THE RHIZOBIUM–PEA SYMBIOSIS AS AFFECTED BY HIGH TEMPERATURES

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(with a summary in Dutch)

Proefschrift

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWWETENSCHAPPEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS,
DR. IR. J. P. H. VAN DER WANT,
HOOGLERAAR IN DE VIROLOGIE,
IN HET OPENBAAR TE VERDEDIGEN
OP VRIJDAG 29 OKTOBER 1976
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IN DE AULA
VAN DE LANDBOUWHOGESCHOOL TE WAGENINGEN.

H. VEENMAN & ZONEN B.V. – WAGENINGEN – 1976

STELLINGEN

I

Bij onderzoek over symbiose van *Rhizobium* en Vlinderbloemigen wordt het microbiologische aspect vaak ten onrechte verwaarloosd.

Dit proefschrift.

II

Yao en Vincent (1976) concluderen tot het bestaan van een dialyseerbare stof die vertakking van wortelharen veroorzaakt en een niet dialyseerbare stof die kromming van wortelharen veroorzaakt; beide stoffen zouden worden uitgescheiden door de bijpassende *Rhizobium*-soort. Dit onderscheid is voorbarig en berust op onvolkomenheden in hun proefopstelling.

YAO, P. Y. and J. M. VINCENT, Plant and Soil **45** (1976) 1–16.
Dit proefschrift.

III

Het is niet waarschijnlijk dat lectine van de erwt van belang is bij het verklaren van de specificiteit van *Rhizobium leguminosarum*. Het is niet uitgesloten dat deze verbinding een algemene rol speelt bij de hechting van de bacteriën aan de wortels.

VAN WAUWE, J. P., F. G. LOONTIENS, C. K. DE BRUYNE, Biochem. Biophys. Acta, **379** (1975) 456–461.
ZEVENHUIZEN, L. P. T. M., J. Gen. Microbiol. **68** (1971) 239–243.
ZEVENHUIZEN, L. P. T. M., Carbohydrate Research **26** (1973) 409–419.
DAZZO, F. B. and D. H. HUBBELL, Appl. Microbiol. **30** (1975) 1017–1033.

IV

Het is niet te verwachten dat ethyleen een belangrijke rol speelt bij de remming door het eerst verschijnende knolletje van de vorming van nieuwe wortelknolletjes.

DART, P. J. In: The biology of nitrogen fixation. ed. A. Quispel. North Holland Publishing Company, Amsterdam, 1974, 381–427.
NUTMAN, P. S., Ann. Bot. **16** (1952) 79–101.

V

De eindigheid van de voorraad beschikbare fossiele brandstoffen voor de productie van stikstofmeststoffen is een beter uitgangspunt om de betekenis in de landbouw van Vlinderbloemigen als stikstofbinders te bestuderen, dan de filosofieën van aanhangers van de 'alternatieve' landbouw.

Interimrapport Commissie Onderzoek Biologische landbouwmethoden. PUDOC, Wageningen, 1974.

VI

Er is geen oorzakelijk verband tussen de kans op het optreden van resistentie tegen een biocide en het aantal plaatsen waarop deze stof ingrijpt in de stofwisseling van een cel.

HAMILTON-MILLER, J. M. T., Adv. Appl. Microbiol. **17** (1974) 109–134.
HILL, P. J., J. Gen. Microbiol. **70** (1972) 243–252.

VII

De definitie van symbiose dient te luiden: het in nauw verband samenleven van ongelijksoortige organismen, die wederzijds van elkaar profijt trekken.

Commissie voor de Terminologie van de Nederlandse Planteziektenkundige Vereniging, Ned. Tijdschr. Pl. Ziekt. **74** (1968) 65–84.

VIII

Microbiologie leent zich bij het voortgezet onderwijs uitermate goed voor het bestuderen van verschillende onderdelen van de biologie.

IX

Het kiezen van een vakkenpakket bij het voortgezet onderwijs, gebaseerd op beroepskeuze, voldoet niet.

X

Het verdient aanbeveling de academische promotieplechtigheid te wijzigen in een openbare inleiding tot het proefschrift, te houden door de promovendus, en leidende tot een discussie.

XI

Het door Gilbert gelegde verband tussen episoden in James Joyce's Ulysses en organen van het menselijk lichaam berust op een oppervlakkige biologische kennis.

S. GILBERT. James Joyce's Ulysses.
Penguin Books Ltd. Harmondsworth 1952.

XII

Het specialist zijn is een verfijnde vorm van beperktheid.

Proefschrift van J. F. J. FRINGS
Wageningen, 29 oktober 1976.

WOORD VOORAF

Het beëindigen van een proefschrift is wel een dankzegging waard aan al diegenen die dit mogelijk hebben gemaakt.

In de eerste plaats geldt mijn dank mijn ouders, die me hebben laten studeren.

Mijn promotor, Professor Mulder, dank ik voor de intense aandacht en het stimulerende enthousiasme bij het bespreken van resultaten en manuscript. De belangstelling en hulp van de medewerkers van het Laboratorium voor Microbiologie bij mijn werk was zeer stimulerend, zowel tijdens de onderzoeksperiode als tijdens de schrijfperiode, waarin ik gebruik heb mogen maken van de faciliteiten van het laboratorium, terwijl ik elders mijn werkkring had; dit geldt wel heel in het bijzonder voor Dr. Middelhoven, die me menigmaal over een dood punt heen heeft geholpen en tevens gastvrijheid heeft verleend. Mijn grote waardering voor de assistentie die ik mocht ondervinden van Mevr. van Joolen-de Moel, Mevr. Jansen-Jurcovičova, Mevr. van Laar-Meintz en de Heer Möller is bekend.

De Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek wil ik mijn dank betuigen voor de financiële steun.

Mijn Nijmeegse collega's ben ik dankbaar voor de wijze waarop ze me gesteund en, als dat nodig was, vervangen hebben.

Tenslotte wil ik mijn vrouw bedanken, die alles mogelijk maakte door een bijkans vaderloos gezin te bestieren.

CONTENTS

1. GENERAL INTRODUCTION

Nitrogen fixation by the legume-*Rhizobium* symbiosis is accomplished after a series of previous stages of nodule development. The *Rhizobium* cells multiply in the rhizosphere (VAN EGERAAT, 1972), causing root hair deformations as branching and tip curling (HAACK, 1964; YAO and VINCENT, 1969). Subsequently, the infective rhizobia enter the plant via a tube, the infection thread, which, according to NUTMAN (1965) is formed by redirected growth of part of the cell wall. The involvement of cell wall-degrading enzymes in this process has been reported (e.g. by FÅHREUS and LJUNGGREN, 1959; LJUNGGREN, 1969; MUNNS, 1969) and contradicted (e.g. by LILLICH and ELKAN, 1968; MACMILLAN and COOKE, 1969; SOLHEIM and RAA, 1971). The infection thread growing into the root's cortex, induces some cortex cells to mitotic activity (LIBBENGA, 1970), which leads eventually to a macroscopically visible nodule on the root. Within the root nodules, most of the bacteria leave the infection thread, surrounded by a membrane (DIXON, 1967). They become pleomorphic (bacteroids), and at the same time leghaemoglobin is formed (KEILIN and WANG, 1945; VIRTANEN, 1945), giving a pink colour to the nodules, which now are able to fix gaseous nitrogen (VIRTANEN et al., 1947). More extensive descriptions of the nodulation process are given by several authors (e.g. STEWART, 1966).

Since the early studies of JONES and TISDALE (1921), temperature, among other environmental factors, has been recognized to influence symbiosis, and to affect both nodule formation and nitrogen fixation (e.g. MES, 1959; ROUGHLY and DART, 1969; PANKHURST and GIBSON, 1973; WILSON, 1975). A review of the literature on this subject has been given by GIBSON (1971) and by LIE (1975).

Nodulation of pea plants is known to be adversely affected by exposing the plants to 30 °C (DIENER, 1950; LIE, 1964). In contrast to the sensitivity of the association, the nodule bacteria as well as the plants, supplied with adequate combined nitrogen, are able to grow satisfactorily at this temperature. Although environmental conditions may affect all stages of nodulation, a discernable sensitivity of specific stages of the nodulation process was demonstrated with respect to acidity by MULDER et al. (1966) and LIE (1969) for *Pisum sativum*, and by MUNNS (1968, 1970) for *Medicago sativa*. With respect to high temperatures, similar results were obtained by GIBSON (1967) for *Trifolium subterraneum* and by LIE (1971) for *Pisum sativum*.

GIBSON (1967) divided the nodulation time, i.e. the period between inoculation and time of appearance of the first nodule, into two parts. During a period until 36 hours after inoculation the sensitivity of the association to high temperatures would be different from that in the following period. His suggestion that the two stages involved concerned infection and beginning of nodule growth (nodule initiation) were not supported by direct observations.

LIE (1971) reported that the nodulation of pea cv 'Iran' requires a temperature

of 26 °C during the 2nd or 3rd day after inoculation. If this requirement is met with, nodulation at 20 °C for the remaining time is far better than at a constant exposure to 26 °C. At a constant temperature of 20 °C, no nodulation takes place.

From the above-mentioned results it can be seen that high temperatures during different periods of nodulation may affect nodulation in a different way. It is not clear, however, which stage or stages in nodulation are involved. The aim of the present investigation was (a) to localize temperature-sensitive periods during nodulation, (b) to determine which stages are involved, and (c) to find out in what way high temperatures affect the sensitive stages.

2. MATERIAL AND GENERAL METHODS

2.1. PLANT MATERIAL

The experiments were carried out with pea plants, *Pisum sativum* L., cultivar Rondo.

Selected seeds were surface-sterilized by shaking in 3% H_2O_2, containing a drop of detergent (Teepol) per 25 ml, for 15 minutes. The seeds were transferred, without washing, to Petri dishes containing 1% agar in tap water and left to germinate at 25°C for about a week. When the roots had attained a length of about 5 cm, the seedlings were transferred aseptically to jars containing a nutrient solution of the following composition: K_2HPO_4, 0.36 g; KH_2PO_4, 0.12 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $CaSO_4$, 0.25 g; Fe(III)-citrate, 30 mg; $MnSO_4 \cdot 4H_2O$, 1 mg; $ZnSO_4 \cdot 7H_2O$, 0.25 mg; $CuSO_4 \cdot 5H_2O$, 0.25 mg; H_3BO_3, 0.25 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.05 mg, per 1000 ml of tap water. The seedlings were wrapped in sterile cotton wool and placed with their roots in tubes of 4 × 30 cm, containing 200 ml nutrient solution. The plants were grown in a controlled-environment cabinet using artificial light (45 W/m^2, photoperiod 12 hours) at a temperature of 22°C, unless otherwise stated.

2.2. BACTERIA

The plants were usually inoculated with *Rhizobium leguminosarum*, strain PRE. The bacteria were maintained on yeast agar slopes of the following composition: Difco yeast extract, 1 g; mannitol, 10 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; NaCl, 0.1 g; $CaCO_3$, 3 g; Davies agar, 10 g, per 1000 ml of tap water. In the earlier experiments, 1–2 drops of a slightly turbid cell suspension in sterile water, made from a 7-day old slope culture of *R. leguminosarum*, were applied per plant. In later experiments, the bacteria were grown in Erlenmeyer flasks of 300 ml capacity containing 100 ml of yeast-extract glucose medium (yeast extract 7 g, glucose 10 g per 1 of tap water). Incubation took place on a shaker at 30°C. The bacteria were harvested during the exponential growth phase and counted in a counting chamber. The plants were inoculated with about 10^4 bacteria per plant.

2.3. NODULATION TIME AND NODULE NUMBER

The roots were inspected at daily intervals, beginning 3 days after inoculation and ceasing when all the plants had formed nodules. The period between inoculation and appearance of the first nodule was defined as nodulation time. Nodules were counted usually 14 days after inoculation. When it was not clear

whether something protruding was a rootlet or a nodule, no nodule was reported.

2.4. Temperatures

Plant roots were exposed to high temperatures by placing the plants in a water bath, controlled with a Grant thermostat. The temperatures were regularly checked with the aid of a gauged thermometer.

3. LOCALIZATION OF THE HIGH TEMPERATURE EFFECT ON NODULATION

3.1. THE POSSIBILITY OF A TRANSPORTED COUMPOUND

3.1.1. *Introduction*

The absence of nodulation at high temperatures might be brought about by several mechanisms. One of the possibilities is the involvement of a substance, synthesized either locally of elsewhere. If this compound would be translocated, the shoot or the root meristems would be the most probable sites of synthesis. The hypothetic agent might be either a thermolabile compound, essential for nodulation, or an inhibitor formed at high temperatures.

3.1.2. *Experimental*

For growing the plants, S-shaped tubes were used. Tilting of such a liquid-filled tube 90° counterclockwise creates two compartments, both of them filled with liquid, separated by air (Fig. 1). These compartments can be independently subjected to the desired temperatures. Plants used in these experiments were somewhat older than usual, because the root system had to be present in the lower compartment at the beginning of the experiment. Bacteria were added just before tilting the tube.

3.1.3. *Results and Discussion*

The results of this experiment are given in Table 1. In both compartments, nodules were formed only at 22 °C. If the upper part of the root system was

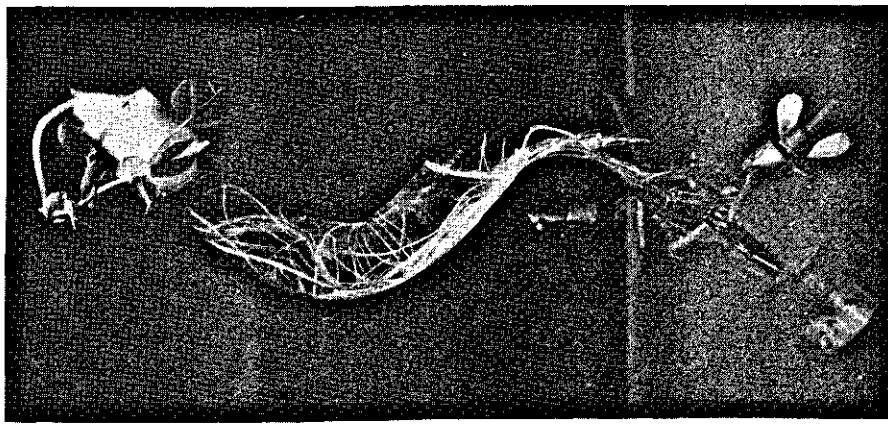

FIG. 1. S-tube in horizontal position containing a pea plant. The left-hand compartment has been exposed to 30 °C, the right-hand compartment to 22 °C. Nodules can be seen in the air compartment, indicating that nodulation was not affected by subjecting a zone of the root system between shoot and nodulation site to 30 °C.

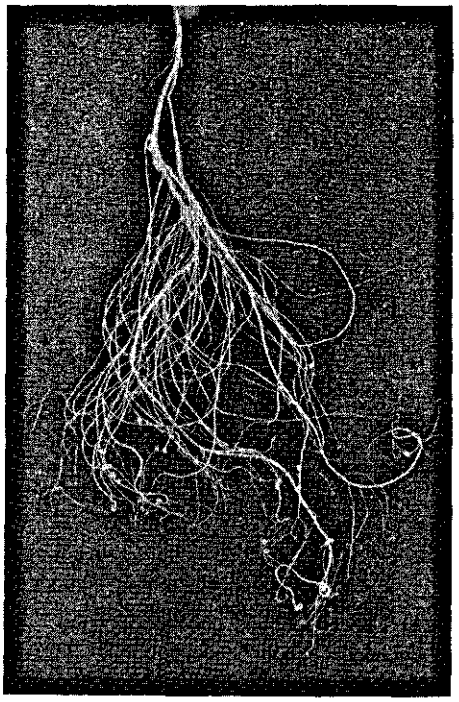

FIG. 2. Nodulated root system of a pea plant, kept at 22°C and photographed 14 days after inoculation with *R. leguminosarum*. During that time the shoot was exposed to 30°C (compare Figs. 3, 5, 6).

TABLE 1. The relationship between nodulation and incubation temperatures of the upper and lower parts of pea roots.

Temperature		Nodulation	
Upper part	Lower part	Upper part	Lower part
22°C	22°C	+	+
22°C	30°C	+	–
30°C	30°C	–	–
30°C	22°C	–	+

exposed to 22°C and the lower part to 30°C, nodules were only formed on the former roots. Although most nodulated roots were entirely present in the 22°C-compartment, nodules were also found on the upper parts of roots which had their meristem exposed to 30°C. If the upper part of the root system was incubated at 30°C, only the lower part of the root system was nodulated.

These results show that the adverse effect of high temperatures on nodulation is not due to the elimination of a translocated thermolabile compound, synthesized in the shoot or the meristem or to a translocated inhibitor, synthesized at high temperatures outside the nodulation site. That such an inhibitor is not

FIG. 3. Non-nodulated root system of a pea plant, exposed to 30°C during 14 days from inoculation with *R. leguminosarum* and photographed after that period.

produced in the shoot and translocated to the nodulation site was shown by the results of the following experiment. Inoculated plants were grown in a 30°C cabinet with their root systems in a thermostate at 22°C. Nodulation occurred both when the shoots were kept at 30°C or at 22°C, provided the root system was exposed to 22°C (Fig. 2). No nodules appeared when both root and shoot were kept at 30°C (Fig. 3).

From the results obtained in this section, it can be concluded that the adverse effect of high temperatures on nodulation is localized at the nodulation site. The possibility that this effect is due to the elimination of a compound, essential for nodulation, or to a nodulation-inhibiting compound, produced at a high temperature, has not to be excluded.

3.2. ESTIMATION OF OPTIMUM INOCULATION TIME

3.2.1. *Introduction*

Several processes are involved in the nodulation of legume roots (GIBSON, 1971). To elucidate which of these processes is inhibited at high temperatures, it is important to know at what time between inoculation and appearance of

the nodule, inhibition occurs and which processes proceed at that time. THORNTON (1929) was the first to notice a link between the unfolding of the first true leaf of lucerne and appearance of the root nodules. Although legume roots at first are resistant to infection (NUTMAN, 1958), it is common practice in legume investigations to inoculate seeds at sowing or a few days afterwards (e.g. LIBBENGA, 1970; ROUGHLEY, 1970). To find out at which plant age nodulation processes are likely to proceed at an optimum rate, the following experiment was carried out.

3.2.2. *Experimental*

Pea plants were inoculated at different periods after sowing (i.e. at different inoculation times). The interval between inoculation and appearance of the first nodules, the nodulation time, was recorded.

3.2.3. *Results and Discussion*

As Fig. 4 shows, the minimum nodulation time was found to occur when inoculation took place shortly before the unfolding of the first true leaf. Until that time of inoculation, nodules always appeared at the same plant age. When

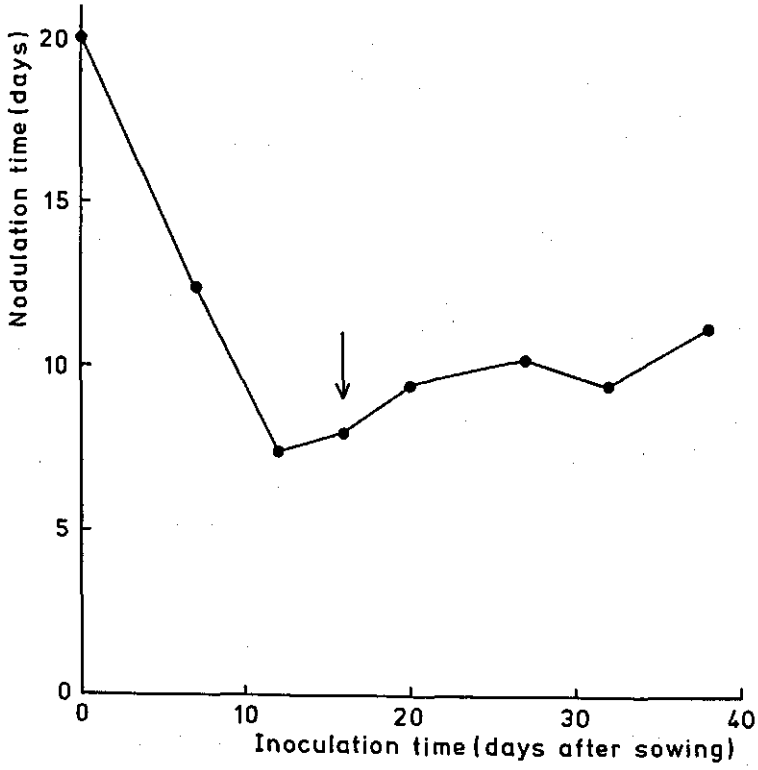

FIG. 4. Relationship between age of pea plants, when inoculated with *R. leguminosarum*, and nodulation time. Arrow indicates unfolding of the first true leaf.

inoculation occurred after the unfolding of the first true leaf, the period between inoculation and nodule appearance increased somewhat. Although in some experiments a slightly shorter average nodulation period was found, the minimum nodulation time was always found when inoculation occurred shortly before or sometimes at the time of unfolding of the first true leaf. The latter phenomenon appears to be a clear physiological indication of an easy accessibility of *Rhizobium* cells to the plant of that age. Hence it was considered in subsequent experiments as an indicator of the optimum inoculation time.

3.3. Determination of the nodulation sites in relation to root age

3.3.1. *Introduction*

The older the plant at inoculation, the greater is the distance from the hypocotyl to the sites of nodulation (Figs. 5 and 6). Pea plants inoculated at sowing on water agar and afterwards transferred to a liquid medium, usually nodulated at similar sites to those of Fig. 5. It is furthermore clear from these photographs that nodules appear in groups. To study the relationship between the ability of the root to nodulate and root age, a method was developed to distinguish between newly grown and older roots.

3.3.2. *Experimental*

Roots present at a certain date can be distinguished from those appearing at a later date by dipping the root system into a charcoal suspension which turns the roots black. After shaking off the excess of charcoal and returning the plant to the usual medium, the roots keep their black colour, but they continue their growth in the normal way. The newly formed parts of the roots are white, so that it can be clearly seen which parts of the root are formed after dipping.

3.3.3. *Results and Discussion*

When simultaneous dipping and inoculation of root systems occurred, most nodules appeared on the white parts, but some were seen on the blackened parts of the roots (Table 2, day 0). When blackening of the roots had occurred at one day before inoculation, all of the nodules appeared on the white roots (Table 2, day –1; see also Fig. 7). Consequently, nodulation occurred on parts of the root system being at most one day old at the time bacteria and roots came into contact. Whether the root system had been subjected to 30 or 22°C until inoculation, did not have any influence on the distribution pattern of the nodules (Figs. 8 and 9). This shows that high temperatures affect nodulation only when applied after inoculation. Possible influences of high temperatures on features of a one day old part of a growing root are obviously not affecting the start of symbiosis. When the roots had been dipped into the charcoal suspension three days after inoculation, all of the nodules originated from the black parts of the roots (Table 2). When the charcoal treatment had taken place two days after inoculation some nodules still appeared on the white parts.

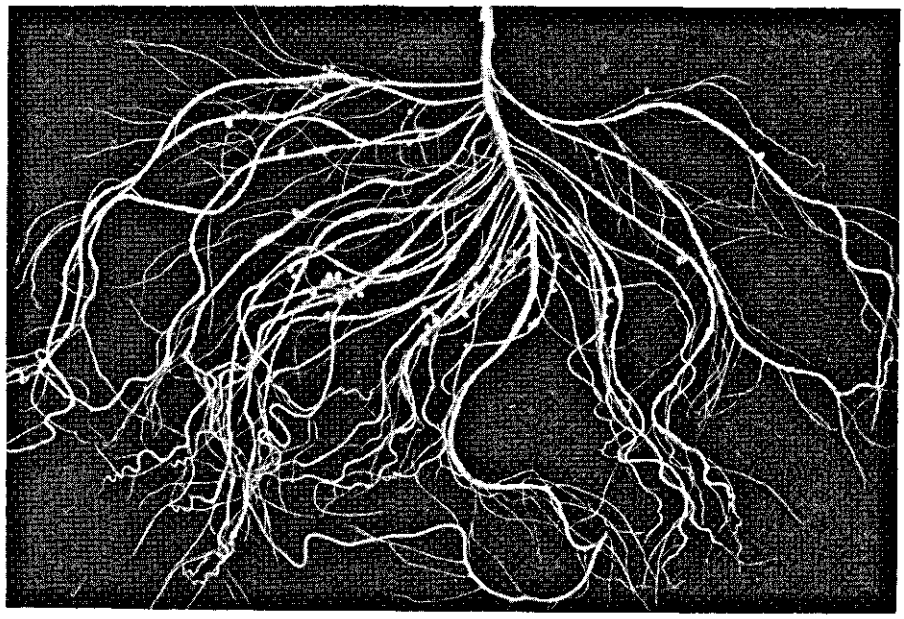

FIG. 5. Root system of a pea plant inoculated 7 days after sowing.

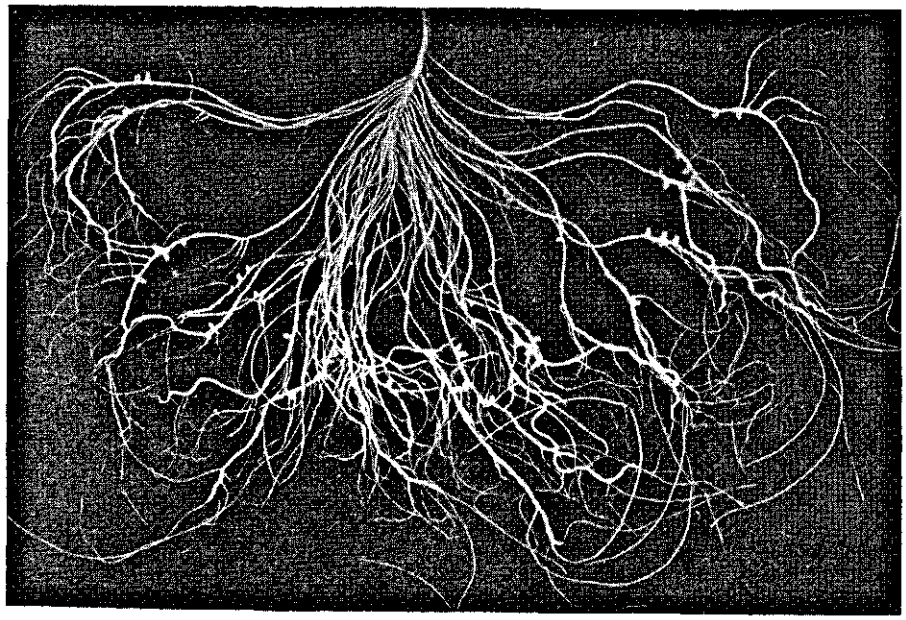

FIG. 6. Root system of a pea plant inoculated 16 days after sowing.

TABLE 2. Number of nodules perceptible on either black or white parts of the roots marked at the indicated day by dipping of the root system into a charcoal suspension.

Time of dipping into a coal suspension (days after inoculation)	Number of nodules on		% of nodules on	
	black parts	white parts	black parts	white parts
−1	0	211	0	100
0	27	122	18	82
1	31	66	33.5	66.5
2	92	2	98	2
3	168	0	100	0

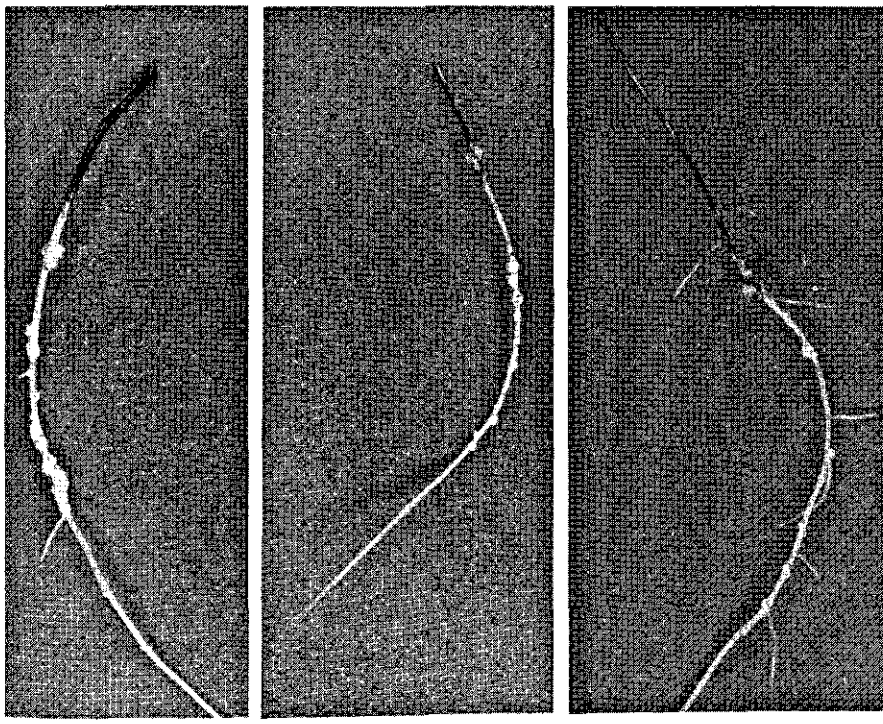

FIG. 7. Root of a pea plant dipped into a charcoal suspension one day before inoculation. All of the nodules were formed on the white part of the root grown after dipping time. Plant exposed continuously to 22°C.

FIG. 8. Root of a pea plant dipped into a charcoal suspension at inoculation time. Some nodules were formed on part of the root existing at the time of inoculation (black). Plant exposed continuously to 22°C.

FIG. 9. Similar to Fig. 8 with the exception that prior to inoculation, the root system had been exposed to 30°C for 3 days (from inoculation the incubation temperature was 22°C). Localization of the nodules was similar to that of Fig. 8.

From these results it can be concluded that the nodulation sites are situated on the younger parts of the root system, formed from one day before to three days after inoculation.

This limitation of nodulation (which is responsible for the occurrence of nodules in distinct groups) towards the higher side of the root is related to the root age. As far as it concerns the lower side this limitation is presumably caused by the developing nodules on that part of the root grown at least 4 days earlier. The latter assumption is in agreement with the results of NUTMAN (1952) with red clover and ROUGHLEY et al. (1970) with subterranean clover. These authors found that the first-formed nodule adversely affected further nodulation, apparently by suppressing the early stages of nodule formation. Exceptions are only seldom found. One or two nodules may sometimes appear on older parts of a root, usually close to a lateral root, due to the fact that the opening made in the cortex by the developing lateral root enables the rhizobia to penetrate into the root giving rise to nodule formation (v. EGERAAT,1972).

Concerning the effect of charcoal on nodulation, the following remark can be made. In the literature it has been reported that activated charcoal may reduce (VANTASIS and BOND, 1950) or stimulate (TURNER, 1955) nodule numbers. To check these observations, it was tested whether or not under the conditions of the present investigation nodulation was affected by the added active coal. As nodules on black roots are not as clearly perceptible as those on white roots, charcoal was not brought on to the root, but added to the medium in concentrations comparable to those found in literature. As Table 3 shows, no significant influence of charcoal on nodulation was found. From this observation together with the fact that active coal brought on to the root did not inhibit nodulation, it can be concluded that the used dipping method is merely a marking method, not affecting the nodulation process in a significant way.

TABLE 3. The influence of active coal and root temperature on number of nodules per plant (averages of 5 plants).

Grams of active coal per plant	Temperature	
	22°C	30°C
0	312 ± 54	0
0.2	260 ± 72	0
2.0	246 ± 55	0

3.4. SUMMARY

1. A method was developed which enables the exposure of different parts of a root system to different temperatures.
2. No translocated thermolabile compound required to initiate nodulation or translocated compound synthesized at a high temperature preventing nodulation is involved in the absence of nodulation at high temperatures.

3. High temperatures do not exert any influence on the ability of a root to nodulate when applied before inoculation time.
4. Roots of pea plants growing in nutrient solution are liable to infection by *Rhizobium* cells from about the time that the first true leaf unfolds.
5. A method was developed to mark the age of nodule-forming root parts relative to the time of inoculation.
6. Nodules develop on parts of the root formed from one day before to three days after inoculation.

4. EFFECT OF TEMPERATURE AND THE PRESENCE OF RHIZOBIA ON MORPHOLOGICAL CHARACTERS OF THE ROOT SYSTEM

4.1. INTRODUCTION

GIBSON (1967) studied the effect of high temperatures on nodulation of subterranean clover and found that number of nodules and nodulation rate were affected. A similar study was made by the present author with pea plants and the results are reported in section 4.2.

In the previous chapter, the adverse influence of high temperatures on nodulation was shown to be due to a local effect. The purpose of the investigation reported in the present chapter was to obtain more details concerning this effect. As seen earlier, the temperature-sensitive area is localized in that part of the root which has been formed between one day before and three days after inoculation. To mark this zone in a growing root, the distances from both the upper and the lower sides of the sensitive zone to the growing root tip should be known. These distances can be calculated if the root-growth rate and the time of inoculation are known. As root growth may be affected by both temperature (KUNG and WEST, 1968) and the presence of *Rhizobium* cells, this aspect is dealt with in some detail in section 4.3. The effect of both variables on number of roots, easily determined in the same experiments, is dealt with in the same section.

4.2. THE EFFECT OF TEMPERATURE ON NUMBER OF NODULES AND NODULATION TIME

4.2.1. *Experimental*

Pea plants were grown at 22°C until the first true leaves unfolded (see 3.2.3.). Then they were inoculated with *Rhizobium* cells and exposed to different temperatures. In the first experiment, 3 *Rhizobium leguminosarum* strains were used viz PRE, PF_2 and S310. In subsequent experiments, strain PRE only was employed. For determining nodulation time, the root systems were inspected daily until the first nodule became visible. Nodule numbers were counted 14 days after inoculation.

4.2.2. *Results and Discussion*

From the results of these experiments, recorded in Table 4 (experiment I), it can be seen that nodule numbers obtained with three different *Rhizobium* strains were affected by temperature in the same way. Therefore, in subsequent experiments only strain PRE was used as inoculant. In experiment II the effect of temperature on nodulation was studied at smaller intervals. In both the

TABLE 4. Influence of temperature on number of nodules and nodulation time.

Experiment	I			II	III	IV
R. leguminosarum Strain	PRE	PF_2	S310	PRE	PRE	PRE
Temperature (°C)						
A. Number of nodules per plant						
20	315 ± 15	200 ± 60	275 ± 64	100 ± 70	–	–
22	–	–	–	170 ± 70	35 ± 16	85 ± 35
25	250 ± 60	200 ± 50	250 ± 80	75 ± 14	60 ± 25	–
28	–	–	–	20 ± 12	–	95 ± 40
30	0	0	0	0	–	–
B. Nodulation time (in days)						
20				5.8 ± 1.1	–	–
22				5.0 ± 0.4	5.8 ± 0.6	4.2 ± 0.4
25				6.0 ± 0.8	8.7 ± 1.8	–
28				12.3 ± 2.9	–	8.8 ± 1.6
30				no nodules	–	–

first and second experiment, no nodules were found when the plants were incubated at 30°C. The optimum temperature for nodulation seemed to be 22°C. The nodules appeared earlier and in greater numbers than at other temperatures. This result was not confirmed by the nodule counts of experiments III and IV which were highest at 25 and 28°C, respectively. As to the nodulation time, the data of the three experiments are in agreement. In all three instances the first nodules appeared earliest when the plant had been incubated at 22°C.

As the consequence of these results, in subsequent experiments the adverse effect of high temperatures on nodulation phenomena was studied at 30°C with control plants exposed to 22°C. In rare instances nodule-like structures were found at 30°C.

4.3. EFFECT OF TEMPERATURE AND THE PRESENCE OF *R. LEGUMINOSARUM* ON ROOT GROWTH AND ROOT NUMBERS

4.3.1. *Experimental*

Plants were exposed to 22, 25 or 30°C after the usual initial growing period at 22°C (see 3.2.3.). At this time half of the plants of each temperature treatment were inoculated with *R. leguminosarum*, strain PRE. At daily intervals, two plants of each experimental group were harvested and the lengths of all of their roots measured. Root length is defined as the distance between root tip and base of the root. Growth rate was determined as the average daily in-

TABLE 5. Root-growth rates (cm/day) as influenced by *Rhizobium* cells and by temperature.

Temperature	Without rhizobia	Inoculated with rhizobia
22°C	1.09 ± 0.12	0.88 ± 0.11
25°C	1.91 ± 0.25	1.13 ± 0.18
30°C	1.54 ± 0.14	1.15 ± 0.17

crease of length of the 20 longest roots measured during a period of 7 days. This was done by calculating the regression coefficient of root length against time.

4.3.2. *Results and Discussion*

The results concerning growth rate are given in Table 5. Of the three temperatures tested, root growth was slowest at 22°C. In the absence of *Rhizobium* cells a marked optimum was found to exist at 25°C. In the presence of *R. leguminosarum*, lower growth rates were found at all temperatures, whilst no differences occurred between plants incubated at 25°C and 30°C. By using root-growth rates (Table 5) and measuring the distance from a point on the root to the root tip, the age of this point on the root may be estimated.

Considering the distribution of roots of different length, the large number of roots up to 1 cm at 22°C in the presence of *Rhizobium* cells is striking (Figs. 10a and b). This may partly have been due to the counting of developing nodules as well as small roots, as it is often difficult to distinguish between very young roots and very young nodules. In almost all other root-length classes, root numbers of inoculated plants were lower than those of uninoculated control plants. In both groups of plants, highest numbers of roots were found in the 1–5 cm class. This is particularly true at 22°C. An exception has to be made for uninoculated plants at 30°C.

As to numbers of roots per plant, an effect of the presence of rhizobia as well as of temperature was found. This is clearly shown in Table 6 which contains the increase in root numbers during 7 days after inoculation. This increase was highest in the uninoculated control plants, particularly at 22°C. The adverse effect of rhizobia on increase of root number is not necessarily the same as that on slowing down the growth rate of the roots. KLASOVA et al. (1972) found evidence that branching of the root and extension growth are independent. Rhizobia may influence both of them. The negative effect of *Rhizobium* cells on root numbers is well-known. NUTMAN (1948) suggested the existence of a physiological similarity of nodules and rootlets in order to explain this phenomenon. The sum of number of nodules and number of roots of nodulated plants should be about equal to the number of roots of uninoculated plants. Nutman's hypothesis does not explain our results at 30°C where usually no nodules are found.

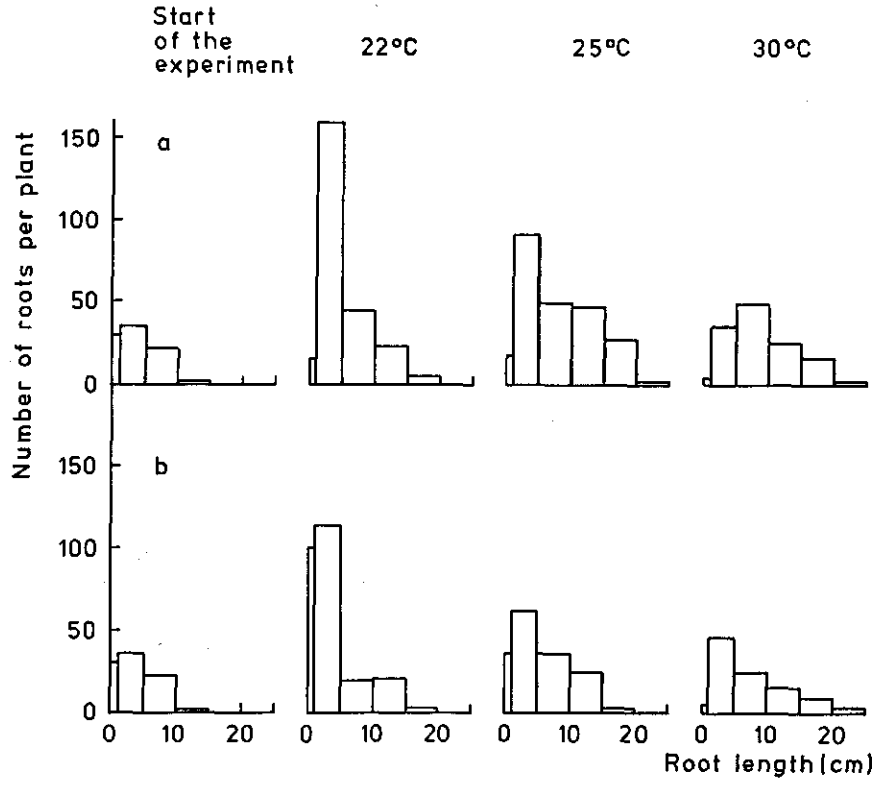

FIG. 10. Distribution of roots of different lengths of pea plants at the start and the conclusion of a 7 days period during which the plants had been exposed to different temperatures. (a) no rhizobia present, (b) plants inoculated with *R. leguminosarum*, strain PRE, at the start of the experiment.

TABLE 6. The increase of the total number of roots during 7 days after inoculation.

Temperature	Inoculated with *R. leguminosarum* PRE	Control plants (uninoculated)
22°C	175	198
25°C	77	159
30°C	21	45

4.4. SUMMARY

1. Nodules of pea plants inoculated with *Rhizobium leguminosarum* are formed at temperatures below 30°C. The numbers of nodules formed were variable.

2. Nodulation time in pea plants was shortest at 22°C. It increased with rising incubation temperature.
3. Root-growth rate at 30°C was found to be higher than at 22°C; it slowed down in the presence of *R. leguminosarum*.
4. Formation of lateral roots of inoculated and control plants at 22°C was much higher than at 30°C. Inoculation drastically reduced root numbers.

5. THE INFLUENCE OF HIGH TEMPERATURE PERIODS ON NODULATION OF PEA PLANTS

5.1. TEMPERATURE-SENSITIVE STAGES IN THE FORMATION OF NODULES

5.1.1. *Introduction*

The observations on nodule formation reported in this section were made before the outset of nitrogen fixation. Within this period, a number of different stages in the formation of the nodules can be distinguished (NUTMAN, 1952). Some of the stages are affected by high temperatures more seriously than others, as it was shown for *Trifolium subterraneum* by GIBSON (1952). Therefore, the effect of high temperatures on nodulation might be expected to be more pronounced if the exposure of the inoculated plants would occur during a temperature-sensitive stage or during part of such a stage. Exposure during an insensitive stage has presumably no effect on nodulation. To find the different stages as to sensitiveness to high temperatures, inoculated pea plants were exposed to 30°C for different periods at various times after inoculation.

5.1.2. *Effect of transfer to 30°C on nodule number and nodule growth*

5.1.2.1. Experimental

Pea plants were grown and inoculated in the usual way. One series of plants was exposed to 22°C during the entire experimental period; other series were transferred to 30°C at different times (from 0 to 10 days) after inoculation and were kept at this temperature for the remaining time of the experiment. Final nodule numbers were counted 14 days after inoculation; nodule size was estimated by measuring the lengths of the protruding parts of 100 nodules at 14 days after inoculation.

5.1.2.2. Results and Discussion

As it was expected, plants transferred to 30°C immediately after inoculation did not nodulate. Transfer within 3 days after inoculation gave the same result (Fig. 11). Obviously during this period at least one key process in nodulation was adversely affected by high temperature. This temperature-sensitive process might be the infection of the root hairs by rhizobia, or the development of the infection thread. Approximately 20 nodules were formed on the roots of plants transferred to 30°C at the 4th day after inoculation (transfer time $t=4$); nodule numbers increased with delayed transfer to 30°C. This increase continued for about 5 days, so that at $t=8$, maximum nodule numbers were obtained. Transfer beyond that time gave no further significant increase, presumably owing to the suppressing effect of developing nodules on nodulation of the same roots (cf. 3.3.). Nodules on plants transferred to 30°C never turned pink.

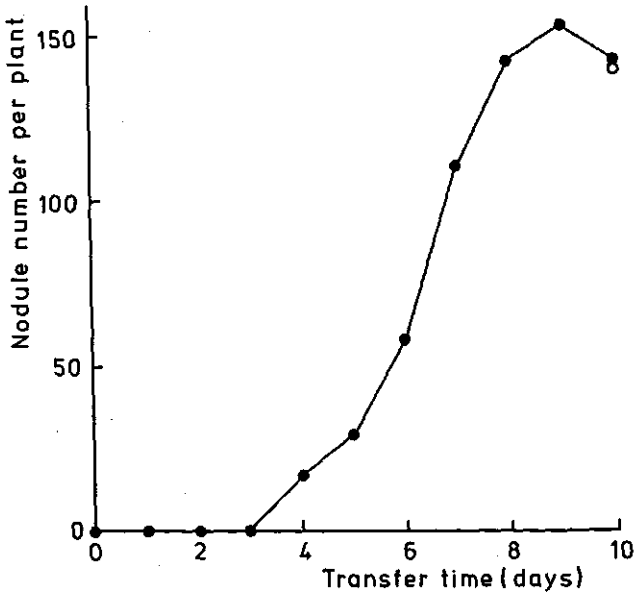

FIG. 11. Final numbers of nodules (counted 14 days after inoculation) of pea plants transferred to 30°C at different transfer times (days after inoculation) o – control. (For nodule lengths cf Fig. 14).

The nodules of plants transferred to 30°C at t = 4 developed on parts of the roots formed during the day before inoculation, those of plants transferred at t = 5 developed on parts of the roots formed from 1 day before to 1 day after inoculation (Fig. 12). The nodules on most plants transferred at t = 7 and later developed on parts of roots which had been formed from 1 day before to 3 days after inoculation. Some of the plants transferred to 30°C at 6, 7, or 8 days after inoculation showed many very small nodules on parts of the roots formed more than 3 days after inoculation, apparently due to the fact that the developing nodules had not completely suppressed nodulation on the younger parts of the roots. In these cases nodules on parts of roots formed between one day before and 3 days after inoculation were also very small.

In a subsequent experiment the plants were transferred to 30°C at the 4th, 5th, 6th and 7th day, respectively, after inoculation and the nodules counted daily until the 14th day after inoculation. As seen in Fig. 13, nodule numbers were higher with retarded transfer and increased after transfer. This increase depended on the time of transfer. When the plants were exposed to 30°C at 4 days after inoculation, numbers of nodules increased during about 2 days. However, when the plants were transferred at the 7th day after inoculation, the increase was only slightly below that of the control plants maintained throughout the experiment at 22°C and proceeded for several days. This extended rise of nodule numbers suggests that a number of nodule initiations, no more sensitive to 30°C treatment at t = 7, had developed to visible nodules

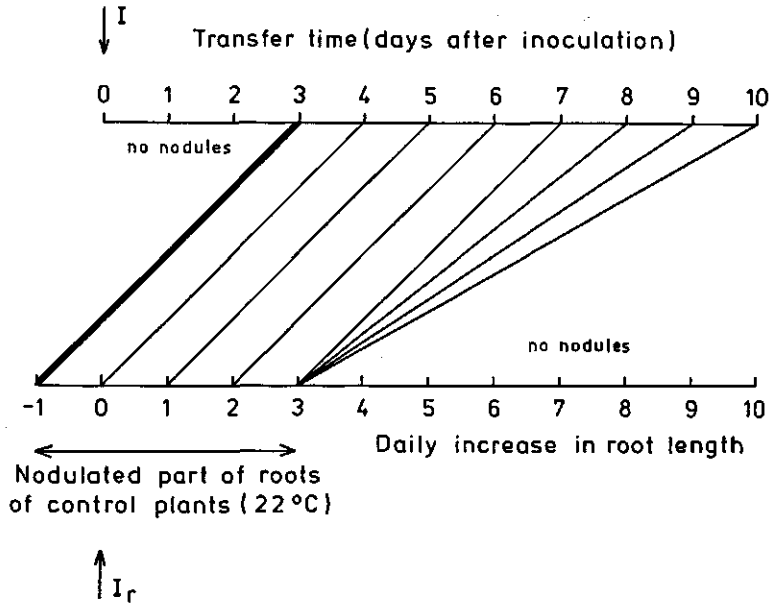

FIG. 12. Relationship between time of transfer to 30°C of pea plants, and lengths of roots bearing nodules. I – inoculation time, I_r – tip of root at inoculation time.

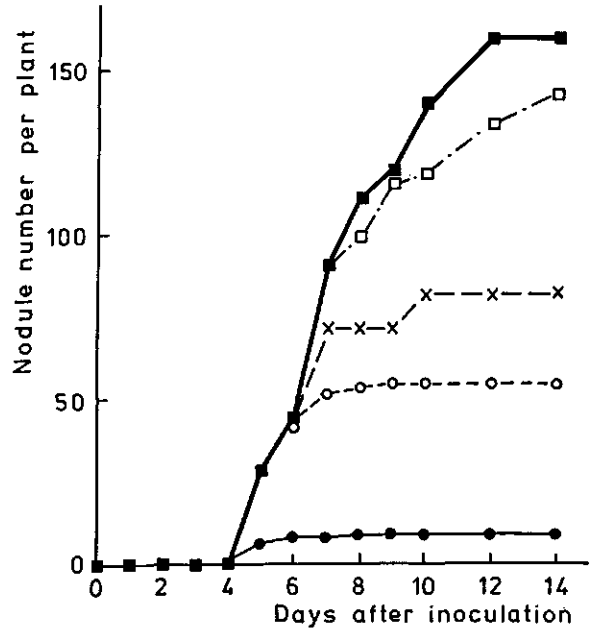

FIG. 13. Numbers of nodules of pea plants, transferred to 30°C at different days after inoculation, present on the indicated days after inoculation. t = 4 (●), 5 (○), 6 (x), 7 (□), control (■).

at 4–5 days after transfer, indicating a very slow development of these nodules. Nodulation occurs generally on a length of root, formed from 1 day before to 3 days after inoculation time. As some nodules develop faster than others, the increase in nodule numbers continues for more than 4 days after the appearance of the first nodule.

In addition to final nodule number, final size of the nodules was related to time of transfer of the plants to 30 °C. Final nodule length was used as a measure of size as collecting and weighing, particularly of the tiny young nodules, was no accurate method. When the plants had been exposed to 30 °C from 10 days after inoculation, the nodules were smaller than those of the control plants at 14 days after inoculation (Fig. 14) although nodule numbers of both series of plants were equal (Fig. 11).

In addition to the experiments described above, some experiments were

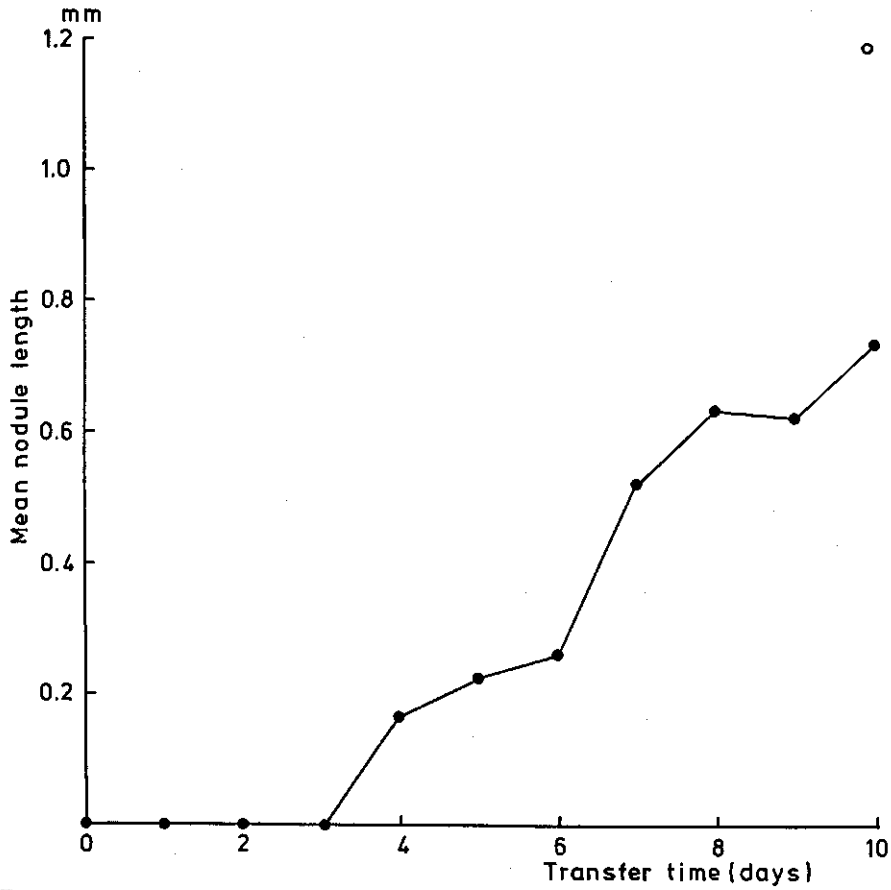

FIG. 14. Final length of nodules (measured 14 days after inoculation) of pea plants transferred to 30 °C at different transfer times (days after inoculation) O – control. (For nodule numbers cf Fig. 11).

TABLE 7. Nodulation of pea plants grown in perlite after transfer from 22°C to 30°C on various days after inoculation. Nodules were counted 13 days after inoculation on series of 5 plants.

Transfer to 30°C	Number of nodules per plant	Number of nodulated plants per series
At inoculation time	0.4 ± 0.9	1/5
4 days after inoculation	4 ± 6	2/5
7 days after inoculation	11 ± 11	4/5
Control	160 ± 40	5/5

performed with pea plants growing in perlite supplied with plant nutrient medium. Plants, pregrown at 22°C in perlite, were transplanted at inoculation time into this material, mixed homogeneously with a suspension of *R. leguminosarum*, strain PRE. Series of 5 plants, apart from the control series, were transferred to 30°C at 0, 4, or 7 days after inoculation; nodules were counted 13 days after inoculation. As it is shown in Table 7, one out of the five plants, placed at 30°C immediately after inoculation, had 2 nodules, resulting in a mean nodule number of 0.4 per plant. Nodule numbers of the control plants were comparable to those of the culture solution experiment shown in Fig. 11. The same was true of plants transferred 4 days after inoculation, but plants transferred to 30°C at 7 days after inoculation gave less satisfactory results. The nodules of the transferred plants were small. Maintenance of the root temperature was a difficulty in these experiments owing to the pronounced evaporation of water from the perlite particles. As the moisture level of the climate cabinet fluctuated considerably during the experiment, evaporation of water and as a consequence temperature of the rooting medium also varied considerably. Because of the complexity of the root's environment, the technical difficulties and because nodulation of roots growing in perlite cannot easily be followed, this method was left.

5.1.3. *Transfer of the inoculated pea plants to 30°C, followed by return to 22°C*

5.1.3.1. Experimental

Pea plants were grown and inoculated in the usual way. Series of 10 plants were transferred to 30°C at different times after inoculation and returned to 22°C at ten days after inoculation. Control plants were maintained at 22°C throughout the experimental period. The nodulation time of each plant was noted.

5.1.3.2. Results and Discussion

The control plants were found to nodulate between 5 and 7 days after inoculation (mean nodulation time 6.2 ± 0.6 days; Fig. 15a). When exposure to 30°C started one day after inoculation, the average nodulation time was 15.2 ± 0.8 days (Fig. 15b); when it started two days later, nodulation time was 15.0 ± 1.1

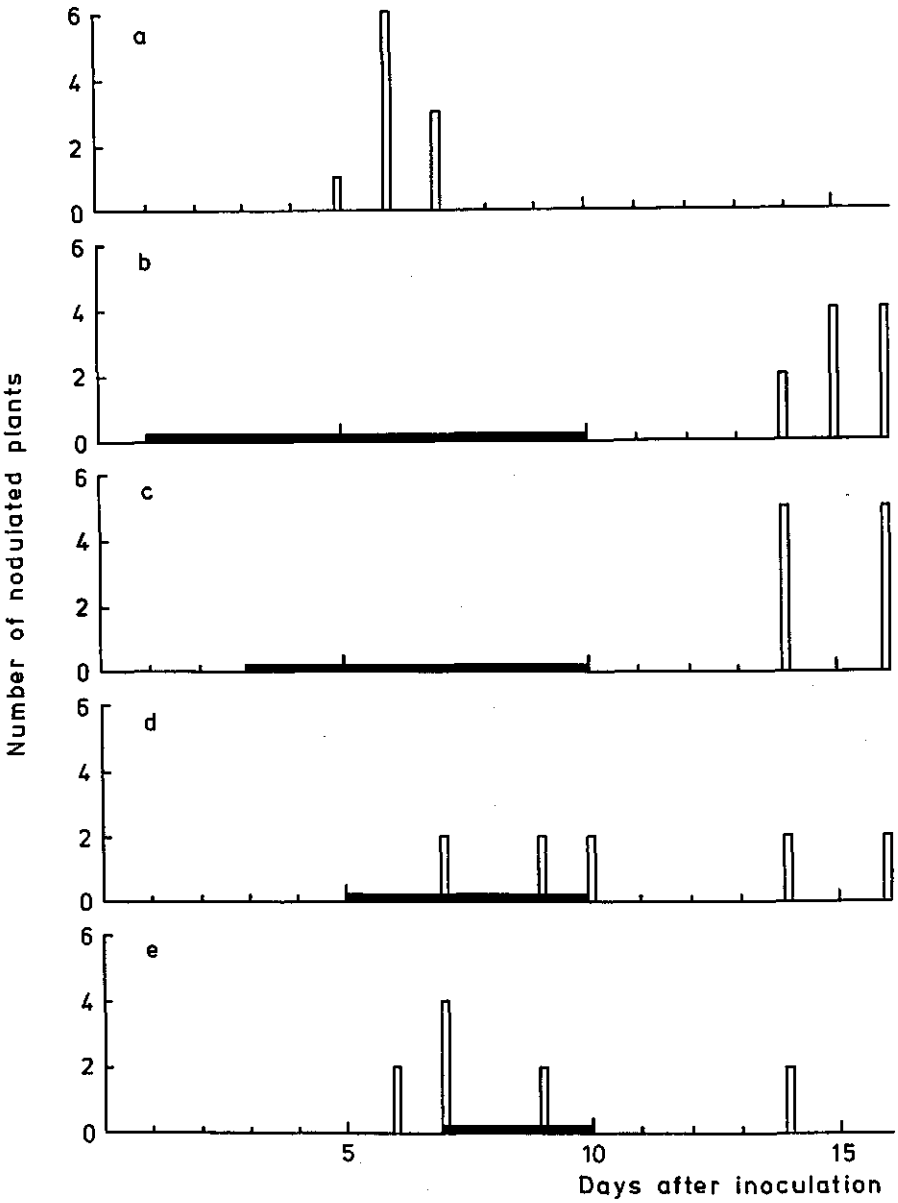

FIG. 15. Influence of period between inoculation and transfer to 30°C on nodulation time. ▬ period at 30°C. Plants returned to 22°C at 10 days after inoculation.

days (Fig. 15c). Assuming that the early stages of nodulation (up to those occurring three days after inoculation) were eliminated by the high temperature treatments, the entire nodulation process had to start again when the plants were returned to the 22 °C environment. However, in both cases the first nodules

were seen 4 days after return to 22°C, which is 1 day earlier than it occurred with the control plants. This suggests that after a high-temperature period, nodulation is not only resumed without any delay but some processes involved in nodulation even proceed more readily. It must be noted, however, that the observed differences are not significant.

After introducing a high-temperature period from the 5th day to the 10th day, the overall nodulation time was 11.2 ± 3.5 days (Fig. 15d). The high standard deviation was due to the fact that two groups of plants occurred. One group nodulated during the period of enhanced temperature (average nodulation time 8.7 ± 1.4 days). The other group nodulated 15 days after inoculation (average nodulation time 15.0 ± 1.2 days) similar to the plants of the preceding experiments (Figs. 15b and c).

The ability of pea plants to form nodules at 30°C had already been shown in an earlier experiment (cf section 5.1.2). It occurs when an incubation period of 4 days at 22°C immediately after inoculation precedes the transfer to 30°C. The experiments of Fig. 15d show that even an incubation period of 5 days at 22°C following inoculation does not guarantee a complete nodulation. The first nodules of four plants became visible 4 and 6 days after return to 22°C. In these plants the entire nodulation process had to be completed after the 30°C treatment. Even two plants incubated from the 7th day to the 10th day at 30°C behaved similarly (Fig. 15e); their first nodules appeared at 4 days after replacing to 22°C. The average nodulation time of this group of plants was 8.6 ± 3.1 days. Nodulation time of the plants forming nodules at 30°C or before exposure to 30°C was 7.3 ± 1.2 days, whereas the remaining plants nodulated 4 days after replacing at 22°C.

The results of these experiments give rise to the following observations. (1) As the average period between replacing the plants from 30° to 22°C and appearance of the nodules is somewhat shorter than the nodulation time of the control plants, one or more early stages of the nodulation process may be insensitive to high temperatures. (2) The large variations in nodulation time of plants incubated for a prolonged period at 22°C, before temporarily being incubated at 30°C, suggest the existence of a temperature-sensitive stage some days before the appearance of the nodules. Plants forming nodules during the exposure to high temperatures must have passed this stage during the low-temperature period.

5.2. The adverse effect of high temperatures on leghaemoglobin content and acetylene-reducing activity

5.2.1. *Introduction*

Nodules, present on plants transferred to 30°C failed to turn pink (5.1.2), whilst pink nodules lost their pigment at high temperatures. From these data it was concluded that high temperatures affect the synthesis and degradation of leghaemoglobin (cf Roponen et al., 1970).

VIRTANEN et al. (1947) have shown that a highly positive correlation exists between the concentration of haemoglobin in root nodules of legumes and nitrogen fixation. BERGERSEN and GOODCHILD (1973) showed that in developing nodules the acetylene-reducing activity is detectable a few days after the appearance of leghaemoglobin. Several workers found a highly positive correlation between leghaemoglobin concentration and acetylene-reducing activity (SCHWINGHAMER et al., 1970; SCHIFFMAN and LÖBEL, 1973; JOHNSON and HUME, 1973).

In this investigation, attention was paid to the correlation between leghaemoglobin and acetylene-reducing activity by using the sensitiveness of leghaemoglobin to high temperatures. The acetylene-reducing activity of free-living nitrogen-fixing bacteria of the genus *Klebsiella* is not inhibited at 30°C (BESSEMS, 1973). High temperatures were found to have an adverse effect on the acetylene-reducing activity of legume nodules; the degree of this effect is dependent on plant species (DART and DAY, 1971) as well on bacterial strain (GIBSON, 1971).

5.2.2. *Methods*

Pea plants were grown at 22°C for different periods after inoculation, before being transferred to 30°C. One series (A) was transferred to 30°C at 10 days after inoculation when white nodules had been formed. Leghaemoglobin content and acetylene-reducing activity of these nodules were determined 14 and 16 days after inoculation, respectively. A second series of plants (B) was transferred to 30°C at 21 days after inoculation, when pink nodules were present. The nodules of these plants were tested for leghaemoglobin content and acetylene-reducing activity at 22 and 23 days after inoculation, respectively. Control series of plants were maintained at 22°C throughout the experiment.

Determination of the leghaemoglobin content of the nodules was performed according to WILSON and REISENAUER (1963); the values obtained are expressed in mg/root system. The acetylene-reducing activity of nodulated root systems was measured according to HARDY and KNIGT (1968).

5.2.3. *Results and Discussion*

The leghaemoglobin content and the acetylene-reducing activity of the nodulated root systems of plants kept throughout the experimental period at 22°C increased until the end of the experiment (Table 8). When the plants were transferred to 30°C at the 10th day after inoculation, the leghaemoglobin content and the acetylene-reducing activity remained around the limit of detection. When the exposure to 30°C began at the 21st day after inoculation, the leghaemoglobin content decreased only slightly but the acetylene-reducing activity fell to about one third of its initial value within 2 days. The correlation coefficient between leghaemoglobin content and acetylene-reducing activity was determined for both sub-experiments. In both cases the correlation was found to be significant at the 1% level. When transfer had taken place 10 days after inoculation the correlation coefficient equalled 0.97, when the plants had

TABLE 8. Leghaemoglobin content and acetylene-reducing activity of nodules of pea plants grown at 22°C for 10 (A) or 21 days (B) after inoculation, and subsequently exposed to 30°C. Control plants were maintained at 22°C throughout the experimental period.

	Days after inoculation	mg leghaemoglobin/root system		nmol C_2H_2 reduced/min. root system	
		22°C	30°C	22°C	30°C
A	10	0.00	0.00	0.02	0.02
	14	0.11	0.005	1.53	0.00
	16	0.22	0.01	6.05	0.02
B	21	0.29	0.29	46.52	46.52
	22	0.32	0.26	67.30	20.18
	23	0.41	0.24	80.24	15.17

* Different plants were used for the determinations of leghaemoglobin and actylene-reducing activity.

** A and B represent different experimental series.

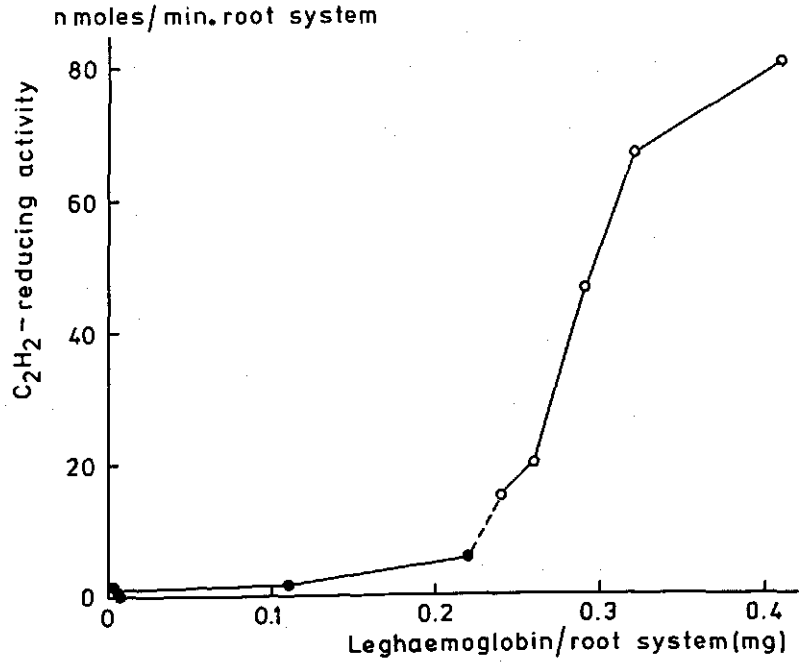

FIG. 16. Relationship between leghaemoglobin content and acetylene-reducing activity of nodules of pea plants, ● at the onset of nitrogen fixation (2 weeks after inoculation) ○ during functioning symbiosis (3 weeks after inoculation).

been transferred 11 days later, this value was 0.93. These results suggest that not only the appearance of leghaemoglobin and acetylene-reducing activity are closely related, as it was also shown by BERGERSEN and GOODCHILD (1973), but that the disappearance of the two are also linked. In Fig. 16 the values of leghaemoglobin content per root system were plotted against the values of nitrogenase-activity, measured on plants grown under the same conditions. Although the data shown were derived from two different experiments, they suggest that at the onset of nitrogen fixation a considerable amount of leghaemoglobin had to be formed before nitrogenase could start its activity. Hereafter a relatively small increase of leghaemoglobin content coincided with a sharp rise in nitrogenase acitivity; a further increase of leghaemoglobin was accompanied by a relatively slight increase in nitrogenase activity.

5.3. SUMMARY

1. Nodules of pea plants may be formed at 30°C, provided that the plants are exposed for more than 3 days to lower temperatures immediately after inoculation.
2. Nodule growth was retarded at high temperatures.
3. The results obtained suggest that high temperatures adversely affect nodulation already shortly after inoculation.
4. Indications have been obtained that before appearance of the nodules a temperature-sensitive stage of nodulation (possibly the infection of the root hair or the growth of the infection thread) is terminated.
5. Leghaemoglobin synthesis as well as initiation of nitrogenase activity were inhibited at 30°C.
6. Once present, leghaemoglobin was less sensitive to high temperatures than nitrogenase activity.

6. THE INFLUENCE OF HIGH TEMPERATURES, LIGHT INTENSITY AND DAY LENGTH ON NODULATION OF PEA PLANTS

6.1. Effect of alternating high and low temperatures on nodulation

6.1.1. *Introduction*

The influence of day length on nodulation has been investigated by several authors (Sironval et al. 1957; Mes, 1959a and b; Gibson, 1967). In some cases the temperature during the light period was several degrees higher than the night temperature (e.g. Mes et al. 1957), in other cases the temperature regime was not made clear (e.g. Doku, 1970). It has been argued that in nature the temperature fluctuates (Joffe et al., 1961), so that by fluctuating temperatures during the experimental treatment one may simulate natural circumstances. Gibson (1967), however, showed that a diurnal fluctuation of the root temperature had a strongly stimulatory effect on nodulation. Moreover, Potts and Ormod (1969) showed that an abrupt temperature increase influenced the rate of internode elongation in peas as well as the content of inorganic phosphorus; the latter in turn may influence nodulation (Trigoso and Fassbender, 1973; Gates and Wilson, 1974; Frings, unpubl.).

In the present investigation an attempt was made to study the effect of the duration of a daily warm period with a constant light regime, as well as the effect of day length with a constant temperature regime, in order to be able to differentiate between both environmental factors.

6.1.2. *Experimental*

Series of 40 pea plants each were exposed daily to a temperature varying between 30° and 33°C for 0, 8, 11, 12 or 16 hours and between 20° and 25°C for the remaining time. The plants received light during 12 hours a day. The middle of the light period coincided with the middle of the period of high temperature. The experiments were performed simultaneously at two light intensities viz. 30 and 125 W/m^2. The plants were inspected for the presence of nodules at 7 and 14 days after inoculation. As only one climate cabinet was available, the experiments with different temperature periods had to be performed after each other.

6.1.3. *Results and Discussion*

The percentage of plants nodulated at 7 days after inoculation decreased rapidly with increased duration of the daily exposure to high temperatures (Fig. 17). At high light intensity more plants nodulated than at low light intensity. The effect of light was most pronounced at daily periods of 8 hours at 30°C: 15 and 70% of the plants being nodulated at low and high light intensities, respectively. One week later the effect of light on the percentage of no-

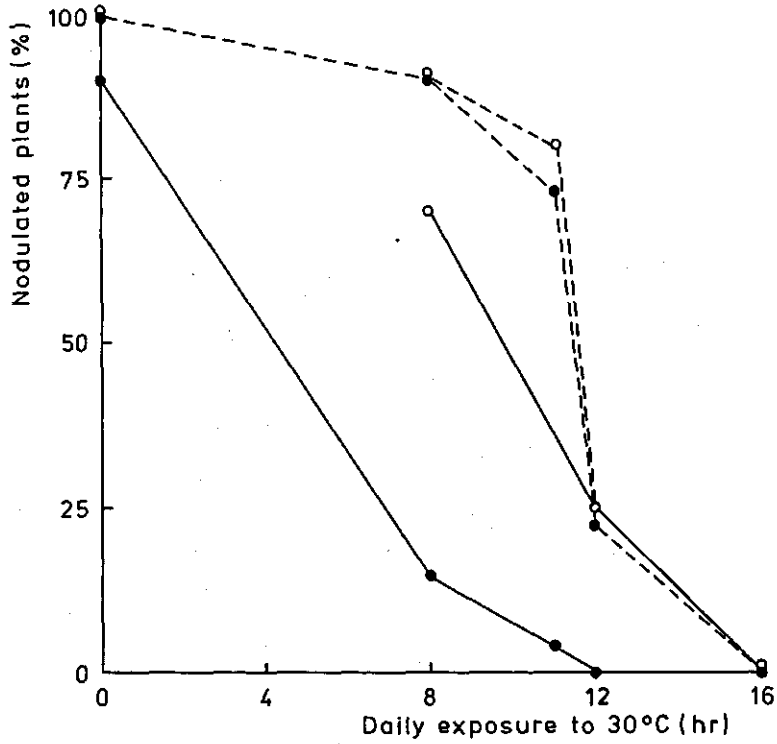

FIG. 17. Influence of the length of the daily periods of exposure to 30°C (variation 30°C–33°C) on the percentages of nodulated pea plants. The lower temperature varied between 20°C and 25°C. The experiments were performed at 2 light intensities corresponding with 30 and 125 W/m²; day length 12 hr. Nodulated plants counted at 7 and 14 days after inoculation.
●——● 30 W/m²; 7 days. ●– – –● 30 W/m²; 14 days.
○——○ 125 W/m²; 7 days. ○– – –○ 125 W/m²; 14 days.

dulated plants at both light intensities had disappeared. A daily exposure to 30°C of 8 and 11 hours brought about a percentage of nodulated plants of 90 and 80, respectively, whereas an exposure of 12 hours decreased this percentage more drastically to 25% (Fig. 17). This refers to the high light intensity; at low light intensity the corresponding values were somewhat lower. When the daily exposure to 30°C was extended to 16 hours, at both light intensities no plants were found to be nodulated.

The results obtained suggest that pea plants need a minimum amount of organic substrates in order to enable nodulation. When the plants are grown at high temperatures, less substrate is available, because under these conditions a greater part is respired (cf. LIE, 1969).

6.2. Effect of high temperatures during light and dark periods

6.2.1. *Experimental*

A series of 40 pea plants, pre-cultivated and inoculated as usual, were grown at 22°C during illumination (12 hours) and at 30°C during the dark period (12 hours). The plants of two control series were grown at 22°C and 30°C, respectively, throughout the experimental period. Light intensity was 30 W/m². Nodules and nodulated plants were counted 14 days after inoculation. Data concerning plants incubated at 30°C during illumination and to 22°C during the dark period were derived from the experiment described in 6.1.2.

6.2.2. *Results and Discussion*

Nodulation was more severely inhibited by high-temperature treatment during the dark period than during illumination (Table 9). This result is in agreement with the fact that high temperatures favour both respiration and photosynthesis. When exposed to 30°C during the light period, the higher rate of respiration is counterbalanced by the higher rate of photosynthesis; when exposed to 30°C during the dark period, respiration only is stimulated. The probability of a plant of being nodulated is apparently correlated with the available substrate which is lowered when the exposure to high temperatures occurs during the dark period (Table 9).

Table 9. Effect of a high temperature during the light or the dark period on nodulation. Each period lasted 12 h.

Temperature during		Percentage of nodulated plants	Average number of nodules
illumination	darkness		
22°C	22°C	100	150 ± 50
30°C	22°C	22[1]	n.d.
22°C	30°C	6	10 ± 37
30°C	30°C	0	0

[1] Value taken from the experiment shown in Fig. 17. Nodules counted at 14 days after inoculation.

6.3. Effect of day length on nodulation

6.3.1. *Introduction*

In the present section the combined influence of day length and light intensity on nodulation is reported. In order to enhance the expected response to the period of illumination, the experiments were carried out at a temperature slightly below the critical temperature of 30°C, viz. 28°C.

6.3.2. *Experimental*

After inoculation, the plants were subjected to daily periods of illumination of 8, 12, 14 and 16 hours. The light intensities used were equal to 30 W/m²

and 125 W/m². Numbers of nodulated plants were recorded at 14 days after inoculation. The temperature was maintained at 28 °C throughout the experimental period.

6.3.3. *Results and Discussion*

Light intensity as well as day length had a stimulatory effect upon nodulation (Fig. 18). The effect of light intensity was much more pronounced at a daily light period of 8, 12 and 14 hours than at a day length of 16 hours. It can be concluded that the harmful influence of short days on nodulation can be eliminated by increasing the light intensity. These results suggest (conformable to those of section 6.1) that nodulation occurs at photosynthetic activities above a certain minimum level.

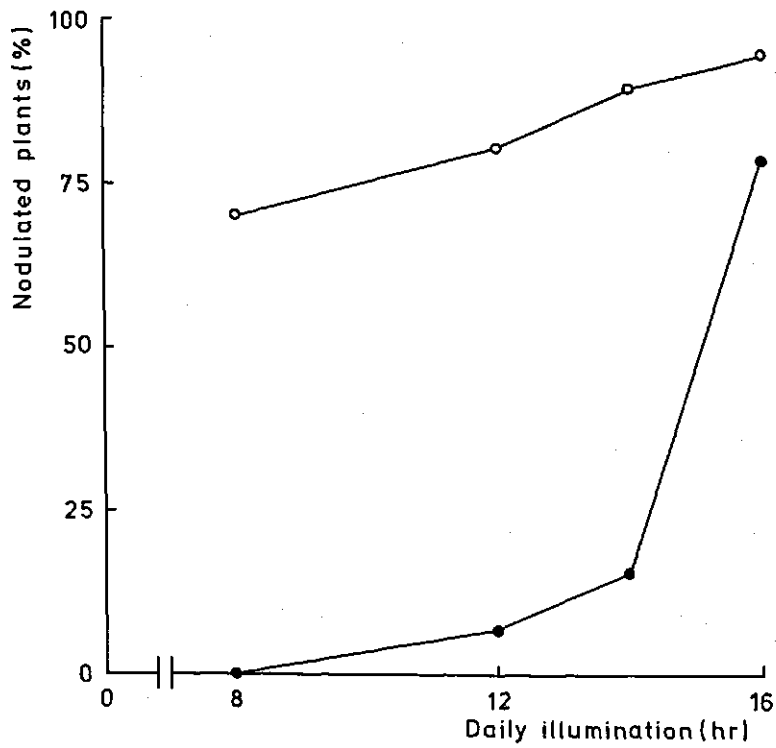

FIG. 18. Influence of day length and of light intensity on the percentages of nodulated pea plants. Temperature 28 °C. Nodulated plants counted at 14 days after inoculation. 30 W/m² (●), 125 W/m² (○).

6.4. SUMMARY

1. A daily warm period of 11 hours or less during the light period does not significantly affect nodulation.
2. Increase of photosynthetic activity increases the probability of a plant of being nodulated; increase of respiratory activity has the adverse effect.

7. INHIBITION BY HIGH TEMPERATURES OF PROCESSES OCCURRING DURING THE INITIAL PHASE OF THE SYMBIOSIS

7.1. THE FIRST INHIBITED STAGE

7.1.1. *Introduction*

The results described in Chapter 5 show that the nodulation process can be affected by high temperatures during at least three distinct periods viz: 1. During three days after inoculation. 2. During nodule growth. 3. During the synthesis of leghaemoglobin.

The picture of the first period is rather vague. FÅHREUS (1957) reported that the earliest infection threads in the root hairs of *Trifolium* spp were observed on the 2nd day following inoculation; it may be assumed that the infection threads of pea roots do not appear at an earlier date. From experiments described earlier (5.1.3.2, Fig. 15) it might be concluded that a high temperature exerts its inhibitory effect from about one day after inoculation. This would mean that the first detectable adverse effect of high temperatures occurs prior to infection of the root hairs by the rhizobia and presumably at 1 day following inoculation. As it is generally assumed that rhizobia have to multiply in the rhizosphere to considerable numbers before being able to invade the root hairs (MUNNS, 1968), reduced multiplication might be involved in this early type of inhibition. However, *Rhizobium* cells are capable to grow at 30°C, whilst in Chapter 3 it has been shown that at this temperature the roots do not exude inhibiting compounds. These arguments would predict that the period prior to infection would not be affected by high temperatures. On the other hand, the experiments reported in Chapter 5 (Fig. 15) point to the contrary. The purpose of the experiments, described in the present chapter, was to establish at what time temperature-sensitivity of the nodulation process becomes perceptible.

7.1.2. *Experimental*

Pea plants grown at 22°C and inoculated with *Rhizobium leguminosarum*, strain PRE, as described in Chapter 3, were kept at high temperatures starting at inoculation for different periods before being returned to 22°C. Several experiments, each comprising 90 plants, were performed at different temperatures (25, 28 and 30°C). The nodulation time was observed. For the purpose of interpreting the results, it was supposed that the first stage of the nodulation process consists of a period A, during which the associative growth is not affected by high temperature, followed by a temperature-sensitive period B. If the plants would be transferred to the high temperature and returned to 22°C during period A, no influence of the adverse temperatures on nodulation would be expected and the plants should nodulate like the control plants.

the high-temperature period, or part of it, would fall during period B, nodulation, as compared to that of the control plants, should be postponed. This delay in nodulation should be more extended the longer the plants would have been exposed to the high temperature.

The delay of nodulation as compared to the nodulation of the control plants, according to the above-mentioned supposition, depends on the time during which the inoculated plants are incubated at 30 °C. If this period (p) is situated within period A, the nodulation-delay time (d) will be zero (first function). The second function (valid for period B) can be expressed as $d = a + bp$. For $d = 0$ in the latter equation (the intersection with the p-axis), a value for p will be found, which gives the duration of period A. The slope, b, of the line may provide information about the degree of the adverse-temperature influence. The equation of the latter function is obtained by determining the regression line through the experimentally found values.

7.1.3. *Results and Discussion*

Table 10 contains equations of the regression lines found after exposing the plants to 25 °C, 28 °C and 30 °C, respectively, and returning them to 22 °C from the third day after inoculation. The standard deviation of the slope, s_b, is also given, as well as the result of substituting $d = 0$ in the regression equation found. Period A is not significantly different from 0 (days) at all of the temperatures. It may be concluded that according to this method a period during which the legume-rhizobium association is unsensitive to high temperatures, does not exist or is at most very short. As a consequence, it may be suggested that high temperatures affect the association as soon as rhizobia and leguminous plant come into contact. This conclusion is at variance with the results of the experiment recorded in 5.1.3.2 where indications were obtained concerning the existence of a temperature-insensitive period following inoculation.

From the regression equations in Table 10 it can also be seen that the slope value of the lines is larger according as the temperature is higher. This phenomenon suggests that the investigated inhibition by high temperature is also present at temperatures below 30 °C but to a lesser extent.

TABLE 10. The influence of the length of a period (p) of high temperature after inoculation on delay of nodulation (d) compared to nodulation time at 22 °C. S_b = the standard error of the regression coefficient. The non-delay period (A) is obtained from the intersections of the regression lines and the t-axis.

Temperature	Equation of regression line	s_b	Period A (days)
25 °C	$d = 0.0 + 0.46\,p$	0.16	0.0 ± 0.8
28 °C	$d = 0.4 + 0.74\,p$	0.20	-0.5 ± 0.7
30 °C	$d = 0.1 + 0.95\,p$	0.28	-0.1 ± 0.6

7.2. Bacterial attachment to the root

7.2.1. *Introduction*

From the results described in section 7.1.3. (Table 10) it is concluded that if an insensitive period would exist immediately after inoculation, it may last at most 0.7 days (17 hours). Because this is a rough estimation, it was tried experimentally to obtain some more detailed information concerning the temperature sensitiveness of this period which comprises the attachment of the rhizobial cells to the root surface and the multiplication of the rhizobia in the rhizosphere. To that purpose plants were inoculated by dipping their root systems into a suspension of *Rhizobium* cells. From preliminary experiments it was found that the bacteria attach to the roots within 1 second. If the attachment of the bacteria would be temperature-sensitive, it may be assumed that the first detectable inhibition of the symbiosis by high temperatures occurs immediately after inoculation.

7.2.2. *Experimental*

Rhizobium leguminosarum, strain PRE, was grown in 100 ml yeast-extract glucose medium in 300 ml Erlenmeyer flasks. The cells were harvested during the exponential growth phase, counted in a counting chamber and diluted to the appropriate concentration. Before inoculation, the bacterial suspension as well as the root systems of the plants were allowed to attain the experimental temperatures (22 °C, 25 °C, 28 °C or 30 °C) by transferring them to the appropriate temperature for at least 15 minutes before dipping.

Whole root systems of pea plants were dipped into suspensions of *Rhizobium* cells of a given concentration for 1 second. Hereafter, the roots were rinsed in 0.02 M $Na_4P_2O_7$ (Augier and Lavergne, 1958) for half a minute, followed by rinsing in tap water for half a minute. All fluids had the same temperature. After rinsing, the plants were placed at 22 °C and observed daily for nodule formation. In some experiments, the plants were not inoculated by dipping but by pipetting known numbers of bacteria per root system.

7.2.3. *Results and Discussion*

7.2.3.1. Influence of temperature on attachment

When the plant roots had been dipped into a suspension of 1.25×10^7 cells/ml at the lower temperatures (22 °C, 25 °C, 28 °C), all of the plants had nodulated at 14 days after inoculation (Table 11). However, if the dipping had been performed at 30 °C, only 3 out of 5 plants had nodulated. When using a suspension of 1.25×10^6 cells, no nodulation was found if the procedure had taken place at 30 °C and only one out of 5 plants had been nodulated at 14 days if the plants had been inoculated at 28 °C. At the lowest two temperatures all of the plants were found to be nodulated. After dipping the root system into a suspension containing 1.25×10^5 cells/ml at all temperatures some plants had failed to nodulate, but most failures were found at the highest temperature.

TABLE 11. Ratio of plant numbers nodulated at 14 days after inoculation and total numbers of inoculated plants. The plants had been inoculated by the dipping method under the circumstances indicated.

	Numbers of rhizobial cells/ml		
Temperature	1.25×10^7	1.25×10^6	1.25×10^5
22°C	4/4	5/5	4/5
25°C	5/5	5/5	2/5
28°C	5/5	1/5	2/5
30°C	3/5	0/5	0/5

When plants become nodulated, at least one bacterium must have attached itself so firmly to the root that it was not rinsed off by the pyrophosphate-containing solution. On the other hand, no bacteria should be attached to the non-nodulating plants, or the attachment should have been so loosely that the bacteria were rinsed off. The results obtained indicate that the attachment of the rhizobia to the roots of pea plants is temperature-dependent.

Numbers of nodules formed on root systems inoculated at different temperatures did not vary very much (Table 12).

TABLE 12. Average numbers of nodules on plants inoculated by dipping into a *Rhizobium*-suspension at temperatures and cell concentrations as indicated. Nodules were counted 14 days after inoculation. Only nodulated plants were included in the calculation of the average numbers of nodules.

	Numbers of rhizobial cells/ml		
Temperature	1.25×10^7	1.25×10^6	1.25×10^5
22°C	205 ± 25	250 ± 40	140 ± 40
25°C	225 ± 35	235 ± 40	110 ± 40
28°C	210 ± 35	325	200 ± 10
30°C	215 ± 50	–	–

7.2.3.2. Influence of bacterial density of the inoculum on nodulation time

Nodulation time is prolonged (Table 13) and numbers of nodulated plants reduced (Table 11) after dipping the root systems of pea plants into suspensions of lower density and of higher temperature. PURCHASE and NUTMAN (1957), inoculating clover plants with mixtures of low numbers of virulent and high numbers of avirulent rhizobia, concluded that only few virulent bacteria are required for nodule formation. This result is not necessarily in contradiction with the results of MUNNS (1968), who found that large numbers of rhizobia have to be present in the rhizosphere before infection can occur. The large population of either virulent or avirulent rhizobia may be needed to provide the

TABLE 13. Nodulation time of plants inoculated by the dipping method at different temperatures and with suspensions of different densities.

Temperature	Numbers of rhizobial cells/ml		
	1.25×10^7	1.25×10^6	1.25×10^5
22°C	5.3 ± 0.3	6.2 ± 0.5	8.0 ± 0.4
25°C	6.0 ± 0.4	7.2 ± 0.7	10.5 ± 0.5
28°C	6.6 ± 0.2	11	9.5 ± 1.5
30°C	8.0	–	–

environment at the root surface to enable few virulent cells to infect. According to this assumption, the probability of nodulation is lowered by reducing the number of bacteria in the inoculum.

The minimum number of bacteria in the inoculum required for nodule formation was estimated by pipetting known volumina of a dilution series of a suspension of the virulent PRE-strain of *R. leguminosarum* into the rooting medium. An inoculum containing about 200 bacteria per root system was found to be required for obtaining a normal nodulation time (Fig. 19). Nodule num-

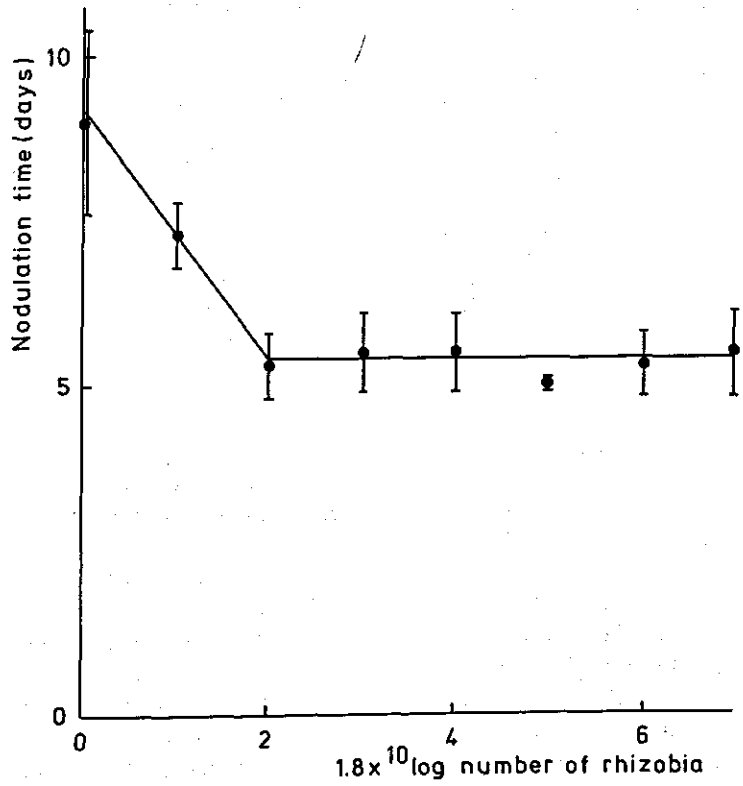

FIG. 19. Effect of bacterial density of the inoculum on nodulation time. Temperature 22°C.

bers of all of these plants amounted to about 300. Plants inoculated by dipping into a rhizobial suspension of 1.25×10^5 bacteria/ml at 22°C gave a delay of nodulation of 2 days as compared to the nodulation time of plants treated in the same way with a rhizobial suspension of higher density (Table 13, 22°C). The same nodulation time (8.0 days) was observed when the root systems had been dipped at 30°C into a bacterial suspension containing 1.25×10^7 bacteria/ml (Table 13) i.e. 100 times as many cells as had to be present at 22°C. This means that at 30°C probably 100 times as many bacteria were rinsed off as compared to room temperature.

7.2.3.3. Counting of *Rhizobium* cells attached to the root

In section 7.2.3.1 it was suggested that at 30°C rhizobia are washed off more easily from the roots of pea plants than at 22°C. This was tried to check by counting the number of bacteria present on the root system after washing. To this purpose, root systems were homogenized in a mortar. The resulting suspension was diluted and plate countings were made. The numbers of rhizobia were very low, probably due to adsorption of the bacteria by the crushed root tissue as was also found by VAN EGERAAT (1972). As can be seen from Table 14, lower rhizobial numbers were generally found to be attached to the roots after the 30°C-treatments as compared to the 22°C-treatments.

TABLE 14. Numbers of *Rhizobium* cells counted on the root systems of pea plants after inoculation by dipping, followed by washing in 0.02 M $Na_4P_2O_7$ at 22°C or at 30°C.

Experiment	Bacteria/ml in dipping suspension	Counted bacteria/root system	
		22°C	30°C
1	8.8×10^6	1.5×10	3.0×10
2	2.6×10^8	3.0×10^3	1.6×10^3
3	2.4×10^8	3.0×10^3	3.3×10^2
4	5.0×10^8	5.3×10^3	1.3×10^2

7.2.3.4. Discussion

The results obtained in the present investigation on attachment of rhizobia to pea roots may be explained in the light of a study on lectin by BOHLOOL and SCHMIDT (1974). These authors found that soybean lectin binds to *Rhizobium japonicum* cells. They suggest that legume lectins may present a site on the legume root surface which interacts specifically with a distinctive polysaccharide on the surface of the appropriate *Rhizobium* cell as a prelude to nodulation. The observations made in the present study on attachment of *R. leguminosarum* to pea roots might be described in terms of a lectin-polysaccharide interaction. As the attachment of the rhizobia to the roots was found to be influenced by temperature, it is suggested that the lectin-polysaccharide binding is temperature-dependent.

7.3. SUMMARY

1. The first effect of high temperatures on nodulation of pea plants occurs immediately after inoculation of the pea roots with *Rhizobium* cells.
2. Nodulation is delayed when the inoculum contains less than circa 200 bacteria per pea plant.
3. A procedure was introduced to inoculate plants by dipping the root systems into a bacterial suspension.
4. Attempts to rinse the rhizobia from the root system, after inoculation by dipping, resulted in absence or delayed appearance of the nodules when dipping and rinsing occurred at a high temperature.
5. The results obtained suggest that the attachment of *Rhizobium* cells to the roots of pea plants is the first stage of nodulation affected by high temperatures.

8. INFLUENCE OF HIGH TEMPERATURES ON NODULATION AS AFFECTED BY RHIZOBIAL GROWTH

8.1. Estimation of the pre-infection period by using antibiotics

8.1.1. *Introduction*

To differentiate between rhizobia in the rhizosphere and those having entered the root, use was made of antibiotics, which suppress the proliferation of these bacteria. If bacteria after having invaded the root would be protected to antibiotic action, infections completed at the time of addition of the antibiotic would give rise to nodule formation.

8.1.2. *Experimental*

Chloramphenicol (20μg/ml) or streptomycin (10 μg/ml) were added to the nutrient solution of pea plants at different periods after inoculation. The plants were inoculated with ca 10^6 bacteria per plant at unfolding of the first true leaf. The bacteria were grown in yeast-extract glucose medium and harvested during the exponential growth phase.

8.1.3. *Results and Discussion*

Chloramphenicol affected the growth of both roots and bacteria. It was impossible to count nodules because of distortions of the roots; the presence of nodules was only detected after the appearance of leghaemoglobin. Streptomycin influenced root growth to a lesser extent. Nevertheless, it became clear that both antibiotics inhibit nodulation completely when added up to the second or third day after inoculation (Table 15). When streptomycin was added 4 days after inoculation, only one out of 3 plants failed to nodulate. After adding chloramphenicol at this time all of the 5 plants tested nodulated. Nodule numbers of control plants and of plants having received streptomycin at the

Table 15. Effect of chloramphenicol (20 μg/ml) and streptomycin (10 μg/ml), added at different periods after inoculation of root systems of pea plants on nodulation.

Days after inoculation	Chloramphenicol Presence of nodules	Streptomycine			
		Nodule numbers			Mean nodule number
0	−	0	0	0	0
1	−	0	0	0	0
2	−	0	0	0	0
3	−	0	0	8	2.7
4	+	0	29	65	31.3
5	+	16	20	84	40.0
control	+	14	44	46	34.7

5th day from inoculation, were equal. These results show that after having invaded the roots, rhizobia are no more accessible to the added antibiotics. From the results obtained it may be concluded that under the conditions of this experiment, infection of pea roots takes place 3–4 days after inoculation.

8.2. The influence of high temperatures on the multiplication of rhizobia in the rhizosphere

8.2.1. *Introduction*

In chapter 7 it has been shown that high temperatures affect the association as soon as the bacteria are added to the root system. Indications were obtained that temperature influences the attachment of the bacteria to the root system, but it is not clear in what way this probably short-lasting process could affect the association in undisturbed environments. In the previous section it was shown that rhizobia do not invade pea roots prior to the 3rd day from inoculation, whilst in 5.1.2. it was shown that transfer from 22 °C to 30 °C up to the 3rd day inhibits nodulation completely. This indicates that the pre-infection stages are more severely affected by high temperatures than the post-infection stages. The attachment stage is probably the first of a number of sensitive stages. It is generally assumed that prior to infection the bacteria have to multiply. In a glass vial, multiplication of rhizobia is not inhibited at 30 °C, the average generation time of *R. leguminosarum* being 5.2 h as contrasted to 7.2 h at 22 °C. For this reason the bacteria used in this investigation were grown at 30 °C.

Subsequently the effect of a high temperature on multiplication of rhizobia in the rhizosphere was investigated. As in earlier experiments direct countings of the rhizobia in the rhizosphere had been shown to be unsatisfactory, an indirect method for estimating rhizobial multiplication in the rhizosphere at 30 °C was developed. This method is based on the observed influence of number of inoculated bacteria on nodulation time (Fig. 19), and on the delay of nodulation time upon temporary exposure to 30 °C. The data presented in Fig. 19 show that at 22 °C a small inoculum causes a delay in nodulation time as compared to a large inoculum. Transfer of plants to 30 °C immediately after inoculation delays nodulation for a period as long as the exposure to this temperature is lasting. Consequently, if multiplication of the rhizobia in the rhizosphere is inhibited at 30 °C the delay of nodulation time of lightly inoculated plants, temporarily exposed to 30 °C will be the sum of the period of incubation at 30 °C and the delay of nodulation of the lightly inoculated control plants. If multiplication in the rhizosphere takes place at 30 °C, the delay of nodulation will only be due to the period of exposure to 30 °C.

8.2.2. *Experimental*

Pea plants were grown as usual. Bacteria were grown in a yeast-extract glucose medium and harvested at the beginning of exponential growth. Inoculation was carried out with either 2.64×10^4 or 2.64×10^0 bacteria per

TABLE 16. Nodulation time of pea plants after inoculation with 1 ml per plant of suspensions of *R. leguminosarum* of indicated strength in combination with different periods of exposure to 30°C, immediately after inoculation.

Exposure (days)	Nodulation time (days)	
	2.64×10^4 bact/ml	2.64 bact/ml
0	6.4 ± 1.1	8.1 ± 0.3
1	7.0 ± 0.7	7.0 ± 0.0
2	8.5 ± 0.5	8.3 ± 0.5

plant. Immediately following inoculation, some plants were placed at 30°C for 1 or 2 days.

8.2.3. *Results and Discussion*

Table 16 shows that the delay in nodulation due to the small inoculum was 2 days. A 2-days incubation period at 30°C of the heavily inoculated groups was also responsible for a delay of nodulation of 2 days. The lightly inoculated plants that had been exposed to 30°C during 2 days did not show further delay, so that the bacteria must have multiplied at 30°C. When the lightly inoculated plants were exposed for 1 day to 30°C, they nodulated one day earlier than the lightly inoculated control plants, showing that the multiplication rate in the rhizosphere at 30°C was faster than at 22°C.

8.3. THE INFLUENCE OF SIZE AND ORIGIN OF THE INOCULUM ON NODULE NUMBER

8.3.1. *Introduction*

In the experiments described in section 8.2. in which the inoculum consisted of cells harvested at the initial phase of the exponential growth, a large inoculum gave 60–80 nodules per plant, whereas a small inoculum resulted in the formation of between 200 and 400 nodules per plant. In experiments performed with bacteria harvested during the exponential growth phase this influence of the inoculum size on nodule numbers was not observed. These observations suggested an influence of the age of the bacterial culture, used as inoculum. Therefore a more detailed experiment on the effect of age of the rhizobia culture used for inoculation was set up.

8.3.2. *Experimental*

Erlenmeyer flasks of 300 ml capacity containing 100 ml yeast-extract glucose medium were inoculated with *R. leguminosarum* strain PRE. After periods of 24, 36 and 48 hours (beginning, middle and end of exponential growth, respectively) *Rhizobium* cells were harvested and, after counting in a counting chamber, diluted to densities of ca 10^4 and 10^0 cells/ml. From each

of these dilutions, 1 ml was pipetted on to the roots of series of 10 pea plants that had just unfolded their leaves. Nodulation time was determined and 14 days after inoculation the nodules were counted.

8.3.3. *Results and Discussion*

Nodulation time was not significantly affected by the age of the bacterial culture. Plants inoculated with the dilute rhizobial suspension nodulated 2 days after those supplied with the dense suspension (Fig. 20).

Nodule numbers were clearly affected by the age of the rhizobial culture used for inoculation (Fig. 21). When ca 10^4 *Rhizobium* cells were added per root system the resulting nodule numbers were higher with increasing age of the bacterial cultures. On the contrary, nodule numbers resulting from small inocula were lower with increasing age of the rhizobial cultures. Both 'age' lines are crossing each other shortly after the mid-logarithmic phase which means that nodule numbers are not affected by the size of the inoculum, when cultures of moderate age are used. These results were confirmed several times. In most of the experiments, in which the rhizobia were precultivated in culture

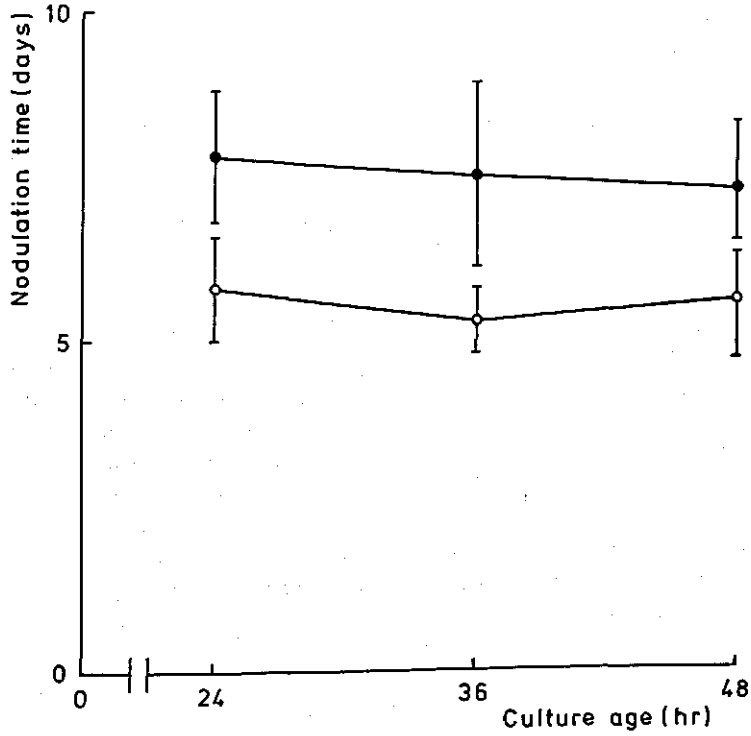

FIG. 20. Influence of number of bacteria in the inoculum and age of the culture used for inoculation on nodulation time. The inoculum contained 2.6, 1.5, and 1.4x either 10^4 (O) or 10^9 (●) cells (at culture ages of 24, 36 and 48 hr, respectively).

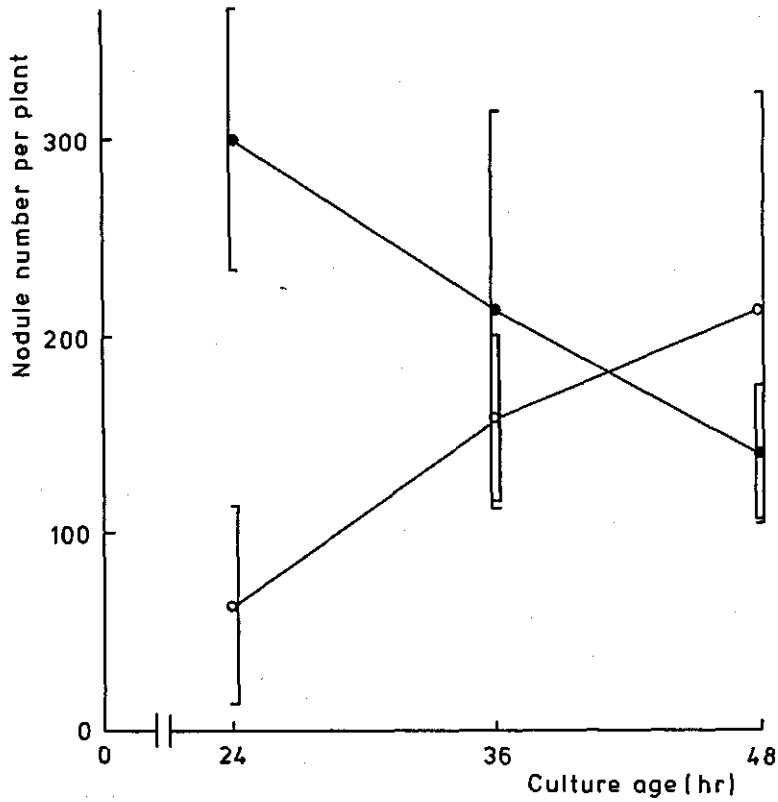

FIG. 21. Influence of number of bacteria in the inoculum and age of the culture used for inoculation on numbers of nodules. Explanations as in Fig. 20.

solutions, the cells were harvested in the middle or towards the end of the logarithmic growth phase. This explains why the effect of the inoculum size on nodule numbers was not detected at an earlier event. One of the differences between inocula of small and large sizes concerns the amount of organic material introduced. When small numbers of 48 h old rhizobia were introduced into the culture solutions of the pea plants, only 6 out of 10 plants formed nodules. Part of this phenomenon may be ascribed to the death of many rhizobia in the relatively old culture. It is striking that only the numbers of nodules caused by infection of roots with bacteria from young cultures show significant differences.

8.4. SUMMARY

1. Nodulation is prevented when chloramphenicol or strephtomycin is added to the rooting medium up to 3–4 days after inoculation. After this period,

rhizobia presumably have entered the root and are no more accessible to these antibiotics.

2. Multiplication of *R. leguminosarum*, strain PRE, in the rhizosphere of pea roots is not inhibited by high temperatures. Owing to this, the prolonged nodulation time of lightly inoculated pea plants was shortened to the nodulation time of heavily inoculated plants, by exposure of both series of plants to 30 °C during one day (Table 16).
3. The number of *Rhizobium* cells of an inoculum and the age of the culture used for inoculation were repeatedly found to affect nodule numbers of pea plants. An explanation of this phenomenon can not be given.

9. THE EFFECT OF BACTERIA, PARTICULARLY *R. LEGUMINOSARUM*, AND TEMPERATURE ON ROOT HAIRS

9.1. THE MORPHOLOGY OF ROOT HAIRS

9.1.1. *Introduction*

Roots of pea plants growing in a liquid medium are forming many root hairs. However, parts of roots or even entire roots may be found, which are completely deprived of root hairs. *Rhizobium* cells, present on the root surface, bring about root-hair deformations (e.g. SAHLMAN and FÅHREUS, 1963; HAACK, 1964; YAO and VINCENT, 1969) before entering the roots, usually via the deformed root hairs which they pass in an infection thread. The influence of temperature and the combined effects of temperature and rhizobia on the appearance of root hairs are not known. Therefore, a study was made of the effect of *R. leguminosarum* and some other soil bacteria on root hair deformation.

According to HAACK (1964), *Rhizobium* species are able to cause deformations of root hairs of plants which they can infect and of closely related plant species. Root-hair deformations of leguminous plants caused by other soil bacteria are unknown.

9.1.2. *Experimental*

Pea plants were grown at 22°C as described before. At unfolding of the first true leaf, the plants (except the control plants) were inoculated and placed either at 22°C or 30°C. The bacteria were grown on agar slants. Duplicate plants were inoculated with a few drips of a bacterial suspension, made by suspending a loopful of bacteria in a few milliliters of sterile water. Some roots of these plants were collected 3 days after inoculation and scanned microscopically for root hairs. Representative parts of the roots were photographed.

The following strains of bacteria were used: *Rhizobium leguminosarum*, strains PRE, PF2 and S310a; *R. trifolii*, strain K8; *R. japonicum*, strain A102; *Micrococcus denitrificans; Pseudomonas aeruginosa; P. fluorescens; Arthrobacter simplex*, strain AC1; and *A. globiformis*, strain AC8.

9.1.3. *Results and Discussion*

Root hairs of uninoculated pea plants differed largely from those of plants inoculated with *R. leguminosarum* (PRE) (Figs. 22 and 23). In the presence of *Rhizobium* cells, they were branched, curled or deformed in some other way. Root hairs of uninoculated plants may show slight deformations but mostly they were straight and longer than those of inoculated plants. This applies to the situation at 22°C. At 30°C, root hairs were rarely seen on uninoculated plants (Fig. 22b) but in the presence of *R. leguminosarum* they occurred more frequently. In the latter case they were usually swollen (Fig. 23b). These

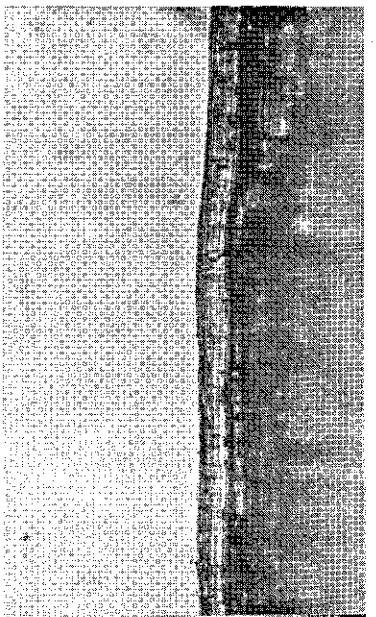

FIG. 22b.

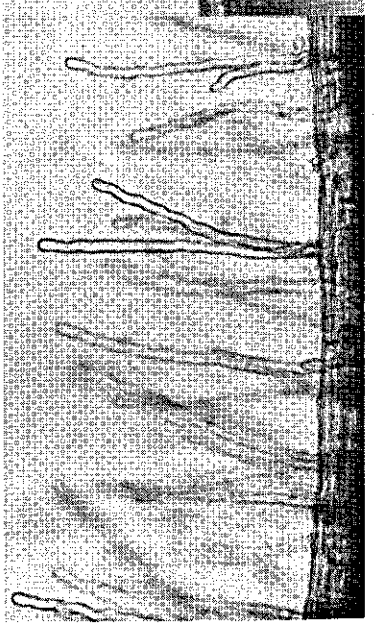

FIG. 22a.

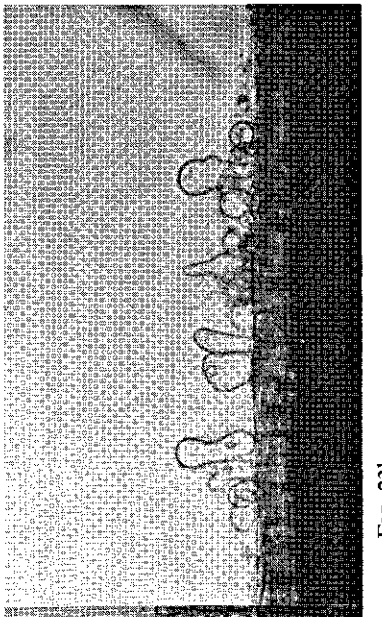

FIG. 23b.

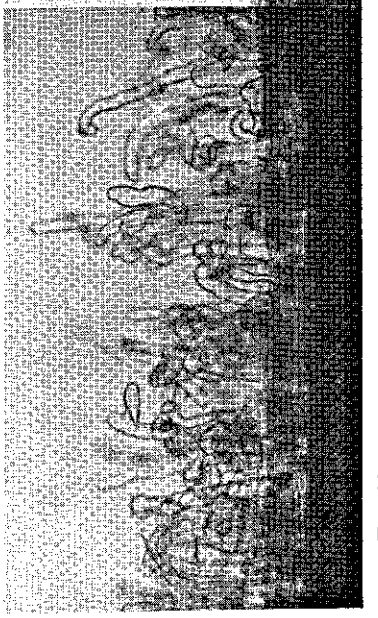

FIG. 23a.

FIG. 22a. Root surface of a pea plant, without rhizobia, temperature 22°C.
FIG. 22b. Root surface of a pea plant, without rhizobia, temperature 30°C. Note absence of root hairs.
FIG. 23a. Root hairs of a pea plant, 3 days after inoculation with *R. leguminosarum*, strain PRE. Temperature 22°C.
FIG. 23b. The same as Fig. 23a. Temperature 30°C.

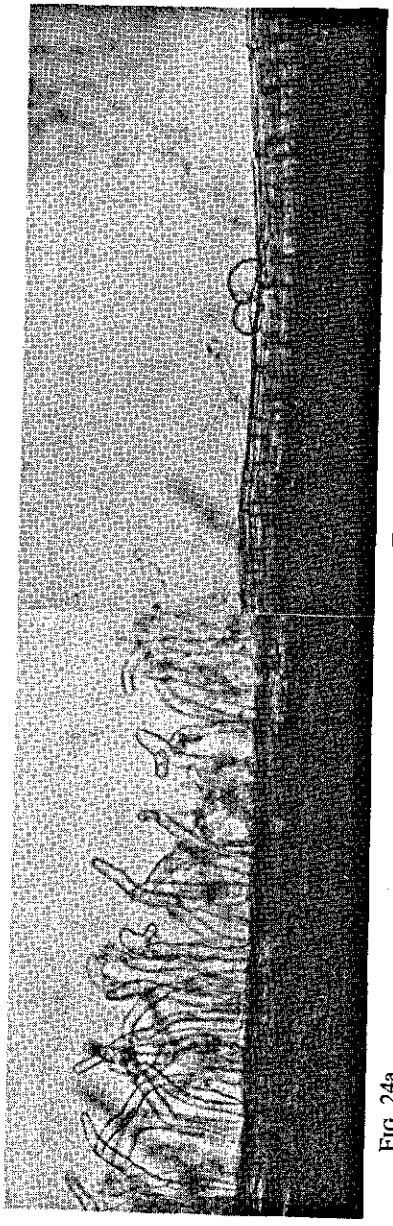

FIG. 24b.

FIG. 24a.

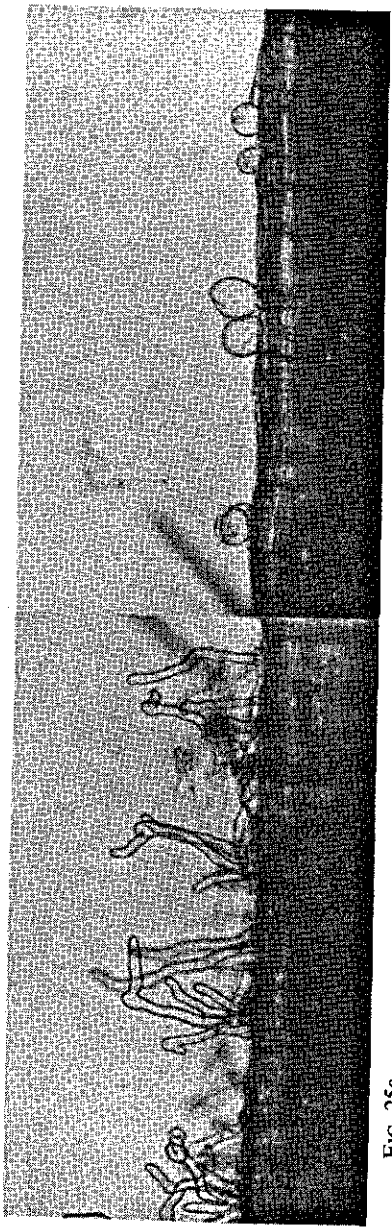

FIG. 25b.

FIG. 25a.

FIG. 24a. Root hairs of a pea plant, 3 days after inoculation with *R. leguminosarum*, strain PF2. Temperature 22°C.
FIG. 24b. The same as Fig. 24a. Temperature 30°C.
FIG. 25a. Root hairs of a pea plant, 3 days after inoculation with *R. leguminosarum*, strain S310. Temperature 22°C.
FIG. 25 . The same as Fig. 25a. Temperature 30°C.

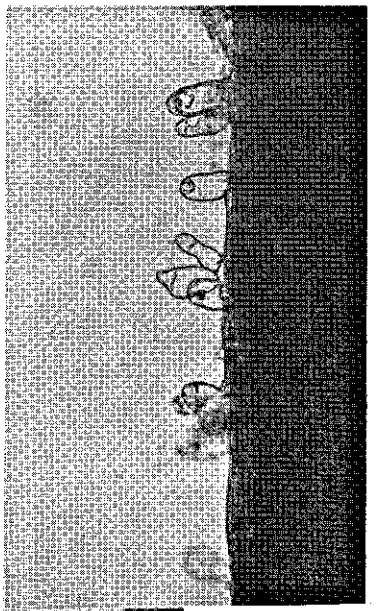

FIG. 26b.

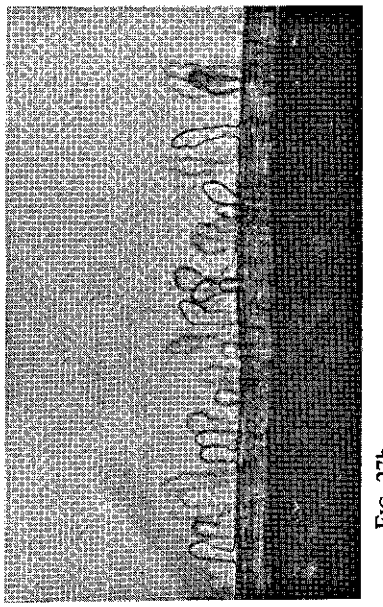

FIG. 27b.

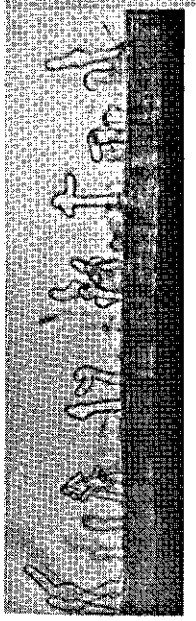

FIG. 26a.

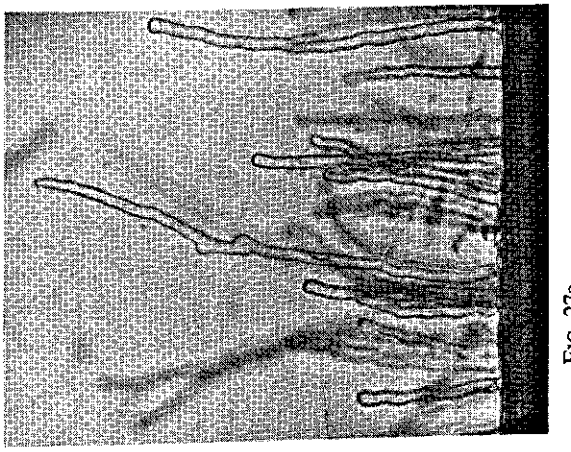

FIG. 27a.

FIG. 26a. Root hairs of a pea plant, 3 days after inoculation with *R. trifolii*, strain K8. Temperature 22°C.
FIG. 26b. The same as Fig. 26a. Temperature 30°C.
FIG. 27a. Root hairs of a pea plant, 3 days after inoculation with *R. japonicum*, strain A102. Temperature 22°C.
FIG. 27b. The same as Fig. 27a. Temperature 30°C.

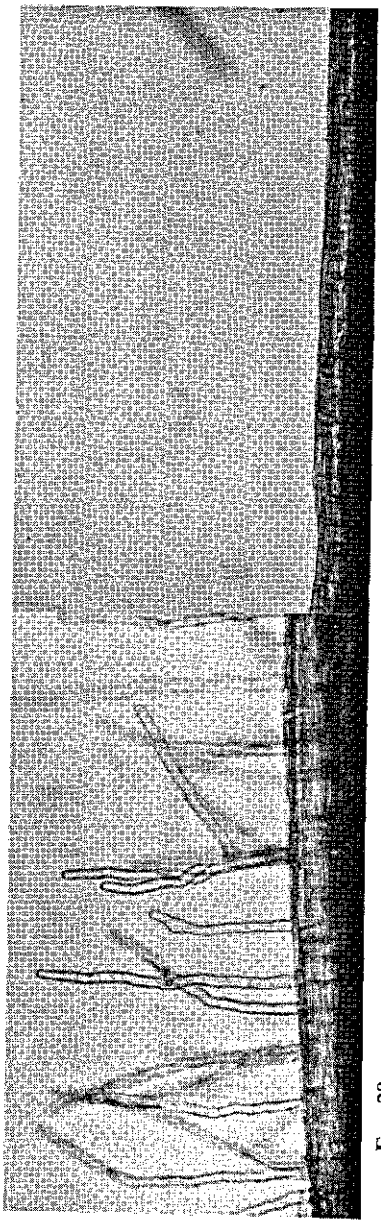

FIG. 28b.

FIG. 28a.

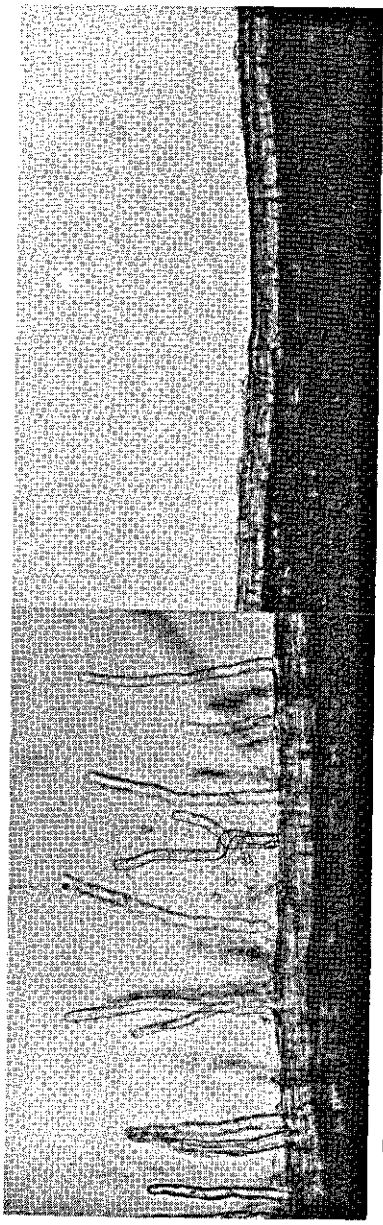

FIG. 29b.

FIG. 29a.

FIG. 28a. Root hairs of a pea plant, 3 days after inoculation with *Micrococcus denitrificans*. Temperature 22°C.
FIG. 28b. Absence of root hairs at 30°C, 3 days after inoculation with *M. denitrificans*.
FIG. 29a. Root hairs of a pea plant, 3 days after inoculation with *Pseudomonas aeruginosa*. Temperature 22°C.
FIG. 29b. Absence of root hairs at 30°C, 3 days after inoculation with *P. aeruginosa*.

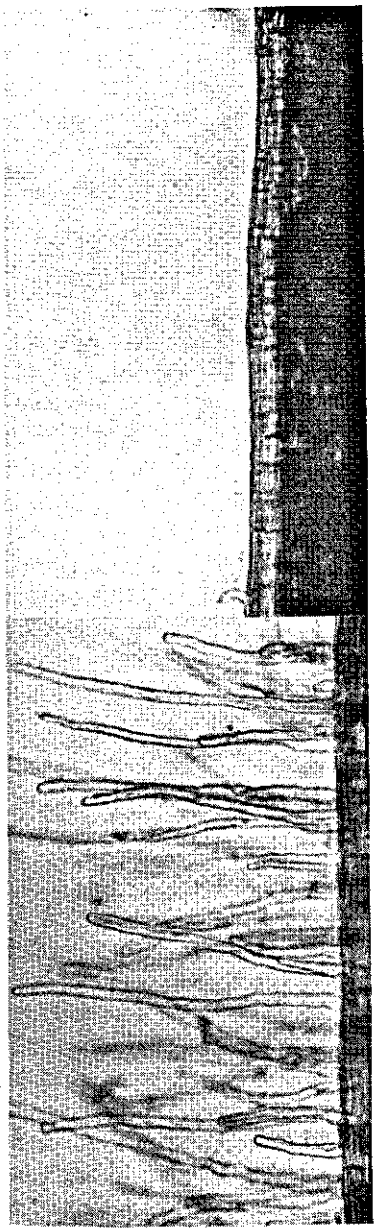

FIG. 30b.

FIG. 30a.

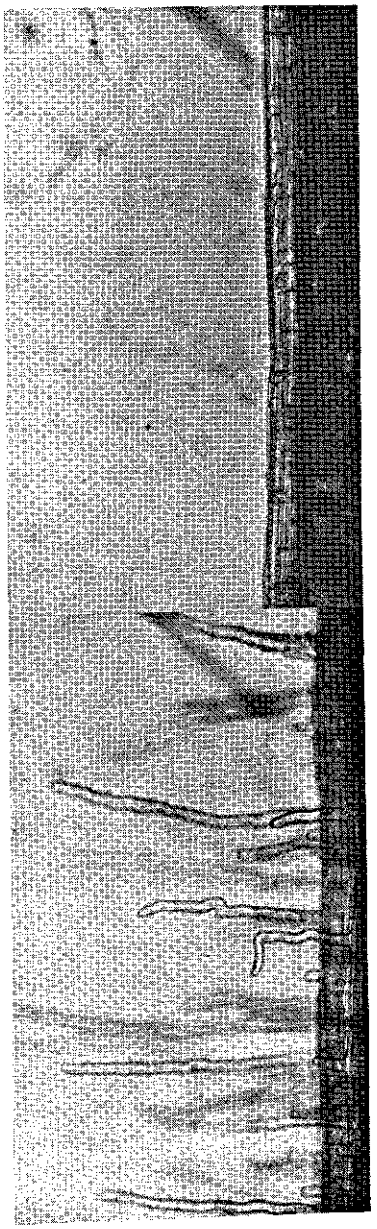

FIG. 31b.

FIG. 31a.

FIG. 30a. Root hairs of a pea plant, 3 days after inoculation with *P. fluorescens*. Temperature 22°C.

FIG. 30b. Absence of root hairs at 30°C, 3 days after inoculation with *P. fluorescens*.

FIG. 31a. Root hairs of a pea plant, 3 days after inoculation with *Arthrobacter simplex* AC1. Temperature 22°C.

FIG. 31b. Absence of root hairs at 30°C, 3 days after inoculation with *A. simplex* AC1.

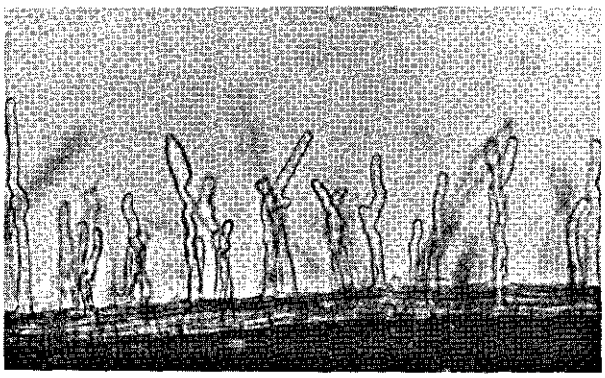

FIG. 32a. Root hairs of a pea plant, 3 days after inoculation with *A. globiformis* AC8. Temperature 22°C. Compare deformations of root hairs to those found after inoculation with *Rhizobium* spp. (Figs. 23–27).

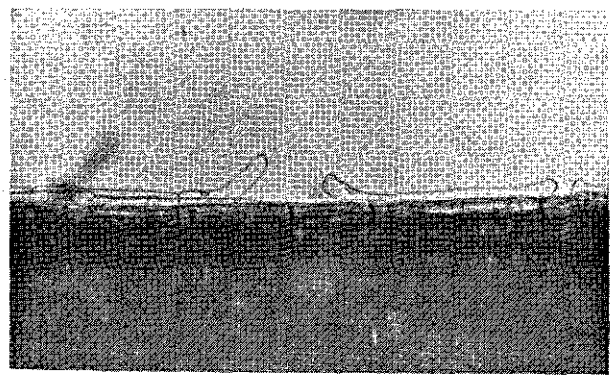

FIG. 32b. Absence of root hairs at 30°C, 3 days after inoculation with *A. globiformis* AC8.

phenomena are very much alike the appearance of root hairs upon treatment with indoleacetic acid (SAHLMAN and FÅHREUS, 1962).

From Figs. 24 and 25 it can be seen that all of the *R. leguminosarum* strains tested affected the root hairs of the pea plants in the same way. This was found to be also true of *R. trifolii*, strain K8, (Fig. 26), but *R. japonicum* caused hardly any deformation of the root hairs at 22°C, except a beginning branching (Fig. 27a). However, at 30°C, root hair formation was found to occur in the presence of this *Rhizobium* species. The root hairs were swollen, though not as pronounced as with the other rhizobia tested (Fig. 27b). *Micrococcus denitrificans* (Fig. 28), *Pseudomonas aeruginosa* (Fig. 29), *P. fluorescens* (Fig. 30) and *Arthrobacter simplex* AC1 (Fig. 31) showed no effect on the appearance of the root hairs. *A. globiformis* AC8 at 22°C caused deformations such as branching (Fig. 32a), similar to those brought about by *Rhizobium japonicum* (Fig. 27) however, no swollen root hairs were observed at 30°C (Fig. 32b). The skate-like epidermis cells, as shown in this figure, may also be found on uninoculated roots.

Apart from the peculiar effect of *A. globiformis* AC8 at 22°C, which deserves closer investigation, it can be concluded that the deformation of root hairs of a particular legume is brought about by specific rhizobia (present study) while on the other hand a particular *Rhizobium* strain gives rise to this phenomenon only on specific leguminous plants (HAACK, 1964).

9.2. NUMBERS OF ROOT HAIRS AND OF ROOT-HAIR DEFORMATIONS AS AFFECTED BY THE PRESENCE OF *RHIZOBIUM* CELLS AND BY TEMPERATURE

9.2.1. *Introduction*

The results reported in the previous chapter show that infection of the root hairs took place about 3 days after inoculation and that multiplication of the rhizobia in the rhizosphere, one of the stages preceding infection, was not inhibited by high temperatures. In addition to the attachment of the bacteria to the root, the development of root hairs is affected by high temperatures, as is shown in section 9.1. (Figs. 23–25). YAO and VINCENT (1969), in a quantitative examination of root-hair deformations of leguminous plants caused by *Rhizobium* spp., differentiated between several categories of deformations, permitting an objective record of the conditions of root hairs. This procedure was adopted in the present investigation in order to obtain quantitative data concerning the influence of *R. leguminosarum* and a high temperature on the root hairs of pea plants.

9.2.2. *Experimental*

Pea plants were grown at 22°C as described before. At unfolding of the first true leaf, the plants were divided into four treatment series: I. inoculated with *R. leguminosarum*, strain PRE, 22°C; II. uninoculated, 22°C; III. inoculated with *R. leguminosarum*, strain PRE, 30°C; IV. uninoculated, 30°C. The inoculated plants received per plant ca 10^6 bacteria, collected in the exponential phase of the bacterial culture, grown in a yeast-glucose medium at 30°C.

Two plants of each series were harvested daily and 5 roots of each plant were collected for root-hair studies. Of each daily root sample, parts of the roots, formed from one day before the start of the experiment (at inoculation) until the day of sampling, were cut and used for calculating the average number of root hairs per cm of root length. To investigate root-hair distribution on parts of roots, grown during 4 or 5 days, these parts were cut into 1-cm length pieces which were numbered from the root-tip piece. The mean age of each length of root was calculated by using the data of Table 5. The various root samples were scanned systematically in order to classify all root hairs that could be clearly seen in the medium optical plane.

The following classification was made: category B, bubble-shaped (Fig. 33); category C, root hairs with curled tips (Fig. 34); category D, otherwise deformed at or near the tips, usually branched (Fig. 34); category E, erect, at least not tip-deformed or branched root hairs (Figs. 33 and 34). C corresponds

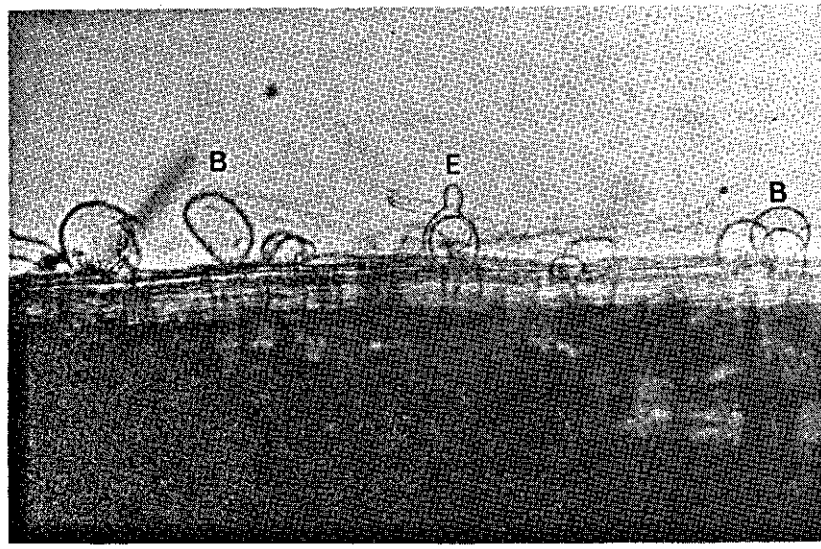

FIG. 33. Root hairs of a pea plant, 4 days after inoculation with *R. leguminosarum*, strain PRE, temperature 30°C. B- bubble-shaped; E- erect (tip not deformed).

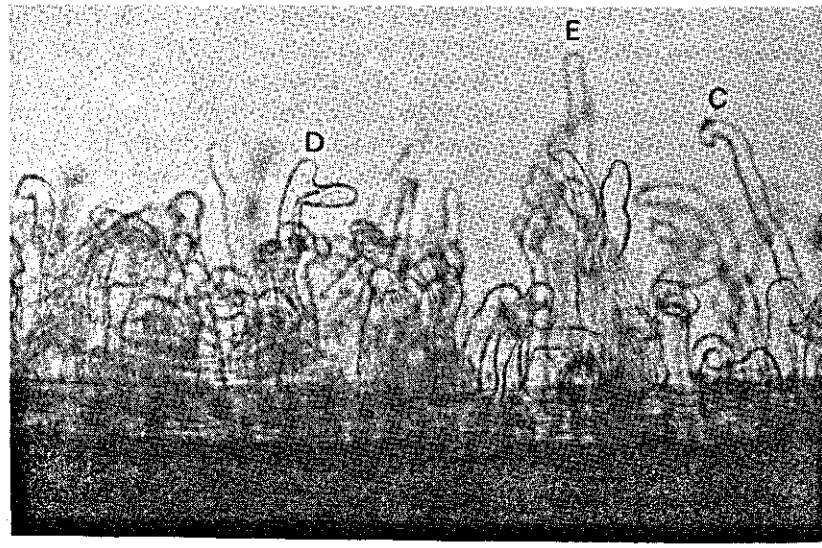

FIG. 34. Root hairs of a pea plant, 3 days after inoculation with *R. leguminosarum*, strain PRE. Temperature 22°C. C- curled, D- deformed, E- erect.

with the combination of Yao and Vincent's catgories 'curled' and 'markedly curled', D corresponds with Yao and Vincent's category 'branched'.

9.2.3. *Results and Discussion*

From the results obtained (Table 17), it can be seen that the average numbers of root hairs per cm of root of inoculated plants exposed to 22°C had increased most substantially on the second day after inoculation. From this time it rose to about 200, which is by far exceeding the corresponding values of the uninoculated plants. It should be stressed that a pronounced variation in root hair number occurred.

On the first day after inoculation no differences were found between the inoculated and the uninoculated plants at 22°C. In only one case a deformed root hair was found on the uninoculated plants, all other root hairs were erect. Even on the inoculated plants, deformed root hairs occurred in minority, most of them being branched (category D). Bubble-shaped root hairs were found to be exceptions at 22°C.

TABLE 17. Effect of temperature (22° or 30°C) and inoculation with *R. leguminosarum* PRE on root-hair formation of pea plants. Two plants of each series, I, II, III, IV, were harvested daily and 10 roots were used for counting numbers of root hairs on root pieces grown from one day before inoculation until the day of harvesting. Numbers of root hairs are given as average values per cm of root length collected. According to their morphology, root hairs were classified in the following categories: B, bubble-shaped, C, curled, D, otherwise deformed and E, erect root hairs.

Days after inoculation	B	C	D	E	Total
	I. Incubation temperature 22°C, *R leguminosarum* present				
1	0	0.1	0	8.9	9.0
2	0.1	2.5	12.4	164.6	179.6
3	0	0.8	4.5	187.2	192.5
4	0	2.2	7.0	239.3	248.5
	II. 22°C, no *Rhizobium* present				
1	0	0	0	11.0	11.0
2	0	0	0	12.7	12.7
3	0	0	0	42.7	42.7
5	0	0.1	0	61.0	61.0
	III. 30°C, *R. leguminosarum* present				
1	0	0	0	0	0
2	2.6	0	0	0.8	3.4
3	2.3	0	0	0	2.3
5	18.3	0	0	2.1	20.4
	IV. 30°C, no *Rhizobium* present				
1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	0	0	0
5	0	0	0	2.9	2.9

Uninoculated plants exposed to 30 °C virtually bore no root hairs. The few root hairs found on some of the roots of plants harvested on the 5th day had probably grown before transferring these plants to 30 °C, as in all cases they were found on the oldest part of the roots examined which differed sharply from the hairless younger parts. Inoculated plants exposed to 30 °C formed moderate numbers of root hairs, showing that also at this temperature the presence of *Rhizobium* cells stimulated root-hair formation (Table 17). The majority of these root hairs belonged to category B.

In all cases the average numbers of root hairs were lowest shortly after the start of the experiment. This was due to the fact that at that time the root tip, which is always hairless for the first half of a centimeter or more, was a major component of the root pieces examined.

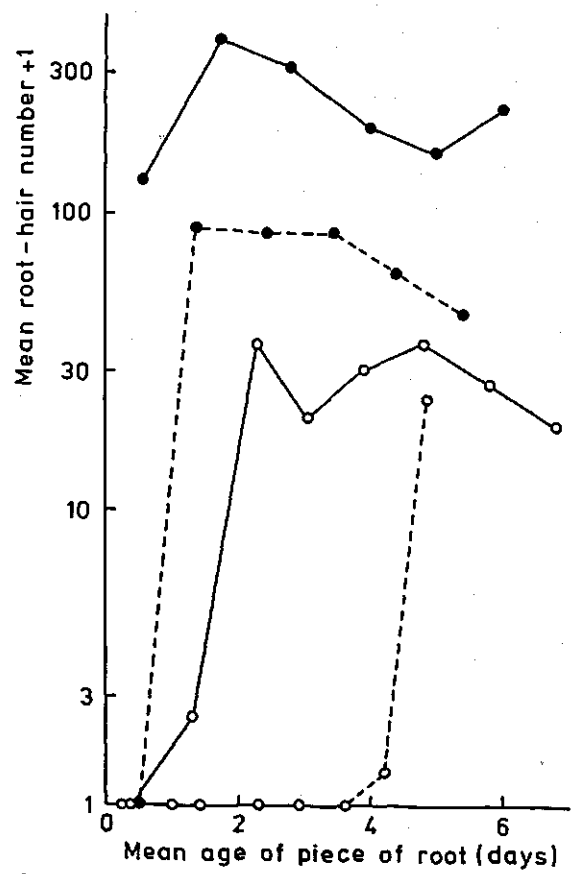

FIG. 35. Effect of temperature and presence of *R. leguminosarum* on root-hair distribution on pea roots. Root hairs were counted on 1 cm root pieces, 4 days (22 °C, *Rhizobium* present) or 5 days (other experimental conditions) after the start of the experiment. ●——● 22 °C, *Rhizobium* present; ●– – –● 22 °C, no *Rhizobium*; O——O 30 °C, *Rhizobium* present; O– – –O 30 °C, no *Rhizobium*.

When comparing the distribution of the root hairs on parts of roots formed until the 4th day after inoculation (Fig. 35) with the average root-hair numbers recorded in Table 17, it will be seen that in the former case root-hair numbers of the various parts of the roots of inoculated plants exposed to 22 °C were fairly constant with the exception of the youngest piece which had slightly lower root hair numbers. The latter is in contrast to the low number of root hairs on the young 1-cm parts of roots formed during the first day after inoculation (Table 17) when the rhizobia apparently had exerted hardly any effect because they were poorly established in the rhizosphere and were present in lower numbers than on the young parts of root formed 3 days after inoculation.

The pronounced effect of an established *Rhizobium* culture on early root-hair

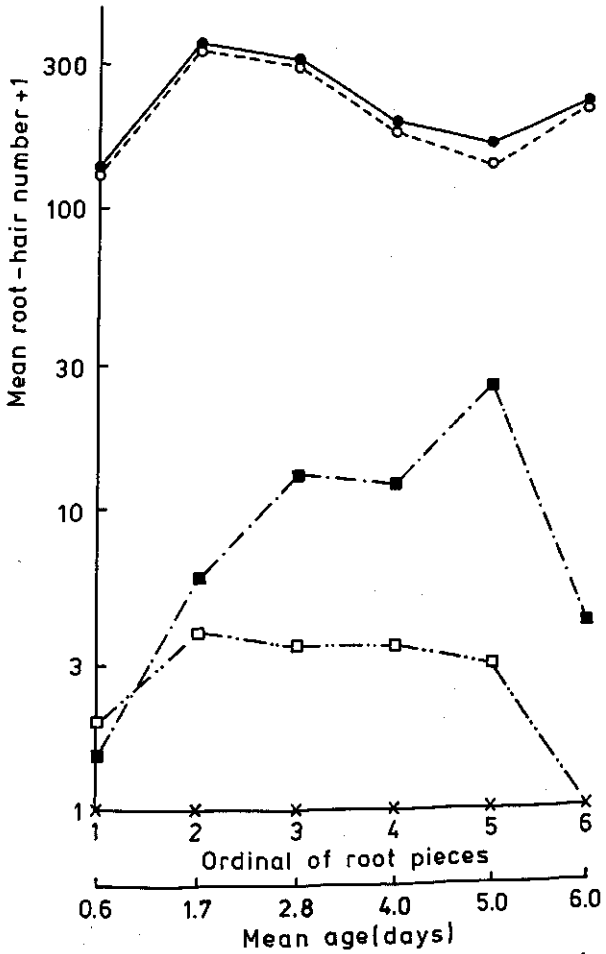

FIG. 36. Distribution of different types of root hairs on the roots of pea plants, 4 days after inoculation with *R. leguminosarum*. Incubation temperature 22 °C. (●) Total number, (○) non-deformed, (■) branched, (□) curled, (x) bubble-shaped root hairs.

formation is clearly seen when comparing root-hair numbers of the youngest root pieces of inoculated and uninoculated plants (Fig. 35). Roots of inoculated plants already contained many root hairs, whereas those of uninoculated plants contained none on 1-day old root pieces. Root-hair numbers on root pieces of approximately two days old and older from uninoculated plants were also fairly constant, although clearly lower than those of inoculated plants. This stimulatory effect of the presence of rhizobia on root-hair numbers occurred also on parts of root which contained already many root hairs at inoculation time.

The formation of root hairs at 30°C, if any, in the presence of *Rhizobium* cells seemed to take more time than at 22°C, as final numbers were reached at a later age. As uninoculated roots exposed to 30°C possessed root hairs only on the oldest parts, it may be assumed that in the absence of rhizobia no root-hair growth at 30°C occurred; root hairs formed at lower temperature and transferred to 30°C remained intact (Fig. 35).

The distribution of various types of root hairs on inoculated plants after exposure to 22° and 30°C is given in Figs. 36 and 37, respectively. Numbers of undeformed root hairs (E) closely followed total numbers in the 22°C treatment.

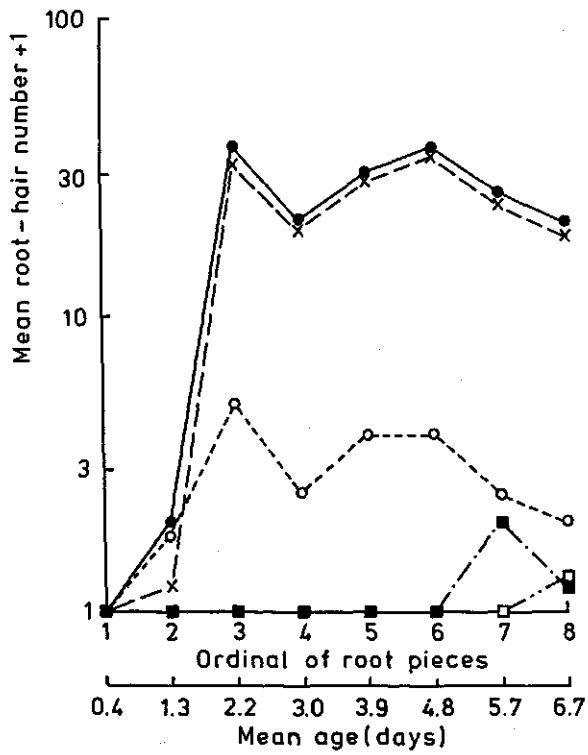

FIG. 37. Distribution of different types of root hairs on the roots of pea plants, 5 days after inoculation with *R. leguminosarum*. Incubation temperature from inoculation 30°C. (●) Total number, (O) non-deformed, (■) branched, (□) curled, (x) bubble-shaped root hairs.

Numbers of branched and similarly deformed root hairs (D) increased with increasing root age. Numbers of curled root hairs (C) were low but occurred fairly constantly on the whole root examined, except the oldest part, although the total number of root hairs remained high. On such older parts of the roots usually no nodules were found. These observations indicate that only root hairs growing in the presence of *Rhizobium* cells can be deformed and infected. Most of the root hairs occurring on roots exposed to 30°C with *Rhizobium* cells present were of the bubble-shaped type (Fig. 37). With the exception of the youngest two pieces, the numbers were constant. Root hairs not deformed at the tip were occasionally found evenly distributed over the root. Only on the oldest two pieces (circa 6 and 6½ days old respectively), the other types of deformations were found. In this zone young root hairs were presumably growing when the plants were transferred from 22° and 30°C.

9.3. Relationship between root hairs and infection

9.3.1. *Introduction*

The striking effect of high temperatures on the appearance of root hairs might be an important factor in the inhibition of nodulation under such conditions. Swollen root hairs might be unsuited for processes involved in nodulation. To obtain more details concerning the relationship between nodulation and type of root hair, observations on root hairs were made at 25°C, where nodulation proceeds readily (Table 4). Furthermore, root-hair morphology was studied of pea plants growing in perlite where sometimes nodulation occurred at 30°C (cf Table 7).

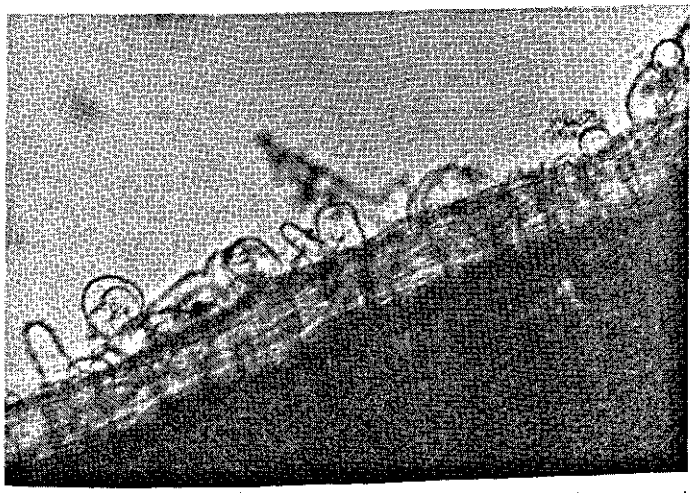

Fig. 38. Root hairs of a pea plant after inoculation with *R. leguminosarum*, strain PRE, at 25°C.

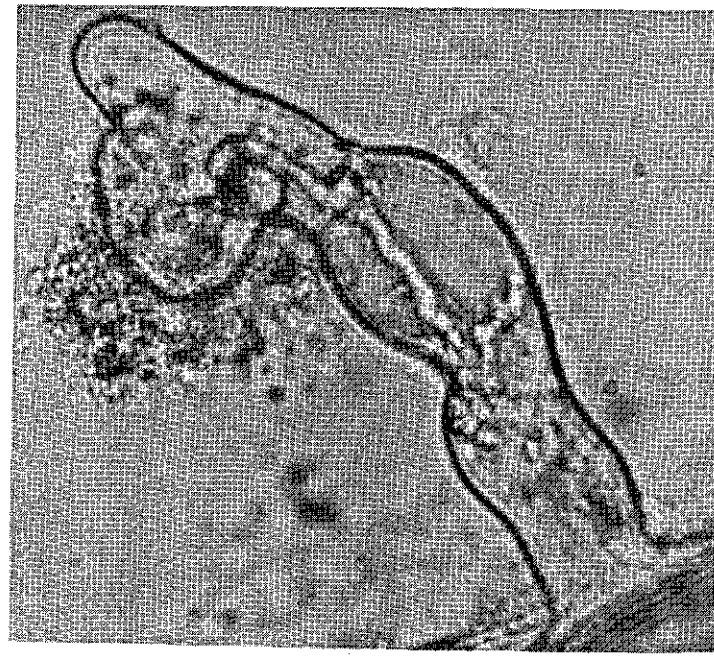

FIG. 39. Root hair with infection thread at 25 °C.

9.3.2. *Results and Discussion*

Root hairs of plants exposed to 25 °C resembled those of plants growing at 30 °C (cf Figs. 38 and 23b). However, nodulation was only moderately reduced at 25 °C, as contrasted to 30 °C, where it was almost completely suppressed. Although this result might suggest that nodulation of pea plants via bubble-shaped root hairs could occur, no infected root hairs of this type have been observed. On the other hand, when infection threads were seen at 25 °C they always occurred in more or less normal root hairs (Fig. 39), which were sporadically observed at 25 °C but never at 30 °C. It is believed that such root hairs were responsible for the nodulation at 25 °C.

Plants growing in perlite or soil at 30 °C were found to bear root hairs (Fig. 40). Nodulation of such plants at 30 °C was found to occur sporadically (Table 7), but more frequently than in culture solution at that temperature.

The results obtained suggest that the presence of more or less normal root hairs is a prerequisite for nodulation.

9.4. INFLUENCE OF A RHIZOBIAL COMPOUND ON THE DEFORMATION OF ROOT HAIRS

9.4.1. *Introduction*

Some authors have provided evidence that an extra-cellular rhizobial compound is involved in the deformation of legume-root hairs (FÅHREUS and

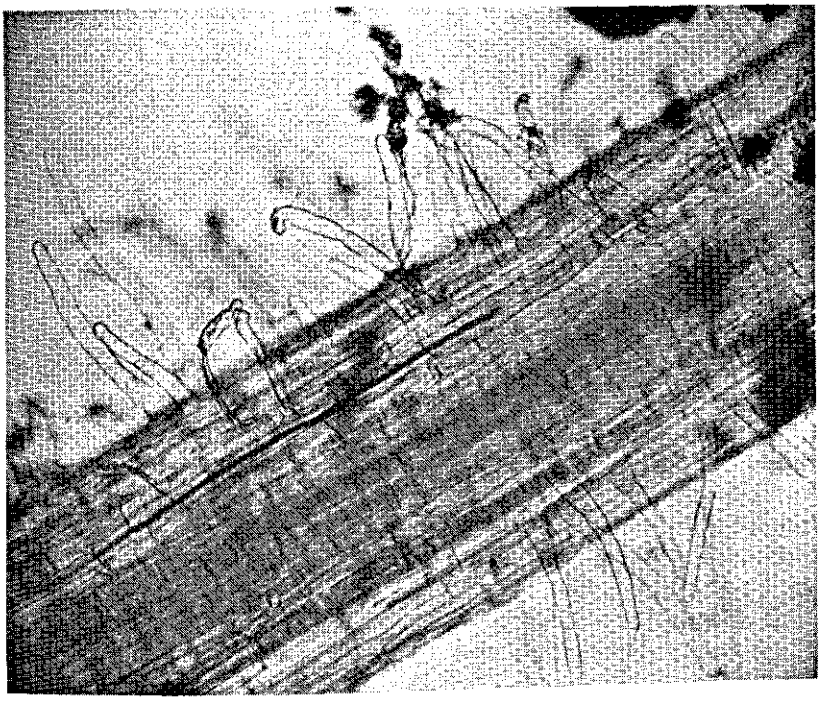

FIG. 40. Root hairs grown in soil at 30 °C.

LJUNGGREN, 1959; YAO and VINCENT, 1969; HUBBELL, 1970; SOLHEIM and RAA, 1973). HUBBELL (1970) as well as SOLHEIM and RAA (1973), using different techniques, obtained curling of legume-root hairs by exposing the root hairs to extra-cellular compounds derived from *Rhizobium* cultures. SOLHEIM and RAA found a non-dialysable compound to be responsible whereas YAO and VINCENT (1969) obtained some evidence that a diffusible factor is involved.

Deformation of root hairs has taken place during growth, which includes cell wall synthesis. Cell-wall growth of root hairs is invariably associated with the near presence of the host-cell nucleus (NUTMAN, 1959). The initiation of the infection thread and its continued growth are also closely connected with the near presence of the host-cell nucleus, as was first demonstrated by FÅHREUS (1957). Both observations suggest that the synthesis of cell-wall material of root hairs as well as that of infection threads is controlled by the nucleus of the plant cell. From the appearance of root-hair deformations and formation of infection threads it might be concluded that in some way the nuclear control of cell-wall synthesis is affected by one or more compounds of rhizobial origin. This compound would have to pass through the cell wall and the cytoplasmic membrane.

9.4.2. *Experimental*

Extracellular rhizobial polysaccharides containing small amounts of protein

were collected as described by ZEVENHUIZEN (1971) from liquid cultures of *R. leguminosarum* grown in a yeast extract-glucose medium.

A rhizobial suspension, containing ca 10^7 cells/ml was pipetted into a dialysis bag, taking care that the culture medium of the pea plants was kept free from *Rhizobium* cells. The bag was placed in the plant-culture solution at unfolding of the first true leaf of the pea plants. After 2 or 3 days the bag was removed and the plant roots were examined for the presence of root-hair deformations.

9.4.3. *Results and Discussion*

The polysaccharide preparation obtained by the method of ZEVENHUIZEN (1971) which had been submitted to dialysis, caused root-hair deformations at 22 °C (Fig. 41). Such deformations were also found at 22 °C in the presence of the dialysis bag, containing a culture of *R. leguminosarum* (Fig. 42). In none of these cases, nodulation followed. It was striking that only root hairs occurring on parts of the root adjacent to the dialysis bag showed deformations, mostly branching.

From the results obtained it follows that a dialysable factor from a *Rhizobium* culture was able to bring about root-hair deformations. Dialysed polysaccharides from *R. leguminosarum* had the same effect. It is suggested that the deform-

FIG. 41. Curled root hair of a pea plant, found one day after the application of dialysed rhizobial polysaccharides.

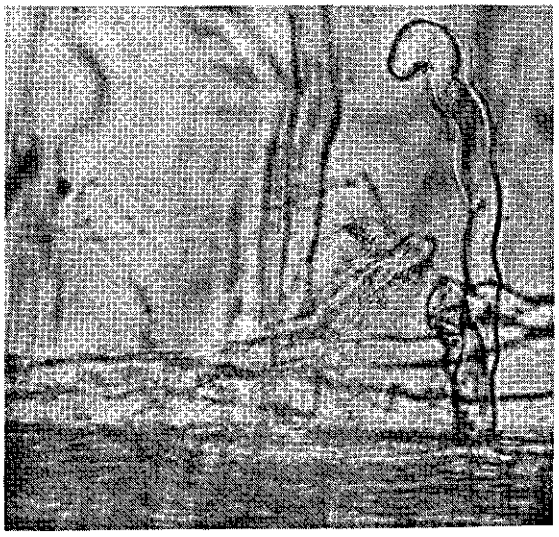

FIG. 42. Curled root hair of pea plant, found in the neighbourhood of a culture of *R. leguminosarum* contained in a dialysis bag, two days after placing the bag between the roots.

ing factor is firmly attached to the polysaccharides. If the pea root would exude lectins (i.e. proteins capable of binding sugars, LIS and SHARON, 1973), it is possible that the rhizobial polysaccharides, carrying the supposed deforming factor, are attached to the plant root, in this way attaching the deforming factor to the root.

9.5. Summary

1. Root-hair formation of pea plants growing in nutrient solution was strongly suppressed at 30°C. In the presence of *Rhizobium* cells, root hairs were formed at this temperature, be it in much lower numbers than at 22°C. Most of these root hairs were spheroid (Fig. 23b).
2. Three strains of *R. leguminosarum* and one strain of *R. trifolii* and *R. japonicum* each caused deformations of root hairs at 22°C and growth of spheroid root hairs at 30°C of pea plant growing in nutrient solution.
3. *Arthrobacter globiformis* caused root-hair deformations at 22°C, but did not stimulate root-hair growth at 30°C.
4. The number of root hairs of pea plants increased 1 day after inoculation with *R. leguminosarum*.
5. A minority of the root hairs grown at 22°C in the presence of *Rhizobium* cells were deformed, some of them being curled.
6. The ability of a plant to nodulate was found to be correlated with the presence of straight root hairs. Spheroid root hairs containing infection threads have not been observed.

7. *Rhizobium* cells produced a dialysable compound which apparently was responsible for the root-hair deformations.
8. Nodule formation occurred only on newly grown parts of roots (grown between 1 day before and 3 days after inoculation) on which root-hair formation took place in the presence of *R. leguminosarum* cells.
9. The main cause of the absence of nodulation of pea plants at high temperatures is assumed to be due to the presence of relatively low numbers of root hairs which in addition are spheroid. Such root hairs apparently are unable to give rise to the formation of normal infection threads.

10. GENERAL DISCUSSION

From the results obtained in the present investigation, it is concluded that nodulation of pea plants includes the following sequence of stages: 1. Attachment of *Rhizobium leguminosarum* to the roots in numbers of at least 200 cells per root system (Table 14; Fig. 19); 2. Increase of the rhizobia in the rhizosphere of the pea plants; 3. Development of root hairs in the presence of the rhizobia which stimulate growth and deformation of the root hairs by producing a dialysable compound, typical of *Rhizobium* cells (Figs. 22–32, 35, 42, Table 17); 4. Infection of a minor percentage of the root hairs by a minority of the *Rhizobium* population present in the rhizosphere, about 3–4 days after the rhizobia population has reached the required density (Table 16, Fig. 36); 5. Nodulation, usually occurring on a length of root formed from 1 day before until 3 days after inoculation (Table 2, Fig. 7); 6. Nodule growth, synthesis of leghaemoglobin, and beginning of the nitrogenase activity (Table 8).

High temperatures may affect several of the above-mentioned processes. Indications were obtained that attachment of the rhizobia to the roots is adversely affected by such temperatures. This is concluded from the fact that root systems of pea plants, dipped into a rhizobial suspension, upon rinsing with an alkaline $Na_4P_2O_7$ solution of pH 9, at 30 °C lost 10–100 times more of the attached rhizobia than at 22 °C (section 8.2). Treatment with buffers of pH 9, nor pretreatment with a pyrophosphate solution before dipping had any effect on the attachment of the cells. Treatment with EDTA or salt solutions of various composition and strength gave no reproducible results. The temperature-dependent attachment of rhizobia by legume roots might be explained by assuming that a lectin-polysaccharide reaction is involved (cf BOHLOOL and SCHMIDT, 1974). The binding of lectin (on the root surface) to polysaccharides (surrounding the bacteria) might be prevented by high temperatures. RITTENHOUSE et al. (1974), investigating the influence of temperature on this type of reaction, reported dramatic alterations in binding at low temperatures. So far, it is unknown to what extent the reduced attachment of *R. leguminosarum* to pea roots may contribute to the adverse effect of high temperatures on nodulation.

By using the charcoal treatment method (Chapter 3), it was shown that nodules develop usually on those parts of the pea roots, which were formed during 3 days after and one day before inoculation (Table 2). No root hairs were present on the latter part of the roots at inoculation time, as contrasted to parts of the roots formed at an earlier date (Fig. 35) which, however, developed no nodules. From these results it is concluded that nodulation occurs only if, at 22 °C, the root hairs are formed in the presence of rhizobia. Only under these conditions, deformation of root hairs and infection by *R. leguminosarum* may be expected. Deformation of root hairs apparently takes place under the influence of an unknown compound derived from rhizobial cells.

This compound also stimulates root-hair formation (Figs. 35, 36).

The adverse influence of high temperatures on root-hair growth is presumably the main reason for the failure of nodulation of pea plants incubated at 30°C. This is concluded from the following experimental results: (a) The adverse effect of high temperatures on nodulation is localized at the sites of nodulation (Table 1). (b) Exposure of uninoculated pea plants to 30°C prevents the formation of root hairs. Inoculated plants form root hairs under such conditions which, however, are spherical and do not give rise to nodule formation. At 25°C, nodulation was found to proceed normally in spite of the fact that most of the root hairs were spherical. It is assumed that nodulation at this temperature resulted from the normal root hairs which were present sporadically rather than from infected bulb-shaped root hairs. (c) Roots of pea plants growing in perlite or in soil, at 30°C, may bear root hairs (Fig. 40) whilst nodules may sporadically be formed (Table 7). (d) Incubation of plants for some days at 30°C prior to inoculation, at 22°C, did not interfere with nodule formation on parts of roots grown 1 day before inoculation (Fig. 9). This is explained by the fact that root hairs were formed in the presence of rhizobia which is leading to nodule formation.

To obtain more detailed information about the main temperature-sensitive stage (root hair formation and deformation) in the nodulation process, pea plants grown for different periods after inoculation at 22°C were transferred to 30°C and kept at that temperature until 14 days after inoculation after which nodule numbers were counted. From these countings it appeared that nodules develop at 30°C on the roots of those plants which have grown for more than 3 days at 22°C before being transferred to 30°C (Fig. 11). This demonstrates that the main temperature-sensitive stage terminated 3 days after inoculating the plants with *R. leguminosarum*. So far, it is unknown whether this stage ends when the bacteria have infected the root hair or when the infection thread enters the plant root. From the fact that the rhizobia were sensitive to antibiotics during three days after inoculation (Table 15), it might be concluded that the temperature-sensitive stage terminates when the rhizobia have entered the root hairs, assuming that bacteria within root hairs are no more sensitive to the antibiotic. If, however, bacteria within infection threads in root hairs are sensitive to antibiotics, the temperature-sensitive stage may be extended to the period of growth of the infection thread within the root hair.

Information concerning the beginning of the main temperature-sensitive stage was obtained by incubating pea plants at 30°C for different periods, starting at inoculation. After these periods at 30°C, the plants were returned to 22°C. From the results obtained (Table 10) it may be concluded that the temperature-sensitive stage of the symbiosis starts shortly after inoculation. However, from the experiment recorded in Fig. 15 it might be concluded that immediately after inoculation a temperature-insensitive stage exists during which the rhizobia in the rhizosphere readily multiply at 30°C, so that after replacing the plants at 10 days after inoculation to 22°C, nodulation time is shortened with $^1/_2$–1 day. These results may be explained by the observed influence of high temperatures

and of *Rhizobium* cells on root-hair formation. As soon as the pea plants are exposed to high temperatures, the formation of root hairs and consequently nodulation are adversely affected. However, the proliferation of *Rhizobium* cells at such temperatures is stimulated, resulting in the accumulation of rhizobial compounds after prolonged incubation at high temperatures. It may be assumed that after transfer to lower temperatures this material favours the formation and deformation of root hairs and shortens nodulation time. This conclusion is in agreement with the results of MULDER and VAN VEEN (1960) who found improved nodulation of clover plants in acid soils following the addition of sterilized bacterial material. The adverse effect of pH on nodulation, which also depends on some process in the pre-infection period, is not due to the absence of root hairs (MUNNS, 1970), and therefore is different from the effect of high temperatures.

Simultaneously with the termination of the main temperature-sensitive stage (approximately 3 days after inoculation), the development begins of part of the roots where no further nodulation occurs (Fig. 12). The latter phenomenon could be due to the suppressing effect of growing infection threads on some early process in root-hair formation on the younger parts of the roots, or to the inhibitory effect of developing nodules on early processes of nodulation as it was described by NUTMAN (1952) and ROUGHLEY et al. (1970).

In addition to the suppressing effect of high temperatures on the formation, deformation and infection of root hairs, nodule growth and a number of other processes are adversely affected by such temperatures. Owing to the reduced nodule growth, the ultimate size is strongly negatively correlated with the length of time during which the nodules have grown at 30°C (Fig. 14). The average nodule size (measured at the conclusion of the experiment) of plants transferred at the 8th day after inoculation to 30°C was even less than half the size of nodules of control plants. Numbers of nodules of these plants did not deviate from those of the control plants (Fig. 11).

Other processes suppressed at 30°C were found to be leghaemoglobin formation and development of nitrogenase activity (Table 8). Nodules of plants, grown until 10 days after inoculation at 22°C before being transferred to 30°C, formed practically no leghaemoglobin as contrasted to nodules of the control plants which were kept continuously at 22°C. The same was true of the acetylene-reducing activity. High temperatures not only suppressed the synthesis of leghaemoglobin, but they also favoured the breakdown of this compound and the decline of nitrogenase activity.

To explain the adverse effect of high temperatures on various processes involved in nodulation, as observed in the present investigation, the results obtained with inoculated pea plants growing under various conditions as to photosynthesis and respiration (chapter 6) may be used. In these experiments, it was shown that nodulation of pea plants growing at a high temperature was enhanced by increasing the light intensity or by extending the illumination period (Fig. 18), i.e. by improving the carbohydrate supply of the plant. Similar results were obtained by extending the diurnal period at 22°C, particularly

during the dark period. This undoubtedly has resulted in a decreased respiration and, consequently, in an improved carbohydrate supply of the plant.

Although it is unlikely that all of the phenomena occurring in inoculated pea plants incubated at a high temperature can be explained by the reduced carbohydrate supply, it is suggested that this condition is involved in the growth of root hairs, infection threads and nodules.

11. SUMMARY

A study has been made concerning the effect of high temperatures on the symbiosis of *Rhizobium leguminosarum* and pea plants (*Pisum sativum*). At 30°C, no nodules were found on the roots of plants growing in nutrient solution after inoculation with the appropriate bacteria. This is in contrast to the ready nodulation at lower temperatures, and to the observation that at the high temperature both partners of the symbiosis, when growing separately, develop normally, provided that they dispose of adequate amounts of combined nitrogen.

From the results of experiments recorded in Chapter 3 it was concluded that the inhibition of nodulation by high temperatures occurs only when the sites of nodulation are exposed to such temperatures. Incubation of other parts of the plant at 30°C (Table 1), or incubating the entire plant during several days before inoculation at that temperature, had no effect.

The unfolding of the first true leaf of pea plants was found to be correlated with the onset of the liability of the root system to infection by *R. leguminosarum*. By blackening root systems with charcoal at different times, it was found that nodulation occurred usually on parts of the roots that were formed from one day before until 3–4 days after inoculation (Figs. 7, 8, 9. Table 2).

An inventarisation of morphological characters of pea roots, affected by high temperatures and by the presence of rhizobia, is given in Chapter 4. Numbers of nodules were found to be dependent not only on temperature, but also on unknown factors that could not be controlled (Table 4a). To a lesser extent, the same was true of the nodulation time (Table 4b). For this reason, the main part of the present investigation was restricted to a comparison of nodulation phenomena at two temperatures viz. 22°C at which nodulation took place readily, and 30°C at which usually no nodules were formed. Root-growth rate and formation of lateral roots were found to be affected by temperature as well as by the presence of rhizobia (Tables 5 and 6, Fig. 10).

By transferring pea plants from 22 to 30°C at different times after inoculation, the temperature sensitivity of various stages of nodule formation was ascertained. Nodulation was found to be suppressed when the 22°C-incubation ended at 3 days after inoculation, but a number of nodules were formed when the 22°C-period was terminated at 4 days, demonstrating that between 3 and 4 days after inoculation, a highly temperature-sensitive stage of nodulation was terminated (Fig. 11). Although nodule initiations beyond this stage developed to nodules at 30°C, the size of the latter remained considerably smaller than those of control plants incubated continuously at 22°C (Fig. 14), whereas leghaemoglobin and acetylene-reducing activity did not develop (Table 8). This demonstrates that in addition to the main temperature-sensitive stage, later stages of nodulation are adversely affected by high temperatures.

Chapter 6 contains the results of experiments in which it was shown that

enhancement of the photosynthetic activity of pea plants (by increasing the light intensity or by extending the illumination period) raised the probability of such plants of being nodulated when growing at moderately high temperatures (Fig. 18). The same effect was obtained by inserting a diurnal period at 22 °C, particularly during the dark period, which decreases the respiratory activity and consequently enhances the carbohydrate content of the plants (Table 9).

In Chapter 7 experiments on the first contact between *R. leguminosarum* and pea roots are recorded. Attachment of the rhizobia to pea roots was found to be adversely affected by high temperatures (Table 14). Once attached to the root, rhizobial growth in the rhizosphere at 30 °C is faster than at 22 °C (Table 16).

Chapter 8 contains the results of experiments with antibiotics added to *R. leguminosarum*-pea plant associations at different periods after inoculation to find out at what time the bacteria were no more susceptible to the antibiotics. This was found to be the case at 3–4 days after inoculation (Table 15), i.e. the time at which the main temperature-sensitive period ended (Fig. 11).

The main effect of high temperatures during the first 3 days after inoculation is connected with root-hair formation and root-hair deformation (Chapter 9). Pea plants at 22 °C form many root hairs but deformations do not occur (Fig. 22a). *R. leguminosarum* and also other *Rhizobium* species tested stimulate root-hair development and deformation of root hairs (curling, branching) (Figs. 23a to 27a, Table 17) which precede infection. This depends on the formation by the rhizobia of a dialysable compound (Fig. 42). Pea plants without rhizobia, incubated at 30 °C, were found to form no root hairs (Fig. 22b; Table 17). In the presence of *R. leguminosarum* or a number of other *Rhizobium* species, root hairs at 30 °C were formed in relatively large numbers, but then they had an abnormal, spheriod shape (Figs. 23b to 27b) and did not give rise to nodule formation.

In conclusion, it can be stated that a number of processes are involved in causing the adverse effect of high temperatures on nodulation and nitrogen fixation of the *Rhizobium*-pea association. Interference with the formation and deformation of root hairs in the presence of an established flora of *R. leguminosarum* was shown to be responsible for the absence of nodules. Reduced growth of the nodules, absence of leghaemoglobin formation, absence of nitrogenase activity (both characters probably being linked) are further important processes, adversely affected by high temperatures.

SAMENVATTING

DE INVLOED VAN HOGE TEMPERATUREN OP DE SYMBIOSE VAN *RHIZOBIUM LEGUMINOSARUM* EN ERWT

Bacteriën van het geslacht *Rhizobium* zijn in staat om met planten van de familie der *Vlinderbloemigen* (*Leguminosae*) een symbiose aan te gaan. In de knolletjes, die hierbij aan de wortels van de plant gevormd worden, wordt stikstof uit de lucht vastgelegd.

De symbiose tussen erwteplanten (*Pisum sativum* L.), groeiend in een voedingsoplossing, en de erbij behorende bacterie (*Rhizobium leguminosarum* Frank.) komt bij 30°C niet tot stand; er worden dan geen wortelknolletjes gevormd. Niettemin kunnen zowel de planten als de bacteriën afzonderlijk bij die temperatuur goed groeien mits voldoende assimileerbare stikstofverbindingen aanwezig zijn.

Dit proefschrift geeft een beschrijving van een onderzoek naar de invloed van hoge temperaturen op het tot stand komen van de bovengenoemde symbiose. In het eerste hoofdstuk is een kort overzicht gegeven van de processen die tot een functionerende symbiose leiden en van de invloed van hoge temperaturen op deze processen. Verder is hier de probleemstelling geformuleerd. Materiaal en methoden, voor zover die gelden voor het gehele onderzoek, zijn vermeld in hoofdstuk 2.

In hoofdstuk 3 zijn proeven beschreven waaruit kon worden geconcludeerd dat vorming van wortelknolletjes alleen wordt geremd op plaatsen van de wortel waar een hoge temperatuur heerst (Tabel 1). Verder bleek dat de knolvorming het snelst verloopt wanneer de bacteriën worden toegevoegd aan wortelstelsels van planten op het moment dat het eerste blad zich ontvouwt (Fig. 4). Door de wortels op verschillende tijdstippen zwart te maken met een suspensie van kool, werd gevonden dat slechts dat gedeelte van de wortel gewoonlijk in aanmerking komt voor knolvorming dat ontstaat tussen één dag voor het enten tot en met ongeveer 3 dagen er na. Of de plant voor het enten al dan niet enige tijd bij hoge temperatuur had gestaan bleek van geen belang (Fig. 8 en 9).

Gezien de slechte reproduceerbaarheid van de invloed van hoge temperaturen op aantallen knolletjes (Tabel 4A) en nodulatietijd (Tabel 4B) is het grootste deel van dit onderzoek beperkt tot een vergelijking van twee temperaturen, nl. 22°C, waarbij de knolvorming goed plaats vond, en 30°C, waarbij gewoonlijk geen knolvorming optrad. De groeisnelheid van de wortel (Tabel 5) en de vorming van de zijwortels (Fig. 10; Tabel 6) bleken te worden beïnvloed zowel door de temperatuur als door de aanwezigheid van rhizobia.

Het voorkomen van voor hoge temperaturen gevoelige perioden in de loop van de knolvorming werd onderzocht door op gezette tijden erwteplanten van de ene naar de andere temperatuur te verplaatsen (Hoofdstuk 5). Bij een

verplaatsing van 22 °C naar 30 °C gedurende de eerste 3 dagen na het enten trad uiteindelijk geen knolvorming op, terwijl dit bij latere verplaatsingen wel het geval was (Fig. 11 en 13). Hieruit werd de conclusie getrokken dat 3 à 4 dagen na het enten een voor hoge temperaturen zeer gevoelig stadium van de symbiose werd beëindigd. De knolletjes die verschenen na de latere verplaatsingen bleven klein (Fig. 14), vormden geen leghaemoglobine en vertoonden geen nitrogenase-activiteit (Tabel 8). Hieruit blijkt dat, behalve het eerste gevoelige stadium, ook latere stadia van de knolvorming ongunstig worden beïnvloed door hoge temperaturen.

In hoofdstuk 6 worden proeven beschreven waarbij incubatieperioden bij lage en hoge temperatuur van verschillende duur per etmaal werden afgewisseld bij een constante lichtperiode. Bij een tweede serie planten werden de lichtperiode en de lichtintensiteit gevarieerd bij een constante temperatuur. Hierbij werd aangetoond dat de kans op knolvorming stijgt met toename van de fotosynthetische activiteit van de erwteplant (Fig. 18). Hetzelfde effect werd bereikt door het inschakelen van een voldoend lange koele periode (Fig. 17), vooral 's nachts, waardoor de ademhaling minder werd (Tabel 9). In beide gevallen werd de kans op knolvorming dus bevorderd door verhoging van het koolhydraatgehalte van de plant.

In de laatste drie hoofdstukken is de aandacht geconcentreerd op de drie dagen durende gevoelige beginfase van de symbiose. In hoofdstuk 7 is het onderzoek naar het begin van deze periode vermeld (Tabel 10). Met behulp van een daartoe ontwikkelde methodiek werd gevonden dat de hechting van *R. leguminosarum* aan wortels gevoelig is voor hoge temperatuur (Tabel 14). Uit onderzoek met behulp van antibiotica, op verschillende tijdstippen na het enten aan het voedingsmedium toegevoegd, bleek dat 3 à 4 dagen na het enten de bacteriën niet meer gevoelig waren voor de antibiotica, vermoedelijk omdat de infectie van de erwtewortel had plaats gevonden (Hoofdstuk 8; Tabel 15). Dit tijdstip komt overeen met het einde van de voornaamste, voor hoge temperaturen gevoelige, periode. De groei van de bacteriën rond het wortelstelsel bleek bij 30 °C sneller te verlopen dan bij 22 °C (Tabel 16).

Uit de resultaten, vermeld in hoofdstuk 9, bleek dat in de beginperiode van de symbiose de invloed van hoge temperaturen in verband staat met de vorming en vervorming van wortelharen. Bij 22 °C worden in aanwezigheid van *R. leguminosarum* en andere geteste *Rhizobium*-soorten door erwteplanten meer wortelharen gevormd dan door niet geënte controleplanten, terwijl dan bij enkele wortelharen vervormingen van de top worden aangetroffen, zoals krommingen en vertakkingen (Fig. 22a t/m 27a). Deze vervormingen, die doorgaans vooraf gaan aan de infectie door *R. leguminosarum*, bleken te worden veroorzaakt door een van de bacteriën afkomstige, dialyseerbare stof (Fig. 42). Bij 30 °C vormen de erwtewortels in voedingsoplossing geen wortelharen, tenzij rhizobia aanwezig zijn (Fig. 22b t/m 32b). De in dat geval gevormde wortelharen zijn bijna alle blaasvormig (Fig. 37; Tabel 17). Ook bij 25 °C werden blaasvormige wortelharen aangetroffen (Fig. 38); infecties werden dan echter uitsluitend waargenomen aan min of meer rechte wortelharen die sporadisch

voorkwamen (Fig. 39). Blijkbaar is er niet veel kans op infectie via een blaasvormig wortelhaar, hetgeen kan verklaren dat bij 30 °C doorgaans geen infecties, dus geen knolvormingen, optreden.

Samenvattend kan worden geconstateerd dat het mislukken van de symbiose bij een hoge temperatuur wordt veroorzaakt door een remmende invloed van deze temperatuur op een aantal processen. De rol van de hechting is in dit opzicht niet duidelijk, en zou kunnen worden gecompenseerd door de versnelde groei van de bacteriën bij de hoge temperatuur. Door de afwezigheid van geschikte wortelharen, ondanks aanwezigheid van een rhizobiumflora, is bij een hoge temperatuur de kans op knolvorming erg klein. Als er toch infectie optreedt, is het uitgroeien van een knolletje geremd, terwijl geen leghaemoglobine gevormd wordt en, waarschijnlijk in verband hiermee, de nitrogenase niet tot functioneren komt.

REFERENCES

AUGIER, J. and D. LAVERGNE. Numération de la microflore totale et microstructure des sols. II. Conséquences pratiques. Annls. Inst. Pasteur, Paris. **94** (1958), 766–774.

BERGERSEN, F. J. and D. J. GOODCHILD. Cellular location and concentration of leghaemoglobin in soybean root nodules. Aust. J. Biol. Sci. **26** (1973) 741–756.

BESSEMS, E. P. M. Nitrogen fixation in the phyllosphere of Gramineae. Thesis, Wageningen (1973).

BOHLOOL, B. B. and E. L. SCHMIDT. Lectins: a possible basis for specificity in the Rhizobium-legume root nodule symbiosis. Science **185** (1974) 269–271.

DART, P. J. and J. M. DAY. Effects of incubation temperature and oxygen tension on nitrogenase activity of legume root nodules. Plant and Soil, Spec. Vol. (1971) 167–184.

DIENER, T. Ueber die Bedingungen der Wurzelknöllchenbildung bei Pisum sativum L. Phytopath. Z. **16** (1950) 129–170.

DIXON, R. O. D. The origin of the membrane envelope surrounding the bacteria and bacteroids and the presence of glycogen in clover root nodules. Arch. f. Mikrobiol. **56** (1967) 156–166.

DOKU, E. V. Effect of day-length and water on nodulation of cowpea (*Vigna unguiclata* (L.) Walp.) in Ghana. Expl. Agric. **6** (1970) 13–18.

EGERAAT, A. W. S. M. VAN Pea-root exudates and their effect upon root-nodule bacteria. Thesis, Wageningen (1972).

FÅHREUS, G. The infection of clover root hairs by nodule bacteria studied by a simple glass-slide technique. J. Gen. Microbiol **16** (1957) 374–381.

FÅHREUS, G. and H. LJUNGGREN. The possible significance of pectic enzymes in root hair infection by nodule bacteria. Physiol. Plant. **12** (1959) 145–154.

GATES, C. T. and J. R. WILSON. The interaction of nitrogen and phosphorus on the growth, nutrient status and nodulation of *Stylosanthes humilis* H.B.K. (Townsville stylo). Plant and Soil **41** (1974) 325–333.

GIBSON, A. H. Physical environment and symbiotic nitrogen fixation. IV. Factors affecting the early stages of nodulation. Aust. J. biol. Sci. **20** (1967) 1087–1104.

GIBSON, A. H. Factors in the physical and biological environment affecting nodulation and nitrogen fixation by legumes. Plant and Soil, Spec Vol (1971) 139–152.

HAACK, A. Über den Einfluss der Knöllchenbakterien auf die Wurzelhaare von Leguminosen und Nichtleguminosen. Zbl. Bakt II **117** (1964) 343–366.

HARDY, R. W. F. and E. KNIGT JR. Biochemistry and postulated mechanisms of nitrogen fixation. In: Progress in phytochemistry, Vol I (eds L. Reinhold and Y. Liwschitz). Interscience Publishers, London, New York, Sydney. (1968) 407–489.

HUBBELL, D. H. Studies on the root hair 'curling factor' of Rhizobium. Bot. Gaz. **131** (1970) 337–342.

JOFFE, A., F. WEYER and S. SAUBERT. The role of root temperature in symbiotic nitrogen fixation. S. Afric. J. Sc. **57** (1961) 278–280.

JOHNSON, H. S. and D. J. HUME. Comparisons of nitrogen fixation estimates in soybeans by nodule weight, leghemoglobin content, and acetylene reduction. Can. J. Microbiol. **19** (1973) 1165–1168.

JONES, F. R. and W. B. TISDALE. Effect of soil temperature upon the development of nodules on the roots of certain legumes. J. Agr. Res. **22** (1921) 17–31.

KEILIN, D. and Y. L. WANG. Haemoglobin in the root nodules of leguminous plants. Nature **155** (1945) 227–229.

KLASOVA, A., J. KOLEK and J. KLAS. Time dynamics of primary root branching in *Pisum sativum* L. Biol. Plant **14** (1972) 249–253.

KUNG, H. K. and S. M. WEST. The effect of temperature treatments on growth and the metabolism of ribonucleic acid in relation to cell division and cell elongation of *Pisum sativum* roots. Physiol. Plant. **21** (1968) 827–832.
LIBBENGA, K. R. Nodulatie bij Leguminosae, Thesis, Leiden (1970).
LIE, T. A. Nodulation of leguminous plants as affected by root secretions and red light. Thesis. Wageningen (1964).
LIE, T. A. The effect of low pH on different phases of nodule formation in pea plants. Plant and Soil **31** (1969) 391–406.
LIE, T. A. Symbiotic nitrogen fixation under stress conditions. Plant and Soil, Spec Vol. (1971) 117–127.
LIE, T. A. Environmental effects on nodulation and symbiotic nitrogen fixation. In: The biology of nitrogen fixation (ed A. Quispel) (1975) 555–582.
LILLICH, T. T. and G. H. ELKAN. Role of polygalacturonase in invasion of root hairs of leguminous plants by Rhizobium spp. Can. J. Microbiol. **14** (1968) 617–625.
LIS, H. and N. SHARON. The biochemistry of plant lectins (phytohemagglutinins). Ann. Rev. Biochem. **42** (1973) 541–574.
LJUNGGREN, H. Mechanism and pattern of *Rhizobium* invasion into leguminous root hairs. Physiol. Plant Suppl. V (1969).
MACMILLAN, J. D. and R. C. COOKE. Evidence against involvement of pectic enzymes in the invasion of root hairs by *Rhizobium trifolii*. Can. J. Microbiol. **15** (1969) 643–645.
MES, M. G., S. SAUBERT VON HAUSEN and N. O. VAN GLYSWYK. A comparative study of the growth and yield of a number of Crotolaria species suitable for green manuring. S. Afr. J. Sci. **53** (1957) 181–189.
MES, M. G. Influence of temperature on the symbiotic nitrogen fixation of legumes. Nature **184** (1959) 2032.
MES, M. G. The influence of night temperature and day length on the growth, nodulation, nitrogen assimilation and flowering of *Stizolobium deeringianum* (Velvet Bean) S. Afr. J. Sci. **55** (1959) 35–39.
MULDER, E. G. and W. L. VAN VEEN. Effect of pH and organic compounds on nitrogen fixation by red clover. Plant and Soil **13** (1960) 91–113.
MULDER, E. G., T. A. LIE, K. DILZ and A. HOUWERS. Effect of pH on symbiotic nitrogen fixation of some leguminous plants. IX International Congress for Microbiology, Moscow (1966) 133–151.
MUNNS, D. N. Nodulation of *Medicago sativa* in solution culture. I. Acid-sensitive steps. Plant and Soil **28** (1968) 129–146.
MUNNS, D. N. Enzymatic breakdown of pectin and acid-inhibition of the infection of Medicago roots by Rhizobium. Plant and Soil **30** (1969) 117–120.
MUNNS, D. N. Nodulation of *Medicago sativa* in solution culture. V. Calcium and pH requirements during infection. Plant and Soil **32** (1970) 90–102.
NUTMAN, P. S. Physiological studies on nodule formation. I. The relation between nodulation and lateral root formation in red clover. Ann. Bot. N.S. **12** (1948) 81–96.
NUTMAN, P. S. Studies on the physiology of nodule formation III. Experiments on the excision of root tips and nodules. Ann. Bot. N. S. **16** (1952) 79–101.
NUTMAN, P. S. The physiology of nodule formation. In: Nutrition of the legumes. (ed. E. G. Hallsworth) Butterworths Sci. Publ. London (1958) 87–107.
NUTMAN, P. S. Some observations on root-hair infection by nodule bacteria. J. exp. Bot. **10** (1959) 250–263.
NUTMAN, P. S. Symbiotic Nitrogen Fixation. In: Soil nitrogen (eds. W. V. Bartholomew and F. E. Clark) American Society of Agronomy, Madison, Wis. USA (1965) 360–383.
PANKHURST, C. E. and A. H. GIBSON. Rhizobium strain influence on disruption of clover nodule development at high root temperature. J. Gen. Microbiol. **74** (1973) 219–231.
POTTS, J. R. M. and D. P. ORMOD. The effect of abrupt temperature increase on growth and phosphorus fraction of *Pisum sativum*. Can. J. Bot. **47** (1969) 1199–1203.

PURCHASE, H. F. and P. S. NUTMAN. Studies on the physiology of nodule formation. VI. The influence of bacterial numbers in the rhizosphere on nodule initiation. Ann. Bot. N. S. **21** (1957) 439–454.

RITTENHOUSE, H. G., R. E. WILLIAMS, B. WISNIESKI and C. FRED FOX. Alterations of characteristic temperatures for lectin interactions in LM cells with altered lipid composition. Biochem. Biophys. Res. Comm. **58** (1974) 222–228.

ROPONEN, I., E. VALLE and T. ETALLA. Effect of temperature of the culture medium on growth and nitrogen fixation of inoculated legumes and rhizobia. Physiol. Plant **23** (1970) 1198–1205.

ROUGHLEY, R. J. and P. J. DART. Reduction of acetylene by nodules of *Trifolium subterraneum* as affected by root temperature, *Rhizobium* strain and host cultivar. Arch. f. Mikrobiol. **69** (1969) 171–179.

ROUGHLEY, R. J. The influence of root temperature, Rhizobium strain and host selection on the structure and nitrogen-fixing efficiency of the root nodules of *Trifolium subterraneum*. Ann. Bot. N. S. **34** (1970) 631–646.

ROUGHLEY, R. J., P. J. DART, P. S. NUTMAN and P. A. CLARKE. The infection of *Trifolium subterraneum* root hairs by *Rhizobium trifolii*. J. Exp. Bot. **21** (1970) 186–194.

SAHLMAN, K. and G. FÅHREUS. An electron microscope study of root-hair infection by Rhizobium. J. Gen. Microbiol. **33** (1963) 425–427.

SCHIFFMANN, J. and R. LÖBEL. Seasonal changes in symbiotic nitrogen fixation and haemoglobin content in nodules of peanuts. Plant and Soil **39** (1973) 329–340.

SCHWINGHAMER, E. A., H. J. EVANS and M. D. DAWSON. Evaluation of effectiveness in mutant strains of Rhizobium by acetylene reduction relative to other criteria of N_2 fixation. Plant and Soil **33** (1970) 192–212.

SIRONVAL, C., C. BONNIER and J. P. VERLINDEN. Action of day-length on nodule formation and chlorophyll content in soybean. Physiol. Plant. **10** (1957) 697–707.

SOLHEIM, B. and J. RAA. Evidence countering the theory of specific induction of pectin-degrading enzymes as basis for specificity in Rhizobium-Leguminosae associations. Plant and Soil **35** (1971) 275–280.

SOLHEIM, B. and J. RAA. Characterization of the substances causing deformation of root hairs of *Trifolium repens* when inoculated with *Rhizobium trifolii*. J. Gen. Microbiol. **77** (1973) 241–247.

STEWART, W. D. P. Nitrogen fixation in plants. The Athlone Press, London (1966).

THORNTON, H. G. The role of the young lucerne plant in determining the infection of the root by nodule-forming bacteria. Proc. Roy. Soc. Lond. B104 (1929) 481–492.

TRIGOSO, R. and H. W. FASSBENDER. Effect of applications of Ca + Mg, P, Mo and B on yield and nitrogen fixation in four tropical legumes. Turrialba **23** (1973) 172–180.

TURNER, E. R. The effect of certain adsorbents on the nodulation of clover plants. Ann. Bot. N. S. **19** (1955) 151–160.

VANTASIS, J. T. and G. BOND. The effect of charcoal on the growth of leguminous plants in sand culture. Ann. Appl. Biol. **37** (1950) 159–168.

VIRTANEN, A. I. Symbiotic nitrogen fixation. Nature **155** (1945) 747–748.

VIRTANEN, A. I., J. JORMA, H. LINKOLA and A. LINNASALMI. On the relations between nitrogen fixation and leghaemoglobin content of leguminous root nodules. Acta Chem. Scand. **1** (1947) 90–111.

WILSON, D. O. Nitrogen fixation by soybeans as influenced by inoculum placement: greenhouse studies. Agron. J. **67** (1975) 76–78.

WILSON, D. O. and H. M. REISENAUER. Determination of leghemoglobin in legume nodules. Anal. Biochem. **6** (1963) 27–30.

YAO, P. Y. and J. M. VINCENT. Host specificity in the root hair 'curling factor' of Rhizobium spp. Aust. J. Biol. Sci. **22** (1969) 413–423.

ZEVENHUIZEN, L. P. T. M. Chemical composition of exopolysaccharides of *Rhizobium* and *Agrobacterium*. J. Gen. Microbiol. **68** (1971) 239–243.

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