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Browning Prevention Method in Kepel (*Stelechocarpus burahol* (Blume) Hook.f. & Thomson) in Vitro Culture

Devi Sonti Sibarani, Ratih Restiani(*), Aniek Prasetyaningsih

Department of Biology, Faculty of Biotechnology, Kristen Duta Wacana University
Dr. Wahidin Sudirohusodo No.5-25, Kotabaru, Gondokusuman,
Daerah Istimewa Yogyakarta 55224 Indonesia

***Corresponding Author:** ratih.restiani@staff.ukdw.ac.id

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Abstract

Browning of explants is a common problem in Kepel (Stelechocarpus burahol (Blume) Hook.f. & Thomson) in vitro culture, resulting in low regeneration of Kepel explants. Using nodal explants, the effect of explant immersion in ascorbic acid, the addition of ascorbic acid to the media, as well as dark and light incubation conditions were investigated. The browning prevention method was selected based on the delayed browning appearance time, the lowest browning intensity, and the highest percentage of callus. This present research used a completely randomized design with treatment variations: immersion explant in ascorbic acid 100 mg/L, addition of ascorbic acid 100 and 200 mg/L to the media, addition of ascorbic acid 100 and 200 mg/L and activated charcoal 1 g/L to the media, and incubation in dark and light conditions for 28 days. The results showed that the combination of the addition of ascorbic acid 200 mg/L to MS media and incubation in dark conditions were effective browning prevention methods in inhibiting browning appearance time (5 DAP), significantly reducing browning intensity (0.3), and increasing callus growth (100%) of kepel nodal explants during 28 days of culture. The results of this research are useful in establishing protocols for in vitro culture of Kepel plants, especially at the initiation stage, and can be expected to support the successful conservation of Kepel plants through in vitro propagation

Keywords: *Ascorbic acid, Activated charcoal, Browning, In vitro culture, Stelechocarpus burahol*

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INTRODUCTION

Stelechocarpus burahol (Blume) Hook.f. & Thomson, also known as Kepel, is one of the fruit plants native from Indonesia and include to the Annonaceae family (Handayani et al., 2020; Angio & Firdiana, 2021). All parts of Kepel have several health benefits, such as preventing body odor (Angio & Firdiana, 2021), preventing mouth odor, urination, and kidney inflammation, anti-implantation, source of antioxidants, anti-

hyperuricemia or prevention of arthritis caused by increased uric acid levels in the blood, and anti-cancer (Rofiqah et al., 2021).

Despite its various health benefits, the cultivation of kepel plants remains relatively low due to several factors. These factors include the low economic value resulting from the relatively smaller size of the fruit compared to its seed, the difficulty in propagation through cuttings and grafts, and the high failure rate. Propagation through seeds is also relatively difficult due to the long dormancy period (4-6 months) and low germination rate (Handayani et al., 2020; Hutabarat et al., 2022). High utilization along with inadequate propagation provoke the declining population of this plant, thus causing it to be categorized as conservation-dependent (Handayani et al., 2020; Angio & Firdiana, 2021).

Ex situ conservation attempts using in vitro culture can be used to effectively propagate this plant. Several studies related to in vitro culture of Kepel plants have been conducted with several focuses, including optimization of sterilization methods for leaf explants (Habibah et al., 2013), nodes (Hutabarat et al., 2022) and endosperm (Handayani et al., 2022), optimization of PGR that supports callus growth of Kepel seed and fruit mesocarp explants (Habibah et al., 2016, 2017, 2019), and nodal explant (Sekar et al., 2023), and optimization of flavonoid production from callus and cell suspension cultures of Kepel plants (Habibah et al., 2016, 2019). However, in vitro propagation of Kepel plants still faces obstacles, including the browning of explants. Research on in vitro cultures of Kepel nodes by Sekar et al.(2023), Hutabarat et al.(2022), and *S. burahol* seed endosperm explants by Handayani et al. (2021) showed that although callus induction in explants can be produced, browning is still one of the inhibiting factors in callus regeneration, resulting in a low percentage of *S. burahol* live callus.

Browning is a common problem in woody plant in vitro culture, resulting in low regeneration of explants (Admojo & Indrianto, 2016) and can cause necrotic to the explant tissue. Browning can be overcome by several methods, such as soaking explants in antioxidant compounds (citric acid, ascorbic acid, and PVP), adding antioxidant compounds to the media, adding activated charcoal as an absorbent of toxic compounds to the media, and incubating the culture in a dark room, which plays a role in reducing PPO enzyme activity (Admojo & Indrianto, 2016; Kumar & Jakhar, 2018; Jakhar et al., 2019; Cai et al., 2020; Amente & Chimdessa, 2021). Therefore, this research aimed to determine the optimal browning prevention method for node explants in in vitro culture of kepel plants through immersion of explants in ascorbic acid, addition of ascorbic acid and activated charcoal in culture media, as well as incubation of cultures in light and dark conditions. The results of this research are expected to serve as fundamental information for more effective in vitro propagation of kepel plants to support the success of ex situ conservation of kepel plants.

METHOD

Tools and Materials

Tools used in this research were an analytical balance, pH meter, bottle culture, autoclave, oven, Laminar Air Flow (LAF), bunsen, stereo microscope, and digital camera. Materials used in this research were young and healthy nodal segment

(explant) of *S. burahol* obtained from Laboratory of Biotechnology, Faculty of Biotechnology UKDW, Murashige and Skoog (MS) media supplemented with BAP 1 mg/L dan IAA 5 mg/L, vitamin, agar, myo-inositol, Clorox 10%, Ethanol 70%, Carbendazim 5% (fungicide), tween 80, aquadest, liquid detergen, activated charcoal 1 g/L, and ascorbic acid 100 and 200 mg/L.

Research Design

This research used completely randomized design with treatment variations: immersion explant in ascorbic acid 100 mg/L, addition of ascorbic acid 100 and 200 mg/L to the media, addition of ascorbic acid 100 and 200 mg/L and activated charcoal 1 g/L to the media, and incubated in dark and light conditions for 28 days. The number of treatments used was 12 treatments, each repeated three times, resulting in a total of 36 treatment units (Table 1).

Table 1. Browning Prevention Treatment

Treatment code	Treatment	Immersion of Explant	Incubation condition	
K1	-	-	Light	Dark
K2	-	Ascorbic acid 100 mg/L	Light	Dark
AA1	Ascorbic acid 100 mg/L		Light	Dark
AA2	Ascorbic acid 200 mg/L		Light	Dark
Aa1	Ascorbic acid 100 mg/L + activated 1g/L		Light	Dark
Aa2	Ascorbic acid 200 mg/L + activated 1g/L		Light	Dark

Note: (-) No treatment

Procedure

Prior to inoculation, the explants were washed using running water to remove dust and dirt on the nodes, then immersed in a mixture of liquid detergent (3 ml), distilled water (27 ml), and three drops of tween 80, then vigorously shaken for 45 seconds. Explants were rinsed using distilled water three times thoroughly and continued sterilization in a laminar air flow using a combination of 10% chlorox, 70% ethanol, and 5% carbendazim fungicide for 5 minutes and 30 seconds, respectively. Explants were then rinsed using sterile distilled water three times until thoroughly clean (Hutabarat et al., 2022). Sterile explants were continued with browning prevention treatment with the treatment variation (Table 1) and inoculated into solid MS media supplemented with BAP 1 mg/L dan IAA 5 mg/L (Sekar et al., 2023). Cultures were incubated at 23–25 °C under dark and light conditions for 28 days. Browning appearance time, browning percentage, browning intensity (Ginting et al., 2023), and callus growth percentage (Sekar et al., 2023) were observed in this research. The data obtained were analyzed using Analysis of Variance (ANOVA) at the 5% level. If the results show a significant difference, it will be continued with the DMRT test (Duncan's Multiple Range Test) at the 5% confidence level.

RESULT AND DISCUSSION

Browning Initiation Stages of *Stelechocarpus burahol* Nodal Explants

The appearance of browning is characterized by a change in color in the explants to brownish or blackish. This condition is caused by the accumulation of phenol compounds in the plant cells due to the wounding of the explants during cutting at the inoculation stage. Phenol compounds will undergo oxidation to form quinones, causing brown to blackish discoloration of the explants and leading to the death of *S. burahol* explants if the condition is not overcome. The process of browning on *S. burahol* node explants in treatments K1 (explants not immersed in ascorbic acid) and K2 (explants immersed in ascorbic acid) incubated in dark and light conditions for 28 DAP (days after planting) is shown in Figure 1.

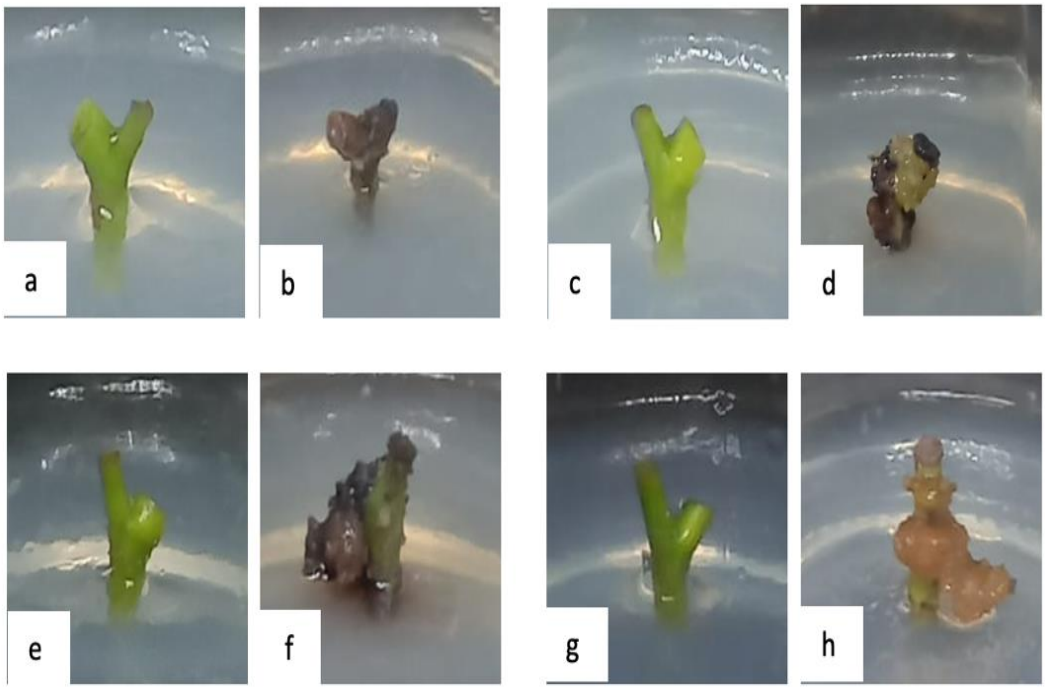

Figure 1. Browning in the nodal explants

Description: K1 light incubation (a) Day 0, (b) Day 28. K1 dark incubation (c). Day 0, (d). Day 28. Control 2 light incubation (e). Day 0, (f). Day 28. Control 2 in dark incubation (g). Day 0, (h). Day 28

Based on the results, the treatment of K1 and K2 in dark and light incubation showed a change in the color of the nodal explants from green to brown to blackish. This indicates that immersion of explants in ascorbic acid prior to inoculation and incubation in dark and light conditions has not been able to prevent browning. The difference that can be observed between both conditions is the intensity of browning color between explants that were not immersed in ascorbic acid (K1) and incubated under light conditions, which showed a change in the color of the explants to black and relatively small callus growth around the nodes (Figure 1b) compared to the K1 treatment under dark conditions and the K2 treatment under light and dark conditions (Figure 1d, f and h). The nodal explants were immersed in ascorbic acid (K2) and incubated in dark conditions, although the explants showed relatively less change in

color intensity to brown and relatively larger callus growth compared to treatment K1 in dark and light conditions and treatment K2 in light conditions (Figures 1a, d, and f). This different response could be due to the absence of anti-browning (ascorbic acid), which acts as an antioxidant to inhibit free radicals derived from cellular respiration and thus prevent browning (Amente & Chimdessa, 2021). In addition, light incubation also contributes to the increase in polyphenol oxidase (PPO) enzyme activity, which plays a role in increasing the speed of the enzymatic browning process in explants (Tarampak et al., 2019). The results of this research are in line with (Dolonseda et al., 2021), who proved that immersion of *Dendrocalamus asper* node explants in ascorbic acid and incubation in light conditions caused an increase in the intensity and percentage of browning compared to those incubated in dark conditions.

Browning Appearance Time

The measurement of browning appearance time aimed to determine the effectiveness of browning prevention methods measured based on the rate of browning appearance on the kepel node explants. Based on the ANOVA results, the variation of browning prevention methods did not have a significant effect on the browning appearance time presented in Figure 2.

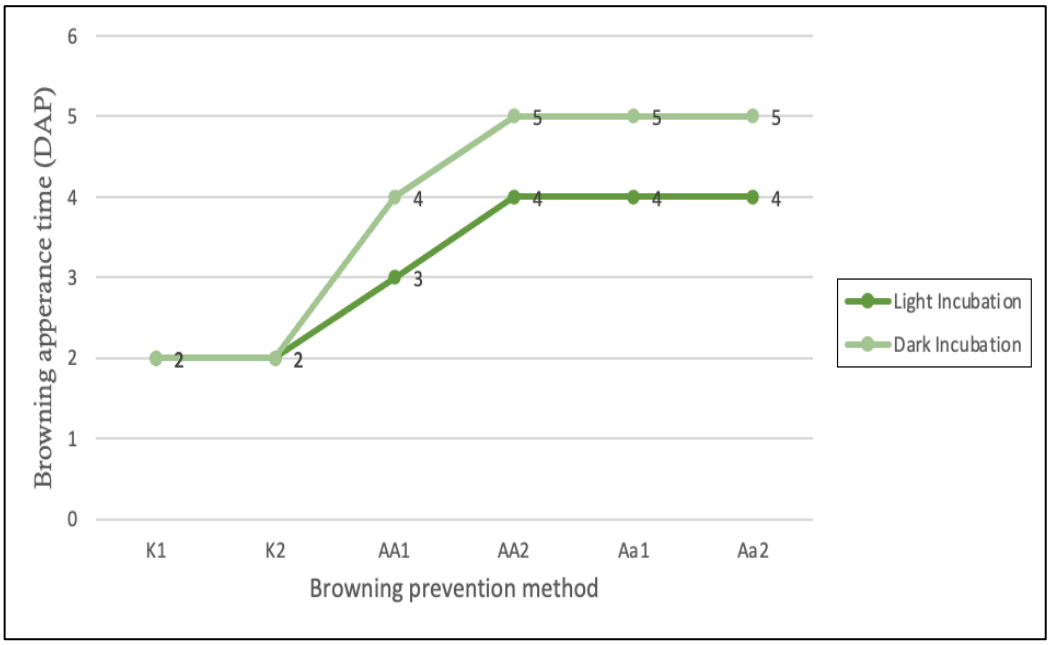

Figure 2. Browning appearance time of Kepel nodal explants

Description: K1 (Control without immersion in ascorbic acid), K2 (Explants immersed in ascorbic acid), AA1 (Ascorbic acid 100 mg/L), AA2 (Ascorbic acid 200 mg/L), Aa1 (Ascorbic acid 100 mg/L + activated charcoal 1 g/L), Aa2 (Ascorbic acid 200 mg/L + activated charcoal 1 g/L).

Based on the results in Figure 2, the addition of an antioxidant (ascorbic acid) or in combination with an absorbent (activated charcoal) to the media was more effective in inhibiting the browning appearance time (3–5 DAP) compared to the control and the treatment of soaking the explants in ascorbic acid (2 DAP). In addition, incubation in dark conditions was more effective in producing a longer browning time

(4–5 DAP) compared to light conditions (3–4 DAP). According to Ginting et al.(2023), browning in in vitro cultures can be affected by light. Light can increase the activity of enzymes involved in phenol biosynthesis and phenol oxidation. In contrast, dark conditions can reduce the activity of PPO enzymes, hence slowing down the formation of browning in explants and reducing the intensity of browning in explants.

Although there was no significant difference, the addition of ascorbic acid at a concentration of 200 mg/L and a combination of ascorbic acid (100–200 mg/L) and activated charcoal (1 g/L) into MS media proved to be more effective in reducing the speed of browning. The results of this research are in line with Helena et al.(2022), who reported that the addition of tomato extract and ascorbic acid at 150 mg/L to MS media resulted in the longest browning time (9 DAP) of *Dendrocalamus asper* node explants. In addition, Dolonseda et al.(2021) also reported that the addition of ascorbic acid 200 mg/L and activated charcoal 0.5 g/L to MS media was effective in inhibiting the appearance of browning of *Dendrocalamus asper* nodal explants (6 DAP) under dark and light conditions. The addition of ascorbic acid and activated charcoal to the culture medium proved to be more effective in delaying the appearance of browning on the explants compared to the immersion of the explants since the contact time of the antioxidants and absorbents with the explants is longer when they are in the medium compared to the contact time when the explants were immersed.

Browning Percentage and Intensity

The observation of the percentage of browning aimed to evaluate the effectiveness of browning prevention methods in reducing the level of browning in the explant population, while the intensity of browning provided more detailed information on the color change or level of browning in each explant. Based on the statistical analysis result, all browning prevention methods showed no significant difference in reducing the percentage of browning in kepel node explants (100%). However, the variation of browning prevention methods significantly affected the browning intensity value of the kepel nodal explants (Figure 3). The treatment of adding ascorbic acid 200 mg/L (AA2) and a combination of ascorbic acid 200 mg/L and activated charcoal 0.1 g/L (Aa2) to the media and incubation in dark conditions significantly reduced the intensity of browning (0.3) compared to the control and other browning prevention treatments. The results of this research are in line with Jakhar et al.(2019), who proved that the addition of ascorbic acid 200 mg/L and activated charcoal 100 mg/L into the media effectively produces low browning intensity in the node explants of the medicinal plant Gulgul (*Commiphora wightii* (Arnott)). The same results were also reported by (Admojo & Indrianto, 2016), rubber (*Hevea brasiliensis* Muell Arg Pb 330) leaf explants immersed in ascorbic acid and incubated in dark conditions effectively produced the lowest percentage of browning intensity among other treatments (7.5%). In addition, Dolonseda et al. (2021) also reported that immersion of Bamboo Petung nodal explants in a combination of 200 mg/L ascorbic acid and 0.5 g/L activated charcoal and incubation in a dark room resulted in a significantly lower percentage of browning intensity (14%) compared to incubation in bright conditions and other treatments.

The results showed that the combination of browning prevention treatment by adding an antioxidant (ascorbic acids), an absorbent (activated charcoal), and incubation in dark conditions was more effective in reducing the intensity of browning. This might be due to the role of ascorbic acid in preventing phenol oxidation and neutralizing quinones, while activated charcoal plays a role in absorbing phenol compounds and activating peroxidase enzymes, and dark conditions reduce the activity of enzymes that play a role in the formation of browning, i.e., PPO (Polyphenol Oxidase) and POD (Peroxidase) (Tarampak et al., 2019; Amente & Chimdessa, 2021; Abdalla et al., 2022).

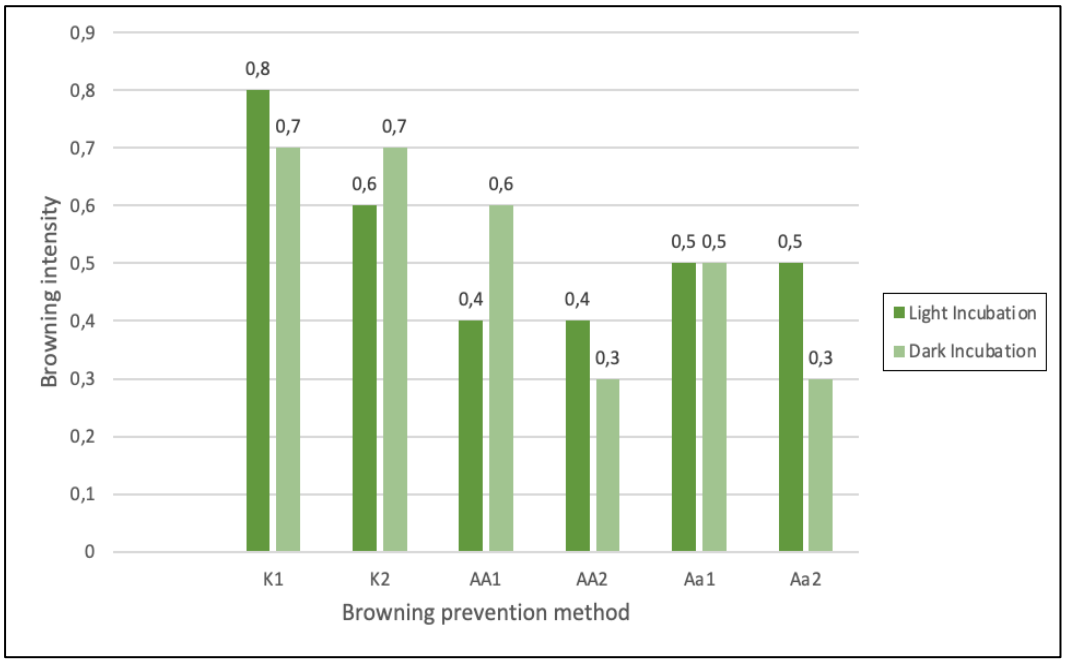

Figure 3. Browning Intensity of Kepel Nodal Explants

Description: K1 (Control without immersion in ascorbic acid), K2 (Explants immersed in ascorbic acid), AA1 (Ascorbic acid 100 mg/L), AA2 (Ascorbic acid 200 mg/L), Aa1 (Ascorbic acid 100 mg/L + activated charcoal 1 g/L), Aa2 (Ascorbic acid 200 mg/L + activated charcoal 1 g/L)

Callus Percentage

The observation of callus percentage in kepel node explants aimed to determine how effectively the browning prevention method reduced browning intensity and supported explant growth. Callus growth on nodal explants is one of the indicators of growth in in vitro culture. Browning in explants can generally cause a loss of regeneration ability in explants, and more extreme conditions can cause death in explants (Bhat et al., 2022). Based on observations of explant growth, all browning prevention methods could support callus growth (70–100%), as shown in Figure 4.

Although all browning prevention methods resulted in a browning percentage of 100% in all nodal explants, these methods proved to be able to maintain the regeneration ability of the nodal explants. In addition to the addition of antioxidants and absorbents into the media, the addition of the right ratio of auxin (IAA) and cytokinin (BAP) concentrations (5 mg/L IAA + 1 mg/L BAP) also helped in supporting callus growth from kepel node explants. Based on the ANOVA analysis,

the browning prevention method did not significantly affect the callus growth of kepel nodal explants. However, when observed based on the callus percentage, explants that were cultured on MS media with the addition of 200 mg/L ascorbic acid in dark and light conditions produced the highest callus growth percentage (100%) compared to the control and other treatments. The callus growth percentage on media added with ascorbic acid and activated charcoal showed a lower callus growth percentage of 70%.

The result of this research is in line with Das & Srivastav (2015), who reported the effect of inhibiting the growth of shoots from *Aloe vera* stem explants in vitro on culture media added with activated charcoal (0.5–2 g/L) compared to the control (without the addition of activated charcoal). However, this result is in contrast to the research of Dolonseda et al. (2021), who reported that the addition of ascorbic acid 150 mg/L and activated charcoal 0.5 g/L effectively produced in vitro shoot growth from *Dendrocalamus asper* node explants. These results indicate that the effectiveness of browning prevention methods in supporting explant growth is not only influenced by the type of browning prevention treatment but also affected by the type of explant, genotype, and the combination of PGR added to the media.

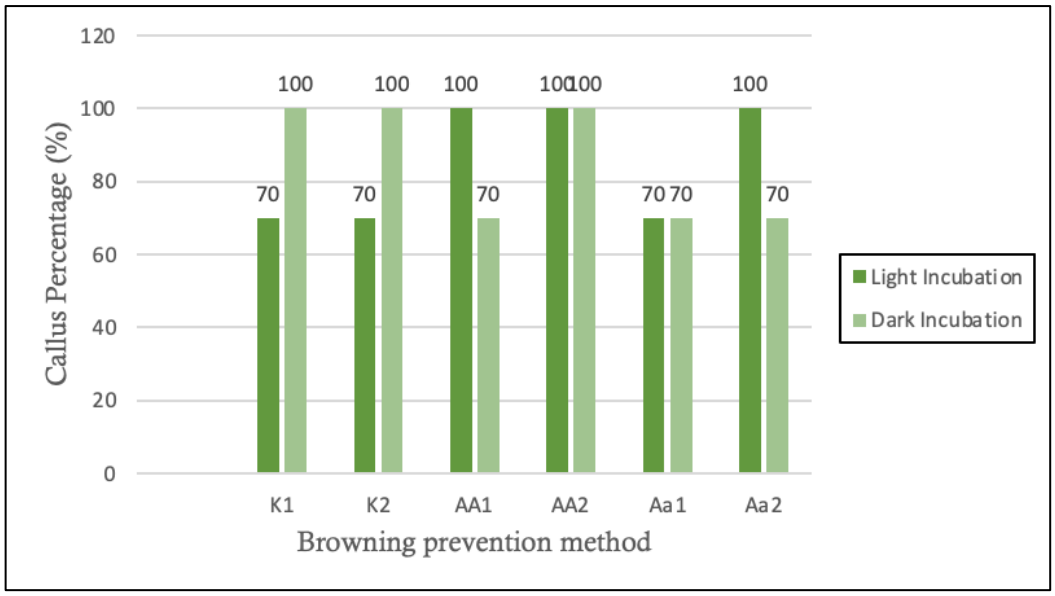

Figure 4. Callus Percentage of Kepel Nodal Explants

Description: K1 (Control without immersion in ascorbic acid), K2 (Explants immersed in ascorbic acid), AA1 (Ascorbic acid 100 mg/L), AA2 (Ascorbic acid 200 mg/L), Aa1 (Ascorbic acid 100 mg/L + activated charcoal 1 g/L), Aa2 (Ascorbic acid 200 mg/L + activated charcoal 1 g/L)

Although activated charcoal is one of the absorbents that is often used in in vitro culture as it absorbs toxic compounds produced by explants during incubation, the absorption of activated charcoal to various compounds present in the culture medium (phenol compounds from cutting explants, antioxidants, and PGR) is not selective, so appropriate concentration optimization is needed to reduce browning but also not inhibit explant growth. Based on the results of this research, the addition of 1 g/L activated charcoal to the media containing ascorbic acid and PGR was proven to reduce the percentage of callus growth in kepel node explants. This effect might be due

to the non-selective absorption activity of activated charcoal towards ascorbic acid and PGR (IAA and BAP) present in the culture medium.

CONCLUSION

In conclusion, the incorporation of ascorbic acid 200 mg/L in MS media and incubation in dark conditions was an effective browning prevention method in inhibiting the appearance time of browning (5 DAP), significantly reducing the intensity of browning (0.3) and increasing callus growth (100%) of kepel nodal explants during 28 days of culture. The results of this research are beneficial in determining the protocol of in vitro culture of kepel plants, especially at the initiation stage and can be expected to contribute in supporting the successful conservation of kepel plants through in vitro propagation.

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