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Review

Tuning Up In Vitro Growth and Development of *Cannabis sativa*: Recent Advances in Micropropagational Approach

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Abstract: *Cannabis sativa* is used for multiple purposes, notably for its medicinal properties. It produces various secondary metabolites, including cannabinoids, terpenes, and flavonoids, which have therapeutic value and typically produce high amounts in female plants. The growth of the global cannabis market has led to intensive breeding efforts to develop elite cultivars with enhanced secondary metabolite profiles. As a dioecious and anemophilous plant, it produces staminate and pistillate inflorescences on separate plants and relies on wind for pollination, rendering traditional propagation methods challenging owing to high genetic recombination in progeny. Consequently, asexual propagation (micropropagation) is commonly employed to maintain female clones entirely. Micropropagation/direct organogenesis is a tissue culture technique that produces numerous disease-free clone plants in vitro more rapidly than traditional rooted cuttings. Factors such as sterilization, hormonal balance, explant type, nutrient additives, carbon source, pH, and environment influence the success of cultivar-specific micropropagation. In this review, we discussed how these factors affect cannabis micropropagation based on recent findings, emphasizing the importance of optimizing cultivar-specific protocols for long-term germplasm conservation and efficient breeding based on a mechanistic background.

Keywords: *Cannabis*; secondary metabolites; in vitro; micropropagation protocols; direct organogenesis; hyperhydricity mitigation; photoautotrophic micropropagation

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1. Introduction

Cannabis sativa L. is an annual, dioecious plant belonging to the Cannabaceae family. This plant synthesizes over 120 compounds, including cannabinoids, terpenes, and flavonoids, which exhibit therapeutic properties, thus supporting its medicinal application. The growth of the worldwide cannabis market has been a key factor in propelling advancements in the cannabis sector [1], including intensive breeding programs for elite cultivars with enhanced cannabinoid profiles to improve therapeutic efficacy, meet consumer preferences, and increase yields [1]. The commercial value of cannabis is primarily attributed to the production of cannabinoids and terpenes within glandular trichomes, which are densely located in the bracts of female flowers [1]. The diversity in cannabinoid composition amongst flower clusters poses a challenge arising from disparate exposure

to factors and fluctuations in source–sink dynamics. One potential strategy to address this variability is utilizing tissue culture systems such as cell suspensions for the mass production of key secondary metabolites [1].

The liberalization of legislation and the expansion of cannabis markets have led to increased cannabis research. As more nations legalize cannabis for medicinal, recreational, and industrial purposes, global cannabis markets have experienced growth [2]. The medicinal cannabis industry relies on high-quality cannabinoid-rich products from only female cultivation, with a preference for trimmed flower bud extracts [2]. The escalating requirement for *Cannabis* propagated under aseptic conditions necessitates scalable cultivation approaches, such as vertical agriculture, plant production complexes, and technologically enhanced farming systems [3].

Cannabis breeding presents complexities owing to several factors: (1) Flowering induction necessitates controlled light regimes, which complicates agricultural practices. (2) Stringent regulations in numerous countries mandate security measures, limit growing spaces, and specify the maximum number of plants [1]. (3) Cannabis is predominantly dioecious, with separate male and female plants, resulting in high genetic recombination and heterozygosity in the offspring [1]. Cultivation from seed leads to inconsistent vegetative growth and flowering owing to trait segregation risks [1]. As an anemophilous species, male flowers produce substantial amounts of pollen, potentially fertilizing female inflorescences-reduced secondary metabolite production [1]. Consequently, asexual propagation is frequently employed to produce and maintain female clones, necessitating the strict isolation of male plants to prevent undesired fertilization, which limits the number of feasible crosses in breeding programs [1]. Thus, the cannabis industry predominantly relies on clonal propagation, which is typically accomplished by taking cuttings from mother plants [2]. This traditional technique is employed even by large-scale producers, with mother plants maintained in a constant vegetative state occupying approximately 15% of the growing area [2]. However, this approach is inefficient and makes the industry vulnerable to potential germplasm loss and decreased production owing to diseases and pests [2].

Micropropagation is an in vitro tissue culture technique that asexually propagates plant material, producing numerous identical clones from a mother plant tissue. This methodology, which has been favored in horticultural practices for decades, is preferred over conventional vegetative propagation techniques for high-value crops due to its aseptic nature, spatial efficiency, and rapid multiplication capabilities [4]. This approach facilitates the mass production of genetically identical plants at a higher rate than traditional methods. Plants are cultured in bottles in a well-illuminated culture room, allowing for dense cultivation in a limited space. Sterile-medium cultivation ensures disease-free plants [1,3]. In vitro flowering has been observed across various species, proving helpful in studying floral mechanisms toward breeding programs, including cannabis [1]. Flower induction in micropropagation systems also offers controlled cannabinoid production and enhances breeding by enabling numerous crosses within a confined facility [1]. In tissue culture systems, factors such as plant growth regulators, light intensity, photoperiod, and temperature are meticulously regulated, enabling researchers to examine flower initiation and development [1]. These systems facilitate swift clonal propagation through either direct or indirect organogenic pathways [2]. The direct approach involves the immediate formation of plant organs from explanted tissues, whereas the indirect method requires an intermediate callus stage before complete plant regeneration [2]. Considerable progress has been made in developing protocols for direct organogenesis, with a focus on diverse explant types and optimization techniques [2].

Micropropagation/direct organogenesis comprises several stages. Stage 0 involves selecting and cultivating stock plants for harvesting various explants before disinfection and establishment in stage I [2]. Stage I establishes aseptic cultures by using disinfecting agents. Stage II is the multiplication phase, where plantlets are cultivated and subdivided until the desired number of propagules is achieved. Stage III involves root induction, and stage IV acclimatizes plants to their growth environment [2–8]. Efficient micropropagation requirements vary among species, and numerous variables, such as disinfection protocols, media inputs, and photoautotrophic and environmental conditions, can be optimized at each stage. Currently, consistent and successful micropropagation protocols for hemp are insufficient [2–8].

Developing a reliable micropropagation protocol for hemp has presented significant challenges owing to hyperhydricity during Stage I and the inability to maintain high-quality shoot growth over extended periods [4,6,8]. Prolonged culture in the multiplication stage frequently results in vitrification/hyperhydricity caused by the gelling agent, plant growth regulators, media composition, and leaf desiccation or necrosis, thereby complicating sustained multiplication [2,4,8]. Trials assessed factors influencing contamination rates and establishment success in different stages: basal nutrient formulations, explant types, sucrose concentrations, pH adjustments, and various auxins and cytokinin concentrations. This information underpins successful hemp micropropagation, which mitigates vitrification/hyperhydricity [2–4,7,8]. The present review aimed to evaluate media components and methodologies to optimize the hemp micropropagation stages.

2. Methodology

This review evaluated contemporary studies on techniques for clonal micropropagation aimed at improving plant growth and development, with a focus on alterations to media formulations and the use of plant growth regulators. The efficacy and limitations of *C. sativa* micropropagation provide insights into practical applications. This review employed various academic databases, including Google Scholar, EBSCOhost, PubMed, and Scopus, to identify the relevant literature. Pertinent publications were retrieved from 2021–2024 using keywords "Clonal micropropagation, *Cannabis sativa*"; and "direct organogenesis, *Cannabis sativa*".

3. Major Factors Determining In Vitro Micropropagation Establishment in *Cannabis sativa*

Recent advancements in understanding in vitro plant regeneration mechanisms, coupled with the rapid evolution of specialized equipment and strategies, have garnered significant attention from plant scientists and industries. Nevertheless, enhancing the methodologies for selecting and regenerating superior genotypes is necessary to improve reproducibility. Refinement of protocols is crucial in this context. Plant tissue culture (PTC) encompasses various regenerative pathways and has been predicted in empirical studies. The effectiveness of PTC depends on several factors, including proper nutrient balance and suitable cells that can undergo epigenetic and molecular reprogramming. These cells must be capable of inducing stem cell formation and precisely controlling the synthesis and distribution of endogenous hormone [9].

3.1. Hormonal Regulation in Organogenesis

Plant cells and tissues exhibit totipotency, allowing them to regenerate into completely fertile plants under suitable in vitro conditions. This capability makes in vitro cultures crucial to plant biotechnology. These methods are rapidly evolving to refine processes and harness plant phenotypic plasticity for various applications in agriculture, industry,

conservation, and research [10–12]. The regeneration of genetically identical shoots from diverse tissue explants typically requires the addition of plant hormones to the culture media, particularly cytokinins (CKs) and auxin [10–12]. Modifying the CK or auxin signaling pathways can markedly improve shoot regeneration, even in tissues that are traditionally difficult to regenerate [10–12].

Under laboratory conditions, processes are initiated by wounding; incubation on an appropriate medium; or supplementation with phytohormones, typically auxins and cytokinins. Plant cells perceive these signals and subsequently alter their metabolic and genetic configurations to initiate reprogramming. However, the precise mechanisms by which these stimuli modulate development patterns and determine optimal regeneration pathways remain unclear [12]. The initial stage of plant regeneration involves the wounding of the explants. Various wound signals, including electric current, hydraulic pressure, Ca^{2+}, reactive oxygen species (ROS), and metabolic alterations, play a vital role in this process (Figure 1A). Although the examination of downstream genes of wound signaling indicates a significant impact on plant regeneration, comprehensive information regarding its effect on in vitro regeneration remains limited [11]. Research has shown that oxidative stress contributes to plant regeneration, influencing phenomena such as programmed cell death, phytohormone signaling pathways, and cell differentiation through ROS [11]. Organogenesis, a form of plant regeneration following tissue injury, involves the regeneration of organs, such as shoots and roots, from the site of the wound and detached organs. This process comprises three steps: (1) signaling to stimulate regeneration, (2) phytohormone, and (3) cell fate transition [11,12].

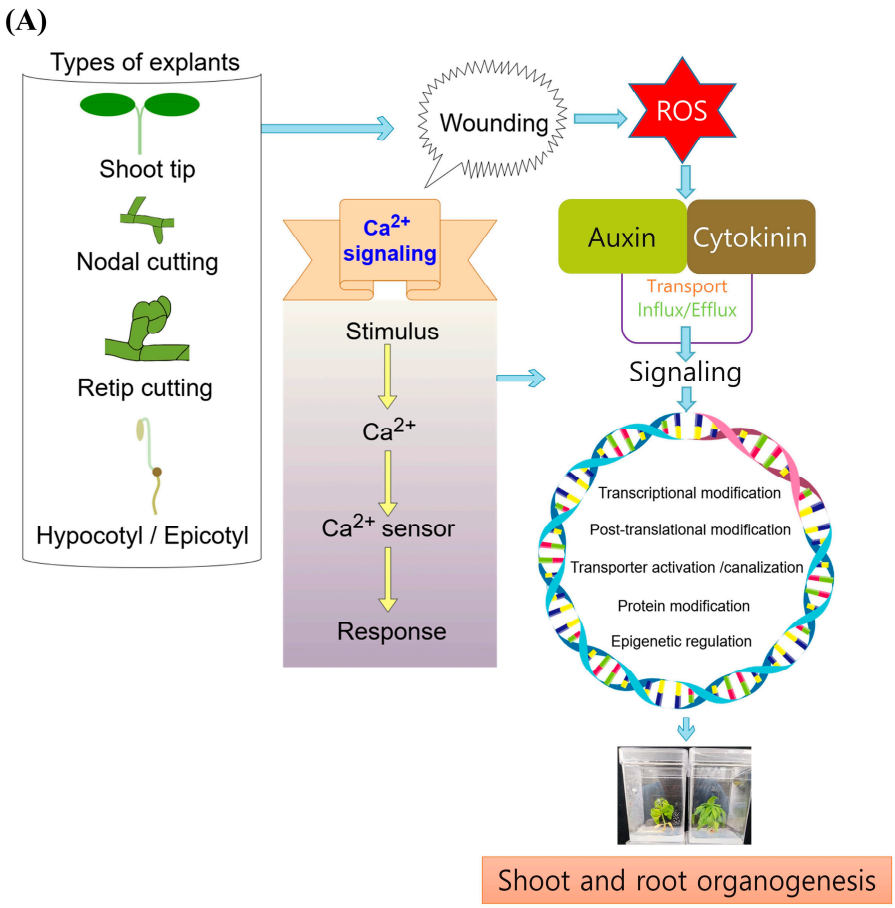

Figure 1. *Cont.*

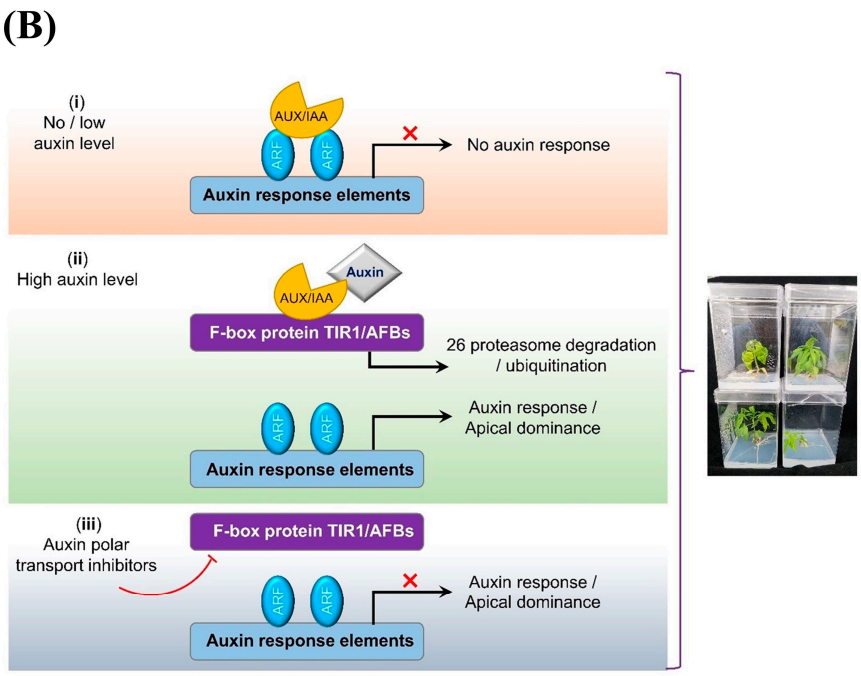

Figure 1. (**A**) Wound stresses and internal stimuli elevated [Ca^{2+}], which is then interpreted by Ca^{2+} sensors, activating downstream regulatory processes and eliciting an appropriate cellular response. Interconnected with auxin transport, cytokinin and ROS signaling pathways regulate growth, development, and plant morphology in response to wound-induced stress. (**B**) A simplified model of the plant nuclear auxin signaling pathway: Auxin binds to the TIR1/AFB auxin receptors, components of ubiquitin ligase complexes with a weak basal affinity for Aux/IAA proteins, significantly enhanced by auxin. This interaction promotes Aux/IAA ubiquitination and proteasomal degradation. The degradation of Aux/IAA lifts repression of auxin response factors (ARFs), enabling them to activate transcription of auxin-inducible genes with auxin-responsive elements (AREs).

Auxin, uniquely among phytohormones, exhibits the capacity for polar transport and polarity induction, rendering it a critical regulator of in vitro plant morphogenesis, whereas cytokinin may induce auxin biosynthesis [9,10]. Shoot and root formation is supported by auxin canalizing through vascular tissues, whereas shoot development occurs when there is a balance between auxin production and active transport via carriers [9,10]. The IPA-mediated auxin synthesis pathway is vital in cell induction [9,10].

Shoot regeneration is influenced by endogenous hormones and environmental conditions, including exogenous plant growth regulators (PGRs) [10]. The signaling pathways of auxin and CK (as an auxin biosynthesis inducer) interact not only with each other but also with other pathways [10]. CK and auxin signaling are crucial for micropropagation, with auxin predominating in early organogenesis stages and CK predominating in later stages (shooting) [10,12]. Root growth is enhanced when the ratio of auxin to CK is elevated, whereas shoot development is promoted when the CK-to-auxin ratio is high [10,12]. Organogenesis is genetically complex and involves numerous genes, such as transcription factors, hormonal response regulators, transporters, efflux–influx carrier genes and epigenetic regulators involved in hormonal metabolism and transport, and cell-cycle genes, all of which are influenced by the concentration and distribution of endogenous hormones [10–13]. The reduced regeneration capacity with age may result from decreased phytohormone responsiveness [11].

Physiologically, roots exhibit a natural means of canalization of the shoot-derived auxin. Successful rooting necessitates elevated auxin production in specific xylem-associated stem cells [13]. Vessels (sieve elements) are crucial for rooting, as they establish robust channels for auxin transport and facilitate root canalization [9,12,13]. Various in vitro

root induction protocols employ pulse treatments with high auxin concentrations (IBA) and darkness [9,12,13]. These protocols address natural rooting challenges by increasing auxin content in vessel element-rich tissue, thereby inducing a transient auxin peak to activate cell division in xylem-pole cells for root induction [9,12,13].

In recent years, synthetic auxin and cytokinin derivatives, including meta-topolin (mT), indole 3-butyric acid (IBA), thidiazuron (TDZ), 6-benzylaminopurine (BAP), and 1-naphthalene acetic acid (NAA), have been evaluated to enhance tissue growth control, thus demonstrating the potential of novel synthetic phytohormone derivatives in cannabis clonal propagation (Table 1) [3–5,14,15].

Table 1. Different factors influencing direct clonal micropropagation of *Cannabis sativa*.

Cultivar/Species	Factors Considered for In Vitro Growth and Development for Micropropagation of *Cannabis sativa*	Crucial Findings	References
TA5, Sky 1, Magic 9 (female cultivars), and Bt (male plant)	Light, carbon source, and ventilation	✓ Plantlet-produced inflorescences and viable seeds in vitro completed their full life cycle under low-light conditions. ✓ Photoautotrophic growth occurs in plants with DKW medium containing 2% sucrose in a filtered vessel.	[1]
TJ's CBD	Disinfection, gelling agents, explant, pH, Temperature, carbon source, and hormone for shooting and rooting	✓ Concentrations of 20%, 40%, and 60% bleach (7.5% sodium hypochlorite) applied for 10 min demonstrated equivalent efficacy in cleaning explants without causing damage. ✓ pH levels of 5.8, 6.0, and 7.0 generated more lateral nodes. ✓ Sucrose concentrations of 1.5% and 3.0% significantly increased fresh weights, shoot lengths, and quality ratings. ✓ Growth room temperatures 28 and 26 °C resulted in greater fresh weights, shoot lengths, and numbers of nodes. ✓ 5.0 µM TDZ and 2ip generated greater fresh weights and numbers of lateral nodes. ✓ 2.5 µM IBA treatment resulted in a higher root response.	[5]
Ten accessions used in Oregon CBD's essential oil-type hemp include "F1 hybrid" and "selections"	Nutrient media and carbon source	✓ DKW medium with glucose and hormone meta-topolin produced the most vigorous plantlets in vitro. ✓ Hybrids were more vigorous than nonhybrid selections in vitro.	[7]
'Abacus' and 'Wife'	Explant and age of culture	✓ The retip cuttings of 'Abacus' and 'Wife' varieties, from cultures aged 6, 9, and 12 weeks, demonstrated rooting success rates at 76% to 81% without rooting hormone.	[8]
'Wife' and 'Dinamed CBD'	Medium with varying nutrients, and vitamins	✓ Compared to the control, the use of MS supplemented with vitamins, as well as MS enriched with both vitamins and additional mesos (calcium chloride (anhydrous), magnesium sulfate (anhydrous), and potassium phosphate (monobasic)), resulted in higher production of retip microcuttings and reduced hyperhydricity.	[4]

Table 1. *Cont.*

Cultivar/Species	Factors Considered for In Vitro Growth and Development for Micropropagation of *Cannabis sativa*	Crucial Findings	References
'Abacus' and 'Wife'	Medium and age of subculturing	✓ Shoot multiplication rate and explant height were greater for 'Abacus' in LBC than DKW. ✓ For 'Wife', at 9 weeks, it was more significant in LBC, as LBC provided more nutrients and water than cultures had received in MS medium with ventilated microboxes. ✓ Culture medium did not influence ex vitro rooting success. ✓ Microboxes were not adequate to control this hyperhydricity.	[6]
High-CBD cultivars	Type of explant, media composition, surface sterilization, and PGR	✓ 30 min 1% NaOCl treatment was identified as optimal, with longer treatments causing significantly higher mortality and shorter treatments, resulting in too-high contamination. ✓ Microshoot tip explants exhibited the best overall results, with very low contamination levels and vitrification. ✓ Excessive presence of cytokinins (meta-topolin and TDZ) significantly increased vitrification. ✓ Proper amounts of vitamins and nutrient elements decreased vitrification.	[2]
'Cheungsam'	PGR, medium type, and medium components	✓ All concentrations of 2iP showed better results compared with BA and mT. ✓ DKW medium containing 1.0 mg/L 2iP and 0.5 mg/L of IBA in ½ DKW medium showed the best shooting and rooting rate, respectively. ✓ Medium with 0.5 g/L activated charcoal did not overcome hyperhydricity.	[3]
'Diana', 'Finola', and 'Fedora 17'	N-1-naphtylphtalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA) with TDZ (PGR)	✓ TDZ with NPA (1–5 mg L^{-1}) and TDZ with TIBA (0.5–2.5 mg L^{-1}) increased the response of explants in a genotype-dependent manner.	[16]

Abbreviation: TDZ—thidiazuron; DKW—Driver and Kuniyuki Walnut medium; LBC—Lubell-Brand Cannabis medium; IBA—indole 3-butyric acid; 2iP—6-(γ,γ-dimethylallylamino)purine; mT—meta-topolin.

Fiber-type hemp exhibits apical dominance [16], promoting the development of taller plants with elongated fibers that flourish at higher densities. Consequently, hemp with prominent apical dominance was cultivated selectively. Apical dominance, characterized by the suppression of axillary bud growth by the main shoot, is a significant factor contributing to the challenge associated with shoot proliferation in industrial hemp [16]. The development of buds is controlled by a sophisticated interplay between internal hormones, including auxin and cytokinins. Auxin, produced in young leaves at the apical meristem, is employed by the shoot apex and moves downward through the polar auxin pathway. This process inhibits the lateral buds by limiting their ability to access this transport pathway [16].

The auxin signaling pathway involves nuclear Transport Inhibitor Response 1/Auxin Signaling F-box protein (TIR1/AFB) auxin receptors, which interact with auxin and act as F-box ubiquitin ligases to facilitate ubiquitination and breakdown of Aux/IAA transcriptional suppressors [15]. These suppressors control auxin response factors (ARFs), which recognize auxin response elements (AREs) in the promoter regions of genes regulated by auxin [15]. Recent findings indicate that certain processes previously thought to be governed by the TIR1/AFB pathway, such as root growth regulation, occur too swiftly to involve transcription and protein synthesis, suggesting the existence of a potential

non-transcriptional branch of TIR1/AFB signaling (Figure 1B). Specific auxin antagonists, "including 4-(2,4-dimethylphenyl)-2-(1H-indol-3-yl)-4-oxobutanoic acid (auxinole) and 2-(1H-indol-3-yl)-4-oxo-4-phenylbutanoic acid (PEO-IAA) [15], can bind to TIR1, inhibiting the formation of the TIR1/AFB–IAA–Aux/IAA complex" and consequently suppressing the expression of auxin-responsive genes [15].

Two recent studies explored the combination of auxin polar transport inhibitors, including "N-1-naphtylphtalamic acid (NPA), 2,3,5-triiodobenzoic acid (TIBA), and PEO-IAA, with the cytokinin N-benzyl-9-(tetrahydro-2H-pyran-2-yl) adenine (BAP9THP)". This combination was employed to boost shoot regeneration in *Cannabis sativa* by inhibiting apical dominance [15,16].

3.2. Carbon Source

Carbon (C) is essential for the majority of macromolecules in plants, including cell walls, proteins, DNA, and RNA, and carbon deprivation is lethal for seedlings [9]. In vitro plant cells, tissues, and organ cultures require carbohydrates in the culture medium because of the lack of complete autotrophy/heterotrophy. These carbohydrates are essential for maintaining osmotic potential and act as reservoirs of energy and carbon, supporting energy-intensive processes such as shoot proliferation, root induction, embryogenesis, organogenesis, and cryopreservation [17]. The requirement for a carbon source in plant culture media is attributed to the heterotrophic nature of cultured cells and cannot be substituted by any other element [17]. Plants utilize various sugars in their metabolic processes, including monosaccharides, disaccharides, trisaccharides, and sugar alcohols [17]. These exogenous sugars are crucial for plant growth and development because they influence nutritional value, osmotic potential, and cell division [18]. They supply energy to explants that are incapable of photosynthesis under heterotrophic or mixotrophic in vitro conditions [17].

Exogenous carbohydrate supplements enhance cell division in plant embryos by promoting cell expansion and reserve accumulation, mediated by various sugar-sensing systems such as (1) hexokinase (HXK); (2) hexose transport-associated sensor (SUCROSE-NONFERMENTATION1-RELATED PROTEIN KINASE1 (SnRK1); (3) TARGET OF RAPAMYCIN (TOR) kinase; and (4) the Suc-specific pathway, possibly involving a signaling Suc transporter (Figure 2) [17]. Studies have demonstrated crosstalk between phytohormone signaling, particularly auxin and cytokinin biosynthesis [10] and sugar sensing, regulating developmental processes at transcriptional, post-transcriptional, and post-translational levels [9,10]. Sugars can also influence phytohormone responses by modifying their levels, localization, and transport [9]. Recent developments in plant propagation techniques, including photoautotrophic micropropagation, have offered a novel framework with numerous beneficial features. These include CO_2 enrichment within culture vessels, reduction or elimination of sugars from growth media, and optimization of in vitro culture conditions for effective micropropagation [17]. Carbohydrates are primarily utilized as transport sugars in phloem sap and have distinct effects on morphogenesis through vascular differentiation, necessitating the evaluation of their specific influences on different species and growth stages. The effectiveness of a carbon source is influenced by its nature, quantity, and interactions, and inappropriate applications may result in slowed morphogenesis and physiological abnormalities, such as vitrification/hyperhydricity [9,17,18].

An examination of various sucrose concentrations (0, 1.5, 3.0, 4.5, and 6.0 wt/vol) demonstrated that *Cannabis sativa* plants achieved superior fresh weight, shoot lengths, and overall quality when grown in 1.5% and 3.0% (Table 1) [5]. In a separate study, researchers successfully developed a technique for inducing in vitro flowering induction in cannabis by utilizing DKW medium supplemented with 2% sucrose in a filtered vessel (Table 1) [1].

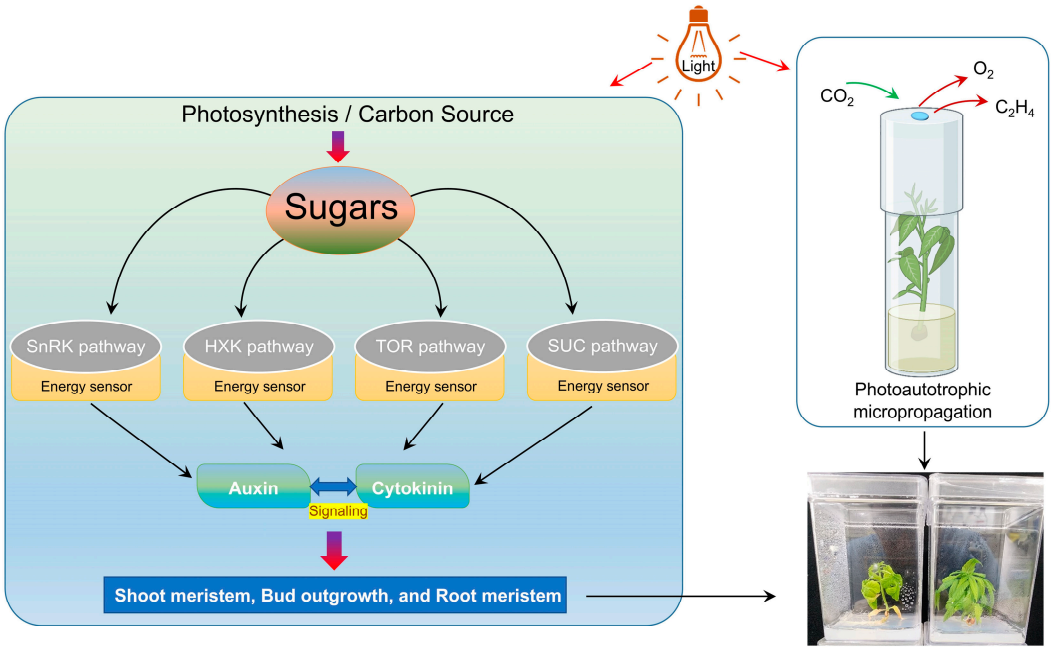

Figure 2. In addition to their function as an energy source, sugars are signaling molecules that regulate auxin–cytokinin hormonal interplay, which is involved in various plant metabolic and developmental processes (including shoots, buds, and roots). This regulation is facilitated by a well-established sugar-sensing system comprising four components: (1) the hexokinase (HXK)-sensing system; (2) a hexose transport-associated energy sensor (SUCROSE-NONFERMENTATION1-RELATED PROTEIN KINASE1 (SnRK1); (3) TARGET OF RAPAMYCIN (TOR) kinase); and (4) the Suc-specific pathway, which may involve a signaling Suc transporter. The photoautotrophic micropropagation (PAM) system, characterized by using sugar-free medium and ventilated vessels, significantly enhances plant physiological processes, such as photosynthesis and transpiration, while reducing hyperhydricity.

3.3. Mineral Additives

Despite substantial progress in understanding the molecular mechanisms of plant regeneration, the role of mineral nutrients as morphogenic stimulants in culture media is often underestimated. Each ion has potential morphogenic properties. Recent research has shown that epigenetic regulation influences most nutrient signaling pathways. Appropriate nutrition is vital for thriving plant tissue culture, and media selection should consider the morphogenic impact of individual elements and ions, drawing upon the principles of classical plant physiology [19].

Nitrogen (N) is essential for plant nutrition, being the second most abundant element after carbon, and it is a significant constituent of amino acids and other biological macromolecules [9]. The optimal form of N for plants is significantly dependent on the pH [9]. Predominantly absorbed as nitrate and ammonium from the soil, nitrogen influences plant growth and cell division, with high crop yields heavily dependent on N fertilization [19]. Key regulatory processes, including auxin-, cytokinin-, and strigolactone-mediated cell division in the shoot apical meristem and gibberellin-regulated shoot organogenesis, govern plant architecture in response to N supply [9,19]. Furthermore, amino acids transport, as a signaling molecule, has been implicated in the regulation of plant architecture [9,19].

In a recent study on *Cannabis sativa*, microcutting cultures with 500 mgL^{-1} ammonium nitrate demonstrated optimal shoot extension and leaf development [4]. Nitrogen additives also enhance shoot culture performance and mitigate vitrification issues [2]. Potassium (K), although not a structural element like nitrogen, is crucial for plant development, functioning as a symporter with hydrogen and facilitating auxin transport. The relationship between auxin distribution, initially identified in 1962 [9], was subsequently found to be

significantly affected by potassium deficiency via various transporters [9]. Potassium is also utilized for agar solidification, reducing hyperhydricity and promoting cell division, particularly under auxin deficiency [9].

Deficiency of both magnesium (Mg) and sulfur (S) severely impairs plant development because Mg functions as a cofactor and S serves as a structural component, as in amino acids and thiols [9]. Calcium (Ca) operates as a signal transducer, regulating kinases, cell wall structure, and responses to stimuli, thus playing a crucial role in plant tissue culture [9] through various signaling processes [9]. For macroelements (excluding carbon), it is recommended to utilize potassium nitrate (KNO_3), ammonium phosphate monobasic ($NH_4H_2PO_4$), calcium nitrate ($Ca(NO_3)_2$), and magnesium sulphate ($MgSO_4$) to achieve optimal ion concentrations in the medium (Figure 3) [9], including for in vitro micropropagation in *Cannabis sativa* (Table 1) [4].

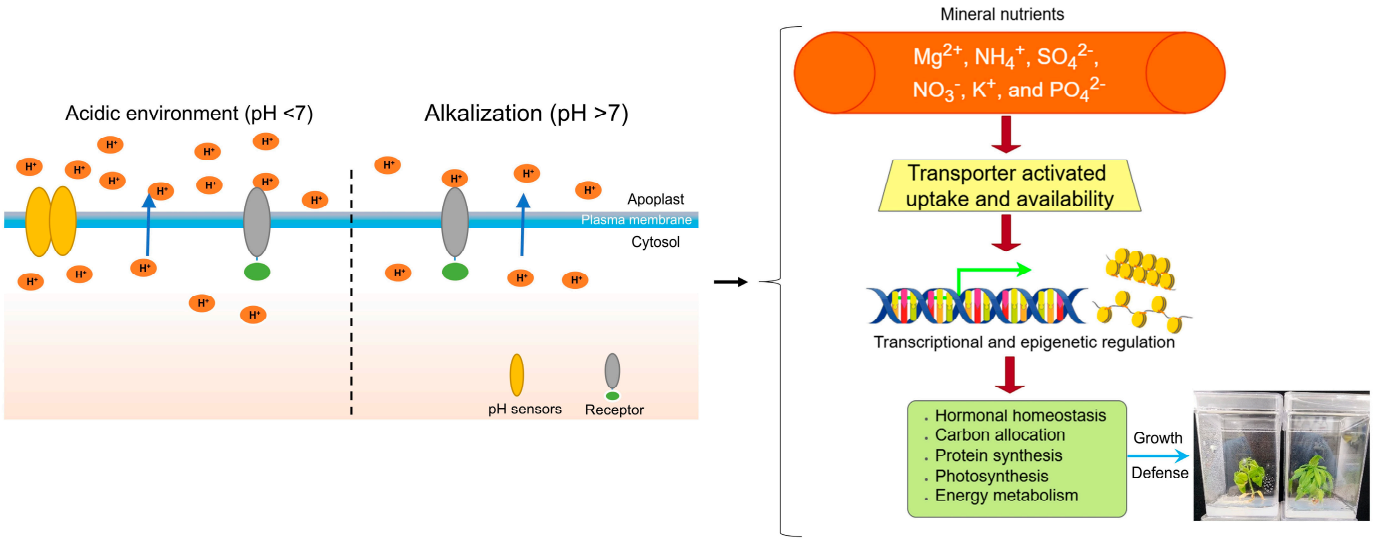

Figure 3. Maintaining an appropriate pH sensed by sensors and receptors is essential for in vitro plant growth, as it affects mineral nutrient availability via transcriptional and epigenetic mechanisms, a key aspect of controlled-environment cultivation.

3.4. pH of Medium

The pH level heavily influences plant development, particularly its impact on auxin transport. Most plant cellular processes are sensitive to pH, making it crucial to maintain a precise pH equilibrium. PTC has three pH types: cytoplasmic (approximately 7, highly buffered), extracellular (medium), and vacuolar (Figure 3). Three distinct vacuolar types are categorized based on their pH levels: alpha type (PSV, protein storage associated with stem cells), beta type (found in dividing cells), and gamma type (lytic, present in expanding/apoptotic cells) [9]. Shoot stem-cell formation is linked to the transition from beta- to alpha-type vacuoles [9]. Local auxin accumulation may induce PSV, whereas chloride promotes lytic vacuole formation, acidifying the vacuole and increasing water uptake [9]. The medium pH is the most adjustable, and artificial acidification enhances plant morphogenesis [9]. The classification of plants as acidic, neutral, or alkaline is determined by their preferred growing conditions, with most plants requiring an acidic environment to facilitate nutrient absorption under typical circumstances [9]. Recent studies have emphasized the critical role of pH homeostasis in plant morphogenesis [9]. In plant tissue culture, chemical buffers maintain the pH of the media within the physiological range [9]. During micropropagation of *Cannabis sativa*, various media pH levels (4.0, 5.0, 5.8, 6.0, and 7.0) were examined. The results showed no significant differences in fresh

weights, shoot lengths, or quality ratings across these pH levels. Nevertheless, 5.8, 6.0, and 7.0 pH values yielded more lateral nodes (Table 1) [5].

3.5. Choosing the Right and Effective Explants

Explants play a vital role in the efficacy of PTC. Significant differences exist between monocotyledons and dicotyledons; monocotyledon cells rapidly differentiate terminally after leaving the meristematic zone. In dicotyledon plants, leaves/cotyledons provide auxin, which translocates from the leaf tips to the stem/hypocotyl and the roots. Auxin is a primary regulator in in vitro plant morphogenesis, and cytokinins potentially induce auxin biosynthesis. The impact of endogenous auxin depends on its biosynthesis pathway, type of cell, and transport pathway. Vascular tissue-mediated auxin transport promotes root development, whereas active transportation by auxin carriers encourages shoot formation when auxin production and transportation are in equilibrium. Auxin accumulation triggers new shoot organ development, and stress-impeded auxin outflow contributes to this process. Auxin governs shoot and root morphogenesis in vitro through transport in systems lacking xylem and via xylem-facilitated transport [9].

The regeneration capability of cotyledons is limited to a brief before shoot apical meristem formation, during which they act as the primary source of auxin for root development [9]. The hypocotyl, situated between the cotyledons and roots of young seedlings, serves as a potential source of adventitious roots and shoots. It exhibits localized auxin metabolism and functions as a "sink" for cotyledon-derived auxin. This regeneration process is commonly employed in plant tissue cultures. Research on gene expression during hypocotyl regeneration has revealed that auxin production and directional transport are crucial for new embryo and shoot formation. Initially, auxin from the hypocotyl stimulates—adventitious root growth; subsequently, cytokinins produced by these roots can trigger new auxin synthesis, accumulation, and cellular reprogramming in the upper portion of the hypocotyl. Direct cytokinin application can directly induce shoot formation in competent epidermis-attached cells [9,11]. The success of hypocotyls, epicotyls, shoot tips, nodal cuttings, microcuttings, and retip cuttings (Figure 1A) (from recently micropropagated plants) has influenced the expansion of liner production in *Cannabis sativa* micropropagation [2–5,8,15,16].

3.6. Surface Sterilization

Plants harbor diverse endophytic/epiphytic microbes that colonize plant tissues without compromising plant health [20]. Recently, plant–endophyte associations have garnered significant attention owing to their applications in enhancing plant growth, mitigating biotic and abiotic stresses, and producing valuable metabolites [20]. Endophytes also provide mechanisms against pathogens with varying virulence. However, they can contaminate culture media [21], presenting a challenge for successful cultivation. Effective sterilization protocols are essential to minimize endophytic harm and phytotoxicity. Typical surface sterilization involves washing the plant material; chemical sterilization with ethanol, sodium hypochlorite, and mercuric chloride; rinsing; and inoculating sterile explants on suitable media. Protocols vary based on plant parts, endophytic, sterilant, concentration, exposure time, and tissue type. Commonly, sterilants include sodium hypochlorite, ethanol, mercuric chloride, formaldehyde, and, more recently, hydrogen peroxide [3,22]. Combinations of sterilants and surfactants such as Triton X-100, Tween 80, Tween 20, and Teepol enhance disinfection efficacy by oxidizing cellular components and denaturing proteins and lipids [20].

Cannabis (*Cannabis sativa* sub sp. sativa) also hosts beneficial microbial communities in its tissues and seeds [23]. Stage I disinfection with 20%, 40%, and 60% bleach (7.5%

sodium hypochlorite) for 10 min demonstrated no differences in contamination rates and caused no damage to explants in micropropagation studies [5].

3.7. Hyperhydricity

Disturbances in tissue structure and biochemical changes in hyperhydric plants primarily affect gas exchange and photosynthesis [24–26]. In vitro cultivation exposes plants to elevated humidity, insufficient illumination, excess sugars and minerals, hormonal imbalances, and poor aeration (accumulation of CO_2 and ethylene), leading to various disorders [24–26]. The most common abnormality observed in plants cultivated in vitro is hyperhydricity, also known as vitrification or glassiness. This condition is characterized by thickened stems; short internodes; and fragile, elongated, and twisted leaves [24–26]. This condition also manifests through various indicators, including a deficiency in chlorophyll, oversized starch particles within plastids, excessive intercellular fluid, reduced cellular adhesion, hypolignification, fewer epicuticular layers on leaves, altered enzyme activity, and disrupted protein synthesis [24–26]. Plants affected by hyperhydricity also exhibit membrane damage, reduced cell wall thickness, decreased mitochondria, enlarged spaces between cells, increased vacuole formation, and deterioration of vascular tissues. Another symptom is reduced lignin content due to decreased enzyme activity in its biosynthesis and increased phenolic compounds [24–26]. The causes and solutions for hyperhydricity are species-specific [24–26]. Approaches include adjusting the medium's mineral and hormonal composition, utilizing exogenous additives, improving aeration, and specific lighting; however, not all methods are universally effective [24–26]. Reducing ethylene levels with silver nitrate [9], increasing air exchange, balancing nutrition by lowering nitrogen and chloride, and reducing exogenous cytokinin to decrease auxin biosynthesis are potential methods [9]. Hemp micropropagation faces challenges such as hyperhydricity and culture decline of microshoots [6].

Activated carbon (AC) is extensively utilized for gas and water treatment in various industries globally, due to its efficacy, reliability, and accessibility [27,28]. It is frequently produced from lignocellulosic biomass-based agricultural waste such as coconut shells, rice husks, and palm kernel shells [27,28]. A significant advantage of activated carbon is its customizable pore structure, which can be achieved through chemical activation [27,28]. AC is highly porous, rendering it efficient for adsorbing various chemicals [27,28]. The adsorption mechanism is primarily attributable to micropores and weak Van der Waals forces that attract impurities [27]. The extensive microporous structure of AC renders it suitable for diverse industrial applications, including chemical treatment, protective suits, water and air filtration, and environmental remediation (Figure 4) [27]. The primary mechanism by which AC enhances in vitro morphogenesis is through the permanent binding of inhibitory substances in the growth medium, thereby diminishing toxic metabolites, phenolic secretions, and accumulation of brown exudates [28]. Moreover, AC participates in various stimulatory and inhibitory processes, including the emission of growth-enhancing compounds; modification and darkening of culture media; and adsorption of vitamins, metal ions, and plant growth regulators, such as abscisic acid and ethylene [28]. The effect of AC concentration differs across plant species, media types, explants, and outcomes. The stimulatory or inhibitory role of AC is contingent on multiple factors [28].

A recent study developed an in vitro micropropagation protocol for *Cannabis sativa* 'Cheungsam'. During the experimental procedure, the shoot tips and nodes exhibited blackening, and hyperhydricity increased. AC was incorporated into the medium to mitigate necrosis and hyperhydricity, which was hypothesized to be caused by ROS and phenolic compounds. Treatment with 0.5 g/L charcoal did not alleviate hyperhydricity and demonstrated no significant difference from the control [3]. The addition of AC inhibited

plant growth in all treatments compared with those without charcoal, suggesting that charcoal adsorbs both growth-inhibiting substances and essential nutrients, resulting in reduced growth [3,27,28].

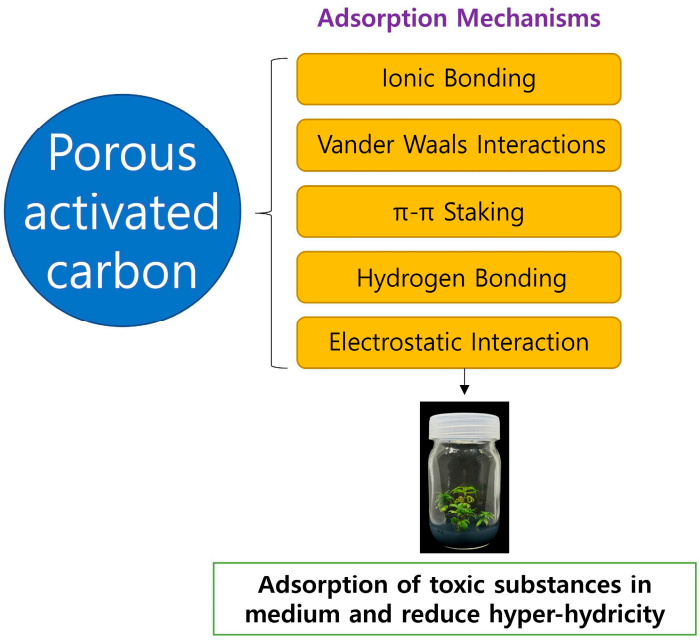

Figure 4. Varying adsorption mechanisms of activated carbon (AC) within in vitro environments.

3.8. Photoautotrophic Micropropagation

Ventilation, quantified as the air change rate per hour, represents the ratio of the total air exchanged between a zone and the external environment per unit of time to the zone's volume, indicating air diffusion. Natural ventilation in smaller culture vessels occurs via gas-permeable films (0.2 to 0.5 μm pore size) and vessel–lid gaps, facilitating higher air change rates and maintaining sterility by preventing microorganism entry [29]. An increased air change rate enhances CO_2 availability to explants, which is crucial for photosynthesis, photorespiration, and hormone biosynthesis, particularly photoautotrophic micropropagation (PAM) systems dependent on CO_2-derived carbohydrates [29]. In photoautotrophic tissue culture, explant growth occurs on a sugar-free medium through photosynthesis and inorganic nutrient uptake [29]. In cannabis micropropagation, the presence of axillary buds on the shoot indicates potential new shoot development, with the multiplication ratio being a key factor for efficiency [29]. Elevated CO_2 resulting from increased air change rates can enhance axillary buds, leaf, and node numbers [29]. Higher air change rates also reduce vessel humidity, enhance transpiration via water vapor diffusion, and mitigate inhibitory gases such as ethylene, thereby promoting explant growth [29].

Photoautotrophic (sugar-free medium) micropropagation, introduced over two decades ago, remains a promising and evolving technology [30]. The research and advancements in PAM emphasize the in vitro environmental features that enhance plant growth (Figure 2) [30]. PAM concepts and methodologies for optimizing in vitro environments to promote photosynthesis and biomass accumulation highlight the advantages of omitting organic carbon sources and utilizing various types of ventilated vessels with natural ventilation, as reported in several studies [30].

Elevated CO_2 levels led to increased plant dry mass and more leaves. PAM offers several advantages over conventional tissue culture methods, including reducing culture time by approximately 30%, mitigating issues such as hyperhydricity, and a nearly 100% improvement in ex vivo survival rates. Additionally, PAM results in reduced labor and

material expenses, making it superior to traditional tissue culture techniques [29]. PAM also facilitates simultaneous root and shoot development, shortening the acclimation period and improving efficiency. Previous research on cannabis did not evaluate the use of small shoots or effective ventilation methods. For other species, various explant sizes have been used in PAM [29].

The increased internode length observed with higher air change rates in PAM indicates that cannabis explants thrive under particular conditions, suggesting that elevated air exchange may require increased light for the photosynthetic process. The similarity in the total dry mass in PAM and shoot dry mass in TC (tissue culture) across various air change rates implies comparable growth rates between PAM and photomixotrophic (sugar and photosynthesis) TC. Enhanced photosynthesis in PAM, achieved through greater light exposure and CO_2 levels, could surpass that in TC. This assessment compares different air change rates by comparing traditional tissue cultures with PAM [29].

For plants, light plays a vital role, affecting photosynthesis and governing various molecular, biochemical, and morphological processes that are fundamental to their growth and development. Key light attributes, such as fluorescence rate, wavelength regions, duration, and direction, drive photosynthesis and photomorphogenesis by selectively activating light receptors/photosensors. These photoreceptors process complex biochemical structures, capture photons, and detect light properties, transducing this information into biochemical and biological responses that regulate growth through hormonal signaling and homeostasis. Artificial light sources are exclusively used in plant tissue cultures [31,32].

In tissue culture, sucrose provides essential carbon for energy and growth, with light intensity promoting mixotrophic growth from both sucrose and photosynthesis. A recent study demonstrated that higher light intensity during flowering-promoting photoperiods resulted in fewer flowers owing to light-induced stress or photoinhibition [1].

A groundbreaking study showed that *Cannabis* can complete its entire life cycle in TC, yielding inflorescences and viable seeds in vitro. The study identified DKW medium with 2% sucrose in a filtered vessel with a lower relative humidity as the most effective condition. Notably, low light intensity during flower induction was essential for maximizing production. The plant's moderate growth in a sugar-free medium suggests that it cannot achieve full photoautotrophy under passive ventilation, likely due to CO_2 limitation. CO_2 concentrations within filtered vessels containing plants fluctuated between 150 and 250 mmol/mol during the photoperiod, depending on the filter quantity, while ambient CO_2 levels ranged from 350 to 400 mmol/mol. Future research should investigate cannabis performance in forced ventilation systems [1].

4. Conclusions and Future Prospect

Direct organogenesis or clonal micropropagation facilitates the rapid production of uniform, disease-free, high-quality planting materials, irrespective of weather or seasonal constraints. However, in vitro methods require specialized equipment, skilled personnel, and expensive plant materials. Consequently, optimization of in vitro protocols is essential. *Cannabis sativa* L., being dioecious, exhibits genetic variability, necessitating specific environmental and media considerations for micropropagation. Critical factors include contamination management, genotype, explant selection, nutrition, plant growth regulators (PGR) type and concentration, carbon source, medium composition, additives and gelling agents, and abiotic/biotic elicitors. Hyperhydricity (vitrification) impedes micropropagation; nevertheless, photoautotrophic culture with improved ventilation can address air exchange-related hyperhydricity. Further research is required to support laboratories lacking facilities and expertise, potentially enhancing plant productivity via tissue-culture techniques to meet cannabis secondary metabolites demand. Optimized micropropagation

and acclimatization methods provide a foundation for cannabis research, sterile secondary metabolites production, and efficient breeding of this recalcitrant species. Micropropagation is crucial for preserving rare and endangered species and augmenting traditional ex situ conservation methods by establishing bioresource collections, encompassing in vitro culture, callus cultures, DNA banks, cryopreserved samples, seed libraries, synthetic seed production, and databases detailing rare and endangered *Cannabis sativa* genotypes.

These factors primarily influence somaclonal variations, both heritable (genetic) and non-heritable (epigenetic). While beneficial for new genotype selection, they are undesirable for germplasm preservation, necessitating elite genotype multiplication to maintain distinct ones. Assessing genetic diversity and purity is vital for developing germplasm conservation programs. Genetic homogeneity of in vitro propagated plants could be validated at genetic, morphological, physiological, and biochemical levels. DNA-based markers are promising tools for confirming clonal fidelity of micropropagated cannabis plants, as they are unaffected by environmental factors.

Temporary immersion systems (TISs) have been extensively employed in plant biotechnology. TISs offer advantages over continuous liquid-phase bioreactors for micropropagation and secondary metabolite production. The structure, operation mode, and configuration type of bioreactors require optimization to create hybrid bioreactors suitable for *Cannabis sativa*. Understanding factors affecting plant growth, including biological phenomena and changes and factors influencing TIS components' function and biological processes, is crucial for scaling up biomass and secondary metabolites' production in cannabis.

With increasing medicinal and recreational interests in *Cannabis sativa* L., research to optimize in vitro practices is necessary to enhance current methods and elucidate underlying physiological processes. Due to numerous factors influencing tissue culture, existing optimization approaches are tedious and time-consuming. New computational methodologies, such as machine learning and optimization algorithms, offer the potential for developing improved protocols more efficiently. Predictions of in vitro conditions to refine growth responses were examined in a validation experiment, demonstrating no significant differences between predicted optimized values and observed data.

Further investigation is required to recommend a more efficacious algorithm to study dynamic plant responses to multivariable stimuli in vitro, develop novel methods, and optimize current protocols. Instead of traditional statistics for evaluating large datasets, effective machine learning strategies for optimizing micropropagation protocols should be assessed as alternatives or in conjunction with conventional statistical approaches for precision tissue culture practices.

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