

MICROPROPAGATION OF THE CANNABIS ‘MANGO HYBRID’ CULTIVAR VIA TISSUE CULTURE TECHNIQUES

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Abstract

Cannabis is a high-value crop with potential for commercial cultivation. In this study, micropropagation was explored as a technique for the mass production of healthy, disease-free plantlets. Seed germination efficiency of the Mango Hybrid cannabis variety was assessed using both peeled and unpeeled seeds on MS medium. Results showed that peeling significantly enhanced germination, reaching 98%, compared to 66% in unpeeled seeds. The impact of various plant growth regulators (PGRs) on shoot and callus induction from nodal explants was also investigated. Among the treatments, TDZ at 3.0 mg L⁻¹ led to the most prolific shoot formation, averaging 3.7 ± 1.3 shoots per explant, which was notably higher than the outputs from BA (2.5 ± 0.5 shoots), 2iP (2.1 ± 0.5 shoots), and the control (2.1 ± 0.7 shoots). TDZ also promoted larger callus formation compared to other PGRs. For root development, the optimal response was observed on half-strength MS medium supplemented with 1.0 mg L⁻¹ NAA. Following successful root induction, the regenerated plantlets were acclimatized, and after one week, they exhibited stable growth under greenhouse conditions. This protocol demonstrates potential for scalable plant production and provides a foundation for future conservation and propagation strategies in cannabis.

Keywords: Cannabis; mutation; Shoot induction; Callus; Root induction

Introduction

Cannabis (*Cannabis sativa* L.), a dioecious species in the Cannabaceae family, is widely valued for its bioactive compounds including tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA), which have applications in medicine, food, and cosmetics (ElSohly and Slade, 2005; Tanney *et al.*, 2021; Duangdamrong *et al.*, 2022). While female flowers are particularly rich in cannabinoids and terpenes,

large-scale and consistent production remains challenging due to heterozygosity, genotype-dependent traits, and the risk of disease transmission through conventional propagation methods (McPartland, 2018; Holmes *et al.*, 2021).

Micropropagation offers an attractive alternative for producing uniform, disease-free plantlets. However, in cannabis, *in vitro* culture responses are notoriously genotype-dependent, and

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successful callus-mediated regeneration has been inconsistent across varieties (Lata *et al.*, 2016; Adhikary *et al.*, 2021; Monthony *et al.*, 2021). These limitations hinder the development of robust, reproducible protocols suitable for large-scale applications. Cytokinins such as thidiazuron (TDZ) and 6-benzylaminopurine (BA) are key regulators of shoot induction, yet their effectiveness varies significantly between genotypes and explant types (Chandra *et al.*, 2020; Monthony *et al.*, 2021). Similarly, auxins such as naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA) play critical roles in root development, but optimal concentrations for efficient acclimatization remain unclear (Chaohua *et al.*, 2016; Monthony *et al.*, 2021).

To address these challenges, this study focused on the 'Mango Hybrid' variety, which contains approximately 8% THC and negligible CBD (Hazekamp *et al.*, 2016; Chandra *et al.*, 2020). This cannabinoid profile underpins its selection as a micropropagation model in this study, as varieties rich in THC and associated terpenes are widely recognized for medicinal applications, particularly in pain management and neurological disorders (Andre *et al.*, 2016; Livingston *et al.*, 2020). Specifically, we tested three key hypotheses: (1) seed coat removal significantly enhances germination efficiency, (2) different cytokinins (TDZ, BA, and 2iP) have distinct effects on nodal shoot proliferation, and (3) auxin supplementation in half-strength MS medium improves rooting and acclimatization. The quantitative objectives were to optimize germination, evaluate comparative cytokinin responses, and establish an effective rooting protocol. Collectively, these findings aim to contribute to more reproducible and scalable cannabis micropropagation systems.

Materials and Methods

Plant Materials

The perfect cannabis seeds of the 'Mango Hybrid' variety that were produced in the greenhouse at the Thailand Institute of Nuclear Technology (TINT), a public organization located in Nakhon Nayok, Thailand. These seeds had been stored at room temperature, 25°C, for one month before being used.

Sterilization and culture conditions

The cannabis seeds, characterized by their robustness and distinctive brown color with patterns on the outer shells, underwent a careful removal of the outer covering to reveal a greenish layer enveloping the white embryo. Special care was

taken to avoid damaging this layer, as detachment could negatively affect seed germination and growth. Control seeds, on the other hand, did not require the removal of their outer covering (Figure 1a). Both peeled seeds and unpeeled control seeds underwent surface sterilization, beginning with immersion in 70% (v/v) ethanol for 1.5 minutes, followed by immersion in 15% (v/v) Clorox solution containing 6.6% sodium hypochlorite (NaOCl) for 15 minutes, followed by three rinses with autoclaved deionized water. The seeds were then placed on paper (Figure 1b) and transferred to glass bottles containing a germination medium composed of Murashige and Skoog (MS), supplemented with 30 g L⁻¹ sucrose and solidified with 2.5 g L⁻¹ gellan gum, adjusted to a pH of 5.8. After germination, both seedlings and explants were maintained under controlled environmental conditions at 25 ± 2°C with a photoperiod of 16 hours light and 8 hours darkness. (Figure 1c). Shoot formation was monitored by counting the number of shoots per explant at 10 and 30 days after culture initiation. (Figure 1d).

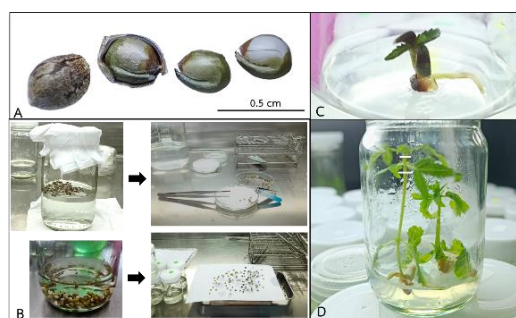


Figure 1. Seed preparation and in vitro germination of Cannabis 'Mango Hybrid' (A): Intact and peeled seeds (B): Surface-sterilized seeds prior to culture (C) Germination and seedling development at 10 days on MS medium (D) Seedling growth at 30 days on MS medium under 25 ± 2°C and a 16 h photoperiod

Shoot and callus induction

Nodal segments measuring approximately 1 cm and containing axillary buds were excised from seedlings and employed as explants to initiate shoot culture. Two nodal segments were placed together in the vessel media. The MS cultured medium contains 30 g/L sucrose, solidified with 2.5 g L⁻¹ gellan gum, and adjusted to a final pH of 5.8. Shoot and callus induction were assessed using various concentrations of plant growth regulators (PGRs), including 6-benzyladenine (BA), thidiazuron (TDZ), and 6-(γ,γ-dimethylallylamino)

purine (2iP), applied at 0.5, 1.0, and 3.0 mg.L⁻¹. A control group without PGR supplementation was also included. The experiment was performed in triplicate, with 10 explants per treatment in each replicate, and the number of shoots and callus generated per explant was recorded following 30 days of *in vitro* cultivation.

Root induction

The experiment utilized nodal segments from seedlings; explants with axillary buds approximately 1 cm in length were selected for the initiation of root induction experiments. Three nodal segments were grouped in the vessel media. The rooting medium was composed of half-strength Murashige and Skoog ($\frac{1}{2}$ MS) formulation, supplemented with 30 g L⁻¹ sucrose and 2.5 g L⁻¹ gellan gum, with the pH adjusted to 5.8. Root induction was facilitated using naphthaleneacetic acid (NAA) hormone at concentrations of 1.0 and 5.0 mg L⁻¹, alongside a control group cultivated on PGRs-free MS medium. The experiment was replicated four times (n = 12 per trial), and the number of roots induced by each explant was recorded after 30 days of culture.

Acclimatization

Following root development, the 10 plantlets were washed to remove residual gel and transferred to pots containing a cocopeat:peat moss mixture (1:1). To maintain a favorable atmosphere during the initial acclimatization phase, the pots were covered with transparent plastic vessels and kept under continuous illumination at 25 - 28°C and ~60% relative humidity. Plantlets were monitored for 7 days post-transplantation.

Experimental design and statistical analysis

A completely randomized design (CRD) was employed for all experiments. Statistical analysis was performed using ANOVA, followed by LSD (Least Significant Difference) tests in R software.

Results and Discussion

The first crucial step in tissue culture is the preparation of the sample for sterilization. The propagation of cannabis is usually through seed or by vegetative propagation. In this experiment, we used the seeds. The results of seed germination for the Mango Hybrid of cannabis in tissue culture, involving both peeled seeds and unpeeled control seeds, indicated that peeled seeds achieved a germination rate of 98% within 10 days of culture, which was higher than that of the unpeeled seeds (66%) (Data not shown). However, after 30 days in

culture, the successful growth of plantlets from peeled seeds decreased to 90%, and some abnormal growth was observed at 8%. This abnormal growth could potentially be attributed to injuries sustained during the husk removal process. The improved germination of peeled seeds can be attributed to the facilitation of water imbibition and gas exchange, as the seed coat often acts as a physical barrier to both (Bewley *et al.*, 2013). In cannabis, the thick pericarp and seed coat have been reported to restrict germination and contribute to dormancy-like behavior (De Meijer and Keizer, 1996). Removal of the coat also reduces the risk of microbial contamination harbored on the seed surface, thereby improving seedling establishment under *in vitro* conditions (Chaohua *et al.*, 2016; Monthony *et al.*, 2021). The growth characteristics of cannabis seedlings cultured on MS medium (Figure 1c-d) demonstrated robust growth, including the development of cotyledons and the emergence of true leaves, typically with two leaves and visible root hairs at 10 days (Figure 1C), and the emergence of 2-3 shoot tips at 30 days (Figure 1D). This is notably faster and more efficient compared to germination methods involving unpeeled seeds, which generally required 5-15 days for seedling emergence in cannabis (Wielgus *et al.*, 2008), our germination method involving peeled seeds achieved seedling development in a shorter period (10 days) with a high germination rate. Hence, seed coat removal proved to be a more effective approach in enhancing the germination rate of cannabis seeds because it can promote the process of breaking dormancy in the endosperm, leading to a higher germination rate in *Viburnum sargentii* (Zhang *et al.*, 2019).

Micropropagation efficiency is influenced by numerous factors, broadly categorized into environmental conditions and the presence of plant growth regulators (PGRs). Among these, PGRs are key biochemical agents that regulate physiological and developmental pathways critical for *in vitro* propagation (Mir *et al.*, 2016). In this study, the effect of different types of cytokinins on shoot and callus induction from nodal segments was evaluated using MS medium supplemented with either BA, TDZ, or 2iP, in comparison with a control group cultivated on MS medium without PGRs. After 30 days of culture, the MS medium containing 3.0 mg/L TDZ exhibited the most pronounced shoot proliferation, producing an average of 3.7 ± 1.3 shoots per explant. This was significantly higher than those obtained from BA ($3.0 \text{ mg L}^{-1} = 2.5 \pm 0.5$ shoots), 2iP ($1.0 \text{ mg L}^{-1} = 2.1 \pm 0.5$ shoots), and the hormone-free MS control (2.1 ± 0.7 shoots) (Table 1 and Figure 2). Furthermore, TDZ at the same concentration also induced the formation of

visibly larger callus masses compared to the other treatments (Figure 3). Which, from the measurement of callus diameter, was found to be the largest at 0.54 ± 0.01 cm (Table 2). These findings are consistent with previous reports indicating that TDZ is among the most effective PGRs for promoting shoot proliferation in *C. sativa*. For instance, axillary nodes derived from a one-year-old drug-type cannabis mother plant cultured on MS medium with $0.5 \mu\text{M}$ TDZ yielded as many as 12.6 shoots per explant (Lata *et al.*, 2009). Cotyledons of *C. sativa* were observed to undergo callus and shoot induction by 0.4 mg L^{-1} TDZ (Chaohua *et al.*, 2016). Certain studies have indicated that concentrations of TDZ exceeding 1.1 mg L^{-1} can inhibit shoot regeneration (Lata *et al.*, 2009). However, in the present study, among all treatments, 3.0 mg/L TDZ resulted in the highest shoot induction rate. This leads to the conclusion that different cannabis varieties and specific plant parts may exhibit varied responses to PGR dosages (Adams *et al.*, 2021).

Table 1. Effect of PGRs on the number of shoots of cannabis, after culturing for 30 days

PGRs	Concentration (mg L^{-1})	No. of shoots
BA	0.5	1.9 ± 0.8^b
	1.0	1.8 ± 0.8^b
	3.0	2.5 ± 0.5^b
TDZ	0.5	2.4 ± 0.7^b
	1.0	2.3 ± 0.7^b
	3.0	3.7 ± 1.3^a
2iP	0.5	1.6 ± 0.6^b
	1.0	2.1 ± 0.5^b
	3.0	1.9 ± 0.6^b
MS (control)	-	2.1 ± 0.7^b

Table 2. Effect of PGRs on callus diameter of cannabis, after culturing for 30 days

PGRs	Concentration (mg L^{-1})	Callus Diameter (cm)
BA	0.5	0.21 ± 0.09^d
	1.0	0.25 ± 0.07^d
	3.0	0.48 ± 0.00^{ab}
TDZ	0.5	0.52 ± 0.02^a
	1.0	0.50 ± 0.07^{ab}
	3.0	0.54 ± 0.01^a
2iP	0.5	0.40 ± 0.03^{bc}
	1.0	0.38 ± 0.10^{bc}
	3.0	0.39 ± 0.03^{bc}
MS (control)	-	0.28 ± 0.01^{cd}

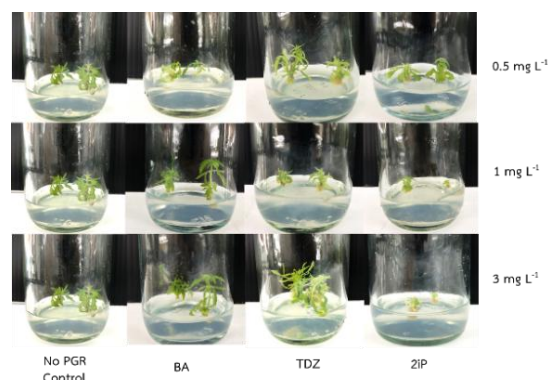


Figure 2. Effects of varying concentrations of plant growth regulators (PGRs) on shoot induction from cannabis nodal explants after 30 days of culture. Abbreviations: PGRs - Plant Growth Regulators; BA - 6-benzyladenine; TDZ - thidiazuron; 2iP - 6-(γ,γ -dimethylallylamino) purine

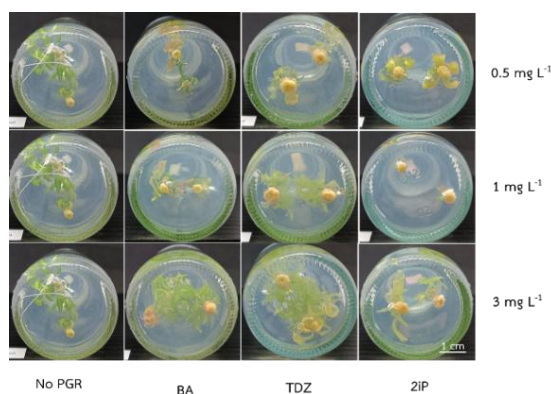


Figure 3. Callus formation responses observed in cannabis explants cultured for 30 days under different concentrations of plant growth regulators. Abbreviations: PGRs - Plant Growth Regulators; BA - 6-benzyladenine; TDZ - thidiazuron; 2iP - 6-(γ,γ -dimethylallylamino) purine

Additionally, root induction results revealed that the most successful root development occurred in explants cultured on half-strength MS medium supplemented with 1.0 mg L^{-1} NAA, showing the highest rooting percentage and number of roots, $33.33 \pm 0.00\%$ and 3.17 ± 2.25 , respectively

(Table 3). In contrast, no root formation was observed in hormone-free MS medium or when 5.0 mg L⁻¹ NAA was applied (Figure 4). Some studies present that the best root length of cannabis was obtained in various auxins (IAA, IBA, NAA) (Lata *et al.*, 2010). Hairy root cultures in cannabis by supplementing B5 medium with 4.0 mg/L NAA, highlighting the role of auxins in root morphogenesis. (Farag and Kayser, 2015). Another study reported that the highest average number and length of roots per shoot were observed when the culture media were supplemented with 4.0 µM IBA or NAA (Ioannidis *et al.*, 2020).

Table 3. Effect of PGRs on rooting percentage (%) and average number of roots per explant in cannabis, after culturing for 40 days

PGRs	Rooting percentage (%)	No. of roots
½ MS+1NAA	33.33±0.00 ^a	3.17±2.25 ^a
½ MS+5NAA	0.00±0.00 ^b	0.00±0.00 ^b
MS (control)	0.00±0.00 ^b	0.00±0.00 ^b



Figure 4. Root induction efficiency under various NAA concentrations after 30 days in culture. Treatments included (A) MS medium without NAA, (B) ½ MS supplemented with 1.0 mg L⁻¹ NAA, and (C) ½ MS supplemented with 5.0 mg L⁻¹ NAA

Acclimatization stands as a crucial final phase following root induction, marking the essential conclusion of a micropropagation protocol. The delicate plantlets, maintained under sterile conditions, undergo a critical transition from *in vitro* to *ex vitro* that requires careful attention. The cannabis plantlets undergo acclimatization in a soil mixture and are placed in a plant growth chamber.

After 7 days of this acclimatization process, our cannabis tissue plantlets demonstrate successful growth 100% (Figure 5). This outcome underscores the success of micropropagating the cannabis ‘Mango Hybrid’ variety.

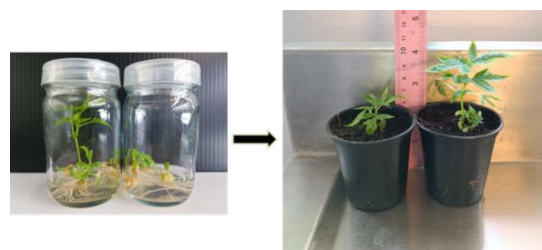


Figure 5. The survival of acclimatized plants derived from rooted plantlets

Conclusions

Cannabis ‘Mango Hybrid’ variety with high THC was selected for micropropagation. This protocol is designed for the expansive production of this cannabis variety, spanning from *in vitro* conditions to a soil substrate under *ex vitro* environments. The seedlings in tissue culture were initiated from seeds, and the removal of seed coating before the sterilization process resulted in an impressive germination rate of 98% at 10 days post-culture. During the tissue culture process, shoot and callus formation were induced using MS medium containing 3.0 mg L⁻¹ TDZ, while root formation was most effective on ½ MS medium supplemented with 1.0 mg L⁻¹ NAA. Following acclimatization, the plantlets with roots displayed growth and survivability. This tissue culture system for the ‘Mango Hybrid’ variety of cannabis holds potential not only for propagation but also for genetic conservation and preservation of selected varieties, as genetic alterations can introduce variations in cannabis.

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