The effect of DNA isolation methods on genetic similarity of winter wheat (*Triticum aestivum* L.) lines using RAPD-PCR techniques

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ABSTRACT

The aim of this study was to compare the effect of 4 DNA isolation methods on the results of analyses using molecular markers of Random Amplified Polymorphic DNA – Polymerase Chain Reaction (RAPD-PCR) in winter wheat lines. DNA was isolated using the method proposed by Thomson and Henry, the column method with a Quiagen DNeasy Plant Kit, a Genomic Mini AX Plant Kit by A&A Biotechnology and the Maxwell® 16 LEV Plant DNA Kit by Promega.

After isolation RAPD–PCR analyses were carried out. The molecular study was an evaluation indicator of the DNA isolation methods. The best isolation method was that using the Quiagen DNeasy Plant Kit. Isolation using this kit provided the best DNA quality and the greatest legibility of electropherograms. However, it is the most time- and cost-intensive method of DNA isolation, which limits its applicability at a large number of tested genotypes. In terms of electrophoretic image quality good DNA isolation was provided by the method using a Genomic Mini AX Plant Kit. A rapid and cheap method was that proposed by Thomson and Henry; unfortunately, it was less efficient than the former ones. The greatest variation in the amounts of obtained DNA was observed for isolation using the Maxwell kit.

Wpływ metod izolacji DNA na podobieństwo genetyczne linii pszenicy ozimej (*Triticum aestivum* L.) przy zastosowaniu techniki RAPD-PCR

Słowa kluczowe: izolacja DNA, pszenica ozima, polimorfizm markerów RAPD

STRESZCZENIE

Celem badań było porównanie wpływu 4 metod izolacji DNA na wynik analizy markerami molekularnymi RAPD-PCR (ang. Random Amplified Polymorphic DNA – Polymerase Chain Reaction) linii pszenicy ozimej. Przeprowadzono izolację DNA metodą Thomsona i Henry'ego, metodą kolumienkową przy pomocy kitu Quiagen DNeasy Plant Kit, przy pomocy kitu Genomic Mini AX Plant firmy A&A Biotechnology oraz zestawu Maxwell® 16 LEV Plant DNA Kit firmy Promega.
Po izolacji przeprowadzono analizy molekularne RAPD – PCR, które posłużyły jako wskaźnik do oceny metod izolacji DNA. Najlepszą metodą izolacji okazała się metoda z wykorzystaniem Quiagen DNeasy Plant Kit. Jakość DNA otrzymanego po izolacji tym kitem była najwyższa, a otrzymane elektroforogramy były najbardziej czytelne. Jest to jednak metoda izolacji DNA najdłuższa i najdroższa, co ogranicza jej wykorzystanie przy dużej liczbie badanych genotypów. Dobrą metodą izolacji DNA ze względu na jakość otrzymanych obrazów elektroforetycznych była metoda izolacji kitem Genomic Mini AX Plant. Metodą szybką i tanią okazała się metoda Thomsona i Henry'ego, niestety mniej wydajną w porównaniu do poprzednich. Metodą o największym zróżnicowaniu ilości otrzymanego DNA była izolacja z wykorzystaniem zestawu Maxwell.

1. INTRODUCTION

The primary aim of DNA isolation is to obtain large amounts of high quality genomic DNA from numerous samples at a simultaneous purification of specimens to remove proteins and enzyme inhibitors. The number of samples is frequently a factor limiting the scope of analyses. Despite the considerable range of various methods of nucleic acid isolation new procedures are constantly being developed [1, 2]. Their objective is to provide a possibly rapid and effective DNA isolation, making it possible to reduce costs of analyses and to provide the largest possible amounts of uncontaminated DNA. It needs to be remembered that DNA extraction from plant tissues varies depending on the used material and further use of extracted DNA. In most isolation methods plant tissue is ground in a mortar or comminuted in a homogenizer in order to destroy cell walls. DNA is purified from solid particles by centrifugation, while soluble proteins and other molecules are separated by chloroform extraction and centrifugation. Next DNA should be precipitated from the aqueous phase and rinsed thoroughly to remove salts contaminating the sample. Molecular markers are applied in several ways in plant breeding, with their main application being in marker assisted selection (MAS), which is used to select specimens with desirable traits. RAPD markers are used first of all to investigate genetic diversity and to identify cultivars, lines or clones [3]. Advantages of RAPD markers include simplicity and rapid performance of the analysis, low unit costs and small amounts of input DNA required as well as their limited costs. Main drawbacks are connected with problems with result repeatability and the dominant character of detected loci [4, 5].
The aim of this study was to compare the effect of 4 DNA isolation methods on results of DNA polymorphism analyses for winter wheat lines, provided by RAPD-PCR molecular markers.

2. EXPERIMENTAL MATERIAL AND METHODS

The plant material used for analyses comprised 25 genotypes of winter wheat coming from the collection of the Department of Genetics and Plant Breeding, the Poznań University of Life Sciences. A total of 10 randomly selected plants were collected from each genotype for further analyses.

2.1 DNA isolation according to Thomson and Henry

Genomic DNA from winter wheat lines was isolated using the modified method by Thompson

and Henry [6]. A bulk sample was produced from 2 mm^2 leaf discs from 10 analyzed plants and it was subsequently treated with 200 ml TBS buffer of 100 mM Tris HCl at pH 9.5, 1 M KCl, 10 mM EDTA and deionized H_2O (made up to 50 ml). Samples were incubated in Eppendorf tubes in a water bath at 95°C for 15 min.

2.2 DNA isolation by the column method using a Quiagen DNeasy Plant Kit

Plant material comprising leaf discs of 50-100 mg from 10 analyzed plants was placed in Eppendorf tubes. In order to obtain homogeneous plant tissue the samples were immersed in liquid nitrogen. After they were taken out of liquid nitrogen each sample was ground in a mortar and next supplemented with 400 ml AP1 buffer, mixed until homogeneous consistency was obtained, and then supplemented with 3 ml RNAse at 100 mg/ml. The entire volume was incubated for 30 min. at room temperature and for 30 min. at 65°C. After incubation all test tubes were supplemented with 130 ml AP2 buffer and mixed, and next the samples were placed on ice for 15 min. After removal from ice the samples were centrifuged in a centrifuge by Heraeus Biofuge Pico for 5 min. at 10956 RCF (relative centrifugal force). Subsequently the obtained supernatant was carefully transferred to previously autoclaved test tubes. Each sample was supplemented with 250 ml AP3 buffer and 500 ml ethanol. The entire solution volume was transferred to a column with a DNA binding membrane and it was centrifuged for 2 min. at 3578 RCF. Columns with bound DNA were transferred to clean test tubes and treated with 450 ml AW buffer and next centrifuged for 1 min. at 3578 RCF. Again columns were transferred to clean test tubes and they were treated with 50 ml AE buffer heated to 65°C and incubated for 20 min. at room temperature. Elution with AE buffer was repeated twice.

2.3 DNA isolation using a Genomic Mini AX Plant Kit by A&A Biotechnology

A sample of plant material (approx. 100 mg fresh weight) was placed in an Eppendorf tube and supplemented with 0.9 ml LS lysis buffer and 20 ml protease solution. The entire volume was mixed and incubated at 50°C for 30 min. Following incubation the sample was shaken vigorously for 15 s and next centrifuged for 5 min. at 10956 RCF. During centrifugation the columns were equilibrated with K1 solution. Supernatant was transferred onto equilibrated columns and the columns were rinsed by adding 1.5 ml K2 rinsing solution. The column were supplemented with 0.25 ml K3 elution solution. The next stage consisted in the transfer of the column to a new test tube and repeated supplementation with K3 elution solution. The eluate containing DNA was supplemented with 0.8 ml PM precipitation mixture. The entire volume was mixed and centrifuged at 3578 RCF. After centrifugation the supernatant was removed (a light-blue precipitate was visible at the bottom of the test tube) and dried, and then rinsed in 70% ethanol. After drying the precipitate was suspended in sterile distilled water.

2.4 Automatic DNA isolation using a Maxwell® 16 LEV Plant DNA Kit by Promega

Isolation was performed according to the manufacturer's protocol. The method is based on the application of a synthetic composite magnetic bed, selectively binding DNA and RNA nucleic acids. Plant material of 20 mg, coming from fresh leaves from 10 analyzed plants, was placed on the bottom of a 1.5 ml vial, next the composite magnetic bed was added along with 300 µl Tail Lysis Buffer (TLB). In the next stage the apparatus was switched on using the DNA and Plant protocol option, with the time and velocity settings as recommended by the manufacturer. Next test tubes with the extract were centrifuged for 2 min. at 10956 RCF, then the samples were supplemented with 300 µl water (nuclease free water). Liquid lysate from each sample was transferred to new test tubes.

2.5 Analysis of polymorphisms of RAPD markers

Concentrations and purity of DNA extracts were measured in the obtained preparations using a Nanodrop apparatus. These spectrophotometers are characterized by a reduced optical path length, while maintaining very high accuracy of measurements and requiring limited amounts of material for analyses. Table 1 presented concentrations of DNA in tested samples in ng/µl and the absorbance ratios at 260 nm and 280 nm. Next, the obtained DNA extracts were diluted to 25 ng/µl. In each of the 4 tested methods of DNA isolation samples were stored at -20°C.

Tabela 1 Zawartość genomowego DNA i absorbancja przy dwóch długościach fali dla różnych genotypów pszenicy
Table 1 The genomic DNA content and the ratio of two wave lengths absorbance of various genotypes of wheat

Genotype of wheat Genotyp pszenicy	Quiagen DNeasy Plant Kit		Genomic Mini AX Plant		Maxwell® 16 LEV Plant DNA Kit		Thomson & Henry	
	DNA content (ng/µl) Zawartość DNA	Absorbance 260/280 Absorbancja	DNA content (ng/µl) Zawartość DNA	Absorbance 260/280 Absorbancja	DNA content (ng/µl) Zawartość DNA	Absorbance 260/280 Absorbancja	DNA content (ng/µl) Zawartość DNA	absorbance 260/280 Absorbancja
1	246.7	1.82	253.2	1.78	198	1.78	101.3	1.67
2	352.8	1.79	357.8	2.01	196.4	1.79	145	1.75
3	221	1.85	421.2	1.88	211.5	1.99	121.3	1.67
4	263.7	1.99	126.2	1.89	96.3	1.84	98	2.1
5	122.9	1.85	198.1	1.76	189.2	1.87	100	1.8
6	328.6	1.91	98.6	1.79	175.5	1.78	91	1.69
7	398.3	1.99	251.9	1.78	162.5	2.12	89	1.87
8	411.3	1.86	179.6	1.81	289.5	2.43	201	2.4
9	276	1.82	276.1	1.69	163.7	1.98	211	2.25
10	354	1.84	190	1.87	111.2	1.91	98	1.89
11	198.9	1.77	176	1.9	79.2	1.99	123	1.9
12	212.6	1.89	201.1	1.98	99.12	2.02	201	1.59
13	362.6	1.86	176.9	1.72	164.2	2.01	176	1.99
14	98.2	1.88	245.8	1.87	111.4	1.99	123	1.67
15	342.6	1.95	164.9	1.86	67	1.69	79	2.01
16	189.1	1.79	199.5	1.80	89.1	1.76	135	1.78
17	321.3	1.8	90	1.86	143	1.79	198	1.9
18	78.2	1.84	154.7	1.97	253	1.88	76	1.99
19	421.6	1.92	199.2	1.99	110.2	2.01	17	2.1
20	244.1	1.88	311	2.2	112.1	2.08	124	1.68
21	189.4	1.92	178.9	1.9	88	1.79	34	1.9
22	334.8	1.76	254.8	2.14	199	1.89	24	1.78
23	321.3	1.78	143.7	2.16	234.1	1.81	178	1.69
24	96	1.91	173.9	1.78	179	1.84	100	1.67
25	355.2	1.89	80.9	1.64	165	1.9	45	1.55
Mean Średnia	236.75	1.86	191.75	1.88	161.50	1.91	115.91	1.85

Each RAPD-PCR reaction was run in 12.5 µl mixture containing deionized water, DreamTaq PCR Master Mix (2X) by ThermoScientific, a primer by Operon Technologies at 20 pm/µl, and DNA extract at 25 ng/µl. Amplification was performed in a T Professional Basic Gradient thermocycler by Biometra. Conditions of RAPD-PCR are given in Table 2. Amplification was repeated twice for each sample, with the list of results presenting only repeatable analyses.

In each case electrophoresis of PCR products was conducted in 1.5% agarose gel with an addition of 1 µl ethidium bromide solution for 2 h at 100 V. In order to identify molecular weights an O'RangeRuler 100 bp size marker by Fermentas was applied with the range of molecular weight

Tabela 2 Warunki reakcji RAPD-PCR
Table 2 Conditions for RAPD-PCR reaction

stage	Temperature (°C)	time (s)
Initial Denaturation	94	60
Denaturation	94	30
Primer Annealing	35	30
Elongation	72	30
Number of cycles 10		
Denaturation	94	30
Primer Annealing	37	30
Elongation	72	60
Number of cycles 30		
Final synthesis	72	180
Storage	4	∞
Increase in temperature	1°C/s	

determination from 100 to 1500 bp. Separated DNA fragments were visualized under ultraviolet light and recorded on images. Based on the image analysis molecular weights of RAPD-PCR amplification products were determined using the UVI*Band* v. 12.14 program.

2. RESULTS

Among the 50 tested oligonucleotide primers 10 generated the greatest numbers of amplification products were selected (Table 3). These primers were analyzed twice for each of the 4 DNA isolation methods. The number of amplification

Table 3 Mean number of amplification products generated by each of the primers used depending on the method of DNA isolation
Tabela 3 Średnia liczba produktów amplifikacji wygenerowanych przez każdy z użytych starterów w zależności od zastosowanej metody izolacji DNA

Primer no. Nr startera	Sequences 5'-3' of primers Sekwencja nukleotydowa 5'-3' starterów	Quiagen DNeasy Plant Kit	Genomic Mini AX Plant	Maxwell® 16 LEV Plant DNA Kit	Thomson & Henry
OPA 04	AATCGGGCTG	7,1	6,2	6,4	3,2
OPA 07	GAAACGGGTG	5,2	5,3	5,2	4,7
OPA 10	GTGATCGCAG	6,5	6,1	4,8	2,5
OPA 14	TCTGTGCTGG	8,5	4,3	7,1	5,1
OPB 04	GGACTGGAGT	8,2	7,5	3,6	5,3
OPB 17	AGGGAACGAG	5,7	6,8	4,5	3,9
OPF 12	ACGGTACCAG	9,1	4,9	4,9	5,7
OPG 12	CAGCTCACGA	7,5	7,1	3,8	2,9
OPH 20	GGGAGACATC	9,3	4,5	4,0	5,0
OPJ 08	CATACCGTGG	9,6	7,5	4,5	2,6
Mean Średnia		**7,67**	**6,02**	**4,88**	**4,09**

products depended on the applied method of DNA isolation. On average the greatest number of amplification products was obtained using the column method of DNA isolation with a Quiagen DNeasy Plant Kit, while it was slightly lower for the Genomic Mini AX Plant Kit by A&A, whereas the lowest number of products was provided by the Maxwell kit and the Thomson and Henry method [6]. Additionally, the number of products was closely connected with the primer sequence. Example electropherograms presenting amplification products, obtained from DNA isolation using different methods, are presented in Fig. 1.

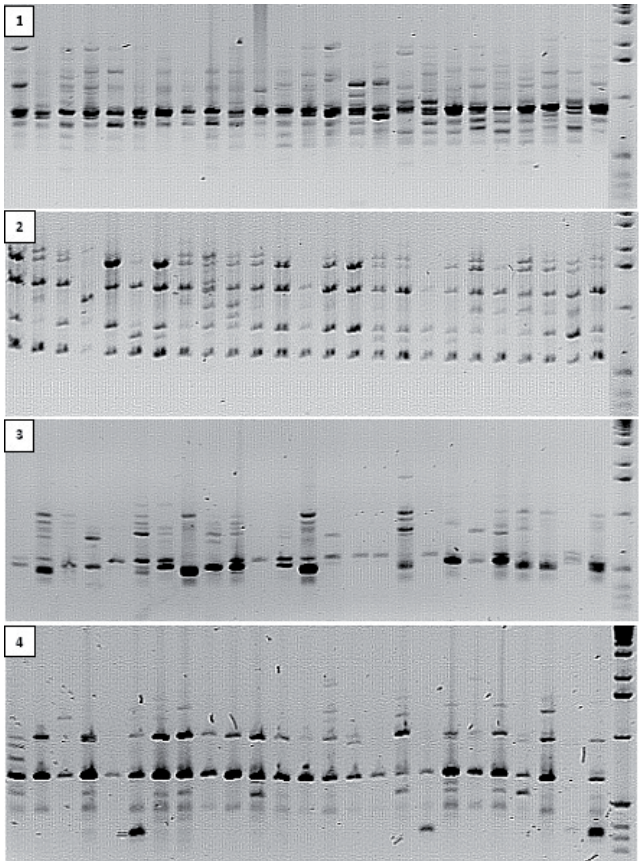


Figure 1 The electropherogram of DNA amplification products yielded by the RAPD-PCR method using DNA isolated with a Quiagen Dneasy Plant Kit (1), according to Thomson and Henry (2), using the Mini AX Plant from A&A Biotechnology (3), and using a Maxwell set (4)

Rysunek 1 Elektroforogram produktów amplifikacji DNA wykonany techniką RAPD-PCR z zastosowaniem DNA izolowanego metodą kolumienkową przy pomocy kitu Quiagen DNeasy Plant Kit (1), metodą Thomsona i Henry'ego (2), metodą kolumienkową Genomic Mini AX Plant firmy A&A Biotechnology (3), przy pomocy zestawu Maxwell (4)

Obtained dendrograms of genetic similarity between winter wheat lines depended on the applied DNA isolation method. At the application of a Quiagen DNeasy Plant Kit genetic similarity between the analyzed treatments ranged from 45% and 88%, for the method according to Thomson and Henry [6] it was from 42% to 93%, while for the column method it ranged from 27% to 94% and when using the Maxwell set it was from 28% to 90% (Fig. 2).

Despite the application of identical primers the adopted method of DNA isolation influenced the number and composition of genetic similarity groups of the tested winter wheat lines. Using the Quiagen DNeasy Plant Kit two main groups of genetic variation may be distinguished, with only 4 genotypes comprising the first group and with three large subgroups covering the other 21 winter wheat genotypes. Following DNA isolation according to Thomson and Henry [6] 25 tested genotypes formed 2 groups of comparable size, while line 19 turned out to be genetically different from the other tested objects. At the application of the column method two groups were visible on the dendrogram: one composed of 9 genotypes and the other consisting of 15 genetically similar lines and line 4, different from all the others. The automatic DNA isolation with the Maxwell set yielded a group composed of two genotypes (18 and 24) and a group consisting of several subgroups comprising all the other analyzed lines.

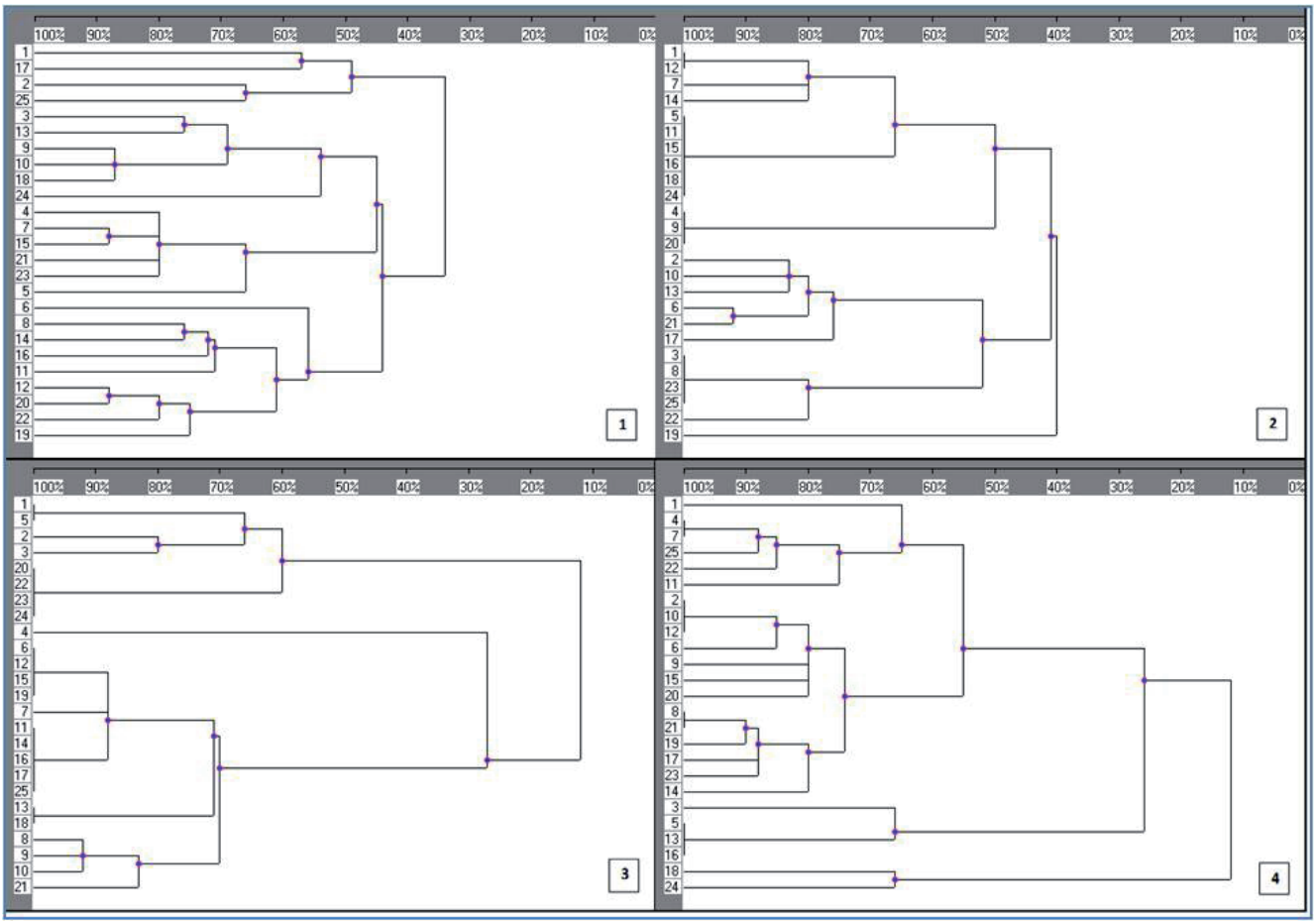
4. DISCUSSION

The problem with the selection of a DNA isolation method has been discussed in literature for several years. The decision on the choice of a specific isolation method is influenced by such factors as the number of analyzed samples, the importance of quality of produced DNA, costs of a single analysis and the time required for the performance of isolation. Obviously methods of DNA isolation are being constantly improved, aiming at the development of cheap methods, yielding large amounts of high quality DNA within a possibly short time at low labor consumption.

Varma et al. [7] specified the main factors affecting the efficiency of DNA isolation as the source of plant material (the type and age of tissue, collection method and storage), procedure (mainly homogenization) and the presence of contaminants (polysaccharides, polyphenols, proteins, RNA and extracellular DNA coming from chloroplasts and mitochondria). The authors indicated that the application of ready-to-use kits for

Figure 2 Dendrograms of genetic similarity between the analyzed objects – analysis performed using DNA isolated by a Quiagen Deasy Plant Kit (1), according to Thomson and Henry (2), using the Mini AX Plant from A&A Biotechnology (3), and using a Maxwell set (4)

Rysunek 2 Dendrogramy podobieństwa genetycznego pomiędzy analizowanymi obiektami – analiza wykonana przy użyciu DNA izolowanego metodą kolumienkową przy pomocy kitu Quiagen DNeasy Plant Kit (1), metodą Thomsona i Henry'ego (2), metodą kolumienkową Genomic Mini AX Plant firmy A&A Biotechnology (3), przy pomocy zestawu Maxwell (4)



the isolation of genomic DNA is preferred by molecular biologists, since they are easy to use and provide good quality DNA rapidly. Moreover, DNA after isolation is well-purified, as the kits contain lytic buffers and RNAse, while proteins and polysaccharides are removed by precipitation and centrifugation. The selection of the DNA isolation method should consider the size of the analyzed population, isolation time, the complexity of the procedure and the intended application of DNA isolate [8].

Among the many available DNA isolation methods some are very popular, such as e.g. those proposed by Murray and Thompson [9], Doyle and Doyle [10], Rogers and Bendich [11] and Lodhi et al. [12]. Unfortunately, none of the methods is universal and none may be applied for all plant species [7]. This is connected e.g. with the presence of cell walls, pigments or various secondary metabolites. Frequently different isolation methods are used for mono- and dicotyledonous plants. Williams and Ronald [13] described a method of DNA isolation from leaves with no tissue homogenization required, i.e. a method useful in a situation when leaves of a given species are fibrous and homogenization is very labor-intensive.

Zhang et al. [13] proposed a method of DNA extraction based on recycling. The primary assumption of this method is for a single sample of plant tissue to return to DNA extraction up to four times, with the respective DNA precipitation repeated 4 times. This method has been applied to such species as wheat, sorghum, barley, corn and rice, at a high yield and good quality of DNA samples.

The available DNA isolation kits are produced by e.g. Quiagen, Promega, Epibio, Cartagen and Roche. In the Maxwell set by Promega a specially selected composition of binding and elut-

ing buffers makes it possible to obtain DNA with a very high quality and purity. Isolated DNA is not fragmented during the purification process. This method is very efficient even in the case of isolation from a small amount of tissue. The lack of inhibitors of consecutive reactions makes it possible to directly apply purified nucleic acids in other molecular biology techniques, such as DNA sequencing (automated and manual), dephosphorylation, phosphorylation, ligation, analyses of DNA-protein interactions, or digestion with restriction enzymes. Magnetic beds, in contrast to standard beds applied in DNA isolation and purification, facilitate complete automation of this process. The procedure does not require specialist equipment such as centrifuges or vacuum pumps.

By using of isolation kits, pure DNA may be obtained within an hour; however, most of these products are relatively expensive. Due to the high prices of commercial kits for DNA isolation or relatively low amounts of obtained DNA it is attempted to optimize methods of plant DNA isolation. The isolation of genomic DNA applied by Pietrusińska and Czembor [8], i.e. the Quiagen kit and the rapid NaOH method, did not cause degradation of nucleic acid under the influence of temperature and the number of obtained PCR products was identical. Moreover, intensity of reaction products (bands) was very high. Xin and Chen [1] in their study analyzed a method of DNA isolation being a combination of CTAB with the MagAttract Kit. They obtained large amounts of high quality DNA for such species as sorghum, wheat, corn and cotton. An additional advantage of this modification was connected with the large number of isolations performed by a single operator – the authors declared that within 1 day a single laboratory worker may process two plates with 96 samples each.

Dobrzycka and Broda [15] showed that the concentration of DNA obtained from isolation according to Thomson and Henry ranges from 2147 µg/ml to 2513 µg/ml, while in the case of DNA isolated by the column method using a Quiagen DNeasy Plant Kit the DNA concentration ranges from 2492 µg/ml to 3465 µg/ml. Tamari et al. [2] observed that the mean concentration of isolated DNA depended on the applied method and it was higher for the Edwards method (300.0 µg/µl to 1558.3 µg/µl) than for the commonly applied CTAB, in which DNA content ranged from 341.7 µg/µl to 897.2 µg/µl. In that study the amount of obtained DNA depended on the isolation method and it was highest for the Quiagen DNeasy Plant Kit and lowest for the method according to Thomson and Henry [6]. Moreover, Tamari et al. [2] showed that DNA isolation from generative or mitotically active tissues improves the efficiency of the process, since these tissues contain greater amounts of genomic DNA, with the highest efficiency of DNA isolation found for flower buds four days before pollination.

The most frequent problem at the application of RAPD-PCR is connected with low repeatability of results. At present it is generally accepted that in order to obtain repeatable sets of profiles on gels it is necessary to maintain constant reaction conditions [16]. Numerous studies showed that the amount and repeatability of products obtained in RAPD-PCR depend on the concentrations of magnesium chlorides, Taq polymerase, DNA concentration or the adopted method of DNA isolation [17, 18, 19, 20, 21]. Dobrzycka and Broda [15] showed that the number of products obtained from RAPD-PCR was dependent on the method of DNA isolation, e.g. the use of a Quiagen DNeasy Plant Kit provided an almost two-fold greater number of bands than isolation according to Thomson and Henry [6], and it affects the image of RAPD-PCR products and the result of analyses of genetic similarity conducted on lucerne, presented in dendrograms. Starke et al. [22] indicated that the results of pyrosequencing are influenced by the method of DNA isolation and the selection of the primer. This was confirmed by the recorded results, as the highest polymorphism of PCR products was observed after isolation of genomic DNA from winter wheat using the Quiagen DNeasy Plant Kit and the OPJ 08 primer. The aim of studies conducted by Gurudeeban et al. [23] was to minimize and simplify the procedure of DNA isolation to assess genetic diversity among plants of *Suaeda* ssp. using RAPD-PCR markers. These plants contain relatively high amounts of polyphenols, tannins and secondary metabolites such as alkaloids, flavonoids and phenols, potentially disturbing the DNA isolation procedure. The authors by modifying the methods of extraction obtained from 2 to 12 reaction products, depending on the used primer.

5. CONCLUDING REMARKS

In terms of the quality of obtained electrophoretic images the best method of DNA isolation was isolation using the Genomic Mini AX Plant Kit. The technique proposed by Thomson and Henry is a rapid and cheap method, while the greatest variation of obtained DNA was observed for isolation with the use of the Maxwell kit. Quality of DNA yielded by isolation with the Quiagen DNeasy Plant Kit was greater, and this isolation method provided the highest quality electropherograms with the simultaneous greatest number of products. However, this method of DNA isolation is the most time- and cost-intensive, which limits its applicability at a large number of tested genotypes.

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