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ESTABLISHMENT OF *Strobilanthes crispus IN VITRO* CULTURE AND ASSESSMENT OF TARAXEROL CONTENT FROM FIELD AND *IN VITRO* GROWN PLANTS

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ABSTRACT. *Taraxerol is a pentacyclic triterpenoid with several reported biological activities and is naturally accumulated by Strobilanthes crispus, a medicinal plant commonly known as Pecah Beling. However, its availability from conventional plant material is limited, highlighting the potential of plant tissue culture as a controlled alternative for plant propagation and secondary metabolite production. This study aimed to establish an in vitro culture system for S. crispus using axillary shoot and nodal explants, evaluate the effects of cytokinin treatments on shoot multiplication and development, and compare taraxerol accumulation between field-grown and in vitro-grown tissues. Axillary shoot explants showed the highest establishment success, with 78.0 ± 2.74% survival following sterilization with 20% (v/v) Clorox™ for nine minutes, whereas field-derived nodal explants did not survive the tested sterilization treatments. Subsequent micropropagation using in vitro nodal explants showed that the combination of 0.5 mg/L kinetin (KN) and 0.1 mg/L thidiazuron (TDZ) produced 9.4 ± 2.8 shoots per explant with an 86.4 ± 17.24% regeneration frequency after 30 days. Transfer to hormone-free MS medium promoted shoot development, achieving 100% shoot elongation and a mean shoot length of 6.0 ± 1.15 cm. Taraxerol assessment showed the highest content in field-grown leaves (48.83 ± 0.18 mg/g dw), followed closely by leaves of in vitro plantlets (45.17 ± 1.25 mg/g dw) and field-grown axillary shoots (26.39 ± 1.14 mg/g dw). The establishment of an in vitro propagation system capable of maintaining substantial taraxerol accumulation in S. crispus leaves provides an important foundation for further optimization of controlled biomass and taraxerol production.*

INTRODUCTION

Natural products remain important sources of bioactive compounds for pharmaceutical and therapeutic applications. Among these compounds, taraxerol is a pentacyclic triterpenoid that has attracted considerable interest because of its reported biological activities, including anti-inflammatory and anti-tumour-promoting properties (Mus et al., 2022). *Strobilanthes crispus* (L.) Blume, commonly known in Malaysia as Pecah Beling, is a medicinal plant that naturally accumulates taraxerol (Koay et al., 2013). The plant has traditionally been used for various medicinal purposes, while more recent studies have reported antioxidative and wound-healing (Ban et al., 2022), antidiabetic (Fauziah et al., 2022), and anticancer activities (Baraya et al., 2022). Collectively, these properties highlight *S. crispus* as a potentially valuable plant source of biologically active secondary metabolites, including taraxerol.

Despite its potential value, obtaining taraxerol through conventional plant harvesting presents several limitations. Taraxerol occurs as a relatively minor constituent in plant tissues, requiring substantial quantities of plant material for extraction. In addition, its structural complexity presents challenges for chemical synthesis and may constrain economical and sustainable production at larger scales (Mus et al., 2022). Consequently, alternative approaches that permit controlled plant biomass production while maintaining the biosynthesis of taraxerol are desirable.

Plant tissue culture offers such an alternative by enabling plant material to be propagated under controlled conditions independently of seasonal and environmental variation. *In vitro* systems have also been explored for the production of valuable plant secondary metabolites and may provide a more consistent and controllable source of biomass than conventional harvesting (Sharma et al., 2023; Chandran et al., 2023). Previous studies have demonstrated the feasibility of producing secondary metabolites through *in vitro* systems, including taraxerol and related triterpenoids in other plant species, as well as triterpenic acids from *Malus domestica* and cytotoxic secondary metabolites from *Jatropha curcas* (Swain et al., 2012; Geană et al., 2021; Adriano-Anaya et al., 2016). These examples demonstrate the potential of plant tissue culture for secondary-metabolite production.

However, despite these advances in other plant systems, information specifically linking the establishment and micropropagation of *S. crispus in vitro* cultures with their capacity to accumulate taraxerol remains limited. In particular, there is a need to establish suitable explant and plant growth regulator conditions for efficient *S. crispus* propagation and to determine whether tissues produced under *in vitro* conditions can maintain taraxerol accumulation at levels comparable to field-grown plants. Addressing these aspects is important for evaluating the feasibility of *S. crispus* tissue culture as a controlled platform for subsequent optimization of taraxerol production.

Therefore, the present study aimed to establish an *in vitro* culture system for *S. crispus* using axillary shoot and nodal explants; evaluate the effects of individual and combined cytokinins on multiple shoot induction and subsequent shoot development; and compare taraxerol accumulation among tissues obtained from field-grown plants and *in vitro* plantlets. By integrating micropropagation with comparative taraxerol assessment, this study provides a foundation for the further development and optimization of controlled *S. crispus* cultures for taraxerol production.

MATERIALS AND METHODS

Plant Materials

Healthy *Strobilanthes crispus* plant materials were purchased from local sources in Likas, Kota Kinabalu, Sabah, Malaysia. Upon arrival at the laboratory, the plant materials were thoroughly washed under running tap water to remove adhering soil and debris. The cleaned plant materials were then maintained in the laboratory with their basal portions immersed in tap water until new axillary shoots developed. Young axillary shoots and nodal segments produced during this laboratory maintenance period were subsequently excised and used as explants for the establishment of *in vitro* cultures.

The plant material was authenticated by Mr. Johny Sigil, a botanist at the Institute for Tropical Biology and Conservation (ITBC), Universiti Malaysia Sabah. A voucher specimen was deposited at the BORNEENSIS Herbarium, ITBC, under voucher number BORH 2481.

Surface Sterilization and Establishment of Aseptic Cultures

Nodal segments (approximately 1 year old) and young axillary shoots (approximately 3–4 weeks old) of *Strobilanthes crispus* were excised using sterile blades. The explants were initially washed thoroughly with Dettol solution (1:20, v/v) and subsequently placed under running tap water for 30 min to remove adhering surface debris. The explants were then blotted dry using sterile tissue paper and transferred to a laminar-flow cabinet for subsequent surface sterilization.

Under aseptic conditions, the explants were rinsed five times with sterile distilled water before being subjected to Clorox™ treatments at different concentrations and exposure durations. For axillary shoot explants, Clorox™ concentrations of 10, 20, 30, and 40% (v/v) were evaluated at exposure durations of 5, 7, 9, and 12 min. For nodal explants, Clorox™ concentrations of 10, 20, and 30% (v/v) were evaluated at exposure durations of 10, 20, and 30 min. Following each sterilization treatment, the explants were rinsed five times with sterile distilled water to remove residual sterilant. The treatment combinations correspond to those reported in Tables 1 and 2.

Each sterilization treatment consisted of five replicates, with 10 explants per replicate, giving a total of 50 explants per treatment (n = 50). Following sterilization, the explants were cultured on Murashige and Skoog (MS) basal medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. Cultures were maintained at 25 ± 2 °C and 45% relative humidity under a 16-h photoperiod provided by cool-white fluorescent lamps at a light intensity of 36 µmol m^{-2} s^{-1}.

Aseptic culture establishment was evaluated after seven days of culture based on explant survival, contamination, and mortality. The percentages of surviving, contaminated, and dead explants were calculated for each treatment and used to evaluate the effectiveness of the sterilization conditions.

In Vitro Multiple Shoot Induction and Development

Successfully established aseptic *Strobilanthes crispus* cultures were maintained on basal Murashige and Skoog (MS) medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar and subcultured at 45-day intervals. Plantlets maintained for two consecutive subculture cycles were used as the source of nodal and leaf explants for the multiple shoot-induction experiments. Cultures were maintained at 25 ± 2 °C under a 16-h photoperiod, and the culture medium was adjusted to pH 5.7.

For multiple shoot induction, nodal and leaf explants were cultured in Petri dishes containing MS medium supplemented separately with 6-benzylaminopurine (BAP), kinetin (KN), or thidiazuron (TDZ) at concentrations of 0.5, 1.0, 2.0, or 4.0 mg/L. MS medium without plant growth regulators served as the control. The effects of combined cytokinins were further evaluated by supplementing the MS medium with either 0.5 mg/L BAP or 0.5 mg/L KN in combination with TDZ at concentrations of 0.05, 0.1, 0.2, or 0.4 mg/L. Each treatment consisted of three replicates with six explants per replicate, giving a total of 18 explants per treatment (n = 18). Morphogenic responses were monitored for 30 days, with observations recorded every six days. The parameters evaluated included the time required for shoot induction, shoot regeneration frequency, number of shoots produced per explant, shoot length, and other morphological responses, including callus formation and tissue browning.

For subsequent shoot development, nodal explants that had produced more than two shoots, with an initial shoot length of approximately 0.7 ± 0.5 cm, were selected and transferred to fresh MS medium in 250-mL conical flasks. The shoots were cultured on MS medium supplemented separately with BAP or KN at concentrations of 0.5, 1.0, 2.0, or 4.0 mg/L, while hormone-free MS medium served as the control. Each treatment consisted of three replicates with six explants per replicate, giving a total of 18 explants per treatment (n = 18). Shoot development was evaluated over a 30-day culture period based on the percentage of shoots showing elongation, number of newly formed shoots, shoot length, number of leaves, and occurrence of callus formation.

Extraction of Taraxerol from *In Vitro* and Intact Plants

Taraxerol content was quantified using reverse-phase high-performance liquid chromatography (RP-HPLC) based on the method of Sharma and Zafar (2014), with slight modifications. An authentic taraxerol standard (Sigma) was used for calibration and peak identification. A stock solution of 200 µg/mL was prepared by dissolving 2 mg of taraxerol in 10 mL of analytical-grade methanol (99.9% purity; Fisher), followed by sonication for 30 min to ensure complete dissolution. The stock solution was subsequently diluted to prepare a five-point calibration series consisting of 200, 100, 50, 25, and 12.5 µg/mL taraxerol.

RP-HPLC analysis was performed using an Agilent Series 200 HPLC system (Model G131104) equipped with a diode-array detector (DAD) and a Supelco C18 column. HPLC-grade acetonitrile and methanol were used as the mobile phase at a ratio of 70:30 (v/v) under isocratic conditions. The mobile phase was sonicated for 10 min before analysis. Prior to HPLC analysis, the taraxerol standard solutions were filtered through a 0.45-µm membrane filter and sonicated for 6 min. Chromatographic detection was performed at 210 nm with a total run time of 5 min.

A five-point calibration curve was generated by plotting chromatographic response area against taraxerol concentration. The relationship between response area and taraxerol concentration was described by the equation **y = 23.684x**, where *y* represents the chromatographic response area (mAU) and *x* represents the taraxerol concentration (µg/mL). Linearity was assessed using the coefficient of determination (R^2), with an R^2 value greater than 0.9985 considered indicative of an acceptable linear fit.

Plant extracts were dissolved in analytical-grade methanol, filtered through a 0.45-µm membrane filter, and sonicated for 6 min before HPLC analysis. Samples were analysed under the same chromatographic conditions used for the authentic taraxerol standard. Taraxerol peaks in the samples were identified by comparing their retention times with those of the authentic standard, which ranged from **1.970 to 2.220 min** under the chromatographic conditions used.

Taraxerol concentration in each sample was calculated from the chromatographic response area using the calibration equation and subsequently normalized to the dry weight of the extracted plant material. Results were expressed as milligrams of taraxerol per gram of dry weight (mg/g dw). All HPLC analyses were performed in triplicate.

Statistical analysis

All statistical analyses were performed using **IBM SPSS Statistics version 25** (IBM Corp., Armonk, NY, USA). Data are presented as mean ± standard deviation (SD), and statistical significance was determined at $p < 0.05$.

For the surface-sterilization experiments, the effects of Clorox™ concentration, exposure duration, and their interaction on explant survival, contamination, and mortality were evaluated using analysis of variance (ANOVA). Each treatment consisted of five replicates with 10 explants per replicate (n = 50 explants per treatment). Where significant differences were detected, treatment means were separated using Duncan's Multiple Range Test (DMRT) at $p < 0.05$.

For the multiple shoot-induction and shoot-development experiments, treatment effects on shoot regeneration frequency, number of shoots per explant, shoot length, shoot elongation, and number of leaves were evaluated using ANOVA followed by DMRT for multiple comparisons. Both experiments consisted of three replicates with six explants per replicate (n = 18 explants per treatment).

Taraxerol content among the different field-grown and *in vitro* plant tissues was analysed using one-way ANOVA followed by DMRT at $p < 0.05$. HPLC measurements were performed in triplicate, and taraxerol concentrations are expressed as mean ± SD in mg/g dry weight.

For all multiple-comparison analyses, means assigned different lowercase letters were considered significantly different at $p < 0.05$. Available *F*-statistics and exact *p*-values from the original statistical analyses are reported where applicable.

RESULTS AND DISCUSSION

Establishment of *S. crispus* Aseptic Culture

The establishment of aseptic *Strobilanthes crispus* cultures was strongly influenced by explant type, Clorox™ concentration, and exposure duration. Axillary shoot explants responded more favourably to surface sterilization than field-derived nodal explants, indicating that both explant developmental stage and sterilization intensity were important determinants of successful culture establishment.

For axillary shoot explants, treatment with 10% (v/v) Clorox™ for 5–7 min was insufficient to control contamination, resulting in a contamination rate of 89.0 ± 2.24% and a survival rate of only 6.0 ± 2.24%. Increasing the Clorox™ concentration to 20% (v/v) substantially improved aseptic establishment. The numerically highest survival rate of 78.0 ± 2.74% was obtained following treatment with 20% (v/v) Clorox™ for 9 min, with contamination and mortality rates of 14.0 ± 2.24% and 8.0 ± 2.74%, respectively (Table 1). Treatment with the same Clorox™ concentration for 12 min resulted in 73.0 ± 4.47% survival, which was not significantly different from that obtained after 9 min. Therefore, treatment with 20% (v/v) Clorox™ for 9 min provided a favourable balance between reducing microbial contamination and maintaining explant viability.

Table 1. Effect of Clorox™ concentration and exposure time in establishment of aseptic culture on axillary shoot explant after 7 days of culture.

Clorox™ concentration (% v/v)	Exposure time (min)	Survival rate (%)	Contamination rate (%)	Death rate (%)
10	5	6.0 ± 2.24^{g}	89.0 ± 2.24^{a}	5.0 ± 0.0^{g}
	7	6.0 ± 2.24^{g}	89.0 ± 2.24^{a}	5.0 ± 0.0^{g}
	9	11.0 ± 2.24^{g}	79.0 ± 4.18^{b}	10.0 ± 3.54^{g}
	12	11.0 ± 2.24^{g}	79.0 ± 6.52^{b}	10.0 ± 5.00^{g}
20	5	36.0 ± 6.52^{d}	53.0 ± 7.58^{c}	11.0 ± 5.48^{g}
	7	62.0 ± 5.71^{b}	33.0 ± 5.71^{d}	5.0 ± 0.0^{g}
	9	78.0 ± 2.74^{a}	14.0 ± 2.24^{ef}	8.0 ± 2.74^{g}
	12	73.0 ± 4.47^{a}	17.0 ± 4.47^{e}	10.0 ± 0.0^{g}
30	5	50.0 ± 3.53^{c}	13.0 ± 4.47^{efg}	37.0 ± 6.71^{f}
	7	39.0 ± 6.52^{d}	9.0 ± 4.18^{fgh}	52.0 ± 2.74^{e}
	9	23.0 ± 9.75^{ef}	8.0 ± 2.74^{gh}	69.0 ± 10.84^{d}
	12	23.0 ± 8.37^{ef}	5.0 ± 0.00^{hi}	72.0 ± 8.37^{d}
40	5	28.0 ± 8.37^{e}	0^{i}	72.0 ± 8.37^{d}
	7	20.0 ± 7.07^{f}	0^{i}	80.0 ± 7.07^{c}
	9	12.0 ± 4.47^{g}	0^{i}	88.0 ± 4.47^{b}
	12	5.0 ± 0.00^{g}	0^{i}	95.0 ± 0.00^{a}

Values represent mean ± standard deviation from five replicates of 10 explants each (n = 50 explants per treatment). Means sharing the same letter are not significantly different at $p < 0.05$ according to Duncan's Multiple Range Test.

Increasing the Clorox™ concentration beyond 20% (v/v) progressively reduced contamination but was accompanied by a marked increase in explant mortality. At the most severe treatment, 40% (v/v) Clorox™ for 12 min, no contamination was detected, but 95.0 ± 0.00% of the explants died. This inverse relationship between contamination and survival demonstrates that increasing sterilant intensity beyond an optimal level can become phytotoxic. Thus, complete elimination of microbial contamination does not necessarily represent successful aseptic establishment when explant viability is severely compromised. Clorox™ concentration and exposure duration significantly influenced the responses of axillary shoot explants, with their combined effects further demonstrating the importance of optimizing both factors simultaneously.

The greater establishment success of axillary shoots may be associated with their relatively young developmental stage. Young axillary shoots and buds are exposed to the external environment for a shorter period than mature stem tissues and may therefore carry a lower microbial load. Appropriate optimization of sodium hypochlorite concentration and exposure duration is essential because the sterilant must sufficiently suppress surface microorganisms while minimizing tissue injury, browning, and loss of viability (Eliwa et al., 2024; Nour Athiroh et al., 2024). The present findings therefore indicate that moderate sterilant concentration and exposure duration were more suitable for axillary shoots than either weak treatments, which failed to adequately control contamination, or severe treatments, which caused excessive tissue damage.

In contrast, none of the tested Clorox™ treatments successfully established aseptic cultures from field-derived nodal explants. Survival remained at 0% across all treatment combinations. At the lowest treatment intensity, 10% (v/v) Clorox™ for 10 min resulted in 100% contamination without explant mortality. Increasing the concentration and exposure duration progressively reduced contamination but increased mortality, culminating in treatment with 30% (v/v) Clorox™ for 30 min, which eliminated detectable contamination but resulted in 100% explant mortality (Table 2). These findings indicate that increasing sterilization intensity alone was insufficient to establish viable aseptic cultures from mature field-derived nodal explants.

Table 2. Effect of Clorox™ concentration and exposure time in establishment of aseptic culture on nodal explants after 7 days of culture

Clorox™ concentration (% v/v)	Exposure time (min)	Survival rate (%)	Contamination rate (%)	Death rate (%)
10	10	0	100^{a}	0
	20	0	86.0 ± 5.48^{b}	14.0 ± 5.48^{e}
	30	0	86.0 ± 5.48^{b}	14.0 ± 5.48^{e}
20	10	0	76.0 ± 8.95^{b}	24.0 ± 8.95^{e}
	20	0	60.0 ± 10.0^{c}	42.0 ± 8.37^{d}
	30	0	46.0 ± 11.4^{d}	54.0 ± 11.4^{c}
30	10	0	36.0 ± 11.4^{d}	64.0 ± 11.4^{c}
	20	0	16.0 ± 8.95^{e}	84.0 ± 8.95^{b}
	30	0	0	100^{a}

Values represent mean ± standard deviation from five replicates of 10 explants each (n = 50 explants per treatment). Means sharing the same letter are not significantly different at $p < 0.05$ according to Duncan's Multiple Range Test.

The poor response of nodal explants may be associated with their morphology, physiological age, and exposure history. The axillary bud of a nodal segment is situated within a V-shaped region formed between the stem and petiole, which may trap environmental debris and microorganisms while limiting effective penetration of the sterilizing solution. Older nodal tissues may also harbour more deeply established contaminants that are difficult to eliminate through surface sterilization alone (Anđelić et al., 2024; Gu et al., 2022). Differences in donor-plant growing conditions may further influence the microbial load carried by explants, particularly when material is collected from open-field environments rather than controlled growing conditions (Martini et al., 2022).

Overall, young axillary shoots were more suitable than mature field-derived nodal segments for establishing aseptic *S. crispus* cultures. Among the treatments evaluated, 20% (v/v) Clorox™ for 9 min provided the most favourable practical balance between contamination control and explant survival. Once aseptic cultures were successfully established, nodal tissues derived from these *in vitro* plantlets could subsequently be used as explant material for the micropropagation experiments.

Effect of Single Cytokinin on Leaf and Nodal Explant of *S. crispus*

Successfully established aseptic cultures of *S. crispus* were maintained on basal MS medium and subcultured for two consecutive cycles before nodal and leaf explants were used for multiple shoot induction. The two explant types exhibited markedly different morphogenic responses to single-cytokinin treatments, with nodal explants showing shoot-regeneration capacity whereas leaf explants predominantly underwent callus formation.

For nodal explants, the hormone-free control and all concentrations of BAP and KN maintained a 100% shoot-regeneration frequency after 30 days of culture (Table 3; Figure 1). At the lower concentrations of BAP and KN, shoot initiation occurred relatively early and the resulting shoots showed comparatively greater elongation. BAP at 0.5 and 1.0 mg/L produced mean shoot lengths of 4.92 ± 0.93 and 5.18 ± 1.34 mm, respectively, while KN at 0.5 and 1.0 mg/L produced shoot lengths of 5.13 ± 1.23 and 4.82 ± 0.84 mm, respectively. However, these treatments generally produced approximately two shoots per explant, indicating that although BAP and KN supported shoot regeneration and elongation, their ability to promote shoot multiplication when applied individually was limited.

Table 3. Effect of single cytokinin on multiple shoot induction from nodal explant of *S. crispus* after 30 days of culture.

Cytokinin concentration (mg/L)			Days required for shoot induction	Shoot regeneration frequency (%)	No. of shoot/s per explant	Length of shoots (mm)	Other morphological changes
BAP	KN	TDZ					
MSO (control)			5	100^{a}	2.00 ± 0.0^{b}	4.92 ± 0.93^{a}	None
0.5	-	-	3	100^{a}	2.00 ± 0.0^{b}	4.92 ± 0.93^{a}	None
1.0	-	-	4	100^{a}	2.00 ± 0.0^{b}	5.18 ± 1.34^{a}	None
2.0	-	-	5	100^{a}	1.93 ± 0.26^{b}	3.21 ± 0.56^{b}	Callus formation
4.0	-	-	5	100^{a}	2.07 ± 0.46^{b}	2.95 ± 0.53^{b}	Callus formation
-	0.5	-	3	100^{a}	2.00 ± 0.00^{b}	5.13 ± 1.23^{a}	None
-	1.0	-	4	100^{a}	1.80 ± 0.41^{b}	4.82 ± 0.84^{a}	None
-	2.0	-	5	100^{a}	1.60 ± 0.51^{bc}	3.13 ± 1.23^{b}	Callus formation
-	4.0	-	5	100^{a}	1.33 ± 0.49^{cd}	2.77 ± 0.57^{b}	Callus formation
-	-	0.5	4	100^{a}	3.13 ± 0.49^{a}	1.92 ± 0.66^{c}	Callus formation
-	-	1.0	4	100^{a}	2.93 ± 0.80^{a}	2.14 ± 0.79^{c}	Callus formation
-	-	2.0	5	73.3^{b} ± 16.89	1.20 ± 0.41^{d}	1.55 ± 0.50^{cd}	Callus formation
-	-	4.0	5	59.8^{c} ± 11.86	1.00 ± 0.00^{d}	1.05 ± 0.56^{d}	Callus formation

Values represent mean ± standard deviation from three replicates of six explants each (n = 18 explants per treatment). Means sharing the same letters are not significantly different ($p < 0.05$) using Duncan's Multiple Range Test.

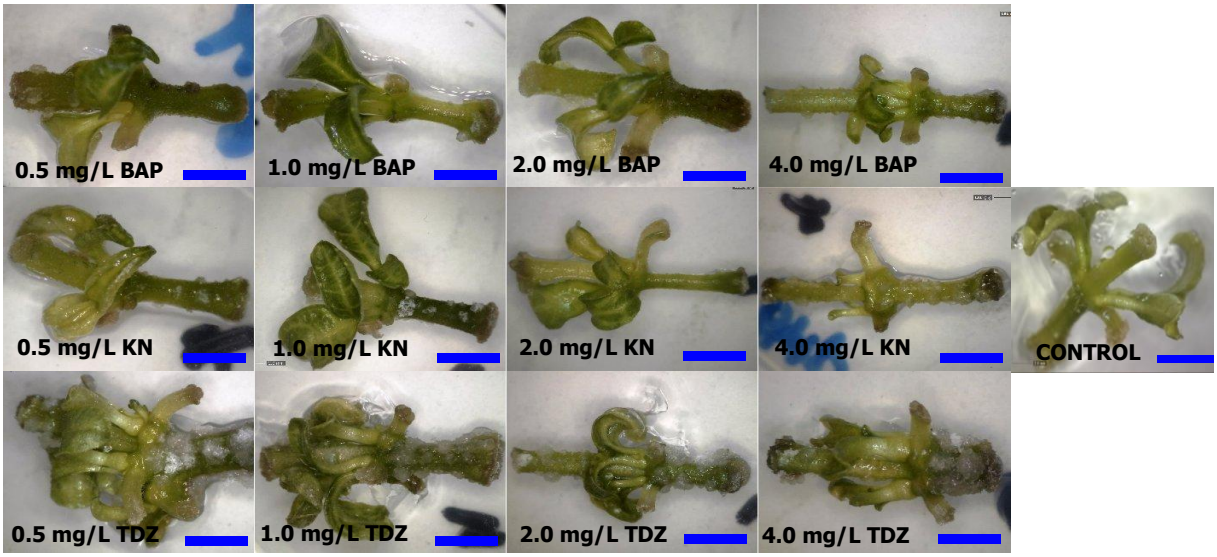

Figure 1. The effect of single cytokinin on multiple shoot induction from nodal explant of *S. crispus* after 30 days of culture in MS medium supplemented with 3% (w/v) sucrose, 0.8% (w/v) agar, pH 5.7 at temperature 25 ± 2°C with 16 h photoperiod. (Bar=5mm)

Among the three cytokinins evaluated, TDZ was more effective in promoting shoot multiplication at relatively low concentrations. TDZ at 0.5 and 1.0 mg/L produced 3.13 ± 0.49 and 2.93 ± 0.80 shoots per explant, respectively, while maintaining 100% shoot-regeneration frequency. Nevertheless, the shoots produced under these treatments were shorter than those obtained with BAP, KN, or the hormone-free control. This response illustrates an important functional difference among the cytokinins: BAP and KN were more favourable for maintaining shoot elongation, whereas low concentrations of TDZ favoured the initiation of a greater number of shoots. The strong multiplication response to TDZ is consistent with its potent cytokinin-like activity and its ability to influence endogenous cytokinin metabolism (Ali et al., 2022).

Increasing the cytokinin concentration generally reduced the quality of the morphogenic response. At 2.0 and 4.0 mg/L, BAP and KN produced shorter shoots and promoted callus formation, whereas TDZ induced callus formation at all concentrations tested. The inhibitory effect was particularly evident with TDZ at 2.0 and 4.0 mg/L, where shoot-regeneration frequency declined to 73.3 ± 16.89% and 59.8 ± 11.86%, respectively, accompanied by reductions in both shoot number and shoot length. These findings indicate that increasing cytokinin concentration does not necessarily improve multiplication efficiency. Instead, excessive cytokinin exposure can shift the developmental response away from organized shoot formation towards callogenesis and reduced shoot growth. High or prolonged exposure to potent cytokinins such as TDZ has been associated with abnormal tissue proliferation and impaired shoot development (Suminar et al., 2024).

In contrast to the nodal explants, leaf explants failed to produce shoots in response to any of the single-cytokinin treatments evaluated (Figure 2). BAP and KN at 0.5–2.0 mg/L predominantly induced compact, white callus, while increasing their concentrations to 4.0 mg/L resulted in tissue necrosis. TDZ similarly promoted callus formation across all tested concentrations without inducing shoot regeneration. The contrasting responses of the two explant types highlight the importance of explant origin in determining morphogenic competence. Nodal explants contain pre-existing axillary meristems that can respond directly to cytokinin stimulation, whereas leaf tissues lack these established meristematic structures and require more extensive cellular reprogramming before shoot organogenesis can occur (Hesami et al., 2023; Pasternak & Steinmacher, 2024).

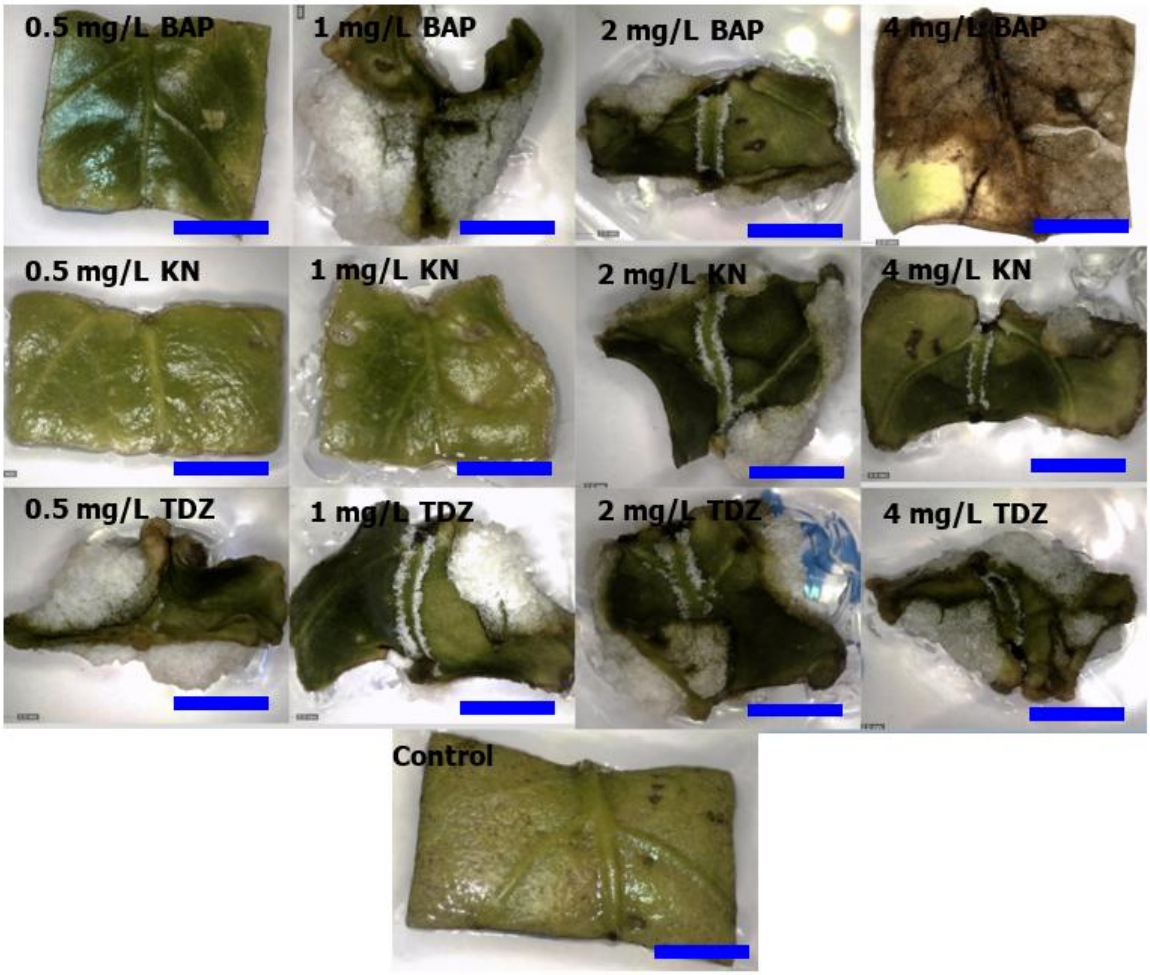

Figure 2. The effect of single cytokinin on multiple shoot induction from leaf explant of *S. crispus* after 30 days of culture in MS medium supplemented with 3% (w/v) sucrose, 0.8% (w/v) agar and grown at 25 ± 2°C with 16 h photoperiod. (Bar = 3mm)

Overall, nodal explants were clearly more suitable than leaf explants for direct shoot regeneration in *S. crispus*. However, the relatively low multiplication rate obtained with BAP or KN alone and the reduced shoot growth observed at higher TDZ concentrations indicate that no single cytokinin simultaneously optimized shoot regeneration, multiplication, and elongation. Low concentrations of TDZ improved shoot multiplication, but this benefit was accompanied by shorter shoots and an increased tendency toward callus formation. These findings provided the rationale for subsequently evaluating cytokinin combinations to enhance shoot multiplication while maintaining acceptable morphogenic development.

Effect of Cytokinin-Cytokinin combination on Leaf and Nodal explant of *S. crispus*

Following the relatively low multiplication rates obtained with individual cytokinins, combinations of cytokinins were evaluated to improve multiple shoot induction in *S. crispus*. BAP or KN at 0.5 mg/L was combined with TDZ at concentrations ranging from 0.05 to 0.4 mg/L. The combined treatments markedly improved shoot multiplication from nodal explants compared with the hormone-free control and the corresponding single-cytokinin treatments, although the response depended strongly on the concentration of TDZ.

Axillary bud break was observed within 4–5 days in most cytokinin-combination treatments, whereas nodal explants cultured with 0.5 mg/L BAP or KN alone initiated shoots as early as day 3. The hormone-free control maintained a 100% shoot-regeneration frequency but produced only 2.07 ± 0.00 shoots per

explant, demonstrating that a high regeneration frequency alone did not necessarily correspond to efficient shoot multiplication (Table 4, Figure 3). Similarly, 0.5 mg/L BAP or KN alone produced approximately two shoots per explant.

Table 4. Effect of cytokinin-cytokinin combination on multiple shoot induction from nodal explant of *S. crispus* after 30 days of culture.

Cytokinin concentration (mg/L)			Days required for shoot induction	Shoot regeneration frequency (%)	No. of shoots per explant	Length of shoots (mm)
BAP	KN	TDZ				
MSO (control)			5	100^{a}	2.07 ± 0.0^{d}	4.92 ± 0.90^{a}
0.5	-	-	3	100^{a}	2.00 ± 0.0^{d}	4.92 ± 0.88^{a}
0.5	-	0.05	4	93.2 ± 14.08^{ab}	2.07 ± 0.72^{d}	3.38 ± 1.01^{b}
0.5	-	0.1	4	86.4 ± 17.24^{ab}	5.27 ± 1.75^{c}	5.05 ± 1.52^{a}
0.5	-	0.2	5	79.8 ± 27.79^{bc}	6.60 ± 1.35^{b}	4.27 ± 1.81^{ab}
0.5	-	0.4	5	66.2 ± 21.93^{cd}	2.0 ± 0.0^{d}	2.01 ± 0.50^{c}
-	0.5	-	3	100^{a}	2.0 ± 0.0^{d}	5.11 ± 1.20^{a}
-	0.5	0.05	4	93.2 ± 14.08^{ab}	5.60 ± 1.92^{bc}	3.49 ± 1.73^{b}
-	0.5	0.1	4	86.4 ± 17.24^{ab}	9.40 ± 2.75^{a}	5.11 ± 0.62^{a}
-	0.5	0.2	5	79.8 ± 27.79^{bc}	10.27 ± 2.57^{a}	4.28 ± 1.55^{ab}
	0.5	0.4	5	59.6 ± 25.89^{d}	2.07 ± 0.26^{d}	2.15 ± 0.49^{c}

Values represent mean ± standard deviation from three replicates of six explants each (n = 18 explants per treatment). Means sharing the same letters are not significantly different ($p < 0.05$) using Duncan's Multiple Range Test.

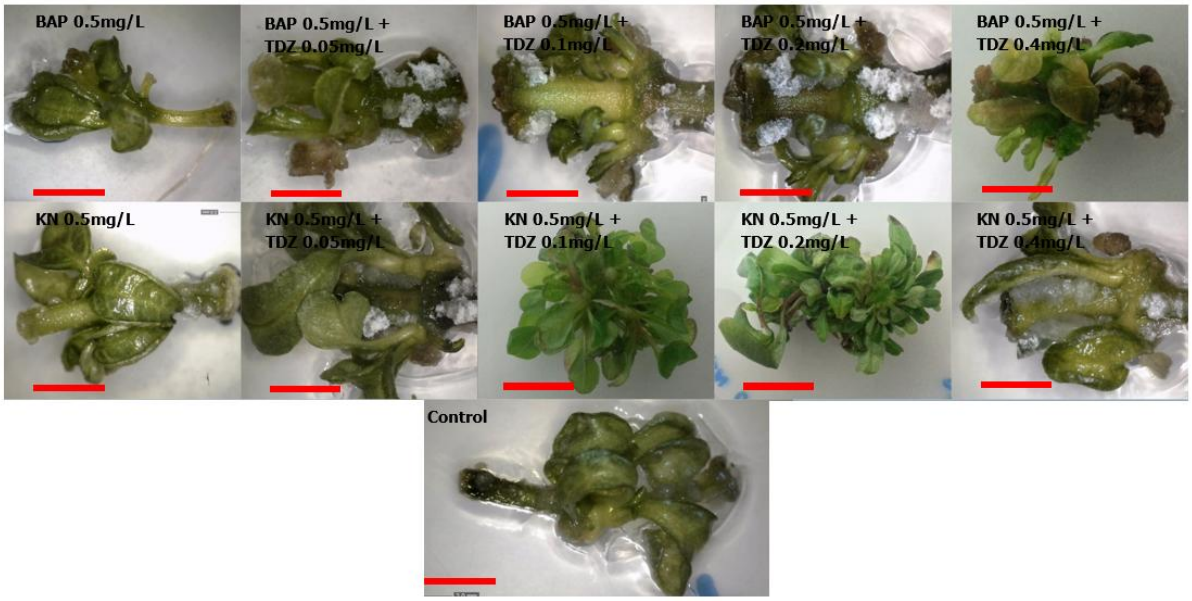

Figure 3. The effect of cytokinin-cytokinin combination on multiple shoot induction from nodal explant of *S. crispus* after 30 days of culture. (Bar = 3 mm)

The addition of low concentrations of TDZ substantially enhanced shoot multiplication, particularly when combined with KN. Treatment with 0.5 mg/L KN + 0.1 mg/L TDZ produced 9.40 ± 2.75 shoots per explant, with an 86.4 ± 17.24% regeneration frequency and a mean shoot length of 5.11 ± 0.62 mm. Increasing TDZ to 0.2 mg/L in combination with 0.5 mg/L KN resulted in the numerically highest shoot number, 10.27 ± 2.57 shoots per explant, although the regeneration frequency decreased to 79.8 ± 27.79% and the mean shoot length declined to 4.28 ± 1.55 mm. The two KN-TDZ treatments shared the same statistical grouping for shoot number, indicating that their multiplication responses were not significantly different.

Considering shoot number together with regeneration frequency and shoot length, 0.5 mg/L KN + 0.1 mg/L TDZ provided the most favourable overall response among the combinations evaluated. Although the 0.2 mg/L TDZ combination produced a slightly greater numerical shoot number, the 0.1 mg/L TDZ treatment maintained a higher proportion of regenerating explants and produced longer shoots. This distinction is biologically relevant for micropropagation because an effective multiplication treatment should not maximize shoot number at the expense of regeneration capacity or subsequent shoot development.

BAP-TDZ combinations also enhanced shoot multiplication compared with BAP alone, although the response was less pronounced than with KN-TDZ. Treatment with 0.5 mg/L BAP + 0.1 mg/L TDZ produced 5.27 ± 1.75 shoots per explant with 86.4 ± 17.24% regeneration, whereas increasing TDZ to 0.2 mg/L produced 6.60 ± 1.35 shoots per explant but reduced regeneration frequency to 79.8 ± 27.79%. These results indicate that combining a relatively low concentration of TDZ with either BAP or KN can enhance multiplication, but KN was more effective than BAP when used in combination with TDZ under the conditions evaluated.

The beneficial effect of KN combined with low concentrations of TDZ may reflect complementary cytokinin activities during shoot proliferation. TDZ is a potent cytokinin-like regulator capable of promoting vigorous cell division and shoot initiation, partly through its effects on cytokinin metabolism, whereas excessive TDZ exposure can negatively affect subsequent morphogenic development (Ali et al., 2022). The present results therefore suggest that maintaining TDZ at a relatively low concentration was important for obtaining enhanced multiplication without severely compromising regeneration and shoot growth.

This concentration-dependent response became particularly evident at 0.4 mg/L TDZ. When combined with 0.5 mg/L BAP, this treatment reduced shoot regeneration to 66.2 ± 21.93%, with only 2.00 ± 0.00 shoots per explant and a mean shoot length of 2.01 ± 0.50 mm. Similarly, 0.5 mg/L KN + 0.4 mg/L TDZ resulted in 59.6 ± 25.89% regeneration, 2.07 ± 0.26 shoots per explant, and a shoot length of 2.15 ± 0.49 mm. Thus, increasing TDZ concentration beyond the optimal range effectively reversed the multiplication advantage observed at lower concentrations. This finding reinforces the importance of optimizing the concentration of a potent cytokinin such as TDZ rather than assuming that increasing its concentration will further enhance shoot production.

In contrast to nodal explants, leaf explants failed to regenerate shoots under all cytokinin-cytokinin combinations tested and instead produced callus (Figure 4). Higher TDZ exposure, particularly 0.4 mg/L TDZ combined with 0.5 mg/L BAP, also caused pronounced browning of the leaf explants. This response was consistent with the single-cytokinin experiment and further demonstrates that increasing cytokinin complexity or concentration was insufficient to confer shoot-regeneration competence on *S. crispus* leaf tissues.

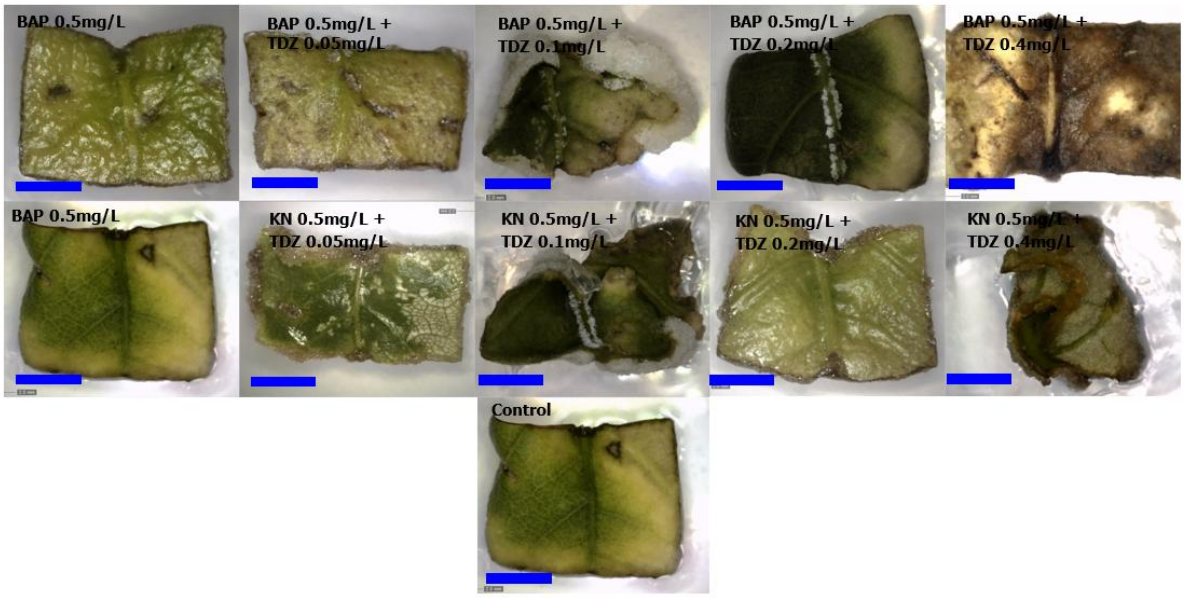

Figure 4. The effect of cytokinin-cytokinin combination on multiple shoot induction from leaf explant of *S. crispus* after 30 days of culture. (Bar = 3 mm)

The contrasting responses of nodal and leaf explants reinforce the importance of explant-specific morphogenic competence. Nodal segments contain pre-existing axillary meristems that can respond directly to cytokinin stimulation, whereas leaf tissues lack established meristematic regions and require more extensive cellular reprogramming before de novo shoot organogenesis can occur. Under the conditions evaluated in this study, cytokinin-only combinations therefore favoured callus formation rather than shoot regeneration from leaf explants.

Overall, combining KN with a low concentration of TDZ substantially improved the multiplication efficiency of *S. crispus* nodal explants compared with single-cytokinin treatments. Among the treatments evaluated, 0.5 mg/L KN + 0.1 mg/L TDZ offered the most favourable balance between shoot multiplication, regeneration frequency, and shoot length, while increasing TDZ to 0.4 mg/L markedly inhibited morphogenic performance. These findings identified nodal explants and the KN-TDZ combination as suitable components of the subsequent *S. crispus* micropropagation protocol.

Effect of Cytokinin on Shoot Development of *S. crispus*

Following multiple shoot induction, nodal explants bearing more than two shoots with an initial shoot length of approximately 0.7 ± 0.5 cm were transferred to fresh MS medium containing BAP or KN at 0.5–4.0 mg/L, together with a hormone-free control, to evaluate subsequent shoot development. After 45 days of culture, shoot development differed markedly among the treatments, demonstrating that the hormonal requirements for shoot elongation were distinct from those required during the preceding multiplication stage.

The hormone-free MS medium produced the most favourable overall shoot-development response. All shoots elongated in the control treatment, which also produced the highest mean number of new shoots (6.60 ± 1.90) and the greatest mean shoot length (6.0 ± 1.15 cm). The control additionally produced 4.6 ± 1.35 leaves per shoot (Table 5; Figure 5). These findings indicate that continued supplementation with exogenous cytokinin was not required once sufficient shoot multiplication had been achieved. Instead, transfer to hormone-free medium favoured further shoot growth and elongation.

Table 5. Effect of cytokinin on multiple shoot development from nodal explant containing shoots more than 2 and shoot length of 0.7 ± 0.5 cm after 30 days of culture.

PGR concentration (mg/L)		Percentage of shoots elongated (%)	Average number of new shoots ± SD	Average shoot length ± SD (cm)	Average number of leaves ± SD	Callus formation
BAP	KN					
Control		100[a]	6.60 ± 1.90[a]	6.0 ± 1.15[a]	4.6 ± 1.35[ab]	No
0.5	-	0[d]	0	0	0	Yes
1	-	0[d]	0	0	0	Yes
2	-	0[d]	0	0	0	Yes
4	-	0[d]	0	0	0	No
-	0.5	97.0 ± 6.75[ab]	1.8 ± 0.79[c]	2.98 ± 0.66[c]	5.3 ± 0.95[a]	No
-	1	97.0 ± 9.49[ab]	3.2 ± 0.79[b]	4.0 ± 0.68[b]	4.1 ± 1.37[bc]	No
-	2	94.0 ± 10.75[bc]	2.1 ± 0.74[c]	1.82 ± 0.48[d]	4.0 ± 1.63[bc]	No
-	4	91.0 ± .76[c]	2.1 ± 0.99[c]	1.22 ± 0.52[e]	3.4 ± 1.43[c]	No

Values represent mean ± standard deviation from three replicates of six explants each (n = 18 explants per treatment). Means sharing the same letters are not significantly different ($p<0.05$) using Duncan's Multiple Range Test.

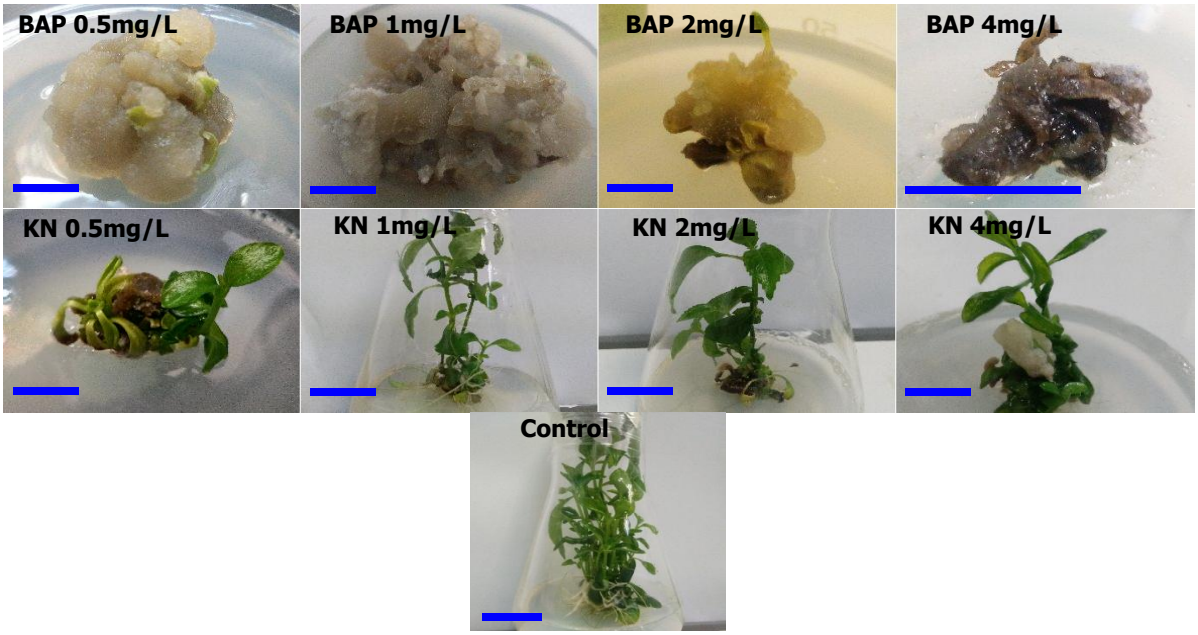

Figure 5. The effect of cytokinin on multiple shoot elongation from nodal explant containing shoots more than 2 and shoot length of 0.7 ± 0.5 cm after 30 days of culture.

The strong response in the hormone-free medium also demonstrates an important practical distinction between the multiplication and development phases of the micropropagation protocol. Cytokinin supplementation was beneficial during the earlier shoot-induction stage, particularly for increasing shoot number, but its continued presence during the development phase did not improve shoot elongation. Removal of exogenous cytokinin may therefore allow shoots to proceed from active proliferation towards elongation and normal vegetative development. This is consistent with reports that prolonged exposure to relatively high cytokinin activity can suppress internode elongation, whereas transfer to cytokinin-free medium can promote subsequent shoot growth (Faisal et al., 2023).

KN supported shoot elongation at all concentrations tested, but its overall performance remained below that of the hormone-free control. Shoot-elongation frequency declined moderately from 97.0 ±

6.75% at 0.5 mg/L KN to 91.0% at 4.0 mg/L. The greatest mean shoot length among the KN treatments was obtained at 1.0 mg/L (4.0 ± 0.68 cm), whereas increasing KN to 2.0 and 4.0 mg/L reduced shoot length to 1.82 ± 0.48 and 1.22 ± 0.52 cm, respectively. The number of leaves also declined from 5.3 ± 0.95 leaves per shoot at 0.5 mg/L KN to 3.4 ± 1.43 at 4.0 mg/L. Thus, although KN maintained a relatively high proportion of elongated shoots, higher concentrations were increasingly unfavourable for shoot length and leaf development.

The response of the number of newly formed shoots to KN was less clearly concentration-dependent. KN at 1.0 mg/L produced 3.2 ± 0.79 new shoots per explant, compared with 1.8 ± 0.79 at 0.5 mg/L and 2.1 shoots per explant at both 2.0 and 4.0 mg/L. Therefore, the principal inhibitory effect of increasing KN concentration was more evident in shoot elongation and leaf development than in the number of newly formed shoots. This distinction is important because shoot-development quality, rather than continued multiplication alone, is required to obtain plantlets suitable for subsequent culture and analysis.

In contrast, BAP was unsuitable for shoot development under the conditions evaluated. None of the BAP concentrations tested promoted shoot elongation. At 0.5–2.0 mg/L BAP, the explants instead produced callus, whereas the highest concentration, 4.0 mg/L BAP, did not promote elongation and was associated with tissue browning. The contrasting responses to BAP and KN indicate that cytokinins with different physiological activities cannot necessarily be used interchangeably between the multiplication and shoot-development phases of *S. crispus* culture.

The callus formation observed following BAP treatment may have been influenced by residual hormonal effects from the preceding multiplication phase, during which the shoots had been exposed to cytokinin combinations containing TDZ. The original study similarly proposed a possible TDZ carry-over effect during subsequent subculture. However, because residual TDZ concentrations within the tissues were not measured and a specific carry-over experiment was not conducted, this mechanism should be regarded as a plausible explanation rather than a demonstrated causal effect. TDZ is known to persist in plant tissues and prolonged exposure can influence subsequent morphogenic responses, including callogenesis and impaired shoot elongation (Ali et al., 2022; Faisal et al., 2023).

Overall, the shoot-development experiment demonstrated that the hormonal conditions favourable for shoot multiplication were not necessarily favourable for subsequent shoot elongation. Although KN permitted further shoot development, particularly at lower concentrations, the hormone-free MS medium produced the best overall combination of elongation frequency, shoot length, and continued shoot growth. Consequently, transfer to hormone-free MS medium after the multiplication phase was the most suitable treatment among those evaluated for developing *S. crispus* shoots before subsequent culture or analysis.

Taraxerol Content Assessment from Field Grown Plant and *In Vitro* Culture

Following establishment of the *S. crispus* micropropagation protocol, taraxerol accumulation was assessed in leaves, nodes, stems, axillary shoots, and roots obtained from field-grown plants and *in vitro* plantlets. Taraxerol content varied considerably among tissues and between plant sources, indicating a strong tissue-dependent pattern of accumulation (Table 6). The highest taraxerol concentration was detected in leaves from field-grown plants at 48.83 ± 0.18 mg/g dry weight (dw), followed closely by leaves from *in vitro* plantlets at 45.17 ± 1.25 mg/g dw. Field-grown axillary shoots contained the next highest concentration at 26.39 ± 1.14 mg/g dw.

Table 6. Taraxerol content assessment between field grown plant and *in vitro* culture

Tissue	Field-grown (mg/g dw)	*In vitro* plantlet (mg/g dw)
Leaf	48.83 ± 0.18^{a}	45.17 ± 1.25^{b}
Nodes	15.18 ± 0.46^{e}	2.19 ± 0.41^{g}
Stems	4.73 ± 0.09^{f}	2.19 ± 0.41^{g}
Axillary shoots	26.39 ± 1.14^{c}	22.43 ± 0.78^{d}
Roots	2.52 ± 0.26^{g}	5.53 ± 0.13^{f}

Values represent mean ± standard deviation of triplicate HPLC measurements. Means sharing the same lowercase letter are not significantly different according to Duncan's Multiple Range Test at the 5% significance level.

Although the field-grown and *in vitro* leaves were statistically separated in the multiple-comparison analysis, the magnitude of the difference was relatively small. The difference between the two leaf sources was only 3.66 mg/g dw, corresponding to approximately 7.5% lower taraxerol content in the *in vitro* leaves. Thus, *in vitro* leaves retained approximately 92.5% of the concentration measured in field-grown leaves. This is biologically important because it demonstrates that leaf tissues developed under controlled *in vitro* conditions retained substantial capacity for taraxerol accumulation rather than showing the pronounced reduction observed in some of the other vegetative tissues.

The relatively high taraxerol accumulation in leaves from both sources also indicates that the distribution of taraxerol within *S. crispus* is strongly tissue dependent. Field-grown nodes, stems, axillary shoots, and roots contained 15.18 ± 0.46, 4.73 ± 0.09, 26.39 ± 1.14, and 2.52 ± 0.26 mg/g dw, respectively, whereas the corresponding *in vitro* tissues contained 2.19 ± 0.41, 2.19 ± 0.41, 22.43 ± 0.78, and 5.53 ± 0.13 mg/g dw. The comparatively high concentrations detected in leaves and axillary shoots suggest that taraxerol accumulation is associated more strongly with particular differentiated tissues than with plant biomass in general.

Such tissue-dependent accumulation of plant secondary metabolites has been associated with differences in cellular differentiation and the presence of specialized biosynthetic or secretory structures. Previous studies have shown that secondary metabolite accumulation can be restricted to particular tissues or specialized structures, while terpenoid biosynthesis is also associated with plastid-containing cells and related precursor pathways (Mallón et al., 2016; McCaskill et al., 1992; Kongduang et al., 2012; Swain et al., 2012). The original study similarly proposed that the greater abundance of photosynthetically active tissues may contribute to the higher taraxerol concentrations observed in leaves. Therefore, the relatively high taraxerol content retained by *in vitro* leaves may reflect preservation of the differentiated cellular structures and metabolic capacity required for triterpenoid biosynthesis.

Differences between field-grown and *in vitro* tissues were nevertheless tissue specific. For example, taraxerol concentrations were substantially lower in *in vitro* nodes and stems than in their field-grown counterparts, whereas *in vitro* roots contained more taraxerol than field-grown roots. Axillary shoots showed a smaller difference, with 26.39 ± 1.14 mg/g dw in field-grown shoots compared with 22.43 ± 0.78 mg/g dw in *in vitro* shoots. These contrasting responses indicate that the effect of the culture environment on taraxerol accumulation cannot be generalized across all plant organs. Instead, the capacity to maintain taraxerol accumulation under *in vitro* conditions appears to depend on tissue type.

The field versus *in vitro* comparison should, however, be interpreted cautiously because the tissues were not matched for developmental age. The field-grown plants were approximately one year old, except for the approximately four-week-old axillary shoots, whereas all tissues collected from the *in vitro* plantlets were approximately 45 days old. Consequently, differences between the two sources may reflect not only environmental conditions but also developmental stage and tissue maturity. The available records also do not establish a standardized leaf position between the two sources. Thus, the

present comparison demonstrates the capacity of *in vitro* tissues to accumulate taraxerol but should not be interpreted as a strictly age-matched comparison between field and *in vitro* plants.

From a practical perspective, the small difference between field-grown and *in vitro* leaves is more informative than statistical significance alone. The retention of approximately 92.5% of the field-leaf taraxerol concentration suggests that micropropagated *S. crispus* can maintain substantial taraxerol biosynthetic capacity under controlled culture conditions. This supports the use of *in vitro* plantlets as a potential controlled source of taraxerol-containing biomass and provides a basis for subsequent optimization of culture conditions. However, the present experiment evaluated taraxerol concentration rather than total taraxerol productivity per culture cycle; therefore, commercial or large-scale production efficiency cannot be concluded without additional assessment of biomass yield, multiplication rate, cultivation duration, extraction efficiency, and scale-up performance.

Overall, leaves represented the most favourable tissue for taraxerol accumulation in both field-grown and *in vitro S. crispus*. Importantly, the relatively small difference between field-grown and *in vitro* leaves indicates that the micropropagation process preserved a substantial proportion of the taraxerol-accumulating capacity of leaf tissues. These results establish *in vitro* leaves as a promising material for further studies aimed at optimizing taraxerol production under controlled culture conditions, while additional analytical validation and developmentally matched comparisons are required before conclusions regarding production equivalence or large-scale yield can be made.

CONCLUSION

This study established an *in vitro* culture and micropropagation approach for *Strobilanthes crispus* and demonstrated that micropropagated plantlets retained the capacity to accumulate taraxerol. Young axillary shoots were more suitable than field-derived nodal explants for establishing aseptic cultures, with 20% (v/v) Clorox™ for 9 min providing the most favourable balance between contamination control and explant survival. During shoot multiplication, nodal explants responded better than leaf explants, and the combination of 0.5 mg/L KN with 0.1 mg/L TDZ provided a favourable balance between shoot multiplication, regeneration frequency, and shoot growth. Subsequent transfer to hormone-free MS medium was most suitable for shoot development, producing 100% shoot elongation, 6.60 ± 1.90 new shoots per explant, and a mean shoot length of 6.0 ± 1.15 cm.

Taraxerol accumulation was strongly tissue dependent, with leaves containing the highest concentrations in both field-grown and *in vitro* plants. Field-grown leaves contained 48.83 ± 0.18 mg/g dry weight, whereas *in vitro* leaves contained 45.17 ± 1.25 mg/g dry weight. Although these values were statistically differentiated, the absolute difference was only 3.66 mg/g dry weight, with *in vitro* leaves retaining approximately 92.5% of the taraxerol concentration measured in field-grown leaves. This relatively small difference indicates that the micropropagation process preserved substantial taraxerol-accumulating capacity in leaf tissues and supports the use of *in vitro S. crispus* plantlets as a promising controlled source of taraxerol-containing biomass.

Nevertheless, the comparison between field-grown and *in vitro* tissues was limited by differences in developmental age, and the original HPLC analysis did not include complete analytical validation parameters such as recovery, LOD, LOQ, and intra- and inter-day precision. The absolute taraxerol concentrations should therefore be interpreted cautiously. Future studies should employ a fully validated analytical method together with developmentally matched plant material and evaluate total taraxerol yield per culture cycle, including the effects of targeted elicitation, to determine whether the established *in vitro* system can be further developed for efficient controlled production.

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