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Plant regeneration of *Dimocarpus scandens* (Winit & Kerr) Boonsuk & Chantar. (Lamyai-Thao) by tissue culture technique

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Abstract The minimum contamination percentage (25%) was recorded in the experiment using 0.2% mercuric chloride ($HgCl_2$) for 20 min, which was observed to be a 50% germination rate after 2 weeks of culture. The maximum germination rate (68%) was obtained on Murashige and Skoog (MS) solid medium supplemented with 1.0 mg/L 6-benzylaminopurine (BAP), giving a shoot length of 48.00 mm. and root length of 31.40 mm. The optimal condition of nodal segment sterilization was recorded in the experiment using 0.1% $HgCl_2$ for 10 min, which gave 80% survival explants. For shoot induction, it was worked on MS solid medium with 0.5 mg/L 6-benzylaminopurine (BAP) that induced the highest average length of 11.31 mm. The highest root formation frequency (50%) and root length (3.0 mm) were achieved on an MS medium containing 2.0 mg/L indole-3-butyric acid (IBA). The plantlets were acclimatized and established in the soil.

Keywords: *Dimocarpus scandens*, Plant induction, Seed germination

Introduction

The longan (*Dimocarpus longan* Lour.), a member of the Sapindaceae family, is a fruit of substantial economic importance. It is exported in various forms including fresh, dried, frozen and canned, contributing billions of baht to the annual revenue. Rich in energy, longan flesh contains glucose, fructose, sucrose, and essential minerals such as phosphorus, calcium, and iron. In traditional Chinese medicine, dried longan is prized for its nourishing properties, particularly for the blood, nerves, and heart, making it beneficial for individuals with weakened health (Shahrajabian *et al*., 2019). In Thailand, longan, locally known as "Lamyai," includes several economically significant varieties, such as E-dor, Chompoo, Biew Kiew, and E-haew. Additionally, a species known as

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"Lamyai-Thao," with the scientific name *Dimocarpus scandens* (Winit & Kerr) Boonsuk and Chantar, 2017 (Boonsuk and Chantaranothai, 2017), is native to eastern Thailand, particularly in Chonburi Province, where it is often cultivated for ornamental purposes. This species was previously known as *Dimocarpus longan* var. *obtusus* and is also referred to by the synonym *Euphoria scandens* Winit & Kerr, 1941 (Winit and Kerr, 1941). Lamyai-Thao differs from *D. longan* as it grows as a vine, featuring reddish-brown bark, compound pinnate leaves, and fragrant white or light-yellow flowers, with round fruit encased in a pink to reddish-brown peel that develops from March to November. The aril or flesh is pinkish, sweet and more aromatic, with a large seed ranging from red-brown to brown (Boonsuk and Chantaranothai, 2017). Past research has focused on longan phenology (Pham *et al.,* 2015). The study during this period examined the relationship between various constituents and bioactive compounds in extracts from longan aril, peel, and seeds (Hsieh *et al*., 2008; Panyathep, 2013; Nitteranon, 2018). This includes the study of the biological effects of Lamyai-Thao (Chantarasaka *et al*., 2022) and its application as a jam (Nitteranon and Aroonkamonsri, 2018). While studies on longan tissue culture (Thu *et al*., 2017; Jiafu and Bizhu, 2000; Lai *et al*., 2000), none focus specifically on Lamyai-Thao, an endemic Thai species. The research aimed to improve *in vitro* propagation and refine suitable tissue culture techniques for Lamyai-Thao.

Materials and methods

Cultural media and conditions

Murashige and Skoog (MS) medium was used as the base in all experiments, with 2.6 g/L gellan gum, 30 g/L sucrose, and various plant growth regulators (PGRs) added. The pH was adjusted to 5.8 ± 0.2, then autoclaved at 121 °C and 15 psi for 15 minutes. Cultures were kept at 25 ± 2-°C, under a 16-hour light and 8-hour dark cycle in controlled plant growth room.

Surface sterilization and seed germination

The seeds of Lamyai-Thao were collected from fruits harvested between March and November. The fruits were washed with tap water, then peeled, and their flesh was separated from the seeds. The seeds were rinsed again before being utilized in the experiments. Five experimental setups were designed to evaluate effective surface sterilization methods, employing various sterilizing agents at different concentrations and exposure times, as outlined in Table 1.

Table 1. The various experiments of surface sterilization on the Lamyai-Thao seed

Experiments	First sterilizing agents	Exposure period (min)
E1	0.1% $HgCl_2$	20
E2	0.2% $HgCl_2$	20
E3	0.2% $HgCl_2$	10
E4	0.3% $HgCl_2$	20
E5	4% NaOCl	20

In all experiments, seeds were agitated in 70% ethanol for 1 minute and then immersed in the sterilizing agents shown in Table 1. This step was conducted alongside a 0.1% antibiotic antimycotic, 0.1% plant preservative mixture (PPM), 0.1% cefotaxime, and three drops of Tween-20. The procedure was then repeated for 10 minutes without using $HgCl_2$ or sodium hypochlorite (NaOCl). After this, the seeds were rinsed thrice in sterile distilled water for 5 minutes each. All procedures were carried out with shaking at 250 RPM. Following sterilization, the seeds were germinated on MS medium without plant growth regulators (PGRs) as a control and supplemented with gibberellic acid (GA3) or 6-benzylaminopurine (BAP) at concentrations of 0.5, 1.0, 2.0 and 3.0 mg/L. The minimum contamination percentage, survival rates and germination percentages were recorded after 2-4 weeks of culture.

Sterilization for nodal explants

Nodal explants were collected from healthy trees, ensuring they were completely free of injuries or damage. Five experiments were conducted to find the optimal sterilization conditions. The explants were first shaken in 70% ethanol for 1 minute, then immersed in the sterilizing agents listed in Table 2, along with a 0.1% antibiotic antimycotic solution, 0.1% PPM, 0.1% cefotaxime, and three drops of Tween-20. This procedure was then repeated without the use of $HgCl_2$ or NaOCl for an additional 5 minutes. The explants were rinsed thrice in sterile distilled water for 5 minutes each. Throughout all processes, shaking was maintained at 200 RPM. The explants were cultured on MS medium without PGRs. After 2 weeks of culture, survival rates were recorded.

Table 2. The various sterilization experiments on nodal explants of Lamyai-Thao.

Experiments	First sterilizing agents	Exposure period (min)
E1	0.1% $HgCl_2$	20
E2	0.1% $HgCl_2$	10
E3	0.2% $HgCl_2$	20
E4	0.2% $HgCl_2$	10
E5	4% NaOCl	10

Shoot induction

This research utilized nodal explants from two distinct sources for shoot induction. The first source involved harvesting nodal explants from naturally healthy trees, which were then sterilized using a specific method. The second source consisted of sterilized seedlings obtained from *in vitro* seed germination. The nodal explants were cut into 1-1.5 cm segments and cultured on MS medium with various concentrations of 6-benzylaminopurine (BAP), kinetin, thidiazuron (TDZ), and meta-topolin (*m*T) at 0.5, 1.0, 2.0, and 3.0 mg/L. Subculturing was performed every 4 weeks.

Rooting and acclimatization

The shoots were transferred to an MS medium with indole-3-butyric acid (IBA) at concentrations of 1.0, 1.5, and 2.0 mg/L. The cultures were maintained under the same conditions as in the preceding subculture, with transfers occurring every 4 weeks. Well-developed plantlets were rinsed with running water to remove residual agar and then immersed in a 0.1% fungicide solution for 30 minutes to prevent contamination. After cleaning, the plants were placed in plastic pots with vermiculite and placed in a controlled plant growth room. They were rinsed weekly with MS solution. After four weeks, the plants were transferred to pots with soil and grown in a greenhouse.

Analysis of data

All experiments were conducted at least in triplicate, with each replication comprising ten explants. Statistical analyses were conducted using IBM SPSS software (version 29), facilitating the execution of ANOVA and mean comparison analysis using Duncan's Multiple Range Test (DMRT, 1955). A significance level of $P < 0.05$ was established, indicating statistical significance for the results obtained.

Results

Surface sterilization technique

The seeds were grown on MS medium without plant growth regulators for 2 weeks, and experiment E4 showed the lowest contamination rate at 16%. However, it also exhibited a low survival rate of 34% and a germination rate of only 6%. In contrast, experiment E2 showed a contamination rate of 24%, but it achieved the highest survival rate at 90% and a germination rate of 50% (Table

3). Therefore, the optimal experiment is the E2 condition; the seeds were agitated in 70% ethanol for 1 minute. This was followed by treatment with 0.2% $HgCl_2$ as the initial sterilizing agent, alongside a 0.1% antibiotic antimycotic solution, 0.1% PPM, 0.1% cefotaxime, and three drops of Tween-20. The procedure was then repeated for an additional 10 minutes without $HgCl_2$. Afterward, the seeds were rinsed thrice in sterile distilled water for 5 minutes each. All procedures were conducted with shaking at 250 RPM.

Table 3. Effect of various sterilization techniques on seed germination of Lamyai-Thao culture on MS medium without PGRs for 2 weeks

Experiments	Contamination (%)	Survival (%)	Germination (%)
E1	64	90	14
E2	24	90	50
E3	36	90	37
E4	16	34	6
E5	30	40	26

Nodal explants from parent trees were cultured on MS medium without plant growth regulators for 2 weeks under various sterilization conditions. This resulted in a maximum survival rate of 80% observed in the E2 condition (Table 4). Therefore, the optimal experiment is the E2 condition; the explants were first shaken in 70% ethanol for 1 minute, then immersed in a sterilizing solution containing 0.1% $HgCl_2$, 0.1% antibiotic-antimycotic solution, 0.1% PPM, 0.1% cefotaxime, and three drops of Tween-20, with continuous shaking for 10 minutes. This procedure was repeated for 5 minutes in the same sterilizing solution without $HgCl_2$. Finally, the explants were rinsed three times with sterile distilled water for 5 minutes each, with all steps performed under constant shaking at 200 RPM.

Table 4. Effect of various sterilization techniques for nodal explants of Lamyai-Thao culture on MS medium without PGRs for 2 weeks

Experiments	Number of explants	Number of survival explants	Survival (%)
E1	30	21	70
E2	30	24	80
E3	30	19	64
E4	30	17	57
E5	30	13	43

Seed germination

Seed germination was conducted on MS medium without PGRs (control) and on media with GA_3 or BAP at concentrations of 0.5, 1.0, 2.0, and 3.0 mg/L. The highest germination rate, 68%, occurred on MS medium with 1.0 mg/L BAP, with optimal shoot and root lengths of 48.00 mm and 31.40 mm, respectively, as shown in Table 5 and Figure 1.

Table 5. Effects of MS medium enriched with varying concentrations of GA_3 or BAP on seed germination for 4 weeks

PGRs (mg/L)	Germination (%)	No. of shoots (shoot/seed)	Shoot length (mm)	Root length (mm)
MS (Control)	50	1.14	10.56^{d}	12.70^{c}
GA_3 0.5	63	1.13	17.71^{c}	15.89^{b}
GA_3 1.0	43	1.06	14.36^{c}	15.50^{b}
GA_3 2.0	37	1.00	13.98^{c}	14.83^{b}
GA_3 3.0	37	1.00	14.14^{c}	13.71^{c}
BAP 0.5	38	1.27	23.60^{b}	21.30^{b}
BAP 1.0	68	1.50	48.00^{a}	31.40^{a}
BAP 2.0	37	1.07	20.10^{b}	16.20^{b}
BAP 3.0	57	1.00	22.80^{b}	14.20^{b}

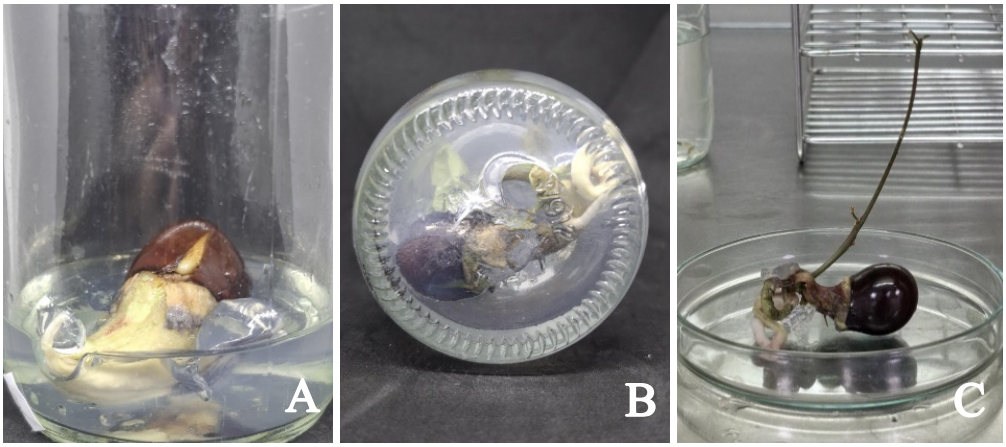

Figure 1. Seed development of Lamyai-Thao on MS medium with 1.0 mg/L BAP: (A) after 2 weeks of culture, (B) roots emerged after 4 weeks, and (C) shoots developed after 4 weeks

Effect of shoot induction

After sterilization, shoot induction was performed on nodal explants cultured on MS medium supplemented with BAP, kinetin, TDZ, or mT at

concentrations of 0.5, 1.0, 2.0, and 3.0 mg/L. The results in Table 6 indicate that the MS solid medium with 0.5 mg/L BAP produced the highest average shoot length of 11.31 mm and generated an average of 1.3 shoots per explant (Figure 2).

Table 6. Effects of MS medium enriched with different concentrations of various cytokinins on shoot induction of Lamyai-Thao for 4 weeks

PGRs (mg/L)	No. of shoots (shoot/explant)	Shoot length(mm)
MS (Control)	1	5.53^{bcd}
BAP 0.5	1.3	11.31^{a}
BAP 1	1	6.95^{bc}
BAP 2	1	5.69^{bcd}
BAP 3	1	5.43^{cd}
*m*T 0.5	1	6.23^{bcd}
*m*T 1	1	5.02^{cd}
*m*T 2	1	5.26^{cd}
*m*T 3	1	4.14^{d}
kinetin 0.5	1	7.67^{b}
kinetin 1	1	6.43^{bc}
kinetin 2	1	5.73^{bcd}
kinetin 3	1	4.87^{cd}
TDZ 0.5	1	7.64^{b}
TDZ 1	1	5.14^{cd}
TDZ 2	1	5.23^{cd}
TDZ 3	1	5.72^{bcd}

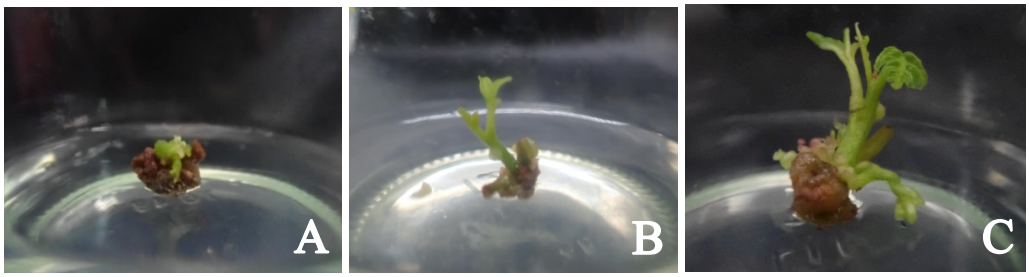

Figure 2. Shoot induction of Lamyai-Thao on MS medium supplemented with 0.5 mg/L BAP: (A) after 2 weeks of culture, (B) after 4 weeks, and (C) after 8 weeks

Root induction and acclimatization

The shoots were transferred to MS medium supplemented with IBA at concentrations of 1.0, 1.5, and 2.0 mg/L. The highest rooting percentage, reaching 50% after 4 weeks of cultivation, was recorded on the medium with 2.0 mg/L IBA, which also promoted the most extended average root length of 3.0 mm and resulted in an average of 3 roots per shoot (Table 7 and Figure 3). Plantlets with well-developed shoots and roots were gently removed from the medium and rinsed with running water to eliminate residual agar. They were then immersed in a 0.1% fungicide solution for 30 minutes to prevent fungal contamination. After cleaning, the plants were transferred to plastic pots filled with vermiculite and placed in a controlled growth chamber to ensure optimal growth conditions. They were rinsed weekly with MS solution. After four weeks, the plants were moved to pots containing soil and cultivated in a greenhouse.

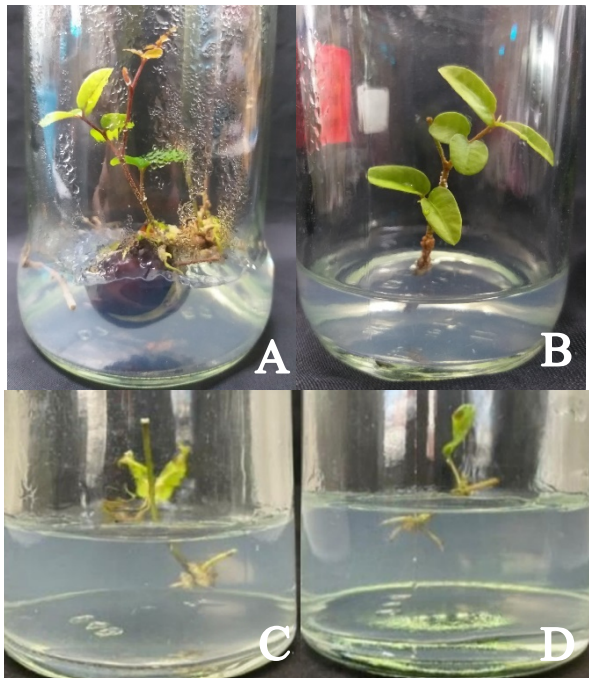

Figure 3. Development of Lamyai-Thao on MS medium with 2.0 mg/L of IBA: (A) healthy plantlet from seed germination after 8 weeks, (B) well-developed shoots moved to MS medium with 2.0 mg/L of IBA, (C and D) after 4 weeks of culturing

Table 7. Effects of MS medium enriched with varying concentrations of IBA on root formation of Lamyai-Thao for 4 weeks

PGRs (mg/L)	No. of roots (root/explant)	Root length (mm)
MS (Control)	0	0
IBA 1	0	0
IBA 1.5	2	3.5
IBA 2	3	3

Discussion

The plant regeneration of Lamyai-Thao using tissue culture techniques commenced with a focus on sterilization methods, which are crucial for the success of the experiments. Various sterilizing agents commonly used to eliminate microorganisms from the explants were evaluated, including NaOCl, ethanol, $HgCl_2$ and Tween-20. The sterilization method has been adapted from Younesikelaki *et al.* (2016) and Ahmed (2022). In this study, the most effective sterilizing agent identified was mercuric chloride for both seeds and nodal explants, aligning with findings from previous research on optimizing explant surface sterilization in *D. longan* (Ahmed, 2022). Seed germination is a critical plant life cycle phase (Al-Namazi *et al*., 2020). Researchers advocate for in vitro seed germination as an effective method to overcome physical barriers and enhance germination rates. Tissue culturists commonly employ this technique as an initial step for extracting explants and regenerating multiple *in vitro* plants from a single seed (Rehman and Park, 2000). GA_3 is a recommended hormone for managing primary dormancy during seed development, as it promotes germination. Additionally, treatment with GA_3 fosters shoot growth, leading to the development of robust plants. The concentration of BAP significantly influences seed germination under *in vitro* conditions, as demonstrated in studies of *Madhuca longifolia* var. *longifolia* ("Mee") (Karunarathna *et al*., 2023). Cytokinins, including BAP, kinetin, TDZ, and *m*T, are the most essential factors in the shoot induction process. In this study, the optimal conditions for establishing nodal regeneration and promoting shoot length in Lamyai-Thao were achieved using MS medium enriched with 0.5 mg/L of BAP. Additionally, significant research on *D. longan* has focused on the influence of various concentrations of BAP and 2iP during the multiplication stage, highlighting their effects on growth and development (Ahmed, 2022). Adjusting auxin levels in the

rooting medium has been shown to enhance root production. Auxin is essential for forming adventitious roots, and different concentrations of endogenous auxin facilitate various physiological stages of the rooting process. For example, adding IBA or NAA at concentrations of 1.0–1.5 mg/L significantly increased the number of roots per plantlet and the overall root length (Mohamad *et al*., 2022). The final step in plant tissue culture is acclimatization, which occurs after the roots have been induced from *in vitro* culture. According to Hiregoudara *et al*. (2003). After cleaning, the plants were transferred to plastic pots with vermiculite and placed in a controlled plant growth room. They were rinsed weekly with MS solution. After four weeks, the plants were moved to pots containing soil and grown in a greenhouse.

This research is represented a significant advancement in tissue culture techniques for Lamyai-Thao. The findings demonstrated effective shoot induction and plant regeneration methods, highlighting the potential for optimizing cultivation practices in this economically important species. Moreover, the results are provided a foundation for further exploration into the genetic variations, which could enhance traits such as disease resistance and improve fruit quality. These insights are contributed to the scientific understanding of this species and open avenues for future research which would be aimed at sustainable agricultural practices and the conservation of genetic resources.

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