

THE IN VIVO AND IN VITRO NITRATE REDUCTASE ACTIVITY
IN TEF (ERAGROSTIS TEF (ZUCC.) TROTTER)
UNDER DIFFERENT ASSAY CONDITIONS

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A B S T R A C T

The presence, substrate inducibility and regulations of the enzyme nitrate reductase (NADH: Nitrate Oxidoreductase, E.C. 1.6.6.1)(NR) of both dark-grown and light-grown tef plants (Eragrostis tef (Zucc.) Trotter) were studied. The relative merits of the in vivo and in vitro methods of assay for the enzyme were evaluated. Major factors affecting nitrate reductase activity and the distribution of the enzyme in both the dark- and light-grown plants were investigated.

Nitrate and urea induced the formation of nitrate reductase in 5-day-old dark-grown tef plants; and the activity of the enzyme was predominantly associated with the roots. In the glasshouse-grown plants, however, both the ammonia-type and the nitrate-type nitrogen sources elicited NR activity although nitrate was still superior in enzyme induction; and the leaves were found to be the major centers for nitrate reduction.

A pH of 7.6 was found to be optimal for an in vivo NR activity of tissue obtained from leaves of light-grown tef plants.

Diurnal variations in NR activity were observed, with peak activity at mid-day, suggesting that the enzyme is under the control of light.

INTRODUCTION

Logging tef plants for 11 days after 10 days of maturation did not substantially affect the enzyme activity. It was observed that tef seedlings discriminate between ammonium and nitrate during their early stages of development by preferentially assimilating the former when given in combination with the latter. These findings suggest that tef plants can, at least at their early stages of development, successfully withstand reductive soil conditions.

forms of life which can fulfill their nitrogen requirements only from an exogenous source of organic nitrogen and ammonia for they are incapable of converting the oxidized states of inorganic nitrogen to the reduced level. Therefore, green plants and a large number of microorganisms, by virtue of possessing the necessary complements of enzymes for carrying out nitrate assimilation, are ultimately responsible for providing nitrogen to many heterotrophic forms of life on our planet (Nason, 1962).

Nitrogen, because it is found in a variety of compounds, has a complex metabolism. Nitrate assimilation represents the biological reduction of nitrate to ammonia or the amino level with the end products being used for the biosynthesis of nitrogen-containing cell constituents such as proteins and nucleic acids; and the conversion of nitrate to nitrite in the process of nitrate assimilation is the first step in the enzymatic pathway of nitrate reduction (Beever, 1976). Hence, the

I N T R O D U C T I O N

After many decades of studies of nitrogen metabolism in plants and microorganisms, it is now considered that nearly all plants and many microorganisms which are capable of transforming the nitrogen atom from its various oxidized states (other than molecular nitrogen in most cases) to the more reduced forms, namely ammonia and amino groups, represent the base or foundation of a typical ecological pyramid. Dependent upon this base are all other forms of life which can fulfil their nitrogen requirements only from an exogenous source of organic nitrogen and ammonia for they are incapable of converting the oxidized states of inorganic nitrogen to the reduced level. Therefore, green plants and a large number of microorganisms, by virtue of possessing the necessary complements of enzymes for carrying out nitrate assimilations, are ultimately responsible for providing nitrogen to many heterotrophic forms of life on our planet (Nason, 1962).

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organic forms of nitrogen are most often derived by the incorporation of the ammonium ion into amino groups or amide groups. Once it is incorporated into an organic compound, nitrogen can be transferred into many other carbon compounds. Certain compounds including glutamic acid, aspartic acid, glutamine, asparagine and carbamyl phosphate are especially active in these transfer reactions (Metzler, 1977). Thus, these substances seem to have constituted a nitrogen pool from which nitrogen can be withdrawn and to which it can be returned.

Aside from the establishment of the fact that the reduction and assimilation of nitrate belongs to the fundamental biochemical reactions in the plant kingdom, the enzyme catalyzing the first step of nitrate reduction was first obtained by Evans and Nason (1953) and Nicholas and Nason (1954) from the fungus Neurospora crassa. It is believed to be a metalloprotein containing FAD, molybdenum and active sulfhydryl groups, with NADH serving as a hydrogen donor (Kessler, 1964; Beevers and Hagemn, 1964).

As a result of the initial work with soyabeans (Evans and Nason, 1953), nitrate reductase was classified as NAD(P)H-nitrate oxidoreductase, E.C. 1.6.6.2. However, subsequent works have demonstrated that nitrate reductases of many plants have a requirement specific for NADH hence the classification E.C.1.6.6.1 (Beevers et al., 1964; Schrader et al., 1968).

According to the current concept of nitrogen metabolism, therefore, nitrate reductase is the first and crucial enzyme in the pathway of nitrate conversion to amino acids, and as such is the bottleneck in these enzymatic processes (Eilrich and Hageman, 1973; Brunetti and Hageman, 1976).

Nitrate reductases can be broadly classified into two groups: assimilatory and respiratory (Nason, 1962). The respiratory nitrate reductases are mostly bound to membranes, are present in bacteria, and their synthesis is inhibited by oxygen; whereas the assimilatory nitrate reductases, found in fungi, algae and higher plants are soluble, nitrate inducible and are not inhibited by oxygen (Subramanian and Sorger, 1972(a)). Furthermore, many investigators have established that in higher plants, nitrate reductases are NADH-dependent (Ingle et al., 1966; Beevers et al., 1964; Schrader et al., 1968; Redinbaugh and Wilbur, 1981).

It is thus apparent that the assimilatory reduction of the highly oxidized nitrogen in nitrate to the highly reduced nitrogen in ammonia has been both extensively and intensively studied at an organ, cellular, subcellular and molecular levels (Udayakumar et al., 1981; Ramarao, 1981; Fernandez, 1981; Mifflin, 1967; Croy and Hageman, 1970; Rao et al., 1977; Losada et al., 1981; Mendez and Vega, 1981).

Furthermore, it was demonstrated that although variations as to sites of major reduction are immense (Chatterjee et al., 1981(a,b)), almost all plant tissues are capable of reduction and assimilation of nitrate since the enzyme is widely distributed in these tissues (Beevers and Hageman, 1969). However, with the exception of apple trees and related species, it is demonstrated that leaf tissue has a greater capacity to reduce nitrate than has root tissue (Klepper and Hageman, 1969; Rao and Rains, 1976; Kadam et al., 1974).

The activity of nitrate reductase has been related to total nitrogen, grain nitrogen and grain yield (Johnson et al., 1976; Brunetti et al. in Chatterjee et al., 1981). Also, genetic variations in the activity of this enzyme have been found in plant species (Beevers and Hageman, 1969; Brunetti and Hageman, 1976; Deckard et al., 1977; Oh et al., 1980; Warner and Kleinhoffs, 1974). It was also shown that there is a positive correlation between nitrate reductase and grain protein in corn (Deckard et al., 1973) and wheat (Croy and Hageman, 1970; Eilrich and Hageman, 1973; Deckard et al., 1977). It is thus natural, following these observations to make a prompt embarkment upon experimentations which would lead to the selection of the most efficient variant from a given population (Zeiserl et al., 1963; Oh et al., 1980; Pal et al., 1976). Moreover, it is proposed that a high

affinity of nitrate reductase could be a biochemical marker for the capacity of the plant to continue assimilatory nitrate reduction for an extended period during the last stage of growth and development (Baer and Collet, 1981). The potential use of physiological and/or biochemical criteria in breeding programmes for the development of plants with efficient nitrogen utilization is apparent (Croy and Hageman, 1970; Rao et al., 1977; Chevalier and Schrader, 1977; Simons and Moss, 1978). In citrus, for example, the nitrate reductase activity of leaves as a measure of the potential of nitrate assimilation capacity of the tested plant tissue has been suggested for nitrogen requirement determination (Bar-Akiva et al. in Bar-Akiva, 1970). It is also emphasized that to improve the efficiency of nitrogen fertilizers and reduce the concomitant problems (eg. eutrophication, increasing costs) more information is required on the process of nitrogen utilization by plants (Baer and Collet, 1981).

It has become conventional to make a prelude, with an eye to introducing a piece of work in Ethiopia, by starting with phrases like "...little has been done regarding...", "...nothing is known about...", "... only a crude survey is done with respect to...", etc.; and studies made on tef cannot be exceptional, particularly regarding its physiological aspects like enzymology, nutrition, water relations and effects of environmental factors.

Early endeavours towards tef research began with collections and groupings of cultivars based on morphology and qualitative characters (Ebba, 1975; Mengesha, 1964). The nutritional survey that was made in 1959 (Mulugeta, 1978) was yet another attempt to elucidate, based on biochemical and clinical data, the vitality of tef as the life source of Ethiopians, particularly in its importance as a protein source. Data gathered by a survey team and reproduced in Mulugeta (1978) indicated that the daily intake per head of protein was 87 gm, 71 gm of which came from plant sources (cereals, 87.0%; pulses, 6.6%; vegetables, 1.9%; fruits, 0.1%). Of the cereals, it was claimed, 56% of the protein came from tef which also supplied half of the total energy budget. Jansen et al. (in Mulugeta, 1978) reported that tef has a high ratio of essential to non-essential amino acids, and an "excellent balance" among essential amino acids except for lysine. It is also reported that attempts geared towards lysine improvement must take arginine and glycine into account because the former amino acid correlates with the latter two amino acids (Bekele, 1978; Lester and Bekele, 1981).

The sensitivity of the tiny florets of tef to standard hand-emasculatation and pollination methods geared efforts towards developing breeding techniques for tef (Mengesha, 1964; Berehe and Miller, 1976; Berehe and Miller, 1978).

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Bekele (1978) and Bekele and Lester (1981) carried out quite a substantial work on the morphological and biochemical relationships of wild Eragrostis species with E. tef. They studied eleven cultivars of the cereal E. tef and fourteen wild Eragrostis species and attempted to identify the degrees of affinity among these species of the genus. The 51 unit characters of stem, leaves and inflorescence were subjected to rigorous classificatory techniques (including the principal component analysis, to identify the major axes of variations) in order to be able to further corroborate their data obtained from amino acid and secondary compounds analyses of plant tissue. The significance of the work of these investigators lies in the fact that a major breakthrough could be made to improve the deficiencies of E. tef reflected by the lower contents of amino acids like lysine and hence upgrade the quality of protein production.

Mulugeta (1978) studied the morphogenesis of tef flowers, the effect of temperature on growth and development of tef, and analysed component nutrients and their distribution in the seeds of the cultivars he studied. He furnished corroborative evidence against Mengesha's claim (1966) that tef has exceptionally high iron content. It thus appears that not only all the hitherto offered explanations based on the unique nature of tef as a source of iron and hence a remedial for

this study would initiate more work on the biochemistry and physiology of tef.

anaemia needs a re-evaluation, but also a meticulous search for sources of such variability in the nutrient distribution of the cereal tef must be undertaken.

Better accounts of the historical perspectives, the importance of tef to Ethiopia, its biology and distribution are well documented elsewhere (Mulugeta, 1978; Bekele, 1978; Berehe, 1974; "Summary of tef Research", 1981, Debre-Zeit). Here, in this paper, suffice it to quote Harlan (1977), who eloquently puts tef at the "base of the nutritional pyramid" for the millions of Ethiopians:

"...Tef is the staff of life for some millions. It is a royal, if not actually a sacred, grain as is planted on more acreage than any other crop in Ethiopia... The grain is ground whole; the flour is fermented and dough baked into a remarkably palatable and nutritious flat bread called "injera". To Ethiopians, this is basic to life...but the crop has been little studied..."

Although statements like: "Very little work seems to have been done, except for the selection of good hay types in South Africa" (Purseglove, 1976) are no longer true, the extreme dearth of information on the physiological aspects of tef must, naturally, given an impulsive vehemence towards gearing endeavours on these lines. The primary objective of this thesis is, therefore, to make a modest effort towards elucidating the way tef metabolises nitrogen. It is hoped that this study would initiate more work on the biochemistry and physiology of tef.

M A T E R I A L S A N D M E T H O D S

I. Dark-room Experiments

Enzyme Induction

White tef seeds, variety DZ-01-354 (1981-82 harvest) obtained from Debre Zeit Agricultural Research Centre, were surface sterilized with 0.1% mercuric chloride for one minute and washed with distilled water. These were then evenly distributed and germinated over Whatman No. 1 filter paper in sterilized Petridishes in darkness in an incubator of $30 \pm 2^{\circ}\text{C}$ for the required number of days*. The germination media consisted of solutions of the following nitrogen sources: 8mM of KNO_3 or urea, or $(\text{NH}_4)_2\text{SO}_4$ or NH_4Cl , buffered at pH 6.6. Experimental seedlings in each medium were given the corresponding solutions ad lib.

In Vitro Nitrate Reductase Activity (NRA)

At the required time of assay*, the seedlings were collected into a 100 ml beaker, thoroughly washed with distilled, sterile water and were blotted dry. One gram of the seedlings (parts of whole) was then triturated in an iced porcelain mortar in 4 ml phosphate buffer (pH 6.6) for five minutes. The triturate was then poured into a test

*The age of seedlings at which assay is done is given with the data.

activity was expressed as μM of nitrite formed during the tube and made upto 20 ml by adding distilled sterile water. Each triturate belonging to the corresponding medium was divided into two equal lots: one lot was boiled for 15 minutes, the other was left unboiled. After adding 2 ml of M/10 KNO_3 to each, samples, were incubated at $30 \pm 2^\circ\text{C}$ for 1.5 to 2.0 hrs, with occasional stirring. Five drops of 10 $\mu\text{g/ml}$ of chloramphenicol were included into each test tube to arrest microbial activities. After the completion of incubation, the reaction mixture was centrifuged, using a clinical centrifuge, at 3000 rpm for three minutes and filtered through a glass fibre filter paper. The reaction, in the final filtrate of 5 ml, was stopped by adding 0.5 ml of 1% (W/V) sulfanilamid in a mixture of 25 ml conc. HCl and 225 ml of distilled water. After 2-8 minutes of a reaction period, 0.25 ml of 0.01% (W/V) of N-(1-naphthyl)-ethylenediamine dihydrochloride was added and the tube was shaken well by hand. The nitrite concentration was determined through the formation of a reddish azo dye by the coupling of diazotized sulfanilic acid with N-(1-naphthyl)-ethylenediamine dihydrochloride. Spectrophotometric readings were made at 540 nm using Bausch and Lomb spectronic 20. The corresponding boiled filtrates served as blanks to zero the instrument. Amount of nitrite of each sample was calculated from the standard curve (Fig. 1). The enzyme

activity was expressed as μM of nitrite formed during the incubation period, and this in turn was expressed as μM nitrite per gram fresh weight per hour.

In Vivo NRA Assay

One g. of seedlings, washed in distilled and sterile water, was placed in a Buchner flask of 250 ml containing 20 ml of $\text{M}/10 \text{KNO}_3$ and 10 ml of phosphate buffer (pH 6.6). Five drops of 10 $\mu\text{g}/\text{ml}$ chloramphenicol were added to control microbial interference. Then, air in the flasks was vigorously removed by evacuation using oil pump and the seedlings were thus infiltrated by a repeated process of infiltration and re-infiltration. Upon the completion of the infiltration, partial anaerobiosis was created as the tissue in all cases was visibly moistened and submerged below the surface of the medium. The flasks were then incubated in the dark at $30 \pm 2^\circ\text{C}$ for 1.5 - 2 hrs. At timed-intervals, 5 ml of the aliquote was removed and the amount of nitrite was determined following the in vitro procedure described above.

II. Glasshouse Experiments

Enzyme Induction

Scoraceous "sand" was purchased and sieved to get sand-sized particles. The sand was then leached in a large

aquarium with occasional washing for 15 days using tap water. Following this it was again soaked in distilled water with occasional washing to avoid a possible nitrate contamination from the tap water. Two kgs of the final washed sand was allotted into pots (ECAFCO makes) of mean diameter 15 cm. The pots were divided into four groups based on the nitrogen sources to be given to the plants, i.e.

Group I. Plants utilizing ammonium sulfate as a nitrogen source.

Group II. Plants utilizing ammonium chloride as a nitrogen source.

Group III. Plants utilizing urea as a nitrogen source.

Group IV. Plants utilizing potassium nitrate as a nitrogen source.

The potassium nitrate group was further divided into water-logged and freely-drained groups to see if there is a difference in nitrate reductase activity under these two conditions. The tef plants were said to be under a logged condition when the solution completely covers the surface of the sand in the pot. The apparent disparity between the freely-drained and logged pots, while irrigating with nutrient solution, was compensated for by the addition of distilled water in the logged lot to maintain a logged condition. To minimize the edge effect, tef plants were grown in plastic bags in soil and kept all around the experimental pots.

Tef seeds were then prepared as described above for the dark room experiments and were sown in the prepared pots. The pots were thoroughly irrigated every day with Hogland No. 1 solution adopted from Hewitt (1966) and modified to contain higher amounts of the corresponding nitrogen sources (i.e. twice the amount added in Hogland No. 1 solution). Soaking the leached sand with Hogland nutrient solution for a day or two prior to sowing was found to be expedient: as the tef seedlings, without presoaking, developed deficiency symptoms presumably because of the diminutiveness of the seeds and hence the rapid depletion of the reserve food.

In Vivo NRA assay under Light Condition.

Leaf Tissue

Leaves were removed from plants using a clean pair of scissors, thoroughly washed with distilled water, blotted dry, cut into 8-10 mm pieces and thoroughly mixed. The fresh weight was then determined immediately.

Root Tissue

The sand in the pot, upon which the plants were maintained, was completely soaked in distilled water and individual plants were carefully uprooted

RESULTS AND DISCUSSIONS

manually. Samples were washed and then weighed immediately to determine fresh weight. For all the experiments, tef samples were taken from a single pot to minimize errors due to variations among pots. The in vivo NR activity assays of both the leaf and the root tissues were carried out as described earlier.

Leaf seedlings maintained in the buffer, although not differentiated into roots and shoots, showed a significant activity. This activity might be due partly to the presence of nitrate in the xylem and partly to the slow activity of the enzyme in the imbibing but not actively germinating seeds. Khan (1964) observed that the activity of nitrate reductase of cotton seeds germinated in a medium containing nitrate was not zero and presumed that nitrate present in dry seeds was responsible for the slight activity. Other investigators have also reported that, using nitrogen deficient isolated barley seedlings, NR activity was present in seedlings cultured in distilled water and the activity was predominantly associated with roots (Beavers and Johnson, 1969). Beavers *et al.* (1969), however, demonstrated that ammonium failed to induce NR in corn seedlings. A similar failure of the induction of NR activity by ammonia was also observed in wheat (Afifi and Hewitt, 1964).

R E S U L T S A N D D I S C U S S I O N S

I. Adaptive Formation of Nitrate Reductase in Tef

When tef seedlings were maintained in 8 mM of the different nitrogen sources (i.e. ammonium chloride, ammonium sulfate, urea and potassium nitrate) it was found that the maximum activity was developed in those seedlings germinated in a nitrate medium as a nitrogen source (Table 1). Results also indicate that urea stimulates the synthesis of the enzyme. Tef seedlings maintained in the buffer, although not fully differentiated into roots and shoots, showed a significant enzyme activity. This activity might be due partly to the presence of nitrate in the seeds and partly due to the slow turnover of the enzyme in the imbibing but not actively metabolizing seeds. Radin (1974) observed that the activity of nitrate reductase of cotton seeds germinated in a medium without nitrate was not zero and presumed that nitrate present in the dry seeds was responsible for the slight activity. Other investigators have also reported that, using nitrogen deficient etiolated barley seedlings, NR activity was present in seedlings cultured in distilled water and the activity was predominantly associated with roots (Beever and Hageman, 1969). Beever et al. (1965), however, demonstrated that ammonium failed to induce NR in corn seedlings. A similar failure of the induction of NR activity by ammonium was also observed in wheat (Afridi and Hewitt, 1964).

Shen (1969) reported that excised embryos and germinating seedlings of rice grown in media containing no added nitrate had very low NR activity. He alluded to the possibility of some of the NR as being a constitutive enzyme in rice. Here in this paper we report, although very small and ephemeral, the presence of NR activity in seedlings of tef maintained in media containing ammonium salts (Table 2). Furthermore, besides furnishing corroborative evidence for the fact that tef nitrate reductase is adaptive, the results given in Fig. 2 reveal that there is NR activity even in those 5-day-old seedlings grown in distilled water.

The fact that urea induces NR activity in tef appears as if the adaptive formation and hence activity of this enzyme were not completely specific for nitrate as an inducer. Analogous observations were made by Ingle et al. (1966) where nitrite was demonstrated to cause induction of the enzyme in higher plants.

The results of this investigation (Table 2) also show that the rate of enzyme formation increases with time upto the 96th hour at which time the experiment was stopped. It is interesting, however, to make note of the fact that this holds only for those seedlings maintained in the nitrate media. Seedlings maintained in distilled water, ammonium chloride, ammonium sulfate had their initial NR activity progressively declining

with time. This seems to show that nitrate is essential not only for the induction of the enzyme but also for its stabilization (Shaner and Boyer, 1976; Subramanian et al., 1974, Afridi et al., 1964; Udayakumar et al., 1981).

From these experiments it is also noted, that in 5-day-old tef seedlings maintained in 8 mM of nitrate or urea, the root appears to be the most active center for nitrate reduction (Table 3). Under the assay conditions employed, root NR activity was more than seven-fold when compared to shoot NR activity in nitrate-grown seedlings, while it was more than nine-fold in urea-grown seedlings. In both cases, therefore, the roots of dark-grown five-day-old tef seedlings turn out to be the major center of nitrate reduction. This might be due to the fact that the substrate was more easily available to the roots than to the shoots since the whole of the roots were immersed in the medium or due to the fact that the substrate not only induces the enzyme but also stabilizes it (Whitelam and Johnson, 1980).

Attempts to reproduce the dark-room experiments in the greenhouse, i.e. the substrate induction of NR activity in tef, were unsuccessful. All the nitrogen sources provided to the plants elicited, with varying degrees, NR activity although the use of nitrate as a nitrogen source was still superior in induction (Table 4). The reason why the ammonia-

type plants developed NR activity could not be due to the sand, as it was negative for nitrate, but, perhaps, might be due to the possible microbial conversion of ammonium to nitrate as the cultures were not sterile. Similar problems were encountered by Klepper et al., (1969) and Barker et al., (1971).

From Table 4 and Fig. 3 it is noted that the shoots of tef grown in light are by far superior to the roots with respect to their NR activity. The shoot: root ratios of activity per unit fresh weight ranged from 6.67 to 20. These values appear to agree with those obtained for barley NR activity (Lewis et al., 1982). The time course study (Fig. 3) indicates that the 120th minute shoot NR activity is more than 42-fold when compared with that of the root.

We have demonstrated that in dark-room grown tef seedlings it is the root system which is predominantly associated with higher NR activity. This must be true since it is believed that in non-photosynthetic tissue, nitrate influences oxygen consumption; and the existence of NADH-dependent nitrate reductase implies that respiratory metabolism supplies the electron donors for nitrate reduction (Beever and Hageman, 1969; Ramarao et al., 1981). If this were the case, it appears that there should be no disparity between the shoot and the root with respect to metabolites supply as they

withdraw these metabolites from the common pool - the reserve food of the seed. Hence the disproportionately higher level of NR activity of the roots of dark-room grown tef seedlings might be due to the constant bathing and continuous influx of nitrate into the root. It might also be due to the fact that the roots have not yet developed NR activity inhibitor which is normally present in light-grown plants (Kadam et al., 1974).

In the light-grown tef plants, however, the shoot must be the center of nitrate reduction for the following three major reasons.

1. There is a copious generation of a reducing power, namely, NADH, either through glycolysis (Klepper et al., 1971) or from the citric acid cycle (Ramarao et al., 1981) as well as a constant synthesis of the enzyme (Sawhney et al., 1972).
2. Since the light-grown tef plants were maintained on a high nitrate level, and since light enhances nitrate uptake and translocation (Whitelam and Johnson, 1980; Beevers et al., 1965; Udayakumar et al., 1981; Rufty et al., 1981) it must be possible that most of the nitrate is transported towards the leaves where it elicits higher NR activity.

3. It is possible that tef roots might have a nitrate reductase inhibitor which was shown to be the case for rice seedlings (Kadam et al., 1974; Leong and Shen, 1980).

TABLE 1

The other important feature of Fig. 3 is the revelation, by the shoot NR activity, of a lag period between roughly 0 and 30 minutes. The length of the lag period was, however, variable from assay to assay rarely exceeding 30 minutes. This seems to indicate that there is an active and nitrate-inducible permease system in tef leaf tissue. Lag periods were observed by Dusky and Galitz (1977) in their effort to determine the in vitro reaction rates for the eight grass species they studied.

Control	0.0
CO ₂	0.0
NO ₂	0.4
NO ₃	1.0
Buffer	3.3
CO ₂	9.3

TABLE 1

ACTIVITY OF NITRATE REDUCTASE FROM TEF SEEDLINGS
SUBSTRATE DEPENDENT INDUCIBILITY OF NITRATE REDUCTASE
IN TEF. (AN IN VITRO ASSAY OF NITRATE REDUCTASE
ACTIVITY (NRA) OF 5-DAY-OLD TEF SEEDLINGS GROWN :
IN DIFFERENT NITROGEN SOURCES IN THE DARK.)

Nitrogen Source	$\mu\text{M NO}_2^-/\text{g.fr.wt/hr}$
Distilled Water (control)	0.0
NH_4Cl	0.0
$(\text{NH}_4)_2\text{SO}_4$	0.4
Urea	1.8
Buffer	3.3
KNO_3	9.3

TABLE 2

IN VITRO ACTIVITY OF NITRATE REDUCTASE FROM TEF SEEDLINGS GERMINATED IN THE DARK AS A FUNCTION OF AGE AND DIFFERENT NITROGEN SOURCES. ACTIVITY IS EXPRESSED IN TERMS OF $\mu\text{M NO}_2^-/\text{G.FR.WT.}/\text{HR.}$

Nitrogen Source	Age (hrs.)			
	24	48	72	96
Distilled Water (control)	0.25	0.00	0.00	0.00
NH ₄ Cl	0.40	0.10	0.00	0.00
(NH ₄) ₂ SO ₄	0.35	0.05	0.00	0.00
Urea	1.15	1.75	2.50	3.70
Buffer	0.80	1.10	2.40	3.50
KNO ₃	0.50	3.50	2.25	7.75

TABLE 3

COMPARISON OF IN VITRO NR ACTIVITY OF ROOTS AND SHOOTS OF DARK-GROWN 5-DAY-OLD TEF SEEDLINGS. ACTIVITY IS EXPRESSED IN TERMS OF $\mu\text{M NO}_2^-/\text{G.FR.WT/HR.}$

Nitrogen Source*	Plant Part		
	Root	Shoot	Root/Shoot
Urea	4.875	0.500	9.8
KNO_3	29.500	4.000	7.4

* Seedlings grown in Urea and KNO_3 were used since they were well differentiated into roots and shoots and had higher NR Activity,

TABLE 4

IN VIVO ASSAY FOR THE ADAPTABILITY OF NITRATE REDUCTASE IN LIGHT-GROWN, 15-DAY-OLD TEF PLANTS UTILIZING DIFFERENT SALTS AS NITROGEN SOURCES. ENZYME ACTIVITY IS EXPRESSED IN TERMS OF $\mu\text{M NO}_2^-/\text{G.FR.WT.}/\text{HR.}$

Nitrogen Source	Plant Part		
	Root	Shoot	Shoot/root
NH_4Cl	0.3	5.0	20.0
$(\text{NH}_4)_2\text{SO}_4$	0.5	7.0	14.0
Urea	0.5	7.5	14.7
KNO_3	3.0	20.0	6.7

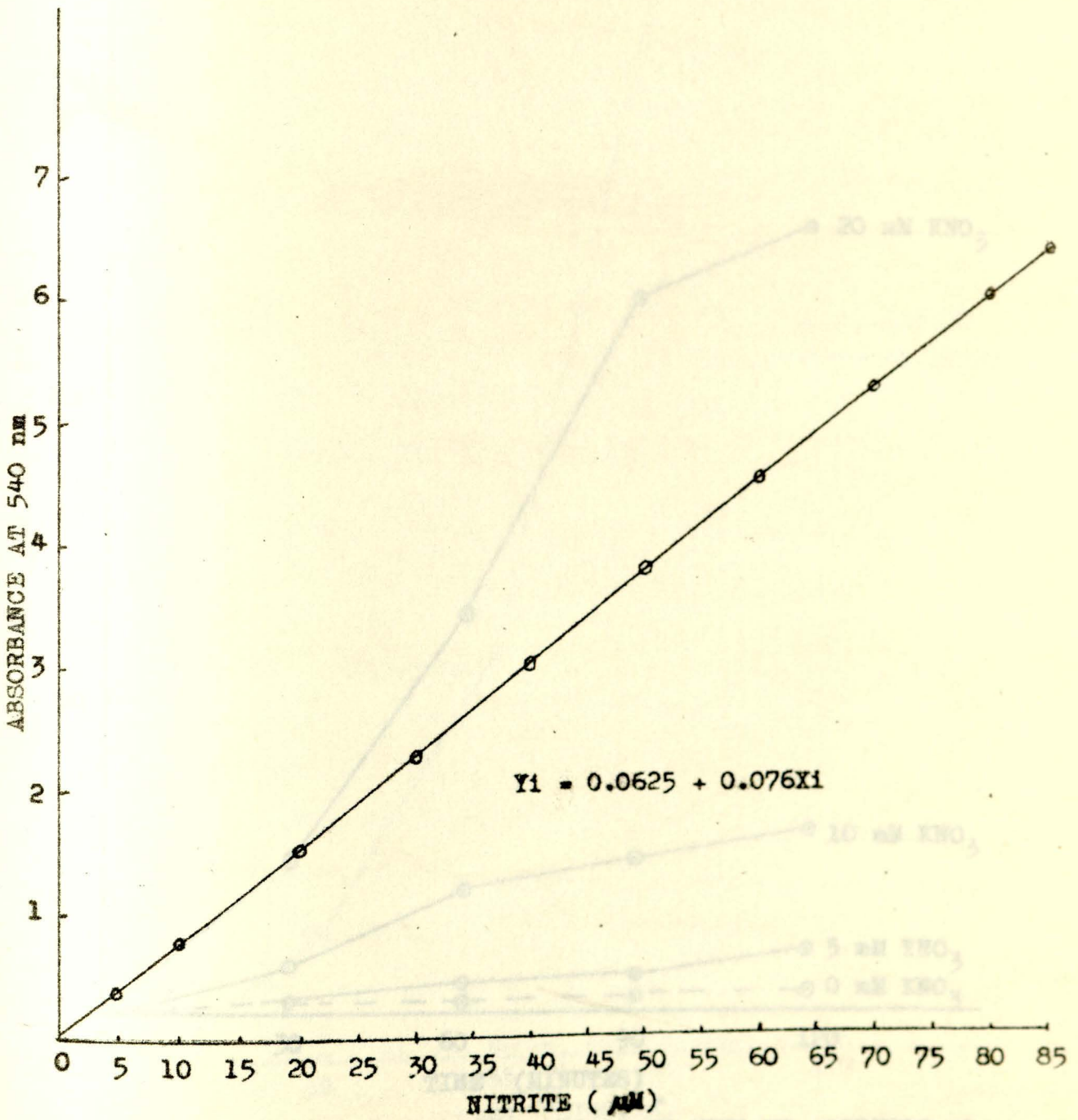


FIG. 1. A TYPICAL STANDARD CURVE FOR NITRITE DETERMINATION

MEDIA OF 5-DAY-OLD TSP BIRNBERG

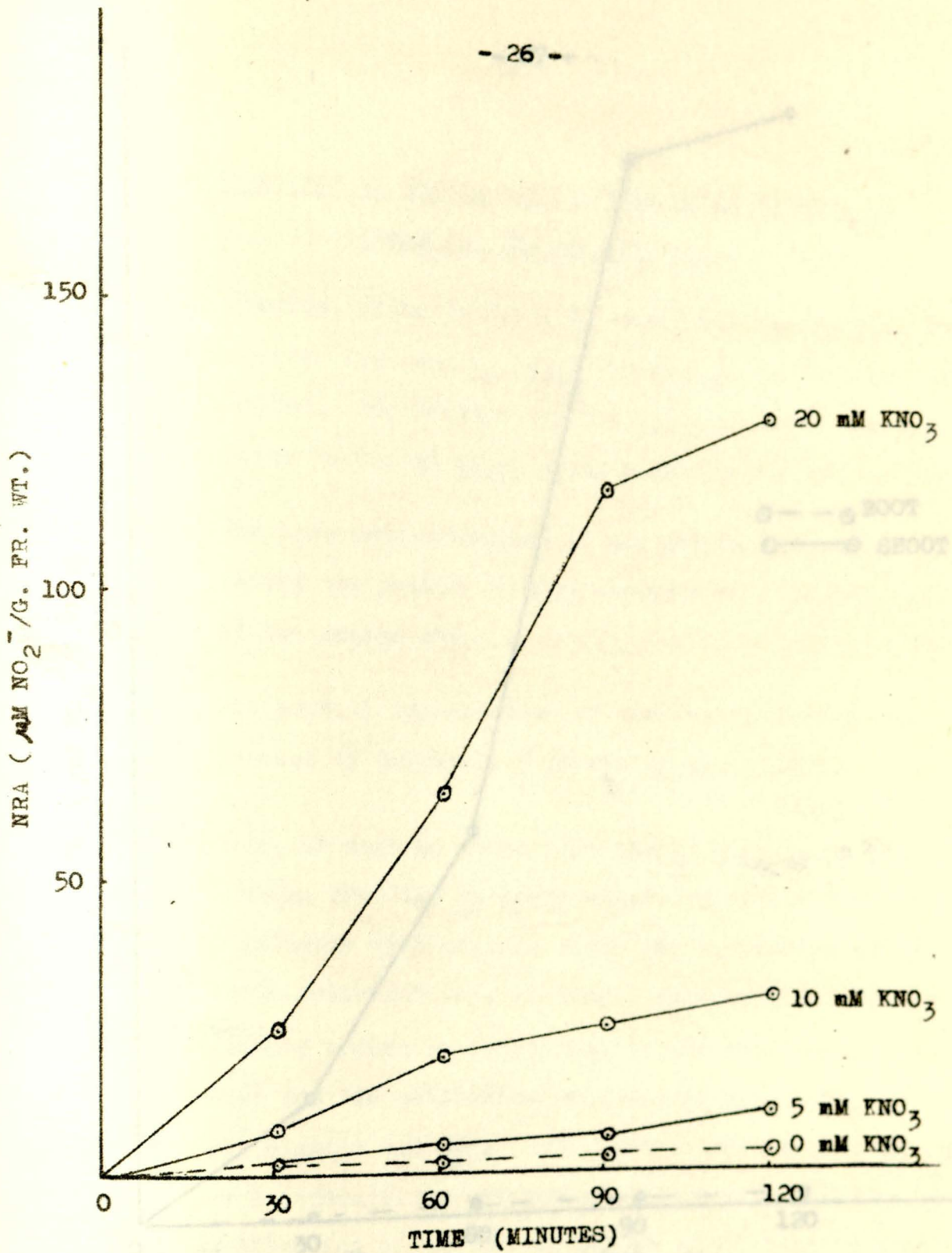


FIG. 2. TIME COURSE STUDIES OF IN VIVO NR ACTIVITY AS A FUNCTION OF NITRATE LEVELS IN THE GERMINATION MEDIA OF 5-DAY-OLD TEF SEEDLINGS IN THE GLASS-BOWLS UNDER LIGHT CONDITION

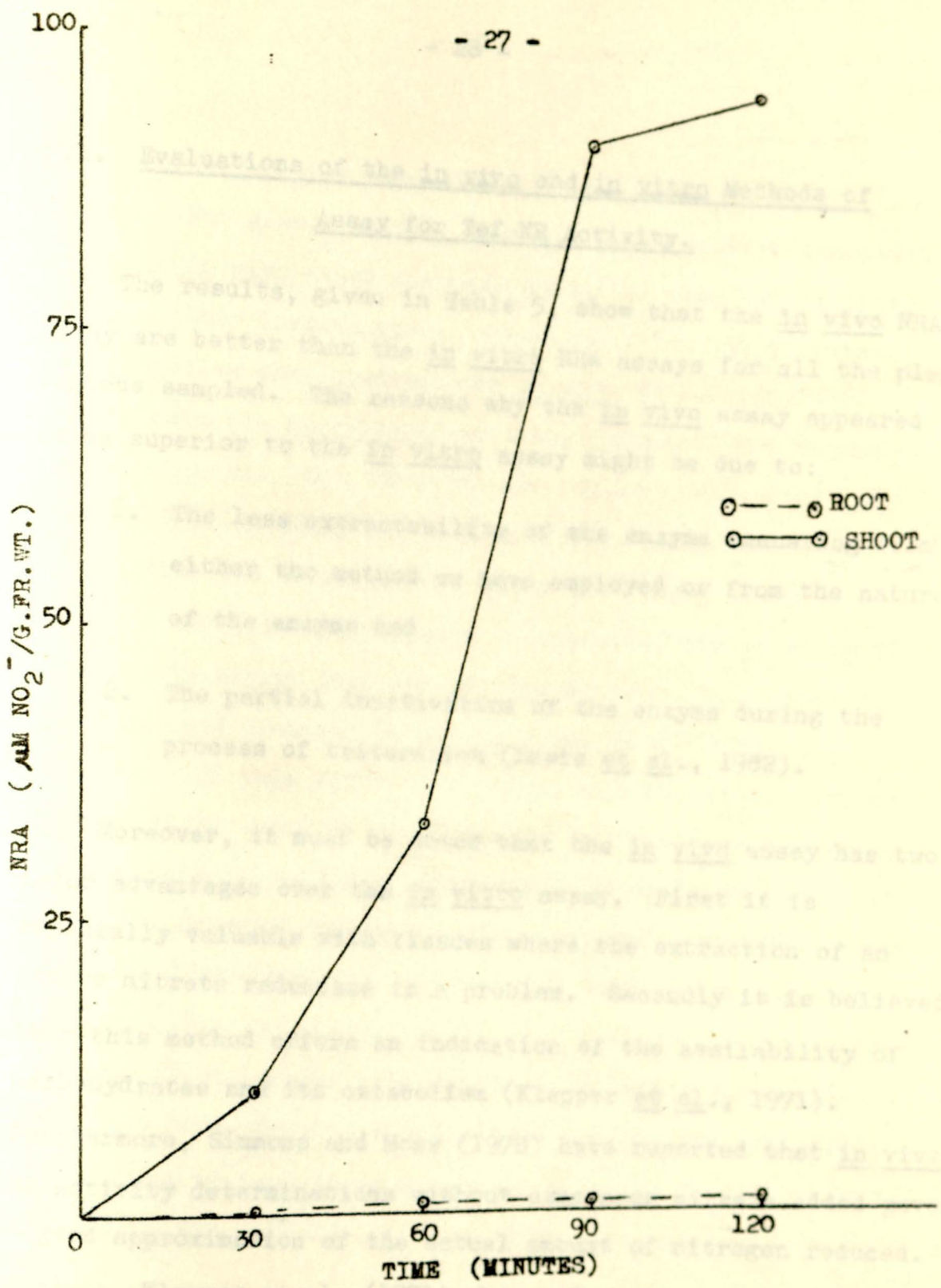


FIG. 3. TIME COURSE STUDIES OF IN VIVO ACTIVITY IN ROOTS AND SHOOTS OF 10-DAY-OLD TEF PLANTS GROWN IN THE GLASS-HOUSE UNDER LIGHT CONDITION

II. Evaluations of the *in vivo* and *in vitro* Methods of Assay for Tef NR Activity.

The results, given in Table 5, show that the *in vivo* NRA assay are better than the *in vitro* NRA assays for all the plant regions sampled. The reasons why the *in vivo* assay appeared to be superior to the *in vitro* assay might be due to:

1. The less extractability of the enzyme emanating from either the method we have employed or from the nature of the enzyme and
2. The partial inactivation of the enzyme during the process of trituration (Lewis et al., 1982).

Moreover, it must be noted that the *in vivo* assay has two major advantages over the *in vitro* assay. First it is especially valuable with tissues where the extraction of an active nitrate reductase is a problem. Secondly it is believed that this method offers an indication of the availability of carbohydrates and its catabolism (Klepper et al., 1971). Furthermore, Simmons and Moss (1978) have reported that *in vivo* NR activity determinations without exogenous nitrate added gave a good approximation of the actual amount of nitrogen reduced. However, Klepper et al. (1971) observed, using comparable corn and pigweed tissues, that the *in vitro* assays for NR activity

in these two species were always higher than the in vivo assays. For giant ragweed, however, these workers demonstrated that the in vivo assay was more sensitive than the in vitro assay. Other investigators found that, for the different plant species they studied, the in vivo NR activity was higher than the in vitro NR activity (Brunetti and Hageman 1973; Chisholm, 1975; Streeter and Bosler, 1972; Baer and Collet, 1981). Dusky and Galitz (1977), in an attempt to standardize the techniques of assay for eight grass species, observed that, in the presence of cysteine, the in vitro NR activity is higher than the in vivo NR activity. Since we have not used cysteine in our in vitro NR activity, our evaluation of this method for tef NR activity needs further refinement in order to establish the relative usefulness of the two methods and hence standardize NR activity assays in tef. For example, the in vivo/in vitro value of 9.0 for the roots of light-grown tef plants (when compared to 1.3 for the dark-grown tef roots), might be a reflection of the weakness of the manual trituration (i.e. since the roots used were from 18-day-old tef plants and so were resistant to grinding by hand it is suspected that the in vitro assay might not have revealed the true state of affairs).

Table 5 also offers a good picture as to the gradient in the distribution of NR activity of dark-grown tef seedlings.

The enzyme decreases progressively from the root towards the shoot. The "transition region", defined here as the region between the "true" root and "true" shoot, occupies in its NR activity, a middle position thus corroborating the observation (discussed elsewhere) that the root and the shoot of a dark-room grown tef seedling represent two extreme ends of the processes of nitrate reduction.

Plant part	Method of assay		
	<u>in vivo</u>	<u>in vitro</u>	<u>in vivo/in vitro</u>
Shoot	6	3	2.0
Transition Region	40	32	1.3
Root	55	40	1.3
Shoot	25	-	-
Root	4.5	0.5	9.0

III. The Effect of Temperature on Nitrate Reduction in Tef

TABLE 5

COMPARISON OF THE METHODS OF IN VIVO AND IN VITRO NR ACTIVITY ASSAYS OF 5-DAY-OLD TEF SEEDLING GROWN IN DARKNESS AND 18-DAY-OLD PLANTS GROWN IN GREENHOUSE. ACTIVITY IS EXPRESSED IN TERMS OF $\mu\text{M NO}_2^-/\text{G.FR.WT.}/\text{HR.}$

Experimental Condition	Plant part	Method of Assay		
		<u>in vivo</u>	<u>in vitro</u>	<u>in vivo/in vitro</u>
Darkness	Shoot	6	3	2.0
	Transition Region	40	32	1.3
	Root	53	40	1.3
Light	Shoot	23	-	-
	Root	4.5	0.5	9.0

III. The Effect of Vacuum-Infiltration Upon Nitrate Reduction in Tef

The time course follow-ups of NR activity as a function of aerobic or anaerobic condition is given in Fig. 4. The results clearly show that anaerobiosis enhances the production and excretion of nitrite into the reaction medium. It was observed that when tef leaf-pieces were vacuum-infiltrated with nitrate they were degassed and hence sank below the surface of the medium thereby increasing the permeability of the membrane to nitrate. When incubated in the dark, the NO_2^- produced, as a result of NR activity, diffused into the surrounding medium. As can be seen from Fig. 4, the difference in the rate of nitrite excretion between the two conditions is more pronounced up to the 60th minute, with a progressive decrease of this difference thereafter. On the 30th minute, for example, the amount of nitrite produced under the anaerobic condition is 3-fold more than that produced under aerobic condition. On the 120th minute, however, the nitrite content of both systems appear to be essentially the same. This becomes obvious when one notes the fact that starting from the 60th minute the medium under which the tissue is submerged decreases in volume thus exposing it to the air in the flask. Since, most probably, the rate of enzyme turnover is the same for both conditions, it cannot be invoked for the almost equal rates of nitrate reduction at the 120th minute.

The enhancement of nitrite production under an aerobic condition was observed by Klepper et al. (1971) but was not explained. It is reported that inhibition of the oxidation of NADH in the mitochondrial electron transport chain is important for the in vivo nitrate reduction in green leaves (Sabalakshmi et al., 1979(a); Sawhney et al., 1978(a,b)). Wallsgrove et al., (1978) proposed that when NADH oxidation is blocked by specific inhibitors or by anaerobic conditions, it becomes available for NR which is probably located in the cytoplasm. It thus appears that competition between oxygen and nitrate for NADH seems to regulate the rate of nitrate reduction in leaves. Similar explanations are invoked for the observed differences in the rates of nitrite production in *tef* under anaerobic conditions.

Time (minutes)	Series 1 (Solid Circles)	Series 2 (Solid Squares)
30	~1.5	~1.0
60	~3.5	~2.5
90	~5.5	~4.0
120	~7.5	~5.5

TIME (MINUTES)

EFFECTS OF VACUUM-INFILTRATION COMPARED WITH FLOATING LEAF PIECES ON THE INCUBATION MEDIUM OF LEAF TISSUE FROM 25-DAY-OLD *TEF* PLANTS.

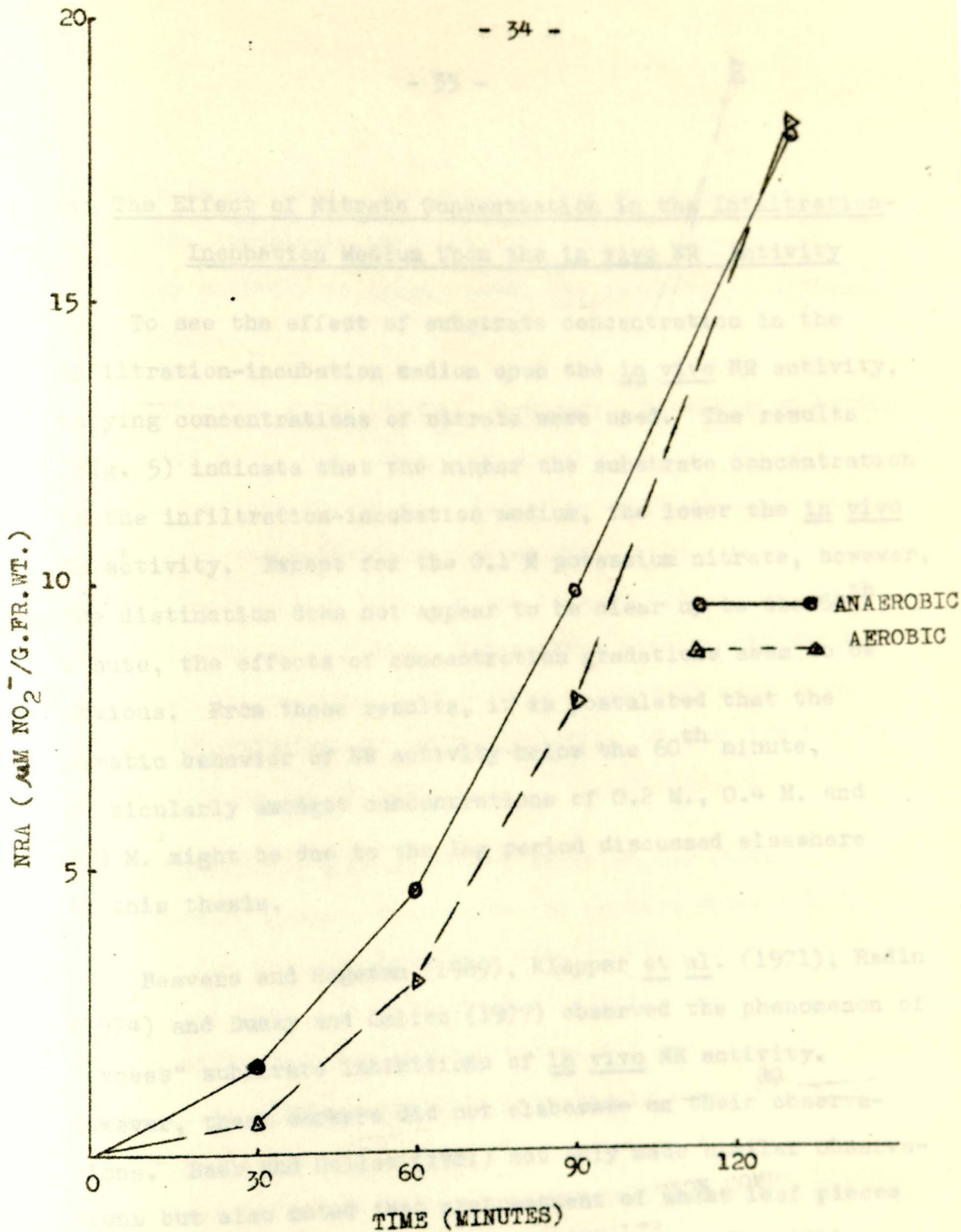


FIG. 4. EFFECTS OF VACCUM-INFILTRATION COMPARED WITH FLOATING LEAF PIECES ON THE INCUBATION MEDIUM ON LEAF TISSUE FROM 25-DAY-OLD TEF PLANTS.

IV. The Effect of Nitrate Concentration in the Infiltration-Incubation Medium Upon the in vivo NR Activity

To see the effect of substrate concentration in the infiltration-incubation medium upon the in vivo NR activity, varying concentrations of nitrate were used. The results (Fig. 5) indicate that the higher the substrate concentration in the infiltration-incubation medium, the lower the in vivo NR activity. Except for the 0.1 M potassium nitrate, however, the distinction does not appear to be clear up to the 60th minute, the effects of concentration gradations seem to be obvious. From these results, it is postulated that the erratic behavior of NR activity below the 60th minute, particularly amongst concentrations of 0.2 M., 0.4 M. and 1.0 M. might be due to the lag period discussed elsewhere in this thesis.

Beevers and Hageman (1969), Klepper et al. (1971), Radin (1974) and Dusky and Galitz (1977) observed the phenomenon of "excess" substrate inhibitions of in vivo NR activity. However, these workers did not elaborate on their observations. Baer and Collet (1981) not only made similar observations but also noted that pretreatment of wheat leaf pieces by nitrite did not result in an inhibition of NR activity. Based on their observation, the workers argued that the possibility of feed-back inhibition of NR activity by end

products is unlikely. According to the above argument, therefore, it appears that inhibition of in vivo nitrate reductase activity by excess substrate seems to be a plausible explanation.

Chatterjee et al. (1981), however, proposed that during the period of in vivo assay, fresh synthesis of nitrate reductase is not envisaged. They then postulated that the decrease in the activity of the enzyme due to a substrate-induced inactivation is ruled out because the enzyme has a high affinity for nitrate. The workers thus concluded that conceivably some other form of regulation such as the availability of reductant or release/blockage of some inhibitors may be influencing NR activity.

To conclude, without losing sight of the above arguments, the following general statements can be made about tef NR based on the data obtained from this investigation. In view of the fact that a high affinity of nitrate reductase for nitrate might be a biochemical marker for the capacity of the plant to continue assimilatory nitrate reduction (Baer and Collet, 1981) and in view of the fact that different plants have different critical concentrations beyond which their NR activities are inhibited (Klepper et al., 1971) it is proposed that a comparative study of this aspect among tef cultivars could reveal interesting features of the enzyme nitrate reductase in tef.

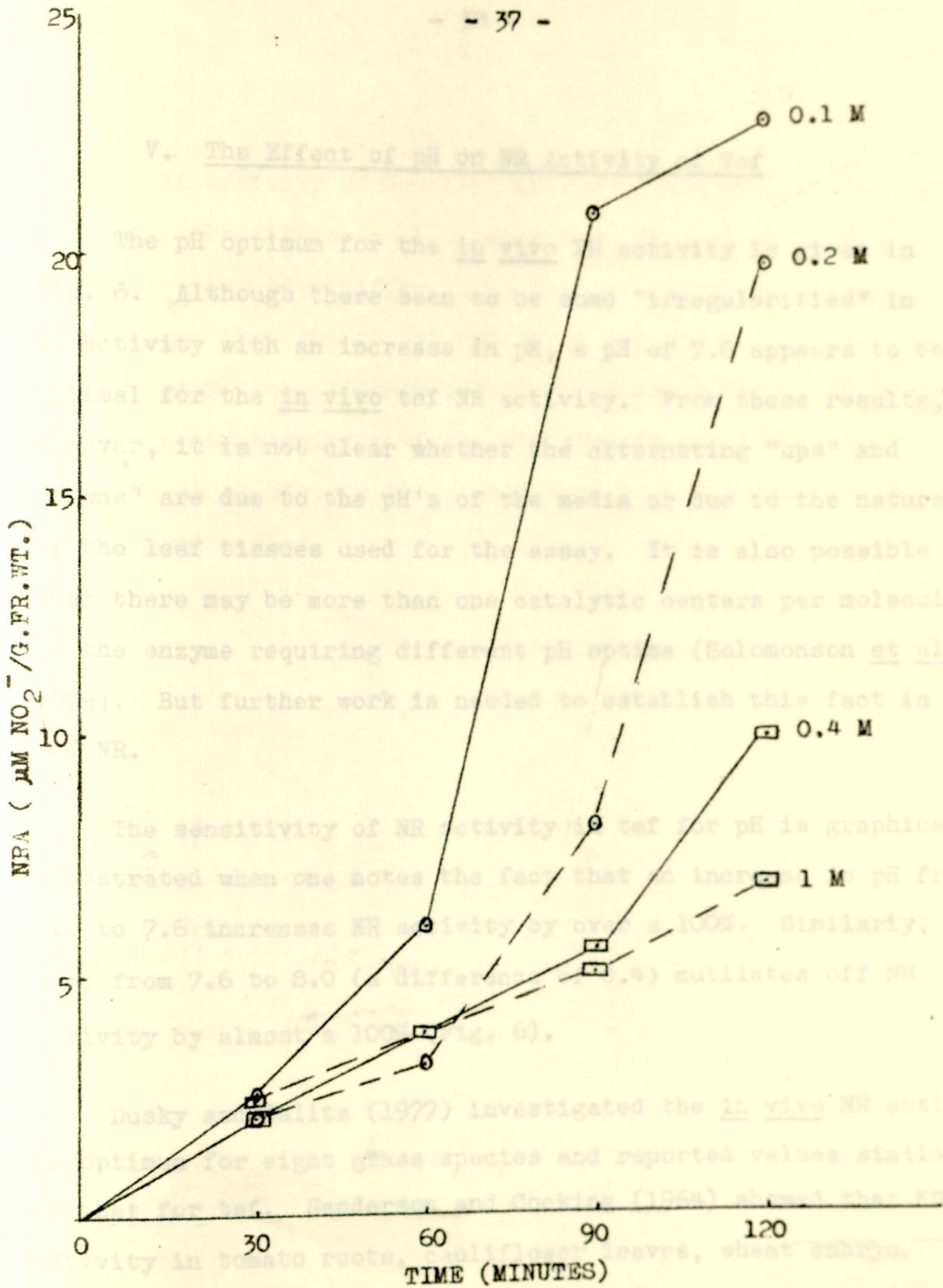


FIG. 5. THE EFFECTS OF NITRATE LEVELS IN THE INFILTRATION-INCUBATION MEDIA UPON AN IN VIVO NITRATE REDUCTION OF 25-DAY-OLD TEF PLANTS.

V. The Effect of pH on NR Activity of Tef

The pH optimum for the in vivo NR activity is given in Fig. 6. Although there seem to be some "irregularities" in NR activity with an increase in pH, a pH of 7.6 appears to be optimal for the in vivo tef NR activity. From these results, however, it is not clear whether the alternating "ups" and "downs" are due to the pH's of the media or due to the nature of the leaf tissues used for the assay. It is also possible that there may be more than one catalytic centers per molecule of the enzyme requiring different pH optima (Solomonson et al., 1979). But further work is needed to establish this fact in tef NR.

The sensitivity of NR activity in tef for pH is graphically illustrated when one notes the fact that an increase in pH from 7.0 to 7.6 increases NR activity by over a 100%. Similarly, a jump from 7.6 to 8.0 (a difference of 0.4) mutilates off NR activity by almost a 100% (Fig. 6).

Dusky and Galitz (1977) investigated the in vivo NR activity pH optimum for eight grass species and reported values similar to that for tef. Sanderson and Cocking (1964) showed that NR activity in tomato roots, cauliflower leaves, wheat embryo, tomato leaves, maize leaves and marrow leaves has a pH optimum of 7.5. Beevers et al. (1964), however, noted that the optimal

pH for NR activity of Soyabean leaves is 6.25-6.50. In view of these observations, it appears that NR's of different plant species have different pH optima. It is postulated, therefore, that the so far collected and identified cultivars of tef might have nitrate reductases with different pH optima, although the magnitude of the differences may not be so drastic as to dictate these cultivars to occupy different ecological niches.

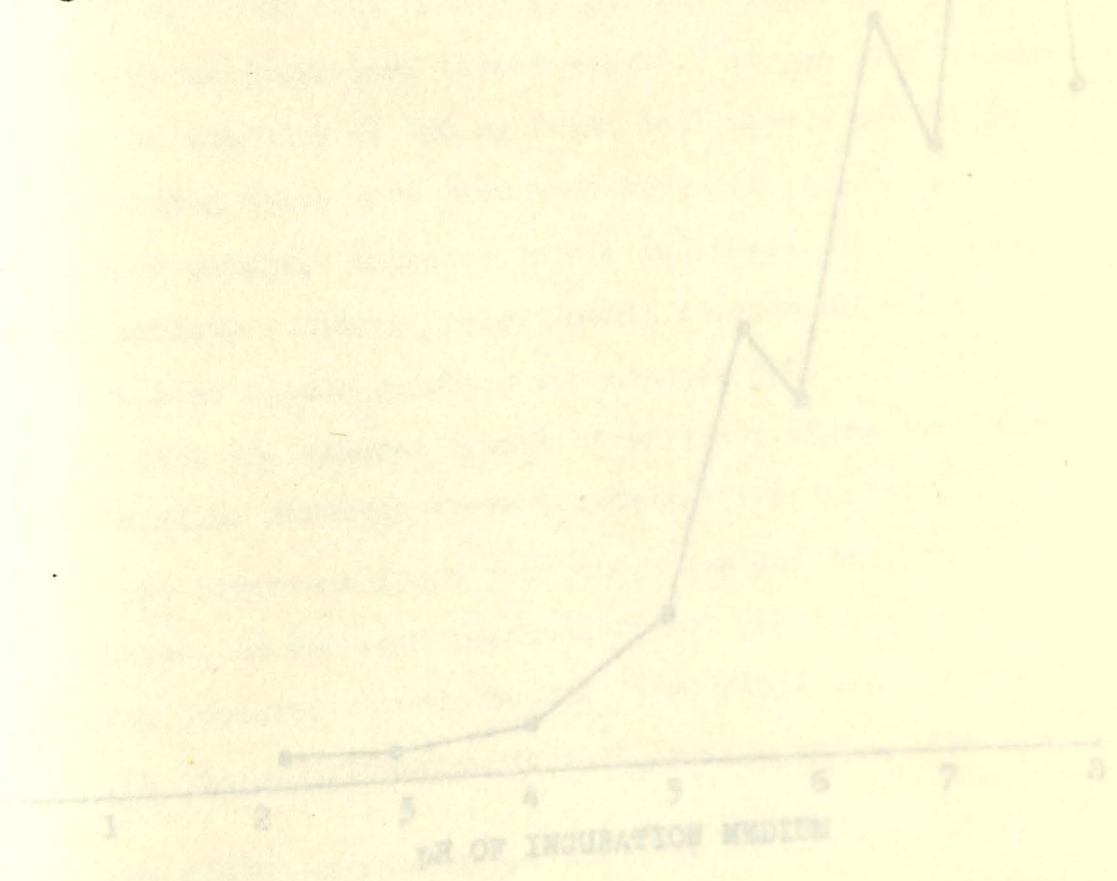


FIG. 5. EFFECT OF pH OF INCUBATION MEDIUM ON THE IN VIVO NR ACTIVITY OF 15-DAY-OLD TEF LEAF TISSUE.

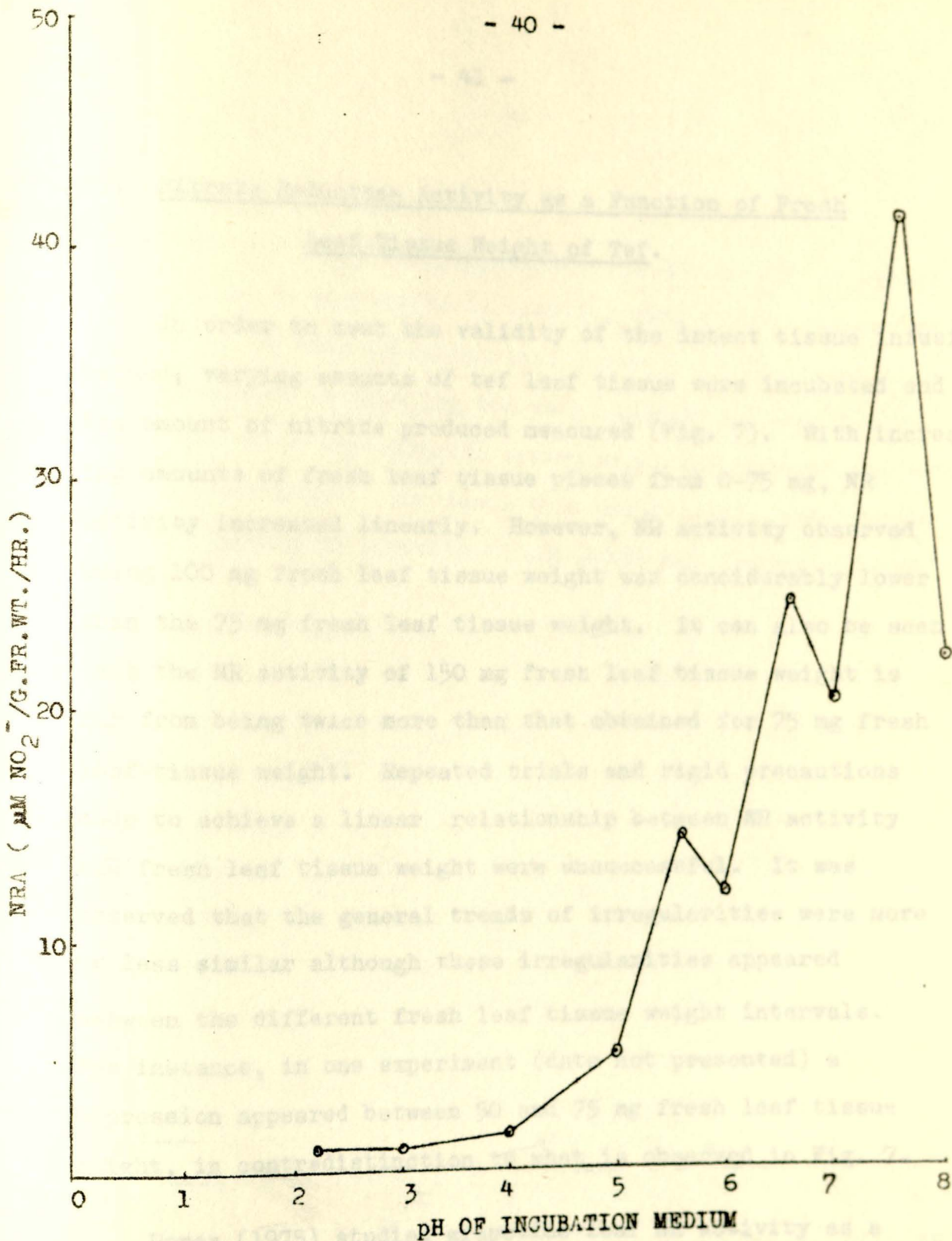


FIG. 6. EFFECT OF pH OF INCUBATION MEDIUM ON THE IN VIVO NR ACTIVITY OF 15-DAY-OLD TEF LEAF TISSUE.

VI\ Nitrate Reductase Activity as a Function of Fresh
Leaf Tissue Weight of Tef.

In order to test the validity of the intact tissue infusion method, varying amounts of tef leaf tissue were incubated and the amount of nitrite produced measured (Fig. 7). With increasing amounts of fresh leaf tissue pieces from 0-75 mg, NR activity increased linearly. However, NR activity observed using 100 mg fresh leaf tissue weight was considerably lower than the 75 mg fresh leaf tissue weight. It can also be seen that the NR activity of 150 mg fresh leaf tissue weight is far from being twice more than that obtained for 75 mg fresh leaf tissue weight. Repeated trials and rigid precautions made to achieve a linear relationship between NR activity and fresh leaf tissue weight were unsuccessful. It was observed that the general trends of irregularities were more or less similar although these irregularities appeared between the different fresh leaf tissue weight intervals. For instance, in one experiment (data not presented) a depression appeared between 50 and 75 mg fresh leaf tissue weight, in contradistinction to what is observed in Fig. 7.

Perez (1975) studied grapevine leaf NR activity as a function of fresh leaf tissue weight and observed more or less similar deviations from linearity. He concluded that

the failure for achieving linearity might be due to experimental errors or to difficulty in handling such a small amount of tissue. In this investigation, however, it is argued that difficulties in handling the small amounts of leaf tissue or some other experimental errors might not account for such large deviations. And hence this observation gave impetus for investigating NR activity of the different laminae of tef.

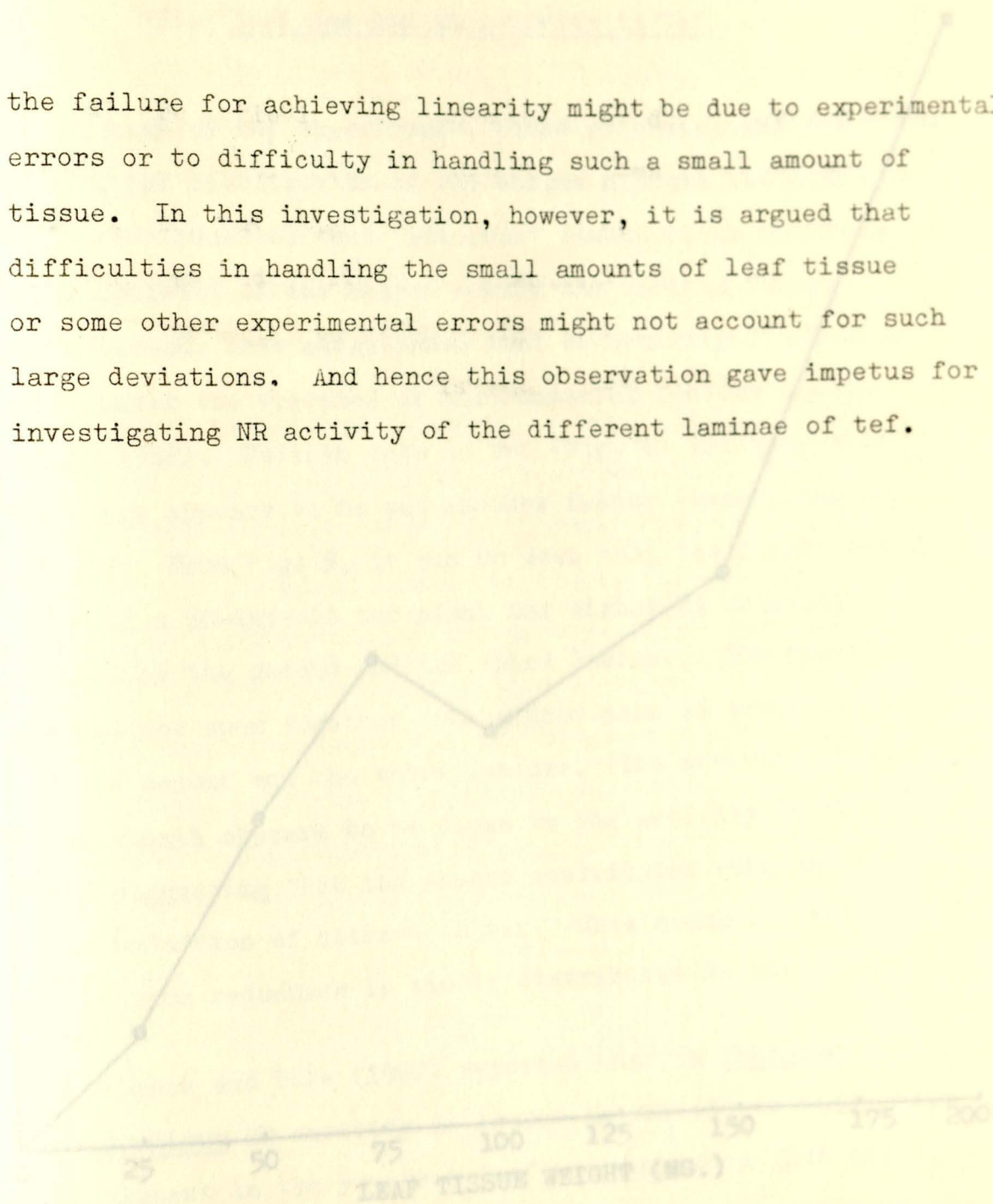


FIG. 7. NITRATE REDUCTASE ACTIVITY AS A FUNCTION OF FRESH LEAF TISSUE WEIGHT OF 30-DAY-OLD TEF PLANTS GROWN IN THE GLASSHOUSE UNDER LIGHT CONDITION.

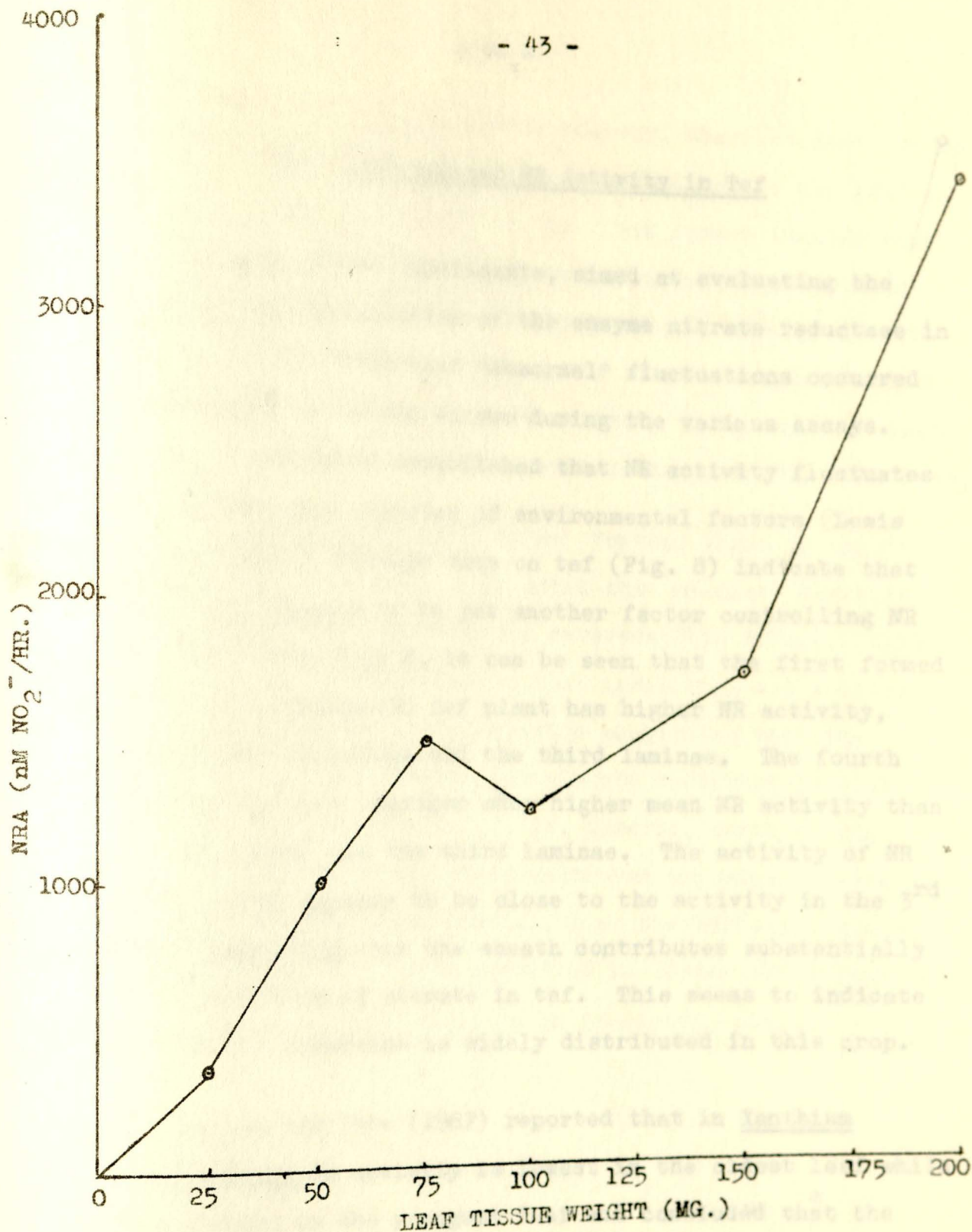


FIG.7. NITRATE REDUCTASE ACTIVITY AS A FUNCTION OF FRESH LEAF TISSUE WEIGHT OF 30-DAY-OLD TEF PLANTS GROWN IN THE GLASSHOUSE UNDER LIGHT CONDITION.

VII. Leaf Age and NR Activity in Tef

In many of the experiments, aimed at evaluating the behavior and distribution of the enzyme nitrate reductase in tef, it was observed that "abnormal" fluctuations occurred in the activity of the enzyme during the various assays. It has already been established that NR activity fluctuates widely under the vagaries of environmental factors (Lewis et al., 1982). Further data on tef (Fig. 8) indicate that tissue age appears to be yet another factor controlling NR activity. From Fig. 8, it can be seen that the first formed lamina of a 50-day-old tef plant has higher NR activity, followed by the second and the third laminae. The fourth leaf and the apex together show higher mean NR activity than both the second and the third laminae. The activity of NR in the sheath appears to be close to the activity in the 3rd lamina suggesting that the sheath contributes substantially to the reduction of nitrate in tef. This seems to indicate that nitrate reductase is widely distributed in this crop.

Wallace and Pate (1967) reported that in Xanthium pennsylvanicum NR activity is lowest in the oldest leaf while it is highest in the youngest leaf and concluded that the latter contributed immensely to the enzymatic potential of the plant. Their results appear to be antithetical to the findings of this investigation.

Chatterjee et al., (1981), however, observed that enzyme activity is directly proportional to the age of the leaf of barley, being the highest in the first formed laminae with a decline in the subsequently formed ones. Because this was similar to the pattern followed in the case of nitrate concentration in these laminae, the workers concluded that the disparities observed in NR activity among the different laminae were due to the differential supply of the substrate.

Ramarao et al. (1981) threw further light on this phenomenon indirectly. They noted that malonate inhibits nitrate reduction and fumarate reverses this effect. With older leaves, it was observed that inhibition was achieved at a higher malonate concentration suggesting that the potential of older laminae for nitrate reduction is higher than the younger laminae. It is, however, worth noting that the results of the above mentioned experiments were one of temporal rather than spatial, i.e. the workers compared the inhibition of nitrate reduction in 10-day-old leaves of rice plants with that observed in 13-day-old plants.

VIII. Diurnal Variations of Nitrate Reductase Activity in Light-Grown Plants

The fluctuations in NR activity under a glasshouse... The peak activity is... It is apparent... increases the root NR act... between 6 a.m. and 12 noon... than 500% while root... during the same interval... activity and their... were not... although... activity...

It is shown that... had a diurnal... activity of the enzyme... environmental or phyto...

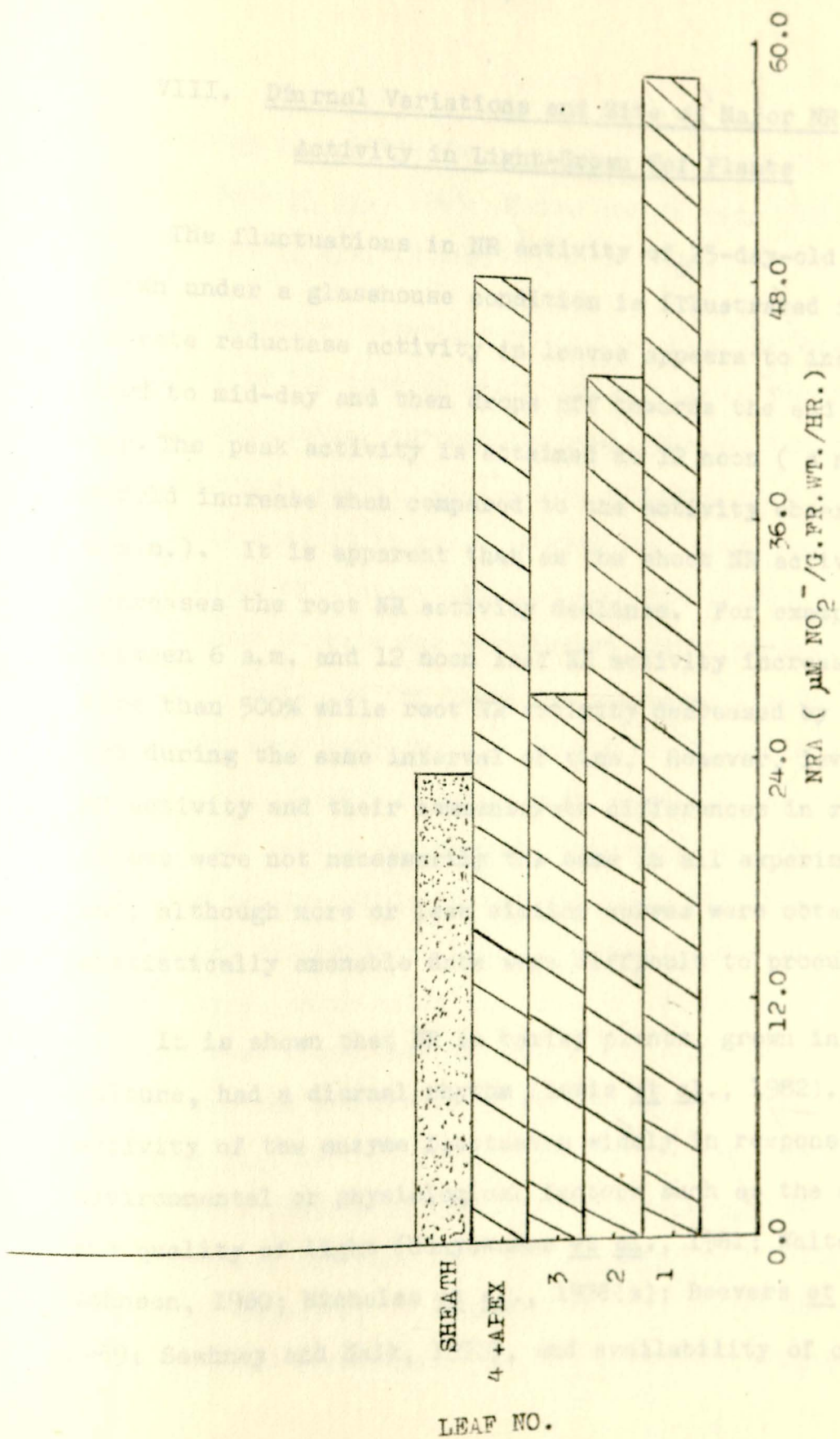


FIG. 8. RELATIONSHIP BETWEEN LEAF AGE AND NR ACTIVITY OF 50-DAY-OLD TEP PLANTS. LEAVES ARE NUMBERED FROM BASE.

LEAF NO.

VIII. Diurnal Variations and Site of Major NR
Activity in Light-Grown Tef Plants

The fluctuations in NR activity of 25-day-old tef plants grown under a glasshouse condition is illustrated in Fig. 9. Nitrate reductase activity in leaves appears to increase from dawn to mid-day and then drops off towards the end of the day. The peak activity is attained at 12 noon (a more than 6-fold increase when compared to the activity observed at 6 a.m.). It is apparent that as the shoot NR activity increases the root NR activity declines. For example, between 6 a.m. and 12 noon leaf NR activity increased by more than 500% while root NR activity decreased by about 90% during the same interval of time. However, levels of NR activity and their commensurate differences in roots and shoots were not necessarily the same in all experiments; and, although more or less similar curves were obtained, statistically amenable data were difficult to procure.

It is shown that NR in barley plants, grown in water culture, had a diurnal rhythm (Lewis et al., 1982). Moreover, activity of the enzyme fluctuates widely in response to many environmental or physiological factors such as the quantity and quality of light (Udayakumar et al., 1981; Whitelam and Johnson, 1980; Nicholas et al., 1976(a); Beevers et al., 1969; Sawhney and Naik, 1972), and availability of cofactor

(Klepper et al., 1971; Jones et al., 1981; Ramarao et al., 1981; Losada et al., 1965; Atkins and Cavins, 1975; Chatterjee et al., 1981(b)).

Furthermore, several workers have proposed that nitrate reduction in the root is reciprocally related to the rate of nitrate transported through the root symplast (Rufty et al., 1981; Beevers, 1976). Dale (1976) observed that the amount of nitrate reduced by roots is much less when compared to that reduced by leaves. Wallace and Pate (1967) noted that in Cocklebur nitrate was assimilated almost exclusively in the shoot, while in the field pea, at lower levels of nitrate, it was the root which was the major site of reduction. Recently, Martin et al. (1981) reported that, based on the xylem sap analyses of organic nitrogenous compounds, in conifers nitrate reduction occurs mainly in the roots and the upward translocation occurs primarily as organic nitrogenous compounds especially as amino acids and amides. Moreover, it has been observed that roots have NR inhibitors which would obviously account for the low levels of NR activity in these organs (Kadam et al., 1974). Mifflin (1967), however, contends that NRA per unit protein of barley roots is almost equal to that of leaves, and that the low in vitro NR activity is due to lack of employing the appropriate procedures and optimum conditions for extraction. He thus concludes that cell free

homogenates of root tissue contain a level of nitrate and nitrite reductase activities equivalent to cell-free homogenates of leaves. In light of this latter report, therefore, it is apparent that our tef root NR activity evaluation could actually be an underestimation. For the possible reasons of leaves for being the major centers of nitrate reduction, however, the reader is referred to section I of this thesis.

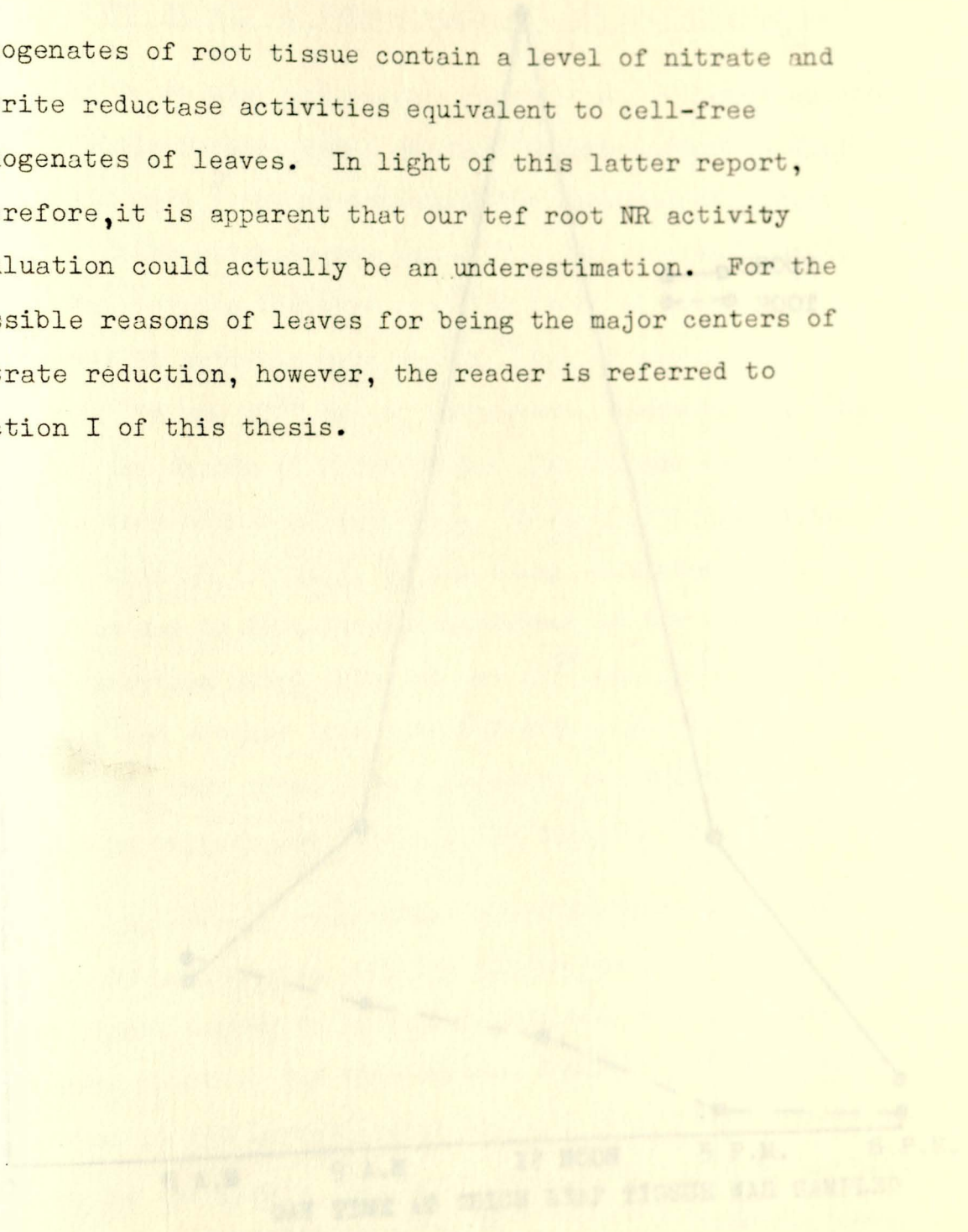


FIG. 9. DIURNAL VARIATIONS IN NR ACTIVITY OF 20-DAY-OLD LEAF AND BLOW TISSUES OF TEF PLANTS (IN 1000 UNITS)

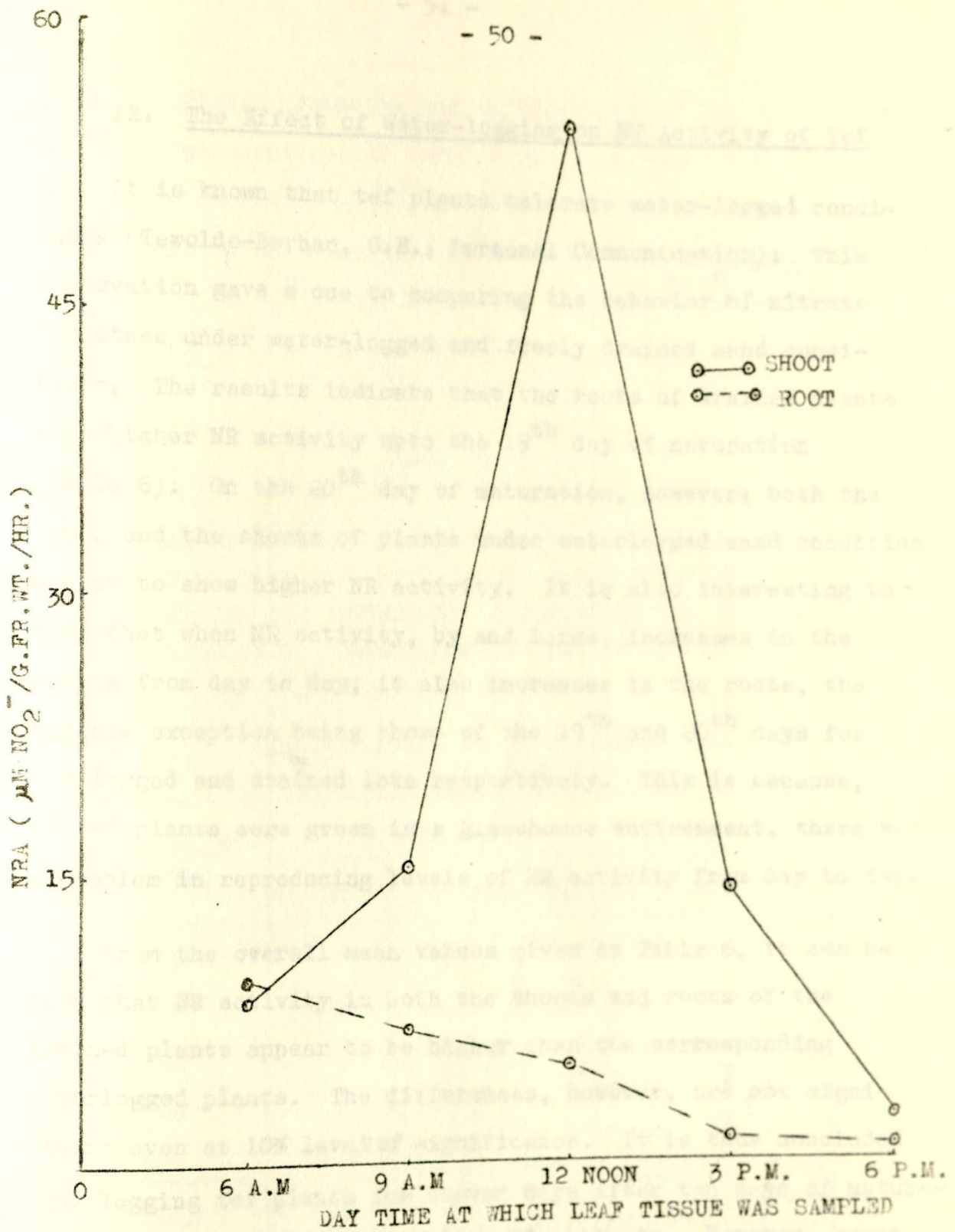


FIG. 9. DIURNAL VARIATIONS IN NR ACTIVITY OF 20-DAY-OLD LEAF AND ROOT TISSUES OF TEF PLANTS (AN IN VIVO ASSAY).

IX. The Effect of Water-logging on NR Activity of Tef

It is known that tef plants tolerate water-logged conditions (Tewolde-Berhan, G.E., Personal Communication). This observation gave a cue to comparing the behavior of nitrate reductase under water-logged and freely drained sand conditions. The results indicate that the roots of drained plants show higher NR activity upto the 19th day of maturation (Table 6). On the 20th day of maturation, however, both the roots and the shoots of plants under waterlogged sand condition appear to show higher NR activity. It is also interesting to note that when NR activity, by and large, increases in the shoots from day to day, it also increases in the roots, the notable exception being those of the 19th and 20th days for the logged and drained lots respectively. This is because, as tef plants were grown in a glasshouse environment, there was a problem in reproducing levels of NR activity from day to day.

From the overall mean values given in Table 6, it can be seen that NR activity in both the shoots and roots of the drained plants appear to be higher than the corresponding waterlogged plants. The differences, however, are not significant even at 10% level of significance. It is thus concluded that logging tef plants for eleven days after ten days of maturation does not substantially alter NR activity. However, great caution has to be exercised in evaluating the significance of

the numerical treatments and implications of the results in view of the constraints imposed by the nature of the enzyme, i.e. the enormous variability and sensitivity of nitrate reductase to the multiples of uncontrollable environmental factors. An S_D^2 of 893.89 for the shoots is a graphic testimony of this fact (Table 6).

TABLE 6

COMPARISON OF NR ACTIVITY IN 21-DAY-OLD TEF PLANT GROWN UNDER DRAINED AND WATER-LOGGED SAND CONDITIONS. THE LOGGED LOT WAS COMPLETELY LOGGED AFTER TEN DAYS OF MATURATION. NUMBERS IN THE MATRIX ARE IN $\mu\text{M NO}_2^-/\text{G.FR.WT./HR.}$

AGE OF PLANT (days)	P L A N T P A R T			
	ROOT		SHOOT	
	LOGGED	DRAINED	LOGGED	DRAINED
15	1.5	3.6	3.0	11.5
18	3.5	6.0	98.7	98.1
19	3.5	5.5	13.0	59.0
20	21.0	13.0	98.1	65.0
21	10.0	20.0	90.2	117.7
Mean	7.9	9.6	60.6	70.3
S_D^2	40.8		893.9	

X. The Effect of Ammonium Salt on NR Activity of Tef

During the course of the glasshouse experiments, it was noted that tef seedlings utilizing ammonium sulfate as a nitrogen source were more green and vigorous than those utilizing potassium nitrate or urea as a nitrogen source. Following this observation, short term experiments were conducted in the darkroom to see if tef seedlings discriminate between nitrate and ammonium. The investigation was carried out using the following four combinations of the two nitrogen sources:

- (a) 5 mM of ammonium sulfate with 15 mM of potassium nitrate;
- (b) 10 mM of ammonium sulfate with 10 mM of potassium nitrate;
- (c) 15 mM of ammonium sulfate with 5 mM of potassium nitrate; and
- (d) 5 mM of potassium nitrate only.

Results indicate that tef seedlings maintained in combination (a) appear to show higher NR activity than those maintained in combinations (b) and (c) (Fig. 10). This is, naturally, to be expected because the concentration of nitrate decreases from combination (a) towards combination (c); and the level of nitrate reductase activity increases as the level of nitrate in the germination medium increases (Fig. 2).

Furthermore, these results seem to suggest that at higher nitrate levels, NR activity appears to be less sensitive to ammonium, while at lower nitrate levels the enzyme activity seems to be considerably affected. Figure 10 also shows that when 5 mM of potassium nitrate alone is supplied to the germinating seedlings, more NR activity is developed. For example, the 120th minute NR activity of seedlings maintained in 5 mM potassium nitrate alone is more than 33% higher than those maintained in 10 mM of potassium nitrate and 10 mM of ammonium sulfate, although the effective nitrate concentration in both cases (i.e. combinations (b) and (d)) appear to be the same. This might suggest that the presence of ammonium reduces the effectiveness of nitrate as an inducer of the enzyme nitrate reductase in tef.

Tef plants, at least at their early stage, appear to tolerate water-logged soil conditions. It is also established that water-logged soils are reductive (Shen, 1969). Under such reductive soil conditions, therefore, ammonium is likely to be the major source of nitrogen for tef since the essentially anaerobic condition there facilitates the reduction of nitrate to ammonia.

Although tef seedlings can assimilate nitrate from the beginning of germination (Fig. 2), the results of this investigation (Fig. 10), indicate that the utilization of

nitrate might be suppressed by the presence and availability of ammonium suggesting the evolutionary adaptation of tef to reductive soils, at least at its early stage.

Shen (1969) reported that rice seedlings utilize ammonia preferentially if this nitrogen source were given in combination with nitrate. He noted that as soon as ammonium is depleted from the medium, nitrate utilization is resumed by rice seedlings. A similar argument could be advanced for what we have observed in tef, although further work is needed to conclusively demonstrate that exactly the same process is taking place as is the case for rice seedlings. A more recent report (Lewis et al., 1982) revealed that the presence of ammonium, in the nutrient culture supporting barley plants, decreased both the export of nitrate to the xylem and its accumulation in leaves and roots. Consequently, NR activity in leaves decreased commensurately in the presence of ammonium.

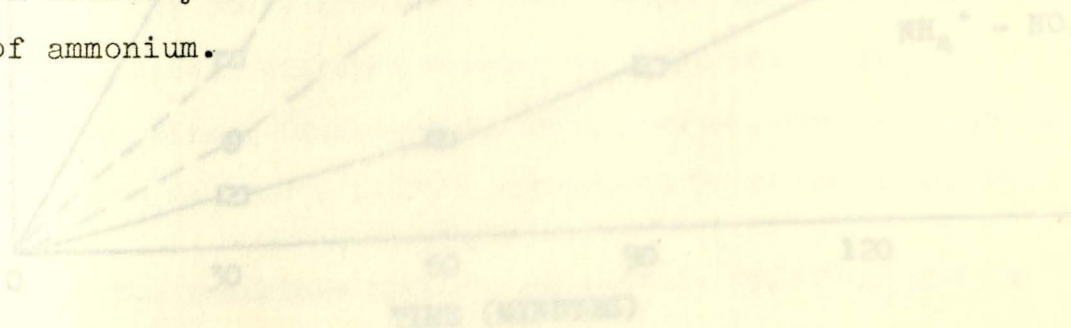


FIG. 10. THE EFFECT OF AMMONIUM SALT IN THE GERMINATION TESTS ON NR ACTIVITY OF TEF.

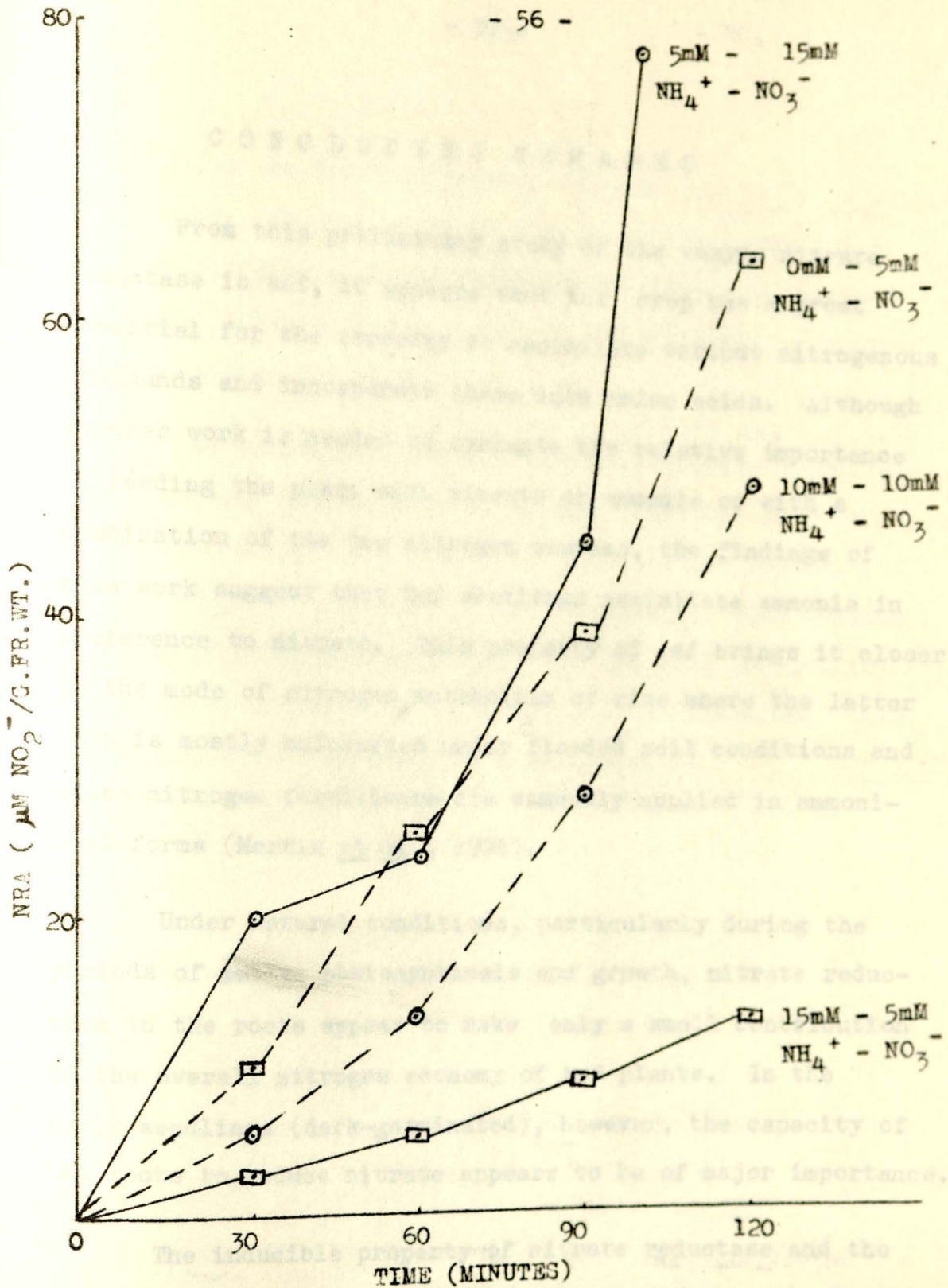


FIG. 10. THE EFFECT OF AMMONIUM SALT IN THE GERMINATION MEDIUM ON NR ACTIVITY OF TEF.

C O N C L U D I N G R E M A R K S

From this preliminary study of the enzyme nitrate reductase in tef, it appears that the crop has a great potential for the capacity to assimilate various nitrogenous compounds and incorporate these into amino acids. Although further work is needed to evaluate the relative importance of feeding the plant with nitrate or ammonia or with a combination of the two nitrogen sources, the findings of this work suggest that tef seedlings assimilate ammonia in preference to nitrate. This property of tef brings it closer to the mode of nitrogen metabolism of rice where the latter crop is mostly maintained under flooded soil conditions and where nitrogen fertilizers are commonly applied in ammoniacal forms (Martin et al., 1976).

Under natural conditions, particularly during the periods of active photosynthesis and growth, nitrate reduction in the roots appear to make only a small contribution to the overall nitrogen economy of tef plants. In the early seedlings (dark-germinated), however, the capacity of the roots to reduce nitrate appears to be of major importance.

The inducible property of nitrate reductase and the dependence of nitrate reductase activity on substrate levels seem to provide tef plants with efficient mechanism of

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controlling the input of reduced nitrogen and hence of protein production. In this context, a study of the correlation between nitrate reductase activity and protein content of this crop appears to be promising for improving the nutritive values of the seeds.

The purported findings of genetic control of nitrate reductase and the association of high enzymatic activities with increased protein production indicate the possibility of a biochemical approach to plant breeding. This becomes particularly relevant when one notes the rich variability of tef in this country. It is postulated, therefore, that work along this line might be rewarding.

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D E C L A R A T I O N

I, the undersigned, declare that this thesis is my work and that all sources of material used for the thesis have been duly acknowledged.

Signed Legesse Negash Legesse Negash
Date June 28/1982