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Inhibition of nitrate reductase and ATPase activities in *Zea mays* roots by tungsten and N, N′-dicyclohexylcarbodiimide

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Abstract

The activity of soluble and membrane-bound ATPase obtained from corn roots was *in vivo* markedly inhibited by N,N' -dicyclohexylcarbodiimide (DCCD) and WO_4^{2-} ions. DCCD (2.5×10^{-5} M) added to the nutrient solution strongly decreased *in vivo* nitrate reductase (NR) activity after 12-h growth of plants while it had no effect in experiments *in vitro* on NR activity. Tungsten in a concentration of 10^{-4} M completely blocked NR activity after 24 h. In the above used concentrations neither DCCD nor WO_4^{2-} inhibited completely NO_3^- absorption by corn roots. The results suggest that there must exist in corn roots another or an additional mechanism of NO_3^- assimilation apart from of that proposed by Butz and Jackson (1977).

INTRODUCTION

Many workers have shown that tungsten is a powerful inhibitor of plant nitrate reductase (Heimer et al. 1969, Wray and Filner 1970, Rao and Rains 1976, Buczek et al. 1980). It probably inhibits incorporation of molybdenum into the nitrate reductase protein (Notton et al. 1974) and thus produces an inactive nitrate reductase analogue. Tungsten also considerably decreases the NO_3^- ions uptake (Buczek et al. 1980), but in short term experiments it has no effect on NO_3^- absorption (Rao and Rains 1976). It has been also found that tungsten in concentrations which inhibit nitrate reductase activity decreases *in vivo* the activity of some plant ATPases (Buczek 1980). DCCD is an inhibitor of membrane-bound ATPase (Leonard and Hodges 1973) of plant roots, but does not inhibit the solubilized form of the enzyme (Abrams and Baron 1970) and is not a potent inhibitor of mitochondrial ATPase, (Leonard and Hotchkiss

1976). The effect of DCCD on the NR activity and NO_3^- absorption was not investigated. However, there is some evidence that the activity of NR reductase is connected with the plasmalemma-bound ATPase. Butz and Jackson (1977) have proposed a model for the uptake and reduction of NO_3^- by plant tissues. This model involves the subunit structure of the enzyme and the participation of membrane-bound ATPase. It seems that DCCD should supress NR activity if the hypothesis of Butz and Jackson is corect.

This paper describes the *in vitro* and *in vivo* effect of tungsten and DCCD on some corn roots ATPase and NR activity and the relationship between NO_3^- absorption and the activity of both enzymes.

MATERIAL AND METHODS

Seeds of corn (*Zea mays* L. *var.* Kb-270) were prepared and treated as described in the preceeding paper (Buczek et al. 1981), after 5 days the seedlings were transferred to the nitrate nutrient solution (Buczek et al. 1981). The plants were grown in this solution for 4 days under a light-dark period (16 h light, 5000 lux) or for 1 day under continuous light.

Extracts of NR were prepared from fresh leaves and roots according to the methods described previously (Buczek 1976), with the exception that 3% caseine was used in the extraction medium (Schrader et al. 1974). NR activity was assayed in the supernatant following 18 000 × g centrifugation of the homogenate, by measuring NADH-dependent production of NO_2^- (Hageman and Flesher 1960). The NR activity was calculated as nmoles of NO_2^- formed per 1 mg of protein per hour or nmoles of NO_2^- produced per gram fresh weight per hour.

Extracts of ATPase were prepared from fresh roots according to the procedure described in the preceeding paper (Buczek 1980). The filtrate was centrifuged at 1500 × g for 10 min. The supernatant from 1500 × g centrifugation was centrifuged at 18 000 × g for 15 minutes. The pellet from the 18 000 × g run is referred to as fraction II (mitochondria-enriched fraction) and the supernatant as fraction III. ATPase activity was measured at 37°C in a 3 cm^3 volume containing 3 mM ATP (Tris salt), 100 mM Tris-maleate (pH 6.5), 25 mM KCl, 2 mM $MgCl_2$, 250 mM sucrose (only for fraction II) and 0.2-0.4 cm^3 of enzyme extracts contaning approximately 0.05-0.1 mg of protein. The reaction was terminated by addition of trichloroacetic acid, and after removal of protein the inorganic phosphate released from ATP was determined by the Fiske and Subbarow (1925) procedure. Substrate blanks were determined and subtracted to calculate all enzyme

activities. The ATPase activity was expressed in μmoles P_i liberated per 1 mg of protein per hour or per gram of fresh weight of tissue.

NADH-cytochrome *c* reductase and cytochrome *c* oxidase were assayed spectrophotometrically according to Hodges and Leonard (1974) and Leonard et al. (1973). The reaction was initiated by the addition of substrate, and absorbance was recorded at 15-sec. intervals for 2 min. with a VSU-2P spectrophotometr. The amount (nmoles) of reduced cytochrome *c* was read from the equation: $\triangle E \times min^{-1} \times 79.0$ at 550 nm. The enzymes activity was expressed in nmoles of cytochrome c $\times$ min^{-1} $\times$ mg^{-1} protein.

Soluble protein was determined according to Lowry et al. (1951). Absorption of nitrate was assayed by measuring the loss of NO_3^- from the medium by spectrophotometric methods (Cawse 1967).

In Figures 1-4 each point represents an average of two experiments, each with four or five replicates. Differences which exceeded twice the standard error of the differences (SE of differences $=\sqrt{(S_1)^2+(S_2)^2}$) were deemed to be significant at a 5 per cent level.

ATP and NADH and cytochrome *c* were purchased from Sigma Chemical Company, Tris from Calbiochem-Behring Corp., other reagent grade chemicals were obtained from POCH-Gliwice.

RESULTS

Table 1 shows the activity of fraction II and fraction III ATPase as well as the activity of NR obtained from 5-day-old corn roots of seedlings growing 12 h in nitrate nutrient solution. DCCD markedly inhibited fraction II ATPase activity when added to the reaction mixture, while it slightly inhibited the activity of fraction III ATPase. DCCD *in vitro* experiments has no or only a slight stimulatory effect on NR activity. The addition of WO_4^{2-} ions to the incubation mixture caused a significant inhibition of both ATPase fractions and the NR activity.

The highest specific activity of cytochrome *c* oxidase (Table 2) a marker for mitochondria (Hodges et al. 1972) occurred in fraction II. It is interesting that this fraction did not show any nitrate reductase activity. The total NR activity was connected with fraction III preparations. Fraction III exhibited also the highest specific activity of NADH-cytochrome *c* reductase which has been ascribed to the plasma membrane in plants (Matile 1968).

Figure 1 shows that addition of DCCD in a concentration of 2.5×10^{-5} M to the nitrate nutrient medium caused a significant inhibition of fraction III ATPase activity and markedly decreased the activity of

Table 1

Effect of DCCD and tungsten in *in vitro* experiments on the activity of ATPase and nitrate reductase extracted from corn roots

Inhibitor (M)	ATPase		Nitrate reductase
	fraction II	fraction III	nmoles NO_2^-
	μmoles P_i mg^{-1} prot. h^{-1}		mg^{-1} prot. h^{-1}
Control	10.2	105.6	166.2
DCCD 3×10^{-5}	9.2	102.4	154.6
3×10^{-4}	7.5	103.5	172.8
5×10^{-4}	4.1	102.4	187.8
10^{-3}	4.6	101.4	199.4
5×10^{-3}	3.3	103.5	172.8
Na_2WO_4 10^{-4}	5.1	31.7	127.9
10^{-3}	4.3	23.2	76.4

The 5-day-old seedlings were grown for 12 h in nitrate nutrient solution. Then the roots were harvested, homogenized and enzyme activity has assayed in particular subcellular fractions (see Material and Methods). Both inhibitors were added to the incubation medium. Each value represents the average for 3 replicates.

Table 2

Distribution of marker enzymes and nitrate reductase activities in corn roots subcellular fractions

Fraction	Specific activity (nmoles/mg protein/min)		
	cytochrome *c* oxidase	NADH-cytochrome *c* reductase	nitrate reductase
II	324.9	96.7	0.0
III	2.4	294.3	1.4

fraction II ATPase from corn roots. The effect of WO_4^{2-} ions on the activity of both ATPase fractions was similar (Fig. 2), but this inhibitor reduced more effectively the activity of fraction III ATPase in roots growing 24 h in nitrate medium with WO_4^{2-}.

Figure 3 shows the effect of DCCD and WO_4^{2-} ions on the time course of NR activity in roots. DCCD (2.5×10^{-5} M) added to the nutrient solution strongly inhibited NR activity after 12-h growth of plants. This inhibitor exerted similar effect on NR in leaves. Tungsten in a concentration of 10^{-4} M has a similar but more powerful effect on NR activity both in leaves and in roots. Both inhibitory compounds affect the absorption of NO_3^- too (Fig. 4), but their effect was different, DCCD decreased the uptake of NO_3^- rather quickly, while WO_4^{2-} reduced the NO_3^- absorption after a lag phase of about 12 h.

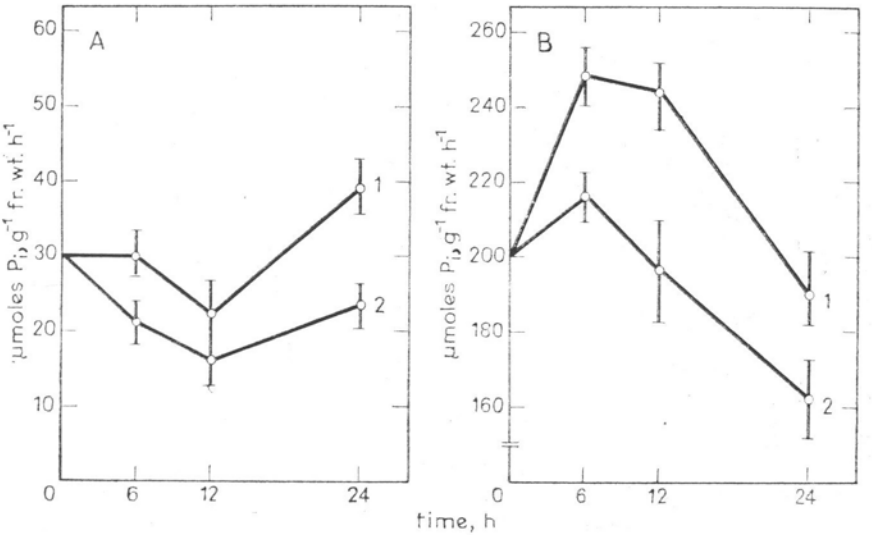

Fig. 1. Effect of DCCD on ATPase activity extracted from corn roots
Five-day-old seedlings were grown 24 h in nitrate nutrient solution with DCCD. At designated times the roots were harvested and enzymic activities were assayed. The bars indicate the 2 SE limits. A — fraction II; B — fraction III; 1 — control; 2 — DCCD 2.5×10^{-5}M

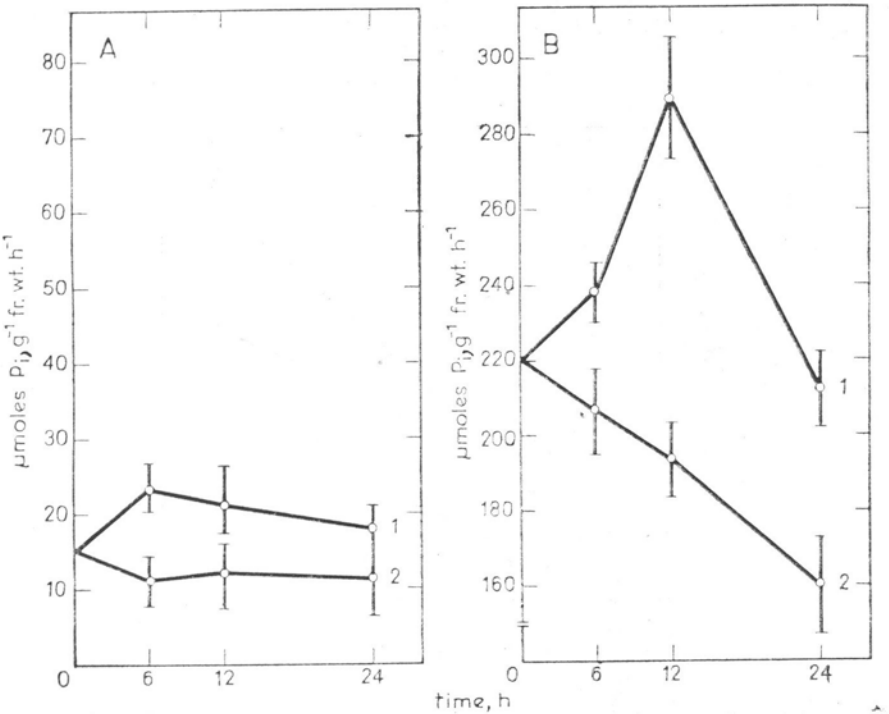

Fig. 2. Effect of tungsten on ATPase activity extracted from corn roots
For explanation see Fig. 1. 1 — control; 2 — Na_2WO_4 10^{-4} M

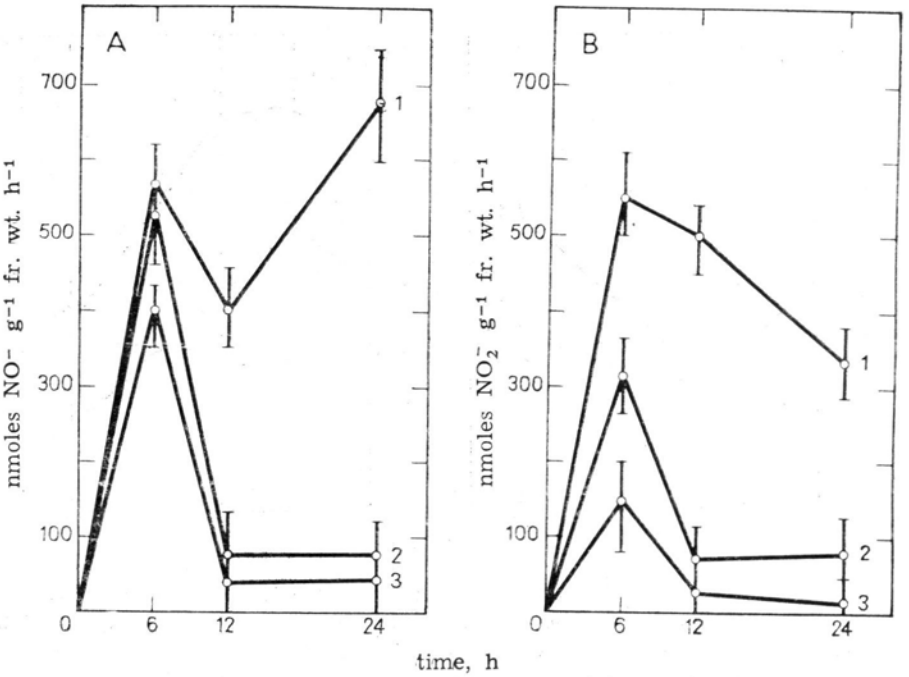

Fig. 3. Time course of nitrate reductase activity in tops (A) and roots (B) as affected by DCCD and tungsten
For explanation see Fig. 1. 1 — control; 2 — DCCD 2.5×10^{-5} M; 3 — Na_2WO_4 10^{-4} M

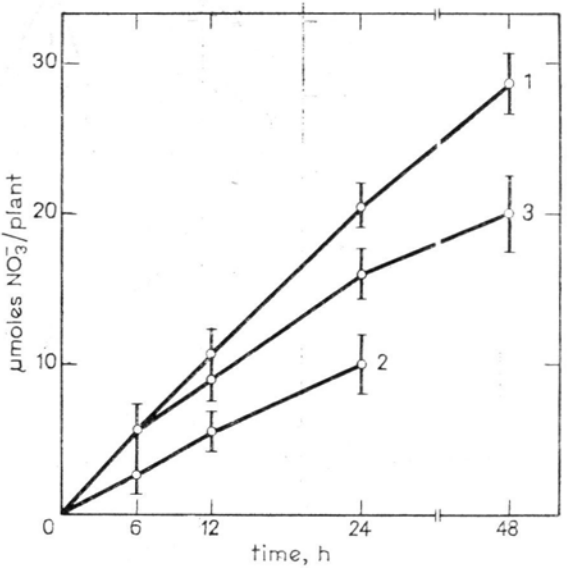

Fig. 4. Effect of DCCD and tungsten on NO_3^- absorption by corn seedlings
For explanation see Fig. 1. 1 — control; 2 — DCCD 2.5×10^{-5} M; 3 — Na_2WO_4 10^{-4} M

Table 3

Effect of tungsten added to the nutrient solution on ATPase and NR activities

Nutrient solution	Period of growth (days)	ATPase activity		Nitrate reductase		Absorption of nitrates (μmoles NO_3^- plant^{-1} 48 h^{-1})
		fraction II	fraction III	leaves	roots	
		μmoles P_i g^{-1} fr. wt. h^{-1}		nmoles NO_2 g^{-1} fr. wt. h^{-1}		
NO_3^-	2	12.8	164.2	316.6	16.6	49.5
$NO_3^-+WO_4^{2-}$		10.4 (81)	122.2 (74)	40.0(13)	0.0	39.0 (79)
NO_3^-	4	15.1	151.1	400.0	16.6	46.3
$NO_3^-+WO_4^{2-}$		13.9 (92)	102.0 (67)	166.6 (42)	0.0	25.4 (54)

Five day-old corn seedlings were grown on nitrate nutrient solution with or without tungsten. A standard assay were used for enzyme measurements. Each value represents the average for 5 replicates. Relative values in brackets.

From the data presented in Table 3 it may be read that the presence of WO_4^{2-} ions in the nutrient solution for a time longer than 24 h, leads to a full decay of NR activity in corn roots and to a marked reduction of NR activity in leaves. The absorption of NO_3^- was slightly decreased by tungsten after a 2-day growth of plants, but after 4 days, the uptake was reduced twice as compared with the control plants. In turn, tungsten markedly inhibited fraction III ATPase activity while WO_4^{2-} ion had a slight inhibitory effect on fraction II ATPase. DCCD has also been tested in similar experiments, but it killed the plants after 2 days.

DISCUSSION

A previous paper (Buczek et al. 1980) showed that NR activity from cucumber roots was fully inhibited after 24-h growth of plants in nitrate nutrient solution with addition of 10^{-4} M WO_4^{2-}, while at the same time the uptake of NO_3^- was reduced by one half only as compared with control plants. Tungsten added to the nutrient solution inhibited fully after 24 h also the NR activity in corn roots, but the uptake of NO_3^- was decreased with a lag phase of about 12 h after NR induction. A decrease of NO_3^- absorption by about 50 per cent was reached after 4 days growth of plants with tungsten. Like in the preceeding paper (Buczek 1980) WO_4^{2-} ions inhibited the activity of fraction III ATPase extracted from corn roots. This may show the relationship of nitrate reduction and some root ATPase, associated mainly with the cytosol fraction of tissues.

According to the hypothesis of Butz and Jackson (1977), between NO_3^- reduction and absorption there exists a direct relationship, viz. reduction ond transport of NO_3^- are brought about by the same enzyme complex, nitrate reductase, closely associated with ATPase and located on the plasma or chloroplast membranes. However, our experiments with corn seedlings do not confirm unresorvedly this hypothesis, since WO_4^{2-}, an inhibitor of NR activity (Heimer et al. 1969) and inhibitor of some root ATPase (Buczek 1980), does not inhibit at the same rate the NR activity and NO_3^- uptake. However, our experiments did not excluded the participation of ATPase in the metabolism of NO_3^- assimilation by corn roots, since tungsten inhibited both NR and fraction III ATPase activities.

This supposition was confirmed by our tests with DCCD, an inhibitor of membrane-boud ATPase (Leonard and Hodges 1973). The experiments showed that DCCD inhibited *in vivo* not only fraction III ATPase, but also NR activity in corn roots was strongly reduced if DCCD was added to the nitrate nutrient solution in a 2.5×10^{-5} M concentration. The fact that DCCD does not inhibit *in vitro* NR activity may suggest an indirect effect of this substance on NR activity. Since DCCD inhibits the plasma-membrane bound ATPase (Leonard and Hotchkiss 1976) in roots and since DCCD decreases markedly in our experiments the uptake of NO_3^- ions, it is not excluded that DCCD inhibits *in vivo* NR activity by reducing the processes associated with the active NO_3^- transport to the root cells. However, DCCD did not decrease completely nitrate uptake, whereas NR activity was practically fully inhibited. This fact suggests that in plant roots there must exist an another mechanism of NO_3^- absorption (Buczek 1980), apart from that proposed by Butz and Jackson (1977).

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Hamowanie aktywności reduktazy azotanowej (NR) i ATPazy w korzeniach Zea mays przez wanad (Na_2WO_4) i N,N'-dwucykloheksylokarbodwuimid (DCCD)

Streszczenie

Badano wpływ Na_2WO_4 i DCCD na aktywność NR i ATPaz w korzeniach kukurydzy oraz wpływ tych inhibitorów na pobieranie NO_3^-. Obydwie substancje w doświadczeniach *in vitro* hamowały aktywność ATPazy frakcji II i frakcji III

wyodrębnionych z korzeni kukurydzy. DCCD w doświadczeniach *in vitro* nie miał wpływu na aktywność NR natomiast WO_4^{2-} wyraźnie hamował aktywność enzymu. DCCD dodany do pożywki w której rosły rośliny, w stężeniu $2{,}5 \times 10^{-5}$ M silnie obniżył aktywność NR, podczas gdy WO_4^{2-} w stężeniu 10^{-4} M zahamował całkowicie aktywność NR w korzeniach. Obydwa inhibitory dodane do pożywki nie zahamowały całkowicie pobierania NO_3^-. Wyniki sugerują, że w korzeniach kukurydzy musi występować dodatkowy mechanizm asymilacji NO_3^-, inny od proponowanego przez Butz i Jacksona (1977).