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ORIGINAL RESEARCH PAPER

The application of plant in vitro cultures in cannabinoid production

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Abstract Cannabinoids have considerable interest in the pharmaceutical industry. However, the production of medicines from hemp (*Cannabis sativa* L.) in most countries is restricted by law. Large-scale, field cultivation of hemp is difficult to control. Cannabinoid content in plants is variable and depends on multiple factors. Therefore, alternative methods of production have been investigated. The development of micropropagation techniques is a necessary step for genetic modification. Promising results have been obtained for certain narcotic genotypes. However, micropropagation of fibre types requires further research. Hemp can be genetically modified which may contribute to the breeding of new varieties in the future. Cell suspension cultures and hairy root cultures of hemp have been used to produce cannabinoids but obtaining cannabinoids from callus and cell suspension cultures has proved impossible. Adventitious roots can, however, deliver small amounts of these metabolites but production ceases over time and is too low for industrial applications.

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Introduction

During the last decade, pharmacological properties of cannabinoids have been intensively studied and new applications of hemp extracts have been suggested. The most important therapeutic indications for hemp use include: neutralising the negative effects of chemotherapy with cytostatic drugs, alleviation of chronic pains associated with cancer, anti-spastic activity in sclerosis multiplex or Tourette's syndrome cases, eating disorders associated with AIDS and anorexia (Grotenhermen and Müller-Vahl 2012), epilepsy (Szaflarski and Martina Bebin 2014) and inflammatory diseases (Borrelli et al. 2013).

The main active components of hemp are cannabinoids, but terpenes and phenolic compounds have also been identified (Flores-Sanchez and Verpoorte 2008). Currently, more than 113 cannabinoids are known (Aizpurua-Olaizola et al. 2016). delta-9-tetrahydrocannabinol (THC), (cannabidiol (CBD) and cannabichromene (CBC) are predominant cannabinoids in plant material. The psychoactive effect of THC is well documented (Matsuda et al. 1990). THC acts through CB1 and CB2 receptors of the endocannabinoid

system. It is a partial agonist of both receptors; however, it exhibits higher affinity for the CB1 receptor, which is believed to be responsible for the psychoactive effect of THC, but also for its analgesic and antispastic action. Anxiety, psychosis, cholinergic deficiency or immunosuppression are the undesirable side effects of THC (Russo 2011). CB1 receptors are mainly located in the central nervous system; however, they are also found in the cells of the immune system, digestive system, reproductive system, heart, lungs, adrenal glands and bladder, which explains its wide spectrum of action (Andre et al. 2016). On the other hand, the CB2 receptor is responsible for modulating the immune system by regulating cytokine activity. Its location overlaps with the peripheral nervous system and immune system, which may be attributed to its analgesic and anti-inflammatory action (Burstein 2015). The existence of a putative third type of cannabinoid receptor ("CB3"), called GPR55, has been suggested (Ryberg et al. 2009; Sharir and Abood 2010). There are some receptors exhibiting the affinity towards cannabinoids: TRPV1 vanilloid receptors (Ross 2003), PPAR peroxisome proliferator-activated receptors (O'Sullivan 2007) and others still unidentified.

CBD is characterised by antipsychotic, antianxietic, anti-inflammatory and antioxidant properties and has no toxic effect on human health in doses from 10 mg up to even 700 mg (Zuardi et al. 2006; Pryce et al. 2015). It has been demonstrated that CBD can limit or alleviate the psychoactive effect of THC (Englund et al. 2013). More recent studies on cannabinoid activity also suggest its antineoplastic action (Velasco et al. 2012; Haustein et al. 2014).

In addition to the dominant THC and CBD, other minor cannabinoids, such as CBC and CBN (cannabinol–THC degradation product) have potential therapeutic applications. CBC inhibits the reuptake of anandamide—an endogenous ligand of CB receptors (De Petrocellis et al. 2011). CBN has twofold lower affinity for the CB1 receptor and threefold higher affinity for the CB3 receptor than THC. Therefore, its positive effect on the immune system is expected (Andre et al. 2016).

Studies on cannabinoid mode of action have contributed to the search for new synthetic ligands or allosteric modulators of CB1/2 receptors. Some synthetic analogues, e.g., nabilone (Cesamet) or dronabinol (Marinol), have been introduced into clinical practice, to treat nausea and vomiting caused by chemotherapy (Bolognini and Ross 2015). Ajulemic acid, another synthetic analogue, selectively interacts with CB2 receptors and shows promising anti-inflammatory activity (Tepper et al. 2014). It is currently in clinical trials under the commercial name Resunab. Another synthetic ligand, Rimonabant (Acompilia), intended for the treatment of obesity was removed from the global market in 2009 due to serious side effects (Bolognini and Ross 2015). The search for selective ligands is mainly focused on the CB2 receptor. Unfortunately, early results of clinical trials are unsatisfactory due to the lack of a strong agonist of the CB2 receptor that will not activate CB1 (Bolognini and Ross 2015). The developed modulators are not sufficiently selective and thus may cause a psychotic effect (Kemp et al. 2016). CB1 contains an allosteric site. Since 2005, numerous positive modulators (PAMs) and negative modulators (NAMs) have been discovered. However, the modes of action of particular PAMs and NAMs are different and their mechanisms are not fully understood, which impedes the development of potential therapeutic agents (Nguyen et al. 2017). Synthetic ligands or allosteric modulators are promising, but they cannot yet replace natural molecules.

The availability of raw material with a high content of cannabinoids is limited due to the legal restrictions. Sativex (GW Pharmaceutics) is a product based on the standardised hemp extract from controlled cultivations. Epidiolex (GW Pharmaceutics) is another example and it consists of the purified CBD plant extract.

Demand for raw material creates a need for new varieties and alternative sources of cannabinoids. Breeding new varieties is a time-consuming process and requires laborious selection of high yielding genotypes. The content and composition of cannabinoids vary between plants and depend greatly on the variety, sex, age, developmental stage, climate and weather conditions, time of harvest, cultivation methods, storage conditions, etc. (Andre et al. 2016). The high variability of cannabinoids limits the availability of standardised raw material; therefore, alternative methods of cannabinoid production are needed. Biotechnology, in particular plant tissue cultures and genetic engineering, offer an opportunity for potentially high yield production of cannabinoids. The main purpose of this review is to provide a comprehensive

overview of hemp in vitro cultures regarding historical perspective and their role in cannabinoid production. In this review, we focused on hemp propagation in in vitro cultures, tissue cultures and transformation as well as hairy and adventitious root cultures.

Cannabinoid biosynthetic pathways

Cannabinoids are produced in glandular hairs located on the above-ground plant parts mainly in female flowers, leaves and buds. Glandular hairs not only excrete cannabinoids, but also synthesize them (Sirikantaramas et al. 2005). Native forms of cannabinoids (e.g., THCA, CBDA) are synthesized in the storage cavity of glandular hairs and enzymes involved in their production follow a sorting pathway from secretory cells to the secretory cavity. Separation of the final products is crucial because cannabinoids are cytotoxic. Cannabinoid cytotoxicity through apoptosis has been demonstrated in the studies on hemp, tobacco and insect suspension cultures (Sirikantaramas et al. 2005). The exogenous addition of THCA and cannabigerolic acid (CBGA—direct precursor of THCA and CBDA) to tobacco and insect cultures resulted in cell death within 24 h. The latter authors did not observe any cytotoxic effect when olivetolic acid was added. This result demonstrated cytotoxic properties of some cannabinoids and suggested their defensive role in hemp (Sirikantaramas et al. 2005).

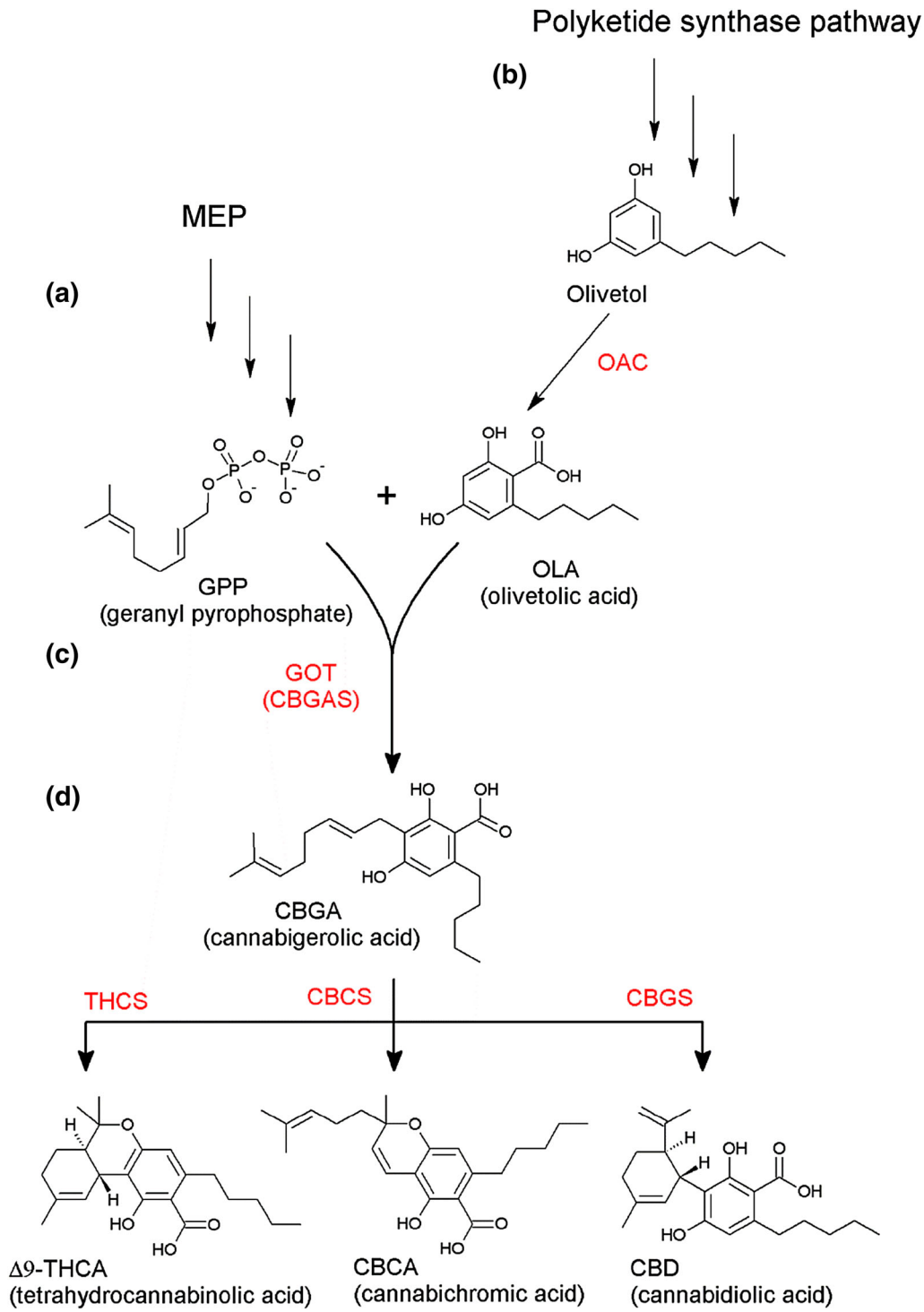
Only three main cannabinoids are synthesized in plants (THCA, CBDA, CBCA). They may serve as precursors of other substances of this group (Fig. 1) (Shoyama et al. 1970). Two independent pathways: the polyketide pathway and the methylerythritol phosphate (MEP) pathway were believed to be involved in cannabinoid biosynthesis (Shoyama et al. 1977). However, geranyl diphosphate (GPP), necessary for the production of terpenoid moiety of cannabinoids, is mainly (> 98%) synthesized by the MEP pathway in plastids, but the rest of GPP (2%) might be derived from the mevalonate pathway (Fellermeier et al. 2001). Olivetolic acid is a carboxylated form of olivetol, synthesized by olivetol synthase and olivetolic acid cyclase (OAC; Taura et al. 2009; Gagne et al. 2012). Olivetolic acid and GPP are alkylated by GPP: olivetolate geranyltransferase, named CBGA synthase (CBGAS), and form CBGA (Fellermeier and Zenk 1998). CBGA is a substrate for various enzymes: THCA synthase (THCAS), CBDA synthase (CBDAS) or CBCA synthase (CBCAS). These enzymes are directly responsible for the synthesis of THCA, CBDA or CBCA (Sirikantaramas et al. 2007). THCAS and CBDAS are oxidoreductases covalently binding FAD, and belong to berberine bridge enzyme family (Kutchan and Dittrich 1995). No data were found concerning THCAS, CBDAS or CBCAS expression regulation.

Hemp micropropagation

Micropropagation allows for the rapid multiplication and large-scale plant production. The great advantage of micropropagation is the possibility to regenerate elite clones and conserve valuable plant genotypes. Establishing effective regeneration protocols is an essential prerequisite for genetic transformation.

Two approaches were considered in developing an efficient hemp micropropagation protocol: direct and indirect organogenesis. Nodal segments containing axillary buds, shoot tips, cotyledons and epicotyls were used in direct organogenesis (Lata et al. 2009b; Wang et al. 2009). Best multiplication rates (12–14 shoots per explant) were obtained for nodal segments of the MX narcotic variety using MS medium with 0.5 μM (equivalent of 0.11 mg) thidiazuron (TDZ) l^{-1} (Lata et al. 2009b; Chandra et al. 2010) or MS mediums with 2 μM (0.48 mg) *meta*topolin l^{-1} (Lata et al. 2016). Shoots were rooted on half-strength MS with 2.5 μM (0.51 mg) indole-3-butyric acid (IBA) l^{-1} (Lata et al. 2009b). Genetic stability of plants obtained with the TDZ-based protocol was assessed and confirmed using inter simple sequence repeat markers (Lata et al. 2010c). The quantity of THC and other cannabinoids obtained from maternal plants propagated in vitro and vegetatively did not differ significantly (8.50–8.8%) (Chandra et al. 2010).

The results for fibrous varieties were considerably lower and the maximum multiplication rate ranged from 3 to 3.2 shoots per explant (Wang et al. 2009; Chaohua et al. 2016). Shoot tips of the Chinese hemp variety (Changdtu) generated 3.2 shoots per tip on MS medium supplemented with 0.2 mg TDZ l^{-1} and 0.1 mg α-naphthaleneacetic acid l^{-1} (NAA). The rooting rate of 85% was achieved on half-strength MS medium with IBA and NAA and 95% of the plants survived further acclimatisation (Wang et al. 2009).



Indirect organogenesis of hemp was less successful although some shoot regeneration via callus was reported (Slusarkiewicz-Jarzina et al. 2005; Wielgus et al. 2008; Lata et al. 2010a, b; Movahedi et al. 2015; Chaohua et al. 2016). First attempts to obtain callus-derived shoots were made by Mandolino and Ranalli

◀**Fig. 1** Main pathways in cannabinoid biosynthesis, (*a*) methylerythritol (MEP) leads to geranyl diphosphate synthesis, (*b*) polyketide synthase-type reactions produce olivetol, which is subsequently condensated by olivetolic acid cyclase (OAC), (*c*) olivetolic acid (OLA) and geranyl pyrophosphate (GPP) are alkylated by GOT [geranyl pyrophosphate–olivetolic acid geranyltransferase, also known as cannabigerolic acid synthase (CBGAS)] to cannabigerolic acid (CBG), and (*d*) CBG is a substrate for synthases producing three main cannabinoids: THC synthase (THCS), CBC synthase (CBCS), CBG synthase (CBGS). Enzymes in the figure are marked in red. Graphic was created using ChemSketch 2016.2 programme

(1999), who obtained only occasional plant regeneration via callus tissue. Feeney and Punja (2003) used leaves, stem petioles and cotyledons of four fibrous hemp varieties to obtain callus. The most efficient medium in terms of callus production was MS medium with B_5 vitamins supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyloaminopurine (BA) or kinetin (KIN). The cultures developed roots after 4 weeks, though shoot regeneration was not achieved. Slusarkiewicz-Jarzina et al. (2005) obtained a callus capable of regenerating shoots using different types of explants (young leaves, petioles, internodes and axillary buds). The highest frequency of callus induction (87%) was acquired from petiole explants on MS medium with 2–3 mg dicamba l^{-1}. Plantlets formed on the same medium after 6 weeks of incubation with a total efficiency of 1.4–2.5%, depending on the variety. Significant influence of hemp variety on the explant reaction and the effect of plant regeneration was also found in the Wielgus study (2008). These authors used different explants for callus induction and concluded that there were no differences between the tested varieties. However, callus obtained from different genotypes exhibited different plant regeneration capacity and its efficiency. The highest regeneration rate (14%) was observed for cotyledon explants. Chaohua et al. (2016) developed a protocol using cotyledons as donor explants and obtained shoot regeneration via callus. The highest (52%) induction frequency and 3 shoots per shoot explant were recorded in MS medium containing 0.4 mg TDZ l^{-1} and 0.2 mg NAA l^{-1}. The age of donor explants was an important factor, as younger cotyledons (2 days after planting) produced a higher number of explants forming shoots (47%) than the older ones (6 days after planting, 11%). IBA was used for rooting with 80% efficiency and 75% of rooted plants were acclimatised. The highest efficiency (96.6%) of plant regeneration from callus was reported by Lata et al. (2010a, b) for the narcotic MX variety. Callus was derived from cotyledons on MS medium with various auxins (NAA, IAA, IBA) in combination with TDZ. MS medium with 0.5 µM (0.11 mg) TDZ l^{-1} was most effective for shoot induction, half-strength MS with 2.5 µM (0.51 mg) IBA l^{-1} was the optimal rooting medium (Lata et al. 2010b). Regeneration via callus from epicotyls was also recorded (Movahedi et al. 2015). The latter study used cotyledons and epicotyls of Iranian hemp. Callus was induced on MS medium with TDZ and IBA. The highest shoot regeneration rate (2 shoots per callus portion) was obtained for callus derived from epicotyl on MS medium with 2 mg BA l^{-1} and 0.5 mg IBA l^{-1}.

Moreover, synthetic seed technology was also developed for multiplication of the narcotic MX-1 hemp variety (Lata et al. 2009a). Axillary buds were encapsulated in sodium alginate and plant regeneration was recorded with a frequency of 77–100% on MS medium with TDZ (Lata et al. 2009a). Similar results were obtained in vivo. The applied technique allowed to limit the amount of MS medium and space required for multiplication (Lata et al. 2009a). Maternal plants and plants micropropagated using axillary bud encapsulation were similar in terms of genetics, chemical profile and cannabinoid content (Lata et al. 2011).

Despite significant progress, simple and efficient regenerative system for fibrous hemp is still needed. The developed propagation protocols are relatively efficient, but limited to selected narcotic varieties. Plant regeneration of fibrous hemp still requires further experiments and improvements in the shoot induction stage for both direct and indirect organogenesis (Slusarkiewicz-Jarzina et al. 2005; Wielgus et al. 2008; Wang et al. 2009; Chaohua et al. 2016). Genotype selection of explant donors and modification of protocols may improve the regenerative capacity of hemp.

Genetic modification

Cannabis sativa L. is relatively resistant to transformation with *Agrobacterium* (Slusarkiewicz-Jarzina et al. 2005). Transformation of hemp remains a

challenge due to low regeneration efficiency and high dependency on variety, tissue, plant age and the lack of transgene stability. The first successful transformation of hemp was described by Mackinnon et al. (2000). Shoot tip explants were selected for transformation with *A. tumefaciens*. More than 50% of hemp plants exposed to the bacterium were susceptible to infection and developed crown galls, indicating that galacturase inhibitory protein genes (PGIPs) (*Botrytis cinerea* resistance) and herbicide resistance genes had been introduced into hemp (Mackinnon et al. 2000).

In another study, callus cells were transformed with the use of *A. tumefaciens* (EHA101) carrying the binary pNOV3635 vector with a gene encoding phosphomannose isomerase (PMI). Callus was capable of expressing the PMI gene but was unable to regenerate organs and plants (Feeney and Punja 2003). The transformation of hemp with the use of *A. rhizogenes* and *A. tumefaciens* was described by Wahby et al. (2013). These authors used 5-day-old seedlings and found that hypocotyls were the most responsive explants for *Agrobacterium* transfection. More than twenty lines of stable hairy roots were established that were able to grow for more than 2 years. Transformation efficiency varied from 43% (*A. rhizogenes* strain AR10GUS) to 98% (*A. rhizogenes* strain R16006) and depended on *Agrobacterium* strain and hemp variety. The authors also obtained transformed callus cultures infected with wild *A. tumefaciens* strains harbouring *pRil185 TL-DNA* with genes *rolA*, *rolB*, *rolC*, alone or in combinations. Plasmid was cloned into the binary *pBin*19 vector. Transformation was confirmed by PCR analysis and histochemical localisation of GUS activity in root tissues.

Hemp is a relatively resistant plant, nevertheless lowering cannabinoid levels in industrial types may increase its susceptibility to fungal infection (Elsohly et al. 1982). Genetic modification can improve its fungal resistance and contribute to developing new varieties designed to synthesize specific cannabinoids that could be used as raw materials for medicine preparation.

Callus and cell suspension cultures

The first attempts of cannabinoid production in vitro were made in the 1980s and resulted in the conversion of CBD and olivetol to cannabielsoin in callus cultures (Loh et al. 1983; Braemer and Paris 1987). Callus was initiated from young leaves on MS (Loh et al. 1983) and B5 medium (Braemer and Paris 1987) supplemented with 2,4-D and KIN. However, cannabinoid production was inefficient and unstable. Callus was unable to produce cannabinoids without the addition of exogenic precursors (CBGA). Subsequent studies demonstrated that undifferentiated callus tissues, even those derived from flowers, were not able to synthesize cannabinoids (Sirikantaramas et al. 2005; Staginnus et al. 2014).

Flores-Sanchez et al. (2009) published an elicitation study using both biotic and abiotic elicitors in cell suspension cultures. Despite using different types of elicitors (fungi extracts: *Pythium aphanidermatum* and *B. cinerea*, signal compounds: salicylic acid, methyl jasmonate, jasmonic acid and metal salts: $AgNO_3$, $CoCl_2{\cdot}6H_2O$, $NiSO_4{\cdot}6H_2O$, and also UVB), enhanced biosynthesis of cannabinoids was not observed. The study confirmed that callus was unable to produce cannabinoids due to its undifferentiated nature. The analysis of THCA gene expression revealed than only flowers and leaves generated trichomes that were able to synthesize cannabinoids (Flores-Sanchez and Verpoorte 2008). Hemp seedlings do not accumulate cannabinoids although low expression of the THCA gene has been reported. This finding suggests that cannabinoid biosynthesis is linked to organ and tissue development and is controlled by genes involved in these processes.

Hairy and adventitious root cultures

Hairy root cultures have many advantages: high biosynthetic capacity for secondary metabolite production, rapid growth and biomass accumulation, high genetic stability and low production costs. Hairy roots can be cultivated in bioreactors, which enable larger scale and make the whole process more profitable.

The first attempt to produce cannabinoids in root cultures was reported by Sirikantaramas et al. (2004). These authors used tobacco hairy root cultures; however, no efficient protocol for hemp modification was available at that time. They isolated THCAS from *C. sativa* leaves (narcotic cultivar), and cloned and sequenced its cDNA. Tobacco hairy roots were transformed with *A. rhizogenes* (15834 strain)

harbouring THCAS in the pBI121 plasmid vector of cauliflower mosaic virus. Transformed root cultures were able to express THCAS and convert exogenously added CBGA to THCA. The maximum conversion rate was only 8.2%. Nearly half of the THCA produced was found in medium, suggesting active intake of CBGA by roots (Sirikantaramas et al. 2004). Farag and Kayser (2015) described a protocol for establishing adventitious roots from callus cultures. Adventitious root cultures were initiated from callus by the addition of auxins (NAA, IBA, IAA) to B5 solid medium after 8 weeks of culture. Satisfactory growth and root differentiation were obtained on a medium with the addition of 4 mg NAA l^{-1} in the dark. Other auxins did not stimulate root growth. Subsequently, root tips were transferred to liquid medium (1/2 B5) with a constant auxin supplementation (IAA, IBA, NAA) and were grown in flasks on shakers. The obtained hairy root cultures required constant medium supplementation with auxins (NAA or IAA). HPLC analysis revealed cannabinoid production at maximum levels of 1 µg THCA g^{-1} dry wt, 1.6 µg CBGA g^{-1} dry wt and 1.7 µg CBDA g^{-1} dry wt. Synthesis of cannabinoids decreased after 28 days. Although root cultures are capable of cannabinoid synthesis, the efficiency was below 2 µg cannabinoids g^{-1} dry wt. Further optimisations are required to develop a more efficient system, however, the authors concluded that scaling up these cultures would be difficult.

Conclusions

There are several strategies that can be used to obtain raw materials for cannabinoid production. They involve both the extraction from plants grown in field, greenhouse, hydroponic cultivation and in vitro production. In field conditions, the yield of THCA obtained from plants may reach 200 mg THCA g^{-1} dry wt (Aizpurua-Olaizola et al. 2016). Currently, various biotechnological strategies have been introduced to cannabinoid production. The approaches used to produce raw materials for cannabinoid extraction are summarized in Table 1.

Efficient regeneration protocols are essential for successful genetic transformation and are needed for micropropagation and germplasm conservation. Micropropagation is also a tool that can indirectly affect yield, allowing developing new varieties and stabilising older ones. In addition to plant tissue cultures, several heterologous systems have been developed to produce enzymes responsible for cannabinoid synthesis. Currently, CBGAS and THCAS are targeted for expression in heterologous systems and the most advanced works in this field seem to be related to *Komagataella phaffii* (former *Pichia pastoris*) (Taura et al. 2007; Lange et al. 2016; Zirpel et al. 2017). None of the present systems provide the solution for the efficient and profitable cannabinoid production. Current micropropagation techniques are sufficiently advanced to develop an efficient mass propagation system, but only for narcotic genotypes. Micropropagation of fibrous hemp varieties needs further improvements. There are two main strategies to improve callus and explant responses and their regenerative capacity: genotype selection of explant donors and medium optimisation. Direct organogenesis may reduce the variability of cannabinoid content in used varieties; indirect organogenesis may be used as a tool for genetic modification of hemp. In both cases, an efficient protocol for shoot induction is required for successful plant regeneration.

Although transformations with the use of *A. rhizogenes* and *A. tumefaciens* have been reported, genetic modification of hemp continues to be a challenging task. It is likely that these methods will soon be replaced by crispr/Cas9-based methods, however, there are no studies yet on this subject. In both cases, an efficient protocol for callus shoot induction is required for successful plant regeneration.

Production of metabolites in bioreactors can be a quick and non-controversial method of obtaining cannabinoids. For this purpose, callus and cell suspension cultures were grown and elicited with various factors. However, they were not able to produce cannabinoids, because biosynthesis of THCA was linked to organ development and tissue differentiation. Low amounts of THCA were obtained from genetically modified callus of tobacco. However, the biosynthesis required CBGA addition and the efficiency was lower compared to other heterologous systems (Sirikantaramas et al. 2004). Adventitious root culture has also been applied for cannabinoid production. However, adventitious roots produced small amounts of cannabinoids, and their production ceased after 28 days. Nevertheless, root culture grew more than 3 years.

Table 1 Biotechnological strategies involved in cannabinoid synthesis

Biotechnological strategy	Methods	Yield	Advantages	Disadvantages
Genetic transformation	*A. rhizogenes* and *A. tumefaciens* transformation	–	Faster development of new varieties, stabilising older ones	Difficulties with transformation, and regeneration from callus
Cell suspension culture	Direct production of cannabinoids or precursor feeding	–	Possibility to grow in bioreactors	No cannabinoid production
Hairy/ adventitious roots		1.04 μg THCA g^{-1} dry wt (Farag and Kayser 2015)		Low yield of cannabinoids, difficulties with scaling up
Heterologous system	Production of enzymes for olivetolic acid (OA) and geranyl pyrophosphate (GPP) conversion	121 g THCA l^{-1} h^{-1} (Lange et al. 2016)	High yield, mass production in bioreactors	Activity of enzymes decreases in time

At present, *C. sativa* L. plants still remain the most efficient source of natural cannabinoids. Therefore, developing new more stable varieties, including genetically modified plants, may be the fastest way to cover growing demand for raw materials.

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