Identification of exudates from callus of Mangrove Plant 
(Rhizophora apiculata BI) in vitro.

Y Nurchayati¹, E Prihastanti¹, R Budihastuti¹

¹Department of Biology, Faculty of Science and Mathematics, Diponegoro University
Jl. Prof. Soedharto, SH, Tembalang, Semarang 50275, Indonesia
E-mail: yulita_yoko@gmail.com

Abstract. *Rhizophora apiculata* is species in mangrove vegetation. The mangroves leaves were the water-storage type. Induction of callus from the leaf of *R. apiculata* faced problems, i.e., browning phenomena and exudates. This research aimed to evaluate the production of exudates and their correlation with the browning of explant and to identify kind of compounds of exudates. The leaf that is used as explants was divided into the base and tip section was grown in Murashige and Skoog (MS) medium with the addition of NAA 1 mg/L, BAP 0,3 mg/L, and activated charcoal 12 g /L. The treatments included 24 hours placed in the dark and 24 hours placed under light, 16 hours placed in the dark and 8 hours under the light, 8 hours placed in the dark and 16 hours under the light; the treatments were repeated four times. Respond of explants were descriptively observed, while the exudates were analyzed by Benedict test. The results showed that light treatment could induce callus formation while 24-hour dark treatment could reduce browning. The novelty of this research lies upon the process of browning prevention using light duration treatment. Therefore, browning could be prevented, and mangrove culture could successfully produce callus. Not at all explants produced the exudates. Light treatment can be used to avoid exudates production. Some of the exudates consisted of carbohydrates. Nevertheless, another one consisted of a salt solution. These exudates made a disturbance in callus initiation of *R. apiculata*.

1. Introduction

*Rhizophora apiculata* BI is one of significant and well-grown mangrove in Indonesia. Mangroves have many benefits not only in improving water quality, feeding ground, spawning ground and nursery ground for species of fish and shrimp, but also play a role in maintaining coastline and river banks from erosion as well as abrasion. Those Mangroves able to remain stability of seawater, controlling seawater intrusion, protecting the area behind mangrove from waves, strong winds and reducing the risk of tsunami hazard [15]. While the population of the last half-century has decreased mangrove area by 30-50%, but to grow it takes a long time is about two months. It becomes an obstacle to meeting the needs of seeds required in mangrove conservation [14].

Plant tissue callus is one of the methods to overcome the mangrove propagation. Callus culture from mangrove leaves can be induced dan regenerated into planets or to get embryogenic cells. There were problems in callus induction of mangroves. *Rhizophora* is one of mangrove which grows in a salty environment, so it contains has a high phenolic compound. This phenolic sometimes makes inhibition for callus proliferation; we called browning. Besides, mangrove’s leaf was water storage that high content of exudates when it was cut. This experiment aims to evaluate the production of exudates from the leaf and their correlation with the browning of explant and to identify kind of
compounds of exudates. Here, a small piece of Rhizophora leaves then growth in MS media was to study the response of callogenesis.

2. Research Methods

2.1 Explant materials and sterilization

Explant was taken from the first sequence leaf of 7 years old Rhizophora apiculata BI trees growing in the area of mangrove forest, Mangkang, Semarang. The explant was washed using detergent, followed by successive sterilization in antibiotic solution, fungicidal solution, bayclinsolution (bleach), 70% alcohol solution.

2.2 Callus Induction Medium

Basic medium used was Murashige & Skoog (MS) supported with 30 g/L sucrose and activated charcoal as much as 12 g/L. There were 2 kinds of medium: i) MS + NAA 1 mg/L + BAP 0.3 mg/L; ii) MS + BAP 1, 2 and 3 mg/L with young and mature leaves. The pH was adjusted by pH-meter at 5.8 and solidified with agar 8 g/L, then sterilized in an autoclave for 15-20 minutes at 121 °C.

2.3 Callus initiation and incubation

Leaf explant was cut in size of 1 cm²; cutting was done in ascorbic acid solution 10 mg/100 ml and planted in callus medium. The culture was incubated by lighting period with 478 lux light intensity and also dark treatment (no light bulbs) by the treatment. The culture was incubated for 30 days.

3. Result and discussion

Callus induction has no optimal response in MS added with charcoal 12 mg/L and lighting period. However, some of the explant showed small clumps at the side of injured in middle leaf section. The color of initiated callus was white and brownish.

Table 1. Color and texture of callus on Rhizophora apiculata BI leaf explant after 30 days of light treatments

| Treatment   | Callus Color | Callus Texture |
|-------------|--------------|----------------|
| D_24        | -            | -              |
| L_16D_8     | Brownish     | Compact        |
| L_24        | Brownish     | Compact        |
| L_8D_16     | White        | Compact        |

Note
- D_24: 2-4 hours placed in the dark
- L_24: 24 hours placed under light
- L_16D_8: 8 hours placed in the dark and 16 hours under light
- L_8D_16: 16 hours placed in the dark and 8 hours under light

The brownish color of the callus is known as the browning phenomenon, which is caused by the metabolism of phenol compounds that are toxic and often arises due to the explant sterilization process. Phenol compounds generally inhibit the growth or even cause tissue death [2]. Zulkarnain (2009) stated that a browning event is a natural event and adaptive change process of plant parts due to physical effects such as stripping and cutting.
Figure 1. Morphological description of *Rhizophora apiculata* BI after 30 days-old in lighting period 1) Dark 24 h; 2) L₈D₁₆ 3) Light 24 h 4) L₁₆D₈ A: Exudate B: callus

Figure 2. Morphological description of *Rhizophora apiculata* BI after 30 days-old from young leaf explant on different concentration of BAP (a) 1 mg/L; (b) 3 mg/L; (c) 5 mg/L .

Ru et al. (2013), explained that browning in the tissues is one of the problems that often occur in woody plant culture. Browning is generally caused by phenolic compounds that usually appear and accumulate when the explants are injured, which is usually caused by the activation of the polyphenol oxidase (PPO) enzyme. An organ opening can cause metabolic imbalance from ROS (Reactive Oxygen Species), peroxidation of the lipid membrane, and loss of integrity of cell membranes that can trigger over accumulation of phenolic compounds and cause browning.

The oxidation of phenol will increase with the presence of light [8]. The oxidation occurring in phenolic compounds may be via autoxidation or enzymatic oxidation reactions. Autoxidation is an oxidation reaction caused by the presence of light and oxygen [3]. Polyphenol oxidase (PPO) enzyme, when it is widespread in the presence of oxygen and light will convert the monophenol group into o-hydroxy phenol then converted again into o-quinone groups. This o-quinone group forms a brown color. Dark treatment (G₂₄) in mangrove leaf explant isolation inhibits browning time compared with other treatments. The lightless conditions resulted in slow oxidation of phenol. This is by the opinion of Corduk (2011) that the increase of phenol occurs when plants are exposed to direct light. Therefore,
24 hours dark treatment (G24), blocking the existence of light that can spur the production of phenol that causes browning.

3.1 Exudate
A section of an explant grown in MS medium showed another response. There was the physiological reaction when plant tissue is injured, caused by cutting effect. One of the physiological reactions in Rhizophora’s leaves section in vitro is the appearance of exudate. Exudate secreted out of sliced mangrove leaf because the leaf of family Rhizophoraceae has water storage [11]. These exudates were kept in leaves of mangrove, without make disturbance in plant growth, as a product of normal metabolisms in Rhizophoraceae. According to Widyati (2013), exudates include compounds with low molecular weight, such as sugars, amino acids, and aromatics. The exudates do not exert a lethal effect on the growth of culture. Exudates produced by explants can be seen in Figure 1-2.

Lighting seemed to be a factor influence in exudates excretion. It supposed that suboptimal of the intensity of the light resulted in less optimal culture growth for mangrove callus. Light also affects the regulation of production of metabolites in cell suspension cultures, both primary metabolites such as enzymes, carbohydrates, lipids, and amino acids or secondary metabolites such as anthocyanins, carotenoids, polyphenols, essential oils, and terpenes. Suspected light intensity <1000 lux is not optimal for the work of several metabolic enzymes in mangrove explant.

The highest amount exudate released was resulted by the L8D16 treatment (8 hours under light treatment, 16 hours in the dark) of 100%, while in L24 treatment (24 hours under light), L16D8 (16 hours under light, 8 hours in the dark), and D24 (24 hour in dark) of exudate produced by 50%, and 100% in L8D16 treatment (8 hours under light treatment, 16 hours in the dark). The released exudate did not affect callus growth because the most exudate was in the L8D16 treatment whereas the most grown callus was in the L16D8 treatment.

Table 2. Biochemical analysis to the exudate of R. apiculate callus by Benedict test

| Treatment                  | Incubation     | The result of Benedict test |
|----------------------------|----------------|----------------------------|
| Young leaf, NAA 1 + BAP 0,3| Light 24 h     | +                          |
| Young leaf, NAA 1 + BAP 0,3| Light 16 h, dark 8 h | +                          |
| Young leaf, NAA 1 + BAP 0,3| Light 8 h, dark 16 h | +                          |
| Young leaf, NAA 1 + BAP 0,3| Dark 24 h      | +                          |
| Young leaf + BAP 1         | Light 24 h     | -                          |
| Young leaf + BAP 3         | Light 24 h     | -                          |
| Young leaf + BAP 5         | Light 24 h     | -                          |
| Mature leaf + BAP 1        | Light 24 h     | +                          |
| Mature leaf + BAP 3        | Light 24 h     | +                          |
| Mature leaf + BAP 5        | Light 24 h     | -                          |

4. Conclusion
Some sentences to give conclusion are leaves of R. apiculate were difficult in inducing callus. These explants released exudates when inoculated in MS medium. This exudates made disturbance in callus initiation of R. apiculate. The exudates was released when the explant gets browning. Some of exudate consisted of carbohydrates, whereas another one was consisted of salt solution.

Acknowledgment
The author would like to team of PNBP Grant from Faculty of Sains and Mathematics Diponegoro University which has funded this research and Mr. Sururi as field supervisor. Thanks to our colleges for preparing and assisting this project.

References
[1] Admojo L and Indrianto A 2016 Indonesian Journal of Natural Rubber Research 34 25
[2] Andaryani S 2010 *Kajian Penggunaan Berbagai Konsentrasi Bap Dan 2,4-D Terhadap Induksi Kalus Jarak Pagar (Jatropha curcas L.) Secara In Vitro*. Surakarta: Fakultas Pertanian Universitas Sebelas Maret.

[3] Andarwulan N and Faradilla R H F 2012 *Senyawa Fenolik pada Sayuran Indigenous Bogor: Southeast Asian Food and Agricultural Science and Technology (SEAFAST) Center Research and Community Service Institution Bogor Agricultural University*.

[4] Corduk N and Aki C 2011 *Romanian Biotechnological Letters* 16 6760.

[5] Chawla H S 2009 *Introduction to Plant Biotechnology Third Edition*. India: Genetics & Plant Breeding Department, G.B. Pant University of Agriculture & Technology.

[6] Eddy S 2008 *Pengelolaan Potensi Hutan Mangrove secara Berkelanjutan Palangbang*: Jurusan Biologi FMIPA Universitas PGRI Palembang.

[7] Hoesen D S H, Witjaksono and Sukamto L A, 2008 *Callus Induction and Organogenesis of in vitro Culture Dendrobium lineage Rolfe* Bogor: Cibinong Science Center (LIPI), Biology News 9 3.

[8] Hutami S 2008 *Jurnal Agro Biogen* 4 83.

[9] Kader A, Sinha, S N, and Ghosh P, 2015 *International Journal of Tropical Plant Research*. 2 192

[10] Kartika L, Atmodjo, P K and Purwijantiningsih L M E 2013 *Callus Induction Rate and Eugenol Content of Red Betel(Piper crocatum Ruiz and Pav.) Treated Using a Variation of The Type and Concentration of Auxsin* Yogyakarta: Atma Jaya University.

[11] Lechtaler S, Robert, E M R, Tonne, N, Prusova, A, Gerkema, E, As, H V, Koedam, N and Windt, C W 2016 *Front Plant Sci.* 7 895.

[12] Manuhara Y S W 2014 *Kapita Selektiva Kultur Jaringan Tumbuhan* Surabaya: Airlangga University Press.

[13] Nasution S S 2013 *Pengaruh Teknik Sterilisasi terhadap Keberhasilan Eksplan Paulownia (Paulownia elongata SY. Hu) secara In vitro* Bogor : Institut Pertanian Bogor.

[14] Ng P K L and Sivatoshi N 2001 A Guide to Mangroves of Singapore. *Volume 1: The Ecosystem and Plant Diversity and Volume 2: Animal Diversity* Singapore: The Singapore Science Centre.

[15] Noor Y R, Khazali, M., Suryadiputra, I N N, 2007 *Panduan Pengenalan Mangrove di Indonesia* Bogor: PHKA/WI-IP.

[16] Pudyastuti S, Habibah, N A, & Sumadi 2012 *Biosaintifikasi Journal of Biology & Biology Education* 4 1.

[17] Putri N I 2008 *Kajian berbagai komposisi media serta kondisi gelap dan terang terhadap induksi kalus tanaman jati belanda (Guazuma ulmifolia Lamk.)* Solo: Universitas Sebelas Maret.

[18] Ru Z, Lai Y, Xu C, and Li L 2013 *Journal of Agricultural Science* 5 57.