

SOME ASPECTS OF THE LEAF NODULE SYMBIOSIS IN *ARDISIA CRISPA*

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SUMMARY

Some data on the leaf nodule symbiosis of *Ardisia crispera* (Thunb.) A.DC. are given.

The nodule containing margins of the leaves contain more nitrogen and less ethanol-soluble sugar than the midrib sections. $^{14}\text{CO}_2$ photosynthesis experiments show a higher activity in the ethanol-soluble as well as in the insoluble fraction of the margin than in those of the midrib section; the activity of the ethanol-soluble sugars was, however, lower in the margin than in the midrib section.

The leaves contain a red pigment of anthocyanic nature. Fixation of atmospheric nitrogen by the symbiont could not be detected.

1. INTRODUCTION

The symbiosis between *Ardisia* species and bacteria living in leaf nodules has been investigated by MIEHE (1913, 1917), VON FABER (1912), NĚMEC (1932), DE JONGH (1938), HANADA (1954) and YAMADA (1960). They found that the symbiont is seed-borne, and that the bacteria are present at all stages of growth. In terminal and axillary buds the bacteria are present between the developing leaves. They infect the leaf via hydathodes and then continue to live in nodules at the leaf margin (fig. 1).

Plants without these bacteria are incapable of normal growth. At the two- or three-leaf stage growth stops and the plant becomes a 'cripple' (figs. 2 and 3).

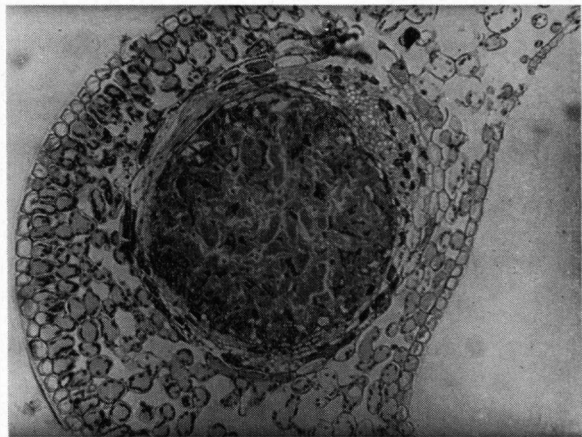


Fig. 1. Leaf nodule of *Ardisia crispera* (750 \times).



Fig. 2. A healthy, bacteria containing, *Ardisia crispa* plant. Age: 11 months.

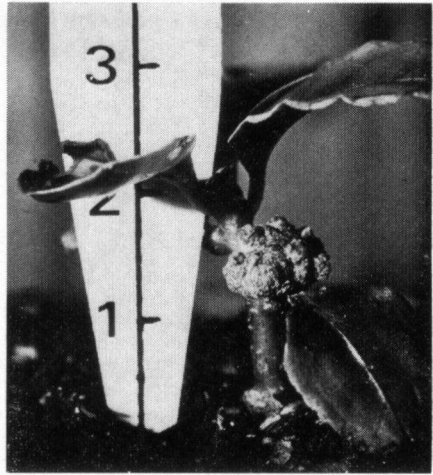


Fig. 3. A malformed bacteria-less *Ardisia* plant. Age: 11 months. (a "cripple")

Axillary buds develop into thick clusters instead of normal leaves. These cripples sometimes occur spontaneously, but it is also possible to obtain them by heating the seeds for 24 hours at 40°C before sowing, thus killing the bacteria without damaging the embryo.

The cause of this crippled growth is not known. HANADA (1954) isolated nitrogen fixing bacteria from *Ardisia hortorum*. MIEHE (1917) and DE JONGH (1938) suggested that *Ardisia crispa* lacked nitrogen fixing bacteria, whereas YAMADA (1960) said that this same species did possess them, although the enrichment in his ^{15}N experiments was about zero. He was unable to conclude that nitrogen fixation was the basic cause of normal growth. According to him, as well as to DE JONGH (1938), growth substances may play a role.

The micro-organism may influence the metabolism of the plant either by synthesizing a compound necessary for growth, by removing a compound harmful to growth, or by creating favourable conditions for growth. If the micro-organism synthesizes a compound necessary for growth, it may be possible to detect this compound by labelling after photosynthesis in $^{14}\text{CO}_2$.

2. EXPERIMENTS

A. Some preliminary experiments were performed in which young leaves were used. One part of the leaves was exposed to $^{14}\text{CO}_2$ after removal of the mar-

gins, the other part was divided into margin and midrib sections after the exposure. Time of exposure to $^{14}\text{CO}_2$ was two minutes, and then 5 or 15 minutes to unlabelled CO_2 . The sections were frozen and powdered in liquid nitrogen and extracted in 80% ethanol. The activity of the ethanol soluble and insoluble fractions was measured in an automatic sample changer and calculated as c.p.m./mg or mm^2 leaf section (table 1). The activity of both fractions proved to be highest in the leaf margins (Ma) and lowest in the margin-less midrib sections (Mi²). For comparison the same experiment was performed with a bacteria-less *Ardisia* species. The figures show that the low activity in the margin-less midrib sections is found in both *Ardisia* species and thus is not caused by the bacteria but by damage due to removal of the margins. A difference in activity between midrib and margin (Mi¹ and Ma¹) was not found in the bacteria-less *Ardisia*, hence the high activity in the margins of *Ardisia crispera* may be caused by the presence of the bacteria.

Table 1. Activity of the 80% ethanol soluble and insoluble fractions of young leaves of *Ardisia crispera* and of a bacteria-less *Ardisia* species after 2 min. in $^{14}\text{CO}_2$ and subsequently 5 or 15 min in unlabelled CO_2 . Mi¹ and Ma¹: the leaves were divided in midrib (Mi¹) and margin (Ma¹) sections after the exposure. Mi²: Margin-less midrib sections were exposed.

	<i>Ardisia crispera</i>			Bacteria-less <i>Ardisia</i>		
	Mi ¹	Ma ¹	Mi ²	Mi ¹	Ma ¹	Mi ²
Activity						
c.p.m./mm ²						
2+15 min Eth. sol.	156	263	77			
2+ 5 min Eth. sol.	101	168	97	193	178	128
2+ 5 min Eth. insol.	90	107	68	54	52	34
Eth. sol. sugars						
Activity in % of tot. act./eth. sol.	65	59		72	72	
Chlorophyll a + b in $\mu\text{g}/\text{mm}^2$	0.155	0.158				

Autoradiographs of chromatograms of the ethanolic extract showed that the higher activity in the margins was correlated with a lower activity in the ethanol-soluble sugar fraction. This corresponds with the data for the total content of ethanol-soluble sugars in young leaves which is also lower in the margin than in the midrib section (table 2).

The higher activity in the margin might be a consequence of a higher rate of photosynthesis, caused by a higher chlorophyll content of the margin sections. Chlorophyll determinations after the method of BRUINSMA (1963) showed, however, about the same content for margin and midrib sections. Thus it may be concluded that the presence of the bacteria in the nodules at the margin of the leaves, which are metabolizing the photosynthates, may play a role here. Experiments are being continued to examine whether further differences in composition of the ^{14}C compounds may be found.

Table 2. Nitrogen and 80% ethanol soluble sugar content in leaves of *Ardisia crispa* and of a bacteria-less *Ardisia* species.

<i>Ardisia crispa</i>	Old leaf		Young leaf		Youngest leaf
Nitrogen content	Mi	Ma	Mi	Ma	
in $\mu\text{g}/\text{mm}^2$	1.24	1.49	0.89	0.99	1.26
in $\mu\text{g}/\text{mg}$ fresh wt.	5.4	6.5	3.9	4.3	5.5
in $\mu\text{g}/\text{mg}$ dry wt.	17.4	19.3	17.0	18.8	25.7
80% eth. sol. sugars					
in $\mu\text{g}/\text{mm}^2$	0.66	0.66	0.43	0.27	0.23
in $\mu\text{g}/\text{mg}$ fresh wt.	2.9	2.9	1.9	1.2	1.0
in $\mu\text{g}/\text{mg}$ dry wt.	9.6	8.6	8.2	5.4	5.0
<i>Bacteria-less Ardisia</i>					
Nitrogen content					
in $\mu\text{g}/\text{mg}$ fresh wt.			4.1	5.0	

B. Until now the data on the nitrogen fixing capacity of the *Ardisia* symbiont are inconclusive (DE JONGH 1938; YAMADA 1960; BETTELHEIM *et al.* 1968). For the *Psychotria* symbiont, nitrogen fixation was claimed by SILVER *et al.* (1963), and some experiments are, therefore, described in which this ability was tested.

1. The total nitrogen content of the leaves was determined after Kjeldahl. The margin sections (Ma) contained more nitrogen than the midrib sections (Mi) in old as well as in young leaves (table 2). This is in accordance with the findings of NĚMEC (1932) and it might point to nitrogen fixation, but the same difference was found in the bacteria-less *Ardisia* species (table 2), and NĚMEC found it also in some other plant species.

2. The leaves of *Ardisia crispa*, especially the very young ones, contained a red pigment. This proved to be of anthocyanic nature, as the ϵ - λ curve of the 1% HCl extract, determined after GEISSMAN (1955), showed. Thus, it had no relation with the leg haemoglobin found in the root nodules of the Leguminosae which is an essential factor for nitrogen fixation.

3. Two methods were used for testing the nitrogen fixing ability of the symbiont.

3a. In the first experiments the ^{15}N method was used. Plants or leaves were placed in an atmosphere of 79.6% argon, 19.9% oxygen and 0.5% carbon dioxide to which 10–15% nitrogen was added, enriched with 30–50% $^{15}\text{N}_2$. The time of exposure was three hours. The enrichment of the material was determined spectrophotometrically after FAUST (1965) with the ^{15}N Analyzer. In some experiments the enrichment was determined in the HCl soluble and insoluble fraction. In all experiments the enrichment was within the accuracy of the ^{15}N Analyzer ($\pm 0.02\%$, table 3).

3b. The acetylene method (HARDY *et al.* 1968) was, therefore, used in a second series of experiments because of its higher sensitivity.¹ Small plants or leaves

¹) The acetylene experiments were performed at the Botanical Laboratory in Leiden. Thanks are due to Dr. A. Akkermans for his assistance.

ASPECTS OF THE LEAF NODULE SYMBIOSIS IN *ARDISIA CRISPA*Table 3. Enrichment in at. %¹⁵N of young and old leaves of *Ardisia crisper* and of a bact.-less *Ardisia* species after a three hour exposure to a gas mixture containing 10% N₂ enriched with 30–50%¹⁵N₂.

<i>Ardisia crisper</i>	Enrichment in at. % ¹⁵ N	
	Young leaf	Old leaf
Midrib	0.01	
Margin	0.02	
Margin, 3% HCl sol.	0.015	0.01
Margin, 3% HCl insol.	0.02	0.015
Bacteria-less <i>Ardisia</i>		
Margin, 3% HCl sol.	0.005	
Margin, 3% HCl insol.	0.01	

were placed in tubes in 79.9% nitrogen, 19.9% oxygen and 0.2% carbon dioxide to which 5% acetylene was added. Time of exposure was three hours. In a second series nitrogen was replaced by argon and different amounts of acetylene were used, 5, 2.5, and 0.5%, respectively. Some of the tubes were placed in the light, others in darkness. In none of the tubes was ethylene production found. It may, therefore, be concluded that the bacteria in the nodule of *Ardisia crisper* do not fix atmospheric nitrogen, neither in the light nor in the dark.

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