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Evaluating the effect of *Sphingobium yanoikuyae* MH394206 and mixed consortia on growth of rice CO 51 in moisture deficit condition

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Abstract
A pot culture study was conducted to evaluate the effect of *Sphingobium yanoikuyae* MH394206 and mixed consortia on the growth of rice CO-51 in flooded and 75% moisture deficit condition. Along with *S. yanoikuyae* MH394206, other plant growth promoting bacteria like *Azospillium brasilense*, multi mineral solubilizing bacterial strain 1 and 2 were used as mixed consortia. Impact of these microorganisms on plant growth was estimated by changes in growth and physiological characteristics of rice grown in flooded and 75% moisture deficit condition. Seed treatment of these bacterial cultures enhanced the growth parameters like plant height, root volume, panicle and tiller quantity and finally increased the fresh weight of the seeds significanlty over uninoculated control. The bacterial inoculation also increased the concentration of osmolytes under stressed condition indicating stress tolerance inducing capacity of the bacterial inoculants in the plant system.

Keywords: rice CO51, moisture stress, *Sphingobium yanoikuyae* MH394206, multi mineral solubilizers, osmolytes, plant growth

Introduction

The current global climatic issue in world's food production is drought. The word "drought" stands for a season without significant rainfall. Mostly drought stress occurs when optimum amount of water in the soil is reduced and atmospheric conditions cause continuous loss of water by transpiration or evaporation. Almost all plants show resistance to drought but its range varies from species to species. Drought stress reduce the plant growth by affecting the physiological and biochemical processes such as photosynthesis, respiration, translocation, ion uptake, carbohydrates, nutrient uptake and phytohormone production (Farooq *et al.*, 2008) [1]. In many developing countries, rice is considered as most famous staple food crop and feeds nearly 50% of the world's population (Zeigler and Barclay, 2008) [2]. To meet the future food requirements, the world rice consuming countries will have to increase the rice productivity up to 50% to face the consumer's demand in 2025 (Salgotra *et al*., 2018) [3]. Rice is normally grown under submerged condition and very sensitive to drought stress compared to other plant species. Rice itself shows some tolerance to water- limited condition (Kamoshita *et al*., 2008) [4]. Most of the rice growing area in the world does not have adequate amount of water to maintain flooded condition and therefore yield is reduced. Water stress at critical stages of crop growth leads to crop failure and yield reduction (Bernier *et al*., 2008) [5].

One of the most probable ways to increase water acquisition or enhance drought tolerance in plants is exploitation of beneficial microbes as bio-inoculants. The rhizosphere bacteria known as plant growth promoting rhizobacteria (PGPR) has the ability to protect the plants from biotic and abiotic stress condition (Azcon and Barea, 2010) [6]. PGPR protects the plants from drought stress through several mechanisms such as production of plant growth hormones (IAA, abscisic acid, gibberelic acid and cytokinin), induction of systemic resistance, exo-polysaccharide production, accumulation of osmolytes, production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase and volatiles (Timmusk *et al*., 2014) [7]. Under drought stress, IAA producing *Azospirilum brasilense* increase the root length, root area compared with uninoculated control in common bean (*Phaseolus vulgaris*) (German *et al*., 2000) [8]. PGPR *Achromobacter piechaudii* ARV8 increase the fresh weight and dry weight of both tomato and pepper seedlings through ACC deaminase activity under drought stress (Mayak *et al*., 2004) [9]. The plants treated with EPS producing bacteria showed resistance to water stress (Bensalim *et al*., 1998) [10].

The microbes to plant signaling compounds like lipochito-oligosaccharides and thuricin enhance the plant growth under stress condition (Zipfel and Oldroyd, 2017) [11]. Well-developed roots help the plants to absorb sufficient amount of water and nutrients that leads to improved crop growth and yield in drought stress (Shakir *et al*., 2012) [12]. Timmusk and Wagner (1999) [13] reported that, inoculation of *Paenibacillus polymyxa* stimulates drought tolerance in *A. thaliana* through the induction of drought responsive gene, ERD15 (Early Response to Dehydration 15). The plants treated with consortium of different beneficial microorganisms improve the crop growth by supplying nutrients and protects the plants from both biotic and abiotic stress conditions (Kang *et al*., 2010) [14]. PGPR increase the symbiotic relationship between plants and microbes in rhizosphere region (Klopper *et al*., 1980) [15] (Vessey *et al*., 2003) [16]. The importance of osmolytes/compatible solutes in drought stress tolerance have been studied extensively. Osmotic adjustment is the major mechanism involved in plants to maintain the turgor pressure under drought stress. Osmolytes such as ammonium compounds (polyamines, glycinebetaine, b-alanine betaine, dimethyl-sulfonio propionate and choline-O-sulfate), sugars and sugar alcohols (fructan, trehalose, mannitol, D-ononitol and sorbitol) and amino acids (proline and ectoine) plays an important role in plants against drought stress. With this background, investigations were done to obtain rice apoplastic fluid endophytes for improving crop growth in moisture stressed conditions. The study resulted in screening 12 endophytic bacteria with *S. yanoikuyae* MH394206 as a potent bacterial culture. This bacterium has the ability to withstand -5.5MPa of PEG 6000, produce exo polysaccharides, growth hormones like gibberelic acid and indole acidic acid and also ACC deaminase activity (Nivitha *et al*., 2019) [17] and it may serve as a potential bacterial inoculant for moisture stress mitigation (Rajavigneshwaran *et al*., 2019) [18]. However, there are certain bottlenecks associated with this bacterium such as unable to fix nitrogen and solubilize insoluble form of minerals like phosphate, potassium and zinc. Thus, to enhance the nutrient availability, it is essential to co-inoculate *S. yanoikuyae* MH394206 with nitrogen fixing and mineral solubilizing bacteria.

Henceforth, a pot culture experiment was conducted to study the effect of *S. yanoikuyae* MH394206 along with mixed bacterial consortia (consisting of associative diazotrophic bacterium *Azospirilum* and multi mineral solubilizing bacteria - phosphate and zinc solubilizing and potassium releasing ability) in moisture deficit condition.

Materials and Methods

Bacterial cultures

For this study, the bacterial cultures like *S. yanoikuyae* MH394206 and multi mineral solubilizing bacteria isolated from paddy rhizophere soil were used. The culture *Azospirillum brasilense* was kindly provided by the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore. The multi mineral solubilizing bacteria having the ability to solublize insoluble phosphate, zinc and release potassium isolated from rice rhizosphere soil along with *A. brasilense* were used as mixed consortia.

The culture *S. yanoikuyae* MH39420, *A. brasilense* and multimineral solubilizing bacteria were grown in LB, nutrient broth respectively at 28 ℃ with 120 rpm until the culture reached log phase with approximately 10^7 to 10^8cfu's mL^{-1}. The characteristics of these cultures are shown in Table 1.

Table 1: Plant growth promoting characteristics of microbial inoculants used for the study

S. No.	Characteristics	***Sphingobium yanoikuiyae*** MH39420	Multimineral solublizer
1.	Nitrogen fixation	-	-
2.	Phosphate solubliztion	-	1.7 cm halozone formation in Pikvoskaya's solid medium
3	Potassium releasing capacity	-	1.4 cm halozone formation in Aleksandrow's agar solid medium
4	Zinc solublization	-	2.3 cm halozone formation in Bunt and Rovira's solid medium
5	IAA (µg mL^{-1})	14.85 (±0.35)	-
6	GA (µg mL^{-1})	695.08(±79.93)	-
7	ACC deaminase activity (nmoles mg-1 protein h	210 (±0.34)	-

Rice cultivar

The rice CO-51 is a short duration variety and the seeds were provided by Paddy Breeding Station (PBS), Tamil Nadu Agricultural University, Coimbatore.

Pot culture experiment

For evaluating the effect of *S. yanoikuyae* MH394206 and mixed consortia on growth parameters of rice-CO51 in moisture deficit condition, a pot culture experiment was carried out in the Department of Microbiology, TNAU, Coimbatore. For mixed consortia, the compatibility among bacteria was test verified under *in situ* conditions. Only compatible cultures were used for this experiment.The treatment details are T_1. Control (Uninoculated and Flooded condition), T_2. Seed inoculation with *S. yanoikuyae* MH394206 (Flooded condition, T_3. *S. yanoikuyae* MH394206 + Mixed consortia (Flooded condition), T_4. Uninoculated + 75% moisture deficit condition, T_5. *S. yanoikuyae* MH394206 + 75% moisture deficit condition and T_6. *S. yanoikuyae* MH394206 + Mixed consortia + 75% moisture deficit condition.

This experiment was carried between February 2020 to May 2020 with surrounding temperature ranging from 28 to 35℃, relative humidity 85%, and photoperiod 12/12 hour day and night. The soil used for pot culture experiment was collected from wetland field, Department of Farm Management, Tamil Nadu Agricultural University, Coimbatore. The soil texture was clay loam, pH 8.2 and EC 0.68 dSm^{-1}. The soil contained 150.15 mg of total nitrogen, 287.67 mg of potassium and 10.14mg of phosphorus per kg of soil sample. The soil was allowed to air dry, mixed uniformly and sterilized thrice by autoclave at 121 ℃ with 15 lbs pressure for 30 minutes. Mud pots of 7kg capacity were filled with sterilized soil, saturated with water and kept overnight. The field capacity of the soil was estimated by gravimetric method. The seeds were surface sterilized with 10% sodium hypochlorite solution for 20 minutes, washed with sterile distilled water for 3 to 4 times and soaked in sterile water overnight for imbibition and pregermination. For seed treatment, the bacterial cultures were prepared by growing the cultures in the respective medium up to log phase with the cell concentration of 10^8 cfu's mL^{-1}. Then, the cultures were centrifuged at 6000 rpm for 10 minutes to collect the cell pellet. The cell pellet was washed with 0.2M phosphate buffer (pH 7.0) and suspended the pellet in 1ml sterile distilled water. The cell pellet of different cultures were collected and mixed based on the treatments to prepare the mixed consortia. The pre-germinated seeds were treated with bacterial suspension @ 1mL/10 seeds for 1 hour. Thereafter, the seeds were sown in the pot (10 seeds/pot); after germination, the extra seedlings were thinned down and retained 5 seedlings per pot. The pots were irrigated continuously with tap water. Drought stress was induced at flowering stage (70 days after sowing) by imposing 75% moisture stress at the field capacity. Weeds were removed manually throughout the growth period. The response of rice CO-51 to different microbial treatments under stress and non-stress conditions was studied by analyzing growth and physiological characteristics of plants at flowering stage.

Analysis of growth parameters

Growth parameters were recorded manually after drought induction. Growth parameters like shoot height, root volume, number of tillers per hill, number of panicles per plant and fresh seed weight were also recorded.

Analysis of compatible osmolytes in rice under drought stressed condition

Estimation of Proline

Accumulation of proline in plant tissue under drought stress was estimated by following the procedure of Bates *et al.* (1973) [19]. About 0.5g leaf tissue was ground with 10 ml of 3% aqueous sulphosalicylic acid using pestle and mortar. The homogenized mixture was centrifuged at 8000 rpm for 10 min and then the supernatant was used for proline estimation. For analysing proline, 2 mL plant extract was taken in a test tube and mixed with 2 mL acid Ninhydrin and 2 mL glacial acetic acid. This mixture was placed in a water bath at 100 ºC for 1 h and then cooled to normal room temperatureby placing the test tubes in ice bath for 10 min. Later, 4 mL toluene was added and mixed vigorously with a test tube stirrer for 20-30s. The supernatant layer was collected and optical density was measured at 520 nm (M/s, Shimadzu, Japan). Toluene was used as a blank. The proline content was expressed as µg of proline g^{-1} of fresh leaf tissue.

Estimation of Glycine betaine

Glycine betaine accumulation in rice leaves was estimated by following the procedure of Greive and Grattan (1983) [20]. Approximately 0.5g leaf sample was ground with 1 mL distilled water and centrifuged at 5000 rpm for 5 min and collected the supernatant. Then, equal volume of 2N H_2SO_4 was added. It was followed by addition of 20 µl of cold KI-I_2 reagent to the reaction mixture. Then the sample was stored at -4 °C for 16 h; later, the samples were centrifuged at 10000 rpm for 15 min. The residues were collected and the supernatant was discarded. To the residue, 900 µL of 1, 2-dichloroethane was added and thoroughly mixed and incubated at room temperature for 2-2.5 h. The absorbance of the solution was measured at 365 nm. The glycine betaine concentration in the sample was measured against the standard curve of glycine betaine (5–500 µg of glycine betaine g^{-1} of fresh tissue) and results were expressed as µg of glycine betaine g^{-1} of fresh tissue.

Estimation of Trehalose

Trehalose content in rice leaves was determined following the method of Li *et al*. (2014) [21]. The leaf sample of 1.0 g was homogenized in 5 mL of 80% (v/v) ethanol. The homogenized mixture was centrifuged at 11,000 rpm for 20 min and the supernatant was collected. The supernatant was dried at 80 °C overnight followed by resuspension in 5 mL distilled water. The resuspended solution (100 µL) was mixed with 150 µL 0.2 N H_2SO_4 and boiled at 100 °C for 10 min then chilled in ice. Then 150 µL of 0.6N NaOH was added to the above mixture and boiled for 10 min and chilled again. To the above mixture, 2.0 ml anthrone reagent (0.2 g anthrone per 100 ml of 95% sulphuric acid H_2SO_4) was added and boiled for 10 min to develop a colour, and chilled again. The absorbance was recorded at 630 nm and trehalose concentration was expressed as µg g^{-1} fresh weight.

Statistical analysis

This experiment was based on completely randomized design. The data were analyzed statistically using SPSS software version 16.0.Significant difference between means was compared using Duncan multiple range test at $p \leq 0.05$.

Results

Growth characteristics of rice CO51 as influenced by *S. yanoikuyae* MH394206 and mixed consortia under moisture stress conditions

Shoot length

Plants grown under flooded condition displayed higher shoot length compared to moisture stressed condition. Among 6 treatments, plants treated with *S. yanoikuyae* MH394206 and mixed inoculants in flooded condition recorded significantly greater shoot length (97.36 cm). It was followed by plants treated with *S. yanoikuyae* MH394206 and mixed inoculants (86.76 cm) in moisture stressed condition. The lowest shoot length (84.06 cm) was observed in plants grown under uninoculated in moisture stressed condition (Table 2).

Root volume

As that of shoot length, plants grown under flooded condition displayed higher root volume compared to plant grown in moisture stressed condition. The highest root volume (61.66 mL) was observed in plants treated with *S. yanoikuyae* MH394206 and mixed inoculants in flooded condition. The same treatment recorded 53.3 ml of root volume in moisture stressed condition. The lowest root volume (33.3 ml) was observed in plants grown under uninoculated under moisture stressed condition (Table 2)

Number of tillers

Plants grown under flooded condition showed more number of tillers/plant compared to moisture stressed condition. Maximum number of tillers per plant (4.66) was observed in plants treated with *S. yanoikuyae* MH39420 and mixed inoculants in flooded condition. It was followed by plants treated with *S. yanoikuyae* MH39420 alone (Table 2). Among moisture stress treatments, plants inoculated with *S. yanoikuyae* MH39420 and mixed inoculants recorded substantial number of tillers (3.66). The minimum number of tillers (2) was observed in plants grown under uninoculated moisture stressed condition.

Number of panicles

Maximum number of panicles (14.6) was observed in plants treated with *S. yanoikuyae* MH39420 and mixed inoculants in flooded condition. Under moisture stressed conditions, the same treatment recorded 12 panicles/plant. The minimum number of panicles (11.6) was observed in plants grown under uninoculated and moisture stressed condition.

Fresh seed weight

Plants grown under flooded condition showed higher seed weight compared to moisture stressed condition. The highest seed weight (23.04g) was observed in plants treated with *S. yanoikuyae* MH39420 and mixed inoculants in flooded condition (Table 2). It was followed by plants treated with *S. yanoikuiyae* MH39420 alone. Among moisture stress conditions, plants treated with *S. yanoikuyae* MH39420 and mixed consortia recorded maximum fresh seed weight of 19.81g per plant. The lowest seed weight (11.94g) was observed in plants grown under uninoculated moisture stress condition.

Table 2: Effects of moisture stress and bacterial inoculants on growth characteristics of rice CO51

Treatments	Shoot length (cm)	Root volume (mL)	Number of tillers	Number of panicles/plant	Fresh weight of the seed (g)
T_1	89.6 ± 2.88^{ab}	55 ± 2.04^{ab}	4 ± 0.0^{a}	13.3± 0.84^{a}	21.0 ± 1.38^{a}
T_2	91.8 ± 1.94^{a}	60 ± 5.40^{a}	4.± 0.40^{a}	14.3 ± 0.84^{a}	21.6 ± 1.85^{a}
T_3	97.3 ±1.33^{a}	61.6 ± 4.24^{a}	4.6 ± 0.62^{a}	14.6 ± 1.84^{a}	23.04 ± 3.38^{a}
T_4	84.1 ± 0.64^{b}	33.3 ± 2.35^{c}	2 ± 0.0^{b}	11.6 ± 0.62^{a}	11.94 ± 1.50^{a}
T_5	85.4 ± 0.45^{b}	41.6± 3.11^{bc}	3.3 ± 0.47^{ab}	12.0 ± 0.24^{a}	14.89 ± 0.62^{a}
T_6	86.7 ± 1.20^{ab}	53.3 ± 2.35^{ab}	3.6± 0.23^{ab}	12.55± 0.70^{a}	19.81 ± 2.77^{a}

Values in each column are mean of three replications ± Standard Error. Mean values in each column followed by same letter(s) are not significantly different at 5% level

T_1. Control (uninoculated and flooded condition), T_2. Seed inoculation with *S. yanoikuyae* MH39420 + flooded condition, T_3. *S. yanoikuyae* MH39420 + Mixed consortia + flooded condition, T_4. Uninoculated + 75% moisture deficit condition, T_5. *S. yanoikuyae* MH39420 + 75% moisture deficit condition and T_6. *S. yanoikuyae* MH39420 + Mixed consortia + 75% moisture deficit condition.

Physiological characteristics of rice CO51 as influenced by *S. yanoikuyae* MH394206 and mixed consortia under moisture stress conditions

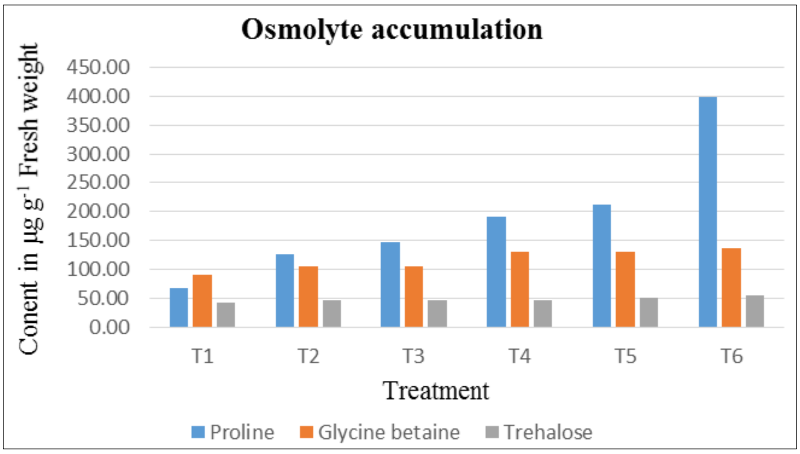

Fig 1: Effect of bacterial inoculants on osmolyte accumulation in leaves of rice CO51 grown in flooded and moisture stressed condition

Proline

The highest proline content was observed *S. yanoikuyae* MH39420 and mixed consortia inoculated rice plants (399.34 ± 0.90 µg g^{-1} fresh weight). The lowest value of 67.74 ± 0.96 µg g^{-1} fresh weight was observed in plants grown under uninoculated flooded conditions. The results of proline for all treatments are represented in Figure 1.

Glycine betaine

The concentration of glycine betaine also increased under stress conditions. The highest value of 137 ± 0.73 µg g^{-1}fresh weight was observed in *S. yanoikuyae* MH39420 and mixed consortia inoculated rice plants. The lowest glycine betaine content of 91.22 ± 1.26 µg g^{-1} fresh weight was observed in uninoculated flooded condition. The results of glycine betaine for all treatments are mentioned in (Figure 1).

Trehalose

Trehalose accumulation also increases under stress condition. The trehalose concentration increases in both T6 (53.88 ± 1.21 µg g^{-1} fresh weight) and T5 (50.29 ± 0.03 µg g^{-1} fresh weight). The trehalose concentration gets decreased in T1 (41.61 ± 0.31 µg g^{-1}fresh weight) followed by T2 (47.40 ± 0.31 µg g^{-1} fresh weight) and T3 (47.54 ± 0.10 µg g^{-1} fresh weight). The results of trehalose for all treatments are mentioned in (Figure. 1).

Discussion

The results indicate that the vegetative growth of rice significantly increased under moisture deficit condition due to bacterial inoculants; the effect was quite significant due to *S. yanoikuyae* MH394206 and mixed consortia of microorganisms. *S. yanoikuyae* MH39420 possess ACCD activity, antioxidants, siderophores and phytohormones production. Mixed consortia might have improved availability of major nutrients like nitrogen, phosphorus, potassium and zinc thereby enhanced the plant growth under moisture stress. Similar plant growth promoting ability of the bacteria were reported in several earlier studies (Compant *et al*., 2005) [22] (Turan, *et al*., 2014) [23] (Karlidag, *et al*., 2010) [24].

Rajavigneshwaran *et al*., 2019 [18] reported that *S. yanoikuyae* MH394206 promotes the growth of rice in moisture-stressed condition. Bano *et al*., 2013 [25] reported that *Azospirillum lipoferum* promotes the plant growth under moisture stress condition.

In this study, in response to microbial inoculation shoot length, root volume, tillers and panicle number, seed weight of rice plants were compared with uninoculated control. The results showed that combination of *S. yanoikuyae* MH39420 and mixed consortia improved the plant growth in both flooded and moisture deficit condition. All the parameters studied were better due to *S. yanoikuyae* MH39420 and mixed consortia. It was followed by *S. yanoikuyae* MH39420 alone. These observations indicate that the performance of *S. yanoikuyae* MH39420 could be improved further by co-inoculating with mixed consortia of nitrogen fixing (*A. brasilense*) and multimineral solubilizing bacteria. It has been reported that seed treatment of rhizobacteria improves the plant growth due to their ability of fixing nitrogen, solubilize phosphate and produce plant growth promoting hormones (Salantur *et al*., 2006) [26] (Karlidag *et al*., 2011) [27]

Accumulation of osmolytes normally increases under stress condition. In this study, among three osmolytes, proline accumulation varied widely with bacterial inoculation and moisture stress treatment and recorded maximum value. Similarly glycine betaine content also varied with treatment. However, trehalose content did not vary with the treatment. In general, bacteria inoculated plants showed higher concentration of proline and glycine betaine compared to un-inoculated control in both flooded and moisture stress conditions. However, the highest concentrations of proline and glycine betaine were recorded under moisture stress condition indicating the mechanism of protection of plants from moisture stress in rice by these bacterial inoculants. Mixed consortia of plant growth promoting rhizobacteria protect the plants under stress conditions through proline accumulation and reduce the damage caused by oxidative stress. Proline accumulation in drought stress in wheat increase the level of antioxidants thereby makes the plants tolerate to drought stress (Valentovic *et al*., 2007) [28]. Naseem *et al*. (2018) [29] reported that bacteria which possess ACC deaminase activity and IAA production increase the proline concentration under osmotic stress condition. In the current study, proline was found in greater quantity in bacteria inoculated treatments and hence played a critical role in plant stress resistance as indicated by Zhang *et al.* (2005) [30]. Jiang *et al*. (2012) [31] reported that enhanced accumulation of glycine betaine improves the relative water content of leaf and plant biomass production. PGPR increases the glycine betaine synthesis in plants thereby protects the plants from moisture stress (Bashan *et al*., 2014) [32].

Conclusion

Application of *S. yanoikuyae* MH394206 and mixed consortia improved the rice growth significantly in moisture stressed condition by altering the morphological and physiological characteristics of the plant. Improved plant osmolytes like proline and glycine betaine as a result of inoculant application would be useful marker for the bacterial effect on the strategies of drought tolerance in the plants. The effect was pronounced due to the treatment of rice with apoplastic fluid associated bacteria *S. yanoikuyae* MH394206. Hence, these bacterial isolates may serve as potential bacterial consortia for improving growth and yield potential of rice CO51 in moisture stress condition.

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