The Storage of the Extracted Plant DNA: Is –20°C Cold Enough?

Čuvanje izolirane biljne DNK: je li –20 °C dovoljno hladno?

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THE STORAGE OF THE EXTRACTED PLANT DNA: IS –20 °C COLD ENOUGH?

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SUMMARY

A high–quality DNA has crucial importance for enzymatic applications in plant molecular biology, including agricultural research. The researchers often need to store the extracted DNA, which has to be suitable for the downstream enzymatic applications, such as a real–time PCR, even after months and years. Storing the DNA as a precipitate in ethanol at –80°C is a commonly used method of preservation. The aim of this research was to determine whether the same was applicable to a temperature of –20°C. In this study, a high-quality DNA from soybean and maize seed was extracted by the widely used CTAB method. The DNA samples were stored at three regimes: water-diluted at +4°C, water-diluted at –20°C, and an undiluted DNA in ethanol at –20°C. The concentration and yield were measured using a spectrophotometer. The impurities and fragmentation were estimated on agarose gel by electrophoresis. Additionally, the DNA was assessed amplifying by the real–time PCR. The quantity and quality of the extracted DNA were assessed prior and subsequent to an annual storage. The results indicated that ethanol provided excellent protection for a short–term DNA storage at –20°C and probably for a long–term storage due to a reduced water content and slightly basic conditions. Thus, such a storage regime could be an appropriate solution when other options are not available.

Keywords: DNA, seed, extraction, ethanol, storage, real–time PCR

INTRODUCTION

The extraction of high–quality genomic DNA is the first step in most methods of plant-oriented molecular biology, as well as in modern agricultural research, and, as such, is of crucial importance for a success of the procedure. There is a large number of different protocols for DNA isolation, and it is crucial to choose a suitable one, depending on a type of the sample as downstream applications. The method must guarantee a sufficient yield and adequate purity of the extraction protocols, since impurities such as proteins, polyphenols, and polysaccharides can interfere with the downstream protocols, while potential contamination by nucleases and the presence of free radicals can cause damage to the DNA molecule (Hanzer, 2019). Once extracted, the DNA often needs to be stored for a longer period of time. A long–term preservation of DNA obtained from the tissue samples is still a great challenge for scientists, and, to some extent, a DNA damage over time is inevitable. The literature describes a large number of different procedures for the preservation of isolated DNA, as well as of the plant tissues, prior to the DNA extraction. The choice of procedure depends primarily on the type of tissue, final purpose, available equipment for isolation and preservation, and downstream applications for which the extracted DNA will be used. The most common approach involves dissolving the isolated DNA in a liquid medium that would not interfere with further enzymatic reactions. The extracted DNA often needs to be stored for a shorter or for a longer period of time; however, none of the DNA preservation procedures will prevent its molecular damage, but a success of the following enzymatic reactions must be equal to use of a fresh (unpreserved) DNA (Pajnič et al., 2019). The environmental factors such as the storage temperature, presence of water, and the pH

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value have a significant influence on the stability of DNA. Although there is a general standpoint that the lower the preservation temperature is, the greater the stability of the stored DNA is as well, some studies indicate a possible damage during cryopreservation, most often caused by the formation of ice crystals (Tan et al., 2021). Once isolated, the DNA for a long–term storage is most often stored at –20°C or at –80°C and, when applicable, at –196 °C, lyophilized at the room temperature. Given that acidic conditions can lead to DNA hydrolysis, for a long–term storage, it is necessary to store the DNA under slightly basic conditions, by dissolving it in Tris-EDTA buffers or as a precipitate in ethanol at –80°C (Gaikwad, 2020). The reduced presence of water therefore favors the long–term preservation of DNA. The DNA molecule in the solid state is more stable than the DNA in a solution due to a reduced molecular mobility and removed water that can accelerate the degradation reactions. Also, the removal of water increases a chemical stability of the DNA by inhibiting hydrolysis and oxidation. Conversely, residual water can act as a plasticizer, as well as a reactant or catalyst (Bonnet et al., 2010).

There are many examples of the preservation of DNA stability by different dehydration procedures, such as ethanol precipitation, air drying, or lyophilization (Anchordoquy and Molina, 2007). Although all these techniques aim to preserve the integrity of the extracted DNA for a longer period of time, each of them has advantages and disadvantages in terms of the achievement of those goals, and the precipitation of DNA pellets in ethanol is certainly the most accessible technique for most researchers. In order to prevent a degradation by chemical and enzymatic processes, storing DNA as a precipitate in ethanol at –80°C is a widely used technique, but often the freezers operating at –80°C, and especially at –196°C, as well as the lyophilizers, are not available to researches. The aim of this research was to determine whether the same is applicable to the storage of DNA pellets in ethanol at –20°C. In the present study, a genomic DNA isolation was performed while using the grinded soybean and maize seed applying the CTAB protocol, and the samples were stored for six and twelve months, respectively, in three different storing regimes. The yield and the quality of the obtained DNA were evaluated by spectrophotometry, real–time PCR, and agarose-gel electrophoresis.

MATERIAL AND METHODS

The soybean and maize seed samples were collected at the Department of Biotechnological Analysis, Mycotoxins, and Pesticide Residues, Croatian Agency for Agriculture and Food. Genomic DNA was isolated from the samples using the cetrimonium bromide method, CTAB (Rogers and Bendich, 1989). The concentration and yield were measured using a spectrophotometer (BioPhotometer, Eppendorf), as were the absorbance and optical density values in ultraviolet light (260/280 ratio, 230/260 ratio, and 320 nm). A method of electrophoresis in agarose gel was applied for additional assessment of the quality and impurities of the extracted DNA in the sample. A DNA acceptability concerning further research and downstream enzymatic reactions was assessed applying a real–time PCR method by amplifying the taxon–specific DNA fragments at four dilution points, 1:4, 1:16, 1:64, and 1:256, and by measuring the deviation within the dilution, and administering the inhibition test (Mazzara et. al., 2008; Hougs et al., 2017). A total of ten different samples were analyzed in 2 x 7 repetitions. Subsequent to the initial measurements, the remaining samples were divided into two groups: in the first group, the DNA was dissolved in the DNase/RNase free water, and in the second group, a DNA precipitate was stored in ethyl alcohol (70% v/v). The samples were then stored at –20°C, whereas one part of the samples was stored at +4°C, as an additional control of the dynamics of degradation. The measurements were performed after six and after twelve months of storage respectively. Prior to the measurement, the extracted DNA was diluted in 100 μL DNase/RNase free water.

RESULTS AND DISCUSSION

DNA Quality and Quantity Assessment Prior to the Storage

The measurement of concentration and purity of DNA solution was performed using a spectrophotometer. The obtained initial results prior to the storage are presented in Table 1. The extracted sample concentrations ranged from 585 μg/mL to 999 μg/mL, with an average concentration amounting to 813.2 μg/mL. Everything above 20 μg/mL of extracted DNA was considered good for further analysis (Sophian and Syukur, 2021), having indicated that the obtained values of DNA concentration were a very good starting point for the downstream applications.

Table 1. Concentration and purity of extracted DNA (260/280, 230/260 ratio and A320 values). Initial values prior storage

Tablica 1. Koncentracija i čistoća izolirane DNK (vrijednosti odnosa 260/280, 230/260 i A320). Početne vrijednosti prije skladištenja

Initial values / *Početne vrijednosti*			
Sample / *Uzorak*	**Concentration** / *Koncentracija* [μg/mL]	**Purity** / *Čistoća*	
1	899	A 260/280 =	1.870
		A 260/230 =	2.060
		A320 =	0.003
2	999	A 260/280 =	1.890
		A 260/230 =	2.190
		A320 =	0.013
3	885	A 260/280 =	1.850
		A 260/230 =	2.070
		A320 =	0.021
4	886	A 260/280 =	1.890
		A 260/230 =	2.090
		A320 =	0.011
5	690	A 260/280 =	1.960
		A 260/230 =	2.020
		A320 =	0.031
6	978	A 260/280 =	1.990
		A 260/230 =	2.160
		A320 =	0.002
7	585	A 260/280 =	1.810
		A 260/230 =	2.140
		A320 =	0.012
8	787	A 260/280 =	1.810
		A 260/230 =	2.040
		A320 =	0.023
9	714	A 260/280 =	1.810
		A 260/230 =	2.090
		A320 =	0.001
10	709	A 260/280 =	2.000
		A 260/230 =	2.030
		A320 =	0.002
Average / *Prosjek*	813.2	A 260/280 =	1.888
		A 260/230 =	2.089
		A320 =	0.012

The purity of DNA samples was assessed by an absorbance measurement at a 260/280 ratio, 230/260 ratio, and at 320 nm. The measured values at the 260/280 absorbance ratio were between 1.810–2.000, with an average of 1.888, while the purity values measured at 230/260 ranged between 2.020–2.190, with an average of 2.089. The absorbance at 320 nm was averagely 0.012. The absorbance ratio 260/280 is most widely used to assess the purity of DNA and RNA. A range between 1.8–2.0 is generally accepted as an indicator of pure DNA even though some researchers allow a more liberal range, from 1.6 up to 2.1 (Abdel–Latif and Osman, 2017; Sophian and Syukur, 2021).

A purity ratio above 1.9 indicates the presence of RNA in the sample. A ratio below 1.7 may indicate the presence of protein, phenol, or other contaminants that are absorbed strongly at, or near, 280 nm (Thermo Fisher Scientific, 2011; Kalmár et al., 2015). It is important to note that both the DNA and RNA solutions partially absorb light at 280 nm, while the proteins partially absorb light at 260

nm; therefore, the 260/280 ratio provides for an estimate of the purity of both DNA and RNA. Even though RNA contamination cannot be assessed in this way, a pure RNA will have a 260/280 ratio significantly above 2.0. By measuring the absorbance at 230 nm, we will gain an insight into the contamination with carbohydrates, polyphenols, and aromatic compounds. A pure DNA is considered to be the one with a 260/230 ratio of approximately 2.2 and the values from 2.0 – 2.2 are acceptable (Somma and Querci, 2006). A 260/230 ratio lower than 2.0 indicates the presence of contaminants which are absorbed at 230 nm. Since nucleic acids and proteins are absorbed minimally at 320 nm, a reading at 320 nm usually serves as background check. Ideally, the reading should be as close to zero as much as possible (Teare et al., 1997). All obtained initial values prior to the storage of spectrophotometric quantity and quality analyses indicate the significantly large amounts of high–quality DNA suitable for further applications.

A real–time PCR method was applied to confirm the absence of PCR inhibitors in the extracted DNA prior to the storage (Table 2). The obtained correlation coefficients (R^2) indicated an efficient PCR amplification, and the differences between the measured and extrapolated Ct values (below 0.5) confirm a low level or absence of inhibition in all tested samples (Mazzara et. al., 2008).

Table 2. The results of an analysis examining the presence of PCR inhibitors in the extracted DNA prior to the storage. The ΔCt values below 0.5 indicates the absence of PCR inhibitors

Tablica 2. Rezultati analize prisutnosti PCR inhibitora u izoliranoj DNK prije skladištenja. Vrijednosti ΔCt ispod 0,5 ukazuju na odsutnost PCR inhibitora

Sample / *Uzorak*	**Slope** / *Nagib*	**R^2**	**Ct measured** / *Ct izmjereno*	**Ct extrapolated** / *Ct ekstrapolirano*	ΔCt
1	–3.3926	0.9985	26.1548	26.104	0.051
2	–3.2905	0.9987	26.2799	26.284	–0.004
3	–3.3862	0.9925	25.0756	24.928	0.1476
4	–3.4218	0.9987	24.4991	24.173	0.3261
5	–3.4327	0.9973	22.3621	22.522	–0.1599
6	–3.429	0.9903	23.6699	23.312	0.36
7	–3.4645	0.996	24.991	25.314	–0.323
8	–3.5321	0.9981	25.32	25.43	–0.11
9	–3.4042	0.9921	26.41	26.36	0.05
10	–3.4296	0.9986	28.68	28.75	–0.07

The quality of the extracted DNA was also assessed by an agarose-gel electrophoresis. The method enables the separation of macromolecules based on their size, charge, and physical properties. The rate of migration of a DNA molecule through a gel of individual molecules depends on the ionic properties and molecular mass. The procedure is standard for the separation of DNA fragments but also for the identification of impurities in the sample. The method enables the identification of presence of larger molecules like proteins, as well as the identification of the smaller ones, like the RNA, by visualization on the gel (Sambrook et al., 1989; Lee et al., 2012). The results obtained by gel electrophoresis showed a high molecular weight of the DNA band, with no or with some indication of protein and RNA contamination and few DNA shearings, both prior and subsequent to the storage.

DNA Quality and Quantity Assessment after Storage

To assess the changes in the quality of DNA, measurements were performed after six and twelve months. Figure 1 shows a decline in the concentration over time for all three storage regimes. As expected, a major decay was evident in the samples stored unfrozen. When compared to the samples stored at –20 °C, the results manifested that most preserved were the samples stored undiluted, as a precipitate in ethanol, respectively. An average measurement of concentration after six months indicated almost no changes when compared to the average initial values, and after 12 months, there was a slight decline in the concentration, but to some extent lesser than the one pertaining to the samples diluted in water.

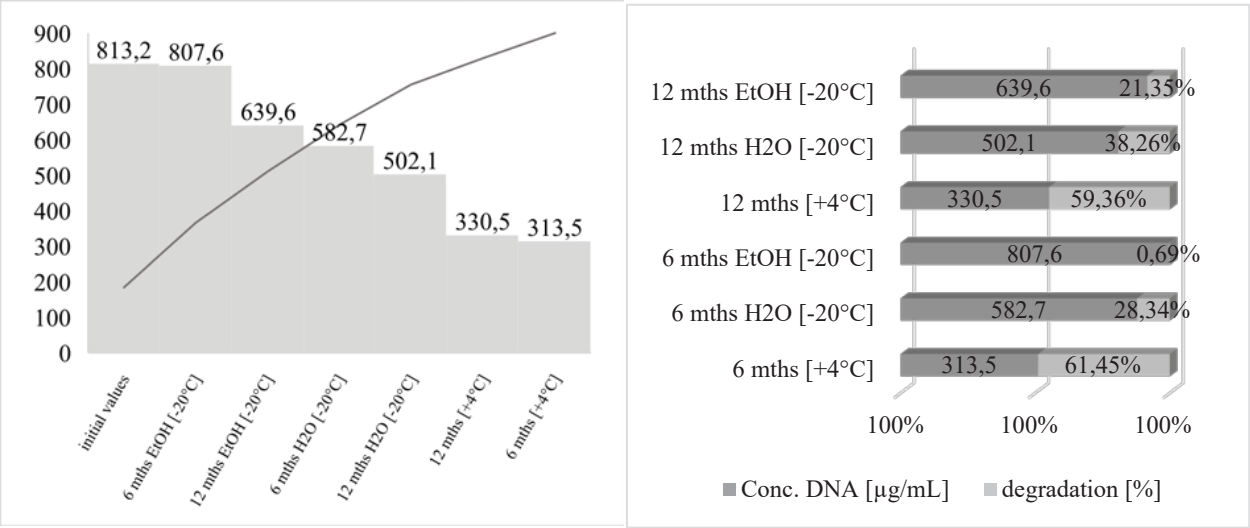

Figure 1. Left: a decline in the concentration of DNA in the solution in relation to the storage regime and the initial value. Right: the dynamics of the DNA molecule decay in aqueous solution and ethanol in relation to the storage regime

Grafikon 1. Lijevo: pad koncentracije DNK u otopini u odnosu režim čuvanja i početnu vrijednost. Desno: dinamika propadanja molekule DNK u vodenoj otopini i etanolu u odnosu režim čuvanja.

According to Piškur and Rupprecht (1995), ethanol has a large effect on biological macromolecules. The aggregated DNA in ethanol solution is thermally relatively stable, most probably because of a strong interhelical interaction within the aggregated DNA. Stability also depends on ethanol concentration. The research preserved the DNA pellet in the 70% ethanol (v/v), indicating the lowest level of degradation dynamics. Ethanol is also known for its ability to deactivate the DNase activity, which additionally favors ethanol as a storage medium (Adams and Zhong, 1999).

DNA stability can be affected by storage time. Depending on the requirements of downstream applications, a suitability of the stored DNA can be questionable, since degradation can lead to a reduction, or to a loss, of structural integrity of cells, as well as to a loss in the quantity and quality of genomic DNA (Lee and Kline, 2013). The results obtained by measuring 260/280 ratio demonstrate the most visible degradation in the samples stored without freezing. The results indicate that quality of those samples was questionable for further analyses. Conversely, the frozen DNA samples manifested excellent quality (Figure 2).

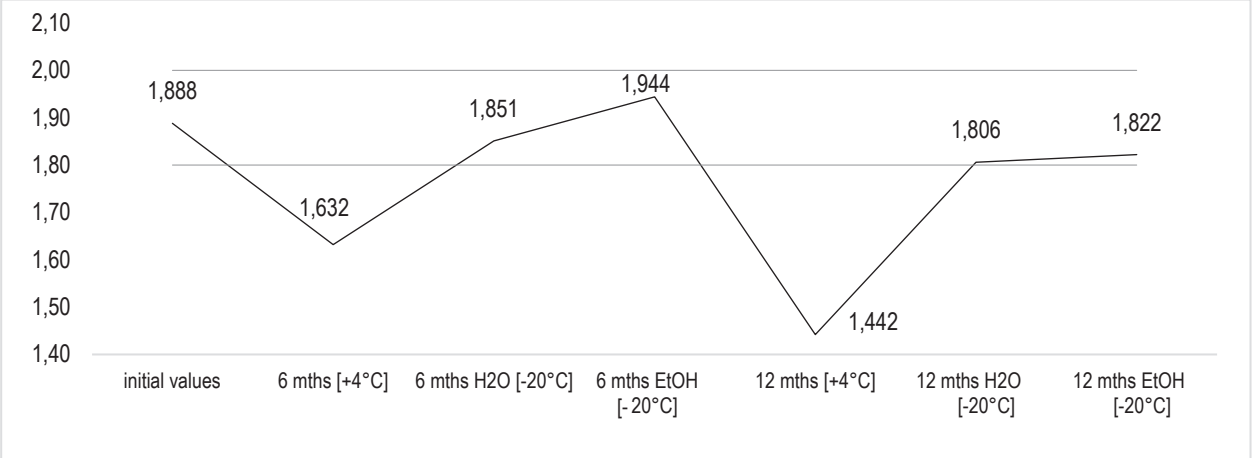

Figure 2. The A260/280 of the measured samples in comparison to a pure DNA value (1.8 to 2.0)

Figure 2. A260/280 izmjerenih uzoraka u usporedbi s vrijednošću čiste DNK (1,8 do 2,0)

The quality of the stored DNA was assessed by visualization using the agarose-gel electrophoresis. The obtained results prove a high molecular weight of the DNA band, with no indication, or with a small indication, of protein and RNA contamination, some shearing, and DNA chain breakage (Figure 3).

The DNA quality and applicability for PCR analysis, as well as the absence of inhibitory substances, were demonstrated by the amplification of taxon-specific genes: lectin gene for soybean samples and alcohol dehydrogenase for the maize samples. The DNA samples were diluted, normalized to 50 μg/mL, and then a fourfold dilution series was prepared at a ratio of 1:4, 1:16, 1:64, and 1:256, respectively. To assess the quality of the DNA Ct, the values of the diluted samples were plotted against a logarithm of the dilution factor, and an equation was calculated by linear regression (Branquinho et al., 2012). The average PCR efficiencies for the maize and soybean endogenous targets using a dilution-series method demonstrated that the samples stored at –20°C, both diluted and undiluted, satisfactory results.

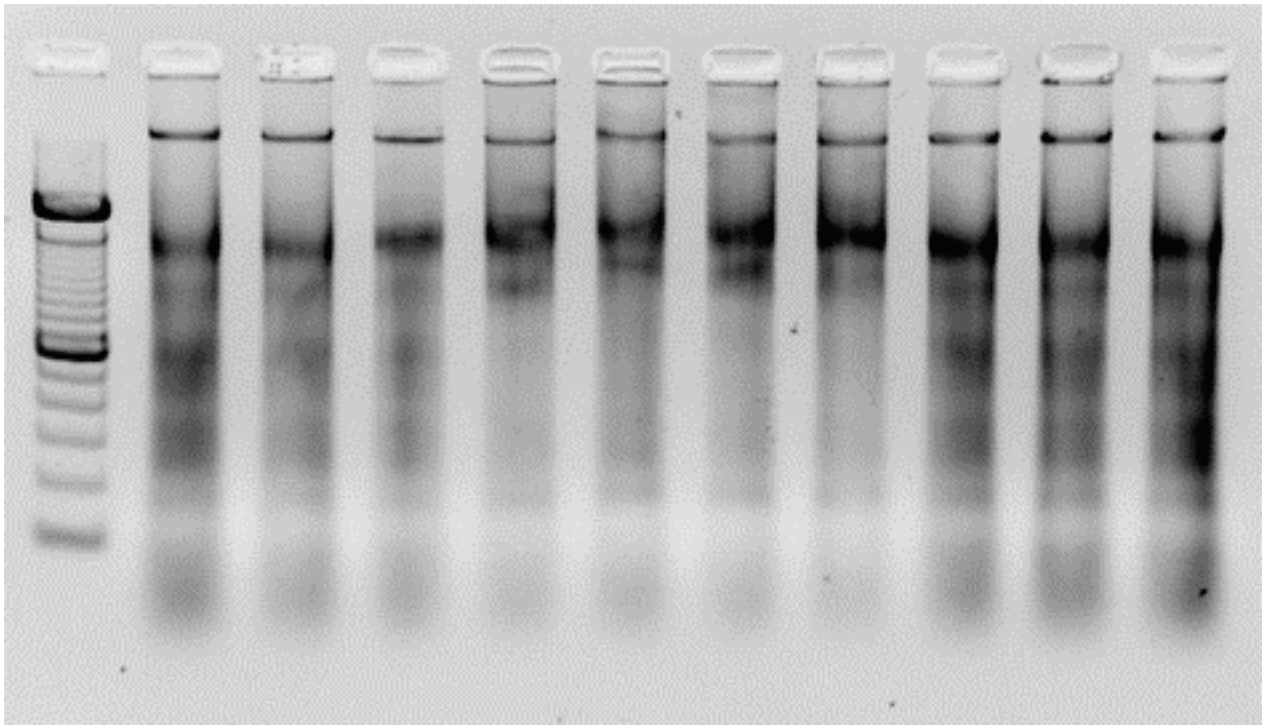

Figure 3. Agarose gel electrophoresis depicting a genomic DNA extracted from the maize and soybean samples stored as a precipitate in ethanol at –20°C, post-storage. From the left to the right: line 1: DNA ladder, lines 2 – 6: maize samples, lines 7 to 11: soybean samples

Grafikon 3. Elektroforeza u gelu agaroze pokazuje genomsku DNK izoliranu iz uzoraka kukuruza i soje pohranjenih kao precipitat u etanolu na –20 °C nakon skladištenja. Slijeva nadesno: linija 1: DNK ljestvica, linije 2 – 6: uzorci kukuruza, linije 7 – 11: uzorci soje

CONCLUSION

In this study, the seed DNA was extracted using the CTAB method to isolate a high-quality DNA that is suitable for downstream enzymatic applications, such as the real–time PCR. The quantity and quality of DNA was assessed prior and subsequent to an annual storage. Preserving a DNA pellet in ethanol indicated the lowest level of degradation dynamics. Even though twelve months could not be considered as a long–term storage, the obtained results still indicated that storing the DNA at –20°C, undiluted in an ethanol solution, could prolong the storage life of the extracted DNA, in comparison to the most commonly used water-diluted DNA. The results indicated that the ethanol-stored DNA samples were of an excellent purity and integrity, and, along with the CTAB extraction method, conserving a DNA pellet in ethanol also presented a convenience of use. Overall, ethanol as a storage media provided excellent protection for a short–term storage of DNA at –20°C and probably a good solution for a long–term storage at the same temperature regime, thus enabling laboratories to prolong the storage period when other options are not available.

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ČUVANJE IZOLIRANE BILJNE DNK: JE LI –20 °C DOVOLJNO HLADNO?

SAŽETAK

Visokokvalitetna DNK od presudne je važnosti za enzimske postupke u biljnoj molekularnoj biologiji, kao i za istraživanja u poljoprivredi. Istraživačima je često potrebno pohranjivanje izolirane DNK, koja i nakon mjeseci i godina mora biti prikladna za nizvodne enzimske metode, kao što je PCR u stvarnome vremenu. Pohranjivanje DNK kao precipitata u etanolu na –80 °C često je korištena metoda konzerviranja. Cilj je ovih istraživanja bilo utvrđivanje je li isto primjenjivo na –20 °C. U ovoj studiji visokokvalitetna DNK iz sjemena soje i kukuruza izolirana je široko korištenom CTAB metodom. Uzorci DNK pohranjeni su u trima režimima: otopljena DNK u vodi na +4 °C, otopljena DNK u vodi na –20 °C i neotopljena DNK u etanolu na –20 °C. Koncentracija i prinos mjereni su s pomoću spektrofotometra. Nečistoće i fragmentiranje procijenjeni su vizualizacijom na agaroznome gelu elektroforezom. Dodatno je DNK procijenjena amplifikacijom s pomoću PCR–a u stvarnome vremenu. Količina i kvaliteta izolirane DNK procijenjena je prije i nakon jednogodišnjega skladištenja. Rezultati pokazuju da etanol pruža izvrsnu zaštitu za kratkotrajno skladištenje DNK na –20 °C i vjerojatno za dugotrajno skladištenje zbog smanjenoga sadržaja vode i blago bazičnih uvjeta. Takav režim skladištenja mogao bi biti dobro rješenje kada druge opcije nisu dostupne.

Ključne riječi: DNK, sjeme, izolacija, etanol, skladištenje, PCR u stvarnome vremenu

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