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In vitro mass propagation of *Cannabis sativa* L.: A protocol refinement using novel aromatic cytokinin meta-topolin and the assessment of eco-physiological, biochemical and genetic fidelity of micropropagated plants

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ABSTRACT

The present study describes a simple, efficient and one step regeneration system for rapid shoot proliferation and *in vitro* rooting of *Cannabis sativa* nodal explants using meta-topolin (mT), an aromatic natural cytokinin. The best response in terms of explants producing maximum number of shoots with maximum shoot length and percent explants producing shoots was recorded on Murashige and Skoog (MS) medium supplemented with 2 μM mT. Shoots multiplied on the same medium for two sub-cultures were able to induce healthy roots within 4–6 weeks. A separate medium containing auxin was not required for root induction. Regenerated plantlets were successfully acclimatized and hardened off in the climatic controlled grow room with 100% survival rate. Genetic fidelity of *in vitro* propagated plants was tested using inter simple sequence repeat (ISSR) markers. Our results show that all the ISSR profiles from *in vitro* propagated plants were monomorphic and comparable to that of the mother plant, thereby confirming the genetic fidelity. Qualitatively and quantitatively, cannabinoid profiles and the content, using gas chromatography-flame ionization detector (GC–FID), in mother plant and *in vitro* propagated plants were found to be similar to each other. Furthermore, regenerated plants were eco-physiologically and functionally comparable to that of the mother plant. The maximized regeneration protocol using mT is thus effective and safe for large scale production of true to type *C. sativa* plants.

1. Introduction

Cannabis sativa L. a member of family Cannabaceae, is a valuable plant with both fiber and medicinal potential (Pertwee, 2014). Among 104 cannabinoids isolated from the plant till date (ElSohly and Gul, 2014), the major biologically active compounds are Δ^9 tetrahydrocannabinol (Δ^9-THC, known for psycho-activity) and cannabidiol (CBD, a non- psychoactive compound with the potential for the treatment of epileptic seizures in children). Other major cannabinoids are tetrahydrocannabivarin (THCV), cannabichromene (CBC), cannabigerol (CBG) and cannabinol (CBN).

Cannabis is generally dioceous and occasionally a hermarphodite, wind pollinated plant. For the production of cannabinoids (or phytocannabinoids) female plants are preferred over male plants for several reasons; firstly, in comparison to male plants, female plants produce higher amount of cannabinoids; secondly, being highly wind pollinated, in presence of males, female plants produce lots of seeds at maturity whereas, seed free plants (sinsemilla, a Spanish word) are preferred to produce higher yield of secondary metabolites and thirdly, if several cannabis varieties are being grown together, cross pollination among the varieties would affect the quality (chemical profile) of the final product. To avoid these situations, removing male plants as they appear, screening of female clones for higher metabolite content and, their conservation and multiplication using biotechnological tools is a suitable way to ensure the consistency in chemical profile of a cannabis crop for the pharmaceutical interest.

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In vitro propagation of plants can be influenced by a variety of factors that are categorized either as environmental or hormonal/plant growth regulators (PGRs). As a vital chemical component, PGRs regulate various physiological and developmental processes during the micropropagation (George et al., 2008). There is a continuous effort aimed to identify new compounds with the ability to stimulate better growth, and to alleviate *in vitro*-induced physiological disorders (Tarkowska et al., 2003). The recent biotechnological advances in the field of phytohormones have significantly facilitated the search for new compounds (Strnad, 1997; Tarkowski et al., 2010). Thus, a new group of aromatic cytokines commonly referred as topolins has been identified (Strnad, 1997). Meta-topolin {6-(3-hydroxylbenzylamino) purine}, first isolated from poplar leaves, is an aromatic cytokinin, differing from isoprenoid cytokinins, such as zeatin and 2-iP, in its biochemistry and biological activity (Strnad, 1997). Topolins have been demonstrated to enhance shoot proliferation, maintain histogenic stability, improve rooting efficiency and alleviate various physiological disorders in micropropagation (Aremu et al., 2012a,b). In *C. sativa*, reports have been available on the shoot regeneration, proliferation and in few cases root formation using various growth regulators (Loh et al., 1983; Richez-Dumanois et al., 1986; Mandolino and Ranalli, 1999; Slusarkiewicz-Jarzina et al., 2005; Bing et al., 2007; Fisse et al., 1981; Feeney and Punja, 2003; Lata et al., 2009a,b, 2010). In the present communication, we report an efficient regeneration protocol for *C. sativa* using novel aromatic cytokinin, meta-topolin. Furthermore, the performance of *in vitro* propagated and hardened plants were evaluated and compared to that of mother plant based upon ecophysiology, photosynthetic pigment content, genetic integrity using ISSR markers and phytocannabinoids content using GC–FID.

2. Material and methods

A step by step schematic diagram of experimental design for one step *in vitro* propagation of *C. sativa* is shown in Fig. 1.

2.1. Plant material

Plants of *C. sativa* were grown from seeds of a high THC yielding Mexican variety in a climatic controlled indoor cultivation facility at Coy–Waller laboratory, School of Pharmacy, University of Mississippi. On flowering, male plants were removed and vegetatively propagated cuttings of selected (based on chemical profile) elite female plants were used for study. This was achieved by a systematic screening process. After a desirable growth, cuttings from seedlings were made at vegetative growth stage. Seedlings were than subjected 12 h photoperiod for flowering till maturity while maintaining cuttings at the vegetative stage under the 18 h photoperiod. On flowering, male plants and their related vegetative cuttings were removed. Samples of flowering female plants were collected and analyzed for their THC concentration using GC/FID. Based on chemical analysis, the elite, highest THC yielding female plant was identified and its related vegetative cuttings were used as high yielding elite 'mother plants' for the current study.

Nodal segments containing axillary buds from selected mother plants were used as an explant for initiation of shoot cultures. Explants were surface-disinfected using 0.5% NaOCl (15% v/v bleach) and 0.1% Tween 20 for 20 min. and washed in sterile distilled water three times for 5 min each, prior to inoculation on the culture medium.

2.2. Shoot regeneration, elongation and rooting

In our previous study, we have reported the effect of different growth hormones on shoot formation and rooting of *C. sativa*. Different concentrations of BA, Kn, TDZ and GA_3 were tested for shoot formation, and IAA, IBA and NAA for rooting. Out of all the growth regulators, best shoot formation were obtained in MS + TDZ whereas, the best rooting were obtained in ½MS + IBA (Lata et al., 2009a,b). Therefore, in the present study, the effect of different concentrations of TDZ (0.05, 0.50, 1.00, 2.00, 3.00, 4.00 and 5.00 μM) with MS medium and IBA (0.05, 0.50, 1.00, 2.00, 3.00, 4.00 and 5.00 μM) with ½MS were tested and compared with similar concentrations (ranging from 0.05 μM to 5.0 μM) of meta-topolin (mT).

Disinfected explants were inoculated on Murashige and Skoog's medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose, 0.8% (w/v) type B agar (Sigma Chemical Co., St. Louis, MO) and 500 mg L^{-1} activated charcoal supplemented with various concentrations of MS + thidiazuron (TDZ, a cytokinin) and ½ MS + Indole-3-butyric acid (IBA, an auxin) and compared with MS + mT ranging from 0.05 to 5.0 μM adjusted to pH 5.7. Sterile medium was dispensed (25 ml) in glass culture vessels (4-cm diameter × 9.5-cm height) with magenta B caps.

All cultures were incubated at 25 ± 2 °C with 16-h photoperiod under fluorescent light with a photon flux of ~52 μmol m^{-2} s^{-1}. All the experiments were repeated three times with ten explants, with one explant per jar. The parameters evaluated were the average number of shoots per explant, average shoot length, percentage of explants producing shoots, average number of roots per explant, average root length, and percentage of explants producing rooted plantlets.

2.3. Acclimatization and ex-vitro propagation

Well-rooted plants were carefully taken out of the medium, washed thoroughly in running tap water to remove all traces of medium attached to the roots. These plants were pre-incubated in coco natural growth medium (Canna Continental, Los Angeles, CA) in thermocol cups for 10 days before transferring in sterile potting mix-fertilome (Canna Continental) in large pots. All these plantlets were kept under controlled environmental conditions (light, ~700 μmmol m^{-2} s^{-1} with 16-h photoperiod, temperature 25–30 °C and relative humidity ~60%) in an indoor cultivation facility. Mother plants and well acclimatized tissue culture raised plants were transferred *ex vitro* for further cultivation.

2.4. Comparison of tissue culture raised plants with mother plants

2.4.1. Eco-physiology

Plants regenerated from tissue culture and their mother plant were compared for photosynthetic characteristics at peak vegetative stage. Measurements were carried out on three upper undamaged, fully expanded and healthy leaves of five randomly selected *in vitro* propagated plants (IVP) and mother plant (MP) using a climatic controlled portable photosynthesis system (Model LI-6400; LI-COR, Lincoln, Nebraska, USA). Throughout the experiment, cuvette temperature and light level were maintained at 25 °C and 1600 μmol m^{-2} s^{-1} photon flux densities (PPFD), respectively, since these conditions were found optimum for the photosynthesis and growth of *C. sativa* following our previous studies (Chandra et al., 2008). Four gas exchange parameters *viz.* photosynthetic rate (P_n), transpirational water loss (E), stomatal conductance (gCO_2) and intercellular CO_2 concentration (C_i) were measured simultaneously at steady state condition. Water use efficiency (WUE) of plants was calculated as a ratio of the rate of photosynthesis (P_n) and transpiration (E).

2.4.2. Pigments content

Leaves used for the photosynthetic gas exchange measurements were later harvested for the determination of pigments content. Chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoid