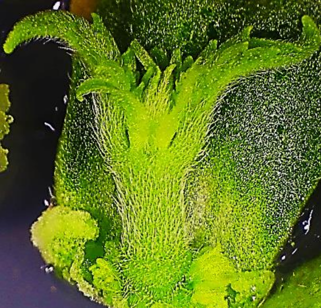

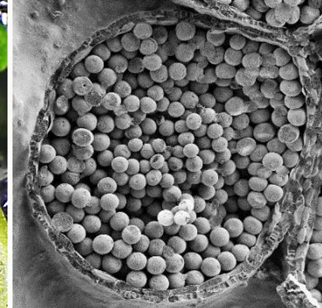
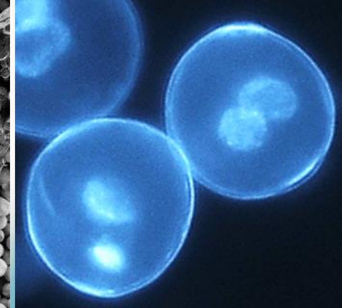

DOCTORAL THESIS

Development of biotechnological tools for the genetic improvement of *Cannabis sativa* L.

Alberto Galán Ávila

Valencia, June 2021

Advisors:

Dr. Francisco Javier Herráiz

Dr. Pietro Gramazio

Tutor:

Dr. Jaime Prohens Tomás

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

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Programa de Doctorado en Biotecnología

TESIS DOCTORAL

Desarrollo de herramientas biotecnológicas para la mejora genética de *Cannabis sativa* L.

DOCTORAL THESIS

Development of biotechnological tools for the genetic improvement of *Cannabis sativa* L.

TESI DOCTORAL

Desenvolupament d'eines biotecnològiques per a la millora genètica de *Cannabis sativa* L.

Presentada por:

Alberto Galán Ávila

Directores:

Dr. Francisco Javier Herráiz

Dr. Pietro Gramazio

Tutor:

Dr. Jaime Prohens Tomás

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“La voluntad separa el grano de la paja”

David Beriain

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Abstract

Cannabis sativa L. (Cannabaceae) is an angiosperm, allogamous and dicotyledonous species that includes short and neutral-day varieties with dioecious specimens (males and females), and monoecious plants. Among its many applications, its industrial and medicinal uses stand out. Despite the fact that cannabis has been used by humans since ancient times and the growing interest that the *C. sativa* therapeutic properties have aroused in researchers around the world, the psychoactivity of some of its varieties, derived from its Δ^9-tetrahydrocannabinol (THC) content, has motivated the prohibition of its cultivation for almost sixty years. The strict control to which cannabis has been subjected has prevented professionals from all over the world from carrying out genetic breeding programs for this species, which has resulted in the absence of uniform varieties.

In this Doctoral Thesis, different biotechnological tools for cannabis genetic improvement have been developed. In the first place, given the lack of reproducibility of some cannabis plant *in vitro* regeneration protocols and the great influence that the genotype exerts on their effectiveness, plant *in vitro* regeneration competence of different explants was evaluated. As a result, an hormone-free protocol from *C. sativa* hypocotyls that presents high regeneration rates (ranging from 32.26% to 71.15%) in all the genotypes evaluated, also presenting a 17.94% of spontaneous rooting rate of regenerants has been developed. At the same time, the polysomatic pattern of different cannabis explants has been studied, and it has been possible to regenerate, from them, a significant percentage of mixoploid specimens (17.65% from cotyledons and 13.33% from hypocotyls) that, as described in the existing literature, could show a greater capacity for cannabinoid synthesis.

On the other hand, given the absence of scientific publications in this regard, and the potential that this technique presents to alleviate the intrinsic variability of this species, the most in-depth study to date on the male floral

biology of *C. sativa* has been developed. Up to 476,903 microspores and pollen grains per male flower, with *in vivo* microspore viability rates from 53.71 to 70.88% have been found. Furthermore, all stages of development of the microgametophyte have been correlated with an easily measurable floral morphological marker such as the bud length, identifying bud length intervals containing mostly vacuolate microspores and young bi-cellular pollen grains in all the phenotypes evaluated. In this way, and although the starch presence in *C. sativa* microspores and pollen grains follows a similar pattern to that observed in species recalcitrant to androgenesis, it has been possible to address the induction of microspore embryogenesis in this species, obtaining for the first time microspore-derived multicellular structures after one week long cold-shock bud pretreatment.

Finally, as a prerequisite for the genetic editing of *C. sativa* by using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas systems, and taking advantage of the *in vitro* plant regeneration protocol which resulted from this Doctoral Thesis, it has been possible to develop for the first time a protocol for the production of stably transformed cannabis plants, which represents a historical milestone in the genetic improvement of the species. After co-culture with *A. tumefaciens* and subsequent culture in antibiotic-containing selective regeneration medium, hypocotyls achieved 23.1% and 5.0% of regeneration and transformation rates respectively.

As a whole, the present Doctoral Thesis provides a range of biotechnological tools that will allow the development of a new generation of high-yield cannabis varieties with uniform traits, resistant to multiple biotic and abiotic stresses, and therefore being suitable for both industrial and medicinal use.

Resumen

Cannabis sativa L. (Cannabaceae) es una especie angiosperma, alógama y dicotiledónea compuesta por variedades de día corto y día neutro que presentan ejemplares dioicos (machos y hembras), y plantas monoicas. Entre sus múltiples aplicaciones destacan tanto su uso industrial como su uso medicinal. A pesar de que el cannabis ha sido empleado por el ser humano desde tiempos ancestrales, y del creciente interés que ha despertado en investigadores de todo el mundo debido a su utilidad terapéutica, la psicoactividad que presentan algunas de sus variedades, derivada de su contenido en Δ^9-tetrahidrocannabinol (THC), ha motivado la prohibición de su cultivo durante casi sesenta años. La estricta fiscalización a la que ha sido sometido el cannabis, ha impedido que profesionales de todo el mundo puedan llevar a cabo programas de mejora genética de esta especie, lo que se ha traducido en la ausencia de variedades uniformes.

En esta Tesis Doctoral se han desarrollado diferentes herramientas biotecnológicas para la mejora genética del cannabis. En primer lugar, dada la falta de reproducibilidad de algunos protocolos de cultivo *in vitro* de cannabis y la gran influencia que el genotipo ejerce en la efectividad de los mismos, se evaluó la capacidad de regeneración *in vitro* de diferentes explantes. Como resultado, se ha desarrollado un protocolo libre de hormonas a partir de hipocótilos de *C. sativa* que presenta altas tasas de regeneración (las cuales oscilan del 32,26% al 71,15%) en todos los genotipos evaluados, presentando además un 17,94% de tasa de enraizado espontáneo de los regenerantes. A su vez, se ha estudiado el patrón polisomático de diferentes explantes de cannabis y se ha conseguido regenerar, a partir de los mismos, un porcentaje significativo de ejemplares mixoploides (17,65% procedentes de cotiledones y 13,33% de hipocotilos) que, tal y como describe la bibliografía existente, podrían mostrar una mayor capacidad de síntesis de cannabinoides.

Por otro lado, dada la ausencia de publicaciones científicas al respecto y el potencial que esta técnica presenta para paliar la variabilidad intrínseca de esta especie, se ha desarrollado el estudio más profundo hasta la fecha relativo a la biología floral masculina de *C. sativa*. Se han descrito hasta 476.903 microsporas y granos de polen por flor masculina, con tasas de viabilidad *in vivo* de las microsporas del 53,71 al 70,88%. Además, se han correlacionado todas las etapas de desarrollo del microgametofito con un marcador morfológico floral fácilmente medible como la longitud de la yema, identificando intervalos de longitud de yema que contienen mayoritariamente microsporas vacuoladas y granos de polen joven bi-celular en todos los fenotipos evaluados. De este modo, y aunque la presencia de almidón en las microsporas y granos de polen de *C. sativa* sigue un patrón similar al observado en especies recalcitrantes a la androgénesis, ha sido posible abordar la inducción de la embriogénesis de microsporas en esta especie, consiguiendo producir por primera vez estructuras multicelulares derivadas de las microsporas tras aplicar sobre las yemas un pretratamiento de frío de una semana de duración.

Finalmente, como requisito previo para la edición genética de *C. sativa* mediante los sistemas Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas, y haciendo uso del protocolo de regeneración *in vitro* de plantas surgido de la presente Tesis Doctoral, se ha conseguido desarrollar por primera vez un protocolo para producir plantas de cannabis transformadas genéticamente de forma estable, lo que supone un hito histórico en la mejora genética de la especie. Después del cocultivo con *A. tumefaciens* y el posterior cultivo en medio de regeneración selectiva con antibióticos, los hipocótilos lograron respectivamente un 23,1% y un 5,0% de tasas de regeneración y transformación.

En su conjunto, la presente Tesis Doctoral proporciona un abanico de herramientas biotecnológicas que permitirán el desarrollo de una nueva generación de variedades de cannabis de alto rendimiento, que presenten caracteres homogéneos, resistentes a múltiples estreses tanto bióticos

como abióticos, y siendo así aptas tanto para un uso industrial como medicinal.

Resum

Cannabis sativa L. (Cannabaceae) és una espècie angiosperma, alógama i dicotiledònia composta per varietats de dia curt i dia neutre que presenten exemplars dioics (mascles i femelles), i plantes monoiques. Entre les seues múltiples aplicacions destaquen tant el seu ús industrial com el seu ús medicinal. Tot i que el cànnabis ha sigut emprat per l'ésser humà des de temps ancestrals, i del creixent interés que ha despertat en investigadors de tot el món a causa de la seua utilitat terapèutica, la psicoactivitat que presenten algunes de les seues varietats, derivada del seu contingut en Δ^9-tetrahidrocannabinol (THC), ha motivat la prohibició del seu cultiu durant gairebé seixanta anys. L'estricta fiscalització a la qual ha sigut sotmés el cànnabis, ha impedit que professionals de tot el món puguen dur a terme programes de millora genètica d'aquesta espècie, la qual cosa s'ha traduït en l'absència de varietats uniformes.

En aquesta Tesi Doctoral s'han desenvolupat diferents eines biotecnològiques per a la millora genètica del cànnabis. En primer lloc, donada la falta de reproducibilitat d'alguns protocols de cultiu *in vitro* de cànnabis i la gran influència que el genotip exerceix en l'efectivitat d'aquests, es va avaluar la capacitat de regeneració *in vitro* de diferents explants. Com a resultat, s'ha desenvolupat un protocol lliure d'hormones a partir de hipocòtils de *C. sativa* que presenta altes taxes de regeneració (les quals oscil·len del 32,26% al 71,15%) en tots els genotips avaluats, presentant a més un 17,94% de taxa d'arrelat espontani dels regenerants. Al mateix temps, s'ha estudiat el patró polisomàtic de diferents explants de cànnabis i s'ha aconseguit regenerar, a partir d'aquests, un percentatge significatiu d'exemplars mixoploids (17,65% procedents de cotilèdons i 13,33% de hipocòtils) que, tal com descriu la bibliografia existent, podrien mostrar una major capacitat de síntesi de cannabinoids.

D'altra banda, donada l'absència de publicacions científiques sobre aquest tema i el potencial que aquesta tècnica presenta per a pal·liar la variabilitat intrínseca d'aquesta espècie, s'ha desenvolupat l'estudi més profund fins hui relatiu a la biologia floral masculina de *C. sativa*. S'han descrit fins a 476.903 microspores i grans de pol·len per flor masculina, amb taxes de viabilitat in vivo de les microspores del 53,71 al 70,88%. A més, s'han correlacionat totes les etapes de desenvolupament del microgametòfit amb un marcador morfològic floral fàcilment mesurable com la longitud de la gemma, identificant intervals de longitud de gemma que contenen majoritàriament microspores vacuolades i grans de pol·len jove bi-cel·lular en tots els fenotips avaluats. D'aquesta manera, i encara que la presència de midó en les microspores i grans de pol·len de *C. sativa* segueix un patró similar a l'observat en espècies recalcitrants a la androgènesi, ha sigut possible abordar la inducció de la embriogènesi de microspores en aquesta espècie, aconseguint produir per primera vegada estructures multicel·lulars derivades de les microspores després d'aplicar sobre les gemmes un pretractament de fred d'una setmana de duració.

Finalment, com a requisit previ per a l'edició genètica de *C. sativa* mitjançant els sistemes Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas, i fent ús del protocol de regeneració *in vitro* de plantes sorgit de la present Tesi Doctoral, s'ha aconseguit desenvolupar per primera vegada un protocol per a produir plantes de cànnabis transformades genèticament de manera estable, la qual cosa suposa una fita històrica en la millora genètica de l'espècie. Després del cocultiu amb *A. tumefaciens* i el posterior cultiu en medi de regeneració selectiva amb antibiòtics, els hipocòtils van aconseguir respectivament un 23,1% i un 5,0% de taxes de regeneració i transformació.

En el seu conjunt, la present Tesi Doctoral proporciona un ventall d'eines biotecnològiques que permetran el desenvolupament d'una nova generació de varietats de cànnabis d'alt rendiment, que presenten caràcters homogenis, resistents a múltiples estressos tant biòtics com abiòtics, i sent així aptes tant per a un ús industrial com medicinal.

General Introduction

1. ***Cannabis sativa L. botany***

Cannabis sativa L. (2n=2x=20) is an angiosperm, dicotyledonous, herbaceous and annual plant that comprises short and neutral-day varieties. *Cannabis sativa* is an allogamous species, generally dioecious (androecious and gynoecious individuals), although monoecious specimens showing male and female flowers in the same plant, and some speciments that occasionally develop hermaphrodite flowers are also found. Since wind is crucial for its pollination, *C. sativa* is described as anemophilous, which explains the absence of corolla and bright colors in their flowers.

The female or pistillate flower of *C. sativa* is composed of a calyx formed by an enveloping sepal or bract that encloses a single ovary from which two hair-shaped styles originate. Its floral formula is: O ♀ ↯ K(1) C(0) A(0) G̲(1) (Salami, 2008).

The male or staminate flower of *C. sativa* is composed of a calyx formed by five non-soldered sepals. The androecium consists of five anthers. Its floral formula is: O ♂ ✳ K(5) C(0) A(5) G(0) (Salami, 2008).

Figure 1. *C. sativa* male individual showing staminate flowers (A) and seed-bearing female plant (B); 1 male flower, enlarged detail; 2 and 3 pollen sac of same from various angles; 4 pollen grain of same; 5 female flower with cover petal; 6 female flower, cover petal removed; 7 female fruit cluster, longitudinal section; 8 fruit with cover petal; 9 same without cover petal; 10 same; 11 same in cross-section; 12 same in longitudinal section; 13 seed without hull (Köhler, 1887)

1.1. *Taxonomy*

There is no unanimity when it comes to categorizing cannabis into different taxa within a hierarchical classification system. The extraordinary variability of this species, as a consequence of its adaptive flexibility, has caused the lack of existing consensus on its taxonomic classification, a controversy that has also been enhanced by the difficulty of species delineation caused by hybridity, as first described by Wiegand (1935). As a result, complications when classifying *Cannabis* based on morphological traits have driven botanical taxonomists to the current discussion concerning *Cannabis* classification.

The first taxonomist to coin the term *Cannabis sativa* was Linnaeus in 1753, specifically in his book '*Species Plantarum'*, accepted internationally as the official beginning of modern botanical nomenclature; although it is also reported that it was firstly named by Fuchs, among others, in 1542 (Fuchs, 1999; Russo, 2019). Afterwards, de Lamarck (1783) described that *Cannabis indica* species presented morphological and phytochemical characters "very distinct" from the species that Linnaeus called *Cannabis sativa*, emphasizing its inebriant potential. More than a century later, Janischevsky (1924) recognized a third species of cannabis while studying wild populations, describing a small and slightly branched plant with wild-type seed characteristics, this being baptized as *Cannabis ruderalis*. As a result of these findings, a classification emerged that establishes three different species within the *Cannabis* genus (Schultes *et al*., 1975):

- *Cannabis sativa* L. (Linnaeus, 1753)
- *Cannabis indica* Lam. (de Lamarck, 1783)
- *Cannabis ruderalis* Jan. (Janischevsky, 1924)

With respect to the Cannabaceae family, some authors believed that it possessed so many similarities with the Ulmaceae, Moraceae and Urticaceae families, that they could be merged into a single family (Gola *et al*., 1965). Other classifications included the family Cannabaceae as a tribe within the

family Moraceae (Chadefaud and Emberger, 1960). Regarding its gender, some researchers consider the gender *Humulus* as the only companion of the *Cannabis* genus within the Cannabaceae family (Schultes *et al*., 1975; Bagci *et al*., 2009).

In the last decades, some molecular and genetic studies have been performed in order to clarify the *C. sativa* taxonomy. Researchers have detected variations in the enzymes responsible for the synthesis of the main cannabinoids, correlating them with the different chemotypes of the plant (Hillig and Mahlberg, 2004; Hillig, 2005). These studies integrated three species into the *Cannabis* genus, giving rise to seven taxa (McPartland and Guy, 2004):

- *Cannabis ruderalis*
- *Cannabis sativa* → *Cannabis sativa* ssp. *sativa*
  → *Cannabis sativa* ssp. *spontaneous*
- *Cannabis indica* → *Cannabis indica* ssp. *indica*
  → *Cannabis indica* ssp. *kafiristanica*
  → *Cannabis indica* ssp. *afghanica*
  → *Cannabis indica* ssp. *chinensis*

On the other hand, some researchers considered *Cannabis* a monospecific genus (Small and Cronquist, 1976; de Meijer *et al.*, 2003; Small, 2015; Zhang *et al.*, 2018), classification supported by little DNA sequence variation found by Gilmore *et al.* (2007) among drug-type, fiber-type and wild populations, suggesting that the different *Cannabis* haplotypes segregated at a rank below that of species, or the low mean values obtained after comparing *C. sativa* and *C. indica* divergence measurements (McPartland and Guy, 2014). In agreement with this hypothesis, McPartland (2018) highlighted that DNA barcode analysis supports the separation of the

sativa and *indica* taxa at a subspecies level, also emphasizing that ubiquitous interbreeding and hybridization of *sativa* and *indica* performed for several thousand years has rendered their distinctions almost meaningless. In this respect, it is noteworthy that some researchers consider *Cannabis* ancestral wild populations as extinct, while extant wild populations are interpreted as weedy derivatives of domesticated populations (Small, 2017). Other studies point that traditional landraces of *sativa* and *indica* are becoming extinct through introgressive hybridization (McPartland, 2017).

Considering the above-mentioned studies, the following taxonomic classification can be derived and will be used in this Thesis:

Table 1. *C. sativa* taxonomic classification

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Hamamelididae
Order	Urticales
Family	Cannabaceae
Genus	*Cannabis*
Species	*Cannabis sativa* L.
Subspecies	*Cannabis sativa* L. ssp. *ruderalis*
	Cannabis sativa L. ssp. *sativa*
	Cannabis sativa L. ssp. *indica*

Different attributes such as leaf morphology, plant growth pattern, flowering duration or photoperiodism, are distinguishable features which have proven useful in order to discern among the different cannabis subspecies. While *C. sativa* ssp. *sativa* tends to be tall, with separated branches, very narrow leaflets and long blooming periods, *C. sativa* ssp. *indica* presents a low stature and a densely branched conical growth habit, with a broad leaflet type and shorter flowering periods. On the other hand, *C. sativa* ssp. *ruderalis* is very small in height, being slightly branched or even unbranched at maturity, with smaller leaves than either of the two other subspecies. It represents neutral-day plants whose flowering is not influenced by day-length.

Figure 2. Morphological and anatomical differences among *C. sativa* ssp. *sativa* (left), *C. sativa* ssp. *indica* (middle) and *C. sativa* ssp. *ruderalis* (right). Image adapted from Anderson (1980)

On the other hand, cannabinoids are considered important chemotaxonomic markers unique to *Cannabis* (Small and Cronquist, 1976; Hillig and Mahlberg, 2004). These compounds are biologically active terpenophenolic metabolites that are responsible for the pharmacological properties of *C. sativa*. Cannabinoids are synthesized in their acid form predominantly in glandular trichomes mainly distributed on the bracts of cannabis female flowers. On the other hand, the main cannabinoid responsible for the narcotic effects of certain *C. sativa* varieties (and, therefore, for the prohibition of its cultivation) is Δ^9-tetrahydrocannabinol (THC), which was first isolated and characterized by Gaoni and Mechoulam

(1964). Based on the THC content, cannabis can be classified as hemp (cultivars mainly developed for fiber and seed production, with low THC content) or marijuana (drug-type cultivars bred for medical and recreational use, with high THC content) (Zhang *et al.*, 2018). From a medical point of view, it is more practical to classify the *C. sativa* plant by its chemotype, making distinctions between *Cannabis* accessions based on their biochemical profile, which is defined by secondary metabolites such as cannabinoids and terpenoids, and their consequent pharmacological properties (Russo, 2019; Reimann-Philipp *et al*., 2020).

2. *Cannabis sativa history, uses and economic potential*

2.1. *Origin, dispersion and domestication*

Although there is no consensus on its place of origin, existing evidence suggests a multiregional origin of cannabis (Long *et al.*, 2017). Paleobotanical studies attest that it was already present during the Holocene epoch about 11,700 years ago (Pisanti and Bifulco, 2019). It seems that the center of origin for wild *C. sativa* is located in central Asia, been described in Altai mountains (mountain range bordering Russia, China, Mongolia and Kazakhstan), in the Tian Shan mountains (which separates Kazakhstan and Kyrgyzstan from northwest China), and near the Irtysch River as it passes through western Siberia, where Vavilov (1926) described populations of cannabis plants growing wild. At this respect, based on pollen studies, ecological proxies and archaeological evidence, the Tibetan Plateau has been proposed as *Cannabis* center of origin (McPartland *et al.*, 2019). More recently, by using whole-genome resequencing of different accessions from worldwide origins, it has been stated that all current hemp and drug cultivars diverged from an ancestral gene pool currently represented by feral plants and landraces in China (Ren *et al*., 2021). However, as declared by Clarke and Merlin (2016), the exact geographical origin of *Cannabis* is unclear today because its location changed repeatedly throughout hundreds of thousands of years due to glacial-interglacial cycles developed during the Pleistocene.

Additionally, Small (2017) argues that, mainly due to the spread and modification performed by humans for millennia, there does not seem to be a reliable means of accurately determining the *C. sativa* original geographical range.

Figure 3: *Cannabis sativa* center of origin described in mountainous regions located in central Asia and bordering Russia, China, Mongolia and Kazakhstan (content shared under Creative Common License in https://en.wikipedia.org/)

The great adaptation of *C. sativa* to different soil and climate conditions facilitated its spread in different temperate and tropical regions. It is thought that climatic and related ecological changes, like the migration of ancient nomadic tribes such as the Scythians, affected the natural and cultivated distribution of *C. sativa* (Clarke and Merlin, 2016). Herodotus (*ca.* 500 BCE) mentions the *Cannabis* recreational use of the Scythian and other Central Asian tribes by breathing the vapors released during seed combustion on stones, and how they “howl with joy for the vapour bath” (Warf, 2014; Clarke and Merlin, 2016). There are also reports on tombs of Caucasoid nobles buried in Xinjiang and Siberia around 2,500 BCE which occasionally include large quantities of mummified psychoactive *C. sativa* (Warf, 2014, and references therein).

Figure 4: 2,700-year-old mummy of a shaman belonging to the Gushi nomad culture found by a team of archaeologists in 2003 in the Xinjiang region, located in the northwest of China. His belongings included bridles, bows, a harp, and 789 grams of cannabis (content shared under Creative Common License in http://foroterraeantiqvae.ning.com/)

While nomadic tribes from Central Asia could be responsible for *Cannabis* introduction in South Asia, the Middle East and Eastern Europe along the various paths that constituted the Silk Road, Arab traders may have introduced *Cannabis* into Eastern Africa, perhaps one to two thousand years ago (Hillig, 2005, and references therein). By the first half of the sixteenth century, *Cannabis* was introduced from Africa to Latin America. Precisely, cannabis seeds were transported to Brazil by slaves coming from Angola who went to work in sugar cane plantations where the landlords let them grow it, and because of this, nearly all the traditional synonyms for marijuana in Brazil had their origin in the Angolan language (De Pinho, 2011). In parallel, in order to avoid the England dependence on foreign hemp, which was a very valuable resource needed to build ships and remain connected

with the rest of the world, in 1611 the king ordered the English colonists at Jamestown, Virginia, to grow hemp (Abel, 1980).

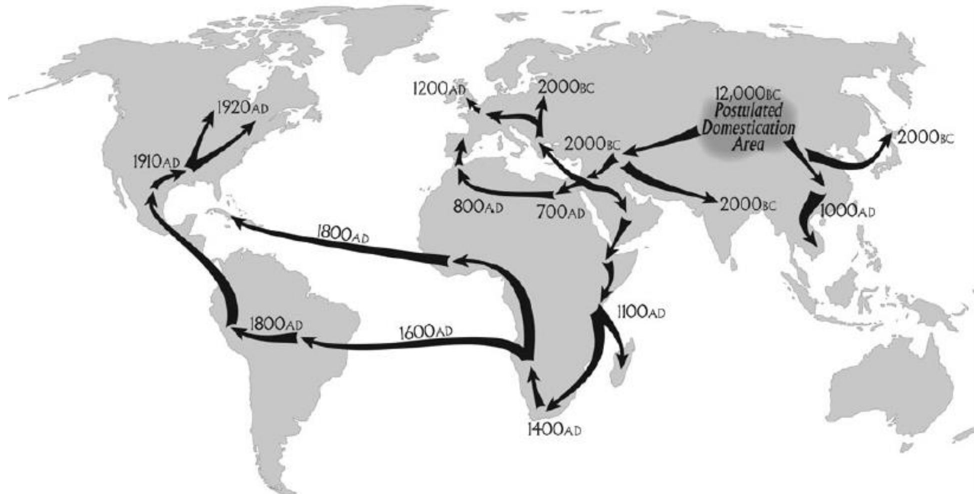

Figure 5: *C. sativa* geographical and historical dispersion (Warf, 2014): arrows suggest humanvectored dispersal from the presumed origin of *Cannabis* in Central Asia

Concerning *Cannabis* domestication, it has been proposed that it was first domesticated in early Neolithic times in East Asia (Ren *et al*., 2021). In this line, and according to archaeological evidences found in Taiwan, it can be concluded that this plant has been used by humans for at least 10,000 years (Abel, 1980). In the past, while in areas of Central and Eastern Europe with temperate, mild, relatively cool, and moist conditions, the ancient farmers selected individuals with relatively limited intoxicant potential, thick and long fibers, and large seeds with a high oil content (cultivars today known as hemp), in semi-tropical and/or very dry regions of India, Pakistan, Nepal or Afghanistan, plants with a copious resin production and, consequently, a considerable intoxicant potential were selected, this being the beginning of the world-famous marijuana varieties. In fact, some researchers assumed that the *sativa* (hemp biotypes) and *indica* (drug-type plants) taxa diverged primarily as a result of this human selection (Small and Cronquist, 1976; Hillig and Mahlberg, 2004).

2.2. *Cannabis: a multipurpose crop*

The selective pressure exerted by the environment together with the domestication carried out by humans has been translated into the great variability currently exhibited by *C. sativa* species. Nowadays, this versatile crop can contribute to reducing the human impact on the environment by means of the production of sustainable resources like biomass and fiber that constitute feedstock for industrial uses such as energy, construction and automotive markets, and for hempseeds that are components of functional foods and animal feeds (Żuk-Gołaszewska and Gołaszewski, 2020). Furthermore, its medical applications are well substantiated, been documented in the most ancient Chinese Pharmacopoea, the '*Shen Nung Pen Ts'ao Ching'* written by Shen Nung (considered the father of Chinese medicine) in the first century BCE, who also warned that too much *Cannabis* causes to "see demons" or "communicate with spirits" (Pisanti and Bifulco, 2019). Afterwards, the Greek Dioscorides, who was an eminent scientist and a physician, published in 70 CE the first copy of *De materia medica*, an important treatise on medicinal herbs, where it appears the description of symptoms and conditions for which *Cannabis*, among other medicinal plants, had proven beneficial. It was the first description of *Cannabis* as a medical remedy in a Western medical text, describing how the juice of its seeds was very beneficial in treating earaches and in diminishing sexual desires (Abel, 1980).

Figure 6: *Cannabis sativa* description and illustration present in '*Illustration of civil life, volume the earth*' published in 1830 (Ahn *et al.*, 2020) (left), and in the facsimile copy of *De materia medica* made in the 19th century that includes the translation from Latin to Spanish, and illustrations made by Andrés Laguna, physician of Pope Julius III (right).

Nowadays, this species is gaining increasing attention due to its medical applications. Cannabinoids are responsible for the therapeutic potential of *C. sativa*. More than one hundred cannabinoids have been identified in the cannabis plant (Calapai *et al.*, 2020), although THC and cannabidiol (CBD) are the most abundant in the plant. Substantial evidence supporting how *Cannabis* derivatives are effective for the treatment of chronic pain, spasticity associated with multiple sclerosis, and chemotherapy-induced nausea and vomiting have been reported (Cascio *et al.*, 2017; Abrams, 2018; Urits *et al*., 2019). Additionally, an increasing number of preclinical studies point to the anticarcinogenic potential of *Cannabis* derived compounds, although there is still a lack of profound safety and efficacy clinical trials to assess the potential benefits and risks of incorporating cannabis pharmaceutical derivatives in clinical care (Śledziński *et al*., 2018).

Recently, and due to the absence of narcotic effects on its consumption, CBD is gaining prominence among researchers. Some indications concerning its therapeutic benefit and medical application in epilepsy and its associated comorbidities such as convulsions, depression, sleep disorders, anxiety and inflammation have been published (Thomas, 2017). Furthermore, CBD is considered as an attractive novel therapeutic option for the treatment of anxiety disorders derived from the newly emerged COVID-19 disease caused by the beta coronavirus SARS-CoV-2 (O'Sullivan *et al.*, 2021), also exhibiting observable antiviral effects against Human Coronavirus (HCoV) strain 229E in *in vitro* experiments (Chatow, *et al.*, 2021).

In addition, it is remarkable how, from a pharmacological perspective, cannabis shows itself considerably safer than opioids and with a broad applicability for palliative care, being an alternative for many patients currently on long-term use of opioids for chronic pain (Carter *et al*., 2011). This alternative is showing promising results in the United States, where states with medical cannabis laws are associated with significantly lower state-level opioid analgesic overdose mortality rates (Carter *et al*., 2011; Bachhuber *et al*., 2014; Hsu and Kovács, 2021). In fact, several studies point to a growing tendency in cannabis patient use to treat pain derived from different ailments, and how opioid consumption has concurrently decreased (Reiman *et al.*, 2017; Vigil *et al.*, 2017; Bradford *et al.*, 2018; Okusanya *et al.*, 2020; Denduluri *et al.*, 2021; López *et al*., 2021; Muacevic *et al.*, 2021).

In this respect, it is necessary to emphasize that it has been described in many studies how THC and CBD can synergize with other compounds of the plant such as terpenoids or other cannabinoids. Therefore, cannabis-based medicinal extracts may result therapeutically more efficient than isolated or synthesized cannabinoids applied separately (Williamson, 2001; Wagner and Ulrich-Merzenich, 2009; Russo, 2011; Gallily *et al*., 2015; LaVigne *et al.*, 2021). It is noteworthy pointing out how cannabis-based medications were approved in 2011 in many European countries (Carcieri *et al*., 2018).

2.3. *Legal context of its cultivation*

Cannabis sativa cultivation and consumption has been a source of discordance since immemorial times. The first documentation of its prohibition dates from 1800, when Napoleon, after Egypt invasion and fearing that cannabis would make them lose their fighting spirit, prohibited his soldiers to smoke or drink the extracts of the plant. He imposed a penalty of imprisonment of three months, thus implementing perhaps the first 'penal law' on cannabis (Ballotta *et al.*, 2008). After that, cannabis was banned for the first time in Brazil in 1830, when the Rio de Janeiro municipal council issued a directive that forbade its sale and use, as well as its presence on any public premises (Bewley-Taylor *et al*., 2014). Following this, the cultivation, use and importation of cannabis were first forbidden in Egypt in 1868, although its prohibition had little effect on its medical and recreational consumption by urban and rural poor from Egypt (Bewley-Taylor *et al*., 2014). Meanwhile, the cannabis control in Europe was focused on regulating its pharmaceutical use, as Germany did in 1872, limiting the sale of Indian hemp to pharmacies through a Pharmacy Ordinance (Ballotta *et al.*, 2008). Subsequently, a law in South Africa in 1887 prohibited cannabis use and possession by Indian immigrants, who along with black people were the main consumers of this drug at that time. Because of this, it is thought that behind this prohibition there were racist motivations (Booth, 2005). However, in what is considered as the first resistance to cannabis prohibition, an extensive 3,000-page report by the 'Indian Hemp Drugs Commission' in 1894, provided an exhaustive analysis of cannabis benefits, risks and harms, bringing the necessary evidence for reject this prohibition. Despite the rejection of a general ban, Indian cities and states issued quotas, tax regimes or restrictions on cannabis (Booth, 2005). In the former report, the Commission concluded that "the moderate use of hemp drugs produces no injurious effects on the mind", also adding that "as a rule these drugs do not tend to crime and violence", and that "moderate use of these drugs is the rule, and the excessive use is comparatively exceptional. The moderate

use produces practically no ill effects" (Indian Hemp Drugs Commission, 1894). Despite the conclusions presented in this report composed of 7 volumes, which contrasted with the absence of arguments beyond the non-supported with data association of cannabis, insanity and crime exposed by those in favour of the ban, cannabis prohibition continued advancing along the whole world.

Cannabis international circulation was firstly negotiated among the international community in the 'International Opium Convention' held in The Hague in 1912. What countries most feared at that time was that the unregulated drug trade could lead to an increase in drug consumption in their territories. However, since most of the states were reluctant to criminalize the non-medical use of these substances, the treaty was mainly focused on the regulation of drug legal trade and its availability for medical uses (Bewley-Taylor *et al*., 2014). Concerned about the smuggling of hashish (a drug derived from the concentration of trichomes from the cannabis plant) into its North African colonies, Italy raised the issue of cannabis international control in this convention, being supported only by the United States of America (Bruun *et al.*, 1975). However, it was not until the celebration of the 'International Opium Convention' in Ginebra in 1925, and especially after World War II that the United States, just based on the cannabis horror stories spread by the American government and media, used its global influence to reach the necessary support to push the international community to ban cannabis in the United Nations 'Single Convention on Narcotic Drugs' signed in New York in 1961, a consolidation of a series of multilateral drug control treaties negotiated between 1912 and 1953. In attendance were representatives of 73 states and a range of international organisations (Bewley-Taylor *et al*., 2014). In this Single Convention, cannabis was included in Schedule I, containing those substances considered most addictive and most harmful, and in the strictest Schedule IV, containing those substances to be the most dangerous and regarded as exceptionally addictive and producing severe ill effects. Thus,

cannabis was classified among the most dangerous psychoactive substances under international control with extremely limited pharmaceutical properties. Cannabis, cannabis resin and extracts and tincture of cannabis were therefore subjected to all control measures foreseen by the Convention (Bewley-Taylor, 2012), even though leaves and seeds were explicitly omitted from the definition of cannabis, which now only referred to the "flowering or fruiting tops of the cannabis plant" (Bewley-Taylor and Jelsma, 2011). In consequence, despite little or no scientific evidence on the alleged connection between cannabis, insanity and crime, and in order to protect the public health and welfare, the Convention stipulated that any signatory should "prohibit the production, manufacture, export and import of, trade in, possession or use of any such drug except for amounts which may be necessary for medical and scientific research only" (Bewley-Taylor *et al*., 2014). The 'Single Convention on Narcotic Drugs' of 1961 was incorporated in the Spanish legislation the 8th of April of 1967 by means of the Spanish law 17/1967.

After that convention, Δ^9-tetrahydrocannabinol (THC) was first isolated and characterized by Gaoni and Mechoulam (1964), and cannabinoids were related with the pharmacological properties of *C. sativa*, which attracted the interest of the pharmaceutical industry and researchers from all corners of the globe. World Health Organization (WHO) Expert Committee on Drug Dependence (ECDD), an independent group of experts in the field of drugs and medicines responsible for assessing psychoactive substances for possible control under the international drug control conventions, recommended in 1990 de-scheduling certain active ingredients of cannabis. This recommendation included the integration of compounds such as THC, as well as their isomers and stereochemical variants, in the Schedule II of the United Nations (UN) 'Convention on psychotropic substances' held in Vienna in 1971, which also included certain amphetamine-type stimulants (ATS) with therapeutic uses. This represented an important step towards the recognition of cannabis therapeutic usefulness. This recommendation was

adopted by the Commission on Narcotic Drugs (CND) in 1991, when dronabinol, a synthetic form of THC, was included in Schedule II of the above-mentioned convention (Bewley-Taylor *et al*., 2014). Following this, many commissions to examine drug use arose in different countries recommending changes in the law on cannabis restrictions, as is the case of the United Kingdom (Report by the Advisory Committee on Drugs Dependence, the so-called Wootton Report, 1969), the Netherlands (The Baan Commission, 1970 and Hulsman Commission, 1971), the U.S. (The Shafer Commission Report, Marihuana: A Signal of Misunderstanding, National Commission on Marihuana and Drug Abuse, 1972), Canada (The Commission of Inquiry into the Nonmedical Use of Drugs, commonly referred to as the Le Dain Commission, 1973) and Australia (Senate Social Committee on Social Welfare, 1977) (Bewley-Taylor *et al*., 2014). This prompted some states within the U.S. to decriminalize cannabis, as is the case of Oregon in 1973, or Alaska and California in 1975 (Bewley-Taylor *et al*., 2014).

Not so long ago, policy shifts towards legally regulated markets within Colorado and Washington in the U.S. in 2012, and in Uruguay (2013), and Canada (2018), paved the way for cannabis legalization (Bewley-Taylor *et al*., 2014). More recently, and after several cannabis de-scheduling attempts promoted by the WHO Expert Committee, a historical moment for cannabis legalization arrived. On the 24th January 2019, Tedros Adhanom, who has served since 2017 as Director-General of the WHO, sent a letter to the Secretary-General of the UN António Guterres communicating its recommendations to decriminalize cannabis after the critical reviews of cannabis and cannabis-related substances carried out in the forty-first meeting of the WHO ECDD. Among others, the WHO ECDD recommendations included the deletion of cannabis and cannabis resin from Schedule IV, together with the deletion of extracts and tinctures from Schedule I of the Single Convention on Narcotic Drugs (1961). Furthermore,

the WHO manifested that pure CBD should not be scheduled within the International Drug Control Conventions.

Although not all of the WHO recommendations were implemented, in the UN Commission on Narcotic Drugs Report on the reconvened sixty-third session, elaborated in Vienna in December 2020, it was decided “To delete cannabis and cannabis resin from Schedule IV of the Single Convention on Narcotic Drugs of 1961 as amended by the 1972 Protocol” (Decision 63/17). This has constituted a historical political recognition of cannabis pharmacological properties which has arrived after 59 years of non-sense cannabis prohibition.

2.4. ***Cannabis market and economic predictions***

As cannabis legalization has been reaching different countries, a potential international market is emerging. Although predictions dramatically vary from source to source, all of them anticipate a multibillion-dollar global cannabis market to be developed in the following years, as cannabis legalization either for medical or recreational use expands in more countries. For example, while the first year of Canada recreational sales was valued at CAD 967 million (Armstrong, 2021), by 2025 the projected market value only for medical cannabis in the United States is estimated to be over USD 13 billion (Mikulic, 2021). In the case of Spain, it is estimated that the medical, recreational and industrial markets value will reach € 6.5 billion by 2028 (Prohibition Partners, 2018). It is also predicted that by 2024 the United Kingdom medicinal and recreational markets would be worth nearly USD 1.3 billion and USD 1.7 billion, respectively (Prohibition Partners, 2019a). Furthermore, assuming that by 2023 all countries profiled in the report have legalized medical cannabis and regulated recreational use, it is expected a potential market worth € 58 billion and € 65 billion by 2028 in Europe for medical and recreational cannabis respectively, so consequently Europe cannabis market is estimated to be worth up to € 123 billion by 2028,

becoming the world largest legal market over the next five years (Prohibition Partners, 2019b).

However, special attention must be paid to the financial bubbles that can arise and burst as a result of highly speculative market predictions. A good example of this was observed in the second half of 2019, when the market value of a Canada-based Canopy Growth (one of the world largest cannabis companies), dropped from USD 18 billion (CAD 24 billion) in April 2019 to about USD 5.5 billion (CAD 7.1 billion) in mid-November (Bewley-Taylor *et al.*, 2020, and references therein).

3. *Biotechnological approaches for C. sativa genetic improvement*

Despite its multiple uses and the multibillion-dollar market predictions related with cannabis, decades of illegalization have been translated into a lack of formal genetic improvement of this species, and the consequent absence of stable varieties with consistent traits. Thus, the allogamous nature of *C. sativa* is transferred into its inherent genetic and phenotypic heterogeneity, which hinders the breeding of this species and results in reduced uniformity for food, fiber, or medical applications (Andre *et al.*, 2016; Onofri and Mandolino, 2017). Regarding the cannabinoid content, the lack of homogeneity in the existing varieties and its concurrent trait variability present important disadvantages, especially in the case of hemp cultivation, as if the THC content of individuals from a specific variety exceeds the legal limit, the crop could be declared illegal, with the consequent damage to the farmer derived from his legal responsibility. On the other hand, regarding medical cannabis, it has been reported the low abundance of some promising minor cannabinoids *in planta* (Andre *et al.*, 2016), which represents an impediment for its extraction, purification and pharmacological evaluation. Furthermore, if cannabis plants are grown from seeds, the type and concentration of cannabinoids can vary among individuals of the same variety, thus hindering the availability of a specific chemotype reproducible after flowering and senescence of plants. So, even

if a particular cannabinoid profile presents in a specific phenotype showed therapeutic potential, it would not be available for further trials or large-scale cannabinoid extraction. For avoiding this, elite clones showing promising chemotypes are selected and vegetatively propagated to preserve them, but mother plants need to be maintained indefinitely, with the consequent expenditure of time and resources.

Thus, breeding programs in hemp are currently focused on fiber content and quality, stem and seed yield, flowering behavior, suitability for different uses and cultivation regions, control of gender, THC content and resistance to pathogens between other traits (Salentijn *et al*., 2015). Conversely, among the most important challenges in medicinal cannabis breeding is the development of varieties resistant to biotic (Hadad *et al*., 2019; Punja *et al*., 2019; Jerushalmi *et al*., 2020), and abiotic stresses (Cosentino *et al*., 2013; Guerriero *et al*., 2017; Gao *et al*., 2018; Landi *et al*., 2019), as well as the development of stable varieties with specific cannabinoid profiles (Lynch *et al*., 2016; Wróbel *et al*., 2018).

The methods commonly used in cannabis breeding have been mass selection, cross-breeding, in-breeding and hybrid breeding. More recently, there are a few examples of the use of molecular markers to assisted breeding (Ranalli, 2004; Salentijn *et al*., 2015). Recently, new advances in *C. sativa* genomics research are also emerging (Hurgobin *et al*., 2020).

3.1. *In vitro plant tissue culture*

As it has been done with other major crops, *in vitro* plant tissue culture can complement cannabis conventional breeding through different approaches. From clonal propagation of already-selected phenotypes to the production of double haploids or gene-edited plants, many alternatives can be exploited through tissue culture to develop homogeneous varieties with specific and improved traits. However, before implementing any of these techniques in *C. sativa* species, it is imperative to fine-tune protocols to

improve their efficacy, which often, and given the absence of scientific publications on the matter, even means starting from scratch.

3.1.1. *Plant in vitro regeneration*

Plant regeneration is considered a crucial step for most *in vitro* culture techniques employed in plant breeding such as the development of double haploids and polyploid plants, or the production of transformed or gene-edited specimens. Classic examples of *in vitro* plant regeneration applications are plant micropropagation, employed to produce a high number of plants genetically identical to the specimen from which they come, or plant sanitation, defined as *in vitro*-production of virus-free plant material.

In the case of *C. sativa*, although high rates of *in vitro* plant regeneration from apical and axillary meristems of the plant (Richez-Dumanois *et al*., 1986; Lata *et al*., 2009, 2016a, b, 2017), young leaves (Lata *et al*., 2010) and cotyledons (Chaohua *et al*., 2016) have already been reported, several studies point out to the high recalcitrance level of *in vitro* shoot regeneration of different tissues. These works include regeneration assays performed on maturing bracts, anther-calyx complexes and vegetative leaves (Hemphill *et al*., 1978), leaves, hypocotyls, cotyledons and roots (Mandolino and Ranalli, 1999), young leaves, petioles, internodes and axillary buds (Lusarkiewicz-Jarzina *et al*., 2005), roots, leaves and stems (Plawuszewski *et al*., 2006), cotyledons, stems and roots (Wielgus *et al*., 2008), cotyledons and epicotyls (Movahedi *et al*., 2015), leaves and hypocotyls (Movahedi *et al*., 2016a, b) hemp transformed roots (Wahby *et al*., 2017), and cotyledons and hypocotyls (Smýkalová *et al*., 2019). After an exhaustive review of the existing literature, it can be concluded that, with some exceptions, the effectiveness of the published *in vitro* plant regeneration protocols for this species is low, or even null in some cases, and greatly varying between explants and varieties, representing a major bottleneck for the application of *in vitro* tissue culture for the improvement

of *C. sativa*. Moreover, in most of the former publications, a small number of varieties were evaluated, not representing all subspecies and reproductive systems present in the species. Furthermore, when shoot regeneration was observed, it developed indirectly through a previous phase of callus formation, which puts at risk the genetic fidelity of the regenerants compared to the donor plants. Additionally, in many of these publications, there is a lack of figures illustrating the process of *in vitro* shoot organogenesis together with a lack of reproducibility of their results. Finally, there is a common feature that can be inferred from previously mentioned studies, which regards the development of *in vitro* shoot organogenesis through the addition of plant growth regulators to the culture medium.

3.2. *Polyploidization*

Polyploid plants are composed of cells with more than two complete sets of chromosomes in their nuclei. Polyploid specimens are associated with enlarged organ sizes, increased biomass yield, phytochemical content and metabolic products, enhanced ability to adapt to biotic and abiotic stresses and changes in gene regulation (Van Hieu, 2019). They are also associated with enhanced levels of secondary metabolites in a large number of species (Iannicelli *et al*., 2019). Among the different improvements that polyploidization brings to plant breeding is the production of tetraploid plants, which in turn are employed for the development of triploid varieties with seedless fruits, as it has been reported in *Citrullus lanatus* (Kihara and Nishiyama, 1947), *Cucumis melo* L. (Adelberg *et al*., 1993) or in *Citrus* spp. (Recupero *et al*., 2005).

Regarding polyploidization in *C. sativa* species and its effects on cannabinoids contents, the scientific literature provides contradictory results. Clarke (1981) related polyploidization with an increase in THC content together with a decrease in CBD and cannabinol (CBN), also reporting that triploids produced fewer cannabinoids than diploids and tetraploids. On the other hand, Mansouri and Bagheri (2017) highlighted

that polyploidization increased significantly the contents of THC in mixoploid plants, which proved superior to tetraploid and diploid plants, also suggesting how mixoploids could be useful to produce THC for commercial use. In the former work, tetraploid specimens showed lower amounts of cannabinoids than diploids. Parsons *et al*. (2019) reported a similar content of cannabinoids among tetraploids and diploids, although significant increases in CBD and sesquiterpenes were associated with tetraploids. As related bibliography reflects, polyploidization in *C. sativa* species has always been induced by treating seeds or explants with chemical microtubule disruptors with a high toxicity grade. More recently, Kurtz *et al.*, (2020) succeeded producing tetraploids after colchicine seed treatment, also developing triploid plants through embryo rescue after crossing tetraploid and diploid specimens, while Crawford *et al.* (2021) produced tetraploids after explant colchicine treatment, also producing triploids after crossing tetraploid and diploid plants. In the former work, tetraploids and triploids showed an increase in cannabigerolic acid (CBGA) concentration as compared to their diploid counterparts, although these differences did not were statistically significant.

3.2.1. *Polysomaty*

The term polysomaty was first coined in the early literature by Langlet (1927). It is defined as the condition of those cells in the somatic tissues of a plant that contain multiples of the typical chromosome number (Ervin, 1941), being first described in *Spinacia oleracea* L. by Stomps (1910). Since its discovery, polysomaty has been described in a wide range of species as diverse as *Cucumis melo* L. (Ervin, 1939), *Beta vulgaris* L. (Sliwinska and Lukaszewska, 2005), *Chenopodium quinoa* Willd. (Kolano *et al*., 2008), or more recently *Solanum melongena* L. (García-Fortea *et al*., 2020). It is thought that polysomaty is promoted by endomitosis or endorreduplication processes (D’amato, 1964; Bubner *et al*., 2006), involved with the growth and differentiation of tissues. It has also been described how plant tissues frequently contain a proportion of endopolyploid cells (Ramsay and Kumar,

1990, and references therein) and that portions of the plant such as storage organs and vessels often contain polyploid cells (Adelberg *et al*., 1993).

Polysomaty was first described in root meristems from *C. sativa* species by Litardière (1925). Following this, Langlet (1927) pointed that the doubled number of chromosomes in root meristems coming from cannabis resulted from two successive cleavages of each chromosome during the prophases, while Breslavetz (1926, 1932) proposed nuclear fusion as the cause of the polysomatic condition described in cannabis roots.

3.3. *Double haploids*

Double haploids are inbred plants 100% homozygous derived from a haploid nucleus of male or female origin. They are obtained in only one *in vitro* generation after spontaneous or induced chromosome doubling, thus allowing for traits fixation and accelerating cultivars development. By means of hybridization of these pure lines, it is possible to exploit the hybrid vigour, obtaining high yielding and uniform F1 hybrid material. In addition, and due to their complete homozygosity, double haploid plants from species whose reproductive system allows autogamy, can be preserved by seed form by self-fertilization without genetic segregation in the offspring, thus eliminating the need for indefinitely keeping previously selected parentals in a perpetual vegetative state. Furthermore, these plants are also useful in basic research, since they can be employed for the identification of recessive traits, efficient mutant selection, easier genetic manipulation at the haploid level, and development of biochemical and physiological studies among other applications (Reinert and Bajaj, 1977; Maheshwari *et al*., 1980; Dunwell, 2010; Ferrie, 2013; Dwivedi *et al*., 2015).

First natural sporophytic haploids were reported in *Datura stramonium* L. by Blakeslee *et al*. (1922), although obtention of haploid embryos with androgenic origin was first time achieved through anther culture of *Datura innoxia* Mill. in the University of Delhi (Guha and Maheshwari, 1964), even though the initial purpose of this experiment was to study the biochemistry

of meiosis (Guha-Mukherjee, 1999). Since then, much progress has been made in the study of double haploids, although its development is an elusive current breeding objective in *C. sativa* species.

3.3.1. *Androgenesis*

In androgenesis, the haploid-derived plant originates from the microgametophyte. It is considered the most effective technique employed to obtain haploid and double haploid plants (Maraschin *et al*., 2005; Srivastava and Chaturvedi, 2008; Wędzony *et al*., 2009). One of the routes that leads to androgenesis is microspore embryogenesis, by which the microspore deviates from its original gametophytic fate and is reprogrammed to a new pathway of embryogenic development. It can be promoted through anther and microspore culture. While most of the early work on haploids has relied on anther culture due to its relative technical simplicity, by microspore isolation prior *in vitro* culture, regeneration of plants from anther somatic tissue can be prevented. Furthermore, among other benefits that microspore culture presents in comparison with anther culture, is the tightest control of the factors affecting induction, better availability of nutrients for microspores, and elimination of deleterious influence of possible inhibitors coming from the anther wall can be mentioned (Maheshwari *et al*., 1982; Heberle-Bors, 1989; Touraev *et al*., 1997; Murovec and Bohanec, 2012; Asif, 2013; Ferrie, 2013; Dwivedi *et al*., 2015). The earliest attempt at *in vitro* culture of isolated pollen grains was carried out in *Ginkgo biloba* L. by Tulecke (1953), who obtained callus tissue without achieving plant regeneration. However, it was not until a couple of decades later when Nitsch and coworkers succeeded in promoting microspore embryogenesis through *in vitro* culture of isolated microspores in *Datura innoxia* Mill. (Nitsch and Norreel, 1973a; Nitsch, 1974a; Sangwan-Norreel, 1977), *Nicotiana tabacum* L. (Nitsch, 1974b), and *Petunia x hybrida* hort. ex E. Vilm. (Sangwan and Norreel, 1975). Since those attempts and with varying induction rates, obtention of haploid and double haploid plants through both anther and microspore culture has been reported in a wide

range of species (Maluszynski *et al*., 2003; Srivastava and Chaturvedi, 2008; Wędzony *et al*., 2009). However, mainly due to species recalcitrance, it has not been possible to implement this technology in the vast majority of plant species, also representing an unexplored territory in most of them.

3.3.2. *Factors influencing androgenesis induction*

Among the most relevant factors affecting microspore embryogenesis, is the microspore and pollen stage development. It is widely accepted how vacuolate microspores and young bi-cellular pollen grains are more sensitive to androgenic induction (Maheshwari *et al*., 1980; Dunwell, 2010; Dwivedi *et al*., 2015; Canonge *et al.*, 2020). In this respect, it has been reported in different species how microspore and pollen stage 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* L. (Pechan and Keller, 1988), bud length and perianth morphological markers in *Lycopersicon esculentum* Mill. (Brukhin *et al*., 2003), pigmentation degree of anthers (Kim *et al*., 2004) and calyx-corolla ratio (Bárány *et al*., 2005) in *Capsicum annuum* L., or more recently, flower bud size in *Stevia rebaudiana* Bertoni (Uskutoğlu *et al*., 2019), and bud length, anther color, and filament length in *Opuntia ficus-indica* L. Mill. (Bouamama-Gzara *et al*., 2020).

On the other hand, stress treatments are also described as highly relevant on microspore embryogenesis (Nitsch and Norreel, 1973a; Maheshwari *et al*., 1980; Touraev *et al*., 1997; Maraschin *et al*., 2005; Murovec and Bohanec, 2012; Dwivedi *et al*., 2015). Different physical and chemical treatments such as low or high temperatures, centrifugation, ethanol, colchicine, carbon or nitrogen starvation, inducer chemicals, high or low medium pH, γ-irradiation or heavy metals, when applied on plants, inflorescences, flower buds, anthers or isolated microspores, can decisively promote the deflection of the gametophytic developmental pathway of microspores and pollen grains towards a sporophytic development

(Shariatpanahi *et al*., 2006). The use of less commonly applied stresses such as electroporation or sonication, have also been suggested as an alternative to promote microspore embryogenesis in recalcitrant legume species (Ribalta *et al*., 2012).

Among the most popular stress treatments above mentioned, cold-shock must be considered, undoubtedly, as the most frequently employed to block the gametophytic development and promote microspore embryogenesis in a huge range of species. Its many effects include cytoskeletal organization disruption and microspore microtubule network reorganization (Zhao *et al*., 2003, and references therein), its possible role in preventing starch formation in Datura proplastids and pollen from *Hordeum vulgare* L. (Sangwan and Sangwan-Norreel, 1987, and references therein), or its influence on Ca^{2+} pathway activation, eliciting an increase in cytosolic free calcium levels in microspores (Zoriniants *et al*., 2005; Żur *et al*., 2008). The latter enhanced the cytosolic concentration, which could lead to increased protein phosphorylation events related to cell division and microspore embryogenesis (Pauls *et al*., 2006). Additionally, Shariatpanahi *et al*. (2006), and references therein, reported that cold slow down the degradation of anther tissues, thus protecting microspores from toxic compounds released in the decaying anthers, and how low temperatures promote the expression of two heat-shock proteins (HSP) genes which possibly can protect cells against chilling injuries. The earliest experiments on the effect of low temperature on microsporogenesis and microgametogenesis were performed by Sax (1935), who studied the influence of temperature in the cell cycle of microspores and pollen grains from *Tradescantia* Ruppius ex L. In the former work, nuclear division abnormalities in microspore development were reported after exposure of donor plants to a three-day long cold treatment. Subsequently, cold treatment applied to flower buds was essential for successful pollen embryogenesis in *Datura innoxia* Mill. (Nitsch and Norreel, 1973a). The efficacy of cold-shock bud pretreatment in promoting microspore embryogenesis continued being reported in *Petunia*

x hybrida hort. ex E. Vilm. (Malhotra and Maheshwari, 1977) and *Nicotiana tabacum* L. (Rashid and Reinert, 1980, 1981). Since then, cold-shock has been successfully employed in the obtention of haploid and double haploid plants in several species as diverse as *Zea mays* L., *Triticum aestivum* L., *Hordeum vulgare* L., and *Oryza sativa* L. among others (Aionesei *et al*., 2005, and references therein). Depending on the species and the explant submitted to the stress pretreatment, a cold-shock can be applied from some days to several weeks at a temperature of about 4-10 ºC (Shariatpanahi *et al*., 2006). In general, cold-shock is more effective in terms of embryogenically-induced microspores when applied directly to the flower buds (Nitsch and Norreel, 1973b; Sunderland and Wildon, 1979; Maheshwari *et al*., 1980, and references therein).

Finally, as it plays a key role in the nutrition and viability of microspores and pollen grains, carbohydrates physiology and metabolism in the androecium is also considered as the determinant for microspore embryogenesis. In particular, amyloplasts act as reservoir plastids, accumulating energy reserves in form of polysaccharides like starch grains which can be employed as an energy source during both the pollen formation and the process of pollination, thus helping the pollen grain to survive during its development and outside the flower. Moreover, plastids contained in the microspores are specific markers used to differ among androgenic and recalcitrant species. Specifically, amyloplast presence is related with irreversible cell differentiation in the microspore (Clément and Pacini, 2001). Starch deposition has also been associated with a drastic change in protein synthesis (Mandaron *et al*., 1990), which suggests the expression of genes involved in the gametophytic pathway and the consequent loss of cellular totipotency. In androgenic species, starch accumulation in microspores and pollen grains starts at the late bi-cellular pollen stage, while in the recalcitrant species, there is an early accumulation of starch during microsporogenesis with an increase during pollen maturation (Sangwan and Sangwan-Norreel, 1987).

3.4. *Plant genetic transformation*

Novel biotechnological approaches such as targeted gene edition based on Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas systems could play a key role in the genetic improvement of *C. sativa* species, as it has been demonstrated in food crops such as *Solanum lycopersicum* L. through the development of a powdery mildew resistant variety (Nekrasov *et al*., 2017), in *Oryza sativa* L. by increasing grain yield (Gao *et al*., 2020), or in *Musa paradisiaca* L. by means of obtention of a β-carotene-enriched cultivar (Kaur *et al*., 2020), and also in other medicinal plant species such as *Papaver somniferum* L. (Alagoz *et al*., 2016), or *Salvia miltiorrhiza* Bunge (Zhou *et al*., 2018), whose plant secondary metabolite pathways have been successfully modified. However, before the implementation of this technique in *C. sativa*, it is imperative to develop an efficient transformation protocol that allows the regeneration of transgenic plants.

Some attempts at *C. sativa* transformation have been published in the last decade. Phosphomannose-isomerase (PMI) gene expression after *Agrobacterium tumefaciens*-mediated transformation of stem and leaf-derived callus suspension cultures has been reported (Feeney and Punja, 2003, 2015), together with β-glucuronidase (GUS) positive staining of hairy root cultures after effective transformation with *Agrobacterium rhizogenes*, and *in vivo* and *in vitro* Ri and Ti plasmid-bearing *Agrobacterium* infection of hypocotyl and cotyledonary node explants (Wahby *et al*., 2013, 2017). Genetic transformation of leaf, male and female flowers, stem, and root tissues through *A. tumefaciens* vacuum infiltration has also been achieved, being verified through subsequent analysis of GUS and green fluorescence protein (GFP) expression in the transformed tissues (Deguchi *et al*., 2020). In the former work, also phytoene desaturase (*PDS*) gene silencing resulting in an albino phenotype in leaves and male and female flowers was carried out. Transient GUS expression in *C. sativa* seedlings after *A. tumefaciens*-

mediated transformation has also been reported (Sorokin *et al*., 2020). Furthermore, transient gene expression after nanoparticle-based transformation of cannabis trichomes and leaf cells, and subsequent transcription of soybean genes and localization of fluorescent-tagged transcription factor proteins has been achieved (Ahmed *et al*., 2020). Finally, induced gene silencing of *PDS* and magnesium chelatase subunit I (*ChlI*) genes in leaves from one hemp variety after transient transformation with *A. tumefaciens* and Cotton leaf crumple virus (CLCrV) (Schachtsiek *et al*., 2019), and GFP-transient expression through polyethylene-glycol (PEG)-mediated protoplast transformation have also been described (Beard *et al*., 2021).

However, despite the successful genetic transformation of different explants, recalcitrance of *C. sativa* to *in vitro* plant regeneration have prevented the recovery of transgenic plants (Feeney and Punja, 2017; Wróbel *et al*., 2018). It was only recently that regeneration of one *C. sativa* transformed plant has been reported (Zhang *et al*., 2021).

Objectives

The following Doctoral Thesis is focused on the development of biotechnological tools for the genetic improvement of *C. sativa*, presenting eight main objectives:

1. Developing a highly-efficient *C. sativa* plant *in vitro* regeneration protocol that makes regeneration less aleatory and genotype-dependent, an essential requirement for the further implementation of other genetic improvement techniques based on biotechnology.

2. Studying the presence of polysomaty in specific *C. sativa* explants and its possible role in the regeneration of polyploid plants.

3. Establishing the most appropriate growth conditions for male flower development that contain a high amount of viable microspores and pollen grains in *C. sativa* as a previous requirement for the further development of a protocol for doubled haploid production through microspore embryogenesis.

4. Characterizing the different microspore and pollen developmental stages and correlate them with an easy-to-measure floral morphological marker as a previous requirement for the further development of a protocol for doubled haploid production through microspore embryogenesis.

5. Determining the androgenic potential of *C. sativa* through the amyloplast pattern observed in microspores and pollen grains during its growth as a previous requirement for the further development of a protocol for doubled haploid production through microspore embryogenesis.

6. Evaluating the effect of cold-shock bud pretreatment on the viability and amyloplast content of microspores and pollen grains, and on the development of multicellular structures of androgenic origin as a previous requirement for the further development of a protocol for doubled haploid production through microspore embryogenesis.

7. Assessing the suitability of kanamycin as a selectable marker for *C. sativa* transgenic plants.

8. Developing an effective protocol for the production of genetically-transformed *C. sativa* plants.

Chapter 1

Development of a direct *in vitro* plant regeneration protocol from *Cannabis sativa* L. seedling explants: developmental morphology of shoot regeneration and ploidy level of regenerated plants

Alberto Galán-Ávila[1*], Edgar García-Fortea[2], Jaime Prohens[2], Francisco Javier Herraiz[2]

PhD candidate contribution

A.G.-A. had a main role in the following activities: conceived and designed the research, performed the experiments, analyzed the results, wrote the manuscript, and was responsible for the verification of the paper.

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[1]Ploidy and Genomics S.L., CEEI VALENCIA, Avda. Benjamin Franklin, nº12, Parc Tecnològic, 46980, Paterna, Valencia, Spain

[2]Instituto Universitario de Conservación y Mejora de la Agrodiversidad Valenciana, Universitat Politècnica de València, Camí de Vera s/n, 46022, Valencia, Spain

* Corresponding author: agalan@ploidyandgenomics.com

1. Abstract

In vitro shoot regeneration can efficiently contribute to the improvement of recalcitrant *Cannabis sativa* L. We aimed at developing a highly efficient protocol for *in vitro* direct regeneration of *C. sativa* plants from different explants (cotyledon, hypocotyl and true leaf) from seedlings of monoecious *C. sativa* short-day varieties Ferimon, Felina32, Fedora17 and USO31, together with dioecious neutral-day variety Finola. Ten regeneration media, including already published protocols, and self-designed combinations of plant growth regulators were tested. The developmental morphology since germination of seeds to the development of rooted plantlets was followed. Additionally, the ploidy level of explants and *in vitro* regenerants was analyzed. We concluded that hypocotyl is the best explant for *in vitro* direct regeneration of *C. sativa* plants with 49.45% of responding explants, while cotyledon and true leaf had a poor response with, respectively, 4.70% and 0.42% of explants developing plantlets. In terms of shoot regeneration, we found significant differences among the culture media evaluated and the varieties studied. Overall, the best regeneration media were ZEA^{RIB} 2.0 (mg/L) and ZEA^{RIB} 1.0 (mg/L) + NAA 0.02 (mg/L) with 66.67% of responding hypocotyls. Amazingly, hypocotyls cultured in medium without plant growth regulators showed an excellent response (61.54% of responding hypocotyls) and spontaneous rooting of regenerants (17.94%). *In vitro* regenerated plants were acclimatized just six weeks after culture initiation. The developmental morphology study suggests that regenerated shoots originate from pericycle cells adjacent to xylem poles. Polysomaty was detected in hypocotyls and cotyledons of all varieties studied, and diploid (>80%) and mixoploid (with diploid and tetraploid cells) plants were regenerated. Our protocol allows a high shoot organogenesis efficiency in different *C. sativa* varieties. The fact that a significant percentage of plants are mixoploid may provide an alternative way to develop polyploids in *C. sativa*. Our results show that direct *in vitro* regeneration may make a

significant contribution to the development of improved *C. sativa* materials for medical applications.

Keywords: Cannabinoids; Hemp; Hypocotyl; Micropropagation; Polyploidization; Polysomaty; Shoot organogenesis

2. Introduction

Cannabis sativa L. (2n=2x=20) is a dicotyledonous species belonging to Cannabaceae family used for multiple purposes (fiber, oil, edible seeds, medicinal, drug) which comprises short and neutral-day varieties. Among its different applications, its use in medicine, derived from its content in cannabinoids (Cascio *et al*., 2017), is raising an increasing interest. Among cannabinoids, Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are generally the most abundant in the plant (Andre *et al*., 2016). Recent research has reported many cannabinoid pharmacodynamic and pharmacokinetic properties, expanding the potential use of cannabinoids in medical therapies (Urits *et al*., 2019), and promoting the development of cannabis improved varieties with specific biochemical profiles. In this respect, *in vitro* culture is a useful tool that has been employed to complement cannabis conventional breeding through large-scale micropropagation of selected elite clones (Lata *et al*., 2017), development of polyploid varieties with enhanced levels of secondary metabolites (Mansouri and Bagheri, 2017; Parsons *et al*., 2019) or genetic transformation of non-regenerating tissues (Feeney and Punja, 2003, 2015, 2017; Wahby *et al*., 2013, 2017). However, there is still a lack of an *in vitro* regeneration protocol efficient in the broad range of genetically diverse materials in the species.

In this respect, plant regeneration is an essential step for most *in vitro* culture techniques employed in plant breeding. High rates of *in vitro* plant regeneration from already developed apical and axillary meristems of the plant (Richez-Dumanois *et al*., 1986; Lata *et al*., 2009, 2016a, 2016b), young leaves (Lata *et al*., 2010) and cotyledons (Chaohua *et al*., 2016) have already been reported in *C. sativa*. However, several studies point out to a high level of recalcitrance of *in vitro* shoot regeneration from different tissues such as maturing bracts, anther-calyx complexes and vegetative leaves (Hemphill *et al*., 1978), leaves, hypocotyls, cotyledons and roots (Mandolino and Ranalli,

1999), young leaves, petioles, internodes and axillary buds (Lusarkiewicz-Jarzina *et al*., 2005), roots, leaves and stems (Plawuszewski *et al*., 2006), cotyledons, stems and roots (Wielgus *et al*., 2008), cotyledons and epicotyls (Movahedi *et al*., 2015), leaves and hypocotyls (Movahedi *et al*., 2016a, 2016b), hemp transformed roots (Wahby *et al*., 2017) and hypocotyl segments (Smýkalová *et al*., 2019). Therefore, the low regeneration efficiency of published *in vitro* plant regeneration protocols for this species, and its wide variation among explant types and varieties represent a major bottleneck for the application of *in vitro* tissue culture to the improvement of *C. sativa*. Moreover, in most of the aforementioned publications, a small number of varieties were evaluated, which not represent all subspecies and reproductive systems present in the species. In addition, in the vast majority of these studies, when shoot regeneration was successful, it developed in an indirect way through a previous phase of callus formation, which may compromise the genetic fidelity of regenerants with respect to the donor plant (Evans and Bravo, 1986; Ramírez-Mosqueda and Iglesias-Andreu, 2015). Finally, a common feature that can be inferred from previously mentioned studies, is that development of *in vitro* shoot organogenesis in this species requires addition of plant growth regulators to the culture medium.

Considering the above mentioned facts, the aim of this study was focused on the development of a highly efficient species-specific protocol for *in vitro* direct regeneration of *C. sativa* plants. For this, we evaluated different explants such as cotyledon, hypocotyl and true leaf coming from seedlings of four monoecious short-day varieties plus a dioecious hemp neutral-day variety, which were employed as donor plants. Explants were cultured on media with different plant growth regulators and hormonal concentrations obtained from already published protocols, together with self-designed combinations of plant growth regulators. The developmental morphology process of *in vitro* shoot organogenesis from cotyledons, hypocotyls and true leaves was followed and registered with images, and

the duration of each of the developmental stages of organogenesis was also recorded. Additionally, due to the lack of studies concerning polysomaty in this species and its potential usefulness to obtain polyploid plants as it has occurred in other species like tomato (Van den Bulk *et al*., 1990), cucumber (Colijn-Hooymans *et al*., 1994), or melon (Ren *et al*., 2013), and more recently in eggplant (García-Fortea *et al*., 2020), authors not only studied the regenerative capacity of cotyledons, hypocotyls and true leaves, but also analyzed the ploidy level of these explants together with that of the *in vitro* regenerants.

3. Material and methods

3.1. Plant material and growth conditions

Seeds from monoecious *C. sativa* short-day varieties Ferimon, Felina32, Fedora17 and USO31, together with seeds from dioecious neutral-day variety Finola were surface sterilized in 75% (v/v) ethanol during two minutes and 30 seconds, followed by immersion in 30 g/L of NaClO with 0.1% (v/v) of Tween 20 during 25 minutes, and finally washed three times in autoclaved deionized water. Once sterilized, seeds were germinated in 9 cm diameter plastic Petri dishes containing previously autoclaved germination medium which composition was ½ MS basal salts and vitamins (Murashige and Skoog, 1962) + 1.5% (w/v) sucrose + 3.5 g/L Gelrite® with a pH value of 5.8. After germination, cotyledons, hypocotyls and true leaves dissected from seven-days-old seedlings were employed as explants. In this species, this stage of seedling development is equivalent to the phenological growth stage coded in *Biologische Bundesanstalt, Bundessortenamt and CHemical industry* (BBCH-scale) by number 11 (Mishchenko *et al*., 2017). Explants were cultured in the different media described in Table 1. Seedlings and explants were grown under controlled conditions at 22ºC ±1ºC and 60% ±1% relative humidity. Photoperiod consisted of 16 hours of light and eight hours of dark. Light was provided by Light Emitting Diode (LED) tubes of 18W and 6000K color temperature, which provided 6,010 lux and 90.15 µmol m^{-2} s^{-1}.

Explants producing shoots and roots, and number of shoots developed on each of responding explants were counted periodically during two weeks of culture. After that time, *in vitro* regenerants were subcultured individually to glass-tubes of 2.5 cm of diameter and 15 cm long, containing the same medium in which shoots were generated.

Table 1: Media tested for *in vitro* shoot induction from cotyledons, hypocotyls and true leaves of *C. sativa*, including plant growth regulators composition and their respective concentrations.

Medium	Plant growth regulators and concentrations (mg/L)	Reference
0	Without plant growth regulators	---
1	TDZ (0.4) + NAA (0.2)	(Chaohua *et al*., 2016)
2	BAP (2.0) + IBA (0.5)	(Movahedi *et al*., 2015)
3	BAP (0.5) + 2,4-D (0.1)	(Movahedi *et al*., 2016a)
4	ZEA^{RIB} (2.0)	(García-Fortea *et al*., 2020)
5	BAP (1.0) + NAA (0.02)	---
6	BAP^{RIB} (1.0) + NAA (0.02)	---
7	TDZ (1.0) + NAA (0.02)	---
8	4-CPPU (1.0) + NAA (0.02)	---
9	ZEA^{RIB} (1.0) + NAA (0.02)	---

When roots were visible, spontaneously-rooted plants were cultured in pots (2 L) with fertilized commercial substrate composed of a mixture of black peat, granulated peat moss and perlite, with a pH value of 6 and a conductivity of 1 mS/cm. Previously, gelled medium was carefully washed from roots. After transplant and during the whole process of acclimatization, the substrate was maintained slightly moist and, twice per day (early in the morning and in late afternoon), regenerants received foliar pulverization with water. To avoid desiccation, the small plants were covered with plastic vessels and were progressively exposed to the environmental humidity. Until complete acclimatization, plants were grown under identical conditions of temperature, photoperiod and light as described above. Plants

employed in this study were grown under license for the cultivation of *C. sativa* for research purposes, issued by the spanish Ministry of Health, Social Services and Equality via Spanish Agency of Medicines and Health Products (Agencia Española de Medicamentos y Productos Sanitarios or AEMPS) to Ploidy and Genomics Ltd.

3.2. *In vitro* shoot organogenesis experiments

In order to promote *in vitro* shoot organogenesis in *C. sativa*, cotyledons, hypocotyls and true leaves dissected from seven-days-old seedlings were cultured in germination medium with the same composition as described above, except for the addition of different plant growth regulators. As a part of this study, we aimed at evaluating with our own genotypes the efficiency of different *in vitro* shoot regeneration published protocols developed for *C. sativa*. Therefore, we selected studies in which different explants, cytokinins and auxins and their respective concentrations were successfully tested. In this way, we tested the media used in a study regarding the regenerative capacity of cotyledons through addition of thidiazuron (TDZ) and α-naphthaleneacetic acid (NAA) to the culture medium (Chaohua *et al*., 2016), one work concerning *in vitro* plant regeneration from cotyledons and epicotyls by means of 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) (Movahedi *et al*., 2015), and another one from leaves and hypocotyls through BAP and 2,4-dichlorophenoxyacetic acid (2,4-D) (Movahedi *et al*., 2016a). Additionally, it was added to our schedule an effective and newly released protocol developed for eggplant in which the use of zeatin riboside (ZEA^{RIB}), provided good results not only in terms of shoot organogenesis, but also in polyploidization of regenerants (García-Fortea *et al*., 2020).

Finally, as it is known that root and shoot development depends on cytokinin:auxin ratio and that high levels of cytokinin supports shoot formation (Skoog and Miller, 1957; Su *et al*., 2011), we tested the effect of media with a cytokinin concentration 50-fold higher than the auxin level,

together with different adenine and phenylurea derivatives like BAP, 6-benzylaminopurine riboside (BAP^{RIB}), TDZ, forchlorfenuron (4-CPPU) and ZEA^{RIB} plus NAA, an auxin commonly employed in protocols for *in vitro* regeneration of shoots (Plawuszewski *et al*., 2006; Wielgus *et al*., 2008; Lata *et al*., 2010; Chaohua *et al*., 2016) and *in vitro* rooting of *C. sativa* (Lusarkiewicz-Jarzina *et al*., 2005; Wang *et al*., 2009; Movahedi *et al*., 2016a; Parsons *et al*., 2019). The different hormonal combinations present in the different shoot induction media evaluated in this work, are detailed in Table 1.

3.3. Developmental morphology of the *in vitro* regeneration process

The whole developmental process of *in vitro* shoot organogenesis, since germination of seeds until acclimatization of plants was followed and registered with images. The time needed for each of the different developmental stages was recorded. High resolution images of the different developmental stages were recorded with an Optika® SZN-6 (OPTIKA S.r.l., Ponteranica, Italy) laboratory stereozoom microscope equipped with an Optika® C-HP (OPTIKA S.r.l.) digital camera.

3.4. Determination of ploidy level of explants and *in vitro* regenerants

Ploidy level of cotyledons, hypocotyls and leaves from *in vitro* grown seven-days-old seedlings was evaluated to verify their polysomatic pattern. The four monoecious short-day varieties Ferimon, Felina32, Fedora17 and USO31, together with dioecious neutral-day variety Finola were analyzed in this experiment. Three seedlings coming from each variety were employed for this assay. On the other hand, young leaves from *in vitro* regenerated plants were also examined. Ploidy level of 35 *in vitro* regenerants (17 from cotyledons, 15 from hypocotyls and three from leaves) was determined. Cell nuclei of explants dissected were mechanically isolated. Sections of approximately 0.5 cm^2 were chopped with a razor blade in a 6 cm diameter glass Petri dish containing 0.5 ml lysis buffer LB01 (pH 7.5) (Dpooležel *et al*.,

1989), and incubated for 5 minutes. Subsequently, the suspension containing nuclei and cell fragments was filtered using a 30 µm CellTrics filter (Sysmex, Sant Just Desvern, Spain). The nuclei in the filtrate were stained with CyStain UV Ploidy (Sysmex) and incubated for 5 minutes. The fluorescence intensity of the homogenate was measured using a CyFlow® Ploidy Analyser Sysmex Partec GmbH, analyzing at least 4,000 nuclei for each sample. Young leaves of diploid plants from all varieties studied were used as control. A diploid control peak was established at 50 points of the arbitrary intensity value of the fluorescence in the histogram. By comparison with this peak, the ploidy of the other tissues evaluated was checked.

3.5. Data analyses

In order to develop a highly efficient protocol for *in vitro* direct regeneration of *C. sativa* plants, we analyzed statistically the effect of different factors such as genotype, explant and culture medium on *in vitro* shoot organogenesis. For each factor, the mean of responding explants was expressed as a percentage (±SE) relative to the total amount of cultured explants. For varieties and media with the best shoot induction rates identified in this study, also the number of shoots per responding explant were statistically evaluated. Data recorded until the second week of culture were employed for the statistical analysis. Additionally, the effect of the explant factor on the ploidy level of *in vitro* regenerants was statistically determined. For each explant, the mean of diploid and mixoploid regenerants was expressed as a percentage (±SE) relative to the total amount of plants submitted to flow cytometry analysis. While the factor genotype was represented through four monoecious short-day varieties (Ferimon, Felina32, Fedora17 and USO31) plus a dioecious hemp neutral-day variety (Finola), the explants evaluated in this assay were cotyledons, hypocotyls, and true leaves coming from seven-days-old seedlings. The 10 different media described in Table 1 constituted the culture medium factor. Each factor was analyzed using at least five biological replicates. Each

biological replicate consisted of a Petri dish containing 3 explants coming from 3 different seedlings of the same variety in the case of hypocotyls, and 6 explants coming from 3 different seedlings of the same variety in the case of cotyledons and true leaves. A total of 2,463 explants were employed in this work (1,000 from cotyledons, 275 from hypocotyls and 1,188 from true leaves). Independence among variables (distribution-plot test), homoscedasticity (Bartlett's test), and normality (Shapiro-Wilk test) were evaluated for the data coming from the experiments. Given that none of the three criteria were met, Kruskal-Wallis non-parametric test followed by pairwise Wilcoxon test ($p<0.05$) was used to evaluate statistical significance of differences between factors. Statistical analysis was carried out using R software (Ihaka and Gentleman, 1996).

4. Results

4.1. Effect of genotype, explant and medium on *in vitro* shoot organogenesis of *C. sativa*

Shoot *in vitro* regeneration was observed in all *C. sativa* varieties, explant types and media tested, resulting in a total of 255 *in vitro* regenerated shoots, although significant differences ($p<0.05$) between the different levels of the three main factors were observed (Table 2). Regarding the factor explant and its effect on the percentage of explants developing shoots, significant differences were detected between cotyledons, hypocotyls, and true leaves. On average, hypocotyl showed the best response in terms of direct plant regeneration, reaching 49.45% of explants with shoot formation, followed by cotyledon with 4.70% and true leaf with 0.42% (Table 2). Also, significant differences between varieties were observed. USO31 was the best variety evaluated in this experiment, with 12.32% of their explants exhibiting shoot organogenesis, while the variety with a lower percentage of regeneration was Finola with only 4.62% (Table 2). Finally, only medium 1 (TDZ 0.4 mg/L + NAA 0.2 mg/L) showed a

significantly higher capacity in promoting shoot organogenesis, achieving an average of 15.78% of induction rate (Table 2).

Table 2: Effect of genotype, explant and medium on direct *in vitro* shoot organogenesis rate of different explants from *C. sativa*.

Factor	Responding explants (%)	Significance[a]	n
Variety			
Ferimon	6.23 ±1.06	bc	514
Felina32	7.37 ±1.03	bc	638
Fedora17	8.55 ±1.36	ab	421
USO31	12.32 ±1.61	a	414
Finola	4.62 ±0.96	c	476
Explant			
Cotyledon	4.70 ±0.66	b	1,000
Hypocotyl	49.45 ±3.02	a	275
Leaf	0.42 ±0.18	c	1,188
Medium (mg/L)			
0 → Without plant growth regulators	6.81 ±1.24	b	411
1 → TDZ 0.4 + NAA 0.2	15.78 ±1.79	a	412
2 → BAP 2 + IBA 0.5	4.61 ±1.25	b	282
3 → BAP 0.5 + 2,4-D 0.1	6.42 ±1.50	b	265
4 → ZEA^{RIB} 2	6.01 ±1.76	b	183
5 → BAP 1 + NAA 0.02	5.50 ±1.54	b	218
6 → BAP^{RIB} 1 + NAA 0.02	6.71 ±1.95	b	164
7 → TDZ 1 + NAA 0.02	4.37 ±1.51	b	183
8 → 4-CPPU 1 + NAA 0.02	8.67 ±2.30	b	150
9 → ZEA^{RIB} 1 + NAA 0.02	5.13 ±1.58	b	195

Mean of responding explants (%), significance and sample size (n) are presented in different columns. For each factor, mean of responding explants is expressed as a percentage (±SE) relative to the total amount of cultured explants.

[a]Different letters among the levels of each of the three factors indicate significant differences between them ($p<0.05$) according to non-parametric Kruskal-Wallis and pairwise Wilcoxon tests.

Since true leaves displayed a weak capacity to induce direct shoot organogenesis, and in order to eliminate the negative effect that they were adding to the variety and medium factors, we analyzed separately data from cotyledons (Table 3) and hypocotyls (Table 4). Regarding cotyledons, significant differences were observed among varieties. USO31 reached the highest rate of shoot organogenesis with 9.29% (Table 3), while Felina32 exhibited the lowest shoot induction rate with 2.10% (Table 3). With respect to the factor medium, medium 1 (TDZ 0.4 mg/L + NAA 0.2 mg/L) was the best, achieving the highest shoot induction rate with a 22.32% of responding explants (Table 3). Medium 0 (without plant growth regulators) and number 9 (ZEA^{RIB} 1 mg/L + NAA 0.02 mg/L) were the worst treatments, without any explant showing response in terms of shoot organogenesis (Table 3).

In addition, the number of shoots developed on each of the responding explants were statistically analyzed for varieties and media with the best shoot induction rates identified in this study. In the case of cotyledons, as varieties USO31, Fedora17 and Ferimon, and media 1 (TDZ 0.4 mg/L + NAA 0.2 mg/L) and 8 (4-CPPU 1 mg/L + NAA 0.02 mg/L) gave the best shoot induction rates, their number of shoots per responding explant were statistically compared (Table 3). Although no significant differences were found between varieties and media in terms of number of shoots per responding cotyledon, Fedora17 showed the best results with 1.42 shoots per responding explant, while medium 1 (TDZ 0.4 mg/L + NAA 0.2 mg/L) reached 1.28 shoots per responding cotyledon (Table 3).

Table 3: Effect of genotype and medium on direct *in vitro* shoot organogenesis rate of cotyledons from *C. sativa*.

Factor	Responding explants (%)	Significance[a]	n	Shoots per responding explant	Significance[a]	n
Variety						
Ferimon	5.50 ±1.61	ab	200	1.09 ±0.09	a	11
Felina32	2.10 ±0.84	c	286	1.17 ±0.17	*	6
Fedora17	6.67 ±1.86	ab	180	1.42 ±0.15	a	12
USO31	9.29 ±2.46	a	140	1.00 ±0.00	a	13
Finola	2.58 ±1.14	bc	194	1.00 ±0.00	*	5
Medium (mg/L)						
0 → Without plant growth regulators	0.00 ±0.00	d	234	-	*	0
1 → TDZ 0.4 + NAA 0.2	22.32 ±3.95	a	112	1.28 ±0.09	a	25
2 → BAP 2 + IBA 0.5	1.85 ±1.30	c	108	1.00 ±0.00	*	2
3 → BAP 0.5 + 2,4-D 0.1	5.56 ±2.42	bc	90	1.00 ±0.00	*	5
4 → ZEA^{RIB} 2	1.28 ±1.28	cd	78	1.00	*	1
5 → BAP 1 + NAA 0.02	1.92 ±1.35	c	104	1.00 ±0.00	*	2
6 → BAP^{RIB} 1 + NAA 0.02	6.25 ±3.04	bc	64	1.00 ±0.00	*	4
7 → TDZ 1 + NAA 0.02	2.56 ±1.80	c	78	1.00 ±0.00	*	2
8 → 4-CPPU 1 + NAA 0.02	14.29 ±5.46	ab	42	1.00 ±0.00	a	6
9 → ZEA^{RIB} 1 + NAA 0.02	0.00 ±0.00	d	90	-	*	0

Mean of responding explants (%), significance and sample size (n) are presented in different columns. For each factor, mean of responding explants is expressed as a percentage (±SE) relative to the total amount of cultured explants. This table also includes the effect of genotype and medium on the number of shoots per responding cotyledon of *C. sativa*. Mean number of shoots per responding explant (±SE), significance and sample size (n) are presented in different columns. Shoots per responding explant from varieties and media with the best shoot induction rates are statistically compared.

[a]Different letters among the levels of each of the two factors indicate significant differences between them ($p<0.05$) according to non-parametric Kruskal-Wallis and pairwise Wilcoxon tests.

*Not analyzed statistically.

Concerning hypocotyls, significant differences were identified between the different varieties and media evaluated in this experiment. Again, USO31 was the best variety evaluated, with 71.15% of its explants developing shoots (Table 4), while Finola and Ferimon were the varieties with the lowest regeneration percentages with, respectively, 35.42% and 32.26% of its explants regenerating shoots (Table 4). In relation to the effect of medium on shoot organogenesis, media number 4 (ZEA^{RIB} 2 mg/L) and number 9 (ZEA^{RIB} 1 mg/L + NAA 0.02 mg/L) resulted in the highest rate of shoot induction with 66.67% of responding explants, followed by medium 0 (without plant growth regulators) and medium 1 (TDZ 0.4 mg/L + NAA 0.2 mg/L) with, respectively, 61.54% and 54.17% of shoot organogenesis rate (Table 4).

Number of shoots developed on each of the responding explants were statistically analyzed for varieties and media with the best shoot induction rates identified in this study. Regarding hypocotyls, since varieties USO31 and Felina32, and media 0 (without plant growth regulators), 1 (TDZ 0.4 mg/L + NAA 0.2 mg/L), 4 (ZEA^{RIB} 2 mg/L) and 9 (ZEA^{RIB} 1 mg/L + NAA 0.02 mg/L) attained the best shoot organogenesis rates, their number of shoots per responding explant were also statistically compared (Table 4). USO31 exhibited the best response in terms of number of shoots per responding hypocotyl, reaching 1.72 shoots per responding explant (Table 4). Furthermore, although no significant differences were found among media tested, medium 4 (ZEA^{RIB} 2 mg/L), closely followed by medium 0 (without plant growth regulators), were the best media evaluated in this experiment with, respectively, 1.60 and 1.54 shoots per responding hypocotyl (Table 4).

Table 4: Effect of genotype and medium on direct *in vitro* shoot organogenesis rate of hypocotyls from *C. sativa*.

Factor	Responding explants (%)	Significance[a]	n	Shoots per responding explant	Significance[a]	n
Variety						
Ferimon	32.26 ±5.98	c	62	1.25 ±0.12	*	20
Felina32	62.50 ±6.09	ab	64	1.20 ±0.06	b	40
Fedora17	44.90 ±7.17	bc	49	1.50 ±0.13	*	22
USO31	71.15 ±6.34	a	52	1.72 ±0.12	a	37
Finola	35.42 ±6.97	c	48	1.59 ±0.12	*	17
Medium (mg/L)						
0 → Without plant growth regulators	61.54 ±7.89	ab	39	1.54 ±0.12	a	24
1 → TDZ 0.4 + NAA 0.2	54.17 ±5.91	ab	72	1.49 ±0.11	a	39
2 → BAP 2 + IBA 0.5	36.67 ±8.94	c	30	1.27 ±0.14	*	11
3 → BAP 0.5 + 2,4-D 0.1	38.71 ±8.89	c	31	1.33 ±0.19	*	12
4 → ZEA^{RIB} 2	66.67 ±12.59	a	15	1.60 ±0.16	a	10
5 → BAP 1 + NAA 0.02	41.67 ±10.27	bc	24	1.40 ±0.16	*	10
6 → BAP^{RIB} 1 + NAA 0.02	43.75 ±12.80	bc	16	1.29 ±0.18	*	7
7 → TDZ 1 + NAA 0.02	40.00 ±13.09	c	15	1.50 ±0.22	*	6
8 → 4-CPPU 1 + NAA 0.02	38.89 ±11.82	c	18	1.43 ±0.20	*	7
9 → ZEA^{RIB} 1 + NAA 0.02	66.67 ±12.59	a	15	1.30 ±0.15	a	10

Mean of responding explants (%), significance and sample size (n) are presented in different columns. For each factor, mean of responding explants is expressed as a percentage (±SE) relative to the total amount of cultured explants. This table also includes the effect of genotype and medium on the number of shoots per responding hypocotyl of *C. sativa*. Mean number of shoots per responding explant (±SE), significance and sample size (n) are presented in different columns. Shoots per responding explant from varieties and media with the best shoot induction rates are statistically compared.

[a]Different letters among the levels of each of the two factors indicate significant differences between them ($p<0.05$) according to non-parametric Kruskal-Wallis and pairwise Wilcoxon tests.

*Not analyzed statistically.

4.2. Developmental morphology of *in vitro* shoot organogenesis in *C. sativa*

First of all, seeds of the different varieties (Fig. 1A) were surface sterilized. Between 24 and 48 h after being cultured in germination medium, seeds started to germinate and the apical root meristem arose from the testa (Fig. 1B). Five days after *in vitro* sowing, seedlings liberated from the testa were visible while emerging (Fig. 1C). On the seventh day from seed plating, the first pair of true leaves was fully expanded (Fig. 1D). When seedlings arrived to this developmental stage, explants needed to continue the experiments were obtained through a clean cut across dissection point (arrow in Fig. 1D). Remaining vegetative shoot apex located on the top of the seedling (arrow in Fig. 1E) was discarded. As can be observed in Fig. 1F, discarded shoot apex preserved the whole shoot apical meristem (SAM) (inset in Fig. 1F).

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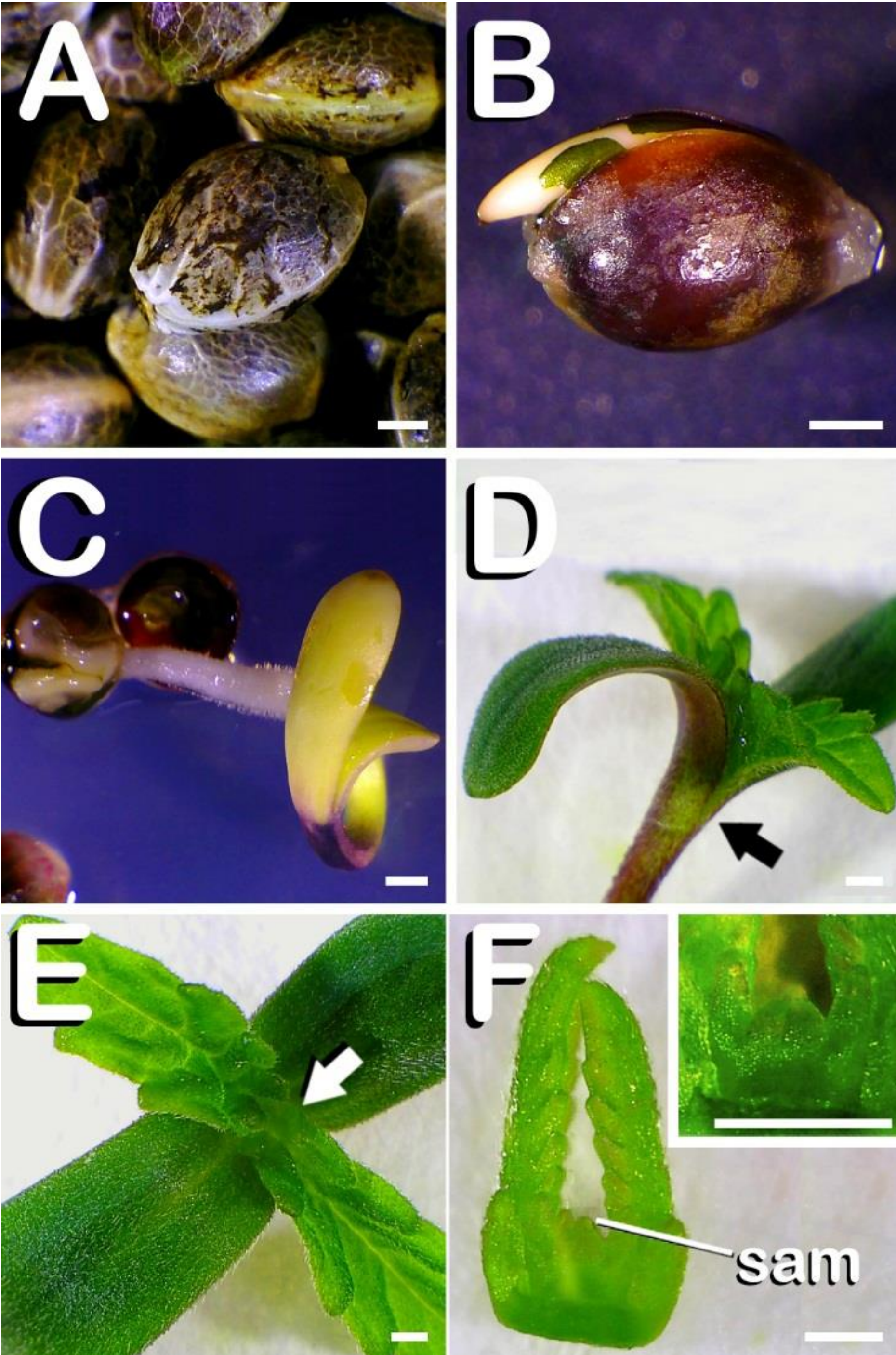
Figure 1: Seed germination of *C. sativa*. The different developmental stages are described as follows: (A) Seeds just before being sterilized. (B) Germinated seed 48 h. after *in vitro* sowing with the root apical meristem arising from testa. (C) Emerging seedling 5 days after seed plating, with testa being visible at the bottom of the image. (D) Seven-days-old seedling with fully expanded first pair of true leaves, which is equivalent to the phenological growth stage coded in this species by number 11 in BBCH-scale: arrow marks dissection point. (E) View of seven-days-old seedling allowing observation of vegetative shoot apex: arrow points shoot apex location on seedling. (F) Remaining vegetative shoot apex after dissection of hypocotyl, cotyledon and true leaves from seven-days-old seedling, with shoot apical meristem (SAM) highlighted on it: detail of SAM (inset in F). Scale bars: 1 mm.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At this stage of seedling development, cotyledon leaves were easily dissected (Fig. 2A). The first steps of direct shoot organogenesis were rapidly visible on the basal zone of responding cotyledons. Shoot primordia formation was centrally located at the proximal part of cotyledons after four days of *in vitro* culture (Fig. 2B). Two weeks after explant transfer to shoot induction medium, vigorous regenerants arising from the proximal edge of cotyledons were observed (Fig. 2C). At this stage of development, since regenerants reached approximately one centimeter in height and in order to avoid their contact with the Petri dish lid, *in vitro* regenerated shoots were subcultured individually in glass-tubes containing the same medium in which they were generated (Fig. 2D). A total of 47 cotyledons responded to the different treatments evaluated, generating a total amount of 54 shoots.

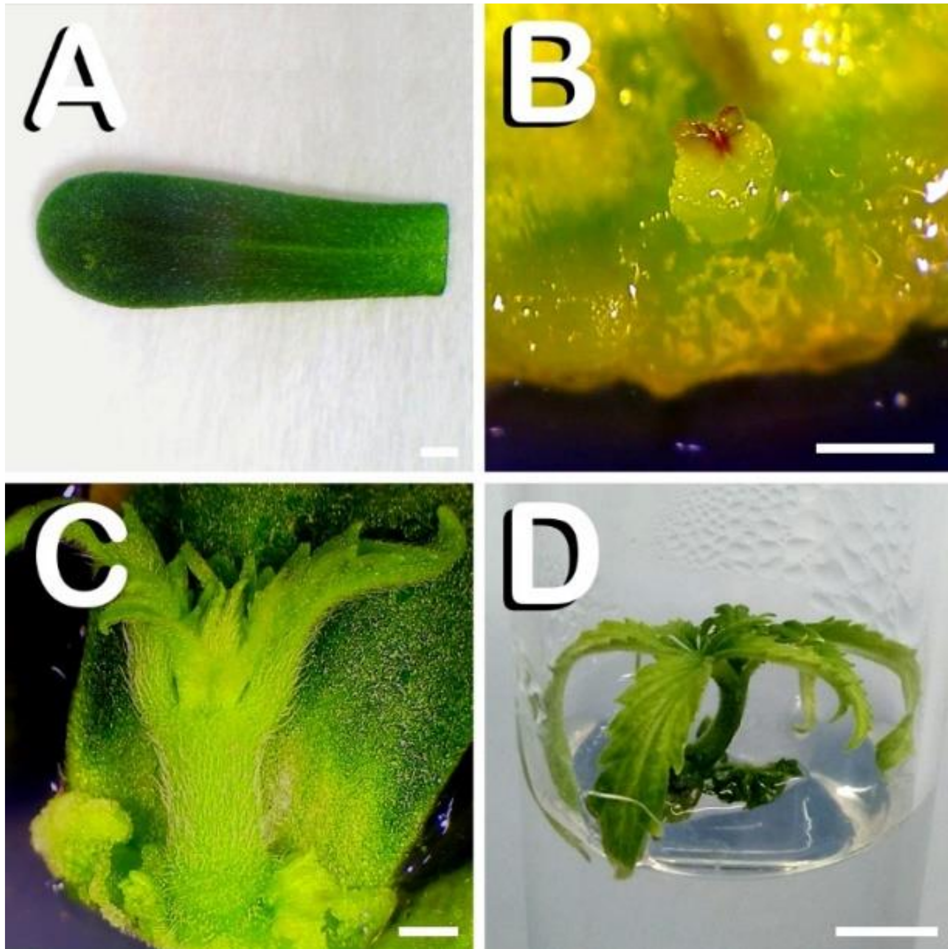

Figure 2: Direct *in vitro* shoot organogenesis from cotyledon leaves of *C. sativa*. The different developmental stages are described as follows: (A) Newly dissected cotyledon leaf from a seven-days-old hemp seedling. (B) Shoot primordium formation at the basal zone of cotyledon leaf after four days of *in vitro* culture. (C) Vigorous shoot arising from the lower part of cotyledon leaf 14 days after exposure to the culture medium. (D) Cotyledon derived plant cultured in a glass-tube 21 days after explant inoculation. Scale bars (A-C): 1 mm. Scale bar (D): 6 mm.

Alternatively, hypocotyls were cut from seven-days-old seedlings. Hypocotyls employed in this experiment measured approximately one centimeter in lenght (Fig. 3A). Transversal section of freshly dissected hypocotyls revealed its internal structure, with different tissue layers such as epidermis, cortex and pith, together with the absence of meristem traces (Fig. 3B). Responding explants exhibited different direct organogenesis patterns. Some of the hypocotyls generated only one primordium on the top of the explant which was originated from the central region of the section (Fig. 3C), and continued its development until becoming a robust plant after two weeks of culture (Fig. 3D). Other explants gave rise to a couple of primordia in the periphery of the organ and on opposite sides (arrows in Fig. 3E). In this last case, there were situations in which development between both primordia was asynchronous, while in other cases, growth and height of both regenerants was coincident, as can be observed in Fig. 3F. In the case embodied in Fig. 3G, both *in vitro* regenerants reached approximately one centimeter in height two weeks after culture initiation, while an overgrowth of the pith (located in the middle of the explant) was also observed. At this stage of organogenic development, shoots were detached from hypocotyls and individually cultured in glass-tubes containing the same medium in which they were generated (Fig. 3H). A total of 136 hypocotyls responded to the different treatments evaluated, producing a total amount of 196 shoots.

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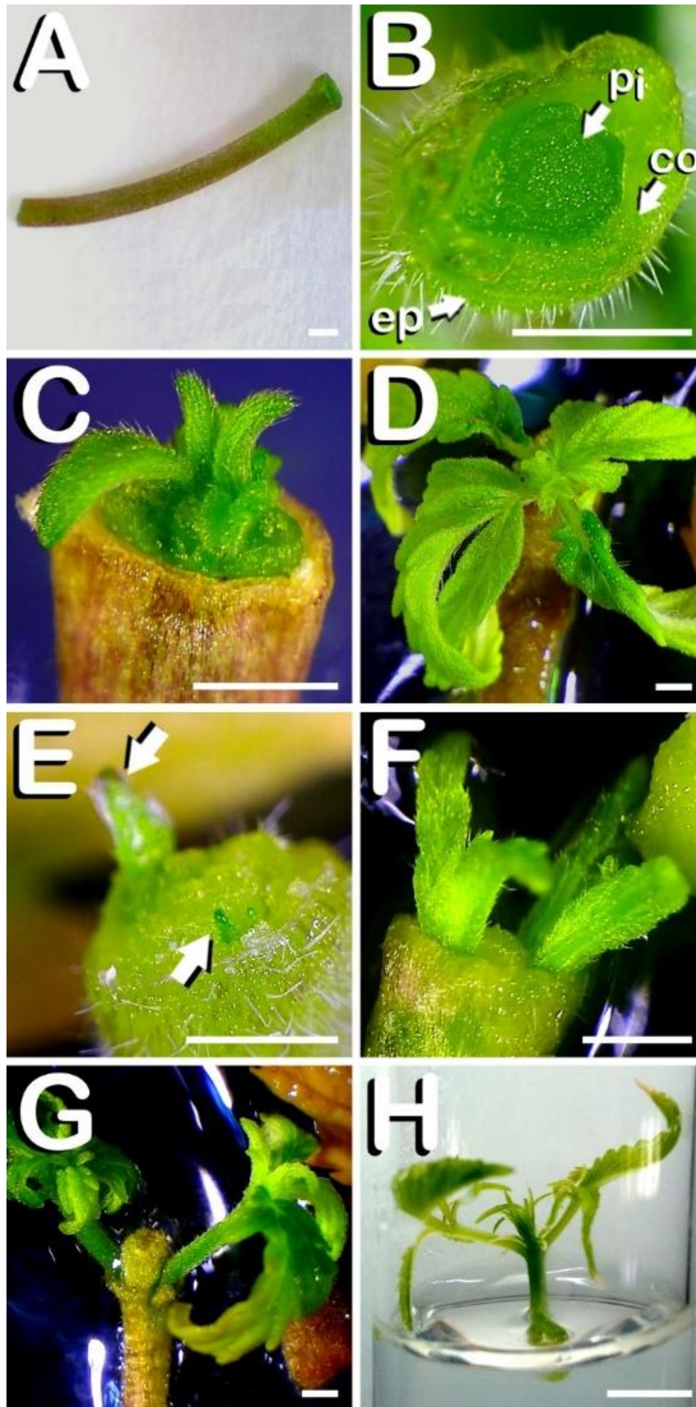
Figure 3: Direct *in vitro* shoot organogenesis from *C. sativa* hypocotyls: (A) Newly dissected hypocotyl from a seven-days-old hemp seedling. (B) Transverse section of newly dissected hemp hypocotyl revealing its different layers: **ep**: epidermis; **co**: cortex; **pi**: pith. (C) Formation of one shoot at the top of the hypocotyl after seven days of *in vitro* culture. (D) Vigorous shoot arising from the upper part of hypocotyl 14 days after exposure to the culture medium. (E) Two primordia arising from the top of the hypocotyl after four days of *in vitro* culture: arrows point both primordia. (F) Two hypocotyl derived plants nine days after explant inoculation. (G) Two hypocotyl derived regenerants ready to be subcultured 14 days after explant culture. (H) Hypocotyl derived plant individually grown in a glass-tube 21 days after culture initiation. Scale bars (A-G): 1 mm. Scale bar (H): 6 mm.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Finally, the regenerative capacity of the first pair of true leaves from seven-days-old seedlings was also studied. For this, each leaf was carefully dissected (Fig. 4A) and cultured in the different media evaluated in this experiment. When *in vitro* plant regeneration occurred, primordia arose always from the base of leaves, specifically from the petiole fragment attached to the leaf, as is the case of Fig. 4B, where a small plantlet arising from the leaf-petiole transition zone can be seen one week after culture. Shoot development continued until regenerated plants reached approximately one centimeter in height (Fig. 4C). Fourteen days after culture initiation, successful excision of shootlets was performed and regenerated plants were individually subcultured in glass-tubes containing the same medium in which they were generated (Fig. 4D). Only five leaves responded to any of the different treatments evaluated, generating a total amount of five shoots.

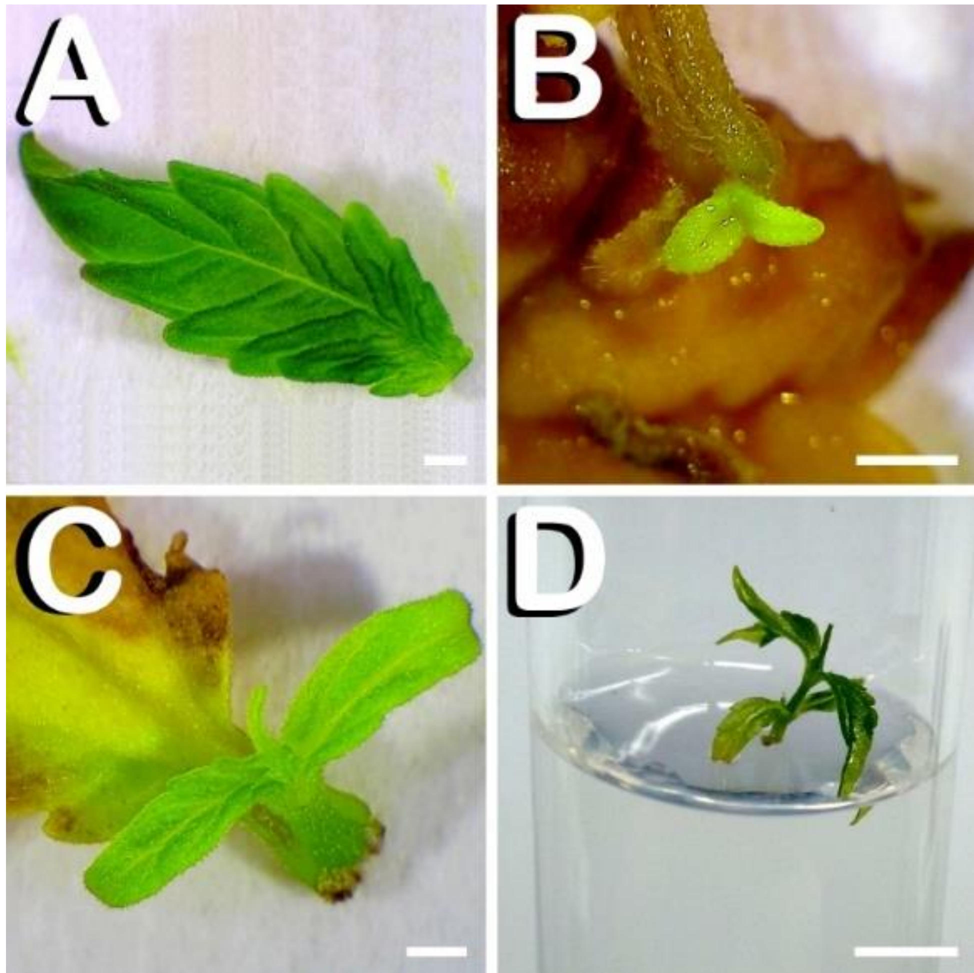

Figure 4: Direct *in vitro* shoot organogenesis from true leaves of *C. sativa*. The different developmental stages are described as follows: (A) Newly dissected leaf from a seven-days-old hemp seedling. (B) Formation of one primordium from leaf-petiole transition zone one week after culture initiation. (C) Two-week-old plantlet of approximately one centimeter in height ready for subculture. (D) Leaf derived plant individually grown in a glass-tube 21 days after culture initiation. Scale bars (A-C): 1 mm. Scale bar (D): 6 mm..

4.3. Rooting of explants and spontaneous rooting of hypocotyl derived plants

Although the present study and its derived experiments were focused on *in vitro* shoot organogenesis, some of the cultured explants developed roots instead of shoots. Specifically, two weeks after culture initiation, 1.09% of cultured hypocotyls developed vigorous roots with root hairs on the lower zone of the explant (arrow in Fig. 5A). The same phenomenon, also located on the proximal part of the explant, was observed in 0.1% of cultured cotyledons two weeks after explant culture (arrow in Fig. 5B). In another way, spontaneous rooting of *in vitro* regenerants only took place in hypocotyl-derived plants cultured in media without plant growth regulators, where 17.94% of cultured hypocotyls developed shoots on its top and roots in its lower part. After 28 days of culture initiation, hypocotyl-derived plants spontaneously rooted were ready for start the acclimatization process (Fig. 5C).

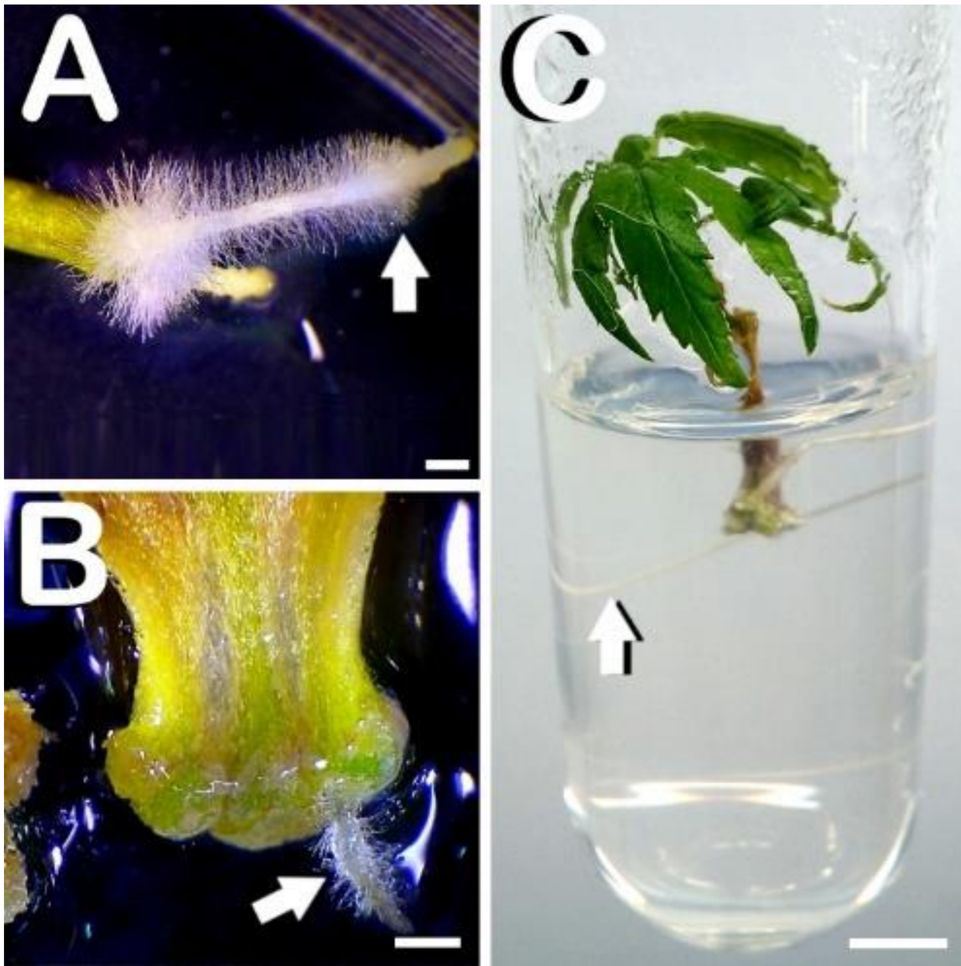

Figure 5: Rooting of explants and spontaneous rooting of hypocotyl derived plants of *C. sativa*. (A) Vigorous root with radicular hairs emerging from the basal zone of the hypocotyl two weeks after culture initiation (arrow). (B) Small root with root hairs arising from the lower part of the cotyledon after 14 days of *in vitro* culture (arrow). (C) Spontaneously rooted hypocotyl derived plant after 28 days of culture initiation with a prominent root (arrow). Scale bars (A-B): 1 mm. Scale bar (C): 6 mm.

4.4. Acclimatization of *in vitro* regenerated plants

Since only plants regenerated from hypocotyls developed spontaneous rooting, exclusively hypocotyl derived plants were submitted to the acclimatization process. The first step consisted of carefully washing the remaining gellified medium from roots. After 28 days of *in vitro* culture, regenerants showed different root morphogenesis patterns as observed in Fig. 6A, where long, medium and short size roots can be visualized, together with a robust main root with a prominent development of secondary roots. Vigorous development of the radicular system guaranteed successful acclimatization of hypocotyl derived plants. At this point, regenerants were ready for transplant in small pots (2 L) with fertilized commercial substrate (Fig. 6B), although placement of transparent plastic vessels was necessary to retain humidity and avoid desiccation of plants (Fig. 6C). However, after one week of progressive exposition of regenerants to the environmental humidity, the acclimatization process ended and hypocotyl-derived plants displayed a vigorous growth, as can be observed in Fig. 6D, where a healthy regenerant stands out six weeks after culture of hypocotyls. In order to verify the proper development of *in vitro* regenerants, acclimatized plants were grown during two additional weeks and were manually pollinated. As shown in Fig. 6E, hypocotyl derived plants showed sexual functionality eight weeks after *in vitro* explant inoculation, as can be deducted from the fact that female flowers developed viable seeds after manual pollination (insets in Fig. 6E). Following this protocol, 100% of hypocotyl-derived plants spontaneously rooted were successfully acclimatized.

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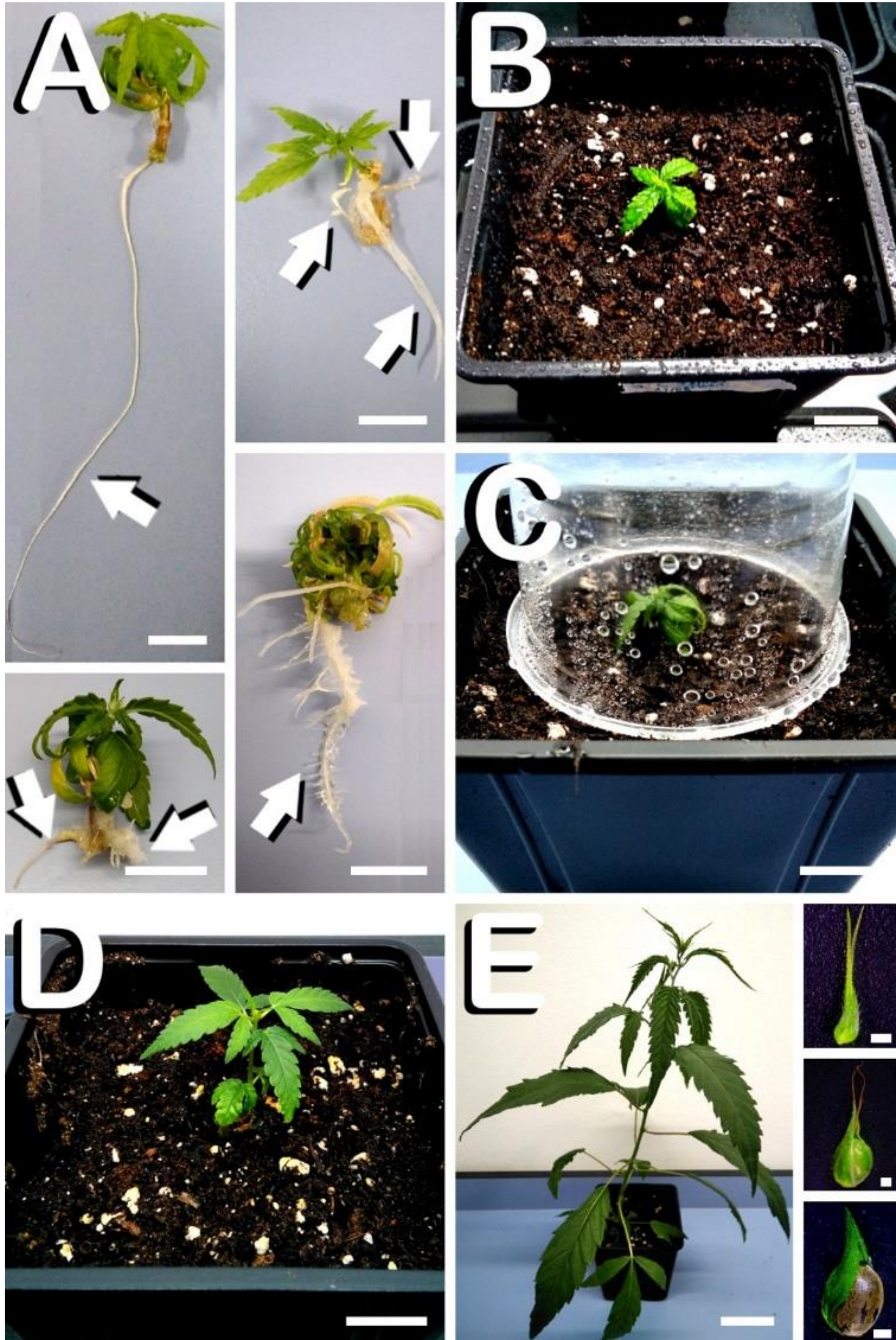
Figure 6: Acclimatization of *C. sativa* hypocotyl derived plants. (A) Roots of hypocotyl derived plants spontaneously rooted 28 days after culture: different root morphogenesis patterns can be observed (arrows). (B) Small plant just after being transplanted to pots. (C) Plastic vessel covering the *in vitro* regenerated plant in order to avoid desiccation. (D) Hypocotyl derived plant exposed to the environmental humidity six weeks after culture. (E) Female hypocotyl derived hemp plant showing sexual functionality eight weeks after *in vitro* explant inoculation (insets illustrates, from top to bottom, unfertilized female flower, fertilized female flower during seed formation and mature seed). Scale bars (A-D): 12 mm. Scale bar (E): 60 mm. Scale bars of insets (E): 1 mm.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4.5. Ploidy evaluation of explants and *in vitro* regenerated plants of *C. sativa*

The analysis of the ploidy level of freshly dissected cotyledons, hypocotyls and true leaves of seven-days-old seedlings of the five varieties evaluated revealed that only true-leaves (green) showed a diploid pattern, while cotyledons (blue) and hypocotyls (red) exhibited a mixoploid pattern (with diploid and tetraploid cells) (Fig. 7). All varieties evaluated in this experiment displayed the same polysomatic pattern for the different explants analyzed.

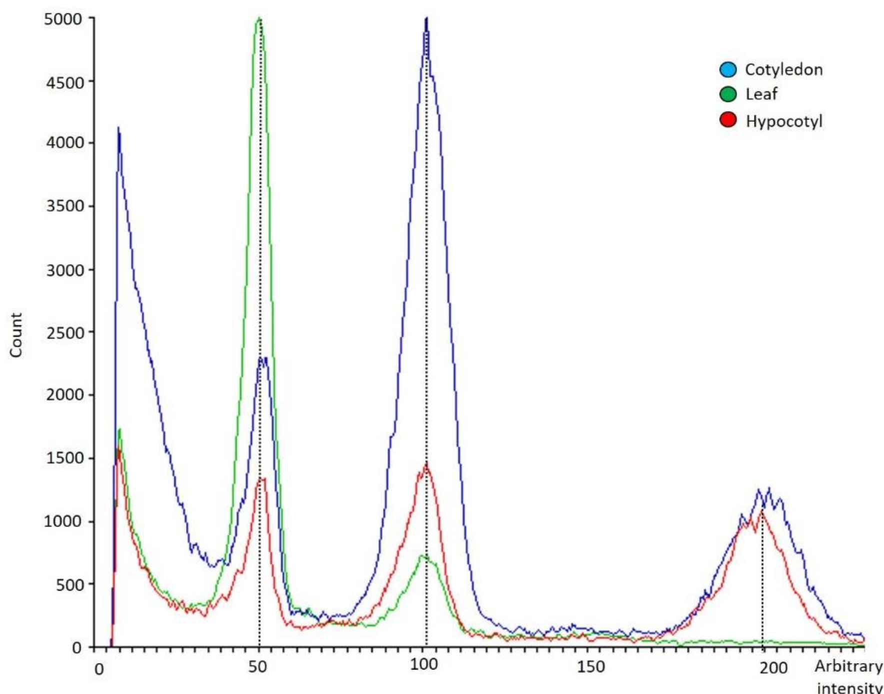

Figure 7: Flow cytometry histogram showing polysomatic pattern in cotyledons (blue), hypocotyls (red) and first pair of true leaves (green) from *C. sativa*. The x-axis represents a fluorescence intensity level proportional to the nuclear DNA content. The peak located at the value 50 corresponds to the diploid nuclei in phase G1, the peak located at the value 100 corresponds to the sum of the diploid nuclei in phase G2 and the tetraploid nuclei in phase G1, while the one at the value 200 represents tetraploid nuclei in G2 phase. The y-axis indicates the number of nuclei analyzed.

A total of 35 *in vitro* regenerated plants (17 from cotyledons, 15 from hypocotyls and three from leaves) were analyzed by means of flow cytometry 28 days after tissue culture initiation. Only diploid and mixoploid plants (with diploid and tetraploid cells) were obtained. Differences in nuclear DNA histogram patterns between diploid (Fig. 8A) and mixoploid (Fig. 8B) plants are represented in a flow cytometry histogram (Fig. 8). As illustrated in Table 5, no significant differences were identified between cotyledon and hypocotyl-derived plants in terms of ploidy level of *in vitro* regenerants. Cotyledons and hypocotyls produced, respectively, 82.35% and 86.67% of diploid regenerants (Table 5). Regarding mixoploid regenerants, both cotyledons and hypocotyls exhibited a significant capacity to generate them, with, respectively, 17.65% and 13.33% of mixoploid *in vitro* regenerated plants (Table 5).

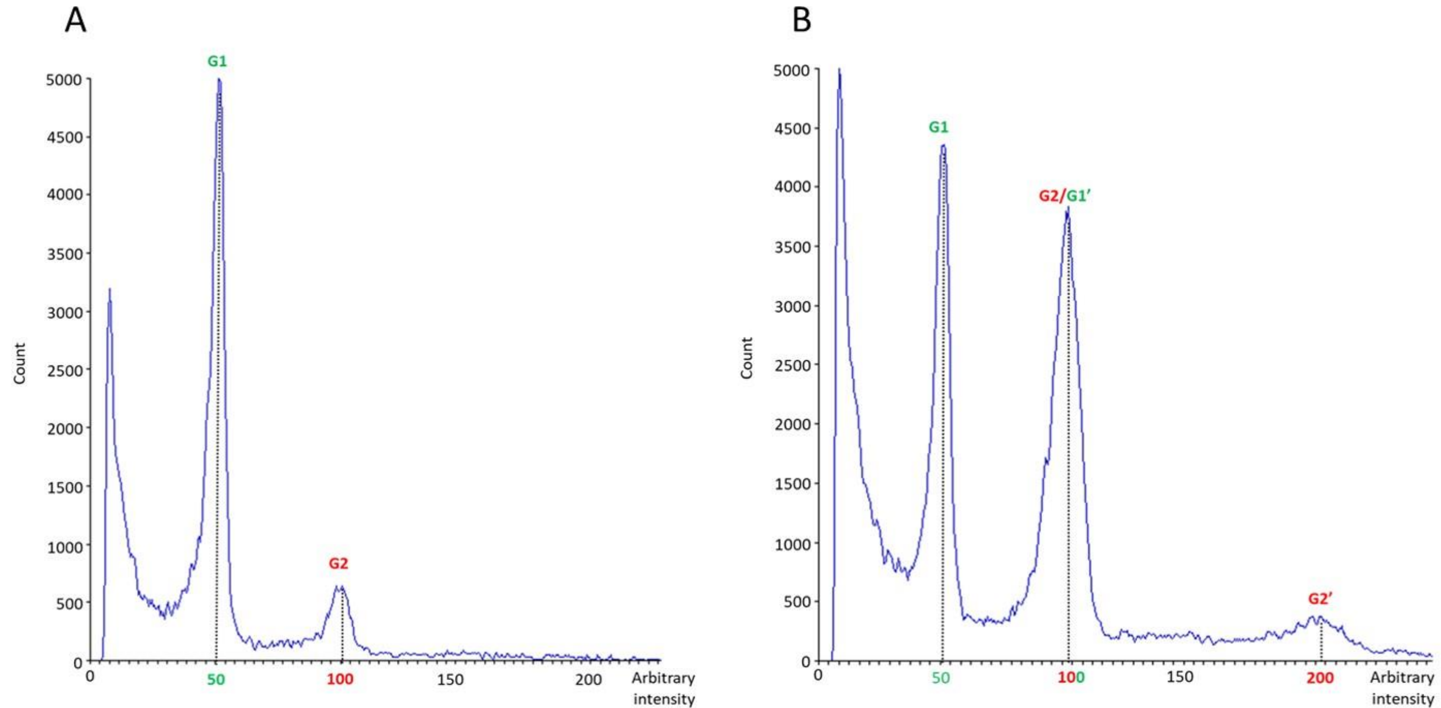

Figure 8: Nuclear DNA histogram patterns of diploid (A) and mixoploid (B) *in vitro* regenerated plants of *C. sativa* analyzed by flow cytometry. The x-axis represents a fluorescence intensity level proportional to the nuclear DNA content. The peak located at the value 50 corresponds to the diploid nuclei in phase G1, the peak located at the value 100 corresponds to the sum of the diploid nuclei in phase G2 and the tetraploid nuclei in phase G1, while the one at the value 200 represents tetraploid nuclei in G2 phase. The y-axis indicates the number of nuclei analyzed.

Table 5: Effect of explant on ploidy level of *in vitro* regenerated plants coming from cotyledons, hypocotyls and leaves of *C. sativa*.

		2 X REGENERANTS		2 X + 4 X REGENERANTS	
Factor	**n**	**Diploid regenerants (%)**	**Significance[a]**	**Mixoploid regenerants (%)**	**Significance[a]**
Explant					
Cotyledon	17	82.35 ±9.53	a	17.65 ±9.53	a
Hypocotyl	15	86.67 ±9.08	a	13.33 ±9.08	a
Leaf[b]	3	100.00 ±0.00		0.00 ±0.00	

Mean of diploid and mixoploid regenerants (%), significance and sample size (n) values are presented in different columns. For each explant, mean of diploid and mixoploid plants is expressed as a percentage (±SE) relative to the total amount of plants submitted to flow cytometry analysis.

[a]Different letters among the levels of explant factor indicate significant differences between them ($p<0.05$) according to non-parametric Kruskal-Wallis and pairwise Wilcoxon tests.

[b]Not analyzed statistically.

5. Discussion

5.1. Hypocotyl presents a high potential for *in vitro* direct regeneration of *C. sativa* plants without addition of plant growth regulators to the culture medium

Although significant differences were found for all factors evaluated, the most relevant differences were observed in the type of explant. Specifically, hypocotyl was significantly better than cotyledons and leaves in terms of shoot organogenic potential, as can be concluded from the fact that hypocotyl resulted in more than a ten-fold increase of shoot induction rate in comparison with cotyledon. In contrast, leaves displayed a poor ability to promote shoot organogenesis. On the other hand, significant differences were detected between the different levels of the factor variety, which demonstrates how genotype affects hypocotyl-derived plant regeneration in *C. sativa*, although even the worst performing variety evaluated in hypocotyl experiments reached a high shoot organogenesis rate. Additionally, despite the inherent genetic heterogeneity among individuals of a single *C. sativa* variety (Lata *et al*., 2016a, 2017), variation between *in*

vitro shoot organogenesis rate of hypocotyls coming from individuals of the same variety was relatively low in our experiments. The fact that low values of variation statistics were observed within each variety suggests that intravarietal variation for hypocotyl regeneration capability is limited in *C. sativa*, which consolidates the high potential that this explant present for plant *in vitro* regeneration in this species. Comparing our results with related bibliography, although Wahby *et al*. (2017) failed to regenerate plants from transformed hemp hypocotyl-derived calli, and Smýkalová *et al*. (2019) suggested that hypocotyls are not suitable explants for hemp multiplication, lacking of organogenic potential probably due to its low concentrations of active cytokinins, it is worth noting that *in vitro* plant regeneration from hypocotyl-derived calli has already been reported in *C. sativa* (Mandolino and Ranalli, 1999; Movahedi *et al*., 2016a, 2016b). However, in the former works, regeneration rate is not quantified and, probably due to the addition of plant growth regulators in the culture medium, a callus formation phase takes place prior to shoot organogenesis, which may compromise the genetic fidelity of regenerants with respect to the donor plant (Evans and Bravo, 1986; Ramírez-Mosqueda and Iglesias-Andreu, 2015). In this context, it should be noted that, to the best of our knowledge, this is the first report of direct *in vitro* regeneration of *C. sativa* plants from hypocotyls, and the first work in which efficient direct *in vitro* shoot organogenesis is promoted on explants lacking already developed meristems and cultured in medium without plant growth regulators. Since age plays a key role in shoot organogenic potential of explants, as has been described in cotyledons of *C. sativa* (Chaohua *et al*., 2016), probably in our case, the use of hypocotyls from seven-days-old seedlings with fully expanded first pair of true leaves, together with suppression of plant growth regulators in shoot induction medium, can make the difference with previously published studies concerning hypocotyl derived plant regeneration in *C. sativa*.

Conversely to what occurs with cotyledons, which are unable to regenerate plants in hormone-free medium, our protocol allows a high rate

of shoot organogenesis from hypocotyls of all tested varieties without addition of plant growth regulators to the shoot induction medium. *In vitro* regeneration of plants from hypocotyls without using plant growth regulators, has been reported in species like *Capsicum annuum* L. (Ezura *et al*., 1993) or *Passiflora setacea* D.C. (Vieira *et al*., 2014). Endogenous hormone levels present in hypocotyls of *C. sativa* could be influencing their ability to generate plants through *in vitro* culture, as can be deducted from the fact that it is possible to promote *in vitro* plant regeneration from hypocotyls even when exogenous supply of plant growth regulators is omitted. In this sense, it has been previously reported how one of the most important factors in adventitious organ formation is the endogenous auxin:cytokinin balance and not the amount of auxin or cytokinin added in a medium (Tanimoto and Harada, 1984). In this respect, it has already been published (Lata *et al*., 2017, and references therein) the influence exerted by the organ from which explants are dissected (and their endogenous level of plant growth regulators) on shoot organogenesis induction rate. From all the above mentioned facts, it can be concluded that the choice of the primary explant and its related endogenous hormonal levels are crucial for *in vitro* newly meristem formation in *C. sativa*, although more research remains to be done in order to clarify this hypothesis, since Smýkalová *et al*. (2019), after analyze endogenous hormone levels of hypocotyls from *C. sativa*, reported how endogenous cytokinin concentrations were below the limit of detection.

On the other hand, dissection of explants is another factor which could be influencing *in vitro* shoot organogenesis in hypocotyls coming from *C. sativa*. Since according to common knowledge, cytokinins are produced predominantly in the root meristem and auxins are synthetized in the shoot meristem, and both types of phytohormones can migrate from roots and shoots to their action site through phloem and xylem (Beck, 1996), segmentation of both shoot and root apical meristems as a result of hypocotyl dissection could modify the endogenous hormonal interaction

between auxins and cytokinins, leading to an appropriate environment for shoot organogenesis development in hypocotyls.

5.2. Hypocotyl derived plants can root spontaneously in hormone-free medium, being completely acclimatized in only six weeks

The same reasoning described above could also explain the fact that, after shoot development in the top of the hypocotyl, auxins produced endogenously in the shoot meristem could promote the spontaneous rooting of *in vitro* regenerants. Regarding this, while *in vitro* spontaneous rooting of regenerated plants has already been reported in other species like *Coleus forskohlii* Briq. (Sairam Reddy *et al*., 2001), *Cotinus coggygria* Mill. (Metivier *et al*., 2007) or *Bambusa vulgaris* Schrad. ex J.C. Wendl. (Furlan *et al*., 2018), its relation with endogenous auxin content of shootlets raised *in vitro* has also been described (Minocha, 1987). On the other hand, root development on the lower portion of hypocotyls of *C. sativa* without apical shoot meristem formation has also been described (LaRue, 1933). In our case, spontaneous rooting of *in vitro* regenerants represents an added advantage of our regeneration protocol, as a separate auxin containing medium is not required for root induction. In any case, although it was necessary to promote rooting of more *in vitro* regenerated plants, there are several published protocols concerning *in vitro* rooting of *C. sativa* shootlets with an efficiency not below 80% (Wang *et al*., 2009; Lata *et al*., 2009, 2010, 2016a, 2016b; Chaohua *et al*., 2016; Parsons *et al*., 2019).

We found that hypocotyls cultured in medium without plant growth regulators reached the third highest shoot induction rate of the evaluated media without presenting significant differences with the other two media with better percentages of shoot organogenesis. In addition, hypocotyls of all tested varieties were able to develop shoots in this medium. This, coupled with the absence of significant differences in terms of number of shoots per responding explant between the four media with the best shoot induction rates, has led us to suggest the combined use of hypocotyls and hormone-

free medium as a suitable combination to obtain hypocotyl derived plants spontaneously rooted and completely acclimatized in just six weeks.

5.3. Pericycle cells adjacent to xylem poles could be the origin of *in vit*ro regenerated plants of *C. sativa*

In order to infer the possible origin of *in vitro* regenerants from cotyledons, hypocotyls and true leaves coming from seven-days-old seedlings, we examined transversal sections of hypocotyls and, while no presence of already developed meristems or its traces was observed on them, pith, cortex and epidermis were easily identified. Our observations are consistent with those documented by Behr *et al*. (2016), who studied the development of the secondary tissues in *C. sativa* hypocotyls, illustrating with great detail cross-sections of hypocotyls during different periods after sowing. As presented in their work, cross-sections of hypocotyls six and nine days after sowing are coincident with our studies, since epidermis, cortex and pith can be easily differentiated and their respective anatomy is also concurrent with our findings, which allows us to discard the presence of already developed meristems or its traces in hypocotyls of *C. sativa*, and reject its possible role in the regeneration processes observed in this study.

The fact that the two primordia emerged from the top of hypocotyls were always distributed in the periphery of the organ and aligned one in front of the other, led us to hypothesize that regenerated plants originated always from the same type of cells. In a work by Miller (1959) concerning secondary growth in the root and seedling vascularization of *Humulus lupulus* L., the only species together with *C. sativa* belonging to Cannabaceae family, the internal structure of roots, hypocotyls and cotyledons of seedlings was studied. In this former work, hypocotyl cross-sections drawings detailed the connection between root and cotyledons of the seedling, describing not only the same regions than in our hypocotyl transversal section, but also two protoxylem poles situated in a peripheral position and distributed in opposite sides, whose location strongly

resembles the regeneration area of hypocotyl derived meristems in our experiments. Furthermore, Miller (1959) also describes how only one protoxylem pole is located in the median strand of the base of each cotyledon. Since in our study, plant regeneration from cotyledons always was located in the central region of the basal zone of the explant, it is reasonable to hypothesize that cotyledon and hypocotyl derived plant regeneration in *C. sativa* originates from xylem poles. We also found another study that supports this theory, in which xylem cells and its peripheral distribution in two distinctly separated xylem traces distributed in opposite sides, were visualized in hypocotyl transversal sections coming from nine-days-old seedlings of *C. sativa* (Behr *et al*., 2018). Again, in this preceding work, there is no trace of already developed meristems in hypocotyl cross-sections. With respect to our observations concerning leaf derived plant regeneration, although in this study only five plants were regenerated from leaves, it is remarkably how all of them were originated from leaf-petiole transition zone, as it has been described in species like *Morus indica* L. (Mhatre *et al*., 1985), previously included together with *C. sativa* in the Moraceae family, or other species like *Beta vulgaris* L. (Detrez *et al*., 1988) or *Tanacetum cinerariifolium* (Trevir.) Schultz-Bip (Hedayat *et al*., 2009). The fact that vascularization also takes place in petioles, as it does also on stems, and that leaf regenerated plants always emerged from petioles, could fit with our hypothesis concerning pericycle-derived *in vitro* shoot organogenesis in this species. This extends the scope of our protocol towards micropropagation purposes, adding the possibility to produce multiple clones genetically identical to the mature elite plants already selected from which they could be derived, although assessment of genetic fidelity by inter simple sequence repeat (ISSR) marker assay is recommended, as it has been performed in other works concerning *C. sativa* micropropagation (Lata *et al*., 2016a, 2016b). In this respect, it is important to emphasize how responding cotyledon and hypocotyl explants and its derived regenerants, while were maintained in glass-tubes, continued producing multiple shoots even two months after culture initiation.

In the line of this hypothesis, Atta *et al*. (2009) describe how pluripotency of *Arabidopsis* xylem pericycle cells is responsible of meristem regeneration from root and hypocotyl explants grown *in vitro*. Finally, it should be noted that Beeckman and De Smet (2014) describe how pericycle cells encircling the xylem pole are considered as an extended meristem which retain the capacity to undergo asymmetric cell division even when other cells have differentiated, and that some pericycle cells surrounded by differentiated cells can still become programmed to begin to proliferate, thus leading to the initiation of a new organ, which could explain how in our work, *in vitro* plant regeneration always developed directly with no need of cell dedifferentiation. Although more research remains to be done in order to validate our hypothesis, the implication of vascular traces on the regenerative capability of the evaluated explants could explain the different shoot organogenesis events observed in our work.

5.4. Polysomaty is present in cotyledons and hypocotyls of *C. sativa* seedlings

The term polysomaty was first applied by Langlet (1927) to the condition of those cells in the somatic tissues of a plant which contain multiples of the typical chromosome number (Ervin, 1941), being first described in the early literature by Stomps (1910) in *Spinacia oleracea* L. Since its discovery, polysomaty has been reported in a wide range of species as diverse as *Cucumis melo* L. (Ervin, 1939), *Beta vulgaris* L. (Sliwinska and Lukaszewska, 2005), *Chenopodium quinoa* Willd. (Kolano *et al*., 2008) or more recently in *Solanum melongena* L. (García-Fortea *et al*., 2020). Endomitosis or endorreduplication are described as possible causes that may lead to polysomaty (D'Amato, 1964; Bubner *et al*., 2006), whose occurrence is related with growth and differentiation of tissues. It has also been reported how plant tissues frequently contain a proportion of endopolyploid cells (Ramsay and Kumar, 1990, and references therein) and how portions of the plant such as storage organs and vessels often contain polyploid cells (Adelberg *et al*., 1993). Concerning polysomaty in *C. sativa*, it

should be noted how it was first described in root meristems of the species by Litardière (1925). Langlet (1927) pointed that the doubled number of chromosomes in root meristems coming from *C. sativa* resulted from two successive cleavages of each chromosome during the prophases, while Breslavetz (1926, 1932) proposed nuclear fusion as the cause of the polysomatic condition described in *C. sativa* roots.

Since there was a lack of literature concerning polysomaty in organs other than roots, we analyzed the ploidy level of cotyledons, hypocotyls and true leaves coming from seven-days-old seedlings of *C. sativa* by means of flow cytometry. To our knowledge, this is the first study describing polysomaty in cotyledons and hypocotyls of *C. sativa*. In light of our results, while leaves preserved the diploid pattern typical of the species, cotyledons and hypocotyls displayed a polysomatic pattern containing diploid and tetraploid cells, and therefore should be considered as mixoploid organs. Our findings concerning polysomaty found in cotyledons and hypocotyls of *C. sativa*, open new opportunities such as the development of polyploids through *in vitro* plant regeneration from these organs.

5.5. Mixoploid plants can be regenerated after *in vitro* culture of hypocotyls and cotyledons coming from seedlings of *C. sativa*

Polyploids are associated with enlarged organ sizes, increased biomass yield, phytochemical content and metabolic products, enhanced ability to adapt to biotic and abiotic stresses, and with changes on gene regulation (Van Hieu, 2019). Additionally, development of polyploid plants, in particular tetraploids, could be useful in plant breeding for development of triploid varieties with seedless fruits, as it has been demonstrated in *Citrullus lanatus* (Kihara and Nishiyama, 1947), *Cucumis melo* L. (Adelberg *et al*., 1993) or in *Citrus* spp. (Recupero *et al*., 2005). Since polyploid nuclei may sometimes be the progenitors of a cell generation, giving rise to a patch of polyploid tissues (D'Amato, 1952) and after being aware of polysomaty in cotyledons and hypocotyls of *C. sativa*, we evaluated if we had obtained

polyploid regenerants which could be useful for cannabinoid production. In this respect, we detected no significant differences between explants in terms of polyploidization of regenerated plants and how cotyledon and hypocotyl were the only explants capable to generate mixoploid plants. It should be noted how polyploidization uses to be associated with enhanced levels of secondary metabolites in a large number of species (Iannicelli *et al*., 2019), although there are also exceptions, as is the case of *Coffea arabica* L. and *Coffea canephora* L. (Silvarolla *et al*., 1999), where polyploidization led to decreased levels of caffeine in leaves. In the case of *C. sativa*, results found in the literature are contradictory. While Clarke (1981) found that polyploidization increased THC levels while decreased CBD and cannabinol (CBN) content, and that triploids proved to be inferior to both diploids and tetraploids in terms of cannabinoid production, Parsons *et al*. (2019) got a similar chemical profile between tetraploids and diploids, although notable increases in CBD and sesquiterpenes were associated with tetraploids. In relation to mixoploids and their cannabinoid content, Mansouri and Bagheri (2017) demonstrated that polyploidization increased significantly the content of THC in mixoploid plants compared with tetraploid and diploid plants, and that tetraploid plants had lower amounts of this substance in comparison with diploids, suggesting that mixoploids could be useful to produce THC for commercial use. Finally, it should be emphasized how polyploidization in *C. sativa* has always been induced by treating apical meristems of the plant with chemical microtubule disruptors with a high toxicity grade, such as colchicine or oryzalin (a less toxic alternative to colchicine), and that chemically induced polyploid plants often revert back to the diploid condition (Clarke, 1981), forcing to test the ploidy level of polyploid plants throughout generations.

6. Conclusion

In conclusion, due the high regenerative capacity of hypocotyl and that only hypocotyl-derived *in vitro* regenerants were able to spontaneously

rooting, together with the absence of significant differences between media with the best shoot induction rates with respect to number of shoots per responding explant, and between cotyledon and hypocotyl derived plants in terms of polyploidization, we suggest the culture of hypocotyls in hormone-free medium as the most suitable of the treatments evaluated in this study. Our protocol makes direct *in vitro* regeneration of *C. sativa* plants less aleatory and genotype-dependent, so it could have important connotations in exploitation of contemporary plant breeding techniques like genome editing (e.g., by using CRISPR/Cas gene edition) or mutagenesis, being also useful for micropropagation and for the development of polyploid varieties with enhanced levels of cannabinoids without using toxic chemical microtubule disruptors.

7. Statements

Data availability statement

The datasets generated for this study are available on request to the corresponding author.

Author contributions

A.G.-A., J.P. and F.J.H. conceived and designed the research. A.G.-A. and E.G.-F. performed the experiments. A.G.-A., E.G.-F., J.P. and F.J.H. analyzed the results. A.G.-A. wrote the manuscript and was responsible for verification of the paper. E.G.-F., J.P. and F.J.H. reviewed and edited the manuscript. All authors have read and approved the manuscript for publication.

Competing interests

The author Alberto Galán Ávila declares that his employer (Ploidy and Genomics Ltd.) is seeking a patent over the protocol presented:

- Patent applicant: Ploidy and Genomics Ltd.

- Name of inventor: Alberto Galán Ávila

- Application number: PCT/EP2020/087829

- PCT Publication number: WO/2021/130342

- Priority claim: 28th of December 2019

- Status of application: The international search authority of the Patent Cooperation Treaty (PCT) has issued the international search report with its corresponding written opinion certifying the novelty, inventive activity and industrial applicability of the protocol described herein.

- Specific aspect of manuscript covered in patent application: Culture of hypocotyl, cotyledon and/or true leaf explants in the different media described in the manuscript for micropropagation of *Cannabis sativa* L. and obtention of polyploid, mutagenized and/or genome-edited plants.
The rest of authors declare no competing interests.

8. References

Adelberg, J. W., Rhodes, Bill B. and Skorupska, H. T. (1993). Generating tetraploid melons in tissue culture. *Acta Hortic.* 336, 373–380. doi: 10.17660/actahortic.1993.336.49.

Andre, C. M., Hausman, J. F., and Guerriero, G. (2016). *Cannabis sativa*: The plant of the thousand and one molecules. *Front. Plant Sci.* 7. doi:10.3389/fpls.2016.00019.

Atta, R., Laurens, L., Boucheron-Dubuisson, E., Guivarc'h, A., Carnero, E., Giraudat-Pautot, V., *et al*. (2009). Pluripotency of *Arabidopsis* xylem pericycle underlies shoot regeneration from root and hypocotyl explants grown *in vitro*. *Plant J.* 57, 626–44. doi:10.1111/j.1365-313X.2008.03715.x.

Beck, E. H. (1996). Regulation of shoot/root ratio by cytokinins from roots in *Urtica dioica*: Opinion. *Plant Soil* 185, 3–12. doi:10.1007/bf02257560.

Beeckman, T., and De Smet, I. (2014). Pericycle. *Curr. Biol.* 24, R378–R379. doi:10.1016/j.cub.2014.03.031.

Behr, M., Legay, S., Žižková, E., Motyka, V., Dobrev, P. I., Hausman, J. F., *et al*. (2016). Studying secondary growth and bast fiber development: The hemp hypocotyl peeks behind the wall. *Front. Plant Sci.* 7. doi:10.3389/fpls.2016.01733.

Behr, M., Sergeant, K., Leclercq, C. C., Planchon, S., Guignard, C., Lenouvel, A., *et al*. (2018). Insights into the molecular regulation of monolignol-derived product biosynthesis in the growing hemp hypocotyl. *BMC Plant Biol.* 18, 1. doi:10.1186/s12870-017-1213-1.

Breslavetz, L. (1926). Polyploide Mitosen bei *Cannabis sativa* L. *Ber. Deutsch. Bot. Ges.* 44, 498–502.

Breslavetz, L. (1932). Polyploide Mitosen bei *Cannabis sativa* L. II. *Ber. Deutsch. Bot. Ges.* 17, 644–649. doi: 10.1007/bf01909774.

Bubner, B., Gase, K., Berger, B., Link, D., and Baldwin, I. T. (2006). Occurrence of tetraploidy in *Nicotiana attenuata* plants after *Agrobacterium*-mediated transformation is genotype specific but independent of polysomaty of explant tissue. *Plant Cell Rep.* 25, 668–675. doi:10.1007/s00299-005-0111-4.

Cascio, M. G., Pertwee, R. G., and Marini, P. (2017). "The pharmacology and therapeutic potential of plant cannabinoids," in *Cannabis sativa L. - Botany and Biotechnology* (Springer International Publishing), 207–225. doi:10.1007/978-3-319-54564-6_9.

Chaohua, C., Gonggu, Z., Lining, Z., Chunsheng, G., Qing, T., Jianhua, C., et al. (2016). A rapid shoot regeneration protocol from the cotyledons of hemp (*Cannabis sativa* L.). *Ind. Crops Prod.* 83, 61–65. doi:10.1016/j.indcrop.2015.12.035.

Clarke, R. C. (1981). "The genetics and breeding of *Cannabis*," in *Marijuana Botany: An Advanced Study: The Propagation and Breeding of Distinctive Cannabis, eds N. Hamel and D. Cross (Berkeley, CA: Ronin Publishing)*, 27–59.

Colijn-Hooymans, C. M., Hakkert, J. C., Jansen, J., and Custers, J. B. M. (1994). Competence for regeneration of cucumber cotyledons is restricted to specific developmental stages. *Plant Cell. Tissue Organ Cult.* 39, 211–217. doi:10.1007/BF00035972.

D'Amato, F. (1952). Polyploidy in the differentiation and function of tissues and cells in plants. *Caryologia* 4, 311–358. doi:10.1080/00087114.1952.10797544.

D'Amato, F. (1964). Endopolyploidy as a Factor in Plant Tissue Development. *Caryologia* 17, 41–52. doi:10.1080/00087114.1964.10796115.

Detrez, C., Tetu, T., Sangwan, R. S., and Sangwan-Norreel, B. S. (1988). Direct organogenesis from petiole and thin cell layer explants in sugar beet cultured *in vitro*. *Journal of experimental botany*, 39(7), 917-926. doi: 10.1093/jxb/39.7.917.

Dpooležel, J., Binarová, P., and Lcretti, S. (1989). Analysis of Nuclear DNA content in plant cells by Flow cytometry. *Biol. Plant.* 31, 113–120. doi:10.1007/BF02907241.

Ervin, C. D. (1939). Polysomaty in *Cucumis melo*. *Proc. Natl. Acad. Sci. U. S. A.* 25, 335–338. doi: 10.1073/pnas.25.7.335.

Ervin, C. D. (1941). A study of polysomaty in *Cucumis melo*. *Am. J. Bot.* 28, 113–124. doi:10.1002/j.1537-2197.1941.tb07950.x.

Evans, D. A., and Bravo, J. E. (1986). Phenotypic and genotypic stability of tissue cultured plants. In *Tissue culture as a plant production system for horticultural crops* (pp. 73-94). Springer, Dordrecht. doi: 10.1007/978-94-009-4444-2_6.

Ezura, H., Nishimiya, S., and Kasumi, M. (1993). Efficient regeneration of plants independent of exogeneous growth regulators in bell pepper (*Capsicum annumm* L.). *Plant Cell Rep.* 12, 676–80. doi:10.1007/BF00233418.

Feeney, M., and Punja, Z. K. (2003). Tissue culture and *Agrobacterium*-mediated transformation of hemp (*Cannabis sativa* L.). *Vitr. Cell. Dev. Biol. - Plant* 39, 578–585. doi:10.1079/IVP2003454.

Feeney, M., and Punja, Z. K. (2015). Hemp (*Cannabis sativa* L.). *Methods Mol. Biol.* 1224, 319–329. doi:10.1007/978-1-4939-1658-0_25.

Feeney, M., and Punja, Z. K. (2017). "The role of *Agrobacterium*-mediated and other gene-transfer technologies in cannabis research and product development," in *Cannabis sativa L. - Botany and Biotechnology* (Springer International Publishing), 343–363. doi:10.1007/978-3-319-54564-6_16.

Furlan, F. C., Gavilan, N. H., Zorz, A. Z., de Oliveira, L. S., Konzen, E. R., and Brondani, G. E. (2018). Active chlorine and charcoal affect the *in vitro* culture of *Bambusa vulgaris*. *Bosque* 39, 61–70. doi:10.4067/S0717-92002018000100061.

García-Fortea, E., Lluch-Ruiz, A., Pineda-Chaza, B. J., García-Pérez, A., Bracho-Gil, J. P., Plazas, M., ... and Prohens, J. (2020). A highly efficient organogenesis protocol based on zeatin riboside for *in vitro* regeneration of eggplant. *BMC Plant Biology*, 20(1), 6. doi: 10.1186/s12870-019-2215-y.

Hedayat, M., Abdi, G. H., and Khosh-Khui, M. (2009). Regeneration via direct organogenesis from leaf and petiole segments of pyrethrum [*Tanacetum cinerariifolium* (Trevir.) Schultz-Bip.]. *Am Eurasian J Agric Environ Sci*, 6(1), 81-87.

Hemphill, J. K., Turner, J. C., and Mahlberg, P. G. (1978). Studies on growth and cannabinoid composition of callus derived from different strains of *Cannabis sativa*. *Lloydia* 41, 453–462.

Iannicelli, J., Guariniello, J., Tossi, V. E., Regalado, J. J., Di Ciaccio, L., van Baren, C. M., *et al*. (2019). The "polyploid effect" in the breeding of aromatic and medicinal species. *Sci. Hortic. (Amsterdam).* 260, 108854. doi:10.1016/j.scienta.2019.108854.

Ihaka, R., and Gentleman, R. (1996). R: A Language for Data Analysis and Graphics. *J. Comput. Graph. Stat.* 5, 299–314. doi:10.1080/10618600.1996.10474713.

Kihara, H., and Nishiyama, I. (1947). An application of sterility of autotriploids to the breeding of seedless watermelons. *Seiken Ziho* 3, 93–103.

Kolano, B., Siwinska, D., and Maluszynska, J. (2008). Endopolyploidy patterns during development of *Chenopodium quinoa*. *Acta Biol. Cracoviensia Ser. Bot.* 51, 85–92.

Langlet, O. (1927). Zur Kenntnis der polysomatischen Zellkerne im Wurzelmeristem. *Sven. Bot. Tidskr.* 21, 397–422.

LaRue, C. D. (1933). Regeneration in mutilated seedlings. *Proceedings of the National Academy of Sciences of the United States of America*, 19(1), 53. doi: 10.1073/pnas.19.1.53.

Lata, H., Chandra, S., Khan, I. A., and ElSohly, M. A. (2009). Thidiazuron induced high-frequency direct shoot organogenesis of *Cannabis sativa* L. *Vitr. Cell. Dev. Biol. - Plant* 45 (1), 12–19. doi: 10.1007/s11627-008-9167-5.

Lata, H., Chandra, S., Khan, I. A., and Elsohly, M. A. (2010). High frequency plant regeneration from leaf derived callus of high Δ9-tetrahydrocannabinol yielding *Cannabis sativa* L. *Planta Med.* 76, 1629–1633. doi:10.1055/s-0030-1249773.

Lata, H., Chandra, S., Khan, I. A., and ElSohly, M. A. (2016a). *In Vitro* Propagation of *Cannabis sativa* L. and Evaluation of Regenerated Plants for Genetic Fidelity and Cannabinoids Content for Quality Assurance. *Methods Mol. Biol.* 1391, 275–88. doi:10.1007/978-1-4939-3332-7_19.

Lata, H., Chandra, S., Techen, N., Khan, I. A., and ElSohly, M. A. (2016b). *In vitro* mass propagation of *Cannabis sativa* L.: A protocol refinement using novel aromatic cytokinin meta-topolin and the assessment of eco-physiological, biochemical and genetic fidelity of micropropagated plants. *J. Appl. Res. Med. Aromat. Plants* 3, 18–26. doi:10.1016/j.jarmap.2015.12.001.

Lata, H., Chandra, S., Khan, I. A., and ElSohly, M. A. (2017). "Micropropagation of *Cannabis sativa* L.-An update," in *Cannabis sativa L. - Botany and Biotechnology* (Springer International Publishing), 285–297. doi:10.1007/978-3-319-54564-6_13.

Litardière, R. D. (1925). Sur l'existence de figures didiploides dans le meristeme radiculaire du *Cannabis sativa* L. *Cellule* 35, 21–25.

Lusarkiewicz-Jarzina, A. S., Ponitka, A., and Kaczmarek, Z. (2005). Influence of cultivar, explant source and plant growth regulator on callus induction and plant regeneration of *Cannabis sativa* L. *Acta Biol Craco Series Bot*, 47(2), 145-151.

Mandolino, G., and Ranalli, P. (1999). "Advances in Biotechnological Approaches for Hemp Breeding and Industry," in *Advances in Hemp Research* (CRC Press), 185–212. doi:10.1201/9781498705820.

Mansouri, H., and Bagheri, M. (2017). "Induction of Polyploidy and Its Effect on *Cannabis sativa* L.," in *Cannabis sativa L. - Botany and Biotechnology.* doi:10.1007/978-3-319-54564-6_17.

Metivier, P. S. R., Yeung, E. C., Patel, K. R., and Thorpe, T. A. (2007). *In Vitro* Rooting of Microshoots of *Cotinus coggygria* Mill, a Woody Ornamental Plant. *Vitr. Cell. Dev. Biol. Plant* 43, 119–123. doi:10.2307/20461658.

Mhatre, M., Bapat, V. A., and Rao, P. S. (1985). Regeneration of plants from the culture of leaves and axillary buds in mulberry (*Morus indica* L.). *Plant cell reports*, 4(2), 78-80. doi: 10.1007/bf00269211.

Miller, R. H. (1959). Morphology of *Humulus lupulus*. II. Secondary growth in the root and seedling vascularization. *Am. J. Bot.* 46, 269–277. doi:10.1002/j.1537-2197.1959.tb07012.x.

Minocha, S. C. (1987). “Plant Growth Regulators and Morphogenesis in Cell and Tissue Culture of Forest Trees” in, 50–66. doi:10.1007/978-94-017-0994-1_4.

Mishchenko, S., Mokher, J., Laiko, I., Burbulis, N., Kyrychenko, H., and Dudukova, S. (2017). Phenological growth stages of hemp (*Cannabis sativa* L.): codification and description according to the BBCH scale. *Žemės ūkio Moksl.* 24. doi:10.6001/zemesukiomokslai.v24i2.3496.

Movahedi, M., Ghasemi-Omran, V. O., and Torabi, S. (2015). The effect of different concentrations of TDZ and BA on *in vitro* regeneration of iranian cannabis (*Cannabis sativa* L.) using cotyledon and epicotyl explants. *Journal of Plant Molecular Breeding*, 3, 20–27.

Movahedi, M., Ghasemi-Omran, V. O., and Torabi, S. (2016a). *In vitro* callus induction and regeneration of medicinal plant *Cannabis sativa* L. *Iranian Journal of Medicinal and Aromatic Plants*, 32, 758–769.

Movahedi, M., Ghasemiomran, V., and Torabi, S. (2016b). Effect of explants type and plant growth regulators on *in vitro* callus induction and shoot regeneration of *Cannabis sativa* L. *Iran. J. Med. Aromat. Plants* 32, 83–96.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant.* 15, 473– 479.

Parsons, J. L., Martin, S. L., James, T., Golenia, G., Boudko, E. A., and Hepworth, S. R. (2019). Polyploidization for the genetic improvement of *Cannabis sativa*. *Front. Plant Sci,.* 10:476. doi:10.3389/fpls.2019.00476.

Plawuszewski, M., Lassocinski, W., and Wielgus, K. (2006). “Regeneration of Polish cultivars of monoecious hemp (*Cannabis sativa* L.) grown in *in vitro* cultures,” in *Renewable resources and plant biotechnology*, 149–154.

Ramírez-Mosqueda, M. A., and Iglesias-Andreu, L. G. (2015). Indirect organogenesis and assessment of somaclonal variation in plantlets of *Vanilla planifolia* Jacks. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 123(3), 657-664. doi: 10.1007/s11240-015-0868-2.

Ramsay, G., and Kumar, A. (1990). Transformation of *Vicia faba* cotyledon and stem tissues by *Agrobacterium rhizogenes*: Infectivity and cytological studies. *J. Exp. Bot.* 41, 841–847. doi:10.1093/jxb/41.7.841.

Recupero, G. R., Russo, G., & Recupero, S. (2005). New Promising *Citrus* Triploid Hybrids Selected from Crosses between Monoembryonic Diploid Female and Tetraploid Male Parents. *HortScience* 40, 516–520. doi: 10.21273/hortsci.40.3.516.

Ren, Y., Bang, H., Gould, J., Rathore, K. S., Patil, B. S., and Crosby, K. M. (2013). Shoot regeneration and ploidy variation in tissue culture of honeydew melon (*Cucumis melo* L. inodorus). *Vitr. Cell. Dev. Biol. - Plant* 49, 223–229. doi:10.1007/s11627-012-9482-8.

Richez-Dumanois, C., Braut-Boucher, F., Cosson, L., and Paris, M. (1986). Multiplication végétative in vitro du chanvre (*Cannabis sativa* L.). Application à la conservation des clones sélectionnés. *Agron. EDP Sci.* 6 (5), 487–495. doi: 10.1051/agro:19860510.

Sairam Reddy, P., Rodrigues, R., and Rajasekharan, R. (2001). Shoot organogenesis and mass propagation of *Coleus forskohlii* from leaf derived callus. *Plant Cell. Tissue Organ Cult.* 66, 183–188. doi:10.1023/A:1010697813852.

Silvarolla, M. B., Mazzafera, P., Lima, M. M. A. de, Medina Filho, H. P., and Fazuoli, L. C. (1999). Ploidy level and caffeine content in leaves of *Coffea*. *Sci. Agric.* 56, 661–663. doi:10.1590/s0103-90161999000300021.

Skoog, F., and Miller, C. O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symp. Soc. Exp. Biol.* 11, 118–30.

Sliwinska, E., and Lukaszewska, E. (2005). Polysomaty in growing *in vitro* sugar-beet (*Beta vulgaris* L.) seedlings of different ploidy level. *Plant Sci.* 168, 1067–1074. doi:10.1016/j.plantsci.2004.12.003.

Smýkalová, I., Vrbová, M., Cvečková, M., Plačková, L., Žukauskaitė, A., Zatloukal, M., ... and Griga, M. (2019). The effects of novel synthetic cytokinin derivatives and endogenous cytokinins on the *in vitro* growth responses of hemp (*Cannabis sativa* L.) explants. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 139(2), 381-394. doi: 10.1007/s11240-019-01693-5.

Stomps, T. J. (1910). *Kerndeeling en synapsis bij Spinacia oleracea L.* Amsterdam: M.J. Portielje.

Su, Y.-H., Liu, Y.-B., and Zhang, X.-S. (2011). Auxin-cytokinin interaction regulates meristem development. *Mol. Plant* 4, 616–25. doi:10.1093/mp/ssr007.

Tanimoto, S., and Harada, H. (1984). Roles of Auxin and Cytokinin in Organogenesis in *Torenia* Stem Segments Cultured *in vitro*. *J. Plant Physiol.* 115, 11–18. doi:10.1016/S0176-1617(84)80046-2.

Urits, I., Borchart, M., Hasegawa, M., Kochanski, J., Orhurhu, V., and Viswanath, O. (2019). An Update of Current *Cannabis*-Based Pharmaceuticals in Pain Medicine. *Pain Ther.* 8, 41–51. doi:10.1007/s40122-019-0114-4.

Van den Bulk, R. W., Löffler, H. J., Lindhout, W. H., and Koornneef, M. (1990). Somaclonal variation in tomato: effect of explant source and a comparison with chemical mutagenesis. *Theor. Appl. Genet.* 80, 817–25. doi:10.1007/BF00224199.

Van Hieu, P. (2019). Polyploid Gene Expression and Regulation in Polysomic Polyploids. *Am. J. Plant Sci.* 10, 1409–1443. doi:10.4236/ajps.2019.108101.

Vieira, L. M., Rocha, D. I., Taquetti, M. F., da Silva, L. C., de Campos, J. M. S., Viccini, L. F., et al. (2014). *In vitro* plant regeneration of *Passiflora setacea* D.C. (Passifloraceae): the influence of explant type, growth regulators, and incubation conditions. *Vitr. Cell. Dev. Biol. - Plant* 50, 738–745. doi:10.1007/s11627-014-9650-0.

Wahby, I., Caba, J. M., and Ligero, F. (2013). *Agrobacterium* infection of hemp (*Cannabis sativa* L.): establishment of hairy root cultures. *J. Plant Interact.* 8, 312–320. doi:10.1080/17429145.2012.746399.

Wahby, I., Caba, J. M., and Ligero, F. (2017). "Hairy root culture as a biotechnological tool in *C. sativa,*" in *Cannabis sativa L. - Botany and Biotechnology* (Springer International Publishing), 299–317. doi:10.1007/978-3-319-54564-6_14.

Wang, R., He, L. S., Xia, B., Tong, J. F., Li, N., and Peng, F. (2009). A micropropagation system for cloning of hemp (*Cannabis sativa* L.) by shoot tip culture. *Pak. J. Bot* 41(2), 603–608.

Wielgus, K., Luwanska, A., Lassocinski, W., and Kaczmarek, Z. (2008). Estimation of *Cannabis sativa* L. tissue culture conditions essential for callus induction and plant regeneration. *J. Nat. Fibers* 5, 199–207. doi:10.1080/15440470801976045.

Chapter 2

Microgametophyte development in *Cannabis sativa* L. and first androgenesis induction through microspore embryogenesis

Alberto Galán-Ávila[1*], Edgar García-Fortea[2], Jaime Prohens[2], Francisco Javier Herraiz[2]

PhD candidate contribution

A.G.-A. had a main role in the following activities: conceived and designed the research, performed the experiments, analyzed the results, and wrote the manuscript.

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[1]Ploidy and Genomics S.L., CEEI VALENCIA, Avda. Benjamin Franklin, nº12, Parc Tecnològic, 46980, Paterna, Valencia, Spain

[2]Instituto Universitario de Conservación y Mejora de la Agrodiversidad Valenciana, Universitat Politècnica de València, Camí de Vera s/n, 46022, Valencia, Spain

* Corresponding author: agalan@ploidyandgenomics.com

1. Abstract

Development of double haploids is an elusive current breeding objective in *Cannabis sativa* L. We have studied the whole process of anther and pollen grain formation during meiosis, microsporogenesis and microgametogenesis and correlated the different microgametophyte developmental stages with bud length in plants from varieties USO31 and Finola. We also studied microspore and pollen amyloplast content and studied the effect of a cold pretreatment to excised buds prior to microspore *in vitro* culture. Up to 476,903 microspores and pollen grains per male flower, with *in vivo* microspore viability rates from 53.71% to 70.88% were found. A high uniformity in the developmental stage of microspores and pollen grains contained in anthers was observed, and this allowed the identification of bud length intervals containing mostly vacuolate microspores and young bi-cellular pollen grains. The starch presence in *C. sativa* microspores and pollen grains follows a similar pattern to that observed in species recalcitrant to androgenesis. Although at a low frequency, cold-shock pretreatment applied on buds can deviate the naturally occurring gametophytic pathway towards an embryogenic development. This represents the first report concerning androgenesis induction in *C. sativa*, which lays the foundations for double haploid research in this species.

Keywords: Amyloplast; Cold-shock bud pretreatment; Double haploids; Microsporogenesis; Microgametogenesis; Pollen embryogenesis

2. Introduction

Cannabis sativa L. is a multipurpose crop used by humans since at least 10,000 years ago (Abel, 1980). It is an allogamous and anemophilous species, which includes short and neutral-day varieties with androecious, gynoecious and monoecious specimens. Nowadays, this species is gaining increasing attention due to its medical applications (Abrams, 2018; Urits *et al*., 2019). Biologically active terpenophenolic metabolites known as cannabinoids are the main compounds responsible for the pharmacological properties of *C. sativa*. The allogamous nature of this species is translated into its inherent genetic and phenotypic heterogeneity, which results in reduced uniformity for food, fiber, or medical applications (Andre *et al*., 2016; Onofri and Mandolino, 2017). Regarding floral biology of *C. sativa*, it has been widely studied from different perspectives. Anatomy of male and female flower is briefly described in several publications (Reed, 1914; Miller, 1970; Zhou and Bartholomew, 2003; UNODC, 2009; Raman *et al*., 2017), and the genetic basis of sexual expression in cannabis has been elucidated (Hirata, 1927; Faux *et al*., 2014). DNA markers have also been reported as linked to *Cannabis* sex expression (Mandolino *et al*., 1999; Toth *et al*., 2020). Sex-reversal through induction of fertile male flowers on female plants is routine (Ram and Sett, 1982). Some detailed microscope studies of the cannabis female flower are also available (Hammond and Mahlberg, 1973; Spitzer-Rimon *et al*., 2019; Livingston *et al*., 2020).

Conversely, despite the key role that the androecium plays in important traits such as crop yield (Frankel and Galun, 2012; Pereira and Coimbra, 2019), especially through the male gametophyte, there is a lack of detailed studies on it in *C. sativa*. Most of the studies have been focused mainly in the process of meiosis carried out in the microsporangium (McPhee, 1924; Heslop-Harrison, 1966; Asanova, 2002), in the tapetum anther layer (Heslop-Harrison, 1962, 1971), or in the exine characterization of the mature pollen grain (Bradley, 1958; Punt and Malotaux, 1984).

Beyond the influence that the pollen grain has in traditional breeding and taxonomy, it takes exclusive prominence in androgenesis. 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. These plants are derived from a haploid nucleus of male origin and after spontaneous or induced chromosome doubling, double haploids are obtained. By means of hybridization of these pure lines, it is possible to exploit the hybrid vigour, obtaining high yielding and uniform F1 hybrid material. 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. Among the most relevant factors affecting microspore embryogenesis, is the microspore and pollen stage of development. It is widely accepted how vacuolate microspores and young bi-cellular pollen grains are more sensitive to the androgenic induction (Maheshwari *et al*., 1980; Dunwell, 2010; Dwivedi *et al*., 2015; Canonge *et al.*, 2020). 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* L. (Pechan and Keller, 1988), bud length and perianth morphological markers in *Solanum lycopersicum* L. (Brukhin *et al*., 2003), pigmentation degree of anthers (Kim *et al*., 2004) and calyx-corolla ratio (Bárány *et al*., 2005) in *Capsicum annuum* L., or more recently, flower bud size in *Stevia rebaudiana* Bertoni (Uskutoğlu *et al*., 2019), and bud length, anther color, and filament length in *Opuntia ficus-indica* L. Mill. (Bouamama-Gzara *et al*., 2020).

Furthermore, stress treatments are also described as highly relevant on microspore embryogenesis (Nitsch and Norreel, 1973a; Maheshwari *et al*., 1980; Touraev *et al*., 1997; Maraschin *et al*., 2005; Murovec and Bohanec, 2012; Dwivedi *et al*., 2015; Testillano, 2019). Different physical and chemical treatments, when applied to plants, inflorescences, flower buds,

anthers or isolated microspores, can decisively promote the deflection of the gametophytic developmental pathway of microspores and pollen grains towards a sporophytic development (Shariatpanahi *et al*., 2006). Among the most popular stress treatments, cold-shock is the most frequently employed to promote microspore embryogenesis in a wide range of species. Its effect on microspore embryogenesis includes cytoskeletal organization disruption, reorganizing microspore and pollen-specific microtubule network thus blocking gametophytic division and promoting sporophytic development (Zhao *et al*., 2003). Other works mentioned its possible effect inhibiting the formation of starch grains in *Datura* proplastids and in pollen from *Hordeum vulgare* L. (Sangwan and Sangwan-Norreel, 1987). It has also been described how low temperature activates Ca^{2+} pathways and elicits an increase in cytosolic free calcium levels in microspores (Zoriniants *et al*., 2005; Żur *et al.*, 2008), which could lead to increase protein phosphorylation events related with cell division and microspore embryogenesis (Pauls *et al*., 2006). Additionally, as reviewed by Shariatpanahi *et al*. (2006), cold slows down degradation processes in the anther tissues, thus protecting microspores from toxic compounds released in the decaying anthers, and low temperatures stimulate the expression of two heat-shock proteins (HSP) genes which possibly can protect cells against chilling injuries. Depending on the species and the explant submitted to the stress pretreatment, a cold-shock can be applied from some days to several weeks at a temperature about 4-10 ºC (Shariatpanahi *et al*., 2006). In general, cold-shock can be considered as more effective in terms of embryogenically-induced microspores when applied directly to the flower buds (Nitsch and Norreel, 1973b; Sunderland and Wildon, 1979; Maheshwari *et al*., 1980; Deswal, 2018).

Finally, as it plays a key role on nutrition and viability of microspores and pollen grains, physiology and metabolism of carbohydrates in the androecium must also be considered as determinant for microspore embryogenesis. Additionally, amyloplasts appear as a marker of irreversible

cell differentiation in the microspore (Clément and Pacini, 2001). Starch deposition has also been associated with a drastic change in protein synthesis (Mandaron *et al*., 1990), which could suggest the expression of genes involved in the gametophytic pathway and the consequent loss of cellular totipotency. In androgenic species, starch accumulation in microspores and pollen grains starts at the late bi-cellular pollen stage while in the recalcitrant species, there is an early accumulation of starch during microsporogenesis with an increase during pollen maturation (Sangwan and Sangwan-Norreel, 1987).

As can be deducted from the existing bibliography, which is mainly focused on the female flower, there are still many aspects of androecium development in *C. sativa* species that have not yet been clarified. Since detailed studies concerning a precise description of the whole process of microspore and pollen grain formation lacked in the related literature, this work is focused on the development of a comprehensive characterization of meiosis, microsporogenesis and microgametogenesis in *C. sativa*. By means of light and fluorescence microscopy on the one hand, and scanning electron microscopy (SEM) and cryo-SEM on the other, special attention was paid to the parallel development of the different layers that compose the anther tissue, together with the ornamentation of the exine of microspores and pollen grains, *in vivo* microspore viability and the different nuclear features observed during cell cycle regulation throughout all stages of pollen formation. On the other hand, in order to develop an experimental microspore culture protocol to induce microspore embryogenesis in *C. sativa,* the correlation of the different developmental stages of microspores and pollen grains with bud length was studied. Furthermore, we also studied the androgenic potential of *C. sativa* through the microscopic analysis of the amyloplasts contained in anthers, microspores and pollen grains. Finally, the effect of a week-long cold pretreatment applied directly on excised buds before microspore culture was evaluated in terms of microspore viability, amyloplast content of microspores and development of multicellular

structures of androgenic origin. Short and neutral-day varieties, together with androecious and monoecious specimens were used in our experiments. Additionally, due to their exclusive capability for cannabinoid synthesis and their influence on breeding of the species, also gynoecious specimens treated with silver thiosulphate anionic complex (STS) for sex-reversal were added to our experimental design. Our work provides new improvements and updates on the morphology and male floral biology of *C. sativa*, laying the foundations for the routine implementation of androgenesis in *C. sativa* breeding.

3. Materials and methods

3.1. Plant material and growth conditions

Staminate floral buds needed for all experiments were collected from androecious, monoecious and gynoecious plants from short-day variety USO31. Additionally, due to the marked dioecious character of the neutral-day variety Finola (which lacked monoecious specimens), only androecious and gynoecious individuals from this variety were used as donor plants. Seeds were germinated in pots (1 L) with fertilized commercial substrate composed of a mixture of black peat, granulated peat moss and perlite, with a pH value of 6 and a conductivity of 1 mS/cm. In order to induce the formation of male flowers in gynoecious individuals, two weeks after germination of the seeds, female plants were sprayed once a day (early in the morning), during five consecutive days, with an aqueous solution of silver thiosulphate anionic complex [(STS); 1 silver nitrate ($AgNO_3$) and 8 sodium thiosulphate ($Na_2S_2O_3$) w/w], as described by Ram and Sett (1982). After spraying, plants were kept in darkness for one hour, and then returned to the growth chamber. Plants were grown under controlled environmental conditions at 25ºC ± 1ºC and 60% ± 1% relative humidity. During the whole cultivation process, photoperiod consisted of 12 hours of light per day. Light was provided by Lumilight® Led Grow Monster LPW-220 (LUMILIGHT LED GROW Ltd., Valencia, Spain), equipped with Light Emitting Diodes (LEDs) of

220W and a color temperature of 2,470K, which supplied 16,700 lumens and 546 µmol m^{-2} s^{-1}. Once a day, plants were watered (75% tap water + 25% osmotized water) through drip-irrigation. Following this protocol, staminate floral buds from all evaluated phenotypes were collected approximately 30 days after seed germination. Plants employed in this study were grown under license for the cultivation of *C. sativa* for research purposes, issued by the Spanish Ministry of Health via Spanish Agency of Medicines and Health Products (Agencia Española de Medicamentos y Productos Sanitarios or AEMPS) to Ploidy and Genomics Ltd.

3.2. Characterization of nuclear dynamics during meiosis, microsporogenesis and microgametogenesis through light and fluorescence microscopy

Male floral buds of different sizes containing anthers in all developmental stages of meiosis, microsporogenesis and microgametogenesis were dissected, and two anthers per bud were separately placed in a glass slide with 10 µL of a 2.5 µg/ml solution of DAPI (4', 6-diamidino-2-phenylindole) to stain nuclear DNA (Kapuscinski, 1995). Anthers were cut in thin sections with a scalpel and their content was exposed to the DAPI solution, while remaining somatic tissue was removed from the slide. Meiocytes, microspores and pollen grains contained in anthers were observed with a Carl Zeiss® Axiovert.A1 (CARL ZEISS MICROSCOPY Ltd., Jena, Germany) inverted microscope equipped with epi-fluorescence excitation LED modules, and images were registered with a Carl Zeiss® Axiocam 305 color (CARL ZEISS MICROSCOPY Ltd.). Measurements on images obtained with the microscope were carried out using ImageJ 1.53a (Schneider *et al.*, 2012). The different developmental stages of meiocytes, microspores and pollen grains observed were classified as follows: microspore mother cell, meiocyte with two nuclei, meiocyte with four nuclei, tetrad, young microspore, mid microspore, vacuolate microspore, young bi-cellular pollen, mid bi-cellular pollen and mature tri-cellular pollen (Crang *et al.,* 2018). For simplification and due to the absence of significant

differences between them, young and mid microspores were merged into a single stage of development.

3.3. Analysis of exine evolution by means of scanning electron microscopy (SEM)

With the aim of studying the ornamentation of the exine of microspores and pollen grains from *C. sativa*, in the first place, their respective stages of development were determined through DAPI staining of one of the anthers contained in each bud, as described above. To avoid mixing microspores and pollen grains at different stages of development, microspore isolation of each developmental stage was performed separately. For each stage, 5-10 buds exclusively containing anthers in a specific developmental stage were selected. The stages of development studied in this experiment ranged from tetrad until mature pollen (as mature tri-cellular pollen grains never composed exclusively the population of a pollen sac, but coexisted with mid bi-cellular pollen grains in the locule, anthers containing both stages of development were merged and classified in this experiment as mature pollen stage). Microspore extraction was carried out under cold conditions (4-8 °C) by gentle squashing of the buds with the plunger of a sterile syringe. Microspores and pollen grains were isolated in MS liquid medium (Murashige and Skoog, 1962) by filtration through two layers of 40 µm nylon filter. Previously, MS liquid medium was sterilized by vacuum filtration through a 0.22 µm polyethersulfone (PES) membrane. Samples were washed with deionized water and centrifuged three times (5 minutes each) at 8 ºC and 110.7 g and, after supernatant was discarded, microspores were deposited in a 11 µm pore size filter bag and exposed to a formaldehyde-glutaraldehyde fixative as described by Karnovsky (1967). Then, samples were washed and kept in cacodylate buffer (0.025 M) at 4 ºC overnight. They were dehydrated through a series of ethanol concentrations in deionized water (70%, 80%, 95%, 100%; one hour each) and, subsequently, ethanol was replaced by CO_2 and intracellular CO_2 was evaporated at 34 ºC and 73.7 bar in a Leica Microsystems® EM CPD300

(LEICA MICROSYSTEMS Ltd., Wetzlar, Germany) automatic critical point drying apparatus. Following this, samples were deposited on a support covered by double-sided tape and were platinum sputtered during 15 seconds prior to visualization in a Carl Zeiss® ULTRA 55 (CARL ZEISS MICROSCOPY Ltd.) scanning electron microscope, with an electron acceleration of 2 kV and a working distance of 4≈8 mm. Images were taken with the software INCA from Oxford Instruments® (OXFORD INSTRUMENTS Ltd., High Wycombe, United Kingdom). Measurements of the images obtained with the microscope were carried out using ImageJ 1.53a (Schneider *et al.*, 2012). Terminology used to define the ornamentation of the exine was extracted from the glossary published by Laín (2004).

3.4. Study of anther wall formation using cryogenic scanning electron microscopy (cryo-SEM)

In order to study the development of the different layers that compose the anther wall during its growth, staminate floral buds of different sizes containing anthers in all developmental stages of meiosis, microsporogenesis and microgametogenesis were dissected. The stage of development of the microspores and pollen grains from one of the five anthers contained in each flower was determined with DAPI staining as described above. The four remaining anthers of each bud were frozen by immersion in slush nitrogen. Water sublimation of the samples was performed at a temperature of -90 ºC during 15 minutes in a Quorum Technologies® PP3010 (QUORUM TECHNOLOGIES Ltd., Laughton, East Sussex, United Kingdom) cryo preparation system for SEM. After freeze-fracture of the anthers, samples were platinum sputtered during 60 seconds and observed with a Carl Zeiss® ULTRA 55 (CARL ZEISS MICROSCOPY Ltd.) scanning electron microscope at a temperature ranging from -150 to -180 ºC, an electron acceleration of 2 kV and a working distance of 4≈8 mm. Images were taken with the software INCA from Oxford Instruments® (OXFORD INSTRUMENTS Ltd.). Measurements of the images obtained with the microscope were carried out using ImageJ 1.53a (Schneider *et al.*, 2012).

Due to the absence of morphological differences between them, stages corresponding to meiocyte with two nuclei and meiocyte with four nuclei were merged together with microspore mother cell in a single meiotic stage of development. Moreover, as mature tri-cellular pollen grains never composed exclusively the population of a pollen sac, but coexisted with mid bi-cellular pollen grains in the locule, anthers containing both stages of development were merged and classified in this experiment as mature pollen stage.

3.5. Histochemical detection of starch through light microscopy

For starch recognition in anthers, meiocytes, microspores and pollen grains from *C. sativa*, buds of different sizes covering all stages of development were dissected and one of the five anthers contained in each flower, was placed in a glass slide and stained with DAPI as described above. After identification of its developmental stage, the four remaining anthers were fixed in Karnovsky solution as described above. Samples were kept in cacodylate buffer (0.025 M) at 4 ºC overnight. After that, samples were dehydrated through a series of ethanol concentrations in deionized water, and infiltrated and embedded in Technovit® 7100 (KULZER Ltd., Wehrheim, Germany) acrylic resin, as described by the manufacturer. Resin sections (1.5 µm) were cut with a glass knife using a Reichert - Jung® (now: Leica Microsystems®) Ultracut E (LEICA MICROSYSTEMS Ltd., Wetzlar, Germany) ultramicrotome, collected on glass slides and exposed to Lugol® (MERCK Inc., Darmstadt, Germany) solution during 5 minutes for iodine-starch complex staining and detection of amyloplasts contained in microspores and anthers. After rinsing in distilled water and drying, preparations were mounted in glycerol and observed in a Carl Zeiss® Axiovert.A1 (CARL ZEISS MICROSCOPY Ltd.) inverted microscope. Histochemical detection of starch was also performed on excised male buds after one week long cold pretreatment at 4 ºC ± 1 ºC. Due to the absence of differences between them, stages corresponding to meiocyte with two nuclei and meiocyte with four nuclei

were merged together with microspore mother cell in a single meiotic stage of development. Moreover, as mature tri-cellular pollen grains never composed exclusively the population of a pollen sac, but coexisted with mid bi-cellular pollen grains in the locule, both stages of development were merged and classified in this experiment as mature pollen developmental stage.

3.6. Correlation of the bud length with the stage of development of microspores and pollen grains

Staminate floral buds from androecious, monoecious and gynoecious plants of short-day variety USO31, and from androecious and gynoecious plants of neutral-day variety Finola, were manually excised and grouped in one-millimeter length intervals ranging from 1.00 to 6.99 mm, covering the whole range of bud development. A minimum of 18 buds coming from at least three different plants were considered for each length interval studied. Buds were observed and dissected with an Optika® SZN-6 (OPTIKA S.r.l., Ponteranica, Italy) laboratory stereo zoom microscope, and images were registered with an Optika® C-HP (OPTIKA S.r.l.) digital camera adapted to the stereoscopic microscope, which allowed for live measurements. Bud measurement was carried out from the pedicel insertion point to the bud tip. As described above, to determine its stage of development and after flower dissection, one anther per bud was placed in a glass slide and its content was exposed to the DAPI solution prior to visualization in the microscope. A minimum of 200 randomly chosen meiocytes, microspores or pollen grains per anther preparation were counted, classifying them following the above described different developmental stages. For each bud length interval, the mean of each developmental stage was expressed as a percentage (±SE) relative to the total sample size of the interval. Stages corresponding to microspore mother cell, meiocyte with two nuclei and meiocyte with four nuclei were merged together in a single meiotic stage of development. As mature tri-cellular pollen grains were observed after

anthesis, it was not possible to scale buds containing this stage, so they were excluded from this experiment.

3.7. *In vitro* microspore culture experiments

Male flowers from androecious, monoecious and gynoecious plants of short-day variety USO31, and from androecious and gynoecious plants of neutral-day variety Finola were used for microspore embryogenesis experiments. For the evaluation of the effect of stress on microspore embryogenesis, we compared isolated microspore cultures coming from non-pretreated staminate buds on the one hand, and from excised male buds exposed to a week-long cold-shock at 4 ºC ± 1 ºC prior to *in vitro* culture on the other in terms of microspore viability, amyloplast content of microspores and development of multicellular structures of androgenic origin. Microspores from different phenotypes were isolated and cultured separately, and experiments were repeated three times. Each microspore culture replicate consisted of 18 mL which were distributed in 6 cm diameter Petri dishes. Microspore density was adjusted to 40,000 microspores/mL, as described by Huang *et al*. (1990). For each culture, 12 buds exclusively containing vacuolate microspores and young bi-cellular pollen grains from at least three different plants were surface sterilized by immersion in 20 g/L of NaClO with 0.1% (v/v) Tween 20 during 10 minutes, and finally washed three times in sterile water for about 1, 4, and 10 minutes each. Vacuolate microspores and pollen grains were isolated under cold (4-8°C) and aseptic conditions as described above. Filter-sterilized MS liquid medium containing 0.04 mg/L of kinetin (KIN) and 1.0 mg/L of indoleacetic acid (IAA) (Murashige and Skoog, 1962), was used for isolation and culture of microspores and pollen grains. Medium pH was adjusted to 5.8 with NaOH. After centrifugation and supernatant discarding, microspore density was calculated by using a haemocytometer counting slide (Neubauer improved cell counting chamber). Microspore cultures were kept in dark and grown under controlled environmental conditions at 25 ºC ± 1 ºC and 60% ± 1% of

relative humidity. Viability measurement of microspores and pollen grains coming from both non-pretreated and cold-pretreated buds was carried out immediately after microspore extraction. Viability quantification was carried out through light and fluorescence microscopy. For this, isolated microspores and pollen grains were stained in a 0.4 M sucrose solution containing fluorescein diacetate (FDA) at a concentration of 4.8 µM, as described by Zottini *et al.* (1997). Data related to microspore density per flower bud and microspore *in vivo* viability obtained from this experiment were used for characterization of the different phenotypes evaluated in the present research. Furthermore, development of multicellular structures of androgenic origin was evaluated by means of light and fluorescence microscopy after staining with 10 µL of a 2.5 µg/ml solution of DAPI. Microspore cultures were examined just after *in vitro* culture establishment and every three weeks during a total period of three months with a Carl Zeiss® Axiovert.A1 (CARL ZEISS MICROSCOPY Ltd.) inverted microscope equipped with epi-fluorescence excitation LED modules.

3.8. Data analyses

Parameters of the different phenotypes evaluated such as plant height, microspores per flower bud, and *in vivo* viability of microspores were statistically analyzed. In order to correlate the different developmental stages of microspores and pollen grains from *C. sativa* with the bud length, percentages of the different stages of development were statistically compared among the different length intervals established for each of the phenotypes evaluated. Additionally, in order to verify the correlation among pollen maturation and the different flower bud length intervals established, Spearman rank correlation coefficient was calculated after data pooling from all *C. sativa* varieties. Finally, viability of microspores from non-pretreated staminate buds on the one hand, and from male buds exposed to a week-long cold-shock at 4 ºC ± 1 ºC prior to *in vitro* culture on the other, were statistically compared. Independence among variables (Durbin-

Watson test), homoscedasticity (Bartlett's test for mean variance analysis or Fligner-Killeen median test), and normality (Shapiro-Wilk test) were evaluated for the data coming from the experiments and, depending on results, ANOVA parametric test followed by Fisher's least significant difference (LSD) test ($p<0.05$), or Kruskal-Wallis non-parametric test followed by pairwise Nemenyi test ($p<0.05$), were used to statistically determine significant differences between levels of each factor evaluated. Statistical analysis was carried out using R software (R Core Team, 2019).

4. Results

4.1. Male reproductive anatomy of *C. sativa*, microspore density per flower bud and *in vivo* viability of microspores

Approximately 30 days after seed germination, all phenotypes studied showed male flowers in all developmental stages of stamen formation, including anthesis. Androecious individuals showed a prominent production of staminate buds, mostly located on the main apical meristem of the plant and arranged in a panicle (Fig. 1A). Regarding monoecious specimens, while the top of the apical meristem was fully covered by pistillate flowers, its middle section produced a high amount of male floral buds (Fig. 1B). Finally, in contrast to what occurred with non-treated gynoecious plants, whose floral apices appeared fully covered by pistillate flowers shaping the female inflorescence of the plant (Fig. 1C), STS treated gynoecious plants yielded a remarkable number of staminate flowers mainly situated on the top of the apical meristem (Fig. 1D).

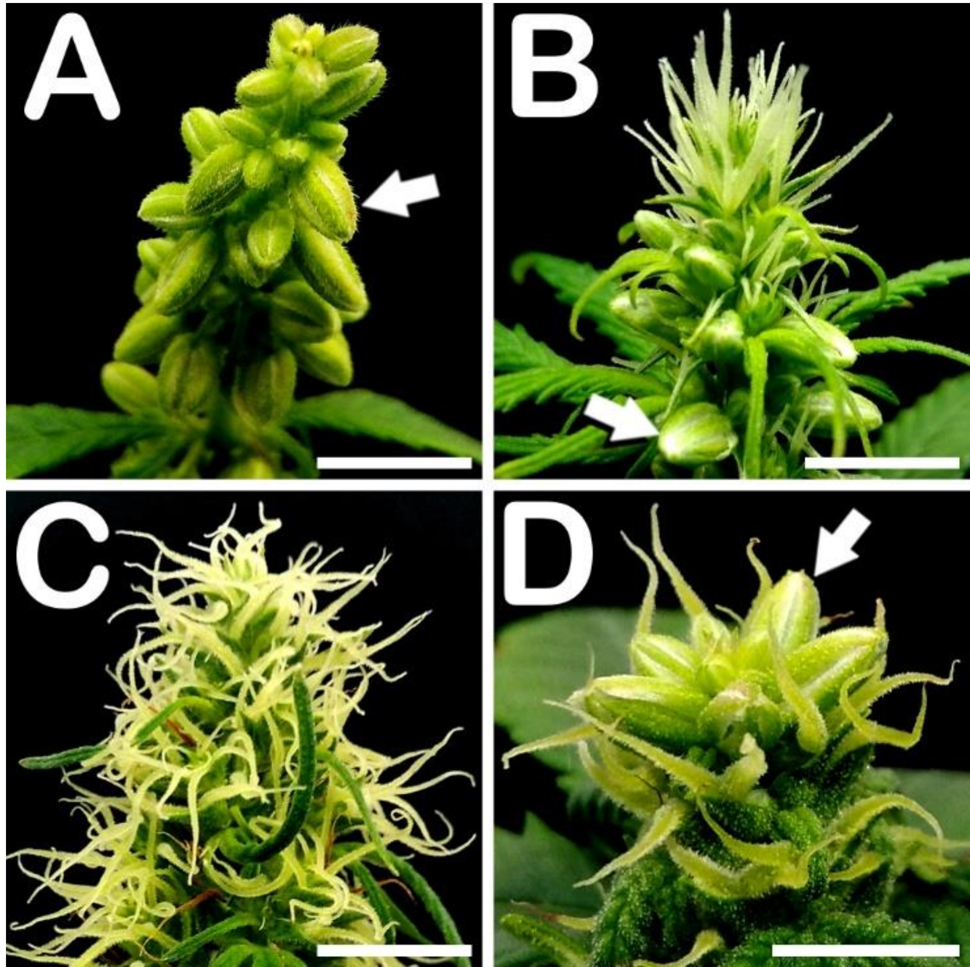

Figure 1: Floral apices of different *C. sativa* phenotypes. (A) Panicle from an androecious plant showing prominent production of staminate flowers: arrow points to a male flower bud. (B) Floral apex from a monoecious specimen fully covered by pistillate flowers on its apical section, and showing male floral buds in its middle section: arrow points to a staminate flower. (C) Apex from a gynoecious plant, showing multiple pistillate flowers. (D) STS treated gynoecious plant showing male bud formation on its apical section coexisting with pistillate flowers: arrow points to a staminate flower. Scale bars: 5 mm.

Statistically significant differences in plant height were detected among the different phenotypes evaluated (Table 1). Specifically, androecious specimens from neutral-day variety Finola were significantly taller than the other varieties tested, reaching a plant mean height of 35.18 cm just 30 days after germination of the seeds, while gynoecious plants had the lowest plant mean height of all the evaluated phenotypes with 17.38 cm (Table 1). Plants from short-day variety USO31 had a similar mean height for androecious, gynoecious and monoecious specimens (Table 1).

Table 1: Evaluation of plant height, microspore and pollen density per flower bud and *in vivo* microspore and pollen viability in different phenotypes of *C. sativa*.

Reproduction	Photoperiodism	Gender	Variety	Plant height (cm)	Microspores per flower bud	Microspore viability (%)
Dioecious	Neutral-Day	♂	Finola	35.18^{a} ±1.93	156,944^{b} ±12,483	53.71^{a} ±4.61
Dioecious	Neutral-Day	♀ (STS)	Finola	17.38^{b} ±1.39	255,494^{ab} ±25,668	59.71^{a} ±4.07
Dioecious	Short-Day	♂	USO31	22.82^{b} ±2.25	476,903^{a} ±64,503	70.88^{a} ±4.27
Dioecious	Short-Day	♀ (STS)	USO31	21.00^{b} ±0.82	303,889^{ab} ±32,961	60.49^{a} ±1.45
Monoecious	Short-Day	♂ + ♀	USO31	19.06^{b} ±0.86	471,865^{a} ±83,205	65.87^{a} ±3.64

Means are expressed as a percentage (±SE) calculated from data coming from at least three replicates.

Different letters among factors indicate significant differences between them ($p<0.05$) according to non-parametric Kruskal-Wallis and pairwise Nemenyi tests.

Not significant differences were detected among factors evaluated in “Microspore viability (%)” column according to parametric ANOVA test.

♂: “Only male flowers present in the plant”.

♀: “Only female flowers present in the plant”.

♂ + ♀: “Male and female flowers present in the plant”.

Significant differences among phenotypes were also observed for microspores and pollen grains produced per flower bud (Table 1). In this case, short-day variety USO31 showed a higher capability for pollen production than neutral-day variety Finola. Androecious plants from USO31 yielded the highest microspore density of this experiment with 476,903 microspores and pollen grains per flower bud, followed by monoecious and gynoecious individuals with, respectively, 471,865 and 303,889 microspores per flower bud (Table 1). Gynoecious and androecious plants from neutral-day variety Finola displayed a lower efficiency in terms of pollen production with, respectively, 255,494 and 156,944 microspores per flower bud (Table 1).

No significant differences were observed among the different phenotypes evaluated for *in vivo* viability of microspores and pollen grains (Table 1). Microspore viability ranged from 53.71% for microspores and pollen grains from androecious plants of neutral-day variety Finola, to 70.88% for microspores and pollen grains coming from androecious specimens of short-day variety USO31 (Table 1). Viability of microspores and pollen grains from STS treated gynoecious plants from both USO31 and Finola varieties, achieved similar viability levels as those from androecious and monoecious specimens (Table 1).

4.2. Cellular characterization of the different developmental stages of meiosis, microsporogenesis and microgametogenesis

Regardless of the anther maturity degree, all evaluated phenotypes presented highly uniform anthers containing microspores and pollen grains in a predominant developmental stage (with the only exception of mature tri-cellular pollen grains, which never composed exclusively the population of a pollen sac, but coexisted with mid bi-cellular pollen grains in the locule). This synchronized development was also observed among different anthers coming from the same bud, which enclosed microspores and pollen grains in the same stage of development. Microsporogenesis initiated when

microspore mother cells (MMCs) proceeded through meiosis. Meiotic stages of development were present in buds ranging from 1.00 to 2.99 mm in the neutral-day variety Finola (Figs. 2A-E), and from 1.00 to 3.99 mm in the short-day variety USO31. Pollen grain formation started from a compact mass of clustered MMCs (Fig. 2A') in which multiple nuclei were observed (Fig. 2A''). This kind of cells were firmly attached one to each other (Fig. 2B'), and underwent meiosis synchronously (Fig. 2B''). Some nuclei exhibited 10 bivalents clearly differentiated and centrally located in the metaphase plate (inset in Fig. 2B"). Isolated MMCs showed a regular polygonal shape, with a rounded cytoplasm encircled by a thick callose layer (Fig. 2C'), and with a large nucleus located in the middle of the cytoplasm (Fig. 2C''). After meiosis I, meiocytes with two nuclei were generated. As observed in MMC stage of development, they also showed a thick callose coat surrounding their rounded cytoplasm (Fig. 2D'), although the main characteristic of this phase was the presence of two prominent nuclei located in the cytoplasm (Fig. 2D''). There were no evidences of wall formation separating nuclei and cytoplasm at meiocyte with two nuclei stage of development. Successively, development of meiosis II gave rise to meiocytes with four nuclei, which still preserved their thick external callose layer and their rounded cytoplasm (Fig. 2E'). They were characterized by the presence of four nuclei clearly distinguishable in their cytoplasm (Fig. 2E''). As in the previous stage of development, no wall separating their nuclei and cytoplasm was observed. All these developmental stages of meiosis were common for the three types of plants and for the two varieties evaluated.

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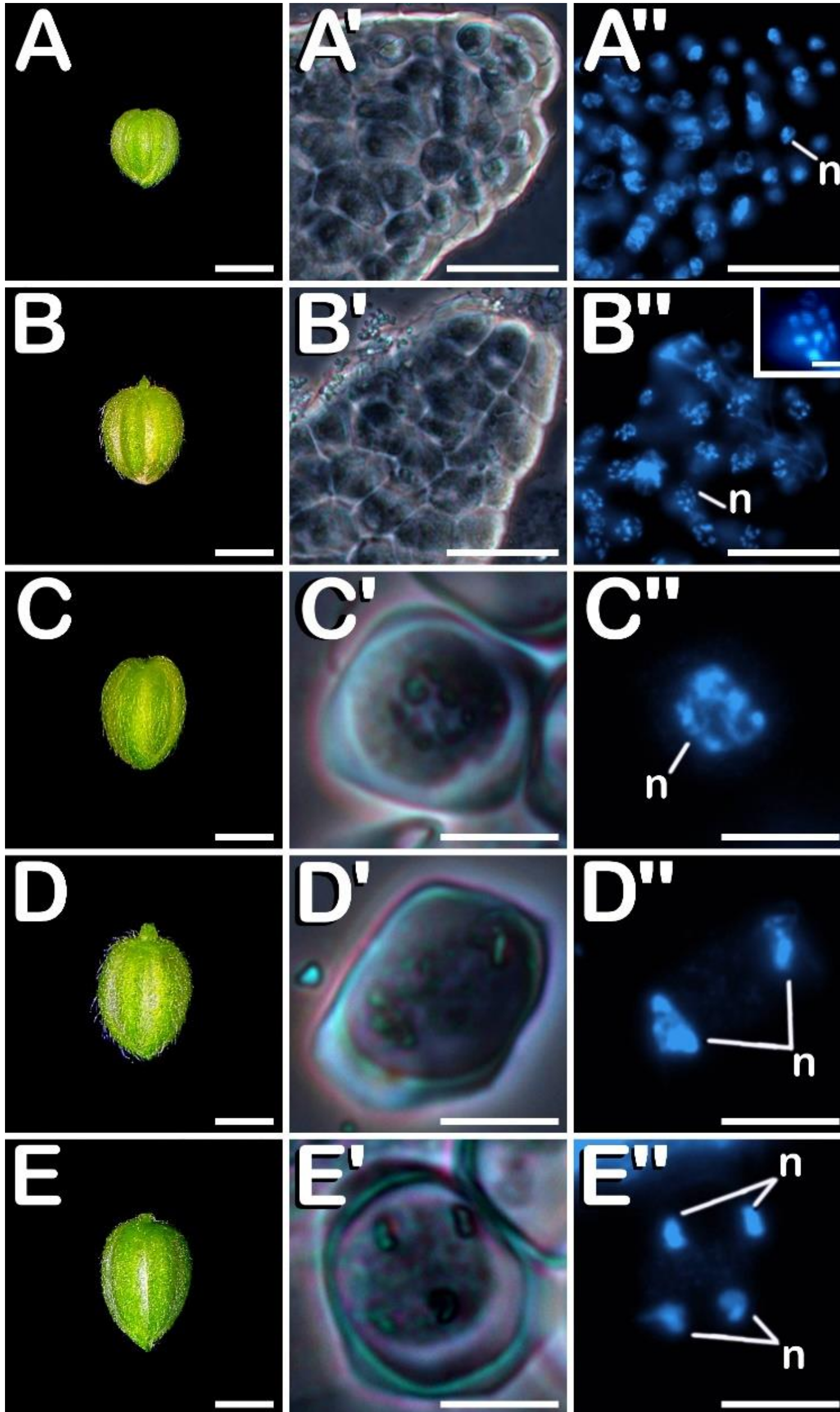
Figure 2: Meiosis development in *C. sativa*: (A–E) *C. sativa* var. Finola (dioecious male) staminate flowers growing in size. (A', A'') Compact mass of clustered microspore mother cells in prophase I. (B', B'') Compact mass of clustered microspore mother cells in metaphase I and individual microspore mother cell showing 10 bivalents (inset in Fig. 2B"). (C', C'') Isolated microspore mother cell. (D', D'') Meiocyte with two nuclei in telophase I. (E', E'') Meiocyte with four nuclei in telophase II. (A'–E') Phase-contrast microscope images. (A''–E'') Fluorescent microscope images after DAPI staining. Scale bars (A–E): 1 mm. Scale bars (A'–B') and (A''–B''): 40 µm. Scale bar (inset in B"): 5 µm. Scale bars (C'–E') and (C''–E''): 10 µm. **Abbreviations** → **n**: nucleus.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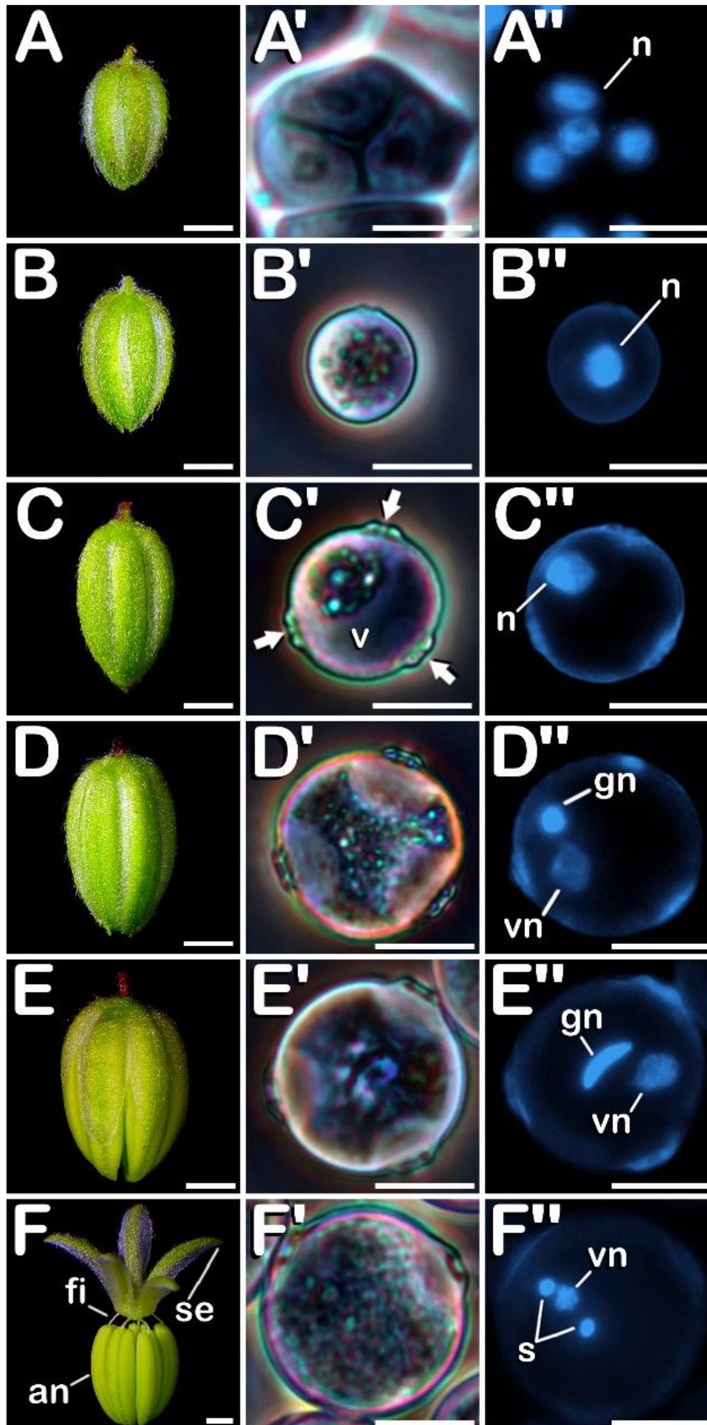
Post-meiotic stages were observed in buds ranging from 1.00 to 5.99 mm in the case of neutral-day variety Finola (Figs. 3A-E), and from 2.00 to 5.99 mm in the case of short-day variety USO31. After meiosis, tetrads (Fig. 3A’) were visualized. They were characterized by the presence of four haploid microspores of polygonal shape constricted in a tetrahedral disposition by a callose wall resembling the thick coat observed in MMC and more advanced meiotic developmental stages. The perspective of the nuclei observed in Fig. 3A’’ revealed the tetrahedral arrangement of microspores inside the tetrad. After degradation of the tetrad wall, the spherical young and mid microspores (Fig. 3B’), with a prominent and centrally located nucleus (Fig. 3B’’), were released. At this phase, microspores reached a diameter of ≈12 µm. During their development, microspores went through a progressive vacuolation process, concluded with the formation of a large vacuole, main characteristic of vacuolate microspores (Fig. 3C’). At this developmental stage, also an increase in exine thickness was observed, which allowed visualization of three prominent apertures distributed throughout the exine (arrows in Fig. 3C’). The vacuole occupied most of the cytoplasm, displacing the nucleus towards the cell periphery (Fig. 3C’’). Commonly considered as one of the suitable developmental stages for the induction of microspore embryogenesis, vacuolate microspores were also characterized by an increase in volume compared with young and mid microspores. The diameter of vacuolate microspores was ≈17 µm. Subsequently, an asymmetric mitotic division of the nucleus was observed. This first pollen mitosis, defines the young bi-cellular pollen stage (Fig. 3D’), determining the completion of microsporogenesis and the start of microgametogenesis. Noteworthy, together with vacuolate microspore, young bi-cellular pollen stage is usually defined as the most sensitive stage of development for androgenic induction in a wide range of species. Its main feature is the presence of two cells of different size and level of chromatin condensation in the cytoplasm. The vegetative cell grew and its chromatin appeared more dispersed, showing weaker fluorescence in comparison with

the generative cell (Fig. 3D''). A slight increase in volume was appreciated, with young pollen grains acquiring a diameter of ≈19 µm. In succession to these stages, mid bi-cellular pollen grains (Fig. 3E') were visualized whose diameter reached ≈21 µm, although the main difference with respect to the previous stage of development was observed by means of fluorescence microscopy. First, the vegetative cell migrated to the center of the pollen grain. After that, generative nucleus moved to the center, close to the vegetative nucleus and acquired a fusiform morphology (Fig. 3E'') which finally defined the mid bi-cellular pollen stage. Until this phase, flower buds increased in size (Figs. 3A-E) as more advanced stages of development were observed. However, only after anthesis (Fig. 3F) and prior to the issuance of the pollen tube, mature tri-cellular pollen grains (Fig. 3F') were visualized. No significant variations in diameter were observed among mid bi-cellular and mature tri-cellular pollen stages of development. Mature tri-cellular pollen grains had a diameter of ≈23 µm. Nonetheless, an important nuclear event took place at this stage. Generative cell, initially inactive as revealed by a high degree of chromatin condensation, entered in cell cycle and underwent second pollen mitosis, which led to the formation of the two spermatid cells (Fig. 3F''), typical from the mature tri-cellular pollen stage of development. It is remarkable how this stage never was exclusively present in a single loculus, always being visualized together with mid bi-cellular pollen grains. This developmental process including characteristics of each stage, was observed for both Finola and USO31 varieties, and also for androecious, monoecious and gynoecious plants. In all evaluated phenotypes, anthers coming from the same bud always showed the same developmental stage.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← **Figure 3**: Microsporogenesis and microgametogenesis developed in *C. sativa*. The different developmental stages are described as follows: (A–E) *C. sativa* var. Finola (dioecious male) staminate flowers growing in size. (F) *C. sativa* male flower after anthesis. (A', A'') Tetrad stage. (B', B'') Young and mid microspore stage. (C', C'') Vacuolate microspore stage: arrows highlight apertures. (D', D'') Young bi-cellular pollen stage. (E', E'') Mid bi-cellular pollen stage. (F', F'') Mature tri-cellular pollen stage. (A'–F') Phase-contrast microscope images. (A''–F'') Fluorescent microscope images after DAPI staining. Scale bars (A–F): 1 mm; Scale bars (A'–F') and (A''–F''): 10 µm. **Abbreviations** → **n**: nucleus; **v**: vacuole; **vg**: vegetative nucleus; **gn**: generative nucleus; **an**: anther; **fi**: filament; **se**: sepal; **s**: spermatids.

4.3. Evolution of the exine throughout microsporogenesis and microgametogenesis

The surface of microspores and pollen grains from tetrad until mature pollen stage of development was studied through SEM. In the early tetrad stage, a callose wall was fully covering the four microspores contained inside of the tetrad (Fig. 4A). At this phase, the tetrad surface presented a regular and smooth texture (Fig. 4A'). As microsporogenesis advanced, the callose coat was progressively degraded until the shape of the microspores contained in the tetrad was noticeable (Fig. 4B). Deterioration of callose wall was characterized by the formation of fissures and holes along the entire tetrad surface (Fig. 4B'), which lost its regular and smooth pattern, giving way to an irregular, disorganized and porous texture. Just before being released, in late tetrad stage, microspores presented a disordered surface of rough appearance and apertures were still covered by callose remnants (Fig. 4C). At this phase, fissures and holes extended in size, which allowed observation of the microspore exine among residual callose fibers (Fig. 4C'). Once liberated, young and mid microspores showed a spherical shape, being partially covered by the last pieces of the callosic layer (Fig. 4D). Their exine exhibited a scabrate pattern characterized by presence of protrusions sticking out less than one micron from a layer full of deep cleavages regularly distributed along its surface (Fig. 4D'). After that, in vacuolate microspore

stage of development (Fig. 4E) the triporate nature of the cannabis pollen grain was revealed. Throughout the exine, three prominent apertures were clearly visible. Each aperture (Fig. 4E') was composed by a circular pore whose diameter reached ≈1.0 µm. It was surrounded by a pore protrusion with a diameter of ≈3.5 µm, which raised up ≈0.8 µm from the exine. Following this phase, at young bi-cellular pollen stage of development (Fig. 4F), some variation in the exine ornamentation was perceived. Mainly, the rifts responsible for the characteristic texture acquired by microspores during their development were not present. Instead of this, a layer resembling sporopollenin seemed to have partially filled the fissures, only keeping visible some protuberances emerging along the entire surface of the pollen grain (Fig. 4F'). Finally, in mature pollen developmental stage (Fig. 4G), a reduction in pore protrusion was observed. It decreased until the annular prominence circumscribing the pore was hardly distinguishable, while exine scabrate pattern was mainly composed by the tip of protuberances arising from a smooth layer of sporopollenin (Fig. 4G'). Regarding exine evolution, no differences were observed neither among Finola and USO31 varieties, nor androecious, monoecious and gynoecious plants.

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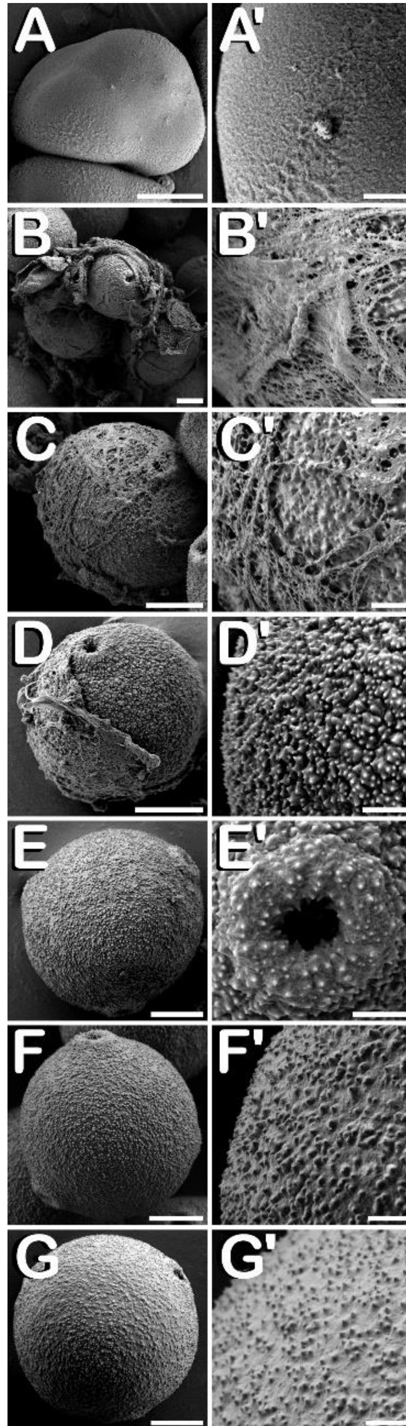
Figure 4: SEM images showing detailed development of the exine ornamentation during microsporogenesis and microgametogenesis in *C. sativa* var. USO31 (monoecious). The different developmental stages are described as follows: (A–C) and (A'–C') Tetrad stage: detail of the callose layer covering the four enclosed microspores can be observed in A'; fissures and holes as a result of callose degradation can be seen in B'; microspore exine appears among callose fibers in C'. (D, D') Young and mid microspore stage. (E) Vacuolate microspore stage. (E') Detail of an aperture of a vacuolate microspore. (F, F') Young bi-cellular pollen stage. (G, G') Mature pollen stage. Scale bars (A–G): 5 µm. Scale bars (A'–G'): 1 µm.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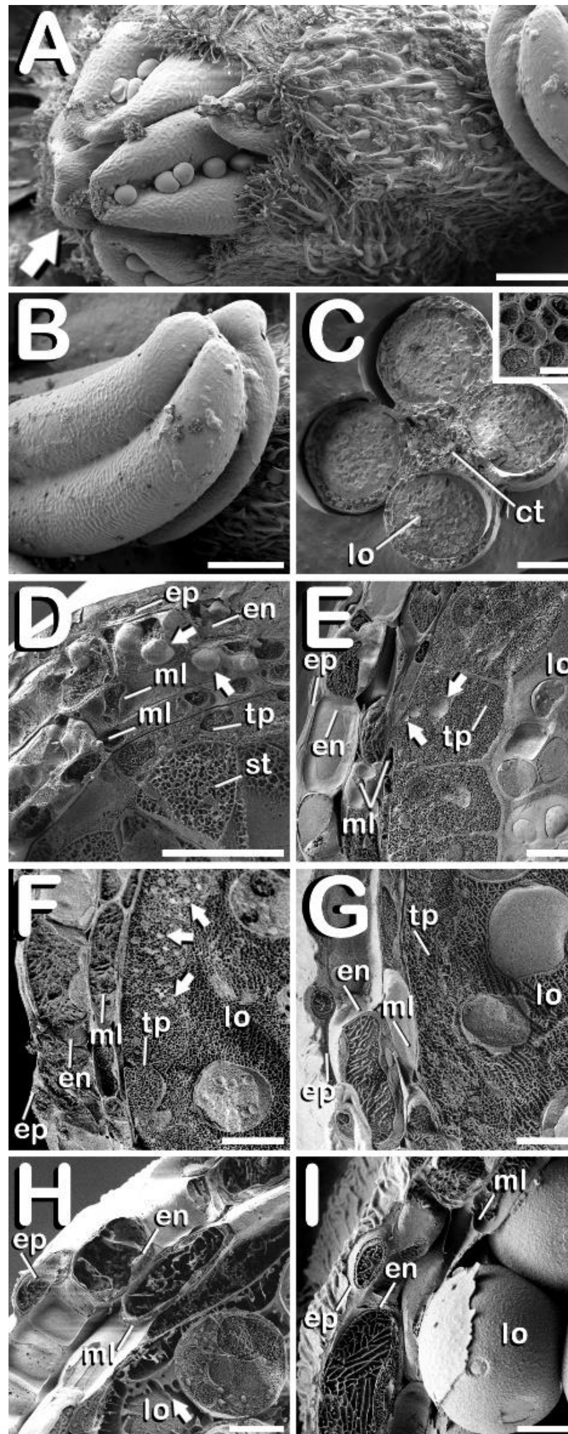
4.4. Development of the different layers that compose the anther wall during stamen formation

The study of fractured anthers through cryo-SEM, allowed for the elucidation of the changes developed in the different tissues that compose the anther wall during the whole process of pollen grain formation. Development of these layers was closely related with the stage of development of the microspores and pollen grains contained in the locule, which denotes a synchronized development between them. *Cannabis sativa* male buds contained five anthers (arrow in Fig. 5A), which were individually excised (Fig. 5B) in order to carry out freeze-fracture of the anthers (Fig. 5C). After that, the four locules of each anther joined by the connective tissue, which was mainly composed by vascular bundles (inset in Fig. 5C) were clearly visible. All the layers of the anther wall were already developed by the time of MMCs differentiation (Fig. 5D). The anther wall was composed by an external epidermis followed by endothecium, two middle layers and tapetum. Endothecium and the outer middle layer presented a high number of rounded plastids ranging in size from 1 to 3 µm in diameter (arrows in Fig. 5D). Total anther wall thickness at this stage was ≈17 µm. The anther wall layers enclosed the sporogenous tissue, which completely filled the loculus. After meiosis, in tetrad stage (Fig. 5E), while epidermis and the inner middle layer were narrowed, tapetum layer reached its maximum thickness. Some bright orbicules of spherical shape embedded on tapetal cells were observed (arrows in Fig. 5E). Their diameter oscillated between 1 and 5 µm. At this phase, total anther wall width extended to ≈45 µm. As microspores progressed in their development, degeneration of tapetum was more severe, being clearly visible from the young and mid-microspore stage of development (Fig. 5F). At this stage, the inner middle layer was completely degraded and some fissures starting from the inner face of tapetal cells advanced perpendicularly to the outer layers of the anther wall, whose total thickness was reduced to ≈28 µm. Degeneracy of tapetal cells allowed observation of the orbicules enclosed in tapetum (arrows in Fig. 5F), which

in this stage ranged in size from 1 to 2 µm in diameter. Thereafter, in vacuolate microspore stage (Fig. 5G), tapetum cells showed strong signs of degradation, being quite difficult to detect. At this phase, anther wall reached a total thickness of ≈24 µm, while in the young bi-cellular pollen stage of development (Fig. 5H), the tapetal layer was absent, and in its place it was observed how tapetum sticky remnants covered the surface of pollen grains (arrow in Fig. 5H), which were in direct contact with the remaining middle layer. In this developmental stage the anther wall reached ≈20 µm in some sections. Finally, the mature pollen stage (Fig. 5I) was characterized by anther locule dehydration, which resulted necessary for subsequent anther longitudinal dehiscence through septum and stomium degeneration. There was no trace of the locular fluid and only mature pollen grains ready for dispersal were visualized. As in the previous phase, only epidermis, endothecium and middle layer were observed. Their total width was ≈15 µm, which was the minimal thickness of the anther wall measured in this study. No differences were observed for anther wall development either in Finola or USO31 varieties, or among androecious, monoecious and gynoecious plants.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← **Figure 5**: Cryo-SEM images showing anther wall development in *C. sativa*. The different developmental stages are described as follows: (A) Staminate flower with its five anthers being visible: arrow points to the anthers. (B) Anther excised from a male bud. (C) Cross-section of an anther after freeze-fracture showing four microsporangia and vascular bundles from connective tissue (inset in Fig. 5C). (D) Microspore mother cell stage: arrows point plastids in endhotecium and outer middle layer. (E) Tetrad stage: arrows point orbicules embedded in tapetum layer. (F) Young and mid microspore stage: arrows point orbicules embedded in tapetum layer. (G) Vacuolate microspore stage. (H) Young bi-cellular pollen stage: arrow points to tapetum sticky remnants covering the pollen grain surface. (I) Mature pollen stage. Scale bars (A, B): 200 µm. Scale bars (C): 100 µm. Scale bar (inset in C): 5 µm. Scale bars (D–I): 10 µm. **Abbreviations** → **lo**: locule; **ct**: connective tissue; **ep**: epidermis; **en**: endothecium; **ml**: middle layer; **tp**: tapetum; **st**: sporogenous tissue.

4.5. Amyloplasts in anthers, microspores and pollen grains from *C. sativa*

Amyloplasts were detected in all the different developmental stages of meiosis, microsporogenesis and microgametogenesis, although in meiotic stages of development, they were only present in some anther wall layers. Specifically, from MMC stage (Fig. 6A) and throughout meiosis, starch deposits were only visualized in endothecium and the outer middle layer of the anther wall (Fig. 6A’). Amyloplasts exhibited a dark purple coloration. MMCs lacked any kind of starch accumulation. After emergence of tetrads (Fig. 6B), amyloplasts showing a red coloration started to be visible within the four microspores contained inside of the callose wall. At this stage, dark purple amyloplasts remained in endothecium and the outer middle layer of the anther wall (Fig. 6B’). In young and mid microspore stages (Fig. 6C), the number and coloration of the starch deposits contained inside of the microspores followed a similar trend as was observed for tetrad stage. Still in this stage, endothecium and the remaining middle layer showed carbohydrates reserves in the form of dark purple stained starch deposits (Fig. 6C’). When microspores reached the vacuolate microspore stage and due to the space restrictions promoted by the development of a large vacuole, red stained microspore amyloplasts were exclusively located in the

periphery of the microspore (Fig. 6D), while dark purple amyloplasts were still present in endothecium and middle layer (Fig. 6D'). After first pollen mitosis (Fig. 6E), the amyloplast content of the pollen grains was increased. They were distributed along the entire pollen grain cytoplasm and presented a red to purple coloration. Simultaneously, energy reserves of the anther tissue were drastically reduced, with the last remnants of dark blue stained amyloplasts being present in endothecium (Fig. 6E'). From this phase onwards, no more amyloplasts were detected in the anther wall. The progressive increase of amylogenesis observed during pollen maturation concluded in mature pollen stage (Fig. 6F). Pollen grains drastically increased their amyloplast content, appearing totally filled with purple stained amyloplasts. No energy reserves in the form of starch grains were observed in the remaining anther layers (Fig. 6F'). It should be noted how tapetum layer did not present organelle-staining in none of the developmental stages studied.

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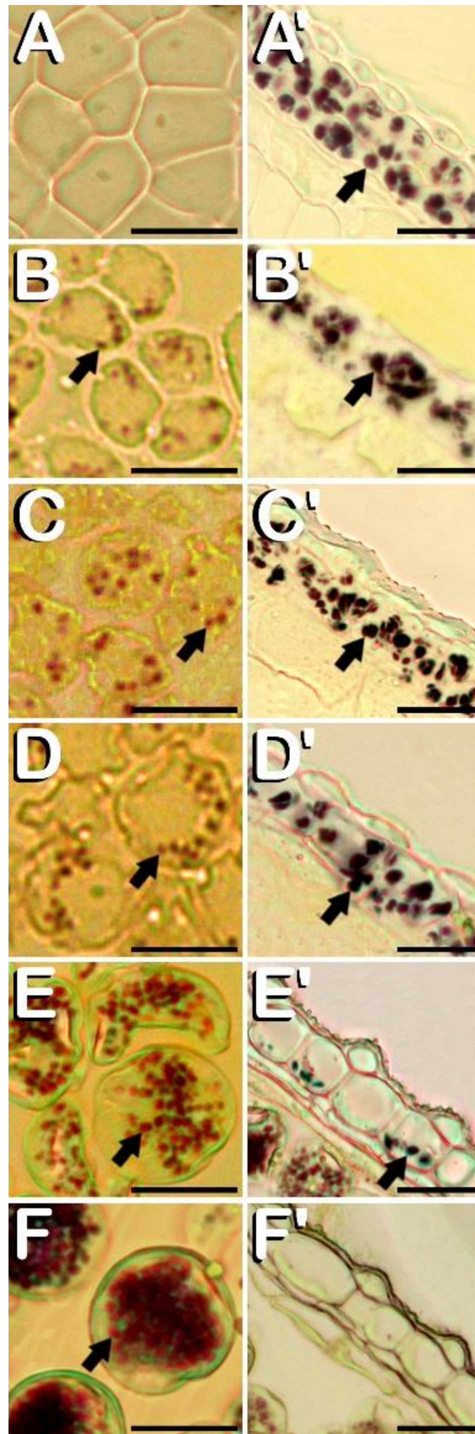
Figure 6: Histochemical detection of the amyloplasts contained in microspores and pollen grains (A–F), and in endothecium and the outer middle layer of the anther wall (A'–F') throughout microsporogenesis and microgametogenesis in *C. sativa*. The different developmental stages are described as follows: (A, A') Microspore mother cell stage. (B, B') Tetrad stage. (C, C') Young and mid microspore stage. (D, D') Vacuolate microspore stage. (E, E') Young bi-cellular pollen stage. (F, F') Mature pollen stage. (A–F) and (A'–F') bright-field microscope images after iodine-starch complex staining. Arrows point to amyloplasts. Scale bars (A–F) and (A'–F'): 20 µm.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4.6. Correlation of the bud length with the different *C. sativa* microgametophyte developmental stages

A correlation between bud length and the different stages of microsporogenesis and microgametogenesis was observed in all phenotypes evaluated. Small buds showed earlier microspore stages of development and, as the buds grew, these stages disappeared and more advanced stages emerged. The Spearman rank correlation coefficient calculated was $\rho = 0.9428$, which denotes a strong positive correlation among bud length interval and pollen maturation. However, some differences with respect to the correlation of the bud length and the developmental stage of microspores and pollen grains contained were detected for the studied cultivars. The main difference observed was the fact that androecious plants from neutral-day variety Finola were the fastest maturing plants of this experiment, as male buds presented more advanced stages of development in less sized buds in comparison with the rest of evaluated phenotypes. Regarding the presence of the commonly considered as suitable stages for androgenesis induction in *C. sativa* male floral buds, significant differences were identified between the different bud length intervals established. The bud length interval which significantly presented the highest percentage of vacuolate microspores (70.00%) coexisting with young bi-cellular pollen grains (15.00%) was 2.00 to 2.99 mm (Table 2). With respect to gynoecious Finola plants, buds oscillating from 4.00 to 4.99 mm were found to contain significantly higher amounts of vacuolate microspores (68.42%) together with the lowest content of young bi-cellular pollen grains (5.26%) (Table 2).

Table 2: Bud length correlation with meiosis, microsporogenesis and microgametogenesis in different *C. sativa* phenotypes. At least 18 buds were used per bud length interval (expressed in mm).

					Microspore and pollen grain developmental stages (%)					
Reproduction	**Photoperiodism**	**Gender**	**Variety**	**Bud Length**	**MEIOSIS**	**TET**	**YM-MM**	**VM**	**YBP**	**MBP**
Dioecious	Neutral-Day	♂	Finola	1.00 – 1.99	40.9^{a} ±10.7	31.8^{a} ±10.2	22.7^{a} ±9.1	4.5^{b} ±4.5	0.0^{c} ±0.0	0.0^{b} ±0.0
				2.00 – 2.99	0.0^{b} ±0.0	10.0^{b} ±6.9	5.0^{b} ±5.0	70.0^{a} ±10.5	15.0^{bc} ±8.2	0.0^{b} ±0.0
				3.00 – 3.99	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	20.0^{ab} ±9.2	55.0^{ab} ±11.4	25.0^{ab} ±9.9
				4.00 – 4.99	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	70.0^{a} ±10.5	30.0^{ab} ±10.5
				5.00 – 5.99	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	50.0^{ab} ±8.0	50.0^{a} ±8.0
Dioecious	Neutral-Day	♀ (STS)	Finola	1.00 – 1.99	100.0^{a} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0
				2.00 – 2.99	66.7^{b} ±11.4	33.3^{a} ±11.4	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0
				3.00 – 3.99	0.0^{c} ±0.0	16.7^{ab} ±9.0	61.1^{a} ±11.8	22.2^{ab} ±10.1	0.0^{b} ±0.0	0.0^{b} ±0.0
				4.00 – 4.99	0.0^{c} ±0.0	0.0^{b} ±0.0	26.3^{ab} ±10.4	68.4^{a} ±11.0	5.3^{b} ±5.3	0.0^{b} ±0.0
				5.00 – 5.99	0.0^{c} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	8.7^{b} ±6.0	39.1^{a} ±10.4	52.2^{a} ±10.6
Dioecious	Short-Day	♂	USO31	1.00 – 1.99	100.0^{a} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{a} ±0.0
				2.00 – 2.99	83.3^{a} ±9.0	16.7^{b} ±9.0	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{a} ±0.0
				3.00 – 3.99	10.5^{b} ±7.2	52.6^{a} ±11.8	31.6^{a} ±11.0	5.3^{b} ±5.3	0.0^{b} ±0.0	0.0^{a} ±0.0
				4.00 – 4.99	0.0^{b} ±0.0	5.3^{b} ±5.3	26.3^{ab} ±10.4	68.4^{a} ±11.0	0.0^{b} ±0.0	0.0^{a} ±0.0
				5.00 – 5.99	0.0^{b} ±0.0	0.0^{b} ±0.0	5.6^{ab} ±5.6	88.9^{a} ±7.6	5.6^{b} ±5.6	0.0^{a} ±0.0
				6.00 – 6.99	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	44.4^{ab} ±12.0	44.4^{a} ±12.0	11.1^{a} ±7.6
Dioecious	Short-Day	♀ (STS)	USO31	1.00 – 1.99	100.0^{a} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0
				2.00 – 2.99	75.0^{b} ±9.9	25.0^{ab} ±9.9	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0
				3.00 – 3.99	0.0^{c} ±0.0	31.6^{a} ±11.0	52.6^{a} ±11.8	15.8^{b} ±8.6	0.0^{b} ±0.0	0.0^{b} ±0.0
				4.00 – 4.99	0.0^{c} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	95.2^{a} ±4.8	4.8^{b} ±4.8	0.0^{b} ±0.0
				5.00 – 5.99	0.0^{c} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	16.0^{b} ±7.5	60.0^{a} ±10.0	24.0^{a} ±8.7
Monoecious	Short-Day	♂ + ♀	USO31	1.00 – 1.99	100.0^{a} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	0.0^{c} ±0.0	0.0^{c} ±0.0	0.0^{c} ±0.0
				2.00 – 2.99	75.9^{b} ±8.1	13.8^{b} ±6.5	10.3^{b} ±5.8	0.0^{c} ±0.0	0.0^{c} ±0.0	0.0^{c} ±0.0
				3.00 – 3.99	3.3^{c} ±3.3	33.3^{a} ±8.7	33.3^{a} ±8.7	30.0^{ab} ±8.5	0.0^{c} ±0.0	0.0^{c} ±0.0
				4.00 – 4.99	0.0^{c} ±0.0	0.0^{b} ±0.0	20.0^{ab} ±7.4	46.7^{a} ±9.3	20.0^{b} ±7.4	13.3^{bc} ±6.3
				5.00 – 5.99	0.0^{c} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	40.0^{a} ±10.0	36.0^{ab} ±9.8	24.0^{ab} ±8.7
				6.00 – 6.99	0.0^{c} ±0.0	0.0^{b} ±0.0	0.0^{b} ±0.0	11.1^{bc} ±7.6	50.0^{a} ±12.1	38.9^{a} ±11.8

The mean of each developmental stage is expressed as a percentage (±SE) relative to the total sample size of the interval.
Stages corresponding to microspore mother cell, meiocyte with two nuclei and meiocyte with four nuclei are merged together in a single meiotic stage of development.
Different letters among bud sizes in each genotype indicate significant differences between them ($p<0.05$) according to non-parametric Kruskal-Wallis and pairwise Nemenyi tests.
Abbreviations → TET: tetrad; **YM-MM:** young and mid microspore; **VM:** vacuolate microspore; **YBP:** young bi-cellular pollen; **MBP:** mid bi-cellular pollen.
♂, ♀, or ♂ + ♀ indicate male, female or both flowers present in the plant.

On the other hand, concerning short-day phenotypes, androecious USO31 plants had a significantly higher quantity of vacuolate microspores (88.89%) coexisting with young bi-cellular pollen grains (5.56%) in male buds fluctuating from 5.00 to 5.99 mm (Table 2), while the best bud length interval for gynoecious USO31 plants was 4.00 to 4.99 mm, which showed significant differences in comparison with the rest of bud length intervals evaluated and contained 95.24% of vacuolate microspores together with 4.76% of young bi-cellular pollen grains (Table 2). Regarding monoecious USO31 plants, the highest percentage of vacuolate microspores (46.67%) was found in buds ranging from 4.00 to 4.99 mm, which also contained microspores in young and mid microspore stage (20.00%), and pollen in young bi-cellular (20.00%) and mid bi-cellular pollen stage (13.33%) (Table 2). It is worth noting that the bud length interval from monoecious plants with more vacuolate microspores, showed the lowest percentage in comparison with the bud length intervals from the rest of phenotypes evaluated which contained the highest percentage of vacuolate microspores.

4.7. Effect of cold-shock bud pretreatment on microspore viability, amyloplast content, and development of multicellular structures of androgenic origin

After merging together data from all evaluated phenotypes, it was found that the cold-shock applied on buds during one week resulted in a statistically significant reduction of microspore viability, which fell from 62.13% of viable microspores and pollen grains under *in vivo* conditions to 46.89% of viability rate for cold treated buds (Table 3). Despite of this, some of the evaluated phenotypes did not show a significant reduction of microspore viability, as is the case of androecious and gynoecious plants from Finola (Table 3). On the other hand, amyloplast content of vacuolate microspores and young bi-cellular pollen grains did not vary after exposure of male buds to a week-long cold-shock at 4 ºC ± 1 ºC, showing the same starch distribution pattern as observed under *in vivo* conditions.

Table 3: Effect of a week-long cold pretreatment (4 ºC ± 1 ºC) applied directly on excised buds from *C. sativa* prior to microspore *in vitro* culture. Mean of microspore viability rates is expressed as a percentage (±SE) calculated from data coming from at least three replicates.

Treatment	All varieties	♂ Finola	♀ (STS) Finola	♂ USO31	♀ (STS) USO31	♂ + ♀ USO31
Flower buds (*in vivo*)	62.1[a] ±2.1	53.7[a] ±4.6	59.7[a] ±4.1	70.9[a] ±4.3	60.5[a] ±1.4	65.9[a] ±3.6
Flower buds at 4 ºC (7 days)	46.9[b] ±2.5	45.6[a] ±3.0	50.3[a] ±6.4	42.4[b] ±9.0	52.3[a] ±5.2	43.8[b] ±5.3

Different letters among treatments indicate significant differences between them ($p<0.05$) according to parametric ANOVA and pairwise Fisher's least significant difference (LSD) tests.
♂: "Only male flowers present in the plant".
♀: "Only female flowers present in the plant".
♂ + ♀: "Male and female flowers present in the plant".

Finally, different cannabis microspore developmental pathways were observed under *in vitro* conditions. Some of the microspores and pollen grains coming from both non-pretreated and cold-pretreated buds were already dead when starting *in vitro* culture (arrows in Fig. 7A), as shown by the lack of enzymatic activity and/or cell-membrane integrity and its consequent absence of fluorescence (Fig. 7A'). It is worth mentioning that FDA vital staining did not penetrate into vacuoles, retaining its fluorescent product in the cytoplasm of viable microspores and pollen grains. Other pollen grains followed their gametophytic developmental pathway with the consequent germination of the pollinic tube (Fig. 7B), through which spermatids circulated behind the vegetative nucleus (Fig. 7B') trying to carry out fertilization of endosperm and egg cell. On the other hand, and although with an extremely low frequency, it should be noted how embryogenic microspores were also observed, but only after cold pretreatment of flower buds. As a consequence of cold-shock bud pretreatment prior to *in vitro* culture, some microspores from androecious and gynoecious USO31 plants deviated from their gametophytic pathway towards a sporophytic development. The first signal of microspore reprogramming was observed just after cold-shock bud pretreatment, which resulted in a striking nuclear disturbance. Instead of generating a vegetative nucleus and a more condensed generative nucleus as a result of first pollen mitosis, a sporophytic microspore from a gynoecious USO31 plant showed two nuclei similar in size and degree of chromatin condensation (Figs. 7C and 7C').

Another multicellular microspore from this replicate containing up to 5 nuclei was also observed (Figs. 7D and 7D'), while 3 weeks after *in vitro* culture, an embryogenic pollen grain from an androecious USO31 plant containing 7≈8 nuclei was found (Figs. 7E and 7E'). In this last case, among the different nuclei observed, one of them seemed to be a generative nucleus. While previous described events constituted the first stages of *Cannabis* microspore embryogenesis, 9 weeks after *in vitro* culture a microspore-derived embryo (Fig. 7F) from the previously mentioned androecious USO31 replicate was identified. Specifically, a heart-shaped embryo which suffered a secondary-embryogenesis event that promoted development of a globular embryo on it was visualized (arrow in Fig. 7F). The embryogenic structure developed protoderm (arrow in Fig. 7F') and showed a well-organized and regular cellular structure (inset in Fig. 7F'). During its development, this embryogenic structure showed oxidation symptoms, as reflected by browning, stopping its development from the moment of its visualization onwards. In summary, both gynoecious and androecious USO31 phenotypes produced 1.11 microspore-derived embryoids per 100 anthers (each phenotype produced two microspore-derived multicellular structures from 180 anthers squashed to perform the microspore culture).

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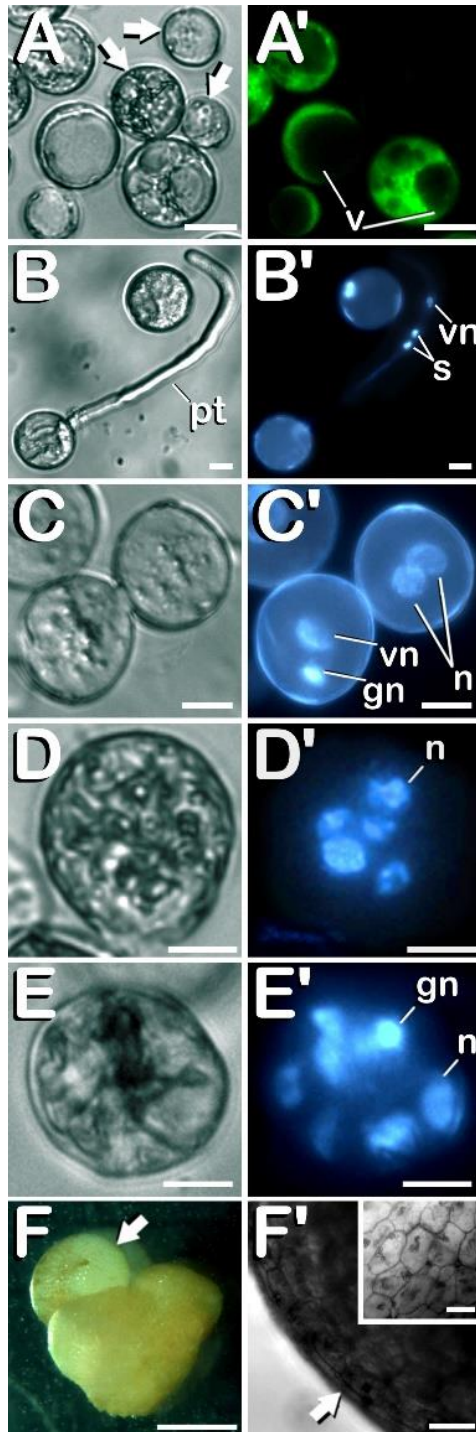
Figure 7: Developmental pathways of *C. sativa* microspores and pollen grains cultured under *in vitro* conditions: (A, A') An enriched population of vacuolate microspores and young bi-cellular pollen grains just after *in vitro* culture stablishment: Arrows point to dead microspores, which does not exhibit any fluorescence. (B, B') Gametophytic pathway developed by a pollen grain, with pollinic tube emission. (C, C') Gametophytic and sporophytic pathways developed after a week-long cold-shock bud pretreatment. (D, D') Microspore under sporophytic development showing five nuclei after cold-shock bud pretreatment. (E, E') Pollen-derived embryogenic structure generated after cold-shock bud pretreatment and three weeks of *in vitro* culture. (F) First *C. sativa* microspore-derived embryos: Secondary embryogenesis event consisting in the development of a globular embryo on a heart-shaped microspore-derived embryo: arrow points to the globular embryo. (F') Protoderm development (arrow) in the embryogenic structure, which showed a well-organized and regular cellular structure (inset in F'). (A–E) Differential Interference Contrast (DIC) microscope images. (A') Fluorescent microscope image after FDA staining. (B'–E') Fluorescent microscope images after DAPI staining. (F) Stereomicroscope image. (F') Bright-field microscope image. Scale bars (A–E) and (A'–E'): 10 µm. Scale bar (F): 1 mm. Scale bars (F') and (inset in F'): 50 µm. **Abbreviations** → **v**: vacuoles; **pt**: pollinic tube; **vn**: vegetative nucleus; **s**: spermatids; **gn**: generative nucleus; **n**: sporophytic nucleus.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5. Discussion

5.1. *Cannabis sativa* is a suitable candidate for androgenesis experiments mainly due to its fast production of viable and potentially inducible microspores and pollen grains

Even though the various phenotypes evaluated showed different sexual systems (dioecious and monoecious), photoperiodism (short and neutral-day) and sex (male and female), direct exposition of seedlings to a photoperiod constituted by 12 hours of light per day resulted in male bud availability in all evaluated phenotypes just 30 days after seed germination. As observed in other *C. sativa* works, male plants were taller than female individuals (Onofri and Mandolino, 2017). The great amount of microspores and pollen grains produced in each flower bud (>150,000), together with the high rates of microspore viability reached for all phenotypes (>50%), makes *C. sativa* a suitable system able to provide, in a very quick period of time (≈30 days after seed germination), huge populations of viable vacuolate microspores and young bi-cellular pollen grains potentially inducible to afford microspore and pollen embryogenesis experiments. Viability rates obtained in our research are similar to the ones reported by Choudhary *et al*. (2014) which, depending on the season, oscillated among 33.3% and 83.0%, while in another work (Zottini *et al*., 1997) a viability rate for mature pollen grains of 92% was reported. This difference among viability rates could be attributable to the microspore isolation procedure, as viability measurements in the former works were performed on mature pollen grains naturally released after anthesis. Regarding gynoecious phenotypes, undoubtedly the most interesting of the species due to their exclusive capability for secondary metabolite production (Small, 2017), it is worth noting how sexual reversion did not influence microspore viability, as compared to androecious and monoecious specimens. This particularity allows to assay microspore embryogenesis protocols with gynoecious plants, which could lead to produce the first cannabis female pure lines 100%

homozygous in only one *in vitro* generation. These double haploids could be used in breeding programs for the development of authentic high-yielding female hybrids, whose biochemical profile could be reliably reproduced through seed. Moreover, taking into account the already known XX female sexual chromosomal inheritance of the species (Hirata, 1927; Faux *et al*., 2014), and how promotion of male flowers on cannabis female plants can be routinely achieved through STS treatment (Ram and Sett, 1982), it would also be possible to self-pollinate the double haploids obtained, thus keeping these genotypes through seed and avoiding the perpetual maintenance of mother plants through vegetative propagation.

5.2. Anthers from *C. sativa* present a high degree of uniformity in the developmental stages of microspores and pollen grains contained

Despite of their biological differences, none of evaluated phenotypes showed significant differences between them throughout the different developmental stages emerged during microsporogenesis and microgametogenesis. Additionally, a high uniformity grade was observed both in the developmental stage of microspores and pollen grains contained in anthers, as well as among anthers coming from the same bud, which presented microspores and pollen grains in the same stage of development. In all phenotypes, MMCs entered in meiosis simultaneously. This synchronized development could be attributable to the presence of cytoplasmic connections between meiocytes, as it has been described in *C. sativa* (Heslop-Harrison, 1966; Mascarenhas, 1975). On the other hand, the present work also certifies the cytokinesis by furrowing already described in this species (Reed, 1914; McPhee, 1924), evidenced by the coexistence of different nuclei in the same cytoplasm after meiosis I and meiosis II, and the polygonal shape of the microspores enclosed by the callosic layer in tetrad stage. Size of mature pollen grains observed in this study was similar to the data published in other works (Punt and Malotaux, 1984; French and Moore, 1986; Shinwari *et al*., 2015; Halbritter, 2016), which indicates that the conditions tested in our experiments did not affect the size of mature pollen

grains. With respect to the second pollen mitosis developed in this species, and in contrast with results published in other *C. sativa* related works (Asanova, 2002), this study demonstrates that it can occur before germination of the pollen tube, as it has been reported in other species such as *Arabidopsis thaliana* L. Heynh. or *Zea mays* L. (Ma, 2005).

Regarding identification of vacuolate microspores and young bi-cellular pollen grain stages of development in *C. sativa*, it should be noted that fluorescence microscopy has been shown to be the most effective technique to discriminate among the different microspore and pollen grain stages of development in this species. This technique allowed a detailed study of nuclear dynamics emerged during the whole process of pollen grain formation, which finally represent the most reliable and easily identifiable events associated with the different developmental stages studied.

5.3. The exine ornamentation of *C. sativa* microspores and pollen grains presents a scabrate sculpture which is almost completely covered by sporopollenin at maturity

After degradation of the callose wall, young and mid microspores appeared spherical and showed their scabrate sculpture, which was slightly modified through progressive addition of sporopollenin on its surface until maturity. It may be noted how sporopollenin deposition and its role in exine pattern and pollen wall formation is preserved across taxa (Wiermann and Gubatz, 1992; Ariizumi and Toriyama, 2011; Borg and Twell, 2011; García *et al.*, 2017). As protrusions observed during microspore development did not stick out more than a micron from the pollen surface in any of its developmental stages, its exine sculpture perfectly fits the scabrate pattern described by Laín (2004). Thus, although our results are in contrast with those reported by Halbritter (2016), who defined the exine of cannabis mature pollen grain as granulate, previously published works are in line with our findings (Bradley, 1958; Punt and Malotaux, 1984; Shinwari *et al.*, 2015). Despite the robust exine exhibited by microspores and pollen grains of *C.*

sativa, it could be traversed by microchannels that, together with apertures, could act as routes for nutrient uptake from loculus into pollen cytoplasm, as has been described in other species like *Olea europaea* L. (Fernández and García, 1990), *Betula verrucosa* Ehrh. and *Chenopodium album* Bosc ex Moq. (Rowley *et al*., 1987), or *Lopezia*, *Gaura*, and *Gelsemium* (Rowley *et al.*, 2003). The fact that microspores and pollen grains of *C. sativa* are surrounded by locular fluid for nutrient supply during great part of their development, coupled with the possible existence of these microchannels distributed in the exine, could also explain the high homogeneity observed in the stage of development of microspores and pollen grains contained in the microsporangium. The fact that desynchronization in development was only detected in mature pollen stage, when mid bi-cellular and mature tri-cellular pollen grains coexisted in the microsporangium (which lacked locular fluid), could confirm this hypothesis.

5.4. The anther wall of *C. sativa* is composed by an external epidermis, endothecium, two middle layers and a secretory type of tapetum

Anther wall composition of *C. sativa* corresponds with the typical pattern commonly described in angiosperms (Clément and Pacini, 2001). In addition, Reed (1914) described the same wall layers in his observations concerning cannabis stamen formation. However, some discordances among results reported by Reed (1914) and our results were detected. Specifically, while in the former work it is described how inner middle layer had already disappeared in tetrad stage, and how only epidermis and endothecium persisted after locule dehydration, we observed remnants of the inner middle layer in tetrad stage and how rests of the outer middle layer were present in mature pollen stage of development. It is necessary to emphasize that in our study, anther dehydration took place at mature pollen stage, in which pollen grains were ready for their dispersal. In this respect, regarding locular fluid desiccation and depending on the species, it could be performed by passive transpiration through anther wall tissues, by reabsorption through stamen filament, or both, being greatly influenced by

environmental factors (Pacini, 1994; Keijzer, 1999). It can be concluded that drastic reduction of the anther wall during pollen grain formation and maturation, together with anther dehydration, facilitates longitudinal dehiscence of the anther and subsequent pollen dispersal through the wind, main vector responsible for pollination in an anemophilous species such as *C. sativa*.

On the other side, it is important to highlight how tapetal cells must be considered essential for the reproductive status of angiosperms, being directly involved in crucial events such as callase supply, which digests the callose wall that encloses microspores in tetrad stage (García *et al*., 2017), nutrition of the microspores and pollen grains developed in the locule, and formation of the exine of mature pollen grains (Heslop-Harrison, 1962; Clément and Pacini, 2001; García *et al*., 2017). In our work, we observed small spheres embedded in the tapetum cell layer from tetrad until young and mid microspore stages of development. With regards to the function of these spheres, which have been previously described in *C. sativa*, some researchers argued that these orbicules could be derived from mitochondria (Heslop-Harrison, 1962), and that during tapetum cell degeneration, they could transport sporopollenin to the microspore exine, a complex of fatty acid derivatives and phenylpropanoids that form an extremely inert biopolymer in the exine to resist physical, biological and chemical attacks (Wang *et al*., 2003; Ariizumi and Toriyama, 2011). Huysmans *et al*. (1998) stated that, although there are some exceptions, presence of orbicules in the tapetal cells can be considered a general feature of a secretory type of tapetum, classifying these structures as Ubisch bodies. Moreover, in the same study, the author categorized the tapetum cell layer as secretory type when it remains *in situ* until its degeneration. With respect to their degeneration, it is well known how tapetal cells of secretory type degenerate by programmed cell death, process generally completed around the first microspore haploid mitosis (Ma, 2005; García *et al*., 2017), as it was

found in the studied phenotypes. Thus, findings from our research support the idea of a secretory type of tapetum in *C. sativa*.

5.5. Starch content of microspores and pollen grains from *C. sativa* is coincident with the amyloplast pattern observed in species recalcitrant to androgenesis

It was found that microspores and pollen grains from *C. sativa* contained amyloplasts from tetrad stage until mature pollen stage. During maturation, microspores and pollen grains experimented a progressive starch accumulation (especially after first pollen mitosis), along with the emptying of the starch reserves from the anther wall. Some authors have suggested how locule external surrounding layers (in particular their plastids) are mainly involved in sugar physiology, storing carbohydrates in form of starch grains (Clément and Audran, 1999; Clément and Pacini, 2001). It is necessary to highlight how our observations strongly suggest that carbohydrates reserves could be transported from microsporangium external surrounding layers into the locule for microspore absorption, as has already been described by other researchers (Bhandari, 1984; Keijzer and Willemse, 1988; Heberle-Bors, 1989; Clément and Pacini, 2001). On the other hand, total absence of amyloplast-staining in tapetum cells during microsporogenesis and microgametogenesis could imply that tapetal cells did not synthetize neither accumulate starch. Instead, other authors have reported that, in anemophilous species, tapetal plastids evolve into elaoiplasts, being responsible for lipid synthesis, accumulation and subsequent secretion into the locule (Clément and Pacini, 2001).

With regards to amyloplast coloration after iodine-starch complex staining, it has been reported how binding of iodine with amylose leads to the formation of deep-blue complexes, while binding with amylopectin results in a reddish-brown color formation, and how when hydrolyzed, both polysaccharides, as far as their chain length is reduced, gradually lose the capacity to be stained with iodine (Bates *et al*., 1943; Bailey *et al*., 1961). The

physical properties of starch, which is mainly conformed by two types of molecules namely amylose and amylopectin, could explain the different colors showed by amyloplasts in our experiments.

Finally, the pattern of plastids contained in microspores and pollen grains from *C. sativa* could help to estimate the androgenic potential of this species. Our observations fit perfectly with the description reported by Sangwan and Sangwan-Norreel (1987) for recalcitrant or nonandrogenic species. As it can be concluded from our results, presence of amyloplasts from tetrad until mature pollen stage of development, along with the strong increase of starch biosynthesis in young bi-cellular pollen stage, points to a high level of recalcitrance of *C. sativa* to androgenic induction.

5.6. The bud length can be used as a floral morphological marker to identify male floral buds containing specific microgametophyte developmental stages

With respect to male bud maturity rate and the slight discrepancies observed among androecious plants from Finola and the rest of evaluated phenotypes, it should be noted how differences observed could be attributable to the fact that neutral-day Finola presented the early-blooming character typical from ssp. *ruderalis* (Callaway and Laakkonen, 1996). As *C. sativa* plants belonging to ssp. ruderalis do not depend on a decrease of light hours in order to start flowering, they have a shorter life cycle than short-day varieties. Particularly, male plants show a faster development which drives them to enter sooner in senescence, which would force microspores to mature sooner to be able to carry out fertilization and continue with species evolution.

On the other hand, since stage of development of microspores and pollen grains is crucial for microspore embryogenesis (Ferrie *et al*., 1995; Dunwell, 2010; Canonge *et al.*, 2020), establishment of the correlation between a simple, accurate and reproducible floral morphological marker

and the different microgametophyte stages of development, must be considered as the first approach in order to study androgenesis induction in a species whose microspores have never been subjected to experimental embryogenesis, as is the case of *C. sativa*, thus increasing the population of specific developmental stages available for the induction. This approach has been successfully applied in model species in which a reliable and highly effective androgenesis induction protocol has been established like *Nicotiana tabacum* L. (Kasperbauer and Wilson, 1979) or *Oryza sativa* L. (Mishra and Rao, 2016). With this aim, this study was focused on finding bud length intervals that contained exclusively the commonly considered as suitable for androgenesis induction vacuolate microspores and young bi-cellular pollen grains. Additionally, an attempt was made to identify the bud length interval where young bi-cellular pollen stage started to appear. In this way, more vacuolate microspores close to go through first pollen mitosis can be present in the interval. If it was not possible and not only vacuolate microspores and young bi-cellular pollen grains were present in the interval, we recommend to choose the bud length interval with these developmental stages coexisting with earlier stages of development, because considering that microspores follow their development during *in vitro* culture, more microspores potentially inducible could be present in the buds. However, it should be noted that in our study, buds with the same length and coming from the same plant, do not necessarily contained microspores and pollen grains in the same developmental stages, as it occurred with buds of the same length belonging to different plants of the same variety. Thus, prior to routinely implementation, it is recommended to adapt this approach to each of the phenotypes with which experiments are going to be developed. Finally, due the absence of studies concerning microspore and pollen embryogenesis in *C. sativa*, we strongly encourage to study and evaluate the androgenic competence of all the microspore and pollen grain stages of development before consider that any of them is the most suitable for androgenesis induction in this species.

In light of the results obtained in this work, bud length is presented as a simple, accurate and reproducible floral morphological marker correlated with the stage of development of the microspores and pollen grains contained, although this method needs to be adjusted for each variety studied to get reliable and reproducible results. Through this methodology, it is possible to avoid additional work like the dissection of buds and the staining of the microspores and pollen grains with DAPI prior to *in vitro* culture for identifying their developmental stage, thus significantly increasing the number of potentially inducible microspores and pollen grains available for androgenesis induction experiments.

5.7. Although with an extremely low frequency, cold-shock bud pretreatment can promote microspore embryogenesis in *C. sativa*

Our research sheds light on the different developmental pathways of *C. sativa* microspores and pollen grains cultured under *in vitro* conditions, and how, although with an extremely low frequency, cold-shock pretreatment applied on buds can deviate the naturally occurring gametophytic pathway towards an embryogenic development. As a result of a week-long cold pretreatment applied directly on excised buds prior to microspore culture, a stress-derived slight decrease in microspore viability was detected. Our findings are in agreement with results published by Choudhary *et al*. (2014), who studied how pollen viability was significantly influenced by seasonal fluctuations in temperature and humidity. Researchers reported a significant decrease of pollen viability at low temperatures, reaching the lowest viability rates in the winter season. Interestingly, in our work, none of the Finola phenotypes evaluated (neither androecious nor gynoecious plants) showed a significant reduction of microspore viability rate after cold-shock bud pretreatment. This could be explained by the fact that Finola is an early-blooming and frost tolerant hybrid developed in Finland (where genetic-selection work was conducted) and derived from Vavilov Research Institute (VIR) accessions descended from *C. sativa* ssp. *ruderalis*, which is thought native to the Altai region of

Siberia (Callaway and Laakkonen, 1996). Furthermore, and although cold treatment has been found to partially or completely inhibit the formation of starch grains in *Datura* proplastids and in pollen from *Hordeum vulgare* L. (Sangwan and Sangwan-Norreel, 1987), this was not observed in vacuolate microspores and young bi-cellular pollen grains coming from a week-long cold pretreated *C. sativa* buds, whose starch distribution pattern was similar to the one observed under *in vivo* conditions.

On the other hand, our research constitutes the first illustration of the early events of microspore embryogenesis ontogeny in *C. sativa*. It is worth noting how analysis of nuclear dynamics through fluorescence microscopy proved to be the best option for unequivocal differentiation among embryogenic and gametophytic development of microspores, being also a useful tool for avoiding wrong diagnoses of different structures which could be incorrectly classified as embryos and/or multicellular structures of microspore origin (Bal *et al*., 2012). This represents a common problem in microspore embryogenesis research, specially with new species which have never been submitted to androgenesis induction experiments. First trace of microspore reprogramming was observed just after the week-long cold-shock bud pretreatment, consisting in the presence of two nuclei with a similar size and low-condensed chromatin. It has been suggested that first symmetrical division of the microspore leads to microspore embryogenesis in model species such as *Brassica napus* L. (Zaki and Dickinson, 1991) or *Nicotiana tabacum* L. (Nitsch, 1974), and also in recalcitrant species like *Antirrhinum majus* L. (Barinova *et al*., 2004), *Solanum lycopersicum* L. (Bal and Abak, 2005) or *Solanum melongena* L. (Bal *et al*., 2009). Furthermore, two multicellular embryogenic microspores containing several nuclei were also observed, although in one of them, the nuclei coexisted with a generative nucleus, which denotes that in this last case, successive divisions of the vegetative nuclei after first pollen mitosis gave rise to this embryogenic structure. This developmental pathway has already been related to microspore embryogenesis and described in model species such

as *Nicotiana tabacum* (Sunderland and Wicks, 1971), or *Datura metel* L. (Iyer and Raina, 1972), and also in less studied species like *Solanum surattense* Burm. fil. or *Luffa cylindrica* L. Roem. (Sinha *et al*., 1978). In this respect, it is worth noting that development of *C. sativa* pollen under low temperatures derived in nuclear disturbances observed during the course of meiosis, as was previously reported by Medwedewa (1935). Finally, more advanced stages of microspore embryogenesis were also identified in our study. Although it suffered a secondary embryogenesis event, showed oxidation signs and stopped its development, a microspore-derived embryo with a compact and regularly distributed cell structure, together with a well-differentiated protoderm was observed. It should be noted that formation of the protoderm is considered a marker for embryo formation (Yeung *et al.*, 1996; Soriano *et al*., 2013). On the other hand, as it has been observed in our work, and as stated by other researchers (Fan *et al*. 1988; Telmer *et al*. 1995), the majority of sporophytic structures stop growing after a few divisions and die, which could explain why only multinuclear structures, which did not develop further, were observed at low frequency. In this respect it is necessary to emphasize that frequency of embryo induction and plant regeneration, together with secondary embryogenesis of the embryoids, are among some of the obstacles for an efficient implementation of microspore culture (Zhou *et al.*, 2002). Finally, addition of auxins to the culture medium (Nehlin *et al.*, 1995), failure to transfer embryos at the right time to regeneration or germination media (Oleszczuk *et al.*, 2014), or the effect of culture temperature (Huang *et al.*, 1991), could be responsible for secondary embryogenesis and growth arrest observed in our work.

6. Conclusion

We have found that *C. sativa* is an appropriate candidate to be submitted to microspore and pollen embryogenesis experiments. The high amount of viable microspores and pollen grains present in its buds, together with the high uniformity grade observed in both the developmental stage of microspores and pollen grains contained in anthers, as well as among anthers coming from the same bud, and the fact that it was possible to correlate the different stages of development with bud length, represent important advantages that can be exploited by researchers who want to advance in this field. However, we recommend to adjust this method for each variety studied to get reliable and reproducible results. Furthermore, although the starch content of *C. sativa* microspores and pollen grains is coincident with the amyloplast pattern observed in species recalcitrant to androgenesis induction, here we demonstrate that it may be possible to deviate the naturally occurring gametophytic pathway towards an embryogenic development through cold-shock bud pretreatment. Although further studies are needed in order to optimize a reliable and effective protocol, here we lay the foundations for androgenesis research in this species, and propose this technique as a promising alternative for genetic standardization of *C. sativa* traits such as cannabinoid content of female inflorescences, avoiding the intrinsic heterogeneity of the species and setting the standards for the future of industrial and medical cannabis.

7. Statements

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Author contribution statement

A.G.-A., E.G.-F, J.P. and F.J.H. conceived and designed the research. A.G.-A. and E.G.-F. performed the experiments. A.G.-A., E.G.-F. and F.J.H. analyzed the results. A.G.-A. wrote the manuscript. J.P. and F.J.H. reviewed and edited the manuscript. All authors have read and approved the manuscript for publication.

Conflicts of interests

AG-Á was employed by company Ploidy and Genomics Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

8. References

Abel, E. L. 1980. Cannabis in the ancient world. In: *Marihuana*, *the first twelve thousands years*, Boston, MA:Springer, 3-35.

Abrams, D. I. 2018. The therapeutic effects of *Cannabis* and cannabinoids: An update from the National Academies of Sciences, Engineering and Medicine report. *European Journal of Internal Medicine*, 49, 7-11. doi: 10.1016/j.ejim.2018.01.003.

Andre, C. M., Hausman, J. F., and Guerriero, G. 2016. *Cannabis sativa*: the plant of the thousand and one molecules. *Frontiers in Plant Science*, 7, 19. https://doi.org/10.3389/fpls.2016.00019.

Ariizumi, T., and Toriyama, K. 2011. Genetic regulation of sporopollenin synthesis and pollen exine development. *Annual Review of Plant Biology*, 62, 437-460. doi: 10.1146/annurev-arplant-042809-112312.

Asanova, D. K. 2002. Specific features of microsporogenesis in the hemp of the Shu valley. *Biology Bulletin of the Russian Academy of Sciences*, 29(6), 545-550.

Bailey, J. M., and Whelan, W. J. 1961. Physical properties of starch I. Relationship between iodine stain and chain length. *Journal of Biological Chemistry*, 236(4), 969-973.

Bal, U., and Abak, K. 2005. Induction of symmetrical nucleus division and multicellular structures from the isolated microspores of *Lycopersicon esculentum* Mill. *Biotechnology & Biotechnological Equipment*, 19(1), 35-42. doi: 10.1080/13102818.2005.10817151.

Bal, U., Ellialtioglu, S., and Abak, K. 2009. Induction of symmetrical nucleus division and multi-nucleate structures in microspores of eggplant (*Solanum melongena* L.) cultured *in vitro*. *Scientia Agricola*, 66(4), 535-539. doi: 10.1590/s0103-90162009000400016.

Bal, U., Shariatpanahi, M. E., Castro, A. J., Emery, D., Clement, C., Dehestani-Ardakani, M., ... and Touraev, A. 2012. Pseudo-embryogenic structures in anther and isolated microspore cultures *in vitro*: a cautionary guide. *Czech Journal of Genetics and Plant Breeding*, 48(2), 51-60. doi: 10.17221/176/2011-cjgpb.

Bárány, I., González-Melendi, P., Fadón, B., Mitykó, J., Risueño, M. C. and Testillano, P. S. 2005. Microspore-derived embryogenesis in pepper (*Capsicum annuum* L.): subcellular rearrangements through development. *Biology of the Cell*, 97(9), 709-722. doi: 10.1042/bc20040142.

Barinova, I., Clément, C., Martiny, L., Baillieul, F., Soukupova, H., Heberle-Bors, E., and Touraev, A. 2004. Regulation of developmental pathways in cultured microspores of tobacco and snapdragon by medium pH. *Planta*, 219(1), 141-146. doi: 10.1007/s00425-003-1202-5.

Bates, F. L., French, D., and Rundle, R. E. 1943. Amylose and amylopectin content of starches determined by their iodine complex formation1. *Journal of the American Chemical Society*, 65(2), 142-148. doi: 10.1021/ja01242a003.

Bhandari, N. N. 1984. The microsporangium. In: *Embryology of Angiosperms*, 53-121. Springer, Berlin, Heidelberg.

Borg, M., and Twell, D. 2011. Pollen: structure and development. In: *eLS*, 1-11. John Wiley & Sons Ltd., Chichester.

Bouamama-Gzara, B., Zemni, H., Zoghlami, N., Gandoura, S., Mliki, A., Arnold, M., and Ghorbel, A. 2020. Behavior of *Opuntia ficus-indica* (L.) Mill. Heat-Stressed microspores under *in vitro* culture conditions as evidenced by microscopic analysis. *In Vitro Cellular & Developmental Biology-Plant*, 56(1), 122-133. doi: 10.1007/s11627-019-10032-4.

Bradley, D. E. 1958. The study of pollen grain surfaces in the electron microscope. *New Phytologist*, 57(2), 226-229. doi: 10.1111/j.1469-8137.1958.tb05308.x.

Brukhin, V., Hernould, M., Gonzalez, N., Chevalier, C. and Mouras, A. 2003. Flower development schedule in tomato *Lycopersicon esculentum* cv. sweet cherry. *Sexual Plant Reproduction*, 15(6), 311-320. doi: 10.1007/s00497-003-0167-7.

Callaway, J. C., and Laakkonen, T. T. 1996. Cultivation of *Cannabis* oil seed varieties in Finland. *Journal of the International Hemp Association*, 3(1), 32-34.

Canonge, J., Philippot, M., Leblanc, C., Potin, P., and Bodin, M. 2020. Impedance flow cytometry allows the early prediction of embryo yields in wheat (*Triticum aestivum* L.) microspore cultures. *Plant Science*, 300, 110586. doi: 10.1016/j.plantsci.2020.110586.

Choudhary, N., Siddiqui, M. B., Bi, S., and Khatoon, S. 2014. Effect of seasonality and time after anthesis on the viability and longevity of *Cannabis sativa* pollen. *Palynology*, 38(2), 235-241. doi: 10.1080/01916122.2014.892906.

Clément, C. and Audran, J. 1999. Anther carbohydrates during *in vivo* and *in vitro* pollen development. In: *Anther and Pollen*, 69–90. Clément, C., Pacini, E., Audran, J.C. (eds), Springer, Berlin. doi: 10.1007/978-3-642-59985-9_7.

Clément, C., and Pacini, E. 2001. Anther plastids in angiosperms. *The Botanical Review*, 67(1), 54-73. doi: 10.1007/bf02857849.

Crang R., Lyons-Sobaski S., and Wise R. 2018. Flowers and male reproductive structures. In: *Plant Anatomy*, 579-613. Springer, Cham.

Deswal, K. 2018. Progress and opportunities in double haploid production in lentil (*Lens culinaris* Medik.), soybean (*Glycine max* L. Merr.) and chickpea (*Cicer arietinum* L.). *Journal of pharmacognosy and phytochemistry*, 7(3), 3105-3109.

Dunwell, J. M. 2010. Haploids in flowering plants: origins and exploitation. *Plant Biotechnology Journal*, 8(4), 377-424. doi: 10.1111/j.1467-7652.2009.00498.x.

Dwivedi, S. L., Britt, A. B., Tripathi, L., Sharma, S., Upadhyaya, H. D., and Ortiz, R. 2015. Haploids: constraints and opportunities in plant breeding. *Biotechnology Advances*, 33(6), 812-829. doi: 10.1016/j.biotechadv.2015.07.001.

Fan, Z., Armstrong, K. C., and Keller, W. A. 1988. Development of microspores *in vivo* and *in vitro* in *Brassica napus* L. *Protoplasma*, 147(2-3), 191-199.

Faux, A. M., Berhin, A., Dauguet, N., and Bertin, P. 2014. Sex chromosomes and quantitative sex expression in monoecious hemp (*Cannabis sativa* L.). *Euphytica*, 196(2), 183-197. doi: 10.1007/s10681-013-1023-y.

Fernández, M. C., and Garcia, M. R. 1990. Passage of lanthanum through the pollen grain wall of *Olea europaea* L. during development. *Plant Cell Reports*, 8(11), 667-671. doi: 10.1007/bf00269989.

Ferrie, A. M. R., Palmer, C. E., and Keller, W. A. 1995. Haploid embryogenesis. In: *In vitro embryogenesis in plants*, 309-344. Springer Netherlands.

Frankel, R., and Galun, E. 2012. *Pollination mechanisms, reproduction and plant breeding*. Vol. 2. Springer Science & Business Media.

French, C. N., and Moore, P. D. 1986. Deforestation, *Cannabis* cultivation and Schwingmoor formation at Cors Llyn (Llyn Mire), Central Wales. *New Phytologist*, 102(3), 469-482. doi: 10.1111/j.1469-8137.1986.tb00823.x.

García, C. C., Nepi, M., and Pacini, E. 2017. It is a matter of timing: asynchrony during pollen development and its consequences on pollen performance in angiosperms - a review. *Protoplasma*, 254(1), 57-73. doi: 10.1007/s00709-016-0950-6.

Halbritter H. 2016. *Cannabis sativa*. In *PalDat - A palynological database*. https://www.paldat.org/pub/Cannabis_sativa/302398;jsessionid=2E9539531BD84E0BEBB72F3CC4A28AB8; accessed 2020-04-29.

Hammond, C. T., and Mahlberg, P. G. 1973. Morphology of glandular hairs of *Cannabis sativa* from scanning electron microscopy. *American Journal of Botany*, 60(6), 524-528. doi: 10.2307/2441375.

Heberle-Bors, E. 1989. Isolated pollen culture in tobacco: plant reproductive development in a nutshell. *Sexual Plant Reproduction*, 2(1), 1-10. https://doi.org/10.1007/BF00190112.

Heslop-Harrison, J. 1962. Origin of exine. *Nature*, 195(4846), 1069-1071. doi: 10.1038/1951069a0.

Heslop-Harrison, J. 1966. Cytoplasmic connections between angiosperm meiocytes. *Annals of Botany*, 30(118), 221-230. doi: 10.1093/oxfordjournals.aob.a084069.

Heslop-Harrison, J. 1971. The pollen wall: structure and development. In: *Pollen,* 75-98. Butterworth-Heinemann.

Hirata, K. 1927. Sex determination in hemp (*Cannabis sativa* L.). *Genetics*, 19(1), 65-81. doi: 10.1007/bf02983117.

Huang, B., Bird, S., Kemble, R., Simmonds, D., Keller, W., and Miki, B. 1990. Effects of culture density, conditioned medium and feeder cultures on microspore embryogenesis in *Brassica napus* L. cv. Topas. *Plant Cell Reports*, 8(10), 594-597. doi: 10.1007/bf02632058.

Huang, B., Bird, S., Kemble, R., Miki, B., and Keller, W. 1991. Plant regeneration from microspore-derived embryos of *Brassica napus*: Effect of embryo age, culture temperature, osmotic pressure, and abscisic acid. *In Vitro Cellular & Developmental Biology-Plant*, 27(1), 28-31. doi: 10.1007/bf00270061.

Huysmans, S., El-Ghazaly, G., and Smets, E. 1998. Orbicules in angiosperms: morphology, function, distribution, and relation with tapetum types. *The Botanical Review*, 64(3), 240-272. doi: 10.1007/bf02856566.

Iyer, R. D., and Raina, S. K. 1972. The early ontogeny of embryoids and callus from pollen and subsequent organogenesis in anther cultures of *Datura metel* and rice. *Planta*, 104(2), 146-156. doi: 10.1007/bf00386991.

Kapuscinski, J. 1995. DAPI: a DNA-specific fluorescent probe. *Biotechnic & Histochemistry*, 70(5), 220-233. doi: 10.3109/10520299509108199.

Karnovsky, M. J. 1967. The ultrastructural basis of capillary permeability studied with peroxidase as a tracer. *The Journal of Cell Biology*, 35(1), 213-236. doi: 10.1083/jcb.35.1.213.

Kasperbauer, M. J. and Wilson, H. M. 1979. Haploid plant production and use. In: *Nicotiana Procedures for Experimental Use*. R. D. Durbin, ed., U.S. Dep. Agric. Tech. Bull, 1586.

Keijzer, C. J. 1999. Mechanisms of angiosperm anther dehiscence, a historical review. In: *Anther and Pollen*, 55-67. Springer, Berlin, Heidelberg.

Keijzer, C. J. and Willemse, M. T. M. (1988). Tissue interactions in the developing locule of *Gasteria verrucosa* during microgametogenesis. *Plant Biology*, 37(4), 475-491. doi: 10.1111/j.1438-8677.1988.tb02155.x.

Kim, M., Kim, J., Yoon, M., Choi, D. I. and Lee, K. M. 2004. Origin of multicellular pollen and pollen embryos in cultured anthers of pepper (*Capsicum annuum*). *Plant Cell, Tissue and Organ Culture*, 77(1), 63-72. doi: 10.1023/b:ticu.0000016506.02796.6ª.

Laín, C. S. 2004. Glosario de términos palinológicos. *Lazaroa*, 25, 93-112.

Livingston, S. J., Quilichini, T. D., Booth, J. K., Wong, D. C., Rensing, K. H., Laflamme-Yonkman, J., ... and Samuels, A. L. 2020. *Cannabis* glandular trichomes alter morphology and metabolite content during flower maturation. *The Plant Journal*, 101(1), 37-56. doi: 10.1111/tpj.14516.

Ma, H. 2005. Molecular genetic analyses of microsporogenesis and microgametogenesis in flowering plants. *Annual Review of Plant Biology*, 56, 393-434. doi: 10.1146/annurev.arplant.55.031903.141717.

Maheshwari, S. C., Tyagi, A. K., Malhotra, K., and Sopory, S. K. 1980. Induction of haploidy from pollen grains in angiosperms - the current status. *Theoretical and Applied Genetics*, 58(5), 193-206. doi: 10.1007/bf00288437.

Mandaron, P., Niogret, M. E., Mache, R., and Moneger, F. 1990. *In vitro* protein synthesis in isolated microspores of *Zea mays* at several stages of development. *Theoretical and Applied Genetics*, 80(1), 134-138. doi: 10.1007/bf00224027.

Mandolino, G., Carboni, A., Forapani, S., Faeti, V., and Ranalli, P. 1999. Identification of DNA markers linked to the male sex in dioecious hemp (*Cannabis sativa* L.). *Theoretical and Applied Genetics*, 98(1), 86-92. doi: 10.1007/s001220051043.

Maraschin, S. D. F., De Priester, W., Spaink, H. P., and Wang, M. 2005. Androgenic switch: an example of plant embryogenesis from the male gametophyte perspective. *Journal of Experimental Botany*, 56(417), 1711-1726. doi10.1093/jxb/eri190.

Mascarenhas, J. P. (1975). The biochemistry of angiosperm pollen development. *The Botanical Review*, 41(3), 259-314. doi: 10.1007/bf02860839.

McPhee, H. C. 1924. Meiotic cytokinesis of *Cannabis*. *Botanical Gazette*, 78(3), 335-341. doi: 10.1086/333400.

Medwedewa, G. B. 1935. The climatic influences upon the pollen development of the Italian hemp. *Zeitschrift für Induktive Abstammungs-und Vererbungslehre*, 70(1), 170-176. doi: 10.1007/bf01741646.

Miller, N. G. 1970. The genera of the Cannabaceae in the southeastern United States. *Journal of the Arnold Arboretum*, 51(2), 185-203. doi: 10.5962/bhl.part.7039.

Mishra, R., and Rao, G. J. N. 2016. *In-vitro* androgenesis in rice: advantages, constraints and future prospects. *Rice Science*, 23(2), 57-68. doi: 10.1016/j.rsci.2016.02.001.

Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, 15(3), 473-497. doi: 10.1111/j.1399-3054.1962.tb08052.x.

Murovec, J., and Bohanec, B. 2012. Haploids and doubled haploids in plant breeding. In: *Plant Breeding*, 87-106. InTech Europe.

Nehlin, L., Möllers, C., and Glimelius, K. 1995. Induction of secondary embryogenesis in microspore-derived embryos of *Brassica napus* L. *Plant Science*, 111(2), 219-227. doi: 10.1016/0168-9452(95)04247-r.

Nitsch, C. 1974. La culture de pollen isolé sur milieu synthétique. *Comptes Rendus de l'Académie des Sciences*, 278, 1031-1034.

Nitsch, C. and Norreel, B. 1973a. Effet d'un choc thermique sur le pouvoir embryogène du pollen de *Datura innoxia* cultivé dans l'anthère et isolé de l'anthère. *Comptes Rendus de l'Académie des Sciences*, 276, 303-306.

Nitsch, C., and Norreel, B. 1973b. Factors favoring the formation of androgenetic embryos in anther culture. In: *Genes, Enzymes and Populations*, 2, 129-144. Springer, Boston, MA. doi: 10.1007/978-1-4684-2880-3_10.

Oleszczuk, S., Tyrka, M., and Zimny, J. 2014. The origin of clones among androgenic regenerants of hexaploid triticale. *Euphytica*, 198(3), 325-336. doi: 10.1007/s10681-014-1109-1.

Onofri, C., and Mandolino, G. 2017. Genomics and Molecular Markers in *Cannabis sativa* L. In: *Cannabis sativa L. - Botany and Biotechnology*, 319-342. Springer, Cham.

Pacini, E. 1994. Cell biology of anther and pollen development. In: *Genetic Control of Self-Incompatibility and Reproductive Development in Flowering Plants*, 289-308. Springer, Dordrecht.

Pauls, K. P., Chan, J., Woronuk, G., Schulze, D., and Brazolot, J. 2006. When microspores decide to become embryos - cellular and molecular changes. *Botany*, 84(4), 668-678. doi: 10.1139/b06-064.

Pechan, P. M. and Keller, W. A. 1988. Identification of potentially embryogenic microspores in *Brassica napus*. *Physiologia Plantarum*, 74(2), 377-384. doi: 10.1111/j.1399-3054.1988.tb00646.x.

Pereira, A. M. and Coimbra, S. (2019). Advances in plant reproduction: from gametes to seeds. *Journal of Experimental Botany*, 70(11), 2933-2936. doi: 10.1093/jxb/erz227.

Punt, W., and Malotaux, M. 1984. Cannabaceae, Moraceae and Urticaceae. *Review of Palaeobotany and Palynology*, 42(1-4), 23-44. doi: 10.1016/0034-6667(84)90061-7.

R Core Team. 2019. R: A language and environment for statistical computing. *R Foundation for Statistical Computing*, Vienna, Austria. URL https://www.R-project.org/.

Ram, H. M., and Sett, R. 1982. Induction of fertile male flowers in genetically female *Cannabis sativa* plants by silver nitrate and silver thiosulphate anionic complex. *Theoretical and Applied Genetics*, 62(4), 369-375. doi: 10.1007/bf00275107.

Raman, V., Lata, H., Chandra, S., Khan, I. A., and ElSohly, M. A. 2017. Morpho-anatomy of marijuana (*Cannabis sativa* L.). In: *Cannabis sativa L. - Botany and Biotechnology*, 123-136. Springer, Cham.

Reed, J. 1914. Morphology of *Cannabis sativa* L. Master of Science thesis, State University of Iowa. http://ir.uiowa.edu/etd/3895.

Rowley, J. R., El-Ghazaly, G., and Rowley, J. S. 1987. Microchannels in the pollen grain exine. *Palynology*, 11(1), 1-21. doi: 10.1080/01916122.1987.9989315.

Rowley, J. R., Skvarla, J. J., and El-Ghazaly, G. 2003. Transfer of material through the microspore exine from the loculus into the cytoplasm. *Canadian Journal of Botany*, 81(11), 1070-1082. doi: 10.1139/b03-095.

Sangwan, R. S. and Sangwan-Norreel, B. S. 1987. Ultrastructural cytology of plastids in pollen grains of certain androgenic and nonandrogenic plants. *Protoplasma*, 138(1), 11-22. doi: 10.1007/bf01281180.

Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, 9, 671-675. doi: 10.1038/nmeth.2089.

Shariatpanahi, M. E., Bal, U., Heberle-Bors, E., and Touraev, A. 2006. Stresses applied for the re-programming of plant microspores towards *in vitro* embryogenesis. *Physiologia Plantarum*, 127(4), 519-534. doi: 10.1111/j.1399-3054.2006.00675.x.

Shinwari, Z. K., Tanveer, M., Yusuf, O., Perveen, A. and Khan, M. 2015. Protein estimation and palynological studies of *Cannabis sativa* L. pollen in relation to respiratory allergies. *Pakistan Journal of Botany*, 47(4), 1517-1520.

Sinha, S., Jha, K. K., and Roy, R. P. 1978. Segmentation pattern of pollen in anther culture of *Solanum surattense*, *Luffa cylindrica* and *Luffa echinata*. *Phytomorphology*, 28, 43-49.

Small, E. (2017). Classification of *Cannabis sativa* L. in relation to agricultural, biotechnological, medical and recreational utilization. In: *Cannabis sativa L. - Botany and Biotechnology*, 1-62. Springer, Cham.

Soriano, M., Li, H., and Boutilier, K. 2013. Microspore embryogenesis: establishment of embryo identity and pattern in culture. *Plant Reproduction*, 26(3), 181-196. doi: 10.1007/s00497-013-0226-7.

Spitzer-Rimon, B., Duchin, S., Bernstein, N., and Kamenetsky, R. 2019. Architecture and florogenesis in female *Cannabis sativa* plants. *Frontiers in Plant Science*, 10, 350. doi: 10.3389/fpls.2019.00350.

Sunderland, N., and Wicks, F. M. 1971. Embryoid formation in pollen grains of *Nicotiana tabacum*. *Journal of Experimental Botany*, 22(1), 213-226. doi: 10.1093/jxb/22.1.213.

Sunderland, N., and Wildon, D. C. 1979. A note on the pretreatment of excised flower buds in float culture of *Hyoscyamus* anthers. *Plant Science Letters*, 15(2), 169-175. doi: 10.1016/0304-4211(79)90070-1.

Telmer, C. A., Newcomb, W., and Simmonds, D. H. 1995. Cellular changes during heat shock induction and embryo development of cultured microspores of *Brassica napus* cv. Topas. *Protoplasma*, 185(1-2), 106-112. doi: 10.1007/bf01272758.

Testillano, P. S. 2019. Microspore embryogenesis: targeting the determinant factors of stress-induced cell reprogramming for crop improvement. *Journal of experimental botany*, 70(11), 2965-2978. doi: 10.1093/jxb/ery464.

Toth, J. A., Stack, G. M., Cala, A. R., Carlson, C. H., Wilk, R. L., Crawford, J. L., ... and Smart, L. B. 2020. Development and validation of genetic markers for sex and cannabinoid chemotype in *Cannabis sativa* L. *GCB Bioenergy*, 12(3), 213-222. doi: 10.1111/gcbb.12667.

Touraev, A., Vicente, O., and Heberle-Bors, E. 1997. Initiation of microspore embryogenesis by stress. *Trends in Plant Science*, 2(8), 297-302. doi: 10.1016/s1360-1385(97)89951-7.

United Nations Office on Drugs and Crime. 2009. *Recommended Methods for the Identification and Analysis of Cannabis and Cannabis Products: Manual for Use by National Drug Testing Laboratories*. United Nations Publications.

Urits, I., Borchart, M., Hasegawa, M., Kochanski, J., Orhurhu, V., and Viswanath, O. 2019. An update of current cannabis-based pharmaceuticals in pain medicine. *Pain and Therapy*, 8(1), 41-51. doi: 10.1007/s40122-019-0114-4.

Uskutoğlu, T., Uskutoğlu, D., and Turgut, K. 2019. Effects on pre-treatment and different tissue culture media for androgenesis in *Stevia rebaudiana* Bertoni. *Sugar Tech*, 21(6), 1016-1023. doi: 10.1007/s12355-019-00722-z.

Wang, A., Xia, Q., Xie, W., Datla, R., and Selvaraj, G. 2003. The classical Ubisch bodies carry a sporophytically produced structural protein (RAFTIN) that is essential for pollen development. *Proceedings of the National Academy of Sciences*, 100(24), 14487-14492. doi: 10.1073/pnas.2231254100.

Wiermann, R., and Gubatz, S. 1992. Pollen wall and sporopollenin. In: *International Review of Cytology*, 140, 35-72. Academic Press.

Yeung, E. C., Rahman, M. H., and Thorpe, T. A. 1996. Comparative development of zygotic and microspore-derived embryos in *Brassica napus* L. cv Topas. I. Histodifferentiation. *International Journal of Plant Sciences*, 157(1), 27-39. doi: 10.1086/297317.

Zaki, M. A. M., and Dickinson, H. G. 1991. Microspore-derived embryos in *Brassica*: the significance of division symmetry in pollen mitosis I to embryogenic development. *Sexual Plant Reproduction*, 4(1), 48-55.

Zhao, J., Newcomb, W. and Simmonds, D. 2003. Heat-shock proteins 70 kDa and 19 kDa are not required for induction of embryogenesis of *Brassica napus* L. cv. Topas microspores. *Plant and Cell Physiology*, 44(12), 1417-1421. doi: 10.1093/pcp/pcg162.

Zhou, W. J., Hagberg, P., and Tang, G. X. 2002. Increasing embryogenesis and doubling efficiency by immediate colchicine treatment of isolated microspores in spring *Brassica napus*. *Euphytica*, 128(1), 27-34.

Zhou, Z. and Bartholomew, B. 2003. Cannabaceae. In: *Flora of China*, 5, 74–75.

Zoriniants, S., Tashpulatov, A. S., Heberle-Bors, E., and Touraev, A. 2005. The role of stress in the induction of haploid microspore embryogenesis. In: *Haploids in Crop Improvement II*, 35-52. Springer, Berlin, Heidelberg.

Zottini, M., Mandolino, G., and Ranalli, P. 1997. Effects of γ-ray treatment on *Cannabis sativa* pollen viability. *Plant Cell, Tissue and Organ Culture*, 47(2), 189-194. doi: 10.1007/bf02318957.

Żur, I., Dubas, E., Golemiec, E., Szechyńska-Hebda, M., Janowiak, F., and Wędzony, M. 2008. Stress-induced changes important for effective androgenic induction in isolated microspore culture of triticale (× *Triticosecale* Wittm.). *Plant Cell, Tissue and Organ Culture*, 94(3), 319-328. doi: 10.1007/s11240-008-9360-6.

Chapter 3

A novel and rapid method for *Agrobacterium*-mediated production of stably transformed *Cannabis sativa* L. plants

Alberto Galán-Ávila[a*], Pietro Gramazio[b], Mily Ron[c], Jaime Prohens[d], Francisco Javier Herraiz[d]

PhD candidate contribution

A.G.-A. had a main role in the following activities: conceived and designed the research, performed the experiments, analyzed the results, wrote the manuscript, and reviewed and edited the manuscript.

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[a]Ploidy and Genomics S.L., CEEI VALENCIA, Avda. Benjamin Franklin, nº12, Parc Tecnològic, 46980, Paterna, Valencia, Spain

[b]Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, 305-8572, Tsukuba, Japan

[c] Department of Plant Biology, University of California, Davis, CA, USA

[d]Instituto Universitario de Conservación y Mejora de la Agrodiversidad Valenciana, Universitat Politècnica de València, Camí de Vera s/n, 46022, Valencia, Spain

* Corresponding author: agalan@ploidyandgenomics.com

1. Abstract

The development of genetically transformed plants is an elusive landmark in *Cannabis sativa* L. breeding. Despite its economic interest, at present, protocols for producing transgenic *C. sativa* plants are scarce. We studied the ability of hypocotyl, cotyledon and meristem explants from six *C. sativa* hemp varieties for transgenic plant regeneration. For this, we firstly evaluated *in vitro* regeneration rates of hypocotyls cultured in medium without plant growth regulators, and cotyledons cultured in medium supplemented with 0.4 mg L^{-1} of thidiazuron (TDZ) and 0.2 mg L^{-1} of α-naphthaleneacetic (NAA). Subsequently, the effect of different kanamycin concentrations (50, 100, 200, 500 and 750 mg L^{-1}) on hypocotyl regeneration rate was determined. Finally, we assessed transformation rates after hypocotyl, cotyledon and meristem co-culture with *Agrobacterium tumefaciens* strain LBA4404 carrying the binary plasmid pBIN19 containing the *β-glucuronidase* (*uidA*) reporter gene and the kanamycin resistance *neomycin phosphotransferase* (*nptII*) genes. Plant transformation was validated through *in vitro* culture of regenerating shoots in kanamycin-containing selective regeneration medium, by GUS histochemical assay for *uidA* expression, and by PCR amplification of *uidA* and *nptII* genes. Our results showed that hypocotyls reached a higher regeneration rate (53.3%) than cotyledons (18.1%) without *Agrobacterium* co-culture. On the other hand, 100 mg L^{-1} kanamycin proved to be the best concentration in terms of regeneration rate (63.3%) and spontaneous rooting rate of hypocotyl regenerating shoots (12.2%), which displayed a 7.1% of albinism rate. After co-culture with *A. tumefaciens* and subsequent culture in antibiotic-containing selective regeneration medium, hypocotyl was the best explant type achieving 23.1% of regeneration rate, which contrasts with the 1.0% regeneration rate detected for cotyledons. Transgenic plants were obtained from all explant types evaluated. Although there were significant differences among varieties evaluated, hypocotyls proved to be superior to already-developed meristems, reaching a transformation rate of 5.0% and 0.8%

respectively. Despite the extremely low regeneration rate of cotyledons after *A. tumefaciens* co-culture, all cotyledon-derived regenerating shoots analyzed were successfully transformed. Our hormone-free protocol doubles the transformation rate of regenerating shoots, also producing transgenic plants three times faster than other already published protocols. This has relevant implications for *C. sativa* breeding, enabling not only genetic transformation, but also the use of new plant breeding techniques such as targeted genome editing by using CRISPR/Cas systems. This may foster the development of *C. sativa* varieties with specific biochemical profiles, or tolerant to biotic and abiotic stresses among others.

Keywords: Cotyledon; Genetic transformation; GUS; Hemp; Hypocotyl; Kanamycin; Meristem; *nptII*; PCR; Transgenesis; *uidA*

2. Introduction

Cannabis sativa L. is a dicotyledonous and angiosperm species with multiple uses belonging to the Cannabaceae family. Cannabinoids are responsible for the pharmacological and psychoactive properties of this species. Since Δ^9-tetrahydrocannabinol (THC) was first isolated and characterized by Gaoni and Mechoulam (1964), the therapeutic properties of *C. sativa* have attracted the interest of researchers around the world. Additionally, hemp (*C. sativa* varieties containing less than 0.3% w/w of THC) is cultivated for biomass and fiber that constitute feedstock for industrial uses such as energy, construction and automotive markets, and for hempseeds that are components of functional foods and animal feeds (Żuk-Gołaszewska and Gołaszewski, 2020).

Biotechnological approaches such as genetic transformation could be an important landmark in *C. sativa* breeding, as demonstrated in other plant species through the development of improved varieties resistant to biotic and abiotic stresses, with better nutritional and processing qualities, or with increased yields among others (Gosal and Wani, 2018). However, before the implementation of this technique in *C. sativa* species, it is imperative to develop an efficient transformation protocol that allows the regeneration of transgenic plants.

In this respect, there have been some attempts at transformation of *C. sativa*. Successful *Agrobacterium tumefaciens*-mediated transformation of stem and leaf-derived callus suspension cultures from four hemp varieties expressing phosphomannose-isomerase (PMI) gene has been reported (Feeney and Punja, 2003, 2015). Additionally, there are reports of effective establishment of *Agrobacterium rhizogenes*-transformed hairy root cultures showing *ß-glucuronidase* (GUS) positive staining from three hemp varieties plus two drug-type varieties (Wahby *et al*., 2013, 2017). These latter studies also reported *in vivo* and *in vitro* Ri and Ti plasmid-bearing *Agrobacterium* infection of hypocotyl and cotyledonary node explants. *Agrobacterium*

tumefaciens-mediated transformation of leaf, male and female flowers, stem, and root tissues from eight hemp varieties using vacuum infiltration has also been achieved, being verified through subsequent detection of GUS and green fluorescence protein (GFP) in the transformed tissues (Deguchi *et al.*, 2020). In the former work, also phytoene desaturase (PDS) gene silencing resulting in an albino phenotype in leaves and male and female flowers was carried out. *Agrobacterium tumefaciens*-mediated transformation of *C. sativa* seedlings from three medical cannabis varieties that transiently expressed GUS gene has also been reported (Sorokin *et al.*, 2020). In addition, nanoparticle-based transient gene transformation of trichomes and leaf cells from one hemp variety, in which transcription of soybean genes and localization of fluorescent-tagged transcription factor proteins were detected, has also been achieved (Ahmed *et al.*, 2020). Finally, transient transformation with *A. tumefaciens* and Cotton leaf crumple virus (CLCrV) induced gene silencing of PDS and magnesium chelatase subunit I (ChlI) genes in leaves from one hemp variety (Schachtsiek *et al*., 2019), and GFP-transient expression through polyethylene-glycol (PEG)-mediated protoplast transformation have also been obtained (Beard *et al.*, 2021). However, despite the successful genetic transformation of different non-regenerating explants, *C. sativa* recalcitrance to plant regeneration have prevented the recovery of transgenic plants (Feeney and Punja, 2017; Wróbel *et al.*, 2018). It was only recently that regeneration of one *C. sativa* transformed plant has been reported (Zhang *et al.*, 2021).

In this work, we aimed at the development of a novel protocol for the regeneration of stably transformed *C. sativa* plants. For this, we assessed the feasibility of different explants for the production of *Cannabis* transformed plants. Consequently, we compared their transformation rates after co-cultivation with *Agrobacterium tumefaciens* strain LBA4404 containing binary plasmid pBIN19 carrying the *β-glucuronidase* (*uidA*) reporter gene and the kanamycin resistance *neomycin phosphotransferase* (*nptII*) genes. While the *nptII* gene (Fraley *et al.*, 1983) confers the ability to

proliferate on a medium containing normally inhibitory levels of kanamycin in transformed cells, the *uidA* gene (Jefferson *et al.*, 1987) encodes for the *β-glucuronidase* (GUS) enzyme that is commonly used as a reporter gene in GUS histochemical assay for validating the integration and expression of foreign DNA in the transformed cells. In our work, plant transformation was verified through the growth of regenerating shoots on kanamycin-containing selective regeneration medium, evaluation of the *uidA* gene expression by GUS assay in regenerant-derived tissues, and amplification of *uidA* and *nptII* genes by PCR, which is routinely employed for quick detection of cell transformation in plant tissues (Lassner *et al.*, 1989). Due to the lack of studies concerning plant growth inhibitory effects of kanamycin in *in vitro* culture of *C. sativa*, we also performed a dose-response experiment with hypocotyls.

3. Materials and methods

3.1. Plant material and growth conditions

Seeds from monoecious *C. sativa* short-day varieties 'Ferimon', 'Felina32', 'Fedora17', 'Futura75' and 'USO31', and from dioecious neutral-day variety 'FINOLA' were used in our experiments. Seeds were surface sterilized by manual shaking in 75% (v/v) ethanol for two min and 30 sec, followed by immersion in 30 g L^{-1} of NaClO with 0.1% (v/v) of Tween 20 for 25 min, and finally washed three times with sterile deionized water. Once sterilized, seeds were germinated in 9 cm diameter plastic Petri dishes containing previously autoclaved semi-solid half-strength MS medium (Murashige and Skoog, 1962) with sucrose (Table 1). Explants were dissected from seven-days-old seedlings grown in plastic Petri dishes with semi-solid germination medium (Table 1) under aseptic conditions. In *C. sativa*, this stage of seedling development is equivalent to the phenological growth stage 11 according to the corresponding BBCH-scale (Mishchenko *et al.*, 2017). Seedlings and explants were grown in a climatic chamber with controlled conditions (25±1ºC and 60±5% relative humidity) under a 16 h

light / 8 h dark photoperiod. The light was provided by Light Emitting Diode (LED) tubes of 18W and a color temperature of 6,000K, which provided 6,010 lux and 90.15 µmol m^{-2} s^{-1}. Plants used in this study were grown under license for the cultivation of *C. sativa* for research purposes, issued by the Spanish Ministry of Health via the Spanish Agency of Medicines and Health Products (Agencia Española de Medicamentos y Productos Sanitarios or AEMPS) to Ploidy and Genomics Ltd.

Table 1: Media tested for the *in vitro* culture experiments with hypocotyls and cotyledons, and for *A. tumefaciens* LBA4404 infection and co-culture of hypocotyls, cotyledons and already-developed meristems and subsequent *in vitro* culture in selective regeneration media. Media pH was adjusted to 5.8 in all protocol stages (except liquid LB medium in which pH was adjusted to 7.0). Semi-solid medium was employed for all protocol stages except for *Agrobacterium* inoculation. All semi-solid media were supplemented with 3.5 g L^{-1} Gelrite®. **Abbreviations → MS**: Murashige and Skoog medium; **LB**: Luria-Bertani medium; **RIF**: rifampicin; **KAN**: kanamycin; **CTX**: cefotaxime; **AcS**: acetosyringone; **CAR**: Carbenicillin; **TDZ**: thidiazuron; **NAA**: α-naphthaleneacetic acid.

Explant	**Protocol stage**	**Media composition**
Seed	Seed germination	½ MS + 1.5% (w/v) sucrose
Hypocotyl	Control	½ MS + 1.5% (w/v) sucrose
	Agrobacterium inoculation	25 g L^{-1} LB + 50 mg L^{-1} RIF + 50 mg L^{-1} KAN + 39.2 mg L^{-1} AcS
	Agrobacterium co-culture	½ MS + 1.5% (w/v) sucrose + 39.2 mg L^{-1} AcS
	Selective regeneration	½ MS + 1.5% (w/v) sucrose + 250 mg L^{-1} CTX + 250 mg L^{-1} CAR + 100 mg L^{-1} KAN
Cotyledon	Control	½ MS + 1.5% (w/v) sucrose + 0.4 mg L^{-1} TDZ + 0.2 mg L^{-1} NAA
	Agrobacterium inoculation	25 g L^{-1} LB + 50 mg L^{-1} RIF + 50 mg L^{-1} KAN + 39.2 mg L^{-1} AcS
	Agrobacterium co-culture	½ MS + 1.5% (w/v) sucrose + 0.4 mg L^{-1} TDZ + 0.2 mg L^{-1} NAA + 39.2 mg L^{-1} AcS
	Selective regeneration	½ MS + 1.5% (w/v) sucrose + 0.4 mg L^{-1} TDZ + 0.2 mg L^{-1} NAA + 250 mg L^{-1} CTX + 250 mg L^{-1} CAR + 100 mg L^{-1} KAN
Meristem	*Agrobacterium* inoculation	25 g L^{-1} LB + 50 mg L^{-1} RIF + 50 mg L^{-1} KAN + 39.2 mg L^{-1} AcS
	Agrobacterium co-culture	½ MS + 1.5% (w/v) sucrose + 39.2 mg L^{-1} AcS
	Selective regeneration	½ MS + 1.5% (w/v) sucrose + 250 mg L^{-1} CTX + 250 mg L^{-1} CAR + 100 mg L^{-1} KAN

3.2. Hypocotyl and cotyledon explant *in vitro* culture, and hypocotyl kanamycin dose-response experiments

Cannabis sativa L. plant *in vitro* regeneration efficiency of already published protocols from hypocotyls (Galán-Ávila *et al*., 2020), and cotyledons (Chaohua *et al.*, 2016) was compared. Regarding kanamycin-resistance of *in vitro* hypocotyl-derived regenerating shoots, a dose-response experiment consisting of adding increasing concentrations (50, 100, 200, 500 and 750 mg L^{-1}) of kanamycin to the semi-solid control medium was performed (Table 1). Culture dishes were examined with an Optika® SZN-6 (OPTIKA S.r.l., Ponteranica, Italy) laboratory stereo zoom microscope equipped with an Optika® C-HP (OPTIKA S.r.l.) digital camera. Explants producing shoots and roots, number of shoots developed on each of responding explants and albino regenerating shoots were counted one month after *in vitro* culture initiation. Media employed as the control for hypocotyl and cotyledon *in vitro* culture experiments are described in Table 1.

3.3. Media used, bacterial strain and transformation vector

The Luria-Bertani medium (LB) (Bertani, 1951) was used for all the processes involved in the liquid bacterial culture, always supplemented with the appropriate antibiotic (50 mg L^{-1} of rifampicin and 50 mg L^{-1} of kanamycin). The *Agrobacterium tumefaciens* strain LBA4404 containing the binary plasmid pBIN19 was employed for transformation experiments. The combination strain/plasmid confers the kanamycin resistance for the presence of the *nptII* gene in the plasmid and the rifampicin resistance for the bacterial chromosome. The reporter gene *uidA* present in the pBIN19 binary vector from *A. tumefaciens* strain LBA4404 is interrupted by an intronic sequence to deduce expression only from eukaryotic cells (Bakhsh, 2020).

3.4. *Agrobacterium* tumefaciens co-cultivation of explants and subsequent culture in antibiotic-containing medium

Hypocotyls, cotyledons and the remaining already developed meristems from seven-days-old *C. sativa* seedlings were employed as explants in transformation experiments. The *Agrobacterium* suspension culture was initiated from frozen 25% glycerol stocks on LB medium preserved at -80ºC. The seeding in 100 ml of fresh LB culture medium containing 50 mg L^{-1} of kanamycin and 50 mg L^{-1} of rifampicin was carried out scratching the surface of the frozen stock with an inoculating loop without thawing, and immediately the tube was dipped in liquid nitrogen to avoid damage in the stock. The bacterial culture was grown for about 24 h at 28ºC under orbital agitation (220 rpm). At optical density at 600 nm (OD600) of ≈1, cells were precipitated by centrifuging at 2,236 g for 15 min at RT and resuspended to an OD600 of 0.5 with the sterile *Agrobacterium* inoculation medium (Table 1). Then, the explants were placed in Petri dishes with the prepared medium for about 40 min for static infection. Immediately following the inoculation, the explants were cultured in Petri dishes with semi-solid co-culture medium (Table 1) for 4 days in the growth conditions indicated above. Subsequently, the explants were cultured in the semi-solid selective regeneration medium (Table 1) for approximately 26 days in equal conditions.

Culture dishes were examined with an Optika® SZN-6 laboratory stereo zoom microscope equipped with an Optika® C-HP digital camera. Explants producing shoots and roots, number of shoots developed on each of responding explants and albino regenerating shoots were counted one month after *in vitro* culture initiation. Media used in the different *C. sativa* transformation protocol stages are described in Table 1.

3.5. Visual analysis of GUS expression in hypocotyls, cotyledons and already-developed meristems after regenerant-derived tissue incubation with X-Gluc

Approximately one month after *in vitro* culture of explants, samples (leaves or newly developed meristems) were taken from the top of regenerated plants, incubated with X-Gluc (0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 100 mM $NaPO_4$ pH 7, 10.0 mM EDTA, 0.1% Triton X-100, 1.0 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) at 37ºC for approximately 12 h as described by Jefferson (1987), and decolored through a graded ethanol series in deionized water (75% EtOH for 2 h + 90% EtOH for 2 h + 100% EtOH o/night). High-resolution images of the different events observed were recorded with an Optika® SZN-6 (OPTIKA S.r.l.) laboratory stereo zoom microscope equipped with an Optika® C-HP (OPTIKA S.r.l.) digital camera.

3.6. Primer design and polymerase chain reaction (PCR) detection of *ß*-glucuronidase (*uidA*) and kanamycin resistance neomycin phosphotransferase II (*nptII*) genes

Approximately one month after explant *in vitro* culture initiation, samples (leaves or newly developed meristems) were taken from the top of regenerated plants and genomic DNA was extracted following the SILEX method (Vilanova *et al*., 2020). DNA quality and integrity were checked by agarose gel electrophoresis and Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). Integration of the T-DNA in the plant genome was confirmed by PCR amplifying a 206-bp fragment of the *uidA* reporter gene (Forward primer 5’-CCCATCATGACCTTGCCAAG-3’, Reverse primer 5’-CAGGGCTACAAAATCACGGG-3’) and a 795-bp fragment of the *nptII* gene (Forward primer 5’-ATGATTGAACAAGATGGATTGCAC-3’, Reverse primer 5’-TCAGAAGAACTCGTCAAGAAGGCG-3’). PCR amplifications were performed in a volume of 25 µL including 19 µL water, 2.5 µL 10 × PCR buffer, 1 µL $MgCl_2$

50 mM, 0.5 µL dNTPs 10 mM each, 0.5 µL of each primer 10mM, 0.5 µL Taq DNA Polymerase (1 U/µL), and 0.5 µL DNA template 100 ng/µL. The PCR program used was the following: 94 °C for 5 min for DNA denaturation, 30 cycles of 20 s at 94 °C followed by 30 s at 60 °C (either for *uidA* and *nptII* genes), and of 20 s at 72 °C and finally 72 °C for 1 min for the last step of extension. Only regenerating shoots that concurrently showed a green phenotype after culture on selective regeneration medium, uniform expression (non-chimeric) of the *uidA* gene after X-Gluc regenerant-derived tissue incubation by GUS histochemical assay, plus amplification of the *uidA* and *nptII* genes by PCR, were considered as transformed regenerating shoots.

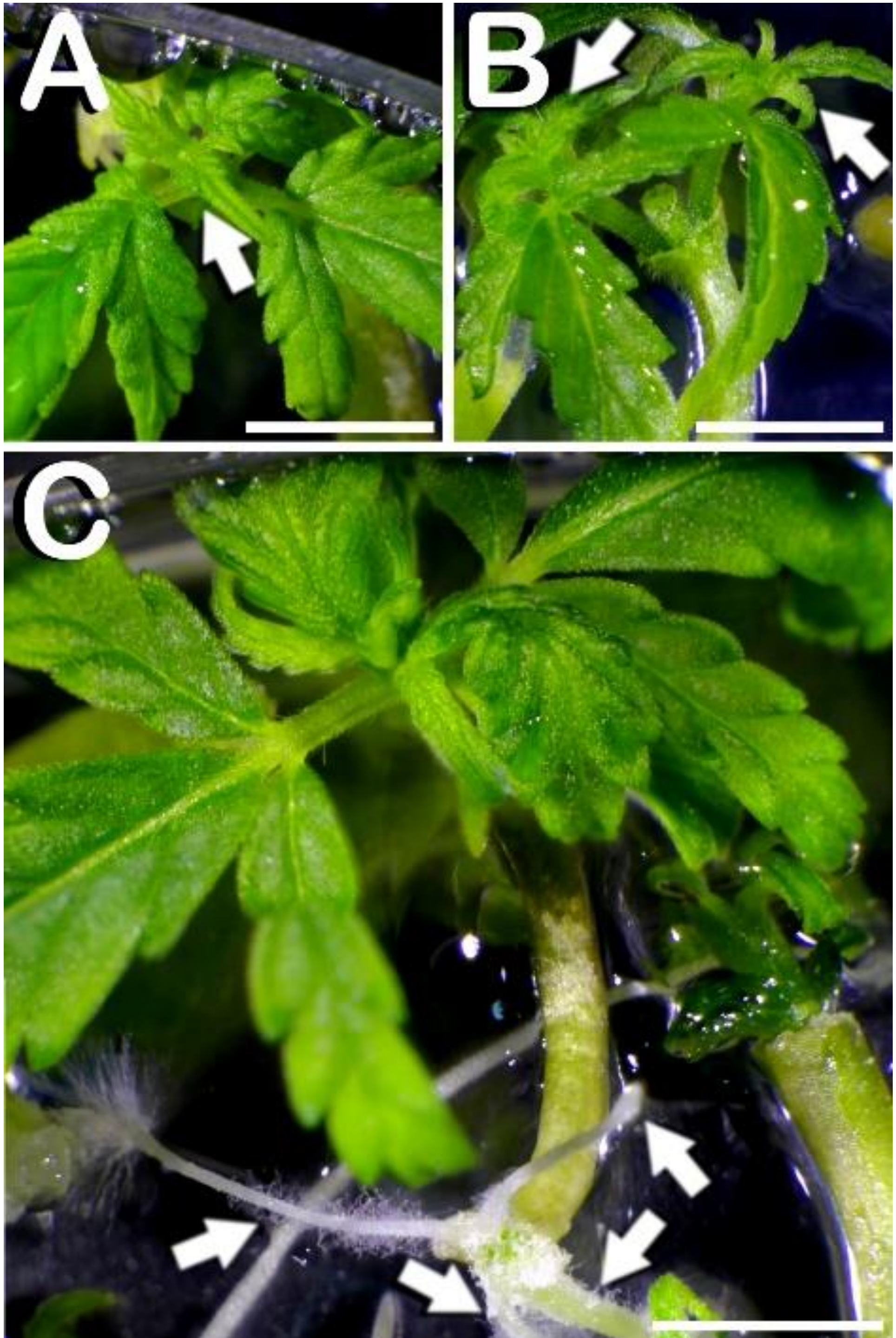
3.7. Data analyses

Depending on the experiment analyzed, the number of shoots per responding explant and plant regeneration, spontaneous rooting, albinism and/or transformation rates were compared for the different factors evaluated (explant type, hemp variety and/or kanamycin concentration). Experiments were repeated at least three times. Each replicate consisted of a Petri dish containing at least 5 explants coming from 5 different seedlings per variety in the case of hypocotyls, and at least 10 explants coming from 5 different seedlings per variety in the case of cotyledons. Independence among variables (Durbin-Watson test), homoscedasticity (Bartlett's test for mean variance analysis or Fligner-Killeen median test), and normality (Shapiro-Wilk test) were evaluated for the data. Subsequently, Kruskal-Wallis non-parametric tests followed by pairwise Wilcoxon or Nemenyi tests ($p<0.05$) were used to statistically determine significant differences between levels of each factor evaluated. Pairwise testing was based on whether analysis comprised more than one pairwise comparison (Nemenyi) or not (Wilcoxon). Statistical analysis was carried out using R software (R Core Team, 2019).

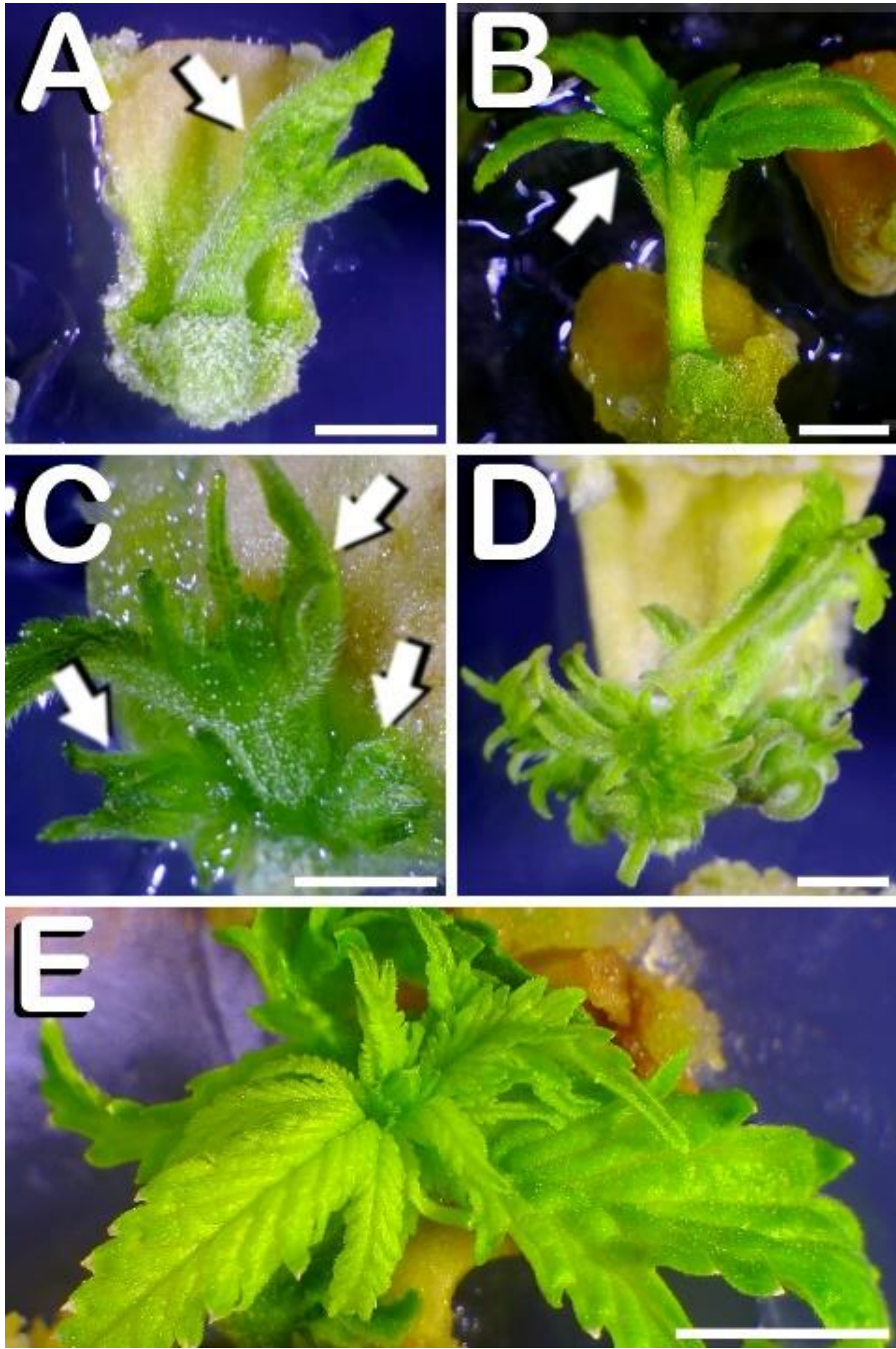
4. Results

4.1. Efficiency comparison of *C. sativa* hypocotyl and cotyledon *in vitro* plant regeneration protocols

We compared the ability of *C. sativa* hypocotyls and cotyledons for plant *in vitro* regeneration without *Agrobacterium* co-culture. The different regeneration patterns observed for hypocotyls and cotyledons are illustrated in Fig. 1 and Fig. 2 respectively. While hypocotyls developed at most one (Fig. 1A) or two shoots per explant (Fig. 1B), they were able to develop roots spontaneously (Fig. 1C). In contrast, single-shoot regeneration (Figs. 2A and 2B), and multiple-shoot regeneration (Figs. 2C and 2D) were observed in cotyledons, although despite the vigorous growth of cotyledon-derived regenerating shoots (Fig. 2E), they were incapable of developing roots spontaneously.


A
B
C

← **Figure 1**: Different *C. sativa* hypocotyl-derived plant regeneration patterns. The different images are described as follows: (A) Single-shoot hypocotyl regeneration three weeks after *in vitro* culture initiation: arrow points to shoot. (B) Regeneration of two shoots from a hypocotyl three weeks after explant *in vitro* culture: arrows point to both shoots. (C) Spontaneous rooting of hypocotyl derived regenerating shoots after three weeks of *in vitro* culture: arrows point to roots. Scale bars (A-C): 4 mm.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← **Figure 2**: Different *C. sativa* cotyledon-derived plant regeneration patterns. The different images are described as follows: (A) Single-shoot cotyledon regeneration one week after *in vitro* culture initiation: arrow points to shoot. (B) Cotyledon-derived regenerating shoot two weeks after *in vitro* culture: arrow points to shoot. (C) Regeneration of three shoots from a cotyledon one week after explant *in vitro* culture: arrows point to shoots. (D) Multiple shoot regeneration from a single cotyledon after two weeks of *in vitro* culture. (E) Shoot development one month after cotyledon *in vitro* culture initiation. Scale bars (A-D): 2 mm. Scale bar (E): 4 mm.

Furthermore, hypocotyls had a significant higher regeneration rate (53.3%) than cotyledons (18.1%) (Table 2). Significant differences were also detected among both types of explant in terms of spontaneous rooting rate. Almost 26% of hypocotyl-derived regenerating shoots developed roots (Table 2), in contrast with cotyledon-derived regenerating shoots, which were unable to develop roots in the evaluated medium. On the other hand, cotyledon-derived regenerating shoots developed more shoots per responding explant compared to hypocotyl-derived regenerating shoots. Both explant types reached 1.6 and 1.3 shoots respectively (Table 2). It should be noted that neither cotyledons nor hypocotyls developed albino plants after *in vitro* culture in control media (Table 2).

Table 2: Regeneration rate (%), shoots per responding explant, spontaneous rooting rate (%) and albinism rate (%) of *C. sativa* hypocotyl and cotyledon-derived regenerating shoots after *in vitro* control, kanamycin and transformation treatments. For each factor, mean is expressed as a percentage (±SE) relative to the total amount of cultured explants.

Treatment	Explant	Responding explants (%)	Shoots/explant	Spontaneous rooting (%)	Albino regenerating shoots (%)
Control	Hypocotyl	53.3^{a} ±4.3	1.3^{a} ±0.1	25.9^{a} ±3.8	0.0^{a} ±0.0
Control	Cotyledon	18.1^{b} ±2.3	1.6^{a} ±0.1	0.0^{b} ±0.0	0.0^{a} ±0.0
0 mg L^{-1} kanamycin	Hypocotyl	53.3^{ab} ±4.3	1.3^{a} ±0.1	25.9^{a} ±3.8	0.0^{b} ±0.0
50 mg L^{-1} kanamycin		60.0^{ab} ±7.0	1.3^{a} ±0.1	4.0^{bc} ±2.8	0.0^{b} ±0.0
100 mg L^{-1} kanamycin		63.3^{a} ±7.0	1.7^{a} ±0.1	12.2^{b} ±4.7	7.1^{b} ±5.0
200 mg L^{-1} kanamycin		64.0^{a} ±6.9	1.7^{a} ±0.1	6.0^{bc} ±3.4	12.5^{b} ±5.9
500 mg L^{-1} kanamycin		56.9^{ab} ±7.0	1.3^{a} ±0.1	0.0^{c} ±0.0	90.9^{a} ±6.3
750 mg L^{-1} kanamycin		40.8^{b} ±7.1	1.2^{a} ±0.1	0.0^{c} ±0.0	93.3^{a} ±6.7
Co-culture+selective regeneration	Hypocotyl	23.1^{a} ±1.3	1.2^{a} ±0.0	2.1^{a} ±0.5	18.0^{a} ±2.6
Co-culture+selective regeneration	Cotyledon	1.0^{b} ±0.2	1.0^{a} ±0.0	0.0^{b} ±0.0	16.7^{a} ±9.0
Control	Hypocotyl	53.3^{a} ±4.3	1.3^{a} ±0.1	25.9^{a} ±3.8	0.0^{b} ±0.0
Co-culture+selective regeneration		23.1^{b} ±1.3	1.2^{b} ±0.0	2.1^{b} ±0.5	18.0^{a} ±2.6
Control	Cotyledon	18.1^{a} ±2.3	1.6^{a} ±0.1	0.0^{a} ±0.0	0.0^{b} ±0.0
Co-culture+selective regeneration		1.0^{b} ±0.2	1.0^{b} ±0.0	0.0^{a} ±0.0	16.7^{a} ±9.0

Different letters among the levels of each factor indicate significant differences between them ($p<0.05$) according to non-parametric Kruskal-Wallis and pairwise Wilcoxon or Nemenyi (for more than one pairwise comparison) tests.

Significant differences were detected among the different varieties studied indicating that there is a genotype effect on hypocotyl-derived plant regeneration. While ‘Fedora17’ had the highest regeneration rate of this experiment with 76.5% of responding explants (Table 3), ‘FINOLA’ displayed the lowest hypocotyl plant regeneration with 36.4% (Table 3). Additionally, some varieties had a significantly higher capacity for spontaneous rooting than others. Again, while ‘Fedora17’ produced the best results of the experiment, ‘FINOLA’ yielded the lower percentages of regeneration. These varieties achieved 47.0% and 13.6% of spontaneous rooting rate respectively (Table 3). The genotype had no effect on cotyledon-derived plant regeneration and no significant differences were detected among the different parameters evaluated (Table 3).

Table 3: Regeneration rate (%), shoots per responding explant, spontaneous rooting rate (%) and albinism rate (%) of *C. sativa* hypocotyl and cotyledon-derived regenerating shoots from different varieties after *in vitro* culturing in their respective control media. For each factor, mean is expressed as a percentage (±SE) relative to the total amount of cultured explants.

Explant	Variety	Responding explants (%)	Shoots/explant	Spontaneous rooting (%)	Albino shoots (%)
Hypocotyl	‘Fedora17’	76.5^{a} ±7.4	1.3^{a} ±0.1	47.1^{a} ±8.7	0.0^{a} ±0.0
	‘Felina32’	39.1^{b} ±10.4	1.2^{a} ±0.1	17.4^{b} ±8.1	0.0^{a} ±0.0
	‘Ferimon’	51.8^{ab} ±9.8	1.2^{a} ±0.1	25.9^{ab} ±8.6	0.0^{a} ±0.0
	‘FINOLA’	36.4^{b} ±10.5	1.6^{a} ±0.3	13.6^{b} ±7.5	0.0^{a} ±0.0
	‘Futura75’	54.5^{ab} ±15.7	1.0^{a} ±0.0	18.2^{b} ±12.2	0.0^{a} ±0.0
	‘USO31’	50.0^{ab} ±12.1	1.6^{a} ±0.2	16.7^{b} ±9.0	0.0^{a} ±0.0
Cotyledon	‘Fedora17’	20.2^{a} ±4.4	1.5^{a} ±0.2	0.0^{a} ±0.0	0.0^{a} ±0.0
	‘Felina32’	11.8^{a} ±5.6	1.2^{a} ±0.2	0.0^{a} ±0.0	0.0^{a} ±0.0
	‘Ferimon’	17.3^{a} ±4.2	1.7^{a} ±0.2	0.0^{a} ±0.0	0.0^{a} ±0.0
	‘FINOLA’	26.3^{a} ±7.2	2.0^{a} ±0.4	0.0^{a} ±0.0	0.0^{a} ±0.0
	‘Futura75’	4.5^{a} ±4.5	1.0* ±*	0.0^{a} ±0.0	0.0* ±*
	‘USO31’	21.4^{a} ±7.9	1.2^{a} ±0.2	0.0^{a} ±0.0	0.0^{a} ±0.0

Different letters among the levels of each factor indicate significant differences between them ($p<0.05$) according to non-parametric Kruskal-Wallis and pairwise Wilcoxon or Nemenyi (for more than one pairwise comparison) tests.

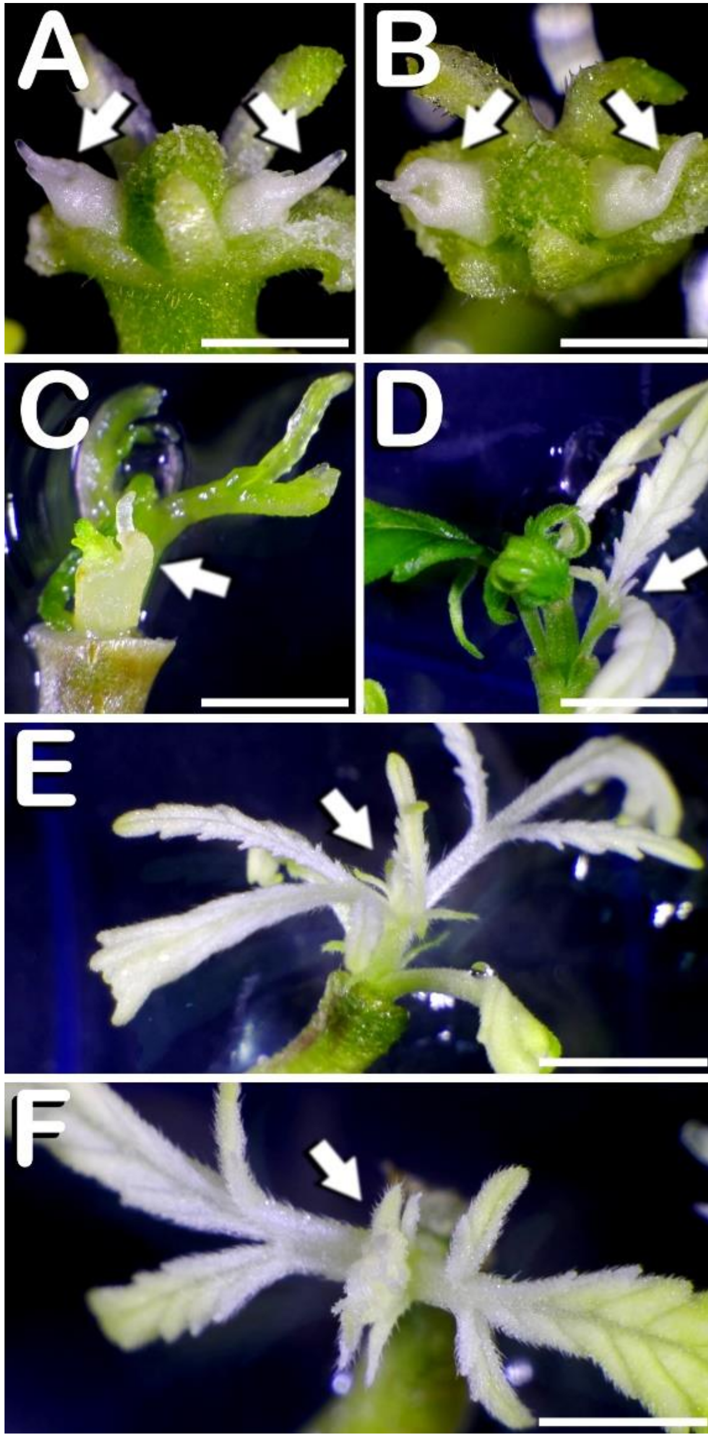
*As only one plant was regenerated, neither mean nor standard error of the mean were calculated and statistically compared with the remaining factor levels.

4.2. Effect of kanamycin on *C. sativa* hypocotyl regeneration in *in vitro* culture

Different parameters were compared after *in vitro* hypocotyl culture in the control medium and after the addition of increasing kanamycin concentrations. Shoot patterns developed during hypocotyl *in vitro* culture in kanamycin containing media can be observed in Fig. 3. While some explants produced synchronous development of albino regenerating shoots (Figs. 3A and 3B), other hypocotyl-derived regenerating shoots, even coming from the same explant, showed different ability for kanamycin resistance (Figs. 3C and 3D). Although hypocotyl-derived regenerating shoots cultured in kanamycin containing medium displayed an albino phenotype, they were able to exhibit a vigorous growth one month after *in vitro* culture initiation (Figs. 3E and 3F).

→

Figure 3: Shoot development patterns observed during hypocotyl *in vitro* culture in different kanamycin concentrations containing media. The different images are described as follows: (A) Albino hypocotyl-derived regenerating shoots developed in medium with 200 mg L^{-1} kanamycin four days after *in vitro* culture initiation (front view): arrows point to both primordia. (B) Albino hypocotyl-derived regenerating shoots developed in medium with 200 mg L^{-1} kanamycin four days after *in vitro* culture initiation (top view): arrows point to both primordia. (C) Kanamycin-resistant and non-resistant regenerating shoots arising from the top of a *Cannabis* hypocotyl one week after *in vitro* culture in medium containing 100 mg L^{-1} kanamycin: arrow points to kanamycin non-resistant shoot. (D) Kanamycin-resistant and non-resistant hypocotyl-derived regenerating shoot developed in medium with 100 mg L^{-1} kanamycin two weeks after *in vitro* culture: arrow points to kanamycin non-resistant shoot. (E) One-month-old hypocotyl-derived albino shoot (front view) developed in medium with 500 mg L^{-1} kanamycin: arrow points to shoot primordia. (F) One-month-old hypocotyl-derived albino shoot (top view) developed in medium with 500 mg L^{-1} kanamycin: arrow points to shoot primordia. Scale bars (A, B): 1.31 mm; Scale bar (C): 2.64 mm; Scale bars (D-F): 4mm.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A decrease in regeneration and spontaneous rooting rates was observed with increasing kanamycin concentrations. Specifically, the highest kanamycin concentration tested (750 mg L^{-1}) decreased the regeneration rate to an average of 40.8% (Table 2). Conversely, compared with the control, 100 mg L^{-1} and 200 mg L^{-1} slightly increased the hypocotyl plant regeneration rate, attaining 63.3% and 64.0% of responding explants respectively (Table 2). The best result for spontaneous rooting rate in kanamycin-containing medium (12.2%), was registered for the concentration of 100 mg L^{-1} (Table 2). Although this concentration caused a significant decrease of the rooting rate with respect to the control treatment (Table 2), it showed the best shoot regeneration and rooting rates on kanamycin, which led us to choose it for the subsequent transformation experiments. Concentrations ranging from 500 mg L^{-1} to 750 mg L^{-1} prevented spontaneous root formation in regenerating shoots (Table 2). On the other hand, an increase in the albinism rate was observed as kanamycin concentrations raised. While no albino plants were detected in 50 mg L^{-1} kanamycin concentration treatment, albino plants were already observed at 100 mg L^{-1}, with a 7.1% of albinism rate (Table 2). The higher kanamycin concentrations tested in our work (500 mg L^{-1} and 750 mg L^{-1}), resulted, respectively, in 90.9% and 93.3% of albino plants (Table 2). Apart from the control, only one of the evaluated treatments showed a significant influence of the genotype in some of the parameters evaluated. Specifically, in the 100 mg L^{-1} kanamycin concentration treatment, regeneration rate and the number of shoots per responding explant significantly differed among *C. sativa* varieties evaluated (Table 4). ‘Felina32’ (83.3%) and ‘Fedora17’ (77.8%) exhibited the best plant regeneration rate, contrasting with ‘Futura75’ which was the worst variety with 16.7% of explants developing shoots (Table 4). ‘FINOLA’ achieved the highest number of shoots per responding explant with 2.5 shoots, while ‘Felina32’ only produced 1.0 shoot per responding hypocotyl (Table 4).

Table 4: Regeneration rate (%), shoots per responding explant, spontaneous rooting rate (%) and albinism rate (%) of *C. sativa* hypocotyl-derived regenerating shoots from different varieties after *in vitro* culturing in control medium supplemented with different kanamycin concentrations. For each factor, mean is expressed as a percentage (±SE) relative to the total amount of cultured explants.

Treatment (mg L^{-1})	Variety	Responding explants	Shoots/explant	Spontaneous rooting	Albino shoots
50 kanamycin	'Fedora17'	77.8^{a} ±14.7	1.8^{a} ±0.2	0.0^{a} ±0.0	0.0^{a} ±0.0
	'Felina32'	28.6^{a} ±18.4	1.0^{a} ±0.0	0.0^{a} ±0.0	0.0^{a} ±0.0
	'Ferimon'	66.7^{a} ±16.7	1.5^{a} ±0.3	11.1^{a} ±11.1	0.0^{a} ±0.0
	'FINOLA'	55.6^{a} ±17.6	1.2^{a} ±0.2	0.0^{a} ±0.0	0.0^{a} ±0.0
	'Futura75'	66.7^{a} ±21.0	1.0^{a} ±0.0	0.0^{a} ±0.0	0.0^{a} ±0.0
	'USO31'	60.0^{a} ±16.3	1.2^{a} ±0.2	10.0^{a} ±10.0	0.0^{a} ±0.0
100 kanamycin	'Fedora17'	77.8^{a} ±14.7	1.4^{ab} ±0.2	11.1^{a} ±11.1	0.0^{a} ±0.0
	'Felina32'	83.3^{a} ±16.7	1.0^{b} ±0.0	0.0^{a} ±0.0	20.0^{a} ±20.0
	'Ferimon'	66.7^{ab} ±16.7	1.7^{ab} ±0.2	22.2^{a} ±14.7	16.7^{a} ±16.7
	'FINOLA'	66.7^{ab} ±16.7	2.5^{a} ±0.2	0.0^{a} ±0.0	0.0^{a} ±0.0
	'Futura75'	16.7^{b} ±16.7	2.0* ±*	0.0^{a} ±0.0	0.0* ±*
	'USO31'	60.0^{ab} ±16.3	2.0^{ab} ±0.4	30.0^{a} ±15.3	0.0^{a} ±0.0
200 kanamycin	'Fedora17'	40.0^{a} ±16.3	1.5^{a} ±0.3	10.0^{a} ±10.0	25.0^{a} ±25.0
	'Felina32'	66.7^{a} ±21.1	1.5^{a} ±0.3	0.0^{a} ±0.0	0.0^{a} ±0.0
	'Ferimon'	66.7^{a} ±16.7	2.0^{a} ±0.4	0.0^{a} ±0.0	0.0^{a} ±0.0
	'FINOLA'	55.6^{a} ±17.6	1.6^{a} ±0.2	0.0^{a} ±0.0	0.0^{a} ±0.0
	'Futura75'	83.3^{a} ±16.7	2.0^{a} ±0.3	0.0^{a} ±0.0	40.0^{a} ±24.5
	'USO31'	80.0^{a} ±13.3	1.6^{a} ±0.3	20.0^{a} ±13.3	12.5^{a} ±12.5
500 kanamycin	'Fedora17'	50.0^{a} ±16.7	1.0^{a} ±0.0	0.0^{a} ±0.0	100.0^{a} ±0.0
	'Felina32'	33.3^{a} ±21.1	2.0^{a} ±1.0	0.0^{a} ±0.0	100.0^{a} ±0.0
	'Ferimon'	55.6^{a} ±17.6	1.0^{a} ±0.0	0.0^{a} ±0.0	50.0^{a} ±28.9
	'FINOLA'	60.0^{a} ±16.3	1.5^{a} ±0.2	0.0^{a} ±0.0	100.0^{a} ±0.0
	'Futura75'	50.0^{a} ±22.4	1.0^{a} ±0.0	0.0^{a} ±0.0	50.0^{a} ±50.0
	'USO31'	80.0^{a} ±13.3	1.2^{a} ±0.2	0.0^{a} ±0.0	100.0^{a} ±0.0
750 kanamycin	'Fedora17'	20.0^{a} ±13,3	1.0^{a} ±0.0	0.0^{a} ±0.0	100. 0^{a} ±0.0
	'Felina32'	50.0^{a} ±22,4	1.3^{a} ±0.3	0.0^{a} ±0.0	66.7^{a} ±33.3
	'Ferimon'	33.3^{a} ±16,7	1.0^{a} ±0.0	0.0^{a} ±0.0	100. 0^{a} ±0.0
	'FINOLA'	62.5^{a} ±18,3	1.4^{a} ±0.2	0.0^{a} ±0.0	100.0^{a} ±0.0
	'Futura75'	33.3^{a} ±21,1	1.0^{a} ±0.0	0.0^{a} ±0.0	100. 0^{a} ±0.0
	'USO31'	50.0^{a} ±16,7	1.0^{a} ±0.0	0.0^{a} ±0.0	75.0^{a} ±25.0

Different letters among the levels of each factor indicate significant differences between them ($p<0.05$) according to non-parametric Kruskal-Wallis and pairwise Wilcoxon or Nemenyi (for more than one pairwise comparison) tests.

*As only one plant was regenerated, neither mean nor standard error of the mean were calculated and statistically compared with the remaining factor levels.

4.3. Effect of *A. tumefaciens* co-culture and subsequent explant culture in antibiotic-containing selective regeneration medium in *in vitro C. sativa* regeneration, spontaneous rooting and albinism rates

Agrobacterium co-culture and successive explant culture in antibiotic-containing selective regeneration medium reduced *in vitro* plant regeneration ability of hypocotyls and cotyledons, although once again, hypocotyl explants displayed a superior efficiency (23.1%) than cotyledons (1.0%) under these experimental conditions (Table 2). Both explant types significantly diminished their respective regeneration rates in comparison with control treatment, from 53.3% to 23.1% in the case of hypocotyls, and from 18.1% to 1.0% in cotyledons (Table 2). Although there were no significant differences among both types of explant in terms of the number of shoots per responding explant, when explants were analyzed separately, this parameter was significantly reduced in comparison with control treatments. While hypocotyl decreased from 1.3 shoots per responding explant under control conditions to 1.2 shoots after culture with *Agrobacterium* and antibiotics, this same trend was observed in cotyledons, which reduced from 1.6 to 1.0 shoot per responding explant (Table 2). Regarding spontaneous rooting of hypocotyl-derived regenerating shoots, both *Agrobacterium* and antibiotics explant exposure promoted a significant rate decrease which ranged from 25.9% in the control treatment to 2.1% after *Agrobacterium* and antibiotic exposure (Table 2). No significant differences were detected among hypocotyl and cotyledon explants in terms of albinism rate, which reached, respectively, 18.0% and 16.7% of albino regenerating shoots (Table 2). The only parameter significantly influenced by the genotype was the albinism rate of hypocotyl-derived regenerating shoots, varying from 45.4% for ‘Futura75’ regenerating shoots, to 4.2% for ‘USO31’ regenerated plants (Table 5).

Table 5: Regeneration rate (%), shoots per responding explant, spontaneous rooting rate (%) and albinism rate (%) of *C. sativa* cotyledon and hypocotyl-derived regenerating shoots from different varieties after co-culture with *Agrobacterium tumefaciens* LBA4404 containing binary plasmid pBIN19 carrying *uidA* reporter gene and the kanamycin resistance *neomycin phosphotransferase* (*nptII*) gene, and culture in their respective selective regeneration media. For each factor, mean is expressed as a percentage (±SE) relative to the total amount of cultured explants.

Explant	**Variety**	**Responding explants (%)**	**Shoots/explant**	**Spontaneous rooting (%)**	**Albino shoots (%)**
Hypocotyl	'Fedora17'	18.2^{a} ±3.3	1.2^{a} ±0.1	2.9^{a} ±1.4	8.0^{ab} ±5.5
	'Felina32'	20.3^{a} ±3.0	1.1^{a} ±0.0	4.5^{a} ±1.6	19.4^{ab} ±6.7
	'Ferimon'	21.8^{a} ±3.2	1.1^{a} ±0.1	1.8^{a} ±1.0	20.6^{ab} ±7.0
	'FINOLA'	24.4^{a} ±3.3	1.2^{a} ±0.1	1.2^{a} ±0.8	14.6^{ab} ±5.6
	'Futura75'	29.1^{a} ±3.8	1.0^{a} ±0.0	0.7^{a} ±0.7	45.4^{a} ±8.8
	'USO31'	24.9^{a} ±3.1	1.3^{a} ±0.1	1.5^{a} ±0.9	4.2^{b} ±2.9
Cotyledon	'Fedora17'	0.7^{a} ±0.5	1.0^{a} ±0.0	0.0^{a} ±0.0	0.0^{a} ±0.0
	'Felina32'	0.3^{a} ±0.3	1.0* ±*	0.0^{a} ±0.0	0.0* ±*
	'Ferimon'	0.3^{a} ±0.3	1.0* ±*	0.0^{a} ±0.0	0.0* ±*
	'FINOLA'	0.3^{a} ±0.3	1.0* ±*	0.0^{a} ±0.0	100.0* ±*
	'Futura75'	0.4^{a} ±0.4	1.0* ±*	0.0^{a} ±0.0	0.0* ±*
	'USO31'	3.2^{a} ±0.9	1.0^{a} ±0.0	0.0^{a} ±0.0	16.7^{a} ±11.2

Different letters among the levels of each factor indicate significant differences between them (p<0.05) according to non-parametric Kruskal-Wallis and pairwise Wilcoxon or Nemenyi (for more than one pairwise comparison) tests.

*As only one plant was regenerated, neither mean nor standard error of the mean were calculated and statistically compared with the remaining factor levels.

4.4. Effect of explant on the production of *C. sativa* transgenic plants after *A. tumefaciens* co-culture and subsequent explant culture in antibiotic-containing selective regeneration medium

Transgenic plants were obtained from all explants evaluated, although hypocotyls, cotyledons and already-developed meristems presented different transformation rates (Table 6). In total, nine transgenic plants were obtained from all the experiments: six transformed plants from hypocotyls (four plants from 'Futura75', one plant from 'Ferimon', and one plant from 'USO31'), two transformed plants from cotyledons (both plants from 'USO31' variety), and one transformed plant from an already-developed meristem (from 'USO31'). Hypocotyls achieved a higher transformation rate than already-developed meristems. While 5.0% of hypocotyl-derived regenerating shoots were successfully transformed (Table 6), only 0.8% of already developed meristems developed a transformed regenerating shoot (Table 6). As only two cotyledon-derived regenerating shoots grew enough to be analyzed, the cotyledon transformation rate was not statistically compared with the transformation rates of the remaining explants. However, both cotyledon-derived regenerating shoots were stably transformed (Table 6).

Furthermore, as only two transgenic plants from cotyledons, and one transformed plant from an already-developed meristem were obtained, the genotype effect on transgenic plant regeneration was only evaluated for hypocotyl explants. Significant differences were detected among varieties studied in terms of the transformation rate of hypocotyl-derived regenerating shoots. 'Futura75' stood out significantly among the rest of the evaluated varieties, reaching 28.6% of hypocotyl-derived transformed regenerating shoots (Table 6), followed by 'Ferimon' and 'USO31' with, respectively, 5.9% and 3.1% of transformation rate (Table 6). No transgenic plants were obtained from 'Fedora17', 'Felina32' and 'FINOLA' hypocotyls (Table 6).

Table 6: Effect of explant on the production of *Cannabis sativa* L. transgenic plants after co-culture of hypocotyls, cotyledons and already-developed meristems with *Agrobacterium tumefaciens* LBA4404 containing binary plasmid pBIN19 carrying *uidA* reporter gene and the kanamycin resistance neomycin phosphotransferase (*nptII*) gene, and subsequent explant culture in their respective selective regeneration media. Only were considered transformed regenerating shoots those that simultaneously exhibited a green phenotype after culture on selective regeneration medium, and uniform expression (non-chimeric) of the *uidA* gene after X-Gluc regenerant-derived tissue incubation by GUS histochemical assay, plus amplification of the *uidA* and *nptII* genes by PCR.

Explant	Variety	Transformation rate (%)	n
Hypocotyl	Pooled varieties	5.0^{a} ±2.0	120
Meristem	Pooled varieties	0.8^{b} ±0.8	130
Cotyledon	Pooled varieties	100.0^{*} ±0.0	2
Hypocotyl	‘Fedora17’	0.0^{b} ±0.0	16
	‘Felina32’	0.0^{b} ±0.0	22
	‘Ferimon’	5.9^{ab} ±5.9	17
	‘FINOLA’	0.0^{b} ±0.0	19
	‘Futura75’	28.6^{a} ±12.5	14
	‘USO31’	3.1^{b} ±3.1	32

Different letters among the levels of each factor indicate significant differences between them ($p<0.05$) according to non-parametric Kruskal-Wallis and pairwise Wilcoxon or Nemenyi (for more than one pairwise comparison) tests.
*Not analyzed statistically

4.5. *uidA* and *nptII* gene expression in *C. sativa in vitro* regenerating shoots

Hypocotyls dissected from seven-days-old *C. sativa* seedlings (Figs. 4A and 4B) were co-cultivated with *A. tumefaciens* LBA4404 for 4 days. During co-cultivation, both single hypocotyl-derived regeneration (Fig. 4C), and synchronized regeneration of various primordia in the periphery of the organ (arrows in Fig. 4D) were observed. After the co-cultivation period, explants were transferred to a selective regeneration medium. Within 16

days after initiation of the hypocotyl *in vitro* culture, we were able to observe the spontaneous rooting of *Cannabis* regenerating shoots developed on selective regeneration medium (Fig. 4E). Approximately one month after *in vitro* culture initiation, samples from regenerating shoots were incubated in X-Gluc and decolored. Leaves coming from non-transformed regenerating shoots were white (Fig. 4F), with no signals of *uidA* gene expression (insert in Fig. 4F). Conversely, *Cannabis* leaves from one-month-old hypocotyl-derived transformed shoots showed different GUS staining patterns. On the one hand, some leaf samples showed non-uniform *uidA* expression (Fig. 4G), being characterized by blue spots with different color intensity irregularly distributed along the putatively chimeric tissue. Furthermore, newly-formed primordia derived from transformed shoots showed strong and uniform GUS staining (Fig. 4H). Even the whole shoot apical meristem (SAM) acquired an intense dark-blue coloration (insert in Fig. 4H). Finally, also some leaf samples coming from one-month-old hypocotyl-derived transformed shoots showed strong and uniform GUS staining (Fig. 4I), which also reached the leaflet outline (insert in Fig. 4I).

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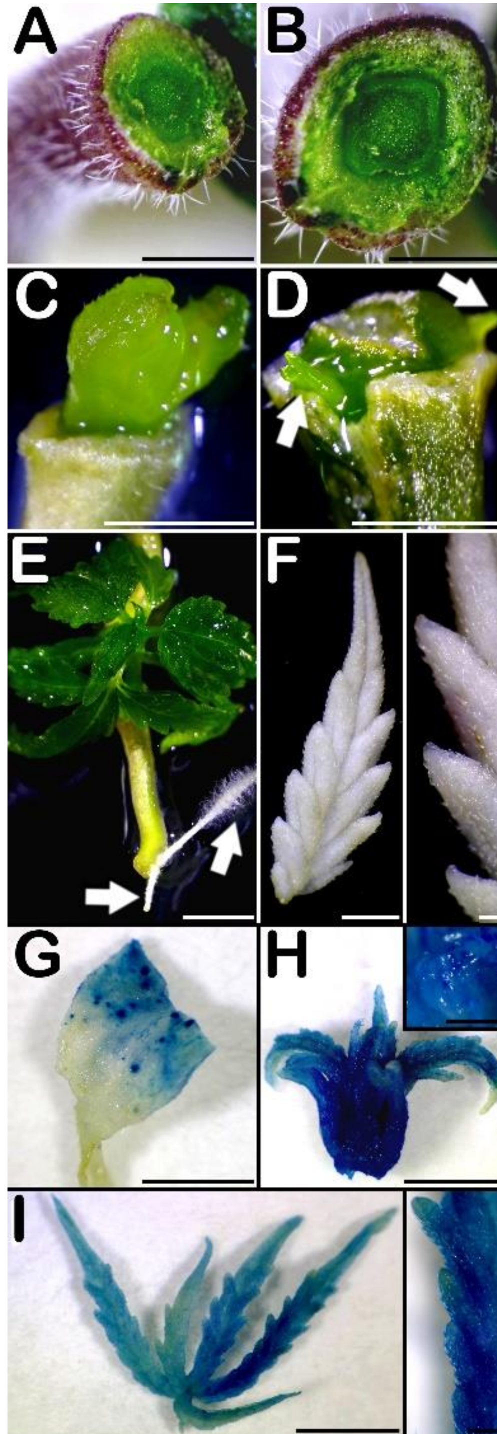
Figure 4: GUS staining of *C. sativa* hypocotyl-derived *in vitro* regenerating shoots: (A) Newly dissected hypocotyl from a seven-days-old hemp seedling. (B) Transversal section of hypocotyl just before co-cultivation with *Agrobacterium*. (C) Shoot *in vitro* regeneration from *Cannabis* hypocotyl after three-day *Agrobacterium* co-culture. (D) Two primordia arising from the top of a *Cannabis* hypocotyl after three-day co-culture with *Agrobacterium*: arrows point to both primordia. (E) Spontaneously-rooted *Cannabis* hypocotyl-derived regenerating shoot 16 days after culture on selective regeneration medium: arrows point to roots. (F) *Cannabis* leaflet from a non-transformed hypocotyl-derived shoot after incubation in X-Gluc and decoloration through a graded ethanol series: detail of leaflet outline (right side in panel F). (G) *Cannabis* leaflet from a one-month-old transformed hypocotyl-derived regenerant showing non-uniform GUS staining after incubation in X-Gluc and decoloration through a graded ethanol series. (H) Shoot apex from a one-month-old transformed hypocotyl-derived regenerant showing strong and uniform GUS staining after incubation in X-Gluc and decoloration: detail of shoot apical meristem (SAM) after GUS staining (insert in panel H). (I) *Cannabis* leaf from a one-month-old hypocotyl-derived transformed shoot showing uniform GUS staining after incubation in X-Gluc and decoloration: detail of leaflet outline (right side in the panel I). Scale bar (A): 1 mm. Scale bar (B): 0.75 mm. Scale bar (C): 2.16 mm. Scale bar (D): 1.31 mm. Scale bar (E): 4 mm. Scale bar (F): 1 mm; Scale bar of insert (F): 0.5 mm. Scale bar (G): 1.73 mm. Scale bar (H): 2.16 mm; Scale bar of insert (H): 0.5 mm. Scale bar (I): 2.64 mm; Scale bar of insert (I): 0.5 mm.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Regarding cotyledon-derived regenerant genetic transformation, explants dissected from seven-days-old *C. sativa* seedlings (Fig. 5A) were co-cultured with *A. tumefaciens* LBA4404 during 4 days. During co-culture, single cotyledon-derived regenerating shoots (Fig. 5B) were observed. After co-culture, explants were transferred to a selective regeneration medium. After 14 days of *in vitro* culture of cotyledons, regenerating shoots reached ~1 cm in length (Fig. 5C). Approximately one month after *in vitro* culture initiation, shoot samples were incubated in X-Gluc and decolored. While leaf samples coming from non-transformed regenerating shoots were white, with no signals of *uidA* expression, some leaf samples from one-month-old cotyledon-derived transformed regenerating shoots displayed strong and uniform GUS staining, acquiring an intense dark-blue coloration, although the tip of some leaflets did not show any signal of GUS staining (Figs. 5D and 5E).

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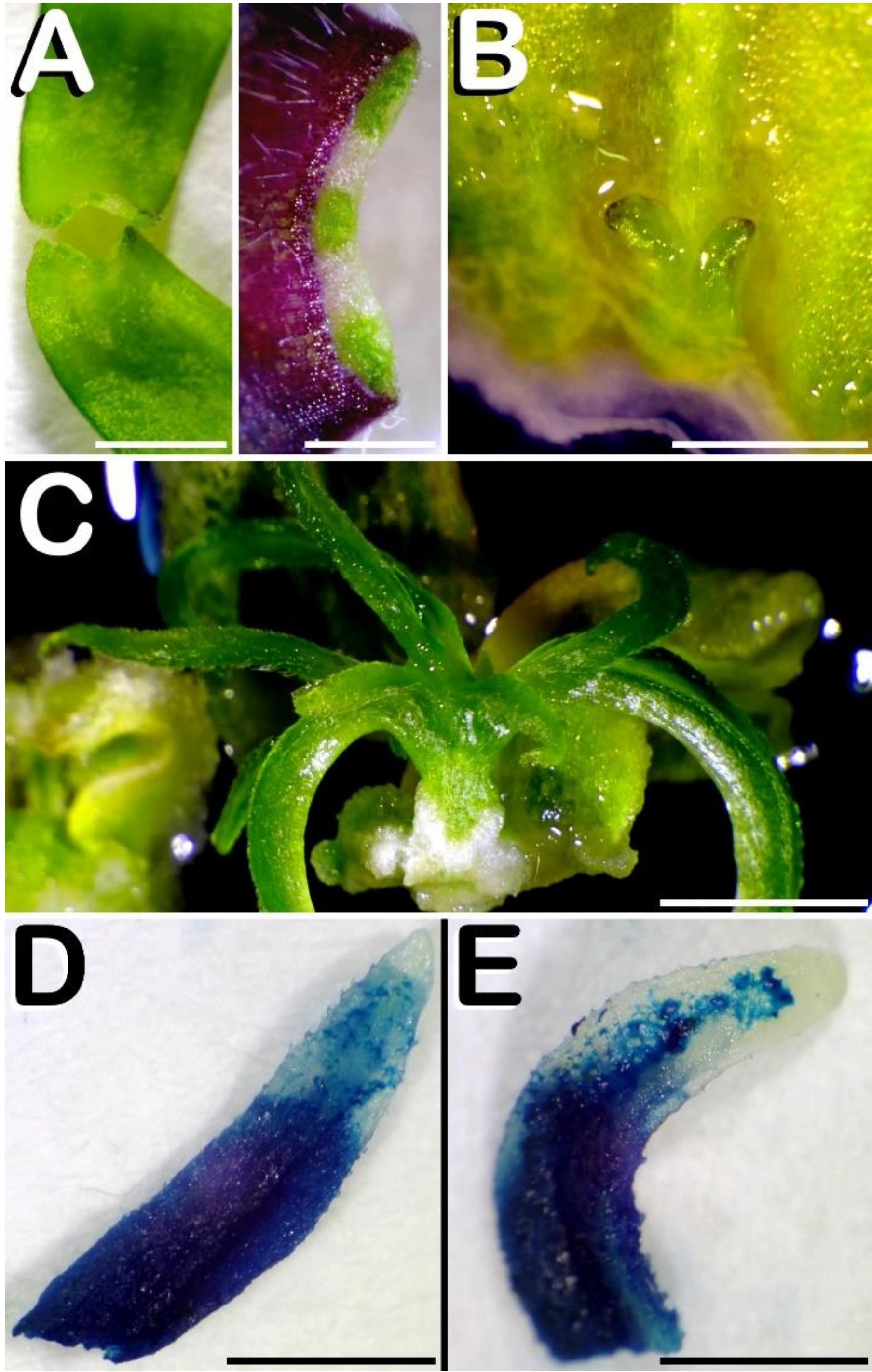
Figure 5: Genetic transformation of *C. sativa* cotyledon-derived *in vitro* regenerating shoots and GUS staining. The different images are described as follows: (A) Newly dissected cotyledons from a seven-days-old hemp seedling: detail of the transversal section of a newly dissected cotyledon just before co-cultivation with *Agrobacterium* (right side in panel A). (B) Shoot *in vitro* regeneration from *Cannabis* cotyledon after three-day co-cultivation with *Agrobacterium*. (C) *Cannabis* cotyledon-derived transformed shoot 14 days after culture on selective regeneration medium. (D) and (E) *Cannabis* leaflets from a one-month-old transformed cotyledon-derived shoot showing non-uniform GUS staining after incubation in X-Gluc and decoloration through a graded ethanol series. Scale bar (A): 2.64 mm; Scale bar of insert (A): 0.65 mm. Scale bar (B): 1 mm. Scale bar (C): 4 mm. Scale bar (D): 1.73 mm. Scale bar (E): 1.73 mm.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Concerning meristem transformation, already-developed meristems dissected from seven-days-old *C. sativa* seedlings (Fig. 6A), which contained the whole shoot apical meristem (SAM) (Fig. 6B), were co-cultured with *A. tumefaciens* LBA4404 for 4 days. After that time, explants were transferred to a selective regeneration medium. Approximately one month after *in vitro* culture initiation, leaf samples were taken from regenerating shoots, which showed a prominent growth (Fig. 6C), incubated with X-Gluc and decolored. While no evidence of GUS staining was detected in samples coming from non-transgenic regenerating shoots (not shown), leaflets coming from the only meristem-derived transformant of the experiment showed a blue coloration distributed along its entire surface (Fig. 6D).

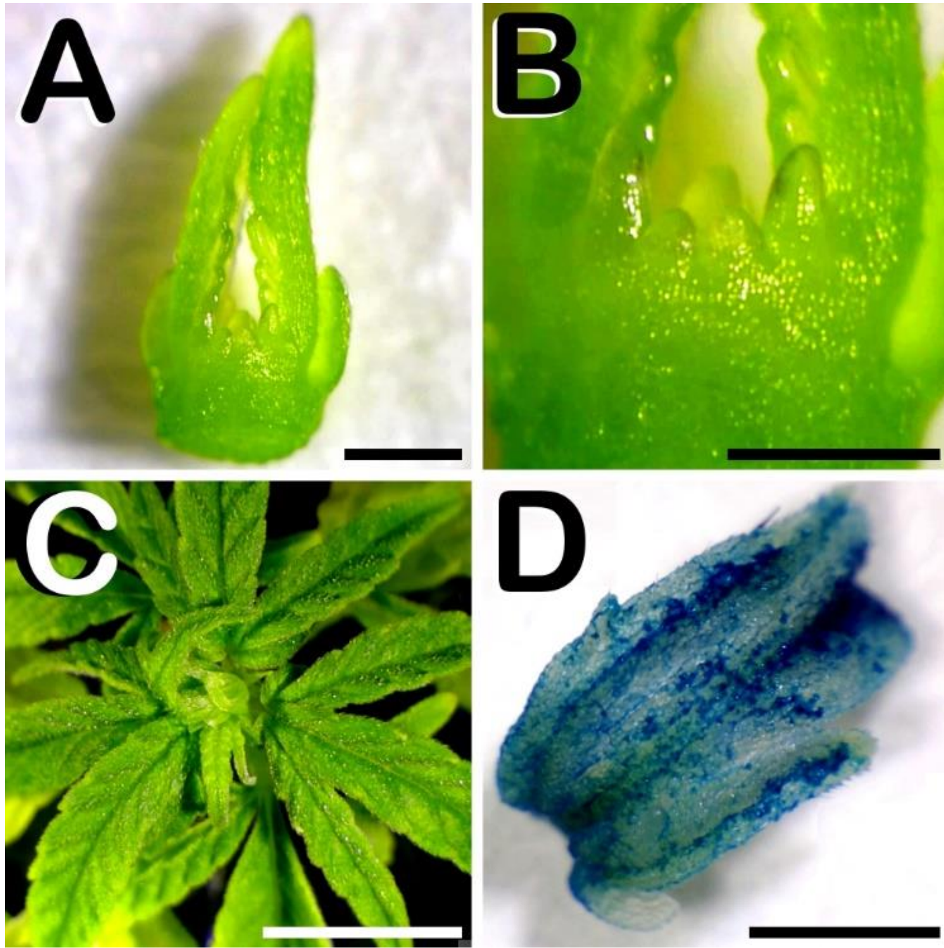

Figure 6: Genetic transformation of *C. sativa* meristem-derived *in vitro* regenerant and GUS staining. The different images are described as follows: (A) Newly dissected meristem from a seven-days-old hemp seedling. (B) Detail of shoot apical meristem (SAM). (C) Meristem-derived transformed regenerant after 26 days in selective regeneration medium. (D) *Cannabis* leaflet from a one-month-old transformed meristem-derived regenerant showing GUS staining after incubation in X-Gluc and decoloration through a graded ethanol series. Scale bar (A): 0.44 mm; Scale bar (B): 0.29 mm; Scale bar (C): 4.00 mm; Scale bar (D): 1.73 mm.

With respect to the response of regenerating shoots after *Agrobacterium* co-culture and culturing in selective regeneration medium containing 100 mg L^{-1} of kanamycin, it should be noted that, regardless of the explant from which they originated, all those transformed regenerating shoots that showed GUS staining signal, developed a normal green phenotype during their development in selective regeneration medium (Fig. 7A). Conversely, some hypocotyls had regenerating shoots with different kanamycin tolerance, as illustrated in Fig. 7B, were kanamycin-resistant (left) and non-resistant (right) regenerating shoots arising from the top of a *Cannabis* hypocotyl can be observed. On the other hand, some kanamycin-non-resistant shoot primordia arising from the basal zone of cotyledons were detected three days after culture on selective regeneration medium (Fig. 7C), although they stopped their development at this stage. In contrast, hypocotyl-derived regenerating shoots showed vigorous growth. Nine-days-old (Fig. 7D), and 20-days-old (Fig. 7E) kanamycin-non-resistant hypocotyl-derived transformants developed on selective regeneration medium and showing an albino phenotype were also observed. Independently of the explant origin of regenerated plants, regenerating shoots were only considered successfully-transformed if they complied with these three conditions: a) green phenotype shown after *in vitro* culture on selective regeneration medium, b) uniform expression (non-chimeric) of the *uidA* gene after incubation of shoot-derived tissue in X-Gluc by GUS histochemical assay, and c) *uidA* and *nptII* gene amplified by PCR (Fig. 8).

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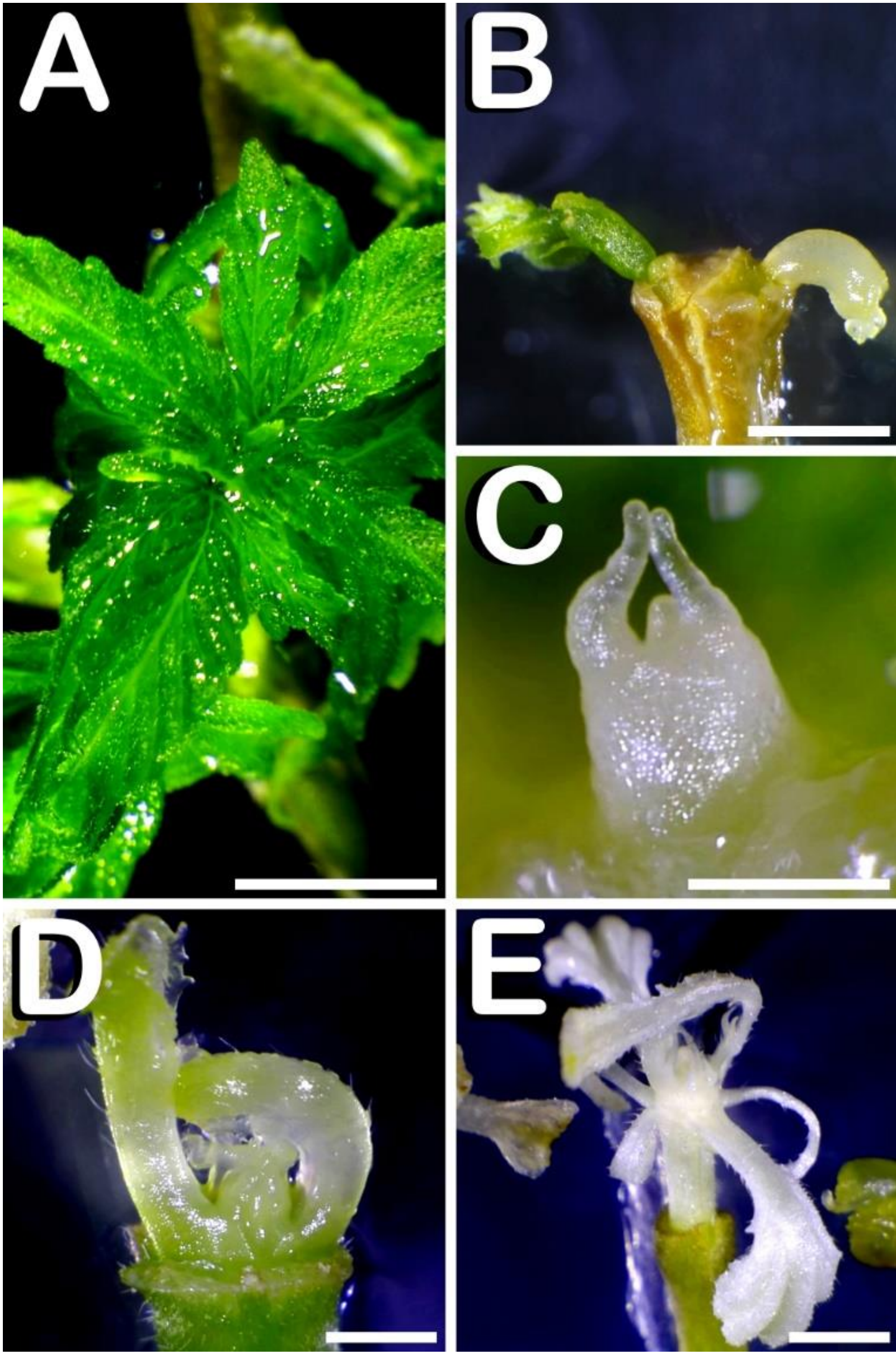
Figure 7: Kanamycin-resistant and non-resistant phenotypes of *C. sativa* transformants. The different images are described as follows: (A) Kanamycin-resistant hypocotyl-derived regenerant 16 days after culture on selective regeneration medium. (B) Kanamycin-resistant (left) and non-resistant (right) regenerating shoots arising from the top of a *Cannabis* hypocotyl three days after culture on selective regeneration medium. (C) Kanamycin-non-resistant shoot primordium arising from the basal zone of a cotyledon three days after culture on selective regeneration medium. (D) Kanamycin-non-resistant shoot primordium arising from the top of a hypocotyl five days after culture on selective regeneration medium. (E) Kanamycin-non-resistant hypocotyl-derived regenerant 16 days after culture on selective regeneration medium.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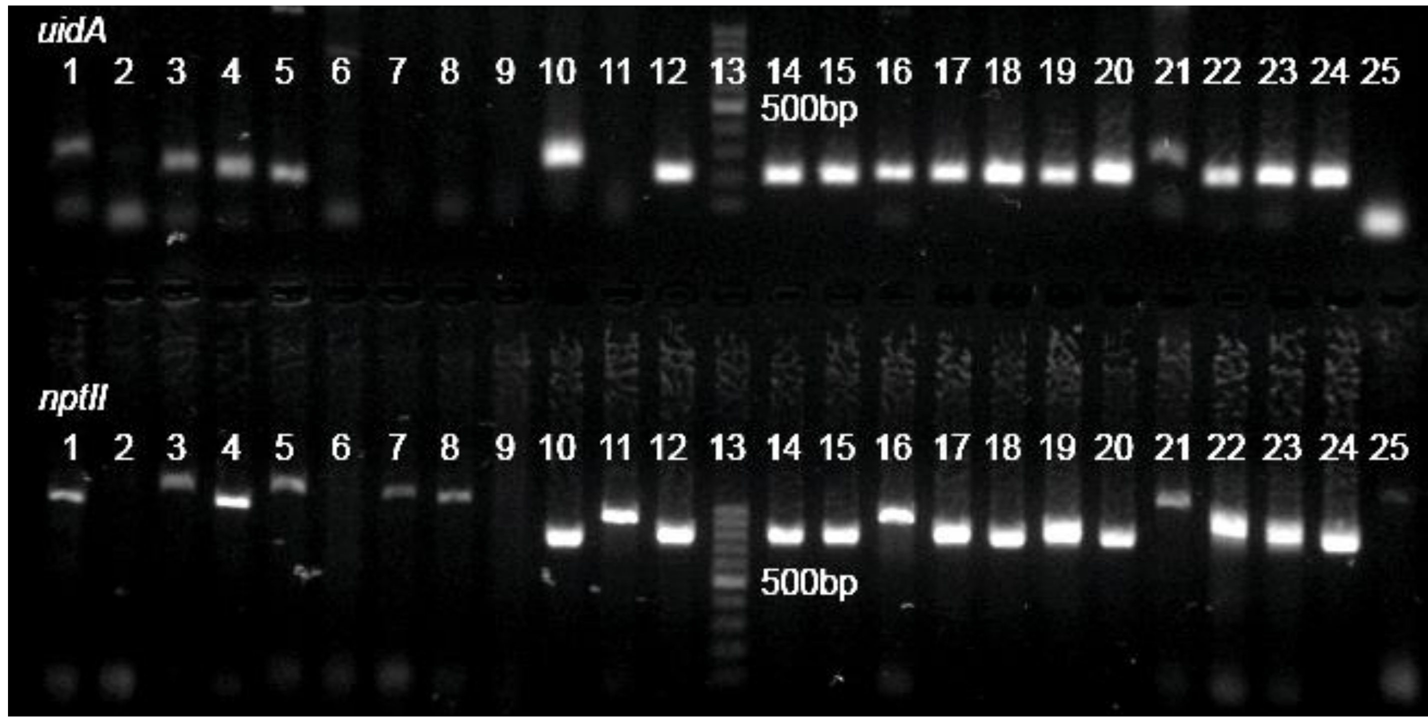

Figure 8: Polymerase chain reaction (PCR) detection of the *ß-glucuronidase* (GUS) (206-bp fragment) and kanamycin resistance neomycin phosphotransferase II (NPTII) (795-bp fragment) genes. Lanes 1-9, 11→ Non-transformed shoots. Lanes 10, 12, 14-20→ Transformed shoots. Lane 13→ 1 kb marker DNA. Lane 21→ DNA from non-transformed control plant. Lanes 22 and 23→ Replicates from lane 10 and lane 18 respectively. Lane 24→ Plasmid. Lane 25→ Negative control.

5. Discussion

In the present work, we have been able to regenerate *C. sativa* transgenic plants. This represents an important landmark in *C. sativa* breeding and research. Even without addition of plant growth regulators in the culture media employed in the different protocol steps, our results indicate that hypocotyls present higher rates of *in vitro* plant regeneration, transformation, and spontaneous rooting of regenerating shoots compared to the rest of the studied explants. Furthermore, by means of our protocol for *Agrobacterium*-mediated transformation and production of *C. sativa* transgenic plants, hyperhydricity of hypocotyl-derived regenerating shoots was prevented to a great extent. Hypocotyl explants have been reported as having a superior ability for *in vitro* plant regeneration (Mandolino and Ranalli, 1999; Galán-Ávila *et al.*, 2020; Zhang *et al.*, 2021), and suitability for genetic transformation (Wahby *et al*., 2013, 2017; Zhang *et al.*, 2021). High plant regeneration ability of *C. sativa* hypocotyls dissected from 7-days-old seedlings, coupled with the putatively unicellular origin of hypocotyl-derived regenerating shoots (Galán-Ávila *et al.*, 2020, and references therein), might have facilitated the genetic transformation of a pluripotent pericycle cell and its subsequent regeneration into a whole transgenic plant, making the difference with previously published attempts concerning transgenic plant regeneration in this species (Feeney and Punja, 2003, 2015; Wahby *et al*., 2013, 2017). In comparison with the only published report concerning the innovative implementation of the CRISPR/Cas system for genome edition in *C. sativa* species (Zhang *et al.*, 2021), our protocol shows not only an improved efficiency in terms of shoot regeneration and transformation rates, but also a faster capability for *C. sativa* transformed plant production.

The transgenic nature of *C. sativa* regenerated plants was confirmed, among other techniques, by means of GUS histochemical assay. Employment of *uidA* as a reporter gene in order to validate *Cannabis* transformation constitutes an appropriate strategy, as its expression in

different *C. sativa* explants has already been evaluated satisfactorily (Wahby *et al*., 2013, 2017; Deguchi *et al*., 2020; Sorokin *et al.*, 2020). In our work, among regenerated plants, putatively chimeric individuals harboring both transformed and non-transformed cells and showing non-uniform GUS staining were also observed. Chimeric transgenic plants have been reported in several species (Ding *et al.*, 2020). Shoot regeneration from a mixture of transformed and untransformed cells, transformed cells conforming only a sector in a shoot, cell cycle arrest of transformed cells, or transient expression of a transgene in some cells of a shoot are possible explanations for the generation of chimeras (Chen, 2011). On the other way, the combined use of *uidA* and *nptII* genes for verifying *Agrobacterium*-mediated plant transformation has been widely implemented in many other species such as *Nicotiana rustica* L. and *Nicotiana tabacum* L. (Hamill *et al.*, 1991), *Solanum lycopersicum* L. (Dan *et al.*, 2006), or more recently, *Gossypium hirsutum* L. (Ahmed *et al.*, 2020) and *Acmella oleracea* L. (Maggini *et al.*, 2021). Regarding kanamycin resistance *nptII* gene, it should be noted that there are no previous works concerning its expression in *C. sativa*. Here we have demonstrated that, among the rest of the evaluated concentrations, the addition of 100 mg L^{-1} of kanamycin to selective regeneration medium yielded the best results of the dose-response experiment, as it ensured both optimal *in vitro* plant regeneration and spontaneous rooting of regenerating shoots. However, despite the recognized biosafety of *nptII*-derived kanamycin resistance (Nap *et al.*, 1992; Das *et al.*, 2020), and although similar concentrations of kanamycin to those used by us have been employed to successfully select transformants in other species such as *Solanum lycopersicum* L. (Chetty *et al.*, 2013), *Antirrhinum majus* L. (Lian *et al.*, 2020), or *Solanum tuberosum* L. (Bakhsh, 2020), due to the low albinism rate achieved under these experimental conditions, kanamycin-resistance proved to be a poor selectable marker for *C. sativa*. A similar situation has been reported for other species like *Glycine max* L. Merr. (Meurer *et al.*, 1998), or *Phelipanche ramosa* L. (Kullačová and Matúšová, 2020). With respect to the rest of the antibiotics present in selective regeneration

medium (250 mg L^{-1} cefotaxime plus 250 mg L^{-1} carbenicillin), their combined use at these concentrations is routinely used for *Agrobacterium* elimination in several *in vitro* plant transformation procedures (da Silva, 2006; Pawar *et al.*, 2013; Yaqoob *et al.*, 2017). Evidence further suggests that antibiotic phytotoxicity can negatively influence explant regenerative ability (Pollock *et al.*, 1983; Qin *et al.*, 2011; Li *et al.*, 2019), as occurs with *Agrobacterium* explant co-culture (Srivastava *et al.*, 2017; de Melo *et al.*, 2020). This might account for the drastic *in vitro* plant regeneration rate reduction registered in our experiments for both *C. sativa* hypocotyl and cotyledon explants.

Cannabis sativa cultivation and research are booming and breeding programs can benefit from the development of transgenic plants, as has occurred with major crops (Herrera-Estrella *et al.*, 2005; Abiri *et al.*, 2015). In addition, new plant breeding techniques such as genome editing offer new avenues for breeding *C. sativa* for medicinal or industrial purposes. Among the most important present challenges is the development of *C. sativa* varieties resistant to biotic (Hadad *et al.*, 2019; Punja *et al.*, 2019; Jerushalmi *et al.*, 2020), and abiotic stresses (Cosentino *et al.*, 2013; Guerriero *et al.*, 2017; Gao *et al.*, 2018; Landi *et al.*, 2019), as well as the development of varieties with specific cannabinoid profiles (Lynch *et al.*, 2016).

6. Conclusions

The present work represents a pioneering study documenting the production of *C. sativa* transgenic plants. Our tissue culture-based procedure for *C. sativa* plant genetic transformation could also enable the implementation of genome editing through CRISPR/Cas systems for *C. sativa* breeding, promoting the development of varieties with enhanced agronomic and medicinal properties with industrial and pharmacological utility.

7. Statements

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Author contribution statement

A.G.-A., P.G, M.R., J.P. and F.J.H. conceived and designed the research. A.G.-A. and F.J.H. performed the experiments. A.G.-A., P.G, M.R., J.P. and F.J.H. analyzed the results. A.G.-A. wrote the manuscript. A.G.-A., P.G, M.R., J.P. and F.J.H. reviewed and edited the manuscript. All authors have read and approved the manuscript for publication.

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Availability of data and material

The datasets used and/or analyzed and plant materials used in the current study are available from the corresponding author on reasonable request.

Conflict of interests

The author Alberto Galán Ávila declares that his employer (Ploidy and Genomics Ltd.) is seeking a patent over the protocol presented:

- Patent applicant: Ploidy and Genomics Ltd.

- Name of inventor: Alberto Galán Ávila

- PCT Application number: PCT/EP2020/087829

- PCT Publication number: WO/2021/130342

- Priority claim: 28th of December 2019

- Status of application: The international search authority of the Patent Cooperation Treaty (PCT) has issued the international search report with its corresponding written opinion certifying the novelty, inventive activity and industrial applicability of the protocol described herein.

- Specific aspect of manuscript covered in patent application: Genetic transformation protocol for the production of *Cannabis sativa* L. transgenic or gene-edited plants.

The rest of authors declare no competing interests.

8. References

1. Abiri, R., Valdiani, A., Maziah, M., Shaharuddin, N. A., Sahebi, M., Yusof, Z. N. B., ... and Talei, D. 2015. A critical review of the concept of transgenic plants: insights into pharmaceutical biotechnology and molecular farming. *Curr. Issues Mol. Biol.* 18, 21-42.

2. Ahmed, H. A., Barpete, S., Uranbey, S., Akdoğan, G., Köm, D., and Özcan, S. 2020. An Efficient *Agrobacterium*-Mediated Genetic Transformation Using Embryonic Axis in Cotton (*Gossypium hirsutum* L.). *Russ. J. Plant Physiol.* 67, 581-587. https://doi.org/10.1134/S1021443720030024.

3. Ahmed, S., Gao, X., Jahan, M. A., Adams, M., Wu, N., and Kovinich, N. 2020. Nanoparticle-Based Genetic Transformation of *Cannabis sativa*. *J. Biotechnol.* 326, 48-51. https://doi.org/10.1016/j.jbiotec.2020.12.014.

4. Bakhsh, A. 2020. Development of efficient, reproducible and stable *Agrobacterium*-mediated genetic transformation of five potato cultivars. *Food Technol. Biotechnol.* 58(1), 57-63. https://doi.org/10.17113/ftb.58.01.20.6187.

5. Beard, K. M., Boling, A. W., and Bargmann, B. O. 2021. Protoplast isolation, transient transformation, and flow-cytometric analysis of reporter-gene activation in *Cannabis sativa* L. *Ind. Crops Prod*. 164, 113360. https://doi.org/10.1016/j.indcrop.2021.113360.

6. Bertani, G. 1951. Studies on lysogenesis I.: the mode of phage liberation by lysogenic *Escherichia coli*[1]. *J. Bacteriol.* 62(3), 293-300.

7. Chaohua, C., Gonggu, Z., Lining, Z., Chunsheng, G., Qing, T., Jianhua, C., ... and Jianguang, S. 2016. A rapid shoot regeneration protocol from the cotyledons of hemp (*Cannabis sativa* L.). *Ind. Crops Prod.* 83, 61-65. https://doi.org/10.1016/j.indcrop.2015.12.035.

8. Chen, G. Q. 2011. Effective reduction of chimeric tissue in transgenics for the stable genetic transformation of *Lesquerella fendleri*. *HortScience.* 46(1), 86-90. https://doi.org/10.21273/HORTSCI.46.1.86.

9. Chetty, V. J., Ceballos, N., Garcia, D., Narváez-Vásquez, J., Lopez, W., and Orozco-Cárdenas, M. L. 2013. Evaluation of four *Agrobacterium tumefaciens* strains for the genetic transformation of tomato (*Solanum lycopersicum* L.) cultivar Micro-Tom. *Plant Cell Rep.* 32(2), 239-247. https://doi.org/10.1007/s00299-012-1358-1.

10. Cosentino, S. L., Riggi, E., Testa, G., Scordia, D., and Copani, V. 2013. Evaluation of European developed fibre hemp genotypes (*Cannabis sativa* L.) in semi-arid Mediterranean environment. *Ind. Crops Prod.* 50, 312-324. https://doi.org/10.1016/j.indcrop.2013.07.059.

11. da Silva, J. A. T. 2006. Chrysanthemum (*Dendranthema × grandiflora*), in: Wang K. (eds.), *Agrobacterium* Protocols, vol. 2. Methods in Molecular Biology, vol. 344. Humana Press Inc., 321-329. https://doi.org/10.1385/1-59745-131-2:321.

12. Dan, Y., Yan, H., Munyikwa, T., Dong, J., Zhang, Y., and Armstrong, C. L. 2006. MicroTom—a high-throughput model transformation system for functional genomics. *Plant Cell Rep.* 25(5), 432-441. https://doi.org/10.1007/s00299-005-0084-3.

13. Das, A., Shukla, A., Thakur, S., Rathore, M., and Singh, N. P. 2020. Estimation of neomycin phosphotransferase-II (NPT-II) protein in vegetative and reproductive tissues of transgenic chickpea (*Cicer arietinum* L.) and biosafety perspectives. *J. Plant Biochem. Biotechnol.* 29(3), 568-570. https://doi.org/10.1007/s13562-020-00562-z.

14. de Melo, B. P., Lourenço-Tessutti, I. T., Morgante, C. V., Santos, N. C., Pinheiro, L. B., de Jesus Lins, C. B., ... and Grossi-de-Sa, M. F. 2020. Soybean embryonic axis transformation: combining biolistic and *Agrobacterium*-mediated protocols to overcome typical complications of *in vitro* plant regeneration. *Front. Plant Sci.* 11:1228. https://doi.org/10.3389/fpls.2020.01228.

15. Deguchi, M., Bogush, D., Weeden, H., Spuhler, Z., Potlakayala, S., Kondo, T., ... and Rudrabhatla, S. 2020. Establishment and optimization of a hemp (*Cannabis sativa* L.) agroinfiltration system for gene expression and silencing studies. *Sci. Rep.* 10(1), 1-11. https://doi.org/10.1038/s41598-020-60323-9.

16. Ding, L., Chen, Y., Ma, Y., Wang, H., and Wei, J. 2020. Effective reduction in chimeric mutants of poplar trees produced by CRISPR/Cas9 through a second round of shoot regeneration. *Plant Biotechnol. Rep.* 14, 549-558. https://doi.org/10.1007/s11816-020-00629-2.

17. Feeney, M., and Punja, Z. K. 2003. Tissue culture and *Agrobacterium*-mediated transformation of hemp (*Cannabis sativa* L.). *In Vitro Cell Dev. Biol. -Plant*, 39, 578-585. https://doi.org/10.1079/IVP2003454.

18. Feeney M. and Punja Z. K. 2015. Hemp (*Cannabis sativa* L.), in: Wang K. (eds), *Agrobacterium* Protocols, vol. 2. Methods in Molecular Biology, vol 1224. Springer, New York, 319-329. https://doi.org/10.1007/978-1-4939-1658-0_25.

19. Feeney, M., and Punja, Z. K. 2017. The role of *Agrobacterium*-mediated and other gene-transfer technologies in cannabis research and product development, in: S. Chandra, H. Lata, and M. A. ElSohly (eds), *Cannabis sativa* L. – Botany and Biotechnology. Springer, Cham, 343-363. https://doi.org/10.1007/978-3-319-54564-6_16.

20. Fraley, R. T., Rogers, S. G., Horsch, R. B., Sanders, P. R., Flick, J. S., Adams, S. P., ... and Woo, S. C. 1983. Expression of bacterial genes in plant cells. *Proc. Natl. Acad. Sci. USA*, 80(15), 4803-4807. https://doi.org/10.1073/pnas.80.15.4803.

21. Galán-Ávila, A., García-Fortea, E., Prohens, J., and Herraiz, F. J. 2020. Development of a direct *in vitro* plant regeneration protocol from *Cannabis sativa* L. seedling explants: developmental morphology of shoot regeneration and ploidy level of regenerated plants. *Front. Plant Sci.* 11:645. https://doi.org/10.3389/fpls.2020.0645.

22. Gao, C., Cheng, C., Zhao, L., Yu, Y., Tang, Q., Xin, P., ... and Zang, G. 2018. Genome-wide expression profiles of hemp (*Cannabis sativa* L.) in response to drought stress. *Int. J. Genom.* 2018, 3057272. https://doi.org/10.1155/2018/3057272.

23. Gaoni, Y., and Mechoulam, R. 1964. Isolation, structure, and partial synthesis of an active constituent of hashish. *J. Am. Chem. Soc.* 86(8), 1646-1647. https://doi.org/10.1021/ja01062a046.

24. Gosal, S. S., and Wani, S. H. 2018. Plant genetic transformation and transgenic crops: methods and applications, in: Gosal S., Wani S. (eds), Biotechnologies of Crop Improvement, vol. 2. Springer, Cham, 1-23. https://doi.org/10.1007/978-3-319-90650-8_1.

25. Guerriero, G., Behr, M., Hausman, J. F., and Legay, S. 2017. Textile hemp vs. salinity: Insights from a targeted gene expression analysis. *Genes*, 8(10), 242. https://doi.org/10.3390/genes8100242.

26. Hadad, L., Luria, N., Smith, E., Sela, N., Lachman, O., and Dombrovsky, A. 2019. Lettuce chlorosis virus disease: a new threat to cannabis production. *Viruses*, 11(9), 802. https://doi.org/10.3390/v11090802.

27. Hamill, J. D., Rounsley, S., Spencer, A., Todd, G., and Rhodes, M. J. 1991. The use of the polymerase chain reaction in plant transformation studies. *Plant Cell Rep.* 10(5), 221-224. https://doi.org/10.1007/BF00232562.

28. Herrera-Estrella L., Simpson J. and Martínez-Trujillo M. 2005. Transgenic Plants: An Historical Perspective, in: Peña L. (eds), Transgenic Plants: Methods and Protocols. Methods in Molecular Biology, vol. 286. Humana Press, 3-31. https://doi.org/10.1385/1-59259-827-7:003.

29. Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. 1987. GUS fusions: *beta-glucuronidase* as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6(13), 3901-3907. https://doi.org/10.1002/j.1460-2075.1987.tb02730.x.

30. Jerushalmi, S., Maymon, M., Dombrovsky, A., and Freeman, S. 2020. Fungal Pathogens Affecting the Production and Quality of Medical Cannabis in Israel. *Plants*, 9(7), 882. https://doi.org/10.3390/plants9070882.

31. Kullačová, D., and Matúšová, R. 2020. Establishment of *Phelipanche ramosa* tissue culture and effect of kanamycin on culture growth. *J. Microbiol. Biotechnol. Food Sci*. 4, 63-65. https://doi.org/10.15414/jmbfs.2015.4.special2.63-65.

32. Landi, S., Berni, R., Capasso, G., Hausman, J. F., Guerriero, G., and Esposito, S. 2019. Impact of nitrogen nutrition on *Cannabis sativa*: An update on the current knowledge and future prospects. *Int. J. Mol. Sci.* 20(22), 5803. https://doi.org/10.3390/ijms20225803.

33. Lassner, M. W., Peterson, P., and Yoder, J. I. 1989. Simultaneous amplification of multiple DNA fragments by polymerase chain reaction in the analysis of transgenic plants and their progeny. *Plant Mol. Biol. Rep.* 7(2), 116-128. https://doi.org/10.1007/BF02669627.

34. Li, J., Zhang, D., Que, Q., Chen, X., and Ouyang, K. 2019. Plant regeneration and *Agrobacterium*-mediated transformation of the miracle tree *Neolamarckia cadamba*. *Ind. Crops Prod.* 130, 443-449. https://doi.org/10.1016/j.indcrop.2019.01.009.

35. Lian, Z., Nguyen, C. D., Wilson, S., Chen, J., Gong, H., and Huo, H. 2020. An efficient protocol for *Agrobacterium*-mediated genetic transformation of *Antirrhinum majus*. *Plant Cell Tiss. Organ Cult.* 142(3), 527-536. https://doi.org/10.1007/s11240-020-01877-4

36. Lynch, R. C., Vergara, D., Tittes, S., White, K., Schwartz, C. J., Gibbs, M. J., ... and Kane, N. C. 2016. Genomic and chemical diversity in *Cannabis*. *Crit. Rev. Plant Sci.* 35(5-6), 349-363. https://doi.org/10.1080/07352689.2016.1265363.

37. Maggini, V., Bettini, P., Firenzuoli, F., and Bogani, P. 2021. An Efficient Method for the Genetic Transformation of *Acmella oleracea* L. (*Spilanthes acmella* Linn.) with *Agrobacterium tumefaciens*. *Plants*, 10(2), 198. https://doi.org/10.3390/plants10020198.

38. Mandolino, G., and Ranalli, P. 1999. Advances in biotechnological approaches for hemp breeding and industry, in: P. Ranalli (eds), Advances in hemp research. Haworth Press, New York, 185-212.

39. Meurer, C. A., Dinkins, R. D., and Collins, G. B. 1998. Factors affecting soybean cotyledonary node transformation. *Plant Cell Rep.* 18(3), 180-186. https://doi.org/10.1007/s002990050553.

40. Mishchenko, S., Mokher, J., Laiko, I., Burbulis, N., Kyrychenko, H., and Dudukova, S. 2017. Phenological growth stages of hemp (*Cannabis sativa* L.): codification and description according to the BBCH scale. *Ž. Moksl.* 24(2), 31-36. https://doi.org/10.6001/zemesukiomokslai.v24i2.3496.

41. Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant*. 15, 473-479. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

42. Nap, J. P., Bijvoet, J., and Stiekema, W. J. 1992. Biosafety of kanamycin-resistant transgenic plants. *Transgen. Res.* 1(6), 239-249. https://doi.org/10.1007/BF02525165.

43. Pawar, B. D., Jadhav, A. S., Kale, A. A., Chimote, V. P., and Pawar, S. V. 2013. Effect of explants, bacterial cell density and overgrowth-control antibiotics on transformation efficiency in tomato (*Solanum lycopersicum* L.). *J. Appl. Hortic.* 15(2), 95-99. https://doi.org/10.37855/jah.2013.v15i02.17.

44. Pollock, K., Barfield, D. G., and Shields, R. 1983. The toxicity of antibiotics to plant cell cultures. *Plant Cell Rep.* 2(1), 36-39. https://doi.org/10.1007/BF00269232.

45. Punja, Z. K., Collyer, D., Scott, C., Lung, S., Holmes, J., and Sutton, D. 2019. Pathogens and molds affecting production and quality of *Cannabis sativa* L. *Front. Plant Sci.* 10:1120. https://doi.org/10.3389/fpls.2019.01120.

46. Qin, Y. H., Da Silva, J. A. T., Bi, J. H., Zhang, S. L., and Hu, G. B. 2011. Response of *in vitro* strawberry to antibiotics. *Plant Growth Regul.* 65(1), 183-193. https://doi.org/10.1007/s10725-011-9587-9.

47. R Core Team. 2019. R: A language and environment for statistical computing. *R Foundation for Statistical Computing*, Vienna, Austria. URL https://www.R-project.org/.

48. Schachtsiek, J., Hussain, T., Azzouhri, K., Kayser, O., and Stehle, F. 2019. Virus-induced gene silencing (VIGS) in *Cannabis sativa* L. *Plant Methods*, 15(1), 157. https://doi.org/10.1186/s13007-019-0542-5.

49. Sorokin, A., Yadav, N. S., Gaudet, D., and Kovalchuk, I. 2020. Transient expression of the *β-glucuronidase* gene in *Cannabis sativa* varieties. *Plant Signal. Behav.* 15(8), 1780037. https://doi.org/10.1080/15592324.2020.1780037.

50. Srivastava, J., Datta, S., and Mishra, S. P. 2017. Development of an efficient *Agrobacterium* mediated transformation system for chickpea (*Cicer arietinum*). *Biologia*. 72(2), 153-160. https://doi.org/10.1515/biolog-2017-0015.

51. Vilanova, S., Alonso, D., Gramazio, P., Plazas, M., García-Fortea, E., Ferrante, P., ... and Prohens, J. 2020. SILEX: a fast and inexpensive high-quality DNA extraction method suitable for multiple sequencing platforms and recalcitrant plant species. *Plant Methods*. 16(1), 110. https://doi.org/10.1186/s13007-020-00652-y.

52. Wahby, I., Caba, J. M., and Ligero, F. 2013. *Agrobacterium* infection of hemp (*Cannabis sativa* L.): establishment of hairy root cultures. *J. Plant Interact*. 8, 312-320. https://doi.org/10.1080/17429145.2012.746399.

53. Wahby, I., Caba, J. M., and Ligero, F. 2017. Hairy root culture as a biotechnological tool in *C. sativa*, in: S. Chandra, H. Lata, and M. A. ElSohly (eds), *Cannabis sativa* L. – Botany and Biotechnology. Springer, Cham, 299-317.

54. Wróbel, T., Dreger, M., Wielgus, K., and Słomski, R. 2018. The application of plant *in vitro* cultures in cannabinoid production. *Biotechnol. Lett.* 40(3), 445-454. https://doi.org/10.1007/s10529-017-2492-1.

55. Yaqoob, U., Kaul, T., and Nawchoo, I. A. 2017. Development of an efficient protocol for *Agrobacterium* mediated transformation of some recalcitrant indica rice varieties. *Ind. J. Plant Physiol.* 22(3), 346-353. https://doi.org/10.1007/s40502-017-0304-1.

56. Zhang, X., Xu, G., Cheng, C., Lei, L., Sun, J., Xu, Y., ... and Su, J. (2021). Establishment of an *Agrobacterium*-mediated genetic transformation and CRISPR/Cas9-mediated targeted mutagenesis in Hemp (*Cannabis Sativa* L.). *Plant Biotechnol. J.* https://doi.org/10.1111/pbi.13611

57. Żuk-Gołaszewska K., and Gołaszewski J. 2020. Hemp Production, in: G. Crini and E. Lichtfouse (eds), *Sustainable Agriculture Reviews*, vol. 42. Springer, Cham, 1-36. https://doi.org/10.1007/978-3-030-41384-2_1.

General Discussion

In recent years, *C. sativa* versatility has promoted the booming of its research, which is mainly focused on its medical properties derived from its content in cannabinoids and other secondary metabolites. This trend is clearly reflected in the large number of recent publications dealing with this crop. After performing an exhaustive search in “PubMed” and “Web of Science”, Treister-Goltzman *et al.* (2019) reported that between the years 2000 and 2017, the number of publications on cannabis increased 4.5-fold, and the number of publications on medical cannabis increased almost 9-fold, while the number of publications on medical cannabis in “Web of Science” increased 10-fold. In that study, the authors also foresaw an even greater increase in the number of publications in this area in the coming years. Furthermore, according to data compiled by the “National Library of Medicine” and “PubMed.gov”, the record number of cannabis scientific papers published in 2020 confirms that scientific interest in cannabis has increased exponentially in recent years (Armentano, 2020).

As its prohibition dwindles, legalization is inevitably bringing an increase in cannabis cultivation and research and, with this, new challenges are emerging. To face them, the development of new biotechnological tools to complement cannabis conventional breeding should be a priority, thus mitigating the research deficit that decades of prohibition have promoted in cannabis breeding in comparison with other crops. In the case of medical cannabis, although nowadays the pharmacological utility of *C. sativa* and cannabinoids is well substantiated in a wide range of pathologies (National Academies of Sciences, Engineering, and Medicine, 2017), the lack of formal genetic improvement of this species is translated in the absence of uniform varieties. This, coupled with cannabis inherent variability, represents an important bottleneck to find a supply of stable cannabis varieties capable of producing the full range of potentially therapeutic cannabinoids (Lynch *et al*., 2016; Wróbel *et al*., 2018).

Connected with this, and from the point of view of cannabis medicinal use, it is necessary to emphasize the need to maintain promising chemotypes over time. It is crucial for researchers to exactly know the biochemical profile of the plant to be able to develop, from this, uniform extractions containing precise concentrations and combinations of cannabinoids, thus being able to quantify, evaluate and guarantee the quality of the preparations and adequately adapt the dose to be prescribed (Carcieri *et al*., 2018). At present, this problem is being addressed by keeping promising parentals in a perpetual vegetative state in indoor growing facilities. Nevertheless, new approaches must be applied, especially when cannabis cultivation is being implemented at a global scale, and reports on the large carbon footprint derived from cannabis indoor cultivation are emerging (Mills *et al.*, 2020; Summers *et al.*, 2021). Thus, it is mandatory to implement environmentally-friendly strategies in this rapid-evolving industry whose foundations are currently beginning to be erected. In this context, biotechnology must play a key role, offering a wide range of applications for the cannabis industry that can help to solve some of the barriers that stand in the way, obstacles such as the current need to implement cannabis genetic improvement. In this sense, it must be emphasized that, although some attempts through different biotechnological approaches such as employment of bioreactors for the production of cannabinoids have been reported (Veliky and Genest, 1972; Itokawa *et al.*, 1977; Hemphill *et al.*, 1978; Heitrich and Binder, 1982; Sirikantaramas *et al*., 2004; Raharjo *et al.*, 2006; Flores-Sanchez *et al.*, 2009; Farag and Kayser, 2015; Gabotti *et al.*, 2019), at present, *C. sativa* plants remain the most efficient source of natural cannabinoids (Wróbel *et al*., 2018). Thus, its genetic improvement presents as the main alternative for enhancing its secondary metabolite production.

Different biotechnological tools, like those studied in the present Doctoral Thesis, could complement conventional breeding in order to improve *C. sativa* species. Thus, each of the different tissue culture-based

procedures developed in this work represent by themselves an important milestone for *C. sativa* breeding. Among the different breakthroughs here reported, the identification of an explant lacking already developed meristems but with a high capability for plant regeneration, as is the case of hypocotyls, represents multiple possibilities for implementation of different plant *in vitro* tissue culture techniques focused on plant genetic improvement. This is particularly relevant in a species previously considered as recalcitrant to the *in vitro* plant regeneration, as is the case of *C. sativa* (Hemphill *et al*., 1978; Mandolino and Ranalli, 1999; Lusarkiewicz-Jarzina *et al*., 2005; Plawuszewski *et al*., 2006; Wielgus *et al*., 2008; Movahedi *et al*., 2015, 2016a, 2016b; Wahby *et al*., 2017; Feeney and Punja, 2017; Wróbel *et al*., 2018; Smýkalová *et al*., 2019). Furthermore, the present study contradicts findings reported in previous cannabis *in vitro* culture works considered as a reference in the field. In particular, our results concerning the poor *in vitro* plant regeneration capability of cannabis leaves, strongly contrast with the extremely high plant regeneration competence reported by Lata *et al.* (2010).

On the other hand, and although more research remains to be done to validate this hypothesis, behind the hypocotyl great capability for the plant *in vitro* regeneration, it could be the putative presence of pluripotent pericycle cells adjacent to vascular poles that, even though being surrounded by differentiated cells, can still lead the formation of a new organ (Beeckman and De Smet, 2014), as demonstrated in other plant species such as *Populus tremula* L. (Vinocur *et al.*, 2000), *Arabidopsis thaliana* L. Heynh. (Atta *et al.*, 2009), *Bixa orellana* L. (da Cruz *et al.*, 2014), *Eustoma grandiflorum* Raf. Shinners (Yumbla-Orbes *et al.*, 2017), or *Passiflora edulis* Sims. (Rocha *et al.*, 2018) among others. Furthermore, pluripotent pericycle cells likely play a key role in the distribution of the newly formed primordia along the whole main stem during plant growth, determining its location and, consequently, its precise arrangement in different spatial patterns conformed by the juxtaposition of the different

meristems around the circumference of the main apical meristem. In this respect, pericycle cell influence on lateral branching and phyllotaxis has already been reported in species from the Piperaceae family such as *Ottonia martiana* Miq. and *Piper diospyrifolium* Kunth. (Souza *et al.*, 2004). It is worth noting that the same plant regeneration pattern observed in our work and consisting of a couple of primordia growing in the periphery of the hypocotyl, strongly resembles the lateral branching pattern which defines cannabis phyllotaxis, being composed by branches emerging from opposite sides of the different internodes developed along the whole cannabis main stem during plant growth.

Also, the identification of polysomaty in *C. sativa* cotyledons and hypocotyls could represent, as demonstrated in the present Thesis, a new approach for the development of polyploids in this species. This is of especial relevance, not only because an increase in DNA content seems to be correlated with enhanced levels of the secondary metabolites involved in cannabis therapeutic use (Clarke, 1981; Mansouri and Bagheri, 2017; Parsons *et al*., 2019; Crawford *et al.*, 2021), but also because this is the first report concerning the regeneration of cannabis plants with an increased ploidy level without explant treatment with chemical microtubule disruptors with a high toxicity grade. This represents a safer procedure for lab technicians and also a more sustainable and environmentally-friendly alternative that also avoids the use of plant growth regulators in the induction process.

In contrast to an increased ploidy level, its reduction can also be exploited to genetically improve *C. sativa* species through double haploid development. This Thesis dealt with this novel approach by studying cannabis androgenesis via microspore embryogenesis. Although the vast majority of cannabis floral biology studies are focused on the female flower, which can be attributed to its crucial role in the therapeutic and psychoactive properties of *C. sativa* species, the cannabis male flower can

play a key role in the breeding of the species, as it has been demonstrated in the present work. Beyond pollination, the pollen grain can contribute to cannabis breeding through androgenesis, which must be considered as a promising alternative for the genetic standardization of *C. sativa* traits and the development of high-yielding and consistent varieties. Undoubtedly, microspore embryogenesis presents itself as an encouraging tool for breeders to avoid the inherent variability of *C. sativa* through double haploid plant development. Furthermore, this technique presents added advantages such as the preservation of the selected parentals through seed, thus avoiding the indefinite maintenance of mother plants in a perpetual state of growth, the consequent expenditure of energy, time and resources, and the environmental impact that this entails. Although no double haploid plants were recovered, this Thesis presents the most in-depth study to date concerning cannabis male floral biology, which includes the correlation of all developmental stages from meiosis to mature pollen grain stages with an easy-to-measure floral morphological marker as the bud length, together with the early events of microspore embryogenesis ontogeny in *C. sativa.* All these data represent valuable information that will help researchers interested in advancing in this promising field.

Finally, probably the most relevant milestone reached in the present Thesis, is the development of the first protocol for the production of stably transformed *C. sativa* plants. Although several attempts at cannabis transformation have been published (Feeney and Punja, 2003, 2015; Wahby *et al*., 2013, 2017; Schachtsiek et al., 2019; Ahmed et al., 2020; Deguchi *et al*., 2020; Sorokin *et al*., 2020; Beard *et al*., 2021), it was only recently that regeneration of one *C. sativa* transformed plant was reported (Zhang *et al*., 2021). In this respect, it is necessary to highlight that, although in the former work the authors claim that they were the first in obtaining a transgenic plant from *C. sativa* species, as it can be read in chapter 3 of the present Thesis, expressly in the “Conflict of interests” section, there is a PCT Application (PCT/EP2020/087829) covering the genetic transformation

protocol for the production of *C. sativa* transgenic or gene-edited plants, whose priority claim dates from 28th of December 2019, confirming that the present study is pioneering in cannabis genetic transformation. In any case, our hormone-free protocol doubles the transformation rate of regenerating shoots of the former work, also producing transgenic plants three times faster. High plant regeneration ability of *C. sativa* hypocotyls dissected from 7-days-old seedlings, coupled with the putatively unicellular origin of hypocotyl-derived regenerating shoots, might have facilitated the genetic transformation of a pluripotent pericycle cell and its subsequent regeneration into a whole transgenic plant, making the difference with previously published attempts concerning transgenic plant regeneration in this species.

The myriad of applications derived from genetic transformation of cannabis can hardly be estimated, but undoubtedly, among its different uses, targeted genome editing by using CRISPR/Cas systems must be highlighted. Genome editing can bring huge advancements for cannabis breeding. From pathogen resistance, to tolerance to drought stress, the possibilities are endless. However, before implementation of this technique in *C. sativa* species, it was imperative to develop an efficient transformation protocol that allowed the regeneration of stably transformed plants, an important breakthrough that has been achieved in this Thesis. The tissue culture-based protocol here described could enable the implementation of genome editing through CRISPR/Cas systems for *C. sativa* breeding, opening the doors of a new cannabis post-legalization era in which biotechnology is called to play a fundamental role.

The results contained in this Doctoral Thesis represent a very significant progress in cannabis research, and will allow a greater efficiency in the breeding of this species, as well as an increase in the genetic variation available for the breeder, with which new varieties with improved traits will be obtained.

Conclusions

1. *Cannabis sativa* hypocotyl explants present a high plant *in vitro* regeneration capability.

2. A highly-efficient and hormone-free *in vitro* tissue culture-based protocol from *C. sativa* hypocotyl explants which has proven useful for direct plant regeneration and spontaneous rooting of regenerants in all genotypes evaluated has been developed.

3. Polysomaty has been identified in *C. sativa* cotyledon and hypocotyl explants, and it has been possible to regenerate from them a significant percentage of mixoploid plants.

4. Staminate flowers from androecious, gynoecious and monoecious *C. sativa* plants have been obtained in only one month by growing plants directly under flowering photoperiod (12 hours of light and 12 hours of dark). Furthermore, the high amount of microspores and pollen grains contained in male flowers have been quantified, which have shown a high viability rate.

5. All developmental stages from meiosis to mature pollen grain stages have been characterized in *C. sativa*, correlating them with an easy-to-measure floral morphological marker as the bud length, and also identifying bud length intervals containing mostly vacuolate microspores and young bi-cellular pollen grains in all the phenotypes evaluated.

6. Based on the amyloplast pattern contained in microspores and pollen grains during their development, *C. sativa* has been classified as recalcitrant to androgenesis.

7. Although a week-long cold pretreatment applied directly on excised buds prior to microspore culture did not change the starch distribution pattern observed under *in vivo* conditions, it promoted a slight decrease in microspore viability. Furthermore, and although with an extremely low frequency, it fostered the development of the first *C. sativa* multicellular structures of androgenic origin which were easily differentiated from gametophytic ones by fluorescence microscopy.

8. Mainly due to the low albinism rate of regenerated plants, kanamycin proved to be a poor selectable marker for *C. sativa* transgenic plants.

9. An effective protocol for the production of stably genetically-transformed *C. sativa* plants derived from hypocotyls has been developed for the first time.

10. The results obtained in this Thesis represent a landmark in cannabis research and breeding and we are confident that they will contribute to the development of a new array of dramatically improved plant materials and varieties.

References (Introduction and General Discussion)

Abel, E. L. (1980). Cannabis in the ancient world. In: *Marihuana*, *the first twelve thousands years*, Boston, MA:Springer, 3-35.

Abrams, D. I. (2018). The therapeutic effects of *Cannabis* and cannabinoids: An update from the National Academies of Sciences, Engineering and Medicine report. *European Journal of Internal Medicine*, 49, 7-11. doi: 10.1016/j.ejim.2018.01.003.

Adelberg, J. W., Rhodes, Bill B. and Skorupska, H. T. (1993). Generating tetraploid melons in tissue culture. *Acta Horticulturae,* 336, 373–380. doi: 10.17660/actahortic.1993.336.49.

Ahmed, S., Gao, X., Jahan, M. A., Adams, M., Wu, N., and Kovinich, N. (2020). Nanoparticle-Based Genetic Transformation of *Cannabis sativa*. *J. Biotechnol.* 326, 48-51. https://doi.org/10.1016/j.jbiotec.2020.12.014.

Ahn, B. S., Kang, S., Lee, K. H., Kim, S., Park, J. S., & Seo, H. S. (2020). A Literature Analysis on Medicinal Use and Research of Cannabis in the Meiji Era of Japan. *Journal of Pharmacopuncture*, 23(3), 142. doi: 10.3831/KPI.2020.23.3.142.

Aionesei, T., Touraev, A. and Heberle-Bors, E. (2005). Pathways to microspore embryogenesis. In: *Haploids in crop improvement II* (pp. 11-34). Springer, Berlin, Heidelberg. https://doi.org/10.1007/3-540-26889-8_2.

Alagoz, Y., Gurkok, T., Zhang, B., and Unver, T. (2016). Manipulating the biosynthesis of bioactive compound alkaloids for next-generation metabolic engineering in opium poppy using CRISPR-Cas 9 genome editing technology. *Scientific reports*, 6(1), 1-9. doi: 10.1038/srep30910 (2016).

Anderson LC. (1980). Leaf variation among Cannabis species from a controlled garden. *Botanical Museum Leaflets, Harvard University,* 28(1): 61–69.

Andre, C. M., Hausman, J. F., and Guerriero, G. (2016). *Cannabis sativa*: the plant of the thousand and one molecules. *Frontiers in Plant Science*, 7, 19. https://doi.org/10.3389/fpls.2016.00019.

Armentano, P. (2020). Record Number of Scientific Papers Published in 2020 About Cannabis. https://norml.org/blog/2020/12/16/record-number-of-scientific-papers-published-in-2020-about-cannabis/

Armstrong, M. J. (2021). Legal cannabis market shares during Canada's first year of recreational legalisation. *International Journal of Drug Policy*, 88, 103028. https://doi.org/10.1016/j.drugpo.2020.103028.

Asif, M. (2013). History, production methods, and types of haploids. In: *Progress and Opportunities of Doubled Haploid Production*. Springer, Heidelberg, 1-6. https://doi.org/10.1007/978-3-319-00732-8_1.

Atta, R., Laurens, L., Boucheron-Dubuisson, E., Guivarc'h, A., Carnero, E., Giraudat-Pautot, V., *et al*. (2009). Pluripotency of *Arabidopsis* xylem pericycle underlies shoot regeneration from root and hypocotyl explants grown *in vitro*. *The Plant Journal*, 57, 626–44. doi:10.1111/j.1365-313X.2008.03715.x.

Bachhuber, M. A., Saloner, B., Cunningham, C. O. and Barry, C. L. (2014). Medical cannabis laws and opioid analgesic overdose mortality in the United States, 1999-2010. *JAMA internal medicine*, 174(10), 1668-1673. doi:10.1001/jamainternmed.2014.4005.

Bagci, E., Bruehl, L., Aitzetmuller, K. and Altan, Y. (2009). A chemotaxonomic approach to the fatty acid and tocochromanol content of *Cannabis sativa* L.(Cannabaceae). *Turkish Journal of Botany*, 27, 141-147.

Ballotta, D., Bergeron, H., and Hughes, B. (2008). *Cannabis* control in Europe. *EMCDDA MONOGRAPHS*, 99.

Bárány, I., González-Melendi, P., Fadón, B., Mitykó, J., Risueño, M. C. and Testillano, P. S. (2005). Microspore-derived embryogenesis in pepper (*Capsicum annuum* L.): subcellular rearrangements through development. *Biology of the Cell*, 97(9), 709-722. doi: 10.1042/bc20040142.

Beard, K. M., Boling, A. W., and Bargmann, B. O. (2021). Protoplast isolation, transient transformation, and flow-cytometric analysis of reporter-gene activation in *Cannabis sativa* L. *Industrial Crops and Products,* 164, 113360. https://doi.org/10.1016/j.indcrop.2021.113360.

Beeckman, T., and De Smet, I. (2014). Pericycle. *Current Biology*, 24, R378–R379. doi:10.1016/j.cub.2014.03.031.

Bewley-Taylor, D., and Jelsma, M. (2011). Fifty years of the 1961 Single Convention on Narcotic Drugs: A reinterpretation. *Series on legislative reform of drug policies*, 12, 1-20.

Bewley-Taylor, D. (2012). *International Drug Control: Consensus Fractured*. Cambridge/New York: Cambridge University Press.

Bewley-Taylor, D., Blickman, T., and Jelsma, M. (2014). The rise and decline of cannabis prohibition. The history of cannabis in the UN drug control system and options for reform. *Amsterdam: Global Drug Policy Observatory*.

Bewley-Taylor, D., Jelsma, M., and Kay, S. (2020). Cannabis Regulation and Development: Fair (er) Trade Options for Emerging Legal Markets. In: *Drug Policies and Development*. Brill Nijhoff, 106-124. doi:10.1163/9789004440494_007.

Blakeslee, A. F., Belling, J., Farnham, M. E. and Bergner, A. D. (1922). A haploid mutant in the Jimson Weed,"*Datura Stramonium*". *Science*, 55(1433), 646-647. doi: 10.1126/science.55.1433.646.

Booth, M. (2005). *Cannabis: a history*. Macmillan.

Bouamama-Gzara, B., Zemni, H., Zoghlami, N., Gandoura, S., Mliki, A., Arnold, M., *et al*. (2020). Behavior of *Opuntia ficus-indica* (L.) Mill. Heat-Stressed microspores under *in vitro* culture conditions as evidenced by microscopic analysis. *In Vitro Cellular & Developmental Biology-Plant,* 56, 122–133. doi: 10.1007/s11627-019-10032-4.

Bradford, A. C., Bradford, W. D., Abraham, A., and Adams, G. B. (2018). Association between US state medical cannabis laws and opioid prescribing in the Medicare Part D population. *JAMA internal medicine*, 178(5), 667-672. doi:10.1001/jamainternmed.2018.0266.

Breslavetz, L. (1926). Polyploide mitosen bei *Cannabis sativa* L. *Ber. Deutsch. Bot Ges*., 44, 498–502.

Breslavetz, L. (1932). Polyploide Mitosen bei *Cannabis sativa* L. II. *Ber. Deutsch. Bot. Ges.* 17, 644–649. doi: 10.1007/bf01909774.

Brukhin, V., Hernould, M., Gonzalez, N., Chevalier, C. and Mouras, A. (2003). Flower development schedule in tomato *Lycopersicon esculentum* cv. sweet cherry. *Sexual Plant Reproduction*, 15(6), 311-320. doi: 10.1007/s00497-003-0167-7.

Bruun, K., Pan, L., and Rexed, I. (1975). *The gentlemen's club: International control of drugs and alcohol* (Vol. 9). Chicago: University of Chicago Press.

Bubner, B., Gase, K., Berger, B., Link, D., and Baldwin, I. T. (2006). Occurrence of tetraploidy in *Nicotiana attenuata* plants after *Agrobacterium*-mediated transformation is genotype specific but independent of polysomaty of explant tissue. *Plant Cell Rep.* 25, 668–675. doi:10.1007/s00299-005-0111-4.

Calapai, F., Cardia, L., Sorbara, E. E., Navarra, M., Gangemi, S., Calapai, G., and Mannucci, C. (2020). Cannabinoids, Blood–Brain Barrier, and Brain Disposition. *Pharmaceutics*, 12(3), 265. https://doi.org/10.3390/pharmaceutics12030265.

Canonge, J., Philippot, M., Leblanc, C., Potin, P., and Bodin, M. (2020). Impedance flow cytometry allows the early prediction of embryo yields in wheat (*Triticum aestivum* L.) microspore cultures. *Plant Science*, 300, 110586. doi: 10.1016/j.plantsci.2020.110586.

Carcieri, C., Tomasello, C., Simiele, M., De Nicolò, A., Avataneo, V., Canzoneri, L., Cusato, J., Di Perri, G. and D'avolio, A. (2018). Cannabinoids concentration variability in cannabis olive oil galenic preparations. *Journal of Pharmacy and Pharmacology*, 70(1), 143-149. https://doi.org/10.1111/jphp.12845.

Carter, G. T., Flanagan, A. M., Earleywine, M., Abrams, D. I., Aggarwal, S. K. and Grinspoon, L. (2011). *Cannabis* in palliative medicine: improving care and reducing opioid-related morbidity. *American Journal of Hospice and Palliative Medicine*, 28(5), 297-303. https://doi.org/10.1177/1049909111402318.

Cascio, M. G., Pertwee, R. G., and Marini, P. (2017). The pharmacology and therapeutic potential of plant cannabinoids. In: *Cannabis sativa L.-botany and biotechnology.* Eds. S Chandra, H. Lata, and M. A. ElSohly, Berlin:Springer, 207–225. doi:10.1007/978-3-319-54564-6_9.

Chaohua, C., Gonggu, Z., Lining, Z., Chunsheng, G., Qing, T., Jianhua, C., ... and Jianguang, S. (2016). A rapid shoot regeneration protocol from the cotyledons of hemp (*Cannabis sativa* L.). *Industrial Crops and Products,* 83, 61-65. https://doi.org/10.1016/j.indcrop.2015.12.035.

Chatow, L., Nudel, A., Nesher, I., Hayo Hemo, D., Rozenberg, P., Voropaev, H., ... and Eyal, N. (2021). *In vitro* evaluation of the activity of terpenes and cannabidiol against Human Coronavirus E229. *Life*, 11(4), 290. https://doi.org/10.3390/life11040290.

Clarke, R. C. (1981). The genetics and breeding of *Cannabis*. In: *Marijuana Botany: An Advanced Study: The Propagation and Breeding of Distinctive Cannabis*. Eds. N. Hamel and D. Cross, Berkeley, CA:Ronin Publishing, 27–59.

Clarke, R., and Merlin, M. (2016). *Cannabis: evolution and ethnobotany*. Univ of California Press.

Clément, C., and Pacini, E. (2001). Anther plastids in angiosperms. *The Botanical Review*, 67(1), 54-73. doi: 10.1007/bf02857849.

Cosentino, S. L., Riggi, E., Testa, G., Scordia, D., and Copani, V. (2013). Evaluation of European developed fibre hemp genotypes (*Cannabis sativa* L.) in semi-arid Mediterranean environment. *Industrial Crops and Products,* 50, 312-324. https://doi.org/10.1016/j.indcrop.2013.07.059.

Crawford, S., Rojas, B. M., Crawford, E., Otten, M., Schoenenberger, T. A., Garfinkel, A. R., and Chen, H. (2021). Characteristics of the Diploid, Triploid, and Tetraploid Versions of a Cannabigerol-Dominant F1 Hybrid Industrial Hemp Cultivar, *Cannabis sativa* 'Stem Cell CBG'. *Genes*, 12(6), 923. https://doi.org/10.3390/genes12060923.

D'Amato, F. (1964). Endopolyploidy as a Factor in Plant Tissue Development. *Caryologia* 17, 41–52. doi:10.1080/00087114.1964.10796115.

da Cruz, A. C. F., Rocha, D. I., Iarema, L., Ventrella, M. C., Costa, M. G. C., Neto, V. B. P., and Otoni, W. C. (2014). *In vitro* organogenesis from root culture segments of *Bixa orellana* L.(Bixaceae). *In Vitro Cellular & Developmental Biology-Plant*, 50(1), 76-83. https://doi.org/10.1007/s11627-013-9580-2.

de Lamarck, J. B. P. A. D. M. (1783). *Encyclopédie méthodique ou par ordre de matières: botanique* (Vol. 1). chez Panckoucke.

de Meijer, E. P., Bagatta, M., Carboni, A., Crucitti, P., Moliterni, V. C., Ranalli, P., and Mandolino, G. (2003). The inheritance of chemical phenotype in *Cannabis sativa* L. *Genetics*, 163(1), 335-346. https://doi.org/10.1093/genetics/163.1.335.

De Pinho, A. R. (2011). Social and medical aspects of the use of cannabis in Brazil. *Spirits, Shamans, and Stars: Perspectives from South America*, 85.

Deguchi, M., Bogush, D., Weeden, H., Spuhler, Z., Potlakayala, S., Kondo, T., ... and Rudrabhatla, S. (2020). Establishment and optimization of a hemp (*Cannabis sativa* L.) agroinfiltration system for gene expression and silencing studies. *Scientific Reports,* 10(1), 1-11. https://doi.org/10.1038/s41598-020-60323-9.

Denduluri, S. K., Woolson, S. T., Indelli, P. F., Mariano, E. R., Harris, A. H., and Giori, N. J. (2021). Cannabinoid and Opioid Use Among Total Joint Arthroplasty Patients: A 6-Year, Single-Institution Study. *Orthopedics*, 44(1), e101-e106. doi: 10.3928/01477447-20200928-02.

Dunwell, J. M. (2010). Haploids in flowering plants: origins and exploitation. *Plant Biotechnology Journal*, 8(4), 377-424. doi: 10.1111/j.1467-7652.2009.00498.x.

Dwivedi, S. L., Britt, A. B., Tripathi, L., Sharma, S., Upadhyaya, H. D., and Ortiz, R. (2015). Haploids: constraints and opportunities in plant breeding. *Biotechnology Advances*, 33(6), 812-829. doi: 10.1016/j.biotechadv.2015.07.001.

Ervin, C. D. (1939). Polysomaty in *Cucumis melo*. *Proc. Natl. Acad. Sci. U. S. A.* 25, 335–338. doi: 10.1073/pnas.25.7.335.

Ervin, C. D. (1941). A study of polysomaty in *Cucumis melo*. *Am. J. Bot.* 28, 113–124. doi:10.1002/j.1537-2197.1941.tb07950.x.

Farag, S., and Kayser, O. (2015). Cannabinoids production by hairy root cultures of *Cannabis sativa* L. *American Journal of Plant Sciences*, 6(11), 1874. doi: 10.4236/ajps.2015.611188.

Feeney, M., and Punja, Z. K. (2003). Tissue culture and *Agrobacterium*-mediated transformation of hemp (*Cannabis sativa* L.). *In Vitro Cell Dev. Biol. -Plant*, 39, 578-585. https://doi.org/10.1079/IVP2003454.

Feeney M. and Punja Z. K. (2015). Hemp (*Cannabis sativa* L.). In: *Agrobacterium* Protocols, vol. 2. Methods in Molecular Biology, vol 1224. Eds. Wang K. Springer, New York, 319-329. https://doi.org/10.1007/978-1-4939-1658-0_25.

Feeney, M., and Punja, Z. K. (2017). The role of *Agrobacterium*-mediated and other gene-transfer technologies in cannabis research and product development. In: *Cannabis sativa L.-botany and biotechnology.* Eds. S Chandra, H. Lata, and M. A. ElSohly, Berlin:Springer, 343-363. https://doi.org/10.1007/978-3-319-54564-6_16.

Ferrie, A.M.R. (2013) Advances in Microspore Culture Technology: A Biotechnological Tool for the Improvement of Medicinal Plants. In: *Biotechnology for Medicinal Plants.* Eds. Chandra S., Lata H., and Varma A. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-29974-2_8.

Flores-Sanchez, I. J., Peč, J., Fei, J., Choi, Y. H., Dušek, J., and Verpoorte, R. (2009). Elicitation studies in cell suspension cultures of *Cannabis sativa* L. *Journal of Biotechnology*, 143(2), 157-168. https://doi.org/10.1016/j.jbiotec.2009.05.006.

Fuchs, L. (1999). *The Great Herbal of Leonhart Fuchs: De Historia Stirpium Commentarii Insignes, 1542 (Notable Commentaries on the History of Plants)*. Stanford, CA: Stanford University Press.

Gabotti, D., Locatelli, F., Cusano, E., Baldoni, E., Genga, A., Pucci, L., ... and Mattana, M. (2019). Cell suspensions of *Cannabis sativa* (var. Futura): effect of elicitation on metabolite content and antioxidant activity. *Molecules*, 24(22), 4056. https://doi.org/10.3390/molecules24224056

Gallily, R., Yekhtin, Z. and Hanuš, L. O. (2015). Overcoming the bell-shaped dose-response of cannabidiol by using cannabis extract enriched in cannabidiol. *Pharmacology and Pharmacy*, 6, 75-85. doi: 10.4236/pp.2015.62010.

Gao, C., Cheng, C., Zhao, L., Yu, Y., Tang, Q., Xin, P., ... and Zang, G. (2018). Genome-wide expression profiles of hemp (*Cannabis sativa* L.) in response to drought stress. *International Journal of Genomics.* https://doi.org/10.1155/2018/3057272.

Gao, Q., Li, G., Sun, H., Xu, M., Wang, H., Ji, J., ... and Zhao, X. (2020). Targeted mutagenesis of the rice FW 2.2-like gene family using the CRISPR/Cas9 system reveals OsFWL4 as a regulator of tiller number and plant yield in rice. *International Journal of Molecular Sciences*, 21(3), 809. https://doi.org/10.3390/ijms21030809

Gaoni, Y., and Mechoulam, R. (1964). Isolation, structure, and partial synthesis of an active constituent of hashish. *Journal of the American Chemical Society*, 86(8), 1646-1647. https://doi.org/10.1021/ja01062a046.

García-Fortea, E., Lluch-Ruiz, A., Pineda-Chaza, B. J., García-Pérez, A., Bracho-Gil, J. P., Plazas, M., ... and Prohens, J. (2020). A highly efficient organogenesis protocol based on zeatin riboside for *in vitro* regeneration of eggplant. *BMC Plant Biology*, 20(1), 6. doi: 10.1186/s12870-019-2215-y.

Gilmore S, Peakall R, Robertson J. (2007). Organelle DNA haplotypes reflect crop-use characteristics and geographic origins of *Cannabis sativa*. *Forensic Science International*, 172, 179–190. https://doi.org/10.1016/j.forsciint.2006.10.025.

Guerriero, G., Behr, M., Hausman, J. F., and Legay, S. (2017). Textile hemp vs. salinity: Insights from a targeted gene expression analysis. *Genes*, 8(10), 242. https://doi.org/10.3390/genes8100242.

Guha, S. and Maheshwari, S. C. (1964). *In vitro* production of embryos from anthers of *Datura*. *Nature*, 204(4957), 497. https://doi.org/10.1038/204497a0.

Guha-Mukherjee, S. (1999). The discovery of haploid production by anther culture. *In Vitro Cellular & Developmental Biology-Plant*, 35(5), 357-360. https://doi.org/10.1007/s11627-999-0048-3.

Hadad, L., Luria, N., Smith, E., Sela, N., Lachman, O., and Dombrovsky, A. (2019). Lettuce chlorosis virus disease: a new threat to cannabis production. *Viruses*, 11(9), 802. https://doi.org/10.3390/v11090802.

Heberle-Bors, E. (1989). Isolated pollen culture in tobacco: plant reproductive development in a nutshell. *Sexual Plant Reproduction*, 2(1), 1-10. https://doi.org/10.1007/BF00190112.

Heitrich, A., and Binder, M. (1982). Identification of (3R, 4R)-gD 1 (6) as an isolation artefact of cannabinoid acids formed by callus cultures of *Cannabis sativa* L. *Experientia*, 38(8), 898-899. https://doi.org/10.1007/BF01953640.

Hemphill, J. K., Turner, J. C., and Mahlberg, P. G. (1978). Studies on growth and cannabinoid composition of callus derived from different strains of *Cannabis sativa*. *Lloydia* 41, 453–462.

Hillig, K.W. and Mahlberg, P.G. (2004). A chemotaxonomic analysis of cannabinoid variation in *Cannabis* (Cannabaceae). *American Journal of Botany*, 91, 966-975. https://doi.org/10.3732/ajb.91.6.966.

Hillig, K. W. (2005). Genetic evidence for speciation in *Cannabis* (Cannabaceae). *Genetic Resources and Crop Evolution*, 53, 161–180. doi: 10.1007/s10722-003-4452-y.

Hsu, G., and Kovács, B. (2021). Association between county level cannabis dispensary counts and opioid related mortality rates in the United States: panel data study. *bmj*, 372. https://doi.org/10.1136/bmj.m4957.

Hurgobin, B., Tamiru-Oli, M., Welling, M. T., Doblin, M. S., Bacic, A., Whelan, J., and Lewsey, M. G. (2020). Recent advances in *Cannabis sativa* genomics research. *New Phytologist*, 230(1), 73-89. https://doi.org/10.1111/nph.17140.

Iannicelli, J., Guariniello, J., Tossi, V. E., Regalado, J. J., Di Ciaccio, L., van Baren, C. M., *et al*. (2019). The “polyploid effect” in the breeding of aromatic and medicinal species. *Scientia Horticulturae,* 260, 108854. doi:10.1016/j.scienta.2019.108854.

Indian Hemp Drugs Commission. (1894). *Report of the Indian Hemp Drug Commission, 1893-94*. Government Central Printing Office, Simla.

Itokawa, H., Takeya, K., and Mihashi, S. (1977). Biotransformation of cannabinoid precursors and related alcohols by suspension cultures of callus induced from *Cannabis sativa* L. *Chemical and Pharmaceutical Bulletin*, 25(8), 1941-1946. https://doi.org/10.1248/cpb.25.1941.

Janischevsky, D. E. (1924). Forma konopli na sornykh mestakh v Yugovostochnoi Rossii. *Uchenye Zapiski Gosudarstvennogo Saratovskogo imeni NG Chernyshevskogo Universiteta. Fisiko-Matematicheskoye otdelenie Pedagogicheskogo Fakul'teta*, 2(2), 3-17.

Jerushalmi, S., Maymon, M., Dombrovsky, A., and Freeman, S. (2020). Fungal Pathogens Affecting the Production and Quality of Medical Cannabis in Israel. *Plants*, 9(7), 882. https://doi.org/10.3390/plants9070882.

Kaur, N., Alok, A., Kumar, P., Kaur, N., Awasthi, P., Chaturvedi, S., ... and Tiwari, S. (2020). CRISPR/Cas9 directed editing of lycopene epsilon-cyclase modulates metabolic flux for β-carotene biosynthesis in banana fruit. *Metabolic Engineering*, 59, 76-86. https://doi.org/10.1016/j.ymben.2020.01.008.

Kihara, H., and Nishiyama, I. (1947). An application of sterility of autotriploids to the breeding of seedless watermelons. *Seiken Ziho*, 3, 93–103.

Kim, M., Kim, J., Yoon, M., Choi, D. I., and Lee, K. M. (2004). Origin of multicellular pollen and pollen embryos in cultured anthers of pepper (*Capsicum annuum*). *Plant Cell Tissue Organ Culture*, 77, 63–72. doi: 10.1023/b:ticu.0000016506.02796.6a.

Köhler, F. E. (1887). Köhlers Medizinal-Pflanzen in naturgetreuen Abbildungen und kurz erläuterndem Texte. *Köhler, Gera, Germany (1883-1914)*.

Kolano, B., Siwinska, D., and Maluszynska, J. (2008). Endopolyploidy patterns during development of *Chenopodium quinoa*. *Acta Biologica Cracoviensia Series Botanica,* 51, 85–92.

Kurtz, L. E., Brand, M. H., and Lubell-Brand, J. D. (2020). Production of Tetraploid and Triploid Hemp. *HortScience*, 55(10), 1703-1707. https://doi.org/10.21273/HORTSCI15303-20.

Landi, S., Berni, R., Capasso, G., Hausman, J. F., Guerriero, G., and Esposito, S. (2019). Impact of nitrogen nutrition on *Cannabis sativa*: An update on the current knowledge and future prospects. *International Journal of Molecular Sciences*, 20(22), 5803. https://doi.org/10.3390/ijms20225803.

Langlet, O. (1927). Zur Kenntnis der polysomatischen Zellkerne im Wurzelmeristem. *Sven. Bot. Tidskr.* 21, 397–422.

Lata, H., Chandra, S., Khan, I. A., and ElSohly, M. A. (2009). Thidiazuron induced high-frequency direct shoot organogenesis of *Cannabis sativa* L. *Vitr. Cell. Dev. Biol. - Plant* 45 (1), 12–19. doi: 10.1007/s11627-008-9167-5.

Lata, H., Chandra, S., Khan, I. A., and Elsohly, M. A. (2010). High frequency plant regeneration from leaf derived callus of high Δ9-tetrahydrocannabinol yielding *Cannabis sativa* L. *Planta Med.* 76, 1629–1633. doi:10.1055/s-0030-1249773.

Lata, H., Chandra, S., Khan, I. A., and ElSohly, M. A. (2016a). *In Vitro* Propagation of *Cannabis sativa* L. and Evaluation of Regenerated Plants for Genetic Fidelity and Cannabinoids Content for Quality Assurance. *Methods Mol. Biol.* 1391, 275–88. doi:10.1007/978-1-4939-3332-7_19.

Lata, H., Chandra, S., Techen, N., Khan, I. A., and ElSohly, M. A. (2016b). *In vitro* mass propagation of *Cannabis sativa* L.: A protocol refinement using novel aromatic cytokinin meta-topolin and the assessment of eco-physiological, biochemical and genetic fidelity of micropropagated plants. *J. Appl. Res. Med. Aromat. Plants* 3, 18–26. doi:10.1016/j.jarmap.2015.12.001.

Lata, H., Chandra, S., Khan, I. A., and ElSohly, M. A. (2017). Micropropagation of *Cannabis sativa* L.-An update. In: *Cannabis sativa L.-botany and biotechnology.* Eds. S Chandra, H. Lata, and M. A. ElSohly, Berlin: Springer, 285–297. doi:10.1007/978-3-319-54564-6_13.

LaVigne, J. E., Hecksel, R., Keresztes, A., and Streicher, J. M. (2021). *Cannabis sativa* terpenes are cannabimimetic and selectively enhance cannabinoid activity. *Scientific Reports*, 11(1), 1-15. https://doi.org/10.1038/s41598-021-87740-8.

Linnaeus, C. (1753). *Species plantarum*. Salvius, Stockholm.

Litardière, R. D. (1925). Sur l'existence de figures didiploides dans le meristeme radiculaire du *Cannabis sativa* L. *Cellule,* 35, 21–25.

Long, T., Wagner, M., Demske, D., Leipe, C., and Tarasov, P. E. (2017). *Cannabis* in Eurasia: origin of human use and Bronze Age trans-continental connections. *Vegetation History and Archaeobotany*, 26(2), 245-258. https://doi.org/10.1007/s00334-016-0579-6.

López, C. D., Boddapati, V., Jobin, C. M., and Hickernell, T. R. (2021). State medical cannabis laws associated with reduction in opioid prescriptions by orthopaedic surgeons in Medicare Part D cohort. *JAAOS-Journal of the American Academy of Orthopaedic Surgeons*, 29(4), e188-e197. doi: 10.5435/JAAOS-D-19-00767.

Lusarkiewicz-Jarzina, A. S., Ponitka, A., and Kaczmarek, Z. (2005). Influence of cultivar, explant source and plant growth regulator on callus induction and plant regeneration of *Cannabis sativa* L. *Acta Biologica Cracoviensia Series Botanica*, 47(2), 145-151.

Lynch, R. C., Vergara, D., Tittes, S., White, K., Schwartz, C. J., Gibbs, M. J., ... and Kane, N. C. (2016). Genomic and chemical diversity in *Cannabis*. *Critical Reviews in Plant Sciences*, 35(5-6), 349-363. https://doi.org/10.1080/07352689.2016.1265363.

Maheshwari, S. C., Tyagi, A. K., Malhotra, K., and Sopory, S. K. (1980). Induction of haploidy from pollen grains in angiosperms - the current status. *Theoretical and Applied Genetics*, 58(5), 193-206. doi: 10.1007/bf00288437.

Maheshwari, S. C., Rashid, A., and Tyagi, A. K. (1982). Haploids from pollen grains – Retrospect and prospect. *American Journal of Botany*, 69(5), 865-879. https://doi.org/10.2307/2442979.

Malhotra, K. and Maheshwari, S. C. (1977). Enhancement by cold treatment of pollen embryoid development in *Petunia hybrida*. *Zeitschrift für Pflanzenphysiologie*, 85(2), 177-180. https://doi.org/10.1016/S0044-328X(77)80293-6.

Maluszynski, M., Kasha, K. J., and Szarejko, I. (2003). Published doubled haploid protocols in plant species. In: *Doubled Haploid Production in Crop Plants.* Springer, Dordrecht, 309-335. https://doi.org/10.1007/978-94-017-1293-4_46.

Mandaron, P., Niogret, M. E., Mache, R., and Moneger, F. (1990). *In vitro* protein synthesis in isolated microspores of *Zea mays* at several stages of development. *Theoretical and Applied Genetics*, 80(1), 134-138. doi: 10.1007/bf00224027.

Mandolino, G., and Ranalli, P. (1999). Advances in Biotechnological Approaches for Hemp Breeding and Industry. In: *Advances in Hemp Research*, CRC Press, 185–212. doi:10.1201/9781498705820.

Mansouri, H., and Bagheri, M. (2017). Induction of polyploidy and its effect on Cannabis sativa L. In: *Cannabis sativa L. – Botany and Biotechnology*. Eds. S Chandra, H. Lata, and M. A. ElSohly. Berlin: Springer, 365-383. doi: 10.1007/978-3-319-54564-6_17.

Maraschin, S. D. F., De Priester, W., Spaink, H. P., and Wang, M. (2005). Androgenic switch: an example of plant embryogenesis from the male gametophyte perspective. *Journal of Experimental Botany*, 56(417), 1711-1726. doi10.1093/jxb/eri190.

McPartland, J. M., and Guy, G. W. (2004). The evolution of Cannabis and coevolution with the cannabinoid receptor - a hypothesis. In: *The Medicinal Uses of Cannabis and Cannabinoids*. Eds. G. W. Guy, B. A. Whittle, and P. J. Robson. London:Pharmaceutical Press, 71–101.

McPartland, JM, and Guy, GW. (2014). A question of rank: Using DNA barcodes to classify *Cannabis sativa* and *Cannabis indica*. In: *Proceedings of the 24th annual symposium on the Cannabinoids. International Cannabinoid Research Society, Research Triangle Park, NC*, 54.

McPartland, J. M., and Guy, G. W. (2017). Models of cannabis taxonomy, cultural bias, and conflicts between scientific and vernacular names. *The Botanical Review*, 83(4), 327-381. https://doi.org/10.1007/s12229-017-9187-0.

McPartland, J. M. (2017). *Cannabis sativa* and *Cannabis indica* versus "Sativa" and "Indica". In: *Cannabis sativa L.-botany and biotechnology.* Eds. S Chandra, H. Lata, and M. A. ElSohly, Berlin:Springer, 101-121. https://doi.org/10.1007/978-3-319-54564-6_4.

McPartland, J. M. (2018). *Cannabis* systematics at the levels of family, genus, and species. *Cannabis and Cannabinoid Research*, 3(1), 203-212. https://doi.org/10.1089/can.2018.0039.

McPartland, J. M., Hegman, W., and Long, T. (2019). *Cannabis* in Asia: its center of origin and early cultivation, based on a synthesis of subfossil pollen and archaeobotanical studies. *Vegetation, History and Archaeobotany*, 28(6), 691-702. https://doi.org/10.1007/s00334-019-00731-8.

Mikulic M. (2021). Current and projected legal cannabis market value in the United States in 2016 and 2025. https://www.statista.com/statistics/765671/value-of-the-us-cannabis-market-current-and-future/.

Mills, E., Zeramby, S., Corva, D., and Meisel, J. (2020). Energy Use by the Indoor Cannabis Industry: Inconvenient Truths for Producers, Consumers, and Policymakers. *The Routledge Handbook of Post-Prohibition Cannabis Research*.

Movahedi, M., Ghasemi-Omran, V. O., and Torabi, S. (2015). The effect of different concentrations of TDZ and BA on *in vitro* regeneration of iranian cannabis (*Cannabis sativa* L.) using cotyledon and epicotyl explants. *Journal of Plant Molecular Breeding*, 3, 20–27.

Movahedi, M., Ghasemi-Omran, V. O., and Torabi, S. (2016a). *In vitro* callus induction and regeneration of medicinal plant *Cannabis sativa* L. *Iranian Journal of Medicinal and Aromatic Plants*, 32, 758–769.

Movahedi, M., Ghasemiomran, V., and Torabi, S. (2016b). Effect of explants type and plant growth regulators on *in vitro* callus induction and shoot regeneration of *Cannabis sativa* L. *Iran. J. Med. Aromat. Plants* 32, 83–96.

Muacevic, A., Adler, J., Takakuwa, K., and Sulak, D. (2021). A Survey on the Effect That Medical Cannabis Has on Prescription Opioid Medication Usage for the Treatment of Chronic Pain at Three Medical Cannabis Practice Sites. *Cureus*, 12(12). doi: 10.7759/cureus.11848.

Murovec, J., and Bohanec, B. (2012). Haploids and doubled haploids in plant breeding. In: *Plant Breeding*, 87-106. InTech Europe. doi: 10.5772/29982.

National Academies of Sciences, Engineering, and Medicine (2017). *The Health Effects of Cannabis and Cannabinoids: The Current State of Evidence and Recommendations for Research*. Washington, DC:The National Academies Press. https://doi.org/10.17226/24625.

Nekrasov, V., Wang, C., Win, J., Lanz, C., Weigel, D., and Kamoun, S. (2017). Rapid generation of a transgene-free powdery mildew resistant tomato by genome deletion. *Scientific Reports*, 7(1), 1-6. https://doi.org/10.1038/s41598-017-00578-x.

Nitsch, C. and Norreel, B. (1973a). Effet d'un choc thermique sur le pouvoir embryogène du pollen de *Datura innoxia* cultivé dans l'anthère et isolé de l'anthère. *C.R. Acad. Sci*. *Paris*, 276, 303-306.

Nitsch, C. and Norreel, B. (1973b). Factors favoring the formation of androgenetic embryos in anther culture. In: *Genes, Enzymes, and Populations*. Springer, Boston, MA, 129-144. https://doi.org/10.1007/978-1-4684-2880-3_10.

Nitsch, C. (1974a). Pollen culture - a new technique for mass production of haploid and homozygous plants. In: *Haploids in Higher Plants - Advances and Potential*. K. J. Kasha, University of Guelph, Guelph, 123-135.

Nitsch, C. (1974b). La culture de pollen isolé sur milieu synthétique. *C.R. Acad. Sci. Paris*, 278, 1031-1034.

O'Sullivan, S. E., Stevenson, C. W., and Laviolette, S. R. (2021). Could cannabidiol be a treatment for coronavirus disease-19-related anxiety disorders?. *Cannabis and Cannabinoid Research*, 6(1), 7-18. https://doi.org/10.1089/can.2020.0102.

Okusanya, B.O., Asaolu, I.O., Ehiri, J.E. *et al*. (2020). Medical cannabis for the reduction of opioid dosage in the treatment of non-cancer chronic pain: a systematic review. *Systematic reviews*, 9(1), 1-8. https://doi.org/10.1186/s13643-020-01425-3.

Onofri, C., and Mandolino, G. (2017). Genomics and Molecular Markers in *Cannabis sativa* L. In: *Cannabis sativa L. - Botany and Biotechnology*. Eds. S Chandra, H. Lata, and M. A. ElSohly. Berlin: Springer, 319-342. https://doi.org/10.1007/978-3-319-54564-6_15.

Parsons, J. L., Martin, S. L., James, T., Golenia, G., Boudko, E. A., and Hepworth, S. R. (2019). Polyploidization for the genetic improvement of *Cannabis sativa*. *Frontiers in Plant Sciences,* 10:476. doi:10.3389/fpls.2019.00476.

Pauls, K. P., Chan, J., Woronuk, G., Schulze, D., and Brazolot, J. (2006). When microspores decide to become embryos - cellular and molecular changes. *Botany*, 84(4), 668-678. doi: 10.1139/b06-064.

Pechan, P. M. and Keller, W. A. (1988). Identification of potentially embryogenic microspores in *Brassica napus*. *Physiologia Plantarum*, 74(2), 377-384. doi: 10.1111/j.1399-3054.1988.tb00646.x.

Pisanti, S., and Bifulco, M. (2019). Medical Cannabis: A plurimillennial history of an evergreen. *Journal of Cellular Physiology*, 234(6), 8342-8351. https://doi.org/10.1002/jcp.27725.

Plawuszewski, M., Lassocinski, W., and Wielgus, K. (2006). Regeneration of Polish cultivars of monoecious hemp (*Cannabis sativa* L.) grown in *in vitro* cultures. In: *Renewable resources and plant biotechnology*, 149–154.

Prohibition Partners. (2018). European cannabis report. 3th ed. https://prohibitionpartners.com/reports/.

Prohibition Partners. (2019a). The UK cannabis report. https://prohibitionpartners.com/reports/.

Prohibition Partners. (2019b). European cannabis report. 4th ed. https://prohibitionpartners.com/reports/.

Punja, Z. K., Collyer, D., Scott, C., Lung, S., Holmes, J., and Sutton, D. (2019). Pathogens and molds affecting production and quality of *Cannabis sativa* L. *Frontiers in Plant Science,* 10:1120. https://doi.org/10.3389/fpls.2019.01120.

Raharjo, T. J., Eucharia, O., Chang, W. T., and Verpoorte, R. (2006). Callus induction and phytochemical characterization of *Cannabis sativa* cell suspension cultures. *Indonesian Journal of Chemistry*, 6(1), 70-74. https://doi.org/10.22146/ijc.21776.

Ramsay, G., and Kumar, A. (1990). Transformation of *Vicia faba* cotyledon and stem tissues by *Agrobacterium rhizogenes*: Infectivity and cytological studies. *Journal of Experimental Botany,* 41, 841–847. doi:10.1093/jxb/41.7.841.

Ranalli, P. (2004). Current status and future scenarios of hemp breeding. *Euphytica*, 140(1-2), 121-131. https://doi.org/10.1007/s10681-004-4760-0.

Rashid, A. and Reinert, J. (1980). Selection of embryogenic pollen from cold-treated buds of *Nicotiana tabacum* var. Badischer burley and their development into embryos in cultures. *Protoplasma*, 105(1-2), 161-167. https://doi.org/10.1007/BF01279858.

Rashid, A. and Reinert, J. (1981). Differentiation of embryogenic pollen in cold-treated buds of *Nicotiana tabacum* var. Badischer burley and nutritional requirements of the isolated pollen to form embryos. *Protoplasma*, 106(1-2), 137-144. https://doi.org/10.1007/BF02115967.

Recupero, G. R., Russo, G., and Recupero, S. (2005). New Promising *Citrus* Triploid Hybrids Selected from Crosses between Monoembryonic Diploid Female and Tetraploid Male Parents. *HortScience* 40, 516–520. doi: 10.21273/hortsci.40.3.516.

Reiman, A., Welty, M., and Solomon, P. (2017) *Cannabis* as a substitute for opioid-based pain medication: patient self-report. *Cannabis and Cannabinoid Research,* 2:1, 160–166. doi: 10.1089/can.2017.0012. https://doi.org/10.1089/can.2017.0012.

Reimann-Philipp, U., Speck, M., Orser, C., Johnson, S., Hilyard, A., Turner, H., ... and Small-Howard, A. L. (2020). *Cannabis* chemovar nomenclature misrepresents chemical and genetic diversity; survey of variations in chemical profiles and genetic markers in Nevada medical cannabis samples. *Cannabis and Cannabinoid Research*, 5(3), 215-230. https://doi.org/10.1089/can.2018.0063.

Reinert, J. and Bajaj, Y. P. S. (1977). Anther culture: Haploid production and its significance. In: *Applied and fundamental aspects of plant cell, tissue and organ culture*. Eds. Reinert, J., and Bajaj, Y. P. S. Berlin-Heidelberg-New York:Springer, 251-267.

Ren, G., Zhang, X., Li, Y., Ridout, K., Serrano-Serrano, M. L., Yang, Y., ... and Fumagalli, L. (2021). Large-scale whole-genome resequencing unravels the domestication history of *Cannabis sativa*. *Science Advances*, 7(29), eabg2286.

Ribalta, F. M., Croser, J. S. and Ochatt, S. J. (2012). Flow cytometry enables identification of sporophytic eliciting stress treatments in gametic cells. *Journal of Plant Physiology*, 169(1), 104-110. https://doi.org/10.1016/j.jplph.2011.08.013.

Richez-Dumanois, C., Braut-Boucher, F., Cosson, L., and Paris, M. (1986). Multiplication végétative in vitro du chanvre (*Cannabis sativa* L.). Application à la conservation des clones sélectionnés. *Agron. EDP Sci.,* 6 (5), 487–495. doi: 10.1051/agro:19860510.

Rocha, D. I., Vieira, L. M., Koehler, A. D., and Otoni, W. C. (2018). Cellular and morpho-histological foundations of *in vitro* plant regeneration. In: *Plant Cell Culture Protocols*. Humana Press, New York, NY, 47-68. https://doi.org/10.1007/978-1-4939-8594-4_3.

Russo, E. B. (2011). Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. *British Journal of Pharmacology*, 163(7), 1344-1364. https://doi.org/10.1111/j.1476-5381.2011.01238.x.

Russo, E. B. (2019). The case for the entourage effect and conventional breeding of clinical cannabis: no “strain,” no gain. *Frontiers in Plant Science*, 9:1969. doi: 10.3389/fpls.2018.01969.

Russo, E. B., Jiang, H. E., Li, X., Sutton, A., Carboni, A., Del Bianco, F., ... and Li, C. S. (2008). Phytochemical and genetic analyses of ancient cannabis from Central Asia. *Journal of Experimental Botany*, 59(15), 4171-4182. https://doi.org/10.1093/jxb/ern260.

Salami, M. (2008). *Cannabis sativa* L. Dr. Grow's production.

Salentijn, E. M., Zhang, Q., Amaducci, S., Yang, M., and Trindade, L. M. (2015). New developments in fiber hemp (*Cannabis sativa* L.) breeding. *Industrial Crops and Products*, 68, 32-41. https://doi.org/10.1016/j.indcrop.2014.08.011.

Sangwan, R. S. and Norreel, B. (1975). Induction of plants from pollen grains of *Petunia* cultured *in vitro*. *Nature*, 257(5523), 222. https://doi.org/10.1038/257222a0.

Sangwan-Norreel, B. S. (1977). Androgenic stimulating factors in the anther and isolated pollen grain culture of *Datura innoxia* Mill. *Journal of Experimental Botany*, 28(4), 843-852. https://doi.org/10.1093/jxb/28.4.843.

Sangwan, R. S., and Sangwan-Norreel, B. S. (1987). Ultrastructural cytology of plastids in pollen grains of certain androgenic and nonandrogenic plants. *Protoplasma*, 138, 11–22. doi: 10.1007/bf01281180.

Sax, K. (1935). The effect of temperature on nuclear differentiation in microspore development. *Journal of the Arnold Arboretum*, 16(3), 301-310.

Schachtsiek, J., Hussain, T., Azzouhri, K., Kayser, O., and Stehle, F. (2019). Virus-induced gene silencing (VIGS) in *Cannabis sativa* L. *Plant Methods*, 15 (1), 157. https://doi.org/10.1186/s13007-019-0542-5.

Schultes, R.E., Klein, W.M., Plowman, T. and Lockwood, T.E. (1975). *Cannabis*: an example of taxonomic neglect. *Botanical Museum Leaflets Harvard University*, 23, 337-367. https://doi.org/10.1515/9783110812060.21.

Shariatpanahi, M. E., Bal, U., Heberle-Bors, E., and Touraev, A. (2006). Stresses applied for the re-programming of plant microspores towards *in vitro* embryogenesis. *Physiologia Plantarum*, 127(4), 519-534. doi: 10.1111/j.1399-3054.2006.00675.x.

Sirikantaramas S, Morimoto S, Shoyama Y et al (2004). The gene controlling marijuana psychoactivity. Molecular cloning and heterologous expression of D1-tetrahydrocannabinolic acid synthase from *Cannabis sativa* L. *Journal of Biological Chemistry*, 279 (38), 39767–39774. https://doi.org/10.1074/jbc.M403693200.

Śledziński, P., Zeyland, J., Słomski, R., and Nowak, A. (2018). The current state and future perspectives of cannabinoids in cancer biology. *Cancer Medicine*, 7(3), 765-775. https://doi.org/10.1002/cam4.1312.

Sliwinska, E., and Lukaszewska, E. (2005). Polysomaty in growing *in vitro* sugar-beet (*Beta vulgaris* L.) seedlings of different ploidy level. *Plant Science,* 168, 1067–1074. doi:10.1016/j.plantsci.2004.12.003.

Small E., and Cronquist A. (1976). A practical and natural taxonomy for *Cannabis*. *Taxon*, 25,405–435. https://doi.org/10.2307/1220524.

Small, E. (2015). Evolution and classification of *Cannabis sativa*, (marijuana, hemp) in relation to human utilization. *The Botanical Review,* 81, 189–294. doi: 10.1007/s12229-015-9157-3.

Small, E. (2017). Classification of *Cannabis sativa* L. in relation to agricultural, biotechnological, medical and recreational utilization. In: *Cannabis sativa L.-Botany and biotechnology.* Eds. S Chandra, H. Lata, and M. A. ElSohly, Berlin:Springer, 1-62. https://doi.org/10.1007/978-3-319-54564-6_1.

Smýkalová, I., Vrbová, M., Cvečková, M., Plačková, L., Žukauskaitė, A., Zatloukal, M., ... and Griga, M. (2019). The effects of novel synthetic cytokinin derivatives and endogenous cytokinins on the *in vitro* growth responses of hemp (*Cannabis sativa* L.) explants. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 139(2), 381-394. doi: 10.1007/s11240-019-01693-5.

Sorokin, A., Yadav, N. S., Gaudet, D., and Kovalchuk, I. (2020). Transient expression of the *β-glucuronidase* gene in *Cannabis sativa* varieties. *Plant Signal. Behav.* 15(8), 1780037. https://doi.org/10.1080/15592324.2020.1780037.

Souza, L. A., Moscheta, I. S., and Oliveira, J. H. G. (2004). Comparative morphology and anatomy of the leaf and stem of *Peperomia dahlstedtii* C. DC., *Ottonia martiana* Miq. and *Piper diospyrifolium Kunth* (Piperaceae). *Gayana Bot*, 61(1), 6-17.

Srivastava, P. and Chaturvedi, R. (2008). *In vitro* androgenesis in tree species: An update and prospect for further research. *Biotechnology Advances*, 26(5), 482-491. https://doi.org/10.1016/j.biotechadv.2008.05.006.

Stomps, T. J. (1910). *Kerndeeling en synapsis bij Spinacia oleracea L.* Amsterdam: M.J. Portielje.

Su, Y.-H., Liu, Y.-B., and Zhang, X.-S. (2011). Auxin-cytokinin interaction regulates meristem development. *Molecular Plant*, 4, 616–25. doi:10.1093/mp/ssr007.

Summers, H. M., Sproul, E., and Quinn, J. C. (2021). The greenhouse gas emissions of indoor cannabis production in the United States. *Nature Sustainability*, 1-7. https://doi.org/10.1038/s41893-021-00691-w.

Sunderland, N. and Wildon, D. C. (1979). A note on the pretreatment of excised flower buds in float culture of *Hyoscyamus anthers*. *Plant Science Letters*, 15(2), 169-175. https://doi.org/10.1016/0304-4211(79)90070-1.

Thomas, B. F. (2017). Cannabidiol as a Treatment for Seizures, Convulsions and Epilepsy. In: *Cannabis sativa L.-Botany and biotechnology.* Eds. S Chandra, H. Lata, and M. A. ElSohly, Berlin:Springer, 249-261. https://doi.org/10.1007/978-3-319-54564-6_11.

Touraev, A., Vicente, O., and Heberle-Bors, E. (1997). Initiation of microspore embryogenesis by stress. *Trends in Plant Science*. 2, 297–302. doi: 10.1016/s1360-1385(97)89951-7.

Treister-Goltzman, Y., Freud, T., Press, Y., and Peleg, R. (2019). Trends in publications on medical cannabis from the year 2000. *Population Health Management*, 22(4), 362-368. https://doi.org/10.1089/pop.2018.0113.

Tulecke, W. R. (1953). A tissue derived from the pollen of *Ginkgo biloba*. *Science*, 117(3048), 599-600. doi: 10.1126/science.117.3048.599.

Urits, I., Borchart, M., Hasegawa, M., Kochanski, J., Orhurhu, V., and Viswanath, O. (2019). An update of current cannabis-based pharmaceuticals in pain medicine. *Pain and Therapy*, 8(1), 41-51. https://doi.org/10.1007/s40122-019-0114-4

Uskutoğlu, T., Uskutoğlu, D., and Turgut, K. (2019). Effects on pre-treatment and different tissue culture media for androgenesis in *Stevia rebaudiana* Bertoni. *Sugar Tech*, 21(6), 1016-1023. doi: 10.1007/s12355-019-00722-z.

Van Hieu, P. (2019). Polyploid Gene Expression and Regulation in Polysomic Polyploids. *American Journal of Plant Sciences*, 10, 1409–1443. doi:10.4236/ajps.2019.108101.

Vavilov, N. I. (1926). The origin of the cultivation of “primary” crops, in particular cultivated hemp. In: *Studies on the origin of cultivated plants*. USSR:Institute of Applied Botany and Plant Breeding, Leningrad, 221-233.

Veliky, I.A., Genest, K. (1972). Growth and metabolites of *Cannabis sativa* cell suspension cultures. *Lloydia*, 35, 450–456.

Vigil, J. M., Stith, S. S., Adams, I. M., and Reeve, A. P. (2017). Associations between medical cannabis and prescription opioid use in chronic pain patients: A preliminary cohort study. *PloS One*, 12(11), e0187795. https://doi.org/10.1371/journal.pone.0187795.

Vinocur, B., Carmi, T., Altman, A., and Ziv, M. (2000). Enhanced bud regeneration in aspen (*Populus tremula* L.) roots cultured in liquid media. *Plant Cell Reports*, 19(12), 1146-1154. https://doi.org/10.1007/s002990000243.

Wagner, H. and Ulrich-Merzenich, G. (2009). Synergy research: approaching a new generation of phytopharmaceuticals. *Phytomedicine*, 16(2-3), 97-110. https://doi.org/10.1016/j.phymed.2008.12.018.

Wahby, I., Caba, J. M., and Ligero, F. (2013). *Agrobacterium* infection of hemp (*Cannabis sativa* L.): establishment of hairy root cultures. *Journal of Plant Interactions,* 8(4), 312–320. doi:10.1080/17429145.2012.746399.

Wahby, I., Caba, J. M., and Ligero, F. (2017). Hairy root culture as a biotechnological tool in *C. sativa.* In: *Cannabis sativa L.-Botany and biotechnology.* Eds. S Chandra, H. Lata, and M. A. ElSohly, Berlin:Springer, 299–317. doi:10.1007/978-3-319-54564-6_14.

Warf, B. (2014). High points: an historical geography of cannabis. *Geographical Review*, 104(4), 414-438. https://doi.org/10.1111/j.1931-0846.2014.12038.x

Wędzony, M., Forster, B. P., Żur, I., Golemiec, E., Szechyńska-Hebda, M., Dubas, E., and Gotębiowska, G. (2009). Progress in doubled haploid technology in higher plants. In: *Advances in Haploid Production in Higher Plants*, Springer, Dordrecht, 1-33. https://doi.org/10.1007/978-1-4020-8854-4_1.

Wiegand KM. (1935). A taxonomist's experience with hybrids in the wild. *Science,* 81, 161–166.

Wielgus, K., Luwanska, A., Lassocinski, W., and Kaczmarek, Z. (2008). Estimation of *Cannabis sativa* L. tissue culture conditions essential for callus induction and plant regeneration. *Journal of Natural Fibers,* 5, 199–207. doi:10.1080/15440470801976045.

Williamson, E. M. (2001). Synergy and other interactions in phytomedicines. *Phytomedicine*, 8(5), 401-409. https://doi.org/10.1078/0944-7113-00060.

Wróbel, T., Dreger, M., Wielgus, K., Słomski, R., (2018). The application of plant *in vitro* cultures in cannabinoid production. *Biotechnology Letters*, 40 (3), 445–454. https://doi.org/10.1007/s10529-017-2492-1.

Yumbla-Orbes, M., da Cruz, A. C. F., Pinheiro, M. V. M., Rocha, D. I., Batista, D. S., Koehler, A. D., ... and Otoni, W. C. (2017). Somatic embryogenesis and de novo shoot organogenesis can be alternatively induced by reactivating pericycle cells in Lisianthus (*Eustoma grandiflorum* (Raf.) Shinners) root explants. *In Vitro Cellular & Developmental Biology-Plant*, 53(3), 209-218. https://doi.org/10.1007/s11627-017-9800-2.

Zhang, Q., Chen, X., Guo, H., Trindade, L. M., Salentijn, E. M., Guo, R., ... and Yang, M. (2018). Latitudinal adaptation and genetic insights into the origins of *Cannabis sativa* L. *Frontiers in Plant Science*, 9:1876. https://doi.org/10.3389/fpls.2018.01876.

Zhang, X., Xu, G., Cheng, C., Lei, L., Sun, J., Xu, Y., *et al*., (2021). Establishment of an *Agrobacterium*-mediated genetic transformation and CRISPR/Cas9-mediated targeted mutagenesis in Hemp (*Cannabis sativ*a L.). *Plant biotechnology Journal*. https://doi.org/10.1111/pbi.13611.

Zhao, J., Newcomb, W., and Simmonds, D. (2003). Heat-shock proteins 70 kDa and 19 kDa are not required for induction of embryogenesis of *Brassica napus* L. cv. Topas microspores. *Plant and Cell Physiology,* 44, 1417–1421. doi: 10.1093/pcp/pcg162.

Zhou, Z., Tan, H., Li, Q., Chen, J., Gao, S., Wang, Y., ... and Zhang, L. (2018). CRISPR/Cas9-mediated efficient targeted mutagenesis of RAS in *Salvia miltiorrhiza*. *Phytochemistry*, 148, 63-70. https://doi.org/10.1016/j.phytochem.2018.01.015.

Zoriniants, S., Tashpulatov, A. S., Heberle-Bors, E., and Touraev, A. (2005). The Role of Stress in the Induction of Haploid Microspore Embryogenesis. In: *Haploids in Crop Improvement II*. Berlin:Springer, 35–52. https://doi.org/10.1007/3-540-26889-8_3.

Żuk-Gołaszewska K., and Gołaszewski J. (2020). Hemp Production. In: *Sustainable Agriculture Reviews*, vol. 42. Eds. G. Crini and E. Lichtfouse. Springer, Cham, 1-36. https://doi.org/10.1007/978-3-030-41384-2_1.

Żur, I., Dubas, E., Golemiec, E., Szechy´nska-Hebda, M., Janowiak, F., and Wêdzony, M. (2008). Stress-induced changes important for effective androgenic induction in isolated microspore culture of triticale (x Triticosecale Wittm.). *Plant Cell, Tissue and Organ Culture*, 94(3), 319–328. doi: 10.1007/s11240-008-9360-6.

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