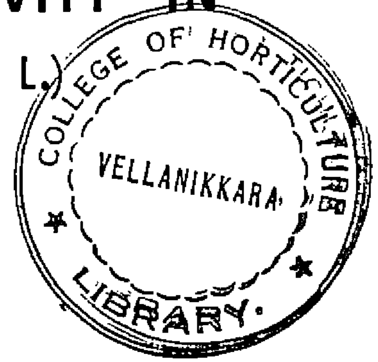
NITRATE REDUCTASE ACTIVITY IN COCOA (*Theobroma cacao* L.)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By

REKHA BHASKAR

THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

Faculty of Agriculture
Kerala Agricultural University

Department of Agronomy
COLLEGE OF HORTICULTURE
Vellanikkara, Thrissur

1991

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DECLARATION

I hereby declare that this thesis entitled "Nitrate Reductase Activity in Cocoa (*Theobroma cacao* L.)" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship of any other similar title, of any other University or Society.

Vellanikkara,
24/10/'91

Rekha Bhaskar
REKHA BHASKAR

CERTIFICATE

Certified that the thesis entitled "Nitrate Reductase Activity in Cocoa (*Theobroma cacao* L.)" is a record of research work done independently by Miss.Rekha Bhaskar under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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We, the undersigned members of the Advisory Committee of Miss.Rekha Bhaskar, a candidate for the degree of Master of Science in Agriculture, agree that the thesis entitled "Nitrate Reductase Activity in Cocoa (*Theobroma cacao* L.)" may be submitted by Miss.Rekha Bhaskar, in partial fulfilment of the requirement for the degree.

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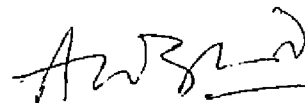


ACKNOWLEDGEMENT

My unreserved gratitude goes to Dr.P.V. Balachandran Associate Professor, Department of Agronomy and Chairman of my Advisory Committee for his expert counsel, unfailing help and affable disposition throughout the course of this investigation. Working under his guidance has been a privilege in itself and his unflagging patience, interest and co-operation have gone a long way towards the satisfactory fulfilment of this endeavour.

The help I received from Dr.E.Tajuddin, Professor and Head, Department of Agronomy, is thankfully acknowledged.

I am deeply obliged to Dr.R.Vikraman Nair, Professor, Department of Agronomy for the generous help he has always accorded me, during the course of this study.

Let me place on record my heartfelt thanks to Dr.P.A.Wahid, Professor, Radiotracer Laboratory, Kerala Agricultural University, Vellanikkara, who, inspite of a busy schedule, made a critical appraisal of this manuscript and offered constructive suggestions for its betterment.

I would be failing in my duty if I were not to acknowledge the help I received from Sri.Madhavan Nair, Associate Professor of Instrumentation by way of granting

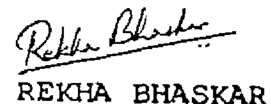
me full use of the Instrumentation laboratory facilities for my research work. I am grateful to him.

My abiding gratitude will remain with the staff members of the Department of Agronomy for their sincere and whole-hearted co-operation, throughout.

It is with immense pleasure that I thank all my friends each of who has contributed in some way or the other towards the completion of this task.

I am forever beholden to my parents who have been the source of inspiration for me at all critical junctures. I acknowledge with a full heart, their boundless affection, incessant encouragement and steadfast confidence in me, which I hope to have justified in some measure by this work.

Last but not the least, I bow my head before God Almighty whose blessings were with me every inch of the way, enabling me to undertake this venture successfully.



REKHA BHASKAR

Dedicated to my parents

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Introduction

INTRODUCTION

Cocoa ranks second only to coffee among the important plantation crops in the world and it is the basic raw material for a wide range of cocoa based products such as chocolates, confectionery, food drinks and ice creams. While cocoa consumption is concentrated primarily in the affluent Western countries, it is grown mainly as a cash crop in the Third World countries such as Ghana, Ivory Coast, Nigeria, Brazil, Malaysia, Indonesia and now in India. It can come up well under Kerala conditions, though its natural home is the lower tree storey of the New World evergreen tropical rain forests. Studies have been conducted on the nutritional requirements of this crop but few attempts have been made to study the physiological processes involved in the assimilation of these nutrients. Nitrogen is one of the most important plant nutrients influencing both vegetative and yield attributes.

Nitrogen is absorbed by plants mostly in the form of nitrate. In the pathway of assimilation of nitrogen, nitrate is first reduced to nitrite and then to ammonia before it is eventually assimilated into amino acid. The first step in this pathway, i.e. the reduction of nitrate to nitrite is regulated by the enzyme, nitrate reductase (NR). Hence nitrate assimilation can be controlled by regulating the activity of the enzyme NR. In green tissues, assimilation of

nitrate is irrevocably linked with photosynthetic reactions not only for the reduction of nitrate to ammonia but also for the generation of carbon compounds which are required for the incorporation of ammonia into amino acids.

Significant correlations have been reported between NR activity and yield in many crops. Such a relationship between the two can be useful for plant breeders if the relationship could be established at the seedling stage itself. Cultivars possessing a higher NR activity at the early vegetative phase are likely to be advantageous as the yield is determined quite early in the life of the plant.

Genotypic differences in NR level have been observed in sorghum, wheat, barley and sudan grass (Nair and Abrol, 1982). Genetic studies reveal that the level of the enzyme is highly heritable with the result that a hybrid could be bred with a predictable enzyme level by selecting parents with known NR activity.

Activity of the enzyme varies widely in different tissues and is influenced by several factors such as growth of the plant, substrate concentration, light intensity, moisture regime etc. Among these, light and nitrate concentration were found to have a profound effect on NR

activity. Further investigation on the effect of shade on NRA is needed as very little work has been done on this aspect.

The activity of this enzyme can be measured both *in vivo* and *in vitro*. The *in vitro* assay is conducted under optimum conditions. However, the presence of an inhibitor or inactivating enzyme in the tissue is likely to cause problems in the enzyme assay. In the *in vitro* assay, extraction media and assay media differ with crop. Hence standardisation of *in vitro* assay method for the crop is a pre-requisite.

Considering all these aspects the present investigation was undertaken with the following objectives:

1. To standardise the nitrate reductase assay in cocoa.
2. To assess the genotypic variability and to test the heritability of nitrate reductase.
3. To study seasonal variation of nitrate reductase activity.
4. To evaluate the influence of shade and moisture stress on nitrate reductase activity.
5. To work out the correlation between yield and NR activity in cocoa.

Review of Literature

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Majority of plants absorb nitrogen in the form of nitrate. Hence nitrate metabolism is one of the most important physiological processes occuring within the plant. Nitrate metabolism is however, dependent upon several extraneous factors, including environmental conditions. Studies have been conducted in many crops to assess the influence of light, moisture stress, mineral nutrients, plant age, genetic make-up, etc. on the process of nitrate reduction. As the published work on the NR activity of cocoa is scanty, the available literature on the subject pertaining to other crops also is briefly reviewed under the following headings:

1. Structure, properties and localization of nitrate reductase
2. Assay methods of nitrate reductase activity
3. Seasonal variations in nitrate reductase activity
4. Genotypic differences in nitrate reductase activity
5. Effect of NO_3-N on nitrate reductase activity
6. Effect of light on nitrate reductase activity
7. Influence of nitrate reductase activity on plant growth and yield

2.1. Structure, properties and localisation of nitrate reductase

Nitrate reductase (NR) is the key enzyme which catalyses the reduction of nitrate to nitrite, the first step in nitrate assimilation by plants. NR is the mandatory step to regulate incorporation of nitrate into amino acids due to its being (a) the first enzyme in the pathway of nitrate assimilation (b) the rate limiting step (c) substrate inducible and (d) relatively unstable both *in vivo* and *in vitro* especially when subjected to water stress, or high temperature (Beevers and Hageman, 1969). According to the specificity for electron donor there are three sub classes of NR. NADH : NR (EC 1.6.6.1) is the most common form in higher plants (Schrader *et al*., 1968). A bispecific NR using either NADH or NADPH is also found in some plants.

The biochemistry of NADH : NR has been extensively investigated with the enzyme isolated from squash, spinach (Nakagawa *et al*., 1985), maize (Nakagawa *et al*., 1984) and barley. More recently, the development of monoclonal antibodies for NR has made possible the use of immunoaffinity chromatography for rapid and direct purification of NR from crude extracts of several different plants. The native enzyme is a dimer of two identical sub units with a size of 210-230 KDa. Each subunit contains three prosthetic groups

which are FAD, heme-Fe and Mo pterin with one of each of these cofactors per subunit. The molybdenum cofactor of NR is a unique combination of pterin (a heterocyclic compound found in folic acid) and Mo (Campbell, 1988a).

Studies on the site of nitrate reduction in plants have indicated varying possibilities. Miflin (1967) reported that NR is present in the roots of barley and it is associated at least in part, with a fraction rich in mitochondria. According to Radin and Sell (1975), ovules of developing cotton bolls had significant NR activity, although considerably less than that in leaves. Developing ears have been found to be the site of nitrate reduction in wheat (Nair and Abrol, 1977). Total NRA of the developing ears equalled that of the leaf blades on the main shoot at low nitrogen (0 and 90 kg ha^{-1}) levels. At high nitrogen (180 kg ha^{-1}) level the total NRA of the leaf blades was significantly higher than that of the developing ears. Hunter _et al_. (1982) reported that soybean cultivars differ in the location of nitrate reduction and in general root reduction dominated in older plants. Higher NRA in shoot as compared to the root was reported in _Lolium perenne_ by Smith and James (1982). Thomas (1990) arrived at the conclusion that even the berries of black pepper exhibited NRA the level of which was found to increase with maturity.

The intracellular localization of NR in the green tissues of higher plants still remains debatable. Although some reports have proved that NR is associated with the chloroplast (Grant et al., 1970; Rathnam and Das, 1974; Rathnam and Edwards, 1976), others have been equally adamant in referring to it as a cytoplasmic enzyme (Dalling et al., 1972; Wallsgrove et al., 1979). There have been convincing reports that NR is attached to the chloroplast envelope membrane (Hewitt, 1975; Rathnam and Das, 1974; Ritenour et al., 1967). The intracellular localization of NR in different genera of green algae was examined by applying an immunocytochemical method. The enzyme was specifically located in the pyrenoid of the chloroplast, in all cases (Lopez-Ruiz et al., 1985). Immunogold labelling technique has revealed that NR appears to be localized exclusively in the cytoplasm of mesophyll cells in maize (Vaughn and Campbell, 1988). Ward et al. (1988) reported the presence of membrane associated NR in plasma membrane fractions isolated from barley roots.

Several experiments by Eilrich and Hageman (1973) and Brunetti and Hageman (1976) have indicated that NR could under some circumstances be the rate limiting step between nitrate and total protein accumulated by vegetation, grain and the entire plant. However, the accumulation of amino

and amide nitrogen that occurs when the plants are provided with excessive nitrate indicates no obligatory control of nitrate reduction over protein synthesis.

2.2. Assay methods of nitrate reductase activity

2.2.1. Extraction and assay media

The enzyme NR has been extracted and assayed by several methods, some of which are reviewed here.

Evans and Nason (1953) extracted crude enzyme from soybean by grinding plant tissue with 3 times its weight of cold 0.1 M KH_2PO_4 buffer (pH 9) and 2 times its weight of alumina powder for 10 minutes at 4°C. Then it was further ground for 3 minutes and centrifuged for 10 minutes at 20,000 times gravity.

It was observed by Mulder *et al*. (1953) that the NR activity of leaf fragments depends on the presence of undamaged cells. Grinding the leaves inactivated the enzyme system, deorganized the electron donor system and also probably led to loss of nitrite. However addition of yeast extract which is a source of FAD restored in some cases, the NRA of macerated leaf.

Maranville (1970) proposed that the addition of nickel (4×10^{-3} M) to the extracting buffer enhanced the

NRA in preparation of young grain sorghum leaf tissue by as much as six fold.

Klepper et al. (1971) used a grinding medium consisting of 25 mM potassium phosphate, 10 mM cysteine, 5 mM EDTA (pH 8.8) or 25 mM triacetate as a substitute for potassium phosphate, in order to extract NR from corn leaf tissue. For soybean, the extraction medium was modified by adjusting pH to 7.8 (Wells and Hageman, 1974).

Eck and Hageman (1974) proved that maximum recovery of NR is sundangrass was obtained with extraction media containing 20 mM glutathione and 4 mM $NiCl_2$. The optimum tissue : extraction medium ratio was 1 : 20. The $NiCl_2$ protected NR from exogenous cyanide upto 7.2 moles g^{-1}.

Schrader et al. (1974) reported that addition of 3% (w/v) BSA or casein to extraction media prevented or retarded the decay of NRA for several hours. Moreover, the presence of BSA or casein in the enzyme homogenate markedly increased NRA upto fifteen fold especially in older leaf tissue. Casein was shown to have such enhancing effect on the yield of NR from maize roots also (Wallace, 1975).

Neyra and Hageman (1975) employed a modified version of Hageman and Hucklesby's (1971) procedure. Their extraction media contained 25 mM buffer (pH 8.8), 5 mM cysteine,

2.5 mM EDTA and 0.1 per cent neutronyx 600. The assay mixture contained in moles - potassium phosphate buffer (pH 7.5) 50; KNO_3-20; NADH 0.4; EDTA and enzyme extract. The reaction was terminated by adding 0.2 ml of 0.5 M zinc acetate + 0.2 ml of phenazine methosulfate (46 mg l^{-1}).

Davies and Ross (1985) reported that NRA in potato leaf was enhanced in the _in vivo_ assay by anaerobic condition and by the inclusion of Triton X-100 and NADH in the incubation medium.

Chalifour and Nelson (1988) suggested the basic extraction medium of 0.1 K mol M-3 HEPES buffer, pH 7.5 containing 1 mol M^{-3} EDTA, 30 kg M^{-3} casein, 5 mol M^{-3} L-cystein and 50 kg M^{-3} of acid washed insoluble PVP. Foaming was suppressed with two drops of a four fold dilution of Balab antiform. The homogenate filtered through four layers of cheesecloth and the filtrate centrifuged at 30000 g for 15 minutes at 20°C.

Lillo and Hendriksen (1984) homogenised leaf tissue with sand in a mortar for 75s with 4 ml 50 mM potassium phosphate buffer at pH 7.5 containing 1% (w/v) casein, 14 mM mercapto-ethanol and 0.1% (v/v) Triton X-100. The extraction buffer was a modification of Lillo (1983) and Lewis _et al_. (1982). Kumar _et al_. (1988) included glycine in the

assay medium and their findings justified the hypothesis that glycine decarboxylation activity is a source of NADH for NRA. Kenis *et al*. (1989) reported that when NR was determined with the *in vitro* assay in oats, H_2O_2 did not seem to affect the activity after 4 h dark treatment, but NR decreased when crude extracts prepared from untreated 14 day old leaves were incubated directly with H_2O_2.

Long and Oaks (1990) reported that NR in maize roots had been stablized *in vitro* by the addition of chymostatin to extraction buffer. Contrary to previous observations, levels of NR were higher in the mature root than in root tip sections when chymostatin was included in the extraction buffer.

2.2.2. Inhibition by phenolic compounds

Conventional techniques to isolate active enzymes from many plant tissues have proved ineffective due to the presence of phenolic compounds. The mechanism of inhibition is that phenols may combine with protein reversibly by H bonding and irreversibly by oxidation followed by covalent condensations. Phenols can be removed by adding large amounts of substances which contain groups similar to the peptide bond. Loomis and Battiale (1966) used insoluble PVP to absorb phenols and thus obtained active soluble enzyme.

Any means of binding tannins (flavolan) the high molecular weight polyphenol, is considered important in extraction and isolation of enzymes from plants containing tannins. This inhibition is believed to be caused by astringent tannin polymer. Badran and Jones (1965) used polyethylene glycol (PEG) to complex the tannins in homogenate of green banana fruit. Methods to remove phenol inhibition include the use of large quantities of buffer, addition of reducing agents, cyanide and metal chelating agents, decolorizing with charcoal, dialysis, gel filtration and addition of PVP, PEG or albumin. PVP proved to be most effective in almost all cases.

Eppley (1978) reported that 5-20 mg PVP removed phenolics in marine phytoplanktons and 0.05 M $MgSO_4$ improved recovery of the enzyme. Lakshmidevi and Maheswari (1979) found that addition of 1.5% PVP in extraction medium enhanced NR recovery from *Lemna paucicostata*.

According to Kanser and Lewis (1984), it is essential to add 2% casein and 1.5 g insoluble PVP per gram material in extraction medium of leaves and roots of *Helianthus annus* while determining *in vitro* NRA.

2.3. Seasonal variations in nitrate reductase activity

Seasonal variations in the level of the enzyme nitrate reductase have been observed in many crops by several workers. Harper *et al.* (1972) reported that mean NRA for the entire leaf canopy was highest in the seedling stage for all maturity groups and declined as the plants matured. Activity of the total plant was maximal at approximately the full bloom stage with all maturity groups, irrespective of the calendar date at which the maturity group attained full bloom. Thus enzyme activity appeared to be closely associated with physiological growth stage. This fact is supported by the report of Harper (1974) which indicated that maximum nitrate utilisation occurs at the full bloom stage in soybean. Farooqi and Sirohi (1976) based on their experiments in Biloxi soybean came to the conclusion that NR activity is indirectly associated with flowering response of the plants.

Eskew *et al.* (1973) studied seasonal pattern of NRA in alfalfa cultivars and reported that in leaflets, NRA and nitrate concentration were high at young vegetative growth stages and declined with maturity during all growth periods. A study conducted by Parkash (1981) revealed that the pattern of NRA observed at different growth stages in wheat was mostly similar in all cultivars but the degree of

activity was strikingly different. Enzyme activity was noticed to be very high at most of the stages in all the three high protein lines, whereas, it was low in local cultivars at most of the stages.

The work of Murti and Balasinha (1983) concluded that potato variety 'Kufri sindhuri' exposed to short day showed tuber initiation by 30 days after transplanting while no tuber initiation was observed under long day. The NRA showed similar pattern till 50 days under the different photo periods with a high initial activity at 20 days, a decreased trend till 40 days, and increased again thereafter till 60 or 70 days.

Desperrier et al. (1986) reported that nitrate reductase activity per gram fresh weight in fenugreek reached a maximum in winter when the temperature was very low. The enzymatic activity also increased during the flowering period. Similar trend was observed in rice by Ramadevi (1986). There was a gradual increase in NRA among the stages upto flowering irrespective of treatments. The peak values were at flowering stage. The activity tends to increase wherever nitrogen content was increased by either organic or inorganic form. The activity tends to decline towards harvest.

The highest NR activity occurred in the seedling stage and declined towards anthesis in cowpea (Rhoden et al., 1987) and in winter wheat (Harper and Paulsen, 1967). Pietila et al. (1989) reported that NR in Scots pine needles had a wide minimum activity (1-10% of the maximum activity) during the summer months. The highest activity was recorded in late autumn or mid-winter. A smaller peak (30-60% of maximum activity) was observed from March to May.

Raju and Rajagopal (1988) observed that NRA in the berries of black pepper increased with the time of development. The leaf NRA of the different plant parts declined as the plant matured. According to Thomas (1990) there was marked variation in NRA in different months and the highest NRA was recorded in November and the least activity in April.

2.4. Genotypic differences in nitrate reductase activity

Genotypic differences in NR levels were reported in many cereals and grasses. Also genetic studies reveal that the level of this enzyme is highly heritable with the result that a hybrid could be bred with a predictable enzyme level by selecting parents with known NR activity (Hageman et al., 1967). The implications of this are of great importance to plant breeders and crop physiologists who are diligently involved in identifying indirect selection criteria for improved productivity of plants.

The work of Zieserl and Hageman (1962) confirmed that the level of NR is under genetic control. Schrader *et al*. (1966) demonstrated through his experiments in maize, that hybrids of the low x low (with respect to seasonal mean NRA) category exhibited heterosis for this character. That is, the level of NRA of the hybrids was significantly higher than the level of the higher inbred parent. Eck and Hageman (1974) reported significant differences in NRA among genotypes of sudangrass cultivars by both *in vivo* and *in vitro* assays.

Results of an experiment by Eck *et al*. (1975) in grain sorghum indicate that the genotype x age variance was essentially equal to the genetic variance for each character, which suggest that genotype x environment interactions are major components of the phenotypic variance. Broad sense heritability of *in vitro* NRA was high at all stages, and narrow sense heritability was moderate to low. Thus significant genetic advance could be expected from selections among pure lines and hybrids, but non additive genetic variance would reduce progress in hybrids based on selection of their parents to a moderate level. Heritabilities, in general, were lower for *in vitro* than for *in vivo* NRA.

Deckard and Busch (1978) established that the mid-parent values for NRA significantly predicted those crosses that produced a higher frequency of high yielding lines. The highest yielding line within a cross could not be differentiated from the lowest yielding line on the basis of NRA by either assay methods. Also, the NRA (*in vitro* and *in vivo*) of the lines from all crosses was not significantly correlated with yield of grain or grain protein. The relationship between *in vitro* NRA and yield of grain or grain protein was improved if crosses involving the more diverse genotypes were detected, but significant relationships generally were not obtained.

Warner *et al*. (1982) suggested that nar 1 is the structural gene for NR while nar 2 is the gene controlling the molybdocofactor component of the enzyme. Both nar 1 and nar 2 exhibit complete dominance and do not appear to be closely linked. The results of Somers *et al*. (1983) also confirmed that nar 1 is the structural gene for NR. Cross-reacting material was not detected in uninduced wild type of mutant extracts, suggesting that NR is synthesized *de novo* in response to nitrate.

The experiment by Messmer *et al*. (1984) culminated in the development of four classes of maize genotypes, viz. high NRA - high RN (reduced nitrogen), high NRA - low RN,

low NRA - high RN and low NRA - low RN. The initial classification of genotypes for high and low NRA or RN was reproducible when the four classes were grown in different environments. The high RN genotypes had greater grain yields than the low RN genotypes. The low NRA genotype also showed a yield advantage over the high NRA; however the low NRA was associated with high RN. The low NRA - high RN class had significantly greater yields than the other three classes. However, no significant genotypic differences in NRA were observed in some barley cultivars by Barneix et al. (1985).

Carroll and Gresshoff (1986) demonstrated stable inheritance of NR 345 and NR 328 phenotypes (NR - deficient mutants) of soybean upto the M_5 generation. Both mutants expressed inducible NR during early development and were sensitive to nitrate inhibition of nodulation.

The work of Rai et al. (1988) in sugarcane revealed varietal difference with respect to NRA. The variety Co 1148 registered higher values for NRA than CoJ 64. Nitrate and amide (urea) form of nitrogen enhanced the activity more than the ammoniacal form.

Thomas (1990) reported that lot of genotypic variation existed among varieties of black pepper. However, the broad

sense heritability of NR was found to be low (0.2) in black pepper.

2.5. Effect of NO_3-N on nitrate reductase activity

The enzyme NR is widely reported to be substrate inducible (Hageman et al., 1967; Campbell, 1988b). When nitrate is given to plants after a lag period, NRA rapidly increases for some hours and then levels off to a steady state. This is the typical pattern of enzyme induction by substrate.

Several workers reported that nitrate content of plant tissue was a major factor in controlling the level of enzyme activity and the induction of NRA on a field scale was achieved by supplemental nitrogen (Hageman and Flesher, 1960; Croy and Hageman, 1970). Deckard et al. (1973) found that each supplemental nitrogen treatment increased leaf blade nitrate concentration which resulted in a corresponding increase in NRA. However, each successive nitrogen application was less effective in increasing and maintaining leaf nitrate content and enzyme activity. Eilrich and Hageman (1973) discovered positive relationship between nitrate content and NRA of leaf tissue which also substantiated the dependence of enzyme activity upon availability of substrate.

Neyra and Hageman (1975) showed that the nitrate uptake pattern as a function of increasing external nitrate concentration followed saturation type kinetics. The reciprocal plot of the data was not linear but hyperbolic indicating that more than one K_m of nitrate uptake can be resolved from the data. This suggests the existence of either one carrier system with changing kinetic constants or the existence of dual uptake systems.

The experiments of Shaner and Boyer (1976) showed that the nitrate flux to the leaves from the roots play a much larger regulatory role than the leaf nitrate content in controlling the level of NRA in intact plants. Bigg and Daniel (1978) in an experiment to demonstrate the effect of nitrate, ammonium and 1:1 mixture of both on the growth of Douglas-fir, found out that Douglas-fir grown in perlite culture showed NRA in the order $NO_3^- >$ 1:1 mixture $> NH_4^+$. Misra _et al_. (1980) reported that the rate of nitrate influx during the period of induction was correlated with the pattern of NRA in shoots but not in roots. Also, studies with inhibitors indicated that NR synthesis was aided by nitrate uptake.

Adams and Attiwil (1982) reported that an increasing proportion of nitrate resulted in increasing NRA in all the species studied. Borah and Johari (1985) also showed that

plant tissues containing higher nitrate levels showed higher NRA in rice. Davies and Ross (1985) further confirmed that the activity of both *in vitro* and *in vivo* assay was inhibited by supra optimal concentration of nitrate.

Chalifour and Nelson (1988) reported that at all times of NO_3^--N application, as the levels of NO_3^--N increased, the proportion of total plant NRA contributed by leaves and roots remained relatively constant in faba bean, but these proportions increased and decreased respectively in pea. Sekhon *et al.* (1988) also demonstrated that NRA and nitrate content increased with increasing nitrogen levels in the nodules and leaves at flowering and pod development stage in moong. Langendorfer *et al.* (1988) opined that in squash, exogenous nitrate increased NRA by increasing steady-state levels of NR protein and by decreasing cellular nitrate concentration in plant cells. The effect of nitrate on enzymes of the nitrate assimilatory pathway are highly specific for these enzymes.

Many workers reported significant positive correlation between NR levels and nitrate content (Liu and Hadley, 1971; Eskew *et al.*, 1973; Adams and Attiwil, 1982; Sekhon *et al.*, 1988; Thomas, 1990). Non-significant correlation between nitrate concentration and NRA was reported by Toman and Pauli (1964) and Guerrier *et al.* (1985). Negative correlation

between the two parameters was reported in maize by Zieserl *et al.* (1963) and in barley by Passama *et al.* (1987).

The results of Goodman *et al.* (1974) showed that NRA was significantly heritable at high nitrogen supply but not at low nitrogen supply when presumably the inducer, nitrate was limiting.

2.6. Effect of light on nitrate reductase activity

Diurnal variations in nitrate reductase activity were first studied by Hageman *et al.* (1961) in corn. Similar variations were later observed in rice seedlings (Shibata *et al.*, 1969), lolium (Bowerman and Goodman, 1971), soybean (Harper and Hageman, 1972), pepper (Steer, 1974), cowpea (Rhoden *et al.*, 1987), barley (Deane-Drummond *et al.*, 1979; Lewis *et al.*, 1982) and in black pepper (Thomas, 1990).

Hageman *et al.* (1961) reported that NRA was reduced proportionally to the reduction in light intensity. Also the diurnal variation of NRA in both the hybrids studied was correlated positively with the water soluble protein content and negatively with nitrate content. The highest level of NRA was observed in plants with the least competitive shading (thin planting rate). The results of Zieserl *et al.* (1963) showed that there was a progressive decrease in leaf NR, protein content and grain yield per plant as the plant

population was increased in maize, due to self-shading. Hageman _et al_. (1967) opined that artificial shading caused both reduction in CO_2 fixation and accumulation of nitrate. Nitrogen metabolism was more affected than carbohydrate metabolism under decreased light intensities. NR activity disappeared in the dark.

Bilal and Rains (1973) reported that light enhanced _in vivo_ enzymatic activity. The activity was highest after 9 h and then decreased steadily for several more hours even in the presence of light. A similar trend observed in cowpea by Rhoden _et al_. (1987) was that NRA increased from 0600 h to 1800 h and then declined to a minimum at 2400 hours.

Nicholas _et al_. (1976) reported that NR activity declined with all assays when plants were exposed to dark. The loss of _in vivo_ NRA in soybean during darkness appeared to be due to the combination of a net loss of enzyme _per se_ and energy depletion. The subsequent light stimulation of NRA was likely due to increased availability of reductant energy as well as a synthesis of the NR enzyme. Rao and Rains (1976) discovered a positive correlation between NRA and light in barley. They opined that illumination increased plant capacity for nitrate absorption, possibly through increased energy supply and or increased NRA.

Smith and James (1982) showed that enzyme activity increased in a linear manner when plotted against the logarithm of the light intensity. Plants grown under high levels of natural illumination did not show a diurnal pattern of change in NRA but those given much lower levels did so, indicating that responses to short term changes in natural but relatively high light intensity did not normally occur. Leong and Shen (1982) have suggested the generation of a NR inhibitor in rice, which is inducible in the shoot of rice seedlings by dark, minus-nitrate or plus-ammonium treatments. Lillo (1983) arrived at the conclusion that the high NRA in barley during the photoperiod was associated with low NR stability both in the extracts and in the plants. On the other hand, the low NRA during the dark period was associated with high stability in the extracts and in the plants.

The results of Lillo and Ruoff (1984) indicated that the decrease of NRA in darkness can be considered as a reversible unimolecular conversion of the active form of NR into an inactive form, forming a negative stabilizing feedback loop. The light induced increase of NRA is related to a positive destabilizing feedback loop.

Rajesekhar <u>et al</u>. (1988) found that after nitrate triggering, NR expression appears to be regulated by light via phytochrome. However, data by Schuster <u>et al</u>. (1989)

document particular kinds of interaction between controlling factors (light, nitrate, ammonium, plastidic factor) which effect gene expression in plants. These intricacies of regulation should be considered in molecular studies on NR gene expression.

2.7. Influence of nitrate reductase activity on plant growth and yield

As nitrate reductase is one of the key enzymes in nitrate assimilation, it is believed to play an important role in the growth and yield of plants. The results obtained by Liu and Hadley (1971) showed that NR activity was strongly linked with vegetative growth and plant maturity. Deckard et al. (1973) observed that the highest correlations between NRA and yields of grain and grain protein were obtained during the stages of ear initiation and development. This suggests that a minimal number (1 to 3) of samplings would be as effective as the laborious full season sampling (12 or more) in selecting individual plants or varieties that have a high potential for grain yields or grain protein production.

Elrich and Hageman (1973) reported that seasonal NR correlated significantly and positively with grain yield in wheat. This was further confirmed by Deckard et al. (1977) and Nair and Abrol (1982).

Dykstra (1974) however opined that the expression of NRA was not an index of nitrogen assimilation ability but may be a useful index of growth potential when nitrate ion does not limit NR synthesis.

Johnson *et al.* (1976) examined the possibility of using NR assays early in the development of the crop as a predictor of ultimate grain yield. Although the NR measurements do not correlate perfectly with final yield, they provide a useful test for future yield except at the highest nitrogen levels and there is at present no remotely reliable alternative. NR activity has been suggested as an indirect selection criterion for green yield in maize by Fakorede and Mock (1978). The experiments of Deshmukh and Srivastava (1983) concluded that NRA at pre-flowering stage of sunflower had significant positive correlation with seed yield under normal condition. However, no correlations of NRA with either seed yield or seed protein were obtained in the case of soybean (Harper *et al.*, 1972) and barley (Young *et al.*, 1980).

From the results of Remadevi (1986) in rice, it would be safe to assume that NR which catalyzes the reduction of nitrate to nitrite assumes importance in the proper growth and development phenomenon right from early stages in most plants.

Materials and Methods

MATERIALS AND METHODS

The experiments undertaken to study nitrate reductase activity (NRA) of cocoa during the course of this investigation were as follows:

1. Field experiments

    1. Genotypic differences in nitrate reductase activity
    2. Influence of shade levels on nitrate reductase activity of cocoa under irrigated and rainfed situations
    3. Seasonal influence on nitrate reductase activity
    4. Heritability of the levels of nitrate reductase activity

The plants used in this study belong to the Cadbury-KAU Co-operative Cocoa Research Project. The details of the experimental field are as follows: It is located on moderately sloping terrain which enjoys a typical humid tropical climate. The average altitude of the site is 22.25 m above mean sea level. The management practices followed were as per the Package of Practices Recommendation (KAU, 1989). The data on the soil characters of the experimental site are presented in Appendix-1.

2. Pot culture experiment

This was undertaken to study the effect of NO_3^--N on nitrate reductase activity. The plants used were cocoa

budlings of the Cadbury-KAU Co-operative Cocoa Research Project.

3. Laboratory study for standardisation of *in vitro* assay of nitrate reductase in cocoa

3.1. Field experiments

3.1.1. Genotypic differences in nitrate reductase activity

To study the genotypic variations in NRA, a total of thirty plants belonging to Germplasm VI were assayed at intervals of 3 months for a period of one year. Out of these, ten plants belonged to the high yielding category, another ten were medium yielders and the remaining ten were poor yielders. The assays for NRA were done at 11 AM during the second weeks of July 1990, October 1990, January 1991 and April 1991. For the assay, the first hardened leaf from the topmost branch exposed to sunlight was used and each assay was carried out in triplicate, employing the method of Klepper *et al*. (1971). Statistical analysis of the data was done as a split plot design in CRD taking genotypes as whole plots and the four seasons as sub-plots.

3.1.1.1. Germplasm VI

This collection of budded plants field established since 1983 arises from nearly all the cocoa types introduced into the country from time to time and maintained at the

CPCRI Regional Station, Vittal; Cadbury Farm, Thamarassery; Regional Agricultural Research Station, Pilicode and CPCRI Sub-Station, Kannara. The list of the types used and their genetic nature are given in Table 1.

3.1.2. Influence of shade levels on nitrate reductase activity of cocoa under irrigated and rainfed situations

In this experiment, cocoa plants subjected to four different shade levels and under two different moisture regimes (irrigated and rainfed) were used. The different intensities of shade were 0, 25, 50 and 75 per cent respectively. These shade levels were maintained by the selective thinning of the rubber trees amidst which the cocoa trees were planted.

Plants under the different shade levels were subjected to different moisture regimes, viz. irrigated and rainfed. Irrigation was done by sprinkler twice a week, during summer. During this period, the plants under rainfed situation experienced moisture stress.

For the nitrate reductase assay, 5 plants under each shade level (for both rainfed and irrigated situations) were randomly selected. Sampling was done in the second weeks of June 1990, September 1990, December 1990 and March 1991 ie. at intervals of three months for a period of one year. The assay procedure was same as above.

Table 1. List of types of Germplasm VI

Accession No.	Parentage	Nature of material	Yielding category
2	C 42	Seedling	High
7	P3 x P4	Seedling	High
9	P3 x P1	Seedling	High
19	W6/56(T63/970)	Seedling	High
22	P12 x P2	Seedling	High
44	Landas 357	Seedling	High
49	SCA 6	Seedling	High
53	MOQ 413	Budded	High
56	EET 272	Budded	High
94	Landas 36	Seedling	High
1	ICS 1	Seedling	Medium
5	C 83	Seedling	Medium
14	C78	Seedling	Medium
26	P1 x P7	Seedling	Medium
35	PA7 x Na 32	Seedling	Medium
40	Jerangau Amel x Na 33	Seedling	Medium
42	Jerangau PA7 x Na 32	Seedling	Medium
46	Na 33	Seedling	Medium
48	ICS 6	Seedling	Medium
51	IMC 67	Cutting	Medium
11	C 79	Seedling	Low
13	T30-10 x Na 32	Seedling	Low
20	T86/2	Seedling	Low
24	W5/15(T63/884)	Seedling	Low
30	T85/5 x Na 32	Seedling	Low
52	Na 31	Cutting	Low
68	P7c	Budded	Low
74	ICS 45 x ICS 39	Seedling	Low
85	Landas 18	Seedling	Low
100	Landas 50	Seedling	Low

In this experiment, the interaction effect of shade and moisture on NRA could not be studied as the treatments were not replicated. Hence the data were analysed as CRD separately for irrigated and rainfed situations.

3.1.3. Seasonal influence on nitrate reductase activity

Leaf NRA of ten cocoa plants belonging to the Germplasm VI were assayed at monthly intervals from June 1990 to May 1991. The plants used were accession Nos.2, 7, 9, 19, 22, 1, 5, 14, 26 and 35. Sampling was done at 11 AM in the third week of each month. Statistical analysis of the data was done as a split plot design in CRD taking the twelve months as sub-plots and the genotypes as main plots. The meteorological data for the sampling period are presented in Appendix-2.

3.1.4. Heritability of the enzyme nitrate reductase in cocoa

To study heritability of levels of nitrate reductase in cocoa, leaf NRA of twenty hybrid plants and their parents were assayed at intervals of three months for a period of one year. The assays were carried out in the last weeks of August 1990, November 1990, February 1991 and May 1991.

Details of the hybrids and their parents used for the study are presented in Table 2.

Table 2. List of hybrids and their parents

Hybrids (F_1)	Parents
H-1	G II 20/4 x M 9/16
H-2	G II 20/4 x M 16/9
H-3	G II 19/5 x M 16/9
H-4	V 10/3 x G VI-54
H-5	V 10/3 x G VI-56
H-6	V 10/3 x G VI-61
H-7	V 5/9 x G VI-54
H-8	V 5/9 x G VI-55
H-9	V 5/9 x G VI-61
H-10	V 4/8 x M 16/9
H-11	V 4/8 x G VI-54
H-12	V 15/5 x G VI-55
H-13	V 15/5 x G VI-54
H-14	M 13/12 x V 5/9
H-15	M 16/9 x G VI-56
H-16	V 15/5 x G VI-56
H-17	V 15/5 x G VI-59
H-18	V 9/6 x GVI-68
H-19	V 9/6 x G VI-51
H-20	V 9/6 x G VI-61

The hybrids were planted under the shade of rubber and were raised without irrigation.

3.2. Pot culture experiment

3.2.1. Effect of NO_3^--N on nitrate reductase activity

A pot culture experiment was conducted in CRD using budded cocoa plants of type V15/5 to examine the effect of nitrate-nitrogen on NRA. The plant is of Amazonian origin and was brought to India from the Cocoa Research Institute of Ghana.

The treatments consisted of four levels of nitrogen replicated thrice. Earthern pots of dimension 35 x 35 x 25 cm were filled with 23 kg of potting mixture (1:1:1 sand:soil: cowdung) and one budling was planted in each pot on 6-3-1991. Four levels of nitrogen viz., 0, 200, 400 and 800 kg N ha^{-1} were applied as calcium nitrate. Sampling was done at fortnightly intervals thereafter. NRA was assayed using the method of Klepper *et al.* (1971) and the index leaf used was the first hardened leaf from the top.

3.3. Laboratory study for standardisation of *in vitro* assay of nitrate reductase in cocoa

For the standardisation of the *in vitro* assay, extraction media composed of the different combinations and concentrations of potassium phosphate buffer of different pH.

EDTA, dithiothreitol, casein, bovine serum albumin (BSA), FAD, polyvinylpyrrolidone, etc. were used.

One gram of plant sample was mixed with 8 ml of the extraction medium and was homogenised. This was then centrifuged at 2000 g for ten minutes at 0-4°C. The supernatant was then used for the assay. NR was assayed in triplicate for 15 minutes at 27°C in the medium of Bar Akiva and Saigiv (1967): 0.5 ml 5 M potassium phosphate buffer pH 7.5, 10 µm FAD and 5 mM EDTA; 0.1 ml 1 mg ml^{-1} NADH; 0.1 ml 0.1 M KNO_3 and 0.1 ml plant extract. The reaction was stopped by adding 1 ml of 1 per cent (w/v) sulphanilamide in 3 M HCl followed by 1 ml 0.01% (w/v) N-(1-naphthyl) ethylene diamine dihydrochloride solution. After standing for 30 minutes the mixture was centrifuged at 2000 g for 5 minutes and nitrite measured by absorbance at 540 nm using a calibration chart prepared by adding nitrite to similar extracts without NADH.

3.4. Analytical procedures

3.4.1. *In vivo* nitrate reductase assay

The procedure of Klepper *et al.* (1971) employed for the *in vivo* assay was as follows: Leaves were cut into 8-10 mm length and weighed before infiltration. 0.3 g of the leaf tissues were placed inside injection bottles containing 5 ml of infiltration medium (0.2 M KNO_3 and 1 mM potassium

phosphate at pH 7.5). The bottle containing the media and tissue was evacuated (6 mm Hg pressure) for 30 seconds, the vacuum was released and the process was repeated. During the infiltration process, the tissues in all cases were visibly wetted and sank below the surface of the medium. The bottles were then incubated in a B.O.D. incubator for one hour at 33°C with gentle shaking at 15 minutes interval.

After one hour the bottles were placed in boiling water bath for 5 minutes to arrest the reaction. After cooling 0.4 ml of the medium was removed for the nitrite determination. One ml of colouring reagent (1% sulphanilamide in 3 M HCl and 0.02% naphthyl ethylene diamine dihydrochloride in equal volumes) was added and solution made upto 6 ml. The colour was read at 540 nm in Spectronic-20 spectrophotometer. Each sample was done in triplicate.

3.4.2. Nitrate

The nitrate content of dried leaves of the Field and Pot culture experiments were estimated using the method of Singh (1988).

3.5.1. Statistical analyses

The statistical analyses were done adopting the method suggested by Panse and Sukhatme (1954).

3.5.2. Heritability

The data were analysed for the analysis of variance as described by Panse and Sukhatme (1954) for a split plot design in CRD.

The formulae used in the estimation of variability at genotypic and phenotypic levels are:

$$\text{Genotypic variance} = \frac{\text{Mean square due to genotype} - \text{Mean square due to error}}{\text{Replications}}$$

$$\text{Phenotypic variance} = \text{Genotypic variance} + \text{error variance}$$

Heritability in the broad sense was estimated by the formula suggested by Burton and Devane (1953).

$$h^2(b) = \frac{\text{Genotypic variance}}{\text{Phenotypic variance}}$$

3.5.3. Heterosis

Heterosis over better parent (heterobeltiosis) (Briggle, 1963) and mid-parent (relative heterosis) (Hayes *et al.*, 1965) were calculated.

The formulae used were:

$$\text{Heterobeltiosis} = \frac{\bar{F}_1 - \overline{BP} \times 100}{\overline{BP}}$$

$$\text{Relative heterosis} = \frac{\bar{F}_1 - \overline{MP} \times 100}{\overline{MP}}$$

where $\bar{F}_1$, $\overline{BP}$ and $\overline{MP}$ are the mean performance of F_1 hybrid, better parent and mid-parent respectively.

Results

RESULTS

4.1. Genotypic differences in nitrate reductase activity

The NRA of the different genotypes showed significant variations and the activities ranged from 1.22 to 2.68 mmoles $NO_2^- g^{-1}$ fwt h^{-1}. The highest activity was recorded by G VI-52 and the lowest by G VI-94 (Table 3). However, the activities of G VI-52 and G VI-48 were on par, as were G VI-48 and G VI-46. The accessions G VI-14, 56, 68, 42 and 26 also had activities that were on par. There were no significant differences among the NRA of G VI-68, 42, 26 and 9, as also between G VI-42 and G VI-44 and also between G VI-26 and G VI-74. The activities of G VI-44 and G VI-51 were not significantly different as also G VI-85 and G VI-20. The accession G VI-20 recorded an NRA which was on par with that of G VI-100. The activities of G VI-40 and G VI-12 were on par as were those of G VI-30 and G VI-11. The genotypes G VI-35 and G VI-24 also recorded NRA which were on par. There were no significant differences among the NRA of G VI-11 and G VI-53, G VI-24 and G VI-5 and between G VI-53 and G VI-13. The activities of G VI-19 and G VI-1 as also that of G VI-1 and G VI-7 were on par. However, the NRA of the accession G VI-94 which was the lowest recorded was significantly lower than that of all the other accessions.

Table 3. Mean NRA (mmoles NO_2^- g^{-1} fwt h^{-1}) of cocoa accessions over 4 months during the year 1990-91 (CD (0.05) = 0.11)

G VI 52	G VI 48	G VI 46	G VI 14	G VI 56	G VI 68	G VI 42	G VI 26	G VI 9	G VI 44	G VI 85	G VI 74	G VI 51	G VI 20	G VI 40
2.68	2.60	2.55	2.39	2.35	2.33	2.31	2.28	2.27	2.20	2.19	2.18	2.15	2.08	2.03

G VI 30	G VI 100	G VI 2	G VI 35	G VI 22	G VI 11	G VI 24	G VI 53	G VI 49	G VI 5	G VI 19	G VI 13	G VI 1	G VI 7	G VI 94
2.00	1.98	1.96	1.93	1.93	1.90	1.84	1.81	1.76	1.76	1.70	1.70	1.64	1.53	1.22

Table 4. Mean NRA (mmoles NO_2^- g^{-1} fwt h^{-1}) of cocoa accessions for each of the 4 months during the year 1990-91 (CD (0.05) = 0.06)

OCTOBER	JULY	JANUARY	APRIL
2.57	2.46	1.73	1.40

Table 5. Effect of genotype on mean NRA (mmoles NO_2^- g^{-1} fwt h^{-1}) of cocoa accessions during the year 1990-91

Treatments	Mean NRA
Genotypes	
G VI - 2	1.96
G VI - 7	1.53
G VI - 9	2.27
G VI - 19	1.70
G VI - 22	1.93
G VI - 44	2.20
G VI - 49	1.76
G VI - 53	1.81
G VI - 56	2.35
G VI - 94	1.22
G VI - 1	1.64
G VI - 5	1.76
G VI - 14	2.39
G VI - 26	2.28
G VI - 35	1.93
G VI - 40	2.03
G VI - 42	2.31
G VI - 46	2.55
G VI - 48	2.60
G VI - 51	2.15

Contd.

Table 5. Continued

Treatments	Mean NRA
G VI - 13	1.70
G VI - 20	2.08
G VI - 24	1.84
G VI - 30	2.00
G VI - 52	2.68
G VI - 68	2.33
G VI - 74	2.18
G VI - 85	2.19
G VI - 100	1.98
F test	Sig.
SEm±	0.06
CD (0.05)	0.11
Seasons	
July	2.46
October	2.57
January	1.73
April	1.40
'F' test	Sig.
SEm±	0.03
CD (0.05)	0.06

4.2. Effect of shade on nitrate reductase activity and nitrate content

This study was undertaken with the objective of finding the effect of varying shade levels on NRA of cocoa under irrigated and rainfed moisture regimes.

4.2.1. Nitrate reductase activity under irrigated condition

Significant difference in NRA was observed at different shade intensities only during June (Table 6). The highest NRA was recorded in the open (0% shade level) and this was significantly superior to the NRA noted at 25% and 75% shade levels.

During March also the same trend was observed, but differences were not significant. During September and December, though the differences were not significant highest NRA was observed at 50% shade level. With respect to the mean NRA over the entire period of observation, highest activity was observed at 0% shade level followed by that at 50% and 75% shade levels. The least activity was observed at 25% shade level.

4.2.2. Nitrate content under irrigated condition

There was significant difference in nitrate content at different levels of shade intensity only in June (Table 6). Maximum nitrate was recorded at 0% shade intensity and was

Table 6. NRA (mmoles NO_2^- g^{-1} fwt h^{-1}) and NO_3^- (ppm) content of leaves of irrigated cocoa at different shade intensities during four months of the year 1990-91

Months	June		September		December		March		Mean	
Shade intensity levels (%)	NRA	NO_3	NRA	NO_3	NRA	NO_3	NRA	NO_3	NRA	NO_3
0	3.61	3154	2.05	1239	1.50	1164	1.60	1164	2.19	1680
25	2.03	1427	2.49	1352	1.49	976	1.09	901	1.78	1164
50	2.71	1803	2.78	1840	1.71	1427	1.47	1126	2.17	1549
75	2.33	1539	2.56	1502	1.64	1765	1.24	1389	1.94	1549
F test	Sig.	Sig.	NS	NS	NS	NS	NS	NS	NS	NS
SEm±	0.44	571	0.42	775	0.36	675	0.34	463	0.20	315
CD (0.05)	0.94	1210	0.90	1642	0.77	1393	0.71	980	0.42	668

significantly superior to the rest. The next highest nitrate concentration was seen at 50% shade level but was on par with that at 75% and 25% shade levels.

In September though the nitrate content did not vary significantly, the 50% shade level recorded the highest nitrate content followed by 75% shade level. In December and March, though the differences were non significant the highest nitrate content was noticed at 75% shade level. During all four months of observation, the least nitrate concentration was obtained at 25% shade level. With respect to mean nitrate content over the entire period of observation, the highest nitrate content was recorded at 0% shade and the least at 25% shade level. The mean nitrate contents at 50% and 75% shade intensities were at par.

4.2.3. Nitrate reductase activity under rainfed condition

Significant difference in NRA was observed at different intensities of shade in June and September (Table 7). In June, the activities at 0% and 25% shade levels were on par and superior to the activities at 50% and 75% shade levels which were on par.

However, in September, the highest activity was observed at 75% shade level but was on par with those at 25% and 50%

shade levels. These were significantly superior to the NRA at 0% shade level.

In December and March, NRA did not vary significantly at the different shade levels. When mean NRA was considered, though differences were not significant, the highest activity was observed at 75% shade level followed by those at 25%, 0% and 50% shade levels.

4.2.4. Nitrate content under rainfed condition

There was significant difference in nitrate content at different shade intensities only during June and September (Table 7). In June, highest nitrate content was observed at 25% shade level followed by those at 0% and 75% shade levels and all these were significantly superior to nitrate content at 50% shade level.

However, in September, the nitrate contents at 50% and 75% shade intensities were on par and superior to the contents at 0% and 25% shade levels which were also on par. In December and March, significant differences were not observed. The mean nitrate content over the entire period of observation was maximum at the 75% shade level, followed by those at 0%, 50% and 25% shade levels.

4.3. Seasonal variations in nitrate reductase activity

The nitrate reductase activities of the ten accessions of cocoa studied showed significant differences during the

Table 7. NRA (mmoles NO_2^- g^{-1} fwt h^{-1}) and NO_3^- (ppm) content of leaves of rainfed cocoa at different shade intensities during four months of the year 1990-91

Months / Shade intensity levels (%)	June		September		December		March		Mean	
	NRA	NO_3	NRA	NO_3	NRA	NO_3	NRA	NO_3	NRA	NO_3
0	3.14	2553	1.26	901	1.92	1464	1.15	789	1.87	1477
25	3.25	2591	2.22	563	1.37	1051	0.86	826	1.92	1258
50	1.58	901	2.39	2328	1.60	1352	1.13	1051	1.68	1408
75	2.32	1765	2.77	2516	1.62	1014	1.05	1014	1.94	1577
F test	Sig.	Sig.	Sig.	Sig.	NS	NS	NS	NS	NS	NS
SEm±	0.38	396	0.37	514	0.31	438	0.21	376	0.13	174
CD (0.05)	0.80	840	0.79	1089	0.65	929	0.43	797	0.27	369

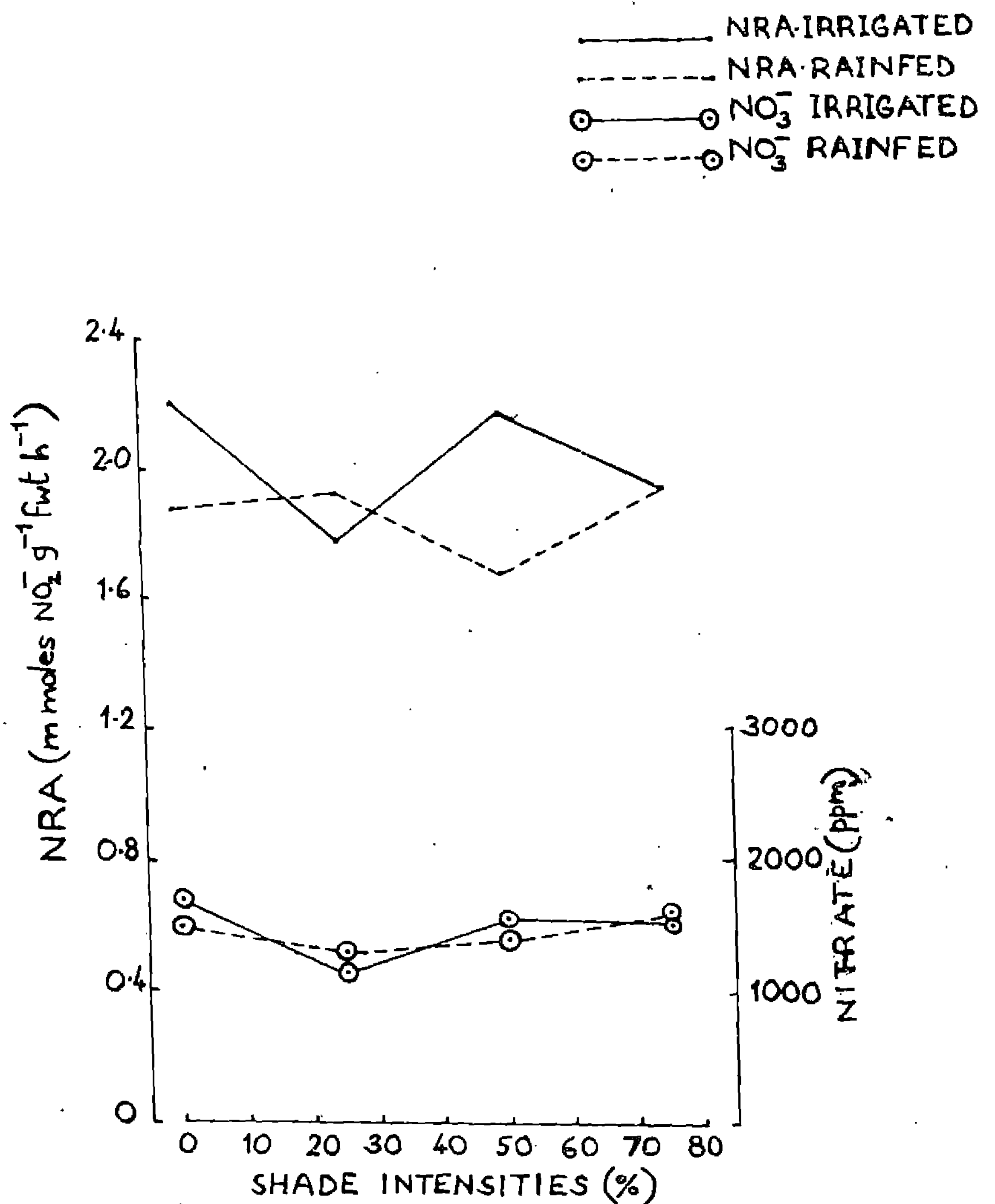
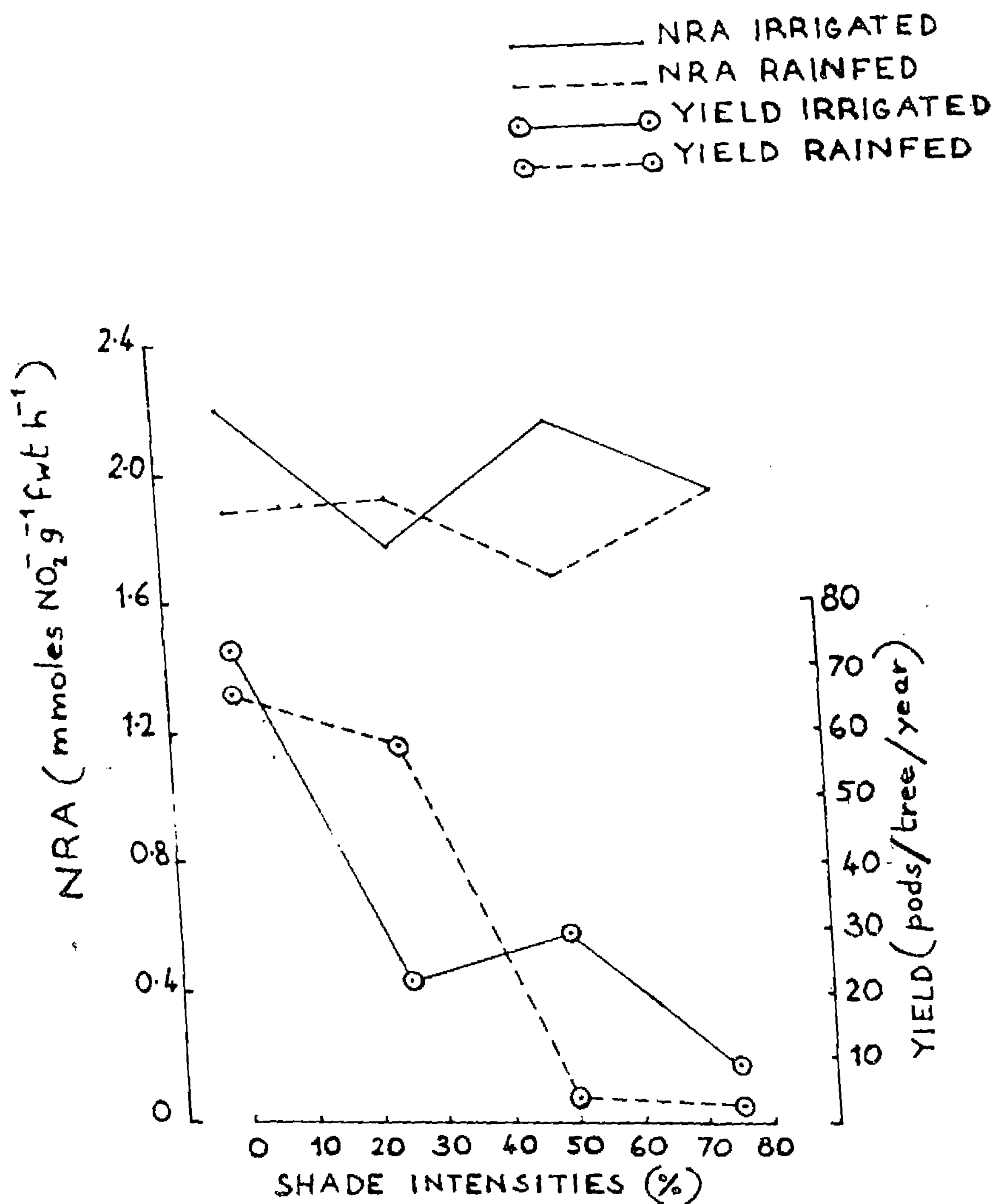


FIG. 1. FOLIAR LEVELS OF NITRATE REDUCTASE ACTIVITY AND NITRATE IN COCOA AS INFLUENCED BY SHADE AND MOISTURE



FIG·2· FOLIAR LEVELS OF NITRATE REDUCTASE ACTIVITY AND MEAN YIELD OF COCOA AS INFLUENCED BY SHADE AND MOISTURE

twelve months during which sampling was done. The maximum activity of 3.05 mmoles NO_2^- g^{-1} fwt h^{-1} was recorded during October and the minimum activity of 0.84 mmoles NO_2^- g^{-1} fwt h^{-1} during May (Table 8).

Significant differences in NRA were also obtained among the ten genotypes studied. The activities ranged from 1.58 to 2.45 mmoles NO_2^- g^{-1} fwt h^{-1}. Maximum NRA was recorded in the case of the accession G VI-9 and minimum NRA in the case of G VI-2 (Table 8). The season x genotype interaction was also found to be significant.

The accessions G VI-2, 7, 10 and 1 recorded maximum NRA during the month of June (Table 11). Maximum NRA was recorded in G VI-9 during August and October, G VI-22, G VI-5 and G VI-26 during August and G VI-5, G VI-14 and G VI-35 during October. On an overall basis, the highest NRA of 3.86 mmoles NO_2^- g^{-1} fwt h^{-1} was exhibited by G VI-5 in the month of October whereas the least NRA of 0.37 mmoles NO_2^- g^{-1} fwt h^{-1} was seen in G VI-2 during the month of November.

4.4. Nitrate reductase activity in different parts of cocoa plant

Nitrate reductase activities in the leaf, petiole and pod were studied during July 1990, October 1990, February 1991 and May 1991. Only the leaf exhibited nitrate reductase

Table 8. Effect of season on mean NRA (mmoles NO_2^- g^{-1} fwt h^{-1}) for cocoa accessions

Treatments	Mean NRA	Treatments	Mean NRA
Genotypes		Seasons	
G VI - 2	1.58	June	2.94
G VI - 7	2.25	July	1.81
G VI - 9	2.45	August	2.79
G VI - 19	1.95	September	2.28
G VI - 22	2.17	October	3.05
G VI - 1	2.06	November	1.96
G VI - 5	2.19	December	1.58
G VI - 14	2.26	January	2.24
G VI - 26	2.16	February	1.90
G VI - 35	2.00	March	1.90
'F' test	Sig.	April	2.00
SEm±	0.02	May	0.84
CD (0.05)	0.04	'F' test	Sig.
		SEm±	0.03
		CD (0.05)	0.05

Table 9. Mean monthly NRA (mmoles NO_2^- g^{-1} fwt h^{-1}) of cocoa accessions during the period June 1990 to May 1991

OCT.	JUN.	AUG.	SEP.	JAN.	APR.	NOV.	FEB.	MAR.	JUL.	DEC.	MAY
3.05	2.94	2.79	2.28	2.24	2.00	1.96	1.90	1.90	1.81	1.58	0.84

CD (0.05) = 0.05

Table 10. Mean NRA (mmoles NO_2^- g^{-1} fwt h^{-1}) of cocoa accessions during the period June 1990 to May 1991

G VI-9	G VI-14	G VI-7	G VI-5	G VI-22	G VI-26	G VI-1	G VI-35	G VI-19	G VI-2
2.45	2.26	2.25	2.19	2.17	2.16	2.06	2.00	2.95	1.58

CD (0.05) = 0.04

Table 11. Mean NRA (mmoles NO_2^- g^{-1} fwt h^{-1}) of ten accessions during the period from June 1990 to May 1991

Accession No.	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May
G VI - 2	2.95	1.22	2.83	1.68	2.55	0.37	0.85	1.64	0.83	1.81	1.29	0.90
G VI - 7	3.47	2.28	3.29	2.16	2.83	2.53	1.17	3.13	2.14	1.08	2.26	0.67
G VI - 9	3.64	1.29	3.68	2.85	3.68	3.26	2.36	2.35	2.26	2.64	1.93	0.50
G VI - 19	3.38	2.58	1.93	1.36	2.12	2.28	1.68	2.09	1.84	1.29	2.26	0.64
G VI - 22	1.89	2.12	3.45	2.07	3.34	1.68	2.00	2.21	2.39	1.71	2.21	0.97
G VI - 1	3.45	2.14	2.19	1.93	3.33	2.28	1.04	1.93	2.16	1.10	2.46	0.64
G VI - 5	3.24	0.99	1.40	2.39	3.86	1.04	1.73	3.19	2.85	2.16	1.64	1.82
G VI - 14	2.44	2.30	3.38	3.11	3.66	2.55	1.71	2.02	1.06	2.12	2.19	0.60
G VI - 26	2.62	1.63	3.66	2.92	2.26	2.44	1.95	2.14	1.61	2.33	1.54	0.78
G VI - 35	2.28	1.57	2.12	2.35	2.85	1.17	2.35	1.73	1.81	2.74	2.23	0.83

CD at 5% level for comparing two main plot (accession) means at same level of sub plot (month) = 0.16

CD at 5% level for comparing two subplot means at same level of main plot = 0.12

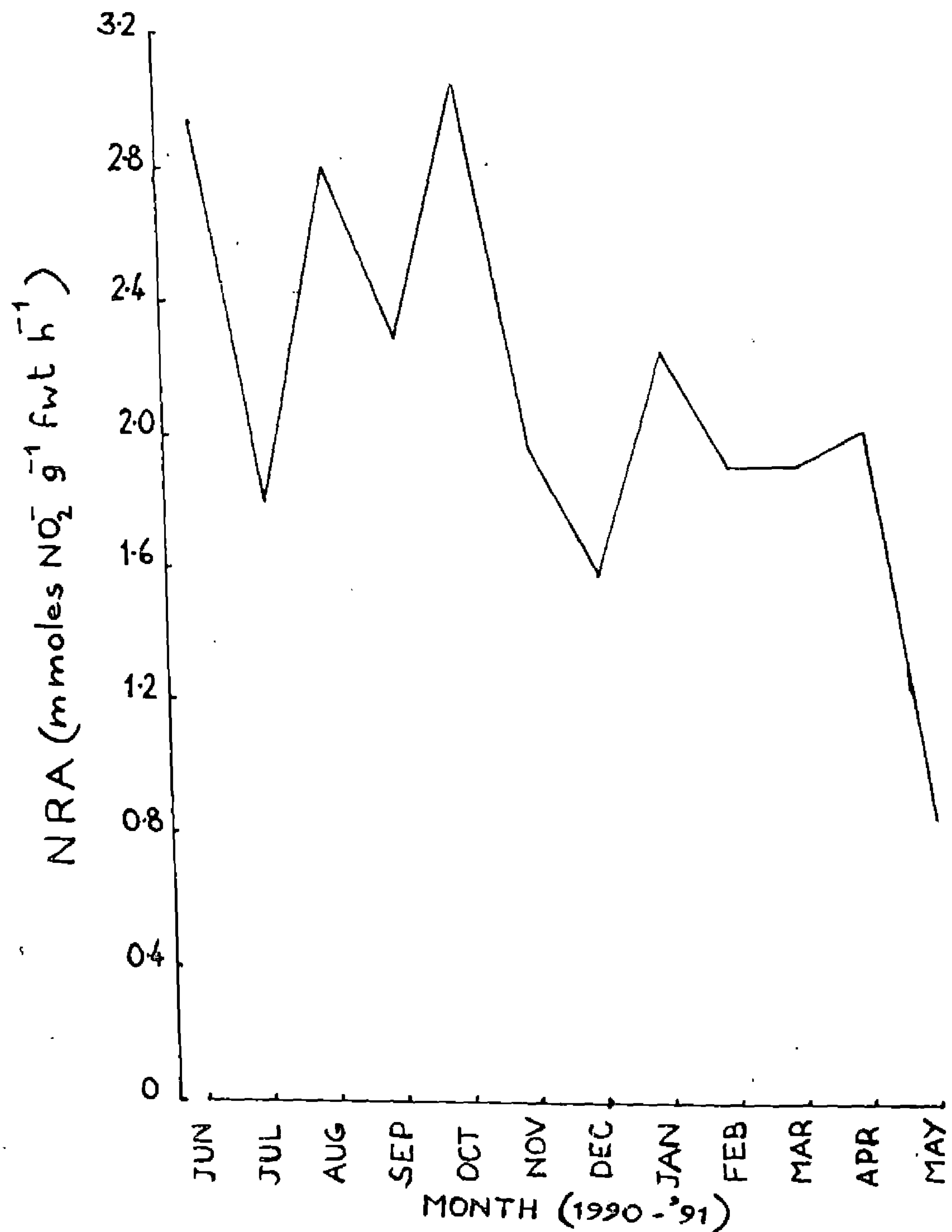


FIG.3. SEASONAL VARIATION IN FOLIAR LEVELS OF NITRATE REDUCTASE ACTIVITY IN COCOA

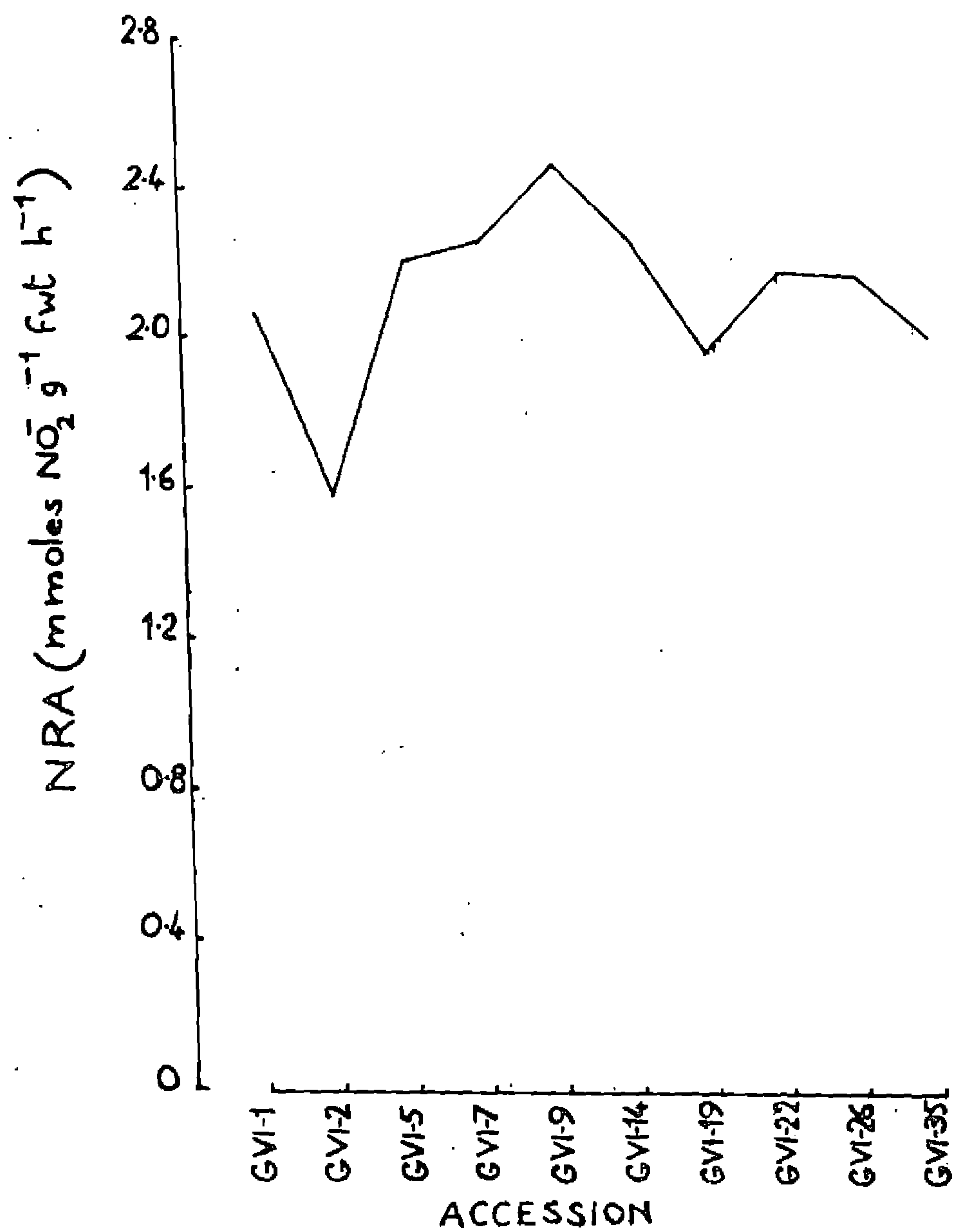


FIG.4. MEAN FOLIAR NITRATE REDUCTASE ACTIVITY IN TEN ACCESSIONS OF COCOA FOR THE PERIOD JUNE 1990 - MAY 1991

activity. No activity was observed in either petiole or pod at any stage.

4.5. Heritability of nitrate reductase and heterosis in hybrids of cocoa

4.5.1. Heritability of nitrate reductase

Analysis of variance for thirty cocoa genotypes was done for a split plot design in CRD and heritability in the broad sense was worked out by the formula suggested by Burton and Devane (1953).

$$\text{Genotypic variance} = \frac{\text{Mean square due to genotype} - \text{Mean square due to error}}{\text{Replications}}$$

$$= \frac{1.40 - 0.02}{3} = 0.46$$

$$\text{Phenotypic variance} = \text{Genotypic variance} + \text{Error Variance}$$

$$= 0.46 + 0.02 = 0.48$$

$$\text{Heritability } h^2(b) = \frac{\text{Genotypic variance}}{\text{Phenotypic variance}}$$

$$= \frac{0.46}{0.48} = 0.96$$

The heritability of nitrate reductase in cocoa was estimated to be 0.96.

4.5.2. Heterosis and heterobeltiosis

Relative heterosis (deviation from mid-parental value) and heterobeltiosis (deviation from better parent) were

calculated. Out of the 20 hybrids evaluated over four months all exhibited negative heterobeltiosis. Similarly negative relative heterosis was exhibited by all hybrids, except H-20 which exhibited a relative heterosis of + 0.57% (Table 12).

4.6. Effect of NO_3^--N on leaf nitrate reductase activity

The study was undertaken to find out the effect of different levels of applied NO_3^--N on leaf NRA and nitrate content in cocoa.

4.6.1. Nitrate reductase activity

There was significant difference in NRA at different levels of nitrate during the first three fortnights of the sampling period (Table 13).

In the first fortnight after nitrate application, plants which received 800 kg N ha^{-1} recorded the highest NRA. Though it was on par with the NRA observed at 200 kg N ha^{-1}, it was significantly superior to the activities noticed at 400 and 0 kg N ha^{-1}. Both the levels, 200 and 400 kg N ha^{-1} also showed significantly superior NRA as compared to the control (0 kg N ha^{-1}).

In the second fortnight, the highest level of 800 kg N ha^{-1} recorded significantly higher NRA than the lower levels of 400, 200 and 0 kg N ha^{-1}. In the third fortnight the highest NR activity was recorded at 0 kg N ha^{-1} and was

Table 12. Relative heterosis and heterobeltiosis with respect to NRA (mmoles NO_2^- g^{-1} fwt h^{-1}) in cocoa hybrids during the year 1990-91

Hybrid	Mean NRA of hybrid	Mean NRA of parents			Relative heterosis (%)	Hetero-beltiosis (%)
		P_1	P_2	Mid-parent		
H - 1	1.54	2.18	2.05	2.12	-27.4	-29.4
H - 2	1.56	2.18	2.28	2.23	-30.1	-31.6
H - 3	1.54	2.16	2.28	2.22	-30.6	-32.5
H - 4	1.95	2.16	2.26	2.21	-11.8	-13.7
H - 5	1.39	2.16	1.94	2.05	-32.2	-35.7
H - 6	1.75	2.16	1.55	1.85	-5.4	-19.0
H - 7	1.56	1.13	2.26	1.70	-8.2	-31.0
H - 8	1.03	1.13	2.37	1.75	-41.1	-56.5
H - 9	1.18	1.13	1.55	1.34	-11.9	-23.9
H - 10	2.05	2.02	2.28	2.15	-4.7	-10.1
H - 11	1.20	2.02	2.26	2.14	-43.9	-46.9
H - 12	1.73	2.44	2.37	2.41	-28.2	-29.1
H - 13	1.98	2.44	2.26	2.35	-15.8	-18.9
H - 14	1.13	1.48	1.13	1.31	-13.7	-23.7
H - 15	1.35	2.28	1.94	2.11	-36.0	-40.8
H - 16	1.74	2.44	1.94	2.19	-20.6	-28.7
H - 17	1.99	2.44	1.61	2.02	-1.5	-18.4
H - 18	1.02	1.92	1.58	1.75	-41.7	-46.9
H - 19	1.36	1.92	1.36	1.64	-17.1	-29.2
H - 20	1.75	1.92	1.55	1.74	+0.57	-8.9

significantly superior to the NR activity exhibited at the higher levels of N. The activities at 200 and 800 kg N ha^{-1} were on par. In the fourth fortnight, NRA at the different levels of N did not show significant variation. With respect to mean NRA over the different sampling intervals, the highest activity was recorded at 800 kg N ha^{-1} followed by that at 400, 200 and 0 kg N ha^{-1}, which were on par.

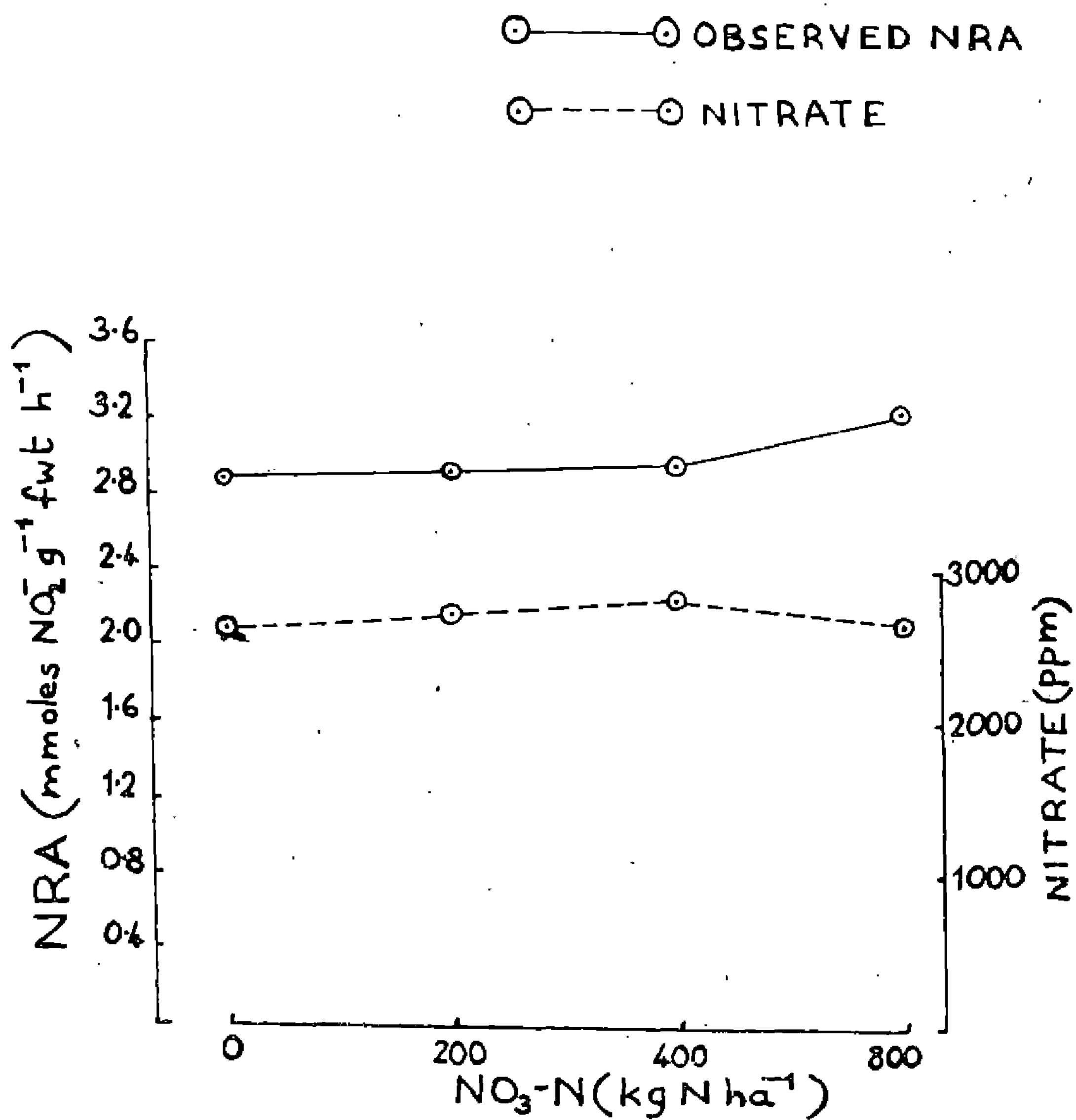
4.6.2. Nitrate

Significant difference in nitrate content at different levels of nitrate application was observed only in the first three fortnights after nitrate application (Table 13). In the first fortnight, nitrate content at all four nitrate levels differed significantly. The maximum nitrate content was recorded at 400 kg N ha^{-1} followed by those at 200, 800 and 0 kg N ha^{-1}. In the second fortnight, the maximum nitrate content was recorded at 800 kg N ha^{-1} which was however on par with 200 and 400 kg N ha^{-1}, and these were significantly higher than the nitrate content recorded at 0 kg N ha^{-1}.

In the third fortnight, maximum nitrate content was noticed in the case of 0 kg N ha^{-1} followed by 200, 400 and 800 kg N ha^{-1} which were on par. In the fourth fortnight, no significant variation in nitrate content was observed for the different levels of N. When mean nitrate content

Table 13. NRA (mmoles NO_2^- g^{-1} fwt h^{-1}) and foliar NO_3^- (ppm) content of cocoa budlings at different NO_3-N levels at fortnightly intervals after fertilizer application

Fortnights after application	I		II		III		IV		Mean	
NO_3 level (kg N ha^{-1})	NRA	NO_3	NRA	NO_3	NRA	NO_3	NRA	NO_3	NRA	NO_3
0	2.99	2378	2.23	2190	3.50	3442	2.85	2503	2.89	2628
200	3.68	3379	2.26	2816	2.78	2566	2.95	2378	2.92	2785
400	3.50	3880	2.90	2816	2.37	2378	2.97	2503	2.94	2894
800	3.80	2941	3.31	2941	2.95	2316	2.85	2566	3.23	2691
F test	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	NS	NS	Sig.	Sig.
SEm±	0.07	108	0.10	125	0.08	89	0.12	177	0.04	57
CD (0.05)	0.16	250	0.23	289	0.19	204	0.27	408	0.09	130



FIG·5· EFFECT OF APPLIED NO_3^--N ON MEAN FOLIAR NITRATE REDUCTASE ACTIVITY AND NITRATE CONTENT IN COCOA

over the different sampling intervals was considered, the highest nitrate content was seen in the case of 400 kg N ha^{-1} followed by 200, 800 and 0 kg N ha^{-1}.

4.7. Influence of nitrate reductase activity on yield

The mean NRA of the thirty genotypes over four months and its correlation with yield are presented in Table 14. The mean activity of NR ranged from 1.22 to 2.68 mmoles NO_2^- g^{-1} fwt h^{-1} while the average yield ranged from 2 to 75.5 pods/tree/year. A non-significant negative correlation between NRA and yield was observed in the present study.

4.8. Standardisation of *in vitro* assay of nitrate reductase in cocoa

Nitrate reductase was extracted from the plant sample in each of the following media:

a) 5 mM potassium phosphate buffered at pH 7.5 and 1 mM EDTA
b) a + 1 mM dithiothreitol
c) b + 2% casein
d) b + 1% Bovine Serum Albumin
e) b + 2% BSA
f) e + 10 µM FAD

The extract was then centrifuged at 2000 g for 10 minutes at 0-4°C and then the supernatant was used for the

Table 14. Mean NRA (mmoles NO_2^- g^{-1} fwt h^{-1}) of genotypes of cocoa and their correlation with mean yield (pods/tree/year) for the year 1990-91

Accession No.	Mean NRA	Mean yield
G VI - 1	1.64	10.3
G VI - 2	1.96	33.4
G VI - 5	1.76	29.0
G VI - 7	1.53	52.0
G VI - 9	2.27	42.0
G VI - 11	1.90	16.0
G VI - 13	1.70	25.0
G VI - 14	2.39	41.3
G VI - 19	1.70	42.4
G VI - 20	2.08	7.4
G VI - 22	1.93	41.2
G VI - 24	1.84	71.5
G VI - 26	2.28	16.5
G VI - 30	2.00	23.5
G VI - 35	1.93	26.0
G VI - 40	2.03	33.6
G VI - 42	2.31	8.3
G VI - 44	2.20	75.5
G VI - 46	2.25	21.3
G VI - 48	2.60	13.0
G VI - 49	1.76	37.5
G VI - 51	2.15	23.2
G VI - 52	2.68	23.8
G VI - 53	1.82	19.0
G VI - 56	2.35	33.4
G VI - 68	2.33	17.5
G VI - 74	2.18	2.0
G VI - 85	2.19	46.0
G VI - 94	1.22	33.5
G VI - 100	1.98	9.5
Correlation coefficient	–0.19	

assay following the method of Lewis *et al.* (1982). Instead of the characteristic pink colour browning of the assay medium resulted, in all the above mentioned extraction media. As the browning is often attributed to phenolic compounds polyvinylpyrrolidone (PVP) was used in the extraction medium. Though different amounts of PVP viz., 0.1 g and 1 g were tried, browning still persisted.

The increase of the enzyme extract in the assay medium or that of the assay medium itself also did not help to overcome the browning of the medium.

Discussion

DISCUSSION

5.1. Genotypic differences in nitrate reductase activity

The thirty accessions of cocoa showed a lot of genotypic variation with activities ranging from 1.22 mmoles NO_2^- g^{-1} fwt h^{-1} in G VI-94 to 2.68 mmoles NO_2^- g^{-1} fwt h^{-1} in G VI-52. Based on the nitrate reductase activity the different genotypes may be grouped into three categories of low, medium and high activity.

1. Low NRA ($<$ 1.71 mmoles NO_2^- g^{-1} fwt h^{-1}) :

   G VI-1; G VI-7; G VI-13; G V-19 and G VI-94.

2. Medium NRA (1.71-2.20 mmoles NO_2^- g^{-1} fwt h^{-1}) :

   G VI-2; GVI-5; G VI-11; G VI-20; G VI-22; G VI-24; G VI-30; G VI-35; G VI-40; G VI-44; G VI-49; G VI-51; G VI-53; G VI-74; G VI-85 and G VI-100.

3. High NRA ($>$ 2.20 mmoles NO_2^- g^{-1} fwt h^{-1}) :

   G VI-9; G VI-14; G VI-26; G VI-42; G VI-46; G VI-48; G VI-52; G VI-56 and G VI-68.

Such genotypic differences in NR levels have been reported in maize (Deckard *et al*., 1973), wheat (Goodman *et al*., 1974), sudan grass (Eck and Hageman, 1974) and barley (Chaterjee *et al*., 1977). As all the genotypes

studied are of very diverse origin and the level of nitrate reductase is under genetic control (Zieserl and Hageman, 1962), it is natural that the levels of NRA in the various genotypes were strikingly different.

Genotypic differences in the capacity to accumulate reduced nitrogen have also been found to vary due to the difference in NRA (Deckard et al., 1973). It could be worthwhile to make changes in the nitrogen management practices to suit the nitrogen assimilation capacity of the plant. In this regard, it is essential to consider the seasonal variation of NRA pattern of different genotypes while formulating nitrogen management practices.

5.2.1. Effect of shade on nitrate reductase activity under irrigated condition

The NRA was fairly high both in the open as well as at 50% shade intensities but was low both at 25% and 75% shade intensities. The pattern of leaf nitrate content also followed a similar trend, thus proving its role as inducer of nitrate reductase.

The high NRA observed in the open could be due to complete exposure of leaves to light. Positive correlation between NRA and light was observed by Rao and Rains (1976) in barley. Moreover, as the plants are irrigated they do

not experience any moisture stress which would otherwise limit their innate physiological processes. The decrease in NRA at 25% shade intensity was probably due to the blocking of this full exposure of leaves to light. At 50% shade there is an increase in the activity and this may be due to the fact that for cocoa the optimum shade level is 50% (Wessel, 1985). The leaf nitrate content also is higher at this shade level leading to an increased NRA. At 75% shade intensity (heavy shade), photosynthesis or carbon dioxide assimilation is affected adversely which would in turn adversely affect the nitrogen assimilation also, both being interrelated (Cresswell et al., 1979).

The mean yield recorded (at the Cadbury-KAU Co-operative Cocoa Research Project) at the four shade levels of 0%, 25%, 50% and 75% were 72.8, 22.4, 29.0 and 9.4 pods/tree/year respectively (Fig. 2). It could be observed that the influence of shade on both NRA and yield followed more or less a similar pattern.

5.2.2. Effect of shade on nitrate reductase activity under rainfed condition

In general the pattern of NRA under rainfed condition was a reversal of that under irrigated condition. Also opposite trends in NRA and leaf nitrate were observed which

seem to point to the possibility that here, nitrate acts as a substrate rather than inducer, for the enzyme.

Fairly low NRA was observed both in the open and at 50% shade level. The NRA at 25% and 75% shade intensities were fairly high. The low NRA in the open could be attributed to full exposure of leaves to sunlight under moisture stressed condition as plants were rainfed which led to hampering of normal physiological processes within the plant. Hence, NRA registered a slight increase under the low shade of 25%. Though there was a decrease in NRA at 50% shade, the activity showed a considerable increase at 75% shade. This may be due to less stress experienced by the rainfed plants under the heavy shade (75%).

The mean yield recorded (at the Cadbury-KAU Co-operative Cocoa Research Project) at the four different shade levels were 65.6, 58.0, 3.8 and 3.4 pods/tree/year, respectively (Fig.2). It could be seen that though the yield recorded was highly in the open, the NRA exhibited by the plants was fairly low. However, the yield as well as NRA was fairly low at 50% shade level. At 75% shade level, though there was an increase in NRA, a similar increase in yield was not observed. Thus the effect of shade is different on NRA and yield under the rainfed situation.

5.3. Seasonal variations in nitrate reductase activity

The highest NRA was observed in October with two lesser peaks in June and August respectively. There was a sharp decline in activity during November and December, followed by a slight increase in January. Thereafter, the activity declined upto May. The extremely low activity observed in May could be due to the development of a new flush which led to a demand on nutrients, including nitrogen which was probably met by translocation from the older leaves (hardened leaves). As nitrate reductase is a substrate induced enzyme, limitation of substrate viz., nitrate, in hardened leaves could be responsible for the decreased activity.

The peak activity observed in October was in conformity with the findings of Pietila _et al._ (1989) who reported that the highest activity in Scots pine needles was recorded in late autumn. Desperrier _et al._ (1986) had also reported similar maximal nitrate reductase activity in fenugreek during winter when the temperature was very low. The relatively high activity observed during June and October could also be due to the fact that the trees received the N fertilizer application during May-June and September-October.

5.4. Nitrate reductase activity in different parts of cocoa plant

Out of the three plant parts studied, viz., leaf, leaf petiole and pod of cocoa, only the leaf exhibited nitrate reductase activity. This is in conformity with the finding of Vaughn and Campbell (1988) which established that NRA was found exclusively in the cytoplasm of mesophyll cells of the leaf when maize tissue was tested for NRA. However, significant NR activity was observed in many parts of black pepper vine, viz., runner shoot, plageotrope, orthotrope, flag leaf, geotrope and berry, by Thomas (1990).

From the results of the present study it would be logical to presume that only the laminar tissue largely contributes to nitrogen assimilation in cocoa.

5.5. Heritability and heterosis

The broad sense heritability of NR was found to be high in cocoa (0.96) in the present study. This is in conformity with the findings of Eck *et al.* (1975) and Warner *et al.* (1969) who obtained high values for broad sense heritability of NRA in sorghum and corn, respectively. The implication of this fact is that sufficient genetic advance could be expected in cocoa from mere selection among pure lines.

Negative relative heterosis and heterobeltiosis for NRA were observed in the hybrids studied. Therefore, it would be logical to presume that no advantage could be gained by hybridisation in cocoa with regard to NR activity. However, the effect of hybridisation on other attributes such as yield, etc. would have to be investigated before concluding that it is not advantageous.

5.6. Effect of NO_3-N on nitrate reductase activity

In the present study NRA increased with increase in NO_3-N levels.

As nitrate is both inducer and substrate for nitrate reductase it seems logical that nitrate would regulate the enzyme. In the present study though such a concomitant increase in leaf nitrate and NRA was observed with increase in nitrate application the relationship did not hold good at high doses (800 kg N ha^{-1}) during the later stages of observation. Though there are many reports that leaf nitrate content directly influences the NRA (Aslam et al., 1973; Travis et al., 1970; Sekhon et al., 1988), there is another school of thought that it is the flux of nitrate into the cells rather than the total nitrate content of the leaves that regulate the activity of NR (Shaner and Boyer, 1976; Gaudinova, 1983). These studies led to the concept of

two pools, metabolic and storage of nitrate. Whether the metabolic pool serves as both inducer and substrate is not clear. Hence in the present study, changes in NRA cannot be explained solely on the basis of leaf nitrate concentration as the flux of nitrate into the metabolic pool is not known. However the increase in NRA, even at low leaf nitrate content indirectly supports the concept that flux of nitrate into the cells may have some influence on NRA.

5.7. Influence of nitrate reductase activity on yield

Negative correlation was observed between NRA and yield of the thirty genotypes of cocoa studied. Harper *et al.* (1972), based on his experiments in soybean reported that NR activity did not correlate with either seed yield or seed protein content. Fakorede and Mock (1978) also found that selection for improved grain yield in the half-sib selection program in maize was associated with decreased NRA.

However, significant positive correlations between NRA and yield were observed in wheat (Deckard *et al.*, 1977), barley (Johnson *et al.*, 1976), sunflower (Deshmukh and Srivastava, 1983) and black pepper (Thomas, 1990).

On the one hand, it has been established that there are particular kinds of interaction between controlling factors (light, NO_3, etc.) and NR-gene expression in plants

(Schuster *et al.*, 1989). On the other, yield is not the result of any single factor, but is controlled by many genetic as well as environmental factors. Therefore, a comprehensive study of plant characteristics such as canopy spread, stem girth, etc. as well as environmental factors has to be undertaken before concluding the NRA has no correlation with yield in cocoa in the present study. Also cocoa in general performs better under shaded conditions which is not congenial for optimal nitrate reductase activity.

5.8. Standardisation of *in vitro* assay of nitrate reductase in cocoa

In vitro assay involves disruption of the tissues with consequent mixing of substances which in the living organisms were rigidly compartmentalised. This may result in inactivation of enzyme. In the present study though different extraction techniques were employed not much success could be obtained in the *in vitro* assay of nitrate reductase.

In the extraction medium casein and bovine serum albumin (BSA) were used to prevent the activity of proteases and to prolong the enzyme activity. Sherrard and Dalling (1978) had observed that use of 2% casein in the extraction medium resulted in a significant increase in nitrate reductase of wheat. This was attributed to the stability of enzyme following addition of casein. Similar results have been

reported following addition of BSA also (Lewis _et al_., 1982). However, in the present study, though both casein and BSA were tried at different concentrations, not much success could be attained. In accordance with reports by Lewis _et al_. (1982) that sulphydryl protective reagents had been found to prolong stability of NRA, a sulphydryl compound (dithiothreitol) was included in the present study but was not found very effective.

Interference by phenolic compounds in the isolation of plant enzymes had been reported by several workers (Eppley, 1978; Kanser and Lewis, 1984). In the present study it was likely that the inhibitors were the phenolic compounds which are responsible for the browning reaction. Phenolic compounds combine with protein reversibly by hydrogen bonding and irreversibly by oxidation followed by covalent condensation. Techniques for isolating enzymes from plants which contain phenolic compounds should specifically separate the phenols from the proteins and at the same time prevent oxidation of the phenols. Those phenols which from hydrogen bonded complexes with protein can be effectively removed by substances which contain groups similar to peptide linkage. Polyvinylpyrrolidone (PVP) has been reported to be a satisfactory agent for this purpose in the isolation of soluble enzymes (Kanser and Lewis, 1984).

In the present investigation though various concentration of PVP was used browning still persisted. It is probable that some phenolic compounds which do not form strong hydrogen bonded compound with PVP are readily oxidised to quinones leading to browning reaction.

In the light of the present study it may be worthwhile to isolate and identify the individual compounds present in the enzyme extract. The study on the character of these compounds may help to know the nature of inhibition, which in turn could consequently help to overcome the interference in colour development.

Summary

SUMMARY

An investigation was conducted at the College of Horticulture, Vellanikkara during the period 1990-1991 to study the nitrate reductase activity in cocoa (*Theobroma cacao* L.). Forty plants belonging to the shade trial, thirty accessions of the G VI series and twenty hybrids and their parents were used in the field experiments. The pot culture experiment made use of twelve cocoa budlings of type V. 15/5. All the cocoa plants used belonged to the Cadbury-KAU Co-operative Cocoa Research Project. The following aspects were studied during the course of this investigation.

1. Genotypic differences in nitrate reductase activity
2. Effect of shade on nitrate reductase activity
3. Seasonal variations in nitrate reductase activity
4. Nitrate reductase activity in different parts of cocoa plant
5. Heritability of nitrate reductase activity in cocoa
6. Effect of NO_3^--N on leaf nitrate reductase activity
7. Influence of nitrate reductase activity on yield
8. Standardisation of *in vitro* assay of nitrate reductase in cocoa

The results of the investigation are summarized below:

The thirty accessions of cocoa showed a lot of genotypic variation with activities ranging from 1.22 mmoles NO_2^- g^{-1} fwt h^{-1} in G VI-94 to 2.68 mmoles NO_2^- g^{-1} fwt h^{-1} in G VI-52.

Based on the NRA the different genotypes may be grouped into three categories of low, medium and high activities.

Significant differences in NRA and foliar nitrate content were observed at different shade intensities under irrigated condition during the month of June. The mean NRA over the entire period of observation was highest at 0% shade level followed by that at 50% and 75% shade levels. The least activity was observed at 25% shade level. Maximum nitrate was recorded at 0% shade intensity followed by that at 50% shade level which was on par with those at 75% and 25% shade levels. Under rainfed condition, significant differences in NRA and nitrate were observed at different shade intensities during June and September. Mean NRA was highest at 75% shade level followed by those at 25%, 0% and 50% shade levels. Mean nitrate content was maximum at 75% shade level, followed by those at 0%, 50% and 25% shade levels.

The nitrate reductase activities of the ten accessions of cocoa showed significant differences during twelve months. The maximum activity of 3.05 mmoles NO_2^- g^{-1} fwt ha^{-1} was recorded during October and the minimum activity of 0.84 mmoles NO_2^- g^{-1} fwt h^{-1} during May. The season x genotype interaction was found to be significant. On an overall basis, the highest NRA of 3.86 mmoles NO_2^- fwt h^{-1} was

exhibited by G VI-5 in the month of October whereas the least NRA of 0.37 mmoles NO_2^- g^{-1} fwt h^{-1} was seen in G VI-2 during the month of November.

Among the plant parts studied, viz., leaf, petiole and pod, nitrate reductase activity was exhibited only by the leaf.

Heritability in the broad sense was found to be high (0.96) in cocoa. In general, negative relative heterosis as well as heterobeltiosis were observed in all the hybrids studied, except H-20 which exhibited a relative heterosis of +0.57%.

There was significant increase in NRA with increase in NO_3^--N application in cocoa. With respect to mean NRA over the different sampling intervals, the highest activity was recorded at 800 kg N ha^{-1} followed by that at 400, 200 and 0 kg N ha^{-1} which were on par.

No significant correlation between NRA and yield could be obtained in this study.

In the _in vitro_ assay, though different extraction media were tried, not much success could be obtained due to browning of the media. Use of bovine serum albumin, casein, dithiothreitol and polyvinylpyrrolidone at different concentrations and combinations could not prevent the browning.

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*Originals not seen

Appendices

Appendix-1. Physico-chemical properties of the soil of the experimental field

1. Mechanical composition

Soil form	Fraction (per cent composition)			Procedure adopted
	Sand	Silt	Clay	
Cocoa field	44	10	48	Hydrometer method (Bouyoucos, 1962)
Pot culture	44	14	44	

2. Chemical properties

Soil characteristic	Soil of cocoa field	Soil used for pot culture	Method used
Organic carbon (per cent)	1.17	0.82	Walkley and Black method (Piper, 1950)
Total N (per cent)	0.115	0.154	Microkjeldahl method (Jackson, 1958)
Available P (ppm)	41	21	Chlorostamnous reduced molybdophosphoric blue colour method (Jackson, 1958)
Available K (ppm)	180	75	Flamephotometric method (Jackson, 1958)
pH	5.8	4.5	Elico pH meter (Jackson, 1958)
Eh (mV)	+320	+410	Model RM-IK oxidation reduction potential meter of TOA Electronics Ltd., Japan
CEC (me 100 g^{-1} soil)	4.0	3.9	Ammonium acetate method (Jackson, 1958)

Appendix-2. Weather data (weekly average) for the cropping period (June 1990 - June 1991)

Week No.	Month and date		Rainfall (mm)	Temperature (°C)		Relative humidity(%)		Sunshine hours	
				Maximum	Minimum	Forenoon	Afternoon	Total	Average
1	2		3	4	5	6	7	8	9
23	June	4-10	72.4	29.9	23.1	93	75	17.8	2.5
24		11-17	215.3	29.1	23.1	95	80	20.1	2.9
25		18-24	87.5	29.7	23.3	94	80	24.3	3.5
26	July	25-1	98.7	30.6	23.6	93	73	42.0	6.0
27		2-8	265.6	27.7	22.1	94	85	9.3	1.3
28		9-15	190.1	28.6	22.4	94	85	11.3	1.6
29		16-22	198.1	27.6	22.4	95	87	10.5	1.5
30		23-29	78.0	29.3	22.5	93	71	29.7	4.2
31	August	30-5	114.0	28.9	23.0	95	78	18.9	2.7
32		6-12	91.7	28.0	22.5	95	80	8.7	1.2
33		13-19	121.6	28.5	23.3	94	77	18.8	2.7
34		20-26	28.3	29.7	23.1	94	72	30.3	4.3
35	September	27-2	14.7	30.6	23.6	92	65	51.8	7.4
36		3-9	60.9	30.0	23.1	94	74	27.5	3.9
37		10-16	-	30.9	24.0	91	64	53.7	7.7
38		17-23	6.9	31.0	23.4	90	65	46.5	6.6
39		24-30	16.6	31.1	23.1	89	69	45.3	6.5

Contd.

Appendix-2. Continued

1	2		3	4	5	6	7	8	9
40	October	1-7	26.9	30.6	22.5	93	70	44.4	6.3
41		8-14	14.4	32.4	23.7	92	63	61.8	8.8
42		15-21	22.3	33.5	23.2	88	62	51.3	7.3
43		22-28	133.9	31.8	23.3	92	78	38.3	5.5
44	November	29-4	184.2	29.1	22.4	95	76	22.0	3.1
45		5-11	-	31.2	21.1	89	62	54.4	7.8
46		12-18	0.6	31.1	22.8	92	65	37.2	5.3
47		19-25	-	33.1	23.2	84	54	53.5	7.6
48	December	26-2	0.8	31.8	23.4	75	52	40.3	5.8
49		3-9	1.8	31.9	24.8	71	48	51.7	7.4
50		10-16	-	31.9	22.3	70	43	57.9	8.3
51		17-23	-	32.7	22.0	76	46	61.4	7.7
52		24-31	-	32.5	23.7	69	44	65.2	8.2
1	January	1-7	3.9	33.1	22.1	83	50	54.6	7.8
2		8-14	-	33.4	21.8	75	44	65.1	9.3
3		15-21	-	33.4	23.6	72	44	58.5	8.4
4		22-28	-	34.2	22.1	66	28	68.4	9.8
5	February	29-4	-	34.5	21.4	76	39	64.2	9.2
6		5-11	-	35.2	21.4	66	23	71.1	10.2
7		12-18	-	36.4	21.0	77	22	73.5	10.5
8		19-25	-	36.5	22.0	73	27	74.1	10.6

Appendix-2. Continued

1	2		3	4	5	6	7	8	9
9	March	26-4	-	36.6	24.4	83	42	66.4	9.5
10		5-11	1.8	35.3	24.7	91	50	54.1	7.7
11		12-18	-	35.9	25.0	89	53	59.8	8.5
12		19-25	-	36.1	24.6	80	51	65.0	9.3
13	April	26-1	-	38.3	25.4	78	38	61.6	8.8
14		2-8	23.0	36.1	24.2	80	51	65.5	9.4
15		9-15	24.4	36.1	24.7	85	52	55.6	7.9
16		16-22	1.4	35.5	25.8	81	56	65.2	9.3
17		23-29	35.0	34.7	23.4	86	59	61.7	8.8
18	May	30-6	-	34.6	25.7	87	54	47.4	6.8
19		7-13	1.4	35.5	26.1	85	51	60.0	8.6
20		14-20	1.2	36.4	26.3	82	54	49.8	7.1
21		21-27	39.3	35.1	25.0	88	56	60.0	8.6
22	June	28-3	132.6	33.3	23.9	89	65	43.5	6.2

Appendix-3. Analysis of variance of NRA of thirty different genotypes of cocoa over four seasons

Source	df	SS	MS	F
Main plot (Genotype)	29	40.51	1.40	70**
Error (a)	60	1.22	0.02	
Sub plot (Season)	3	90.28	30.09	752.25**
Interaction	87	106.19	1.22	30.5**
Error (b)	180	7.82	0.04	

Appendix-4. Analysis of variance of seasonal NRA of ten accessions of cocoa over twelve months

Source	df	SS	MS	F
Main plot (Genotype)	9	17.92	1.99	331.7
Error (a)	20	0.11	0.006	
Sub plot (Month)	11	125.71	11.43	1270**
Interaction	99	98.41	0.99	110**
Error (b)	220	1.91	0.009	

** Significant at 1% level

Appendix-5. Analysis of variance of nitrate reductase activity of irrigated cocoa under different shade levels at intervals of three months during the period June 1990-May 1991

Source	df	Mean square				
		I	II	III	IV	Mean
Treatment	3	2.36*	0.47	0.06	0.26	0.20
Error	16	0.49	0.45	0.33	0.28	0.10
CD (0.05)		0.94	NS	NS	NS	NS

Appendix-6. Analysis of variance of nitrate reductase activity of rainfed cocoa under different shade levels at intervals of three months during the period June 1990-May 1991

Source	df	Mean square				
		I	II	III	IV	Mean
Treatment	3	3.04**	2.04**	0.26	0.08	0.08
Error	16	0.36	0.35	0.23	0.11	0.04
CD (0.05)		0.80	0.79	NS	NS	NS

* Significant at 5% level
** Significant at 1% level

Appendix-7. Analysis of variance of nitrate content of irrigated cocoa under different shade levels at intervals of three months during the period June 1990-May 1991

Source	df	Mean square				
		I	II	III	IV	Mean
Treatment	3	3183172*	340607	585245	199644	248593
Error	16	814968	1499552	1079315	534640	248050
CD (0.05)		1210	NS	NS	NS	NS

Appendix-8. Analysis of variance of nitrate content of rainfed cocoa under different shade levels at intervals of three months during the period June 1990-May 1991

Source	df	Mean square				
		I	II	III	IV	Mean
Treatment	3	3181042**	4881895**	246857	87007	89994
Error	16	392094	658993	480092	351150	75805
CD (0.05)		840	1089	NS	NS	NS

* Significant at 5% level
** Significant at 1% level

Appendix-9. Analysis of variance of nitrate reductase activity of cocoa budlings at different NO_3^--N levels at fortnightly intervals after fertilizer application

Source	df	Mean square				
		I	II	III	IV	Mean
Treatment	3	0.38**	0.82**	0.65**	0.01	0.07**
Error	8	0.0075	0.02	0.01	0.02	0.003
CD (0.05)		0.16	0.23	0.19	NS	0.09

Appendix-10. Analysis of variance of nitrate content of cocoa buddlings at different NO_3^--N levels at fortnightly intervals after fertilizer application

Source	df	Mean square				
		I	II	III	IV	Mean
Treatment	3	1224885**	344584**	816863**	18571	40301**
Error	8	17625	23563	11750	46922	4795
CD (0.05)		250	289	204	NS	130

* Significant at 5% level
** Significant at 1% level

NITRATE REDUCTASE ACTIVITY IN COCOA (*Theobroma cacao* L.)

By

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ABSTRACT OF A THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

Faculty of Agriculture
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Vellanikkara, Thrissur

1991

ABSTRACT

An investigation on the nitrate reductase activity in cocoa (*Theobroma cacao* L.) was conducted at the College of Horticulture, Vellanikkara during the period 1990-1991. Forty plants belonging to the shade trial, thirty accessions of the H VI series and twenty hybrids and their parents were used for the study. All the plants used in the study belonged to the Cadbury-KAU Co-operative Cocoa Research Project. Genotypic and seasonal variations in NRA and NRA in different parts of cocoa plant were studied. The effect of NO_3^--N and shade on NRA were also investigated. Heritability of NR, heterosis and relationship between NRA and yield were also worked out.

Based on genotypic differences in NRA, the thirty accessions were grouped into three categories of low, medium and high activity. The highest NRA was observed in October with two lesser peaks in June and August respectively. The season x genotype interaction was found to be significant. Among the plant parts studied, viz., leaf, petiole and pod, nitrate reductase activity was exhibited only by the leaf.

There was significant increase in mean NRA with increase in NO_3^--N levels. Under irrigated condition, NRA increased with decrease in shade intensity. NRA at 0% shade

intensity was superior to that at 50% and 75% shade levels, while the least activity was observed at 25% shade level. Under rainfed condition, high NRA was observed at 75% shade level followed by those at 25%, 0% and 50% shade levels.

Heritability in the broad sense was found to be high in cocoa. In general, all the hybrids studied showed negative relative heterosis and heterobeltiosis. No significant correlation between NRA and yield could be obtained in this study.