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EFFECT OF THIDIAZURON ON CANNABIS SATIVA L. PLANTLETS AND ISOLATION OF CANNABINOIDS USING CENTRIFUGAL PARTITION CHROMATOGRAPHY

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Abstract: Cannabis (*Cannabis sativa L.*) is a plant native to Thailand. The ability of cannabis to exert effects on health varies depending on different amounts of the active compounds, cannabinoids. These compounds accumulate in the inflorescence of the female plant. When the plant is grown from seed, it is susceptible to genetic instability. However, growing clones using tissue culture guarantees the desired genetics. This study examined the effects of the plant growth regulator thidiazuron (TDZ) on growth and cannabinoids in cloned cannabis plantlets. Moreover, the primary cannabinoids, Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) isolation from seized 400 g of dried cannabis were optimized using Centrifugal Partition Chromatography (CPC), and they were used as the reference standard for compounds quantification. The result showed that 1 g of cannabis extract provided 50 mg CBD and 20 mg THC within 2 h. Moreover, it was found that at TDZ 1.0 µM, the plantlets produced the highest number of multiple shoots after 4 weeks, while the cannabinoids profile under UV detector (220 nm) was no different compared to control without TDZ supplemented. No difference was found in the amounts of CBD and THC from both groups; CBD and THC content was around 0.12 and 0.05 %w/w, respectively, in the plantlets supplemented with 1.0 µM TDZ, while CBD and THC content was around 0.13 and 0.05 % w/w, respectively, in the control group. Treatment with 1.0 µM TDZ can be used to boost cannabis clones. This provides an alternative cloning method for commercial cannabis growers.

Keywords: Cannabis, Cannabinoids, Centrifugal Partition Chromatography, Thidiazuron, Tissue culture

INTRODUCTION

Cannabis, also known as marijuana, is a plant native to the Indochinese Peninsula. Historians have long argued over when the plant was first used for medical purposes in this region. An inscription at Prasat Phra Khan in Cambodia suggests that it was used as a herbal medicine since the King Jayavarman VII era ("Transcribe the inscription," 2020). In Thailand, a well-documented record dating from the time of King Narai the Great in the 17th century describes the use of cannabis in many medical formulas. The recorded herbal recipes were used to treat symptoms such as muscle pain, anorexia, nausea, vomiting, stress, insomnia, and more (Chokevivat, 2019). The later identification of cannabinoids expanded the knowledge of how these compounds react in the body. The most abundant cannabinoids in cannabis are cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (THC) in the acid form. CBD and THC were first isolated from cannabis and studied for their pharmacological activity in the early 1940s; cannabinoid receptors were discovered in humans in the mid-1980s (Pertwee, 2006).

Generally, cannabinoids are synthesized and concentrated in the glandular trichomes of inflorescence in female plants. The types and amounts of cannabinoids depend on the cannabis strain and growing environment. A wide variety of cannabis strains are found in Thailand, such as Hang Krarok, Foi Thong, Wild Thailand, KD KT, ISSARA 01, and more; most are THC dominant strains. The cannabis plant can reproduce either sexually or asexually. However, the likelihood of mutation significantly increases if the plant is grown from seed (in which a genetic contribution is made by both a mother and father). Growing from tissue culture or cloning using micropropagation are options to preserve the plant's character (Wróbel *et al*., 2017). In this process, the explant, which can be a shoot or a stem, is collected from the mother plant and placed in a sterile container containing gelling media. The media, which is supplemented with nutrients, provides for the plantlets' shoot and root development. After the plantlets have grown sufficiently, they are transferred into soil pots. Since the tissue culture method provides a controlled environment, the effect of parameters on cannabis growth and cannabinoid profile can be observed.

In this study, the effects of a plant growth regulator on development and cannabinoid profile were investigated in cannabis plantlets. Many plant growth regulators such as 6-benzylaminopurine (BAP), 1-naphthaleneacetic acid (NAA), and thidiazuron (TDZ) can be used for shoot induction in cannabis cultures (Lata *et al*., 2017). It was reported that TDZ showed high efficiency for shoot induction than other plant growth regulators (Lata *et al*., 2017; Wang *et al*., 2009). The study findings can be used to increase the commercial production of cannabis.

MATERIALS AND METHODS

Plant tissue culture

Cannabis seeds of unknown strain were collected from dried cannabis seized by police and legally collected at College of Pharmacy, Rangsit University. The seeds were immersed in 70% ethanol for 30 min and disinfected with 30% Clorox for 30 min. They were then washed three times and immersed in sterile distilled water for 24 h. The seeds were placed on Murashige and Skoog (MS) medium with the vitamin in a downward position. After six weeks, the plantlets were ready to be sub-cultured.

The plantlets were dissected into pieces (around 1.5 cm. long) on the x-axis. Explants were inoculated on MS medium supplemented with 1 mg/L pyridoxine HCl, 1 mg/L nicotinic acid, 10 mg/L thiamine HCl, and 100 mg/L myo-inositol. Concentrations of TDZ were varied to 0.1, 0.5, and 1.0 µM. The temperature was fixed at 25 ± 2 °C under 18 h of fluorescent light. Plantlets' weight and the number of shoots were recorded for four weeks. The experiment was performed in five replicates.

Cannabinoid determination

After four weeks, cannabis plantlets were collected and dried in a hot air oven at 50 °C for 24 h. The sample (100 mg) was extracted in ethanol (5 mL) using sonication for 30 min. Cannabis extract was obtained after solvent evaporation. The extract was reconstituted with 1 mL methanol and filtrated through a 0.22 µm nylon filter before analysis. The cannabinoids in the sample were analyzed using a Dionex UltiMate™ 3000 High-Performance Liquid Chromatography-Ultraviolet (HPLC-UV). An Acclaim™ 120 C18 chromatography column (3 µm, 2.1 mm x 150 mm) was used as a stationary phase. Analyses were performed in an isocratic mode using aqueous 0.1% formic acid and acetonitrile at 15% and 85%, respectively (Saingam & Sakunpak, 2018). The instrument temperature was 25 °C with a flow rate of 0.2 mL/min. A 5 µL aliquot sample was then injected onto the column. The cannabinoid profile (at 220 nm) was obtained from DataAnalysis software. CBD and THC content in the samples were analyzed

by comparison to reference standards purified in our laboratory. Calibration curves prepared in methanol ranged from 0.78 to 200 ng/mL for CBD and 3.13 to 100 ng/mL for THC.

Cannabinoid isolation

The dried cannabis material (400 g) was ground and extracted in ethanol (2 L) using maceration for 3 days and repeated 3 times. The % yield of the ethanolic extract was 10% w/w. After evaporation, only 1 g of ethanolic extract was injected into a Centrifugal Partition Chromatography (CPC) column, a silica-free separation technique, for CBD and THC isolation. A PLC 2250 Purification System controlled a Gilson CPC 250 PRO column, and the solvent system was ethanol/water/n-hexane/ethyl acetate. Elution and extrusion rates were 12 mL/min and 30 mL/min, respectively. The rotation speed was set at 2,000 rpm, and the detection wavelength was 280 nm. The compounds from each fraction were identified by mass spectrometry (MS). A Bruker AmaZon SL MS instrument was set in positive mode and recorded using a mass range of m/*z* 100-2,000. The capillary voltage was 4,500 V, and the drying gas temperature was 200 ^{o}C with a flow rate of 7.0 L/min. Nebulizer pressure was set at 2 bars. Data were processed using the Compass 1.3 SR2 program.

RESULTS AND DISCUSSION

Effect of TDZ on cannabis growth

Since the seeds were from seized dried cannabis, only 4% were active. After the plants had grown for 6 weeks, the explants were cut and inoculated in the medium. Plant response in all treatment conditions was recorded every week after culturing. No changes were observed after 1 week. After 2 weeks, signs of shoot proliferation were observed in all treatment conditions. The shoot was light green, and multiple shoots appeared. The MS medium supplemented with 1.0 µM TDZ showed higher growth than the medium containing 0.1 and 0.5 µM TDZ after 3 weeks.

Moreover, the medium with 1.0 µM TDZ showed the greatest shoot induction after 4 weeks; the average number of shoots was 9.7±2.0, the average shoot length was 6.2±2.8 cm, and all explants had produced shoots. TDZ, which possesses cytokinin-like activity, can increase cell division and break apical buds (Huetteman & Preece, 1993). In this study, multiple shoot initiation occurred in the presence of a low level of cytokinin. This result is congruent with a previous study of the cannabis strain MX-1, which showed a greater number of shoot induction at 0.5-2.5 µM TDZ (Lata *et al.*, 2008). Our study used a different strain, based on the size and shape of leaves, which had a narrower palmate-shape (Figure 1-3). Thus, this study confirmed that TDZ had the same effect on shoot multiplication in different cannabis strains.

Effect of TDZ on cannabinoid profile

Using a plant growth regulator to increase the number of cannabis clones requires not only the same morphological response, but the same active compound profile. To our knowledge, this is the first study examining the effect of TDZ on cannabinoid profiles. Cannabis ethanolic extract was analyzed by HPLC-UV, which is a highly selective method. The absorption wavelength was set at 220 nm since it provides high sensitivity for cannabinoid detection. The optimized method provided separation of all analytes within a run time of 16 min. The chromatographic fingerprints did not differ between control and TDZ-supplemented groups (Figure 4). Six cannabinoids were identified based on their mass spectra and in comparison to previous literature (Mandrioli *et al*., 2019). The peaks and retention time were represented as cannabidiolic acid (CBDA) at 4.6 min, CBD at 5.2 min, cannabinol (CBN) at 7.6 min, THC at 9.4 min, cannabichromene (CBC) at 11.5 min, and tetrahydrocannabinolic acid (THCA) at 12.5 min. These results confirmed that cannabinoid biosynthesis had been triggered

in the 4-week-old plantlets. However, CBN is a byproduct of THC degradation and is not produced by plant metabolism (Mander & Liu, 2010); the trace amount of CBN found suggested that THC can be converted to CBN even at a low drying temperature under air oxidation (50 ^{o}C, 24 h).

Effect of TDZ on THC and CBD in cannabis plantlets

Quantitative data regarding cannabinoids in the cannabis plantlets was also examined. To determine the amount of CBD and THC, reference standards were needed. CPC is a preparative purification technique that can be used on an industrial scale. The method was used previously to isolate cannabinoids from cannabis (Hazekamp *et al*., 2004). In this study, the separation was achieved within 2 h; CBD was in the fraction 31-37 and THC was in the fraction 6-7 (Figure 5). From 1 g extract, 50 mg CBD and 20 mg THC can be isolated. The two isolates can be identified by comparison of mass spectra to literature data (Figure 6) (McRae & Melanson, 2020). However, the MS spectra of both compounds are the same; thus, the retention time was also used in identification (Saingam & Sakunpak, 2018). The purity was calculated based on MS data, and the purities of CBD and THC were determined to be 99% and 95%, respectively.

Due to incomplete decarboxylation, CBDA and THCA appeared in the sample. However, our material for cannabinoid isolation was seized 400 g of dried cannabis. All CBDA and THCA were changed to CBD and THC, so it was impossible to isolate those acid form cannabinoids and use them as the reference standards. Since the purpose is to compare whole CBD and THC contents in the plantlets, a rough calculation for CBDA and THCA is needed. The calibration curves of CBD and THC were used instead since their chromophores are almost similar. This experiment was performed with a five-point calibration curve. Excellent linearity was presented in both CBD and THC curves (R^2 0.9997 and 0.9996, respectively). It was noticed that the peak area responses of CBD and THC were similar at 220 nm. The results obtained from the analysis of cannabis plantlets were presented. CBD content in the plantlets growing in MS medium and MS medium supplemented with 1.0 µM TDZ was approximately 0.13% and 0.12 %w/w, respectively, while THC content was around 0.05 %w/w in both groups. It was suggested that 1.0 µM TDZ did not affect CBD and THC amounts. In the plantlets, CBD content was higher than THC content, indicating the possibility of mixing cannabis seeds other than the native Thai strains in this lot.

Although low-level TDZ did not alter the cannabinoid profile (CBDA, CBD, CBN, THC, CBC, and THCA), it may affect other metabolites. A recent metabolomics study suggested that low-level TDZ involved steroid biosynthesis and sugar metabolism in African violet. Moreover, high-level TDZ altered sesquiterpene biosynthesis, which can cause the same effect on cannabinoids in cannabis (Erland *et al*., 2020). However, a high concentration of TDZ may inhibit shoot growth (Huetteman & Preece, 1993) and multiplication (Lata *et al*., 2009).

Figure 1. Shoot multiplication of *C. sativa* on MS supplemented with 1 µM TDZ.

Figure 2. Shoot multiplication of *C. sativa* on MS supplemented with 0.5 µM TDZ.

Figure 3. Shoot multiplication of *C. sativa* on MS supplemented with 0.1 µM TDZ.

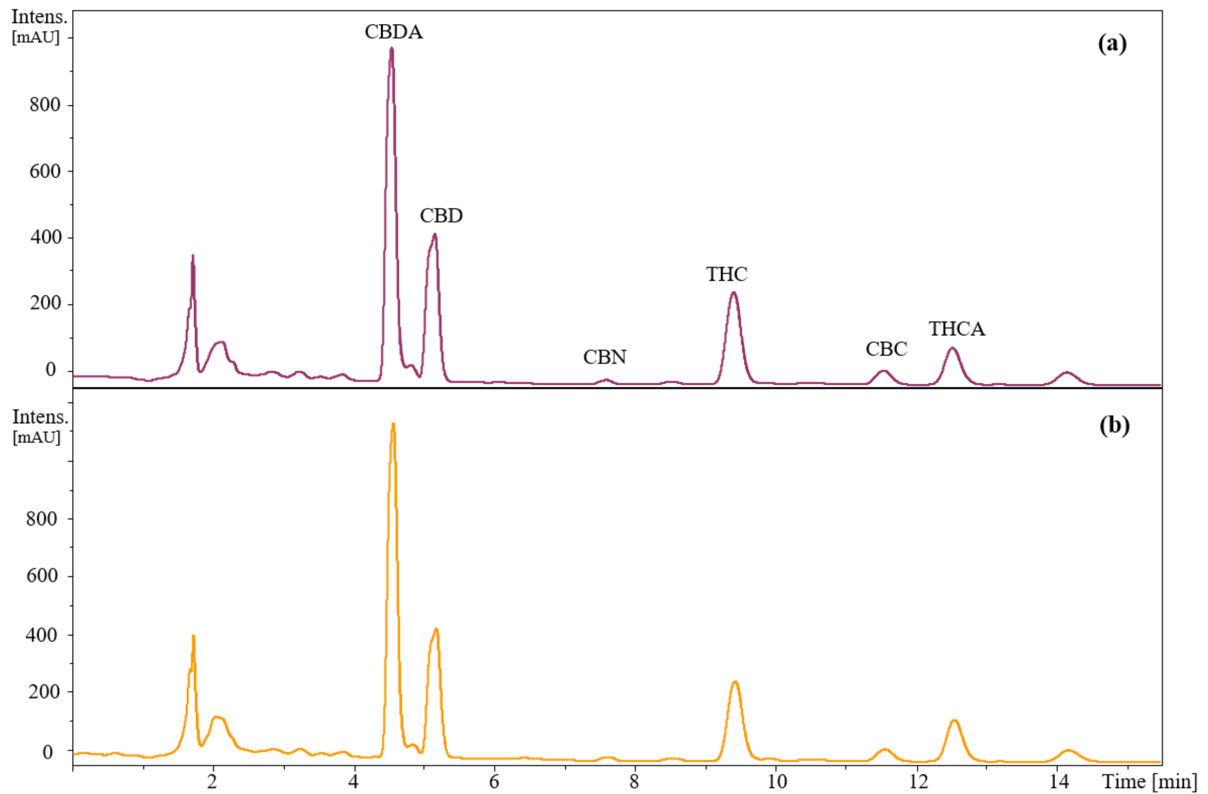

Figure 4. HPLC-UV chromatographic fingerprints of (a) cannabis plantlet in MS medium supplemented with 1.0 µM TDZ (b) control.

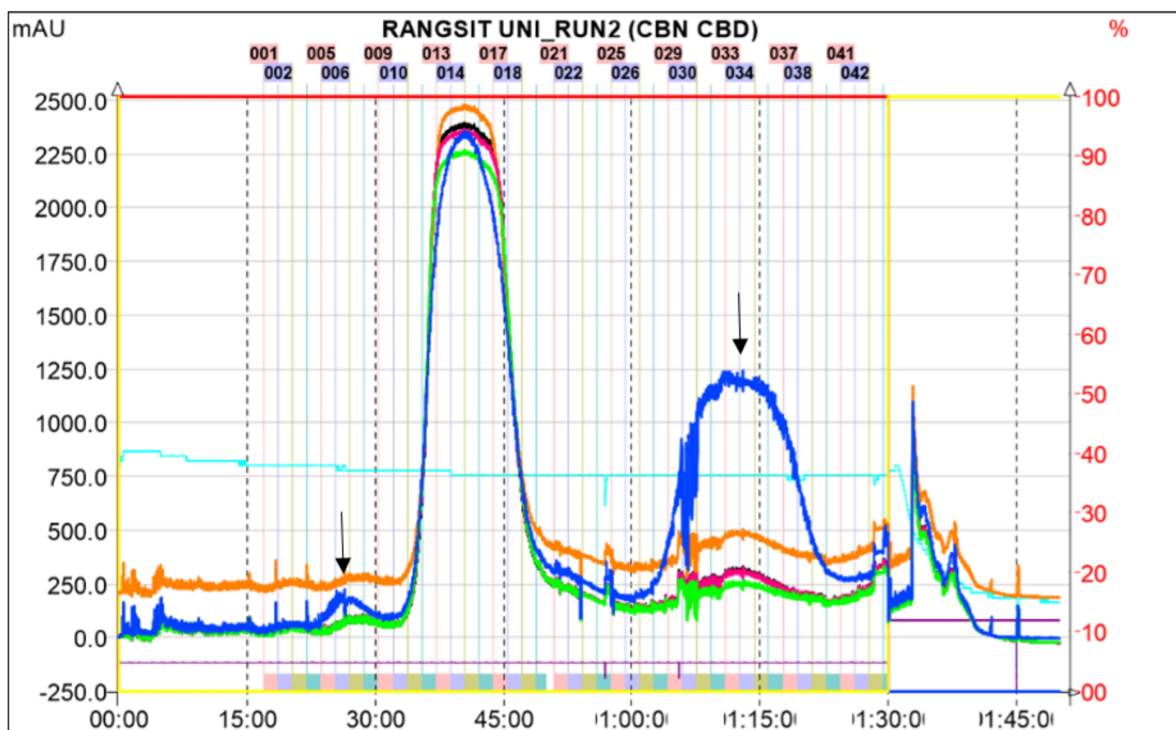

Figure 5. CPC chromatogram of cannabis extract from dried cannabis. The blue line was used, and it was indicated that cannabinoids were detected at 220 nm.

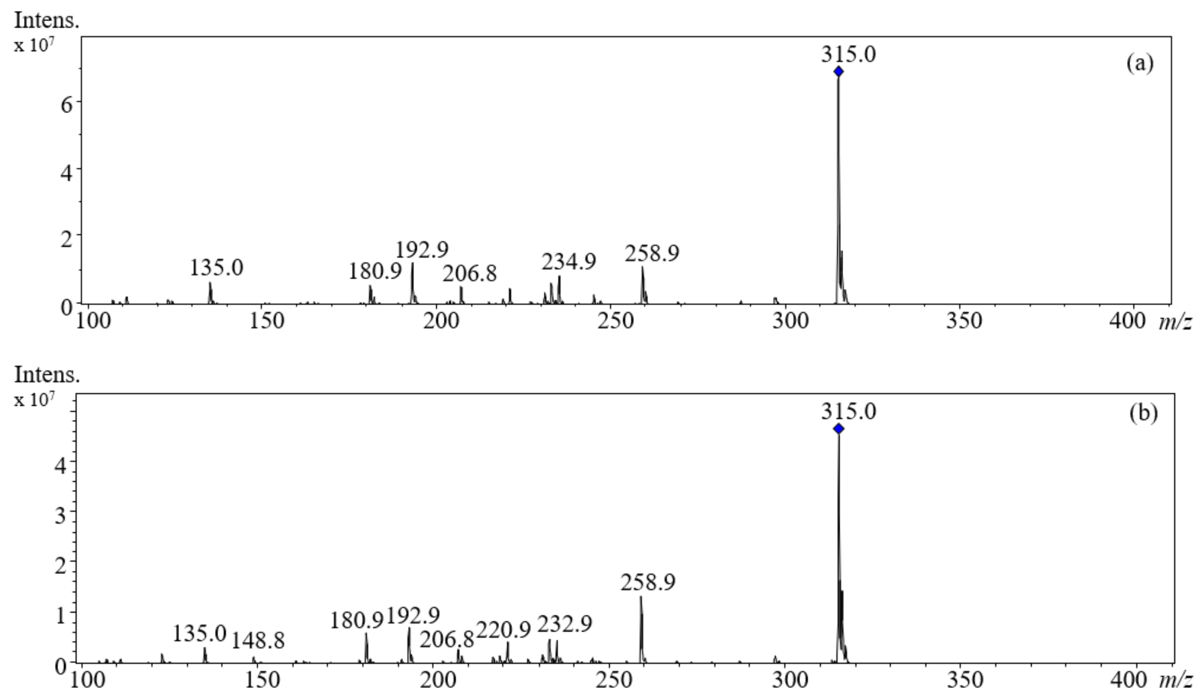

Figure 6. Mass Spectra of (a) CBD (b) THC purified from cannabis extract.

CONCLUSION

CPC, a silica-free separation technique, was used to isolate high yields of CBD (5% w/w) and THC (2% w/w) from discarded cannabis within a short time. Moreover, this study is the first report about the effect of TDZ on the growth and cannabinoids of cannabis plantlets. MS medium supplemented with 1.0 µM TDZ was efficient in cannabis shoot multiplication without altering cannabinoids. TDZ can be used to boost cannabis clones significantly. This method allows growers to cultivate plants that are uniform and high in quantity and quality.

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