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RESEARCH PAPER

Genetic characterization of rhizobium bacteria isolated from bean (*Phaseolus vulgaris L.)* nodules and its effect on growth

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Abstract

Biological nitrogen fixation (BNF) is an important nitrogen source, providing a variety of legumes and pasture plants. Rhizobia is soil bacteria that can form nitrogen-fixing nodules on legumes. In this study, we have isolated 10 bacteria from root nodules of sugar beans from the Gembos plain Derebucak district of Konya, Turkey. The morphological and metabolic characteristics of the isolates were tested under laboratory conditions. According to molecular identification, eight bacterial isolates were identified as *Agrobacterium tumefaciens,* and two isolates (F4DC and F6DC) were identified as *Rhizobium gallicum.* Field experiments were carried out to compare the effect of one native rhizobia (*Rhizobium gallicum* F4DC), chemical fertilizer, and non-fertilizer control in SFWRRI Sarayköy Research and Application Station in Ankara. Our results show that sugar bean (*Phaseolus vulgaris L.*) inoculation with *Rhizobium gallicum* F4DC (MZ156852) induced a significant increase in the number of nodules, grain yield, number of pods, and plant height compared to the control. These results show that *Rhizobium gallicum* F4DC is a suitable choice for use in symbiotic association with beans to work as a biofertilizer.

Introduction

It is well recognized that nitrogen is an essential element for the growth and development of plants. Chemical fertilizers are necessary for intensive agricultural methods to produce large yields, but they are also expensive and may have negative environmental effects. Biological nitrogen fixation (BNF) is an important nitrogen source, providing a variety of legume and pasture plants with about 2170–300 kg of nitrogen per hectare per year (Tu et al., 2016). Especially in regions of the world where nitrogen fertilizer imports are intense, legumes are of particular importance due to their unique ability to fix atmospheric nitrogen and being self-sufficient in nitrogen demand, as well as their capacity to supply nitrogen to the soil. Rhizobia are alpha-proteobacteria that live in a symbiotic relationship with a wide range of leguminous plants by forming nodules in roots where these bacteria fix atmospheric nitrogen. A total of seven Alphaproteobacteria genera, including Rhizobium, Azorhizobium, Allorhizobium, Bradyrhizobium, Mesorhizobium, Sinorhizobium, and Methylobacterium contain about 40 rhizobia species (Lemaire et al., 2015). Rhizobia bacteria play an important role in agro-ecosystem services as they perform biological nitrogen

fixation by establishing symbiotic relationships with a wide variety of leguminous plants (Orrell and Bennett, 2013).

N_2 fixing microorganisms are able to enhance the nitrogen fixation performance and also may increase the nutrient level in soil through phosphate solubilization, water uptake, and the production of substances like hormones, and siderophores. These bacteria also work to mitigate agro-environmental issues while improving soil fertility (Kumar et al., 2012). Since rhizobia-legume symbiosis is a host-specific relationship, it is necessary to identify the strains and diversity of rhizobia associated with a particular species of legume in order to better utilize the advantages of rhizobia biofertilizers (Batista et al., 2015). The symbiosis between Rhizobium bacteria and legumes is facultative and is determined by the host plant's nitrogen requirement. In this mutual symbiosis, bacteria supply nitrogen to the plant in exchange for photosynthesis products from the host (Wang et al., 2018).

The most significant grain legume for direct human consumption worldwide is the bean. Beans are an excellent supplier of protein and are also a good source of vitamins, minerals, and minerals (particularly iron and zinc). Rhizobium and beans (*P. vulgaris L.*) work together in a symbiotic relationship to create root nodules that catalyze the fixation of nitrogen from the air (Baginsky et al., 2015). The species that establish a symbiotic relationship with the bean are the generally fast-growing Rhizobiums, which include *Rhizobium phaseoli, Rhizobium leguminosarum, Rhizobium gallicum*, *Rhizobium tropici, Rhizobium fleshy*and and *Sinorhizobium meliloti* (Adhikari et al., 2013).

Biofertilizers containing various strains of rhizobia are being developed for use in legumes to increase soil fertility and symbiotic nitrogen fixation. A key strategy in sustainable agriculture is the use of efficient rhizobial strains as biofertilizers to increase the production of legumes. The use of native isolates in biofertilizer production; is preferred primarily due to its adaptation to environmental conditions and higher competitiveness with local strains.

The aim of this research was to isolate and identify native rhizobium bacteria and to investigate the effects of the obtained biofertilizer on bean plant growth parameters.

Materials and methods

Collection of nodules

The nodules were collected from ten different sugar bean (*Phaseolus vulgaris* L.) growing areas in Gembos plain Derebucak district of Konya, Turkey. The plants were carefully uprooted and transferred to the laboratory and stored at 4°C until isolation. The nodules that were healthy, unbroken, and especially pink was chosen for rhizobia isolation.

Isolation of Rhizobia from the Root Nodules

Nodules were thoroughly washed under tap water to remove the adhering soil particles from the nodule surface and then carefully severed from the root with sterile forceps. Healthy and undamaged root nodules were subjected to surface sterilization by washing in 3-5% hydrogen peroxide solution for 4-5 minutes, followed by repeated rinsing with sterile distilled water. Subsequently, they were washed with sterile distilled water after being treated with 70% ethyl alcohol (Somasegaran and Hoben, 1994). After surface sterilization, the nodules were crushed and extracted in an environment containing a small amount of sterile distilled water. Yeast Extract Mannitol Agar (YEMA) plates were streaked with a loop full of nodule suspension and cultured for 3-5 days at 28°C. (Vincent, 1970). Following the incubation period, single colonies were selected and re-streaked on YEM agar for purity. A total of 10 isolates were obtained and all purified isolates were preserved at -80 °C in YEM containing 40 % glycerol.

Morphological and Metabolic Characterization of the Isolates

Gram staining was carried out with 3–4 days old cultured on YEMA were smeared on clean microscope slides. According to Beck et al. (1993) instructions, the wet smears were air dried, heat fixed, and then Gram stained. On a compound light microscope, the produced slides were examined under immersion oil.

The isolates were incubated in YEMA medium containing Congo red for detection of absorption congo red (Somasegaran et al., 1994). Then, rhizobia isolates were cultured in glucose peptone agar plates supplemented with bromocresol purple, incubated at 28°C for 4 days in the dark. In YEMA medium containing bromothymol blue (YEMA-BTB), the formation of acid or alkali was determined. The plates were incubated at 28°C for 5 days in the dark and color changes of the medium were recorded. The isolates that turned YEMA-BTB from green to yellow were found to generate acid and grow fast. Slow-growing isolates that turned the YEMA-BTB medium blue were recognized as alkaline producers. Isolates were checked on the basal medium of YEMA supplemented 2% NaCl as 2% NaCl is inhibitory for most rhizobial isolates it can serve as an identification tool (Koskey et al., 2018).

Molecular Identification of Isolates

The genomic DNA of the bacterial isolates was extracted using the CTAB (cetyltrimethylammonium bromide) technique (Wilson, 2001). A Microvolume spectrophotometer was used to measure the concentration of DNA, which was then kept at -20°C until use. With the use of the universal primers 27F (5'-AGAGTTTGATC(AC)TGGCTCAG-3') and 1492R (5'-

CGG(CT)TACCTTGTTACGACTT-3'), 16S rDNA regions of genomic DNA isolated from bacteria were amplified. (Khan et al., 2014). The 50 µl reaction volume used for the PCR amplification contained 3 µL of template genomic DNA, 5 µL of 10xTaqbuffer, 1 µL of 10 mM dNTP, 1 µL of each primer, 0.25 µL of 5 U Taq polymerase, and 4 µL of 25 mM $MgCl_2$. The following conditions were used for the PCR reaction: a 2-minute denaturation period, 35 cycles of temperature cycling (95°C for 45 s, 57°C for 45 s, and 72°C for 2 min), and a final 7-minute extension period at 72°C (Özdoğan, D.K. et al., 2022). Amplified fragments were checked, then purified and sequenced at BM Labosis (Ankara, Turkey). The NCBI-BLAST server is used to analyze 16S rDNA sequences. To perform molecular phylogenetic and evolutionary relationship analysis, isolates 16S rDNA gene sequences were aligned by ClustalW using four reference strains (Saitou and Nei, 1987) and the phylogenetic tree was conducted using Neighbor-Joining method with MEGA software v.10.0.5 (Kumar et al., 2016).

Amplification of nif H gene from isolates

polF (5'-TGCGAYCCSAARGCBGACTC-3') and polR (5'-ATSGCCATCATYTCRCCGGA -3') primers were used for application of Preserved region of the *nif H* gene fragment (Poly et al. 2001). For this reaction *Azotobacter vinelandii* was used as positive control.

Field experiment

Field experiment was conducted to examine the impact of the native rhizobia, chemical fertilizer, and a non-fertilizer control on nodulation, growth, and plant yield of the common bee. The experiment was carried out in SFWRRI Sarayköy Research and Application Station (Ankara, located at 36.46_N, 52.56_E, and 25 masl) in a fully randomized design trial with three replicates in 2020. The test subjects were seeds without bacterial treatments and without chemical fertilization (C0), microbial fertilizer prepared using *Rhizobium gallicum* F4DC strain (MF), and seeds without bacterial treatments but treated with optimal chemical fertilization (CF). According to the soil analysis results, triple super phosphate fertilizer (9.6 kg/da) with planting was given to microbial fertilizer and optimum fertilizer subjects, and the nitrogen requirement of CF subject was divided into two parts, half of it was applied in the form of ammonium sulfate (7 kg/da) during planting and the other half during flowering period. Each plot measured 2 × 3 m and a spacing of 2 m between the plots was left to minimize inter-plot interference. The soil of the experimental area is classified as clay soil according to the USDA texture classification (Gerakis and Baer, 1999) and the amount of organic matter is very low. The soil reaction of the area, which is medium in terms of lime content, is slightly alkaline and moderately salty.

The bacteria were grown in a yeast extract mannitol broth and added to milled peat in the ratio of 1 bacteria inoculum to 2 parts peat. The rhizobium in peat was prepared and applied as a seed treatment at one kg per 100 kg seed. During the flowering stage, three plants from each plot were selected randomly and harvested for assessment of nodulation in the first five cm and after the harvesting plant height, number of pods per plant, 100 grain weight, grain yield, and protein ratio were measured.

One-way analysis of variance (ANOVA) was performed on the experimental data using the SPSS-22 software package, and Duncan's multiple range test was used to determine the significance of treatment effects at the $p < 0.05$ level.

Morphological and Metabolic Characterization of the Isolates

In the present study, ten strains were isolated from root nodules of sugar beans. According to microscopic investigation all isolates are gram negative, rod shaped. Gram staining results preliminary confirmed the standard morphological characteristics of *Rhizobium* as described by Vincent (1970) and Somasegaran and Hoben (1994). All isolates grew on 2% NaCl supplemented YEMA. Rhizobium is inhibited by high salt concentrations like 2% NaCl, however, practically all of the strains we obtained grow at this concentration (Figure 1b), supporting the research done by (Dubey et al., 2010). Bromothymol blue-supplemented yeast mannitol agar (YMA) plates were used to differentiate rapid (acid-producing) growing strains from slow (non-acid-producing or alkali-producing growing rhizobia). (Somasegaran et al., 1994). Isolates F4DC, and F6DC can not absorb the Congo red dye (Figure 1a). It is known that most rhizobia can absorb the congo red only weakly whereas contaminants including Agrobacterium, can absorb strongly (Beck et al., 1993). Out of 10 isolates, 8 isolates were turned by the BTB indicator from blue to yellow and not growth on glucose peptone agar. While 8 isolates were acid producers and fast growers, two isolates (F4DC, F6DC) did not turned BTB color so they indicated as slow growers and they also were not grown on glucose peptone agar (Figure 1c, 1d). The little or no growth on the glucose peptone agar without altering the pH of the media was a clear indication of the presence of *Rhizobium*. The strains F4DC and F6DC did not grow on glucose peptone agar. It is known that contaminants like Agrobacterium, show growth on the glucose peptone agar with a distinct change in pH (Pervin et al., 2017). Ten pure isolates were placed into two distinct groups based on glucose peptone growth, BTB reaction and absorption of congo red dye (Table 1). According to the results we obtained in our study, growth in 2% NaCl is not a distinguishing feature for Agrobacterium and Rhizobium.

Molecular Identification of Isolates

Table 1. Morphological and Metabolic Characterization of the Isolates

Isolates	F1DC	F2DC	F3DC	F4DC	F5DC	F6DC	F7DC	F8DC	F9DC	F10DC
Gram reaction	-	-	-	-	-	-	-	-	-	-
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
CR absorption	A	A	A	NA	A	NA	A	A	A	A
BTB reaction	Y	Y	Y	B	Y	B	Y	Y	Y	Y
Glucose peptone growth	G	G	G	NG	G	NG	G	G	G	G
Growth on 2% NaCl	G	G	G	NG	G	NG	G	G	G	G

A: absorption NA: No absorption Y: Yellow B: Blue G: Growth NG: No growth CR: Congo red BTB: bromothymol blue

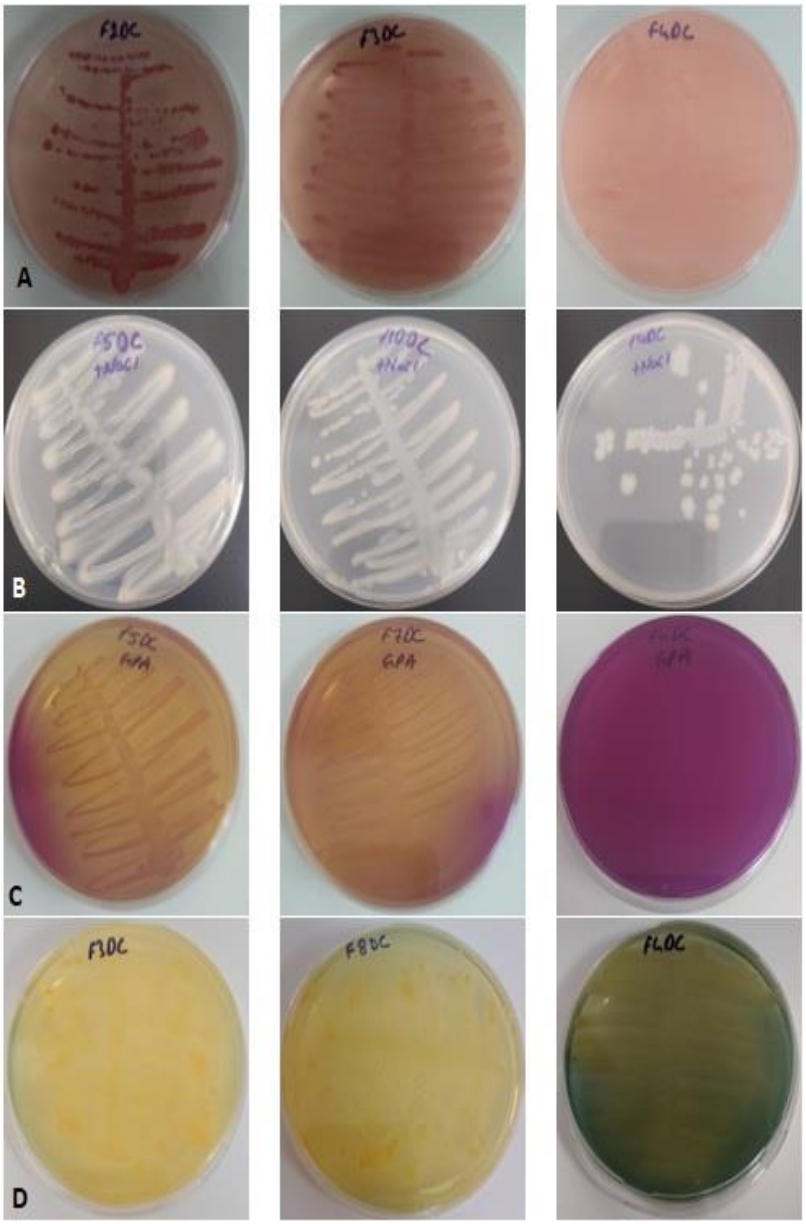

Figure 1. Growth of selected isolates at A. on YEMA with Congo red, B. YEMA supplemented with 2%NaCl C. Glucose Peptone Agar D. YEMA supplemented with BTB

The PCR amplification of the 16S rDNA region produced a single band with a size of roughly 1500 bp. 16S rDNA gene sequences of bacterial isolates compared with previously deposited bacterial sequences in the NCBI GenBank database. According to the BLAST results, eight of the bacterial isolates were identified as *Agrobacterium tumefaciens,* and two isolates (F4DC and F6DC) identified as *Rhizobium gallicum*. The GenBank accession numbers of *Rhizobium gallicum* F4DC (MZ156852) and *Rhizobium gallicum* F6DC (MZ156854) are given in Figure 2. The neighbor-joining method was used to create the phylogenetic tree. The phylogenetic tree was formed by the neighbor-joining method. According to the phylogeny of 16S rRNA genes, one group belongs to the genera Rhizobium, and the other one belongs to the genera *Agrobacterium.* As in our study, Agrobacterium strains have been isolated from root nodules of beans and other legumes in many previous studies (De Lajudie et al., 1999; Mhamdi et al., 2002; Hameed et al., 2004). Similar to metabolic characterization according to molecular identification, isolates are divided into two groups as Agrobacterium and Rhizobium. The ability of non-nodulating Agrobacterium to colonize the root nodules of common beans was demonstrated by Mhamdi et al. (2005). In the infected nodules, Rhizobium and Agrobacterium coexisted. The mechanism by which these isolates integrated nodules is still not known. More recently, Mrabet et al. (2006) discovered that in non-sterile soil samples and in vitro antibiosis experiments, nodulation by *Rhizobium gallicum* was selectively reduced by Agrobacterium strains derived from root nodules of common beans.

Field experiment

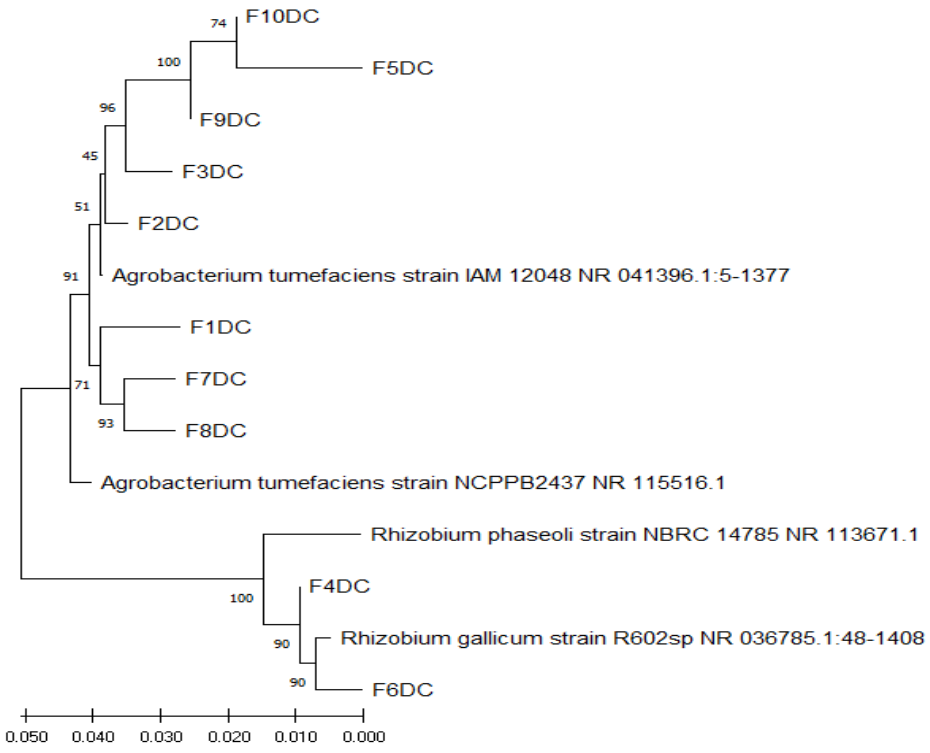

Figure 2. Phylogenetic tree of isolates and reference strains from GenBank database based on sequence of 16S rDNA constructed by maximum likelihood method.

Rhizobium are nitrogen-fixing bacteria that process the nif gene, responsible for the nitrogenase enzyme that converts atmospheric nitrogen (N2) to ammonia. The *nifH* gene amplification of the F4DC and F6DC bacteria resulted in an amplified segment of approximately 360 bp that resembled the positive control *Azotobacter vinelandii*. The dinitrogenase reductase subunit of the nitrogenase enzyme, which is responsible for biological nitrogen fixation, is encoded by the *nifH* gene and is employed as a biomarker to research the ecology and evolution of nitrogen-fixing bacteria. In our study, *nifH* genes of *Rhizobium gallicum* F4DC and F6DC strains were detected in the gel image shown in Figure 3. In many studies amplification of the *nifH* gene fragment was detected for Rhizobium strains (Akter et al., 2016; Khalid et al., 2020).

Figure 3. PCR amplification of *nifH* gene from genomic DNA of isolates. M: molecular size marker, P: positive control (*Azotobacter vinelandii*), N: negative control (water)

In the field experiment, the effect of inoculation with *Rhizobium gallicum* F4DC (MZ156852) on sugar beans was studied. In order to control nodulation, 5 plants were randomly selected from each plot during the flowering period and the number of nodules in the first 5 cm was recorded. Plant height, number of pods per plant, 100 grain weight, grain yield, protein ratio was determined in all plots after harvest. The SPSS-22 package program was used to do a one-way analysis of variance (ANOVA) on the experimental data, and the Duncan multiple comparison test was used to determine the significance of the effects of the applications at the $p < 0.05$ level.

F4DC strain induced a significant increase in grain yield, number of pods and plant height compared to the control and also induced a significant increase in the number of nodules both compared to the control and chemical fertilizer treatments. (Table 2). Also, the highest protein ratio recorded in the F4DC treatment however, there was no significant increase.

It was found that *P. vulgaris* inoculated with different rhizobial isolates showed substantial changes in the shoot, root, and total dry biomass. One of the primary approaches for evaluating the symbiotic efficiency of rhizobial isolates is to look at the major changes in growth parameters, which revealed clear disparities in the ability of the isolates to fix nitrogen. (Sharma et al., 2003). *Rhizobium sp.* inoculation of seeds prior to planting has reportedly been found to be a significant impact in boosting early emergence, product viability, and high grain production. (Figueiredo et al., 2008; Otieno et al., 2009) Similar to our study Bambara and Ndakidemi (2010); also reported high sugar bean seed yield inoculation of seeds with Rhizobium bacteria compared to the uninoculated control. Koskey et al. (2018) reported that inoculating climbing beans with rhizobium dramatically increased nodule and shoot dry biomass, number of pods per plant, seed yields, and nitrogen content in shoot in the field.

Conclusion

Table 2. Effects of MF (F4DC) on yield parameters of bean under field condition

Treatments	Plant height cm	Number of pods/plants	100 grain weight g	Grain yield kg/da	Number of nodules	Protein ratio %
MF	61,23 a	17,87 a	21,79	107.5 a	38,67 a	26,00
CF	61,67 a	16,03 a	23,47	112,0 a	6,00 b	24,88
C0	48,27 b	14,63 b	21,11	84,75 b	7,00 b	24,35

In this study, 10 rhizobia isolates were obtained from the root nodules of sugar bean identification based on morphological, metabolic, and molecular characteristics. We found that molecular techniques are more sensitive in identifying Agrobacterium and Rhizobium strains than their morphological and metabolic characteristics. In addition, this study showed that the bacteria isolated from the root nodules of sugar beans cause a significant increase in the number of nodules, grain yield, number of pods and plant height compared to the control. Based on the results obtained, it can be concluded that *Rhizobium gallicum* F4DC could potentially be used as microbial fertilizer.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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Author Contribution

DKO: conducted fieldwork, collected plant and nodule specimens and prepared for analysis, drafted 17 (review and editing) **CS**: conducted fieldwork, collected plant and nodule specimens and prepared for 18 analysis, drafted (review and editing) **Vİ:** conducted fieldwork, Performed soil analysis of the trial area 19 **AP:** conducted fieldwork, Performed soil analysis of the trial area **EK**: conducted fieldwork, Performed 20 plant analysis of the trial area.

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