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Studies on nitrate reductase in *Mangifera indica* L.

T. V. ANANTHANARAYANAN AND ELIAS K. CHACKO

Indian Institute of Horticultural Research (ICAR), Experimental Station, Hessarghatta Lake P.O., Hessarghatta, Bangalore 560 089, India.

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Abstract

An investigation has been made into the properties of nitrate reductase in a typical tree species like *Mangifera indica*. The effect of growth regulators on the induction of the enzyme has been studied. NR activity exhibited a decline with leaf ontogenesis. Mango leaf has a much higher nitrate reducing potential than other tropical fruit plants like banana and guava. Nitrite reductase activity has also been demonstrated in mango leaf.

Key words : Nitrate reductase, growth regulators, leaf ontogenesis, nitrite reductase.

1. Introduction

Nitrate reductase (EC 1.6.6.1) has been studied from a number of sources. Being a key enzyme in the assimilation of nitrogen, it has attracted widespread attention both from the fundamental point of view as well as its bearing on applied aspects, as applicable to plant productivity. However, most of the investigations have been confined to annual crops, cereals, or algae[1–5]. The present study aims to understand the properties of this enzyme in a perennial tree crop like mango. The investigation under report is an offshoot of studies aimed at understanding the role of certain key regulatory enzymes involved in carbon and nitrogen assimilation.

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2. Materials and methods

The plant material used in these investigations were obtained from the experimental farm of the Indian Institute of Horticultural Research, Bangalore. For routine investigations leaves from Dasehari variety were used.

2.1 '*In vivo*' assay

Around 50 mg of leaf discs were suspended in a screw cap vial containing 5 ml of a medium consisting of 0·1 M phosphate buffer, pH 7·5, 0·1 M KNO_3 and 1% propanol. The components of the reaction mixture were purged with nitrogen to ensure anaerobic condition. The discs were vacuum infiltrated twice for a duration of 2 min each with a short interval, ensuring that the discs were completely submerged in the medium. These operations were done in a vacuum desiccator. The desiccator was flushed with nitrogen and the vacuum infiltration repeated. The vials were sealed and incubated in the dark at 30° C in a metabolic shaking incubator water-bath. Incubation in the dark under anaerobic conditions ensured an accumulation of nitrite ions in the medium. Nitrite released into the medium was determined at zero time, and at various time intervals during the preliminary investigations. Thereafter it was found that an incubation period of 20 h was necessary in order to ensure a measurable spectrophotometric reading at 540 nm. The reaction was terminated by placing the vials in a boiling water-bath for 2 min. The reaction mixture was allowed to cool and 0·4 ml aliquots were removed and treated with 0·3 ml each of 1% sulphanilamide in 3N HCl and 0·02% of N-1 naphthylethylene diamine HCl. After 20 min, 4 ml of water was added and the optical density read at 540 nm.

Enzyme activity was expressed as nanomoles of nitrite formed per gram of leaf tissue per hour.

Nitrite reductase was assayed by monitoring the disappearance of nitrite with time. In this case KNO_2 was substituted for KNO_3 in the reaction mixture.

The effect of chloramphenicol on the induction of NR was studied by incorporating 100 μg of chloramphenicol in the reaction medium used in the NR assay.

Investigations on the effect of growth regulators on NR activity were made by incubating the leaf discs for 3h in the respective solutions of growth regulators. A

Abbreviations used :

NR = Nitrate reductase
PMS = Phenazine methosulphate
PVP = Polyvinylpolypyrrolidone
EDTA = Ethylenediaminetetra acetate
GA = Gibberellic acid.

vacuum infiltration was done as described previously. At the end of 3 h the tissues were carefully removed from the solution, blotted free of any adhering solution and suspended in the NR assay medium.

For monitoring the changes in NR activity during leaf ontogenesis, the leaves were tagged immediately on emergence and samples taken at three weekly intervals and assayed for NR activity.

2.2 *Assay in cell-free extract*

The *in vitro* assay was done by preparing 10% homogenate of leaves in a grinding medium consisting of 0·1 M phosphate buffer, pH 7·5, 1 mM EDTA and 15 mM cysteine. 20% w/v of polyclar AT (PVP) was added. The tender leaves were finely cut and suspended in the chilled (0°–4° C) grinding medium. The homogenate was prepared in an Eberbach Waring Blender equipped with a jacketed jar assembly, the temperature being maintained at 4° C by circulating 50% methanol. The blending was done as quickly as possible in two short bursts of 40s each at low speed. The crude homogenate was filtered over four layers of muslin cloth and the filtrate centrifuged at 10,000 g, for 10 min. The pH of the supernatant was adjusted to 7·5 with dilute ammonia. This was used as the crude enzyme. Due to the extreme lability of the enzyme, it was ensured that all operations were performed as quickly as possible and at 4° C.

The NADH-dependent NR was assayed in 2 ml of a reaction mixture consisting of 0·1 M phosphate buffer, pH 7·4, 0·1 M KNO_3, 1 mM NADH and the enzyme. The reaction was initiated by the addition of enzyme. Appropriate blanks were run by omitting NADH in the reaction mixture and by running a zero time control. The mixture was incubated at 30° C for 15 min and terminated by placing the tubes in a boiling water-bath. Any precipitate was removed by centrifugation. Post-assay treatments were done by the addition of 30 nm PMS. After 20 min, nitrite colour development was done by the addition of 1 ml each of 1% sulphanilamide in 3 N HCl and 1 ml of N-1-naphthylethylene-diamine HCl (0·02%). The absorption was read at 540 nm and the nitrite quantitated by reference to a standard calibration curve obtained with KNO_2.

Enzyme activity was expressed as micromoles of nitrate reduced per minute at 30° C.

Protein was estimated by the method of Bradford[6].

Nitrate concentration in the leaves was determined by the method of Johnson *et al*[7] using phenoldisulphonic acid.

3. Results and discussion

Being a substrate–inducible enzyme, the enhancement of nitrate reductase activity by nitrate is to be expected and it does so optimally at a concentration of 100 mM. Table I shows the result obtained with growth regulators on the induction of NR. A perusal of the effect of various growth regulators on the NR activity reveals that benzyladenine has a pronounced effect on NR activity even at a concentration of 10^{-12}M. This is followed by kinetin, although the effect is not of the same order. GA, however, would seem to have a very marginal effect even at a concentration of 10^{-3}M.

The response of NR activity to nitrate and cytokinins has been earlier investigated by Kende *et al*[8] in the excised embryos of *Agrostemma githago*. The effect of GA has also been studied in tobacco leaves[9]. The effect of these growth regulators, however, appears to be a slower process in mango leaves, compared to a fast biochemical reaction in *A. githago* embryos.

Table I

Effect of nitrate, benzyladenine, gibberellic acid and kinetin on nitrate reductase activity in mango leaves

Sl. No.	Treatment	Concentration	NR activity* (nm of No_2/1 gm/hr)
1.	KNO_3	50 mM	30·62
		75 mM	31·87
		100 mM	55·89
		200 mM	42·48
2.	Benzyladenine (BA)	10^{-12}M	68·16
		10^{-9}M	68·92
		10^{-6}M	59·14
		10^{-3}M	56·74
3.	Gibberellic Acid (GA)	10^{-12}M	...
		10^{-9}M	...
		10^{-6}M	56·22
		10^{-3}M	56·52
4.	Kinetin	10^{-12}M	61·07
		10^{-9}M	65·30
		10^{-6}M	57·92
		10^{-3}M	57·99

* All values are mean of triplicate determinations.

The slower response may also be attributed to the permeability factor. This is easily evidenced by the fact that despite the incorporation of 1% propanol in the reaction medium for *in vivo* assay and vacuum infiltration, there is an abnormal time lag before the NR activity could be discerned. Since the release of nitrite ions into the medium is initiated within one hour and shows a gradual rise with time, the possibilities of bacterial enzymes catalysing the reaction are minimised. Figure 1 shows the time course of activation of NR in the *in vivo* assay.

Table II reveals data on the changes in nitrate reductase activity with leaf ontogenesis. There is a burst of activity in the new flush with a progressive decline with age. Although the fall in activity was very pronounced in the initial phase, it showed a steady trend during the later stages of leaf maturity.

Experiments were also conducted to ascertain the endogenous levels of nitrate in the leaf tissue. It was found that the average nitrate levels ranged from 149 to 355 ppm per gm of leaf tissue, at any stage of maturity. Hence the nitrate reducing potential of the leaf is actuated by the presence of exogenously added nitrate. The precise effect of foliar application of nitrate in the induction of flowering, if any, is yet to be determined. It is however known from an earlier observation[10] that enhanced amino acids and carbohydrates are required during flower initiation. It is possible that the physiological response in the plant is mediated by NR induction and the consequences thereof.

A comparison has been made in Table III of the nitrate reductase activity of mango and certain other tropical fruit plants like guava and banana. In all the cases, tender

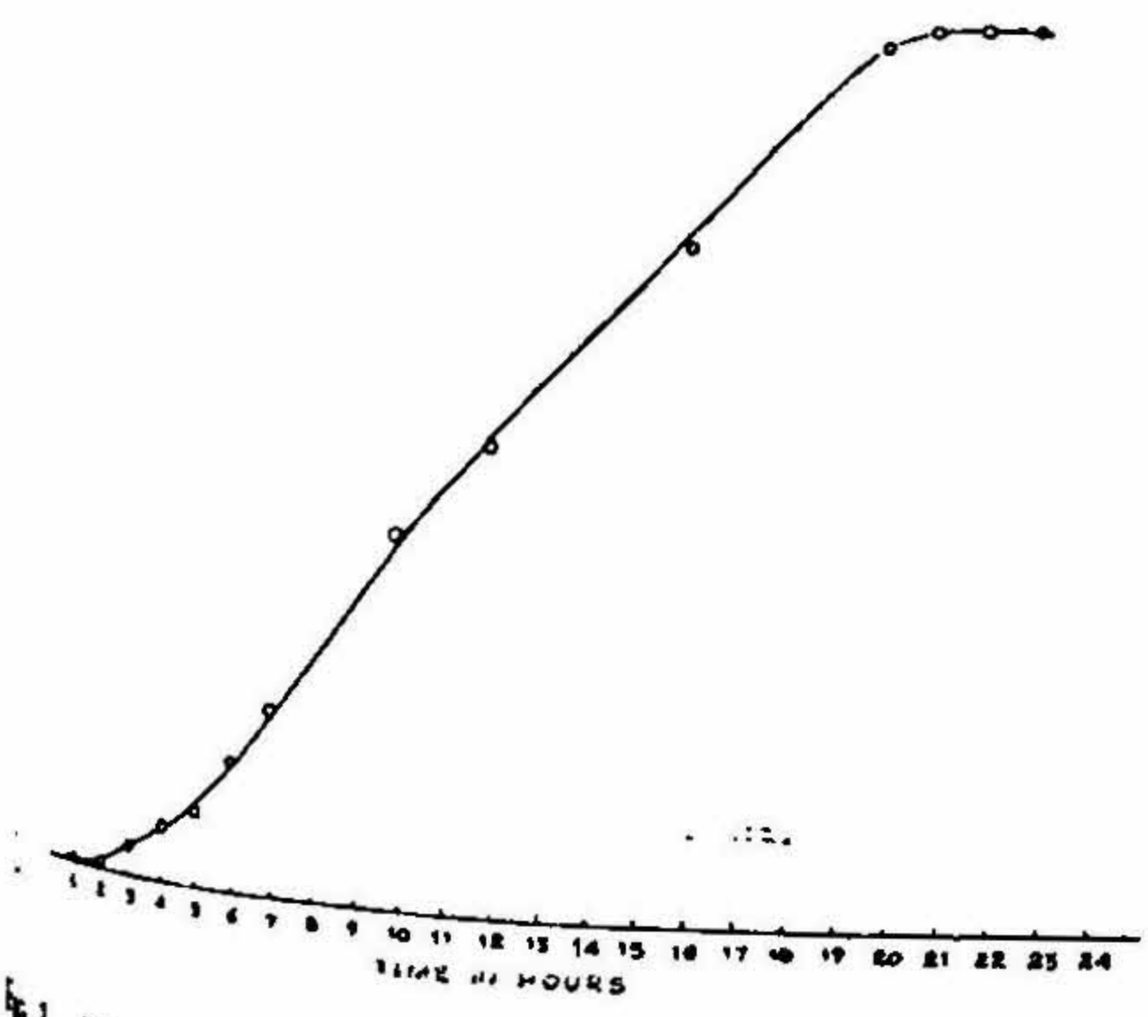

Fig. 1. Time course of activation of nitrate reductase in *Mangifera indica* L. (*in vivo* assay). Enzyme activity in nanomoles of nitrite formed per gram per hour. Time in hours.

Table II

Nitrate reductase activity during leaf ontogenesis (Dasehri variety)

Age of leaf	NR activity* nm of No_2^-/gm/hr
New flush	69·26
3 weeks	54·59
6 weeks	19·52
9 weeks	6·57
12 weeks	5·48
15 weeks	4·88
18 weeks	4·50

* Values based on triplicate determinations.
Nitrate content in leaf tissue (Dasehri).
ppm No_3^- in 1 gm of leaf tissue : 148·77.

Table III

Nitrate reductase activity of tropical fruit plants (mango, guava and banana)

Sl. No.	Sample	NR activity (nm of No_2^-/gm/hr)
1.	*Mango*	
	Variety Langra	57·50
	Variety Dasehri	68·92
	Variety Kalapad	54·12
	Chloramphenicol treated (Daschri)	13·78
	Percentage of original activity	20·00%
2.	*Guava*	36·49
	Chloramphenicol treated	13·61
	Percentage of original activity	37·30%
3.	*Banana*	23·62
	Chloramphenicol treated	9·51
	Percentage of original activity	40·26%

Table IV

Nitrite reductase activity* (μg of No_2^- in the reaction medium)

Zero time	125·72
3 hours	93·16
5 hours	80·25
30 hours	1·08

* Mean of triplicate determinations.
* Decrease in No_2^- concentration with time-index of utilisation of nitrite by plant tissue—proportional to nitrite reductase activity.

fully expanded leaves were used for the determination of activity. It is found that mango has greater nitrate reducing potential than either guava or banana. It is further seen that all of them are subjected to inhibition by treatment with chloramphenicol ($P<0.05$). The effect of other protein synthesis inhibitors is yet to be investigated.

Table IV shows data on the nitrite reductase activity on the leaf. It is seen that there is a rapid consumption of nitrite ions produced as a consequence of NR activity.

From the above observations it would appear that higher plant nitrate reductase with special reference to tree crop species like mango shows a close parallelism with the enzymes studied from other sources. The rapid inducibility of this enzyme by growth regulators like benzyladenine and kinetin might lead one to believe the important role that the activity of the enzyme may play in the overall regulation of nitrogen metabolism during such important physiological events like flower initiation and fruit setting/development. It remains to be ascertained as to how critical the NR activity is in triggering these events in the life of the plant, as also the impact/influence that it might have in determining the yield pattern or productivity in mango.

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