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Plant regeneration of *Bauhinia purpurea* by tissue culture technique

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Abstract Results showed that the seed of *Bauhinia purpurea* germination was archived 100% from half-strength MS medium supplemented with 3.0 mg/L Gibberellic acids (GA3) and gave 71.137 mm for the highest seedling. The optimal condition of nodal explants sterilization was E5, which gave 92% of survival explants. For the shoot induction method, the results showed that 24.270 mm was the highest proliferation of shoots achieved from MS medium supplemented with 1.0 mg/L 6-benzylaminopurine (BAP). The highest number of roots was 2.867 roots, and the highest root length was 33.453 mm, obtained at half-strength MS medium complemented with 0.25 mg/L Indole-3-acetic acid (IAA). The 73.33% of plantlets were survived after being transferred to plastic pot.

Keywords: *Bauhinia purpurea*, Shoot induction, Sterilization

Introduction

Bauhinia purpurea is a member of flowering plant, belonging to Fabaceae (Leguminosae), and also known as Purple Bauhinia, Butterfly Tree, Orchid Tree and many more names. This tree is located in the Southern region of China (including Hong Kong), the Southeastern region of Asia and may be found across India, ascending to an altitude of 1300 meters in the Himalayas (Khare *et al.,* 2004). *B. purpurea* is a medium to small-sized tree that reaches a standing height of 5 to 10 meters. It has a round, symmetrical, moderately thick crown. The bark is light grey-brown, and the twigs are thin and light green. Its heartwood is dark brown, hard and substantial. Leaves with equally broad rounded to shallow-cordate bases that are equally wide, an entire border, smooth surfaces, and small stipules 1-2 mm. in length, 2.5–3.5 cm. long petioles which are puberulous to

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glabrous, and leaves are also 4.5–11 centemeters long. Their flowers are pink to almost white colour or at least purple dots, followed by approximately 30 centimeters long, slim, brown, unruffled seed pods, which usually persist on the tree throughout the winter. The fruits are brown colour, strap-shaped, not septate, elongated dehiscent pods, 15 to 30 centemeters long and maturing in spring and summer (Orwa *et al*., 2009).

Bauhinia purpurea is commonly planted as a garden tree in backyards or parks, along the street, due to its stunning redolent pink or purple blooms. The leaves can be used as a decoration or to season fish and other meats. Their seeds are abundant with fiber, crude lipid, crude protien, minerals, carbohydrates and essential amino acids (Vadivel *et al*., 2011) Sometimes the seeds are eatable and the leaves are applied as cough remedy (Dhyani *et al*., 2016). The plant's bark is applied as an astringent for treating indigestion. Its infusions are suggested as an effective ulcer wash solution. *Bauhinia purpurea* is commonly known as a component of Bauhinia purpurea agglutin (BPA), a lectin concerning a binding capacity to galactose and lactose, extensively used in immunochemical, biochemical and histochemical research (Orwa *et al*., 2009). Additionally, this plant has been traditionally used to treat septicemia, convulsions, delirium, pain, rheumatism, and dropsy. Flavone gylcosides, 6-butyl-3-hydroxy flavanone, foliar flavonoids, amino acids, phenyl fatty ester, lutine, and β-sitosterol were all reported to be found in the plant's aerial parts. The plant's medicinal properties were discovered relating to these chemical compounds (Shreedhara *et al*., 2009). Furthermore, there are more studies about the dehydrated leaves' nutrient and anti-nutritional composition and content of antioxidants. They discovered that the dehydrated leaves were rich in iron (21.73 mg/100 g), calcium (240mg/100g), carbohydrate (66.82 g/100 g), and energy (365 kcal/100 g) (Dhyani *et al*., 2016). *B. purpurea* had several advantages and enemies, such as the rodents and larvae of various insects that consume their seeds and plantlets. The report only mentioned two diseases that the trees were susceptible to: leaf spot and leaf scorch. The viruses that the trees were subjected with were Clitoria yellow vein tymovirus and Turnip rosette sobemovirus. (Orwa *et al*., 2009). All of these problems reduced the growth index of the tree and increased the chances of its extinction in the future.

For a long time, studies of plant tissue culture from explants have been published in many different plant species, including *Glycyrrhiza Glabra* (Mousa *et al.,* 2006), *Medicago sativa* (Li *et al*., 2009) and *Ceratonia siliqua* (Ahmed *et al.,* 2021). The objective was to estimate the number of *B. purpurea* rapidly and without mutation from various explants.

Materials and methods

Seed germination

The seeds of *B. purpurea* were obtained from Chiang Rai, northern Thailand and were cleaned by running tap water for 15 minutes. The cleaned seeds were soaked with 20% sodium hypochlorite for 20 minutes and then washed with distilled water 2 times. Finally, the seeds were sterilized with 0.2% fungicide for 15 minutes and soked with distilled water 3 times. Forceps opened the seed coat of the seeds before being inoculated on half-strength Murashige and Skoog (1962) medium containing 3% sucrose supplemented with different concentrations (1.0, 2.0, 3.0 and 4.0 mg/L) of GA_3 and the pH of the medium was adjusted to 5.6- 5.8 before autoclaving at 121ºC for 15 minutes. The medium were solidified by adding 8% of agar. The cultures were sustained at 25 ± 2ºC with 16 h of photoperiod.

Surface sterilization

The nodal explants collected from naturally healthy trees were studied to find the optimal condition for surface sterilization. There are six experiments in this study (E1 to E6). For all experiments, the explants were cleaned with 20% Teepol detergent solution for 10 minutes in the pre-sterilizing process and then were rinsed in distilled water for 5 minutes. Afterward, washed with 0.1% fungicide for 15 minutes after that rinsed 2 times with distilled water, followed by 10% (E1, E2 and E3) or 20% (E4, E5 and E6) sodium hypochlorite for 10 minutes, rinsed in distilled water for 5 minutes and then lastly, washed with 1.0% (E2 and E5) or 2.0% (E3 and E6) $HgCl_2$ for 5 minutes and the rest of experiments (E1 and E4) were washed without $HgCl_2$. All experiments were rinsed 2 times with distilled water (Table 1).

Shoot induction

The research used nodal explants from two sources for shoot induction. Firstly, the nodal explants were collected from naturally healthy trees and sterilized using the method. The second source of the nodal explants was sterilized seedlings from inoculated seeds. Both of the sterilized nodal explants were cut into 1-1.5 cm per segment and then inoculated on MS medium supplemented with various amounts (1.0, 2.0, 3.0 and 4.0 mg/L) of BAP or different concentrations (1.0, 2.0, 3.0 and 4.0 mg/L) of Kn for shoot induction. The cultures were incubated under 16 h of photoperiod. The temperature was maintained at 25 ± 2ºC and subculture every 4 weeks in the same condition.

Table 1. The various experiments of surface sterilization method on nodal explants of *B. purpurea*

Experiments	First sterilizing agents	Exposure period (min)	Second sterilizing agents	Exposure period (min)	Third sterilizing agents	Exposure period (min)
E1	0.1% fungicide	15	10% NaOCl	10	-	5
E2	0.1% fungicide	15	10% NaOCl	10	1.0% $HgCl_2$	5
E3	0.1% fungicide	15	10% NaOCl	10	2.0% $HgCl_2$	5
E4	0.1% fungicide	15	20% NaOCl	10	-	5
E5	0.1% fungicide	15	20% NaOCl	10	1.0% $HgCl_2$	5
E6	0.1% fungicide	15	20% NaOCl	10	2.0% $HgCl_2$	5

Root induction

Following four weeks of the culture, regenerated shoots were moved into the following method, root induction. In this study, the root induction medium was applied was a ½ strength MS medium enriched with various auxin, including Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), and 1-Naphthaleneacetic acid (NAA) at 0.25, 0.50, and 1.0 mg/L concentrations. The cultures were incubated for 16 h of photoperiod. The temperature was maintained at 25 ± 2ºC and subculture every 4 weeks in the same condition.

Acclimatization

For acclimatization, the healthy plantlets from *in vitro* culture were washed with tap water for removed medium and agar, followed by soaking with 0.1% fungicide for 20 minutes. The cleaned plantlets were planted in the plastic pot, filled with soil and perlite in 2:1 ratio, wrapped in a perforated plastic bag for 2-4 weeks, and inoculated at 25 ± 2ºC with a photoperiod of 16 h. The grown plants were transferred to a green greenhouse 2 weeks later for further hardening.

Statistical analysis

The information was analyzed through the one-way analysis of variance (ANOVA) by using the IBM SPSS Statistics program version 29 (SPSS Inc.,

USA), and the multiple data were evaluated via Duncan's at the significant differences ($P<0.05$).

Results

Surface sterilization

Surface sterilization is one of the most crucial processes in *in vitro* propagation. The nodal explants were investigated in this study to find optimal conditions and suitable concentration of sterilizing agent for the surface sterilization method. The highest percentage of nodal explants, which were healthy and free of infection, was 92 percent (46 of 50 explants) treated on E5, as shown in Table 2 and Figure 1. The E5 condition included pre-sterilized for 10 minutes with 20% Teepol detergent solution and then soked with distilled water for 5 minutes. Then washed with 0.1% fungicide for 15 minutes, rinsed twice with distilled water, followed with 20% sodium hypochlorite for 10 minutes, rinsed twice with distilled water, and finally with 1.0% $HgCl_2$ for 5 minutes (Table 1).

Table 2. The effects of various sterilization methods on nodal explants of *B. purpurea* for 2 weeks inoculation

Experiments	Number of explants	Number of survival explants	Percentage of survival explants
E1	50	32	64
E2	50	34	68
E3	50	36	72
E4	50	42	84
E5	50	46	92
E6	50	34	68

Seed germination

The seed germination was germinated on a half-strength MS medium enhanced with GA3 (1.0-4.0 mg/L), so the seed germination procedure did not require more than 7 days. The highest germination percentage was 100% achieved from half-strength MS medium applyed with 2.0, 3.0 or 4.0 mg/L of GA_3. However, the maximum length of the seedling was 71.14 mm, and it grew from a half-strength MS medium supplemented with 3.0 mg/L of GA_3 in only 3 days, as shown in the Table 3.

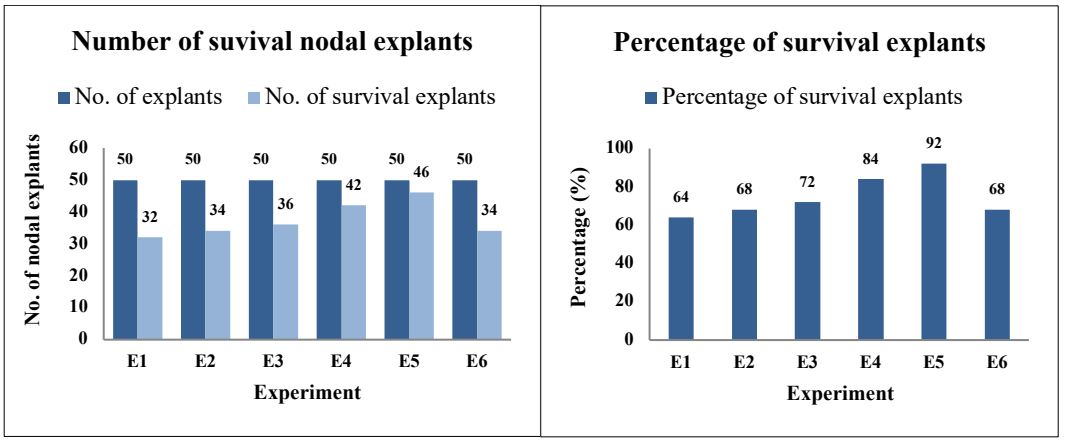

Figure 1. The effects of various sterilization methods on nodal explants of *B. purpurea* for 2 weeks inoculation

Table 3. The effects of ½ strength MS medium supplemented with several concentrations of GA_3 on seed germination of *B. purpurea* for 2 weeks

Concentration of GA_3 (mg/L)	Seedling length (mm) Mean±SD	% Germination	Days of germination
1.0	55.05±0.552[d]	93.33	5
2.0	60.80±0.483[c]	100	5
3.0	71.14±0.528[a]	100	3
4.0	63.17±0.321[b]	100	4
Control	48.90±0.507[e]	80	7

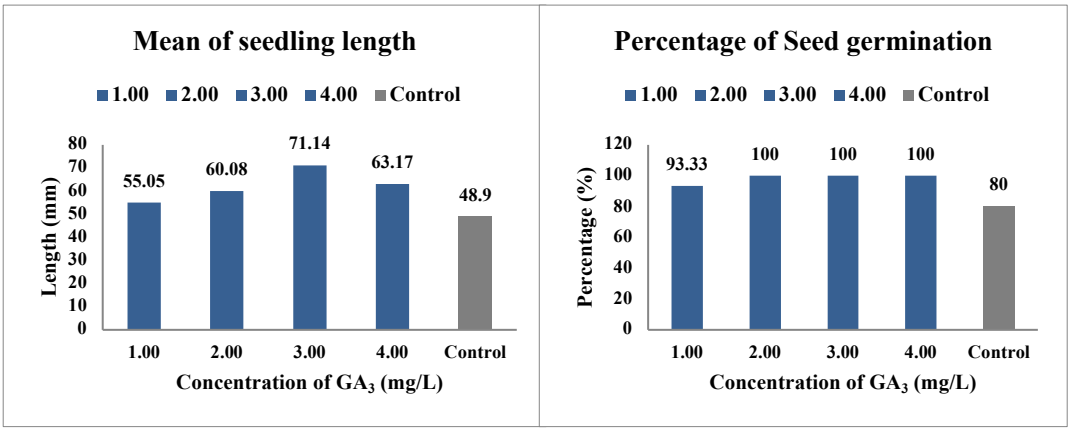

Figure 2. The effects of half-strength MS medium supplemented with various concentrations of GA_3 on seed germination of *B. purpurea* for 2 weeks

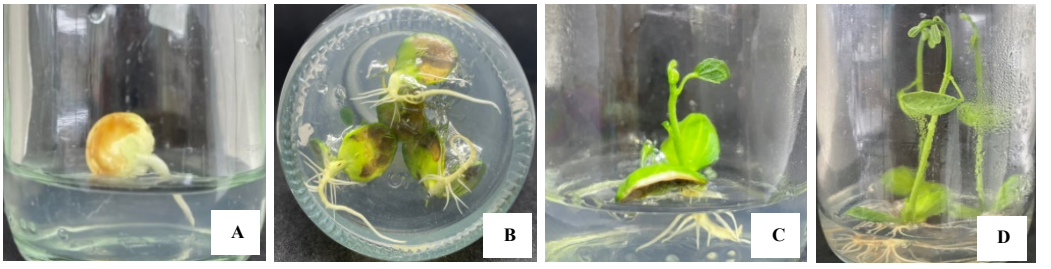

Figure 3. Development of *B. purpurea*'s seeds on half-strength MS medium supplemented with 3.0 mg/L of GA_3. (A) After 3 days of cultured, (B) After 5 days of cultured, (C) After 5 days of cultured and (D) After 2 weeks of cultured

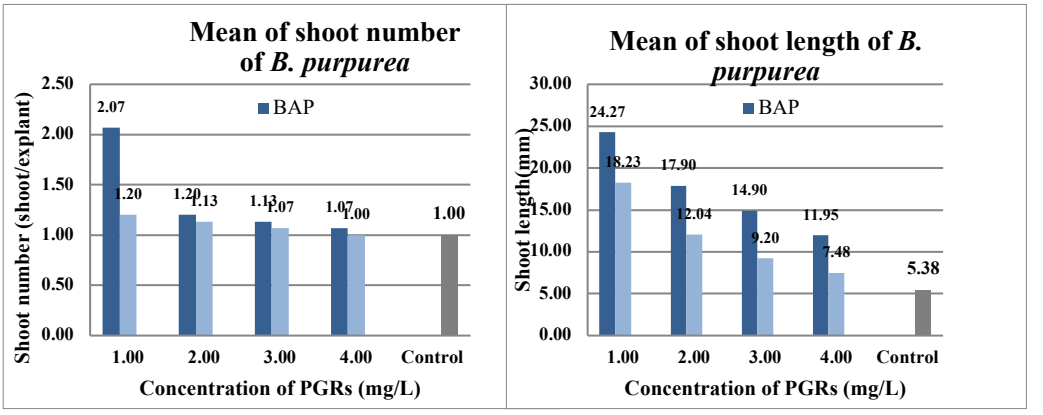

Figure 4. The effects of MS medium supplemented with multiple concentrations of different cytokinin on shoot induction of *B. purpurea* for 4 weeks. (A) Mean of shoot number and (B) Mean of shoot length

Shoot induction

After sterilization, the shoot induction was applied to the nodal explants and inoculated on MS medium supplemented with various concentrations of BAP (1.0, 2.0, 3.0, and 4.0 mg/L) or Kn (1.0, 2.0, 3.0, and 4.0 mg/L). The shoots were elongated on MS medium supplemented with 1.0 mg/L of BAP as the shoot induction medium for a 2-week culture period. Within 4 weeks, the shoots were promoted with all of BAP and Kn concentrations. According to the results reported in Table 4, MS media supplemented with 1.0 mg/L of BAP produced the most significant average amount of shoots per explant and the highest length of shoots as 2.07 shoots and 24.27 mm, respectively (Figure 4 and Figure 5).

Table 4. The effects of MS medium supplemented with various concentrations of different cytokinin on shoot induction of *B. purpurea* for 4 weeks

Cytokinin	Concentration (mg/L)	Mean of shoot no. per explant	Shoot length (mm) Mean±SD
BAP	1.0	2.07±0.799[a]	24.27±0.984[a]
	2.0	1.20±0.414[b]	17.90 ±0.968[b]
	3.0	1.13±0.352[b]	14.90±0.511[c]
	4.0	1.07±0.258[b]	11.95±0.847[d]
Kn	1.0	1.20±0.414[b]	18.23±0.403[b]
	2.0	1.13±0.352[b]	12.04±0.493[d]
	3.0	1.07±0.258[b]	9.20±0.525[e]
	4.0	1.00±0.000[b]	7.48±0.615[f]
Control		1.00±0.000[bc]	5.38±0.731[g]

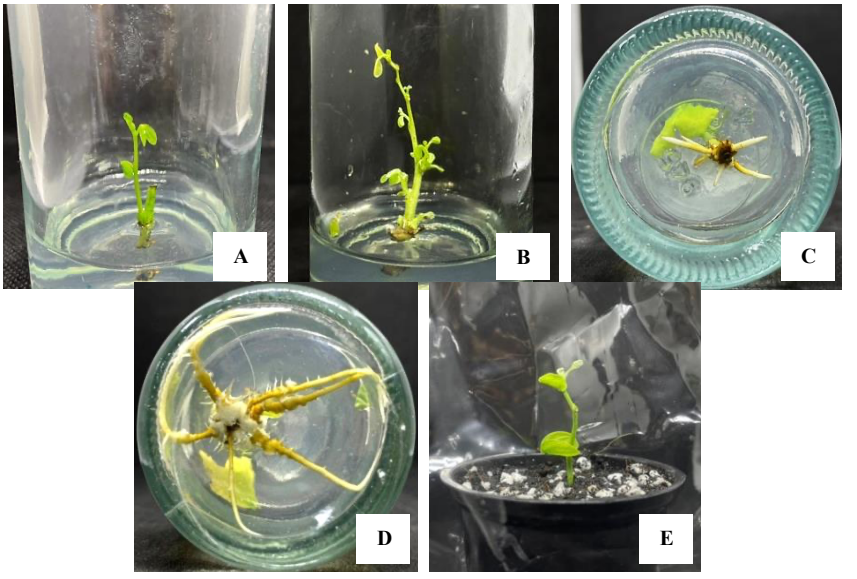

Figure 5. Plant tissue culture of *B. purpurea*. (A) Nodal explants cultured on MS medium supplemented with 1.0 mg/L of BAP for shoot induction in 2 weeks, (B) Nodal explants cultured on MS medium supplemented with 1.0 mg/L of BAP for shoot induction in 4 weeks, (C) Root formation cultured on half-strength MS medium supplemented with 0.25 mg/L of IAA after 2 weeks, (D) Root formation cultured on half-strength MS medium supplemented with 0.25 mg/L of IAA after 4 weeks and (E) Acclimatization of *B. purpurea*

Root induction

This study added several auxins, such as IAA, IBA, or NAA, to half-strength MS medium at varied concentrations (0.25, 0.5, and 1.0 mg/L) as root induction mediums. However, after 4 weeks of culture, *in vitro* rooting of *B. purpurea* was only enhanced by adding IAA as a plant growth regulator. Table 5 showed that at half-strength MS medium supplemented with 0.25 mg/L of IAA, the percentage of root development was 100%. However, the percentage of root formation dropped as the concentration of IAA increased. Table 6 showed that 3.60 roots per explant and 33.45 mm were the most significant root length and the greatest average number of roots per explant, respectively.

Table 5. Effects of half-strength MS medium supplemented with different concentrations of auxin on root formation of *B. purpurea* for 4 weeks

Auxin	Concentration (mg/L)	% Root formation
IAA	0.25	100
	0.50	84
	1.0	70
IBA	0.25	0
	0.50	0
	1.0	0
NAA	0.25	0
	0.50	0
	1.0	0
Control		0

Table 6. Effects of half-strength MS medium supplemented with different concentrations of IAA on root induction of *B. purpurea* for 4 weeks

IAA (mg/L)	Mean of root no. per explant	Root length (mm) Mean±SD
0.25	3.60±0.737[a]	33.45±0.401[a]
0.50	2.53±0.743[b]	26.94 ±0.131[b]
1.00	2.40±0.910[b]	24.66±0.412[c]
Control	0.00±0.000[c]	0.00±0.000[d]

Acclimatization

The following technique used in this study was acclimatization, while the root induction approach was accomplished. According to the acclimatization procedure, 73% of plantlets survived after being transferred to plastic pot, filled

with soil and perlite in 2:1 ratio and wrapped in a perforated plastic bag for 2 weeks. After that, the small trees were transferred to the glasshouse for 2 weeks. Percentage of survival plantlets result was shown in table 7 and figure 6.

Table 7. The percentage of survival plantlets of *B. purpurea* after being transferred to a greenhouse for 2 weeks

No. of acclimating plantlets	No. of survival plantlets	Percentage of survival plantlets (%)
15	11	73.33

Figure 6. The 2-week-old plantlets were cultured in plastic bags before being transferred to the greenhouse

Discussion

The study of plant regeneration of *B.purpurea* by tissue culture technique was begun with the most important method, sterilization. Several popular sterilizing agents for removing microorganisms from explants such as mercuric chloride ($HgCl_2$) and sodium hypochlorite (NaOCl). In this study, the most efficient condition of the sterilizing agent was NaOCl, as in the study of optimization of explant surface sterilization in *Phanera sirindhorniae* (Sirimat and Sakulsathaporn, 2019). The most important factor in the shoot induction process is cytokinin, for example, BAP, Kn, and *m*T or a combination. In this study, the optimal conditions for development for establishing nodal regeneration and increasing shoot length of *B. purpurea* were MS medium added with 1.0 mg/L of BAP alone. On the other hand, the most compelling studies in *B. variegate* produced the highest number of nodes, shoot length, and ö calli on MS medium attended with 1.0 M BAP and 0.5 M NAA (Singh *et al.,* 2012). So, the findings suggested that different species performed differently to cytokinin paired with auxin or cytokinin alone. The application of auxins plays a major role in the adventitious rooting process, where numerous reports ascribed the initiation and

division of adventitious roots to exogenous or endogenous auxins (Abdullahil *et al*., 2010).

Furthermore, it has been observed that the effectiveness of several auxins for induction of adventitious roots is variable for disparate species (Hussein *et al*., 2012). For example, NAA and IBA were efficient at root induction of *Bauhinia vahlii* (Upreti and Dhar, 1996), while IBA alone showed excellent results to root induction of *Bauhinia cheilantha* (Gutiérrez *et al*., 2011). The final process of plant tissue culture was acclimatization. According to Jaime (2013), the next method was acclimatization after the roots from in vitro culture were induced. The acclimatization of *Bauhinia* species was survived while planted in a combination of 1:1 sand and soil; however, in the study of *Bauhinia purpurea,* the plantlets survived in a mixture of soil and perlite in 2:1 ratio.

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