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Review Article

Factors affecting micropropagation of *Cannabis sativa* L. : A review

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ABSTRACT

Cannabis sativa L. is a well-known herb that has been used for medicines and textile fibers for thousands of years. Its biomass and phytochemicals are precious sources for pharmaceuticals. Various preparations featuring bioactive compounds from *C. sativa* including tetrahydrocannabinol and cannabidiol have been reported to link with a wide range of health benefits. Micropropagation is an alternative method for production of these resources as the field-cultivation of *C. sativa* is strictly regulated in many countries. This review highlights the conditions for micropropagation of *C. sativa.* Factors affecting establishment of *in vitro* cultures including selection of explants and disinfection protocols, media and culture conditions, plant growth regulators used for cultivation of *C. sativa,* and protocols for acclimatization are reviewed and discussed. These successful established protocols for micropropagation of *C. sativa* are advantageous for further applied approaches such as mutation breeding, genetic engineering, somaclonal variation, and *in vitro* production of valuable bioactive components.

1. INTRODUCTION

Cannabis sativa L. is an annual herb of the family Cannabaceae extensively recognized as source of precious phytochemicals. The plant has been used for medicines and textile fibers throughout the history. During the past decade, *C. sativa* has attracted great interests in as much as attempts to legalize uses of *C. sativa* in many parts of the world. To date, medical cannabis is legal in some countries including Netherlands, Germany, Canada, and in some states of the United States.

Various preparations of *C. sativa* have been used in associated with several pharmacological disorders including pain, nausea and vomiting, anxiety, colitis, sleep disorder, multiple sclerosis Alzheimer's disease and Parkinson's disease[1]. The most recognized phytochemicals produced in *C. sativa* are cannabinoids, namely tetrahydrocannabinol (THC), cannabidiol (CBD), and up to 90 different minor cannabinoids[2]. Other active components that may contribute to pharmacological activities of *C. sativa* are terpenes and phenolic compounds.

Cannabis sativa is conventionally propagated via seeds and cuttings. Limitations of field cultivated *C. sativa* plants are

their heterozygosity and their susceptibility to diseases and pests. Furthermore, the cultivation of *C. sativa* in many countries is strictly regulated. Therefore, micropropagation using plant tissue culture is a promising alternative for propagation of pathogen-free true-to-type *C. sativa* stock plants. In this review, we focused on factors affecting micropropagation of *C. sativa* from the establishment of *in vitro* cultures, media and culture conditions, plant growth regulators for shoot multiplication and rooting, as well as protocols for acclimatization.

2. ESTABLISHMENT OF *IN VITRO* CULTURE

2.1. Selection of plant materials

The efficient clonal multiplication of desired true-to-type plants starts with the healthy mother plants. Selection of explants as starting materials to establish an *in vitro* culture is a crucial step for micropropagation. Generally, meristematic tissues are used for direct regeneration. Protocols for *C. sativa* micropropagation by direct organogenesis using cotyledons, axillary buds, and shoot tips as explants were established[3-5] (Table 1). For callus-mediated organogenesis, various explants are used for callus induction before shoot multiplication and rooting. Slusarkiewicz-Jarzina et al. reported that, among young leaves, petioles, internodes, and axillary buds, the highest callus frequency was obtained from petioles and young leaves[6]. Another study reported the effect of *C. sativa* explant types on callus induction and plant regeneration. Although there was no significant difference in callus induction among the explants tested, cotyledon-derived callus had the highest regeneration efficiency when compared with callus originated from stems and roots[7]. In addition to explant types, different cultivars also had different effects on callus formation and plant regeneration[“6-7”].

Table 1. Micropropagation protocols for C. sativa

Micropropagation strategy	Steps	Explants used (Explant disinfection)	Culture medium /PGRs	Culture conditions	Optimal experimental outcome	References
Direct regeneration	Shoot induction	Nodal segments containing axillary buds (0.50% sodium hypochlorite for 20 min)	MS medium/ 0.05 - 9.0 μM BA or 0.05 - 9.0 μM KN or 0.05 - 9.0 μM TDZ With or without 7.0 μM GA3	25 ± 2°C, 16/8 h photoperiod	0.5 μM TDZ resulted in 100% response (average 12.6 shoots)	Lata et al., 2009
	Rooting		½ MS medium/ 2.5 - 5.0 μM IAA or 2.5 - 5.0 μM IBA or 2.5 - 5.0 μM NAA with 500 mg/L activated charcoal	25 ± 2°C, 16/8 h photoperiod	2.5 μM IBA resulted in 94% response (average 4.8 roots)	
Direct regeneration	Culture initiation	Seeds (75% (v/v) ethanol for 30 s and 0.1% mercuric chloride for 10-15 min)	1/2 MS medium (1%(w/v) sucrose)	25±1°C, 16/8 h photoperiod		Wang et al., 2009
	Regeneration	Shoot tips	MS medium/ 1.0 - 5.0 mg/L BA or 1.0 - 5.0 mg/L KN or 0.1 – 0.5 mg/L TDZ with or without 0.05 - 0.5 mg/L NAA	25±1°C, 16/8 h photoperiod	0.2 mg/L TDZ and 0.1 mg/L NAA showed the highest auxiliary bud multiplication rate (3.22 buds per shoot tip)	

Table 1. Micropropagation protocols for C. sativa (cont.)

Micropropagation strategy	Steps	Explants used (Explant disinfection)	Culture medium /PGRs	Culture conditions	Optimal experimental outcome	References
	Rooting		MS medium/ 0.1 - 0.5 mg/L IBA with 0.05 - 0.25 mg/L NAA or 0.05 - 0.25 mg/L IAA	25±1°C, 16/8 h photoperiod	0.1 mg/L IBA and 0.05 mg/ L NAA showed the highest rooting (85%)	
Direct regeneration	Culture initiation	Seeds (sulfuric acid for 20 s tap water 20 min, 75% (v/v) ethanol for 2 min, 3% (w/v) sodium hypochlorite for 20 min)	MS medium	22±2◦C, 16/8 h photoperiod		Cheng et al., 2016
	Shoot induction	*In vitro* cotyledons (2-6 day old)	MS medium/ 0.1 - 0.6 mg/L TDZ with or without 0.2 - 0.6 mg/L NAA	22±2◦C, 16/8 h photoperiod	3-day old cotyledon on MS medium + 0.4 mg/L TDZ + 0.2 mg/L NAA resulted in 51.7% induction frequency (3.0 shoots per explant)	
	Rooting		1/2 MS medium/ with or without 0.2 -2.0 mg/L IBA	22±2◦C, 16/8 h photoperiod	0.5 - 2 mg/ L IBA show 80% rooting	
Direct regeneration	Shoot induction/ rooting	Nodal segments containing axillary buds (0.50% sodium hypochlorite +0.1%Tween 20 for 20 min)	MS medium/ 0.05 - 5.0 µM TDZ or 1/2 MS medium/ 0.05 - 5.0 µM IBA or MS medium/ 0.05 - 5.0 µM mT	25 ± 2°C, 16/8 h photoperiod	MS medium + 2.00 µM mT showed 100% shoot response, average 13.4 shoot and 96% root response, 13.8 roots)	Lata et al., 2016
Indirect regeneration via callus	Culture initiation	Seeds (5% calcium hypochlorite for 6, 8, 15 min)	MS medium	22°C, 12/12 h photoperiod		Slusarkiewicz-Jarzina et al., 2005
	Callus induction and indirect regeneration	*In vitro* young leaves, petioles, internodes, and axillary buds	MS medium/ 1.0 - 4.0 mg/L KN or 0.5 - 2.0 mg/L NAA or 2.0 - 4.0 mg/L 2,4-D or 2.0 - 3.0 mg/L DIC	24°C, Darkness for 2-3 weeks callus induction, then, 16/8 h photoperiod for indirect regeneration	Petiole explants with 2.0-3.0 mg/ L DIC showed highest frequency of callus formation (82.7% of explants) and plant regeneration (6.0% of callus from all cultivars)	

Table 1. Micropropagation protocols for C. sativa (cont.)

Micropropagation strategy	Steps	Explants used (Explant disinfection)	Culture medium /PGRs	Culture conditions	Optimal experimental outcome	References
	Rooting		MS medium / 1.0 mg/L IAA and 1.0 mg/L NAA	24°C, 16/8 h photoperiod	MS medium/ 1.0 mg/L IAA and 1.0 mg/L NAA	
Indirect regeneration via callus	Culture initiation	Seeds (10 s 70% ethanol and 1% sodium hypochlorite for 20 min)	DARIA medium	24°C, darkness for 4-7 days		Wielgus et al., 2008
	Callus induction	Cotyledons, stems, and roots	DARIA medium/ 1 mg/L KN and 0.05 mg/L NAA	24-26°C, 16/8 h photoperiod	Cotyledon explants showed the highest callus induction efficiency and stem explants showed the highest regeneration percentage	
	Indirect regeneration		DARIA medium/ 0.2 mg/L BA and 0.03 mg/L NAA	24–26°C, 16/8 h photoperiod	DARIA medium/ 0.2 mg/L BA and 0.03 mg/L NAA	
	Rooting		DARIA medium/ 2.0 mg/L IAA	24–26°C, 16/8 h photoperiod	DARIA medium/ 2.0 mg/L IAA	
Indirect regeneration via callus	Callus induction	Young leaves (0.50% sodium hypochlorite +0.1% Tween 20 for 20 min)	MS medium/ 0.5 - 2.0 μM IAA or 0.5 - 2.0 μM IBA or 0.5 - 2.0 μM NAA or 0.5 - 2.0 μM 2,4-D or 1.0 μM TDZ	25 ± 2°C, 16/8 h photoperiod	0.5 μM NAA + 1.0 μM TDZ resulted in 93% response and the highest amount of callus	Lata et al., 2010
	Shoot induction		MS medium/ 0.5 - 10.0 μM BA or 0.5 - 10.0 μM KN or 0.5 - 10.0 μM TDZ	25 ± 2°C, 16/8 h photoperiod	0.5 μM TDZ resulted in 93.3% response (12.3 shoots)	
	Rooting		MS medium/ 0.5 - 10.0 μM IAA or 0.5 - 10.0 μM IBA or 0.5 - 10.0 μM NAA	25 ± 2°C, 16/8 h photoperiod	2.5 μM IBA resulted in 96.6% response (10.0 roots)	
Indirect regeneration via callus	Culture initiation	Seeds (30 s 70% alcohol, 2% sodium hypochlorite + 1 drop of tween 20 for 20 min, 5 min 0.05% mercuric chloride	MS medium	25°C, 16/8 h photoperiod		Movahedi et al., 2015

Table 1. Micropropagation protocols for C. sativa (cont.)

Micropropagation strategy	Steps	Explants used (Explant disinfection)	Culture medium /PGRs	Culture conditions	Optimal experimental outcome	References
	Callus induction	*In vitro* cotyledon and epicotyl	MS medium/ 0.1 - 3 mg/L BA or 0.1 - 3 mg/L TDZ with or without 0.5 mg/L IBA	25°C, 16/8 h photoperiod	Cotyledon explants treated with 2 mg/L TDZ + 0.5 mg/L IBA showed the highest callus fresh weight	
	Shoot induction		Same media as callus induction	25°C, 16/8 h photoperiod	Callus from epicotyl explant treated with 2 mg/L BA + 0.5 mg/L IBA showed the highest shoot regeneration rate	
	Rooting		MS medium/ 0.1 - 1 mg/L NAA or 0.1 - 1 mg/L IBA	25°C, 16/8 h photoperiod	0.1 mg/L IBA showed the highest rooting rate	

Abbreviations : PGRs plant growth regulators, MS Murashige and Skoog medium, BA benzyladenine, KN kinetin, TDZ thidiazuron, GA3 gibberellic acid, IAA indole-3-acetic acid, IBA indole-3-butyric acid, NAA 1-naphthaleneacetic acid, mT meta-topolin, 2,4-D 2,4-dichlorophenoxyacetic acid, DIC 3,6-dichloroanisic acid

2.2. Disinfection

To initiate contamination-free *in vitro* culture, plant materials from field cultivation need to be surface-sterilized. Several studies germinated seeds of *C. sativa* on culture medium and used seed-derived *in vitro* explants for further experiments. Generally, seeds were washed thoroughly with running tap water and optional 70-75% ethanol followed by a disinfection reagent. The prepared seeds were then rinsed with distilled water before transferred to the culture medium for germination. Commonly used disinfectants for seeds sterilization are 1-3% sodium hypochlorite[5,7]. In addition, 5% calcium hypochlorite[6] and 0.1% mercuric chloride[4] were also used. In some studies, cultivated nodal segments and young leaves were directly surface sterilized to establish the *in vitro* cultures. The concentration of disinfectant was lower, i.e. 0.5% sodium hypochlorite with 0.1% Tween 20 as surfactant[3,"8,9"], since these tissues were more delicate than seeds.

3. MEDIA AND CULTURE CONDITIONS

3.1. Culture media

Artificial media for *in vitro* cultures are generally composed of macronutrients, micro-nutrients, carbon sources, and vitamins, with optional gelling agent and some additives or plant growth regulators. The most frequently used basal medium for cultivation of *C. sativa* is Murashige and Skoog (MS) medium[10] with 3% (w/v) sucrose as carbon source. Modified MS media were also reported. Wang et al. used half-strength MS medium with 1% (w/v) sucrose for seed germination and transferred the *in vitro* explants to full-strength MS medium with 3% (w/v) sucrose supplemented with various plant growth regulators for shoot and root induction[4]. Only one study used Daria ind medium, which the authors explained as a modification of Knopp's solution medium, for seed germination and Daria medium supplemented plant growth regulators for regeneration via callus of *C. sativa*[7].

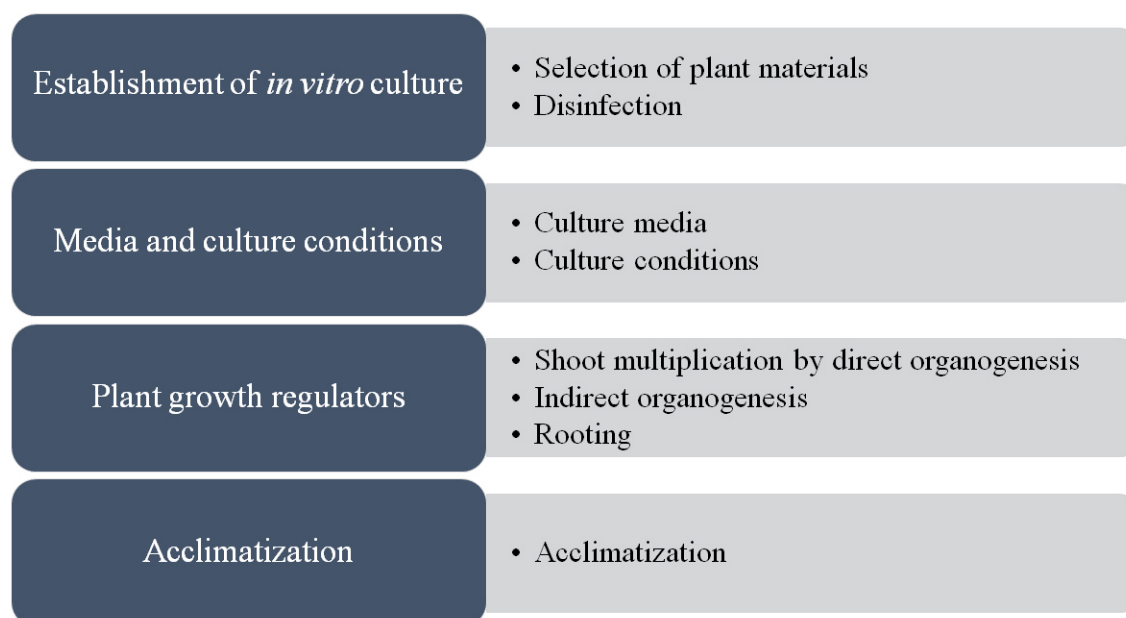

Figure 1. Schematic diagram of steps in micropropagation

3.2. Culture conditions

In most studies, *in vitro C. sativa* cultures were cultivated at 22-26 ºC with a 16-h photo period. However, seed germination and callus induction were carried out in darkness in some studies[6-7]. Details on effects of culture conditions and light sources on *C. sativa* plant regeneration were limited. However, a study in field-cultivated *C. sativa* clonally propagated via cuttings suggested that bud biomass and phytochemical profiles were improved when treated with supplemental red-blue and red-green-blue subcanopy lights[11]. Therefore, future studies could pay attention on effects of different light spectra and intensities on *in vitro* development of *C. sativa.*

4. PLANT GROWTH REGULATORS

Plant growth regulators, especially auxins and cytokinins, play a very important role on *in vitro* development, morphogenesis, and growth of plant tissue cultures[12]. Shoots can be induced directly from explants (direct organogenesis) or via callus (indirect organogenesis), which generally involves appropriate combination of auxins and cytokinins. After shoot elongation and multiplication, adventitious root formation is stimulated. Plant growth regulators used in micropropagation of *C. sativa* are discussed according to stages of culture development.

4.1. Shoot multiplication by direct organogenesis

To establish a protocol for propagation of elite *C. sativa* clones, Lata et al. studied the effects of different concentrations (0.05 - 9.0 µM) of cytokinins- benzyladenine (BA), kinetin (KN), and thidiazuron (TDZ)- on multiplication and proliferation of nodal explants containing axillary buds[3]. Among the three cytokinins, TDZ showed the highest response of shoot induction. Shoots were proliferated on all explants within 14 d when cultured on MS medium supplemented with 0.5 µM TDZ, with average 13 shoots per explant and average 7.1 cm of shoot length. Slight increase, although statistically insignificant, in shoot growth was observed when transferring cultures to MS medium supplemented with 0.5 µM TDZ in combination with 7.0 µM gibberellic acid (GA3)[3]. Although cytokinin alone is sufficient for shoot multiplication, studies showed that adding a low concentration of auxin is beneficial in some cases[12]. In this matter, Wang et al. studied effects of BA, KN, and TDZ, either alone or in combination with auxin 1-naphthaleneacetic acid (NAA), on bud formation of shoot tip explants[4]. The best response was obtained from explants cultured on MS medium containing 0.2 mg/L TDZ and 0.1 mg/L NAA, with the highest auxiliary bud multiplication rate of 3.22 per shoot tip. Similarly, Cheng et al. found that MS medium supplemented with 0.4 mg/L

TDZ and 0.2 mg/L NAA yielded the best response of shoot regeneration from cotyledon explants, with 51.7 ± 7.2 % of explant forming shoots and average 3.0 ± 0.4 shoots per explants. Recently, efficient micropropagation of *C. sativa* was achieved using novel aromatic cytokinin, meta-topolin (mT). The best response of shoot formation was obtained from nodal segments cultured on MS medium supplemented with 2 µM mT, with 100% of explant producing shoots, average 13.44 ± 1.38 shoots per explant, and average shoot length of 11.44 ± 0.80 cm[13].

4.2. Indirect organogenesis

Indirect organogenesis requires shoot regeneration from morphogenic callus. Slusarkiewicz-Jarzina et al. studied influence of plant growth regulators–KN, NAA, 2,4-dichlorophenoxyacetic acid (2,4-D), and 3,6-dichloroanisic acid (DIC)– on callus induction and plant regeneration of *C. sativa*. The results showed that, of all media used, MS medium supplemented with 2.0 – 3.0 mg/L DIC yielded the highest frequency of callus formation (82.7% of explants). However, plant regeneration rate from all media tested were very low (1.4% of plated callus)[6]. Successful callus-mediated *C. sativa* regeneration was reported by using combination of auxin and cytokinin[“7-8”, 14]. In order to initiate callus cultures, leaf explants were cultured on MS medium supplemented with different concentrations of various auxins, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), NAA and 2,4-D, in combination with cytokinin TDZ. High callus amount with 93% callus response were obtained from explants cultured on MS medium containing 0.5 µM NAA and 1.0 µM TDZ. Then the shoots were induced by MS medium containing 0.5 µM TDZ[8]. Similarly, Movahedi et al. obtained high callus formation from cotyledon explants cultured on MS medium supplemented with 0.5 mg/L IBA in combination with 3 mg/L TDZ[14]. Another group of researchers also obtained successful *C. sativa* plant regeneration using Daria medium supplemented with combination of auxin and cytokinin – 0.05 mg/L NAA and 1 mg/L KN for callus induction, and 0.03 mg/L NAA and 0.2 mg/L BA for shoot regeneration[7].

4.3. Rooting

Unlike shoot and callus induction, root induction is generally achieved by supplementation of auxin alone whereas exogenous cytokinins often inhibit root induction[12]. For micropropagation of *C. sativa,* the most frequently used auxins are IBA[“3-5”, 8] and IAA[“6-7”]. In some studies, highest rooting frequencies were obtained by using combination of natural auxin and synthetic auxin such as NAA[“4,6”]. Interestingly, recent study reported that a separate rooting step by medium supplemented with auxin was not required for *C. sativa* regenerated on MS medium containing mT, a novel aromatic cytokinin, as the healthy roots were able to regenerate from the shoots multiplied within 4-6 weeks[13].

5. ACCLIMATIZATION

Acclimatization is a crucial step when transferring plants from *in vitro* cultures into filed or greenhouse. In the *in vitro* cultures, plants are cultivated under special conditions including availability of nutrient and growth regulators, aseptic condition, and high humidity[15]. Efficient acclimatization process provides environmental adaptation for minimal damage of plants before being transferred to soil. To increase survivability of *C. sativa in vitro* plantlets, protocols for acclimatization had been established and reported. Wang et al. deflasked *C. sativa* plantlets when the intro roots reached 0.5-1.0 cm length and cultured in the bottle poured with sterile water. After a week, the plantlets were washed in running tap water to remove any remained medium followed by 0.2% (w/v) Bavistin1, a fungicide solution. The plantlets were transferred to be cultured on perlite and hardened by an extra 3-4 weeks in controlled conditions of culture room, followed by 2 weeks cultured in semi controlled conditions in the shade house, and finally transplanted in field, after which allowed 95% of *in vitro* plantlets to survive for 3 months[4]. Another acclimatization protocol using coco natural growth medium, a commercially available medium for hydroponic plants made from coconut fibers, was reported. In this protocol, *C. sativa* rooted shoots were taken out of the *in vitro* medium and washed thoroughly in running tap water. The plantlets were preincubated coco natural growth medium in a thermocol cup covered with polythene bags to maintain humidity for 10 day. After that, they were acclimatized in ferti-

lome, a sterile potting mix, in large pots. The plants reached 14-to 16-cm height within 6 week and exhibited 95% survival rate at 8 wk after transfer[3].

6. CONCLUSIONS

As *C. sativa* conventional field propagation proved difficult by its susceptibility to plant pathogens, variation in bioactive components, and a strict regulatory in many countries, *in vitro* cultures can be a potent alternative for production of elite *C. sativa* plant materials in the controlled conditions. Fortunately both direct and indirect plantlet regeneration were reported to be highly successful using various explant types. Most study used MS medium as the basal medium for *in vitro* cultures. Combination of both auxins and cytokinins as plant growth regulators were suitable for callus induction and shoot multiplication in most studies while rooting step generally required only auxin alone. Despite the successful of *C. sativa in vitro* culture establishment, the further challenging step lies in the improvement of active compound accumulated in *C. sativa in vitro* cultures.

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