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Nitrogen fixation in the phyllosphere of Gramineae

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Abstract

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The investigation was carried out with *Zea mays*, grown under temperate conditions, and with *Tripsacum laxum* Nash, grown in the tropics. The conditions for nitrogen fixation were found to be unfavourable in the leachate, obtained by spray irrigation of the aerial plant parts of both experimental plants, but were favourable in the water present between sheath and stem. The ability of the phyllosphere micro-flora to fix nitrogen was proved qualitatively with the acetylene-reduction technique. The amount of nitrogen fixed by the micro-organisms in the phyllosphere of guatemala grass (260–400 g/ha per year) was estimated by exposing entire plants to ^{15}N in situ. The nitrogen-fixing micro-organisms belonged mainly to the *Enterobacteriaceae* (genus *Klebsiella*).

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Contents

1 Introduction

The influence of permanent pastures on soil fertility in the tropics is still an unsolved problem. Many experiments have been carried out to investigate the gains of nitrogen in the absence of legumes in tropical soils. Based on literature review, Moore (1966) stated that the nitrogen gains are the results of numerous micro-organisms fixing small quantities of nitrogen. The International Biological Programme has stimulated further investigations on the significance of nitrogen fixation. My part in this program was concerned with nitrogen fixation by free-living micro-organisms on the leaf surface of *Zea mays*, grown under temperate conditions of Western Europe, and of *Tripsacum laxum* Nash, grown under tropical conditions. *Zea mays* is a well-known fodder plant, and *Tripsacum laxum* Nash is a tropical grass, growing to 2 m or more in height, but cut for fodder at a younger stage.

The term 'phyllosphere' was proposed for the environment provided by the wet leaf surface and enabling microbial development (Last, 1955; Ruinen, 1956). Ruinen observed that, together with a considerable variety of other micro-organisms, species of *Beijerinckia* and *Azotobacter* occurred on the surface of the leaves of a wide range of trees and shrubs under humid tropical conditions. She suggested that these organisms might fix an appreciable amount of nitrogen, utilizing carbohydrates excreted by the leaves, and that the vegetation might benefit by foliar uptake of this fixed nitrogen. Based on the carbohydrate concentration of the liquid in the space between the stem and the leaf sheath, she calculated that the grass sheath of *Tripsacum laxum* Nash might be a site for nitrogen fixation (1971). Jones (1970) reported that a considerable proportion of the annual requirement of nitrogen by *Douglas Fir* may be provided by nitrogen-fixing bacteria on the leaves and in the soil.

In the following sections a survey of published work, pertinent to this study, will be given. In the last section of this chapter the scope of the study will be defined in more detail.

1.1 Micro-flora of the phyllosphere of living plants

Although earlier workers (Beijerinck, 1888; Winkler, 1899) have isolated bacteria from plant leaves, Burri (1903) first showed that a variety of plants support an abundant leaf-surface micro-flora. Bacterial populations were observed to range from several millions to more than a hundred million per gram fresh leaf material. In 1904, Düggeli reported similar observations. Several workers (Huss, 1907; Gruber, 1909; Löhnis, 1910; Wolff, 1913) recognized that the micro-flora on the aerial plant parts

differed quantitatively from the micro-flora in the soil.

Phyllosphere populations as determined by several investigators show that great variability exists in the phyllosphere population not only of different plant species but also of individual species sampled at different dates or in different places (Allen, 1937; Thomas & McQuillin, 1952; Kroulik, 1955; Di Menna, 1959; Leben, 1961; Ruinen, 1961). Wieringa (1955) found pectine-decomposing micro-organisms on the leaf surface of several West-European plants. Mulder (1938) studied the micro-flora of the chaffs of wheat plants and found a large number of *Pseudomonas trifolii* spp. Grainger & Keddie (1963) isolated micro-organisms belonging to the *Corynebacteriaceae* from several grasses. Gram-negative, yellow-pigmented bacteria appear to be highly characteristic in the phyllosphere. Yeasts are also common inhabitants of the grass leaves. The yeasts dominant in the phyllosphere are not similar to those dominating in the underlying soil (Di Menna, 1959). Other micro-organisms encountered in the phyllosphere include the following: coliform bacteria (Allen et al., 1937; Graham & Hodgkess, 1967), lactic acid bacteria (Keddie, 1959), aerobic spore formers, micrococci, *Beijerinckia* spp., *Azotobacter* spp., spirilla, actinomycetes, fungi, protozoa (Wolff, 1913; Ruinen, 1956, 1961; Last et al., 1965; Bhat et al., 1971). Perhaps the simplest explanation of the existence of a micro-flora, adapted to the condition of the phyllosphere, is that sugars and organic acids are prominent components of the leaf exudates so that fast-growing, sugar-utilizing micro-organisms, including yeasts, are preferentially encouraged (Clark & Paul, 1970).

1.2 Leaching of nutrients from plants

Leaching of plants is defined by Tukey & Tukey (1962) as the loss of organic and inorganic metabolites from aerial plant parts by the leaching action of an aqueous solution, including rain, mist and dew. The term leaching as used by these authors thus also includes the release (excretion) of compounds which accumulate on the leaf surface, giving rise to the development of the phyllosphere. In this thesis the term leaching is used only when the released compounds in aqueous solution run off the leaves (leachate). The leaching phenomenon is well documented, beginning with de Saussure in 1804 and Hales in 1927. Mes (1954), Long et al. (1956), Tukey et al. (1958) showed with radioactive isotopes that labelled metabolites, which had been introduced into plants, may be leached again and collected.

The leached substances include various materials (Morgan & Tukey, 1964). Inorganic nutrients leached include all the essential major and minor elements found in the plant. In addition, large amounts of organic substances may be leached, including sugars and pectic substances. All of the amino acids found in plants and many of the organic acids have been detected in the leachates. Kozel & Tukey (1968) have shown that growth-regulating compounds, such as the gibberellins, can be leached from the plants as well as vitamins (Wasicky, 1958), alkaloids (Böde, 1958) and phenolic substances (Kozel & Tukey, 1968).

In spite of the large amounts of inorganic nutrients which can be leached (Wallace, 1930; Arens, 1934; Tamm, 1951; Dalbro, 1956), organic substances, principally carbohydrates, account for the major quantity of leached materials. Dalbro (1956) has calculated that losses of carbohydrates from apple trees may amount to 800 kg per ha per year. Up to 6% of the dry weight may be leached each day from young bean leaves, mainly in the form of carbohydrates (Tukey, 1958). No quantitative estimates of losses of amino acids and organic acids have been reported.

Tukey & Morgan (1964) compiled a list of plants from which substances have been leached. This list includes a wide variety of representative species of trees, grains, grasses, tropical plants, in all 180 different plant species. Leaching is widespread in nature and in fact no plant has yet been studied which cannot be leached to some degree.

1.3 Nitrogen fixation by free-living micro-organisms

Biological nitrogen fixation is a characteristic of certain micro-organisms, which may be free-living or occur in symbiotic association with higher plants. Many workers described heterotrophic micro-organisms that could fix N_2. For example, Campbell, et al. (1967) reported that 23 out of 150 isolates from sub-arctic soils fixed nitrogen. Meiklejohn & Weir (1968) isolated 83 bacterial strains from Rhodesian soils and concluded that about half of these could fix nitrogen. Line & Loutit (1971) made several hundred aerobic and anaerobic isolations from some New Zealand tussock grassland soils. Only 1 anaerobic and 5 facultatively anaerobic species were able to fix nitrogen. The Russian workers Federov & Kalininskaya (1961) and Kalininskaya (1967) have studied nitrogen-fixing mycobacteria. Some actinomycetes (Metcalfe & Brown, 1957; Fiuczeck, 1959), yeasts (Metcalfe, 1954; Németh, 1959; Campbell et al., 1967) and fungi (Brown & Metcalfe, 1954; Campbell et al., 1967) have been reported to fix nitrogen. However, Hill & Postgate (1969) recently rechecked some of these organisms and obtained negative results. So far, no conclusive evidence is available about the fixation of elementary nitrogen by eucaryotic micro-organisms.

Heterotrophic nitrogen fixation depends on the availability of carbohydrates or various other carbon compounds, including organic acids, alcohols etc. In general, large quantities of carbon compounds are required for high rates of nitrogen fixation, because nitrogen-fixing heterotrophs are inefficient users of carbon compounds. When combined nitrogen is available, there is much competition between nitrogen-fixing and non-nitrogen-fixing organisms. Approximate efficiency values in terms of mg nitrogen fixed per gram carbohydrate consumed are: *Azotobacter* 10–20, *Clostridium* 2–7, *Klebsiella* 5 and *Bacillus* 12. The amount of assimilable carbon compounds available in most soils is insufficient to support high levels of nitrogen fixation.

Ruinen (1966, 1971) has drawn attention to *Azotobacter* and *Beijerinckia* strains, occurring on the leaf surface of tropical trees and grasses. These organisms obtain carbon and energy from the carbohydrates excreted by the leaves. Her findings are supported by the work of Döbereiner & Alvahydo (1959) who observed that washings

of the shoots and leaves of sugar cane stimulate the growth of *Beijerinckia* in soils, and by the work of Vasantharajan & Bath (1968, 1971) who found that the phyllosphere of mulberry, sandal and dolichos may support a heavy growth of *Azotobacter*.

The oxygen levels have a significant effect on the efficiency of nitrogen fixation by aerobic micro-organisms. Parker (1954) found this efficiency of *Azotobacter* at a pO_2 of 0.04 atm (where p is partial pressure) to be 3 times greater than at 0.2 atm. High pO_2 levels (Schmidt-Lorenz & Rippel-Baldes, 1957) and excessive aeration (Postgate, 1969) inhibit nitrogen fixation. Dalton & Postgate (1968, 1969) studied the influence of oxygen on nitrogen fixation of *Azotobacter* and *Mycobacterium flavum* by using the continuous-culture technique. They concluded that the high respiration rate of the *Azotobacteriaceae* represents a physiological mechanism for protecting the nitrogen-fixing site from damage by oxygen. This hypothesis was confirmed by the findings of Yates (1970) and was supported by previous experiments with purified cell-free extracts of free-living nitrogen fixers. These extracts fix nitrogen only under strict anaerobic conditions (Carnahan et al., 1960). Oppenheim & Marcus (1970) and Oppenheim et al. (1970) have shown that N_2-grown *Azotobacter* possesses an extensive internal membrane network, which is not present in NH_4^+-grown cells and the formation of which is repressed bij NH_4^+. This membrane protects an insoluble nitrogenase from damage by oxygen.

There is much literature showing that combined nitrogen inhibits nitrogen fixation. This inhibition has usually been attributed to the fact that nitrogen is reduced to ammonia and that the presence of free ammonia inhibits nitrogenase activity. Some workers reported that inhibition does not always occur and that the degree and the type of inhibition depend on the level of supplied combined nitrogen. Strandberg & Wilson (1968) showed that cell-free extracts of *Azotobacter* continue to fix nitrogen normally in the presence of ammonium-nitrogen levels up to 40 mg per litre and that concentrations of 150 mg per litre play a role in intact cells, not by inhibiting nitrogenase activity but by repressing nitrogenase synthesis. This hypothesis was in fact suggested by the work of Pengra & Wilson (1958), who noted a lag-phase of several hours before cells, previously grown on combined nitrogen, resumed fixation on transfer to a medium free from combined nitrogen. The data of Stewart et al. (1968) on the alga *Nostoc muscorum* confirm the findings of Wilson's group. All these results suggest that the levels of combined nitrogen in most natural ecosystems are sufficiently low to prevent inhibition of nitrogen fixation.

The presence of low levels of combined nitrogen in nature may actually be advantageous to nitrogen-fixing leguminous plants by preventing the occurrence of extreme nitrogen deficiency in a young growing stage. Such plants may be more vigorous and may sometimes fix more elementary nitrogen than is achieved with N_2 as the sole nitrogen source. This has been noted previously for nodulated non-legumes (McConnel & Bond, 1957; Stewart & Bond, 1961).

1.4 Scope of this study

From the literature survey it follows that very little is known about nitrogen fixation in the phyllosphere of *Gramineae*. Only a few reports indicate that it may occur, but no data are available on the amount of nitrogen fixed. To study nitrogen fixation quantitatively, first an attempt was made to localize those parts of the phyllosphere where the conditions for nitrogen fixation are favourable.

The chemical composition of the excreted material was studied extensively because of its suitability of serving as substrate in biological nitrogen fixation. Data on the micro-flora of the phyllosphere were collected by isolating large numbers of micro-organisms which were tested for a number of characteristics, including nitrogen fixation.

The experiments were carried out with *Zea mays* under temperate conditions in the Netherlands and with *Tripsacum laxum* Nash (guatemala grass) under humid tropical conditions in Surinam.

To study nitrogen fixation in the phyllosphere under natural conditions, use was made of the acetylene-reduction technique. Furthermore, experiments with ^{15}N were carried out in situ with guatemala grass.

2 Methods

2.1 Growth of plants

Growth of plants was measured by estimating height, number of leaves, leaf surface, and contents of nitrogen, phosphorus (P_2O_5), potassium (K_2O), and water-soluble carbohydrates calculated on dry matter.

The leaf surface of *Zea mays* plants was estimated by spreading out the cut leaves on photographic paper (Dalco-N.C.S.), covering them with a glass plate, and exposing them to light for 30 seconds. The prints were developed and the leaf surface determined.

The leaf surface of guatemala grass plants was estimated by cutting all the green leaves of one plant and measuring maximum length (l) and maximum breadth (b) of each leaf. Leaf surface $= 0.75\ l \times b$ (Stickler, 1961).

2.2 Bacteriological methods

2.2.1 Total viable counts

Plate counts were done on glucose yeast extract agar, soil extract azotobacter agar and azotobacter agar. All plates were incubated at 30°C. The colonies on glucose yeast extract agar were counted after 2 days and those on the other media after about 1 week. The countings were carried out in triplicate.

2.2.2 Identification of micro-organisms

Slides were Gram-stained according to common procedure (Adamse, 1970).

Flagella were stained by Bailey's method (1929). The slides were examined under a Wild-M20-phase contrast microscope. Preparations were made of actively growing cultures.

Generally, the method of Hugh & Leifson (1953) gave information on the oxidative or fermentative metabolism of carbohydrates with relation to the identity of micro-organisms.

Cultures grown in glucose-peptone solution were subjected to the Voges-Proskauer and methyl-red tests after incubation for 3 days at 30 °C to indicate the production of acetylmethylcarbinol and of acid (O'Meara, 1931).

The oxidase test was carried out according to the method of Kovacs (1956).

2.2.3 *Media*

Glucose yeast extract agar: glucose, 10 g; yeast extract, 7 g; agar, 12 g; tap water, 1000 ml.

Soil extract azotobacter agar: soil extract, 1000 ml (Chan, Katznelson, Rouatt, 1963); sucrose, 5 g; $MgSO_4.7H_2O$, 0.2 g; KH_2PO_4, 0.2 g; K_2HPO_4, 0.8 g; $FeSO_4.7H_2O$, 0.04 g; $CaCl_2$, 0.15 g; $Na_2MoO_4.2H_2O$, 0.005 g; agar, 12 g.

Azotobacter agar: glucose, 20 g; $MgSO_4.7H_2O$, 0.5 g; K_2HPO_4, 1 g; $CaCO_3$, 20 g; agar, 12 g; tap water, 1000 ml.

Hugh & Leifson medium: glucose, 10 g; peptone, 2 g; K_2HPO_4, 0.3 g; NaCl, 5 g; bromothymolblue, 0.03 g; agar, 3 g; tap water, 1000 ml; pH = 7.1.

Voges-Proskauer and methyl red medium: A) glucose, 5 g; peptone, 7 g; NaCl, 5 g; tap water, 1000 ml. B) NaOH, 20 g; tap water, 100 ml. C) creatin, 1 g; tap water, 100 ml.

Kovacs reagent: ascorbic acid, 0.1 g; tetramethylparaphenylene diamine dihydrochloride, 1 g; tap water 100 ml.

2.3 Chemical methods

2.3.1 *Analysis of the dry matter of the experimental plants*

In the pot experiments, the plants were harvested at different times and, immediately after harvesting, dried. To analyse for nitrogen, phosphorus and potassium, the dry matter was digested with sulphuric acid, salicylic acid and perhydrol (Lindner & Harly, 1942). In the digest, potassium was measured photometrically with an Eppendorf flame-photometer (Schuknecht & Schinkel, 1963), phosphate as the blue phosphomolybdate complex (Murphy & Riley, 1962) while nitrogen was estimated by steam distillation of the ammonia into 2 per cent boric-acid solution and titration with potassium biiodate (van Schouwenburg & Wallinga, 1971). The water-soluble carbohydrates of the dry matter of *Zea mays* were estimated according to the method of van der Plank (1936).

The determinations of nitrogen, phosphorus and potassium were carried out at the Laboratory of Soils and Fertilizers of the Agricultural University.

2.3.2 *Analysis of the soluble fraction of maize plants*

In some field experiments, carbohydrates and nitrogenous compounds of the soluble fraction of maize plants were determined. Several plants were harvested, weighed and cut into small pieces before the determinations (Bell, 1955).

Carbohydrate determination: Samples of 10 g of the cut fresh material were mixed with about 200 ml ethanol for 5–7 min. The mixture was filtered and washed with 50–70% ethanol. The filtrate was concentrated to approximately 100 ml by vacuum rota evaporation. Proteins were precipitated by adding 15 ml of a 20% trichloroacetic-

acid solution. After 15 min, the liquid was filtered through celite powder. The filtrate was desalted by ion exchange with the resins Amberlite IR-120H-AG and IR-450H-AG and concentrated to 20 ml by vacuum rota evaporation. In 1 ml of the concentrated sample the carbohydrates were determined colorimetrically by the anthron method of Trevelyan & Harrison (1952). The reference standard glucose was run with every series of samples.

Nitrogen determination: Samples of 10 g of finely cut fresh material were ground and washed into 100 ml Erlenmeyer flasks, with about 50 ml water. The flasks were then placed in boiling water for 15 min, cooled and supplied with 2.5 ml of 20% trichloroacetic-acid solution to separate proteins and soluble non-protein nitrogen. The mixture was filtered and washed with 10 ml of a 1% trichloroacetic-acid solution. The filtrate was brought into 250 ml Kjeldahl flasks and digested with 2.5 ml sulphuric acid and 2 ml digestion solution. The composition of the digestion solution was: 2 g SeO_2, 1 g $CuSO_4$, 500 g $KHSO_4$ and 1000 ml distilled water. The ammonia in the digest was estimated by nesslerization according to Polly (1954). In each series a reference standard of ammonium sulphate was run simultaneously.

2.3.3 Analysis of sheat and leaf water of the experimental plants

In most field experiments and in some pot experiments, samples of the sheath water (the liquid occurring between the leaf sheath and the stem) and of the leaf water (the liquid present on the surface of the leaves) of the experimental plants were collected for the determination of the amount of carbohydrates and ninhydrin-positive compounds.

The carbohydrates were determined colorimetrically with the anthron method of Trevelyan & Harrison (1952) in samples of 1 ml of collected water.

The ninhydrin-positive compounds were determined in samples of 1 ml of the collected water according to the prescription of Rosen (1957). Leucine was used as reference standard.

2.4 Gas-chromatographical analysis

2.4.1 Measurement of the reduction of acetylene

The reduction of acetylene to ethylene was measured according to Stewart et al. (1967) and Hardy et al. (1968). The produced ethylene was measured with a gas-chromatograph (Unigraph-F, type 407, Becker, Delft, the Netherlands).

Specifications:
detector: flame ionization detector
column: Poropak R, 80–100 mesh, length 100 cm, I.D. 4 mm
carrier gas: commercial nitrogen (Loos and Co, Amsterdam)
gas flow: 40 ml/min.

One hundred µl of acetylene were injected per analysis. The time for one analysis

was 3 min. The ethylene content was calibrated with a standard gas. Under constant conditions, the height of the peaks is linearly related to the concentration of C_2H_2 and C_2H_4 (Hardy et al., 1968).

2.4.2 Measurement of hydrogen and nitrogen

The hydrogen and nitrogen contents of a gas mixture were measured with a Multigraph type 409 gas-chromatograph, Becker, Delft, the Netherlands.

Specifications:
detector: thermal conductivity detector.
column: Molecular sieve 13X (Becker, Delft, the Netherlands), 60–80 mesh A.D. 432, length 200 cm, I.D. 4 mm.
carrier gas: argon (Loos and Co, Amsterdam).
gas flow: 40 ml/min.
One hundred µl gas were injected per analysis.

2.5 Measurement of ^{15}N fixation

Guatemala grass was exposed to elementary nitrogen enriched with ^{15}N in situ. The enrichment of the isotope in several parts of the plant was determined with the NaBrO method of Faust (1967) and measured with the ^{15}N analyser, type Stratron NO I–4 (Isocommerz, DDR). The apparatus used for the preparation of the gas samples was well described by Akkermans (1971).

The intensities of the emission bands at 298.3 nm and 297.7 nm were measured corresponding to the 2–0 transition of the second positive system of ^{14}N–^{15}N and ^{14}N–^{14}N. Based on the intensities of the bands, the enrichment of ^{15}N can be calculated. A calibration curve was made by measuring a series of standard samples, which had been previously analysed with a mass spectrometer.

3 The relation between micro-flora and substrates released into the phyllosphere of Zea mays grown under temperate conditions

3.1 Introduction

The purpose of the experiments described in this chapter was to study the composition of the substrates released into the phyllosphere, to count total numbers of micro-organisms, and to identify the dominating micro-organisms of this environment. For collecting the released substrates, use was made of spray irrigation on the aerial plant parts. The experiments were carried out in pots and in the field with *Zea mays*.

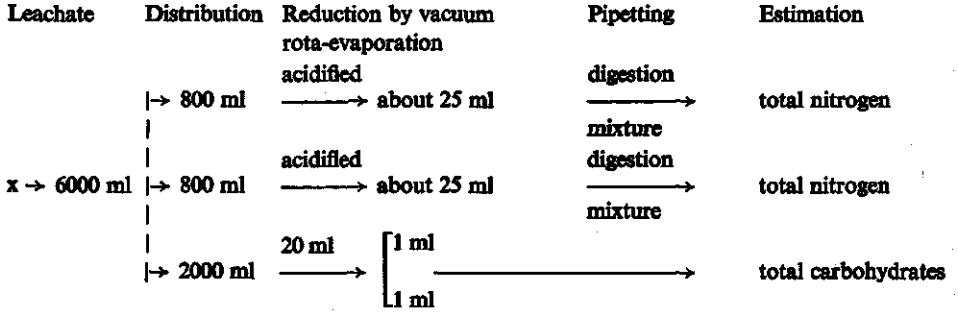
3.2 Leaching experiments with Zea mays grown in pots

3.2.1 Weekly leaching of tops of maize (1967)

Pots of 10 litre were filled with soil and sown with *Zea mays*, each pot containing 3 plants. Nitrogen, as ammonium nitrate, was added in quantities of 0.75 and 4.5 g N per pot, corresponding with 50 and 300 kg N per hectare. All the experiments were duplicated. The spray irrigation was applied when the plants were about 50 cm. Tap water, after being filtered through a 'Fulvo' filter to remove traces of iron, was used. Two pots of each nitrogen level were sprayed once a week, between 8–9 a.m., by placing the plants under a fixed spray-installation. During this treatment the soil in the pots was covered with plastic; each pot was placed in a plastic reservoir of 1 m^2, which served for collecting the leachate. The amount of water sprayed represented a shower of 6 mm, corresponding with an amount of leaching water of 6 l. The leachate was used for the analyses of carbohydrates and nitrogen as shown in Fig. 1. After the spray irrigation, the plants of the two pots were harvested and the height of the plants, the number of leaves and the leaf surface estimated. In the dry plant material nitrogen, phosphorus, potassium and water-soluble carbohydrates were estimated (see Chapter 2).

Samples of the water in the space between stem and leaf sheath, the so-called 'sheath water', were collected from all the leaves of all the plants before spray irrigation. In 1 ml of this sheath water total numbers of micro-organisms, after dilution, were counted on three different agars: glucose yeast extract (rich); soil extract azotobacter (poor in nitrogen); azotobacter (without added nitrogen). From these 3 media about 15 strains were isolated and identified with Bergey's Manual of Determinative Bacteriology.

Fig. 1. Scheme of the distribution of leachates for the carbohydrate and nitrogen analyses.



x is pot submitted to spray irrigation (each treatment included 2 pots).

Growth of Zea mays The growth was measured weekly. The maximum number of leaves was reached at the same date at both nitrogen levels (Table 1). After this date, the plants still grew, but there was no increase in leaf number as the oldest leaves died. The leaf surface of the plants grown at the higher nitrogen level was approximately 15% larger than that of the low-nitrogen plants. When the panicle became visible, the total leaf surface decreased, due to the dying-off of the oldest leaves.

Table 1. Average height of plants, number of leaves and size of the leaf surface of *Zea mays*, grown in pots at two different nitrogen levels (corresponding with 50 and 300 kg N per ha).

Date of harvest	50 kg N/ha			300 kg N/ha		
	height (cm)	number of leaves	leaf surface (cm^2/plant)	height (cm)	number of leaves	leaf surface (cm^2/plant)
10/7	61	10	1241	48	8	1232
17/7	63	11	1482	66	10	.
24/7	86	12	1675	72	11	1893
31/7	115	13	1799	90	13	2037
7/8	123	14	1961	125	14	2222
14/8	130	14	1837	130	14	2291
21/8	130	14	1852	135	14	2165

The results of the analyses of mineral components and water-soluble carbohydrates in the dry matter are given in Fig. 2. It will be seen that plants dressed with the highest amount of nitrogen were considerably higher in nitrogen and phosphate, but lower in potassium and water-soluble carbohydrates. With increasing age, K and N contents displayed a drop (particularly at the highest N dressing), P (at high N supply) and carbohydrates remained at the same level, whereas % dry matter rose.

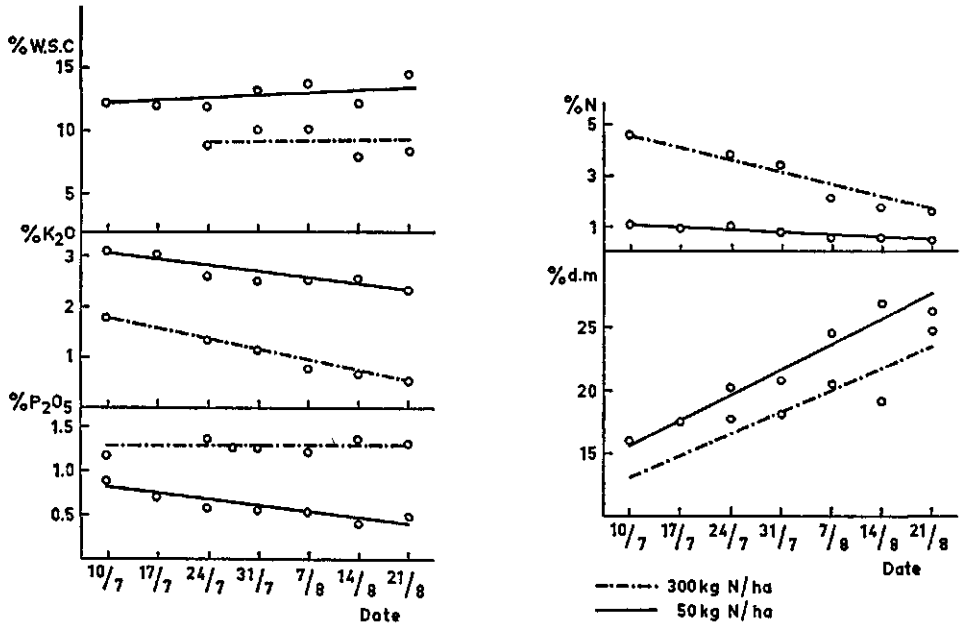


Fig. 2. Analysis of the dry matter of *Zea mays*, grown in pots at two different nitrogen levels (corresponding with 50 and 300 kg N/ha).

Carbohydrate and nitrogen contents in the leachates Leachate of plants grown with the high nitrogen dressing contained more carbohydrates than that from low-N plants (Table 2). This was mainly due to the larger leaf surface of the former plants (Table 1). If the amounts of carbohydrates in the leachates are calculated per cm^2 leaf surface no significant differences between plants with different N treatment are seen (Table 3). With increasing age of the plants, the amount of carbohydrates leached per cm^2 leaf

Table 2. Carbohydrates and total nitrogen (mg/plant) in the leachates of *Zea mays*, grown in pots at two different nitrogen levels (corresponding with 50 and 300 kg N per ha) after a weekly spray irrigation of 6 mm in 60 min.

Date of harvest	Carbohydrates		Nitrogen	
	50	300	50	300
10/7	7.0	5.2	1.4	2.5
17/7	4.2	.	1.9	.
24/7	6.8	7.8	1.4	1.8
31/7	6.8	8.6	0.9	2.5
7/8	6.2	6.8	0.9	1.7
14/8	6.6	9.4	0.9	1.5
21/8	5.4	7.4	0.9	1.1

surface was maintained at the same level with the high-nitrogen plants, but was lower with the low-nitrogen plants.

As total nitrogen in the leachate of 300-N plants was higher than that in the leachate of 50-N plants (Table 2), the nitrogen in the leachate was correlated with the nitrogen content of the dry matter. In contrast to carbohydrates, these differences did not disappear when the amount of nitrogen was calculated per cm^2 leaf surface (Table 3).

The amount of nitrogen leached per cm^2 leaf surface decreased with ageing of the plants. This reduction was strongest with plants well supplied with nitrogen. Since the carbohydrate content of the leachate of these plants hardly decreased with ageing, the

Table 3. Carbohydrates and total nitrogen (μg) leached per cm^2 leaf surface of *Zea mays* grown in pots at two different nitrogen levels (corresponding with 50 and 300 kg N per ha) after a weekly spray irrigation of 6 mm in 60 min.

Date of harvest	Carbohydrates		Nitrogen	
	50	300	50	300
10/7	5.6	4.2	1.1	2.0
17/7	2.8	.	1.3	.
24/7	4.1	4.1	0.8	1.0
31/7	3.8	4.2	0.5	1.2
7/8	3.2	3.1	0.5	0.8
14/8	3.6	4.1	0.5	0.7
21/8	2.9	3.4	0.5	0.5

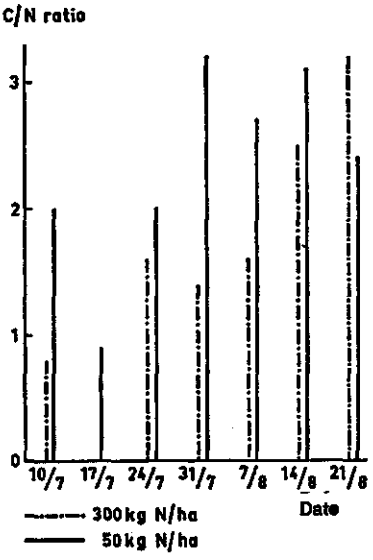


Fig. 3. C/N value of the leachate of *Zea mays*, grown in pots at two different nitrogen levels (corresponding with 50 and 300 kg N/ha) after a spray irrigation of 6 mm in 60 minutes.

C/N value of the leachate increased with the age of the plants (see Fig. 3). The C/N value of a medium may be an indication for biological nitrogen fixation. The highest values of the C:N ratio were about 3. This value is far below the limit above which nitrogen fixation may proceed in natural environments like the phyllosphere.

Assuming that the nitrogen as well as the carbohydrates of the leachate are assimilable by the non-nitrogen-fixing micro-flora of the phyllosphere, 1 mg of N, enabling the synthesis of 10 mg cell material, will require 25–50 mg carbohydrates (containing 40% C). This would correspond with a C/N value of 10–20 in the leachate. Carbohydrates in excess of this amount can only be assimilated by nitrogen-fixing bacteria. Only when nitrogen compounds contain a certain amount of carbon or are less available than the carbon compounds, can nitrogen fixation occur at a lower C/N value. The compounds containing nitrogen, released into the phyllosphere, undoubtedly contained a certain amount of carbon, so that the C/N values calculated in this study should be increased by a few units.

Micro-flora of the sheath water The numbers of bacteria, counted on the three agar media used, are given in Table 4. In sheath water of both 300-N plants and 50-N plants, the numbers of bacteria increased with ageing of the plants. The highest numbers of bacteria were counted in the sheath water of the plants grown at the rate of 300 kg N per ha. The large differences in bacterial numbers of sheath water, derived from low and high-nitrogen plants, which occurred in the young plants, had more or less disappeared during the period of maximum growth of the plants, but re-appeared when the plants were ageing.

Although the numbers of micro-organisms grown on azotobacter agar were considerably lower than those grown on glucose yeast extract agar, relatively large numbers were counted on the former agar medium. From a more detailed study of the

Table 4. Numbers[1] of micro-organisms (in millions) in 1 ml sheath water of *Zea mays*, grown in pots at two different nitrogen levels (corresponding with 50 and 300 kg N per ha).

Counting medium	N dressing of maize	Sampling date						
		10/7	17/7	24/7	31/7	7/8	14/8	21/8
Glucose yeast extract agar	50	0.001	0.008	11	52	150	180	1.4
	300	10	.	24	100	130	1900	480
Soil extract azotobacter agar	50	0.003	0.02	14	.	8.6	820	1.2
	300	10	.	9.8	12	15	1600	100
Azotobacter agar	50	0.001	0.004	9	20	0.8	72	2.6
	300	34	.	8	10	4.2	540	92

1. Averages of triplicate plate countings.

Predominant micro-organisms in sheath water of *Zea mays*, grown in pots at two different nitrogen levels (corresponding with 50 and 300 kg N/ha).

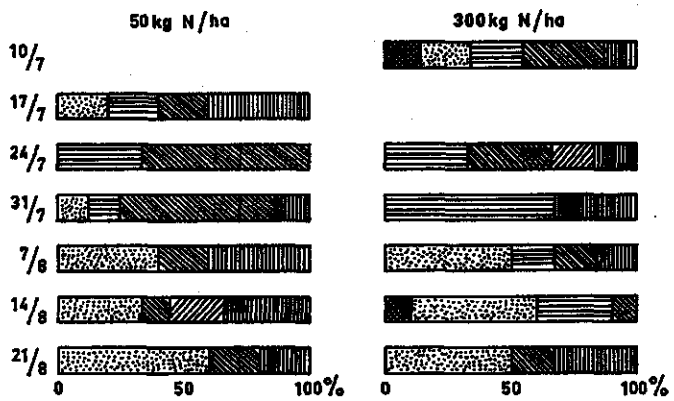


Fig. 4. Countings on glucose yeast extract agar.

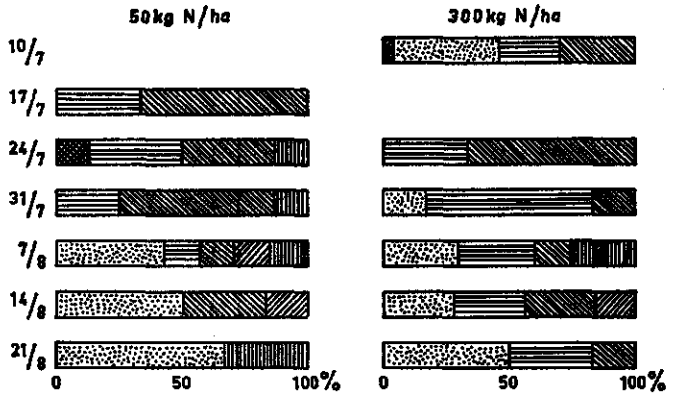


Fig. 5. Countings on soil extract azotobacter agar.

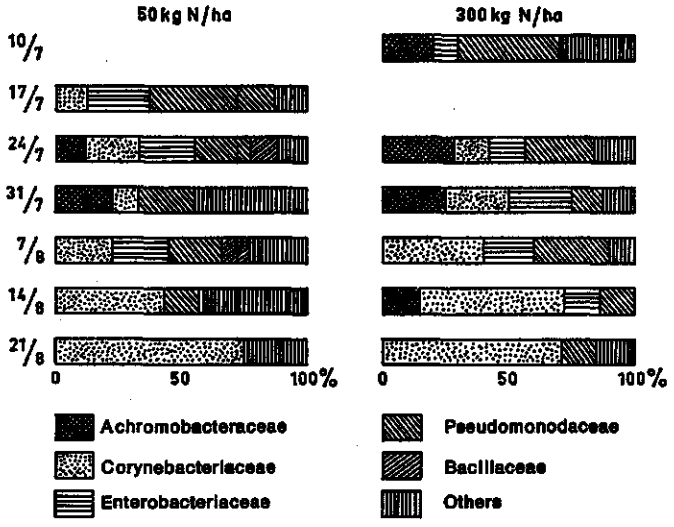


Fig. 6. Countings on azotobacter agar.

bacteria it was found that these organisms were unable to fix nitrogen but apparently utilized the traces of nitrogenous compounds which were contained as impurities in the azotobacter agar. The additional small amounts of nitrogen that came from the added soil extract resulted in more colonies on soil extract azotobacter agar than on azotobacter agar alone.

Details about the micro-flora of the phyllosphere are given in figs 4–6. In the phyllosphere of young plants, bacteria belonging to the *Pseudomonadaceae* dominated. With ageing of the plants, these bacteria decreased and the *Corynebacteriaceae* increased. This shift was observable on all three media. *Enterobacteriaceae* were more frequent in the sheath water of young plants, particularly low-N plants, where these bacteria apparently were superseded by corynebacteria with ageing of the plants. Maize plants dressed with the high amount of N maintained a much larger proportion of enterobacteria, almost until the end of the vegetation period. Bacteria belonging to the *Achromobacteraceae* occurred in irregular numbers, slightly more frequently in plants dressed with the highest amount of nitrogen. Bacilli and 'others', including agrobacteria, micrococci and yeasts, also occurred in irregular numbers.

3.2.2 Daily leaching of tops of maize (1968)

In a second pot experiment with *Zea mays*, the aerial parts of the plants were spray-irrigated daily. Methods and material of this experiment were the same as those of the previous experiment (3.2.1) except for the nitrogen dressing which corresponded with an amount of 150 kg N per ha. In addition to the chemical analyses of the daily leachates, numbers of micro-organisms in the sheath water of the sprayed plants were counted once a week, samples being taken before spray irrigation. About 15 representative micro-organisms were isolated from the proper dilution of each counting agar and identified according to Bergey's Manual of Determinative Bacteriology.

Carbohydrates and nitrogen contents of the leachate The results of the chemical analyses of the leachates show that carbohydrates and nitrogen were leached daily from the tops of the maize plants (Figs 7–8). Thus under natural conditions a constant supply of substrates to the phyllosphere organisms may occur. Younger plants lost more carbohydrates and nitrogen per cm^2 leaf surface by leaching than older plants. This result differs from those of the weekly spraying of the first experiment, as far as it concerns the carbohydrate content of the high-N plants, but is in agreement with the previous experiment in the case of total nitrogen. The C/N value in the leachate was very low and must have been unfavourable to nitrogen fixation.

Micro-flora in the sheath water Although the numbers of micro-organisms counted were much lower than those of the first experiment (3.2.1) probably because of the daily spray irrigation, the general trend of the first experiment i.e. the rise in numbers per ml sheath water with ageing of the plants, was more or less confirmed (Table 5). This increase was particularly true of the numbers counted on a rich nutrient medium

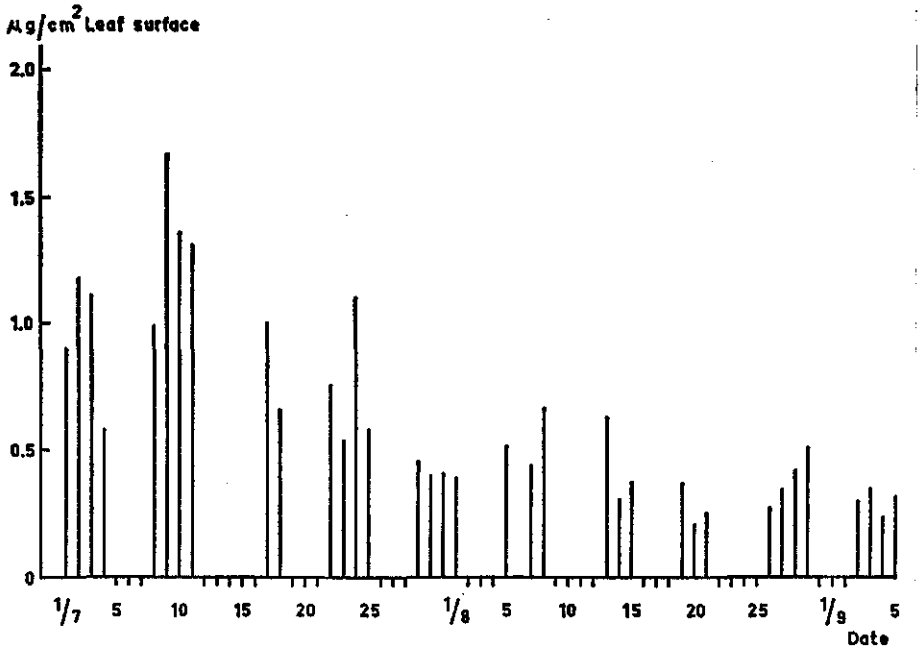


Fig. 7. Carbohydrates (µg) leached per cm² leaf surface of *Zea mays*, grown in pots with a nitrogen dressing corresponding to 150 kg N per ha, after a daily spray irrigation of 6 mm in 60 minutes.

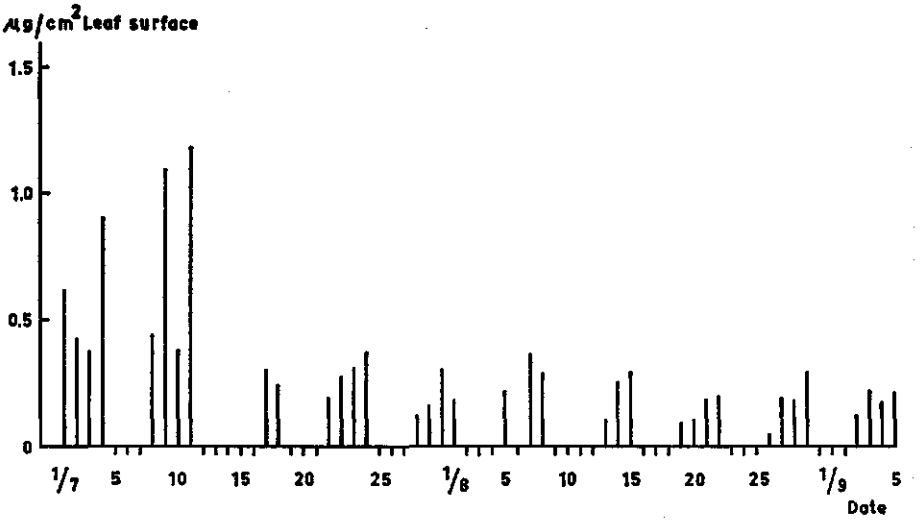


Fig. 8. Nitrogen (µg) leached per cm² leaf surface of *Zea mays*, grown in pots with a nitrogen dressing corresponding to 150 kg N per ha, after a daily spray irrigation of 6 mm in 60 minutes.

(glucose yeast extract agar). Similar to the first experiment, the lowest numbers were counted on the azotobacter medium which was very low in available nitrogen. Although growth on the latter medium might mean that the organisms fixed N_2, this was not the case. In fact, neither *Azotobacter* nor any other nitrogen-fixing bacterium was isolated from the phyllosphere of *Zea mays* in this experiment. In spite of the very low content of combined nitrogen in the azotobacter medium, certain types of non-

Table 5. Numbers[1] of micro-organisms (in millions) in 1 ml sheath water of daily sprayed *Zea mays*, grown in pots at a nitrogen dressing corresponding with 150 kg N per ha.

Counting medium	Sampling date									
	5/7	12/7	19/7	26/7	2/8	9/8	16/8	23/8	30/8	6/9
Glucose yeast extract agar	0.2	0.7	0.4	5	3.4	8	7	1.6	20	25
Soil extract azotobacter agar	12	62	50	24	50	14	6	.	27	2
Azotobacter agar	2	5	4	5	5	1	0.4	2	15	8

1. Averages of triplicate plate countings.

Predominant micro-organisms in the sheath water of *Zea mays*, grown in pots with a nitrogen dressing corresponding to 150 kg N per ha.

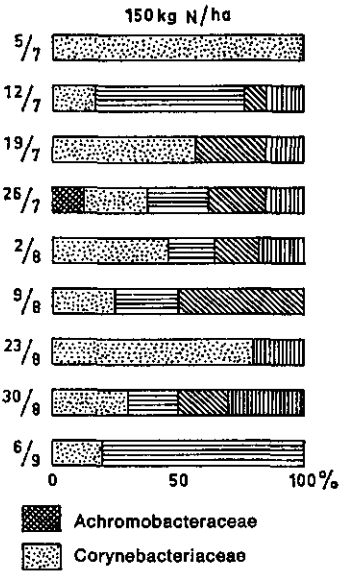


Fig. 9. Countings on glucose yeast extract agar.

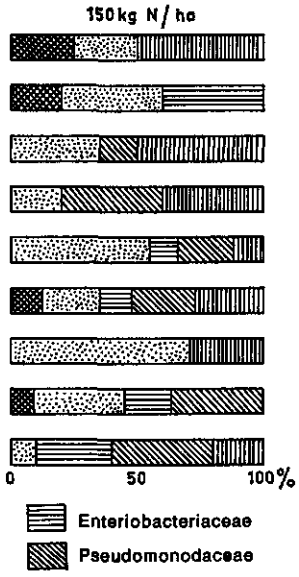


Fig. 10. Countings on soil extract azotobacter agar.

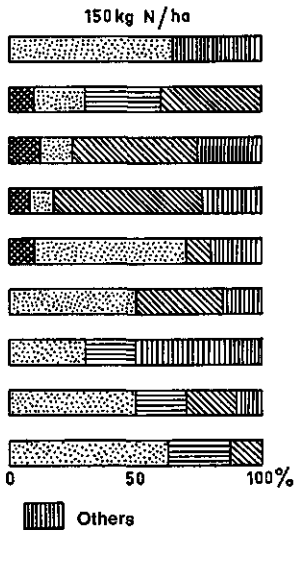


Fig. 11. Countings on azotobacter agar.

nitrogen-fixing bacteria did grow on this medium.

Addition of soil extract to the azotobacter medium gave a striking increase in the numbers counted, presumably due to the introduction of traces of nitrogen.

Although the same main groups of bacteria were found to occur as in the previous experiment (3.2.1) i.e. organisms belonging to the *Corynebacteriaceae*, *Pseudomonadaceae*, *Enterobacteriaceae* and *Achromobacteraceae* (Figs 9–11), the distribution of these different types of organisms in the various periods of development of the maize plants was not equal to that of the first experiment. The shift of pseudomonads to coryneform bacteria, which was very clear in the first experiment, was hardly visible in the present countings. Corynebacteria which were almost absent in the phyllosphere of young plants in the preceding experiment (3.2.1), made up a very large part of the first counting in the present test. A further difference was that the *Enterobacteriaceae* were present in approximately equal proportions throughout the entire growing period and not preferentially in the sheath water of young plants, as found in the preceding experiment 3.2.1. The deviation in the micro-flora in the 2 experiments may have been due to differences in frequency of spraying or/and in weather conditions during the two experimental years.

3.3 Leaching experiments with Zea mays grown under field conditions (1969)

Maize was sown in the centre of plots of 1 m^2 laid down in two rows on a sandy soil. Each row consisted of 5 plots of which the central one was left unplanted and served as control (see Plate 1). The basal dressing of these plots consisted of superphosphate, at the rate of 120 kg P_2O_5 per ha and K_2SO_4 mixed with $MgSO_4$, at the rate of 100 kg K_2O per ha. Nitrogen was supplied as ammonium-nitrate limestone at the rate of 50 and 200 kg N per ha. The soil of the plot was covered with a plastic foil to prevent the leachate from flowing into the soil. The fixed spray installation used for the pot experiment was also used in the field experiment.

The plants were simultaneously sprayed daily between 8–9 a.m. with an amount of water corresponding with a shower of 6 mm and giving 12 l of leachate per 2 duplicate plots, of which 6 l was collected in 10 litre flasks. In the leachate the total amounts of carbohydrates and nitrogen were estimated (see Fig. 1).

From the results of this experiment it will be seen that the amount of *carbohydrates* in the daily leachates showed hardly any relationship with the age of the plants (Fig. 12). The amounts of carbohydrates (calculated as averages per growing period) in the daily collected leachate of plants, dressed with 50 and 200 kg N per ha, were 1.8 and 2.1 mg/plant respectively.

The leaching of *nitrogenous compounds* behaved similarly to that of carbohydrates (Fig. 13). In the field experiment the amounts (calculated as averages per growing period) of total nitrogen in the daily leachates of plants dressed with 50 and 200 kg N per ha equalled 0.80 and 0.94 mg/plant, respectively.

From these data it can be derived that the *C/N value* of the leachates of plants

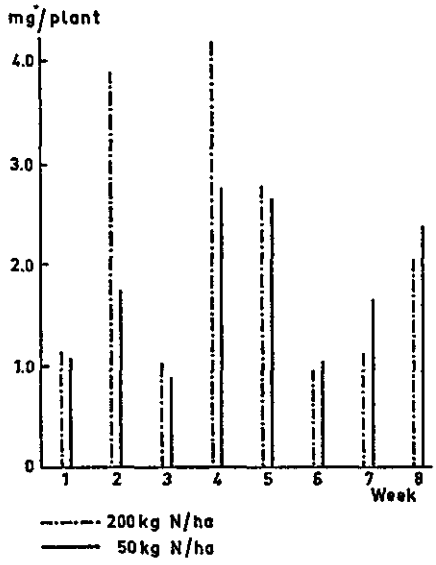


Fig. 12. Carbohydrates in mg per plant (calculated as weekly averages) leached from *Zea mays*, growing under field conditions at two different nitrogen levels (corresponding to 50 and 200 kg N per ha) after a daily spray irrigation of 6 mm in 60 minutes.

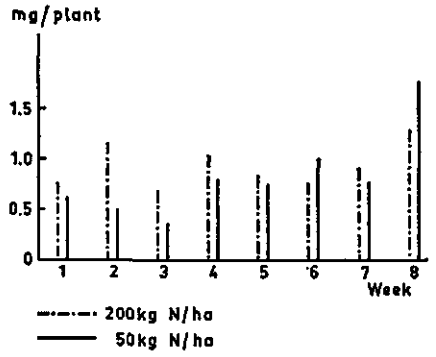


Fig. 13. Nitrogen in mg per plant (calculated as weekly averages) leached from *Zea mays*, growing under field conditions at two different nitrogen levels (corresponding to 50 and 200 kg N per ha) after a daily spray irrigation of 6 mm in 60 minutes.

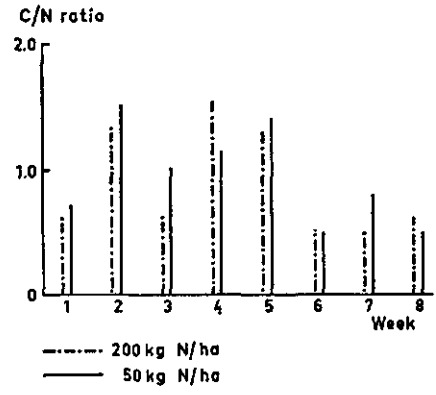


Fig. 14. C/N values (calculated as weekly averages) of the leachates of *Zea mays*, grown under field conditions at two different nitrogen levels (corresponding to 50 and 200 kg N per ha) after a daily spray irrigation of 6 mm in 60 minutes.

grown under field conditions are similar to those of plants grown in pots except that the values of the last 3 leachings were considerably higher in the case of the pot-grown plants. Nevertheless, the C:N ratios of the leachates of both types of plants were too low to expect biological fixation of elementary nitrogen in the phyllosphere (Figs 14 and 3).

It may be assumed that the amounts of leached carbohydrates and nitrogenous compounds are connected with the amounts of these compounds in the soluble fraction of the plant. Therefore experiments were carried out to compare the amounts of carbohydrates and nitrogenous compounds released by the tops of maize plants with those of the soluble fraction of these tops. In these experiments, sheath water was used as the medium representative for compounds released by the aerial plant parts of maize.

3.4 The carbohydrate and nitrogen contents of the soluble fraction and of sheath water of maize plants grown under temperate conditions

For these experiments, use was made of a series of experimental plots, laid down on a light sandy soil in the garden of the Laboratory of Microbiology at Wageningen about 50 years ago and since that time dressed in a different way with N, P and K fertilizers or with farmyard manure (Table 6).

On each plot the maize was sown in one row of 9 groups of 4 plants each at distances of 65 cm. For the analyses 2 representative plants of each plot were harvested. The preparation of samples used for collecting the soluble fraction of the maize plants, and the analyses of these samples were described in section 2.3.2. All the plants of an experimental plot, of which two plants were analysed weekly, were used for collecting sheath water twice a week. The nitrogen content of the sheath water was based on the estimation of ninhydrin-positive compounds.

Table 6. Manuring of the plots used for growing *Zea mays*.

pH of soil	Manuring rate (kg/ha)		
	N^1	$P_2O_5{}^2$	K_2O^3
5.3	150	100	150
5.3	150	0	150
5.3	150	100	0[4]
5.5	0	0	0
5.5	0	100	150
5.5	farmyard manure about 25000 kg		

1. N as ammonium-nitrate limestone.
2. P_2O_5 as superphosphate, granulated.
3. K_2O as potassium sulphate mixed with magnesium sulphate.
4. Compensated for Mg by adding $MgSO_4$ (50 kg MgO per ha).

Table 7. Average height (cm) of *Zea mays* grown with different dressings.

Date of harvest	Farmyard manure	N.P.K.	No manure	No nitrogen	No phosphorus	No potassium
14/7	75	71	39	33.5	48	35
21/7	100	80	51	50.5	51	47.5
4/8	120.5	136	75	92	105	87.5
11/8	181.5	154	118	122	127.5	88.5
18/8	190	167.5	125	130	143	102.5
25/8	195	168	125	140	145	112
1/9	195	174	125	139.5	147.5	112
8/9	195	174	125	140	147	113

Table 8. Weight of green leaves (g) per plant of *Zea mays* grown with different dressings.

Date of harvest	Farmyard manure	N.P.K.	No manure	No nitrogen	No phosphorus	No potassium
7/7	40.8	37.4	18.0	13.6	23.7	24.2
14/7	63.1	39.5	18.0	15.9	21.6	24.5
21/7	103.4	75.8	34.7	35.1	43.2	33.0
28/7	124.3	100.9	60.6	58.3	45.1	48.4
4/8	163.2	129.6	59.5	77.2	87.3	97.1
11/8	214.0	144.1	65.0	85.0	87.5	84.5
18/8	166.2	130.7	71.5	89.5	76.1	82.0
25/8	144.0	112.0	76.0	80.0	86.5	77.0
1/9	140.2	109.0	71.5	74.0	78.7	75.0
8/9	110.5	88.7	64.0	66.5	72.0	67.0

Growth of plants As will be seen from the data of tables 7 and 8, the best growth and the highest yields of tops of the maize plants were obtained on the plots with farmyard manure. Omission of either N, P or K from the dressing badly affected the growth of the plants.

Soluble fraction As shown in Table 9, the highest amounts of *carbohydrates* were found in the plants without any dressing and in those without potassium dressing. This result is in accordance with observations made in other plants, e.g. potatoes, that potassium deficiency raises the carbohydrate content of the plant tissue. The relatively high carbohydrate content of the unfertilized plants in this experiment agrees with the observation that potassium deficiency was the main deficiency symptom of these plants. Fully dressed plants, either with farmyard manure or with a complete fertilizer treatment, were only slightly lower in carbohydrate content of the soluble fraction during the period of maximum development, but at the end of the growing period the differ-

Table 9. Carbohydrates (mg per gram fresh material) in the soluble fraction of *Zea mays*, grown on plots with different dressings.

Date of harvest	Farmyard manure	N.P.K.	No manure	No nitrogen	No phosphorus	No potassium
7/7	5.7	5.9	12.6	6.2	5.2	12.4
14/7	15.8	12.3	15.5	12.0	10.2	20.4
21/7	14.7	10.4	21.9	14.8	13.0	23.8
28/7	14.1	10.1	16.0	12.6	11.9	18.6
4/8	13.6	11.6	15.1	11.8	11.6	18.0
11/8	17.0	16.1	17.8	12.6	11.6	16.1
18/8	16.6	16.2	16.5	12.2	11.1	18.1
25/8	10.8	8.5	8.1	9.9	9.1	16.0
1/9	10.4	9.6	11.5	8.9	8.4	15.1
9/9	9.5	8.0	6.3	8.6	8.0	14.8

Table 10. Nitrogen (mg per gram fresh material) in the soluble fraction of *Zea mays*, grown on plots with different dressings.

Date of harvest	Farmyard manure	N.P.K	No manure	No nitrogen	No phosphorus	No potassium
7/7	1.2	1.5	1.2	1.3	1.9	1.5
14/7	1.3	1.4	1.1	1.3	1.4	1.5
21/7	1.1	1.0	0.9	0.9	1.1	1.4
28/7	1.0	0.7	0.6	0.8	0.7	1.1
4/8	1.1	0.8	0.7	0.8	0.9	1.3
11/8	1.1	1.0	0.7	1.0	1.3	1.1
18/8	1.5	1.4	.	1.4	.	1.3
25/8	1.4	1.9	1.6	1.3	1.5	1.4
1/9	1.6	1.7	1.3	1.4	1.7	1.5
9/9	1.5	1.5	1.5	1,6	1.0	1.4

ences with the K-deficient plants were again larger. It is remarkable that plants with nitrogen deficiency were not higher in carbohydrate content of the soluble fraction than those fully dressed. The same was true of plants without added phosphate.

From the data of Table 10 it follows that the *nitrogen content* of the soluble fraction was lowest in the plants growing without nitrogen or in those without any dressing, whereas the highest value was obtained in the K-deficient plants during the first half of the growing period.

The *C/N value* of the soluble fraction of tops of maize plants varied between 2 and 5 in both young (first 2 sampling dates) and old plants (last 3 sampling dates). From the 3rd to the 7th sampling date (i.e. during the period of maximal growth intensity) it varied between 4 and 11 (Table 11). The highest values were found in the unfertilized

plants. In spite of their relatively high values of soluble carbohydrates, potassium-deficient plants had no exceptionally high C:N ratios, because of their high content of soluble nitrogenous compounds.

Table 11. C/N values of the soluble fractions of *Zea mays*, grown on plots with different dressings.

Date of harvest	Farmyard manure	N.P.K	No manure	No nitrogen	No phosphorus	No potassium
7/7	1.9	1.6	4.2	2.0	1.1	3.3
14/7	4.9	3.5	5.6	3.7	3.0	5.4
21/7	5.3	4.2	9.7	6.6	4.7	6.8
28/7	5.6	5.8	10.7	6.3	6.8	6.8
4/8	4.9	5.8	8.6	5.9	5.1	5.5
11/8	6.2	6.4	10.2	5.0	3.6	5.8
18/8	4.3	4.6	.	3.5	.	5.6
25/8	3.1	1.8	2.0	3.1	2.4	4.6
1/9	2.6	2.3	3.5	2.5	2.0	4.0
9/9	2.5	2.1	1.7	2.2	3.2	4.2

Table 12. Carbohydrates (μg/ml) in the sheath water of *Zea mays*, grown on plots with different dressings.

Date of sampling	Farmyard manure	N.P.K	No manure	No nitrogen	No phosphorus	No potassium
7/7	82	74.4	66.1	45.2	103.1	84.5
14/7	71.7	93.0	61.9	45.6	110.7	64.9
17/7	67.7	91.0	69.5	55.5	96.4	53.3
21/7	99.5	118.5	111.1	63.5	132.5	65.5
24/7	253.9	296.6	343.2	240.0	268.8	201.1
28/7	106.7	110.7	343.6	108.0	90.6	84.0
31/7	157.1	270.6	271.7	260.2	183.2	103.2
4/8	88.9	73.3	101.1	112.1	135.0	87.7
7/8	397.0	113.8	109.1	93.2	143.3	81.1
11/8	69.4	48.8	40.0	59.3	82.8	51.9
14/8	56.0	89.1	58.9	40.3	59.3	86.2
18/8	24.7	36.7	67.2	36.5	46.4	66.9
21/8	42.1	53.3	56.6	51.3	44.9	51.9
25/8	19.3	27.6	36.6	40.9	39.4	69.3
28/8	7.1	14.0	18.3	17.3	15.5	22.2
1/9	24.7	58.5	55.0	53.9	129.5	66.2
4/9	52.0	89.5	58.2	67.0	79.0	113.7
8/9	62.2	93.5	41.5	64.8	70.2	50.7
11/9	37.5	40.0	29.8	38.6	33.8	37.3

Sheath water of Zea mays From Table 12 it appears that the sheath water of maize plants was relatively rich in *carbohydrates*. Values between 300–400 μg carbohydrates per ml sheath water were no exception. Although the variation in carbohydrate concentration at different sampling dates was considerable, these data clearly show that the sheath water of young plants contained more carbohydrates than that of old plants. The highest values were obtained from 21 July–7 August, i.e. during the period of intense growth. As to the effect of different dressings, it can be stated that the highest carbohydrate contents occurred in the sheath water of unfertilized plants. Potassium-deficient plants which contained the highest amount of carbohydrates in the soluble fraction of the plant tissue, belonged to the lowest as to carbohydrate content in the sheath water. Phosphorus-deficient plants, however, which were low in carbohydrate content of the soluble fraction, released considerable amounts of carbohydrates so that relatively high values occurred in the sheath water. This different behaviour of K-deficient and P-deficient plants can be demonstrated more clearly when the ratio of the average carbohydrate contents of the soluble fraction of both types of plants (Table 9) is compared with the ratio of the carbohydrate contents of sheath water. These values equalled 1.73 and 0.77, respectively.

Nitrogenous compounds, estimated as ninhydrin-positive compounds, with leucine as standard, were very low in young plants and relatively high in old plants (Table 13)

Table 13. Ninhydrin-positive compounds[1] (μg/ml) in the sheath water of *Zea mays*, grown on plots with different dressings.

Date of sampling	Farmyard manure	N.P.K	No manure	No nitrogen	No phosphorus	No potassium
17/7	4.8	6.7	8.1	4.1	6.7	14.5
21/7	12.3	6.3	10.5	6.3	17.7	13.9
24/7	10.7	11.5	6.2	6.6	12.2	6.3
28/7	13.2	16.6	13.3	15.3	15.0	21.4
31/7	23.0	21.8	7.3	10.4	18.9	23.1
4/8	17.6	23.6	18.2	13.0	14.7	16.8
7/8	38.6	16.7	6.3	8.2	16.1	12.5
11/8	35.1	35.0	22.8	33.8	20.9	20.4
14/8	19.0	24.0	40.2	13.7	21.1	19.5
18/8	11.4	13.2	13.5	8.4	11.6	12.7
21/8	9.0	11.4	10.6	14.9	11.9	11.3
25/8	16.3	15.7	8.1	14.6	15.9	10.4
28/8	6.6	8.2	10.1	5.4	8.5	11.2
1/9	87.7	47.4	52.4	38.0	58.3	67.8
4/9	27.0	31.8	31.6	47.0	52.0	38.5
8/9	30.0	38.8	33.2	29.4	67.4	34.7
11/9	45.1	50.9	49.5	52.5	48.3	48.7

1. For the estimation of these compounds, leucine, containing 10% N, was used as the standard. Consequently, total nitrogen is 10% of the values presented in this table.

Growth of guatemala grass Plants dressed with the highest amount of nitrogen had a larger total leaf surface, depending on increased number of leaves as well as on larger leaves (Table 15). Yields of dry matter of tops were considerably higher; N and P contents and even K contents were higher during the first half of the growing period (Table 16). During the second half of the growing period, there was a tendency towards lower contents of N, P and K in the tops of plants with the highest N dressing.

Table 15. Average height of plants, number of leaves and size of the leaf surface of *Tripsacum laxum* Nash, grown in pots at two different nitrogen levels (corresponding with 50 and 200 kg N per ha).

Date of harvest	50 kg N/ha			200 kg N/ha		
	height (cm)	number of leaves	leaf surface (cm^2/plant)	height (cm)	number of leaves	leaf surface (cm^2/plant)
11/7[1]	75	34	9184	75	46	10612
18/7	90	48	12144	90	47	12517
25/7	90	43	12529	110	56	17728
1/8	105	41	17750	110	54	19257
8/8	110	43	18248	115	45	20862
22/8[2]	112	46	19108	115	50	22024
29/8	118	48	19820	.	.	.
5/9	120	51	20666	130	53	22085
12/9	125	39	17695	.	.	.
18/9	130	37	15785	130	46	20383

1. Corresponding with 2nd week of Table 17.
2. Corresponding with 8th week of Table 17.

Table 16. Yield, dry matter, and N, P_2O_5 and K_2O contents of the dry matter of *Tripsacum laxum* Nash, grown in pots at two different nitrogen levels (corresponding with 50 and 200 kg N per ha).

Date of harvest	50 kg N/ha				200 kg N/ha			
	tops, dry matter (g)	N (%)	P_2O_5 (%)	K_2O (%)	tops, dry matter (g)	N (%)	P_2O_5 (%)	K_2O (%)
11/7	75	2.5	1.2	7.4	85	2.6	1.3	7.4
18/7	92	2.0	1.2	6.5	111	2.6	1.4	7.2
15/7	112	1.6	1.0	5.5	175	2.1	1.1	6.7
1/8	217	1.5	1.1	5.5	261	1.7	1.1	6.0
8/8	175	1.2	0.9	5.0	183	1.1	0.9	5.0
22/8	200	1.4	1.0	5.7	256	1.2	0.9	5.3
5/9	211	1.3	1.0	5.3	254	0.9	0.9	4.3
18/9	209	1.1	0.9	4.8	264	1.2	1.0	4.6

Carbohydrate and nitrogen contents of leachate and of sheath water The leachates were collected from 2 pots each containing 1 plant, sprayed simultaneously; the analyses of the leachates as recorded in the tables are averages of the separate values of these 2 plants. The effect of the nitrogen dressing on the carbohydrate content of the leachate was different from that on the carbohydrate content of the sheath water (Table 17). Leachates from 200-N plants contained more carbohydrates than those from 50-N plants as contrasted to sheath water that mostly contained more carbohydrates with the low N-dressing. A further difference between leachate and sheath water is that, in general, the amount of carbohydrates leached per cm^2 of leaf surface was highest in young plants, whereas the concentration of carbohydrates in the sheath water was lowest in young plants. This result indicates that the components of the sheath water are not or only partly released from the laminae, but mainly from the sheath and (or) the stem.

Nitrogen was estimated as total nitrogen in the leachate and as ninhydrin-positive compounds, assumed to have an average N-content of 10%, in the sheath water. Since not all of the soluble N in sheath water occurred as α-amino nitrogen, the values of total soluble N may have been slightly higher than those calculated from the ninhydrin-positive compounds. There was no much difference in nitrogen content of the sheath water from plants with different nitrogen dressings, but the nitrogen content of the

Table 17. Carbohydrates, calculated as weekly averages, in the leachate (mg per plant and μg per cm^2 of leaf surface) after spray irrigation of 6 mm in 60 min of *Tripsacum laxum* Nash, grown in pots at two different nitrogen levels (corresponding with 50 and 200 kg N per ha) and in sheath water (μg/ml[1]) of these plants sampled before spraying.

Week	50 kg N/ha			200 kg N/ha		
	leachate		sheath water	leachate		sheath water
	(mg/plant)	(μg/cm^2 leaf surface)	(μg/ml)	(mg/plant)	(μg/cm^2 leaf surface)	(μg/ml)
1	11.4	.	867	4.2	.	575
2	7.8	0.9	1330	9.0	0.9	992
3	7.2	0.6	427.5	10.8	0.9	340
4	4.2	0.4	336	9.6	0.5	324
5	4.8	0.3	1475	12.0	0.6	1231
6	24.6	1.3	1480	15.6	0.7	1517
7	13.8	.	1131	18.0	.	1213
8	6.6	0.3	1087	12.6	0.6	1220
9	6.0	0.3	802	12.0	.	774
10	8.4	0.4	1576	11.4	0.5	1077
11	7.8	0.4	1862	20.4	.	2215
12	8.4	0.5	1412	13.2	0.6	1340

1. The amount of sheath water in guatemala grass varied from approximately 0.1–1.0 ml per leaf.

Table 18. Total nitrogen, calculated as weekly averages, in the leachate (mg per plant and μg per cm^2 leaf surface) after spray irrigation of 6 mm in 60 minutes of *Tripsacum laxum* Nash, grown in pots at two different nitrogen levels (corresponding with 50 and 200 kg N per ha) and ninhydrin-positive compounds in the sheath water (μg/ml) of these plants, sampled before spraying.

Week	50 kg N/ha			200 kg N/ha		
	leachate		sheath water	leachate		sheath water
	mg/plant	μg/cm^2 leaf surface	μg/ml[1]	mg/plant	μg/cm^2 leaf surface	μg/ml[1]
1	1.08	.	12.5	1.68	.	9.6
2	0.54	0.06	23.7	1.08	0.11	14.3
3	0.48	0.04	7.5	0.90	0.07	10.4
4	0.90	0.07	5.1	2.40	0.14	5.4
5	1.08	0.06	23.0	3.00	0.16	22.1
6	0.78	0.04	28.4	1.50	0.07	25.7
7	1.02	.	17.4	1.08	.	25.7
8	0.60	0.03	10.5	1.20	0.05	15.6
9	0.48	0.02	28.8	1.26	.	17.4
10	0.54	0.03	12.1	1.02	0.05	19.7
11	0.84	0.05	46.0	1.08	.	51.2
12	1.26	0.08	11.6	1.32	0.07	9.8

1. Total N is 10% of n.p.c. values (cf. Table 13).

Table 19. C/N values, calculated as weekly averages, in the leachate after spray irrigation of 6 mm in 60 min of *Tripsacum laxum* Nash, grown in pots at two different nitrogen levels (corresponding with 50 and 200 kg N per ha) and in the sheath water of these plants, sampled before spraying.

Week	50 kg N/ha		200 kg N/ha	
	leachate	sheath water	leachate	sheath water
1	4.2	277	1.0	240
2	5.8	224	3.4	277
3	6.0	228	4.8	131
4	1.9	263	1.6	240
5	1.8	257	1.6	223
6	12.6	208	4.1	236
7	5.4	260	6.6	189
8	4.4	414	4.2	313
9	5.0	111	3.8	178
10	6.2	521	4.5	219
11	3.7	162	7.5	173
12	2.7	487	4.0	547

leachates clearly responded to the different N-supply (Table 18).

An enormous difference was found to exist between the C/N value of the sheath water and that of leachates, confirming the observations made earlier on the different origin of the components of both types of liquid (Table 19). The very high C/N values of the sheath water which occurred throughout the growing period of guatemala grass were thought to enable nitrogen fixation in the sheath water of both high and low-nitrogen plants during the entire growing period (see Chapter 6).

Micro-flora in the sheath water The numbers of micro-organisms, counted in the sheath water of guatemala grass, grown under tropical conditions (Table 20), were considerably larger than those counted in the sheath water of maize, grown under temperate conditions (Table 4). In the former case these numbers were in the order of 10^8–10^9 and, in some cases, even 10^{10} per ml sheath water, whereas in the sheath water of the maize plants they were much lower: about 10^7 per ml. In both cases these numbers increased with age, attaining maximum values at the beginning of August. The highest numbers of micro-organisms were counted on azotobacter agar supplemented with soil extract. This may indicate that the phyllosphere organisms occurring inside the sheath of guatemala grass are adapted to very low concentrations of combined nitrogen or are able to fix elementary nitrogen.

Details on the different groups of micro-organisms are given in Figs 15, 16 and 17. It will be seen that the *Achromobacteraceae* were the predominating type of organism, followed by the coryneform bacteria. Pseudomonads occurred only sporadically. This result is in contrast with that of sheath water of maize plants grown under temperate conditions where the pseudomonads belonged to the predominating bacteria, particularly in the earlier stages of development of the plants. Achromobacters occurred only sporadically in the sheath water of maize, but corynebacteria were found in large numbers in the phyllosphere of both guatemala grass and maize. *Enterobacteriaceae* were counted in rather large numbers in the sheath water of low-N plants, streaked on glucose yeast extract agar. Bacilli (particularly *B. polymyxa*) were found in consider-

Table 20. Numbers of micro-organisms (in millions) in 1 ml sheath water of *Tripsacum laxum* Nash, grown in pots at two different nitrogen levels (corresponding with 50 and 200 kg N per ha).

Counting medium	N dressing of plants	Sampling date							
		11/7	18/7	25/7	1/8	8/8	15/8	29/8	12/9
Glucose	50	120	150	150	800	1200	355	2250	2100
yeast extract agar	200	285	200	300	510	1700	925	750	1100
Soil extract	50	55	1100	4600	5000	5000	9000	230	650
azotobacter agar	200	19	550	4000	10000	10000	6500	4000	300
Azotobacter	50	37	50	100	250	500	1300	700	950
agar	200	200	400	300	1300	3500	2500	2050	4500

Predominant micro-organisms in sheath water of guatemala grass, grown in pots at two different nitrogen levels.

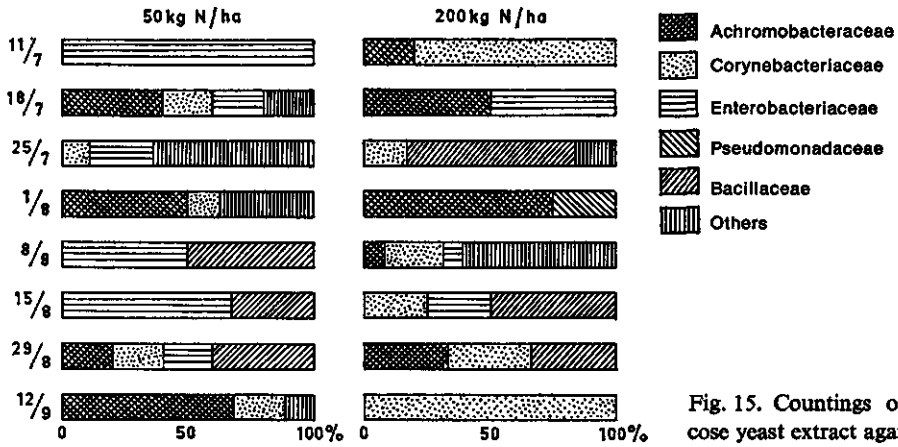


Fig. 15. Countings on glucose yeast extract agar.

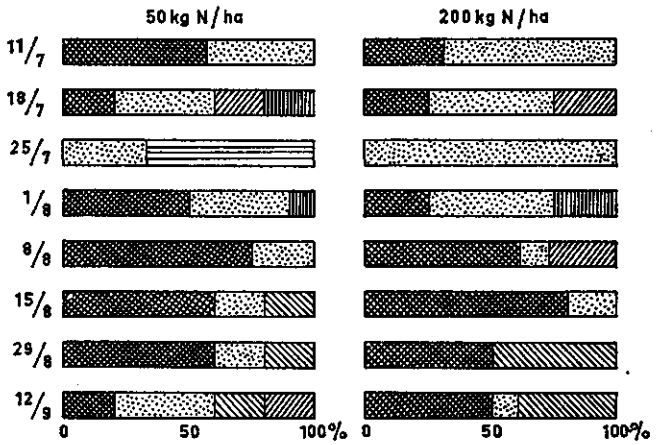


Fig. 16. Countings on soil extract azotobacter agar.

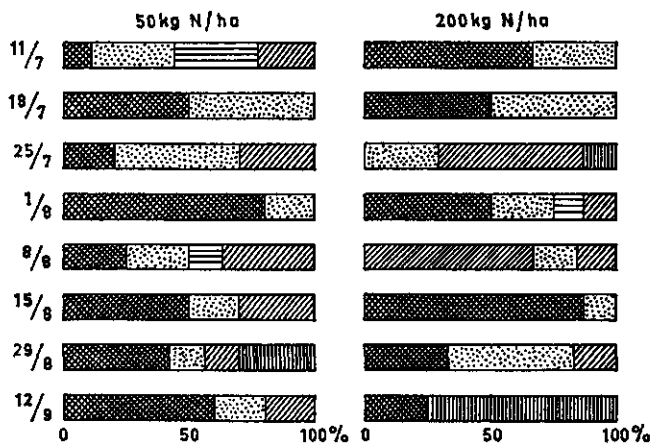


Fig. 17. Countings on azotobacter agar.

Plate 1. Spray irrigation of *Zea mays* in the field.

Plate 2. Guatemala grass in the field.

ably larger numbers than in the case of maize. In addition to the above-mentioned groups of bacteria, small numbers of *Rhizobiaceae, Micrococcaceae,* yeasts and fungi were found. These organisms are designated as 'others' in Figs 15–17.

The pot experiment with guatemala grass was repeated under similar weather conditions. The results obtained confirmed those of the first pot experiment. This concerns the carbohydrate and nitrogen analyses of the leachates as well as the numbers of micro-organisms and the composition of the micro-flora of the sheath water.

4.3 Leaching experiments with guatemala grass, grown under field conditions in the tropics

The method used in the field experiment with *Zea mays* was also employed in the field experiment with guatemala grass. In the centre of each 8 plots a slip of this grass was planted. All the plots received a basal dressing of superphosphate (120 kg P_2O_5/ha) and potassium sulphate (100 kg K_2O/ha). Nitrogen was supplied as ammonium-nitrate limestone in amounts of 50 and 200 kg N per ha. The plants were sprayed simultaneously, 3 times a week, between 7–8 a.m., with an amount of water corresponding with a shower of 6 mm. Six litre of the leachate was collected in 10 litre flasks. In the leachate the total amounts of carbohydrates and nitrogen were estimated according to Fig. 1.

Micro-organisms were counted in sheath water collected weekly before the spraying started, using 3 different media (cf. 4.2). After counting, about 15 micro-organisms were isolated from each medium and identified according to Bergey's Manual of Determinative Bacteriology.

Carbohydrate and nitrogen contents of the leachate After spraying for 1 hour, the amount of carbohydrates leached from the guatemala grass, grown under field conditions (Table 21), was lower than that leached from the plants in the corresponding pot experiment (Table 17). In the field experiment the nitrogen dressings of 50 and 200 kg N per ha corresponded with average leachings of 4.6 and 2.5 mg carbohydrates per plant, respectively, whereas in the pot experiment these values were 9.2 and 12.4 mg, respectively.

Similar to the carbohydrates, lower amounts of nitrogen were released in the field experiment (average values 0.63 and 0.54 mg/plant) than in the pot experiment (average values 0.80 and 1.46 mg/plant, respectively, of plants dressed with low and high amounts of nitrogen).

The C/N values of the leachates (Table 21) in the field experiment were lower than those in the pot experiment. This difference was mainly due to the lower release of carbohydrates in the field experiment. The highest C/N values were found in the leachates of the low-N plants, confirming the findings in the pot experiment.

Micro-flora in the sheath water The total numbers of micro-organisms in the sheath water were comparable to those of the pot experiment (Table 22). Similar to the pot

Table 25. C/N values (calculated as weekly averages) of leaf water and of sheath water of guatemala grass, grown in the field at three different nitrogen levels.

Week	Leaf water			Sheath water		
	kg N/ha added			kg N/ha added		
	0	100	200	0	100	200
1	6.5	8.0	22.9	124.0	99.7	115.2
2	10.4	5.2	15.9	187.8	112.4	118.7
3	2.5	5.7	5.3	100.7	54.8	73.4
4	5.1	3.7	6.4	59.8	35.7	91.8
5	8.2	3.9	16.4	124.6	76.9	104.7
6	7.1	4.0	4.8	157.1	68.6	65.1
7	8.9	6.8	11.5	208.9	81.3	147.8
8	36.0	15.6	44.7	321.5	336.9	277.4
9	5.9	10.7	7.9	294.7	204.3	63.7
10	11.5	12.9	9.5	323.4	291.8	168.9
11	.	.	.	305.8	168.5	95.5
12	.	.	.	252.2	261.7	68.5
13	.	.	.	204.3	117.4	27.3
14	4.1	3.7	2.1	157.5	100.0	193.0
15	5.5	3.0	6.1	225.0	164.8	80.8
16	8.0	2.7	3.2	771.4	212.8	120.0
17	.	.	.	266.6	69.5	138.3
18	.	.	.	233.1	64.8	17.5
19	.	.	.	657.1	55.5	110.5
20	6.0	4.9	6.2	502.8	125.9	57.7
21	4.1	4.2	4.5	125.0	121.7	103.7
22	3.1	7.9	5.6	55.4	107.3	72.1

tion, enormous differences occurred between both types of liquid (Table 25). Owing to the high carbohydrate contents and the low n.p.c. contents, very high values occurred in sheath water. Since the n.p.c. values of sheath water were lower with ageing of the plants and higher with improved nitrogen supply, maximum C/N values were found in the second part of the experimental period of plants without added nitrogen.

As leaf water had much lower values of carbohydrate content and higher n.p.c. contents than sheath water, the C/N values were low. Although these values varied to a rather high degree, the variation was independent of age and nitrogen supply of the plants.

To see whether the high C/N value of the sheath water of guatemala grass also occurred in other *Gramineae*, growing in the field under tropical conditions, the carbohydrate and n.p.c. contents of the sheath water of *Zea mays*, *Sorghum bicolor* and *Echinochloa polystachya* were estimated daily as for guatemala grass and weekly averages of the C/N values calculated. These values were also high, particularly those

Table 26. C/N values as weekly averages of sheath water of *Zea mays*, *Sorghum bicolor* and *Echinochloa polystachya*.

Week	*Zea mays*	*Sorghum bicolor*	*Echinochloa polystachya*
1	654	38	12
2	198	30	26
3	36	84	30
4	180	500	28
5	230	640	52
6	50	226	48
7	40	180	96
8	48	146	138
9	204	86	56
10	212	68	56
11	390	126	86
12	158	88	28
13	372	142	42
14	112	190	30

of *Zea mays* and *Sorghum bicolor* (Table 26).

The carbohydrate analyses of the sheath water of guatemala grass, recorded in Table 23, have clearly shown that the carbohydrate contents were highest in the youngest plants, and gradually decreased during the second half of the experimental period. Since only the total amount of sheath water collected from all the plants was analysed, a more detailed experiment was carried out in which the sheath liquid of each leaf of one plant was collected and analysed separately. For this purpose single plants were harvested on different sampling dates.

From Table 27 it can be seen that the carbohydrate contents of sheath water of different leaves of one plant fluctuated considerably. The highest concentrations were found in the sheath water of young unexpanded leaves.

From the results obtained in this chapter it may be concluded that the sheath water of tropical grasses would be an excellent medium for the growth of nitrogen-fixing bacteria. Its carbohydrate content is high, whereas its nitrogen content is low, resulting in a high C/N value. Leaf water offers much less favourable conditons for the growth of such bacteria, because of its much lower carbohydrate content and its higher nitrogen content, resulting in low C/N values. A further reason for the leaf surface being a less favourable habitat for nitrogen-fixing bacteria than the leaf sheath is that leaf water was present on the leaf surface only during part of the night and a few hours in the morning. For most of the day the leaf surface was dry, with temperatures in the experimental field approximately 50 °C between 11 a.m. and 16 p.m. Light may also adversely affect the growth of micro-organisms on leaf surfaces.

From the widely diverging composition of sheath water and leaf water it is concluded that both types of liquid have not the same origin, viz. the leaf surface, but are

Table 27. Carbohydrate content (μg/ml) of sheath water of different leaves of one guatemala grass plant, per harvest.

Leaf[1]		Date of harvest of test plant														
		10/3	19/3	24/3	24/3	30/3	20/4	20/4	22/4	23/4	24/4	25/5	25/5	12/6	29/6	14/7	7/8
Old	1	1083	950	w[2]	w	1050	1125	817	800	2050	2600	675	430	180	183	183	575
	2	1025	2833	w	w	.	8000	3750	519	2200	1900	166	4900	40	125	92	700
	3	2800	655	w	w	1225	7750	3000	1200	980	475	23	280	100	267	100	750
	4	1025	1031	w	w	925	3166	3875	537	900	450	135	.	15	142	117	630
	5	1036	160	w	w	1450	594	1060	4500	1300	1350	400	400	200	117	200	460
	6	.	165	w	w	2200	1120	4250	2500	320	220	120	3100	825	100	87	750
	7	4600	200	w	w	2375	1063	2667	1925	165	800	750	1850	3400	83	250	3150
	8	3250	395	w	w		656	5450	5250	134	600	800	2150	2100	50	437	4750
	9	4750	733	w	w	3000	1200		4500	130	200	4400	.	3600	600		2450
	10		1466	1416	550					330		2650	7600		975	2550	
	11		4822	1070	500					465	1350	3700	7750				6000
	12			710	.												
	13			1875	790					3050							
	14			4450	840												
	15			4600	1062												
	16																
	17			7000[3]	5900												
Young	18																

1. Rising number denotes decreasing age of leaf.
2. Withered.
3. Sheath water of youngest 2–3 leaves could not be sampled separately.

derived from different sources (cf. section 4.2).

As 10^8 bacteria are normally present in sheath water and they undoubtedly consume the carbohydrates present, it is very unlikely that sheath water has to be considered as excess leaf water coming down to the leaf sheath when an excessive production of dew takes place. It is more probable that the sheath of guatemala grass, like that of other *Gramineae* tested, has its own source of carbohydrates and amino compounds, different from that of the leaf surface. This source apparently replenishes the carbohydrates and amino compounds of the sheath water when these nutrients have been utilized by the micro-organisms.

4.5 Origin of the carbohydrates in the sheath water of guatemala grass

To demonstrate that the carbohydrates (and other compounds of the sheath water) are released by the stem and/or leaf sheath and are not derived from excessive dew formed on the leaf blades, experiments were carried out in which most of the sheath water was washed out and the subsequent recovery of the carbohydrate and amino acid content was measured.

To remove the constituents of the sheath water, a litre of sterile water was introduced twice into a particular leaf sheath, with a siphon. In this way most of the carbohydrates and other constituents were leached out. Samples of the sheath water were taken immediately before the first washing with 1 l water, 15 minutes thereafter, immediately before the second washing with 1 l water and 15, 45, 105, 165 and 225 minutes, respectively, after the second washing. The samples were analysed for carbohydrates and ninhydrin-positive compounds.

In a subsequent experiment the leaf blade of one leaf was detached to investigate whether the remaining leaf sheath and/or stem were able to release carbohydrates into the sheath water. The first sample was taken immediately before the lamina was detached, the second sample 45 minutes after cutting. Immediately hereafter the sheath water was leached out by introducing twice 1 litre of sterile water with an interval of 15 minutes. Samples of the sheath water were taken as indicated in Table 30 and analysed for carbohydrates and ninhydrin-positive compounds.

The introduction of sterile water into the sheath water of entire plants drastically reduced the carbohydrate concentration (Table 28). However, a ready recovery occurred so that the original concentration was obtained 45 minutes after the second washing. This rise continued in subsequent samples so that a few hours after the leaching much higher values were found than those originally present. This ready restoration of the high carbohydrate concentration clearly shows that the leaf sheath and/or stem are responsible for the release of carbohydrates.

The concentration of ninhydrin-positive compounds also readily recovered after washing with sterile water, but not always as fast as that of carbohydrates (Table 29). Therefore the C/N value was higher than before the washing. Such increased C/N values may occur after tropical rain showers, thus improving the conditions for ni-

Table 28. Recovery of the carbohydrate concentration (μg/ml) of sheath water of leaf sheaths of entire guatemala grass plants after washing out the sheath contents with sterile water.

Sample[1] of sheath water	Date of treatment											
	12/8	14/8	19/8	20/8	21/8	26/8	28/8	1/9	3/9	4/9	7/9	8/9
1	740	660	1080	375	2640	1390	1790	1790	605	640	465	680
2	380	500	180	215	357	535	410	286	107	142	392	357
3	220	145	785	125	232	430	385	125	53	53	285	340
4	880	96	640	820	750	321	3070	322	410	965	475	1570
5	410	355	4800	2120	1140	1290	4300	1360	410	2570	640	2070
6	700	.	.	.	.	.	.	.	5430	.	2000	6430
7	3800	.	.	.	.	.	.	.	.	.	.	.

1. (1) Immediately before the washing of one leaf sheath of a particular plant with 1 litre sterile water, (2) 15 min after the first washing, immediately followed by a second washing with 1 litre sterile water, (3) 15 min, (4) 45 min, (5) 105 min, (6) 165 min, (7) 225 min after the second washing.

Table 29. Recovery of the ninhydrin-positive compounds[1] (μg/ml) of sheath water of leaf sheaths of entire guatemala grass plants after washing out the sheath contents with sterile water.

Sample[2] of sheath water	Date of treatment											
	12/8	14/8	19/8	20/8	21/8	26/8	28/8	1/9	3/9	4/9	7/9	8/9
1	10.0	9.7	23.4	6.7	.	39.5	46.0	6.6	6.6	5.0	5.0	5.0
2	8.2	3.2	5.0	5.1	8.3	18.0	21.4	8.3	3.3	8.3	10.0	10.0
3	6.5	3.2	6.7	1.7	5.0	11.5	4.9	6.6	1.6	6.6	5.0	8.3
4	.	13.0	.	30.4	6.7	16.4	46.0	20.0	8.3	3.3	5.0	10.0
5	10.0	.	.	.	13.3	19.7	19.7	13.0	3.3	10.0	1.6	10.0
6	.	.	.	.	.	49.4	.	.	.	.	13.3	6.7

1. Total N is 10% of n.p.c. values (cf. Table 13).
2. See Table 28.

trogen fixation.

Detaching the leaf blades from the sheath increased the carbohydrate content of the sheath water (Table 30). This increase occurred in the short period (about 45 min) between cutting of the leaf blade and washing of the sheath contents with sterile water. The ninhydrin-positive compounds decreased or slightly increased during this period (Table 31), so that the C/N value of the sheath water increased. The results of this experiment confirm the conclusion of the preceding experiment (4.4) that the constituents of the sheath water are released by the leaf sheath and/or stem.

Table 30. Carbohydrate content (μg/ml) of the sheath water of leaf sheaths of entire guatemala grass plants, immediately before and 45 min after detaching the leaf blades and washing out the sheath contents with sterile water.

Sample[1] of sheath water	Date of treatment											
	5/8	18/8	20/8	26/8	28/8	1/9	2/9	3/9	4/9	7/9	8/9	9/9
1	120	1465	1140	860	860	322	555	285	214	555	750	642
2	760	2360	1790	1640	1570	1145	2790	1350	447	680	2430	1210
3	380	390	500	555	410	161	268	71	53	357	357	214
4	128	267	232	500	215	71	125	71	35	286	250	178
5	34	680	3360	1570	1930	1140	1950	372	555	357	715	1350
6	240	2440	.	3800	.	.	10720	.	6780	428	2780	.

1. (1) Immediately before detaching the leaf blades, (2) 45 min after detaching the leaf blades, immediately followed by the first washing with 1 litre sterile water, (3) 15 min after the first washing, immediately followed by the second washing with 1 litre sterile water, (4) 15 min, (5) 45 min, (6) 105 min after the second washing.

Table 31. The ninhydrin positive compounds[1] (μg/ml) of sheath water of leaf sheaths of entire guatemala grass, immediately before and 45 min after detaching the leaf blades and washing out the sheath contents with sterile water.

Sample[2] of sheath water	Date of treatment											
	5/8	18/8	20/8	26/8	28/8	1/9	2/9	3/9	4/9	7/9	8/9	9/9
1	44.0	5.0	27.0	29.6	32.9	10.0	10.0	10.0	10.0	10.0	6.6	3.3
2	8.8	1.5	.	.	39.5	1.6	1.6	6.7	8.3	15.0	13.3	20.0
3	53.0	6.7	10.0	19.7	6.5	1.6	1.6	8.3	5.0	10.0	11.6	0
4	22.0	3.5	8.4	26.3	14.8	5.0	5.0	1.6	3.3	6.6	3.3	6.7
5	11.0	17.0	.	.	.	10.0	10.0	.	6.6	11.6	16.7	3.3
6	55.0	.	.	.	.	.	.	.	21.7	16.7	6.7	.

1. Total N is 10% of n.p.c. values (cf. Table 13).
2. (1) Immediately before detaching the leaf blades, (2) 45 min after detaching the leaf blades, immediately followed by the first washing with 1 litre sterile water, (3) 15 min after the first washing, immediately followed by the second washing with 1 litre sterile water, (4) 15 min, (5) 45 min, (6) 105 min after the second washing.

5 Growth of micro-organisms in sheath water of guatemala grass and nitrogen-fixing capacity of bacteria isolated from several Gramineae

5.1 Growth of micro-organisms in the sheath water of guatemala grass

The growth of micro-organisms in the sheath water of guatemala grass under natural conditions was studied by taking samples of the sheath water of particular full-grown leaves at intervals of 1 or 2 hours throughout the day. These samples were tested for carbohydrates and numbers of micro-organisms, counted on glucose yeast extract agar.

Table 32 shows that the micro-organisms in the sheath water of full-grown leaves generally increased in numbers during the experimental period. In about 40% of all the leaves tested for bacterial numbers, no increase in number took place (Table 33).

The data of tables 32 and 33 show that no correlation existed between numbers of micro-organisms and carbohydrate content of the sheath water; the latter varied greatly during the day. This lack of correlation may be explained by assuming that an unknown growth factor, required for a rapid growth of the micro-organisms, was missing or was present in inadequate amounts in the sheath water. The presence of growth-inhibiting compounds has also not to be excluded.

Assuming that the average capacity of 1 bacterial cell is 1 μm^3, 1 cm^3 of bacterial cells contains 10^{12} organisms, equivalent to approximately 1 g fresh weight and 0.2 g dry weight. To synthesize this amount of cell material, approximately 1 g of carbohydrates is required. The number of bacteria counted in the sheath water was about 4.10^8/ml, which have required about 400 μg of carbohydrates per ml for synthesis. As the carbohydrates, upon their consumption by micro-organisms, were readily replenished by the plant (cf. Table 30), it may be assumed that the phyllosphere micro-organisms within leaf sheaths of guatemala grass have at their disposal excessive amounts of carbohydrates. Thus the conditions as to carbon and energy supply are favourable to nitrogen fixation.

In general the number of bacteria in the sheath water increased in the day time (Table 32). To see what happened overnight, the number of bacteria in the sheath water of specific leaves was counted every 2 hours during a period of 24 hours. These experiments were carried out almost one year after those recorded in tables 32 and 33, during a period of much less rain. Some examples of these countings have been recorded in Table 34. Although the numbers found are relatively low, and do not increase in the day time, they show a clear drop during the night to 1/3–1/5 of the original values, followed by a rise at 4–8 a.m. If we assume that the nightly drop represents the death of bacterial cells, followed by decomposition of the cell material, a rough calculation can be made of the amount of nitrogen which becomes available

Table 32. Numbers of micro-organisms (in millions) and carbohydrate content in the sheath water of full-grown leaves of guatemala grass, growing under natural conditions.

Time	Numbers/ml				Carbohydrates (μg/ml)			
	16/7	23/7	20/8	15/9	16/7	23/7	20/8	15/9
7 a.m.	127	225	25	90	437	82	125	357
9	132	220	25	.	287	40	770	1710
11	125	225	50	115	544	70	715	4350
1 p.m	107	250	55	100	52	94	205	1465
2	167	250	.	45	25	75	625	1666
3	175	350	10	75	67	312	322	250
4	180	300	95	.	27	350	965	214
5	300	275	100	390	70	550	337	482
6	325	275	85	315	50	163	302	238
7	350	400	.	.	100	137	257	1515

Table 33. Numbers of micro-organisms (in millions) and carbohydrate content in the sheath water of full-grown leaves of guatemala grass, growing under natural conditions.

Time	Numbers/ml				Carbohydrates (μg/ml)			
	7/7	14/7	30/7	25/8	7/7	14/7	30/7	25/8
7 a.m	92	15	250	115	37	362	60	855
9	112	20	250	165	100	200	150	1070
11	93	10	200	155	30	125	70	4700
1 p.m	128	10	200	150	55	69	85	2360
2	110	25	350	.	37	175	70	465
3	125	25	350	140	31	75	240	1280
4	120	20	200	145	125	37	120	1280
5	123	65	200	.	62	250	300	392
6	122	20	200	140	87	133	120	357
7	124	10	210	125	65	75	100	642

to the green plant. The average drop in cell numbers, to be derived from the data of Table 34, equals 4.10^7 cells/ml sheath water/night, corresponding with 0.8 μg N/ml (1 g bacteria fresh weight contains 10^{12} cells with approximately 20% dry weight and 10% N in the dry material). This corresponds with a yearly production of $5 \times 10^4 \times 365 \times 0.8 = 14.6$ g N/ha (5 ml sheath water/plant/night; number of plants 10^4/ha). Since only some of the micro-organisms in the leaf sheath are nitrogen fixers, the amount of nitrogen derived from nitrogen fixation is still lower than that calculated above. These rough calculations suggest that the amount of nitrogen in the phyllos-

Table 34. Numbers of micro-organisms (in millions) in the sheath water of particular leaves of guatemala grass, growing under natural conditions and sampled with intervals of 2 hours during a 24-hour period.

Time	Numbers/ml				
	22/6	25/6	29/6	12/7	9/8
08.00	49	48	112	104	35
10.00	43	40	93	99	44
12.00	41	31	78	94	38
14.00	40	30	85	122	39
16.00	31	33	87	93	39
18.00	27	28	75	.	15
20.00	30	06	87	128	11
22.00	.	12	84	124	07
24.00	.	11	44	146	07
02.00	14	24	43	76	14
04.00	44	23	75	52	09
06.00	83	40	110	101	15
08.00	140	44	109	110	32

phere, derived from nitrogen-fixing micro-organisms, is very low.

As stated earlier, excessive amounts of carbohydrates are contained in the sheath water of guatemala grass, even in the presence of 5.10^8 bacteria/ml of sheath water. This disagreement between number of bacteria and amount of carbohydrates in sheath water can also easily be shown when the maximum number of bacteria is calculated, which correspond with the amounts of carbohydrates present (2–4 mg/ml; Table 27), supposing that nitrogen and other nutrients are present in optimum concentration in the sheath water. Assuming that 1 g of the carbohydrates gives rise to the formation of 0.25 g cell material, (corresponding with approximately 10^{12} bacteria), then 2–4 mg carbohydrates may give rise to the synthesis of $2–4.10^9$ cells, a number which is 10–100 times higher than the numbers virtually counted. These deviating results may be explained by the absence of an essential nutrient or growth factor and /or the presence of growth-inhibiting factors in sheath water.

5.2 Sheath water as growth medium for micro-organisms

To test sheath water for the absence of essential nutrients or/and the presence of inhibiting compounds, the following experiment was carried out in triplicate several times during the growth period of the grass. Tubes of 20 ml capacity were filled with 6 ml soil extract azotobacter solution (s.a.s.), supplemented with: 6 ml distilled water (A 1); 6 ml sheath water of guatemala grass (A 2); 6 ml water + 6 mg $(NH_4)_2SO_4$ (B 1); 6 ml sheath water + 6 mg $(NH_4)_2SO_4$ (B 2). Tube C received 6 ml sheath water + 6 ml water but no s.a.s. One of the micro-organisms (a *Pseudomonas* strain),

isolated from sheath water, was used for inoculation of the tubes and the bacterial growth was measured nephelometrically.

The results of this experiment showed (Fig. 21) that the addition of sheath water, instead of distilled water to the basal solution affected the growth of the inoculated bacterium only slightly. The addition of $(NH_4)_2SO_4$ to the basal solution, however strongly promoted the growth of the organism showing that the medium was poor in

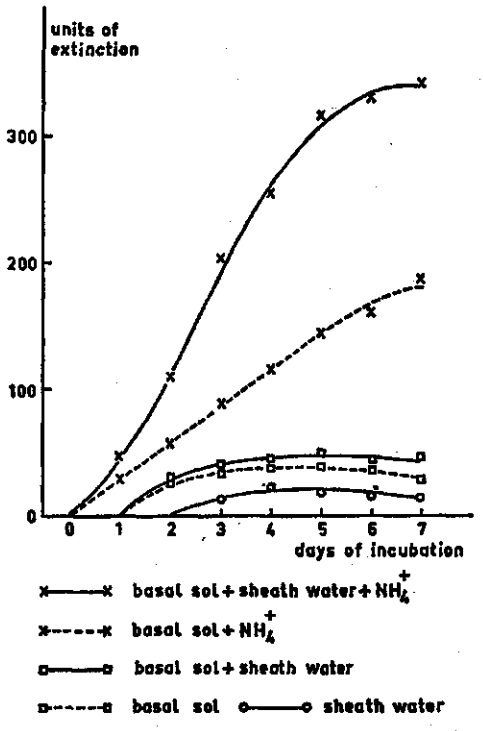


Fig. 21. The growth of a *Pseudomonas* strain, isolated from the sheath water of guatemala grass, in soil extract azotobacter solution supplemented with sheath water, $(NH_4)_2SO_4$ and sheath water + $(NH_4)_2SO_4$, respectively.

Table 35. Growth of micro-organisms in collected sheath water of guatemala grass, supplemented with $(NH_4)_2SO_4$, yeast extract or soil extract.

Days of incubation	Growth (nephelometric units)			
	Control	$(NH_4)_2SO_4$	Yeast extract	Soil extract
0	35	35	35	35
1	44	46	80	38
2	45	86	144	36
3	42	119	151	38
6	38	131	125	37
7	40	110	120	36

Table 36. C_2H_4 production (nmol/culture) of a number of *Klebsiella* strains, grown on azotobacter agar, isolated from sheath water of guatemala grass.

Strain	Approx. initial pO_2 (atm)	Hours after injection of C_2H_2							
		0	24	47	71	95	168	220	360
1	0.20	0	44	.	60	242	735	.	.
	0.05	0	52	.	104	948	1833	.	.
10	0.20	0	181	383	553	857	.	.	.
	0.05	0	406	778	1074	1145	.	.	.
13	0.20	0	288	746	1227	1533	2567	.	8674
	0.05	0	1027	1943	2883	3397	4621	.	11968
16	0.20	0	98	320	387	656	1043	.	.
	0.05	0	15	56	394	758	2465	.	.
17	0.20	0	5	7	.	190	1485	4298	.
	0.05	0	19	164	.	1130	2733	6952	.

with 45 ml argon which replaced 45 of the 70 ml air above the cultures. Thereupon 8 ml C_2H_2 was injected and the pressure of the mixture reduced to atmospheric pressure.

All of the isolates found to be able to reduce acetylene (11 strains) belonged to the *Enterobacteriaceae* (genus *Klebsiella*). These bacteria were isolated from the sheath liquid of guatemala grass, sorghum and maize. Examples of the course of acetylene reduction after the injection with C_2H_2 of the *Klebsiella* cultures growing on azotobacter agar slants are given in Table 36. It will be seen that lowering the initial pO_2 strongly favoured the acetylene reduction of these organisms.

6.2.2 Acetylene-reducing activity of sheath water of guatemala grass

To decide if the micro-flora of sheath water of guatemala grass had the capacity to reduce acetylene or enabled the enrichment of nitrogen-fixing bacteria, tubes of 10 ml capacity were supplied with 1 ml of this liquid collected from different plants. After closing the tubes with Suba seal caps, different amounts of argon were injected into the tubes, with a polythene syringe, replacing different amounts of air which were released through a needle in the stopper giving pO_2 values of 0.2, 0.15, 0.10, 0.05 and 0.0 atm, respectively. For complete removal of the air the tubes were flushed with argon for about 1 minute. Subsequently, 1 ml C_2H_2 was injected into the tubes through the rubber stopper and the ethylene production measured at definite time intervals. All the tubes were incubated at room temperature (approximately 30 °C).

The reduction of acetylene in sheath water was not detectable in 3 experiments; only in 1 experiment were the micro-organisms in the sheath water able to grow and to

Table 37. C_2H_4 production (nmol) per ml of sheath water, collected from guatemala grass at different pO_2.

Initial pO_2 (atm)	Hours after injection of C_2H_2						
	0	1	6	12	21	24	28
0.20	0	0	0.9	2.7	0.4	1.1	1.3
0.15	0	0.2	0.2	2.7	1.1	.	.
0.10	0	0	0.2	0.2	0.2	0.5	0.9
0.05	0	0	0.4	0.5	4.5	7.2	8.1
0.0	0	2.1	1.1	1.4	1.1	8.2	17.6

reduce C_2H_2. The results of this experiment, given in Table 37, showed that the ethylene production by the sheath-water micro-flora was very small and hardly increased with time. Only under anaerobic conditions did a slight ethylene production occur which increased with time.

6.2.3 Acetylene-reducing activity of populated parts of guatemala grass plants

For this experiment, which was carried out three times during the growth period of the guatemala grass, use was made of populated parts of stems detached from grass plants, grown in the field. These stem parts, which contained leaf sheaths, were brought into 250-ml tubes. The total volume of the plant parts, present in 1 tube, was about 100 ml. The tubes were closed with Suba seal caps and injected or flushed with different amounts of argon, so that oxygen tensions of 0.2, 0.1 and 0.0 atm were obtained. Hereafter 15 ml C_2H_2 was injected. All the experiments were duplicated and incubated in the dark at room temperature (approximately 30 °C).

The production of ethylene was detectable in all the tubes of this experiment. The results, given as averages of 6 values (Table 38), show that the acetylene-reducing activity was slight; it was not increased by lowering the initial oxygen pressure.

During one experiment, samples of the gas mixture were analysed by gas chromato-

Table 38. C_2H_4 production (nmol) of populated parts of guatemala grass (about 100 g fresh wt), at different pO_2.

Initial pO_2 (atm)	Days after injection of C_2H_2					
	0	1	2	3	4	5
0.2	0	17	45	87	125	234
0.1	0	19	53	108	158	184
0.0	0	9	31	53	143	331

Table 39. H_2 (‰) and N_2 (%) contents of the gas mixture in tubes containing parts of guatemala grass at different pO_2.

Initial pO_2 (atm)	Days after injection of C_2H_2											
	0		1		2		3		4		5	
	H_2	N_2	H_2	N_2	H_2	N_2	H_2	N_2	H_2	N_2	H_2	N_2
0.2	0	80	0.2	80	1.1	73	1.7	72	2.6	69	.	.
0.1	0	65	0.06	64	0.4	63	0.8	61	1.4	60	2.7	60
0.0	0	47	0.06	46	0.5	46	1.4	46	2.7	46	5.3	44

graphy for H_2 and N_2. The results, given in Table 39, show that H_2 was produced 1 day after the commencement of the experiment. The H_2 production increased with incubation time and ran parallel with the acetylene-reducing activity, indicating that the N_2-fixing bacteria (which were identified as *Klebsiella*) were responsible for its formation.

6.2.4 Acetylene-reducing activity of the phyllosphere of entire plants, growing under field conditions in the tropics

Several young plants of about 1.5 meter high were separately enclosed by a transmitting bag, sealed with 'saran' plastic of a gas-tight quality. About 15 cm above the soil surface the bag was fixed around the stem between 2 copper strips, pressed together with 2 clips, making it gas-tight by using a ceramic lute 'kachelkit' (Hercules) and plasticine. The air inside the bag was removed by suction, so that the bag became flattened. When after about 30 min the pressure had not changed, the bag was filled with a gas mixture containing 70% Ar, 25% O_2 and 5% CO_2, which was removed by suction. This procedure was repeated 3 times whereupon the bag was filled with 3 litre of the gas mixture and closed with a stopcock. Then 400 ml C_2H_2 was injected through a rubber septum in the wall of the bag and ethylene formation was measured

Table 40. Acetylene-reducing activity (n mol/plant) of the phyllosphere micro-flora of entire guatemala grass plants.

Plant	Hours after injection of C_2H_2						
	0	19	30	45	68	91	116
1	0	0	53	142	303	587	854
2	0	36	53	71	151	481	
3	0	0	36	89	196	498	659

twice a day for 5 days.

The results obtained with 3 guatemala grass plants show (Table 40) that acetylene reduction by the phyllosphere organisms had occurred but at a low level. After 5 days the nitrogen-fixing micro-organisms of the phyllosphere of plant 1 had produced 854 nmol ethylene, corresponding with $854 \times 28/3 \times 10^{-6} = 8 \times 10^{-3}$ mg fixed nitrogen per plant. However, it should be stressed that the conditions for nitrogen-fixing bacteria in the sheath water for some unknown reason are unfavourable. If this limiting factor could be eliminated, the gains of fixed nitrogen would presumably be considerably higher.

6.3 Experiments with Zea mays, grown under temperate conditions in the Netherlands

6.3.1 Acetylene-reducing activity of sheath water of maize

The results of the experiments recorded in 3.4, have shown that the most favourable conditions for nitrogen fixation in the sheath water of maize, grown under temperate conditions, occurred in plants without any dressing and in those without nitrogen dressing. Therefore, acetylene reduction by incubated sheath water of such plants was tested several times during the growth period of the plants. One ml liquid was added to tubes of 10 ml capacity, closed with Suba seal caps and injected with 1 ml C_2H_2. For measuring the acetylene-reducing activity under anaerobic conditions, the tubes were flushed with argon and then injected with 1 ml C_2H_2. All the tubes were incubated at 30°C.

No ethylene production was found in any sample of sheath water tested either aerobically or anaerobically. This experiment was repeated one year later with the same negative results.

6.3.2 Acetylene-reducing activity of populated plant parts of maize, grown under temperate conditions

Similar to the experiments with guatemala grass under tropical conditions, young parts of maize plants, used for the sheath-water experiment (6.3.1), were tested for the presence of nitrogen-fixing bacteria. During the growth period of the maize plants parts

Table 41. C_2H_4 production (nmol) of populated parts of young maize plants (about 100 g fresh wt), grown under temperate conditions.

Plants	Days after injection of C_2H_2				
	0	2	6	8	10
Unfertilized	0	0	46	101	162
Without N	0	0	29	46	74

of stems, surrounded by the leaf sheath and with a volume of about 100 ml, were tested three times for acetylene reduction, as described earlier. Each experiment was duplicated and incubated in the dark at 30 °C.

The results, given in Table 41 as averages of 6 values, show that parts of maize plants grown under temperate conditions, when kept for a prolonged period of time in the laboratory, produced small amounts of ethylene. Similar results were obtained with parts of maize plants tested one year later.

6.3.3 Acetylene-reducing activity of the phyllosphere micro-organisms of entire maize plants, growing in the field under temperate conditions

Similar to the experiment with guatemala grass, recorded earlier, 3 young maize plants, growing without added nitrogen, were separately enclosed by a transmitting bag as described earlier and tested for acetylene reduction. During 8 days the ethylene production was measured.

As average of the values of 3 incubated plants, 80 nmol of ethylene was found to be produced by the phyllosphere micro-organisms after an incubation period of 8 days. This amount corresponds with 0.45 g fixed nitrogen per ha assuming a growing period of 120 days and 4×10^4 maize plants per ha.

7 ^{15}N experiments with guatemala grass in the field under tropical conditions

7.1 Introduction

The experiments with guatemala grass recorded in the previous chapter have shown that small amounts of nitrogen may be fixed in the phyllosphere of these plants. To obtain more quantitative data on this nitrogen fixation, experiments with labelled N_2 were carried out. For this purpose, guatemala grass, growing in the field under tropical conditions in Surinam, was exposed to elementary nitrogen enriched with ^{15}N and the amount of ^{15}N fixed and present in various plant parts estimated.

7.2 Materials and methods

Three young unfertilized plants, about 1.5 m high, were separately enclosed by a transmitting bag as described in Section 6.2.4. The air in the bag was removed by suction, so that the bag flattened and leakages could readily be observed. The bag was then filled through a stopcock with a gas mixture, containing 5% CO_2, 25% O_2 and 70% Ar which afterwards was sucked out. This procedure was repeated three times. Then the three bags were filled with 2 l of this gas mixture supplemented with 0.5 litre N_2, enriched with 52.3% ^{15}N (bags 2 and 3) and with 200 ml N_2, enriched with 96.8% ^{15}N (bag 1). The three plants were incubated for 2, 3 and 4 days, respectively. Hereafter they were harvested and divided into the following parts: sprouts, consisting of blades, sheaths and stem, main stem (axis), adventitious roots, main roots and lateral roots (see Fig. 22). About 250 mg of the dry matter of these plant parts was used for the determination of nitrogen according to the Kjeldahl method. After the distillation of the NH_3 into boric acid and its titration, the ammonia was steam-distilled into 5 ml 0.1 N HCl. The rest of the dry matter of the plant parts was digested and the NH_3 distilled into the same acid solution, which was reduced by vacuum rota evaporation to a volume of 5 ml and used for the ^{15}N analysis. This was carried out with an emission spectrometer after the conversion of ammonia to N_2 with sodium hypobromite (see Chap. 2).

7.3 Results and discussion

The results, given in Table 42, show that the micro-organisms in the phyllosphere of guatemala grass fixed measurable amounts of nitrogen. The plants had taken up 222, 230 and 287 μg nitrogen in 2, 3 and 4 days, respectively. Based on these results,

Table 42. N_2 fixed by phyllosphere micro-organisms in guatemala grass plants exposed to ^{15}N in the field under tropical conditions

Plant part (see Fig. 22)	Days of incubation								
	2 (plant 1)			3 (plant 2)			4 (plant 3)		
	Tot. N (mg)	At.% ^{15}N excess	Fixed N µg	Tot. N (mg)	At. % ^{15}N excess	Fixed N µg	Tot. N (mg)	At. % ^{15}N excess	Fixed N µg
1*Blades	44.6	0.025	11.5	22.7	0.011	4.8	16.2	0.052	16.0
Sheaths	57.3	0.075	44.3	46.8	0.008	7.1	52.2	0.035	34.8
Stem	62.2	0.000	0.0	26.6	0.010	5.2	.	.	.
2 Blades	46.4	0.041	19.6	19.0	0.018	6.5	38.2	0.080	5.9
Sheaths	50.2	0.060	31.0	22.3	0.008	3.4	87.6	0.090	15.1
Stem	80.3	0.013	14.4	8.0	0.011	1.7	85.5	0.030	5.0
3 Blades	75.6	0.060	46.8	70.5	0.010	13.6	7.5	0.099	14.1
Sheaths	90.2	0.041	38.4	70.1	0.038	50.8	11.4	0.059	12.8
Stem	155.6	0.010	16.1	103.4	0.000	0.0	19.8	0.033	12.4
4 Blades				53.4	0.028	28.5	13.9	0.100	26.5
Sheaths				42.0	0.023	18.5	21.3	0.122	45.4
Stem				55.9	0.000	0.0	.	.	.
5 Blades				45.9	0.015	13.2	16.3	0.161	50.0
Sheaths				44.5	0.020	17.0	18.3	0.113	39.5
Stem				65.1	0.009	11.1	15.0	0.013	9.4
6 Blades				53.5	0.015	15.3			
Sheaths				90.3	0.019	32.9			
Stem				.	.	.			
Axis	81.0	0.000	0.0	622.4	0.000	0.0	82.4	0.000	0.0
Adv. roots	19.5	0.000	0.0	49.2	0.000	0.0	18.4	0.000	0.0
Lat. roots	33.5	0.000	0.0	96.7	0.000	0.0	18.6	0.000	0.0
Main roots	46.9	0.000	0.0	38.9	0.000	0.0	58.4	0.000	0.0
Fixed N_2 in plants:			222.1			229.6			287.0

The biological standard contained 0.365 atom % ^{15}N.
* Figures refer to sprout number.

it is possible to calculate the amount of nitrogen fixed by the phyllosphere micro-organisms and taken up by the guatemala grass during one year. After 4 days, plant 3 contained 287 µg of fixed nitrogen. Assuming a continuous cultivation of guatemala grass, this corresponds to an amount of 263 g N per ha. The benefit to the plant is the total amount of fixed nitrogen minus the nitrogen present in the micro-organisms of the phyllosphere. Assuming the presence of 10^9 micro-organisms per ml sheath water and of 5 ml sheath water per plant, the total number of micro-organisms occurring in the phyllosphere of one plant is approximately 5×10^9. This number corresponds to

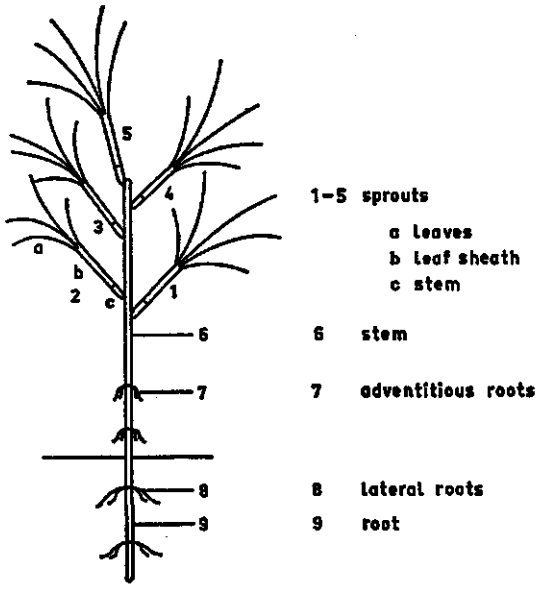


Fig. 22. The parts of a guatemala grass plant, exposed to ^{15}N.

1 mg dry matter, containing about 0.1 mg N per plant and with 1 g per ha, assuming the presence of 10^4 guatemala grass plants per ha. The difference, 262 g of the fixed nitrogen, is available to the growth of the guatemala grass. Similarly, for plant 1 and 2 values of 403 and 280 g per ha can be calculated.

That the nitrogen fixation occurred mainly in the sheath water may be concluded from the fact that the highest amounts of ^{15}N were mostly found in the sheaths and to a smaller degree in the blades. The stem of the sprouts contained considerably lower amounts of labelled N, whereas outside the sprouts no ^{15}N was detected. The fact that considerable amounts of ^{15}N occurred in blades and stem, shows that a considerable proportion of the ^{15}N had been released from the bacterial cells and was absorbed by the plant.

Summary

A study was made of the occurrence of nitrogen-fixing micro-organisms in the phyllosphere of *Zea mays* and *Tripsacum laxum* Nash (guatemala grass) and of the contribution of such micro-organisms to the nitrogen supply of the plants. These investigations were carried out within the framework of the 'International Biological Programme' (IBP). The phyllosphere is the environment of micro-organisms on the wet leaf surface and in the space between leaf sheath and stem. These micro-organisms utilize the substrates released from the leaves and from the stem. Several factors, including type of plant, age and nutritional conditions of the plant, affect amount and type of substrate. Nitrogen fixation may occur when the carbohydrate content exceeds that of nitrogenous compounds many times; otherwise the nitrogen-fixers are superseded by fast-growing non-nitrogen-fixing bacteria that use both carbohydrates and N compounds for growth. Generally it can be assumed that the latter type of bacteria occurs when the C/N value of the assimilable substrates is below 20. Some reports on nitrogen fixation in the phyllosphere may be found in the literature, but so far no quantitative data on the subject are available.

In this study, amount and composition of the substrates, released from some types of plants, were related to the micro-organisms occurring in the phyllosphere. The experiments were carried out under temperate conditions in the Netherlands and under tropical conditions in Surinam. In both cases plants were grown in pots and in the field with different dressings. The ability to fix elementary nitrogen was investigated with isolated micro-organisms under laboratory conditions and with the natural micro-flora occurring in collected sheath water, while in some cases entire plants were tested for nitrogen fixation.

The substrates released by leaves and stem into the phyllosphere were mostly collected by spray irrigation of the aerial plant parts. To study the micro-flora of the phyllosphere, the sheath water was used i.e. the water between leaf sheath and stem present before spraying. Initially it was assumed that the sheath water was representative of the substrates released under natural conditions by the blades into the phyllosphere, its 'overflow' being caught by the leaf sheath. Experiments on the chemical composition of the liquid released by the blades as well as that of the sheath water learnt that this was probably not the case, the sheath water being mainly released by the leaf sheath and the stem. In subsequent experiments, the composition of the sheath water was estimated as well, so that a better comparison between substrates and microflora of the phyllosphere could be made.

Chapter 3 contains the results of experiments with maize grown in pots as well as on experimental plots in the garden of the Laboratory of Microbiology, Wageningen. In the first experiment the spray irrigation (corresponding with a shower of 6 mm) was carried out weekly for 1 hour. In this experiment the influence of the nitrogen dressing and of the age of the plants on the leaching of substrates and on the micro-flora of the phyllosphere were studied. The plants with the highest nitrogen dressing had the highest N and P content (Fig. 2). The amount of carbohydrates leached from the aerial parts of the plants by the spray irrigation was small (< 10 mg/plant) and was hardly affected by an increased nitrogen dressing or by the age of the plants. The nitrogen content of the leachate was clearly higher with increased nitrogen supply and decreased with ageing of the plants (Tables 2 and 3). The C/N value of the leachate was low and presumably did not allow nitrogen fixation in the phyllosphere.

The numbers of micro-organisms in the sheath water increased during the growth of the plants, except for the last counting (Table 4). These numbers were often considerably higher in the phyllosphere of plants grown at the high nitrogen dressing. The predominant micro-organisms of the phyllosphere mainly belonged to the *Pseudomonadaceae*, *Corynebacteriaceae*, *Enterobacteriaceae* and *Achromobacteraceae*. Initially the pseudomonads were counted as the largest group, but they decreased with ageing of the plants, whereas the coryneforms increased (Figs. 4–6).

In a second pot experiment with maize the plants were sprayed daily. The carbohydrate and nitrogen contents of the leachate were lower than those of the weekly spraying, but they remained more or less at the same level, indicating a constant supply of small amounts of substrates to the phyllosphere organisms (Figs. 7 and 8). In this experiment the C/N value of the leached substrates was also very low.

Although the total numbers of bacteria counted were considerably lower than those of the first pot experiment (Table 5), the same types of organisms were identified (Figs 9–11). The coryneforms were the most important group from the beginning of the experiment.

The same method for collecting leachate as used in the pot experiments, was employed for maize growing on experimental plots. The plants were sprayed daily for 1 hour with an amount of water corresponding with a shower of 6 mm. In the first field experiment, plants dressed with a low and with a high amount of nitrogen were compared. The amount of carbohydrates released did not change very much with ageing of the plants; low-N plants leached slightly less carbohydrates than high-N plants. Due to the relatively high nitrogen content of the released substrates, very low C/N values were calculated in this experiment (Figs 12–14).

In the second field experiment with maize, use was made of a series of experimental plots with different N, P and K dressings in the garden of the Laboratory of Microbiology, Wageningen (Tables 6–8). In this experiment the composition of the water-soluble fraction of the plant material was compared with the amounts of carbohydrate and nitrogen in the sheath water during the growth of the plants. The highest amount of carbohydrates was found in the soluble fraction of plants deficient in potassium (Table 9); the nitrogen content of these plants was also high. Nitrogen-deficient plants

contained the lowest amounts of soluble N (Table 10). The highest C/N value in the soluble fraction was found in plants without any dressing (Table 11). In this case values of about 10 were found during the period of maximum growth of the plants as contrasted with about 5 in plants with the other dressings. With all of the dressings, lower C/N values in the soluble fractions of the plant material were found in the beginning and at the end of the growth period.

The sheath water of young maize plants of this experiment was rich in carbohydrates (Table 12). The highest values occurred in plants without dressing. K-deficient plants had relatively low and P-deficient plants relatively high values. This was in contrast with the carbohydrate contents of the soluble fractions of plants deficient in K and P, respectively.

This difference in effect of K and P deficiency is clearly shown when the ratio of the carbohydrate contents of the soluble fraction of K and P-deficient plants (1.73) is compared with the corresponding value in the sheath water (0.77).

The nitrogen content of the sheath water, estimated as ninhydrin-positive compounds, was relatively low (Table 13). It clearly increased with ageing of the plants. Since the carbohydrate content decreased, the C/N value of the sheath water was highest in young plants (Table 14). Values between 100 and 200 were found, indicating favourable conditions for nitrogen fixation in this medium.

Large differences existed between the C/N values of the soluble fraction of maize plants and those of the sheath water.

Chapter 4 gives the results of experiments on the phyllosphere of guatemala grass (*Tripsacum laxum* Nash) grown under tropical conditions in Surinam (Plate 2 and Fig. 22). Similar to the experiments with maize in the Netherlands, a comparison was made of the micro-flora and the amounts of carbohydrates and nitrogenous compounds leached from the leaves of plants of different age and nitrogen supply.

Data on the growth and the chemical composition of this grass grown in pots have been given in tables 15 and 16. In this experiment the composition of the leachate was compared with that of sheath liquid, sampled before spraying. The amounts of carbohydrates leached from the plants were higher than those leached from maize under temperate conditions (cf. tables 17 and 2). The carbohydrate content of the sheath water was also considerably higher than that of maize plants, reaching values of 2 g per litre. The nitrogen content of the leachate was relatively high, that of the sheath water was low (Table 18). Consequently, the C/N values of the leachate were low, but those of the sheath water were very high. These results indicated favourable conditions for nitrogen fixation.

The large differences in carbohydrate and nitrogen contents between leachate and sheath liquid and the different response of these contents to age and nitrogen supply of the plants indicate a different origin of the substrates of both types of liquid.

The number of micro-organisms in the sheath water of guatemala grass was much higher than that counted in sheath water of maize, growing under temperate conditions (Tables 20 and 4). Bacteria belonging to the *Achromobacteraceae* were predominant

in the sheath water, followed by coryneforms. Pseudomonads, one of the most important groups of bacteria of maize, occurred only sporadically in the phyllosphere of guatemala grass (Figs 15–17).

The results of this pot experiment were confirmed by those of a field experiment with guatemala grass in which the plants were sprayed 3 times a week. The amounts of carbohydrates in the leachate were even considerably lower than those of the pot experiment. Since the nitrogen contents were somewhat higher, the C/N values were still lower than in the pot experiment (Table 21).

The total numbers of micro-organisms in the sheath water of guatemala grass growing in the field were comparable to those of the pot experiment (Table 22). The composition of the micro-flora, mainly achromobacters and coryneforms and a few pseudomonad strains, resembled that of the pot experiment (Figs 18–20).

In a subsequent field experiment with guatemala grass a comparison was made of the carbohydrate and nitrogen contents of leaf water (i.e. water present on the blades, resulting from dew) and of sheath water. Although the carbohydrate content of the sheath water was considerably lower than that of the sheath water of the previous pot experiment, it was much higher than that of the leaf water (Table 23). In the first half of the experimental period considerably higher values for carbohydrate content were found than in the second half. Leaf water did not show these differences.

In contrast with the carbohydrate content, nitrogenous compounds occurred in considerably lower amounts in the sheath liquid than in the leaf water. The lowest nitrogen content was found in the sheath water of mature plants; the nitrogen content of leaf water hardly changed during the growing period of the grass (Table 24). The divergency of leaf and sheath water excludes the existence of a common origin.

In consequence of the high carbohydrate content of the sheath water and the low nitrogen content, very high C/N values were found (Table 25). The highest values (600–700) were calculated for sheath water of plants, growing with the lowest N-supply, during the second part of the experimental period. In leaf water, the C/N values were found to be very low viz. mostly < 10. These results indicate that the conditions for nitrogen fixation are favourable in the phyllosphere of the leaf sheath and unfavourable in that of the leaf blade.

As in the sheath water of guatemala grass, high C/N values were also found in the sheath water of some other *Gramineae* (*Zea mays*, *Sorghum bicolor* and, to a lower degree, *Echinochloa polystachya*; Table 26).

To study the effect of the age of the leaf on the carbohydrate content of the sheath water, separate analyses were made of the sheath water of each leaf of particular plants. This experiment was repeated several times with different plants in the course of the growth period of guatemala grass. The highest concentrations were found in the sheath water of young unexpanded leaves (Table 27).

From the results of the experiments discussed so far, it was concluded that the substrates of sheath water and leaf water (dew) of guatemala grass are derived from different sources. That the sheath water was excreted by the leaf sheath and/or stem was shown in the following experiments. Sheath water was diluted and then immediately

washed out with sterile water. Afterwards carbohydrates and nitrogenous compounds in the sheath water were periodically measured. A ready recovery of these substrates occurred, the values found being much higher than those originally present. The recovery of nitrogenous compounds was not as fast and complete as that of carbohydrates, resulting in increased C/N values with maximum values > 1000 (Tables 28 and 29). The release of substrates also occurred after cutting the lamina but before washing of the sheath contents. Detaching the leaf blades considerably stimulated the release of carbohydrates and nitrogenous compounds (Tables 30 and 31).

In Chapter 5 the growth of micro-organisms in the phyllosphere of guatemala grass under natural conditions was studied. In spite of the presence in sheath water of large numbers of micro-organisms, the occurrence of considerable amounts of carbohydrates in this liquid pointed to a low activity of these bacteria. This low activity might indicate a poor growth of the micro-organisms due to the absence of an essential nutrient or the presence of a growth-inhibiting factor in the sheath liquid.

To check this hypothesis, sheath water of particular leaves of guatemala grass was sampled at intervals of 1–2 hours and tested for carbohydrates and number of micro-organisms. In about 60% of the leaves tested the numbers of micro-organisms increased during day-time (Table 32); in the remaining 40% no alteration of the bacterial numbers occurred (Table 33). In both cases excessive amounts of carbohydrates were found but no correlation occurred between number of bacteria and carbohydrate content. In a further experiment bacterial countings in the sheath water were made during a period of 24 hours. The numbers of micro-organisms of the phyllosphere of the leaf sheath were shown to decrease during the night (Table 34). When the reduction of cell number was taken as a basis for the amount of bacterial nitrogen that became available to the plant, the annual amount of nitrogen was calculated to be approximately 15 g/ha. It was assumed that this process continued throughout the year and that all the bacteria were nitrogen fixers or obtained their nitrogen from nitrogen fixers.

To investigate if the suggested poor bacterial growth in the sheath water of guatemala grass was caused by a deficiency of a particular nutrient or by an excess of an inhibiting compound, laboratory experiments were carried out with a nutrient solution poor in nitrogen and supplemented with $(NH_4)_2SO_4$, sheath water, and $(NH_4)_2SO_4$ + sheath water, respectively. A non-nitrogen-fixing *Pseudomonas* strain, isolated from sheath water, was used as the test organism. Without added $(NH_4)_2SO_4$, no growth of the bacteria occurred; addition of $(NH_4)_2SO_4$ gave good growth, but sheath water had hardly any effect, showing that assimilable N-compounds (Fig. 21) occurred in very low concentrations. When in addition to $(NH_4)_2SO_4$, sheath water had been added to the basal medium, bacterial growth was much increased. This increase shows (a) the absence of growth-inhibiting compounds and (b) the good assimilability of the carbohydrates of the sheath water.

The importance of nitrogen deficiency as the growth-limiting factor of non-nitrogen-fixing bacteria in the phyllosphere of the leaf sheath of guatemala grass was clearly

shown in the following experiment with sheath water as nutrient solution for the above-mentioned *Pseudomonas* strain. Without added nitrogen almost no growth occurred; addition of $(NH_4)_2SO_4$ or yeast extract gave a normal growth of the inoculated bacterium (Table 35).

Since the results of the experiments with sheath water suggested that nitrogen-fixing bacteria were able to grow in that medium, several micro-organisms, isolated on azotobacter agar and originating from the sheath water of guatemala grass and rice plants, were tested for their nitrogen-fixing capacity. Approximately 20 isolates, mainly *Klebsiellas*, were found to fix small amounts of nitrogen (2–6 mg N/g saccharose); one *Klebsiella* strain fixed 10 mg and one *Pseudomonas* strain 20 mg N/g saccharose. Low amounts of N were fixed when sheath water was incubated in soil extract azotobater medium. When sheath liquid was incubated in Erlenmeyer flasks, growth of nitrogen-fixing bacteria was found in only a few instances. This indicates the absence of a compound required for nitrogen fixation.

In Chapter 6 experiments were described, concerning the nitrogen-fixing capacity of a number of micro-organisms, isolated from the phyllosphere of guatemala grass, sorghum and maize, and measured with the acetylene-reduction technique. The reduction of acetylene was studied at atmospheric as well as at low oxygen pressure (pO_2 about 0.05 atm). All the strains found capable of reducing acetylene to ethylene belonged to the genus *Klebsiella*. An example of the course of C_2H_2 reduction of a number of these strains is given in Table 36. From these results it will be seen that lowering of the oxygen pressure strongly promoted acetylene reduction.

Sheath water of guatemala grass, incubated at different oxygen pressures, showed only in 1 of the 4 experiments a small acetylene reduction. Lowering the oxygen pressure (< 0.05) slightly increased this effect (Table 37).

In a subsequent series of experiments, populated parts of the stem of guatemala grass, surrounded by the leaf sheath, were exposed to acetylene at different pO_2. Although production of ethylene was always detectable, the activity was small; reduction of the oxygen tension did not affect this activity (Table 38). Except C_2H_2-reduction, production of H_2 by the nitrogen-fixing bacteria of the phyllosphere (mainly *Klebsiellas*) was found during this experiment (Table 39).

Finally, entire plants of guatemala grass, growing in the field, were enclosed by a plastic bag, sealed gas-tight from the air and supplied with C_2H_2. The production of C_2H_2 in the bag was measured during several days. Though a clear acetylene reduction was detected, the calculated amount of nitrogen fixed was only 8 μg per plant. This low figure was mainly due to the fact that in spite of the excess of carbohydrates and the favourable C/N value, the activity of nitrogen-fixing phyllosphere-bacteria, for some unknown reason, was very low. If this limiting factor could be eliminated, the amounts of fixed nitrogen would presumably be considerably higher. A further reason for the low values may be that during the transport of the ethylene-containing tubes from Surinam to the Netherlands losses of ethylene may have occurred (cf. Chap. 7).

Similar experiments on the acetylene-reducing capacity of the phyllosphere as done in Surinam with guatemala grass, were carried out with maize, growing under temperate conditions in the Netherlands. Sheath water of plants, grown without any dressing or without nitrogen dressing, having the most favourable C/N value for nitrogen fixation, was incubated aerobically as well as anaerobically under laboratory conditions after addition of C_2H_2. Though this experiment was repeated many times during the growth period of maize, positive results were never obtained.

By using populated parts of the stem of maize, surrounded by the leaf sheath, small amounts of acetylene were found to be reduced (Table 41). A small but clearly detectable activity was also found when the acetylene-reducing capacity of entire maize plants, growing in the field, was measured, showing that under temperate conditions N_2 fixation by the phyllosphere organisms is also possible.

In Chapter 7 the results of some experiments with ^{15}N were given. Guatemala grass, growing under natural conditions in Surinam, was enclosed in a plastic bag and exposed to labelled N_2. After incubation for some days, the plants were harvested and divided into different parts which were analysed for total N and ^{15}N. For ^{15}N an emission spectrophotometer was used.

From the results it can be seen that measurable amounts of ^{15}N were found in the plant material (Table 42). This nitrogen was mainly present in the sheaths and to a smaller degree in the leaf blades. The stem of the sprouts contained low amounts of ^{15}N while outside the sprouts no ^{15}N was detected. These results show that fixation of nitrogen occurs through the phyllosphere flora of the leaf sheats.

References

Adamse, A. D., 1970. Some characteristics of Arthrobacters from a dairy waste activated sludge. Wat. Res. 4: 797–803.

Akkermans, A. D., 1971. Nitrogen fixation and nodulation of *Alnus* and *Hippophae* under natural conditions. Thesis Leiden.

Allen, L. A., J. Harrison, S. J. Watson & W. S. Ferguson, 1937. A study of the chemical and bacteriological changes occurring in grass silage. J. agric. Sci. 27: 271–293.

Arens, K., 1934. Die kutikuläre Exkretion der Laubblätter. Jb. wiss. Bot. 80: 248–300.

Bailey, H. D., 1929. A flagella and capsule stain for bacteria. Proc. Soc. exp. Biol. Med. 27: 111.

Bell, D. J., 1955. Mono- and oligosaccharide and acidic monosaccharide derivatives. In: Moderne Methoden der Pflanzenanalyse: 1–55. Eds K. Peach & M. Tracey. Springer-Verlag Berlin.

Beyerinck, M. V., 1888. Die Bakterien der Papilionaceenknöllchem. Bot. Ztg 46: 742–750.

Bhat, J., K. Limaye & V. Vasantharajan, 1971. The effect of leaf surface microflora on the growth and root exudation of plants. In: Ecology of leaf surface micro-organisms: 581–595. Eds T. F. Preece & C. H. Dickinson. Academic Press London.

Bode, H. R., 1958. Beiträge zur Kenntnis allelopathischer Erscheinungen bei einigen Junglandaceen. Planta 51: 440–480.

Brown, M. E. & G. Metcalfe, 1957. Nitrogen fixation by a species of *Pullularia*. Nature, Lond. 180: 282.

Burri, R., 1903. Die Bakterien Vegetation auf der Oberfläche normal entwickelter Pflanzen. Zentbl. Bakt. ParasitKde (Abt II) 10: 756–757.

Campbell, N. E. R., R. Dular & H. Lees, 1967. The production of $^{13}N_2$ by 50-MeV protons for use in biological nitrogen fixation. Can. J. Microbiol. 13: 587–599.

Carnahan, J. E., L. E. Mortenson, H. F. Mower & J. E. Castle, 1960. Nitrogen fixation in cell-free extracts of *Clostridium pasteurianum*. Biochim. biophys. Acta 38: 188–189.

Chan, E. C. S., H. Katznelson & J. W. Rouatt, 1963. The influence of soil and root extracts on the associative growth of selected soil bacteria. Can. J. Microbiol. 9: 187–197.

Clark, F. E. & E. A. Paul, 1970. The microflora of grassland. Adv. Agron. 22: 375–426.

Dalbro, S., 1956. Leaching of nutrients from apple foliage. Proc. 14th int. hort. Congr. (Paris): 770–778.

Dalton, H. G. & J. R. Postgate, 1969. Effect of oxygen on growth of *Azotobacter chroococcum* in batch and continuous culture. J. gen. Microbiol. 54: 463.

Di Menna, M. E., 1959. Yeasts from the leaves of pasture plants. N.Z. Jl agric. Res. 2: 394–405.

Dilworth, M. J., 1966. Acetylene reduction by nitrogen-fixing preparations from *Clostridium pasteurianum*. Biochim. biophys. Acta 127: 285–294.

Döbereiner, J. & R. Alvahydo, 1959. Sôbre a influência da cana-de-açúcar na ocorrência de 'Beyerinckia' no solo. II. Influência das diversas partes do vegetal. Rev. Brasil. Biol. 19: 401–412.

Düggeli, M., 1904. Die Bakterienflora gesunder Samen und daraus gezogener Keimpflanzen. Zentbl. Bakt. ParasitKde (Abt II) 12: 602–614.

Faust, H., 1967. Probenchemie ^{15}N-markierter Stickstoffverbindungen im Mikro- bis Nanomolbereich für die emissionsspektrometrische Isotopenanalyse. Isotopenpraxis 3: 100–103.

Federov, M. V. & T. A. Kalininskaya, 1961. Relationships of nitrogen fixing *Mycobacterium* to various supplements of carbon and supplementary growth factors. Microbiologiya 30: 681–686.

Fiuczek, M., 1959. Atmospheric nitrogen fixation in pure culture of *Streptomyces alni*. Acta mikrobiol. Polon. 8: 283–287.

Graham, D. C. & W. Hodgkiss, 1967. Identity of Gram negative, yellow pigmented, fermentative bacteria isolated from plants and animals. J. appl. Bacteriol. 30: 175–189.

Grainger, J. M. & R. M. Keddie, 1963. Nutritional studies on coryneform bacteria from soil and herbage. J. gen. Microbiol. 31: VIII.

Gruber, Th., 1909. Die Bakterienflora von Runkelrüben, Steckrüben, Karotten, von Milch während der Stallfütterung und des Weideganges einschliesslich der in Streu, Gras und Kot vorkommenden Mikroorganismen und deren Mengenverhältnisse in den 4 letzten Medien. Zentbl. Bakt. ParasitKde (Abt II) 22: 401–416.

Hales, S., 1727. 'Vegetable Staticks'. Eds. W. and J. Innys & T. Woodward. London.

Hardy, R. W. F., R. D. Holsten, E. K. Jackson & R. C. Burns, 1968. The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. Pl. Physiol. 43: 1185–1207.

Hill, S., 1971. Influence of oxygen concentration on the colony type of *Derxia gummosa* grown on nitrogen-free media. J. gen. Microbiol. 67: 77–83.

Hill, S. & J. Postgate, 1969. Failure of putative nitrogen-fixing bacteria to fix nitrogen. J. gen. Microbiol. 58: 277–285.

Hugh, R. & E. Leifson, 1953. A taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. J. Bacteriol. 66: 24–26.

Huss, H., 1907. Morphologisch-physiologische Studien über zwei aromabildende Bakterien. Zentbl. Bakt. ParasitKde (Abt II) 19: 50–70.

Jones, K., 1970. Nitrogen fixation in the phyllosphere of the Douglas Fir, *Pseudotsuga douglassii*. Ann. Bot. 34: 239–244.

Kalininskaya, T. A., 1967. Utilization of different carbon sources by nitrogen-fixing bacterial associations. Mikrobiologiya 36: 621–625.

Keddie, R. M., 1959. The properties and classification of lactobacilli isolated from grass and silage. J. appl. Bacteriol. 22: 403–416.

Kovacs, N., 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature, Lond. 178: 703.

Kozel, P. C. & H. B. Tukey Jr., 1968. Loss of gibberellins by leaching from stems and foliage of *Chrysanthemum morifolium* 'Princes Anne'. Am. J. Bot. 55: 1184–1189.

Kroulik, J. T., L. A. Burkey & H. G. Wiseman, 1955. The microbial populations of the green plant and of the cut forage prior to ensiling. J. Dairy Sc. 38: 256–262.

Last, F. T., 1955. Seasonal incidence of *Sporobolomyces* on cereal leaves. Trans. Brit. mycol. Soc. 38: 221–239.

Last, F. T. & F. C. Dieghton, 1965. The non-parasitic microflora on the surfaces of living leaves. Trans. Brit. mycol. Soc. 48: 83–99.

Leben, C., 1961. Micro-organisms on cucumber seedlings. Phytopathology 51: 553–557.

Lindner, R. C. & C. P. Harly, 1942. Rapid method for the determination of nitrogen in plant tissue. Science, N.Y. 96: 565–566.

Line, M. A. & M. W. Loutit, 1971. Non-symbiotic nitrogen-fixing organisms from some New Zealand tussock-grassland soils. J. gen. Microbiol. 66: 309–318.

Löhnis, F., 1910. 'Handbuch der landwirtschaftliche Bakteriologie'. Borntraeger, Berlin. 6–96.

Long, W. G., D. V. Sweet & H. B. Tukey, 1956. Loss of nutrients from plant foliage by leaching as indicated by radioisotopes. Science, N.Y. 123: 1039–1040.

MacConnell, J. T. & G. Bond, 1957. A comparison of the effect of combined nitrogen on nodulation in non-legumes and legumes. Pl. Soil 8: 378–388.

Meiklejohn, J. & J. B. Weir, 1968. Nitrogen-fixers, pseudomonads and other aerobic bacteria, from Rhodesian soils. J. gen. Microbiol. 50: 487–496.

Mes, M. G., 1954. Excretion of phosphorus and other mineral elements under the influence of rain. S. Afr. J. Sci. 50: 167–172.

Metcalfe, G. & M. Brown, 1957. Nitrogen fixation by new species of *Nocardia*. J. gen. Microbiol. 17: 567–572.
Metcalfe, G., S. Chayen, E. R. Roberts & T. G. G. Wilson, 1954. Nitrogen fixation by soil yeasts Nature, Lond. 174: 841–842.
Moore, A. W., 1966. Non-symbiotic nitrogen fixation in soil and soil-plant systems. Soils Fertil. 29: 113–128.
Moore, S. & W. H. Stein, 1954. Procedures for the chromatographic determination of amino acids on four percent cross-linked sulfonated polystyrene resins. J. biol. Chem. 211: 893.
Morgan, J. & H. B. Tukey, 1964. Characterization of leachate from plant foliage. Pl. Physiol. 39: 590–593.
Mulder, E. G., 1938. Over de beteekenis van koper voor de groei van planten en micro-organismen. Thesis Wageningen.
Murphy, J. & J. R. Riley, 1962. A modified single solution method for the determination of PO_4 in natural waters. Analytica chim. Acta 27: 31–36.
Németh, G. Y., 1959. A new nitrogen fixing micro-organism producing a red pigment. Nature, Lond. 183: 1460–1461.
O'Meara, R. A. Q., 1931. A simple delicate and rapid method of detecting the formation of acetylmethylcarbinol by bacteria fermenting carbohydrate. J. Path. Bact. 34: 401.
Oppenheim, J. & L. Marcus, 1970. Correlation of ultrastructure in *Azotobacter vinelandii* with nitrogen source for growth. J. Bacteriol. 101: 286–291.
Oppenheim, J. & L. Marcus, 1970. Induction and repression of nitrogenase and internal membranes in *Azotobacter vinelandii*. Bact. Proc.: 149.
Parker, C. A., 1954. Effect of oxygen on the fixation of nitrogen by *Azotobacter*. Nature, Lond. 173: 780–781.
Pengra, R. M. & P. W. Wilson, 1958. Physiology of nitrogen fixation by *Aerobacter aerogenes*. J. Bacteriol. 75: 21–25.
Plank, J. E. van der, 1936. The estimation of sugars in the leaf of the Mangold (*Beta vulgaris*). Biochem. J. 30: 457–483.
Polly, J. R., 1954. Colorimetric determination of nitrogen in biological materials. Anal. Chem. 26: 1523.
Postgate, J. R., 1970. Biological nitrogen fixation. Nature, Lond. 226: 25–27.
Rosen, H., 1957. A modified ninhydrin colorimetric analysis for amino acids. Arch. Biochem. Biophys. 67: 10–15.
Ruinen, J., 1956. Occurrence of *Beyerinckia* species in the 'phyllosphere'. Nature, Lond. 177: 220–221.
Ruinen, J., 1961. The phyllosphere. I. An ecologically neglected milieu. Pl. Soil 15: 81–109.
Ruinen, J., 1971. The grass sheath as a site for nitrogen fixation. In: Ecology of leaf surface microorganisms: 567–581. Eds. T. F. Preece & C. H. Dickinson. Academic Press London.
Saussure, N. T. de, 1804. 'Récherches chimiques sur la végétation'. Gauthier-Villars, Paris.
Schmidt-Lorenz, W. & A. Rippel-Baldes, 1957. Wirkung des Sauerstoffs auf Wachstum und Stickstoffbindung von *Azotobacter chroococcum*, Beyk. Arch. Mikrobiol. 28: 45–68.
Schöllhorn, R. & R. H. Burris, 1966. Study of intermediates in nitrogen fixation. Fedn Proc. Fedn Am. Socs exp. Biol. 25: 710.
Schöllhorn, R. & R. H. Burris, 1967. Acetylene as a competitive inhibitor of N_2 fixation. Proc. natl. Acad. Sci. U.S.A. 58: 213–216.
Schouwenburg, J. Ch. van & I. Wallinga, 1971. Methods of analysis for plant materials. M.Sc course on soil science and water management. University Wageningen, the Netherlands.
Schuknecht, W. & H. Schinkel, 1963. Beitrag zur Beseitigung der Anregungsbeeinflussung bei flammenspektralanalytischen Untersucchungen. Z. anal. Chem. 194: 161.
Stewart, W. D. P. & G. Bond, 1961. The effect of ammonium-nitrogen on fixation of elemental nitrogen in *Alnus* and *Myrica*. Pl. Soil 14: 347–360.

Stewart, W. D. P., G. P. Fitzgerald & R. H. Burris, 1967. *In situ* studies on N_2 fixation using the acetylene reduction technique. Proc. natl. Acad. Sci. U.S.A. 58: 2071–2078.

Stewart, W. D. P., G. P. Fitzgerald & R. H. Burris, 1968. Acetylene reduction by nitrogen-fixing blue-green algae. Arch. Mikrobiol. 62: 336–348.

Stickler, F., S. Wearden & A. Pauli, 1961. Leaf area determination in grain Sorghum. Agron. J. 53: 187–188.

Strandberg, G. W. & P. W. Wilson, 1968. Formation of the nitrogen-fixing enzyme sytem in *Azotobacter vinelandii*. Can. J. Microbiol. 14: 25–31.

Tamm, C. O., 1950. Growth and plant nutrient concentration in *Hylocomium proliferum* L. in relation to tree canopy. Oikos 2: 60–64.

Thomas, S. B. & J. McQuillin, 1952. Coli-Aerogenes bacteria isolated from grass. Proc. Soc. appl. Bacteriol. 15: 41.

Trevelyan, W. E. & J. S. Harrison, 1952. Study on yeast metabolism. I. Fractionation and microdetermination of cell carbohydrates. Biochem. J. 50: 298.

Tukey, H. B. & J. V. Morgan, 1964. The occurrence of leaching from above-ground plant parts and the nature of the material leached. Proc. 16th int. hort. Congr. (Brussels) 4: 146–153.

Tukey, H. B. & H. B. Tukey, 1962. The loss of organic and inorganic materials by leaching from leaves and other above-ground plant parts. In: Radioisotopes in Soil-Plant nutrition studies. Intern. Atomic Energy Agency, Vienna: 289–302.

Tukey, H. B., S. H. Wittwer & H. B. Tukey, 1958. Loss of nutrients by foliar leaching as determined by radioisotopes. Proc. Am. Soc. hort. Sci. 126: 120–121.

Vasantharajan, V. & J. Bhat, 1968. Interrelations of micro-organisms and Mulberry. II. Phyllosphere microflora and nitrogen fixation in leaf and root surfaces. Pl. Soil 28: 258–267.

Wallace, T., 1930. Experiments on the effect of leaching with cold water on the foliage of fruit trees. J. Pomol. 8: 44–60.

Wasicky, R., 1958. Durch Regen bewirktes Auswaschen von Vitamine C und andere Substanzen aus oberirdischen Pflanzenorganen. Scientia pharm. 26: 100–103.

Wieringa, K. T., 1955. Der Abbau der Pektine; der erste Angriff der organischer Pflanzensubstanz. Z. PflErnähr. Düng. Bodenk. 69: 151–155.

Winkler, W., 1899. Untersuchungen über das Wesen der Bakterien und deren Einordnung im Pilzsystem. Zentbl. Bakt. ParasitKde (Abt II) 5: 569–579.

Wolff, A., 1913. Zur Frage nach den Beziehungen zwischen Bakterienflora der Milch und der Weide. Zentbl. Bakt. ParasitKde (Abt II) 39: 411–419.

Yates, M. G., 1970. Control of respiration and nitrogen fixation by oxygen and adenosine nucleotides in N_2-grown *Azotobacter chroococcum*. J. gen. Microbiol. 60: 393–401.