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Increasing *Rhynchostylis retusa*'s Callus Formation by Immersing Explant in Ascorbic Acid and Planting on Activated Charcoal Contained Medium

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Abstract. The aimed of this research is to increase *Rhynchostylis retusa* callus formation through immersing explants in ascorbic acid and planting it on activated charcoal contained medium. It's an experimental research that consist of three factors; i.e. immersion explants in ascorbic acid (immersed and not immersed), kind of in-vitro culture medium (not-contained (as control), pro-analysis activated charcoal contained and commercial activated charcoal contained), and the type of expants (leaf and root). The source of explants used are 18 months after planting (map) *Rhynchostylis retusa* orchids plants in in-vitro cultures. The explants condition (the level of browning) and callus formation (the percentage of callus forming explants, the time of callus appearance, and callus diameters) were observed at 8 weeks after planting (wap). The result show that callus formation can be induced by medium that contain activated charcoal of both kind (pro-analysis or commercial). Combination of the treatment with explants immersion in ascorbic acid before planting can increase the callus formation. The root explants form higher callus diameters (0,563 mm) than leaf (0,298 mm).

keywords: callus, ascorbic acid, activated charcoal, *Rhynchostylis retusa*, in-vitro culture

1. Introduction

Indonesia has around 5,000 orchid species from 30,000 natural orchid species spread throughout the world [1]. The squirrel tail orchid (*Rhynchostylis retusa*) is one of the epiphytic orchid species that is widely distributed on the island of Java. *Rhynchostylis retusa* (*R. retusa*) has a fragrant floral aroma and attractive shape and color. The content of secondary metabolites in orchids has health potential as a medicinal plant [2]. The superiority causes *R. retusa* to be favored by orchid developers.

Propagation of orchids can be done vegetatively or generatively through conventional or in vitro culture techniques. Conventional vegetative propagation requires a long time and the number of tillers is limited [3], so that to get large amounts of seedlings and short time can be done in vitro culture.

Reproduction through in vitro culture can be done through callus culture which will produce tillers with relatively low genetic diversity [4]. Callus induction can be done for the purpose of somatic embryo propagation or the production of secondary metabolites. Callus produces higher secondary metabolites than those from plants [5].

Constraints that commonly occur in callus induction in addition to contamination also occur browning on explants. These constraints will reduce the ability of explants to grow and develop [6]. According to [7] the browning level in the callus induction phase is still high. Browning is caused by phenol compounds which accumulate when explants are injured. Browning levels in young tissues are generally lower than those of older tissues [8] [9]. Browning can be overcome by adding antioxidants such as vitamin C and activated charcoal.

The use of activated charcoal with a concentration of 2 g.L⁻¹ can eliminate the negative effects of phenol oxidation on the growth of *Disa* spp. [10]. Research conducted by [11] showed that vitamin



C can not only reduce browning but also can improve regeneration of the roots. Immersion of explants (leaf bone / midrib) of rubber PB 32 clones in 100 mg.L⁻¹ of vitamin C for 30 minutes can inhibit browning up to 30% in callus induction culture [7].

The types of activated charcoal that are commonly used in tissue culture are active pro-analyst charcoal. The pro-analyst active charcoal is difficult to obtain and has a relatively expensive price so that alternative ingredients are needed that are more affordable and easily found on the market. Based on this, this study was conducted to find the right method for increasing *R. retusa* callus formation through explant immersion in vitamin C and addition of activated charcoal in in vitro culture medium.

2. Materials and Methods

2.1 Types of research

This research is an experimental research. The treatment of the research carried out is as follows:

Table 1. Matrix of treatment

Activated Charcoal Vitamin C	- (AC)	+ Pro-analis (AC-PA)	+ Commercial (AC-C)
- (C0)	C0 -AC	C0 +(AC-PA)	C0 +(AC-C)
+ (C)	C -AC	C +(AC-PA)	C +(AC-C)

Note :

C0 : without immersing in vitamin C

C : with immersing in vitamin C

- AC : planting explant on medium without activated charcoal

+ AC-PA : planting explant on medium with pro-analysis (PA) activated charcoal

+ AC-C : planting explant on medium with commercial (C) activated charcoal

2.2 Time and Place

The study was conducted in November 2017 - April 2018 at In-Vitro Culture Laboratory of Mathematics and Science Faculty, Universitas Negeri Yogyakarta, Indonesia.

2.3 Materials

Eighteen months after planting (map) *Rhynchostylis retusa* orchids plants in in-vitro cultures were use as the source of explant.

2.4 Procedure

2.4.1 Medium preparation

One g.L⁻¹ activated charcoal (PA and C) were added in the basic medium (NP + 150 ml. L⁻¹ coconut water + 2 mg. L⁻¹ 2,4-D). The medium adjusted at pH 5.8 and solidified with agar (0.7 %).

2.4.2 Callus Induction

Leaves and roots were cut into 1 cm long. For Vitamin C immersion treatment, explant were immersed in 100 ppm of vitamin C for 30 minutes with 100 rpm agitation on shaker before planting. For treatment without vitamin immersion, explants directly planted on each medium (Basic Medium, +AC-PA, +AC-C)

2.5 Data analysis

Data obtained included data on browning rates, percentage of explants that formed callus, time of callus emergence, and callus size. Data is taken every week until 8 weeks after planting (wap). The data were then analyzed using One Way Anova test with 5% error. If there are significantly differences, it continued with the DMRT test.

3. Results

3.1 The Effect of Vitamin C Immersion and Activated Charcoal contained medium on Browning Levels of *R. Retusa* Explants

All treatments show an increase in the level of browning each week. The results showed that the addition of active pro-analyst and commercial charcoal in NP medium could prevent increasing browning levels. The addition of vitamin C immersion was able to prevent browning in both root and leaf explants (Figure 1 and Figure 2).

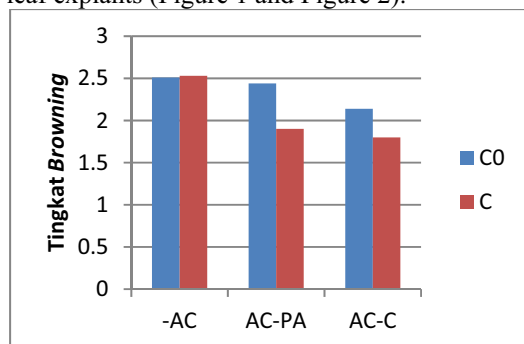


Figure 1. Browning level of *R. retusa* root explants at 8 wap

In leaf explants, treatment of vitamin C immersion and addition of AC-PA resulted in the lowest browning rate at 8 wap. The treatment proved to be able to prevent the occurrence of browning better.

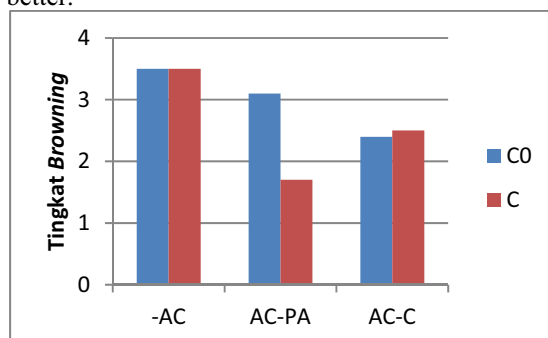


Figure 2. Browning rate of *R. retusa* leaf explants at 8 wap

3.2 The Effect of Explant Immersion in Vitamin C and Activated Charcoal contained medium on Callus Formation

Callus was only formed on media with activated charcoal addition. Both types of activated charcoal (PA and C) can induce callus formation with or without explants immersion in vitamin C.

There are no differences in the time of callus appearance between treatments. Callus from root explant in all treatment appeared at 3 wap. The optimum treatment yielded with immersing explants in vitamin C and planting it on commercial activated charcoal contained medium. The treatment can produce highest callus diameter (0.563 mm) (Table 2).

Table 2. Callus induction from root explants at 8 wap,

Treatment Code	The percentage explant induced callus (%)	Time of callus appearance (wap)	Average callus diameters (mm)
C0 –AC	0	-	0.000 a
C0 +(AC-PA)	8.33	3	0.113 a
C0 + (AC-C)	83.33	3	0.443
C –AC	0	-	0.000 a
C + (AC-PA)	100	3	0.432 b
C + (AC-C)	91.67	3	0.563 b

Similar to root explants, callus from leaves explants were induced by immersing it in vitamin C and planting it on medium which contained activated charcoal. The highest callus diameters from leaves (0.298 mm) were yielded on AC-PA medium at 2 wap (Table 3).

Table 3. Callus induction from leaves explants at 8 wap,

Treatment Code	The percentage of callus grows (%)	The appearance of callus (wap)	Average callus length (mm)
C0 –AC	0	-	0.000 a
C0 + (AC-PA)	16.67	3	0.040 a
C0 + (AC-K)	83.33	3	0.263 bc
C –AC	0	-	0.000 a
C + (AC-PA)	100	2	0.298 c
C + (AC-C)	58.33	4	0.163 b

4. Discussion

4.1 The Effect of Vitamin C Immersion and Activated Charcoal contained medium on Browning Levels of *R. retusa* Explants

Browning begins with a change in the explant's color from green turns brown on the open area. Brownish explants indicate that the cells have died [12]. Ozyigit *et al.* [13] explained that cut explants cause cytoplasmic and vacuole contents to be mixed and exited so that phenol compounds can be oxidized by air. The process occurs enzymatically between the enzyme polyphenoloxidase (PPO)

and peroxidase (POD) with the polyphenols that form quinon which is then polymerized to produce brown color (Figure 3).

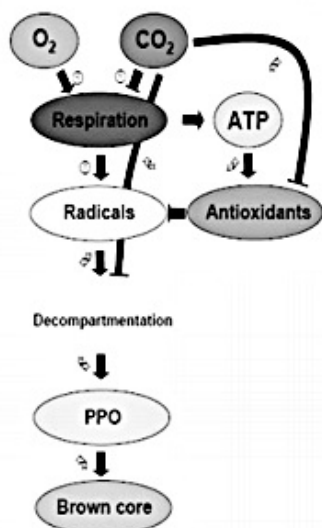


Figure 3. Browning prevention mechanism by antioxidant compounds [14] [15].

Browning reactions will occur when the substrate and enzymes mix and involve oxygen in the reaction [16].

Explant response with treatment of activated charcoal (AC-PA / AC-C) showed good results in slowing the occurrence of browning. These results are consistent with [17] study that activated charcoal is able to reduce phenol released by explants. Activated charcoal will absorb compounds released by explants due to toxic (exudate) injury.

The addition of AC-C gives better results than AC-PA due to its lower light intensity which can be seen from the color of the blacker media. In accordance with the statement of [13] that phenol synthesis is influenced by genetic and environmental factors. One way to reduce browning on explants can be done by reducing light exposure [18].

Better results for the prevention of explant browning are shown by the addition of vitamin C soaking in media containing activated charcoal. Vitamin C plays a role in capturing free oxygen so as to prevent decompartmentalization (rupture of vacuoles and plastids in cells) that can activate PPO enzymes [14] [7].

4.2 The Effect of Explant Immersion in Vitamin C and Activated Charcoal contained medium on Callus Formation

According to [19], callus growth can be inhibited in the presence of light. The intensity of light can degrade auxin through a photooxidation process so that the chemical structure of auxin will change which causes the enzyme to become inhibited. In accordance with [3] statement that auxin in plant tissues can work optimally in dark conditions.

The callus formed on the explant appears on the part that gets injured. Callus growth is characterized by the appearance of small clumps of cells, white on the opening (Figures 4 and 5).

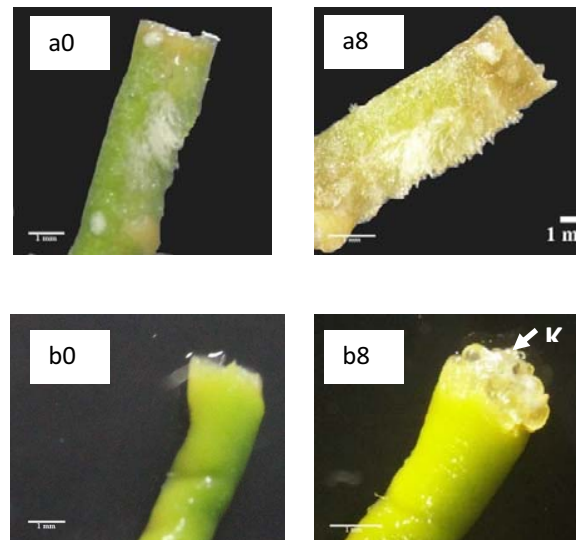


Figure 4. Callus formed on the root extract of *R. retusa* aged 0 wap (a0 & b0) and 8 wap (a8 & b8) in control explants (a) and explants treated with vitamin C + (AC-C) (b). K: callus.

The existence of injury to the explant causes exogenous ZPT to diffuse more easily with endogenous ZPT to form callus by stimulating cell division, especially cells in the open area [3]. [20] Auxin initiates cell elongation by affecting the flexibility of cell walls and stimulating certain proteins in the plasma membrane to pump H^+ ions to the cell wall, resulting in easing of the cell wall and then cell division [21].

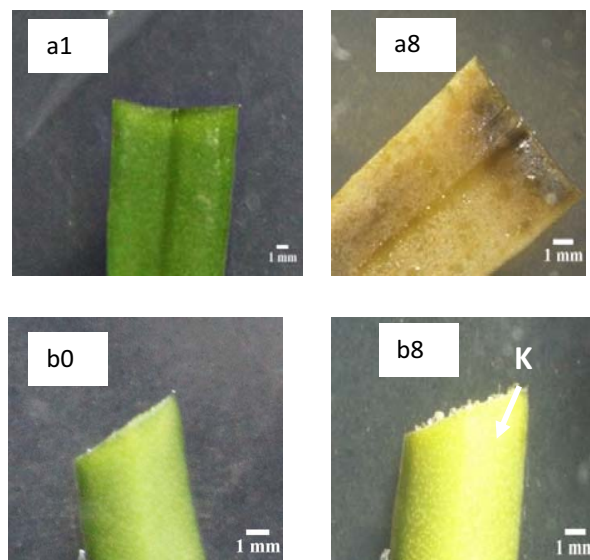


Figure 5. Callus formed on *R. retusa* leaf eskplan aged 0 wap (a0 & b0) and 8 wap (a8 & b8) on control explants (a) and explants treated with vitamin C + AC-PA (b). K: callus.

Callus growth increased with a combination of vitamin C soaking treatment and addition of activated charcoal. Aside from being an antioxidant in preventing browning, vitamin C plays an important role in cell metabolism. The formation of callus indicates that the process of absorption of nutrients can run well. Research from [22] shows that the addition of vitamin C can increase the growth of pule pandak callus (*Rauwolfia serpentina* Benth.), As well as [23] who reported that pre-treatment of explant immersion in *Platanus occidentalis* L. in antioxidant vitamin C 10 g. L⁻¹ could effectively reduce contamination and browning. Vitamin C is able to bind free radicals produced when explants are injured and prevent oxidation, while activated charcoal will adsorb inhibitor compounds found in the media so as to reduce the metabolic activity of toxic compounds and prevent the accumulation of exudates [24].

R. retusa leaf explants are able to form callus faster than root explants. This is related to the process of nutrient absorption. The results of the study by [25] show that the roots of epiphytic orchids (the genus *Liparis*) have about two layers of epidermis and the cell wall is thickened. The structure of the cell wall causes cells not to quickly absorb nutrients such as cells in the leaves. This causes the callus initiation time from the roots to be longer than that of leaf explants.

According to [12] small and thin explants have fewer cell counts. It is suspected that the number of cells contained in explants is insufficient to divide, so the callus size produced from leaf explants is smaller. According to [26], the resulting callus size varies, this is due to the ability of the tissue in the process of diffusion, osmosis and turgor pressure. Supported by the statement of [27] that the ability of tissues to form callus and callus growth rate depends on the medium, the growth regulating substances used and other environmental factors.

In conclusion, explant immersion in vitamin C which was not followed by explant planting on medium containing activated charcoal was not able to induce callus and prevent browning on explants, while planting on medium containing activated charcoal was able to prevent browning explants *R. retusa* better. Planting explants (both roots and leaves) on medium with the addition of activated charcoal capable of producing callus, explant immersion combination treatment with vitamin C and activated charcoal (AC-PA and AC-C) will increase callus formation on *R. retusa* explants. *R. retusa* root explants can produce the largest callus.

5. Acknowledgements

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