Production of cannabinoids using tissue culture and suspension culture

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Abstract:

The primary effective ingredients of hemp are cannabinoids. Cannabinoid derived drugs are used as palliative, anti-inflammatory, appetite stimulant, antibiotic and anti-cancer. Tissue culture technology is a biotechnological tool for multiplication and genetic enhancement of medicinally plants. The in vitro culture of *Cannabis sativa* has advantage over traditional methods of multiplication not only due to high propagation rate and production of disease-free elite plant but also in dealing with the problems of heterozygosity because of its allogamous. In this study we plan to stablish an optimized medium culture to produce cannabinoids using tissue culture and suspension culture.

Introduction

For thousands of years, *Cannabis sativa* has been used both as a stem source and edible seeds (Chandra & Lata, 2017). It produces a distinctive category of terpenophenolic compounds called cannabinoids (Chandra & Lata, 2017) which is the principle active components of hemp, other compounds like terpenes and phenolic have also been reported (Josefina & Verpoorte, 2008). In recent years, the pharmacological characteristics of cannabinoids have been widely studied and new applications of cannabis extracts have been proposed (Wróbel, Dreger, Wielgus, & Słomski, 2018).

Based on the production source, cannabinoids have been categorized into three groups; phytocannabinoids, endogenous cannabinoids, and synthetic

cannabinoids (Roberto, 2018). Resources have reported nearly 565 Cannabis constituents in *Cannabis sativa* , out of which 120 are phytocannabinoids some of which have been extensively explored as potentially therapeutic. The predominant cannabinoids in plant material are delta-9-tetrahydro-cannabinol (THC), cannabidiol (CBD) and cannabichromone (CBC) (Wróbel et al., 2018). Table 1 depicts, the importance therapeutic effects of various phytocannabinoids

Due to the medicinal value of cannabinoids, growing cannabis is being a flourishing industry. The leading countries in the marketplace are The USA, Canada and Australia with several investments in both cultivation and manufacturing facilities.

Table 1- The therapeutic effects of phytocannabinoids

Phytocannabinoids	Therapeutic Effects
delta-9-tetrahydro-cannabinol (THC)	Modulation of pain, spasticity, sedation, appetite and mood; Bronchodilator; Neuroprotective and antioxidant;

	Antipruritic in cholestatic jaundice;
	Anti-inflammatory (power: 20x aspirin and 2x hydrocortisone);
	Analgesic;
CANNABIDIOL (CBD)	Anticonvulsive;
	Anti-inflammatory and immunosuppressive;
	Neuroprotective and antioxidant;
	Antipsychotic;
	Counteracts the intoxicating effects of cannabis;
	Anxiolytic;

	Addiction treatment;
	Muscle relaxant;
	Analgesic;
	Modest antifungal;
	Antineoplastic;
	Antidepressant;
CANNABIGEROL (CBG)	Inhibition of keratinocyte proliferation in psoriasis;
	Antibiotic activity against methicillin-resistant Staphylococcus aureus (MRSA);
	Analgesic;
CANNABICHROMENE (CBC)	Anti-inflammatory;
CANNABINOL (CBN)	Sedative;

	Anticonvulsive; Anti-inflammatory; Antibiotic (with anti-MRSA activity); Inhibition of keratinocyte proliferation; Promotion of bone formation;
TETRAHYDROCANNABIVARIN (THCV)	Promotion of weight loss; Anticonvulsive; Suppression of hyperalgesia and inflammation; Appetite suppression; Counteracts the intoxicating

	effects of cannabis;
	Immunomodulatory;
TETRAHYDROCANNABINOLIC ACID (THCA-A)	Anti-inflammatory;
	Neuroprotective;
	Antineoplastic
CANNABIDIVARIN (CBDV)	Anticonvulsant;

Cannabis is an allogamous plant thus its progeny display considerable heterozygosity. Although, seed is the predominant means of propagation technique of cannabis, however, preserving the elite cultivar/clone by seed is impossible and the majority of the resulting offsprings in this way are male plants.

Thanks to greater levels of THC and CBD in female plants, they are more favored for cultivation rather than male plants. To preserve uniformity and genetic purity, majority of the cannabis used for therapeutic purposes is cultivated in green houses using vegetative techniques. The fact is that, cutting is a labor intensive and time-consuming method, also, indoors grown crops are prone to pests that reproduce quickly (spider, mites and aphids). Biotechnology, in particular plant tissue culture and cell suspension techniques are substitute to germplasm collections and breeders. These

promising techniques not only play a prominent role in quick propagation of cultivars with desirable traits but also helps in the large-scale production of disease-free plants and offer an opportunity for high yield cannabinoids production (Lineberger 1983).

Developing an in vitro system for cannabinoids production in *C. sativa* is vital. Cannabinoid drugs derived from the female cannabis plants cannot compete with in vitro tissue or cell culture in terms of reliability and reproducibility, for drug production, in vitro systems would be desired. The production of in vitro secondary metabolites can be possible through plant cell cultures. The technology represents an efficient model to combat many problems linked to traditional agriculture like variations in the crop quality due to environmental factors: drought, flooding and other abiotic stresses and/or biotic stresses as diseases or pest attacks. Additionally drops in storage and handling might result in a decrease production of the secondary metabolites and it is hard to prevent (Flores-Sanchez et al., 2009).

This study aims to explore the best possibilities to produce cannabinoids from cannabis by efficient and comprehensive *in vitro* culture and cell suspension culture protocols. To achieve this, the following sub-aims are established.

1. To assess the production of cannabinoids by using different commercial and cost-effective *in vitro* culture protocols of Cannabis;
2. To investigate the difference of cannabinoids production by in-vitro propagating of various explants derived from a mother plant;

3. To determine the effect of elicitors on inducing and enhancing the cannabinoids production in suspension culture.

Literature Review

Studies have suggested seedling parts (radicle, hypocotyl, epicotyl and cotyledon), to start the multiplication studies, however, Chandra et al. 2010 have used, nodal parts having axillary buds, this part has genetic uniformities among the clones.

Micropropagation as a rapid multiplication method, is fundamental in the preservation of elite clones and novel germplasm. Chandra & Lata, 2017 developed in vitro protocols for cell suspension cultures, callus production, agrobacterium mediated hairy root manipulation and plant regeneration. However, these techniques have been widely applied over the past years, the regeneration in Cannabis plantlets via in vitro propagation, still remains a big challenge.

Mostly, the propagation of Cannabis has been achieved by two distinct organogenesis ways namely direct and indirect. The Murashige and Skoog (MS) medium is the most frequently used formulation for in vitro propagation of Cannabis (Murashige and Skoog 1962). However, the use of media such as Millers medium, DARIA ind, MB medium and B5 have also been reported (Feenay and Punja 2003; Plawuszewski et al. 2006; Wieglus et al. 2008).

Direct propagation

For direct organogenesis, nodal parts with axillary buds, cotyledons, shoot tips and epicotyls were used (Lata et al. 2009b; Wang et al. 2009). Best MX

narcotic variety multiplication rates were obtained for nodal parts using MS medium with thidiazuron (Lata et al. 2016; Chandra et al. 2010) used MS mediums with metatopolin. plantlets were rooted on half-strength MS with IBA (Lata et al. 2009b). Chandra et al. 2010 reported that the quantity of cannabinoids extracted from female plants propagated in vitro and vegetative method did not show significant difference.

The results for fibrous varieties were lower considerably (Wang et al. 2009; Chaohua et al. 2016). Shoot tips of the Chinese cannabis variety generated 3. 2 shoots per tip on MS medium supplemented with TDZ and NAA. Wang et al. (2009) reported an 85% rooting rate on half-strength MS medium containing IBA and NAA

Indirect; Callus/Cell suspension

Recourses have reported some shoot regeneration via callus, they showed that indirect organogenesis of Cannabis was less fortunate (Slusarkiewicz-Jarzina et al. 2005; Wielgus et al. 2008; Lataetal. 2010a, b; Movahedi et al. 2015; Chaohua et al. 2016). Mandolino and Ranalli's study on hemp (1999) was the first attempt to obtain callus-derived shoots. They reported occasional plant regeneration via callus tissue. Feeney and Punja (2003) used cotyledons, stem petioles and leaves of four fibrous cannabis varieties to obtain callus. The MS medium with B5 vitamins supplemented with 2, 4-D and BA or KIN was the most efficient medium in terms of callus production, roots developed with no shoot. Slusarkiewicz-Jarzina et al. (2005) usied various explants (internodes, axillary buds, young leaves and petioles) to acquire a callus capable of regenerating shoots. The highest frequency of

callus induction was obtained from petiole explants on MS medium with dicamba. After 6 weeks of incubation and depending on the variety, Plantlets formed on the same medium with a total efficiency of 1. 4–2. 5%. Hemp variety has a Significant influence on the explant reaction and Wielgus study (2008) found the effect of plant regeneration. In the abovementioned studies different explants for callus induction was used and they reported no differences between the tested varieties. However, callus obtained from different genotypes showed various plant regeneration capacity. The highest regeneration rate was observed for cotyledon explants. Chaohua et al. (2016) by using cotyledons as donor explants devised a protocol to obtaine shoot regeneration via callus. They reported that the highest induction frequency was recorded in MS medium containing thidiazuron and naphthalene acetic acid. The age of donor explants was an important factor, as younger cotyledons produced a higher number of explants forming shoots than the older ones. Regeneration via callus from epicotyls was also recorded (Movahedi et al. 2015). The latter study used epicotyls and cotyledons of Iranian cannabis. Callus was induced on MS medium with TDZ and Indole-3-butyric acid. The highest shoot regeneration rate was obtained for callus derived from epicotyl on MS medium with BA and Indole-3-butyric acid.

The first in vitro cannabinoid production happened in the 1980s and resulted in the conversion of olivetol and CBD to cannabielsoin in callus cultures. Callus was initiated from young leaves on MS (Loh et al. 1983) and B5 medium (Braemer and Paris 1987) containing 2, 4-D and KIN. They reported inefficient and unstable cannabinoid production.

External precursors (CBGA) had to be added to the medium in order to induce cannabinoids production in callus did not produce. Further studies showed that undifferentiated callus tissues, derived from flowers, did not synthesize cannabinoids (Sirikantaramas et al. 2005; Staginnus et al. 2014). Callus were used to set up cell suspension cultures. By using both biotic and abiotic elicitors, Flores-Sanchez et al. (2009) devised an elicitation study in cell suspension cultures. Despite using various types of elicitors, enhanced biosynthesis of cannabinoids did not occur. This study confirmed that callus could not produce cannabinoids thanks to its undifferentiated nature. The analysis of THCA gene expression showed than only flowers and leaves generated trichomes that were able to synthesize cannabinoids (Flores-Sanchez and Verpoorte 2008).

Hemp seedlings do not produce cannabinoids but low expression of the THCA gene has been reported. It can be inferred from these reports that biosynthesis of cannabinoids is related to tissue and organ development it is said to be controlled by several genes.

Fibrous cannabis still needs a simple and efficient regenerative system. The developed protocols are efficient, to some extent, but limited to few narcotic varieties. The problem with fibrous hemp is that plant regeneration in these varieties still needs further studies and shoot induction stage for both direct and indirect organogenesis should be improved (Slusarkiewicz-Jarzina et al. 2005; Wielgus et al. 2008; Wang et al. 2009; Chaohua et al. 2016). Improvements in regenerative capacity of cannabis should consider genotype selection of explant donors and modification in protocols.

Agrobacterium Mediated Transformation

Combining gene manipulation techniques with an efficient protocol of callus formation and cell suspension culture may result in a reliable cannabinoids production.

Hairy root cultures technology has fundamentally changed the role of plant cell culture technology in secondary metabolite synthesis (Toivonen 1993). It offers a substitute in vitro source for the accumulation of fine biological chemicals as compared to plant suspension cultures thanks to more biochemical and genetic stability (Liu et al. 1998; Farag and Kayser 2015). Feeney and Punja were the pioneers in cannabis transformation (2003). The agrobacterium mediated transformation resulted in well-developed calli on MS medium with B5 vitamins supplemented with 2, 4 D and KIN. However, the obtained callus did not regenerate. In 2006, Wahby et al used *A. rhizogenes* for root infection. They identified secondary metabolites in Cannabis roots. Later on, they continued the study in 2013, and used two different Agrobacterium species namely A. *tumefaciens* (for calli) and A. *rhizogenes* (for hairy root). The work suggested that hypocotyl of intact seedlings can be the best choice for the establishment of C. sativa hairy root cultures, however, shoots did not regenerate. In 2015, Farag and Kayser have reported hairy root cultures of C. sativa from callus induced using B5 medium supplemented with naphthalene acetic acid under dark conditions, for the production of cannabinoids. They reported very low amount of cannabinoids.

Heterologous expression of cannabinoids

Insect (*Spodoptera frugiperda*) expression systems were the first non-plant ectopic hosts for expression of a recombinant THCA synthase and CBDA synthase (Sirikantaramas et al. 2004; Taura et al. 2007a, b). Although the activity of the recombinant THCA synthase was like native enzyme, the recombinant CBDA synthase showed fairly low levels of activity.

Secreted recombinant THCA synthase was also obtained from Pichia pastoris (Taura et al. 2007a, b). Zirpel et al. (2015) managed to produce an intracellular THCA synthase in *Saccharomyces cerevisiae* and *Pichia pastoris* they used a signal peptide from the vacuolar protease proteinase A. These studies demonstrate an efficient system designed for recombinant production of THCA. Recombinant THCA production can also be accomplished in transgenic tobacco (*Nicotiana tabacum*). In 2004 Sirikantaramas used hairy roots technology for ectopic expression of the gene THCA. They obtained THCA upon CBGA feeding, representing only 8. 2% bioconversion from CBGA. This could be due to the toxicity of both the substrate and product to tobacco hairy roots. These results confirm that heterologous expression of cannabinoids is possible.

CRISPR/Cas9-based technology has opened new venues in this regard but there are no studies reported on this subject.

Secondary metabolite production in bioreactors can be a quick and non-controversial method to obtain cannabinoids. For this goal, callus and cell suspension cultures were grown and elicited with various elicitors. However, they did not manage to produce cannabinoids, due to the fact that biosynthesis of THCA is tighten to organ development and cell differentiation

Extraction

Apparently, there are three extraction methods to purify cannabinoids from Cannabis plant;

1. Supercritical Fluid extraction-FLE
2. Hydrocarbon extraction
3. Alcohol extraction

Supercritical Fluid extraction-FLE (CO_2) seems to be more expensive and time consuming method, Hydrocarbon extraction (Butane, Propane and Pentane) is reported to have flammability risk (Rovetto & Aieta, 2017) and Alcohol extraction with less combustible and highest efficiency is more desirable method to extract cannabinoids in less time (Nn, 2015).

Methodology

1. Search with various keywords i. e. “ tissue culture”, “ hairy root”, “ suspension culture”, “ Cannabis”, “ cannabinoids”, “ medicinal values”, “ secondary metabolites”, “ metabolite engineering”, “ heterologous expression”, “ ectopic expression” and etc.
2. Literature review to stablish a robust inclusion/exclusion selection criteria in terms of direct/indirect in vitro culture, explant selection, formulating an optimized medium culture, elicitor selection (biotic/abiotic), analysis (GC-MS, HPLC, NMR or GC-Mass) and extraction methods (Supercritical Fluid extraction-FLE, Hydrocarbon extraction or Alcohol extraction) and etc.;

3. Experimental design to start the study and develop reproducible and replicable procedure to establish an efficient in vitro solid and liquid culture system for biosynthesis of cannabinoids;
4. Evaluating the results, data extraction, data analysis, and presenting the results.

Outcome

Devising an efficient protocol for high yield cannabinoids production via in vitro culture.

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