Journal of Bioscience and Bioengineering
VOL. 128 No. 4, 463–467, 2019

www.elsevier.com/locate/jbiosc

Cobalt-dependent inhibition of nitrite oxidation in *Nitrobacter winogradskyi*

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Received 13 November 2018; accepted 1 April 2019
Available online 24 April 2019

***Nitrobacter winogradskyi* is an abundant, intensively studied autotrophic nitrite-oxidizing bacterium, which is frequently used as a model strain in the two-step nitrification of ammonia (NH_3) to nitrate (NO_3^-) via nitrite (NO_2^-), either in activated sludge, agricultural field studies or more recently in artificial microbial consortia for organic hydroponics. We observed a hitherto unknown cobalt ion-dependent inhibition of cell growth and NO_2^- oxidation activity of *N. winogradskyi* in a mineral medium, which strongly depended on accompanying Ca^{2+} and Mg^{2+} concentrations. This inhibition was bacteriostatic, but susceptible to natural chelators. L-Histidine effectively restored cell growth and NO_2^- oxidation activity of *N. winogradskyi* in mineral media containing Co^{2+} with >90% recovery. Our results suggest that Co^{2+} competed with alkaline earth metals during uptake and that its toxicity was significantly reduced by complexation.**

[**Key words:** *Nitrobacter winogradskyi*; Nitrification; Heavy metal toxicity; Cobalt; Calcium antagonism; Histidine]

Carbon dioxide (CO_2) and nitrous oxide (N_2O) are the most abundant greenhouse gases and their emission is often brought in direct connection to the ongoing global warming and climate change (1). Although extensive use of fertilizer and manure is frequently brought into context of the major environmental burden of modern agriculture (2–5), their impact on the biological carbon and nitrogen cycles is not fully understood. This is partially due to the complexity of the soil microbiome (6) and its different populations based on available nutrition (7) or the presence of organic pollutants (8). Ammonia-oxidizing bacteria (AOB) were extensively studied in recent decades (9) owing to the significantly higher accumulation of ammonia (NH_3) over nitrite (NO_2^-) in nature, which led to the general regard of NH_3 oxidation as the rate-limiting step of nitrification (10), despite the ability of AOB for denitrification (11–13), which would also impede NO_2^- accumulation (14,15) and lead to N_2O emission while simultaneously restricting the nutrients for nitrite-oxidizing bacteria (NOB). It was recently highlighted that NOB are of equal importance for the nitrogen cycle, but have not been sufficiently characterized, while their diversity and natural abundance have become more and more apparent (16). In an effort to better understand these autotrophs and how to increase their relative abundance in nitrifying communities, we examined *Nitrobacter winogradskyi* as a NOB model strain with a focus on novel growth-promoters or inhibitors. During the course of studies by one of the authors, it was found that the colony size of *N. winogradskyi* was increased when adding a trace element solution (TES) to a mineral medium (17). To the best of our knowledge, only a few studies have examined transition metal effects on pure cultures of *Nitrobacter*, mostly with a focus on increased NO_2^- consumption (18–20). This prompted us to analyse the effects of each single metal ion in the TES in more detail. Hence, we hereby found that cell growth and NO_2^- oxidation activity of *N. winogradskyi* were inhibited by certain heavy metals whose toxic effects have not been characterized for the genus *Nitrobacter*. We further searched for chemical factors contributing to heavy metal resistance in *N. winogradskyi* that might improve nitrification efficiency in view of reducing nitrogen loss by N_2O formation in natural or artificial microbial communities.

MATERIALS AND METHODS

Materials All chemicals were purchased in high purity from either Tokyo Chemical Industry (Tokyo, Japan), Wako Pure Chemical Industries (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan) and were used without further purification. Glassware was treated with 4 M HCl for several hours to remove trace metal impurities, kept in 2% (v/v) HNO_3 until use and rinsed three times with reverse osmosis-treated water before dry heat sterilization (160 °C, 2 h). Type-strain *N. winogradskyi* NBRC 14297 was obtained from Biological Resource Center, National Institute of Technology and Evaluation (NITE) (NBRC, Chiba, Japan).

Culture media and growth conditions *N. winogradskyi* NBRC 14297 was grown in a mineral medium (21) composed of 34 mM $NaNO_2$, 18 mM Na_2HPO_4, 2 mM KH_2PO_4, 40 μM $MgSO_4$ and 3.6 μM $FeSO_4$. In an appropriate experiment, trace elements (TES; 90 μM $CaCl_2$, 0.41 μM Na_2MoO_4, 1.0 μM $MnCl_2$, 8.4 nM $CoCl_2$, 0.35 μM $ZnSO_4$ and 80 nM $CuSO_4$ as final concentrations) were added from a 1000-fold concentrated solution after autoclaving. Sub-cultivation was performed at 28 °C and 100 rpm rotary shaking in the dark. Contamination was checked regularly by spreading 50 μL aliquots on solid medium plates selective for heterotrophic bacteria (Luria–Bertani (LB) medium; 15 g L^{-1} agar), nitrogen-fixing bacteria (N-free Burk's medium (22); 15 g L^{-1} agar) or NOB (mineral medium with TES; 6 g L^{-1} gellan gum (23,24)). Colonies that appeared on the plates were evaluated after 3 weeks of incubation at 28 °C in the dark.

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1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2019.04.001

Measurement of NO_2^- oxidation activity Quantitative analysis of NO_2^- and NO_3^- concentrations was performed via Griess assay using vanadium chloride (VCl_3) for homogeneous reduction of NO_3^- to NO_2^- (25). One unit (U) of NO_2^- oxidation activity was defined as the amount of cells that oxidize NO_2^- to NO_3^- at a rate of 1 μmol min^{-1}.

Colony-forming units analysis The viable cell count of samples was determined via plate counting of a serial dilution in the mineral medium containing TES. Aliquots (50 μL) of each sample, diluted with fresh medium (10^2- to 10^5-fold), were spread onto gellan gum plates (6 g L^{-1}, deep petri dishes, 50 mL mineral medium with TES) and incubated at 28 °C in the dark, under 0.4% CO_2 and 100% humidity. Under these optimized conditions, colonies grew to diameters of 0.3–0.6 mm within 2–3 weeks, which was sufficient for enumeration by using a counter pen with the unaided eye (Fig. S1).

Antimicrobial susceptibility tests Determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of metal ions was performed in 96-well plates according to the standard method for aerobically growing bacteria (26) with the following alterations. Due to the low turbidity and extended lag phase of NOB cultures, growth inhibition was estimated from the NO_2^- oxidation activity after 48 h of incubation at 28 °C and 500 rpm. Each well contained 100 μL of metal-containing mineral medium with or without TES and 10 μL of a fresh NOB subculture (3–4 days), corresponding to 2.4×10^6 cells mL^{-1}. Each metal ion was analysed in triplicates of six serial twofold dilutions and one 0.1 μM control sample. Samples with strongly inhibited NO_2^- oxidation were examined in serial tenfold dilutions to give plates with 250–300 colonies for the standard inoculum and MIC. Samples giving less than three colonies at the same dilution were defined as the MBC.

Trace metal effects Inhibition studies were performed in 18.5 × 1.6 cm test tubes with paper plugs (Heinz Herenz Medizinalbedarf GmbH, Hamburg, Germany) and each test tube contained 10 mL of the mineral medium with or without TES inoculated with 2.0% (v/v) of a fresh subculture (3–4 days), corresponding to 1.6×10^6 cells mL^{-1}. Each experiment was done in triplicate and incubated for at least 48 h at 30 °C and 200 rpm reciprocal shaking in the dark to reach the logarithmic growth phase. Unless otherwise stated, all experiments were analysed in 24 h intervals for NO_2^- oxidation activity. IC_{50} values were calculated with the software GraphPad Prism 7 (version 7.04) using a non-linear regression curve fit (Hill equation) with four parameters and a confidence interval of 95%.

RESULTS

During reproduction of the initial findings that the TES increased the colony size of *N. winogradskyi* (17), we further found that NO_2^- oxidation activity in liquid cultures with TES was markedly enhanced in comparison to cultures without TES (Fig. S2A). As NO_2^- oxidation is directly coupled to ATP generation in this strain, it is usually regarded as proportional to cell growth, which we also confirmed by colony-forming units (CFU) analysis (Fig. S2B). Using the mineral medium without TES, we first analysed the effect of each metal in the TES on NO_2^- oxidation activity of *N. winogradskyi*. When we increased the dosage of Co^{2+} from 8.4 nM to 200 nM, we observed a strong inhibition of NO_2^- oxidation, accompanied by a decrease of turbidity. Other trace metals like Zn^{2+} and Cu^{2+} were significantly weaker inhibitors. MoO_4^{2-} and Mn^{2+} did neither increase nor decrease NO_2^- oxidation activity up to 200 μM. Surprised by this strong Co^{2+} effect on *N. winogradskyi* with no indications in the literature, we subsequently performed the experiments in the mineral medium with TES. However, in the presence of the TES, Co^{2+} did not show any signs of inhibition of NO_2^- oxidation activity or cell growth at 200 nM or even at 1.0 μM. After determining the IC_{50} values of each metal, an increased Co^{2+} and Zn^{2+} tolerance in the presence of the TES became apparent (Table 1, entries 1 and 3), while the results for Cu^{2+} were inconclusive (Table 1, entry 2). Other metals did not inhibit the NO_2^- oxidation activity of *N. winogradskyi* in the tested ranges.

The Co^{2+}-inhibited cultures with TES (Table 1) nevertheless continued to consume NO_2^- slowly and were able to fully convert it to NO_3^- within 10 days, suggesting that the inhibition did not kill the cells. We confirmed this hypothesis by determining the antimicrobial susceptibility of *N. winogradskyi* for the metal ions that inhibited NO_2^- oxidation in our mineral medium (Table 2). Based on the results, Co^{2+}, Cu^{2+} and Zn^{2+} were bacteriostatic inhibitors in the presence of TES. Similar to the inhibition of NO_2^- oxidation, Co^{2+} toxicity increased in the absence of TES and became bactericidal at 100 μM.

It was still not clear why the Co^{2+} resistance increased dramatically when other metal ions (TES) were added to the medium. We therefore tested combinations of each metal in the TES with Co^{2+} in a dose-dependent manner and found that none of the transition metals could restore the NO_2^- oxidation activity inhibited by Co^{2+} (Table S1). The answer was found with the remaining component of the TES, Ca^{2+}, which showed a logistic dose-response for restoration of NO_2^- oxidation activity inhibited by Co^{2+}, conferring to a nearly 4-fold increase in Co^{2+} resistance of *N. winogradskyi* (Fig. 1). Among the metals already included in the mineral medium, Mg^{2+} was found to show a similar response (Fig. S3).

Although crucial for reducing Co^{2+} toxicity, further increases of the Ca^{2+} concentration could not fully eliminate the inhibition of NO_2^- oxidation. Higher concentrations of Ca^{2+} (>90 μM) precipitated as insoluble phosphate salts from the medium. We thus changed our focus towards complexation of Co^{2+} with organic compounds. Cyanocobalamin (vitamin B_{12}), the most important cobalt complex in nature, did not inhibit *N. winogradskyi* (Table 3, entry 2). We therefore tested several natural and non-natural chelators for Co^{2+} complexation and their effects on NO_2^- oxidation activity of this strain. Among the natural chelators, L-histidine almost completely eliminated the inhibition by Co^{2+} (Table 3, entry 8). Other chelators with catechol-type (Table 3, entries 3 and 5), carboxylate- (Table 3, entry 6) or α-amino acid-based ligands (Table 3, entries 4, 7 and 9) had only little or no effect for

TABLE 1. Inhibitory concentration (IC_{50}) of various metal ions for NO_2^- oxidation activity in *N. winogradskyi* in the mineral medium.

Entry	Metal ion	Tested range (μM)[a]	IC_{50} (μM)[b]	
			without TES	with TES
1	Co^{2+}	0.01–1000	0.25 ± 0.065	14.7 ± 0.26
2	Cu^{2+}	0.08–130	>130	35.1 ± 1.76
3	Zn^{2+}	0.35–1000	14.0 ± 0.88	61.5 ± 1.87
4	Ca^{2+}	0.10–90.0	>90.0	>90.0
5	Fe^{2+}	3.60–90.3	>90.3	>90.3
6	Mn^{2+}	1.00–100	>100	>100
7	Mg^{2+}	40.6–129	>129	>129
8	MoO_4^{2-}	0.41–206	>206	>206

[a] Lower limits (except for Ca^{2+}) correspond to the metal concentration in mineral medium with TES. Upper limits depended on the concentration in experiments which showed an immediate formation of precipitate after metal addition.
[b] Defined as the concentration of metal ion at which NO_2^- oxidation activity of *N. winogradskyi* was reduced to 50% of its activity in the mineral medium with or without TES, respectively. Values are given in mean ± SD as calculated by the curve fitting model from five concentrations with each three replicates.

TABLE 2. Antimicrobial susceptibility of *N. winogradskyi* for metal ions inhibiting NO_2^- oxidation in the mineral medium.

Entry	Metal ion	TES	Tested range (μM)[a]	MIC[b] [μM (CFU mL^{-1})]	MBC[c] [μM (CFU mL^{-1})]
1	Co^{2+}	–	0.1–200	25 (1.5×10^5)	100 (<8×10^3)
2	Co^{2+}	+	0.1–200	100 (2.4×10^6)	>200
3	Cu^{2+}	–	0.1–100	100 (1.8×10^6)	>100
4	Cu^{2+}	+	0.1–100	50 (1.6×10^6)	>100
5	Zn^{2+}	–	0.1–200	200 (6.8×10^5)	>200
6	Zn^{2+}	+	0.1–200	>200	>200

[a] Upper limits correspond to the metal concentrations which did not lead to precipitate formation in the mineral medium. Six serial twofold dilutions and one control sample containing 0.1 μM of the corresponding metal ion were analysed.
[b] Minimal inhibitory concentration. Defined as the concentration of metal ion at which the CFU (48 h) did not exceed the inoculum of 2.4×10^6 cells mL^{-1}. Values are the mean from three replicates.
[c] Minimal bactericidal concentration. Defined as the concentration of metal ion at which the CFU (48 h) were below 1% of the inoculum of 2.4×10^6 cells mL^{-1}. Values are the mean from three replicates.

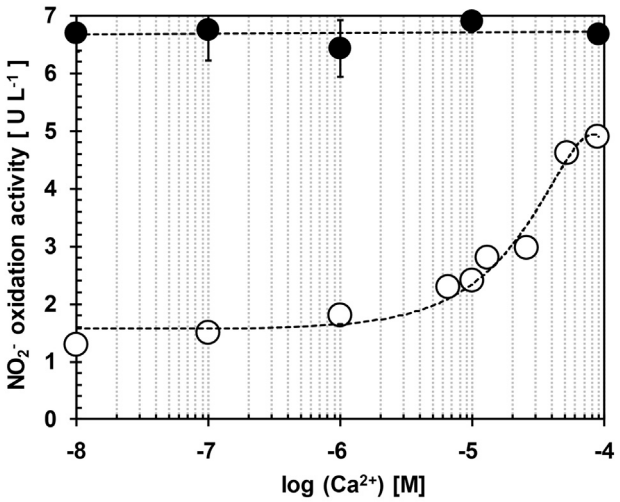

FIG. 1. Effect of co-existing Ca^{2+} on the Co^{2+}-dependent inhibition of NO_2^- oxidation in *N. winogradskyi*. Ca^{2+} was added to the mineral medium containing TES and (filled circles) 0 μM or (hollow circles) 25.8 μM Co^{2+}, omitting $CaCl_2$. Owing to the strong inhibition the depicted NO_2^- oxidation activities (hollow circles) were calculated from interpolated cultivation times (58–221 h) to reach 50% NO_2^- consumption. Markers represent the mean values of three replicates. Error bars represent the SD and may be smaller than the diameter of the markers.

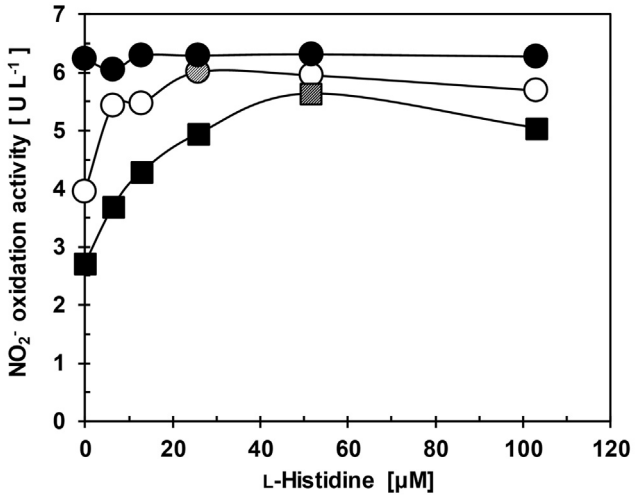

FIG. 2. Dose-dependent recovery of NO_2^- oxidation activity in *N. winogradskyi* by L-histidine complexation of Co^{2+}. Complexation was performed by mixing L-histidine with Co^{2+} concentrations of 0 μM (filled circles), 12.9 μM (hollow circles) or 25.8 μM (filled squares) at pH 8.0 prior to addition to the mineral medium with TES. Experiments were analysed after 90 h of incubation. Markers represent the mean of three replicates. Error bars representing the SD are smaller than the diameter of the markers. Oblique markers show the experiments with an L-His/Co^{2+} molar ratio of 2:1.

detoxification. Dose-dependent experiments with varying Co^{2+} concentrations further revealed that a molar ratio of 2:1 for L-histidine was particularly effective in maintaining high NO_2^- oxidation activity and cell growth (Fig. 2).

DISCUSSION

There are several contradictions in the literature as to how heavy metals influence the cell growth and NO_2^- oxidation activity of *N. winogradskyi*. In very early studies, sufficient cell mass of axenic cultures was achieved by using large fermenters in which at least one part, e.g., the container itself or the stirrer, was made from metal (21). In addition, the use of tap water, sterilized by boiling or deionized by distillation, could still contain sufficient metal impurities to affect nitrifying bacteria (27). This problem has been pointed out regularly in the literature when several cultures or isolates of nitrifying autotrophic bacteria became inactive for no apparent reason (28,29). The majority of studies regarding heavy metal toxicity during nitrification were performed on microbial communities in contaminated soil (30), waste water treatment facilities (31), or nitrification reactors (32). Although many of these studies might provide a more realistic picture of the consequences of heavy metal stress on autotrophic microbial communities, there is still a need to identify the general mechanisms of heavy metal toxicity in axenic cultures of AOB or NOB to improve strategies for detoxification. Our results suggest that Co^{2+}, Cu^{2+} and Zn^{2+} reduce both the cell growth and NO_2^- oxidation activity of *N. winogradskyi*. Interestingly, a similar inhibition was reported in a study on nitrifying sludge starting from NH_4^+, but could not be rationalized owing to the use of an undefined mixed culture (32). Due to their bacteriostatic nature, in situ treatment of metal-inhibited NOB with TES might resuscitate NO_2^- oxidation activity.

However, there are no reports explaining the increased Co^{2+} resistance we observed after adding the TES. By varying each metal concentration individually, we were able to identify Ca^{2+} as the crucial factor in stabilizing *N. winogradskyi* from its Co^{2+}-dependent inhibition. Antagonism between these metals is mostly found in divalent cation transporters, which tend to become inaccessible to heavy metals when saturated by alkaline earth metal ions like Ca^{2+} or Mg^{2+} (33,34). It is also reported that Ca^{2+} reduced the Co^{2+}-dependent inhibition of an H^+ efflux pump in *Saccharomyces cerevisiae* (35) and that oyster shell lime, containing mostly $CaCO_3$, recovered NO_2^- oxidation activity in organic hydroponic media (36). Although Co^{2+} is known vice versa to exhibit toxicity by blocking Ca^{2+} channels in eukaryotes, such an activity is usually inferior to Cu^{2+} or Mn^{2+} (37). Residual inhibition by Co^{2+} in Ca^{2+}-saturated medium and additional dependence on Mg^{2+} concentration suggest the existence of other transport systems in *N. winogradskyi* which might be more

TABLE 3. Natural chelators for the complexation of Co^{2+} and recovery of NO_2^- oxidation activity in *N. winogradskyi* cultures in the mineral medium with TES.

Chelator	Molar ratio (ligand/Co^{2+})[a]	Relative NO_2^- oxidation activity (%)[b]	
		without Co^{2+}	with Co^{2+}
None	–	100	54
Natural chelator			
Cyanocobalamin[c]	–	100	–
Kojic acid	1:1	90	41
Glutathione[d]	1:1	106	43
Caffeic acid[e]	1:1	98	53
Citric acid[e]	1:1	104	65
Glycine	3:1	110	30
L-Histidine[f]	1:1	111	92
L-Tryptophan	1:1	100	58
Non-natural chelator			
EDTA	1:1	48	44
2,6-Dipicolinic acid[e]	1:1	19	12

[a] Co^{2+} (25.8 μM) was mixed with the chelator before addition to the culture.
[b] Relative to a NO_2^- oxidation activity of 5.8 ± 0.77 U L^{-1}after 68 h of cultivation without Co^{2+}. Values represent the mean of three replicates with SD values of ≤2%.
[c] Cyanocobalamin containing Co^{3+} was used.
[d] Added in reduced form.
[e] Added as sodium salt.
[f] Added as hydrochloride salt.

specific for Co^{2+}, such as Nramp homologues (38), NiCoT family transporters (39), NhlF (40), or the more recently described CbiMNQO (41). These transporters are in general also selective for Ni^{2+} and we confirmed in this study that Ni^{2+} is a bactericidal inhibitor (IC_{50} = 0.64 ± 0.044 μM, MBC = 12.5 μM, mineral medium with TES) of cell growth and NO_2^- oxidation activity in *N. winogradskyi*, although it is not included in the TES. Genome sequencing actually indicated the presence of homologues of Nramp and MgtE transporters in *N. winogradskyi*, as well as several other ABC transporters (42). However, owing to the difficulty in cultivation of this autotroph, biochemical characterizations of these transporters are not yet available.

Complexation of Co^{2+} to further reduce or completely eliminate its effect on *N. winogradskyi* in liquid medium was surprisingly specific for the natural amino acid L-histidine at a molar ratio of 2:1. Citric acid in high excess was also effective, but still to a much lesser extent. These two ligands are known for heavy metal complexation in hyper-accumulating plants (43) and yeast (44). Other known natural metal chelators like caffeic acid, kojic acid or glutathione (reduced form) did not show any beneficial effects. Neither L-tryptophan nor glycine could reduce Co^{2+} toxicity. Given the fact that cyanocobalamin did not inhibit *N. winogradskyi*, it is reasonable to expect that siderophores and more complex natural chelators might also be effective ligands. Non-natural chelators like EDTA and 2,6-dipicolinic acid were toxic for this strain, even in the absence of Co^{2+}. Studies are underway to clarify if this beneficial effect of L-histidine is also applicable to the nitrification of organic nutrients with natural or artificial microbial communities.

In conclusion, we identified a hitherto unknown Co^{2+} toxicity in the chemolithoautotroph *N. winogradskyi*, which is bacteriostatic and inhibited NO_2^- oxidation, a crucial process for ATP generation in this microorganism. Detrimental effects on cell viability were only observed at significantly higher concentrations in the absence of TES, suggesting that Co^{2+} inhibits NO_2^- oxidation directly by interacting with proteins in the nitrite oxidoreductase cluster (45,46). Co^{2+} uptake seems to be closely associated with divalent cation transport proteins selective for Ca^{2+} and Mg^{2+}. Addition of L-histidine in a molar ratio of 2:1 almost completely eliminated Co^{2+} toxicity, indicating that detoxification involves the formation of the well-known Co[L-His]$_2$ complex (47). In the absence of such a chelator and with sufficient Ca^{2+} and Mg^{2+} available, the IC_{50} value of Co^{2+} was still 14.7 ± 0.26 μM. Although complementary data of a potential Co^{2+}-dependent inhibition of NH_3 oxidation in AOB are not available, early work states that some inhibition was observed in an AOB isolated from effluent of an activated-sludge plant (48). Thus, in the presence of Co^{2+} concentrations in the lower micromolar range, NO_2^- oxidation by *N. winogradskyi* could be partially interrupted and lead to accumulation of NO_2^- and consequently higher N_2O emissions (49). This is of particular interest as the excessive application of manure in agriculture and effluents from mining and manufacturing industries steadily increase the content of $Co^{2+/3+}$ in soil and water (50). Future research on NOB should clarify whether Co^{2+} is binding to active proteins or interrupts the biosynthetic machinery of proteins necessary for NO_2^- oxidation activity.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.04.001.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Makoto Shinohara (Institute of Vegetable and Floriculture Science, NARO, Japan), a pioneer of organic hydroponics, for useful discussions and comments on this study. This work was supported by the Japan Science and Technology Agency under the Advanced Low Carbon Technology Research and Development Program.

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