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The Effects of Heavy Metals and Chelating Agents on Phage Development and Enumeration of *Rhizobium* by Phage Counting in Different Soils

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Abstract: The effect of heavy metals and chelating agents on phage development was investigated using *Rhizobium phaseoli* and *Rhizobium leguminosarum* and their related phages. *Rhizobium phaseoli* phages were sensitive to Na^{+}, Mn^{2+}, Mg^{2+}, Fe^{3+}, Mo^{2+}, $HAsO_4^{2-}$, Ni^{2+} and Pb^{2+}. The invasiveness of the phages was reduced, but these materials had stimulatory effect on *Rhizobium leguminosarum* phages. Cr^{2+} and Cd^{2+} both had stimulatory effects on both sets of phages. It seems that the presence of different elements in soil unequally affects stability of *Rhizobium* phages. Agents such as $Na^{+}$and Mn^{2+} have no inhibitory effects on *Rhizobium phaseoli*, but effective on *Rhizobium leguminosarum*. The number of the phages is higher in area where the host is isolated. It is speculated that in farm soils, in addition to the presence of the host bacterium, phage stimulatory factors such as acidic pH, free ions like Mn^{2+}, Mg^{2+} and Fe^{3+} are effective in the changes of the number of phages. The highest number of phages is also indicative of the highest number of *Rhizobium* in each soil.

Key words: Phage · *Rhizobium* · Stimulatory · Inhibitory · Enumeration · Metal · Chelating agents

INTRODUCTION

For increasing the nitrogen fixation of the host legume, some strains of *Rhizobia* are commonly added to agricultural soil annually. The bacterial population of the soil is affected by many biological factors including predators and phages. Protozoa play key role in controlling bacterial number in the rhizosphere. It is shown where Lucerne crops were grown for several years, phages destroyed *Rhizobium* bacteria in soil and resulting in a widespread Lucerne sickness [1]. Phages and bacteria were found simultaneously in alfalfa root nodules [2]. It is suggested that selection or elimination of certain types of *Rhizobium* bacteria by phages, influences the evolution of bacterial populations [1]. The effects of Mn^{2+} on phage T7 and herpes simplex type-1 have already been reported by Tabor and Richardson [3] and Villiani *et al.* [4]. Partial disintegration of viral membrane of retroviruses by chelating factors (EDTA) [5], the action of ascorbate (Vitamin C) on suppression of human immunodeficiency virus [6] and the inhibitory effect of Cd^{2+} on phage T4D infection [7] is documented. The influence of molybdenum in the reduction of pathogenicity of tobacco mosaic virus [8], the role of cadmium cyanide as an adsorption cofactor for phages T2 and T4 [9], the toxicity of mercury to phages (11M15 of *Staphylococcus aureus* and P1 of *Escherichia coli*) [10] and inhibition of plaque production of Φ80 by ferrichrome at micro molar concentrations [11] are already reported. It seems that in addition to bacterial host, the soil elements also may affect the phage number present. Therefore, for biological control of phages by adding inhibitory agents or avoiding the use of soils with stimulatory factors, the effects of different elements with different concentrations on *Rhizobium* phages was investigated.

MATERIALS AND METHODS

1. Enrichment and isolation procedure: *Rhizobium phaseoli* and *Rhizobium leguminosarum* were isolated from healthy root nodules of phaseolus (bean) and pisum (pea) plants according to procedure explained by Jordan [12]. Yeast extract-manitol agar (YMA) containing manitol, 10.0g; KH_2PO_4, 0.5 g; $MgSO_4.7H_2O$, 0.2 g; $CaCO_3$, 4.0 g; Yeast water, 100 ml; Yeast extract (Merck), 0.4 g; agar, 15.0, g and distilled water, 1 liter; pH 6.8-7, sterilized at 121°C for 15 min, was used as culture medium.

2. Testing of stimulatory and inhibitory effects: Fifty micro liter of different concentrations of sterilized NaCl

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(0.8%, 1% and 1.2%), $MgSO_4$ (0.12, 0.24 and 0.36%), $MnSO_4$ (0.01, 0.1 and 0.5%), $FeCl_3$ (2, 3 and 4%), EDTA (1.2, 4, 5 and 10%), Vitamin C (20%, 22.5% and 25.0 %), $HgCl_2$ (0.1% and 0.05%), $Na_2MoO_4.2H_2O$(0.05%), $Na_2HAsO_4.2H_2O$(0.05%), Ni SO_4 (0.05%), $PbCl_2$(0.05%), $K_2Cr_2O_7$ (0.05%) and $Cd(NO_3)_2$ were inoculated in the center of lawn culture of each isolated *Rhizobium* (concentration: OD = 1.9, λ = 520 nm). Controls of bacteria plus each element without phage inoculation and phage alone were included in each test. Each concentration was tested in triplicate. The diameter of the plaques induced after four days was used as criteria for determination of inhibitory and stimulatory effects.

3. Phage preparation and counting: Soil sample (10g) was mixed with Calgon solution (Sodium metahexaphosphate 0.455 g, Sodium carbonate 0.045 g and 10 ml of distilled water), shaked and left on the table for 10 minutes. Then it was shaked for 7 minutes and also placed in ultrasonic batch (Transsonic 660/H, Germany) for 5 minutes and then centrifuged (Sigma 3E1, Germany) for 10 minutes at 2000 rpm. The upper solution was collected and centrifuged at 4°C at 13000 rpm (5810 Eppendorf, Germany). Two and half milliliter (OD = 1.9, λ = 520 nm) of *Rhizobium leguminosarum* isolated from Isfahan (region I), 5 ml of viral suspension and 5 ml of 1.2 % agar (Merck) were mixed under sterilized conditions and poured off on a plate containing nutrient agar [13].

4. Bacterial counting: Bacterial count was calculated according to procedure explained by Greenberg and Trussell [14] for estimation of total coliform number by using the following formula:

Total Rhizobium:

$$\text{Log } Y = 0.627 (\text{Log } X) + 1.864$$

Where:

Y= Total *Rhizobium*/100 ml and X=Rhizobiophage/100 ml

RESULTS AND DISCUSSION

According to plaque diameter, the stimulatory and inhibitory effects of different agents on *Rhizobium phaseoli* and *Rhizobium leguminosarum* and their phages are shown in Table 1 and 2,respectively. *Rhizobium phaseoli* phages were sensitive to Na^+, Mn^{2+}, Mg^{2+}, Fe^{3+}, Mo^{2+}, $HasO_4^{2-}$, Ni^{2+} and Pb^{2+}. The invasiveness of the phages was reduced, but these materials (except Mg^{2+}) had stimulatory effect on *Rhizobium leguminosarum* phages. Cr^{2+} and Cd^{2+} both had stimulatory effects on both sets of phages. EDTA had stimulatory effects on *Rhizobium leguminosarum* phages but its effect on *Rhizobium phaseoli* phages was not interpretable. Na^+, Mn^{2+} and Mg^{2+} had no effects on both bacteria while in the case of Fe^{3+}, it had negative effects on the growth of the bacteria. The effects of ascorbate on the two sets of phages were not interpretable. Hg^{2+} had no effects on *Rhizobium phaseoli* phages while showed stimulatory effects on *Rhizobium leguminosarum* phages. Mo^{2+} had inhibitory effects on *Rhizobium phaseoli* phages while

Table 1: The stimulatory and inhibitory effects of different agents on bacteriophages of *Rhizobium phaseoli*

		Mean of plaque diameter (cm)		
Substance	%	S+Bac.	Phage+Bac.	S+Phage+Bac.
NaCl	0.80*	0.00 (0.00) $	4.00 (0.36)	1.50 (0.45)
	1.00*	0.00 (0.00)	4.00 (0.36)	1.50 (0.26)
	1.20*	0.00 (0.00)	4.00 (0.36)	0.00 (0.00)
$MnSO_4$	0.01*	0.00 (0.00)	4.00 (0.36)	0.00 (0.00)
	0.10*	0.00 (0.00)	4.00 (0.36)	0.00 (0.00)
	0.50*	0.00 (0.00)	4.00 (0.36)	0.00 (0.00)
$MgSO_4$	0.12‡	2.25 (0.30)	4.00 (0.36)	0.50 (0.20)
	0.24*	0.00 (0.00)	4.00 (0.36)	0.70 (0.10)
	0.36*	0.00 (0.00)	4.00 (0.36)	0.75 (0.08)
$FeCl_3$	2.00*	1.40 (0.20)	4.00 (0.36)	1.90 (0.23)
	3.00‡	3.30 (0.26)	4.00 (0.36)	2.00 (0.17)
	4.00*	2.25 (0.18)	4.00 (0.36)	2.90 (0.20)
EDTA	1.20‡	3.70 (0.26)	4.00 (0.36)	0.60 (0.17)
	4.00‡	6.25 (0.18)	4.00 (0.36)	4.65 (0.47)
	5.00‡	7.20 (0.20)	4.00 (0.36)	4.70 (0.26)
	10.00‡	7.80 (0.26)	4.00 (0.36)	6.00 (0.26)
Vitamin C	20.00‡	10.00 (0.00)	4.00 (0.36)	3.35 (0.31)
	22.50‡	3.50 (0.30)	4.00 (0.36)	3.00 (0.30)
	25.00†	3.20 (0.36)	4.00 (0.36)	3.00 (0.34)
$HgCl_2$	0.10†	4.50 (0.26)	4.00 (0.36)	4.40 (0.20)
	0.05†	1.25 (0.08)	4.00 (0.36)	1.50 (0.36)
$Na_2MoO_4.2H_2O$	0.05*	0.00 (0.00)	4.00 (0.36)	2.50 (0.20)
$Na_2HAsO_4.2H_2O$	0.05*	0.00 (0.00)	4.00 (0.36)	3.25 (0.22)
$NiSO_4$	0.05*	0.00 (0.00)	4.00 (0.36)	2.30 (0.26)
$PbCl_2$	0.05*	2.25 (0.13)	4.00 (0.36)	2.60 (0.17)
$K_2Cr_2O_7$	0.05**	3.50 (0.20)	4.00 (0.36)	6.50 (0.17)
$Cd(NO_3)_2$	0.05**	6.00 (0.17)	4.00 (0.36)	7.25 (0.22)

S= Substance, Bac.= Bacterium, *= Inhibitory effects, †= No effect, **= Stimulatory effects, ‡= Not interpretable, $= Values in parentheses are standard deviations.

Table 2: The stimulatory and inhibitory effects of different agents on bacteriophages of *Rhizobium leguminosarum*

		Mean of plaque diameter (cm)		
Substance	%	S+Bac.	Phage+Bac.	S+Phage+Bac.
NaCl	0.80*	0.00 (0.00) $	2.25 (0.15)	0.00 (0.00)
	1.00**	0.00 (0.00)	2.25 (0.15)	10.00 (0.00)
	1.20**	0.00 (0.00)	2.25 (0.15)	10.00 (0.00)
$MnSO_4$	0.01*	0.00 (0.00)	2.25 (0.15)	0.00 (0.00)
	0.10**	0.00 (0.00)	2.25 (0.15)	10.00 (0.00)
	0.50**	0.00 (0.00)	2.25 (0.15)	10.00 (0.00)
$MgSO_4$	0.12*	0.00 (0.00)	2.25 (0.15)	0.00 (0.00)
	0.24*	0.00 (0.00)	2.25 (0.15)	0.00 (0.00)
	0.36*	0.00 (0.00)	2.25 (0.15)	0.00 (0.00)
$FeCl_3$	2.00**	1.60 (0.17)	2.25 (0.15)	10.00 (0.00)
	3.00**	2.10 (0.13)	2.25 (0.15)	5.50 (0.26)
	4.00**	2.50 (0.17)	2.25 (0.15)	10.00 (0.00)
EDTA	1.20**	0.00 (0.00)	2.25 (0.15)	10.00 (0.00)
	4.00**	2.00 (0.26)	2.25 (0.15)	10.00 (0.00)
	5.00**	2.20 (0.26)	2.25 (0.15)	10.00 (0.00)
	10.00**	2.30 (0.26)	2.25 (0.15)	7.00 (0.43)
Vitamin C	20.00‡	10.00 (0.00)	2.25 (0.15)	8.00 (0.52)
	22.50‡	10.00 (0.00)	2.25 (0.15)	10.00 (0.00)
	25.00‡	10.00 (0.00)	2.25 (0.15)	10.00 (0.00)
$HgCl_2$	0.10**	4.80 (0.45)	2.25 (0.15)	10.00 (0.00)
	0.05**	0.00 (0.00)	2.25 (0.15)	4.50 (0.17)
$Na_2MoO_4.2H_2O$	0.05**	0.00 (0.00)	2.25 (0.15)	3.50 (0.27)
$Na_2HAsO_4.2H_2O$	0.05**	0.00 (0.00)	2.25 (0.15)	3.80 (0.20)
$NiSO_4$	0.05**	0.00 (0.00)	2.25 (0.15)	4.20 (0.17)
$PbCl_2$	0.05**	0.00 (0.00)	2.25 (0.15)	4.50 (0.26)
$K_2Cr_2O_7$	0.05**	4.00 (0.41)	2.25 (0.15)	4.50 (0.60)
$Cd(NO_3)_2$	0.05**	0.00 (0.00)	2.25 (0.15)	4.00 (0.60)

S= Substance, Bac.= Bacterium, *= Inhibitory effects, †= No effect, **= Stimulatory effects, ‡= Not interpretable, $= Values in parentheses are standard deviations

Table 3: The number of phages and the estimated number of bacteria in the soils from different areas studied

Regions	No. of phage/g	Estimated No. of *Rhizobia*
Hssan-Sara farm soil	1370	2819
Jir-Gavabar farm soil	340	1175
Roudsar garden soil	160	742
Najvan farm soil	80	479
Isfahan(region I) garden soil	1630	3163

showed stimulatory effects on *Rhizobium leguminosarum* phages. Cd^{2+} had stimulatory effects on both phages. Pb^{2+} and $HAsO_4^{2-}$ had inhibitory effects on *Rhizobium phaseoli* phages while had stimulatory effects on the other. Ni^{2+} had inhibitory and stimulatory effects on *Rhizobium phaseoli and leguminosarum* phages, respectively. Among the tested materials, Mn^{2+} had the most inhibitory effects on *Rhizobium phaseoli* phages. The most stimulatory effects on *Rhizobium phaseoli* phages resulted from Cr^{2+}. The most inhibitoty effects on *Rhizobium phaseoli* resulted from Vit C(20%), while the most stimulatory effects on *Rhizobium phaseoli* was resulted from Na^+, Mn^{2+}, Mg^{2+}(0.24, 0.36%), Mo^{2+}, $HAsO_4^{2-}$ and Ni^{2+}. Mg^{2+}, Mn^{2+}(0.01%) and NaCl(0.8%) had the most inhibitory effects on *Rhizobium leguminosarum* phages. The most stimulatory effects on *Rhizobium leguminosarum* phages resulted from Na^+(1%,1.2%), Mn^{2+}(0.1,0.5%), Fe^{3+}(2, 4%), EDTA(1.2,4, 5%), Vit C(22.5,25%) and Hg^{2+}(0.1%). The most stimulatory effects on *Rhizobium leguminosarum* resulted from Na^+, Mn^{2+}, Mg^{2+}, EDTA(1.2%), Hg^{2+}(0.05%), Mo^{2+}, $HASo_4^{2-}$, Ni^{2+} and Pb^{2+}, while the most inhibitory effects on *Rhizobium leguminosarum* was resulted from Vit C. In enumeration of phages using Calgon solution, *Rhizobium leguminosarum* isolated from Isfahan (region I) was used as host. Two farm soils from Hassan-Sara and Jir-Gavabar and a garden soil from *Roudsa*r city in north of *Iran*, one farm soil from Najvan and one garden soil from Isfahan in center of Iran were tested. The number of the phages and the calculated count of bacteria are shown in Table 3. As it is shown, the highest the number of the phages, the highest the number of Rhizobia. The number of the phages is higher in area where the host is isolated.

In the case of iron and its effects on interaction and growth of the phages and their hosts, in contrast to many organisms such as bacteria which produce chelating factors that posses high affinities for iron as either Fe^{2+} or Fe^{3+} to suppress the cytotoxic potential of iron, viruses have not evolved mechanisms for actively scavenging host iron. Therefore, DNA viruses are directly dependent on iron for their replication as a result of the essential role that iron plays in catalytic center of ribonucleotide reductase (RR) which is supportive in the production of the dNTPs required for viral DNA synthesis [15]. Therefore, proper concentration of iron has activatory or stimulatory effects on viruses [15]. In this study the effects of different concentrations of Fe^{3+} was different in two sets of phages and their bacteria. It had inhibitory and stimulatory effects on phages of *Rhizobium phaseoli* and *Rhizobium leguminosarum*,respectively. Fe^{3+} had relatively negative effects on the growth of both bacteria.

In the case of magnesium, studies indicate the substitution of Mg^{2+} with Mn^{2+} promotes the replicative DNA polymerase of herpes simplex virus to bypass of bulky lesions which is not the case for phage T4 or δ polymerases. On the other hand this kind of substitution inhibits the 3' to 5' exonuclease activity of catalytic

subunit (UL30) of the replicative DNA polymerase of herpes simplex virus, but has lesser effect on that of T4 DNA polymerase. Manganese also induces conformational changes in the structure of UL30 [4]. On the other hand substituting Mn^{2+} for Mg^{2+} reduces the discrimination against dideoxynucleotides approximately 100-fold for DNA polymerase I and 4-fold for T7 DNA polymerase [3]. So the effects of Mn^{2+} on phages and other viruses seem to be contravening. In the current study Mn^{2+} revealed different inhibitory (on *Rhizobium phaseoli* phages) and stimulatory (on *Rhizobium leguminosarum* phages except for 0.01% concentration) effects, respectively.

After chelating of ions (Ca^{2+} and Mg^{2+}) by EDTA, the integrity of the retroviral membrane either disrupt or become permeable for the exogenous template of reverse transcriptase and hence reduce infectivity of it (inhibitory effect) [5]. The obtained results in this study revealed that EDTA has different effects on the two sets of phages. In the case of phages of *Rhizobium phaseoli* the effects were not interpretable, while it was stimulatory for the other.

The reports indicate that ascorbate (Vitamin C) mediates anti-HIV effect by diminishing viral protein production in infected cells and extra cellular virions [6]. In present study the effects of ascorbate on the two sets of phages were not interpretable.

The toxicity of heavy metals such as mercury for phages (11M15 *Staphylococcus aureus* and P1 *Escherichia coli*) has already been reported. The toxicity was less in sea water than in lake water. It is postulated that the lower toxicity of Hg in sea water is a result of formation of HgClSUB-3 SUB--/ HgClSUB-4 SUP-2- complexes [10]. In this study Hg^{2+} had no effects on *Rhizobium phaseoli* phages while showed stimulatory effects on *Rhizobium leguminosarum* phages.

The influence of Molybdenum in reduction of pathogenicity (inhibitory effects) of tobacco mosaic virus has already been reported by Sidhu [8]. In current study Mo^{2+} had inhibitory effects on *Rhizobium phaseoli* phages while showed stimulatory effects on *Rhizobium leguminosarum* phages.

It is shown that heavy metal Cd^{+2} has inhibitory effects on phage T4D infection [7]. It is also shown that if T4 is incubated with either an antibody specific for the sheath of the phage or an ionic species of Cadmium cyanide that causes contraction of the sheath, the phage particles will thereafter attach to a bacterial surface in the absence of tryptophan [9]. So the effects of Cd^{2+} on different phages are different. In this study Cd^{2+} had stimulatory effects on both phages.

Ferrichrome at micro molar concentration strongly inhibited plaque production by Φ80. The phage was not inactivated by preincubation with ferrichrome. It was suggested that a component of the ferrichrome uptake system may reside in the outer membrane of *Escherichia coli* K-12 and may also function as a component of the receptor site for phage Φ80 and that ferrichrome inhibition of the phage represents a competition for this common site [11]. So an inhibitory effect is involved. This was not the case in this study which stimulatory effects was observed in the two sets of phages studied.

Phage counting in different soils and their comparison showed the number of phages is higher in areas where the host is isolated. Phage counts of up to1370 PFU/gram was observed. It is speculated that in farm soils, in addition to the presence of the host bacterium, phage stimulatory (activatory) factors such as acidic pH, free ions like Mn^{2+}, Mg^{2+} and Fe^{3+} are effective in changes of the number of phages. On the other hand the highest number of phages is also indicative of the highest number of *Rhizobium* in each soil.

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