# NODULATION OF LEGUMINOUS PLANTS AS AFFECTED BY ROOT SECRETIONS AND RED LIGHT

(MET EEN SAMENVATTING IN HET NEDERLANDS)

Dit proefschrift met stellingen van

LIE TEK AN

landbouwkundig ingenieur, geboren te Padang (Indonesia), 11 december 1932, is goedgekeurd door de promotor, Dr. Ir. E. G. MULDER, hoogleraar in de Microbiologie.

*De Rector Magnificus der Landbouwhogeschool,*
W. F. EYSVOOGEL

*Wageningen*, 6 mei, 1964

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWKUNDE
OP GEZAG VAN DE RECTOR MAGNIFICUS IR. W. F. EIJSVOOGEL
HOOGLERAAR IN DE HYDRAULICA, DE BEVLOEIING,
DE WEG- EN WATERBOUWKUNDE EN DE BOSBOUWARCHITECTUUR,
TE VERDEDIGEN TEGEN DE BEDENKINGEN
VAN EEN COMMISSIE UIT DE SENAAT
VAN DE LANDBOUWHOGESCHOOL TE WAGENINGEN
OP WOENSDAG 10 JUNI 1964 TE 16 UUR

DOOR

T. A. LIE

H. VEENMAN EN ZONEN N.V. – WAGENINGEN – 1964

*Aan mijn Ouders*

# STELLINGEN

I

De vorming van wortelknolletjes bij leguminosen wordt beïnvloed door het rood-infrarood systeem (het phytochroom).

Dit proefschrift.

II

De remmende werking van het infrarood op de vorming van wortelknolletjes is niet het gevolg van een verhoogde strekkingsgroei van de bovengrondse delen van de plant.

Dit proefschrift.

III

De afscheiding van stoffen door de wortel wordt in hoge mate beïnvloed door de leeftijd van de plant en klimaatsfactoren.

ROVIRA, A. D. (1959). Plant & Soil **11**, 53–64.
Dit proefschrift.

IV

De gunstige werking van schaduwbomen voor de cacao en koffie, kan voor een deel worden toegeschreven aan de gunstige klimaatsomstandigheden voor het tot stand komen van de fyllosfeer.

RUINEN, J. (1961). Plant & Soil **15**, 81–109.
CUNNINGHAM, R. K. & P. W. ARNOLD (1962). J. Sci. Food & Agric. **4**, 213–221.

V

Veranderingen in het gedrag van plantecellen, onder invloed van galvormende microörganismen en vernaliserende lage temperaturen, kunnen alleen plaats vinden indien delende cellen beschikbaar zijn.

BRAUN, A. C. (1962). Ann. Rev. Plant Physiol. **13**, 533–558.
WELLENSIEK, S. J. (1962). Nature **195**, 307–308.

VI

Het tegengaan van eiwitafbraak in afgesneden bladeren door beworteling, moet worden toegeschreven aan de produktie van een stof in de wortel met een werking analoog aan die van het kinetine.

MOTHES, K. (1961). Ber. Dtsch. Bot. Gesellsch. **74**, 24–42.
KULAEVA, O.N. (1962). Soviet Plant Physiology (Fiziologiya Rastenii) **9**, 182–189.

Dissertatie T. A. LIE
*Wageningen*, 10 juni 1964

VII

De afwezigheid van virus in het topmeristeem van planten is niet het gevolg van een ontsnapping van de plantecellen aan het virus door een sterke celdeling.

BRANTS, D. H. (1961). Acta Bot. Neerl. **10**, 113–163.

VIII

In proeven met watercultures, waarin het ijzer in de vorm van Fe(III)EDTA wordt gegeven, moet rekening worden gehouden met de werking van het EDTA op het wortelstelsel van de plant.

HEATH, O. V. S. & J. E. CLARK (1960). J. Exp. Bot. **11**, 167.
HEATH, O. V. S. & J. E. CLARK (1964). Nature **201**, 585.

IX

Bij de topsterfte van cultuurgewassen moet rekening worden gehouden met het feit, dat symbiontische schimmels van epiphyten, onder bepaalde omstandigheden, als parasiet kunnen optreden van de planten, waarop de epiphyt groeit.

MULLER, H. R. A. (1936). Arch. Koffiecultuur **10**, 279–349.
RUINEN, J. (1953). Annales Bogoriensis **1**, 101–158.

X

Resistentie tegen aantasting door *Fusarium oxysporum* bij erwten en bananen kan het gevolg zijn van een afscheiding van stoffen door de wortels, die de kieming van de schimmelsporen remmen.

BUXTON, E. W. (1957). Trans. Mycol. Soc. **40**, 145–154.
BUXTON, E. W. (1962). Ann. Appl. Biol. **50**, 269–282.

# VOORWOORD

Gaarne maak ik van deze gelegenheid gebruik om allen te bedanken die betrokken zijn bij het tot stand komen van dit proefschrift.

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## CONTENTS

CHAPTER 1

# INTRODUCTION

## A. SURVEY OF THE LITERATURE

Symbiotic nitrogen fixation in leguminous plants depends on the presence of well-developed root nodules. Formation and functioning of the latter are the results of a highly specialized cooperation between the higher plant and bacterial cells of the genus *Rhizobium*.

### 1.1. FORMATION OF ROOT NODULES

Five different stages in the process leading to symbiosis may be distinguished *viz.* increase of bacterial cells in the rhizosphere, infection of the plant root by the bacterial cells, growth of the nodules, functioning of the nodules and maintenance of the symbiosis. The literature pertaining to the first three stages will be given in some detail.

*Rhizobium* cells as well as other bacteria multiply more readily in the rhizosphere than in the remainder of the root medium. This increase in number of *Rhizobium* cells, which is a prerequisite for the association of bacteria and leguminous plants, is due to the excretion of organic compounds by the plant roots (KATZNELSON *et al.*, 1956; ROVIRA, 1956, 1959; RADEMACHER, 1959). Apart from serving as nutrients for the bacteria, some of the excreted compounds may also have a specific function in the infection process. For instance, indoleacetic acid which is synthesized from excreted tryptophan by *Rhizobium* as well as by other bacteria (KEFFORD *et al.*, 1960) may have some specific bearing on the invasion of the roots through its ability to increase the plasticity of the cell walls (NUTMAN, 1958). The functioning of pectic enzymes in the infection process may be anticipated since these enzymes are detected only in the root secretions of plants inoculated with nodule bacteria able to infect them (FÅHRAEUS & LJUNGGREN, 1959). This is of special interest since neither cellulase nor pectic enzymes are synthesized by *Rhizobium* cells grown in pure culture (FRED *et al.*, 1932).

The pattern of root-hair infections, as studied in clovers (NUTMAN, 1958, 1962) reveals the following features. During the first period after seed germination, the roots are resistant to infection, although root hairs are present (THORNTON, 1929; NUTMAN, 1958). Infection starts at a few well separated points (foci) on the roots (NUTMAN, 1958). These points become the centres of subsequent infections. The course of the infection is similar in all clover species studied and consists of two distinct phases. The first phase ends when, or shortly before, the first nodule becomes visible. Hereafter the infection rate decreases considerably (NUTMAN, 1958, 1962; LIM, 1963). Usually only a small percentage of the root hairs is infected.

The entrance of the *Rhizobium* cells into the root hairs is characterized by

an intense cytoplasmatic streaming and the nucleus of the host cell moves to the site of infection (FÅHRAEUS, 1957; NUTMAN, 1959). The invading bacteria are enclosed in an envelop of cellulose deposited by the host cells, and so a special structure, the infection thread, is formed, which enters the cortical cells.

The normal root cells degenerate when they are penetrated by the infection thread. If, on the other hand a disomatic cell, i.e. a cell with a double chromosome number, is invaded, cell division is stimulated, presumably owing to some hormonal factor excreted by the infection thread. In these cells the bacteria are released from the infection thread. These dividing disomatic cells initiate the formation of root nodules (WIPF, 1939; WIPF & COOPER, 1940).

### 1.2. NUMBER OF ROOT NODULES

The number of nodules formed on the root system of a leguminous plant depends, firstly on the genetic constitution of the host plant (NUTMAN, 1959 b), secondly on the *Rhizobium* strain used (CHEN, 1941) and thirdly on the environmental conditions of growth (FRED *et al.*, 1932; WILSON, 1940; NUTMAN, 1956). With some exceptions (NUTMAN, 1957) it is assumed that nodules capable of fixing nitrogen (effective nodules) are large, red-coloured and located on the upper part of the root system. They originate from the first infections and largely prevent subsequent infections. This has been shown by NUTMAN (1952) by excising a number of the nodules present on the roots. New ones then appear and their number is proportional to the number excised. The inhibition is connected with the meristematic apex, since excision of nodule apices or even partial isolation of these apices by a transverse incision also reduces the inhibition exerted by these nodules.

On the other hand, nodules incapable of fixing nitrogen (ineffective nodules) are small, white-coloured and they are formed continuously on the entire root system. Apparently here no inhibition is exerted by the first formed nodules. This has been confirmed by the fact that no stimulation was observed after removal of a number of the ineffective nodules in contrast with the excision of the effective ones (NUTMAN, 1952). The small size of the ineffective nodules is ascribed to the short period of meristematic activity of the nodule apex (CHEN & THORNTON, 1940; DART & PATE, 1959). Since the apex of the nodule was found to be the centre of inhibitory action, the lack of inhibition by ineffective nodules may well be explained. The association between inhibition and meristematic tissue is supported by the fact that excision of the root tips also increases subsequent nodulation (NUTMAN, 1952).

The inhibitory effect of existing root nodules on subsequent nodule formation may well be due to the production of an inhibitor by the nodule apices (NUTMAN, 1952, 1953, 1957; DART & PATE, 1959). To exert its influence, however, this substance must be transported to the root-hair zone, known to be the locus of new infections. Such a transport can be mediated either by backward diffusion inside the roots, or by secretion into the culture medium,

or along both ways. Backward diffusion of the inhibitor into the root is suggested by the fact that partial excision of the nodule apex, i.e. preventing the transport to and from the meristem, has the same effect as the entire removal of the nodule tip (NUTMAN, 1952). On the other hand, it has been shown by THORNTON (1929), LUDWIG & ALLISON (1935), NUTMAN (1953, 1957) and ELKAN (1961) that secretions influence nodulation of leguminous plants. Certainly a part of this effect can be ascribed to the presence of non-specific substances like sugar (LUDWIG & ALLISON, 1935) or nitrate (GIBSON & NUTMAN, 1960). The presence of substances having a specific influence on nodulation, however, can not be ruled out. In this respect, the presence of indoleacetic acid (KEFFORD *et al.*, 1960) and of substances showing gibberellin-like activity (RADLEY, 1961) in the root medium may be significant.

### 1.3. GROWTH SUBSTANCES IN ROOT NODULES

Root nodules are exceedingly rich in auxins (THIMANN, 1936, 1955). No differences in content occur between the apical and basal parts of the nodules (THIMANN, 1936; PATE, 1958). By using the *Avena* coleoptile straight-growth test, combined with paper chromatography, PATE (1958) has found five distinct growth substances in an ethanol extract of root nodules of *Pisum arvense* L. and *Ulex europaeus* L. Three of these were classed by him as promotors ($A_1$, $A_2$, $A_3$) and two as inhibitors ($I_1$, $I_2$) of coleoptile elongation. A comparison of the content of these growth substances in nodules of different ages (young white, young red and old red nodules) revealed that $A_1$ appeared just after the nodules turned red and remained detectable, although at low concentrations, during further stages of nodule development. $A_2$, identified as indoleacetic acid (I.A.A.), was detected in high amounts at all stages, and diminished only slightly when the nodules were senescent. The third promotor $A_3$, probably identical with indoleacetonitrile (I.A.N.) was present in large amounts only in young red nodules but was absent in young white and old ones. The amounts of inhibitors were difficult to evaluate since in the above-mentioned technique the action of $I_1$ and $I_2$ was partly masked by $A_2$ and $A_3$, respectively. The results of PATE (1958), however, indicate that the inhibitors appeared predominantly in the older nodules. In pea roots, $A_1$ and $A_2$ were only present in low amounts, approximately 40–60 times lower than in nodule tissue, but the inhibitors $I_1$ and $I_2$ occurred in high amounts (PATE, 1958).

RADLEY (1961) found that root nodules from peas and beans contained large amounts of substances acting physiologically like gibberellic acid. The same substance could be detected in the culture solutions of bean plants inoculated with *Rhizobium* but not in that of nodule-free plants. She concluded that the inhibitor, as proposed by NUTMAN (1953, 1957), might well be a gibberellin.

## 1.4. The possible role of growth substances in root-nodule formation

So far, three groups of natural growth substances have been distinguished, the auxins, the gibberellins and the kinins, the action of which is usually interdependent (Kefford, 1963; Thimann, 1963). It appears that the growth process can be precisely regulated by a proper balance of these substances. A good example was given by Wright (1961) in the analysis of the response to indoleacetic acid (I.A.A.), gibberellic acid (G.A.) and kinetin (K) at different stages of growth of wheat coleoptiles. The response to G.A. was the highest during the period of rapid expansion of the cells, 18 hours after sowing. An increase of cell number was observed 30–36 hours after sowing. During this period, coleoptile growth was considerably increased by kinetin. The effect of I.A.A. was absent in the early period and appeared to be optimal 54 hours after sowing, when the effect of G.A. and kinetin was slight.

Kinetin in combination with I.A.A. was found to be indispensable for the continuous growth, i.e. cell division, of tobacco pith cells (Miller *et al.*, 1955; Strong, 1958; Miller, 1961). With regard to root nodules in leguminous plants the cytological study of pea-root callus tissue is of special interest (Torrey, 1961). The addition of kinetin specifically enhanced the division of the tetraploid cells present in pea roots. After 7 days, approximately 80% of the cells in division belonged to the tetraploid class (Torrey, 1961).

It may be recalled that root nodules originate from tetraploid cells present in low numbers in the root (Wipf & Cooper, 1940). Such tetra- and polyploid cells originate from endomitosis, i.e. chromosome doubling without subsequent cell division (review see Geitler, 1953). They have lost the capacity to divide, but by appropriate treatment as wounding (see Geitler, 1953), application of growth substances (Levan, 1939; Torrey, 1961) and infection with *Rhizobium* (Wipf & Cooper, 1940) this capacity can be restored.

Continuous growth of tobacco-pith tissue was found after transformation of the cells by *Agrobacterium tumefaciens*. Such tissue was found to synthesize kinetin-like substances (Braun, 1956; Wood & Braun, 1961) and auxins (Braun, 1956). Moreover a strain of tobacco tissue was isolated which was able to synthesize a kinetin-like substance (Fox, 1962, 1963). This tissue contained cells belonging predominantly to the tetraploid class, whereas the pith tissue, from which they were derived, contained cells with chromosome numbers varying from 40 (diploid) up to higher than 221 (Fox, 1963).

In root nodules high amounts of I.A.A. and substances with G.A. activity were detected, but so far no substances similar to kinetin were found. The similar action of kinetin and *Rhizobium* on the activation of tetraploid cells suggests that the same mechanism may be involved, presumably by the production of a kinetin-like substance during the nodulation process. The production of pseudo-nodules by kinetin on roots of tobacco (Arora, 1959) and tomato (Wittwer & Dedolph, 1963) also points into the same direction.

## B. SCOPE OF THE INVESTIGATIONS

The aim of the present investigations was a) to study the specific effect of root secretions on nodule formation in leguminous plants, b) to characterize the substance responsible for the effects observed and c) to study its relationship with kinetin.

CHAPTER 2

# MATERIAL AND GENERAL METHODS

## A. PLANT MATERIAL

### 2.1. INTACT PLANTS

Nodule formation was studied with pea plants, *Pisum sativum* L. variety Rondo and three bean varieties, *Phaseolus vulgaris* L. The bean varieties Amerikaanse dubbele zonder draad (Amerikaanse) and Beka were used in the early experiments, but later on the variety Walcherse Witte was used exclusively. The number and size of the nodules when inoculated with the same *Rhizobium* strain were different in the three bean varieties. When *Rhizobium* strain Bokum was used, the highest numbers of nodules were found with Beka, the lowest with Amerikaanse and intermediate with Walcherse Witte (table 1). Large nodules were formed on Amerikaanse and Walcherse Witte whereas small nodules were obtained on Beka.

TABLE 1. Numbers[1] of nodules formed in *Phaseolus vulgaris* L. varieties Amerikaanse, Beka and Walcherse Witte, inoculated with *Rhizobium* strain Bokum

| Bean variety | Number of nodules per plant | | | | |
|---|---|---|---|---|---|
| | Exp. 1 | Exp. 2 | Exp. 3 | Exp. 4 | Exp. 5 |
| Amerikaanse | 31.6 | 34.6 | 26.3 | 39.5 | – |
| Beka | – | – | 112.0 | – | 129.0 |
| Walcherse Witte | 49.0 | 51.0 | – | 53.0 | – |

[1] Mean values per plant of 6 replicates.

Selected seeds were surface-sterilized by shaking in 3% $H_2O_2$ with a drop of detergent (Teepol) during twenty minutes. The seeds were transferred, without washing, to Petri dishes containing 2% agar in tap water, and left to germinate at 25°C for about 5 days. When the roots had attained a length of ca. 5 cm, the seedlings were transferred to jars containing a nutrient solution of the following composition:

$K_2HPO_4$, 0.36 g; $KH_2PO_4$, 0.12 g; $MgSO_4$. 7 $H_2O$, 0.25 g; $CaSO_4$, 0.25 g; Fe+++-citrate, 30 mg; $MnSO_4$. $4H_2O$, 1 mg; $ZnSO_4$. 7 $H_2O$, 0.25 mg; $CuSO_4$. 5 $H_2O$, 0.25 mg; $H_3BO_3$, 0.25 mg; $Na_2MoO_4$. 2 $H_2O$, 0.05 mg; 1000 ml tap water.

a). *Sterile cultures.* The seedlings were wrapped in sterile cotton wool and placed with their roots in Erlenmeyer flasks containing 300 ml nutrient solution sterilized at 105°C for 20 minutes. The nutrient solution was not changed during the experiment and sterile solution was added under aseptic conditions if necessary.

b). *Semi-sterile cultures.* The aim of this method was to prevent the plants from becoming infected with *Rhizobium* until the start of the experiment. The nutrient solution was only boiled and poured into the jars while still hot.

The pea seedlings were wrapped in sterile cotton wool and the root system inserted in tubes containing 180 ml of nutrient solution. Two weeks after germination, at the start of the experiment, the plants were transferred to 360 ml preservation jars containing 300 ml of nutrient solution.

The bean seedlings were immediately planted in preservation jars of 360 ml and in some cases of 1.5 l capacity. When the experiment was started the nutrient solution was renewed and the solution was changed again 7–10 days afterwards when the first nodules were visible.

In some experiments the nutrient solution was aerated. For practical reasons, and to avoid infections in the sterile plant cultures, this procedure was usually omitted. In the latter case the jars were two thirds-filled with nutrient solution, leaving the upper part of the root system in moist air. Plant growth and nodulation were satisfactory under these conditions.

### 2.2. Plant systems with limited shoot growth

In some experiments where differences in shoot growth had to be avoided, bean plants var. Walcherse Witte, were treated in the following ways:

#### 2.2.1. *Decapitated plants*

The emerging shoots of plants grown for two weeks in nutrient solution, and all axillary buds appearing during the experimental period were removed, so that only primary leaves were left on the stem.

#### 2.2.2. *Stem cuttings*

Stem cuttings were obtained from two weeks old plants by severing them just above the cotyledons. The cuttings were placed in jars containing a nutrient solution of $^1/_{10}$ normal strength. To reduce evaporation, the cuttings were covered with transparent polythene, approximately 1 cm above the leaves. Light was supplied by white fluorescent lamps (Philips, TL 33) at a light intensity of 20 000 ergs/cm$^2$ sec. at plant level. The temperature was maintained at 22–25°C. The roots appeared 5–7 days after cutting and after another 7–10 days, the cuttings could be used. The experiments were carried out with only the two primary leaves on the stem. The same nutrient solution as for intact plants was used.

#### 2.2.3. *Leaf cuttings*

Primary leaves of two weeks old bean plants were cut near the stem and placed with the petioles in jars containing a nutrient solution of $^1/_{10}$ strength during the first week and afterwards of $^1/_4$ strength. The same light and temperature conditions as for stem cuttings were used. Roots appeared within a

week and the rooted leaves were used for the experiments after a further week. No buds appeared on the leaf cuttings.

Root formation in stem and leaf cuttings were easily obtained with bean plants grown in the greenhouse during the spring, autumn and winter. But in the hot summer months callus tissue instead of roots appeared at the base of the stems and the petioles. The use of auxins to promote root formation was avoided since these compounds may interfere with root-nodule formation. Leaf cuttings were rather difficult to handle due to rapid senescence of the leaves, especially at high light intensities.

### B. *RHIZOBIUM* STRAINS

The strains used in the present investigations are recorded in table 2. The bacteria were maintained on agar slopes of the following composition: Difco yeast extract, 1 g; mannitol, 10 g; $K_2HPO_4$, 0.5 g; $MgSO_4$. $7H_2O$, 0.25 g; NaCl, 0.1 g; $CaCO_3$, 3 g; Davies agar, 10 g; water, 1000 ml. For inoculation, 1–2 drops (ca. $^1/_{20}$ ml) of a 7-days old culture, suspended in 50 ml of sterile water, was applied to each plant.

TABLE 2. Some symbiotic characteristics of *Rhizobium* strains used

| Bacterial strain | Host plant | Effectivity | Size and colour of the nodules | Nodule number |
|---|---|---|---|---|
| PRE | pea | effective | large, red | low |
| $PF_2$ | pea | effective | large, red | low |
| H VIII | pea | ineffective | small, white | high |
| P8 | pea | ineffective | small, white | high |
| Bokum | bean | effective | large, red | low |
| $WH_3$ | bean | ineffective | large, pale red | low |
| S460 | bean | ineffective | small, white | high |

### C. CULTURAL CONDITIONS OF THE PLANT MATERIAL

The plants were grown either in the greenhouse or in light cabinets with artificial light.

### 2.3. GREENHOUSE

The temperature varied during the year, and in the summer the temperature sometimes exceeded 30°C for some hours in the afternoon. In late autumn, winter and early spring the greenhouse was heated and a fairly constant temperature of 23–25°C was maintained during the day. During the night the temperature dropped 7–10°C below the day temperature.

During late spring and summer only natural light was available for the plants. On warm sunny days the greenhouse had to be shaded to prevent excessive heating and the floor of the greenhouse was sprayed with water, two or three times a day, to increase humidity. The amount of light entering the greenhouse was about 50–75% of that outside.

From October until April supplementary illumination was provided with super-high-pressure mercury-vapour lamps, two lamps per m² mounted at 1 m above plant level, to make a photoperiod of ca. 18 hours. In 1959 the HO 450 (Philips) type of lamps afterwards, another type, HPL 400 (Philips), was used.

### 2.4. Light cabinets

The plants were precultivated for approximately 10 days in white fluorescent light (TL 33, Philips) at a light intensity of 30 000 ergs/cm² sec. and a temperature of 22–25°C during the day. Hereafter they were transferred to the cabinets with white light or to cabinets with light of defined spectral regions.

#### 2.4.1. *White-light cabinet*

During the day the temperature was controlled and kept at 26–28°C. The temperature during the night was about 18–20°C. Light was applied from above, using white fluorescent lamps (TL 33, Philips). The light intensity measured at plant level was about 30 000 ergs/cm² sec. (cosine corrected photocell, Hartig & Helwig, 1955).

#### 2.4.2. *Cabinets with light of defined spectral composition (coloured-light cabinets)*

These cabinets are the high-energy cabinets used previously by Stolwijk (1954) and de Lint (1960) at the Laboratory of Plant Physiological Research. Light of defined spectral composition was obtained from 'monophosphor' fluorescent lamps in combination with filters of specific transmission. The spectral composition of the light in the different cabinets, as derived from de Lint (1960) is presented in fig. 1. The light intensities measured at plant

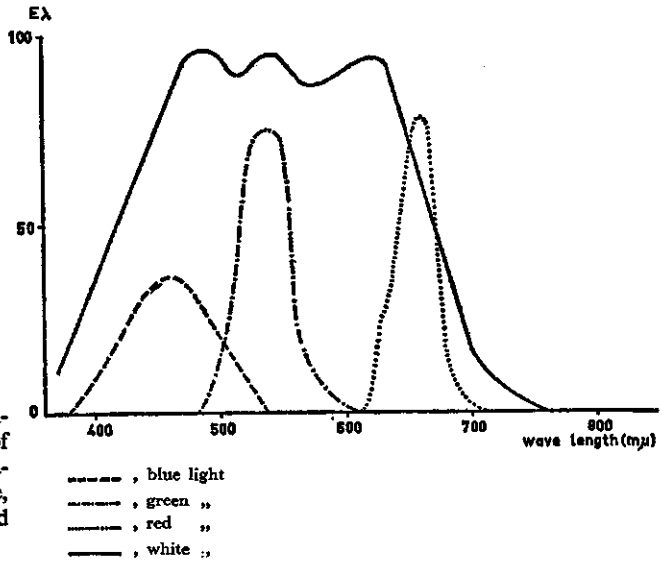



Fig. 1. Energy-distribution spectra of incident irradiation of blue, green, red and white light.

level, using a cosine corrected photocell (HARTIG & HELWIG, 1955) were chosen in such a way that cabinets 1 (blue), 2 (green) and 3 (red) had light of approximately the same number of quanta. Besides these cabinets, three other cabinets 4 (white), 5 (red) and 6 (blue) were available (table 3), of which cabinets 4 (white) and 5 (red) had approximately the same energy level as 1 (blue) while 6 (blue) had a lower energy level than 1 (blue).

TABLE 3. Coloured light cabinets

| No. of cabinet | Light quality | Maximum transmission (mμ) | Light intensity (ergs/cm² sec.) |
|---|---|---|---|
| 1[1] [2] | Blue | 450 | 17000 |
| 2[1] | Green | 540 | 15000 |
| 3[1] | Red | 650 | 12000 |
| 4[2] | White | – | 16000 |
| 5[2] | Red | 650 | 16000 |
| 6 | Blue | 450 | 13000 |

[1]) Approximately equal numbers of quanta.
[2]) Approximately equal energy level.

These cabinets were placed in a room the temperature of which was controlled by central heating and by a fan supplying cool air from outdoors. Average temperature was 25°C.

### 2.4.3. *Equipment for far-red irradiation*

Far-red light was obtained by filtering incandescent light (Comptalux 150 W, Philips) through a layer of 10 cm water and a blue and a red filter. Approximately 75% of the filtered light had a wave length between 7 and 800 mμ, whereas 25% was above 800 mμ. Far red was supplied either in special boxes (experiments in white-light cabinet) or in the coloured-light cabinets in which the plants were growing. In the first case the plants had to be transported over a short distance in darkness or in subdued light, which took only a few seconds.

## D. EXPERIMENTAL PROCEDURES

The experiments were started two weeks after germination of the seeds or, when cuttings were used, two weeks after excising, when the roots were susceptible to infection with *Rhizobium*. The nutrient solution was replaced by a fresh one and the plants were inoculated with *Rhizobium*. Simultaneously, growth substances were applied and exposure to light of defined spectral regions started. The nutrient solution was changed again after 7–10 days, when the first nodules appeared, and no growth substances were added to the fresh solution. In all experiments age of the plants is defined as number of days after inoculation.

## E. ANALYSIS

The first countings of the nodules were made 8–10 days after inoculation when the nodules were about 1 mm in diameter and the final ones at harvest, i.e. 16–20 days after inoculation. For fresh-weight determination the sizable nodules were detached with forceps and the adherent water removed by blotting with filter paper. For practical reasons the small nodules were not included in the weight determination.

At harvest the shoots and roots were blotted with filter paper and weighed separately. For dry-weight determination the plant material was dried for at least one day at 50°C followed by 30 minutes at 105°C.

Total nitrogen was determined according to the Kjeldahl method. The dried material was milled and samples of at least 100 mg digested with concentrated $H_2SO_4$. Some drops of $H_2O_2$ (30%) were added until the solution was clear and the mixture was heated for another 10–15 minutes. The ammonia was distilled into boric acid (2% solution, containing bromocresol green-methyl red indicator) and titrated with 0.1 N HCL.

CHAPTER 3

# INFLUENCE OF ROOT SECRETIONS ON ROOT-NODULE FORMATION OF LEGUMINOUS PLANTS

A considerable amount of evidence has been provided concerning the importance of root secretions for the growth of plants and soil microorganisms (RADEMACHER, 1959; VISSER, 1961). An extensive account of the organic compounds secreted by plant roots is given by VISSER (1961). Apart from sugars, amino acids and organic acids, usually present in the root exudates, substances also of higher molecular weight like enzymes, vitamins and toxins have been detected (see VISSER, 1961). Environmental conditions *viz.* light, temperature and soil moisture have been found to influence the root secretions quantitatively (ROVIRA, 1959; KATZNELSON *et al.*, 1955).

The nutritive value of root exudates is demonstrated by the rhizosphere effect, i.e. the pronounced increase of numbers of microorganisms in the immediate surroundings of the roots as compared with those of the soil. The composition of the exudates exerts a preferential stimulation on certain groups of microorganisms. Those requiring amino acids and vitamins are more abundant in the rhizosphere than others which synthesize these compounds.

Many examples are known of more specific relationships between plants and other organisms. Susceptible plants attract and stimulate the growth of their parasites e.g. the hatching of parasitic nematodes (WIDDOWSON, 1958) and the enhanced growth of *Fusarium* through root secretions of susceptible plants (BUXTON, 1957). Resistant plants either lack this character or even inhibit the growth of the parasites (BUXTON, 1957). Germination of phanerogamic root parasites (e.g. *Striga, Orobanche*) only takes place in the presence of root exudates from susceptible plants (BROWN *et al.*, 1949; WILLIAMS, 1961). It is noteworthy that the exudates can be replaced by either a pentose (BROWN *et al.*, 1949) or kinetin (WILLIAMS, 1961). Plant associations are often balanced by the mutual influence of root secretions. DELEUIL (1951a, 1951b) has found that the phytotoxic substances, secreted by the plant roots of the *Rosemarine-Erica* association, prevent other plants from surviving with the exception of leguminous plants and hemiparasites. This apparently is due to the presence of specialized organs on the root of these plants *viz.* root nodules in legumes and root suckers in the hemiparasites, which neutralize the above-mentioned toxins.

As early as 1929, THORNTON reported that root secretions may influence nodule formation. He observed that lucerne seedlings nodulate earlier when growing in the neighbourhood of older lucerne plants. The same stimulating effect was obtained using an extract of soil in which lucerne plants had grown previously. The extensive experiments carried out by LUDWIG & ALLISON (1935) suggested that under summer conditions nodulation of soybean plants was stimulated by older soybean or corn plants, grown previously in the sand

medium (preplanting). Since small amounts of sugar or nitrogen also stimulated nodulation, these authors believed that similar substances, present in the root exudates, were responsible for the observed effects. No such stimulation, however, was observed in the experiments carried out in October-November. Nodulation was even inhibited in the presence of older plants, probably due to light deficiency.

Working under bacteriologically-controlled conditions, NUTMAN (1953, 1957) verified the previous observations that preplanting with leguminous or even with non-leguminous plants shortened the time of appearance of the first nodule. The ultimate number of nodules formed, however, was reduced. Unfortunately it turned out that the tap water used contained small amounts of nitrate, a powerful inhibitor of nodulation. Although some stimulation was occasionally observed when highly purified medium was used, it was concluded that the greater part of the 'preplanting effect' was due to the removal of traces of nitrogen (GIBSON & NUTMAN, 1960).

In recent years it was found that a non-nodulating line of soybean exuded substances able to suppress nodulation in a normally nodulating soybean or in other leguminous plants (ELKAN, 1961). A bacterial count of *Rhizobium* in the rhizosphere of these plants eliminated the possibility that the exudates of the non-nodulating plant contained antibiotics against *Rhizobium* (ELKAN, 1962).

### 3.1. PRESENCE OF NODULATION-STIMULATING SUBSTANCES IN USED CULTURE SOLUTIONS OF NODULATED PEA AND BEAN PLANTS

Under hot summer conditions, nodule formation of bean plants, (*Phaseolus vulgaris* L.), grown in water culture was found to be poor and irregular. The growth of the plants was satisfactory, however, when combined nitrogen had been supplied. No improvement of nodulation was achieved by raising the inoculation density, by adding a suspension of killed *Rhizobium* cells, yeast or soil extracts, or by aeration of the nutrient solution. A significant increase in nodulation was observed, however, when beans were grown in a nutrient solution precultivated with nodulated legumes. Typical examples are shown in Plate I.

The influence of the concentration of used culture solution was tested with two bean varieties *viz.* Amerikaanse and Beka. Different amounts of a nutrient solution in which effectively nodulated pea or bean plants (donor plants) had grown before were made up to 1 liter by using fresh medium. The range covered 0, 100, 250, 500, 800 and 1000 ml of the preplanted medium. The supply of inorganic salts was brought up to the fresh-medium level in all vessels. Three aseptically grown bean seedlings (5 days old) were transplanted into each of a number of jars of 1.5 liter capacity containing the nutrient solution, and subsequently inoculated with *Rhizobium* strain Bokum. The nutrient solution was aerated to ensure the necessary oxygen supply. The nodules were counted twice, firstly 12 days after inoculation and subsequently

at the time of harvest, 12 days later. The fresh weight of the nodules was also determined (table 4). The beneficial effect of the used culture solution on the nodulation of the plants is clear. Both the number and the weight of the nodules increased with increasing amounts of used medium, either from nodulated peas, or from beans. Nodulation of the bean variety Amerikaanse, however, was inhibited by undiluted used medium of bean plants indicating a supra-optimal concentration of the stimulating agent. No such an inhibitory effect was detected when undiluted medium from pea plants was used. The same stimulating effect of used solution on nodulation was observed in early and late countings. Therefore the increased nodule number can not have been due to an inhibition of nodulation in the early periods. This would have resulted in higher numbers of root nodules at harvest as reported for delayed inoculation (NUTMAN, 1949; PATE & DART, 1958).

TABLE 4. The effect of different amounts of used culture solution of pea and bean plants, bearing effective nodules, on nodulation[1]) of bean plants varieties Amerikaanse and Beka, inoculated with *Rhizobium* strain Bokum. Greenhouse experiment; time of inoculation 22th September 1959.

| Donor plant | Used medium (ml/liter) | Test plant | | | |
|---|---|---|---|---|---|
| | | Bean variety | Number of nodules at | | Fresh wt. of nodules (g) |
| | | | 12 days | 17 days | |
| Bean | 0 | Amerikaanse | 2.3 | 4.0 | 0.03 |
| | 100 | | 9.5 | 38.0 | 0.14 |
| | 250 | | 17.9 | 40.3 | 0.21 |
| | 500 | | 11.6 | 42.0 | 0.21 |
| | 1000 | | 8.7 | 15.0 | 0.14 |
| | 0 | Beka | 1.9 | 16.0 | 0.03 |
| | 800 | | 66.0 | 257.0 | 0.63 |
| Pea | 0 | Beka | 4.2 | 22.0 | 0.03 |
| | 250 | | 6.2 | 54.0 | 0.10 |
| | 500 | | 8.5 | 57.0 | 0.12 |
| | 1000 | | 16.7 | 117.0 | 0.36 |

[1]) Mean values per plant of 6–9 replicates.

### 3.2. PRODUCTION CENTRES OF THE ACTIVE SUBSTANCE (A.S.)

Experiments were carried out to locate the production centre of the active substance (A.S.). Nodules and roots of pea plants, inoculated with the effective *Rhizobium* strain PRE, were collected separately and an aqueous extract was made from both parts by mashing in a mortar. Water was added and after filtration the solutions were assayed with bean plants, inoculated with *Rhizobium* strain Bokum. Two concentrations of each extract were compared, equivalent to 50 and 250 mg fresh weight of tissue per test plant. Since at that time of the year, sun light was becoming limiting for plant growth in the greenhouse, supplementary light was given. The experiment was started on 12th October and the plants were harvested on 9th November 1959. The mean

number and the fresh weight of the nodules per test plant were determined and the results compared with those of control plants not supplemented with the extracts (table 5).

TABLE 5. The effect of aqueous extracts of roots and of effective nodules from pea plants on nodulation[1]) of bean plants variety Beka, inoculated with *Rhizobium* strain Bokum. Greenhouse experiment; time of inoculation 23th Oct. 1959

| Pea plant extract applied per test plant equivalent to | Test plant | |
|---|---|---|
| | Number of nodules | Fresh wt. of nodules |
| None | 129 | 0.18 |
| 50 mg (root tissue) | 103 | 0.23 |
| 250 mg (root tissue) | 66 | 0.16 |
| 50 mg (nodule tissue) | 116 | 0.19 |
| 250 mg (nodule tissue) | 162 | 0.35 |

[1]) Mean values per plant of 9 replicates.

It may be seen that the number of nodules of the control plants was rather high apparently due to the late season (see also fig. 10). The lowest concentration of the nodule extract had no influence on nodulation but when the extract derived from 250 mg of nodules was added, both the number and the weight of nodules increased. Small amounts of root extract did not effect the nodulation and at higher concentration even inhibition occurred.

This experiment clearly demonstrates that root nodules are the main producers of A.S. Furthermore it shows that the beneficial effect of preplanting on nodulation was not due to the removal of inhibitory substances from the nutrient solution.

### 3.3. INFLUENCE OF A.S. ON BEAN PLANTS INOCULATED WITH DIFFERENT *RHIZOBIUM* STRAINS

In previous experiments two bean varieties were used successfully for the assay of A.S., but only one *Rhizobium* strain, Bokum, was tried. It seemed worth while to include other bacterial strains in the experiments. Therefore, two ineffective strains were combined with bean plants var. Walcherse Witte, a variety which responded similarly to A.S. like the bean varieties used in the preceding experiments. *Rhizobium* strain $WH_2$ produces large but ineffective nodules, whereas strain S460 forms large numbers of small ineffective nodules. An uninoculated series was included in the experiment.

The test plants were grown singly in 500 ml flasks with the roots under sterile conditions in the white-light cabinet. Half of the plants received a certain amount of an ether-soluble fraction (see chapter 5) of effective bean nodules equivalent to 200 mg of fresh tissue, which was added aseptically after sterilization through a Seitz filter. At harvest, 24 days after inoculation, the number and the fresh weight of the nodules were determined (table 6,

TABLE 6. The effect of extracts from effective bean nodules on root growth[1]) and nodulation[1]) of bean plants, variety Walcherse Witte, uninoculated or inoculated with the ineffective *Rhizobium* strains $WH_2$ or S460. White-light cabinet; photoperiod 16 hours, 30000 ergs/cm² sec.

| Mg of fresh nodules used for extracting A.S., applied per plant | *Rhizobium* strain used for inoculation | Test plant | | |
|---|---|---|---|---|
| | | Nodules | | Roots |
| | | Number | Fresh wt. (g) | Fresh wt. (g) |
| 0 | Uninoculated | – | – | 5.25 |
| 200 | | – | – | 5.69 |
| 0 | $WH_2$ | 75 | 0.38 | 3.69 |
| 200 | | 130 | 1.05 | 4.43 |
| 0 | S460 | 113 | – | 3.80[2]) |
| 200 | | 180 | – | 5.80[2]) |

[1]) Mean values per plant of 5 replicates, [2]) roots + nodules

Plate II). The fresh weights of the roots are also recorded, but those of the shoots were omitted since the latter are erratic due to abscission of the primary leaves.

The results of this experiment show that A.S. also stimulated nodulation with ineffective *Rhizobium* strains. Both number of nodules and nodule weight of the test plants inoculated with $WH_2$ increased notably after A.S. had been added. Nodule numbers obtained with strain S460 also clearly increased upon addition of A.S. Fresh weight was not determined since the nodules formed by this strain were too small to separate them from the roots. No differences in size between A.S.-treated and control nodules were observed. Root growth was slightly stimulated by the nodule extract in the absence of *Rhizobium* but considerably more in its presence.

### 3.4. INFLUENCE OF THE *RHIZOBIUM* STRAIN ON THE FORMATION OF A.S. IN THE DONOR PLANTS

The production of A.S. by effective and ineffective strains was studied by comparing nodule extracts and exudates from nodulated roots. From the experiments with nodule extracts differences in internal concentration of A.S. may be detected, whereas with the second approach an impression may be gained of the release of A.S. The relatively large nodules of bean plants were easy to handle and they therefore were used for the preparation of the extract. The root exudates of pea plants were employed for the experiments with exudates, because of the uniformity of these donor plants and the ease of cultivating them under sterile conditions.

#### 3.4.1. *Nodule extracts*

For the preparation of the extracts, nodules were obtained from bean plants grown under bacteriologically controlled conditions. The plants inoculated with the effective *Rhizobium* strain Bokum were 30 days old when the nodules

were harvested, those inoculated with $WH_2$ and S460 were 40 days old. An ether extract was prepared as described in chapter 5 and the extracts were assayed with bean plants, inoculated with strain Bokum. Four concentrations, equivalent to 50, 100, 200 and 400 mg of fresh weight of nodules per test plant were used. A control which was run simultaneously received no extract. The plants were harvested 35 days after inoculation and the average number and fresh weight of nodules determined (table 7). A.S. proved to be present in both type of nodules. The effective root nodules contained more A.S. than the ineffective ones when compared on a fresh-weight base. With the former the optimum effect was reached with 50 mg of tissue whereas with ineffective nodules the extract of 100 mg was required to obtain a maximal effect. With increasing concentration of the extract, nodulation was reduced and no differences existed between the extracts of effective and ineffective nodules.

TABLE 7. The effect of extracts from effective or ineffective bean nodules on nodulation[1]) of bean plants inoculated with *Rhizobium* strain Bokum. White-light cabinet; photoperiod 16 hours, 30000 ergs/cm² sec.

| Nodules obtained with *Rhizobium* strain | Mg. of fresh nodules used for extracting A.S., applied per plant | Test plants | |
|---|---|---|---|
| | | Nodules | |
| | | Number | Fresh wt. (g) |
| Control | 0 | 4 | 0.05 |
| Bokum (eff.) | 50 | 38 | 0.68 |
| | 100 | 20 | 0.24 |
| | 200 | 16 | 0.39 |
| | 400 | 26 | 0.44 |
| $WH_2$ (ineff.) | 50 | 9 | 0.08 |
| | 100 | 21 | 0.25 |
| | 200 | 11 | 0.13 |
| | 400 | 10 | 0.17 |
| S460 (ineff.) | 50 | 8 | 0.03 |
| | 100 | 41 | 0.45 |
| | 200 | 28 | 0.54 |
| | 400 | 11 | 0.14 |

[1]) Mean values per plant of 4 replicates.

### 3.4.2. *Root exudates*

Root exudates were obtained from pea plants grown in 300 ml Erlenmeyer flasks in the greenhouse. Two weeks after inoculation, these plants were removed and the nutrient solution assayed for A.S. At this time about half of the water had been used up by the pea plants. Before the assay started, the culture solution was made up to 300 ml using fresh nutrient medium. Experiments were carried out under conditions unfavourable and favourable for nodulation of the control test plants.

Experiments 1 and 2 were run in the autumn of 1959, during a hot sunny period when nodulation of the control bean plants was poor. Root exudates,

obtained, either from uninoculated pea plants, or from plants inoculated with PRE or a mixture of P8 and H VIII, were assayed with bean plants, varieties Beka and Amerikaanse, inoculated with *Rhizobium* strain Bokum. These experiments were carried out with an interval of two weeks. In both experiments the same root exudates from pea plants were investigated. In experiment 1 the used solutions were assayed immediately after removal of the donor plants. In experiment 2, however, root exudates were stored at 4°C before being used.

Similar experiments (3 and 4) were carried out in the spring of 1960, when nodulation of the control test plants was satisfactory. Root exudates, obtained from uninoculated pea plants or from plants inoculated with one of the following strains: PRE, $PF_2$, P8 and H VIII, were assayed with bean variety Walcherse Witte, inoculated with *Rhizobium* strain Bokum.

The results of the experiments carried out under conditions unfavourable (table 8) and favourable (table 9) for nodulation seem conflicting. A certain trend, however, appears to be present in both series of experiments. Only slight effects or none at all could be detected in media preplanted with unin-

TABLE 8. The effect of root exudates of pea plants, inoculated with different *Rhizobium* strains, on nodule numbers[1]) of bean plants, inoculated with *Rhizobium* strain Bokum. Greenhouse experiment.

| Bean variety used for the assay | Control (without added exudates) | Root exudates of pea plants | | |
|---|---|---|---|---|
| | | Uninoculated | Inoculated with | |
| | | | H VIII + P8 (ineff.) | PRE (eff.) |
| *Exp. 1, inoculated at 16th October 1959* | | | | |
| Amerikaanse | 2.8 | 2.8 | 27.0 | 21.5 |
| Beka | 31.8 | 27.6 | 111.0 | 97.0 |
| *Exp. 2, inoculated at 12th November 1959* | | | | |
| Beka | 30.6 | – | 47.3 | 94.0 |

[1]) Mean values per plant of 5 replicates.

TABLE 9. The effect of root exudates of pea plants, inoculated with different *Rhizobium* strains, on nodule numbers[1]) of bean plants, inoculated with *Rhizobium* strain Bokum. Greenhouse experiment.

| Bean variety used for the assay | Control (without added exudates) | Root exudates of pea plants | | | | |
|---|---|---|---|---|---|---|
| | | Uninoculated | Inoculated with | | | |
| | | | P8 (ineff.) | H VIII (ineff.) | PRE (eff.) | $PF_2$ (eff.) |
| *Exp. 3, inoculated at 10 Februari 1960* | | | | | | |
| Walcherse Witte | 121.3 | – | 114.0 | 111.5 | 49.0 | 58.8 |
| *Exp. 4, inoculated at 18 Februari 1960* | | | | | | |
| Walcherse Witte | 107.3 | 128.2 | 43.3 | 47.0 | 17.0 | 24.4 |

[1]) Mean values per plant of 5 replicates.

oculated plants. Obviously the association with *Rhizobium* is needed for the production of A.S. When nodulation of the control test plants was poor (table 8), preplanting with nodulated pea plants stimulated nodulation. In experiment 1 slight differences existed between the exudates of effective and ineffective nodules, but in experiment 2 those from ineffective nodules apparently were less active than those from effective ones. When nodulation of the control plants was satisfactory (table 9), root exudates from nodulated plants reduced nodulation. The inhibitory effect was more pronounced with exudates from effective than with those from ineffective nodules, and in experiment 4 more pronounced than in experiment 3.

The conflicting results may have been due to a difference in internal concentration of A.S. in the assay plants grown under different climatical conditions. During a hot sunny spell the internal concentration of A.S. apparently is low and stimulation is obtained after supplying additional amounts of A.S. In contrast, in spring the internal concentration of A.S. may be high and consequently nodulation will be satisfactory. A further supply of A.S. will result in a supra-optimal concentration of this compound in the plant. The latter concept would be in accordance with previous experiments in which high concentrations of nodule extract or exudates reduced nodulation.

### 3.5. Influence of the age of the donor plants on the exudation of A.S.

In order to gain some information on the exudation of A.S. in relation to the age of the plants, the following experiments were designed. Pea plants, used as donor plants, were inoculated at an age of two weeks. Nodules appeared after 5–7 days and in the case of effective nodules, red pigmentation occurred after another week.

In the first experiment half of the pea plants had been inoculated with the effective *Rhizobium* strain PRE, whereas the other half had been left uninoculated. The precultivated nutrient solutions of the donor plants of both groups were assayed for A.S. at 6, 12, 19 and 26 days, respectively, after inoculation. The used nutrient solution of one donor plant, after being made up to 300 ml with fresh nutrient medium, was employed for one test plant. A control series of bean plants, grown in fresh nutrient solution only, was run simultaneously. The results expressed as the average number and the fresh weight of nodules per test plant are shown in table 10.

In a subsequent experiment the ineffective *Rhizobium* strain P8 was used for inoculation of the donor plants. In this case the root exudates were collected at 7, 14 and 25 days, after inoculation respectively. Nodule numbers of the test plant are given in table 11.

The salient features of these two experiments are:

When uninoculated plants had been used as donor plants, no stimulation and in some instances even inhibition was observed in the bean assay. With increasing age of the donor plants the inhibitory effect of the root exudates increased (cf. also tables 12 and 13).

TABLE 10. The effect of the age of the donor plants (peas, uninoculated and inoculated with the effective *Rhizobium* strain PRE) on the exudation of A.S. as measured by number[1]) and fresh weight[1]) of nodules per plant in the bean assay. Greenhouse experiment; time of inoculation 18th March 1960.

| Age of donor plants (days after inoculation) | Nodulation of test plants | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Donor uninoculated | | Donor inoculated | | Inoculated / Uninoculated × 100 | | Control | |
| | Number | Fresh wt. (g) | Number | Fresh wt. (g) | Number | Fresh wt. | Number | Fresh wt. (g) |
| | | | | | | | 107 | 0.12 |
| 6 | 81 | 0.13 | 66 | 0.11 | 82 | 85 | | |
| 12 | 58 | 0.11 | 120 | 0.20 | 207 | 182 | | |
| 19 | 59 | 0.10 | 34 | 0.04 | 58 | 40 | | |
| 26 | 84 | 0.12 | 0 | 0.0 | 0 | 0 | | |

[1]) Mean values per plant of 4 replicates.

TABLE 11. The effect of the age of the donor plants (peas, uninoculated and inoculated with the ineffective *Rhizobium* strain P8) on the exudation of A.S. as measured by number[1]) of nodules per plant in the bean assay. Greenhouse experiment; time of inoculation 8th March 1960

| Age of donor plants (days after inoculation) | Number of nodules of test plants | | | |
|---|---|---|---|---|
| | Donor uninoculated | Donor inoculated | Inoculated / Uninoculated × 100 | Control |
| | | | | 106 |
| 7 | 119 | 138 | 116 | |
| 14 | 104 | 140 | 135 | |
| 25 | 84 | 128 | 152 | |

[1]) Mean values per plant of 4 replicates.

Root exudates from pea plants bearing effective nodules gave a maximal stimulation when the peas had grown for twelve days after inoculation. When the donor plants had grown for a longer period of time, nodulation of the test plants was reduced, and at 26 days it was entirely inhibited. Pea plants with ineffective nodules exuded small amounts of A.S. during the entire preplanting period.

It is tempting to relate the exudation process to the nodulation pattern of the *Rhizobium* strains used. Effective nodules are only formed in the early period and this may be reflected in the exudation of stimulating substances being restricted to the first two weeks. The constant exudation of small amounts of stimulating substances in the ineffective symbiosis may bear a relationship with the prolonged formation of new, but small and ineffective nodules.

Two explanations may be given for the observed inhibition by exudates from old plants with effective nodules. Either the same active substance is exuded throughout the preplanting period, or different substances are exuded in the course of ageing, a stimulating one in the first period and an inhibitive one afterwards. The first explanation requires the assumption that the sti-

mulating substance, when exceeding the optimal concentration inhibits nodulation.

The technique employed so far to obtain root exudates has been to grow the donor plants until a certain age in the same nutrient solution. This method gives information about the accumulated exudates, but not about exudation in successive periods of growth. Therefore in the following experiment the nutrient solution of part of the plants was renewed weekly.

Pea plants inoculated with strain PRE, or left uninoculated, respectively, were allowed to grow for 27 days after inoculation in a culture solution which was renewed every 7 days. The exudates of the successive intervals were compared with the exudates of plants grown for 7, 14, 21 and 27 days, respectively, in the same medium. A series of test plants grown in fresh nutrient medium only, served as a control. The results, expressed as the mean number of nodules formed in the bean assay, are given in table 12. It will be seen that exudation of A.S. by nodulated plants was highest during the first week and less during the second week after inoculation. Exudates of the third and fourth week exerted an inhibitory effect on nodulation of the test plants. Uninoculated plants excreted inhibitory substances throughout the growing period.

TABLE 12. Comparison of the activity of root exudates, collected at weekly intervals or at the end of the growing period (donor: pea plants, uninoculated or inoculated with *Rhizobium* strain PRE; activity measured as number[1]) of nodules in the bean assay). Greenhouse experiment; time of inoculation 20th May 1960

| Period of exudation (days after inoc.) | Donor inoculated with PRE | Period of exudation (days after inoc.) | Donor uninoculated | Donor inoculated with PRE | Control (no pre-planting) |
|---|---|---|---|---|---|
| | | | | | 35.7 |
| 0–7 | 46.8 | 0–7 | – | 46.8 | |
| 0–14 | 60.3 | 7–14 | 12.3 | 39.3 | |
| 0–21 | 30.0 | 14–21 | 26.0 | 20.8 | |
| 0–27 | 24.3 | 21–27 | 7.8 | 14.5 | |

[1]) Mean values per plant of 4 replicates.

By continuous growth in the same medium an accumulation of A.S. took place during the first two weeks. The inhibitory effect of the exudates of later stages was partly counteracted by the A.S. accumulated during the preceding period (compare columns 2 and 5 of table 12).

In conclusion, it can be stated that different substances are excreted by the nodulated root at different ages. Therefore the assumption that inhibition is due to an overdose of stimulating substances has to be discarded.

### 3.6. INFLUENCE OF DILUTION AND HEATING OF ROOT EXUDATES OF DIFFERENT AGES

The results described in the previous section clearly show that stimulating substances were exuded in the early period of nodule formation of pea plants,

whereas inhibitors were present in exudates of later periods. Some experiments have been carried out to compare the biological activity of these two types of exudates after different treatments. The effect of dilution was studied in order to learn the effect of concentration of the stimulating or inhibiting substances on nodulation. Heating was introduced into the experiment because it might affect both substances in a different way.

Root exudates of pea plants infected with *Rhizobium* strain PRE were assayed at two concentrations, equivalent to the amounts excreted by one or half a donor plant. The exudates were tested at 8, 14, 16 and 20 days after inoculation of these plants. For comparison, root exudates of uninoculated plants of the same age were assayed at concentrations equivalent to the exudates of one donor plant. A control series without preplanting was run simultaneously (table 13).

TABLE 13. The effect of dilution on the activity of root exudates of pea plants of different ages, measured as number[1]) of nodules per plant in the bean assay. (Donor: pea plants, uninoculated and inoculated with *Rhizobium* strain PRE) Greenhouse experiment; time of inoculation 16th March 1960.

| Age of donor plants (days after inoc.) | Root exudates from | | | Control (no preplanting) |
|---|---|---|---|---|
| | 1 donor uninoc. | 1 donor inoc. | ½ donor inoc. | |
| | | | | 201 |
| 8 | 187 | 219 | 208 | |
| 14 | 207 | 263 | 243 | |
| 16 | 192 | 176 | 216 | |
| 20 | 145 | 161 | 231 | |

[1]) Mean values per plant of 4 replicates.

The results obtained with uninoculated plants are in agreement with the data of tables 11 and 12 which show that with increasing age inhibiting substances are exuded by the roots of pea plants. Exudates of 14 days old plants with effective nodules, which consisted of root and nodule secretions, stimulated nodulation of the test plants. At half concentration nodule stimulation was slightly reduced. Used nutrient solutions of 16 and 20 days old pea plants reduced nodulation of the test plants when used undiluted, an observation which is in agreement with previous experiments. At half concentration, however, no inhibition and even a slight stimulation occurred. Obviously the concentration of the inhibitors but not that of the stimulating substances was lowered below the activity level by dilution of the used nutrient solution.

In previous experiments, it was found that heat treatment lowered the stimulating effect of root exudates (cf. chapter 5). Since different components in the exudates were likely to behave differently, it seemed worth while to test the thermostability of root exudates of different ages. Use was made of culture media of pea plants grown for 14 and 27 days after inoculation with *Rhizo-*

*bium* strain PRE. Aliquots of each used culture medium were exposed for 30 minutes to 60° and 80°C, respectively. Treated and untreated solutions in amounts equivalent to the amount of exudates of one pea plant were assayed. Root exudates of uninoculated pea plants and of plants inoculated with the ineffective *Rhizobium* strain P8 were included. As a control, test plants without any addition were run simultaneously (table 14).

TABLE 14. The effect of heating on the biological activity of root exudates, derived from pea plants of different ages; activity measured as number[1]) of nodules per plant in the bean assay. Greenhouse experiment; time of inoculation 25th May 1960.

| Age of donor plants (days after inoc.) | Root exudates from | | | | | Control (no pre-planting) |
|---|---|---|---|---|---|---|
| | Donor uninoc. | Donor inoc. with P8 (ineff.) | Donor inoc. with PRE (eff.) | | | |
| | | | Untreated | 60°C | 80°C | |
| | | | | | | 27 |
| 14 | 6.5 | 40.8 | 37.8 | 43.0 | 19.3 | |
| 27 | 5.5 | 17.8 | 9.0 | 45.0 | 12.0 | |

[1]) Mean values per plant of 4 replicates.

A severe inhibition was obtained with root exudates of uninoculated plants of both ages. Stimulating substances were present in root exudates of 14 days old pea plants inoculated with an ineffective or an effective *Rhizobium* strain.

The used nutrient solution of older donor plants (27 days), however, exerted an inhibitory effect. It is noteworthy that in order of magnitude the root exudates of the uninoculated plants contained the highest amount of inhibitors and those of the plants with ineffective nodules the lowest. As an explanation it may be suggested that, 27 days after inoculation, the exudates of nodulated plants contained stimulating substances from the nodules and inhibitors from the roots (see tables 10, 11, 12). At this time the exudation of stimulating substances by the effective nodules had stopped, whereas that of the ineffective nodules still continued (see tables 10 and 11).

Heating affected the root exudates of young and old plants with effective nodules in a different way. In both cases heating at 60°C increased stimulation, particularly with the exudates from the 27 days old donor plants. Apparently at this temperature treatment the inhibitory substance was eliminated to a large extent, whereas the stimulative one was affected only slightly or not at all. Heating at 80°C, however, gave numbers of nodules even considerably lower than those of the control plants. The effect of heating at this temperature may have been partly due to the destruction of the stimulating substances (of exudates of 14 days old donor plants), partly to the presence of inhibitory substance. The latter may have been part of the originally present inhibitor or it may have been formed during the heat treatment.

A subsequent experiment was carried out under conditions favourable for nodulation of the control assay plants. Root exudates were obtained from pea plants inoculated with *Rhizobium* strain PRE, 16 days after inoculation. Amounts of root exudates excreted by one, two or three donor plants were

tested for A.S. Aliquots of the exudates were treated during 30 minutes at 60° and 80°C, respectively, and amounts as excreted per single pea plant were assayed. A series of bean plants, without preplanting, served as a control.

It may be seen from table 15 that the root exudates, when used at a concentration equivalent to that excreted by one or two donor plants, did not influence nodulation of the test plants, but at a higher concentration reduced nodulation. Heating of the root exudates gave a considerably increased stimulation of nodulation, especially when treated at 60°C. These results suggest that in these root exudates stimulatory and inhibitory substances were present in such a ratio that no activity was shown in the bean assay. Heating at 60°C reduced the activity of the inhibitory substances more than that of the stimulatory, whereas at 80°C part of the stimulatory substances was also inactivated.

TABLE 15. The effect of concentration and heating on the biological activity of root exudates from pea plants, 16 days after inoculation with *Rhizobium* strain PRE; activity measured as number[1]) of nodules per plant in the bean assay. Greenhouse experiment; time of inoculation 27th April 1960.

| Heat treatment | Root exudates from | | | Control (no preplanting) |
|---|---|---|---|---|
| | 1 donor plant | 2 donor plants | 3 donor plants | |
| Untreated | 120 | 124 | 66 | 124 |
| 60°C | 203 | – | – | |
| 80°C | 172 | – | – | |

[1]) Mean values per plant of 4 replicates.

The results obtained in the above-mentioned experiments clearly demonstrate the complexity of factors affecting the biological activity of root exudates from pea plants. The absence or presence of nodules, the effectiveness of the latter, and the age of the nodules and the root system play an important role. Strong evidence was obtained that at least in the early period of nodule formation, i.e. up to two weeks after inoculation, one or perhaps more than one, stimulatory substance is exuded by the nodules. This substance is inactivated at 80°C. Nodulation-inhibiting substances are also secreted in the medium approximately two weeks after inoculation. These inhibitors are also formed in the absence of *Rhizobium* and therefore they are assumed to be formed largely by the roots. They are more sensitive to heat treatment than the stimulating ones and partly inactivated at 60°C.

CHAPTER 4

# INFLUENCE OF SOME GROWTH SUBSTANCES, COMBINED NITROGEN AND BORON ON NODULATION OF BEAN PLANTS

Growth substances are supposed to be involved in root-nodule formation. Therefore the effect of addition of a number of such compounds on nodulation was studied and compared with the effect of A.S. Since it is well known that combined nitrogen and boron affect nodule formation, these nutrients were included in the experiments.

## 4.1. INDOLEACETIC ACID

Root nodules (THIMANN, 1936; PATE, 1958) and root exudates (KEFFORD *et al.*, 1960) of nodulated plants are known to contain auxins, especially indoleacetic acid (I.A.A.). When supplied at low concentrations ($< 10^{-6}$M) to bean plants, grown under climatic conditions associated with poor nodulation, no stimulation by I.A.A. was observed by the present author. At higher concentrations, nodulation was inhibited. This was also true under conditions of satisfactory nodulation.

The interaction of high concentrations of I.A.A. and A.S. was studied in the following experiments. At first bean plants inoculated with the ineffective *Rhizobium* strains $WH_2$ and S460 were used. The ether-soluble fraction (A.S.) from 100 mg of fresh bean nodules, and I.A.A. ($5 \times 10^{-6}$M) were given either singly or in combination. The number of nodules determined at harvest, 3–4 weeks after inoculation, is given in table 16.

TABLE 16. The effect of I.A.A. ($5 \times 10^{-6}$M) and A.S. (equivalent to 100 mg fresh bean nodules) on nodulation[1]) of bean plants, inoculated with the ineffective *Rhizobium* strains $WH_2$ and S 460. Greenhouse experiments; times of inoculation 28th Jan. 1961 (Exp. 1 and 3), 9th Febr. 1961 (Exp. 2 and 4).

| Treatment | Strain $WH_2$ | | Strain S 460 | |
|---|---|---|---|---|
| | Exp. 1 | Exp. 2 | Exp. 3 | Exp. 4 |
| Control | 110 | 60 | 106 | 60 |
| I.A.A. | 30 | 5 | 77 | 49 |
| A.S. | 86 | 106 | 109 | 61 |
| I.A.A. + A.S. | 82 | 53 | 93 | 64 |

[1]) Mean number of nodules per plant of 4 replicates.

It may be seen that nodule number was reduced by I.A.A. The effect of A.S. was somewhat variable, but in all cases A.S. partly eliminated the inhibitory action of high concentrations of I.A.A.

The interaction of I.A.A. and A.S. was also found in bean plants inoculated

with the effective strain Bokum. The amounts of A.S. and I.A.A. applied were similar to those used in the previous experiment; the plants were harvested 3 weeks after inoculation (table 17). The results of exp. 5 were similar to those obtained with plants inoculated with the ineffective *Rhizobium* strains *viz.* inhibition by I.A.A. and elimination of this effect by A.S. In exp. 6, however, no effect of added I.A.A. was observed, but severe inhibition was found in the plants treated with A.S. In agreement with former experiments, however, I.A.A. counteracted the effect of A.S.

From the results obtained in these experiment it may be concluded that A.S. is not identical to I.A.A.

TABLE 17. The effect of I.A.A. ($5 \times 10^{-6}$M) and A.S. (equivalent to 100 mg of fresh bean nodules) on nodule number[1]) of bean plants, inoculated with the effective *Rhizobium* strain Bokum. Greenhouse experiment; time of inoculation 28th Februari 1961 (Exp. 5) and 6th March 1961 (Exp. 6).

| Treatment | Exp. 5 | Exp. 6 |
|---|---|---|
| Control | 121 | 60 |
| I.A.A. | 97 | 57 |
| A.S. | 110 | 26 |
| I.A.A. + A.S. | 119 | 42 |

[1]) Mean values per plant of 4 replicates.

## 4.2. KINETIN

Nodulation is not stimulated by kinetin when supplied to the roots of intact plants (KEFFORD *et al.*, 1960) or to isolated root cultures (RAGGIO *et al.*, 1959). At higher concentration it is even inhibited.

In the following experiment the effect of kinetin on bean plants was tested separately or in combination with I.A.A. The latter was included since it was found that kinetin is only active in combination with I.A.A. (see STRONG, 1958). Three concentrations of kinetin ($10^{-7}$; $5 \times 10^{-7}$ and $25 \times 10^{-7}$M) and two concentrations of I.A.A. ($10^{-6}$ and $2.5 \times 10^{-6}$M) were used. For inoculation the *Rhizobium* strain $WH_2$ was used.

It may be seen from table 18 that nodule numbers and nodule weight were

TABLE 18. The effect of kinetin and I.A.A. on nodulation[1]) of bean plants, inoculated with *Rhizobium* strain $WH_2$. Greenhouse experiment; time of inoculation 17th Febr. 1961.

| Concentration of kinetin ($10^{-7}$M) | Number of nodules | | | Fresh wt. of nodules (g) | | |
|---|---|---|---|---|---|---|
| | Concentration of I.A.A. ($10^{-6}$M) | | | Concentration of I.A.A. ($10^{-6}$M) | | |
| | 0 | 1 | 2.5 | 0 | 1 | 2.5 |
| 0 | 109 | 75 | 55 | 0.84 | 0.65 | 0.62 |
| 1 | 69 | 70 | 89 | 0.72 | 0.67 | 0.62 |
| 5 | 61 | 57 | 107 | 0.72 | 0.64 | 0.76 |
| 25 | 50 | 58 | 71 | 0.53 | 0.64 | 0.48 |

[1]) Mean values per plant of 4 replicates.

reduced when kinetin or I.A.A. was supplied separately to the plants; this effect was more pronounced at higher concentrations. When both substances were given in combination, the depressing effect of kinetin on nodule number was partly eliminated by the highest concentration of I.A.A., and the depressing effect of the highest concentration of I.A.A. by various concentrations of kinetin. In the case of fresh weight of nodules the interaction of kinetin and I.A.A. was less clear. The depressing effect of kinetin and I.A.A. was confirmed in other experiments, but the interaction between both compounds was not always as pronounced as in the above-mentioned experiment.

### 4.3. Gibberellic acid

Inhibition of nodulation in bean plants by gibberellic acid (G.A.) was found by Thurber *et al.* (1959). This effect was only observed when G.A. was applied onto the leaves and absent when applied to the soil. An inhibitory effect of G.A. on nodulation of *Vicia villosa* was observed in plants treated in the early growth phase but not in older plants (Mess, 1959). According to this author the decreased nodulation in young plants treated with G.A. may be due to poor growth of the roots as a result of increased shoot growth, and not to some specific action of the growth substance on nodulation. This assumption is in agreement with the finding of Raggio *et al.* (1959) that no influence on nodulation was found when G.A. was given to the base of excised roots.

A search for G.A. in root nodules of leguminous plants revealed that nodules of beans and peas are rich in substances with G.A.-activity (Radley, 1961). In contrast, little or nothing was detected in the roots. When bean plants were grown in water culture, G.A.-like substances were detected only in the culture solution of nodulated plants, but not in that of unnodulated ones (Radley, 1961).

In the present investigations several experiments were carried out with bean plants sprayed with G.A. The plants were cultivated in white fluorescent light (30000 ergs/cm²sec.) at 24–26°C. Two weeks after germination, one primary leaf of a number of plants was sprayed with G.A. (50 ppm G.A. as a Na-salt). In one of the experiments a series of plants was included in which one primary leaf per plant was sprayed with A.S. equivalent to 100 mg fresh bean nodules. One primary leaf of the control plants was sprayed with water. The cotyledons were removed at the time of spraying and at the same time the plants were inoculated with *Rhizobium* strain Bokum.

After 24 hours the primary leaves sprayed with G.A. were distinctly larger than the opposite untreated leaves. The increase in length of the petioles and the stems of the G.A.-treated plants after 48 hours was larger than that of the control plants. At harvest, two weeks after treatment, the shoots of the G.A.-treated plants were 44 cm long, whereas shoots of the controls were only 12 cm. Pronounced differences existed in the root system of the treated and untreated plants. Instead of the thin, branched roots of the control plants, rather thick roots with short laterals appeared on the treated plants. In the plants

sprayed with A.S. no response like in G.A.-treated plants was observed. The number of nodules, determined at harvest, is given in table 19. The inhibitory effect of G.A. on nodulation is clearly demonstrated. Nodule extract applied to the leaves had no influence on nodulation. From the results obtained it may be concluded that A.S. is not identical with G.A.

TABLE 19. The effect of spraying with gibberellic acid (G.A., 50 ppm as Na-salt) and A.S. (equivalent to 100 mg of fresh bean nodules) on nodule number[1]) of bean plants inoculated with *Rhizobium* strain Bokum. White-light cabinet; photoperiod 16 hr, 30000 ergs/cm² sec.

| Treatment | Number of nodules | | |
|---|---|---|---|
| | Exp. 1 | Exp. 2 | Exp. 3 |
| Water | 23.4 | 225.4 | 90.0 |
| G.A. | 12.4 | 130.2 | 35.3 |
| A.S. | – | – | 95.1 |

[1]) Mean values per plant of 16 (Exp. 1), 8 (Exp. 2) and 10 (Exp. 3) replicates, respectively.

### 4.4. PURINES, PYRIMIDINES, YEAST AND SOIL EXTRACTS

These substances were added to the nutrient solution of bean plants, grown under conditions associated with poor nodulation. Adenine, adenosine, uracil, guanine and xanthine, at concentrations of 0.25–1.25 $\times$ $10^{-5}$M, were inactive when applied separately or in combination with I.A.A. (5 $\times$ $10^{-6}$M). Benzimidazole, supposed to be an antagonist for adenine (GALSTON *et al.*, 1953), inhibits nodulation considerably and its inhibiting effect was increased when added in combination with I.A.A. (table 20).

Yeast extract, found to be stimulatory on nodulation of clover in acid soil (MULDER & VAN VEEN, 1960) and soil extract were also found to be inactive.

TABLE 20. The effect of benzimidazole and I.A.A. on nodule number [1]) of bean plants inoculated with *Rhizobium* strain Bokum. Greenhouse experiment; time of inoculation 10th April 1961.

| Benzimidazole ($10^{-4}$M) | I.A.A. | |
|---|---|---|
| | 0 | $5\times10^{-6}$M |
| 0 | 82 | 31 |
| 2.5 | 81 | 16 |
| 5.0 | 23 | 14 |
| 12.5 | 22 | 14 |

[1]) Mean values per plants of 4 replicates.

### 4.5. COMBINED NITROGEN

When combined nitrogen is given to leguminous plants at low concentrations, nodulation is enhanced but at higher concentrations it is severely inhibited. Since small amounts of amino acids are known to be excreted by the roots (ROVIRA, 1956; KATZNELSON *et al.*, 1955; VISSER, 1961) it would be

possible that the stimulating effect of root exudates is due to the small amounts of nitrogen. To investigate this, the effect of root secretions and nodule extract was compared wtih that of ammonium nitrate. Root secretions, heated at 110°C for 20 minutes, were included for comparison. Since the pH of the root secretions was about 6, nitrogen losses by evaporation during heating will have been improbable. The amount of root secretions tested was equivalent to 100 ml used nutrient solution of bean plants with effective nodules; the amount of nodule extract given was equivalent to 100 mg fresh bean nodules per plant. Nitrogen, 10 mg N per liter, was given as ammonium nitrate. The number of nodules, counted 18 days after inoculation, is recorded in table 21. Nodulation of the control plants was poor, but was considerably enhanced in the presence of root secretions and nodule extract. No such stimulation was found after heating of the used nutrient solution and even inhibition occurred. When combined nitrogen was given, nodulation was completely inhibited. From these data it may be concluded that the stimulating effect of root secretions and nodule extract is not due to the presence of combined nitrogen.

TABLE 21. The effect of combined nitrogen, root secretions and nodule extract on nodulation[1]) of bean plants, inoculated with *Rhizobium* strain Bokum. Greenhouse experiment; time of inoculation, 21th October 1959.

| Treatment | Nodules | |
|---|---|---|
| | Number | Fresh wt. |
| Control, no substances added | 16 | 0.02 |
| $NH_4NO_3$, 10 mg N | 1 | – |
| Root secretions (equivalent to 100 ml used culture solution) | 44 | 0.05 |
| Root secretions, heated at 100° C | 6 | – |
| Nodule extract, equivalent to 100 mg fresh nodules | 56 | 0.13 |

[1]) Mean values per plant of 6 replicates.

## 4.6. BORON

The necessity of boron for the growth of meristematic tissue in plants is well established (see HEWITT, 1963). The typical symptoms of boron deficiency (dying of the growing tips of shoot and root system) may be interpreted as a lack of carbohydrates in these systems, attended by an excessive accumulation of sugar in the leaves and stems. GAUCH & DUGGAR (1959) have put forward the hypothesis that boron acts as a carrier for the translocation of sugar due to the formation of a sugar-boron complex. Alternatively, boron deficiency might be the cause of cessation of growth of the meristematic tissue. Lack of sugar transport would then be the result and not the cause of growth inhibition. The following observations are in favour of the latter hypothesis. In root tips of *Vicia faba,* cell division stopped within 24–48 hours after transfer to a boron-deficient medium (WHITTINGTON, 1959). Nucleic acid synthesis, a prerequisite for cell division, is correlated with the amount of boron present (SHKOL'NIK & KOSITSIN, 1962) and some transient improvement in growth was

obtained by applying nucleic acid to boron-deficient sunflower (SHKOL'NIK & SOLOV'EVA, 1962, 1963). In view of these results, the inhibition of nodule growth in boron-deficient plants (BRENCHLEY & THORNTON, 1925; MULDER, 1949) may be due to reduced cell division.

Since both boron and A.S. affect nodulation, it could be suggested that the beneficial effect of nodule extract and root secretions depends on the presence of boron. Therefore, some experiments with boron-deficient bean plants were conducted.

Boron deficiency was easily obtained by growing bean plants under high temperature and light conditions. Chemically pure salts, dissolved in water, purified through ion-exchange resins, proved to be satisfactory to obtain boron deficiency. The vessels used were rinsed with dilute HCl to remove traces of boron. The plants were grown in boron-deficient nutrient solution for two weeks under aseptic conditions in the white-light cabinet at 28°C.

In the first experiment half of the plants received boron (50 μg/l) at inoculation with strain Bokum, whereas the others remained boron-free. A.S., equivalent to 100 mg fresh bean nodules per plant, was given to one set of plants receiving boron and to one set of boron-deficient plants. The numbers of nodules were counted 9 and 15 days after inoculation; at the latter date the plants were harvested and weight of nodules, shoots and roots determined (table 22). When boron was absent no nodules were formed either in the presence or in the absence of A.S. Evidently A.S. contained no boron or only in low amounts. However, when boron was present, nodulation was satisfactory and A.S. further stimulated nodulation.

TABLE 22. The effect of boron and A.S. on nodulation[1]) of bean plants, inoculated with *Rhizobium* strain Bokum. White-light cabinet; photoperiod 16 hr of white light, 30000 ergs/cm² sec.

| Boron (μg) | 9 days after inoculation | | 15 days after inoculation | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Number of nodules | | Number of nodules | | Fresh wt. of nodules (g) | | Fresh wt. of shoots (g) | | Fresh wt. of roots (g) | |
| | – | A.S. | – | A.S. | – | A.S. | – | A.S. | – | A.S. |
| 0 | 0 | 0.2 | 0 | 0.2 | – | – | 3.3 | 3.0 | 1.1 | 1.0 |
| 50 | 6.2 | 12.5 | 14.8 | 18.8 | 0.06 | 0.08 | 3.1 | 2.8 | 1.4 | 1.3 |

[1]) Mean values per plant of 6 replicates.

In a subsequent experiment the effect of different amounts of boron was studied with or without A.S. At inoculation with *Rhizobium* strain Bokum, half of the plants received A.S. equivalent to 100 mg fresh bean nodules. Plants of each group received boron in amounts of 0, 5, 10, 50 and 1000 μg/l. At harvest, 20 days after inoculation, the number and fresh weight of the nodules were determined (fig. 2). Nodules were virtually absent in plants grown in solutions containing no or 5 μg of boron per liter, while no effect was observed upon addition of A.S. At boron concentrations of 10 and 50 μg/l both number

and fresh weight of the nodules were increased. At these concentrations of boron, nodulation was highly stimulated by A.S. At 1000 μg boron per liter a slight inhibition of nodulation was observed in the plants without added A.S.

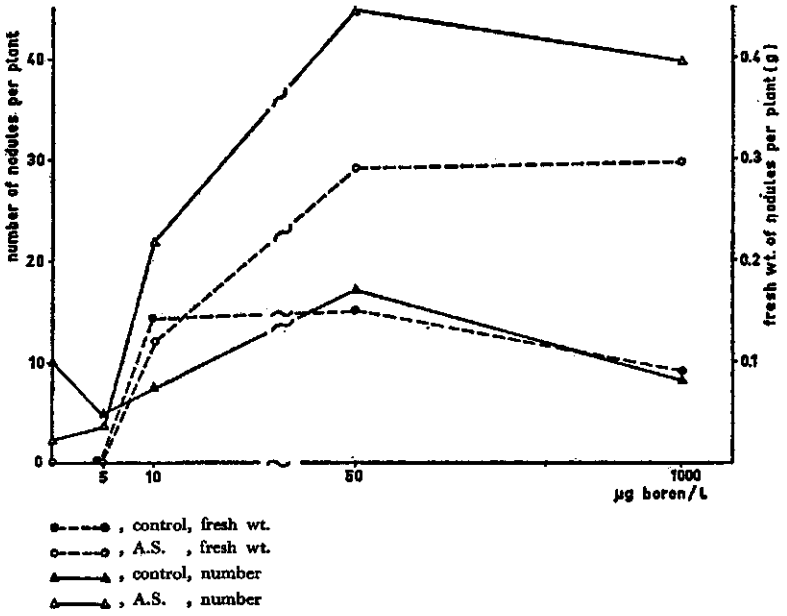



FIG. 2. Effect of A.S. and of different amounts of boron on nodulation of bean plants, inoculated with Rhizobium strain *Bokum*.

From these experiments it is evident that the stimulating effect of A.S. can not be ascribed to the presence of boron in the nodule extract or in the root secretions; A.S. is only active in the presence of boron.

CHAPTER 5

# PURIFICATION OF ROOT EXUDATES AND NODULE EXTRACTS; SOME CHARACTERISTICS OF THE ACTIVE SUBSTANCE (A.S.)

It has been well established by the experiments in chapter 3 that substances stimulating and inhibiting nodulation in bean plants are present in root exudates as well as in root nodules. However, the experimental results obtained in different seasons may vary considerably. It is conceivable that environmental factors such as light and temperature affect the production and the exudation of the active substance by the donor plant, as well as the response of the test plant to A.S.

It was assumed that by using one large batch of exudates or nodule extracts for many experiments, the variability of the donor plant would be eliminated. The remaining variability thus could be attributed to variation in response to A.S. of the test plants under different conditions. A further step would be to concentrate and to isolate A.S. The present chapter deals with results of tentative purification experiments.

For the preparation of a standardized extract, root exudates or root nodules from a large number of cultures were collected. Two criteria were used to follow the successive steps of the purification process *viz.* the ultra-violet light absorption and the biological activity, as measured in the bean assay (bean variety Walcherse Witte, inoculated with *Rhizobium* strain Bokum). As to the former the suggestion was made that A.S. is related to kinetin, so that nodule extracts and root exudates like kinetin would have a maximum absorption at ca. 260 mμ. It is noteworthy that the active fractions of coconut milk (STEWARD & SHANTZ, 1959) and immature corn seeds (MILLER, 1962), both containing kinetin-like substances, have also a strong absorption at ca. 260 mμ.

As a preliminary step, a comparison was made of the absorption spectra in U.V. light of adenine, root exudates, extracts of root nodules and of root tips. For root exudates the used nutrient solution of bean plants with effective nodules, 16 days after inoculation, was concentrated to 1/100 the original volume by evaporation at 45°C. To obtain the nodule extract effective bean nodules were crushed in water and filtered. The extract of root tips was obtained as follows: pea seeds were germinated on agar for 7 days under aseptic conditions at 25°C. Five-mm root tips were crushed and extracted with water, and the solution after filtration was extracted overnight with ether. The ether-soluble fraction was taken up in water and used for spectrophotometrical determination. The U.V. light absorption of adenine (5 mg/ml) in 0.1 N HCl was also recorded.

From fig. 3 it may be seen that adenine and the extract of root tips had similar absorption spectra with a maximum value at 260–265 mμ and a minimum at 230–235 mμ. The root exudates showed a maximum at 250–255 and a minimum at 245 mμ. No such distinct maximum and minimum values were

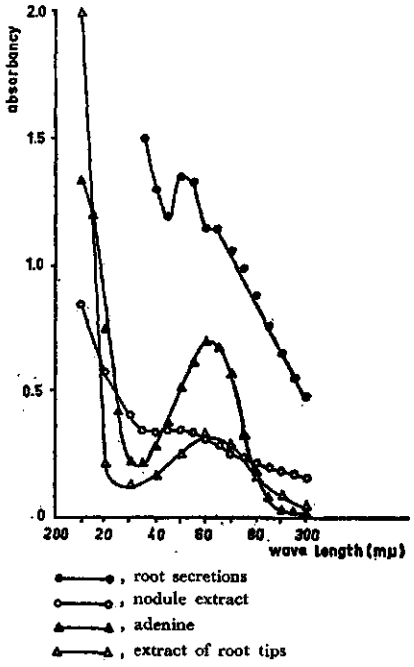



FIG. 3. U.V.-light absorbtion spectra of root secretions, nodule extract, extract of root tips and adenine.

observed in the spectrum of root nodules, but a slight flattening of the curve occurred at 240–255 mμ.

### 5.1. CONCENTRATION OF A.S. BY EVAPORATION

It was shown in chapter 3 that the activity of the stimulating substance was not eliminated by heating up to 60°C. It therefore seemed possible to concentrate the active substance by evaporation of the used nutrient solution at low temperatures. The medium of bean plants, 16 days after inoculation with Bokum, was used. It was filtered to remove root debris and precipitated salts and evaporated at 45°C at reduced pressure using a film evaporator. The precipitated salts were again removed by filtration and the clear yellow solution assayed for A.S.

In the first experiment the used nutrient solution was concentrated to 1/5 the original volume and the biological activity of the treated solution compared with that of the untreated one. For the assay of A.S., 200 ml used nutrient solution was applied per test plant. The concentrated solution was assayed in two amounts *viz.* 40 and 80 ml per test plant. The results, expressed as number and fresh weight of nodules per test plant, are given in table 23. It appears that the biological activity of the root exudates was not reduced after concentration at 45°C. Evidently no inactivation took place at this temperature. When the amount of applied used nutrient solution was doubled (80 ml, 5×

TABLE 23. The effect of concentration by evaporation at 45°C on the content of A.S. of root exudates of bean plants with effective nodules. Greenhouse experiment; time of inoculation 18th March 1960.

| Used nutrient solution applied per plant (ml) | Nodules[1]) | |
|---|---|---|
| | Number | Fresh wt. (g) |
| None (control) | 34.5 | 0.05 |
| 200 ml, not concentrated | 63.8 | 0.13 |
| 40 ml, 5 × concentrated | 80.8 | 0.15 |
| 80 ml, 5 × concentrated | 28.3 | 0.06 |

[1]) Mean values per plant of 4 replicates.

concentrated), a reduction in nodulation was observed, indicating that a supra-optimal concentration was reached.

In a subsequent experiment the effect of different amounts of the concentrated used nutrient solution was investigated in more detail. Nutrient solution of nodulated bean plants, 16 days after inoculation with Bokum, was concentrated to 1/28 of the original volume as previously described. Different amounts (0; 0.5; 1; 2; 5 and 10 ml) of this preparation were added to each test plant. The results are presented in table 24. In agreement with the results of the previous experiments, no inactivation of A.S. was observed after concentration of the root exudates. Both number and fresh weight of the nodules produced per test plant increased with increasing amounts of added solution.

TABLE 24. The effect of different amounts of concentrated (28 ×) used nutrient solution on nodulation[1]) of bean plants, inoculated with *Rhizobium* strain Bokum. Greenhouse experiment; time of inoculation 9th July 1960.

| Concentrated used nutrient solution, applied per plant (ml) | Nodules | |
|---|---|---|
| | Number | Fresh wt. (g) |
| 0 | 65.0 | 0.15 |
| 0.5 | 67.0 | 0.18 |
| 1.0 | 106.8 | 0.42 |
| 2.5 | 97.8 | 0.53 |
| 5.0 | 148.8 | 1.59 |
| 10.0 | 183.0 | 1.64 |

[1]) Mean values per plant of 4 replicates.

A clear stimulation was already obtained after the addition of 1 ml of this preparation. No reduction of nodulation was observed with the highest amount of preparation used in this experiment (equivalent to 280 ml used nutrient solution).

In conclusion it can be safely assumed that concentration of A.S. at 45°C does not affect its biological activity.

## 5.2. Concentration of A.S. by extraction with organic solvents

The water-solubility of A.S. is evident from its presence in the nutrient solution and in the aqueous extract of root nodules. The second step, after concentrating by evaporation, was to investigate the solubility of A.S. in some organic solvents with low affinity for water. It was conceivable that the solubility in an organic solvent might be used for the separation of A.S. from the other water-soluble components present in root exudates and root nodules, and for the further concentration of A.S. It may be recalled that kinetin was isolated from 'aged' D.N.A. by extraction with ether (Strong, 1958). The extraction of A.S. was carried out with root exudates and root nodules.

### 5.2.1. *Root exudates*

Culture medium of bean plants, collected 16 days after inoculation with strain Bokum, was concentrated to 1/100 of the original volume by evaporation (see section 5.1.). If not used immediately, the preparation was stored at –20°C.

Extraction with chloroform was performed by shaking equal volumes of the above-mentioned concentrated solution and of chloroform intermittently for 30 minutes. The treatment was repeated three times with fresh chloroform. Subsequently, the water fraction was filtered through wetted filter paper to remove the trace amounts of chloroform. The pooled chloroform fractions were evaporated at 45°C to dryness and the dark residue taken up in water. Both fractions, the chloroform-soluble and insoluble fractions, were stored at –20°C before being assayed.

For the extraction with ether, 50-ml samples of the concentrated culture medium were percolated overnight with ethyl ether. After addition of 1 ml water to the pooled extracts, the ether was removed by evaporation. The resultant aqueous solution of the ether-soluble fraction was filtered through wetted filter paper to remove lipid material. The remaining culture medium, containing the ether-insoluble substances, was heavily aerated to remove traces of ether. Both the ether-soluble (called ether extract) and ether-insoluble fractions were stored at –20°C.

Aliquots of the ether- and chloroform-soluble and insoluble fractions were standardized so that 1 ml represented 100 ml of the original nutrient solution. The biological activity of the fractions was determined in the bean assay. To each test plant 1 ml of these fractions was added. The results are summarized in table 25. The U.V. absorption spectra of the fractions were determined with a Beckman modell D.U. spectrophotometer against water. The results are presented in fig. 4.

The data of table 25 clearly demonstrate that A.S. is highly soluble in ether and slightly soluble in chloroform. Extraction with ether overnight removed A.S. completely from the root exudates. The U.V. absorption spectrum displayed a maximum at 250–255 mμ and a minimum at 245 mμ. The ether extract, which contained A.S., had a strong absorbancy at 260 mμ whereas no

TABLE 25. The effect of ether and chloroform extracts of exudates from bean roots with effective nodules on nodulation [1]) of bean plants, inoculated with *Rhizobium* strain Bokum. Greenhouse experiment; time of inoculation 25th June 1960.

| Treatment | Nodules | |
|---|---|---|
| | Number | Fresh wt. (g) |
| Control (without added root exudates) | 9 | 0.02 |
| Ether-soluble fraction | 226 | 0.55 |
| Ether-insoluble fraction | 8 | 0.01 |
| Chloroform-soluble fraction | 45 | 0.20 |
| Chloroform-insoluble fraction | 137 | 0.51 |

[1]) Mean values per plant of 4 replicates.


FIG. 4. U.V.-light absorbtion spectra of untreated root secretions, ether-soluble, ether-insoluble, chloroform-soluble and chloroform-insoluble fractions.

such absorbancy was found in the biologically inactive ether-insoluble fraction. There was a certain shift in the maximum and minimum values of the ether extract when compared with the original solution. Maximal absorbancy now occurred at ca. 260 mμ and minimal at 240 mμ, resembling those of adenine or other purines. No typical absorbancy was present in the chloroform-soluble fraction, whereas a slight inclination can be observed between 245–265 mμ in the spectrum of the chloroform-insoluble fraction. A.S. was mainly present in the latter and only slightly in the former fraction. The results of this experiment suggest that the biological activity of the fractions coincides with the occurrence of a maximal absorbancy at ca. 260 mμ.

Subsequently, a comparison was made between root exudates before and after extraction with ether. The fractions thus obtained were assayed separately and in combination. The same used nutrient solution as described previously was used, but the fractions per ml contained substances equivalent to 28 ml used nutrient solution. These fractions were assayed in portions of 1 ml and 5 ml per test plant.

The data recorded in table 26 confirmed the previously described observation that A.S. is completely extracted with ether. Some activity was lost during the procedure, as was especially demonstrated by the reduced activity of the lowest concentration used. The ether-insoluble fraction had no influence on nodulation. The combination of the soluble and insoluble fractions enhanced nodulation, but the number of nodules formed was slightly less than that obtained with the ether extract only.

TABLE 26. The effect of the ether-soluble and ether-insoluble fractions of exudates from bean roots with effective nodules on nodulation[1]) of bean plants, inoculated with *Rhizobium* strain Bokum. Greenhouse experiment; time of inoculation 9th July 1960.

| Treatment | Nodules | |
|---|---|---|
| | Number | Fresh wt. (g) |
| Control (without root exudates) | 67.0 | 0.15 |
| 1 ml untreated solution | 106.8 | 0.42 |
| 5 ml untreated solution | 148.8 | 1.59 |
| 1 ml ether-soluble fraction | 85.8 | 0.42 |
| 5 ml ether-soluble fraction | 128.8 | 1.15 |
| 1 ml ether-insoluble fraction | 64.0 | 0.39 |
| 5 ml ether-insoluble fraction | 75.3 | 0.42 |
| 5 ml ether-soluble + 5 ml ether insoluble fraction | 113.0 | 0.98 |

[1]) Mean values per plant of 4 replicates.

### 5.2.2. *Root nodules*

The extraction procedure used for root exudates was also applied to the isolation of A.S. from root nodules. In chapter 3 it has been shown that root nodules represent a good source of A.S.

Nodules from bean plants, inoculated with the effective strain Bokum, were collected 2–3 weeks after inoculation and stored at –20°C. The frozen material was crushed in water and the red aqueous solution filtered to remove plant debris (fraction I in table 27). The turbid solution, representing 100 mg fresh nodules per ml, was shaken with equal amounts of chloroform to remove fatty substances and to denature the soluble proteins (HOTCHKISS, 1957). The chloroform and precipitated protein were separated from the aqueous solution by centrifugation at 4000 g and discarded. The resultant clear, red solution (fraction II) was used for further extractions.

In the extraction procedure with root exudates, the pH value of the used nutrient solution was about 6.0–6.5. Since it was conceivable that the pH of

the solution might influence the extraction of A.S., the ether extraction was carried out at low, neutral or high pH. The aqueous solution (fraction II) had a pH value of about 6.5. The pH of two aliquots was adjusted to 2.1 or 10.8 by adding HCl and NaOH, respectively. These two preparations and the solution of pH 6.5 were extracted overnight with ether as described before (fractions III A, III B, III C).

The biological activity of all these fractions was determined in the bean assay at concentrations equivalent to 200 mg (Exp. 1) or 50 and 200 mg (Exp. 2) of nodule tissue per test plant. A control series without added nodule extracts served as a control. The results of this experiment are summarized in table 27. It may be seen that the aqueous nodule extract (I) enhanced nodu-

TABLE 27. Biological activity of different fractions of bean nodules as measured by number[1]) and fresh weight[1]) of nodules per plant in the bean assay. Greenhouse experiment; time of inoculation, 17th July 1960 (Exp. 1) and 30th July 1960 (Exp. 2).

| Treatment | Mg of fresh nodules used for extraction, applied per plant | Exp. 1 | | Exp. 2 | |
|---|---|---|---|---|---|
| | | Number | Fresh wt. (g) | Number | Fresh wt. (g) |
| Control, no extract added | – | 86 | 0.22 | 123 | 0.68 |
| Aqueous extract of | 50 | – | – | – | – |
| nodules (I) | 200 | 137 | 0.77 | – | – |
| Aqueous extract of | 50 | – | – | 164 | 1.08 |
| nodules after chloroform treatment (II) | 200 | 172 | 0.40 | 97 | 0.90 |
| Ether extract of (II) at: | | | | | |
| pH 2.1 (III A) | 50 | – | – | 123 | 0.56 |
| | 200 | 50 | 0.16 | 110 | 0.63 |
| pH 6.5 (III B) | 50 | – | – | 176 | 1.23 |
| | 200 | 101 | 0.33 | 137 | 1.08 |
| pH 10.8 (III C) | 50 | – | – | 120 | 0.89 |
| | 200 | 111 | 0.32 | 113 | 0.88 |

[1]) Mean values per plant of 4 replicates.

lation and its biological activity was not reduced after chloroform treatment (II). The number of nodules obtained with the chloroform-treated solution (II) was even higher than that obtained with the untreated aqueous extract (I), indicating that some inhibitor was removed by the chloroform extraction. No stimulation (exp. 2) and even slight inhibition (exp. 1) was observed by adding the ether-soluble fraction from the acid solution (III A). Nodulation was clearly stimulated upon addition of the ether-soluble fraction obtained under neutral conditions (III B). The beneficial effect was higher at the lower concentration, indicating an inhibitory effect on nodulation of excessive amounts of A.S. The results obtained with the ether-soluble fraction from the alkaline solution (III C) were inconclusive. Nodule number was increased with this extract in the first experiment, but not in the second one; nodule weight, however, was always stimulated.

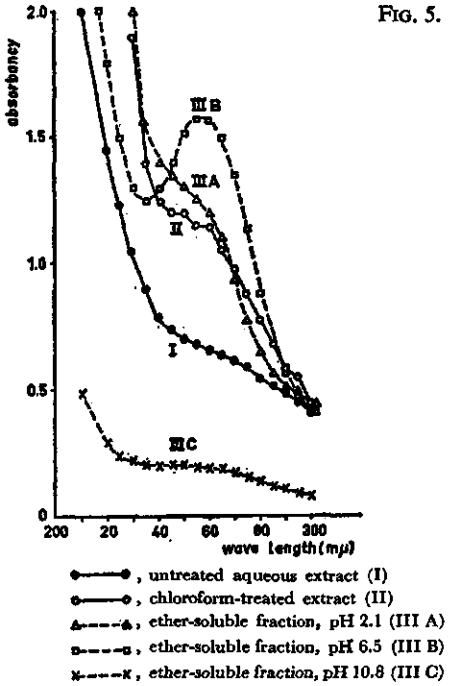



FIG. 5. U.V.-light absorbtion spectra of untreated aqueous extract of nodules (I); chloroform-treated (II); and ether-soluble fractions of (II), extraction performed at pH 2.1 (III A), pH 6.5 (III B) and pH 10.8 (III C), respectively.

The U.V. absorption spectra of the different fractions are presented in fig. 5. The untreated aqueous solution (I) contained substances derived from 7.7 mg, the chloroform-treated solution (II) from 20 mg and the ether-soluble fractions (III A, B and C) from 200 mg of nodules per ml. These concentrations were chosen to obtain good readings. The absorption curve of the aqueous extract (I) showed a flattening at 245–260 mμ, which was more pronounced after purification with chloroform (II). Pronounced differences were found to exist between the curves of the ether-soluble fractions. The curve of that obtained from the acid solution (III A) did not show any typical characteristics, although some deviation could be observed in the slope of the curve between 240–260 mμ. In contrast, a typical maximum at 255–260 mμ and a minimum at 230–235 mμ occurred in the absorption spectrum of the ether-soluble fraction obtained under neutral conditions (III B). The absorbancy of the ether extract from the alkaline solution (III C) was very low in comparison with that of the others.

A comparison of the biological activity of the fractions and the absorbancy in ultra-violet light again suggests that the biological activity coincides with the presence of an absorption peak at ca. 260 mμ.

In a subsequent experiment a comparison was made between the biological activities of the ether-soluble and ether-insoluble fractions, obtained under neutral conditions. The amounts assayed were equivalent to 10,50 and 100 mg of fresh bean nodules per test plant (table 28). In contrast to the root exudates

TABLE 28. Biological activity of the ether-soluble and ether-insoluble fractions of effective bean nodules as measured by numbers[1]) of nodules per plant in the bean assay. Greenhouse experiment; time of inoculation 9th July 1960.

| Mg of fresh nodules used for extraction, applied per plant (mg) | Ether-soluble fraction | Ether-insoluble fraction | Ether-soluble + insoluble fractions | Control |
|---|---|---|---|---|
| None | | | | 65 |
| 10 | 217 | 189 | 201 | |
| 50 | 188 | 253 | 171 | |
| 100 | 239 | 187 | 179 | |

[1]) Mean values per plant of 4 replicates.

(table 26) nodulation-stimulating substances were present in both the ether-soluble and insoluble fractions. These two fractions may have contained either completely different active substances or different modifications of the same compound. In the latter case the ether-insoluble fraction might contain the precursor of the ether-soluble A.S. present in the root nodule and in root exudates.

### 5.3. THE EFFECT OF HEATING ON THE BIOLOGICAL ACTIVITY AND THE ABSORBANCY OF ULTRA-VIOLET LIGHT OF A.S.

No perceptible decline in biological activity was observed in root exudates stored for several months at room temperature. To test the thermostability of A.S. at first a comparison was made of the activity of root exudates and that of an aqueous extract of root nodules after heating at different temperatures. Aliquots of used nutrient solution from bean plants with effective nodules, and of the aqueous extract obtained by crushing these nodules, were heated for 30 minutes at 40°, 60°, 80° and 110°C, respectively. The treated and untreated solutions were assayed in the bean assay in amounts equivalent to 100 ml used nutrient solution and 100 mg fresh nodules per plant, respectively. The results, recorded in table 29, show that in accordance with the results obtained in table

TABLE 29. The effect of heating on the biological activity of root exudates and of an aqueous extract of nodules of bean plants, inoculated with *Rhizobium* strain Bokum; activity measured as number[1]) of nodules per plant in the bean assay. Greenhouse experiment; time of inoculation 10th February 1960.

| Treatment | Added to test plants | |
|---|---|---|
| | Root exudates | Root-nodule extract |
| Control, no substances added | 34.6 | 34.6 |
| No heating | 65.5 | 78.0 |
| 40°C | 62.0 | 68.7 |
| 60°C | 63.5 | 59.0 |
| 80°C | 35.5 | – |
| 110°C | 49.0 | 85.3 |

[1]) Mean values per plant of 4 replicates.

14, exposure to 80°C and higher temperatures reduced the biological activity of root exudates. In contrast, no such effect was found with the aqueous extract of root nodules; the activity remained even after exposure to 110°C.

However, when the ether-soluble fraction of root nodules, equivalent to 200 mg fresh tissue, was exposed to 60° and 80°C (table 30) the same results were found as with root exudates, i.e. inactivation at 80°C.

TABLE 30. The effect of heating on the biological activity of the ether-soluble fraction of effective bean nodules; activity measured as number[1]) and fresh weight[1]) of nodules per plant in the bean assay. White-light cabinet; photoperiod 16 hr, 30000 ergs/cm² sec.

| Treatment | Nodules | |
|---|---|---|
| | Number | Fresh wt. (g) |
| Control, no substances added | 41 | 0.09 |
| No heating | 126 | 0.45 |
| 60° C | 138 | 0.28 |
| 80° C | 71 | 0.11 |

[1]) Mean values per plant of 4 replicates.

These results suggest that in the aqueous extract of bean nodules two types of nodulation-stimulating substances are present. The first one, soluble in ether and inactivated by heat treatment at 80°C, may be identical with that present in root exudates. The second one, however, is insoluble in ether and is heat-resistant.

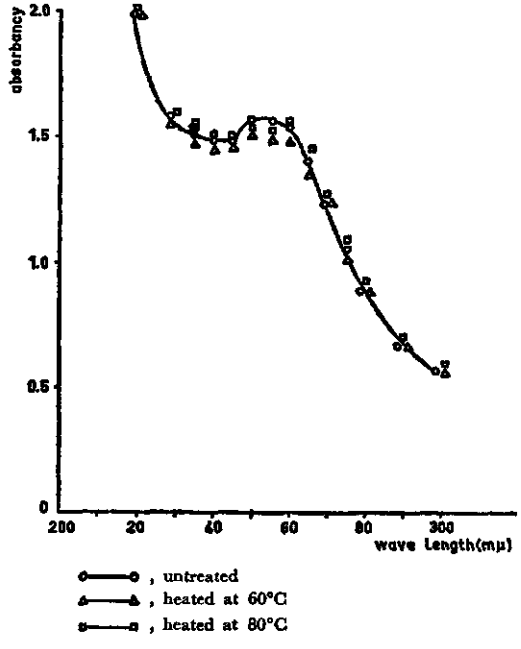



FIG. 6. The effect of heat treatment on the U.V.-light absorption spectra of A.S.

Absorption spectra of the untreated and heated aqueous solutions obtained from the ether-soluble fraction of root nodules are presented in fig. 6. It may be seen that no differences occurred between the spectra of the heated and untreated solution.

### 5.4. The effect of pH on the absorbancy of the ether-soluble fraction of nodules

The importance of pH-control in measuring absorbancy of purines and pyrimidines is well known (Beaven *et al.*, 1955). Therefore, it seemed logical to perform the measurements at different pH-values. The ether-soluble fraction of effective bean nodules (equivalent to 100 mg tissue per ml solution), known to possess biological activity in the bean assay, was adjusted to pH 2.6 and pH 10.9 by using HCl and $NH_4OH$, respectively. The untreated solution had a pH of 6.0.

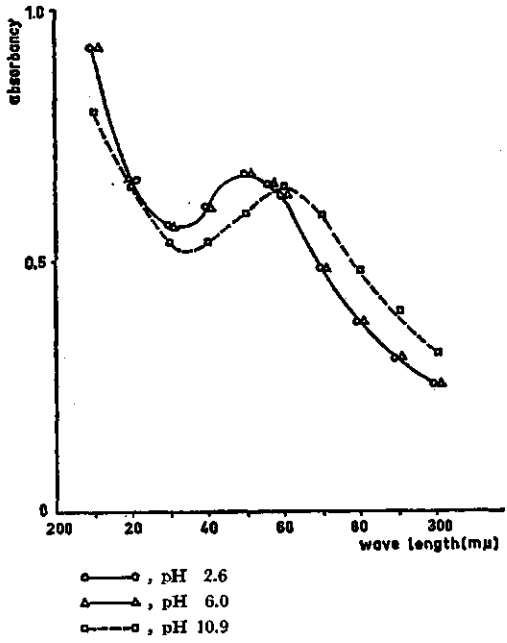



Fig. 7. The effect of pH on the U.V.-light absorption spectra of A.S.

The results presented in fig. 7 clearly show that no differences occurred between the curves of the neutral and acid solutions. Under alkaline conditions, however, there was a shift of the maximum value towards higher wave lengths; the absorption maximum was less pronounced. These results indicate that the component of the ether-soluble fraction, responsible for the absorption in U.V. light, is dissociated at high pH but remains undissociated under neutral and acid conditions (Beaven *et al.*, 1955). It may be recalled that a very low U.V. absorption was obtained with the ether-soluble fraction of alkaline nodule extract, as compared with that of the acid and neutral solutions( cf. fig. 5).

This may have been the result of the dissociation of the U.V.-absorbing substances under alkaline conditions, preventing them from being taken up by the ether.

### 5.5. Some other characteristics of the ether-soluble fraction

The ether-soluble fraction, either from nodules or from root exudates, has a bluish fluorescence when examined in ultra-violet light. It contained no nitrate or amino-nitrogen. When examined for sugars, no reaction was obtained with Fehlings' solution, but when heated at 90°C with Dische reagent (cysteine in N HCl), a red colour appeared, indicating that a deoxypentose might be present.

### 5.6. Fractionation of the ether-soluble fraction of root nodules on ion-exchange resins

To isolate A.S., a study was made of the behaviour of the ether-soluble fraction on ion-exchange resins. The use of different resins enabled a separation of cations, anions and non-ionic substances. Since in general, different ions possess different affinities for a given ion-exchange resin, further separation could be effected by using ion-exchange chromatographical methods.

#### 5.6.1. *Column chromatography on Amberlite IRC-50 ($H^+$) and IR-45 ($OH^-$)*

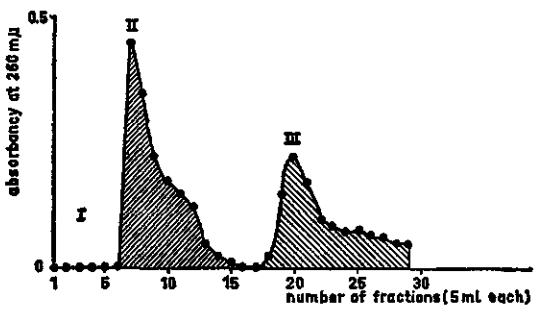
An experiment was conducted to fractionate the ether-soluble fraction on columns of the weakly acidic cation-exchange resin Amberlite IRC-50 ($H^+$), 100–200 mesh, and the weakly alkaline anion-exchange resin Amberlite IR-45 ($OH^-$), 100–200 mesh. The columns, 10 × 1 cm, were washed thoroughly with N HCl and N $NH_4OH$, care being taken that for the final washing of these resins HCl and $NH_4OH$, respectively, were used. Excessive acid and base were removed by treatment with water. One ml of the ether-soluble fraction, derived from 2 g of fresh bean nodules, was applied on top of the column and subsequently eluted with the following solutions:

IRC-50 ($H^+$), 25 ml water, 25 ml N NaCl, 25 ml N HCl, 50 ml N $NH_4OH$, 10 ml water.

IR-45 ($OH^-$), 25 ml water, 25 ml N NaCl, 25 ml N $NH_4OH$, 50 ml N HCl, 25 ml water.

Fractions of 5 ml were collected and the acidity inspected with lacmoid. After determination of the absorbancy at 260 mμ, the neutral, alkaline and acid fractions were pooled and after acidification with HCl, extracted overnight with ether. The ether extracts of each group, taken up in water, were divided into 5 portions and assayed with 5 bean plants. The results of the biological assay and the absorbancy at 260 mμ of the fractions obtained are presented in fig. 8a and 8b.

The first neutral fractions (1–8) from the cation-exchange resin IRC-50 ($H^+$) showed a distinct absorption at 260 mμ but did not contain A.S. The



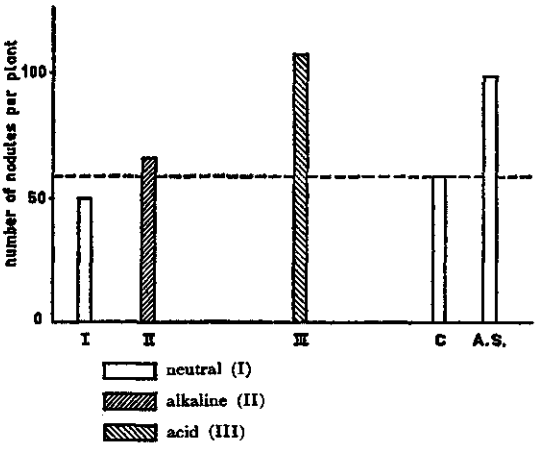
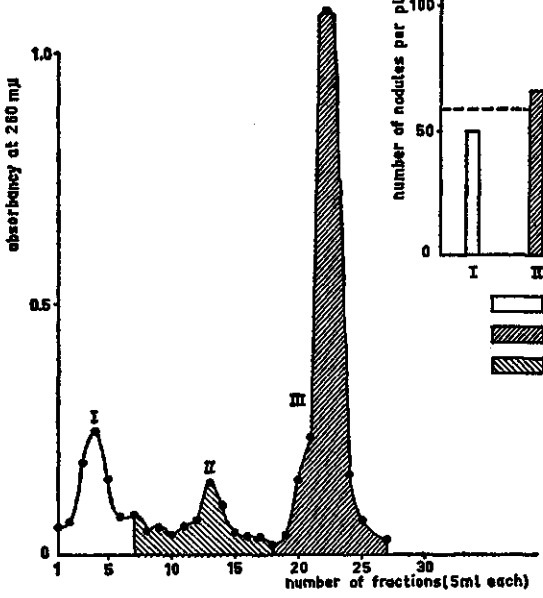




FIG. 8B. Comparison of the absorbancy at 260 mμ and the biological activity of neutral (I), alkaline (II) and acid (III) fractions from A.S. obtained by ion-exchange chromatography on Amberlite IR-45 ($OH^-$). C, control without any addition; A.S., extract from 100 mg fresh bean nodules.



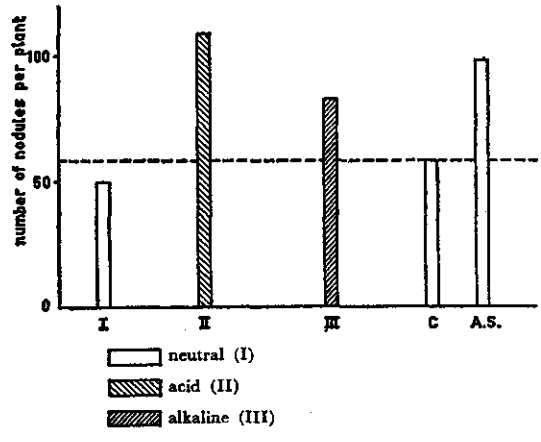




FIG. 8A. Comparison of the absorbancy at 260 mμ and the biological activity of neutral (I), acid (II) and alkaline (III) fractions from A.S. obtained by ion-exchange chromatography on Amberlite IRC-50 ($H^+$). C, control without any addition; A.S., extract from 100 mg fresh bean nodules.

acid fractions (9–18) on the contrary showed a distinct absorbancy and a strong biological activity. The alkaline fractions (19–27) although containing substances with a strong absorption at 260 mμ, contained A.S. only in low concentrations.

No biological activity and no U.V. light-absorbing substances were present in the neutral fractions (1–5) from the anion-exchange resin IR-45 ($OH^-$). The alkaline fractions (6–17) with a strong absorption at 260 mμ only showed slight biological activity. In contrast, high biological activity was found in the acid fractions (18–27) which had a lower absorbancy in U.V. light than those of the alkaline group.

In general, these results were confirmed by a number of similar experiments. This was also the case when the sequence of acid and alkaline elution was changed. No activity was present in the first neutral fractions obtained by elution with water from both resins. A strict comparison of the biological activities of the alkaline and acid fractions was not possible since the amount of substances in each group of fractions was unknown. But if the activity was calculated per unit of U.V. light-absorbing substances, assuming that the absorbancy was directly related to the amount of these substances, the highest activity was found in the fractions from both resins eluted with HCl.

*5.6.2. Column chromatography on IMAC-C12 ($H^+$)*

In a subsequent experiment the ether-soluble fraction from root nodules was fractionated on the strongly acidic cation-exchange resin IMAC-C12 ($H^+$), 0.3-2 mm. One ml of the extract, representing 2 g of nodules, was applied on top of the column (20 × 1 cm) of this resin and subsequently eluted with 50 ml water, 250 ml N $NH_4OH$, 100 ml water, 200 ml N HCl. Five-ml fractions were collected and their acidity and absorbancy at 260 mμ were determined. The fractions were pooled and divided in 7 groups: two neutral groups (I no. 1–19; II no. 20–42), then three alkaline groups (III no. 43–49; IV no. 50–72; V no. 73–85) and finally two acid groups (VI no. 86–100 and VII no. 101–112). Each group, after acidification, was extracted overnight with ether and the ether extracts taken up in water. The extracts were divided each into 5 portions for testing with 5 plants in the bean assay. The results are shown in fig. 9.

No biological activity was detected in the first two neutral fractions, although a distinct absorbancy at 260 mμ was observed in both groups (I and II). Three peaks in absorbancy were present in the alkaline ones and the groups (III, IV and V) showing these maxima contained A.S. The biological activity of these fractions was proportional to the absorbancy at 260 mμ. The remaining two acid groups, especially group VI, also contained U.V. light-absorbing substances. When the fractions of group VI were applied to the test plants, nodulation was severely inhibited, whereas with those of group VII nodulation was highly stimulated. The inhibitory effect of group VI might be ascribed either to the presence of inhibitors or to a supra-optimal concentration of stimulating substances. The results obtained with this strongly acidic

resin resembled those of the weakly-acidic resin IRC-50, but the separation of the different components was more complete and seven peaks, probably corresponding to 7 substances, could be observed in the effluent of the column.

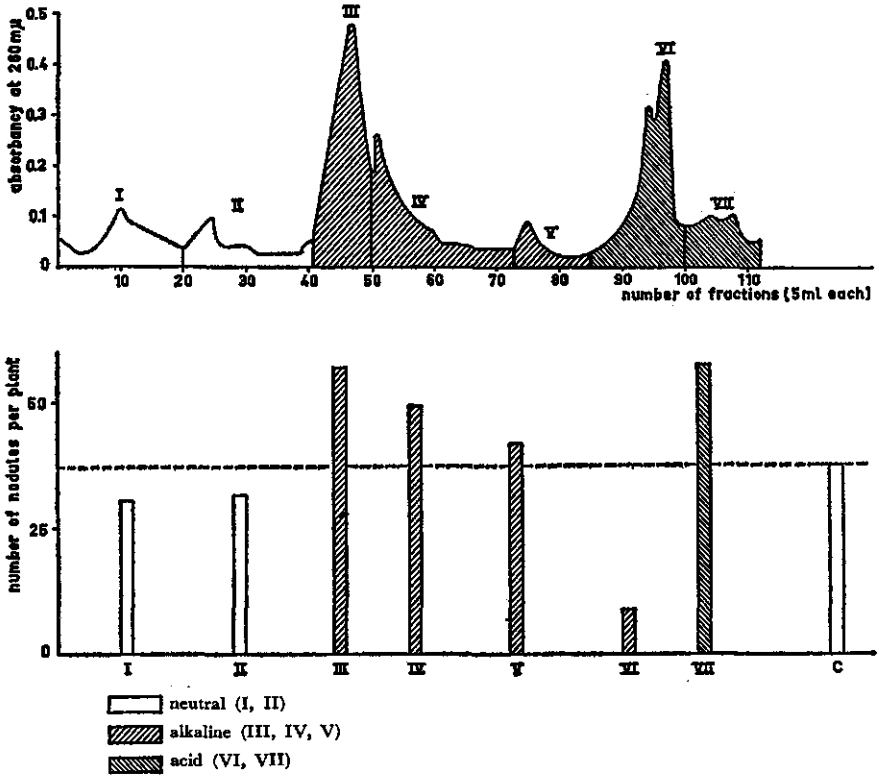



FIG. 9. Comparison of the absorbancy at 260 mμ and the biological activity of neutral (I, II), alkaline (III, IV, V) and acid (VI, VII) fractions from A.S. obtained by ion-exchange chromatography on IMAC-C12 ($H^+$). C, control without any addition.

CHAPTER 6

# THE INFLUENCE OF THE ENVIRONMENTAL CONDITIONS ON NODULATION OF BEAN PLANTS AND THEIR RESPONSE TO A.S.

Nodulation of leguminous plants depends on environmental conditions. Even in the greenhouse, in which the experiments described so far were carried out, great variation in the number of nodules formed in different seasons was observed. A more complete account of these experiments relative to seasonal variation will be given below.

The seasonal influence on nodulation of bean plants and their response to added A.S. is clearly demonstrated in the experiments carried out from August 1959-January 1961. In 1959 the bean variety Amerikaanse was the test plant, but later on the variety Walcherse Witte was used. The A.S. supplied to the plants was initially crude root exudates of bean plants with effective nodules and later on an ether extract of root exudates or of effective bean nodules. In all experiments the amount of A.S. supplied per plant was equivalent to 100 ml used nutrient solution or to 100 mg fresh nodules.

In fig. 10 the mean number of nodules on the bean plants, with or without added A.S., has been plotted against the data of inoculation. This date was chosen in view of the fact that environmental conditions, particularly those during the period following inoculation, affect nodulation (QUISPEL, 1958;

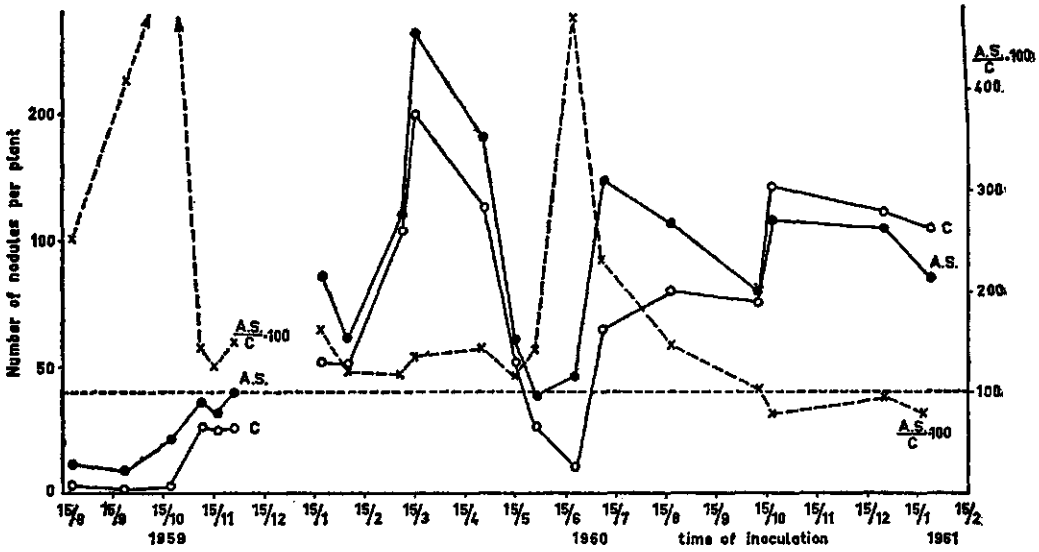



FIG. 10. Seasonal variation of nodulation of bean plants, inoculated with *Rhizobium* strain Bokum, with or without added A.S. Bean varieties used: Amerikaanse (1959) and Walcherse Witte (1960, 1961).

BARRIOS *et al.*, 1963). Percentage increase of nodule number due to A.S. has also been plotted.

Intense light and high temperature conditions (mean maximum air temperature 25–28°) were prevailing from August until October 1959 and the nodulation of the control bean plants was poor. Under such conditions nodulation was highly stimulated by A.S. (400–700%). In November and December 1959 nodulation of the control bean plants was satisfactory and only a slight stimulation due to A.S. was observed (20–40%). The graph for nodule number of the control bean plants in 1960 and early 1961 exhibits two maxima *viz.* in early spring (March) and late fall (October). Again a minimum was reached during a short spell of warm sunny weather in June (mean maximum air temperature 28–34°C). Only slight stimulation was obtained with A.S. in the spring (20–40%) but in June nodulation was considerably increased after application of A.S. (200–400%). Small effects were produced with A.S. in September and October 1960, and in the following months nodulation was even slightly depressed by nodule extract.

Additional experiments carried out during hot sunny spells in 1961 and 1963 confirmed the above-mentioned results, that under such climatic conditions nodulation of bean plants is poor and response to added A.S. is high. The mean maximum air temperatures during these two periods were 28–32°C and 26–30°C, respectively. During these periods the mean numbers of nodules of the control bean plants were 4.0 and 17.4 per plant and those of the plants treated with A.S. 53.8 and 48.0 per plant, respectively.

Summarizing the results, it can be said that the highest effect of A.S. was obtained when nodulation was poor *viz.* under hot, sunny conditions. The two main factors, which determine the seasonal differences seem to be temperature and light. Therefore, in the subsequent experiments these factors were investigated in more detail.

### A. TEMPERATURE

The effect of temperature on the growth of organisms can usually be ascribed to a non-specific effect on various processes. In some instances, the adverse effect of extreme temperatures can be overcome by the addition of inorganic ions, amino acids, vitamins, yeast extract or beef extract (see LANGRIDGE, 1963). Most studies, recorded in the literature, have been carried out with microorganisms, but only few data are available concerning higher plants. It has been reported by GALSTON (1957) that adenine increased the growth of pea plants, cultivated at temperatures deviating from the optimum values, but this report could not be confirmed by others (LANGRIDGE & GRIFFING, 1959). Subsequent experiments revealed that biotin or cytidine were able to prevent the high-temperature lesions in certain strains of *Arabidopsis*, grown under aseptic conditions (LANGRIDGE & GRIFFING, 1959). Substantially increased growth of pea plants at high temperatures was obtained upon spraying with sugar, ribosides and B-vitamins (KETELLAPPER, 1963). The response of corn plants to

I.A.A. was found by GALSTON (1957) to depend on the temperature of cultivation. The above-mentioned results suggest that at high temperatures the plants become heterotrophic for one or more compounds, which are normally synthesized.

A study of the effect of temperature on root-nodule formation revealed that optimal nodulation coincides with the optimum temperature for plant growth (see FRED *et al.*, 1932). In contrast, PATE (1961, 1962) found two maxima, at or near the extremes of the temperature range of plant growth, taking as an index the number of nodules formed on the primary root. Using the isolated-root-culture technique (BARRIOS *et al.*, 1963), it was shown that optimal nodulation of bean roots occurred at 25°C. Growth of the roots was not reduced at 17° and 30°C but nodulation was found to be diminished to 30%. Inhibition of nodulation by high temperature was confined to a period of three days after inoculation; no such effect was observed when the roots were transferred to 30°C after this period. Similar results have been obtained by the present author using intact pea plants (unpublished results).

The effect of A.S. on nodulation of bean plants at different temperatures was studied in the phytotron of the 'Instituut voor Biologisch en Scheikundig onderzoek van landbouwgewassen'. The plants were precultivated for ten days in a greenhouse and after inoculation with the Bokum strain, transferred to cabinets of the following temperatures: 18°, 20°, 25° and 30°C. By shading with cheese cloth, the light intensities in the first three cabinets were adjusted to 50 000, 35 000 and 20 000 ergs/cm² sec. At 30°C only the highest and the lowest light intensities were applied. Light was supplied by high-pressure mercury lamps (HPL 400, Philips). The plants were grown under continuous aeration in 1 liter jars, one plant per jar. One half of the plants received A.S. equivalent to 250 mg of fresh bean nodules. Due to differences in growth rate at different temperatures, the plants were harvested when the nodules had reached approximately their ultimate size. The plants grown at 30° and 25°C were harvested 14 days after inoculation and those grown at 20° and 18°C, 21 and 27 days, respectively, after inoculation. It may be seen from table 31 that with all light intensities used, maximum nodulation occurred at 25°C. At lower temperatures the number of nodules was reduced, whereas at 30°C nodulation was almost completely inhibited. The effect of A.S. on

TABLE 31. The effect of A.S. on nodulation[1]) of bean plants, inoculated with *Rhizobium* strain Bokum, grown at different temperatures and light intensities.

| Temperature (°C) | Light intensity (ergs/cm² sec.) | | | | | |
|---|---|---|---|---|---|---|
| | 20000 | | 35000 | | 50000 | |
| | None | A.S. | None | A.S. | None | A.S. |
| 18 | 48 | 49 | 43 | 47 | 74 | 59 |
| 20 | 77 | 106 | 78 | 89 | 73 | 93 |
| 25 | 93 | 58 | 132 | 118 | 135 | 120 |
| 30 | 3 | 11 | – | – | 6 | 11 |

[1]) Mean number of nodules per plant of 8 replicates.

nodule number was rather small. Some stimulation was observed at 20°C and 30°C and no effect at 18°C. At 25°C, the optimum temperature for nodulation, nodule number was even depressed by A.S.

### B. LIGHT

Two types of light reactions can be distinguished in green plants *viz.* the photo-energetic reactions and the photo-stimulus ones (WASSINK, 1954). In photosynthesis, which belongs to the first group, high light intensities are required for obtaining measurable effects and, within certain limits, a linear relationship exists between the energy supplied and the effects produced. In contrast herewith, processes belonging to the second group can be initiated with very low light intensities. Photomorphogenesis, i.e. the light control of development and differentiation of a plant, belongs to the latter group.

Nodulation in leguminous plants depends on photosynthesis for the supply of carbohydrates. When the light intensity decreases, nodulation is reduced, whereas more light (WILSON, 1940), an increase of $CO_2$ (WILSON, 1940) or a supply of sugar to the leaves (VAN SCHREVEN, 1959) favour nodulation. On the other hand, nodulation is reduced when nitrogen is applied to the roots (WILSON, 1940) or to the leaves (VAN SCHREVEN, 1959; CARTWRIGHT & SNOW, 1962). To explain these facts, the hypothesis has been put forward that the ratio of carbohydrates to nitrogen in the plant acts as a controlling mechanism for nodulation (WILSON, 1940). According to this hypothesis, the effect of light on nodulation can be mainly explained as the effect of light on photosynthesis. The effect of light intensity on nodulation was shown in table 31. At temperatures deviating from the optimum (25°C), light intensity had little influence on nodulation. At 25°C nodulation was enhanced with increased light intensity up to 35 000 ergs/cm² sec. With further rise in light intensity, no increase in nodule number was observed. No effect of light intensity on the response of nodulation to A.S. was observed.

Photosynthesis is independent of wave length of light, provided that the same number of quanta is absorbed by the plant. In contrast to photosynthesis, photomorphogenesis is wave-length-dependent. A useful method to separate these two processes is the application of far-red radiation (700–800 mμ), which is only slightly active in photosynthesis, but is of considerable importance in photomorphogenesis (MOHR, 1962). The latter is thought to be due the presence within plants of a growth-controlling factor, phytochrome, a bright blue protein, having two interconvertible forms, $P_{660}$ and $P_{730}$, with absorption maxima in the red part of the spectrum at 660 mμ and in far red at 730 mμ. The conversion reaction is:

$$P_{660} \underset{730\ m\mu}{\overset{660\ m\mu}{\rightleftharpoons}} P_{730}$$

As stated earlier (see Introduction) kinetin or kinetin-like substances may be involved in root-nodule formation. A number of widely different metabolic effects has been claimed for kinetin (review see STRONG, 1958; MILLER, 1961; VAN EYCK, 1963), but special mention should be made of the apparently similar results obtained with kinetin and red light, and the enhancement of kinetin of red light effects (MILLER, 1956, 1958; SCOTT & LIVERMAN, 1956; ROMBACH, 1960). Therefore, it seemed logical to investigate the requirement for red light in the nodulation process. For this reason the plants were grown in light of different spectral regions. To avoid differences due to photosynthesis, a comparison was made of plants grown in light of approximately equal numbers of quanta. Plants grown in light of different spectral regions exhibited a different degree of stem elongation (see e.g. STOLWIJK, 1954; DE LINT, 1960). To circumvent this complication, additional experiments were designed with plant systems with limited shoot growth *viz.* rooted leaves and decapitated plants. Since red-light effects in photomorphogenic processes could be reversed by treatment with far-red light, the effect of the latter was also studied in the nodulation process. Finally the effects of A.S., kinetin and I.A.A. were studied in nodulation of plants with or devoid of red light.

### 6.1. THE EFFECT OF LIGHT OF DIFFERENT SPECTRAL REGIONS

Firstly the growth and nodulation of pea plants were studied in light of different spectral regions. The plants were precultivated under aseptic conditions in white light (20 000 ergs/cm² sec.) in a nitrogen-free medium for two weeks. Then the nutrient solution was renewed and either combined nitrogen (first experiment), or *Rhizobium* (second experiment) were added to the nutrient solution. The plants were transferred to cabinets, in which they were exposed to equal numbers of quanta of blue (1), green (2) or red (3) light. Cabinets with white (4) and red (6) light of an intensity approximately similar to that of blue (1) were included to compare the growth of plants at the same energy level. An additional cabinet with blue light (5) of an intensity approximately similar to red (3) was also used.

#### 6.1.1. *Pea plants with combined nitrogen*

Ammonium nitrate, 100 mg N per liter, was given to pea plants two weeks after germination, when the plants were transferred to the above-mentioned cabinets (coloured-light cabinets). The plants were harvested after a further two weeks and the dry weight of the shoots and roots determined. To study the effect of light of different spectral regions on stem elongation, the total length of the shoots and the number of internodes were recorded (table 32). Approximately the same dry-matter production was obtained with light of different wave lengths at equal quantum level. When the plants had been exposed to red (6) and white (4) at approximately the same energy level as blue (1), considerably higher values for dry weight were obtained with the former two light treatments. Slight differences existed in shoot length and number of internodes of plants grown at equal number of quanta.

TABLE 32. The effect of light of different spectral regions on the growth[1]) of pea plants with combined nitrogen.

| Number of cabinet | Light quality | Light intensity (ergs/cm² sec.) | Dry wt. (g) of | | Length of the shoot (mm) | Number of internodes |
|---|---|---|---|---|---|---|
| | | | Shoot | Root | | |
| 1 | Blue[2]) | 17000 | 0.24 | 0.09 | 173 | 11.2 |
| 2 | Green[2]) | 15000 | 0.26 | 0.10 | 194 | 12.5 |
| 3 | Red[2]) | 12000 | 0.25 | 0.09 | 175 | 12.6 |
| 4 | White | 16000 | 0.27 | 0.14 | 161 | 11.9 |
| 5 | Blue | 13000 | 0.22 | 0.10 | 156 | 10.0 |
| 6 | Red | 16000 | 0.37 | 0.11 | 219 | 13.6 |

[1]) Mean values per plant of 6–8 replicates.
[2]) Approximately equal numbers of quanta.

Therefore, to detect photomorphogenic effects of light on nodulation, the plants had to be grown in light of equal numbers of quanta.

### 6.1.2. *Inoculated pea plants*

In a subsequent experiment two series of pea plants were inoculated with an effective (PRE) and an ineffective (P8) *Rhizobium* strain, respectively. No combined nitrogen was given to the plants. At harvest, 15 days after inoculation, the number and the dry weight of leaves, stem and roots were determined. The nitrogen content and the number of lateral roots of the second order of the plants infected with strain P8 were determined in addition. The results are recorded in tables 33 A and 33 B.

When a comparison is made of plants grown at equal quantum levels, it will be seen that the highest number of nodules occurred when red light was supplied (cabinet 3); green light (2) gave intermediate and blue light (1) the lowest number. That these differences did not depend on the differences in dry-matter

TABLE 33A. The effect of light of different spectral regions on number[1]) of nodules and of lateral roots of the 2th order of pea plants, inoculated with *Rhizobium* strains PRE and P8, respectively.

| Number of cabinet | Light quality | Light intensity (ergs/cm² sec.) | Rhizobium strain | | | |
|---|---|---|---|---|---|---|
| | | | PRE | P8 | | |
| | | | Nodule number | Nodule number | Root number | $\frac{\text{Nodules}}{\text{Nod.}+\text{Roots}} \times 100$ |
| 1 | Blue[2]) | 17000 | 57 | 73 | 6 | 92 |
| 2 | Green[2]) | 15000 | 63 | 95 | 40 | 70 |
| 3 | Red[2]) | 12000 | 67 | 177 | 73 | 71 |
| 4 | White | 16000 | 71 | 140 | 53 | 73 |
| 5 | Blue | 13000 | 50 | 63 | – | – |
| 6 | Red | 16000 | 96 | 179 | – | – |

[1]) Mean values per plant of 6 replicates.
[2]) Approximately equal numbers of quanta

TABLE 33B. The effect of light of different spectral regions on dry weight[1]) and nitrogen content of pea plants inoculated with *Rhizobium* strains PRE and P8, respectively.

| Number of cabinet | Light quality | Light intensity (ergs/cm² sec.) | Rhizobium strain | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | PRE | | | | P8 | | | | | | |
| | | | Dry wt. (mg) | | | | Dry wt. (mg) | | | | % N | | |
| | | | Leaves | Stems | Roots | Total | Leaves | Stems | Roots | Total | Leaves | Stems | Roots |
| 1 | Blue[2]) | 17000 | 80 | 62 | 55 | 197 | 93 | 63 | 58 | 214 | 5.6 | 3.4 | 2.9 |
| 2 | Green[2]) | 15000 | 96 | 78 | 64 | 238 | 97 | 62 | 63 | 222 | 5.4 | 2.5 | 3.0 |
| 3 | Red[2]) | 12000 | 90 | 68 | 58 | 216 | 90 | 50 | 70 | 210 | 5.5 | 3.0 | 2.7 |
| 4 | White | 16000 | 122 | 70 | 70 | 262 | 98 | 50 | 60 | 218 | 4.8 | 2.7 | 2.8 |
| 5 | Blue | 13000 | 80 | 68 | 66 | 214 | 72 | 66 | 63 | 201 | 5.5 | 3.1 | 3.0 |
| 6 | Red | 16000 | 123 | 80 | 105 | 308 | 97 | 72 | 73 | 242 | 4.9 | 1.8 | 2.6 |

[1]) Mean values per plant of 6 replicates.
[2]) Approximately equal numbers of quanta.

production, as caused by light treatment, may be seen from table 33 B. Plants inoculated with the ineffective strain P8, grown in blue (1) and red (3) had nearly equal values for total dry weight. Nevertheless, the number of nodules and lateral roots of the second order of plants grown in red light were twice and twelve times, respectively, higher than those of plants treated with blue light (33 A). In the case of plants with effective nodules, considerably bigger nodules were present on plants grown in red light than those in blue. Since the former nodules already turned red, which indicates nitrogen fixation, the small difference in dry weight between these plants may have been due to a different nitrogen supply.

### 6.1.3. *Rooted bean leaves*

The effect of light quality on nodulation was also investigated in rooted bean leaves. With this system only slight changes due to light treatment occurred in the leaves and photomorphogenic influences may only affect the root system. Three weeks old leaf cuttings were inoculated with strain Bokum and transferred to cabinets blue (1), red (3) and white (4). The nodules were counted after 25 and 30 days and at the latter date the weights of nodules and roots were determined (table 34).

TABLE 34. The effect of light of different spectral regions on nodulation[1]) and root growth[1]) of rooted bean leaves, inoculated with *Rhizobium* strain Bokum.

| Number of cabinet | Light quality | Light intensity (ergs/cm² sec.) | Number of nodules at | | Fresh wt. of nodules (mg) | Dry wt. of roots (mg) |
|---|---|---|---|---|---|---|
| | | | 25 days | 30 days | | |
| 1 | Blue[2]) | 17000 | 2.4 | 4.6 | 17 | 59 |
| 3 | Red[2]) | 12000 | 6.3 | 10.0 | 29 | 58 |
| 4 | White | 16000 | 9.3 | 13.5 | 48 | 69 |

[1]) Mean values per leaf of 14 replicates.
[2]) Approximately equal numbers of quanta.

The results obtained confirm those obtained with intact pea plants. Again nodulation was poor in blue and considerably higher in red light (see plate III). The leaves in white light were exposed to a higher number of quanta than those in blue and red light. Consequently, more roots and nodules were formed due to a higher level of photosynthesis.

Summarizing, it may be concluded that red light seems to be important for root nodule formation.

### 6.2. The effect of supplementary irradiation with red and far-red light on nodulation of leguminous plants growing in blue light

The experiments in previous sections suggest that red light has a specific effect on nodule formation. The experiments carried out in light of different spectral regions, however, have the disadvantage that in addition to nodule formation, other effects e.g. stem elongation etc. can be observed. Since it is known that in a number of photomorphogenic processes, red-light reactions can be reversed by far-red light, it seemed worth while to study the interaction of red and far-red light on nodule formation.

At first the effect of supplementary irradiation of red and far-red light was studied in bean plants growing in blue light. Decapitated bean plants, inoculated with strain Bokum, were transferred to a cabinet with blue light (17 000 ergs/cm² sec.), photoperiod 20 hours (exp. 1) or 16 hours (exp. 2 and 3). In all experiments decotylized bean plants were used, but in the latter a comparison was also made between the plants with or without cotyledons. Immediately after the blue light period, the plants were exposed daily to 15 minutes of red light (8 000 ergs/cm² sec.), or to far-red light (18 000 ergs/cm² sec.) or to both light treatments in succession. Since photosynthesis took

Table 35. The effect of supplementary irradiation with red (15 min. 8000 ergs/cm² sec.) and far-red (15 min. 18000 ergs/cm² sec.) light on nodulation[1]) of bean plants, inoculated with *Rhizobium* strain Bokum. Photoperiod 20 hr. of blue light, 17000 ergs/cm² sec.

| Light treatment | Number of nodules | | | | | |
|---|---|---|---|---|---|---|
| | Exp. 1 | Exp. 2 | | Exp. 3 | | |
| | 20 days | 15 days | 21 days | 11 days | 16 days | 18 days |
| Decotylized plants | | | | | | |
| C | 3.8 | 2.0 | 2.0 | 3.5 | 6.7 | 7.5 |
| R | – | 5.5 | 9.5 | 2.7 | 8.7 | 13.3 |
| I | 1.8 | – | – | 1.3 | 3.5 | 6.7 |
| R–I | – | – | – | 5.2 | 6.2 | 9.3 |
| I–R | – | 4.5 | 7.0 | – | – | – |
| With cotyledons | | | | | | |
| C | – | – | – | 10.8 | 18.4 | 20.4 |
| I | – | – | – | 6.4 | 7.5 | 8.6 |
| R–I | – | – | – | 9.2 | 13.4 | 18.6 |

[1]) Mean values per plant of 6 replicates. C = control, not irradiated; I = far red; R = red.

place during the 15 minutes of red light treatment, all the plants not treated with red light were exposed for another 15 minutes to blue light.

Root nodules appeared slowly in plants grown in blue light. The first nodules became visible about 11 days after inoculation, in contrast with about 7 days in white or red light. The nodules remained small and at harvest no weight determination could be made. From table 35 it is obvious that far red reduced nodulation to about 50%, whereas red light significantly increased it. Red light partially eliminated the effect of far-red and the reverse was also true. The presence of cotyledons increased the number of nodules formed.

### 6.3. The effect of supplementary irradiation with far-red light on nodulation of leguminous plants growing in red light

The effect of supplementary far-red light was also studied in leguminous plants growing in red light. The plants were precultivated in white light up till two weeks after germination, then inoculated and transferred to a cabinet with red light (8 000 ergs/cm² sec.). Far-red light was given at an intensity of 18 000 ergs/cm² sec.

#### 6.3.1. *The effect of cotyledons on the response to far-red light*

In preliminary tests it had been observed that only slight effects of far red on nodulation were obtained in plants with cotyledons. Therefore in a subsequent experiment a comparison was made between intact pea plants and plants decotylized at the time of light treatment. At the same time the plants were inoculated with *Rhizobium* strain PRE. Half of the plants received 30 minutes of far-red light at the end of the light period. The number of nodules were counted 7 and 14 days after treatment (table 36). The inhibitory effect of far red on nodulation of plants grown in red light is clearly demonstrated.

Table 36. The effect of supplementary irradiation with far-red light (30 min. 18000 ergs/cm² sec.) on nodule number[1]) of intact and decotylized pea plants, inoculated with *Rhizobium* strain PRE. Photoperiod 20 hours of red light, 8000 ergs/cm² sec.

| Far red | Cotyledons | Number of nodules at | |
|---|---|---|---|
| | | 7 days | 14 days |
| – | – | 14.2 | 30.5 |
| + | – | 2.2 | 9.3 |
| – | + | 23.5 | 37.8 |
| + | + | 11.4 | 32.4 |

[1]) Mean values per plant of 12–16 replicates.

It is of interest that the presence of cotyledons reduces the inhibitory action of far-red light. The effect of far red is very pronounced in the first counting but less clear later on, especially in the presence of cotyledons. These results sug-

gest that substances, which eliminate the effect of far-red light may be present or produced by the cotyledons and the early-formed nodules.

The inhibitory effect of far red was also found in pea plants, inoculated with the ineffective *Rhizobium* strain P8. In such an experiment the mean number of nodules in the control plants was 37 and that of plants treated with far-red light only 25.

### 6.3.2. *The effect of far-red light, applied at different periods of time after inoculation*

So far, the far-red treatment was applied daily for 30 minutes during the entire experimental period. A subsequent experiment was conducted to investigate the effect of far-red light applied in different amounts: 0, 5, 10 and 30 minutes daily at the beginning of the night, throughout the experimental period; or in the same amount (30 minutes) during the first week after inoculation only, or during the remaining period only. The experiment was conducted with decotylized pea plants inoculated with *Rhizobium* strain PRE. The mean numbers of nodules, determined 14 and 19 days, respectively, after inoculation are given in table 37. It will be seen that the saturation level of far red was already reached at the lowest level applied, five minutes of irradiation inhibited nodulation to the same degree as 30 minutes. Far red applied during the first week had almost the same effect as irradiation during the entire period (see number of nodules at harvest). This could mean that all the nodules, counted on the control plants at harvest time, had been initiated during the first seven days after inoculation. An alternative explanation would be that nodule initiation also occurred after this period and that exposure to far-red light during 7 days had an after-effect, inhibitory to further nodulation. Evidence supporting the latter view may be derived from the fact that treatment with far-red light from the 8th day after inoculation seriously reduced nodule number at harvest time.

TABLE 37. The effect of supplementary irradiation with far-red light (18000 ergs/cm² sec.) on nodulation of decotylized pea plants, inoculated with *Rhizobium* strain PRE. Photoperiod 20 hours of red light, 8000 ergs/cm² sec. Far red was applied either in different amounts during the entire experimental period or in one amount during different parts of the experimental period.

| Far-red light in minutes per day | Period of irradiation in days after inoculation | Number of nodules at | |
|---|---|---|---|
| | | 14 days | 19 days |
| 0 | – | 19.0 | 60.0 |
| 5 | 0–19 | 13.7 | 28.0 |
| 10 | 0–19 | 10.6 | 34.8 |
| 30 | 0–19 | 15.1 | 23.7 |
| 30 | 0–7 | 11.2 | 22.8 |
| 30 | 8–19 | 18.4 | 27.3 |

[1]) Mean values per plant of 10 replicates.

### 6.3.3. *The effect of far-red light, applied at the beginning or at the end of the dark period*

It is well known that far-red light eliminates the photomorphogenic effects induced by red light when applied immediately after the latter. When a dark period is inserted, the degree of reversal depends on the length of the dark period (DOWNS, 1959; TOOLE, 1959). In view of these results, the effect of far red on nodulation was studied when applied at the beginning or at the end of the dark period. The experiments were carried out in two cabinets with red light of approximately equal light intensity (8 000 ergs/cm² sec.). Each cabinet was divided into two compartments, one receiving only red light and the second one in addition receiving far-red light from above, either at the beginning or at the end of the dark period. Far-red light was given during 30 minutes at an intensity of 18 000 ergs/cm² sec.

In the first experiment decapitated bean plants, inoculated with strain Bokum, were treated according to the following irradiation scheme:

Cabinet 1, R-I-D: red (16 hr), far red (0.5 hr), dark (7.5 hr)
R-D : red (16 hr), dark (8 hr)
Cabinet 2, R-D-I: red (16 hr), dark (7.5 hr), far red (0.5 hr)
R-D : red (16 hr), dark (8 hr).

At harvest, three weeks after inoculation, the number and fresh weight of the nodules were determined (table 38). It may be seen that far red given at the beginning of the dark period inhibited nodulation more strongly than when applied at the end. In the latter case the inhibition was small or even absent.

TABLE 38. The effect of far-red light (30 min. 18000 ergs/cm² sec.), applied either at the beginning or at the end of the dark period, on nodulation[1]) of decotylized bean plants, inoculated with *Rhizobium* strain Bokum. Photoperiod 16 hr of red light, 8000 ergs/cm² sec.

| | Cabinet 1 | | | Cabinet 2 | | |
|---|---|---|---|---|---|---|
| | R–D | R–I–D | $\frac{\text{R–I–D}}{\text{R–D}} \times 100$ | R–D | R–D–I | $\frac{\text{R–D–I}}{\text{R–D}} \times 100$ |
| Number of nodules at | | | | | | |
| 9 days | 8.8 | 5.5 | 62 | 8.2 | 9.2 | 112 |
| 15 days | 10.7 | 7.0 | 67 | 15.5 | 13.8 | 89 |
| Fresh wt. of nodules at | | | | | | |
| 15 days | 0.029 | 0.018 | 62 | 0.047 | 0.039 | 83 |

[1]) Mean values per plant of 6 replicates.

The above-mentioned results were confirmed by experiments with rooted bean leaves. Primary bean leaves were inoculated with strain Bokum two weeks after excision and transported to the cabinets, where they were exposed to light as described in the previous experiment. The numbers of nodules were counted 9 and 15 days, respectively, after inoculation (table 39). The data

TABLE 39. The effect of far-red light (30 minutes, 18000 ergs/cm² sec.) applied either at the beginning or at the end of the dark period, on nodulation[1]) of rooted bean leaves, inoculated with *Rhizobium* strain Bokum. Photoperiod 16 hours of red light, 8000 ergs/cm² sec.

| Light treatment | Cabinet 1 | | | Cabinet 2 | | |
|---|---|---|---|---|---|---|
| | R–D | R–I–D | $\frac{\text{R–I–D}}{\text{R–D}} \times 100$ | R–D | R–D–I | $\frac{\text{R–D–I}}{\text{R–D}} \times 100$ |
| Number of nodules at | | | | | | |
| 9 days | 5.0 | 1.8 | 37 | 7.2 | 6.8 | 94 |
| 15 days | 16.8 | 8.3 | 49 | 10.8 | 12.4 | 115 |

1) Mean values per plant of 6 replicates

obtained confirmed those of the experiment with decapitated bean plants *viz.* a pronounced effect of far red when applied immediately at the beginning of the dark period, and almost none when it was given at the end of the dark period.

### 6.4. THE EFFECT OF SUPPLEMENTARY IRRADIATION WITH RED AND FAR-RED LIGHT ON NODULATION OF LEGUMINOUS PLANTS GROWING IN WHITE LIGHT

The results obtained in previous sections clearly demonstrate that the reversal of the effect of red light on nodulation by far-red light depends on the duration of the dark period between the red and far-red light. It seemed of interest to determine the critical length of this period in more detail. For this purpose the plants were grown in white light, which contains red light (see fig. 1) at a higher light intensity (30 000 ergs/cm² sec.) in a shorter photoperiod, 8 hours. Consequently the dark period was prolonged to 16 hours.

#### 6.4.1. *The effect of far-red light, applied after different periods of darkness, on nodulation of bean plants*

Decotylized bean plants, inoculated with strain Bokum, were irradiated with far-red light (15 minutes, 50 000 ergs/cm² sec.) at the beginning of the dark period ($I_0$), 4 hours later ($I_4$), in the middle of the night ($I_8$) and at the end of the night ($I_{16}$). To study the effect of cotyledons one series of plants with cotyledons was treated with far red at the beginning of the dark period ($+I_0$). Far red light was given daily during the week after inoculation. The plants were harvested 13 days after inoculation and the number and fresh weight of the nodules determined (table 40). It is evident that the inhibition of nodulation by far red was most pronounced when applied immediately after the light period ($I_0$). This effect gradually diminished when the dark period, before treatment with far red, increased to 8 hours. At variance with the results of a number of similar experiments (tables 38, 39 and 41 and some unpublished results) far red supplied at the end of the dark period ($I_{16}$) gave a strong inhibition on nodulation. In agreement with previous experiments, only slight inhibition was observed with far red when the cotyledons were present. The

TABLE 40. The effect of far-red light (15 minutes, 50000 ergs/cm² sec.) applied after different periods of darkness (0, 4, 8 and 16 hours) on nodulation[1]) of decotylized and intact bean plants, inoculated with *Rhizobium* strain Bokum. Photoperiod 8 hours of white light, 30000 ergs/cm² sec.

| Light | Cotyledons | Number of nodules | In % of control | Fresh wt. of nodules (g) | In % of control |
|---|---|---|---|---|---|
| C | – | 16.5 | – | 0.038 | – |
| $I_0$ | – | 7.5 | 46 | 0.025 | 66 |
| $I_4$ | – | 10.2 | 62 | 0.030 | 79 |
| $I_8$ | – | 13.3 | 86 | 0.030 | 79 |
| $I_{16}$ | – | 11.3 | 69 | 0.018 | 47 |
| +C | + | 18.0 | – | – | – |
| $+I_0$ | + | 17.7 | 98 | – | – |

[1]) Mean values per plant of 6 replicates.

C = control, no far-red; $I_{0-16}$ = far-red after 0, 4, 8 and 16 hours, respectively, of darkness; + = no cotyledons removed.

nodules formed on the plants with cotyledons were smaller than those formed on decotylized plants and difficult to collect. Therefore the fresh weight of the nodules was not determined for the intact plants.

### 6.4.2. *The effect of far-red light, applied after different periods of darkness, on nodulation of decotylized pea plants*

The experiment concerning the inhibitory effect of far-red light, applied after different periods of darkness, was repeated with decotylized pea plants, inoculated with strain PRE. Cotyledons were removed 2 weeks after germination, but inoculation and light treatment were performed 4 days later since the plants were rather small. Light treatment was similar to that of the previous experiment *viz.* 8 hours/day white light and far red given at 0, 4, 8 and 16 hours after the light period. The plants were inspected for nodules 7 days after

TABLE 41. The effect of far-red light (15 minutes, 50000 ergs/cm² sec. applied after different periods of darkness (0, 4, 8 and 16 hours) on nodulation[1]) of decotylized pea plants, inoculated with *Rhizobium* strain PRE. Photoperiod 8 hours of white light, 30000 ergs/cm² sec.

| Light treatment | Nodulated plants (%) at 7 days | Nodules | | | |
|---|---|---|---|---|---|
| | | Number at 11 days | In % of control | Number at 14 days | In % of control |
| C | 67 | 15.3 | – | 19.0 | – |
| $I_0$ | 33 | 4.9 | 32 | 15.0 | 74 |
| $I_4$ | 50 | 6.6 | 43 | 11.5 | 61 |
| $I_8$ | 67 | 6.3 | 41 | 21.3 | 112 |
| $I_{16}$ | 67 | 18.4 | 120 | 21.4 | 114 |

[1]) Mean values of 12 replicates.

C = control, no far-red, $I_{0-16}$ = far-red after 0, 4, 8, 16 hours respectively, of darkness.

inoculation and from this day the treatment with far red was stopped. Numbers of nodules were counted after a further 4 and 7 days, respectively (table 41). The results obtained with peas were almost similar to those with beans. Inhibition of nodulation by far red was most pronounced when applied at the beginning of the dark period and still strong when far red was given after 4 hours of darkness ($I_4$). Treatment in the middle of the night gave a considerable reduction of nodule numbers at the first counting but a slight increase in the second one. Far-red treatment at the end of the night slightly increased nodulation in both countings.

Summarizing, it can be concluded that the inhibitory effect of far red on nodulation is only visible when applied within the first 8 hours of the dark period.

6.4.3. *The reversibility of the effect of red and far-red light on nodulation*

In the preceding sections, it has been shown that far-red light eliminates the stimulatory effect of red light on nodulation. The same photoreversibility has been established for other plant systems, e.g. germination of lettuce seeds, expansion of etiolated bean leaves, growth of *Lemna minor* (see HENDRICKS & BORTHWICK, 1963). When these systems are exposed alternately to red and far-red light, the final irradiation will determine the ultimate result. It therefore seemed of interest to investigate if the same holds true for root-nodule formation.

In the following experiments, decotylized pea and bean plants received daily 8 hours of white light (30 000 ergs/cm² sec.) and were then exposed to red (8 000 ergs/cm² sec.) and far-red light (50 000 ergs/cm² sec.) according to the following scheme:

C, control, no supplementary irradiation.
I, 5 min. far-red light
I-R, 5 min. far-red, 5 min. red light
I-R-I, 5 min. far-red, 5 min. red, 5 min. far-red light
I-R-I-R, 5 min. far-red, 5 min. red, 5 min. far-red, 5 min. red light.

This scheme of irradiation was maintained during the first week after inoculation. One experiment with peas and two with beans were carried out (table 42). In all experiments the first treatment with far-red light considerably reduced nodule number, whereas red light applied afterwards partly abolished the inhibiting effect of far-red light. However, when far-red light was given once more, no inhibition was observed in the first countings and even stimulation occurred in the second ones. Slight stimulation was again observed when red light was applied hereafter indicating that red light counteracted the effect of far-red again. No explanation can be given for the higher numbers of nodules found in the treatments I-R-I and I-R-I-R.

More clear-cut results were obtained when the experiment was repeated with rooted bean leaves. Primary bean leaves, two weeks after rooting, were inoculated with *Rhizobium* strain Bokum, and treated with red and far-red

TABLE 42. Photoreversibility by red and far-red light on nodulation[1]) of decotylized pea and bean plants, inoculated with *Rhizobium* strains PRE and Bokum, respectively. Photoperiod 8 hours of white light, 30000 ergs/cm² sec.

| Light treatment | Peas | | Beans | | |
|---|---|---|---|---|---|
| | | | Exp. 1 | Exp. 2 | |
| | Number of nodules at | | Number of nodules at | Number of nodules at | |
| | 8 days | 14 days | 10 days | 8 days | 14 days |
| C | 15.3 | 19.0 | 16.5 | 12.7 | 28.5 |
| I | 4.9 | 15.0 | 7.5 | 9.2 | 16.3 |
| I–R | 6.9 | 15.3 | 13.3 | 10.0 | 23.2 |
| I–R–I | 6.8 | 27.0 | – | 10.3 | 31.2 |
| I–R–I–R | 7.7 | 26.8 | – | 12.3 | 37.7 |

[1]) Mean values per plant of 10 (peas) and 6 (beans) replicates

C = control, I = 5 min. far red, R = 5 min. red.

light as described in the previous experiments. However, the exposure to red and far-red light was 10 minutes instead of 5. The nodules were counted three times and at the last one the leaves were harvested (table 43).

From the results obtained it will be seen that the response of nodule weight to red and far-red light was more pronounced than that of nodule number. Treatment with far-red light depressed nodulation considerably. Subsequent treatment with red light clearly restored this process which was again depressed by subsequent treatment with far red, and restored by a further treatment with red light. No measurable effect of irradiation on dry weight of leaves and roots occurred.

TABLE 43. Photoreversibility by red and far-red light on nodulation[1]) of rooted bean leaves inoculated with *Rhizobium* strain Bokum. Photoperiod 8 hours of white light, 30000 ergs/cm² sec.

| Light treatment | Number of nodules at | | | Fresh wt. of nodules (g) | Dry wt. of | | |
|---|---|---|---|---|---|---|---|
| | 8 days | 11 days | 15 days | | leaves | petioles | roots |
| C | 0.9 | 1.5 | 3.8 | 0.012 | 0.12 | 0.02 | 0.06 |
| I | 0 | 1.0 | 4.4 | 0.004 | 0.11 | 0.03 | 0.06 |
| I–R | 0.2 | 1.3 | 2.2 | 0.009 | 0.11 | 0.03 | 0.06 |
| I–R–I | 0 | 0.7 | 0.8 | 0.002 | 0.12 | 0.03 | 0.06 |
| I–R–I–R | 0.3 | 1.7 | 4.3 | 0.006 | 0.11 | 0.03 | 0.06 |

[1]) Mean values per leaf of 10 replicates

C = control, I = 10 min. far red, R = 10 min. red.

## 6.5. The effect of growth substances on nodule formation of leguminous plants growing in light of different spectral regions

From the results obtained in previous sections it seems well established that red light participates in nodule formation. Since the light treatment is given to the shoot only, it is likely that some compound essential for nodulation is produced in the leaves, under the influence of red light, and transported to the root system. In the absence of red light the production of this substance is apparently curtailed, resulting in poor nodulation.

In the following experiments an attempt was made to substitute some growth substance for the requirement of red light in the nodulation process. Since nodule extract has been found in the present study to stimulate nodulation of bean plants, it seemed worth while to study its effect on plants growing in light of different spectral regions.

### 6.5.1. *Bean plants with cotyledons*

At first intact bean plants, with cotyledons, were used. At inoculation time, half of the plants received A.S. in an amount equivalent to 100 mg fresh bean nodules, and then they were transferred to cabinets with blue (1), green (2), red (3) and white (4) light, respectively. Number of nodules, estimated 17 days after inoculation, is recorded in table 44. In accordance with the results obtained with pea plants (table 33 A) it will be seen that more nodules were formed in red than in blue light. The response to A.S. was absent in blue and green, and slight in red and white light.

Table 44. The effect of A.S. (equivalent to 100 mg fresh bean nodules/plant) on nodulation[1]) of bean plants (cotyledons not removed), inoculated with *Rhizobium* strain Bokum. Photoperiod 20 hours of blue, green, red and white light, respectively.

| Number of cabinet | Light quality | Light intensity (ergs/cm² sec.) | Growth substances added | Nodules | |
|---|---|---|---|---|---|
| | | | | Number | Fresh wt. (g) |
| 1 | Blue[2]) | 17000 | None | 55.6 | 0.325 |
| | | | A.S. | 54.5 | 0.421 |
| 2 | Green[2]) | 15000 | None | 41.5 | 0.242 |
| | | | A.S. | 42.7 | 0.262 |
| 3 | Red[2]) | 12000 | None | 76.9 | 0.380 |
| | | | A.S. | 85.0 | 0.397 |
| 4 | White | 16000 | None | 119.7 | 0.663 |
| | | | A.S. | 141.7 | 0.847 |

[1]) Mean values per plants of 6 replicates
[2]) Approximately equal numbers of quanta.

### 6.5.2. *Decotylized bean plants*

In a subsequent experiment bean plants with cotyledons removed were used. Decotylization and inoculation with *Rhizobium* strain Bokum took place 11 days after germination when the plants were transferred to cabinets with light

of different spectral regions; blue (1), green (2) and red (3) of equal numbers of quanta and two cabinets one with blue (5) of an intensity similar to red (3) and one with red (6) light of an intensity similar to blue (1). At the same time two growth substances *viz.* A.S. (equivalent to 100 mg fresh bean nodules per plant) and kinetin (10 μg/plant) were added to a number of plants of each light treatment. The plants were harvested three weeks after inoculation, and the number and fresh weight of nodules determined (table 45). When exposed to light of equal numbers of quanta, nodulation in blue light was considerably lower than that in green and red light. With blue light at a lower light intensity (cabinet 5) practically no nodules were formed. Red light applied at an increased intensity (cabinet 6) gave much higher numbers of nodules than that of cabinet 3. At both intensities of blue light A.S. as well as kinetin gave a large increase of number and fresh weight of nodules. In green and red light, however, no such effect was observed.

TABLE 45. The effect of A.S. (equivelent to 100 mg of fresh bean nodules/plant), and kinetin (10 μg/plant) on nodulation[1]) of decotylized bean plants, inoculated with *Rhizobium* strain Bokum. Photoperiod 20 hours of blue, green and red light, respectively.

| Number of cabinet | Light quality | Light intensity (ergs/cm² sec.) | Growth substances added | Nodules | |
|---|---|---|---|---|---|
| | | | | Number | Fresh wt. (g) |
| 1 | Blue[2]) | 17000 | None | 5.0 | 0.028 |
| | | | A.S. | 15.8 | 0.137 |
| | | | Kinetin | 15.2 | 0.113 |
| 2 | Green[2]) | 15000 | None | 17.3 | 0.130 |
| | | | A.S. | 12.7 | 0.122 |
| | | | Kinetin | – | – |
| 3 | Red[2]) | 12000 | None | 18.3 | 0.170 |
| | | | A.S. | 16.7 | 0.173 |
| | | | Kinetin | 16.7 | 0.153 |
| 5 | Blue | 13000 | None | 1.8 | 0.030 |
| | | | A.S. | 6.8 | 0.038 |
| | | | Kinetin | 12.3 | 0.087 |
| 6 | Red | 16000 | None | 40.2 | 0.297 |
| | | | A.S. | 39.5 | 0.280 |
| | | | Kinetin | 42.0 | 0.242 |

[1]) Mean values of 6 replicates.
[2]) Approximately equal numbers of quanta.

### 6.5.3. *Bean cuttings*

The experiment with bean plants had the disadvantage that striking differences existed in the size of the plants grown in light of various spectral regions. Plants grown in blue light were long, those in red short and those in green light intermediate. To eliminate this complication, the same experiment was repeated with stem cuttings of bean plants.

Two weeks old stem cuttings of bean plants were inoculated with strain Bokum and transferred to cabinets with blue (1), green (2), red (3) and white

(4) light. To prevent the growth of the shoot, all buds were periodically removed. In each light treatment a number of cuttings received either kinetin (10 μg/cutting) or A.S. (equivalent to 100 mg fresh nodules/cutting). The number and fresh weight of the nodules, determined 15 days after inoculation, are presented in table 46.

TABLE 46. The effect of A.S. (equivalent to 100 mg fresh bean nodules/plant) and kinetin (10 μg/plant) on nodulation[1]) of stem cuttings of bean plants, inoculated with *Rhizobium* strain Bokum. Photoperiod 20 hours of blue, green red and white light.

| Number of cabinet | Light quality | Light intensity (ergs/cm² sec.) | Growth substance added | Nodules | |
|---|---|---|---|---|---|
| | | | | Number | Fresh wt. (g) |
| 1 | Blue[2]) | 17000 | None | 2.5 | 0.009 |
| | | | A.S. | 14.8 | 0.057 |
| | | | Kinetin | 28.7 | 0.088 |
| 2 | Green[2]) | 15000 | None | 14.5 | 0.038 |
| | | | A.S. | 19.2 | 0.072 |
| | | | Kinetin | 30.0 | 0.095 |
| 3 | Red[2]) | 12000 | None | 17.5 | 0.097 |
| | | | A.S. | 21.0 | 0.072 |
| | | | Kinetin | 25.0 | 0.117 |
| 4 | White | 16000 | None | 15.2 | 0.112 |
| | | | A.S. | 27.6 | 0.137 |
| | | | Kinetin | 10.8 | 0.070 |

[1]) Mean values per plant of 6 replicates.
[2]) Approximately equal numbers of quanta.

The results with stem cuttings confirm those obtained with decotylized bean plants. In agreement with the previous experiments, nodulation of rooted stem cuttings was poor in blue light as contrasted to that in green and red light. Response to A.S. and to kinetin was highest in blue light; however, in contrast with decotylized plants, a pronounced stimulation of nodulation by kinetin was also observed in green and red light. The response to A.S. was moderate in green and red light. In white light, A.S. stimulated nodulation whereas kinetin reduced it. The latter is in accordance with the experiments with kinetin carried out in the greenhouse.

The results of this section support our hypothesis that the production of a substance, necessary for nodulation, is low in the absence of red light. Under such conditions apparently A.S. and kinetin can be substituted for the hypothetical substance.

### 6.6. THE EFFECT OF GROWTH SUBSTANCES ON NODULATION OF LEGUMINOUS PLANTS GROWING IN BLUE LIGHT

In subsequent experiments the effect of growth substances on nodulation of leguminous plants, cultivated in blue light, was studied in more detail.

6.6.1. *Decotylized bean plants*

At first, four concentrations of kinetin (5, 10, 15 and 20 μg/plant) and one concentration of A.S. (equivalent to 100 mg of fresh bean nodules) were tested. Inoculation and decotylization took place when the plants were transferred to the cabinet with blue light (1). The number of nodules, determined three weeks after inoculation, is given in table 47. Unlike in previous experiments kinetin stimulated nodulation at a concentration of 15 μg/plant. No effect was observed at 5 or 10 μg/plant and at 20 μg/plant inhibition of nodulation occurred. The stimulating effect of A.S. was comparable to that of 15 μg kinetin.

TABLE 47. The effect of A.S. (equivalent to 100 mg fresh bean nodules/plant) and kinetin (5, 10, 15 and 20 μg/plant) on nodule number[1]) of decotylized bean plants, inoculated with *Rhizobium* strain Bokum. Photoperiod 20 hours of blue light, 17000 ergs/cm² sec.

| Control | A.S. | Kinetin (μg/plant) | | | |
|---|---|---|---|---|---|
| | | 5 | 10 | 15 | 20 |
| 28.4 | 35.8 | 26.8 | 24.3 | 35.5 | 15.3 |

[1]) Mean value per plant of 10 replicates.

Subsequently, the effect of I.A.A. was investigated at two concentrations *viz.* 8.5 and 17 μg/plant. Numbers of nodules, counted 20 and 32 days after inoculation, are given in table 48. It may be seen that I.A.A. stimulated nodulation at both concentrations in the first counting, but only at the highest (17 μg/plant) when the plants were inspected after 32 days of treatment. These results suggest that the amount of I.A.A. or I.A.A.-like substances is suboptimal for nodulation in blue light.

TABLE 48. The effect of I.A.A. on nodule number[1]) of decotylized bean plants, inoculated with *Rhizobium* strain Bokum. Photoperiod 20 hours of blue light, 17000 ergs/cm² sec.

| I.A.A. added (μg/plant) | Number of nodules at | |
|---|---|---|
| | 20 days | 32 days |
| 0 | 0 | 18.4 |
| 8.5 | 2.4 | 18.2 |
| 17.0 | 11.4 | 41.0 |

[1]) Mean values per plant of 6 replicates.

6.6.2. *Decotylized pea plants*

The effect of kinetin (10 μg/plant) and I.A.A. (8.5 μg and 17 μg/plant) separately or in combination was studied on decotylized pea plants, inoculated with *Rhizobium* strain PRE. The numbers were counted 26 days after inoculation. Since a number of plants had formed no nodules, the results are presented in terms of the means of all plants and also as the means of the nodulated plants only (table 49).

TABLE 49. The effect of kinetin and I.A.A. on nodule number[1]) of decotylized pea plants, inoculated with *Rhizobium* strain PRE. Photoperiod 20 hours of blue light, 17000 ergs/cm² sec.

| | Control | Kinetin (10 μg) | I.A.A. | | Kinetin (10 μg) | |
|---|---|---|---|---|---|---|
| | | | 8.5 μg | 17 μg | + 8.5 μg I.A.A. | + 17 μg I.A.A. |
| All plants | 5.8 | 33.0 | 15.0 | 11.4 | 11.8 | 12.4 |
| Nodulated plants only | 10.0 | 33.0 | 16.4 | 12.4 | 17.6 | 14.9 |

[1]) Mean values of 12 replicates. The number of the nodulated plants varied between 7–12.

Subsequently the effect of kinetin (10 μg/plant), I.A.A. (8.5 μg/plant) and A.S. (eq. to 100 mg nodules per plant) was studied on decotylized pea plants, inoculated with *Rhizobium* strain P8. The number of nodules, counted after 21 days, is given in table 50.

TABLE 50. The effect of A.S. (equivalent to 100 mg fresh bean nodules/plant). kinetin (10 μg/plant) and I.A.A. (8.5 μg/plant) on nodule number[1]) of pea plants inoculated with *Rhizobium* strain P8. Photoperiod 20 hours of blue light, 17000 ergs/cm² sec.

| | Control | A.S. | Kinetin | I.A.A. |
|---|---|---|---|---|
| All plants | 28.2 | 54.3 | 52.9 | 34.1 |
| Nodulated plants only | 47.0 | 68.0 | 52.9 | 48.7 |

[1]) Mean values per plant of 10 replicates. The number of nodulated plants varied between 6–10.

It may be seen from tables 49 and 50 that nodule numbers were increased by kinetin and A.S., whereas the stimulating effect of I.A.A. was less pronounced than that of the afore-mentioned growth substances. The stimulatory effect of kinetin was reduced by I.A.A. A similar interaction of kinetin and I.A.A. was observed in bean plants grown in the greenhouse (cf. table 18).

### 6.7. THE EFFECT OF A.S. AND KINETIN ON NODULATION OF LEGUMINOUS PLANTS, IRRADIATED WITH SUPPLEMENTARY FAR-RED LIGHT

In the preceding sections it has been shown that the stimulatory effect of A.S. and kinetin on nodulation of leguminous plants occurred in plants growing in blue light i.e. in the absence of red light. It was also established that the effect of red light can be partly eliminated by subsequent treatment with far red. It therefore seemed of interest to investigate if the inhibitory effect of far red can be counteracted by the addition of growth substances.

The effect of A.S. was investigated in decotylized bean plants, growing in red light (20 hr, 12 000 ergs/cm² sec.). Half of the plants were treated with far-red light (18 000 ergs/cm² sec.) for 30 minutes at the end of the photo-

period. A.S., equivalent to 100 mg fresh bean nodules per plant, was added to the nutrient solution when the plants were inoculated with *Rhizobium* strain Bokum. The plants were harvested 21 days after inoculation and the number and fresh weight of the nodules determined (table 51). It is clearly shown that in red light, in which nodulation is optimal, no stimulation and even slight inhibition occurred when A.S. was supplied. It may be assumed that by adding A.S., supra-optimal concentrations of this substance were attained, already shown to be inhibitory for nodulation. Far-red light significantly reduced nodulation, an observation which is in agreement with earlier results. Under the latter conditions, nodulation was increased by adding A.S. Apparently the production of A.S. within the plants is reduced by treatment with far-red light. As a consequence the addition of A.S. restores the nodulation process.

TABLE 51. The effect of A.S. (equivalent to 100 mg fresh bean nodules/plant) on nodulation[1]) of bean plants, inoculated with *Rhizobium* strain Bokum. Plants grown in red light (20 hours daily, 12000 ergs/cm² sec.) with or without far-red light (30 minutes, 18000 ergs/cm² sec.)

| Far-red light applied (minutes) | Number of nodules | | Fresh wt. of nodules (g) | |
|---|---|---|---|---|
| | No A.S. | with A.S. | No A.S. | with A.S. |
| 0 | 90 | 79 | 0.16 | 0.16 |
| 30 | 36 | 55 | 0.11 | 0.14 |

[1]) Mean values per plant of 6 replicates.

The effect of kinetin on plants receiving far-red light was investigated in decotylized pea plants, inoculated with *Rhizobium* strain PRE. The plants were grown under the same conditions as in the previous experiment. For each light treatment, three concentrations of kinetin (5, 10 and 20 μg per plant) were tested. The mean number of nodules, observed 19 days after inoculation, is presented in table 52. Kinetin inhibited nodulation of plants grown in red light and in red light with supplementary far-red. Inhibition was even more severe when far red was applied to the plants.

TABLE 52. The effect of kinetin and supplementary far-red light (30 minutes, 18000 ergs/cm² sec.) on nodule number[1]) of decotylized pea plants, inoculated with *Rhizobium* strain PRE. Photoperiod 20 hours of red light, 12000 ergs/cm² sec.

| Kinetin (μg/jar) | Control, no far red | With far red |
|---|---|---|
| 0 | 39.7 | 34.9 |
| 5 | 28.8 | 17.4 |
| 10 | 19.3 | 7.2 |
| 20 | 9.8 | 8.1 |

[1]) Mean values of 10 replicates.

Kinetin also inhibited nodulation in decotylized pea plants, inoculated with the ineffective *Rhizobium* strain P8. The plants receiving far red only had a mean number of nodules per plant of 25 whereas those receiving kinetin in addition had 9.

CHAPTER 7

# THE EFFECT OF A.S. ON OTHER BIOLOGICAL SYSTEMS

In previous chapters the effect of A.S. on nodulation was recorded. In additional experiments the effect of A.S. on some other biological processes was studied and compared with that of known growth substances.

## 7.1. The effect of A.S. on germination of lettuce seeds in the dark

The germination of lettuce seeds is known to be controlled by the red-far-red system (Toole, 1959). In the dark at high temperatures (Haber & Tolbert, 1959; Toole, 1959) germination is poor, but it is considerably enhanced by small amounts of red light (Miller, 1956). Far red eliminates the favourable effect of red light. Kinetin and gibberellic acid also stimulate germination in darkness, but the compounds have different temperature ranges for promoting germination (Haber & Tolbert, 1959).

The following experiments with lettuce seeds have been performed to investigate the effect of A.S. on germination and to compare its effect with that of kinetin and gibberellic acid. In the first experiments the influence of light, temperature, kinetin (K) and gibberellic acid (G.A.) was determined with lettuce seeds variety Meikoningin.

About 100–125 seeds were evenly distributed in a Petri dish of 11 cm diameter on three layers of filter paper, wetted either with 5 ml water or with a solution of the compound under investigation. The dishes were kept in light-tight boxes for 60–72 hours. Hereafter the germinated seeds were counted and the results presented as percentage germination. For each treatment three dishes were used.

### 7.1.1. *The effect of red and far-red light*

As lettuce seeds are insensitive to light in the early period of germination, the seeds were left moistened with water for 6 hours. Then one series was irradiated for 5 minutes with red light (8 000 ergs/cm² sec.) whereas a second, after the red-light treatment, received 5 minutes of far-red light (18 000 ergs/cm² sec.). After incubation at 26.3°C, the percentage of germinated seeds was determined. Without irradiation 3.7% of the seeds had germinated. Red light increased germination to 19.0%, whereas far-red light, applied after red, inhibited germination completely (1%).

### 7.1.2. *The effect of kinetin and gibberellic acid at different temperatures*

At first one concentration of kinetin (2 μg/ml) and one of gibberellic acid (2.5 μg/ml) were tested with lettuce seeds, incubated at four different temperatures (20.0°; 24.3°; 28.0°; 30.1°C). The results are presented in table 53. In a subsequent experiment only two temperatures (24.3° and 26.2°C) were

tested, whereas the concentration of the growth factors was varied: kinetin 1, 2 and 4 μg/ml and gibberellic acid 2.5 and 5 μg/ml (table 54).

The effect of temperature on germination of lettuce seeds in the dark is obvious. At 20°C nearly all the seeds germinated, only 25% at 24°C, whereas no germination took place at 28° and 30°C. At 24°–28°C germination was highly stimulated by kinetin, but the highest response was found at 26°C. The effect of G.A. was slight at all temperatures used.

TABLE 53. The effect of kinetin (2μg/ml) and gibberellic acid (2.5 μg/ml) on germination of Meikoningin lettuce seeds, grown at different temperatures in the dark (% germinated seeds).

| Substance added | Temperature (°C) | | | |
|---|---|---|---|---|
| | 20.0 | 24.3 | 28.0 | 30.1 |
| None | 97.3 | 25.0 | 0 | 0 |
| Kinetin | 96.0 | 48.0 | 9.5 | 3.3 |
| Gibberellic acid | 95.0 | 21.1 | 1.0 | 0.3 |

TABLE 54. The effect of kinetin and gibberellic acid on germination of Meikoningin lettuce seeds at 24.3° and 26.2°C (% germinated seeds).

| Substance added (μg) | 24.3°C | 26.2°C |
|---|---|---|
| None | 30.0 | 3.0 |
| Kinetin 1 | 63.0 | 32.7 |
| 2 | 70.6 | 66.7 |
| 4 | 86.7 | 54.0 |
| Gibberellic acid 2.5 | 27.3 | 6.7 |
| 5.0 | 27.3 | 2.3 |

### 7.1.3. *The effect of A.S.*

In the first experiment, one concentration of A.S. (equivalent to 20 mg of fresh nodules/ml) and in the second, four (equivalent to 10, 20, 40 and 100 mg fresh nodules/ml) were tested. For comparison, seeds treated with water and with kinetin (2 μg/ml), respectively, were present. The first experiment was conducted at 26.2° and the second at 24.3°C.

It will be seen from table 55 that in both experiments germination was sig-

TABLE 55. The effect of A.S. and kinetin on germination of Meikoningin lettuce seeds at 24.3°C and 26.2°C (% germinated seeds).

| Substances added | | Experiment 1 | Experiment 2 |
|---|---|---|---|
| | | 26.2°C | 24.3°C |
| None | | 10.4 | 34.9 |
| Kinetin 2 μg/ml | | 36.9 | 47.8 |
| A.S. (equivalent | 10 mg | – | 40.6 |
| to fresh bean | 20 mg | 11.8 | 40.2 |
| nodules/ml) | 40 mg | – | 38.9 |
| | 100 mg | – | 38.9 |

nificantly increased by the addition of kinetin. Nodule extract, at all concentrations used, stimulated germination only slightly. From these data it can be concluded that in the germination of lettuce seeds, kinetin can not be replaced by A.S.

### 7.2. The effect of A.S., I.A.A. and kinetin on rooting of bean cuttings in light of different spectral regions

In earlier experiments it was observed that the number of roots formed on pea plants in blue light was lower than that of plants in red light. From rooting experiments with *Coleus* leaves it is known that the number of roots formed is proportional to the amounts of auxins applied to the roots (van Raalte, 1951). Consequently, the low numbers of roots formed by the plants grown in blue light may be the result of a low concentration of auxins in the plant. In view of these results, it seems of interest to investigate the effect of I.A.A. and some other growth substances on rooting in light of different spectral regions.

#### 7.2.1. *The effect of light of different spectral regions on rooting of stem cuttings*

At first the effect of light quality on rooting was investigated. Stem cuttings were obtained by severing 14 days old bean plants, grown in the greenhouse, just above the cotyledons. Only fully expanded primary leaves were present. The apical buds and all axillary buds, appearing in the following experimental period, were removed. The cuttings were placed in jars containing $^1/_{10}$ strength nutrient solution. Blue, green and red light were applied at approximately equal quantum numbers; white light had the same energy level as blue light. The numbers of roots were counted after 8 days, and the mean values of 8 replicates are recorded in table 56. It will be seen that the number of roots is

Table 56. The effect of light of different spectral regions on root formation of stem cuttings. Photoperiod 20 hours.

| Light colour | Light intensity (ergs/cm² sec.) | Number[1]) of roots/cutting |
|---|---|---|
| Blue[2]) | 17000 | 26.0 |
| Green[2]) | 15000 | 38.3 |
| Red[2]) | 12000 | 42.8 |
| White | 16000 | 33.3 |

[1]) Mean values per cutting of 8 replicates.
[2]) Approximately equal quantum level.

lowest in blue, highest in red and intermediate in green light. In blue light, roots appeared only at the base of the cutting, whereas in the other light treatments they appeared in the zone 1–1.5 cm above the cut. The low numbers of roots formed in blue light suggest that the amount of root-forming substances is lower than in green and red light.

### 7.2.2. *The effect of A.S., I.A.A. and kinetin on rooting of leaf cuttings in blue light.*

To reduce the variability in the plant material, the lamina of primary leaves of 14-days old been plants was reduced to 12.6 cm², using a borer of 4 cm diameter. The leaves were detached in the afternoon and with their petioles, cut down to 2.5 cm, placed in small vessels, containing either distilled water or an aqueous solution of the growth substances under investigation. The leaves were kept overnight in a dark moist chamber. The next morning the petioles were rinsed with water and placed through a plastic gauze in jars containing $^1/_{10}$ strength solution. For each treatment 12–25 leaves were used, evenly distributed on the gauze of two jars, containing 500 ml of nutrient solution each. To prevent drying of the leaves, a sheet of transparent polythene was placed on the laminae during the first three days. Roots usually appeared 5–7 days after cutting and the leaves were harvested 2–3 days later. The numbers of roots and root initials were counted and their total length determined after excising. Numbers of roots per leaf, length of the individual root and total root length per leaf were calculated. Table 57 contains the results of three experiments (I, II, III). It will be seen that the number of roots of the control leaves was low. Nodule extract (A.S.) increased root number only in the first, but not in the following two experiments. Considerable increase in root numbers was found in the petioles of leaves treated with I.A.A. The roots induced by I.A.A. were formed in a zone of ca. 1 cm above the base of the petiole. This is in contrast to the controls, where they occurred predominantly near the base. Slight stimulation of root number was observed after treatment with kinetin. Kinetin and to a smaller degree A.S. reduced the effect of I.A.A. on root number.

TABLE 57. The effect of A.S., I.A.A. and kinetin (K) on root formation of excised primary bean leaves (12.6 cm²) in blue light. Photoperiod 20 hours 17000 ergs/cm² sec.

| Treatment | Number[1]) of roots/leaf | | | Mean length[1]) of the individual root (mm) | | | Total length[1]) of all roots/leaf (mm) | | |
|---|---|---|---|---|---|---|---|---|---|
| | I | II | III | I | II | III | I | II | III |
| Control | 1.5 | 3.3 | 1.4 | 3.1 | 3.2 | 4.9 | 4.7 | 10.6 | 6.9 |
| A.S. (eq. to 100 mg fresh nodules) | 4.8 | 2.3 | 1.7 | 4.3 | 6.4 | 5.9 | 20.4 | 14.6 | 10.0 |
| I.A.A. $10^{-3}$M | – | – | 8.4 | – | – | 4.0 | – | – | 33.9 |
| I.A.A. $2\times10^{-3}$M | 6.3 | – | 7.9 | 6.6 | – | 5.2 | 41.6 | – | 41.1 |
| I.A.A. $4\times10^{-3}$M | 8.9 | 5.5 | – | 4.6 | 4.2 | – | 40.9 | 23.1 | – |
| A.S. + I.A.A. $10^{-3}$M | – | – | 4.7 | – | – | 4.0 | – | – | 18.8 |
| A.S. + I.A.A. $2\times10^{-3}$M | – | – | 8.4 | – | – | 4.3 | – | – | 36.1 |
| A.S. + I.A.A. $4\times10^{-3}$M | 8.9 | 1.7 | – | 4.4 | 2.5 | – | 39.2 | 4.3 | – |
| K $2\times10^{-7}$M | 2.1 | – | 3.3 | 5.2 | – | 5.1 | 10.9 | – | 16.9 |
| K $4\times10^{-7}$M | 3.0 | 3.6 | 3.5 | 6.2 | 6.1 | 6.1 | 18.6 | 22.0 | 21.4 |
| A.S. + K $4\times10^{-7}$M | 0.5 | – | – | 5.8 | – | – | 2.9 | – | – |
| I.A.A. $10^{-3}$ M + K $4\times10^{-7}$M | – | – | 3.4 | – | – | 4.5 | – | – | 15.3 |
| I.A.A. $2\times10^{-3}$M + K $4\times10^{-7}$M | – | – | 2.3 | – | – | 5.3 | – | – | 12.2 |

[1]) Mean values of 12–25 leaves.

The length of the individual root was considerably increased after treatment with kinetin and to a smaller degree also by A.S. No such increase was found with the leaves treated with I.A.A. The stimulatory effect of kinetin and A.S. on root growth was reduced by I.A.A.

### 7.2.3. *The effect of A.S., I.A.A. and kinetin on rooting of leaf cuttings in red light with or without supplementary far-red light*

Leaf cuttings after being treated as described in the previous section were placed in red light (8 000 ergs/cm² sec., photoperiod 16 hours). Half of the leaves received daily 30 minutes of far-red light (18 000 ergs/cm² sec.) immediately at the end of the light period. The leaves were harvested after 8 days (table 58). From the data obtained, it is evident that the effect of far-red on root number and root length was negligible. The response to I.A.A., kinetin and A.S. was not affected by far-red treatment. In both red and red + far-red light, root numbers were approximately doubled by I.A.A.-treatment, whereas in blue light (table 57) application of I.A.A. increased root numbers about five times.

TABLE 58. The effect of A.S. (eq. to 100 mg fresh nodules), I.A.A. ($10^{-5}$M), and kinetin ($4\times10^{-7}$M) on root formation of excised primary bean leaves in red light (16 hours, 8000 ergs/cm² sec.) with or without supplementary irradiation with far-red light (30 min. 18000 ergs/cm² sec.).

| Treatment | Exp. 1 | | Exp. 2 | | Exp. 3 | | Exp. 4 | | Exp. 5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | – | I | – | I | – | I | – | I | – | I |
| *Number*[1]) *of roots/leaf* | | | | | | | | | | |
| Control | 2.7 | 2.7 | 5.5 | 4.3 | 3.1 | 4.6 | 5.1 | 7.7 | 9.4 | 10.0 |
| A.S. | – | – | – | – | 6.9 | 8.0 | – | – | – | – |
| I.A.A. | – | – | 10.7 | 8.1 | 10.4 | 10.7 | – | – | 19.1 | 17.4 |
| Kinetin | – | – | – | – | 3.4 | 5.5 | 3.3 | 5.1 | – | – |
| *Length*[1]) *of the individual root (mm)* | | | | | | | | | | |
| Control | 3.0 | 3.7 | 6.6 | 5.7 | 4.3 | 4.0 | 8.9 | 10.5 | 10.2 | 10.5 |
| A.S. | – | – | – | – | 7.1 | 7.4 | | | | |
| I.A.A. | – | – | 7.5 | 6.4 | 3.5 | 3.7 | – | – | 10.5 | 8.0 |
| Kinetin | – | – | – | – | 3.2 | 4.5 | 8.0 | 7.8 | – | – |
| *Total length*[1]) *of the roots/leaf (mm)* | | | | | | | | | | |
| Control | 8.1 | 9.9 | 36.5 | 24.7 | 13.2 | 19.2 | 45.4 | 77.4 | 96.0 | 105.0 |
| A.S. | – | – | – | – | 49.0 | 59.0 | – | – | – | – |
| I.A.A. | – | – | 80.0 | 52.2 | 36.0 | 40.9 | – | – | 200.7 | 138.1 |
| Kinetin | – | – | – | – | 10.8 | 24.8 | 26.6 | 39.8 | – | – |

[1]) Mean values of 12–25 replicates.

I = far-red light.

### 7.3. The effect of A.S. on tobacco pith tissue

For the biological assay of substances stimulating cell division, tobacco-pith tissue is successfully used (Miller *et al.*, 1955; Bottomley *et al.*, 1963). Pith tissue consists of rectangular cells without meristematic activity. When I.A.A. is applied, the cells start to enlarge but no cell division takes place. The latter may be initiated when in addition to I.A.A., kinetin or a kinetin-like substance is added. Clusters of small cells of irregular form appear between the rows of rectangular cells. After a certain period, lumps of callus tissue protrude from the pith tissue. In view of this result, the effect of A.S. on pith tissue was tested.

Stems of tobacco plants, *Nicotiana tabacum* var. White Burley, grown in the greenhouse were harvested when 75–100 cm high. After removal of the leaves, the stems were swabbed with 95% alcohol and stem segments of 1–1.5 cm cut aseptically. Blocks of pith tissue, free of cambial and vascular tissue, were obtained by cutting at right angles parallel to the stem axis. Perpendicular to the axis, the blocks were sliced into slabs 2–3 mm thick. Each slab was cultivated in 25 ml flasks containing 5 ml of basal medium (Jablonski & Skoog, 1954) of the following composition (in mg/liter):

$KH_2PO_4$, 37.5; $Ca(NO_3)_2$, 100; $KNO_3$, 80; $MgSO_4.7H_2O$, 35; KCl, 65; KI, 0.5; $MnSO_4.4H_2O$, 4.4; $Fe_2(SO_4)_3$, 2.5; $ZnSO_4.7H_2O$, 1.5; $H_3BO_3$, 1.6; glycine, 2; thiamine. HCl, 0.1 nicotinic acid, 0.5; pyridoxine. HCl, 0.5 and glucose, 20 g/l; Difco agar 10 g/l.

I.A.A. (1 mg/l), kinetin (0.2 mg/l) and A.S. were sterilized through a Seitz filter and mixed with the melted agar medium at 45–50°C. A.S. was tested (in amounts equivalent to 0, 5, 10, 25, 50 and 100 mg fresh bean nodules per flask) in the basal medium or in the basal medium supplied with I.A.A. A number of additional cultures received I.A.A. and kinetin. The flasks were placed in white light of fluorescent lamps (TL 33, 10 000 ergs/cm² sec., photoperiod 16 hours) at room temperature (20–22°C). The cultures were inspected regularly during two months, and at the end of the first month, hand sections were prepared and examined microscopically. Except in the cultures with kinetin, no growth was observed with all treatments. In the former a white callus tissue appeared, which gradually turned to light green. Clusters of small dividing cells irregularly scattered between the old pith cells were present. Good growth was obtained after transfer to a fresh medium containing kinetin. No cell division took place in the other series and the tissue gradually turned brown.

These negative results, however, do not exlude the possibility that a cell-division factor is present in nodules. Firstly, inhibitors, present in the nodule extract, may have interfered in the biological assay. Such an inhibitor was found in pea seedlings (Zwar & Skoog, 1963). Secondly, A.S. may be inactive in stimulating cell division of tobacco-pith cells.

CHAPTER 8

# GENERAL DISCUSSION

Nodulation of leguminous plants depends on several factors, e.g. type of plant and *Rhizobium*, supply of nutrient elements, pH of the rooting medium etc. An effect of organic substances, excreted by the roots of leguminous and even non-leguminous plants, has been claimed by some authors to play an important role in nodule formation (THORNTON, 1929; LUDWIG & ALLISON, 1935; NUTMAN, 1953, 1957; GIBSON & NUTMAN, 1960; ELKAN, 1961). An internal supply of inhibitory substances originating from the nodule apex is thought by some authors (NUTMAN, 1952; DART & PATE, 1958) to be responsible for the resistance of leguminous plants to new infections by *Rhizobium*, once nodules are appearing on the roots.

## 8.1. ROOT SECRETIONS

The results obtained in the present investigation clearly show that nodule and root secretions may have a pronounced effect on nodulation. The variable and often contradictory results described in the literature may be ascribed to a large extent to the presence of (a) both nodulation-stimulating and inhibiting substances in the root secretions, and (b) to the pronounced influence of environmental conditions on the response of the plants to the root exudates. By using the nodulation of bean plants as an assay, it was shown in the present paper that the stimulating effect of root secretions was only found under conditions of poor nodulation. Such conditions, yet undefined, occurred in the greenhouse during hot sunny periods, and in artificial light devoid of red light.

Root secretions of nodulated plants consist of a mixture of substances released from the roots and nodules. The substances exuded may vary qualitatively and quantitatively, depending on the age of the plants, and on the environmental conditions, as shown for root exudates by KATZNELSON *et al.* (1955), ROVIRA (1959) and BUXTON (1957).

In contrast to an earlier report by NUTMAN (1953), in the present study a pronounced influence of the *Rhizobium* on exudation was observed. In general, root exudates from young uninoculated peas had no influence on nodulation of bean plants; inhibitors were found to be present in the nutrient solution of older pea plants. However, when the pea plants were inoculated with an effective *Rhizobium* strain, nodulation-stimulating substances were exuded during the first two weeks after inoculation; later on inhibitors appeared in the medium. Plants bearing ineffective nodules, however, excreted small amounts of stimulating substances throughout the entire experimental period.

A comparison of the biological activity of the root secretions at different stages of nodulation of the donor plants, suggests that nodulation-stimulating substances were secreted during active growth of the nodule. Under the conditions of our experiments, pea plants inoculated with an effective *Rhizobium*

strain formed nodules predominantly during the first week; the growth of the nodules was completed within a further week. Plants with ineffective nodules continued to produce small amounts of nodulation-stimulating substances, a phenomenom which may be directly related to the continuous formation of small nodules. It would be of interest to investigate if the substances dealt with in these experiments are produced by the meristematic apex of the nodules, as was demonstrated for inhibitors of nodulation in clover plants (NUTMAN, 1952).

Nodulation-stimulating substances (A.S.) were also obtained by aqueous extraction of nodules. No differences were found in extracts from root nodules produced by effective or ineffective *Rhizobium* strains, indicating that the production of A.S. is not linked to nitrogen fixation. A.S. was present in both the ether-soluble and ether-insoluble fractions from root nodules. With root exudates on the other hand, A.S. was present only in the ether-soluble fraction. These results suggest that the ether-soluble substance (A.S.) of nodules and that of root exudates are identical. The ether-insoluble fraction of nodules, which is not inactivated by heating at 80°C, may be a precursor of A.S. or may be a quite different compound with the same activity in the bean test as A.S.

No inhibitors were found in nodule extract as were detected by PATE (1958) with the *Avena*-coleoptile test. This discrepancy may have been due to the fact that only young nodules were used for extraction of A.S. in our experiments or that the biological assays used are not comparable. However, the fact that root secretions from ageing plants inhibited nodulation indicates that the inhibitors may be derived from senescent nodules and roots.

GIBSON & NUTMAN (1960) ascribed the beneficial effect of preplanting on nodulation of leguminous plants to the presence in their nutrient solution of small amounts of nitrate, which exerted an inhibitory effect on nodulation. Removal of the nitrate by preplanting would be the explanation of the preplanting effect. That such an explanation would not hold for the experiments dealt with in the present investigation can be easily seen from the fact that addition of concentrated exudations or an ether extract of exudates or root nodules to fresh medium has the same effect as preplanting.

### 8.2. RED-LIGHT REQUIREMENT OF ROOT-NODULE FORMATION

One of the most striking observations in the experiments was that the response of bean plants, growing in the greenhouse, to added A.S. was most pronounced under conditions of poor nodulation. A more detailed investigation, in which the leguminous plants were grown in light of various spectral regions, revealed that nodulation was poor in the absence of red light. The importance of red light for the nodulation process was especially demonstrated when the plants were growing in blue light. Small amounts of red light supplied to such plants enhanced nodulation; this could be eliminated by subsequent irradiation with far-red light. The reversibility of red and far-red light was also

ascertained for plants growing in red and white light. From these experiments it may be concluded that the red/far-red system, known to regulate photomorphogenic processes, also affects nodulation.

In recent years these processes have been found to be controlled by phytochrome (P), a pigment system with absorption maxima in red (660 mμ) and far-red light (730 mμ). The active form $P_{730}$ is formed after irradiation with red light. Far-red light immediately converts $P_{730}$ to $P_{660}$; the same reaction takes place in darkness but the conversion time is about 4 hours (HENDRICKS & BORTHWICK, 1963). The conversion reaction is

$$P_{660} \underset{730\ m\mu}{\overset{660\ m\mu}{\rightleftharpoons}} P_{730} \xrightarrow{\text{darkness}} P_{660}$$

Additional support for the hypothesis that the phytochrome system functions in the nodulation process, was obtained from experiments in which the plants were treated with far-red light after different period of darkness. The most severe reduction was found to occur when the plants were treated immediately after the light period or within 4 hours. When treated after 8 hours, nodulation was only slightly reduced and no inhibition was observed when the plants were treated at the end of the dark period. Similar results have been obtained in the light control of seed germination (TOOLE, 1959) and stem elongation (DOWNS, 1959).

From the experiments with blue light and with supplementary far-red light it might be concluded that in both instances the conditions for nodulation were similar. (In the first case red light is absent whereas in the second one the photomorphogenic effects of red light are inactivated by subsequent irradiation with far red.) In both cases nodulation was poor and it was stimulated by adding A.S. and by not removing the cotyledons of the test plant. However, upon further analysis, some differences were observed. In blue light kinetin favoured nodulation whereas in plants exposed to supplementary far-red light, it exerted an inhibitory effect. Such differences were also observed in the rooting of leaf cuttings and their response to added growth substances. In blue light the number of roots was low, whereas treatment with far red had little effect on the number of roots formed. In blue light root elongation was stimulated by kinetin and no such effect was observed in the roots of leaves treated with far-red light.

### 8.3. THE ROLE OF COTYLEDONS IN ROOT-NODULE FORMATION

The requirement for red light in nodule formation seems to be at variance with the fact that nodulation can take place in etiolated seedlings (RAGGIO & RAGGIO, 1956) and root cultures (RAGGIO *et al.*, 1957). However, cotyledons have to be present on the dark-grown seedlings in order to obtain nodulation (RAGGIO & RAGGIO, 1956), indicating the supply of some unknown growth

factor. So far, nodulation in isolated root cultures has been obtained with root tips, directly isolated from the seedlings. Therefore the transport of substances from the cotyledons to the root tips can not be ruled out.

In the present investigations, nodulation of plants growing in blue light was higher when the cotyledons were present than when removed. The presence of cotyledons also protected the plants against the depressing effect on nodulation of far-red light. Apparently substances present or produced in the cotyledons may be substituted for the requirement of red light in the nodulation process.

### 8.4. The possible nature of A.S.

So far, the active principle (A.S.) responsible for the stimulation of nodulation has not been isolated and chemically defined. A number of purification treatments, combined with measuring of the biological activity in the bean assay, revealed that A.S. has an absorption peak in U.V. light at ca. 260 mμ, that it is soluble in ether (an ether-insoluble fraction with biological activity derived from root nodules may contain a precursor), and is insoluble in chloroform. The ether-soluble fractions of root exudates and that of nodule extract are inactivated by heat treatment at 80°C, but the ether-insoluble fraction of nodule extract even resists a treatment at 110°C. Fractionation using column chromatography of the ether-soluble fraction revealed the presence in nodule extract of several nodulation-promoting substances.

The low amounts of biologically active substance required to obtain stimulatory effects, and its thermolability, suggests that A.S. belongs to the growth-substances group.

Up till now, three groups of plant-growth substances have been assumed to occur and to operate in the plant *viz.* the auxins, the gibberellins and the kinins (Kefford, 1963; Thimann, 1963).

The auxins present in root nodules consist largely of indoleacetic acid and indoleacetonitrile, which is known to be convertible to I.A.A. The concentration of auxins in root nodules is high, about 40–60 times higher than that of the roots, from which they originate (Pate, 1958). I.A.A. is not identical to A.S., since the bean plants responded differently to exogeneously applied I.A.A. and A.S. When applied together, A.S. even reduced the inhibitory effect of high concentrations of I.A.A. A different effect of I.A.A. and A.S. on rooting of bean leaves was also observed; and a slight interaction occurred when these substances were applied simultaneously.

Only few experiments were conducted with gibberellic acid, but from the data obtained it is unlikely that A.S. is identical to gibberellic acid.

The hypothesis that a kinetin-like substance is involved in root-nodule formation is attractive in view of the following considerations. Root nodules originate from divisions of disomatic cells. Such cells and other polyploid ones are known to have lost the capacity to divide, unless they are exposed to certain stimuli (Geitler, 1953) including kinetin (Torrey, 1961). Comparable phenomena occur in tobacco-pith cells transformed by *Agrobacterium tume-*

*faciens* to crown-gall tissue. Although normal pith tissue only grows upon addition of kinetin and I.A.A., profuse growth of crown-gall tissue takes place in the absence of these growth substances. The latter tissue was found to produce kinetin-like substances and I.A.A. (BRAUN, 1956). FOX (1963) isolated a strain of callus tissue from tobacco pith cells capable of producing a kinetin-like substance. This tissue consists largely of tetraploid cells.

Kinetin was found to simulate the action of red light in a number of processes, but when their actions were analysed more critically, it turned out that different reactions were involved (MILLER, 1956, 1958; POWELL & GRIFFITH, 1960). Part of the reaction sequence, however, may be common to both kinetin and red light, so that similar end results may be obtained. In *Lemna minor* the growth enhancement of kinetin was considerably increased by low light intensities of red light (ROMBACH, 1960). In view of these results it is of interest to note that red light apparently is necessary for nodule formation (see chapter 6). In blue light, i.e. in the absence of red light, both kinetin and A.S. increased nodulation, suggesting that these substances may replace the requirement for red light. This is in agreement with the stimulatory effect of kinetin on bud formation of *Pohlia nutans* (MITRA & ALLSOPP, 1959); in the absence of kinetin, buds were formed readily in red but not in blue light (MITRA *et al.*, 1959).

The enhancement of nodulation by the cotyledons in plants, growing in blue light or receiving supplementary far-red light, is important, since it has been found that germinating seeds may contain substances stimulating cell division (ZWAR & SKOOG, 1963). The presence of cotyledons also diminished the response of bean plants to A.S. In view of these results it is possible that red light, cotyledons and A.S. affect nodulation in the same way.

A.S. resembled kinetin in being soluble in ether and having strong absorption in U.V. light at ca. 260 mμ. The latter was also observed in fractions containing a cell-division factor extracted from immature corn seeds (MILLER, 1962), coconut milk (STEWARD & SHANTZ, 1959). However the following facts are against the assumption that A.S. is identical to kinetin it self. No enhancement of cell division in tobacco-pith tissue or of germination in lettuce seeds was detected upon application of A.S., whereas kinetin proved to be active. Nodulation of leguminous plants, reduced by supplementary far-red treatment, was found to be stimulated by A.S. but not by kinetin. Moreover, A.S. was inactivated by heat treatment, whereas kinetin is known to be heat-resistant. Although A.S. and kinetin are not identical, part of the results obtained in the present investigations still supports the assumption that A.S. belongs to the group of kinins. More experimental evidence is needed, however, to confirm this hypothesis.

So far, no efforts have been made to explain the original greenhouse observations concerning the response to A.S. under certain climatic conditions. A comparison of the results obtained in the greenhouse and those of the light cabinets is difficult since large differences occurred between e.g. the light intensities used and presumably the light quality in both environments.

## SUMMARY

An investigation was made of the nodulation of bean plants, *Phaseolus vulgaris* L., growing in water culture under greenhouse conditions and under controlled light and temperature conditions. During periods of hot sunny weather, plants grown in the greenhouse nodulated poorly. No improvement in nodulation was obtained by increasing the inoculum or by the addition of I.A.A., kinetin, gibberellic acid, different purines and pyrimidines, yeast or soil extract, ammonium nitrate or boron. However, nodulation was enhanced upon the addition of used culture solution of nodulated pea or bean plants.

No influence on nodulation of bean plants was observed when root secretions from uninoculated young pea plants were added, but inhibition was observed when exudates of older plants were used. Nodulation-stimulating substances were obtained from pea plants, inoculated with an effective *Rhizobium* strain, during the first two weeks after inoculation, but again inhibitors appeared in the medium of older plants. Pea plants infected with an ineffective *Rhizobium* strain yielded small amounts of nodulation-stimulating substances even three weeks after inoculation.

Nodulation-stimulating substances were also obtained by an aqueous extraction of both effective and ineffective nodules from bean plants.

The active substance (A.S.) of root secretions can be completely extracted with ether. In contrast, a nodulation-stimulating substance was present in the ether-soluble and insoluble fractions from root nodules. The substance present in the ether-soluble fraction was soluble in water but insoluble in chloroform. It was inactivated by heating at 80°C. On the other hand, the ether-insoluble fractions from nodules, maintained its biological activity even after heating at 110°C.

Fractionation of the ether-soluble fraction by ion-exchange chromatography revealed that at least two and probably five nodulation-stimulating components ware present. The fractions showing biological activity had a strong U.V.-light absorption at ca. 260 mμ. However, not all the fractions showing U.V.-light absorption stimulated nodulation. Heat treatment inactivated the biological activity of A.S., but had no influence on the absorption spectra of the treated solutions.

A.S. stimulated nodulation in bean plants under conditions associated with poor nodulation in the greenhouse. Under conditions of optimal nodulation no effect or even inhibition was observed upon application of A.S. Inhibition was also observed when high concentrations of A.S. were applied to the plants.

An analysis of the environmental factors supposed to influence the response of nodulation to added A.S. revealed that no specific effect was exerted by temperature and light intensity. However, light quality was found to be of considerable importance. Nodulation was poor in blue light and optimal in red light. Red and far-red light acted oppositely, far-red inactivating the stimulatory effect of red light. Small amounts of red light stimulated nodulation of plants growing in blue light.

The inhibitory effect of far-red light on nodulation was also observed in rooted bean leaves and decapitated bean plants, i.e. in plants lacking stem elongation. Treatment with far-red light during the first week after inoculation had an almost similar effect to treatment during the entire experimental period. The most pronounced inhibition by far red was observed when it was applied after the daily photoperiod or within four hours afterwards. After eight hours the effect was less and at the end of the night almost no effect was observed.

A.S. and kinetin stimulated nodulation of bean plants growing in blue light. Plants treated with far-red light responded favourably to A.S. but not to kinetin. The effect of I.A.A. on nodulation in blue light was visible, but the effect was less than that of A.S. or kinetin. When kinetin and I.A.A. were applied simultaneously, the effect of kinetin was reduced by I.A.A.

The above-mentioned effects of light quality and growth substances were found to obtain with decotylized plants. The presence of cotyledons on the plants gave higher numbers of nodules and slight effects or none at all were observed upon irradiation with far-red light. The response to A.S. was also less in plants bearing cotyledons.

Application of A.S. to leaf cuttings in blue light increased root elongation but unlike I.A.A. it did not affect root numbers. An almost similar effect was observed with low concentrations of kinetin. The effect of far-red light on rooting of leaf cuttings was virtually absent.

Unlike kinetin, A.S. stimulated neither germination of lettuce seeds nor cell division of tobacco-pith cells.

Although A.S. present in root exudates and root nodules has not yet been isolated and chemically defined, it is assumed that A.S. belongs to the kinins.

# SAMENVATTING

Aanleiding tot dit onderzoek vormde de waarneming dat de vorming van wortelknolletjes bij boneplanten (*Phaseolus vulgaris* L.) sterk kon worden beinvloed door wortelexcreties van leguminosen voorzien van wortelknolletjes. Dit effect doet zich voor bij planten die in kassen groeien gedurende de zomermaanden tijdens perioden met warm en zonnig weer, wanneer de knolvorming slecht is. Bij andere klimatologische omstandigheden is de vorming van wortelknolletjes bevredigend en heeft een toevoeging van wortelexcreties geen of zelfs een remmende werking. Het gunstige effect van wortelexcreties kan niet worden verkregen door toevoeging van respectievelijk indolazijnzuur, kinetine, gibberelline, enkele purinen en pyrimidinen, gist- en grondextract, ammoniumnitraat en borium.

Door de toeneming van het aantal en het gewicht van de wortelknolletjes van boneplanten, na toediening van wortelexcreties, als maatstaf te gebruiken (bonetest) kon een onderzoek worden verricht naar de aard en werking van de biologisch actieve stof.

In wortelexcreties was de vorming van A.S. afhankelijk van de aanwezigheid en de aard van de *Rhizobium* stam. Wortelexcreties van jonge, ongeënte erwteplanten gaven geen stimulerende werking in de bonetest. Bij oudere erwteplanten werd zelfs een remstof afgescheiden door wortels. Erwteplanten geënt met een effectieve *Rhizobium* stam scheidden gedurende de eerste twee weken na enting een stof uit, die de vorming van knolletjes bij bonen sterk stimuleerde. Later werden in de voedingsoplossing ook remstoffen afgescheiden. Erwteplanten geënt met een ineffectieve *Rhizobium* stam scheidden geringe hoeveelheden A.S. uit gedurende een veel langere periode dan bij planten met effectieve knolletjes het geval was. Een stimulerende werking op de wortelknolvorming bij bonen werd ook verkregen door een waterig extract van effectieve en ineffectieve wortelknolletjes van bonen.

De werkzame stof uit wortelexcreties kon door een aetherextractie volledig uit de voedingsoplossing worden verwijderd. Deze stof was onoplosbaar in chloroform. Daarentegen vertoonden zowel de in aether oplosbare als in aether onoplosbare fracties van een waterig extract uit wortelknolletjes een stimulerende werking in de bonetest. De in aether oplosbare fracties van wortelexcreties en van wortelknolletjes werden geïnactiveerd na verhitting bij 80°C, terwijl de in aether onoplosbare fractie van wortelknolletjes zelfs na verhitting bij 110°C nog zijn activiteit had behouden.

Door gebruik te maken van kolomchromatographie met ionenuitwisselaars kon de aanwezigheid van twee en misschien zelfs vijf componenten met biologische activiteit worden aangetoond. De biologisch actieve fracties vertoonden een sterke absorptie van het U.V. licht bij ongeveer 260 mμ. Het omgekeerde was echter niet het geval.

Zoals reeds eerder vermeld, is de reactie van de boneplant op het toegevoegde extract sterk afhankelijk van de weersomstandigheden. Bij een nader onderzoek van de klimaatsfactoren bleken de temperatuur en de lichtintensi-

teit geen specifieke invloed in de bonetest uit te oefenen. De lichtsoort speelde echter een belangrijke rol bij de vorming van wortelknolletjes en de reactie van de plant op A.S. De vorming van wortelknolletjes was slecht in blauw en optimaal in rood licht. Toediening van geringe hoeveelheden rood licht aan planten die in blauw licht groeiden, bevorderde de vorming van wortelknolletjes terwijl infrarood licht een sterke remming vertoonde. De werking van het rood en het infrarood licht was reversibel. De remmende werking van infrarood licht op de vorming van knolletjes was ook te zien bij bewortelde bladstekken en bij gedecapiteerde planten, d.w.z. in systemen die geen of weinig stengelstrekking vertoonden. Bestraling met het infrarood gedurende de eerste week na enting met *Rhizobium* had ongeveer dezelfde werking als bestraling gedurende de gehele proefperiode. De sterkste remming door het infrarood werd verkregen wanneer de planten aan het begin van de donkere periode of binnen de eerste 4 uren bestraald werden. Na 8 uur was het effect veel geringer en toegepast aan het eind van de nacht was de werking practisch gesproken nihil.

In blauw licht bevorderden zowel A.S. als kinetine de vorming van wortelknolletjes. Planten bestraald met infrarood licht reageerden gunstig op het toegevoegde extract, maar ongunstig op kinetine wat betreft de knolvorming. Ook indolazijnzuur stimuleerde de vorming van wortelknolletjes bij planten opgekweekt in blauw licht maar ten opziche van A.S. en kinetine was de werking vrij gering. Kinetine en I.A.A. werkten antagonistisch op de vorming van wortelknolletjes zowel in blauw als in wit licht.

De bovengenoemde resultaten gelden alleen voor planten waarvan de zaadlobben voor de proef waren verwijderd. De aanwezigheid van zaadlobben verhoogde het aantal knolletjes in de planten in blauw licht, terwijl ook de werking van het infrarood er sterk door werd verminderd. Als gevolg hiervan reageerden planten met zaadlobben, opgekweekt in blauw licht, nauwelijks op A.S.

A.S. en kinetine stimuleerden de lengtegroei van wortels bij bladstekken van bonen. Daarentegen werd het aantal wortels er niet door verhoogd zoals bij de behandeling met I.A.A. Infrarood had geen invloed op de beworteling van bladstekken.

In tegenstelling tot het kinetine vertoonde A.S. geen stimulerende werking op de kieming van slazaden en de groei van weefselculture van tabaksmergweefsel.

Hoewel de biologisch werkzame stof uit wortelexcreties en wortelknolletjes nog niet is geïsoleerd en geïdentificeerd, zijn uit dit onderzoek aanwijzingen gevonden, dat deze stof behoort tot de groep van groeistoffen, die kininen worden genoemd.

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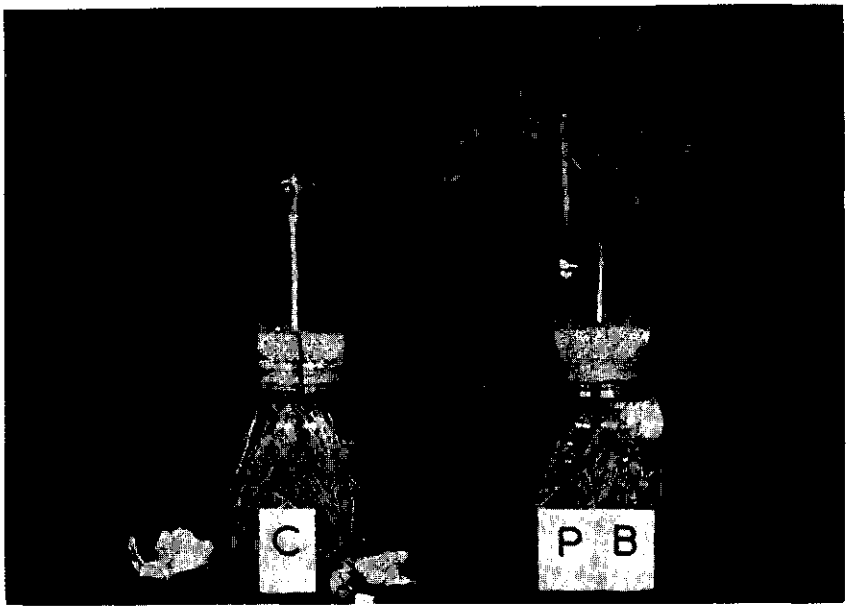


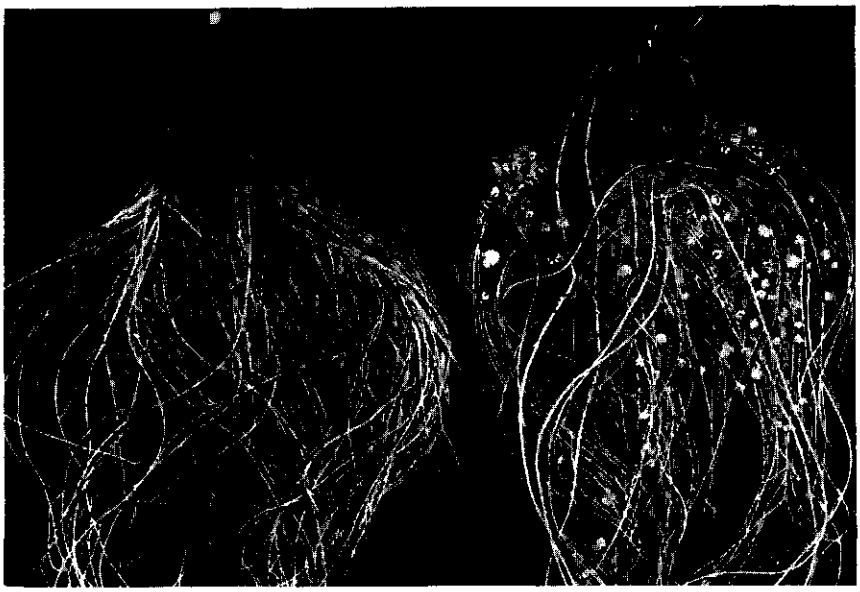

PLATE I.

Effect of preplanting on nodulation of bean plants, inoculated with the effective *Rhizobium* strain Bokum, under climatical conditions extremely unfavourable for nodulation of the control plants (August 1959). Donor: bean plants, inoculated with *Rhizobium* strain Bokum. Left control, right preplanted.

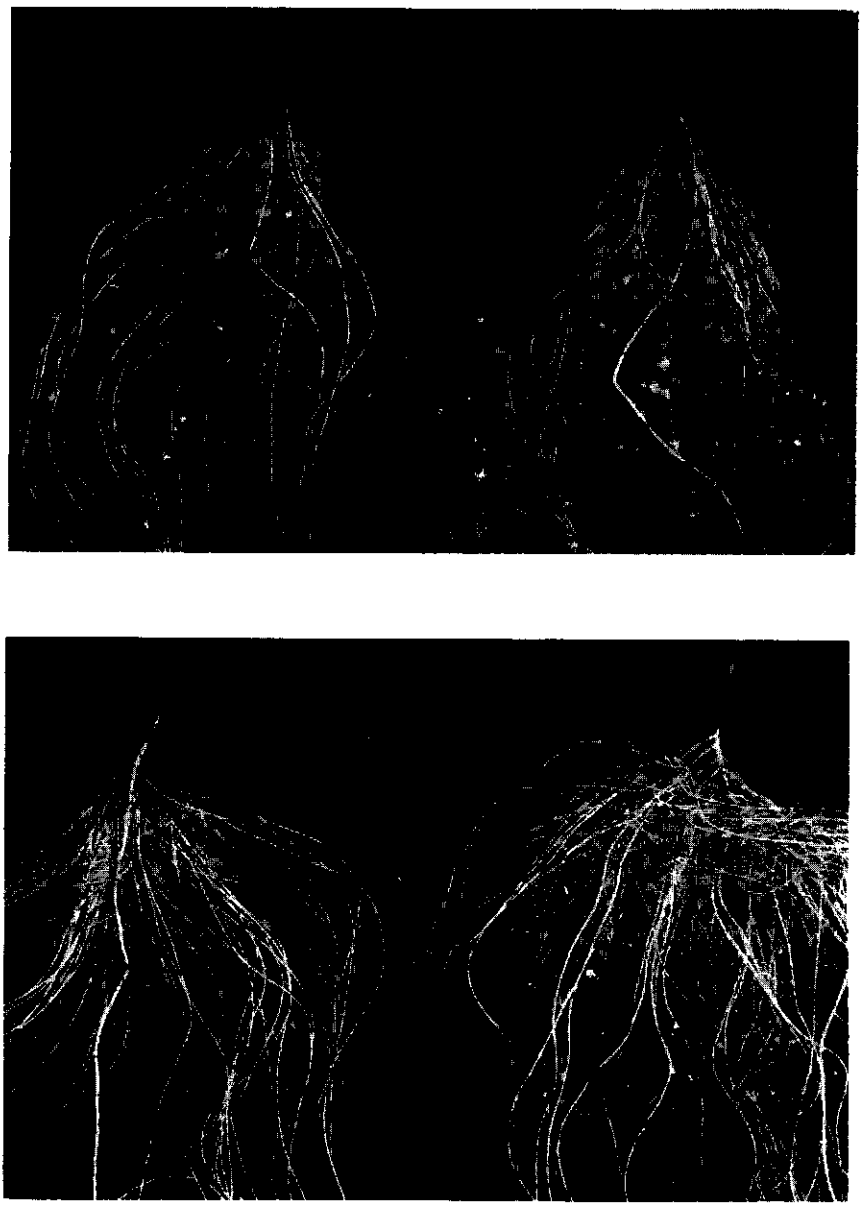

PLATE II.

Effect of A.S. on nodule formation of bean plants, inoculated with the ineffective *Rhizobium* strains $WH_2$ (above) and S460 (below). Left untreated, right supplied with A.S., equivalent to 200 mg fresh bean nodules per plant (cf. table 6).

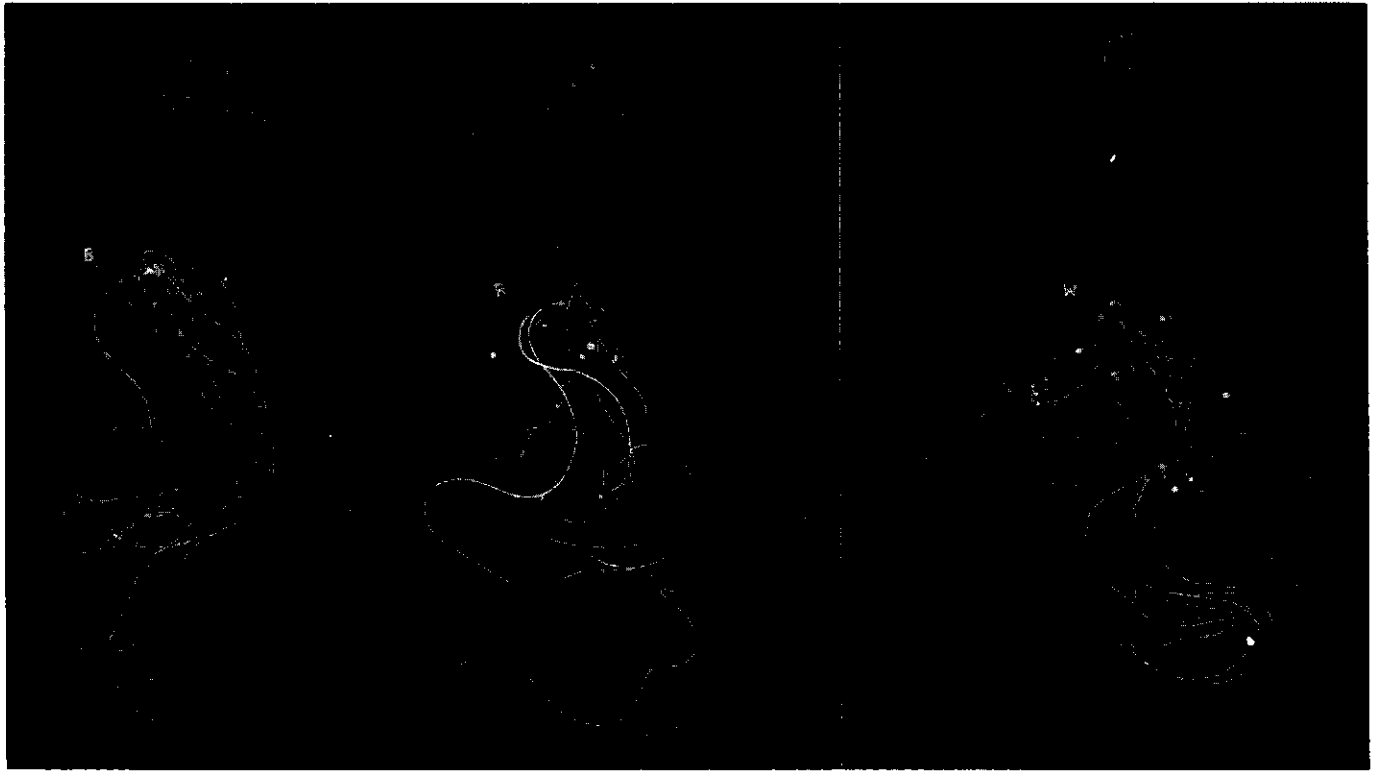


PLATE III.

Effect of light of different spectral regions on nodulation of rooted bean leaves, inoculated with *Rhizobium* strain Bokum. B = blue (17000 ergs/cm² sec.); R = red (12000 ergs/cm² sec.) and W = white (16000 ergs/cm² sec.) (cf. table 34).