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Original scientific paper

STANDARDIZATION AND REPRODUCIBILITY OF RANDOM MARKER BASED ANALYSIS OF MICROPROPAGATED CRIMSON BEEBALM

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Genetic analysis of the stability in micropropagated plant material are required to be perform by effective and reproducible techniques. When no specific DNA based markers are available for certain species, such as for crimson beebalm, standardized and optimized protocols can be used even for random based markers. Here we report for the first time the optimization of PCR conditions for RAPD analysis for the purposes of the establishment of fast, reliable and reproducible method for screening of genetic stability in *Monarda dydima*, L. micropropagated cultures. RAPD protocol was optimized based on the use of 50 or 100 ng of template DNA and annealing temperature of 36°C, resulted optimal amplification. Setting the standardized PCR conditions, primers used in experiments generated reproducible and distinguishable fingerprinting patterns for micropropagated cultures of crimson beebalm and is suitable for further analysis of studying genetic variation of this medicinal plant.

Key words: crimson beebalm, *Monarda dydima*, L., RAPD optimization, reproducibility

INTRODUCTION

Monarda dydima, L. (crimson beebalm) originates in the eastern region of the U.S., where it can be found from the state Michigan to New York and from North Georgia to southern Tennessee. *Monarda* was introduced as an ornamental plant in Europe, too.In half of 18th century, pioneer colonists brought the seeds of the crimson beebalm to England and from there were spread across Europe (SMALL, 2006). Crimson beebalm is a medicinal plant belonging to family *Lamiaceae*. Actually, it is used mainly for tea called Eswego –tea, as a supplement drinks wine, soft drinks, juices, jellies and cheeses and essential oils, which are obtained from monarda, called bergamot oil, are added as a flavoring ingredient in perfumes (PRATHER *et al*., 2002; SMALL, 2006).

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Here, the setting up the RAPD (randomly amplified polymorphic DNA) conditions is reported for the purposes of sreening the stability of micropropagated *Monarda dydima*, L. The range of used DNA markers is still very limited for *Lamiaceae* (SKOULA *et al*., 1999; VIEIRA *et a*l., 2003; FRACARO *et al*., 2005; VERMA *et al*., 2006; TRINDADE *et al*., 2009; YUZBASIOGLU *et al.*, 2008; SOSTARIC *et al.*, 2012; ZHANG *et al*., 2012; RODRIGUES *et al*., 2013; RATHORE *et al.,* 2014) and no specific markers actually exist for *Monarda dydima*, L..

RAPD method is still used in various plant DNA polymorphism studying, including inter and intraspecific variability or species identification. It is still one of the most efficient molecular method, as no specific genomic information are obligated to know about analysed plant species (ŠTEFÚNOVÁ and BEŽO, 2003; MILELLA *et al*., 2011; PAVLOVIČ *et al.,* 2012; HUO *et al.,* 2013; BOJOVIČ *et al.,* 2013). Beside RAPD technique, such as independency from genomic data knowledge posses microsatelite and retrotransposon length polymorphism based techniques (ŽIAROVSKÁ *et al*., 2013 a); ŽIAROVSKÁ *et al*., 2013 b); SENKOVÁ *et al*., 2013;)

In spite of its ongoing popularity in plant germplasm evaluation, RAPD technique is limited by low repeatebility of the amplification patterns (SKORIČ *et al.,* 2012). The reproducibility of RAPD banding pattern is only achieve by optimization of all PCR components, sucha s type and concentration of Taq polymerase, Mg^{2+} ions, dNTPs, primer concentration, purity and concentration of template DNA and individual parameters of cycling program.

The aim of the study was to standardize components of RAPD for DNA based polymorphism analysis of *Monarda dydima*, L. and to develop fully reproducible protocol for RAPD screening of genetic stability in micropropagated crimson beebalm.

MATERIALS AND METHODS

Plant material

Micropropagated plant material was prepared in the Laboratory of plant tissue culture of the Department of Crop Science and Agroforestry of the Czech University of Life Sciences. Nodal segments of *Monarda didyma* were cultivated on MS (MURASHIGE and SKOOG, 1962) medium in cultivation box under 16/8 h photoperiod with illumination 2500 lx and temperature 25/20°C. Before starting the RAPD optimization, multiplication of the plant material was realised using nodal segments. For optimized RAPD protocol, two variants of micropropagated plants were tested for reproducibility - variant growing on basal MS medium (MURASHIGE and SKOOG, 1962) without plant growth regulators and variant growing on MS medium with the addition of BAP (6-benzylaminopurine) in the concentration of 0,5 mg/L.

DNA isolation

DNA extraction and molecular analysis were performed in the Laboratory of genetic technologies of the Department of Plant Genetics and Breeding of the Slovak University of Agriculture. DNA was isolated from micropropagated plants of crimson beebalm. The samples about 100 mg weight were taken for DNA extraction. A modified cetyl trimethyl ammonium bromide (CTAB) extraction method was used according the ROGERS *et al*. (1994). In total, three independent samples were used for DNA extraction. The yield of extracted DNA was measured using a Nanodrop™, where the purity of DNA was determined by calculating the absorbance ratio at 260 nm to that of 280 nm. The purity and concentration of extracted DNA was also inspected by running the samples on 1% agarose gel comparing them to samples of human genomic DNA with known concentration (Human Genomic DNA, Promega, 245 $\mu g \times ml^{-1}$).

RAPD optimization

In total, five decamer primers (Operon Technologies; table 1) and isolated DNA of *Monarda dydima*, L. were used to optimize the RAPD protocol for this species. The initial PCR reaction mixture consisted of 60 ng of template DNA, 2,5 mmol.dm^{-3} $MgCl_2$, 2U Taq DNA polymerase (DreamTaq polymerase, ThermoScientific), 200 µmol.dm^{-3} dNTPs of each and 8 pmol.dm^{-3} primer in 1X reaction buffer supplied with the polymerase in a total volume of 25 µl. PCR amplification were performed on a C1000 thermocycler (BioRad) with following time regime: an initial denaturation step 94°C for 5 minutes, followed by 45 cycles of 94°C 1 minute, 35°C 1 minute and 72 °C 2 minutes. The final extention was performed 10 minutes at 72°C and 4°C was hold at the end.

The optimization analysis were performed with different amounts of template DNA (10 ng, 50 ng and 100 ng); concentrations of primer (0,8; 1,2; 2 and 4 µmol.dm^{-3}), concentrations of $MgCl_2$ (1,5; 2; 2,5 and 3 mmol.dm^{-3}), concentrations of dNTPs (0,1; 0,2 and 0,3 mmol.dm^{-3}). In total, three annealing temperatures were tested for every combination (35, 36 and 37 °C).

The possible experiment error in all combinations was prevented by preparing master mixes of all components and only the varying components were added individually. All PCR reactions were repeated at least twice.

Table 1. Decamers tested in RAPD standardization for Monarda dydima, L.

Primer name	Sequence
OPB2	5´TGATCCCTGG 3´
OPB4	5´GGACTGGAGT 3´
OPB10	5´CTGCTGGGAC 3´
OPB12	5´CCTTGACGCA 3´
OPB14	5´TCCGCTCTGG 3´

RAPD profiles reproducibility

Obtained amplification products were separated by electrophoresis on 1,5% agarose gels in 1× TBE buffer at 55 V for 3,5 hod. Gels were stained with GelRed™ and visualized using Gene:Box UV transilluminator. The 250 bp ladder (Fermentas) was used for determination of amplified fragments sizes.

Those concentrations of PCR components that resulted in the highest number of amplified fragments of different lenght were selected as the most appropriate for further analysis. These PCR mixture parameters were used for RAPD profile reproducibility testing analysis. Selected optimized RAPD parameters were as followed: 1U of Taq polymerase, 3 mmol.dm-3 $MgCl_2$, 0,1 mmol.dm-3 dNTPs, 0,4 µmol.dm-3 of decamer primer and 50 ng of template DNA in 1 × reaction buffer. Banding patterns repoducibility was tested in triplicates by comparing the obtained RAPD profiles and only those decamers, where no differencies were found are suitable for analysis of stability in micropropagated crimson beebalm plants.

RESULTS AND DISCUSSION

Since the RAPD technique was prooved as to be very effective in the studies of genetic diversity in the populations of many plant or animal species (BEŽOVÁ *et al*., 2002; RAŽNÁ and HRUBÍK 2009; VIVODÍK *et al*., 2013), still is used not only for this purposes, but it became a very

usable in analysis of genetic stability in plant tissue cultures, too (BINDLYA and KANWAR, 2003.; RASHEED *et al*., 2005; de MELLO *et al.,* 2006).

The main disadvantage of the RAPD technique is a sensitivity to any deviation from optimized PCR conditions that results in non-reproducible banding pattern. The alterations in the different parameters tested that had varying degrees influence on the RAPD profiles and their reproducibility was and still are reported (KUTA *et al*., 2005; VURAL *et al*., 2009; SAHASRABUDHE and DEODAR, 2010; WANG *et al.,* 2010; SKORIČ *et al.,* 2012).

To obtain reproducible, well defined and consistent RAPD amplicons, following PCR paramaters were optimized in this study: concentration of Taq polymerase, $MgCl_2$, dNTPs, primer concentration and the amount of template DNA.

Optimization of template concentration is one of the crucial steps in getting the optimal RAPD patterns. The effect of template DNA concentration is reported as not very variable in literature. SKORIČ *et al*. (2012) reported in their study, that an efficient RAPD protocol is resistant to variations in template DNA concentration and is reported reported, that RAPD patterns changed only quantitatively withing a 200-fold change of DNA concentrations. Results of different amount of template DNA of *Monarda dydima*, L. are in concordance with the previous findings, as only changes in quantity in banding pattern was observed for different amounts of template DNA in PCRs (figure 1).

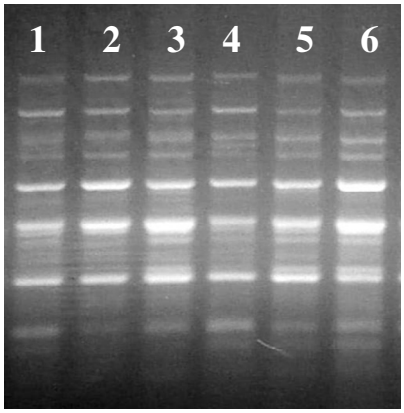

Figure 1. Optimization of DNA concentration for RAPD analysis of crimson beebalm for primer OPB 4. From left to right: 1,2,3 - 50 ng; 4,5,6 - 100ng of template DNA in triplicate.

Another characteristic of DNA templates that directly affect amplification of PCR products is quality of DNA. For *Monarda dydima*, L. no problems resulted in the low quality of DNA were observed and both, quantity and quality expressed by A260/A280 were of high standart.

Primer concentration, as a factor that has an significant influence on RAPD reproducibility, was optimized for concentrations of 0,8; 1,2; 2 and 4μmol.dm^{-3}. Optimization of this PCR component was performed, as higher concentrations of primer often result in nonspecific

priming and formation of primer dimers and lower concentrations affect the PCR efficiency (GRUNENWALD, 2003).

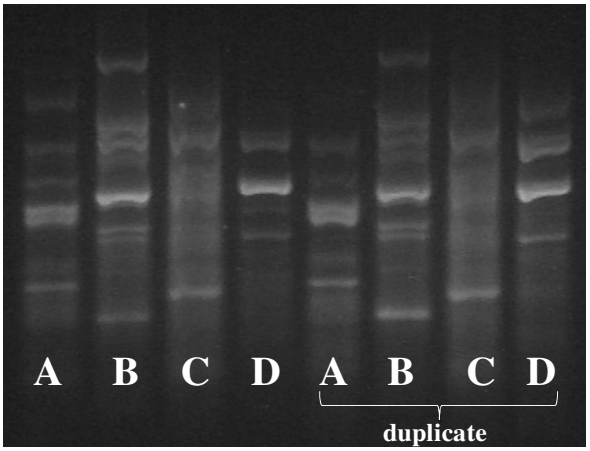

Figure 2. Optimization of primer concentration for RAPD analysis of crimson beebalm for primers OPB14. From left to right: 2 (A) - 4 (B) - 0,8 (C) - 1,2 (D) μmol.dm-^{3} of primer concentration

In our study, the final concentration of each primer was found to be optimal as 0,4 μmol.dm-3 for all tested primers (table 2).

Table 2. Changes in the number of detected RAPD bands according to the concentration of primer.

Primer name	Number of bands for appropriate primer concentration in PCR			
	0,8 μmol.dm^{-3}	1,2 μmol.dm^{-3}	2 μmol.dm^{-3}	4 μmol.dm^{-3}
OPB2	3	6	8	11
OPB4	5	9	10	13
OPB10	4	6	6	7
OPB12	3	7	8	10
OPB14	2	4	6	9

The quality of amplified PCR products corelates directly with the concentration of $MgCl_2$ as the Taq DNA polymerase co-factor. Magnesium concentration changes effect is is reported widely (KUTA *et al.,* 2005; SAHASRABUDHE and DEODAR, 2010; SKORIČ *et al*., 2012) for RAPD technique. As affecting the DNA polymerase activity and fidelity, primer annealing and the basic

PCR specifity following concentrations were tested for $MgCl_2$: 1,5; 2; 2,5 and 3 mmol.dm^{-3} $MgCl_2$. For crimsom beebalm RAPD analysis, concentration of $MgCl_2$ of 3 mmol.dm^{-3} was optimal what is in concordance with polymorphism analysis practice where $MgCl_2$ concentrations higher than 1 mmol.dm^{-3} are sufficient for plant genomic DNA in length polymorphism techniques. The same optimal concentration as reported here was found to be optimal for RAPD analysis of *Echinaceae purpurea*, L. Moench by VURAL et DAGERI (2009).

Another optimized factor of RAPD analysis of *Monarda dydima*, L. was the concentration of dNTPs. In total, three different concentrations were tested: 0,1; 0,2 and 0,3 mmol.dm^{-3}. Generally, the concentration of dNTPs out of optimal affect the yield and specifity of PCR amplification (GRUNENWALD, 2003). Concentration of 0,1 mmol.dm^{-3} dNTPs was found as optimal in this study for crimson beebalm. The same concentration was used in RAPD analysis of West African fonio (KUTA *et al.,* 2005).

Next, various annealing temperatures (figure 3) were checked for RAPD analysis of *Monarda dydima*, L. In literature, two quite different annealing temperature approaches are reported. Most often annealing temperature range from 34°C up to the 38°C is reported for RAPD (KUTA *et al.,* 2005; SARASHABUDHE and DEODAR, 2010). In our analysis, 36°C was found as to be optimal for RAPD analysis of crimson beebalm (table 3). But, annealing temperatures varying from 41°C up to the 50°C are reported as well (SOMPONG, 2004; FERNANDEZ, 2002). Such as increasing of annealing temperature is usually used together with addivives such as Triton-X100 or gelatin for impoving of specifity or yield of RAPD.

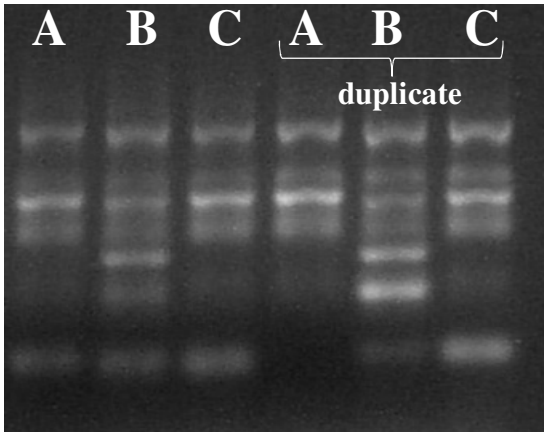

Figure 3. Optimization of primer annealing temperature for RAPD analysis of crimson beebalm for primer OPB10. From left to right: 35°C (A) - 36°C- (B) - 37°C (C).

Another type of additive such as TBT-PAR is used when the quality of extracted DNA is not optimal (SAMARAKOON *et al.,* 2013), special for recalcitrant, woody or secondary metabolite rich plants. For *Monarda dydima*, L., in spite of its chemical composition (MAZZA *et al.,* 1993;

TABORSKÝ *et al.,* 2012), CTAB based extraction yielded in DNA of suitable quality as well as quantity.

Table 3. Optimization of RAPD parameters

RAPD parameter	Analysed range	Optimum value
template DNA	10 – 100 ng	50 ng
primer concentration	0,8 – 4 μmol.dm-^{3}	4 μmol.dm-^{3}
$MgCl_2$ concentration	1,5 – 3 mmol.dm-^{3}	3 mmol.dm-^{3}
dNTPs concentration	0,1 – 0,3 mmol.dm-^{3}	0,1 mmol.dm-^{3}
annealing temperature	35 – 37 °C	36 °C

Finally, optimized RAPD protocol was tested for reproducibility of banding pattern for two different types of crimson beebalm micropropagated samples - variant growing on basal MS medium (MURASHIGE and SKOOG, 1962) and variant growing on MS medium with the addition of BAP in the concentration of 0,5 mg/L. For both of them, reproducibility and the same quality of banding patterns were confirmed (figure 4).

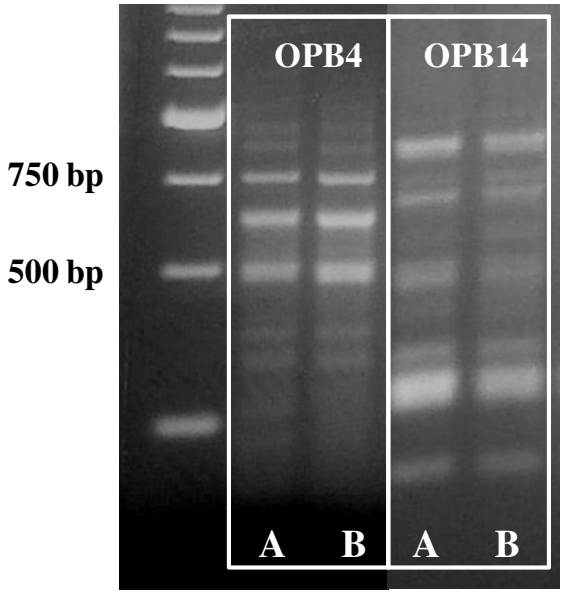

Figure 4. RAPD profile of crimson beebalm for decamers OPB4 and OPB14. A - basal MS medium; B - MS medium with the addition of BAP.

As RAPD works in nonstringent conditions, it is very sensitive to changes of every individual PCR parameter and lack of reproducibility is one of its disadvantages. Different consequences of failing of RAPD reproducibility are reported in literature (COLAGAR *et al.,* 2010; SKORIČ *et al.,* 2012; HUO *et al*., 2013), but all of them can be avoided by standardization of RAPD parameters, such as reported here.

CONCLUSION

In the experiment presented here, identical RAPD profiles were obtained for *Monarda dydima*, L. random marker based analysis using a following range of RAPD parameters: 1U of Taq polymerase; 3 mmol.dm-3 $MgCl_2$; 0,1 mmol.dm-3 dNTPs; 4 μmol.dm-3 primer and 50 ng of template DNA in PCR. This standardized protocol enables RAPD analysis by well defined and scorable amplified PCR products. This can be used as a rapid molecular based tool for different aims related to genetic diversity analysis of crimson beebalm without any prior knowledge about its genome specifities.

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STANDARDIZACIJA I REPRODUKTIVNOST ANALIZA ZASNOVANIH NA SLUČAJNO ODABRANIM MARKERIMA NA BILJKAMA DOBIJENIM MIKROPROPAGACIJOM MONARDE (*Monarda dydima*, L)

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Izvod

Genetičke analize stabilnosti biljnog materijala dobijenog mikropropagacijom zahteva efektivne i reproduktivne metode. Prikazani su rezultati optimizacije protokola za PCR uslove u korišćenju RAPD markera u cilju razvoja brze, pouzdane i reproduktivne metode za ispitivanje genetičke stabilnosti *Monarda dydima,* jer za neke vrste nisu dostupni specifični DNK markeri. U tom slučaju mogu da se koriste standardizovani i optimalni protokoli. Dobijeni rezultati su među prvima kod *Monarda dydima,*L. U sandardizovanim PCR uslovima korišćeni prajmeri su dali reproduktivne i različite rezultate identifikacije za kulture *crimson beebalm* dobijene mikropropagacijom i pogodne su za ispitivanja genetčke varijabilnosti ove lekovite biljke.

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