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Analysis of Somaclonal Variation in *Cinchona succirubra* Using Random Amplified Polymorphic DNA (RAPD)

Analisis Variasi Somaklonal pada *Cinchona succirubra* Menggunakan Random Amplified Polymorphic DNA (RAPD)

Titin PURNANINGSIH[1)]
[1)]Department of Biology, Palangka Raya University, Indonesia
Email : titinpurnaningsih@yahoo.co.id

Abstract. Regenerants of *C. succirubra* were examined by RAPD analysis to determine the occurrence and extent of somaclonal variation. Plantlets were regenerated by nodal culture. DNA was extracted from the parent plant and regenerants of *C. succirubra*. Fifteen base synthetic oligonucleotides from Operon were chosen that gave multiple polymerase chain reaction (PCR) amplification products using cinchona DNA from parent plants grown in the field and plantlets after 4 and 5 passages or 4 and 5 month old in vitro culture. The results showed that all of morphological characters on regenerated plants were clonally uniform and all of the RAPD profiles were generally monomorphic, stable, and similar to parent plants. These results demonstrated that RAPD can be used successfully to determine the somaclonal variation among regenerated plants.

Key words: Somaclonal Variation, *C. succirubra*, RAPD

Abstrak. Tanaman hasil regenerasi *C. succirubra* diuji dengan analisis RAPD untuk menentukan terjadinya variasi somaklonal. Tanaman anakan diregenerasi dengan menggunakan kultur nodul. DNA diekstraksi dari tanaman induk dan regeneran dari *C. succirubra*. Lima puluh basa oligonukleotida sintetik dari Operon dipilih yang memberikan hasil amplifikasi polymerase chain reaction (PCR) menggunakan DNA kina dari tanaman induk yang tumbuh di lapangan dan anakan tanaman setelah 4 dan 5 subkultur atau 4 dan 5 bulan dalam kultur *in vitro*. Hasil penelitian menunjukkan bahwa semua karakter morfologi pada tanaman regenerasi adalah seragam secara klonal dan semua profil RAPD umumnya monomorfik, stabil, dan mirip dengan cirri-ciri tanaman induknya. Hasil ini menunjukkan bahwa RAPD dapat digunakan untuk menentukan variasi somaklonal diantara tanaman-tanaman turunanya.

Kata kunci: Variasi Somaklonal, *C. succirubra*, RAPD

INTRODUCTION

Cinchona succirubra is one of very important pharmaceutical plants used in the medicine. Cinchona contains quinolin as the source of pharmaceutical compounds for malaria and cardiac arrhythmic disease. Cinchona can be widely applied in the industry as bitter agent and military industry. Plant regeneration from *in vitro* culture very important as a source of material for quinolin and has long been recognized as an efficient tool for rapid clonal multiplication. Regeneration of plants through tissue culture technique will produce the clones that are phenotypically and genetically identical to the material from which the explants were originally derived. However, in some cases namely the effect of growth regulator added to the media and the culture period may cause deviation from the parent type. According to Larkin & Scowcroft (1981) this phenomenon was called somaclonal variation, which was sometimes inherited.

Several types of somaclonal variation happened *in vitro* culture have been reported i.e. the variety in the number of chromosomes (Karp, 1991) or the number of gene copy (Brettel *et al*., 1986; Zheng *et al*., 1987), DNA mutation (Muller *et al*., 1990), transpositional changes (Peschke & Phillips, 1991), change in the structure and amplification of mitochondrial genome

(Shizadegan *et al*., 1991) and the changes in the structure of chloroplast DNA (Dunford & Walden, 1991).

So far, the somaclonal variation in Cinchona plantlets derived from tissue culture can be recognized when they have grown in the field. Some strategies can be used to detect the genetic variability of the plantlets that derived from *in vitro* tissue culture i.e. cytology analysis, isoenzym analysis, and DNA analysis with Restriction Fragment Length Polymorphism (RFLP). However, some of them have limitation because they need a lot of plant tissue, a long period and high cost of analysis. Polymerase Chain Reaction (PCR) with short primer which forms oligonucleotide, as the molecular marker of the Random Amplified Polymorphis DNA (RAPD), can be used to identify the plant species in a short period and relatively cheap (Williams *et al*., 1990)

The advantages of RAPD technique namely are a number of samples can be analyzed economically and quickly, the material used for analysis is only a little, the DNA printing is very specific which forms independent ontogeny expression. Besides, all genomes can be analyzed using unlimited number of markers. This technique can be used also to test the somaclonal variation on culture callus of *Prunus persica* (Hashmi *et al*., 1997) and somaclonal variation on *Oryza sativa* var. indica (Godwin *et al*., 1997), and analysis of the genetic stability of plantlet *Populus deltoids* derived from micropropagation (Rani *et al*., 1995).

In this research, we confirmed the somaclonal variation of regenerated plants using RAPD. We have developed a method for micro-propagation of *Cinchona succirubra* through formation buds from nodal culture. In pursuing the objective, we applied RAPD technique to analyze of somaclonal variation in regenerated plantlets.

METHODS

Plant Material. The seeds of *C*. *succirubra* were originated from the Indonesian Tea and Cinchona Research Institute, Gambung. The mature seed washed with tap water, surface sterilized with Tween 80% for 10 min, then 70% EtOH for 30 s and finally thoroughly washed twice with sterilized water. The seedling was incubated on MS Medium without plant growth regulator.

Tissue Culture. The explants were taken from nodes from *in vitro* germinated seedling (2 segments each) and every explant was cultured in MS medium supplemented with 5 mg/l BAP. The cultures were incubated at temperature of 25 ± 2^{o}C and irradiance of 1500 lux (cool, white fluorescent lamps) in 16 h photoperiod for 24 weeks. The cultures were subcultured at 4-week intervals to fresh medium for buds initiation and multiplication. The plantlets were analyzed to know somaclonal variation and were taken from 5 subcultured.

DNA Isolation. DNA was extracted from leaves of regenerated plants (4 passages and 5 passages) and field grown mother plants of *C. succirubra* according to the method of Orozco-Castillo *et al.* (1994) with some modification by adding antioxidant polyvinylpolypirrolidone (PVPP) during the sample was grinded, and adding mercaptoethanol into the extract buffer. 0,4 g of fresh leaves for each sample of regenerated plants were ground to a powder in liquid nitrogen and 0,2% PVPP using a mortar and pestle. The powder tissue was transferred into 1.5 ml sterile microcentrifuge tube containing preheated (65^{o}C) extraction buffer. The extraction buffer consisted of 4% (w/v) CTAB supplemented with 50 µL mercapto-ethanol.

DNA purification was conducted by using mixture of chloroform: isoamyl alcohol (24: 1v/v). The emulsion was centrifuged at 10,000 g for 10 minutes, The aqueous phase was transferred to another tube and 1/10 volume of ammonium acetate 3 M (ph 5.2) and 2x volume of cold absolute ethanol was added, and then it was centrifuged at 25,000 g for 15 minutes. The precipitate was rinsed with ethanol 70% and resuspended in 200 TE (10 m Tris, 10 EDTA, pH 8.0). The DNA concentration was determined using spectrophotometer UV and electrophoresis agarose 1.4 %.

PCR Reaction. Random decamer primers (Operon Technologies, Alameda, California), OPH-19 (CTGACCAGCC), OPC-10 (TGTCTGGGTG), OPC-08 (TGGACCGGTG),

OPA-04 (AATCGGGCTG), SC10-20 (ACTCGTAGCC) were used for PCR amplification to produce polymorphism. Amplification by PCR was performed in 25 L reaction mixture containing 200 M dNTPs (1 : 1 : 1: 1) mixture of dATP, dCTP, dGTP, and dTTP, 10 pMol Primer, 2.5 mM MgCl2 and 50 ng DNA sample in 1 x PCR buffer and 1 unit Taq polymerase. Mineral oil was added into the Ependdorf tube to prevent evaporation during the amplification reaction. The amplification of DNA was conducted using thermal cycler, Thermolyne I Amplitrone for 45 cycles consisting of 1 minute at 94^{o}C (denaturation), 1 minute at 37^{o}C (annealing), and 2 minutes at 72^{o}C (extension) with a final extension step at 72^{o}C for 4 minutes.

The Products of amplification was separated using electrophoresis 1.4% agarose gel containing ethidium bromide 0.5 gm/l as the dying. The separation of the sample with electrophoresis was run at 50 V for 3 hours, while 1 kb Ladder DNA was used as the molecule weight marker to determine the fragment size of the amplification products. The products of DNA amplification was visualized using UV transluminator (312 nm) and documented with Polaroid camera.

The genetic stability of regenerated plantlets of *Cinchona succirubra* was determined by comparing the DNA band of the mother plant as the source of the explant with the DNA band of plantlets of fourth and fifth subculture.

RESULTS AND DISCUSSION

Effect of BAP to Buds Multiplication. Plant regeneration was obtained when the regenerated buds had developed after 4th week in MS medium with the addition of 5 mg/l BAP. The growth regulator 5 mg/l BAP affected to buds formation significantly and the effect of BAP concentration are very significant at 12th week (Table 1).

Table 1. Effect of BAP on Bud Multiplication of *Cinchona succirubra*

Treatment of BAP (mg/l)	Number of Bud		
	4th week	8th week	12th week
5	2	11,8	41,0

New multiple buds developed on this medium grew vigorously and elongated to 1 - 2 cm within 1 month of culture. The new buds were continuously formed, and the buds were subcultured for multiplication every 4 weeks. The results showed that the multiplication rates of *C. succirubra* were not significantly different, i.e. 7 - 9 shoots/4 weeks. The observation on the morphology of plantlets after 5 passages or 5 month old *in vitro* culture did not show signs of morphological alterations. Five month old micro propagated plants appeared morphologically uniform with leaf form, shape, and growth patterns (Figure 1).

Figure 1. Buds multiplication of *Cinchona succirubra*

RAPD Analysis of Regenerated Plants. The five primers was used on the genetic analysis of regenerated plants. The variation of monomorphic bands in micropropagated plants by using different primers has been reported earlier (Rout and Das, 2002). The result showed that the primers OPA-04, OPC-08, OPC-10, OPH-19, and SC10-20 produced amplification which monomorphic across all regenerated plants and the mother plant (Figure 2). The size of the monomorphic DNA fragments, ranged from 200 – 2300 pb. This indicated that the genetic stability of *C. succirubra* through tissue culture technique was stable until 5th passage or 5 month *old in vitro* culture. The plants have normal phenotype and genotype and as the as their mother plants.

Shenoy and Vasil (1992) reported that micropropagation through meristem culture is generally associated with low risk of genetic instability because the organized meristems are generally more resistant to genetic changes that might occur during cell division. The DNA amplification products, which represent one allele per locus, could be resulted from changes in either sequence of the primer binding sites or changes which alter the size and the occurrence of the successful amplification of DNA target. In this research, the amplified products exhibited monomorphisms among all the *in vitro* regenerated plants and were similar to those from mother plant.

The RAPD technique is simple and the results are reproducible, because only micro-amounts of material are necessary. This approach can be used to assess tissue at several stages of *in vitro culture*. Furthermore, the genome is most probably randomly sampled without the influence of ontogeny. It can be concluded that in commercial scale the RAPD technique is very ideal to analyze genetic variation of *C. succirubra* plantlets in which there is no abnormality on Cinchona plants produced through tissue culture technique. This technique was widely used to detect the somaclonal variation on various plants.

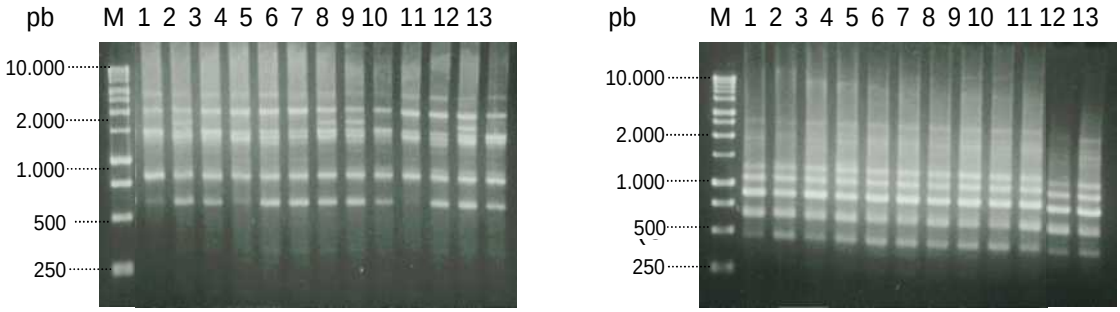

Figure 2. RAPD patterns was obatined from *Cinchona succirubra* DNA with primers OPC-10 and OPH-19 (M) 1 kb DNA Ladder, (1) mother plant, (2-7) regenerated plants from fourthth culture,(8-13) regenerated plants from fiveth culture

CONCLUSIONS

RAPD technique is very suitable for analysis somaclonal variation of *C. succirubra* plants produced through tissue culture. The genetic variability of the plants can be maintained until 5 passages or 5 month old *in vitro* culture.

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