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In vitro Anther culture and Production of Haploids in *Cannabis sativa*

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Abstract

Cannabis sativa has been used for thousands of years for recreational, medicinal, or religious purposes. Another culture technique is the most viable and efficient method of producing homozygous doubled haploid plants within a short period. The most widely extended approaches to obtain doubled haploids (DHs) have traditionally been based on the use of haploid cells of male or female origin to induce their development as haploid embryos by the application of different stresses under *in vitro* conditions. They are the so-called *in vitro* approaches. Thus, the long process of conventional breeding methods can be reduced by homozygosity in early generations. The recessive alleles could be obtained and selected earlier due to the homozygosity of doubled haploids (DHs) lines. Double haploid technology (DH) is an essential tool in plant breeding, enabling the rapid production of homozygous lines. However, doubled haploids (DH) were not highly relevant in plant breeding until researchers at the **Department of Botany in the University of Delhi, India**, reported a major breakthrough in the production of haploids from anther culture in *Datura innoxia* (Guha and Maheshwari, 1964, 1966). Their research revolutionized the use of doubled haploid (DH) technology in plant breeding worldwide. However, the practical application of this technology in *Cannabis sativa* improvement is still limited by various factors that influence culture efficiency. *Cannabis sativa* L. has been categorized as recalcitrant to doubled haploid (DH) induction and androgenesis induction, although very few embryos can be developed. However, the potential of *in vitro* anther culture in *Cannabis sativa* is yet to be completely exploited mainly due to the recalcitrant genetic backgrounds in *Cannabis sativa.*

Keywords: Anther Culture; Androgenesis; Double Haploid; Cannabis; Gynogenesis; Haploids; In Vitro Culture; Microspore; Totipotency

1. Introduction

The wild noxious weed *Cannabis sativa* L. belongs to the family *Cannabaceae* is a dioecious plant, producing male and female flowers on separate unisexual individuals, a trait regulated by an XY chromosome sex determination system [1-37]. Cultivation and use of cannabis plants for recreational, medical, and industrial use were strictly banned and severely limited the scientific research in the field [1-37]. Owing to strict legal regulations, the plant remained unexplored for its incredible potential in drug discovery for an extended period until it was legalized for medical use in many countries around the globe [1-38]. Nowadays, *Cannabis* is the centre of many scientific studies, which mainly focus on its chemical composition and medicinal properties [1-38]. The CANNUSE database (Database URL: http://cannusedb.csic.es) provides an organized information source for scientists and general public interested in different aspects of *Cannabis* use [39]. The main aim of the CANNUSE database is to gather and organize the abundant information on traditional *Cannabis* use in a simple manner [39].

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Cannabis sativa and *Cannabis indica* are the native of Indian origin found as wild noxious weed in the foothills of Indian Himalayan Region and other parts of India, China, Nepal, Bhutan, Sri Lanka, Pakistan, Afghanistan, Persian, Iran, and Morocco and plains of Pamir (a high mountain range centered in eastern Tajikistan with extensions into Afghanistan, the Republic of China and Kyrgyzstan) [1-38, 262-265]. *Cannabis sativa* is cultivated as a crop in different regions of India but it is also found as a weed in different crops [1-38, 262-265]. It is a common weed species of different kharif crops (Dhillon, 2024) [41-43]. It is also documented as a weed species in wheat crop (rabi crop) fields in the state of Punjab, India [41-43]. This noxious weed *Cannabis sativa* is a biggest problem for the agriculture farming in India. However, the money spent and labour is very expensive to remove this weed than agriculture farming in India. Now days *Cannabis sativa* is a globally domesticated, cultivated and introduced species occurring in North and South America, Europe, Africa, Australia, Asia and other parts of world [1-38]. These cannabis species are hybrid varieties and known for very high levels of THC (0.3 to 38%) as compared to wild noxious weed found in all the parts of India [1-38, 23-265].

Female *Cannabis sativa* flowers have densely packed glandular structures called trichomes that store the phytocannabinoids, tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA) which must be decarboxylated by heat to produce Δ9-tetrahydrocannabinol (THC: intoxicating) and cannabidiol (CBD: non-intoxicating) [1-38]. The two cannabinoids, the most well known for their therapeutic properties are, Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) [1-35].

Today *Cannabis sativa* continues to be the most used drug in the world [1-38, 262-265]. Research showed that *cannabis* use is associated with a wide range of adverse health consequences that may involve almost every physiological and biochemical system including respiratory/pulmonary complications such as chronic cough and emphysema, impairment of immune function, and increased risk of acquiring or transmitting viral infections such as HIV, HCV, and others [1-38]. Both Medical *Cannabis sativa* (Marijuana or drug type) and Industrial *Cannabis sativa* (hemp or fiber type) are used for controlling numerous diseases, such as chronic pain, asthma, rheumatoid arthritis (RA), wound healing, constipation, multiple sclerosis (MS), cancer, inflammation, glaucome, neurodegenerative disorders (Epilepsy-seizure disorder, Alzheimer's disease, Parkinson's disease, dengue viral disease, Huntington's disease, Tourette's syndrome, Dystonia, Lennox-Gastaut Syndrome (LGS) and Dravet Syndrome (DS), Obesity, weight loss, anorexia, and emesis, osteoporosis, schizophrenia, cardiovascular disorders, sleep disorders, Traumitic brain injury (TBI), Post traumetic stress injury, drug addiction (Marijuana), AIDS Wasting syndrome, Amyotrophic lateral sclerosis (ALS), depression and anxiety, diabetes, migraine (headache disorder), Covid-19 (SARS-CoV-2), Leishmaniasis (Kala-Azar), dengue fever, monkeypox, Nipah virus, Lumpy skin vital disease of cattle, and metabolic syndrome related disorders, are being treated or have the potential to be treated by cannabinoid agonists/ antagonists/cannabinoid-related compounds [1-38].

Anther culture technique is the most viable and efficient method of producing homozygous doubled haploid plants within a short period [43-103-116]. However, the practical application of this technology in *Cannabis sativa* improvement is still limited by various factors that influence culture efficiency [104, 106]. *Cannabis sativa* L. has been categorized as recalcitrant to doubled haploid (DH) induction [104, 106]. Double haploid technology (DH) is an essential tool in plant breeding, enabling the rapid production of homozygous lines. This experimental pathway was first discovered by Guha and Maheswari in 1964, while working with *in vitro* cultured anthers of *Datura innoxia* [43, 44, 45]. The plants derived from doubled haploid (DH) techniques are completely homozygous breeding lines that can be produced by anther or microspore culture within a year, instead of waiting for more than five generations of inbreeding cycles [43-103-116]. Moreover, theoretically, no further segregation can be expected from the developed doubled haploid (DH) plants, which makes them useful as a fixed homozygote mapping population for different molecular genetic studies [43-103-116]. Each plant developed through *in vitro* anther culture could be a potential homozygous line, which can be useful to study phenotypic variation for desirable traits [43-103-116]. The double haploid (DH) lines are also ideal for genetic mapping of agro-morphological and complex traits [43-103-116]. Advantages of doubled haploid (DH)s are quickest homozygosity and uniformity [43-103-116]. However, there are numerous drawbacks, including segregation ratio distortion, the incidence of albinism, a limited and frozen crossover, which significantly limits their application [43-103-116]. However, the potential of *in vitro* anther culture in *Cannabis sativa* is yet to be completely exploited mainly due to the recalcitrant genetic backgrounds in *Cannabis sativa* [104, 106]. Among the several factors, the genotype of the explants, growing conditions of the donor plants, media composition including macro-and micronutrients, vitamins, carbohydrates, organic adjutants, and growth regulators have been identified to influence the culture efficiency [43-103-116]. In the following section, the application of *in vitro* anther culture and double haploid (DH) production has been updated and discussed.

2. *In vitro* Anther culture and double haploid (DH)

Another culture (AC) techniques can produce homozygous doubled haploid (DH) lines within one generation [43-103-116]. Thus, the long process of conventional breeding methods can be reduced by homozygosity in early generations [43-103-116]. The recessive alleles could be obtained and selected earlier due to the homozygosity of doubled haploid (DH) lines [43-103-116]. Doubled haploid (DH) plant production methods have improved and led to accelerating the breeding of new varieties and hybrids [43-103-117]. However, doubled haploids (DH) were not highly relevant in plant breeding until researchers at the Department of Botany in the University of Delhi, India, reported a major breakthrough in the production of haploids from anther culture in *Datura innoxia* (Guha and Maheshwari, 1964, 1966) [43, 44, 45]. Their research revolutionized the use of doubled haploid (DH) technology in plant breeding worldwide [43, 44, 45]. Thereafter through the major discovery of induction of haploids through interspecific crosses followed by embryo culture as a promising method for obtaining haploids in barley (*Hordeum vulgare* L.) (Kasha and Kao, 1970) [80]. To date, doubled haploid (DH) technology has been used in cultivar development in self fertilizing species, or in inbred line development for their further use in producing hybrids of out crossing species [43-103-116]. The *in vitro* procedure using androgenesis (anther or microspore culture) and gynogenesis (unfertilized egg cell) has been used to produce doubled haploids (DH) [43-103-116]. Androgenesis refers to culturing immature anther or microspores from the immature pollen grain in artificial media to isolate haploid cells that are then chromosome doubled using colchicine, oryzalin, caffeine, trifuralin, or phosphoric amides) or gaseous i.e. nitrous oxide to develop DH [43-103-116]. However, haploid production by in vitro culture is a highly technical procedure; labour-intensive, time-consuming and costly; and more importantly, species- and genotype-dependent [43-103-116]. Other constraints associated with use of this technology are the low rate of embryogenesis and regeneration, high frequency of albinism, segregation distortion, and the low frequency of chromosome doubling to obtain DH [43-103-116]. This technology has been standardized and routinely used for production of DH in barley, brassica, oat, rice, and triticale [43-103-116]. These methods are widely used in many crop plants such as barley, rapeseed, and maize etc [43-52-103-116]. The culture conditions of anther culture (induction medium, growth regulators, carbon source, temperature etc.) influence the efficiency of anther culture [43-103-116]. Double haploids (DH) have become a powerful tool to assist in different basic research studies, and also in applied research [52].

Isolated microspores, when given the optimal combination of culture conditions and stresses, can be diverted from the normal gametophytic developmental pathway to a sporophytic pathway, and subsequently produced embryos and haploid or doubled haploid (DH) plants [43-103-117]. The production of doubled haploid (DH) plants from microspores is an important technique used in plant breeding and basic research [43-103-117]. Doubled haploid (DH) technology is a rapid method for developing homozygous lines, which can be used to accelerate crop improvement programs [43-103-117]. Commercial varieties developed through doubled haploid (DH) protocols have been reported for many crops, such as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), triticale (x *Triticosecale* Wittm.), rice (*Oryza sativa* L.), *Brassica* spp., eggplant (*Solanum melongema* L.), pepper (*Capsicum annuum* L.), asparagus (*Asparagus officinalis* L.), and tobacco (*Nicotiana tabacum* L.) [43-103-117]. A plethora of other uses for isolated microspore culture has arisen and this subject has been reviewed [43-103-117].

This experimental pathway was first discovered by Guha and Maheswari in 1964, while working with *in vitro* cultured anthers of *Datura innoxia* [43, 44, 45]. Later on, many different research groups have reproduced their findings in many other species and genera, making this experimental phenomenon a powerful and widespread tool to produce DHs [51]. However, not all the species respond equally to the induction of this process [43-103-116]. Some species, considered models for the study of this phenomenon, respond fairly well [51]. This is the case of certain lines of rapeseed (*Brassica napus*), tobacco (*Nicotiana tabacum*), or barley (*Hordeum vulgare*) [43-103-116]. Others, considered recalcitrant, present a low or very low response, and in other cases, a protocol to efficiently induce this process is still pending to be developed, as for scientifically or agronomically important species such as *Arabidopsis thaliana* or tomato (*Solanum lycopersicum*), respectively [51]. Many other species are in between these two extreme situations, being possible to induce microspore embryogenesis, but with yet improvable protocols [51]. Woody species are good examples of materials where some success has been achieved, but there is still a large room for improvement [43-103-116].

Even within a species, there will be varieties, lines and even individuals that respond differently [43-103-116]. This strong influence of the genotype, together with the fact that this trait is transmitted across generations and segregates in the hybrids offspring indicates that it is under genetic control [43-103-116]. Furthermore, it was proposed that, at least for *Brassica napus*, the embryogenic competence of microspores is controlled by two loci with additive effects [43-103-116]. The gene or genes involved, however, remain to be elucidated [43-103-116].

Anther culture is the most universal method to produce DHs [43-103-116]. It is technically simple, consisting basically of the steps: (1) flower bud collection, (2) isolation of anthers from flower buds, (3) inoculation and *in vitro* culture in

agar-based culture medium, (4) isolation of embryos, (5) regeneration of plants, and (5) analysis of regenerants [43-103-116]. Few weeks (months in many cases) after, microspore-derived embryos may be seen to emerge from anther walls, in parallel to the degradation and necrosis of these walls [43-103-116]. In general, a given anther under optimal culture conditions may give rise to several tens of microspore-derived embryos during several months of culture [43-103-116]. The presence of these walls (the tapetum principally) during the first stages of anther culture may protect and help microspores to undergo the first stages of haploid development, in a way similar to how they assist normal microspore development *in vivo* [43-103-116]. Perhaps, this is the reason why anther culture works in many different species, including those where other DH methods do not work [43-103-116].

However, anther cultures are not devoid of limitations [43-103-116]. Perhaps, the main limitation comes from the fact that microspores are cultured together with anther walls [43-103-116]. Anther walls (the tapetal layer mostly) may secrete molecules that may protect microspores or promote their growth, but it may also secrete inhibitory or even toxic compounds, as is the case of necrosing anther tissues [43-103-116]. In any case, this secretary effect is uncontrollable in essence, and makes difficult a strict control of culture conditions. Moreover, when exposed to growth regulators, these walls are able to proliferate *in vitro*, producing calli [43-103-116]. Indeed, some parts of the anther, such as the filament insertion, are especially prone to form calli when *in vitro* cultured [43-103-116]. Therefore, can not rule out the possibility of occurrence of somatic embryos (very rare but possible) and calli (much more frequent) from anther walls [43-103-116]. This implies that for every single plant confirmed as diploid (2C DNA content) by flow cytometry [43-103-116].

3. Anther culture in *Cannabis sativa*

Anther culture is an efficient biotechnological tool in modern plant breeding programs to produce new varieties and parental lines in hybrid seed productions [43-52-103-116]. Double haploids (DH) plant production methods are widely used in crop breeding and research programs because of their ability to produce genetically pure lines in one generation [43-52-103-116]. The production of doubled haploids (DH) in cannabis would be highly advantageous, as it would be possible to produce female pure lines in one generation [43-52-103]. Haploid plants have in other plant species been produced via androgenesis (anther or microspore culture), gynogenesis, parthenogenesis or wide hybridization-chromosome elimination [43-52-103-116]. Later chromosome duplication in the haploid plants is performed, either spontaneously or by chemical treatment, colchicine, oryzalin, caffeine, trifuralin, or phosphoric amides) or gaseous i.e. nitrous oxide [43-52-103]. Although double haploids (DH) production via microspore culture has been investigated in cannabis, successful double haploids (DH) production has so far not been established [105]. Cannabis seems to be recalcitrant to androgenesis induction, although very few embryos can be developed [106]. The method used for successful doubled haploid production seems to be species dependent where for also the other methods should be investigated for their usefulness in cannabis [104, 105, 106]. Recently, CRISPR/ Cas have been used to develop haploid-inducer lines in both monocot and dicot plants [49]. As also suggested by others, this method might be very useful in cannabis [43-49- 104-108].

The study reported by Tonolo and Ambra (2024) [104] have examined two cultivars, a THCA-dominant cultivar and a CBDA-dominant line for the diploid haploid (DH) regeneration [104]. Callus induction success varied, with 29.48% for the THCA cultivar and 71.08% for the CBDA genotype with a regeneration success of 14.45% in 17 weeks for the latter. Mixoploidy in the callus indicated spontaneous genome doubling, while genetic testing confirmed DH nature of the regenerants [104]. This is the first report documenting the successful induction of DH. *C. sativa* plants through *de-novo* indirect organogenesis [104]. These findings have profound implications for the *C. sativa* breeding sector by potentially improving efficiency of genome editing and hybrid development in this economically significant species [104].

The study reported by Tonolo and Ambra (2024) [104] successfully induced callus growth from *C. sativa* anther culture, induce indirect *de-novo* shoot and root organogenesis from the obtained callus, and ultimately regenerate and acclimatize several plants with this system [104]. The investigations based on the ploidy tests could tell us more about the underlying processes occurring during the *in-vitro C. sativa* culture. Indeed, while the ploidy measurements did not reveal haploid cells, the results were fundamental for documenting and understanding the shifts of ploidy levels necessary for this process to be successful [104]. On the other hand, the genetic test was fundamental to confirm the DH nature of the obtained plants [104]. To the best of our knowledge, this is the first report on the successful induction of double haploids in *C. sativa* leveraging protocols that can regenerate plants via the indirect *de-novo* organogenesis pathway [104]. Overall, the designed culture system has several advantages, making it an extremely valuable asset for the *C. sativa* breeding sector [104].

There are two main examples of totipotency, somatic or gametophytic, each of which can take two different developmental routes: the embryogenesis or the *de-novo* organogenesis pathway [104]. The main differences are

determined by the type of cells that can proliferate and the developmental route which leads to a fully regenerated plant. The origin cells can be either gametes or somatic cells [104]. At the same time, the developmental route can either involve the generation of an embryo or the differentiation of the meristematic center in different organs [104]. In the case of somatic regeneration, the cells originate from a diploid vegetative tissue [104]. The regenerated plant generally presents the same genetic profile and ploidy level as the donor plant, although this process can also contribute to generating plants with new characteristics due to somaclonal variation [104]. Therefore, this culture system is a gateway to unlock the potential of modern genome editing techniques on *Cannabis sativa*, enabling the development of new cultivars at a quicker pace and more cost-effectively while at the same time providing the much-needed genetically healthy and stable starting material for F1 breeding pipelines [104].

On the other hand, gametophytic proliferation is a form of totipotency based on the proliferation of the male or female haploid gametes and the associated cells [43-49- 104-108-116]. In this case, the cells that proliferate are derived from meiosis, and therefore, they represent the haploid segregant progeny of the donor plant [43-49- 104-108-116]. Apart from having a unique genetic profile, these cells have a different ploidy level as they are generated by a haploid reproductive cell [43-49- 104-108-116]. These ploidy levels and genetic profile changes have been used in several ways to advance the human understanding and exploitation of plants' survival strategies [43-49- 104-108-116]. Indeed, once a haploid plant is generated and it undergoes genome doubling spontaneously or artificially, a so-called double haploid (DH) is obtained. Because of this process, the resulting DH plant is completely homozygous and obtained in just one generation [43-49- 104-108-116]. The double haploid plants have been exploited by scientists to develop immortalized molecular mapping populations, to fix traits obtained through genome editing techniques quickly, or to simplify genome sequencing by eliminating heterozygosity [43-49-104-108-116]. Moreover, DH technology has proven paramount for the breeding sector, given the quickness in the generation of homozygous lines for F1 hybrid production, the rapid fixing and introgression of new traits and the exploitation of the gametoclonal variation and *in-vitro* selection system to decrease time, labour, and costs of plant-breeding programs significantly [43-49- 104-108-116].

The most widely extended approaches to obtain double haploids (DH) have traditionally been based on the use of haploid cells of male and female origin to induce their development as haploid embryos by the application of different stresses *in vitro* and their subsequent *in vitro* culture [43-52-108-116]. They are the so-called *in vitro* approaches [43-52-103-116]. The production of haploid/DH plants from male haploid cells is commonly known as induction of *in vitro androgenesis*, whereas production of haploid/ double haploids (DH) plants from female haploid cells is commonly known as induction of *in vitro gynogenesis* [43-52-103-116]. The different strategies have in common the blockage of the normal development of these cells, whose natural fate is the production of functional gametes or accessory cells, and their *in vitro* reprogramming towards a different developmental fate, which is to become embryos without fertilization [43-52-103-116]. This way, haploid and/or double haploids (DH) individuals can be produced *in vitro* [43-52-108-116].

To be induced to embryogenesis, microspores/pollens must be stressed. The need for application of physicochemical stress treatments seems common to all inducible species. The variety of responses, depending principally on the genotype but also on the developmental stage of the microspore/pollen, makes that each species has its own specific inductive treatments to trigger the developmental switch. Some of these stresses (heat, cold or starvation) are common to many species, whereas others need more specific stressors or combinations of them [37]. As a rule of thumb, the more recalcitrant a species is, the more combined and more intense stresses are needed. Typically, induction of microspore embryogenesis produces microspore-derived.

Galán-Ávila et al., (2021) [106] confirmed that the pollen grain has traditional breeding and taxonomy, it takes exclusive prominence in androgenesis [43-52-106]. Through this technique, it is possible to obtain 100% homozygous inbred lines in only one in vitro generation, thus allowing for fixation of traits and accelerating cultivars development [43-52-106-116]. These plants are derived from a haploid nucleus of male origin and after spontaneous or induced chromosome doubling, double haploids are obtained [43-52-106-116]. By means of hybridization of these pure lines, it is possible to exploit the hybrid vigor, obtaining high yielding and uniform F1 hybrid material [43-52-106-116]. One of the routes that leads to androgenesis is microspore embryogenesis, by which the microspore deviates from its original gametophytic fate and it is reprogrammed to a new pathway of embryogenic development [43-52-106-116]. Galán-Ávila et al., (2021) [106] also reported that the most relevant factors affecting microspore embryogenesis, is the microspore and pollen stage of development [43-52-106]. It is widely accepted how vacuolate microspores and young bi-cellular pollen grains are more sensitive to the androgenic induction [43-52-106-116]. On the other hand, it has been demonstrated in different species how microspore and pollen stage of development can be correlated with some features of the flower, as is the case of bud length, pedicel length, anther length and petal to anther ratio in *Brassica napus*, bud length and perianth morphological markers in *Solanum lycopersicum*, pigmentation degree of anthers and

calyx-corolla ratio in *Capsicum annuum*, or more recently, flower bud size in *Stevia rebaudiana* Bertoni, and bud length, anther color, and filament length in *Opuntia ficus-indica* L. Mill [43-52-106-116].

Furthermore, stress treatments are also described as highly relevant on microspore embryogenesis [43-52-106]. Among the most popular stress treatments, cold shock is the most frequently employed to promote microspore embryogenesis in a wide range of species [43-52-106-116]. The low temperatures stimulate the expression of two heat-shock proteins (HSP) genes which possibly can protect cells against chilling injuries [43-52-106]. In general, cold-shock can be considered as more effective in terms of embryogenically induced microspores when applied directly to the flower buds [43-52-106].

On the other hand, in order to develop an experimental microspore culture protocol to induce microspore embryogenesis in *Cannabis sativa*, the correlation of the different developmental stages of microspores and pollen grains with bud length was studied [43-52-106]. Furthermore, Galán-Ávila et al., (2021) [106] also studied the androgenic potential of *Cannabis sativa* through the microscopic analysis of the amyloplasts contained in anthers, microspores and pollen grains [43-52-106].

Galán-Ávila et al., (2021) [106] confirmed that *Cannabis sativa* is an appropriate candidate for microspore and pollen embryogenesis [106]. Galán-Ávila et al., (2021) [106] also reported that the presence of starch in *Cannabis sativa* microspores and pollen grains follows a similar pattern to that observed in species recalcitrant to androgenesis [106]. Although at a low frequency, cold-shock pre-treatment applied on buds can deviate the naturally occurring gametophytic pathway toward an embryogenic development [106]. This represents the first report concerning androgenesis induction in *Cannabis sativa*, which lays the foundations for double haploid research in this species [106].

4. Tissue Culture Studies in *Cannabis sativa*

On the basis of literature survey, most of the tissue culture studies on *Cannabis sativa* has reported as recalcitrant [118-152]. The good news is that the literature on tissue culture studies of *Cannabis sativa* is slowly warming up [118-152]. There are studies highlighting successful *Cannabis sativa* organogenesis but the commercial scale production is still a problem [118-152]. Till today there are no reports on the induction of somatic embryogenesis in *Cannabis sativa*. Direct *de-novo* organogenesis often appears to yield good results, while indirect regeneration via callus formation generally has regeneration rates that are absent or relatively low [118-152]. The failures of the last 20 years and the challenges faced by the scientists, therefore, prompted the researchers to classify *Cannabis sativa* as a recalcitrant species to plant regeneration and DH induction [118-152].

Tissue culture technique depends mainly on the concept of totipotentiality of plant cells, which refers to the ability of single cell to express the full genome by cell division [153-262, 266-270]. The totipotency of somatic plant cells is a specific and scientifically exciting phenomenon, which is based on the developmental program of plants [153-262-270]. It can be best demonstrated in an *in vitro* system where somatic plant cells can regain their totipotency and are capable of forming embryos through the developmental pathway of somatic embryogenesis [153-262, 266-270]. Under *in vitro* conditions, one or a few somatic cells of the plant or explants have to be competent to receive a signal (endogenous or exogenous) [153-262-270]. This triggers the reprogramming of plant cells into the pathway of embryogenic development (commitment) leading to somatic embryo formation [153-262-270]. The controlled conditions provide the culture of explants on a defined nutrient medium with the source of carbohydrate in an environment conducive for their growth and multiplication [153-262-270]. These conditions include proper supply of nutrients, source of carbohydrate, pH of the medium, adequate temperature, proper gaseous and liquid environment [153-262-270].

Recalcitrant is very common in many plant species under *in vitro* conditions [153-262]. But many recalcitrant plant species have been cloned successfully via organogenesis or somatic embryogenesis [153-262-270]. This could be possible only by reprogramming the cell pathway towards somatic embryogenesis [153-262-270]. There are many signalling molecules which can re-programme the dipoloid (somatic cell) cell to somatic embryogenesis [153-262-270]. The totipotency of somatic plant cells is a specific and scientifically exciting phenomenon, which is based on the developmental program of plants [153-262-270]. Many of the recent studies showed that signaling molecules such as butenolide, calcium ions, salicylic acid, antioxidants, amino acids, triacontanol, melatonin, and 24-epibrassinolide all play an important role in the conversion of somatic cells into an embryogenic pathway in many recalcitrant pines, and tree species [153-262-270]. It can be best demonstrated in an *in vitro* system where somatic plant cells can regain their totipotency and are capable of forming embryos through the developmental pathway of somatic embryogenesis [153-262]. Another important factor is that one has to develop natural or synthetic precursor molecules which can trigger and reprogramming of the cells towards somatic embryogenesis [153-262-270]. These precursor molecules can break the recalcitrant nature of plant cells and resulted in successful organogenesis or somatic embryogenesis [153-262].

However, the interaction studies of new precursor molecules with plant cells under *in vitro* conditions is a long term study which needs funding, good laboratories facilities, well trained scientists particularly in the field of somatic embryogenesis and challenging too [153-262-270]. Some times, these studies might end up as experimental models and commercialization is still a bottleneck [153-262]. Therefore, commercialization of plant tissue protocols in many plant species is a major problem and challenging too [153-262-270]. Artificial neural networks (ANNs) are widely used in science and technology, and have been successfully applied in cannabis plant tissue cultures [16, 17]. Furthermore Artificial neural networks (ANNs) can also simulate the growth of plants under different *in vitro* conditions [16, 17]. However, very few and limited *in vitro* regeneration protocols have been developed in cannabis and existing protocols highlights only organogenesis [118-152]. Therefore, there is a golden opportunity for the development of new *in vitro* regeneration protocols particularly induction of somatic embryogenesis, cryopreservation, protoplast isolation and culture, genetic transformation, production of synthetic seeds, and anther culture for the production of haploids in cannabis [153-262].

5. Conclusion

Anther culture is an efficient biotechnological tool in modern plant breeding programs to produce new varieties and parental lines in hybrid seed productions. However, some bottlenecks— low induction rate, genotype dependency, albinism restrict the widespread utilization of *in vitro* anther culture in *Cannabis sativa* breeding, especially in Medical *Cannabis sativa* and hemp fibre type genotypes, while an improved efficient protocol can shorten the process of breeding. However, the practical application of this technology in *Cannabis sativa* improvement is still limited by various factors that influence culture efficiency. The most widely extended approaches to obtain double haploids (DHs) have traditionally been based on the use of haploid cells of male and female origin to induce their development as haploid embryos by the application of different stresses *in vitro* and their subsequent *in vitro* culture. They are the so-called *in vitro* approaches. The production of haploid/DH plants from male haploid cells is commonly known as induction of *in vitro androgenesis*, whereas production of haploid/DH plants from female haploid cells is commonly known as induction of *in vitro gynogenesis*. Furthermore, haploid cells of both male and female origins have been used to produce double haploids (DHs) *in vitro*, although with different success rates. In general, the haploid cells where *in vitro* haploid/DH induction has been the most successful are male microspores and female egg cells. In particular, *in vitro* production of androgenic double haploids (DHs) has been more successful than production of gynogenic DHs. Double haploids (DH) research has advanced considerably and facilitated the release of large number of cultivars, mostly in *Brassica* and cereals. Research led to great understanding of the genetics and mechanisms of haploid induction, identifying factors influencing haploid induction, finding useful markers (morphological, biochemical and DNA markers) to detect putative haploids, and increasing genetic gains through the use of DH technology in plant breeding.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed

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