

Effect of 2,4-Dichlorophenoxyacetic Acid on Callus Induction from *Eurycoma Longifolia* Leaves

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Abstract

Eurycoma longifolia Jack (Simaroubaceae), known as Tongkat Ali, is a slow-growing Southeast Asian medicinal tree whose root quassinoids are of pharmacological interest. Pressure on wild populations has increased demand for in vitro propagation. This study determined the optimum 2,4-dichlorophenoxyacetic acid (2,4-D) concentration for callus induction from juvenile leaf explants of *E. longifolia* and evaluated proliferation during the first subculture cycle. Leaf segments and primary callus were cultured on Murashige and Skoog (MS) medium with six concentrations of 2,4-D (0 to 3.0 mg/L), in a completely randomised design with 10 replicate vessels per treatment.

Hormone concentration significantly affected callus induction, fresh weight, and volume, but not dry weight. The hormone-free control produced no callus. The best response occurred at 1.0 mg/L 2,4-D, giving the highest induction (88.65%), fresh weight (2.55 g) and volume (2.64 cm³); dry matter content ranged from 4.05 to 9.18%. During subculture, this concentration remained optimal (47.15% proliferation, 1.38 g fresh weight, 1.02 g dry weight), though biomass was lower than in primary culture. Colour-quality scores were highest at 1.0 mg/L and declined at 2.5-3.0 mg/L, coinciding with visible browning. These results identify 1.0 mg/L 2,4-D as the most effective concentration for callus induction, proliferation, and visual quality.

Keywords: Callogenesis, Medicinal Plant, Subculture, Tongkat Ali, Woody Plant Tissue Culture

1. Introduction

Eurycoma longifolia Jack (Simaroubaceae) is a slender medicinal tree native to the lowland forests of Peninsular Malaysia, Indonesia, Thailand, Vietnam, and the Philippines, known locally as Tongkat Ali, Pasak Bumi, or Pla-Lai-Phueak. The roots of mature trees accumulate a rich array of quassinoids (eurycomanone, eurycomanol, eurycomalactone), canthin-6-one and beta-carboline alkaloids, squalene-type triterpenoids, and biphenyl neolignans. Several of these compounds display antimalarial, anti-

inflammatory, cytotoxic, and pro-androgenic activities [1, 2]. Standardised root extracts are now widely incorporated into functional foods and male-fertility products, and global demand has increased steadily over the past two decades [3].

This commercial interest contrasts sharply with the plant's biology. *E. longifolia* is a slow-growing understorey tree that requires 4 to 7 years for its taproot to reach harvestable size. Seed germination is uneven, leading to poorly established seedlings under field conditions. Concerns about over-exploitation of wild populations have therefore stimulated the development of in vitro alternatives, including shoot multiplication and micropropagation [17], somatic embryogenesis [18], adventitious-root culture [4], and undifferentiated callus and cell-suspension cultures aimed at quassinoid production [3, 5, 6].

Callus culture is a versatile platform. It can serve as the starting material for cell-suspension culture, as a source of regenerable somatic embryos, and as a tool for studying secondary-metabolite biosynthesis under controlled conditions [3, 7]. The successful establishment of any such platform begins with the identification of an explant-auxin combination that reliably yields friable, proliferative callus. Synthetic phenoxyacetic auxins, particularly 2,4-dichlorophenoxyacetic acid (2,4-D), are the most widely used callogenic agents because of their stability and their potent capacity to drive cellular dedifferentiation, often in the absence of exogenous cytokinin [7, 8]. However, the optimum concentration is strongly dependent on genotype, explant type, and plant population. Previous studies on *E. longifolia* have used 1.0 to 2.0 mg/L 2,4-D for root and stem-derived callus, and 1.0 mg/L as the optimum for leaf explants [15], and various combinations of 2,4-D with 1-naphthaleneacetic acid (NAA) or 6-benzylaminopurine (BAP) for petiole and leaf explants from Indonesian populations [16].

Two specific gaps in the literature motivated the present work. First, although leaf explants of *E. longifolia* have been used for callus induction, most existing reports rely on leaves harvested from greenhouse- or field-grown plants, which are subject to seasonal physiological variability, surface contamination, and the need for repeated surface-sterilisation [3, 6]. Leaves obtained directly from in vitro-germinated seedlings represent a more uniform, microbiologically clean source of juvenile tissue and have not been used systematically for 2,4-D dose-response work in this plant. Second, callus systems of woody medicinal plants are known to lose proliferative vigour during repeated subculture even on the same medium [7, 9], yet quantitative estimates of the magnitude of this decline at the first subculture cycle are rarely reported for *E. longifolia*. The present study was therefore designed to determine the effect of 2,4-D concentration on callus induction frequency, biomass, and colour quality from juvenile in vitro-derived leaf explants of *E. longifolia* from a southern Thai population, and to evaluate the proliferation and biomass accumulation of primary callus after transfer to the same hormone series during the first subculture cycle.

2. Materials and Methods

2.1 Plant Material and Explant Source

Mature seeds of *E. longifolia* were collected from the experimental plot of the Faculty of Agriculture, Princess of Naradhiwas University, Narathiwat, southern Thailand (6°25'N, 101°49'E). Seeds were rinsed in running tap water for 15 minutes, immersed in 70% ethanol for 1 minute, surface-sterilised in 15% commercial sodium hypochlorite (final available chlorine approximately 0.9%) supplemented with two drops of Tween-20 per 100 mL for 10 minutes with gentle agitation, and rinsed five times with sterile distilled water. The sterilised seeds were germinated in vitro on hormone-free Murashige and Skoog (MS) medium [14] at 25 ± 1 °C under an 8 hour photoperiod, and the resulting aseptic seedlings were maintained under the same conditions for 8 to 12 weeks. Healthy, fully expanded young leaves from these in vitro

seedlings were used as the explant source. Because explants originated from already-sterile seedlings, no further surface-sterilisation was required, ensuring uniform physiological status and elimination of microbial contamination as a source of variability.

2.2 Culture Media and Hormone Treatments

The basal medium consisted of full-strength MS macronutrients, micronutrients, and vitamins [14] supplemented with 30 g/L sucrose and solidified with 8 g/L agar (Bacteriological grade, HiMedia, India). The pH was adjusted to 5.8 ± 0.05 with 1 N NaOH or 1 N HCl prior to autoclaving at 121 °C and 1.05 kg/cm² for 15 minutes. 2,4-D (2,4-dichlorophenoxyacetic acid; analytical grade, Sigma-Aldrich, USA) was dissolved in a small volume of 1 N NaOH, brought up to volume with distilled water, filter-sterilised through a 0.22 micrometre membrane, and added to autoclaved media that had cooled to approximately 50 °C, to give final concentrations of 0 (control), 0.5, 1.0, 2.0, 2.5, and 3.0 mg/L.

2.3 Experiment 1: Callus Induction from Leaf Explants

Leaf laminae from in vitro seedlings were cut aseptically into rectangular segments of approximately 0.5 cm by 0.5 cm and placed adaxial-side up on the medium surface. Each culture vessel contained three leaf-segment explants. Cultures were maintained at 25 ± 1 °C under cool-white fluorescent illumination of 57.5 micromol/m²/s with a photoperiod of 16 hours light and 8 hours dark. The experiment was arranged as a completely randomised design with six hormone treatments and 10 replicate vessels per treatment. Cultures were observed monthly. After 3 months of culture, callus induction frequency was recorded for each vessel as the percentage of its three explants that showed visible callus formation, and the ten per-vessel values were used as the replicate observations in all statistical analyses. Callus fresh weight was determined immediately after harvest on an analytical balance, callus volume by water displacement in a graduated micro-cylinder, and dry weight after drying to constant weight at 60 °C in a hot-air oven; fresh weight, dry weight, and volume were recorded per vessel as the pooled mass of the callus produced in that vessel. Cultures were then maintained for a further month, and callus colour-quality was scored at 3 months as described below.

2.4 Experiment 2: Subculture and Proliferation

Pieces of friable callus produced in Experiment 1, originating from the 1.0 mg/L treatment that produced the highest-quality callus, were transferred to fresh medium of identical composition and re-exposed to the same hormone series. The experiment was arranged as a completely randomised design with 10 replicate vessels per treatment and one callus inoculum per vessel. Cultures were maintained under the same physical conditions as in Experiment 1. Proliferation frequency, fresh weight, dry weight and callus volume were recorded after 4 months of subculture.

2.5 Colour-Quality Scoring

In addition to biomass measurements, callus quality was evaluated at the end of each 3 month culture cycle using a 5 point ordinal scoring scale based on visual morphological characteristics. The assessment considered callus colour, size, texture, freshness, degree of proliferation, uniformity, and the presence of browning or necrosis. The scoring scale was defined as follows: 1, very poor, dark brown to black, necrotic tissue with no clear proliferation; 2, poor, brown or light brown, weakly developed tissue with visible browning; 3, moderate, pale yellow, cream, or slightly green tissue with visible but not abundant growth; 4, good, light yellow, cream, or light green, healthy, well-developed tissue with clear proliferation and little browning; and 5, excellent, abundant, fresh, uniform, light yellow to greenish tissue that was actively proliferating and free from browning. Scoring was performed by two independent observers, and the scores were averaged. The mean score per replicate was used for statistical analysis.

2.6 Statistical Analysis

Data are reported as means $\pm$ standard deviation. Because the hormone-free control produced no callus in either experiment, including it in a parametric analysis of variance would violate the homogeneity-of-variance assumption; the control was therefore treated as a descriptive reference point and was not entered into the inferential model. Each experiment was analysed separately as a one-way analysis of variance on the five hormone-containing treatments (0.5, 1.0, 2.0, 2.5, and 3.0 mg/L) as the fixed factor, using the agricolae package [10] in R version 4.3.1. Treatment means were compared by the least significant difference test at P less than 0.05; means sharing the same lowercase letter within a column do not differ significantly. Coefficients of variation are reported for each response variable. Percentage data were arcsine-square-root transformed before analysis and back-transformed for presentation. Normality and homogeneity of variance among the five active treatments were verified by Shapiro-Wilk and Levene's tests, respectively, prior to analysis of variance.

3. Results and Discussion

3.1 Visible Morphology of Callus

Visible swelling at the cut edges of leaf explants was observed within 2 to 3 weeks on all hormone-supplemented media, but not on the hormone-free control. On the control medium, leaf segments remained green during the first 3 to 4 weeks but progressively turned chlorotic and senesced by month 2, with no visible callus formation throughout the 3 month culture period.

Friable callus emerged from the explant margins by month 2 in the 0.5 to 2.0 mg/L treatments and continued to expand throughout the culture period (Figure 2). The callus colour in these treatments corresponded to Royal Horticultural Society (RHS) Colour Chart codes 154D to 154B and was visually described as pale yellow to yellow, consistent with the higher visual colour-quality scores recorded in Table 3. At higher concentrations, callus colour shifted to RHS 153D to 152B, appearing light brownish yellow to brown; these treatments also showed lower colour-quality scores. The browning observed at higher concentrations may be associated with phenolic oxidation, which is commonly reported in callus cultures of woody medicinal plants. This response is consistent with the dose-dependent effect of 2,4-D, in which low to moderate concentrations promote cellular dedifferentiation and callus formation, whereas excessive auxin levels may reduce tissue quality through auxin-induced physiological stress, including enhanced ethylene production, reactive oxygen species accumulation, and reduced mitotic activity [7, 8].

3.2 Callus Induction from In vitro Leaf Explants

Hormone concentration had a highly significant effect on callus induction frequency, fresh weight, and callus volume of *E. longifolia* leaf explants after 3 months of culture (P less than 0.01; Table 1), but did not significantly affect dry weight (P greater than 0.05). The hormone-free control produced no visible callus and is presented for reference only. Callus induction was highest on MS medium supplemented with 1.0 mg/L 2,4-D ($88.65 \pm 18.63\%$). The response decreased as the concentration was increased beyond this level, reaching $44.30 \pm 9.31\%$ at 3.0 mg/L. However, callus induction at 1.0 mg/L did not differ significantly from that at 2.0 mg/L.

Fresh weight and callus volume were also highest at 1.0 mg/L, reaching 2.55 ± 0.47 g and 2.64 ± 0.45 cm³, respectively; these values were significantly greater than at the other concentrations. The optimum concentration identified in this study was therefore 1.0 mg/L, which agrees with previous work reporting high callus induction from *E. longifolia* leaf explants at the same concentration [15]. The lower optimum concentration observed here compared with auxin-cytokinin combinations reported for petiole and leaf

explants of Indonesian populations [16] may be related to differences in explant type, physiological age, and tissue competence. In contrast, dry weight did not differ significantly among 2,4-D concentrations (P greater than 0.05), ranging narrowly from 0.07 ± 0.02 g at 3.0 mg/L to 0.14 ± 0.14 g at 1.0 mg/L. Dry matter content was correspondingly low and relatively uniform across treatments, ranging from 4.05% at 2.5 mg/L to 9.18% at 0.5 mg/L, consistent with the friable, water-rich nature of the callus produced under all 2,4-D treatments.

3.3 Callus Proliferation Under Subculture

When primary callus was transferred to fresh MS medium supplemented with different concentrations of 2,4-D, all measured parameters during the first subculture cycle were significantly affected by the treatment (P less than 0.01; Table 2). No measurable callus proliferation was observed in the PGR-free control, which was included for reference only. Among the 2,4-D treatments, 1.0 mg/L gave the best overall response, with the highest proliferation frequency ($47.15 \pm 9.83\%$), fresh weight (1.38 ± 0.25 g), callus volume (1.03 ± 0.20 cm³) and dry weight (1.02 ± 0.19 g). The response declined when the 2,4-D concentration was increased above 1.0 mg/L, with the lowest values recorded at 3.0 mg/L. Fresh weight and callus volume were consistently lower than in the primary induction experiment at every concentration, indicating an overall decline in biomass accumulation during subculture (Figure 1).

The reduction in growth during subculture suggests that although 1.0 mg/L was effective for primary callus induction, the same treatment did not fully maintain callus proliferation after transfer to fresh medium. A requirement for an appropriate auxin-cytokinin balance has also been reported in *E. longifolia* tissue culture; embryogenic callus formation has been reported to improve on medium containing 1.0 mg/L 2,4-D together with 0.5 mg/L kinetin, with activated charcoal further increasing somatic embryo production [18]. The decline observed here may also reflect the loss of physiological support from the original leaf explant and the stress associated with prolonged in vitro culture under a single auxin-dominated medium; in vitro tissue-culture systems are known to impose physiological and genetic stress, which can affect growth competence and tissue quality during repeated culture [7, 9]. Further optimisation of the maintenance medium should evaluate cytokinin co-supplementation, partial replacement with weaker auxins, activated charcoal or other anti-browning additives, and adjustment of the subculture interval.

One design feature should be considered when interpreting the subculture data. The callus inocula used for subculture originated from primary callus induced under the optimum treatment and were then transferred to media containing different hormone concentrations. Thus, the subculture results describe the response of callus with a common physiological origin to different maintenance auxin levels, rather than the self-renewal capacity of callus continuously maintained at each respective concentration. Future studies comparing concentration-matched induction and maintenance treatments would help clarify possible carry-over or adaptation effects.

3.4 Callus-Quality Score of Explants and Callus

Callus-quality scoring supported the visible morphological observations after 4 months of culture (Table 3). For callus derived from leaf explants, the highest quality score was recorded at 1.0 mg/L (3.8 ± 0.91), statistically comparable to 0.5 mg/L (3.3 ± 0.79) and 2.0 mg/L (3.5 ± 0.84), but significantly higher than 2.5 mg/L (2.6 ± 0.62) and 3.0 mg/L (1.9 ± 0.45). For subcultured callus, a similar pattern was observed, although absolute scores were lower than for leaf-derived callus; the highest subcultured callus score was recorded at 1.0 mg/L (2.7 ± 0.70), and the lowest at 3.0 mg/L (1.5 ± 0.39).

The decrease in colour-quality score at 2.5 to 3.0 mg/L indicates reduced visual quality and increased browning at higher concentrations. Browning is a common limitation in plant tissue culture and is frequently associated with the accumulation and oxidation of phenolic compounds, particularly following explant wounding and culture stress [11, 12]. In the present study, this interpretation was based on visible colour changes and colour-quality scoring rather than direct biochemical measurement of phenolic compounds or oxidative enzymes, and should therefore be regarded as an indicator of possible browning-related stress rather than direct evidence of phenolic oxidation. The agreement between the highest visual scores and the highest biomass responses at 1.0 mg/L suggests that visual scoring can provide a practical supplementary tool for monitoring callus quality during routine culture work, consistent with similar yellowish callus responses reported previously in this plant [17]. Further optimisation using anti-browning additives such as ascorbic acid, citric acid, activated charcoal, and polyvinylpyrrolidone may be useful in subsequent culture improvement [11, 12, 13].

3.5 Future Perspectives

The protocol established in this study provides a useful starting point for further optimisation of *E. longifolia* callus culture. First, factorial trials combining 2,4-D with cytokinins such as BAP or kinetin at low concentrations should be conducted to identify a suitable maintenance medium for reducing the biomass decline observed during the first subculture cycle. Second, biochemical characterisation of the callus is required to determine whether leaf-derived callus accumulates pharmacologically relevant secondary metabolites, including total phenolic content, antioxidant capacity assays, and chromatographic quantification of marker compounds such as eurycomanone. Third, transition to liquid suspension culture, together with elicitation using methyl jasmonate or yeast extract, should be evaluated as a potential strategy for improving biomass production and secondary metabolite accumulation.

4. Conclusion

In vitro callogenesis from juvenile leaf explants of *E. longifolia* derived from aseptic seedlings was successfully achieved on medium supplemented with 2,4-D. Among the concentrations tested, 1.0 mg/L was identified as the most effective treatment for both primary callus induction and callus proliferation during the first subculture cycle, giving the highest induction frequency, biomass accumulation, callus volume, and visual colour-quality scores. Higher concentrations, particularly 2.5 to 3.0 mg/L, reduced callus induction, proliferation, and visual quality, and were associated with increased browning. Although 1.0 mg/L remained the best treatment during subculture, biomass values were lower than during primary induction, suggesting that this hormone alone may not be sufficient for maintaining vigorous callus proliferation beyond the initial culture stage. This protocol provides a contamination-controlled baseline for future optimisation of cytokinin co-supplementation, subculture maintenance, suspension culture, and secondary metabolite production in this medicinally important plant.

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Table 1: Effect of 2,4-D Concentration on Callus Induction Frequency, Fresh Weight, Dry Weight, Callus Volume, and Dry Matter Content of Callus Produced from Eurycoma Longifolia Leaf Explants after 3 Months of Culture on MS Medium

2,4-D (mg/L)	Induction (%)	Fresh Weight (g)	Dry Weight (g)	Volume (cm ³)	Dry Matter Content (%)
0.5	68.00 +/- 14.30 b	1.10 +/- 0.21 c	0.10 +/- 0.06	0.96 +/- 0.18 c	9.18
1.0	88.65 +/- 18.63 a	2.55 +/- 0.47 a	0.14 +/- 0.14	2.64 +/- 0.45 a	5.45
2.0	75.85 +/- 15.94 ab	2.17 +/- 0.40 b	0.12 +/- 0.04	1.03 +/- 0.18 c	5.31
2.5	51.20 +/- 10.76 c	2.05 +/- 0.38 b	0.08 +/- 0.05	1.66 +/- 0.29 b	4.05
3.0	44.30 +/- 9.31 c	1.09 +/- 0.20 c	0.07 +/- 0.02	0.46 +/- 0.08 d	6.42
F-test	**	**	ns	**	-
CV (%)	21.03	19.35	73.74	19.75	-

Values are means $\pm$ standard deviation; n equals 10 replicate vessels per treatment, with three explants per vessel. The hormone-free control (0 mg/L) produced no callus and is shown for reference only. Means within a column followed by different lowercase letters differ significantly at P less than 0.05 according to the least significant difference test. ** indicates significance at P less than 0.01; ns indicates not significant (P greater than 0.05). Dry matter content was calculated as (dry weight divided by fresh weight) times 100. CV in this and subsequent tables denotes coefficient of variation.

Table 2: Effect of 2,4-D Concentration on Callus Proliferation Frequency, Fresh Weight, Callus Volume and Dry Weight of Leaf-Derived Eurycoma Longifolia Callus after 4 Months of Culture during the First Subculture Cycle

2,4-D (mg/L)	Proliferation (%)	Fresh Weight (g)	Dry Weight (g)	Volume (cm ³)
0	0.00	0.000	0.000	0.000
0.5	30.02 +/- 6.26 c	0.91 +/- 0.17 b	0.42 +/- 0.09 c	0.26 +/- 0.06 c
1.0	47.15 +/- 9.83 a	1.38 +/- 0.25 a	1.02 +/- 0.19 a	1.03 +/- 0.20 a
2.0	38.30 +/- 7.98 b	1.05 +/- 0.19 b	0.63 +/- 0.12 b	0.66 +/- 0.12 b
2.5	27.92 +/- 5.82 c	0.69 +/- 0.12 c	0.26 +/- 0.05 d	0.14 +/- 0.03 d

3.0	17.98 +/- 3.75 d	0.37 +/- 0.07 d	0.14 +/- 0.03 e	0.04 +/- 0.01 e
F-test	**	**	**	**
CV (%)	20.85	17.60	19.92	22.90

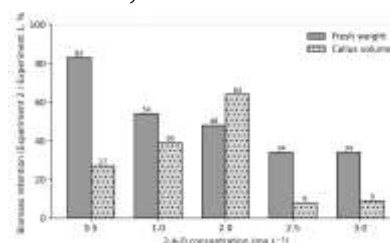
Values are means $\pm$ standard deviation; n equals 10 replicate vessels per treatment, with one callus inoculum per vessel. Proliferation frequency, fresh weight, callus volume and dry weight were recorded after 4 months of subculture. The hormone-free control (0 mg/L) produced no measurable proliferation and is shown for reference only. Means within a column followed by different lowercase letters differ significantly at P less than 0.05 according to the least significant difference test. ** indicates significance at P less than 0.01. CV denotes coefficient of variation.

Table 3: Effect of 2,4-D Concentration on the Visual Colour-Quality Score of Leaf Explants and Subcultured Callus of Eurycoma Longifolia after 3 Months

2,4-D (mg/L)	RHS Colour Code	Visual Colour Description	Leaf-Derived Callus Score	Subcultured Callus Score
0.5	154D	Pale Yellow	3.3 +/- 0.79 a	2.1 +/- 0.55 bc
1.0	154C	Light Yellow	3.8 +/- 0.91 a	2.7 +/- 0.70 a
2.0	154B	Yellow	3.5 +/- 0.84 a	2.4 +/- 0.63 ab
2.5	153D	Light Brownish Yellow	2.6 +/- 0.62 b	1.8 +/- 0.47 cd
3.0	152B	Brownish Yellow to Brown	1.9 +/- 0.45 c	1.5 +/- 0.39 d
CV (%)	-	-	23.92	26.08

Score scale: 5, excellent; 4, good; 3, moderate; 2, poor; 1, very poor. Values are means $\pm$ standard deviation.

Figure 1: Biomass Retention of Leaf-Derived Callus of Eurycoma Longifolia at the First Subculture Cycle, Expressed as the Experiment 2 Mean as a Percentage of the Corresponding Experiment 1 Mean at Each 2,4-D Concentration



Bars show retention of fresh weight (solid) and callus volume (dotted); the dotted line marks 100 percent (no change between cycles). Dry weight retention is not shown because 2,4-D concentration did not significantly affect dry weight in the primary induction experiment (Table 1). Values are derived from the treatment means in Tables 1 and 2.

Figure 2: Representative Photographs of Callus Produced from Juvenile Leaf Explants of *Eurycoma Longifolia* after 4 Months on Medium with Different 2,4-D Concentrations



(a) 0.5 mg/L



(b) 1.0 mg/L



(c) 2.0 mg/L



(d) 2.5 mg/L



(e) 3.0 mg/L

Panels show increasing 2,4-D concentration from (a) 0.5 mg/L, limited pale-yellow friable callus, to (e) 3.0 mg/L, compact brown callus showing visible browning. The hormone-free control (0 mg/L) produced no callus and is not shown. Scale bar equals 1 cm.