J. BIOL. ENVIRON. SCI.,
2012, 6(18), 253-261

Original Research Article

Isolation and Characterization of Nickel and Cadmium Tolerant Plant Growth Promoting Rhizobacteria from Rhizosphere of *Withania somnifera*

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Received: 15.08.2012; Accepted: 11.10.2012; Available Online: 25.01.2013

ABSTRACT

Plant growth promoting rhizobacteria PGPR are known to influence plant growth by various direct or indirect mechanisms. The present study aimed to isolate both nickel and cadmium tolerant plant growth promoting rhizobacterial communities of *Withania somnifera.* The representative PGPR strains of *Bacillus, Pseudomonas* and *Azotobacter* were tested for plant growth promoting activities and heavy metal tolerance pattern. The test rhizobacterium P-35 both metal tolerant with multiple PGP activities like IAA, ammonia, HCN, catalase etc. was subjected to seed germination test. Present study showed that seed inoculation with test bacterium significantly enhanced seed germination, root and shoot growth.

Key Words*: Ammonia, W. somnifera*, HCN (Hydrogen Cyanide), PGPR (Plant Growth Promoting Rhizobacteria), Heavy metal tolerance, Indole Acetic Acid (IAA).

INTRODUCTION

Withania somnifera (Ashwagandha) a wonder herb of India, also known as Indian Ginseng, is one of the most valuable herbs in the Ayurvedic and indigenous medical systems dating back more than 3,000 years. Ashwagandha, cultivated as an annual crop, is erect, 30-150 cm high and have fleshy and whitish-brown roots. *Withania somnifera* is anti-carcinogenic and is specific for wide range of conditions including arthritic inflammation, anxiety, insomnia, respiratory disorders including emphysema, asthma bronchitis and coughs, nervous disorders and has antioxidant properties (Ganzera et. al., 2003). In view of its varied therapeutic potential, it is the subject of considerable modern scientific attention

Pollution with heavy metals has received great attention in the last few years. Excessive accumulation of heavy metals is most toxic to most medicinal plants and results in decreased soil microbial activity and soil fertility. Among heavy metals pollutants nickel and cadmium need special attention due to their widespread occurrence and potential for their toxicities. Although nickel is considered as an essential micronutrient for plants, but is strongly phytotoxic at higher concentrations (Boominathan and Doran, 2002). Soil contamination with nickel has become a world-wide problem, leading to losses in agricultural yield and hazardous health effects as it enters the food chain (Guo and Marschner, 1996; Salt et al., 1999). The worldwide use of nickel in different branches of industries exposes environment to its uncontrolled emission into atmosphere and soil. Its influence on microbiological properties of soil is less recognized than that of other heavy metals.

Besides nickel, cadmium- significant pollutant due to its high toxicity, is of great concern in the environment. Contamination of soil with cadmium can negatively affect biodiversity and the activity of soil microbial communities (Chen et al., 2003). Cadmium can inhibit root and shoot growth, affect nutrient uptake and is frequently accumulated by agriculturally important crops (Sanita di Toppi and Gabrielli,1999). Studies on long term exposure of heavy metals showed decrease in microbial diversity and metabolic processes (Smit et al., 1997; Kojdroj and Van Elsas, 2001). Plants roots and soil microbes and their interaction improves metal bioavailability in rhizosphere (Sarvanan et al., 2007). Soil bacteria can be found in the rhizosphere and have been considered to promote plant growth directly or indirectly. Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that actively colonize plant roots and increase plant growth and yield (Wu et al., 2005). The mechanisms by which PGPRs promote plant growth are not fully understood, but are thought to include: - the ability to produce phytohormones (Egamberdiyeva, 2007; Shaharoona et al., 2006) - asymbiotic N2 fixation (Mrkovacki et al., 2001; Salantur et al., 2006)-against phytopathogenic microorganisms by production of

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siderophores, the synthesis of antibiotics, enzymes and/or fungicidal compounds (Ahmad et al., 2006; Bharthi et al., 2004; Jeun et al., 2004) and also - solubilisation of mineral phosphates and other nutrients (Cattelan et al., 1999).The common traits (Glick *et al.,* 1998) include production of plant growth regulators (plant hormones; auxin gibberellin, ethylene etc.), siderophore, HCN, NH_3 and antibiotics. In the present study, we studied the effect of heavy metal contamination particularly nickel and cadmium on abundance, diversity and plant growth promoting traits of rhizobacteria associated with rhizosphere of *Withania somnifera.* The present study helped us in obtaining more meaningful and realistic knowledge of distribution, diversity and composition of nickel and cadmium tolerant microbial communities associated with *Withania somnifera.*

MATERIALS AND METHODS

Sampling

The rhizospheric soil samples were collected from both normal and Ni-Cd treated pots growing *Withania somnifera* (Ashwagandha) from west of Sam Higginbottom Institute of Agriculture, Technology and Sciences, Allahabad, India. Randomly selected plants were uprooted carefully and the excess of soil was removed by gentle shaking and the soil adhering to roots formed composite samples. The collected samples were placed in plastic bags and kept at 4^0C in the laboratory until processed.

Isolation of rhizobacteria

Soil samples were serially diluted in upto 10^{-6} in sterile phosphate- buffered saline (Hi-media, Ph 7.2) and plated on the appropriate medium for isolating different rhizobacteria. All bacterial strains were isolated on their respective media; *Bacillus* was isolated on Nutrient agar medium, *Pseudomonas* on King's B medium (King et al., 1954) and *Azotobacter* on Ashby's medium (Norris and Chapman, 1968). Bacterial cultures were maintained on the respective slants. After incubation at 28-30^0 Cfor 2-3 days, bacterial colonies were counted; representative colonies were selected based on distinct types observed according to the morphological characteristics including pigments; colony form, elevation and margin; texture; and opacity (Simbert *et al.,* 1981).

Biochemical characterization of rhizobacteria

Selected isolates of *Bacillus* (50), *Pseudomonas* (45) and *Azotobacter* (30) were biochemically characterized by Gram's reaction, carbohydrate fermentation, oxidase test, O-F test, H2S production, IMVIC tests, NO2 reduction, and starch and gelatin hydrolysis as per the standard methods (Cappuccino and Sherman, 1992).

Characterization of rhizobacteria for PGP traits

Selected rhizobacterial isolates were characterized for plant growth promoting characteristics based on the standard procedures.

Production of Indole acetic acid

The bacterial strains were cultured in test tubes for 24h containing LB medium amended with 50 mg ml^{-1} of tryptophan. After incubation 2 ml of cell suspension was centrifuged at 1000 rpm for 10 min and 2-3 drops of orthophosphoric acid was added to the supernatant along with 4 ml of Solawaski's reagent. The tubes were kept at room temperature for 20 min. IAA production was indicated but the development of pink colour. Optical density was read at 530 nm and level of IAA production was estimated by standard IAA graph (Bric *et al.,* 1991).

Ammonia Production

Bacterial isolates were grown in peptone water at $30^{0\ C}$ for 4 days and 1 ml of Nessler's reagent was added. Production of ammonia was represented by development of faint yellow colour (Bakker *et al.,* 1987).

Siderophore production

Siderophore production was detected by the universal method of Schwyn and Neilands (1987) using blue agar plates containing the dye chrom azurol S (CAS). Orange halos around the colonies on blue were indicative for siderophore production.

Catalase production

Bacterial cultures were grown in nutrient agar medium for 18-24h. The cultures were mixed with appropriate amount of H_2O_2 on a glass slide to observe the evolution of oxygen.

Phosphate Solubilization Activity

All the bacterial isolates were grown on Pikovskaya medium and Phosphate Solubilization activity was indicated by the formation of a clear halo zone around the bacterial growth after 3 days of incubation.

HCN Production

The isolates were streaked on King's B medium amended with $4.4gl^{-1}$ of glycine. The plates were covered with sterile filter paper impregnated with 0.5% picric acid in 2% sodium carbonate, sealed with parafilm and incubated for 4 days (Bakker *et al.*, 1987). Development of yellow colour on the filter paper indicates the positive results.

Heavy metal and physicochemical analysis of soil

For determination of heavy metals content of soil samples were acid digested and Cd, Ni, Pb and Cr were estimated using a Direct Current Plasma (DCP) spectrophotometer. Soil samples were analysed for physicochemical parameters like pH, conductivity, total organic carbon, total nitrogen, etc. Organic carbon was analysed by the Walkely and Black dichromate oxidation method (Blakemore et al., 1972). Soil pH was measured in soil: water (1:2.5) slurry using a glass electrode. Nitrogen was measured by Kjeldahl method.

Heavy metal tolerance

The selected bacterial strains were tested for their resistance to heavy metals by agar dilution method (Cervantes et al., 1986). Freshly prepared agar plates were amended with various soluble heavy metal salts namely Cd, Ni, Co, Zn, Hg, Cu, Cr, Ag, As and Pb at various concentrations ranging from 25 to 400 µg ml-1 were inoculated with overnight grown cultures. Heavy metal tolerance was determined by the appearance of bacterial growth after incubating the plates at room temperature for 24-48h.

Seed germination test

Surface sterilized and uniform size Ashwagandha's (*Withania somnifera)* seeds dipped in gum acacia were inoculated with test bacterium P35 and placed in petridsihes. Germination test was carried out by the paper towel method. Treated and untreated seeds were kept for germination in dark for two days in an incubator at 27 ± 2^0 C. Number of germinated seeds was counted. After 7 days of incubation, the seedlings were taken out for various studies like seed germination percentage, vigor index, root and shoot length and the data were recorded.

Vigor index was calculated by following formula:

Vigor index = (Mean Root Length + Mean shoot length) × % germination

RESULTS AND DISCUSSION

Heavy metals are known to alter the functional diversity of soil, microbial community and impair specific pathways of nutrient cycling (Ramteke et al., 2012). In the present investigation slight depletion of rhizobacterial population was noticed in Ni-Cd treated rhizosphere as compare to normal rhizosphere of *Withania somnifera* (Table 1). The bacterial population (cfu gm^{-1}) ranged from 3.5×10^6 of *Bacillus* spp., 4.5×10^6 of *Pseudomonas* spp. and 2.0×10^6 of *Azotobacter* spp. in the normal rhizosphere while 1.4×10^6 of *Bacillus* spp., 1.8×10^6 of *Pseudomonas* spp. and 0.8×10^6 of *Azotobacter* spp. in Ni-Cd exposed rhizosphere. The population of

Pseudomonas dominated in both the rhizosphere. Previous studies have documented that heavy metal contamination results in the reduction of bacterial diversity, biomass and metabolic activity (Ellis et al., 2003; Ramteke et al., 2012). It is known that heavy metal pollution causes selection and/or development of tolerant microorganisms.

Table 1. Microbiological analysis of soil samples collected from rhizosphere of *Withania somnifera*.

Soil type	Total viable count (cfu $g^{-1} \times 10^6$)		
	Bacillus	*Pseudomonas*	*Azotobacter*
Normal soil	3.5	4.5	2.0
Ni-Cd treated soil	1.4	1.8	0.8

Rhizobacterial isolates *Bacillus* (25), *Pseudomonas* (28) and *Azotobacter* (17) from both normal and Ni-Cd treated rhizosphere, were selected for detail study. Morphological and cultural characteristics of rhizobacterial isolates is given in Table 2. The organic content in soil samples is considered as one of the key determinants driving the microbial community structure (Roane and Kellogg, 1996; Zhou et al., 2002). Soil with high heavy metal content also had a high organic content, which can probably explain the maintaince of the microbial community diversity due to lack of competition, as suggested by others authors (Zhou et al., 2002; Branco et al., 2005; Ramteke et al., 2012). There was a slight increase in soil health by PGPR even in the presence of high Ni and Cd levels (Table 3).

Table 2. Morphological and cultural characteristics of rhizobacteria associated with rhizosphere of *Withania somnifera*.

Morphological and Biochemical Characterization	***Bacillus* (25)***	***Pseudomonas* (28)***	***Azotobacter* (17)***
Grams reaction	G +ve	G –ve	G -ve
Shape	Rods	Rods	rods
Pigment	-	+	+/-
Lactose	+	-	+
Dextrose	+	+	+
Sucrose	+	+	+
Mannitol	+	-	+
Oxidase	-	+	+
OF test	-	+	+
H_2S production	-	+	-
Indole	-	-	+
Methyl red	-	-	-
Vogues Proskauer	+	-	+
Citrate utilization	+	+	+
Nitrate reduction	+	+	+
Starch hydrolysis	+	+	+
Gelatin hydrolysis	-	-	-

Table 3. Heavy metal content and physicochemical analysis of soil samples.

Parameters	Normal Soil	Ni-Cd Treated Soil
pH	6.6	7.5
Conductivity (µm cm^{-1})	0.33	0.27
Total organic carbon (%)	0.37	0.73
Organic matter	1.06	1.23
Nitrogen availability		
Nitrate kg ha^{-1}	50.65	50.62
Ammonical kg ha^{-1}	14.62	18.83
Heavy metals (mg/kg)		
Cadmium	1.08	8.56
Nickel	1.03	8.07
Lead	0.50	0.61
Copper	0.78	0.70
Mercury	2.08	2.01
Arsenic	2.07	2.18

Microorganisms have developed the mechanisms to cope with a variety of toxic metals for their survival in the environment enriched with such metals (Martin-Laurent et al., 2004). As expected, tolerance to heavy metals was found more predominant among rhizobacteria from Ni-Cd treated soil as compared to normal (Table 4). Among all the rhizobacteria studied, tolerance to heavy metal was observed less frequently in *Azotobacter* spp. (Table 4). None of the isolates from normal rhizosphere were tolerant to most of the heavy metals upto 100µgml^{-1}. *Pseudomonas* isolates dominates in tolerating most of the heavy metals upto 200µgml^{-1}. However, many isolates from normal rhizosphere also exhibited metal tolerant activity such as *Bacillus* (PB, Co) and *Pseudomonas* (Zn, Co, Pb).

Table 4. Heavy metal tolerance of selected of rhizobacterial isolates associated with rhizosphere of *Withania somnifera.*

			Heavy Metal Tolerance (µg ml^{-1})									
Organism	**Culture No.**	**Rhizosphere**	**Co**	**Zn**	**Hg**	**Cu**	**Cr**	**Ag**	**Cd**	**Ni**	**As**	**Pb**
Bacillus	B 5	Ni-Cd	200	400	100	100	100	200	200	400	200	400
Bacillus	B 11	"	200	400	50	50	100	200	100	400	200	200
Pseudomonas	P 9	"	200	200	50	200	50	100	100	200	100	400
Pseudomonas	P 28	"	200	200	100	50	50	100	100	200	50	400
Pseudomonas	P 35	"	200	200	100	200	100	200	200	200	200	200
Pseudomonas	P 40	"	200	100	100	100	200	200	100	200	100	400
Pseudomonas	P 42	"	200	200	100	100	200	100	100	100	100	200
Azotobacter	A 5	"	50	50	60	100	50	100	25	50	50	50
Azotobacter	A 9	"	50	50	50	100	50	50	25	25	100	50
Bacillus	B 15	Normal	200	100	50	50	200	100	100	200	200	200
Bacillus	B 18	"	200	100	25	50	100	100	100	200	100	100
Pseudomonas	P 15	"	50	100	50	50	100	50	25	50	50	100
Pseudomonas	P 30	"	100	100	100	50	100	100	25	50	100	100
Pseudomonas	P 38	"	100	200	100	100	100	100	100	200	100	100
Pseudomonas	P 45	"	200	100	100	100	100	200	100	200	100	100
Azotobacter	A 10	"	25	50	100	50	50	50	50	100	50	25
Azotobacter	A 12	"	50	25	50	50	25	50	50	100	25	100

Burd and co-workers (1998) found that by decreasing heavy metal toxicity, PGPR increase plant growth. Several workers reported adverse effect of heavy metal pollution on PGP characteristics. In the present study rhizobacteria tolerant to multiple heavy metals also exhibited two or more PGP activities (Table 5).

Table 5. Plant Growth Promoting Characteristics of rhizobacteria associated with rhizosphere of *Withania somnifera*.

			Plant Growth Promoting Characteristics				
Organism	**Culture No.**	**Rhizosphere**	**IAA**	**CATALASE**	**HCN**	**AMMONIA**	**SIDEROPHORE**
Bacillus	B 5	Ni-Cd	+++	+	++	+++	+
Bacillus	B 11	"	++	+	++	++	-
Pseudomonas	P 9	"	+++	+	+++	+++	-
Pseudomonas	P 28	"	+++	+	+++	++	+++
Pseudomonas	P 35	"	++++	+	+++	+++	+++
Pseudomonas	P 40	"	++	+	++	+++	-
Pseudomonas	P 42	"	+++	+	+	++	+
Azotobacter	A 5	"	-	+	-	-	-
Azotobacter	A 9	"	+	+	+	++	-
Bacillus	B 15	Normal	++	+	+	-	+
Bacillus	B 18	"	++	+	++	++	-
Pseudomonas	P 15	"	+++	+	-	++	-
Pseudomonas	P 30	"	++	+	++	++	+
Pseudomonas	P 38	"	-	+	+	++	-
Pseudomonas	P 45	"	++	+	+	++	-
Azotobacter	A 10	"	-	+	-	-	-
Azotobacter	A 12	"	+++	+	++	+	-

+ : Poor ; ++ : Fair; +++ : Good; ++++ : Excellent

It is also apparent from the table that more PGPR isolates tolerant to elevated levels of heavy metals were obtained from Ni-Cd treated rhizosphere. It is clear that Ni-Cd exposure do not have profound inhibitory influence on PGP characteristics of rhizobacteria. On the other hand more isolates from Ni-Cd exposed soil exhibited PGP activities as compared to their counterparts from normal. All the rhizobacteria isolated from both normal and Ni-Cd exposed rhizosphere of *Withania somnifera* are found to produce plant growth hormone like IAA, HCN, Catalase etc. (Fig. 1). Bacterial IAA plays a major role in root elongation, it may promote root growth directly by stimulating plant cell elongation or cell division or indirectly by influencing bacterial ACC deaminase activity. Higher number of isolates of *Pseudomonas* spp. and *Bacillus* spp. from Ni-Cd exposed rhizosphere of *Withania somnifera* showed more IAA production as compared to their counter parts from normal soil. Catalase activity in the bacterial strains may be potentially very advantageous and bacterial strains showing catalase activity must be highly resistant to environmental, mechanical and chemical stress. All isolates from both the rhizosphere were able to produce catalase. Another important trait of PGPR that may indirectly influence the plant growth is the production of ammonia. Mostly all the isolates from both the rhizosphere were able to produce ammonia. However, ammonia production was observed less frequently in *Azotobacter* spp. as compared to other rhizobacteria. Majority of the isolates from both rhizosphere were significant HCN producers. Siderophores may directly stimulate the biosynthesis of other antimicrobial compounds by increasing the availability of these minerals to the bacteria. Production of Siderophore and Phosphate solubilizing ability was comparatively less frequent than other traits. Siderophore and phosphate solubilizing ability was detected significantly higher among isolates of *Pseudomonas* spp. Three isolates of *Pseudomonas* spp were strong siderophore producers and out of them two were from Ni-Cd exposed rhizosphere. Several studies have established a correlation between bacterial antibiotic resistance and metal tolerance (Ramteke, 1997; Verma et al., 2001).

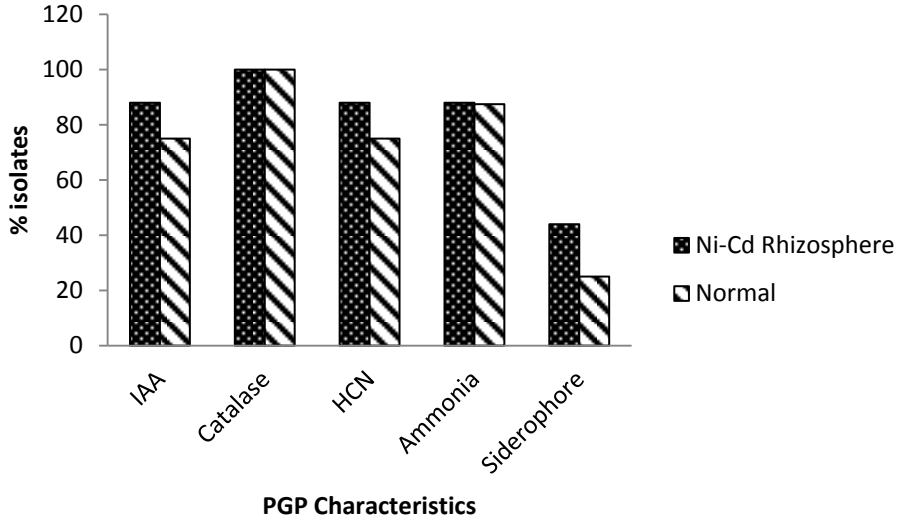

Figure 1. Plant growth promoting characteristics of rhizobacterial isolates associated with rhizosphere of *Withania somnifera.*

In the present study, one of the potential bacterial isolate P 35 tolerant to elevated level of multiple heavy metals (As, Pb, Ni Cd etc.) and efficient IAA, siderophore and Ammonia producer was subjected to elucidate its role in stimulation of root and shoot growth in presence of heavy metals and improved seed germination, seedling vigor, seedling emergence and seed stand over the control. The root growth promotion by beneficial strains of *Pseudomonas* has often been related to their active but low level of auxins secretion in the rhizosphere (Patten and Glick, 2002). From the results, it is apparent that test bacterium had significant impact on stimulation of root and shoot growth (Table 6). Roots from seeds treated with bacterial cultures were an average 40% longer than untreated control seeds after 9 days. Influence of PGP activity on root and shoot length in the presence of heavy metals are presented in Table7 and Table 8 respectively. Similar findings were supported by other workers (Gholami et al., 2009; Ramteke et al., 2012).

Table 6. Effect of P 35 on shoot and root growth of *Withania somnifera*.

Shoot/ Root length	Days after germination	Length (cm)*	
		Control	Seed coated with PGPR strain
Shoot Length	3rd	1.8	2.5
	5th	2.2	3.5
	7th	3.8	6.2
	9th	5.7	6.6
Root Main Lateral	9th	5.7	9.5
1st		3.6	5.3
2nd		3.0	4.8
3rd		2.8	4.2
4th		1.2	2.7

* Average of triplicate

Table 7. Effect of P 35 on root main lateral in Ashwagandha in presence of heavy metals 9 days after germination.*

Root Main Lateral (cm)*	Control		Arsenic		Cadmium		Nickel	
	Normal	Treated	Normal	Treated	Normal	Treated	Normal	Treated
Main Root	6.2	9.5	4.1	6.8	4.8	7.1	6.0	9.5
1st	4.1	5.6	3.0	6.0	3.2	6.8	4.0	5.4
2nd	4.0	5.1	2.8	5.2	3.0	5.9	3.8	5.0
3rd	3.8	4.6	2.5	4.8	2.8	4.6	3.5	4.5
4th	2.2	3.0	1.2	2.6	1.8	2.8	2.1	3.0

* Heavy metals 100 µg ml^{-}

Table 8. Effect of P35 on shoot growth in Ashwagandha in presence of heavy metals.*

Days After Germination	Control		Arsenic		Cadmium		Nickel	
	Normal	Treated	Normal	Treated	Normal	Treated	Normal	Treated
3rd	2.5	4.8	1.8	4.1	2.3	3.7	2.3	4.6
5th	5.7	7.2	4.2	7.0	4.6	7.1	5.4	6.8
6th	8.3	10.2	6.1	9.6	7.2	9.8	8.1	9.8
7th	11.0	13.5	8.2	11.5	8.6	12.0	10.2	13.0

* Heavy metals 100 µg ml^{-}

Heavy metals exert toxic effects on the microorganisms through various mechanisms and metal tolerant bacteria could be isolated and selected for their potential application in the bioremediation of contaminated soil. Heavy metals resistant microorganisms which grow not only under contaminated environment but also possess growth promoting properties are of particular importance for the degraded and polluted land use practices (Joseph et., al 2007; Ramteke et al., 2012).

CONCLUSIONS

The present study indicates that the rhizobacterial population was slightly affected by Ni-Cd exposure. However, majority of the isolated strains f both the rhizosphere possessed one or more PGP traits. Isolates tolerant to elevated levels of heavy metals and test bacterium P35 are outstanding for PGP potential. Selection of microorganisms both metal tolerant and efficient in producing PGP compounds can be useful to speed up the recolonization of the plant rhizosphere in polluted soils. Analysis of genetic structure of a rhizobacterial community is of practical importance and further evaluation of the heavy metal tolerant PGPR on soil-plant system is needed to understand their potential efficacy.

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