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Study of the Diversity in Different Cultivars of *Pistacia vera* L. Resistant to Drought and Salinity: Comparing Protein Patterns Using SDS-PAGE Method

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Abstract

Pistachio is one of the most important agricultural products that have always been associated with Iran, and its production has a long historical background in our country. In this research, protein patterns of 10 cultivars of *Pistacia vera* L. were compared in which cultivars grown in normal conditions where compared with cultivars grown in salinity and water shortage to determine diversity. For this purpose, after extraction of storage proteins of seeds, the density of proteins was determined using the Bradford method. Then, the SDS-PAGE method was used to separate extracted proteins. In order to analyze electrophoretic data, we allocated 0 to absence and 1 to presence of each of bands. The results showed that stresses resulted in expression or loss of expression of some of the bands in cultivars, and the total distance between bands in each of the cultivars from origin of bands' movements from the gels was considered as one of the studied characteristics. The data was analyzed using cluster and Principal component analysis using SPSS software. Analysis of variance showed that average protein concentration of species was 19.59% and that the maximum protein percentage belongs to the Badami cultivar of Rafsanjan with 22.09%, and that the minimum protein percentage belongs to the Fandoghi cultivar of Rafsanjan with 13.98%. There was a significant difference among the amounts of proteins at the 5% level. In qualitative analysis of storage proteins of seeds, a total of 18 bands were observed. The maximum number of protein bands belongs to Kalehghochi and Fandoghi cultivars of Sirjan (saline area). In cluster analysis, species were classified in 10 different groups.

Keywords: Electrophoresis, *Pistacia vera* L, Seed storage proteins.

Introduction

Pistacia vera L. belongs to Anacardiaceae. Plants of this family include 75 genera and 600 species; of these, the pistachio genus has 11 species all of which discharge turpentine (Ahmed *et al*., 2010). Pistachio is one of the important agricultural products which have always been associated with Iran and its production has a long historical background in our country. The origin of the pistachio plant is still unknown, but most of the experts agree that this plant is probably native to central Asia (Sheibani *et al*., 1995). Among non-oil exports, pistachio has the first place in Iran. The major reasons for spread of its cultivation are likely its high economic value (because of export and uniqueness) and its resistance to drought and salinity of water and soil. Generally, three factors can influence resistance of plants to salinity and environmental stresses (Abiotic stress) at the molecular level: difference in genome structure, quantitative and qualitative differences in

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level of gene expression and difference in level and structure of proteins (Kosova *et al*., 2013). Every living creature lives in special environmental conditions. Since plants cannot move, stress is inevitable for them. Generally, the response to environmental conditions will lead to expression and induction of a set of proteins called stress proteins that protect the living creature against cell damage. Abiotic stressors include heat, low temperature, drought, salinity, lack of nutrients, ozone, heavy metals, UV-B rays, visible light, chemical toxicity, and oxidative stress, and pose serious threats to agricultural products. Abiotic stress usually causes protein dysfunction (Kim *et al*., 2001). In addition, a study of acorns shows that identified proteins can be classified into two major groups: stored proteins and stress or defense proteins (Meghani *et al*., 2003; Osborne, 1924).

In this study, the diversity of some cultivars of *Pistacia vera* resistant to salinity and drought was investigated by comparison of protein patterns using SDS-PAGE.

Materials and Methods

Plant Material

Samples were selected from five cultivars of *Pistacia vera* in Sirjan (a saline area) in which pistachio trees are irrigated just once in 45 days (drought stress) with irrigation water having a conductivity (Ec) of 13.4 dS/m and five other cultivars from Rafsanjan (without drought and salt stress) irrigated with water of Ec=1.9 dS/m which are irrigated once in 8-12 days. Pistachio seed samples Ahmadaghaei, Fandoghi, Kallehghouchi, Badami48 cultivars from Sirjan and Ahmadaghaei, Fandoghi, Kalehghochi, Badami 48 cultivars from Rafsanjan were collected. Samples were collected from each of the above-mentioned cultivars, peeled manually, and dried to a moisture content of 4-6 percent. Then, they were kept in a refrigerator at 4°C. Next, 15 samples were separated; the seed coatings were peeled off. Then the coat attached to the seed was eliminated and cotyledons and embryonic axes were powdered in a mortar and degreased using acetone. Then 0.2-g samples of dried powders were separately weighed and poured in micro-tubes, and SDS-PAGE electrophoresis was used to compare protein patterns. In order to do this, after extraction of storage proteins, Protein concentrations were determined by the Bradford method.

Seed protein extraction

1 ml of buffer, 50 mM Tris containing 2 mM EDTA, 1 mM DTT, 2 mM mercaptoethanol, pH=7.5, (weight/volume ratio of 1/20) was added (Khoshroo *et al*., 2013: Ehsanpour *et al*., 2010) and shaken in a shaker without refrigeration at room temperature at 500 rpm. Then, suspension was centrifuged at 4°C and 14000 rpm for 25 minutes. In the next stage, the upper layer, which is a clear solution, was removed and upper remaining layer was separated from sediment and poured in micro-tube. Then, as much as 5 time its volume of cold acetone was added and Vortexed. Next step was putting it in a freezer at -20 for an hour so that proteins float in solution as white flakes. At this point, we centrifuged the solution at 12000 rpm for 10 minutes and extracted the upper solution. Then, pure ethanol was added to the precipitated protein, after which the mixture was shaken and centrifuged again at 12000 rpm for 10 minutes. Again, the upper layer of liquid was removed and the tube was kept at room temperature until ethanol was completely evaporated. Protein was precipitated as a white powder. This sample was kept in a freezer at -20°C until the electrophoresis experiment was done (Ehsanpour *et al*., 2010; Grafin 2003).

Polyacrylamide Gel Electrophoresis

In this research, the SDS-PAGE method, electrophoresis in polyacrylamide gel in presence of sodium dodecyl sulfate, was used for electrophoresis. SDS-PAGE in 12.5% separating gel and 5% stacking gel was conducted using the Laemmli method (Laemmli, 1970). After extraction of seed proteins, the

amount of protein was measured using the Bradford method (Bradford 1976). Electrophoresis was conducted with 150V and 20-30 A (Kakaei 1389). Each sample was separately loaded on gel. A ladder with molecular weight markers of 10-250 kDa was used in a 20-40% gel (Burnette 1981).

Polyacrylamide gel staining

After electrophoresis, gel staining was performed using Coomassie Brilliant Blue R-250. In this method, fixation and staining of proteins were done simultaneously. After 2 hours of fixation and staining, removal of excess stain was done solution with methanol: acetic acid: distilled water (9:2:9) until gel was clear, after which it was then scanned (Grafin 2003).

Statistical analysis

The presence or absence of a band in proteomics of different cultivars was indicated by 1 and 0, and a comparison matrix was formed using this information. The comparison of protein percentage of cultivars was performed based on Duncan Method at significance levels of 1% and 5% (P-value -1% and 5%) using SPSS software. Analysis of variance was applied to survey the significance of protein percentages. In order to perform qualitative analysis of protein bands corresponding to each of the cultivar and to construct a table showing their similarities, TotalLab software was used. In order to compare the different cultivars with each other, Principle Component and Cluster Analysis were applied using Statistical software.

For comparing correlation between different cultivar based on the number of protein bands, the Pearson correlation coefficient (r) was used. This coefficient may vary between -1 and 1 ($-1 \leq r \leq 1$) (Khoshroo *et al*., 2013: Ehsanpour *et al*., 2010).

Results

In this research, in order to compare protein patterns, after extraction of storage proteins of pistachio seeds, the profiles of the seed storage proteins were compared after electrophoresis using Total Lab software. The results showed that seed storage protein profiles for different cultivar formed 18 bands, most of which (13 bands) correspond to the Kalehghochi and Fandoghi cultivars from saline areas, and the least number of bands (10 bands) belong to Badami and Ahmadaghaei cultivars from non-saline areas(Fig. 1 and Table 1). Results also showed that there is enough diversity in seed proteins of cultivars and that none of the cultivars are similar to the others. Protein band weights of the cultivar from saline areas are between 12-140 kDa and band weights of those in non-saline areas is between 15-141 kDa. Bands of all cultivars were evaluated based on the presence or absence and the distance from origin and presence of band No. 9 (except for the Kalehghochi cultivar); the stability of band No. 2 in all cultivars was observed, which could be used as an identification characteristic in the study of cultivars. Expression of bands No. 14 and 18 in stressed cultivars (except for one of them) and the lack of expression and absence of the protein bands No. 10, 13 and 15 in some cultivars from saline areas, in comparison with those of non-saline areas, confirms the results of previous studies and suggests that stress in some cultivars causes an increase and in some other cultivars causes a decrease in number of proteins and expression or absence of them. Comparison of Badami cultivars in saline areas and non-saline areas shows that presence of bands No. 5, 6, 8, 14 and 18 with molecular weights of 88.46, 80.46, 53 and 23.78 kDa, and absence of bands No. 10 and 15 with molecular weights of 41.46 and 20.43 kDa in saline area cultivars in comparison with non-saline area cultivars show the consequences of lack of expression of some proteins and an increase in expression of some others (Fig. 1).

In addition, the absence of bands No. 5 and 17 with molecular weights of 88.46 and 16.48 kDa and presence of protein bands No. 6, 9 and 14 with molecular weights of 84.10 and 24.70 kDa showed the difference between Kalehghochi cultivar of saline and none-saline areas.

Comparison of storage protein bands of Badami Sirjan showed that this cultivar has 12 protein bands with molecular weights between 14 and 140 kDa. Badami Sirjan lacks protein bands No. 1, 10, 11, 13, 15 and 17 and the relative motion is between 0.121-0.777.

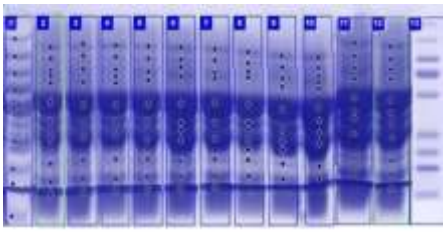

Fig. 1. Proteomics resulting from Storage protein electrophoretic pattern of pistachio seed.
(Left to right: Molecular weight ladder injected in the gel, Badami Rafsanjan, Kaleghuchi Sirjan, Kaleghuchi Rafsanjan, Fandoghi ghi Rafsanjan, Ahmadaghaei Rafsanjan and Ahmadaghaei Sirjan, Fandoghi 48 Sirjan, Fandoghi 48 Rafsanjan, Molecular weight ladder)

According to fig. 2, pistachio cultivars could be placed in 5 groups, giving results very similar to the dendrogram of cultivars.

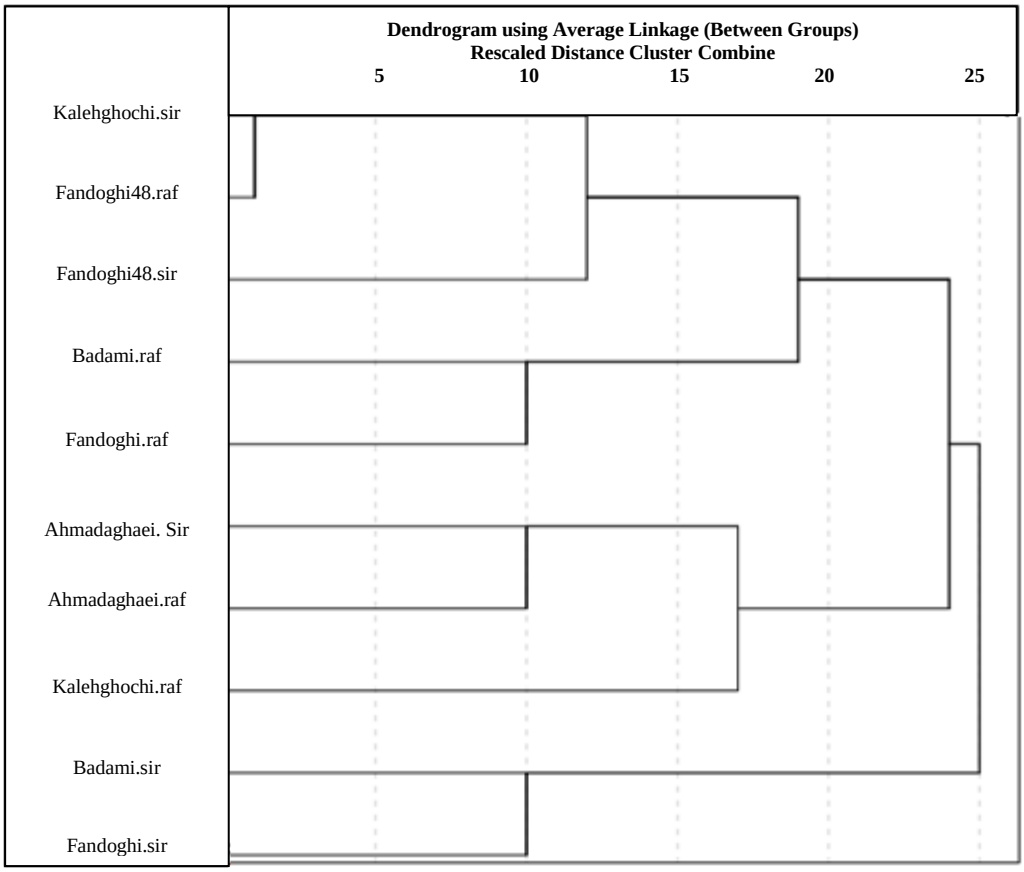

Fig. 2. Classification of pistachio cultivars using cluster analysis average linkage method

For comparing correlation between different cultivar based on number of protein bands, the Pearson correlation coefficient (r) was used (Table 1).

Table 1. Correlation between different pistachio cultivars based on number of protein bands, Pearson correlation coefficient (r).

	Badami Sirjan	Badami Rafsanjan	Kalehghochi Sirjan	Kalehghoch Rafsanjan	Fandoghi Sirjan	Fandoghi Rafsanjan	Ahmadaghaei Sirjan	Ahmadaghaei Rafsanja	Fandoghi48 Sirjan	Fandoghi48 Rafsanjan
Badami Sirjan	1									
Badami Rafsanjan	.079	1								
Kalehghochi Sirjan	.351	.194	1							
Kalehghoch Rafsanjan	-.081	.204	.269	1						
Fandoghi Sirjan	.351	-.055	-.108	.014	1					
Fandoghi Rafsanjan	-.322	.433	.269	.299	.014	1				
Ahmadaghaei Sirjan	.161	-.255	.269	.299	.014	.065	1			
Ahmadaghaei Rafsanja	-.158	.100	-.055	.204	-.055	.433	.433	1		
Fandoghi48 Sirjan	.079	.100	.194	-.255	.194	.204	-.025	.100	1	
Fandoghi48 Rafsanjan	.079	.100	.693**	.204	-.055	.433	-.025	.100	.550*	1

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Variance of the test results for cultivars shows that the highest percentage of average protein, 19.71%, belongs to Sirjan cultivar (Table 1). Among cultivars chosen from Sirjan, the highest percentage of average protein is 21.39%, seen in the Fandoghi48 cultivar, and the lowest amount is 18.21%, seen in the Badami cultivar. The average percentage of protein for different cultivars from Rafsanjan is 19.47%. The highest percentage of average protein for cultivars of Rafsanjan is 22.09% that belongs to Badami cultivar and the lowest amount is 16.91% that belongs to Fandoghi cultivar. Analysis of variance showed that average protein concentration of cultivars was 19.59% regardless of the city and shows a significant difference of 5%. Nevertheless, considering cities, no significant difference shown by analysis of variance and in dual comparison of cultivar difference was observed. The comparison of protein percentages of cultivars based on the Duncan Method, at a significance level of 5%, showed that there is no significant difference in protein percentage, and that the Fandoghi48 cultivar showed the highest and Fandoghi cultivar showed the lowest amount of protein regardless of cities.

The results of the test conducted in Sirjan, where there is very high salinity; in comparison with those of the test conducted in Rafsanjan conform to previous studies because salinity and drought stress lead to an increase in the amount of protein in stressed cultivars in comparison with the normal situation. The reason for this increase is the effect of stress on the gene expression that occurs to adapt the plant to these stresses. Since it has been a long-term stress, plant has adapted itself to the situation.

Comparison between storage protein bands of Badami cultivar from Rafsanjan showed that this cultivar has 10 protein bands with molecular weights

between 18-141 kDa. The Badami cultivar of Rafsanjan lacks protein bands no. 1, 5, 6, 11, 14, 17 and 18 and the relative motion is between 0.119 and 0.708. This cultivar has 10 protein bands and in comparison with Badami cultivar of Sirjan, this cultivar lacks bands No. 5, 6, 8, 14 and 16, but it has two protein bands with molecular weights of 56 and 43 kDa which are not expressed in saline area cultivars. These two cultivars have bands No. 2, 3, 4, 6, 8, 9, 12 and 16 in common.

Comparison of the storage protein bands in the Kalehghochi cultivar from Sirjan shows that this cultivar has 13 protein bands with molecular weights between 13 and 139 kDa. The Kalehghochi cultivar of Sirjan lacks protein bands No. 1, 5, 12, 15 and 17 and relative the motion is between 0.124 and 0.800.

Comparison of storage protein bands of the Kalehghochi cultivar from Rafsanjan showed that this cultivar has 11 protein bands with molecular weights between 16 and 141 kDa. The Kalehghochi cultivar of Rafsanjan lacks protein bands no. 1, 6, 9, 12, 14, 15 and 18 and the relative motion is between 0.119 and 0.732. In comparison with the Kalehghochi cultivar of Sirjan, this cultivar lacks bands No. 6, 9, 14 and 18, but it has protein band No. 5 that is not expressed in the Sirjan cultivar. These two cultivars have bands No. 2, 3, 4, 7, 8, 10, 11, 13 and 16 in common.

Comparison of the storage protein bands in the Fandoghi cultivar from Sirjan shows that this cultivar has 13 protein bands with molecular weights between 14 and 135 kDa. The Badami cultivar of Sirjan lacks protein bands No. 1, 6, 13 and 16 and its relative motion is between 0.132 and 0.784.

Comparison of storage protein bands of the Fandoghi cultivar from Rafsanjan showed that this cultivar has 11 protein bands with molecular weights between 16 and 139 kDa. The Fandoghi cultivar of Rafsanjan lacks protein bands no. 1, 5, 8, 12, 14, 16 and 18 and its relative motion is between 0.123 and 0.732. In comparison with the cultivar of Sirjan, this cultivar lacks bands No. 5, 8, 12, 14 and 18, but it has protein bands No. 6, 10, and 13 that have not been expressed in Sirjan cultivars. This two cultivar are similar in not having protein bands No. 1 and 16.

Comparison of the storage protein bands in the Ahmadaghaei cultivar from Rafsanjan shows that this cultivar has 10 protein bands with molecular weights between 15 and 140 kDa. The Ahmadaghaei cultivar of Sirjan lacks protein bands No. 1, 4, 7, 11, 12, 14, 16 and 18 and its relative motion is between 0.121 and 0.757. This cultivar has protein bands No. 10 and 15 that were not expressed in the Sirjan cultivar.

Comparison of storage protein bands of the Ahmadaghaei cultivars from Sirjan showed that this cultivar has 11 protein bands with molecular weights between 12 and 132 kDa. Ahmadaghaei cultivar of Rafsanjan lacks protein bands no. 1, 4, 7, 10, 12, 14 and 15 and its relative motion is between 0.139 and 0.827. In comparison with the Ahmadaghaei cultivar of Rafsanjan, this cultivar lacks bands No. 10 and 15, but it has protein bands No. 11 and 16 that are not expressed in Rafsanjan cultivars. These two cultivars are similar in not having protein bands No. 1, 4, 7, 12 and 14.

Comparison of the storage protein bands in the Fandoghi 48 cultivar from Sirjan shows that this cultivar has 10 protein bands with molecular weights between 20 and 104 kDa. The Fandoghi48cultivar of Sirjan lacks protein bands No. 1, 2, 5, 7, 10, 16, 17 and 18 and its relative motion is between 0.191 and 0.742. This cultivar lacks protein bands No. 7 and 11 that were expressed in the Rafsanjan (non-saline area) cultivar.

Comparison of storage protein bands of the Fandoghi48 cultivar from Rafsanjan showed that this cultivar has 11 protein bands with molecular weights between 20 and102 kDa. The Fandoghi48 cultivar from Rafsanjan lacks protein bands no. 1, 2, 5, 13, 16, 17 and 18 and its relative motion is between 0.198 and 0.778. In comparison with Fandoghi48cultivar of Sirjan, this cultivar lacks band No. 13.

Band No. 2 in all cultivars remains unchanged and the expression of band No. 18 in saline areas in comparison with other cultivar can show that this band has been expressed because of stress. It can be inferred, among common cultivar of the two areas, that most changes happen in bands of the Fandoghi cultivar and the fewest changes relate to those of the Badami cultivar.

It seems that bands No. 5, 6 and 7 are expressed with greater intensity in stressed cultivars.

Discussion

Pistachio tree is resistant to drought and salinity, and results of the test conducted in Sirjan, which has a high salinity, in comparison with test results of Rafsanjan cultivars conformed to previous findings. Because drought and salt stress causes an increase in the amount of protein in stressed cultivar, this increase is the effect of stress on gene expression that makes the plant capable to resist against these stresses. Because it is a long-term stress, plant has become adapted.

A study conducted by Hamilton *et al.* (2000) to analyze the reaction of plants to stress showed that plants are exposed to a wide range of stress in their life cycle. One of the ways to understand plants abilities to tolerate environmental stresses is by identifying the changes induced by stress, such as their amounts of protein, considering that adaption to stress is a result of change in gene expression. Protein synthesis changes in response to environmental stresses such as heat stress, anaerobic conditions, drought stress, osmotic shock, wound, LTS and salinity. Such stresses lead in an increase in synthesis of some proteins and a decrease in synthesis of some others. It seems that proteins induced by salinity can be effective in tolerating this stress. So far, some proteins have been identified which have salinity resistant genotypes. Resistance to salinity is a result of cooperation between some physiologic and biochemical factors (Hamilton *et al*., 2000).

A study performed by Halal *et al.* (1975) on salt stress in tobacco and maize showed that one of these factors can be the ability of ions to bind to proteins and different proteins which are induced after salt treatment. Protein synthesis changes as a response to salt stress. This change can be an increase in some of them and a decrease in some others. These two situations have been demonstrated in tobacco and maize tissue culture. Salt stress changes translatable mRNAs in barley roots and increases specific proteins significantly (Halal *et al*., 1975).

In a study conducted by Seyyedi *et al* (2010) on the application of seed storage protein in intra-specific variation in three population of *Pistacia atlantica* Desf, the average protein concentration of populations was 13 percent and there were significant differences at the 5% level, and the seed storage protein profile showed 21 bands which could be separated into 3 populations (Seyydi *et al*., 2010).

A study performed by Mighati *et al*. (2010) on some wheat cultivars showed that wheat cultivars, like other glufits (sensitive to salinity), responsed to salinity by changing gene expression. A short time after contact with salt, mRNA is stored in wheat root and reaches a maximum level after 6-12 hours. Generally, salts have two incompatible effects on proteins: a) they tend to break electrostatic bonds b) they increase hydrophobic interactions. The study was conducted on two wheat cultivars--the Ghods cultivar, which is sensitive to salinity, and the Bolani cultivar, which is resistant to salinity and it showed that in this stage, elimination and fading of protein bands occur. In research on protein changes in wheat leaf as a response to salinity, the 26 and 28 kDa bands become paler than others (Mighani *et al.,2003*).

In a study by Ahmad *et al.* (2010) performed on wheat proteins using SDS-PAGE, they found that the main effect of salinity is reduction of protein synthesis.

These negative effects include degradation of transcription and translation mechanisms.

In a study conducted by Galic *et al.* (1992) on *Lophopyrum elongatum*, they were led to believe that contact of this plant with salt, can increase or decrease the amount of specific proteins Sal. Salinity and drought stress has caused much agricultural loss throughout the world. Plant biology together with plant physiology and the use of biochemical and genetic methods is studying plants' response to biological stress in order to find ways to overcome the stresses. Biochemical indices data of the cells can be applied as a selection criterion for the resistance of plants to salt in agricultural products. We may be able to achieve a comprehensive solution and a complete analysis of defense mechanism of plants against biological and abiotic stresses using the above-mentioned classification. After some stresses, the reaction of protein, protein-protein interaction and post-translational term were identified (Kim *et al*., 2013).

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