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PCR-RAPD OPTIMIZATION FOR HOSPITAL WASTEWATER GENOTOXIC INFLUENCE ANALYSIS ON *ALLIUM CEPA ROOT* MERISTEM CELLS

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Abstract

PCR-RAPD (Polymerase Chain Reaction – Random Amplified Polymorphic DNA) is a PCR based method used for biodiversity, phylogenetics, genotoxicity and cancerogenesis research. It possesses several advantages, such as no necessity for information about DNA sequence or multiple primers usage for generating a great deal of fingerprints data. But on the other hand PCR-RAPD is known to be unrepeatable and generating unspecific PCR products, if PCR is not optimized for a particular biological material. The aim of this experiment was to optimize PCR-RAPD with a modified Taguchi method for its usage for genotoxicity analysis. The research was performed on DNA isolated from onion (*Allium cepa*) root meristem cells. The plants were grown on medium containing an increasing concentration of hospital wastewater in order to observe its genotoxic influence on eukaryotic DNA. The wastewater derived from Hospital for Phthisiatry and Lung Diseases in Pilchowice, Poland, where large amount of anti-tuberculosis and anti-cancer drugs are used. The research proved modified Taguchi method being useful for optimal PCR mixture. In the experiment repeatable RAPD results with no additional nonspecific PCR products were received. It was also stated that PCR-RAPD is useful for genotoxicity analysis performed with plant DNA.

Streszczenie

PCR-RAPD (Polymerase Chain Reaction – Random Amplified Polymorphic DNA) jest odmianą PCR wykorzystywaną w badaniach bioróżnorodności, filogenezy, genotoksyczności oraz kancerogenezy. Do jej zalet zalicza się brak konieczności znajomości badanej sekwencji DNA oraz możliwość użycia wielu rodzajów starterów reakcji. Z drugiej strony jednak wyniki uzyskiwane tą metodą są uznawane za niepowtarzalne, a w reakcji PCR generowane są często niespecyficzne produkty, jeśli metoda nie jest zoptymalizowana do badanego materiału. Celem tego eksperymentu była optymalizacja techniki PCR-RAPD za pomocą zmodyfikowanej metody Taguchi, wykorzystanej do badań nad genotoksycznością ścieków szpitalnych. Badania prowadzono na DNA izolowanym z komórek merystemów korzenia cebuli (*Allium cepa*), hodowanych na pożywce zawierającej rosnące stężenia ścieków szpitalnych, pochodzących ze Szpitala Chorób Płuc w Pilchowicach, uznawanych za potencjalnie genotoksyczne w stosunku do komórek eukariotycznych, ze względu na wysoką zawartość pozostałości leków przeciwgruźlicznych i antynowotworowych. Przeprowadzone analizy udowodniły użyteczność zmodyfikowanej metody Taguchi do optymalizacji składu mieszaniny reakcyjnej do techniki PCR-RAPD. Uzyskane wyniki były powtarzalne, a w trakcie reakcji nie generowano niespecyficznych produktów PCR. Stwierdzono również użyteczność metody PCR-RAPD do analiz genotoksyczności z użyciem roślinnego DNA.

Keywords: ***Allium cepa*; Hospital wastewater genotoxicity; Modified Taguchi method; PCR-RAPD optimization; Ecotoxicological usage.**

1. INTRODUCTION

1.1. Polymerase Chain Reaction (PCR) and its modification – Random Amplified Polymorphic DNA (RAPD)

Polymerase Chain Reaction (PCR) was discovered in 1980s by Karry Mullis and this tool appeared to be crucial for the molecular research. Most of up-to-date molecular methods performed in a wide range of research are based on PCR amplification. This technique enables a particular DNA fragment multiplication in millions of copies, even from a minute DNA sample [1].

PCR mixture contains deoxynucleotides (dNTPs), PCR primers (short DNA fragments flanking the amplification target site and necessary for DNA polymerase performance), thermostable DNA polymerase and reaction buffer with magnesium cations. Amplification is based on cyclic temperature changes in 3 steps, repeated 20-35 times:

- denaturation, in ca. 95°C, when DNA helix is separated and each single strand DNA (ssDNA) is used for DNA amplification,
- annealing, step in which primers link with ssDNA to flank the amplification site,
- elongation, when DNA polymerase builds a new DNA strand adding dNTPs on the basis of DNA sample.

PCR sensitivity is both, advantage and disadvantage of the reaction. Theoretical possibility of DNA amplification in millions of copies enables the use of a PCR product as a material for other sorts of analysis. On the other hand the reaction requires sterile environment for PCR preparation to avoid reaction mixture contamination [1].

One of the commonly used PCR modification is Random Amplified Polymorphic DNA (RAPD). This method was first used by Welsh, McClelland and Williams in 1990. In RAPD short oligonucleotides with random sequence are used as primers for PCR reaction. The PCR product, separated electrophoretically, generates fingerprint pattern characteristic for analyzed DNA sample and it contains as many DNA bands as the primer specific sites present in the research material. In other words, if during the experiment in analyzed DNA a mutation occurs and it changes primer binding site (or creating a new one), the fingerprint pattern of this sample changes. These fingerprints analysis will lead to sequence diversity estimation and in case of ecotoxiciology research it will give the information about a number of mutation modifying DNA in primer sites. Also the presence or absence of particular DNA bands is used for samples similarity and diversity analysis [2].

The most common DNA changes altering RAPD profiles are mutations, such as DNA disruptions and adducts, point mutations and large DNA fragment modifications. These situations occur more often in areas between primer sites. Breakage of DNA strand between two primer sites usually generates DNA band in a RAPD profile in comparison to the control sample, while point mutations or translocations can both, generate or eliminate DNA bands from the profiles. The research performed by Jones and Kortenkamp [3] revealed that DNA bands in RAPD profile occurring in consequence of the mutation in primer site appeared not before 1/50 of DNA sample will be mutated. More probable fingerprints changes detected by RAPD are caused by DNA translocation, especially in case of when the new DNA band is appearing. Point mutations can influence RAPD fingerprint but the probability of their appearing in primer sites is lower than translocation-based changes.

DNA bands intensity differences can be caused by DNA adducts or DNA methylation. It is worth mentioning that during PCR high GC content DNA fragments possess strong secondary structure which can be insufficiently denaturated before being amplified in PCR. That is why DNA mutation stabilizing or destabilizing secondary structure of double stranded DNA can be a reason why RAPD fingerprints differ if they are not located on primer sites. The analysis performed by Atienzar and Jha [2] also proved that individual differences in genes expression and metabolic changes can influence RAPD profiles, but mutation and DNA damages are the most common factors causing differences among RAPD fingerprints.

RAPD is a popular method for various research. Originally it was used to analyze polymorphism in the area of genetic map construction, plant and animal variety identification, resistance genes identification in pests and sexual markers research. Nowadays RAPD is commonly used for genotoxicity and cancerogenesis study [2]. In ecotoxicology this method is used in diagnostics, enabling to estimate the differences between DNA profiles on the basis of DNA bands intensity and their presence or absence in the profile. These changes can be caused by DNA damages, mutations or DNA rearrangement, but RAPD analysis doesn't reveal the cause or the level of such damages [4]. RAPD can be a tool for finding muta-

tions if they are present in at least 2% of DNA analyzed, but they need to be in a particular sites of the genome [2].

No requirements for DNA sequence knowledge and small DNA samples analysis are RAPD advantages. The method is relatively cheap, it doesn't require expensive and highly specialized equipment [5]. In the same time it is sensitive and gives reliable results if the optimization of the method is performed [2].

RAPD possesses several disadvantages, appearing in situation when no PCR optimization is performed. In such cases false PCR product is generated in negative control and often the technique is not repeatable. It is important to know that different types of mutations and DNA damages can give the same result in fingerprint as non optimized PCR reaction, so it is impossible to find the cause of this change. In RAPD it should be assumed that DNA bands size similarities are linked with DNA homology in compared fingerprints. But it is known that this stipulation is false. It is also significant that RAPD generates qualitative and semi-quantitative data thus their interpretation is difficult [2].

1.2. PCR-RAPD optimization

There is no universal protocol for PCR-RAPD of all types of samples due to the individual characteristics and primers used in their study. That is why each reaction need to be optimized to particular type of material and primers for analysis. The most frequent with PCR are: no visible PCR product, low reaction efficacy and generation of non-specific PCR products [6]. DNA presence in negative control (with no DNA added) exclude this data from the analysis. Such situation occurs when the PCR mixture components concentration or annealing temperature is improper [7]. To avoid this problem the optimization of PCR reaction should be performed for each experiment, by determination of proper reaction components concentration [8] and the right annealing temperature usage [9]. Optimized PCR reaction should by highly effective, repeatable and it shouldn't generate additional PCR product in negative control. PCR-RAPD particularly requires such an optimization, because of short and random primers use and their low melting temperature. These primers are able to link with non-complimentary sites in DNA sample and give non-specific fingerprints.

One of the commonly used optimizing techniques for PCR-RAPD is modified Taguchi method [8]. It enables the assessment of the optimal reaction conditions with the lowest possible number of the experiments. First of all, the factors responsible for PCR efficacy should be determined prior to the PCR optimization. Then their combination for the optimal effectiveness should be assessed. Standard procedure requires separate testing of each possible reaction mixtures. Such test performed at 3 levels of reaction components concentration requires 81 separate experiments ($3^4 = 81$). Taguchi method reduces the number of the samples to nine. The number of the reaction in Taguchi experiment is calculated according to the equation (1):

$$E = 2k + 1 \tag{1}$$

where k is a value of factors tested.

If the number calculated with this equation is the multiplication of 3, then the number of the experiment required is equal to the next such a number. Next step is to prepare orthogonal table where the columns represent the mixture components and the lines correspond with their concentrations. Each component is present in this method at one of three concentration levels: A, B and C, chosen to estimate its influence on the reaction efficacy. When the concentration is chosen it is required to know the average range of the concentration used. The compounds of the reaction and their concentrations are set in orthogonal table where in each column the level combination (A, B and C) appears with the same frequency. Table 1 is an example of orthogonal table for 4 components at 3 concentration levels.

Table 1.
An example of orthogonal table for 4 components at 3 concentration levels

Reaction no	component 1	component 2	component 3	component 4
1	A	A	A	A
2	A	B	B	B
3	A	C	C	C
4	B	A	B	C
5	B	B	C	A
6	B	C	A	B
7	C	A	C	B
8	C	B	A	C
9	C	C	B	A

After all PCR-RAPD reactions agarose gel electrophoresis is performed and the reaction efficacy is estimated. RAPD profiles are evaluated according to the equation (2):

$$P = (n \times s) + 1 \tag{2}$$

where:

P is the product efficacy,

n is the number of DNA bands,

s it the range of DNA bands presence, with $s = 1$ for DNA profiles up to 1 kbp and $s = 2$ for DNA profiles up to 2 kbp.

The reaction with no product or smear DNA are scored as 1. The values obtained this way are used for further calculations.

In Taguchi method the product efficacy from each of the reaction is used to estimate the influence of particular component on amplification reaction. For this aim the signal-to-noise ratio (SNL) is calculated according to the equation (3):

$$SNL = -10 \log\left(\frac{1}{n}\sum_{i=1}^{n}\frac{1}{P_i^2}\right) \tag{3}$$

where:

SNL is signal to noise ratio,

n is the reaction number,

P is reactions efficacy.

For each reaction component the optimal conditions are retained when SNL is the highest. The results undergo polynomial regression ($y = ax^2 + bx + c$) and the optimal concentrations are equal to the maximum the diagrams obtained. Then the results acquired in the method can be accepted to further research or used as narrowed initial data to search for more precise optimal values.

1.3. PCR-RAPD data analysis

PCR-RAPD fingerprints can be analyzed in ecotoxicological research when toxic compounds influence on organisms is observed. The similarity of RAPD profiles is commonly described by the equation (4) [2]:

$$S = \frac{2\,n_{ij}}{n_i + n_j} \tag{4}$$

where:

S is the similarity,

n_{ij} is the number of common DNA bands in the fingerprints,

i and j are DNA fingerprints,

n_i and n_j are the total DNA bands number for i and j fingerprints, respectively.

The S value is in the range between 0 and 1 adequately to the situation, when the fingerprints don't have common DNA bands or when they are identical.

In population biodiversity analysis using PCR-RAPD Shannon-Wiener (H') or Simpson (D) indexes are used. These indexes are calculated according to the equations (5) and (6):

$$D = \sum_{i=1}^{k}\left(\frac{n_i}{N}\right)^2 \text{ lub } D = \sum_{i=1}^{k}\left[\frac{n_i(n_i-1)}{N(N-1)}\right] \tag{5}$$

$$H' = -\sum_{i=1}^{k} p_i \ln(p_i) \tag{6}$$

where:

k is DNA bands number in RAPD,

p_i is the frequency of i-DNA band presence,

n_i is the number of fingerprints with i-DNA band,

N is the number of analyzed fingerprints.

Both indexes differ at the level of detection of the common and separated features. Simpson index is more sensitive to the common DNA bands while Shannon-Wiener index is more sensitive to fingerprints differences [4].

The interpretation of the PCR-RAPD fingerprints in genotoxicity analysis is difficult due to the influence of the mutation and DNA damages on RAPD profiles. The electrophoretic pictures from the control and experimental samples can be compared with the samples recovered after the toxic exposition during the experiment. Such comparison enables to find the DNA bands appearing or disappearing by mutation or DNA damage [2].

RAPD profiles analysis in genotoxic research is based on genetic stability of the sample analyzed in comparison to the control sample. The stability is given as a percent and calculated according to the equation (7) [10]:

$$100 - (100\,a/n) \tag{7}$$

where:

a is the number of polymorphic DNA bands (appearing or disappearing in the sample) in the sample analyzed,

n the number of all DNA bands in the control sample.

It should be mentioned that RAPD usage for genotoxicity research requires careful selection of the proper test organism characterized with a low genetic diversity, which should be checked before the experiment beginning. If RAPD profile based on DNA from several individuals from one species are repeatable it is possible to compare DNA profiles of these organisms after toxic exposure with a control sample. Otherwise it is necessary to isolate DNA *in vivo* and compare each organism separately [2].

The aim of this study was to optimize PCR-RAPD method for genotoxic research performed on DNA isolated from onion (*Allium cepa*) root meristem cells. The plant seeds were grown on increasing concentration of hospital wastewater found to be potentially toxic to genetic material. The optimization was performed with modified Taguchi method in order to obtain repeatable results.

2. MATERIALS AND METHODS

2.1. Plant material preparation and DNA isolation

PCR-RAPD was performed to analyze genotoxic effect of hospital wastewater derived from Hospital for Phthisiatry and Lung Diseases in Pilchowice, Poland, on onion (*Allium cepa*) DNA. The onion seeds were geminated and grown for 96 h in wastewater in distilled water solution with wastewater concentrations: 100, 75, 50, 25, 12.5 and 6.25%. As positive control *A. cepa* seeds were grown in ethyl methanesulfonate (EMS, Sigma, 10 mg/l) and 3% solution of hydrogen peroxide. Onion seeds grown on tap water were the negative control in the experiment. After the incubation root meristems, ca. 1-2 cm in length, were cut off and used to DNA isolation with Genomic Mini AX Plant Spin Kit (A&A Biotechnology) according to manufacturer's instruction.

2.2. PCR-RAPD test reaction

PCR-RAPD test reaction was performed according to Qari [10] with 30 PCR cycles using OPA04 primer (5'-AATCGGGCTG-3', Genomed). The PCR product was electrophoretically separated in 1% (w/v) agarose gel) with ethidium bromide (10 μg/ml) in 1× TBE (Tris, boric acid, EDTA, pH = 8.2) at 80 V, 75 min, and visualized under UV light. The amount of DNA was measured fluorometrically with Qbit (Invitrogen).

2.3. PCR-RAPD optimization according to modified Taguchi method

In order to optimize PCR-RAPD the Taguchi method modified by Cobb and Clarkson [3] was used. The initial concentrations of DNA sample, OPA04 primer and dNTPs (Promega) were as in Table 2.

Table 2.
The initial concentrations of PCR- RAPD mixture components

Mixture component	level A	level B	level C
dNTPs (10 mM, Promega)	0.1 mM	0.2 mM	0.4 mM
OPA04 (100 μM, Genomed)	0.1 μM	0.2 μM	0.4 μM
$MgCl_2$ (25 mM, Promega)	1 mM	2 mM	4 mM
DNA (40 ng/μl)	5 ng	10 ng	20 ng

On the basis of components tested (k = 4) the number of required reaction was calculated (E = 2 × 4 + 1 = 9). In each reaction Green GoTaq Flexi Buffer (Promega) and GoTaq DNA Polymerase (Promega) in the final concentration of 1× and 2 U respectively were used. The concentrations of the other reaction components were chosen on the basis of orthogonal table (Table 1). The PCR reaction was performed in final volume of 30 μl. The concentration of PCR mixture components during optimization was used as in Table 3.

Table 3.
PCR-RAPD mixture components concentration used in modified Taguchi optimization

no.	buffer	$MgCl_2$ [mM]	dNTPs [mM]	OPA04 [μM]	GoTaq [U]	DNA [ng/μl]
1	1×	1	0.1	0.1	2	5
2	1×	2	0.2	0.1	2	10
3	1×	4	0.4	0.1	2	20
4	1×	2	0.4	0.2	2	5
5	1×	4	0.1	0.2	2	10
6	1×	1	0.2	0.2	2	20
7	1×	4	0.2	0.4	2	5
8	1×	1	0.4	0.4	2	10
9	1×	2	0.1	0.4	2	20

Table 4.
The assignment of PCR- RAPD components during optimization

	A			B			C		
OPA04	9	11	13	11	6	6	25	8	17
DNA	9	11	25	11	6	8	13	6	17
$MgCl_2$	9	6	8	11	11	17	13	6	25
dNTPs	9	6	17	11	6	25	13	11	8

The amplification of DNA isolated from *A. cepa* root meristem cells was performed in C-1000 thermocycler (BioRad). The reaction consisted of: (1) DNA predenaturation (12 min, 95°C), (2) DNA denaturation (95°C, 1 min), (3) annealing (37°C, 1 min), (4) DNA elongation (72°C, 90 sec) and (5) final elongation (72°C, 10 min). Steps 2-4 were repeated 30 times. The PCR product was electrophoretically separated using 1.5% (w/v) agarose gel with ethidium bromide (10 mg/ml) with ×1 TBE, at 80 V, 75 min, and visualized under UV light. The obtained fingerprints were scored according to the number and range of DNA bands. The SNL values were calculated and the optimal PCR reaction mixture components concentration were assigned. The PCR-RAPD was performed in triplicate.

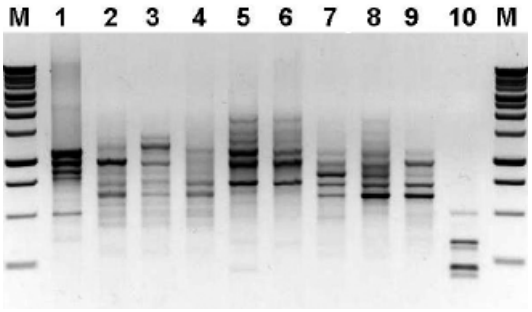

Figure 1.
PCR-RAPD fingerprint performed before Taguchi method optimization
M – 1kb DNA ladder (Promega), 1-9 – *A. cepa* DNA samples, 10 – negative PCR control

3. RESULTS

PCR-RAPD was performed according to Qari [10]. DNA samples derived for onion (*Allium cepa*) root meristem cells were grown on medium containing an increasing concentration of hospital wastewater. DNA isolated from onion grew on 10 mg/l EMS solution and 3% hydrogen peroxide solution were used as positive control, while negative control was DNA isolated from onion grew on tap water. Figure 1 presents the results of PCR-RAPD before optimization with Taguchi method. There is non-specific DNA in negative control (line 10).

Next step was the PCR optimization with modified Taguchi method according to Cob and Clarkson [3]. The results obtained after the optimization are presented in Figure 2. The PCR-RAPD was performed in triplicate, in all cases the results were repeatable.

The reaction efficacy was calculated according to the equation (2). The results were averaged and assigned to each of PCR reaction mixture components at the particular level of concentration (Table 4). For example, points for PCR mixtures numbered: 2, 4 and 9 were assigned to $MgCl_2$ at concentration B, that correspond to the orthogonal table (Table 1). Than SNL was calculated according to the equation (3). The results obtained are presented in Table 5.

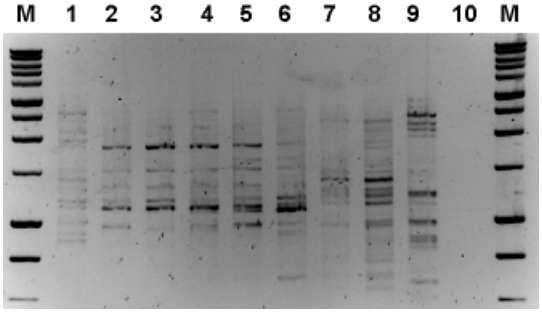

Figure 2.
PCR-RAPD fingerprint performed after Taguchi method optimization;
M – 1kb DNA ladder (Promega), 1-9 – *A. cepa* DNA samples, 10 – negative PCR control

Table 5.
SNL values calculated for PCR- RAPD optimized components; the highest values for particular components are bold

	A	B	C
OPA04	20.53	16.72	**21.61**
DNA	**21.31**	17.64	19.07
$MgCl_2$	17.31	**21.76**	19.29
dNTPs	18.38	19.01	**20.03**

The optimal parameters for PCR reaction with the buffer concentration of 1× and *Taq* polymerase concentration of 2 U were the PCR-RAPD mixture component concentrations with the highest SNL values: 0.4 μM OPA04, 5 ng DNA, 2 mM $MgCl_2$ and 0.4 mM dNTPs. The reactions performed with these concentrations of components enabled repeatable results and absence of non-specific PCR products.

PCR-RAPD was performed on DNA isolated from *A. cepa* according to PCR mixture components concentration optimized with modified Taguchi method. The results obtained after the optimization are presented in Figure 2. The PCR-RAPD was performed in triplicate, in all cases the results were repeatable.

4. DISCUSSION AND CONCLUSION

In this experiment it was proved that direct usage of the method described by Qari, [10] for genotoxicity analysis in *Allium cepa* failed. The RAPD profiles obtained in this research gave unrepeatable results and non-specific product in negative PCR control. Such data are useless from the point of further analysis. In the experiment Qari [10] did not observe such situation, probably because of solutions and thermocycler from different manufacturer usage [2]. PCR-RAPD for genotoxic analysis for DNA isolated from onion seeds grown on hospital wastewater in this study required optimization. The optimization technique – modified Taguchi method [8] enabled to obtain RAPD fingerprints with absence of non-specific DNA in negative PCR control and repeatable results for the material used. It was revealed that such optimization should be performed each time for new biological material or solutions and equipment used. Such optimizations were performed previously in several PCR-based experiments [11, 12, 13] where the method presented high level of discrimination for prokaryotic and eukaryotic analysis.

RAPD is widely known as unrepeatable method. This is usually caused by low quality of DNA sample used or lack of the PCR optimization [2]. High quality DNA, without inhibitors and contamination is crucial for repeatable PCR results obtainment. The amount of DNA sample should be also carefully added to obtain as many separated DNA bands as possible well. In this study it was revealed that relatively low magnesium ions concentration protects primers against improper linking during PCR reaction. Repeatability of the fingerprints should be checked at least two times with the amount of DNA sample differing twice [2].

After its optimization and checking the repeatability PCR-RAPD is a useful tool for genotoxicity and genetic instability analysis in plant material, such as *Allium cepa*. RAPD results are perfect to supplement the information about population genetics, toxicity and cancerogenesis obtained by means of different methods. Several RAPD applications have been developed which have the potential to be useful in ecotoxicological research [4]. As a DNA based method, PCR-RAPD can be easily used in ecotoxicology research, because this tool is able to generate a nearly unlimited number of markers due to the random primers usage helpful in a large number of genome loci analysis [14, 15].

In comparison to the other mutagenicity tests, such as Ames test, RAPD is less sensitive. But in the same time it enables to find mutations in the sites where no other technique is able to come across it. In some research, PCR-RAPD presented higher sensitivity than the other commonly used genotoxicity method – comet assay [16]. RAPD is relatively inexpensive and gives a great deal of data even without the knowledge about genome sequence. All these features make PCR-RAPD an useful technique for ecotoxicological research (not only for initial), screening genotoxicity and toxicity tests.

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