The changes of DNA sequence in callus, cell agregates and cell suspension of Catharanthus roseus in the prolonged subculture in bioreactor

by Parluhutan Siahaan 20

Submission date: 28-Dec-2020 05:27AM (UTC+0700)
Submission ID: 1481547357
File name: The_changes_of_DNA_sequence_in_callus,_cell.pdf (1.49M)
Word count: 6651
Character count: 34422

ISSN: 1811-9506

B R

Bioscience Research

An international quarterly journal of ISISnet

Innovative

Informative

Excelling

Worldwide

ISISnet©

Volume : 16 No. 2
May: 2019

Editorial board

TOTAL ON BOARD EDITORS = 22

Editors (22)

Field Chief Editor (s)

Dr. Arruje Hameed
Assistant Professor (Biochemistry)
Department of Biochemistry, Government College University Faisalabad, **Pakistan**
Interests: Biological chemistry, nano-biotechnology, Plant Biology, abiotic stresses, environmental Biology, antioxidants, diabetes,
Special Issues and Collections in ISISnet journals
Website

Managing editor

Zahida Parveen Malik
In-HOUSE Editorial Incharge
Innovative Scientific Information & Services network. Email: meditorbr@yahoo.co.uk

Specialty Chief Editor (s)

Dr. Wafaa Choumane*
Professor,
Head of the Department of Fundamental Sciences
Consultant in biotechnology at ICARDA in Aleppo, **Syria**
Faculty of Agriculture, Tishreen University,
P.O.Box 2099, Lattakia, Syria ,
Website:
Interests:
Special Issues and Collections in ISISnet journals
*Biotechnology

Available online freely at www.isisn.org

Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2019 16(2): 2389-2401. OPEN ACCESS

The changes of DNA sequence in callus, cell agregates and cell suspension of *Catharanthus roseus* in the prolonged subculture in bioreactor

Dingse Pandiangan[1*], Beivy Kolondam[1], Parluhutan Siahaan[1], Nelson Nainggolan[2] and Fenny Dwivany[3]

[1]Department of Biology Mathematics and Science Faculty of Sam Ratulangi University Manado 95115, **Indonesia.**
[2]Department of Mathematics Sam Ratulangi University Manado 95115, **Indonesia.**
[3]Institut Teknologi Bandung, **Indonesia**.

***Correspondence:** dingsepan@unsrat.ac.id **Accepted:** 04 May 2019 **Published online:** 30 June 2019

The objective of the research is to obtain scientific information regarding the effects of prolonged subculture that resulted in changes in DNA sequences by *matK* Barcode DNA methods. The steps of study are include a callus, aggregate and suspension cell culture, DNA extraction, DNA isolation, PCR and identification by electrophoresis. Geneous software v5.6 was used for data analysing changes in DNA chromatogram and editing the DNA sequencing results. New callus induction, new aggregate and new cell subculture are still stable DNA sequences until subcultured 8 month to 1 year. Callus and cells culture prolonged culturing for 3 years, experience changes in DNA that are very different from *C. roseus* leaves and callus induction results. The similarity of *matK* gene between cell or callus subculture for 3 years reached 93.28% with all other samples after BOLD system analysis. Meanwhile, other samples (new induction callus, new aggregate, cell suspension) whose culture period is less than 3 years still show similarity with 100% with leaves explan leaves. The alignment of all DNA sequences of the *matK* gene from leaf explants, calluses, cells and aggregates and cells or callus subcultured for 3 years from *C.roseus* showed differences in 33 nukleotides. The results of DNA sequencing with *matK* barcode showed the presence of very large genetic variation changes from the initial leaves *C. roseus*. The DNA sequence that changed *matK* gene is the nucleotid sequence 109th to 233rd, which is about 33 nucleotides. This difference indicates a nucleotide change or change in DNA. The changes of DNA sequence occured in callus and cell suspension of *Catharanthus roseus* in the prolonged subculture about 3 years.

Keywords: Barcode *matK*, Prolonged Culture, Genetic Variation, DNA, *C. roseus*

INTRODUCTION

The Madagascar periwinkle (*Catharanthus roseus* (L) G. Don), is characterized by the large variety of monoterpenoid indole alkaloids that it produces (Faccini and DiCosmo 1994). Among these many structures, vinblastine and vincristine are of particular importance because of their wide use in cancer chemotherapy. These alkaloids are produced *in vitro* by the condensation of vindoline and catharanthine (Zhao et al., 2001). The pharmaceutical values of these dimeric alkaloids, their low abundance, and their cost of production have prompted extensive efforts to generate cost efficient high-yielding methods.

This study is the part of the biotechnology research to found the new anticancer compounds by utilizing secondary metabolites of plants, such as catharanthine in *Catharanthus roseus* (L.) G. Don (Edress 1994, Faccini and DiCosmo 1994). The advantages of biotechnology technique is that

it can control cell growth and regulate of metabolic processes automatically for increased productivity (Endress et al., 1994, Verpoorte et al., 2000). There has been much previous research which examined how much catharanthine content in the plant tissue culture, starting from callus up to the aggregate level and cell suspension (Zhao et al., 200, Wilken et al., 2005, El Sayed et al., 2004, Pandiangan et al. 2006 and 2008). Besides that, there have been improvements of various methods such as the addition of NAA growth regulators, precursor tryptophan, elicitation and immobilization (Pandiangan et al., 2008). There were encouraging results to produce catharanthine by using the result of the previous research (Pandiangan et al., 2008).

The study was repeated with the same treatment, it showed a different catharanthine content even if the same treatment was given to another experiment, except the subculture callus resources for the aggregates and cell suspension cultures that are different (Pandiangan et al., 2010 and 2011). Research about callus stock from the same explant has different subcultures ranging from 4th to 16th subculture, or about the age of the callus stock from 3 months to 13 months (Pandiangan et al., 2008, 2010, 2011). The question that appears is how the catharanthine change could happen at the same condition where the culture was at the same bioreactor but the metabolite content are different (Pandiangan et al., 2015). This condition raises doubts of the researches to produces catharanthine on a large scale in the bioreactor or before the causes of the difference was known.

Based on the condition that has already been explained above, another analysis of the protein changing and the activity of enzymes involved in the synthesis catharanthine includes enzyme tryptophan decarboxylase (TDC) which showed changes in activity and protein profile (Pandiangan et al., 2011). The TDC is the mayor enzyme in the biosynthesis of catharanthine through the change of triptophan to triptamine (Gaines, 2004, El Sayed et al., 2004). The change of growth, protein profile, TDC activity and cell morphology treated tryptophan (Pandiangan et al. 2011), related with the catharanthine content and another secundary metabolite (Pandiangan et al., 2015). The change of protein profile and TDC activity and catharanthine content shows the possibility of a change in DNA. However, research is still needed to prove it.

DNA *Barcode* is a method that is commonly used to identify a species or cultivars (Bruni et al., 2012). This initiates a study on biodiversity and the identification of offspring, kinship, and strengthens forensic analysis (Kress et al., 2005). The main purpose of DNA *Barcode matK* was not for building a phylogenetic tree, but for providing a quick and accurate identification of an organism using the DNA barcode which has been recorded in the data genebank sequences. Ideally, *barcode* connect the method of changed DNA identification which is convincing because it has a variety of sequence enough between the species and intraspecific low variation (Kress and Erickson, 2007). This method may be more effective for identification and analyzing of change DNA sequence in callus, cell aggregate and cell suspension culture.

There are difficulties in the selection of a specific gene to becoming a plant barcode. This is due to mithochondial genes of plants which are very slow to evolve. The research of the DNA plant *Barcode matK* focused on the gene in chloroplast genome (Kolondam et al., 2012), and few of the proposed candidates such as *accD*, *atpF-atpH*, *matK*, *nhdJ*, *psbK-psbI*, *rbcL*, *rpoB*, *rpoCl*, and *trnH-psbA* (Hollingsworth et al., 2011). From the gene candidates for the barcodes, *matK* was the most promising one for becoming DNA barcode. *MatK* gene has a length of approximately 1570 base pairs, coding maturase protein, contained in the chloroplast genome, and in many cases reduced maternal. *MatK* gene had the right size, faster improvement about three times faster than *rbcL* gene and *atpb*, high substitution speed, large proportion in first and second codon variations, low transition-transversion ratio, and the presence of a mutation that protects sector. The evolution speed of *matK* gene makes it useable for detecting the changing of DNA of species (Galimberti et al., 2014). The purpose of this study is to obtain the scientific information regarding the effects of prolonged subculture to change the DNA sequence used by *matK* Barcode DNA methods.

MATERIALS AND METHODS

Culture and Subculture of Callus, Aggregates and Cells Suspensions of *C. roseus*

The plant used as the source of explants in the experiment was white flower Vinca (*Catharanthus roseus* (L.) G. Don). The *C. roseus* grown in greenhouse Faculty of Agriculture Sam Ratulangi University. The leaves still actively growing at 3-4 leaves from the apex shoots were used as the explants (CRP). Callus induction used

MS solid media with 2,4-D 2 mg/L and kinetin 0.2 mg/L at the subculture I and II namely induction medium.The first callus induction namely new callus as KB sample. Callus cultures and subcultures were employed to maintain the stock callus and subcultured one time a month, which was added media and nutrients to increase the callus growth. The next callus subculture (III, IV, etc) used MS solid media too with NAA 2 mg/L, kinetin 0.2 mg/L and 150 mg/L tryptophan namely production medium (Pandiangan et al., 2006). Liquid culture (aggregate culture and suspension culture) using MS medium by using the same composition with callus production medium without using agar (Pandiangan et al., 2015). The medium of liquid culture in Erlenmeyer and bioreactor is also the same production medium.The culture was placed in sterile room with a temperature of 25°C and lighting 15 Lux for along culture (Pandiangan et al., 2011).

Callus subculture was also used as a stock which replicates research. Subculture was done by moving the callus induction results on a media that was similar to the induction media by decreasing the size of the callus to experience good growth (Pandiangan et al., 2015). The subculture was carried out continuously to multiply the callus as the source of explants on cell aggregate cultures in Bioreactor. The subculture of callus was conducted every 21 days and the callus has been subcultured for 3 year in solid medium. Aggregate cultures were carried out in a 250 mL Erlenmeyer flask. Each flask contained 25 mL of liquid MS medium with the same combination of PGR ie. NAA 2 mg/L, Kinetin 0.2 mg/L and 150 mg/L tryptophan as used for callus production. The callus was about 1 month old subcultured, collect as sampel AB and 1 years old as sample AL. The callus, aggregate and cell cultures were incubated at room temperature and agitated at a speed of 120 rpm. Subcultures were made after 14 days by replacing the old liquid medium with new liquid medium of the same nutrient composition.

Sample Collecting

Sampling is done at each stage by dividing the sample into two.Partially samples were collected for DNA analysis and partly to continue the subculture continuously for the next type of culture. Explants or source leaves of *C. roseus*, with white flowers referred to as CRP. CRP namely as control. As jungtification red flowering collecting as CRM. The callus induction as new callus or KB was collecting too. Callus subculture becoming a sample for a year is called KL collected and freeze dried. After the stage of KL, then it subcultured into a liquid medium namely as AB or cell aggregates collected and freeze dried. The stage of AB subcultured in liquid medium about four times in Erlenmeyer called old cell aggregates (AL) sample. The AL sample collected and freeze drying. Old cell aggregates (AL) continued in subcultures to form a cell suspension (SB) and samples taken. The suspension cultures subcultured continuously up to 3 years in liquid MS medium (Pandiangan et al., 2015), which is referred to as SL.

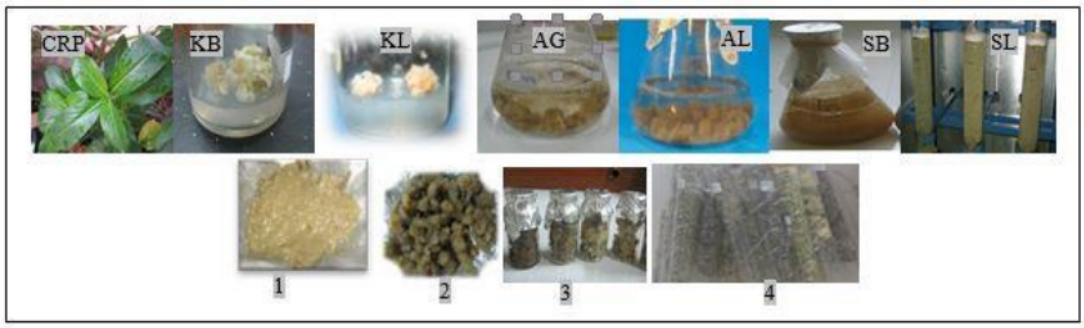


Figure 1; Samples were taken for the analysis of changes in DNA; leaf explants (CRP or CRM), callus induction or new callus (KB), 1 year subcultured Callus (KL), new Aggregate (AB), old Aggregate (AL), cell suspension cultures of new (SB), old suspense culture cells in bioreactor 1 L (SL) of *C. roseus* in prolonged culture; 3 year old cell culture bioreactor that has been harvested and analyzed (1), old culture cells Aggregate harvested from *C. roseus* (2), some samples of cell aggregates and callus in freeze drying (3), the results of callus, aggregate and *C.roseus* cells that have been dried by freze drying (4).

The aggregate and the cell suspension sample were dried from the medium by using the suction paper 3 times until no trace of fluid on the suction paper.

All of the samples taken gradually. All of sample was collected about 1 g ww, and then dried with the freeze dryer. The dried sample is stored in a sealed and labeled sample bottle and then placed in the refrigerator or freezer at 4^0C.

DNA Extraction

The DNA was extracted using a Multisource Genomic DNA Miniprep Kit (Axygen) according to the protocol already modified by Kolondam et al., (2012). The dried sample (Figure 1) about 1 mg dw, was extracted. All of sample was placed into an Eppendorf tube. All samples added 350 μL PBS and 0,9 μL Rnase A and then crushed with pestel (homogenized). It was added 20 μL Proteinase K and 150 Buffer C-L (lysis buffer), then put in the vortex for about 1 minute. It was then incubated for 1 hour at the temperature of 56˚C. Then, the sample was added into Buffer P-D (protein precipitation buffer) 350 μL, vortex for 30 seconds, then centrifuged at 12,000 x g for 10 minutes. The miniprep column that has been provided was placed in 2 mL in Eppendorf tubes. The supernatant samples were pipetted into the column, and then centrifuged at 12,000 x g for 1 minute. The filtrate in the 2 mL tube was removed and placed by Miniprep column back to its original position. W1 buffer (washing buffer) 500 μL was pipetted into the column and centrifuged at 12,000 x g for 1 minute. The miniprep colum filtrate was discarded and placed back on its original position, then 700 μL Buffer W2. Buffer W2 was pipetted into the column and centrifuged (This step is repeated 2 times). The filtrate in 2 mL tube was removed and the Miniprep column was placed back to its original position and then centrifuged. The Miniprep column was transferred to a new tube, 1.5 mL Eppendorf tubes. The DNA was eluted by adding 100 μL of deionized water in the central part of the membrane and centrifuging. The total DNA capable of being detained was as many as 20 μg silica membrane. Then final concentration was about 0.2 μg/μL.

***MAtK* Gene Amplification by PCR Technique**

Primar for *matK* genes used were *matK*-3F (5'-CGT ACA GTA CTT TTG TGT TTA CGA G-3') and *matK*-1R (5'-ACC CAG TCC ATC TGG AAA TCT TGG TTC-3'). This primers is primary universal and producted by Integrated DNA Technologies (IDT) Singapore. Primers *matK* genes were still in the powder form rehydrated with nuclease-free water. The concentration of the primary stock solution was diluted to 100 pmol/μL then in the aliquot to 10 pmol/μL. Optimal reaction conditions used the optimization of previous studies conducted by Kolondam (2012). The components were mixed ie 10 μL 5X Master Mix, 1μL forward primer, 1μL reverse primer, 3 μL DNA template, and 35μL deionized water. The conditions of the end of each 50 μL PCR reaction were 1.25 units Taq DNA polymerase, 0.2 mM each dNTPs, 1.5 mM MgCl2, 0.2 mM of each primer and approximately 0.6 μg of DNA samples. Setting the temperature for the PCR machine started with an initial denaturation at 95˚C for 2 min followed by 35 cycles of denaturation stage, adhesion primer and DNA extension. The temperature and time were set consecutively to 95˚C for 30 seconds, 52ºC for 30 seconds, and 72˚C for 1 minute.

DNA Electrophoresis

Agarose of 0.75 grams in 50 mL TBE buffer (Tris-Boric EDTA) 1X boiled to obtain 1% agarose gel to be used. Then the agarose gel is printed together with comb to make wells. After hardening, the gel is placed in an electrophoresis device and flooded with a 1X TBE Buffer. A 5 µL PCR product containing loading dye was piped directly into agarose gel wells. The approximate size of the DNA band in agarose gel is used 1 kb Ladder DNA The Ladder DNA of 5 µL is piped into the gel well. Electrical voltage is given through 100 volts electrophoresis for 30 minutes and visualized using UV-Transilluminator. Documentation is done using digital cameras and UV light filters. The remaining PCR product is shipped with the primary pair for the sequencing process. All processes including PCR clean up (DNA extraction of the gel) are performed by the sequencing service provider.

Data Analysis

The DNA sequencing results were edited using Geneious software v5.6 (Drummond et al., 2012). The 30 first DNA sequences were trimmed and nucleotides erroneous readings were corrected based on the level of accuracy legible. Reversed process was then performed to complement the results of sequencing using reverse primer, which was then combined with the forward primer sequencing results using MUSCLE (Multiple Sequence Comparison by Log-Expectation) integrated into Geneious. The accuracy of the amplification of target genes was

tested by predicting the amino acid sequence based on the *matK* sequence. The goal was to see where the stop codon sequences amid the active genes in order to know for sure that the amplified gene was not apparent (pseudogene), located in another part of the cell. Sequences that had been tested had changed in any forma t FASTA (fast alignment) for comparison. DNA and amino acid sequences were lined up alongside each other to see the uniqueness, including alleged insertions / deletions and point mutations resulted in amino acid sequence changes. DNA Sequences of genes *matK* in FASTA format each sample were identified from BOLD (Barcode of Life Database) Systems (www.boldsystems.org). The result matches (hit) produces table describing 7 samples had a score of coverage and the percentage of the highest sequence similarity. The juxtaposition of the sample was sequenced with the search results displayed to illustrate the difference and the gap between the two DNA sequences. Then, the DNA sequences *matK C. roseus* consensus with DNA sequences in the sample were lined up and changes in DNA were seen. Then the nucleotide change table is arranged in the observation table.

RESULTS AND DISCUSSION

The Growth of *Catharanthus roseus* Callus, Aggregates and Cells

The qualitative observations of callus, aggregates and cells from culture results can be seen in Figure 2. This Figure only shows observation on day harvested. The cell growth could also be seen from the color change of cell aggregates. Well grown cells tend to have flaxen color to yellowish, while the color of those not well grown will be yellow to brown. Callus cultures given 150 mg / L tryptophan grow well. But the cultures of aggregates and suspensions given tryptophan there is a difference from visual observation. The tryptophan treatment appears to be yellow (T) browned than without tryptophan (K) in Fig. 2.

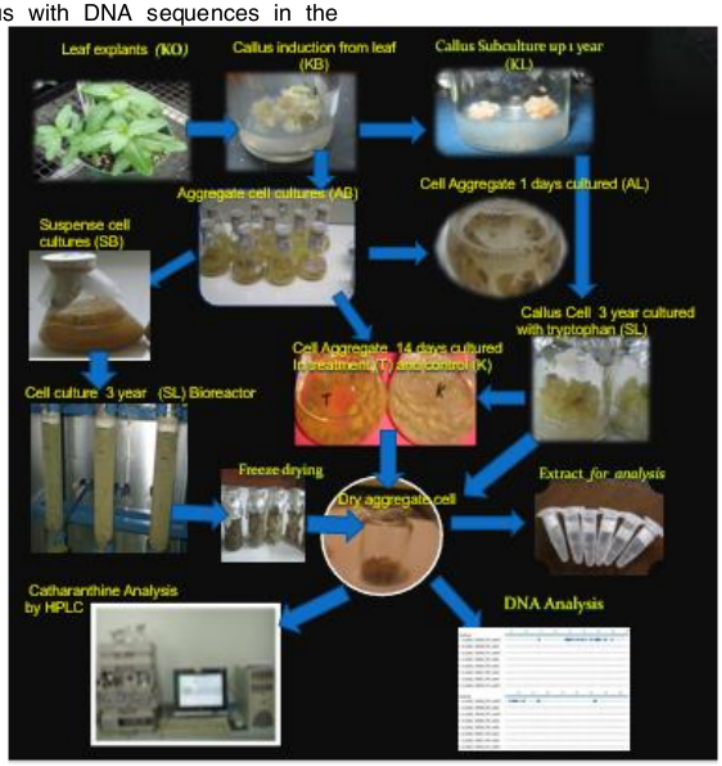


Figure 2; The appearance of the calus, aggregates and cell *C. roseus* culture results during the research process.

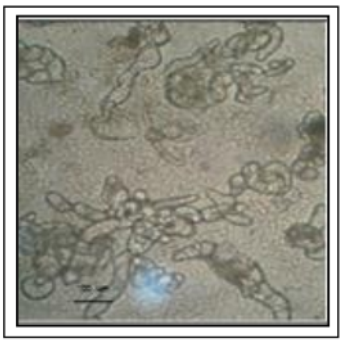
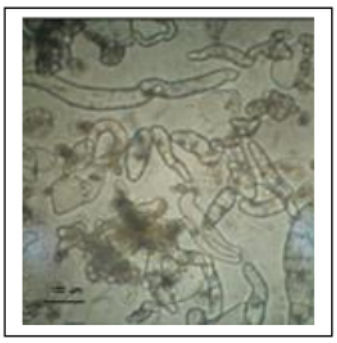
Control (0 mg/L tryptophan) 3 years subculture	Tryptophan 150 mg/L 3 years subculture
The spherical cell shape (small cell) up to 53% in control medium (no treatment) in bioreactor	The length cell shape obout 75% after tryptophan added in bioreactor
	
Cell ratio (length/width) 1,5 to 5	Cell ratio (length/width) 1,5 to 12
The cell color: white-blackish	The cell color: gray-white yellowish

Figure 3; The cell morphological observation *C. roseus* cell suspention that measured day 14th after treated with tryptophan. 0 mg/L tryptophan with small round and small cell shape and 150 mg/L with cell shape lengthwise and large.

The morphological microscopic observation of the cells produced in cell aggregate cultures also showed a significant effect of tryptophan treatment. The effect was in the cell specialization. The results can be seen in Figure 3. The morphology of the cells in the control group was dominated by spherical cells (the length: width ratio is small), while the number of long cells in the tryptophan treatment groups higher than control. The length: width ratio could reach 1.5 to12. The morphology of long cells in medium with added with 150 mg/L tryptophan seems to relate with the activity of Tryptophan Decarboxylase (TDC), protein content, and catharanthine content. Thomas et al., (1995) reported that TDC can changed tryptophan to tryptamine or alkaloid in *C. roeus*. This reinforces the results of research on the determination of cataharanthine content in repetition of research showed a real change. Through this analysis also suspected changes in cathanthine content because there is a change in DNA that leads to changes in functional proteins and enzymes that play a role including encim involved in cathanthine synthesis (Dahap et al., 2006).

The Result of Analysis DNA Calli, Aggregates and Cells *C. roseus*

The fragment *matK* gene was successfully amplified from all samples using universal primers (Figure 4). Strong bands were observed by agarose gel electrophoresis. Amplified fragments varies in length from 887 bp to 908 bp (alignment not shown). There were 50 bp of primer sequences trimmed from both end of the fragments prior to identification using BOLD Systems.

Sequencing of amplified fragment showed high-quality results. The HQ% chromotogram values read in Geneious v5.6 show values 88% to 90.5% (Table 1). The CRP, SL cells, SB sample has 977 bp of the matK sequence. The AL, AB, KB sample has 903 bp of the matK sequence. The KL, CRM, SL callus sample has 864 bp of the matK sequence (Table 1).

The analyzed samples were 9 kinds (Table 1). The concentration and purity of the isolated DNA can be observed (data not shown). Isolates DNA callus, and cell aggregates have the highest purity (R> 1.70). Furthermore, they were selected for amplified using PCR. The purity of the isolated DNA was used to determine the success of PCR amplification (Hillis et al. 1996).

This study used two primers: primer 1 (*matK*-3F) with a DNA sequence 5'-CGT ACA GTA CTT TTG TGT TTA CGA G-3' and primer 2 *matK*-1R (*matK*2) with a DNA sequence 5'-ACC CAG TCC ATC TGG AAA TCT TGG TTC-3'. *matK* DNA gene extraction samples and the applification

process by PCR, all produced results shown in gure 4. The universal primers *matK* included *matK*-3F-R and *matK*-1R-F attached to the gene *matK* on all sample resulting banding pattern clearly visible. Based on the size of the DNA Ladder (1K), it appears that all the samples are in the size between 853 bp and 977 bp (Figure 5).

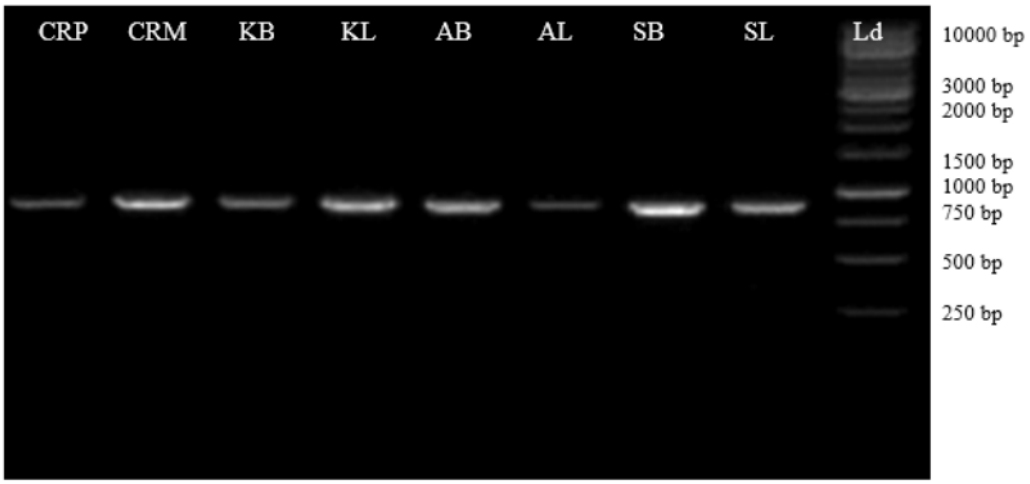


Figure 4; Electrophoresis PCR Product in 1 % agarose gel as visualization of results amplified gene fragment *matK* of callus samples, aggregates, cells *Catharanthus roseus* subcultured prolonged in the bioreactor. Description Ld: DNA ladder (MBI 100 bp DNA ladder plus), leaf explants were white-flowered *C. roseus* (CRP or KO), callus or new callus induction (KB), Long Calli about 1 year subculture (KL), a new Aggregate (AB), Long Aggregate (AL), a new suspension culture cells (SB), cell culture long suspense in bioreactor 1 L (SL) of *C. roseus* in prolonged culture.

Table 1; Identity DNA sequencing results which have been amplified with the primers *matK*

Sample Name	HQ %	Sequence	Post-trimmed	Topology	
CRP	88.0%	977	977	Linear	DNA
SL cells	88.0%	977	977	Linear	DNA
SB	88.0%	977	977	Linear	DNA
AL	82.2%	903	903	Linear	DNA
AB	82.2%	903	903	Linear	DNA
KB	82.2%	903	903	Linear	DNA
KL	90.5%	864	864	Linear	DNA
CRM	90.5%	864	864	Linear	DNA
SL Callus	90.5%	864	864	Linear	DNA

Note: Leaf explants were white-flowered *C. roseus* (CRP), callus or new callus induction (KB), Long Calli about 1 year subculture (KL), a new Aggregate (AB) , Long Aggregate (AL), a new suspension culture cells (SB), cell culture long suspense in bioreactor 1 L (SL) of *C. roseus* in prolonged culture.

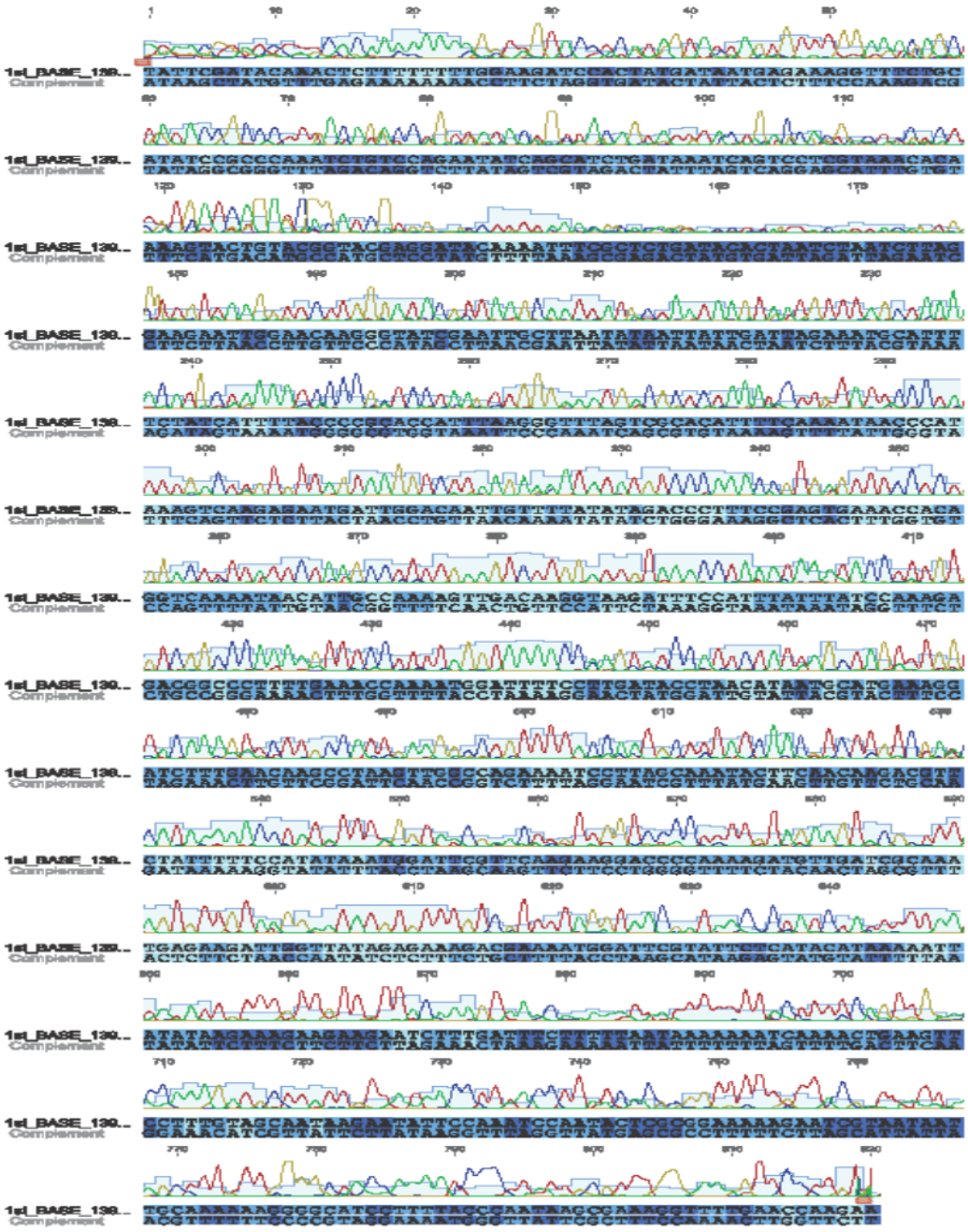


Figure 5; Sample SL DNA sequence chromatograms (callus and cells of *C. roseus* 37eady survived in three years subculture on MS medium with 2 38mes, the first subculture by 2.4 D 2 mg/L kinetin and 0.2 mg/L and then sub-cultured with NAA 2 mg/L kinetin and 0.2 mg/L).

The one of chromatogram sample that changing with DNA sequencing others is shown at Figure 5. Sequencing the form of chromatogram read within Geneious v5.6 for all of the samples were of high quality with HQ% value of approximately 88-90% (Table 1). The average length of all DNA samples is about 864 bp (Table 1). The juxtaposition of all the DNA sequences of gene *matK* samples used Multalin software (Corpet 1988). The results of the juxtaposition that SL sample chromatogram of DNA sequence showed differed from all the other sample chromatogram (Figure 6). The differences of DNA sequence SL sample with other samples at 109th to 233th sequences, can be seen in Figure 6. In that sequence nucleotide changes were discovered. The nucleotides that changed were about 33 nucleotide bases (Table 2). The SL samples (cells that are already subcultured prolonged for 3 years) are very different from the sample KB (leaf *C. roseus*). However, the KB sample (new callus induction) is not different from or similar to the sample of CRP (leaf *C. roseus* white flowers) and CRM (leaf *C. roseus* red flower).

Variations occurred in the SL DNA sequence. The gene similarity matrix barcode *matK* of callus, and cell aggregates *C. roseus* prolonged subculture up 3 years (Table 2). The results of sequencing *matK* DNA barcode gene shown are one example of the results of DNA sequencing with *matK* cell, while the results of the other samples have been closer, but have differences in certain DNA sequences. Here is the DNA sequence of gene *matK* callus cells of *C. roseus* subculture for three years in a MS solid medium labeled sample SL (sub-cultured cells in the long term). The SL samples have the following nucleotide sequence of gene barcode *matK* :

GAGGATACAAAATTTCGCTCTGATACACTA
ATCTAATCTTAGGAAGAATTGGAACAAGGGTA
TCGAATTGCTTAATATAATTATTGATTAGAAAT
GCATTTTCTATCATTTTACCCCGCACCATTTAA
GGGTTTAGTCGCACATTTTCAAAATAACCCAT
AAAGTCAAGAGAATGATTGGACAATTGTTTTAT
ATAGACCCTTTCCGAGTGAAACCACAGGTCAA
AATAACATTGCCAAAAGTTGACAAGGTAAGAT
TTCCATTTATTTATCCAAAGAGACGGCCCTTTT
GAAACCAAAATGGATTTTCCTTGATACCTAAC
ATAATGCATGAAAGGATCTTTGAACAAGCCTA
AGTTGGCCAGAAAATCCTTAGCAAATACTTCA
ACAAGACGTTCTATTTTTCCATATAAATGGATT
CGTTCAAGAAGGACCCCAAAAGATGTTGATCG
CAAATGAGAAGATTGGTTATAGAGAAAGACGA
AAATGGATTCGTATTCTCATACATAAAAATTAT
ATAAGAAAGAAGAAGAATCTTTGATTTCTTTTT
AATAAAAAAATCAAAACTGAAGTTCCTTTGTAG
CAATAAGAATATTCCAAATCCAATACTCGCGG
AAAAAGAATCGTAATAATTGCAAAAAAGGGGC
ATCCTTTTACCCAATAAGCGAAAGGTTTTGAA
CCAAGAA

DNA sequence chromatograms subcultured prolonged of C. *roseus* callus (SL), as follows in Figure 4. The DNA sequence of the DNA gene change *matK* leaves white flowers and red flowers after juxtaposed together (Table 1 and Figure 6). The changes are likely due to the callus induction and subculture in the early use 2.4-D (2,4-Dichlorophenoxiacetic acid). The 2,4-D is genotoxic or mutagenic and the mutation is heritable permanently in plants (Kumari and Vaidyanath 1989). Therefore, it is reported that its mutation was caused by aberration. Manabe et al. 1989 reported that tryptophan pyrolysis is carcinogenic. This study used precursor tryptophan, which might undergo pyrolysis during sterilization and culture. Possible changes in the gene sequence of DNA nucleotides *matK* is an indication that there are other changes in DNA associated with the synthesis catharanthine (Pandiangan et al., 2012).

The result of the juxtaposition of the entire DNA sequence of genes *matK* callus, cells and aggregates as well as leaves *C. roseus* show the difference to the DNA sequence 109th to 233rd (Figure 6). Pandiangan et al., 2010 reported that changes have occured in protein pattern by PAGE electrophoresis or occurring genetic variation and somaclonal of callus, aggregates and cell during prolonged subculture (Figure 4). The different sequences in the image are dark or dark blue. These results suggest that prolonged subculture results in differences or genetic changes or no changes in the DNA of *matK* genes (Bruni et al. 2012). This suggests the possibility of a change in the DNA sequence of other genes as well. It is possible that the genes associated and enzymes for catharanthine biosynthesis are also changing (Dahap & El Aziz, 2006). Therefore, the production of catharanthine which has experienced changes may be also caused by the duration of the cells in culture during the culture period (Hvoslef-Eide et al., 2005).

Nucleotide gene barcodes or markers that have been identified are about 33 nucleotides that change along the DNA sequence of about 853 bp to 977 bp. Therefore, it is expected that other genes may also experience changes. This changes affect the sustainability of the culture and the secondary metabolite content will change.

Table 2;Similarity analysis through matrix of genetic distance between leaf explants (CRP) new callus (KB), callus 1 year subculture (KL), new aggregate (AB), old aggregate (AL), new cell suspension culture (SB), suspension culture old cells in a 1 L (SL) biorector of *C. roseus* in 3 years prolonged cultures.

	leaf explants (CRP)	new Callus (KB)	long Calli (KL)	new Aggregate (AB)	long Aggregate (AL)	new sus pension (SB)	long 3 yr cell (SL)
leaf explants (CRP)		100%	100%	100%	100%	100%	93.2%
new Callus (KB)	100%		100%	100%	100%	99.9%	93.2%
long Calli (KL)	100%	100%		100%	100%	100%	93.2%
new Aggregate (AB)	100%	100%	100%		100%	100%	93.2%
long Aggregate (AL)	100%	100%	100%	100%		100%	93.2%
new suspension (SB)	100%	100%	100%	100%	100%		93.2%
long 3 year cell (SL)	93.2%	93.2%	93.2%	93.2%	93.2%	93.2%	

Figure 6. The difference in the *matK* DNA genes sequences 3 years subcultured callus (SL, old cell culture) and other samples. The juxtaposition of the *matK* DNA gene sequences SL, SB, AL, AB, KL, KB and CRM or CRP shows the differences in DNA sequences order 109-233 nucleotide (dark blue or dark).

Table 3; Changes in the nucleotide sequence of the *matK* gene from subcultured cells for 3 years (SL) of C. *roseus* callus and cells culture with leaves or consensus DNA sequence.

No	Sequence of Nukleotide	DNA Consensus or *Catharanthus roseus*	Callus or cell subcultured 3 years or prolonged culture
1	109	A	C
2	127	C	T
3	128	A	C
4	129	A	G
5	130	C	T
6	131	C	A
7	133	G	A
8	135	T	A
9	136	T	C
10	138	C	A
11	139	T	A
12	141	A	G
13	144	G	C
14	145	G	T
15	146	A	G
16	148	G	A
17	149	T	C
18	150	C	G
19	151	C	G
20	154	A	C
21	155	T	G
22	159	T	A
23	174	T	C
24	176	A	G
25	177	C	A
26	178	G	T
27	179	-	A
28	180	A	C
29	184	G	A
30	193	A	T
31	194	G	T
32	232	G	T
33	233	C	A

Chromatogram DNA sequence of *C.roseus* callus that has been prolonged subcultured (SL) is as in Figure 5. The sequence of DNA in the image is different from the DNA of the explant leaf *matK* gene (without subculture) and other samples. It can be seen from the results of the similarity analysis in Table 3. All the analysis samples ranging from leaf eksplan, callus induction, callus subculture, aggregate, and cells that have not 3 years subculture shows 100% equation through BOLD System analysis. The difference occurs only in a culture that has 3 years both on callus and cell culture in bioreactor. The change is probably due to the induction of callus and subculture at the start of induction and subculture II and III using 2.4- Mutagenic Dichlorophenoxide acetate and other unknown causes.

This reinforces the results of research on the determination of cataharanthine content in repetition of research showed a real change (Pandiangan et al., 2011). Through this analysis also suspected changes in catharanthine content because there is a change in DNA that leads to changes in functional proteins and enzymes that play a role including encim involved in cathanthine synthesis (Dahap and El-Azis, 2006), Van Hofste et al., 2005).

Prolonged cell culture or culture for 2 years changes the *matK* DNA gene, which is very different from *C. roseus* leaves and callus from the induction results (not in the subculture). *MatK*

DNA gene sequencing results showed enormous genetic variations and its changes from the initial samples of the white flowering *C. roseus*. The *matK* gene DNA sequence similarity of explants and callus subculture for three years has 93.28% commonality (Tabel 1). DNA sequences that are changing were the sequence order 109 to 233 bp (Figure 5), approximately 33 nucleotide changes (Table 3). *MatK* DNA gene changes indicated that there may be changes of genes and enzymes involved in the synthesis of catharanthine, which certainly affect the content of catharanthine changes during subcultures. Meanwhile, other samples (newly inducted and new aggregate) still show similarities with the explants or leaves of the white flowers and red flowers with 100% similarity. DNA changes in *C. roseus* may be related to changes in the content of catharanthine.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

The authors would like to thank the Ministry of Education and Culture of the Republic of Indonesia for funding this research through Sam Ratulangi University 2016 budget SP-DIPA No. 042.06.1.401516/2016 dated December 07, 2015.

REFERENCES

Bruni I, De Mattia F, Martellos S, Galimberti A, Savadori P, Casiraghi M, Nimis PL, Labra M. 2012. DNA barcoding as an effective tool in improving a digital plant identification system: a case study for the area of Mt. Valerio, Veste (NE Italy). PloS one, 7(9) http://dx.doi.org/10.1371/journal.pone.00432 56

Corpet F. 1988. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res16:10881-10890. http://dx.doi.org/10.1093/nar/16.22.10881

Dahab TAMA and El-Azis NGA, 2006. Physiological effect of diphenylamin and tryptophan on the growth and chemical constituents of *Philodendron erubescens* plants. *World* Journal of Agricultural Sciences 2 (1): 75-81

Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Duran C, Field M, Heled J, Kearse M, Markowitz S, Moir R, Stones-Havas S, Sturrock S, Thierer T, dan Wilson A. 2012. Geneious v5.6. Biomatters. New Zealand.

El-Sayed M, Choi YH, Frederich M, Roytrakul S, Verporte R. 2004. Alkaloid accumulation in *Catharanthus roseus* cell suspension cultures fed with stemmadenine. *Biotechnology Letters* 26: 793-798.

Endress, R. 1994. Plant Cell Biotecnology. Springer-Verlag Berlin Heidelberg. New York. p.67.

Facchini PJ and DiCosmo F. 1991. Secondary metabolite biosynthesis in cultured cells of *Catharanthus roseus* (L.) G. Don immobilized by adhesion to glass fibres. Appl Microbiol Biotechnol. 35:382–392.

Gaines, J. 2004. Increasing alkaloid production from *Catharanthus roseus* suspension through methyl jasmonate elisitation. Parmaceutical Engineering 24:24-35.

Galimberti A, Labra M, Sandionigi A, Bruno A, Mezzasalma V, and De Mattia F. 2014. DNA Barcoding for Minor Crops and Food ceability. *Advances in Agriculture*. http://dx.doi.org/10.1155/2014/831875

Hillis DM, Moritz C, Mable BK. 1996. Molecular systematics (2nd ed.) Sinauer Associates, Sunderland, MA

Hollingsworth, PM, Graham SW, dan Little DP. 2011. Choosing and using a plant DNA barcode. *PLoSONE* (6): e19254.

Hvoslef-Eide AK, Olsen OAS, Lyngved R, Munster C and Eyerdahl P. 2005. Bioreactor design for propagation of somatic embryos. In: Hvoslef-Eide AK and Preil W (Eds) Liquid Culture Systems For In Vitro Plant Propagation. Springer Netherlands. pp.41-60.

Kolondam BJ, Lengkong E, Polii-Mandang J, Pinaria A, Runtunuwu A. 2012. DNA Barcode of Payus Limondok Orchid (*Phaius tancarvilleae*) Based on the rbcL and *matK* genes. JURNAL BIOSLOGOS 2(2): 55-62 (In Indonesian)

Kolondam BJ, Lengkong E, Polii-Mandang J, Pinaria A, Runtunuwu, S. 2012. DNA

Barcode of Payus Limondok Orchid (*Phaius tancarvilleae)* Based on the rbcL and *matK* genes. Journal Bioslogos 2(2): 55-62 (in Indonesia)

Kress WJ, dan Erickson DI. 2007. A Two-Locus Global DNA Barcode for Land Plants: The Coding *rbcL* Gene Complements the Non-Coding *trnH-psbA* Spacer Region. PLoSONE (6): e508.

Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, dan Janzen DH. 2005. Use of DNA Barcodes to Identify Flowering Plants. Proceedings of the National Academy Sciences of the United States of America. 102(23): 8369-8373.

Kumari TS and Vaidyanath K. 1989. Testing of genotoxic effects of 2,4-dichlorophenoxyacetic acid (2,4-D) using multiple genetic assay systems of plants. *Mutat Res.* 226 (4): 235-238.

Manabe, S , Uchino, E , Wada, O. 1989. Carcinogenic tryptophan pyrolysis products in airborne particles and rain water. Mutation Research Letters 226(4): 215-221

Pandiangan D, Esyanti RR, Lestiany V and Wulansari W. 2008. Production of Catharanthine in *Catharanthus roseus* Aggregate Cell Cultures by Feeding, Elicitation and Immobilization Method," In: Proceedings of the International Conference on Mathematics and Natural Sciences (ICMNS), Bandung, Indonesia, 2008, pp. 379-386.

Pandiangan D, Rompas D, Aritonang H, Esyanti RR and Marwani E. 2006. The Influence of Tryptophan on Growth and Catharanthine Content in *C. roseus* Callus Culture," Jurnal Matematika dan Sains11: 111-118 (in Indonesian).

Pandiangan D, Tilaar W and Nainggolan N. 2012. The Correlation of Cell Specialization with IAA Content of *Catharanthus roseus* Cell Culture by the Addition of Tryptophan. Eugenia, vol. 18, pp. 140-149 (in Indonesian).

Pandiangan D, Tilaar W, Karyono, Esyanti RR and Subarnas A. 2010. The Effect of Tryptophan on Growth, Protein Content and TDC Activity of *Catharanthus roseus* (L) G. Don Cell Aggregate Culture in the *Airlift* Bioreactor. International Conference on Mathematics and Natural Sciences Proceedings. November 23-25, 2010 Bandung-Indonesia.

Pandiangan D, Tilaar W, Karyono, Esyanti RR, and Subarnas A. 2011. The Growth, Protein Content and TDC Activity Responses of *Catharanthus roseus* (L) G. Don Cell Aggregate Culture that Feeding Tryptophan. Bionatura (13):16-25 (in Indonesian).

Pandiangan D, Tilaar W, Nainggolan N, Wahyudi L. 2015. Relations between Catharanthine Content Enhancement with the Other Associated Secondary Metabolites in *Catharanthus Rose* Cell Culture that Treated Tryptophan. International Journal of Science and Research (IJSR) 4 (11): 2208-2212

Rijhwani SK and Shanks JV. 2008. Effect of subculture cycle on growth and indole alkaloid production by *Catharanthus roseus* hairy root cultures. Enzyme and Microbial Technology 22: 606–611.

Thomas_JC, Adams DC, Nessler CL, Brown JK, and Bohnert HJ. 1995. Tryptophan Decarboxylase, Reproduction of the Tryptamine, and Whitefly. Plant Physiol. 109: 71 7-720

Verpoorte R, van der Heijden R and Memelink J. 2000. Engineering the plant cell factory for secondary metabolite production. Transgenic Research 9: 323–343.

Wilken D, Gonzalez EJ, Hohe A, Jordan M, Kosky RG, Hirschmann GS and Gerth A. 2005. Comparison of secondary plant metabolite production in cell suspension, callus culture and temporary immersion system. In: Hvoslef-Eide AK and Preil W (Eds) Liquid Culture Systems For In Vitro Plant Propagation. pp.525-538. Springer Netherlands.

Zhao J, Hu Q, Guo YQ, Zhu WH. 2001. Effects of stress factors, bioregulators, and synthetic precursors on indole alkaloid production in compact callus clusters cultures of *Catharanthus roseus*. Appl Microbiol Biotechnol. 55(6):693-698.

The changes of DNA sequence in callus, cell agregates and cell suspension of Catharanthus roseus in the prolonged subculture in bioreactor

ORIGINALITY REPORT

20%	19%	14%	%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	www.isisn.org Internet Source	2%
2	docplayer.net Internet Source	1%
3	link.springer.com Internet Source	1%
4	www.plantphysiol.org Internet Source	1%
5	repository.unair.ac.id Internet Source	1%
6	pdfs.semanticscholar.org Internet Source	1%
7	smujo.id Internet Source	1%
8	www.researchgate.net Internet Source	1%
	journal.ipb.ac.id	

9	Internet Source	1 %
10	"Cannabis sativa L. - Botany and Biotechnology", Springer Science and Business Media LLC, 2017 Publication	1 %
11	academic.oup.com Internet Source	1 %
12	revista.ibp.co.cu Internet Source	1 %
13	Guyon Francois, Vaillant Fabrice, Montet Didier. "Traceability of fruits and vegetables", Phytochemistry, 2020 Publication	<1 %
14	scielo.sld.cu Internet Source	<1 %
15	www.banglajol.info Internet Source	<1 %
16	slidelegend.com Internet Source	<1 %
17	dc.etsu.edu Internet Source	<1 %
18	www.cambridge.org Internet Source	<1 %
	innspub.net	

19	Internet Source	<1%
20	Tasiu Isah, Shahid Umar, Abdul Mujib, Maheshwar Prasad Sharma, P. E. Rajasekharan, Nadia Zafar, Arajmand Frukh. "Secondary metabolism of pharmaceuticals in the plant in vitro cultures: strategies, approaches, and limitations to achieving higher yield", Plant Cell, Tissue and Organ Culture (PCTOC), 2017 Publication	<1%
21	sith.itb.ac.id Internet Source	<1%
22	www.koreascience.or.kr Internet Source	<1%
23	www.tandfonline.com Internet Source	<1%
24	hdl.handle.net Internet Source	<1%
25	media.neliti.com Internet Source	<1%
26	library.wur.nl Internet Source	<1%
27	web.ics.purdue.edu Internet Source	<1%

28	203.130.254.138 Internet Source	<1 %
29	www.oncotarget.com Internet Source	<1 %
30	X H Wan, E H Lee, H J Koh, J Song, E K Kim, C Y Kim, J B Lee, S-Y Kim, K Yao, J H Lee. "Enhanced expression of transglutaminase 2 in anterior polar cataracts and its induction by TGF- in vitro", British Journal of Ophthalmology, 2002 Publication	<1 %
31	web.stanford.edu Internet Source	<1 %
32	www.agrobiology.ru Internet Source	<1 %
33	"Production of Plant Derived Natural Compounds through Hairy Root Culture", Springer Science and Business Media LLC, 2017 Publication	<1 %
34	orbit.dtu.dk Internet Source	<1 %
35	shodhganga.inflibnet.ac.in Internet Source	<1 %
36	sinta3.ristekdikti.go.id Internet Source	<1 %

37	academicjournals.org Internet Source	<1 %
38	Karen Kaul, Pritam S. Sabharwal. "Kinetin-induced Changes in δ-Aminolevulinic Acid Dehydratase of Tobacco Callus", Plant Physiology, 1974 Publication	<1 %
39	Steve Hisiger, Mario Jolicoeur. "Analysis of Catharanthus roseus alkaloids by HPLC", Phytochemistry Reviews, 2007 Publication	<1 %
40	repository.upi.edu Internet Source	<1 %
41	duepublico.uni-duisburg-essen.de:443 Internet Source	<1 %
42	www.sciepub.com Internet Source	<1 %
43	"Liquid Culture Systems for in vitro Plant Propagation", Springer Science and Business Media LLC, 2005 Publication	<1 %
44	H. Hasegawa, D. Tarore. " n. sp. and (Yamaguti 1935) (Nematoda Oxyuridae) collected from (Gray 1867) (Rodentia Muridae) in North Sulawesi, Indonesia ", Tropical Zoology, 1996 Publication	<1 %

45	krishikosh.egranth.ac.in Internet Source	<1%
46	B J Takaendengan, U Paputungan, R R Noor, A Adiani. "Live Weight Estimation by Chest Girth, Body Length and Body Volume Formula in Minahasa Local Horse", Media Peternakan, 2012 Publication	<1%
47	Bharat Singh, Ram Avtar Sharma. "Secondary Metabolites of Medicinal Plants", Wiley, 2020 Publication	<1%
48	repository.lppm.unila.ac.id Internet Source	<1%
49	"Medicinal Plants - Recent Advances in Research and Development", Springer Science and Business Media LLC, 2016 Publication	<1%
50	"Plant Cell and Tissue Differentiation and Secondary Metabolites", Springer Science and Business Media LLC, 2021 Publication	<1%

Exclude quotes	Off	Exclude matches	Off
Exclude bibliography	Off		