EFFECT OF LEVELS OF APPLIED NITROGEN ON NITRATE REDUCTASE ACTIVITY AND GRAIN PROTEIN IN MAIZE (*Zea mays* L.)

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By

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D E C L A R A T I O N

I declare that this work is originally mine and has not been hitherto part of any presentation for any qualification. I hereby present it in partial fulfilment for the award of M.Sc degree by Ahmadu Bello University, Zaria.

5/9/88

a. u. Sheikh (tilde).

D E D I C A T I O N

To

Professor A. A. Khan

"

Who served for many years in this Department and who tried to teach all his students one thing: Hardwork.

ABSTRACT

The patterns of activity «f nitrate reductase (NR) in leaves and the development of crude protein in grains (GPC) were studied in tv/o genotypes of maize (HYBRID 8321-21 and TZB Farz-21) under four levels of soil-applied N (0, 80, 120, 160 kgN/ha), two N-sourceS(UREA and CAN) and at various stages of plant growth.

' Plants v/ere raised in completely randomized plots. The plants were sampled fortnightly for leaf nitrate reductase activity (NRA), weekly for ear leaf NRA during grain filling, and at 10 days intervals for grain protein. Results were analysed using Analysis of Variance for 2 x 2 x 4 x 9 x 3 factorial design.

There were no significant differences in NRA between the two varietes. The TZB however had significantly higher GPC at 0 and 80KgN/ha. Urea treated plants had a significantly higher NRA than CAN treatments, whereas their difference in effect on GPC was not significant on GPC. In both varieties, \ both NRA and GPC were found to increase with increase in soil-applied N-level; the increase being significant in NRA while it was not significant in GPC at 120 and 160 KgN/ha regardless of N-source and sampling date, NRA tended to increase with increase in vegetative growth up to flov/ering after which it became erratic. The ear leaf contained a lower NRA than the vegetative leaf in both varieties, and its (ear leaf's) NRA was not a direct function of N-level. The difference in GPC at 10, 20, and 30 days after anthesis was not significant in both varieties but a peak was observed midway during filling. GPC was significantly correlated

with NRA at nil the three stages of grain frilling in both varieties at all sampling dates for NRA, and hence the latter could be used effectively to predict grain quality in the two varieties.

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CHAPTER ONE

INTRODUCTION

Maize (Zea mays L.) (n= 10) is the third most important cereal in the world after wheat and rice (Purseglove 1968). Worldwide, maize is used for three different purposes: human consumption, livestock feed, and as a raw material for many industrial products.

As cereals form the major source of protein in the Third world countries, researches on the economic and nutritional qualities of maize are becoming increasingly important. One important area of such researches is breeding for new genotypes with higher grain yield and quality (the latter usually expressed in terms of percentage (%) grain protein content), level of essential amino acids and mineral elements.

In Nigeria there has been an increase in demand for maize. Apart from its traditional role as a staple source of carbohydrates, it is being used as a substitude for imported wheat and barley for making bread, poultry feed and beer.

It is in response to this demand that the International Institute for Tropical Agriculture (IITA), Ibadan, introduced a hybrid maize for cultivation in 1984. However, the main aim of producing the hybrid in 1983 was to maximize grain yield and not grain quality.

Consequently, there is not much said about the quality of the grain of this hybrid in the literature.

Recent trends in plant breeding indicate that enzyme activity can be used as a criterion for selecting cultivars in terms of grain yield and quality (Deckard et al 1973). One of such enzymes, nitrate reductase, which is also among the most widely investigated in plants has been found to be important in determining grain yield and protein content of cereals (Elrich, 1968, Croy and Hageman, 1970; Deckard, et al, 1973; Elrich and Hageman, 1973; Ajakaiye, 1981).

Many authors have also reported a positive relationship between leaf NRA and grain protein content (Elrich and Hageman, 1973; Ajakaiye, 1981). This is probably because NR catalyses the first step reaction of nitrate reduction which culminates in the production of amino acids, the structural units of proteins.

Furthermore, the level of nitrogen in the soil is an important edaphic factor that influences the grain quality of crops (Tiwari et al., 1981; Novikov et al., 1982).

The investigation being reported here was therefore carried out to:

(1) evaluate both vegetative and ear leaf NRA;

(2) evaluate protein level of each genotype at three stages of grain development;

(3) determine the effects of levels and sources of nitrogen on the parameters in (1) and (2);

(4) establish a relationship (if any) between source and level of nitrogen and NRA, and also between nitrogen source, level and grain protein at three stages of grain development.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction to Nitrate Reductase Activity

The process of denitrification, which is the first step in nitrogen metabolism in most higher plant tissues, starts with the reduction of nitrate to nitrite followed by a series of reactions culminating in the production of ammonia from which amino acids are formed.

The enzyme involved in the reduction of nitrate to nitrite is nitrate reductase (NR). It belongs to a group of enzymes commonly termed molybdenum - multicentre redox enzymes (Hewitt and Notton, 1979). Others in the same group include nitrite reductase, sulfite oxidase xanthine dehydrogenase and nitrogenase.

The enzyme has been reported to be bound to cell membranes in higher plants (Izmailov et al., 1982), fungi and bacteria (Hackett and Macgregor, 1981; Premakumar et al., 1980), to chromatophores and periplasm in Rhodopseudomonas sphaerioides (Kerber and Cadenas, 1982; Urata et al., 1982), or mitochondria (Cadenas and Garland, 1979; Suzuki et al., 1981).

In Digitaria janguinalis, a plant which goes through Hatch and Slack pathway (C4), NR and nitrite reductase (NiR) are localized in the mesophyll cells and the other enzymes (of nitrogen metabolism) in the bundle sheath, analagous to the position of the photosynthetic pathway enzymes (Moore and Black, 1979).

2.2 Distribution of nitrate reductase activity between different plant organs

The potential of a plant part to reduce nitrate is considered a function of its fresh weight and the amount of NR per unit fresh weight (Chatterjee *et al.*, 1980). Consequently, the shoot is observed to have higher NRA than the root (Smith and James, Fädeeva *et al.*, 1982). Roots may possess quite a substantial amount of NRA depending on the light conditions under which the plant is grown. Barley roots, for example, accounted for 40% of the nitrate reduction when grown in dark against 20% in light because leaves reduce less nitrate in darkness (Aslam and Huffaker, 1982).

Within the shoot, comparatively differential performance in NRA of the various parts is expected to follow this pattern: first leaves, then stem and finally fruits. In ten varieties of cowpea, NRA was found to be highest in petioles and young stems rather than in the lamina (Okali, 1976). In leaves of some plants the leaf blade has been found by various workers to have a higher NRA than other organs (Suen, *et al.*, 1979; Rao *et al.*, 1979), and the laminae to have a higher NRA than leaf sheath (Smith and James, 1982). Similar differences occur between leaf blade of the flag leaf and two immediate lower leaves, and between upper leaves of a canopy and lower ones (Patil *et al.*, 1982). Leaves usually show a progressive increase in NRA

as they increase in size, attaining the highest rate just before reaching full size in the case of cucumber (Balhova and Sagata, 1980).

2.3 NRA as affected by various stages of plant development.

As demands for nutrients and metabolites vary in plants from one stage of development to another, NRA also exhibits a marked difference in levels of activity at these stages of plant or organ development.

Consequently, the greatest activity is found in young leaves (Alaa-el-Din, et al., 1978; Fadeeva et al., 1982; Macknown et al., 1982) especially when they are just about to reach maximum size (Balhova and Segata, 1980) and continue to decrease rapidly in lower leaves after full expansion (Al'aa el-Din et al., 1978; Fadeeva et al., 1982; Streit and Feller, 1982; Winter et al., 1982). In roots of kidney bean, however, NRA did not change significantly with changes in the vegetative phase of the plant (Vakhaniya and Nutsubize, 1979).

Increases in fresh weight were found to be accompanied by increases in NRA in various parts of some plants. Subbiaih and Balasimha (1983) for example, observed high rates of NRA during fresh weight accumulation of cashew pedicel. The same is true at flower and pod initiation, grain development and tillering (Yuan and Shein, 1980; Dany and Murty, 1982; Streit and Feller, 1980).

During grain development, two peaks of NRA were observed in Lolium perenne one after three weeks and the other after fourth week of flowering (Streit and Feller, 1982).

Recent reports on levels of NRA during senescense seem to vary from one species to another. For example Streit and Feller (1982) observed low NRA in senescing leaves and pods of dwarf beans (Phaseolus valgaris) whereas in Lolium vulgare senescense was not accompanied by marked changes in in vivo NRA (Smith and James 1982).

2.4. Effect of Environmental Factors on NRA

2.4.1 Effect of N-source on Induction of activity

When more than one form of nitrogen is supplied to the growth medium, then NRA in the plant tissue growing in the medium is either stimulated or inhibited

Studies carried out on various plants, e.g. cucumber, corn, Helianthus, Cascuta, barley and wheat have shown that NRA is directly proportional to the concentration of the exogenous nitrate which is the form of nitrogen that controls the level of activity of their enzyme better than any other nitrogen form (Jordan and Fletcher, 1979; Vijayaraghavan et al., 1979; Baer and Collet, 1981; Lewis et al., 1982; Metha and Srivastava, 1982; Bose et al., 1982; Adams and Attiwal, 1982 , Quetz, et al., 1982; Smith and Rice, 1983; Mengel et al., 1983).

Urea, like ammonium, caused large decreases in NRA of Cauliflower, turnip and raddish (Notton et al., 1979) and induced remarkable nitrate accumulation in growing fresh vegetables (Lin and Yen, 1980). Where urea was the absolute source of nitrogen, as reported by Hamdi et al (1978), a positive correlation was observed between its concentration and NRA and total nitrogen of plants.

2.4.2. Light and NRA

The positive influence of light on NRA has been extensively studied. Photoinduction of the enzyme appears universal. Among recent researches on photoinduction are those carried out by Llobell et al., (1980), Datta et al., (1981), Ninnerman (1982). Malik and Kapur (1979) were the first to report its induction in germinating pollen grains.

Furthermore, induction of NR by far-red light has been reported in corn (Duke and Duke, 1979).

2.4.3. Water Stress and NRA

Reports of various researches are unanimous on the fact that water stress (dehydration) is accompanied by significant decreases in NRA (Holst and Yopp, 1979; Garg, et. al., 1981; Nielson and Muller, 1981; Singh and Vijayakumar, 1981; Aparicio-Tejo and Sanhez-diaz, 1982; Patil et al., 1982; Taylor et al., 1982; Leclenc and Robin, 1983; Meguro and Megalhaes, 1983).

The response however, would vary with the level of substrate available. Aparicio-Tejo and Sanhez-Dias (1982), for example, found that during drought, leaf NRA of Medicago sativa decreased significantly particularly in plants treated with nitrate. The decrease in NRA during water stress may be accompanied by decrease or increase in activity of other nitrogen-metabolism enzymes. For example, in Pterium sanguisorba shoot, increase in NRA was accompanied by an increase in the level of glutamine synthetase (Taylor et al., 1982), whereas in wheat decrease in NRA during dehydration led to decreases in levels of soluble amino acids especially proline (Singh and Vijayakumar, 1981).

2.4.3 Temperature and NRA

Temperature is also known to play an important role in determining the level of NRA in plant tissues. A negligible amount of activity is observed at very low (about 5%) and freezing temperatures due to stress (Blahova, 1982; Bakker and Hussett, 1982). However, measurable NRA starts from temperatures as low as 5°C (Harmer and Lee, 1981) with activity progessively increasing with increasing temperature up to about 40°C Mendel et al., 1982). Temperatures higher than 50°C usually denature the enzyme (Theodarites and Pearson, 1982). On the other hand, temperatures as high as 50°C may be required to activate NRA as reported in red alga Porphyridium aerugineum by Rigano et al. (1979).

2.5. Effect of Chemical Elements and Compounds on NRA

2.5.1. Mineral elements and NRA

The effect of mineral elements like potassium, calcium, sodium, copper, mercury, cadmium, zinc molybdenum, etc., made available to the plant in their ionic form have been reported. Potassium has appeared to have a marked enhancement effect on NRA next to nitrogen and molybdenum (Buczek and Burzynsk; 1979; Fattah, et al., 1980). Calcium ions have been observed to have effect similar to that of potassium through uptake of nitrate.

Salts of sodium have also been found to stimulate NRA (Buckzek, 1979; Smith et al., 1980).

Manganese deficiency also reduces NRA in relation to its role in oxygen evolution and promotion of enzyme transformations (Hewit and Notton, 1979).

Phosphorus enhances the activity of NR. It is an intergral part of electron donors and acceptors of NR and is centrally involved in all cellular processes involving energy transformations. Similarly, molybdeum for its central position in the structure of NR is found to enhance the activity of the enzyme especially when applied as spray (Fernandez and Almeida, 1982).

2.5.2. Effect of ammonium and amino acids on NRA

Most reports concerning ammonium supplied in different forms accentuates the existence of a

Amino acids incorporated into culture solutions decreased *in vivo* NRA (Fukunaga and King, 1982; Vijayaraghavan *et al.*, 1982). Cysteine behaved as an inhibitor for the crude extract and purified NR of barley (Oji *et al.*, 1982) and *Datura inonia* (Fakunaga and King, 1982); glutamine and asparagine were similarly inhibitory in corn seedlings (Oaks *et al.*, 1979; Premakumar *et al.*, 1980, Weidner and Kiefer, 1981) while histidine, methionine, lysine and phenylalanine inhibited growth and NRA in the same crop (corn) (Srivastava *et al.*, 1979; Fukunaga and King, 1982).

Proteinase inactivated NRA in maize root (Wallace and Shanon, 1981) and Scutellum (Batt and Wallace, 1983). Effect of α-Ketoglutarate and citrate were

negative relationship between its concentration and *in vivo* NRA. However, depending on the concentration, form of application, species, and NR system, this effect of ammonium is found to vary.

Inhibitory effect of ammonium has been reported in various plants; in corn seedlings (Oaks *et al.*, 1979), cucumber (Buczek, 1979; Buczek and Burzynski, 1979), *Moricandia arvensis* (Winter et al., 1982), *Chlorella vulgaris* (Di - martino - Rigano et al., 1983) and thermophilic unicellular algae (Fuggi *et al.*, 1981). The ammonium solutions in these works were either soil-applied (in case of crops) or incorporated in the growth medium (in case of the unicellular algae).

found to vary with plant species. Contrary to the case in tobacco cell cultures, where the two acids severely inhibited development of *in situ* NRA, yet, they were able to stimulate the *in situ* activity in wheat cells (Fukanaga, *et al.*, 1980).

2.5.3 Effect of Herbicides, Pesticides and growth substances.

Grant and Prague (1982) found that dalapon, a phenoxy - compound, at a concentration of 10kg/ml inhibited denitrification in salt marsh sediments but at concentrations of 100-250kg, ml , dalapon and 2.2 - dichloropropionic acid inhibited both NR and NIR. These authors observed that the action of the two enzymes was inhibited by aerobic conditions. They suggested that delapon probably has the capacity only to slow down the progress of but not to completely suppress, denitrification. Other harbicides found to be inhibitory to NRA are 2,4 - D in pea (Shen *et al.*, 1982), norflurazon in soybean (Duke *et al.*, 1982), organophosphates and benomyl in leaves of dwarf beans (Rozeck, 1982 a,b), atrazine in maize (Fedina *et al.*, 1979; Lawrence *et al.*, 1980; Buczek *et al.*, 1982) and wheat (Churchill and Lowell, 1979).

Much of the effect of these herbicides is attributed to their effect on photosynthetic reaction especially photophosphorylation. Benomyl (0.1%) was also found inhibitory in dwarf bean leaves. The inhibition of benomyl (0.1%) is particularly pronounced during flowering and pod

setting stages. Contrarily, Basagran was found stimulatory to NR (Petkov, 1979).

Some growth stimulators have been found to stimulate NR and other enzymes of nitrogen metabolism, a particular case is indole acetic acid (IAA) in cuttings of Phaseolus vulgaris (Burzynski et al., 1982).

Abciscic acid (ABA) in Agrostemma githago (Munkoev et al., 1981; Cronewald and Hansen, 1982) has been found to block BAP and cytokinin induced NRA. In maturing seeds of wheat (Singh and Vijayakumar, 1981) decrease in NRA was correlated with accumulation of ABA. In experiments examining the effect of inhibitors on protein and nucleic acid synthesis in potato (Palmer, 1982), ABA was found to suppress their inhibitory effects thereby enhancing NRA. The effect of ABA in this case is on the inhibitors not on the enzyme.

2.6 NRA in the Different Genotypes

Another important aspect of the enzyme's activity is the variation found in various genotypes and strains of the same species. Ajakaiye (1981), for example, tested three genotypes of sorghum and found that the HYBRID which had a higher grain protein also had a higher NRA. Similar differences were obtained between the rhizosphere of three plantago species (Smit and Woldendorp, 1981). Differences in NRA were also

reported in many varieties of wheat (Relly, 1979; Nair and Abrol, 1979), common beans (Felix et al., 1981), Capsicum (Steer, 1982), Agrostis (Harris and Wittington, 1983), mustard (Childyal et al., 1981), Brachia (Pereira, et al., 1981), Chlorococcales (Mathew, 1981) and various strains of Chlostridium perfringens (Hewitt , 1979).

Genotypes are found to respond differently to various methods of NR assay. For example, Harmer and Lee (1981) found relatively similar rates of induced activities using in vivo technique of assaying NR between upland and lowland population of several common pasture grasses whereas in vivo activity of the enzyme from upland population was relatively greater than lowland population.

Variation in NRA between nodules of different leguminous species has been reported (Naives et al., 1982). Sometimes variation can be at a particular level of soil nitrogen as suggested by the report of Nair and Abrol (1979) on wheat cultivars.

2.7. Nitrogen Fertilization and Yield Quality

Total crude protein content (N x 6.25) is markedly influenced by levels and method of nitrogen fertilization in various crops. Quality of wheat grains are the most widely studied in this respect. Tiwari et al., (1981) Studied the productivity of dwarf wheat, Triticum aestivum, as influenced by foliar application of urea.

They found not only an increase in grain yield but also in total nitrogen content. The influence of root and foliar applications of nitrogen to wheat and wheat-grass hybrids has also been reported by Navikov et al. (1982). In their study, certain hybrids were found to give a better positive response in their grain quality to nitrogen application.

In rice, applications of nitrogen and zinc increased the quality of grains in terms of normally non-available amino acids especially tryptophan (Singh, 1981). As in other cereals, the relationship between soil nitrogen and yield quality or quantity is not infinite, a limit is reached beyond which no further increase in yield occurs. This is explicitly reported by Singh and Singh (1979).

The response of other cereals like sorghum (Lantewar and Khot, 1978; Ajakaiye, 1981), maize (Stoyanov, 1983), barley (Moravcik and Balson, 1982) to nitrogen fertilization in terms of yield quantity and quality have been similar to those discussed above in other cereals. Beet (Filip'er et al., 1982), perennial fodder grasses (Storozhenko, 1983) and red-clover grass (Keane, 1982) also showed increases in both yield and crude protein content with different levels of soil nitrogen.

Under dryland farming, soil nitrogen is of secondary influence to soil moisture in determining grain yield. Dubetz (1977) found that with rela-

tively high rates of granular nitrogen on irrigated land, yield responses are not always obtained but protein content is usually increased with increased nitrogen.

3. MATERIALS AND METHODS

3.1 Treatments and Planting

Seven beds were prepared ($3m^2$) with a one meter spacing in between.

The beds were mixed thoroughly with 70kg N/ha each of P and K using single superphosphate and muriate of potash as sources.

Nitrogen N was applied before planting in two forms: either in the form of calcium ammonium nitrate (CAN) or UREA. Four levels of N were used: 0, 80, 120 and 160 kg N/ha. How these rates are calculated is shown in Appendix 4.

One bed was left as control (0 kg N/ha) to which no N was applied. The remaining six beds were divided into two: three for CAN treatments and three for UREA, such that to each level of N was allocated one bed. The beds were randomized.

Six rows of planting were made on each bed: three for TZB and three for HYBRID in alternating sequence. Each of the three was a replicate.

3.2 Determination of NRA (in vivo)

Method: The method used in determining in vivo NRA was adopted from the standard methods of Radin (1973) and Jaworski (1971).

Procedure:

Preparation of incubation medium: Equal volumes (300ml) of 0.1M KH_2PO_4 buffer (pH 7.5), 0.1M KNO_3, and 1% propanol to which was added chloramphenicol ~~(2%)~~ 5mg/ml at the rate of 2 drops per 5 ml of incubation medium) were mixed to form the incubation medium.

Five ml of this medium were pipetted into 43 labelled incubation bottles: one as a blank and 42 for the various replicates of treatments.

Sampling: Leaf samples of the second and third topmost leaves of at least three plants per replicate were taken and immediately placed in labelled polythene bags and placed in ice. These were transferred to the laboratory where they were washed and dried between filter papers. Using a leaf punch, leaf discs (2mm in diameter) were obtained from each replicate sample avoiding the midribs 0.3g of the leaf discs were weighed from each sample and placed in their respective incubation medium for 30 mins at 30°C.

Reaction was stopped at the end of 30 mins by adding and mixing thoroughly with, 1% sulfanilamide (in 3N HCl) at the rate of 0.3ml to 0.5ml aliquot of the incubation medium.

Colour was developed by adding 0.02% N-1-Naphthylene diamide dihydrogen chloride ~~naphthylamine~~ again at the rate of 3ml to every 5ml of incubation medium.

After 10 mins, absorbance was read for each sample using a corning colorimeter at 540nm after setting the instrument at zero using the blank.

Reading were converted to µmoles No_2^- /g fr. wt/h using the calibration curve prepared as follows:

Preparation of calibration curve for NRA

A calibration curve was prepared using different concentrations of nitrite (in form of potassium nitrite) solutions between 0 - 200 µM .
Five ml of each concentration was treated with reagents as described above for the enzyme assay.

The absorbance of the solutions were read at 540nm and a calibration curve of absorbance against concentration was plotted. Both the curve and equation of the line have been presented under Appendix 1.

3.3 Determination of GPC in Seeds.

Method: The standard micro-kjeldahl method was used for determining grain protein content in maize.

Procedure: 100 mg sample was placed in digestion flask. One gm of catalyst powder mixture (99.0g of K_2SO_4, 4.1g of HgO and 0.8g of $CuSO_4$) and 2ml of concentrated sulfuric acid were added.

This was digested for 40 - mins, then cooled, and a minimum quantity of water was added to dissolve solids.

The digest was transferred to distillation apparatus and rinsed 5 to 6 times with 1-2 ml portions of distilled water.

And erlenmeyer flask with 6ml of boric acid solution and 3 drops of mixed indicator solution was placed under condenser with tip extending below surface of solution.

Eight ml NaOH - $Na_2S_2O_3 . 5H_2O$ solution was added to the flask and steam distilled until about 20ml of distillate collects.

This was titrated to pink end point with 0.1N HCl. A blank determination was made using same quantity of reagents and same digestion period as for determination.

Percentage N is calculated thus:

$$\% N = \frac{\text{ml HCl used in titration} \times \text{Normality} \times 14.007 \times 100}{\text{mg sample}}$$

$$\% \text{ Protein} = \% N \times 6.25$$

4. RESULTS

4.1 Nitrate Reductase Activity (NRA).

4.1.1 Effect of N-level on leaf NRA

The effect of soil-applied N level was found to be significant (at 5% level). A summary of the pattern of variation between the four levels is given in Table I below:

Table I. NRA as affected by applied N level irrespective of N-source, genotype and sampling date

N-level	0	80	120	160	(KgN/ha)
NRA	105.4a	134.1b	145.3c	153.0d	

L.S.D(5%) = 5.18

4.1.2. Effect of N-source on NRA

Table II below shows that plants grown with UREA had significantly higher NRA than those grown with CAN irrespective of genotype and sampling date.

Table II. Mean Values for NRA as affected by N-source x N level, irrespective of genotype and sampling date.

N-source	U R E A				C A N			
N-level (kgN/ha)	0	80	120	160	0	80	120	160
NRA	154.5e	212.4bc	226.2b	238.1a	154.7e	190.1d	209.7c	222.7bc

L.S.D. (5%) = 7.33. Two figures followed by the same letter are not significantly different by DMRTS.

The interaction between N-source and stage of growth (sampling date) in both vegetative and ear leaf is presented in Table III below (next page).

Table III. Mean values for NRA as affected by source of N - Date of sampling but irrespective of genotype.

NRA (μMNO_2^- g fr. wt.$^{-1}$ h^{-1}

N-source	UREA	C A N
Vegetative leaf (wks after planting)		
* 4th	192.4ab	117.9c
6th	146.7de	150.2d
8th	112.8fg	115.5fg
10th	178.8bc	175.1c
* 12th	106.3fg	72.9i
Ear leaf (wks after anthesis)		
* 1st	101.1gh	133.5e
2nd	118.9f	107.fg
* 3rd	197.5a	177.9c
* 4th	94.5h	69.5i

L.S.D. (5%) = 11.0. Two figures followed by the same letter are not significantly different (at 5% level) by DMRTS.
* Variations in NRA between the two sources of N were significant in weeks with asterik.

UREA treated plants showed significantly higher NRA than CAN treatments in the 4th and 12th weeks after planting in the vegetative leaf and in the 3rd and 4th week after anthesis in the ear leaf. CAN treated plants exhibited a significantly higher NRA than UREA treatments only during the 1st week after anthesis.

4.1.3 Effect of sampling date on NRA

NRA varied significantly from one week to another throughout the period of plant growth. In the vegetative leaf it was highest when the plant was fully vegetative at 4th week of growth and 10th week (when flowering was complete); it was lowest at the onset of flowering (8th week after planting) (Table IV below).

In the ear leaf however, lowest NRA was observed in the 4th week after anthesis whereas it was highest during the 3rd week after anthesis.

Table IV mean values for NRA as affected by sampling date but irrespective of N-level, N-source and genotype

Vegetative leaf(wks after planting)					Ear leaf (wks after anthesis)			
Wk	4	6th	8th	10th	12th	1st	2nd	3rd	4th
NRA	185.2ab	148.5c	112.1d	117.0b	89.6e	116d	112.9d	187.7a	82.0f

L.S.D. (5%) = 3.66 two figures followed by the same letter are not significantly different by DMRTS.

The variation in NRA in both vegetative and ear leaves of each treatment level as affected by sampling date (sampling date x N-level) is presented in Table V below.

Table V. The interaction effect of sampling date and N-level on NRA in maize leaf tissue.

Sampling dates (wks)	N-Level (kg/ha) 0	80	120	160
	NRA in vegetative leaf (Um NO_2^-/g/hr)			
4th	84.0n	184.0efg	122.52	250.1ab
6th	87.2n	148.8 ij	173.8fgh	183.9 def
8th	47.60	134.2 jh	144.6 ij	121.kl
10th	127.3 kl	205.3 cd	198.0 de	155.hi
12th	148.8 ij	68.8 n	65.70	75.in
	- NRA in Ear leaf (µm NO_2^-/g/hr)			
1st	68.3 n	122.9 kl	112.0 im	163.9 ghi
2nd	87.0 n	117.8 nlm	144.7 ij	102.1 m
3rd	125.3 hl	172.6 fgh	187.2 def	265.7a
4th	151.6 i	52.1 o	59.3 o	64.1 o

L.S.D. (5%) = 7.33. Two figures followed by the same letter are not significantly different by DMRTS.

In the vegetative ard ear leaf respectively, the control plants gave lowest NRA except during the last week of sampling. NRA increased with increase in N level up to 8 weeks after planting. In subsequent samplings however, the response of NRA to applied N was not consistent (Table V).

4.1.4 Effect of genotype on NRA

The HYBRID consistently had higher NRA than TZB with an overall mean of 169.3 and 120.0 μM NO_2^- g^{-1}fr. wt. h^{-1} respectively for HYBRID and TZB, the difference was not significant though.

None of the interactions between factors involving genotype (i.e. genotype x N-source, genotype x N-level, genotype x sampling date, genotype x N-level x N-source, or genotype x N-level x sampling date) gave a significant variation in NRA. This means that the genotype did not have any significant effect on the parameters considered.

4.1.5. Overall trend in NRA (genotype x N-source x N-level x sampling date)

In vegetative leaf: The overall trend in NRA involving all factors is presented in figures 1 and 2 for HYBRID and TZB maize respectively. Upto the 6th week after planting (when the plants were purely vegetative) plants grown with no additional N gave the lowest NRA. This difference (i.e. between the

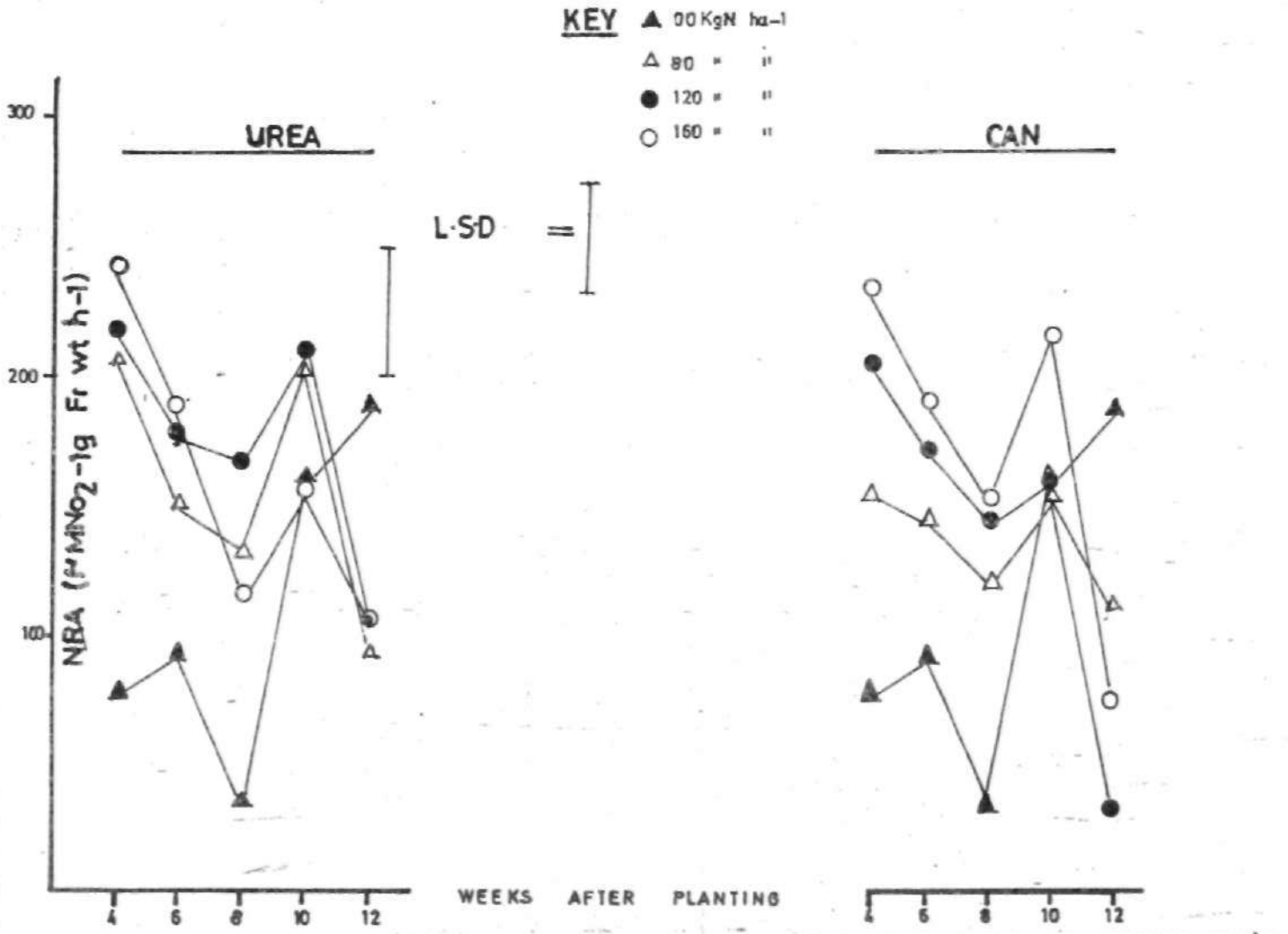


FIG. 1. NITRATE REDUCTASE ACTIVITY IN VEGETATIVE LEAF OF HYBRID MAIZE

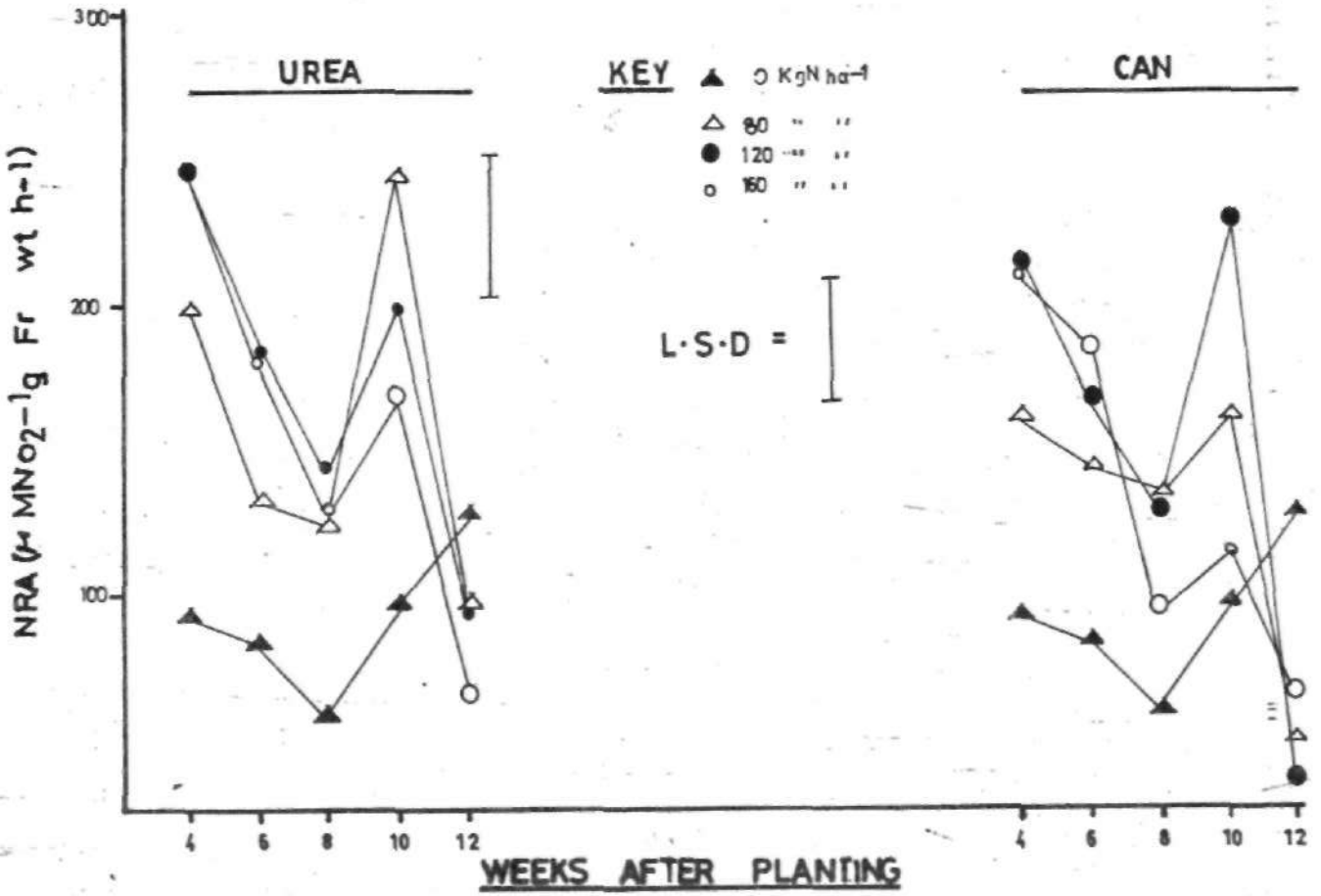


FIG 2. NITRATE REDUCTASE ACTIVITY IN VEGETATIVE LEAF OF TZB MAIZE

controls and the treatments) in NRA in both varieties was significant; it was highest at the 4th week after planting. NRA in the control plants increased significantly in both genotypes between 10th and 12th weeks of growth (Table VI). For instance, in HYBRID, NRA increased from an average value of 38.6 (in the 8th week) to 171 μM NO_2^-g fr. $wt^{-1}h^{-1}$ (in the 12th week) (Fig.1), while in TZB, it increased from 56.6 to 125.8 in the 8th and 12th week of growth respectively (Fig.2). Furthermore, the NRA in the control plants of both genotypes in the 12th week was higher than that in the treatments.

The ranking of NRA (in increasing order) in both CAN and UREA treated HYBRID plants followed this order: 80 < 120 < 160 < kg N/ha, the actual respective values of which are 177,213 and 257.3 μM NO_2^- g fr. $wt.^{-1}$ h^{-1}. This pattern of difference was maintained up to the 8th week after which NRA did not increase with increasing N concentration (Fig.1).

In TZB however, there was no significant differences in vegetative leaf NRA between 120 and 160 kg N ha^{-1} treatments in CAN treatments. So it was also in UREA treated plants except in the 10th week when the plants treated with 120 kgN ha^{-1} had a significantly higher NRA than the 160kg N ha^{-1} (Fig.2)

In Ear Leaf

The response of the ear leaf NRA in the two genotypesto N application differred significantly from that of vegetative leaf NRA. For example plants without any additional N showed higher ear leaf NRA than 120 kg N ha^{-1} but less than 80 and 160 kg N ha^{-1} in the first week after ear formation. Again at the 4th week after ear formation, the controls had a higher ear leaf NRA than 80 and 120 kg N ha^{-1} (Fig.4). Furthermore, both HYBRID and TZB plants grown without additional N gave the highest NRA at the 4th week of ear leaf sampling (Fig.3).

The vegetative leaf of both genotypes were generally superior in their NRA to ear leaf in all treatments except in HYBRID treated with CAN - 120 kg N ha^{-1} and the controls of TZB maize. This difference was significant during the 10th week after planting (i.e. 2nd week after ear formation). However, the pattern of NRA were sometimes similar. For example, when the level of NRA in the 10th week of the ordinary leaf declined, that of the ear leaf similarly declined at the same time (2nd week after ear formation) in both genotypes and at all treatments. This similarity was also observed in vegetative leaf NRA at 12 weeks after planting and the corresponding ear leaf NRA at 4th week after ear formation.

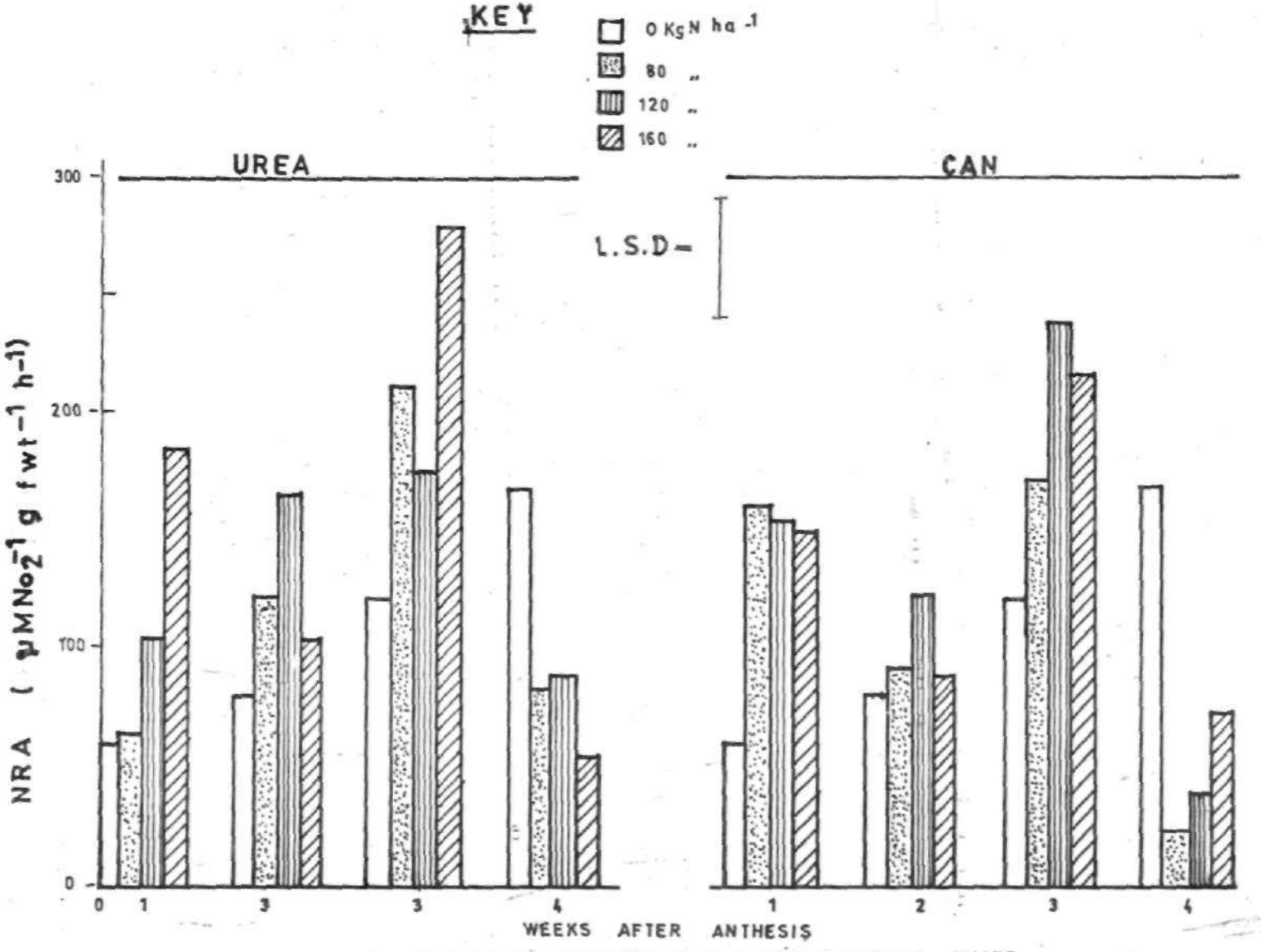


FIG. 3. NITRATE REDUCTASE ACTIVITY IN EAR LEAF OF HYBRID MAIZE

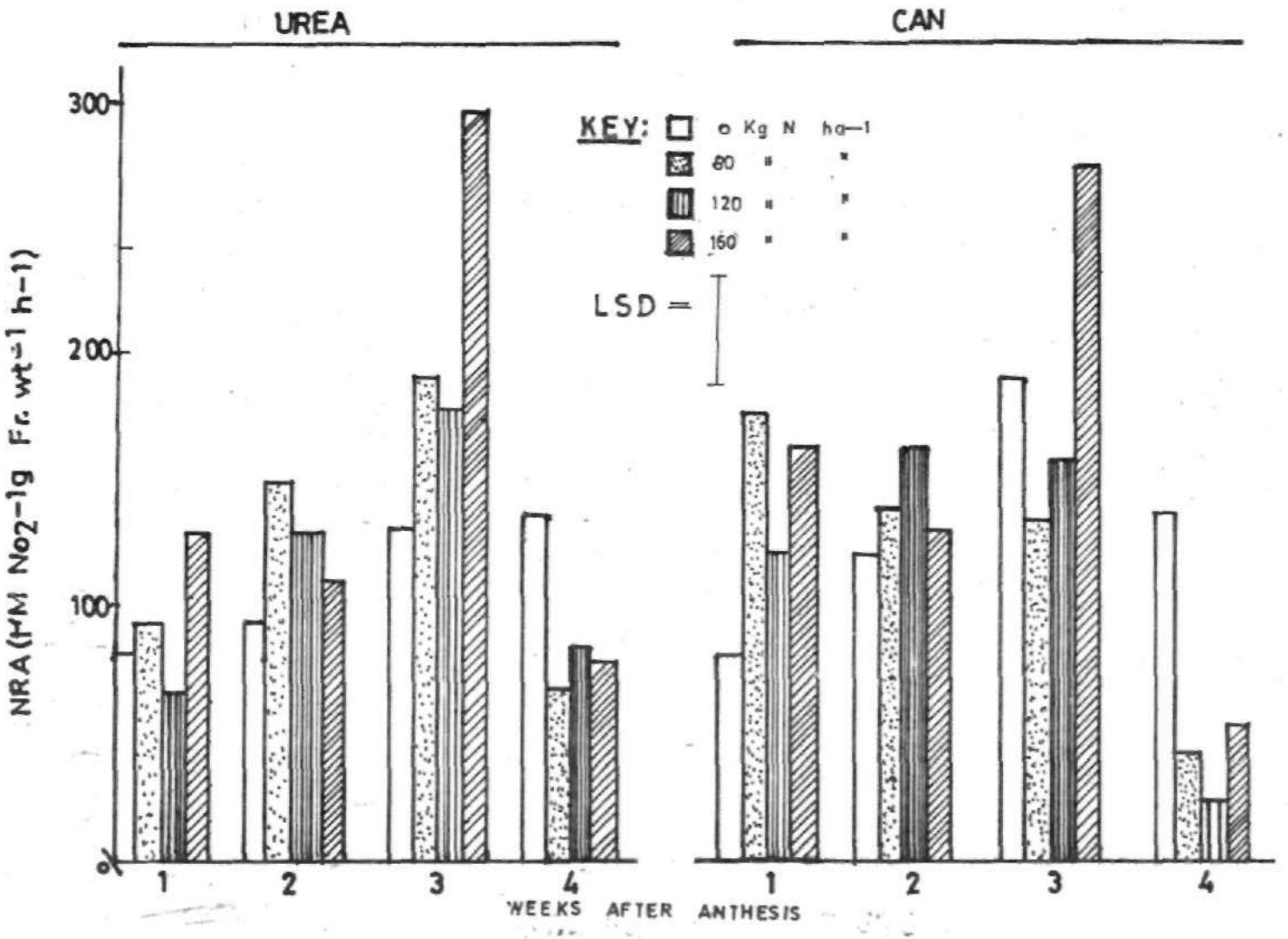


FIG.4. NITRATE REDUCTASE ACTIVITY IN EAR LEAF OF TZB MAIZE

4.2. Grain Protein Content (GPC)

4.2.1 Overall pattern of GPC.

Grain protein level in the grains of both genotypes and in all treatments was highest at 20 days after anthesis. The interaction of the four factors (genotypes x N-source x N level x sampling date) gave a significant variation between the various treatments (L.S.D. (5%) = 1.46) (see ANOVA Table II). Figs. 5 and 6 show the pattern of this interaction as it affects GPC in the HYBRID and TZB maize respectively. Plants in all the treatments had in common is the existence of a peak in GPC at 20 days after ear formation.

4.2.2. Effect of applied-N level on GPC

GPC was significantly affected by level of soil applied-N. The mean values for each level of applied-N is shown in Table VI below.

The grain protein concentration of genotypes increased significantly with increase in level of soil applied-N up to 120 kgN ha^{-1} level. Grains of plants grown with 160kg N ha^{-1} however did not differ significantly from those treated with 120 kg N ha^{-1} though it was significantly higher than that of 0 and 80 kg N ha^{-1}.

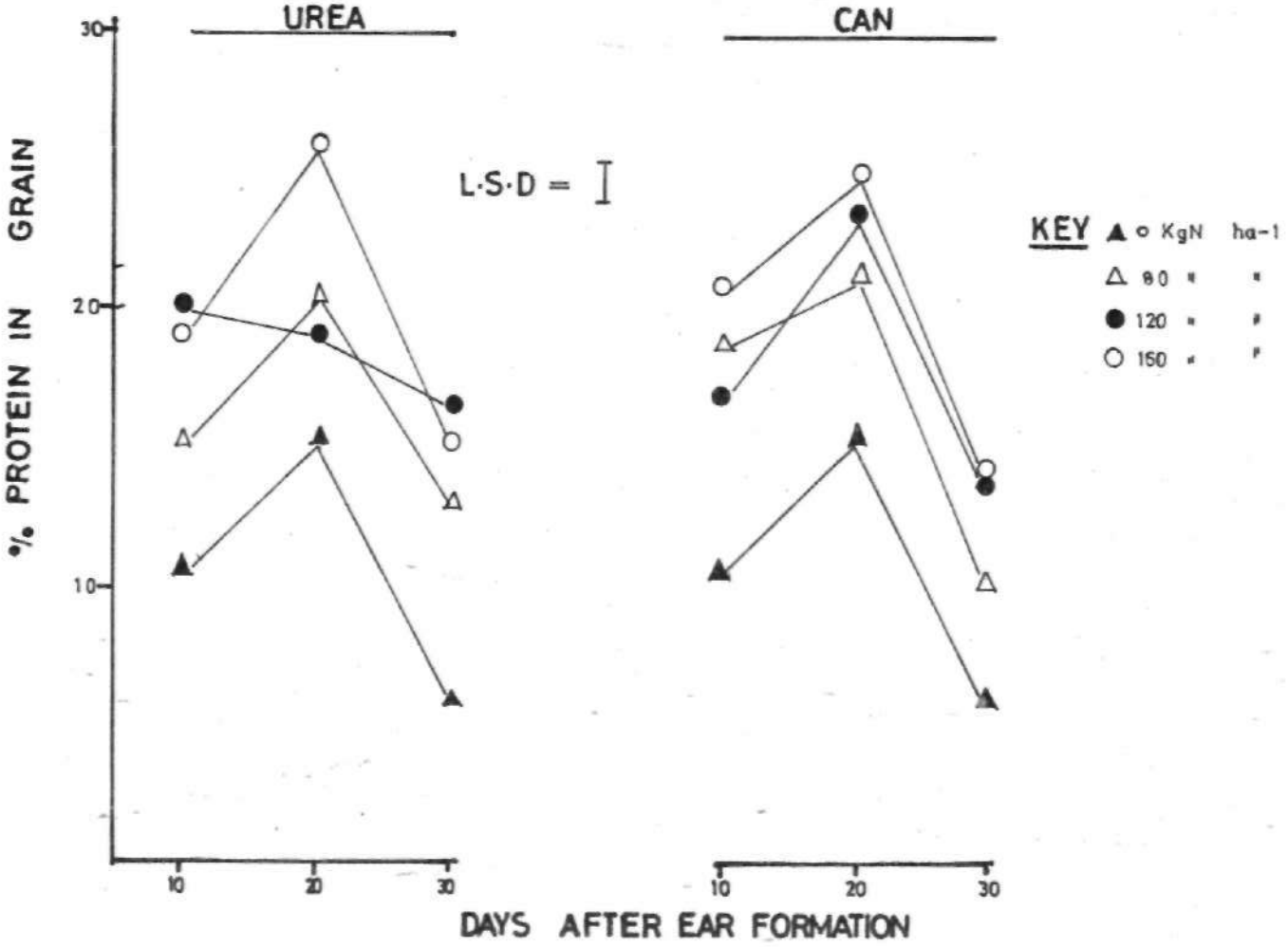


FIG.5. GRAIN PROTEIN CONTENT (%) DEVELOPEMENT IN HYBRID MAIZE

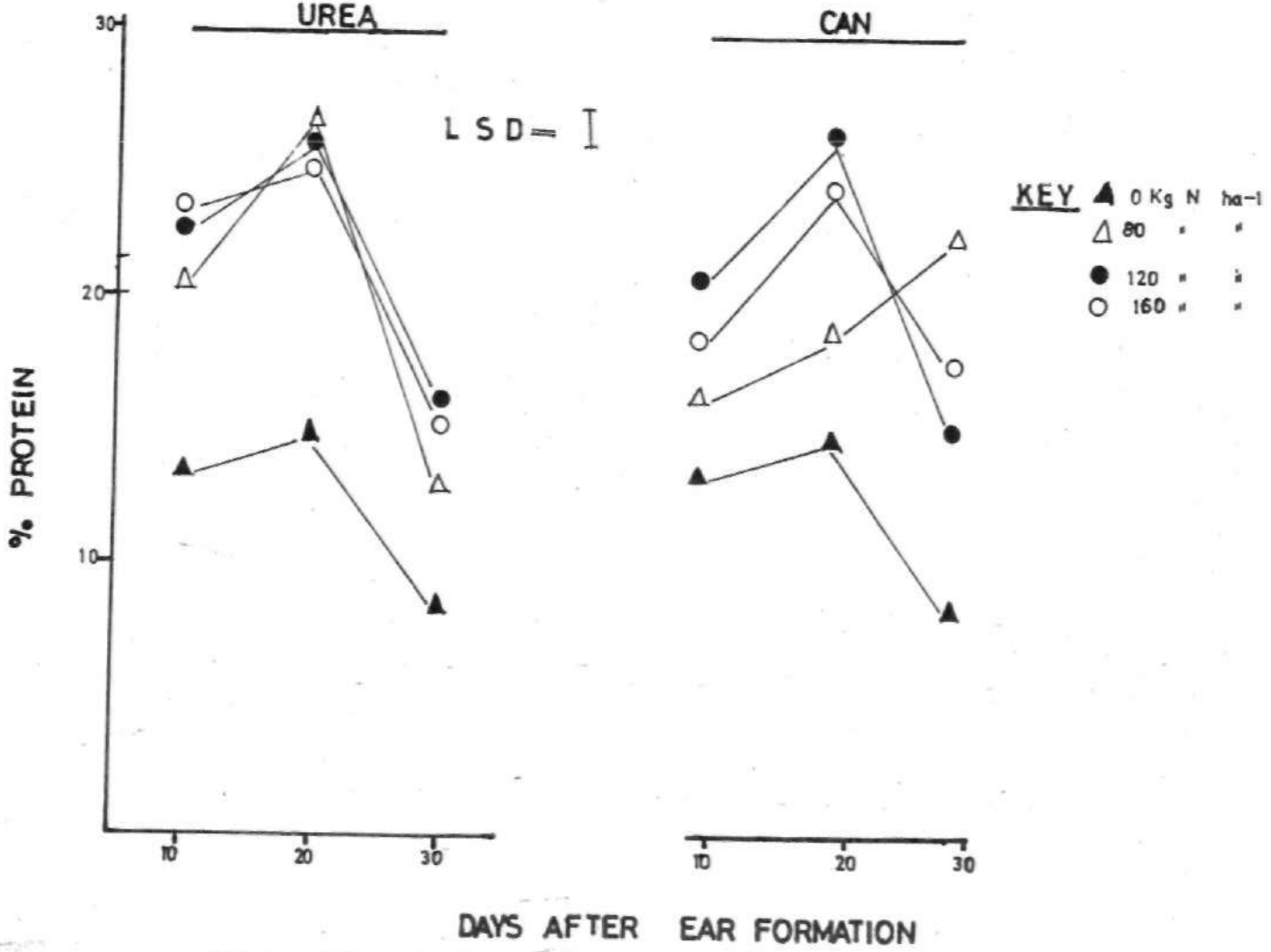


FIG. 6. GRAIN PROTEIN CONTENT (%) DEVELOPEMENT IN TZB MAIZE

Table VI. Effect of N level on GPC irrespective of genotype sampling date and N-source.

N-level	0	80	120	160 (kgN/ha)
GPC	14.58c	17.87b	19.28ab	20.05a

L.S.D (5%) = 1.68. Two figures followed by the same letter are not significant by DMRTS.

4.2.3. Effect of genotype on GPC

TZB maize was found to be significantly higher ($\bar{x}$ =19.8%) than HYBRID ($\bar{x}$=16.2%) in grain protein.

The effect of genotype was however not significant in its interaction with all other factors except with levels of applied-N and a combination of genotype x N-level x N-source.

The relationship between genotype and soil-applied N in GPC is shown in Table VII below:

Table VII. Mean values for effect of genotype x N-level on GPC.

Genotype	HYBRID				TZB			
N-levels (Kg N/ha)	0	80	120	160	0	80	120	160
GPC	11.3c	16.2b	18.0ab	19.7a	18.0ab	19.5a	20.5a	20.1a

L.S.D. (5%) = 2.38. Two figures followed by the same letter are not significantly different by DMRTS.

TZB plants grown without applied N contained significantly higher protein than those of the HYBRID; the same is true also for treatments with 80kg N ha^{-1}

However at 120 and 160 kg N ha^{-1} the difference in GPC between the two genotypes was not significant. Genotype x N-source x N level had no significant effect on GPC except for the controls of HYBRID which were significantly lower than all other treatments (Table VIII).

4.2.4. Effect of N-source on GPC

There was no significant difference in GPC between UREA and CAN treatments. Also N-source did not significantly affect other factors except for N-source x N-level x sampling date (see ANOVA Table II Appendix).

4.2.5. Effect of sampling date on GPC

There were significant changes in the level of protein in the course of grain development in the two genotypes. For instance, mean values for GPC for the three sampling dates were 17.6, 22.06 and 14.09% for 10, 20, 30 days after anthesis respectively. This indicates the existence of a peak at 20 days after anthesis in protein accumulation in the grain.

Table VIII. Effect of genotype x N-source x N-level on GPC.

Genotype:	HYBRID		TZB	
N-source:	UREA	CAN	UREA	CAN
N-level (kgN/ha)				
0	11.3b	11.3b	17.9a	17.9a
80	16.0a	16.0a	19.8a	19.1a
120	18.3a	18.3a	20.4a	20.7a
160	19.7a	19.7a	20.8a	20.0a

L.S.D. (5%) = 3.37. Two figures followed by the same letter are not significantly different by DMRTS.

4.3. Correlation between GPC and NRA

Grain protein content at all sampling detes (10, 20, and 30 days after anthesis) was found to be significantly correlated with NRA at all the different stages of growth in both vegetative and ear leaf. (Table IX).

Table IX correlation cefficient (r) values for NRA and GPC in Maize

	Days after ear formation		
	10	20	30
VEGETATIVE LEAF (wks after planting)			
4th	0.93	0.76	0.72
6th	0.89	6.81	0.71
8th	0.88	0.77	0.78
10th	0.68	0.78	0.56
11th	0.78	0.66	0.82
EAR LEAF (wks after anthesis)			
1st	0.81	0.77	0.79
2nd	0.79	0.79	0.60
3rd	0.73	0.71	0.71
4th	0.71	0.73	0.65

All 'r' values are significant at 1% level.

5. DISCUSSION

Nitrate Reductase Activity

The increase in NRA found in both genotypes with increase in levels of nitrogen is similar to the observations of earlier investigators (Harnandez et al, 1974; Ajakaiye, 1981). This is so because the enzyme is substrate - inducible. Recently, a number of reports have shown that NRA is directly proportional to the concentration of exogenous nitrate in various crops (Hamdi et al., 1978; Smith and Rice, 1983; Mengel et al., 1983).

The report that higher NRA is obtained when the plant is fully vegetative (but before the onset of flowering) (Al'aa el-Din et al., 1978; Vakhaniya and Nutsubize, 1979; Blahova and Sagata, 1980; Fadeeva et al., 1982; Streit and Feller, 1982; Winter et al., 1982) is further supported by results obtained in this work. The 4th and 6th weeks after planting were the weeks when dry matter accumulation was probably fastest and highest values for NRA were recorded then excluding the 10th weeks of sampling. This shows not only the existence of a relationship between the enzyme and the plant's ontology but it also suggests that the best period for selection purposes involving NRA should be immediately after seedling stage especially if N is applied once only (i.e. at the seedling stage or before) because, as earlier reported by Ajakaiye (1981), split application of N fertilizers increases NRA at later stages of plant growth.

The positive and significant values obtained for correlation between leaf NRA (in both ordinary and ear leaf) and GPC at various stages of grain development confirms the reports of other workers on various plants like wheat (Elrich and Hageman, 1973), Corn (Croy and Hageman, 1970), Sorghum (Ajakaiye, 1981), etc. This shows that in both genotypes, NRA could be used to predict treatments with higher GPC even before the grain formation.

The progressive decline in NRA in the successive weeks (except for the 10th week) could be due to various reasons. First, it may be due to the change in the plant's physiology from vegetative to flowering, a period of growth which is often accompanied by a fall in NRA possibly due to fluctuation in the ration of nitrate uptake to assimilation as reported by Dany and Murty (1982). Secondly, earlier rate of fresh matter accumulation apparently couldn't be maintained. This brings about a decrease in NRA which is positively correlated with fresh matter accumulation (Subbiaih and Balasimha, 1983). Thirdly, senescense might have accounted for the very low NRA during the 12th week. Senescense as earlier suggested (Ajakaiye, 1981) is said to be accompanied by high proteinase activity in the flag leaf.

The two N sources used, CAN and UREA, contain calcium ammonium nitrate and urea-N respectively. One would have expected a higher amount of NRA in CAN treated plants since the enzyme is substrate inducible following the results of earlier investigators. For example, Shraeder et al, (1972) found higher level of NRA in corn plants supplied with a combination of both ammonium and nitrate as compared to nitrate alone. On the contrary, UREA treated plants in the investigation reported here showed significantly higher NRA than CAN treatments at all levels of applied-N. The superiority of UREA here could be due to inhibitory effect of ammonium ions (from CAN) on nitrate uptake (Mcknown et al., 1982; Buczek, 1979) or on NRA itself (Oaks et al., 1979).

The vegetative leaves used throughout have been the second or third topmost leaf except the flag leaf which was used during the 12th week. These leaves are sites where vegetative activity is highest and hence sites of probable highest NRA in the plant. The ear leaf on the other hand is a much older leaf at about the middle of the plant's height. By virtue of its age, the ear leaf is expected to have a lower NRA as explained above.

Grain Protein

Increase in level of N above 80kg N/ha has caused increases not only in final GPC but also in earlier samplings (at 10 and 20 days after ear formation). The levels of GPC in treatment plants of both genotypes were significantly higher than those of plants grown without additional N.

Increases in GPC resulting from increases in soil-N content was also earlier reported by Yesevych et al (1980), Singh and Singh (1979), Nair and Abrol (1982), Campell et al. (1983), etc.

The absence of significant responses between the N-level of 120 and 160 kg N/ha suggests that the effect of soil-N level on GPC is not infinite. This shows that there is a threshold beyond which it is not economical to increase N application to crops.

That TZB maize had an overall significantly higher GPC in the absence of any such difference in NRA could be due to TZB probably having greater translocation efficiency of reduced- N to the grain than the HYBRID. The efficiency of the transport system hypothesis was postulated long ago by Croy and Hageman (1970) to explain the variation in GPC recorded in corn genotypes with similar NRA.

The occurence of a peak in GPC preceding grain maturity has earlier been reported in cereals like wheat (Jennings and Morton, 1963), barley (Rhodes and Mathers, 1974), and recently in sorghum varieties (Ajakaiye, 1984).

This work is probably the first of its type in corn. The occurence of such a peak has been previously explained in two ways: first, the proteolytic enhancement of hydrolysed N to the embryo and the dilution effect of starch that is normally used to fill the grains. The dilution effect is probably responsible for the decline in GPC recorded in both genotypes at 30 days after anthesis.

The observations made in some reports (Yuan and Shein, 1980; Dany and Murty, 1982; Streit and Feller, 1982) that the high rates of NRA occur during flower and pod initiation, grain development and tillering, could be related to the occurence of a peak in GPC during grain development. This most probably explains the increase in NRA during the 10th week (when the grains were half full and experienced the highest GPC). It will be interesting, for instance, to find out whether the increase in NRA was initiated by a demand for additional protein in the grain or vice-versa.

6. SUMMARY AND CONCLUSION

Both HYBRID maize and TZB exhibited similar pattern of response to soil - applied N in their vegetative leaf NRA. The controls were significantly lower than the treated. NRA continued to increase significantly with increase in level of applied N. This was common to all samplings up to the 10th wk of planting after which NRA became erratic.

Urea treated plants generally showed higher NRA than CAN treated plants.

NRA in both varieties varied at various stages of plant growth. Higher NRA was recorded at initial stages of sampling (at 4th and 6th wk after planting) when the plants were fully vegetative. In both vegetative and ear leaves, NRA sharply declined at the beginning of senescense.

No significant difference was found in NRA between the two genotypes, HYBRID and TZB maize, even though the HYBRID recorded a higher mean NRA values in the vegetative leaves of both genotypes were generally higher than those of the ear leaves

The interaction of the four factors (genotypes x N-level x N-source x sampling date) caused significant differences in NRA of various samplings. Generally, amount of reduced N increased with increase in soil-N level up to 120kgN ha. This was the same in both HYBRID and TZB; UREA or CAN; and all sampling dates.

TZB was found to be significantly higher in grain quality especially at 0 and 80 kg N/ha than the HYBRID.

Generally, there was no significant difference in GPC between UREA and CAN treatments.

Highest grain protein was recorded in both varieties at about 20 days after ear formation.

GPC at all sampling dates were found to be significantly correlated to NRA of both vegetative and ear leaf at all different stages of growth in both genotypes.

CONCLUSION

The two varieties of maize used, HYBRID 8321 - 21 and TZB Farz -21 are similar in their patterns of NRA and grain protein development. In both varieties, NRA and grain quality can be significantly increased with increase in soil - applied N. For good grain quality especially, additional application of N to soil is necessary. However, TZB appears to be more promising in grain quality.

In both varieties, NRA was found to be higher before the onset of flowering. At the set of flowering and thereafter, the enzyme activity becomes hardly predictable. Future research may need to focus attention on the definite cause of this fluctuation.

If only grain quality is required, say for preparation of feeds for cattle, sheep and chickens, grains of maize should be harvested before they are maximally filled about 20 days after signs of their formation have appeared.

Lastly, under adequate rainfall, NRA in the two varieties can be used at any stage of their growth to predict their grain quality.

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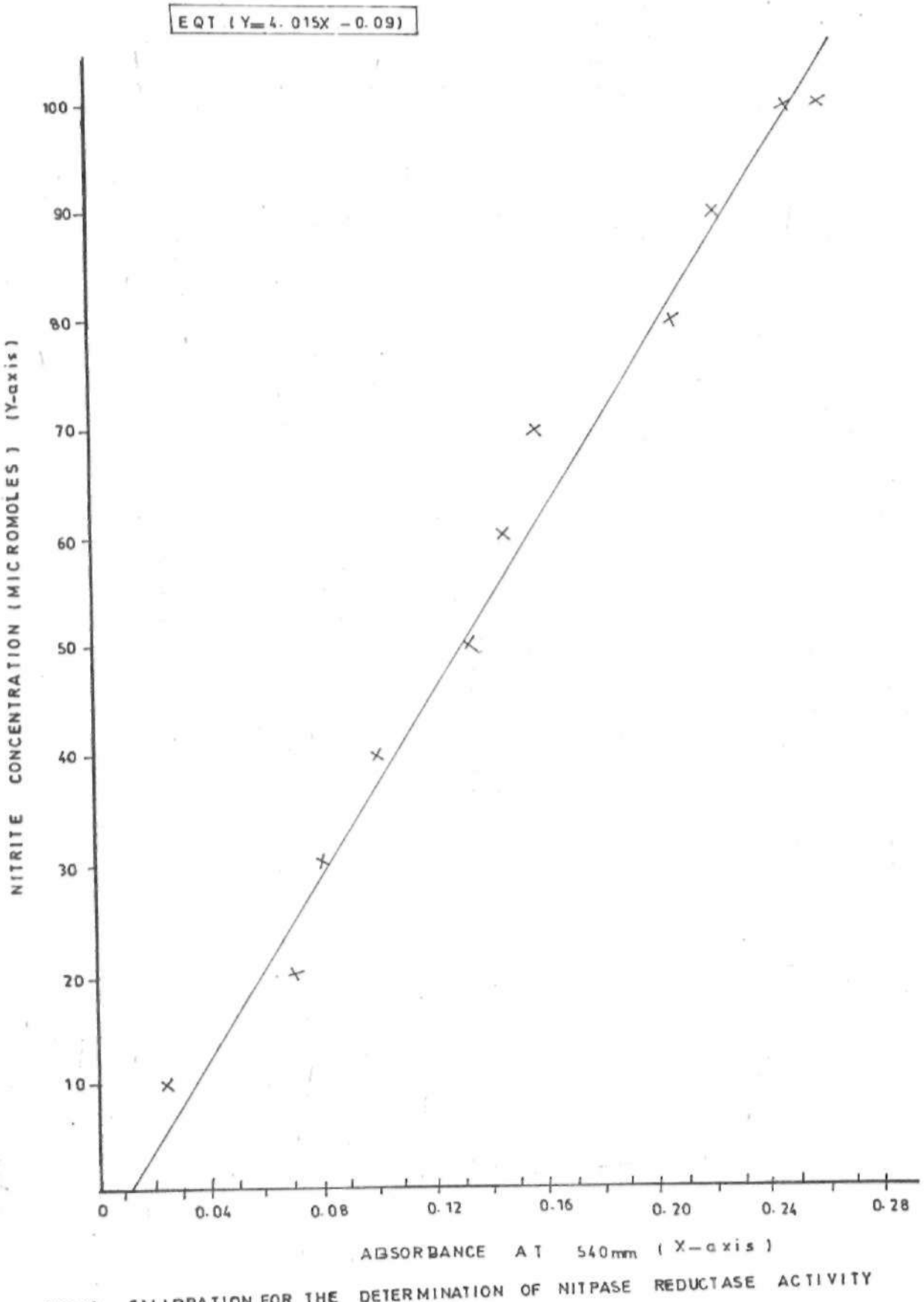


FIG. 13. CALIBRATION FOR THE DETERMINATION OF NITPASE REDUCTASE ACTIVITY

APPENDIX 2: ANOVA TABLES

1. ANOVA FOR NITRATE REDUCTASE ACTIVITY

Source of Error		DF	SS	MSS	F
Blocks (r=3)	(r-1) = 2		733.6	366.8	
Treatment					
Genotype (g=2)	(g-1) = 1		2001.61	2001.61	3.74
N-Source (s=2)	(s-1) = 1		6676.41	6676.41	12.46**
Sampling Date (d=9)	(d-1) = 8		645558.85	80694.86	150.65**
N-Level (l=4)	(l-1) = 3		143460.34	47820.11	89.27**
GS	(g-1) (s-1) = 1		49.18	49.18	0.09
SD	(g-1) (d-1) = 8		7643.79	955.47	1.78
GL	(g-1) (l-1) = 3		638.30	212.77	0.40
SD	(s-1) (d-1) = 8		36333.59	4541.70	8.48**
SL	(s-1) (l-1) = 3		6075.07	2025.02	3.78**
LD	(l-1) (d-1) = 24		548670.81	22861.28	42.68**
GSL	(g-1) (s-1) (l-1) = 3		4430.30	1476.77	2.76*
GLD	(g-1) (l-1) (d-1) = 24		41531.03	173.04	0.32
SLD	(g-1) (l-1) (d-1) = 24		55711.39	2321.30	4.33*
GSD	(g-1) (s-1) (d-1) = 8		4575.39	571.98	1.07
GSLD (g-1)	(s-1) (l-1) (d-1) = 24		32071.39	1336.31	2.49**
Error	(r-1) (gsld-1) = 286				
Total	(gsldr-1) = 431		153542.10		

2. ANOVA TABLE FOR GRAIN PROTEIN CONTENT

Source of Error				df	SS	MSS	F
Blocks	(r=3)			(r-1) = 2	3.02	1.51	0.12
Treatments							
Genotype	(g=2)			(g-1) = 1	383.17	383.17	29.40**
N-Source	(s=2)			(s-1) = 1	1.25	1.25	0.10
Sampling date	(d=3)			(d-1) = 2	1526.58	763.30	58.72**
N-level	(l=4)			(l-1) = 3	630.23	210.08	16.16**
GS			(g-1)	(s-1) = 1	0.61	0.61	0.47
GD			(g-1)	(d-1) = 2	52.68	28.34	2.03
GL			(g-1)	(l-1) = 3	161.94	53.98	4.15**
SD			(s-1)	(d-1) = 2	23.96	11.98	0.92
SL			(s-1)	(l-1) = 3	1.11	0.37	0.03
LD			(l-1)	(d-1) = 6	65.40	10.90	0.84
GSD		(g-1)	(s-1)	(d-1) = 2	92.78	46.39	0.57
GSL		(g-1)	(s-1)	(l-1) = 3	6.17	2.07	0.15
GDL		(g-1)	(d-1)	(l-1) = 6	204.96	34.16	2.63**
SDL		(s-1)	(d-1)	(l-1) = 6	133.76	22.29	1.72
GSDL	(g-1)	(g-1)	(s-1)	(l-1) = 6	185.46	30.91	2.38*
Error			(r-1)	(gsld-1)= 94	1222.78	13.01	
Total				(gsldr-1)= 143	4657.00		

APPENDIX 3

Regression value

For regression of y on x,

$$y = \bar{y} + b (x - \bar{x})$$

Where $b = \frac{\Sigma(dx \cdot dy)}{\Sigma dx^2}$

Correlation coefficient value ('r')

$$r = \frac{\Sigma dx \cdot dy}{\sqrt{(dx^2 \cdot dy^2)}}$$

ANOVA

Standard procedure for analysis of variance for 2x2x4x9x3 factorial design was used.

$$L.S.D. = t_{(5\%)} \times \sqrt{\frac{2MSE}{N}}$$

APPENDIX 4

CALCULATION OF RATES OF NITROGEN/ HECTRE

Example: How many grams of UREA fertilizer should be applied on each bed (Size: 3 x 1) to meet the concentration of 80kg N/ha?

1st Step: Find the concentration of N in Urea fertilizer. This concentration is written on the bag = 46% N. This implies that for every Kg of UREA, there is 0.46kg N.

80kg N/ha of urea is found by simple ratio and proportion arithmetic:

Fertilizer : N

1kg : 0.46kg

x : 80 kg.

$$x = \frac{80 \times 1}{0.46} = 174 kg/ha.$$

So if 174kg of UREA, is applied per hectre ($10000 m^2$) to meet a concentration demand of 80Kg N/ha, the quantity for 3sq. meter is calculated as:

$174kg : 10000m^2$

$x : 3m^2$

$$x = \frac{174 \times 3.0}{10000} = \frac{522.0}{10000}$$

$$= .0522kg = \underline{52.2g}$$

For CAN (concentration of N = 26%), 80kgN/ha will be

$$\frac{80 \times 1}{20} = 300kg/ha$$

So for $3m^2$, CAN (at 80kgN/ha) is calculated as:

$$\frac{300 \times 3}{10000} = 90g.$$