Journal of Medicinal and Aromatic Plant Sciences 45(1-2), 2023, 1-16

REVIEW ARTICLE

Exploring methodologies in Cannabis tissue-culture and genetic transformation: Opportunities and obstacles

SHUKLA D*

Article History

Received: May 12, 2023
Revised: June 17, 2023
Accepted: June 27, 2023

Key Words

Cannabis sativa
Genetic transformation
Hemp
Regeneration
Tissue culture

ABSTRACT

In recent years, the growing interest in Cannabis sativa L., particularly its medicinal and aromatic properties, has propelled advancements in its tissue culture and genetic transformation techniques. This review delineates the significant strides and persistent challenges in the field, offering a comprehensive overview of the current methodologies and their implications. It discusses the synergistic effects of Thidiazuron (TDZ) and Naphthaleneacetic acid (NAA) in the Murashige and Skoog (MS) medium as well as the use of meta-topolin (mT). This synthetic cytokinin (mT) facilitates a high induction frequency and many shoots per explant. It introduces a time-efficient and resource-optimized pathway for Cannabis micropropagation and germplasm conservation. The genetic transformation in Cannabis was predominantly facilitated through Agrobacterium-mediated transformation, a cornerstone technique that enabled the integration of foreign genes into the plant genome. Regulatory implications associated with gene editing in Cannabis sativa are highlighted. Despite these advancements, the field grapples with several challenges, including the recalcitrant nature of Cannabis, especially regarding in vitro propagation or genetic transformation, the genotypic specificity of regeneration protocols, and the reproducibility of existing methods. The complexity of the Cannabis genome, characterized by a high degree of polymorphism and multiple copies of specific genes, further exacerbates these challenges. Moreover, the current research landscape is marred by a lack of standardized protocols and variable responses among different Cannabis varieties, necessitating more robust and universally applicable protocols. This review underscores the pressing need for further research to optimize protocols for higher efficiency and to develop suitable systems for in-vitro plantlet regeneration.

INTRODUCTION

In recent years, the scientific community has witnessed a resurgence in the interest surrounding *Cannabis sativa* L., a plant species historically cultivated for a myriad of purposes, including its utilization in the food, oil, and fibre industries, as well as a source of medicinal and recreational substances (Adhikary *et al*., 2021; Clarke and Merlin, 2016; ElSohly *et al*., 2017; Feeney and Punja, 2003; Monthony *et al*., 2021a; Musio *et al*., 2018; Zhang *et al*., 2021). It is important to note that *Cannabis sativa* L.

*Tissue-culture and Transformation Facility, Plant Biotechnology Division, CSIR – Central Institute of Medicinal and Aromatic Plants, Picnic Spot road Kukrail, Lucknow – 226015, Uttar Pradesh, India; Email: devesh.shukla@cimap.res.in
Institutinal Communinacation No. CIMAP/PUB/ 2023/154
Doi: https://doi.org/10.62029/jmaps.v45i1.shukla

can be classified into two primary types: Hemp and Cannabis. While Hemp is primarily cultivated for industrial uses like fibre and has a low THC content (below 0.3%), Cannabis is grown for its higher THC levels and medicinal properties (Adhikary *et al*., 2021). This resurgence is particularly noticeable in regions such as Canada, where there is a renewed focus on expanding its cultivation for fibre and seed crop production, thereby promoting demand for improved Cannabis varieties with enhanced traits (Adhikary *et al*., 2021; Feeney and Punja, 2003; Musio *et al*., 2018; Slusarkiewicz-Jarzina *et al*., 2005). The development of these new Cannabis cultivars is significantly facilitated through advanced breeding and biotechnological strategies, including tissue culture and genetic transformation techniques (particularly CRISPR-based), which have gained prominence in recent years as pivotal tools in the exploration of the phytochemical profiles found in *Cannabis sativa* (Adhikary *et al*., 2021; Feeney and Punja, 2003; Galán-Ávila *et al*., 2020, 2021; Monthony *et al*., 2021a; Raharjo *et al*., 2010; Slusarkiewicz-Jarzina *et al*., 2005). These strategies have proven instrumental in understanding the metabolic pathways and developing protocols for the *in-vitro* tissue culture of this plant species, a development that holds significant implications for the pharmaceutical industry (Chandra *et al*., 2011; Lata *et al*., 2009b, 2009a; Monthony, *et al*., 2021; Page *et al*., 2020; Raharjo *et al*., 2010).

Furthermore, the cultivation and utilization of *Cannabis sativa* have garnered significant attention due to its potential applications in various sectors, including pharmaceuticals, textiles, and energy generation. The plant is renowned for its unique class of compounds called cannabinoids, primarily accumulated in the glandular trichomes of the female plant, which have significant pharmacologic and therapeutic potential (Adhikary *et al*., 2021; Lata *et al*., 2016; Monthony *et al*., 2021a; Smýkalová *et al*., 2019). However, the dioecious and allogamous nature of *C. sativa* poses challenges in maintaining consistency in its chemical profile and cannabinoid content when propagated through seeds. This has led to a surge in research focusing on developing reliable *in-vitro* propagation techniques to facilitate large-scale production of true-to-type Cannabis plants with desirable pharmacological active chemotypes (Lata *et al*., 2016; Smýkalová *et al*., 2019). Despite the advancements, the existing body of literature on Cannabis tissue culture presents certain limitations, including the prevalent use of Cannabis as a surrogate for drug-type Cannabis and a notable lack of reproducibility across different genotypes. This review offers a comprehensive and critical analysis of the current state of Cannabis tissue culture, exploring the historical trajectory, methodological advancements, and the challenges encountered in the field of Cannabis genetic transformation. Moreover, the recent developments in gene editing technologies hold promising avenues for the breeding and propagation of elite cannabis varieties, potentially reforming cannabis breeding and propagation (Adhikary *et al*., 2021; Galán-Ávila *et al*., 2021; Monthony *et al*., 2021a).

Recent studies have introduced groundbreaking methods for producing genetically transformed *Cannabis sativa* L. plants at a significantly faster rate than existing protocols. These methods, utilizing different explants and the *Agrobacterium tumefaciens* strain LBA4404, promise to expedite the development of new Cannabis varieties with better traits (Galán-Ávila *et al*., 2021). These studies are significant milestones in *Cannabis sativa* breeding, presenting novel and rapid methods for producing stably transformed plants and outlining detailed methodologies for achieving this, including assessing different kanamycin concentrations and validation techniques (Galán-Ávila *et al*., 2021). The implications of these studies are vast, potentially nurturing the development of Cannabis cultivars with specific biochemical profiles or increased tolerance to various stresses (Galán-Ávila *et al*., 2021). Overall, the resurgence in the interest surrounding the therapeutic potential of cannabinoids has propelled *Cannabis sativa* to the forefront of medical and agricultural research. The plant, historically renowned for its ability to induce a relaxed state, now holds promise in the growing medical Cannabis market and is projected to be worth $43 billion by 2025 (https://www.forbes.com/sites/irisdorbian/2021/06/18/legal-cannabis-market-projected-to-rack-up-43-billion-by-2025-says-new-study). This review seeks to elucidate the advancements and challenges in the tissue culture and genetic transformation of *Cannabis sativa*, offering a comprehensive overview of the progress, challenges, and prospects in this dynamic field of research. Future research must focus on developing optimized protocols for tissue culture techniques, which could significantly aid in elite Cannabis breeding and propagation, thereby

fostering a new era of advancements in plant science research.

Historical Perspective of Cannabis Tissue Culture and Genetic Transformation

Cannabis sativa, commonly known as Hemp, has been a plant of significant interest due to its myriad applications in the medicinal, industrial, and pharmaceutical sectors. Its utility has spanned thousands of years, encompassing various industries such as textiles, paper, and medicine (Clarke and Merlin, 2016). Historically, *Cannabis sativa* has been a plant of multifaceted importance, significantly so in India, where its utility can be traced back around 5,000 years (Abel, 1980; Chopra and Chopra, 1957; Iversen, 2008). In Ayurvedic medicine, an ancient Indian system of natural healing, Cannabis has been employed as a therapeutic agent to treat a range of conditions such as stress, insomnia, and digestive issues (Abel, 1980; Chopra and Chopra, 1957; Clarke and Merlin, 2016; Kuddus *et al*., 2013; Iversen, 2008). Beyond its medicinal applications, it has cultural relevance in rituals and ceremonies (Clarke and Merlin, 2016; Kuddus *et al*., 2013). For example, "Bhang," a traditional Indian beverage made from Cannabis leaves and flowers, is consumed during the festival of Holi. Such enduring and varied uses highlight the integral role *Cannabis sativa* L. has played, not just in industrial and medicinal arenas globally but also in cultural and traditional contexts in India (Abel, 1980; Chopra and Chopra, 1957; Clarke and Merlin, 2016; Kuddus *et al*., 2013; Iversen, 2008). The legalization of Cannabis has gained momentum globally, particularly in Canada, Europe, and some US states. Canada federally legalized the crop in October 2018 (Cannabis Act), becoming the second country after Uruguay in 2013, leading to increased commercial production. In the US, 12 states have legalized recreational use, while another 22 permits medical use (Adhikary *et al*., 2021; Adinoff and Reiman, 2019). A well-defined legal framework and a balanced approach considering potential benefits and risks are crucial to guide research and applications in this field. Collaborative efforts between researchers, policymakers, and industry stakeholders are necessary to support the responsible development and application of gene editing technologies in Cannabis.

The journey of Cannabis genetic transformation has been marked by significant milestones and periods of stringent regulations, which have invariably stifled research and innovation. In the early stages, the propagation of *Cannabis sativa* was primarily through conventional methods. However, the allogamous nature of the plant posed challenges in maintaining potency and efficacy when propagated from seeds. Traditional breeding methods were supplemented with biotechnological approaches to enhance Cannabis breeding, focusing on the development of tissue culture techniques and genetic transformation protocols (Adhikary *et al*., 2021; Feeney and Punja, 2003; Monthony *et al*., 2021a; Slusarkiewicz-Jarzina *et al*., 2005) and the late 20th century marked the advent of molecular biology and biotechnology, ushering in a new era in Cannabis research. Scientists began exploring the genetic transformation of Cannabis to enhance its desirable traits and minimize undesirable characteristics (Adhikary *et al*., 2021; Monthony *et al*., 2021a). Initially, research was centred on conventional breeding techniques, which gradually transitioned to more advanced genetic transformation methods, including Agrobacterium-mediated genetic transformation and CRISPR/Cas9-mediated targeted mutagenesis. A pivotal study by Raharjo *et al*. (2010) is a critical reference in understanding the limitations and challenges of producing cannabinoids from *Cannabis sativa* cell cultures. This period also saw the establishment of protocols for the regeneration of *Cannabis sativa* from leaf explants, with a notable study by Lata *et al*. (2010) reporting a high response rate. However, the reproducibility of this method has been brought into question, paving the way for alternative approaches and a deeper understanding of genotypic variations in response to transformation protocols. In recent years, the field of Cannabis tissue culture has navigated through liberalization in regulations globally, developing a conducive environment for research. This shift is expected to recuperate the gaps in research due to overregulation and criminalization of Cannabis, paving the way for a rich body of literature that explores the nuances of Cannabis micropropagation and regeneration. As we move forward, it is anticipated that the integration of modern biotechnological tools with traditional breeding methods will significantly enhance the production of pharmacologically active compounds and facilitate the development of transgenic plants with improved fibre elasticity for the production of biodegradable plastics and other biopolymers.

METHODS OF TISSUE CULTURE AND GENETIC TRANSFORMATION

Cannabis Micropropagation and Agrobacterium-mediated Transformation

Micropropagation remains an indispensable technique for the clonal propagation of elite plant varieties, including *Cannabis sativa*. Micropropagation techniques have been employed to induce callus formation and shoot regeneration, utilizing different explants such as cotyledons and epicotyls and employing various growth regulators (Figure 1). The protocols generally involve stages of disinfection, inoculation on growth mediums supplemented with specific growth regulators, and acclimatization under controlled conditions (Chaohua *et al*., 2016; Lata *et al*., 2009a; Monthony *et al*., 2021b; Movahedi *et al*., 2015). Piunno *et al*. (2019) made a remarkable contribution to the field by exploring the use of floral explants for the micropropagation of *Cannabis sativa* (Table 1). It was the first study that successfully regenerated shoots from *C. sativa* in its reproductive phase, presenting a significant advancement in breeding programs and clonal propagation. Smýkalová *et al*. (2019) contributed by elaborating on the properties of synthetic cytokinin derivatives concerning the *in-vitro* growth of Cannabis. Their research led to an optimized Cannabis *in-vitro* regeneration protocol, and multiple shoot cultures open avenues for developing new Cannabis cultivars with improved traits, thus impacting the textile and pharmaceutical industries. Page *et al*. (2020) brought attention to optimizing basal media for the micropropagation of *Cannabis sativa*. Their work suggested that the Driver and Kuniyuki Woody (DKW) basal salt mixture was superior to the commonly used MS basal salt mixture, particularly for callogenesis and plant regeneration (Table 1). The DKW and MS (Murashige and Skoog) media are both widely utilized basal media in plant tissue culture, each with unique compositions that cater to the growth and development of plant tissues *in vitro*. The essential differences between these media, mainly as discussed in the research by Page *et al*. (2020), are that DKW basal salts significantly enhanced micropropagation and callogenesis in several commercial cultivars of *Cannabis sativa* compared to MS basal salts. Specifically, explants grown on DKW medium displayed higher multiplication rates and a larger canopy area.

Furthermore, callogenesis (callus formation) was superior on DKW compared to MS medium under varying concentrations of 2,4-D (Page *et al*., 2020). The research also indicated that MS medium was associated with excessive callus formation, hyperhydricity (water-soaked appearance), lower multiplication rates, and higher mortality rates in *Cannabis sativa*, whereas DKW medium resulted in healthier plants. This finding is crucial for standardizing micropropagation practices, making the process more efficient and reproducible.

Callus induction is pivotal in tissue culture and plant biotechnology for plant regeneration and genetic transformation (Figure 1). Various studies have utilized MS basal medium added with varying concentrations of plant growth hormones to induce callus formation from different explants, including leaves, stems, and axillary buds (Feeney and Punja, 2003; Lata *et al*., 2009a, 2009b; Slusarkiewicz-Jarzina *et al*., 2005) (Figure 1). The selection of appropriate explants and culture conditions is vital in establishing successful cell culture lines, as highlighted in the study by Raharjo *et al*. (2010) (Table 1). Notably, a study by Monthony *et al*. (2021b) cast a critical eye on the reproducibility of callus induction protocols in *Cannabis sativa*. Their study attempted to replicate a previously successful high-response protocol across multiple drug-type *C. sativa* genotypes. Surprisingly, the team found that not only was the original protocol non-reproducible across the tested genotypes but also that callus induction was highly genotype-specific, which has significant implications for developing universal methods in Cannabis tissue culture, suggesting that protocols may need to be tailored to individual genotypes for effective callus induction.

In terms of hardening, the transition from *in-vitro* to *ex-vitro* conditions is a critical phase. Ioannidis *et al*. (2022) offered an alternative *in-vitro* propagation protocol that improved rooting traits and had a high survival rate during the hardening process. Their method involved peat moss-based sponges and specific concentrations of indole-3-butyric acid (IBA), demonstrating that their system could benefit both propagation and hardening, especially for industrial-scale applications. These authors have significantly advanced our understanding of the complexities involved in the micropropagation and hardening of *Cannabis sativa*. Their work underscores the importance of optimizing culture conditions and

Table 1: Comparative analysis of explant types and plant growth regulator combinations for optimized in-vitro morphogenesis: A literature survey

Explant	Best Growth Regulators	Best Conc.	Best Response	Author
hypocotyl, epicotyl, cotyledons, petioles, leaves, and immature flower buds	2,4-D+Kinetin	5 µM +1 µM	Callogenesis	Feeney and Punja 2003
Young leaves, Petioles, Internodes, & Axillary buds	IAA+NAA	1.0 mg L^{-1}	Root formation	Slusarkiewicz-Jarzina *et al*., 2005
	2,4-D	2-4 mg L^{-1}	Callogenesis	
	Kinetin+NAA	1 mg L^{-1}+0.5 mg L^{-1}	Callogenesis	
	DICAMBA	2-3 mg L^{-1}	Shooting	
Flowers, leaves, and 4-day-old seedlings	2,4-D	1 mg L^{-1}	Callogenesis	Raharjo *et al*., 2006
Nodal segments containing axillary buds (1 cm in length)	TDZ	0.5 µM	Shooting	Lata *et al*., 2009a
	IBA	2.5 µM	Rooting	
	Gibberellic acid	7.0 µM	More Shooting if added with TDZ	
Young leaf explants from vegetative cuttings	TDZ+NAA	1.0 µM +0.5 µM	Callogenesis	Lata *et al*., 2010
	TDZ	0.5 µM	Shooting	
	IBA	2.5 µM	Rooting	
Cotyledon and epicotyl	BA+IBA	2+0.5 (mg L^{-1})	Shooting	Movahedi *et al*., 2015
	IBA	0.1 mg L^{-1}	Rooting	
Cotyledon	TDZ+NAA	0.4 mg L^{-1} + 0.2 mg L^{-1}	51.7% regeneration frequency	Chaohua *et al*., 2016
	IBA	0.5-2 mg L^{-1}	80% Rooting	
Nodal segments	TDZ	0.5 µM	Shooting	Lata *et al*., 2016
	Activated charcoal + IBA	500 mg L^{-1} + 2.5 µM	Rooting	
Nodal segments containing axillary buds	meta-Topolin	2 µM	Shooting	Lata *et al*., 2016
	meta-Topolin	2 µM	After 2 subculture in the same media induces rooting	
Immature and mature floral explants	Activated charcoal + Kinetin + NAA	0.03%+1.86 µM + 0.54 µM	Shooting	Piunno *et al*., 2019
Leaf	DKW (Basal salt mixture) + TDZ	DKW + 0.5 µM	Best *in-vitro* Multiplication and callogenesis	Page *et al*., 2020
Seedling explants (Cotyledon, hypocotyl, and true leaf)	ZEA RIB	2.0 (mg/L)	Shoot organogenesis. (hypocotyl gives the best).	Galan-Avila *et al*., 2020
	ZEA RIB + NAA	1.0 (mg/L)+ 0.02 (mg/L)		
Cotyledons	TDZ+NAA	0.4+0.2 (mg L^{-1})	Shooting	Galan-Avila *et al*., 2021
Hypocotyl	Kanamycin	100 mg L^{-1}		
4 cm long stem tips	meta-Topolin + Gibberellin	0.5+0.1 (mg L^{-1})	Shoot initiation & multiplication	Lubell-Brand *et al*., 2021
Immature grains (Immature embryos)	2,4-D + Kinetin	1.0 + 0.25 (mg L^{-1})	Callogenesis	Zhang *et al*., 2021
	TDZ+6-BA+NAA+IAA	0.5+0.3+0.2+0.2 (mg L^{-1})	Regeneration medium	
	NAA+IBA+ZeaRIB	0.2+0.5+0.01 (mg L^{-1})	Rooting medium	

protocols tailored to specific genotypes for efficient micropropagation and successful hardening. These contributions are instrumental in accelerating the cultivation of *Cannabis sativa* varieties for medicinal, industrial, and pharmaceutical applications.

Agrobacterium-mediated transformation has been a cornerstone in the genetic transformation of Cannabis, facilitating the integration of foreign genes into the plant genome. This method has paved the way for developing transgenic Cannabis varieties with desirable pest and disease-resistance traits. Feeney and Punja (2003) investigate Cannabis's

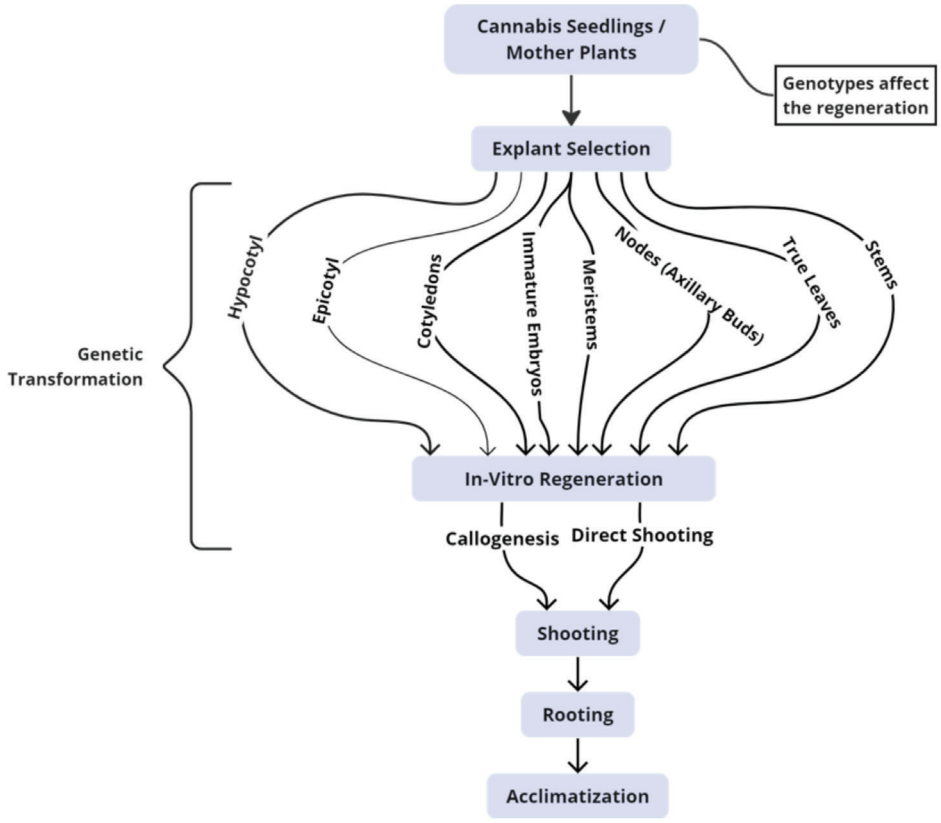

Figure 1: Schematic representation of the plant tissue culture and transformation process, detailing the journey from explant selection to acclimatization. The flowchart commences with the explant selection stage, where various types of explants such as hypocotyl, epicotyl, cotyledons, meristems, nodal segments with axillary buds, true leaves, immature embryos, and stems are considered. These explants are then directed towards the regeneration stage. The shooting can occur via two primary pathways: One is callogenesis, in which first callus is formed and subsequently shooting induced, and the other is direct shooting, in which a direct shoot regeneration can occur from the explants under suitable media conditions. Further, the progress is made through the rooting stage and culminates at the acclimatization stage.

propagation culture and standardize a protocol for Agrobacterium-mediated transformation of foreign genes (Table 1). The results of this study show that a significant amount of callus was generated within four weeks for all cultivars of Cannabis using the media recipe with hormone composition of MS medium with Gamborg B5 vitamins added with 5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 µM kinetin, 3% sucrose, and 8 g/L agar (Table 1) (Feeney and Punja, 2003). Despite numerous attempts, no treatments proved effective for the regeneration of plantlets. Cells in suspension were genetically modified using the *Agrobacterium tumefaciens* EHA101 strain, which carried the binary construct pNOV3635 containing the phosphomannose isomerase (PMI) gene. The genetically altered callus was isolated using a medium enriched with 1-2% mannose. Expression of the PMI gene was confirmed, and its presence was validated through PCR and Southern blot techniques (Feeney and Punja, 2003). The number of gene copies varied across cell lines, ranging from one to four. The paper concludes that the propagation of Cannabis in tissue culture can be achieved using the media recipe with varying hormone composition. However, the study did not find a successful protocol for plantlet regeneration. The study provides a foundation for further research on developing new Cannabis cultivars with improved traits using biotechnological strategies.

Slusarkiewicz-Jarzina *et al*. (2005) examined the impact of various combinations of phytohormones on the initiation of callus formation and the regeneration of plants across five cultivars of *Cannabis sativa* L. (Table 1). They found that callus was produced from explants of the five different

varieties, and calli were diverse, ranging from friable compact to watery, with colour ranging from pale yellow to green and white—petiole explants of cv. Fibrimon-24 exhibited the highest frequency of callus induction. Regeneration of plants was achieved across all examined cultivars, with the maximum number of regenerated plants observed in callus tissues derived from petiole explants when cultured on MS medium supplemented with DICAMBA (Table 1). A total of 46 plants (1.35% of callus) were regenerated. The study achieved significant improvement in Cannabis plant regeneration *in vitro*.

In the study by Raharjo *et al*. (2010), a significant stride was made in developing cell lines and callus induction from *Cannabis sativa* explants and seedlings (Table 1). The researchers established four distinctive cell culture lines, identifying flowers as the optimum explant source for callus induction and spotlighting the superiority of light conditions over dark in enhancing the induction process. Following this foundational work, the study ventured into the biochemical aspect to investigate the secondary metabolic pathways operative in these developed cell cultures, particularly on cannabinoid biosynthesis. However, the phytochemical analysis, facilitated through HPLC and 1H-NMR, unveiled a pronounced limitation - the non-presence of cannabinoids and related flavonoids in the cell suspension cultures. This deficiency was correlated with an absence of polyketide synthase activity, a crucial element in cannabinoid biosynthesis. Consequently, this study not only illuminates the complexities of cannabinoid production in cell cultures but also underscores an urgent need for further investigative endeavours at the enzymatic level for potential breakthroughs in the metabolic engineering of *Cannabis sativa*.

In the notable study conducted by Lata *et al*. (2009a, 2009b), a significant advancement in the domain of *Cannabis sativa* research was achieved through the development of an efficient protocol for *in-vitro*-tissue culture, specifically focusing on nodal segments containing axillary buds (Table 1). This pioneering work set the foundation for establishing a secure and stable *in-vitro* collection of superior medicinal plant genetic resources, thereby promising a steady supply of pharmacologically active chemotypes in the future. The authors meticulously addressed the core issue by initiating surface-disinfecting the explants, followed by inoculation on MS medium supplemented with varying cytokinins and gibberellic acid concentrations. Thidiazuron (TDZ) demonstrated superiority in encouraging high-frequency shoot regeneration compared to other agents like benzyl adenine or kinetin. This successful tissue culture method facilitated a remarkable 95% rooting success rate when elongated shoots were transferred to a specific medium containing activated charcoal and indole-3-butyric acid (Table 1). Following soil acclimatization, these *in-vitro* propagated plants exhibited growth parameters comparable to *ex-vitro* vegetatively grown counterparts, especially regarding gas and water vapour exchange characteristics under varied light intensities. Consequently, the study establishes a rapid and fruitful plant regeneration methodology and underscores its potential in fostering mass multiplication of high-yielding *C. sativa* varieties. Further, In another extensive research by Lata *et al*. (2010), a concerted effort to comprehend and map the variations in cannabinoid content across different growth stages and plant types of *Cannabis sativa* was undertaken. The study, which notably encompassed micropropagation and hardening processes of Cannabis plants, endeavoured to unravel the biochemical consistency and yield of cannabinoids in *in-vitro* propagated plants (IVP), vis-a-vis conventionally grown and mother plants. Utilizing a robust methodology, the research described a significant correlation between the growth stages and the variations in THC concentration, pinpointing the peak reproductive/budding stage as the phase registering the highest concentration of THC. Lata *et al*. (2010) had achieved a significant breakthrough in *Cannabis sativa* tissue culture. The research centred around developing and optimizing a robust tissue culture protocol, specifically focusing on the regeneration of plants from leaf-derived callus, a technique showcasing the substantial potential for the mass propagation and *in-vitro* conservation of chemically elite varieties of this medically significant plant (Table 1). The investigators astutely addressed the need for high Δ9-THC yielding clones, developing a methodology that facilitated the propagation of male plants and catered to other genotypes within this species. Moreover, the study made strides in identifying a female clone of *Cannabis sativa* with a high yield of Δ9-THC, a critical component for pharmaceutical applications. The tissue culture-raised plants

exhibited remarkable conformity with the mother plant regarding cannabinoid profile and Δ9-THC content, demonstrating no significant morphological variations, thereby confirming the protocol's efficacy and reproducibility. This research stands as a monumental contribution to tissue culture, offering an alternative yet efficient pathway for the regeneration of *Cannabis sativa* plants, promoting the production of plants with high Δ9-THC content that aligns with the chemical profile of the mother plant underscores the significant advancements in tissue culture techniques, particularly highlighting the approach and success in utilizing leaf-derived callus to propagate *Cannabis sativa* with enhanced pharmacological potential. In the pioneering study conducted by Movahedi *et al*. (2015), a significant exploration into the realms of tissue culture and micropropagation of Iranian Cannabis was undertaken, leveraging cotyledon and epicotyl explants as pivotal elements in the research (Table 1). The study pioneers using these explants alongside plant growth regulators (PGRs) such as BA and TDZ, combined with IBA, to facilitate callus induction and plant regeneration. A meticulous analysis revealed that the cotyledon explants exhibited a higher proclivity for callus formation, especially when nurtured in MS medium supplemented with specific concentrations of TDZ and IBA, thus distinguishing themselves as superior candidates for generating substantial callus volumes. Conversely, epicotyl explants demonstrated an enhanced propensity for regeneration, offering a promising avenue for achieving optimal shoot formation and regeneration. The study illuminated the superior quality of calli produced through the employment of MS medium enriched with TDZ hormone compared to its BA-incorporated counterpart. This study provides a noteworthy contribution to the field by establishing a protocol for the micropropagation and *in-vitro* regeneration of Cannabis in Iran (Movahebi *et al*., 2015). In a study conducted by Chaohua *et al*. (2016), a significant leap in tissue culture techniques for *Cannabis sativa* L. was documented, wherein a swift and effective protocol for *in-vitro* shoot regeneration using Cannabis cotyledons was introduced (Table 1). This method leveraged the synergistic effects of Thidiazuron (TDZ) and Naphthaleneacetic acid (NAA) in Murashige and Skoog (MS) medium, establishing a potent medium that notably facilitated a high induction frequency and a substantial number of shoots per explant, especially in the MS medium enriched with 0.4 mg/l TDZ and 0.2 mg/l NAA. The study underscored the potency of younger cotyledons (2-3 days post-planting) as superior explants, showcasing markedly higher regeneration frequencies than their older counterparts (5-6 days post-planting). This protocol offers a time-efficient and resource-optimized pathway for Cannabis micropropagation and germplasm conservation but also opens up promising avenues for genetic transformations in Cannabis, setting a new benchmark in tissue culture and propagation techniques for *Cannabis sativa* L. These findings vividly illustrated the advancements in tissue culture protocols, potentially steering a new direction in research and cultivation practices in *Cannabis sativa* L.

Lata *et al*. (2016) elucidate a refined protocol for the efficient and large-scale propagation of *Cannabis sativa*, leveraging the novel cytokinin meta-topolin (mT) (Table 1). This research outlines a streamlined one-step process that significantly nurtures adventitious shoot formation and root induction in nodal explants of *Cannabis sativa*. The paramount findings indicate a maximized shoot proliferation rate and a 100% survival frequency of acclimatized plants, highlighting the protocol's efficacy in encouraging eco-physiologically and functionally similar to the mother plant. Notably, the clonal fidelity of the micro-propagated plants was confirmed through inter simple sequence repeat (ISSR) markers, showcasing an analogous qualitative and quantitative cannabinoid profile to the mother plants. The protocol, which stands as a cornerstone for the commercial propagation of high-yield elite varieties, particularly for pharmaceutical applications, was initiated by utilizing segments with axillary buds harvested from chosen maternal plants as explants. These were disinfected and cultivated on MS medium supplemented with 2 µM mT, nurturing optimal shoot proliferation and length, eliminating the necessity for a separate auxin medium for root induction. This study thus lays a robust foundation for the mass production of genetically uniform, disease-free plantlets, promising significant advancements in the conservation and propagation of *Cannabis sativa* L. for both medicinal and industrial spheres. This protocol promises a stable *in-vitro* clonal resource of superior medicinal plant germplasm. It assures the future availability of pharmacologically potent chemotypes, marking a significant stride in the domain of plant tissue culture

techniques (Lata *et al*., 2016). A notable endeavour was made to enhance the efficiency of *Cannabis sativa* breeding programs and clonal propagation, mainly focusing on day-neutral cultivars (Piunno *et al*., 2019). The study carved a novel pathway in the propagation sphere by targeting floral explants as a viable source for micropropagation. It marks its inaugural appearance in the field of the species in its reproductive phase (Piunno *et al*., 2019) (Table 1). The findings highlighted the potential of both immature and mature floral explants in promoting shoot development when cultured on thidiazuron (TDZ), ushering in a new horizon where clonal propagation can potentially extend up to the harvest day, a feat previously unattained (Table 1). Despite a variation in the regeneration capacity among different cultivars, the study witnessed the successful development of green shoot-like structures from mature inflorescences within 18 days, which later morphed into phenotypically typical plants, displaying successful rooting and acclimatization (Piunno *et al*., 2019). However, the authors underscore the necessity for further refinements in the protocol to fully harness its potential, indicating that the current findings, albeit promising, mark only the inception of a more extensive investigative journey. This study, therefore, stands as a promising harbinger of advancements in the micropropagation techniques for *Cannabis sativa*, potentially developing breeding programs and clonal propagation strategies for day-neutral cultivars in the foreseeable future. The study conducted by Smýkalová *et al*. (2019) significantly contributes to plant tissue culture, mainly focusing on the *in-vitro* growth responses of Cannabis (*Cannabis sativa* L.). The team meticulously optimized protocols for Cannabis's *in-vitro* regeneration and multiple shoot culture, utilizing new synthetic cytokinin derivatives and endogenous cytokinins. The research describes an optimized protocol that involves selecting a primary explant containing either an apical or axillary meristem and developing successful regeneration. These explants are cultured on a medium enriched with the novel cytokinin derivative BAP9THP, which promotes substantial shoot multiplication. This stage is followed by repeated cultures on media supplemented with BAP9THP and the auxin antagonist PEO-IAA, promoting the development of balanced multiple shoot cultures with a promising multiplication coefficient of up to 1:10. Furthermore, this innovative protocol ensures the reliable rooting of the newly formed shoots, establishing robust plantlets ready for acclimatization. This technique, therefore, presents a potential tool for the efficient *in-vitro* multiplication of technical Cannabis, potentially catalyzing advancements in genetic transformations and other *in-vitro* applications in Cannabis, including the production of transgenic plants. Therefore, Smýkalová *et al*. (2019) not only bridge the gap in establishing reliable *in-vitro* regeneration systems for Cannabis but also pave the way for the development of new Cannabis cultivars with enhanced traits, steering a new direction in the commercial cultivation.

The research undertaken by Page *et al*. (2020) significantly advances the field of tissue culture in *Cannabis sativa* L. by focusing on optimizing basal media to enhance micropropagation and callogenesis (Table 1). The study hypothesized that the commonly utilized MS basal salt mixture might not be conducive to Cannabis micropropagation and sought to identify a more proficient alternative. The findings substantiated the hypothesis by highlighting the superior efficacy of the DKW basal salt mixture in facilitating healthier plant growth and a markedly increased multiplication rate across most tested genotypes compared to the MS basal salt mixture. Moreover, the DKW mixture demonstrated a preferable environment for callogenesis, promoting a significantly accelerated growth and larger callus mass. These promising results propose the DKW basal salt mixture as a potential standardized medium, paving the way for more efficient and expansive micropropagation practices in *Cannabis sativa* L. However, the study also suggests the necessity for further research to unequivocally establish the benefits of DKW in plant regeneration processes. This study, therefore, stands as a pivotal contribution to establishing refined and standardized micropropagation protocols in Cannabis cultivation, potentially nurturing a broader spectrum of genetic propagation. The Galán-Ávila *et al*. (2020) study focuses on advancing the *in-vitro* tissue culture methodologies for *Cannabis sativa*, aiming at developing a highly effective protocol for direct regeneration from various explants and analyzing the ploidy levels of both explants and *in-vitro* regenerates. The research identifies the hypocotyl as the most viable explant for direct regeneration, indicating a potential avenue to

stand-in high-shoot organogenesis efficiency across diverse *C. sativa* varieties (Table 1). The introduction underscores the critical role of *in-vitro* culture as a potent tool to supplement traditional breeding and facilitate the development of polyploid varieties with heightened secondary metabolite levels or to enable genetic transformations of non-regenerating tissues. Despite its potential, the existing protocols demonstrate low regeneration efficiency, representing a significant impediment to leveraging *in-vitro* tissue culture for *C. sativa* enhancement. The study evaluates the response of various explants, including cotyledons, hypocotyls, and true leaves, to ten different regeneration mediums. The research offers substantial data to substantiate the protocol developed through meticulous observation of developmental morphology from seed germination to rooted plantlet formation, coupled with a detailed analysis of ploidy levels. The findings affirm the superiority of the hypocotyl as an explant for direct *in-vitro* regeneration, showcasing a starkly better response than cotyledons and true leaves. The developmental morphology study hypothesizes the origination of regenerated shoots from pericycle cells adjacent to the xylem poles. It reveals the presence of polysomy in all examined varieties of hypocotyls and cotyledons. Notably, the protocol unveiled in this study demonstrates a significant promise to facilitate the creation of polyploids in *Cannabis sativa*, thereby potentially contributing immensely to the development of medically superior *C. sativa* materials. The study marks a significant step in tissue culture advancements, outlining a protocol that promises high shoot organogenesis efficiency across various *Cannabis sativa* varieties, potentially paving the way for the development of polyploids in *C. sativa* and contributing significantly to the enhancement of *C. sativa* materials for medical applications. Another study by Galán-Ávila *et al*. (2021) meticulously evaluated the potential of different explants from six *C. sativa* varieties for transgenic plant regeneration. A novel, rapid method for cultivating genetically transformed *Cannabis sativa* L. plants is introduced, utilizing various explants and the *Agrobacterium tumefaciens* strain LBA4404, remarkably accelerating the transformation rate of regenerating shoots and producing transgenic plants three times faster than previously published protocols (Table 1). The study evaluates the regeneration potential of hypocotyl, cotyledon, and meristem explants from six distinct *C. sativa* varieties, paving the way for breakthroughs in plant breeding techniques, including targeted genome editing using the CRISPR/Cas systems. The research offers a detailed, hormone-free protocol that doubles the efficiency of the transformation process, holding significant implications for *C. sativa* breeding and nurturing the development of improved varieties with resistance to various stresses, enhanced nutritional profiles, and increased yields. The study emphasizes the versatile applications and therapeutic properties of *C. sativa*, a dicotyledonous species from the Cannabaceae family. It emphasizes its role in industrial sectors and as a component in functional foods and animal feeds. Acknowledging the scarcity and limitations of existing protocols for transgenic *C. sativa* plants, the paper advocates developing proficient transformation protocols to leverage this technique extensively in *C. sativa* species. Employing this methodology, the study examines the *in-vitro* regeneration rates of different explants in varied culture mediums, scrutinizes the impact of diverse kanamycin concentrations on regeneration rates, and validates plant transformation through comprehensive assays and PCR amplification of uidA and nptII genes (Galán-Ávila *et al*., 2021). Notably, the research reveals that hypocotyls manifest a higher regeneration rate than cotyledons, with 100 mg/L being the optimal kanamycin concentration facilitating the regeneration of shoots, thus substantiating the efficacy of the newly devised hormone-free protocol. The paper suggests that this protocol represents a significant step forward in *C. sativa* breeding, potentially facilitating the development of varieties with particular biochemical characteristics or improved tolerance to environmental stresses, indicating a notable progression in plant science research.

In the context of addressing persistent challenges in Cannabis (*Cannabis sativa*) micropropagation, such as hyperhydricity, poor shoot extension, and ineffective *ex-vitro* rooting, Lubell-Brand *et al*. (2021) present an *in vitro-ex vitro* micropropagation system designed to address the challenges of hyperhydricity, poor shoot extension, and unsuccessful *ex-vitro* rooting in *Cannabis sativa* (Table 1). The authors introduce a culture initiation method that mitigates shoot hyperhydricity by utilizing a retipping technique. Additionally, the study demonstrates that by adjusting the composition of the culture medium, they improved shoot extension and maintained healthy growth over extended periods.

The article concludes by suggesting that the methods developed can serve as a basis for a commercial-scale micropropagation system for Cannabis, offering potential advancements in tissue culture protocols for this economically important crop.

The study by Zhang *et al*. (2021) showed the development of a CRISPR-Cas9-based mutant of *C. sativa* for the first time. To improve regeneration in *C. sativa*, they used developmental and growth regulators in the media. Still, the regeneration of transformants could have been better. Nevertheless, they have established an Agrobacterium-mediated genetic transformation and CRISPR/Cas9-targeted mutagenesis protocol for Cannabis (*Cannabis sativa* L.), focusing on studying genes implicated in cannabinoid biosynthesis (Table 1). Utilizing the DMG278 strain as a model due to its high shoot induction rate, the team enhanced shoot regeneration efficiency by overexpressing the Cannabis developmental regulator Chimaera. They also applied CRISPR/Cas9 technology to edit the phytoene desaturase gene, generating four edited seedlings with an albino phenotype. This work not only provides a reliable methodology for Cannabis genetic transformation but also validates the functional genomics of critical genes involved in cannabinoid biosynthesis, thereby laying the groundwork for the development of new therapeutic drugs and improved Cannabis varieties. A review paper by Adhikary *et al*. (2021) serves as a comprehensive repository of the latest scientific findings in Cannabis tissue culture, shedding light on the advancements and potential applications of optimized techniques in the propagation, regeneration, and transformation of Cannabis plants. The study shows the progress in various facets of tissue culture, encompassing micropropagation, transformation, and regeneration of medicinal Cannabis and industrial Cannabis transformants. It proposes potential trajectories for future research aimed at fostering elite Cannabis breeding and propagation.

The genetic transformation methods in Cannabis have evolved significantly, encompassing a range of techniques that aim to enhance gene transformation and plant regeneration. These advancements promote the development of novel genetic variants exhibiting preferred characteristics. Augmented secondary metabolite production paving the way for further research and development in this field.

Synthetic Seed Technology

Synthetic seeds, often made from encapsulated somatic embryos or axillary buds, present an innovative approach to plant breeding, particularly for Cannabis. These artificial seeds are easier to handle transport and are pathogen-free, making them ideal for large-scale clonal propagation. Given the cross-pollinating nature of Cannabis, traditional breeding methods require a minimum distance of 5 km between nurseries to prevent unwanted genetic mixing, a logistical challenge in areas with high Cannabis production. In contrast, synthetic seeds offer a viable alternative, often encapsulated in a hydrogel like calcium alginate, which provides essential nutrients and growth regulators. Lata *et al*. (2009b) demonstrated that synthetic seeds, specifically encapsulated axillary buds, could be propagated both *in-vitro* and *in-vivo* in specific media conditions, thus mitigating tight scheduling constraints tied to the long growth cycles of cannabis plants. The research not only emphasized the cost-effectiveness of this method for preserving germplasm and potentially enhancing planting material quality but also showed its promising applications in the pharmaceutical sector. Importantly, it opens avenues for further research on the nutritive conditions in planting substrates and encapsulation matrices and suggests the potential for automation to improve efficiency further. The technology holds promise for revolutionizing germplasm storage and mass propagation of elite, high-yielding variants of *Cannabis sativa*. (Chand and Singh, 2004; Lata *et al*., 2009a; Rai *et al*., 2008; Rihan *et al*., 2017).

CHALLENGES IN CANNABIS GENETIC TRANSFORMATION

Genotypic Specificity and Reproducibility

Cannabis genetic transformation is currently grappling with significant challenges, primarily revolving around the genotypic specificity of regeneration protocols and the reproducibility of existing methods. Studies have indicated that the protocols seem genotype-dependent, necessitating customization based on the specific varieties of *Cannabis sativa* being propagated (Feeney and Punja, 2003; Monthony *et al*., 2021b). Furthermore, the significant variation in response among different genotypes calls for a more nuanced approach to developing transformation methods, focusing on

creating more robust and universally applicable protocols (Monthony *et al*., 2021b).

Efficiency in Secondary Metabolite Production

Secondary metabolites in *Cannabis sativa* have garnered significant attention due to their diverse pharmacological properties. These metabolites primarily include cannabinoids, flavonoids, and terpenes, each with unique biosynthetic pathways and functions. The production of these secondary metabolites is influenced by various factors such as genotype, environmental conditions, and plant growth stage. Cannabinoids like THC and CBD are the most studied secondary metabolites in Cannabis. They are synthesized in glandular trichomes, and their levels can vary significantly among different strains. Recent studies have employed tissue culture techniques to manipulate cannabinoid biosynthesis. For instance, Raharjo *et al*. (2010) study highlighted the limitations in producing cannabinoids and flavonoids in cell suspension cultures, indicating a need for further research to enhance the production efficiency of these valuable compounds. Flavonoids and terpenes are other critical secondary metabolites with potential therapeutic benefits. However, their production in *in-vitro* cultures remains a challenge. The study by Feeney and Punja (2003) pointed out the unsuccessful attempts to promote plantlet regeneration via somatic embryogenesis, which is crucial for metabolite production. Advancements in genetic transformation methods, such as Agrobacterium-mediated transformation and CRISPR/Cas9 technology, offer promising avenues for enhancing secondary metabolite production. However, challenges persist, including low transformation efficiency and genotype-dependent responses, as indicated in recent studies like Zhang *et al*. (2021) and Ioannidis *et al*. (2022). The transformation efficiency, however, was limited, with only a small percentage of calli generating white seedlings and most transgenic shoots forming chimeric tissues, highlighting the challenges in achieving robust regeneration in *C. sativa* (Zhang *et al*., 2021).

In summary, while tissue culture and genetic transformation techniques hold promise for the targeted production of secondary metabolites in *Cannabis sativa*, several challenges must be addressed. These include optimizing culture conditions, improving transformation efficiency, and developing genotype-independent protocols. Future research should focus on overcoming these challenges to harness the medicinal potential of Cannabis secondary metabolites.

Complexity of the Cannabis Genome

The complexity of the Cannabis genome presents a substantial hurdle in the genetic transformation of Cannabis. Characterized by a high degree of polymorphism and multiple copies of specific genes, the Cannabis genome poses challenges in achieving high transformation rates. *Cannabis sativa* typically has a diploid chromosome number of 2n = 20. The genome size of *Cannabis sativa* has been estimated to be approximately 820 Mb. The regeneration efficiency of transformed cells remains a significant bottleneck, often resulting in low transformation rates (Feeney and Punja, 2003).

Lack of Standardized Protocols

The field faces a lack of standardized protocols, which has resulted in ambiguous results and a marked lack of reproducibility across different genotypes. This has been exacerbated by the prevalent use of Cannabis as a proxy for drug-type Cannabis, limiting the scope of research and necessitating a shift towards more inclusive and precise research methodologies.

Despite the advancements (Galán-Ávila *et al*., 2021; Zhang *et al*., 2021), there is a pressing need to optimize protocols for higher efficiency, including determining the optimal medium composition and the most effective combinations of plant growth regulators. Moreover, addressing the challenges in promoting plantlet regeneration via somatic embryogenesis or organogenesis remains a priority, as highlighted in the Feeney and Punja (2003) study. Future research should focus on developing suitable systems for *in vitro* plantlet regeneration, fostering more inclusive and precise research methodologies.

Gene Editing Technologies in Cannabis sativa L.

Recent advancements in plant science have seen a surge in research focusing on the genetic modification of *Cannabis sativa*, a plant renowned for its industrial and medicinal applications (Deguchi *et al*., 2020; Galán-Ávila *et al*., 2020). Various studies have embarked on exploiting gene editing technologies such as the CRISPR/Cas9 system and Agrobacterium-mediated genetic transformation to manipulate the genome of *Cannabis sativa* to enhance traits such as yield, resistance to pests, and medicinal

properties (Zhang *et al*., 2021). Notably, establishing agroinfiltration as a viable method for transient gene expression in Cannabis has opened new avenues for functional genomics studies, a stepping stone toward more advanced research in this domain (Deguchi *et al*., 2020). Furthermore, integrating Agrobacterium-mediated genetic transformation with the CRISPR/Cas9 system has emerged as a powerful tool for precise gene editing, facilitating targeted modifications to enhance various traits of interest in *Cannabis sativa* (Zhang *et al*., 2021). This dual system represents a significant advancement, providing a robust platform for exploring and manipulating the *Cannabis sativa* genome, potentially encouraging the development of varieties with improved properties. However, the research also highlights the necessity for further optimization to enhance the efficiency and applicability of these techniques (Galán-Ávila *et al*., 2021). Despite the promising advancements, the field is not devoid of challenges. The recalcitrance of *Cannabis sativa* to de novo regeneration is a significant barrier, hindering successful regeneration and transformation processes (Galán-Ávila *et al*., 2021). Studies have emphasized the need for focused research to develop strategies to overcome these challenges, promoting advancements in *Cannabis sativa* research. Collaborative approaches involving researchers from various disciplines are advocated to address the complex challenges associated with regeneration and transformation in *Cannabis sativa*.

Moreover, the research underscores the importance of considering the regulatory and ethical implications associated with gene editing (Galán-Ávila *et al*., 2020). The current research landscape in gene editing technologies in *Cannabis sativa* is dynamic and evolving. While significant progress has been made, a broad scope exists for further research to fully realize the potential of gene editing technologies in *Cannabis sativa*. Future directions involve exploring new frontiers in gene editing technologies, addressing ethical considerations, and fostering collaborative approaches to overcome existing challenges and unlock new commercial prospects for Cannabis, including the development of varieties with enhanced properties for industrial, medicinal, and recreational uses (Clarke and Merlin, 2016; Galán-Ávila *et al*., 2020; Zhang *et al*., 2021).

Recent developments have introduced promising techniques like morphogenic genes, computational approaches, and CRISPR/Cas9-equipped Agrobacterium-mediated genome editing. These methods aim to enhance gene transformation and plant regeneration, speeding the development of new genotypes with desirable traits and augmented secondary metabolite production (Ioannidis *et al*., 2022; Zhang *et al*., 2021).

Regulation and Biosafety Concerns

The growing developments in the genetic transformation of Cannabis necessitate a keen focus on concurrent regulatory and biosafety concerns. The pharmaceutical applications of genetically modified Cannabis accentuate the urgency of establishing robust frameworks to govern its cultivation and utilization, ensuring environmental and consumer safety (Adhikary *et al*., 2021; Hesami *et al*., 2021). The global community is gradually acknowledging the necessity of governing the cultivation and utilization of *Cannabis sativa* through stringent international frameworks. International organizations are at the forefront of establishing guidelines safeguarding biodiversity and human health, as evidenced by the ongoing discussions in various research communities (Monthony *et al*., 2021a; Wawrosch and Zotchev, 2021). On a national front, different countries are adopting diverse strategies to regulate the cultivation and research of genetically edited Cannabis. These policies, primarily driven by a country's socio-political stance and the current state of research, aim to foster a conducive environment for research while ensuring biosafety (Hesami *et al*., 2021; Zhang *et al*., 2021).

Applications of Genetically Modified Cannabis

Genetically modified Cannabis, a pivotal frontier in the Cannabis industry, harbours the potential to revolutionize various sectors, including medicine, agriculture, and industry. Leveraging genetic modification, techniques can promote the development of Cannabis varieties with enhanced biochemical profiles and improved resistance to biotic and abiotic stresses, signalling a promising trajectory for the Cannabis sector.

Recent studies underscore the remarkable potential of genetically modified Cannabis in the pharmaceutical sector. Genetic manipulations have facilitated advancements in cannabinoid biosynthesis, paving the way for developing new therapeutic drugs to combat human diseases. For instance, the optimization of transformation and gene editing protocols, as illustrated in the research conducted by Zhang *et al*. (2021), can be

extended to other Cannabis genotypes, potentially revolutionizing agricultural practices and crop improvement strategies. Furthermore, the study by Adhikary *et al*. (2021) accentuates the prospective applications in plant tissue culture and the prospects of medical Cannabis. In agriculture, genetically edited Cannabis promises to usher in a new era of sustainable practices. The development of pest-resistant varieties, as indicated in the findings of Wawrosch and Zotchev (2021), can mitigate reliance on chemical pesticides, fostering an eco-friendly approach to agriculture. Moreover, introducing high-yielding varieties can enhance the quality and yield of Cannabis products, contributing to germplasm conservation and facilitating the production of genetically uniform plants, as highlighted in the study by Ioannidis *et al*. (2022).

The industrial sector stands to benefit substantially from the innovations spurred by genetically edited Cannabis. As Monthony *et al*. (2021a) suggested, enhancing fibre qualities can foster textile and construction development. Furthermore, the potential to manipulate terpene profiles and optimize CBD-to-THC ratios can herald a new epoch in the production of therapeutic and biodegradable material and other biopolymers, a potential underscored in the research by Lata *et al*. (2016). Genetically edited Cannabis presents many applications, signalling a promising trajectory for the Cannabis industry. The innovations in genome-edited techniques are poised to catalyze advancements in medicinal, industrial, and recreational sectors, encouraging a new era of research and development. As the field continues to evolve, researchers and practitioners must delve deeper into the potential applications and prospects of genome-edited Cannabis, paving the way for a sustainable and prosperous future.

CONCLUSION

The recent surge in studies regarding Cannabis tissue culture and genetic transformation underscores a pivotal transformation in *Cannabis sativa* research. These studies, including those by Adhikary *et al*. (2021) and Galán-Ávila *et al*. (2021), offer illuminating insights into the protocols and methodologies of Cannabis tissue culture and genetic transformation, notably in callus induction and Agrobacterium-mediated transformation. These advancements mark a significant stride in fostering the advancement of novel cultivars of industrial Cannabis and medicinal Cannabis featuring enhanced traits. However, the journey is fraught with challenges, particularly in establishing successful protocols for plantlet regeneration, a crucial phase in the development of transgenic Cannabis varieties, as underscored in earlier studies (Feeney and Punja, 2003; Slusarkiewicz-Jarzina *et al*., 2005). Despite these hurdles, the field stands on the cusp of reform, with the potential to profoundly influence various industries by developing Cannabis varieties boasting specific biochemical profiles or heightened tolerance to diverse stresses. The increasing comprehension of the Cannabis genome and advancements in gene editing technologies herald a promising future, paving the path for improved Cannabis varieties. This burgeoning field necessitates concerted efforts and collaboration among scientists, policymakers, and industry stakeholders to harness the potential of genetically modified Cannabis fully.

Moreover, a critical focus on optimizing existing protocols and exploring the potential of gene editing technologies in *Cannabis sativa* is imperative. This review encapsulates the dynamic and evolving landscape of Cannabis tissue culture, providing a critical perspective to navigate the past, present, and future trajectories of Cannabis genetic transformation. It aims to guide researchers and producers in the Cannabis industry, steering them toward a future characterized by innovation, growth, and the fruition of enhanced therapeutic potentials of *Cannabis sativa* in both medical research and industry. Though marked by challenges of reproducibility and genotypic variation, this journey promises to unfold innovative approaches to meet the current demands of the Cannabis industry, thus ushering in an era of substantial growth and transformation.

FUTURE PROSPECTS

Looking ahead, the field of Cannabis tissue culture and genetic transformation is at a juncture of substantial evolution and innovation, underpinned by a growing body of literature and the relaxation of global regulations, nurturing enhanced research opportunities. The studies reviewed herein underscore the imperative for continuous research focusing on optimizing existing protocols for tissue culture techniques and exploring the potential of gene editing technologies, including CRISPR/Cas systems, for the genetic enhancement of *Cannabis*

sativa. Future research endeavours should primarily concentrate on overcoming the persistent challenges associated with plantlet regeneration and further delve into the potential of introducing novel genes or traits into Cannabis through established tissue culture and transformation protocols. These endeavours should also encompass evaluating the consequential effects of these protocols on the growth and development of the resultant Cannabis plants. Moreover, the upcoming research trajectory should develop Cannabis varieties endowed with tailored cannabinoid profiles, superior agronomic traits, and augmented industrial applications. A concerted effort to integrate omics technologies and computational biology will be instrumental in fostering a profound understanding of the Cannabis genome, thereby facilitating more precise and efficient genetic transformations.

Furthermore, prospects gleam with the anticipation of a rich corpus of research exploring the intricacies of Cannabis micropropagation and regeneration, which encompasses the development of protocols for somatic embryogenesis, organogenesis, and haploid production, particularly pivotal for gene transformation and genome editing studies. Thus, the field is poised for robust growth and innovation, potentially revolutionizing the Cannabis industry.

ACKNOWLEDGMENTS

The Director, CSIR Central Institute of Medicinal and Aromatic Plants, Lucknow, India, is acknowledged for providing the facilities and support.

CONFLICT OF INTEREST

The author declares no conflict of interest.

REFERENCES

Abel EL. 1980. Marihuana: The First Twelve Thousand Years. Plenum Press, New York, USA.

Adhikary D, Kulkarni M, El-Mezawy A, Mobini S, Elhiti M, Gjuric R, Ray A, Polowick P, Slaski JJ, Jones MP, Bhowmik P. 2021. Medical Cannabis and Industrial Hemp Tissue Culture: Present Status and Future Potential. *Front Plant Sci* **12**: 627240.

Adinoff B, Reiman A. 2019. Implementing Social Justice in the Transition from Illicit to Legal Cannabis. *Am J Drug Alcohol Abuse* **45**: 673-688.

Chand S, Singh AK. 2004. Plant Regeneration from Encapsulated Nodal Segments of Dalbergia sissoo Roxb., a Timber-Yielding Leguminous Tree Species. *J Plant Physiol* **161**: 237-243.

Chandra S, Lata H, Khan IA, ElSohly MA. 2011. Photosynthetic Response of *Cannabis sativa* L., an Important Medicinal Plant, to Elevated Levels of CO2. *Physiol Mol Biol Plants* **17**: 291-295.

Chaohua C, Gonggu Z, Lining Z, Chunsheng G, Qing T, Jianhua C, Xinbo G, Dingxiang P, Jianguang S. 2016. A Rapid Shoot Regeneration Protocol from the Cotyledons of Hemp (*Cannabis sativa* L.). *Ind Crops Prod* **83**: 61-65.

Chopra IC. and Chopra, RN. 1957. The use of cannabis drugs in India. *Bulle Narc* **9**: 4-29.

Clarke RC, Merlin MD. 2016. Cannabis Domestication, Breeding History, Present-day Genetic Diversity, and Future Prospects. *Crit Rev Plant Sci* **35**: 293-327.

Deguchi M, Bogush D, Weeden H, Spuhler Z, Potlakayala S, Kondo T, Zhang ZJ, Rudrabhatla S. 2020. Establishment and optimization of a Hemp (*Cannabis sativa* L.) Agroinfiltration System for Gene Expression and Silencing Studies. *Sci Rep* **10**: 3504.

ElSohly MA, Radwan MM, Gul W, Chandra S, Galal A. 2017. Phytochemistry of *Cannabis sativa* L. In: Kinghorn AD, Falk H, Gibbons S, Kobayashi J, (Eds.), *Phytocannabinoids*, Springer International Publishing, pp 1–36.

Feeney M, Punja ZK 2003. Tissue Culture and Agrobacterium-mediated Transformation of Hemp (*Cannabis sativa* L.). *In Vitro Cell Dev Biol Plant* **39**: 578-585.

Galán-Ávila A, García-Fortea E, Prohens J, Herraiz FJ. 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.

Galán-Ávila A, Gramazio P, Ron M, Prohens J, Herraiz FJ. 2021. A Novel and Rapid Method for Agrobacterium-mediated Production of Stably Transformed *Cannabis sativa* L. Plants. *Ind Crops Prod* **170**: 113691.

Hesami M, Baiton A, Alizadeh M, Pepe M, Torkamaneh D, Jones AMP. 2021. Advances and Perspectives

in Tissue Culture and Genetic Engineering of Cannabis. *Int J Mol Sci* **22**: 5671.

Ioannidis K, Tomprou I, Mitsis V. 2022. An Alternative *In-vitro* Propagation Protocol of *Cannabis sativa* L. (Cannabaceae) Presenting Efficient Rooting, for Commercial Production. *Plants* **11**: 1333.

Iversen LL. 2008. The Science of Marijuana. Oxford University Press, Oxford, UK.

Kuddus M, Ginawi I, AlHazimi A. 2013. *Cannabis sativa*: An Ancient Wild Edible Plant of India. *Emirates J Food Agric* **25**: 736.

Lata H, Chandra S, Khan IA, ElSohly M. 2010. High Frequency Plant Regeneration from Leaf Derived Callus of High Δ9- Tetrahydrocannabinol Yielding *Cannabis sativa* L. *Planta Medica*, **76**: 1629–1633.

Lata H, Chandra S, Khan IA, ElSohly MA. 2009a. Propagation Through Alginate Encapsulation of Axillary Buds of *Cannabis sativa* L. —An Important Medicinal Plant. *Physiol Mol Biol Plants* **15**: 79-86.

Lata H, Chandra S, Khan IA, ElSohly MA. 2009b. Thidiazuron-induced High-frequency Direct Shoot Organogenesis of *Cannabis sativa* L. *In-Vitro Cell Dev Biol Plant* **45**: 12-19.

Lata H, Chandra S, Khan IA, ElSohly MA. 2016. *In-vitro* Propagation of *Cannabis sativa* L. and Evaluation of Regenerated Plants for Genetic Fidelity and Cannabinoids Content for Quality Assurance. In: Jain SM (Ed.) *Protocols for In Vitro Cultures and Secondary Metabolite Analysis of Aromatic and Medicinal Plants*, Springer New York, pp. 275–288.

Lubell-Brand JD, Kurtz LE, Brand MH. 2021. An *in-vitro–Ex-Vitro* Micropropagation System for Hemp. *Hort Technol* **31**: 199-207.

Monthony AS, Kyne ST, Grainger CM, Jones AMP. 2021. Recalcitrance of *Cannabis sativa* to De Novo Regeneration; A Multi-genotype Replication Study. *PLOS ONE* **16**: e0235525.

Monthony AS, Page SR, Hesami M, Jones AMP. 2021. The Past, Present and Future of *Cannabis sativa* Tissue Culture. *Plants* **10**: 185.

Movahedi M, Ghasemi-Omran V, Torabi S. 2015. The Effect of Different Concentrations of TDZ and BA on *In-vitro* Regeneration of Iranian Cannabis (*Cannabis sativa*) Using Cotyledon and Epicotyl Explants. *J Plant Mol Breeding* **3**.

Musio S, Müssig J, Amaducci S. 2018. Optimizing Hemp Fiber Production for High Performance Composite Applications. *Front Plant Sci* **9**: 1702.

Page SRG, Monthony AS, Jones AMP. 2020. DKW basal salts improve micropropagation and callogenesis compared to MS basal salts in multiple commercial cultivars of *Cannabis sativa Plant Bio* [Preprint].

Piunno KF, Golenia G, Boudko EA, Downey C, Jones AMP. 2019. Regeneration of shoots from immature and mature inflorescences of *Cannabis sativa. Can J Plant Sci* **99**: 556-559.

Raharjo TJ, Eucharia O, Chang W-T, Verpoorte R. 2010. Callus induction and phytochemical characterization of *Cannabis sativa* cell suspension cultures. *Indones J Chem* **6**: 70-74.

Rai MK, Jaiswal VS, Jaiswal U. 2008. Encapsulation of shoot tips of guava (Psidium guajava L.) for short-term storage and germplasm exchange. *Scientia Horticult* **118**: 33-38.

Rihan H, Kareem F, El-Mahrouk M, Fuller M. 2017. Artificial Seeds (Principle, Aspects and Applications). *Agronomy* **7**: 71.

Slusarkiewicz-Jarzina A, Ponitka A, 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**: 145-151.

Smýkalová I, Vrbová M, Cvečková M, Plačková L, Žukauskaitė A, Zatloukal M, Hrdlička J, Plíhalová L, Doležal K, 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, Tiss Org Cul* **139**: 381-394.

Wawrosch C, Zotchev SB. 2021. Production of bioactive plant secondary metabolites through *in-vitro* technologies—Status and outlook. *Appl Microbiol Biotechnol* **105**: 6649-6668.

Zhang X, Xu G, Cheng C, Lei L, Sun J, Xu Y, Deng C, Dai Z, Yang Z, Chen X, Liu C, Tang Q, Su J. 2021. Establishment of an Agrobacterium-mediated genetic transformation and CRISPR/Cas9-mediated targeted mutagenesis in Hemp (*Cannabis sativa* L.). *Plant Biotechnol J* **19**: 1979-1987.