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Reduction of nitrates in *Cucumis sativus* L. seedlings

I. Influence of tungsten and vanadium on absorption and reduction of nitrates

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Abstract

Investigations were conducted on the influence of tungsten (Na_2WO_4) and meta-vanadate ($NaVO_3$) on uptake and reduction of nitrates by cucumber seedlings. Tungsten and vanadium almost completely inhibited nitrate reductase activity (NR) after treating the plants for 24 hours with nitrates in the presence of 10^{-4} M WO_4^{-2} and 10^{-3} M VO_3^-. Uptake of NO_3^- in the presence of WO_4^{-2} in this same period of time was lowered by about 50%, while in the presence of VO_3^- by seventy percent. The ratio of NO_3^- absorbed to that reducted in control plants was around 3, whereas in the presence of WO_4^{-2} and VO_3^- 9 and 8 respectively. The effect of NR inhibiting activity by WO_4^{-2} and VO_3^- was significant but somewhat weaker if both inhibitors were applied to the plants 24 hours after formation of the NR system, whereas NO_3^- uptake was subject to a slight lowering. The above data suggest that WO_4^{-2} and VO_3^- after a 24 hour application of these inhibitors to plants, primary inhibit biosynthesis and activity of NR and to a lesser extent effect NO_3^- absorption. On the basis of results reached the Butz and Jackson (1977) hypothesis concerning the model of NO_3^- uptake and reduction by plant tissues is discussed.

INTRODUCTION

Tungsten is a known inhibitor of nitrate reductase in higher plants (Heimer et al., 1969; Wray, Filner, 1970; Rao, Rains, 1976). Tungsten is generally supposed to inhibit NR biosynthesis preventing incorporation of molybdenum into subunit structure of enzyme (Wray, Filner, 1970; Vega et al., 1971; Notton et al., 1974), or by effecting structural changes of the whole enzyme (Heimer et al., 1969). In short term experiments tungsten does not influence uptake of nitrates (Heimer et al., 1969; Rao, Rains, 1976). Vanadium salts in higher plants show a similar effect on NR activity as the influence of tungsten (Buczek, 1973). The effect of vanadium refers to inhibiton of NR activity both *in vivo* as well as *in vitro*, whereas its influence on NO_3^- uptake is not known.

The inductive enzyme nitrate reductase, according to the data of Ritenour et al. (1976), is localized in the cytoplasma and a continuous influx of NO_3^- ions is indispensable for its functioning (Beevers, Hageman, 1969; Hewitt, 1975). Buts and Jackson (1977) recently put the hypothesis forward that NR is linked with plasmatic and chloroplast membranes, fulfilling the function of reductase and at the same time that of carrier in the absorption of NO_3^-. In accordance to the hypothesis of the above mentioned authors, reduction of NO_3^- takes place only during the transport of nitrates.

It appears that since NR induction and activity are linked with the uptake of NO_3^-, use of certain NR inhibitors such as tungsten and vanadium renders it possible to determine a certain correlations between these processes. This aspect was the subject of the present study with consideration being given to the effect of WO_4^{-2} and VO_3^- present in a nutrient medium on uptake and reduction of nitrates on cucumber seedlings. The studies likewise included the correlation between NR activity in cotyledons and roots, and uptake of nitrates in the presence of inhibitors.

MATERIAL AND METHODS

Plant material

Five day cucumber (*Cucumis sativus* L.) seedlings of the Monastyrski variety were prepared and cultivated as described in a previous study (Buczek, 1979). After three days of growth of the seedlings on an initial nutrient medium consisting of 0.89 mM K_2SO_4; 0.22 mM $Ca(H_2PO_4)_2$; 0.37 mM $MgSO_4$ with an addition of microelements (Buczek, 1979), they were placed in an induction nitrate nutrient containing the same macro- and microelements with the exception of K_2SO_4, which was substituted by 3.75 mM KNO_3 and 1.25 $NaNO_3$. The pH of the induction nutrient was brought to 5.5. The seedlings developed in a photothermostat under constant light (6.83 W/m²) at 27°C and in constant air humidity (48 %). The induction nutrient was exchanged after 24 hours if the experiment lasted more than 48 hours. Tungsten as $Na_2WO_4 . 2H_2O$ and vanadium as $NaVO_3 \cdot H_2O$ were added into nutrient in amounts as indicated for particular experiments.

Analytical method

Nitrate reductase extracts were prepared from fresh cotyledons and roots according to the procedure described in a previous study (Buczek, 1976). NR activity was determined by measuring NADH-dependent production of NO_2^- (Hageman, Flesher, 1960). Enzyme activity was expressed in nmoles of NO_2^- produced in one hour per 100 mg of fresh tissue matter.

The amount of NO_3^- taken up was determined by measuring the loss of nitrates from the nutrient solution by the phenoldisulphonic acid method (Johnson, Ulrich,

1950). Cotyledons, hypocotyls and roots dried at 70°C were used for analysis of NO_3^- accumulated. Reduced nitrogen was computed from the difference between NO_3^- taken up and accumulated. Tissues were extracted with 10 ml of water at 90°C for three hours and then 10 minutes in a boiling water bath. NO_3^- was determined in the filtrate as above.

The reagents used were of analytical grade and were supplied by POCH (Gliwice), NADH from the firm Siga Chemical Co.

The results are the averages of 4 to 6 repetitions. Each experiment was repeated three times. Statistical calculations were carried out by means of the method presented in the previous study (Buczek, 1969).

RESULTS

Table 1 contains the results of the influence of tungsten and vanadium on NR activity and uptake of nitrates by cucumber seedlings. The plants grew for 24 hours in an induction nutrient containing inhibitors and followed by determining NR activity and uptake of NO_3^-. WO_4^{-2} in concentration of 10^{-3} and 10^{-4} completely inhibited NR activity, while uptake of nitrates was lowered by one half as compared to the controls. Although VO_3^- in concentrations of 10^{-3} M and 10^{-4} M inhibited NO_3^- uptake somewhat more effectively than tungsten, NR activity was fully inhibited only in the presence of 10^{-3} M $NaVO_3$. Hence in the further studies 10^{-4} M Na_2WO_4 and 10^{-3} M $NaVO_3$ were used. As can be noted from the data presented in Table 1 the inhibitors did not effect dry matter increment in the plants.

Table 1

Effect of tungstate and metavanadate concentrations on dry weight, aborption of NO_3^- and NR activity

Treatment mM		Dry mass mg per 20 plants	Uptake µmoles NO_3^- absorbed		Nitrate reductase activity nmoles $NO_2^- \cdot 100\ mg^{-1}$ fr. wt. $\cdot\ hr^{-1}$	
			per plant	per g dr. wt.	cotyledons	roots
Control		462	19.3	833.7	500.0	20.8
Na_2WO_4	10^{-5}	422	13.1	618.0	133.3	0
	10^{-4}	458	10.1	443.0	29.2	0
	10^{-3}	461	10.4	452.5	5.6	0
$NaVO_3$	10^{-5}	472	13.5	533.6	416.7	12.0
	10^{-4}	472	8.3	358.5	295.8	10.1
	10^{-3}	442	8.5	383.7	6.2	0

NR activity and uptake of NO_3^- were measured after 24 hr growth of seedlings in nitrate (induction) medium. Each value represents the average for 5 replications

The investigations on the influence of tungsten and vanadium on absorption of NO_3^- and NR activity were further continued by placing the plants first in an induction nutrient containing WO_4^{-2} or VO_3^- for 24 hours, and then for a further

Table 2

Effect of 10^{-4} M WO_4^{-2} and 10^{-3} M VO_3^- on NO_3^- absorption and nitrate reductase activity in cucumber seedlings

Pretreatment	NO_3^- absorbed[1]	NR activity[2]		Treatment	NO_3^- absorbed[1]	NR activity[2]		Total NO_3^- absorbed
		cotyledons	roots			cotyledons	roots	
NO_3^-	827	379	7	NO_3^-	1191	550	17	2018
				$NO_3^-+WO_4^{-2}$	767 (64)	185 (34)	5 (29)	1594 (79)
				$NO_3^-+VO_3^-$	893 (75)	97 (17)	5 (29)	1720 (85)
$NO_3^-+WO_4^{-2}$	438 (52)*	33 (9)	0	NO_3^-	567 (48)	220 (40)	0	1005 (50)
				$NO_3^-+WO_4^{-2}$	450 (38)	82 (15)	0	888 (44)
$NO_3^-+VO_3^-$				NO_3^-	715 (60)	324 (59)	9 (53)	980 (48)
				$NO_3^-+VO_3^-$	488 (41)	221 (41)	0	733 (37)

* Values in brackets reflect the effect of the treatment in relation to the control

Absorption[1] — μmoles NO_3^- absorbed per g dry wt. per 24 hr

NR activity[2] — nmoles NO_2^- formed per 100 mg fresh wt. per hr

24 hours in the same nutrient with or without an addition of WO_4^{-2} and VO_3^-. The results presented in Table 2 show that constant presence of WO_4^{-2} or VO_3^- in the induction nutrient completely inhibited NR activity after 24 hours and NO_3^- uptake by 50 and 70 percent respectively. After 48 hours absorption of NO_3^- in the presence of WO_4^{-2} did not change in comparison to the first 24 hours of reaction of the inhibitors, on the other hand a twofold increase was noted in the presence of VO_3^-. However as compared to controls the inhibitors significantly lowered uptake of nitrates (after 48 hours). NR activity after 48 hours of reaction of the inhibitors was indeed significantly inhibited as compared to the controls, but in comparison to activity in the first day of treatment it increased distinctly in the cotyledons, especially in the presence of VO_3^-. By adding WO_4^{-2} or VO_3^- to the nutrient only after 24 hours after formation of the NR system, both WO_4^{-2} and VO_3^- lowered NO_3^- uptake by only relatively little (by about 30 percent), but distinctly decreased NR activity in both types of tissue. Lack of WO_4^{-2} in the nutrient after previous 24 hour treatment of plants with this inhibitor in the presence of NO_3^- only slightly effected changes in the uptake of nitrates, but NR activity was distinctly inhibited. However removal of VO_3^- from the induction nutrient had a distinct influence on increasing NO_3^- uptake and NR activity. Neverthelass both inhibitors reduced the level of enzyme activity as compared to NR activity of the control plants.

It hence appears that the effect of tungsten and vanadium on assimilation of nitrates is to some extent different and depends distinctly on the already formed NR system. The consecutive experiment, the results of which are presented in Table 3, confirms the above mentioned assumptions.

Table 3

Effect of WO_4^{-2} and VO_3^- pretreatment on NO_3^- absorption and nitrate reductase activity

Treatment		NO_3^- absorbed µmoles $NO_3^- \cdot g^{-1}$ dry wt.	Nitrate reductase activity nmoles $NO_2^- \cdot 100\,mg^{-1}$ fr. wt. hr^{-1}	
Pretreatment	Absorption solution		cotyledons	roots
	NO_3^-	1248 a	792 a	89 a
VO_3^-	NO_3^-	661 b (53)	324 b (41)	57 b (64)
	$NO_3^- + VO_3^-$	474 c (38)	8 c (1)	2 c (2)
WO_4^{-2}	NO_3^-	649 b (52)	235 d (30)	48 b (54)
	$NO_3^- + WO_4^{-2}$	373 d (30)	36 e (4)	13 d (15)

The seedlings were pretreated 24 hr in medium without nitrates and subsequently (for next 24 hr) they were transferred to the absorption solution with NO_3^-. The concentration of WO_4^{-2} and VO_3^- (where present) in both pretreatment and absorption solutions were 10^{-4} M and 10^{-3} M respectively. The values are the average of 5 repetitions. The differences between 2 values denoted with different letters within each column is significant at 5% level.

Three day cucumber seedlings were initially treated for 24 hours with WO_4^{-2} and VO_3^- and then transferred to an induction nutrient containing or lacking both inhibitors. It was found that uptake of NO_3^- with the lack of WO_4^{-2} and VO_3^- in the induction nutrient was relatively high as compared to absorption of NO_3^- by

seedlings which grew continuously in the presence of the inhibitors, but was, however, lower by one half as compared to the controls. It is of interest to note that pretreatment of the seedlings with tungsten and vanadium, and an addition of the inhibitors to the induction nutrient did not totally inhibit uptake of NO_3^-, whereas NR activity was inhibited completely. It was next noted that NR activity in plants growing in an induction nutrient lacking inhibitors but previously treated with WO_4^{-2} or VO_3^- was relatively high, but lower by one half than in the control plants.

It therefore appears that WO_4^{-2} and VO_3^- in the first place influence biosynthesis and the level of NR activity, and to a lesser degree effect uptake of nitrates. The experiment, results of which are presented in Table 4, more or less confirm the above assumptions. Data show that uptake of NO_3^- from an external solution and its reduction in plant tissue are not processes closely correlated, this being indicated by the results of calculations on the relation of NO_3^- taken up to that reduced. Although both inhibitors distinctly lower NO_3^- uptake as compared to the controls, that is tungsten by twofold and vanadium by threefold, the amount of reduced NO_3^- nevertheless dropped fivefold and sixfold respectively.

Table 4

Absorption, accumulation and reduction of NO_3^- by cucumber seedlings as effected by tungstate and metavanadate ions

Treatment	Absorption	Accumulation	Reduction	Absorbed / reduced
	μmoles $NO_3^- \cdot g^{-1}$ dry weight			
NO_3^-	871	589	282	3.1
$NO_3^-+WO_4^{-2}$	487 (56)	431 (73)	56 (19)	8.8
NO_3^-	1190	850	340	3.3
$NO_3^-+VO_3^-$	402 (34)	349 (41)	53 (16)	7.6

Absorption and accumulation of NO_3^- was measured after 24 hr induction of seedlings in nutrient medium containing 10^{-4} Na_2WO_4 or 10^{-3} M Na_2VO_3. The values are average of 6 repetitions.

DISCUSSION

The results of the investigations described above show that WO_4^{-2} and VO_3^- almost completely inhibit NR activity, whereas to a much lesser extent reduce uptake of nitrates. An analysis of NO_3^- absorption and simultaneous accumulation of nitrates and their reduction (Table 4) after 24 hours of treating the cucumber seedlings with nitrates in the presence of WO_4^{-2} and VO_3^- showed a five to sixfold lowering of reduced NO_3^- as compared to the controls. In this same time NO_3^- uptake in the presence of WO_4^{-2} dropped more or less twofold and in the presence of VO_3^- threefold.

It is therefore difficult on the basis of the above given data to explain inhibition of NO_3^- reduction as being due to lowered intake of nitrates in the presence of WO_4^{-2},

since with almost total disappearance of NR activity, uptake is inhibited more or less at the rate of 50% (Tables 1 and 2). It does not likewise appear that the relatively greater drop in the uptake of nitrates in the presence of VO_3^- had an influence on total inhibition of NR activity. The above data hence do not show any close correlation between NO_3^- uptake and their reduction, which is rather contrary to the Butz and Jackson hypothesis (1977). The mentioned authors proposed an inter-dependence to exist between functioning of NO_3^- transport and its reduction in participation with the reductase nitrate system, which at the same time fulfills the role of NO_3^- carrier in transporting to cells, and the function of reductase.

It is assumed that WO_4^{-2}, as an analogue of Mo, inhibits NR biosynthesis (Heimer et al., 1969; Wary, Filner, 1979), having no effect on the already formed enzyme (Rao, Rains, 1975), but can, however, inhibit later synthesis of nitrate reductase. On the other hand, vanadium probably causes certain unstable changes in the NR structure inactivating its ability to reduce NO_3^- (Buczek, 1973). Our experiments showed that transferring the cucumber seedlings from a nutrient lacking nitrogen to a nitrate one with an addition of WO_4^{-2} almost completely inhibited development of an active NR system in cotyledons, and totally inhibited it in roots, whereas NO_3^- uptake was lower by only around 50 percent. On the other hand, introduction of WO_4^{-2} to the nitrate nutrient 24 hours after formation of the NR system had a weaker inhibiting effect both on NR activity as well as on nitrate uptake, as compared to the effect of WO_4^{-2} if applied to the plants at the moment of enzyme induction. However in comparison to the controls NR activity was highly inhibited (by about 70 percent), whereas NO_3^- uptake declined by only 36 percent. Similar correlations were still more distinct in the presence of VO_3^-.

The above data hence indicate that despite inhibiting reduction by the inhibitors under study, nitrate absorption continues, which fact negates existance of any close correlation between the two processes. If, therefore, the Butz and Jackson (1977) hypothesis is correct, there should be a distinct correlation between transport of nitrates from outside to the plant, and their reduction. Meanwhile in our experiment the ratio of NO_3^- absorbed to that reduced 24 hours after treating the plants with WO_4^{-2} or VO_3^- was 8 and 9 respectively, and around 3 in the control. Hence introducing the two inhibitors into the induction nutrient does in fact lower absorption of nitrates, but one cannot accept this as fully explaining total inhibition of NR, at least in the presence of WO_4^{-2}.

The results reached can, therefore, suggest that either NO_3^- absorption is a process independent of the functioning of the NR system, or according to the suggestion of Rao and Rains (1976) there are two ways of NO_3^- absorption of which one would be positively correlated with the NR system. On the basis of the results reached it is not possible to solve the question of inter-dependence or non-dependence of nitrate reduction in association with simultaneous NO_3^- transport according to the Butz and Jackson hypothesis due to the lack of data concerning the reaction of WO_4^{-2} and VO_3^- on the mechanism of nitrate absorption. However results of these investigations distinctly indicate that both inhibitors effect both reduction and transport

of NO_3^- to the roots. The problem with reference to the Butz and Jackson hypothesis remains open, the more so that one of our experiments appears to agree with this hypothesis.

In plants which were transferred to a nitrate induction nutrient after being initially treated for 24 hours with WO_4^{-2} and VO_3^- without an addition of nitrates, there was a fairly distinct correlation between the level of NR activity and the amount of NO_3^- assimilated, at a simultaneously partial inhibition of NR. The above data can suggest a dependence of reduction upon transport of NO_3^- to the roots and the leaves. Hence pretreatment of the plants with these salts exerted an influence on later NR induction and uptake of NO_3^-. Since in this case both inhibitors lowered NR activity by almost one half, and likewise lowered uptake, it can be assumed that development of the NR system was limited by transport of NO_3^- from the external solution. One likewise cannot exclude the effect of WO_4^{-2} and VO_3^- on internal cell translocation of NO_3^- between the so called non-metabolic storage pool and the nitrate metabolic one proposed by Ferrari et al. (1973), Heimer and Filner (1973), Martin (1973) and Aslam and Oaks (1975, 1976). In line with assumptions put forward by the cited authors only nitrates from the metabolic pool can induce NR, or can be taken advantage of in the process of reduction.

To conclude it is obvious that WO_4^{-2} and VO_3^- in the first place inhibit development of active NR and also lower NO_3^- uptake. Inhibition of NR activity cannot be explained by lowered absorption of nitrates. The problem requires further studies referring both to the mechanism of inhibiting NO_3^- uptake by WO_4^{-2} and VO_3^-, as also to that of inhibiting the NR system.

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Redukcja azotanów w siewkach Cucumis sativus L.

I. Wpływ wolframu i wanadu na pobieranie i redukcję azotanów

Streszczenie

Badano wpływ wolframu (Na_2WO_4) i metawanadu ($NaVO_3$) na pobieranie i redukcję azotanów przez siewki ogórka. Wolfram i wanad hamowały prawie całkowicie aktywność reduktazy azotanowej (NR) po 24 godzinach traktowania roślin azotanami w obecności 10^{-4} M WO_4^{-2} i 10^{-3} M VO_3^-. W tym samym czasie pobieranie NO_3^- w obecności WO_4^{-2} było obniżone o około 50 procent, natomiast w obecności VO_3^- w 70 procentach. Stosunek NO_3^- pobranych do zredukowanych u roślin kontrolnych wynosił około 3, podczas gdy w obecności WO_4^{-2} i VO_3^- odpowiednio 9 i 8. Efekt hamowania aktywności NR przez WO_4^{-2} i VO_3^- był istotny, ale nieco słabszy, jeżeli obydwa inhibitory podano roślinom w 24 godziny po wykształceniu systemu NR, pobieranie NO_3^- uległo natomiast nieznacznemu obniżeniu. Powyższe dane sugerują, że WO_4^{-2} i VO_3^-, po 24 godzinach traktowania roślin tymi inhibitorami, hamują głównie biosyntezę i aktywność NR a w mniejszym stopniu wpływają na pobieranie NO_3^-. W oparciu o uzyskane wyniki dyskutowano hipotezę Butz i Jacksona (1977) dotyczącą modelu dla pobierania i redukcji NO_3^- przez tkanki roślin.