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Plant Regeneration and Antioxidant Activity of *Hippobroma longiflora*

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Abstract
Hippobroma longiflora, a medicinal plant in the Campanulaceae family is valued for its therapeutic qualities by Asian communities. This study examined its antioxidant properties and phytochemical composition in *in vitro* propagated plants and those grown naturally. *H. longiflora* has diverse medicinal attributes, including antioxidative, anti-inflammatory, and antimicrobial activities. In Indonesia, its flowers and leaves are traditionally used for ocular conditions like cataracts. Seeds were cultured on Murashige and Skoog medium with 2 mg/L BAP and varying NAA concentrations. Antioxidant activity was assessed through DPPH scavenging and FRAP assays. Immature white seeds showed 50% germination in 7 weeks. The treatment involving 2 mg/L BAP and 0.3 mg/L NAA exhibited the most favorable *in vitro* propagation results, achieving 100% shoot regeneration and a 0.70 multiplication rate. The combination of 2 mg/L BAP with 0.5 mg/L NAA displayed the highest antioxidant activity, with inhibition of 51.42% in the DPPH assay and a FRAP value of 10559.40 µM Fe^{2+}/g. These findings highlight the potential therapeutic application of *H. longiflora*, particularly for ocular ailments like cataracts.
Keywords: *Hippobroma longiflora*; Shoot Induction, DPPH, FRAP, Antioxidant Activity

Introduction

Hippobroma longiflora is under scrutiny for its potential medicinal applications, notably in cataract treatment. Cataract surgery is limited by the absence of true accommodative ability (Imelda et al., 2022). Secondary metabolites from *H. longiflora* exhibit a range of properties, including antitumor, anti-insecticide, anticancer, antibacterial, antimicrobial, antifungal, antioxidant, anti-inflammatory, antimalarial, and antiseptic effects (Imelda et al., 2022). The focus has recently shifted to cultivating *H. longiflora* through in vitro plant tissue culture, driven by its manifold advantages (Yee & Abd Samad, 2022). Successful cultivation using this approach depends on multiple factors, with plant growth regulators (PGRs) playing a pivotal role.

Metabolite production can diverge based on plant origin and culture conditions. Comparative studies comparing compounds from tissue-cultured plants to those in the wild reveal distinctions in their phytochemical profiles. Plant tissue culture has ascended as a prime avenue for generating secondary metabolites, attributing to its diverse merits (Yee & Abd Samad, 2022). Earlier studies have evidenced that 2 mg/L of BAP, a type of cytokinin, fosters *H. longiflora* growth without compromising secondary metabolite and phytochemical production (Yee & Abd Samad, 2022). In light of this context, this study aimed to contrast the antioxidant activity of in vitro plants, utilizing a blend of 2 mg/L BAP and varying NAA concentrations, with field-grown *H. longiflora*.

Materials and methods

The immature (white) and mature (brown) seeds acquired from Agrotani at Universiti Teknologi Malaysia (UTM) as explants for tissue culture and shoot initiation. For the comparison between field-

grown and *in vitro* propagated plants, vegetative cuttings of *H. longiflora* leaves were collected. Murashige and Skoog (MS) medium served as the culture medium.

To eliminate contamination, *H. longiflora* seeds were soaked in DYNAMO® detergent, followed by rinsing under tap water for 30 minutes. The seeds were then soaked in 70% ethanol for 1 minute and rinsed with distilled water to remove ethanol. The sterilization process continued with a 50% bleach solution and three drops of Tween 20 added to a conical flask. The flask with explants was shaken at 130 rpm for 30 minutes. After rinsing with distilled water five times, explants were cultured on MS free media and 2 mg/L BAP. All sterilization was performed in a laminar flow cabinet to maintain aseptic conditions (Yee & Abd Samad, 2022). Capsules of *H. longiflora* were opened to extract seeds after surface sterilization. Immature and mature seeds were treated in Petri dishes with control MS free media and MS media with 2 mg/L BAP. After 7 weeks, observations were made on shoot emergence, length, and overall growth, providing data to analyze the treatment effects on shoot induction in *H. longiflora*. The seeds with the highest germination proceeded to the next step (Yee & Abd Samad, 2022).

Following seed germination, small plantlets were transferred from Petri dishes to jam jars with seven different treatment samples. These included negative control (MS free media), positive control (2 mg/L BAP), and treatments with 2 mg/L BAP combined with varying NAA concentrations (0.1, 0.2, 0.3, 0.4, 0.5) mg/L. Plantlets were subcultured every two weeks for ten weeks to maintain growth. Cultures were observed for responses in a controlled room at 25 ± 2℃ with a 16-hour photoperiod provided by cool white, fluorescent tubes (3000 lux). Three replicates for each concentration ensured result reliability. After ten weeks, parameters like average shoot length, average shoots per explant, percentage of shoot regeneration, and shoot multiplication rate were recorded and analyzed, with the multiplication rate calculated using a specified equation (Sharma and Ramamurthy, 2000).

$$\text{Multiplication rate} = \frac{Number\ of\ shoots\ after\ culturing}{Number\ of\ shoots\ at\ beginning}$$

For plant extraction, samples were collected from both in vitro tissue culture plants and field-grown leaves of *H. longiflora*. The leaves were washed with tap water to eliminate external contaminants, followed by cutting into small pieces. These leaf fragments were weighed and crushed into a powder using liquid nitrogen. About 1 g of the powdered plant material was mixed with 20 ml of 70% ethanol. The mixture underwent maceration in a conical flask for three days within an incubator shaker operating at 100 rpm and 30℃. After this period, the ethanolic solutions were filtered to remove solid particles using filter papers. The obtained ethanol filtrates were stored in Falcon tubes and subjected to vacuum evaporation using an IKA® RV 10 auto rotary evaporator. The concentrated solution was subsequently freeze-dried to obtain a powdered form, which was stored at 4℃ for further investigations such as DPPH assay and FRAP assays (Yee & Abd Samad, 2022).

Fresh DPPH solution was prepared by dissolving 4 mg of DPPH powder in 100 ml of absolute ethanol. In a 96-well microplate, 100 µl of DPPH solution was added to different concentrations of *H. longiflora* extract (ranging from 1.95 µg/ml to 1000 µg/ml) through 2-fold dilution. Control samples included a DPPH solution in ethanol and a blank of 70% ethanol. Ascorbic acid served as a positive control for antioxidant activity, with the same procedure conducted for comparison. After standing for 30 minutes in a dark room, the 23 solutions in microplates were tested for absorbance at 517 nm using a SPECTROstar Nano microplate reader. A standard curve was constructed using the absorbance at 517 nm against different concentrations of ascorbic acid (1.95, 3.91, 7.81, 15.63, 31.25, 62.50, 125, 250, 500, and 1000 µg/ml). Antioxidant activity of H. longiflora extract and ascorbic acid was calculated and expressed as inhibition percentage (%) and IC_{50} value (Yee & Abd Samad, 2022). The percent inhibition value is derived using the following equation:

% Inhibition = [(A control−A sample) A control] × 100

A control = Absorbance of the control
A sample = Absorbance of the sample

To prepare the FRAP reagent, a combination of 50 ml of 0.3 M acetate buffer at pH 3.6, 5 ml of 10 mM tripyridyltriazine (TPTZ) solution in 40 mM HCl, and 5 ml of 20 mM ferric chloride solution was made (Mohd Usri & Abd Samad, 2020). This FRAP reagent was freshly prepared and pre-warmed in an oven to 37°C before application. For the standard solution, 100 µl of various concentrations (ranging from 0 to 100 µM) of 1 mM ferrous (II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) was mixed with 200 µl of the freshly prepared FRAP reagent. In wells containing 100 µl of 1 mg/ml *H. longiflora* ethanolic extract and absolute ethanol as a blank, 200 µl of FRAP reagent was added. The solutions within the microplate were mixed and then incubated for 30 minutes at 37°C in a dark environment. The absorbance of the standard solution, the samples, and the blank were measured and recorded at 593 nm using a SPECTROstar Nano microplate reader at room temperature (Yee & Abd Samad, 2022).

To ensure data reliability, each experiment was performed in triplicate for each treatment and sample concentration. Significant distinctions were identified through statistical analysis employing SPSS Statistics (Statistical Package for the Social Sciences), with results presented as mean standard error (SEM).

Results and discussion

The study involved collecting *H. longiflora* specimens from Agrotani UTM in two distinct forms, whitish-yellow and brown, which served as indicators of seed ripeness. Whitish-yellow denoted unripe seeds, while brown signified ripe seeds, as explained by Ero (2015). It was observed that immature stage embryos displayed the highest potential for sorghum tissue culture, as reported by Ahmed et al. (2021). In this experiment, it was noted that immature seeds exhibited a higher germination percentage compared to mature seeds. Moreover, the most effective treatment for seed germination was found to be the MSO treatment, which did not involve the application of any hormones in the MS media.

Based on the information presented in Table 1, this experiment evaluated the germination percentages of *H. longiflora* seeds, focusing on both white and brown seeds. The treatments included a control group (MSO) and a treatment group with 2 mg/L BAP, a synthetic cytokinin hormone. The results revealed that the control treatment (MSO) displayed higher germination percentages compared to the treatment with 2 mg/L BAP. In the case of white seeds, the control treatment resulted in a germination percentage of 50%, whereas the BAP treatment led to a lower germination percentage of 35%. Similarly, for brown seeds, the control treatment exhibited a germination percentage of 20%, while the BAP treatment had a lower germination percentage of 15%.

Table 1: Percentage of seeds germination of *H. longiflora*

Seeds germination percentage of *H. longiflora* (%)		
Treatments	**Immature seeds (white)**	**Mature seeds (brown)**
0 (Control/MSO)	50	20
2 mg/L BAP	35	15

Data were expressed as N=3 replicates.

The observations indicated that both the control group (MSO) and the treatment group with 2 mg/L BAP displayed a higher germination percentage in immature seeds compared to mature seeds. This discovery challenges the conventional belief that seed maturity is a prerequisite for successful germination. To sum up, the findings suggest that white seeds, representing immature seeds, exhibited the highest germination rate. Notably, seeds treated with MSO, the hormone-free medium, showed the most favorable germination results. Figure 1 illustrates small plantlets derived from both immature and mature seeds under two different treatments: media without plant growth regulators (PGRs) and media supplemented with 2 mg/L BAP.

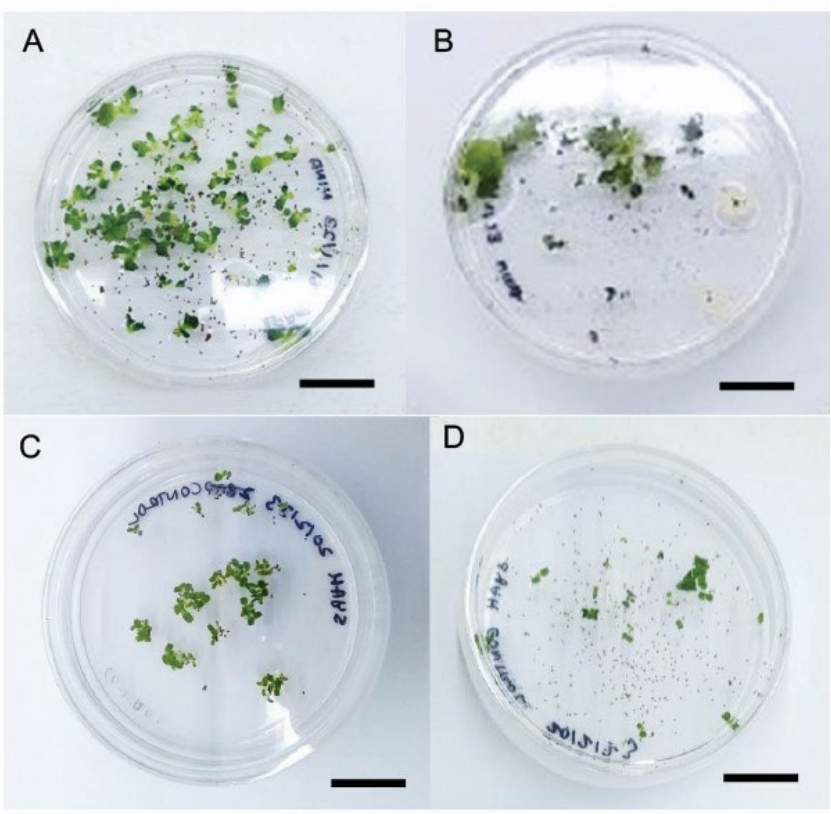

Figure 1 Small plantlets originating from both immature and mature seeds, with the following conditions: A) Immature seeds cultivated on MS-free media; B) Immature seeds subjected to 2 mg/L BAP treatment; C) Mature seeds grown on MS media; and D) Mature seeds treated with 2 mg/L BAP. Scale bars: 1 cm.

Auxins and cytokinins are two essential subgroups of plant growth regulators (PGRs) crucial for successful tissue culture. In contrast to auxin, which influences various aspects of plant development, such as stem elongation, root formation, differentiation, apical dominance, phototropism, fruit development, and geotropism, cytokinins serve as hormones that control cell division, regulate developmental processes, and boost metabolic activity in plants (Alfarisi, 2019). In this particular experiment, BAP (benzylaminopurine) was intentionally maintained at a fixed concentration of 2 mg/L. This choice was based on a prior study that established 2 mg/L BAP as the most optimal concentration for promoting the growth of *H. longiflora* (Yee & Abd Samad, 2022). On the other hand, NAA (naphthaleneacetic acid) was used as an auxin and tested at five different concentrations: 0.1, 0.2, 0.3, 0.4, and 0.5 mg/L. The inclusion of these hormones in the culture media is a common practice due to their proven ability to effectively enhance explant growth (Pamela et al., 2014). The experiment aimed to determine the specific concentrations of these plant growth regulators that would yield optimal results in terms of shoot production and morphological attributes. Table 2 presents the impact of the combination of BAP and NAA on shoot regeneration.

Table 3 provides a comprehensive analysis of the effects of different treatment combinations involving BAP and NAA on shoot regeneration percentage (%), the number of shoots per explant, the length of shoots per explant (cm), and shoot characteristics in tissue culture. All data are expressed as the mean ± standard error of the mean (SEM) of the analysis (n=3). The significance level between means is indicated as ($p<0.05$) for significant differences or ($p>0.05$) for no significant difference between the means. The dataset consistently shows a 100% shoot regeneration percentage across all treatment combinations. However, notable disparities are evident in the number of shoots regenerated per explant and the length of shoots per explant among the different treatments.

Table 2: Effect of combination of BAP and NAA on shoot regeneration

Treatments combination (mg/L)		Percentage of shoots regeneration (%)	No. of shoots per explant ($\bar{x}$ ± SEM)	Length of shoots per explant (cm) ($\bar{x}$ ± SEM)
BAP	**NAA**			
0	0	100	2 ± 0.00	4.5
2	0	100	6 ± 0.88	1.4
	0.1	100	7 ± 1.00	1.1
	0.2	100	6 ± 0.67	1.27
	0.3	100	8 ± 0.57	1.77
	0.4	100	6 ± 1.45	1.03
	0.5	100	5 ± 0.58	1.03

Table 3: Shoot multiplication rate of *H. longiflora* after 10 weeks of culture

Treatment combinations (mg/L)		Number of shoots produced per explant	Multiplication Rate per 10 weeks
BAP	**NAA**		
0	0	2 ± 0.00	0.07
2	0	6 ± 0.88	0.53
	0.1	7 ± 1.00	0.59
	0.2	6 ± 0.67	0.53
	0.3	8 ± 0.57	0.70
	0.4	6 ± 1.45	0.53
	0.5	5 ± 0.58	0.41

The control group, characterized by the absence of BAP and NAA concentrations, demonstrates the lowest average number of shoots per explant (2 ± 0.00). In contrast, the treatment involving 2 mg/L BAP combined with 0.3 mg/L NAA exhibits the highest shoot count (8 ± 0.57). Regarding shoot length, the control group showcases the highest average length of shoots per explant at 4.5 cm. Notably, the treatment comprising 2 mg/L BAP with 0.3 mg/L NAA attains the highest shoot length value of 1.77 cm compared to the other combination treatments (1.4 cm, 1.1 cm, 1.27 cm, 1.03 cm, and 1.03 cm for 2 mg/L BAP with 0 mg/L, 0.1 mg/L, 0.2 mg/L, 0.4 mg/L, and 0.5 mg/L NAA, respectively). The data presented in Table 3 demonstrate that after a span of 10 weeks, noticeable differences in shoot growth become apparent across various treatment rates. The treatment group with 2 mg/L BAP and 0.3 mg/L NAA demonstrates the highest multiplication rate of 0.70, indicating a substantial increase in shoot numbers over the 10-week period.

All data were expressed as mean ± standard error mean (SEM) of analysis (n=3). The significant level between means at ($p<0.05$) or no significant difference between the means ($p>0.05$). These findings shed light on the variations in shoot regeneration potential and shoot length observed across the treatment combinations. While the control group displays lower

shoot production, it remarkably yields longer shoots. Conversely, the specific combination of 2 mg/L BAP with 0.3 mg/L NAA results in the highest shoot count and relatively longer shoot length among the tested concentration combinations. These findings contribute significantly to our comprehension of the

role played by plant growth regulators (PGRs), particularly BAP and NAA, in the development and multiplication of shoots in tissue culture systems. These growth regulators exert diverse physiological effects on various plant species, influenced by factors such as their type, concentration, and the presence or absence of other growth regulators. Notably, higher doses of a growth regulator that induces a favorable response in one tissue may hinder the same physiological response in another (Tyub et al., 2021).

The introduction of auxins to the culture media resulted in an overall increase in auxin levels, leading to significant apical dominance that suppressed shoot proliferation. However, when these auxins interact with cytokinins, they can promote shoot proliferation (Rasool et al., 2009). These results underscore the potential of specific treatment combinations to enhance shoot production, which could have implications for the propagation and cultivation of various plant species in diverse applications, such as horticulture, agriculture, and conservation efforts. Figure 2 illustrates the shoot explants treated with 2 mg/L BAP at different concentrations of NAA.

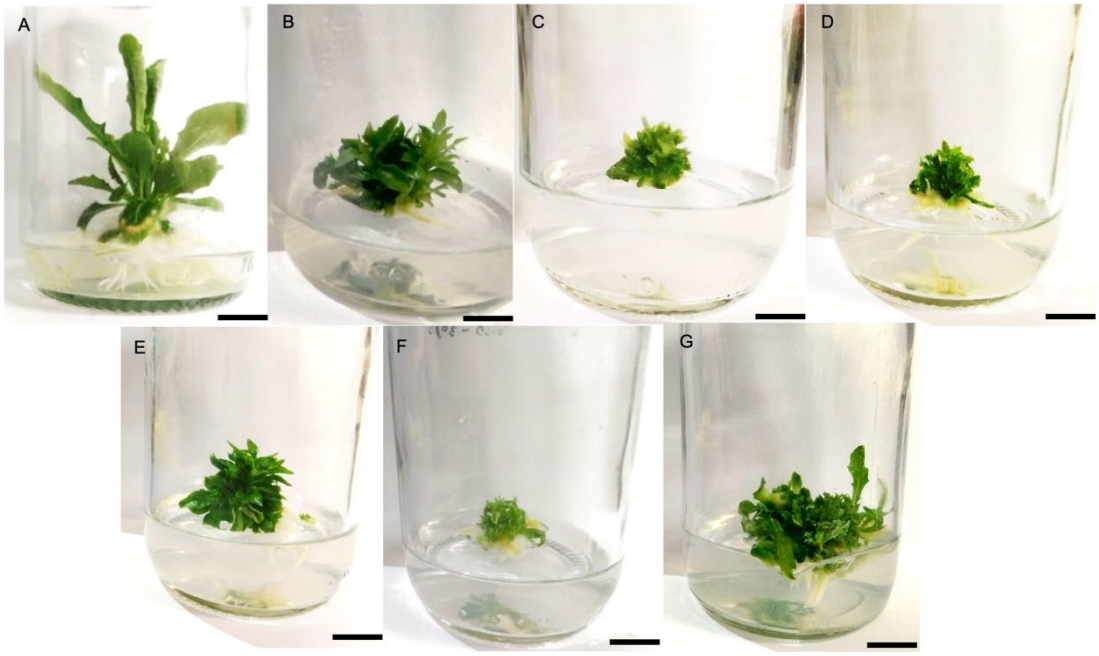

Figure 2 Shoot explants treated with 2 mg/L BAP with different concentrations NAA. (A) Control (MSO) (B) 2 mg/L BAP (C) 2 mg/L BAP + 0.1 mg/L NAA (D) 2 mg/L BAP + 0.2 mg/L NAA (E) 2 mg/L BAP + 0.3 mg/L NAA (F) 2 mg/L BAP + 0.4 mg/L NAA (G) 2 mg/L BAP + 0.5 mg/L NAA. Scale bars: 1.5 cm

Table 4 reveals that applying various plant growth regulators (PGRs) to tissue-cultured *H. longiflora* resulted in varying levels of antioxidant activity, as evidenced by the DPPH inhibition percentage. The data demonstrated a clear association between increasing concentrations (ug/ml) ranging from 100 to 1000 ug/ml and higher inhibition values. The control sample exhibited the highest inhibition percentage value, measuring 69.40 ± 0.19%. Following that, the sample treated with 2 mg/L BAP as a positive control demonstrated an inhibition percentage value of 63.48 ± 0.23%. When the samples treated with 2 mg/L BAP were combined with various concentrations of NAA, it was observed that the sample treated with 0.5 mg/L NAA displayed the highest inhibition percentage value of 51.42 ± 0.17% at a concentration of 1000 ug/ml. Conversely, the samples treated with 0.1, 0.2, 0.3, and 0.4 mg/L NAA exhibited inhibition percentage values of 47.35 ± 0.12%, 30.48 ± 0.18%, 29.55 ± 0.27%,

Table 4: DPPH inhibition percentage of field- grown and tissue-cultured *H. longiflora*

Samples extract of plant		% Inhibition	IC_{50} (µg/ml)
Ascorbic Acid		90.14 ± 0.16	100.22 ± 0.17
Field-grown		67.14 ± 0.12	373.49 ± 19.61
Treatment combination			
BAP	**NAA**		
0	0	69.40 ± 0.19	N.D
2	0	63.48 ± 0.23	N.D
	0.1	47.35 ± 0.12	N.D
	0.2	30.48 ± 0.18	N.D
	0.3	29.55 ± 0.27	N.D
	0.4	48.73 ± 0.11	N.D
	0.5	51.42 ± 0.17	N.D

and 48.73 ± 0.11%, respectively. Notably, the sample of *H. longiflora* treated with 2 mg/L BAP and 0.3 mg/L NAA displayed the lowest inhibition percentage value of 29.55 ± 0.27%.All data were expressed as mean ± standard error mean (SEM) of analysis (n=3). The significant level between means at ($p<0.05$) or no significant difference between the means ($p>0.05$).

The previous study documented an inhibition percentage of 46.47 ± 0.49% for the control (MSO) and 25.74 ± 0.68% for 2 mg/L BAP (Yee & Abd Samad, 2022). Interestingly, the results obtained in this experiment revealed higher inhibition percentage values for both the control and 2 mg/L BAP samples. This discrepancy could potentially be attributed to errors in the preparation of plant extraction or in the calculation of sample concentrations. It is imperative to conduct a comprehensive review and investigation of these potential error sources to ensure the accuracy and reliability of future experiments.

As per the data in Table 5, the extracts demonstrated varying reducing abilities, measured within the range of 9258.94 ± 55.97 to 10942.94 ± 35.12 µM Fe^{2+}/g. Notably, the FRAP values of the *in vitro* extracts of *H. longiflora* were notably lower compared to the field-grown extracts of *H. longiflora* (10942.94 ± 35.12 µM Fe^{2+}/g). Specifically, the reducing abilities of tissue-cultured H. longiflora cultivated in the control (MS0) and media supplemented with 2 mg/L BAP extract as a positive control were recorded at 10776.27 ± 2.92 and 10534.75 ± 29.64 µM Fe^{2+}/g, respectively.

Table 5: Ferric ion reducing antioxidant power (FRAP) of field-grown sample extracts and *in vitro* propagated sample extracts of *H. longiflora*

Samples extract of plant		FRAP Value (µM of Fe^{2+}equivalent per gram of dry sample)
Field-grown		10942.94 ± 35.12
BAP	NAA	
0	0	10776.27 ± 2.92
2	0	10534.75 ± 29.64
	0.1	10300.67 ± 33.79
	0.2	10112.21 ± 35.37
	0.3	9258.94 ± 55.97
	0.4	10433.34 ± 43.54
	0.5	10559.40 ± 71.30

However, among the extracts derived from tissue-cultured *H. longiflora* samples cultivated in MS media supplemented with 2 mg/L BAP and various concentrations of NAA, it was determined that 2 mg/L BAP with 0.5 NAA exhibited the highest value, measuring 10559.40 ± 71.30 µM Fe^{2+}/g. Conversely, the micropropagated plants grown in 2 mg/L BAP with NAA concentrations of 0.1, 0.2, 0.3, and 0.4 mg/L displayed slightly weaker ferric reducing abilities compared to the plants grown in MS

media without supplementation (10300.67 ± 33.78, 10112.21 ± 35.37, 9258.94 ± 55.97, 10433.34 ± 43.54). In general, it can be inferred that the FRAP reducing ability may have been influenced by the growth rates of the cultured tissue, as well as the quantities of auxins or cytokinins in the media. These factors can impact the production rate of phenolic and flavonoid compounds in the plants (Yee & Abd Samad, 2022). The reduction properties of FRAP are directly linked to the presence of compounds that function by breaking the free radical chain through the donation of H+ atoms (Singh et al., 2023).All data were expressed as mean ± standard error mean (SEM) of analysis (n=3). The significant level between means at ($p<0.05$) or no significant difference between the means ($p>0.05$).

Conclusion

In conclusion, the study revealed that immature seeds (white in color) achieved the highest germination rate of 50% within 7 weeks. Regarding *in vitro* propagation, the treatment involving *H. longiflora* treated with 2 mg/L BAP and 0.3 mg/L NAA resulted in the highest percentage of shoot regeneration at 100%, with an average of 8 ± 0.57 shoots per explant. Additionally, the combination of 2 mg/L BAP with 0.5 mg/L NAA exhibited the highest percentage of antioxidant activity, with a DPPH inhibition percentage of 51.42 ± 0.17% and an FRAP value of 10559.40 ± 71.30 μM Fe^{2+}/g. The reduced antioxidant activity observed in the *in vitro* propagated plants may be attributed to the fact that the application of BAP and NAA primarily stimulated shoot multiplication in *H. longiflora*, without significantly influencing the antioxidant activity of the *in vitro* plants.

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