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COMPARISON OF FOUR GENOMIC DNA ISOLATION METHODS FROM SINGLE DRY SEED OF WHEAT, BARLEY AND RYE

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Abstract

Modern breeding programs are based on the differences that distinguish one plant from another one, differences encoded in the plant's genetic material, the DNA. Genotypic selection, particularly at the DNA level, can be exploited in Marker Assisted Selection (MAS) to identify desirable recombinants among segregating populations. Successful DNA amplification is vital for the detection of specific DNA targets, and this depends on the ability of DNA isolation methods to produce good quality DNA.
DNA isolation from plant tissues remains difficult because of the presence of a rigid cell wall surrounding the plant cells. DNA isolation methods are affected by several factors like the amount of tissue needed and its availability, the number of steps involved and the chemicals used.
In this study four different DNA isolation methods, based on CTAB and SDS (three methods), applied on single dry seed of wheat, barley and rye, were tested and compared. The quality of DNA was assessed by spectrophotometric measurements, gel electrophoresis and PCR reactions. The results of the experiments showed that DNA isolated by all four isolation methods is not excessively fragmented, A260/A280 ratio was between 1.6-1.9 and the concentration ranged between 20-194 ng/µl. The quality of the DNA was good and allowed the amplification of specific fragments by PCR. CTAB method had better A260/A280 ratio followed by SDS1, SDS2 and SDS3. Electrophoretic pattern showed better results with SDS2 method, followed by SDS3, SDS1 and CTAB. Furthermore, the CTAB and SDS1 methods need more time than SDS2 and SDS3, even though the number of steps of each method are almost equal (±2 steps). An estimative cost per sample showed that the cheapest method is the SDS3, the other three having similar costs.

Key words*: DNA isolation, single dry seed, wheat, barley, rye.*

INTRODUCTION

Modern plant breeding, due to advancements in genetics, molecular biology and tissue culture, is being carried out by using molecular genetics tools. Genotypic selection, particularly at the DNA level, can be exploited in Marker Assisted Selection (MAS) to identify desirable recombinants among segregating populations.
PCR allows the selective amplification of specific segments of DNA in a mixture of other DNA sequences. Isolation of DNA would be the first step in such analytical methods. Successful DNA amplification is vital for the detection of specific DNA targets, and in return this depends on the ability of DNA isolation methods to isolate DNA of reasonable quantity, purity, integrity and quality and is often the most time consuming step of a DNA-based detection method (Singh, 2009).
Typical plant DNA isolation methods must go through some basic steps like breaking the cell wall, usually done by grinding the tissue, disruption of the cell membrane, using a detergent like SDS (sodium dodecyl sulfate) or CTAB (cetyltrimethyl ammonium bromide), protection of DNA from the endogenous nucleases with EDTA (chelating agent that binds magnesium ions, generally considered a necessary cofactor for most nucleases), removal of proteins from the buffer/tissue mixture using chloroform or phenol to denature and separate the proteins from DNA, precipitation of DNA with either ethanol or isopropanol (Rogers and Bendich, 1989).
The presence of a rigid cell wall surrounding the plant cells, polysaccharides, proteins, and DNA polymerase inhibitors (tannins, alkaloids,

and polyphenols) makes the DNA isolation from cereals a difficult task. Polysaccharides, the most common contaminants found in the plant DNA isolation, make DNA pellets slimy and difficult to handle. The anionic contaminants inhibit restriction enzymes and affect enzymatic analysis of the DNA. The presence of these compounds reduces the quality and quantity of DNA which often makes the sample non-amplifiable. Furthermore, DNA isolation methods are affected by several factors like the amount of tissue needed and its availability, the number of steps involved, and the chemicals used (Chaves et al., 1995).

DNA isolation performed on seed instead of leaf tissue allows MAS to be carried out independently of the growth season, and the time and glasshouse space needed for growing the plants are saved. Most importantly, the seed can be analyzed during the non-field season, selected and prepared for the next breeding cycle. Furthermore it is possible to send seed samples internationally for comparative studies, this being difficult for leaf samples which have to be kept on ice or lyophilized (Von Post, 2003).

The isolation of high-quality DNA from plant tissue is time consuming, laborious, and quite expensive due to multiple steps and the cost of reagents used. High quality DNA is characterized by predominantly high molecular weight fragments with an A260/280 ratio between 1.8 and 2.0 and the lack of contaminating substances (Abdel-Latif and Osman, 2017).

A fast, simple, and reliable DNA isolation method, which does not require long incubations, multiple steps or expensive commercial kits, that could meet in the PCR, sequencing and next-generation library preparation requirements, will be invaluable to plant research. Therefore, the aim of this study was to compare quality and quantity of DNA isolated using four different isolation methods from a single dry seed of three cereal species, wheat, barley and rye. Furthermore, we compared the isolation methods regarding the number of steps, time and price/sample.

MATERIALS AND METHODS

Plant material was obtained from NARDI Fundulea, Romania, and consisted of seeds from three cereal species, wheat (Izvor – cultivar with the *Lr34* resistance allele), barley (Scânteia cultivar) and rye (Harkovskaya cultivar). 16 individual seeds (four seeds / isolation method, DNA was isolated from each seed - four repetitions) were used from each cultivar. The seeds were dry crushed using a mortar and pestle. The amount of crushed sample obtained from each seed ranged between 30-60 mg.

DNA isolation was performed using four different modified methods (CTAB; SDS 1; SDS 2 and SDS 3).

Table 1. The isolation buffers for each method are shown in

CTAB	SDS 1	SDS 2	SDS 3
100 mM Tris	100 mM Tris	100 mM Tris	100 mM Tris
700 mM NaCl	-	500 mM NaCl	-
50 mM EDTA	50 mM EDTA	50 mM EDTA	50 mM EDTA
2% CTAB	1.5% SDS	1% SDS	1.25% SDS
-	-	2.5% D-Sorbitol	-
-	-	2% N-Lauroylsarcosine sodium salt	-
140 mM β-mercaptoethanol	-	-	-

- CTAB - based on Murray & Thompson, 1980;
- SDS 1 - based on Mohammadi, http://shigen.nig.ac.jp/ewis/article/html/118/article.html;
- SDS 2 - Cristina et al., 2015;
- SDS 3 - based on Chao & Somers, 2012.

The protocols steps for DNA isolation using the above mentioned methods are shown in Table 2. Removal of proteins from the buffer/tissue mixture, in case of CTAB and SDS 1 methods, was done with dichloromethane:isoamyl alcohol (24:1) instead of chloroform:isoamyl alcohol.

Dichloromethane offers a cheaper, less toxic alternative to chloroform in protocols for DNA isolation (Chaves, 1995).

Table 2. Protocols for DNA isolation

Step	CTAB	SDS 1	SDS 2	SDS 3
Isolation buffer	600 µl (freshly made)	500 µl	500 µl (freshly made)	500 µl
Incubation 65°C	60’	60’	-	30’
Cooling samples	2-3’	2-3’	-	-
Incubation 4-7°C	-	-	-	15’
Vortex	-	-	1’	-
DNA purification	Mixed with 1:1 vol. Dichloromethane:Isoamyl alcohol (24:1, V:V)	Mixed with 270 µl Potassium acetate 3M (final conc. 1.62M)	200 µl Potassium acetate 3M (final conc. 1.2M)	Mixed with 250 µl Ammonium acetate 6M (final conc. 3M)
Vortex	-	-	2’	-
Incubation 4-7°C	-	-	-	15’
Centrifugation	12’ 9500 RCF	15’ 7690 RCF	15’ 16055 RCF	15’ 7690 RCF
DNA purification	>500 µl supernatant mixed with 1:1 vol. Dichloromethane:Isoamyl alcohol (24:1, V:V)	>400 µl supernatant mixed with 1:1 vol. Dichloromethane:Isoamyl alcohol (24:1, V:V)	-	-
Centrifugation	12’ 9500 RCF	10’ 7690 RCF	-	-
RN-ase treatment	45’			
DNA precipitation	5µl NaCl (5M) per 100µl sample + 2 vol. EtOH (kept at -20°C) *optional, for better DNA precipitation, incubate samples on ice for 2-3’			
Centrifugation	6’ 16055 RCF			
DNA pellet wash	200-300 µl Wash buffer (76% EtOH, 10 mM NH_4OAc)			200 µl EtOH 70%
Centrifugation	5’ 18620 RCF			
Pellet drying	10-30’ at room temperature			
DNA dissolving	100 µl TE (TE should be adjusted according to pellet dimension) > samples are kept overnight for dissolving at 4-7°C			
Optional step	If the pellet is not dissolved in the next day the upper faze could be transferred into new tubes before quality and quantity checking.			

Gel electrophoresis
Both the genomic DNA and PCR products were analysed by agarose gel electrophoresis using 0.8% agarose gel for the genomic DNA and 1.2-1.5% agarose gels for PCR products**.**

Spectrophotometric measurements.
DNA purity (A260/A280 ratio) and quantity analysis were performed with a Beckman Coulter Life Sciences DU 730 spectrophotometer.

Evaluation of DNA amplification
In order to evaluate if the DNA is amplifiable, all of the samples analysed were subjected to PCR amplification using the following markers: cssfr5 – functional marker for Lr34 gene selection in wheat (Lagudah, 2009), HvBM5A-exon2 – barley VRN-H1 genotype assays (Zlotina, 2013) and SCM9 – rye SSR (Saal and Wricke, 1999). Also, we used ISSR 17898B, UBC818 and UBC 876 for all samples.
One sample of wheat, barley and rye from each method has been chosen and diluted to a working concentration 25 ng/µL. PCR amplifications were performed in ABI ProFlex™ 3 x 32-well PCR System.

PCR amplification with functional marker cssfr5 was performed using KAPA2G Fast Multiplex PCR Kit (KAPA Biosystems) in a 10 µL final reaction volume containing 1X Multiplex Mix, 0.2 mM each primer, 2 µL DNA sample (40-50 ng). PCR programme was: initial denaturation at 95°C for 3 min, followed by 30 cycles of (95°C – 15 s, 62°C – 30 s, 72°C - 30 s) and a final extension at 72°C for 7 min. PCR product was analyzed on 1.2% agarose gel.

PCR reactions performed with primers HvBM5A-exon2 (barley), SCM9 (rye), 17898B and UBC 818 were carried out using MyTaq™ Red DNA Polymerase (Bioline).

PCR conditions were as follows:
- HvBM5A-exon 2 -15 µL final reaction volume containing 1X reaction buffer, 0.5 mM primers, 0.6U DNA polymerase and 3 µL DNA sample (60-80 ng). PCR programme: initial denaturation at 95°C for 1 min, followed by 35 cycles of (95°C – 15 s, 60°C – 15 s, 72°C - 10 s) and a final extension at 72°C for 5 min. PCR product was analyzed on 1.2% agarose gel.

- SCM9 -10 µL final reaction volume containing 1X reaction buffer, 0.5 mM primers, 0.3U DNA polymerase and 2 µL DNA sample (40-50 ng). PCR programme: initial denaturation at 95°C for 1 min, followed by 40 cycles of (95°C – 15 s, 60°C – 15 s, 72°C - 10 s) and a final extension at 72°C for 5 min. PCR product was analyzed on 1.5% agarose gel.

- 17898B -25 µL final reaction volume containing 1x buffer, 0.28 mM primer, 2U DNA polymerase, 1 µL wheat and rye DNA sample (20-30 ng), 1.5 µL barley DNA sample (30-40 ng). PCR programme: initial denaturation at 95°C for 3 min, followed by 35 cycles of (95°C – 15 s, 44°C – 15 s, 72°C - 30 s) and a final extension at 72°C for 5 min. PCR product was analyzed on 1.2% agarose gel.

- UBC 818 -25 µL final reaction volume containing 1x buffer, 0.28 mM primer, 1U DNA polymerase, 0.5 µL DNA sample (10-15 ng). PCR programme: initial denaturation at 95°C for 3 min, followed by 40 cycles of (95°C – 15 s, 50°C – 15 s, 72°C - 30 s) and a final extension at 72°C for 5 min. PCR product was analyzed on 1.2% agarose gel.

RESULTS AND DISCUSSIONS

The quality and quantity of the template DNA are critical factors for the successful PCR analysis. The efficiency of the DNA extraction steps can be critical for successful amplification since there are many compounds that inhibit DNA amplification that can be co-purified with the DNA, such as polysaccharides, lipids and polyphenols or extraction chemicals.
The comparative analysis of electrophoretic patterns of genomic DNAs (Figure 1) revealed visible DNA bands for all isolation methods applied. DNA isolation methods SDS 2 and SDS 3 overall had better electrophoretic profile, followed by SDS 1 and CTAB.

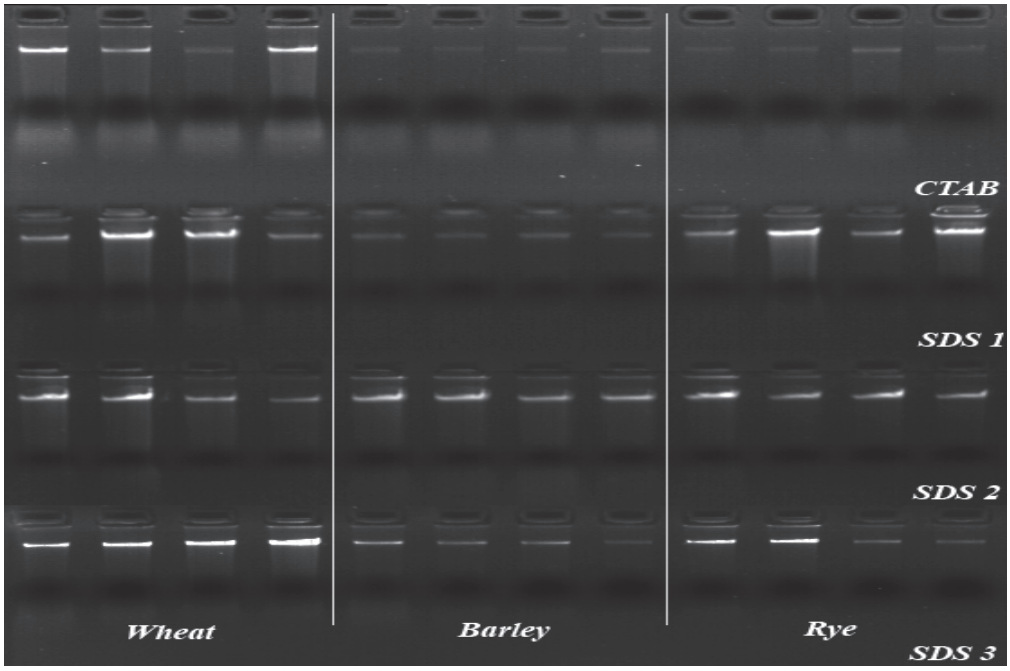

Figure 1. Agarose gel electrophoresis of genomic DNA

Spectrophotometric assessment of DNA quality revealed good results for all isolation methods applied (Table 3).

Table 3. Spectrophotometric results

Sample	CTAB		SDS 1		SDS 2		SDS 3	
	Ratio	ng/µl	Ratio	ng/µl	Ratio	ng/µl	Ratio	ng/µl
Wheat 1	1.767	89	1.693	46	1.617	155	1.736	49
Wheat 2	1.934	76	1.758	113	1.623	195	1.746	34
Wheat 3	1.883	85	1.814	77	1.784	35	1.613	54
Wheat 4	1.859	70	1.701	67	1.612	37	1.701	30
Min.	**1.767**	**70**	**1.693**	**46**	**1.612**	**35**	**1.613**	**30**
Max.	**1.934**	**89**	**1.814**	**113**	**1.784**	**195**	**1.746**	**54**
Barley 1	1.878	82	1.742	59	1.836	115	1.702	85
Barley 2	1.928	112	1.744	63	1.671	140	1.635	50
Barley 3	1.850	60	1.718	51	1.843	168	1.735	49
Barley 4	1.858	80	1.731	43	1.792	120	1.663	20
Min.	**1.850**	**60**	**1.718**	**43**	**1.671**	**115**	**1.635**	**20**
Max.	**1.928**	**112**	**1.744**	**63**	**1.843**	**168**	**1.735**	**85**
Rye 1	1.890	59	1.850	63	1.733	136	1.669	47
Rye 2	2.146	57	1.860	48	1.702	40	1.615	36
Rye 3	1.846	64	1.766	38	1.778	50	1.569	21
Rye 4	1.859	39	1.849	57	1.763	66	1.638	27
Min.	**1.846**	**39**	**1.766**	**38**	**1.702**	**40**	**1.569**	**21**
Max.	**2.146**	**64**	**1.860**	**63**	**1.778**	**136**	**1.669**	**47**

According to the results, CTAB method had overall better A260/A280 ratio (1.767-2.146), followed by SDS 1 (1.693-1.860), SDS 2 (1.612-1.843) and SDS 3 (1.569-1.746). DNA concentrations ranged between 39-112 ng/µl with CTAB method, 38-113 ng/µl - SDS 1, 35-195 ng/µl - SDS 2 and 20-85 ng/µl - SDS 3.

Electrophoretic and spectrophotometric results showed some differences regarding the DNA purity and concentration: in some samples the DNA concentration determined

spectrophotometrically was registered as high, but the electrophoretic analysis did not confirm the results, suggesting the presence of contaminants that affect the measurements and/or the electrophoresis results.

PCR amplification

Cultivar Izvor carries *Lr34* resistance allele to leaf rust (Ciuca et al., 2015) meaning that PCR with cssfr5 primers results in a PCR product amplification of 751bp (Figure 2).

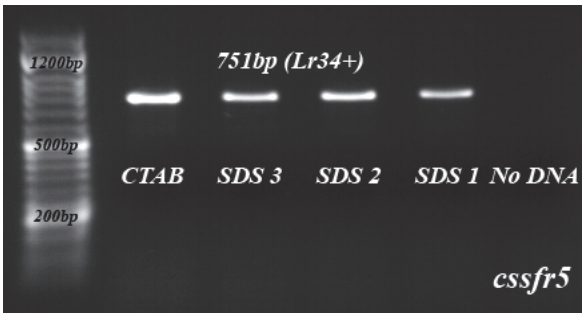

Figure 2. Agarose gel electrophoresis of PCR product obtained with cssfr5 functional marker

All DNA isolation methods applied for wheat cultivar gave good amplification results and no significant differences were observed.

PCR for barley DNA samples (cultivar Scânteia), amplified a 616bp PCR product with HvBM5A-exon 2 primers (Figure 3).

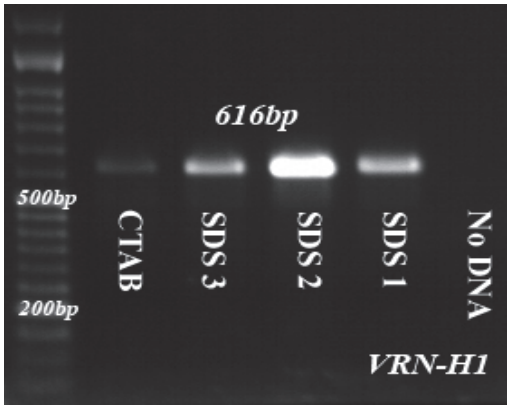

Figure 3. Agarose gel electrophoresis of PCR product obtained with HvBM5A-exon 2 primers

Barley DNA obtained with CTAB isolation method had weaker amplification signal compared to the other methods. DNA obtained with SDS 1 and SDS 3 methods gave good and similar amplification products. The best results seem to be obtained with DNA isolated by SDS 2 method, but the concentration of DNA template was higher (Figure 3).

PCR with SCM9 primers amplified in rye DNA samples (Harkovskaya cultivar) a 220bp product and no product in wheat cultivar Izvor (no-rye reference sample) (Figure 4).

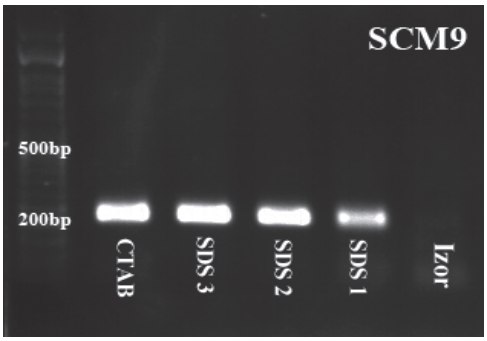

Figure 4. Agarose gel electrophoresis of PCR product obtained with SCM9 primer

All rye DNA samples were amplified but DNA sample obtained with SDS 1 method had a weaker amplified product than all other isolation methods. Nevertheless, DNA obtained with SDS 1 method gave good amplification if we compare it with DNA ladder intensity. As expected, wheat reference sample had no PCR product.

ISSR PCR with 17898B primer gave better results for barley DNA samples, but the amplification for wheat and rye DNA was weaker (Figure 5).

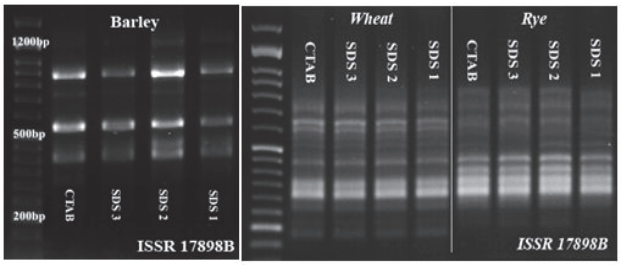

Figure 5. Agarose gel electrophoresis of PCR product obtained with 17898B primer

The next PCR using UBC 818 ISSR primer has shown good amplification for wheat DNA samples whatever the method used. The poorest results were observed in case of barley DNA obtained by CTAB method: no clear amplicons were detected. The best results were recorded with barley DNA samples obtained by SDS 2 and SDS 3 methods.

For rye DNA samples, weaker amplification was detected in the case of samples isolated with SDS 2 method (Figure 6).

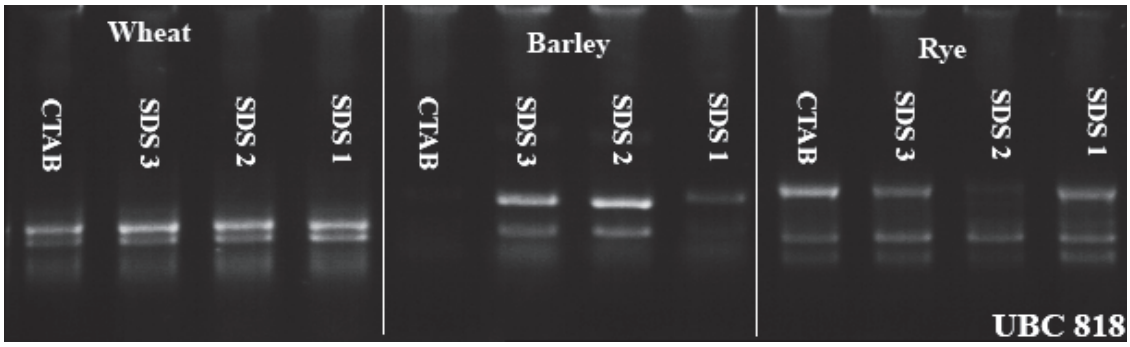

Figure 6. Agarose gel electrophoresis of PCR product obtained with UBC 818 primer

Regarding the cost/sample for each method it was found that SDS 3 method is the cheapest variant (Table 4). The cost/sample was calculated using the online available prices for reagents from Sigma-Aldrich (http://www.sigmaaldrich.com). Furthermore, we took in consideration the time needed to process 10-16 DNA samples: SDS 2 and SDS 3 are the shortest methods. For SDS 3 method it can be added that the extraction buffer does not need to be freshly made (Table 4), making this method suitable for large number of samples.

Table 4. Time, number of steps and the cost/sample

	CTAB	SDS 1	SDS 2	SDS 3
Time (average)	3-4h	3-4h	**2-3h**	**2-3h**
Number of steps	14	14	**12**	13
Cost/sample (€)	0.113	0.113	0.114	**0.107**

CONCLUSIONS

The methods used for the extraction of genomic DNA have a great influence on both quality and quantity of the recovered DNA. Furthermore, time, cost of reagents, amount of biological material needed and its availability are key factors that must be taken into consideration before choosing the best DNA isolation method. Differences among the methods tested related to the plant species were observed.

Barley DNA obtained with CTAB method, presented in this study, had weaker amplification in some cases making this method not suitable for this type of cereal.

The quality of DNA obtained with the methods applied allowed good amplification results when specie-specific primers were used. Contrary, depending on the isolation method used, differences among the DNA samples were detected with ISSR primers.

The best results regarding the DNA quality and quantity, the amplification results, the cost and the time it takes to DNA extraction protocol were obtained with SDS 3 method, making this method suitable for large number of samples.

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