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Research Article

Thin Cell Layer Cultures for *In Vitro* Shoot Regeneration and Proliferation of *Kaempferia parviflora*

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ABSTRACT

Kaempferia parviflora contains bioactive compounds with beneficial effects on health, including the management of obesity, diabetes, and cardiorespiratory disorders, as well as anticancer properties. However, *in vitro* propagation of this species remains challenging due to the limited availability of rhizomes as explants, which is caused by the prolonged dormancy period and low rate of rhizome splitting. Therefore, this study aimed to evaluate the suitability of thin cell layer (TCL) cultures as an alternative explant source for the shoot regeneration of *K. parviflora*. TCLs of different thicknesses (1.0, 2.0, 3.0, 4.0 & 5.0 mm) were assessed to determine the optimum explant size using *in vitro* aerial shoots. The effects of various concentrations of BAP (1.0–5.0 mg/L), kinetin (0.5–4.0 mg/L), TDZ (0.5–4.0 mg/L), NAA (0.5–4.0 mg/L), and 2,4-D (0.2–1.0 mg/L) applied individually were tested for shoot regeneration. Subsequently, the effect of 1.0 mg/L BAP in combination with 2,4-D or TDZ (0–0.8 mg/L) was evaluated. TCLs of 5.0 mm thickness produced the highest shoot regeneration rate (46.33 ± 11.64%). Among the cytokinins, BAP at 1.0 mg/L induced the highest percentage of TCL-forming shoots (44.4 ± 15.7%) with an average of 2.38 ± 0.75 shoots per explant. Supplementing BAP with TDZ further enhanced shoot regeneration, with the combination of 1.0 mg/L BAP and 0.6 mg/L TDZ producing the highest regeneration rate (70.83 ± 8.33%). After 5 weeks of acclimatisation, 90% of plantlets survived in a soil and burnt rice husk mixture (1:1). These findings demonstrate that TCLs are effective explants for the *in vitro* regeneration of *K. parviflora*, reducing dependency on rhizomes and supporting large-scale propagation of this medicinal species.

Key words: *Kaempferia parviflora*, micropropagation, plant growth regulator, thin cell layer

INTRODUCTION

Kaempferia parviflora Wall. Ex Baker (Black Ginger), also known as Kra-chai-Dum in Thailand, is a species of the family Zingiberaceae with deep purple rhizomes that are morphologically similar to common ginger (Nopporncharoenkul *et al*., 2017). The rhizomes contain various bioactive components, including volatile oils, phenolic glycosides, and various flavonoids (Yenjai *et al*., 2004). The plant has been traditional used to treat a wide range of ailments (Wattanasri *et al*., 2016), such as obesity and metabolic disorders including diabetes and inflammatory bowel disease (Toda *et al*., 2016; Lee *et al*., 2018; Yoshino *et al*., 2018; Elshamy *et al*., 2019), to enhance cardiorespiratory and longevity physical fitness (Toda *et al*., 2016; Wattanathorn *et al*., 2023), as an antimicrobial and therapeutic agent (Supian *et al*., 2022; Azrihan, 2023; Krongrawa *et al*., 2023), and for its anticancer properties (Hossain *et al*., 2012; Atun & Arianingrum, 2015; Chen *et al*., 2018; Asamenew *et al*., 2019; Sun *et al*., 2021).

In Malaysia, the cultivation of *K. parviflora* is constrained by the limited availability of planting materials (Suhaimi *et al*., 2024). The species undergoes a dormancy of approximately 5 to 7 months (November to May), depending on the climatic conditions (Techaprasan *et al*., 2010). This species exhibits a 3-month vegetative phase and a 2-month reproductive phase. The prolonged dormancy period disrupts cropping cycles and prevents year-round cultivation, thus hindering the commercial production of this medicinal plant (Labrooy *et al*., 2016). Furthermore, conventional propagation through rhizome division is inefficient, as only a few buds (two to four) can be obtained simultaneously from a single plant (Chirangini *et al*., 2005).

Given the constraints of obtaining planting materials, an alternative propagation strategy is needed to support the large-scale cultivation of this species. Micropropagation offers a promising solution for the rapid production of disease-free planting materials (Gaikwad *et al*., 2017). Plant tissue culture typically employs small or large fragments of plant material—cells, tissues, or entire organs—as explants for *in vitro* regeneration through organogenesis (Pietropaolo, 2023). Several studies have investigated the micropropagation of *K. parviflora* using rhizomes as conventional explants (Dheeranupattana *et al*., 2003; Laipaitong, 2017; Khairudin *et al*., 2020; Labrooy *et al*., 2020; Park *et al*., 2021). However, due to the limited availability of plant resources, the use of thin cell layer (TCL) cultures as alternative explants warrants exploration.

The TCL system employs ultrathin sections ranging from 0.1 to 2.0 mm in thickness (da Silva & Dobránszki, 2019) and, in some cases, up to 5.0 mm for transverse TCLs (Rout *et al*., 2006). TCL explants, when cultured on a suitable medium, can express diverse multiple or individual morphogenetic programs, including callus formation, shoot regeneration, rooting, flowering, or somatic embryogenesis (Murthy & Kondamudi, 2011). Previous studies have shown that TCL technology is particularly promising for clonal propagation, as it enables faster shoot regeneration using the TCL approach compared with conventional

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explants (Yulianti *et al*., 2017).

The efficiency of TCL cultures depends on several factors, including the TCL explant size (Aswathi *et al*., 2022) and the type of plant growth regulators (PGRs) used in the medium (Jing *et al*., 2014). Explant size significantly influences the rate of shoot regeneration (Nhut *et al*., 2006). Thinner TCLs have been reported to possess greater regeneration owing to a higher proportion of morphogenic cells and more efficient nutrient transfer between the medium and the explant (Van, 2003).

The regenerative potential of TCLs also depends on the response of cells to PGRs, which varies among plant species (Media, 2023). For instance, Thingbaijam and Huidrom (2014) reported that TCL explants of ginger, with a thickness of 0.5 mm, produced maximum callus proliferation on medium supplemented with 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). Similarly, *Chrysanthemum indicum* L. TCLs derived from calyx tissue (0.5 to 1.0 mm thick) exhibited optimal callus formation and shoot regeneration on modified MS medium containing 0.5 mg/L BAP, 0.2 mg/L kinetin, and 0.2 mg/L NAA (Viet, 2017). Likewise, TCLs of nodal and internodal segments (1.0–2.0 mm thick) of *Ceropegia spiralis* L. cultured on MS medium supplemented with 3.0 mg/L BAP and 0.1 mg/L NAA produced the highest number of shoots (Murthy & Kondamudi, 2011). The TCL approach has also been successfully applied to several orchid species, such as *Brasilidium forbesii* (Hook.) (Gomes *et al*., 2015), *Hadrolaelia grandis* (Vudala *et al*., 2019), *Paphiopedilum callosum* var. *sublaeve* (Wattanapan *et al*., 2018), and *Phalaenopsis Hwafeng Redjewel* × *Phalaenopsis New Cinderella* (Lo *et al*., 2022). In *Ficus carica* cv. Violette de Soillès, TCLs derived from stem sections of 0.5 to 0.8 mm exhibited maximum callus induction on medium supplemented with 1.0 mg/L BAP and 0.5 mg/L NAA (Sriskanda *et al*., 2022). Meanwhile, a combination of 2.0 mg/L BAP and 0.5 mg/L NAA was found to be the best phytohormone combination for shoot bud induction for seedlings of sesame using tTCL sections (0.5–1.0 mm thick) (Chattopadhyaya *et al*., 2010).

The advantages of the TCL technique are substantial, as it requires only small explants and can utilise tissues from various plant organs (Sharma *et al*., 2023). This method is particularly advantageous for species with limited plant material during the establishment or subcultures stages (da Silva & Dobránszki, 2019).Given the success of TCL techniques in numerous plant species, the potential of using TCL in the tissue culture of *K. parviflora* should be explored. Thus, the objective of this study was to evaluate the suitability of TCL as explants for the micropropagation of *K. parviflora*.

MATERIALS AND METHODS

Preparation of mother stock plant materials and surface sterilisation

Fresh, mature *K. parviflora* rhizomes (> 9 months old) were purchased from Jaya Trading, Kelantan. The rhizomes were cleaned to remove soil debris, soaked in 0.05% (w/v) Benomyl 50% (BENEX, IMASPRO Corporation Sdn. Bhd., Malaysia) for 24 hr, placed on plastic trays lined with clean towels, and kept in a shaded area for 3-4 weeks to allow for bud formation. Subsequently, the rhizome buds were surface-sterilised with 75% (v/v) ethanol for 45 s, 5% (v/v) hydrogen peroxide for 10 min, 20% (v/v) Clorox® with one drop of Tween 20 for 10 min, and 1 g/L chlorine dioxide with one drop of Tween 20 for 20 min. Rhizome buds were cultured on Murashige & Skoog's medium without hormone (MS0) supplemented with 3% (w/v) sucrose and semi-solidified with 0.8% (w/v) agar. The medium pH was adjusted to 5.8 before autoclaving at 121°C for 15 min, and dispensed into 175 mL glass jars. The cultures were maintained for 30 days under a 16-hr photoperiod at 24 ± 2°C. These *in vitro* plantlets were used as the source of TCL for subsequent experiments.

Determination of TCL size as explants

Aerial shoots of *in vitro K. parviflora* plantlets (30 days old) derived from MS0 medium were excised and transversely sectioned into thin cell layers (tTCL) of five sizes: 1.0, 2.0, 3.0, 4.0, and 5.0 mm. The tTCLs cultured transversely on semi-solid MS0 (control) and MS media supplemented with 1.0 mg/L BAP, based on findings from our preliminary study. All media were supplemented with 3% (w/v) sucrose with 0.8% (w/v) agar. The pH was adjusted to 5.8 before autoclaving at 121°C for 15 min, and the media were dispensed into 9.0 cm Petri dishes. Each dish contained six tTCLs, and each treatment was replicated four times.

Effect of plant growth regulators on multiple shoot induction

The effects of PGRs, applied individually or in combination, on shoot regeneration and multiplication were evaluated using 5.0 mm transverse thin cell layers (tTCL) derived from aerial shoots. The tTCL explants were cultured on MS media supplemented with various concentrations of BAP (1.0, 2.0, 3.0, 4.0 & 5.0 mg/L), kinetin (0.5, 1.0, 2.0, 3.0 & 4.0 mg/L), thidiazuron (0.5, 1.0, 2.0, 3.0 & 4.0 mg/L), NAA (0.5, 1.0, 2.0, 3.0 & 4.0 mg/L), and 2,4-D (0.2, 0.4, 0.6, 0.8 & 1.0 mg/L), each tested individually. For combination treatments, the MS medium was supplemented with a constant concentration of BAP (1.0 mg/L) together with varying concentrations (0, 0.2, 0.4, 0.6, and 0.8 mg/L) of either 2,4-D or thidiazuron at various concentrations. MS medium without PGRs (MS0) served as the control. All media were supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar. The pH was adjusted to 5.8 before autoclaving at 121°C for 15 min, and the media were dispensed into 9.0 cm Petri dishes. Each dish contained six tTCLs, and each treatment was replicated four times.

All cultures were incubated in a growth chamber (CONVIRON GEN 1000) under a 16-hr photoperiod at 25 ± 2°C. Subculturing was carried out every 4 weeks. The percentage of explants forming shoots or roots, the number of shoots, roots, and leaves, root lengths, and the number of days required for shoot and root formation were recorded.

Percentage of explant-forming shoot or root (%)

$$\frac{\text{Number of explant producing adventitious shoots (or roots)}}{\text{Total number of explant cultured}} \times 100$$

Number of shoots

$$\text{Average number of shoots per explant} = \frac{\text{Total number of shoots}}{\text{Total number of responsive explants}}$$

Number of leaves

$$\text{Average number of leaves per explant} = \frac{\text{Total number of leaves}}{\text{Total number of responsive explants}}$$

Number of roots

$$\text{Average number of roots per explant} = \frac{\text{Total number of roots}}{\text{Total number of responsive explants}}$$

Root length (cm)

$$\text{Average root length} = \frac{\text{Total root length}}{\text{Total number of roots measured}}$$

Shoot regeneration and rooting

After 8 weeks of culture in shoot regeneration and multiplication media, adventitious shoots (approximately 2.0 cm in length) regenerated from TCL explants were aseptically excised and transferred to MS0 medium in glass jars for shoot and root development. The cultures were maintained for 5 weeks at 25 ± 2°C with a 16-hr photoperiod. Data on shoot and root length, as well as the number of leaves and roots, were recorded.

Acclimatisation

Acclimatisation was carried out following the methods described by Zahid *et al*. (2021), with minor modifications. *In vitro*-rooted plantlets bearing 3-4 leaves were carefully removed from the jars. The rooted plantlets were dipped in 0.05% (w/v) fungicide (Benomyl 50%) for 5 min before being transplanted into black polybags (5 × 8 inches) containing a 1:1 mixture of autoclaved soil and burnt rice husk. he polybags were covered with transparent plastic bags and placed in a shaded area at 29°C. The survival rate and morphological characteristics of the plantlets were recorded after 5 weeks.

Survival rate for acclimatisation (%)

$$\frac{\text{Total number of surviving plantlets}}{\text{Total number of plantlets acclimatised}} \times 100$$

Experimental Design and Data Analysis

The experiments were designed using a completely randomised design (CRD), and all data were presented as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) was performed to evaluate significant differences in the morphogenetic responses of TCL explants among treatments. Treatment means were compared using Duncan's Multiple Range Test (DMRT) at a significance level of $p<0.05$, using IBM SPSS Statistics version 29.

RESULTS AND DISCUSSION

Determination of TCL size as explants

Growth initiation in the TCL explants was observed approximately 1 week after culture establishment. During the 1st week, the explants showed swelling (Figure 1a), and by the 2nd week, adventitious buds began to form along the wounded margins (Figure 1b). These buds subsequently enlarged (Figure 1c). By the third week, the buds developed into rolled shoots (Figure 1e, indicated by the yellow arrow), accompanied by the emergence of adventitious roots (Figure 1e). After 5 weeks of culture, the shoots had unrolled and expanded, forming small leaf blades, while additional new buds appeared on the explants (Figure 1f; yellow arrows). By the seventh week, the shoots had developed into aerial shoots or leaves (Figure 1h), morphologically resembling those produced by rhizome-derived plants under *ex vitro* conditions.

The results of TCL explant size determination are presented in Table 1. In the control medium (MS0), only the 5.0 mm explants regenerated shoots (16.70% ± 0.0) after 8 weeks of culture, showing a significant difference ($p<0.05$) compared with other sizes, which failed to regenerate. The 1.0 mm–4.0 mm TCL explants turned brown and died within 4 weeks of culture. Plants naturally contain endogenous hormones that regulate morphogenesis, and exogenous hormones primarily act as modulators of these endogenous activities (Pasternak & Steinmacher, 2024). In MS0 medium, the endogenous hormone levels in small TCL (<5.0 mm) may have been insufficient to induce cell division in the absence of exogenous PGRs. On MS media supplemented with 1.0 mg/L BAP, shoot generation increased with explant size.

The smallest explants (1.0 mm) failed to regenerate shoots, whereas 5.0 mm TCLs produced the highest percentage of explants forming shoots (46.33 ± 11.64%). Explants with 4.0 mm and 5.0 mm thickness yielded 42.50 ± 8.48% and 46.33 ± 11.64% shoot-forming explants, respectively. Although the difference between these two sizes was not statistically significant, both showed significantly higher regeneration than thinner slices (1.0, 2.0 & 3.0 mm) (Table 1).

Shoots regenerated from TCL explants produced roots within 24 days of culture. These observations indicated a positive relationship between TCL size and the percentage of root formation, whereby larger explants exhibited higher rooting frequencies. The 5.0 mm TCLs recorded the highest percentage of root formation (38.90 ± 19.23%), followed by 4.0 mm (33.33 ± 16.65%) and 3.0 mm (22.23 ± 9.58%). Although the 2.0 mm explant regenerated shoots, no roots were formed. No significant differences were observed in the mean number of roots and root length among the 3.0, 4.0, and 5.0 mm TCL explants.

According to Van (2003), the shape and size of TCLs depend on the organ from which they are derived and the orientation of sectioning, either longitudinal or transverse. The regeneration capacity of TCL explants is generally higher than that of conventional thicker explants, largely due to the greater proportion of morphogenic cells and improved transport of nutrients between the medium and cells. Similar observations were reported by Hany *et al*. (2023), who found that smaller TCLs exhibited a higher morphogenic capacity than larger explants. Furthermore, smaller TCLs possess a larger surface area in contact with the culture medium, enhancing the efficiency of nutrient transfer from the medium to the cells (Sharma *et al*., 2023).

The present study demonstrated that a TCL thickness of 5.0 mm yielded optimal shoot regeneration for *K. parviflora*. This value represents the upper range of TCL thickness. However, several other species have also been reported to require thicker TCLs for successful shoot regeneration. For instance, *Vanilla planifolia* produced optimal regeneration from TCLs sectioned into 2.0-5.0 mm segments (Jing *et al*., 2014). Similarly, *Lilium longiforum* exhibited the highest shoot regeneration when 3.0 mm and 4.00 mm receptacle sections were cultured on MS medium supplemented with NAA and BAP (Nhut *et al*., 2001). In *Allium ampeloprasum* L., transverse segments of 5.0 mm flower stalks resulted in the highest percentage of regenerative explants and the greatest number of adventitious shoots per explant (Silvertand *et al*.,1995). The thickness or size of TCL explants may influence the combined effect of endogenous and exogenous plant hormones on growth and development. According to Pietropaolo (2023), auxins and cytokinins are the most frequently used PGRs in plant tissue culture, and the ratio of their concentrations determines the type of organ produced from cultured cells or tissues.

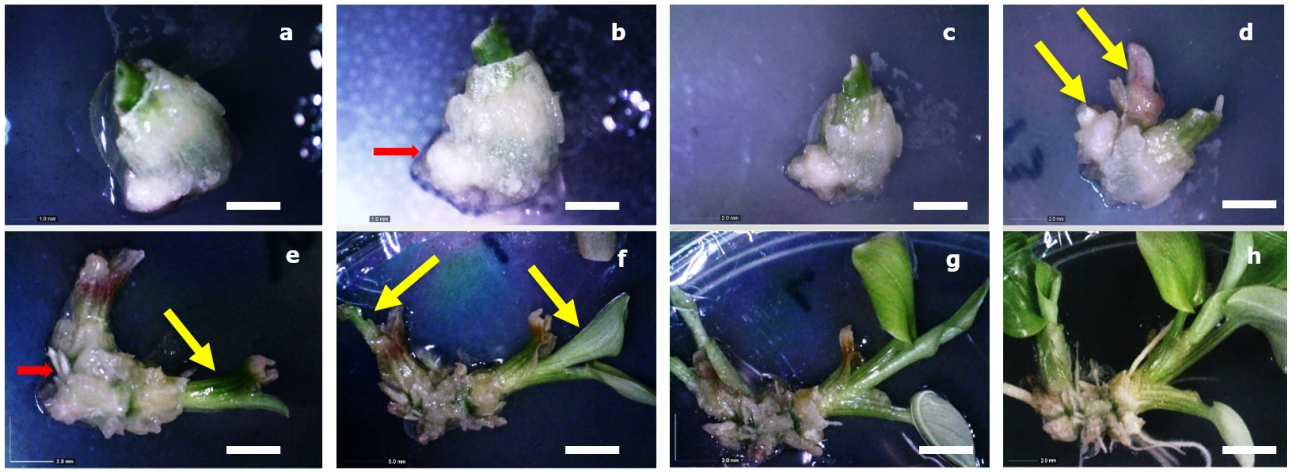

Fig. 1. Response of *K. parviflora* thin cell layer (5.0 mm) cultured on MS medium containing 1.0 mg/L BAP over 8 weeks. (a) Swelling of TCL explant (Bar = 2.0 mm); (b-c) formation of an adventitious bud (red arrow) (b: Bar = 2.0 mm, c: Bar = 0.5 cm); (d) enlargement of adventitious buds (Bar = 0.5 cm); (e-f) development of fine adventitious roots (red arrow) and shoot formation (yellow arrow) (Bar = 0.5 cm); (g) proliferation of multiple adventitious shoots (Bar = 2.0 cm); (h) well-developed, healthy, adventitious shoots with roots from TCL explant before transfer to the shoot development media (Bar = 2.0 cm).

Table 1. Shoot and root regeneration responses of aerial shoot-derived thin cell layers of *K. parviflora* at different thicknesses

Treatment	Explant size (mm)	Explants forming shoots (% ± SD)	Number of shoots per responsive explant (Mean ± SD)	Number of leaf (Mean ± SD)	Explants forming roots (% ± SD)	Number of roots per responsive explant (Mean ± SD)	Roots length(cm) (Mean ± SD)
MS0 (Comparison)	1.0	0.00 ^{d}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}
	2.0	0.00 ^{d}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}
	3.0	0.00 ^{d}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}
	4.0	0.00 ^{d}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}
	5.0	16.70 ± 0.00 ^{c}	1.33 ± 0.58 ^{b}	0.00 ^{c}	16.70 ± 0.00 ^{b}	2.33 ± 1.53 ^{ab}	1.67 ± 0.42 ^{ab}
MS + 1.0 mg/L BAP	1.0	0.00 ^{d}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}
	2.0	18.47 ± 3.06 ^{c}	1.17 ± 0.29 ^{b}	1.00 ± 0.00 ^{b}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}
	3.0	31.47 ± 3.18 ^{b}	1.42 ± 0.38 ^{b}	1.22 ± 1.06 ^{ab}	22.23 ± 9.58 ^{b}	3.33 ± 0.58 ^{a}	1.44 ± 0.54 ^{ab}
	4.0	42.50 ± 8.48 ^{a}	1.67 ± 0.29 ^{b}	2.00 ± 0.00 ^{a}	33.33 ± 16.65 ^{ab}	2.78 ± 1.54 ^{ab}	1.99 ± 0.21 ^{a}
	5.0	46.33 ± 11.64 ^{a}	2.33 ± 0.58 ^{a}	1.94 ± 1.00 ^{a}	38.90 ± 19.23 ^{a}	1.80 ± 1.06 ^{ab}	1.47 ± 0.61 ^{ab}

Values represent the means ± SD of 4 experimental replicates. Data were recorded after 8 weeks of culture. Means within each column followed by the same letters did not differ significantly at $p < 0.05$ according to Duncan's Multiple Range Test (DMRT).

Effect of plant growth regulators (PGRs) on shoot regeneration and multiplication

When the effects of individual plant growth regulators (PGRs) on shoot regeneration were evaluated, BAP, kinetin, and TDZ exhibited similar growth patterns. In all treatments involving these three cytokinins, shoot production by TCLs was inversely proportional to PGR concentration; the highest shoot induction occurred at the lowest concentration tested. At 1.0 mg/L BAP, 44.4 ± 15.7% of the TCL explants produced shoots, whereas 0.5 mg/L kinetin and TDZ resulted in 29.2 ± 8.3% and 16.7 ± 0.0% shoot-forming explants, respectively (Table 2). At higher concentrations (2.0– 5.0 mg/L), both BAP and kinetin induced shoot formation at markedly lower frequencies. In contrast, TDZ completely inhibited regeneration when its concentration exceeded 0.5 mg/L.

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Table 2. Effects of different types of plant growth regulators (PGRs) on shoot regeneration and proliferation of *K. parviflora* using 5.0 mm TCL explants

PGR	Concentration (mg/L)	Explants forming shoots (% ± SD)	Number of shoots per responsive explant (Mean ± SD)	Number of leaf (Mean ± SD)	Explants forming roots (% ± SD)	Number of roots per responsive explant (Mean ± SD)	Roots length(cm) (Mean ± SD)
MS0	0	16.7 ± 0.0 ^{cd}	1.75 ± 0.96 ^{bcd}	1.88 ± 0.85 ^{a}	12.5 ± 8.4 ^{c}	4.75 ± 3.30 ^{abc}	1.84 ± 1.24 ^{bcd}
BAP	1.0	44.4 ± 15.7 ^{a}	2.38 ± 0.75 ^{ab}	1.83 ± 1.31 ^{a}	37.5 ± 8.4 ^{a}	4.00 ± 2.45 ^{abcd}	1.64 ± 0.16 ^{bcde}
	2.0	20.9 ±8.3 ^{bcd}	2.00 ± 0.82 ^{bc}	1.75 ± 0.50 ^{ab}	20.9 ± 8.3 ^{bc}	3.59 ± 2.04 ^{abcd}	1.59 ± 0.84 ^{bcde}
	3.0	20.9 ± 8.3 ^{bcd}	1.50 ± 0.58 ^{bcde}	1.50 ± 0.58 ^{ab}	16.7 ± 0.0 ^{c}	3.00 ± 0.82 ^{bcd}	1.31 ± 0.38 ^{cde}
	4.0	16.7 ± 0.0 ^{cd}	1.25 ± 0.50 ^{cde}	1.50 ± 1.00 ^{ab}	16.7 ± 0.0 ^{c}	2.75 ± 0.96 ^{bcd}	1.45 ± 0.44 ^{bcde}
	5.0	16.7 ± 0.0 ^{cd}	1.00 ± 0.0 ^{de}	1.38 ± 0.48 ^{ab}	12.5 ± 8.4 ^{c}	2.25 ± 1.50 ^{de}	0.75 ± 0.50 ^{de}
KINETIN	0.5	29.2 ± 8.3 ^{b}	2.25 ± 0.50 ^{ab}	2.25 ± 0.35 ^{a}	29.2 ± 8.3 ^{b}	2.33 ± 0.53 ^{cde}	4.53 ± 4.28 ^{a}
	1.0	25.0 ± 9.6 ^{b}	2.13 ± 0.85 ^{abc}	2.04 ± 1.40 ^{a}	20.8 ± 15.9 ^{bc}	3.75 ± 3.30 ^{abcd}	2.95 ± 1.56 ^{bc}
	2.0	20.9 ± 8.3 ^{bcd}	2.13 ± 0.63 ^{abc}	2.03 ± 0.70 ^{a}	20.9 ± 8.3 ^{bc}	3.83 ± 1.67 ^{abcd}	3.05 ± 0.74 ^{b}
	3.0	16.7 ± 0.0 ^{cd}	1.83 ± 0.58 ^{bcd}	2.03 ± 0.74 ^{a}	16.7 ± 0.0 ^{c}	4.88 ± 2.95 ^{ab}	2.25 ± 0.96 ^{bcd}
	4.0	16.7 ± 0.0 ^{bcd}	2.00 ± 1.41 ^{bc}	1.75 ± 1.26 ^{ab}	16.7 ± 0.0 ^{c}	5.83 ± 2.96 ^{a}	1.50 ± 0.48 ^{bcde}
TDZ	0.5	16.7 ± 0.0 ^{cd}	3.00 ± 1.15 ^{a}	1.33 ± 0.47 ^{ab}	12.5 ± 8.4 ^{c}	1.75 ± 1.26 ^{de}	1.30 ± 0.90 ^{cde}
	1.0	0.0 ^{e}	0.00 ^{f}	0.00 ^{c}	0.0 ^{d}	0.00 ^{e}	0.00 ^{e}
	2.0	0.0 ^{e}	0.00 ^{f}	0.00 ^{c}	0.0 ^{d}	0.00 ^{e}	0.00 ^{e}
	3.0	0.0 ^{e}	0.00 ^{f}	0.00 ^{c}	0.0 ^{d}	0.00 ^{e}	0.00 ^{e}
	4.0	0.0 ^{e}	0.00 ^{f}	0.00 ^{c}	0.0 ^{d}	0.00 ^{e}	0.00 ^{e}
	0.5	0.0 ^{e}	0.00 ^{f}	0.00 ^{c}	0.0 ^{d}	0.00 ^{e}	0.00 ^{e}
NAA	1.0	16.7 ± 0.0 ^{cd}	1.50 ± 0.58 ^{bcde}	0.00 ^{c}	0.0 ^{d}	0.00 ^{e}	0.00 ^{e}
	2.0	0.0 ^{e}	0.00 ^{f}	0.00 ^{c}	0.0 ^{d}	0.00 ^{e}	0.00 ^{e}
	3.0	0.0 ^{e}	0.00 ^{f}	0.00 ^{c}	0.0 ^{d}	0.00 ^{e}	0.00 ^{e}
	4.0	0.0 ^{e}	0.00 ^{f}	0.00 ^{c}	0.0 ^{d}	0.00 ^{e}	0.00 ^{e}
	0.2	16.7 ± 0.0 ^{cd}	1.75 ± 0.50 ^{bcd}	1.50 ± 1.00 ^{ab}	12.5 ± 8.4 ^{c}	1.50 ± 1.00d ^{e}	0.93 ± 0.65 ^{de}
	0.4	25.0 ± 9.6 ^{bc}	1.00 ± 0.00 ^{de}	0.00 ^{c}	0.0 ^{d}	0.00 ^{e}	0.00 ^{e}
2,4-D	0.6	16.7 ± 0.0 ^{cd}	1.00 ± 0.00 ^{de}	0.75 ± 0.50 ^{bc}	0.0 ^{d}	0.00 ^{e}	0.00 ^{e}
	0.8	12.5 ± 8.4 ^{d}	0.75 ± 0.50 ^{ef}	0.00 ^{c}	0.0 ^{d}	0.00 ^{e}	0.00 ^{e}
	1.0	0.0 ^{e}	0.00 ^{f}	0.00 ^{c}	0.0 ^{d}	0.00 ^{e}	0.00 ^{e}

Values represent the mean ± SD of 4 experimental replicates. Data were recorded after 8 weeks of culture. Means within each column followed by the same letters did not differ significantly at $p<0.05$ according to Duncan's Multiple Range Test (DMRT).

The results for other parameters, including the number of shoots, number of roots, and root length, followed a similar pattern. Shoot regeneration induced by the auxins NAA and 2,4-D was markedly less efficient than that induced by cytokinins. Under the influence of 2,4-D, shoot formation increased with rising concentrations of the auxin, reaching a maximum at 0.4 mg/L 2,4-D with 25.0 ± 9.6% of the TCL producing shoots, and a minimum at 0.8 mg/L, with 12.5 ± 8.4%. When the 2,4-D concentration was increased to 1.0 mg/L or higher, the explants failed to produce shoots and turned brown, eventually dying by the 5th week of culture. In contrast, NAA induced shoot formation only at 1.0 mg/L, with 16.7% of the explants producing shoots.

Although the percentage of explant-forming shoots was higher in the BAP treatment, the number of shoots produced per explant was higher on the medium supplemented with 0.5 mg/L TDZ (3.0 ± 1.15), followed by 1.0 mg/L BAP (2.38 ± 0.75) and 0.5 mg/L kinetin (2.25 ± 0.5). However, no significant differences (p<0.5) were observed in the number of shoots among the different types and concentrations of PGRs.

A total of 37.5 ± 8.4% of the explants formed roots in the medium supplemented with 1.0 mg/L BAP, followed by 0.5, 1.0, and 2.0 mg/L kinetin (29.1 ± 8.32%, 20.9 ± 8.3%, & 20.8 ± 15.9%, respectively). Treatment with 0.5 mg/L of kinetin produced the longest roots (4.53 ± 4.28 cm), which were significantly different (p<0.05) from those in other treatments, followed by 1.0–4.0 mg/L kinetin and 1.0–2.0 mg/L BAP. Explants cultured on MS0 medium also developed long roots in the absence of PGRs, presumably due to endogenous hormonal activity. In contrast, no root formation was observed on the media supplemented with 0.4–1.0 mg/L 2,4-D. Similar rooting results were obtained with TDZ and NAA treatments. Rooting during shoot production has also been reported in the micropropagation of *K. parviflora* using rhizomes (Khairudin *et al*., 2020; Labrooy *et al*., 2020).

Previous studies on the micropropagation of *K. parviflora* have primarily used rhizomes as explants, with BAP being the most extensively used cytokinin for shoot regeneration and proliferation on MS media. For instance, shoot buds of *K. parviflora* were successfully cultured in MS medium supplemented with 3.0 mg/L BAP (Zuraida *et al*., 2015; Laipaitong, 2017; Kitwetcharoen *et al*., 2020; Rahman *et al*., 2021). Khairudin *et al*. (2020) reported that 1.5 mg/L BAP increased the shoot induction rate of *K. parviflora* after 8 weeks, yielding an average of 1.4 shoots per explant. Alveno (2012) obtained the highest number of *K. parviflora* shoots (an average of 1.77 shoots per explant) on MS medium supplemented with 3.75 mg/L BAP, while Rahman *et al*. (2014) initiated *K. parviflora* cultures using 5.0 mg/L BAP. In the present study, a lower concentration of BAP (1.0 mg/L) was sufficient to stimulate shoot regeneration from TCL explants of *K. parviflora*. These findings demonstrate that the effectiveness of TCLs in producing shoots is comparable to that of rhizomes when an appropriate explant size and a low concentration of BAP are used.

Cytokinins play an important role in the elongation and differentiation of plant cells. In the present study, explants cultured on media containing kinetin and BAP exhibited greater shoot elongation and a higher number of leaves than those treated with TDZ. Furthermore, as the concentration of BAP increased, both shoot number and length decreased. This observation aligns with the findings of Labrooy *et al*. (2020), who reported that increasing BAP concentrations reduced the number and length of *K. parviflora* shoots. The ability of BAP to stimulate shoot growth in the current study may be attributed to its resistance to cytokinin oxidase cleavage, which enhances its stability in the culture medium (Kieber & Schaller, 2018).

According to Teixeira da Silva and Dobranszki (2015), the TCL concept involves *in vitro* reprogramming, enabling the regeneration of organs or embryos by isolating one or a few (3–6) layers of differentiated cells from any organ or tissue. TCLs are multicellular systems derived from small explants (generally <1–2 mm) prepared from differentiated plant organs, which maintain inherent temporal and spatial organisation. Media (2023) reviewed that the TCL is more efficient for total plantlet production than conventional *in vitro* methods due to the small explant size, which facilitates the diffusion of nutrients from the medium into the tissues.

TCL explants display unique morphogenetic traits, with direct organogenesis enabling shoot regeneration from *K. parviflora* tissue cultures. Organogenesis is particularly valuable for the clonal propagation of cultivars that lack sexual reproduction, allowing the production of genetically uniform planting material (Gallego, 2021). The results of this study demonstrate that different types of PGRs significantly influence the morphogenetic responses of TCL explants, with 1.0 mg/L BAP producing the most effective shoot formation compared to other PGRs.

Effect of PGR combinations on shoot regeneration and proliferation of *K. parviflora*

The growth responses of the TCL explants were consistent with previous observations, with the plantlets forming over 8 weeks. Combinations of BAP with 2,4-D or TDZ were evaluated based on the response of TCL explants to single PGR applications on MS media. In these treatments, BAP was maintained at a constant concentration of 1.0 mg/L, while 2,4-D and TDZ were tested at 0, 0.2, 0.4, 0.6, and 0.8 mg/L. The results revealed that the addition of 2,4-D to BAP inhibited both shoot and root regeneration. As the concentration of 2,4-D increased, regeneration rates were significantly reduced (p<0.5); shoot regeneration decreased by 1.8-fold, from 45.83 ± 15.96% in the absence of 2,4-D to 25.00 ± 9.62% in its presence, while root regeneration declined by 1.3-fold from 33.33 ± 13.61% to 25.00 ± 9.62% under the same PGR conditions (Table 3). When the concentration of 2,4-D exceeded 0.2 mg/L, the regeneration capacity of the TCLs was completely inhibited. A similar inhibitory trend was observed for the number of shoots and roots per explant (Table 3), with an average of 1.50 ± 0.58 shoots, 2.88 ± 0.85 roots, and a mean root length of 1.29 ± 0.36 cm.

In contrast, combining TDZ with BPA enhanced the regeneration of shoots and roots from TCL explants. As the TDZ concentration increased, the percentage of explants forming shoots and roots also rose, reaching a maximum at 0.6 mg/L TDZ, with 70.83 ± 8.33% of explants forming shoots and 33.33 ± 13.6% forming roots. However, when TDZ concentration increased to 0.8 mg/L, shoot regeneration decreased to 62.50 ± 20.97%. The number of leaves and roots per explant followed a similar trend, as shown in Table 3, with the highest numbers recorded at 0.6 mg/L TDZ (3.26 ± 1.09 leaves and 14.35 ± 5.15 roots per explant).

These findings demonstrate that the combination of PGRs significantly influences the regeneration and multiplication ability of TCL explants. Regeneration was suppressed by 2,4-D, likely due to its herbicidal properties, which can cause abnormal growth and plant mortality (Chepkoech, 2021). Excessive 2,4-D levels disrupt physiological processes such as photosynthetic carbon reduction, fluorescence characteristics, and non-photochemical quenching mechanisms (Sachu *et al*., 2022, as cited by Sarkar *et al*., 2024).

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Table 3. Effect of the combination of BAP (1.0 mg/L) with different concentrations of 2,4-D or TDZ on the shoot regeneration and proliferation of *K. parviflora* using 5.0 mm TCL explants

Treatment (mg/L)		Explants forming shoots (% ± SD)	Number of shoots per responsive explant (Mean ± SD)	Number of leaf (Mean ± SD)	Explants forming roots (% ± SD)	Number of roots per responsive explant (Mean ± SD)	Roots length (cm) (Mean ± SD)
MS0		20.83 ± 8.34 ^{cd}	1.25 ± 0.50 ^{c}	2.25 ± 0.5 ^{abc}	20.66 ± 8.45 ^{abc}	2.00 ± 0.82 ^{ef}	1.18 ± 0.29 ^{ab}
BAP	2,4-D						
1.0	0	45.83 ± 15.96 ^{ab}	2.75 ± 0.96 ^{a}	2.25 ± 0.50 ^{abc}	33.33 ± 13.61 ^{a}	3.50 ± 1.00 ^{def}	1.56 ± 0.35 ^{a}
1.0	0.2	25.00 ± 9.62 ^{cd}	1.50 ± 0.58 ^{bc}	2.38 ± 0.48 ^{ab}	25.00 ± 9.62 ^{abc}	2.88 ± 0.85 ^{def}	1.29 ± 0.36 ^{ab}
1.0	0.4	0.00^{d}	0.00 ^{d}	0.00 ^{d}	0.00 ^{d}	0.00 ^{f}	0.00 ^{e}
1.0	0.6	0.00^{d}	0.00 ^{d}	0.00 ^{d}	0.00 ^{d}	0.00 ^{f}	0.00 ^{e}
1.0	0.8	0.00^{d}	0.00 ^{d}	0.00 ^{d}	0.00 ^{d}	0.00 ^{f}	0.00 ^{e}
BAP	TDZ						
1.0	0	37.50 ± 20.97 ^{bc}	1.00 ± 0.00 ^{c}	0.00	0.00	0.00	0.00
1.0	0.2	54.17 ± 20.97 ^{ab}	1.18 ± 0.24 ^{c}	1.30 ± 1.25 ^{bc}	16.67 ± 13.61 ^{bc}	7.13 ± 5.11 ^{cd}	0.70 ± 0.50 ^{cd}
1.0	0.4	62.50 ± 34.36 ^{ab}	1.31 ± 0.22 ^{c}	2.23 ± 0.95 ^{abc}	29.17 ± 8.33 ^{ab}	10.00 ± 1.78 ^{bc}	0.95 ± 0.19 ^{bc}
1.0	0.5	70.83 ± 15.96 ^{a}	1.39 ± 0.35 ^{bc}	2.11 ± 1.02 ^{bc}	25.00 ± 9.62 ^{abc}	15.75 ± 5.72 ^{a}	1.06 ± 0.22 ^{bc}
1.0	0.6	70.83 ± 8.33 ^{a}	1.99 ± 0.64 ^{b}	3.26 ± 1.09 ^{a}	33.33 ± 13.61 ^{a}	14.38 ± 5.15 ^{ab}	1.27 ± 0.22 ^{ab}
1.0	0.8	62.50 ± 20.97 ^{ab}	1.28 ± 0.25 ^{d}	1.18 ± 0.62 ^{c}	12.50 ± 8.33 ^{cd}	5.75 ± 4.65 ^{cde}	0.36 ± 0.30 ^{de}

Values represent the mean ± SD of 4 experimental replicates. Data were recorded after 8 weeks of culture. Means within each column followed by the same letters did not differ significantly at $p<0.05$ according to Duncan's Multiple Range Test (DMRT).

In plants, growth hormones, such as auxins, cytokinins, gibberellins, ethylene, and abscisic acid, interact synergistically and antagonistically. Their effects are optimised when balanced; however, hormonal imbalance can alter signalling pathways, leading to the suppression or activation of particular physiological functions. Auxins regulate cell division, root formation, and tropic responses, whereas cytokinins promote cell division, lateral bud development, and delay senescence (the ageing of organs and plant tissues) (Sosnowski *et al*., 2023).

The combination of BAP and TDZ enhanced shoot multiplication in TCL explants of *K. parviflora*. TDZ functions as a plant growth regulator with both auxin- and cytokinin-like activity (Murthy *et al*., 1998), but exhibits a stronger cytokinin effect (Nisler, 2018). The cytokinin-like activity promotes enhanced cell division and confers anti-senescence and anti-stress properties (Nisler, 2018). The observed enhancement of shoot regeneration in the present study supports the findings of Saensouk *et al*. (2016), who reported that supplementing BAP with TDZ increased the number of shoots per explant in the *in vitro* propagation of *Kaempferia marginata*. Similarly, TDZ has been shown to promote regeneration in other species (Pai & Desai, 2018).

Comparable results were observed in a TCL study by Le *et al*. (1998) on *Digitaria sanguinalis,* where a combination of low concentration of BAP and 2,4-D effectively generated a complete plant directly (without an intermediate callus phase) with high efficiency, eliminating the need for subculture. These plants originated from pseudo-embryogenic structures (Le *et al*., 1998). Likewise, Thingbaijam and Huidrom (2014) reported high-frequency shoot proliferation in *Zingiber officinale* var 'Nadia' using TCL derived from microrhizomes with on MS medium containing 6.0 mg/L BAP and 0.2 mg/L 2,4-D. The study indicated that lower concentrations of 2,4-D in combination with BAP were most effective for redifferentiation of ginger shoots.

Plantlet development and acclimatisation

Adventitious shoots excised from the TCL explants developed into fully formed leaves and roots (Figures 2a & 2b). Data obtained after 5 weeks of culture are shown in Table 4. The average shoot height was 6.55 ± 1.78 cm and 3.05 ± 0.29 leaves per plantlet. The number and length of the roots also increased, averaging 5.04 ± 1.5 cm and 4.58 ± 1.31 cm, respectively. After 5 weeks, plantlet survival reached 90 ± 7.07% with a mean shoot length of 9.49 ± 1.63 cm (Table 5). Acclimatised plantlets exhibited normal characteristics (Figures 2c & 2d).

The composition of plant media is crucial for optimal root development during acclimatisation. The media used for acclimation consisted of organic soil and burnt rice husks in a 1:1 ratio. This mixture provides excellent water-holding capacity, while the burnt rice husks contain silica, enhancing soil fertility. Ahmad and Mohamad (2022) reported similar findings in ginger cultivation using the same media at MARDI. Previous studies have used various substrates during acclimation, including autoclaved perlite (Labrooy *et al*., 2020) and peat moss (Roslan *et al*., 2023).

Table 4. Shoot and root development of *K. parviflora*

Type of media	Shoot length (cm) (Mean ± SD)	No. of leaf (Mean ± SD)	No. of roots (Mean ± SD)	Root length (cm) (Mean ± SD)
MS0	6.55 ± 1.78	3.05 ± 0.29	5.04 ± 1.5	4.58 ± 1.31

Values represent the mean ± SD of 5 experimental replicates. Data were recorded after 5 weeks of culture.

Table 5. Acclimatisation of TCL-derived plantlets

Type of media	Number of plantlets	% survival (Mean ± SD)	Root length (cm) (Mean ± SD)
Organic soil: Burnt rice husk (1:1)	50	90.0 ± 7.07	9.49 ± 1.63

Values represent the means ± SD of 5 experimental replicates. Data were recorded after 5 weeks of culture.

Fig. 2. Development and acclimatisation of *K. parviflora*. (a) Well-developed shoots and roots from TCL explants after 8 weeks of culture (Bar = 2.0 mm); (b) adventitious shoot in MS0 medium for shoot and root development (Bar = 1.49 cm); (c) plantlets after 5 weeks of acclimatisation; (d) plantlets after 2 months of growth (Bar = 2.6 cm).

CONCLUSION

This study demonstrates that TCLs derived from aerial shoots can serve as effective explants for the micropropagation of *Kaempferia parviflora*. Optimal culture conditions for *in vitro* plantlet production were identified, with 5.0 mm TCL thickness found to be most suitable. Supplementation of the propagation medium with 1.0 mg/L BAP yielded the highest shoot regeneration (44.4 ± 15.7%), producing 2.38 ± 0.75 shoots per TCL explant. The combination of BAP and 0.6 mg/L TDZ further enhanced regeneration, achieving the highest percentage of TCL explants forming shoots (70.83 ± 8.33%). The use of TCLs offers a practical and efficient alternative to rhizome-based propagation, reducing the dependency on rhizomes as explant sources and facilitating large-scale production of *K. parviflora* planting materials for commercial cultivation.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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