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Role of *Azospirillum* and *Rhizobium* in Bio-Remediating Cd and Zn Polluted Soil Cultivated with Wheat Plant

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Abstract: A greenhouse experiment was conducted to investigate the role of each of *Azospirillum lipoferum* and the endosymbiont *Rhizobium leguminosarum*bv. trifolii in bio-remediating the harmful effects of cadmium and zinc heavy metals on wheat growth parameters and its content of macro-nutrients and both tested heavy metals. The obtained results show that no significant differences were recorded in root dry weight due to the tested treatments in comparison with the positive control. For shoot dry weight, it was found that heavy metals as such negatively affected shoot dry weight. In contrast, significant increases in shoot dry weight were demonstrated owing to the treatments of *Azospirillum*, *Azospirillum* + *Rhizobium* and *Rhizobium* + 200ppm Zn. Combined application of *Azospirillum* and *Rhizobium* in the presence of 300ppm Cd was the only treatment that induced highly significant increase in comparison with the positive control. The other treatments (except one treatment of *Azospirillum* + *Rhizobium* + 300ppm Cd) significantly inhibited root length in comparison with the positive control. The treatment of *Rhizobium* in the presence of 300ppm Zn and *Azospirillum* + 300ppm Zn were the only treatments that significantly increased shoot length.Irrespective of the treatments, the macro-nutrients ranged from 0.76 to 1.33 % for N content, 0.11 to 0.85 % for phosphorus and 0.12 to 0.42% for potassium in roots; while zinc amounts in roots ranged from 15.3 to 174 ppm and cadmium ranged from not detected amounts to 242.7 ppm. The shoots nutrients content ranged from 1.01 to 2.1%, 0.10 to 0.53% and 0.26 to 0.70% respectively for N, P and K irrespective of the treatments. Application of bio-remediating microorganisms *Azospirillum* and *Rhizobium* neutralized the adverse effect of heavy metals on wheat growth.

Key words: Bio-remediation, heavy metals polluted soil, *Azospirillum*, *Rhizobium*, wheat growth parameters.

INTRODUCTION

Although heavy metals are natural components of the Earth's crust, they considered to be a dangerous group of chemical pollutants. Cadmium, copper, lead, zinc and chromium are among the most hazardous heavy metals and are included in the EPA's list (Environmental Protection Agency) of priority pollutants (Cameron, 1992). As trace elements, some heavy metals are essential to maintain the metabolism of human body. Heavy metals such as Cu, Zn and Fe are essential for normal growth of living microorganisms. However, at higher concentrations they can be harmful to any biological function (Jarup, 2003). Human activities such as waste disposal, mining and smelting have led to a substantial release of toxic heavy metals in the environment, which can badly affect the ecosystem, plant, animal and human health (Wu *et al.*, 2006).

Pollution is a major problem all around the world. Contamination of soil, ground water, sediments, surface and air with hazardous materials and toxic chemicals are growing threat to the environment. The large-scale production of variety of chemical compounds, however, has caused a global deterioration of environmental quality (Boopathy, 2000 and Iwamoto and Nasu, 2001). Heavy metals have recently received great attention of researchers; mainly because they cannot be degraded or destroyed to a small extent and they enter our bodies via food, drinking water and air (Adarsh*et al.*, 2007). Heavy metals contamination of soil is the main issue in many countries. The study of soils polluted with heavy metals is important because soils effectively act as a reservoir which, after temporary storage of metals, can act as a source under certain conditions. Therefore, soil is both source and sink of metal pollutants (Doumett*et al.*, 2008).

Bioremediation can be used to stabilize, extract or reduce the toxicity of soil containing heavy metals; bioremediation uses biological agents, mainly microorganisms i.e., yeast, fungi or bacteria to clean up contaminated soil and water (Strong and Burgess, 2008). Microorganisms are recently identified as one of the vectors of metal dissemination in soils, thanks to their high sorption capacity. Soil microorganisms such as bacteria which are suggested to be the most active biological part in soil, are surface charged and able to secrete various organic compounds such as low-molecular organic acids, carbohydrates and enzymes (Huang *et al.*, 2002). This property appears useful for polluted soil bio-remediation. Bacteria play an important role in the

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environmental fate of toxic metals (Gadd, 2000). Soil rhizobacteria, with activity and a high surface area-to-volume ratio because of their small size and therefore providing a large contact area, may have the potential to act as microbial chelates associated with phytoremediation (Anderson *et al*., 1993 and Kärenlampi*et al*., 2000).Hanjun*et al.* (2010) stated that the endophytic Bacillus sp. could specifically uptake 75.78%, 80.48% and 21.25% of Cd (II), Pb (II) and Cu (II) under the initial concentration (10 mg/L).

Recent studies have established that *Rhizobium*, the well-known nitrogen fixing root nodules endosymbiont of legume plants also develops a natural, beneficial endophytic association with cereal roots growing in the same crop rotation (Yanni*et al*., 1997& 2001 and Dazzo*et al*., 2003). Several field inoculation trails have indicated that certain strains of these rice adapted clover rhizobia can promote vegetative and reproductive growth of the rice crop resulting in significant increases in grain yield and agronomic fertilizer N-use efficiency, with less dependence of chemical N-fertilizer (Yanni*et al*., 2001).

The work within hand aims at investigating the role of two different types of biofertilizers i.e., *Rhizobiumleguminosarum*bv. trifolii and/or *Azospirillumlipoferum* in reducing the harmful effects of two heavy metals i.e., cadmium and zinc on wheat plants grown under stress of high concentrations of these heavy metals.

MATERIALS AND METHODS

Collection Of Soil Samples For Isolation Of microorganisms Used In Bio-Remediation:

For isolation of Azospirillum, soil samples were collected from the rhizosphere of each of Watercress (*Nasturtium officinale*) and Molokhia (*Corchorusolitorius*) cultivated in agriculture land in Asfan where the plants were uprooted and shacked to get adjacent soil to the plant roots. Roots of clover plants (*Trifoliumalxandrium*) with their surrounding soil were brought from Egypt for isolation of the *Rhizobium* strain.

Isolation of Azospirillum:

Tubes containing semi-solid N-deficient medium (Dobereiner*et al*., 1976) were inoculated with 1 ml aliquots of previously prepared serial dilution of the rhizosphere soil. The inoculated tubes were shacked well then incubated at 32 °C for 72 hours. The positive tubes were recognized by the presence of subsurface white fine pellicles. Microscopic examination of the positive tubes confirmed the predominance of typical Azospirillum cells of characteristic spiral movement (Gomaa, 1989).

Isolation of Rhizobium:

Nodulated Egyptian clover plants (*Trifolium alxandrium*) were brought from Egypt with the surrounding soil. In the lab, the surrounding soil to the plant roots was carefully removed then clover roots were washed under running tap water to get rid of the adjacent soil to the roots. Clover root nodules were carefully separated from the roots and collected in a screw cap test tube. The root nodules were washed with tap water supplemented with few drops of liquid soap, and then washed many times to get rid of the remnant liquid soap. The root nodules were sterilized in a mixed sterilizing solution composed of ethyl alcohol (95%) and hydrogen peroxide (5%) (1:1 v/v) where the nodules washed three times (1 minute per each). Finally, the nodules were rinsed with sterilized distilled water many times to remove the remnant of the mixed sterilizing solution. In a sterilized Petri dish, some nodules were crushed in few drops of sterile distilled water, after that the nodules juice were streaked on yeast mannitol agar medium (Fred *et al*., 1932 and Rangaswami and Bagyaraj, 1993).

Determination Of Heavy Metals And Nutrient Content Of Soil And Plant Samples:

Heavy metals (Cd & Zn) and nutrient contents (N, P, K) of soil and plants were determined according to Chapman and Pratt (1961), wherethe soil and plant materials were wet ached and digested in $H_2SO_4.H_2O_2$ mixture.

Effect Of Heavy Metals And Bio Remediation On Wheat Growth:

A pot experiment was carried out in the greenhouse of the faculty of science at King Abduaziz Univ., KSA in the winter season of 2011. The temperature was adjusted to 25oC and plastic pots of 25 cm diameter were filled with 10 kg/pot of soil which brought from Asfan governorate, KSA. Tables (1) and (2) indicate the physical and chemical analysis of the soil. Each treatment was replicated 3 times.

Heavy metals treatments of each of zinc (as zinc sulfate) and cadmium (as cadmium sulfate) were applied at 0, 200, 300ppm for each. The calculated quantity of each concentration was mixed well with the soil before wheat seeds cultivation according to the treatment.

Table 1: The mechanical analysis of soil.

Soil ingredient	Silt	Clay	Sand	W.H.C	Organic matter
Quantity (%)	4.20	10.25	85.03	25.10	0.07

Table 2: The chemical analysis of soil.

EC (dS/m)	pH	Cations (ml/100 mg meque./100 gm)				Anions (ml/100 mg meque./100 gm)				Nutrients and heavy metals				
										Total (%)		Available (ppm)		
		^{+}K	^{+}Ca	^{+}Na	^{+}Mg	$^{--}CO3$	$^{-}HCO3$	^{-}Cl	$^{--}SO_4$	N	P	K	Cd	Zn
1.1	7.92	0.52	0.20	0.03	0.02	0.7	0.1	---	0.19	0.023	0.25	3.2	---	10

Determination Of Heavy Metals And Nutrient Content Of Soil And Plant Samples:

Soil, roots, shoots and seeds of wheat plants in both flowering and fruiting stages were wet ached and digested in H_2SO_4-H_2O_2 mixture and the total content of zinc and cadmium and mineral contents (N, P, K) in soil and wheat plant extract was determined according toChapman and Pratt (1961).

Wheat seeds (*Triticumaestivum* var. Sakha98) were sown randomly in each pot then inoculated with the respective tested biofertilizer (*Azospirillum* and/or *Rhizobium*); where each pot received 50 ml of each culture according to the treatment. Wheat seeds were covered with a thin layer of soil then irrigated with tap water without water excess. After germination, wheat plants were thinned to 15plant/pot.

The following treatments were investigated:

1. Control.
2. Zinc sulfate (200 ppm).
3. Zinc sulfate (300 ppm).
4. Cadmium sulfate (200 ppm).
5. Cadmium sulfate (300 ppm).
6. *Azospirillum lipoferum.*
7. *Rhizobium leguminosarum.*
8. *Azospirillum lipoferum* + *Rhizobium leguminosarum.*
9. Zinc sulfate (200 ppm) + *Azospirillum lipoferum.*
10. Zinc sulfate (200 ppm) + *Rhizobium leguminosarum.*
11. Zinc sulfate (200ppm) + *Azospirillum lipoferum*+ *Rhizobium leguminosarum.*
12. Zinc sulfate (300 ppm) + *Azospirillum lipoferum.*
13. Zinc sulfate (300 ppm) + *Rhizobium leguminosarum.*
14. Zinc sulfate (300 ppm) +*Azospirillum lipoferum*+ *Rhizobium leguminosarum.*
15. Cadmium sulfate (200 ppm) + *Azospirillum lipoferum.*
16. Cadmium sulfate (200 ppm) + *Rhizobium leguminosarum.*
17. Cadmium sulfate (200 ppm) + *Azospirillum lipoferum*+ *Rhizobium leguminosarum.*
18. Cadmium sulfate (300 ppm) + *Azospirillum lipoferum.*
19. Cadmium sulfate (300 ppm) + *Rhizobium leguminosarum.*
20. Cadmium sulfate (300 ppm) + *Azospirillum lipoferum*+ *Rhizobium leguminosarum.*

Statistical Analysis:

The obtained results were statistically analyzed using the computer program SPSS 17(2007). ANOVA test (one way) was applied to differentiate between the various means of treatments. The experimental design was completely randomized block design.

Results:

Isolation of Azospirillum and Rhizobium species:

Isolation of *Azospirillum* was achieved according to (Dobereiner*et al.,* 1976 and Gomaa, 1989). Plate (1) indicates the typical characteristic growth of *Azospirillum* in N-Deficient Semi-Solid edium of Dobereiner*et al.* (1976). For the *Rhizobium* isolation, it was isolated from the root nodules of Egyptian clover using the yeast mannitol agar medium (Fred *et al.*, 1932).

Identification of Azospirillum and Rhizobium:

The isolated *Azospirillum* strain was identified according to Tarrand*et al.*(1978) and Peter *et al.* (1980) as *Azospirillum lipoferum*. The main morphological and physiological characteristics of this strain were: cell morphology was vibrio containing poly β-hydroxybuterate, very active motile with spiral movement, negative to gram stain and capable of using glucose as a sole carbon source. Furthermore, the strain was able to produce acid from sucrose, fructose, galactose, lactose and sorbitole as a sole carbon source. With regard to the *Rhizobium* sp., it was identified as *Rhizobium leguminosarum* bv. trifolii.

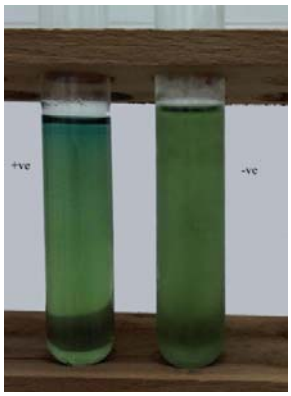

Plate 1: The Typical growth of *Azospirillum* in N-Deficient Semi-Solid medium of Dobereiner*et al.* (1976).

The Biological Analysis Of Soil:

Serial dilution method was used, after that the dishes were incubated at 37°C for 5 days; the results were recorded as the following: total count 1.3×10^4, Actinomycetes 5.9×10^3 and fungi 1.9×10^2 CFU/g dry weight.

Effect Of The Tested Treatments On Growth Parameters At Flowering Stage:

Table (3) illustrates the effect of different tested treatments on dry weight (g/plant), plant height (cm) and leaves number per plant. No significant differences were recorded in root dry weight due to the tested treatments. Regarding shoot dry weight, it was foundthat heavy metals as such negatively affected shoot dry weight in comparison with the positive control treatment. On the other hand, significant increases in shoot dry weight were demonstrated owing to the treatments of *Azospirillum*, *Azospirillum*+*Rhizobium* and *Rhizobium*+200ppm Zn; while the combined inoculation of *Azospirillum* and *Rhizobium* in the presence of 300ppm Cd was the only treatment that induced a highly significant increase in comparison with the positive control.

For root length, Table (3) also shows that the combined inoculation with *Azospirillum* and *Rhizobium* was the only treatment that induced significant increase over the positive control treatment. In contrast, the other treatments (except one treatment of *Azospirillum*+ *Rhizobium*+300ppm Cd) significantly inhibited root length in comparison with the positive control. Furthermore, the treatment of *Rhizobium* in the presence of 300ppm Zn and *Azospirillum* +300ppm Zn were the only treatments that significantly increased shoot length. As to the leaf number/plant, *Rhizobium* in the presence of Zn at 200 or 300ppm and *Azospirillum*+ 300ppm Zn treatments were the only ones that significantly overcame the positive control. No significant differences were found in leaves number between the various treatments except the treatments of *Rhizobium* in the presence of Zn at 200 or 300ppm and *Azospirillum* + 300ppm Zn that significantly surpassed the other treatments.

Table 3: Effect of various tested treatments on dry weight, plant height and the number of leaves of wheat plants at flowering stage.

Element / Treatment	Dry weight (g/plant)		Lengths (cm)		Number of leaves/plant
	Root	Shoot	Root	Shoot	
Control	0.05	0.47	24.2	49.0	4
Rhizobium (Rh.)	0.06	0.51	20.2	50.8	4
Azospirillum (Azo.)	0.05	0.48	19.6	48.7	4
Rh. +Azo.	0.05	0.51	26.5	50.4	4
Zn 200ppm	0.04	0.36	16.4	44.3	4
Zn 300ppm	0.05	0.36	20.3	45.2	4
Cd 200ppm	0.06	0.35	18.1	40.2	4
Cd300ppm	0.06	0.34	18.8	48.7	4
Rh.+ Zn200ppm	0.06	0.51	21.7	50.1	5
Rh.+ Zn300ppm	0.05	0.43	19.7	51.5	5
Rh.+ Cd200ppm	0.07	0.41	19.3	44.5	4
Rh.+ Cd300ppm	0.08	0.40	20.9	42.4	4
Azo.+ Zn200ppm	0.05	0.44	19.4	43.8	4
Azo.+ Zn300ppm	0.05	0.45	16.4	51.5	5
Azo.+ Cd200ppm	0.05	0.40	13.3	44.8	4
Azo.+ Cd300ppm	0.08	0.42	20.5	46.4	4
Rh.+Azo.+Zn200ppm	0.07	0.46	21.9	46.5	4
Rh.+Azo.+Zn300ppm	0.05	0.42	20.7	43.7	4
Rh.+Azo.+Cd200ppm	0.07	0.48	20.1	47.8	4
Rh.+Azo.+Cd300ppm	0.10	0.55	23.9	45.6	4
L.S.D at 0.05	N.S	0.03	2.2	2.5	0.42
L.S.D at 0.01	N.S	0.05	3.0	3.3	0.56

N.S: Not significant

Table (4) shows the effect of various treatments on tested macro- and micro-nutrients content of wheat roots. The ranges of these macro-nutrients ranged from 0.76 to 1.33 % for N content, 0.11 to 0.85 % for phosphorus and 0.12 to 0.42% for potassium. It is worthy to mention that no significant differences were recorded between the different tested treatments for each of N, P and K in comparison with the positive control.

For both tested heavy metals Zn and Cd, Table (4) also indicatesthat irrespective of the treatment, the zinc amounts in roots ranged from 15.3 to 174 ppm while cadmium ranged from not detected amounts to 242.7 ppm. Furthermore, the wheat roots significantly accumulated Zn element in comparison with the positive control except the *Rhizobium* treatment. As to Cd element, it was recorded that this heavy element was accumulated in significant amounts in the roots treated with cadmium only. On the other hand, the treatments that do not receive cadmium, the roots had cadmium content ranged from not detected amounts to 2.00 ppm.

Table 4: Effect of various treatments on wheat roots content of tested macro-elements and heavy metalsat flowering stages.

Treatments	Macro-elements (%)			Heavy metals (ppm)	
	N	P	K	Zn	Cd
Control	1.33	0.36	0.39	15.7	N.D
Rhizobium (Rh.)	1.15	0.31	0.31	14.7	N.D
Azospirillum (Azo.)	1.10	0.28	0.31	32.0	N.D
Rh. + Azo.	1.19	0.29	0.28	19.0	N.D
Zn 200ppm	1.17	0.68	0.35	58.3	1.00
Zn 300ppm	1.15	0.23	0.28	97.7	N.D
Cd 200ppm	0.82	0.11	0.21	38.0	157.0
Cd 300ppm	0.81	0.15	0.28	15.3	195.0
Rh.+ Zn 200ppm	1.16	0.41	0.40	69.3	2.00
Rh.+ Zn 300ppm	1.13	0.27	0.42	105.3	N.D
Rh.+ Cd 200ppm	0.91	0.28	0.16	60.3	165.7
Rh.+ Cd 300ppm	0.84	0.11	0.12	22.7	224.7
Azo.+ Zn 200ppm	1.30	0.80	0.17	56.3	1.00
Azo.+ Zn 300ppm	1.13	0.32	0.28	174.0	N.D
Azo.+ Cd 200ppm	0.92	0.16	0.17	28.7	155.3
Azo.+ Cd 300ppm	0.92	0.18	0.18	28.7	242.0
Rh.+Azo.+Zn 200ppm	1.22	0.85	0.17	42.0	N.D
Rh.+Azo.+Zn 300ppm	1.11	0.71	0.23	63.3	N.D
Rh.+Azo.+Cd 200ppm	0.76	0.75	0.13	18.0	134.7
Rh.+Azo.+Cd 300ppm	0.68	0.76	0.13	72.3	213.0
L.S.D at 0.05	N.S	N.S	N.S	1.80	1.54
L.S.D at 0.01	N.S	N.S	N.S	2.27	2.00

N.S: Not significant; N.D: Not detected

For the impact of both heavy metals either alone or in combination with the remediating microorganisms (*Azospirillum* and/or *Rhizobium*) on wheat shoots contents of macro- nutrients, Table (5) demonstrates that no significant differences were recorded between the control and the various tested treatments. The shoots nutrients content ranged from 1.01 to 2.1%, 0.10 to 0.53% and 0.26 to 0.70% respectively for N, P and K irrespective of the treatments.

With regard to wheat shoots content of both heavy metals zinc and cadmium, Table (5) also shows that,in general, application of bio-treatments lowers the zinc quantity in shoots in comparison with the non bio-treated treatments. Concerning cadmium, it was found that the high significant quantities were recorded with the treatments that received such heavy metal at both its studied concentrations. It is worthy to mention that Zinc quantities in wheat shoots ranged from 4ppm (positive control) to 47ppm in the treatment that received 300ppm Cd as such; while the quantities of cadmium in ranged from not detected amounts to 24.3ppm.

Table 5: Effect of various treatments on wheat shoots content of tested macro-nutients and heavy metalsat flowering stage.

Treatment	Macro-elements (%)			Heavy metals (ppm)	
	N	P	K	Zn	Cd
Control	1.27	0.35	0.66	4	N.D
Rhizobium (Rh.)	1.13	0.27	0.60	15	N.D
Azospirillum (Azo.)	1.10	0.30	0.52	15	N.D
Rh. + Azo.	1.37	0.35	0.59	22	N.D
Zn 200ppm	1.27	0.31	0.59	19	N.D
Zn 300ppm	1.42	0.26	0.58	42	N.D
Cd 200ppm	2.10	0.26	0.42	18	1.11
Cd 300ppm	1.80	0.10	0.49	47	1.10
Rh.+ Zn 200ppm	1.19	0.37	0.70	21	3.00
Rh.+ Zn 300ppm	1.42	0.33	0.59	22	N.D
Rh.+ Cd 200ppm	1.10	0.14	0.33	26	7.00
Rh.+ Cd 300ppm	1.01	0.34	0.33	16	7.00
Azo.+ Zn 200ppm	1.40	0.42	0.46	23	N.D

Azo.+ Zn 300ppm	1.30	0.36	0.44	30	N.D
Azo.+ Cd 200ppm	1.16	0.14	0.42	18	18.7
Azo.+ Cd 300ppm	1.08	0.45	0.28	15	23.3
Rh.+Azo.+Zn 200ppm	1.49	0.47	0.44	24	N.D
Rh.+Azo.+Zn 300ppm	1.34	0.53	0.51	22	4.70
Rh.+Azo.+Cd 200ppm	1.06	0.42	0.35	8	11.3
Rh.+Azo.+Cd 300ppm	1.01	0.48	0.26	25	24.3
L.S.D at 0.05	N.S	N.S	N.S	1.40	1.40
L.S.D at 0.01	N.S	N.S	N.S	1.90	2.00

N.S: Not significant; N.D: Not detected

Discussion:

Hazardous organic and metallic residues or by-products can enter into soil,plants and sediments from processes associated with domestic, municipal, agriculture, industrial and military activities. Handling, ingestion, application to land or other distributions of the contaminated materials into the environment might render harm to humans, livestock, wild life, crops or native plants (Allen *et al.*, 2002).Recent studies in many continents have established that *Rhizobium*, the well-known nitrogen-fixing root-nodule endosymbiont of legume plants, also develops a natural, beneficial endophyticassociation with cereal roots growing in the same crop rotation (Dazzo*et al.*, 2003).With regard to wheat plants dry weight, either roots or shoots, it was observed that inclusion of both heavy metals bio-remediating*Azospirillum* and *Rhizobium* mitigated the detrimental effect of both heavy metals on wheat growth, where the treatment of *Rhizobium* + *Azospirillum* + Cd 300ppm induced increasing percentages reached 40 and 61.8 respectively for root and shoot in comparison with their corresponding treatments that received Cd 300ppm as such. In another meaning, application of the tested bio-remediation microorganisms neutralized the adverse effect of heavy metals on wheat growth. The obtained results are in agreement with those obtained by Gille*et al.* (1998), Elsgaard*et al.* (2001) and Filip (2002) who stated that bacteria can augment the remediation capacity of plants or reduce the phytotoxicity of the contaminated soil.Regarding the macro-nutrients content(N, P, K) of roots and shoots, it was found that no significant differences were recorded between the various tested treatments and the positive control.This means biological treatments inhibited the harmful effects of Cd and Zn at both applied concentrations. Concerning the tested heavy metals content of roots and shoots, it was found that both heavy metals were accumulated in the root system more than shoots. This finding is in agreement with that obtained by Liu *et al.* (2009) who studied the accumulation of toxic heavy metals by winter wheat and found that heavy metals content in roots was significantly higher than the aerial parts (stem, leaves and grains).

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