NITRATE REDUCTASE ACTIVITY IN A MEMBER OF THE RESTIONACEAE

(*Thamnocortis hanna*)

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ABSTRACT

Nitrate reductase activity in stalks and roots of *T. hanna* plants grown in nitrate solutions of increasing concentration were assayed by the *in-vivo* method after 5, 10 and 27 hours growth. Maximum induction at 10ppm nitrate and the failure of 50 and 100ppm nitrate solutions to induce greater nitrate reductase activity indicate that this plant is poorly adapted to deal with high levels of nitrate. This, in accordance with the low levels of nitrate in natural soils and since total nitrogen of these soils is low, could be a major factor contributing towards the slow growth rate of this species.

CONTENTS

1. INTRODUCTION

1.1 Ecology of the Restionaceae

The Mediterranean climate of the South Western Cape supports one of the richest floral kingdoms in the world. In the Cape Peninsula alone there are well over 2500 species distributed in over 700 genera making up this immensely diverse vegetation type. The vegetation is commonly referred to as the fynbos (or macchia) because many of the elements are sclerophyllous. It occurs between latitudes 32^{o} - 35^{o} and longitudes 18^{o} - 20^{o} (Acocks, 1975).

The family Restionaceae is a characteristic element of the fynbos of S.W. Cape, growing in mountainous regions as well as sandy flats. It represents an important element in the seral succession of the fire adapted fynbos flora: after a fire the Restionaceae predominate along with fire-resistant perennials, geophytes and annuals. As the succession proceeds initial colonizers are replaced by Ericas and thence by Proteas. The Restionaceae persist throughout the seral succession (although becoming progressively less dominant), thus representing a vital element of the fynbos flora.

The soils of the S.W. Cape are of particular interest as, in spite of the tremendously rich and diverse flora supported by them, they are nutritionally very poor. Most of the soils are originally derived from Table Mountain Sandstone and have poor water and nutrient retention properties. Soils are shallow and total phosphate is often below 0,3% (Low, 1975). Nitrogen, the most important growth limiting element in terms of protein production, is always below 0,1% (total nitrogen).

1.2 Nitrate Reductase

In view of the low nitrogen levels in fynbos soils, the assimilation of inorganic nitrogen by the Fynbos flora represents a particularly interesting and, as yet, a very much unstudied field of research. On this basis it was decided to investigate the levels of nitrate reductase in a member of the important Restionaceous element of the Fynbos vegetation. The particular example chosen was Thamnocortis hanna.

Generally in plants, apart from those species that have a symbiotic association with nitrogen fixing bacteria, the bulk of the plant nitrogen arises from the reduction of nitrate taken up from the soil. Since almost all organic-nitrogen compounds contain, or are made from fully reduced nitrogen, absorbed nitrate must be reduced in order to make it available for organic synthesis.

It is in the first step of this reduction series that nitrate reductase is involved and it mediates the reduction of nitrate to nitrite (NO_3 to NO_2). Indeed, it appears that the control of nitrate reduction is mediated through a regulation of this important enzyme. Nitrate reductase is the logical point to effect regulation of the input of reduced nitrogen for the plant because it is (a) the first enzyme in the pathway; (b) the rate limiting step; (c) substrate inducible (i.e. greater enzyme activity is induced by an increased substrate concentration); and (d) relatively high turnover rate. Further the toxic effects of excess levels of nitrite and ammonium ions also indicate the desirability of regulating their production. The enzyme occurs widely in both roots and leaves of plants.

Evidence that nitrate reductase is inducible by nitrate has been provided by many workers (see references Beevers et al, 1969). A feature of

these observations is that different levels of nitrate are required for optimum induction in different species and in some cases high concentrations of nitrate are needed. These differences probably indicate differences in rate of uptake between species because with a given species the level of enzyme is dependent, within limits, upon the concentration and rate of supply of nitrate to the tissue.

That the level of nitrate required for optimum induction is under genetic control has been shown in corn and wheat inbreds and hybrids (Cove et al, 1963) as well as several species of cauliflower. Apparently different plants are genetically preadapted to cope with different levels of nitrate. That this genetic control is real and of practical significance has been established with wheat by showing that the seasonal input of reduced nitrogen to the plant, computed on an acreage basis from the nitrate reductase enzyme assay, was related in a linear manner to the protein accumulated in the vegetation and grain.

In summary, then, nitrate reductase is an important enzyme responsible for the reduction of nitrate to nitrite, is known to be substrate inducible, the optimum level of induction being genetically controlled and levels of the enzyme show a linear correlation with protein accumulation.

The only work on nitrate reductase in members of the Fynbos flora to date is that by W. Stock (1971) on members of the Proteaceae. Although root material was not investigated, nitrate reductase was found to be present in leaves at very low levels and greater enzyme activity could not be induced by increasing nitrate levels fed to shoots. Since ammonia levels in the soil are themselves very low, low nitrate reductase activity could have an important effect on protein production.

In the course of these experiments, nitrate reductase activity was investigated in both roots and stalks of Thamnocortis hanna.

2. METHODS

2.1 Plant material

Four Thamnocortis hanna plants were obtained from Kirstenbosch Botanical Gardens. These had been removed from the field with minimal root disturbance and grown in pots of the sand from which they were collected for two growing seasons. The roots were thus well established and fully recovered from any disturbance experienced during transplanting to pots.

At the start of the experiment the sand was carefully washed from the roots of the plants and three were placed respectively in 10 ppm, 50 ppm and 100 ppm aerated nitrate solutions (KNO_3). The fourth plant was used to determine natural levels of nitrate reductase activity.

2.2 Nitrate content of soils

Soils were analysed for nitrate content by means of a nitrate electrode.

2.3 Determination of nitrate reductase activity

Nitrate reductase activities in stalks and roots of all four plants were assayed by the in-vivo method* described below. Tissues from plants grown in nitrate solutions were assayed after five, ten and twenty-seven hours respectively.

i. Duplicate samples of 1g root and 1g stalk were cut into ± 1mm

*After Klepper, et al. 1971.

segments, cutting across the organs.

ii. The one gram samples were placed into separate test tubes each containing 10ml incubating medium (0,1M Potassium phosphate buffer pH 7,5 and 0,1M Potassium nitrate) and infiltrated under vacuum for 2 minutes. Infiltration was repeated once.

iii. Tubes were wrapped in aluminium foil to exclude light, thus preventing the reduction of nitrite formed, and placed in a 30°C shaking water bath. The incubating medium was kept in complete darkness at all times except when removing samples.

iv. Immediately after infiltration, 0,2ml incubating medium was pipetted from each tube into test tubes containing 2ml distilled water. 1ml sulphanilamide solution (1% in 1,0N HCl) to stop the enzyme action was added as well as 1ml colour reagent (0,01% N (1-napthyl)-ethylenediamine hydrochloride).

v. After 60 minutes, a further 0,2ml aliquot of each incubating solution was pipetted into a test tube containing 2ml water and was treated as in step iv.

vi. After allowing the colour of the last aliquot to develop for at least 15 min., the optical density of each aliquot was read in a Spectronic 20 absorptiometer set at 540nm.

vii. A standard curve (fig. 1) was prepared by pipetting 0,2ml of standard nitrite solutions into test tubes containing 2ml distilled water (in duplicate) and treating as in steps iv and vi.

From the standard graph the quantity of nitrite produced in each tube was read off and the average rate of colour production (i.e. nitrate

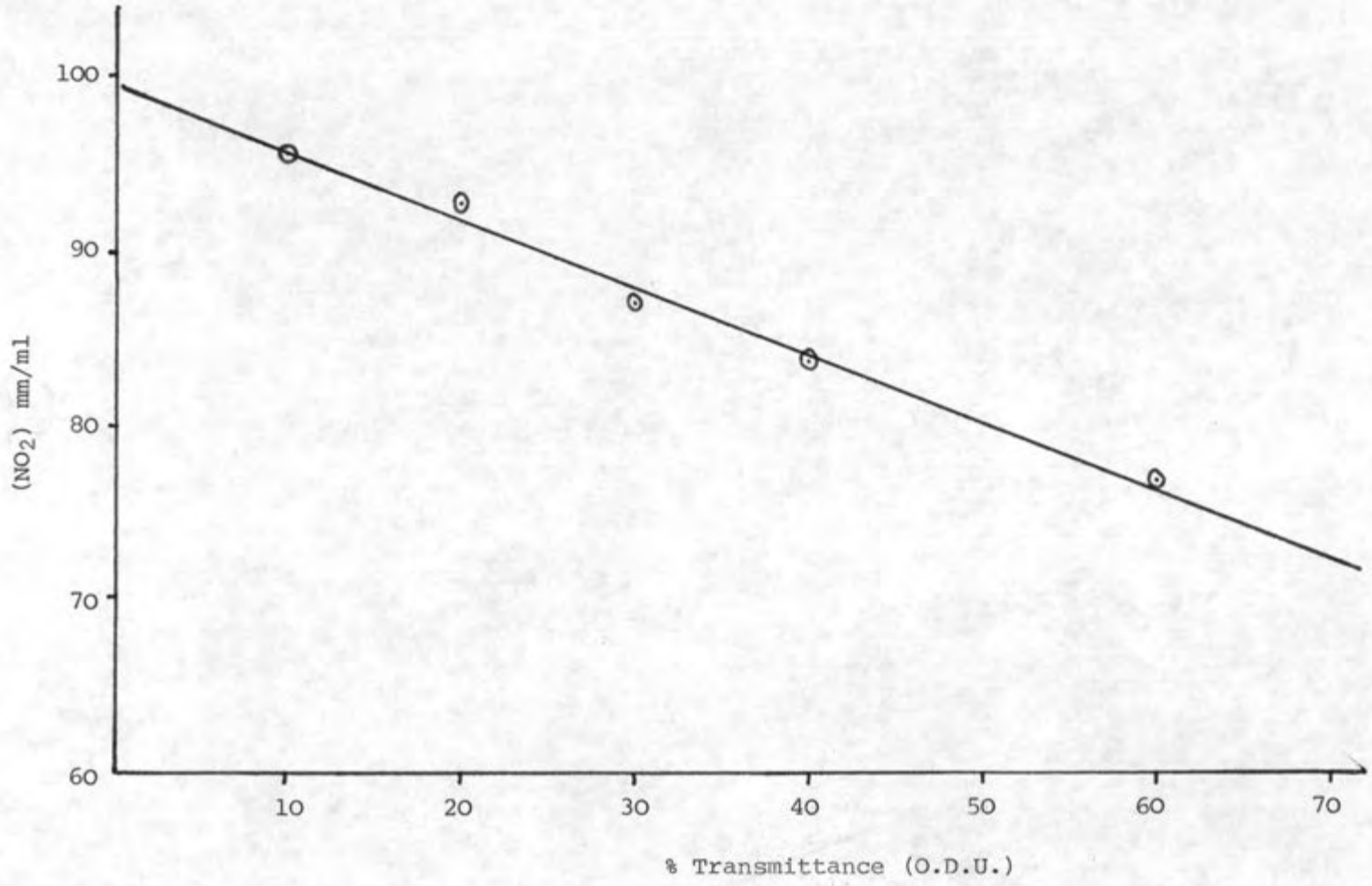

FIGURE 1: Standard curve: NO_2 concentration vs. % transmittance (O.D.U.)

reductase activity) in nmoles nitrite/ml/hr/g fresh weight for each tissue and feeding treatment was calculated.

3. RESULTS

3.1 Nitrate content of soils

Nitrate content of the soils, as measured by a nitrate probe, were found to be 0,34 ppm.

3.2 Enzyme activity

Nitrate reductase activity in plants growing in natural soils were found to be 6,2 and 5,6 nm NO_2/ h /g for roots and stalks respectively (Appendix 1).

Nitrate reductase activities in plants growing in 10 ppm, 50 ppm and 100 ppm nitrate solutions, as assayed after 5, 10 and 27 hours are presented in Table 1. Graphic representation of these data appear in Figures 2 and 3.

In the case of the stalks of plants grown at 10 ppm, a higher level of nitrate reductase activity is induced after 5 hrs in the nitrate solutions relative to enzyme activity in plants not grown in nitrate solutions (5,6 to 19,3 nM_2NO / g/h). After 10 and 27 hours there is, however, no further increase in activity; the enzyme appears to be maximally induced. In the root of this plant induction continues for the first 10 hours.

TABLE 1

Nitrate reductase activity in stalks and roots of Thamnocortis hanna plants grown in different NO_3 solutions and sampled after 5, 10 & 27 hours.
(Derived from Appendix Tables 2 and 3)

Tissue	NO_3 fed for	Nitrate reductase activity (nM NO_2/g.fw/h)		
		10 ppm	50 ppm	100 ppm
STALKS	5 h	19,3±3,0	28,9±0,8	25,6±0,4
	10 h	13,7±1,0	4 ±1,0	2,4±0,3
	27 h	20,2±1,4	2 ±0,4	1 ±0,2
ROOTS	5 h	18,2±2,0	20,8±0,5	27 ±0,4
	10 h	27 ±0,8	6,6±1,2	2,8±1,5
	27 h	18,4±1,2	10,2±2,8	10,0±0,8

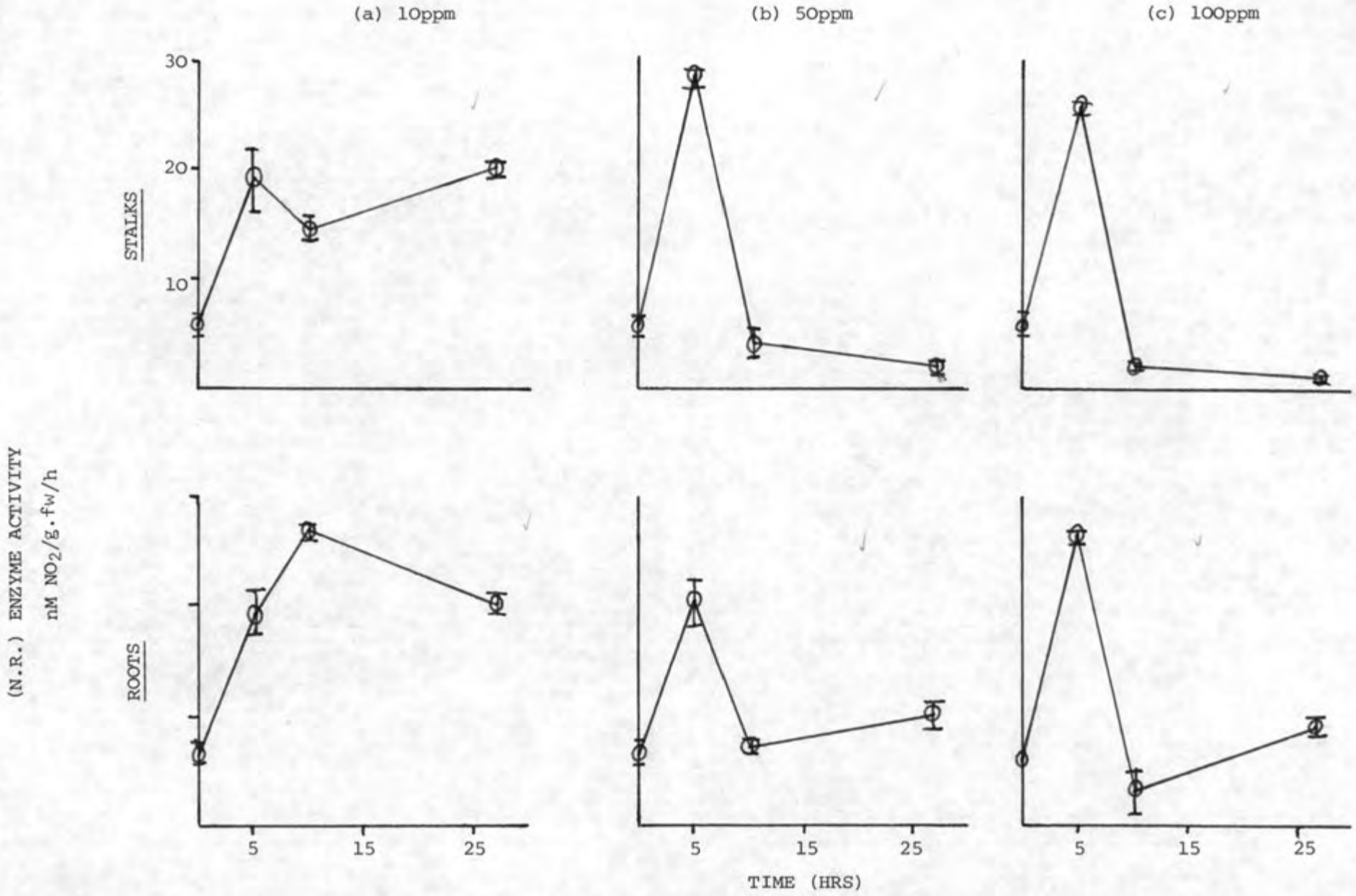

FIGURE 2: Nitrate reductase activity of roots and shoots of T. hanna after 5, 10 and 27 hours growth in nitrate solutions of 10, 50 and 100ppm.

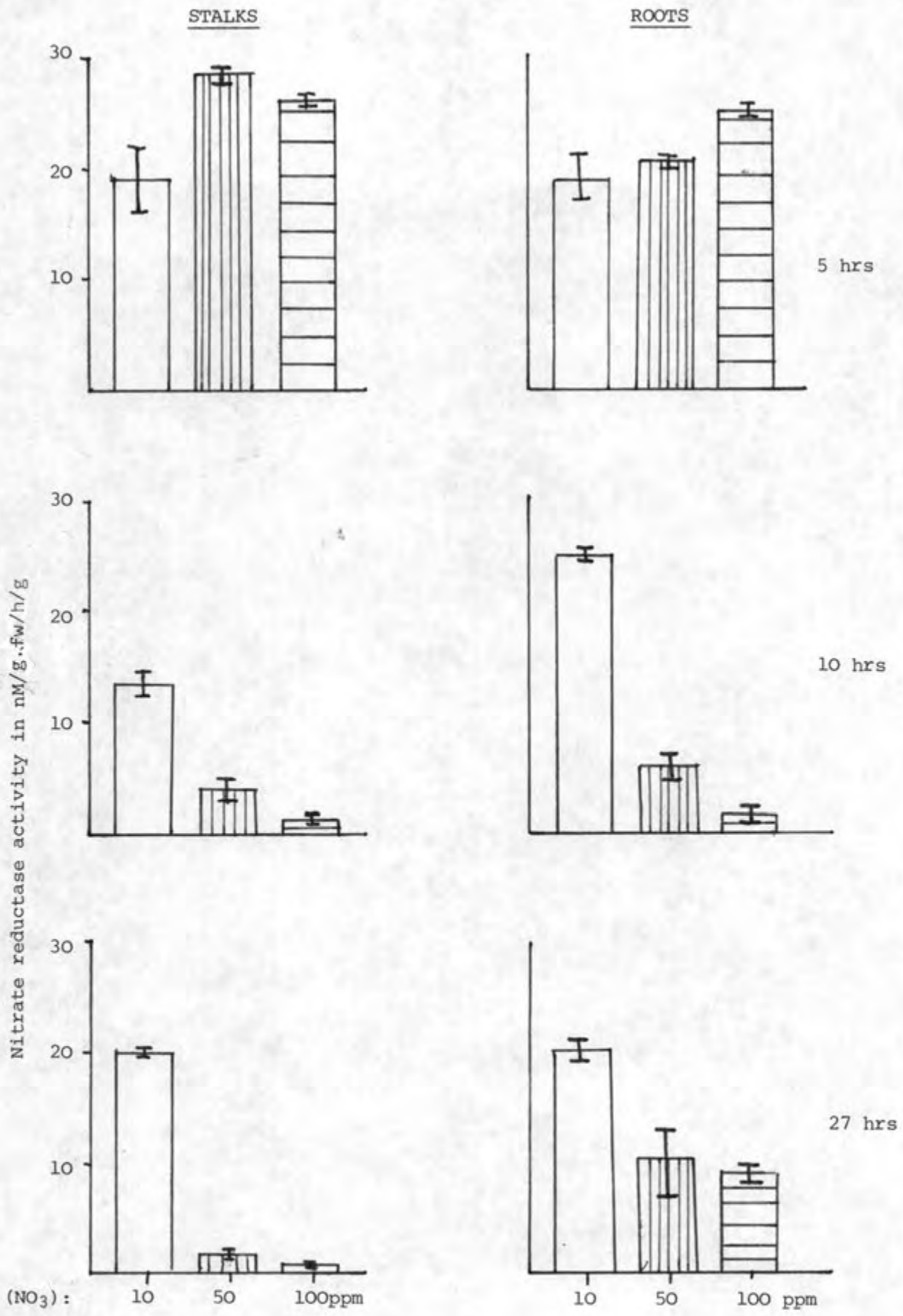

FIGURE 3: Nitrate reductase activity of roots and shoots of T. hanna after 5, 10 and 27 hours growth in nitrate solutions of 10, 50 and 100ppm.

In plants grown at 50 and 100 ppm nitrate, greater levels of nitrate reductase activity in the stalks are induced during the first 5 hours (relative to 10 ppm soil samples, see figs. 2a and 3a). There is, however, a dramatic decrease in activity to a level lower than that found naturally in the plants after 10 hours growth in nitrate solutions of this concentration.

In the case of the roots of these plants the overall trend is the same except that the decrease in nitrate reductase after 5 hours is somewhat less marked (see nitrate reductase activity in roots and stalks after 27 hours in figure 2b and c and figure 3c).

From Figure 3a it is apparent that after 5 hours in nitrate solutions the level of nitrate reductase activity in the stalks is lowest at 10 ppm, and only slightly higher at 50 and 100 ppm nitrate.

After 5 hours growth in nitrate solution a similar, slight overall increase in nitrate reductase activity with increased nitrate concentration occurs in the roots.

After 10 and 27 hours there is a progressive decrease in nitrate reductase activity in stalks with increasing nitrate concentration (Fig. 3b and c). Similar trends are observed in the roots.

The maximum nitrate reductase activity observed in roots and stalks were 28,9 and 27 nm NO_2/ml/g/hr respectively.

4. DISCUSSION

The low value for maximum nitrate reductase activity (28,9 and 27 nm NO_2/ml/g/hr for roots and stalks respectively) indicates that T.hanna

is adapted to deal only with low levels of nitrate. The negligible induction of enzyme activity in roots and stalks with increasing nitrate concentration after 5 hours (Fig. 3a) along with the near maximum induction of the enzyme after five hours at 10 ppm with no marked increase in activity with time (Fig. 2a) confirms this. That T. hanna is poorly adapted to deal with (1) high nitrate concentrations in accordance with the low levels of nitrate naturally present in the soil as well as (2) the fact that it, as a fynbos species, has a renowned slow growth rate (bearing in mind that ammonia, as an alternative nitrogen source, is also exceptionally low in Fynbos soils (Low, 1975)). These results relfect those obtained by W. Stock for nitrate reductase activity in the Proteaceae.

The explanation for the marked decrease in nitrate reductase activity observed in roots and stalks after time periods of greater than five hours at higher nitrate concentrations is, however, more speculative.

It can be suggested to be due to limiting levels of other nutrients required by the plant but not present in the nitrate solutions, particularly PO_4 (in terms of its importance in the production of energy rich substances such as ATP, for example) and Molybdenum (an important element in terms of the nitrate reductase system). It has been demonstrated that in molybdenum-deficient plants grown in the presence of nitrate, molybdenum can serve as an inducer for nitrate reductase. Since no increase in enzyme activity can be induced by adding molybdenum to cell-free extracts of deficient plants (Afridi et al, 1964), it appears that the metal is involved in the induction process and is not merely activating pre-existing protein. Furthermore, the induction of nitrate reductase by molybdenum shows the same sensitivity to inhibitors of protein synthesis as does induction by

nitrate (Afridi et al, 1965).

Even if nutrients such as PO_4 and molybdenum were limiting nitrate reductase activity, which is unlikely - as discussed below, it would merely accentuate the fact that these plants are poorly adapted to deal with high nitrate concentrations since nutrients such as molybdenum are themselves present at very low levels in the nutrient poor Fynbos soils.

Attractive though this nutrient-limiting hypothesis may seem, it is unlikely that nutrients such as PO_4 and Mo would become limiting after + 5 hours growth in 50 ppm nitrate solution, if they fail to do so after 27 hours growth at a nitrate concentration only five times less than 50 ppm (see Fig. 2a and b). Furthermore, overall decreases in nitrate reductase activity in roots after 5 hours growth in 50 ppm and 100 ppm appear to be less drastic than those in the stalks (see Fig. 2b and c). That a nutrient deficiency is limiting nitrate reductase activity within the time limits of this experiment thus fail to provide a complete and adequate explanation for the observed decrease in enzyme activity after + 5 hours growth at higher nitrate concentrations.

A further factor to be taken into account is the possibility of end product repression. Filner (quoted by Beevers et al, 1969) has demonstrated that casein hydrolysates and 11 amino acids, added individually, would repress the synthesis of nitrate reductase of cultured tobacco pith cells. The amino acids arginine, lysine, leucine, glutamate, as well as orinithine and ammonium ions have also been shown to effect repression of enzyme induction, but none of these compounds actually inhibited nitrate reductase activity. This may explain the levelling off of nitrate reductase activity observed in stalks and roots at 10 ppm nitrate.

The secondary metabolites of nitrogen metabolism, coumarin, trans-cinnamate and trans-o-hydroxycinnamate do inhibit induction of nitrate reductase and accumulation of these substances may play a role in causing decreased enzyme activity in stalks and roots after + 5 hours at 50 and 100 ppm nitrate solutions. It is not known, however, whether the decreased enzyme activity is due to enzyme degradation, or merely inactivation due to change in enzyme configuration due to end or secondary product repression.

CONCLUSIONS

The maximum induction of nitrate reductase in stalks and roots of T. hanna at 10 ppm nitrate, as well as negligible induction of the enzyme with increasing nitrate concentrations after five hours growth indicate that this species is poorly adapted to deal with high nitrate concentrations. In view of the low total nitrogen levels in Fynbos soil, this could be a major contributing factor towards the slow growth rate observed in the species.

The reasons for the marked decrease in nitrate reductase activity observed in stalks and roots after + 5 hours at higher (50 and 100 ppm) nitrate concentration are more speculative and probably involve repression of the enzyme by accumulation of end or biproducts of this phase of nitrogen metabolism.

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APPENDIX

APPENDIX 1: Table showing details relating to tissue samples taken from plants growing in natural soils.

Tissue	% transmittance (O.D.U.)						nM NO_2/ g.fw		nM NO_2/g.fw/hr
	*1: 60 min.			0 min.			0 min.	60 min.	
	Samples		Ave	Samples		Ave			
Stalks	95	94	94,5	96	97	96,5	10,4	16	5,6
Root	87,6	89,2	88,4	91	91	91	23,2	29,5	6,2

*1: time of sampling from incubation medium pH 7,5 containing 1g plant tissue, during enzyme assay.

APPENDIX 2: Table showing % transmittance of samples taken during assays for NO_2 production by 1gm portions of root and stalk tissues.

			% Transmittance (O.D.U.)								
			10 ppm			50 ppm			100 ppm		
	(NO_3)		Samples		Ave	Samples		Ave	Samples		Ave
*2 5 hrs.	Min*1 0	Root	91,8	92,5	92,2	92,8	90,5	91,7	94,8	94	94,8
		Stalk	94,3	96,8	95,5	97,2	97,6	97,4	97,9	96,1	97
	60	Root	81,5	88,5	85	83	84	83,5	84	83	83,5
		Stalk	80,8	93,8	87,3	87	84,4	85,9	86,8	86,3	86,5
10 hrs.	0	Root	85	87	85	80	89	84,5	82,5	86,5	84,5
		Stalk	91	94	92,5	88,2	91	89,7	95	94,5	94,8
	60	Root	74	77	75,5	86	78	82,8	79,5	88	83,8
		Stalk	91	84,2	87,7	91,3	91,3	96,5	93,5	94,3	93,4
27 hrs.	0	Root	85	89,8	87,4	85	75	80	84	80	82
		Stalk	97	98	97,5	99	98,5	98,8	98	98,5	98,3
	60	Root	78,3	81,5	79,88	70	80	75	78	79	78,5
		Stalk	89	90	89,5	98,3	97,5	97,9	98	97,5	97,8

*2 time of growth of whole plant in nitrate solution.
*1 as for Appendix 1.

APPENDIX 3: Table showing amount of NO_2 present during assays at different times on tissues from plants grown at different NO_3 concentrations.

	(NO_3)		nM NO_2/g.fw		
			10 ppm	50 ppm	100 ppm
*2 5 hrs.	Min.*1 0	Root	19,6	21,2	13,2
		Stalk	13,2	6,5	8,4
	60	Root	37,8	42	41
		Stalk	32,5	35,4	34
10 hrs.	0	Root	35,4	39,2	39,2
		Stalk	18,6	26	13
	60	Root	62,4	45,8	42
		Stalk	32,3	22	15,4
27 hrs.	0	Root	32,6	50,8	45,8
		Stalk	6,4	3	4,4
	60	Root	51	61	55,8
		Stalk	26,6	5	5,4

*1 As in Appendix
*2 As in Appendix