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Toward scalable micropropagation of *Cannabis sativa* (chemotype III): Protocol optimization and preliminary evaluation of cannabinoid stability

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ABSTRACT

A micropropagation protocol was optimized for *Cannabis sativa* L. cv. Charlie's Dream (chemotype III; Δ9-THC ≪ CBD) to generate clonal plants for cannabinoid stability assessment. Axillary buds were cultured on Murashige and Skoog (MS) medium supplemented with benzylaminopurine (BAP) or thidiazuron (TDZ) (0.5–2.5 μM), with or without vitamin supplementation. Shoot number, shoot length, and foliar area were evaluated. Rooting responses were tested using 1-naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA) under both *in vitro* and *ex vitro* conditions. Resins extracted from dried flowers of donor and micropropagated plants were analyzed for CBD and Δ^9-THC contents by gas chromatography coupled to mass spectrometry (GC/MS). Vitamin supplementation did not improve growth, whereas TDZ and BAP showed differential effects over time. At 15 days post-initiation of micropropagation (dpim), explants treated with 0.5 μM TDZ produced more shoots and greater foliar area than those treated with BAP. By 30 dpim, explants treated with BAP achieved values similar to those with TDZ, whereas 2.5 μM TDZ promoted callus formation and vitrification. Shoot elongation was significantly enhanced when gibberellin (7 μM) or red light was combined with 0.5–1 μM BAP, but not with TDZ. *In vitro* rooting was unsuccessful; however, *ex vitro* rooting improved after pretreatment with 2.5 μM IBA. Based on these findings, the optimized micropropagation workflow consisted of: (i) shoot multiplication on MS + BAP (0.5–1 μM) for 30 days; (ii) shoot elongation under MS + BAP (0.5–1 μM) supplemented with gibberellin (7 μM) or red light; and (iii) *ex vitro* rooting after IBA (2.5 μM) pretreatment. GC-MS analysis demonstrated that the CBD/Δ^9-THC ratio remained stable between donor, first-generation (G1), and second-generation (G2) plants, supporting chemotype stability. This approach reduces medium complexity, improves scalability, and provides a reproducible framework suitable for industrial applications in high-value crops.

1. Introduction

Cannabis sativa is a multipurpose species that has been cultivated for millennia due to its value as a source of fiber, seed oil, and bioactive compounds with medicinal properties (Hesami et al., 2020; Sandler and Gibson, 2019). In recent decades, renewed scientific and clinical interest has focused on its therapeutic potential, which is largely attributed to a diverse array of secondary metabolites, including more than 100 phytocannabinoids and numerous terpenes (ElSohly et al., 2014; Fathordoobady et al., 2019). These compounds have been pharmacologically documented for their efficacy in the management of neurological, inflammatory, and neurodegenerative disorders, as well as epilepsy, chronic pain, nausea, and multiple sclerosis, leading to the development of approved cannabinoid-based medicines (Adams et al., 2021). The

Abbreviations: BAP, benzylaminopurine; CBD, cannabidiol; Dpim, days post-initiation of micropropagation; G1, first-generation plants; G2, second-generation plants; GC/MS, gas chromatography coupled to mass spectrometry; IBA, indole-3-butyric acid; MS, Murashige and Skoog medium; MSS, vitamin-supplemented Murashige and Skoog medium; NAA, 1-naphtalenacetic acid; PGRs, plant growth regulators; TDZ, thidiazuron; Δ^9-THC, Δ^9-tetrahydrocannabinol.

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growing demand for standardized medicinal cannabis has highlighted the need for uniform plant material with stable chemotype profiles (Chandra et al., 2020; Pepe et al., 2021).

C. sativa is mainly dioecious and allogamous, resulting in high genotypic and phenotypic variability (Chandra et al., 2017a). This inherent variability hinders the consistent production of uniform, high-quality plants from seed that comply with regulatory and market standards (Lata et al., 2010). For this reason, cannabis for medicinal use has been propagated through clonal methods. Traditionally, clonal propagation is performed by cutting female plants with high cannabinoid levels. Generally, large quantities of plants can be produced from a single donor plant using this propagation technique, as the cannabis plant is relatively easy to root (Campbell et al., 2019). However, this procedure involves allocating between 10% and 15% of the ground space for a single commercial operation. Furthermore, under these cultivation conditions, donor plants are susceptible to attack by insects, viruses, fungi, and bacteria that can be transmitted to the cuttings and seriously affect the production and traceability of the crop. This is of great relevance in cannabis, as there are currently very few pathogen control options registered for cultivation, and, in addition, there is a strong preference for pesticide-free consumption (Caplan et al., 2018, 2017).

In vitro micropropagation represents an alternative approach to conventional clonal propagation, enabling large-scale multiplication under strictly controlled, aseptic conditions. The sterile environment inherent to this technique facilitates the production of pathogen-free plantlets, thereby reducing biotic stress (Monthony et al., 2021). In the clonal micropropagation of *C. sativa*, a primary challenge is to develop robust methods that ensure reproducibility of protocols across different genotypes and chemotypes (Adams et al., 2021). To date, most micropropagation protocols for the cannabis plant are based on the multiplication of shoots from apical and axillary nodes. The most widely adopted and effective approaches involve the use of commercial plant growth regulators (PGRs) (Campbell et al., 2019; Ioannidis et al., 2020; Kodym and Leeb, 2019; Monthony et al., 2021). Nonetheless, variability in morphogenic responses among cultivars remains a challenge, hindering efficient (Ioannidis et al., 2020) and reliable production (Monthony et al., 2021).

The development of protocols for large-scale production of cannabis with minimal somaclonal variation and pathogen incidence has become an emerging research focus. Only a limited number of protocols have been described worldwide, addressing factors such as explant choice and decontamination, direct and indirect organogenesis, rooting, acclimatization, and even some aspects of genetic engineering. Early and recent studies have explored the use of different basal media, cytokinins, auxins, and environmental conditions to improve shoot multiplication and plantlet quality, consistently highlighting strong genotype-dependent responses (Adams et al., 2021; Chandra et al., 2020; Ioannidis et al., 2020; Kastelec et al., 2025; Lata et al., 2016b). Since cannabis micropropagation systems are still relatively recent, combinations of plant growth regulators need to be tested to improve both organogenesis and acclimatization stages, while ensuring that plants remain true-to-type and industrially suitable. In this context, recent integrative and comparative studies emphasize that protocol robustness and scalability remain major bottlenecks, particularly during elongation and rooting phases (Kastelec et al., 2025; Pepe et al., 2021). Moreover, most studies lack post-culture analyses of phytochemical integrity after acclimatization, a key step if protocols are to be adopted by the pharmaceutical industry (Adams et al., 2021).

Likewise, due to genetic and varietal diversity within *C. sativa*, bottlenecks persist during the regeneration, growth, and rooting stages of *in vitro* cultivation, limiting the development of rapid, reliable, and efficient production systems. As recent reviews emphasize, the dioecious nature, open pollination, and high heterozygosity of cannabis strongly contribute to genotypic and phenotypic heterogeneity, which in turn complicates breeding and standardization efforts. These biological constraints have been repeatedly identified as major obstacles to protocol transferability across cultivars and chemotypes, and they are further reinforced by environmental and legal challenges that affect conventional cultivation. This highlights the need for reliable clonal systems to ensure uniform chemotype production (Ndlovu et al., 2024).

Therefore, it is crucial to investigate and optimize *in vitro* culture protocols to establish standardized micropropagation methods tailored to each chemotype and/or genotypic/phenotypic variety (Dreger et al., 2025). In this context, recent analyses also suggest that integrating tissue culture advances with broader biotechnological tools, including functional genomics, metabolomics, and next-generation gene editing, could accelerate the development of standardized cannabis propagation platforms for both pharmaceutical and industrial applications (Boonsnongcheep and Pongkitwitoon, 2020; Kastelec et al., 2025). Here, we focused on the chemotype III variety Charlie's Dream (Δ^9-tetrahydrocannabinol (Δ^9THC) ≪ cannabidiol (CBD)) and evaluated the combined effect of vitamins, benzylaminopurine (BAP), thidiazuron (TDZ), gibberellin, and red light on shoot proliferation and elongation, together with *ex vitro* rooting strategies and acclimatization. In addition, we carried out a preliminary assessment of cannabinoid profiles across donor, first-generation (G1), and second-generation (G2) plants, aiming to explore whether clonal micropropagation can preserve chemotype stability in this cultivar.

2. Materials and methods

2.1. Chemicals and reagents

Ethanol (96°) was purchased from Purocol (Burzaco, Buenos Aires, Argentina). Sodium hypochlorite (NaOCl) solution was purchased from Anedra (Cat. No. 9300; Tigre, Buenos Aires, Argentina). Tween 20 (Cat. No. 2000200307) and sucrose (Cat. No. 9789.08) were obtained from Biopack (Zárate, Buenos Aires, Argentina). Murashige and Skoog (MS) basal medium was purchased from PhytoTechnology Laboratories (Cat. No. M519; Shawnee Mission, KS, USA), and agar-agar was obtained from Britania (Cat. No. B0101406; Buenos Aires, Argentina). Nicotinic acid (Cat. No. N0761), pyridoxine (Cat. No. P8666), thiamine (Cat. No. T3902), thidiazuron (TDZ; Cat. No. P6186), benzylaminopurine (BAP; Cat. No. B3408), gibberellin (Cat. No. G7645), 1-naphthaleneacetic acid (NAA; Cat. No. N0640), and indole-3-butyric acid (IBA; Cat. No. I5386) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The solvents chloroform (Cat. No. 1116110) and methanol (Cat. No. 897110) were purchased from CICARELLI (San Lorenzo, Santa Fe, Argentina). Cannabidiol (CBD) was obtained from Cayman Chemical Company (Cat. No. 90080, Ann Arbor, Michigan, USA), and Δ^9-tetrahydrocannabinol (Δ^9-THC) was obtained from the National Institute of Industrial Technology (INTI, Cat. No. INTI-MRC 032, San Martín, Buenos Aires, Argentina).

2.2. Plant material

Two seeds of the *Cannabis sativa* variety Charlie's Dream (chemotype III, Δ^9-THC ≪ CBD) were purchased commercially and germinated on filter paper moistened with distilled water inside Petri dishes in a culture chamber at 23 °C ± 2 °C in the dark. Germinated seeds were transplanted into pots containing a 1:1 mixture of perlite and soil and grown in a culture room maintained at 23 °C ± 2 °C, under a 22-h light/2-h dark photoperiod and a photon flux density of 200 μmol m^{-2} s^{-1} provided by 6000 K LED lighting. Both seedlings were grown until maturity, and their inflorescences were harvested for cannabinoid analysis (hereafter referred to as Donor 1 and Donor 2). To preserve genetic fidelity and ensure clonal uniformity, only Donor 1 was used as the source plant for all micropropagation experiments described in this study (Fig. S1). Micropropagation of Donor 1 yielded independent first-generation clonal plants (G1). Four G1 plants, considered independent biological replicates, were randomly selected and used as explant sources to

generate second-generation plants (G2). Fifteen G2 plants were randomly selected from the resulting population for cannabinoid analysis, representing multiple independent clonal lines derived from all four G1 individuals within the same donor background. (Fig. S1).

2.3. Explants

The evaluated explants (~1 cm in length) consisted of axillary and apical buds (Fig. 1a). The outer tissue was cut with a scalpel, and two sets of primordial leaves were kept to protect the meristem from excessive damage during disinfection. Approximately 20 explants were taken per plant per treatment, both from the Donor 1 and from the four G1 plants. Each treatment was replicated at least three times, and the results shown are representative of the average of each treatment.

2.4. Disinfection

Explants were surface-disinfected by immersion in 80% ethanol for 1 min with agitation, followed by treatment with 0.5% w/v NaClO solution with 0.1% Tween 20 for 20 min. Subsequently, they were rinsed three times (5 min each) with sterile distilled water (Fig. 1b), dried on sterile filter paper in a laminar flow hood, and transferred to the corresponding culture media.

2.5. Culture media

MS medium contained the composition of macro- and micronutrients described by Murashige and Skoog (1962), sucrose (30 g L^{-1}), and agar-agar (8 g L^{-1}). Vitamin-supplemented Murashige and Skoog medium (MSS) contained nicotinic acid, pyridoxine, and thiamine, each at a final concentration of 0.01 g L^{-1}. Both media were adjusted to pH 5.8 and autoclaved for 20 min at 121ºC. The plant growth regulators evaluated for vegetative growth were TDZ and BAP, each tested at concentrations of 0.5, 1.0, and 2.5 μM (Fig. 1c). Culture media were dispensed into sterilized glass jars, each initially containing four to five explants. Following the first subculturing, two explants were maintained per jar.

2.6. Growth of axillary and apical buds

The explants were incubated in culture chambers with a 16-h photoperiod, LED lights with a photon flux density of 200 μmol m^{-2} s^{-1}, and a temperature range of 23C ± 2C. The explants were repotted every 15 days. At 30 days post-initiation of micropropagation (dpim), gibberellin (7 μM) was added to the MS medium containing the respective growth regulators, or the explants were exposed to red LED light (Interelec; peak emission at 665 nm; FWHM ≈ 78 nm; photon flux density of 56 μmol m^{-2} s^{-1}; Fig. S2) under a 22-h light/2-h dark photoperiod to promote shoot elongation. Shoot length, number of shoots per explant, and foliar area were quantified from digital images using the ImageJ software. These parameters were assessed at 15, 30, and 45 dpim (Fig. 1c).

2.7. Rooting promotion and acclimatization

For *in vitro* root induction, shoots were transferred to MS and MSS media supplemented with either NAA or IBA, at concentrations of 2.5 and 5.0 μM (Fig. 1d). Additional media formulations were tested, containing half- and quarter-strength micro- and macronutrients as described by (Murashige and Skoog, 1962), along with sucrose (30 g L^{-1}) and agar-agar (8 g L^{-1}), in the presence and absence of the auxins NAA or IBA at concentrations of 2.5 and 5.0 μM. For *ex vitro* rooting, shoots were transferred to pots filled with 1:1 perlite:soil substrate. Before transplantation, the basal ends of the shoots were immersed in a solution of NAA or IBA (2.5 or 5 μM) for 30 s. Root length was measured after four weeks for each treatment.

2.8. Resin extraction and cannabinoid analysis

Dried inflorescences (5 g per sample) were stored at –80 °C for resin extraction. Cannabinoid analysis was performed on the Donor 1 and Donor 2 plants, the four randomly selected G1 plants, and the fifteen randomly selected G2 plants obtained from explants of the four G1 plants. The G1 and G2 plants used for cannabinoid analysis were obtained from the *in vitro* micropropagation treatments evaluated in this study, including the different hormonal conditions tested. For each sample, 250 mL of 96° ethanol (pre-chilled at –80 °C) was added, and the mixture was stirred for 15 min without prior grinding. Extracts were filtered twice through filter paper. Solvent removal was initially performed in a water bath at 45 °C, followed by evaporation of the residual solvent in a SpeedVac concentrator at 45 °C (Fig. 1f). Gas chromatography coupled to mass spectrometry (GC–MS) analyses were carried out at the GC–MS Analysis Laboratory, Faculty of Biochemical and Pharmaceutical Sciences, National University of Rosario (Rosario, Argentina). Resin samples were diluted in a 1:1 (v/v) mixture of

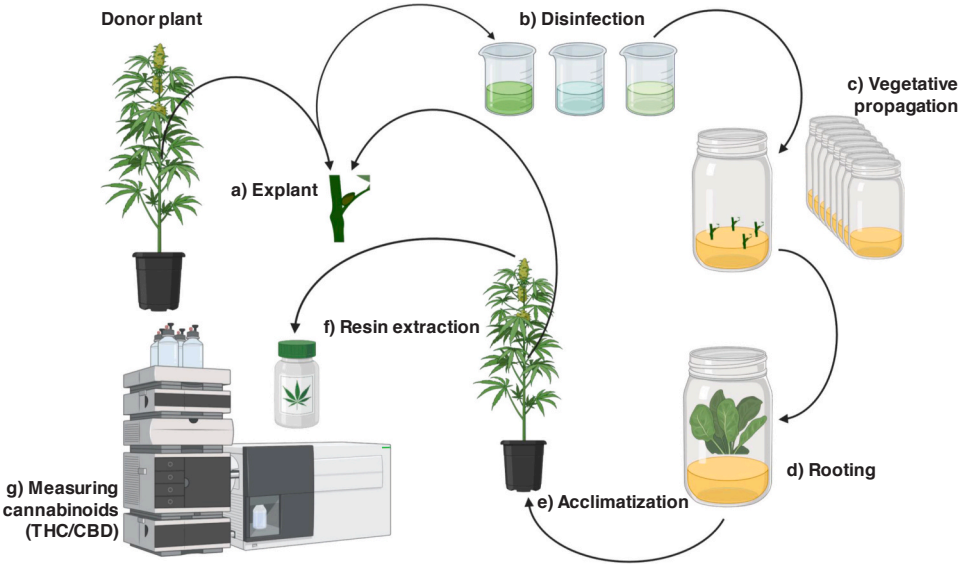

Fig. 1. Schematic representation of the micropropagation protocol used to obtain cloned seedlings from a donor plant of the Charlie's Dream variety of *Cannabis sativa*.

chloroform and methanol. A 1 μL aliquot of each sample was injected in split mode (1:20) into an Agilent 7890B gas chromatograph coupled to an Agilent 5977 A mass spectrometer, equipped with an HP-5MS UI capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). The injector temperature was set at 250 °C. The oven temperature program started at 120 °C (held for 2 min), increased at a rate of 8 °C min^{-1} to 270 °C, and was held for 3 min, resulting in a total run time of 25.75 min. Mass spectrometric detection was carried out in full scan mode over a mass range of *m/z* 50–500. Compound identification was performed by comparison of mass spectra with the NIST 2011b library. Quantification of cannabidiol (CBD) and Δ⁹-tetrahydrocannabinol (Δ⁹-THC) was carried out using calibration curves generated with the corresponding certified reference standards (Fig. 1g). All GC–MS analyses were performed on independent biological samples (individual plants).

2.9. Statistical analysis

Statistical data analysis was performed using a two-way ANOVA followed by Tukey's multiple comparison test. Normality was assessed using the Shapiro–Wilk test, and homogeneity of variances was evaluated using the Brown–Forsythe test, as implemented in GraphPad Prism. A two-tailed Student's *t*-test for independent samples was employed to evaluate differences in the CBD Δ⁹-THC ratios between G1 and G2 plants. Differences were considered statistically significant at $p < 0.05$. GraphPad Prism version 8.0.2 was used for the analysis.

3. Results

3.1. Effect of supplementation with vitamins nicotinic acid, pyridoxine, and thiamine

The effect of the vitamins: nicotinic acid, pyridoxine, and thiamine (included in vitamin-supplemented Murashige and Skoog medium, MSS) on shoot length, number of shoots per explant, and foliar area was assessed in the presence of the growth-promoting hormones thidiazuron (TDZ) and benzylaminopurine (BAP) at three different concentrations (0.5, 1, and 2.5 μM).

The results indicated that the supplementation with the three vitamins in combination with TDZ did not enhance any of the parameters evaluated (Fig. 2A). At 15 days post-initiation of micropropagation (dpim), explants cultured in MSS medium supplemented with 2.5 μM TDZ negatively affected the foliar area of the explants in comparison with those cultured in Murashige and Skoog medium (MS) with 0.5 μM TDZ ($p = 0.025$) (Fig. 2A). In addition, the increasing TDZ concentrations in MS significantly reduced the number of shoots per explant (MS + 0.5 μM TDZ vs MS + 2.5 μM TDZ, $p = 0.027$) and the foliar area (MS + 0.5 μM TDZ vs MS + 1 μM TDZ, $p = 0.0064$) (Fig. 2A). At 30 dpim, explants in MSS with 2.5 μM TDZ showed a smaller foliar area compared to those in MS medium at any TDZ concentration (MSS + 2.5 μM TDZ vs MS + 0.5 μM TDZ, $p = 0.0013$; MSS + 2.5 μM TDZ vs MS + 1 μM TDZ, $p = 0.0061$; MSS + 2.5 μM TDZ vs MS + 2.5 μM TDZ, $p = 0.011$). It is worth mentioning that the use of 2.5 μM TDZ in both media induced callus formation (data not shown).

The addition of BAP to MS and MSS media enabled the evaluation of vitamin supplementation effects on shoot number and foliar area (Fig. 2B). At 30 dpim, explants cultured in MS with 1 μM BAP exhibited a higher number of shoots per explant than those in MSS medium with BAP at any concentration (MS + 1 μM BAP vs MSS + 0.5 μM BAP, $p = 0.037$; MS + 1 μM BAP vs MSS + 1 μM BAP, $p = 0.0005$; MS + 1 μM BAP vs MSS + 2.5 μM BAP, $p = 0.030$) (Fig. 2B). Likewise, foliar area was greater in explants from MS with 1 μM BAP compared to those from MSS with the same BAP concentration ($p = 0.016$). Overall, vitamin supplementation negatively affected explant development when BAP was used as a vegetative growth-promoting hormone.

3.2. Effect of TDZ and BAP hormones

Since the MSS medium did not enhance any of the three parameters evaluated, a comparison between the growth-promoting hormones TDZ and BAP was performed using the MS medium. At 15 dpim, explants cultured in MS with 0.5 μM TDZ showed more shoots compared to explants grown in MS with 0.5 μM BAP medium ($p = 0.028$) (Fig. 3A). Similarly, explants treated with 0.5 μM or 2.5 μM TDZ exhibited greater foliar area development compared to those treated with the same concentrations of BAP (MS + 0.5 μM TDZ vs MS + 0.5 μM BAP, $p = 0.0001$; MS + 2.5 μM TDZ vs MS + 2.5 μM BAP, $p = 0.015$) (Fig. 3A). However, at 30 dpim, the explants grown in the presence of BAP exhibited improvements across all three parameters evaluated, reaching values comparable to those obtained with TDZ (Fig. 3A, Fig. S3).

3.3. Effect of Gibberellin and LED red light

Given that no significant increase in shoot length was observed between 15 and 30 dpmi, the MS + growth-promoting hormones medium was supplemented with gibberellin, and its effect was compared with that of incubation under red light, as both treatments have been reported to influence shoot elongation under *in vitro* conditions (Lata et al., 2016b; Yun et al., 2023). The results showed that both gibberellin supplementation and red-light exposure enhanced shoot length, but only when combined with BAP at 0.5 or 1 μM (Fig. 4A). In contrast, when these treatments were combined with TDZ (0.5 or 1 μM), shoot length was negatively affected (gibberellin: 0.5 μM BAP vs 0.5 μM TDZ, $p = 0.034$; 1 μM BAP vs 1 μM TDZ, $p = 0.003$; red-light exposure: 0.5 μM BAP vs 0.5 μM TDZ, $p = 0.007$; 1 μM BAP vs 1 μM TDZ, $p = 0.027$) (Fig. 4A, Fig. S4).

An increase in the number of shoots per explant was observed with both gibberellin and red-light treatments (Fig. 4B), compared to the previously described results (Fig. 3B). However, explants cultured in the presence of red light and 1 μM TDZ produced fewer shoots than those treated with red light and BAP at the same concentration ($p = 0.013$) (Fig. 4B). Moreover, combining 2.5 μM BAP with red LED light negatively affected both shoot length (2.5 μM BAP vs 0.5 μM BAP, $p = 0.005$) and the number of shoots per explant (2.5 μM BAP vs 0.5 μM BAP, $p = 0.012$; 2.5 μM BAP vs 1 μM BAP, $p = 0.003$) (Fig. 4A, B). Finally, treatments with red light and gibberellin promoted an increase in the foliar area (Fig. 4C). However, no significant differences were observed between the two hormones when shoots were treated with gibberellin (Fig. 4C). In contrast, treatment with red light and TDZ significantly increased foliar area compared to red light and BAP (0.5 μM BAP vs 0.5 μM TDZ, $p = 0.045$; 2.5 μM BAP vs 2.5 μM TDZ, $p = 0.0008$) (Fig. 4C).

3.4. Effect of the hormones IBA and NAA

The rooting of the obtained shoots was evaluated through two treatment groups. Rooting was assessed under *in vitro* culture conditions or through *ex vitro* rooting. For *in vitro* root induction, shoots were randomly placed in different glass flasks containing MS, half-strength or quarter-strength MS media, which were either supplemented or not with the root growth-promoting hormones 1-naphthaleneacetic acid (NAA, 2.5 μM or 5 μM) or indole-3-butyric acid (IBA, 2.5 μM or 5 μM); however, rooting was not induced under any of the tested conditions. For *ex vitro* rooting, 45-dpim shoots were transplanted into pots after being pre-treated or not with NAA or IBA at the previously mentioned concentrations (Fig. 5). Significantly greater root length was observed in shoots that were pre-treated with 2.5 μM IBA compared to control shoots (without hormone treatment) (Control vs 2.5 μM IBA, $p = 0.032$) and those pre-treated with NAA at either concentration (2.5 μM NAA vs 2.5 μM IBA, $p = 0.017$; 5 μM NAA vs 2.5 μM IBA, $p = 0.017$) (Fig. 5).

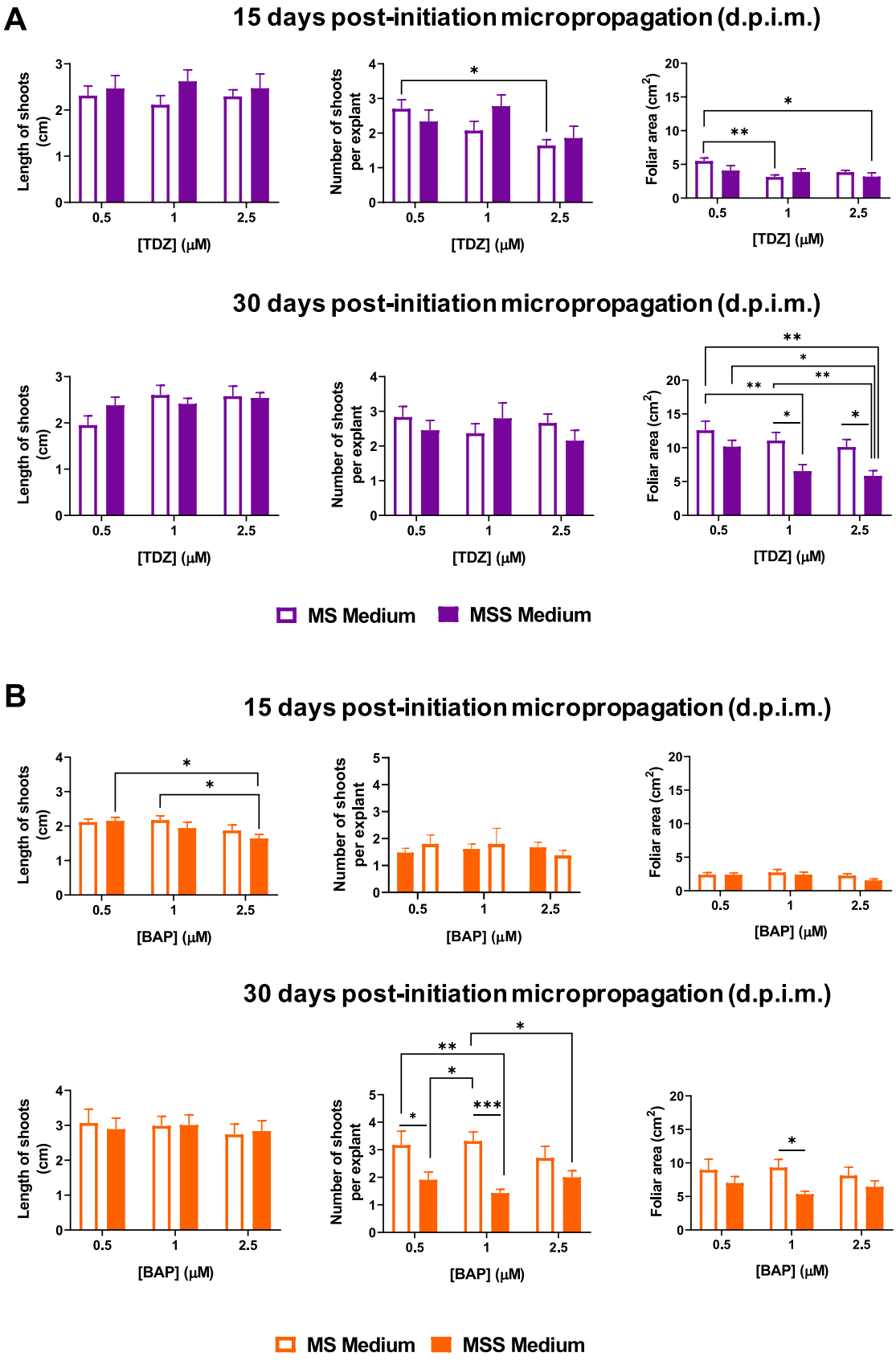

Fig. 2. Analysis of the effect of the nicotinic acid, pyridoxine, and thiamine vitamins on the shoot length, number of shoots per explant, and foliar area. A) Effect of vitamin supplementation combined with the growth-promoting hormone thidiazuron (TDZ) at 15- and 30-day post-initiation micropropagation. B) Effect of vitamin supplementation combined with the growth-promoting hormone benzylaminopurine (BAP) at 15- and 30-day post-initiation micropropagation. Bars represent mean + SEM (n = 22 independent explants per treatment). A two-way ANOVA followed by the Tukey multiple comparison test was used. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

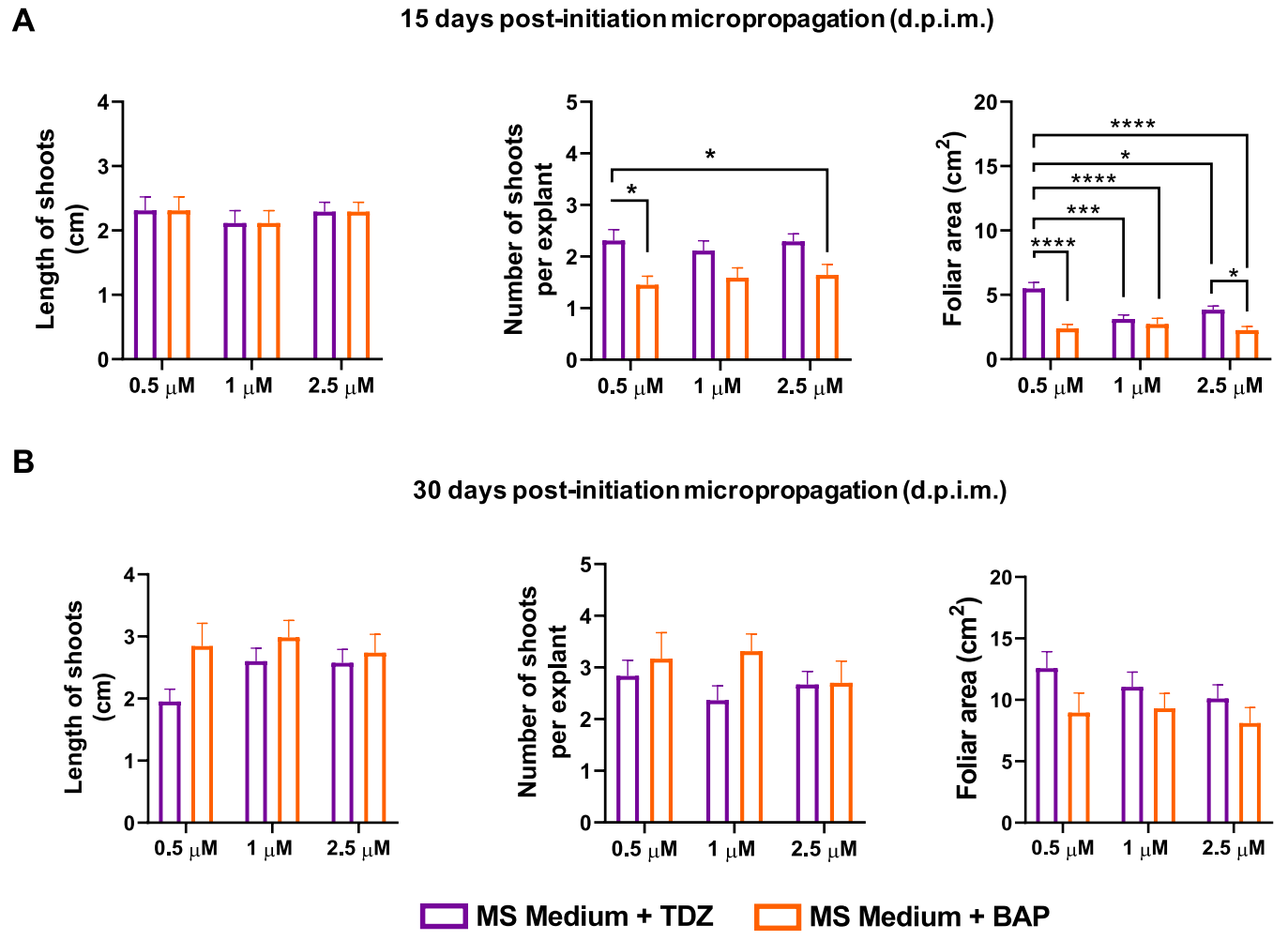

Fig. 3. Analysis of the effect of the growth-promoting hormones thidiazuron (TDZ) and benzylaminopurine (BAP) on shoot length, number of shoots per explant, and foliar area at 15 (A) and 30 days (B) post-initiation micropropagation. Bars represent mean + SEM (n = 21 independent explants per treatment). A two-way ANOVA followed by the Tukey multiple-comparison test was used. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$.

3.5. Analysis of cannabinoid profile

Resins were extracted from the two seed-derived donor plants (Donor 1 and Donor 2). In addition, Donor 1 was selected as the source for the micropropagation experiments, giving rise to first-generation plants (G1). Four of these G1 plants, randomly selected, were subsequently used as explant sources yielding second-generation plants (G2). Random selection was performed within the population of plants generated through the *in vitro* treatments evaluated in this work. Cannabinoid content (CBD and Δ⁹-THC) was therefore evaluated in: (i) both donor plants, (ii) the four randomly selected G1 plants derived from Donor 1, and (iii) the fifteen randomly selected G2 plants obtained from the G1 generation (Table 1, Fig. S5). In addition, the relative percentages of CBD, Δ^9-THC, and other cannabinoids were determined in the resin from donor plant 1 and from the micropropagated plants (G1 and G2) (Table 2). The results showed that the CBD/Δ^9-THC ratio and the relative percentages of cannabinoids remained similar between the donor plant 1 and the micropropagated plants (G1 and G2). Specifically, the CBD/Δ⁹-THC ratio averaged 15.75 ± 2.06:1 in G1 plants (n = 4) and 16.00 ± 3.53:1 in G2 plants (n = 15), indicating no statistically significant differences between G1 and G2 plants ($p = 0.881$). Likewise, markedly different CBD/Δ^9-THC ratios were observed between the samples from both donor plants (Donor 1, 19:1, and Donor 2, 4:1; Table 1), suggesting that greater variability in cannabinoid content between plants of the same chemotype would be generated by seed propagation. Together, these data provide preliminary quantitative support for short-term chemotype stability under the specific micropropagation conditions tested. It is worth noting that, on average, a resin yield of 15 ± 2 g per 100 g of dried flowers was obtained under our flowering conditions using a single resin extraction procedure.

4. Discussion

In this work, the effect of vitamin supplementation (nicotinic acid, pyridoxine, and thiamine) in Murashige and Skoog medium (MS) medium with thidiazuron (TDZ) and benzylaminopurine (BAP) on the growth and development of axillary and apical buds from the Charlie's Dream variety of *C. sativa* (chemotype III, Δ^9-tetrahydrocannabinol (Δ^9THC) ≪ cannabidiol (CBD)) was evaluated. The number of shoots per explant, shoot length, and the foliar area were analyzed as development parameters of vegetative development. Differentiated effects were observed in both the short and long term, providing a more detailed understanding of how these hormones and vitamins influence cell proliferation and plant vegetative development.

At higher concentrations of TDZ, a negative effect on growth was observed, with a significant reduction in the number of shoots per explant. These effects may be explained by the capacity of TDZ to induce cell de-differentiation at high concentrations, thereby inhibiting shoot proliferation and promoting callus formation (Baek et al., 2024; Chandra et al., 2020, 2017b; Lata et al., 2009). Similar callogenic responses

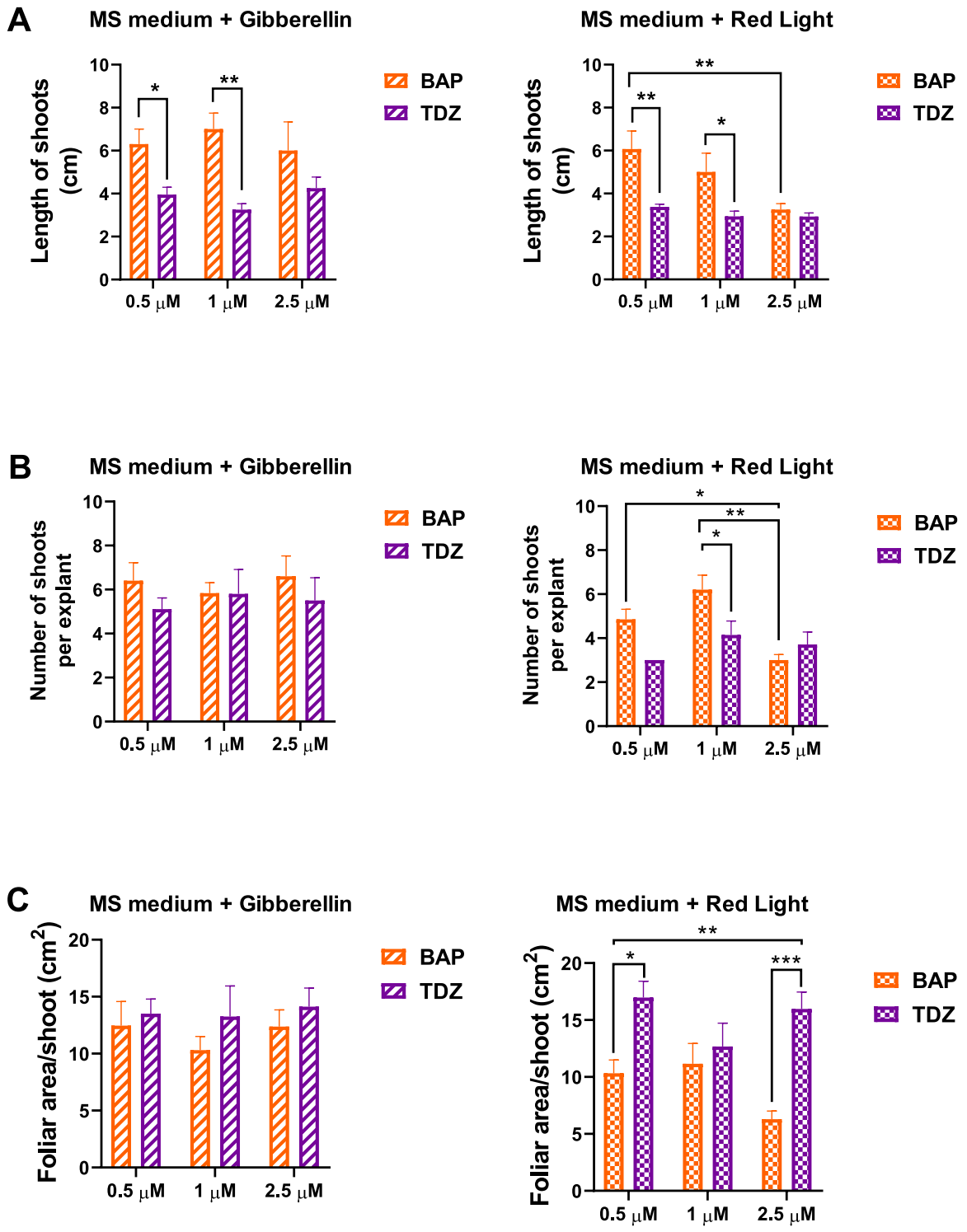

Fig. 4. Analysis of the effect of the gibberellin hormone and red light on shoot length (A), number of shoots per explant (B), and foliar area per shoot (C) at 45 days post-initiation of micropropagation. Bars represent mean + SEM (n = 12 independent explants per treatment). A two-way ANOVA followed by the Tukey multiple-comparison test was used. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

have also been described with other plant growth regulators, such as 2, 4-dichlorophenoxyacetic acid and its combinations with kinetin or 1-naphthaleneacetic acid (NAA), which yielded strongly organogenetic calli in *C. sativa* explants (Holmes et al., 2021; Ioannidis et al., 2022; Slusarkiewicz-Jarzina et al., 2005). Here, the negative effect of TDZ was not counteracted by the use of a vitamin-supplemented Murashige and Skoog medium (MSS), as vitamin supplementation did not enhance cell proliferation or differentiation. On the other hand, BAP treatment also resulted in variable responses depending on the concentration used. Our results are consistent with previous studies reporting that a concentration of 1 μM BAP is the most suitable for promoting shoot proliferation in other *C. sativa* varieties (Chandra et al., 2020; Galán-Ávila et al., 2020; Lata et al., 2016a, 2016b). The differential responses observed between TDZ and BAP are consistent with previous reports describing TDZ as a potent cytokinin regulator that promotes rapid initial shoot induction but frequently leads to callogenesis, vitrification, and reduced shoot

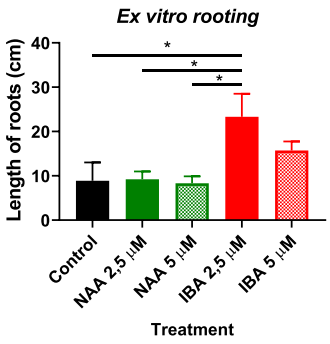

Fig. 5. Effect of indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) on root length. Bars represent mean + SEM (n = 15 shoots per treatment). Statistical analysis was performed using a two-way ANOVA followed by the Tukey multiple comparison test. * $p < 0.05$.

Table 1
Quantification of CBD and THC contents per gram of resin in donor plants and in first- (G1) and second-generation (G2) plants obtained by micropropagation from Donor plant 1.

Plants	mg CBD / g resin	mg THC / g resin	CBD: THC
Donor plant 1	260.80	13.56	19: 1
Donor plant 2	283.46	76.84	4: 1
Micropropagated plant 1 (G1)	328.15	18.10	18: 1
Micropropagated plant 2 (G1)	345.00	20.55	17: 1
Micropropagated plant 3 (G1)	429.43	30.13	14: 1
Micropropagated plant 4 (G1)	349.33	25.03	14: 1
Micropropagated plant 5 (G2)	514.43	29.8	17: 1
Micropropagated plant 6 (G2)	572.29	33.05	17: 1
Micropropagated plant 7 (G2)	415.79	27.34	15: 1
Micropropagated plant 8 (G2)	513.59	39.92	13: 1
Micropropagated plant 9 (G2)	461.51	38.4	12: 1
Micropropagated plant 10 (G2)	587.75	32.54	18: 1
Micropropagated plant 11 (G2)	441.58	28.1	16: 1
Micropropagated plant 12 (G2)	418.06	30.65	14: 1
Micropropagated plant 13 (G2)	356.92	16.65	21: 1
Micropropagated plant 14 (G2)	339.79	14.63	23: 1
Micropropagated plant 15 (G2)	468.26	21.90	21: 1
Micropropagated plant 16 (G2)	595.14	40.1	15: 1
Micropropagated plant 17 (G2)	541.57	38.61	14: 1
Micropropagated plant 18 (G2)	557.74	50.1	11: 1
Micropropagated plant 19 (G2)	574.89	44.18	13: 1

quality when applied at higher concentrations or for extended periods (Adams et al., 2021; Baek et al., 2024; Ioannidis et al., 2022).

In contrast, BAP-based protocols have been reported to support more stable shoot proliferation and improved explant morphology over longer culture periods in several cannabis cultivars (Pepe et al., 2021; Shi et al., 2024; Stephen et al., 2023). The improved performance observed in this study at 30 days post-initiation of micropropagation (dpim) using 0.5–1 µM BAP highlights its suitability for sustained multiplication, particularly when the objective is scalable micropropagation rather than rapid but unstable shoot induction. However, explants grown in the MSS medium exhibited reduced foliar development and shoot number, suggesting that the vitamins in MSS may have interfered with the action of BAP on cell proliferation. Although the underlying mechanism was not investigated in this study, this effect likely reflects alterations in endogenous metabolic balance and/or cytokinin responsiveness induced by vitamin supplementation under these specific culture conditions.

Vitamin supplementation is commonly included in standard micropropagation media; recent studies indicate that its contribution to shoot multiplication in cannabis is highly genotype-dependent and often marginal when optimal growth-promoting hormone concentrations are used (Kastelec et al., 2025; Lavie et al., 2024). In this sense, the absence of positive effects of MSS observed in our study supports the simplification of the protocol, reducing medium complexity and costs without compromising shoot proliferation efficiency, which represents a practical advantage for large-scale propagation systems. These findings are particularly relevant for the optimization of micropropagation protocols, as they indicate that vitamin supplementation may not be required when appropriate concentrations of BAP are used. The behavior of BAP may be associated with its role in promoting cell differentiation and organogenesis, facilitating balanced growth throughout developmental phases (Galán-Ávila et al., 2020). These findings also highlight the importance of adjusting growth-promoting hormone concentrations (in this case, BAP and TDZ) to prevent adverse effects and to maximize cell proliferation without inducing dedifferentiation.

Gibberellin has been used to stimulate shoot elongation and organ differentiation in micropropagation protocols (Du et al., 2022; Song et al., 2023). In addition to hormonal regulation, environmental factors such as light quality are also known to affect plant development (Cardoso-Magaña et al., 2024; Wang et al., 2024). Recent integrative and computational approaches have further highlighted the central role of light quality and intensity in directing *in vitro* cannabis development, underscoring the complexity of light-hormone interactions during micropropagation (Pepe et al., 2021). In the present study, shoot elongation was enhanced by gibberellin supplementation and red-light exposure, particularly when explants were cultivated in MS medium supplemented with BAP at 0.5 or 1 µM. These findings align with previous reports, which indicate that exposure to red light and gibberellin promotes shoot elongation under *in vitro* conditions (Cavallaro et al., 2022; Lata et al., 2016b). However, when these treatments were combined with TDZ, shoot elongation was negatively affected, consistent with the inhibitory action of TDZ on shoot formation and differentiation (Chandra et al., 2017b; Lata et al., 2010). Furthermore, explants exposed to TDZ under red light showed a reduced number of shoots per explant compared to those exposed to red light with BAP, reinforcing the idea that TDZ may hinder shoot proliferation. The reduced shoot elongation observed when using 2.5 µM BAP combined with red light further emphasizes the need for precise hormone concentration optimization to support *in vitro* plant growth and development. Synergistic effects between light quality and hormonal signaling have been reported in recent cannabis studies, where red light enhanced shoot elongation and physiological performance, particularly under cytokinin-based regeneration systems (Kastelec et al., 2025; Shi et al., 2024). The present results support these observations by showing that gibberellin-mediated elongation is strongly dependent on the cytokinin background, being effective under BAP but not TDZ treatments, thereby emphasizing the importance of coordinated hormonal and environmental optimization.

Concerning rooting, our results point to this stage as one of the main bottlenecks in *C. sativa* micropropagation. The effects of the hormones indole-3-butyric acid (IBA) and NAA on the rooting of shoots derived from axillary and apical buds of the Charlie's Dream variety were evaluated under both *in vitro* and *ex vitro* rooting conditions. Under *in vitro* conditions, root development was not achieved under any treatment tested, and the shoots remained at the callus stage. Comparable

Table 2
Relative percentages of cannabinoids in Donor plant 1 and in first- (G1) and second-generation (G2) plants obtained by micropropagation from this donor plant.

Cannabinoids	Δ9-Tetrahydrocannabivarin	Cannabidiol	Cannabichromene	Δ9-Tetrahydrocannabinol	Cannabigerol	Cannabinol
Donor plant 1	0.00	88.26	5.39	4.97	1.20	0.19
Plant 1 (G1)	0.00	89.69	4.23	5.36	0.71	0.00
Plant 2 (G1)	0.00	86.36	7.09	5.58	0.82	0.16
Plant 3 (G1)	0.00	88.81	3.94	5.47	1.49	0.29
Plant 4 (G1)	0.00	87.00	5.50	6.76	0.59	0.15
Plant 5 (G2)	0.00	91.24	4.06	3.67	0.78	0.26
Plant 6 (G2)	0.00	90.60	4.79	3.74	0.71	0.17
Plant 7 (G2)	0.00	91.57	3.59	3.92	0.79	0.13
Plant 8 (G2)	0.00	90.58	4.76	3.81	0.70	0.15
Plant 9 (G2)	0.00	91.74	3.47	3.94	0.67	0.18
Plant 10 (G2)	0.00	91.65	3.98	3.65	0.58	0.14
Plant 11 (G2)	0.00	90.27	5.07	3.81	0.74	0.10
Plant 12 (G2)	0.00	91.50	3.53	3.76	1.13	0.08
Plant 13 (G2)	0.00	89.69	5.21	4.03	0.90	0.17
Plant 14 (G2)	0.00	91.23	4.37	3.78	0.61	0.00
Plant 15 (G2)	0.00	92.62	3.45	3.44	0.40	0.10
Plant 16 (G2)	0.00	88.19	5.49	5.73	0.27	0.32
Plant 17 (G2)	0.00	88.77	4.33	6.10	0.41	0.39
Plant 18 (G2)	0.00	89.56	4.49	5.04	0.62	0.28
Plant 19 (G2)	0.00	89.75	4.60	4.93	0.45	0.27

difficulties in achieving consistent *in vitro* rooting have been reported across multiple cannabis genotypes (Adams et al., 2021; Dreger et al., 2025; Kastelec et al., 2025). Although *in vitro* rooting of *C. sativa* has been reported under specific conditions, including reduced-strength MS media (Baek et al., 2024; Chaohua et al., 2016), genotype-dependent recalcitrance remains a frequent limitation. In our case, shoot necrosis occurred within one week of culture in both half- and quarter-strength MS medium. Moreover, although red LED light irradiation has been shown to improve *in vitro* rooting in hard-to-propagate species (Wu and Lin, 2012), no root development was observed under this treatment either.

In contrast, under *ex vitro* conditions, greater root length was observed when shoots were pretreated with 2.5 μM IBA compared to untreated controls or those treated with NAA (Chandra et al., 2020, 2010; Favero et al., 2023; Lata et al., 2010). Rooting performance in our study aligns with the broader literature on *C. sativa*, where IBA is consistently reported to be more effective than NAA in promoting root number and length, although specific outcomes vary depending on medium strength and explant type (Ioannidis et al., 2020). The *ex vitro* rooting appears to have been favored by the direct contact of shoots with the substrate. In this framework, the successful *ex vitro* rooting achieved following IBA pretreatment represents a pragmatic alternative aligned with protocols proposed for commercial-scale propagation, where reducing *in vitro* stages can improve plantlet survival, lower costs, and enhance operational feasibility (Pepe et al., 2021; Stephen et al., 2023). Recent work by Kastelec et al. (2025) further supports this strategy, demonstrating that simultaneous *ex vitro* rooting and acclimatization can significantly improve propagation efficiency while shortening the overall micropropagation timeline. Together with our results, these findings reinforce the concept that *ex vitro* rooting is not merely a fallback option when *in vitro* rooting fails, but rather a scalable and biologically robust strategy for cannabis micropropagation when combined with appropriate auxin pretreatments such as IBA. Importantly, *ex vitro* rooting with IBA pretreatment relies on simple and standardized handling steps, supporting its reproducibility and scalability under both research and commercial propagation settings.

Finally, the cannabinoid profile of micropropagated plants was compared with that of the donor plants. Similar exploratory approaches have been used in previous studies to assess chemotype fidelity during early stages of protocol development (Chandra et al., 2010; Dreger et al., 2025; Ioannidis et al., 2022). Our results indicated that the CBD/Δ^9-THC ratio and the relative percentages of other therapeutically relevant cannabinoids remained stable across two generations of micropropagation. These results are consistent with previous reports, which show that the cannabinoid profiles of *in vitro* propagated plants, conventionally grown plants, and mother plants remain qualitatively similar, with only minor, non-significant differences in concentrations (Dreger et al., 2025; Ioannidis et al., 2022). Such findings further support the suitability of micropropagation as a strategy to maintain clonal fidelity of *in vitro* propagated plants and support the use of micropropagation for the large-scale production of true-to-type plants for pharmaceutical purposes (Chandra et al., 2010). Although only the short-term cannabinoid profile was assessed in this study, future work will be necessary to determine whether the cannabinoid profile remains stable over successive micropropagation cycles.

Taken together, the optimized combination of BAP-based shoot proliferation, gibberellin- with red light-mediated elongation, and *ex vitro* rooting with IBA pretreatment provides a coherent and reproducible framework for micropropagation of chemotype III cannabis. Compared to existing protocols, the present approach emphasizes stability, reduced medium complexity, and practical scalability, which are key requirements for translational and industrial applications. In addition, our findings underscore the importance of optimizing reliable micropropagation protocols for *C. sativa*, as current recalcitrance to regeneration limits the establishment of industrially viable systems. As emphasized by Adams et al., (2021), overcoming these limitations would open the door for routine applications of genetic engineering and gene editing in cannabis, including the integration of fast-evolving "omics" technologies to accelerate the identification of genes and mechanisms underlying cannabinoid biosynthesis. Such advances would facilitate the development of standardized, large-scale propagation systems combining clonal fidelity with metabolomic stability, a prerequisite for the future of pharmaceutical and industrial cannabis production. Beyond its immediate application to chemotype III cannabis, this work contributes to the broader field of plant biotechnology by demonstrating how coordinated optimization of hormonal, nutritional, and environmental factors can enhance protocol robustness while preserving phytochemical integrity, a central challenge in the propagation of high-value medicinal and industrial crops.

5. Conclusion

The present study demonstrated that the growth and development of axillary and apical buds from the *C. sativa* Charlie's Dream variety are significantly influenced by the concentrations of thidiazuron (TDZ) and benzylaminopurine (BAP), as well as by the vitamin content in the culture medium. Although TDZ initially promoted shoot proliferation, its prolonged exposure induced callus formation and vitrification,

limiting its suitability for scalable propagation. In contrast, BAP at 0.5–1 μM supported sustained shoot multiplication without morphological abnormalities and was therefore selected as the basis of the optimized protocol. The findings underscore the importance of optimizing hormonal and nutritional conditions to promote effective shoot proliferation and elongation during *in vitro* propagation.

Based on our results, a clearly defined and reproducible micropropagation protocol for this *C. sativa* variety was established. The optimized workflow consists of: (i) shoot multiplication on MS medium supplemented with BAP (0.5–1 μM) for 30 days, with subculture at day 15; (ii) shoot elongation for an additional 15 days under the same BAP concentrations, combined with either gibberellin (7 μM) or red light (56 μmol m^{-2} s^{-1}; 22 h light/2 h dark); and (iii) *ex vitro* rooting following a 30-second basal immersion in 2.5 μM IBA before transplantation into a 1:1 perlite:soil substrate. Clonal micropropagation of Charlie's Dream variety was validated as a reliable strategy for maintaining the chemical stability of cannabinoid profiles across generations, as the CBD/Δ9-THC ratio remained stable between donor, G1, and G2 plants. This finding is particularly relevant given the potential influence of *in vitro* hormonal regimes on secondary metabolite production.

Despite these advances, several future challenges remain. The protocol requires validation across additional genotypes and chemotypes to account for genotype-dependent responses. In addition, future studies should address long-term culture effects and broader metabolomic profiles to more comprehensively evaluate phytochemical stability. Given the challenges observed during the rooting stage, particularly under *in vitro* conditions, future investigations should consider the role of soil microbiota, particularly plant growth-promoting bacteria, in contributing to *ex vitro* rooting through phytohormone production or other growth-stimulating mechanisms (Glick, 2020). Overall, these results contribute to the development of standardized micropropagation protocols tailored to specific chemotypes, representing a crucial step toward scalable and reproducible production systems in medicinal cannabis.

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CRediT authorship contribution statement

Analía Sannazzaro: Writing – review & editing, Formal analysis, Conceptualization. **Estefanía Butassi:** Writing – review & editing, Methodology, Formal analysis. **Melina Di Liberto:** Writing – review & editing, Methodology, Formal analysis. **Eliane Pérez Sanchidrian:** Visualization, Methodology. **Marina Clemente:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Conceptualization. **M. Micaela González:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Gabriel Yañuk:** Writing – review & editing, Methodology, Formal analysis. **Mónica Hourcade:** Writing – review & editing, Supervision, Methodology, Formal analysis. **Laura Svetaz:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis. **Manuel A. Sánchez:** Methodology.

Institutional Review Board Statement

Neither humans nor animals were utilized in this study.

Informed Consent Statement

Not applicable

Author Contributions

M.G. and G.Y.: Experimental design, data analysis, and figure design; A.S.: Experimental design and data analysis. M.H., E.B., M.D.L., and L.S.: Chemical analysis and data analysis. E.P.S., and M.A.S: Experimental design; M.C.: Experimental design, data analysis, manuscript writing, and editing. All authors read the manuscript and contributed to the writing and final editing.

Declaration of Competing Interest

None of the authors has any conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2026.123040.

Data availability

Data will be made available on request.

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