Journal of **BIOLOGICAL RESEARCHES**
Berkala Penelitian Hayati

ISSN: 08526834 | E-ISSN:2337-389X
Volume 22 | No. 1 | December | 2016

Original Article

Growth and accumulation of flavan-3-ol in *Camellia sinensis* through callus culture and suspension culture method

Sutini Sutini[1*], Susilowati Susilowati[2], M. Rasjad Indra[3], Djoko Agus Purwanto[4]
[1] Department of Agrotechnology, Faculty of Agriculture, Universitas Pembangunan Nasional
[2] Department of Chemical Engineering, Faculty of Industrial Technology, Universitas Pembangunan Nasional
[3] Department of Physiology, Faculty of Medicine, Brawijaya University
[4]Department of Pharmaceutical, Faculty of Pharmacy, Airlangga University

Abstract

This study was aimed to assess flavan-3-ol biomass in *C. sinensis* through callus cultures and suspension cultures derived from leaf explants. Callus initiation of both cultures were using Murashige and Skoog medium were enriched with plant growth regulators Naphthalene Acetic Acid 3.0 mg/L and kinetin 2.0 mg/L. The procedures in this study were: (1) callus initiation by cutting the leaves of *C. sinensis* shoots then planted on Murashige and Skoog medium that were enriched with plant growth regulators, (2) sub callus culture on fresh medium that enriched with the same growth regulators, (3) suspension culture initiation of liquid callus, (4) growth examination of callus and suspension cultures in week 12, (5) examination of qualitative-quantitative content of flavan-3-olin suspension cultures at week 4. The results show that suspension cultures contain biomass flavan-3-ol that increase in the same manner of the increase of callus age and weight

Key words: biomassa flavan-3-ol, *C. sinensis*, Murashige and Skoog, naphthalene acetic acid, kinetin.

Received: 31 August 2015 Revised: 26 January 2017 Published: 26 February 2017

Introduction

Flavan-3-ol is one of the secondary metabolites contained in *C.* sinensis, which can be utilized in various fields including medical industry. This compound is classified into phenol group which can performs anti-oxidant activity (Giri et al., 2012; Vinha et al., 2013; El-kassas 2014; Khalaf et al., 2008; Makanjuola et al., 2015). In agricultural industry, flavan-3-ol monomer in the form of catechins can be used as allelochemical (Inderjit, 2008; Castells E. 2008). It also *trimethyl xanthina* is utilized in food industries to supports food and beverage becoming functional (Sutini et al., 2011; 2014; 2016; Ferruzzi, 2012; Astri, 2009; Ivan, 2005).

Flavan-3-ol can be harvested from *C. sinensis* plant in disturbed farming area after 3-5 years of plantation with special treatments (Ryo, 2009; Asri, 2010). *In vitro* culture method has been used widely for various purpose such as secondary metabolites production, obtaining identic clone of *C. sinensis*, obtaining Jatropha that is resistant to drought, and obtaining sugar cane with abundant yield (Sumaryono et al., 2005; Sandal et al., 2005; Mochamad et al., 2012; PTPN. 2014). Callus cultures form was crumb and varies, so it should be kept in optimum condition that corresponds to research purposes. Production of flavan-3-ol through *in vitro* culture (callus cultures and suspension) has several advantages, namely less time consuming compare to field production, more efficient (laboratory scale results that can provide industrial needs), and to free from climatic change effect. Therefore, this study was purposed to evaluate the biomass of flavan-3-ol in callus cultures and suspension cultures derived from leaf explant of *C. sinensis*.

* Corresponding Author:
Sutini
Department of Agrotechnology, Faculty of Agriculture, Universitas Pembangunan Nasional, Veteran.
East Java. Surabaya 60115, Indonesia
telp : +628123503771
e-mail : tien_basuki@yahoo.com

Methods

Initiation of Callus

Initiation of callus was done by cutting *C. sinensis* leaves then planted on Murashige and Skoog medium (MS) enriched with plant growth regulators Naphthalene Acetic Acid (NAA) and kinetin (Aljabari et al., 2014; Farzana et al., 2011; Nikolaeva et al., 2009; Orihara and Furuya, 1990). *C. sinensis* leaves were washed with running water for 30 minutes, and then dipped in mix solution of fungicide-bactericide 3% and 5% of calcium hypochlorite. The leaves then were rinsed with distilled water and soaked in ascorbic acid 3% for 15 minutes in culture tubes. Sterilization was done by soaking the leaves in 5% of sodium chlorine solution for 30 minutes then rinsed three times with sterile distilled water. Leaves were cut in sterile area for about 1 cm with forceps. Leave cuts were initiated in solid medium MS enriched with NAA 3 mg/L kinetin and 2 mg/L at laminar air flow cabinet. At last, they were stored in temperature 20-25 ^{o}C (Sutini et al., 2008, 2012).

Callus Subculture

Calluses were cut into 2-4 pieces sub-callus using forceps and needles. The sub-calluses were subsequently transferred to the fresh medium that has similar composition as initiation medium. Sub-calluses were stored at the same temperature as previous step or 20-25^{o}C (Sutini et al., 2008, 2012).

http://dx.doi.org/10.23869/bphjbr.22.1.20166
Published by © PBI East Java. Open Access www.berkalahayati.org

Suspension Culture Initiation

Suspension cultures were initiated in liquid Murashige and Skoog medium. Suspension culture was weighed around 0.5-1 g callus to put in 20 ml of liquid MS medium inside 100 ml Erlenmeyer bottle that has been added with PGR. The bottles were then shaken in rotary shaker at 100 rpm at 20-25^{o} C of temperature (Arif, 2009).

Callus and Suspension Culture Growth Monitoring

Both callus and suspension culture growths were monitored for 12 weeks. Weight of each culture was measured in the beginning of culture and in the harvesting time.

Qualitative-Quantitative Test of Flavan-3-ol Content

Both qualitative and quantitative analysis was conducted at week 4 of culture. Qualitative analysis was conducted by observing morphological characteristic of cultures using three-ocular microscope then photograph the features. Following this, the extraction of flavan 3-ol was conducted. Furthermore, this content was analyzed through *High Performance Liquid Chromatography* (HPLC) chromatogram.

Results

Callus Initiation

Callus initiation effects can be observed at 12 weeks of treatment. Physiological changes that could be observed were explants became swollen, brighter colour, curved shape, have calluses on the edge that become wider (Figure 1). Callus sub-cultures appeared at 12 weeks of plantation with 1-2cm of diameter (Figure 2). In addition, callus of suspension cultures in liquid MS medium after plantation are shown in Figure 3.

Callus Growth Examination

Callus growth was monitored from callus weight after 4-12 weeks of plantation (Table 1). Moreover, the growth of suspension culture from week 0 to week 4 after plantation can be seen in Table 2.

Table 1. Callus weight in week 4 up to week 12

Weeks	Weight (mg)
4	500
6	600
8	900
10	1700
12	1800

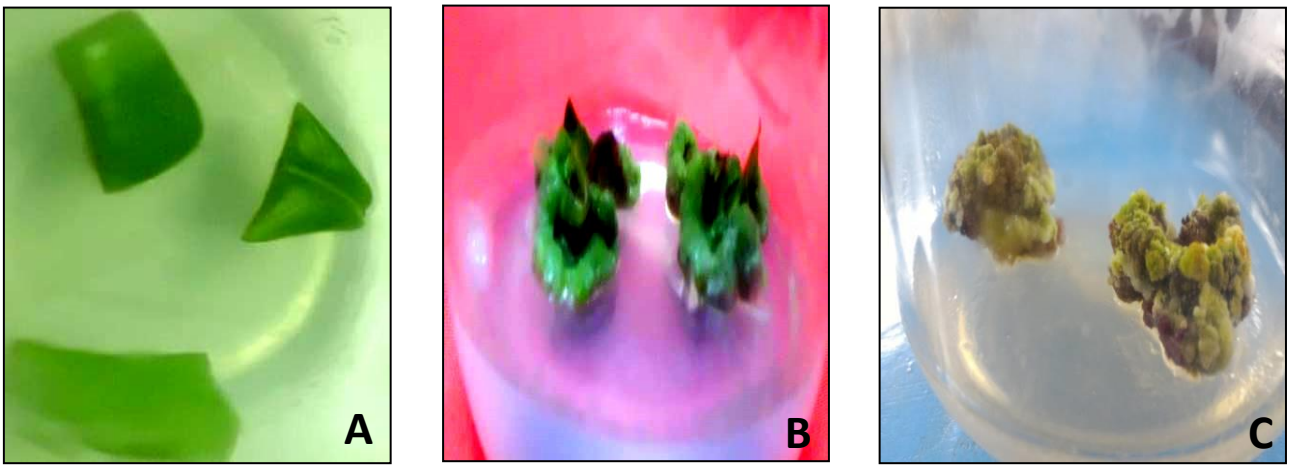

Figure 1. Explant physiological change characteristics: A. soft, swell. B. growing callus on the edge. C. compact textured callus, bars 5 mm.

Figure 2. Sub-cultured callus in week 12

Figure 3. The suspension cultures on liquid MS medium

Table 2. Suspension culture weight after plantation

Weeks	Weight (mg)
1	500
2	1100
3	1500
4	2500

Qualitative and Quantitative Analysis of Flavan-3-ol Content

Qualitative analysis of flavan-3-ol content in callus was done through stereo microscopes observation, binoculars or three occulars microscope observation were shown in Figure 4. Quantitative analysis result shows that flavan-3-ol content was 3.48 ppm in week 4 (Figure 5).

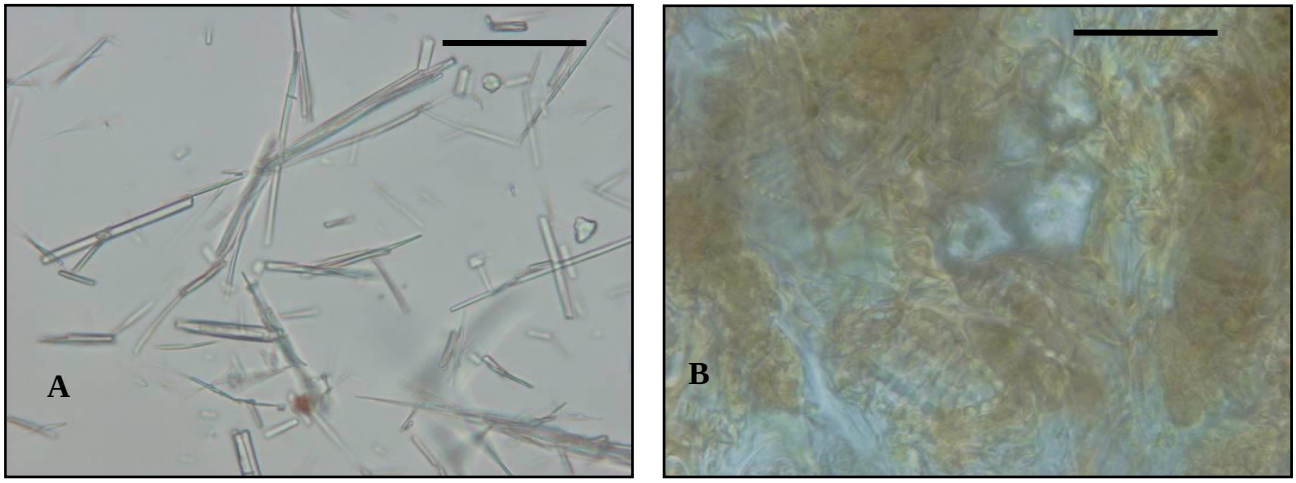

Figure 4. Flavan-3-ol content in catechin standard cells (A), flavan-3-ol callus cells (B), bars 5 mm.

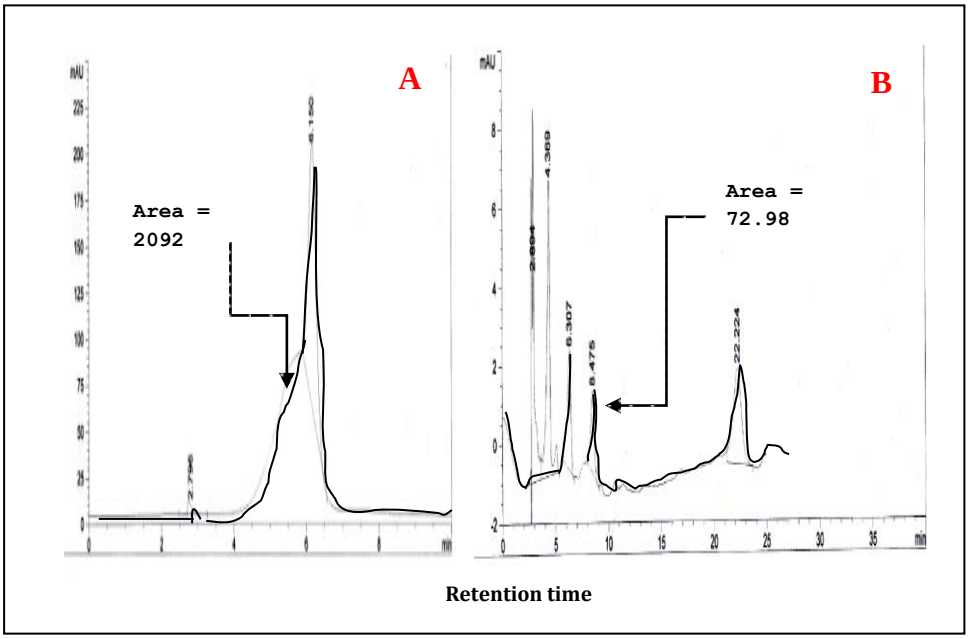

Figure 5. Chromatogram of flavan–3–ol – catechin content in *C. sinensis*: (A) flavan-3-ol-catechin standard, (B) flavan-3-ol-catechin sample.

Discussion

Callus initiation should be performed during in vitro culture as the early stage of culture. Good quality of callus resulted in good quality of culture. Callus that was produced at the initial stage will free from contamination or browning. Response of injured tissues due to nutrition reduction and NAA addition are shown in Figure 1. This response is relevant to the research by Maharik et al. (2009) and Semiarti et al. (2014) that described the addition of NAA and 2,4-dichlorophenoxyacetic acid / 2,4-D can promote callus growth. Chavan et al. (2013) reported that Initiation on *Ceropegia panchganiensis* by adding 2,4-dichlorophenoxyacetic acid, 9 M could induct 95% of callus. Initiation of callus on in vitro culture of use of plant growth regulators, as well as the number of concentrations used and the origin of the use of plant eksplan influence the direction of research data research purposes as follows.

Verbenacea callus initiation by Soumahoro et al. (2015) using NAA 1 µM has obtained callus by 56%. Furthermore, Bhagya et al. (2013) study on *Justicia gendarussa* Burm f. with addition of NAA 1 mg/L initiates callus growth while somatic embryos was produced with

addition of NAA 0.1 mg/ L. Callus subculture is a stage in which cell multiplication happens in culture process. The replacement of growth medium with the fresh one will provide more nutrients that are required to grow. Sheena, et al. (2015) use the method in the proliferation stage of sub-culture, so the discoloration process of callus from row yellowish-green to brown and from compact callus and friable can be clearly observed. Calluses were sub-culturred to be kept for further study. Research conducted by Rahayu et al. (2003) explained that MS medium enriched with PGR kinetin and 2,4-D has successfully increased *Acalypha indica* callus mass. In accordance with research by Petel et al. (2014), BAP inducted 3-4 the growth of *Caralluma edulis* sprout .

The cells of suspension culture were separated in liquid medium to form cell aggregates. The aggregates are useful for metabolytes production. As research conducted by Maria et al. (2013), tea suspension culture in the medium with shicimic acid addition can directly produce polyphenols. Likewise, Hariprasth et al. (2015) stated that sub-culture can accelerate the process bioactive compound isolation from culture.

Callus growth during in vitro culture is influenced by nutrients in medium, PGR addition, also external environment such as irradiation and room temperature of the culture chamber. In the same way, Srivasta and Chaturvedi (2008) described that callus growth depends on temperature, nutrition combination in the medium, and types of explant. Bidarigh and Azarpour (2013) induced tea bud growth with addition 3mg/L of PGR 6-Benzylaminopurine.

Suspension culture grows in shorter time than callus culture. In this study, suspension cultures only needed two to three weeks to be harvested. It is corelated to the research conducted by Zakiah et al. (2003) on suspension culture of *Azadirachta indica*, which the Azadirahtin metabolites can be harvested after 20 days of culture. Relevant with research by Seran et al. (2007) that shows the growth of culture suspense could be used to gain monocell embryo as genetic transformation agent.

Quantitative examination of flavan-3-ol was done through High Performance Liquid Chromatography/ HPLC methods using solvents that have been optimized in advance to prevent much loss. In accordance with this, the research conducted by Argawal and Kamal (2007) and Baranek et al. (2012) that successfully got flavonoids and naphthoquinone from in vitro culture of *Momordica charantia* and *A. euchroma* (Royle) Johnst. Research by Wei et al. (2012) shows that HPLC analysis could determine cathecin from cultivar plant albino tea plant and normal tea plant. Study conducted by Gupta (2012) revealed that flavan-3-ol quantitative analysis using HPLC obtained 15% of catechin content. It can be concluded that HPLC analysis is effective, efficient and suitable for flavan analysis

Acknowledgment

The author would like to acknowledge the Indonesian Directorate General of Higher Education, which has funded this research trough Competition Grants 2013-2015.

References

Aljabari Z., Alzeer J., and Arafeh R. 2014. Catechin detection in callus and in vitro cultures of the Eastern strawberry tree, arbutus andrachne l., an Endangered medicinal tree in palestine. *Global J Res. Med. Plants & Indigen. Med*. 3: 196-205.

Argawal M., and Kamal R. 2007. Study on flavanoid production using *in vitro* cultures of *Momordica charantia L. Indian Journal of Biotechnology*. 6:277-279.

Arif N. 2009. Plant tissue culture Theory and Applications. The graduate program of University Brawijaya, Malang. 87-93

Astri DA. 2009. The antioxidant activity and characteristics Organoleptik functional drinks green tea (*Camellia sinensis*) instant spice. Essay. Nutrition and Family Resources. Department of Agriculture. Institute of Agricultural Technology, Bogor.

Asri YP. 2010. The report intern in units Tambi tea plantation, tea PT.Perkebunan Tambi, Wonosobo (black tea production process).Essay . Agricultural Technology Department of Agriculture . Sebelas Maret University, Surakarta.

Bhagya N., Chandrashekar KR., and Karun A. 2013. Plantlet regeneration through indirect shoot organogenesis and somatic embryogenesis in Justicia gendarussa Burm. f., a medicinal plant. *J.Plant Biochemistry and Biotechnology*. 22: 474-482.

Baranek KS., Pietrosiuk A., and Naliwajski MR. 2012. Secondary Metabolism. Effect of L phenylalanine on PAL activity and production of naphthoquinone pigments in suspension cultures of Arnebia euchroma (Royle) Johnst. *J. In Vitro Cell.Dev.Biol.* 48:555-564.

Bidarigh S and Azarpour E. 2013. Study effect of ba hormone levels on length shoot in-vitro Culture of tea (*C. sinensis* l.). *Journal of Agricultural and Biological Science*. 8: 86-89.

Chavan JJ., Gaikwad, NB., and Yadav, SR. 2013. High multiplication frequency and genetic stability analysis of Ceropegia panchganiensis, a threatened ornamental plant of Western Ghats: Conservation implications. *J. Scientia Horticulturae* 161: 134-142.

Castells E. 2008. Indirect Effects of Phenolics on Plant Performance by Altering Nitrogen Cycling: Another Mechanism of Plant-Plant Negative Interactions. *Proc. Allelopathy in Sustainable Agriculture and Forestry*. 160: 137-157.

El-kassas FB., Ali, AM., and Mostafa SE. 2014. Phenolic compounds as antioxidants of some products manufactured from two cultivated Egyptian varieties of seedless grapes. *J. Annals of Agricultural Science*. 59: 195-199.

Farzana B C., Safiul A., and Hassan M. 2011. Studies with Callus Induction of *Vitex negundo*: an Aromatic Medicinal Plant. *J. Sustain. Agric. 5:* 6-14.

Ferruzzi MG., Bordenave N., and Hamaker BR. 2012. Does flavor impact function?Potential consequences of polyphenol-proteininteractions in delivery and bioactivity of flavan-3-ols from foods. J. Physiology & Behavior. 107: 591-597.

Giri L., Dhyani P., and Rawat S., 2012. In vitro production of phenolic compounds and antioxidant activity in callus suspension cultures of *Habenaria edgeworthii*: A rare Himalayan medicinal orchid. *Industrial Crops and Products*.39:1-6.

Gupta Ashray. 2012. "Extraction, Purification, Identification and Estimation of Catechins from *C. sinensis*" (Dissertation). Himachal Pradesh: Institute of Himalayan bioresource and technology Palampur.

Hariprasath L., Jegadeesh R., and Arjun P. 2015. In vitro propagation of Senecio candicans DC and comparative antioxidant properties of aqueous extracts of the in vivo plant and in vitro-derived callus. *South African Journal of Botany*. 98: 134-141.

Inderjit, Jarrod L. and Pollock. 2008. Phytotoxic Effects of (6)-Catechin *In vitro*, in Soil, and in the Field. *PLoS ONE*. 3: 25-36.

Ivan A R. 2005. Medicinal Plants *of the* World. Chemical Constituents, Traditional and Modern Medicinal Uses. Humana Press. Totowa, New Jersey. 1-27.

Khalaf NA., Ashok K., and Shakya. 2008. Antioxidant Activity of Some Common Plants. *Turk J Biol*. 32: 51-55.

Maharik N., Elgengaihi S., and Taha H. 2009. Anthocyanin production in callus Cultures of *crataegus sinaica* boiss. *International Journal Of Academic Research*. 1: 30-34.

Makanjuola SA., Enujiugha V N., and Omoba OS. 2015. Optimization and prediction of antioxidant properties of a tea- ginger extract. *J. Food Science and Technology*. 3: 443-452.

Maria JM., Nagella P., and Nagella M. 2013. Enhancement of the Productivity of Tea (*C. sinensis*) Secondary Metabolites in Cell Suspension Cultures Using Pathway Inducers. *J. Crop Sci. Biotech.* 16: 143-149.

Mochamad SF., Thomy Z., and Harnelly E. 2012. *In-Vitro* Effect of Combined Indole Butyric Acid (IBA) and Benzil Amino Purine (BAP) on the Planlet Growth of *Jatropa curcas* **L.** *Jurnal Natural l.* 12: 27-31.

Murashige T and Skoog FA. 1962. A revised medium for rapid growth bioassays with tobacco tissue cultures. *Physiol Plant.* 15: 473-497.

Nikolaeva TN., Zagoskina NV., and Zaprometov MN. 2009. Production of phenolic compounds in callus cultures of tea plant under the effect of 2,4-D and NAA. *Russ. J. Pl.Physiol*. 56: 45-49.

Orihara, Y. and Furuya, T. 1990. Production of theanine and other glutamyl derivatives by *C. sinensis* cultured cells. *Plant Cell Rep*. 9: 65-68.

Patel AK., Phulwaria M., and Rai MK. 2014. In vitro propagation and ex vitro rooting of Caralluma edulis (Edgew.) Benth & Hook. f.: An endemic and endangered edible plant species of The Thar Desert. *J. Scientia Horticulturae*. 165 : 175-180.

Rahayu B., Solichatun., and Anggarwulan**.** 2003. The effect of 2,4-dichlorophenoxyacetic acid (2,4-D) on callus growth and production flavonoid content on culture callus *Acalypha indica* L. *Biofarmasi 1*. 1:1-6.

Ryo FT. 2009. Identifikasi sistem produksi teh di PT Perkebunan Nusantara IV Kebun Bah Butong. (Undergraduate Thesis). Medan: Universitas Sumatra Utara.

Sandal I., Kumar A., and Bhattacharya A. 2005. Gradual depletion of 2,4-D in the culture medium for indirect shoot regeneration from leaf explants of *Camellia sinensis* (L.) O. Kuntze. *J. Plant Growth Regulation*. 47:121-127

Semiarti E., Purwantoro A., and Indrianto A. 2014. In Vitro Culture Of Orchids: The Roles Of Class-1 In Shoot Development. A Review. *Journal of Biological Researches*. 20: 18-27.

Sheena and Gabriel JJ. 2015. In vitro propagation of Orthosiphon stamineus Benth (Lamiaceae) an important medicinal plant using nodaland leaf explants. *J. The Pharma Innovation*. 4: 6-10

Soumahoro BA., Mongomaké K., and Kouassi KM. 2015. Effects of plant growth regulators and carbohydrates on callus induction and proliferation from leaf explant of Lippia multiflora Moldenke (Verbenacea) . *J. Agriculture and Crop Sciences*. 8: 118-127

Sumaryono, Riyadi I., and Tahardi JS. 2005. Morphological variations during the development of somatic embryos of tea (*C. sinensis* L.) *in vitro*. *J. Menara Perkebunan*. 69:46-57.

Sutini, TW., Widoretno, W., and Sumitro SB. 2008. Enhancing *flavan 3-ol* production derived from *C. sinensis l callus*. using elicitor: ion cu^{2+}. *Journal of Biological Research.* 14: 39-44.

Sutini. 2011. Production of epigallocatechingallate on *in vitro* culture of callus *C. sinensis* as a functional food candidate. *RekaPangan*. 5: 92-100.

Sutini., Indra R., Wardiyati T., and Sumitro, BS. 2012. Production methods of epigallocatechin gallate through *invitro* callus culture *C. sinensis L.* Ministry of Justice and Human Rightsof Indonesia Directorate General of Intellectual Property Rights. Director General of the patent. Tangerang.

Sutini., Susilowati., and Djoko AP. 2014. Development of the production of flavan-3-ol via cell suspension culture Camellia sinensis l To the inhibition of adipose cell differentiation. The annual report of research grants competence. University of Pembangunan Nasional "Veteran" Jawa Timur. Surabaya. 1-110.

Sutini, Susilowati, and Djoko AP. 2016. The extraction process of *trimethyl xanthina* in vitro Culture of callus *C. sinensis* With ethyl acetate solvent. *MATEC*. 58: 1-4.

Srivastava P and Chaturvedi R. 2008. In vitro androgenesis in tree species: An update and prospect for further research. *J. Biotechnology Advances*. 26: 482-491.

Seran TH., Hirimburegama K., Gunasekare. 2007. Production of embryogenic callus from leaf explants of *Camellia sinensis* (L). *J. natrn. Sci. Foundation sri lanka*. 3: 191-196.

Vinha AF., Sérgio VP., and Castro A. 2013. Comparison Between the Phytochemical and Antioxidant Properties of Plants Used in Plant Infusions for Medicinal Purposes. *Journal of Agricultural Science*. 5:11-19.

Wei K., Wang LY., and Zhou J. 2012. Comparison of catechins and purine alkaloids in albino and normal green tea cultivars (*Camellia sinensis* L.) by HPLC. *J. Food Chemistry*. 130:720-724

Zakiah Z., Marwani E., and Arbayah HR. 2003. Enhancing Azadirahtin Production in Cell Suspension Culture *Azadirachta indica* A.Juss through the addition of squalene. *Journal of Mathematics and Science*. 8: 141-146