TWO ASPECTS OF C-4 PLANTS

1. EFFECTS OF LIGHT INTENSITY ON PHOTOSYNTHESIS

2. A NITROGEN-FIXING ASSOCIATION

by

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ABSTRACT

Part 1 concerns the effect of light intensity on the leaves of *Gomphrena globosa*, a C-4 plant.

Plants were grown under three light intensities. The structure and function of the photosynthetic apparatus of newly matured leaves were examined by a number of techniques. These were electron microscopy, infra-red fluorescence photomicrography, dye (TNBT) reduction and $^{14}CO_2$ feedings. Contrary to conclusions from the literature, light intensity did not affect the membrane configuration of the chloroplasts. Metabolite levels seemed to vary with light intensity. With respect to its photosynthetic properties *G. globosa* seems to occupy an intermediate position in a range of other C-4 plants.

Part 2 describes some aspects of the relationship between a grass, *Paspalum notatum*, and a bacterium, *Azotobacter paspali* which is found mainly on the root surface of the grass. These organisms form a nitrogen fixing association in South-Eastern and Central Brazil. Both were studied separately because of the difficulty of establishing the association in the laboratory.

An apparatus was built in which photosynthesis and root function of *P. notatum* could be monitored. $^{14}CO_2$ was fed to the leaves and ^{14}C appearing in the roots and root exudate was analyzed. *P. notatum* appears to exude sufficient substrate to support nitrogen fixation by *A. paspali* but the amount and nature of exudate proved difficult to ascertain.

The association is found in acid soils but nitrogen fixation by *A. paspali* is inhibited by low pH. It was found that roots of *P. notatum* were able to neutralize increases in acidity in their environment. A low pH inhibited $N_2[C_2H_2]$-fixing activity of *A. paspali* grown in continuous culture at high and low concentrations of carbohydrate and oxygen.

Other nitrogen fixing bacteria, e.g. *Beijerinckia spp* and *Derxia gummosa*, are found in relatively low numbers in the rhizosphere of *P. notatum*. Nitrogen fixation by *A. paspali* may be less prone to oxygen inhibition than it is in these other bacteria, thus allowing *A. paspali* to compete more efficiently for carbohydrate.

It seems that *A. paspali* lives with *P. notatum* because of the favourable pH that is maintained in the root environment. The carbohydrate and oxygen concentration are probably also favourable for nitrogen fixation.

How can the association be easily established in the laboratory? Before further research can be done, a way must be found to answer this question.

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PART I

EFFECT OF LIGHT INTENSITY ON PHOTOSYNTHESIS

Chapter 1

EFFECT OF LIGHT INTENSITY ON THE PHOTOSYNTHETIC APPARATUS OF GOMPHRENA GLOBOSA

Foreword

The work described in this chapter was done in 1970 and reported (21) at a conference on photosynthesis and photorespiration held at Canberra, Australia, 23 November - 5 December 1970. It is discussed using the knowledge of C-4 photosynthesis available at that time, which is contained in the proceedings of the conference. An addendum contains comments made on the basis of relevant papers which have appeared since the conference.

Introduction

Gomphrena globosa is a C-4 plant (12). These plants are characterized by a number of properties which include C-4 dicarboxylic acids as the major products of short-term

photosynthesis and leaf photosynthetic tissue consisting of mesophyll and bundle sheath cells (11). The chloroplasts of the bundle sheath show various degrees of granal development (11).

Downton (10) has divided C-4 plants into "malate formers" and "aspartate formers." The bundle sheath chloroplasts of "malate formers" have no or rudimentary grana, and malic acid is a major product of short-term photosynthesis. When $^{14}CO_2$ was fed to *G. globosa* for six seconds, 70% of the carbon fixed into C-4 dicarboxylic acids was found in malic acid. The rudimentary grana of the bundle sheath chloroplasts consist of only two stacked thylakoids (10,33). Downton placed *G. globosa* in the "malate formers" group (10).

There is evidence to suggest that bundle sheath chloroplasts which are agranal or have only rudimentary grana are deficient in photosystem II (PS II); which would result in a low capacity to reduce NADP (23,40) (the problem of the relationship between PS II activity and the presence of grana is not yet resolved; there are a number of reports (19,24,31) showing that PS II activity is not dependent on grana formation). It was expected therefore, that if they lacked, or were deficient in, PS II, *G. globosa* bundle sheath chloroplasts would not be able to reduce the dye tetranitro blue tetrazolium (TNBT) as its reduction requires non-cyclic electron flow. However, preliminary experiments in this laboratory indicated

that bundle sheath chloroplasts of *G. globosa* were able to reduce TNBT in the absence of an exogenous electron donor.

A possible reason for these unexpected results was that the plants used in the various experiments may have had functional differences because they were grown in different environments. The experiments discussed here were done to show whether the photosynthetic apparatus of *G. globosa* was modified by the light intensity during growth.

The structure and function of the photosynthetic apparatus under the various conditions were measured by TNBT reduction, electron microscopy, infra-red fluorescence photomicrography and $^{14}CO_2$ feedings.

Materials and Methods

Gomphrena globosa var. globe amaranth was grown in vermiculite, and watered with half-strength Hoagland's solution (15). The plants were grown from seed in a growth chamber at 10,000 lux, 16 hour day, and 24°C/18°C day/night temperature. When approximately six leaves were present, some plants were transferred to a light intensity of 2,000 lux in the growth chamber and some outside for three months during the summer where the average maximum light intensity was 50,000 lux.

Leaves used in the experiments had grown more than 95% of their length in the environment indicated and had just stopped elongating.

Electron microscopy.

Samples from the mid-sections of leaves were taken at approximately 09.00 hours. Pieces 1 to 2 mm square were fixed for one hour at 4°C in 2.5% v/v glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 6.8, and post-fixed in 1% w/v OsO_4 in 0.1 M sodium cacodylate buffer for one hour at 4°C. The pieces were dehydrated in a graded ethanol series and embedded in Spurr's medium (38). Sections were cut with a glass knife on a Sorvall MT-1 ultra microtome, post-stained with uranyl acetate and lead citrate (35) and viewed in a Zeiss EM-9A microscope.

Before ultrastructural variation in cells or organelles can be ascribed to some treatment, the variation must be statistically significant. Unfortunately, the micrographs in this study were not taken with the intent of doing a statisticalaanalysis, making it difficult to apply an analysis when the need for one was realized. To see if there was any variation in bundle sheath chloroplast ultrastructure with light intensity, an analysis of variance (ånova) was performed

on the terms: (a) grana per chloroplast, (b) thylakoids per granum and (c) $\frac{\text{partition length x number of partitions}}{\text{chloroplast area}}$

Term (c) is an estimate of the total partition length per thin section of chloroplast on a per unit area chloroplast basis. These factors were chosen for analysis because they should reflect any changes in chloroplast configuration or ultrastructure.

Dye reduction

The ability of hand-cut transverse leaf sections to reduce TNBT was studied using a test solution containing 1 part TNBT (1 mg/ml), 1 part 0.1M phosphate buffer, pH 6.0, and 3 parts 0.33M sucrose (13). The three controls used were (a) darkness, (b) no TNBT, and (c) the addition of ascorbate-dichlorophenol indophenol, at 6×10^{-3}M and 1.5×10^{-4}M respectively, as the alternate electron donor. The leaf tissue was examined as described by Downton *et al.* (13) A Zeiss Ultraphot II microscope was used. If reduction of TNBT was going to occur it usually did so in freshly mounted sections within three to four minutes; if it had not occurred within 10 minutes reduction usually did not occur after a longer period. As Downton *et al.* (13) also found, controls stored in darkness reduced the dye more quickly when they were

returned to light (less than two minutes) as the dye had penetrated the section during storage.

Infra-red fluorescence photomicrography

The technique described here was brought to our attention by Lynne Elkin (16).

Fluorescence from hand cut transverse leaf sections and from isolated bundle sheath strands was studied. For comparative purposes the fluorescence from sections and bundle sheath strands of the monocotyledons *Zea mays*, *Sorghum sudanense*, *Saccharum sp* and *Panicum spp*, was observed.

The bundle sheath strands were isolated using a method similar to that described by Berry (5). Sections from the mid-lamina were cut into 1 cm pieces and ground with a pestle in a mortar containing 5 ml of glass beads (0.25 - 0.30 mm) and 10 ml of grinding medium (0.1M Tris buffer, 3×10^{-4}M EDTA, pH 7.0). Grinding was continued until the mesophyll became translucent, leaving the green bundle sheath around the veins. The fragments of tissue were then transferred to a small Ten-Broeck homogenizer which was used to loosen the strands from the vascular tissue and remove any remaining mesophyll cells. Both the bundle sheath strands and transverse sections were mounted on microscope slides in grinding medium.

Blue light was used to excite the pigments. The light source was a high pressure Philips HBO200 mercury lamp housed in a Zeiss Ultraphot II microscope. The light passed through a deep blue BG3 exciter filter before it reached the specimen slide. Dark field illumination was used. Before reaching the film, the fluorescence passed through an X16 planar objective and a yellow barrier filter (Kodak Wratten No. 12). The fluorescence was recorded on Kodak Ektachrome Infra-red Aero film. Exposure time (usually two to three minutes) was determined automatically by a device in the microscope after it was calibrated using test exposures. It was found that *G. globosa* chloroplasts photobleached quite quickly. Although the material was photographed as quickly as possible, the length of exposure necessary to take a photograph was often sufficient to result in photobleaching (see Discussion).

The film was developed using the Kodak E-3 process for Ektachrome film. Developing instructions were followed precisely except that the developing tank was kept at room temperature and the water for washing came directly from the cold tap. A reverse exposure of 15 s was given under a Sylvania No. 2 Superflood lamp.

The response of the film to red and far-red light was determined by placing a camera body, without the lens,

in the path of the sample beam of a Unicam SP800A UV spectrophotometer. Light from 440 - 750 nm, at 10 nm intervals, was used to expose successive frames of the film. From 700 to 750 nm the film was exposed with and without a deep-blue filter (Kodak Wratten No. 47B) in the beam path.

$^{14}CO_2$ feedings

Leaves were detached, recut under water, and illuminated for at least 20 minutes. They were then placed in a Plexiglas chamber (120 ml) and illuminated at 20,000 lux for a further 10 minutes while the chamber was flushed with air. Approximately 10 μc of $^{14}CO_2$ were then fed to the leaves and after six seconds they were killed in liquid nitrogen.

The leaves were extracted in boiling 80% v/v ethanol and then in 0.2N formic acid in 20% v/v ethanol. Descending chromatography of the combined extracts were performed using 4 x 46 cm Whatman No. 1 paper strips with phenol (liquefied, 90% v/v), water, acetic acid, 1M EDTA (840: 160: 10: 1) as a solvent (5). In this system malic and aspartic acids are separated from one another and from other early products of photosynthesis, the acids having Rf values of 0.32 and 0.23 respectively. The ^{14}C content of malic and aspartic acids was determined using autoradiography followed by liquid

scintillation counting or by the use of a radiochromatogram scanner.

Results

Plants at 10,000 lux showed the fastest growth. At 2,000 and 10,000 lux the leaves were bright green and about 10 to 15 cm long when mature. The plants grown outside (50,000 lux) had more lateral branches than the others and the leaves were leathery and shorter, about 4 to 8 cm. The plants grown outdoors also had a red pigment in many of the leaves mainly about the midrib and the edges of the lamina. Some of the leaves had pale tips.

Electron microscopy

The ultrastructure of the photosynthetic tissue was similar to that described by other workers (10,33). At all light intensities, the mesophyll chloroplasts had prominent grana and peripheral reticula. Many, though not all, mesophyll chloroplast profiles showed a few starch grains. A typical mesophyll chloroplast section (from 50,000 lux) is illustrated in Figure 1.

The anova (Table 1) of the bundle sheath chloroplast characteristics showed there was significant variation ($P < 0.05$)

Table 1

Bundle sheath chloroplast characteristics.

Means and standard deviations

	Lux		
	50,000	10,000	2,000
Grana/chloroplast	112.4 ± 7.2	11.8 ± 2.1	27.3 ± 17
Thylakoids/granum	2.5 ± 0.7	3.5 ± 1.1	4.0 ± 1.0
$\frac{PL \times PP}{Area}$[1]	103.9 ± 134.0	38.6 ± 23.3	186.0 ± 212.8

[1] $\frac{\text{Partition length x number of partitions}}{\text{chloroplast area}}$

Significant variation ($P < 0.05$) among treatments for grana per chloroplast but not for other terms.

among light intensities for grana per chloroplast but insignificant variation for thylakoids per granum and $\frac{\text{partition length x number of partitions}}{\text{chloroplast area}}$. The thylakoid overlaps (Figure 2) characteristic of *G. globosa* bundle sheath chloroplasts (10) were seen at all light intensities. Some chloroplasts had grana of more than two thylakoids particularly at 10,000 and 2,000 lux (Figures 3 and 4); the mean number of thylakoids per granum for 50,000, 10,000 and 2,000 lux were 2.54, 3.48 and 3.96 respectively. Many of the chloroplasts at 50,000 lux showed dilation of the intra-thylakoid space (Figure 5); this was also sometimes present in chloroplasts at 10,000 lux. A peripheral reticulum was present in all cases.

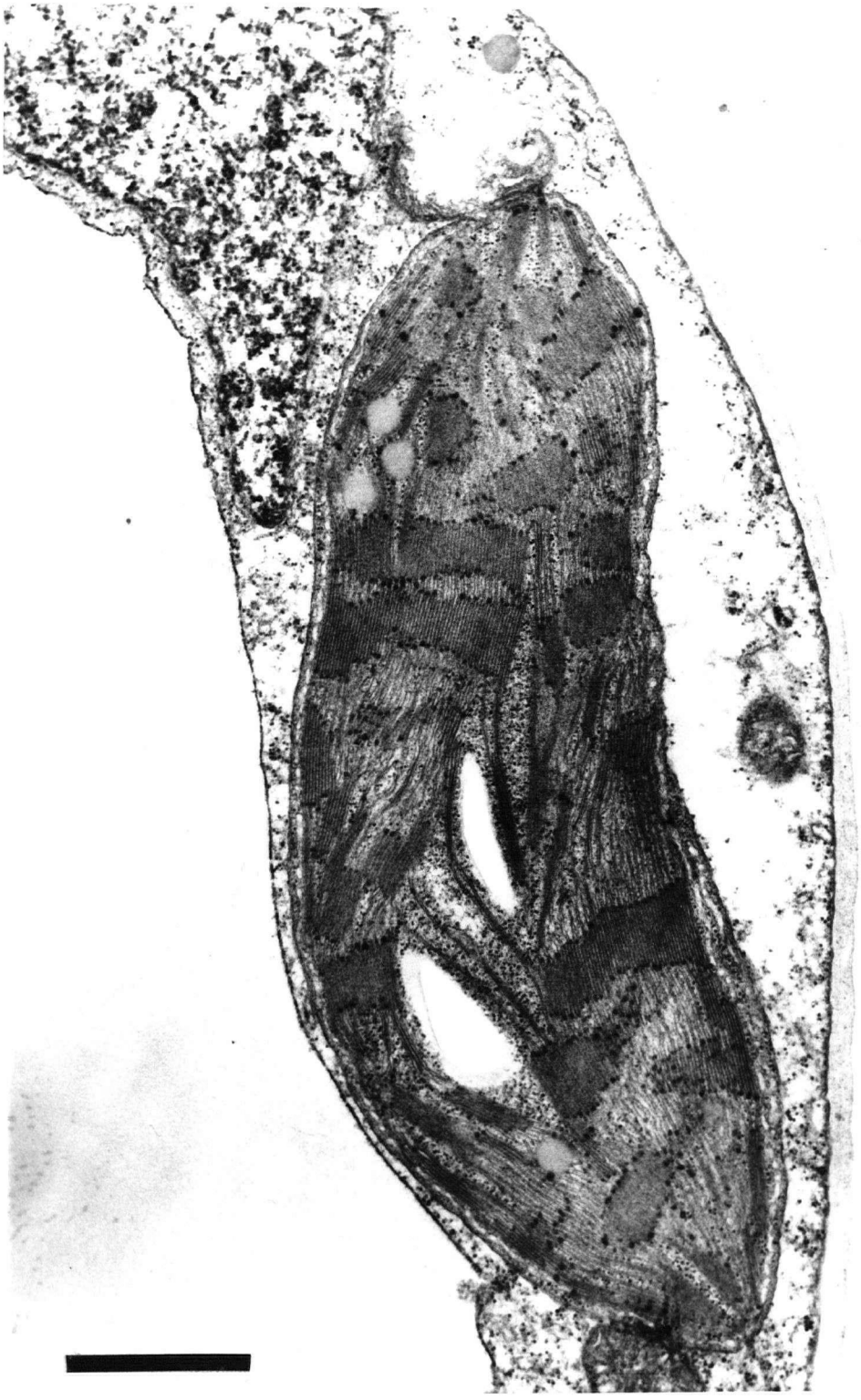
Starch grains were a prominent feature of the bundle sheath chloroplasts, particularly those from 50,000 and 10,000 lux. The starch grains in chloroplasts from 10,000 lux were sometimes so large as to almost completely exclude the internal membranes (Figure 6).

Dye reduction

In all cases mesophyll chloroplasts reduced TNBT in the light. No reduction of TNBT by either bundle sheath or mesophyll chloroplasts occurred in the dark. Table 2 shows the response of the bundle sheath chloroplasts to TNBT.

The black bar on each plate represents 1μm

Figure 1. Mesophyll chloroplast. 50,000 lux. Large granal stacks. X26,000.



①

Figure 2. Portion of a bundle sheath chloroplast showing thylakoid overlaps. 50,000 lux. X54,800.

2

Figure 3. Bundle sheath chloroplast. Grana of more than two thylakoids are arrowed. 10,000 lux. X37,000.

3

Figure 4. Bundle sheath chloroplasts. Some of the grana of more than two thylakoids are arrowed. 2,000 lux. X19,900.

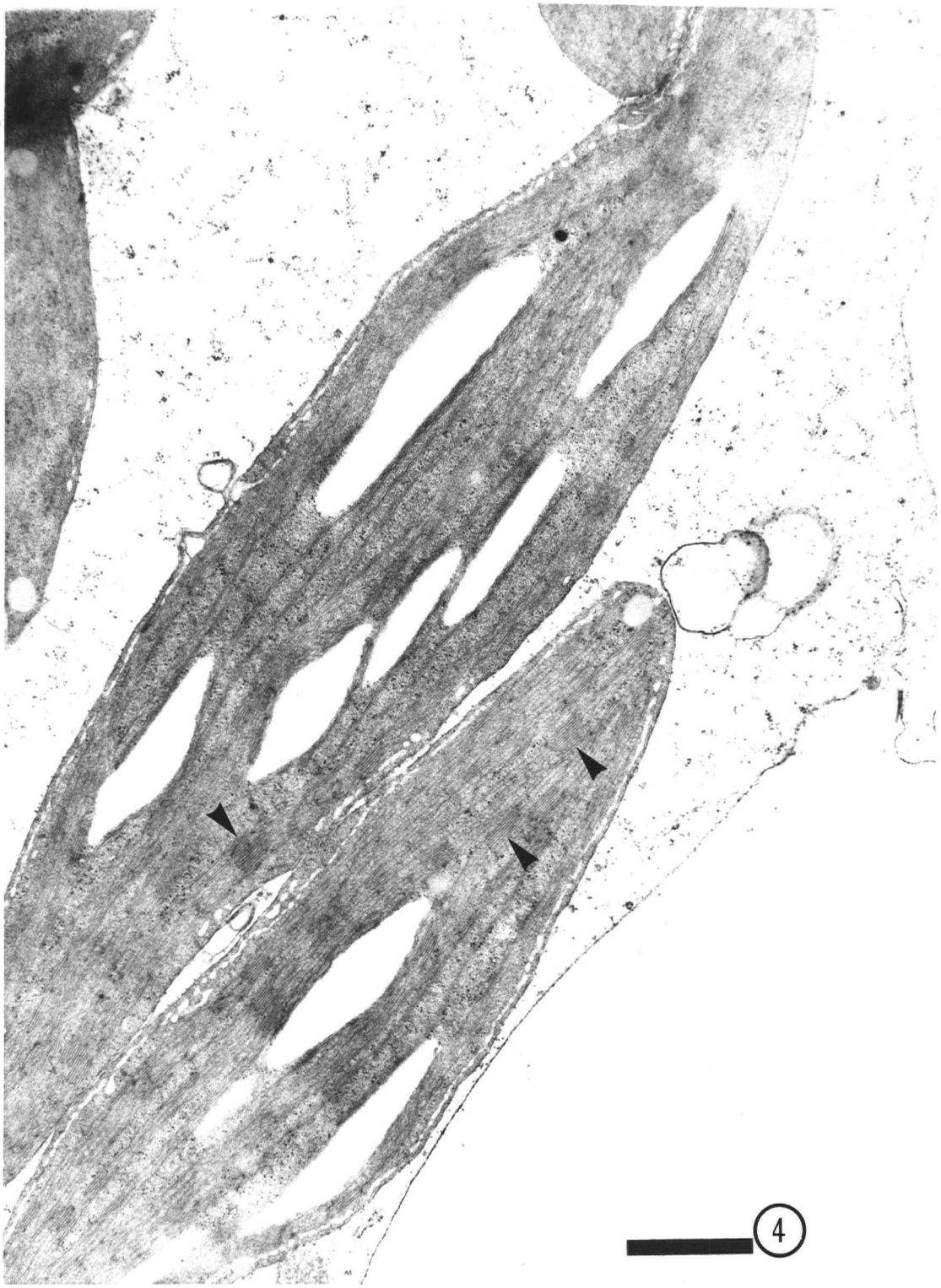

4

Figure 5. Bundle sheath chloroplast with dilated thylakoids. 50,000 lux. Large starch grains. X20,100.

5

Figure 6. Bundle sheath chloroplast. 10,000 lux. Starch grains occupy nearly all chloroplast. X20,400.

6

Table 2

TNBT reduction in light by bundle sheath chloroplasts from leaves grown at different light intensities

	Light intensity (lux)		
Test Conditions	50,000	10,000	2,000
TNBT + light	Infrequent darkening	Mosaic	Mosaic
TNBT + light + ascorbate - DCIP[1]	Mosaic	Mosaic	+

[1] DCIP: dichlorophenol indophenol.

TNBT is colourless and becomes blue-black if it is reduced within the chloroplasts. "Infrequent darkening" means that reduction occurred in only some of the several tests.

"Mosaic" means that, consistently, chloroplasts reduced TNBT in only some cells of the bundle sheath.

Infra-red fluorescence photomicrography

Infra-red Aero film portrays visible red light as yellow and invisible far-red light as red (the hue varies somewhat according to the source).

In all cases, the mesophyll chloroplasts appeared bright red as they emitted visible red fluorescence. Depending on whether they fluoresced in the red or far-red region of the spectrum, bundle sheath chloroplasts appeared red or were almost invisible, and yellow or red on the film respectively.

Figures 7 to 10 show the fluorescence of *G. globosa* chloroplasts. Figures 11 and 12 show fluorescence from *Panicum maximum* and *Sorghum sudanense* and are representative of monocotyledon fluorescence.

Bundle sheath chloroplasts in transverse sections from leaves of *G. globosa* grown at all three different intensities emitted far-red fluorescence (Figures 7, 8 and 9); some visible fluorescence was discernible at times. Isolated bundle sheath strands from leaves grown at 50,000 lux showed a red fluorescence. Strands from leaves grown at 10,000 and 2,000 lux (Figure 10) emitted far-red fluorescence together with some red wavelengths.

$^{14}CO_2$ feedings

Table 3 gives the results of the $^{14}CO_2$ feedings.

Figure 7. Photograph of fluorescence from leaf of *G. globosa* T.S. 50,000 lux. Colour of fluoresence from bundle sheath (arrow on protective flap) is not much different from that of the mesophyll; difference is more marked on original colour slide.

Figure 8. Fluorescence from leaf of *G. globosa* T.S. 10,000 lux. Bundle sheath fluorescence (arrow) is at far-red wavelengths. Mesophyll fluorescence is visible.

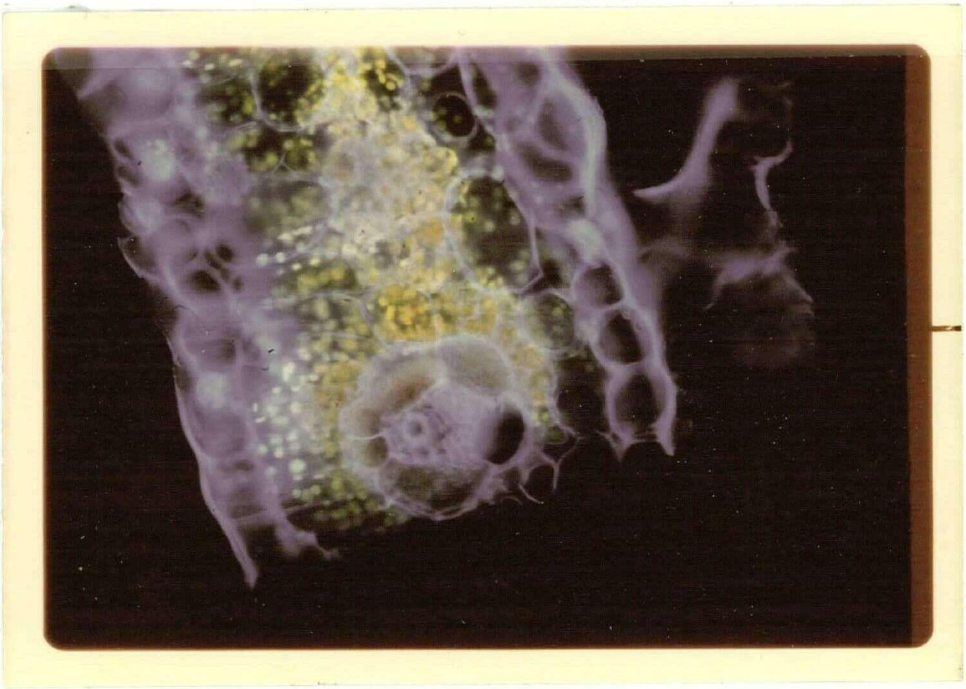
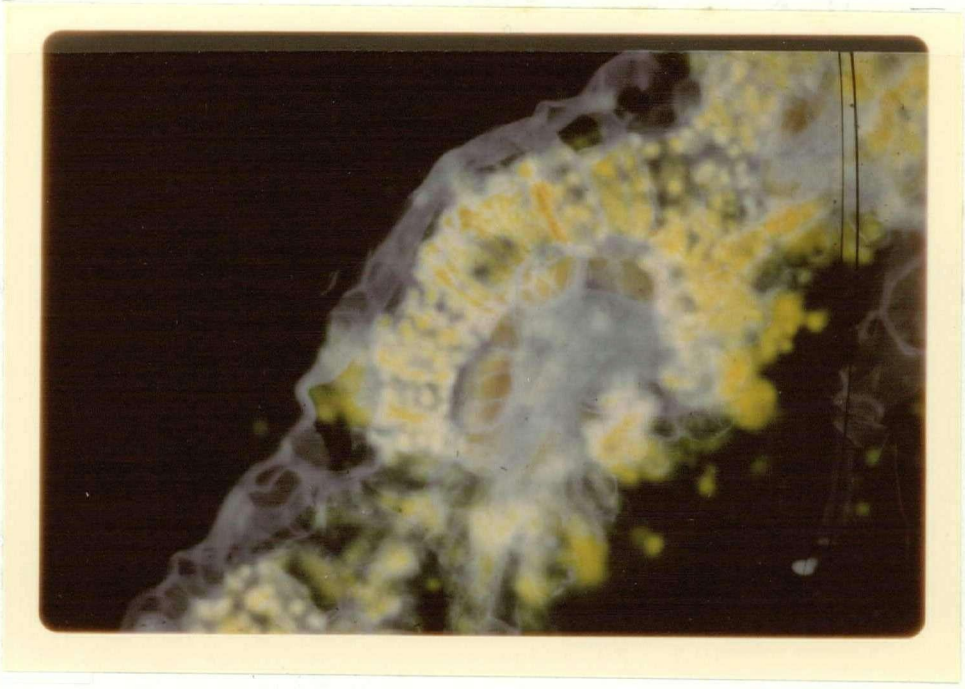


Figure 9. Fluorescence from leaf of *G. globosa*. T.S. 2,000 lux. Far-red fluorescence from bundle sheath (arrow), visible from mesophyll.

Figure 10. Isolated bundle sheath strand of *G. globosa* T.S. 2,000 lux. The bundle sheath cells (small arrow) emit mostly far-red wavelengths. The pure yellow areas (large arrows) are mesophyll cells.



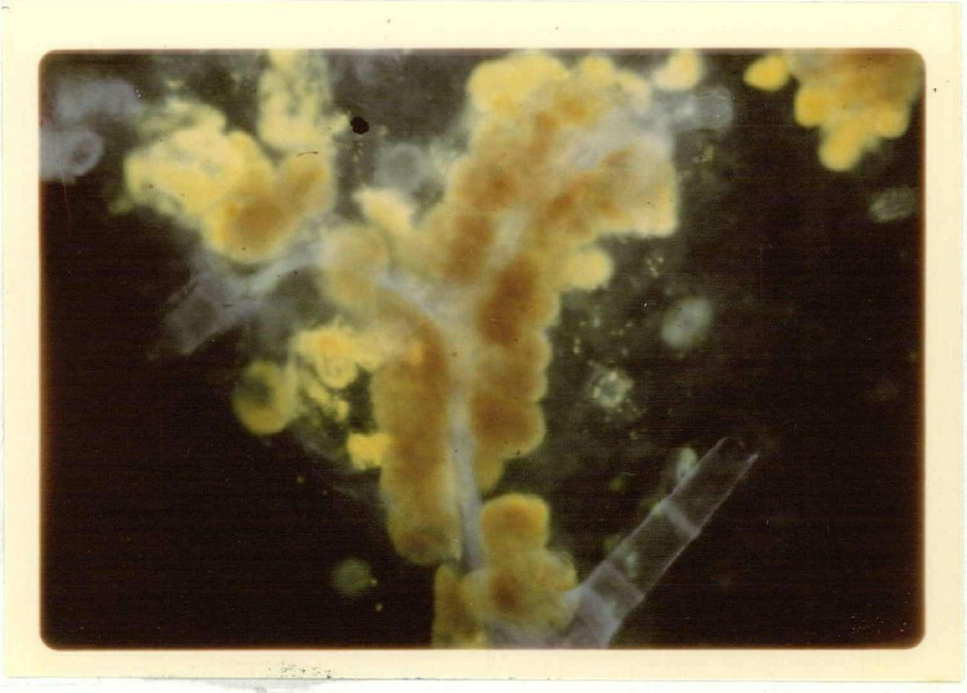
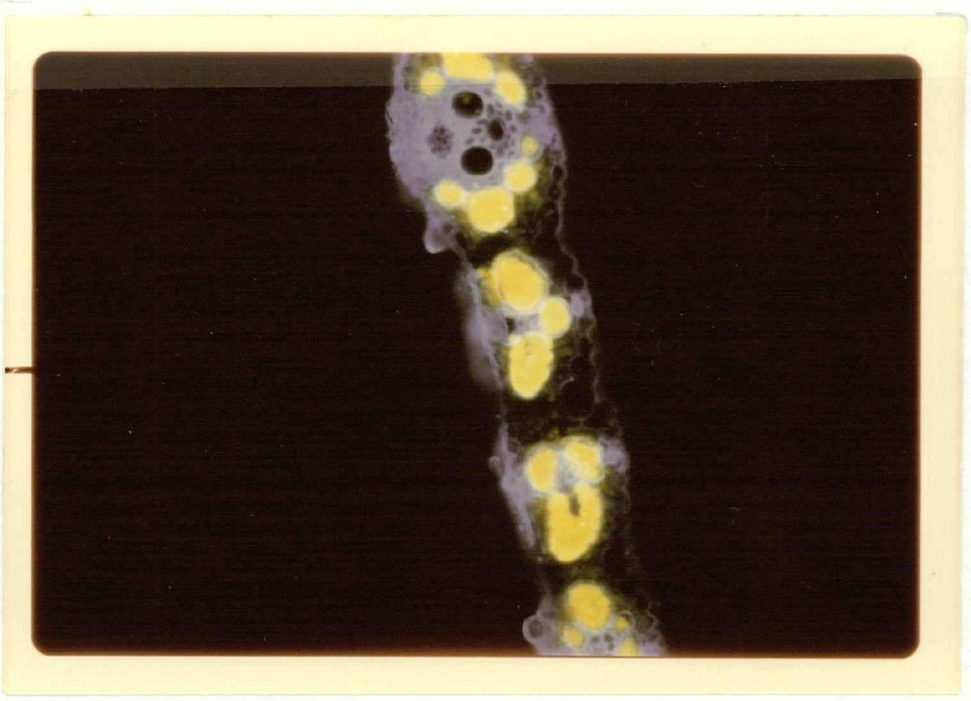


Figure 11. Fluorescence from leaf of *Panicum maximum*. The large bundle sheath cells (arrow) emit only visible fluorescence, as do the mesophyll cells.

Figure 12. Fluorescence from leaf of *Sorghum sudanense*. Far-red fluorescence is emitted from the bundle sheath cells (arrows); visible fluorescence from the mesophyll.



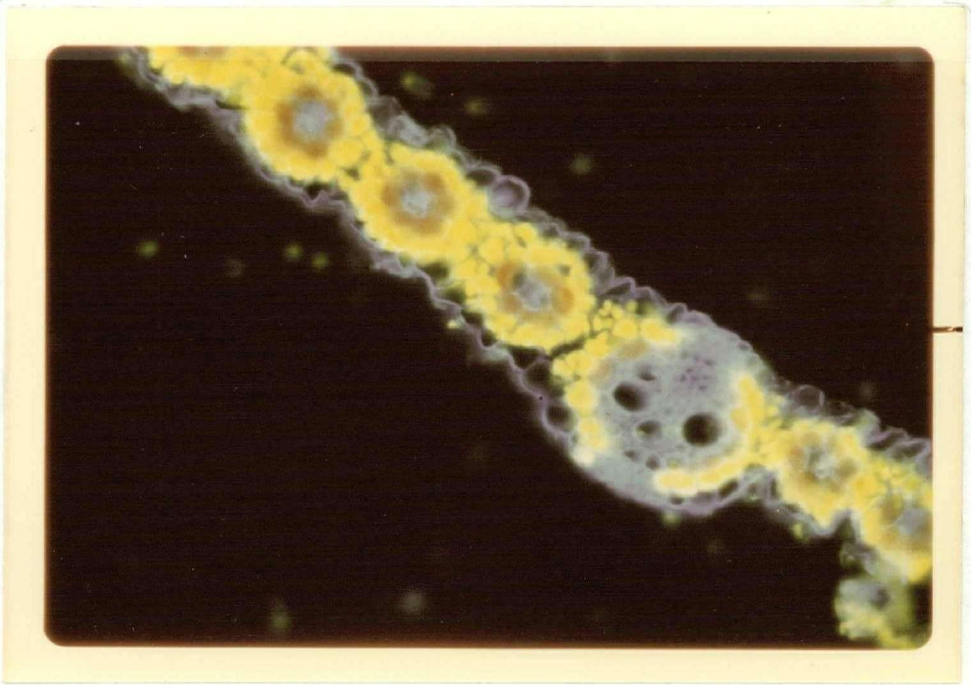


Table 3

Ratio of ^{14}C in malic acid to that in aspartic acid at different light intensities

	Light intensity (lux)		
	50,000	10,000	2,000
Ratio	0.9 ± 0.2	2.4 ± 0.9	4.5 ± 0.5

Discussion

Electron microscopy

Reports of experiments in which the effect of light intensity on the membrane structure of mature chloroplasts has been studied, indicate a tendency towards an agranal condition at high light intensity. Ballantine and Forde (3) found that in leaves of soybean grown at high light intensity (220 $W.m^{-2}$ in the 400-700 nm range, 420 $W.m^{-2}$ approximately equals 10,000 ft.c., 1 ft.c. = 10 lux) the granal stacks were reduced to two or three appressed thylakoids; the effect was even more marked at low temperature (20°C/12.5°C day/night) the grana being confined to occasional thylakoid overlaps. *Amaranthus lividus* (a C-4 dicotyledon (27)) has granal chloroplasts in both the mesophyll and bundle sheath (27). At high light intensity (approximately 145 $W.m^{-2}$) only a few rudimentary grana were present in chloroplasts from both tissues (27). The degree of granal stacking increased markedly at lower intensities. When maize was grown under continuous fluorescent light (60 $W.m^{-2}$) (32), mesophyll chloroplasts had smaller grana than under full natural daylight (light intensity not actually measured). Under full daylight the bundle sheath chloroplasts had the usual lamellar structure but under continuous fluorescent light there was a repression of the "lamellar condition" together with increased lipid

deposition. It was expected then that chloroplasts from leaves grown at high light intensity would show less granal development, and hence, assuming that agranal chloroplasts are deficient in PS II function, have a lower ability to reduce NADP.

Light intensity did not seem to affect granal development of the mesophyll chloroplasts; large granal stacks were present at all light intensities. This is in contrast to the situation in *Amaranthus lividus* (see above). There is some indication from the anova that bundle sheath chloroplasts from the two highest light intensities contained fewer grana but there was no significant variation in the total partition length. In view of this and the fact that the anova was performed on micrographs not taken for this purpose, I cannot conclude that there were differences in the ability of bundle sheath chloroplasts to photo-reduce NADP among the three light intensities.

Bundle sheath chloroplasts of C-4 plants have often been observed to contain large quantities of starch (25). Judging by published photographs (25), the bundle sheath chloroplasts at the two highest light intensities contained unusually large amounts of starch, especially those grown at 10,000 lux.

For the plants grown at 50,000 lux a possible reason for the large amount of starch in the bundle sheath chloroplasts

has its origin in the fact that at low night temperature (10°C) transport of starch out of mesophyll chloroplasts of *Digitaria decumbens* was severely inhibited (22). *D. decumbens* accumulates starch in both mesophyll and bundle sheath chloroplasts whereas it is usually in the bundle sheath of C-4 plants (17). In summer in Vancouver, minimum daily temperatures, which occur at night, sometimes fall below 10°C and more frequently below 15°C, Table 4. It seems probable that this would result in an inhibition of starch transport out of the bundle sheath, if the extrapolation from C-4 monocotyledons to C-4 dicotyledons can be made. Such an inhibition of carbohydrate translocation may have contributed to the slower growth of these plants.

It might also be noted that the mean maximum and mean daily temperatures are only slightly above the temperatures, 10°C to 15°C, at which photosynthesis and growth of maize and other tropical grasses are very low. They are well below the temperatures, 30°C to 40°C, which are considered to be optimum for C-4 photosynthesis (11). These low temperatures may also be a factor in the slower growth of plants outside compared to the ones in the growth chamber at 24°C. However, this is only a tentative suggestion as air temperatures measured meterologically are often considerably different from leaf surface temperatures (36).

Table 4

Summary of temperatures recorded at the Plant Science weather station, University of British Columbia, Vancouver during the summer, 1970 (°C)

	Mean Max.	Mean Min.	Max.	Min.	Mean Daily
June	20.0	12.2	30.0	8.3	16.1
July	20.5	12.6	27.2	9.4	16.5
August	20.0	12.7	24.4	9.4	16.1
Sept.	16.3	9.4	21.7	-1.7	12.9

Number of days per month in which the temperature fell below:

	10°C	15°C	18°C
June	7	26	29
July	3	28	31
August	2	31	31
Sept.	17	30	30

The very large, unusually shaped starch grains in the bundle sheath chloroplasts from leaves grown at 10,000 lux are something of an enigma. I would not have expected an inhibition of starch translocation under their temperature regime (24°C/18°C) nor that photosynthesis would be so rapid as to produce such apparently large quantities of starch.

The marked formation of β-cyanin in leaves grown at 50,000 lux may be due to their high starch content. Anthocyanin formation seems to be commonly associated with accumulation of sugars in plant tissues. Any environmental factor such as high light intensity, low temperature, drought or low nitrogen supply, which favours an increase in the sugar content of a given plant tissue, often favours synthesis of anthocyanin in that tissue (29). In view of these comments it is surprising that visible pigment formation did not occur at 10,000 lux; 10,000 lux is also intermediate in the range of light intensities (approximately 3000 to 24,000 lux) used by Downs and Siegelmann (9) to study photocontrol of anthocyanin synthesis in *Sorghum vulgare* seedlings.

Dye reduction

The results of the TNBT reduction tests indicate that chloroplasts from leaves grown at 50,000 lux had a low non-cyclic electron flow which is associated with a low PS II

activity. This would fit in with expectations about the effect of light intensity on chloroplast structure and function derived from the literature but in this study electron microscopy did not provide concrete evidence for less granal development at high light intensity. Some reserve is needed in interpreting TNBT results as the dye has difficulty penetrating intact cell walls (the "mosaic" observed in some cases may be due to variable rates of TNBT penetration into different cells). Also, after this work was completed it was reported that TNBT is one of the least satisfactory dyes for in vivo demonstration of PS I and PS II activity (25); no explanation for this statement was given.

Infra-red fluorescence photomicrography

Elkin (16) found, using infra-red fluorescence photomicrography, that on excitation with blue light, granal chloroplasts emitted visible red fluorescence and agranal chloroplasts emitted invisible far-red fluorescence. I decided to use the technique to see if fluorescence from mesophyll and bundle sheath chloroplasts would change with the environment, and correlate with changes in the granal structure. The hypothesis was that under high light intensity, bundle sheath chloroplasts would show more far-red fluorescence and under lower light intensities more red fluorescence.

For those monocotyledons where I had prior knowledge of the chloroplast structure, there was, as Elkin found, a correlation for bundle sheath chloroplasts between the occurrence of grana and the type of fluorescence; two examples are shown in Figures 11 and 12. *G. globosa* bundle sheath chloroplasts gave more variable results (see Figures 7 to 10). These might be due to environmental differences but may also be due to photobleaching to which *G. globosa* chloroplasts seem particularly prone. Elkin (16) noted too, that *G. globosa* bundle sheath chloroplasts seemed unusually sensitive to photobleaching. She also found that, depending on the species used, chloroplasts with double or triple thylakoids or even a small number of well developed grana gave red or far-red fluorescence, i.e. the correlation between fluorescence and structure broke down. At the moment environmental studies using this technique should be confined to those species where there is a definite correlation between fluorescence and chloroplast structure. In view of Elkin's comments it may not be suitable even in these cases, as in chloroplasts where the granal structure was changing, e.g. young *Sorghum bicolor* bundle sheath chloroplasts (14), the fluorescence may not reflect granal structure as it does in mature chloroplasts. It would be interesting to see if other C-4 dicotyledon chloroplasts behaved similarly to those of *G. globosa*.

What is the origin of the fluorescence seen using this technique? There is a correlation, for some species, between the red and far-red fluorescence and the presence or absence of grana (my own and Elkin's (16) observations). Further, as noted previously in the Introduction and Discussion, there is evidence for granal chloroplasts having both PS I and PS II whereas agranal chloroplasts are deficient in PS II. As a result Elkin (16) has considered that the far-red fluorescence is from PS I, the red fluorescence being from PS II (a conclusion which is not entirely incompatible with the ample evidence for the two photosystems fluorescing at different wavelengths, e.g. (34)).

Concerning the actual wavelength of the fluorescence from sections of C-4 leaves there are two pertinent papers. Boardman *et al.* (7) showed with subchloroplast fragments enriched in PS I or PS II that a fluorescence emission band at 735 nm (far-red) originated mainly from PS I and bands at 683 and 695 nm (red) came from PS II. It was then shown by Woo *et al.* (40) that mesophyll chloroplasts of *Sorghum bicolor* had emission bands at 683, 695 and 735 nm whereas the agranal bundle sheath chloroplasts emitted 95% of their fluorescence in the 735 nm band. Elkin (16) and also Laetsch (25) have apparently equated the 735 nm fluorescence, and hence PS I, with the long wavelength fluorescence from sections of C-4 leaves. However the spectra studied by Boardman *et al.*

(7) and Woo *et al.* (40) were obtained at 77°K, not at room temperature. Room temperature spectra do not show the large 735 nm band seen at 77°K; in fact, system I particles are "weakly fluorescent" at room temperature (see Figure 13). Even if it is assumed that the visible fluorescence seen in the leaf sections at room temperature is from PS II, then it seems that the fluorescence from PS I would hardly be sufficient to form an image of comparable intensity on film to that formed by the fluorescence from PS II. I have to conclude that the nature and origin of the fluorescence from the leaf sections and isolated bundle sheath chloroplasts is still uncertain, there being no direct proof that the two kinds of fluorescence come from PS II and PS I.

$^{14}CO_2$ feedings

The ratios of malic acid to aspartic acid show that at high light intensity more ^{14}C was initially fixed into aspartic acid, while at the lower intensities most of the ^{14}C was fixed into malic acid. This was somewhat surprising as electron microscopy (see above) showed there was no appreciable difference in chloroplast ultrastructure between light intensities. The unexpected ratios may be due to difference in the pool sizes of the dicarboxylic acids at the time of feeding.

Assays of malic enzyme activity which catalyses the decarboxylation of malic acid (11) would have been more illuminating.

Conclusion

There was no effect of light intensity on partition length (an indicator of PS I function) of bundle sheath chloroplasts, a conclusion which is strengthened by the lack of an effect of light intensity on mesophyll chloroplast structure. This was unexpected as the literature indicated there would be greater granal development at lower intensities (3,27,32).

There was some variation in metabolite levels among plants grown at different light intensities. Starch formation seemed to be controlled by light intensity and possibly by stress, although the importance of this latter factor is not clear. Variation in metabolite levels was also indicated by β-cyanin formation at 50,000 lux and the ratios of malic to aspartic acid.

I have collected the results of this work together in Table 5. For the mesophyll chloroplasts at all light intensities the ability to reduce TNBT, the occurrence of grana and the red fluorescence correlate, as expected. At 10,000 and 2,000 lux the bundle sheath chloroplasts show the expected correlation between far-red fluorescence, thylakoid overlaps and high malic acid content but the apparent capacity to reduce

Table 5

Summary of experimental results

	TNBT	Fluorescence	Malate / Aspartate	E.M.
50,000 lux				
Bundle sheath	Infrequent reduction	Far-red; red in isolated cells	0.9 ± 0.2	Thylakoid overlaps
Mesophyll	+	Red		Grana
10,000 lux				
Bundle sheath	Mosaic	Far-red	2.4 ± 0.9	Thylakoid overlaps
Mesophyll	+	Red		Grana
2,000 lux				
Bundle sheath	Mosaic	Far-red	4.5 ± 0.5	Thylakoid overlaps
Mesophyll	+	Red		Grana

TNBT does not agree. For the bundle sheath chloroplasts at 50,000 lux the low capacity to reduce TNBT, the mainly far-red fluorescence and the thylakoid overlaps correlate together but the high aspartate content does not.

Perhaps with its lack of structural response to light intensity, grana of two stacked thylakoids only and lack of correlation between characters which are known to do so for other plants, *G. globosa* occupies an intermediate position between an extreme agranal type such as *Sorghum bicolor* and a typical granal type such as *Amaranthus lividus*. Pyliotis *et al.* (33) place *G. globosa* between typically agranal and granal types in a table comparing total partition length per unit area of chloroplast and chlorophyll a to chlorophyll b ratio. *G. celosoides* was found to be intermediate in malic enzyme and aminotransferase activity between a group containing *Sorghum sp* and another including *Amaranthus sp* (20).

Addendum

Studying the effect of climatic stress on *Sorghum sp*, Taylor and Craig (39) found that when the night temperature was dropped to 10°C a pronounced increase in starch in bundle sheath chloroplasts occurred. After three days of treatment two-thirds of the sectional plane through the chloroplast appeared as starch grain. In a later paper (8) Brooking and

Taylor observed a greater proportion of ^{14}C fixed into aspartic acid at 6 s in *Sorghum* leaves after 6.5 hours at 10°C and at 30 hours nearly 80% of the ^{14}C remained in aspartic acid after one minute of chasing with $^{12}CO_2$. The higher proportion of ^{14}C found in aspartic acid in leaves of *G. globosa* grown outside may bear some relation to this observation, although the low temperature stress occurred at night, not during the day.

There now seems to be a general consensus on the question of whether agranal chloroplasts, in particular bundle sheath chloroplasts, contain PS II. In agranal bundle sheath chloroplasts an intact electron transport chain including both photosystems has been demonstrated, mainly by the use of Hill oxidants (1,24,37), the dependence of cytochrome f and b oxidation on the wavelength of the exciting red light (4,6) and by measuring fluorescence excitation and emission spectra (4). Isolated maize bundle sheath chloroplasts would only reduce NADP in the presence of added plastocyanin (37) indicating that plastocyanin was lost during isolation of the chloroplasts. These agranal bundle sheath chloroplasts are deficient in PS II however, there being about a three-fold higher PS I to PS II ratio in these chloroplasts than in mesophyll chloroplasts (4). It is likely that the size of the deficiency varies between species. On the basis of experiments using different species (1,2,4,6,28,37) I would

put *Atriplex spongiosa* (a granal type) *G. globosa, Digitaria sanguinalis* (crab-grass), maize and *Sorghum sp* as a series showing increasing PS II deficiency.

To my knowledge no work has been published which gives a definite clue to the nature and origin of the far-red fluorescence seen in the bundle sheath chloroplasts. An interesting paper on chlorophyll fluorescence in a system I - chlorophyll a - protein complex and system II particles has been published by Mohanty *et al.* (30). Using dilute suspensions (chlorophyll concentration 2.0 - 3.0 mg/ml) prominent fluorescence peaks were found at 685 nm in system I particles as well as in system II particles at 77°K (see Figures 14 and 15A). It has been considered, egg. (7), that system I fluorescence at 77°K is predominantly at 735 nm. When a thick suspension (chlorophyll concentration 40 mg/ml) was used the 685 nm band was present only as a small shoulder, most of the fluorescence being in the 735 nm band (see Figure 15B). This was thought to be due to reabsorption of the 685 nm band by neighbouring particles. It should be noted that Boardman *et al.* (7) who found the majority of system I fluorescence at the 735 nm band at 77°K diluted their fractions to an absorbance of 0.1 at 436 nm to minimize reabsorption of emitted light (the chlorophyll concentration as such as not given). Either Mohanty *et al.* (30) diluted their suspension even more, or there is a genuine difference in results.

Returning to the results of Mohanty *et al.* (30), Figure 16 shows a room temperature emission spectrum of a dilute suspension of system II particles. There is a large F685 band with small ill-defined bands at 720 nm and 740 nm. A spectrum, Figure 17, obtained similarly for PS I had an F685 band with a shoulder at 692 nm and bands at 720 nm and 740 nm somewhat larger than the corresponding ones in PS II.

Intact chloroplasts may be regarded as "thick suspensions" and I propose that in agranal bundle sheath chloroplasts, which are deficient in PS II, any 685 nm fluorescence is absorbed and reappears with the F720-740 band (which at room temperature would still not be as large as the 735 nm band seen at 77°K). In granal chloroplasts the F685 and F695 bands are probably strong enough to mask any far-red fluorescence on the film and the chloroplasts would thus appear to fluoresce only visibly.

In a paper published by Bazzaz and Govindjee (4) emission spectra of mesophyll and bundle sheath chloroplast fragments at room temperature are practically identical (and very similar to the spectrum shown in Figure 17) both spectra showing a large band at 685 nm. This may well be evidence against my proposal as reabosrption of the F685 band obviously did not occur. However these spectra are from fragments in which transfer of fluorescence may not have been able to occur between PS II and PS I.

Lastly, to end on a somewhat dampening note, I would like to quote from an article on fluorescence from the 1972 edition of the Annual Review of Plant Physiology (18) where it is suggested that,

> *. . . the increase in F725 due to cooling is caused by an increase in energy transfer to a long wave chlorophyll form. This form is usually present in low concentration but has a high intrinsic fluorescence yield. It is probably not identical to P700, the energy trap of photosystem I. . . . Possibly the chlorophyll form emitting F725 acts as an energy sink, protecting the photochemical systems from photo-oxidation under unfavorable conditions.*

Figure 13. Approximate emission spectra of system I fraction of spinach chloroplasts at 293°K and 77°K. Drawn from Figure 3, Boardman *et al.* (7).

Figure 14. Approximate emission spectrum of system II particles of maize chloroplasts at 77°K. Chlorophyll concentration approx. 3.0 µg/ml. Drawn from Figure 3, Mohanty *et al.* (30).

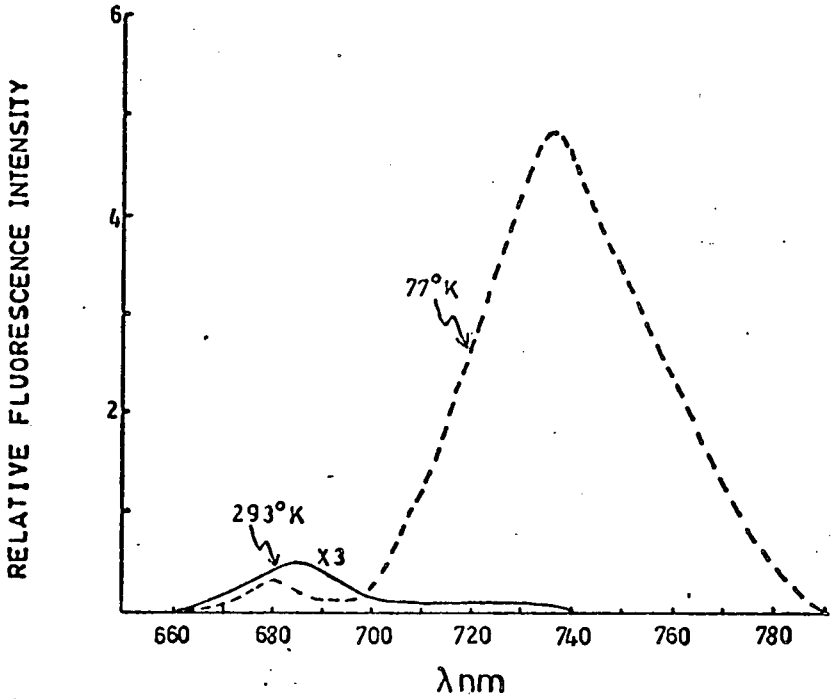


Figure 13

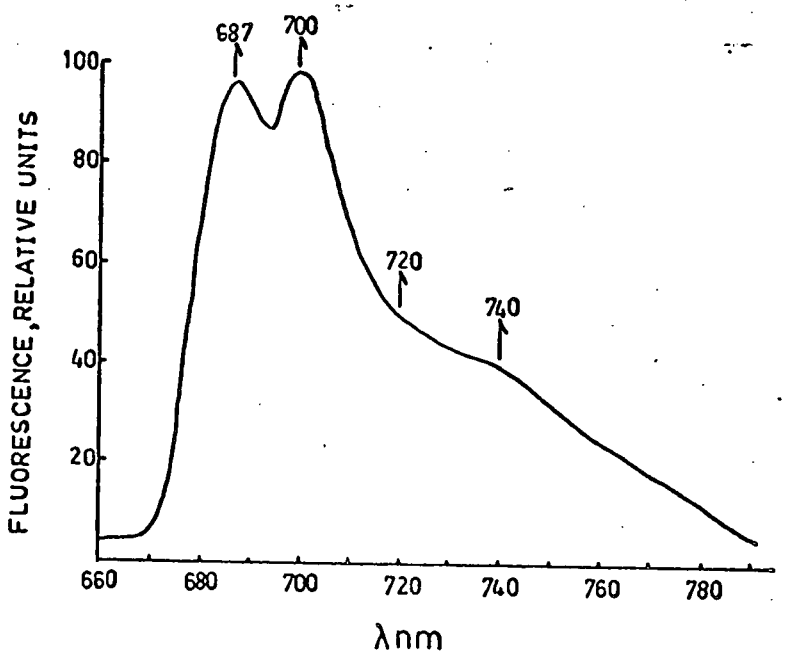


Figure 14

Figure 15. Approximate emission spectra at 77°K of system I chlorophyll a-protein complex of maize.

A. Thin suspension, chlorophyll conc. approx. 2.0 µg/ml.

B. Thick suspension, chlorophyll conc. approx. 50 µg/ml.

Drawn from Figure 4, Mohanty *et al.* (30)

Figure 16. Approximate emission spectrum at room temperature of system II particles of maize. Chlorophyll conc. approx. 3.0 µg/ml. Drawn from Figure 2, Mohanty *et al.* (30).

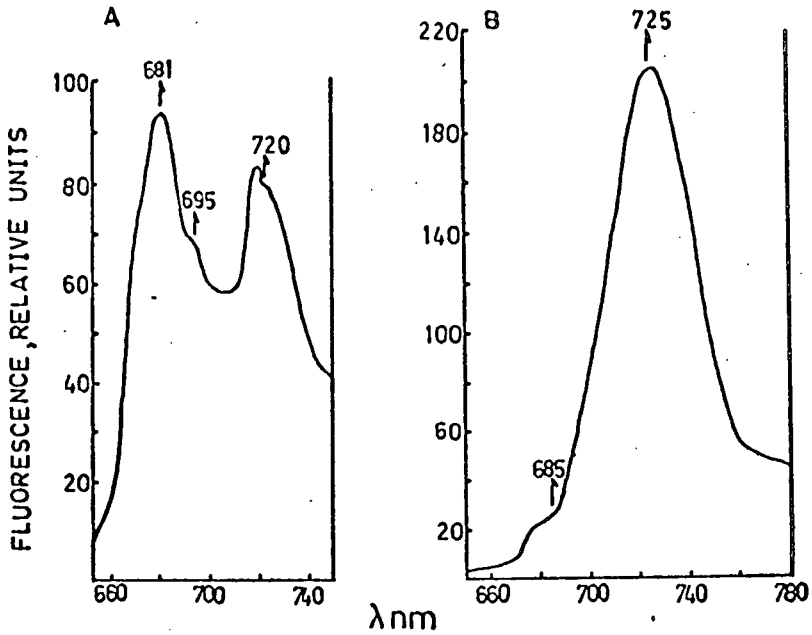


Figure 15

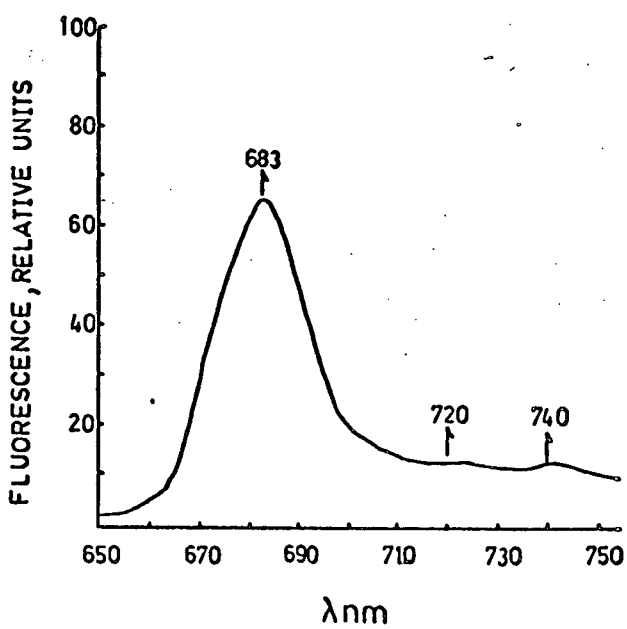


Figure 16

Figure 17. Approximate emission spectrum at room temperature of system I chlorophyll a-protein complex of maize. Chlorophyll conc. approx. 2.0 µg/ml. Drawn from Figure 2, Mohanty *et al.* (30).

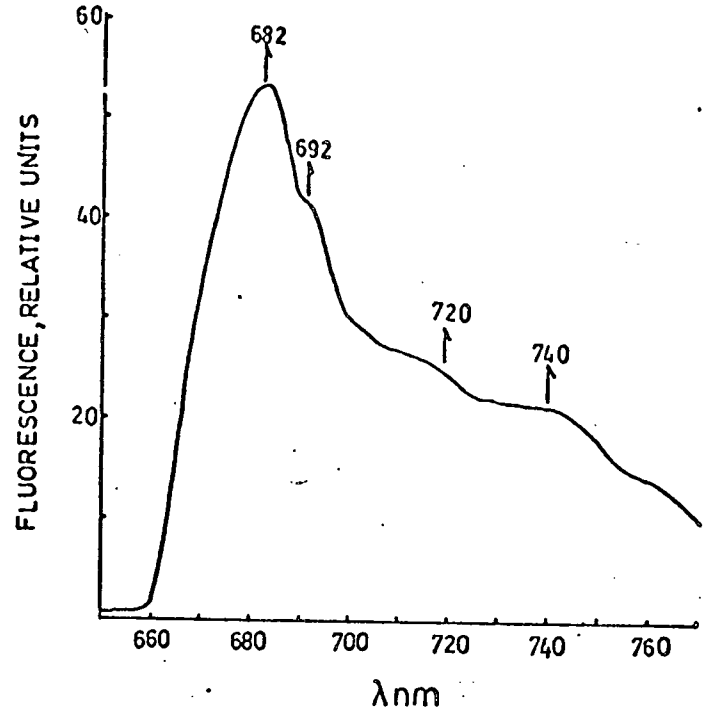

60
40
20
FLUORESCENCE, RELATIVE UNITS
682
692
720
740
660
680
700
720
740
760
λnm

Figure 17

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PART II

A NITROGEN FIXING ASSOCIATION: *PASPALUM NOTATUM* AND *AZOTOBACTER PASPALI*

TERMINOLOGY

The term "nitrogen" is commonly used as a generic term for any of a variety of nitrogen-containing compounds such as ammonia or nitrate. "Nitrogen" is also used in the specific sense as referring to the gas, N_2. Where confusion may exist in the intended meaning, the term "dinitrogen" is used to refer to the N_2 molecule in accordance with recent chemical convention (2).

I have used the following terminology of Hardy *et al.* (1) to indicate the analytical origin of data on nitrogen fixation:

"$N_2[C_2H_2]$-fixing activity" - the data have been derived from tests of the ability of a system to reduce acetylene (C_2H_2), and these data were interpreted as a measure of the system's ability to reduce dinitrogen.

"N_2ase$[C_2H_2]$-activity" - this term is reserved to indicate the ability of a preparation of the dinitrogen-fixing enzyme, nitrogenase, to reduce C_2H_2. Some authors have used "nitrogenase activity" or some similar term to indicate the C_2H_2 reduction activity of, for example, a root-soil system. I feel this is misleading.

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PROLOGUE

All known biological agents of nitrogen fixation are prokaryotes (23). They are generally thought of as one of the partners of a symbiosis or as free living, i.e. able to function without a particular relationship with some other organism. Free living nitrogen fixers have been found in a number of terrestial habitats, particularly the soil (23).

The bulk soil usually contains less bacterial activity than the rhizoplane or the rhizosphere (25). Nitrogen-fixing activity has been found to be particularly associated with the rhizosphere of a number of plants, the degree of fixation often being much higher than that usually associated with asymbiotic soil nitrogen fixers.

By way of comparison, nitrogen fixation by so-called free living soil bacteria has been estimated as falling within the range 0.04 to 15.0 $KgN.ha^{-1}yr.^{-1}$ (20). Some examples are 1 $KgN.ha.^{-1}yr.^{-1}$ and 2 $KgN.ha.^{-1}yr.^{-1}$ for a natural grassland ecosystem in Saskatchewan, Canada (27), 0-3 $KgN.ha.^{-1}yr.^{-1}$ for tussock grassland soils in New Zealand (18), 4-5 $KgN.ha.^{-1}yr.^{-1}$ for non-rhizosphere fixation in a wilderness site at Rothamsted, England (15). The amount of nitrogen fixed may be increased by amending soils, i.e. by adding carbohydrate (1,13,19). A

notable exception to these relatively low rates are Egyptian soils which support dense populations of *Azotobacter spp* and *Clostridium spp* and fix large amounts of nitrogen particularly when an adequate supply of carbohydrate is present (20,23).

As a result of a survey of research on nitrogen-fixing bacteria Mishustin and Shil'nikova (19) concluded that *Azotobacter spp* occurred frequently in the rhizosphere. However, they also noted Rovira's comment (26), that *Azotobacter spp* constitute not more than 10% of the population of the root zone. Facultative anaerobic nitrogen fixing bacteria of the *Klebsiella-Aerobacter* group have been isolated consistently from the surfaces of roots and nodules of soybean plants; similar types of bacteria were also isolated from alfalfa and clover (12). Nitrogen fixation, in the near absence of legumes, occurred at the rate of 55 $KgN.ha.^{-1}yr.^{-1}$ on a site at Rothamsted, England, left uncultivated since 1882 (15). A survey of about 40 plant species showed considerable nitrogen fixing activity associated with the roots of *Heracleum sphondylium* L. (hogweed), *Anthriscus sylvestris* Hoff. (cow parsley), *Mercurialis perennis* L. (dogs mercury), *Rumex acetosa* (sorrel), *Convolvulus arvensis* L. (bindweed), *Viola canina* L. (dog's violet) and *Stachys sylvatica* L. (hedge woundwort). Roots of *S. sylvatica*, free of loose soil, gave a maximum rate of C_2H_2 reduction of 1.31 ± 0.20 nmoles C_2H_2. $g.^{-1}h.^{-1}$ at pO_2 0.04 atm. Plants with an intact soil-root system gave rates of C_2H_2 reduction

per gram dry weight of root two to three times higher than this. Three nitrogen-fixing isolates were obtained, but the organisms were not identified. Assuming that there are about 5 g of roots (fresh weight) per dm^2 of ground area, 1.31 nmoles $C_2H_2.g.^{-1}h.^{-1}$ is about equivalent to 0.5 $KgN.ha.^{-1}yr.^{-1}$. This value is about 0.01 of the 55 $KgN.ha^{-1}yr.^{-1}$ noted above, and is probably an overestimate as it was calculated on the basis of continuous nitrogen fixation every day of the year. The discrepancy between the amount of nitrogen fixed estimated from $N_2[C_2H_2]$=fixing activity and that from nitrogen accumulated in the soil over many years needs further investigation.

Nitrogen fixing activity has also been found in the rhizospheres of a number of tropical members of the Gramineae and Cyperaceae. Nitrogen is continually available to rice (*Oryza sativa* L.) for many years in flooded soil even in the absence of fertilizer (30). Yoshida and Ancajas (29) showed that nitrogen was fixed by bacteria in the root zones of rice in flooded soil. In a field experiment (30) these authors found the following rates of soil nitrogen fixation in the wet and dry season.

Wet season: planted, flooded soil 51.7 $KgN.ha.^{-1}$; unplanted, flooded soil 22.2 $KgN.ha.^{-1}$; planted, upland soil 7.0 $KgN.ha.^{-1}$; unplanted, upland soil 3.0 $KgN.ha.^{-1}$.

Wet season

Dry season: planted, flooded soil 63.3 $KgN.ha^{-1}$; unplanted, flooded soil 28.3 $KgN.ha.^{-1}$; planted, upland soil

5.0 KgN.ha.$^{-1}$; unplanted, upland soil 2.8 KgN.ha.$^{-1}$. Dommergues *et al.* (10) measured $N_2[C_2H_2]$-fixing activity in the rhizospheres of rice, maize and a number of other tropical grasses and sedges. The species and associated C_2H_2 reduction rates are given in Table 1. The $N_2[C_2H_2]$-fixing activity in the rice rhizosphere was one-tenth of that of symbiotic systems. An anaerobic bacterium, *Enterobacter cloacae*, was isolated from the root system of maize (24) although in this case the degree of nitrogen fixation was estimated to be less than 0.5 KgN.ha.$^{-1}$yr.$^{-1}$. Sugar cane, like rice, can be grown for long periods without nitrogenous fertilizer. In Brazil, sugar cane has been cultivated for 100 years without fertilizer (9). Experiments with $^{15}N_2$ in Hawaii showed that even when 165 KgN.ha.$^{-1}$ was applied to sugar cane as fertilizer, 70% of the nitrogen in the crop came from other sources (9). It has been found that the obligate aerobe *Beijerinckia* is consistently stimulated in the rhizosphere of sugar cane (5,9). Dobereiner *et al.* (9) estimated that the rhizosphere of soil of one sugar cane site produced 1.51 nmoles C_2H_4. h.$^{-1}$g.$^{-1}$ soil which extrapolated to 67 KgN.ha.$^{-1}$yr.$^{-1}$ fixed in the top 20 cm of soil. *Beijerinckia* is also stimulated in the rhizosphere of *Hiparrhenia rufa, Digitaria decumbens, Panicum maximum, Cynodon dactylon, Setaria sphacelata,* rice, *Panicum purpurescens* and *Paspalum notatum* (all tropical grasses) and *Cyperus rotundus* although less consistently than in sugar

Table 1

$N_2[C_2H_2]$-fixing activity in the rhizospheres of tropical grasses. These values are taken from Table 2, Dommergues *et al.* (10)

Plant species	Plant age (months)	$N_2[C_2H_2]$-fixing activity*
Rice	1	1800 ± 274
	1	1190 ± 140
	1	1430 ± 205
	0.3	6153 ± 1516
Eleusine coracana	1	374 ± 165
	1	1.3 ± 0.5
Paspalum virgatum	4	528 ± 48
	8	345 ± 163
Pennisetum purpureum	2	4 ± 0.7
Panicum maximum	2	10 ± 1.1
Cyperus zollingeri	3	162 ± 47
	6	110 ± 25
Cyperus obtusiflorus	6	352 ± 66

*nmoles $C_2H_2.h.^{-1}(g.\ dry\ root)^{-1}$

1000 nmoles $C_2H_4.h.^{-1}g.^{-1}$ are about equivalent to 20 $KgN.ha.^{-1}yr.^{-1}$.

cane (5,9). $N_2[C_2H_2]$-fixing activity was also associated with roots of *Pennisetum purpureum* and *Cymbopogon citratus* (9).

In examining the rhizosphere of a number of tropical plants for nitrogen fixing activity, Dobereiner (4) found large numbers of a species of *Azotobacter* associated with *Paspalum notatum* var. "batatais" in Brazil. Recent work (8) has shown that this association may fix up to 93 $KgN.ha^{-1}yr.^{-1}$.

Dobereiner's work is interesting for a number of reasons. The species of *Azotobacter* is separate from the *A. chroococcum-vinelandii* group. The cell form and colony type are different (4) as are the CG% values of its DNA (6).

It is found associated exclusively with broad-leaved, tetraploid (8) varieties of *P. notatum*, these being known as "Common Bahia Grass" or "batatais" (6). This grass, which is a poor pasture grass (6), invades the sward in large areas of extremely poor latosols in South Eastern and Central Brazil (7). The bacterium has also been isolated from *P. notatum* var. "batatais" in Florida, southern Brazil and at the Amazonian mouth in Belem, Para (Brazil). Only sporadic occurrence of the bacterium with narrow leaved, diploid varieties (which are better pasture grasses) of *P. notatum* such as "pensacola bahia" and with *P. plicatulum*, *P. dilatatum* and *P. virgatum* has been observed (6). It was never found in 200 root soil samples of 31 other species of Graminae, 8 species of

Leguminosae and several other unidentified plants. In view of its exclusive association with *P. notatum* var "batatais," Dobereiner has named the new bacterium *Azotobacter paspali* (4).

In culture, rapid growth of *A. paspali* on N-free media was restricted to a pH range of 6.7 to 7.0 although it occurred abundantly in the rhizosphere of *P. notatum* in soils at pH 4.9 to 7.8 (6). There is normally a higher pH on the root surface of *P. notatum* than in the surrounding soil (4).

The association has proved difficult to establish in the laboratory. Dobereiner (6) found that the bacterium did not develop well in the rhizosphere in sterile sand and she was unable to measure nitrogen fixation by the association. Kass *et al.* (16) inoculated plants of *P. notatum* var "batatais" growing in initially sterile sand, with *A. paspali*, 4 and 12 weeks after planting. At 16 weeks *A. paspali* was isolated from rhizospheres in only two out of six jars (25 plants per jar). Consistent establishment of *A. paspali* did occur in those jars where glucose (41 mg per jar) was added at the first inoculation. The nitrogen gain associated with *A. paspali* establishment was quite small, about 0.5 mgN per jar or 0.6 ppm. Increases in nitrogen of the same magnitude were also observed in jars containing plants alone, and in jars containing neither plants nor added bacteria. In plants from jars in which *A. paspali* had become established, significant increases in nitrogen were observed in the roots but not in the shoots, and

it was suggested that the higher levels of root nitrogen resulted from an accumulation of *A. paspali* on the root surface rather than an uptake of fixed nitrogen. Rates of C_2H_2 reduction by soil samples containing the *P. notatum-A. paspali* association were very small: 67 and 59 pmole. $hr.^{-1}$ $(g.\ soil)^{-1}$. In pure culture, 10^8 cells of *A. paspali* reduced 1.29 nmoles $C_2H_2.hr^{-1}$. The low rates are possibly due to the soils being incubated in 0.21 atm O_2. It was concluded that the association probably fixed less than 10 $KgN.ha^{-1}yr.^{-1}$. (Cf. estimate of 93 $KgN.ha.^{-1}yr.^{-1}$ obtained by Dobereiner *et al.* (8).)

The association has been established in field experiments but only after about a year. Significant numbers of *A. paspali* were found in the rhizosphere of *P. notatum* var "batatais" after 10 months following a) transplatation of *P. notatum* from a long established lawn into soil free of *A. paspali* and b) transplantation of *P. notatum* with or without *A. paspali* into soil containing *A. paspali* (7).

The $N_2[C_2H_2]$-fixing activity associated with the roots is indirectly related to photosynthesis. Shading a lawn of *P. notatum* var "batatais" established for many years resulted in a decrease in the number of micro-colonies of *A. paspali* isolated from the rhizosphere (7). The $N_2[C_2H_2]$-fixing activity of an intact shoot-root-soil system almost ceased after 45 hours in darkness. When the system was returned to the light, activity started quickly, returning almost to the original rates after 15 hours (7).

Cells of *A. paspali* appear to be concentrated on the root surface. Unwashed roots and rhizomes had only a slightly higher $N_2[C_2H_2]$-fixing activity than when they were washed; in another experiment vigorous washing under a strong jet of tapwater removed only half the activity (8). Light microscopy of field grown roots (8) showed a prominent mucigel layer associated with colonies of bacteria. The mucigel layer may prevent the bacteria from being washed off and also help provide a suitable gaseous environment (see below) for nitrogen fixation (8).

Experiments with detached roots and rhizomes (8) showed that maximum rates of C_2H_2 reduction occurred at a pO_2 of about 0.04 atm. In contrast to roots freed from soil, the $N_2[C_2H_2]$-fixing activity of intact root-soil systems was not inhibited by an external pO_2 of 0.20 atm. This indicates that at the sites of C_2H_2 reduction within the root-soil system there was a favourable gaseous environment.

As a result of the work of Postgate and others (e.g. 21, 22, 23, 231, 3) it has become clear that the pO_2 can be a very important factor in nitrogen fixation by the Azotobacteraceae. Nitrogen fixation is a reductive process, and the nitrogenase enzyme is severely inhibited by oxygen (23). Extensive investigations of the physiology of the Azotobacteraceae have shown that in vivo, the nitrogenase is probably protected from oxygen in two ways (21):

1. respiratory protection: assuming that substrates are not limiting, respiration acts to keep the oxygen concentration within the organism very low. This is reflected in the high Qo_2 and maintenance coefficients characteristic of the Azotobacteraceae (21) arising from diversion of carbon compounds from biosynthesis to respiratory protection (21).

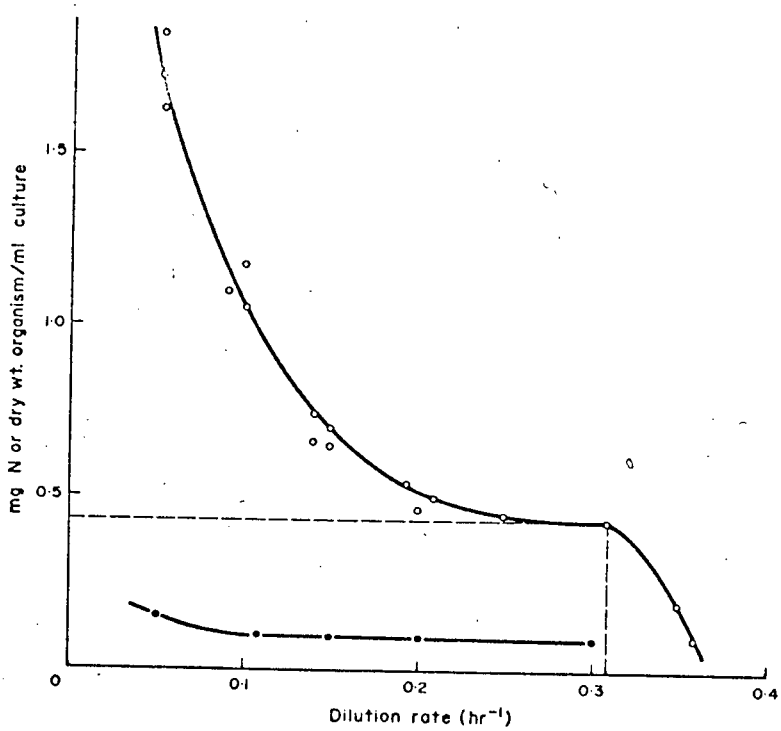
2. conformation protection: when respiration is not adequate to provide a low pO_2 within the cell, the nitrogenase enzyme assumes a conformation in which it is insensitive to oxygen. In this conformation it is not able to fix nitrogen. The conformation can quickly change to the dinitrogen fixing (and oxygen sensitive) state. Drozd and Postgate (11) were able to show a "switch on-switch off" effect by varying the oxygen supply to a continuous culture of *A. chroococcum*.

Work with cell-free preparations of nitrogenase has shown that it is a complex composed of two proteins (23). One has a relatively high molecular weight (200,000-300,000) and contains iron, molybedenum (10 or 20:1) and sulphur; this protein is relatively insensitive to oxygen. The other is smaller, has less iron and sulphur and no molybdenum. It is irreversibly damaged by oxygen.

The ideas that have developed about the effect of oxygen on nitrogenase, and the protective mechanisms, have enabled comments to be made about the nutritional status of

cultures of *Azotobacter spp.* With chemostat cultures of *A. chroococcum* and *A. vinelandii*, Dalton and Postgate (3) found that, in media containing an excess of all soluble nutrients and free dissolved oxygen, the bacterial density was inversely related to the dilution rate (see Figure 1). They explained the shape of the curve by proposing that the yield was limited by a gaseous component of the environment. It was concluded that the gas was dinitrogen, and that the organism was intrinsically limiting the rate at which it fixed nitrogen. This intrinsic limitation was considered to be part of the respiratory protection mechanism; it seems that at a given pO_2 only as much of the nitrogenase that can be protected by respiration is in the oxygen sensitive, dinitrogen fixing state (21). (Presumably, the probability of any one molecule being in the dinitrogen fixing conformation is the same throughout the population of nitrogenase molecules.) Such a population is said to be "N_2-limited." A non-nitrogen fixing population, limited by the supply of, for example, NH_4^+, is said to be "N-limited." So-called "ordinary" laboratory cultures are N_2-limited; in the field organisms are likely to be carbon limited (21).

A carbon limited (C-limited) culture may be defined operationally as one in which the organism concentration is halved if the carbon concentration in the inflowing medium is halved, and the organism concentration is not altered when



Population densities, contents of fixed N and growth rates of *Azotobacter chroococcum* in continuous culture. o: mg dry wt. organisms/ml culture; • mg N/ml culture. A 'theoretical' curve for an ideal, 'maintenance'-free, carbon-limited chemostat culture of $\mu max = 0.31\ h^{-1}$ is dotted in (- - -).

Figure 1. Growth curve for an N_2-limited culture of *A. chroococcum*. Taken from Postgate (21).

the concentration of all other medium components is doubled (3). C-limited, nitrogen fixing cultures are difficult to establish as they are hypersensitive to oxygen (3); respiration is not adequate to protect the nitrogenase.

Phosphorus limited (P-limited) cultures are defined in a similar manner (3). They are also hypersensitive to oxygen when fixing nitrogen (3). There is evidence to suggest that respiration in *A. chroococcum* is controlled by the ATP/ADP ratio (28). On the basis of this Dalton and Postgate (2) proposed that if respiration were suddenly increased to overcome an increase in pO_2 all the available ADP would be converted to ATP. The ATP/ADP ratio would then be shifted to favour decreased respiration, so negating the respiratory protection. However there is also evidence for a forked electron transport pathway in *Azotobacter* (23). One path is associated with respiratory phosphorylation. In the other, electrons are transferred from substrates to oxygen without, or with limited, phosphorylation. Under conditions of high oxygen, the non-phosphorylating pathway could use up the oxygen without excessive generation of ATP (23). If this is the case, there seems to be no reason why P-limited organisms should be unduly hypersensitive to high oxygen. There is a need for further clarification of the problem.

Nitrogen-fixing cultures may also be O_2-limited. In these the biomass is inversely related to the dilution rate,

due to the increased synthesis of polyhydroxybutyrate and polysaccharide at the lower dilution rates (17). When the oxygen supply was increased, O_2-limited cultures were able to increase their respiration to keep the level of dissolved oxygen low, and became N_2-limited cultures (17).

The Azotobacteraceae characteristically form a lot of slime (2). Polysaccharide formation is typical of most types of N-limited bacteria (21) and Postgate suggested that the slime of *Azotobacter spp* was an expression of their usual nutritional status, one of N_2-limitation (21). Gel, such as the slime, has a high oxygen diffusion resistance. Consequently the slime may have survival value in limiting the diffusion of oxygen to cells. It would be of particular advantage to C-limited populations (21).

The mucigel layer which is present on the roots of *P. notatum* var "batatais" is possibly involved in preventing rapid equilibration of soil oxygen at the sites of nitrogen fixation. Both the plant and *A. paspali* may contribute to the layer. Using axenic and non-axenic plants Greaves and Darbyshire (14) found that the mucilaginous layer on plant roots was derived both from micro-organisms and the plant.

When the work reported in this thesis was started, little was known about the physiology of the relationship between *A. paspali* and *P. notatum*. In view of the importance

of carbohydrate and oxygen concentration to nitrogen fixation by *Azotobacter spp* it was decided to study root exudation under different root oxygen regimes. As the work continued, the emphasis of my experiments eventually shifted away from exudation to a study of the buffering capacity of *P.nnotatum* roots and of the effect of pH and oxygen concentration on the growth of *A. paspali* in culture.

For two organisms such as these it is obviously preferable to study them when they are associated. However, in view of the difficulty of establishing the association in the laboratory it was necessary to grow and study the organisms separately.

It was also decided to work with non-sterile plants of *P. notatum*. The reason was a pragmatic one in that, as they were not already available, it was felt to be too complicated to build facilities for gnotobiotic culture of the large plants used here. There are some advantages in using non-sterile plants in that sterile plants are in the nature of an experimental artifact, plants in the field always functioning in the presence of micro-organisms. The main disadvantage, which admittedly outweighs many of the advantages, is that the presence of micro-organisms makes it practically impossible to study root exudation unaltered by microbial activity.

This thesis, then, is concerned with some aspects of the physiology of the relationship between *A. paspali* and *P. notatum* var. "batatais."

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Chapter 1

ROOT EXUDATION BY PASPALUM NOTATUM

Introduction

Paspalum notatum var "batatais" and *A. paspali* are in close association with one another in the field (11) and therefore it is reasonable to assume that *A. paspali* makes use of organic compounds exuded from the roots of the grass; it is also possible that sloughed off cellular material is a source of nutrients.

In the field the carbohydrate supply to *A. paspali* may usually be high enough for the bacteria to be N_2-limited. If the carbohydrate supply were low, then the bacteria might be C-limited, in which case nitrogen fixation would be prone to oxygen inhibition. As the carbon and oxygen supplies are of crucial importance to nitrogen fixation it was of interest to examine root exudation at different root oxygen regimes.

Since the oxygen environment of the roots could affect leaf function (16) a preliminary investigation was made of the response of photosynthesis to the root oxygen regime.

In the course of this investigation, it was unexpectedly found that root anoxia had no effect on photosynthesis. Consequently the oxygen treatments were combined with low temperature treatments to demonstrate that inhibition of root function would be reflected in CO_2 uptake measurements. Photosynthesis was measured in an open circuit apparatus, which was also used in the exudation experiments.

The amounts of material exuded by plants within convenient experimental time periods are minute but can be estimated and analyzed by radio-tracer techniques. In this study $^{14}CO_2$ was fed to the leaves of the plant and the ^{14}C in the roots and root medium was measured and analyzed. A similar approach has been used by several other workers (23,24,26,27,28). Seedlings have often been used to study root exudation (e.g. 23,24,26,28) but it was decided to use adult plants, particularly as field and laboratory experiments (10,11) involving the occurrence and establishment of *A. paspali* have been done using adult plants.

A number of experiments were done in which exudation occurred but in further experiments, exudation occurred at very low levels for no apparent reason. The pO_2 seemed not to be related to the amount of exudation, so an attempt was made to determine at least some of the other factors controlling exudation. This caused the emphasis of the work to move away from the effects of different root oxygen regimes.

A study was made of the role of young nodal roots in exudation, and of the effect of the pH of the root medium, ethanol and the composition of the root medium. These last three factors were studied for their effect on root permeability. Ethanol was chosen as data (32) indicated that ethanol would increase the permeability of the root cell membranes. The pH of the root medium proved to be important (although not, apparently, with respect to permeability); this is discussed further below and in the next chapter.

Materials and Methods

Germination

Seeds of *P. notatum* Flügge var "batatais" are difficult to germinate. Two methods of germination were used [1]. Seeds were placed in sulphuric acid (S.G. 1.84) for 15 minutes, then washed thoroughly with distilled water, care being taken not to let the temperature rise (9). [2] The hull (lemma and palea) was removed using sharp-pointed forceps (1). The scarified seeds or seeds without the hull were then placed in sterile sand in an incubator at 35°C, with distilled water being applied every day because germination is inhibited by the presence of inorganic ions (9). The germination percentage was very low. In later work, due to the difficulty of germinating seed, plants were obtained by cloning.

Growth conditions

Young seedlings were transferred to pots of sand in a growth room at 9 x 10^4 ergs.cm.$^{-2}$sec.$^{-1}$, 16 hour day and 30°/30°C day/night temperature. Light was provided by 400W Deluxe White GE mercury fluorescent lamps. The plants were watered every day, and Hoagland's solution (12) was given twice weekly. Plants were used in experiments when 4 to 5 tillers were present; plants grown from seed were about 3 months old at this stage.

Experimental apparatus

Figure 1 is a schematic diagram of the apparatus used. It was designed with three main functions in view: [1] monitoring photosynthesis, [2] controlling the root oxygen regime and [3] monitoring root exudation and collecting the exudate at the end of the experiment.

The plant was placed in the system as shown in Figure 1. The root medium was circulated between the root chamber (P) and the mixing tower (X). The direction of flow of medium in the tower was opposite to that of the root gas. After leaving the mixing tower, the root gas passed into the root chamber via the overflow line (Z) and then into the shoot chamber (C). The level of medium in the root chamber was maintained by use of the valve (H_c). The root medium circulating system enabled the oxygen regime to be replicated from day to day.

Figure 1. Schematic diagram of open circuit apparatus used to study photosynthesis and root exudation.

Key to Figure 1

A - lamps
B - heat shield
C - plexiglas chamber
D - shoot gas input
E - pump
F - flow gauge
G - shoot gas output
H - valves: H^a - ingoing chamber gas
H^b - outgoing chamber gas
H^c - intake for root medium
H^d - drain for root medium
I - water vapour trap
J - infra-red gas analyzer
K - strip chart recorder
L - cooling coil
M - fan
N - heating element
O - leaf temperature probe
P - root chamber
Q - root temperature probe
R - telethermometer
S - temperature controlled water bath
T - pH electrode
U - pump circulating root medium
V - sample port
W - cooling jacket
X - mixing tower
Y - oxygen electrode
Z - overflow line from mixing tower
a - root gas input

The connecting tubes of the root medium circulating system are represented by solid black lines. The direction of medium flow is shown by the solid arrows. The direction of flow of the root gas is shown by the dotted arrows. The small arrow-heads show the direction of flow of the shoot gas. The arrowhead just above the root-shoot junction indicates that, at this point, the root and shoot gases merge.

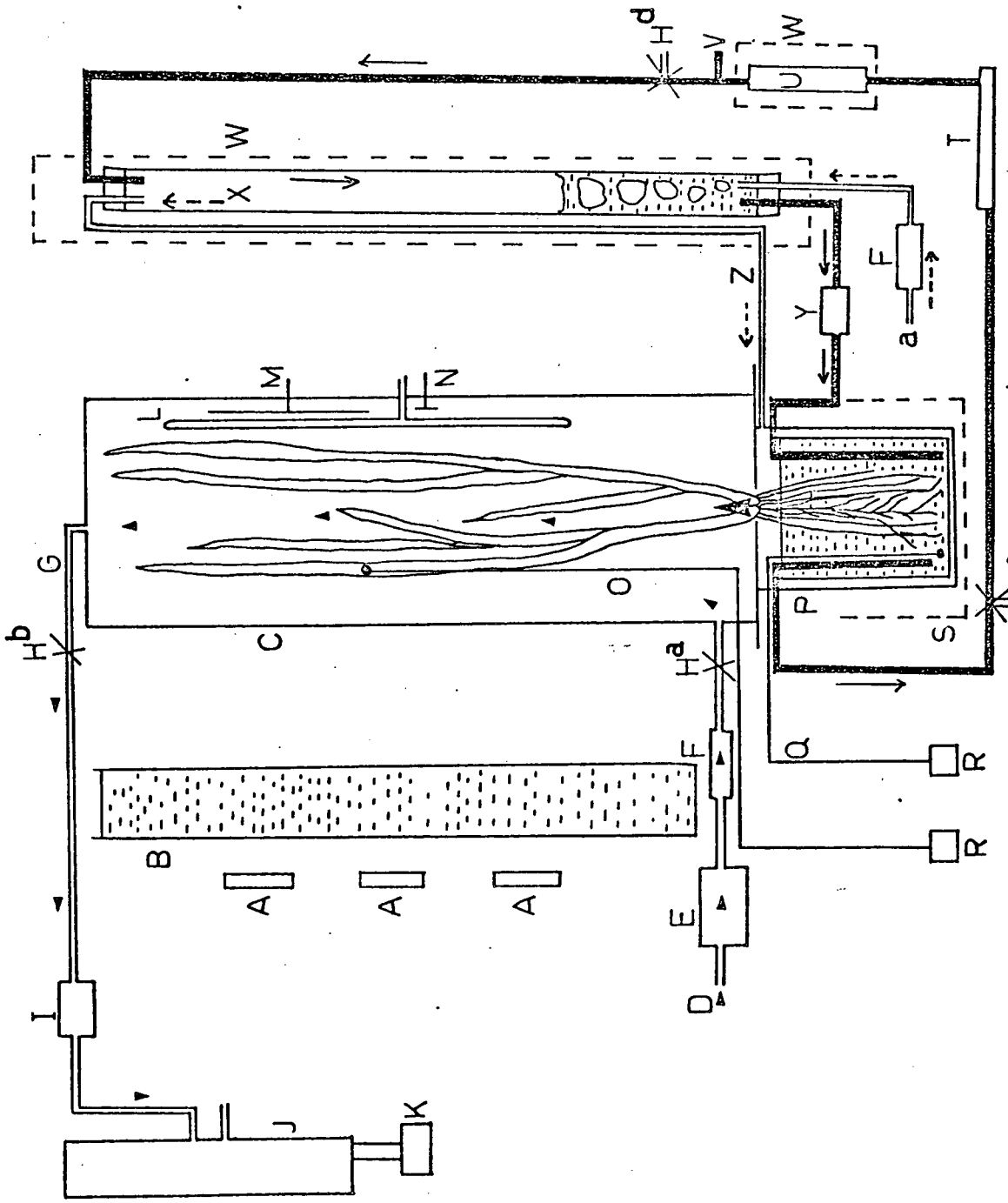


Figure 1

With the gas for the roots flowing into the apparatus at 'a' a gas mixture of 0.99 atm N_2, 0.01 atm CO_2 diluted with N_2 or 0.98 atm N_2, 0.02 atm O_2 was let into the shoot chamber via the path D → E → F → H to give an ingoing CO_2 concentration of 450-500 ppm, as measured by the infra-red gas analyzer (J).

The temperature of the root medium was controlled by a water bath (S) and also, when necessary, by cooling jackets (W) around the pump (U) and mixing tower. Shoot chamber temperature was adjusted using the cooling coil (M) and heating element (N) so that the temperature shown on the telethermometer (R) connected to the leaf temperature probe (O) was 30°C.

Three 300W Coolbeam GE lamps provided an average light intensity of 3.8×10^5 ergs.cm.$^{-2}$ sec.$^{-1}$ at the face of the shoot chamber.

An oxygen electrode (Y) was used to determine the concentration of dissolved oxygen in the root medium because the pO_2 of the solution will not be equal to the pO_2 of the gas supply. Table 1 gives the oxygen solution rates, determined by the method of Cooper *et al.* (6), corresponding to the pO_2 of the root gas supply. It shows that the nitrogen used to provide oxygen-free conditions contained traces of oxygen below the sensitivity of the oxygen electrode.

Table 1

Oxygen Solution Rates (mmole $l^{-1}hr^{-1}$) Corresponding to pO_2 of Root Gas Supply. Gas Flow Rate = 1.2 $l.min^{-1}$

pO_2 (atm)

	0.0(N_2)	0.02	0.04	0.20
Solution rate	0.9*	2.3	5.0	15.3

*This value is at the limit of detection

In the open circuit system described above the rate of plant photosynthesis is given by $PPS = C_1F_1 - C_2(F_1 + F_2)$

where PPS = rate of plant photosynthesis

C_1 = ingoing CO_2 concentration

C_2 = outgoing CO_2 concentration

F_1 = rate of flow of ingoing shoot gas

F_2 = rate of flow of ingoing root gas

Photosynthesis under different root oxygen regimes

Photosynthesis was monitored for up to 8 hours when the pO_2 in the ingoing root gas was 0.20 atm, 0.02 atm and 0.01 atm. Nitrogen was the other component of the root gas. Hoagland's solution (12) was used as root medium. The root temperature was varied between 30°C and 5°C. The particular sequences and combinations of pO_2 and temperature to which the roots were subjected are indicated in the Results section.

$^{14}CO_2$ feedings and root exudation

The design of the open circuit apparatus was such that part of any volatile exudate was lost due to agitation of the root medium. In order to estimate the degree of loss, the apparatus was set up without a plant. With the medium in the root circulatory system at pH 6.8, 2 µc of $NaH^{14}CO_3$ were injected into the medium, and samples were taken every 15 minutes for 90 minutes to determine the activity remaining in solution. The pH was then lowered to 3.9 and samples taken for a further 90 minutes.

For experiments on root exudation, the apparatus was set up with the plant in the chamber. Initially Hoagland's solution (12) was used as root medium; later 0.5 mM $CaSO_4$ was used (see Results). The temperature of the medium was adjusted to 30°C; the gas regimes are given in the results. Photosynthesis was allowed to come to steady state, after which the shoot chamber was isolated from its gas supply and 50 µc of $^{14}CO_2$ were fed to the shoot. After five minutes, unfixed $^{14}CO_2$ was flushed from the chamber, and the chamber was reconnected to the gas supply.

At hourly intervals, 1 ml of root medium was taken at the sample port (V) and placed in a scintillation vial with approximately 15 ml of Aquasol liquid scintillation fluid. In some experiments a further 1 ml of root fluid was

acidified with approximately 0.05M HCl and bubbled with nitrogen for 15 minutes before the Aquasol was added.

At the end of the experiment, normally eight hours after feeding the $^{14}CO_2$, the root medium was collected and ethanol was added to make the final ethanol concentration 20%. The medium was stored at 5°C until analyzed. In later experiments the medium was treated to estimate the size of the volatile fraction before adding the ethanol. It was acidified with 2M HCl and bubbled with nitrogen for 20 minutes, the carbon dioxide being trapped in 10 ml of 1M NaOH. Then, 5 ml of 0.1M Na_2CO_3 was added to the NaOH as a carrier and 5 ml of 0.1M $BaCl_2$ was added to precipitate $BaCO_3$. Aliquots (3.5 ml) of the $BaCO_3$ suspension were counted as a gel in Aquasol liquid scintillation fluid.

The roots were washed twice, cut from the shoot and both shoots and roots were killed in liquid nitrogen. Shoots and roots were freeze-dried and stored at -15°C.

A comparison of the distribution of ^{14}C in young nodal roots and the main root mass was made in two ways: [1] roots were blotted dry and placed between two layers of Saran-wrap (Dow Chemical Co.), and then against a sheet of Kodak No-Screen Medical NS54T X-ray film. The film was stored at -15°C and developed after three days to obtain an autoradiograph. [2] short lengths of roots from the main root mass and young nodal roots were placed in liquid scintillation

vials with Aquasol and counted. Correction for quenching was made using the channels ratio method (30).

In order to study the effect of low pH on exudation, the pH of the root medium was lowered to 4.0, four hours after feeding $^{14}CO_2$ to the shoot. The pH was kept at 4.0 ± 0.2 by the addition of dilute HCl for the remainder of the experiment.

The effects of ethanol were studied by placing plants in the open circuit apparatus and four hours after feeding $^{14}CO_2$ to the shoot, exposing the roots to ethanol. The root medium was bubbled throughout with nitrogen containing 0.20 atm O_2; in order to ensure that the gas supply to the roots was saturated with ethanol, the gas was first passed through ethanol solutions of the appropriate concentrations.

In the first experiment, the ethanol concentration in the root medium was brought to 2.5% at four hours, and increased through 5% and 7.5% to 10% at hourly intervals. In the second experiment, the ethanol concentration was brought to 7.5% at four hours. At 5.5 hours the root was removed, and replaced with medium without ethanol. The amount of ^{14}C in hourly, 1 ml samples of the root medium was taken as a guide to the degree of root exudation in both experiments.

Analysis of root tissue and exudate. Roots from each plant were divided into two samples, and extracted in 75 ml of boiling solvent for one hour. The extraction solvent contained 95% v/v ethanol: water: 90% v/v formic acid (33: 7: 2) (2). The extract was separated from insoluble material by filtration through Whatman No. 1 filter paper. The residue was washed with 3 x 5 ml lots of extraction solvent, 2 x 5 ml lots of 95% v/v ethanol and 2 x 5 ml lots of water. The washings and extract were transferred to a round-bottomed flask and evaporated to dryness in vacuo at 50°C. Residual formic acid was removed by passing a stream of nitrogen gas over the dried extract. Prior to analysis the dried extract was taken up in 1 to 2 ml of water.

The extracts were fractionated on cation (Dowex-50W, 50 x 8 - 400) and anion (Dowex -1, 1 x 8 - 400) exchange resins. Preparation of the resins and fractionation of the extract

followed the procedure of Atkins and Canvin (2) except that only three fractions were obtained: the neutral fraction (eluted with water), the basic fraction (eluted with 2M NH_4OH) and the acidic fraction (eluted with 2M HCl). The radioactivity of the fractions was determined by liquid scintillation counting.

Amino acids in the basic fraction were separated by two-dimensional thin layer chromotography. Samples were applied to plates coated with Avicel (19 x 19 cm, 250μ thick layer) and developed in phenol (liquefied 90% v/v): ammonium hydroxide: water (178:0.6:21.4). After drying for 18 hours the plates were developed in n-propanol: ethyl acetate: water (140:20:60) at right angles to the first solvent (4). Sugars in the neutral fraction were separated by one-dimensional descending chromatography on 46 x 57 cm Whatman No. 1 paper with ethyl acetate: acetic acid: water (14:3:3) for 30 hours (2). Organic acids in the acid fraction were separated by two-dimensional paper chromatography. Samples were applied to 20 x 20 cm Whatman No. 3MM paper and developed in ethanol: water: ammonium hydroxide (140:52:8). After drying for 12 hours the papers were run in ethyl acetate: acetic acid: water: sodium acetate (100:56:50:240 mg sodium acetate) (22). Autoradiography was used to locate radioactive compounds. Spots were identified from standard maps.

Most of the ^{14}C in the medium from the root chamber was volatile, and it proved to be difficult to obtain

qualitative and quantitative identification of the non-volatile fraction. The following procedure gave the best recovery of ^{14}C in the non-volatile fraction. The volume of fluid collected from the root chamber was about 300 ml; this was evaporated in vacuo at 40°C leaving a residue consisting partly of a white deposit (presumably $CaSO_4$). The residue was taken up in 10 ml of 70% v/v ethanol and filtered through 1 g of celite to remove the white deposit. The celite was washed with 2 x 10 ml lots of 70% v/v ethanol and 10 ml of acetone. After being quantitatively transferred to a round-bottomed flask, the filtrate was evaporated in vacuo at 40°C. The dried extract was taken up in 1 to 2 ml of water and fractionated using ion-exchange resins as described previously. Attempts to chromatograph the various fractions were not successful because of inadequate amounts of ^{14}C.

Results

Photosynthesis under different root oxygen regimes

Table 2 compares the photosynthetic rates obtained at different partial pressures of oxygen in the root gas supply.

Table 2

Rates of Photosynthesis of *P. notatum* Under Different Root Oxygen Regimes

	pO_2 (atm)			
	0 (N_2)	0.02	0.04	0.20
mg CO_2.hr.$^{-1}$ (g.dry wt)$^{-1}$	26.0 ± 5.1	29.5[1]	19.3 ± 5.8	16.2 ± 3.0
mg CO .hr.$^{-1}$ plant^{-1}	50.7 ± 5.5	61.4 ± 11.7	43.9 ± 9.4	57.7 ± 15.5

[1] one determination only

The rates varied between treatments, but photosynthesis was not inhibited by a low pO_2 around the roots. Figures 2 and 3 illustrate the effect of the temperature of the root medium. Photosynthesis became very low or stopped when the temperature was lowered to 5°C, or when the roots were immersed directly in medium at 5°C.

Root exudation: Whether Hoagland's solution, pH 5.4, or 0.5 mM $CaSO_4$ solution, pH 6.9, was used did not seem to have any effect on exudation. Once this knowedge was established, $CaSO_4$ was used as it is the solution of choice for studies of root exudation (13).

Figure 2. Effect of low root temperature on photosynthesis. Roots at 5°C throughout. Roots supplied with nitrogen.

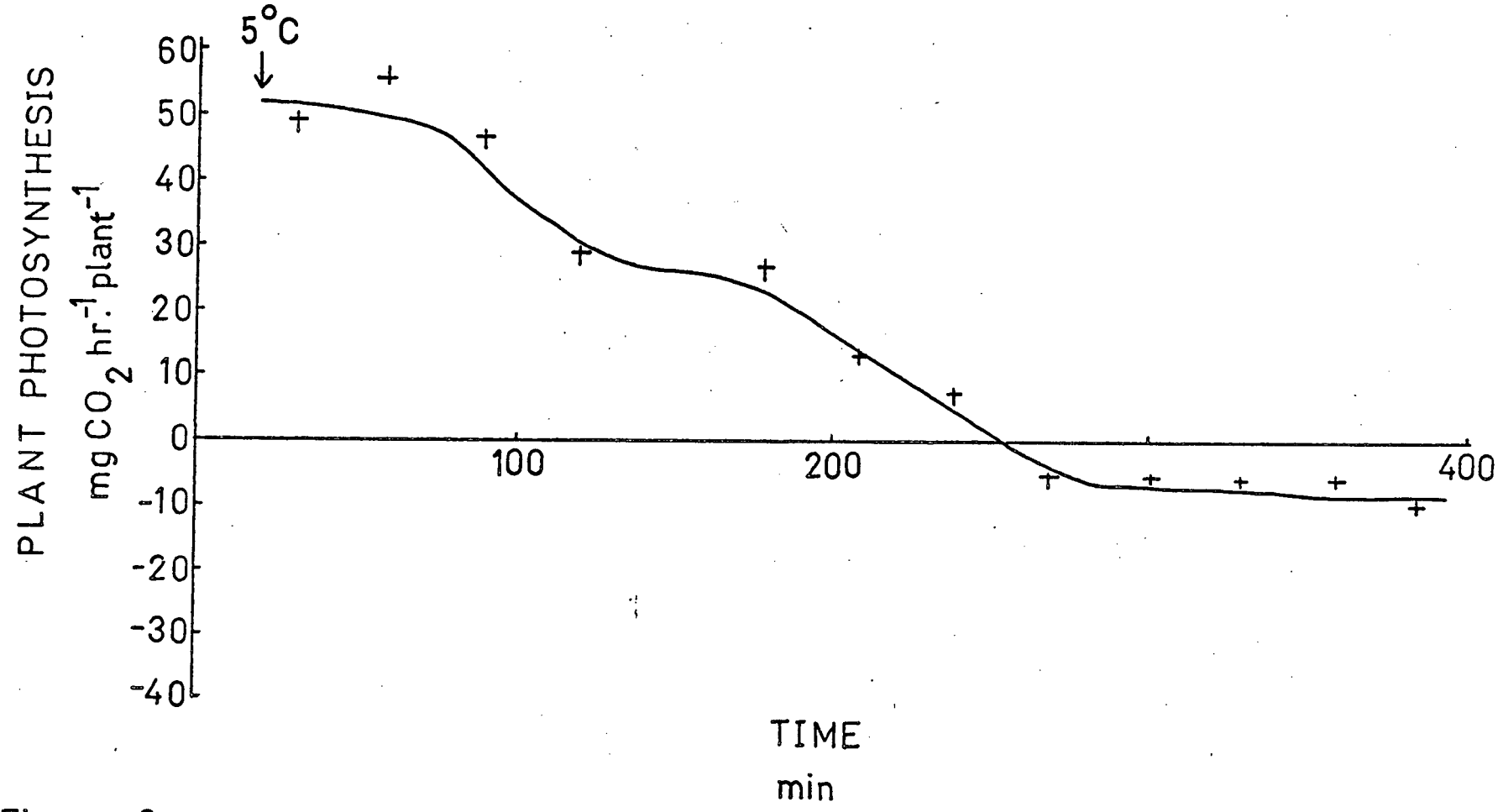

5°C
60
50
40
30
20
10
0
-10
-20
-30
-40
100
200
400
PLANT PHOTOSYNTHESIS
mg CO2 hr.-1 plant-1
TIME
min

Figure 2

Figure 3. Effect of low root temperature on photosynthesis. R@Roots cooled as indicated by arrows. Roots supplied with nitrogen.

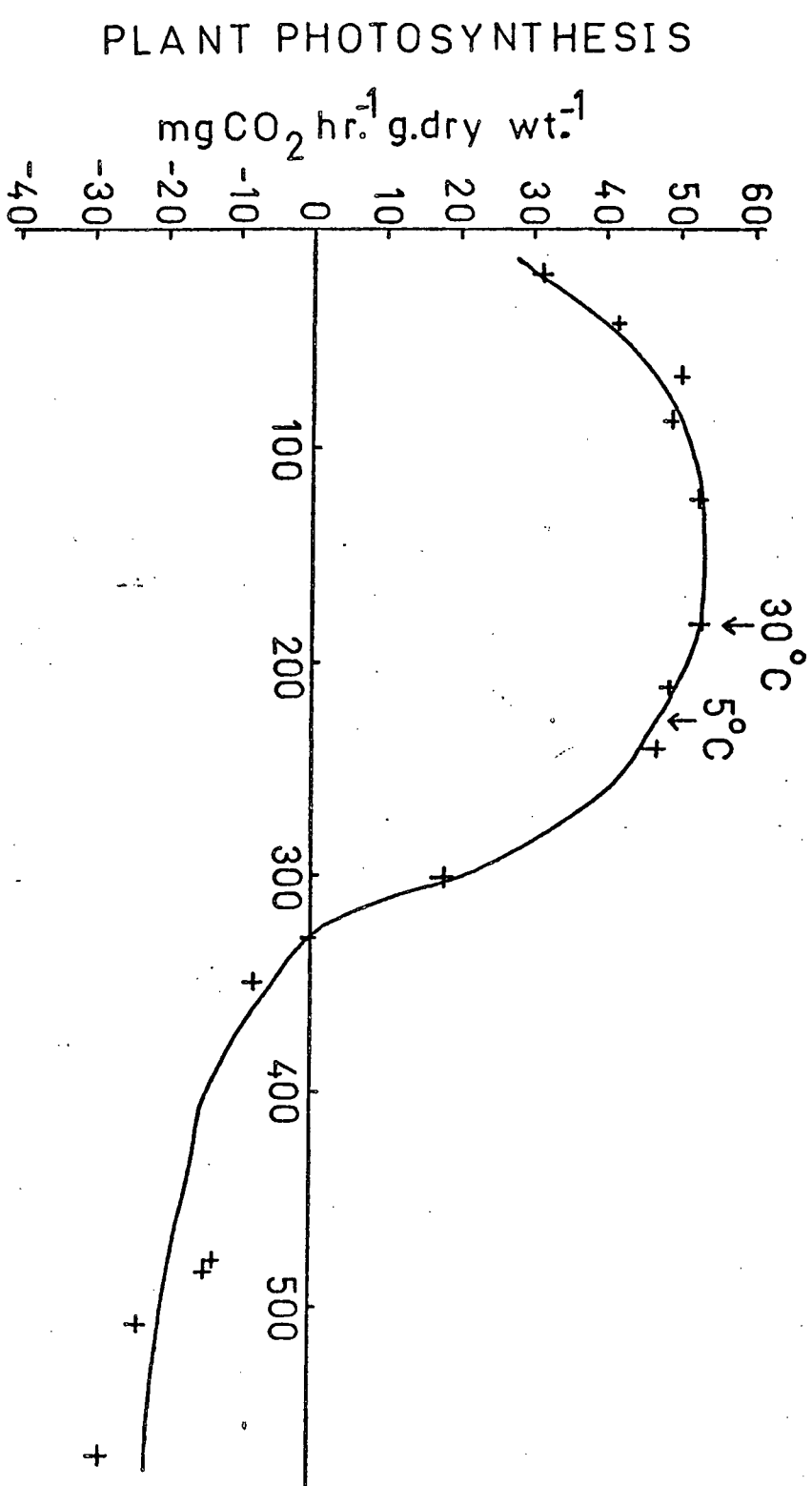

PLANT PHOTOSYNTHESIS
mg CO2 hr.-1 g.dry wt.-1
-40
-30
-20
-10
0
10
20
30
40
50
60
100
200
300
400
500
TIME
min
30°C
5°C

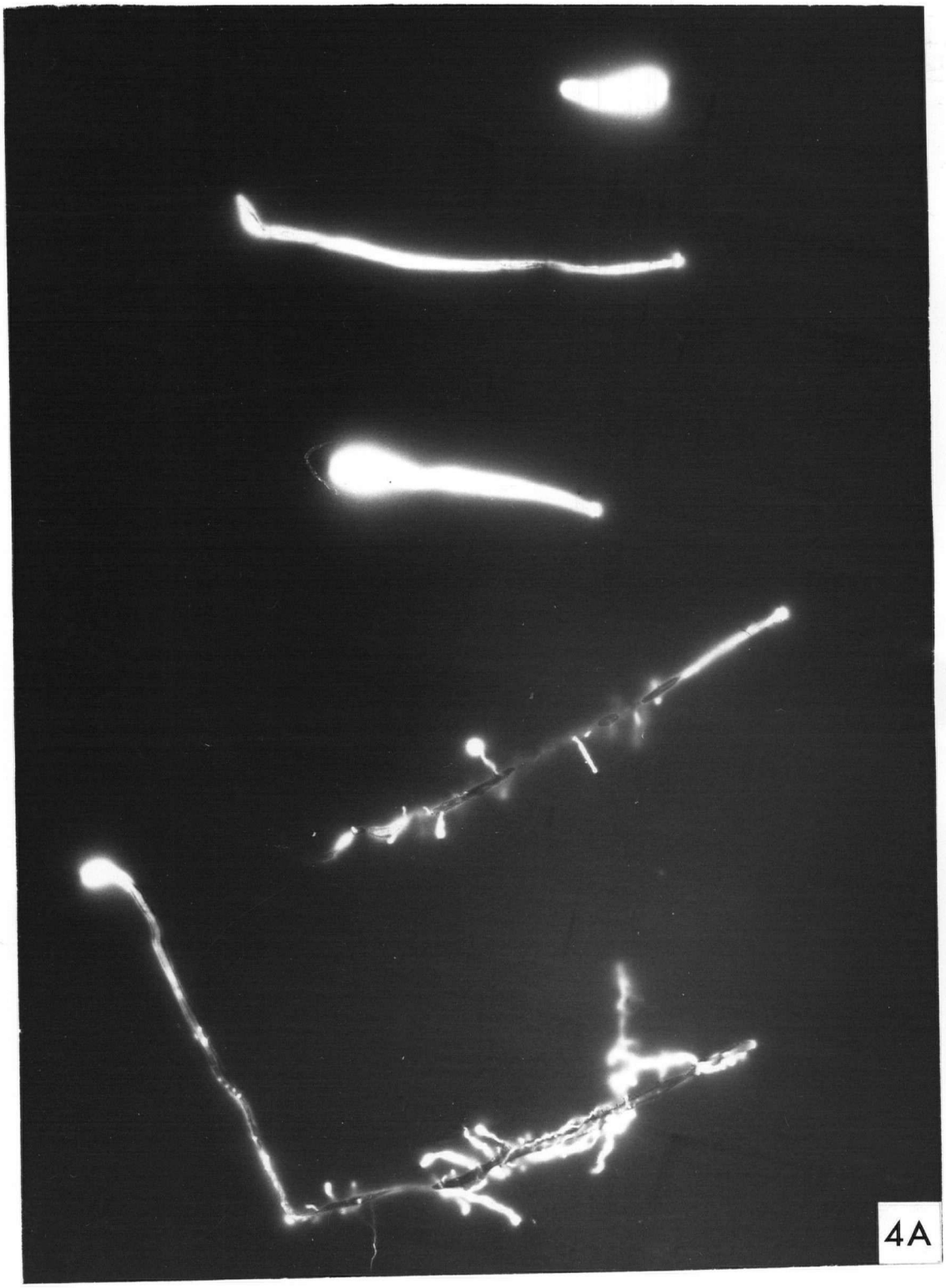
Figure 3

The root system consisted mainly of branched, fibrous nodal roots and the seminal roots. (No seminal roots were present, of course, in plants obtained by cloning). When young, the nodal roots were white, unbranched and of larger diameter than the older, branched, fine roots which had turned a yellowish colour. (New nodal root formation was stimulated by the presence of moist sand at the stem nodes.) The presence of several of the younger, unbranched roots was necessary to obtain detectable levels of exudation. Figures 4a and 4b are copies of autoradiographs of the root system. Figure 4a shows young nodal roots, some of which have developed lateral branches. Figure 4b shows the rest of the root system. It is clear that the younger roots contain more ^{14}C than the older ones. Table 3 compares the ^{14}C content of samples of young nodal roots (as in Figure 4a) and of older ones. The mean activity of the young roots is significantly different ($P < 0.01$) from that of the older ones.

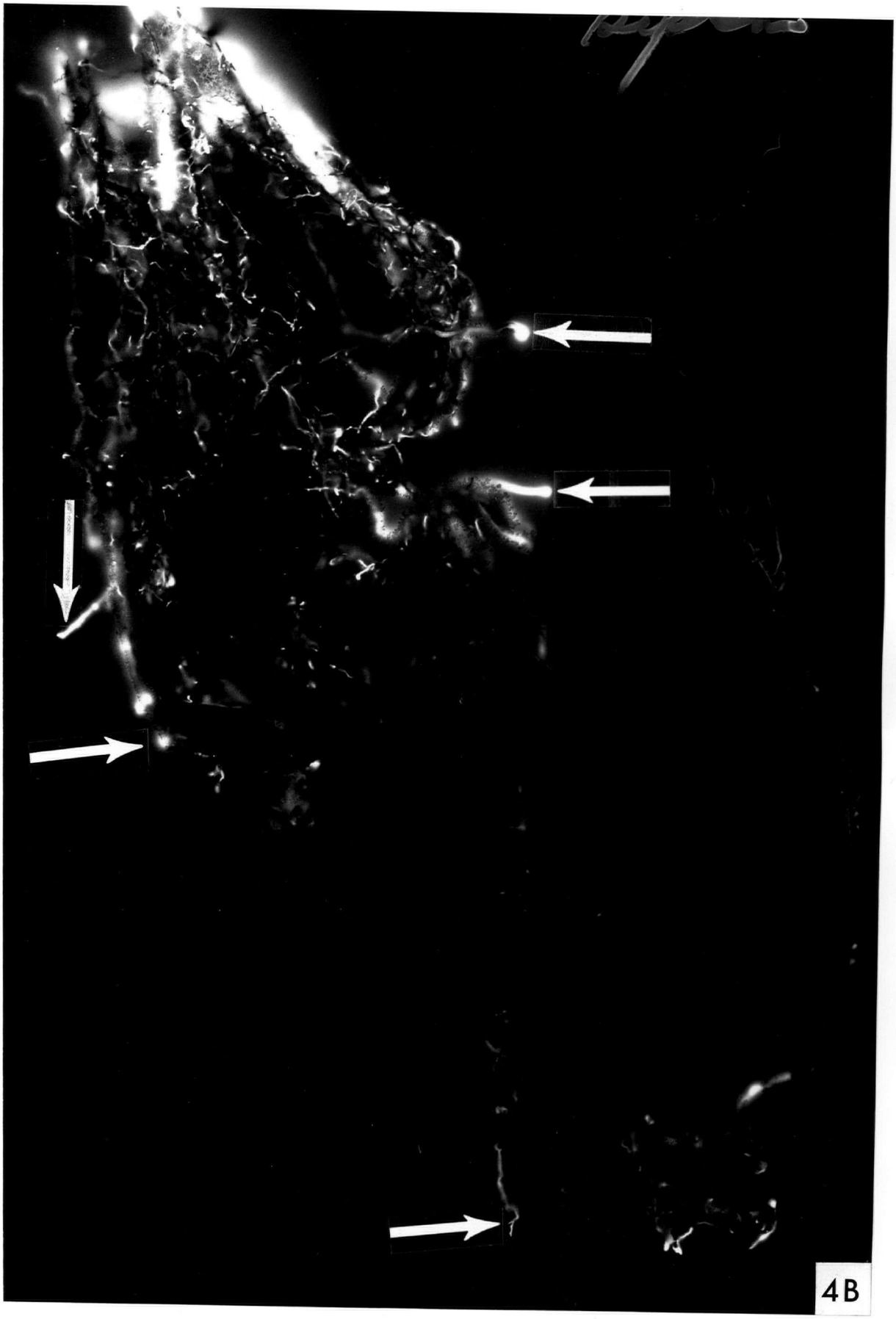
Figure 5 shows the loss of ^{14}C from the root medium after injection of $NaH^{14}CO_3$. The loss can be described by first order reaction kinetics. When the pH of the root medium was lowered to 3.9, the ^{14}C content dropped rapidly to very low levels. A complex calculation would be required to correct for this loss in estimates of the amount of ^{14}C exuded from the roots. However it is clear that graphs (see

Figure 4. A. Autoradiograph of young nodal roots. The two lower roots have developed some lateral branches. The branches have a high ^{14}C content.

B. Autoradiograph of the bulk of the root system (comprised of fine, branched nodal roots and the seminal roots). The ^{14}C tends to be concentrated in the apices of the lateral branches of the older roots; the arrows point to some of the apices.



4A



4B

Table 3

Comparison of radioactivity in samples of young nodal roots and older, fine, branched roots. Activity is dpm of ^{14}C per g. fresh wt. x 10^6

	Young Roots	Older Roots
	1.28	0.24
	1.46	0.12
	0.64	0.14
Mean:	1.13 ± 0.43	0.17 ± 0.06

Figure 5. Graphs of the loss of ^{14}C from the root medium after injection of $NaH^{14}CO_3$ (No plant present). When the percentage of ^{14}C remaining in solution is plotted linearly against time, it shows an exponential decline (A). When the log of the percentage is plotted against time, a straight line is obtained confirming that the loss of ^{14}C follows first order kinetics (B).

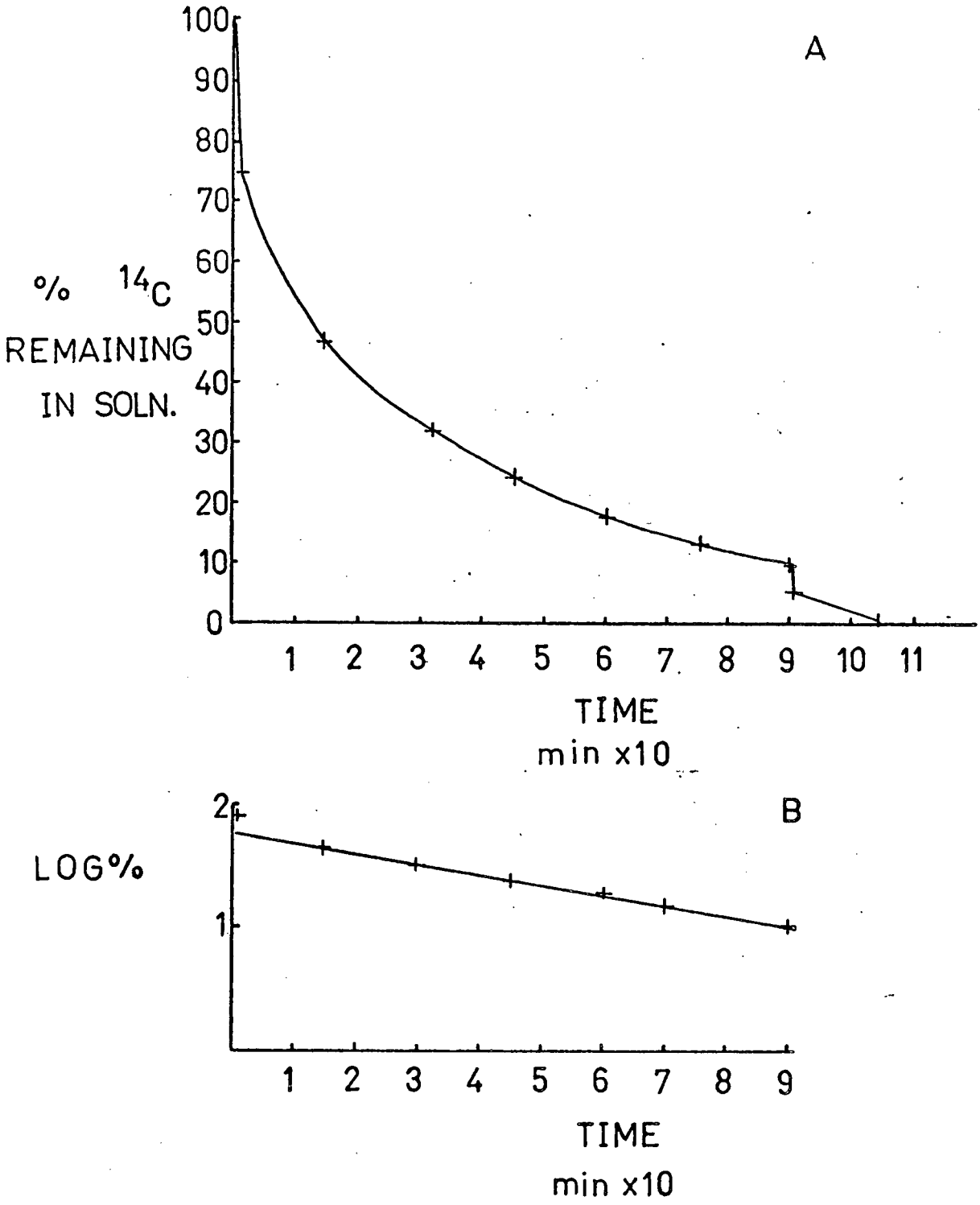

A
100
90
80
70
60
50
40
30
20
10
0
% ^{14}C REMAINING IN SOLN.
1 2 3 4 5 6 7 8 9 10 11
TIME
min x10
B
2
1
LOG%
1 2 3 4 5 6 7 8 9
TIME
min x10

Figure 5

below) where the amount of ^{14}C in the root medium is plotted against time underestimate the amount of ^{14}C exuded from the roots.

A representative graph of the accumulation of ^{14}C in the root medium with time is shown in Figure 6. The ^{14}C was detected in the medium one hour after feeding $^{14}CO_2$ to the shoot. The amount increased with time although it sometimes decreased between six and eight hours. Curve A in figure 6 is derived from roots supplied with gas containing 0.15 atm O_2; curve B is derived from roots supplied with 0.04 atm O_2. The maximum in curve A represents about 0.3% of the ^{14}C in soluble compounds of the root eight hours after feeding $^{14}CO_2$, and the maximum in curve B represents about 1.4% of the soluble ^{14}C of the root.

Figure 7 shows the accumulation of ^{14}C in the root medium as indicated by acidified and non-acidified samples. The maximum of the dotted line (total activity in the root medium) represents about 2.5% of the ^{14}C in the soluble compounds of the root, and the solid line (non-volatile ^{14}C) represents about 0.3% of the soluble compounds of the root. Some estimates of the size of the volatile fraction of root exudates are in Table 4. Direct measurements of the size of the volatile fraction made by trapping CO_2 in NaOH (plants Y and Z, Table 4) gave values considerably lower than those

Figure 6. The accumulation of ^{14}C in the root medium with time. Each curve is derived from a separate plant.

Curve A: Roots supplied with 0.15 atm O_2.

B: Roots supplied with 0.04 atm O_2.

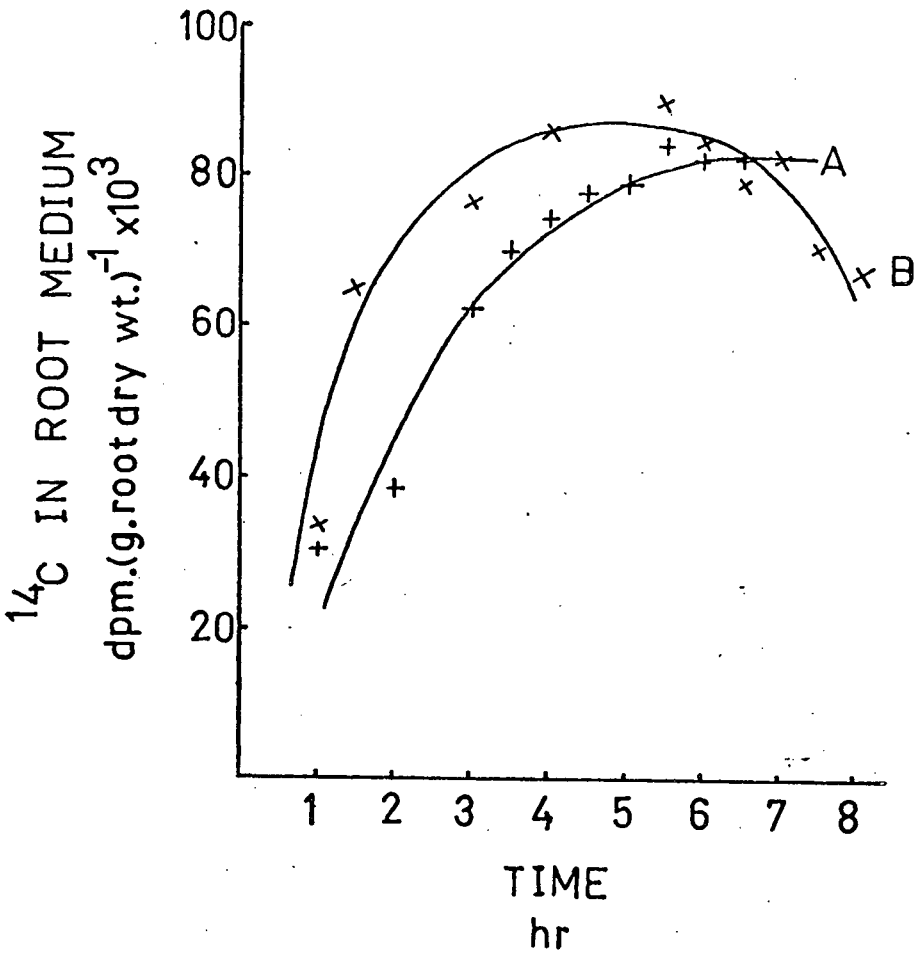

100
80
60
40
20
^{14}C IN ROOT MEDIUM
dpm.(g.root dry wt.)$^{-1}$ x10^3
A
B
1
2
3
4
5
6
7
8
TIME
hr

Figure 6

Figure 7. The accumulation of ^{14}C in the root medium.

Dotted line: Total activity in medium (from non-acidified samples).

Solid line: Non-volatile fraction (from acidified samples).

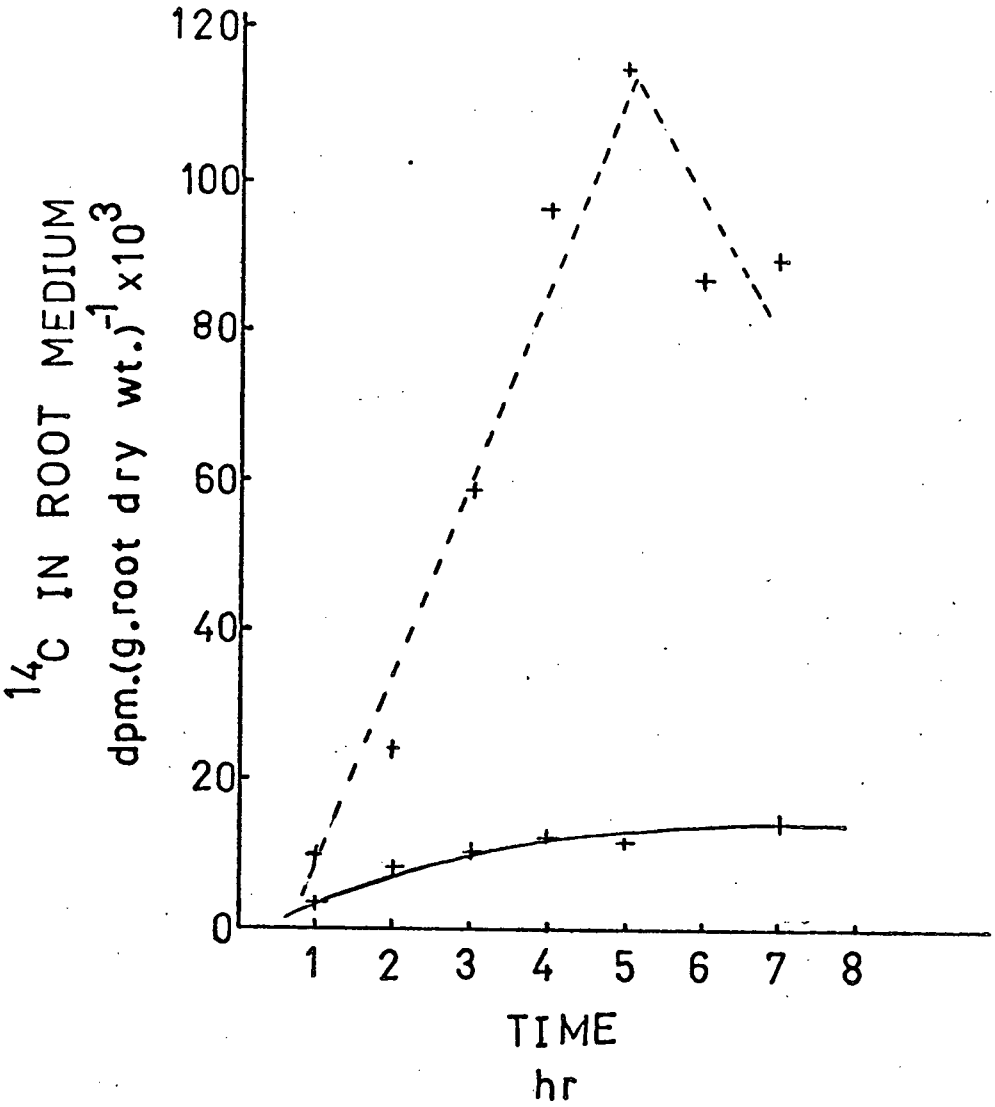

120
100
80
60
40
20
0
^{14}C IN ROOT MEDIUM
dpm.(g.root dry wt.)$^{-1}$ x10^3
1
2
3
4
5
6
7
8
TIME
hr

Figure 7

Table 4

Estimates of the size of the volatile and non-volatile fractions of the root exudate

Plant	Total Activity	Activity in non-volatile fraction	Activity in $Ba^{14}CO_3$
V	75500	12400a (83.6)	-
W	57700	2400a (95.7)	-
X	22900	20800a (9.2)	-
Y	88200	14000b (84.2)	23000 (26.3)
Z	10500	55200b (65055)	3360 (32.0)

'Total activity' is the activity ($dpm^{14}C$) in root medium at end of experiment. The figures in brackets in the third and fourth columns are the percentages of the activity in the volatile fraction.

a. The activity in the root medium extract after evaporation and filtration of the bulk medium.

b. The activity has been calculated from the activity remaining in a 1 ml sample of the root medium after acidification, and bubbling with nitrogen.

The %activity in the volatile fraction for a. and b. was calculated from:

$$\left[100 - \frac{\text{Activity in residue}}{\text{Total activity}}\right] \times 100$$

obtained by indirect estimates. The assumption behind measurement of the volatile fraction by trapping CO_2 is that all the volatile fraction is CO_2. If this assumption is right then the low values may have resulted from incomplete removal of the CO_2 from the bulk medium. Alternatively, all the volatile fraction is not CO_2; some other volatile compounds were present which were not trapped by the NaOH.

Lowering the pH of the root medium caused a sharp drop in the ^{14}C content (Figure 8). The ^{14}C content increased only very slightly during the remainder of the experiment.

In the first experiment with ethanol, exudation was low and remained low throughout, the ethanol apparently having no effect. The results of the second experiment are shown in Figure 10. After the addition of 7.5% ethanol, the amount of ^{14}C in the root medium did not increase. The ^{14}C content of the fresh root medium five minutes after being introduced into the system was low, although well above background levels, but appeared to rise in both instances over the next 30 minutes. For the remainder of the experiment, a decline in ^{14}C content was observed for plant 1 and an increase for plant 2. In both cases the ^{14}C level remained below that reached before the addition of ethanol. At the end of the experiment, the amount of ^{14}C in the soluble compounds of the roots of plant 1 (3.5×10^6 dpm. (g. root dry wt.)$^{-1}$) was similar to that of other plants not treated with

Figure 8. The effect of lowering the pH to 4.0 (at arrow) on the ^{14}C content of the root medium.

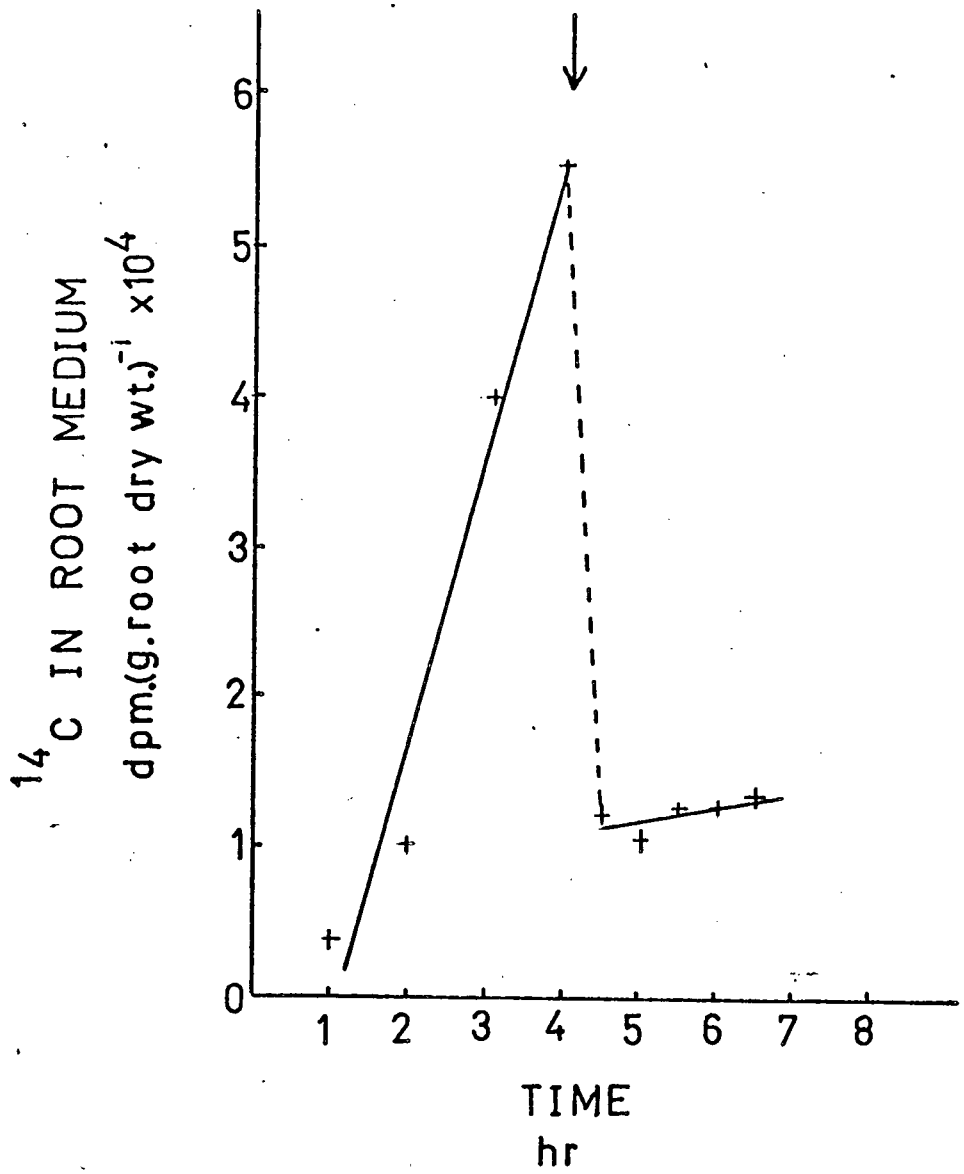

^{14}C IN ROOT MEDIUM
dpm.(g.root dry wt.)$^{-1}$ x10^4
0
1
2
3
4
5
6
1
2
3
4
5
6
7
8
TIME
hr

Figure 8

Figure 9. The effect of 7.5% ethanol on ^{14}C in the root medium of *P. notatum*. The ethanol was added at 4 hours (solid arrows). The ethanolic solution was replaced by fresh medium at 5.5 hours.

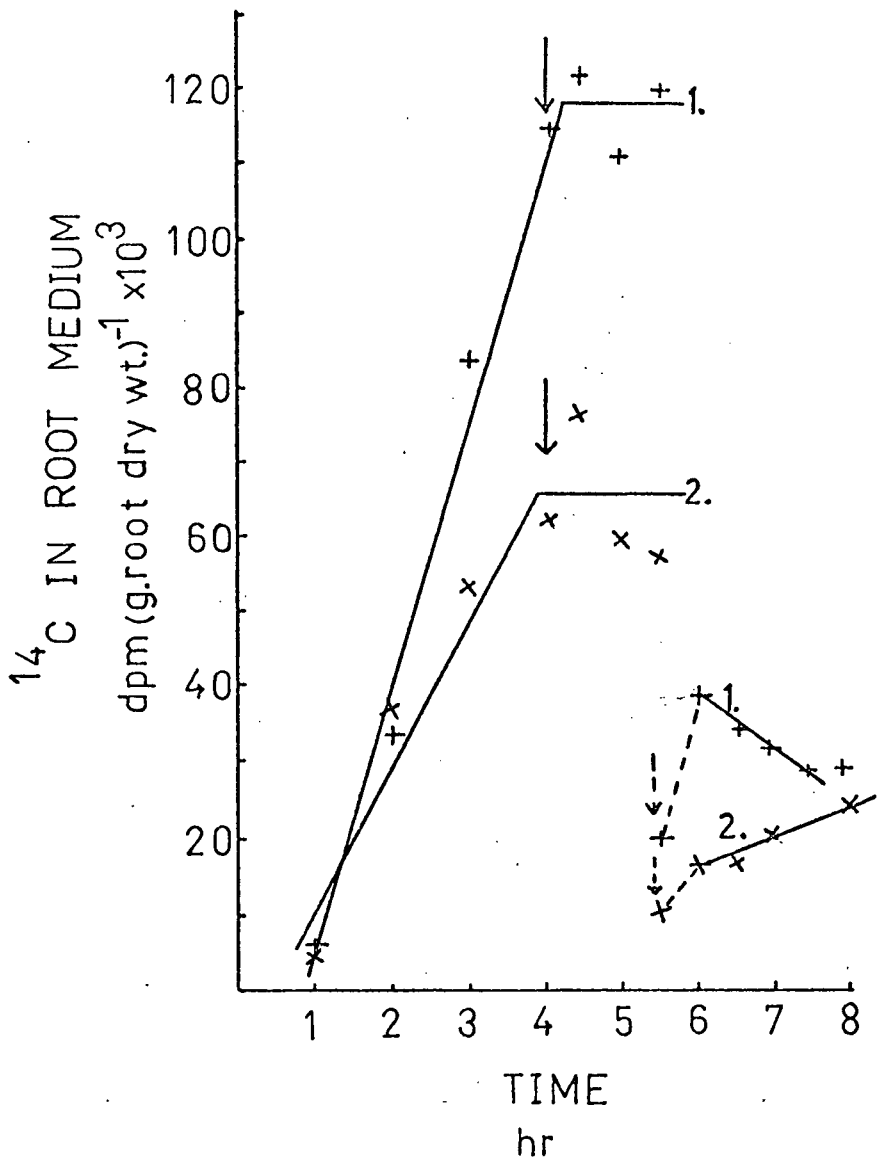

^{14}C IN ROOT MEDIUM
dpm (g.root dry wt.)$^{-1}$ x10^3
120
100
80
60
40
20
1.
2.
1.
2.
1
2
3
4
5
6
7
8
TIME
hr

Figure 9

ethanol (see Table 5, Chapter 1). The ^{14}C in the root medium of plant 1 when the ethanol was added was about 3.4% of that in the soluble compounds. Wilting, and a decrease in the rate of photosynthesis, occurred 1.5 hours after the addition of ethanol.

Anälysis of root tissue and exudate. Table 5 gives the distribution of radioactivity among the fractions of the soluble extract of several root samples.

Glucose, fructose and sucrose appeared consistently in chromatograms of the neutral fraction; no other labelled compounds were present (Table 6). Control analyses showed that the glucose and fructose did not result from acid hydrolysis of sucrose during evaporation of the eluate from the cation exchange column.

Up to seven compounds were found in the acidic fraction. Compounds identified as malic acid, citric acid and a compound tentatively identified as glyceric acid were always present; the occurrence of the other compounds was

Table 5

Distribution of activity among fractions of soluble extract of roots of *P. notatum* dpm ^{14}C (root g.dry wt.)$^{-1}$. Each value of activity is the mean of two samples

		Neutral		Basic		Acidic		
Plant No.	Total Activity	Activity	% Total	Activity	% Total	Activity	% Total	Percent Recovery
1	1.11×10^7	0.84×10^7	75.1	0.18×10^6	6.39	0.29×10^6	10.6	92.1
2	2.35×10^7	1.78×10^7	76.0	0.04×10^6	0.72	0.43×10^6	7.67	84.4
3	3.48×10^7	1.82×10^7	52.2	0.97×10^6	2.77	1.31×10^6	17.4	72.4
4	5.25×10^6	0.43×10^7	81.9	0.26×10^6	4.38	0.44×10^5	7.35	93.6
5	0.67×10^7	0.52×10^7	77.5	0.46×10^6	6.8	0.51×10^6	7.6	91.9
6	0.61×10^7	0.46×10^7	75.1	0.56×10^6	9.1	0.71×10^6	11.6	95.8

Table 6

Percentage of total activity in each spot of chromatograms of neutral fractions. Total activity is that found on the chromatogram

Plant No.	Sucrose	Glucose	Fructose
1	26.2	38.7	33.1
2	41.0	30.3	28.7
4	15.0	43.8	41.2
5	32.1	34.0	33.9
6	17.3	42.3	40.4

Table 7

Percentage of total activity in each spot of chromotograms of acidic fractions.
Total activity is that found on chromatogram

Plant No.	Origin	X_1	Citrate	Malate	Glycerate?	X_2	Succinate	Glycolate?
1	16.7	10.5	16.7	49.4	-	3₹8	-	6.8
2	17.0	13.3	18.9	35.8	-	3₹8	2.1	9.0
3	9.9	18.9	15.2	46.3	9.7	-	-	-
7	5.5	11.7	17.2	46.4	10.2	5.5	3.6	-
8	9.7	7.9	10.7	50.9	9.4	4.0	3.1	4.3

more variable. Table 7 shows the distribution of radioactivity among the compounds of the acidic fraction. Figure 10 shows the positions of the compounds on a chromatogram.

In the basic fractions, radioactive aspartic and glutamic acids, alanine and some unidentified compounds, were always present. Sometimes asparagine was present as well. Judging from autoradiograph spot density glutamic acid and alanine contained the largest amounts of ^{14}C. The unknown compounds contained only nominal amounts of ^{14}C.

As noted in the Methods section it was difficult to obtain a satisfactory analysis of the non-volatile fraction of the root medium. In some cases it was not possible to elute significant quantities of ^{14}C from resins, and even in the cases, see Table 8, where this was achieved, adequate chromatograms of the fractions were not obtained. Compounds were detected by autoradiography only in the basic fraction of one sample. The spots on the autoradiograph did not match with any of the spots on standard maps.

Table 8

Distribution of Radioactivity Among Fractions of Non-volatile Residue of Root Medium

Plant	Neutral	Basic	Acidic
V	39.0	27.3	11.7
X	42.8	9.1	30.7

Figure 10. A composite chromatogram of the acid fraction of the soluble root extract.

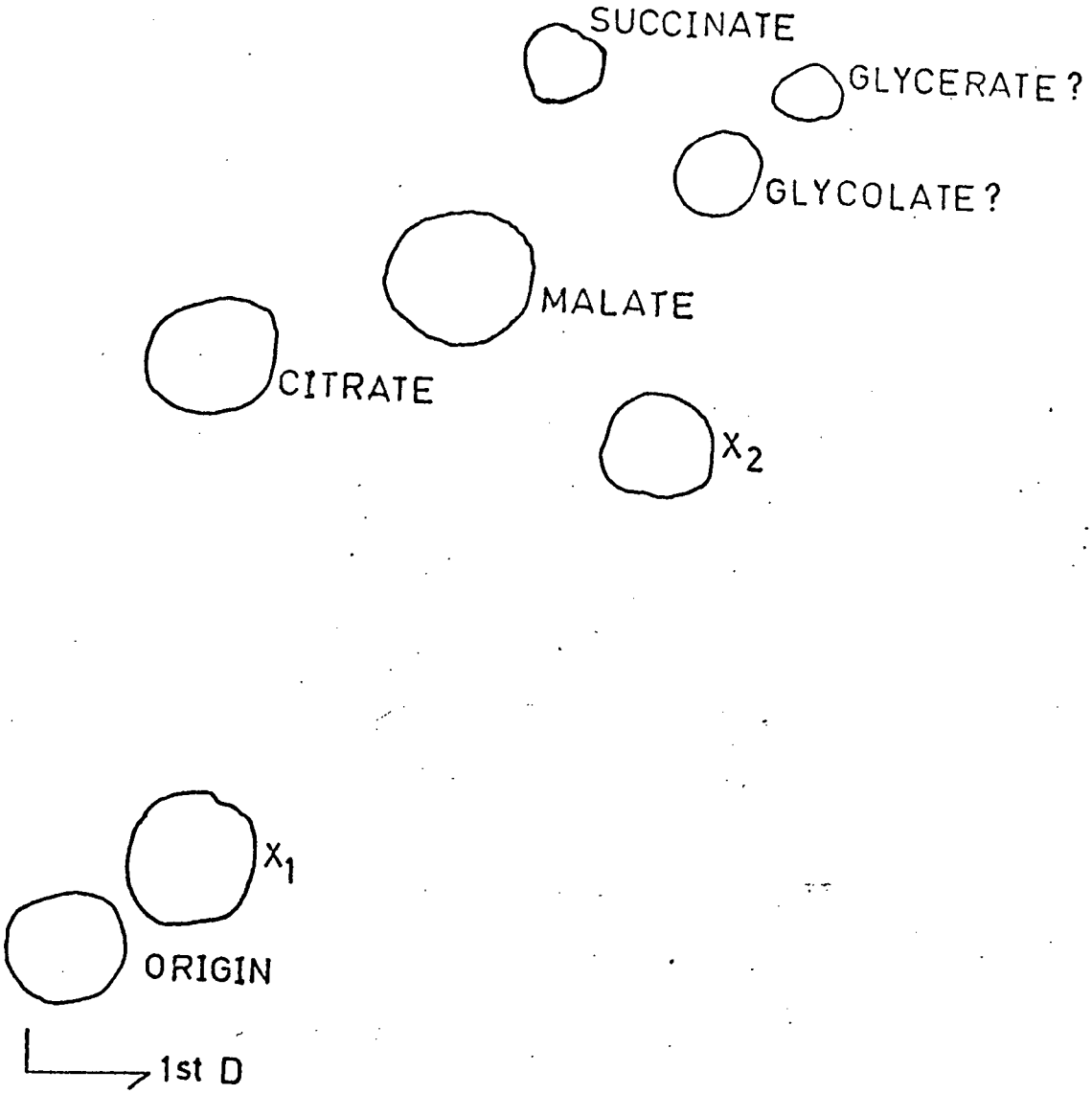

SUCCINATE
GLYCERATE?
GLYCOLATE?
MALATE
CITRATE
X2
X1
ORIGIN
1st D

Figure 10

Discussion

P. notatum plants were able to function, at least for eight hours, when the roots were provided with very low amounts of oxygen. There is little justification for extrapolating these results to longer periods of time but it is probable that the grass can grow for long periods of time under low root oxygen. In an analysis of the oxygen requirements of roots, Greenwood (17) concluded that aerobic metabolism would be unaffected by oxygen deficiency when the pO_2 at the root surface was 0.01 atm (provided the diffusion coefficient in the roots was greater than $1.2 \times 10^{-4} cm.^2 sec^{-1}$).

It has been shown (17,18) that mesophytic plants (as well as, for example, bog plants) can meet at least some of the root oxygen requirements by oxygen diffusion from the aerial parts. In my experiments this is unlikely to have occurred as the pO_2 in the shoot chamber was less than 0.02 atm, and there was a positive gas pressure in the root to shoot chamber direction.

A small value for oxygen solution rate (0.9 mmole. $l^{-1} hr.^{-1}$) was obtained when nitrogen was used indicating that traces of oxygen were available to the root. If this amount of oxygen is below that necessary to support aerobic respiration then *P. notatum* may be able to function for short periods of time under root anoxia. In view of the

many observations (16) of the importance of an adequate root oxygen supply (although as pointed out above the pO_2 can be very low) for shoot and root growth, it is unlikely that *P. notatum* can function adequately under prolonged root anoxia. With respect to exudation, anaerobiosis probably results in loss of compounds from the root; it was observed to do so in cotton radicles (5). However this represents an abnormal state. It is important that at low oxygen concentrations exudation be fully under control (also, *A. paspali* is an obligate aerobe).

The inhibition of photosynthesis at a root temperature of 5°C is probably due to reduced water absorption leading to a reduced leafe water content. Retardation of water absorption is a characteristic result of low root temperature (21). Ehrler (14) found that whether alfalfa roots were placed in a precooled water bath or whether the root temperature was lowered over two hours, a 5°C treatment caused wilting and a 70% reduction in water uptake in 24 hours. A rapid transient drop in the water content of maize leaves occurred when the root temperature was lowered to 5°C (20).

The autoradiographs of roots, Figure 4, and Table 3 show greater amounts of ^{14}C in the young nodal roots than in the rest of the root system. This will be due partly to their proximity to the shoot but their fast growth rate implies a greater demand for photosynthate. Radioactivity can

sometimes be seen to be concentrated at the apices of lateral roots (arrows in Figure 4b); a similar distribution was seen in wheat seedlings by McDougall and Rovira (24) who considered that the bulk of exudation occurred from lateral root apices.

Some ^{14}C was present in the root medium one hour after feeding $^{14}CO_2$ to the shoot. The movement of ^{14}C appears to be faster than in wheat seedlings, where translocation of ^{14}C to the primary root tips took two hours, exudation taking place one to two hours later (23).

The absolute amount of ^{14}C in the root medium did not vary much (8 to 10 x 10^4 dpm. (g. root dry wt.)$^{-1}$) between root oxygen regimes, although there were differences in the amounts of ^{14}C exuded expressed as a proportion of the ^{14}C in the soluble fraction of the roots. Unfortunately, because of the variation in exudation from plant to plant, it was not possible to demonstrate an effect of pO_2 on exudation.

The absence of an increase in ^{14}C content of the root medium in the presence of ethanol was unexpected. The high soluble ^{14}C content of the roots of plant 1 at the end of the experiment indicates that the ethanol did not extract compounds from the roots. Apparently, the effect of the ethanol was not to increase permeability; it changed the roots in such a way as to prevent further exudation. The wilting observed in the second experiment suggests that the effect of ethanol on the roots was the same as that observed by

Allerup (31) in barley seedlings. Transpiration showed a transient increase followed by a steep decline within 30 minutes when the barley roots were exposed to nutrient solution containing 10% ethanol (it follows there may have been a transient increase in permeability of *P. notatum* roots on exposure to ethanol). A less steep decline occurred at 5% ethanol but transpiration containued to be affected at 3%, 2% and 1% ethanol. These changes in transpiration were attributed to changes in the water permeability of root cells. At 5% and 10% ethanol the changes were thought to be irreversible. The increase in ^{14}C content observed in the fresh root medium may indicate that the changes in *P. notatum* roots were not wholly irreversible. On the otherhand, the ^{14}C of the fresh root medium may have been ^{14}C adhering to the root surfaces, after removal of the ethanolic solution.

It was thought that lowering the pH of the root medium would increase the permeability of the roots, causing an increase in the amount of ^{14}C in the medium but this did not happen in my experiments (Figure 8). Most of the decrease in ^{14}C is due to the loss of $^{14}CO_2$ from the medium but no increase in the non-volatile ^{14}C occurred. The two most likely sources of the $^{14}CO_2$ are the root and microbial respiration utilizing exudedn non-volatile compounds. Rovira and

Ridge (28) reported a 65% decrease in radioactivity of the solution around the roots of non-sterile wheat seedlings fed $^{14}CO_2$ compared with the solution around sterile roots.

The decrease in ^{14}C content of the root fluid observed in some cases after six hours, Figure 6, may be due to the cessation of ^{14}C exudation combined with loss of $^{14}CO_2$ due to microbial respiration and agitation of the root medium. In order for the amount of ^{14}C in the root medium to increase, exudation had to occur at a sufficient rate to overcome losses due to agitation and microbial respiration, and thus the ^{14}C exudation was larger than indicated by Figures 6, 7 and 8.

In spite of the discrepancies between direct and indirect estimates of the size of the volatile fraction (Table 4) there is little doubt that most of the exudate is volatile. Most of the exudate from wheat seedlings was also volatile; McDougall (23) found that acidification and aeration of the exudate solution removed approximately 80% of the radioactivity.

In the soluble extract of the roots 70 to 90% of the ^{14}C was in the sugars and organic acids which is compatible with carbohydrate making up the bulk of the root exudate. It does not necessarily follow that these compounds will be represented to the same degree in root exudate, although in those instances where an estimate of the size of

the exudate fractions was obtained 51 to 74% of the label was in the neutral and acidic fractions.

About 50% of the ^{14}C in the acidic fraction of the root tissue was in malic acid. It is possible that some of this was formed by fixation of respiratory $^{14}CO_2$ (29). An inducible glucose-specific uptake system is present in membrane vesicles of *A. vinelandii* (3). The uptake of glucose is coupled to an FAD-linked L-malate dehydrogenase since the addition of L-malic acid to a preparation of membrane vesicles resulted in a 25-fold stimulation of both the rate and steady-state level of glucose accumulation. The efficient uptake of carbohydrate is likely to be of critical importance to *A. paspali* and it is tempting to speculate that if malic acid is also present in the root exudate it is involved in the uptake of carbohydrate by *A. paspali*.

Amino acids are commonly found in root exudates (26). Radioactivity was found in the basic fraction of the exudate solutions analyzed, so it is possible amino acids are present in exudate of *P. notatum*. It is difficult to say if they would have any function in relation to *A. paspali*.

The amount of carbon exuded from roots is small, and the non-volatile fraction is even smaller. Rovira and Ridge (28) concluded that the total radioactivity of the root fluid around wheat seedlings was probably less than 0.5% of the applied $^{14}CO_2$. Some examples of the amounts of carbon

exuded by various plants are given by Rovira (26); these include corn: 8.2 and 8.4 mg of reducing sugars over 20 days and wheat: 2.6 to 22.5 mg over two months.

These figures and the small amount of exudate found in my experiments raise the question as to how much non-volatile exudate as opposed to sloughed off cellular material is available to *A. paspali* under field conditions. Assuming that *A. paspali* can compete sufficiently well so that the numbers of other micro-organisms are low, there may be more non-volatile exudate available to it than is indicated here. The availability of adequate levels of soluble carbohydrate is suggested by the relationship between photosynthesis and $N_2[C_2H_2]$-fixing activity in an intact *P. notatum-A. paspali* association (11). Reduction of C_2H_2 was unaffected by day-night changes but was much diminished after 45 hours of darkness, increasing rapidly upon a return to light. This indicates a dependence of nitrogen fixation on relatively recent exudation.

Calculations based on rates of C_2H_2 reduction and photosynthesis, and on the carbohydrate requirements of *Azotobacter* for nitrogen fixation also suggest that *P. notatum* is able to provide sufficient exudate to support nitrogen fixation by *A. paspali*.

Dobereiner *et al.* (11) found the $N_2[C_2H_2]$-fixing activity in two soil cores to be:

143.2 nmole C_2H_2.hr.$^{-1}$ and 398.3 nmole C_2H_2.hr.$^{-1}$

assuming an equivalence between 3 C_2H_2 and 1 N_2.

These rates are equivalent to 1334 µg N /hr and 3.72 µg N_2/hr. An N_2-limited culture of *A. chroococcum* fixed N_2 with an efficiency of 38 mgN fixed per g mannitol consumed (8). Hence, the mannitol required to fix the amounts of nitrogen above are 0.035 mg/hr and 0.098 mg/hr. If a photosynthetic rate of 57.7 mg CO_2.hr.$^{-1}$ plant^{-1} (Table 2) is taken for *P. notatum*, and it is assumed that 0.5% (27) of the carbon fixed is exuded as glucose (M.W. 180 cf. mannitol M.W. 182) then the amount of glucose exuded is approximately 0.2 mg glucose.hr.$^{-1}$plant^{-1}. This quantity is sufficient to meet the carbohydrate requirement of the $N_2[C_2H_2]$-fixing activity of the soil cores.

By extrapolating the $N_2[C_2H_2]$-fixing activity of soil cores to larger areas, Dobereiner *et al.* (11) obtained values for nitrogen fixation by the *P. notatum-A. paspali* association including one of 93 KgN.ha.$^{-1}$yr.$^{-1}$ Kass *et al.* (19) found the nitrogen content of *P. notatum* tops to be 0.4% on a dry weight basis. If *A. paspali* is taken to be the sole source of nitrogen for *P. notatum*, then the dry weight of *P. notatum* corresponding to 93 KgN.ha^{-1}yr.$^{-1}$ is 23250 KgDW.ha.$^{-1}$yr.$^{-1}$.....a. An N_2-limited culture of *A. chroococcum*

fixed N_2 with an efficiency of 38 mg N fixed per gram of mannitol consumed (8). Plant dry matter is about 40% carbon, hence the amount of carbon that has to be exuded from the plant to fix 93 KgN.ha.$^{-1}$yr.$^{-1}$ is 2450 KgDW.ha.$^{-1}$yr.$^{-1}$.....b. If 30% of the carbon fixed in photosynthesis is lost as respiration, then respiratory losses are equivalent to 9964 KgDW.ha.$^{-1}$yr.$^{-1}$.....c. The total productivity of the aerial parts in terms of dry weight is therefore 33664 KgDW.ha.$^{-1}$yr.$^{-1}$ (the sum of a, b and c). Expressed as a percentage, the amount exuded for nitrogen fixation, 2450 KgDW.ha.$^{-1}$yr.$^{-1}$, is 6.9%. The conclusion that can be drawn from this calculation is that exudation has to be higher than the 0.5% of the fixed carbon mentioned previously. Alternatively, if exudate is only about 0.5%, then *A. paspali* can act only as a partial supplier of the nitrogen requirements of *P. notatum*.

In a review of root exudates Rovira (26) states,

> *In studying the effects of plants upon specific micro-organisms . . . it is unlikely that the ubiquitous sugars, amino acids, and organic acids will make a significant contribution, but rather it will be the balance of these compounds and/orrthe presence of the rarer exotic compounds peculiar to a particular plant species that will be important.*

A. paspali, however, is able to grow quickly in a simple medium and it seems unlikely that it is dependent on a particular balance of compounds or some exotic molecule. I suggest that

it makes relatively unspecific use of whatever soluble carbohydrate is available, except possibly malic acid, if it appears in the exudate.

My experiments show that *P. notatum* plants absorb $^{14}CO_2$ and quickly transfer the fixed ^{14}C to the root environment by root exudation. These experiments using low root oxygen provide circumstantial evidence that controlled exudation can occur at oxygen levels suitable for nitrogen fixation by *A. paspali*. The analysis of the nature and amount of the labelled compounds in the roots indicate that these were of a type compatible with a carbohydrate based exudate for *A. paspali*.

In retrospect, the difficulty of collecting non-volatile exudate under non-sterile conditions was underestimated. There was also a certain incompatibility between exudation studies and the method of control of oxygen concentration. Until a carbon and nitrogen budget is drawn up for the *P. notatum-A. paspali* association it will not be possible to decide whether *A. paspali* in the rhizosphere is normally C- or N_2-limited (to this end it is imperative that a convenient means be found for establishing the association in the laboratory).

Whether they are P-limited is also a question of prime interest. Of relevance to this question is the observation by Dobereiner *et al.* (11) of hyphae and spores of *Endogone*, a mycorrhizal fungus, in the outer cortex of *P. notatum* roots. Its presence becomes fascinating in view of a report

(25) on the effects of *Endogone* on growth of *P. notatum* var "batatais." In two Brazilian soils, deficient in available phosphorus, infection of the grass with *Endogone* improved growth almost to the extent of added phosphate. Lime was added in some cases, as some *Endogone* strains do not grow well in acid soils (the pH of the unlimed soils was 4.5 and 5.2), and the improvements in growth of the grass were greater in these than in unlimed soils (25). In the field, *Endogone* can be envisaged as mobilizing phosphorus both for *P. notatum*, which without an adequate phosphorus supply would not respond to nitrogen (25), and for *A. paspali*. The buffering capacity of *P. notatum* roots (see next chapter) may also be of benefit to *Endogone* as well as to *A. paspali*.

Presumably the external supplies of oxygen and dinitrogen are not limiting. There is a need for a description of the gaseous phase in the rhizosphere. A comment by Dalton and Postgate (7) is relevant to the problem of the nutritional status of *A. paspali* populations:

> *The concept of limitation by single nutrients in chemostats thus becomes rather involved in a circumstance in which oxygen concentration is critical and access of nitrogen to nitrogenase is limited by some intracellular mechanism.*

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Chapter 2

THE IMPORTANCE OF pH TO THE PASPALUM NOTATUM-AZOTOBACTER PASPALI ASSOCIATION

Introduction

Species of *Azotobacter* are capable of growing within a pH range of 4.5 to 9.0 if the medium contains fixed nitrogen, but nitrogen fixation occurs only within a range of 5.5 to 7.2. They are not usually encountered in soils with a pH below 5.6 (10). *A. paspali* was observed to occur abundantly in the rhizosphere of *P. notatum* in soils ranging in pH from 4.9 to 7.8 (4). In culture medium, however, good growth occurs only above pH 5.5 (4), and nitrogen fixation is restricted to a narrow range of pH 6.7 to 7.0 (5). *A. paspali* produces acid itself, both in liquid and on solid, nitrogen-free media (4); in a liquid medium with sucrose, growth stopped when the pH reached 5.2 because of acid production (4). Dobereiner (4) found a higher pH on the root surface than in surrounding acid soil, and suggested that the occurrence of *A. paspali*

in the rhizosphere of *P. notatum* in acid soils was due to a buffering effect of the plant roots (6).

In studies of root exudation (previous chapter) I observed that when the pH of the root medium was lowered, it rose again fairly quickly. Also, if the pH of the root medium at the beginning of an experiment was, for example, 6.6 then the pH normally increased to about 6.9. In such cases, significant levels of ^{14}C were found in the root medium. When the increase in pH did not occur, less ^{14}C was found in the root medium. In one experiment, the pH dropped to 5.4 over four hours from an initial pH of 6.6. When the pH was lowered further to 4.0 by the addition of acid, the pH remained at 4.0 for the remainder of the experiment (another four hours). Part of the reason for the low ^{14}C levels at the lower pH values is due to increased loss of the $^{14}CO_2$ from the root medium, but there seemed to be a correlation between root exudation and the capacity to buffer the root medium.

In view of these observations and the findings of Dobereiner and other workers on the response of *Azotobacter spp.* to pH, experiments were carried out to determine more precisely the buffering capacity of *P. notatum* roots. In addition, chemostat cultures of *A. paspali* were set up to determine the effect of low pH and other nutritional conditions on the $N_2[C_2H_2]$-fixing activity.

Materials and Methods

Buffering capacity of roots

Plants of *P. notatum* were set up in the experimental apparatus described previously (Chapter 1). The root medium at 30°C was bubbled with compressed air at the rate of 1.2 $l.min^{-1}$. Photosynthesis was allowed to come to steady state.

After 30 minutes to an hour of steady state photosynthesis, the pH of the root medium was lowered to 5.0 by the addition of 0.05M HCl. The subsequent increase in pH was noted for about an hour. The pH was then brought to 6.9, and after 30 minutes, the pH was lowered to either 4.0 or 5.0, and the increase in pH noted again. Control experiments were done in which the apparatus was set up without a plant and the rise in pH noted after lowering the pH of the medium in the cirulatory system.

In further experiments, the 'alkali' causing the increase in pH was titrated with dilute HCl. To do this, the pH was lowered to 5.0 from 6.8 and maintained between 5.0 and 5.1 for 30 minutes by the addition of 0.05M HCl. The pH was then brought back to 6.8 for 30 minutes, by the addition of NaOH, before repeating the procedure. After this had been done three times, the pH was lowered to 4.2 and kept between 4.2 and 4.3. The results of these experiments indicated that

the buffering capacity of the roots decreased with continued stress, so in some experiments the pH of the medium was lowered to 4.2 from the beginning. The pH of the root medium was returned to 6.8 for 30 minutes between successive trials as before.

pH and $N_2[C_2H_2]$-fixing activity of *A. paspali*

Azotobacter paspali, strain Ax 4, was kindly provided by Dr. Dobereiner. It was maintained on a yeast extract and sucrose medium. For experiments, it was grown on the nitrogen-free medium described in Table 1.

Table 1
Nitrogen-free medium used to grow *A. paspali*

Components	Concentration (g/l)
K_2HPO_4	0.05
KH_2PO_4	0.15
$MgSO_4.7H_2O$	0.20
FeEDTA	0.027
$NaMoO_4.2H_2O$	0.002
Sucrose	20 or 2

For solid media, bromothymol blue (5 ml/l of a 1% alcohol solution) and 15 g/l agar were added. The pH of solid and liquid media was adjusted to 6.9 ± 0.1.

Figure 1 is a schematic diagram of the simple chemostat used (a more satisfactory and elaborate chemostat is described in a paper by Baker (1)). The culture vessel (C) was a round-bottomed 500 ml Pyrex flask with ports for an oxygen electrode (H,O) and a weir-type overflow (D). The culture volume was 200 ml. A Quickfit MF28/3/500 fitting, modified to allow the entry of the medium (A), gas (L) and thermocouple probe (J), was attached to the top of the flask. The culture medium was run into the vessel through an intravenous injection set (B), the rate of flow being controlled by the height of the medium reservoir and a screw clamp on the injection set. Details of the oxygen electrode port are shown at O in Figure 1. A piece of millipore membrane filter (b), pore size 0.22 μ, was glued to the end of a Beckman oxygen electrode cap (c) which was then glued into the electrode port (a) of the culture vessel. A Beckman oxygen electrode (d) was screwed into the cap after sterilization of the vessel. It was necessary to determine the response of the electrode to oxygen in solution with and without the filter on the cap. The chemostat was housed in a temperature controlled cupboard.

The culture vessel containing 190 ml of medium and a stirring bar, and with the gas line, effluent line (D) and thermocouple probe in place, was sterilized by autoclaving. The medium and effluent (F) vessels were autoclaved separately.

Figure 1. Schematic diagram of chemostat.

Key to Figure 1

A - medium reservoir

B - Saftiset, Volutrole intravenous injection set. Cutter Labs, Inc., Berkeley, Calif., U.S.A.

C - culture vessel

D - effluent line

E - sample port

F - effluent receiver

G - magnetic stirrer

H - Beckman oxygen electrode

I - Beckman oxygen amplifier

J - temperature probe

K - YSI telethermometer

L - gas supply

M - flow meter

N - Whatman Gamma-12 inline filter unit with 12-03(3μ) filter tube.

O - detail of oxygen electrode port:

a - wall of culture vessel

b - millipore filter glued to electrode cap

c - electrode cap glued to vessel

d - oxygen electrode

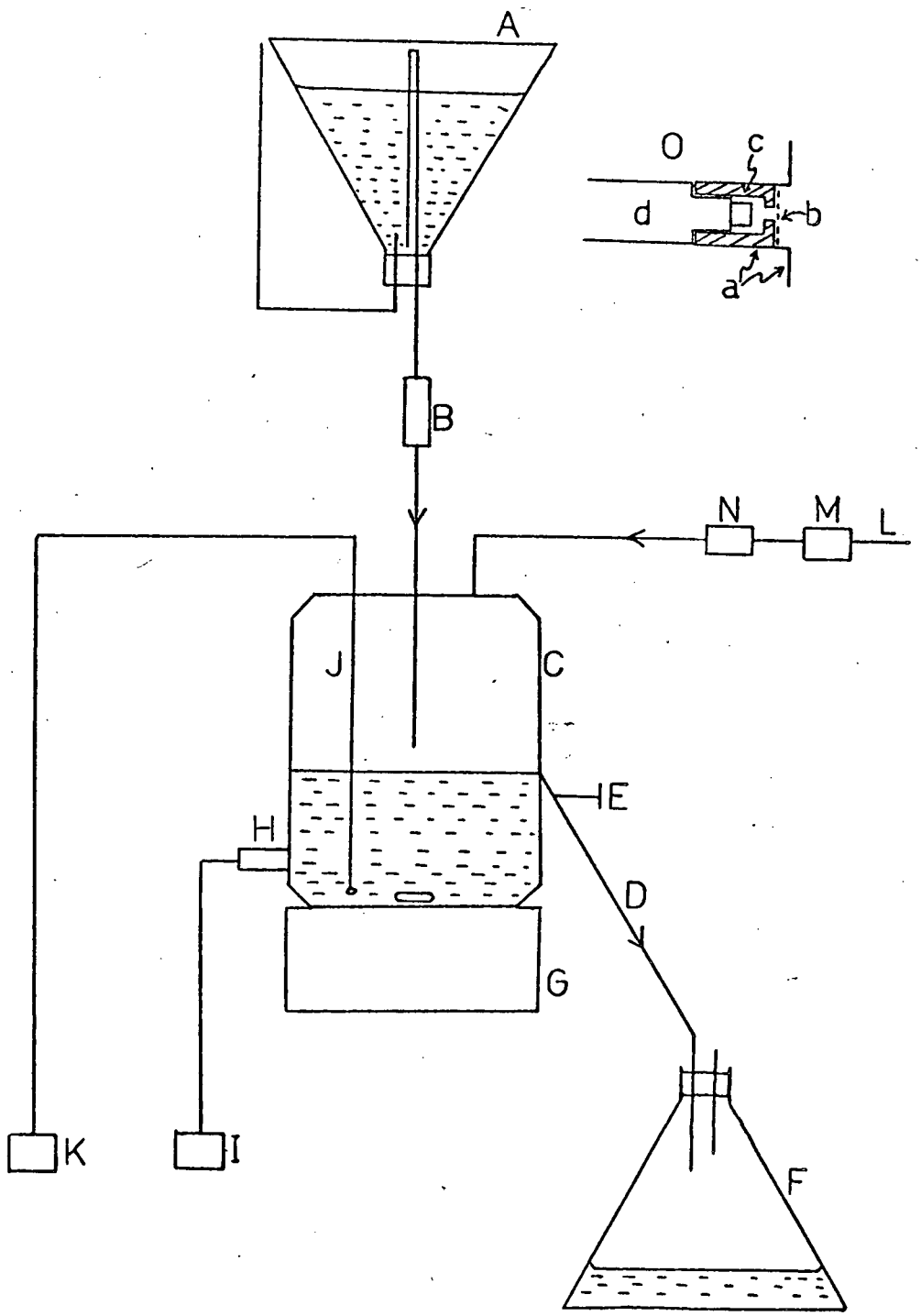

Figure 1

The sterile medium reservoir, intravenous injection set and effluent vessel were attached under sterile conditions.

To start a culture in the chemostat, 10 ml of bacterial suspension, $O.D._{600}$ = 0.12, was made by mixing liquid medium with bacteria grown on solid medium for four days. The suspension was injected into the culture vessel, the magnetic stirrer started, and the bacteria grown as a batch culture for approximately 24 hours (preliminary experiments showed that 24 hours corresponded to about the middle of the exponential phase of growth under the conditions used in these experiments). Medium was then allowed to drip in from the reservoir at the rate of 40 $ml.hr.^{-1}$ ($D = 0.2\ hr.^{-1}$), and the gas flow was started at a flow rate of 1.2 $l.min^{-1}$. The temperature in the culture, throughout the batch and continuous phases, was kept at 35 ± 1°C. Dry weight of bacteria was determined by correlation with measurements of the optical density at 600 nm of cultures grown at 0.04 atm O_2, 20 g/l sucrose. The oxygen solution rate was determined by the m method of Cooper *et al.* (2).

One of the primary reasons for culturing organisms in a chemostat is to maintain populations in a steady state. When they are in this state, the nutritional status, for example, can be studied without complications caused by the population going through the growth curve typical of batch cultures. With the chemostat used in these experiments it was difficult to keep the cultures in perfect steady state

as judged by changes in optical density. However every effort was made to keep the optical density constant for some hours before doing experiments with samples of the cultures.

The effect of lowering the pH on the $N_2[C_2H_2]$-fixing activity of cultures grown under the conditions shown in Table 2 was measured. Sucrose at 20 g/l was used as this was the concentration used by Dobereiner; one-tenth of this amount was taken as constituting a low carbohydrate concentration. Dobereiner *et al.* (7) found that a pO_2 of 0.04 atm was optimal for $N_2[C_2H_2]$-fixing activity. This was taken as a low oxygen concentration.

Table 2
Chemostat culture conditions for *A. paspali*

N-free Dobereiner's Medium (Table 1) T = 35 ± 1°C, D = 0.2 $hr.^{-}$, initial pH = 6.9 ± 0.1, culture volume - 200 ml
0.04 atm O_2, 0.96 atm N_2, 20 g/l sucrose 2 g/l sucrose O_2 solution rate = 5.9 mmole $l^{-1}hr.^{-1}$
0.20 atm 0 , 0.80 atmN , 20 g/l sucrose 2 g/l sucrose O_2 solution rate = 24.4 mmole $l^{-1}hr.^{-1}$

Erlenmeyer flasks, 58 ml in volume were fitted with rubber caps, flushed with argon and used to hold 15 ml samples of culture. In some of the samples, the pH was lowered to 5.2 by the addition of 0.05M HCl. Acetylene and oxygen were then injected to give a final pC_2H_2 of 0.1 atm, and a pO_2 of 0.04 or 0.20 atm. The flasks were incubated at 35°C for one hour in a water bath with a shaking amplitude of 3.5 cm and a frequency of 0.7 Hz. Every 15 minutes, a 1 ml sample of the gas phase was removed and 1 ml of the original gas mixture was injected.

The ethylene content of the gas samples was determined using a Wilkins Aerograph gas chromatograph fitted with a flame ionization detector and a 182.5 cm x 0.63 cm od Porapak R column. The column was operated at 45°C with helium as carrier gas flowing at 30 $ml.min^{-1}$.

Results

Buffering capacity of plant roots

Figure 2 shows the increases in pH, for two plants, after lowering the pH of the root medium. Table 3 shows the quantity of acid required to lower, and maintain the pH at either 5.0 or 4.2.

Figure 2. The increase in pH of the root medium after lowering the pH of the medium from 6.9 to the value given in the figure. Plant A had a root dry wt. of 0.2g. Plant B had a root dry wt. of 0.8g. For each plant the data for the lower curve was obtained after that for the upper.

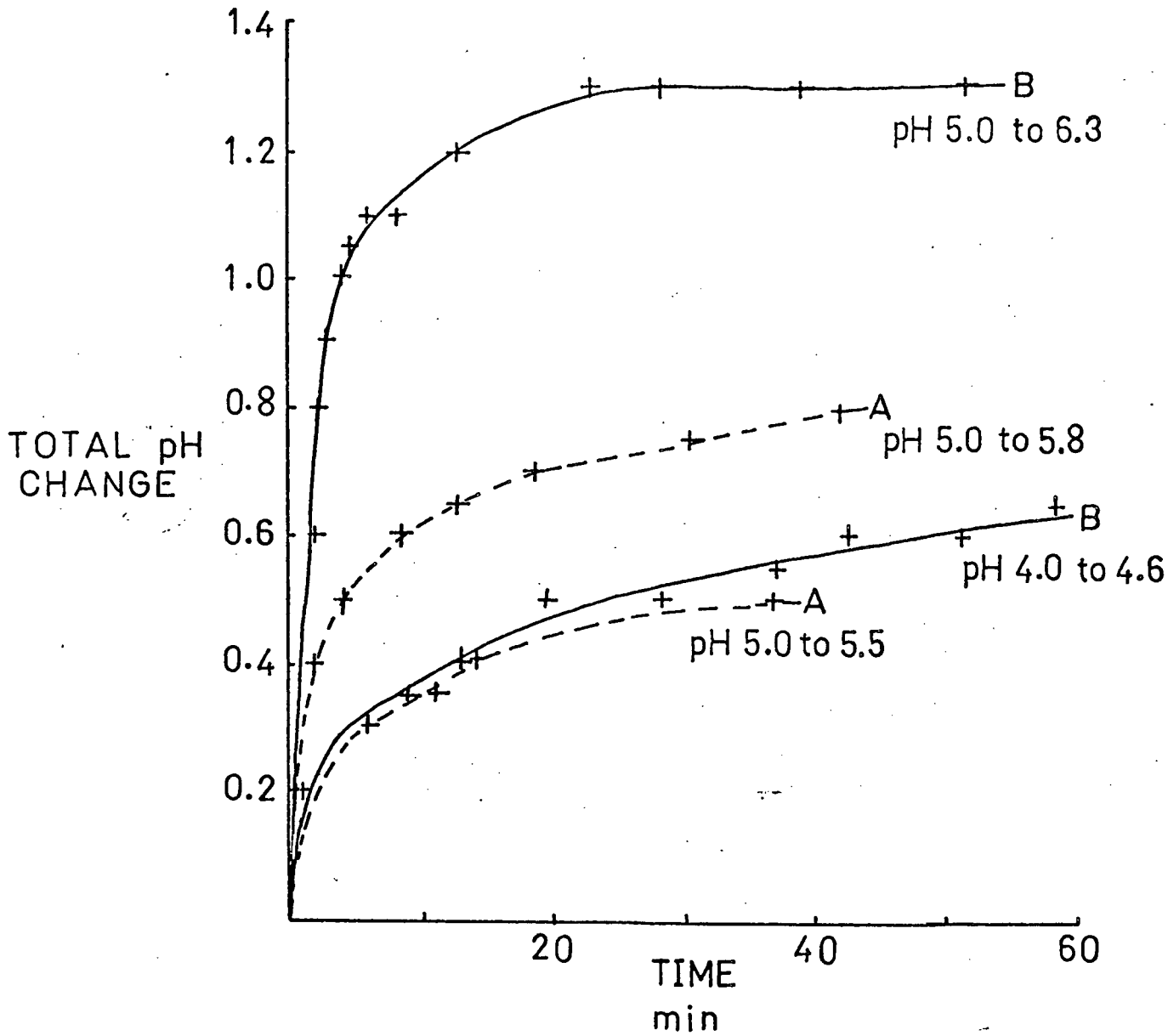
1.4
1.2
1.0
0.8
0.6
0.4
0.2
TOTAL pH CHANGE
B
pH 5.0 to 6.3
A
pH 5.0 to 5.8
B
pH 4.0 to 4.6
A
pH 5.0 to 5.5
20
40
60
TIME
min

Figure 2

Table 3

Quantity of acid, μmoles H^+.(g.root dry wt.)$^{-1}$, required to lower pH and maintain the root medium at low pH for 30 minutes. The data in each column were obtained successively. Initial pH was 6.8

	Acid required to lower pH	Acid required to maintain pH
Plant		
C to pH 5.0	225	108
	211	131
	92	97
to pH 4.2	478	369
	492	306
	769	28
D to pH 5.0	316	163
	183	23
	50	137
to pH 4.2	1588	960
	1388	67
E to pH 4.2	1171	471
	1092	667
	1327	157
F to pH 4.2	534	686
	964	672
	-	134
Control[1]		
to pH 5.0	35[2]	0
to pH 4.2	223[3]	70

[1] $CaSO_4$ solution without roots.

[2] 27-70% of the absolute amount of acid required to lower pH with the plant present.

[3] 25-57% of the absolute amount of acid required to lower pH with the plant present.

The ability to resist the lowering of pH seems to decrease with continued stress in the form of low pH. In Figure 2 the increases in pH after lowering the pH for the second time are smaller than after the first decrease in pH; for plant B the smaller increase may also result from the pH being lowered to 4.0 rather than 5.0. The data in Table 3 show a similar effect. With successive trials the amount of acid required to lower the pH to 5.0 decreased, as did the amount needed to keep the pH low. Similarly, the amount of acid needed to keep the roots at 4.2 decreased with repeated stress. After three trials in which the pH had been lowered to 5.0, the amount of acid needed to lower the pH to 4.2 was less than that required to lower the pH to 4.2 in plants which had not been stressed.

Most of the acid needed to lower the pH to 4.2 was needed in the region 4.5 to 4.2. A similar effect was seen in control experiments in which the amount of acid required to lower the pH of $CaSO_4$ in the absence of a plant was measured, so it seems the $CaSO_4$ solution used as root medium has buffering properties of its own in this region.

For plants C and D, the absolute amount of acid required to maintain the pH at 5.0 and 4.2 has been plotted against the time at which the acid was added after lowering the pH (Figure 3). Most of the acid had to be added within

Figure 3. The absolute amount of acid required to maintain the pH of the root medium at 5.0 and 4.2 for plants C and D (see text and Table 3). Solid lines represent amount of acid required to keep pH at 5.0; dotted lines the amount required to keep pH at 4.2.

137A

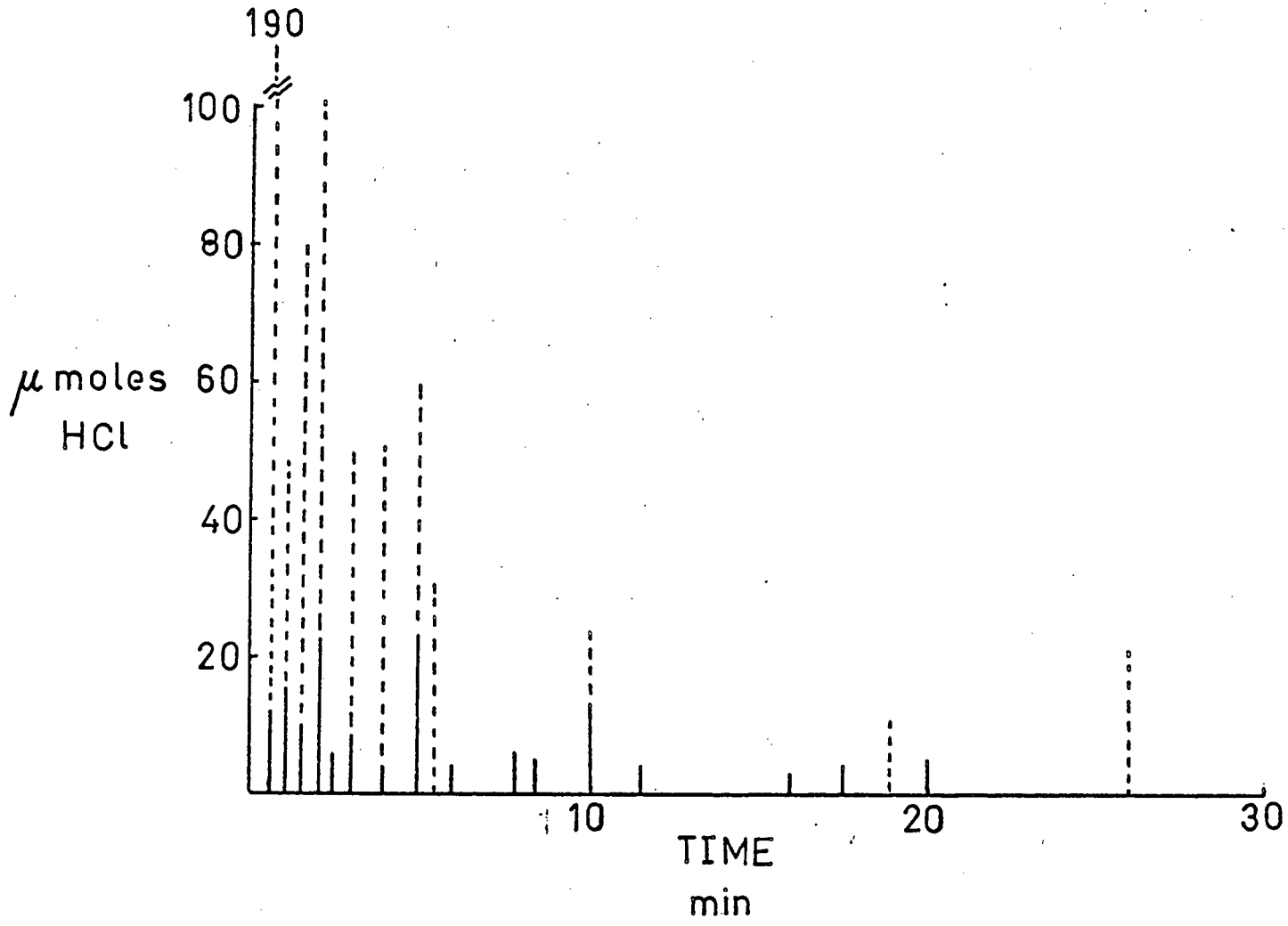

Figure 3

10 minutes of lowering the pH; the same trend was observed for other plants.

pH and $N_2[C_2H_2]$-fixing activity of *A. paspali*

Table 4 gives the pH and O.D. of the various cultures. The bacteria produced acid in culture and continued to do so while being tested for $N_2[C_2H_2]$-fixing activity. All the cultures appeared to contain dissolved oxygen at a concentration of about 10μM, except for the ones at 0.20 atm O_2, 20 g/l sucrose where the concentration was about 30μM.

Table 5 shows the $N_2[C_2H_2]$-fixing activity of samples at the control pH and low pH. A low pH inhibited $N_2[C_2H_2]$-fixing activity in all cultures except those grown at 0.04 atm O_2, 2 g/l sucrose.

Table 4
pH and O.D. of Chemostat Cultures of *A. paspali* Approximating to Steady State Conditions

	0.04 atm O_2, 2 g/l sucrose	0.04 atm O_2, 20 g/l sucrose	0.20 atm O_2, 2 g/l sucrose	0.20 atm O_2, 20 g/l sucrose
pH	5.80 ± 0.12[1]	6.24 ± 0.13	5.60 ± 0.17	5.22 ± 0.13
O.D.	0.24 ± 0.02	0.24 ± 0.04	0.34 ± 0.10	0.53 ± 0.05
mg dry wt.ml^{-1}	1.2	1.2	1.7	2.7

[1]Standard deviation.

Table 5

The effect of low pH on C_2H_2 reduction by *A. paspali*

The amounts of C_2H_2 reduced are nmoles C_2H_2.(mg dry wt)$^{-1}$. Rates are nmoles C_2H_2.(mg dry wt)$^{-1}$ min^{-1}. Means with the superscript 'a' are significantly different ($P < 0.05$) from the corresponding control means. Means without a superscript are not significantly different.

	Culture conditions			
	0.20 atm O_2, 20 g/l sucrose			
	Control[1]		pH 5.2	
	30 min	60 min	30 min	60 min
Amount	211 ± 90	461 ± 295	166 ± 87[a]	331 ± 205[a]
Rate	7.0	7.7	5.5	5.5
	0.20 atm O_2, 2 g/l sucrose			
	Control, pH 5.6		pH 5.2	
	30 min	60 min	30 min	60 min
Amount	181 ± 58	407 ± 209	104 ± 35[a]	252 ± 129[a]
Rate	6.0	6.8	3.5	4.2
	0.04 atm O_2, 20 g/l sucrose			
	Control, pH 6.2		pH 5.2	
	30 min	60 min	30 min	60 min
Amount	295 ± 119	641 ± 94	229 ± 41[a]	346 ± 76[a]
Rate	9.8	10.6	7.6	5.8
	0.04 atm O_2, 2 g/l sucrose			
	Control, pH 5.8		pH 5.2	
	30 min	60 min	30 min	60 min
Amount	362 ± 354	578 ± 522	255 ± 127	671 ± 409
Rate	12.1	9.6	8.5	11.2

CONTINUED

Table 5 (Continued)

[1]As the pH of the culture was already 5.2, the pH of the control samples was brought to 6.9 by the addition of NaOH.

A t-test was used to test the differences between means, the formula for paired comparisons being used where there was large variation between successive experiments under the same conditions.

Discussion

Buffering capacity of plant roots

It is clear from the results that *P. notatum* has the ability to neutralize increases in acidity in the root environment, at least in the system used here.

The decrease in buffering ability under repeated stress suggests that 30 minutes was not long enough for the buffering mechanism to recover. The pH changes induced in these experiments were relatively large and abrupt. In the field, the plant may be concerned with neutralizing lower rates of addition of acid over longer periods of time. However in the acid soils where the association is found, large changes in the pH may occur from time to time; after a rain shower, for example.

There are a number of mechanisms by which the root might resist increases in acidity. Protons may exchange with cations, e.g. Ca^{+2}, K^{+} bound to carboxyl groups of the cell wall. Figure 3 which shows the most of the acid necessary to keep the pH low had to be added within a short time, provides supportive evidence for an exchange site mechanism. The protons may be neutralized by OH' ions transported actively out of the cell in exchange for counter-ions, e.g. PO_4^{-3}, SO_4^{-2}. Alternatively, the protons may be taken into

the cell in exchange for ions giving rise to an alkaline pH in aqueous solution. The use of radio-isotopes under appropriate conditions would be a suitable approach to distinguish between passive and active processes. It has the advantage of allowing the study of both organic (e.g. malate) and inorganic ions.

pH and $N_2[C_2H_2]$-fixing activity

A low pH inhibited the $N_2[C_2H_2]$-fixing activity of samples taken from cultures grown at three of the four sets of conditions used. The lack of inhibition in samples from cultures grown at 0.04 atm O_2, 2 g/l sucrose was unexpected; further experimentation may have shown an inhibition. It seems that nitrogen fixation in continuous culture is inhibited by low pH, as it is when *A. paspali* is grown on solid medium or in liquid batch culture (4). This conclusion could have been stated with more certainty had significant inhibition been found under all conditions. Bearing in mind this proviso, the inhibition of $N_2[C_2H_2]$-fixing activity at low pH emphasizes the importance of the buffering capacity of the plant roots.

Dobereiner noted that good growth of *A. paspali* occurred only above pH 5.5 (4). In my experiments (see Table 4) the pH of cultures was as low as 5.2 and $N_2[C_2H_2]$-fixing activity took place at this pH (see Table 5). Dobereiner

used batch cultures; in chemostat culture the continuous supply of nutrients may have mitigated the effect of low pH.

Cultures grown at low carbon concentrations, particularly 0.20 atm O_2, g/l sucrose, might be hypersensitive to oxygen and thus unstable. However the cultures were stable and not so sensitive to oxygen as to be washed out from the culture vessel. If the amount of carbon available to these cultures had been increased, then their biomass may have been larger but under the conditions used in my experiments the supply of carbon was sufficient to support nitrogen fixation at atmospheric oxygen concentrations at both optimal and low pH. These observations indicated that *A. paspali* may be relatively resistant to oxygen inhibition of nitrogen fixation, particularly with respect to other nitrogen fixers such as *Beijerinckia spp* and *Derxia sp* found in the rhizosphere of P. notatum. It is shown in the next chapter that *A. paspali* is less sensitive to oxygen than *Derxia gummosa*.

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Chapter 3

INFLUENCE OF OXYGEN CONCENTRATION ON COLONY MORPHOLOGY OF AZOTOBACTER PASPALI

Introduction

When grown in air on N-free agar medium *A. paspali* forms copious quantities of slime. Growth is very rapid, and if the inoculum is large, raised mucilaginous streaks cover nearly all of the petri dish surface within 24 to 48 hours. Slime formation is characteristic of the Azotobacteraceae and it has been suggested that the slime plays a role in keeping the oxygen concentration below levels inhibitory to nitrogen fixation (5).

In some of the Brazilian soils from which *A. paspali* was isolated, another member of the Azotobacteraceae was found, *Derxia gummosa* (1). *D. gummosa* is sensitive to the oxygen concentration in liquid culture (3,4) and its colony morphology is markedly influenced by the oxygen concentration (3).

In air on N-free agar medium, *D. gummosa* formed small, "thin" colonies after two days. These colonies did not reduce C_2H_2. After four to nine days, scattered "massive" colonies appeared either from individual "thin" colonies or from confluent growth of "thin" colonies. "Massive" colonies were mucilaginous and reduced C_2H_2. The formation of "massive" colonies was stimulated by lowering the concentration of oxygen in the gas phase. At 0.05 atm O_2 all the colonies were of the "massive" type. When plates incubated at 0.20 atm O_2 were transferred to 0.05 atm O_2, the "thin" colonies developed into the "massive" type.

The low growth and lack of $N_2[C_2H_2]$-fixing activity of the "thin" colonies was considered to be due to oxygen inhibition; the "thin" colonies were thought to develop only to the limit of nitrogenous impurities in the medium. Formation of the few "massive" colonies in air was thought to be due to the initiation of nitrogen fixation either after a period of growth on nitrogenous impurities, or, in cases where the "thin" colonies were densely packed, after competition between the organisms for oxygen had lowered the local oxygen concentration sufficiently to allow some of them to initiate nitrogenase activity. It was suggested that the viscous slime of the massive colonies protected the cells from oxygen. When colonies grown at 0.05 atm O_2 were transferred to 0.20 atm O_2, they did not show inhibition of $N_2[C_2H_2]$-fixing activity.

It was clear from my own observations that, in air, at least at high carbohydrate concentrations (20 g/l sucrose), *A. paspali* did not form "thin" and "massive" colonies like those of *D. gummosa*. Although, as discussed in the previous chapter, 2 g/l sucrose may have been sufficient to prevent oxygen inhibition in chemostat culture under the conditions used, the apparent lack of inhibition of $N_2[C_2H_2]$-fixing activity at 0.20 atm also suggested that the oxygen response of *A. paspali* may be different from *D. gummosa*. *A. paspali* was incubated at high and low oxygen concentration, using techniques similar to those described by Hill (3), in order to gain more information about its oxygen response and to compare its colony morphology to that of *Derxia gummosa*.

Methods and Materials

A. paspali, strain AAx 4, was grown on Dobereiner's medium (2) containing sucrose at 20 g/l or 2 g/l, and on modified Burk's medium (3) containing sucrose or glucose at 1 g/l. Bromothymol blue was included in both Burk's and Dobereiner's medium. Dobereiner's medium will be referred to as D medium and the modified Burk's medium as B_5 medium (3).

Bacteria were grown in air at 35°C for four days on D medium containing 20 g/l sucrose. A dilute suspension was then made of these bacteria in liquid D medium. An inoculating loop was dipped into the suspension and used to streak agar plates containing the medium given above. The plates were incubated at 35°C in polyethylene bags which were flushed continuously with nitrogen containing 0.04 atm or 0.20 atm oxygen.

Results

On B_5 medium the bacteria grew quickly. At three days the colony morphology was identical on all plates whether incubated at high or low oxygen or whether sucrose or glucose was the carbon source. Figure 1 shows photographs of typical plates incubated under various conditions. Most of the colonies were convex and mucilaginous, the cells within the mucilage being grouped into a reticulum. (The reticulum was easy to see because of the yellow colour produced by the bromothymol blue on reaction with the acid produced by the bacteria). Some smaller colonies were present which were hemispherical and mucilaginous but did not contain any reticulum, being entirely translucent.

Similar results were obtained using D medium containing 2 g/l sucrose. Under both oxygen concentrations, the

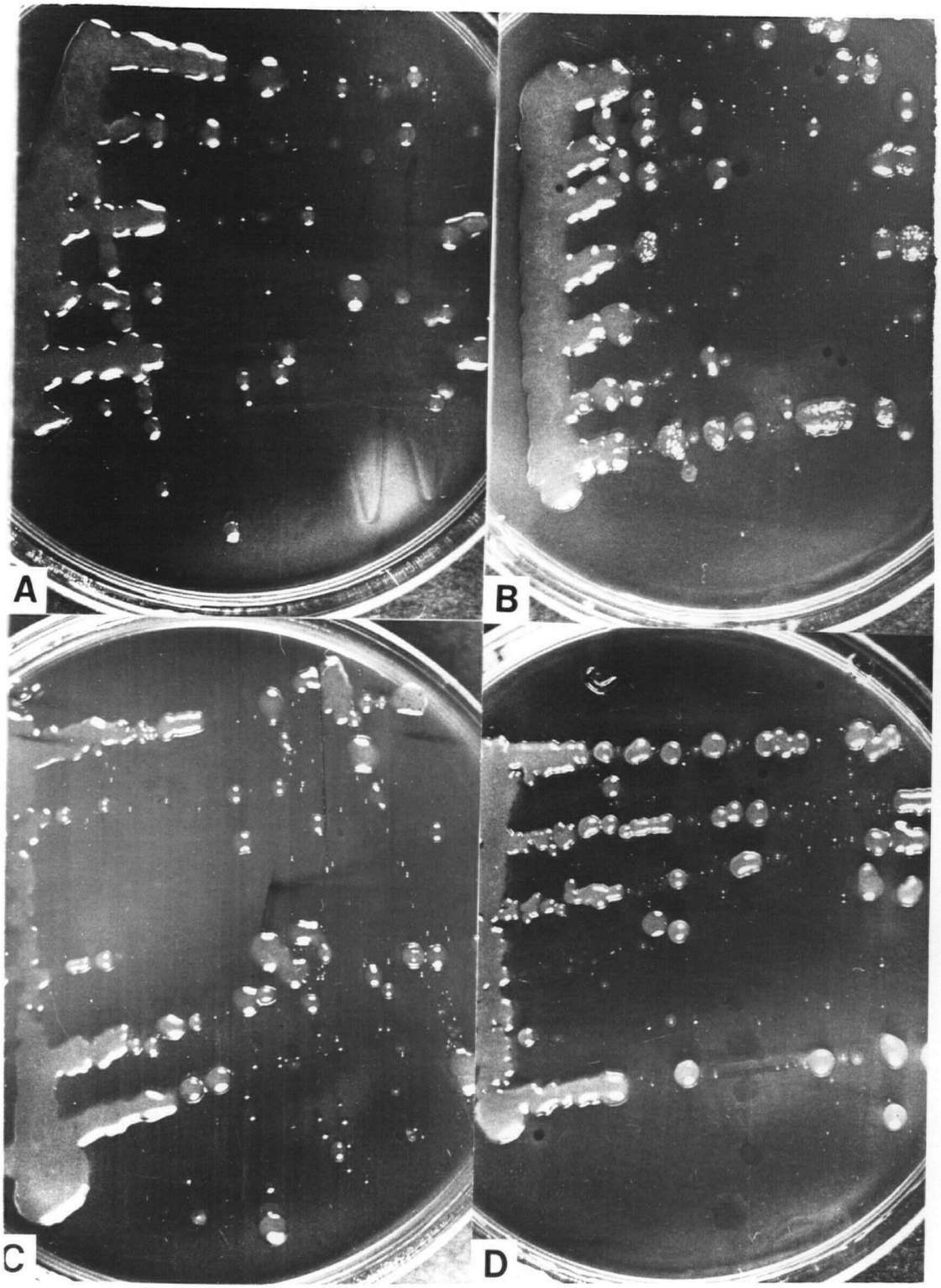
Figure 1. *A. paspali* grown on solidified B_5 medium with sucrose or glucose at 1 g/l, and at 0.04 atm or 0.20 atm O_2.

A. 0.04 atm O_2, sucrose

B. 0.20 atm O_2, sucrose

CC. 00.04 atm O_2, glucose

D. 0.20 atm O_2, glucose


A
B
C
D

growth at three days on all plates was identical, the cells being grouped into reticula within the convex mucilaginous colonies. At four days the small translucent colonies were apparent. When these were streaked onto plates of D medium containing 20 g/l sucrose and incubated in air, large mucilaginous streaks formed by seven days. The cells were not grouped into reticula.

Colonies with reticula were also formed on medium containing 20 g/l sucrose. Some interesting observations were made in one instance when the bacterium was incubated at 0.20 atm O_2. At six days large translucent colonies were present, arising from underneath and breaking through the older colonies or growing on top of them. Gram staining of the two types of colony showed that the reticula were made up of densely packed gram negative rods with many macrocysts. The translucent colonies consisted of long, thin rods sparsely distributed among the mucilage. Formation of translucent colonies of this nature were not observed in other experiments. In older colonies the reticula generally became more concentrated towards the centre of the mucilage, thus resulting in a more extensive mucilaginous shell.

Discussion

From these results it appears that *A. paspali* is not as sensitive to oxygen inhibition as *D. gummosa*. One possible explanation for this is that it is able to make efficient use of the carbon available, 1 g/l of sucrose or glucose being sufficient to provide adequate respiratory protection. All the colonies were probably N_2-limited. In comparison, *D. gummosa* showed oxygen inhibition at a mannitol concentration of 10 g/l (3). Postgate (6) has made the comment, "It seems likely that *Derxia* has relatively inefficient respiratory or conformational protection mechanism compared with *Azotobacter*."

The growth rate of *A. paspali* is higher than that of *D. gummosa*. *A. paspali* always formed large colonies within three days whereas *D. gummosa* took up to 13 days to produce what appears (from Hill (3), Figure 1) to be similar amounts of growth.

The nature and occurrences of the translucent colonies of *A. paspali* are difficult to explain. They are possibly slow growing mutants. As they were formed under both high and low oxygen, their presence does not seem to be related directly to the oxygen concentration.

More work needs to be done on the oxygen response of *A. paspali*. In particular, cultures should be grown for

longer periods of time, and the $N_2[C_2H_2]$-fixing activity of each colony type determined. The experiments reported here establish the high growth rate, and lack of response of the cultures as a whole to oxygen concentration, in contrast to the response of *D. gummosa*.

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EPILOGUE

Why is the distribution of *A. paspali* in the field restricted to an exclusive association with the rhizophere of *P. notatum*? *A. paspali* can grow, divide and fix nitrogen in a simple medium, so obvious hypotheses such as a requirement for an exotic nutrient are apparently not applicable, although at low carbohydrate concentrations malic acid produced by the plant may be important as a stimulator of carbohydrate uptake. The work reported here shows that the roots of *P. notatum* provide a favourable pH for nitrogen fixation in the rhizosphere. Growth and nitrogen fixation by *A. paspali* are inhibited by low pH. The capacity of *P. notatum* roots to neutralize increases in acidity in the root environment would prevent decreases in pH due to acid production by the bacterium. It also explains the distribution of the association in acid soils.

The bacterium probably utilizes material recently formed by photosynthesis as a carbon source (3) and the demonstration of rapid translocation and exudation of photosynthate (Chapter 1) is circumstantial evidence for this.

Unfortunately I did not succeed in estimating accurately the amount of non-volatile material exuded. An exact description of the gaseous phase of the rhizosphere does not exist but the mucigel probably maintains oxygen concentrations suitable for nitrogen fixation in the rhizosphere.

Some other members of the Azotobacteraceae, *Beijerinckia indica, B. derxii, B. fluminensis* and *Derxia gummosa* are found in the same soils as the *P. notatum-A. paspali* association (1). In some instances *Beijerinckia spp* and *Derxia sp* have been found in the rhizosphere of *P. notatum* although *A. paspali* apparently always predominates, the numbers of *A. paspali* being very large (greater than 10,000 micro-colonies per gram of soil adhering to the roots (2)). *Beijerinckia spp* and *Derxia sp* have a greater tolerance to low pH than *A. paspali* (it is interesting that *B. derxii*, which appears to be the species found in the rhizosphere of *P. notatum* (13) is the most sensitive to low pH of the three species of *Beijerinckia* found in Brazilian soils). I think the reasons why they do not form an equally large proportion of the bacterial population of *P. notatum* are as follows.

Provided the pH is optional, *A. paspali* has a higher growth rate than *Derxia sp* (see Chapter 3) or *Berijerinckia* (9), as it is able to make more efficient use of available carbohydrate. This will be particularly important at low carbohydrate concentrations. *A. paspali* also has a higher

optimal temperature for growth than *Beijerinckia*, though not *Derxia* (1). Consequently it is able to compete successfully against other bacteria in the rhizosphere of *P. notatum*. In a discussion of the rhizosphere effect Macura (8) noted that bacteria adapt to a set of conditions in such a way that their growth is as fast as possible. In an ecosystem like the rhizosphere it is the most rapidly growing bacteria that are selected.

The difficulty of establishing *A. paspali* in the rhizosphere and the length of time required to do so, is a puzzling problem. If *A. paspali* is a good competitor against other organisms by virtue of its efficient use of carbohydrate, it seems unlikely it would take a long time to become established. Perhaps in low numbers, it faces heavy competition from heterotrophic bacteria, and cannot compete successfully for its niche in the rhizosphere until, eventually, its numbers increase.

The exclusive association of *A. paspali* with *P. notatum*, the location of the bacteria in the mucigel, the relationship between photosynthesis and $N_2[C_2H_2]$-fixing activity, the demonstration of root exudation, the buffering of the rhizosphere and the pH requirements of $N_2[C_2H_2]$-fixing activity all suggest at least a partially symbiotic association. I do not think it can be regarded as wholly so, as

the bacterium can live and fix nitrogen in the absence of the plant. Further, as yet, no uptake of bacterially fixed nitrogen by the plant has been demonstrated.

Parker (12) has proposed that the stages in the evolution of nodule symbioses are as follows: casual associations of N_2-fixing bacteria and plants → loose associations on plant surfaces → symbiosis within the plant in cortical tissues → symbiosis in organized, highly adapted tissues. Light micrographs (3) show *A. paspali* embedded in mucigel on the root surface. There is no apparent penetration of the bacteria into the cortex. The association can hardly be described as "loose," so it seems to represent a stage about midway between a loose association and the penetration of the bacterial partner into the cortical tissues.

Beijerinckia and the plants in the rhizospheres of which it is sometimes found, represents a stage equivalent to the loose associations. Its distribution does not appear to be related to a particular plant but to those rhizospheres where there is an abundant supply of carbohydrate. For example, large populations of *Beijerinckia* have been observed in the rhizosphere of sugar cane which has a sucrose-rich exudate (1).

Many of the tropical plants which have been noted as having relatively high rates of $N_2[C_2H_2]$-fixing activity in their rhizosphere have the C-4 pathway of photosynthesis (5).

Plants with this pathway have a high photosynthetic productivity (4), especially in the conditions prevailing in the savannas (high solar radiation, high temperatures). Consequently nitrogen fixing bacteria in the rhizospheres may have access to relatively large amounts of carbohydrate. In temperate zones, $N_2[C_2H_2]$-fixing activity has also been found in the rhizosphere of Calvin cycle plants (6) but the activity is not as high.

The *P. notatum-A. paspali* association highlights the importance of the rhizosphere to so-called asymbiotic nitrogen fixation, particularly in tropical ecosystems. It is possible that, in unamended soils, high rates of nitrogen fixation only occur within the rhizosphere. Field surveys to estimate nitrogen fixation should be done with cognizance of this possibility. Where high rates are obtained efforts should be made to identify the plant concerned so that tests of the $N_2[C_2H_2]$-fixing activity of the rhizosphere can be made, particularly at sub-atmospheric oxygen levels.

There is a desperate need to increase the productivity of these ecosystems underpinning mans' welfare. Since one of the factors limiting productivity is the availability of fixed nitrogen any association of organisms that is capable of fixing atmospheric nitrogen is of considerable interest. In itself, the *P. notatum-A. paspali* association is not very promising as a system which could be incorporated easily

into agriculture, particularly intensive agriculture. *P. notatum* var "batatais" is a poor pasture grass. It is not a cereal, so advantage cannot be taken of grain with a high nitrogen content. The association is slow to establish and the exclusivity of the association mitigates against the inoculation of the rhizosphere of other plants with *A. paspali*. Further research should be concerned with modifying the association tomake it more suitable for agricultural use. Alternatively, it represents a potentially valuable source of components which could be incorporated into other systems to enhance their nitrogen-fixing capacity. I will outline some possible, and highly speculative, strategies for carrying out these broad alternatives.

Substitution of a single chromosome pair in cultivars of spring wheat markedly changes the nature of the rhizosphere population (10,11). Perhaps there is a chromosome pair (for buffering of the rhizosphere?) in *P. notatum* var "batatais" which contributes to a rhizosphere favourable for growth of *A. paspali*. If this pair could be incorporated into the genome of those varieties of *P. notatum* which are good pasture grasses, e.g. the diploid Pensacola Bahia types, then *A. paspali* might grow in their rhizospheres.

The problem still remains of the slowness of establishment of *A. paspali*. Bacteria of the *A. chroococcum-A. vinelandii* growth readily in many rhizospheres (9). Jackson &

Brown (7) induced *A. chroococcum* to multiply in the rhizospheres of wheat and peas. It is possible to transfer genes from one bacterium to another by conjugation, transformation or transduction (14). If the gene(s) responsible for good growth of *A. chroococcum-A. vinelandii* in rhizospheres could be located, perhaps they could be transferred to *A. paspali*, so resulting in its rapid establishment in the rhizosphere of "Penscola Bahia-grass." Figure 1 is a summary of this strategy.

Another approach might be to work with a cereal such as wheat. Varieties could be chosen, or developed, which have high rates of photosynthesis and exudation and the ability to buffer the rhizosphere. The rhizosphere could then be inoculated with *A. paspali* having the "broad host range" factor (see Figure 1). Alternatively bacteria of the *A. chroococcum-A. vinelandii* group containing a "symbiont" gene derived from *A. paspali* could be used as an inoculum. I have speculated on the existence of a "symbiont" gene since *A. paspali* is capable of forming a close association with *P. notatum* whereas members of *A. chroococcum-A. vinelandii* group do not appear to form associations of a symbiotic nature. Figure 2 summarizes these ideas.

Figure 1. Hypothetical scheme for development of *P. notatum* var "Pensacola Bahia" having *A. paspali* in its rhizosphere.

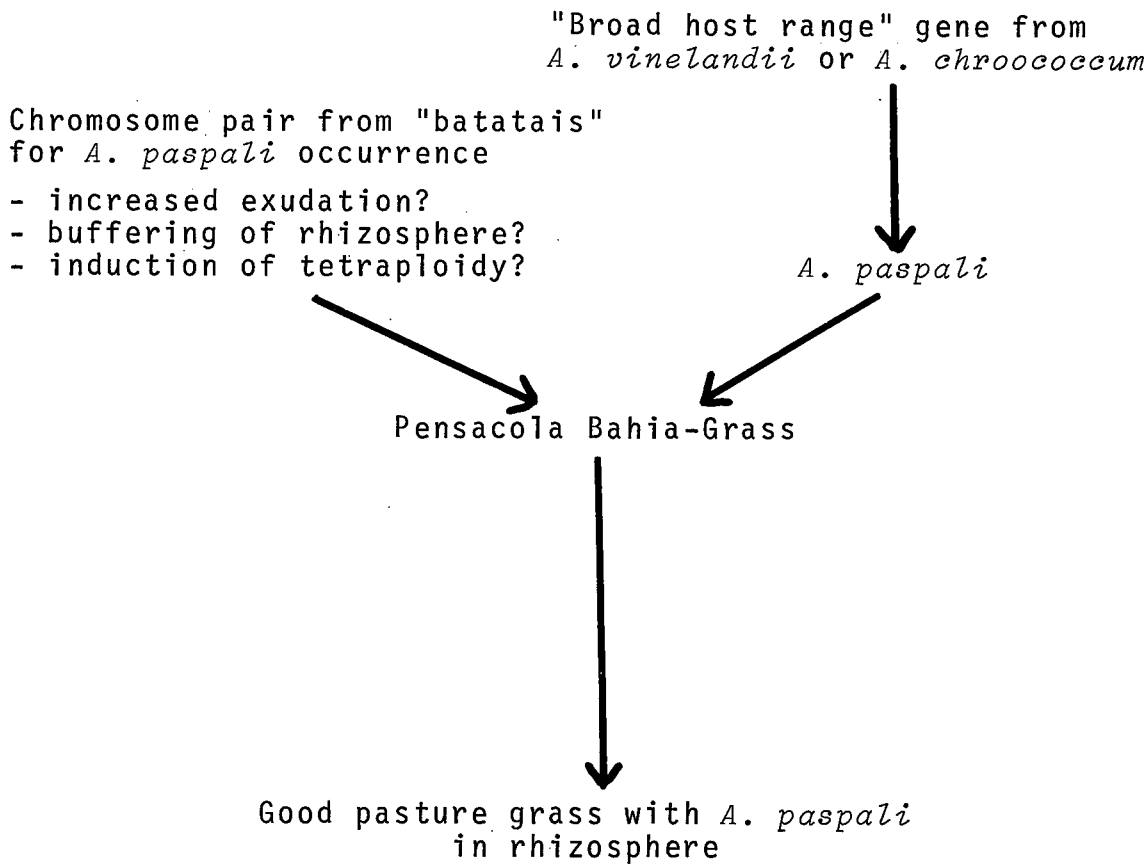
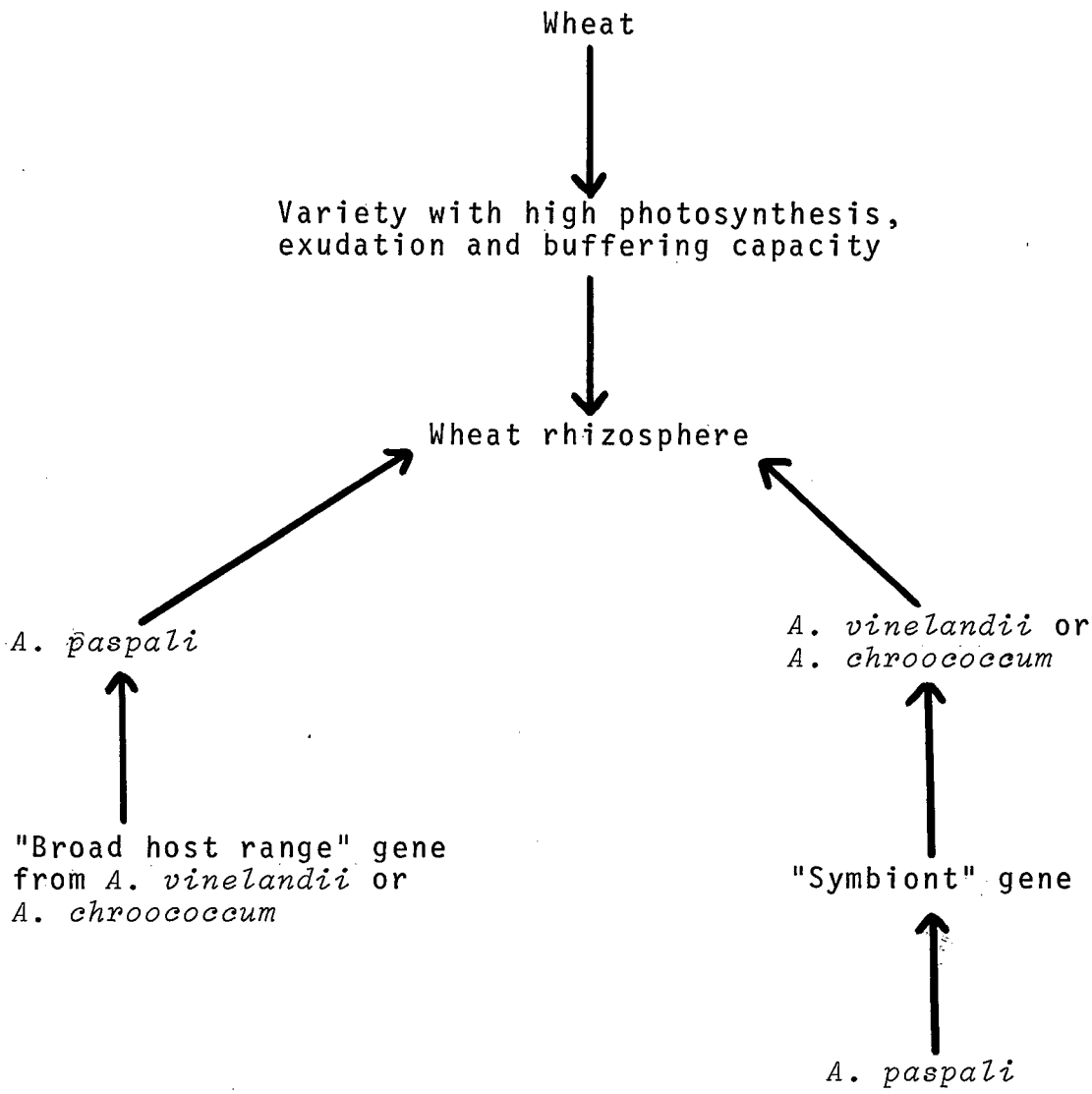

"Broad host range" gene from
A. vinelandii or A. chroococcum
Chromosome pair from "batatais"
for A. paspali occurrence
- increased exudation?
- buffering of rhizosphere?
- induction of tetraploidy?
A. paspali
Pensacola Bahia-Grass
Good pasture grass with A. paspali
in rhizosphere

Figure 2. Hypothetical scheme for development of a wheat rhizosphere with modified strains of either *A. paspali* or *A. vinelandii-A. chroococcum*.


Wheat
Variety with high photosynthesis, exudation and buffering capacity
Wheat rhizosphere
A. paspali
"Broad host range" gene from A. vinelandii or A. chroococcum
A. vinelandii or A. chroococcum
"Symbiont" gene
A. paspali

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