CORRELATION BETWEEN DARK RESPIRATION AND *IN VIVO* NITRATE REDUCTASE ACTIVITY IN MAIZE

by

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1. ABSTRACT

Dark respiration rates using intact leaves, and nitrate reductase activity using those same leaves after excision (using the *in vivo* assay after Klepper et.al. (1971)) were determined using the second leaves of 10-day old *Zea mays* c.v. SR 52 seedlings. Regression analysis was carried out between the dark respiration and nitrate reductase activities. Although the regression co-efficients (r^2) are below 0,13, the results promise that further work may well demonstrate a good correlation between these two metabolic processes.

2. INTRODUCTION

There is, at present, great interest in developing ways of predicting yield in plant crops because of the food, economic, and fuel problems in the world. It is very desirable that crops cultivated be high yielding relative to the cost of production, and that crops grown in a particular area are the best suited to that area. Plant breeders need to know characteristics for which they can select to develop ideal crop cultivars for high yield, and cultivars best suited to given areas.

Because of the importance of nitrogen (N) in the plant, being incorporated into proteins such as grain storage proteins and enzymes, nitrate reductase (NaR), the first enzyme involved in nitrate (NO_3^-) assimilation, and the enzyme which limits the rate of NO_3^- reduction to ammonia, has been used as a selection characteristic in plant breeding. Close positive correlation between NaR activity and grain yield has been reported by Johnson et. al. (1976), Deckard et. al. (1973), and others. Two methods which are extensively used to assay NaR activity are the *in vivo* assay and the *in vitro* assay. The results obtained in these two assays are, however, not always closely correlated. Klepper et. al. (1971) report that the *in vitro* assay shows levels 2,5-20 fold higher than those for the *in vivo* assay for most plant species, but Jaworski (1971) reports that the results obtained using the *in vivo* assay were always higher, but that the result of the *in vivo* assays are proportional with those of the *in vitro* assays with respect to ranking genotypes for nitrate reducing potential of a given

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species. Brunetti and Hageman (1976) also report that the in vitro assay yielded higher results than did the in vivo assay when using wheat seedlings. The former values were 4,1 times higher than the actual reduced N accumulated in the whole plant while the in vivo assay results were closer to the actual amount of reduced N, being 0,9 times that for the whole plant, than those obtained using the in vitro assay. Streeter and Bosler (1972) referring to work with soyabeans, suggest that this may be because the in vitro assay is less efficient. Deckard and Busch (1978) did not give a close correlation between the two methods when using spring wheat. A description of NaR activity is essential at this stage to show how these assays are used to estimate NaR activity.

Both of the assays use the accumulation of nitrite (NO_2^-) as a measure of NaR activity, NO_2^- being formed by reduction of NO_3^-. NaR reduces NO_3^-, and obtains this reducing power from NADH (reduced nicotinamide adenine diphosphate). This NADH is available in green tissues in the light, as reduced carbon compounds are released from the chloroplast, for example glycolate is converted to glyoxylate, generating NADH, and the Glyoxylate returns to the chlorplast. (Canvin and Atkins (1974)). This NADH is oxidised as NaR reduces Nitrate. NADPH (reduced nicotinamide adenine triphosphate, or $NADPH_2^+$) is available, in those cases where the NaR is NADPH dependent (as Hageman and Hucklesby (1971) report to be the case in some maize cultivars where either or both of NADH and NADPH dependent NaR may be present), at the terminal end of the photosynthetic electron transport chain. In the in vitro assay, however, intact chloroplasts are present, presumably with some reserve NADH, but that would only be in limited supply because the assay is carried out in dark conditions to prevent the reduction of nitrite which takes place in the light. Another supply of NADH is also present; however, as the mitochondria generate NADH in the respiratory processes. This $NADH_2^+$ from mitochandria is the source of reducing power in non chlorophyllous tissues such as roots. Another requirement of the in vivo assay is that oxygen be expelled from the system as it competes very strongly with the NO_3^- in the dark. (Canvin & Atkins (1974)). This means that lack of O_2, the final electron acceptor in the oxidative phosphorylation electron transport chain, would cause an accumulation of Tricarboxylic acid (TRA) cycle intermediates which would eventually cause a slowing of

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the repiratory processes. In such cases NO_3^- would probably act as in terminal ectron acceptor as is the case in some micro-organisms in anaerobic or partially anaerobic conditions where Lehninger (1975) reports that NaR receives electrons from cytochrome b as in Escherechia coli. Cresswell (pers. comm.= reports that workers in his laboratory have shown that uncouplers of oxidative phosphorylation, which result in increased electron flow in the mitochondria, stimulate NO_2^- production. This would indicate that NO_3^- may be acting as a terminal electron acceptor, and that the use of uncouplers enhances this contribution to the NO_2^- production. The question arises as to whether the accumulating NO_2^- may not act as the electron acceptor, but no reports of this are known to the author. It would result in underestimation of NaR activity, however, since it is the accumulation of NO_2^- which is the measured criterion. The important matter is that, if repiratory NADH is the source of reducing power for NaR in the in vivo nitrate reductase assay, an alternative terminal electron acceptor must replace the oxygen, otherwise NaR activity would be reduced as the respiratory activity decreases. Furthermore - Sawhney et. al. (1978) report that carbon monoxide (CO), which inhibits oxidation of NADH by the electron transport chain in the mitochondria, does not inhibi NO_2^- formation, indicating that NO_2^- is not formed only as NO_3^- acts as terminal electron acceptor to this chain. Cresswell and his co-workers (pers. comm.) report that it is in fact the glycolytic fermentation pathway which appears to be involved in supplying NADH, and this pathway operates normally in anaerobic conditions. This being the case, an alternative eleectron acceptor to replace O_2 would not be required. In fact, in their work they have found that 3 Phosphoglyceraldehyde, which is an intermediate in the glycolytic pathway, and also in the Calvin cycle of photosynthesis, can act as a reductant in the light. They have also found that glycolytic substrates stimulate NaR activity in the in vivo assay while TCA cycle intermediates have no effect. TCA inhibitors also have no effect. This indicates that the NADH used for NaR as electron donor is not supplied by the TCA cycle. This agrees with results reported by Klepper et. al. (1971) where the oxidation of Glyceraldehyde-3-Phosphate was ultimately the source of NADH for Nitrate reduction.

It would appear, then, that reducing power, in the in vivo assay, is supplied both by the glycolytic pathway as NADH, and by the cytochromes in the oxidative phosphorylation electron transport.

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chain as NO_3^- acts as the terminal electron acceptor. NADH and NADPH from the chloroplast would rapidly be used up, after which, if NADPH dependent NaR is present, contributing to the NO_2^- production, this contribution would cease unless an alternative NADPH supply is served. Only NADH dependent NaR would then act to reduce NO_3^-. Butz (1977), in discussing the in vivo assay as a predictive test for grain yield, indicates that, in that the electron donor in this assay has never been identified, and also that it is uncertain as to whether it is the same as the donor in the intact plant, the in vivo assay cannot be used as a good predictive test for crop yield. Johnson et.al. (1977), however, report that although the electron donor has not been identified, the fact that in vivo assay results have been shown to be correlated with yield, means that the in vivo assay can be used to predict crop yield.

Cresswell and his co-workers (pers. comm.) feel that the in vivo nitrate reductase assay is, in the light of the above information, a reflection of respiratory activity as seen in the interaction of dark respiration with NaR under the experimental conditions of the assay, and not a reflection of the activity of NaR in situ. Some workers, (e.g. Canvin and Atkins (1974)), dispute a link existing between NaR and respiratory NADH because NaR is associated with the chloroplast, and the localisation of NaR and dark anaerobic reduction of NO_3^- are consistent with each other.

Dark respiration is linked with productivity, as pointed out by Penning De Vries (1975[a]), with a relationship of 531g CO_2 produced for 1 000g end product in maize. M^{c}Cree and Van Bovel (1977) report that computer modelling is currently being used successfully, to relate respiration to crop production. Evans(1975) points out how these models in early years assumed that respirtion was proportional to dry weight accumulation, but that in later years it has been found that dark respiration is in fact proportional to photosynthesis rather than to dry weight.

It has been found that dark respiration is the sum of two terms. The first is the basic cost of metabolism, or maintenance costs. This term is related to dry weight and is dependent on temperature. The second term is that of the respiratory cost of growth or synthesis. The latter term is not dependent on temperature or species,

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provided that it has coupled oxidative phosphorylation taking place, and is proportional to gross photosynthesis.

Maintenance respiration thus brings an excessive depletion of photosynthate above what is used for growth, and hence yield. Penning De Vries (1975b) reports that this maintenance, which involves the turnover of proteins, is absent in rapidly growing maize. In another work (Penning De Vries (1975a)) he reports that maintenance respiration in maize is generally not high, e.g. $\frac{1}{3}$ the rate in sunflower.

Synthetic respiration involves the release of energy from various high energy substrates such as glucose, and this energy is used in growth. It would then not be surprising that a close positive correlation should exist between dark respiration and potential crop yield.

The aim of this project was initially to find a correlation between dark respiration rates and NaR activity in maize seedlings using the in vivo assay. If such a correlation were found, a correlation would then be sought between the dark respiration rates and yields of various maize cultivars, but this stage was not reached, as a good correlation was not found in the first phase of the experiment.

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3. MATERIALS AND METHODS

3.1 Plant Material

Seeds of Zea mays cv. SR 52 were obtained from the Summer Grain Centre at Potchefstroom and were germinated individually in polystyrene vending cups as shown in Fig 1A. They were grown in a phytotron growth chamber with 16 hours daylength, 8 hour night, temperatures 30°C in the day, 23°C in the night, relative humidity 60% in the day, 72% in the night. The light intensity was 300 microeinsteins. m^{-2}. sec^{-1}.

In order to germinate the seeds, they were placed, with river sand from Frankenwald, in the vending cups, and placed in a shallow tray of water thereby keeping the sand wet, until the coleoptile appeared. Thereupon the cups were removed from the water tray and were supplied daily with nutrient solution as per Hewitt (1966) using a 200 ppm. NO_3^- combination. Components of this solution are shown in table 1.

TABLE 1: Components of the nutrient solution applied to the maize seedlings. After Hewitt (1966).

Chemical	Concentration ($g.\ell^{-1}$)	Molarity (mM)
KNO_3	0,505	0,005
$Ca(NO_3)_2$	0,820	0,005
Fe EDTA	0,03	0,00008
$NaH_2PO_4 2H_2O$	0,208	1,3
$MgSO_4 7H_2O$	0,369	3
$MnSO_4 4H_2O$	0,00223	0,01
$CuSO_4\ 5H_2O$	0,00024	0,001
$ZnSO_4\ 7H_2O$	0,00029	0,001
H_3BO_3	0,00186	0,033
$(NH_4)_6\ MO_7O_{24} 4H_2O$	0,00003	0,002
$CoSO_4\ 7H_2O$	0,00002	0,0001
NaCl	0,00585	0,1

Imbibition was allowed to commence at the beginning of the dark period.

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The coleoptile emerged in 4 or 5 days, and, by the commencement of the tenth dark period (the tenth day) the seedlings were of a reasonable size for use, the second leaf being some 10 to 12 cm. from sheath to tip. Hageman and Hucklesby (1971) also report that there is a peak of NaR activity 7-10 days after planting for maize seedlings, so that this age plant was suitable for both dark respiration rate determination and use in the in vivo NaR assay.

Excess seeds were used to allow for failure of some to germinate (sometimes one in 10 or 15 did not germinate), and so that plants of very similar size could be used each time, although on some occasions the plants used were small compared with the average, and this can be detected by the small leaf area used. The last application of Nutrient solution was 2 hours before the plants were used in the determination for repiration rates.

3.2 Dark Respiration (DR) Measurement

At the commencement of the tenth dark period the plants were removed to a laboratory with an Infra Red Gas Analyzer (I.R.G.A.) which was used to analyze the quantity of CO_2 evolved in dark Respiration in the leaf. After measuring the respiratory activity the plants were returned to the phytotron chamber.

The dark respiration rates were determined for the leaves after they had been in the dark for an hour. There is controversy as to whether respiration takes place in the light or not, as some workers (e.g. Cresswell (pers. comm.)) feel that only photorespiration takes place in the light, while others report respiration for the light period. For example M^{c}Cree and Troughton (1966) report that repiration has a maximum at midday and a minimum in the second half of the night since the readings the present work were done in the dark, it was hoped that they would be most representative of the actual rates if the readings were done when the biological rhythm registered that it was night time.

The second leaf of a seedling was inserted through a slit in the front of the cuvette as shown in Fig. 1A, and the slit sealed off with

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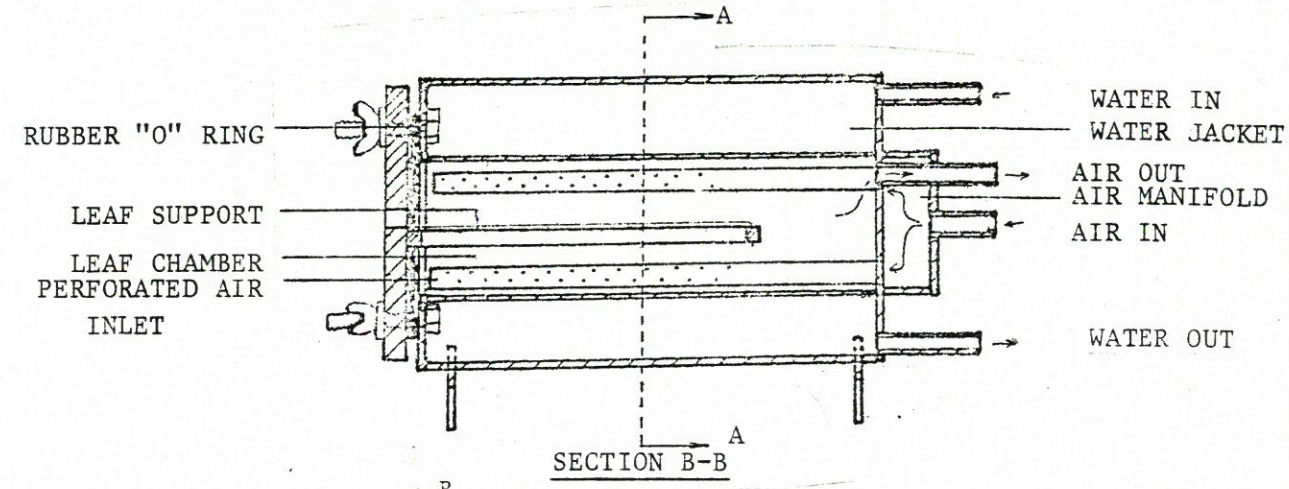
FIG. 1A Cuvette with leaf inserted showing Pres-stik sealing slit



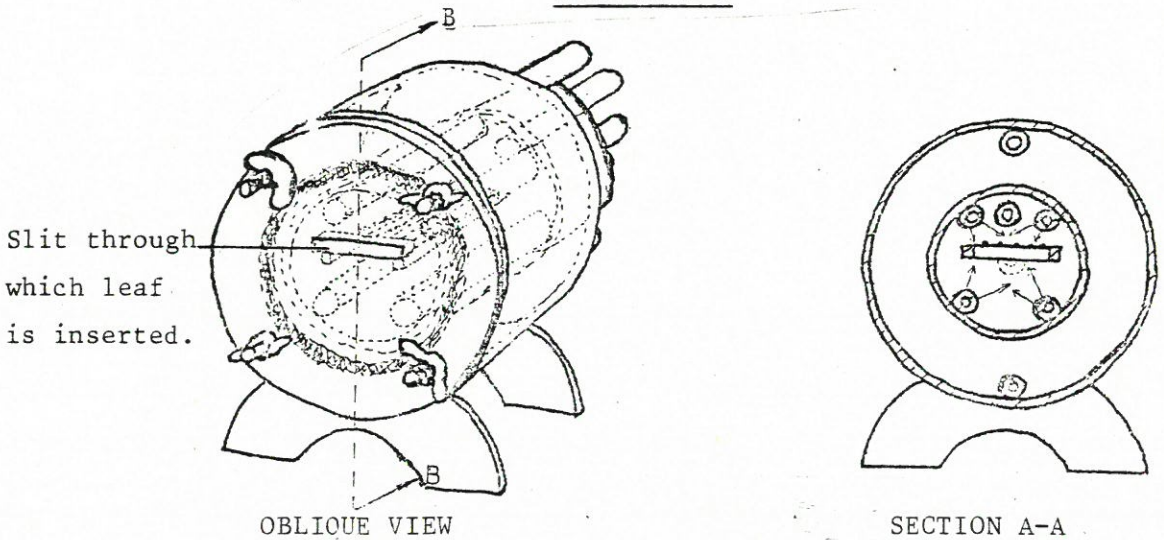


FIG 1B Detail of cuvette in figure 1A

FIG 1 CUVETTE USED TO DETERMINE DARK RESPIRATION RATES OF LEAVES

A: with leaf inserted B: Detail of cuvette

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Bostik Pres-Stik. A cuvette designed to take a leaf, attached to the plant was used so that the same leaf could be used fifteen hours later for the NaR assay. The design of this cuvette is shown in Fig. 1B. The plant and cuvette were then covered with black polythene sheeting to keep the light out, and in the interim a minimun of exposure to light was experienced by the plant. Ideally the work should have been carried out with exposure to only monochromatic safe-light, rather than with white light, and, in the phytotron chamber where the growth conditions of temperature and humidity would have been controlled.

A test was carried out to show the effect of light on the respiration rate. As can be seen in Fig. 2, when the plant excluding the leaf in the cuvette was exposed to light of 650 microeinstein. m^{-2}. hr^{-1}, while the cuvette was wrapped in aluminium foil to exclude light, CO_2 went from the darkened leaf presumably to supply the photosynthetic demand for CO_2 in the plant parts in the light, rather than escaping to the space within the cuvette. (See Fig. 2, plants A and B). For this reason care was taken to exclude all light. This also shows that despite care taken to seal the slit in the cuvette, CO_2 could leak from the cuvette within the leaf itself. If the entire plant were in the dark this CO_2 leakage should have been minimal as the CO_2 distribution would presumably be in equilibrium within the leaf.

Figure 3 shows the apparatus used in the determination of dark respiration rates. The cuvette, as can be seen in Fig. 1B was so designed as to dispel the boundary layer of air on the leaf surface as air was released in several directions from the air inlet, as shown by arrows. The pumps for air and water were switched on and time allowed for the air to equilibrate in the cuvette, and for the water temperature to equilibrate to 25°C. The IRGA was calibrated using test gases of given concentrations of CO_2, as shown in Fig. 2, e.g. 367 and 285 vpm CO_2. The large buffering tank shown in Fig. 3B was used as fluctuations in the CO_2 concentration of the atmosphere were quite sharp, but the buffering tank smoothed the fluctuations out considerably.

An open, or flow, system was used in preference to a closed circuit system for measuring the respiration rates. In an open system

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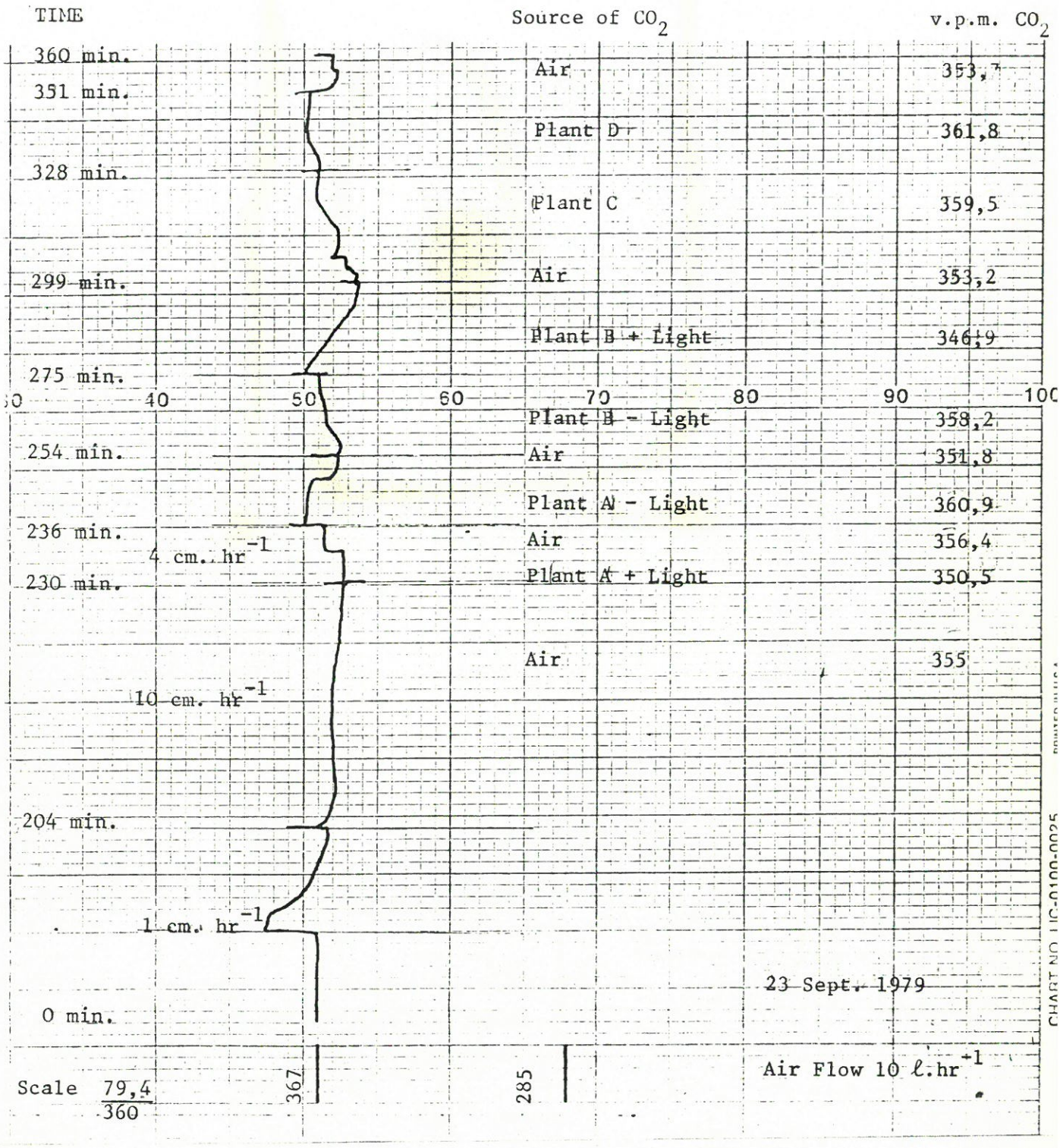

Fig. 2. A copy of one trace showing changes in v.p.m. CO_2 for air supply from atmosphere, and after passing over a leaf. Plants A and B were placed in 650 microeinstein m^{-2} hr^{-1} light while the leaf was in the cuvette, wrapped in aluminium foil In this case CO_2 was lost to the rest of the plant and not to the air in the cuvette.

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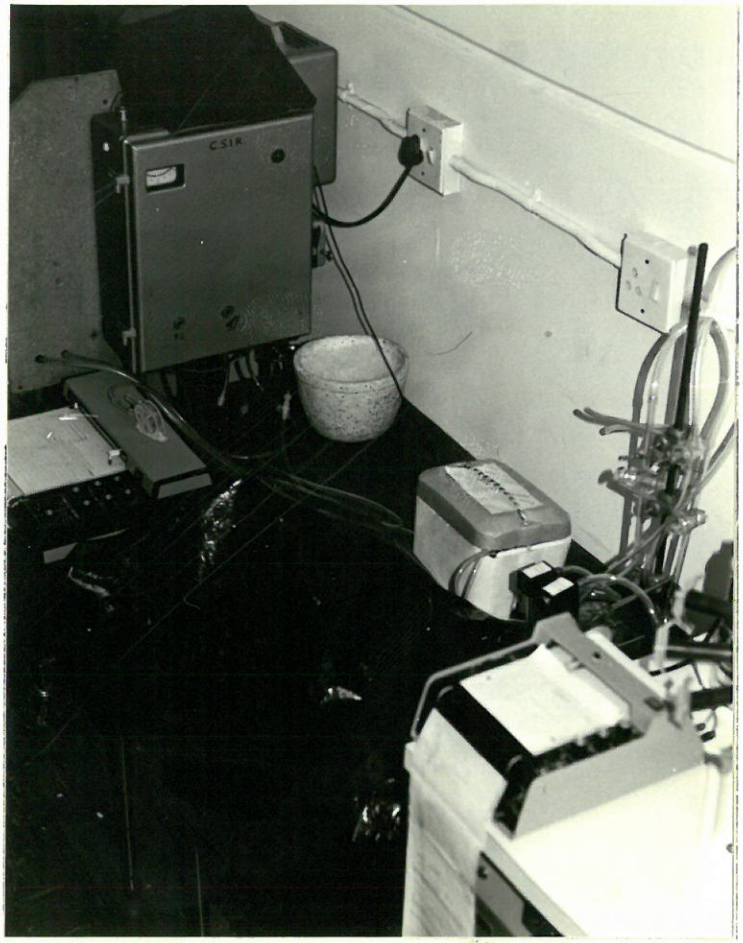
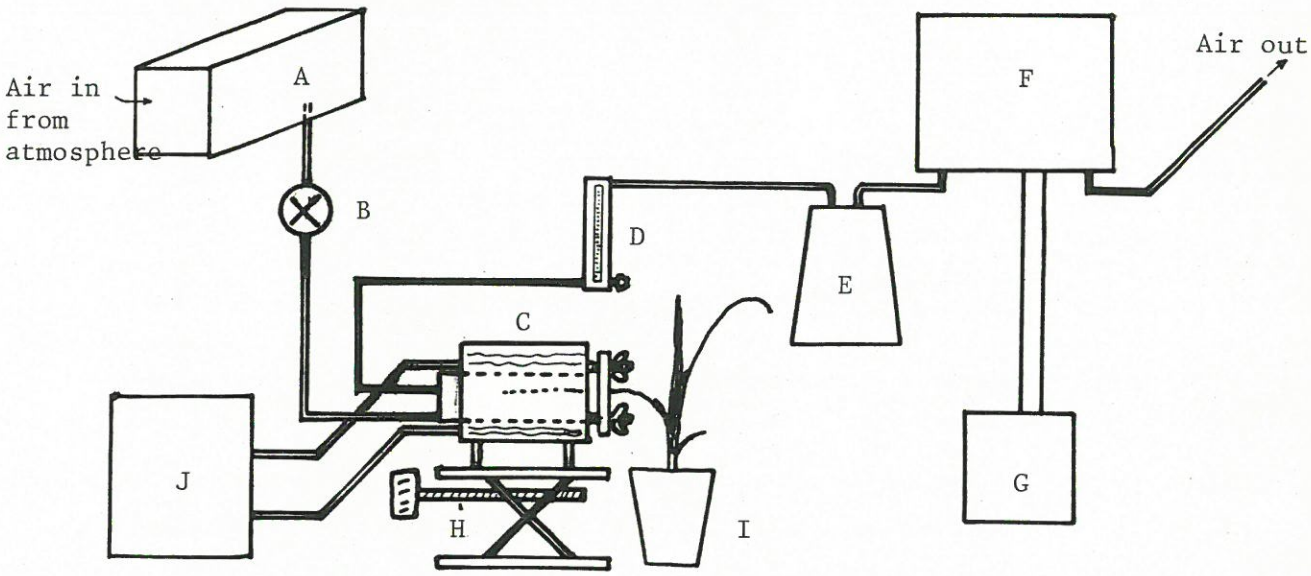


A. Layout of some of the apparatus shown in B.



B. Circuit diagramme.

Fig. 3 Apparatus used for measuring dark respiration rates in maize seedlings. A: buffering tank (see text). B: Air pump. C: Leaf cuvette. D: Flow rate meter. E: Ice trap to remove water vapour. F: Infra Red Gas analyzer. G: Linear trace recorder. H: Adjustable Laboratory stand. I: Plant in polystyrene cup. J: Controlled temperature water bath.

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atmospheric air is passed over the leaf, thereby having as small a gradient of CO_2 concentration, between the leaf and outside, as possible, thereby not creating abnormal diffusion gradients which may bias the readings - giving overestimate if the air had lower CO_2 concentration than normal, or underestimate if higher than normal. In a closed system the same air is circulated, and may increase in CO_2 concentration above normal levels, Furthermore, there is greater likelihood of leakage and exchange with air outside the system. In an open system as used, there is always a positive pressure so that air might leak out, but none would leak in. Another reason for opting for an open system was that intact leaves could be used while this often creates leakage in a closed system if the opening around the leaf is not sealed correctly.

The rate of air flow in the cuvette was kept constant by monitoring the flow rate using an air flow-meter as shown in Fig. 3B. The flow-meter was positioned after the cuvette in the circuit because there was great resistance to air flow in the cuvette, and it the flow meter preceeded the cuvette, the resistance could cause leakage in the joints between the flow meter and the cuvette. Being positioned as it was, and with it registering the same rate of air flow, each time, as when the slit was sealed with no leaf in, indicated that the slit was sealed off properly and the air flow constant. In some 5% of the cases the air flow may have been different, this being before the methodology was corrected. Which readings were affected, however, are not known. The air flow was at a rate of 10 ℓ. hr^{-1}.

The cuvette was kept at 25^{o}C by water kept at this temperature, circulating in the water jacket as shown in Fig. 1B.

The difference in CO_2 concentration was read directly off the IRGA, or else off a trace as shown in Fig. 2. As can also be seen from the trace, the CO_2 concentration in the cuvette stabilized in about 10 minutes, and the reading taken after 15 minutes to ensure that the concentration was stable. Plain air from the atmosphere was then passed through the IRGA and the difference in v.p.m. CO_2 represents the amount of CO_2 evolved in the respiring leaf. This value was then used in a calculation where correction was made

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for temperature (25°C) and pressure (read on a barometer) and the results expressed as milligrammes CO_2 evolved at Standard Temperature and Pressure (S.T.P.). The calculation is as follows:-

$$V_s = \frac{V\ h_s - T_s}{h_t}$$

V_s = ℓ. hr^{-1} CO_2 at STP

h_s = 760 mm Hg

h_t = mm Hg read off barometer

T_s = 273,2°K

V = ℓ. hr^{-1} CO_2 produced (10ℓ. hr^{-1} X vpm)

This gives ℓ. CO_2 produced per hour which can be converted as follows to mg CO_2. hr^{-1}.

$$m = \rho CO_2 - Vs$$

m = mass in g

ρCO_2 = density of CO_2 = 1,977 g. ℓ^{-1} at S.T.P.

V was determined using the URAS 1 I.R.G.A. which has an accuracy of 0,5 v.p.m.

There was apparently little physiological effect done to the leaves by using pres-stik as described. Some 75% of the leaves were clearly physically damaged, the most common damage incurred being the bending and breaking of the mid-rib as the leaf was removed from the cuvette. This is shown in Fig. 4A,B,C; Fig. 4B shows where a little piece of epidermis was peeled off. Fig. 4D has similar damage, and Figures 4E,F show tearing of the leaf, all occurring

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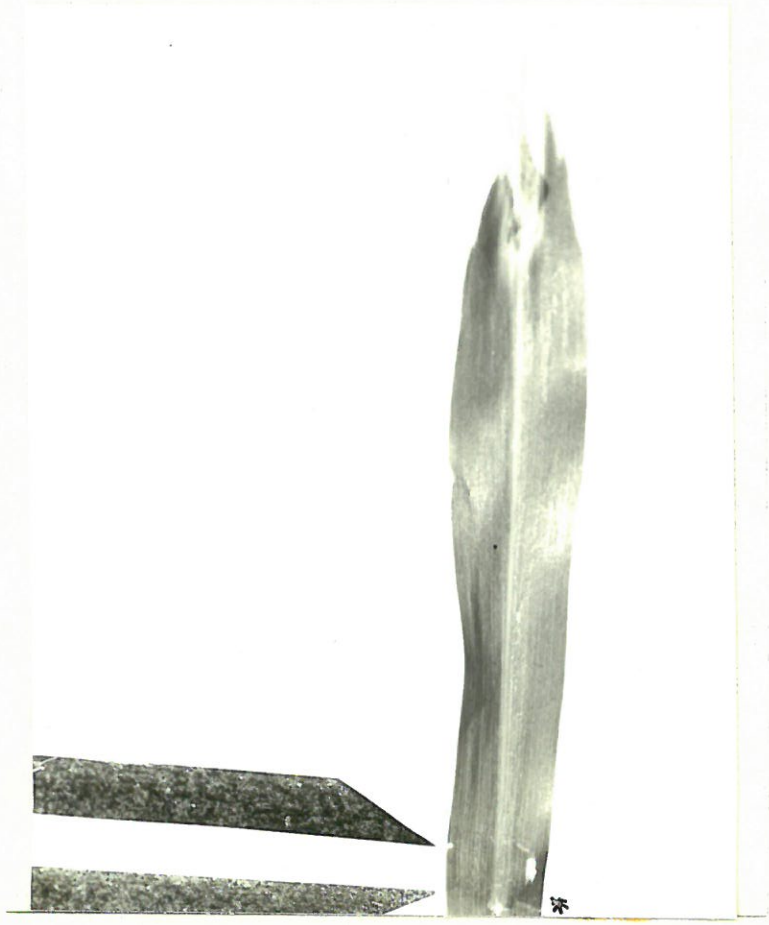
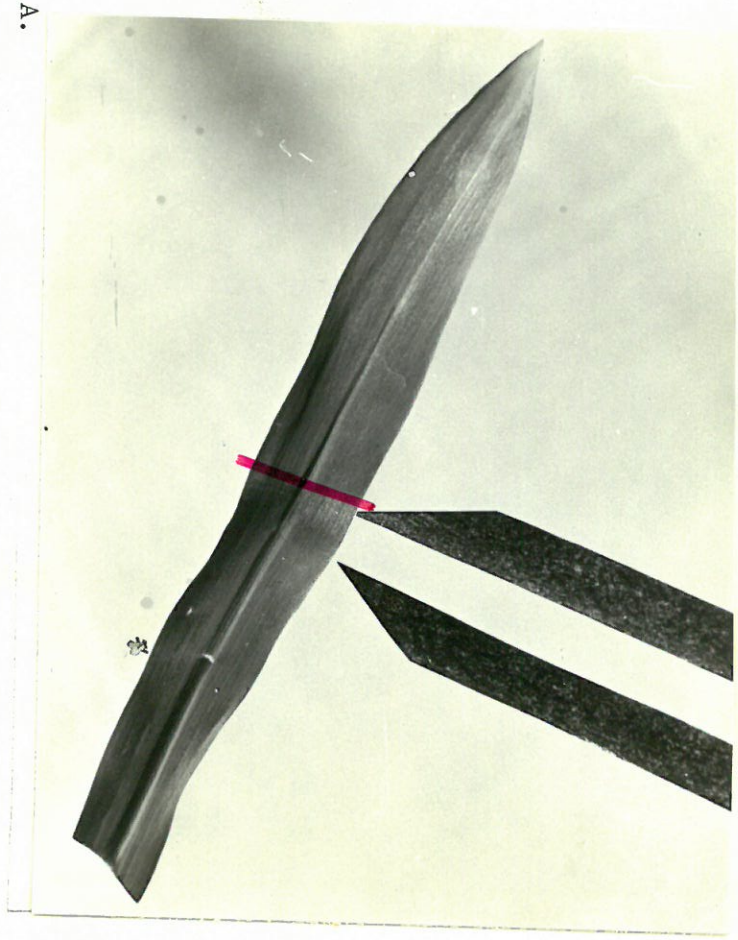
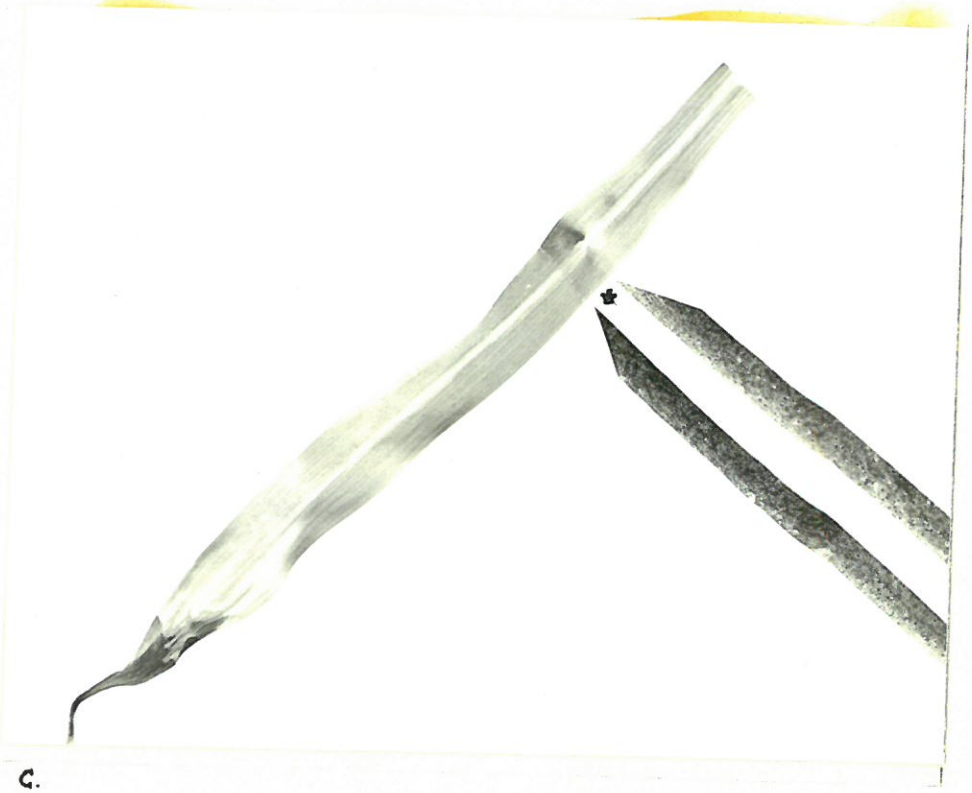
Fig. 4. Maize leaves 5 days after being placed in leaf cuvette. Area between pointed bars indicates position where Pres-stik was applied. Damage by bending and breaking of mid-rib (*). Piece of epidermis stripped off leaf (**). Leaf blade torn (***) A-E, G-I Adaxial surfaces. F: Abaxial surface of E.

B.



A.





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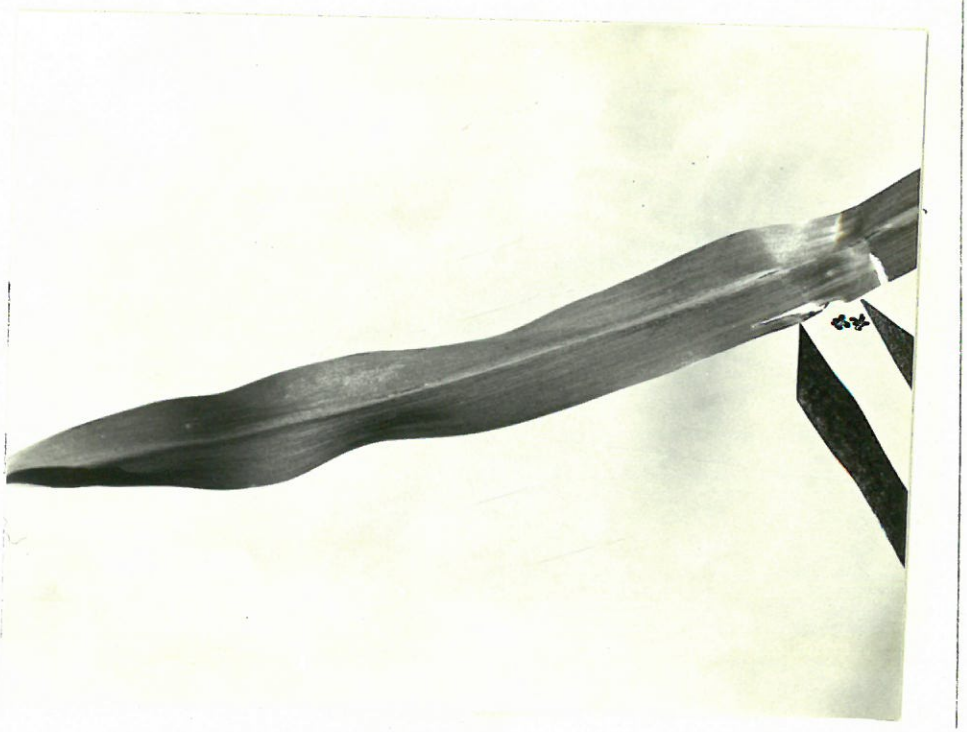

D.

Fig. 4 Contd.

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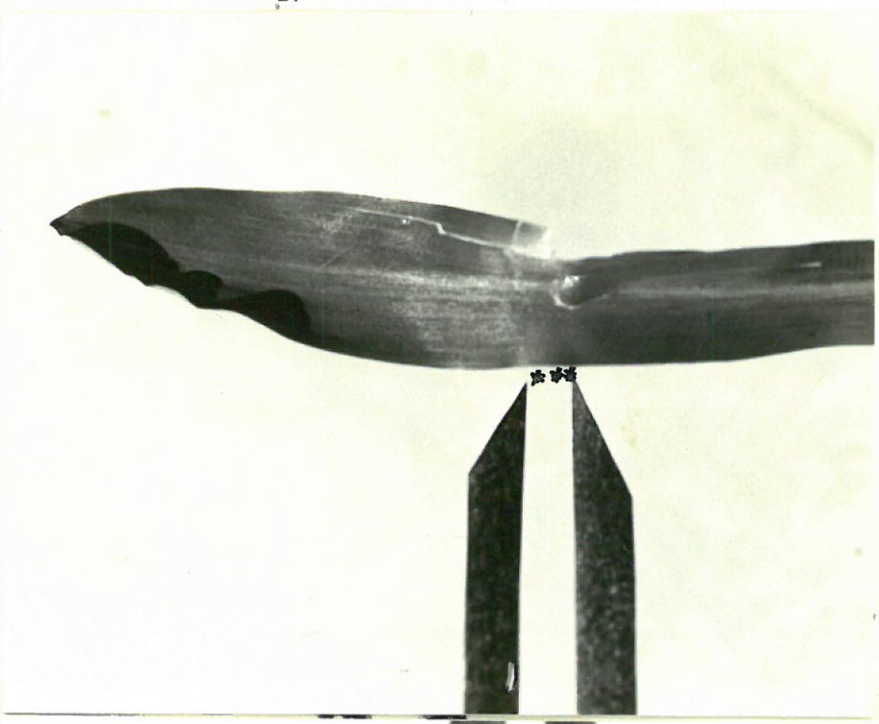
E.



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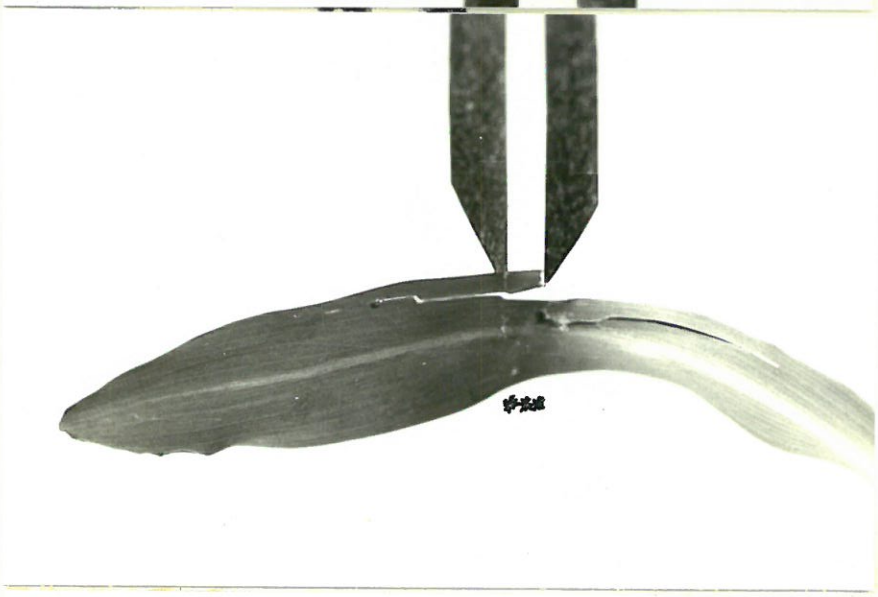
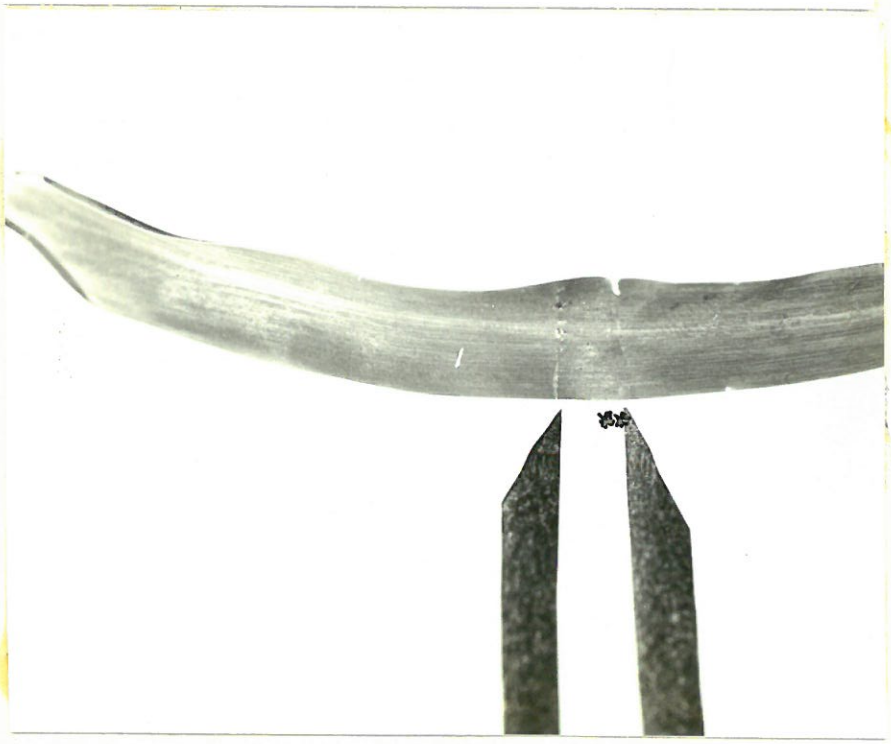


G.



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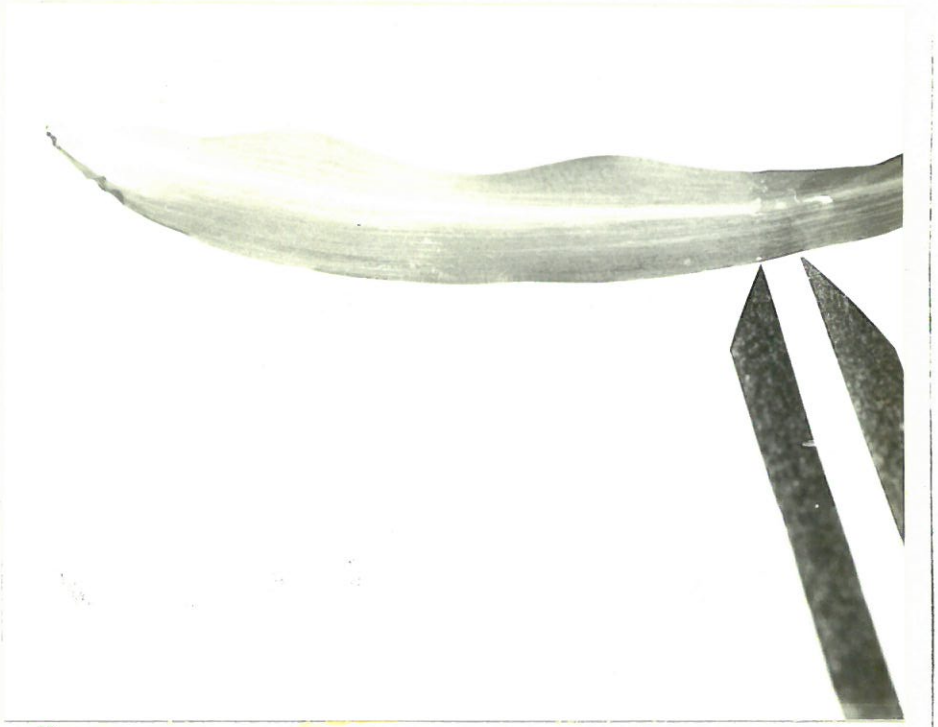
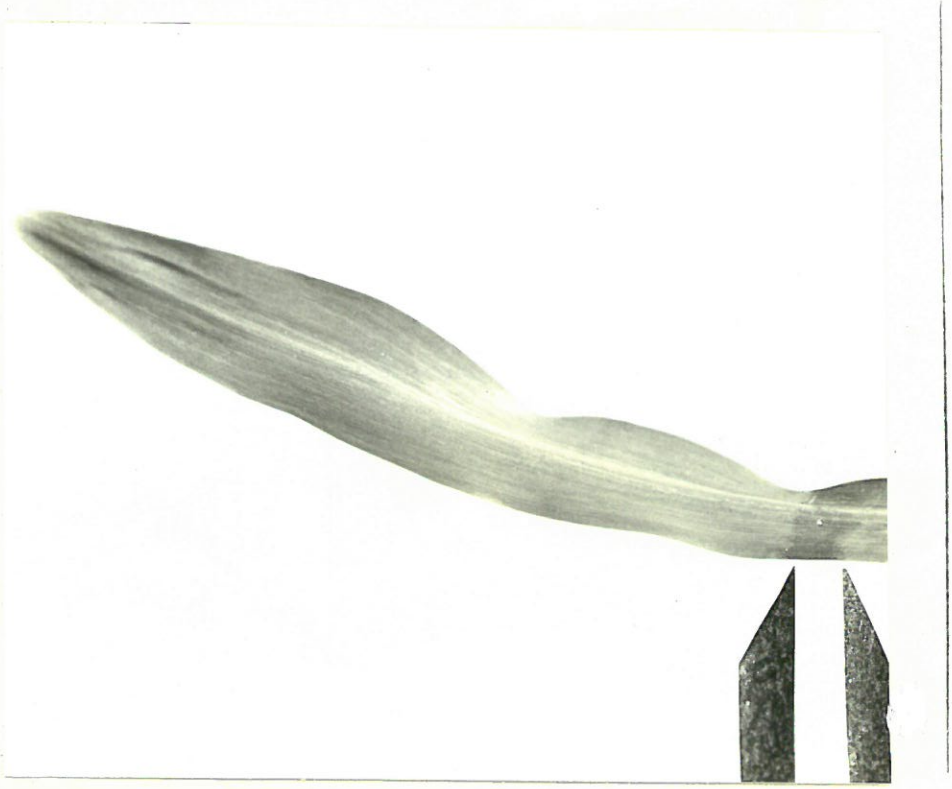


H.



I.

Fig. 4 Contd.

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when the Pres-stik was removed from the respective leaf. Leaves in Figures 4G,H,I show practically no evidence of their having been handled. The area where the pres-stik was applied can be seen clearly in Fig. 4G, being delineated by light bands, and in Fig 4C there is a broad dark band. It was observed that the epidermis reflected the light differently and was slightly darker green in colour where the pres-stik was applied, but otherwise there was little marking of the leaf. Despite the damage as shown the leaves still looked healthy, as these photographs show, as they were taken 5 days after the leaves were placed in the cuvette. No microscopic observations were carried out to see the epidermis in greater detail. Laquer varnish impressions could have been used, or else scanning electron microscopy used. However, as the epidermal integrity was not relevent to this work, such tests were not carried out. Analysis of variance was used to see if the NaR activity in leaves placed in the cuvette were significantly different from NaR activity in control leaves, but the mean rates were not significantly different. It would appear, from this comparison, that the metabolism, or at least the NaR activity which is being considered here, is not significantly affected by the treatment to the leaves during the respiration measurement.

3.3 The *in vivo* assay for Nitrate Reductase Activity, (NRA)

12-15 Hours later the plants used above, plus 50% more were removed to the laboratory where the *in vivo* NaR assay was carried out. This allowed recovery time of about 4 hours in the light for NaR to reach high rates of activity after depletion during the dark period. Shaner and Boyer (1976) report that NaR recovers full activity after about 3 hours of light and that the activity then changes less than 10% until the 9th hour of light. The readings in this work were taken during this period of high and stable activity of NaR.

The leaves as used in the cuvette for measuring respiratory activity were cut at the position as indicated in Fig. 4A, being the portion which was in the space within the cuvette. The increase in leaf area in the intervening 15 hours between the

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times of recording the respiratory activity and of cutting, was not sufficient to detect by measuring.

Each leaf was weighed, and traced out on graph paper so that the area could be determined. The piece of leaf between the first cut and near the sheath was cut off and weighed, then dried in an oven at 85°C for 48 hours, giving the ration of fresh weight to dry weight so that the dry weight of the leaf material used could be estimated. This work with the leaf was done quickly and the leaf placed on ice as soon as possible after cutting. Johnson et.al. (1976) report that NaR activity in leaves stored at ambient temperature for a few hours showed no significant altering of enzyme activity, so that measuring these parameters in this work should not have affected NaR activities.

The in vivo assay was based on that used by Klepper et. al (1971). The leaf material was cut into small pieces of about 4 mm and that from each leaf was placed in a separate test-tube containing 10 ml. 0,01 M KNO_3 buffer (PH 7.5). (Fresh buffer was mixed each day when used). The test-tube was wrapped in aluminium foil to keep light out, and the open ends also covered with foil. The leaves were vacuum infiltrated until the medium bubbled, for 15 seconds, as though boiling. This was done with the test tubes on ice. 3x 0,2ml aliquots of medium was then taken from each sample and placed in each of three test tubes with 1,8ml distilled water, and NO_2^- content determined using 1 ml each of the colour reagents, 1% sulphanilamide in IN HCI and 0.01% aqueous naphthyl ethylenediamine dihydrochloride and measuring absorbance at 540 n.m. using a spectrophotometer.

The test-tubes were then transferred to a mechanical shaker in a water bath regulated at 30°C, where incubation proceeded for 30 min. Thereafter the test-tubes were transferred to ice, and further 3x 0,2 ml aliquots taken for NO_2^- determination. The leaf material was then blotted dry and homogenized in a chilled mortar and pestle with 10 ml distilled water, and from this 3x 0,2 ml aliquots taken for NO_2^- determination, mixed with the water and colour reagents, shaken up with 0,5 ml. chloroform, and centrifuged for 6 minutes to remove chlorophyll, then the absorbance was determined for each of these samples, after pipetting the aqueous fraction off from the chloroform fraction.

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The quantity of Nitrate present in the 0,2 ml aliquot was determined by multiplying the absorbance value by the slope of a standard curve for quantity of NO_2^- vs. absorbance. This quantity was then multiplied by 47* to obtain the amount of NO_2^- in the 9,4 ml. sample, which is the true amount of NO_2^- formed by the leaf material. (This multiplication step is necessary; since absorbance registers quantity and not concentration of NO_2^-). The NaR activity was then determined as follows, for the differance in absorbance for initial sample and for 30 min. sample:

$$NRA = \frac{94 \cdot A \cdot slope}{time}$$

NRA = NaR activity

94 is the product of 47 (multiplication factor as mentioned) and 2 to convert 30 minutes to 1 hour.

A = absorbance at 540 nm.

Slope= slope from NO_2^- vs. absorbance slope

$$\frac{u\ moles}{Absorbance}$$

* It must be noted at this point that, in removing 3 x 0,3 ml. (or 0,6 ml.) from the incubating medium at the commencement of the incubation period, the volume of medium is decreased by 6%. This means that the final value for NO_2^- formed is an overestimate of about 5% if multiplied by 50 which would give the value for 10ml. of medium. Multiplying by 47 corrects for this error.

The activity of NaR was determined for NO_2^- released to the incubation medium (NRA_{med}) and for NO_2^- accumulated in the tissue (NRA_{tis}). Since these both represent activity of NaR, in fact as the only means of NO_3^- reduction, these two values were added to give total NO_2^- formed (NRA_{tot}).

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3.4 Presenting the data

The rates obtained for respiratory and NaR activities, in order to be meaningful, were presented in terms of leaf area, fresh and dry weights. As can be seen from Fig. 5, these parameters are reasonably proportional, with the best relationship being between leaf area and fresh weight. Because the dry weight values were evidently not as accurate as they should have been, the later data was expressed only in terms of leaf area and fresh weight. Published rates are generally presented as μ moles NO_2^- formed. g fr wt^{-1} (freshweight). hr^{-1} for NRA, and as mg CO_2. dm^{-2} hr^{-1} for respiration, although respiration is apparently determined in many cases using O_2 uptake instead of CO_2 release.

3.5 Statistical Analysis of Data

An analysis of the interrelationships of these quantitative data was undertaken by using regression analysis after Parker (1973), and using the curve-fitting facilities of the Hewlett • Packard 67 calculator for calculating linear regressions.

Regression analysis was done to analyze the dependence of NRA on dark respiration. Multiple regression analysis was carried out for NRA_{tot} to see which contribution of NRA_{med} and NRA_{tis} is the more meaningful. Other multiple regression analysises were carried out as discussed below.

Since the regression co-efficients (r^2 values) were below 0,13, indicating that the interrelationships were poor, analysis of variance was used, also as in Parker (1973) to determine if the variability between the means of the different sets of data is significant, thereby indicating that the data from different days is not really comparable. Gray (pers. comm.) indicates that he found variability in in vivo assay data using maize on consecutive days to be a regular feature. For this, the simultaneous testing procedure (re. Bradu (pers. comm.)) was used to test the data, to see if certain sets of data is sufficiently different to say that it does not fit in a mornal distribution as for the results for

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a single population. An example of this test is given in appendix C.

One set of data for NRA_{tis} was found to be significantly different (data for 27 July (●) , Fig. 7 in Results section) and doing regression analysis for NRA_{tis} g fr wt^{-1} excluding this days data gave an r^2 value of 0,39.

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4. RESULTS

4.1 Interrelationship of leaf Fresh weight, dry weight, and leaf area

These parameters were compared to see how closely correlated each is with the other. These relationships are shown in Fig. 5. Regression coefficients are presented.

4.2 Interrelationship of NRA_{med} and NRA_{tis}

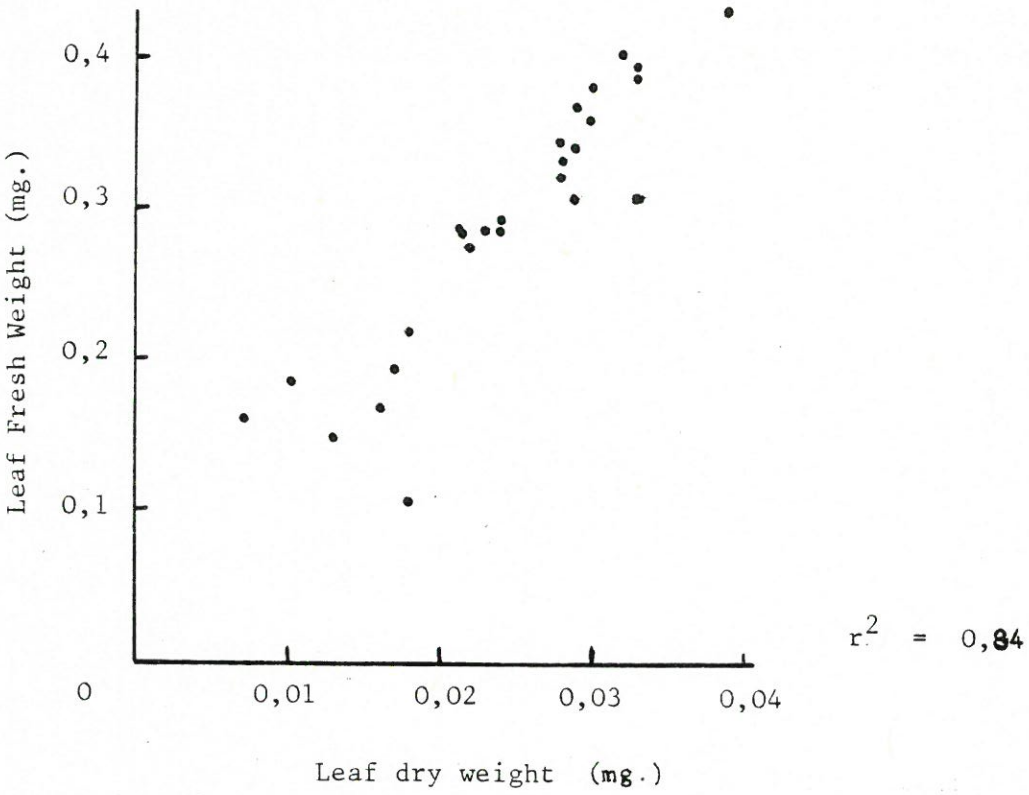
This is shown in Fig. 6, and in Appendix B. Regression co-efficient is given for the data presented in Fig. 6.

4.3 Interrelationship of NRA to Dark Respiration (DR)

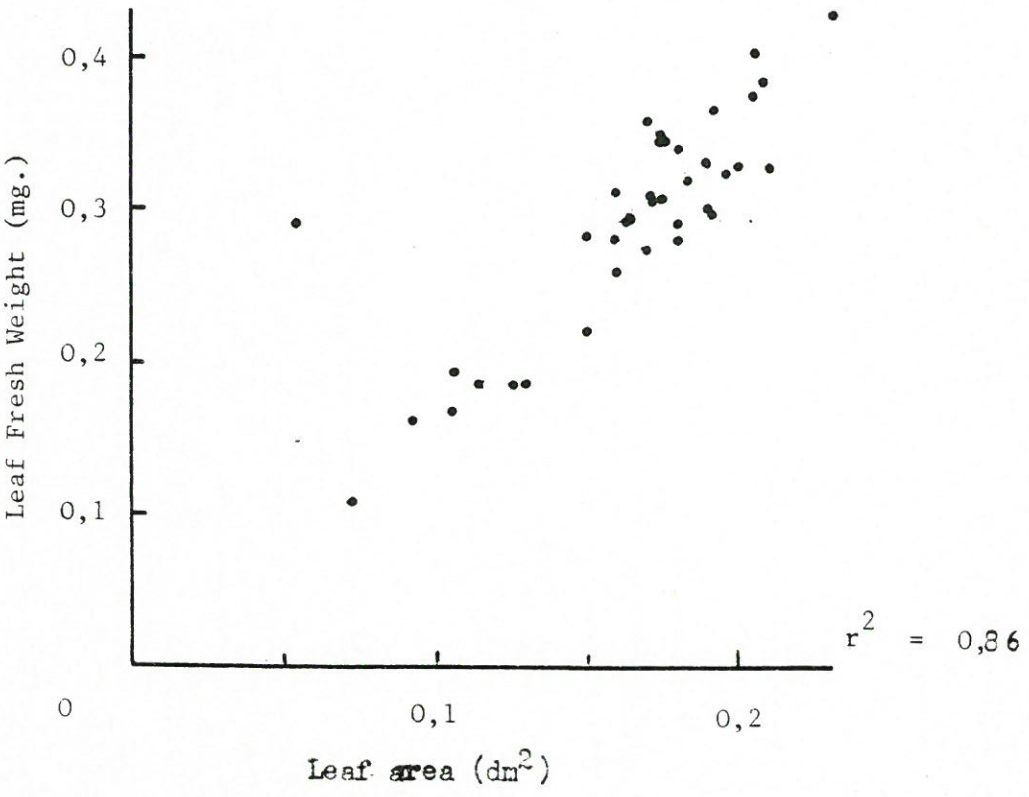
Fig. 7 shows the data for NRA and DR, and comparisons are shown for NRA_{med}, NRA_{tis}, NRA_{tot} vs. DR. In each case NRA is presented in terms of fresh weight of the leaves (NRA g fr. wt^{-1}) and DR is presented both in terms of fresh weight (DR g fr. wt^{-1}) and leaf area (DR dm^{-2}). The latter is, as discussed, the manner in which data is regularly presented in the literature, but using only fresh weight eliminates the variability due to variation between fresh weight and leaf area. Appendix A contains comparisons of NRA in terms of each parameter (fresh, dry weight, leaf area) vs. DR, in terms of each parameter other than those presented here.

4.4 Relationship between leaf area and activity observed

A comparison was made between each activity recorded and the size of the leaf used to see if any interrelationship exists between these two factors. That is, to see if using smaller leaves in 10 ml. incubation medium for the *in vivo* assay yielded either lower or higher results than using large leaves in the same volume of medium. This is shown for NRA ($dm.^{-2}$) vs. leaf area and DR ($dm.^{-2}$) in Fig. 8.

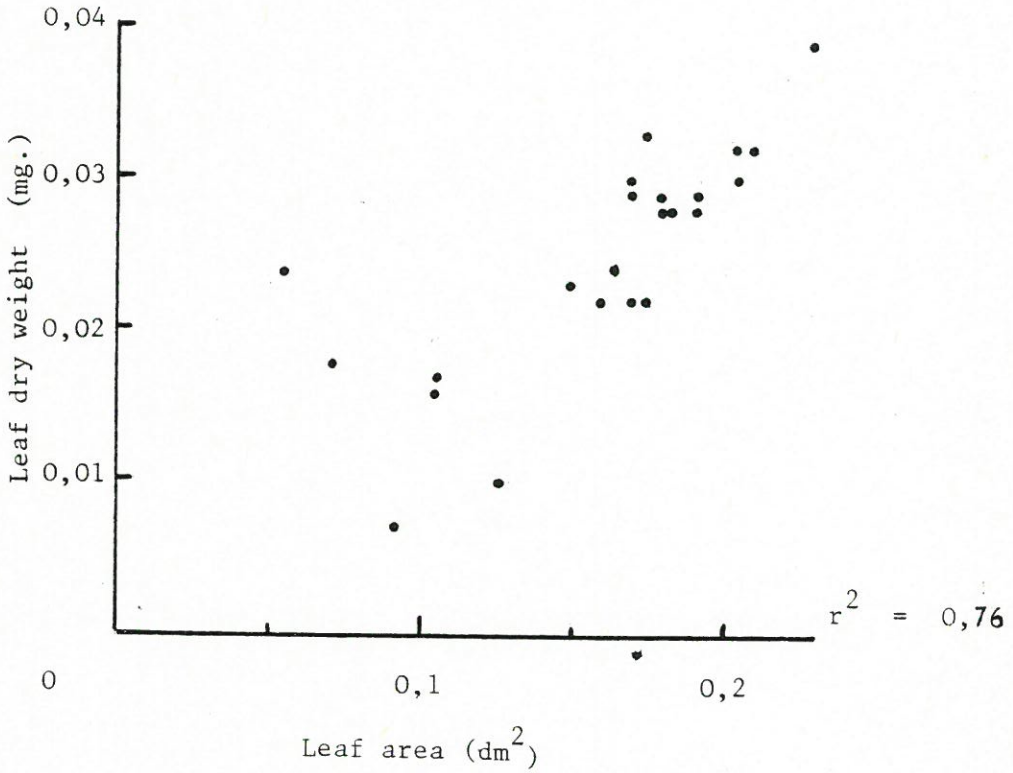

Leaf Fresh Weight (mg.)
0,4
0,3
0,2
0,1
0
0,01
0,02
0,03
0,04
Leaf dry weight (mg.)
r^2 = 0,84

A.


Leaf Fresh Weight (mg.)
0,4
0,3
0,2
0,1
0
0,1
0,2
Leaf area (dm^2)
r^2 = 0,86

B.

27 .....



C.

Fig. 5 contd.

28 .....

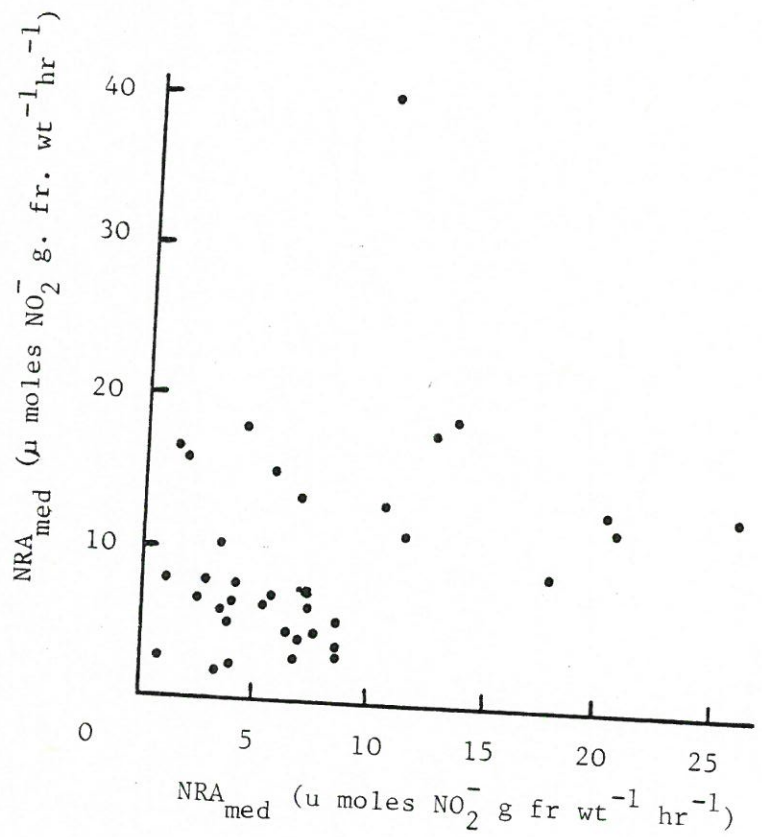
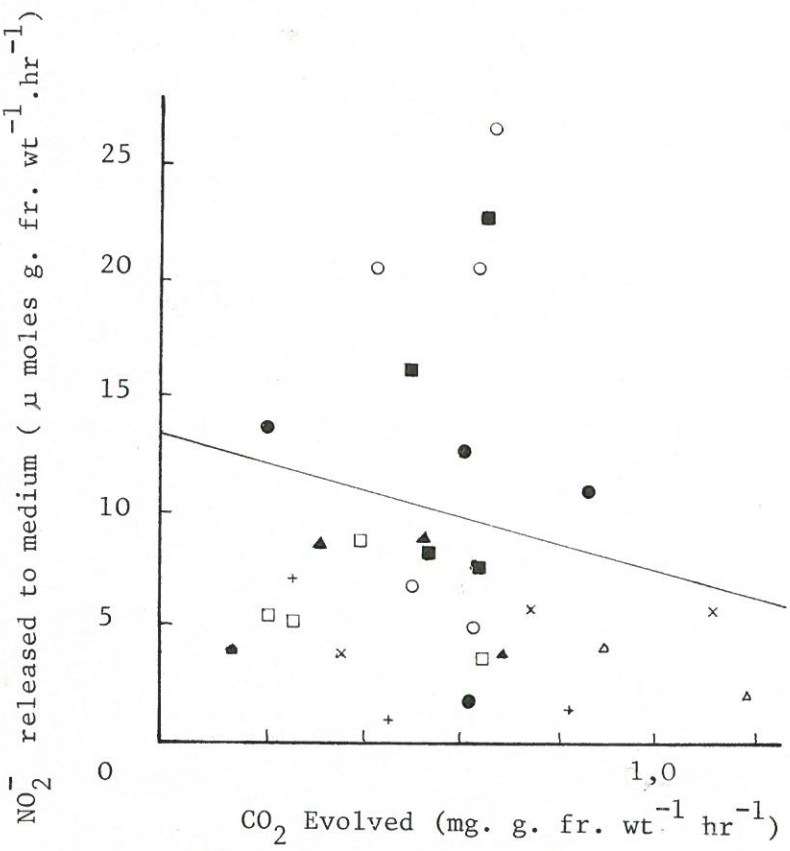
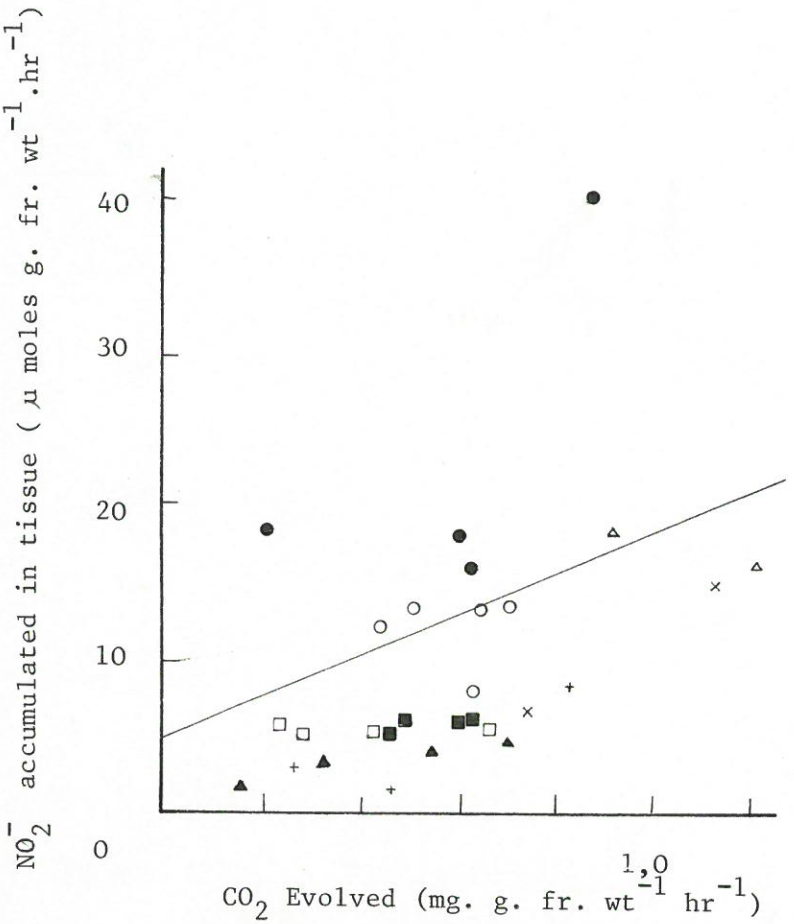


Fig. 6 Relationship between NAR_{tis} and NAR_{med}.



r^2 = 0,03

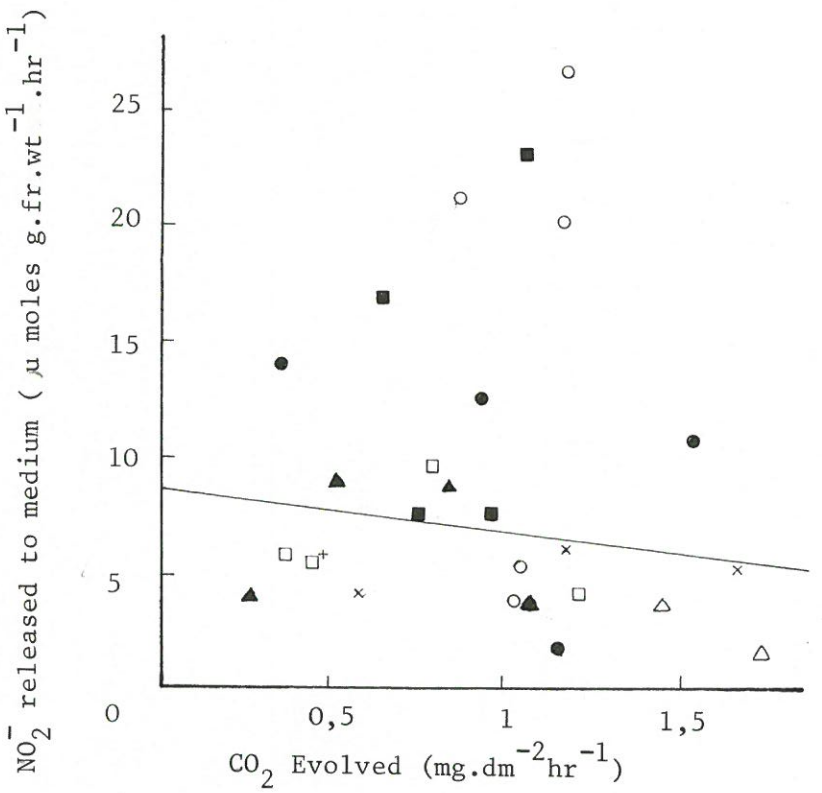
A.



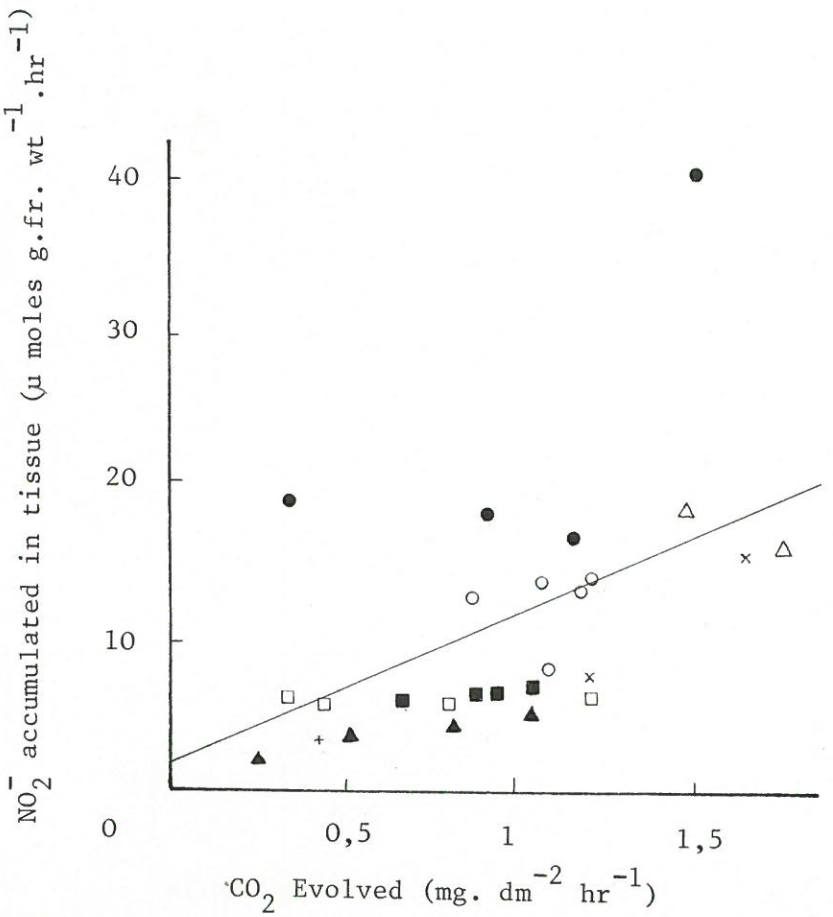
r^2 = 0,13

B.

31 .....



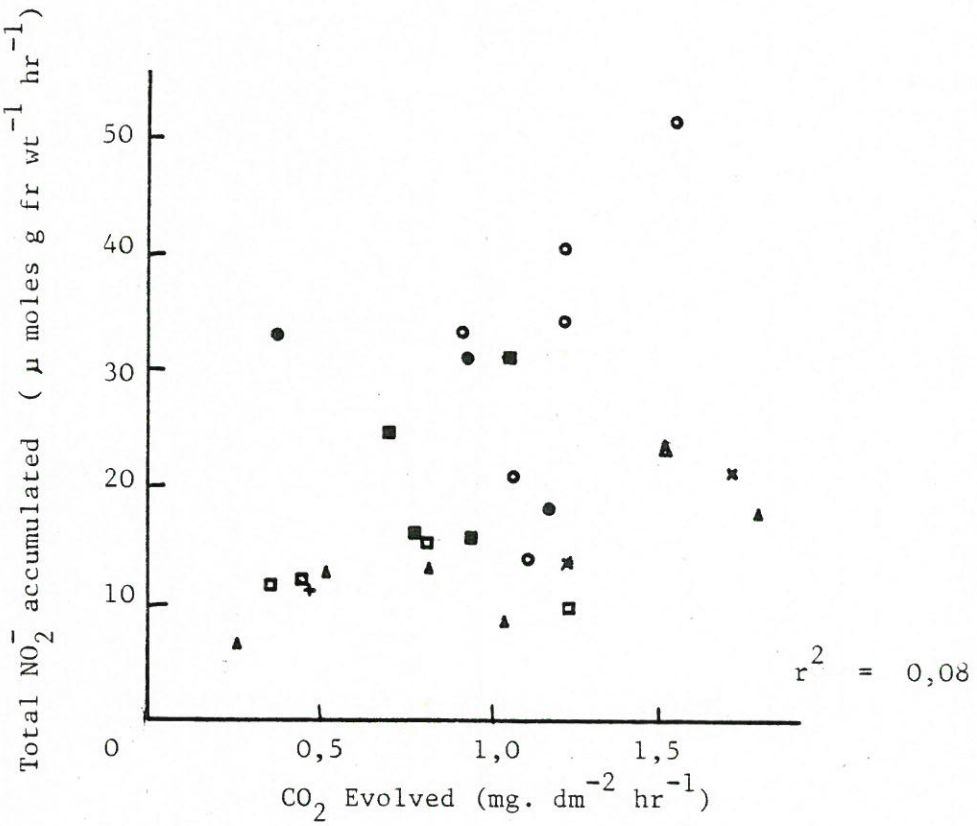
C.



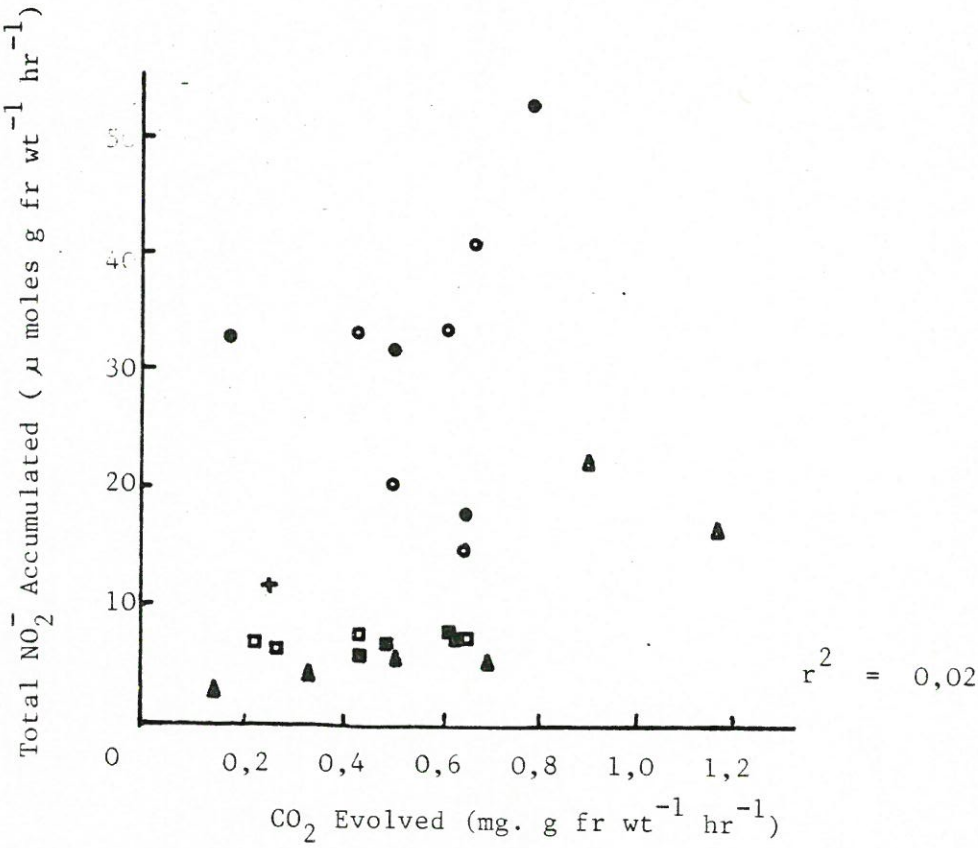
D.

Fig. 4 contd.

32 .....



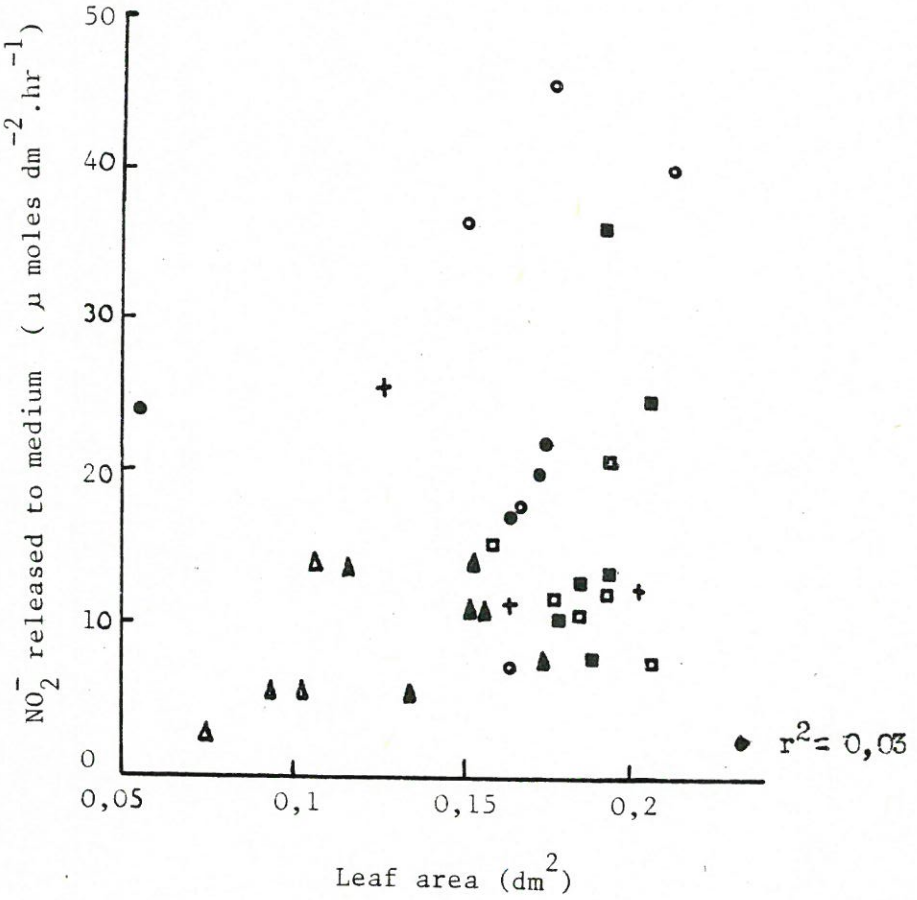
E.



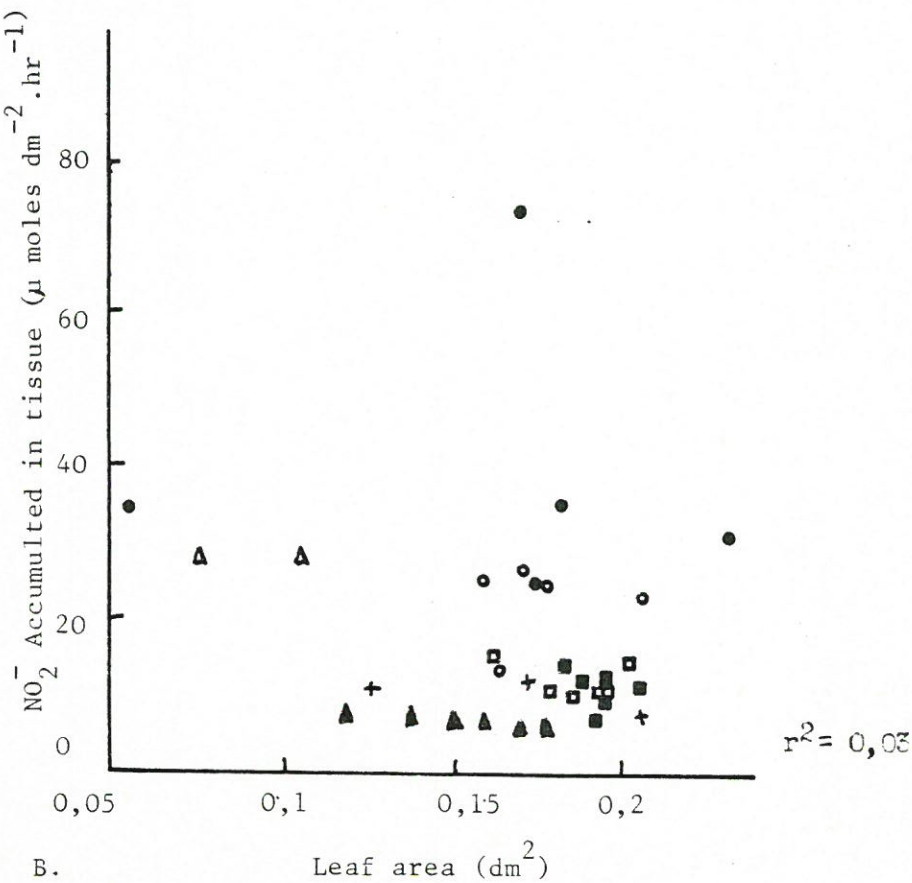
F.

Fig. 7 contd.

33 .....


50
40
30
20
10
0
NO_2^- released to medium (μ moles $dm^{-2}.hr^{-1}$)
0,05
0,1
0,15
0,2
Leaf area (dm^2)
r^2= 0,03

A.


80
60
40
20
0
NO_2^- Accumulted in tissue (μ moles $dm^{-2}.hr^{-1}$)
0,05
0,1
0,15
0,2
Leaf area (dm^2)
r^2= 0,03

B.

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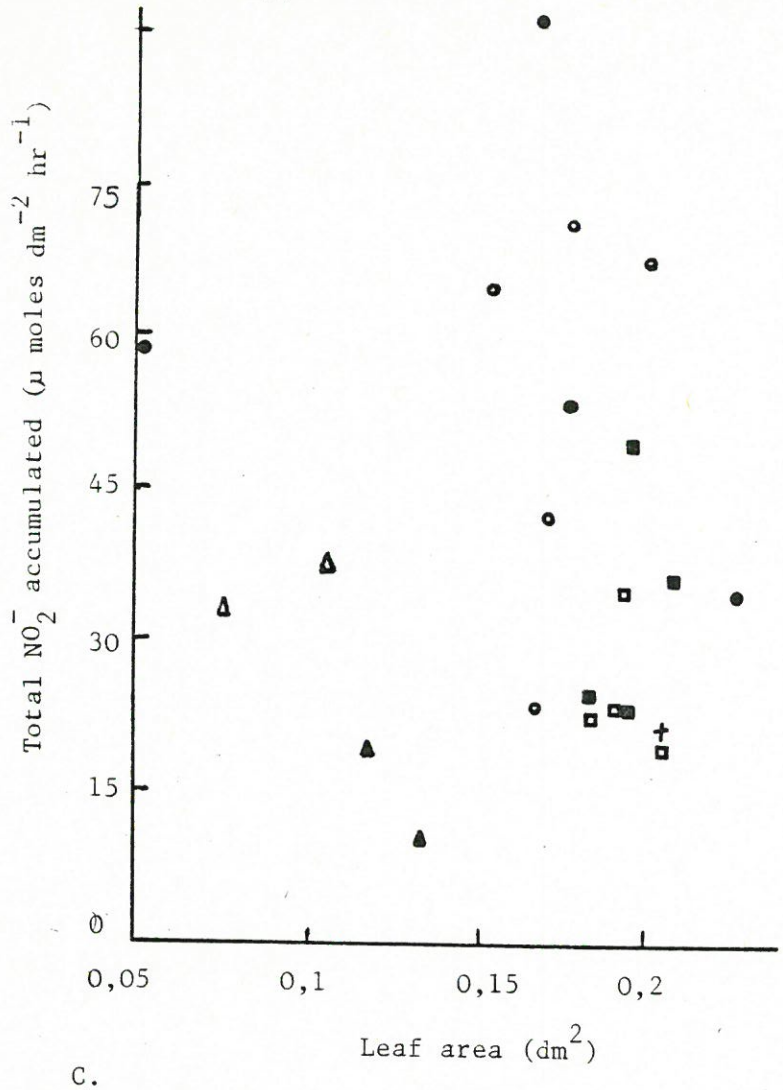
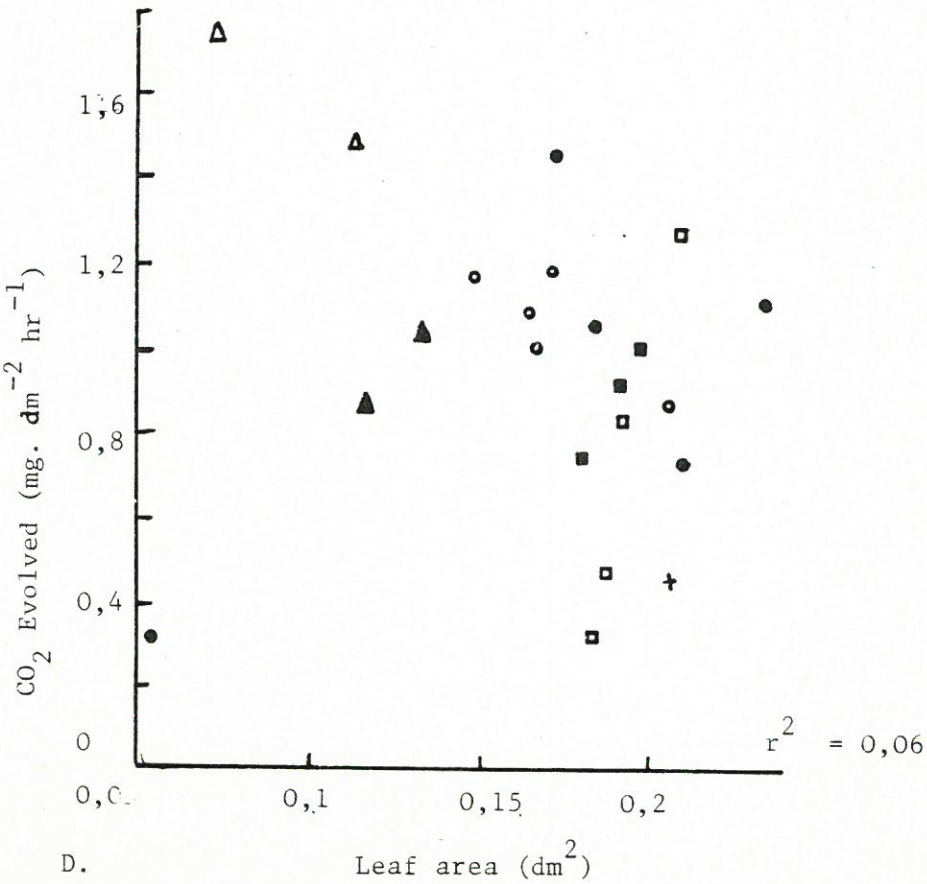


C.



D.

Fig 8 contd.

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4.5 Regression analysis

Table 2 gives r^2 values for the sets of data compared.

4.6 Analysis of variance

Analysis of variance, using the F test after Parker (1973) was used to test for significant differences between the means of the data from each day's work.

4.6.1 Variance in NRA_{med} g fr wt^{-1}

There is between 10 % and 20% chance that each set of data represents data from the same population.

4.6.2 Variance in NRA_{tis} g fr wt^{-1}

There is between 5% and 10% chance that each set of data is from the same population. Using the simultaneous testing procedure (S.T.P.) at 10% level of significance, after Bradu (pers. comm.) the data from 27 July is significantly different (symbol o in Fig. 7 B,D). See Appendix C for calculations.

4.6.3 Variance in NRA_{tot} g fr wt^{-1}

There is between 1% and 5% chance that each set of data is from the same population. Using the S.T.P it was not possible to separate any one set of data which is significantly different from the others at the 5% level of significance.

4.6.4 Variance in DR g fr wt^{-1}

There is between 5% and 10% chance that each set of data is from the same population. STP at 10% level of confidence does not single out any one set of data as being significantly different from the other sets of data.

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TABLE 2: Regression and multiple regression co-efficients (r^2) for interrelationships between various sets of data
Positive or negative slopes are also indicated for regression lines.

Dependent Variable	Independent Variable	r^2	Slope
NRA_{med} g fr wt^{-1}	DR g fr wt^{-1}	0,03	-
NRA_{tis} g fr wt^{-1}	"	0,13	+
NRA_{tot} "	"	0,02	+
NRA_{tot} "	DR dm^{-2}	0,10 (85% level of significance)	+
NRA_{med} + 10 NRA_{tis}	DR g fr wt^{-1}	0,11	+
" + 4 "	"	0,09	+
" + 2 "	"	0,06	+
" + 0,5 "	"	0,00017	+
NRA_{tis} g fr wt^{-1}	DR (u moles CO_2 g fr $wt^{-1}.hr^{-1}$)	DR g fr wt^{-1}	0,13	+
" 2 "	"	0,12	+
NRA_{tot} g fr wt^{-1}	"	0,01	+
" 0,5 "	"	0,02 (46% level of significance)	+
$\frac{NRA_{med} \text{ g fr } wt^{-1}}{NRA_{tis} \text{ g fr } wt^{-1}}$	DR g fr wt^{-1}	0,12	-
NRA_{med} dm^{-2}	Leaf area	0,03	+
NRA_{tis} dm^{-2}	"	0,03	-
NRA_{tot} dm^{-2}	"	0,0002	-
DR DM^{-2}	"	0,06	-

FOOTNOTE: mg CO_2 converted to μ moles so that the relation was worked stoichiometrically as molar quantities of NO_2^- were compared with molar quantities of CO_2.

4.6.5 Variance between means for NRA in those leaves used and those not used for DR measurements.

For means of NRA_{med} there is a greater chance than 20% ($F = 0,12 \ll F_{(1,30)\alpha=0,20} = 172$) that the means are the same.

The means of NRA_{tis} there is a greater chance than 20% ($F = 0,05 \ll 1,72$) that the means are the same.

For means of NRA_{tot} there is a greater chance than 20% ($F = 1,67 < 1,72$) that the means are the same.

5. DISCUSSION

In considering the hypothesis that the reduction of NO_3^- is dependent on the production, in respiratory metabolism, of NADH, (or at least in the dark conditions under which the in vivo NaR assay is carried out), one might anticipate three possibilities, as described in Fig. 9 and its legend.

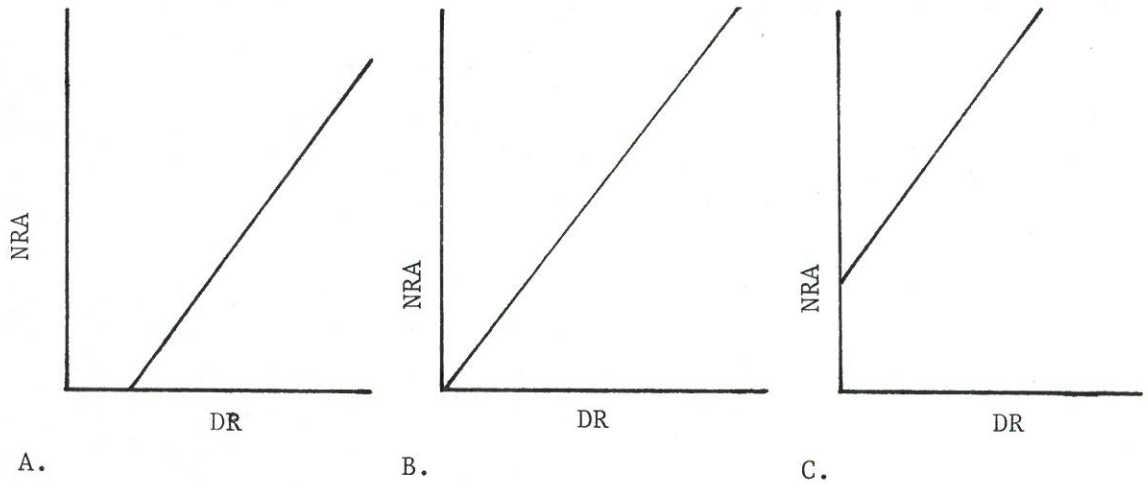

Fig. 9 : Three possible relationships between NRA and DR.
A: NRA proceeds only when the NADH demands by other metabolic activities are met, or else NaR has a stronger competitive ability for NADH, than do the other metabolic reactions concerned. The curve has a negative y- intercept.
B: NADH availability allows NRA to proceed in correlation with DR. The curve passes through the origin.
C: NRA proceeds independently of DR, using NADH available within the chloroplast in addition to that from the mitochondria. This curve has a positive y- intercept.

The possibility mentioned in Fig. 9C is the more likely, since there is NADH in the chloroplast, although these reserves would be expected to disappear quite rapidly, beyond which point NRA would proceed in proportion with DR. If there were no correlation between NRA and DR, the curve shown in Fig. 9C would be expected horizontal (no change in NRA as DR increases).

If NRA is measured in terms of NO_2^- accumulation and some NO_3^- is reduced by cytochromes as Beevers (1961) reports for some micro-organisms, and in non-chlorophyllans tissue, then NO_2^- accumulation can be due either to cytochrome, or to both NaR using NADH from glycolysis and cytochrome reducing power. Such a situation, with both glycolysis and cytochrome giving the reducing power, would have additive effects. Supposing that reduction via cytochrome proceeds immediately, but via glycolytic NADH is as in Fig. 9A, a biphasic curve would be expected, as in Fig. 10.

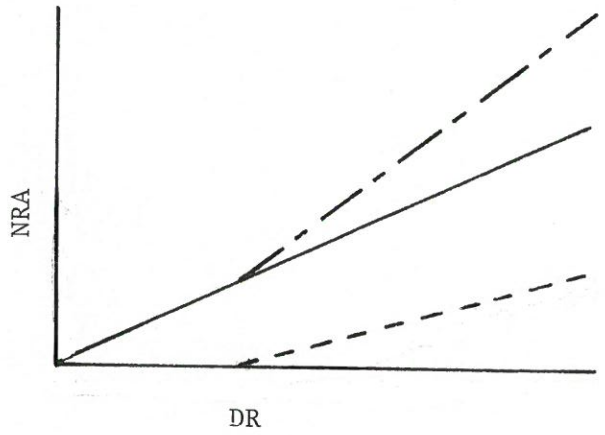

Fig. 10: NRA dependence on DR where reducing power is obtained via cytochromes (solid line,———), but supplemented by glycolytic NADH (dashed line, ----), when the other metabolic requirements in the cell are met, or if NaR competes more strongly for NADH than do the other metabolic reactions, thus giving the half broken line (——— - ———).

This situation would be further complicated if NADH reserves in the chloroplast were used where the initial curve (cytochrome reducing curve)would have a positive y- intercept value.

In each of the above situations straight line (or biphasic straight line) correlations would be expected. However, the correlation between NRA and DR when observed over time, would give a curve as in Fig. 11, where the ratio NRA:DR would exceed, but approach a constant value (suppose unity if the relationship is simple) as chloroplastic NADH reserves are used up.

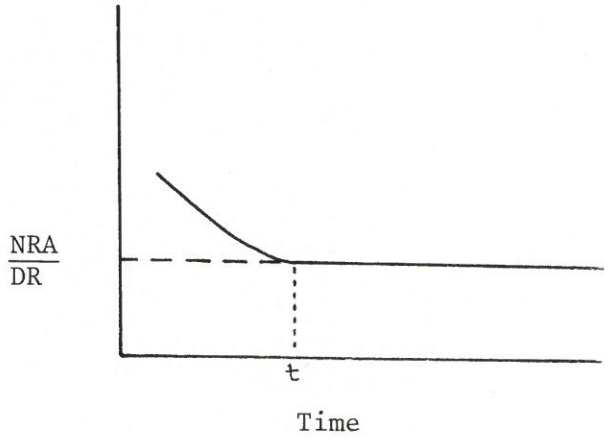

Fig. 11: Ratio of NRA:DR decreases as NADH reserves in the chloroplast are depleted, to stabilize as the only reducing power available is from DR.

It can be seen from Fig. 11 that any correlation sought between NRA and DR should be sought beyond time t as shown, when all reserve NADH is used, and all NRA is dependent on DR. The time required to utilize all NADH reserves may vary in different cells. This initial increased reduction of NO_3^- would not be detected in the assay as carried out since only two samples were taken, i.e. an initial sample prior to incubation, and a final sample. No allowance was made for avoiding recording NRA prior to time t. The amount of reserve NADH may be dependent on the amount of light on the plant (which should have been essentially similar).

While the attempt was made to keep growing conditions uniform, the transport of plants between phytotron and each laboratory varied in length of time, and temperature, humidity and light conditions varied. This may have had a small effect on DR as well as on NRA results.

For this reason the analysis of variance was carried out as outlined, and it is interesting to note that NRA_{tis} has the highest r^2 values (see Fig. 7), with NRA_{med} and NRA_{tot} having lower values. Looking at Fig. 7 B,D, it can be seen that NRA_{tis} data for several different days has very close correlation with DR (e.g. data for 31 July (+) r^2 = 0,79; 17 August (▲) r^2 = 0,99; 23 Sept. (■) r^2 = 0,92. Yet for the data for 26 July (o) r^2 = 0,01 and

24 August (□) r^2 = 0,99 which appear to have the straight curves the r^2 values indicate that there is no covariance of NRA with DR, while the former 4 show an almost definite covariance of NRA with DR.

One would expect a positive correlation to exist where NRA would increase as DR increases. This is seen for NRA_{tis} and NRA_{tot}, but not for NRA^{med} where the slope of the regression line is negative, implying a negative correlation. Even if a direct correlation between NRA and reducing power from respiratory processes does not exist, NO_3^- uptake would probably be more active with higher respiratory activity. Beevers(1961) points out the close link between high NO_3^- concentrations in an external medium and high respiration rates. However, this is most noticeable in root or storage tissue, and Amory (pers. comm.) reports that no salt respiration has been detected in maize leaf material with vacuum infiltration of KNO_3.

The NRA_{med} data suggests that more NO_2^- leaks to the medium when respiratory activity is low. This was tested by regression analysis, plotting the ratio of NRA : NRA_{tis} vs DR, but this shows a low r^2 value of 0,12, and the slope is negative. Therefore, it can be suggested from the data that NO_2^- leaks more readily into the medium when respiratory activity is low, as an NRA_{med}:NRA_{tis} vs DR relationship may suggest a negative correlation.

It is interesting that the higher correlation for NRA_{tot} vs DR is seen in Fig. 7E, and not 7F, and this is obviously linked to the high r^2 value shown for NRA_{tis} g fr wt^{-1} vs DR dm^{-2} in Fig. 7D. This may suggest that the regression analysis is picking out the correlation between the fresh weight and the area of the leaves. This is strange when considered in the light of the fact that Figs. 8 and 9 do not show a definite relationship between leaf area and NRA, or between leaf area and DR. Negative slopes were obtained for each of these regression lines. Furthermore, one would expect that using the two variables, viz. leaf area and leaf dry weight, would introduce a factor which would add to the variability, there-by lowering the r^2 value below that for

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NRA_{tis} g fr wt^{-1} vs DR g fr wt^{-1} where the fresh weights cancel out so that it is simply NRA_{tis} vs DR.

Finally, in connection with the interrelationship between NRA and DR, it is evident from the above data, and discussion, that a correlation has not yet been determined. A regression co-efficient of at least $r^2 = 0,5$ would be desirable in order to feel confident of a relationship. Even this value is arbitrarily selected as a value of $r^2 = 0,9$ is usually required for a conclusive argument. Furthermore, using single classification of variance, or multiple regression, it was found (see table 2) that as more weight was placed on NRA_{tis}, and lesson NRA_{med}, the r^2 value increased, to a maximum when NRA_{tis} is used alone in the regression with DR. This r^2 value was the greatest ($r^2 = 0,13$) of all in table 2, while that for NRA_{tis} - DR (μ moles CO_2 g fr wt^{-1}) vs DR g fr wt^{-1} also has $r^2 = 0,13$, if going beyond 2 decimal places it is probably smaller than that for NRA_{tis} vs DR, as it can be seen that the r^2 value decreases as less weight is put on NRA_{tis}, and more on DR (μ moles CO_2 g fr wt^{-1}. hr^{-1}).

The values for NRA and DR agree with those published in the literature For example, the DR (0,2 - 1,8 mg CO_2 dm^{-2} hr^{-1}) results are in the same range as those obtained by Amory (pers. comm.), McCree and Troughton (1966) report an average respiration rate for white clover of 5 mg CO_2 dm^{-2} hr^{-1}. The <u>in vivo</u> assay gave NaR activity ranging from 8 to 50 μ moles NO_2^- g fresh weight hr^{-1} for NRA_{tot}, 2-25 μ moles g fr wt^{-1} hr^{-1} for NRA_{med}, and 2-40 μ moles g fr $wt^{-1}hr^{-1}$ for NRA_{tis}. Deckard et. al (1973) report activity in the range 6-12 μ moles g fr $wt^{-1}hr^{-1}$, and Klepper et. al (1971) report NaR activity in maize, also using the <u>in vivo</u> assay, of about 2,8 μ moles g fr wt^{-1} hr^{-1}.

From this data, what may possibly be safely said is, the hypothesis that there is a dependence of NaR on respiratory activity for reducing power, cannot be rejected. The values obtained in this work are of the same order of magnitude as are results from the literature, but there is a great deal of variability within the data, and between the different sets of data, NRA_{tis} shows the

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most promising regression coefficient, and individual sets of data, on the whole, show positive and consistent correlations with DR. From the analysis of variance, the data of 27th July was separated as being significantly different. Regression analysis for NRA^{tis} vs DR excluding those 4 data points gives an r^2 value of 0,39, and a positive slope for the curve. If the differences between the means for the sets of data were reduced, a good positive correlation would quite probably be found, such as for the data of 31st July r^2 = 0,79, 23rd September r^2 = 0,92, or even that for 17th August (r^2 = 0,99). Even the data for 27th July, which is separated off as being significantly different has an r^2 value of 0,54, and the one point in that set of data is clearly at an extreme, being almost twice as high as the other 3 values.

If experimental error were reduced, and more samples used each day, it is almost certain that a better correlation would be found between NRA and DR. Work in the future can include DR determination as done in this work, as well as with anaerobic conditions. This would determine if glycolysis, or fermentation, is more closely related to NRA, and, if there is a contribution via the cytochromes as NO_3^- acts as terminal electron acceptor, then lower results should be obtained using O_2 free air.

While O_2 should be excluded for this test, CO_2 free air should not be used (i.e. N_2 gas alone) as unnatural CO_2 diffusion gradients would be formed, drawing O_2 from the leaf at a greater rate, until equilibrium is reached, than normal. This would result in an overestimate of DR.

Other work should include DR readings followed by the <u>in vivo</u> assay for non-chlorophyllous tissue, where NaR is known to be linked with DR. It would be very enlightening to see what correlation is found for this tissue while using the same experimental techniques. If no correlation, or a poor correlation, is observed, it may suggest a fault in the tehnique.

It would also be worthwhile using about 10 leaf chambers in parallel,

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and feeding simultaneously into the same IRGA. The CO_2 evolved by all ten leaves can be determined, and the in vivo assay done on all 10 leaves together, as one sample. This will mean that four or more sets of ten leaves can be used in one day, eliminating a great deal of variability by using the average rate for ten plants as one sample. Gray (pers. comm.) points out that this will give more meaningful direction to the plant breeder in selecting genetic material from a population. Care would then need to be taken to ensure that the air flow in each cuvette is the same.

A refinement of the in vivo assay method, as used by Gray (pers. comm.), is to homogenize the leaf material in the incubation medium before taking the final aliquots for NO_2^- determination. Care must be taken to ensure, however, that the chlorophyll is all properly removed in the centrifugation step. This would give a far more accurate value for the amount of NO_2^- produced, as some would otherwise be lost prior to sampling.

Another important test would be to simply test the DR activity of each of several cultivars of maize and to then test for a correlation between yield for that cultivar, and dark respiration. If such a correlation is found, an idea can be obtained as to the similarity of this correlation and that between yield and NRA. If the correlation between yield and DR is either positive or negative, it may give a clue as to how a correlation between NRA and DR may be found if it is not found using the ideas presented above.

The measurements of dark respiration could also quite simply be carried out in the field, and the plants left intact, and the yields of the same plants recorded as they mature.

46 .....

6. CONCLUSION

Since nitrate reductase in non-green plant tissues receives reducing power from the respiratory metabolic pathways, and in carrying out the in vivo nitrate reductase assay with green tissues, the activated enzyme is suddenly placed under dark conditions, it would be expected that if it continues to function, it must be receiving most of the reducing power from the respiratory pathways. Since the in vivo assay is also carried out in the absence of oxygen, it is reasonable to assume that the fermentation pathway of glycolysis may be operating, unless nitrate is functioning as the terminal electron acceptor for the electron transfer chain, thereby allowing a flux through the TCA cycle. Beevers (1961) explains that these respiratory, or modified respiratory processes occur as a regular process in some micro-organisms, and while the enzyme in maize would be slightly different from that in these micro-organisms, it would probably be capable of using the same source of reducing power.

The writer proposes that there are three sources of reducing power for NaR in situ if dark respiration takes place in green tissue in the light. At least, in non-green tissues, or in green tissue in dark and anaerobic conditions, reducing power is obtainable via the cytochromes of the oxidative phosphorylation electron transport chain, and NADH produced in glycolytic fermentation. This is outlined in Fig. 12

For this reason, a correlation was sought between NaR activity and Dark respiratory activity in maize seedlings. The data suggests that such a correlation may exist, but further refinement of the technique is necessary before a definite statement to this effect can be made. The best regression co-efficient found was $r^2 = 0,39$ for NO_2^- accumulated in the tissues vs Dark respiration when a significantly different set of data was excluded. When this data was included, r^2 was 0,13, which is still higher then any other regression coefficient. These results are promising although not yet conclusive.

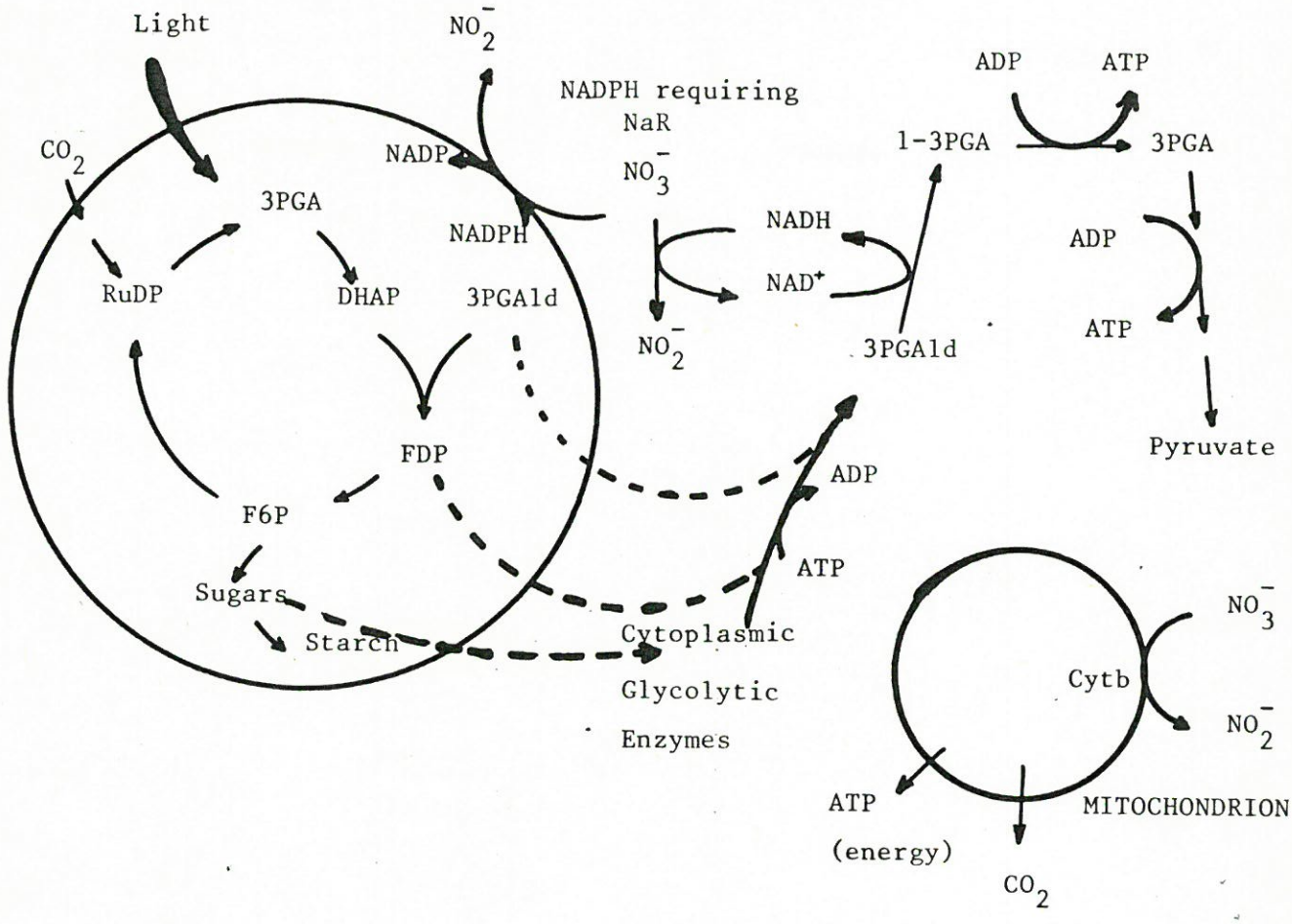

Fig. 12: Interrelationship between light, glycolysis, cytochromes and nitrate reduction. The $NADH_2^+$ generated would be used by other $NADH_2^+$ requiring cytoplasmic reactions as well as for nitrate reduction. In the in vivo nitrate reductase assay NO_3^- can also be reduced by the cytochromes in the mitochondrial cytochrome chain, and by Glycolate dehydrogenase generation of NADH in or near the chloroplast. $NADPH_2^+$ requiring NaR, if present, can function only when light generates $NADPH_2^+$ in the photosynthetic electron transport chain.
1,3 PGA: 1,3-diphosphoglyceric acid; 3 PGA : 3-phosphoglyceric acid, 3 PGAld: 3-phosphoglyceraldehyde, DHAP: Dihydroxy Acetone Phosphate; FDP = Fructose 1,6-di-phosphate, F6P: Fructose-6-Phosphate; RuDP: Ribulose-1,5-diphosphate; Cyt b = Cytochrome b ; ADP: Adenosine diphosphate; ATP: Adenosine triphosphate. (Based on Klepper et.al.(1971))

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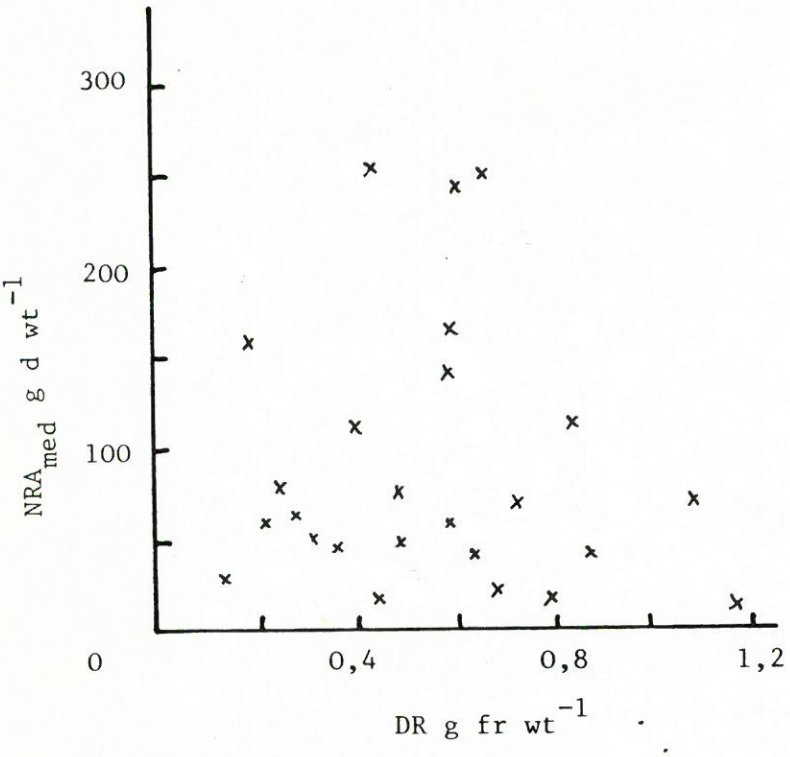
- Cresswell, Prof. C.F. Dept. Botany and Microbiology
- Gray, Vincent Dept. Botany and Microbiology.

50 .....

APPENDIX A

51 .....



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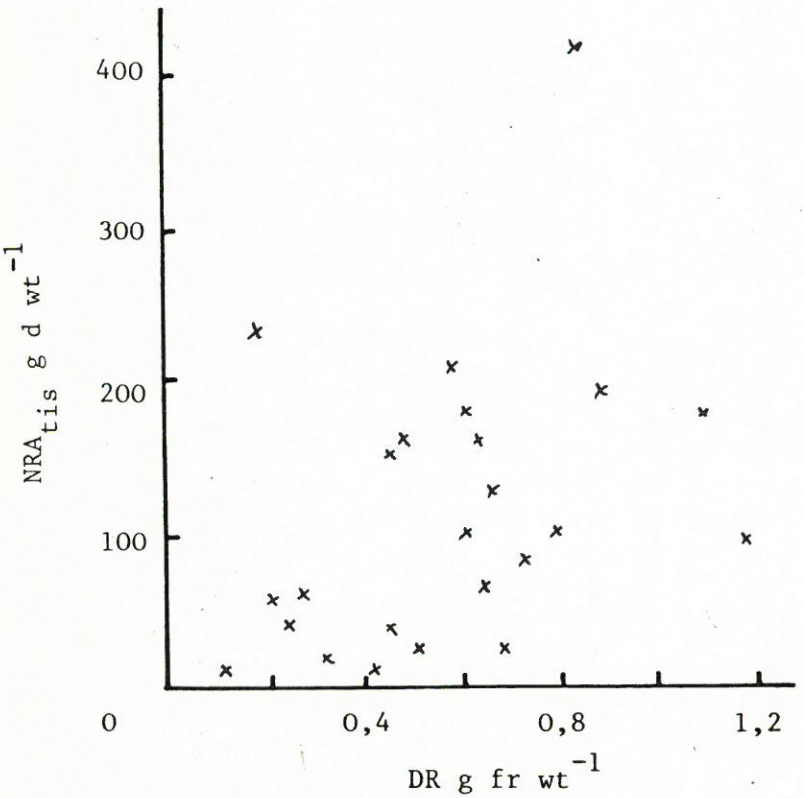
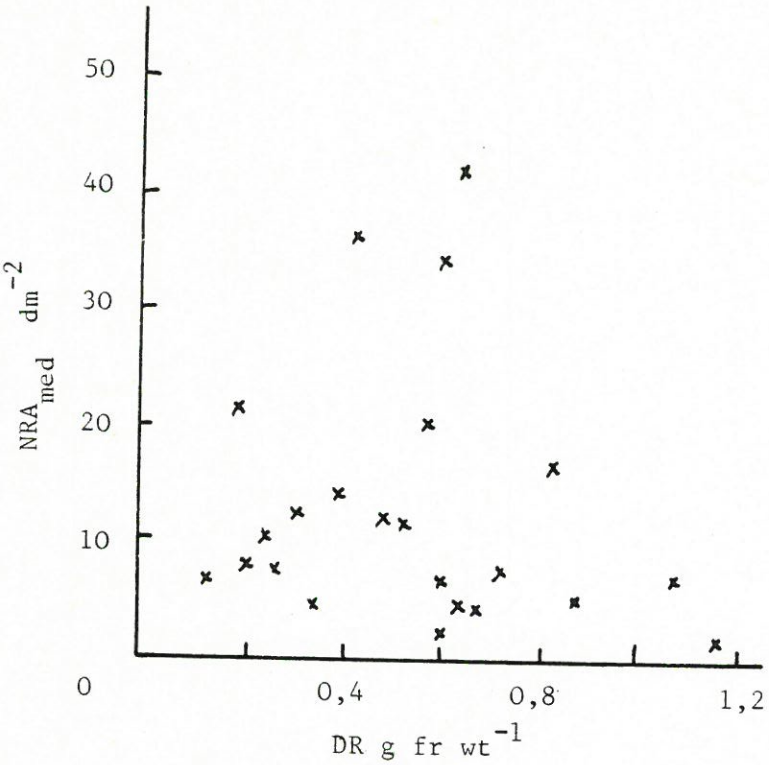


B.

Fig. 13 : Relationships between NRA in μ moles NO_2 produced per hour against DR in mg CO_2 evolved per hour.

52 .....



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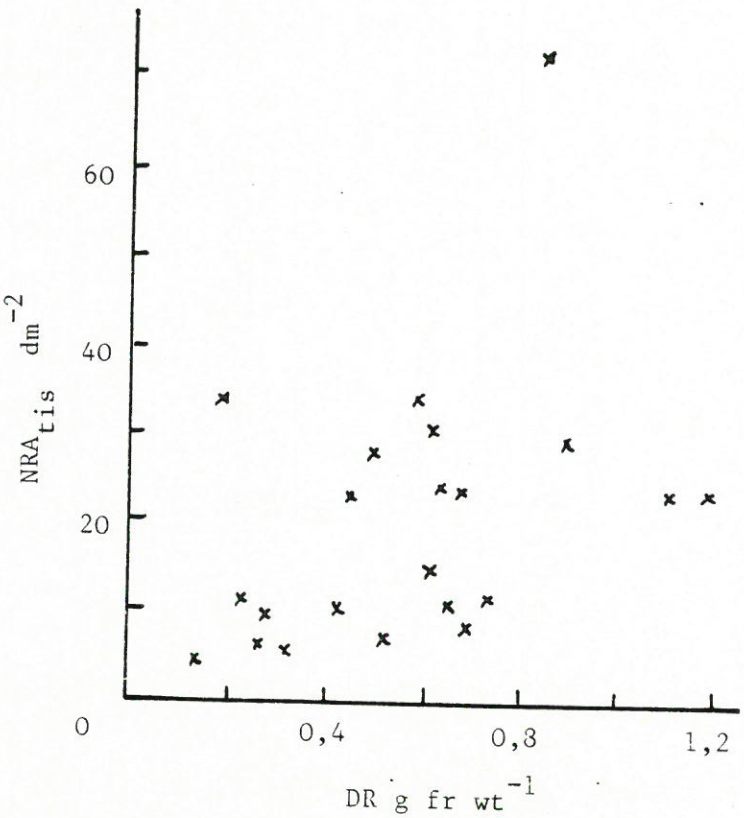
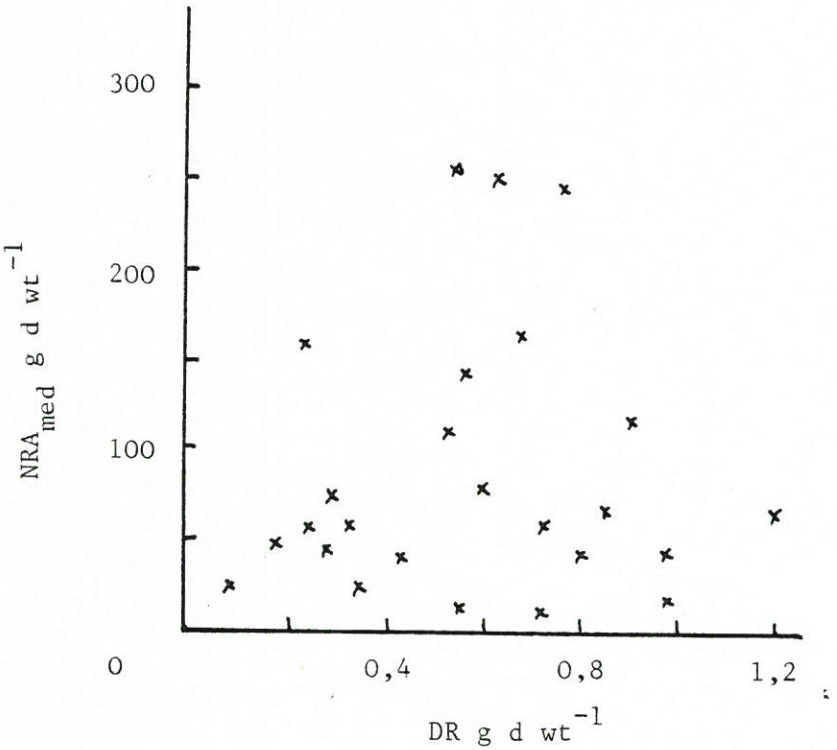


D.

53 .....

Fig. 13 contd.



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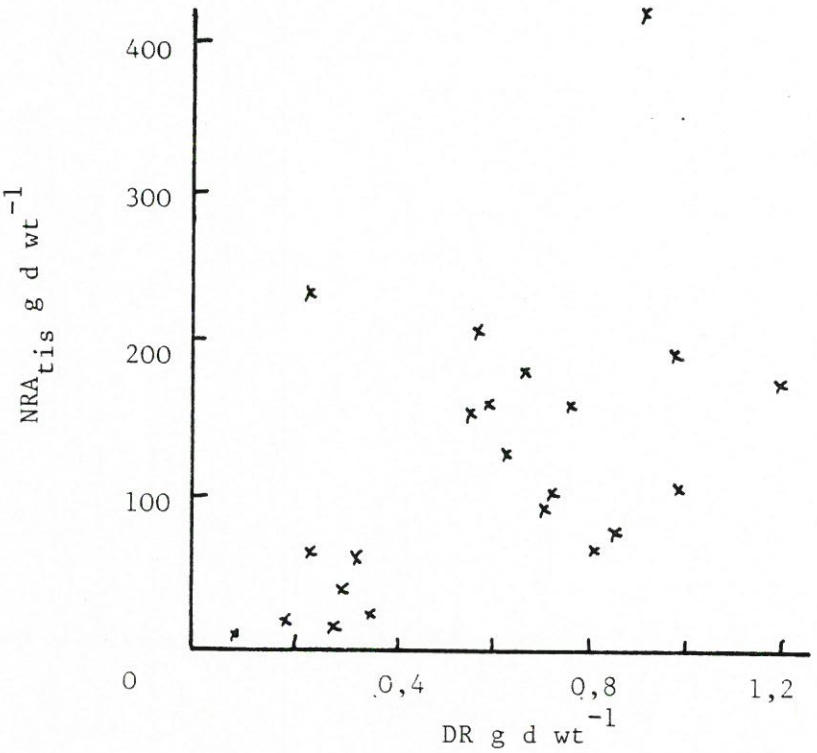

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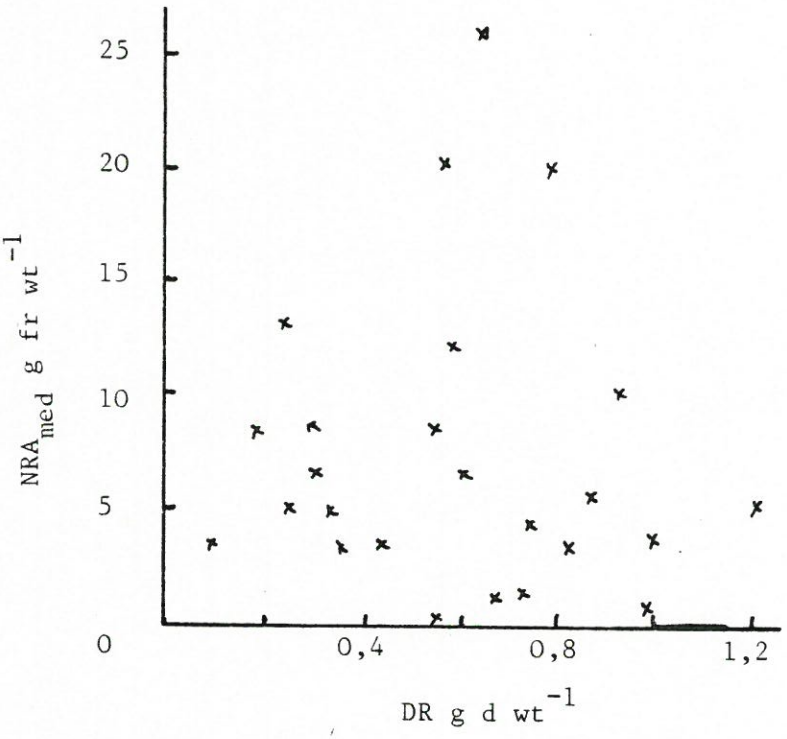
Fig. 13 contd.

·54 .....



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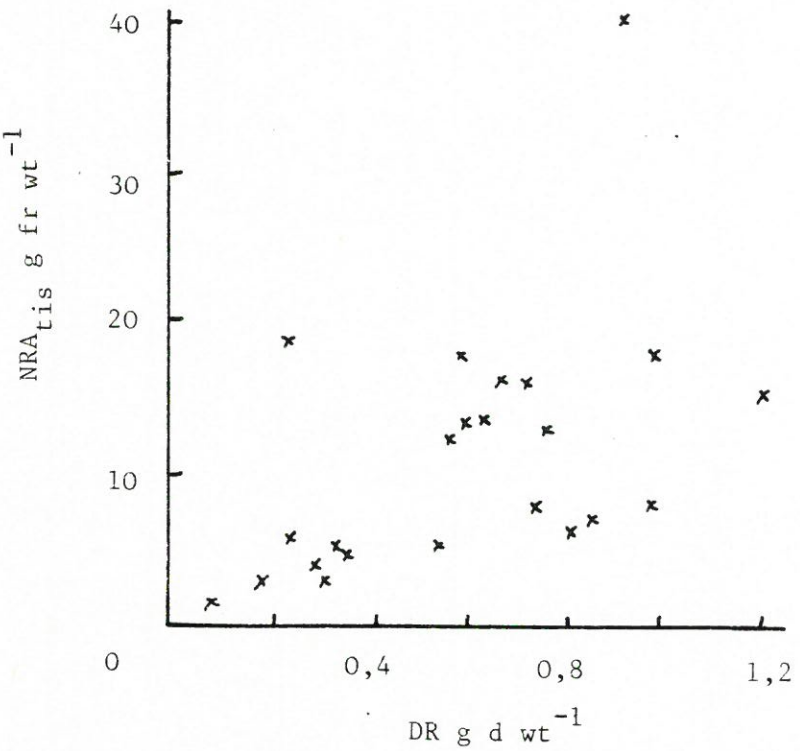
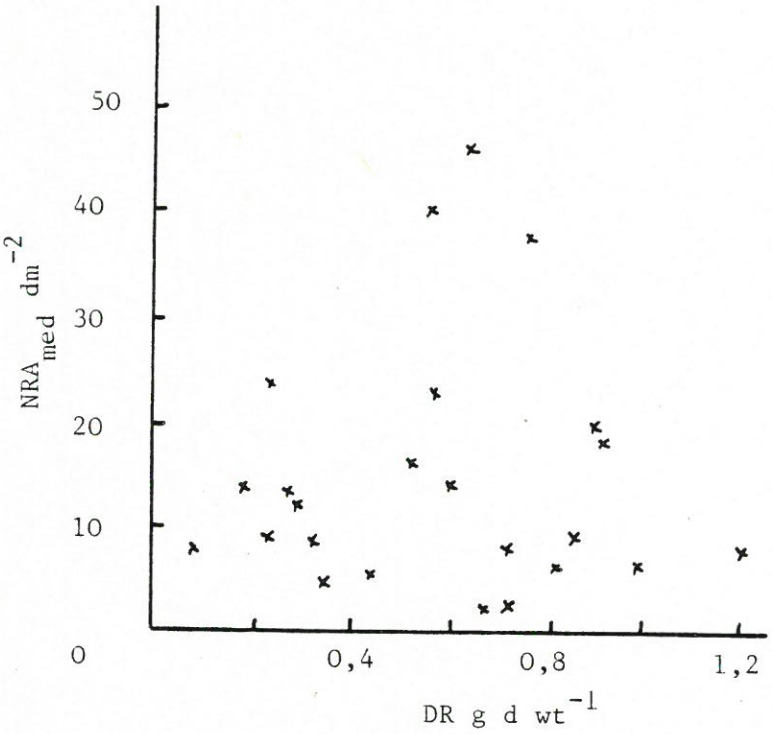


H.

.55 .....

Fig. 13 contd.



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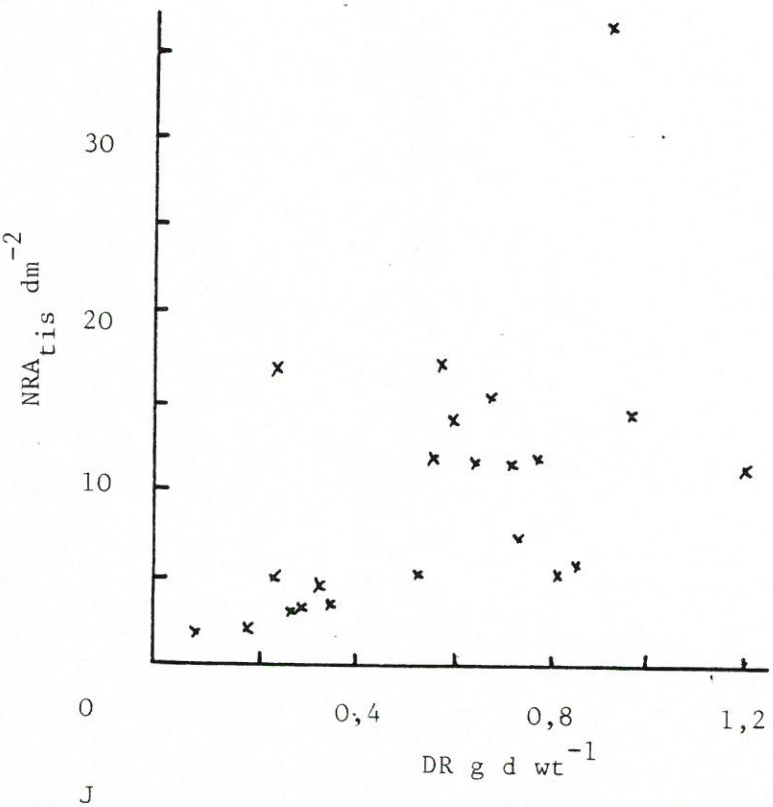
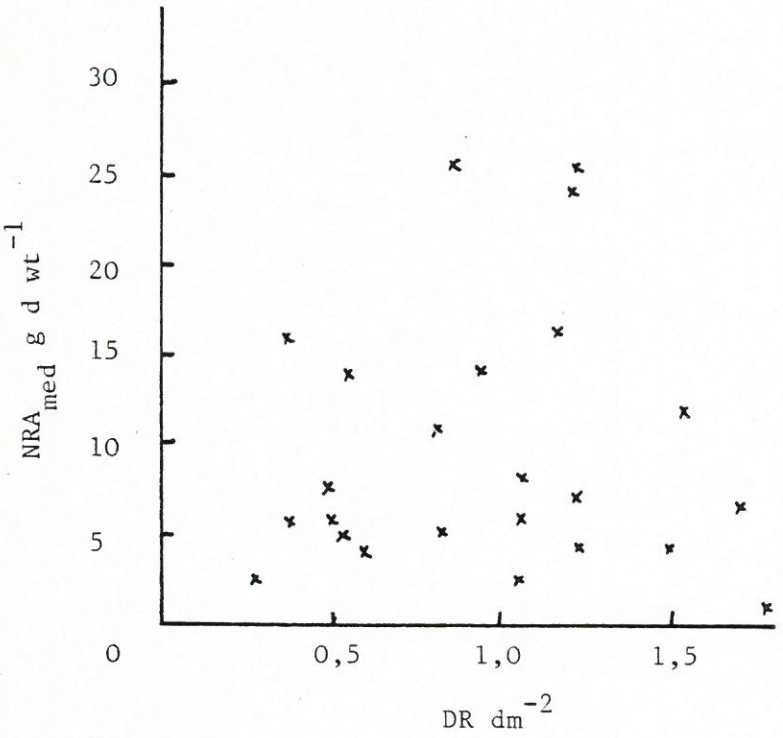


J

Fig. 13 contd.

56 .....



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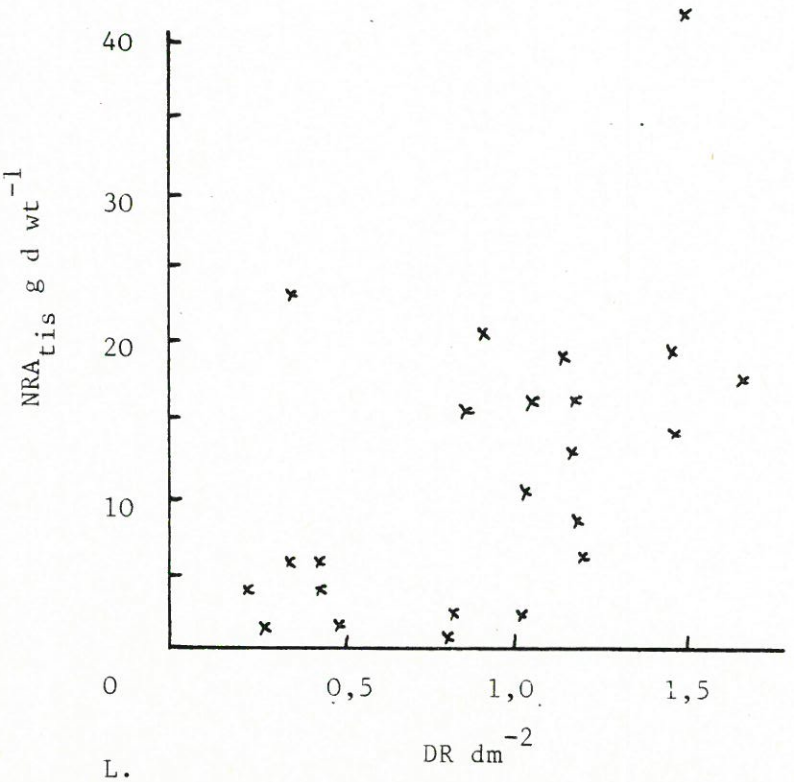
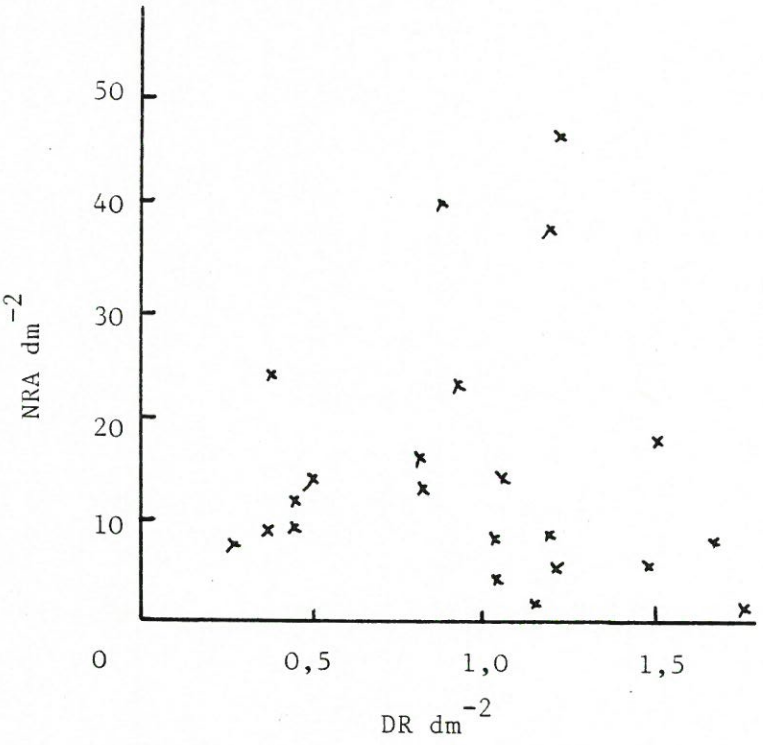


L.

Fig. 13 contd.

.57 .....



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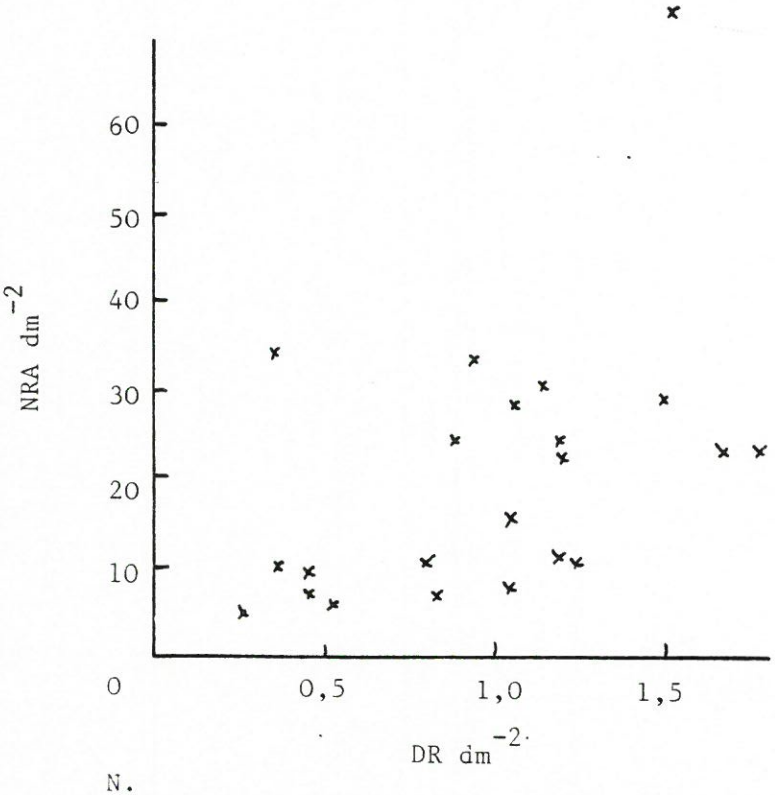
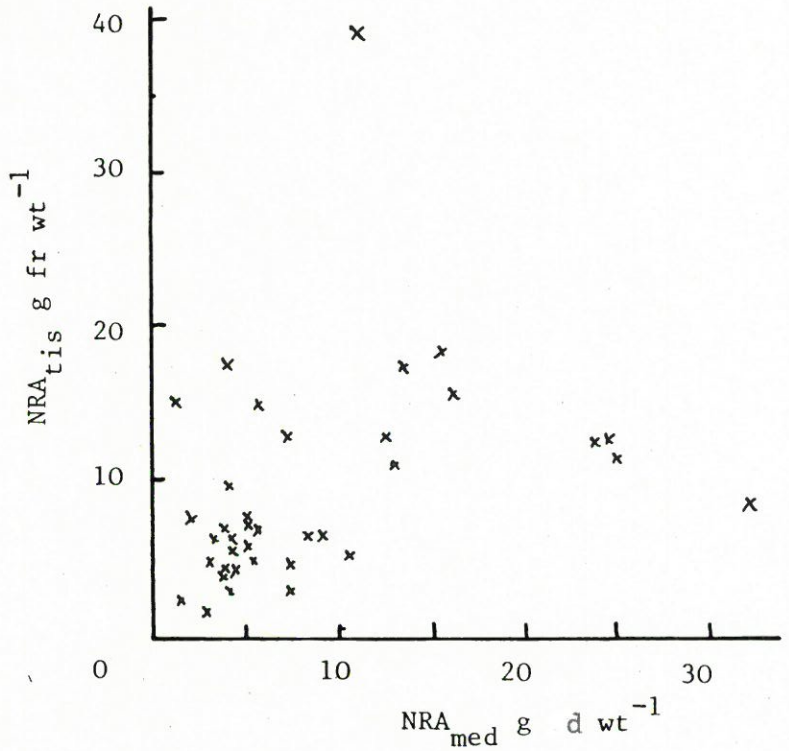


N.

Fig. 13 contd.

58 .....

APPENDIX B



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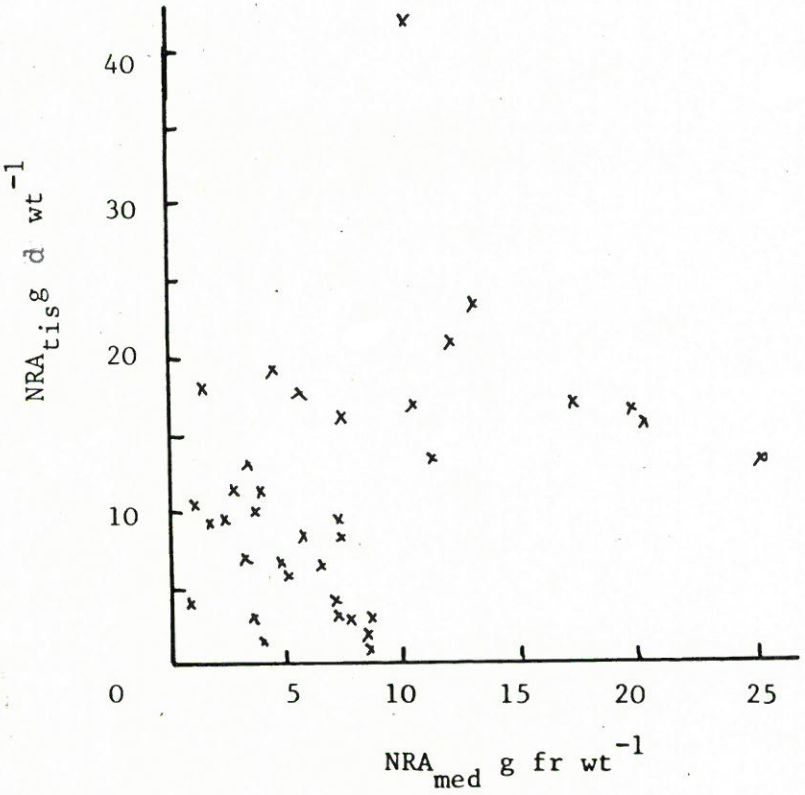
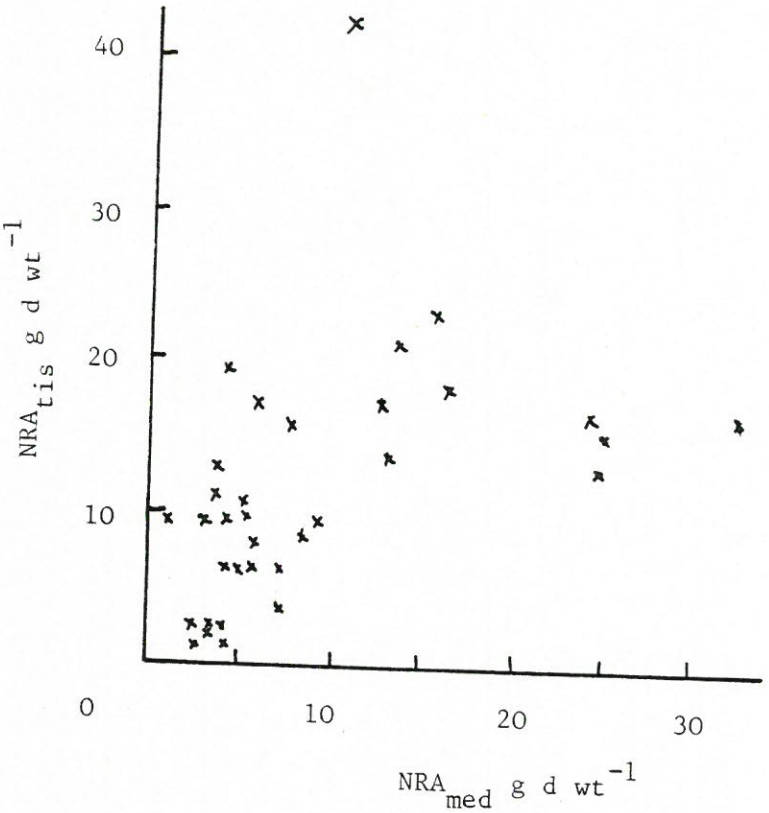


B.

Fig. 14 : Relationships between NO_2^- accumulated in the tissue, and that released to the incubation medium in the <u>in vivo</u> NaR assay.

60 .....



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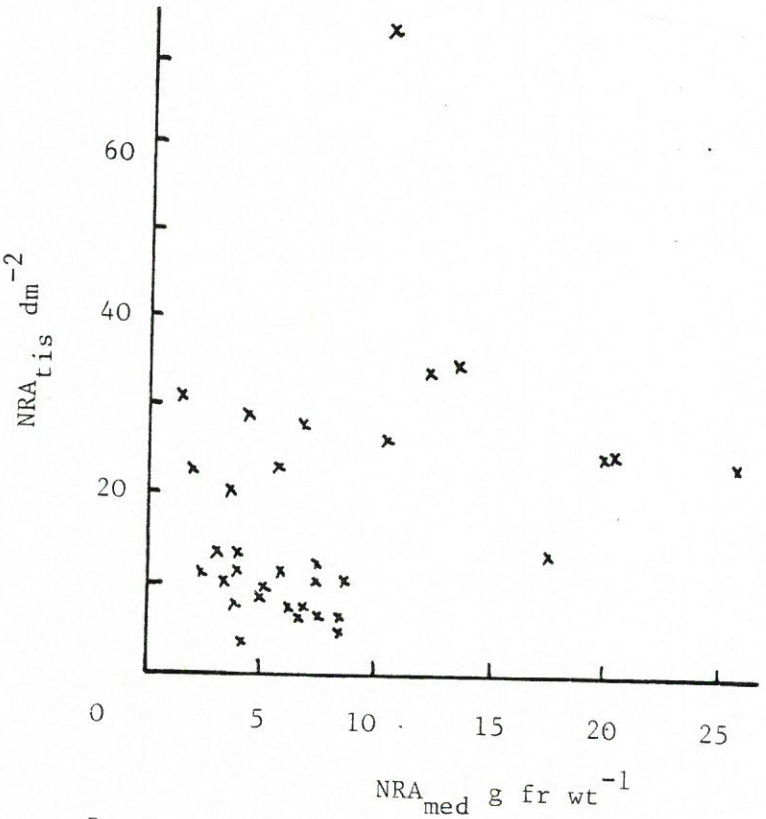
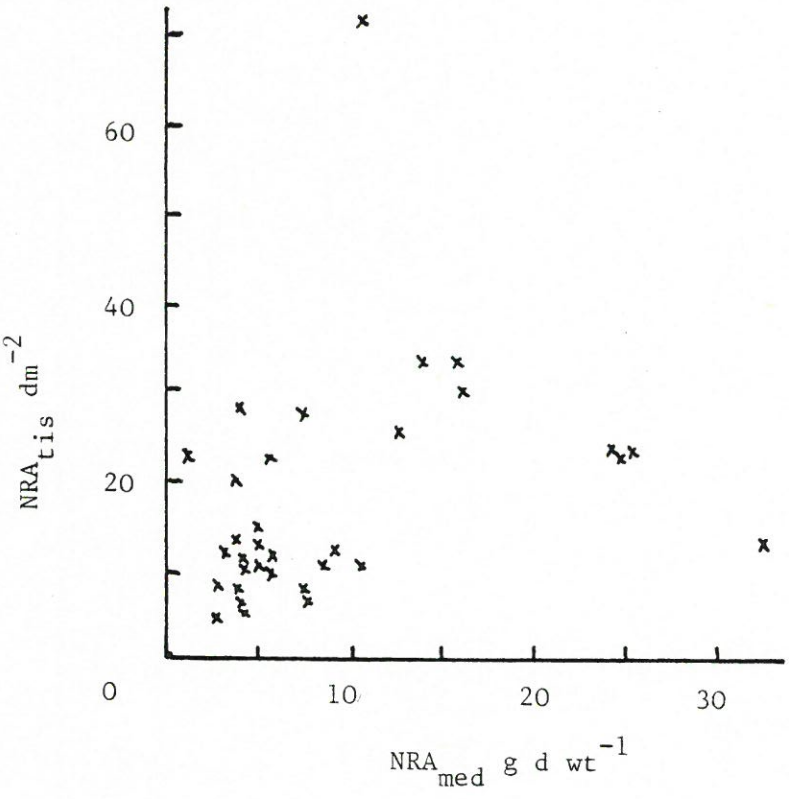


D.

Fig. 14 contd.

61 .....



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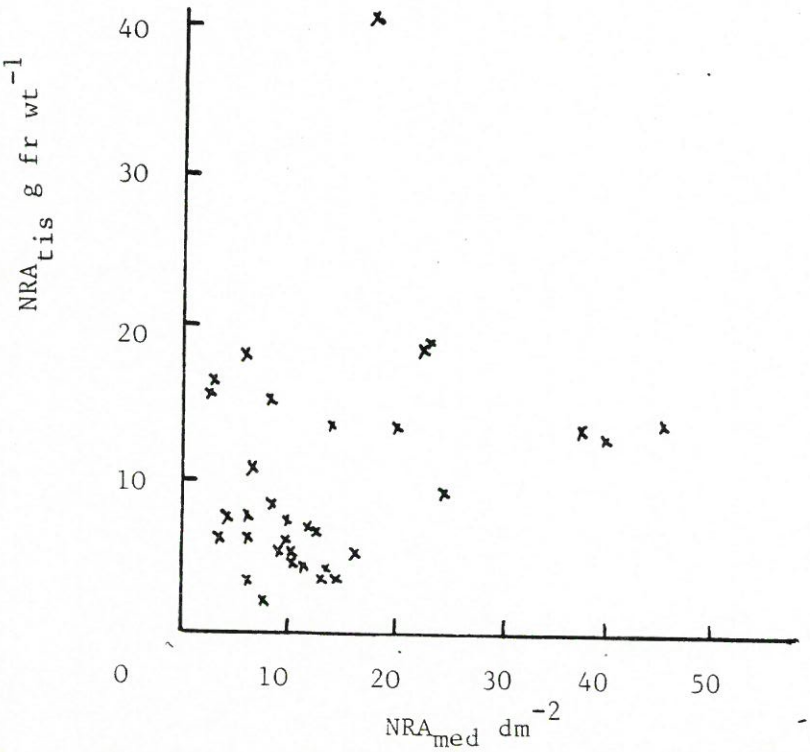
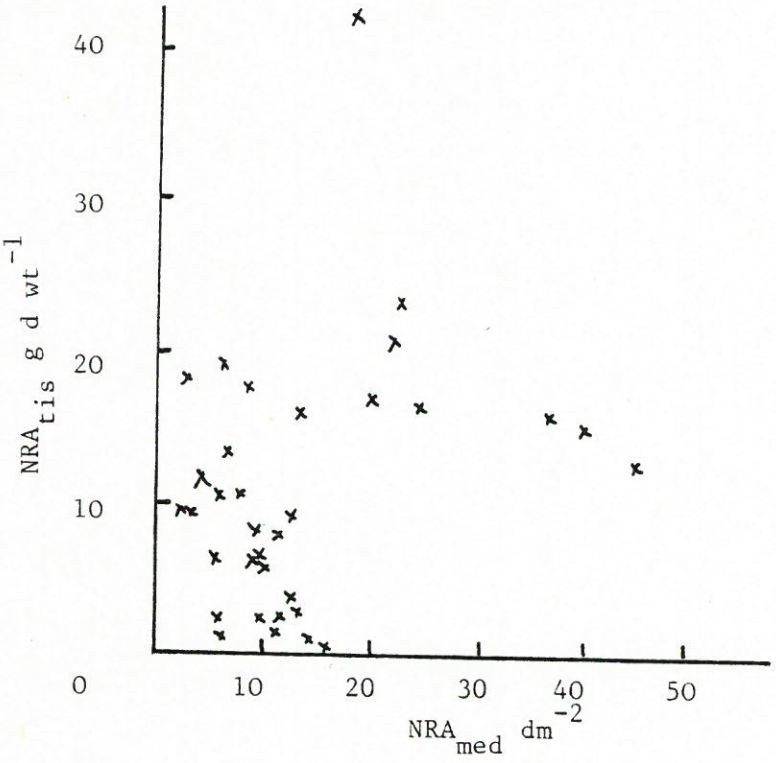


F.

62 .....

Fig. 14 contd.



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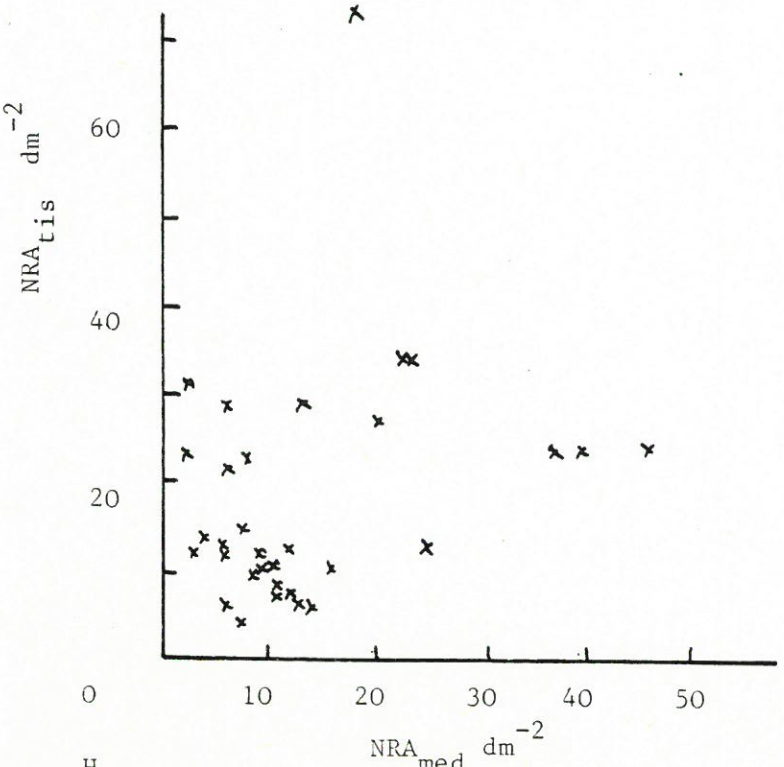

H.

Fig. 14 contd

63 .....

APPENDIX C

An example of the analysis of Variance, after Parker (1973) using the data for NRA_{tis} g fr wt^{-1}.

1) H_o: The means of each group of data are the same.
   H_a: The means are not the same.

2) $\alpha = 0{,}05$

3) Statistic $\frac{SSG}{SSE}\ \frac{N-r}{r-1}$ = f value

   SSG = Group sum of Squares = $\frac{\Sigma(\Sigma x)^2}{n} - \frac{(\Sigma\Sigma x)^2}{N}$

   SSE = Error sum of Squares = SST_o - SSG

   SST_o = Total sum of Squares = $\Sigma\Sigma(x^2) - \frac{(\Sigma\Sigma x)^2}{N}$

   N = Total number of observed values

   r = Number of data sets

   N-r = Error degrees of freedom

   r-1 = Groups degrees of freedom

   If H_o is true, $\frac{SSG}{SSE}\ \frac{N-r}{r-1} = f_{(r-1;N-r)}$ is f distributed with (r-1) degrees of freedom for numerator and (N-r) degrees of freedom for denominator.

4) H_o is rejected if $f_{(r-1;N-r)} \geq f_{(r-1;N-r)\alpha}$
   Where $f_{(r-1;N-r)} \geq C$: C is chosen such that $PH_o [\, f_{(r-1;N-r)} \geq C \,] = \alpha$

5)

	26 July	27 July	31 July	11 Aug	17 Aug	24 Aug	23 Sept
	275 267 274 251 173	334 812 368 382 272	79 65 172 231 184 141	370 322	73 52 90 106 89 100	159 151 149 157 185 126	158 144 138 151 109 190
$\frac{x}{(\Sigma x)^2}$ $\frac{}{n}$ $\Sigma(x)^2$	1240 307520 314920	2168 940045 1126232	872 126731 147148	692 239432 240584	510 43350 45290	927 143222 145033	890 132017 135526

$\lesssim\lesssim x = 7299$

$\lesssim\lesssim x^2 = 2154733$

$\frac{(\lesssim\lesssim x)^2}{N} = 1479872$

$SST_o = 2154733 - 1479872$

$= 674861$

$SSG = 1932316 - 1479872$

$= 452443$

$SSE = 674861 - 452443$

$= 222417$

$\frac{SSG}{SSE} \frac{N-r}{r-1} = 9,83$

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F value
Groups	452443	6	75407	9,83
Error	222417	29	7670	
Total	674860	35		

$F\ (6,29)\ 0,05 = 2,43$

$9,83 > 2,43$

6) Thus the H_o must be rejected, as the means are significantly different.

Fisher and Yates (1953) gives the F value at $\alpha = 0,001$ as 5,18. This is still smaller than the calculated F value of 9,83. This means that there is less than a 0,1% chance that each group of plants for which NRA_{tis} as determined was drawn from the same population.

This test can now be followed by the simultaneous testing procedure (STP), testing each group of data at the $\alpha = 0,001$ level of significance and comparing each group with the others to see if any group has a significantly different mean.

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	1	2	3	4	5	6	7
x	1240	2168	872	692	510	927	890
n	5	5	6	2	6	6	6
$\bar{x}$	248	434	145	346	85	155	148

For this S.T.P., each group is compared with each other, some will be similar, others may be significantly different. The test statistic is

$$\frac{(\bar{x} - \bar{x})^2}{(\frac{1}{n} + \frac{1}{n})} \geqslant (r-1)\ \text{MSE.}\ F_{(6;29)\ 0,05}$$

$$\geqslant 111822,8$$

Where MSE is mean square of error.

Firstly, the two most extreme groups are compared with each other: e.g.

[2:5] : 331423,5 $\geqslant$ 238 371,2

Similarly, a table can be drawn up comparing each group:

	1	2	3	4	5	6
7	27109,3	221989,3*	27,0	58336,3	1178,9	113
6	23842,5	212445,8*	248,9	54734,7	14206,6	
5	72460,9	331423,5*	10694,4	100895,5		
4	13720	10962,5	60120,1			
3	28765,3	226682,4*				
2	86118,4					

* Significant difference between groups.

So it can be seen that Group 2 is significantly different from 4 other groups, namely 3,5,6 and 7, at the 95% level of confidence.

APPENDIX D

67 .....

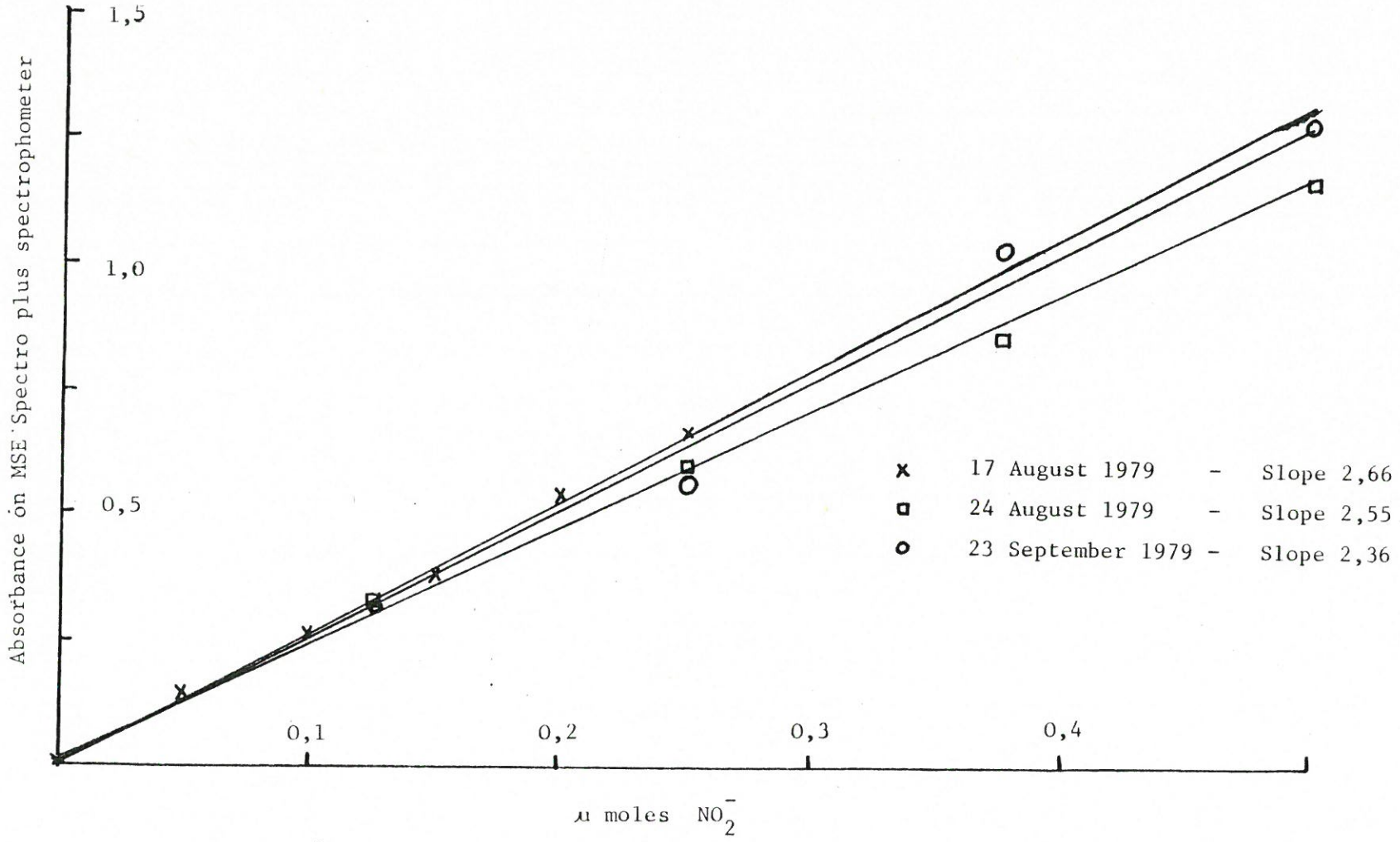

Fig. 15 : Standard curve for Absorbance in spectrophotometer vs. μ moles NO_2^- present.