Scientific Papers. Series B, Horticulture. Vol. LXVI, No. 2, 2022
Print ISSN 2285-5653, CD-ROM ISSN 2285-5661, Online ISSN 2286-1580, ISSN-L 2285-5653

SWEET CHERRY (*Prunus avium* L.) RANDOM AMPLIFICATION OF POLYMORPHIC DNA ANALYSIS OPTIMIZATION

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Abstract

Prunus avium L. is a fruit trees species belonging to the Rosaceae family, cultivated worldwide in temperate climates. This study presents the genomic DNA extraction and RAPD method optimisation using the 'Severin' sweet cherry cultivar. For genomic DNA genomic DNA extraction was optimized the method for breaking the cell wall. The parameters optimized for RAPD reaction were genomic the annealing temperature, DNA concentration and primer concentration. The best method for breaking the cell wall was Method 1, grinding the tissue with liquid nitrogen. The optimum annealing temperature was determined to be 30°C. The concentration of genomic DNA in the RAPD reaction varied between 0.05 ng/µl and 1.00 ng/µl, and that of the primer varied between 0.1 µM and 2.0 µM. The optimum concentration for the genomic DNA proved to be 0.05 ng/µl and that of the primer 2.0 µM. These results will be applied in a future experiment that will study the genetic variability of Romanian sweet cherry cultivars present in the USAMV of Bucharest orchard collection.

Key words*: genomic DNA extraction; polymerase chain reaction; RAPD optimization.*

INTRODUCTION

Even though cherries have been used by humans for more than 6000 years, domesticated by ancient Greeks and introduced to the rest of Europe by Romans in the 1st century, due to the fruits' perishability, cherry breeding started much later, during the 18th century (Blando & Oomah, 2019). Nowadays, new sweet cherry cultivars are created and evaluated for economically important traits such as fruit weight and colour, resistance to cracking, fertility, resistance to low winter temperatures (Iurea et al., 2019; 2020; Palubiatka et al., 2021).

In Romania, sweet cherry (*Prunus avium* L.) is cultivated traditionally. Local sweet cherry cultivars are the main sources of biodiversity in agriculture, so their recognition, identification and conservation is of particular importance.

To be able to implement the necessary conservation measures, it is very important to know the genetic characteristics of the varieties and their degree of relatedness (Parra-Quijano et al., 2012). Recent sequencing techniques and genome sequencing have brought to light new data that can be used for a variety of purposes, including the identification of molecular markers linked to traits of interest, and molecular marker-assisted selection (Soundararajan et al., 2019).

Since a minimum of 4-5 years are needed for cherry trees to produce the first fruits, a lot of time and space are required to develop new varieties. In this case, marker - assisted selection employing molecular markers such as RAPD, SSR, AFLP, reduces considerably both the time and space required in the breeding process, as the plants carrying the desired trait can be selected from the seedling phase (Jayasankar & Kappel, 2011). The selection at young stage can be done not only for cultivars, but also for rootstocks (Quero-Garcia et al., 2017), because cultivar/rootstock combination impacts the growth and development of the plants, affecting economically important traits such as yield and fruit size (Asănică et al., 2013).

Random amplification of polymorphic DNA (RAPD) is a PCR technique that uses an arbitrary single oligonucleotide primer that can anneal to template DNA sequences on both DNA strands and amplify sequences between

the annealing sites under low stringency conditions, creating a "fingerprint" of a particular genome (Babu et al., 2021). RAPD has been used in studies of genetic diversity, plant breeding, germplasm management, cultivar and hybrid verification, taxonomic studies, phylogenetic studies, study of genetic marker–trait association, etc. (Antić et al., 2020; Babu et al., 2021; I. V. Berindean et al., 2016; Khadivi-Khub, 2014; Sharma et al., 2012; Zarei et al., 2017).

RAPD techniques has a high versatility, low cost, but a low reproducibility due to low quality of DNA template, and variations in template DNA concentration (Babu et al., 2021). Therefore, optimization of genomic DNA extraction, as well as optimization of template DNA concentration, RAPD primer concentration, and primer annealing temperature are three parameters that should be optimised to get the best results in a RAPD reaction.

The objective of this study was to optimise the genomic DNA extraction conditions as well as the RAPD reaction conditions before proceeding to the genetic variability study of sweet cherry cultivars present in the USAMV of Bucharest orchard collection.

MATERIALS AND METHODS

Plant material

Leaves from sweet cherry cultivar 'Severin' were used for this study. The leaves were harvested from the University of Agronomic Sciences and Veterinary Medicine of Bucharest orchard.

Genomic DNA extraction optimization

Young leaves, harvested from the top of the shoots were washed with distilled water, weighed, placed in 50 ml tubes, and stored at -70°C.

Cell wall breaking was performed using the InnuPREP Plant DNA I KIT IPC 16 (Analitik Jena) by three different methods: 1. Grinding the tissue with liquid nitrogen using a mortar and pestle, then adding SLS lysis solution and proteinase K; 2. Grinding the tissue with a micro pestle in a 1.5 ml tube containing the SLS lysis solution and proteinase K; 3. Tissue frozen at –70°C for 24 hours in 2 ml tubes containing steel balls was homogenized using the SpeedMILL PLUS homogenizer, then SLS lysis solution and proteinase K were added. Thereafter, all three protocols continued with the external lysis of the cells, according to the InnuPREP Plant DNA I KIT IPC 16 manufacturer instructions. Briefly, samples were homogenized, then incubated at 65°C for one hour. Thereafter, the samples were filtered to remove tissue debris, treated with RNase A, and transferred to plate for automated DNA extraction. Automatic extraction was performed using InnuPURE C16 System with the Ext_Lysis_200_C16_04 program. Following extraction, samples were stored at –20°C.

Measurement of DNA quantity and quality

DNA concentration and the DNA quality based on the A260/A280 and A260/A230 absorbance ratios were measured using the NanoDrop 1000 spectrophotometer (Biorad).

RAPD optimization

Polymerase chain reaction (PCR) was done with the Platinum™ II Hot-Start PCR Master Mix (2X) (Invitrogen) according to the manufacturer's instructions regarding the extension temperature and the amounts of PCR master mix and Platinum GC enhancer used. PCR setup was done according to Table 1.

Table 1. PCR reaction setup

Component	Volume	Final concentration
Nuclease-free water	to 10 µl	-
Platinum™ II Hot-Start PCR Master Mix (2X)	5 µl	-
10 µM Primer P59	0.1-2 µl	0.1-2.0 µM
0.5-10 ng/µl Template DNA	1 µl	0.05-1 ng/µl
Platinum GC Enhancer	2 µl	-
Total	10 µl	-

The sequence of primer P59 was 5'-GTTGGTGGCT-3'. For genomic DNA, the concentration varied between 0.5 ng/µl and 10 ng/µl. The concentration of the primer varied between 0.1 µM and 2 µM, and the annealing temperature gradient was between 30°C and 34°C. PCR program is visible in Table 2.

The online tool *Oligo Calc: Oligonucleotide Properties Calculator* was used to determine the range of annealing temperature to be tested

(Kibbe, 2007). The basic Melting Temperature of the primer was calculated using the formula Tm= (wA+xT) * 2 + (yG+zC) * 4, where w, x, y, z are the number of the bases A, T, G, C in the sequence, respectively, since the length of the primer is below 14 bp (Marmur & Doty, 1962).

Table 2. PCR program used for RAPD reactions

Temperature	Time	Cycles
94°C	2 min	1
94°C	15 sec	40
30-34°C	15 sec	
68°C	30 sec	
68°C	2 min	1
4°C	HOLD	

The PCR products were visualized using Molecular Imager® PharosFX™ and PharosFX™ Plus Systems after migration in a 2% agarose gel at 100 V for 45 min.

Data analysis

Data for genomic DNA extraction optimization was analysed using the Excel for Microsoft 365 software.

RESULTS AND DISCUSSIONS

Genomic DNA extraction optimization

The three parameters considered to obtain the optimum method of genomic DNA extraction were the yield (DNA concentration), and the A260/A280 and A260/A230 ratios.

DNA yield

The genomic DNA yield varied between 30.9 ng/µl (Method 2) and 40.3 ng/µl (Method 3) (Figure 1). The DNA yield obtained by Method 1 (39.51 ng/µl) was similar to the one obtained by Method 3.

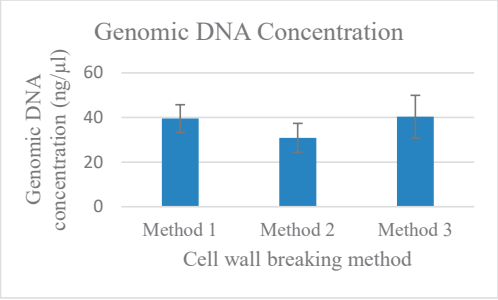

Figure 1. Genomic DNA concentration obtained with three different methods for breaking the cell wall. Error bars represent standard error of the mean

DNA purity

Genomic DNA purity was assessed based on the A260/A280 and A260/A230 ratios. For genomic DNA, an ideal A260/A280 ratio is ~1.8, and A260/A230 ratio should be between 2.0-2.2 (Matlock, 2015).

Methods 1, using liquid nitrogen, and Method 3, using prior deep freezing of the tissue before homogenization with SpeedMill Plus, yielded A260/A280 absorbance ratio values over 1.5 (1.72 for Method 1 and 1.63 for Method 3), whereas when using Method 2, the A260/A280 absorbance ratio was much lower, 1.14 (Figure 2).

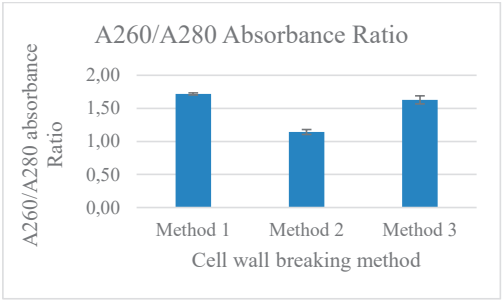

Figure 2. DNA purity measured using the A260/A280 absorbance ratio for the three different methods used to break the cell wall. Error bars represent standard error of the mean

In a study to optimize genomic DNA extraction protocol for twelve rosaceous species, Antanaviciute et al. (2015) observed an A260/A280 ratio of 1.53 for sweet cherry, one of the lowest ratios noted among the species studied. The low ratios indicate contaminants absorbing at 280 nm or less (Matlock, 2015).

When looking at A260/A230 ration, all samples have low values, indicating carbohydrate carryover (Matlock, 2015), however the best results were observed for Method 1 with an almost double value when compared to the other two methods (Figure 3).

Taking into consideration the results obtained, the optimum Method that was used to extract genomic DNA to be used further in the RAPD reaction optimization was Method 1, breaking of the cell walls using liquid nitrogen, with the highest yield and the purest genomic DNA extracted.

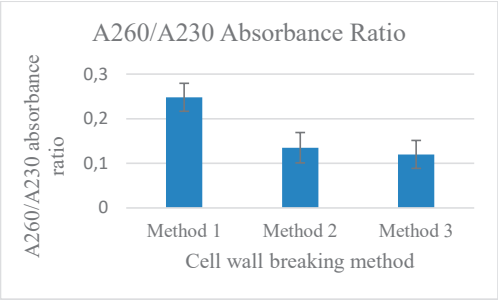

Figure 3. DNA purity measured using the A260/A230 absorbance ratio for the three different methods used to break the cell wall. Error bars represent standard error of the mean

RAPD reaction optimization

The three parameters optimised for the RAPD analysis were DNA template concentration, RAPD primer concentration, and primer annealing temperature.

Annealing temperature optimization

Based on the basic melting temperature of the primer of 32°C, the range of annealing temperatures tested was between 30.0°C and 34.0°C. The clearest amplification of the PCR products was observed at 30°C (Figure 4). The low value of the annealing temperature is due to the short length of the primer, thus in RAPD reactions annealing temperatures are usually below 40 °C (Antić et al., 2020; I. V. Berindean et al., 2016; Guan et al., 2014; Sevindik et al., 2020; Sharma et al., 2012; Thakur et al., 2018).

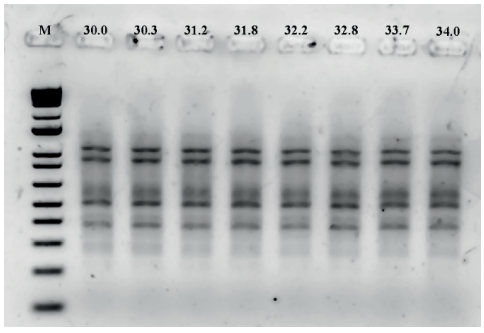

Figure 4. Effect of annealing temperature optimization on RAPD amplification patterns. M-1Kb Plus DNA Ladder (Invitrogen). Numbers represent the annealing temperature in °C

Genomic DNA concentration optimization

The range of genomic DNA concentrations tested was between 0.5 ng/µl and 10.0 ng/µl. The best results were obtained with the initial concentration of 0.5 ng/µl (final concentration of 0.05 ng/µl) for template DNA (Figure 5). Williams et al., who developed the RAPD technique in 1990, used a template DNA concentration of 1 ng/µl, however they recommended reducing the template DNA concentration to obtain distinct bands as opposed to a smear (Williams et al., 1990).

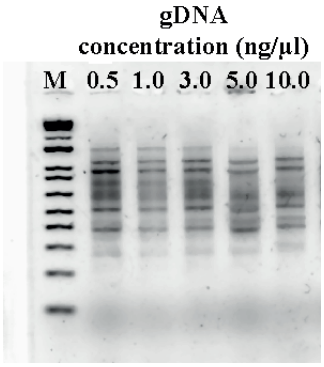

Figure 5. Effect of genomic DNA concentration on RAPD amplification pattern. Numbers represent the initial concentrations of genomic DNA

Primer concentration optimization

The range of final concentrations for the analysed primer P59 was between 0.1 µM and 2.0 µM and it was noticed that the optimum concentration was 2.0 µM, (Figure 6). Final primer concentration in RAPD reactions are usually below 1 µM (Antić et al., 2020; Berindean & Itu, 2019; Eimert et al., 2012; 2016; Zamani et al., 2012)

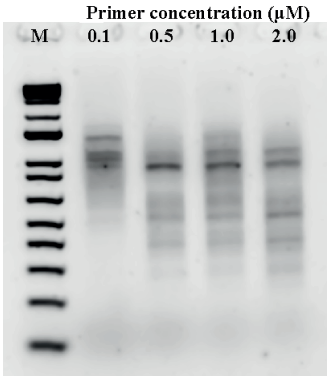

Figure 6. Effect of primer concentration on RAPD amplification pattern. Numbers represent the final concentrations of the primer in the reaction

The results obtained in this study will be applied in a future experiment that will study the genetic variability of cherry varieties present in the USAMV Bucharest orchard.

CONCLUSIONS

In conclusion, in the present study were optimised the method for breaking the cell wall prior to genomic DNA extraction and three RAPD reaction parameters as follows:

1. The best method for breaking the cell walls was Method 1, grinding the tissue with liquid nitrogen with a mortar and pestle, as it gave the best DNA yield and the purest DNA.
2. Optimum annealing temperature was 30°C.
3. Optimal concentration of genomic DNA was 0.05 ng/µl.
4. Optimum primer concentration was 2.0 µM.

ACKNOWLEDGEMENTS

This research work was supported by a grant of the University of Agronomic Sciences and Veterinary Medicine of Bucharest, project number Cod 2021-5/2021.07.16, acronym MicroSatCherry, within Joint Junior Competition 2021 and project FDI-2022-0634.

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