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GRAPEVINE TRUNKS DECLINE ASSOCIATED WITH *MACROPHOMINA PHASEOLINA*

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ABSTRACT

Macrophomina phaseolina fungi were isolated from infected grape branches and it was identified using morphological and molecular characteristics. *M. phaseolina* colonies were observed on PDA medium with a distinct color gradient between dark gray and black, and formed microsclerotia after 4-5 days .Molecular identification of the ITS 1,4 region of the two *M. phaseolina* isolates (grape1 and B66) was deposited in the NCBI database with accession numbers OQ802810 and PQ587052, which showed 99-100% identity with reference sequences in NCBI. The phylogenetic tree showed a clear evolutionary affinity between grape1 and BS6 isolates with minor differences due to environmental or geographical diversity. Chlorite susceptibility test revealed three growth patterns (dense, feathery, restricted). This reflects variation in their resistance or sensitivity and in the optimal temperature for fungal growth, with the maximum development of the two M. phaseolina isolates occurring at 30°C. In contrast, growth was poor at low temperatures (15-20°C) or at high temperatures (35 °C). The pathogenicity test showed that the two M. phaseolina isolates caused clear disease symptoms, represented by general deterioration and wilting of the inoculated branches.

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INTRODUCTION

Grape plant *Vitis vinifera* is one of the genera of the Vitaceae family, which includes more than 700 species and 10,000 cultivars widely cultivated in different parts of the world (Alimam and Hasan,2023; Faizy *et al.,* 2024). Grapes are one of the most important types of fruit and have great economic importance. A large portion of grapes is consumed as fresh fruit or dried raisins and is used in many food industries, such as juice production, grape molasses, grape seed oil, ethanol production, and anthocyanin production (Abdul-Qader, 2009; Yuldashev and Iminov, 2022). Fungi are among the most important plant pathogens, causing significant losses in agricultural products (Ismaiel and Ibrahim, 2023; Ibrahim *et al.,* 2024). Grapes are susceptible to many diseases, the most important of which are vine decline, which causes a decrease in vine production and early, sudden, or gradual death of vines, including Esca disease caused by Haeomoniella sp., Chlamydospora sp., Phaeoacremonium minimum, and *Fomitiporia mediterranea*. Dead arm disease caused by *Eutypa lata*. Dieback disease caused by *Botryosphaeria* sp. (Ye *et al.,*

2021, Kenfaoui *et al.,* 2022; Berbegal *et al.,* 2024). *M. phaseolina* is a soil-borne fungus with a wide host range that includes no less than 500 plant species belonging to more than 100 families (Shirai and Eulgem, 2023).

M. phaseolina produces a wide range of enzymes that degrade the plant cell wall, enabling it to penetrate the host tissues and overcome plant defenses, and cause stem and root rot, charcoal rot, and seedling wilt (Marquez *et al.,*2021).

M. phaseolina is a pathogen associated with the decline of grapevines in several areas in Iran, the average of disease incidence reached 25% in the surveyed grapevines. Symptoms initially appeared as partial leaf necrosis, which progressed to necrosis of the entire leaf area and drying of some infected branches. The development of the disease leads to the decline and wilting of the entire vine (Abed-Ashtiani *et al.,*2018). Symptoms of decline appeared in approximately 100 Selma Pete raisin grape vines of the type *Vitis vinifera* in two vineyards in Fresno County, California, USA. Wedge-shaped cankers and dark brown wood discoloration were observed in the branches and trunks of the infected vines. Black spherical microsclerotia were also observed in the bark tissues, and the fungus was identified as M. phaseolina (Nouri et al., 2018). Beyond grapevines, *M. phaseolina* has been linked to decline and disease in many other fruit trees. During surveys conducted in apricot orchards in Hatay Province, Turkey, 30 isolates of M. phaseolina were recovered from apricot trees exhibiting symptoms of chlorosis, wilting, and root rot (Pekgoz and Tok, 2018). *M. phaseolina* causes gum disease in orange trees *Citrus reticulata*, characterized by gum exudation with trunk blisters and orange to pink discoloration of the underlying wood below these blisters appear in color (Das *et al.,* 2010). *M. phaseolina* has been linked to tree decline in pistachio trees in California (Nouri *et al.,*2020). and is a known pathogen affecting various fruit trees*, including grapevines in Australia, Iran, South Africa, Spain, and California, olives in Australia, cankers in almond trees in California (Sergeeva et al.,2005;Agustí-Brisac, 2022),* and wilting of pistachio trees in Turkey(Aydin *et al.,*2024). This study aimed to characterize *M. phaseolina* isolates obtained from grape vines showing decline symptoms from the grape gene bank College of Agriculture and Forestry, University of Mosul and evaluate their sensitivity to the chlorite test. This evaluation contributed to classifying the isolates by their growth patterns, to understand their physiological diversity and its impact on the disease.

MATERIALS AND METHODS

Pathogen Isolation

A total of 50 Samples were collected from grapevine branches planted in the grape gene bank, College of Agriculture and Forestry, University of Mosul (seedless variety). The samples were cut into small pieces of 0.5-1 cm length, superficially sterilized with sodium hypochlorite solution (1%) for 3 minutes, washed with sterile

distilled water, and dried with sterile filter papers. Five pieces were transferred to sterile plastic Petri plates of 8.5 cm diameter containing Potato Dextrose Agar (PDA) medium, sterilized by autoclaving at 121 °C and a pressure of 1.5 kg/cm2 for 20 minutes, and supplemented with the antibiotic Ampicillin at 150 mg/L. The plates were incubated at 25 °C for five days. Plates were examined for fungal growth, and the fungal colonies were purified and identified to the species level based on colony characteristics. Following the classification keys prepared by (Marquez *et al.,* 2021).

Molecular Diagnosis

According to the manufacturer's instructions, DNA was extracted using the ZR Fungal/Bacterial/Yeast DNA MiniPrep™ Extraction Kit (catalog number: D6005) produced by Zymo Research Corporation, USA. The Nano Drop device was used to estimate DNA concentration and assess its purity.

The ITS1 and ITS4 genes were detected using PCR using forward primer (ITS1 F: 5′- TCCGTAGGTGAACCTGCGG -3′) and reverse primer (ITS4 R: 5′ TCCTCCGCTTATTGATATGC-3′) produced by IDT, Canada. PCR was performed at a volume of 25 µl containing 1.5µl DNA, 5µl Master mix Taq PCR PreMix produced by Intron, Korea, and 1 µl of each primer. Thermal cycling conditions were Denaturation at 94°C for 3 min, followed by 35 cycles (94°C for 45 sec, 52°C for 45 sec, and 72°C for 45 sec), with a final incubation at 72°C for 7 min. Products were separated by electrophoresis on a 1.5% agarose gel. These were followed by sequencing using the same forward and reverse primers used in the PCR by Pishgam Biotech. The obtained DNA sequences were deposited in NCBI. (Ahmed et al.,2023). The generated consensus sequences were compared with other sequences in the NCBI database using BLAST to determine the identities of the fungal isolates. A phylogenetic tree was constructed using multiple maximum likelihood (ML) sequence alignments in MEGA 11, based on a substitution model, and the strength of clusters and branches was assessed using 1000 bootstrap iterations.

Chlorate sensitivity

Discs of 0.5 cm diameter were transferred from the edge of a 7-day-old fungal culture to the middle of a Petri dish containing PDA medium containing 120 mmol potassium chlorate. The plates were incubated for 1 week at 27°C, and the control treatment consisted of growing the fungus on PDA medium. Colonies that showed discontinuous growth and light color were classified as (feathery), Colonies that showed circular growth and completely black colonies were classified as (dense), Colonies that showed no growth or showed limited growth were classified as (restricted) (Rayatpanah, *et al.,* 2012).

Temperature sensitivity

Discs of 0.5 cm diameter were transferred from the edge of a 7-day-old colony of *M. phaseolina* (grape 1 and B66) to the middle of a Petri dish containing PDA. The dishes were incubated for a week at different temperatures (15, 20, 25, 30, 35,

40°C). (Akhtar, *et al.,* 2011). Three Petri dishes were used for each temperature; the results were taken by calculating the area of the colonies using the Image J software to draw a chord diagram.

Pathogenicity Test

The pathogenicity test was performed by inoculating healthy stems of two-year-old potted grapevines (Kishmish variety) with discs of two isolates of *M. phaseolina* (grape 1 and B66), with three replicates for each isolate. Disease development and symptoms were evaluated after 60 days of inoculation. (Abed-Ashtiani *et al.,*2018).

RESULTS AND DISCUSSION

Diagnostic results

Their morphological characteristics identified Macrophomina. Colonies on PDA showed a distinct color range from dark gray to black, a characteristic feature of *Macrophomina* species. Mycelium was transparent to brown in color, with gradations appearing as colony age increased. Mycelium branches showed sharp-angle ramifications, a typical morphological feature of *Macrophomina*. Hyphae were thin to medium in thickness, with regular cells and septate hyphae were clearly observed with time. An increase in the intensity of black color was observed due to the accumulation of Microsclerotia after 4-5 days. The microsclerotia, round to irregular in shape and characterized by a rough or irregular surface, distributed uniformly within and around the colony and measured 105–185 µm long (mean 135 µm) and 61–95 µm wide (mean 85 µm). No conidia or conidiophores were observed in the colony. These morphological features were identical to *Macrophomina phaseolina* according to (Poudel and Vaghefi, 2023).

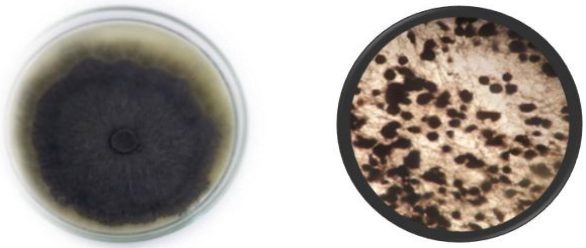


Fig 1: (A) colony of *M. phaseolina* on PDA medium showing dark pigmentation and irregular borders after 7 days of incubation at 25°C. (B) Microsclerotia at 100x magnification, appearing as dark, round to oval structures within the hyphae.

Molecular Diagnosis

The fungal isolates were identified as *M. phaseolina* by gene sequence analysis. The ITS regions of rRNA were amplified using primers ITS1/ITS4, and the DNA sequences of two representative isolates, Grape 1 and B66, were registered in the NCBI database with accession numbers OQ802810 and PQ587052.The BLAST search results showed 99%-100% identity with the reference sequences of M. phaseolina on NCBI. The phylogenetic tree Fig 2 revealed that there is evolutionary closeness between the isolates, with minor differences supported by bootstrap values. Stronger relationships are seen in branches with high bootstrap values (66-69) compared to branches with lower bootstrap values (47-55) and show a clear division of isolates into evolutionary groups Clades supported by varying bootstrap values. Isolates OP599770.1 and OQ802810.1 showed the highest. evolutionary support (bootstrap 69), making them the closest evolutionary within the tree and indicating a very strong evolutionary relationship between them. The relatively low bootstrap values (47-55) in some branches indicate slight differences between isolates, perhaps due to their environmental or geographic diversity. Also, all listed Macrophomina isolates are more closely related to each other than to the Fusarium verticillioides isolate (Outgroup), which strengthens the validity of the tree arrangement and confirms that all Macrophomina phaseolina isolates belong to the same species but show internal diversity.

Table 1. shows the percentage of conformity of Iraqi *M. phaseolina* isolates with global isolates registered in NCBI.

Description	Quarry cover	percent	ACCESSION	country
Macrophomina phaseolina strain BRIP 68039	100%	100	**MK968305.1**	Australia
Macrophomina phaseolina isolate DENI MacPha013	100%	100	**OQ305124.1**	Hungary
Macrophomina tecta isolate PRS3	99%	99	**PP352063.1**	Iran
Macrophomina phaseolina isolate grape	100%	100	**OQ802810.1**	Iraq
Macrophomina phaseolina isolate B66	100%	100	**PQ587052.1**	Iraq
Macrophomina phaseolina strain P1BR2	100%	100	**JN672592.1**	Philippines
Macrophomina phaseolina isolate CRUSASF1001	100%	100	**KF432177.1**	Saudi Arabia
Macrophomina phaseolina isolate Gaziantep18/1	100%	100	**PQ644267.1**	Turkey
Macrophomina phaseolina isolate CUZF16HIK	100%	100	**OM980256.1**	Turkey
Macrophomina phaseolina strain KARE1045	100%	100	**MH577343.1**	USA
Macrophomina phaseolina strain BRIP15497	100%	100	**MW596110.1**	USA
Macrophomina phaseolina isolate Yuma	100%	100	**OP599770.1**	USA

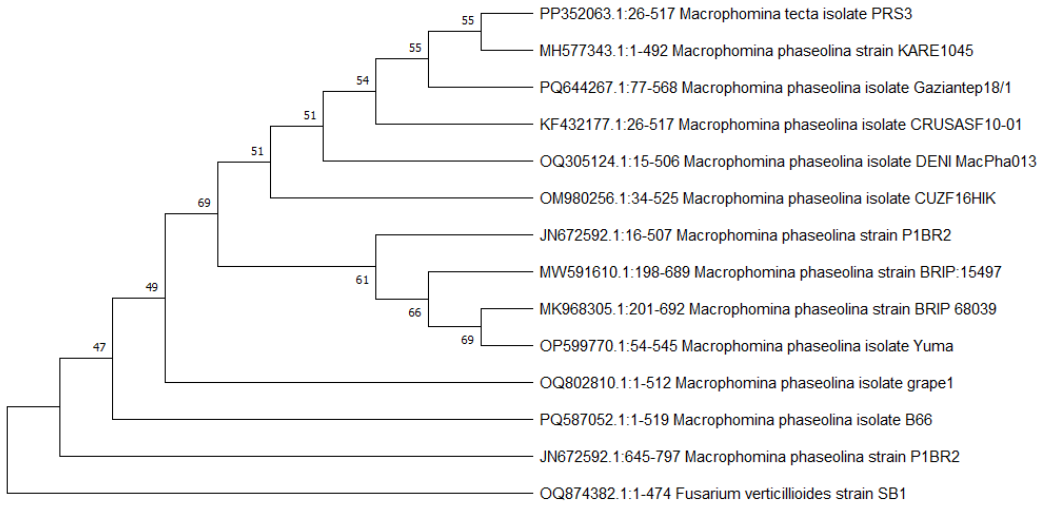


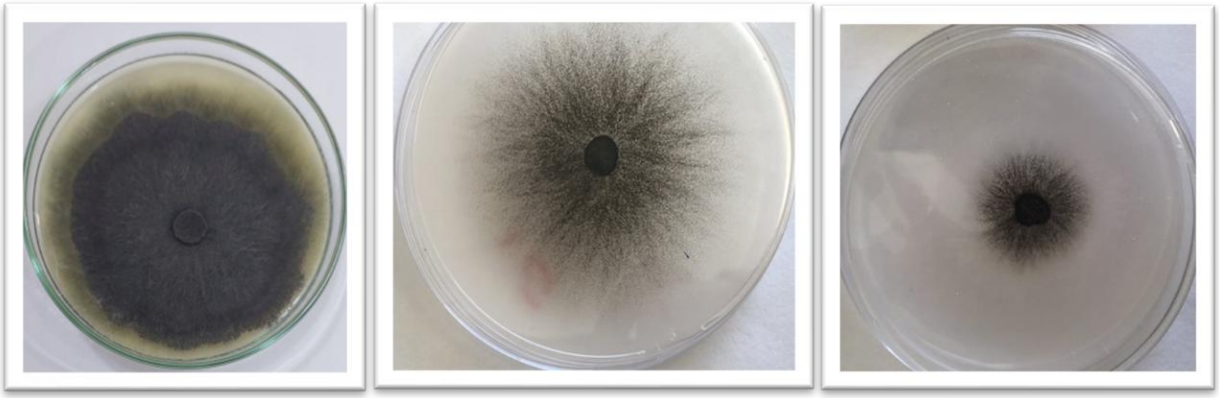
Figure 2. Phylogenetic tree of *M. phaseolina* isolates (isolate A: B66 and isolate Grape1), constructed based on the ITS1,4 region sequence using the nearest neighbor joining method and supported by bootstrap values of 1000 iterations.

Chlorate sensitivity

Results in Table 2 and Figure 3 indicate the diversity of phenotypes of *M. phaseolina* isolates when grown in chlorate-containing media. Out of 10 isolates analyzed, two isolates showed a Dense growth pattern, five isolates showed a Feathery growth pattern, and three isolates had a Restricted growth pattern. The Dense pattern indicates that these isolates can grow vigorously in the presence of chlorate and have a high tolerance to chlorate. The Branching pattern showed an intermediate ability to adapt to chlorate, and the Restricted pattern represents the isolates with the least ability to grow in the presence of chlorate.

Table 2 Growth patterns of *M. phaseolina* isolates growing in chlorate media

Growth Pattern	Number of Isolates	Interpretation
Dense	2	Vigorous growth and high chlorate tolerance
Feathery	5	Intermediate adaptation ability
Restricted	3	Lowest growth ability in chlorate presence

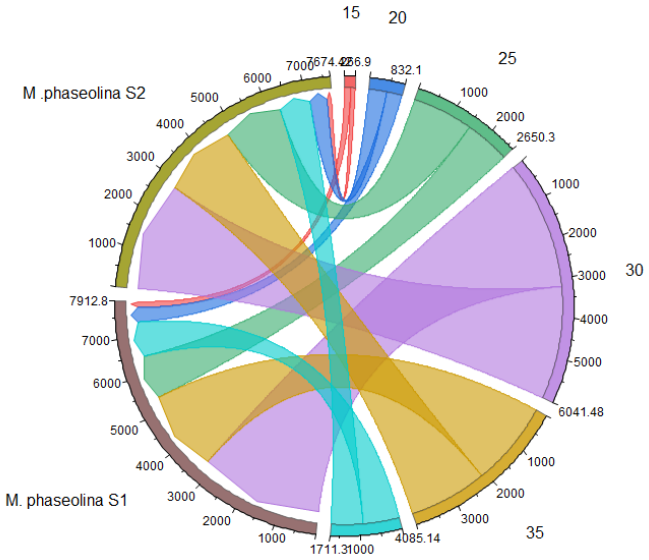


A **B** **C**

Figure 3 *M. phaseolina* Growth chlorate patterns A: Dense pattern B: Feathery pattern C: Restricted pattern

Temperature Sensitivity

Figure 4 shows that *M. phaseolina* isolates growth is low at low temperatures of 15 and 20°C. The links between 15°C and the colony area are small and narrow. Growth is very limited at 20°C, colony area increases slightly but growth is still weak. At 25°C, it marks a transition point at which colonies begin to grow significantly. The links become wider and longer at higher temperatures, and fungal growth responds accordingly. Fig. 4. Chord diagram of M. phaseolina isolates 'growth (S1 B66, S2 Grape 1) at different temperatures. The growth abundance is expressed as a colony



area. The line indicates that the thicker the line, the more colony growth.

Figure 4 Chord diagram of *M. phaseolina* isolates growth (S1 B66,S2 Grape 1) in different temperatures The growth abundance is expressed as a colony area. The line indicates that the thicker the line, the more colony growth.

Pathogenicity Test

Pathogenicity test of *M. phaseolina* isolates (grape 1 and B66) on grapevines confirmed that the isolates caused clear disease symptoms. The symptoms appeared 60 days after inoculation and were similar to those observed on naturally infected plants. The symptoms included dry leaves that remained green and general plant wilting, giving them a decline in appearance. When examining the inoculated branches, lesions were observed extending to 27–35 cm, with clear necrosis in the wood in longitudinal and transverse sections. Color changes under the bark also appeared, indicating the spread of the infection. The uninoculated plants (control) did not show any

disease symptoms. The fungus was re-isolated only from infected tissues, not from healthy plants, confirming that *Macrophomina phaseolina* is the cause of the symptoms. These results support the validation of Koch's hypotheses and the pathogenicity of the isolates *M. phaseolina* isolates (grape 1 and B66) on grapevines. *M. phaseolina* has been reported as a grapevine decline in Australia, Hawaii, Malawi, South Africa, Spain, the United States and Iran. (Gonzalez and Tello, 2011; Farr and Rossman, 2017; Abed-Ashtiani *et al.2018,* Nouri et al., 2018). *M. phaseolina* isolates exhibits a wide diversity of morphological appearances, with colonies ranging in color from dark to black with brown gradations with age. Microsclerotia are round to irregular and have a rough surface (Marquez, *et al.,* 2021). Molecular analysis using the ITS region sequencing and PCR revealed high genetic similarity among isolates, with minor variations associated with geographic and environmental variation. Previous studies have suggested that approximately 4% of the fungal genome contains transposable elements, which enhance genetic diversity through the introduction of new mutations, gene duplication and horizontal gene transfer (Shirai, and Eulgem, 2023). The sensitivity of *Macrophomina phaseolina* isolates to chlorate is an effective means of classifying them due to the great intraspecific differences in morphology and pathogenicity. Chlorate is an analogue of nitrate and can be toxic to plants and fungi when reduced to chlorite via the nitrate reduction pathway. This pathway plays an important role in determining the sensitivity or tolerance of fungi to chlorate (Sánchez, *et al.,*2017). The presence or absence of this pathway can be a marker for identifying host-specific strains (Tok, 2019; Aydogdu and Kurbetli, 2024).The chlorate phenotype was used to classify *M. phaseolina* isolates, showing that isolates with dense growth on chlorate medium had higher tolerance to chlorate. In contrast, isolates with pinnate or restricted patterns were more susceptible. This classification not only provides insight into isolates' ability to adapt to environmental conditions but also indicates their specificity towards the host and pathogenicity (Pekgöz and Tok, 2018; Pandey *et al*., 2020; Viejobueno *et al.,* 2022). To our knowledge, this is the first record of *M. phaseolina* associated with grapevine branch decline in Iraq.

CONCLUSIONS

M. phaseolina isolated from grapevine branches morphologically and molecularly, colonies appeared in color ranging from dark gray to black, with microsclerotia. Genetic sequencing results confirmed that the isolates were 99-100% identical to the reference sequences. The phylogenetic tree showed high genetic closeness with diversity among the isolates. The chlorate test showed diverse phenotypes and yielded the best growth at 30°C. Pathogenicity tests confirmed the ability of the isolates to infect grapevines, causing disease symptoms such as leaf drying, cankers, and wood necrosis, supporting the role of Macrophomina phaseolina isolates in the infection.

. ACKNOWLEDGMENT

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CONFLICT OFINTEREST

There is no conflict of interest, the authors state.

تدهور كرمات العنب المرتبط بالفطر *Macrophomina phaseolina*

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الخلاصة

تم عزل الفطر *Macrophomina phaseolina* من أغصـــان كرمات العنب التي ظهرت عليها اعراض التدهور. وشـــخص الفطر باســـتخدام الخصـــائص المورفولوجية والجزيئية. نمت مســـتعمرات الفطر *M. phaseolina* على وســـط PDA بتدرج لوني مميز بين الرمادي الداكن والأســـود وكونت الاجســـام الحجرية الصـــغيرة بعد 4–5 أيام. أظهر التشـــخيص الجزيئي لمنطقة ITS لعزلتي الفطر عزلتي *M. phaseolina* (Grape1 و B66) اللتين تم إيداعهما في قاعدة بيانات NCBI بأرقام انضمام OQ802810 و PQ587052 تطابقًا بنسبة 99–100٪ مع التسلسلات المرجعية في قاعدة بياناتNCBI ، أظهرت شجرة التقارب الوراثي تقاربًا تطوريًا واضـــحًا بين عزلتيGrape1 وB66 مع اختلافات طفيفة بســـبب التنوع البيئي أو الجغرافي. أظهر اختبار حساسية الكلوريت ثلاثة أنماط للنمو (كثيف، ريشي، مقيد) وأظهر اختبار درجة الحرارة المثلى لنمو الفطريات أن أقصـى نمو لعزلتي الفطر *M. phaseolina* تحقق عند 30 سـيليزية، في حين كان النمو

ضعيفًا عند درجات حرارة منخفضة 15–20 سيليزية أو عالية 35 سيليزية. أظهر اختبار القدرة المرضية أن عزلتي *M. phaseolina* أظهرتا أعراض مرضية واضحة تتمثل في التدهور العام وذبول النباتات الملقحة.

الكلمات المفتاحية: العنب، الكلورايت، تدهور، *M. phaseolina*.

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