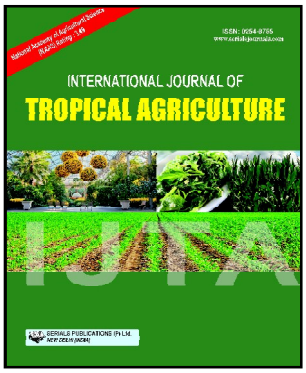

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In Vitro Propagation of *Cannabis sativa* L.: Advancements during the Last Decade (2009 to 2019)

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Abstract: Cannabis belonging to family Cannabaceae, has a long history of an important cultivated crop known for recreational and therapeutic use. Cannabis is a dioceous plant with male and female flowers appearing on two different plants. Being allogamous in nature, it is difficult to maintain the chemical profile of biomass product, if grown from seeds. Plant to plant variation is often observed even though plants are grown from seeds obtained from a single female plant. Therefore, to maintain the consistency in the end product, elite female plants are screened and multiplied using vegetative propagation and/or tissue culture. Micropropagation has been embraced as an important biotechnological tool for conservation and rapid multiplication of elite germplasm of cannabis. On the other hand, it can also be used in genetic modification for the enhanced cannabinoid production. Research on *in vitro* propagation of cannabis has resulted in the development of protocols for callus production, cell suspension cultures, agrobacterium mediated hairy root cultures and regeneration of plants. This review is an overview of advancements made in the field of *in vitro* propagation of cannabis and addresses the current applications of modern biotechnology for the propagation of elite cannabis plants.

CANNABIS PLANT: BOTANY, CHEMISTRY AND LIFE CYCLE

Cannabis is one of the oldest plant cultivated by human. It is a multi-purpose species, which has been grown for seed oil, recreational drug, medicinal purposes and industrial hemp. Origin of cannabis is referenced somewhere in Central China where it was initially grown for Hemp fiber. From there, it travelled to Middle East, Europe and to American

subcontinent in the 16th century. Nowadays, it is grown in most of the parts of the world from Alpine foothills to temperate and tropical areas.

Cannabis, on the other hand, is a rich source of traditional medicine. As a plant cannabis has been used to treat a variety of ailments including glaucoma, nausea, pain, asthma, depression, insomnia and neuralgia (Mechoulam et al., 1976; Duke and Wain, 1981). The therapeutic values of Cannabis derivatives have also been highlighted against HIV/AIDS (Abrams et al., 2007) and multiple sclerosis (Pryce and Baker, 2005).

Cannabis produces cannabinoids or phytocannabinoids, a group of terpeno-phenolic compounds unique to this plant. So far, more than 550 constituents are reported in cannabis, out of which 120 are cannabinoids (ElSohly et al., 2017). Among cannabinoids, Δ^9-tetrahydrocannabinol (D^9-THC), commonly known as THC, is the major biologically and most important psychologically active compound, which accumulates mainly in the glandular trichomes of the plant (Hammond and Mahlberg, 1977). The pharmacologic and therapeutic potency of preparations of *Cannabis sativa* L. and Δ^9-tetrahydrocannabinol (THC) has been extensively reviewed (Grinspoon and Bakalar, 1993; Mattes et al., 1994; Brenneisen et al., 1996; Long et al., 2005; Sirikantaramas et al., 2007). Δ^8-THC is another closely related isomer of Δ^9-THC which is much less abundant (thought to be an artifact) and less potent than Δ^9-THC (Small and Marcus, 2003). Besides THC, cannabidiol (CBD) is another important compound which is non-psychoactive and highlighted for its activity against childhood epilepsy syndromes and other disorders. Besides THC and CBD, other major cannabinoids are Tetrahydrocannabivarin (THCV), Cannabichromene (CBC), Cannabigerol (CBG) and Cannabinol (CBN).

Based on its numerous natural constituents, cannabis is considered as a chemically complex species (ElSohly and Slade 2005). The concentration of Δ^9-THC in the dried inflorescence (leaves and buds) is used to determine its psychoactivity. Quantitative and qualitative analysis of cannabis can be employed to characterize its phenotype and phytocannabinoid profile (Mandolino et al., 2003). Based on the chemical profile cannabis can be divided in three distinct categories, (a) Drug type- with high THC content, (b) Intermediate type- with presence of THC and CBD both and (c) Fiber type- very low in cannabinoids (THC below 0.3%, by dry weight).

Cannabis is an annual plant in nature, sprouting starts with the melting of snow or with arrival of warm weather. During 'long day' it continues to grow vegetatively. Cannabis is a short day plant, it starts flowering when days start getting shorter. An increase in Δ^9-THC and CBD content is observed with age of the plant. A jump on cannabinoids content is noticed with days getting shorter and plant starting flowering. A plateau in cannabinoids content is found at the peak flowering stage for 8 to 10 days. Plant eventually dies in nature if it is not harvested.

Cannabis, in general, is a dioceous plant. However, cannabis sometimes also shows monoecious/hermaphrodite characteristics with male and female flowers appearing on same plant. For the production of cannabinoids, female plants are preferred over male plants for several reasons- (a) female plants produce more cannabinoids as compared to the male plants, (b) females are more robust and produces more biomass, males are normally skinny and tall (c) in the presence of male plants, females produces seedswhereas, for the production of drug sinsemilla (seedless plants) are preferred and (d) if several varieties of cannabis are growing together from seeds, due to cross pollination at the end none of the variety will be pure.

To avoid this situation and to maintain the genetic and chemical homogeneity of the cultivated plants for the production of consistent phytopharmaceuticals, male plants are removed from

grow sites. Backup cuttings are made from randomly selected healthy plants (mother plants) at the vegetative stage and those cuttings are kept under the vegetative light conditions (18 hours light). Mother plants are than exposed to short light hours (< 12 hours). Under short day conditions, cannabis plant starts flowering in 10 - 15 days. In general, male plants flowers appear earlier than female flowers and removed from the growing area. The relative cuttings of those plants are also removed from the growing area. Remaining female plants are allowed to mature till flowering and ultimately upto the budding stage, where cannabinoids content reaches to the peak value. Biomass sample from fully mature plants are then taken and tested for their cannabinoids profile and content. Monitoring cannabinoids content for genetic material selection is carried out by one of several analytical methods such as GC-FID (Ross et al., 1995), HPLC (Gul et al., 2015), UPLC (Wang et al., 2018).

Further, based on the suitable chemical profile mother plants are selected and their backup cuttings (kept at vegetative stage) are used as mother plants for the future cultivation. For mass-propagation, selected mother plants are multiplied by either vegetative propagation or through micropropagation.

PROPAGATION OF CANNABIS

As described earlier, seed raised plants of cannabis have a disadvantage of producing seeds (in presence of male plants) and therefore, less cannabinoids. However, to maximize cannabinoids production, male plants are removed and based on their chemical profile, high yielding female plants are selected. These plants can be multiplied using vegetative propagation or micropropagation to maintain the consistency in producing phytopharmaceuticals.

Micropropagation is another form of vegetative propagation that can regenerate elite clones of same biochemical profile and genetics and offers advantages for multiplication and large-scale production of plants. Mostly, the propagation of Cannabis has been achieved by following two different routes *i.e.* direct and indirect organogenesis. The Murashige and Skoog (MS) formulation is the most commonly used medium for *in vitro* propagation of Cannabis genotypes (Murashige and Skoog 1962). However, the use of other media such as DARIA ind, Millers medium, B5 and MB medium has also been reported (Feenay and Punja 2003; Plawuszewski et al., 2006; Wieglus et al., 2008). In the last decade, several studies are published on cannabis micropropagation that describe regeneration of cannabis explants using biotechnological tools (Table 1). This review addresses the advancements made in past decade in the field of cannabis micropropagation.

INDIRECT ORGANOGENESIS AND AGROBACTERIUM MEDIATED TRANSFORMATION

Most of the earlier studies of indirect organogenesis in *Cannabis sativa* have involved production of callus, cell suspension cultures and biotransformation of cannabinoids (Itokawa et al., 1975; John et al., 1978; Francoise and Vincent 1981; Fisse et al., 1981; Heitrich and Binder 1982; Verzar-Petri et al., 1982; Loh et al., 1983; Braut-Boucher et al., 1985; Fisse and Andres 1985). Further, callus cultures using seed explants of different hemp varieties have also been studied and reported (Mandolino and Ranalli, 1999; Feeney and Punja, 2003; Slusarkiewicz-Jarzina et al., 2005; Wielgus et al., 2008).

In their early work Feeney and Punja (2003) have reports of Cannabis bio-transformation using agrobacterium mediated transformation approach (in MS + B5 vitamins supplemented + 5 µM 2, 4 D and 1 µM KIN) that has resulted in well-developed calli but the cultures were unresponsive to plant regeneration. Whereas, Wahby et al., (2006 and 2013) reported their work on hairy root cultures, however, no regenerated shoots were observed.

In an another study, fourteen percent shoot

Table 1
***In vitro* studies on *Cannabis sativa* L. during the last decade (2009 to 2019)**

Title	*Explant*	*Medium*	*Response*	*Reference*
Evaluation of media for hemp (*Cannabis sativa* L.) *in vitro* propagation	Meristems	Formula β based medium	Shoot regeneration	Casano and Grassi (2009) Italus Hortus, 16: (2) 109-112
Elicitation studies in cell suspension cultures of *Cannabis sativa* L.	Leaf	MS+ B5 vitamins+1mg/l 2,4-D+1mg/l KIN	Cell culture	Flores et al., (2009) Journal of Biotechnology, 143 (2): 157-168.
Thidiazuron induced high-frequency direct shoot organogenesis of *Cannabis sativa* L.	Nodal segments with axillary buds	MS+ 0.5µM TDZ MS+ 2.5 µM IBA	Direct organogenesis	Lata et al., (2009) In Vitro Cellular & Developmental Biology - Plant, 45 (1): 12–19
Propagation through alginate encapsulation of axillary buds of *Cannabis sativa* L. an important medicinal plant.	Nodal segments with axillary buds	MS+ 0.5µM TDZ MS+ 2.5 µM IBA	Shoot and root regeneration	Lata et al., (2009) Physiology and Molecular Biology of Plants, 15 (1) 79–86
A Micropropagation system for cloning of Hemp (*Cannabis sativa* L.) by shoot tip culture.	Shoot tips	MS+ 0.2 mg/l TDZ + 0.1 mg/l NAA MS+ 0.1 mg/l IBA+ 0.05 mg/l NAA	Shoot and root regeneration	Wang et al., (2009) Pakistan Journal of . Bot., 41(2): 603-608, 2009.
High frequency plant regeneration from leaf derived callus of high delta(9)-tetrahydrocannabinol yielding *Cannabis sativa* L.	Leaf	MS+ 0.5 µM NAA+ 1.0 µM TDZ	Callus culture	Lata et al., (2010) Planta Medica, 76 (14): 1629-33
In vitro germplasm conservation of high delta (9)-tetrahydrocannabinol yielding elite clones of *Cannabis sativa* L. under slow growth conditions.	Nodal segments with axillary buds	MS+ 0.5µM TDZ MS+ 2.5 µM IBA	Synthetic seed	Lata et al., (2012) Acta Physiologiae Plantarum , 34: 743–750
Agrobacterium infection of hemp (*Cannabis sativa* L.): establishment of hairy root cultures.	Hypocotyl	A. rhizogenes and A. tumefaciens strains	Hairy root cultures	Wahby et al., (2013) Journal of Plant Interactions 2013 8(4): 312-320
Cannabinoids production by hairy root cultures of *Cannabis sativa* L	Seedling	B5+ 4 mg/l NAA	Hairy root cultures from callus	Farag and Kayser (2015) American Journal of Plant Sciences, 6: 1874-1884
Preliminary studies on the tissue culture of *Cannabis sativa L.* (Industrial Hemp).	Internodes	MS+1 mg/l BAP+ 0.5 mg/l NAA	Callus culture	Jiang et al., (2015) Agricultural Science and Technology. 16 (5) 923 - 925

contd. table 1

Title	*Explant*	*Medium*	*Response*	*Reference*
The effect of different concentrations of TDZ and BA on in vitro regeneration of Iranian Cannabis (Cannabis sativa) using cotyledon and epicotyl explants.	Epicotyl	MS+ 2mg/l BAP+ 0.5 mg/l IBA	Callus culture	Movahedi et al., (2015) Journal of Plant Molecular breeding, 3(2): 20-27
A rapid shoot regeneration protocol from the cotyledons of hemp (Cannabis sativa L.)	Cotyledons	MS+0.4 mg/l TDZ+ 0.5 mg/l IBA	Shoot and root regeneration	Chaohuaet al. , (2016) Industrial Crops and Products, 83: 61-65
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.	Nodal segments with axillary buds	MS+ 2µM m-topolin	Direct organogenesis; Shoot and root regeneration	Lata et al. , (2016) Journal of Applied Research on Medicinal and Aromatic Plants, 3: 18-26.
Effect of different phytohormones on growth and development of micropropagated Cannabis sativa L.	Shoot tips	MS+ 0.1 mg/l TDZ+ 2.5 mg/l GA3 MS+ 0.5 mg/l mT	Shoot and root regeneration	Grulichova et al. , (2017) Mendel Net, 618 - 623
Determination of Optimal Hormone and Mineral Salts Levels in Tissue Culture Media for Callus Induction and Growth of Industrial Hemp (Cannabis sativa L.)	Leaf tissue	MS salts, MB5D1K and MTSU formulations	Callus culture	Thacker et al., (2018) Agricultural Sciences, 9(10):1250-1268
Back to the roots: protocol for the autotrophic micropropagation of medical Cannabis	Shoot tips, Nodal cuttings	Canna Aqua Vega Fertilizer	Shoot and root regeneration	Kodym et al., (2019) Plant Cell Tissue and Organ Culture. 138(2):399-402
Regeneration of shoots from immature and mature inflorescences of Cannabis sativa	Floral explants	MS+TDZ	Shoot and root regeneration	Piunno et al., (2019), Canadian Journal of Plant Science, 99(4):556-559
Cryopreservation of shoot tips of elite cultivars ofCannabis sativa L. By droplet vitrification	Shoot tips	Droplet vitrification, MS	Shoot and root regeneration	Uchendu et al. , (2019) Medicinal Cannabis and Cannabinoids, 2:29–34

regeneration from calli was reported by Weiglus et al., (2008) using different explants from cotyledons, stem and root on DARIA medium. It was observed that highest regeneration was achieved with cotyledons and the lowest for stem explants. Casano and Grassi (2009), reported a higher micropropagation rate of meristem of selected clones of Cannabis in a medium composed of Murashige and Skoog (MS) inorganic salts and Gamborg (B5) vitamins supplemented with 3% sucrose, 1 mg/l IBA, 0.25 mg/l GA_3, 0.1% activated charcoal, 0.8% agar (MS-based medium). Further, Flores-Sanchez et al., (2009) employed elicitation using biotic and abiotic elicitors on cannabinoid production in *C. sativa* cultures.

In our laboratory, young leaf tissues were used as an explant for obtaining callus on MS medium supplemented with different concentrations (0.5, 1.0, 1.5, and 2.0 µM) of IAA, IBA, NAA, and 2,4-dichlorophenoxyacetic acid (2,4-D) in combination

with 1.0 µM TDZ for the callus production. The optimum callus growth and maintenance was in 0.5 µM NAA plus 1.0 µM TDZ (Lata et al., 2010). On the other hand, Jiang et al., (2015) have used internodes of the new cultivar Long-ma of *C. sativa* as explants for tissue culture. In another recent study conducted by Movahedi et al., (2015), the best callus were obtained using cotyledon explant treated with 2 mg/1 TDZ and 0.5 mg/1 IBA.

In a recent work, Farag and Kayser (2015) have reported hairy root cultures of *C. sativa* (in callus using B5 medium + 4 mg/l NAA, under dark conditions) for the production of cannabinoids. However, a very low amount of cannabinoids have been detected.

DIRECT ORGANOGENESIS

Studies on organogenesis (direct or indirect) in Cannabis show the predominance of use of Thidiazuron (TDZ), a substituted phenylurea (N-phenyl-1,2,3-thidiazol-5-ylurea) with intrinsic cytokinin like activity (Huetteman and Preece, 1993). Compared to most other compounds, with cytokinins activity, TDZ can stimulate better shoot proliferation and regeneration (Lata et al., 2009a; 2009b; Parveen and Shahzad 2010).

For a hemp variety of cannabis, Wang et al., (2009) used shoot tips as explants for obtaining axillary bud induction using MS medium supplemented with different cytokinins (benzyladenine, BA; kinetin, KN and Thidiazuron, TDZ). Among the cytokinins tested by them, TDZ (0.2 mg/l) was found to provide the best shoot induction. For root induction different media, full strength MS, half strength MS, B5 and NN were tested. The best rooting and elongation was obtained on 0.1 mg/l IBA and 0.05 mg/l NAA on MS media with 85% rate of success in root development.

In our laboratory, we have successfully established a direct organogenesis protocol for drug type cannabis variety using nodal segments containing axillary buds as explants(Lata et al., 2009a). The quality and quantity of regenerants were better with thidiazuron (0.5 µM than with benzyladenine (BAP) or kinetin (Kn). Adding 7.0 µM of gibberellic acid (GA3) into a medium containing 0.5 µM thidiazuron slightly increased shoot growth. Elongated shoots when transferred to half-strength MS medium supplemented with 500 mg/L activated charcoal and 2.5 µM indole-3-butyric acid(IBA) resulted in maximum rooting.

Movahedi et al., (2015) used cotyledon and epicotyl explant on MS medium supplemented with various combinations of BA, TDZ or alone, to investigate micropropagation in *C. sativa* L. Both, callus formation (direct organogenesis) and direct shoot formation (indirect organogenesis) was observed. However, the callus formation was dominant over direct regeneration with cotyledon giving higher callus frequency and volume in TDZ (3.0 mg/l) in combination with IBA (0.5 mg/l), whereas, epicotyl showed better regeneration than cotyledon. Both BAP and TDZ, were individually effective in shoot formation and no significant differences were observed. Roots were obtained on 0.1 mg/l IBA. The highest shoot regeneration rate was achieved in calli produced from epicotyl treated with 2 mg/l BAP and 0.5 mg/l IBA. More recently, cotyledons were used as explants for the initiation of cultures (Chaohua et al., 2016). TDZ in MS medium was reported to be more efficient in inducing shoots than BAP or trans-zeatin (ZT). Based on their results 80% of shoots were able to develop roots on MS medium supplemented with 0.4 mg/l TDZ and 0.5 mg/l IBA.

Using meta-topolin (*m*T) in our laboratory, we have reported an effective one step regeneration system for *Cannabis sativa* L. (Lata et al., 2016). Nodal segments containing axillary buds from a selected vegetatively propagated plant (mother plant) were used as explants for initiation of shoot cultures. The maximum number of shoots was obtained in the treatment with 2.0 µM *m*T with maximum shoot

length. All the explants were capable of producing shoots. Most of the shoots were rooted in various concentrations of *m*T, however, the optimal concentration for rooting was obtained on MS medium supplemented with 2.0 µM *m*T, on which 100% of the regenerated shoots developed roots with an average of 18.7 roots per shoots within 4 weeks of transfer to fresh medium.

In a study conducted by Grulichova et al., (2017) shoot tips from 10-18 days old plantlets were used as explant. Plant grown in TDZ in combination with GA3 were reported to have tallest stems, plants grown with NAA + TDZ had highest fresh weight and plants grown with NAA + BAP had highest number of nodes.

Recently, Kodym et al., (2019) also have used shoot tips for the propagation and reported that in three weeks 97.5% rooting rate and successful acclimatization was achieved in glass vessels with passive ventilation. Concurrently, a study conducted by Piunno et al. (2019) provides micropropagation from floral tissues in *C. sativa*. Immature (three cultivars) and mature (one cultivar) floral explants were cultured on thidiazuron and shoot development was observed in both the immature and mature explants till maturity.

ELITE GERMPLASM CONSERVATION FOR FUTURE USE

Once a desirable elite germplasm is screened and selected, conservation of plant germplasm can be achieved by *in vitro* technology including micropropagation. Short term and long term conservation of elite germplasm has been practiced in many medicinal plant species (Narula et al., 2007; Faisal and Anis, 2007; Ray and Bhattacharya, 2008; Lata et al., 2009a; 2009b; 2010; 2012; 2016; 2019 and Uchendu et al., 2019).

For the short term conservation, growth of cultures are generally slowed down by reducing the temperature, by modifying culture media with supplements of osmotic agents, by the use of growth inhibitors or by removing growth promoters (Dodds and Roberts 1995). This techniqueh as the potential of prolonging the subculture interval, thereby maintaining the elite germplasm and reducing the overall cost of labor. However, for an efficient germplasm storage, the protocols should reflect maximum survival rate as well as chemical and genetic stability with minimum subculture frequency.

In our laboratory at National Center for Natural Product Research (NCNPR), School of Pharmacy, The University of Mississippi, we have successfully encapsulated axillary buds isolated from aseptic multiple shoot in calcium alginate beads to form 'synthetic seeds'. The best gel complexation was achieved using 5% sodium alginate with 50 mM $CaCl_2.2H_2O$. The highest rate of re-growth of seeds was achieved in Murashige and Skoog medium supplemented with thidiazuron (TDZ 0.5 µM) and Plant Preservative Mixture (PPM) (0.075%) under *in vitro* conditions (Lata et al., 2009b). After 6 months of storage at 15 °C 'synthetic seeds' were successfully re-grown and produced homogeneous and genetically stable cannabis plants (Lata et al., 2012).

Cryopreservation allows a safe and cost-effective long-term conservation method for important plant germplasm. For the long term conservation of cannabis germplasm two different systems are developed by our group at the NCNPR (Lata et al., 2019 and Uchendu et al., 2019).

In the first study, we have developed an efficient protocol for the cryopreservation of axillary buds of *Cannabis sativa* elite cultivars (MX, a high THC yielding variety and V1-20, High CBD yielding variety) by the V-cryoplate droplet-vitrification technique. The survival and regrowth rates of cryopreserved axillary buds of cultivar MX was reported to be 42 to 45% whereas, it was 44 to 47% for the cultivar V1-20. After cryopreservation, re-grown plants appeared normal, without any callus formation or morphogenetic

variation. On maturity, mother plants and re-grown cryopreserved plants were comparable in terms of Δ^9-THC and CBD content (Lata et al., 2019).

In an another study, Uchendu et al., (2019) have reported 55% regrowth of cryopreserved cannabis explants. This study was focused on the evaluation of three droplet vitrification protocols for long-term conservation of shoot tips in liquid nitrogen. Shoot tips (~0.5 mm) were excised from 3- to 4-week-old *in vitro*-grown shoots of three different cultivars (high THC yielding, Intermediate THC~CBD and High CBD yielding). These shoot tips are pretreated on 5% dimethyl sulfoxide agar plates for 48 h. The shoot tips were then vitrified in liquid nitrogen using three separate cryoprotectant (plant vitrification solutions *i.e.* PVS2, PVS3, PVS4) droplets on an aluminum cryoplate. Fifteen to 20 min of exposure to cryoprotectant PVS2 was reported to be most suitable for cryopreservation and for the regrowth (55%) of these varieties.

Thus, micropropagation is an important application of plant biotechnology and now is being used effectively for the propagation of cannabis. This review will allow researchers to use the updated information that has been documented to establish more efficient protocols for the propagation and conservation of their own cannabis varieties or germplasm.

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