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Screening of *Trichoderma spp and Pseudomonas spp*. for their Biocontrol Potential against Phytopathogens of Vanilla

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Abstract

Fungal pathogens of vanilla such as *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotium rolfsii* were isolated from naturally infected vanilla plants and an attempt were made to minimize the damage caused by the pathogen using biocontrol agents *Trichoderma harzianum*, and *Pseudomonas fluorescens* isolated from soil. The combined inoculation of of *Trichoderma harzianum* with *Pseudomonas fluorescens* treatment showed maximum disease suppression followed by the single inoculation of *Pseudomonas fluorescens*, *Trichoderma harzianum*, *Pseudomonas putida*, *Trichoderma virens*, respectively in decreasing order. The results clearly indicated that these bio-control agents suppressing the disease incidence. Concerning the interaction effect between used antagonistic microorganisms and method of treatments, there was a highly significant effect. These results suggested that using of *Trichoderma harzianum* with *Pseudomonas fluorescens* through soil mixing plus root dipping treatment could be provided not only additional protection against crop loss due to *Fusarium* diseases but also significantly increased vegetative growth of vanilla. The mechanism of biocontrol involved the production of volatile and non volatile organic acids, siderophore, chitinase, peroxidise and salicylic acid. Application of biocontrol agents for crop protection is very significant as it has several advantages such as possibility of multiple pathogen suppression, low cost and promotion of soil fertility.

Keywords: Antagonism, Bio-agents, Root rot disease, Siderophore.

Introduction

Biological control of plant pathogens is considered as a potential control strategy in recent years, because chemical control results in accumulation of harmful chemical residues, which may lead to serious ecological problems. At present, effective management of plant diseases & microbial contamination in several agricultural commodities is generally achieved by the use of synthetic pesticides. Vanilla is the second most expensive spice after Saffron and is still big money spinners. It is often referred to as green gold and princess of spices. In India it is grown in an area of 2545 hectares covering Karnataka, Kerala and Tamilnadu with production of about 100 metric tonnes (Kuruvilla *et al.,* 2004). Chemical and physical methods are widely used to control the disease. Wide spread use of chemical to control plant diseases has undesirable results.

However it disturb soil environment, contaminate underground water, result in the development of resistant races of pathogen and cause health risk to human. An alternative to these chemicals is the use of certain biocontrol agent, which are inexpensive, and ecofriendly, and has no harmful effect on human population. The use of biocontrol agent besides enhancing the growth also triggers defence mechanisms in plants. (Kloepper *et al*., 1992).

Materials and Methodology

Isolation of Plant Pathogens

Pathogens causing wilt and rot diseases of vanilla namely *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotium rolfsii* were isolated from naturally infected vanilla plants using standard isolation techniques (Riker and Riker, 1936). The infected plant parts were collected and brought to the laboratory. Samples were washed under tap water and dried using blotting paper. Small bits of infected portions along with healthy areas were surface sterilized with 1% sodium hypochloride and then washed repeatedly with sterile water. The sterilized bits were then placed in sterile petridishes containing oatmeal agar medium and incubated at 28 ± 2°C. Mycelial bits were transferred to sterile petridishes containing Carrot agar (CA) medium; later it was purified by hyphal tip method and transferred to Potato sucrose Agar (PSA) and Potato dextrose agar (PDA) slands and pure cultures of the pathogens were maintained for further studies.

Isolation of *Trichoderma* sp. and *Pseudomonas* spp.

The rhizosphere soils of vanilla along with roots were collected from vanilla growing areas in kerala state and were used for the isolation of *Trichoderma* by serial dilution Figure techniques (Johnson and Curl 1972), using Martin's Rose Bengal Streptomycin agar medium and malt extract agar medium. For this 10^{-3} and 10^{-4} dilution of soil samples were used. *Trichoderma harzianum* obtained from microbial culture collection centre (MTCC 801) Chandigarh was used as reference strain.

Pseudomonas spp. were isolated form soil using King's B (KB) agar medium following serial dilution and plating techniques. The Figures were incubated at 30°C for 48 hours. Colonies that came up on KB Figures were observed under UV light on a transilluminator. The green fluorescent colonies under UV light were picked up, purified by repeated streaking on the same medium and checked for their fluorescens. *P. fluorescens* obtained from Microbial Culture Collection Centre (MTCC 1748) Chandigarh was used as reference strain.

Screening of biocontrol agents

In vitro *screening of the isolated* Trichoderma *spp. for their biocontrol activity*

All the 20 isolates were tested *In vitro* for their biocontrol activity against three fungal pathogens viz. *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotium rolfsii* by dual culture method outlined by Skidmore and Dickinson (1976). Mycelial discs (6 mm) of pathogen from seven day old culture grown on PDA was inoculated aseptiecally on one side of petridishes containing PDA and incubated at 28 ± 2°C for 24 hours. After this 6 mm disc of *Trichoderma* isolates were inoculated in the same petridishes 3.5 cm away from the pathogen and incubated for 5 days. Three replications were maintained for each isolate. Pathogen and Trichoderma isolates grown in monoculture served as control. Growth measurements were taken at regular intervals of 24 h of inoculation. Zone of inhibition was calculated and compared with control Figures. Inhibition of mycelial growth of the pathogen was calculated using the formula suggested by Vincent, (1976).

$$\text{Percent inhibition (PI)} = \frac{T}{C} \times 100$$

C – Growth of pathogen in control

T – Growth of pathogen in dual culture

In vitro Screening of *Pseudomonas* spp. for their biocontrol activity

Ten predominant rhizobacteria isolated from different locations along with standard cultueres of *P. fluorescens* were tested for their antagonist effect against three fungal pathogens viz. *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotium rolfsii* by dual culture method.

Evaluation of biocontrol potential of isolates against fungal pathogens of Vanilla under green house condition

A pot culture experiment was conducted to assess biocontrol potential of the isolates *T.harzianum* ,*T . virens* ,*P.fluorescens* and *P.putida* against fungal pathogens of vanilla by dual culture inoculation of the pathogens and biocontrol agents (Ganeshan and Gnana Manickam, 1987). The trials with vanilla cuttings were carried out in two phases by cross inoculation methods. For seedling

inoculation, the aqueous inocula of pathogen and fungal antagonists were prepared by macerating the respective agar cultures in a mixer grinder using distilled water. For bacterial antagonists, the inocula used were the broth cultures. The concentration of the pathogen and antagonists was estimated using dilution Figure technique. The experiment was conducted with 27 treatments consisting of 4 isolates, two reference strains, three fungal pathogens and control with only pathogens. The soil was having a pH of 7.2, 0.18%, organic carbon 127 kg/ha available nitrogen, 26 kg/ha of available phosphorous and 346 kg/ha of potassium. The soil had bacterial population of $4.1x\ 10^5$ cfu/g, fungi $3.45x\ 10^3$ cfu/g and actinomycetes $2.54x10^3$ cfu/g. The experiment was conducted with nine treatments with three replications.

Aqueous inocula of the bio agents were first drenched at the base of the cuttings followed by cross inoculation with the pathogens, *F.oxysporum ,Rhizoctonia solani* and *sclerotium rolfsii* in the respective control plants after seven days. The experiments was done in three replicates in each treatment as detailed in table 2

Design : CRD

Treatments : 27

Replication : 3

The plants were allowed to grow up to 60 days and observation on the percent leaves infection was calculated by using the formula

Percentage of leaves infection = No.of leaves infected x 100

Total number of leaves

Results and Discussion

Isolation of Phytopathogens

The pathogens causing wilt and rot disease of vanilla *Fusarium oxysporum, Rhizoctonia solani* and *Sclerotium rolfsii* were isolated from naturally infected vanilla plants using standard isolation technique. They were purified by hyphal tip method, transferred to Potato Sucrose Agar and PDA slants and pure cultures of the pathogens were maintained for further studies.

Identification of *Trichoderma* spp.

The colonies were wooly and green. Conidiophores were branched like a pyramidal arrangement. Conidia were unicellular, round or ellipsoidal and were grouped in sticky heads at the tips of the phialides which were the characters of *Trichoderma* spp. (Figures 1a &1b). The dendogram are show*ing* evolutionary relationship of *Trichoderma spp* JN 863298 and *Trichoderma harzianum* JN000305 with some related organisms. From the phylogenitic tree (Table1) it is evident that the isolate *Trichoderma spp* JN 863298 is closely related to *Hypocrea lixii* strain DAOM 231412.

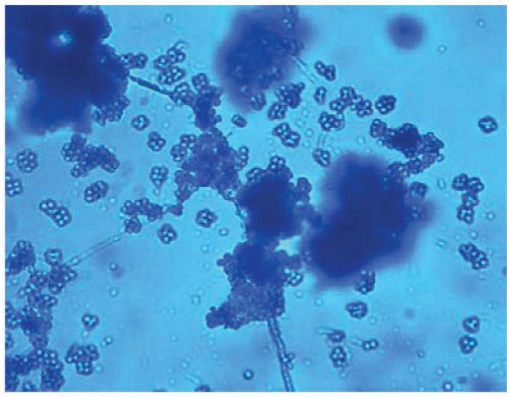
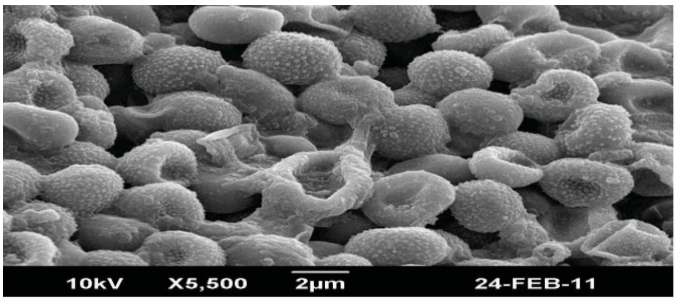
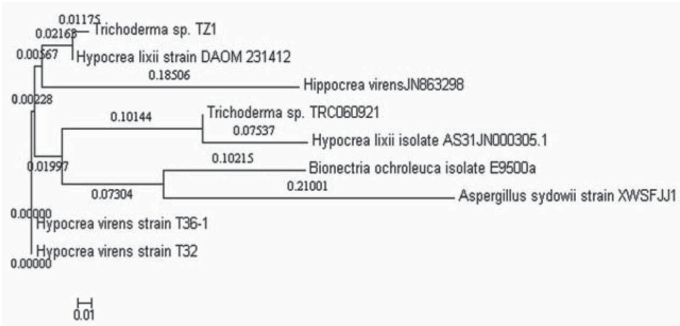


Figure 1a: *Trichoderma* spp.



1b: The spores of Trichoderma in Scaning Electron Microscope at 5,500x

Table1: Phylogenetic tree showing relationship between *Trichoderma spp* and *Trichoderma harzianum* with the nearest neighbours



Identification of *Pseudomonas* spp.

For the identification of efficient antagonist rhizobacteria morphological, cultural, biochemical and molecular tests were done and identified according to Bergey's manual of systematic bacteriology.

Molecular Identification of *Pseudomonas* spp.

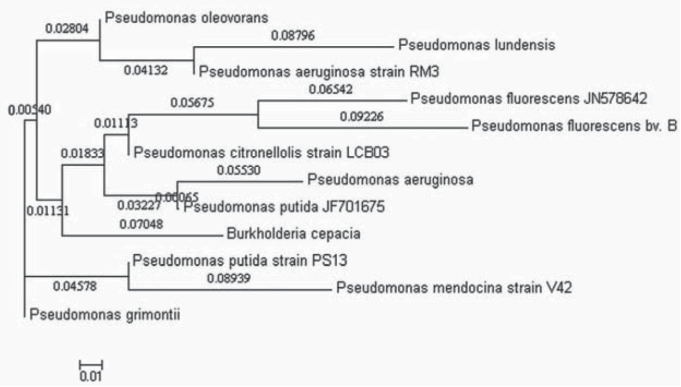
Antagonistic rhizobacteria were subjected to 16S rDNA analysis (Figure2). The sequence generated from the analysis were compared with some related organisms with Basic Local Alignment search Tool (BLAST) using the program BLASTIN 2.2.24+ NCBI. The isolate P4 showed close similarity with *Pseudomonas putida* and the isolate P7 showed close similarity with *Pseudomonas fluorescens.* The sequences of the isolated organisms were deposited in the NCBI gene bank and culture collection centre and got the accession number **JF701675** for *P. putida* (P4) and **JN578642** for the isolate *P. fluorescens* (P7)

The figure (1)shows the dendogram showing evolutionary relationship of *Pseudomonas fluorescens* JN578642 and *Pseudomonas putida* JF701675with some related organisms. From the phylogenitic tree it was evident that the isolate *P. fluorescens* JN578642 is closely related to *Pseudomonas fluorescens* bv. B and the isolate *Pseudomonas putida* JF701675 is closely related to *Pseudomonas aeruginosa* HQ271084 (Table 2).

Figure 2: Agarose gel electrophoresis of DNA M Pf7 P4

M : Marker DNA
Pf 7 : *Pseudomonas fluorescens*
P4 : *Pseudomonas putida*

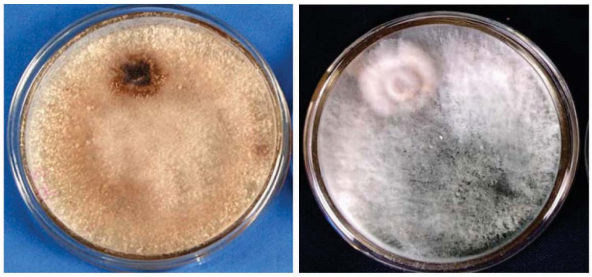
Table 2: Phylogenetic tree showing relationship between *Pseudomonas fluorescens* and *Pseudomonas putida* with the nearest neighbours



Screening of *Trichoderma spp* against phytopathogens

Antagonistic effect of Trichoderma isolates against phytopathogens were tested by dual culture method outlined by Skidmore and Dickinson (1976). For this, 10 predominant isolates of *Trichoderma spp* isolated fromvanilla growing areas were used (Table 3). The *Trichoderma* isolate Tv5 showed maximum inhibition against *Fusarium oxysporum* (87.77%) *Rhizoctonia solani* (87.88%) and *Sclerotium rolfsii* (80.67%) and were selected for further studies. Tv8 showed least inhibition against *Fusarium oxysporum* (11.11%) and Tv6 showed the least inhibition against *Rhizoctonia solani* (12.78%). All the isolates except Tv3 showed inhibition against *Sclerotium rolfsii* after 5 days of inoculation (Figure 3a,3b, 3c).

Figure 3: Screening of *Trichoderma spp* against phytopathogens
3a: *Invitro* inhibition of *Fusarium oxysporum by Trichoderma spp.*



3b: *Invitro* inhibition of *Rhizoctonia solani* by *Tricho**derma spp***

3c: *Invitro* inhibition of *Sclerotium rolfsii* by *Trichoderma spp.*

Table 3: *In vitro* screening of *Trichoderma spp* against Phytopathogens (Percentage of inhibition after 5days)

Sl. No	Biocontrol agent	*Fusarium oxysporum*	*Rhizoctonia solani*	*Sclerotium rolfsii*
1.	Tv1	44.44	76	63.333
2.	Tv2	22.22	71.11	71.111
3.	Tv3	60	16.333	0
4.	Tv4	65.555	21.111	13.333
5.	Tv5	87.77	87.88	80.67
6.	Tv6	23.33	12.78	58.88
7.	Tv7	68.88	62.22	24.44
8.	Tv8	11.111	24.44	27.90
9.	Tv9	83.00	67.777	66.67
10.	Tv10	13.33	34.44	55.55

*Values are average of three replicates

Screening of *Pseudomonas* spp. against phytopathogens

All the 10 isolates of *Pseudomonas* spp. were tested for their biocontrol potential against three fungal pathogens and the results are presented in Table (4). The *Pseudomonas* spp. isolate P7 showed maximum inhibition against *Fusarium oxysporum* (60.23%) *Rhizoctonia solani* (58.67%) and *Sclerotium rolfsii* (54.19%). The isolate P4 showed 55.55% inhibition against *Fusarium oxysporum*,

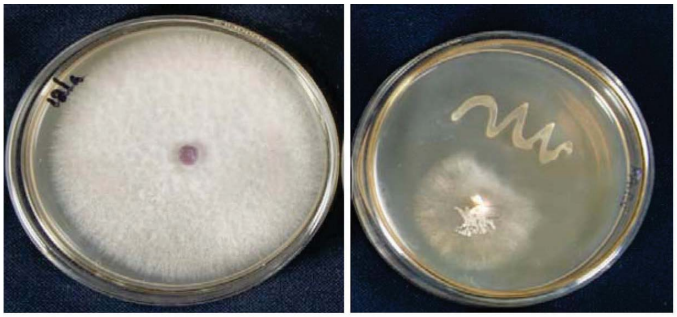
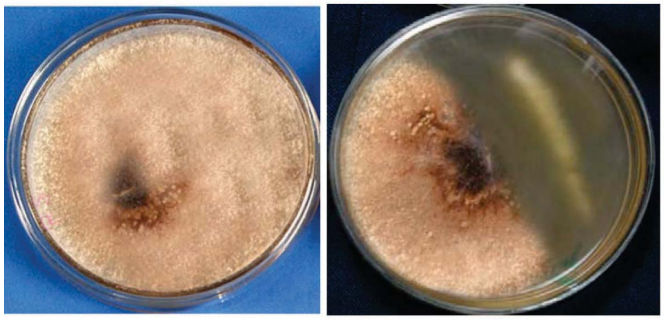


Figure 4: Screening of *Pseudomonas* spp. against phytopathogens
4a: *In vitro* inhibition of *Fusarium oxysporum by Pseudomonas* spp.



4b: *In vitro* inhibition of *Rhizoctonia solani by Pseudomonas* spp.

4c: *In vitro* inhibition of *Sclerotium rolfsii* by *Pseudomonas* spp.

57.77% against *Rhizoctonia solani* and 48.89% against *Sclerotium rolfsii*. These two isolates were selected for further studies (Figure 4a,4b,4c)

Table 4:*In vitro* screening of *Pseudomonas* spp. against Phytopathogens (Percentage of inhibition after 5days)

SI. No	Biocontrol agent	*Fusarium oxysporum*	*Rhizoctonia solani*	*Sclerotium rolfsii*
1.	P1	38.88	43.67	48.889
2.	P2	46.744	44.44	22.22
3.	P3	36.470	36.66	47.77
4.	P4	55.55	57.77	48.89
5.	P5	43.00	49.87	48.89
6.	P6	51.11	54.285	50
7.	P7	60.23	58.67	54.19
8.	P8	49.00	56.66	46.66
9.	P9	45.55	42.22	38.11
10.	P10	40	34.00	36.66

*Values are average of three replicates

Evaluation of biocontrol potential of isolates against fungal pathogens of Vanilla undergreen house condition

Based on *In vitro* performance of the *Trichoderma* spp. and *Pseudomonas* spp., four effective antagonistic isolates and two reference strains were screened under pot culture for

their biocontrol potential against fungal pathogens with vanilla as test plant and the results are presented in Tables (5).

Visible symptoms started to appear from the fifth day after inoculation with *F.oxysporum*, *Rhizoctonia solani* and *Sclerotium rolfsii* in the respective control plants. The symptoms were in the form of leaf yellowing which later turned to leaf rotting. The rotting extended to leaf sheath and rarely to the pseudostem also. Observations were recorded in terms of number of leaves infected and the severity was recorded as the total number of leaves infected in all plants in each treatment. In control plants inoculated with *F.oxysporum*, *Rhizoctonia solani, Sclerotium rolfsii* alone the infection rate was very high and severity was near 90%. Besides leaf yellowing and leaf rotting, root rotting followed by wilting and dying of seedlings were also noticed. In all cases, where bioagents were inoculated first (in first phase experiment) and later cross inoculated with phytopathogens , disease symptoms were not visible even after 15-20 days after inoculation.

Trichoderma harzianum and *Pseudomonas fluorescens* were proved to be better biocontrol agents (Table 5). *T. virens* and *Pseudomonas putida* were found to be less effective in controlling fungal diseases of Vanilla. Single inoculation of *Trichoderma harzianum* were found to be statistically on par with dual applications of *Trichoderma harzianum* and *Pseudomonas fluorescens* where as were statistically superior than the single inoculation treatments with *Pseudomonas fluorescens*. *Trichoderma harzianum* inoculatnt was proved to be efficient biocontrol agent than *Pseudomonas fluorescens* in controlling vanilla pathogens.

The minimum percentage of leaf infection were observed in (in both phase experiment) the combination of *Trichoderma harzianum* with *Pseudomonas fluorescens* treatment followed by *Pseudomonas fluorescens*,

Table 5: Evaluation of microbial antagonists against phytopathogens of vanilla plants

Treatments	Pre inoculation With bio- control agents	Cross inoculation with pathogen	Percentage of leaves infection*
T1	*T.virens*	*Fusarium oxysporum*	12.37
T2	*T.virens*	*Rhizoctonia solani*	14.10
T3	*T.virens*	*Sclerotum rolfsii*	15.87
T4	*T .harzianum*	*Fusarium oxysporum*	8.49
T5	*T.harzianum*	*Rhizoctonia solani*	7.34
T6	*T.harzianum*	*Sclerotum rolfsii*	10.95
T7	*P.flourescens*	*Fusarium oxysporum*	7.18
T8	*P.flourescens*	*Rhizoctonia solani*	10.31
T9	*P. flourescens*	*Sclerotum rolfsii*	11.31
T10	*P.putida*	*Fusarium oxysporum*	20.74
T11	*P. putida*	*Rhizoctonia solani*	30.54
T12	*P. putida*	*Sclerotum rolfsii*	39.17
T13	*P.flourescens+T.harzianum*	*Fusarium oxysporum*	7.03
T14	*P. flourscens+T. harzianum*	*Rhizoctonia solani*	7.41
T15	*P.flourscens+ T. harzianum*	*Sclerotum rolfsii*	9.77
T16	Control(no biocontrol agent)	*Fusarium oxysporum*	90.27
T17	Control	*Rhizoctonia solani*	93.26
T18	Control	*Sclerotum rolfsii*	88.40
T19	*T .harzianum* (std)	*Fusarium oxysporum*	8.50
T20	*T.harzianum* (std)	*Rhizoctonia solani*	7.66
T21	*T.harzianum* (std)	*Sclerotum rolfsii*	10.43
T22	*P. fluorescens* (std)	*Fusarium oxysporum*	8.37
T23	*P. fluorescens* (std)	*Rhizoctonia solani*	10.17
T24	*P. fluorescens* (std)	*Sclerotum rolfsii*	12.48
T25	*P.flourescens+T.harzianum* (std)	*Fusarium oxysporum*	8.42
T26	*P. flourscens+T. harzianum*(std)	*Rhizoctonia solani*	8.01
T27	*P.flourscens+ T. harzianum*(std)	*Sclerotum rolfsii*	10.10
	VR = ** 1429.135, CD(5%) =1.95		

*Values are mean of three replicates

Figure 5a: Evaluation of biocontrol potential of isolates against *Fusarium oxysporum* of vanilla plants

5b: Evaluation of biocontrol potential of isolates against *Rhizoctonia solani* of vanilla plants

5c: Evaluation of biocontrol potential of isolates against *Sclerotium rolfsii* of vanilla plants

Trichoderma harzianum, *Pseudomonas putida*, *Trichoderma viren*, respectively in decreasing order. Control with no biocontrol agents showed maximum percentage of leaves infection of vanilla

It can be concluded that the present study revealed the antagonistic property of *Trichoderma* spp. and *Pseudomonas* spp. against *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotium rolfsii*. The biocontrol agents work by triggering the plant's natural defence system to protect it from more harmful pests and diseases or by competing with pathogens for space and nutrients. Because these bacteria and fungi are not toxic to other organisms, they pose a low risk to the environment.

The above proposed study will provide us in depth knowledge on the influence of the biocontrol agents like *Pseudomonas fluorescens* and *Trichoderma harzianum* on

the control of the various diseases of Vanilla crop biologically. The beneficial side of the studies will be a great boon to the vanilla growers of Kerala state. These biocontrol agents were also found to produce growth promoting substances, thereby enhancing the growth and bio mass of the Vanilla. In short the study will able to provide us the efficient disease management of Vanilla crop by using biocontrol agents which are very cost effective and eco-friendly in the present contest of sustainable agriculture.

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