International Journal of Agricultural Technology 2024 Vol. 20(3):1237-1246
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Effects of growth regulators on *in vitro* propagation of *Sophora tomentosa* L. (Necklace pod)

Rungprateeppaiboon, N.[1], Chareonsap, P. P.[2], Pongtongkam, P.[3], Poeaim, A.[1*] and Poeaim, S.[1]

[1]Department of Biology, School of Science, King Mongkut's Institute of Technology Ladkrabang (KMITL), Ladkrabang, Bangkok 10520, Thailand; [2]Plant Genetic Conservation Project, Chitralada Villa, Dusit, Bangkok 10303, Thailand; [3]128/13 Moo 5 Tambon Sano Loi, Bang Bua Thong, Nonthaburi, 11110. Thailand.

Rungprateeppaiboon, N., Chareonsap, P. P., Pongtongkam, P., Poeaim, A. and Poeaim, S. (2024). Effects of growth regulators on *in vitro* propagation of *Sophora tomentosa* L. (Necklace pod). International Journal of Agricultural Technology 20(3):1237-1246.

Abstract Seeds of *Sophora tomentosa* were sterilized and germinated on MS medium with BAP (1 mg/L), which was observed to be 75% germination rate with the highest amount of multiple shoots (3 shoots per seed) and an average length of 29.56 mm. The germination rate was reduced to 33.33% by 1% NaCl. Multiple shoot induction was worked on MS medium with 2.0 mg/L of TDZ that induced the highest shoot bud formation rate (46.67%) with the highest amount of shoot bud (10.71 buds per culture), while 1.0 mg/L GA_3 showed the highest average length of 34.13 mm. For root induction, 20% rooting was formed in 1/2 MS medium with a combination of 0.5 mg/L IAA and BAP with 0.1% AC, with 3 roots per culture and an average root length of 15.36 mm. After acclimatization for 16 weeks, 70% of the plantlets successfully survived.

Keywords: *Sophora tomentosa*, Multiple shoot induction, Root induction, Acclimatization

Introduction

Sophora tomentosa is known as a necklace pod or silver brush. This plant was classified in the Fabaceae family. It is native to Florida and Texas. It is found worldwide on tropical beaches. It is commonly a thicket-forming shrub. The bark is yellowish-brown and has corky lenticels. The leaves are alternate and odd-pinnate. The corolla is pea-like and flowers throughout the year but is most abundant in fall. Both flowers and immature pods appear simultaneously on the lengthening raceme. Constricted pods appear as beads on a necklace (Brown and Cooprider, 2009), so in Thailand, it was known as Sara phat phit and could be found in the north, south and southeast, growing along the coast, for instance, in Phetchaburi province (Smitinand, 2001). All parts of *S. tomentosa* are herbs; for example, leaves were used to stun fish. Aly *et al.* (2020) have reported that the extract from leaves of *S. tomentosa* was partially effective against *Staphylococcus aureus* and active against *E. coli*. Seed, root and bark could relieve dysentery and diarrhea (Gardner *et al.*,

[*]**Corresponding Author**: Poeiam, A.; **Email**: anurug.po@kmitl.ac.th

2016). According to this reason, many seeds were reaped for treatment and selling. The dried seed cost 1,000 – 1,500 THB/kg (about 28 USD/kg), and the seed from wild nature would be moldy and drilled by bugs since it was inside the pod.

Seeds or cuttings can propagate necklace pods. The seed is completely covered by a waxy seed coat, making it difficult for water to enter and imbibe the seed. Mechanical or chemical scarifications are usually recommended to produce effective germination (Jara-Peña and Marín-Bravo, 2023). Good air circulation is needed to avoid fungal infection. Thus, a vast space would be required. Propagation by seed could get only 1 seedling, while *in vitro* propagation could get more seedlings in a confined space.

However, *in vitro* propagation is easily attainable for many plant species, and this method could increase many plants under aseptic conditions. Depending on the species, many factors affect the propagation of this plant, such as seed surface sterilization, plant growth regulators (PGRs) and acclimatization. The most important classes of the PGRs used in tissue culture are auxins and cytokinins. PGRs show dramatic effects depending on the concentration used, even though they are used in deficient concentrations in the media. Auxins play a role in many developmental processes, such as cell elongation and adventitious root formation. Generally, in the presence of low concentrations, root initiation occurrs. The most common auxins used in this study are Indole-3-acetic acid (IAA), 3-indolebutyric acid (IBA) and α-Naphthaleneacetic acid (NAA). Cytokinin such as 6-Benzylaminopurine (BAP), *meta*-Topolin (*m*T), Thidiazuron (TDZ) and kinetin were used to promote cell division and stimulate initiation and growth of shoots *in vitro*. They induce adventitious shoot formation at high concentrations but inhibit root formation. Gibberellins help to stimulate the elongation of internodes and seed germination (Trigiano and Gray, 2000). Gibberellic acid (GA_3) is used in this research.

After *in vitro* propagation, the plantlets might be acclimated before growing in an open environment for more survival. When plantlets are transferred from *in vitro* to an open environment, they are exposed to biotic stresses, altered temperature, light intensity and humidity conditions. So, keeping them in the greenhouse with similar conditions in which they were cultured in the lab is necessary. Maintaining high humidity for plants and keeping them in the shade is essential to protect them from water loss. The change in media from organic to inorganic nutrients will activate the photosynthesis mechanisms and prepare them to withstand low humidity. The acclimatization was adjusted to depend on the plant (Singh, 2021).

This research aimed to investigate the suitable PGRs for germinating, multiplying shoots, and increasing the shoot length and rooting. After *in vitro* propagation, the plantlet was adjusted to survive in the open environment.

Materials and method

Surface sterilization

The pods of *S. tomentosa* were collected from Koh Yao Noi, Phang Nga Bay, in southern Thailand. Seeds were removed from the pod and washed with dishwashing liquid and running water for dirt cleaning, then surface sterilized with 70% ethanol for 1 minute. Therefore shaking at 225 rpm in a solution that included 0.1% Mercuric chloride ($HgCl_2$), 0.1% plant preservative mixture (PPM), 0.1% Cefotaxime and a few drops of tween-20 for 15 minutes, then repeating this step without $HgCl_2$ and rinsing them in the sterilized water for 5 minutes. After sterilization, the seed coat was cut slightly for easier germination.

Seed germination and multiple shoot induction

Murashige and Skoogs (1962) MS medium supplemented with sucrose (30 g/L), gellan gum (2.6 g/L) and 1.0 mg/L of BAP, GA_3, *m*T, TDZ or kinetin were used for seed germination and multiple shoot induction. The pH of medium was adjusted to 5.6-5.8 by NaOH and HCl before autoclaving, except for GA_3 which was filtrated. Seeds were grown in a photo period (16 hours/day) at 25±2 °C. Shoot formation was observed every week for 4 weeks, then the amount of shoots per seed was counted, and the germination rate and average shoot length were calculated.

Effect of NaCl on germination and multiple shoot formation

Seeds were cultured on MS medium supplemented with 1.0 mg/L of BAP, which was added by 0, 0.5, 1.0, 2.0 and 3.0% of NaCl, and grown under the same photo-period at 25±2 °C. Germination and multiple shoot formation were observed for 4 weeks, then the amount of shoots per seed was counted, and the germination rate and average shoot length were calculated.

Shoot elongation

Shoot buds were separated to increase the amount and height by cultured on MS medium, which was added by each of the following BAP, GA_3, *m*T, TDZ or kinetin at the concentrations of 1.0, 2.0 and 3.0 mg/L. The buds were cultured under 16 hours/day photo-period at 25±2 °C. After 8 weeks, the shoot bud formation rate was recorded with an average number of shoot buds per culture and the height of the shoot was measured by Vernier caliper and presented as the average shoot length.

Root induction

The shoot with 20-30 mm. was transferred to treatment on half-strength MS (1/2 MS) medium with and without 0.1% AC supplemented by 0.25, 0.5, 0.75 and 1.0 mg/L of IAA, IBA or NAA alone and the combination of 0.5 mg/L BAP and 0.5 mg/L IAA, IBA or NAA. The cultures were kept under the same conditions as the previous subtitle. Root formation and root length were marked after 8 weeks.

Acclimatization

The plantlets with well-developed roots from seed culturing were removed medium from the root by running tap water before putting in the plastic pot with sterilized peat moss and perlite (2:1) and watering by 1/4 MS. They were covered by a transparent plastic bag and kept at 25±2 °C. After 8 weeks, the plastic bag was removed, and the plantlets were maintained in the greenhouse. The plantlets were moved to an open environment, and the survival rate was calculated in the 16^{th} week of acclimatization.

Data analysis

All the experiments were tested in triplicates with 5 explants per replication. Data were analyzed using analysis of variance (ANOVA). Duncan's multiple range tests (DMRT) at $\alpha = 0.05$ was used for mean separation utilizing IBM SPSS Statistics 25 software.

Results

Seed germination and multiple shoot induction

Seed cultured on MS medium supplemented with 1.0 mg/L BAP had 75% germination rate and an average length of 29.56±0.95 mm. with the maximum number of shoots per seed (3 shoots per seed), but rooting was not well. However, MS medium supplemented with GA_3 had 92.86% germination rate but shot up only 1 shoot per seed with fine rooting and the highest average length of 50.83±0.81 mm. Moreover, MS medium supplemented with *m*T, kinetin and TDZ had 1 shoot per seed with germination rate of 21.43%, 31.25% and 46.15%, respectively, as shown in Table 1. The seed germinated on BAP had thicker shoots than GA_3 and kinetin, which was tall and slim, while shoots from *m*T and TDZ were stout and short (Figure 1 A-E).

Effect of NaCl on germination

Seed could germinate at 1.0% NaCl and induce 1.33 shoots per seed with an average shoot length of 16.68±0.55 mm. (Figure 1F), but the

germination rate was reduced to 33.33%, while at 0.5%, it has a little effect on germination. And there is no germinating at concentrations of 2.0 and 3.0% NaCl (Table 1).

Table 1. Seed germination rate and shoot formation after culturing onto MS medium supplemented with PGRs for 4 weeks

PGRs (mg/L)	Germination rate	No. of shoots (shoot/seed)	Shoot length (mm.)
MS (control)	84.62%	1.00	44.25±1.08[b]
BAP 1.0	75.00%	3.00	29.56±0.95[c]
GA_3 1.0	92.86%	1.00	50.83±0.81[a]
kinetin 1.0	31.25%	1.00	15.86±0.85[e]
*m*T 1.0	21.43%	1.00	11.83±0.83[f]
TDZ 1.0	46.15%	1.00	3.40±0.67[g]
BAP 1.0 + 0.5% NaCl	60.00%	2.33	24.54±0.94[d]
BAP 1.0 + 1.0% NaCl	33.33%	1.33	16.68±0.55[e]
BAP 1.0 + 2.0% NaCl	0.00%	0.00	0.00[h]
BAP 1.0 + 3.0% NaCl	0.00%	0.00	0.00[h]

*Data are presented at the mean ± SD.
Means followed by different letters in the same column show significant differences at $p \leq 0.05$

Shoot elongation

MS medium supplemented with 1.0 mg/L of GA_3 could elongate the shoot bud to have an average length of 34.13±0.46 mm. The stem was slimmer when compared to the stem treated by BAP. In addition to 2.0 mg/L TDZ, the optimum shoot bud formation rate (46.67%) was exhibited with the highest amount of shoot bud (10.71±0.76 buds per culture) but did not increase the height of the shoot. The result is shown in Table 2. (Figure 2. A-K)

Root induction

Each formula of the root induction medium did not show any rooting response. Except for 1/2 MS medium supplemented with a combination of 0.5 mg/L IAA and BAP with 0.1% AC, that was observed to have 20% rooting and 3 roots per culture with an average root length of 15.36±0.67 mm. Roots were lean and weak (Figure 2L). The shoots treated by IAA and IBA were fresh green but had thicker stems when combined with BAP. While NAA was presented in the medium, leaves and shoot tips turned yellow and withered.

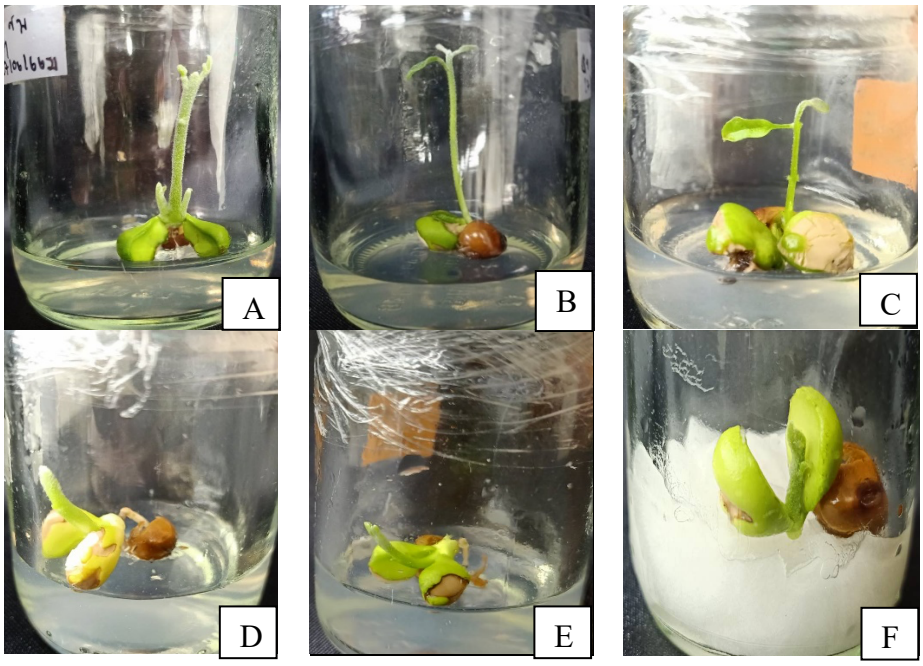

Figure 1. Characteristics of the shoot after 4 weeks of culturing seed on MS with BAP 1.0 mg/L (A), MS with GA_3 1.0 mg/L (B), MS with kinetin 1.0 mg/L (C), MS with *m*T 1.0 mg/L (D), MS with TDZ 1.0 mg/L (E) and MS with BAP 1.0 mg/L added by 1% NaCl (F)

Table 2. Effect of PGRs on shoot bud formation and shoot elongation after cultivating for 8 weeks

PGRs (mg/L)	Shoot bud formation rate	No. of shoot buds (bud/culture)	Shoot length (mm.)
MS (control)	20.00%	3.67 ± 0.58^{cd}	11.13 ± 1.52^{d}
MS + BAP 1.0	20.00%	2.33 ± 0.58^{e}	10.18 ± 1.60^{d}
MS + BAP 2.0	20.00%	3.33 ± 0.58^{de}	15.60 ± 0.92^{c}
MS + GA_3 1.0	20.00%	3.67 ± 0.58^{cd}	34.13 ± 0.46^{a}
MS + GA_3 2.0	26.67%	3.75 ± 0.50^{cd}	20.77 ± 0.74^{b}
MS + kinetin 1.0	20.00%	3.00 ± 0.00^{de}	10.24 ± 0.51^{d}
MS + kinetin 2.0	20.00%	3.67 ± 0.58^{cd}	-
MS + *m*T 1.0	26.67%	4.75 ± 0.96^{c}	11.78 ± 1.41^{d}
MS + *m*T 2.0	26.67%	6.75 ± 0.50^{b}	10.16 ± 1.00^{d}
MS + TDZ 1.0	33.33%	6.00 ± 0.71^{b}	-
MS + TDZ 2.0	46.67%	10.71 ± 0.76^{a}	-

* Data are presented at the mean ± SD
Means followed by different letter in the same column show significant differences at $p\leq0.05$

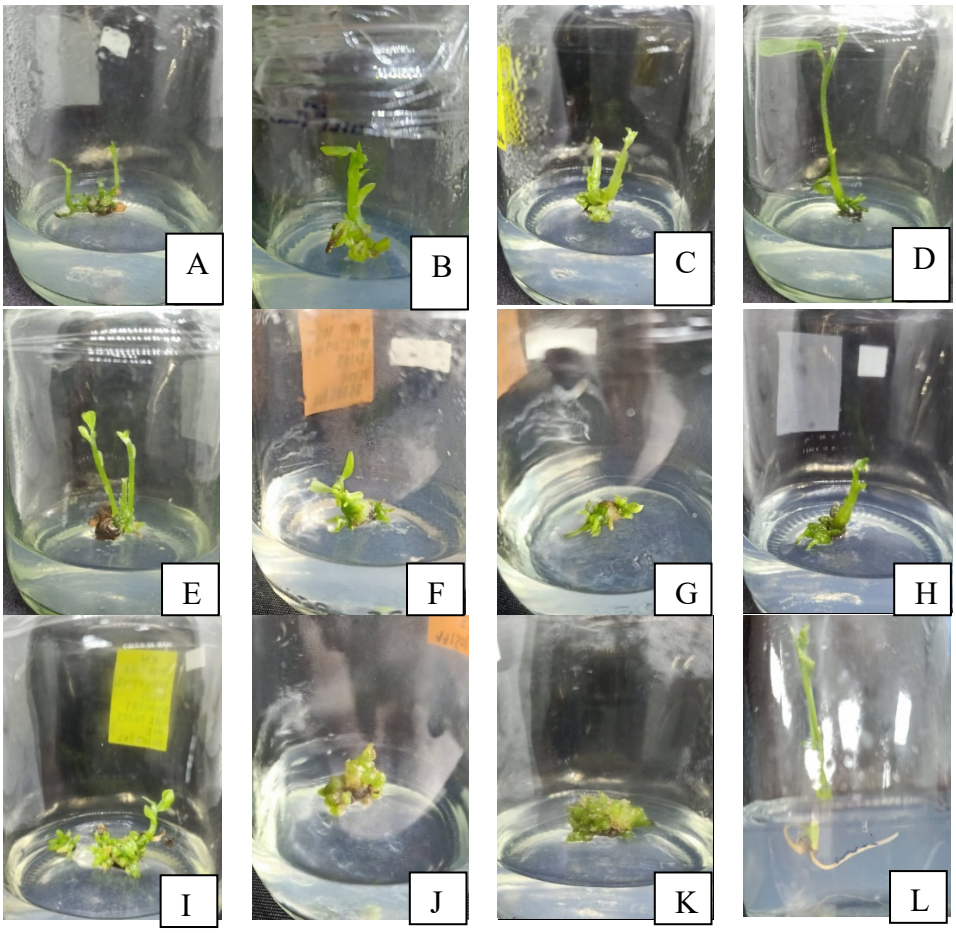

Figure 2. Characteristics of the shoot after 8 weeks of cultivating shoot bud on MS (control) (A), MS with BAP 1.0 mg/L (B), MS with BAP 2.0 mg/L (C), MS with GA_3 1.0 mg/L (D), MS with GA_3 2.0 mg/L (E), MS with kinetin 1.0 mg/L (F), MS with kinetin 2.0 mg/L (G), MS with *m*T 1.0 mg/L (H), MS with *m*T 2.0 mg/L (I), MS with TDZ 1.0 mg/L (J) and MS with TDZ 2.0 mg/L (K) and Characteristics of root after culturing on 1/2 MS medium with 0.1% AC supplemented with 0.5 mg/L of IAA and BAP for 8 weeks (L)

Acclimatization

Plantlets with well-developed roots from seed culturing (Figure 3A) were transferred to grow in potting media with soil and perlite (2:1) under greenhouse conditions. After 8 weeks, the plantlets of 70-80 mm length (Figure 3B) were taken out of the plastic bag and kept in the nursery for another 8 weeks. After all, 70% of plantlets successfully survived in an open environment with an average shoot length of 109.75±1.09 mm (Figure 3C).

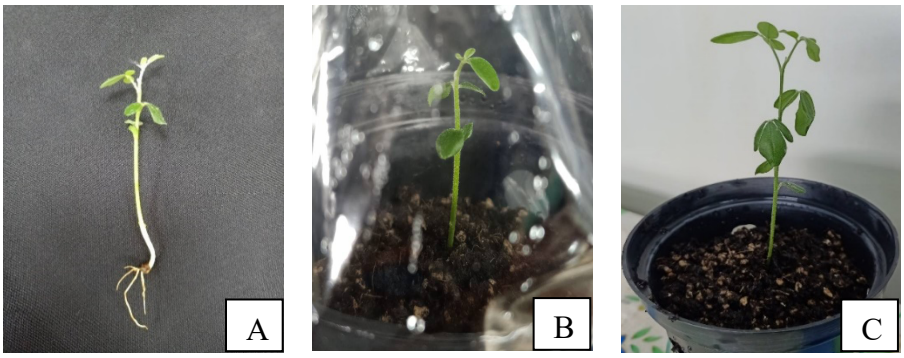

Figure 3. Plantlets with well-developed roots from seed culturing (A), Plantlet after acclimatization for 8 weeks (B) and 16 weeks (C)

Discussion

This study showed that MS medium supplemented with 1.0 mg/L BAP proved to have the maximum number of shoots per seed (3 shoots per seed) among all PGRs used with a shoot length of 29.56±0.95 mm. This finding accorded with the *in vitro* study on *Sophora mollis*, in which the 96.27% shoot formation was achieved with 25.32 mean shoot number per culture and 4.5 cm. shoot length in MS medium enriched with 8.9 µM BAP (Bhandari *et al*., 2021). GA_3 at a concentration of 1.0 mg/L showed the highest germination rate of 92.86%, which is related to the work of Mensah *et al.* (2020) that found GA_3 promoted seed germinations of *Sesbania rostrata* and *Sesbania sesban* significantly compared to the control (MS). Furthermore, in the presence of NaCl, the seed remained at 20% germination rate with 1 shoot per seed and 10.74±0.75 mm. shoot length because *S. tomentosa* grew in the coastal forest, so it could tolerate a high concentration of salt (Brown and Cooprider, 2009).

For shoot elongation, shoots cultured on MS medium augmented with 1.0 mg/L GA_3 were observed to have the highest average length of 34.13±0.46 mm. that corresponded to the role of GA_3, which is responsible for stem elongation by increasing internode extension (Davies, 2010). Hakim *et al.* (2010) found that maximum elongation of the carob shoot (4.7±0.48 cm.) was recorded in the combination of 1.0 mg/L of BAP and GA_3 which was better than cultured on BAP alone. MS medium with 2.0 mg/L of TDZ could induce the highest shoot bud formation rate in this work. In the investigation of Singh *et al.* (2014), MS medium supplemented with 3.0 mg/L of TDZ could induce shoot regeneration rates of 89.2% and 13.40±2.04 shoots per explant in *in vitro* propagation of Goa bean. Moreover, Kurup *et al.* (2018) reported that MS medium enhanced with different concentrations of TDZ showed significant differences in bud sprouting and adventitious shoot induction. The highest shoot bud formation of *Haloxylon persicum* (a

hardy woody desert shrub) was recorded on MS medium enhanced with 0.5 µM TDZ.

Rooting was responded to 1/2 MS medium with 0.1% AC and the combination of 0.5 mg/L IAA and BAP, which had the optimal 3 roots per culture with an average root length of 15.36±0.67 mm. after 8 weeks of culture. IAA plays a role in stimulating root initiation on stem cuttings, the development of branch roots and the differentiation of roots in tissue culture (Davies, 2010). Fratini and Ruiz (2003) reported that the combination of 5 µM IAA and 1 µM kinetin induced the highest rooting rate (95.35%) in the culture of lentils. The study of factors affecting the inhibitory effect of IAA on root formation in pea cutting (Eliasson, 1981) showed the addition of activated charcoal to the rooting solution could increase the number of roots developed by adsorbed stimulatory and inhibitory compounds at the surface of the cutting base. The research of Omran *et al.* (2008) studied the effect of BAP on rooting of lentils, the results indicated that the high concentration of BAP (3 – 4 mg/L) inhibited rooting induction.

The efficiency of acclimatization for 16 weeks after moving out the plantlets from *in vitro* by using peat moss and perlite (2:1) as potting media was 70% successfully survived in an open environment. The survival of plantlets was concerned with a ventilated area that avoided fungal infection, and *S. tomentosa* will not tolerate long-term flooding by salt or brackish water (Brown and Cooprider, 2009). Therefore, perlite was used to improve aeration, defy compaction and be well-draining (Grant, 2021).

Acknowledgments

The author is thankful to the School of Science, King Mongkut's Institute of Technology Ladkrabang (KMITL) for providing instruments and a workplace. This project is funded by the National Research Council of Thailand (NRCT) under grant numbers N21A661218 and RE-KRIS (NRCT) 01/67.

References

Aly, S. H., Elissawy, A. M., Eldahshan, O. A., Elshanawany, M. A. and Singab, A. B. (2020). Phytochemical investigation using GC/MS analysis and evaluation of antimicrobial and cytotoxic activities of the lipoidal of leaves of *Sophora secundiflora* and *Sophora tomentosa.* Archives of Phamaceutical Sciences Ain Shams University, 4:207-214.

Bhandari, A., Singh, H., Srivastava, A., Kumar, P., Panwar, G. S. and Mao, A. A. (2021). *In vitro* propagation andcytological analysis of *Sophora mollis* Royle: an endangered medicinal shrub. Journal of Genetics Engineering and Biotechnology, https://doi.org/10.1186/s43141-021-00140-3.

Brown, S. H. and Cooprider, K. (2009). *Sophora tomentosa*. Retrieved from https://silo.tips/download/sophora-tomentosa-necklace-pod-silver-bush-yellow-necklace-pod

Davies, P. J. (2010). Plant Hormones Biosynthesis, Signal Transduction, Action!, New York, Springer, pp.1-15.

Eliasson, L. (1981). Factors affecting the inhibitory effect of indolylacetic acid on root formation in pea cuttings. Physiologia Plantarum, 51:23-26.
Fratini, R. and Ruiz, M. L. (2003). A rooting procedure for lentil (*Lens culinaris* Medik.) and other hypogeous legumes (pea, chickpea and *Lathyrus*) based on explant polarity. Plant Cell Report, 21:726-732.
Gardner, S., Sidisunthorn, P. and Chayamarit, K. (2016). Forest Trees of Southern Thailand Volume 2, Bangkok, Kobfai publishing project, pp.939.
Grant, A. (2021). What is Perlite: Learn About Perlite Potting Soil. Retrieved from https://www.gardeningknowhow.com/garden-how-to/soil-fertilizers/perlite-potting-soil.htm
Hakim, L., Islam, M. R., Mamun, A. N. K., Ahmed, G. and Khan, R. (2010). Clonal propagation of carob (*Ceratonia siliqua* L., Fabaceae). Bangladesh Journal of Botany, 39:15-19.
Jara-Peña, E. and Marín-Bravo, M. (2023). The role of the internal structure of Fabaceae seeds in the processes of dormancy and germination. Production and utilization of legumes Progress and prospects. IntechOpen. Retrieved from http://dx.doi.org/10.5772/intechopen.109627
Kurup, S. S., Thayale Purayil, F., Alkhaili, M. M. S., Tawfik, N. H., Cheruth, A. J., Kabshawi, M. and Subramaniam, S. (2018). Thidiazuron (TDZ) induced organogenesis and clonal fidelity studies in *Haloxylon persicum* (Bunge ex Boiss & Buhse): an endangered desert tree species. Physiology and Molecular Biology of Plants, 24:683-692.
Mensah, S. I., Ekeke, C. and Ibeagi, N. K. (2020). Effect of gibberellic Acid (GA_3) and kinetin on seed germination of *Sesbania sesban* L. and *Sesbania rostrata* L. (Fabaceae). Asian Journal of Agricultural and Horticultural Research, 5:32-41.
Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum, 15:473-497.
Omran, V. G., Bagheri, A. and Moshtagh,i N. (2008). Direct *in vitro* regeneration of lentil (*Lens culinaris* Medik.). Pakistan Journal of Biological Sciences, 11:2237-42.
Singh, A. (2021). How to Acclimate Tissue Culture Plants. Retrieved from https://www.plantcelltechnology.com/blog/how-to-acclimate-tissue-culture-plants/
Singh, V., Chauhan, N. S., Singh, M., Idris, A., Madanala, R., Pande, V. and Mohanty, C. S. (2014). Establishment of an efficient and rapid method of multiple shoot regeneration and a comparative phenolics profile in *in vitro* and greenhouse-grown plants of *Psophocarpus teyragonolobus* (L.) DC. Plant Signaling & Behavior, 9:e970443.
Smitinand, T. (2001). Thai plant names. Bangkok, The Forest Herbarium Royal Forest Department, pp.491.
Trigiano, R. N. and Gray, D. J. (2000). Plant Tissue Culture Concepts and Laboratory Exercises. New York, CRC Press LLC, pp.27-75.

(Received: 16 November 2023, Revised: 30 April 2024, Accepted: 10 May 2024)