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Research Article

Induction of *in Vitro* Germination of Tandui (*Mangifera rufocostata* Kostrem.): Effect of Antioxidants and 2,4-dichlorophenoxyacetic Acid

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ABSTRACT
The bark of tandui (Mangifera rufocostata Kostrem.) is commonly used as a medicine for diabetes. Regeneration of this plant is difficult, and continuous harvesting of the bark leads to a decrease in the plant population. The purpose of this research is to apply tissue culture techniques for the propagation of tandui. Different antioxidants (KNO_3, polyvinylpyrrolidone (PVP), Murashige and Skoog (MS) media + PVP, and MS + ascorbic acid) and varying concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (0.8, 1.0, 1.2, and 1.4 mg L–1) were tested for embryogenic tandui. The application of KNO_3 and PVP suppressed the formation of browning in the media and explant of tandui. Soaking the explants in KNO_3 and PVP reduce browning to 35% and 20%, respectively. The results also showed that increasing the concentration of 2,4-D enhanced the percentage of embryogenic tandui. Supplementing the media with 1.4 mg L–1 2,4-D resulted in 90% of embryogenic tandui. This study demonstrates that pre-soaking explants in antioxidants significantly reduces media browning, and supplementation of MS media with 2,4-D enhance embryogenic process. Thus, micropropagation of tandui could be achieved on a commercial basis.

Keywords: *Germination; In vitro; Micropropagation; Phenolic Compounds; Somatic Embryogenesis.*

Introduction

Mangifera rufocostata Kostrem. is a plant native to the lowland forests of Kalimantan, Sumatra and the Malay Peninsula [1]. It is traditionally used as a medicinal plant [2], with local communities believing that its bark can cure diabetes [3], [4]. However, continuous harvesting of tandui bark has led to the death of many trees. Additionally, tandui is often cut down for its large trunk diameter, which is highly valued for wood [5]. The International Union for Conservation of Nature (IUCN) lists tandui as vulnerable on its Red List. This classification suggests that while tandui survives under current conditions, it is at risk of becoming critically endangered in the future. The species is projected to experience an 80% decline over the next ten years, due to factors such as the area of occupancy, extent of occurrence, and habitat quality [6]. A study in the Hulu Sungai Selatan Regency, South Kalimantan Province, Indonesia, revealed that tandui is in the critical group and considered threatened, with only 10 trees recorded in 2010, decreasing to just 4 trees by 2014 [7].

These studies highlight the urgent need for tandui conservation to prevent extinction.

The decline in plant populations typically results from an imbalance between the number of plants dying and the number of new plants growing, whether naturally or through cultivation efforts [8], [9]. Unlike other *Mangifera* species, tandui bears fruit outside the main fruiting season [10], Consequently, most of its fruit is consumed by wild animals during the flowering and fruiting periods, leaving few to mature into new plants. This situation underscores the need for targeted propagation techniques to increase the tandui population and aid in its conservation.

Plant tissue culture is one of the most commonly used strategies for the micropropagation of exotic and endangered plants [11], [12]. This technique encompasses seed germination, meristem culture, and callus culture, all conducted under sterile conditions [13]. Various plant tissues, such as shoot buds, axillary buds, roots, shoot eyes, leaves, and embryos, can be utilized for propagation [14]. This method allows for rapid propagation of genetically identical plants free from pests and diseases [15]. Tissue culture provide benefits in micropropagation that can address challenges in tandui regeneration. However, propagating *Mangifera* species is more complex than propagating shrubs and herbs. Numerous challenges arise from explant collection to the establishment of in vitro cultures, with one major issue being browning caused by the oxidation of phenolic compounds during explant preparation, which can hinder successful plant propagation [16], [17]. Additionally, successful plant germination in tissue culture requires plant growth regulators (PGRs), with specific PGR requirements varying by species [18–20]. These challenges highlight the importance of preliminary research to optimize treatments such as sterilization pre-treatment and PGR selection, to improve propagation success.

Previous studies have highlighted the potential of tissue culture as a rapid and large-scale propagation system for superior plant genotypes [21], [22]. Several studies have successfully employed tissue culture techniques to propagate *Mangifera* species [23], [24]. For example, Conde *et al* [25] demonstrated that in vitro germination of *M. indica* seeds led to a greater increase in the number of developing embryos compared to ex vitro conditions. They also found that using cotyledonary nodes is an efficient method for mass propagation of *M. indica*. Amente & Chimdessa [26], reported that presoaking explants in an antioxidant solution or incorporating antioxidants into the medium effectively suppresses browning during *M. indica* micropropagation. Additionally, Meenashree *et al* [27], recommended the use of PGRs to enhance the success rate of *M. indica* micropropagation. These findings suggest that tissue culture has been effective and is extensively utilized for the micropropagation of *M. indica*. However, there is still limited information regarding in vitro techniques for the propagation of tandui. Therefore, this study aimed to explore tissue culture application for the micropropagation of tandui. The study was conducted in two stages: (1) assessing the impact of different antioxidants on browning in the tissue culture of tandui, and (2) evaluating the effect of varying concentrations of the plant growth regulator 2,4-dichlorophenoxyacetic acid on the formation of tandui embryos. The hypotheses are: (1) various antioxidants exhibit different capabilities in preventing browning in the tissue culture of tandui, and (2) increasing the concentration of 2,4-dichlorophenoxyacetic acid enhances the embryo percentage of tandui.

Materials and Methods

Plant materials and chemicals

Immature tandui fruits, around 40 days after flowering (4–6 cm in length) were collected from trees growing naturally in the forest in the Balangan Regency,

South Kalimantan Province, Indonesia (Figure 1). The annual rainfall in this area ranges from 2253−2926 mm, with the driest month in August receiving an average of 91.49 mm, and the wettest month in November with 390.38 mm of rainfall. The average temperature is between 27.20−27.73°C, with minimum temperatures ranging from 21.25−22.25°C and maximum temperature from 33.15−33.90°C.

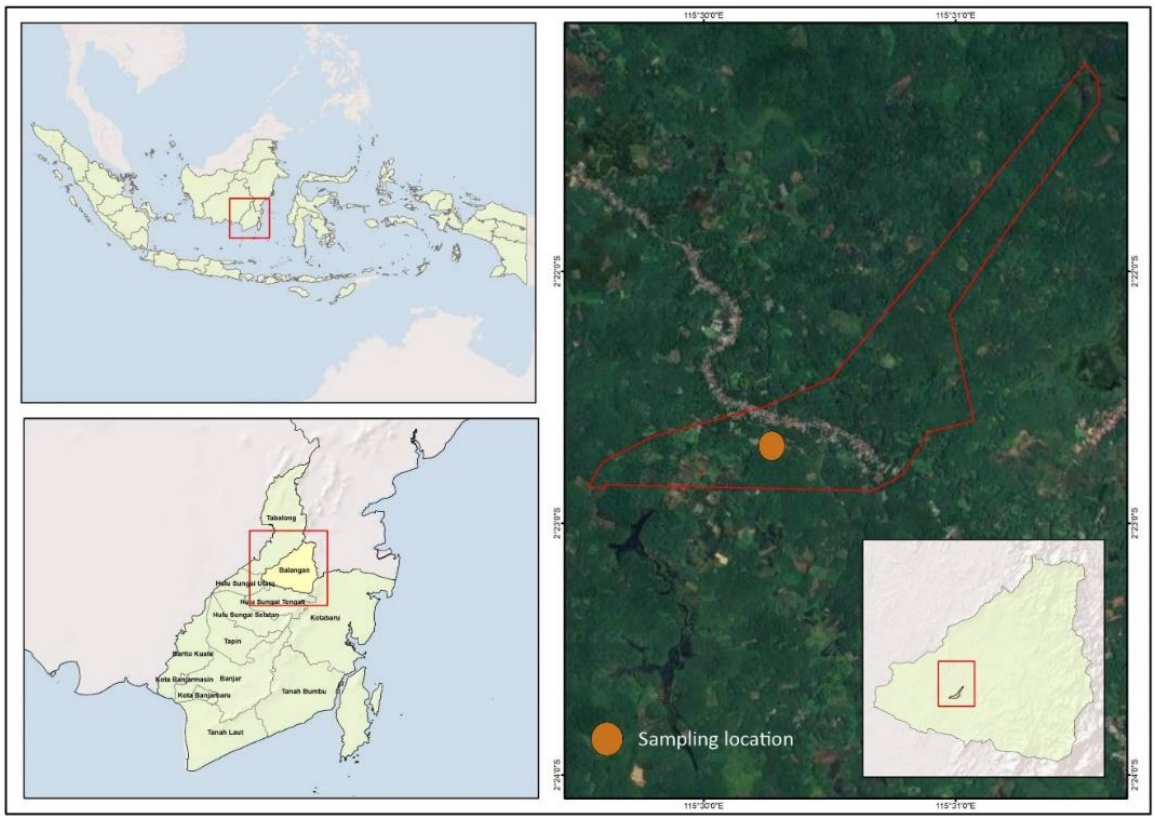

Figure 1. Study site located in Balangan District, South Kalimantan Province, Indonesia.

The fruits used in this study were selected from healthy, brownish-green specimens free of pests and diseases. The fruits were first washed with liquid detergent, then rinsed thoroughly in running tap water. Dissection involved removing the outer skin and flesh of the fruits, then bisecting the seed coat longitudinally to obtain the embryo sac. Seed coats were discarded, and only the nucellus tissues were used for culture.

Agar, Murashige and Skoog Basal Medium, and sucrose powder were purchased from Duchefa Biochemie, Netherlands. Potassium nitrate (KNO_3), 2,4-dichlorophenoxyacetic acid (2,4 D), ascorbic acid, and polyvinylpyrrolidone (PVP) were obtained from Sigma–Aldrich Co., Singapore. All chemicals used in the assays were of analytical grade.

Effect of antioxidants on Browning

This study aimed to determine the effect of sterilization treatment on explants in reducing browning during the induction of germination of tandui using an in vitro method. A single-factor completely randomized design was employed to test treatments aimed at reducing phenol oxidation. The treatments included: (a) soaking explants in a 125 mg L^{-1} KNO_3 solution for 1 minute, (b) soaking explants in a 500 mg L^{-1} PVP solution for 1 minute, (c) soaking explants in a liquid MS medium with 100 mg L^{-1} PVP and agitating at 15 rpm for 24 hours, and (d) soaking explants in a liquid MS medium with ascorbic acid

and agitating at 15 rpm for 24 hours. These treatments were selected based on the effective use of various antioxidants in tissue culture to suppress browning during the micropropagation of Mangifera species [27–30]. Each treatment was replicated five times, resulting in a total of 20 experimental units. Explants (nucellus tissue) of tandui, which had been surface sterilization according to the treatment, were then planted on MS media with an addition of 100 mg L^{-1} PVP. During the experiment, all cultures were stored in a culture room at 25 ± 2°C for 12 weeks. The observed variables included the emergence of browning, percentage of browning, percentage of contaminated, and survival of explants.

Effect of 2,4-Dichlorophenoxyacetic Acid on Tandui Embryo

The aim of this study was to determine the effect of the concentration of plant growth regulator (2,4-dichlorophenoxyacetic acid, 2,4-D) in MS medium on the germination of tandui in tissue culture. The treatments tested in this study involved supplementing MS media with various concentrations of 2,4-D: 0.8, 1.0, 1.2, and 1.4 mg L^{-1}. The concentration range of 2,4-D tested was based on several previous studies on the micropropagation of M. *indica*, which used a maximum dose of 2 mg L^{-1} [31–33]. Each treatment was replicated five times, resulting in 20 experimental units. Explants (nucellus tissue) of tandui, pretreated by soaking in 125 mg L^{-1} KNO_3 solution for 1 minute (from the first experiment), were then grown on MS media + PVP 100 mg L^{-1} supplemented with different concentration of 2,4-D. During the experiment, all cultures were stored in a culture room at 25 ± 2°C for 10 weeks. The observed variables included embryogenic growth time and percentage of embryos.

Experimental design and data analysis

The experimental design used was a single-factor completely randomized design. The effect of the treatments on the observed parameters were quantified using analysis of variance. Prior to the analysis, the data's normality and homogeneity of variance were tested using the Shapiro-Wilk and Bartlett tests, respectively. If the results indicated that the observed data were not normally distributed and exhibits non-homogeneous variance, data transformation was performed to achieve normal distribution and homogeneous variance. When analysis of variance showed a significant effect of the treatment on the observed parameters, the means of the treatments were differentiated using the least significant difference (LSD) test at $P < 0.01$. The GenStat 11th Edition was employed for all statistical analyses.

Results and Discussion

Percentage and Times of Browning

Some plants, particularly tropical species such as those in the *Mangifera* family, contain high concentrations of phenolic substances that are oxidized when cells are wounded or senescent [31], [33]. Browning occurs when phenolics exudate from the cut ends of mango explants oxidize to quinones, which produce brown pigments in wounded tissues. Polyphenol oxidases, a group of copper-proteins, catalyze the oxidation of phenolics to quinones [17], [32], which may inhibit growth, result in a loss of regenerative capacity, and cause cell death in explants [34]. The accumulation and oxidation of phenolic compounds are thought to be the main reasons for growth inhibition and a decrease in the regeneration ability of plant cells [35]. Pretreating explants with chemical compounds helps mitigate the exudation of phenolics from the cut ends. The application of antioxidants can reduce the activity of polyphenol oxidase.

The results of the study showed that different antioxidants had varying abilities to prevent browning in the tissue culture of tandui. Tandui explants soaked in liquid MS media with added PVP 100 mg L^{-1} or ascorbic acid, then agitated for 24 hours at

15 rpm, did not prevent the formation of browning, as all explants in this treatment exhibited browning (Figure 2B). However, the treatment of tandui explant soaked in KNO_3 125 mg L^{-1} for 60 seconds and in PVP 500 mg L^{-1} for 60 seconds successfully suppressed the browning process. Soaking tandui explants in KNO_3 and PVP suppressed browning by 35% and 20%, respectively (Figure 2A).

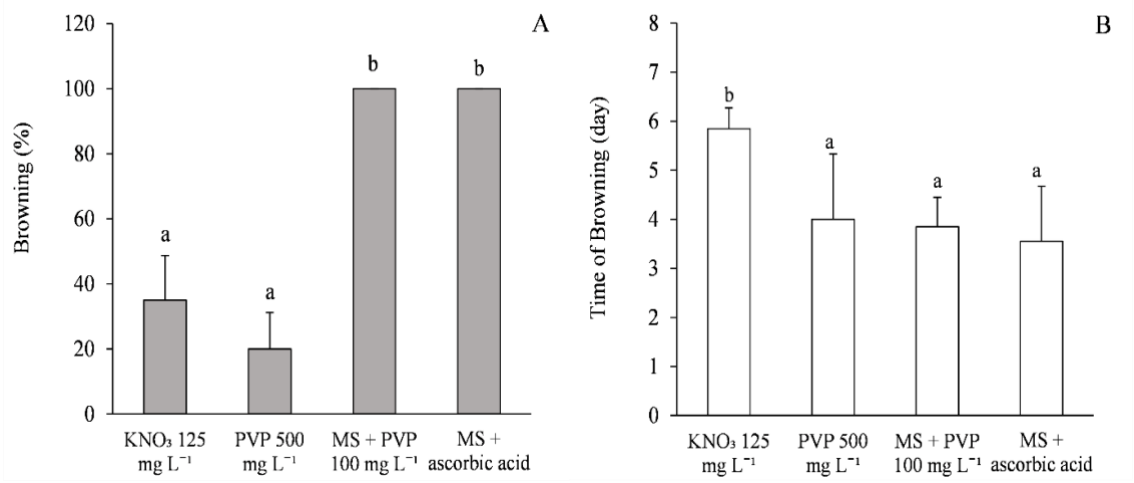

Figure 2. Effect of different antioxidants on % browning (A) and time to browning (B) in tandui (*Mangifera rufocostata* Kostrem) tissue culture, observed 12 weeks after antioxidant application. Lines above the bars represent the standard deviation of the mean (n=5). The same letters above the lines indicate similar effect of treatments based on the least significant difference (LSD) test at P <0.05.

The results of the study also showed that the appearance of browning in tissue cultures of tandui varied based on the applied antioxidants. The appearance of browning in the explant soaking treatments with PVP 500 mg L^{-1}, liquid MS + PVP 100 mg L^{-1} and liquid MS + ascorbic acid agitated for 24 hours occurred at relatively the same time, ranging from 5 hours to 96 hours after antioxidant application (Figure 2B). In contrast, soaking plant explants in KNO_3 125 mg L^{-1} resulted in slower browning formation, i.e., 140 hours after antioxidant application (Figure 2B). The results of previous studies also showed that immature mango embryos inoculated in MS medium supplemented with 100 mg L^{-1} of cysteine, 0.5 mg L^{-1} of gibberellic acid (AG3), and 30 g L^{-1} sucrose showed a high oxidation rate of 60% after 72 hours [23].

Contamination rates and survival of plant explants

Observation of the percentage of contamination at 12 weeks after antioxidant application showed that all tandui explants with the agitation treatment for 24 hours in a solution of MS + PVP 100 mg L^{-1} and liquid MS + ascorbic acid were contaminated (100% contaminated) (Table 1). However, explants soaked in KNO_3 solution of 125 mg L^{-1} and PVP 500 mg L^{-1} were able to reduce explant contamination to 20% and 35%, respectively (Table 1). The results of this study indicate the importance of antioxidant treatment in tissue culture to reduce explant contamination.

The results also showed that different antioxidant applications resulted in different responses to the survival percentage of explants. No tandui explants survived after 12 weeks of antioxidant application in the treatments with MS + PVP 100 mg L^{-1} and MS + ascorbic acid (Table 1). However, explants soaked in KNO_3 125 mg L^{-1} and PVP 500 mg L^{-1} had survival percentages of 85.00% and 65%, respectively (Table 1).

The experime ntal results indicated that soaking explants in KNO_3 125 mg L^{-1} was the most promising techniques to reduce the oxidation of phenolic compounds. The effect of KNO_3 and PVP application in suppressing the oxidation process of phenolic compounds, which causes browning in tissue cultures of several plants, has also been reported in previous studies. Research conducted by Shirazi *et al* [28], showed that tissue culture of hazelnut (*Corylus avellana* L.) using Nas and Read (NR) media with the addition of 500 mg dm^{-3} of PVP produced the least amount of browning (9.15%). PVP is known to be the most potent antioxidant, as it absorbs phenols through hydrogen bonding and thus prevents their oxidation [36]. A study by Meenashree *et al* [27], showed that the application of 1.5 g L^{-1} KNO_3 completely reduced browning of calli *Bacopa monnieri*, a medical plant with high economic value. The role of KNO_3 in reducing browning was also reported by Aldaej *et al* [37], who found that decreasing the concentration of KNO_3 in tissue culture of date palm (*Phoenix dactylifera* L.) led to increased browning in explants. Sterilizing pomegranate explants (*Punica granatum* L.) with 3% sodium hypochlorite for 15 min and frequent sub-culturing of the explants on media containing PVP plus silver nitrate was found to be the optimal treatment for controllling oxidative browning and initiating callus [38]. The results of this study highlight the significant role of KNO_3 and PVP in reducing browning in plant tissue culture.

Table 1. Effect of different antioxidants on the survival of Tandui (*Mangifera rufocostata* Kostrem) explants observed 12 weeks after antioxidant application

Antioxidants	Contamination (%)	Survival of Explants (%)
Potassium nitrate 125 mg L^{-1}	20.00 (11.18)* a**	85.00 (37.91) a
PVP 500 mg L^{-1}	45.00 (27.39) b	65.00 (37.08) a
MS + PVP 100 mg L^{-1}	100.00 (0.00) c	0.00 (0.00) b
MS + ascorbic acid	100.00 (0.00) c	0.00 (0.00) b

*Notes: * Numbers in parentheses represent the standard deviation of mean (n=5).*
*** Numbers followed by the same letter in each column indicate that the treatments were not significantly different based on the least significant difference (LSD) test at P <0.05.*

Several other studies, however, indicated different results from to those obtained in this study. For example, the application of different concentrations of KNO_3 to culture media of date palm (*Phoenix dactylifera* L.) did not affect the frequency of browning [39]. The use of ascorbic acid in this study did not reduce browning (Figure 1), in line with the results of a study conducted by Booranasrisak [40], who reported that ascorbic acid and activated charcoal were ineffective in eliminating browning in tissue culture of 8 indigenous Thai mango varieties. However, a study by EL-Gioushy *et al* [41], showed different results, where the application of ascorbic acid of 1 g L^{-1} showed the best results in reducing browning formation in tissue culture of coconut (*Cocos nucifera* L.). Moreover, incorporating an antioxidant agent (100 mg L^{-1} ascorbic acid) for 2-24 h prior culturing in the medium is the most effective treatments for controlling phenol exudation in plant tissue culture [42]. The variation in the effect of ascorbic acid on browning observed in this study compared to previous studies might be attributed to the type of explant used. In studies by Abahmane [39] and El-Gioushy *et al*. [41], where ascorbic acid effectively reduced browning, shoot tips were used as explants. Shoot tips typically exhibit higher metabolic activity and phenolic content, which result in more significant browning.

Ascorbic acid helps mitigate this browning by neutralizing free radicals and reducing the oxidation of phenolic compounds [43]. On the other hand, seeds, which were used as explants in this study, generally have a different composition and lower phenolic content than shoot tips. Consequently, the impact of ascorbic acid on reducing browning in seed explants may not be as pronounced as with shoot tips.

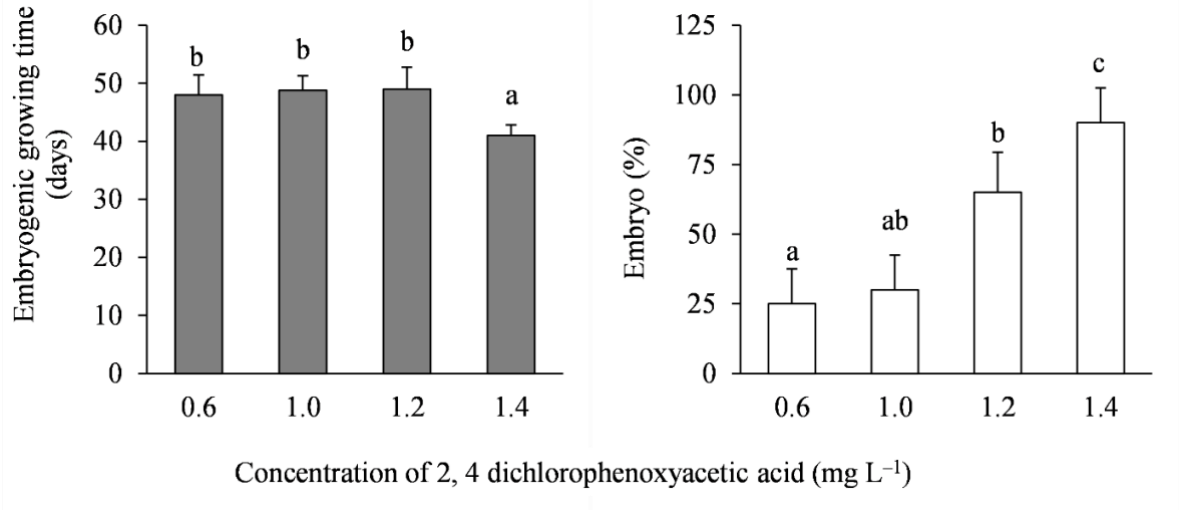

Figure 3. Effect of 2,4-dichlorophenoxyacetic acid (2,4-D) concentrations on the embryogenic growth time (left) and embryo percentage of tandui (*Mangifera rufocostata* Kostrem) tissue culture. Lines above the bars represent the standard deviation of the mean (n=5). The same letters above the lines indicate similar effects of treatments based on the least significant difference (LSD) test at P <0.05.

The effect of antioxidant on browning in tissue culture may vary significantly among different plant species due to differences in their metabolic pathways and phenolic content. Some species, such as apple (*Malus domestica*) and banana (*Musa* spp.), are particularly prone to browning due to their high phenolic content. In these cases, antioxidant are quite effective in reducing browning [44]. This is consistent with previous studies that reported the success of treatments such as pretreatment of explants with antioxidants, incorporation of antioxidants into the culture medium, and the application of absorbent, for reducing browning in tissue cultures. These effects are largely determined by the plant species and the types of explants used [42].

Embryogenic growth

Subsequent research on tissue culture of tandui showed that both the embryogenic growth time and the percentage of embryos were influenced by the concentrations of 2,4-D. Increasing the concentrations of 2,4-D resulted in reduction in embryogenic growth time for tandui. Embryogenic growth times ranged from 48 to 49 days with 0.6–1.2 mg L^{-1} of 2.4-D application, and the time reduced to 41 days when 2,4-D concentration increased to 1.4 mg L^{-1} (Figure 3). The study also showed that increasing the concentrations of 2,4-D led to a higher percentage of embryos. The embryo percentage increased from 25% with 0.6 mg L^{-1} of 2,4-D application to 65% and 90% with 1.2 and 1.4 mg L^{-1} of 2,4-D, respectively (Figure 3).

It is well known that plant growth regulator (PGRs) play an important role in the success of somatic embryogenesis of plants. Micropropagation procedures in tissue culture are based on the main principle that plant growth prior to shoot regeneration, from existing apical and axillary meristems, is controlled by the

presence of PGRs, especially by the balance of auxins and cytokinins in the culture medium [45]. In addition, *in vitro* propagation of plants, such as through somatic embryos (which are basically imitations of zygotic embryos in morphology), requires strong PGR exposure to plant cells [46]. One commonly used PGR that shows high success in plant propagation in vitro is the auxin-like compound 2,4-D [47], [48]. The use of 2,4-D has been reported to promote somatic embryogenesis by influencing plant cell development, root growth, cell growth, cell division, and the formation of adventitious roots [49].

The present study indicates that MS medium supplemented with 2,4-D is necessary for embryo formation. This finding is consistent with results from similar studies on somatic embryogenesis in durian (*Durio zibethinus* Murr.) [49], sugarcane (*Saccharum officinarum* Linn.) [50], and castor bean (*Moringa oleifera* Lam.) [51]. Sajana *et al* [24], reported that embryonic calli of *Mangifera* developed completely on induction medium containing 5 mg L^{-1} of 2,4-D. The application of 4.0 mg L^{-1} 2,4-D to the medium also enhanced callus production in jojoba (*Simmondsia chinensis*) [46]. Moreover, callus induction in finger millet (*Eleusine coracana*) significantly increased with 1.5 mg $L^{-1}$of 2,4-D application [48]. In a propagation study of *Spartium junceum* L. using tissue culture, Taghizadeh and Dastjerdi [52], reported that the highest callus formation and the lowest explant browning were obtained on media supplemented with 0.5 mg L^{-1} 2,4-D under dark conditions. The results of this study demonstrate the crucial role of 2,4-D in the micropropagation of plants.

Conclusion

The results show that different antioxidants have varying effects on the occurrence of browning in the tissue culture of tandui (*Mangifera rufocostata* Kostrem). Soaking the explants in a solution of KNO_3 125 mg L^{-1} and polyvinylpyrrolidone (PVP) 500 mg L^{-1} effectively suppresses browning, while other treatments result in 100% browning in the explants. Additionally, KNO_3 and PVP treatments resulted in a longer browning emergence time, a lower percentage of explants experiencing contamination, and a higher percentage of explant survival compared to other treatments. These findings indicate that KNO_3 125 mg L^{-1} and PVP 500 mg L^{-1} are superior in suppressing the browning process in tissue cultures of tandui, suggesting that this treatment has the potential to be used in pretreatment processes for the in vitro propagation of tandui. Given that KNO_3 may also supply nitrogen for explant growth, this treatment could be employed in sterilization for further research on the tissue culture of tandui. The results also show that the concentration of plant growth regulators significantly affects embryo formation. Increasing the concentrations of 2,4-D result in a reduction in embryogenic growth time and an increase in the embryo percentage of tandui explants. This study demonstrates that pretreating xplants with antioxidants significantly controls lethal browning, while supplementation of the media with plant growth regulators results in efficient micropropagation of tandui.

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Conflict of interest

We declare that there is no conflict of interest.

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