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A Re-evaluation of the Nitrate Reductase Content of the Maize Root[1]

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ABSTRACT

The standard procedure for the *in vitro* extraction of nitrate reductase from the tip region (0–2 cm) of the primary root of the maize (*Zea mays* L.) seedling indicated an activity of the enzyme approximately 5-fold higher than that obtained with an *in vivo* assay. In more mature regions of the primary root the ratio of *in vitro* to *in vivo* activity was much lower and in older seedlings was less than unity. The mature root extracts had a more labile nitrate reductase and a higher level of an inactivating enzyme. The use of phenylmethylsulphonyl fluoride in the extraction medium gave only a partial protection of the nitrate reductase from the old root samples. Casein (3%) resulted in a greatly increased yield of nitrate reductase (36-fold with one sample) and a more constant *in vitro-in vivo* activity ratio for all root samples. With casein in the extraction medium, much higher levels of nitrate reductase were recovered from the mature root zone, and the root content of this enzyme was now shown to be quite a significant proportion of the total in the maize seedling. Casein was shown to inhibit the action of the inactivating enzyme on nitrate reductase. Evidence is also presented for a nitrate reductase inactivating enzyme in the maize scutella and leaf tissues and in the roots and shoots of pea seedlings.

Several reports in the literature indicate that highest levels of root nitrate reductase are found in the apical zone and only low levels of the enzyme occur in older, mature root regions (4, 6, 12). A nitrate reductase inactivating enzyme has now been found in extracts of the mature root region of maize seedlings (13), so that estimates of nitrate reductase activity based on *in vitro* assay data alone may have been an underestimate in mature root samples.

The inactivating enzyme from the maize root has been purified 460-fold and estimated to have a mol wt of 44,000 (14). It had no effect on a range of other enzymes (13) but did seem to cause some degradation of casein (14).

A comparison of the *in vivo* and *in vitro* assay data, presented in this paper, for nitrate reductase in the maize root indicates the magnitude of the interference that could be attributable to the inactivating enzyme. Recently Schrader *et al.* (9) reported that the use of 3% casein or BSA in the extraction medium markedly improved the extraction and stability of leaf nitrate reductase. I tested casein for its protective effect on the root nitrate reductase and action on the inactivating enzyme.

MATERIAL AND METHODS

Seeds of *Zea mays* L. were supplied by the DeKalb Shand Seed Company (Tamworth, 2340, Australia). Unless stated, the hybrid variety DSC1 was used. Seedlings were grown either on 1% (w/v) agar or in pots of sand as described previously (5, 12). For the liquid culture study, the seedlings were germinated on agar and transferred at 3 days to a Perspex support on a one-tenth Hoagland's solution containing 5 mM nitrate and 0.01 μg of Mo/ml. The root medium was aerated and maintained in darkness, employing the same growth conditions as for pot studies. The root-tip sample used in all experiments was a 0 to 2 cm portion of the primary root; the mature root, where not specified, refers to the primary root without the 0 to 2 cm tip zone.

The *in vitro* extraction and assay procedure for nitrate reductase has been described (5, 12, 13). The time between extraction of the sample and commencement of the assay was approximately 30 min. The *in vivo* assay was conducted in a Thunberg tube under anaerobic conditions. The assay mixture was 0.1 M phosphate, 0.1 M KNO_3, and 5% *n*-propanol, a total volume of 5 ml being used for approximately 1 g tissue fresh weight. After an initial lag nitrite production was linear from 30 to at least 150 min with agar-grown root samples and for 30 to 90 min only with liquid culture material. The latter tend to brown more readily during the assay. Both nitrate reductase assays were conducted at 25 C.

The levels of inactivating enzyme reported here, unless stated, were estimated on a fraction precipitated by 40 to 60% saturation with $(NH_4)_2SO_4$ and tested on the nitrate reductase isolated as the 40% $(NH_4)_2SO_4$ fraction of the root tip or scutella of 3-day maize seedlings (14). The inactivating enzyme and nitrate reductase sample were incubated for 1 hr at 25 C before assay of the latter with NADH as electron donor. One unit of nitrate reductase is that amount of the enzyme which produces 1 nmole of NO_2^- hr^{-1}.

PMSF[2] was obtained from the Sigma Chemical Company and dissolved in a minimum volume of isopropyl alcohol (final concentration 2.5% when PMSF was 0.5 mM). Casein (light white soluble grade) was obtained from the British Drug Houses Ltd.

RESULTS AND DISCUSSION

The distribution of nitrate reductase and the inactivating enzyme along the primary root of 5-day maize seedlings is

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[2] Abbreviation: PMSF: phenylmethylsulfonyl fluoride.

Table I. *Distribution of Nitrate Reductase and Inactivating Enzyme along Maize Root*

The primary root of 5-day seedlings grown on agar and approximately 16 cm in length was examined. A sample of 40 seedlings was used for the *in vivo* assay and 120 for the *in vitro* studies on nitrate reductase. The extraction medium for the *in vitro* procedure was 0.05 M phosphate, pH 7.5, containing 5×10^{-4} M EDTA and 5×10^{-3} M cysteine. The inactivating enzyme was measured in the fraction of the crude extract precipitated by 40 to 60% saturation with $(NH_4)_2SO_4$.

Root Sample (Distance from tip)	Fresh Wt	Protein	Nitrate Reductase		*In Vitro/ in Vivo* Activity	*In Vitro* Stability of Nitrate Reductase[1]	Inactivating Enzyme
			In vitro	*In vivo*			
cm	*mg*	*mg g fresh wt*$^{-1}$	*nmoles* NO_2^- *produced hr*$^{-1}$ *g fresh wt*$^{-1}$				*units nitrate reductase inactivated hr*$^{-1}$ *g fresh wt*$^{-1}$
0–2	17	5.8	629	141	4.5	300	22
2–5	31	2.9	180	52	3.5	23	217
5–8	35	3.1	334	160	2.1	27	271
8–11	38	3.3	834	597	1.4	33	262
11–16	74	2.3	409	256	1.6	18	328

[1] $t_{0.5}$ min, 25 C.

shown in Table I. Lateral roots were visible in the oldest section of the roots (approximately 11–16 cm from the root tip). Both an *in vivo* and an *in vitro* assay for nitrate reductase indicated the highest level of the enzyme in the mature-root section (8–11 cm). The *in vitro* assays also showed a high level of the enzyme in the tip region (0–2 cm). In this region the ratio *in vitro-in vivo* activity was 4.5:1. This ratio decreased with distance along the root (Table I) and in the oldest section was less than 2:1. Streeter and Bossler (10), when comparing the *in vivo* and *in vitro* assay with soybean leaves, reported that the extractability of nitrate reductase decreased with the age of the plant.

The nitrate reductase in the root-tip extract was much more stable *in vitro* (25 C) than that from older sections of the root (Table I). These mature root samples had high levels of inactivating enzyme, its activity/g fresh weight increasing with age. It thus seemed likely that the *in vitro* assay procedure underestimated the level of nitrate reductase in the mature root samples. The *in vivo* assay suggests that, whereas higher levels of nitrate reductase are found in the root tip than in adjacent mature-root cells, as previously described (8, 12), even higher levels of the enzyme occur in older zones of the roots of older seedlings. This is appparent for maize seedlings grown on agar (Table I) and in liquid culture (Table III).

A similar distribution of nitrate reductase and the inactivating enzyme along the root axis was found for six maize hybrids grown for 4 days. Compared to the DSC1 variety described above, the mature-root samples of the variety XL361 had a more labile nitrate reductase ($t_{0.5}$ at 25 C, 16 min compared to 34 min) and a 2-fold higher level of the inactivating enzyme. Although the *in vivo* assay for nitrate reductase indicated a 2-fold higher level of the enzyme in the mature root of XL361 than in DSC1, this was not apparent from *in vitro* assay data.

PMSF was shown (14) to inhibit the inactivating enzyme and, when employed in the extraction medium (1 mM), prevented the inactivation of nitrate reductase from the mature root of 3-day maize seedlings. With older seedlings of the same variety (Table II), PMSF, although it did allow some increase in the recovery of nitrate reductase from the root, only partially prevented its inactivation *in vitro*. Higher levels of PMSF (up to 20 mM), which were not completely soluble, only slightly improved the recovery and stability of nitrate reductase in preparations of this older root tissue. The failure of PMSF to protect nitrate reductase in this case may suggest that other factors, in addition to the inactivating enzyme, are responsible for the *in vitro* loss of nitrate reductase activity in extracts of such mature root tissue. It has recently been demonstrated that the inclusion of 3% w/v casein in the extraction medium markedly increased the yield and stability of nitrate reductase from leaves (9). A similar effect was found with the maize root (Table II). In this case, a 16-fold higher level of nitrate reductase was found and, again as reported for leaves, the enzyme was quite stable for a few hours at 0 C. Loss of activity occurred during storage for 24 hr at 0 C (58%) and −15 C (36%). The half-life at 25 C was 70 min. The use of PMSF with casein did not have any additional effect on the recovery or stability of the root nitrate reductase. Omission of cysteine from the casein-containing extraction medium did not alter the initial level of nitrate reductase but did result in its more rapid decay *in vitro*, with a half-life of 20 min at 25 C.

The effectiveness of casein compared to PMSF for the extraction of nitrate reductase is further demonstrated in Table III. The data from the improved *in vitro* assay method with

Table II. *Influence of PMSF and Casein on Extraction of Nitrate Reductase from Maize Root*

A mature root sample with visible lateral roots was harvested from 12-day samples grown on liquid culture. The extraction medium was 0.05 M phosphate, pH 7.5, containing 5×10^{-4} M EDTA and 5×10^{-3} M cysteine.

Addition to Extraction Medium	Nitrate Reductase		
	Initial	2 hr at 0 C	24 hr at 0 C
	μmoles NO_2^- *produced hr*$^{-1}$ *g fresh wt*$^{-1}$		
None	0.46	0.04	
PMSF (1 mM)	1.41	0.51	
Casein (3%, w/v)	7.47	7.50	3.10

Table III. *Effectiveness of Casein in Extraction Medium for Nitrate Reductase in Relation to Age of Root Sample*

Root samples were taken from 8-day seedlings grown in liquid culture. The extraction medium was as in Table II. The mature root samples were sectioned into 3-cm segments for the *in vivo* assay. The *in vitro* activity in the presence of casein is used in the estimation of the *in vitro-in vivo* ratio.

Root Sample	Addition to Extraction Medium	Nitrate Reductase		
		In vitro	*In vivo*	*In vitro-in vivo* activity
		nmoles NO_2^- *hr*$^{-1}$ *g fresh wt*$^{-1}$		*ratio*
Root tip (0–2 cm)	None	3045		
	1 mM PMSF	3385	510	7.0
	Casein (3%, w/v)	3595		
Mature root (approx. 2–14 cm)	None	288		
	1 mM PMSF	1224	936	6.5
	Casein (3%, w/v)	6060		
Mature root with visible laterals (approx. 14–26 cm)	None	88		
	1 mM PMSF	404	586	5.5
	Casein (3%, w/v)	3200		

Table IV. *Influence of Casein in Extraction Medium on Apparent Distribution of Nitrate Reductase in Maize Seedling*

All samples were extracted with 0.05 M phosphate, pH 7.5, with 5×10^{-4} M EDTA. Casein 3% w/v was added as indicated, with 5 mM cysteine for root samples and 20 mM cysteine for axis and shoot samples. The ratio extraction medium to sample fresh weight was 4:1 for root and axis samples and 10:1 for scutella and leaf samples.

Age of Seedling	Organ	Fresh Weight	Nitrate Reductase	
			No casein	Plus casein
days		*mg*	*nmoles NO_2^- produced hr^{-1} $organ^{-1}$*	
4	Root	90	12	92
	Scutellum	69	98	269
7	Root	446	69	1,802
	Scutellum	76	87	650
	Axis	245	279	458
	Leaf	208	1,169	2,050
21	Root	5,550		8,269
	Axis	4,700		4,324
	Leaf	5,300		24,592

casein are also compared with those estimated by the *in vivo* procedure. With the root tip, only a slight enhancement in the recovery of nitrate reductase is found with either casein or PMSF. In both mature root samples there is an approximately 4-fold increase in yield of nitrate reductase attributable to PMSF but 21-fold and 36-fold increases attributable to casein in the 2 to 14 and 14 to 26 cm root samples, respectively. The ratio of the *in vitro* activity with casein in the extraction medium to the *in vivo* activity was now more comparable for each root sample, although decreasing slightly in the older root samples.

Before the identification of an inactivating enzyme in the maize root and the finding that casein markedly improved the recovery of nitrate reductase (results above and ref. 9) it had been concluded for the maize seedling (12) that the root contained only a minor portion of the total activity (<15%) before leaf expansion and 1 to 2% in older seedlings. It now seemed necessary to reexamine the distribution of nitrate reductase in the maize seedling. The results for 4-, 7-, and 21-day maize seedlings extracted in the presence of casein and 4- and 7-day seedlings extracted in its absence are shown in Table IV. In these young maize plants the most marked improvement in the recovery of nitrate reductase was found in the root, a nearly 8-fold increase in activity at 4 days and a 26-fold increase in activity in 7-day seedlings in the presence of casein. A considerable increase was also found in the scutella nitrate reductase, 2.7- and 7.5-fold on days 4 and 7, respectively. The effect of casein on the recovery of nitrate reductase from the maize shoot, *i.e.*, leaf and axis sample, was less than 2-fold in these young maize plants. A more marked effect of casein was found with the leaves of more mature maize plants (9). Negligible losses of nitrate reductase activity occurred in all casein-containing extracts stored for 2 hr at 0 C. A reliable comparison of nitrate reductase activity in different plant parts (Table IV) can thus be made with casein-containing extracts.

When casein is included in the extraction medium, a much higher level of nitrate reductase is found in the root than in the absence of casein: 36% of the total compared to 4% at 7 days (Table IV). At 21 days, 22% of the activity found in the maize plant is still in the root. However, before leaf expansion, the scutella is still the major location of nitrate reductase.

The level of inactivating enzyme in the most mature root sample at 5 days (Table I) was 328 units of nitrate reductase inactivated hr^{-1} g fresh $weight^{-1}$. In 23-day maize plants the total root activity of inactivating enzyme (same unit as above) was 1151, while that for an apparently similar inactivating enzyme in a sample of the older leaves was 689. In 19-day plants of the field pea (*Pisum arvense* L.), grown under the same conditions as the maize, the level of an apparent inactivating enzyme in the root was 283 units and in the shoot, 182 units. Again, the level of inactivating enzyme increased with seedling age. A nitrate reductase inactivating enzyme was also found in the maize scutella, its level increasing markedly after 4 days, apparently resulting in a corresponding increase in the lability of the nitrate reductase in the scutella extract. In a scutella sample of 6-day seedlings, the activity of the inactivating enzyme was 278 units of nitrate reductase inactivated hr^{-1} g fresh $weight^{-1}$. The activities reported above for inactivating enzyme were measured on the cell fraction precipitated by 40 to 60% saturation with $(NH_4)_2SO_4$ and tested on a sample of 3-day maize scutella nitrate reductase. In all cases, when samples containing the inactivating enzyme were incubated at 70 C for 10 min, there was a complete loss of activity. Boiling the samples for the same period resulted in the appearance of some nonprotein factor which did cause some inactivation of nitrate reductase when incubated with it at 25 C.

I have reported (14) that the fraction of the mature root containing a 460-fold purified sample of the inactivating enzyme degraded casein to trichloroacetic acid-soluble products. Figure 1 shows that casein markedly inhibits the action of the inactivating enzyme, with complete inhibition occurring with 1.5% casein in the assay. It seems likely that at least part of the action of casein in stabilizing nitrate reductase is to prevent the action of the inactivating enzyme. In all cases where the nitrate reductase inactivating enzyme was detected,

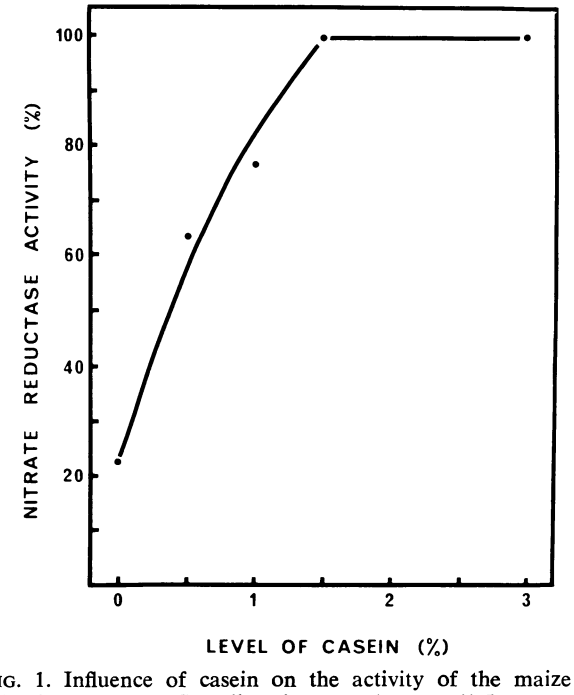

FIG. 1. Influence of casein on the activity of the maize root inactivating enzyme. Scutella nitrate reductase (1.5 mg protein precipitated by 40% saturation with $(NH_4)_2SO_4$) was incubated for 1.5 hr with the inactivating enzyme (40 μg protein, CM32 fraction in ref. 14) and casein as shown. The total volume was 1.8 ml. Nitrate reductase activity remaining is shown as a percentage of that in the scutella sample incubated with the same level of casein but with inactivating enzyme omitted.

the inclusion of casein in the extraction medium resulted in an increased recovery of nitrate reductase.

When casein is used in the extraction medium for nitrate reductase in the maize root, the *in vitro* assay for nitrate reductase indicates a consistently higher level of activity (5.5- to 7-fold) than the *in vivo* procedure. A similar relationship between *in vitro-in vivo* nitrate reductase activity has been reported by others (1, 3).

With the *in vivo* assay procedure used in the present study on the maize root, it was found that the optimal level of *n*-propanol and nitrate for both root-tip and mature root samples was similar to that employed with other plant tissues (2, 7). Dimethylsulfoxide was found to be an ineffective substitute for *n*-propanol. The following compounds were tested and found to have no significant effect on the *in vivo* assay: 5 mM antimycin A, 6 mM glyceraldehyde-3-P, and 6 mM fructose-1,6 diP (3), and 20 mM malate (11). When the root samples were chopped into mm segments, there was a 50% lower activity.

If the *in vivo* assay for nitrate reductase indicates the true tissue activity for nitrate reductase, the enzyme must either be partially inactive *in vivo* or operating at a lower turnover rate than in the *in vitro* assay. The former explanation was suggested by Ferrari and Varner (1), who used the incorporation of $KN^{15}O_3$ to confirm the relatively low *in vivo* rate of nitrate reduction. Because nitrate reductase is rapidly degraded *in vivo* when nitrate is withdrawn (5), it would seem unlikely that the enzyme would exist *in vivo* in a partially inactive form. Thus, if the *in vivo* and *in vitro* assay data can be reliably compared, it seems that nitrate reductase in the plant cell reduces nitrate at a consistently lower rate than can be demonstrated when the enzyme is assayed *in vitro*.

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