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Nitrate concentration and nitrate reductase activity in the leaves of three legumes and three cereals*

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SUMMARY

Nitrate concentration and nitrate reductase activity (NRA) were studied in the leaves of soybean (*Glycine max*), groundnut (*Arachis hypogaea*) and cowpea (*Vigna unguiculata*) and sorghum (*Sorghum bicolor*), pearl millet (*Pennisetum americanum*) and maize (*Zea mays*) at three nitrogen fertiliser levels in two field experiments.

Higher nitrate concentrations were detected in the leaves of groundnut, cowpea and pearl millet than in sorghum and maize. Nitrate content in the leaves and leaf NRA were not related across crop species, nor was a generalised pattern of leaf NRA and leaf nitrate observed within legumes or within cereals. Nitrogen application resulted in higher nitrate availability in the leaves, with varied leaf NRA.

INTRODUCTION

The reduction of nitrate to nitrite, catalysed by the enzyme nitrate reductase (EC 1·6.6·1, NADH: nitrate oxidoreductase), is believed to be the rate-limiting step in mineral nitrogen utilisation in plants and is an inducible enzyme by substrate nitrate in the majority of plants investigated (Beevers & Hageman, 1969; Srivastava, 1980). In some plants, however, both inducible and constitutive enzymes are detected, for example in soybean (Nelson, Streit & Harper, 1986). Although nitrate reductase is detected in most plant parts, including roots, most of the nitrate is reduced in the leaves when exogenous nitrate is taken up (Beevers & Hageman, 1969; Goodman, 1979). We have earlier reported (Nambiar, Rego & Srinivasa Rao, 1986) that groundnut and sorghum differ in leaf nitrate reductase activity and leaf nitrate concentration. The concentration of nitrate in leaves was lower in sorghum than in groundnut, while leaf NRA was higher in sorghum than in groundnut. The present investigation examines whether these differences hold true for other cereals and legumes.

MATERIALS AND METHODS

Plants. The legumes tested were soybean (*Glycine max* L.) (genotype JS 7244), cowpea (*Vigna unguiculata* (L.) Walp) (EC 6216), groundnut (*Arachis hypogaea* L.) (nodulating ICGS 11) and a non-nodulating groundnut (Non-nod). The cereals tested were maize (*Zea mays* L.) (DH 103), pearl millet (*Pennisetum americanum* L. Leeke) (BK 560), and sorghum (*Sorghum bicolor* L. Moench) (CSH 8R). Groundnut and cowpea were nodulated well by existing rhizobia, while soybean did not form nodules without inoculation. Hence, soybean was tested with and without *Rhizobium* (strain TAL 102) inoculation. The experiments were conducted

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on Alfisol fields at ICRISAT Center, near Hyderabad, India, as described previously (Nambiar *et al.*, 1986) and summarised below

Expt 1. The seeds were sown during the post-rainy season on 29 November 1985 on raised beds, 1·5 m wide, with three rows per plot for cereals and four for legumes. Plant spacing within the row was 20 cm. All crops were fertilised with three levels of nitrogen (0, 100, 200 kg N ha^{-1} as urea), applied in four equal applications, 11, 31, 52, 73 days after sowing (DAS). Leaf samples for nitrate reductase activity (NRA) and nitrate estimations were collected at 66, 80, 94, 109 and 129 DAS. Owing to poor seed germination, observations on the pearl millet crop were not made in this season.

Expt 2. The same crops were sown during the rainy season on 24 June 1986 on four ridges 60 cm apart on a plot 4 m long. All crops were fertilised at three levels of nitrogen (0, 100, 200 kg N ha^{-1} as urea) applied in four equal applications (15, 36, 55, 76 DAS). Leaf samples for NRA and nitrate estimations were collected at 35, 56, 70, 84, 98 DAS. Owing to damage by shoot fly, observations on sorghum were not made in this season.

Table 1. *Effect of nitrogen application on leaf nitrate concentration of crop species during the 1985–86 post-rainy season*

	Nitrogen applied (kg ha^{-1})			
	0	100	200	Mean
	Nitrate content ($\mu g\ NO_3\ g^{-1}$ dry leaf)			
66 DAS (Days after sowing)				
1. Groundnut (Non-nod)	*2032 (3·25)	2896 (3·11)	5290 (3·64)	3406 (3.33)
2. Groundnut (ICGS 11	2487 (3·39)	3239 (3·16)	3646 (3·15)	3124 (3·23)
3. Cowpea	1837 (3·20)	3958 (3·00)	6931 (3·64)	4242 (3·28)
4. Soybean (Uninoc.)	519 (2·60)	359 (1·97)	1184 (2·35)	687 (2·31)
5. Soybean (Inoc.)	1002 (2·82)	656 (2·43)	2267 (3·28)	1308 (2·85)
6. Maize	1074 (2·90)	983 (2·10)	1788 (2·50)	1282 (2·50)
7. Sorghum	693 (2·78)	518 (2·28)	878 (2·30)	696 (2·45)
S.E. ± a = (0·1639); b = (0·1073); c = (0·2838)				
80 DAS				
1. Groundnut (Non-nod)	293 (2·18)	1126 (2·70)	2488 (3·34)	1303 (2·74)
2. Groundnut (ICGS-11)	1173 (2·97)	2232 (3·34)	2926 (3·45)	2110 (3·25)
3. Cowpea	398 (2·31)	1628 (3·16)	2593 (3·37)	1540 (2·95)
4. Soybean (Uninoc.)	64 (1·29)	361 (2·49)	920 (2·57)	448 (2·12)
5. Soybean (Inoc.)	106 (1·72)	740 (2·79)	1124 (2·72)	657 (2·41)
6. Maize	63 (1·28)	321 (2·47)	757 (2·79)	380 (2·18)
7. Sorghum	103 (1·63)	224 (2·34)	685 (2·71)	337 (2·23)
S.E. ± a = (0·1242), b = (0·0813); c = (0·2150)				
94 DAS				
1. Groundnut (Non-nod)	154 (1·55)	861 (2·86)	2204 (3·32)	1073 (2·58)
2. Groundnut (ICGS 11)	458 (2·63)	1123 (3·03)	1478 (3·17)	1020 (2·95)
3. Cowpea	281 (1·98)	1355 (3·05)	2347 (3·34)	1328 (2·79)
4. Maize	135 (1·49)	236 (2·33)	350 (2·18)	240 (2·00)
5. Sorghum	122 (1·42)	153 (1·73)	139 (1·49)	138 (1·55)
S.E. ± a = (0·1469), b = (0·1138), c = (0·2545)				

* Means calculated from original values. Data analysed after $\log_{10}(X + 1)$ transformation are given in parentheses. Inoc. = inoculated with *Rhizobium*; uninoc. = uninoculated.

S.E. ± (a) = for comparing the means among the crop species, (b) = for comparing the means among the nitrogen levels; (c) = for comparing the means of the crop species × nitrogen levels.

Table 2. *Effect of nitrogen application on leaf nitrate concentration of crop species during the 1986 rainy season*

	Nitrogen applied (kg ha^{-1})			
	0	100	200	Mean
	Nitrate content (μg nitrate g^{-1} dry leaf)			
35 DAS				
1. Groundnut (Non-nod)	181 (2·22)	468 (2·63)	1296 (3·07)	648 (2·64)
2. Groundnut (ICGS 11)	552 (2·71)	732 (2·85)	1180 (3·06)	821 (2·88)
3. Cowpea	416 (2·62)	572 (2·75)	828 (2·90)	605 (2·76)
4. Soybean (Uninoc.)	217 (2·20)	369 (2·55)	792 (2·85)	459 (2·53)
5. Soybean (Inoc.)	336 (2·49)	600 (2·73)	1144 (3·05)	693 (2·76)
6. Maize	116 (2·02)	552 (2·63)	656 (2·79)	441 (2·48)
7. Pearl millet	668 (2·00)	2976 (3·50)	5440 (3·71)	3028 (3·06)
S.E. ± a = (0·1395); b = (0·0854); c = (0·2415)				
56 DAS				
1. Groundnut (Non-nod)	120 (1·50)	132 (2·10)	188 (2·30)	147 (1·96)
2. Groundnut (ICGS 11)	184 (2·24)	256 (2·41)	476 (2·66)	305 (2·44)
3. Cowpea	100 (1·94)	276 (2·39)	421 (2·56)	266 (2·30)
4. Soybean (Uninoc.)	68 (1·84)	132 (2·11)	224 (2·26)	141 (2·07)
5. Soybean (Inoc.)	104 (1·99)	120 (2·07)	272 (2·43)	165 (2·17)
6. Maize	40 (1·53)	72 (1·85)	172 (2·18)	95 (1·85)
7. Pearl millet	168 (1·88)	412 (2·59)	205 (2·25)	262 (2·24)
S.E. ± a = (0·1301); b = (0·0852); c = (0·2254)				
70 DAS				
1. Groundnut (Non-nod)	132 (2·07)	196 (2·27)	396 (2·60)	241 (2·31)
2. Groundnut (ICGS 11)	196 (2·28)	364 (2·53)	606 (2·78)	389 (2·53)
3. Cowpea	184 (2·25)	160 (2·18)	584 (2·63)	309 (2·35)
4. Soybean (Uninoc.)	96 (1·96)	152 (2·17)	140 (2·11)	129 (2·08)
5. Soybean (Inoc.)	92 (1·90)	84 (1·90)	76 (1·79)	84 (1·87)
6. Maize	108 (2·01)	132 (2·07)	96 (1·96)	112 (2·01)
7. Pearl Millet	76 (1·84)	84 (1·92)	388 (2·52)	183 (2·09)
S.E. ± a = (0·0721); b = (0·0472); c = (0·1249)				

For details see Table 1.

Leaf NRA. Discs of 8 mm diameter were cut from each leaf and incubated in sodium phosphate buffer (0·1 M sodium phosphate, pH 7·5, 5% n-propanol and 0·02 M KNO_3; approximately 2 ml buffer/each disc). The discs were subjected to vacuum infiltration for 2 min at 1×10^3 Pa and incubated at 30 °C for 30 min. The incubated mixture was filtered through a nitrate-free Whatman No. 1 filter paper and nitrite content was estimated using Szechrome NIT (Hunter, Fahring, Olsen & Porter, 1982).

Leaf nitrate content. The separated leaves were dried at 60 °C for 48 h and finely ground to pass through a 1 mm sieve. A sample of 0·1 g leaf powder from the 1985 - 1986 post-rainy samples was mixed with nitrate-free, activated charcoal in a ratio of 1:2. The nitrate was extracted into 20 ml of distilled water and estimated using Szechrome NAS (Hunter *et al.*, 1982).

A sample of 0·5 g leaf powder from the 1986 rainy season samples was mixed with nitrate-free, activated charcoal in a ratio of 1:2. The nitrate was extracted into 30 ml of distilled water and estimated using phenol disulphonic acid (Donald & Nason, 1957).

Table 3. *Effect of nitrogen application on leaf NRA of crop species during the 1985 86 post-rainy season*

	Nitrogen applied (kg ha^{-1})			
	0	100	200	Mean
	Nitrate reductase activity (nmoles NO_2^- g^{-1} h^{-1})			
66 DAS				
1. Groundnut (Non-nod)	1067 (2·96)	2313 (3·33)	2152 (3·25)	1844 (3.18)
2. Groundnut (ICGS 11)	1401 (3·10)	1125 (3·00)	1588 (3·18)	1371 (3·10)
3. Cowpea	4479 (3·62)	5569 (3·69)	6278 (3·76)	5442 (3·69)
4. Soybean (Uninoc.)	867 (2·87)	3678 (3·51)	2596 (3·31)	2380 (3·23)
5. Soybean (Inoc.)	1144 (2·90)	6219 (3·59)	5530 (3·69)	4298 (3·39)
6. Maize	2089 (3·27)	3330 (3·45)	2998 (3·44)	2806 (3·39)
7. Sorghum	1514 (3·07)	1764 (3·16)	2247 (3·17)	1842 (3·13)
S.E. ± a = (0·0525); b = (0·0344); c = (0·0909)				
80 DAS				
1. Groundnut (Non-nod)	382 (2·56)	797 (2·88)	790 (2·89)	656 (2·78)
2. Groundnut (ICGS 11)	559 (2·71)	631 (2·78)	701 (2·83)	630 (2·77)
3. Cowpea	1477 (3·15)	2211 (3·33)	2561 (3·36)	2083 (3·28)
4. Soybean (Uninoc.)	695 (2·80)	1834 (3·25)	2710 (3·41)	1746 (3·16)
5. Soybean (Inoc.)	1200 (3·05)	2734 (3·38)	2348 (3·36)	2094 (3·26)
6. Maize	1568 (3·18)	1839 (3·25)	1759 (3·22)	1722 (3·22)
7. Sorghum	1630 (3·19)	2302 (3·32)	3050 (3·47)	2328 (3·33)
S.E. ± a = (0·0262); b = (0·0171); c = (0·0454)				
94 DAS				
1. Groundnut (Non-nod)	316 (1·78)	609 (2·47)	795 (2·89)	573 (2·38)
2. Groundnut (ICGS 11)	533 (2·69)	736 (2·56)	929 (2·94)	733 (2·73)
3. Cowpea	1619 (3·18)	2544 (3·37)	2995 (3·45)	2386 (3·33)
4. Maize	1685 (3·21)	2210 (3·34)	3024 (3·46)	2306 (3·34)
5. Sorghum	1677 (3·17)	3420 (3·52)	2866 (3·42)	2654 (3·37)
S.E. ± a = (0·1032); b = (0·0799); c = (0·1788)				

For details see Table 1.

RESULTS

Data of only the first three samplings are presented. Some of the crop species matured by this time and, in general, results from the later samplings do not contradict those from the earlier dates. In general, application of nitrogen fertiliser increased leaf nitrate and leaf NRA (Tables 1 to 4) although results of all N level treatments are presented in tables, only mean values of N treatments are discussed.

Leaf nitrate content. During the post-rainy season, nitrogen application increased leaf nitrate content in all crop species (Table 1). Groundnut (nodulating) and cowpea had higher and maize had lower nitrate content at all the three sampling times. Similar results were observed during the rainy season although, in general, nitrate concentrations were lower (Table 2). Very high nitrate levels were detected in pearl millet, especially at 35 DAS.

Leaf NRA. During the post-rainy season the highest leaf NRA was observed in cowpea. Next highest were soybean, maize, Non-nod, sorghum, and nodulating groundnut in that order at 66 DAS (Table 3). Groundnut with 200 kg ha^{-1} N had only one-fourth the leaf NRA of soybean. During the 1986 rainy seeason inoculated soybean had highest NRA at 35 DAS (Table 4), followed by pearl millet, maize and cowpea. Groundnut had the lowest leaf NRA throughout the samplings.

Table 4. *Effect of nitrogen application on leaf NRA of crop species during the 1986 rainy season*

	Nitrogen applied (kg ha^{-1})			
	0	100	200	Mean
		Nitrate reductase activity (nmoles NO_2^- g^{-1} h^{-1})		
35 DAS				
1. Groundnut (Non-nod)	620 (2·73)	873 (2·92)	999 (2·95)	830 (2·87)
2. Groundnut (ICGS 11)	415 (2·58)	768 (2·86)	536 (2·69)	573 (2·71)
3. Cowpea	651 (2·77)	1127 (3·01)	964 (2·90)	914 (2·90)
4. Soybean (Uninoc.)	1462 (3·05)	4053 (3·51)	4810 (3·54)	3442 (3·37)
5. Soybean (Inoc.)	2805 (3·20)	5924 (3·58)	6273 (3·63)	5001 (3·47)
6. Maize	1608 (2·83)	1621 (3·16)	1512 (3·17)	1580 (3·05)
7. Pearl millet	1866 (2·94)	2441 (3·34)	2098 (2·25)	2135 (3·18)
S.E. ± a = (0·0949), b = (0·0581), c = (0·1644)				
56 DAS				
1. Groundnut (Non-nod)	107 (1·97)	348 (2·47)	472 (2·60)	309 (2·34)
2. Groundnut (ICGS-11)	205 (2·28)	341 (2·47)	455 (2·63)	334 (2·46)
3. Cowpea	606 (2·74)	883 (2·93)	1162 (3·04)	884 (2·90)
4. Soybean (Uninoc.)	763 (2·70)	1516 (3·08)	1287 (3·01)	1189 (2·93)
5. Soybean (Inoc.)	1004 (2·85)	1456 (3·03)	1530 (3·12)	1330 (3·00)
6. Maize	811 (2·87)	1416 (3·14)	1553 (3·16)	1260 (3·06)
7. Pearl millet	792 (2·87)	1336 (3·10)	1359 (3·03)	1162 (3·00)
S.E. ± a = (0·0335), b = (0·0219), c = (0·0580)				
70 DAS				
1 Groundnut (Non-nod)	104 (1·44)	256 (1·95)	367 (2·55)	243 (1·98)
2 Groundnut (ICGS 11)	150 (1·76)	183 (1·62)	334 (2·51)	222 (1·96)
3 Cowpea	1169 (3·03)	1175 (3·06)	1447 (3·15)	1264 (3·08)
4. Soybean (Uninoc.)	496 (2·14)	647 (2·79)	798 (2·88)	647 (2·61)
5 Sorghum (Inoc.)	635 (2·69)	990 (2·97)	1097 (3·02)	907 (2·89)
6 Maize	1095 (3·01)	948 (2·99)	1107 (3·04)	1050 (3·01)
7 Pearl millet	911 (2·95)	1325 (3·12)	1604 (3·04)	1280 (3·08)
S.E. ± a = (0·1126), b = (0·0737), c = (0·1951)				

For details see Table 1

DISCUSSION

In addition to its role as substrate, nitrate in the leaf can induce the enzyme nitrate reductase and also has a stabilising effect on the enzyme (Aslam & Oaks, 1976). Jones & Sheard (1975) concluded that only a small fraction of absorbed nitrate is actually involved in enzyme induction. Only small quantities of nitrate are needed to induce the enzyme in cell suspension cultures, and large quantities are needed to stabilise it. In the present study, we did not calculate the NO_3 in different metabolic pools and we assumed that NO_3 concentration in the leaves was an indication of the NO_3 available to the enzyme, even though most of the NO_3 is stored in the vacuole (Ferrari, Yoder & Filner, 1973).

Comparison of leaf nitrate content and leaf NRA among crop species

Within a given genotype of a crop species, increase in leaf nitrate concentration resulted in increased leaf NRA. In wheat, (*Triticum aestivum* L.) *in vivo* data showed that sources of organic carbon were adequate for NRA and that nitrate was the limiting factor for nitrate reductase *in situ* (Hageman, 1979). However, our studies did not find across the crop species a definite quantitative relationship between nitrate content in the leaf and leaf NRA. The above comparisons are made from the general trend across the samplings, but since physiological age of these crop species varies one should be cautious in comparing within a sampling date. It should be considered that other differences like in (i) exploitation by the roots of the soil profile, (ii) rates of NO_3 uptake, (iii) root and shoot nitrate reduction and preferential use of other anions to osmoregulate and to maintain leaf turgor, between species and genera exist.

One of the possible explanations for such large differences across crop species in leaf nitrate content and leaf NRA may be the differences in the affinity of different nitrate reductase for the substrate. This relationship would indicate differences in Km values (the Michaelis-Menton constant), assuming that nitrate available to the enzyme is proportional to leaf nitrate concentration. It is proposed to study the Km of the enzyme in several crop species. Hageman (1979), from the *in vivo* assay in wheat, concluded that "*in situ*, the leaf could assimilate more nitrate into grain protein, if the leaves were supplied with more nitrate, but there is no easy or economical way to maintain high levels of nitrate in the leaf blade throughout the vegetative development." The data presented here indicate that the above situation may not be true in crop species such as pearl millet and groundnut where higher nitrate accumulation does not apparently result in rapid nitrate incorporation into proteins. Shaner & Boyer (1976) suggested that, in 14 day-old maize seedlings grown in the greenhouse, nitrate flux to the leaves from the roots plays a much larger regulatory role than the leaf nitrate content in controlling the level of NRA.. However, it is not known whether this situation is true for plants grown in the field during the entire growth period. Among the legumes there are differences in nitrate accumulation and leaf NRA. Soybean appears to be a more efficient utiliser of available nitrate than groundnut. In soybean only 40 - 60% of the total plant nitrogen at harvest is accounted for by nitrogen fixation and the rest is derived from mineral N (Weber, 1966; Deibert, Bijeriego & Olson, 1979), while in groundnut most of the total plant nitrogen (70 - 90%) at harvest is derived from nitrogen fixation (Giller *et al.*, 1987; Yoneyama *et al.*, in preparation). To conclude, we observed no generalised relationship between leaf nitrate content and leaf NRA in the crop species within legumes, or those within cereals.

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