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Short communication

Biomass production and cannabinoid accumulation in Cannabis (*Cannabis sativa* L.) callus cultures

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Introduction

Cannabis sativa L. (Cannabaceae) is well-known medicinal plant for its pharmacological and therapeutic properties. Secondary metabolites found in *C. sativa* include cannabinoids, flavonoids, stilbenes, terpenoids, alkaloids, and lignans. Cannabinoids are class of natural compounds, with over 70 distinct cannabinoids discovered so far (ElSohly and Slade, 2005). The conventional methods for cannabis propagation by seeds or stem cuttings are not efficient, safe or scalable and could lead to complete crop loss as a result of uncontrollable pathogen outbreak.

Micropropagation is an effective propagation tool for scale-up cannabis production with consistent plants and predictable cannabinoid profile. Other significant benefits of cannabis micropropagation include constant supply of biochemicals and improved compound production. Biotechnological approaches, particularly plant tissue culture, are crucial in the search for alternative methods for production of useful therapeutic molecules from plants.

The main objective of this study was to develop an effective *in vitro* protocol for callus induction from *C. sativa* explants in the presence of various concentrations of cytokinins and auxins. During this study, the following parameters were examined:

• Quantitative determination of biomass production;
• Microscopic analysis of trichomes;
• Identification of cannabinoids using high-performance liquid chromatography coupled with diode array detector (HPLC-DAD).

Materials and methods

The seeds from *C. sativa* were sterilized with 70% ethanol for 1 min and 1% $Ca(OCl)_2$ for 30 min, then rinsed three times with sterile deionized water. Thereafter seeds were cultured on MS macro and oligoelements (Murashige and Skoog 1962), B5 vitamin solution (Gamborg et al. 1968) supplemented with 3% sucrose and solidified with 0.7% agar. Cotyledons as primary explants were excised from seedlings and cultured on MS/B_5 medium, supplemented with plant growth regulators such as 1.0 mg/L thidiazuron (TDZ) and 0.5 mg/L 1-naphthaleneacetic acid (NAA) for induction of callogenesis. Callus cultures were maintained in a growth chamber at 25±1°C under a photoperiod of 16 h light, irradiance at 50 µmol m^2 s^{-1} and 50-60% relative humidity. After three weeks, calli were transferred onto MS/B_5 medium supplemented with various concentrations (0.5, 1.0, 1.5 and 2.0 mg/L) of cytokinins N^6-benzyladenine (BA), kinetin (KIN) and thidiazuron (TDZ). Calli were subcultured on 1.0 mg/L TDZ and several concentrations (0.1, 0.5 and 1.0 mg/L) of auxins 1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA) and 2,4-dichlorophenoxyacetic acid (2,4-D). Biomass production, trichome development and cannabinoid accumulation were analyzed in callus cultures.

Calli were lyophilized and used for cannabinoid extraction. DAB Pharmacopoeial method for assay of cannabis flos was used to determine cannabinoid content. The chromatographic analyses were performed using an

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Agilent 1200 Model HPLC outfitted with a DAD, quaternary pump, column thermostat and autosampler (Agilent Technologies, USA). The InfinityLab Poroshell 120 EC-C 18 chromatographic column (150 mm x 3 mm ID, 2.7 m, Agilent Technologies, USA) was used for separation. The mobile phase consisted of an *o*-phosphoric acid aqueous solution (8.64 g/L) as solvent A and acetonitrile as solvent B. The identification of cannabinoids was performed using a gradient method with a flow rate of 0.7 mL/min and temperature of 40°C. DAD measurements were taken at 225 nm for neutral cannabinoids and 306 nm for acidic cannabinoids.

Results and Discussion

The biomass production in callus cultures was increased from 2.5 to 4.1 g in the presence of high concentrations (up to 2.0 mg/L) of BA in the medium. According to morphological characteristics, cultures showed a light yellow to green color and a compact and nodular structure. The presence of KIN in the medium decreased the biomass of calli from 3.8 to 2.5 g. The cytokinin KIN in the medium induced the appearance of yellow-brown callus with friable consistency. Exogenous application of TDZ in the medium induced the highest callus biomass yield from 11.3 to 18.8 g. In the presence of TDZ, callus cultures have compact consistency and light green color. Based on the outcomes, it can be considered that TDZ significantly increases biomass production in callus cultures when compared to BA and KIN.

The biomass of calluses cultured on medium with 1.0 mg/L TDZ and NAA was reduced from 6.9 to 3.4 g. The callus cultures changed color from green to light yellow as the concentration of NAA in the medium increased. The calluses had a compact and nodular structure. Biomass production of callus cultures containing 1.0 mg/L TDZ and IBA was also reduced from 13.9 to 8.0 g. The callus cultures were dark green in color with friable and nodular structure. When calluses were cultured in the presence of 1.0 mg/L TDZ and 2,4-D, biomass production was not significantly altered. These calluses showed compact structure and color range from green to yellow. In comparison to the presence of NAA and 2,4-D in the medium, the auxin IBA significantly induced biomass production of cannabis calluses.

Results from this study showed that phytohormones significantly influenced the compactness, consistency and color of callus cultures. Callogenesis was successfully induced from cotyledons isolated from cannabis seedlings which is in accordance with previous data (Lata et al., 2010, Chaohua et al., 2015). These authors reported that cotyledons are the best primary explants for induction of green calluses with compact structure. Our results showed that combination of 1.0 mg/L TDZ and 0.5 mg/L IBA in the medium significantly increased biomass production of calluses.

Microscopic analysis showed the formation of trichome-like structures on the surface of cannabis calli cultured on MS/B_5 medium with 1.0 mg/L TDZ and 0.5 mg/L NAA. These trichomes had similar structure like trichomes found on the intact cannabis plant. Outgoing results showed that TDZ is probably a key factor in the differentiation of trichomes in cannabis callus cultures.

Cannabidiolic acid (CBDA) and Δ9-tetrahydrocannabinolic acid (Δ9-THCA) were found in callus extracts using chromatographic analysis. The 9-THCA was noted as the dominant compound. The presence of Δ9-THCA in callus cultures could be due to the regulation role of glandular trichomes in cannabinoid metabolism.

Conclusion

This study presented for the first time the production of biomass and cannabinoids in callus cultures of *C. sativa* depending of exogenously applied cytokines and auxins. The presence of trichomes on the callus surface confirms their ability to produce cannabinoids. The relationship between biomass and cannabinoid production is a vital first step toward understanding the potential mechanisms for usage of *C. sativa* callus cultures as an effective source of bioactive compounds in pharmaceutical industry. Future perspectives should be focused on development of various strategies for stimulation of biomass production and cannabinoid biosynthesis in cannabis callus cultures.

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