 33. Normal scion on cripple stock, 2 months after grafting, showing development of two new leaves. Cotyledonary mass left intact.

Photograph 34. Left side; normal scion on cripple stock, cotyledonary mass removed. 2 months after grafting, showing development of root system as compared with the cripple of equal age as the stock, on the right side.

Photograph 35. Left side; normal scion on cripple stock, one year after grafting. All buds of the normal scion were removed.
Right side; normal scion on cripple stock one year after grafting. Buds of normal scion were allowed to remain.

joint shown in Fig. 14 as a small square. In the detailed drawing original phelleme-formation in the stock is apparent (black zone, lower centre), the

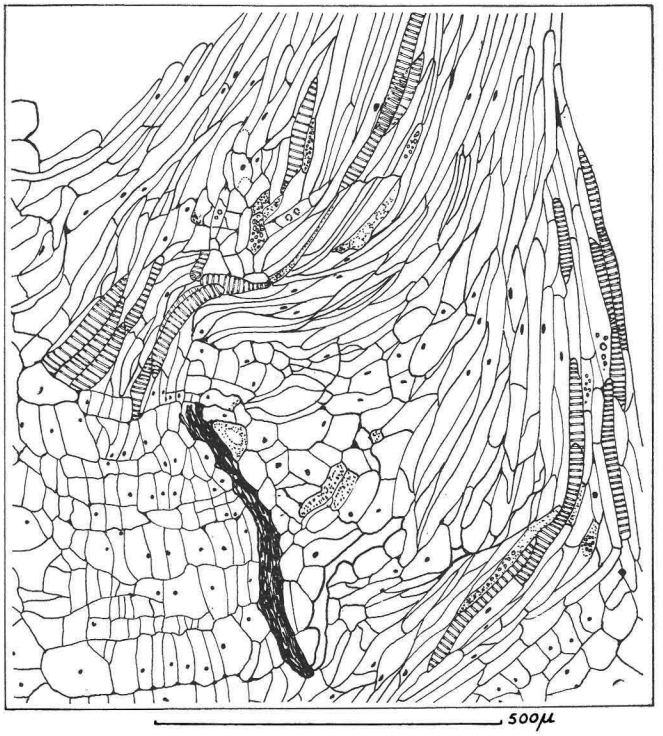

Fig. 15. See Fig. 14. Detail of the joint, described in the text.

phellogen may have reverted to a monogenic cambium, forming originally parenchyma cells (lower left). Prosenchymatous tissue from the scion seems to penetrate into this mass, many of the prosenchymatous elements already converted into ring-tracheids with an indication of the formation of ring vessels. The peripheral prosenchyma seems the more advanced.

The joint is indeed complete. Still both scion and stock kept their respective characteristics for over one year! Only the root-system of the cripple-stock began to branch copiously in all cases. Comparison with a cripple-control of the same age, which control only shows an almost unbranched tap root, brings out the influence very clearly (Photograph 34). The graft "normal scion, cripple stock" therefore shows a tendency to the formation of lateral roots. It is possible that, in this case, root forming-substances (hetero-auxin?) are transported from scion to stock.

In order to stimulate the stock the terminal buds of the scion were removed in three of the grafts. But after 3—4 weeks only the axillary buds of the scion started to develop and formed normal shoots. These shoots were also cut off. After the scion had been systematically bereft from all of its normal buds, its possibilities seemed exhausted, the only available buds being the cotyledonary mass of the stock. These bud-masses

started to increase in girth, but did not form shoots, even after one year the situation remained unchanged. Compared to a similar graft with an intact scion (which scion had formed four new leaves) the only difference between the two lies in the swelling of the cotyledonary bud-mass in the former case (Photograph 35).

c. *Cripple scion, normal stock.*

Experimental- and spontaneous cripples were used as scions. In a few cases the cotyledonary buds were removed prior to grafting. In some instances we used normal plants from the same batch of seeds and of the same age as the scions. 22 Grafts of this type were prepared which number may be divided into three groups, dependent upon the material used.

1. Scion: spontaneous cripples, $1\frac{1}{2}$ years old. Stock: 2 year old normal, belonging to the same series. Cotyledonary bud-mass removed from scion. Of the 8 grafts prepared only 4 succeeded. Bad joints, probably due to insufficient contact, are presumably the cause of this failure. This insufficient contact may be due to the disproportion of the slender scion and the heavy stock, which made secure fastening by means of yarn very difficult. Of the 4 successful grafts the following may be reported; callus-formation occurred after 10 days, and protruded through the yarn, especially on the horizontal plane of the stock. The yarn was removed 2 months after preparation of the graft. The joints seemed perfect. 3—4 Weeks after the graft was made, and for the following year the stock persisted to form shoots from dormant buds. These shoots were removed. After the possibilities of the stock have been exhausted the graft remains stationary. The swollen terminal buds of the scion do not increase in girth nor do they show any tendency to form shoots. This stationary stage is depicted in Photograph 36, taken of a two month-old graft.

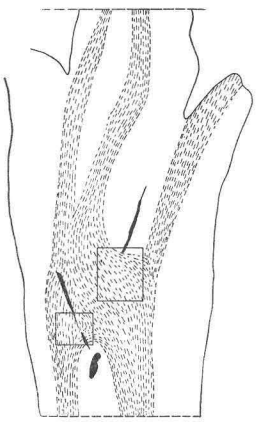

Fig. 16. Longitudinal section of a graft; cripple scion on normal stock 4 ×. The squares are the area's covered by the details given in Figs. 17 and 18.

2. Scion: four spontaneous cripples, one year old (series IV, § 6, Chapter VI). Stock: normal, one year old, from the same batch of seeds as the cripples.

Cotyledonary buds of the scion were allowed to remain. The stocks possessed lateral shoots which would have formed inflorescences next year. The scion was placed high upon the stem in order to keep the lateral axes of the stock intact. The behaviour of these grafts was similar to those described above. The stock formed shoots from dormant buds, which shoots were removed. The cripple did not show any changes, not even after removal of the lateral axes of the stock 3 months after the preparation of the graft. All grafts in this series were successful (Photograph 37).


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PLATE IX.

Cleft-grafts; cripple scion, normal stock.

Photograph 36. Cripple scion (1½ years old) on normal stock (2 years old), 2 months after grafting.

Photograph 37. Cripple scion (1 year old) on normal stock (1 year old), 4 months after grafting. Lateral shoots of stock were allowed to develop.

Photograph 38. Cripple scion (4 months old) on normal stock (1 year old), one year after grafting. All developing shoots removed, the scars still showing.

3. Scion: ten experimental cripples, 4 months old. Stock: normal, one year old. All ten of the grafts were successful. One year after the preparation of these grafts (continually removing shoots formed by the stock) no change whatsoever could be observed (Photograph 38).

Longitudinal sections of these grafts were examined microscopically. Figures 16, 17 and 18. Phelleme-like masses again seem to separate scion and stock. Continuity between the two components follows from the detail drawings. It appears as if the individual cells of the cripple scion are larger

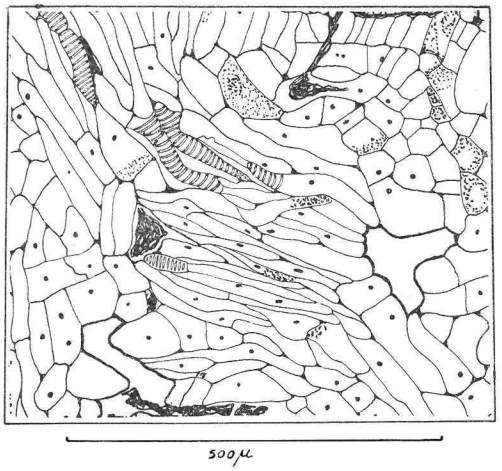

Fig. 17. See Fig. 16 upper square. Detail of joint, described in the text.

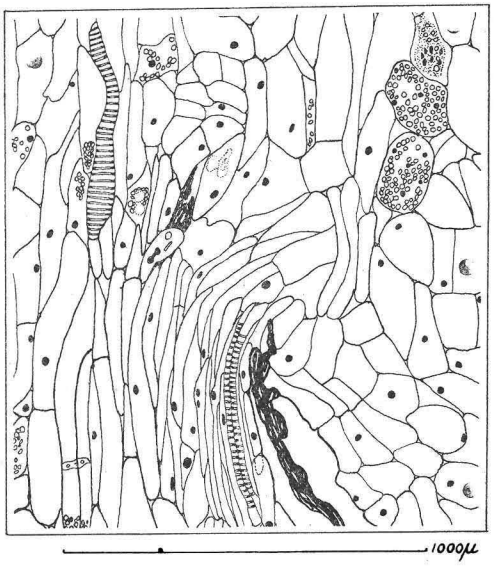

Fig. 18. See Fig. 16 lower square. Detail of joint, described in the text.

than those of the normal stock. The continuity of the tracheids is unmistakable. The lacunae in Fig. 17 represent imperfect contacts which did not even form phelleme.

In spite of the fact that many good grafts were prepared, and the xylem-to-xylem junction was demonstrated microscopically, while also the parenchymatous elements seemed continuous in scion and stock, no influence seemed to emanate from the normal plant. No transport through xylem, phloem or parenchyma of a growth-promoting substance seems therefore to take place. As, according to AVERY (2) growth-promoting substances are readily transported in both directions, either in the xylem or in the phloem, it remains possible that other substances than auxin are the causative agent emanating from the bacterial film.

An experiment which yielded results similar to ours was already performed by MIEHE, who induced the apical part of normal plants to assume a crippled character by means of local heat-treatment. In this case also the lower zones (which remained normal) formed normal shoots.

It must be stated also that the normal leaves in the stock often contained

masses of bacteria, especially when young. Apparently these bacteria exert no influence whatsoever upon either elongation of the internodes or upon the further leaf-development.

§ 4. *Infection-experiments.*

The bacterial strains isolated were used to infect cripples in order to furnish the proof that the bacterium is necessary for the normal development of Ardisia.

In my experience in which several thousand seedlings were examined a spontaneous recovery of a cripple never took place. This is a very lucky circumstance, since the seeds need not to be grown under sterile conditions, for external spontaneous infection seems excluded [1]).

It proved, however, to be a difficult task to infect crippled-plants. For, in the first place, we are ignorant of the susceptible stage of the plant and in which nutrient-medium the bacteria be best suspended. As to the locality of infection we had the choice between the terminal and cotyledonary buds.

Series I. The first series of experiments was started in the autumn of 1937. The following method was used:

The terminal buds of 4 month-old experimental cripples were covered every other day in seven successive treatments with a drop of a two-day old peptone-glucose culture of various white strains and a yellow strain.

These strains were numbered respectively: 95, 90, 94, 54, 51 and 51 yellow.

In total 35 plants were used. Five plants were always treated with the same strain, five plants in total were kept as controls. The controls were treated with a sterile peptone-glucose solution in a similar way. At first the experiment seemed a failure, for no changes could be stated between treated plants and control plants. However, 4½ months after the beginning of the experiment, when new soil was placed in the pots tiny shoots were observed originating from the axillary cotyledonary buds. As, in the original application the terminal bud (which proved refractory to treatment) was moistened it seems that part of the bacterial suspension has flown down the skin and in this fashion had infected the more susceptible cotyledonary bud. (Fig. 19.)

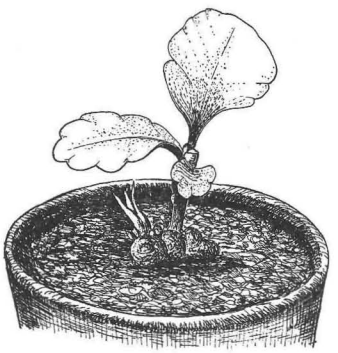

Fig. 19. Experimental cripple infected with bacterial strain 51, 7 months after inoculation. ½ nat. size.

The number of plants in which the tiny shoots appeared was 5, or 1/6

[1]) Ardisia seeds may develop under sterile conditions. We succeeded in raising one cripple and four normal plants on nutrient agar in an Erlemeyer flask. The root-systems showed well through the agar and demonstrated again that the differences are essentially in stem and leaves only.

of the plants treated. Two of these plants were infected with strain 95 and 3 plants with strain 51. The other plants showed no change.

The bacteria, when arrived on the axillary cotyledonary bud might have penetrated this bud through the channels described in the previous Chapter (Photograph 24) and awakened the meristem to renewed activity. The new shoots grow very slowly and we do not as yet know whether the leaves formed contain bacteria! No absolute proof of a successful infection has been obtained as yet from this experiment.

Series II. A second series of infections was started in the spring of 1938. In relation to the results obtained in the former series a copious quantity of bacteria, obtained from a 5-day old culture on pea agar, was smeared on the axillary cotyledonary buds. This treatment was repeated after four days.

The 10 month-old, experimental cripples used in this experiment belonged to the same batch of seeds as those used in the former series. These plants showed already strongly swollen cotyledonary buds. Strains 51, 54, 94, 95 and 51 (yellow) were used as infection-material.

Thirty-two plants were used in these series, of which two plants were kept as controls, while 5 plants each were treated with the same strain. The sixth group of five plants was treated with both strain 51 and strain 51 (yellow). No trace of any change could be observed in the plants four months after infection. At this stage of development (10 month-old) the plant is apparently irreversibly changed.

Series III. Experiments with very young crippled plants seemed the only possibility left. To this end the seed coats were removed from young seedlings. The cotyledons were spread apart and a drop of a two-day old peptone-glucose culture was brought upon the terminal bud. The bacteria were still motile in this medium.

As the seeds were previously heated at 40° C for 24 hours we might expect 100 % cripples both according to MIEHE's and to our own experiences. Strains 51, 95 and 51 yellow served as infection material. Three groups of six cripples were infected with the strains mentioned. Moreover, three plants were infected in a similar way with crushed material from the terminal buds of normal plants. Three plants, treated with a sterile peptone-glucose solution in a similar way, served as controls. Moreover, twelve cripples were allowed to germinate in a normal way. After four months only one plant, infected with strain 51, showed a normal lateral bud, while all other plants, both treated and control, remained crippled.

Three out of six plants infected with strain 51 (yellow) were destroyed by rot, a phenomenon never observed in infection with the colourless bacterium.

Series IV. In this series the seedlings were allowed to shed their seedcoat, and infection was brought about as soon as the terminal bud had freed itself from the rest of the seed. The infection, either with a two-day old culture on peptone-glucose, or four-day old pea agar suspension in

water, was repeated in certain cases. Only strain 51 was used as infection material. The plants were raised from seeds heated for 24 hours at 40° C.

Forty plants were used in this experiment, divided in four groups. One group served as a control, in order to see whether the plants actually developed into cripples. Another group was infected once with a suspension of bacteria in water, two plants serving as control. In the third group the plants were treated twice with this suspension, two plants being kept as control, while in the last group apart from the two controls, the plants were treated 4 times (every 2—3 days) with a two-day old peptone-glucose culture. The controls were similarly treated with a sterile peptone-glucose culture.

After three months the last group yielded one normal plant, the twice-infected group showed three normal plants, while the once infected group showed two. The controls remained stunted throughout.

Although the results are far from complete, they may be considered as positive evidence for successful infection. The "white" strain seems to be active, while the stage of development of the plant seems important. Young crippled plants still may revert to "normals" while older plants seem no longer susceptible to infection. As in the bacterial symbiont, the plant symbiont appears to exhibit, therefore, a reversible and an irreversible stage.

The nature of the infection-material seems equally important. Aqueous suspension of the bacterium seem more efficient than peptone-glucose cultures. It may assumed that, in the latter case, the bacteria still find a favourable milieu in the peptone, without looking for the less favourable environment of the terminal bud.

The quantitative deficiency of the experiments may be accounted for by the age of the strains, which were isolated one year previous to the experiment, and were cultured on artificial substrates. They may have become much weakened by this procedure and as a passage through a natural milieu, as practized with cellulose- or N-fixing bacteria in order to increase the virulence is not possible in this case, it might be advisable to repeat the experiments with newly-isolated bacteria. As the number of factors which apparently determine the successful infection is, apparently, large it still seems worth while to strengthen our proof by means of more exhaustive experiments.

§ 5. *Mutilation of leaves and shoots in normal plants and in "intermediates".*

a. The foliar nodules of one year old plants were removed by means of a puncher and the terminal bud was cut off also. In the controls only the terminal bud was removed. 10 Plants of each series were prepared in this way. As a result the youngest dormant bud started to develop at the same time in both series (3—4 weeks). In both cases a normal shoot

was formed. The role of the bacteria in the leaf margins seems to be quite subordinate. From the above experiment it follows that the presence of these bacteria does not influence the development of dormant buds, which development depends solely upon the removal of other buds (food factor) and the *local* presence of bacteria.

b. From six one year old "intermediates" the normal, lateral shoot (see Chapter VI, § 2) was removed. The intermediates proved capable to develop a new shoot from the "partner" cotyledonary bud which had, thusfar, remained dormant. This proves that in the intermediary form, the axillary cotyledonary buds contain bacteria, while the terminal bud is devoid of them.

Removal of the second lateral shoot yields a cripple, whose swollen terminal bud seems incapable of further development.

§ 6. *Discussion and summary.*

We did not succeed in "curing" cripples by external circumstances, except by means of bacteria. Hetero-auxin only caused root-formation and from this we concluded that nitrogen fixation of the symbiontic entity or of the bacterial component is extremely unlikely. The cripple being apparently able to supply the nitrogen compounds necessary for root-formation. Grafts only supplied us with negative information. Only the cripple stock seems stimulated to root-formation by a normal scion. We should not forget, however, that in the case of normal stock only the (apparently inactive) foliar bacteria were present, the shoots being removed as soon as formed. Notwithstanding the complete continuity between scion and stock and notwithstanding the presence of active bacteria in normal scions, no influence of scion upon stock or vice versa could be detected.

In keeping with its inactive state in the leaves, removal of the nodules was without effect upon the rest of the plant. The localization was further specified in plants with three buds, one year old intermediates, in which one bud seems free of bacteria (the terminal bud), one bud is dormant (2nd axillary cotyledon) and one bud forms a normal shoot (1st axillary cotyledon). Removal of this shoot induces development of its cotyledonary partner.

The infection experiments show that not only we isolated the specific symbiont, but that the symbiontic entity may be synthesized.

The action of the bacteria is extremely local.

CHAPTER VIII.

Discussion of the results.

It is almost inevitable that we should be inclined, at the end of this paper, to indulge in speculations. For the curious partnership between the Ardisia-plant and the bacterium has taught us much less, and at the same time much more, than we expected. Our original hope, to elucidate the problem of the foliar symbiosis, has not been fulfilled. At the other hand, the very elusiveness of our game made us view the relations from all angles. In this way this work may be helpful, as it excluded several possible solutions, propable enough in themselves, but which proved to be fallacious. What remains, apart from the negative results, gives much food for thought. Thought upon the problem of symbiosis both as to its nature and its origin.

a. *The nature of the symbiosis.*

It is with the observations on Lichens that we should start our argument. Prior to SCHWENDENER's work in 1867 we only find very vague concepts as to their nature. It was usually held that, what is now known as the algal component was a fruiting body of the hypha. SCHWENDENER demonstrated the presence of Algae and Fungi in the Lichen, but considered the fungus to be parasitic upon the alga — in the spirit of his time. His work was analytical. It was due to DE BARY's brilliant synthetic thought that we are aware, since 1873, of an intimate inter-dependence of two organisms who mutually derive benefit from their association. The first symbiosis described was a mutual symbiosis, in which both components might, at least potentially, lead independent lives and in which neither of the components became so intimate as to penetrate into the cells of the partner. One might, therefore, further define this form of symbiosis as extracellular.

Amongst the great many forms of symbiosis known to us now we mention the well known endotrophic mykorrhiza, concerning the knowledge of which we are so much indebted to BURGEFF and the bacterial root symbiosis of Leguminous plants. Here the partners quite readily undergo experimental association, as any orchid-grower or modern farmer knows. This form of symbiosis is intracellular. The ectotrophic mykorrhiza, the relation of cellulose bacteria to termites and ruminants are examples of extracellular symbiosis, probably also capable of easy realization. It is curious that the two forms of extracellular symbiosis known as the Lichen and the Ardisia crispa should prove so refractory to experimental synthesis.

Some of the failures of Lichen-synthesis were due to the difficulty of growing the algal, respectively the fungous component in a solitary state, while in other cases the combination did not seem to "click". The symbiosis known as the "normal" Ardisia crispa consists of an energetic bacterium, capable of development on a great variety of media and, on the other hand, of a mutilated, pathological plant which is incapable of development. Synthesis of both symbiontic components, is difficult if at all completely successful. The cripple may be compared to a suckling kid as long, as it is in the first seedling-stage — it still lives on the reserves provided by the mother. But as soon as the kid is weaned and wants to become a goat it has to digest cellulose and in nature a goat free of cellulose-bacteria is unknown. It should be, however, experimentally possible to raise such an animal. But when the cripple, but hitherto normal Ardisia has exhausted the endosperm, when it too is "weaned" the bacterium becomes a vital necessity. But like the fertilized queen of the leaf-cutting ant, which carries the precious Rhozites fungus in its crop, the normal Ardisia seedling, thusfar the very image of its crippled brother, carries the bacteria. And the cripple seems very soon too far gone to recover by the external applications of bacteria.

This strange inter-relationship we shall consider more closely.

If a gardener sees a crippled Ardisia he will most probably call it "a dwarf". It is remarkable how little we know about dwarfs. There may be cases of retarded or totally stagnated development, with persistence of juvenile characters which may be classed as such. This nanism is often caused by a deficiency in a specific substance. However we often meet with an opposite case. Development in most "hunger" dwarfs may be extraordinary rapid, although growth is much impaired. In other cases the balance in development may be upset and we see tiny plants, profusely flowering. Such dwarfs may be caused by excessive radiation but also by starvation (Japanese dwarf-trees).

Still another group of dwarfs are genetically deficient. A certain strain of dwarf-maize differs in one gene from another strain of normal habit, certain gene combinations in Epilobium hybrids show nanism, experimental haplonts may develop into individuals of subnormal stature. It is hard to tell under which group to class the crippled Ardisia plant. It certainly possesses juvenile characteristics. After three years of development it looks like a three months old (normal) plant. But its terminal stem meristems have become inactive, while the cotyledonary buds have formed secondary, tertiary and perhaps even quaternary buds, all crowded together in a large, wart-like mass. The cells of its meristems, present in large numbers cannot elongate. The axial meristem cannot form an inflorescence, the plant cannot propagate itself either sexually or asexually. We find starch amassed, but it cannot be used. Curiously enough, the root-system remains normal, and is as large as we may expect from a plant of that size. Its growth may be stimulated by external influences. It possibly, because of

its healthy appearance, is able to exert sufficient root pressure to feed the cotyledonary buds which are the only other part of the plant in which the vitality has not sunk to almost zero level.

Juvenile dwarfs remind us of deficiency diseases, and deficiency diseases remind us of hormones. It seems indeed plausible, even from macroscopic examination of the plant, to suspect hormonal deficiency and what should sound better than some form of deficiency in growth-hormone? The auxins promote cell elongation, which seems to be totally lacking in the cripple after the seed reserves are exhausted. Moreover it has been repeatedly stated in the literature, that the auxin production in a terminal bud inhibits the development of the axillary buds. In the cases of the Ardisia-cripple the terminal bud is "taken away" in a physiological sense, because it seems totally inert, while the cotyledonary buds remain active for years! (see Photographs 20 and 21). The so-called intermediates might be interpreted from this viewpoint as plants in which the terminal bud is destroyed before the "material" auxin reserve was exhausted. Now it is difficult to say whether the lesion in the cripple is due to a deficiency in- or to a destruction of the growth-substances. One fact seems to point to the latter possibility: the active peroxidase in the terminal bud of the cripple, which seems absent from the terminal bud of the normal plant. And also in dwarf peas an increased peroxidase activity was demonstrated. It was assumed by VAN OVERBEEK and later by DE HAAN and GORTER that in those cases the auxins when formed were oxidized before they could exert their action. It remains obscure, however, whether the presence of the peroxidase is the cause of the dwarfing, or merely a result of the general metabolic upset. Unfortunately, application of hetero-auxin only promoted root-formation in Ardisia, while the stem and leaves remained in their refractory condition. It may be assumed that the hetero-auxin did not reach the meristems, and consequently, only caused the activation of the pericycle, which it could reach. But the much more active axillary cotyledonary buds did not react either. Moreover, auxins when present and in a stable condition in the normal plant, should be transported, and while cleft grafts, using normals and cripples both as scion and stocks, were successful in many cases — no effect was induced, apart from a better development of the root system in the case of the cripple stock. The latter effect may be very well caused by the availability of photosynthates, produced by the active scion which, both in leaf area and in metabolic activity, enormously exceeds the cripple. None of the other efforts to cure the cripple by means of external circumstances yielded any effect. These are the main facts as far as the cripple is concerned.

The other symbiontic partner now makes its appearance. We know that the bacterium, which we suspect to be an organism of common occurrence in tropical soils, may occur in various stages, which stages may be defined according to their motility, activity in the plant, and reversibility

to the active state. Motile forms were not observed on the plant, but only on solid- and in liquid culture media.

In liquid media the bacteria forms a pellicle, and it is also in membranes that the microbe deploys its activity in the plant. On a potential or latent meristem the bacterium may persist for several years. This is the inactive, non-motile, reversible stage, reversible, because development of the microbe reoccurs as soon as the latent meristem becomes active. Finally we meet with the bacteroid stage, which is irreversible, incapable of multiplication.

As the bacterium occurs only near the meristems and in the foliar nodules, and only occurs inter- or extracellularly, it follows that its action is a peculiar one. We shall consider the action in the nodules first.

MIEHE found large rods in the hydathodes up to the exfoliation-stage of the bud. The number of bacteria decreased thereafter and bacteroids appeared. Removal of the nodules does not seem to influence growth and development of the normal plant. Moreover, NĚMEC showed that roots developed copiously from leaves the nodules of which were removed prior to the experiment. Moreover, normal scions or stocks from which all buds were removed, exerted the same influence upon the cripple as the unmutilated grafts. In the mutilated grafts only the leaves contained bacteria. One is driven to the conclusion that the role of the bacteria in the mature nodule is nil, but that in the early development of the leaf the bacteria may play a role.

What is the role the bacteria play near the meristem? The activity must be merely local. Substances, when produced, should be consumed locally, within the growing tips — as the grafting experiments show. Now we may consider either the bacterium or the meristem as the incentive factor, or consider the partnership itself as the most important. In the latter case we might assume special substances to be formed, for we know that symbiosis may breed special products, such as lichen-acids.

Let us consider what possible contributions may be given by the meristem. We know that plant-cells are capable of secretion and excretion, both of inorganic and organic substances. The hairs (Photograph 12 and 26) present almost everywhere, remind us of the glandular hairs in a related family, the Primulaceae, where secretion is demonstrated. It is quite possible that the young hydathodes, which seem to attract bacteria, also may secrete nutrient matter. The bacterium may: 1. Fix atmospheric nitrogen in contact with the plant. We know it does no such thing on artificial media. It is extremely unlikely that it performs this function in combination with the plant, as its total absence from the root meristems (which are normally developed) and the root system in general (which is normally developed) show.

2. The bacterium may form a growth-promoting substance. If it would do so (and this is hardly surprising, in view of the number of micro-organisms excreting growth-substance) the cripple meristem might oxidize this substance. And this may be the reason of the slow reaction of the

cripple to infection with bacteria. The auxins might penetrate into the meristem and temporarely "overpower" the oxidizing enzymes — leaf primordia are formed, but neighbouring cells contribute their enzymes and finally the auxin reaction comes to nought. This may be one explanation of the curious behaviour of an apparently successfully infected cripple which, after development of small but perfectly normal leaves, sunk back into lethargy for more than four months (Fig. 19). We are inclined to believe that actually a growth-promoting substance is secreted by the bacterium but consider, for completeness' sake, two other possibilities.

3. The bacterium forms an antoxidant which is present in normal plants and which is carried by the seed. The supply of intracellular antoxidant is sufficient for about three months. When it is exhausted the plant becomes a cripple when there are no bacteria to furnish a fresh supply.

4. Another possible action was suggested to me by Prof. Dr L. G. M. BAAS BECKING. The bacterial pellicle might act as an oxygen absorbent. BEIJERINCK was able to culture anaerobes under a film of aerobic bacteria and an active bacterial film might lower the oxygen pressure of the underlying tissues. Without mentioning all of the arguments for and against this idea (they are all purely theoretical) the suggestion will be put to test by me in the near future. The root-system also deserves attention in this connection. As far as I am aware, little is known about the oxygen pressure within a bud [1]).

Apart from the above mentioned possibilities the question arises whether the bacterium acts upon the meristem itself or upon its products, such as foliar primordia. This question is difficult to answer. Examination of the cripple-meristem (Photographs 23 and 24) show the "tracings" of the foliar primordia, but not the young leaves themselves. It is hard to tell whether the meristem, when unimpaired by the masses of undefined tissues around it, might not be influenced itself. It seems, however, that the bacteria exert their most important action in cell-elongation and, therefore, upon the development of internodes and primordia. We have seen the bacterium in a reversible- and also in the irreversible stage. Is it possible that the other symbiontic component, the cripple, also shows reversible and irreversible stages. It seems that the infected cripple (Fig. 19) while showing a reaction after inoculation, is unable to revert to the normal stage. There are indications, however, that very young seedlings, in which the tissues are not "poisoned" as yet, show a more complete reversion.

b. *The origin of the symbiosis.*

MIEHE advocated the view that the subgenus Crispardisia in which we

[1]) Experiments undertaken by Miss M. A. BOK M. Sc. show that diminished oxygen tension seems to have a stimulating effect upon the cripples so that the above mentioned hypothesis may ultimately yield a solution of this problem. The results will be reserved for a separate publication.

find bacteriophilous plants exclusively, is of a monophyletic origin. We are rather inclined to the belief, however, that in a great many non-bacterial species or Ardisia an analogous mutation might have occurred, in which the mutants obtained an unfortunate metabolic anomaly, chiefly expressed in an abnormal redox-potential of the meristematic cells [1]). Instances of analogous mutations in allied species are well-known. The chromosome-constellations might, in these species, have one mutable group in common. This type of mutant could not grow unless it became infected by bacteria. According to our experience, the cotyledonary bud is the most probable place in a young — and the only place in an advanced cripple, where bacteria may exert any influence. We found these cotyledonary bud-masses always near or slightly below the surface of the soil, although the hypocotyledon was originally well above ground. It may be that root-contraction causes the lowering of the hypocotyledon. Whatever the cause, the accessible cotyledonary buds are situated at a place where soil bacteria may penetrate. It is quite possible that other Ardisia's should possess different bacterial symbionts. VON FABER described the symbiont of Pavetta as an acid-fast form, while our form did not show any property characteristic of Mycobacteria. The soil bacterium Bacterium foliicola MIEHE shows remarkable characteristics for an inhabitant of the tropics, 24 hours sojourn at 40° C is lethal, prolonged exposure to temperatures between 35—37° C may be exceedingly harmful, and those temperatures are easily realized in the tropics, while the soil temperatures are even lower (see for the climate of Buitenzorg the report of BRAAK). The Ardisia species belonging to the genus Crispardisia therefore live dangerously near the limits of their potential milieu. Crippling should occur frequently.

The bacterium, once on the meristem, has saved the life of the plant. Fortunately this plant forms adventituous embryo's or, it may be that the presence of the bacteria on the integumental wedge induces embryo-formation. Whatever the cause, the bacterium is always at the right spot and after the first cycle has been closed, the future of this new Crispardisia is assured. It may be questioned whether sexual reproduction would allow bacterial infection of the seed, for the bacterium is never intracellular and it is hard to conceive how the bacterium, either by means of the pollentube, or through accidental clefts, might make its appearance between embryo and endosperm. It remains a future task to investigate whether:

1. the various species of Crispardisia show corresponding forms, of similar geographical distributions, with the non-bacterial Ardisiae;
2. whether there occurs sexual reproduction in any of the Crispardisia species.

For those who deny the bacterial symbiosis of these plants there remains

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[1]) The redox-potential is here considered as the cause of the mutilation. It might be equally well considered as its effect.

another possibility. JAENSCH, who observed the apogamy, also saw a dividing egg-cell, while DAHLGREN saw a haploid cell. Polyembryony has also been stated. It may be assumed that a haploid and a diploid embryo may occur, or both together. If a haploid embryo occurs, we obtain a spontaneous cripple. When we keep the seeds at 40° C the diploid embryo, being for some reason more susceptible, dies. The haploid remains; the experimental cripple. In normal cases the diploid crowds out the haploid, and we obtain a normal plant. Unfortunately the cripples were shown by us to be diploid, so that this possibility is ruled out.

The bacterial symbiosis of Ardisia, as conceived by MIEHE, was confirmed by us in almost all particulars. It is not improbable that a careful survey may yield a great number of new cases of this type of symbiosis. To use an analogon taken from zoology; once the bacterial symbiosis was established, such a careful survey yielded an enormous number of bacteria-insect symbiosis, of which nobody had even dreamt.

It seems indeed curious that thusfar only tropical plants should exhibit the phenomenon of foliar symbiosis. If this generalization should prove to hold it does not lighten our problem.

This work was carried out at the Botanical Institute of the Government University, Leyden, Director Prof. Dr L. G. M. BAAS BECKING. At this place I want to express my sincere thanks to Prof. Dr L. G. M. BAAS BECKING for his constant interest in my research and for his criticism and help in the preparation of this publication.

SUMMARY AND CONCLUSIONS.

1. A study of the literature revealed that more than 370 species of plants, belonging to the Myrsinaceae, Rubiaceae and Dioscoreaceae possess symbiontic bacteria in the leaves.

2. Amongst these cases, the symbiosis of Ardisia crispa A. DC. was studied extensively by HUGO MIEHE. The aim of this study is to repeat and extend MIEHE's work, in order to arrive at a conclusion as to the nature of the bacterial symbiosis.

3. For this purpose many thousands of Ardisia plants were grown from seeds and many bacterial strains were isolated from seeds.

4. The bacteria occur in the plant on active- and dormant meristems (the root and anther meristem excepted) and in the foliar nodules.

5. Colourless, motile aerobes were isolated from the seeds. They proved to be non-acid fast, gram negative rods, with a tendency to become non-motile within a mucilaginous membrane and with a tendency to form bacteroids. They are unable to fix nitrogen.

6. Within the plant they are always non-motile. They are active in active stem-meristems, inactive, but reversible to the active stage in dormant buds, where they may renew activity after several years of rest.

7. The bacteria are enclosed within the floral parts at flower-formation. They are locked within the carpels, finally surround the ovules and are entrapped by the growing integuments at the micropylar region between the two parts of the inner integument.

8. At this place, after embryo sac formation, from a wedge-shaped cell mass of the ventral flank of the inner integument the embryogenic tissue originates, in which the embryo is formed adventitiously.

9. The bacteria adhere to the radical pole of the embryo, where they remain in a resting, but reversible state.

10. At germination the bacteria are pushed over the cotyledons, where they infect the axillary bud while a bacterial mass also lands upon the terminal bud.

11. In some cases the terminal bud remains unaffected, but cotyledonary buds show the bacterium.

12. The bacteria-free plant, the "cripple" has a juvenile appearance. Longitudinal growth stops entirely over the entire plant, the root excepted. The individual meristematic cells are larger, the leaf primordia remain undifferentiated.

13. The axillary cotyledonary buds proceed to proliferate for years, forming subsidiary buds. A wart-like mass originates.

14. The cripple proved to be diploid.

15. An apparently haploid, though reduced, egg-apparatus was described.

16. The cripple contains catalase and peroxidase in the terminal bud. The normal plant contains only catalase in the terminal bud.

17. Most batches of seeds showed the presence of cripples (spontaneously cripples), while heating to high temperatures (40° C or higher) cause all of the seeds to develop into crippled plants (experimental cripples). Heating to temperatures below 40° C yields a very low percentage of cripples.

18. The cripples cannot be cured by forcing.

19. Cleft-grafts, using normal and cripple as scion and stock, and vice versa showed the root system of the crippled stock to be stimulated by the normal scion.

20. Application of hetero-auxin caused only increased root-formation.

21. Infection experiments with fullgrown cripples showed much promise, while developing a pointed bud and later a young shoot — but later the development ceased. Very young cripples may be artificially infected, which infection yields the proof both of the identity of the bacterium isolated and of the synthesis of the symbiosis.

22. Several theories may account for the particulars observed. We deem it most likely that a growth-promoting substance is excreted by the bacteria.

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