

## Rapid Micro-Propagation of Disease-Free Hemp (*Cannabis sativa* L.) to Improve Industrial Production

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### Abstract

Hemp (*Cannabis sativa* L.) is widely used in industrial production due to its pharmacological properties, particularly cannabidiol (CBD). For commercial production, it is essential to have a large-scale propagation that is consistently disease-free and rapid. Thus, this study aims to establish a protocol for exponentially increasing the amount of high CBD hemp variety CA-UP1 by nodal segment via tissue culture method. Initially, with 10-minute surface sterilization with 0.1 % HgCl<sub>2</sub>, the microbial contamination rate was reduced to its lowest level (approximately 16.67 %) within 7 days. The nodal segments exhibited optimal response to MS media supplemented with 0.5 mg/L TDZ, resulting in a 100 % shoot multiplication rate. The average number of shoots per explant was  $4.7 \pm 1.57$ , and the average shoot height was  $2.30 \pm 0.42$  cm at 4 weeks. Following the induction of elongation in MS medium supplemented with charcoal, the seedlings were acclimatized and dipped in a 1,000 ppm IBA solution mixed with talcum powder. The cultivation resulted in a 100 % rooting rate, with an average of  $5.6 \pm 1.94$  roots/explant, an average root length of  $5.78 \pm 1.96$  cm, and an average explant height of  $4.32 \pm 1.52$  cm. The tissue culture protocol can produce over 190 times the quantity of hemp plants compared with the stem cutting technique. The researchers anticipate that this micropropagation protocol will be useful as a rapid and economical alternative for the industrial production of effective *C. sativa* L.

**Keywords:** Hemp, Micropropagation, Thidiazuron, Shoot multiplication, Root induction

### Introduction

*Cannabis sativa* L. also known as hemp, this genus is classified within the Cannabaceae family and has a history that extends for more than 8,000 years. Currently, it is widely utilized for its fibers, animal feed, human food, environmental applications, construction components, and bio-energy production [1]. Particularly, hemp is rich in several bioactive compounds that are secondary metabolites for medical purposes [2]. Nevertheless, the compounds most commonly cited in hemp are cannabinoids, specifically CBD (Cannabidiol), due to their prominent pharmacological characteristics and low addictive potential [3]. However, there are still obstacles in industrial hemp propagation that require immediate research and development to obtain high-

quality yields, reduced production time, and high concentrations of active compounds.

Micropropagation by plant tissue culture techniques offers a promising method to improve hemp cultivation and CBD production by generating disease-free plants and genetically consistent planting material. Moreover, this approach can especially optimize plant yields within a brief period, The plants are situated in an environment that is conducive to their growth. Typically, it is commonly used in commercial applications to produce plantlets, biological activities, genetic transformation, and secondary metabolites [4].

Specifically, the production of bioactive compounds such as CBD for medical purposes requires the use of micropropagation to generate a large quantity

of pathogen-free plants with the same genetic chemical type. Moreover, propagation serves as an important technique for conserving valuable plant germplasm [5]. Tissue culture techniques are essential for the production of commercial herbal medicines to adhere with good agricultural and collection practice (GACP), good plant authentication and identification practice (GPAIP), good manufacturing practice (GMP) [6]. Tissue culture micropropagation has been successful in various plant species, such as Hardy kiwi (*Actinidia arguta*), False Shamrock (*Oxalis triangularis*), and Linharn (*Launaea sarmentosa*) [7-9]. In addition, several studies have reported the effective micropropagation of hemp using tissue culture techniques. These studies have identified the optimal conditions determined by several parameters including medium compositions, plant growth regulators, sucrose concentrations, and type of explant for the development of micropropagation protocols. Moreover, the cultivation methods that can efficiently reduce losses caused by outbreaks of bacterial, fungal, and viral infections, including gray mold (*Botrytis cinerea*), and bacterial leaf spots was reported [10-12]. However, the challenges to developing efficient micropropagation procedures include microbe contamination and difficulties in *in vitro* rooting, as well as failures in subsequent acclimatization [13].

The objective is to develop efficient rapid micropropagation protocols via tissue culture techniques. This study aims to investigate the effect of cytokinin (TDZ) on shoot multiplication rate and the effect of auxin (IBA) on root induction and acclimatization processes to maximize the highest survival rate of hemp explants to achieve rapid clonal propagation. Furthermore, the study compares the quantity of hemp plants produced by tissue culture and stem cutting propagation protocols.

## Materials and methods

### Plant material and explant preparation

*Cannabis sativa* L. variety CA-UP1 which has high CBD selected by Rungreungkunpanit Co., Ltd. (Phayao, Thailand), and acts as the donor plant in this research. This hemp possesses the precise properties of the strain and is devoid of any diseases. The growing medium includes coconut coir, peat moss, and perlite in the ratio of 1:1:0.5 (w/w). The hemp plants were grown

in a controlled environment room following the standards of the industry. The temperature was maintained at  $25 \pm 2$  °C, 2520 flux daylight with 16/8 h light and darkness photoperiod for vegetative growth.

The explants utilized in this study consist of nodal segments with axillary buds about 1.5 cm (**Figure 2(A)**), which were trimmed from the branches of the donor plant using a scalpel. A leaf growing near the axillary bud was excised to avoid tissue damage caused by excessive sterilization. A detailed description of the surface sterilization process for the explants will be provided below.

### Study of surface sterilization time

Initially, the explants should be washed under water that has been mixed with a cleansing agent for 60 min. The procedure of disinfection is conducted within a lamina flow hood. The disinfectant used consists of 0.1 % Mercury chloride ( $\text{HgCl}_2$ ) mixed with 2 - 3 drops of Tween 20, applied for 5, 7, and 10 min. Afterward, cleanse the explants thoroughly with sterilized deionized water (autoclaved) at 121°C for 15 min, a process that should be repeated 3 times. The explants were dried on sterilized paper towels and then transferred into Murashige and Skoog medium (MS) [14] that has been adjusted to a pH value of 5.6 - 5.8 by adding 1 N NaOH or 1 N HCl, 3 % sucrose, and 0.85 % agar to the culture bottle (8 oz.). Then, place the culture bottles in a room with a 16/8 h light and darkness photoperiod, and maintain a temperature of  $25 \pm 2$  °C. Conduct 30 repetitions (one explant per culture bottle) and record the contamination percentage after 7 days. The explants without disease were used in the next experiment.

### Effect of TDZ on shoot multiplication

The MS medium was supplemented with TDZ at varying concentrations (0, 0.05, 0.10, 0.50, and 1.00 mg/L) for *in vitro* shoot multiplication. A total of 20 repetitions were performed to inoculate the sterile nodal segments on the prescribed presence of the medium, with one explant per culture bottle. Measurements were recorded after 4 weeks to determine the explants forming multiple shoots rate, average number of shoots, and average shoot length.

### Shoot elongation, root induction, and acclimatization of plantlets

The shoots obtained from the multiplication were examined to determine the optimal concentration of IBA at different concentrations for initiating root formation. Initially, the shoots were transferred into MS medium with 0.1 % activated carbon for 6 to 8 weeks to induce elongation. Then, hemp seedlings measuring around 2 cm in length and with well-established roots were selected. The hemp seedlings were dipped in solutions containing IBA at different concentrations (0, 1,000, 1,500, and 2,000 ppm) in addition to talcum powder (fine white powder of  $Mg_3Si_4O_{10}(OH)_2$ ), 1 mL IBA solution per 0.7 g of talcum powder, applying the technique described by El-Banna *et al.* [15]. After that, the seedlings were cultivated in a growing medium consisting of peat moss and coconut coir in a 1:1 (w/w) ratio, with a total of 20 repetitions. Incubated in a room controlled at a temperature of  $25 \pm 2$  °C, 16/8 h light and darkness photoperiod. After 4 weeks, the percentage of root formation, average number of roots, average root length, and average plant height were measured.

### Statistical analysis

Statistical analysis was performed on the collected data to examine variance and identify differences by evaluating the Analysis of Variance (ANOVA) using Duncan's New Multiple Range Test (DMRT) with a 95 % confidence level, using IBM SPSS Statistics version 26.

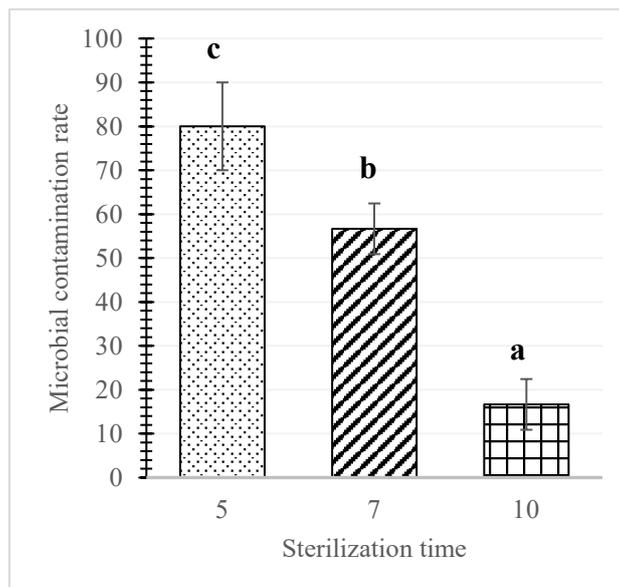
## Results and discussion

### Study of surface sterilization time

The crucial stage in plant tissue culture technique is the standardization of a protocol for explant sterilization with phytotoxic sterilants [16]. The present study investigated the duration of sterilization for

explants surfaces within  $HgCl_2$  in the range of 5 to 10 min. It was observed that with increased sterilization time, the percentage of microbial contamination decreased in **Figure 1**. Sterilization with a 0.1 %  $HgCl_2$  solution for 10 min provided the lowest contamination rate (16.67 %). Previous research has reported the application of  $HgCl_2$  for surface sterilization in several plant species, such as Yadav *et al.* [17] reported the duration suitable for the disinfection of bananas (*Musa paradisiaca* L.), resulted in a contamination rate of 32.24 % after 10 days, and a survival rate of 67.73 % after 25 days. According to Hashim *et al.* [18] consistent with the results of this research study, which indicates the contamination rate related to the duration of surface disinfection. Sterilization of the nodal segment of Sabah snake grass (*Clinacanthus nutans*) was conducted using various disinfectants. Investigation revealed that the use of 0.2 %  $HgCl_2$  for 1 h showed the lowest contamination rate in comparison to other disinfectants (3.33 %).

On the other hand, the application of higher concentrations of  $HgCl_2$  will result in tissue death, inhibited growth, and ultimately mortality due to its toxicity to plant tissues. The potent disinfectant can damage the cell membranes and walls of plants [19], resulting in the loss of water in the cell sap (nutrients), which in turn promotes excessive fungal growth in the tissues after being added to the culture medium. This explosion can transform the fungi in explants into pathogens [20], particularly after long-term exposure to the agent [21]. In addition, Gu *et al.* [22] demonstrate that the application of 0.1 %  $HgCl_2$  for 10 to 15 min leads to a survival rate ranging from 42.2 to 44.4 % for tree spinach (*Cnidioscolus aconitifolius*). The research showed that  $HgCl_2$  has a highly efficient and commonly used disinfectant.



**Figure 1** Comparison of percentage of microbial contamination with difference time of surface sterilization. Data represent mean  $\pm$  SD.

#### Effect of TDZ on shoot multiplication

The response of hemp to TDZ at varying concentrations (0 - 1.00 mg/L) is shown in **Figure 3**. Shoot proliferation was assessed over 4 weeks by measuring the average number of shoots per explant, average shoot height, and the percentage of multiple shoot formation. The explants exhibited shoot formation within 43.33 to 100 %. Furthermore, the concentration of TDZ exhibited significant statistical differences ( $p < 0.05$ ) in the average number of shoots and shoot height (**Table 1**). MS medium supplemented with 0.5 mg/L TDZ optimized hemp tissue proliferation, resulting in the formation of multiple shoots that microscopic examination using stereomicroscope (Olympus SZ61, Tokyo, Japan) at a magnification of 0.6x with the EPview program showed that axillary buds on MS medium supplemented with TDZ produced multiple shoots (**Figure 2(B)**), whereas shoots on MS medium without TDZ did not show multiple shoot development (**Figure 2(A)**). This medium produced an average of  $4.7 \pm 1.57$  shoots per explant, 100 % shoot formation, and an average shoot height of  $2.30 \pm 0.42$  cm. A previous study conducted by Mubi *et al.* [23] revealed that the nodal segments of the MX-CBD-11 and MX-CBD-707 hemp varieties exhibited a positive response to 0.44 and 0.01 g/L TDZ. The average number of shoots per explant on day 52 was  $2.67 \pm 0.30$  and  $2.43 \pm 0.20$ , respectively. Moreover, Wróbel *et al.* [24] discovered

that using the nodal portion of the Epsilon 68 hemp variety in MS media supplemented with 0.1 - 0.5 mg/L TDZ resulted in an average of around  $1.3 \pm 0.72$  shoots per explant. Furthermore, it showed that the application of TDZ for shoot multiplication was more efficient compared to alternative types of plant growth regulators.

In general, Cytokinins are central growth regulators of cell division and development in plants, including apical dominance [25]. A similar, TDZ is a substituted phenyl urea compound that is capable of mimicking both auxins and cytokinins, and it acts a critical role in regulating plant growth [26]. This results in the stimulation of shoot proliferation, organogenesis of callus, elongation of shoots, etc. Previous studies have demonstrated that the application of TDZ promoted the induction of synthesis or accumulation of endogenous cytokinin in plant tissues [27] and caused an increase in endogenous cytokinin-ribosides associated with the regulation of shoot multiplication [28]. In general, the application of a relatively low concentration of TDZ can successfully stimulate the formation of multiple shoots. Nevertheless, excessive concentrations of TDZ may inhibit the development of the apical meristem (located at the tips of shoots and roots). The optimal concentration of TDZ was determined by the specific plant species, type of explants, and the duration that tissues were exposed to it [29].

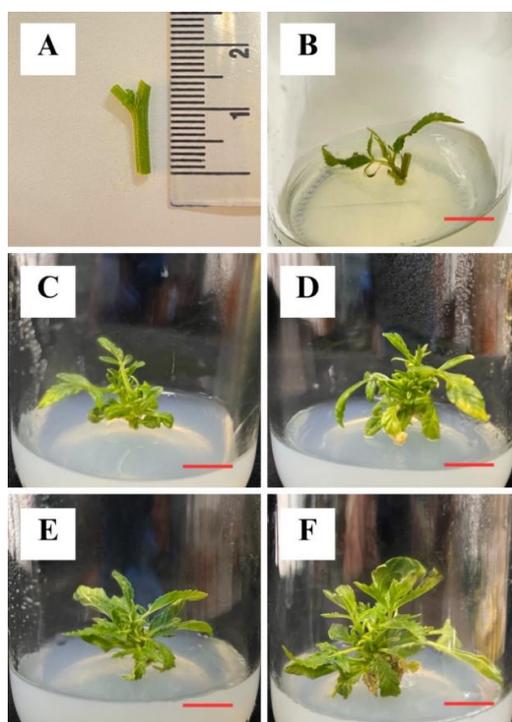
**Table 1** Effect of TDZ on shoot multiplication from the nodal segment of CA-UP1 variety at 4 weeks.

TDZ concentration (mg/L)	Explant-producing shoots (%)	Average number of shoots per explant	Shoot height (cm)
0	0	1.6 ± 0.70 <sup>c</sup>	2.02 ± 0.32 <sup>ab</sup>
0.05	80.00	1.9 ± 1.00 <sup>c</sup>	1.93 ± 0.33 <sup>b</sup>
0.10	93.33	3.8 ± 0.63 <sup>ab</sup>	2.11 ± 0.29 <sup>ab</sup>
0.50	100.00	4.7 ± 1.57 <sup>a</sup>	2.30 ± 0.42 <sup>a</sup>
1.00	90.00	3.2 ± 0.92 <sup>b</sup>	1.88 ± 0.17 <sup>b</sup>

\*Different lowercase letters indicate significant differences within the same column at  $p < 0.05$ .



**Figure 2** Shoot formation from nodal segment of CA-UP1 variety after 1 week (arrow). (A) MS medium without TDZ and (B) MS medium supplemented with 0.5 mg/L (Scale bar = 1 mm).



**Figure 3** Stages of shoots multiplication from explant at different times. (A) nodal segments of CA-UP1 variety. (B) Shoot formation on MS medium without TDZ. (C) - (F) Multiple shoots on MS medium supplemented with 0.5 mg/L TDZ at 1, 2, 3, and 4 weeks (Scale bar = 1 cm).

### Root induction and acclimatization of plantlets

The objective of measuring the optimal concentration of IBA (0 - 2,000 ppm) for root induction was to investigate both the acclimatization of the seedlings and the growth characteristics of the seedlings.

**Table 2** demonstrates that dipping in IBA solutions at concentrations of 1,000 and 1,500 ppm, mixed with talcum powder, obtained the most desirable root response, indicated by a rooting rate of 100 % and average plant heights of  $4.32 \pm 1.52$  and  $4.14 \pm 0.71$  cm, respectively. By dipping the plants in 1,000 ppm IBA solution mixed with talcum powder, the longest roots measured  $5.78 \pm 1.96$  cm (**Figure 4**). In contrast, the control group showed the lowest root response in rooting rate, average number of roots, root length, and seedlings height. According to previous research reported by McLeod *et al.* [30]. A study investigating the development of hemp roots by dipping them in 15 mM IBA solution resulted in a significant increase in the average rate of root formation, reaching up to 94.44 %. The average length of the roots was 0.72 cm, surpassing the rate of the control group that did not receive the hormone. Ioannidis *et al.* [31] reported that the method of dipping hemp in a 15 mM IBA solution resulted in a statistically significant difference in the average number of roots. This method was more effective than the method of using autoclaved IBA with the medium and IBA via syringe filter-sterilization. In addition, IBA is also used with other plant species. However, difference

plant varieties have different responses to the concentrations of auxin in the process of adventitious root induction.

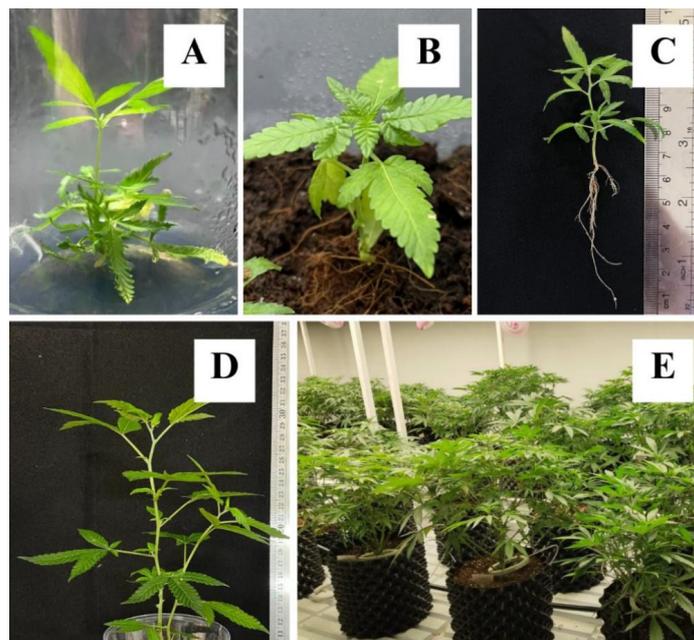
Kumar *et al.* [32] evaluated the effect of IBA on rose-scented geranium (*Pelargonium graveolens*) and discovered that IBA possesses the ability to expedite hydrolytic activity, therefore promoting the production of callus and the conversion of meristematic cells into root primordium. The application of IBA externally stimulates an increase in the internal auxin concentrations within the explants, leading to improved cell division and the generation of multiple root primordia from the regions of root origin. Furthermore, there have been published reports of IBA being used in several plant species, including *Ixora* (*Ixora coccinea*) [33]. A study conducted by El-Banna *et al.* [15] investigated the process of adventitious roots (ARs) development in Chinese Pepper Tree (*Zanthoxylum beecheyanum* K. Koch) by the use of exogenous IBA.

The results indicated 1,500 - 2,500 mg/kg of IBA was successful in stimulating root development in cuttings. Furthermore, this treatment provided the most elevated percentages of root development, root length, root number and both fresh and dry root weight. The effects of IBA stimulation on the formation and development of ARs depend on the translocation and movement of sugars to the base of the cuttings, as well as the motivation of DNA, RNA, and protein synthesis for cell division [34].

**Table 2** Effect of various concentration of IBA on root induction of CA-UP1 variety at 4 weeks.

IBA concentration (g/L)	Root formation (%)	Average number of roots per explant	Root length (cm)	Explant height (cm)
0	20	$1.8 \pm 0.88^b$	$3.86 \pm 1.56^b$	$2.68 \pm 0.35^b$
1.0	100	$5.6 \pm 1.94^a$	$5.78 \pm 1.96^a$	$4.32 \pm 1.52^a$
1.5	100	$5.4 \pm 1.14^a$	$3.42 \pm 0.89^b$	$4.14 \pm 0.71^a$
2.0	70	$3.2 \pm 1.30^b$	$3.20 \pm 1.06^b$	$3.40 \pm 0.83^{ab}$

\*Different lowercase letters indicate significant differences within the same column at  $p < 0.05$ .

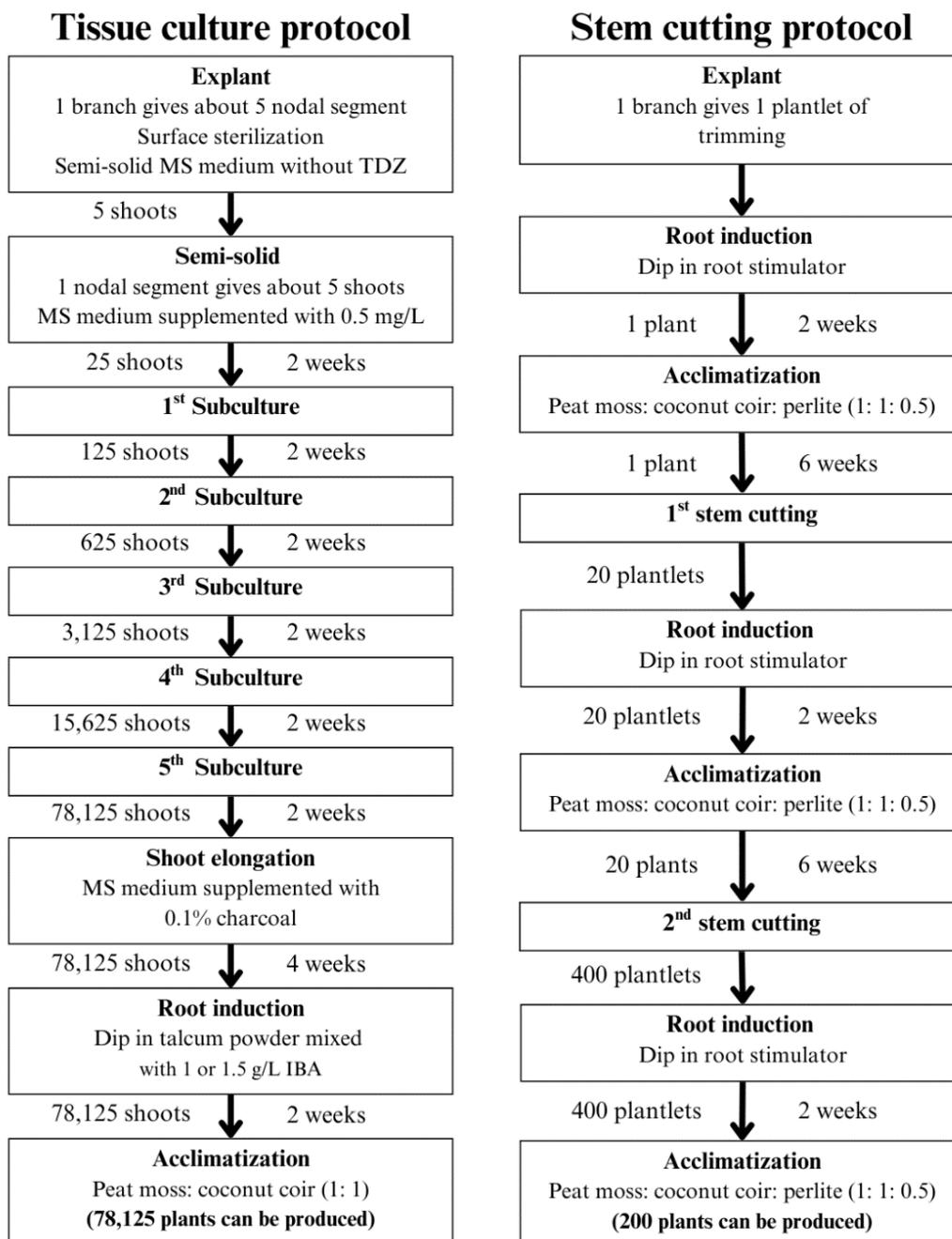


**Figure 4** Root development and acclimatization to produce plants. (A) Shoot after 4 weeks in MS medium with charcoal. (B) Acclimatization of plantlets on growth medium. (C) Root formation from shoot after 2 weeks. (D) Plant of CA-UP1 variety for 2 weeks after acclimatization. (E) Controlled environment room for cultivating hemp.

The protocol in the propagation of the CA-UP1 variety was presented by the comparison between cuttings and tissue culture within 18 weeks (**Figure 5**). Tissue culture protocol begins with the selection of the mother plant, sterilization, induction of shoot and root formation, and acclimatization of the seedlings to produce hemp plants. This process produced approximately 78,125 hemp plants. While, stem cutting propagation, which starts with the selection of the mother plant, induction of root formation, and acclimatization of the seedlings to produce hemp plants, produced approximately 400 hemp plants. The tissue culture protocol produced seedlings about 190 times more than the cutting protocol in the same duration. The result was consistent with those reported by Lubell-Brand *et al.* [35], which found that the retip propagation can yield commercial hemp 9 times more than the stem cutting propagation within 1 cm<sup>2</sup>. Every 2 weeks, the large mother plant provides 50 - 60 cuttings with around an 80 % rooting rate. The stem cutting propagation produces 200 hemp plants within 10 weeks, while the retip micropropagation can hold 67 micropropagated plants in the same 1 cm<sup>2</sup> area, producing nearly 1,800 plants. Moreover, the legal hemp industry for CBD production is a rapidly growing market, and cultivators are turning to advanced scientific methods, such as *in*

*vitro* micropropagation, to reduce production costs, ensure health, and maintain high quality [36]. The traditional method for commercial hemp propagation is stem cuttings, which ensure the rapid propagation of desired genotypes [37]. However, stem cuttings may reduce vigor, as the donor plants might be damaged by fungal infections and viruses that negatively impact their growth and quality [35,38]. The maintenance of mother plants necessitates being kept in a vegetative condition. Although this is readily achievable for the majority of genotypes, it presents difficulties for day-neutral genotypes, as they are unresponsive to photoperiod [39]. Furthermore, sustaining donor plants obtaining stem cuttings can be time-consuming and needs considerable area [40]. Tissue culture propagation rapidly generates numerous genetically identical cloned plants that exhibit uniform vigor and disease resistance, requiring minimal care for the mother plants and requiring less space than traditional cuttings [41].

Therefore, micropropagation by plant tissue culture represents a viable alternative for the multiplication of disease-free and *C. sativa* plants [42]. In the present study, with an aim to develop an efficient and regeneration protocol for mass multiplication of *C. sativa* L. variety CA-UP1. The protocol here is very important for large scale propagation of the hemp.



**Figure 5** Schematic representation of tissue culture and stem cutting production within 18 weeks.

**Conclusions**

In conclusion, this research presents a method for the rapid propagation of disease-free *C. sativa* L. using tissue culture, with the results of this study providing opportunities for the mass propagation of the CA-UP1 variety. We have successfully implemented a protocol in which nodal segment (1.5 cm) can be cultured on MS medium, sterilized with 0.1 % HgCl<sub>2</sub> for 10 min, which 16.67 % contamination rate, shoot multiplication can be

induced by 0.5 mg/L TDZ ( $4.7 \pm 1.57$  shoots/explant), and root formation can be induced by 1,000 ppm IBA with  $5.6 \pm 1.94$  roots/explant in 100 % root formation. Additionally, the tissue culture protocol produces 190 times more hemp plants than the stem cutting protocol within 18 weeks. Implementing this protocol could improve the scalability of *C. sativa* L. production. These findings offer a reliable rapid protocol for efficient

propagation of *C. sativa* L., which could be valuable for commercial applications.

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