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GENETIC STABILITY ANALYSIS OF REGENERATED PLANTS OF *Cinchona ledgeriana* BY RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

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ABSTRACT

Long-term maintenance of plant cell in tissue culture is known to induce somaclonal variation in regenerated plants. Clones of *C. ledgeriana* were micropropagated using nodal culture. The objective of this research was to analyze the genetic stability of C. ledgeriana plantlets from several passages with their parent plants as source of explants by random amplified polymorphic DNA (RAPD). Five arbitrary decamers were used to amplify genomic DNA from *in vitro* and *in vivo* plant material. All of RAPD profiles from regenerated plants after 4 - 5 passage or 4 – 5 month old *in vitro* culture were monomorphic and similar to mother plants. It was indicated that up to five times of subcultures or 5 month in culture genetically plantlets were exactly the same as the mother plant. This result showed that clonal propagation of *C. ledgeriana* up to five times passages or 5 month in culture produce stable genetic integrity of plantlets.

***Keywords**: Genetic stability, RAPD, Tissue culture, Cinchona ledgeriana*

1. INTRODUCTION

Cinchona ledgeriana 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 regeneratiuon 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 regulators added to the media and the culture periode may cause deviation from the parent type. This phenomenon was called somaclonal variation[4)] .

There were some strategy can be used to determine genetic stability of the plantlets derived from tissue culture i.e. cytology analysis, isoenzym analysis, DNA analysis such as Restriction Fragmen Length Polymorphism (RFLP) and Random-amplified polymorphic DNA (RAPD). The advantages of RAPD technique are a number of samples can be analyzed economically and quickly, the genomes can be analyzed using unlimited number of markers. RAPD analysis is often preferred because of reduced complexity and the one of methods that can partly overcome this limitation and be easily adopted to detect the variations in plant material at DNA level during all culture and growth stages. RAPD has been used for a number of crop species to detect genetic diversity among micropropagated plants[1,5,9)] , the genetic stability of regenerated plants from protoplast[11)].

In this research, we confirmed the genetic stability of regenerated plants using RAPD. We have developed a method for micropropagation of *Cinchona ledgeriana*. through formation buds from nodal culture. In pursuing objective, we applied RAPD technique to investigate genetic stability of regenerated plantlets.

2. MATERIALS AND METHODS

Plant Material. The seeds of *C ledgeriana* 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[7)].

Tissue Culture. The explants were taken from nodes from *in vitro* germinated seedling (2 segments each) and explant was cultured in MS medium supplemented with 5 mg/l BAP. The cultures were incubated at temperature of 25±2°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 initiation and multiplication buds. The plantlets was analysed to know genetic stability and was taken from 4 and 5 subcultured.

RAPD Analysis. DNA was extracted from leaves of regenerated plants (every subcultured; 4, 8, 12, 16, 20 passages) and field grown mother plants of *C. ledgeriana* according to the method of Orozco-Castillo[8)] 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 mercaptoethanol.

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 %.

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, 10pMol 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 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 gml-1 as the dying. The separation of the sample with electrophoresis was run at 50 V for 3 hours, while DNA Ladder 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 ledgeriana* 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 fiveth subculture (4 and 5 month)

3. RESULTS AND DISCUSSION

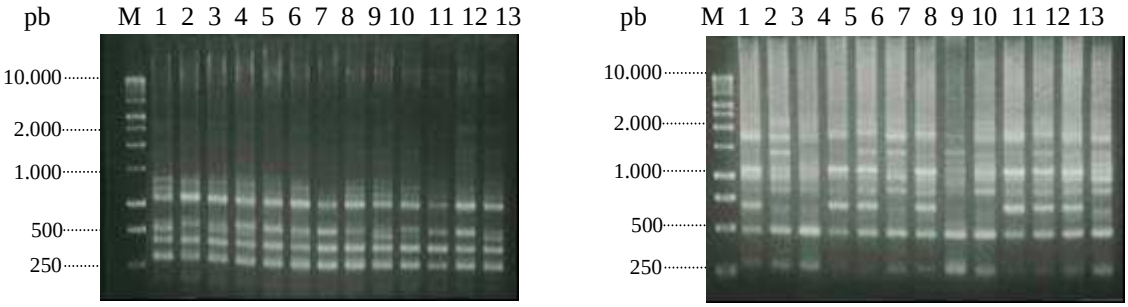
3.1. Cinchona propagation through *in vitro* technique

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 was usually cytokinin which added into the medium and had functioned for multiplication and to accelerate the multiplication. New multiple buds developed on this medium grew vigorously and elongated to 1 – 2 cm within 1 months of culture. New buds were continuosly formed, and every 3-4 weeks the buds were subcultured for multiplication. The results showed that the multiplication rates of *C. ledgeriana* were not significantly different, i.e. 7-9 shoots/4weeks. The observation on the morphology of the plantlets after 4-5 passage or 4-5 month old in vitro culture did not show signs of morphological alterations. Fiveth month old micropropagated plants appeared morphologically uniform with normal leaf form, shape, and growth patterns (Figure 1).

Figure 1. Buds multiplication *C. ledgeriana*

3.2. RAPD Analysis of Regenerated Plants

The five primers (OPA-04, OPC-08, OPC-10, OPH-19, and SC10-20) was used on the analysis genetic stability of regenerated plants. The size of the monomorphic DNA fragmens, ranged from 200 – 2300 pb with 4 – 9 DNA bands. Some primers produced clear DNA bands and the other had unclear DNA bands. The result showed that the all of primers produced monomorphic DNA band, both in the mother plant as the explant source and in the plantlets after 4 and 5 passages or 4-5 month old *in vitro* culture (Figure 2). This indicated that the genetic stability of *C. ledgeriana* through tissue



(A) (B)

Figure 2. RAPD patterns was obtained from *Cinchona ledgeriana* DNA with primers OPA-04 and OPC-08. (M) 1 kb DNA Ladder, (1) mother plant, (2-7) regenerated plants from fourth subculture, (8-13) regenerated plants from fifth subculture.

culture technique was stable until 5 passage or 5 month old *in vitro* culture. The plants have normal phenotype and genotype and same with mother plant. There are several factors as explants source, duration of culture and growth regulators influencing the variation in the culture condition[3].

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[10]. The DNA amplification products, which represent one allele per locus, could result from changes in either sequence of the primer binding sites or changes which alter the size and present the successful amplification of target DNA. In this research, the amplified products exhibited monomorphisms among all the *in vitro* regenerated plants and were similar to those from mother plant.

The use of 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. Futhermore, the genome is most propably randomly sampled without the influence of ontogeny. It can ce concluded that in commercial scale the RAPD technique is very ideal to analyze the genetic stability of *C ledgeriana* plantlets that there will be no abnormality on Cinchona plants produced through tissue culture technique. This technique was widely used to detect the somaclonal variation on various plants. Detected somaclonal variation on the regenerant plantlets of beet produced through tissue culture using RAPD technique[6] and identified somaclonal variants among peach regenerants by RAPD[2].

4. CONCLUSIONS

RAPD technique is very suitable for analysing the genetic stability of *C. ledgeriana* plants produced through tissue culture technique and until 5 passages or 5 month old *in vitro* culture the genetic stability of *C. ledgeriana* can be maintained.

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