IN VITRO CELL CULTURE OF *CANNABIS SATIVA* FOR THE PRODUCTION OF CANNABINOIDS

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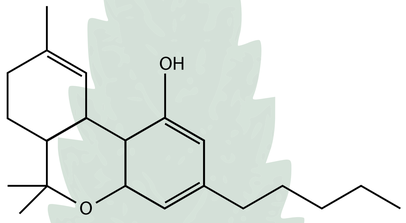
Degree in Biotechnology, Universitat Autònoma de Barcelona, Bellaterra 2014

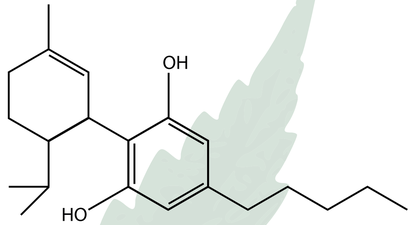
Universitat Autònoma de Barcelona

INTRODUCCION

Cannabis has a long history of medicinal use in the Middle East and Asia, with references as far back as the 6th century BC.
Cannabis sativa contains a unique class of terpeno-phenolic compounds (cannabinoids or phytocannabinoids) which have been extensively studied since the discovery of the chemical structure of tetrahidrocannabinol (Δ9-THC) commonly known as THC, which is the main constituent responsible for the psychoactive effects.
The accumulation of THC is mainly found in glandular trichomes of the flowers of the female plant. A total of 537 Cannabis constituents including 109 phytocannabinoids have been reported in *C. sativa*.
Pharmaceutical research companies are developing new natural cannabinoid formulations and delivery systems that will meet government regulatory requirements. Cannabis is used to relieve nausea and secondary to chemotherapy, pain, vomiting, spasticity in multiple sclerosis and increase hunger in anorexia.



Tetrahydrocannabinol (THC)



Cannibidiol (CBD)

Figure 1. Mollecular structure of THC and CBD

PROCESS

We have proposed a system of *in vitro* cell culture of C. sativa to produce cannabinoids, because potential drugs derived from cannabis would no longer be produced by the extraction of the cannabinoids from of the entire female plant that can't compete the reliability and reproducibility of *in vitro* cell culture and therefore would be desired when it comes to producing a drug.
The production of *in vitro* secondary metabolites can be possible through plant cell cultures. This technology represents a good model to overcome many problems linked to the conventional agriculture such as variations in the crop quality due to environmental factors: drought, flooding and other abiotic stresses and/or biotic stresses as diseases or pest attacks. Moreover crop adulteration, losses in storage and handling may decline the secondary metabolites production, which cannot be prevented by inability of some authorities.


Figure 2. Female flower used as explant


Figure 3. Callus induction of *C. sativa*


Figure 4. Cell suspension in a bioreactor

ELICITORS	
BIOTIC	**ABIOTIC**
chitosan	**sodium orthovanadate**
chitin	**vanadyl sulphate**
elicitin	

Table 1. Proposed elicitors for a cell culture of C. sativa

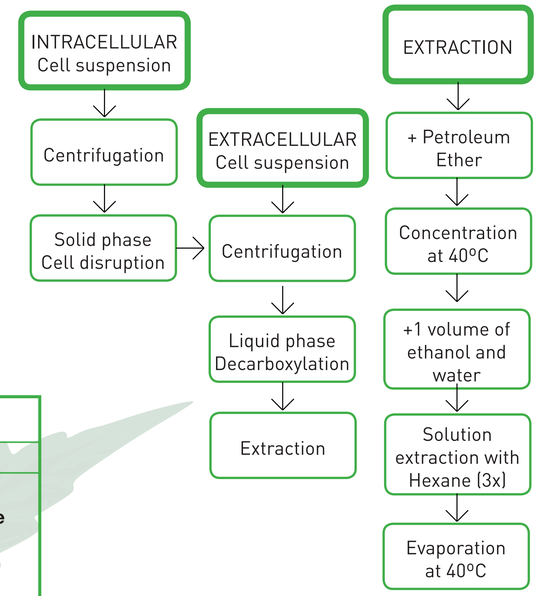


Table 2. Downstream process

Explant

Explant culture is a technique used for the isolation of cells from a piece or pieces of tissue. Tissue harvested in this manner is called an explant. It can be a portion of the shoot, leaves, flowers or some cells from a plant.
The tissue is harvested in an aseptic manner, often minced, and pieces placed in a cell culture dish containing growth media. Over time, progenitor cells migrate out of the tissue onto the surface of the dish. These primary cells can then be further expanded and transferred into fresh dishes.
For plant the material, we propose to use the flowers of the female plant to make explants, as it is in those flowers where the cannabinoids are produced in the plant.

Callus

The first step for obtaining cell culture is inducing callus. This stage involves the dedifferentiation of plant material. Differentiated cells that are part of the plant tissues will lead to an unorganized group of cells that are called callus. These cells can regenerate a new plant, this ability is called totipotency (describes the ability to regulate cell division and give rise to all different cell types of the vegetable body). But for this change of tissue, it is essential a certain combination of growth regulators or phytohormones. In our case, growth regulators are always added to the culture medium of Murashige-Skoog (MS) medium used previously by other authors, for the *in vitro* culture of the plant species
Sterile explants are cut in pieces of about (0.2 x 0.2) cm2.
They are then transferred into petri dishes with solid MS medium.
The medium brings the auxin responsible of the callus induction 2,4-D at 1 mg/L.

Cell suspension

Callus obtained in the petri dishes are used to set up cell suspension cultures. Approximately 1.5 g of callus from each explant is chopped to smaller particles and suspended into 250 mL flask with 50 mL of liquid MS medium.
The suspension cultures are grown at 25ºC under continuous light (intensity 1,200-1,800 lux) on a gyratory shaker at 110 rpm.
After 2 weeks, the contents of each flask is maintained in B5 medium (B5 components, 2,4-D 2.0 mg/L, IAA 0.5 mg/L, NAA 0.5 mg/L, K 0.2 mg/L and sucrose 30 g/L). These cell cultures are then maintained by sub-culturing weekly.

Elicitation

Elicitors have been described as molecules involved in interactions and therefore plant-microbe related to the formation of phytoalexins (molecules produced in response to infection). The characteristic property of these compounds to cell cultures is their ability to activate the secondary metabolism.
The response to elicitors can vary depending on various factors including the specificity of elicitors interval addition, the culture conditions or the concentration of the elicitor. This last factor can affect the intensity of the response, and each may have a different plant at the same concentration of elicitor response, which causes only the effective dose can find empirically.
For now in cannabis cell cultures, cannabinoid biosynthesis was not stimulated or induced by biotic and abiotic elicitors. But it has been used few elicitors, so we propose, other elicitors that they seem to work well with another cell cultures. We propose **chitosan, chitin and elicitins** that are biotic elicitors and **Sodium orthovanadate and vanadyl sulphate** as chemical abiotic elicitors, from here we would have to experiment with these elicitors at different concentrations to test if the cannabinoid biosynthetic pathway is activated.

Downstream

The downstream part of a bioprocess refers to the part where the cell mass from the upstream are processed to meet purity and quality requirements.
Decarboxylation
Cannabinoids are biosynthesized as their acidic forms, characterized by the presence of a carboxyl group attached to the phenolic ring. Acidic cannabinoids can be rapidly converted into their 'neutral' analogues under the influence of heat. The temperature is increased to a temperature for a time until at least 95% conversion of the acid cannabinoids to their neutral form has occurred. It is important not to overheat the material or the THC will be converted to CBN. First we heat the material at 105ºC for 15 minutes and then for 60 minutes at 120ºC.
Extraction
The heated material, is extracted with petroleum ether. This extract is concentrated to a tar on a flash evaporator at 40°C. The tar is dissolved in ethanol and an equal volume of water is added. This solution is extracted three times with hexane. The hexane extracts are pooled and then evaporated on a flash evaporator at 40°C.

CONCLUSIONS

In recent years it has exponentially increased research on the pharmacological properties of cannnabis due to impairment of the prohibition based in economic and political reasons and not for public health reasons. Even that has not yet been achieved cannabinoids elicit an *in vitro* cultivation of *Cannabis sativa*, we have proposed several new elicitors, which have not yet been tested, to fulfill this function.
The future of cannabis in the pharmaceutical industry goes through the cell cultures, the security, efficiency and reproducibility of these *in vitro* cultures make it necessary to approve a drug based on cannabis.

PROCESS IMPROVEMENTS

For industrial production we have to go through a process of gradual scale-up.
We could make a study cellular immobilization, also a hairy root culture and even biotransformation by agrobacterium to enhance the production of cannabinoids.

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