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Nitrate Reductase Activity in Coconut Leaves

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The effect of various assay parameters on in-vivo nitrate reductase (NR) activity in coconut leaf tissue was studied and *n*-propanol was found to inhibit the activity at all levels. The enzyme activity was higher in air than under nitrogen. Addition of NADH to the assay medium did not increase the activity significantly. Light stimulated the activity and the addition of glucose to dark treated leaves did not restore the activity fully. The 14th leaf on the coconut crown is the most appropriate for sampling. The inducible NR activity showed a high positive correlation with annual yield.

1. Introduction

Nitrate reductase (E.C. 1.6.6.1) is a rate limiting enzyme in nitrogen assimilation of plants and is inducible by substrate[1–4] and light[1,2,5–7]. The activity of this enzyme *in vivo* has been correlated with yield and protein content in several cereals.[8,9] Among perennial plantation crops such work is not available except in tea[10] where NR activity has been reported to be positively correlated with yield. The objective of this paper is to report a study of the assay parameters of the enzyme in coconut leaves and investigate the relationship between activity and coconut yield.

2. Experimental

Leaflets from 30–40 years old West Coast Tall (WCT) palms growing in the Institute farm at Kasaragod under rainfed conditions were the material for study. Sampling was done between 10.00 and 11.00 hours.

2.1. NR activity

NR activity was estimated in vivo using the method of Ferrari and Varner[11] with some modifications. The standard assay medium contained 0.5 g leaf discs in a total volume of 5.0 ml containing 1 mM nitrate in 0.8 mM phosphate buffer (pH 7.6). The reaction medium was devoid of *n*-propanol. The discs were vacuum infiltrated for 3 min before incubation in the dark. The length of assay was 1 h after which time the flasks were kept in a boiling water bath for 2 min. Nitrite was estimated after clarifying the medium with activated charcoal.[12] Activity is expressed as nmol nitrite produced h^{-1} g^{-1} fresh weight of leaf.

Activity was induced by placing the petioles of excised leaves under nitrate solution in daylight. Activity measurements were made after induction in nitrate solution for 5 h (unless otherwise stated), as there was no measurable activity in the freshly excised leaves.

When the effectiveness of NADH as electron donor for NR was tested, the NADH was added to the standard assay medium just prior to infiltration at the rate of 2 mg ml^{-1}. Disappearance of NADH was followed by absorbancy loss of aliquots removed at intervals from the incubation mixture.

2.2. Light and dark treatments

Excised leaflets which had been stored in the dark for 16 h in different induction media were transferred to light and dark treatment in the morning. NR activity was estimated at intervals.

2.3. Standardisation of leaf position

To standardise leaf sampling, leaflets were collected from fronds 1, 6, 11, 14, 15, 16, 21 and 28 in order of maturity, leaf 1 being the youngest fully opened frond. Leaflets were collected from both sides of the rachis from the central portion. Each experiment was repeated 4–5 times and the mean experimental values are presented here.

3. Results and discussion

The assay parameters of the enzyme in the coconut leaf tissue were studied at different pHs (6.0–8.0) in combination with phosphate buffer concentrations, P_i (0–2.0 mM) and nitrate, NO_3^- (0–2.5 mM) in the assay medium. Table 1A shows the NR activity values at different pHs with optimum concentrations of nitrate (1 mM) and phosphate (0.8 mM). Table 1B depicts the activity at different P_i concentrations measured at pH 7.6 and with the optimal concentration of nitrate (1.0 mM). In Table 1C, the activity at different nitrate concentrations in the medium at pH 7.6 and with a P_i concentration of 0.8 mM is shown.

The optimum temperature for the activity was at 32°C (Fig. 1). NR activity increased rapidly at this temperature up to 6 h and thereafter declined. There was fairly good activity at other tempera-

Table 1. Effect of pH, phosphate buffer concentration (P_i) and NO_3^- on NR activity *in vivo*

A		B		C	
pH	Activity[a] (% of max)	P_i conc. (mM)	Activity[b] (% of max)	NO_3^- conc. (mM)	Activity[c] (% of max)
6.0	22.8±0.9	0	16.3±0.8	0	54.3±1.2
6.5	32.4±1.2	0.04	28.1±1.5	0.5	72.2±0.4
7.0	51.8±0.7	0.2	33.8±1.2	1.0	100.0±1.8
7.2	68.2±2.1	0.4	84.6±0.6	1.5	68.4±0.6
7.4	76.9±1.3	0.8	100.0±1.1	2.0	54.3±0.9
7.5	89.3±0.6	2.0	30.1±1.3	2.5	41.9±2.2
7.6	100.0±1.6				
8.0	25.7±2.3				

[a] Measured at 0.8 mM P_i and 1.0 mM nitrate.
[b] Measured at pH 7.6 and 1.0 mM nitrate.
[c] Measured at pH 7.6 and 0.8 mM P_i.
The values against ± indicate the limits of variation from the mean value.

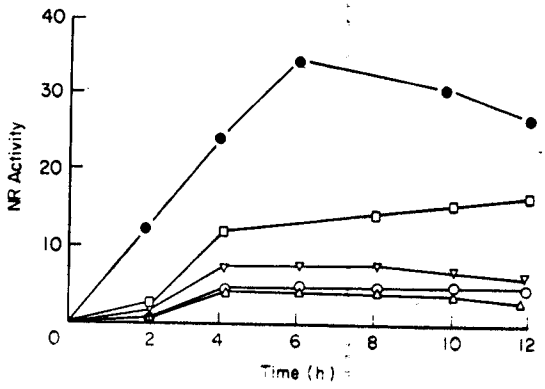

Figure 1. Effect of temperature on NR activity *in vivo*: ●, 32°C; □, 22°C; △, 8°C; ▽, 38°C; ○, 15°C.

Table 2. Effect of propanol concentration and vacuum infiltration on measurable NR activity

1-Propanol (% v/v)	NR activity (% of max)	
	With infiltration	Without infiltration
Zero	100	72.1
0.1	98.5	56.3
0.5	96.1	38.5
1.0	92.0	36.9
1.5	89.5	32.9
2.0	80.0	31.9
3.0	79.1	—
5.0	30.0	—

tures but it was far below the optimum. The high Q_{10} (above 2) between 22 and 32°C probably explains the dependence of the enzyme on light for induction.

Ferrari and Varner[11] have reported the stimulation of activity of the enzyme with alcohols which they attributed to a capacity to cause leakage of nitrate from the metabolically inactive to the active compartment. However, in coconut leaf tissue not only was no stimulation obtained but there was a remarkable inhibition when alcohols were used (Table 2). Inhibition increased with increasing concentration of *n*-propanol. Other alcohols also inhibited the activity to varying levels (Table 3). Maximum inhibition of activity was obtained with amyl alcohol. The inhibition by alcohols may be due to their effect in denaturing the NR protein.

Table 3. Effect of alcohols on NR activity *in vivo*

Solvent[a]	% of max activity
None	100
Acetone	65.1
Methanol	86.3
Ethanol	84.6
Butanol	58.1
2-Propanol	63.4
n-Propanol	92.0
Amyl alcohol	42.8

[a] All solvents used at 1% level.

The NR activity was reduced when the tissues were incubated in an atmosphere of nitrogen (Figure 2). Afridi and Hewitt[13] reported similarly. The exact mechanism by which this happens is not known. Vacuum infiltration of leaf discs in the reaction medium for 3 min before incubation in the dark improved NR activity. The activity without infiltration of discs was only 72.1% of that obtained with infiltration (Table 2).

Addition of NADH to the assay medium with and without nitrate did not enhance the activity appreciably over the respective control (Figure 3). High activity was obtained with only nitrate in the medium and the increase in activity by the inclusion of NADH was only marginal. These observations show that NADH may not be a limiting factor for enzyme activity in the cell.

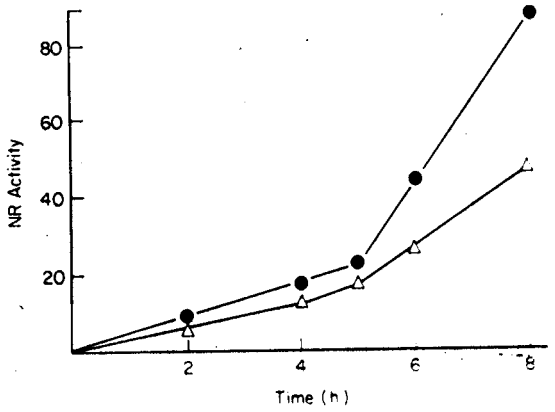

Figure 2. Effect of partial anaerobic conditions on NR activity. The air in the tubes was replaced by nitrogen each time after evacuation during infiltration and incubated in darkness, in an atmosphere of nitrogen. ●, Air; △, nitrogen.

The dependence of the activity on light was known[1,2,5-7] indicating thereby the intimate relationship of the enzyme with photosynthesis. This relationship was investigated in coconut leaf tissue also in the present studies. In an experiment in which the leaf samples were pre-incubated in darkness for 16 h in various induction media and then transferred to light and dark conditions, maximum activity was observed in the case of nitrate treated leaves in light (Figure 4). Those in water showed very little activity. The nitrate treated leaves in the dark had less than half of the maximum activity.

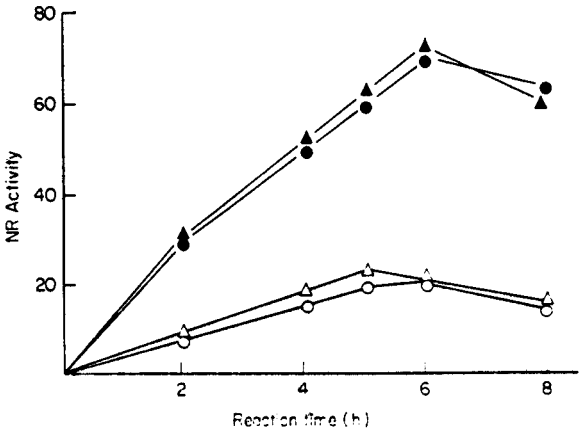

Figure 3. Effect of added NADH on NR activity *in vivo*: ▲, standard assay medium (control); ●, control+NADH; △, control $-NO_3^-$+NADH; ○, control$-NO_3^-$-NADH.

Aslam and Oaks[14] reported that adding glucose to the incubation medium greatly retarded the loss of activity in darkness in barley leaves. Although, in the coconut leaf tissue there was enhancement of activity under darkness with glucose, the level of activity was far below the maximum obtained under light. The highest activity as observed in light under inductive conditions may not be entirely due to availability of carbon source, as supplementary glucose during dark incubation did not restore the activity fully. In darkness with glucose, respiration probably maintained the synthetic phase of the turnover system of NR at a higher level than in the control. Light is also known to promote synthesis of NR protein itself.[15]

The NR activity increased with maturity up to leaf 16 thereafter declining. The highest activity, however, was between leaves 14 and 16. However, since the C.V. % was lowest for leaf 14, this leaf was chosen as the appropriate frond representative of the crown for the study of enzyme activity. The activity was subsequently measured in 50 adult WCT palms each of low, medium and high yield groups. The inducible activity is found positively and significantly correlated with annual

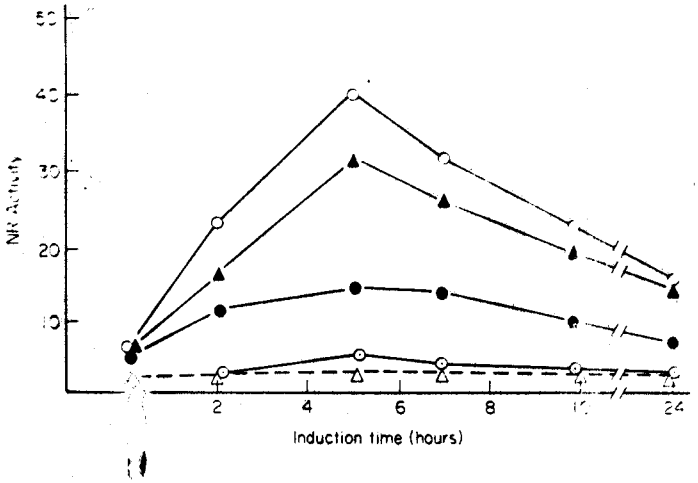

Figure 4. Effect of light and dark conditions on NR activity *in vivo*: . under nitrate in light; ●, under nitrate in dark; ▲, with nitrate and glucose in dark; _. under water in light; ○, under water in dark.

yield of nuts ($r = 0.685$) computed as the average of the previous ten years' yield in the experimental palms (Figure 5).

As may be noted from the Figure 5 the high yielders (> 80 nuts) showed a three-fold higher activity compared to that in low yielders (< 40 nuts).

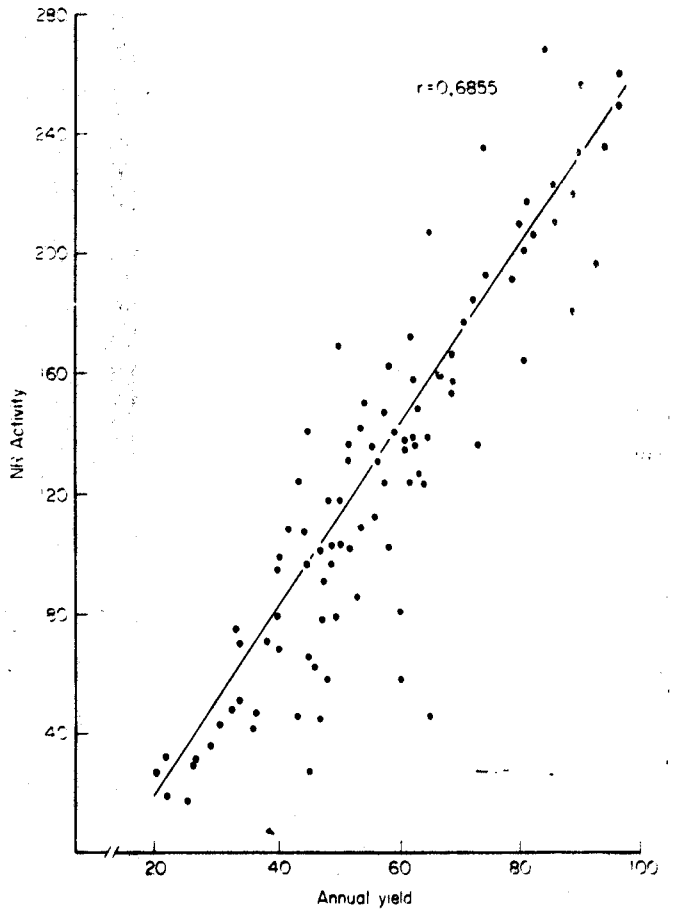

Figure 5. Relationship of inducible NR activity with annual yield. (r = 0.6855).

The high correlation of NR activity with the annual yield of nuts in the coconut palm is important because of the close relationship between level of activity and nitrogen utilisation. Since the central theme of much crop productivity research is to increase the efficiency of carbon and nitrogen assimilation, the present results could be of use in both selection and breeding coconut palms for higher productivity.

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