AL-KAUNIYAH: Jurnal Biologi, 16(1), 2023, 89-97
Website: http://journal.uinjkt.ac.id/index.php/kauniyah
P-ISSN: 1978-3736, E-ISSN: 2502-6720

IN-VITRO CALLUS DEVELOPMENT AND THE BIOACTIVE COMPOUNDS OF TOMATO (*Lycopersicon esculentum* Mill.)

PERKEMBANGAN KALUS SECARA IN VITRO DAN KANDUNGAN BIOAKTIF PADA TOMAT (*Lycopersicon esculentum* Mill.)

Yulita Nurchayati*, Erma Prihastanti, Rini Budihastuti
Department of Biology, Faculty of Sciences and Mathematics, Universitas Diponegoro,
Jl. Prof. H. Rais, Tembalang Semarang 50275
**Corresponding author*: yulita.yoko@gmail.com

Received: 8 July 2021; Revised: 6 November 2021; Accepted: 23 March 2022

Abstract

Bioactive compounds in tomatoes can be produced through the development of callus culture. This study aimed to investigate callus development and observe bioactive compounds and antioxidant activities in explants and callus. The cotyledon and hypocotyl from the sprouts were induced to form callus on Murashige and Skoog (MS) medium supplemented with NAA 2.5 mg/L combined with kinetin 0.5 mg/L and 2,4 D 1 mg/L. All parts of seedling and callus were analyzed for their bioactive compounds and antioxidant activity using Spectrophotometer UV-Vis, whereas the other bioactive compounds were identified by *Gas Chromatography-Mass Spectrophotometry*. This research applied a Completely Randomized Design with sample sources of tomato sprout and callus from cotyledon and hypocotyl, with 3 replicates. The result showed that friable callus was able to be developed from both explants through the addition of NAA-Kin to MS medium. The three compounds were observed in callus and all explants. These calluses produced high antioxidant compounds from their pigments and ascorbic acid. The metabolites will be analyzed according to the perspective of their role. Major groups of compounds from GC-MS are dominated by hydrocarbons. Callus culture has a potential as the source of bioactive compounds.

Keywords: Ascorbic acid; Carotenoid; Chlorophyll; Secondary metabolites, Tissue culture

Abstrak

Senyawa bioaktif pada tomat dapat diproduksi melalui kultur kalus, Penelitian ini bertujuan untuk mengkaji perkembangan kalus dan mengobservasi jenis senyawa bioaktif serta aktivitas antioksidan pada eksplan dan kalus. Kotiledon dan hipokotil dari kecambah diinduksi membentuk kalus di dalam medium MS dengan penambahan NAA 2,5 mg/L dan kinetin 0,5 mg/L, maupun 2,4 D 1 mg/L tunggal. Semua bagian kecambah dan kalus dianalisis kandungan senyawa bioaktif dan aktivitas antioksidannya dengan menggunakan Spektrofotometer UV-Vis. Sementara itu, senyawa bioaktif lainnya diidentifikasi dengan menggunakan Kromatografi Gas-Spektrofotometer Massa. Penelitian ini dilaksanakan dengan Rancangan Acak Lengkap dengan faktor sumber eksplan : kecambah, kalus yang berasal dari kotiledon maupun dari hipokotil disertai 3 ulangan. Hasil penelitian menunjukkan bahwa kalus remah dapat berkembang dari semua bagian kecambah pada medium MS dengan penambahan NAA dan kinetin. Terdapat tiga senyawa yang diobservasi baik pada kalus maupun sumber eksplannya. Kalus tersebut menghasilkan senyawa antioksidan yang tinggi, berdasarkan dari kandungan pigmen dan dari asam askorbat. Metabolit-metabolit tersebut akan dianalisis lebih lanjut terhadap peranannya. Kelompok senyawa yang terbanyak dari hasil GC-MS didominasi oleh hidrokarbon. Kultur kalus memiliki potensi sebagai sumber senyawa bioaktif tanaman.

Kata kunci*: Asam askorbat; Karotenoid; Klorofil; Kultur jaringan; Metabolit sekunder*

Permalink/DOI: http://dx.doi.org/10.15408/kauniyah.v16i1.21565

© 2023 The Author(s). This is an open article under CC-BY-SA license (http://creativecommons.org/licenses/by-sa/4.0/)

INTRODUCTION

Natural substances contained in all types of plants are current concern. Several studies have been carried out to obtain natural substances with nutritious bioactive compounds. Natural ingredients mostly have important role for humans, for instance as dyes and perfumes, while some others are widely used by humans because of their medicinal properties (Dechaux & Boitel-Conti, 2005; Palazon et al., 2000). This includes primary and secondary metabolites (Dewick, 2002). However, collecting bioactive compounds directly from the plant requires abundant biomass, hence it is less effective in supplying raw materials for the pharmaceutical industry. Therefore, the plant tissue culture, including callus culture, is a considerable technique to solve the problem (Olusegun et al., 2012; Osman et al., 2012; Benítez-García et al., 2014).

This study will explore the component of antioxidant compounds of tomato plants using tissue culture technique. Normally, plant tissue culture is an effort of plant propagation by isolating plant parts and grow them in aseptic conditions. This technique encourages propagation of several plant species by shoot culture or somatic embryogenesis (Jabeen et al., 2005; Karuppusamy, 2009). Moreover, the technique also focuses on the ability of plant cells to perform primary and secondary metabolic processes on a nutrient medium enriched with sucrose and other organic supplements. A plant's cell is being manipulated to carry out metabolism for producing secondary metabolites through this technique (Baldi & Dixit, 2008). By applying callus culture, valuable compounds, especially for human health or food supplement, can be obtained in short time. In fact, we do not have to wait for the plant to grow in order to obtain the bioactive compounds. Marchev et al. (2014) stated that in-vitro culture could change secondary metabolisms pathway by increasing phytochemical production. Hence, this technique can be an alternative way to obtain metabolites in greater quantities than the mother plant produces. Tomato is one of vegetable-fruit which contains bioactive compounds for human health. Its activity is related to the content of carotenoid, ascorbic acid (vitamin C), dan tocopherol (Iswari & Susanti, 2016). Some bioactive compounds in tomatoes are sources of antioxidant, such as chlorophyll, carotenoids, and ascorbic acid.

Techniques to increase the production of secondary metabolites in tissue culture are usually carried out in several ways. They are consisted of a selection of culture types, culture medium engineering, and elicitation techniques (Iskandar & Iriawati, 2016; Trigiano & Gray, 2005). However, this study is the initial stage investigate callus formation from the aseptic tomato sprout. The objectives are to examine tomato callus development through the use of different hormones and observe the bioactive compounds from the best callus obtained.

MATERIALS AND METHODS

Plant Material

The seeds of tomato var Permata were collected from Central Java, Indonesia, in 2018. The seeds were sterilized in 30% of sodium hypochlorite for 5 min and then 70% ethanol for 30 sec to be further washed with sterile distilled water three times and germinated aseptically on the hormone-free MS medium for a week. The seedlings emerged were used as explant source.

Callus Induction

Each cotyledon and hypocotyl were cut into small size and planted on the Murashige and Skoog (MS) as the basal medium with the addition of 3% (w/v) sucrose and was further solidified with 0.2% (w/v) gelzan. Later, this basal medium was added with 2.5 mg/L NAA and 0.5 mg/L kinetin, also 1 mg/L 2,4 D as initiation medium. The pH was adjusted to 5.8, then it was sterilized in an autoclave (121 °C, at 1,5 atm for 15 min). Callus cultures were incubated in a growth chamber at 25 °C temperature and a 16 h photoperiod of tube lamp. The cultures were sub-cultured once by transferring callus into the fresh medium after 15 days.

Sample Harvest

Ten-day-old calluses in all treatment were harvested separately. The fresh calluses were cleaned from agar residue by filter paper, then were dried by the oven at 60 °C and subsequently be stored for further analysis.

Extraction

Extraction was done by the maceration method following soxhletation. Sample was discolored with petroleum ether solvent for 2 hours. The residue was taken, dried and macerated with 96% ethanol for 24 hours. The sample was filtered and the filtrate obtained was collected while the residue was re-extracted. The solution was evaporated and further dried in an oven at 40 °C until a concentrated extract was obtained (Jefriyanto et al., 2012).

Determination of DPPH free radical capture activity was done according to Bondet et al. (1997). As much as 0.5 mL of each sample was extracted in 200 µg/mL and later poured into a test tube to be added with 2 mL of 0.2 mM DPPH solution. A blank solution was made by putting 2 mL of 0.2 mM DPPH solution into a test tube and added with 0.5 mL ethanol. The DPPH absorbance was measured using a UV-Vis Spectrophotometer at a wavelength of 517 nm after 30 minutes incubation in the dark room. Samples were taken as samples containing antioxidants. The absorption value was calculated as a percentage of antioxidant activity by the formula.

Callus extraction was conducted by traditional maceration with ethyl alcohol and N-hexane as solutions. Two grams of dried callus were put into 20 mL ethyl alcohol. The solution was covered and kept at a constant temperature of 20 °C. Maceration was carried out for 4 days with occasional stirring. The extracts were filtered through filter paper (no. 1) and dried by a rotary evaporator. The dry residues were dissolved in 10 mL N-hexane. The hexane-phases were collected, evaporated and the residues were ready for GC-MS analysis.

Identification of Chemical Compounds

The chemical compounds of extracts were analyzed by GC-MS (Agilent GC 6890N 5975B MSD). The capillary column was the Agilent 19091S-433 model, HP-5MS 5% phenyl methyl siloxane. The oven temperature was programmed as follows: the initial temperature at 100 °C for 1.00 min, the final temperature at 300 °C for 10.0 min. The conditions of splitless front inlet mode were as follows: the initial temperature at 300 °C, the pressure was 10.45 psi for CE and 9.32 psi for WE, purge flow was 50.0 mL/min, purge time for 0.0 min, total flow of 53.8 mL/min, saver flow for 20.0 mL/min, saver time was 2.0 min, and carrier gas was Helium. The sample was dissolved in pure ethanol and injected using a split technique. Identification of components in the sample was done using Wiley9 database.

Statistical Analysis

The data obtained were further analyzed using the Analysis of Variance (ANOVA) at 95% confidence level for assessing whether a difference between treatment is statistically significant or not. The Duncan's Multiple Range Test (DMRT) was further applied to significantly different results to determine the best effect.

RESULTS

Development of Callus

The sterilized tomato seed was used as starting material for excellent growth and to obtain callus with good quality. The seed germinated well on the hormone-free MS medium after 8 days. Figure 1 shows the germinated seed and the development stage to the emergence of cotyledon, hypocotyl, and primary root. It took 4 days in tissue media and 6 days in agar medium for tomato seed to germinate, with high viability (90%).

Figure 1. The 8 day-old germinated seed of tomato in hormone-free medium

The physiological response following the use of kinetin and NAA was the root growth from the callus of the cotyledon. Both hormones could promote the differentiation stage of the callus (Figure 2). It was suggested that NAA was auxin which can stimulate adventitious root in tomato. Another response was found in cotyledon or hypocotyl on hormone-free MS medium (MS-0) due to the appearance of adventitious roots (Figure 3).

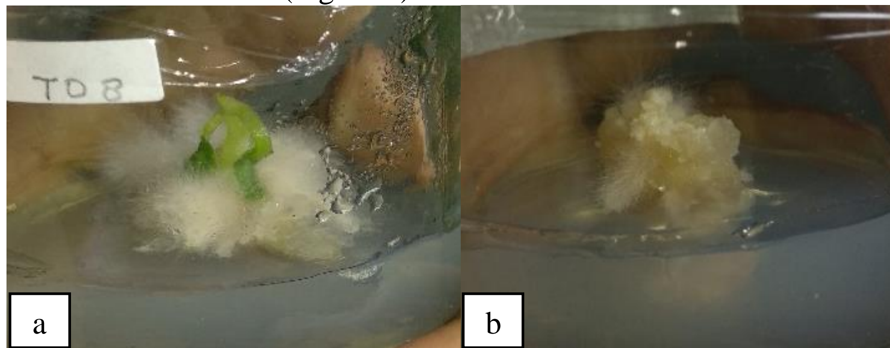

Figure 2. Development of callus from cotyledon (a) and hypocotyl (b) in MS medium supplemented with kinetin and NAA

Figure 3. Root growth from cotyledon on MS medium without hormone

All parts of the seedling including cotyledon and hypocotyl can be induced to callus. The 7-day-old seedling was still a juvenile which consisted of meristematic cells, hence cell division occurred fast (Table 1). In fact, the cotyledon served as food storage of seed that is a rich source of energy for growth and development. The hypocotyl developed into a stem, thus there was no secondary thick wall. The aseptic seedling is used as a source of explants for its benefit and high success rate in producing axenic culture since the seed is easily sterilized.

Table 1. Characteristics of tomato callus from different explants cultured on hormone-added MS medium

Kinds of explant	Kinds of hormone	Callus texture color	Type of callus	Initiation time (day)
Cotyledon	Kin + NAA	Friable, yellow fresh	Non-embryogenic	6
Hypocotyl	Kin + NAA	Friable, yellow fresh	Non-embryogenic	6.7
Cotyledon	2,4 D	Semi compact, yellow-brownish	Non-embryogenic	8
Hypocotyl	2,4 D	Semi compact, white-yellowish	Non-embryogenic	9.3

Analysis of Bioactive Compounds

This research observed pigment content such as chlorophyll and carotenoid from callus formed in Kin-NAA application. Chlorophyll is the main pigment in leaves, including in cotyledon. Moreover, the secondary pigment in leaves known as carotenoid was abundant in tomato fruits. According to this study, another bioactive successfully analyzed by spectrophotometer UV vis was ascorbic acid. To say, tomato callus culture showed the potency of producing these compounds (Table 2).

Table 2. Carotenoid, chlorophyll, ascorbic acid, and antioxidant activity in tomato sample

Samples	Carotenoid (mg/L)	Chlorophyll	Ascorbic acid (g/100)	Antioxidant activity
Cotyledon of sprout	5159.15 ± 121^{a}	0.61 ± 0.02^{a}	7.05 ± 0.1^{b}	4.74^{a}
Hypocotyl of sprout	491.22 ± 21^{b}	0.05 ± 0.005^{c}	5.51 ± 0.07^{c}	3.81^{c}
Callus from cotyledon	176.35 ± 7.7^{c}	0.07 ± 0.005^{b}	9.87 ± 0.5^{a}	3.81^{c}
Callus from hypocotyl	99.46 ± 3.5^{d}	0.07 ± 0.004^{b}	10.23 ± 0.67^{a}	4.42^{b}

Note: Numbers with different superscripts in the same column are significantly different following the Duncan's Test at a 95% confidence level

The preliminary observation of bioactive compounds found in the extract of tomato callus was represented by hexane solvent which aimed to investigate what are organic compounds made of. The results of qualitative tests with GC-MS were presented in Table 3. Two samples from young fruit and in-vitro callus were used to compare the difference of compounds between intact plant and callus.

Table 3. Organic compounds in tomato sample resulted from GC-MS analysis

Metabolite name	19 day-old fruit	Stem-derived callus
Cyclooctane (hydrocarbon)		v
Nitrocyclohexadiene (benzene)		v
Pentadecanone (ketone)		v
Dotriacontane (alkane)		v
Tetracontane (alkane)		v
Tritetracontane (alkane)		v
Cyclododecanone (hydrocarbon)	v	
Nonadecanoic acid (fatty acid)	v	
Cyclotridecanone (ketone)	v	
Methyl commate E (terpenoid)	v	
Oleic acid (fatty acid)	v	
Digitoxigenin (saponin)	v	
Beta-Patchoulene (terpenoid)	v	
Triacontane (alkane)	v	
Tetradecane (alkane)	v	v

DISCUSSION

A study on callus development was carried out on 2 types of MS medium added with different hormones. The first was the application of kinetin 0.5 mg/L combined with NAA 2.5 mg/L, and the second one was the application of 2.4 D 1 mg/L in the basal MS medium. Long-term application of kinetin and NAA may cause callus initiation from cotyledon and hypocotyl of tomato. Cotyledon was swollen on the 6 days after planting, while hypocotyl took a longer time. After subculturing, both explants responded excellently and callus emerged after 10–15 days. Both calli were friable with yellow fresh in colour (Figure 2). This callus was characteristically as non-embryogenic or proliferative callus and indicated as secondary metabolites (Hartman & Kester, 1997; Chawla, 2003). This result was similar to the brownish color of *Catharanthus roseus* callus which produced phenolic compounds (Fitriani, 2003). The use of kinetin along with the NAA also promoted the growth rate of *Pogostemon cablin* callus (Trimulyono et al., 2004) and *Datura metel* L. callus (Nurchayati et al., 2016; Iranbakhsh et al., 2007). The performance of callus growth in the single 2.4 D medium was not as good as that firstly treated with hormone. This callus was compact and yellow-brownish in color. Callogenesis requires more time to ensure proper callus development. In the 2.4-D medium without kinetin, callus growth response in explants was observed.

Moreover, the size of callus was small, hence it took more time to grow. This indicates that tomato sprout callus cannot grow well with the addition of only a single auxin without cytokine

group. According to Kala et al. (2014), the growth regulators cannot induce a callus from *C. parviflorum* leaf explants. However, the combinations of growth regulators resulted in optimum callus production. Callus formation is strongly influenced by the type and concentration of the growth regulator. Zulfiqar et al. (2009) revealed that the growth and morphogenesis of in-vitro plants are controlled by the balance and interaction of the growth regulator absorbed from the medium. Auxins play a role in stimulating the growth of explant cells; thus, auxin tends to form a callus, starting with cell division in the meristematic area. At the beginning of the growth response, auxin triggers the elongation of cells by loosening the cellulosic cell wall. This cell elongation occurs due to the response of 2.4-D, yet cell cannot divide rapidly because of no addition of kinetin. In all explant sources (cotyledons, stems, tubers, and roots), the combination of the growth regulators, 2.4-D, and kinetin was found to result in a larger callus size compared to those treated with 2.4-D only. A similar result was observed in the callogenesis of *Myrmecodia tuberose* (Sari et al., 2018).

Metabolites are only synthesized in callus, indicating that callus could use a different pathway compared to explants (leaves) and other cultures (shoots and sprouts cultures). Endress (1994) mentioned that cells from explants, those are isolated and grown in an aseptic environment, would change the genes in their DNA. The contact between explants and culture medium was obtained from heterotrophic plants, while the cells which composed explants used sucrose from MS medium as their carbon source. According to Trigiano and Gray (2005), the type of organic supplements, including sucrose and its concentration in the culture medium, greatly determined the preservation it performs.

There are other factors besides internal or genetic ones that contribute to the diversity of natural materials within a family (Veerporte & Memmelink, 2002). An aseptic environment with an artificial medium and microclimate is not similar to the intact plant, hence resulting in plant interaction with different external factors which leads to different metabolism process. This shows that in-vitro culture environment results in an increase or decrease in metabolite content, thus inducing new metabolites.

Developed callus can stimulate the synthesis of pigment and ascorbic acid. Based on the result, 30-day-old callus showed its ability to display the chloroplast pigment. In undifferentiated tissue, the living cells carried out their metabolism. Nutrients in MS medium and Kin-NAA hormone support the formation of pigments despite the limited light intensity. The formation of chlorophyll and carotenoids requires not only nutrients but also genes to synthesize pigments that require light as well (Llorente et al., 2019; Sun et al., 2018). Phytoene synthase (PSY) enzyme is an enzyme that determines carotenoid biosynthesis (Baranski & Cazzonelli, 2016; Welsch et al., 2000). Limited light in the culture incubation room has caused the formation of a different type of chromoplast from crystalline chromoplast in carrot roots, which was found to accumulate considerably high carotenoids (Oleszkiewicz et al., 2018). Carotenoids are also produced in sweet potato callus (Othman et al., 2017), while chlorophyll is produced from 60-day-old callus from leaf explants of *Rosa gallica* and *R. hybrida* (Tarrahi & Rezanejad, 2017).

Ascorbic acid is one of the secondary metabolites in plants, which is also an indicator of environmental stress. Formation of ascorbic acid in callus occurs along with the de-differentiation process which begins with callus stimulation. Rosella callus is also able to produce high amount of vitamin C through the addition of a high concentration of sucrose to the medium (Nurchayati & Rahmah, 2010). The compounds have antioxidant activity (Davey et al., 2006; Giovannoni, 2007). Ascorbic acid concentration in the callus was significantly different from that in the explants. Sprouts prioritize their primary growth over secondary growth; hence, the juvenile sprouts were not yet produced a high amount of vitamin C.

In term of GCMS profile, some are hydrocarbons dominated by alkane. Young tomato fruits contain methyl commate E, which is one of resin. This compound was found and isolated from *Commiphora glandulosa* and has antibacterial activity (Motlhanka et al., 2010). These compounds play a variety of roles. Pentadecanone, that is a ketone, is utilized as food additive, fragrance, and anticancer agent. Both samples contained different types of metabolites, indicating that different

tissue/organs in different growth areas will produce different compounds. Endress (1994) mentioned that explant's cell isolated and planted in an aseptic environment will change their gene expression. Interaction between explant and nutrient media resulted in heterotrophic character. In this study, explant's cell used sucrose in the MS medium as the carbon source. According to Trigiano and Gray (2005), organic supplement including sucrose and its concentration in the medium will determine culture metabolism.

This research showed that some metabolites were able to be produced in callus, that is plant tissue derived from organized organ. Callus represents the nature of living cells that absorb nutrients from the medium for metabolism. Calli synthesize several organic compounds to support their life.

CONCLUSION AND SUGGESTIONS

The explants from in-vitro sprouts of tomatoes are easily changed into callus through the dedifferentiation process. Callus derived from tomato sprouts becomes tissues which produce metabolites. This finding is possibly developed to increase metabolite production through modification of basal media. In-vitro tissue has different metabolites from those of intact plants, and this step provides an opportunity to further research about the diversity of organic compounds. Further, each callus derived from defined organ is necessarily analyzed for its ability in producing secondary metabolite and the result has to be compared with the intact plant. Additionally, bioassay of the potential callus will be conducted in suspension culture.

ACKNOWLEDGMENTS

The authors would like to thank the Faculty of Sciences and Mathematics, Diponegoro University (Fiscal Year 2018 No.1754 F/UN7.5.8/PG/2018) for providing a scholarship to support the funding of this research.

REFERENCES

Baldi, A., & Dixit, V. (2008). Enhanced artemisinin production by cell cultures of *Artemisia annua*. *Current Trends in Biotechnology and Pharmacy*, *2*(2), 341-389.

Baranski, R., & Cazzonelli, C. I. (2016). *Carotenoid biosynthesis and regulation in plants*. Chichester, UK: John Wiley & Sons, Ltd.

Benítez-García, I., Vanegas-Espinoza, P. E., Meléndez-Martínez, A. J., Heredia, F. J., Paredes-López, O., & Del Villar-Martínez, A. A. (2014). Callus culture development of two varieties of *Tagetes erecta* and carotenoid production. *Electronic Journal of Biotechnology*, *17*(3), 107-113.

Bondet, V., Brand-Williams, C., & Berset, C. (1997). kinetics and mechanisms of antioxidant activity using the DPPH free radical method, LWT. *Food Science and Technology*, *30*(6).

Chawla, H. S. (2003). *Introduction to plant biotechnology*. New Hemsphire: Science Publishers Inc.

Davey, M. W., Kenis, K., & Keulemans, J. (2006). Genetic control of fruit vitamin c content. *Plant Physiology, 142*, 343-351.

Dechaux, C., & Boitel-Conti, M. (2005). A strategy for overaccumulation of scopolamine in *Datura innoxia* hairy root cultures. *Acta biologica cracoviensia Series Botanica*, *47*(1), 101-107.

Dewick, M. (2002). *Medicinal natural products a biosynthetic approach*. Chichester: John wiley & sons. Ltd.

Endress, R. (1994). *Plant cell biotechnology*. Berlin: Springer-Verlag

Fitriani, A. (2003). Kandungan ajmalisin pada kultur kalus *Catharanthus roseus* (L) G. Don setelah dielistasi homogent jamur *Phytium aphanidermalium* (Tesis master). Institut Pertanian Bogor, Bogor, Indonesia.

Giovannoni, J. J. (2007). Completing a pathway to plant vitamin synthesis. *Proceedings of the National Academy Sciences of the United States of America Journal, 104*(22), 9190-9110. doi: 10.1073/pnas.0703222104.

Hartman, H. T., & Kester, D. E. (1997). *Plant propagation principles and practice ed ke-6*. New Jersey: Prentice Hall.

Iranbakhsh, A. R., Oshagi, M. A., & Ebadi, M. (2007). Growth and production optimization of tropane alkaloids in *Datura stramonium* cell suspension culture. *Pakistan Journal of Biological Sciences, 10*, 1236-1242.

Iskandar, N. N., & Iriawati, P. (2016). Vinblastine and vincristine production on madagascar periwinkle (*Catharanthus roseus* (L.) G. Don) callus culture treated with polethylene glycol. *Makara Journal of Science*, *70*(1), 7-16. doi: 10.7454/mss.v20i1.5656.

Iswari, R. S., & Susanti, R. (2016). Antioxidant activity from various tomato processing. *Journal of Biology & BiologyEducation*, *8*(1), 127-132.

Jabeen, N., Chaundhry, Z., Rashid, H., & Mirza, B. (2005). Effect of genotype and explants type on in vitro shoot regeneration of tomato *(Lycopersicon esculentum* Mill*). Pakistan Journal of botany, 37*(4), 899-903.

Jefriyanto, I., Runtuwene, R. J. M., & Feti, F. (2012). Penentuan total fenolik dan uji aktivitas antioksidan pada biji dan kulit buah pinang yaki (*Areca vestiaria* Giseke). *Jurnal Ilmiah Sains, 12*(2).

Kala, S. C., Mallikarjuna, K., & Aruna, P. (2014). An efficient protocol devised for rapid callus induction from leaf explants of *Biophytum sensitivum* (Linn) DC. *International Journal of Phytopharmacy, 4*(1), 20-24. doi: 10.7439/ijpp.v4i1.74.

Karuppusamy, S. (2009). A review on trends in production of secondary metabolites from higher plants by in vitro tissue, organ and cell cultures. *Academic Journals: Journal of Medicinal Plants Research*, 1222-1239.

Llorente B., Torres-Montilla, S., Morelli, L., Florez-Sarasa, I., Ezquerro, M., D'andrea, L., … Concepcion, R. (2019). Synthetic biogenesis of chromoplasts from leaf chloroplasts. *bioRxiv Preprint- the Preprint Server for Biology*, 1-26. doi: 10.1101/819177.

Marchev, A., Christiane, H., Schulz, S., Georgiev, V., Steingroewer, J., Bley, T., & Pavlov, A. (2014). Sage in vitro cultures: A promising tool for the production of bioactive terpenes and phenolic substances. *Biotechnology Letter*, *36*, 211-221.

Motlhanka, D., Houghton, P., Miljkovic-Brake, A., & Habtemariam, S. (2010). A novel pentacyclic triterpene glycoside from a resin from *Commiphora glandulosa* Bostnawa. *African Journal of Pharmacy and Pharmacology*, *4*(8), 549-554.

Nurchayati, Y., & Rahmah, F. A. (2010). Kandungan asam askorbat pada kultur kalus rosela *(Hibiscus sabdariffa* L.) dengan variasi konsentrasi sukrosa dalam media MS. *Majalah Obat Tradisional, 15*(2), 71-74.

Nurchayati, Y., Santosa, S., Nugroho, L. H., & Indrianto, A. (2016). Growth pattern and copper accumulation in callus of *Datura metel*. *Biosaintifika: Journal of Biology & Biology Education*, *8*(2), 135. doi: 10.15294/biosaintifika.v8i2.5177.

Oleszkiewicz, T., Chodacka, M. K., Hendel, A. M., Zubko, M., Stróż, D., Kurczyńska, E., … Baranski, R. (2018). Unique chromoplast organization and carotenoid gene expression in carotenoid-rich carrot callus. *Planta*, *248*, 1455-1471. doi: 10.1007/s00425-018-2988-5.

Olusegun, A., Makun, H. A., Ogara, I. M., Edema, M., Idahor, K. O., Oluwabamiwo, B. F. & Eshiett, M. E. (2012). We are IntechOpen, the World's leading Publisher of Open Access books Built by scientists, for scientists TOP 1%. *Intech, i(tourism)*, 38. doi: 10.1155/2018/2961767.

Osman, H. A., Taha, H. S., Youssef, M. M. A., El-Gindi, A. Y., Ammen, H. H., & Lushein, A. M. S. (2012). Establishment of calli cultures from different explants of *T. erecta* and *T. patula. Journal of Application Science Research*, *8*(7), 3850-3854.

Othman, R., Kammona, S., Jaswir, I., Jamal, P., & Hatta, F. A. M. (2017). Effect of abiotic stress on carotenoids accumulation in orange sweet potato callus under light and dark conditions. *International Food Research Journal*, *24*, 481-487

Palazon, J., Navarro-Ocana, A., Hernandez-Vazquez, L., & Mirjalili, M. H. (2000). Application of metabolic engineering to the production of scopolamine. *Molecules, 13*, 1722-1742.

Sari, Y. P., Kusumawati, E., Saleh, C., Kustiawan, W., & Sukartiningsih. (2018). Effect of sucrose and plant growth regulators on callogenesis and preliminary secondary metabolic of different explant *Myrmecodia tuberosa*. *Nusantara Bioscience*, *10*(3), 183-192.

Sun, T., Yuan, H., Cao, H., Yazdani, M., Tadmor, Y., & Li, L. (2018). carotenoid metabolism in plants: The role of plastids. *Molecular Plant*, *11*, 58-74.

Tarrahi., & Rezanejad, F. (2017). Callogenesis and production of anthocyanin and chlorophyll in callus cultures of vegetative and floral explants in *Rosa gallica* and *Rosa hybrida* (Rosaceae). *Turkey Journal of Botany, 37*, 1145-1154. doi:10.3906/bot-1205-42.

Trigiano, R. N., & Gray, D. J. (2005). *Plant development and biotechnology*. Boca Raton: CRC Press.

Trimulyono, G., Solichatun, S. D., & Marliana. (2004). pertumbuhan kalus dan kandungan minyak atsiri nilam *(Pogostemon cablin* (Blanco) Bth.) dengan perlakuan asam α-naftalen asetat (NAA) dan kinetin. *Biofarmasi*, *2*(1), 9-14

Veerporte, R., & Memmelink, J. (2002). Engineering secondary metabolite production in plants. *Current Opinion in Biotechnology, 13*, 181-187.

Welsch, R., Beyer, P., Hugueney, P., Kleinig, H., & von Lintig, J. (2000). Regulation and activation of phytoene synthase, a key enzyme in carotenoid biosynthesis, during photomorphogenesis. *Planta*, *211*, 846-54. doi: 10.1007/ s004250000352.

Zulfiqar, B., Abbasi, N. A., Ahmad, T., & Hafiz, I. A. (2009). Effect of explant sources and different concentrations of plant growth regulators on in vitro shoot proliferation and rooting of avocado (*Persea americana* Mill.) cv “Fuerte”. *Pakistan Journal of Botany, 41*(5), 2333-2346.