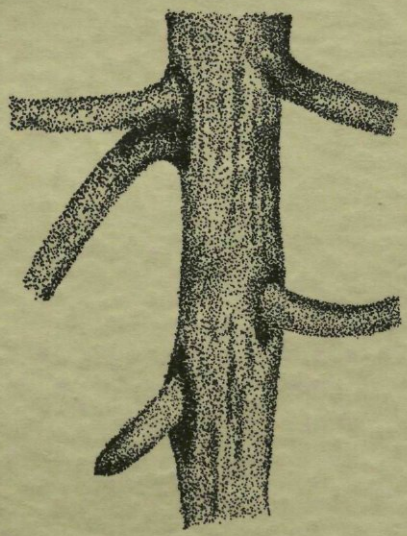
PEA-ROOT EXUDATES AND THEIR EFFECT UPON ROOT-NODULE BACTERIA



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635.656;581.135;631.461.52

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PEA-ROOT EXUDATES AND THEIR EFFECT UPON ROOT-NODULE BACTERIA

(with a summary in Dutch)

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWWETENSCHAPPEN OP GEZAG VAN
DE RECTOR MAGNIFICUS, PROF. DR. IR. H. A. LENIGER,
HOOGLERAAR IN DE TECHNOLOGIE,
IN HET OPENBAAR TE VERDEDIGEN
OP VRIJDAG 8 DECEMBER 1972 DES NAMIDDAGS TE VIER UUR
IN DE AULA
VAN DE LANDBOUWHOGESCHOOL TE WAGENINGEN

H. VEENMAN & ZONEN N.V. – WAGENINGEN – 1972

STELLINGEN

I

De algemeen optredende groeistimulatie van voornamelijk tot het geslacht *Rhizobium* behorende bacteriën in de rhizosfeer van vlinderbloemige planten wordt naar men aanneemt veroorzaakt door de uitscheiding van specifiek *Rhizobium* stimulerende verbindingen door wortels van leguminosen; het bestaan van een groeiremmende factor voor *Rhizobium*, uitgescheiden door wortels van niet-vlinderbloemige planten, is echter evenzeer denkbaar.

II

Homoserine, afgegeven door erwtekiemwortels als gevolg van verwondingen ontstaan bij het uittreden van zijwortels, lijkt selectief de groei van die *Rhizobium*-stammen te bevorderen welke behoren tot de 'cross-inoculation'-groep *Rhizobium leguminosarum*.

III

Het door SCHUPHAN voor mogelijk gehouden verband tussen de toeneming van het aantal gevallen van hart- en vaatziekten en hoge stikstofbemesting van voedingsgewassen lijkt ongegrond.

W. SCHUPHAN: Ernährungs-Umschau **18**, 1971, 148.

IV

In navolging van het bestaande periodieke bevolkingsonderzoek naar longtuberculose zou een periodieke screening op coronaire hartziekten van groot belang zijn voor de volksgezondheid.

V

Bij een studie over de opname van voedingselementen door de plantewortel onder niet steriele omstandigheden dient rekening te worden gehouden met de aanwezigheid van een rhizosfeer-microflora welke de beschikbaarheid van voedingsstoffen voor de plant sterk kan beïnvloeden.

D. A. BARBER: Ann. Rev. Plant Phys. **19**, 1968, 71.
M. LOUTIT e.a.: Soil Biol. Biochem. **4**, 1972, 267.

VI

Naast het binden van stikstof bij leguminosen zouden bacteriën behorend tot het geslacht *Rhizobium* mogelijk ook, door een direct effect op de wortel, een verbeterde opname van voedingselementen uit de grond kunnen bewerkstelligen.

T. A. LIE: Plant and Soil, Special Volume, 1971, 117.
D. A. BARBER: Ann. Rev. Plant Phys. **19**, 1968, 71.

VII

Bij de preventie van voedselvergiftiging door levensmiddelen van dierlijke oorsprong zou een controle van de veestapel van vnl. de grote bedrijven (de bio-industrie) op met name de aanwezigheid van pathogene micro-organismen en op restproducten van grote betekenis kunnen zijn.

VIII

Het toepassen van een directe, selectieve ophopingsmethode voor het aantonen van *Salmonella* in gedroogde voedingsmiddelen geeft een onjuist beeld over de werkelijke mate van besmetting.

M. VAN SCHOTHORST en F. M. VAN LEUSDEN:
Zlb. Bakt. Hyg., I. Abt. Orig. A 221, 1972, 19.

IX

De berekeningen van BEEK betreffende de fosfaatvervuiling van het oppervlaktewater ten gevolge van de verschillende distributieve bronnen (huishoudens, veeteelt, landbouw) zijn zeer aanvechtbaar.

W. J. BEEK: Toekomstbeeld der Techniek **8**, 1971, 11.

X

De fosfaatbelasting van het oppervlaktewater is slechts in belangrijke mate terug te dringen door defosfatering van het effluent van rioolwaterzuiveringsinstallaties. De hoge kosten om dit te verwezenlijken zouden mede moeten worden gedragen door de wasmiddelenindustrie.

XI

Milieubelasting geheven op het huishoudelijk afvalwater als vaste aanslag per woning is onjuist, beter ware het om de drinkwaterrekening met een bepaald percentage, afhankelijk van het verbruik, te verhogen.

Proefschrift van A. W. S. M. VAN EGERAAT
Wageningen, 8 december 1972.

WOORD VOORAF

Gaarne maak ik van deze gelegenheid gebruik om allen te bedanken die aan het tot stand komen van dit proefschrift hebben bijgedragen.

Veel dank ben ik verschuldigd aan mijn te vroeg overleden vader die mij in mijn vlegeljaren zover heeft weten te brengen een studie te beginnen. Mijn ouders hebben zich financiële offers moeten getroosten en hiervoor en voor al hun goede zorgen zeg ik hen nu oprecht dank.

Marianne, je belangstelling, je optimisme en je gave om mij, zo dit nodig was, uit de wetenschappelijke put te halen zijn van onschatbare betekenis geweest. Ik ben je erg dankbaar.

Hooggeleerde Mulder, hooggeachte Promotor, ik dank U voor de gelegenheid die U mij geboden hebt om een proefschrift te bewerken. De grote vrijheid die U mij gelaten hebt, Uw waardevolle suggesties en Uw kritiek worden in dank gememoreerd. Uw warme belangstelling voor ook meer persoonlijke lotgevallen heb ik zeer op prijs gesteld.

Zeergeleerde Lie, beste Tek An, voor je hulp in de beginfase van dit onderzoek en voor je voortdurende kritische belangstelling ben ik je zeer erkentelijk. Ik dank je voor de goede verstandhouding die we in de afgelopen jaren steeds hebben gehad.

Zonder de hulp van vele medewerkers van het laboratorium voor Microbiologie zou dit onderzoek niet mogelijk zijn geweest. Gaarne dank ik de Heer Wessels die met grote deskundigheid vele foto's heeft gemaakt en afgedrukt. Met genoegen denk ik terug aan de tijd dat de aminozuuranalysator nog bij U inwoonde. Veel dank ook aan de Heer van Velzen die met uiterste precisie alle tekeningen en grafieken voor het proefschrift heeft verzorgd. Nooit heb ik vergeefs een beroep op U gedaan. De Heren van de Kolk en Schouten hebben voortreffelijke diensten bewezen bij de voorbereiding van vele experimenten. De Heer Rátz heeft op een uitstekende wijze een groot aantal monsters op de aminozuuranalysator verwerkt terwijl Mej. de Goffau met grote welwillendheid bij een aantal experimenten behulpzaam is geweest.

De Heer Houwers dank ik voor de kritische wijze waarop hij het Engels van een gedeelte van het concept manuscript heeft beoordeeld. De Dames Van der Scheer en Haagen dank ik voor hun altijd tegemoetkomende hulp. Mevr. Möller-Mol ben ik zeer erkentelijk voor de voortreffelijke wijze waarop ze het manuscript heeft getypt en verzorgd.

De medewerkers van de Centrale Bibliotheek dank ik voor hun uitstekende diensten.

CONTENTS

1. INTRODUCTION

A. SURVEY OF THE LITERATURE PERTAINING TO PLANT-ROOT EXUDATES AND THEIR INFLUENCE UPON SOIL MICROORGANISMS

1.1. GENERAL INTRODUCTION

HILTNER (1904) observed that microorganisms were more abundant in soil near plant roots than in distant soil. The zone in which stimulation occurred he termed 'the rhizosphere'. The first indication concerning root exudation was provided by KNUDSON (1920) who found that peas and maize grown under aseptic conditions in sucrose solution produced considerable quantities of reducing sugars. He suggested that the sucrose was absorbed by the roots and converted to reducing sugars, which were subsequently excreted. LYON and WILSON (1921) found that organic nitrogen compounds were released from maize roots growing under sterile conditions.

A specific effect caused by root exudates was first demonstrated by O'BRIEN and PRENTICE (1930). They showed that the cysts of the potato eelworm (*Heterodera schachtii*) hatched in the presence of root washings of potato, but not with washings of beet, lupin, mustard or oat roots.

After these findings, research into the nature and effect of root exudates came to a temporary standstill. This was probably due to difficulties in the identification of organic compounds at the low concentrations at which they occur in root exudates. In the last ten to fifteen years, however, research in this field has considerably increased.

1.2. THE RHIZOSPHERE EFFECT

The rhizosphere may be defined as the zone of about 5 mm surrounding the roots. In this zone there occurs considerable stimulation of growth of microorganisms. The extent of the rhizosphere effect is characterized by the R/S ratio (R = number of microorganisms in the rhizosphere soil, S = number of microorganisms in the soil, without roots). The rhizosphere effect is most pronounced in the case of bacteria: R/S values of 10 to 100 or even higher are reported. Gram-negative non-sporulating rod-shaped bacteria are favoured by the roots of most plants (LOCHHEAD, 1940; SPERBER and ROVIRA, 1959; ROUATT *et al.*, 1963). Actinomycetes and fungi are less strongly stimulated in the rhizosphere (PARKINSON, 1958; PETERSON, 1958), while protozoa and algae are only very slightly stimulated (ROUATT *et al.*, 1960). Nematodes are also found more frequently in rhizosphere soil than in root-free soil (HENDERSON and KATZNELSON, 1961).

The rhizosphere effects of different plants vary in magnitude. In a study of six plants, the highest rhizosphere counts were reported with red clover, followed by flax and oats, while the lowest counts were found with wheat, barley and corn (ROUATT and KATZNELSON, 1961). In general, leguminous plants have larger rhizosphere populations than non-legumes, while root-nodule bacteria are much more strongly stimulated by legumes than other rhizosphere organisms. With legumes, the R/S ratio for *Rhizobium* often exceeds 10^6, whereas for other rhizosphere bacteria ratios fall within the range of 10 to 100 (NUTMAN, 1965). In general, nodule bacteria are little affected by roots of non-legumes. Nodule bacteria are more strongly stimulated by those hosts which they are able to infect than by other legumes (WILSON, 1930). This was again shown recently by ROBINSON (1967), who found that in a mixed pasture of subterranean clover (*Trifolium subterraneum*) and lucerne (*Medicago sativa*), clover only stimulated *Rhizobium trifolii* but not *Rhizobium meliloti*. Lucerne stimulated both species in the rhizosphere. In water cultures or in agar media there is no evidence of differential stimulation (PURCHASE, 1952).

So far nothing is known of the cause of the specific stimulation of *Rhizobium* spp. by the legume roots in general and of the stimulation of individual strains by certain hosts in particular. It may well depend on the root exudates from legumes being more varied than those from other plants (ROVIRA, 1956, 1962). According to ROVIRA (1969), it is unlikely that the ubiquitous sugars, amino acids and organic acids provide the specificity, but rather the balance of these compounds or the presence of exotic compounds peculiar to a particular plant species.

1.3. THE NATURE OF THE EXUDATES

ROVIRA (1965, 1969) has reviewed the nature of plant-root exudates. At least ten sugars have been identified in the exudates of a wide range of plants, glucose and fructose being in general the most abundant. Amino acids are the most frequently studied group of compounds in plant root exudates. Considerable variation has been reported for different plants. Moreover, different exudation patterns for one single plant species has been reported, but this may be due to the different conditions under which the experiments have been conducted. Vitamins have also been found in root exudates. Although in general the levels are low, they may have a considerable effect on vitamin-requiring microorganisms in the rhizosphere. WALLACE and LOCHHEAD (1949) found a high proportion of vitamin-requiring bacteria in the rhizosphere. Exudation of organic acids has been reported by RIVIÈRE (1959, 1960). FRIES and FORSMAN (1951) found nucleic-acid derivatives among the exuded compounds, while exo-enzymes have been identified in the exudates of maize roots (ROGERS *et al.*, 1940, 1942). A wide range of miscellaneous compounds are released from roots, several being toxic to microorganisms, e.g. hydrocyanic acid (TIMONIN, 1941), glycosides (SCHÖNBECK, 1958), and saponins (MISHUSTIN and NAUMOVA, 1955). ELKAN (1961) demonstrated that non-nodulating soybean released compounds which

altered the morphology of *Rhizobium japonicum* and prevented nodulation of the nodulating soybean line. Finally, factors affecting nematodes and fungi are found in root exudates (O'BRIEN and PRENTICE, 1930; KERR, 1956; BUXTON, 1957; WALLACE, 1958; BIRD, 1959).

1.4. FACTORS AFFECTING ROOT EXUDATION

The exudation of organic compounds by roots can be influenced by many factors. The amounts, identity and mutual proportion of compounds in root exudates differ for different plant species (ROVIRA, 1965). Plant age is also important. ROVIRA (1956) found more amino acids and sugars being exuded by peas and oats during the first ten days of growth than during the second ten days. ROVIRA (1959) found an increased exudation of asparagine by tomato roots and subterranean clover with rising temperature. HUSAIN and MCKEEN (1963) also found a temperature-dependent exudation. The light intensity at which plants were grown affected the amount and mutual proportion of compounds exuded into the nutrient solution by tomato and subterranean clover roots (ROVIRA, 1959). BOWEN (1969), working with *Pinus radiata* seedlings, demonstrated a marked effect of plant nutrition on exudation of amino acids by roots. Especially phosphate deficiency caused an increase in amino-acid exudation.

It may be assumed that in the rhizosphere, where a very large development of microorganisms occurs, exudation is affected by the presence of these organisms. ROVIRA (1965) supposed that microorganisms can affect exudation in at least three ways: a. by altering the permeability of root cells, b. by modifying the metabolism of the roots and c. by modifying some of the material released from the roots. LJUNGGREN and FÅHRAEUS (1959, 1961) found that polygalacturonase was released from roots under the influence of compatible *Rhizobium* spp. However, according to SOLHEIM and RAA (1971) such a release would not occur; the observed polygalacturonase activity was assumed to depend on fungal contamination.

Root exudates are affected by the media used for exudation studies. Most studies are done with solution cultures, but exudation of roots growing in soil may be quite different from exudation of roots growing in solution or sand cultures due to the different physical and chemical environments (ROVIRA, 1969). BOULTER *et al.*, (1966) found seven times more amino acids released from roots growing in quartz sand as compared with those growing in nutrient solution. These results, however, are doubtful, because part of the exudates in sand may have been caused by the damaging of root hairs and epidermical cells during removal of the roots from the sand. KATZNELSON *et al.* (1954, 1955) demonstrated that temporary wilting of plants greatly increased the exudation of amino acids into sand or soil. The amino acids were rapidly released from the roots of wilted plants after rewetting. This factor may be important under field conditions when rainfall occurs after a dry period. However, it is likely that the

sudden wetting of the roots damages the root hairs and epidermal cells (author's experiments), which may interfere with the observation of KATZNELSON *et al.*

Apart from factors such as temperature, light, soil moisture and rooting medium, it is possible to affect the exudation by more adjustable means. Attempts have been made to control the rhizosphere microflora by foliar applications. AGNIHOTRI (1965) modified the exudation of wheat by foliar spray of urea. He found a marked increase in the exudation of glucose, fructose, glutamine and α-alanine, and a decrease in the exudation of organic acids. VRANY *et al.* (1962) found an increased exudation of amino acids and sugars by wheat roots after application of chloramphenicol on the leaves. This caused fewer bacteria and more fungi in the rhizosphere. Alteration of the rhizosphere microflora caused by changes in the exudates has also been reported by RAMACHANDRA-REDDY, 1959; VENKATA-RAM, 1960; HORST and KERR, 1962; VRANY *et al.*, 1962; AGNIHOTRI, 1964 and VRANY, 1965. PEACOCK (1966) studied the control of nematodes by foliar sprays. He showed that a nematocide moved from the leaves to the roots of tomato plants, where it reduced the numbers of galls caused by the nematodes or even killed the nematodes in the sand surrounding the roots.

The use of foliar sprays can be very promising in controlling plant diseases caused by root pathogens.

1.5. MECHANISM AND SITES OF ROOT EXUDATION

Little is known about the mechanism of root exudation. The key question is whether exudation is controlled by metabolic processes or is a simple leaking out of compounds (ROVIRA, 1969). BOULTER *et al.* (1966) found that the ratios of compounds of root exudates and root extracts are not the same. They concluded that a selective exudation of compounds by roots takes place, but ROVIRA (1969) questions this conclusion in view of the fact that exudation is probably not uniform along a root, while the cell contents of different parts of the root may differ. In a comparison of root exudates and homogenates of root tips, AYERS and THORNTON (1968) found that the amino acids present in greatest abundance in the root tips were also the most abundant in the exudates. LUNDEGÅRDH and STENLID (1944) linked exudation with respiration and RIVIÈRE (1959) posed that exudation should be considered to be a consequence of cell permeability at a particular stage of growth.

With regard to the sites of exudation, the zone immediately behind the root tip has been considered to be the most important. This was found by PEARSON and PARKINSON (1961) and by SCHROTH and SNYDER (1962) who studied the exudation of ninhydrin-positive compounds by broad beans. Additional evidence of the importance of this zone is that it strongly attracts nematodes (BIRD, 1959) and fungal zoospores (ZENTMYER, 1961).

There is evidence, however, that older parts of the roots also exude organic compounds. FRENZEL (1960) used mutants of *Neurospora* with specific nutrient

requirements and showed that with *Helianthus annuus*, threonine and aspargine came from the root tip and leucine, valine, phenylalanine and glutamic acid from the root-hair zone. SCHROTH and SNYDER (1962), working with beans, found that sugars and amino acids were exuded from the zone where adventitious roots emerged. MCDOUGALL (1968) showed that wheat exuded C^{14}-labeled compounds mainly from the zone of lateral roots after these had fully emerged. Subsequent experiments showed that most of the radioactive material came from the lateral root tips (ROVIRA, 1969; MCDOUGALL and ROVIRA, 1969). BOWEN (1968), working with *Pinus radiata* roots labeled with Cl^{36}, showed that chloride exudation occurred along the entire length of the roots, but the highest proportion of the chloride was exuded from the apical region.

From the above-mentioned data it can be concluded that the sites of root exudation are not the same in different plant species. It is difficult to draw further conclusions, because the experimental work has been done with plants of various ages, under different growth conditions, and by means of different techniques.

B. SCOPE OF THE INVESTIGATIONS

The aim of the present investigations was a. to study the behaviour of *Rhizobium* spp. on and close to the surface of pea-seedling roots in relation to root exudates, b. to study sites and mechanism of exudation of pea-seedling roots and c. to identify root exudates and their possible action upon *Rhizobium* spp.

2. MATERIAL AND METHODS

2.1. PLANT MATERIAL

The experiments were mainly carried out with pea plants, *Pisum sativum* L., cultivar Rondo. For comparison, in certain experiments also other plant species were used (see the relevant experiments in chapters 4 and 7).

Selected seeds were surface-sterilized by shaking in a 3% H_2O_2 solution containing a drop of detergent (Teepol) per 25 ml during twenty minutes. The seeds were transferred, without washing, to Petri dishes containing 2% agar in tap water. For some experiments the agar was covered with a sheet of Whatman nr. 1 filter paper. The seeds were left to germinate at room temperature in the dark for 3–8 days. The seedlings were then treated in different ways depending on the experiments to be carried out.

When fully-grown pea plants were needed, sterile seedlings were transferred to jars containing a nutrient solution of the following composition: K_2HPO_4, 0.36 g; KH_2PO_4, 0.12 g; $MgSO_4.7H_2O$, 0.25 g; $CaSO_4$, 0.25 g; Fe^{+++}-citrate, 30 mg; $MnSO_4.4H_2O$, 1 mg; $ZnSO_4.7H_2O$, 0.25 mg; $CuSO_4.5H_2O$, 0.25 mg; H_3BO_3, 0.25 mg; $Na_2MoO_4.2H_2O$, 0.05 mg, per 1000 ml of tap water. The seedlings were wrapped in sterile cotton wool and placed with their roots in Erlenmeyer flasks containing 300 ml nutrient solution. If necessary, additional nutrient solution was supplied during the experiment. When sterile cultures were needed, the nutrient solution was first sterilized at 105 °C for 20 minutes. The plants were grown in a controlled-environment cabinet using artificial light (circ. 45.000 ergs/cm^2/sec., photoperiod 12 hours) at a temperature of approximately 23 °C.

2.2. *RHIZOBIUM* STRAINS

Two *Rhizobium leguminosarum* strains were mainly used: PRE and PF_2. Both strains are effective with pea plants and are making large, red-coloured nodules. In a few experiments also other *Rhizobium* strains were used (see the relevant experiments).

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; $MgSO_4.7H_2O$, 0.25 g; NaCl, 0.1 g; $CaCO_3$, 3 g; Davies agar, 10 g, per 1000 ml of tap water. For plant inoculation, 1–2 drops (circa 1/20 ml) of a 7-days-old culture, suspended in 50 ml of sterile water, was applied per plant. For growth experiments with *Rhizobium*, 1 loopful of a 7-days-old culture was suspended in 10 ml of sterile water. After diluting 1:10^2, 1–2 drops of this suspension was used for inoculation.

2.3. Bacteriological methods

2.3.1. *Growth of* Rhizobium

The bacteria were grown in Erlenmeyer flasks of 100 ml capacity, containing 25 ml of nutrient solution. Incubation took place on a rotary shaker at 25°C during seven days. In some experiments, culture tubes containing 5 ml of nutrient solution were used instead of the Erlenmeyer flasks. The tubes were placed in an almost horizontal position to ensure good oxygen supply.

2.3.2. *Estimation of bacterial growth*

2.3.2.1. Plate counts

Plate counts were performed on yeast-extract medium (see 2.2) solidified with 1% agar. Amounts of 0.2 ml of suitable dilutions of bacterial suspensions were evenly spread over the agar surface using a bent glass rod. Colonies were counted after 5 days incubation at 25°C.

2.3.2.2. Spot-plate counts

Portions of 0.02 ml of suitable dilutions were pipetted on a yeast extract agar medium (10 spots per plate) using a microscrew attachment. The agar plates were dried by storage at 25°C for 3 days prior to use. The spots were counted after 24 hours incubation at 25°C under a Wild-M5 stereomicroscope.

2.3.2.3. Nephelometric method

The turbidity of bacterial suspensions was measured at 610 mμ using a Kipp nephelometer.

2.4. General and chemical methods

2.4.1. *Collection of root exudates*

A glass jar of 800 ml capacity was filled up until 1 cm below the brim with glass-distilled water or with plant-nutrient solution (see 2.1, 1/5 strength). The jar was wrapped in aluminium foil and the top covered with a perforated glass plate. The jar was then placed in a larger, empty glass jar of 3000 ml capacity and the top of the latter covered with cellophane. After sterilization and cooling, pea seedlings were aseptically transferred from Petri dishes to the glass plate covering the inner jar. They were inserted in the holes, taking care that the root tips were in contact with the solution (see fig. 2.1). Each jar contained 18 seedlings. The cultures were placed under controlled environmental conditions (see 2.1).

2.4.2. *Collection of root-tip exudates*

For these experiments, plastic tubes of 20 ml capacity, partly filled with nutrient solution, were used. Adjustable rubber rings, with a small hole in the centre, were mounted in the tubes. After sterilization and cooling, pea seedlings were placed on the rubber rings with the roots through the holes just in contact

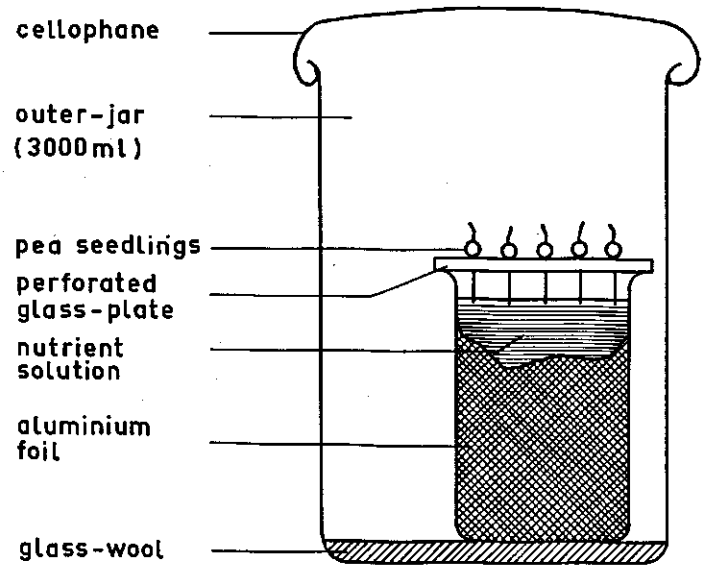


FIG. 2.1. Apparatus used for obtaining root exudates of pea seedlings.

with the solution (see fig. 2.2). Every three hours, the rubber rings were pulled upward so that during the whole experiment only root tips were in contact with the solution.

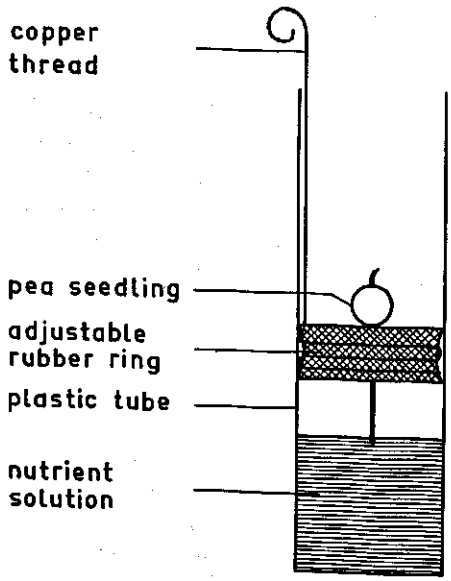


FIG. 2.2. Apparatus used for obtaining root-tip exudates.

2.4.3. *Collection of compounds adhering to the root surface*

Roots of pea seedlings without lateral roots and grown under humid conditions were immersed into glass-distilled water during two hours (perforated glass plates were used to support the cotyledons).

A different method was to scrape the roots using a razor blade. Material derived from several roots was collected in glass-distilled water. Using this method, compounds from epidermal cells and from the outer cortex cells in addition to the compounds adhering to the root were collected.

2.4.4. *Preparation of tissue extracts for the analyses of ninhydrin-positive compounds*

Fresh plant tissue was homogenized in a mortar in a small volume of 70% alcohol. Afterwards more alcohol was added up to 200 ml. The suspension was left overnight at 4°C and then filtered through a Whatman glass-fibre filter GF/C. The residue was washed several times with glass-distilled water. The filtrate and the washings were pooled and evaporated under vacuum at 50°C to a final volume of 25 ml. The ninhydrin-positive compounds were assayed after deproteinization of the extract with sulfosalicylic acid (2.5 mg/10 ml extract) and centrifugation. By adding the sulfosalicylic acid, the pH of the extract dropped to approximately 2.0.

2.4.5. *Treatment of root exudates for the analyses of ninhydrin-positive compounds*

2.4.5.1.

The nutrient solution in which roots had been grown was filtered through a Whatman nr. 1 filter paper to remove root caps and other cell debris and afterwards desalted using the cation-exchange resin Amberlite IR-120 (H^+). The ninhydrin-positive compounds were eluted from the resin with 0.5 N NH_4OH and the eluate was evaporated under vacuum at 50°C until dry. The residue was dissolved in 10 ml of a buffer solution having a pH of 2.2 (composition buffer: see table 2.1).

2.4.5.2.

When ninhydrin-positive compounds adhering to the root surface had been collected, the solution was filtered through Whatman nr. 1 filter paper and afterwards evaporated under vacuum at 50°C to a final volume of 10 ml. Sulfosalicylic acid was added and after centrifugation the samples were assayed for ninhydrin-positive compounds.

2.4.6. *Treatment of root exudates for analyses of U.V.-light-absorbing compounds*

The experiments were performed in glass-distilled water. After filtration and evaporation under vacuum at 50°C until dry, the residue was dissolved in 5 ml 0.1 N HCl.

2.4.7. *Hydrolyses of tissue extracts and root exudates*

2.4.7.1. Ninhydrin-positive compounds

Portions of tissue extracts or root exudates were diluted 20-fold with 6 N HCl. Hydrolysis was carried out in a sealed glass tube during 20 hours at 110 °C. The hydrolysed samples were evaporated under vacuum at 50 °C until dry and the residue was dissolved in a buffer solution of pH 2.2.

2.4.7.2. U.V.-light-absorbing compounds

Portions of the exudates were evaporated until dry. Then 0.3 ml of 72% $HClO_4$ was added and hydrolyses were carried out in a water bath during two hours at 100 °C. After hydrolysis, 3.70 ml 0.1 N HCl was added and afterwards the sample was centrifuged.

2.4.8. *Determination of ninhydrin-positive compounds*

The analyses were made with the aid of a Biocal-200 amino-acid analyser, following the procedure of MOORE and STEIN (1954). Acid and neutral ninhydrin-positive compounds were eluted from a column containing 55 by 0.9 cm Biorad A 6 spherical ion-exchange resin. The basic amino acids were separated on a 25 by 0.9 cm Biorad A 5 ion-exchange resin. The citrate buffers used for the separation were prepared as indicated in table 2.1. The ninhydrin solution contained: methyl cellosolve, 750 ml; sodium acetate buffer (4 N, pH 5.51 $\pm$ 0.03), 250 ml; ninhydrin, 20 g and $SnCl_2.2H_2O$, 400 mg. The identities of the ninhydrin-positive compounds in the samples were determined by comparison with analyses obtained from calibration mixtures of authentic ninhydrin-positive compounds and by adding pure compounds to the samples. The amount of each ninhydrin-positive compound in the sample was determined by measuring the area enclosed by its corresponding peak on the chromatogram and comparing this area with the area found when known concentrations of authentic ninhydrin-positive compounds were used.

TABLE 2.1. Buffers used in the analyses of ninhydrin-positive compounds.

Composition of buffer		pH			
		A	B	C	
		3.25 $\pm$ 0.02	4.25 $\pm$ 0.02	5.28 $\pm$ 0.02	2.20 $\pm$ 0.02
Sodium citrate.$2H_2O$	(g)	19.6	19.6	34.3	19.6
HCl, concentrated	(ml)	12.3	8.4	6.5	16.5
Thiodiglycol	(ml)	5.0	–	–	–
Brij-35 (50 g/100 ml)	(ml)	2.0	2.0	2.0	2.0
N-caprylic acid	(ml)	0.1	0.1	0.1	0.1
Final volume	(l)	1.0	1.0	1.0	1.0

Buffers A and B were used for the separation of acid and neutral ninhydrin-positive compounds, buffer C for the separation of basic ninhydrin-positive compounds. Buffer pH 2.2 was used as starting buffer and for diluting and dissolving of samples.

2.4.9. *Determination of U.V.-light-absorbing compounds*

U.V.-light-absorbing compounds were separated on a cation-exchange resin Zeocarb 225 (H^+), 200–400 mesh. The column (15 by 1 cm) was thoroughly washed with 1.0 N HCl. Excessive acid was removed with water. Separation was achieved by gradient elution with HCl, following the procedure described by DURAND (1966). The initial concentration of HCl was 0.25 N. The highest concentration used to obtain the gradient was 2.5 N.

Fractions of 6 ml were collected using an automatic fraction collector and the absorbancy of each fraction was determined at 260 mμ using a Beckman, model D.U., spectrophotometer. The fractions apparently containing one compound were pooled and evaporated to dryness. The residue was taken up in 0.2 ml 0.1 N HCl and 0.05 ml of this sample was applied to a strip of Whatman nr. 1 filter paper (40 by 6 cm). Authentic compounds were applied on the same strip. Chromatography (ascending method) was carried out using either iso-propanol – conc.HCl – H_2O (170–44–36) or n-butanol – H_2O (86–14) or n-butanol – sat.H_3BO_3 (86–14) as a solvent. After 24 to 36 hours, the paper was dried at room temperature and then examined under U.V.-light. The spots showing absorption were marked and afterwards cut from the paper. The spots were eluted with 5 ml 0.1 N HCl for 5 hours at 37 °C. After centrifugation, absorption spectra were made.

3. THE SITES OF EXUDATION OF PEA-SEEDLING ROOTS

3.1. INTRODUCTION

Several reports deal with sites of root exudation of a wide variety of plant species (see 1.5). In most of the investigations, filter paper was used to absorb the compounds released by the growing roots. After removing the roots from the filter paper, the absorbed compounds could be detected by spraying with specific reagents. When radioactive compounds are released, use may be made of a recording radiochromatogram scanner (BOWEN and ROVIRA, 1967).

In general, the root tip is considered to be the major site of exudation.

The aim of this investigation was to localize the sites of exudation of young pea roots.

3.2. EXPERIMENTAL

To localize the sites of exudation of pea-seedling roots, a modification of the filter-paper technique, first described by PEARSON and PARKINSON (1961) was used. Petri dishes (diameter cover 14 cm) were placed in larger Petri dishes. The space between the two dishes was filled with paper tissue (Kleenex). The smallest Petri dish was covered with a disc (diameter 14 cm) of Whatman nr. 1 filter paper. The filter paper and the tissue were slightly wetted with sterile water. Seedlings with roots of circa 1 cm were placed on the tissue in such a way that the root tips were just in contact with the filter paper. On top of the filter paper disc and covering the root tips, another disc (diameter 14 cm) of moist filter paper was placed. The Petri dishes, each containing 5 seedlings, were placed in the dark at room temperature.

After 24 hours, the upper filter paper was removed and the seedlings were carefully transferred to another similar device for the next 24 hours. The filter paper that was left was air-dried and afterwards sprayed with a solution of ninhydrin, 0.2 g, in 100 ml ethanol and 4 ml collidine to detect ninhydrin-positive compounds. Organic acids were detected by spraying with a 9:1 mixture of n-propanol and conc. ammonia followed by a solution of bromophenol blue, 50 mg, in 100 ml ethanol and 200 mg of citric acid.

3.3. RESULTS AND DISCUSSION

From fig. 3.1 it will be seen that exudation by young pea roots takes place at or just behind the root tips. Lateral roots behave similarly to the main root. The same exudation pattern was found for organic acids. These findings are in agreement with the generally accepted view that the root tip is the major site of exudation.

However, in the present study, additional sites were found to release consider-

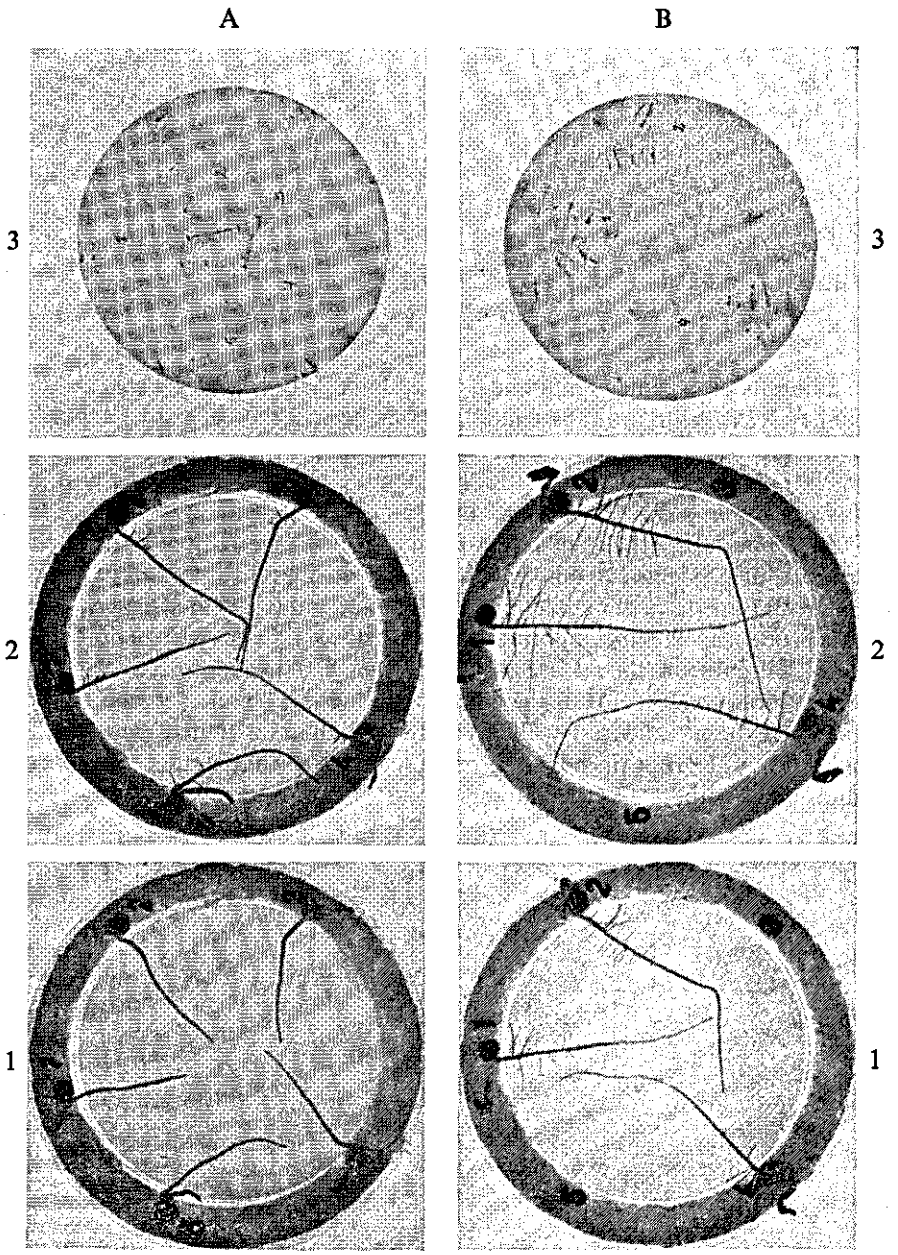


FIG. 3.1. Sites of exudation of ninhydrin-positive compounds by roots of pea seedlings. A: 1, root length at start of experiment; 2, root after 24 h; 3, filter paper after spraying with ninhydrin. B is similar to A, except older roots being used.

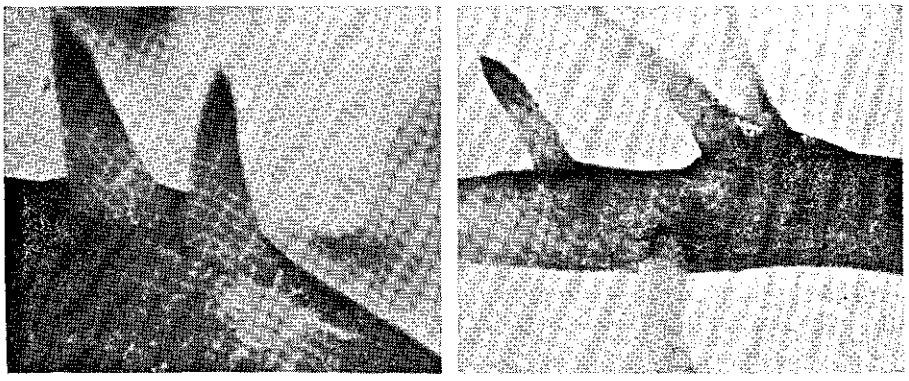


FIG. 3.2. Damage to main root resulting from the emergence of lateral roots.

able amounts of organic compounds. When lateral roots emerge from the main root they leave open wounds which are not healed by the plant (BURSTRÖM, 1965). Fig. 3.2 shows the severe damage of a main root resulting from the emergence of lateral roots. It is clear that leaking of compounds from these wounds can easily occur. Fig. 3.3 shows ninhydrin-positive compounds released from the main root during the emergence of lateral roots. When using the filter-paper technique, this leakage can only be observed if the lateral roots emerge from the main root at the place of contact between the latter and the filter paper.

The process by which compounds are exuded from the root-tip region is completely different from the release following damage of the root. In the latter case all the soluble constituents of the damaged tissue will be released and the composition of these 'exudates' will probably be similar to that of root extracts. Exudation by the root tip might be more selective so that certain specific compounds can be liberated (see chapters 6 and 7).

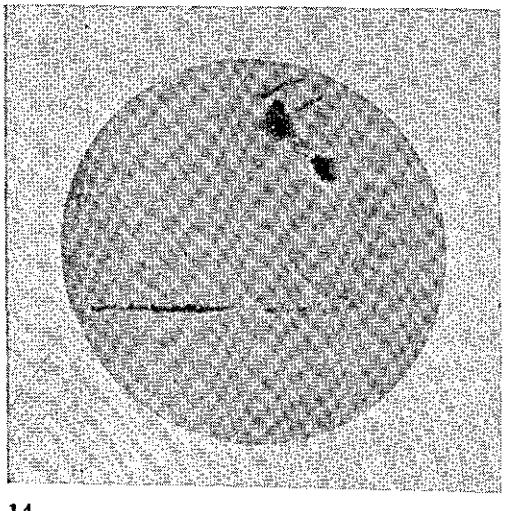


FIG. 3.3. Ninhydrin-positive compounds released from the main root of a pea seedling during the development of lateral roots. Roots grown on filter paper which was afterwards sprayed with ninhydrin.

4. PEA-ROOT EXUDATES AND THEIR ACTION UPON *RHIZOBIUM*

4.1. GROWTH OF *RHIZOBIUM LEGUMINOSARUM* WITH EXUDATES OF YOUNG PEA ROOTS

4.1.1. *Introduction*

The stimulation of microorganisms and in particular of *Rhizobium* spp. in the rhizosphere of leguminous plants is caused by substances exuded by the growing roots (see 1.2). To study the action of these compounds *in vitro*, exudates of young pea roots were collected and used as a medium for *Rhizobium leguminosarum*.

4.1.2. *Experimental*

Pea seedlings with roots of approximately two cm were grown on Whatman nr. 1 filter paper as shown in fig. 4.1. Filter paper A had been in contact with the non-growing part of the root, no laterals were present. Filter paper B had been in contact with the growing root tip and on filter paper C, lateral roots had been grown. Filter paper which had not been in contact with roots was used as a blank (Bl). Of each type (A, B. C and Bl), 25 filter papers were added to Erlenmeyer flasks of 100 ml capacity, containing 10 ml of a mineral salts' medium (K_2HPO_4 .$3H_2O$, 0.5 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2.2H_2O$, 40 mg; $FeCl_3.6H_2O$, 5 mg; $MnSO_4.H_2O$, 0.2 mg; $ZnSO_4.7H_2O$, 0.2 mg; $CuSO_4.5H_2O$, 0.02 mg; $CoCl_2$. $6H_2O$, 0.002 mg; H_3BO_3, 0.2 mg; $Na_2MoO_4.2H_2O$, 0.2 mg; distilled water, 1000 ml). The pH of the medium was adjusted to 6.8. The media were shaken for

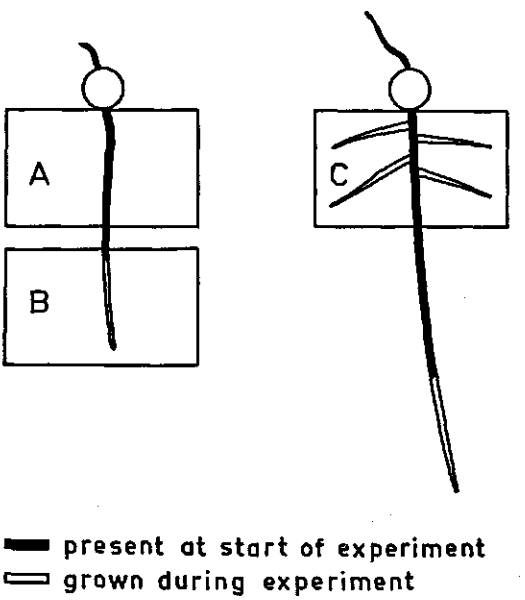


FIG. 4.1. Collection of pea-root exudates. Filter paper A had been in contact with the non-growing part of the root, filter papers B and C with tips of main root and lateral roots respectively.

two hours and left overnight at 4°C. Then they were filtered through a Whatman glass-fibre filter GF/C. Part of the Erlenmeyer flasks was supplied with an additional carbon source (mannitol, 1 g/l). After sterilization the media were inoculated with *Rhizobium leguminosarum*, strain PRE or strain PF_2. Seven days after inoculation, bacterial counts (2.3.2.1) were made.

4.1.3. *Results and discussion*

From the data of table 4.1 it can be seen that the addition of filter papers B or C to the mineral salts' medium considerably promoted the growth of *R. leguminosarum*. Filter paper A had no effect, indicating that no growth-promoting nitrogenous compounds are exuded by non-growing parts of the young root. This observation is in agreement with those found in chapter 3. Addition of a carbon source to the medium gave a considerable increase of the stimulatory effect of the exudates, indicating that excessive amounts of nitrogenous compounds were present in the root exudates during the early stages of growth, whereas assimilable carbohydrates were present in limiting amounts. Addition of a mixture of vitamins to the media had no further effect, indicating that sufficient vitamins apparently were present in the exudates.

In case of root-pressure juices or intact root segments of young pea plants added to the mineral salts' medium, a supplementary carbon source also greatly favoured bacterial growth (fig. 4.2). This means that also in the seedling root itself the nitrogenous compounds were present in excess.

TABLE 4.1. Response of *Rhizobium leguminosarum*, strain PRE, to root exudates of pea seedlings.

Additional C-source added	Filter paper	Bacteria° per ml × 10^6
+	–	16
–	–	5
+	Bl	21
+	A	19
+	B	115
–	B	45
+	C	88
–	C	38

° Values of typical experiments.
A non-growing parts of main roots; B growing root tips; C. lateral roots.

4.2. GROWTH OF *RHIZOBIUM LEGUMINOSARUM* ON THE ROOT SURFACE OF PEA SEEDLINGS

4.2.1. *Introduction*

The purpose of these experiments was to investigate if on the root surface of pea seedlings special areas are present stimulating growth of *Rhizobium*. Such areas when present might be identical with those where infection takes place.

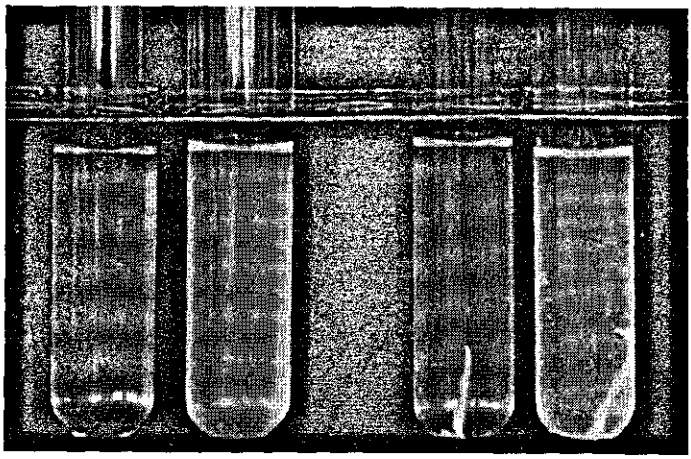


FIG. 4.2. Growth of *Rhizobium leguminosarum*, strain PF_2, in a mineral salts' medium supplemented with pea-root pressure juices or pea-root segments; A. without an additional carbon source, B. with added mannitol.

4.2.2. *Experimental*

Roots of sterile seedlings (root length 2–2.5 cm, no laterals) were inoculated with *Rhizobium leguminosarum*, strain PRE. Inoculation was carried out by dipping the roots for a short period (2–3 seconds) in a suspension of *R. leguminosarum* containing approximately 200,000 bacteria per ml. After inoculation, the seedlings were either directly used for *Rhizobium* countings or they were transferred to sterile Petri dishes containing water agar. In the Petri dishes, the seedlings were not in direct contact with the agar but cotyledons as well as roots were supported by slides. To prevent water shortage of the growing roots, the slides supporting the roots were covered with filter paper which was also in contact with the agar (see fig. 4.3). The seedlings were kept in the Petri dishes in the dark for 6, 24, 48, or 72 hours.

For collecting the *Rhizobium* cells from the roots, the latter were homogenized with the aid of a glass rod in a test tube containing 5 ml of a sterile 1% Na-pyrophosphate solution.

Counts were made by spreading 0.2 ml of the eventually diluted bacterial suspensions over the surface of yeast-extract agar plates and estimating the number of colonies after a five days' incubation period at 25°C.

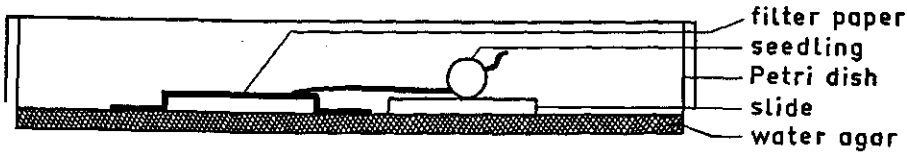


FIG. 4.3. Method used for growing pea seedlings when studying multiplication of *Rhizobium leguminosarum* on the root surface.

4.2.3. *Results and discussion*

The results of the bacterial countings are given in table 4.2. As will be seen, roots homogenized immediately after inoculation gave an average of 2580 bacteria. The actual numbers of several replicates varied from 1600 to 3100 per root. By dipping the roots in the *Rhizobium* suspensions, approximately 0.02 ml of liquid was retained by the roots. This means that the latter were inoculated with about 4000 bacteria. It is not surprising that the countings were lower than the actual numbers of bacteria added because part of them presumably were adsorbed by the crushed root tissue.

To get an idea of the magnitude of such an adsorption, an experiment was carried out in which segments (2 cm) of pea-seedling roots were added to different suspensions of *Rhizobium leguminosarum*, strain PRE. Immediately hereafter the root segments were homogenized and after settling of the root tissue the bacteria were counted. The numbers were compared with those found in suspensions without root tissue. Table 4.3 shows that under these circumstances approximately 20–25% of the bacteria were adsorbed by the root tissue.

The data of table 4.2 show that from the time of inoculation till six hours thereafter a sharp decrease in the number of *Rhizobium* cells on the root surface had occurred, while 24 hours after inoculation even lower numbers were found. The cause of this decrease is not fully understood. It is possible that part of the inoculated bacteria are strongly adsorbed at the root surface and are not detached during the counting procedure. It is unlikely that multiplication of the bacteria has taken place during these 24 hours and this indicates unfavourable growing conditions for the bacteria on the root surface (see also 4.3.3).

At 48 hours after inoculation a strong increase in the number of *Rhizobium* was observed and this continued from 48 to 72 hours. The increase of the *Rhizo-*

TABLE 4.2. Growth of *Rhizobium leguminosarum*, strain PRE, on the root surface of pea seedlings.

Time in hours after inoculation	0	6	24	48	72
Number° of bacteria found per root	2580	490	295	12,920	277,000

° Mean values of 3–5 replicates.

TABLE 4.3. Influence of homogenized root tissue on bacterial numbers found in suspensions of *Rhizobium leguminosarum*, strain PRE, of different density.

Number of bacteria found in 0.1 ml suspension	Number° of bacteria found in 0.1 ml suspension + pea-root tissue
348	262
32	23
4	3

° Mean values of three replicates.

bium cells on the root surface coincided with the formation of lateral roots. At 24 hours after inoculation no lateral roots were present; at 48 hours a few had been formed and at 72 hours several had developed. Multiplication of the bacteria owing to compounds released from the main root during the formation of lateral roots is very plausible (see also 3.3). If this hypothesis is correct, multiplication of the bacteria only takes place on that part of the root where lateral roots are present. To confirm this, main roots were cut into three pieces 72 hours after inoculation. One of the pieces contained all of the lateral roots. The rhizobia present on each of the root segments were counted; the results are given in table 4.4. It is obvious that a strong multiplication of *Rhizobium* had occurred only on that part of the root where lateral roots were present. Additional support for the hypothesis was provided by the observation that wounding with a needle of roots without lateral roots also resulted in a strong increase of the number of *Rhizobium* cells in the vicinity of the wounds.

Few bacteria were found on root segments without lateral roots and as mentioned before it looks as if growth conditions on the surface of intact roots of pea seedlings are not favourable for the multiplication of *Rhizobium*.

TABLE 4.4. Numbers of *Rhizobium leguminosarum*, strain PRE, found on the root surface of a pea seedling, 72 hours after inoculation. Root divided into three segments of equal length.

Segment	Laterals	Number° of bacteria found
1°°	+	325,000
2	−	2,680
3	−	1,090

° Values of a typical experiment.
°° Upper part; several lateral roots present.

4.3. PROMOTION OF THE GROWTH OF *RHIZOBIUM LEGUMINOSARUM* IN AGAR CULTURES BY GROWING ROOTS

4.3.1. *Introduction*

The aim of these experiments was to visualize the growth of microorganisms in the rhizosphere.

Agar, mixed with a suspension of *R. leguminosarum* was used as a medium for root growth of different plant species. It was expected that root exudates would promote bacterial growth and consequently would bring about a zone of increased bacterial growth around the roots.

4.3.2. *Experimental*

4.3.2.1. Roots grown on agar in Petri dishes

Sterile Petri dishes (diameter 11 cm) were supplied with 30 ml of agar of the following composition: mineral salts, see under 4.1.2; mannitol, 1 g; Davies

agar, 12 g; water, 1000 ml. After the agar had cooled down to approximately 45°C, 1 ml of a suspension of *Rhizobium leguminosarum* (strain PRE or strain PF_2), containing about 5.10^5 bacteria, was added. The bacteria were carefully mixed through the agar and after solidification, a sterile slide was placed on top of the agar plate. The Petri dishes were incubated at 25°C for 3 days, to allow the bacteria to multiply (as far as possible) and to become nitrogen-starved.

Seedlings, with roots of circa 1 cm, of lucerne (*Medicago sativa*), red clover (*Trifolium pratense*), white clover (*Trifolium repens*), black medick (*Medicago lupulina*), lupine (*Lupinus luteus*), peas (*Pisum sativum*), green gram (*Phaseolus radiatus*) and maize (*Zea mays*) were transferred to the Petri dishes, taking care that the cotyledons were supported by the slides and that the root tips were in contact with the agar. The Petri dishes were then placed at 22°C and examined daily to detect stimulation of the bacteria in the vicinity of the roots.

In some experiments (see 4.3.3.1.3), only performed with pea seedlings, uninoculated agar was used. After the seedlings had grown for 5 days on this agar, two or three lateral roots were inoculated with *Rhizobium leguminosarum*. This was done by the addition of 0.01 ml of a *Rhizobium* suspension to the surface of the lateral roots on a place where the roots had entered the agar layer.

4.3.2.2. Roots grown in agar in glass tubes

Sterile glass tubes (diameter 4 cm) were partly filled with agar having the same composition as used in the experiments of 4.3.2.1, but in certain cases supplemented with either yeast extract (1 g/l) or glutamic acid (1 g/l) or methylene blue (5 mg/l). When methylene blue had been added, the agar was coloured dark blue. The pH of the agar was always adjusted to 6.0. A suspension of *R. leguminosarum* was mixed through the agar prior to solidification (at about 45°C) and the tubes were incubated at 25°C for three days. Seedlings of black medick, red clover, white clover, green gram and peas were inserted in the tubes with their roots about one cm deep in the agar and placed in the dark or in the light (controlled-environment cabinet) at a temperature of approximately 22°C. The tubes in the light were partly wrapped with aluminium foil to shelter the roots from direct light. When yeast extract or glutamic acid had been added, the agar surface was covered with a one-cm-thick layer of glass beads to prevent contamination of the seed with bacteria.

4.3.3. *Results and discussion*

4.3.3.1. Roots grown in Petri dishes

4.3.3.1.1. Promotion of growth of *Rhizobium leguminosarum* by root-tip exudates of intact seedlings of various leguminous plants with the exception of those of pea plants

In view of the fact that ninhydrin-positive compounds were exuded by growing pea roots, it might be expected that the bacteria in the agar close to the surface of the roots would be stimulated by these compounds. Other plant spe-

cies were used for comparison and it was suggested that pea roots would be able to cause a stronger stimulation than roots derived from smaller seeds.

The results obtained showed that *Rhizobium leguminosarum*, strain PRE and strain PF_2, were stimulated by the root exudates of red clover, white clover, lucerne, black medick, lupine, green gram and maize (fig. 4.4). Contrary to expectation, pea seedling roots were the only ones causing no stimulation (fig. 4.5). Only where lateral roots emerged from the main root was a rather weak stimulation of the rhizobia observed after some time (fig. 4.6). This stimulation was most likely caused by compounds released from the wounds made by the emergence of lateral roots. When, instead of *Rhizobium*, other bacteria (*Pseudomonas* spp., *E. coli*) were mixed through the agar, the same negative results were obtained with pea roots.

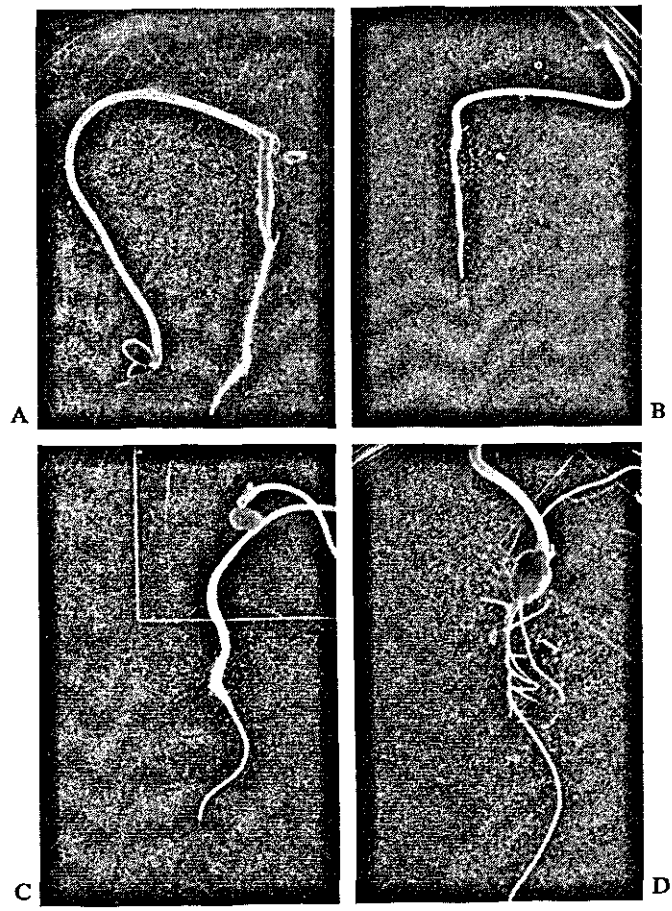


FIG. 4.4. Growth stimulation of *Rhizobium leguminosarum*, strain PRE, by root exudates of white clover (A), red clover (B), lucerne (C) and maize (D). Roots grown on agar (containing mineral salts and a carbon source), mixed with a suspension of the *Rhizobium* strain.

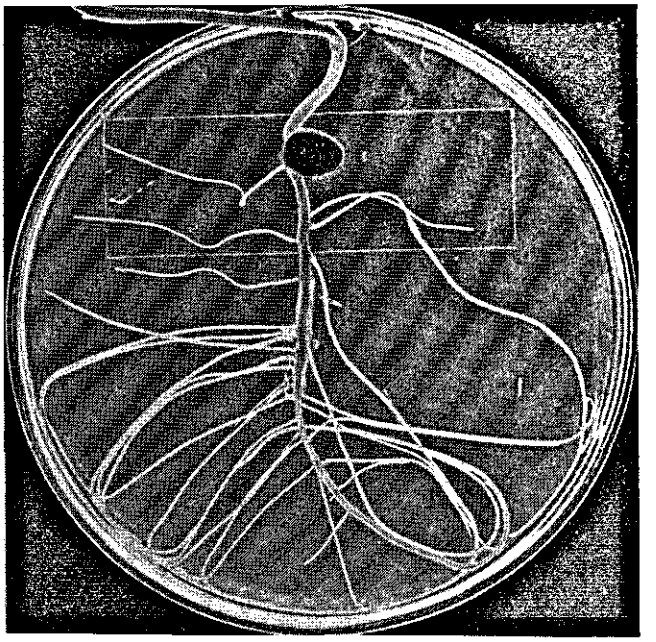


FIG. 4.5. The absence of rhizobial stimulation by root-tip exudates of pea seedlings. Roots grown on agar (containing mineral salts and mannitol), mixed with a suspension of *Rhizobium leguminosarum*, strain PRE.

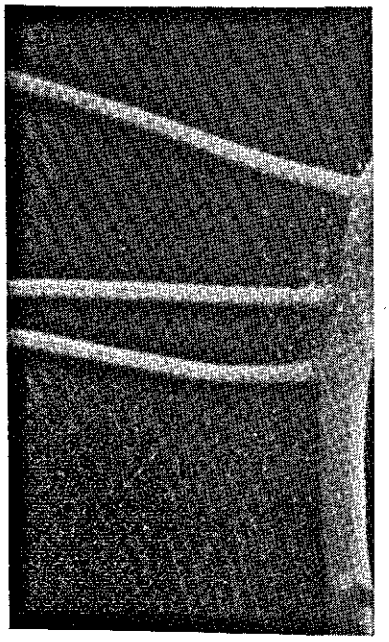


FIG. 4.6. Growth stimulation of *Rhizobium leguminosarum* by compounds released from the main root of a pea seedling after the emergence of lateral roots. The agar contained mineral salts and mannitol and was mixed with a suspension of *Rhizobium leguminosarum*, strain PRE.

The cause of this phenomenon is not clear. Initially it was supposed that the ninhydrin-positive compounds exuded by the pea plants were not assimilable by the bacteria, but this suggestion is in contrast with the finding that *Rhizobium leguminosarum* was stimulated by the amino-compounds exuded by pea roots on filter paper. It is more likely that simultaneously with the growth-promoting substances a compound was exuded by pea-root tips inhibiting bacterial growth to a certain extent (see below).

4.3.3.1.2. Promotion of growth of *Rhizobium leguminosarum* by compounds released from roots of pea seedlings after removing cotyledons and stem

When cotyledons and stem were removed from pea seedlings growing in rhizobia-containing agar, an enormous growth of *R. leguminosarum* around the whole root system was observed within a few days (fig. 4.7). This effect was also observed when sterile pea-root segments were placed on *Rhizobium*-containing agar (fig. 4.8). By removing cotyledons and stem, the whole physiology of the root is disturbed. The sucking action of the stem does not exist any more, no upward transport is possible, root growth ceases and obviously the whole root system then starts to exude. This type of exudation is probably not selective and all the soluble root constituents are released. The action of the apparently inhibiting compound exuded by the growing root tip of intact pea seedlings (fig. 4.5) is eliminated by the stimulating compounds released from the whole root after removing cotyledons and stem.

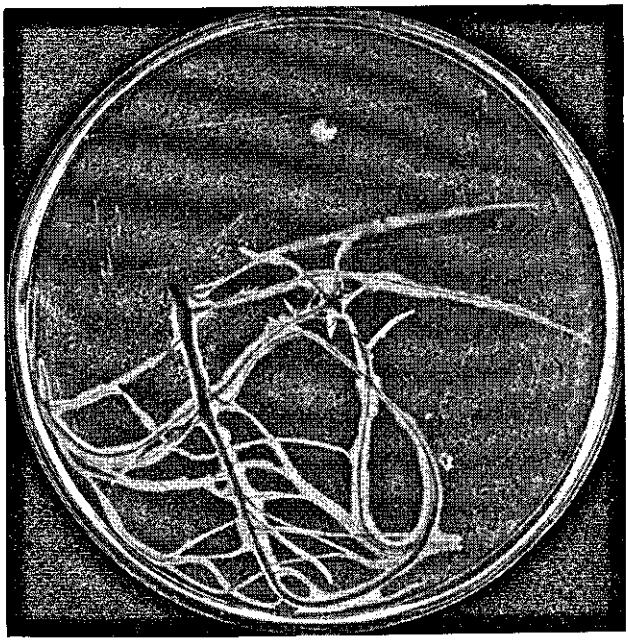


FIG. 4.7. Promotion of growth of *Rhizobium* by nitrogenous compounds exuded from roots of pea seedlings after removing cotyledons and stem. Seedlings grown on agar (containing mineral salts and manintol), mixed with a suspension of *Rhizobium leguminosarum*, strain PRE.

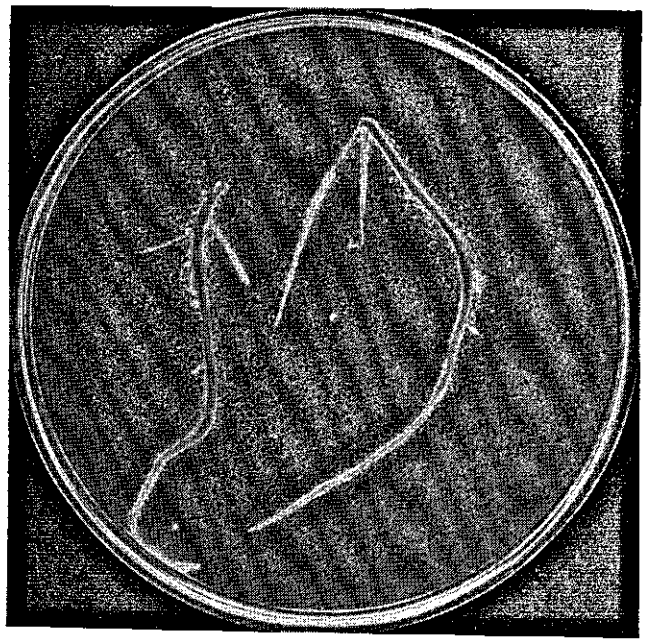


FIG. 4.8. Promotion of growth of *Rhizobium* by nitrogenous compounds exuded from sterile pea-seedling root segments. Seedlings grown under sterile conditions. After removing cotyledons and stem, roots placed on agar (containing mineral salts and mannitol), mixed with a suspension of *Rhizobium leguminosarum*, strain PRE.

From figures 4.7 and 4.8 it will be seen that there was some difference in the type of response of the bacteria to the exudates. When sterile separate roots had been placed on the *Rhizobium*-containing agar, the exudates diffused from the roots over some distance in the agar before being completely consumed by the bacteria, thus causing a rather broad zone of stimulation. When the roots had been grown in the inoculated agar, before being separated from cotyledons and stem, the root surface was covered with bacteria which immediately consumed the compounds released from the roots, as a result of which no broad stimulation zone was built up.

4.3.3.1.3. Inhibition of rhizobial growth close to the surface of roots of pea seedlings

Approximately 5 days after the inoculation of lateral roots of pea seedlings grown on sterile agar, a zone of bacterial growth was visible around those inoculated lateral roots which had been grown between the lower part of the agar and the bottom of the Petri dish. The zone of stimulation was located at some distance from the root (fig. 4.9). To explain this phenomenon, it may be assumed that after inoculation of the lateral roots, the bacteria have spread along these roots via the water film between root surface and agar. When the roots were growing along the bottom of the Petri dish, additional spreading of the bacteria was possible more remote from the root via the water film between the bottom of the Petri dish and the agar. As the zone of maximal bacterial growth was located at a certain distance from these roots, this may indicate the presence of

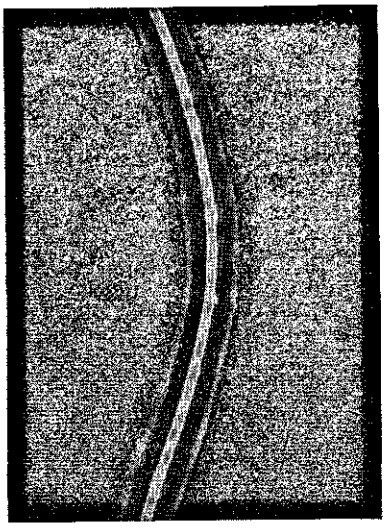


FIG. 4.9. Inhibition of rhizobial growth close to the surface of lateral roots of pea seedlings. Seedling grown on sterile agar (containing mineral salts and mannitol) for 10 days prior to the inoculation of lateral roots with *Rhizobium leguminosarum*, strain PRE.

a zone of inhibited bacterial growth close to these roots. The zone of promoted growth may be explained by assuming that the growth-stimulating substances diffused more readily through the agar than the inhibiting substance, or that the concentration of the inhibitor in the zone of stimulation had become too low to cause inhibition of bacterial growth. The zone of inhibition was clearly visible during several days but afterwards became less pronounced.

4.3.3.2. Roots grown in glass tubes

4.3.3.2.1. Bacterial growth in agar tubes without plants

Three days after mixing a suspension of *Rhizobium leguminosarum* and agar, a clearly visible zone of bacterial growth was observed located approximately 1.5 cm below the surface of the agar. The zone was much more pronounced when the agar was supplemented with yeast extract or glutamic acid. This phenomenon was due to the fact that the best growth of *Rhizobium* takes place under reduced oxygen pressure, an observation already made by ALLEN and BALDWIN (1930) and more recently by APEL and WEICHSEL (1968). According to ALLEN and BALDWIN, the position of this zone is dependent on the redox-potential of the medium and the oxygen requirements of the bacteria.

In the deeper parts of the tube, growth of the rhizobia was absent, indicating a shortage of oxygen. The anaerobic conditions in the deeper parts of the tube were made visible by the addition of methylene blue to the agar. When the agar was supplemented with yeast extract or with glutamic acid, the blue colour disappeared after a couple of hours owing to the reduction of methylene blue to the colourless leuco-methylene blue. Only in the upper two cm of the agar the methylene blue was kept in the oxidized form resulting from the diffusion of oxygen into the agar.

4.3.3.2.2. Promoted growth of rhizobia owing to the excretion of O_2 by plant roots

When pea seedlings were growing with their roots in the agar, no promotion of growth of *R. leguminosarum* was found near the roots, but such a promotion zone of bacterial growth was observed near the roots of the other plant species used, viz. black medick, red clover, white clover and green gram (fig. 4.10). The promotion of growth was found along the entire root system even when the roots had reached the deeper parts of the tube. This promotion of growth cannot only have been caused by the exudation of nitrogenous compounds, but must also have been due to an improved oxygen supply resulting from the release of oxygen from the roots.

To demonstrate the exudation of oxygen by roots, agar supplemented with methylene blue was used. When roots of several seedlings were grown in the decolourized agar, a zone having a blue colour became visible in the surroundings of the roots indicating that leucomethylene blue was oxydized to methylene blue. Pea roots had a very broad blue zone and sometimes the whole tube was coloured blue.

The phenomenon of exudation of oxygen by roots in media having a very low amount of oxygen was already observed by CANNON (1932), VAN RAALTE (1944) and by APEL and WEICHSEL (1968). The last workers concluded from their experiments that oxygen must be transported from the shoot to the root system. They used red clover grown in tap-water agar supplemented with glucose and methylene blue and inoculated with *Rhizobium trifolii*. When the blue zone was

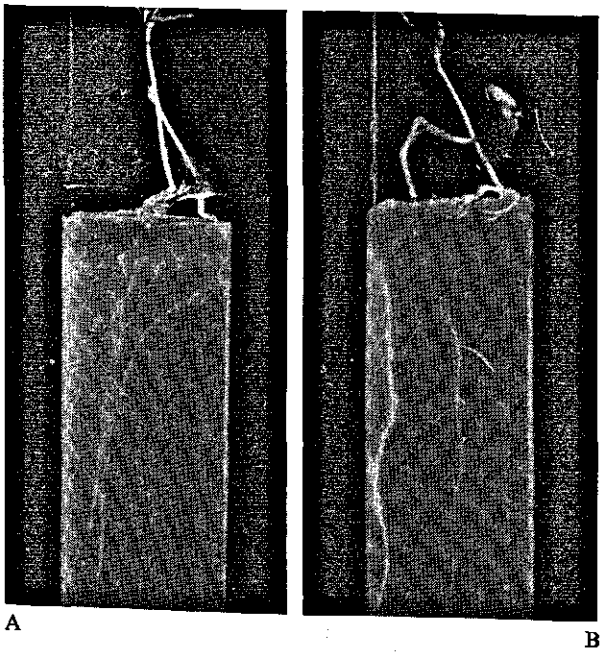
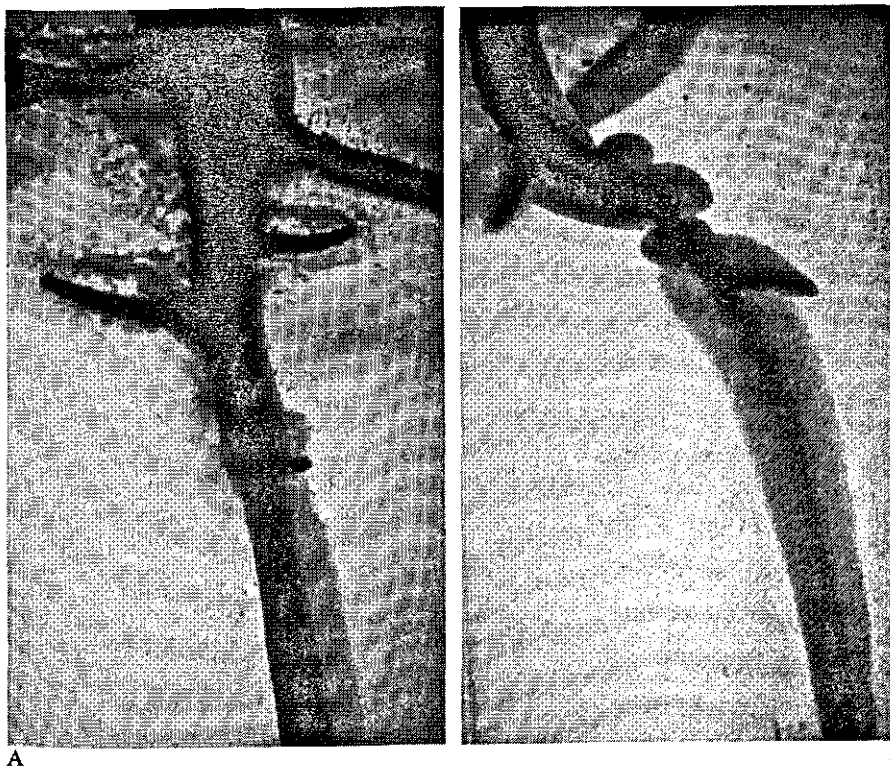


FIG 4.10. Promotion of growth of *Rhizobium* by root exudates of red clover (A) and black medick (B) growing in agar (containing mineral salts and mannitol), mixed with a suspension of *Rhizobium leguminosarum*, strain PRE (red clover) or strain PF_2 (black medick).

clearly visible around the roots, they removed most of the stem from the root system and poured paraffin-oil on top of the agar in such a way that the part of the stem left was fully covered. In less than three hours the agar surrounding the roots was decolourized. In a further experiment APEL and WEICHSEL (1968) removed the stem from the root system but now left the cut uncovered. No decolourization of the agar took place showing that oxygen could obviously enter also the root system via the cut end of the root.

According to VAN RAALTE (1940, 1944) and YIN *et al.* (1966) oxygen in the root tissue is transported via the intercellular spaces.

When in the present experiments with black medick, red clover, white clover and green gram the agar had been supplemented with yeast extract or glutamic acid, a much more intensive stimulation was observed in the surroundings of the roots than without these compounds (fig. 4.11). This was due to the fact that in the presence of yeast extract or glutamic acid the bacteria immediately used these compounds when oxygen was present, being independent of the nitrogen-containing exudates of the roots.



A B

FIG 4.11. Promotion of growth of *Rhizobium* in the vicinity of roots of green gram (A) and black medick (B) owing to the excretion of O_2. Roots grown in agar (containing mineral salts, mannitol and yeast extract), mixed with a suspension of *Rhizobium leguminosarum*, strain PRE (black medick) or strain PF_2 (green gram).

4.3.3.2.3. Inhibition of rhizobial growth by the roots of pea seedlings

When agar was supplemented with one of the nitrogenous compounds, pea plants also promoted the growth of *R. leguminosarum* in the vicinity of their roots, but in contrast to the other plant species used, the zone of promoted growth was not in direct contact with the root surface. An area of hardly visible growth was observed between the root surface and the zone of intensive stimulation (fig. 4.12). This area of inhibitory growth was clearly visible during a couple of days, before disappearing slowly.

From the results of the experiments described in this chapter, it can be concluded that the roots of young pea plants in addition to growth-stimulating substances (4.1, 4.2, 4.3) exude (an) inhibiting compound(s) (4.2, 4.3). This (these) compound(s) can temporarily prevent the growth of *Rhizobium leguminosarum* on or in the immediate vicinity of the roots.

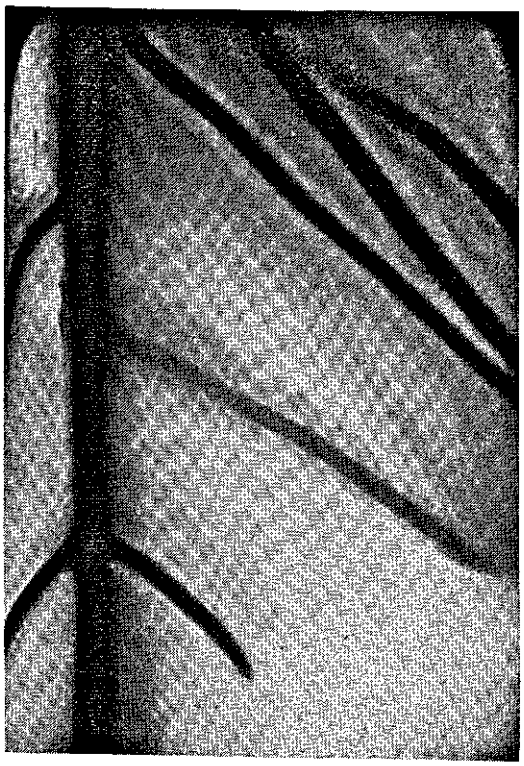


FIG. 4.12. Inhibition of rhizobial growth in the vicinity of roots of pea seedlings. Outside the zone of inhibition, a zone of promoted growth due to the excretion of O_2 by the pea roots is visible. A suspension of *Rhizobium leguminosarum*, strain PRE, was mixed with the agar containing mineral salts, mannitol and yeast extract.

5. A SYNTHETIC MEDIUM FOR THE GROWTH OF *RHIZOBIUM LEGUMINOSARUM*, STRAIN PRE

5.1. INTRODUCTION

In the previous chapter, studies on the behaviour of *Rhizobium leguminosarum* on and near the root surface of young pea plants have been recorded. In the following chapters (6 and 7), the exudation of nucleic-acid derivatives and of ninhydrin-positive compounds by roots of pea seedlings is described.

In order to investigate the exuded compounds for their action upon *Rhizobium* species, a synthetic growth medium was needed.

Rhizobium leguminosarum, strain PRE, which was most frequently used in the present experiments did not grow, in contrast to the other *Rhizobium leguminosarum* strains tested, on the synthetic *Rhizobium* media of NORRIS (1959) and of BERGERSEN (1961).

The purpose of this investigation was to prepare a synthetic medium suitable for growth of *Rhizobium leguminosarum*, strain PRE.

5.2. EXPERIMENTAL

The basal medium (B), derived from the media of NORRIS (1959) and of BERGERSEN (1961), had the following composition: glutamic acid, 1 g; mannitol, 5 g; $K_2HPO_4.3H_2O$, 1 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2.H_2O$, 40 mg; $FeCl_3.6H_2O$, 0.2 mg; $MnSO_4.H_2O$, 0.2 mg; $ZnSO_4.7H_2O$, 0.2 mg; $CuSo_4.5H_2O$, 0.02 mg; $CoCl_2.6H_2O$, 0.002 mg; H_3BO_3, 0.2 mg; $Na_2MoO_4.2H_2O$, 0.2 mg; biotin, 0.05 mg; thiamine, 0.1 mg; distilled water, 1000 ml. The pH of the medium was adjusted to 6.8.

In order to find the compound(s) required for the growth of *Rhizobium leguminosarum*, strain PRE, and not contained in the B medium, the basal medium was supplemented with groups of compounds which were added separately or in combination. The first group consisted of the following vitamins: nicotinic acid, p-aminobenzoic acid, pyridoxine, Ca-pantothenate, riboflavin, folic acid and Vit.B_{12} (Vit.B_{12} 2 μg/l, other vitamins 0.1 mg/l). The second group consisted of amino acids, added as vitamin-free casamino acids (500 mg/l). The third group was composed of purine and pyrimidine bases: adenine, guanine, uracil, cytosine and thymine (10 mg/l). In subsequent experiments separate compounds were added instead of whole groups. For comparison, yeast extract was supplied to the basal medium (200 mg/l).

All additional compounds were sterilized by filtration using a G.5 Seitz filter and added to the heat-sterilized basal medium in appropriate amounts.

The growth experiments were performed as described in chapter 2.

5.3. Results and discussion

5.3.1. *Effect of added sulfhydryl compounds*

No growth of *Rhizobium leguminosarum*, strain PRE, took place in the basal medium B. However, good growth occurred when the basal medium had been supplemented with yeast extract or with casamino acids in combination with purine and pyrimidine bases; vitamins had no effect. In further experiments the vitamins were omitted and individual amino acids were added (100 mg/l). Of the 20 amino acids (L-forms) tested, 4 stimulated growth, viz. leucine, methionine, cysteine and cystine. When concentrations of 10 mg/l of these amino acids were used, equal growth of *R. leguminosarum*, strain PRE occurred with methionine, cysteine and cystine, but leucine stimulated growth only slightly.

In subsequent experiments it was found that methionine gave maximal growth at a concentration of approximately 7 to 10 mg/l whereas leucine had to be added in concentrations of about 110 mg/l to achieve a similar result. Paper-chromatographic analyses of the leucine used revealed that this compound was contaminated with methionine which was responsible for the growth stimulation found with large amounts of leucine.

Further experiments showed that it was possible to replace methionine, cysteine or cystine by glutathione (20 mg/l), Na-thioglycollate (10 mg/l) or even by Na_2S (50 mg/l). The sulphide must be added in relatively large amounts because H_2S disappeared from the medium during the experiments.

From these results it will be clear that *Rhizobium leguminosarum*, strain PRE, is unable to use sulphate as a sulphur source, so that sulfhydryl compounds must be added to medium B to achieve growth. This is in contrast to most other bacteria which are able to utilize sulphate as the S-source. In both instances the sulfhydryl group is coupled with serine to give cysteine (Thompson, 1967).

5.3.2. *Influence of purines and pyrimidines*

During the foregoing experiments, purine and pyrimidine bases (adenine, guanine, cytosine, uracil, thymine) had always been added to the nutrient medium. Omission of this group of compounds resulted in a sharp decrease of bacterial growth. In a subsequent series of experiments, the basal medium, including methionine (10 mg/l), was supplemented with individual purine or pyrimidime bases (10 mg/l). Uracil or cytosine were found to be responsible for the stimulated growth. Adenine and guanine had no effect while thymine at a concentration of 10 mg/l completely inhibited bacterial growth. This inhibition disappeared when uracil or cytosine were added in at least equal amounts as thymine.

Uracil and cytosine are undoubtedly used by *R. leguminosarum*, strain PRE, for the synthesis of pyrimidine nucleotides which normally are synthesized following a different pathway (Magasanik, 1962). In this strain the normal pathway of pyrimidine-nucleotide synthesis apparently is blocked. The inhibition caused by thymine is not clear.

Recently, Scherrer and Dénarié (1971) described pyrimidine-dependent

mutants of an effective strain of *Rhizobium meliloti*, which were able to grow on media supplemented with uracil or cytosine but not on media supplemented with thymine. The authors found that these mutants as well as purine-dependent mutants derived from effective strains of *Rhizobium meliloti* were ineffective apparently due to an inadequate supply of pyrimidines and purines by the host plant.

In the present investigation it was found that uracil or cytosine in some cases slightly increased the growth rate of other strains of *Rhizobium leguminosarum*. Thymine always caused a delay during the first days of bacterial growth. None of these compounds, however, showed any effect on the ultimate numbers of bacteria.

The results of the experiments described in 5.3.1 and 5.3.2 are compiled in table 5.1. Growth differences of *Rhizobium leguminosarum*, strain PRE, in the basal medium (B) and in the basal medium supplemented with uracil and methionine (BUM) are shown in fig. 5.1. For comparison, growth of *Rhizobium leguminosarum*, strain PF_2, in both media is also shown.

TABLE 5.1. Growth of *Rhizobium leguminosarum*, strain PRE, in basal medium B and in medium B supplemented with different compounds.

B supplemented with	—	Methionine	Cysteine	Gluta-thione	Na-thio-glycollate	Na_2S	Yeast extract
—	90	63	62	60	63	64	19
Uracil	90	20	21	19	20	22	18
Cytosine	89	20	22	20	20	21	20
Thymine	96	98	97	95	97	98	19
Uracil + Thymine	90	21	20	–	–	–	–
Adenine + Guanine	89	59	62	–	–	–	–

Concentration of compounds added: methionine, 10 mg/l; cysteine, 10 mg/l; glutathione, 20 mg/l; Na-thioglycollate, 10 mg/l; $Na_2S.9H_2O$, 50 mg/l; yeast extract, 200 mg/l; purine and pyrimidine bases, 10 mg/l.
Growth measured turbidimetrically as percentage transmittance. Transmittance B: 100. Transmittance values of 20 are comparable with 5.10^8–10^9 bacteria per ml.

All the *Rhizobium* strains tested (70 strains belonging to 5 different cross-inoculation groups) showed excellent growth on the synthetic medium prepared for *Rhizobium leguminosarum*, strain PRE, and therefore this medium (BUM), by omitting certain constituents, was used to investigate the action of specific root exudates on growth of *Rhizobium* spp. *in vitro*.

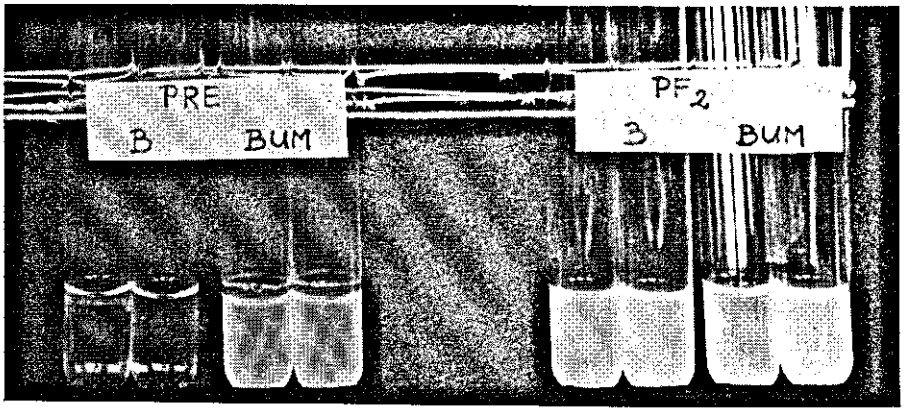
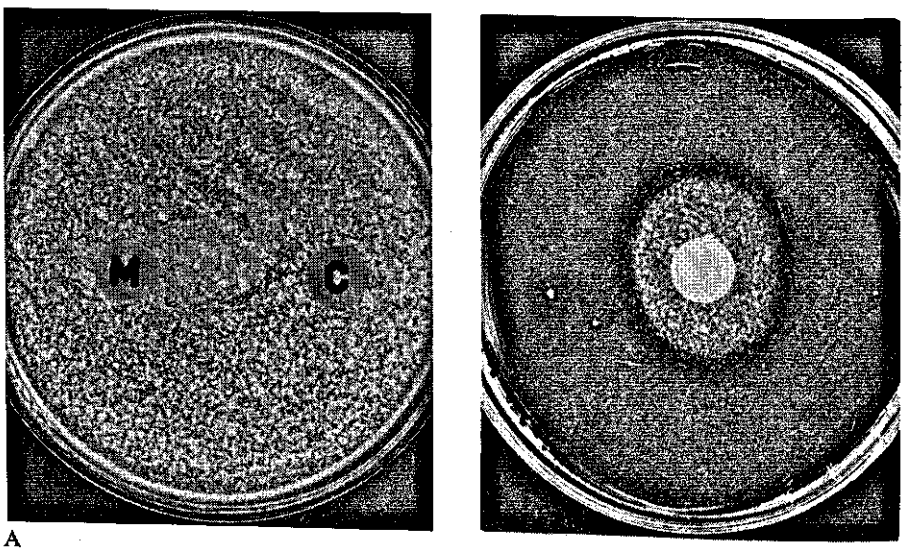


FIG. 5.1. Growth of *Rhizobium leguminosarum*, strain PRE and strain PF_2 in basal medium (B) and in basal medium supplemented with uracil and methionine (BUM).

5.4. GROWTH REQUIREMENT EXPERIMENTS WITH SOLID MEDIA

Rhizobium leguminosarum, strain PRE, was found to form normal colonies on plates containing basal medium (B) supplemented with uracil and solidified with Difco agar. No sulfhydryl compounds had to be added. Obviously, the agar provided reduced sulphur-containing compounds and most likely also other



A B

FIG. 5.2. Growth of *Rhizobium leguminosarum*, strain PRE, on medium B supplemented with uracil and solidified with agar (A) or silica gel (B).
M: methionine, C: cysteine. Central disc of B contained methionine.

growth-effecting substances. Therefore, agar was not used as solidifying agent in growth requirement experiments. When solid media were needed, silica gel was used instead of agar (WIERINGA, 1966).

When *Rhizobium leguminosarum*, strain PRE, was inoculated on the surface of silica-gel plates containing basal medium and uracil but no methionine, growth of this strain was absent. When a filter-paper disc, first dipped in a solution containing methionine, was placed on the surface of the silica gel, growth took place in the vicinity of the disc (fig. 5.2). When instead of methionine other reduced sulphur-containing compounds were used (glutathione, Na-thioglycollate) similar results were obtained.

6. THE EXUDATION OF ULTRA-VIOLET LIGHT-ABSORBING COMPOUNDS BY PEA-SEEDLING ROOTS

6.1. INTRODUCTION

In studying root exudates from pea and oat plants, ROVIRA (1956) found two ultra-violet (U-V)-absorbing compounds exuded by the roots of pea plants growing in nutrient solution. No attempts were made to identify these compounds. Several other workers have reported the release of nucleic-acid derivatives from young pea roots (LUNDEGÅRDH and STENLID, 1944; STENLID, 1947; FRIES and FORSMAN, 1951). These studies, however, were performed with excised roots and in this case most of the compounds are liberated from the cut end of the roots. FRIES and FORSMAN (1951) detected, by means of mutants of *Ophiostoma multiannulatum*, adenine, adenosine, guanine, guanosine and uridine or cytidine in exudates of excised pea-seedling roots while high-molecular phosphorylated U-V-absorbing compounds were also present. BROWN (1962), BROWN and SILVER (1965) and BROWN and MANGAT (1968) have carried out a considerable amount of work in identifying nucleic-acid derivatives in mature pea seeds and in pea seedlings, but no exudates were studied.

The purpose of this investigation was to obtain information about the exudation of U-V-absorbing compounds by intact roots of pea seedlings.

6.2. EXPERIMENTAL

To collect U-V-absorbing compounds exuded by pea roots, seedlings (root length 1–1.5 cm) were grown as described in 2.4.1. The exudates were treated as described in 2.4.6. The separation and identification procedures for the U-V-absorbing compounds can be found in 2.4.9. To obtain permanent records of the paper chromatograms, contact prints (MARKHAM, 1956) can be made. The paper chromatogram is placed on a sheet of Reflex-Contact-Document Paper and exposed to ultra-violet light. The U-V-absorbing spots on the filter paper prevent the passage of U-V light. After developing the Document Paper, white spots corresponding with the U-V-absorbing spots on the filter paper are visible in a dark field.

6.3. RESULTS AND DISCUSSION

6.3.1. *The separation of a standard mixture of nucleic-acid derivatives*

The separation of a mixture of purines (adenine, guanine, xanthine), pyrimidines, (uracil, cytosine, thymine) and nucleosides (adenosine, guanosine, uridine, cytidine, thymidine) on a cation-exchange resin (Zeocarb 225, H^+) is shown in fig. 6.1. Of each compound 100 μg was used. As can be seen, uracil,

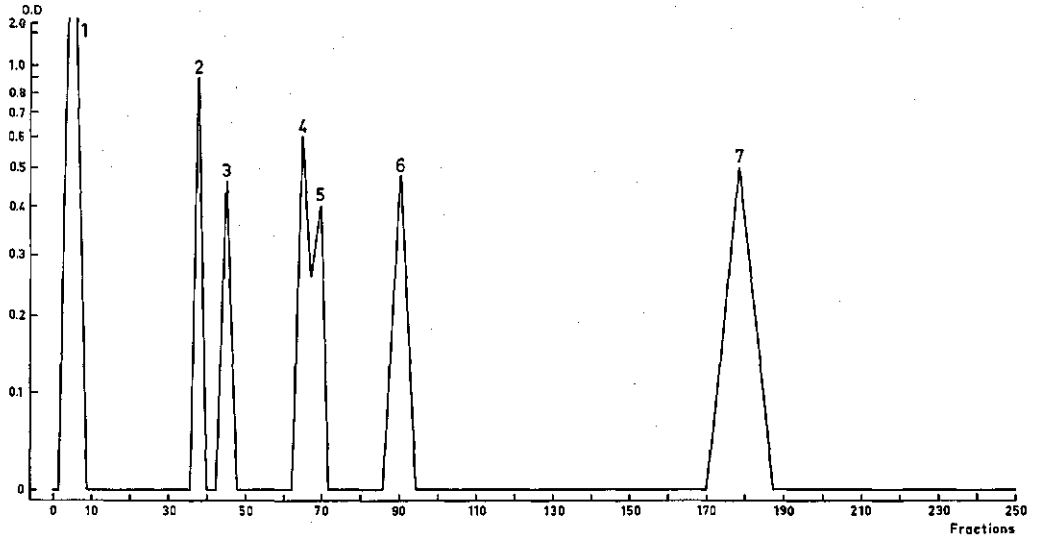


FIG. 6.1. Separation of a mixture of purines (adenine, guanine, xanthine), pyrimidines (uracil, cytosine, thymine) and nucleosides (adenosine, guanosine, uridine, cytidine, thymidine) on a cation-exchange resin (Zeocarb 225, H^+). Elution has been performed with HCl (gradient: 0.25 N – 2.5 N). Absorbancy of each of the (6 ml) fractions was determined at 260 mμ.
1: uracil + uridine + thymine + thymidine, 2: xanthine, 3: cytidine, 4: cytosine, 5: guanosine, 6: guanine, 7: adenine.

uridine, thymine and thymidine are eluted together. Adenosine is not found as this compound is hydrolysed to adenine during the elution.

Uracil, uridine, thymine and thymidine can easily be separated by paper chromatography (cf. 2.4.9). When using n-butanol-H_2O as the solvent, uracil and uridine are separated from thymine and thymidine, which are sticking together in a third spot. After eluting this spot, thymine can be separated from thymidine by using n-butanol-H_3BO_3 (86–14) as the solvent. In this system nucleosides do not move. Fig. 6.2 shows contact prints of paper chromatograms obtained by separation of a mixture of uracil, uridine, thymine and thymidine.

6.3.2. *Ultra-violet-absorbing compounds found in exudates of pea-seedling roots*

In this experiment, use was made of root exudates of 54 pea plants, grown in water during two days. U-V absorption by fractions obtained from these exudates is shown in fig. 6.3. Apparently only two U-V-absorbing compounds were present, which is in agreement with the findings of ROVIRA (1956). Attempts to identify these compounds have only partly been successful (cf. 6.3.4). Therefore, the U-V-absorbing compound appearing first was mentioned UV 1 and the second UV 2. To give some information about the amounts present, optical-density (O.D.) values for both compounds are presented of fractions showing maximal absorption at 260 mμ (UV 1 in fraction 5, UV 2 in fraction 31).

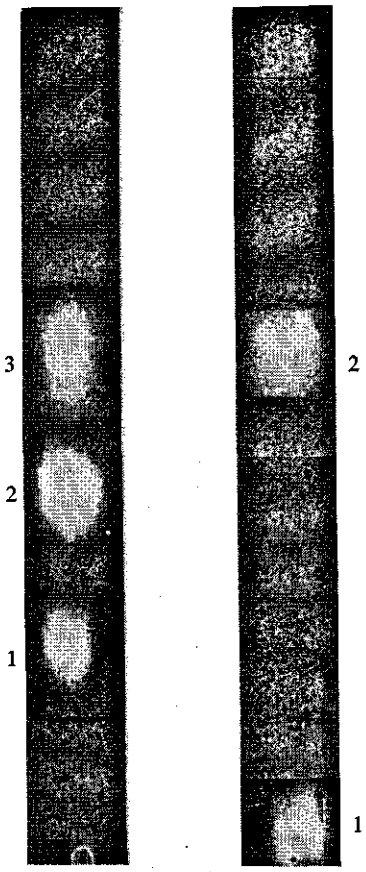


FIG. 6.2. Paper-chromatographic separation of a mixture of uracil, uridine, thymine and thymidine. A. contact print of a chromatogram obtained after separation of a mixture of uracil, uridine, thymine and thymidine. Solvent n-butanol-H_2O (86-14). 1: uridine, 2: uracil, 3: thymine + thymidine. B. contact print of a chromatogram obtained after separation of a mixture of thymine and thymidine. Solvent n-butanol-H_3BO_3 (86–14). 1: thymidine, 2: thymine.

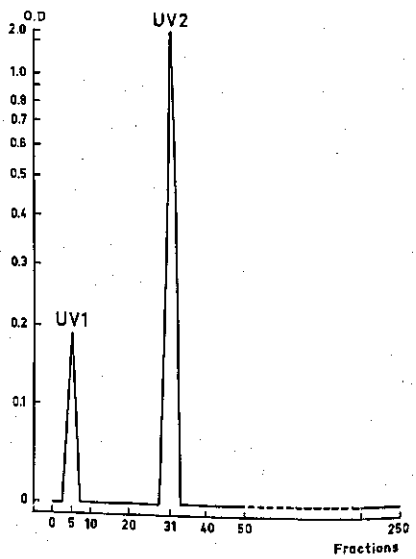


FIG. 6.3. Ultra-violet-absorbing compounds exuded by roots of pea seedlings. Exudate separated on a cation-exchange resin (Zeocarb 225, H^+). Elution had been performed with HCl (gradient: 0.25 N – 2.5 N). Absorbancy of each of the (6 ml) fractions was determined at 260 mμ.

In table 6.1 the O.D. values of compound UV 1 are given as related with time of exudation and presence of lateral roots. As can be seen from these data, exudation of UV 1 considerably increased after formation of lateral roots.

With UV 2 the results initially were highly variable. This was found to be due to the fact that seedlings with their root tips only being dipped in water at the start of an experiment, exuded much less compound UV 2 than those being immersed more deeply. This effect was not found with UV 1 (see table 6.2).

TABLE 6.1. Exudation of U-V-absorbing compound UV 1 by roots of pea seedlings°.

Time of exudation (days)	Lateral roots present	O.D. of fractions showing max. absorption
1	–	0.10
2	–	0.23
4	+	0.70
5	++	1.20
6°°	+++	1.70
10°°	+++++	>>2

° Values of typical experiments, 54 seedlings used.
°° 36 seedlings used.
+ Few lateral roots.
+++++ Many lateral roots present.

TABLE 6.2. Exudation of U-V-absorbing compounds UV 1 and UV 2 by roots of pea seedlings under different conditions°.

Period of exudation* (days)	O.D. of fractions with max. absorption	
	Root tips only in solution	
	UV 1	UV 2
1	0.10	0.38
2	0.23	0.92
4	0.70	2.00
	Root tips + older parts in solution	
	UV 1	UV 2
1	0.11	1.45
2	0.29	1.68

° Values of typical experiments, 54 seedlings used.
* Lateral roots absent except with roots exuding 4 days which contained a few lateral roots.

6.3.3. *Ultra-violet-absorbing compounds* (UV 1 *and* UV 2) *adhering to the root surface and present in the outer-cell layers of pea-seedling roots*

To explain the high amounts of UV 2 released by roots immersed relatively deeply in water, pea seedlings with roots of approximately 4 cm, but having no lateral roots were employed. Prior to use, the seedlings had been grown on water

agar covered with a sheet of Whatman nr. 1 filter paper. Hereafter, the roots of these seedlings (25 per replicate) were completely immersed in sterile water for two hours (cf. 2.4.3.1). In this period, hardly any root growth took place. Hereafter, the seedlings were placed in the same way in fresh water for a second period of two hours. The U-V-absorbing compounds released by the roots under these circumstances were estimated. In addition, root scrapings (cf. 2.4.3.2) of seedlings, grown on water agar were analysed for these compounds (see table 6.3).

Before considering these data, a few remarks should be made. When compounds are exuded by roots growing in moist air, they can only accumulate at the surface of the root itself. After transferring the roots to a liquid medium, it is likely that the adhering compounds leave the root surface. In addition epidermal cells, in particular root hairs, may be damaged by this sudden wetting, as was shown by NICOLAI (1929).

TABLE 6.3. U-V-absorbing compounds released by 25 roots of pea seedlings after transferring from humid air to water.

		Roots in water 0–2 hours	Roots in water 2–4 hours	Root scrapings°
O.D. of fractions with max. absorption	UV 1	0.13	0.01	0.05
	UV 2	1.58	0.04	1.83

° 5 roots used.

Table 6.3 shows that roots of pea seedlings grown under humid conditions and then placed in water for a couple of hours, release relatively small amounts of compound UV 1. This means that this compound was present only in small amounts on the root surface and in the epidermal cells of the seedling root. This was confirmed by experiments in which root scrapings were analysed. When pressure juices of whole seedling roots were studied, UV1 was also found in relatively small amounts. Clearly higher concentrations were found in pressure juices of root tips (2 mm sections).

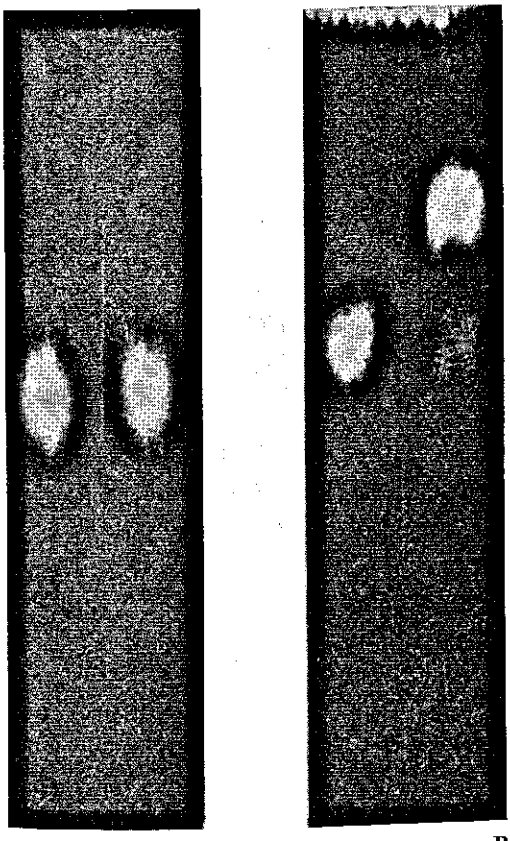
It looks as if compound UV 1 is synthesized in the root-tip region, possibly as an intermediate in nucleic-acid metabolism; overproduction of this compound is exuded. Lateral roots probably behave as the main root, because after formation of lateral roots the amount of UV 1 sharply increased (table 6.1).

When considering compound UV 2, it can be seen from table 6.3 that in contrast with compound UV 1, UV 2 is present in relatively large amounts in root scrapings. In root pressure juices, UV 2 is also found in high quantities. Tables 6.2 and 6.3 show that relatively large amounts of compound UV 2 are found after placing roots, grown under humid conditions, in water. Part of this amount probably is released from damaged root cells, while the rest must have been present on the root surface.

6.3.4. *Attempts made to identify compounds* UV 1 *and* UV 2

The fractions containing compound UV 1 were pooled, treated as described in 2.4.9, whereupon contact prints of the chromatograms were made. When n-butanol-H_2O was used as the solvent, only one spot with a Rf. value similar to that of uracil (Rf. 0.36) appeared (fig. 6.4). When iso-propanol-HCl-H_2O was used, two spots appeared. The weak spot was comparable to that of uracil (Rf. 0.65), while the second much more intensive spot had a Rf. value of 0.84. The compound responsible for the weak spot had the same absorption spectrum as authentic uracil. Maximal absorption occurred at 259.5 mμ and a minimum was found at 227.5 mμ. The second spot gave a maximum at 278 mμ and a minimum at 230 mμ. When the compound giving the latter spot after elution was hydrolysed (2.4.7.2) and afterwards chromatographed, a chromatogram similar to that of uracil was obtained.

When the root exudate was hydrolysed and then separated on the resin, again two peaks were found. The first peak appeared in the same fractions as with unhydrolysed exudate, but maximal absorption of the second peak was now



A B

FIG. 6.4. Paper-chromatograms of U-V-absorbing compound UV 1.
A. contact print of a chromatogram showing UV 1 (right) and uracil (left). Solvent n-butanol-H_2O (86–14).
B. contact print of a chromatogram showing UV 1 (right, two spots) and uracil (left). Solvent iso-propanol-HCl-H_2O (170–44–36).

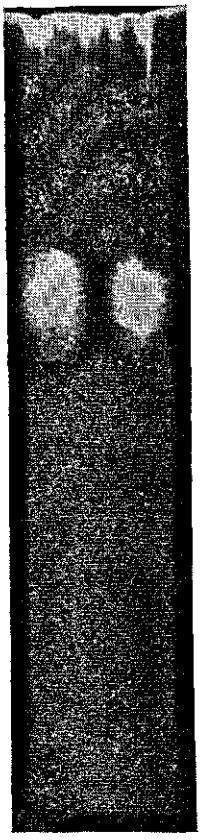


FIG. 6.5. Contact print of a chromatogram of hydrolysed U-V-absorbing compound UV 1 (hydrolysed root exudate) and uracil (left). Solvent iso-propanol-HCl-H_2O (170–44–36).

found in fraction 27 instead of in fraction 31. When the compound giving the first peak was chromatographed, using iso-propanol-HCl-H_2O as the solvent, only one spot, apparently being uracil, appeared (fig. 6.5). From these results it can be concluded, that compound UV 1 consisted of uracil and a derivative of uracil, which after hydrolysis gives uracil.

Compound UV 2 always gave one spot on the chromatograms, but so far this compound has not been identified. In the following chapter (7), in which a report is given on ninhydrin-positive compounds, exuded by pea roots, more information about this compound will be presented.

7. NINHYDRIN-POSITIVE COMPOUNDS IN PEA SEEDLINGS AND THE EXUDATION OF THESE COMPOUNDS BY PEA-SEEDLING ROOTS

7.1. INTRODUCTION

Several reports are dealing with the exudation of amino acids by roots of pea plants. A comparison between the amino acids present in the root tissue and those exuded has only rarely been made. FORSMAN (1955) found amino acids to be released by pea-root tips suspended in distilled water. ROVIRA (1956) found exudation of 27 amino compounds after growing pea plants in nutrient solution. Of these compounds, 11 could not be identified. Homoserine and threonine were the predominant amino acids present. BOULTER, JEREMY and WILDING (1966) studied the exudation by roots of sterile pea plants growing in sand and in culture solution. They found up to a 7 times increased release of certain amino acids in quartz sand as compared with liquid medium. In culture solution, glutamic acid was exuded in the greatest amount, followed by aspartic acid and homoserine. In quartz sand, however, homoserine was the predominant amino acid exuded, followed by aspartic acid and glutamic acid. By using a Technicon amino-acid analyser, these authors detected 29 ninhydrin-positive compounds (n.p.c.) of which 5 were unknown. AYERS and THORNTON (1968) found aspartic acid and serine to be the dominant amino acids exuded by roots of pea plants in culture solution. No homoserine was found by these authors in liquid media, although in sand culture homoserine was the principal amino acid exuded. Changing the root atmosphere by using different gas mixtures affected the exudation of the amino acids. The results of AYERS and THORNTON were based upon the intensities of spots on one-dimensional paper chromatograms which can be regarded as semi-quantitative. SHERROD and DOMSCH (1970) compared the exudation by roots of uninoculated pea plants growing in culture solution with the exudation by roots inoculated with *Gliocladium catenulatum*, a fungus causing severe root necrosis. Employing an amino-acid analyser, 21 amino acids were found to be exuded by healthy plants, the predominant one being homoserine, followed by serine and aspartic acid. In exudates of necrotic roots several amino acids which were present in exudates of healthy plants were lacking or were present in considerably reduced amounts. These authors concluded that the fungus preferentially utilized certain amino acids.

The results of the above-mentioned investigations show a certain disagreement which is probably due to differences in growth conditions of the pea plants and to different techniques of identification of the amino acids. However, it is clear that homoserine must be regarded as the principal amino acid exuded by pea roots while according to several authors aspartic acid would also be a major constituent.

BOULTER *et al.*, (1968) and AYERS and THORNTON (1968) compared the free

amino acids of root extracts with those of root exudates. The former authors found a poor correlation between the amino-acid composition of extracts and that of exudates but AYERS and THORNTON observed a better agreement. These authors compared root exudates with the free amino acids of crushed root tips. Their observation is in agreement with the generally accepted idea that the root-tip is the zone of major exudation.

VIRTANEN *et al.* (1953) first identified homoserine in germinating peas; in ungeeminated seeds it was found to be absent. The presence of homoserine is specific of the genus *Pisum*, little or no homoserine being found in other plants.

The main purpose of the investigations described in this chapter was: a. to study the changes in free ninhydrin-positive compounds (n.p.c.) in pea plants during and after germination as affected by different environmental and nutritional conditions, b. to study the exudation of n.p.c. (composition as well as mechanism) by roots of pea seedlings.

7.2. THE SEPARATION OF A MIXTURE OF KNOWN NINHYDRIN-POSITIVE COMPOUNDS

7.2.1. *Experimental*

The separation of a mixture of 22 amino acids, 2 amides, NH_3 and glutathione was performed on a Biocal 200 automatic amino-acid analyser as described in 2.4.8. Of each compound 0.25 μmole was used. Buffers, temperatures and elution rates used for the separation of these compounds are given in table 7.1.

TABLE 7.1. Buffers, temperatures and elution rates used for the separation of ninhydrin-positive compounds in the amino-acid analyser.

	Buffer*	Temperature	Elution rate
Acid and	A (0–140)°	30°C (0–155)°	60°°
neutral n.p.c.	B (140–320)	55°C (155–320)	60
Basic n.p.c.	C (0–175)	55°C (0–175)	100

* Composition of buffers see table 2.1.
° Time in minutes.
°° ml/hour.

7.2.2. *Results*

The separation of acid and neutral n.p.c. is shown in fig. 7.1. Fig. 7.2 gives the results of the chromatographic estimation of the basic n.p.c. and ammonia. Asparagine and glutamine were not separated when citrate buffers were used. As can be seen, the separation of glutathione and aspartic acid was not satisfactory. By changing the pH of buffer A, complete separation of the latter compounds was achieved (see under 7.3) but now separation of other compounds was less favourable.

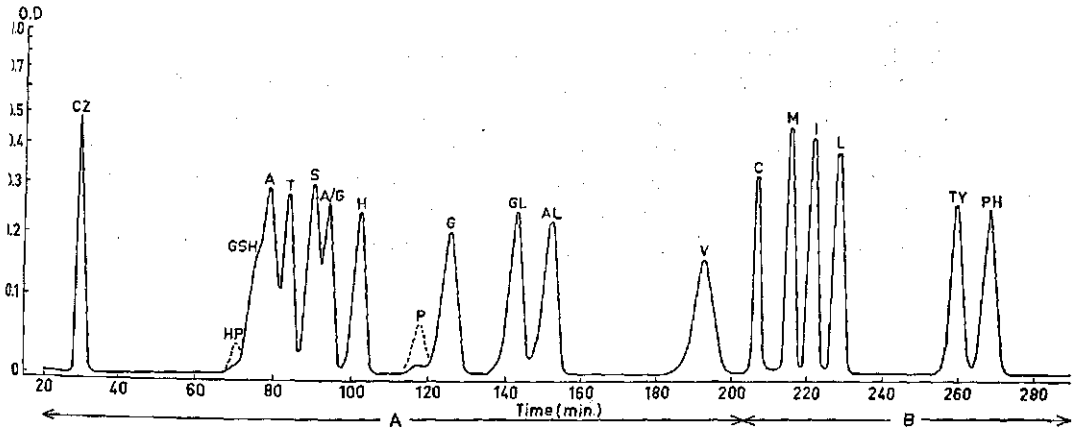


FIG. 7.1. Separation of a standard mixture of acid and neutral amino acids, amides and glutathione (0.25 μmole of each compound) on a column containing 52 × 0.9 cm Biorad A_6 spherical ion-exchange resin. CZ, cysteic acid; HP, hydroxy-proline; GSH, glutathione; A, aspartic acid; T, threonine; S, serine; A/G, asparagine + glutamine; H, homoserine; P, proline; G, glutamic acid; GL, glycine; AL, alanine; V, valine; C, cystine; M, methionine; I, isoleucine; L, leucine; TY, tyrosine; PH, phenylalanine.

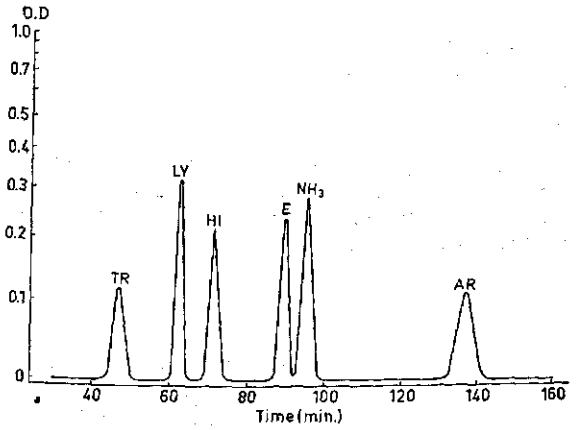


FIG. 7.2. Separation of a standard mixture of basic amino acids and ammonia (0.25 μmole of each compound) on a column containing 22 × 0.9 cm Biorad A_5 spherical ion-exchange resin. TR, tryptophan; LY, lysine; HI, histidine; E, ethanolamine; NH_3, ammonia; AR, arginine.

7.3. FREE NINHYDRIN-POSITIVE COMPOUNDS IN THE ROOTS OF PEA SEEDLINGS

7.3.1. *Experimental*

The ninhydrin-positive compounds (n.p.c.) present in extracts (2.4.4) of pea-seedling roots (seedlings grown on agar, root length approximately 3.5 cm, no lateral roots present) were separated in the amino-acid analyser employing buffers, temperatures and elution rates similar to those used for analysing the standard mixture.

7.3.2. *Results and discussion*

The chromatogram of the acid and neutral n.p.c. of the root extracts is shown in fig. 7.3 and that of the basic n.p.c. in fig. 7.4. When comparing these chromatograms with those of the standard mixture, it will be seen that by far the most important n.p.c. of the root extract was homoserine. This is in agreement with the findings of VIRTANEN *et al.* (1953), AYERS and THORNTON (1968) and PATE (1968). The small peak on the chromatogram appearing just before homoserine is asparagine or glutamine or, more plausible, a mixture of both compounds.

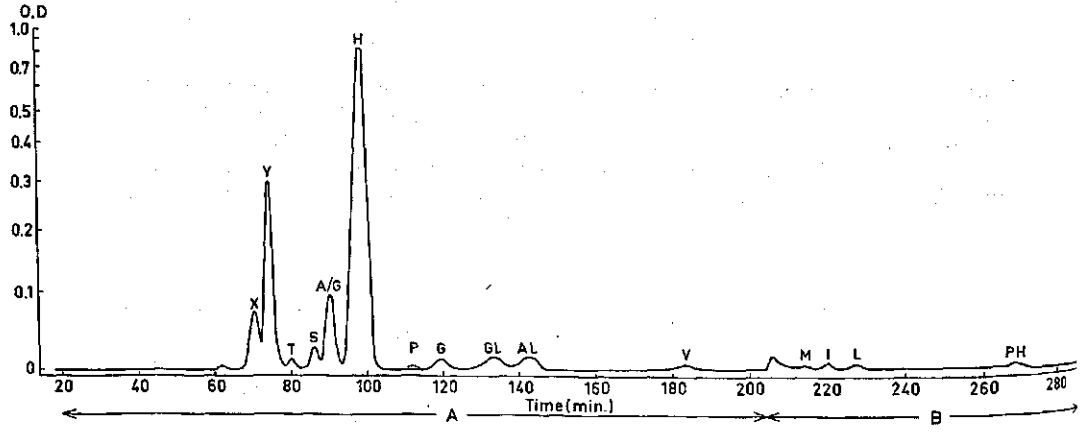


FIG. 7.3. Acid and neutral ninhydrin-positive compounds (n.p.c.) present in extracts of pea-seedling roots. X, unknown; Y, unknown; T, threonine; S, serine; A/G, asparagine + glutamine; H, homoserine; P, proline; G, glutamic acid; GL, glycine; AL, alanine; V, valine; M, methionine; I, isoleucine; L, leucine; PH, phenylalanine.

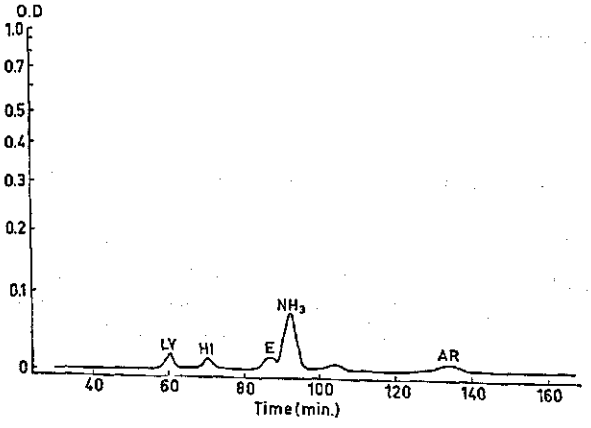


FIG 7.4. Basic ninhydrin-positive compounds (n.p.c.) present in extracts of pea-seedling roots. LY, lysine; HI, histidine; E, ethanolamine; NH_3, ammonia; AR, arginine.

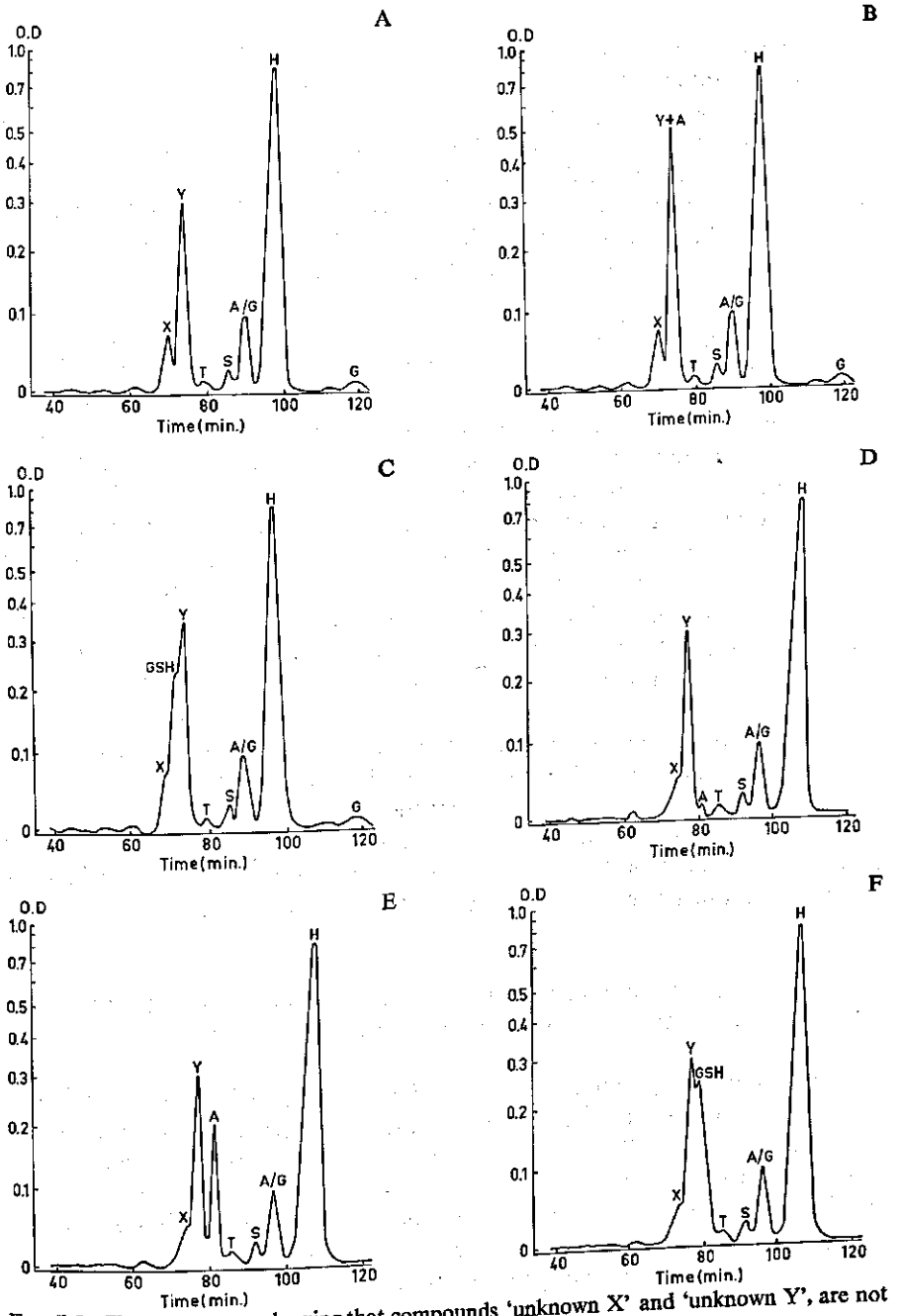


FIG. 7.5. Chromatograms showing that compounds 'unknown X' and 'unknown Y', are not identical with aspartic acid or glutathione.
A. root extract, buffer A, pH 3.25; B. root extract supplemented with aspartic acid, buffer A, pH 3,25; D. root extract, pH 3.25; C. root extract supplemented with glutathione, buffer A, pH 3.18; F. root buffer A, pH 3.18; E. root extract supplemented with aspartic acid, buffer A, pH 3.18.
extract supplemented with glutathione, buffer A, pH 3.18.
X, unknown; Y, unknown; GSH, glutathione, A, aspartic acid; T, threonine; S, serine; A/G, asparagine + glutamine; H, homoserine; G. glutamic acid.

The first two peaks on the chromatogram shown in fig. 7.3 correspond with those of glutathione and aspartic acid on the chromatogram of the standard mixture. This would suggest that these peaks represent glutathione and aspartic acid. However, a more detailed investigation of these peaks by the present author has shown that this hypothesis is not correct. By adding pure glutathione and aspartic acid to root extracts or root exudates and by changing the pH of buffer A, it was clearly demonstrated that neither glutathione nor aspartic acid corresponded with one of these peaks (fig. 7.5 A-F).

It is obvious that the data found in the literature concerning the presence of glutathione and aspartic acid in roots of pea plants and the exudation of these amino compounds by the roots must be regarded with doubt. Aspartic acid has been stated by several investigators to occur in relatively large amounts in pea-root exudates as well as in pea-root extracts (see section 7.1). BOULTER *et al.* (1966), using an automatic amino-acid analyser, concluded that aspartic acid would be the free amino acid which occurred in the largest amount in roots of 14-days-old pea seedlings. In root exudates this amino acid would occur as the second largest amino acid. On the other hand, PATE (1968), investigating the nutritional system of the field pea (*Pisum arvense* L.) with the aid of an amino-acid analyser concluded from his chromatograms that aspartic acid occurred only in minor quantities in root extracts but glutathione would occur in rather large amounts. The presence of this compound was not recorded by earlier investigators.

In view of the results of the present investigation concerning the two first-appearing peaks, it is probable that the 'aspartic-acid peak' and the 'glutathione peak' recorded in the literature represent the two compounds found in this investigation. As the identities of these compounds were unknown, they were indicated as 'unknown X' and 'unknown Y'. Results of attempts to identify these compounds are presented in section 7.7 of this chapter.

7.4. FREE NINHYDRIN-POSITIVE COMPOUNDS PRESENT IN ROOTS OF SEEDLINGS OF SOME OTHER LEGUMINOUS PLANTS

As homoserine is peculiar to the genus *Pisum*, the question arose whether in other leguminous plants specific n.p.c. would also be present. Therefore, n.p.c. in root extracts of seedlings of the following arbitrarily selected legumes were studied: birdsfoot trefoil (*Lotus corniculatus*), red clover (*Trifolium pratense*), soyabean (*Glycine max*), cowpea (*Vigna sinensis*), green gram (*Phaseolus radiatus*), garden bean (*Phaseolus vulgaris*), broad bean (*Vicia faba*), peanut (*Arachus hypogaea*) and sweet pea (*Lathyrus latifolius*).

The acid and neutral n.p.c. present in the respective root extracts are shown in fig. 7.6 A-I. Chromatograms showing the basic n.p.c. are omitted because of the relatively low quantities present and the slight differences found. It will be seen that, with the exception of *Lathyrus*, all other legumes tested contained the amides glutamine and asparagine as the predominant ninhydrin-positive com-

pounds. Aspartic acid was always present in only small quantities, but homoserine was absent, except in *Lathyrus*. The close relationship between *Pisum* and *Lathyrus* is clear. Homoserine and 'unknown Y' are both present in *Lathyrus*. In addition a different (not identified) n.p.c. is present on the chromatogram of the latter between glutamic acid and glycine.

In general, it can be concluded that the ratios of the various n.p.c. in the seedling roots of different leguminous plants are different while in some legumes also specific (not identified) n.p.c. occur (soyabean, peanut, broad bean, sweet pea). Consequently it is acceptable that the exudation patterns of these plant species are also different so that different rhizosphere populations may be built up.

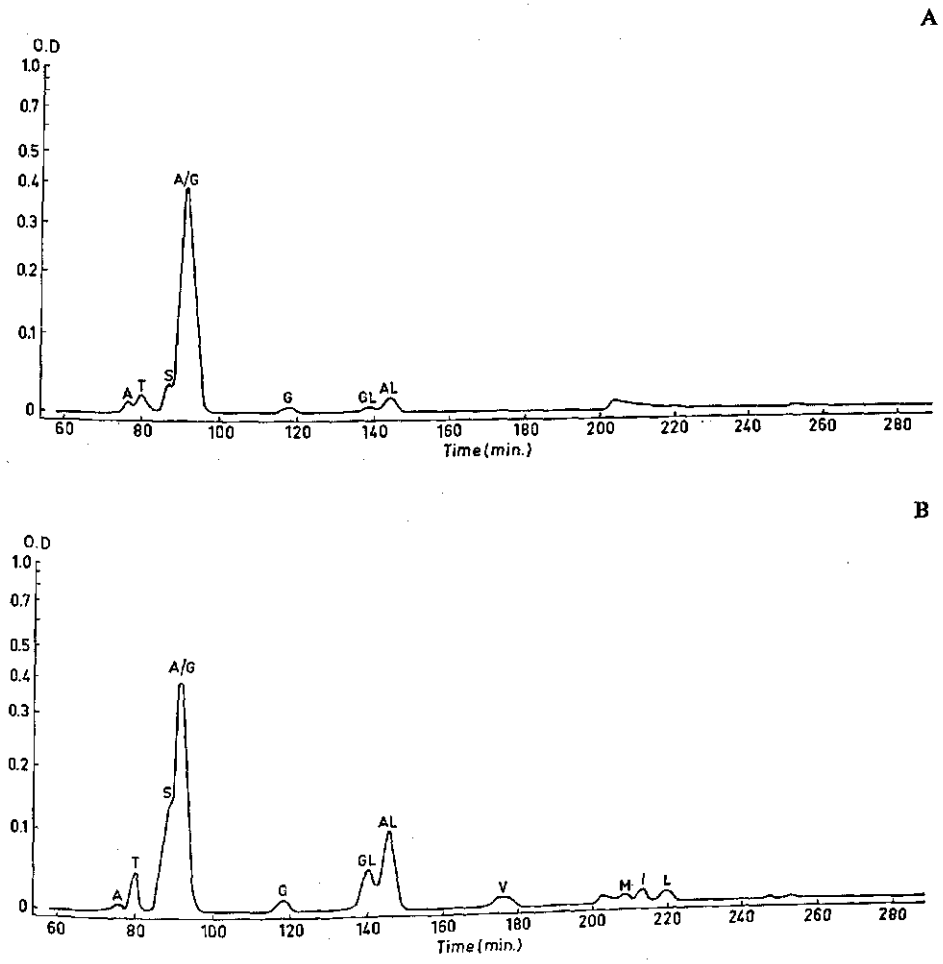


FIG. 7.6. Acid and neutral n.p.c. present in roots of seedlings of various leguminous plants.
A, birdsfoot trefoil; B, red clover.
A, aspartic acid; T, threonine; S, serine; A/G, asparagine + glutamine; G, glutamic acid; GL, glycine; AL, alanine; V, valine; M, methionine; I, isoleucine; L, leucine.

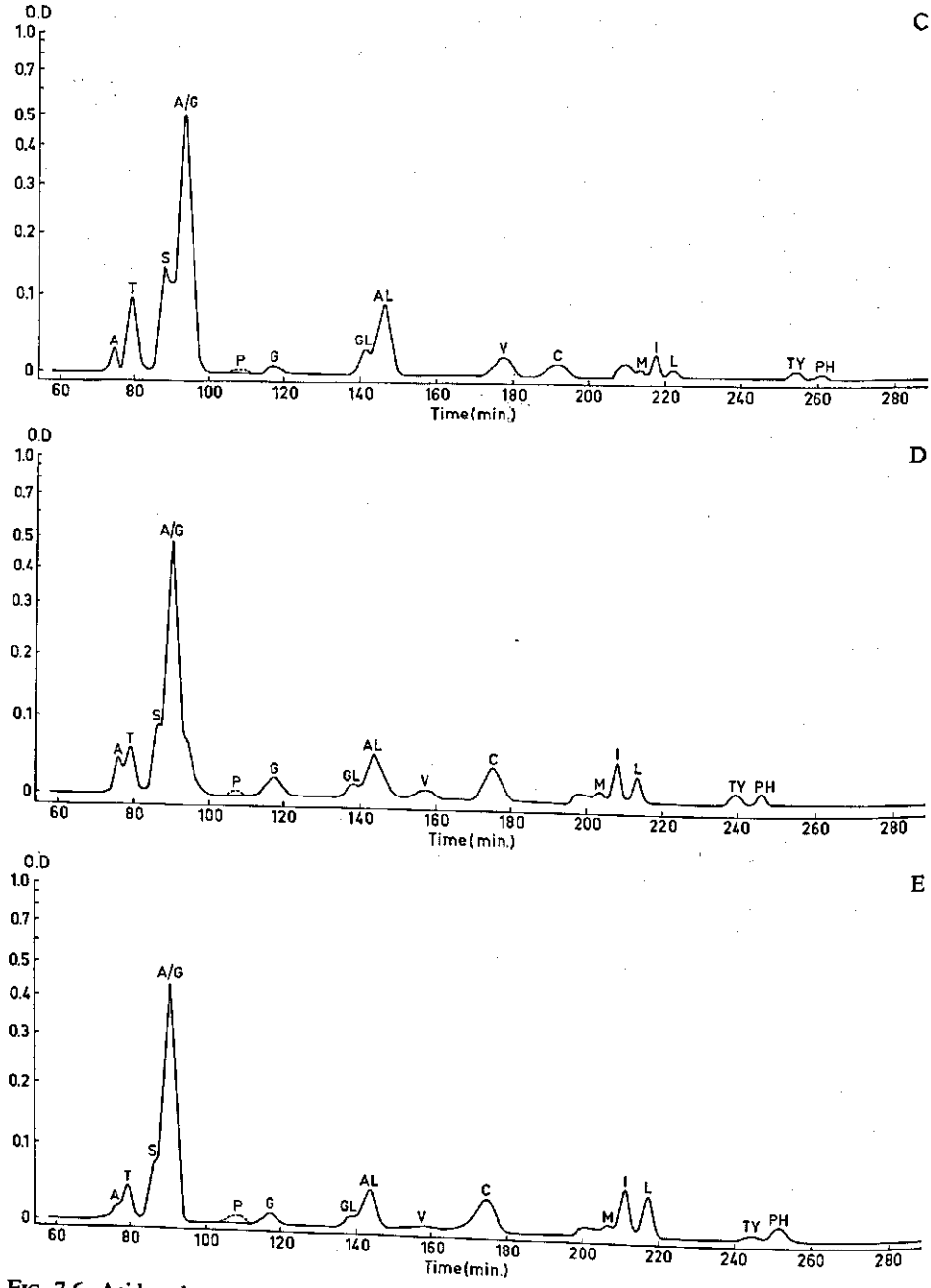


FIG. 7.6. Acid and neutral n.p.c. present in roots of seedlings of various leguminous plants. C, soyabean; D, cowpea; E, green gram.
A, aspartic acid; T, threonine; S, serine; A/G, asparagine + glutamine; P, proline; G, glutamic acid; GL, glycine; AL, alanine; V, valine; C, cystine; M, methionine; I, isoleucine; L, leucine; TY, tyrosine; PH, phenylalanine.

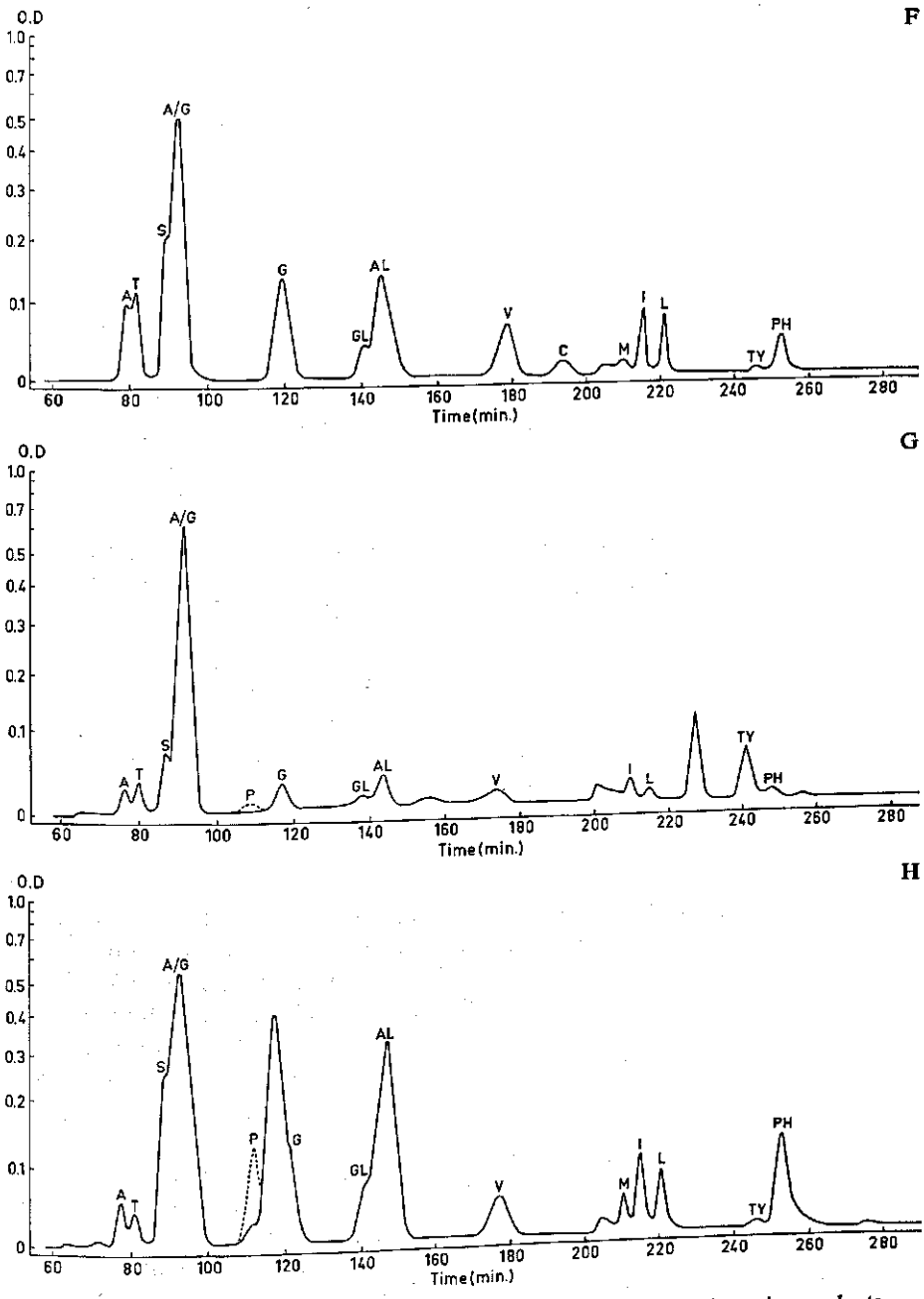


FIG. 7.6. Acid and neutral n.p.c. present in roots of seedlings of various leguminous plants. F, garden bean; G, broad bean; H, peanut.
A, aspartic acid; T, threonine; S, serine; A/G, asparagine + glutamine; P, proline; G, glutamic acid; GL, glycine; AL, alanine; V, valine; C, cystine; M, methionine; I, isoleucine; L, leucine; TY, tyrosine; PH, phenylalanine.

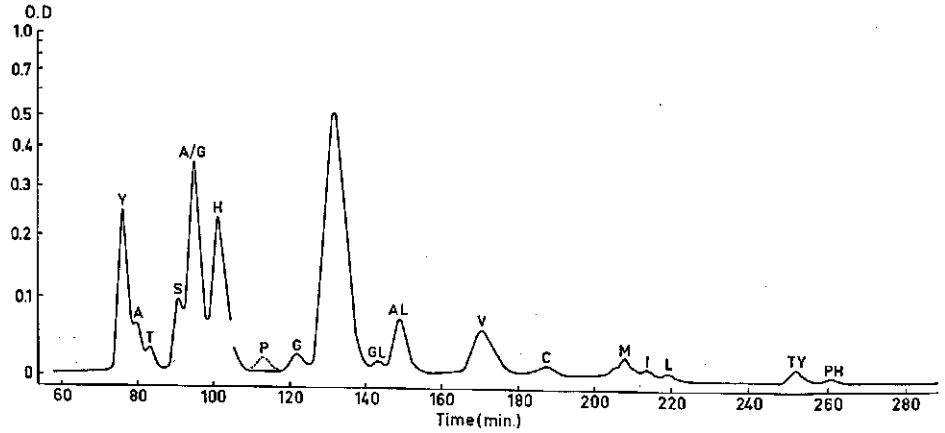


FIG. 7.6. Acid and neutral n.p.c. present in roots of seedlings of various leguminous plants. I, sweet pea.
Y, unknown; A, aspartic acid; T, threonine; S, serine; A/G, asparagine + glutamine; H' homoserine; P, proline; G, glutamic acid; GL, glycine; AL, alanine; V, valine; C, cystine; M' methionine; I, isoleucine; L, leucine; TY, tyrosine; PH, phenylalanine.

7.5. CHANGES IN FREE NINHYDRIN-POSITIVE COMPOUNDS OF COTYLEDONS, ROOTS AND TOPS OF PEA PLANTS AS INFLUENCED BY AGE, ENVIRONMENTAL CONDITIONS AND NUTRITION

7.5.1. *Experimental*

Pea seedlings (derived from selected seeds of approximately equal weight, 300 mg ± 10 mg) were grown on water agar during 6 days and subsequently transferred to Erlenmeyer flasks containing nutrient solution (2.1). After 5 days, the nutrient solution of a number of flasks was supplemented with nitrate (100 mg NO_3^-/l) while part of the plants growing on nutrient solution without added nitrogen were inoculated with *Rhizobium leguminosarum*, strain PRE. The plants were grown under controlled environmental conditions (2.1); part of the plants were kept in complete darkness.

The n.p.c. present in the cotyledons were estimated from the start of germination (wetting the seed) until 24 days thereafter, when the seed was found to be exhausted. The n.p.c. in roots and tops (including epicotyl) were determined until 30 days after wetting the seed. For each analysis, use was made of material derived from 3 plants in the same stage of development and having approximately similar weights. Values given in tables and figures are always calculated for a single plant.

As quantitative values of 'unknown X' and 'unknown Y' are not available, these compounds will be expressed by + (present), tr. (trace amounts), – (not

present). 'Total amounts of free n.p.c. present' does not include the unknown compounds.

7.5.2. *Results and discussion*

7.5.2.1. Pea plants (uninoculated) growing in nutrient solution without added nitrogen under controlled environmental conditions

A survey of the amount of the free soluble ninhydrin-positive compounds (mainly amino acids) in pea plants of different age, calculated as μg n.p.c. per plant, is given in tables 7.2 (cotyledons), 7.3 (roots) and 7.4 (tops). In fig. 7.7 total amounts (in mg) of free n.p.c. in the cotyledons are presented while of a number of amino acids (homoserine, asparagine/glutamine, aspartic acid and glutamic acid) the separate amounts are given. Of homoserine also the percentages, calculated on the total amounts of n.p.c. have been given. Figures 7.8 and 7.9 show similar data of roots and tops.

TABLE 7.2. Free ninhydrin-positive compounds (μg/plant) in cotyledons of uninoculated pea plants of different age, grown without added nitrogen.

Age (days after wetting the seed)	0	1	2	3	4	5	6	7	10	14	18	21	24
'Unknown X'	—	—	—	—	—	—	—	—	—	—	—	—	—
'Unknown Y'	tr.	tr.	+	+	+	+	+	+	+	+	+	+	+
Aspartic acid	50	133	106	225	95	106	145	133	225	212	560	40	tr.
Threonine	tr.	tr.	48	42	42	36	36	36	119	131	320	105	tr.
Serine	tr.	33	168	220	273	220	325	357	378	378	662	115	tr.
Asparagine/-glutamine	90	490	830	620	680	680	740	830	880	800	1925	300	tr.
Homoserine	tr.	tr.	60	350	715	1150	3000	2511	550	350	298	48	tr.
Proline	tr.	tr.	tr.	tr.	35	35	70	35	35	23	23	tr.	tr.
Glutamic acid	265	309	840	660	700	735	760	748	1470	985	690	75	tr.
Glycine	23	18	34	30	30	30	75	75	100	127	200	45	tr.
Alanine	22	22	63	45	40	63	175	186	195	195	285	45	tr.
Valine	tr.	tr.	tr.	tr.	35	35	70	46	47	35	35	tr.	tr.
Cystine	tr.	tr.	84	144	310	335	360	408	600	760	1100	240	tr.
Methionine	tr.	tr.	tr.	tr.	tr.	tr.	45	30	30	30	30	tr.	—
Isoleucine	tr.	tr.	tr.	tr.	78	78	78	131	140	140	300	26	tr.
Leucine	tr.	tr.	tr.	tr.	52	52	78	118	140	150	390	tr.	tr.
Tyrosine	tr.	tr.	tr.	tr.	54	36	36	72	72	90	180	38	tr.
Phenylalanine	tr.	tr.	tr.	tr.	65	82	115	165	230	214	330	65	tr.
γ-Aminobutyric acid	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	56	42	61	30	tr.
Lysine	tr.	tr.	55	64	73	91	100	183	240	240	458	55	tr.
Histidine	tr.	tr.	46	40	62	93	186	155	230	186	420	110	tr.
Ammonia	34	41	24	41	58	90	53	68	60	50	44	44	tr.
Arginine	230	670	735	680	630	730	840	820	840	630	1050	210	tr.

TABLE 7.3. Free ninhydrin-positive compounds (μg/plant) in roots of uninoculated pea plants of different age, grown without added nitrogen.

Age (days after wetting the seed)	5	6	7	14	18	24	31
'Unknown X'	+	+	+	+	+	+	+
'Unknown Y'	+	+	+	+	+	+	+
Aspartic acid	27	40	93	120	200	266	160
Threonine	12	12	47	171	143	167	24
Serine	11	31	52	95	126	179	52
Asp./glut.	118	237	310	148	163	222	59
Homoserine	476	1330	2260	1790	1606	1570	595
Proline	12	34	12	tr.	tr.	tr.	tr.
Glutamic acid	15	44	59	130	220	294	147
Glycine	15	23	23	38	53	60	38
Alanine	18	36	80	71	89	151	45
Valine	tr.	tr.	tr.	tr.	tr.	tr.	tr.
Cystine	24	48	120	72	148	160	tr.
Methionine	tr.	tr.	tr.	tr.	tr.	tr.	tr.
Isoleucine	tr.	tr.	tr.	13	tr.	tr.	tr.
Leucine	tr.	tr.	tr.	13	tr.	tr.	tr.
Tyrosine	tr.	tr.	tr.	tr.	tr.	tr.	tr.
Phenylalanine	tr.	tr.	tr.	tr.	tr.	tr.	tr.
γ-Aminobutyric acid	tr.	tr.	tr.	tr.	tr.	tr.	tr.
Lysine	19	38	55	92	73	54	tr.
Histidine	23	31	31	62	46	36	tr.
Ammonia	tr.	10	15	60	41	68	tr.
Arginine	21	42	32	21	21	tr.	tr.

7.5.2.1.1. Homoserine

As can be seen in table 7.2 and in fig. 7.7, homoserine was practically absent in dry pea seeds. From 2 days after wetting the seed, an increase of this amino acid in the cotyledons took place and its maximum value was reached 6 days after the start of germination. Four days later, the amount of homoserine in the cotyledons had sharply decreased and from the 10th until the 24th day after germination a further decrease took place. VIRTANEN *et al.* (1953) found an increase of homoserine in pea seedlings until 5 or 6 days after wetting the seed, whence it remained constant until the 15th day when the experiment was finished. These results seem to be only partly in agreement with those of the present investigation. However, VIRTANEN analysed whole seedlings and when in the present experiments the homoserine values of cotyledons, roots and tops are added, the present data agree more closely with those of VIRTANEN *et al.*

In roots, homoserine attained a maximum 7 days after the start of germination (table 7.3 and fig. 7.8). Seventy percent of the free n.p.c. ('unknown X' and 'unknown Y' excluded) was made up of homoserine at this stage of development. Hereafter a slow decrease set in, but 24 days after the start of germination homoserine still represented 50% of the total free n.p.c. of the pea roots. After

TABLE 7.4. Free ninhydrin-positive compounds (μg/plant) in tops of uninoculated pea plants of different age, grown without added nitrogen.

Age (days after wetting the seed)	5	6	7	14	18	24	31
'Unknown X'	tr.	tr.	+	+	+	+	+
'Unknown Y'	+	+	+	+	+	+	+
Aspartic acid	13	tr.	27	106	106	93	113
Threonine	tr.	24	36	83	60	24	24
Serine	tr.	31	42	84	84	73	73
Asp./glut.	89	252	592	453	89	74	74
Homoserine	190	595	833	464	143	143	119
Proline	14	12	12	tr.	tr.	tr.	tr.
Glutamic acid	29	47	44	217	323	396	294
Glycine	8	60	75	172	90	45	23
Alanine	9	18	27	133	142	213	160
Valine	tr.	tr.	tr.	tr.	tr.	tr.	tr.
Cystine	tr.	24	72	190	24	tr.	tr.
Methionine	tr.	tr.	tr.	tr.	tr.	tr.	tr.
Isoleucine	tr.	tr.	tr.	tr.	tr.	tr.	tr.
Leucine	tr.	tr.	tr.	tr.	tr.	tr.	tr.
Tyrosine	tr.	tr.	tr.	tr.	tr.	tr.	tr.
Phenylalanine	tr.	tr.	tr.	tr.	tr.	tr.	tr.
γ-Aminobutyric acid	tr.	tr.	tr.	tr.	tr.	tr.	tr.
Lysine	tr.	55	92	92	tr.	tr.	tr.
Histidine	tr.	31	78	78	31	tr.	tr.
Ammonia	tr.	8	11	46	44	66	64
Arginine	tr.	42	42	147	105	tr.	tr.

the seed had been exhausted (24 days after wetting the seed, cf. table 7.2) the pomoserine content of the roots, like that of the other n.p.c., rapidly decreased, hrobably owing to transportation to the tops where it may have been used for the synthesis of insoluble plant material.

From the data of table 7.4 and of fig. 7.9, it will be seen that 7 days after the start of germination, homoserine was also in the tops of pea seedlings quantitatively the most important free amino acid (approximately 45% of the n.p.c. consisted of this compound). Subsequently a decrease set in, more rapidly than in roots, and at the 18th day homoserine was only present in minor quantities.

Little is known about the role of homoserine in pea plants. Although this amino acid is the precursor of threonine and methionine (cf. studies in yeast and bacteria by FOWDEN, 1965), there is no evidence that the large amounts present in peas are metabolized in this way. LARSON and BEEVERS (1965) were unable to find a major conversion of labeled homoserine to other compounds up to 24 hours after its injection into cotyledons of 2 or 10-days old etiolated pea seedlings. PATE *et al.* (1965) found that in the field pea (*Pisum arvense* L.) the carbon from homoserine became mainly incorporated into insoluble non-proteinaceous material. MITCHELL and BIDWELL (1970) concluded from their experiments with

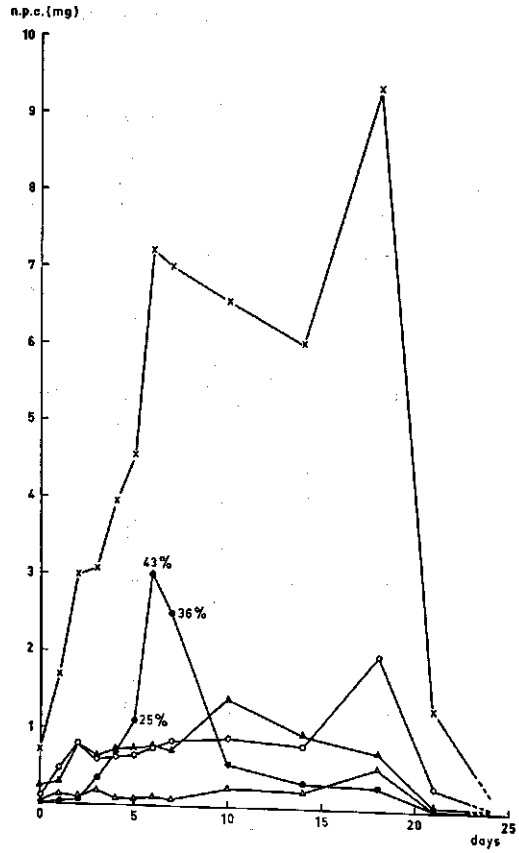


FIG. 7.7. Total amounts (mg per plant) of free ninhydrin-positive compounds (unknown 'Y' excluded) in the cotyledons of uninoculated pea plants grown without added nitrogen during a period from the start of germination until 25 days thereafter. The amounts of homoserine, asparagine/glutamine, aspartic acid and glutamic acid are presented separately.

×——× total amount
●——● homoserine
○——○ asparagine/glutamine
▲——▲ aspartic acid
△——△ glutamic acid

pea seedlings to which ^{14}C-homoserine was added that homoserine would be involved in the mobilization and translocation of carbon and nitrogen reserves after germination.

7.5.2.1.2. Glutamine + asparagine

The amides glutamine and asparagine were found to occur in approximately constant amounts in the cotyledons from 2 until 14 days after the start of germination (table 7.2, fig. 7.7). After this period a pronounced increase took place with a maximum being reached at about the 18th day. At this time the total amount of free n.p.c. in the cotyledons was also highest which, in addition to the increased content of the amides, was due to high levels of several other amino compounds, mainly arginine and cystine. Since asparagine and glutamine as well as arginine may be considered as storage and detoxication products of ammonia,

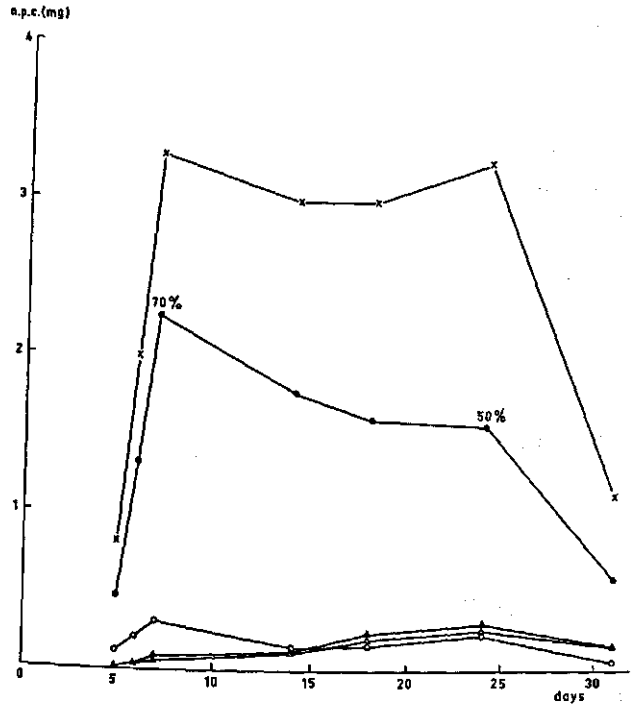


FIG. 7.8. Total amounts (mg per plant) of free ninhydrin-positive compounds (unknowns 'X' and 'Y' excluded) in the roots of uninoculated pea plants grown without added nitrogen from the 5th until the 31st day after the start of germination. The amounts of homoserine, asparagine/-glutamine, aspartic acid and glutamic acid are presented separately.

×——× total amount
●——● homoserine
○——○ asparagine/-glutamine
▲——▲ aspartic acid
△——△ glutamic acid

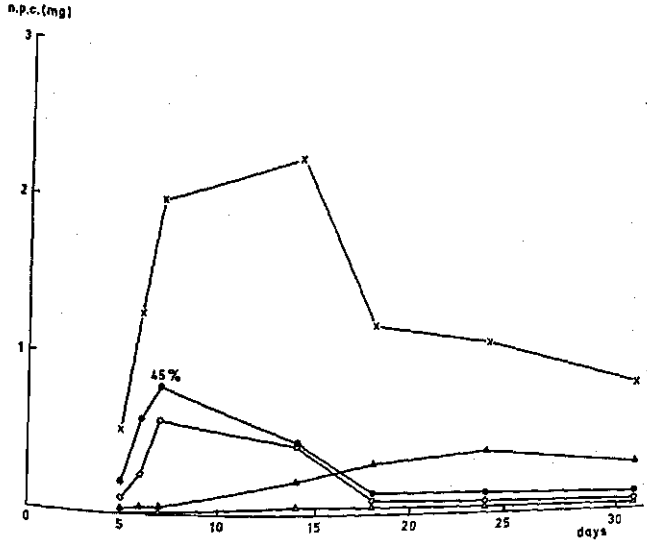


FIG. 7.9. Total amounts (mg per plant) of free ninhydrin-positive compounds (unknowns 'X' and 'Y' excluded) present in the tops of uninoculated pea plants grown without added nitrogen from the 5th until the 31st day after the start of germination. The amounts of homoserine, asparagine/-glutamine, aspartic acid and glutamic acid are presented separately.

×——× total amount
●——● homoserine
○——○ asparagine/-glutamine
▲——▲ aspartic acid
△——△ glutamic acid

accumulation of these compounds means that excessive amounts of ammonia must have been formed, presumably resulting from autolysis and break-down of amino acids (PRJANISCHNIKOW, 1952).

The increase of the amides in pea seedlings was also noticed by VIRTANEN *et al.* (1953) and earlier by PRJANISCHNIKOW. The former authors found a considerable increase of asparagine 15 days after the start of germination. The cotyledons, shortly before being exhausted obviously may provide the plant with a final amount of nitrogen in the form of these storage compounds.

In the roots of pea seedlings the amides are of minor importance (table 7.3 and fig. 7.8). This is in contrast with the roots of seedlings of other leguminous plants where the amides were quantitatively always the most important nitrogenous compounds (section 7.4). In pea seedlings the role of the amides has apparently been taken over by homoserine. This conclusion is in agreement with the results of experiments carried out by MITCHELL and BIDWELL (1970).

7.5.2.1.3. Total amounts of n.p.c.

The total amount of free n.p.c. in roots of pea plants grown under the conditions of the present experiments is more or less constant from the 7th until the 24th day after start of germination (at this final date the seed was exhausted). However, the amounts of the individual n.p.c. were not constant during this period (table 7.3).

The maintaining of the total amount of free n.p.c. in the pea roots at a more

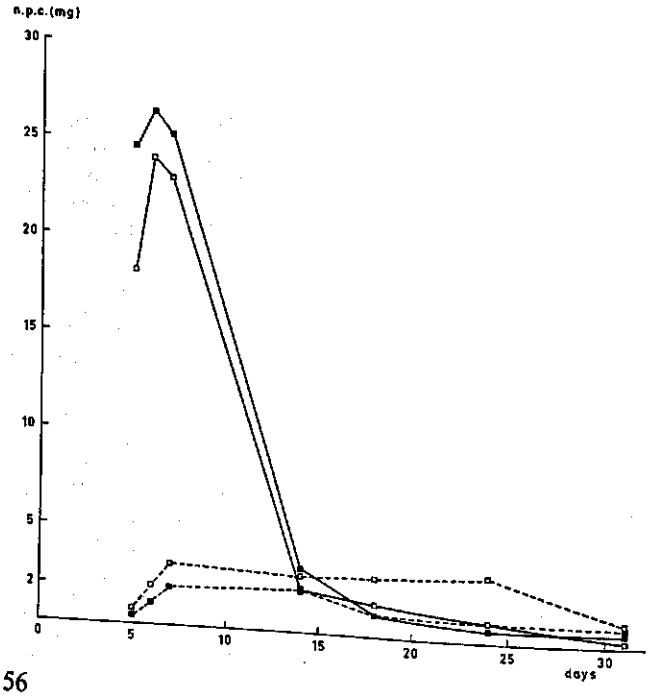


FIG. 7.10. Concentrations (mg/g fresh weight) and total amounts of free ninhydrin-positive compounds (unknowns 'X' and 'Y' excluded) in both roots and tops of pea plants grown without added nitrogen from the 5th until the 31st day after the start of germination.
■—— ■ concentration in tops
□—— □ concentration in roots
■..... ■ total amount in tops
□..... □ total amount in roots

or less constant level implicates a continuous decrease in concentration of these compounds since the root weight is considerably increasing (fig. 7.10). In the tops of the pea plants, the total amount of n.p.c. decreased from the 14th until the 24th day after the start of germination, apparently owing to the utilization of these compounds in the synthesis of cellular material (fig. 7.9). As a consequence, the concentration of n.p.c. in the tops fell even more drastically than that in the roots (fig. 7.10). As can be seen from these curves, the sharpest fall in concentration occurred between the 7th and the 14th day. Maximal concentration of the free n.p.c. in both roots and tops was reached 6 or 7 days after the start of germination.

7.5.2.2. Pea plants (uninoculated) in nutrient solution supplemented with nitrate, under controlled environmental conditions

The total amounts (in mg per plant) of free n.p.c. in roots and tops, respectively, of uninoculated pea plants growing in nutrient solution supplied with nitrate are plotted in figures 7.11 and 7.12. Separate data are given of homoserine, asparagine/glutamine, aspartic acid and glutamic acid.

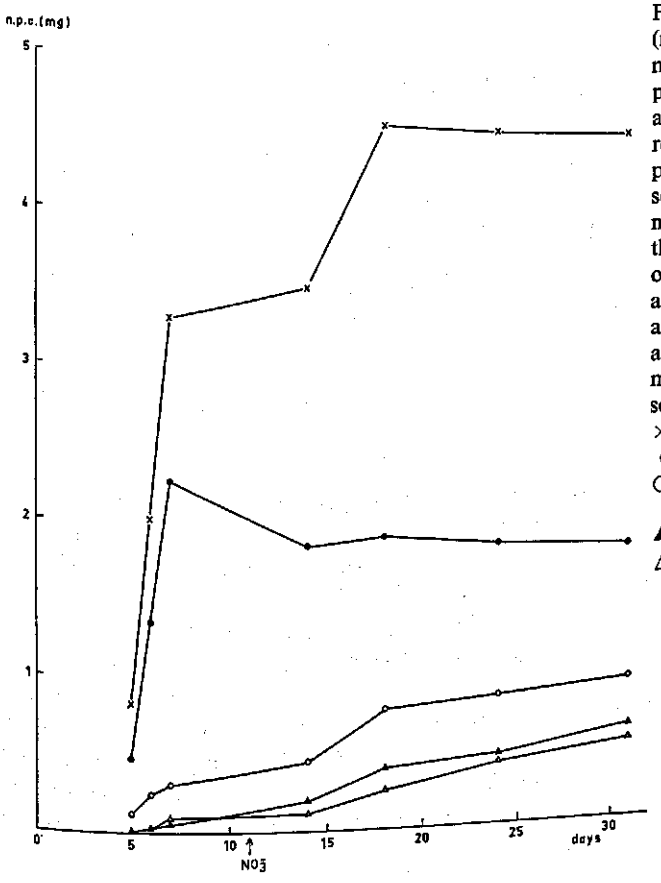


FIG 7.11. Total amounts (mg per plant) of free ninhydrin-positive compounds (unknowns 'X' and 'Y' excluded) in roots of uninoculated pea plants grown in nutrient solution supplied with nitrate from the 5th until the 31st day after start of germination. The amounts of homoserine, asparagine/glutamine, aspartic acid and glutamic acid are presented separately.

×——× total amount
●——● homoserine
○——○ asparagine/-glutamine
▲——▲ aspartic acid
△——△ glutamic acid

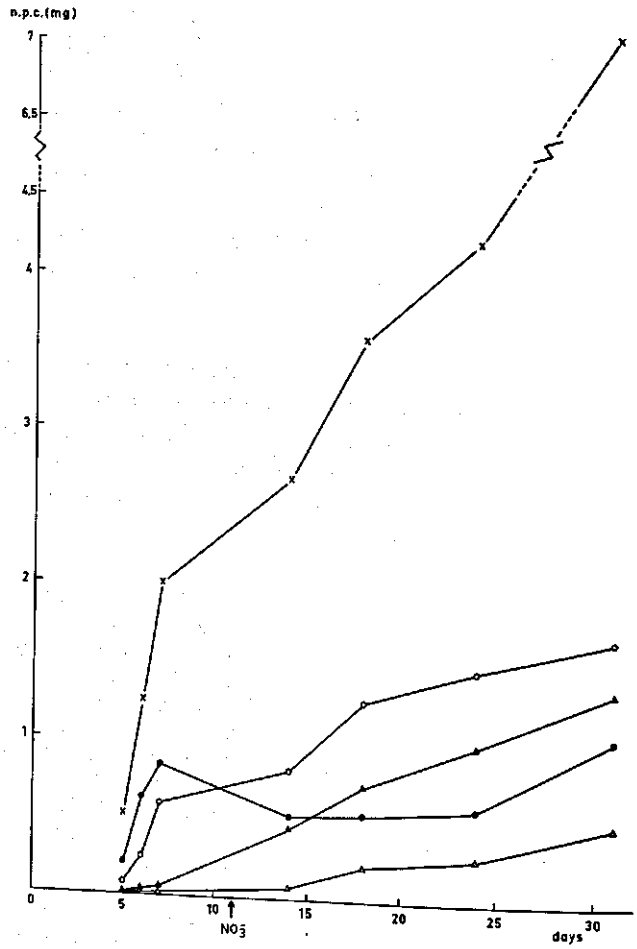


FIG. 7.12. Total amounts (mg per plant) of free ninhydrin-positive compounds (unknowns 'X' and 'Y' excluded) present in tops of uninoculated pea plants grown in nutrient solution supplied with nitrate from the 5th until the 31st day after start of germination. The individual amounts of homoserine, asparagine/-glutamine, aspartic acid and glutamic acid are presented separately.
×——× total amount
●——● homoserine
○——○ asparagine/-glutamine
▲——▲ aspartic acid
△——△ glutamic acid

When comparing these total amounts in roots grown in the presence of nitrate with those in roots of plants without added nitrogen, remarkably small differences will be observed up to 24 days after the start of germination (figures 7.8 and 7.11). In accordance with PATE (1968), it can be concluded that roots of pea plants are not programmed to store large quantities of soluble nitrogen.

Fig. 7.12 shows that the total amount of free n.p.c. in tops was constantly increasing. This was also true of most of the individual amino acids, particularly of glutamic acid, cystine, alanine, proline and homoserine. The increase of the amides glutamine and asparagine was also substantial, mainly between 14 and 18 days after the start of germination. This increase was probably mainly due to the direct supply from the cotyledons where during this period a considerable increase of amides took place (fig. 7.7).

7.5.2.3. Pea plants inoculated with *Rhizobium leguminosarum*, strain PRE, in nutrient solution without added nitrogen under controlled environmental conditions

Six days after transferring the seedlings to Erlenmeyer flasks, the nutrient solution was inoculated with *Rhizobium leguminosarum*, strain PRE. Approximately 12 days after inoculation, the nodules started to fix nitrogen. Until this time (24 days after the start of germination), growth conditions of these plants were identical to those growing without added nitrogen so that during this period no differences in n.p.c. were found between both types of plants (cf. figures 7.8 and 7.9).

From the 24th to the 31st day after the start of germination, the total amount of free n.p.c. in plants inoculated with *Rhizobium* greatly increased. In tops an increase of free n.p.c. of approximately 4 mg was found (1 mg after 24 days, 5 mg after 31 days). This increase was mainly due to the higher amounts of amides (0.1 mg after 24 days, 2.25 mg after 31 days). Most of the other free amino compounds also increased, particularly glutamic acid and homoserine. In the nodulated roots this rise of free n.p.c. was for over 90% due to the increased amount of amides (0.25 mg after 24 days, 2.10 mg after 31 days). Glutamic acid slightly increased, aspartic acid and homoserine remained constant.

When the nodules were analysed immediately after the separation from the roots, 80% of the total amount of free n.p.c. was found to be present as amides (table 7.5). The unknown compounds 'unknown X' and 'unknown Y' did not occur.

The nitrogen fixed in the nodules is thus converted to amide nitrogen and in this form it is transported to the tops.

TABLE 7.5. The predominant ninhydrin-positive compounds in root nodules of pea plants.

Ninhydrin-positive compounds	% of total amount of n.p.c.
Amides	80
Glutamic acid	9
Aspartic acid	3
Homoserine	5
Others	3

7.5.2.4. Pea plants (uninoculated) in nutrient solution in the dark with or without added nitrate

Addition of nitrate to the nutrient solution had no effect on growth and composition (as far as concerns n.p.c.) of etiolated plants. Total amounts of free n.p.c. (in mg per plant) in roots and tops, respectively, are shown in figures 7.13 and 7.14. The amounts of homoserine, asparagine/glutamine, aspartic acid and glutamic acid are presented separately.

In complete darkness, root growth stopped between 2.5 and 3 weeks after the start of germination (fresh root weight approximately 700 mg per plant as compared with approximately 4500 mg in the case of plants grown in the light with-

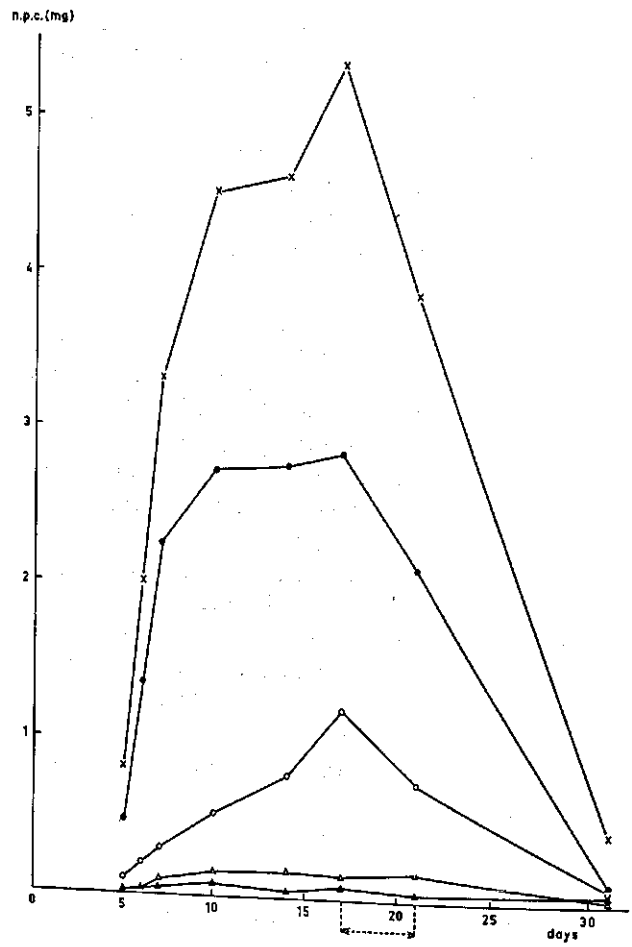


FIG. 7.13. Total amounts (mg per plant) of free ninhydrin-positive compounds (unknowns 'X' and 'Y' excluded) present in roots of uninoculated pea plants growing in nutrient solution in the dark with or without added nitrate from the 5th until the 31st day after start of germination. The individual amounts of homoserine, asparagine/-glutamine, aspartic acid and glutamic acid are presented separately.
×——× total amount
●——● homoserine
○——○ asparagine/-glutamine
▲——▲ aspartic acid
△——△ glutamic acid
<- - -> root growth ceased

out added nitrogen and approximately 4000 mg in the light with added nitrate). Until this time the amount of free n.p.c. had constantly increased so that it had attained a value which was almost twice as high as the amount in roots of plants grown in the light. This means that the concentration of free n.p.c. in the dark was more than 10 times higher than in the light. When the root growth ceased, a sharp drop of the free n.p.c. in the roots occurred and at the 31st day after the start of germination, n.p.c. were almost no more detected in the roots of the etiolated plants (fig. 7.13).

Growth of the tops stopped about 3 weeks after the start of germination (fresh weight approximately 1300 mg as compared with approximately 1700 mg in the case of plants grown in the light without added nitrogen and approximately 3500 mg in the light with added nitrate). In contrast with the roots, the free n.p.c. of the tops continued to increase until the cotyledons were exhausted (shortly before finishing the experiments). At this stage 22.5 mg of free n.p.c. were present of which almost 13 mg consisted of glutamine + asparagine, 4 mg

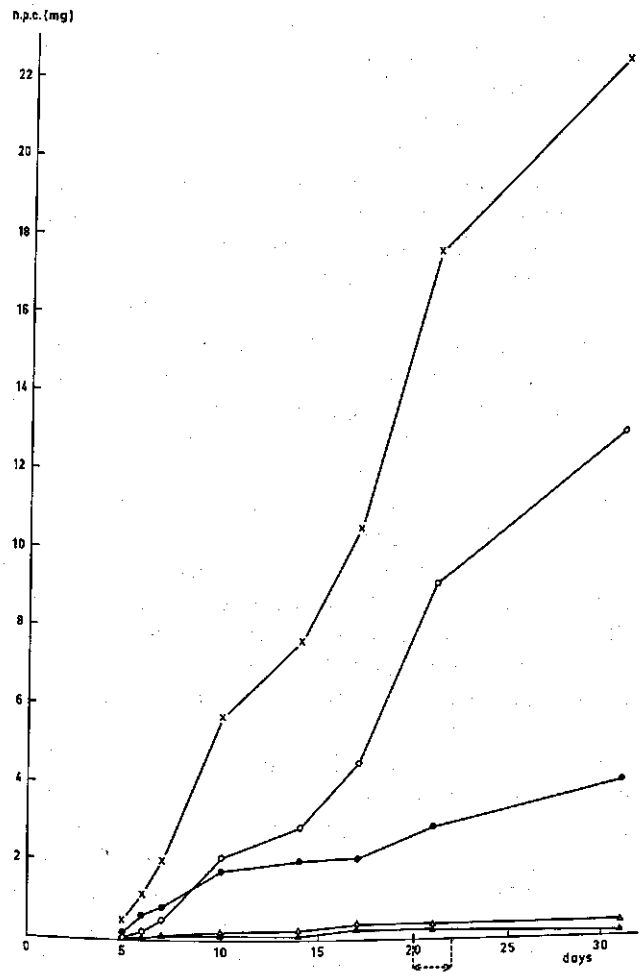


FIG. 7.14. Total amounts (mg per plant) of free ninhydrin-positive compounds (unknowns 'X' and 'Y' excluded) present in tops of uninoculated pea plants growing in nutrient solution in the dark with or without added nitrate from the 5th until the 31st day after start of germination. The individual amounts of homoserine, asparagine/glutamine, aspartic acid and glutamic acid are presented separately.

×——× total amount
●——● homoserine
○——○ asparagine/-glutamine
▲——▲ aspartic acid
△——△ glutamic acid
<- - -> top growth ceased

of homoserine whereas only 5 mg was left for the remaining free n.p.c. of which arginine and cystine were the most important ones.

7.5.3. *Summary and general discussion of section 7.5*

Homoserine, hardly present in dry pea seeds, increased rapidly from the 2nd day of germination. In the cotyledons, its maximum value was reached 6 or 7 days after wetting the seed, whereafter it decreased to a low, fairly stable level (fig. 7.7). Most of the homoserine formed in the cotyledons was transported to the young root because during the first week after the start of germination, growth of the tops was far behind root growth. Afterwards, growth of the shoot became important, but now the supply of homoserine from the cotyledons was already greatly decreased.

In roots, approximately 70% of the total amount of free n.p.c. consisted of

homoserine 7 days after the start of germination (fig. 7.8). At this time, the concentration of free n.p.c. had reached its maximum (the same is true in tops) but then it dropped sharply (fig. 7.10).

Roots of plants growing without added nitrogen lost a small amount of homoserine from the 7th until the 24th day after the start of germination (fig. 7.8). Hereafter a sharp decrease of the amount of homoserine took place due to the transport of this and other amino acids to the tops. This transport occurred because approximately 24 days after the start of germination, the cotyledons were exhausted so that the tops needed the soluble nitrogen of the roots for their development.

In roots of plants fed with nitrate, the amount of homoserine remained more or less constant from the 7th day after the start of germination until the experiments were finished (fig. 7.11). Since the weight of the root tissue constantly increased during this period, the concentration of homoserine was constantly decreasing.

In the tops of the pea plants, homoserine was also quantitatively the most important free amino compound at 7 days after the start of germination. Without added nitrogen, the amount of homoserine in the tops decreased from the 7th day after the start of germination and at the 18th day this amino acid was only present in minor quantities (fig. 7.9). With added nitrate, synthesis of homoserine took place in the tops from about the 24th day after the start of germination (fig. 7.12). However, this amino acid was not transported to the root system, at least not during the experimental period.

From the results of the experiments performed in the dark (figures 7.13 and 7.14), it can be seen, that adding up of the amounts of homoserine in the roots and in the tops gives approximately constant values during the period from 10 until 31 days after the start of germination. LARSON and BEEVERS (1965), after injection of ^{14}C-labeled homoserine in pea seedlings found no conversion of this amino acid in etiolated plants. From the results of the present investigations and from those of LARSON and BEEVERS, it can be concluded that in total from 4 to 5 mg homoserine per plant may be provided by the cotyledons. Seventy percent of this amount is given off by the cotyledons during the first seven days after the start of germination.

As to the amides (glutamine and asparagine), it has been shown that in the roots of seedlings of several leguminous plants these amino compounds were predominant (section 7.4). Only in the roots of pea plants this was not the case.

In the cotyledons of pea seedlings, the amides were quantitatively secondly important after homoserine during the first two weeks after the start of germination (fig. 7.7). Hereafter a sharp increase of the amides took place so that these compounds became predominant and remained so until the cotyledons were exhausted.

Almost all of the glutamine and asparagine was transported to the tops (most of the homoserine derived from the cotyledons was transported to the root system).

Of the n.p.c. derived from the cotyledons, the amides are quantitatively the most important compounds. Almost 13 mg of these compounds were found in the tops of etiolated pea plants (fig. 7.14). In the light this accumulation did not occur because in this case the amides supplied by the cotyledons to the tops are immediately used for the synthesis of non-soluble plant material (e.g. plant proteins); this is particularly true of plants growing without added nitrogen.

When considering the total amounts of free n.p.c. in roots of pea plants grown in nutrient solution without or with added nitrate, it can be seen that there was little difference between these amounts (figures 7.8 and 7.11). Obviously, roots of pea plants are not programmed to store large amounts of soluble nitrogen. After the cotyledons were exhausted, the free n.p.c. present in the roots of plants grown without added nitrogen were transported to the tops.

Root growth of pea plants kept in the dark stopped between 18 and 21 days after the start of germination and thereafter almost all the free n.p.c. was transported to the etiolated shoot (fig. 7.13). All the n.p.c. derived from the cotyledons accumulated in this part of the plant (fig. 7.14).

7.6. Exudation of ninhydrin-positive compounds by roots of pea seedlings

7.6.1. *Experimental*

For collecting root exudates, the seedlings were grown as described in 2.4.1 and in 2.4.2. The exudation of n.p.c. was investigated up to 10 days after the start of germination. During this period, the concentration of the n.p.c. in the root tissue is highest (section 7.5) and it may be expected that the exudates have a decisive influence on the establishment of the rhizosphere microflora.

7.6.2. *Results and discussion*

7.6.2.1. Ninhydrin-positive compounds found in root-tip exudates

Fig. 7.15 shows part of a chromatogram representing the acid and neutral n.p.c. found in exudates of tips of pea-seedling roots. As other amino compounds were only present in relatively small amounts, the remaining part of this chromatogram and the chromatogram representing the basic n.p.c. are omitted. Chromatograms derived from exudates of whole roots, before formation of lateral roots, give similar patterns. This again points to the importance of the root tip as site of exudation.

As can be seen, by far the most important n.p.c. exuded by the root tips is 'unknown Y' while 'unknown X' is quantitatively secondly important. Homoserine is only present in minor quantities. As homoserine in extracts of whole roots of pea seedlings is by far the most important free n.p.c. (fig. 7.3), this means that a selective exudation by the root tip takes place or that the composition of the pool of free n.p.c. in the root tip is different from that in the older

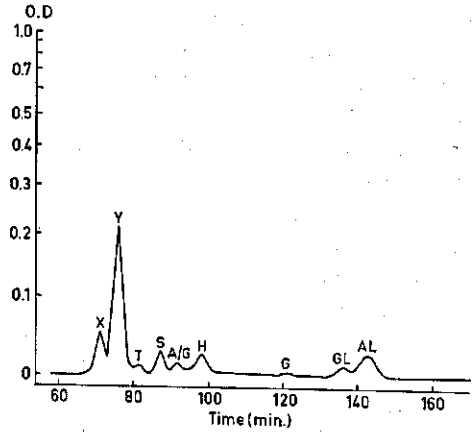


FIG. 7.15. Part of a chromatogram showing ninhydrin-positive compounds exuded by tips of pea-seedling roots.
X, unknown; Y, unknown; T, threonine; S, serine; A/G, asparagine + glutamine; H, homoserine; G, glutamic acid; GL, glycine; AL, alanine.

parts of the root. As AYERS and THORNTON (1968) found a good correlation between amino acids exuded by young roots of pea plants and amino acids present in crushed root tips (cf. section 7.1) n.p.c. analyses of root-tip extracts were made.

7.6.2.2. Ninhydrin-positive compounds present in root-tip extracts

Fig. 7.16A shows part of a chromatogram derived from extracts of 2 mm slices of tips of young pea-seedling roots. As will be seen, 'unknown' Y was predominant in extracts of the first 2 mm slices, whereas in extracts of the second 2 mm

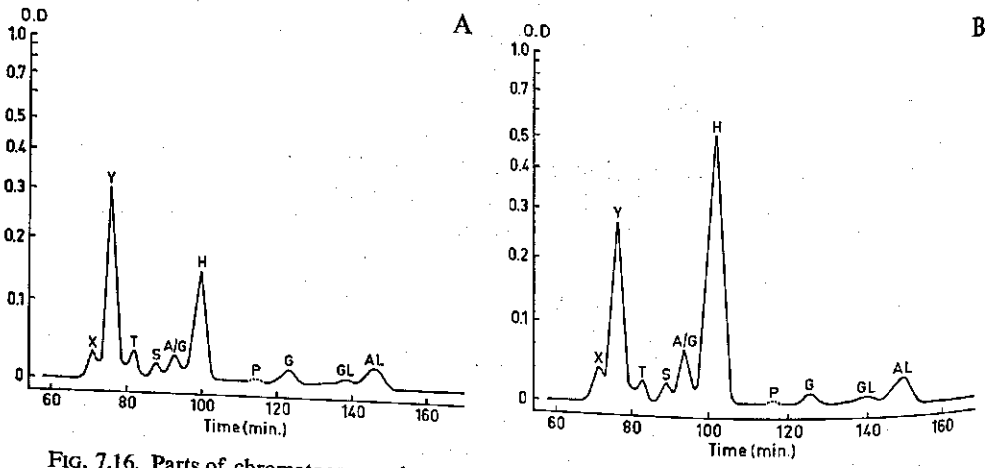


FIG. 7.16. Parts of chromatograms showing ninhydrin-positive compounds in extracts of tips of pea-seedling roots.
A. 0–2 mm slices (including tips); B. 2–4 mm slices.
X, unknown; Y, unknown; T, threonine; S, serine; A/G, asparagine + glutamine; H, homoserine; P, proline; G, glutamic acid; GL, glycine; AL, alanine.

slices (2 tot 4 mm from the root tip), homoserine was quantitatively the most important amino compound (fig. 7.16B). It is likely that 1 mm slices would have given even more striking differences.

These results are in agreement with those of AYERS and THORNTON, but as already stated before (section 7.3), their conclusion concerning the identity of the most important free n.p.c. found in the root tips and exuded by these tips was probably not correct. AYERS and THORNTON identified the compound as aspartic acid, whereas from the results of the present investigation this certainly cannot be underlined.

7.6.2.3. Ninhydrin-positive compounds in exudates of pea-seedling roots with lateral roots

When seedlings were cultivated in nutrient solution up to 10 days after the start of germination, lateral roots had developed. The influence of lateral roots on the composition of the n.p.c. in the root exudates is shown in fig. 7.17. Homoserine was predominant while 'unknown Y' took the second place. Lateral root formation obviously caused exudation of homoserine and this amino acid was most likely liberated from the main root by the wounds originated during the formation of lateral roots (chapter 3, fig. 3.2). In the absence of these wounds, homoserine is practically not exuded by the seedling roots, in spite of the high concentration of this amino acid in the roots (cf. 7.6.2.1, first paragraph).

To obtain further evidence concerning the importance of wounds for exudation, seedling roots (grown in nutrient solution) were deliberately damaged with

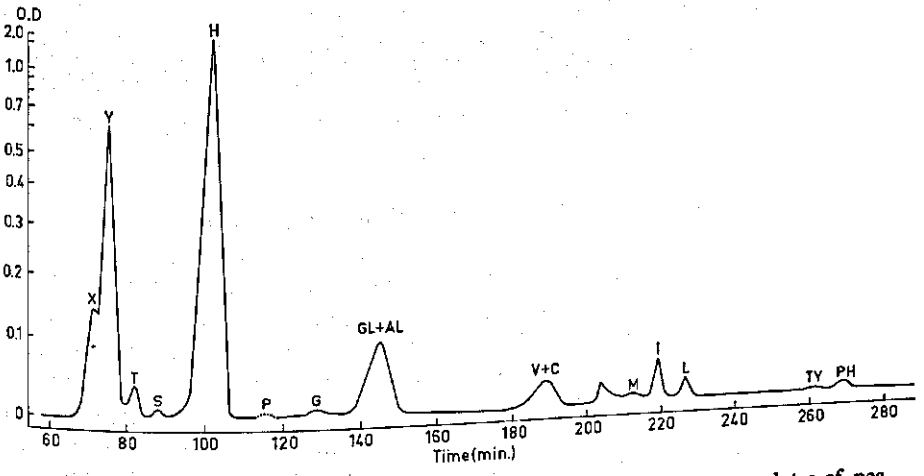


FIG. 7.17. Acid and neutral ninhydrin-positive compounds present in root exudates of pea seedlings (lateral roots present).
X, unknown; Y, unknown; T, threonine; S, serine; A/G, asparagine + glutamine; H, homoserine; P, proline; G, glutamic acid; GL, glycine; AL, alanine; V, valine; C, cystine; M, methionine; I, isoleucine; L, leucine; TY, tyrosine; PH, phenylalanine.

a needle shortly before the formation of lateral roots. After placing these roots in water (for 2 hours), homoserine was by far the most important n.p.c. in the 'exudates'.

Under the conditions of the present investigation, the first lateral roots emerged from the main root 6 or 7 days after the start of germination (fig. 7.18). As seen in section 7.5, the concentration of the free n.p.c., which consisted for approximately 70% of homoserine, was maximal in this period. Therefore, it is clear that during the formation of the first lateral roots considerable amounts of homoserine are released from the main root and that consequently this amino acid might be of utmost importance for the establishment of the rhizosphere microflora of pea plants.

FIG. 7.18. Development of the pea seedling from wetting the seed until 8 days thereafter.

7.6.2.4. Release of ninhydrin-positive compounds after the transfer of pea-seedling roots from humid air to water

When roots of pea seedlings, grown on agar, were placed in water (2.4.3.1) shortly before the formation of lateral roots (approximately 6 days after the start of germination), 'unknown Y' was predominantly found in the 'exudates'. The results of such an experiment are given in fig. 7.19. In this investigation, the whole 'exudate' of one seedling root, placed in water during 2 hours, was used for the analysis. As discussed in chapter 6, section 6.3, release of compounds after transferring roots from humid air to a liquid medium can be caused by two phenomena *viz.* by dissolving compounds adhering to the root surface and by damage of the epidermal cells resulting from the sudden wetting of the root. In 7.6.2.5, experiments are described giving some information about the magnitude of both phenomena.

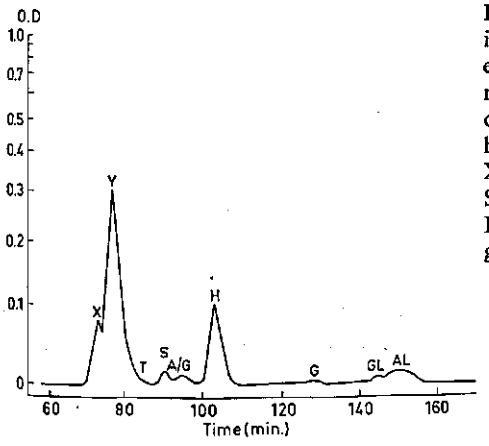


FIG. 7.19. Part of a chromatogram showing ninhydrin-positive compounds 'exuded' by a pea-seedling root (without lateral roots) when placed in water during a period of 2 hours (seedling precultivated under humid conditions).
X, unknown; Y, unknown; T, threonine; S, serine; A/G, asparagine + glutamine; H, homoserine; G, glutamic acid; GL, glycine; AL, alanine.

7.6.2.5. Ninhydrin-positive compounds in root scrapings and root extracts of pea seedlings grown on water agar or in nutrient solution

In root scrapings (2.4.3.1) of seedlings grown on agar, 'unknown Y' was predominant, whereas in scrapings of roots grown in nutrient solution, homoserine was predominant. This was also true of seedlings grown on agar, but placed with their roots for a short period in water before collecting the root scrapings.

Chromatograms derived from root extracts of these three types of seedlings (grown on agar, in nutrient solution, and on agar with a short stay in nutrient solution, respectively) were almost identical. However, there was one striking difference. The ratios of 'unknown Y' to homoserine (the height of the peak representing 'unknown Y' divided by the height of the peak representing homoserine) were not the same. In the case of roots grown in nutrient solution, this ratio was approximately 1/5 whereas in roots permanently grown on agar, this ratio was 1/3 (fig. 7.3).

In a further experiment, the root of a seedling (no lateral roots present) grown on agar was placed in water for 2 hours whereupon an extract was made from this root. The n.p.c. present in the 'exudates' and in the root tissue were estimated, and the total amount of each compound was calculated. It appeared that 25–30% of the total amount of 'unknown Y' was present in the 'exudate' whereas with homoserine this percentage was only 4.

From the above-mentioned experiments it is concluded that 'unknown Y', released from roots of pea seedlings after placing in water, for the greater part was present on the root surface. Release caused by damage of the epidermal cells is of minor importance.

In the present investigation little attention has been paid to the second, quantitatively less important, n.p.c. ('unknown X') exuded by the root tip. This compound has been found to behave similarly to 'unknown Y' as to excretion and presence on the root surface.

7.7. Attempts made to identify the unknown ninhydrin-positive compounds

In order to elucidate the identities of 'unknown X' and 'unknown Y', these compounds were isolated from root scrapings with the aid of the amino-acid analyser. Fig. 7.3 shows, that the unknown compounds are leaving the column first. The exact periods of time during which this occurs, can be easily calculated. By uncoupling the elution column from the apparatus, collection of both compounds was achieved.

However, under normal conditions as to buffer, elution temperature and elution rate (table 7.1), the compounds left the column not fully separated, the results becoming worse when samples with high concentrations of n.p.c. were analysed. Therefore, conditions had to be modified to achieve a better separation. Changing the pH of buffer A from 3.25 to 3.40 enabled the separation of 'unknown X' from the other n.p.c. (also when relatively high concentrations were used). 'Unknown Y', although being completely separated from 'unknown X', was now contaminated with aspartic acid and threonine. To separate 'unknown Y' from the other amino compounds, the pH of buffer A had to be brought down to 2.90, while the temperature had to be risen to 58 °C. Under these conditions, 'unknown Y' instead of 'unknown X' left the column first (fig. 7.20).

The eluate containing 'unknown X' was hydrolysed, evaporated and redissolved (2.4.7) whereupon the sample was eluted, using the normal buffer, elution temperature and elution rate (table 7.1). This gave 3 n.p.c. present in equimolar amounts: glutamic acid, alanine and NH_3, indicating that 'unknown X' would be a dipeptide consisting of glutamine and alanine. However, experiments with pure glutamylalanine gave a peak on the chromatogram coinciding with that of 'unknown X'. So far, it is unknown if this result is in disagreement with the

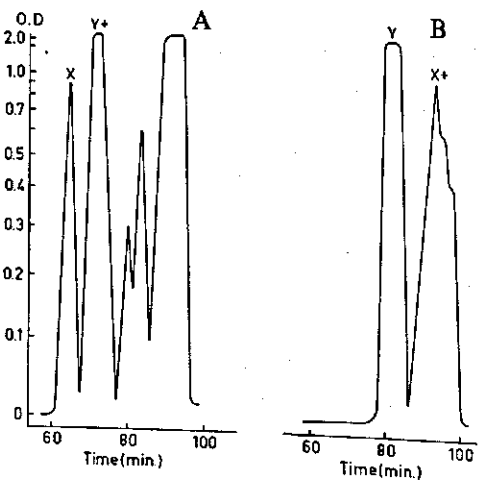


Fig. 7.20. The influence of various elution conditions on separation of the unknown compounds 'X' and 'Y'.
A. Buffer A, pH 3.40; elution temperature, 30 °C.
B. Buffer A, pH 2.90; elution temperature, 58 °C.

suggestion that 'unknown X' would be a dipeptide of glutamine and alanine.

A considerable amount of work has been done to identify 'unknown Y'. When the eluate containing this compound was hydrolysed, the only n.p.c. found in the hydrolysate was NH_3. More information concerning this compound was obtained by determining the ultra-violet-absorption spectra and the infrared spectrum, and by NMR spectroscopy. For these analyses, approximately 15 mg of the purified compound were needed so that the elution procedure had to be repeated 75 times. The eluates ('unknown Y' present in Na-citrate buffer) had to be desalted to avoid interference during infrared and NMR analyses. Desalting was carried out using an Amberlite IR-120 (H^+) ion-exchange resin (2.4.5.1). After evaporation, the residue was dissolved in water and frozen dry.

The results of these experiments, which will not be discussed in detail, showed that 'unknown Y' displayed an U-V-absorption spectrum similar to that of the U-V-absorbing compound UV 2 (cf. 6.3.4). Since the latter compound was found to be also ninhydrin-positive and after analysis in the amino-acid analyser gave a peak on the chromatogram coinciding with that of 'unknown Y', it was suggested that 'unknown Y' and UV 2 would be identical. This conclusion is in agreement with the observation of BROWN and SILVER (1966) concerning the occurrence in pea-seedling extracts of a pyrimidine-amino acid which according to BROWN and MANGAT (1969) would be uracil-alanine. Further experiments are required to decide if the compound of the present study is uracil-alanine.

8. THE INFLUENCE OF HOMOSERINE ON THE GROWTH OF *RHIZOBIUM*

8.1. Introduction

In chapter 7, section 7.6, it has been reported that a considerable amount of n.p.c. is released from the main root during the formation of the first lateral roots (6 or 7 days after the start of germination). Since approximately 70% of the released amino compounds consisted of homoserine (chapter 7, section 7.5), it was suggested that this amino acid would play an important role in the establishment of the rhizosphere microflora of pea plants either as nitrogen source or as nitrogen, carbon and energy source.

Other compounds may be released during the formation of lateral roots (carbohydrates, organic acids, vitamins) but the effect of these compounds on rhizobial growth depends on the presence of a suitable nitrogen source. In addition, the concentration of these compounds may be low during this stage of root growth as it was reported for carbohydrates in 4.1.

Compounds exuded by the tips of young roots may contribute to the development of a rhizosphere microflora but the conditions at, and close to, the surface of intact seedling roots of pea plants are not very favourable for the multiplication of rhizobia owing to the release of inhibiting compounds (cf. 4.2 and 4.3). Only after the formation of lateral roots, a ready multiplication of the *Rhizobium* cells was found to occur at the root surface, be it restricted to the area of lateral root growth (cf. 4.2). At this part of the root almost all of the nodules developed when pea seedlings, after inoculation with *Rhizobium leguminosarum*, were transferred to sterile soil (fig. 8.1).

The above-mentioned considerations suggest that homoserine is one of the main compounds effecting the growth of *Rhizobium* in the rhizosphere of pea seedlings. Therefore, a number of experiments concerning this subject have been carried out.

8.2. Experimental

The experiments were performed in culture tubes containing 5 ml of nutrient solution (2.3.1). BUM medium (chapter 5) without glutamic acid and mannitol was used as basal medium (B), enriched with glutamic acid (1 g/l), homoserine (1 g/l) and mannitol (5 g/l), added separately or in combination.

Forty three strains of *Rhizobium* belonging to 4 different cross-inoculation groups were tested for growth in these media.

Growth was measured turbidimetrically, 8 days after inoculation (2.3.2.3).

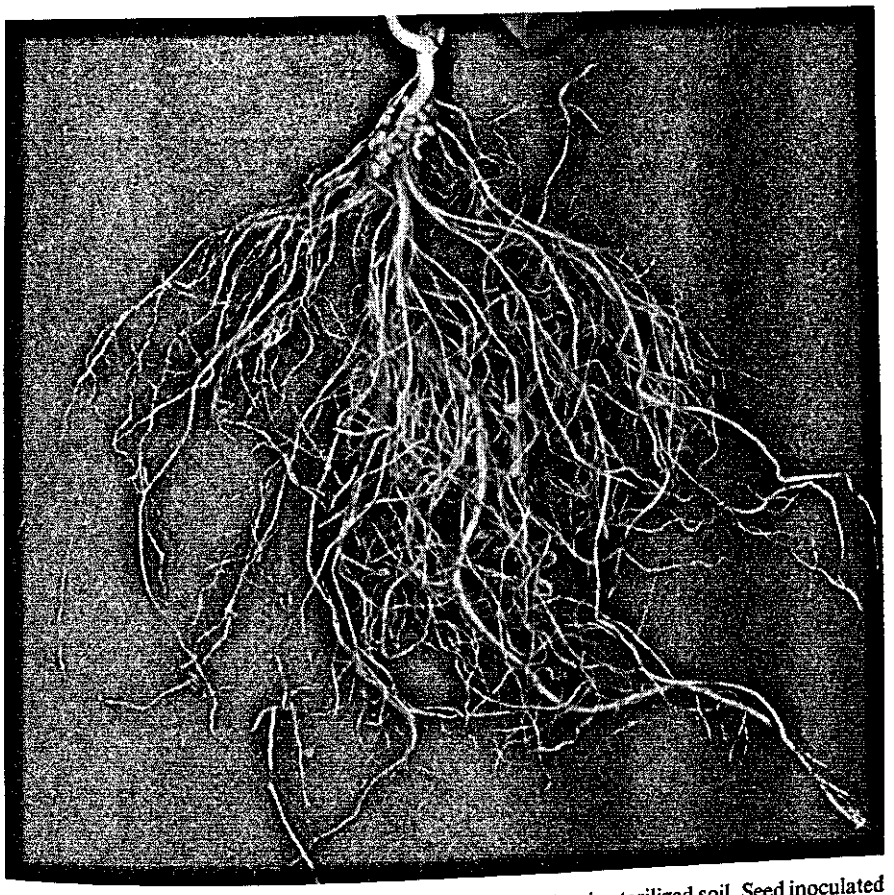


FIG. 8.1. Sites of root-nodule formation on pea plants grown in sterilized soil. Seed inoculated with *Rhizobium leguminosarum*, strain PRE.

8.3. RESULTS AND DISCUSSION

From the results of these experiments (table 8.1; fig. 8.2) it will be seen that almost equally good growth of *Rhizobium leguminosarum* was achieved with glutamic acid or homoserine as the nitrogen source. Similar results were obtained when using these amino acids as the sole source of nitrogen, carbon and energy.

Entirely different results were obtained with strains of *Rhizobium trifolii* and *Rhizobium phaseoli*. With glutamic acid as the only C and N-source, growth was similar to that of *R. leguminosarum*. The same was true of cultures supplied with glutamic acid and mannitol. However, with homoserine as the sole source of nitrogen and carbon, growth was practically absent. When this amino acid was used as nitrogen source only, a slight growth was observed; some strains of *R. trifolii* gave a moderate growth. In the presence of both homoserine and

Table 8.1. The influence of homoserine on growth of rhizobia belonging to different cross-inoculation groups.

Rhizobium strain	Cross-inocul. group	Basal medium					
		Mannitol added			Without mannitol		
		Glutamic acid	Homo-serine	Glutamic a. Homo-serine	Glutamic acid	Homo-serine	Glutamic a. Homo-serine
H 8	Rhiz. leg.	40	45	41	61	62	51
P 8	Rhiz. leg.	32	41	37	61	64	60
P 16	Rhiz. leg.	30	35	40	51	51	57
PF_2	Rhiz. leg.	30	31	39	52	54	49
PRE	Rhiz. leg.	39	40	44	55	59	54
S 310a	Rhiz. leg.	40	44	45	58	60	62
S 310b	Rhiz. leg.	36	46	–	59	63	–
S 313	Rhiz. leg.	29	37	32	53	50	48
317	Rhiz. leg.	32	30	30	51	45	49
B 3	Rhiz. leg.	35	39	37	58	55	55
B 4	Rhiz. leg.	33	37	35	56	58	57
Pn 13	Rhiz. leg.	41	39	43	53	59	56
Pn 27	Rhiz. leg.	40	40	41	57	56	59
Po 32	Rhiz. leg.	36	39	35	94	92	90
H 6	Rhiz. leg.	32	36	33	59	52	60
BPe	Rhiz. meliloti	26	35	35	50	45	48
K 24	Rhiz. meliloti	34	41	48	54	85	77
106	Rhiz. meliloti	38	39	45	60	92	76
A 148	Rhiz. meliloti	31	36	40	50	60	56
A 161	Rhiz. meliloti	32	38	35	49	73	52
A 145	Rhiz. meliloti	30	40	39	46	85	78
Blink	Rhiz. phaseoli	37	82	80	60	95	86
S 460	Rhiz. phaseoli	31	80	73	55	90	83
Bokum	Rhiz. phaseoli	44	80	79	54	93	90
V 23	Rhiz. phaseoli	42	79	85	60	97	90
WH 1	Rhiz. phaseoli	33	72	72	56	94	89
Zijlma	Rhiz. phaseoli	36	88	80	65	97	93
AR 3	Rhiz. trifolii	38	70	65	62	95	90
A 121111	Rhiz. trifolii	41	62	68	61	91	90
CLF	Rhiz. trifolii	38	70	60	52	90	83
Coryn	Rhiz. trifolii	42	71	64	56	92	91
Coryn KL	Rhiz. trifolii	41	75	72	55	90	92
F 12	Rhiz. trifolii	44	74	70	60	89	92
In 1	Rhiz. trifolii	45	76	77	60	88	93
In 2	Rhiz. trifolii	40	78	72	54	92	90
In 30[2]	Rhiz. trifolii	38	80	75	61	94	91
K 8	Rhiz. trifolii	38	84	72	51	96	93
In 32	Rhiz. trifolii	35	64	61	60	92	93
N	Rhiz. trifolii	32	68	64	62	89	92
OB 1a	Rhiz. trifolii	39	70	–	59	91	89
TmZ2	Rhiz. trifolii	40	60	–	58	94	86
A 139	Rhiz. trifolii	38	72	–	55	95	92

Growth measured turbidimetrically as percentage transmittance.
Transmittance basal medium (B), not inoculated, 100.

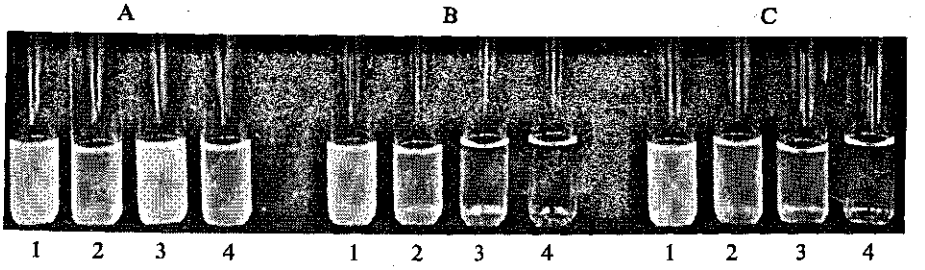


FIG. 8.2. The influence of homoserine on growth of rhizobia belonging to different cross-inoculation groups.
A. *Rhizobium leguminosarum*, strain PF_2; B. *Rhizobium phaseoli*, strain Zijlma; C. *Rhizobium trifolii*, strain CLF.
1. Basal medium (B) supplemented with glutamic acid and mannitol; 2. B + glutamic acid; 3. B + homoserine + mannitol; 4. B + homoserine.

glutamic acid, either with or without mannitol, the growth was strongly reduced as compared with glutamic acid as the sole N-source. In these strains homoserine apparently was toxic.

The response of *R. meliloti* to homoserine was different from that of the other types of *Rhizobium*. When used as the nitrogen source, this amino acid gave almost equally good growth as glutamic acid. However, when serving as both the nitrogen and carbon source, yields, with one exception, were considerably lower than with glutamic acid. When homoserine had been added to cultures supplied with glutamic acid, either in the presence or absence of mannitol, growth was only slightly reduced. This means that homoserine was practically not toxic to *R. meliloti* in the concentrations used.

These experiments have clearly shown that homoserine favours the growth of those *Rhizobium* strains which are capable of producing nodules with peas, i.e. strains belonging to the cross-inoculation group *Rhizobium leguminosarum*. This is true of cultures utilizing homoserine as both the C and N-source. In the presence of adequate amounts of an assimilable C-compound (e.g. mannitol) *R. meliloti* behaved similarly to *R. leguminosarum*. However, it may be assumed that under natural conditions such assimilable C-compounds will hardly be available in the rhizosphere of pea seedlings. This means that the homoserine released from the main root during the formation of (the first) lateral roots selectively stimulates the growth of *Rhizobium leguminosarum* when a mixture of *Rhizobium* strains belonging to different cross-inoculation groups is present in the surroundings of the young pea root. Even, when the rhizobia of other cross-inoculation groups are not inhibited by homoserine and are able to utilize other compounds exuded, *Rhizobium leguminosarum* will accumulate due to its capacity to utilize homoserine as both the C and N-source and because of the proportionally large amounts of homoserine available.

Selective stimulation of *Rhizobium* by the host to be infected has already been found by, among others, WILSON (1930), ROVIRA (1961), NUTMAN (1963),

ROBINSON (1967), FÅHRAEUS and JUNGGREN (1968). However, these authors did not detect the cause of the stimulation. ROVIRA (1965, 1969), stated that the selective stimulation may well depend on the more varied root exudates of legumes as compared with those of other plants. It would be unlikely that the ubiquitous sugars, amino acids and organic acids would provide the specificity, but rather the balance of these compounds or the presence of exotic compounds peculiar to a particular plant species.

In the present investigation, the probable cause of such a specific stimulation is reported for the first time. However, definite proof of this assumption is awaiting inoculation experiments with pea seedlings using mixtures of *Rhizobium* strains belonging to different cross-inoculation groups.

SUMMARY

The main purpose of this investigation was to study the exudation (mechanism, sites) of various compounds by roots of pea seedlings in relation to the growth of *Rhizobium leguminosarum*.

Chapter 1 gives a survey of the literature pertaining to plant-root exudates and their influence upon soil microorganisms. In chapter 2, material and methods used in the investigation are described.

Chapter 3 deals with experiments to localize the sites of exudation of young pea roots. In agreement with the generally accepted view, the tips of the main root as well as of the lateral roots were found to be important sites of exudation (fig. 3.1). Furthermore, a considerable release of ninhydrin-positive compounds occurred during the formation of lateral roots, a process by which the main root is severely damaged (figures 3.2 and 3.3).

In chapter 4, experiments are recorded on the effect of root exudates, obtained from pea seedlings, on the growth of *Rhizobium leguminosarum* in a mineral salts' medium. Furthermore, the behaviour of *Rhizobium leguminosarum* on the root surface (before and shortly after the emergence of the first lateral roots) and in the direct vicinity of roots of pea seedlings is described.

Exudates from growing roots were found to enable the growth of *Rhizobium leguminosarum*. Addition of a carbon source to the medium considerably increased the stimulatory effect of the exudates, indicating that excessive amounts of nitrogenous compounds were present in the root exudates during the early stages of growth, whereas assimilable carbohydrates were present in limiting amounts (table 4.1).

Roots of pea seedlings without lateral roots, inoculated with *Rhizobium leguminosarum*, were unable to support growth of the bacteria on the root surface. Even a considerable drop of bacterial numbers occurred, suggesting the release of growth-inhibiting compounds by the roots (table 4.2). After the formation of lateral roots a strong increase of bacterial numbers on the root surface was observed. This increase was restricted to that part of the main root where lateral roots were present (table 4.4).

When pea seedlings were grown in Petri dishes with their roots on agar (carbon source present), mixed with a suspension of *Rhizobium leguminasorum*, no zone of stimulated bacterial growth was observed in the vicinity of the root (fig. 4.5). This was in contrast with seedlings of a number of other legumes which under the same experimental conditions gave a distinct zone of bacterial growth near the roots (fig. 4.4). Only where lateral roots emerged from the main pea root was a rather weak stimulation of the rhizobia observed after some time (fig. 4.6). Removal of the cotyledons and stems of the pea seedlings growing on the agar resulted in an enormous growth of *Rhizobium leguminosarum* around the entire root system (fig. 4.7).

Lateral roots of pea seedlings growing in sterile agar along the bottom of the Petri dish upon inoculation with *R. leguminosarum* gave a zone of bacterial growth. This zone started at some distance from the root, apparently owing to the presence of a zone of growth inhibition surrounding the roots more closely (fig. 4.9).

When *R. leguminosarum* was grown in tubes in an agar medium supplemented with yeast extract or glutamic acid, growth was confined to the upper part of the agar with a maximum at some distance below the surface of the agar. However, when seedlings of various legumes were present with their roots in the agar, rhizobial growth occurred along the entire root system down to the bottom of the tube as a result of transport of oxygen through the plant and excretion by the roots (fig. 4.11). In the case of pea seedlings, the bacterial growth was present at some distance from the roots, apparently owing to a zone of inhibition surrounding the pea root (fig. 4.12).

When no glutamic acid or yeast extract had been supplied to the agar, a clear zone of bacterial growth was observed surrounding the roots of all the legumes tested (fig. 4.10), except with peas where no zone of stimulated growth occurred.

In chapter 5 the composition of a synthetic medium, suitable for the growth of *Rhizobium leguminosarum*, strain PRE, is given. This strain, frequently used in the present investigations, did not grow on regular *Rhizobium* media. It was found that sulfhydryl compounds together with uracil or cytosine had to be added to the basal medium to achieve optimal yields (table 5.1). A large number of *Rhizobium* strains tested in this medium showed excellent growth. The nutrient solution, by omitting certain constituents, was used to investigate the action of certain specific root exudates on the growth of *Rhizobium* spp. *in vitro*.

In chapter 6 the exudation of nucleic-acid derivatives by roots of pea seedlings, grown in water, is described. Apparently two U-V-absorbing compounds were exuded by the growing roots (fig. 6.3), UV 1 and UV 2. The former consists of uracil and a derivative of uracil which after hydrolysis gave uracil (figures 6.4 and 6.5). UV 2, in subsequent experiments, appeared to be also ninhydrin-positive (see chapter 7).

The amounts of UV 1, exuded by roots without lateral roots, were found to be proportional to time of exudation while a considerable increase of the exudation of this compound was noticed after the formation of lateral roots (table 6.1). This compound is presumably synthesized in the root-tip region (of the main root as well as of the lateral roots), possibly as an intermediate in nucleic-acid metabolism: exudation being a result of overproduction. This agrees with the fact that UV 1 was found in relatively small amounts in pressure juices of whole seedling roots, but in clearly higher concentrations in pressure juices of root tips.

The amount of UV 2 exuded depends on the length of roots being dipped in water at the start of an experiment. Roots of which the tips only were dipped exuded much less compound UV 2 than those which were immersed more deeply (table 6.2). This shows that UV 2 is released by the entire root surface,

rather than by the growing root tips. Relatively high concentrations of UV 2 were found in pressure juices of whole roots. Furthermore, root scrapings (including root hairs, epidermal cells and outer cortex cells) contained large amounts of UV 2. When the roots of pea seedlings (no lateral roots present), grown in humid air, were transferred to water, large amounts of UV 2 were released within a few hours, indicating the presence of UV 2 on the root surface and in epidermal cells, becoming damaged upon transfer to water (table 6.3).

Chapter 7 deals with the free ninhydrin-positive compounds (n.p.c.) of pea seedlings and the exudation of these compounds by the roots. Extracts of roots of 5 or 6-days-old pea seedlings were found to contain 24 n.p.c., two of which were unknown: 'unknown X' and 'unknown Y' (figures 7.3 and 7.4). As the peaks of these unknown compounds on the chromatograms corresponded with those of glutathione and aspartic acid as obtained after separation of mixtures of known n.p.c., it was initially believed that these compounds represented glutathione and aspartic acid (cf. the literature survey in 7.3). However, a more detailed investigation showed that this hypothesis was not correct (fig. 7.5 A-F). 'Unknown X' was found to be a dipeptide, most likely consisting of glutamine and alanine and 'unknown Y' was identical with the U-V-absorbing compound UV 2, which most likely is a pyrimidine-amino acid.

Homoserine was quantitatively the most important free n.p.c. in the root extracts. As this amino acid is specific to the genus *Pisum*, other legumes were tested for the presence of such specific compounds. In some of the legumes studied, this was indeed the case. In all of the legume seedlings tested, except peas and *Lathyrus*, the amides glutamine and asparagine were the predominant amino compounds (fig. 7.6 A-I).

Since it might be expected that the exudation of certain compounds by roots of pea seedlings is closely connected with the occurrence of these compounds in the roots, a number of experiments were carried out in which the free amino compounds in different parts (cotyledons, roots, tops) of the seedlings were estimated during a period of approximately 1 month after the start of germination. The pea plants were grown under different environmental conditions, including nutrition.

Homoserine, hardly present in dry pea seeds, increased rapidly in the germinating seed. In the cotyledons, its maximum value was reached 6 or 7 days after wetting the seed (table 7.2 and fig. 7.7). From the cotyledons the amino acid was transported mainly to the developing root system.

Approximately 70% of the total amount of the free n.p.c. of the seedling root consisted of homoserine at 7 days after the start of germination when the concentration of free n.p.c. had reached its maximum. During the subsequent 2–3 weeks the concentration of homoserine in the roots dropped, owing to increased root weight, but the total amount of this amino acid in the roots decreased only slightly. On the 24th day after the start of germination still 50% of the free n.p.c. of pea roots was present as homoserine. Hereafter a pronounced decrease of the amount of homoserine (as well as of other n.p.c.) of the roots

occurred in those plants which had not been supplied with nitrogen (table 7.3 and fig. 7.8). The soluble amino compounds of the roots of these plants, including homoserine, were needed to supply the growing top with nitrogen.

Plants supplied with nitrate did not utilize the soluble nitrogen compounds of the roots for the growth of the tops. As a result of this, the amount of homoserine remained more or less constant from the 7th day after the start of germination until the experiments were finished (31 days after the start of germination; fig. 7.11).

In the tops of 1-week-old pea plants, homoserine was also quantitatively the most important free amino compound. Without added nitrogen, it decreased rapidly from the 7th day after the start of germination owing to its utilization in the synthesis of insoluble plant material (table 7.4 and fig. 7.9). With added nitrogen, synthesis of homoserine took place in the tops from about the 24th day after the start of germination (fig. 7.12).

The amides glutamine and asparagine were quantitatively the second most important amino compounds in the cotyledons of pea seedlings during the first two weeks after the start of germination. Hereafter a sharp increase of the amides took place so that these compounds became predominant and remained so until the cotyledons were exhausted (fig. 7.7). Almost all of the amides were transported to the tops, where they were used for the synthesis of insoluble cell compounds, mainly proteins. In the tops of etiolated plants this synthesis probably hardly occurred so that large amounts of the amides accumulated (fig. 7.14).

Pea plants, inoculated with *Rhizobium leguminosarum* on the 12th day after the start of germination, and with nodules starting nitrogen fixation on approximately the 24th day after germination, rapidly increased in free amino compounds of both nodulated roots and tops. More than 90% of these N-compounds in the roots and nearly 60% in the tops consisted of glutamine and asparagine.

Of the free amino compounds exuded by the root tip, 'unknown Y' was by far the most important compound while 'unknown X' was quantitatively the second most important (fig. 7.15). Homoserine was only present in minor quantities. When extracts were made of 2-mm slices of tips of young pea-seedling roots, 'unknown Y' was predominant in extracts of the first 2-mm slices, whereas homoserine was quantitatively most important in extracts of the second 2-mm slices (2 to 4 mm from the root tip; fig. 7.16 A and B). These results confirm the generally accepted view that the root tips are important sites of exudation.

When roots, without laterals, grown in humid air, were transferred to water, large amounts of 'unknown Y' were released within a short time. (fig. 7.19). This was found to be mainly due to the presence on the root surface of 'unknown Y'. It is assumed that under the conditions of this experiment, 'unknown Y', excreted by the root tip, is adsorbed by the root surface. After placing the root in water, the compound is released.

Pea seedlings cultivated in nutrient solution during a period in which lateral roots had developed, released predominantly homoserine while 'unknown Y'

came second (fig. 7.17). Lateral-root formation obviously cuased release of homoserine which was most likely liberated from the main root by the wounds originated by the formation of lateral roots. Further evidence concerning the importance of wounds for the release of homoserine was obtained by deliberately wounding seedling roots (grown in nutrient solution) shortly before the formation of lateral roots. After placing these roots in water, homoserine was by far the most important n.p.c. in the 'exudates'.

Under the conditions of the present investigations, the first lateral roots emerged from the main root 6 or 7 days after the start of germination (fig. 7.18). In this period, the concentration of the free n.p.c., approximately 70% of which consisted of homoserine, was maximal. Therefore, it is clear that during the formation of the first lateral roots, considerable amounts of homoserine were released.

In chapter 8, experiments on the influence of homoserine on the growth of *Rhizobium* are summarized. The data recorded in chapter 7 suggested that homoserine would play an important role in the establishment of the rhizosphere microflora of pea plants.

Compounds exuded by the tips of young roots in general will contribute to the development of a rhizosphere microflora, but the conditions on and close to the surface of intact seedling roots of pea plants are unfavourable for the multiplication of rhizobia owing to the release of inhibiting compounds (chapter 4).

The experiments described in chapter 8 showed that *R. leguminosarum* grew equally well with homoserine as with glutamic acid as the nitrogen source or as the sole source of nitrogen, carbon and energy. Strains of *R. trifolii* and *R. phaseoli* behaved entirely differently. With glutamic acid as the only C and N-source, growth was similar to that of *R. leguminosarum*, but with homoserine growth was practically absent. Using this amino acid as the nitrogen source only, slight growth was observed. In the presence of homoserine and glutamic acid, the growth was strongly reduced as compared with glutamic acid as the sole N-source. For these strains homoserine was apparently toxic.

The response of *R. meliloti* to homoserine was different from that of the other types of *Rhizobium*. When serving as both the nitrogen and carbon source, this amino acid gave considerably lower yields than glutamic acid, but when used as the nitrogen source only, it gave almost equally good growth as glutamic acid.

These experiments show that homoserine, functioning as N, C and energy source, enables the growth of those *Rhizobium* strains which are capable of producing nodules with pea plants, i.e. strains belonging to the cross-inoculation group *R. leguminosarum*. This means that homoserine, released from the main root during the formation of the first lateral roots, selectively stimulates the growth of *Rhizobium leguminosarum* when a mixture of *Rhizobium* strains, belonging to different cross-inoculation groups, is present in the surroundings of the young pea root. However, definite proof of this assumption is awaiting inoculation experiments with pea seedlings using mixtures of *Rhizobium* strains belonging to different cross-inoculation groups.

SAMENVATTING

Het voornaamste doel van dit onderzoek was het bestuderen van de uitscheiding (mechanisme, plaats van afgifte) van verschillende verbindingen door wortels van kiemplanten van erwten in verband met de groei van *Rhizobium leguminosarum.*

Hoofdstuk 1 geeft een overzicht van de literatuur die betrekking heeft op worteluitscheidingen en hun invloed op bodemmicro-organismen. In hoofdstuk 2 zijn materiaal en methoden beschreven welke bij dit onderzoek zijn gebruikt.

Hoofdstuk 3 handelt over de plaatsen van uitscheiding bij jonge erwtewortels. In overeenstemming met de algemeen heersende inzichten werd vastgesteld dat worteltoppen van zowel hoofdwortels als zijwortels belangrijke plaatsen van uitscheiding zijn. Bovendien trad een aanzienlijke afgifte van ninhydrine-positieve verbindingen op gedurende de vorming van zijwortels. Bij dit proces wordt de hoofdwortel ernstig beschadigd (figuren 3.1, 3.2 en 3.3).

In hoofdstuk 4 zijn proeven beschreven over de invloed van worteluitscheidingsprodukten van kiemplanten van erwten op de groei van *Rhizobium leguminosarum.* Verder zijn proeven vermeld over het gedrag van *R. leguminosarum* op het worteloppervlak (voor en kort na het doorbreken van de eerste zijwortels) en in de directe omgeving van wortels van erwtekiemplanten.

Uitscheidingsprodukten van groeiende wortels veroorzaakten de groei van *Rhizobium leguminosarum* in een voedingsoplossing met anorganische zouten. Toevoeging van een C-bron aan het medium gaf een aanzienlijke verhoging van het stimulerende effect van de uitscheidingsprodukten hetgeen wijst op de aanwezigheid van een overmaat stikstofhoudende verbindingen in de worteluitscheidingsprodukten van jonge kiemplanten terwijl assimileerbare koolhydraten in groei-beperkende hoeveelheden aanwezig waren (tabel 4.1).

Wortels van erwtekiemplanten zonder zijwortels, geënt met *R. leguminosarum,* waren niet in staat om de groei van deze bacteriën op het worteloppervlak mogelijk te maken. In eerste instantie trad zelfs een belangrijke daling van het aantal bacteriën op wat de afgifte van een groeiremmende stof door de wortel doet vermoeden (tabel 4.2). Na vorming van zijwortels werd een sterke toename van het aantal bacteriën op het worteloppervlak waargenomen. Deze toename was echter beperkt tot dat gedeelte van de hoofdwortel waar zijwortels waren ontstaan (tabel 4.4).

Erwtekiemplanten, groeiend in Petri-schalen met de wortels in contact met agar welke was voorzien van een C-bron en was gemengd met een suspensie van *R. leguminosarum,* vertoonden geen zone van groeibevordering van de bacteriën in de buurt van de wortels (fig. 4.5). Dit was in tegenstelling met kiemplanten van een aantal andere leguminosen die onder dezelfde proefomstandig-

heden een duidelijke zone van bacteriegroei dicht bij de wortels gaven (fig. 4.4). Slechts daar waar zijwortels de hoofdwortel van de erwt hadden doorbroken, werd een vrij zwakke groeistimulatie van de bacteriën waargenomen (fig. 4.6). Nadat van de op agar groeiende erwtekiemplanten, zaadlobben en stengels waren weggenomen, ontstond een enorme groei van *Rhizobium leguminosarum* rond het gehele wortelstelsel (fig. 4.7).

Zijwortels van erwtekiemplanten welke in steriele agar langs de bodem van de Petri-schaal groeiden, vertoonden na enting met *R. leguminosarum* een zone van bacteriegroei. Deze zone begon op enige afstand van de wortel hetgeen waarschijnlijk een gevolg was van de aanwezigheid van een zone van groeiinhibitie dicht bij de wortel (fig. 4.9).

In buizen gevuld met agar, waaraan gistextract of glutaminezuur was toegevoegd, bleef de groei van *R. leguminosarum* beperkt tot het bovenste gedeelte met een maximum op enige afstand onder het agaroppervlak. Echter, wanneer wortels van kiemplanten van leguminosen in deze agarbuizen groeiden, ontstond langs de gehele wortel tot aan de bodem van de buis groei van *Rhizobium* als gevolg van transport en uitscheiding van zuurstof door de wortels (fig. 4.11). Indien erwtekiemplanten waren gebruikt, was groei van *Rhizobium* eveneens aanwezig maar op enige afstand van de wortel, klaarblijkelijk weer te wijten aan de zone van inhibitie rond de wortel (fig. 4.12).

Wanneer gistextract of glutaminezuur niet aan de agar waren toegevoegd, werd toch een duidelijke zone van bacteriegroei rond de gehele wortel waargenomen bij alle onderzochte leguminosen (fig. 4.10) uitgezonderd bij de erwt.

In hoofdstuk 5 is de samenstelling beschreven van een synthetisch medium, geschikt voor de groei van *Rhizobium leguminosarum*, stam PRE. Deze stam, vaak gebruikt in dit onderzoek, groeide niet op een van de bestaande *Rhizobium* media. Sulfhydryl-verbindingen samen met uracil of cytosine moesten aan een basismedium worden toegevoegd om groei te verkrijgen (tabel 5.1). Een groot aantal andere *Rhizobium*-stammen getest op dit medium vertoonde eveneens uitstekende groei. Het medium, met weglating van bepaalde verbindingen, werd gebruikt om de werking van specifieke worteluitscheidingsprodukten op de groei van *Rhizobium* spp. te onderzoeken.

In hoofdstuk 6 is de uitscheiding van nucleïnezuur-derivaten door wortels van erwtekiemplanten beschreven. Door in water groeiende wortels werden ogenschijnlijk 2 ultra-violet-licht absorberende verbindingen uitgescheiden (fig. 6.3), UV 1 en UV 2. De eerste component bleek te bestaan uit uracil en uit een derivaat van uracil, welke na hydrolyse uracil gaf (figuren 6.4 en 6.5). UV 2 was, zoals uit latere proeven bleek, ook ninhydrine-positief (zie hoofstuk 7).

De hoeveelheid UV 1 uitgescheiden door kiemwortels zonder zijwortels nam toe met de tijd van exudatie, terwijl een aanzienlijke vermeerdering van de uitscheiding van deze stof optrad na vorming van zijwortels (tabel 6.1). Waarschijnlijk wordt de verbinding gesynthetiseerd in de worteltop (van hoofdwortels zowel als van zijwortels), mogelijk als een intermediair in het nucleïnezuur-

metabolisme; exudatie zou het resultaat zijn van overproduktie. Dit is in overeenstemming met het feit dat UV 1 in relatief zeer geringe hoeveelheden werd gevonden in perssappen van gehele wortels maar in duidelijk hogere concentraties in perssappen van worteltoppen.

De hoeveelheid UV 2, uitgescheiden door wortels zonder zijwortels, was afhankelijk van de lengte van het stuk wortel dat zich bij het begin van een proef in het water bevond (wortels voorgekweekt in vochtige lucht). Als alleen worteltoppen in contact waren met het water, werd veel minder UV 2 afgegeven dan wanneer de wortels dieper in het water werden gebracht (tabel 6.2). Dit wijst op een afgifte van UV 2 door de gehele wortel. Relatief hoge concentraties van UV 2 werden gevonden in de perssappen van gehele wortels. Wortelschraapsels (epidermiscellen, wortelharen, cellen van buitenste schorslagen) bevatten ook grote hoeveelheden UV 2.

Wanneer wortels van erwtekiemplanten (geen zijwortels aanwezig), gegroeid in vochtige lucht, in hun geheel in water werden geplaatst, werd binnen enkele uren een grote hoeveelheid UV 2 afgegeven. Dit wijst op de aanwezigheid van UV 2 op het worteloppervlak en in de epidermiscellen, welke zeer waarschijnlijk bij de plotselinge plaatsing van de wortel in water worden beschadigd (tabel 6.3).

Hoofdstuk 7 geeft een beschrijving van de vrije ninhydrine-positieve verbindingen (n.p.c.) aanwezig in kiemplanten van erwten en van de uitscheiding van deze stoffen door de wortel. In wortelextracten van 5 of 6 dagen oude erwtekiemplanten kwamen 24 n.p.c. voor; twee ervan waren onbekend 'unknown X' en 'unknown Y' (figuren 7.3 en 7.4). Omdat de pieken van deze onbekende verbindingen op het chromatogram overeenstemden met die van glutathion en asparaginezuur (chromatogrammen verkregen na scheiding van bekende n.p.c.) werd eerst aangenomen dat deze verbindingen identiek waren met glutathion en asparaginezuur (zie literatuuroverzicht onder 7.3). Een meer gedetailleerd onderzoek toonde echter aan dat deze veronderstelling niet juist was (fig. 7.5 A-F). 'Unknown X' bleek een dipeptide te zijn, waarschijnlijk bestaande uit glutamine en alanine en 'unknown Y' was identiek aan de U-V-absorberende stof UV 2 en is hoogstwaarschijnlijk een pyrimidine-aminozuur.

Homoserine was kwantitatief de belangrijkste vrije aminoverbinding in de wortelextracten. Daar dit aminozuur specifiek is voor het geslacht *Pisum*, werden andere leguminosen onderzocht op de aanwezigheid van soortgelijke specifieke verbindingen. In enkele van de bestudeerde leguminosen was dit inderdaad het geval. In de kiemplanten van alle geteste leguminosen, met uitzondering van erwt en *Lathyrus*, waren de amiden glutamine en asparagine kwantitatief de belangrijkste aminoverbindingen (fig. 7.6 A-I).

Daar men mag verwachten, dat de uitscheiding van bepaalde verbindingen door wortels van erwtekiemplanten nauw verbonden is met de aanwezigheid van deze stoffen in de wortel, werd een aantal experimenten uitgevoerd waarbij de vrije amino-verbindingen in verschillende delen (zaadlobben, wortels, bovengrondse delen) van kiemplanten, gegroeid onder verschillende omstandigheden werden bepaald gedurende een periode van 1 maand na het begin van de kieming.

Homoserine, nauwelijks aanwezig in droog erwtezaad, nam zeer snel toe in ontkiemend zaad. In de zaadlobben werd de maximumhoeveelheid bereikt 6 tot 7 dagen na bevochtiging van het zaad (tabel 7.2 en fig. 7.7). Vanuit de zaadlobben werd het aminozuur voornamelijk getransporteerd naar het zich ontwikkelende wortelstelsel.

Zeven dagen na het begin van de ontkieming bestond ongeveer 70 % van de totale hoeveelheid vrije n.p.c. in wortels van erwtekiemplanten uit homoserine. Op dit moment had de concentratie van vrije n.p.c. een maximum bereikt. Gedurende de 2–3 volgende weken zakte de concentratie van homoserine als gevolg van het toenemende wortelgewicht, maar de totale hoeveelheid van dit aminozuur in de wortels daalde slechts weinig. Op de 24ste dag na het begin van de ontkieming maakte homoserine nog de helft van de aanwezige vrije n.p.c. uit. Hierna trad een duidelijke daling van dit aminozuur op (evenals van alle andere n.p.c.) in wortels van planten welke geen stikstof hadden gekregen (tabel 7.3 en fig. 8.7). De oplosbare stikstofverbindingen uit de wortels van deze planten waren nodig om de groeiende bovengrondse delen van stikstof te voorzien.

Planten, welke nitraat hadden gekregen, gebruikten de oplosbare stikstofverbindingen uit de wortel niet. Als gevolg hiervan bleef de hoeveelheid homoserine in de wortel min of meer constant vanaf de 7de dag na het begin van de ontkieming totdat de experimenten werden beëindigd (31 dagen na het begin van de ontkieming; fig. 7.11).

Ook in de bovengrondse delen van 1 week oude erwteplanten was homoserine kwantitatief de meest belangrijke vrije amino-verbinding. Zonder toegevoegde stikstof daalde de hoeveelheid snel vanaf de 7de dag na het begin van de ontkieming als gevolg van het gebruik van dit aminozuur voor de synthese van onoplosbaar plantmateriaal (tabel 7.4, fig. 7.9). Indien nitraat was toegevoegd, vond in de bovengrondse delen vanaf de 24ste dag na het begin van de ontkieming synthese van homoserine plaats (fig. 7.12).

De amiden glutamine en asparagine waren, na homoserine, kwantitatief de belangrijkste amino-verbindingen in de zaadlobben van erwtekiemplanten gedurende de eerste twee weken na het begin van de ontkieming. Daarna trad een sterke stijging van beide amiden op, zodat deze verbindingen de overhand kregen en deze behielden totdat de zaadlobben waren uitgeput (fig. 7.7). Bijna alle glutamine en asparagine werd getransporteerd naar de bovengrondse delen waar het werd gebruikt voor de synthese van onoplosbare celcomponenten vnl. eiwitten. In de bovengrondse delen van geëtioleerde planten vond deze synthese nauwelijks plaats, zodat grote hoeveelheden van beide amiden werden opgehoopt (fig. 7.14).

Bij erwteplanten, geënt met *Rhizobium leguminosarum* op de 12de dag na het begin van de ontkieming en waarbij de stikstofbinding begon op de 24ste dag na ontkieming, vond zowel in de wortels als in de bovengrondse delen een sterke toename plaats van vrije amino-verbindingen. Meer dan 90 % van deze verbindingen in de wortels en bijna 60 % in de bovengrondse delen bestond uit glutamine en asparagine.

Van de vrije n.p.c., uitgescheiden door de worteltoppen van erwtekiemplan-

ten, was 'unknown Y' kwantitatief verreweg de belangrijkste component, terwijl 'unknown X' op de tweede plaats kwam (fig. 7.15). Homoserine was slechts in geringe hoeveelheden aanwezig. Wanneer extracten werden gemaakt van 2-mm schijfjes van topjes van kiemwortels van erwtekiemplanten, was 'unknown Y' overheersend in extracten van de eerste 2-mm schijfjes, terwijl homoserine kwantitatief het belangrijkst was in extracten van de volgende 2-mm schijfjes (2–4 mm van de worteltop; fig. 7.16 A en B). Deze resultaten zijn in overeenstemming met de algemeen heersende inzichten omtrent de betekenis van de worteltop als plaats van uitscheiding.

Wanneer wortels, zonder zijwortels, gegroeid in vochtige lucht, in water werden geplaatst, werd in korte tijd een grote hoeveelheid 'unknown Y' door de wortels afgegeven (fig. 7.19). Dit was voornamelijk te wijten aan de aanwezigheid van 'unknown Y' op het worteloppervlak. De veronderstelling is, dat onder de omstandigheden van het huidige experiment 'unknown Y', uitgescheiden door de worteltop, wordt geadsorbeerd aan het worteloppervlak. Na plaatsing van de wortel in water verlaat deze verbinding het worteloppervlak.

Wanneer erwtekiemplanten werden gecultiveerd in een voedingsoplossing gedurende een periode waarin zich zijwortels ontwikkelden, werd vnl. homoserine afgegeven, terwijl 'unknown Y' op de tweede plaats kwam (fig. 7.17). Zijwortelvorming veroorzaakt klaarblijkelijk afgifte van homoserine, hoogstwaarschijnlijk vrijgekomen uit de hoofdwortel via de verwondingen welke optraden tijdens de vorming van zijwortels. Een verdere aanwijzing omtrent het belang van verwondingen voor het vrijkomen van homoserine werd verkregen door wortels van erwtekiemplanten (gegroeid in voedingsoplossing) kort voor het ontstaan van zijwortels moedwillig te verwonden. Na plaatsing van deze wortels in water werd als verreweg belangrijkste n.p.c. homoserine in de „uitscheidingsprodukten" gevonden.

Onder de omstandigheden van het huidige onderzoek kwamen de eerste zijwortels 6 of 7 dagen na het begin van de ontkieming uit de hoofdwortel te voorschijn (fig. 7.18). In deze periode was de concentratie van de vrije n.p.c., waarvan homoserine ongeveer 70% uitmaakte, maximaal. Daarom is het duidelijk, dat gedurende de vorming van de eerste zijwortels aanzienlijke hoeveelheden homoserine vrijkwamen.

In hoofdstuk 8 zijn experimenten vermeld over de invloed van homoserine op de groei van *Rhizobium*. De waarnemingen uit hoofdstuk 7 wijzen op het belang van homoserine voor het ontstaan van de rhizosfeer-microflora bij erwteplanten. In het algemeen zullen componenten uitgescheiden door de worteltoppen eveneens bijdragen tot het ontstaan van de rhizosfeer-microflora, maar de condities dichtbij en op het worteloppervlak van intacte wortels van erwtekiemplanten zijn niet gunstig voor de vermenigvuldiging van rhizobia als gevolg van de afgifte van remmende verbindingen (hoofdstuk 4).

De experimenten beschreven in hoofdstuk 8 toonden aan dat *R. leguminosarum* evengoed groeide met glutaminezuur of met homoserine als stikstofbron of als enige bron voor stikstof, koolstof en energie. Stammen van *R. trifolii*

en *R. phaseoli* gedroegen zich volkomen anders. Met glutaminezuur als enige bron voor C en N was de groei gelijk aan die van *R. leguminosarum* maar met homoserine trad dan praktisch geen groei op. Wanneer dit aminozuur alleen als stikstofbron werd gebruikt, werd een geringe groei waargenomen. Wanneer glutaminezuur en homoserine beide aanwezig waren, was de groei, vergeleken met die met alleen glutaminezuur als N-bron, sterk gereduceerd. Voor deze *Rhizobium*-stammen werkt homoserine klaarblijkelijk giftig.
Rhizobium meliloti reageerde anders op homoserine dan de andere *Rhizobium*-typen. Wanneer dit aminozuur fungeert als stikstof- en koolstofbron, werden aanzienlijk lagere opbrengsten verkregen dan wanneer glutaminezuur als zodanig werd gebruikt. Wanneer homoserine alleen als stikstofbron werd gebruikt, was de groei bijna even goed als met glutaminezuur.

Deze experimenten tonen aan dat homoserine, fungerend als N-, C- en energiebron, de groei van die *Rhizobium*-stammen mogelijk maakt, welke in staat zijn om met erwteplanten knollen te vormen, dus stammen behorend tot de 'cross-inoculation'-groep *Rhizobium leguminosarum*. Dit betekent dat homoserine, afgegeven door de hoofdwortel gedurende de vorming van de eerste zijwortels, selectief de groei van *R. leguminosarum* stimuleert wanneer een mengsel van *Rhizobium*-stammen, behorend tot verschillende 'cross-inoculation'-groepen, aanwezig is in de omgeving van de jonge erwtewortel. Voor het definitieve bewijs van deze verklaring zijn entingsproeven met erwtekiemplanten nodig waarbij mengsels van *Rhizobium*-stammen, behorend tot verschillende 'cross-inoculation'-groepen, moeten worden gebruikt.

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