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Shoot Induction and Antioxidant Property of *Isotoma longiflora* (L.) C. Presl

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Abstract
Isotoma longiflora is a herbal plant known for medicinal uses by local residences in Asia, notably in Malaysia and Indonesia. To date, effect of plant growth regulators on shoot induction and its antioxidant activity were not fully studied. Thus, in this study, the effect of BAP on shoot induction, antioxidant activity and phytochemical analysis of *vitro* propagated plants and field-grown *I. longiflora* were investigated. Shoots were cultured on MS medium supplemented with various BAP concentrations for shoot regeneration at culture condition (16h photoperiod; 25 ± 2°C; 3000-lux light intensity provided by cool white fluorescent tubes) for five weeks culture. Next, phytochemical analysis using TPC, TFC and antioxidant assays using DPPH and FRAP assays were carried out. Results revealed that 50% (v/v) Clorox was the best sterilizing condition with 100% sterilized seeds. For shoot induction, the highest shooting was achieved at 2 mg/L BAP with 93.33 ± 6.67 % of shoot regeneration and 9.55 ± 0.85 of shoot multiplication rate. Results showed that field-grown flower extracts had higher TPC, TFC, DPPH and FRAP than BAP-treated extracts with 6.60 ± 0.08 mg GAE/g of dry sample, 3.91 ± 0.30 mg QCE/g of dry sample, 71.60 ± 1.25% DPPH inhibition and 20971.05 ± 15.19 µM Fe^{2+}/g of dry sample respectively. In conclusion, shoot generated *I. longiflora* was successfully established *in vitro* and BAP treated shoot possessed antioxidant property. Therefore, future investigation such as the roles of various plant growth regulators on the growth and phytochemicals of *in vitro I. longiflora* will provide better insights on phytochemical constituents responsible for antioxidant property of *I. longiflora.*
Keywords: *Isotoma longiflora*; shoot induction; antioxidant activity; DPPH; FRAP

Introduction

Isotoma longiflora is a traditional plant employed by the locals in Malaysia as their primary medicinal and health care treatment. Metabolites derived from *I. longiflora* have been shown to exhibit variety of pharmacological and therapeutic properties such as anti-inflammatory, antioxidant, anticancer, antidiabetic, antibacterial, antimalarial, antitumor, antimicrobial, antifungal, anti-insecticide, and antiseptic property (Martiningsih et al., 2021). Several studies have been conducted to investigate the properties of I. longiflora, including antimicrobial and antioxidant studies, which show that this plant contains secondary metabolites such as alkaloids, phenolics, flavonoids, triterpenoids, steroids, coumarins, saponins, and tannins, which are responsible for various therapeutic treatments and bioactivity.

This study focused on antioxidant activity of the *I. longiflora* because it is rich in bioactive compounds with proven antioxidants. Plant tissue culture is employed as a continuous source for the development of important bioactive chemicals from plants as it allows for bulk multiplication of plants under regulated environmental settings without regard any environmental constraints, allowing us to maximize the synthesis of metabolites efficiently,

particularly those that are beneficial for our health (Chandran et al., 2020). In this context, the consistency in terms of antioxidant activity and active ingredient of *I. longiflora* could be achieved via tissue culture techniques. Plant growth regulators used in tissue culture also play a significant part in in vitro regeneration and promote the production of bioactive compounds that are responsible for antioxidant activity in many plants. Cytokinin such as 6- Benzylaminopurine (BAP) has been proven to have significant impact in numerous *in vitro* investigations, particularly in shoot generation and multiplication.

According to Mohd Usri & Abd Samad (2020), results revealed that *in vitro P. odorata* that treated with different BAP concentration had a significant result in term of shoot regeneration and antioxidant activity of *P. odorata*. However, to date, less attention has been dedicated to studying the variations in the biological characteristics of tissue-cultured *I. longiflora* plants. Therefore, this study was designed to grow shoots of *I. longiflora in vitro* and compare the antioxidant activity of ethanolic extracts of *in vitro* propagated plants to plants grown in the field. In this research project, we aimed to look at whether the *in vitro* propagated *I. longiflora* in different concentration of BAP treatment could be a possible antioxidant which is comparable to the field-grown *I. longiflora*.

Materials and methods

Seeds with the seed coats of *I. longiflora* plants were obtained from Agrotani of Universiti Teknologi Malaysia (UTM) and used as an explant for initiation of shoot cultures. Vegetative cuttings of *I. longiflora* were also taken for antioxidant analysis and phytochemical analysis for the comparison between field- grown and *in vitro* propagated plants. Meanwhile, a sample of whole *I. longiflora* plant was sent to Forest Research Institute Malaysia (FRIM) for plant identification with specimen voucher number of PID 030322- 03.

The basal media which was MS media was prepared according to (Bhojwani & Razdan, 1996). The basis MS media contained 30 g/L sucrose, 3 g/L gelrite and the pH was adjusted with 1 M sodium hydroxide or 1 M hydrochloric acid to pH 5.7 before autoclaving at 121℃ for 15 minutes.

The surface sterilization was done according to Mohd Nawi and Abd Samad (2012). The seeds with the seat coats of *I. longiflora* were used as explant. Firstly, the explants were soaked in detergent DYNAMO® and then rinsed under running tap water for 30 minutes to remove all contaminants. Then, the explants were immersed in 70% ethanol for 1 minute. After that, the ethanol was discarded and rinsed the explant with sterile distilled water, following with different concentration of Clorox (40%, 50%, 60%, 80% and 100%) and three drops of Tween 20 were added in conical flask. The explants were agitated on an incubator shaker at 130 rpm for 30 minutes. Next, the explants were then rinsed with sterile distilled water for five times to remove any bleach traces. Lastly, the seed coats of *I. longiflora* were cut open using scalpel then the disinfected seeds were taken to be cultured on MS free media. All the sterilization works were carried out in laminar air flow.

Disinfected seeds were inoculated on MS free media prepared earlier for two months for seed germination and shoot induction. Shoots were excised and sub-cultured onto the MS free solid media and MS media supplemented with BAP 1, 2, and 3 mg/L in the jemjar for continuous growing and shoot multiplication (Manivannan et al., 2015). The cultures were observed constantly for any respond for five weeks. All treatment cultures were grown in a culture room at 25 ± 2℃ with a photoperiod of 16 hours at 3000-lux light intensity provided by cool white fluorescent tubes. These experiments were carried out with 3 replicates for each concentration treatment. After five weeks of incubation, the average shoot length (cm) per explant, average number of shoots per explant, average fresh weight of shoots per jar (g), percentage of shoot regeneration and lastly shoot multiplication rate were recorded. The following equation was applied to calculate the multiplication rate of shoots (Sharma & Ramamurthy, 2000):

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

Leaves and flowers of field-grown *I. longiflora* and leaves of tissue-cultured *I. longiflora* were collected. The leaves and flowers were cut into small pieces, weighed and crushed into powder with liquid nitrogen. 1g of plant sample powder were macerated in 20 ml of 70% ethanol (Ratio 1:20) for three days. The maceration process was done in incubator shaker at 100 rpm and 30℃. After three days, all ethanolic solution of plant extraction were filtered, vacuum evaporated with vacuum rotary evaporator IKA® RV 10 auto and freeze-dried into powder and kept at 4℃ until further investigation.

The total phenolic content of *I. longiflora* extract was determined by Folin-Ciocalteu method with minor modification (Baba & Malik, 2015). Briefly, 200 µl of plant extract (1 mg/ml) were mixed with 1 ml of Folin-Ciocalteu (10%) reagent for 5 minutes using vortex mixer, followed by addition of 0.8 ml of 5% (w/v) sodium carbonate. The mixture was incubated for 60 minutes in dark condition. Then, the absorbance was measured at 760 nm using microplate reader (SPECTROstar nano). TPC value was calculated from the calibration curve of standard Gallic acid, and the results were expressed as mg of Gallic acid equivalent per g of crude extract. The experiments were conducted in triplicates. The same procedures were employed for Gallic acid as standard solution and absolute ethanol as blank.

The total flavonoid content of *I. longiflora* extract was determined by the aluminum chloride colorimetric protocol by Baba and Malik (2015) with minimal arrangement. Firstly, 200 µl of plant extract (1mg/ml) was mix with 0.06 ml of 5% sodium nitrite solution for 5 minutes followed by adding 0.06 ml of 10% aluminum chloride solution for another 5 minutes. Then, the mixture was added with 0.4 ml of 1M sodium hydroxide solution and allowed to stand for 5 minutes, before the final volume of mixture was brought up to 2 ml with deionized water. In the next step, the mixture was incubated in dark condition for 60 minutes and the absorbance was measured at 510 nm using SPECTROstar nano microplate reader. Lastly, TFC value was calculated from the calibration curve of standard Quercetin, and the results were expressed as mg of Quercetin equivalent per g of dry weight. The experiments were conducted in triplicates. The same procedures were applied for Quercetin as standard solution and absolute ethanol as blank.

DPPH solution was prepared freshly by mixing 4 mg DPPH powder into 100 ml of absolute ethanol. In a 96-well microplate, 100 µl DPPH solution was added into various concentrations of *I. longiflora* extract (1.95 µg/ml to 1000 µg/ml) using 2-fold dilution. 70% ethanol was employed as the blank, while DPPH solution in ethanol served as the control. On the other hand, the same procedures were repeated using ascorbic acid as a positive control for the antioxidant activity. The solutions in the microplates were then let to stand for 30 minutes in a dark room before being tested for absorbance at 517 nm with SPECTROstar Nano microplate reader. A standard curve was constructed by plotting the absorbance at 517 nm versus different concentration 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 *I. longiflora* extract and ascorbic acid were calculated and measured as percentage inhibition (%) and IC_{50} value. The following equation yields value of inhibition percentage:

$$\% \text{ inhibition} = \frac{\text{A control} - \text{A sample}}{\text{A control}} \times 100$$

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

FRAP reagent was prepared by mixing 50 ml of 0.3 M acetate buffer at pH 3.6, 5 ml of 10 mM tripyridyltriazine (TPTZ) solution prepared in 40 mM HCL and 5 ml of 20 mM ferric chloride solution (Mohd Usri & Abd Samad, 2020). The FRAP reagent was made freshly and warmed in an oven to 37°C before use. 200 µl of the freshly prepared FRAP reagent was added to100 µl of the different concentration (0, 5, 10, 20, 40, 60,80 and 100 µM) of 1 mM ferrous (II) sulfate heptahydrate, $FeSO_4 \cdot 7H_2O$ for standard solution. On the other hand, 200 µl of FRAP reagent was also added into the wells containing 100 µl of 1 mg/ml *I. longiflora* ethanolic extract and absolute ethanol as a blank respectively. After mixing, the solutions in the microplate were incubated at 37°C for 30 minutes under dark condition. Then the absorbance of standard solution, samples and blank were measured

and recorded at 593 nm against the at room temperature using SPECTROstar nano microplate reader. The calibration curve was constructed by plotting the absorbance at 593 nm versus different concentration of $FeSO_4 \bullet 7H_2O$.To ensure the reproducibility of the data, all experiments were set up with five replicates per treatment, and the assays were done in triplicate for each concentration of samples. Using SPSS Statistics (Statistical Package for the Social Sciences), an analysis of variance (ANOVA) was used to identify the significance differences, which were expressed in mean ± standard error (SEM).

Results and discussion

Surface sterilization with varying Clorox concentration was performed in order to collect data that were useful in the selection of an appropriate sterilization method for seeds. This was because inappropriate sterilant concentrations could lead to lethal effect on cell division, limiting explant growth and development. To successfully raise *in vitro* cultures, appropriate sterilant concentrations, combinations, and duration of exposure are required (Bhadane & Patil, 2016). In this experiment, it was observed that when the sterilant concentration increased, the microbiological contamination decreased dramatically.

Based on the findings in Table 1, the percentage of sterilized plate in 40% (v/v) Clorox increased significantly from 33.33 % to 100 % in all cultures with 50, 60, 80 and 100% (v/v) Clorox concentration treatment. On the other hand, successful seed germination and primary shoot generated were observed in different Clorox 40, 50, 60 and 80 % (v/v) at 9^{th} week and 12^{th} week. However, seeds treated with 40% (v/v) Clorox was insufficient for surface sterilization since the seed cultures were contaminated (33.33% sterilized condition) although there was good growth in the petri dish. On the contrary, 50, 60 and 80% (v/v) Clorox were enough as surface sterilizing agents for *I. longiflora* seeds since the seeds successfully germinated and grew with shoots and leaves without contamination and 100% sterilized condition after 9th week. Despite the fact that no contamination occurred in seeds treated with 100% (v/v) Clorox, it was not so suitable for use as a sterilant because late growth was occurred after week 14 in the plate. This could be because the increasing concentration of Clorox might minimize microbial contamination, but it could also kill the culture materials as reported in Hammond et al. (2014). To conclude, best sterilization of seeds of *I. longiflora* was achieved at 50% (v/v) Clorox with best growth observed compared to 60 and 80% (v/v) Clorox (Figure 1).

Table 1: Observation of *I. longiflora* seed cultures in MS free media and percentage of sterilized plate after treating with different Clorox concentration at Week 9 and Week 12

Clorox Concentration % (v/v)	**Observation on seed cultures**		
	Percentage of sterilized plate (%)	**Week 9**	**Week 12**
40	33.33	Very short shoots, and very small leaves	Longer shoots, and leaves
50	100	Very short shoots, and very small leaves	Longer shoots, big and more leaves
60	100	Start gemination	Thin and short shoots, small leaves
80	100	Start germination	Very short shoots, and small leaves
100	100	No germination	Late growth after 14 weeks- start shooting

Data were expressed as n = 3 replicates.

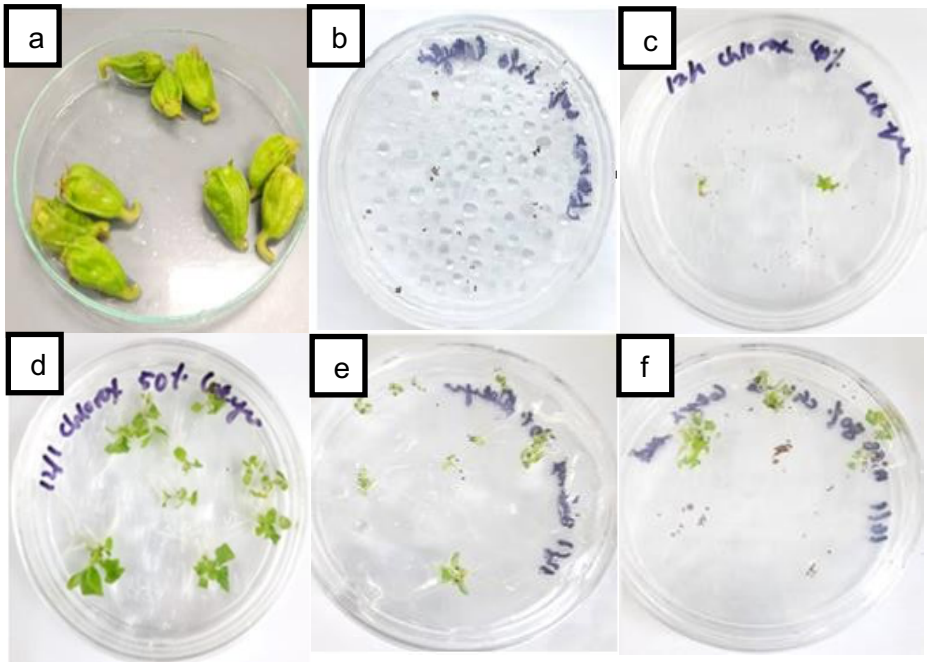

Figure 1 Seedlings from seeds treated with different Clorox concentrations of *I. longiflora*. (a) seed coats; (b) seeds were cultured in MS free media on the first day; (c) 5th week seedlings, 40% (v/v) Clorox; (d) 12th week seedlings, 50% (v/v) Clorox; (e) 12th week seedlings, 60% (v/v) Clorox; (f) 12th week seedlings, 80% (v/v) Clorox

The transfer of primary generated shoots onto MS media treated with varying BAP concentrations led to shoot multiplication and further growth was observed. At the time of subculturing, the shoots were transferred into fresh growth media supplemented with the same BAP concentration. The effect of plant regulated hormones on *Isotoma longiflora* shoot multiplication was summarized in Table 2 and Table 3.

The increment of BAP concentration, the higher number of adventitious shoot percentage and their subsequent proliferation was achieved. By comparing the shoots cultured in 1, 2 and 3 mg/L BAP with that cultured in MS free media without BAP, the percentage of shoot regenetation (%), average shoot number per explant, average shoot length (cm) per explant, average shoot weight of explants (g) per jar cultured in BAP supplemented media were higher. For example, the percentage of shoot regeneration treated with 1, 2 and 3 mg/L BAP was measured at 89.68±5.21 %, 93.33±6.67 % and 84.92±8.29 respectively, which was much higher than that in MS control (87.78±6.19 %). The multiplication rate of shoot in treated with 1, 2 and 3 mg/L BAP also greater (7.64 ± 0.62, 9.55 ± 0.85 and 6.83 ± 0.28 respectively) than the multiplication rate in MS control (3.98 ± 0.52). These indicated that all BAP concentrations enhanced the growth of shoots in *I. longiflora*. Good growth profile of shoots of randomly selected cultures of *in vitro I. longiflora* at different BAP concentration (mg/L) over a period of propagation (5 weeks) were demonstrated in Figure 2.

I. longiflora shoots were produced optimally with very compact shoots and healthy green leaves in MS media supplemented with 2 mg/L BAP. At 2 mg/L BAP, the number of shoots produced per explant, the length of shoots per explant and the fresh weight of shoots per jar were the highest at 59 ± 3.06, 5.17± 0.35 cm and 7.82 ± 0.60 g respectively compared to other BAP concentration. Besides, when comparing the number of shoots primarily generated at the beginning of experiment with the number of shoots generated after 5 weeks of culture, shoots of *I. longiflora* produced after 5 weeks were recorded the highest (59 ± 3.06 shoots), with the highest shoot multiplication rate of 9.55 ± 0.85.

Table 2: The percentage of shoot regeneration (%), average shoot number per explant, average shoot length (cm) per explant, average shoot weight of explants (g) per jar cultured on MS media with different BAP concentration

BAP Concentration (mg/L)	Percentage of shoot regeneration (%)	No. of shoots per explant ($\bar{x}$ ± SE)	Length of shoots per explant (cm) ($\bar{x}$ ± SE)	Fresh weight of shoots (g) per jar ($\bar{x}$ ± SE)
0 (Control)	87.78 ± 6.19 ^{a}	17 ± 1.45^{a}	2.67 ± 0.49 ^{a}	1.14 ± 0.34 ^{a}
1	89.68 ± 5.21^{a}	45 ± 3.18 ^{c}	4.77 ± 0.38 ^{c}	5.92 ± 0.46 ^{b}
2	93.33 ± 6.67 ^{a}	59 ± 3.06 ^{b}	5.17 ± 0.35 ^{b}	7.82 ± 0.60 ^{c}
3	84.92 ± 8.29 ^{a}	40 ± 2.40 ^{d}	2.63 ± 0.03 ^{a}	3.30 ± 0.15 ^{d}

All data were expressed as mean ± standard error mean (SEM) of analysis (n=3). Different letters shown significant level between means at $p<0.05$. Same letter indicates no significant difference between the means ($p>0.05$).

Table 3: Shoot multiplication rate over 5 weeks of culture in randomly selected subcultures

BAP Concentration (mg/L)	Number of shoots		Multiplication Rate
	At beginning	After 5 weeks	
0 (Control)	4 ± 0.88	17 ± 1.45	3.98 ± 0.53^{a}
1	6 ± 0.58	45 ± 3.18	7.64 ± 0.62^{c}
2	6 ± 0.88	59 ± 3.06	9.55 ± 0.85^{b}
3	6 ± 0.58	40 ± 2.40	6.83 ± 0.28^{a}

All data were expressed as mean ± standard error mean (SEM) of a shoot multiplication rate (n=3). Different letters shown significant level between means at $p<0.05$. Same letter indicates no significant difference between the means ($p>0.05$).

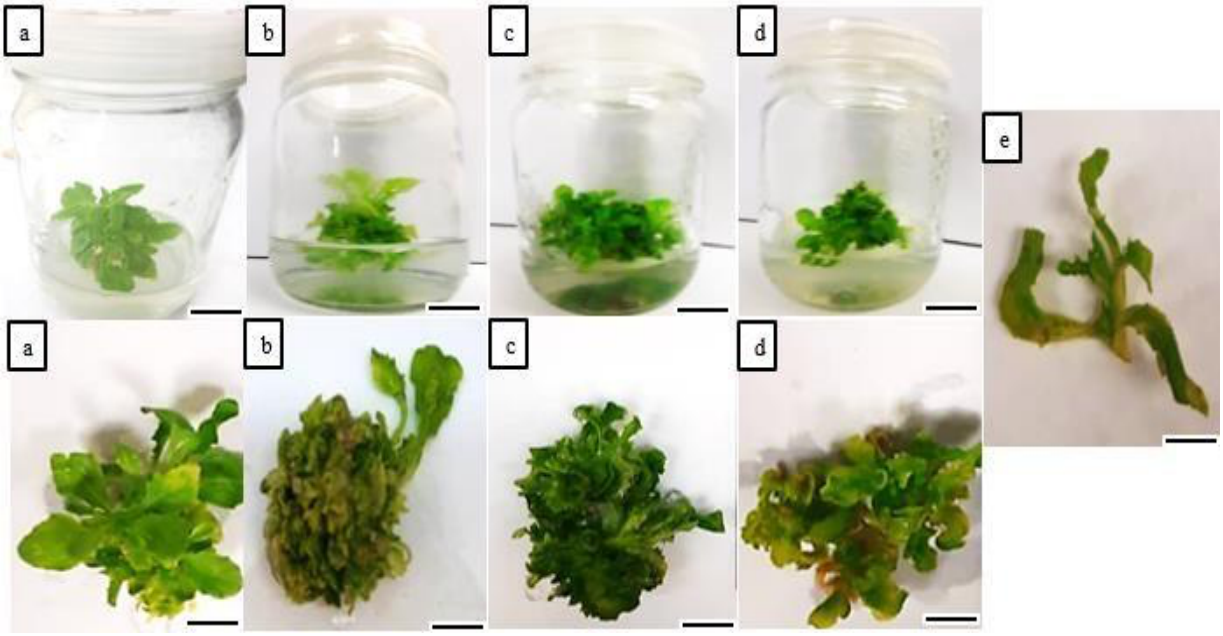

Figure 2 Shooted explants treated with different BAP concentrations. (a) Control; (b) 1 mg/L BAP; (c) 2 mg/L BAP; (d) 3 mg/L BAP; (e) Shoot cuttings from in vitro plant. Scale bars 1 cm = 1.5 cm.

Because phenolic and flavonoid molecules include hydroxyl groups, they have the capacity to eliminate radicals and so play a major role in antioxidant activity. The amounts of phenolic compounds in a plant must be determined in order to estimate the antioxidant ability. Table 4 shows the results of total phenolic (TPC) and flavonoid content (TFC) of field-grown and *in vitro* propagated *I. longiflora.*

Table 4 Total phenolic and flavonoid content of field-grown and *in vitro* propagated *I. longiflora*

Sample	Total Phenolic Content (mg GAE/g of dry sample)	Total Flavonoid Content (mg QCE/g of dry sample)
Field-grown flower	6.60 ± 0.08 ^{b}	3.91 ± 0.30 ^{c}
Field-grown leaf	6.47 ± 0.04 ^{c}	6.24 ± 0.33 ^{b}
MS control	2.05 ± 0.05 ^{a}	2.05 ± 0.29 ^{a}
1 mg/L BAP	1.57 ± 0.02 ^{d}	0.29 ± 0.10 ^{d}
2 mg/L BAP	1.34 ± 0.05 ^{e}	1.01 ± 0.13 ^{a}
3 mg/L BAP	1.40 ± 0.03 ^{f}	0.82 ± 0.048 ^{e}

All data were expressed as mean ± standard error mean (SEM) of a shoot multiplication rate (n=3). Different letters shown significant level between means at $p<0.05$. Same letter indicates no significant difference between the means ($p>0.05$).

As a basis, phenolic compounds present in field-grown and *in vitro* propagated *I. longiflora* was measured using the Folin–Ciocalteu reagent since it has been proven to be preferred compared to other approaches (Dai & Mumper, 2010). The results were derived from Gallic acid calibration curve with y= 5.9721x + 0.0199, R^2= 0.9955 and expressed in Gallic acid equivalents (GAE) per gram dry extract weight (Table 4.4). The total phenolic content in ethanol extracts of *I.longiflora* ranged from 1.34 ± 0.05 to 6.60 ± 0.08 mg GAE/g. Field-grown flower of *I. longiflora* showed the highest phenolic content (6.60 ± 0.08 mg GAE/g), followed by field-grown leaf (6.47 ± 0.04 mg GAE/g), in vitro propagated plant cultivated in MS media without PGR treatment (2.06 ± 0.05 mg GAE/g), in 1 mg/L BAP (1.57 ± 0.02 mg GAE/g), 3 mg/L BAP (1.40 ± 0.03 mg GAE/g) and finally 2 mg/L BAP (1.34 ± 1.40 mg GAE/g). *I. longiflora* in MS media without BAP treatment had a higher TPC than those grown

in cultures with BAP treatment. As a result, total phenolic content present in *in vitro* propagated *I. longiflora* did not show greater total phenolic content that that in field-grown *I. longiflora*.

Further in this study, total flavonoid content in field-grown and *in vitro* propagated *I. longiflora* was analyzed using aluminium chloride in a colorimetric method. The results were derived from the calibration curve (y = 1.4019x + 0.0026, R^2 = 0.9921) of quercetin and expressed in Quercetin equivalents (QCE) per gram dry extract weight (Table 3.4). In ethanolic extracts of *I. longiflora* leaf, the highest TFC of 6.24 ± 0.33 mg QCE/g was recorded. We found that a TFC of 3.91 ± 0.30 mg QCE/g in ethanolic extracts of *I. longiflora* flower. While in ethanolic extracts of tissue-cultured *I. longiflora* in MS free media, 1 mg/L BAP, 2 mg/L BAP and 3 mg/L BAP, lower TFC value were observed compared to the field-grown plant sample, at 2.05 ± 0.29, 0.29 ± 0.01, 1.01 ± 0.13 and 0.82 ± 0.05 mg QCE/g respectively. Plant cultivated in MS free media without BAP treatment displayed a higher TFC than those grown in cultures with BAP treatment. As a result, the *in vitro* regenerated *I. longiflora* plants exhibited lower TFC compared to field-grown plant parts.

In comparison to other studies, micropropagated *C. decussata* had higher total phenolic and flavonoid content found (577.77 ± 15.18 mg GAE/g DW and 179.16 ± 10.92 mg Rutin equivalents/g DW) than the wild-type plant (471.99 ± 0.72 mg GAE/g DW and 165.50 ± 4.94 mg Rutin equivalents/g DW) (Kousalya & Narmatha Bai, 2016). According to Aryal et al., (2019a), the presence of varying amounts of sugars, carotenoids, or ascorbic acid, as well as the duration, geographical variation, or methods of extraction, may affect the amount of phenolics compounds present in plants. Additionally, TFC values may vary slightly due to a variety of factors that may influence secondary metabolite formation, such as plant age, ambient factors, and time of collection (Abdulhafiz et al., 2020). Thus, the values of TPC and TFC in the *in vitro* propagated *I. longiflora* plant differed slightly compared to those in the field-grown *I. longiflora* flower and leaf. Combinations of plant growth regulators can be studied in the future because they had a better influence on phenolic compound production for esample in *S. tebesana* calli than their use alone (Hemmati et al., 2020).

The DPPH method was used to investigate the antioxidant activity of *I. longiflora* since it is a simple, rapid, and easy approach for screening the radical scavenging activity of various substances. The recorded absorbance value in each experiment was used to calculate percentage of DPPH inhibition of every sample. Results in Table 5 revealed that the field-grown plant of *I. longiflora*, including flowers and leaves, as well as the *in vitro* propagated plant of *I. longiflora* in different concentration of BAP treatment of MS media could scavenge DPPH radicals in a concentration-dependent way.

Table 5: DPPH inhibition percentage and IC_{50} (µg/ml) of field- grown and tissue-cultured *I. longiflora*

Sample	% Inhibition	IC_{50} (µg/ml)
Ascorbic acid	99.16 ±0.24 ^{b}	4.93±0.12
Field-grown Flower	71.60 ± 1.25 ^{c}	618.33 ±15.90
Field-grown Leaf	44.46 ±2.67 ^{a}	N.D.
MS control	46.47 ±0.49 ^{a}	N.D.
BAP 1	35.65 ± 1.58 ^{e}	N.D.
BAP 2	25.74 ± 0.68 ^{d}	N.D.
BAP 3	36.70 ±1.35 ^{f}	N.D.

All data were expressed as mean ± standard error mean (SEM) of a shoot multiplication rate (n=3). Different letters shown significant level between means at $p<0.05$. Same letter indicates no significant difference between the means ($p>0.05$). N.D.= IC_{50} was not determined.

It was noticed that the percentage of inhibition increased in tandem with the concentration value of the test sample. This demonstrated that there was a correlation between the increasing concentration of the test sample and the increasing free radical reduction as illustrated in Figure 3.

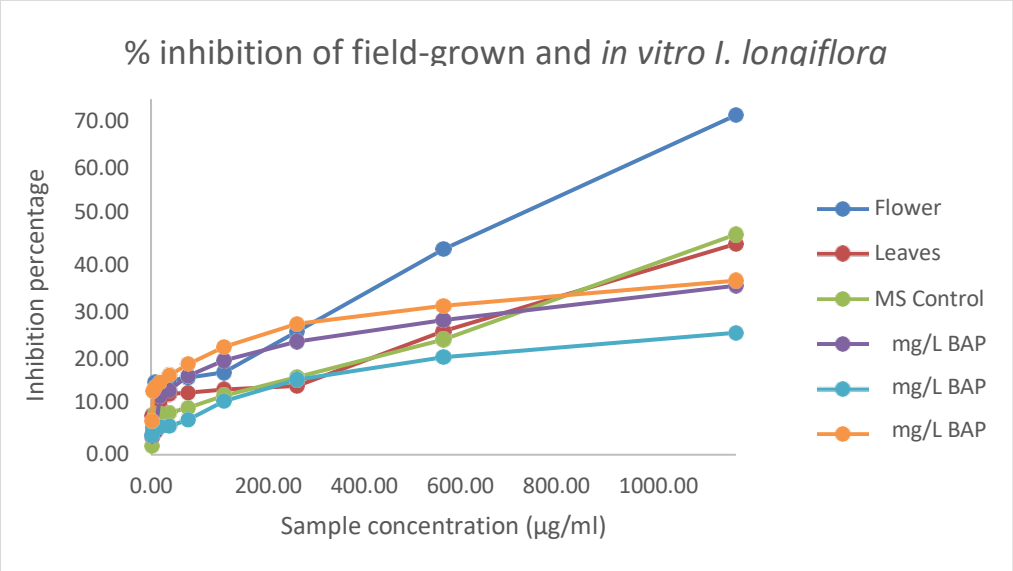

Figure 3 DPPH scavenging activity of BAP treated *in vitro* propagated and field-grown *I. longiflora*

L-ascorbic acid is a known antioxidant used as a control in plotting standard curve for DPPH assay. Figure 3.4 depicts ascorbic acid's capabilities as a strong antioxidant with IC_{50} 4.93±0.12 µg/ml. Ascorbic acid demonstrated constant DPPH inhibition percentages ranging from 97.09 ± 0.01% to 99.16
± 0.24% at concentrations 31.25 µg/ml up to 1000 µg/ml, indicating the attainment of maximum capability in free radical scavenging. When compared to field-grown *I. longiflora* flower and leaf ethanolic extracts, both demonstrated increasing radical scavenging activity as sample concentration increased. The flower extract had IC_{50} value of 618.33 ±15.90 µg/ml. It inhibited DPPH free radicals at 17.41 ± 1.13% at 125 µg/ml, and the inhibition activity gradually rose to 71.60 ± 1.25% at 1000 µg/ml. Furthermore, from 250 µg/ml to 1000 µg/ml, the radical scavenging activity of leaf extract increased from 14.47 ± 0.31% to 44.46
± 2.67%. Since both field-grown extracts increased in DPPH inhibition activity as concentration increased, the concentration range evaluated was insufficient to achieve the maximum radical scavenging activity for field-grown *I. longiflora* flower and leaf ethanolic extract. A larger concentration range is needed in future studies to investigate the inhibitory action of the DPPH radical in field-grown *I. longiflora* sample extracts. However, at 1000 µg/ml sample concentration, field-grown *I. longiflora* flower extract inhibited DPPH more than field-grown *I. longiflora* leaf extract, with 71.60 ± 1.25% and 44.46 ± 2.67% respectively. On the other side, Martiningsih et al. (2021) had carried out different analysis of antioxidant activity using crude ethanol extract and fractions of *I.* longiflora. It was found that the antioxidant activity of the ethanol extract of *H. longiflora* was more potential than the n-hexane and chloroform fractions with lower IC_{50} at 9.57 µg/ml compared to 99.59 µg/ml and 48.54 µg/ml respectively. Thus, the antioxidant activity of plant varies with different sample extracts and fractions. Generally, field-grown *I. longiflora* flower and leaves were proved as a potent antioxidant.

On the other side, it was observed that the antioxidant activity of tissue-cultured *I. longiflora* with different PGR treatment had different DPPH inhibition percentage. Firstly, *in vitro* propagated *I. longiflora* in MS media without any PGR treatment displayed the highest antioxidant activity as it

showed highest inhibition at 46.47 **±** 0.49% at 1000 µg/ml concentration among all tissue-cultured plant. While the *in vitro* propagated *I. longiflora* cultivated in 1, 2 and 3 mg/L BAP had lower antioxidant activity with 35.65 ± 1.58, 25.74 ± 0.68, and 36.70 ± 1.35% DPPH inhibition, respectively compared to that in MS media at the same concentration. *I. longiflora* cultivated in 2 mg/L BAP showed the least inhibition percentage at 25.74 ± 0.68%. In addition, as shown in Figure 3.4, the DPPH inhibition percentage of all *in vitro* propagated *I. longiflora* sample extract increased along with the increased concentration. This revealed that the concentration range evaluated was insufficient to achieve the maximum radical scavenging activity for all sample extract of tissue-cultured *I. longiflora*.

Thus, a larger concentration range of sample is needed in future studies to investigate the inhibitory action of the DPPH radical in tissue-cultured *I. longiflora* sample extracts.Other studies discovered that *in vitro* plant had higher DPPH scavenging compared to field-grown plant. For example, *in vitro* shoot extract of *S. kakudensis* exhibited higher DPPH scavenging (85.25%) compared to *in vivo* shoot (83.93%) (Manivannan et al., 2015). This could be because *in vitro* cultivation can cause stress during polyphenol production, and plant growth regulator treatment during micropropagation stages with cytokinin and auxin may boost phytochemical production in the *in vitro* plants, thus affect the antioxidant activity of a plant (Mohd Usri & Abd Samad, 2020).

The ferric reducing/antioxidant power (FRAP assay) is a novel technique for determining a sample's antioxidant potential by measuring its ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II). The standard graph with equation y= 0.0038x + 0.0081 was used to determine sample solution antioxidant activity by comparing sample's absorbance change at 593nm to a known concentration of Fe^{2+} according to standard curve. Table 6 indicated FRAP (µM of Fe^{2+}equivalent per gram of dry sample) of field-grown sample extracts and *in vitro* propagated sample extracts of *I. longiflora*.

Table 6: Ferric ion reducing antioxidant power (FRAP) of field-grown sample extracts and *in vitro* propagated sample extracts of *I. longiflora*

Sample Extract of Plant	FRAP Value (µM of Fe^{2+}equivalent per gram of dry sample)
Field-grown plant extract	
Flower	20971.05 ± 15.19 ^{b}
Leaf	17269.30 ± 17.54 ^{c}
Tissue-cultured plant extract	
MS 0	10593.86 ± 17.54 ^{a}
1 mg/L BAP	10357.02 ± 8.77 ^{d}
2 mg/L BAP	10488.60 ± 8.77 ^{f}
3 mg/L BAP	11058.77 ± 8.77 ^{e}

All data were expressed as mean ± standard error mean (SEM) of a shoot multiplication rate (n=3). Different letters shown significant level between means at $p<0.05$. Same letter indicates no significant difference between the means ($p>0.05$)

According to Table 6, the reducing ability of the extracts were in the range of 10357.02 ± 8.77 to 20971.05 ± 15.19 µM Fe^{2+}/g. The FRAP values of extracts of tissue-cultured *I. longiflora* were significantly lower than those of the field-grown flower and leaf extract. In particular, the reducing ability of tissue-cultured *I. longiflora* cultured in MS free media, and media supplemented with 1 mg/L BAP extract, 2 mg/L BAP extract and 3 mg/L BAP were recorded at 10593.86 ± 17.54, 10357.02 ± 8.77, 10488.60 ± 8.77 and 11058.77 ± 8.77 µM Fe^{2+}/g of dry sample respectively, which were lesser to that of field-grown flower with 20971.05 ± 15.19 µM Fe^{2+}/g and leaf with 17269.30 ± 17.54 µM Fe^{2+}/g. However, among the sample extracts of tissue-cultured *I. longiflora, I. longiflora* cultivated in MS media supplemented with 3 mg/L BAP displayed the highest FRAP value of 11058.77 ± 8.772

μM Fe^{2+}/g. Meanwhile, micropropagated plant grown in 2 mg/L BAP MS media and 1 mg/L BAP MS media had slightly weaker ferric reducing ability than plants grown in MS free media (10488.60 ± 8.77 <10357.02 ± 8.77 < 10593.86 ± 17.54 μM Fe^{2+}/g respectively). Mohd Usri & Abd Samad (2020) found that *in vitro P. odorata* was proven to be better in reducing activity compared to control treatment. Generally, the FRAP reducing ability might be affected by the growth rates of the cultured tissue as well as the quantities of auxin or cytokinin in the media and varies the rate of production of phenolic and flavonoid compounds in the plants.

As a result, it was concluded that field-grown flower and leaf of *I. longiflora* showed stronger antioxidant activity than tissue-cultured plant treated with different BAP concentrations based on the FRAP value and colour variations of the TPTZ-Fe (III) complex in the samples. This was because the BAP treatment could enhanced only shoot initiation and shoot multiplication of *I. longiflora in vitro*, but not enhancing the phytochemical in micropropagated *I. longiflora*. Therefore, the antioxidant property of *in vitro* propagated *I. longiflora* with various BAP treatment was not comparable to that of the field-grown *I. longiflora*.

Conclusion

In conclusion, best sterilization of 100% sterilized *in vitro* propagated of *I. longiflora* was achieved at 50% (v/v) Clorox concentration. The *in vitro I. longiflora* treated with 2 mg/L BAP had the highest percentage of shoot regeneration of 93.33 ± 6.67 % and shoot multiplication at 9.55 ± 0.85 shoots. On the other hand, there was a strong correlation between total phenolic and flavonoid content, and the antioxidant activity of *I. longiflora* by DPPH and FRAP assay. The antioxidant capacity of *in vitro* propagated *I. longiflora* with various BAP treatments was not that comparable to that of the filed-grown plant. For instance, compared to the tissue-cultured *I. longiflora*, field-grown *I. longiflora* displayed higher TPC, TFC, and higher antioxidant activity in DPPH and FRAP. Additionally, *in vitro* propagated *I. longiflora* cultivated in 1, 2 and 3 mg/L showed no significant differences in DPPH scavenging and ferric reducing activities. This concluded that the antioxidant activity of *in vitro propagated I. longiflora* with BAP treatment was lower to that of the field-grown *I. longiflora.* This could be due to BAP only promoted shoot multiplication (*I. longiflora)* and did not influence the phytochemical content in *in vitro* plants.

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