

***Agrobacterium*-mediated transformation of
Cannabis sativa.**

**A Thesis Presented for the
Master of Science
Degree
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Dedication

This is dedicated to my son, Israel, and to my wonderful partner, Rachel, without whom I would have never finished writing the darn thing to begin with.

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Abstract

Agrobacterium-mediated transformation has long been utilized as a method of producing stably-transformed plants. This project describes an attempt to develop a system of producing stably-transformed *Cannabis sativa* plants. Because of its recalcitrance to *in vitro* tissue culture and regeneration, transformation studies of *C. sativa* have been severely limited. In this study, I combined media components that have been shown to improve tissue culture health and regeneration. Additionally, I used hemp cultivars and explant sources that have been reported to function well in *in vitro* systems. The results showed that the improved media components did not have a significant effect on plantlet regeneration. They did, however, show that the cultivars ‘Futura 75’ and ‘USO 31’ outperformed ‘Fedora 17’ in both the number of explants with shoots (responding explants) and the number of shoots per explant. Explant source was shown to have a significant effect on both the proportion of responding explants and the proportion of shoots per explant, with approximately 51% of hypocotyl explants producing shoots at a rate of 1.32 shoots per explant. Also noted was novel organogenesis in cotyledon explants. Finally, I transformed *Cannabis* with a chimeric growth regulator shown to improve *in vitro* regeneration across several other plant species. This growth regulator was introduced into a transformation vector which also contained mGFP5-ER as a reporter gene. While no regenerated plants were verified to have been transformed, several tissues in culture expressed GFP, with cotyledons exhibiting a low transformation rate of 6%. While these results were unfavorable (as no transformed plant was regenerated), I do believe that these experiments have laid important guidelines for future work in developing a stable transformation system for *C. sativa* (L.).

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Chapter 1

Introduction

Background

Cannabis sativa is a crop that originates from eastern Asia. Since the passing of the 2018 Farm Bill that legalized low-THC hemp, it is now considered an agricultural commodity of high interest in the United States. Hemp has several industrial applications, including textiles, food, paper, bioplastics, insulation, and biofuel. There has also been increasing pharmacological interest in cannabinoids, the secondary metabolites produced in the leaves and flowers of *C. sativa*. The global industrial hemp market size was estimated at USD 4.13 billion in 2021 and is expected to grow at a compound annual growth rate of 16.8% from 2022 to 2030 (Grand View Research, 2022). The total value for US planted *C. sativa* in 2021 was approximately \$824 million and the total acreage planted in 2021 totaled to 54.2 thousand acres (National Hemp Report, 2022). Because of its growing adoption rate as an agronomic crop, genetic engineering will likely be pursued for crop improvement (Andre et al., 2016). Therefore, an efficient method of generating genetically modified *C. sativa* plants is needed.

Agrobacterium-mediated transformation

Agrobacterium-mediated transformation is a cornerstone of plant biotechnology (Tzfira & Citovsky, 2006). Plant genetic transformation is most often performed using the plant pathogen *Agrobacterium tumefaciens* as a method to deliver transgenes into a host plant (Hinchee et al., 1988). This method of transformation integrates a reduced number of transgene copies into plants compared to the biolistic method and has become the industry standard for accomplishing stable expression of transgenes and avoiding transgene silencing (Joyce et al., 2010; Ramesh et al., 2004). Within the nucleus, genomic integration allows for possible inheritance of the DNA construct (containing selectable marker, a reporter gene, and the genes of interest) to the next generation (i.e., stable transformation). This process is facilitated a set of

virulence or *vir* genes on the Ti plasmid of *Agrobacterium tumefaciens*, which is used by the bacteria to infect plants (Schrammeijer et al., 2000). The activity of these genes contributes to the precise excision of the T-DNA from the Ti plasmid. This is a key step in the transfer of DNA from *A. tumefaciens* to the genomes of higher plants (Baker et al., 2005). Additionally, it is believed that, while natural exudates of wounded plants may be enough to activate *vir* genes, the addition of exogenous acetosyringone stimulates *Agrobacterium* to more effectively transfer T-DNA to host cells (Ali et al., 2007; Sheikholeslam & Weeks, 1987). The efficiency of this complex process can be highly dependent on several factors including explant orientation, explant source i.e., plant genotype and *Agrobacterium* strain (Frary & Earle, 1996; Horlemann et al., 2003; Jin et al., 2005; Ślusarkiewicz-Jarzina et al., 2005). Recent studies have shown success with agroinfiltration—a transient leaf-infiltration assay using engineered *Agrobacterium*—in various cultivars of *C. sativa* and transient expression of the GUS reporter (Deguchi, Bogush, et al., 2020). These experiments also showed the effects of *Agrobacterium* strain and cultivar on agroinfiltration in *C. sativa* utilizing vacuum infiltration. Previous studies have shown *Agrobacterium*-mediated transformation of *C. sativa* callus is possible, although shoot regeneration and transgenic plant recovery were unsuccessful (Feeney & Punja, 2003). More recent studies have established stable transformation of *C. sativa*, however with these studies reported regeneration rates of less than 1% (Zhang et al., 2021). Given the lack of publications regarding robust transformation systems, there is a need for an improved, high-throughput transformation protocol for use in genetic engineering.

Optimizing in vitro media components for Cannabis sativa plant regeneration

Plant regeneration is a vital component to *Agrobacterium*-mediated transformation. Traditional, tissue-culture based methods of regeneration are based on the manipulation of plant growth regulators on responsive explants (Sugiyama, 2015). Unlocking the correct growth conditions and combinations of specific growth regulators to facilitate successful regeneration is a time consuming and laborious process that often fails. While many *C. sativa* regeneration protocols have been published, many of these protocols focus on nodal propagation as a method of maintaining *in vitro* germplasm (Lata et al., 2017; Richez-Dumanois et al., 1986). Proliferation of shoots, primarily from meristematic tissue, provide a dependable method of maintaining the genetic fidelity of elite lines and/or tissue-culture responsive lines. However, these methods are of limited use for biotechnological applications, as *de novo* regeneration is a primary pathway to produce stably transformed plants (de Almeida et al., 2012; Deo et al., 2010; Rai & Shekhawat, 2014). *De novo* shoot organogenesis of *C. sativa*, be it direct or indirect, has been the focus of many published studies. However, recalcitrance to regeneration has been shown in much of the existing literature (Feeney & Punja, 2003; Galán-Ávila et al., 2020; Mandolino & Ranalli, 1999; Wielgus et al., 2008). In addition to general recalcitrance, successful regeneration of *C. sativa* depends on a range of factors, including genotype, explant source, and media inputs (Galán-Ávila et al., 2021; Lata et al., 2009; Zhang et al., 2021). As researchers continue to examine the factors determining plant regeneration of *in vitro* cultivated *C. sativa*, new media inputs have emerged that demonstrate the ability to improved regeneration and help to overcome recalcitrance (Monthony et al., 2021). Therefore, there is a need to test how a combination of these factors might improve the regeneration rates of both wild-type and transformed *C. sativa* tissues into complete, healthy *C. sativa* plants.

Transcription factors, growth regulators and plant regeneration

Overcoming the hurdle of recalcitrance has led many researchers to turn to manipulation of transcription factors involved in the signaling responsible for cell reprogramming, determining cell fate and differentiation. These transcription factors, referred to as developmental regulators, control the organized, spatial distribution of cells during the development of organs and embryos (Duclercq et al., 2011; Gaillochet & Lohmann, 2015; Gordon et al., 2007; Ikeuchi et al., 2013; Perez-Garcia & Moreno-Risueno, 2018). Overexpression of these developmental regulators has been shown to improve the regeneration rates of several monocot and dicot plant species (Boutilier et al., 2002; Kong et al., 2020; Lotan et al., 1998; Lowe et al., 2018; Stone et al., 2001; Zuo et al., 2002). While overexpression of these growth regulators can improve plant regeneration efficiency, the increased regeneration rate can come at the cost of negative pleiotropic effects (G. Luo & Palmgren, 2021; Srinivasan et al., 2007). Recent studies have shown that overexpression of GROWTH-REGULATING FACTORS (GRFs), either alone or in chimeras with GRF-INTERACTING FACTOR (GIF), significantly increases the regeneration efficiency of a broad range of species with limited pleiotropic effects that can easily be segregated out (Debernardi et al., 2020; Kong et al., 2020). One of the primary roadblocks present in *C. sativa* biotechnology is the relative recalcitrance of the plant. Previous experiments have shown *C. sativa* regeneration to be dependent on cultivar, tissue, plant age and growth regulator combination (Boonsnongcheep & Pongkitwitoon, 2020; Chaohua et al., 2016; Galán-Ávila et al., 2020). Because of these dependent factors, a reliable regeneration protocol has to be established (Monthony, Kyne, et al., 2021; Salentijn et al., 2019). Recently, a dicot *GRF3-GIF1* chimera was shown to be effective in increasing the regeneration rate of *C. sativa* by 1.7-fold over the empty vector control (Zhang et al., 2021). The recent research concerning the GRF-GIF

chimeric protein provides new opportunities for overcoming recalcitrance in all *C. sativa* varieties.

The goal of the research presented in this thesis was to develop a robust and high-throughput method of stable *C. sativa* transformation. There were two objectives for the research: 1) To transform two types of *C. sativa* explants with a construct containing the chimeric protein *GRF3-GIF1* and utilizing the CaMV 35S promoter and NOS terminator to control expression. The construct contains a visual fluorescent marker (*mGFP5-ER*) to used to estimate transformation efficiency. 2) To compare the effect of improved media components on *de novo* indirect organogenesis to the regeneration rates of a published protocol.

Chapter 2

Utilizing improved media components and over-expression of the chimeric growth regulator, GRF-GIF, to improve *de novo* regeneration in *Cannabis sativa*.

Abstract

Since the passing of the 2018 Farm Bill that legalized low-THC hemp, *Cannabis sativa* has become a species of interest for genetic engineering. *C. sativa* produces many secondary metabolites of pharmacological interest, the most important of these being cannabinoids. The recently mapped genome, as well as the elucidation of cannabinoid biosynthetic pathways, has created increased need for a robust system for the stable transformation of *C. sativa*. However, publications outlining successful protocols for the regeneration and stable transformation of *C. sativa* are few and have not shown functional transgenic plant recovery rates, likely because of the reported recalcitrance of the plant. In this study, I attempted to combine “improved” media components and an auxin antagonist with the overexpression of the GRF-GIF chimera, have all shown to improve shoot regeneration *C.sativa*. Explant source was shown to have a significant effect on regeneration rates and transformation rates, with hypocotyls showing the highest regeneration rate and cotyledons showing the highest transformation rate. Genotype was shown to affect regeneration rates, with the cultivars ‘USO 31’ and ‘Futura 75’ outperforming ‘Fedora 17’ across the board. The improved media components were not shown to have a significant effect on regeneration rate. However, they did exhibit an effect on transformation rates. Ultimately, I was unable to regenerate any transgenic plantlets and, therefore, was unable to analyze the effect of the GRF-GIF chimera on regeneration rates.

Introduction

C sativa is perhaps the most well-known member of the Cannabaceae family, having a well-documented history as being a psychoactive drug source. Historical usage of the plant, not only as a psychoactive compound source, but as a textile, medicine, and food, dates as far back as 2700 BCE (Russo et al., 2008). In the past decade, *C. sativa* has seen a resurgence in interest

as an agronomic crop (Honig, 2022; Mechoulam, 2012; Ren et al., 2019). Mass breeding, as a method of selecting for preferred traits, has created divergent genotypes, differentiated by phytochemical composition (Hennink, 1994). Drug types of *C. sativa* (marijuana) contain high amounts of the psychoactive cannabinoid Δ 9-tetrahydrocannabinol (Δ 9-THC). Hemp types are grown to produce seed, oil and fiber, and contain low amounts of Δ 9-THC. Hemp types also produce higher amounts of cannabidiol (CBD), a non-psychoactive secondary metabolite. Both cannabinoids, as well as the entire cannabinoid suite produced by the plant, are of great interest as potential therapeutics. Researchers have identified several of the genes functioning the cannabinoid biosynthesis pathway (Degenhardt et al., 2017; X. Luo et al., 2019; Rodziewicz et al., 2019; Shoyama et al., 1975; Tahir et al., 2021). These genes, as well as the cannabinoid biosynthesis pathway, must be more carefully understood if *C. sativa* and its cannabinoids are to be fully utilized (Willow et al., 2023). Thus, there is a need for an effective method of performing *C. sativa* genetic engineering.

However, genetic engineering for crop improvement in *C. sativa* has had a somewhat difficult time with functional validation of these genes. This is due to the lack of a published, efficient *Agrobacterium*-mediated transformation and regeneration protocol. Few reports of stable transformation of *C. sativa* exist, due largely to the plant's recalcitrance to *in vitro* regeneration when compared to other model plants. (Chaohua et al., 2016; Galán-Ávila et al., 2020; Lata, Chandra, Khan, et al., 2016; Shiels et al., 2022; Wielgus et al., 2008). *De novo* regeneration in *C. sativa* has shown to be dependent on a range of factors, including genotype, explant source, and media inputs (Adhikary et al., 2021; Li et al., 2022). These studies helped to identify several cultivars ('USO 31', 'Futura 75' and 'Fedora 17') that seem to respond favorably to *in vitro* tissue culture systems, as well as some positively responding explant sources

(hypocotyls and cotyledonary node) (Chaohua et al., Deguchi et al., 2020, 2016; Galán-Ávila et al., 2020, 2021). Few of these publications, however, have addressed the entirety of these factors in pursuit of developing a functional *C. sativa* tissue culture system, be it through direct or indirect organogenesis (Deguchi et al., 2020; Lata et al., 2009; Monthony et al., 2021). Some studies have produced methods that increase the efficiency of shoot regeneration through modification of media components and explant source selection. The synthetic cytokinin meta-Topolin can improve shoot induction in various genotypes of *C. sativa* (Vrundha et al., 2021; Lata et al., 2016a). Use of PEO-IAA, an auxin antagonist, has shown to overcome the higher levels of endogenous auxin that likely produces apical dominance in *C. sativa* explants (Smýkalová et al., 2019a; Žukauskaitė et al., 2023). Hypocotyl- and cotyledonary node-derived explants have shown promising results when utilized in both direct and indirect organogenesis regeneration systems (Chaohua et al., 2016; Zhang et al., 2021). Increased levels of plant tissue culture media micronutrients, also known as mesovitamins, have been shown to reduce the occurrence of tissue hyperhydricity, increase shoot number, and improve shoot elongation in *C. sativa* micropropagation systems (Lubell-Brand et al., 2021; Zarei et al., 2023). These studies have provided keen insights into how *C. sativa* tissue culture efficiency might be increased through utilization of improved media components and explant sources shown to be responsive to *in vitro* tissue culture.

Agrobacterium-mediated transformation, an integral tool utilized in genetic engineering, has also shown to have reduced effectiveness when working with *C. sativa*. (Deguchi, Bogush, et al., 2020; Feeney & Punja, 2017; Zhang et al., 2021). Methods of transformation through agroinfiltration have proven effective to produce transient expression (Deguchi, Bogush, et al., 2020; Türkölmez et al., 2023). These studies provided results vital to optimization of *C. sativa*

transformation protocols through selection of *Agrobacterium* strain and explant source. Stable *C. sativa* transformation through whole plant regeneration has proven more elusive. Some published protocols have shown promise. Researchers have utilized the manipulation of transcription factors functioning as developmental regulators to overcome the general recalcitrance of *C. sativa*. Overexpression of the growth regulator GROWTH-REGULATING FACTOR (GRF), in fused, chimeric transcriptional complex with GRF-INTERACTING FACTOR (GIF), has been shown to improve regeneration rates, via *de novo* organogenesis, in several recalcitrant plant species (Kong et al., 2020). It has also been shown that the forced proximity of the growth regulators, which are normally expressed separately, increases the ability of transcription factors to induce regeneration (Debernardi et al., 2020). A recent study indicated that the GRF-GIF chimeric complex can improve the regeneration of *C. sativa* indirect organogenesis. Researchers in that study verified stable transformation but were only able to recover and acclimatize a single plant from 3500 explants (Zhang et al., 2021). Another protocol showed potential for a high-throughput system of creating stably transformed *C. sativa* plants, derived through direct organogenesis (Galán-Ávila et al., 2021). Unfortunately, this study did not attempt to root and acclimatize transformed plants for verification of stably transformed *C. sativa* plants. Thus, a functional protocol for the stable transformation of *C. sativa* remains to be fully elucidated. These issues, as well as those associated with *de novo* regeneration, have prevented the development of a robust and high-throughput *Agrobacterium*-mediated transformation and regeneration protocol for *C. sativa*.

In this research, I attempted to develop a functional *Agrobacterium*-mediated transformation and regeneration protocol. In that pursuit, I investigated the effect of improved media components that have shown to improve *de novo* regeneration in *C. sativa* tissue culture.

Thus, I compared selected explants cultivated on regeneration media containing increased meso-vitamins, meta-Topolin, and PEO-IAA. Results of these experiments were compared with those of a proposed rapid and high-throughput *C. sativa* transformation and regeneration system published in 2021 (Galán-Ávila et al., 2021).

Materials and Methods

Creation of cloning expression constructs and cloning into vectors.

An effective *C. sativa* GRF3-GIF1 chimera has been previously elucidated (Zhang et al., 2021). The GRF3/GIF1 gene sequence was assembled, using Snapgene™ by GSL Biotech LLC (San Diego, California), from publicly available DNA sequences and synthesized by Integrated DNA Technologies (Coralville, Iowa). For the test vector, the GRF-GIF chimera was driven under the 2x35S promoter of the cauliflower mosaic virus (CaMV) for constitutive expression. For the positive control vector, the late anther tomato promoter, LAT59, was used to drive the green fluorescent protein mEmerald. The neomycin phosphotransferase II gene (*NPTII*) was used to confer resistance to kanamycin for antibiotic selection in both vectors. Additionally, both vectors contained a green fluorescent protein mGFP5-ER, as well as right and left borders to enable *Agrobacterium*-mediated insertion into the genome (Figure 1, all tables and figures are located in an appendix at the end of the document). All vectors were assembled using Golden Gate assembly method of molecular cloning (Engler et al., 2008). Expression cassettes were verified at each level of Golden Gate reaction with both PCR and sequencing. These expression cassettes were cloned into the pAGM4723 binary vector, which was sourced as part of MOCLO Toolkit from the lab of Dr. Sylvestre Marillonnet (Weber et al., 2011). Verified final vectors were then transformed into *A. tumefaciens* strain (AGL1) using the heat-shock method.

Seed sterilization and germination.

Mature hemp seeds from three cultivars ('Futura 75', 'Fedora 17', and 'USO 31') were sourced via commercial means. Seeds were chemically scarified in a solution of 70% (v/v) sulfuric acid for 20 minutes, under agitation. Seeds were then rinsed three times with water and placed into a solution of 50% (v/v) sodium hypochlorite and 1% (v/v) TWEEN® 20 (Croda, Princeton, NJ) for 1 hour, under agitation. Sterile seeds were washed three times with sterilized deionized water. Sterile seeds were added to sterile 250 mL flasks containing 60 mL Germination Media (GM) containing 1% (v/v) H₃O₃ and 2% (v/v) Plant Preservative Mixture (Plant Cell Technology, Washington, DC) (Table 1). Flasks were shaken at 100 RPM for 72 hours (24° C with a 16/8-hour photoperiod) with daily media changes. Seedlings were germinated after 72 hours in the solution (Figure 2A). Germinated seedlings were then placed on sterile GM containing 3.5 g of Gelzan (MillaporeSigma, Burlington, MA) (Figure 2B). Seedlings were allowed to grow for an additional 4 days at 22° C with a 16/8-hour photoperiod (Figure 2C).

***Agrobacterium*-mediated transformation.**

Cultures of *Agrobacterium tumefaciens*, containing plasmid constructs, were grown in 50 ml of yeast extract and peptone (YEP) broth with 500 mg/L of rifampicin and 50 mg/L of kanamycin. Cultures were then incubated at 27° C to stationary phase ($OD_{600} \approx 2.0$) and then centrifuged at 4000 rpm for 10 minutes. The pelleted culture was resuspended in 15 ml of inoculation media (IM) to OD_{600} of 0.6 - 0.8 (Table 1). The *A. tumefaciens*/IM solution was then used for plant transformation experiments.

For explants to be used in transformation experiments, an excision is made just below the apical meristem to remove the shoot apical meristem (SAM) and cotyledons. Another excision is made above the root meristem to remove the meristem and radicle (Figure 2D). The hypocotyl

explants were then selected away from the cotyledons, radical, and meristem tissues (Figure 2E). Cotyledon explants were separated from the cotyledonary node (Figure 2F). Both cotyledons and hypocotyls were retained for transformation experiments. Explants were placed into IM solution and incubated at 22° C for 40 minutes. After 40 minutes, *A. tumefaciens* inoculum was removed and explants were placed on sterile absorbent material for 30 minutes to remove excess *A. tumefaciens* inoculum.

Direct organogenesis and improved media components for *C. sativa* regeneration.

To test the effect of improved media components, all explants, including those not treated with *Agrobacterium* co-cultivation, were given one of two media treatments (Table 1). Media treatment 1 (MT1) utilized the published, direct organogenesis regeneration protocol developed by Dr. Galán Avila. (Galán-Ávila et al., 2020, 2021). Media treatment 2 (MT2) was developed by combining published improvements of several *C. sativa* tissue culture papers. Cotyledon explants given MT1 media were placed on ½ MS-based basal media with vitamins, solid co-cultivation medium (MT1-C-CCM) (Table 1). Hypocotyl explants in MT1 media were placed on solid co-cultivation medium (MT1-H-CCM) (Table 1). All explants given MT2 media were placed on solid co-cultivation medium (MT2-CCM) (Table 1) In addition, All MT2 media contained increased concentrations of plant vitamins and mesonutrients (meso-vitamins) (Tables 1 and 2). Explants to be used as a control were not treated with *Agrobacterium* and instead were placed directly on media containing no antibiotics. All explants on both treatments were maintained under light (16 hr day/8 hr dark, 22 °C) for four days.

After 4 days, after four days explants were removed from co-cultivation media and allowed to dry on sterile paper towels in laminar flow hood for 30 minutes. Explants were then treated with liquid MS media solution containing 6 mg/L timentin and 2% (v/v) Plant Preservative Mixture. Explants in this solution were lightly agitated for 10 minutes before being

rinsed three times in sterile water and allowed to dry for an additional 30 minutes on paper towels in laminar flow hood. Once dried, explants were immediately moved to the shoot induction media correlated with its media treatment (Tables 1 and 2). Hypocotyl and cotyledon explants receiving MT1 were placed on MT1-H-SIM and MT1-C-SIM, respectively. Explants treated with *Agrobacterium* were placed on media containing 100 mg/l kanamycin (KAN) and treatment-specific combinations of antibiotics to be used to control *Agrobacterium* overgrowth (Table 1). Both hypocotyl and cotyledon explants receiving MT2 were placed on MT2-SIM. All explants on both treatments were maintained under light (16 hr day/8 hr dark, 22 °C) for 28 days. Explants were placed on new media every 14 days. Observation of organogenesis, as well as instances of selection-induced tissue death and *Agrobacterium* overgrowth, were recorded every 14 days. Explants were also screened for expression of GFP. New shoots, evidence of organogenesis, and GFP expression were documented using an epifluorescent microscope (Olympus stereo microscope model SZX12, Olympus America, Center Valley, PA) using a GFP filter set (emission 509 nm) and QCapture 10 imaging software, and an Olympus Q-colour 5.

After 28 days, shoots were counted and documented before being excised from explants and moved to rooting media. As the Galán-Avila protocol (MT1) did not test examine root induction, all explants were moved to sterilized Magenta boxes containing previously tested rooting media (RIM) (Table 1). During this portion of the experiment, the only difference between the two media treatments was the combination and concentrations of antibiotics used to control *Agrobacterium* overgrowth. To encourage root induction and to reduce selection pressure, the concentration of kanamycin in this media was reduced from 100 mg/L to 50 mg/L. Explants used as a control were moved to RIM containing no antibiotics for selection or *Agrobacterium* control. All explants on both treatments were maintained under light (16 hr day/8

hr dark, 22 °C) for 28 days (Figure 3). Explants were placed on new media every 14 days. Explants were also screened for expression of GFP via UV lamp excitation. After 28 days, rooted shoots were moved to potting mix in temperature-controlled growth chamber and maintained under light (16 hr day/8 hr dark, 22 °C) for 14 days. Rooted shoots were also maintained under humidity control to acclimatize to *ex vitro* conditions. Acclimatized, rooted plants were moved to greenhouse conditions and maintained under similar light and temperature controls. Explants that showed healthy tissue growth that did not root were maintained on media in *in vitro* conditions for another 14 days.

Verification of *Agrobacterium*-mediated plant transformation via polymerase chain reaction detection of mGFP-ER and fluorescence spectroscopy.

Approximately 74 days after explant transformation, samples (leaves or newly developed meristems) were taken from the top of regenerated plants and genomic DNA was extracted following the CTAB method (Stewart & Via, 1993). Integrity and quality of extracted genomic DNA were quantified via agarose gel electrophoresis and Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). DNA templates were diluted to 100 ng/μL where appropriate. Primers were designed via Primer3 (v. 4.1.0) and verified *in silico* via Snapgene. To verify the integration of the T-DNA in the plant genome, transgene-specific primers were designed, and transgene fragments were amplified via polymerase chain reaction (PCR). Genomic DNA extracted from *E. coli* harboring the binary vector used for transformations was used as a positive control. Nuclease-free water was used as a negative control. PCR reactions were performed utilizing GreenTaq (Thermo Scientific, Waltham, MA, USA). PCR amplifications were performed in a volume of 50 μL including 22 μL nuclease-free water, 25 μL 2 × DreamTaq Master Mix, 1 μL of each primer 10 μM, 1 μL DNA template 100 ng/μL. The PCR protocol accompanying the master mix was the following: 95 °C for 3 min for DNA denaturation, 35

cycles of 30 s at 95 °C, followed by 30 s at 62°C and of 60 s at 72 °C. A final extension was performed at 72 °C for 15 min. PCR amplicons were measured via agarose gel electrophoresis.

Data Analyses

For the majority of the experiments analyzed, the number of explants with shoots ("responding explants"), as well as the number of shoots per responding explants were compared for the various, evaluated factors, i.e., cultivar, explant, and media type. For root induction experiments, responding explants with roots, as well as root number, were compared for media types. Transformation experiments were compared for transformation rates, as well as cultivar, explant, and media type.

The experimental unit for each experiment consisted of a single petri dish containing 6 explants. Explants were excised from 6 different seedlings from a single cultivar per plate. For each experimental treatment, every cultivar/explant combination was repeated at least 4 times. All data was evaluated for homogeneity of variances (Levene's test) and normality (Shapiro-Wilk test). Subsequently, significant differences between factors were evaluated via pairwise Wilcoxon test ($P < 0.05$) and Kruskal–Wallis non-parametric test ($P < 0.05$). Statistical analysis was carried out using the SPSS statistical package (version 29.0; SPSS Inc., Chicago, IL).

Results

Effect of media, explant source and cultivar on *in vitro* tissue culture of *C. sativa*.

Shoot regeneration was observed in both types of media used, in all cultivars, and explant sources (Figure 4). However, media did not have a significant effect on explant response or shoot regeneration (Table 3). Observationally, media treatments produced different morphological responses across explant sources. In control experiments, the MT1 treatment was observed to have induced healthier, more robust plantlets in both cotyledons and hypocotyls,

with shoots induced on MT2 showing signs of tissue necrosis and reduced leaf lamina (Figure 5A,B). Shoot organogenesis produced from MT2-treated hypocotyls did not undergo a callus phase (Figure 5B). While explants produced from MT1-treated hypocotyls did produce callus, shoots did not originate from said callus (Figure 5A). Shoots originating from cotyledons cultured on MT1 did undergo a callus phase, with cotyledons receiving this treatment often producing several shoots per explant (Figure 5C). Shoots induced from cotyledons cultured on MT2 did not exhibit a callus phase, often inducing single shoots (Figure 5D). Though not statistically analyzed, the MT1 control group exhibited spontaneous rooting across almost all plates containing hypocotyls, while this was not observed at the same rate in MT2-hypocotyls (Figure 6). Given that media had no significant effect on shoot regeneration, media treatments were grouped together for data analysis of effect of cultivar, explant source, and co-cultivation treatment.

Regarding the factor of cultivar, there was a significant differences between hypocotyls and cotyledons, in both the number of explants with regenerated shoots (responding explants) and the number of shoots regenerated per responding explants (Table 4). In the control hypocotyls, ‘Futura 75’ was shown to have had the highest proportion of responding explants (0.58 ± 0.04), while ‘USO 31’ had the highest rate of shoots per responding explant (1.52 ± 0.07) (Table 4). In cotyledons treated with co-cultivation and selection, ‘Futura 75’ had the highest rate of shoots per responding explant (0.18 ± 0.05). Across all treatments (control, co-cultivation with selection) ‘Fedora 17’ performed the poorest across explant source in both the proportion of responding explants and the proportion of shoots per responding explants (Table 4). Analysis of the factor of explant source showed significant differences, with hypocotyls exhibiting the highest proportion of responding explants (0.42 ± 0.02), as well as the highest rate of shoots per

responding explant (1.07 ± 0.04) (Table 5). Observationally, different explant sources exhibited varied tissue regeneration patterns. Shoot organogenesis observed in hypocotyls was typically either a single shoot (Figure 7B, D), or two shoots grown oppositely (Figure 7A, C). Shoots regenerated in cotyledons either exhibited a single shoot (Figure 8A, B) or several shoots (Figure 8C-F).

Effect of *Agrobacterium*-mediated transformation and growth regulating factors on an *in vitro* tissue culture transformation system.

Shoots were regenerated from all explants, as well as from all cultivars and media treatments. Additionally, shoots were regenerated from both transformation treatments. Ultimately, of the 2,051 explants treated with co-cultivation, 27 samples survived tissue culture to be analyzed for evidence of transgenes or GFP expression. Callus expressing GFP was observed across explant source, cultivar, media and transformation treatment (Figure 9, Table 6). However, no shoots expressing GFP were observed. To verify, plants that survived acclimatization were measured for GFP expression via Fluorolog fluorescence spectroscopy. As a control, *C. sativa* plants showing transient expression were used (Figure 10A). Again, no GFP expression was observed (Figure 10B). Finally, PCR was run to determine the presence of the transgene via partial insertion. Primers were designed to amplify a 1421 bp fragment of the GRFGIF transgene, as well as a 756 bp fragment of mGFP5-ER (Table 7). In the explants treated with *Agrobacterium* co-cultivation, no amplicon was detected (Figure 10C).

Regarding the effect of media on transformation of explants, MT2 had the highest transformation rate in hypocotyls (0.02 ± 0.01), as no hypocotyls cultured on MT1 expressed GFP (Table 6). Media did not influence responding explants or shoot regeneration (Table 3), however effect of antibiotic selection on shoot regeneration was observed (Figure 11A-C).

Though not statistically analyzed, abnormal regeneration patterns, such as elongated petioles and single-lamina leaves (instead of the normal, palmate leaf structure), were observed in plants treated with *Agrobacterium* co-cultivation (Figure 11D). Explant source had an effect on number of explants expressing GFP (Table 6). Although hypocotyls treated with MT1 had the highest proportion of responding explants and the high proportion of shoot number in explants treated with co-cultivation (Table 6), cotyledons had significantly higher proportion of explants expressing GFP (0.06 ± 0.01) (Table 6). Cotyledons cultured on MT1 had the highest transformation rate, at 0.09 ± 0.02 . Also observed were higher rates of *Agrobacterium* contamination in MT1 than on MT2. Cultivar did not have an effect on transformation rate, though it was shown to have an effect on *Agrobacterium* contamination. However, the higher rates of *Agrobacterium* contamination were shown to be localized to explants cultured on MT1 (Table 6). Finally, cotyledons cultured on MT1 and treated with co-cultivation with the GRFGIF vector had the highest rate of transformation (0.12 ± 0.02), indicating that transformation vector (GRFGIF versus Lat59) influenced transformation rate. However, it is unclear if this effect can be contributed to the vector itself or to the explant source, as cotyledons had a higher transformation rate in general (Table 6). Transformation vector did not have a significant effect on the proportion of *Agrobacterium*-contaminated explants to total explants (Table 6).

Discussion

Cannabis sativa is a globally important agro-economic crop that, in recent years, has become the focus of gene engineering studies. Much work has been published on the myriad of factors affecting stable transformation and plant regeneration, two of most the important tools available to plant biotechnology. In recent years, reports of stable transformation have been published, giving new insights into avenues of advancement. These studies not only provided

two avenues for shoot regeneration (direct and indirect organogenesis) (Galán-Ávila et al., 2021) but also provided a tested growth regulator (the GRF-GIF chimera) that could improve regeneration rates (Zhang et al., 2021). Unfortunately, these studies reported relatively low rates of transformation. Several studies have focused on *in vitro* regeneration by testing individual aspects of plant regeneration media (Monthony, Page, et al., 2021; Žukauskaitė et al., 2023). These studies have produced exciting possibilities for overcoming recalcitrance and improving shoot regeneration (Simiyu et al., 2022). In other studies, there was an effect of cultivar and explant source on regeneration (Adhikary et al., 2021; Li et al., 2022). However, a robust and high-throughput protocol for stable transformation and regeneration has yet to be published (Ingvarsen & Brinch-Pedersen, 2023). Therefore, there is a need for continued research into how these studies may provide insight to further advance the *Agrobacterium*-mediated transformation of *C. sativa*.

In the present work, I combined improved media components with an existing rapid, *in vitro*, direct organogenesis protocol (Galán-Ávila et al., 2021). I then transformed *C. sativa* cultivars reported to be favorable to tissue culture and regeneration with a growth regulator shown to improve shoot regeneration. Unfortunately, I was unable to regenerate stably transgenic *C. sativa* plants, nor was I able to show that the combination of improved media components increased the rate of plantlet/shoot regeneration. However, these experiments provided insight into previous studies by validating experimental results indicating tissue culture-responsive cultivars and explant sources. Experimental results also illuminated shortcomings in previously published *Agrobacterium*-mediated transformation protocols. In addition, these experiments provided possibilities for continued work towards the goal of high-throughput, stable transformation.

In my experiments, I utilized several published media components t proposed to improve *C. sativa* shoot regeneration. The synthetic cytokinin, meta-Topolin, improved shoot regeneration, health, and number in hemp node explants (Krishna Vrundha et al., 2021; Lata, Chandra, Techen, et al., 2016). In a 2020 study, the auxin antagonist PEO-IAA improved shoot regeneration and number in hemp node explants, because it works to antagonistically to endogenous auxin (Dreger et al., 2020; Smýkalová et al., 2019b). Increased *meso*-nutrients, or plant media micronutrients, have been shown to provide improved shoot extension and leaf lamina development while reducing hyperhidrosis, a common problem in plant tissue culture (Lubell-Brand et al., 2021). These components were combined in a single media (MT2) and tested against MT1, a previously published culture system (Dr. Galán-Ávila, 2020). These media were used in all transformation experiments, in both co-cultivation and control groups. No significant difference between media was reported for the proportion of responding explants/total explants, or for the rate of shoots regenerated/responding explants. There were, however, significant differences between media when analyzing the number of explants with *Agrobacterium* contamination; explants cultured on MT1 had a 1.3-fold greater contamination rate than those on MT2. There was no mention of issues with *Agrobacterium* contamination in the publication of this protocol and plant medium were sourced from (Galán-Ávila et al., 2021). Media type also had an effect on transformation efficiency, as cotyledons cultured on MT1 had a 1.8-fold increase in the percentage of explants expressing GFP, when compared to MT2. This rate is difficult to compare to previous *C. sativa* transformation studies, as Zhang et al., 2021 did not transform cotyledons, and Galán-Ávila et al., 2021 did not statistically analyze the transformation rate of treated cotyledons, as the number of explants expressing the transgene was low. It is worth mentioning that no hypocotyls cultured on MT1 expressed GFP. Although not

analyzed, MT1 was observed to have an apparently higher number of spontaneously induced roots, which has been previously observed (Galán-Ávila et al., 2020, 2021). Reduced number of roots observed in MT2 could be explained by the presence of PEO-IAA, an auxin antagonist that has been shown to cause transient reduction in auxin signaling, downregulation of the expression of root cell genes, as well as increased auxin accumulation, in *Arabidopsis* root meristems (Ishida et al., 2010; Takato et al., 2017). Auxin homeostasis is a vital factor in root development and root growth is adapted through the modulation of endogenous auxin levels (Benková & Hejácíko, 2009; Lee & Cho, 2013). As such, presence of PEO IAA in the media could be responsible for the reduced occurrence of spontaneous rooting. Moving forward, it would be worthwhile to test the combinations of the various media components piecewise to determine any interactions that might be negatively affecting shoot regeneration. Finally, although not statistically analyzed, shoots transferred to Magenta boxes for rooting regularly exhibited premature flowering (Figure 3D), with both male and female flowering structures present. This could be attributed to stress-induced flowering, which has been documented to cause *in vitro* flowering (Ramanayake et al., 2001). Alternatively, it is possible that the premature flowering is being induced by the 16 hr/8 hr photoperiod. *Cannabis* flowering is highly dependent on photoperiod (Avci, 2022). Future experiments should be performed to determine optimal growth conditions to prevent *in vitro* flowering. Cultivar had a significant effect on both proportion of responding explants to total explants and the number of shoots per responding explant. ‘Futura 75’ and ‘USO 31’ produced higher numbers of shoots in cotyledons and hypocotyls, respectively than ‘Fedora 17’ (Table 4), and both had had higher rates of explant response than ‘Fedora 17’. The results confirm findings from previous studies that these cultivars (‘Futura 75’ and ‘USO 31’) respond more favorably to tissue culture than ‘Fedora 17’ (Galán-Ávila et al., 2020, 2021).

These results also confirm previous findings that shoot regeneration is dependent on cultivar (Ślusarkiewicz-Jarzina et al., 2005; Wielgus et al., 2008; Wróbel et al., 2020; Zhang et al., 2021a). Given the poor performance of ‘Fedora 17’ (Table 4) in these experiments, future work could focus on testing other cultivars, or focusing on either ‘USO 31’ or ‘Futura 75’. Cultivar did not have a significant effect on transformation rate, which was unexpected. Previous studies have shown otherwise, indicating an effect amongst the cultivars tested here (Deguchi et al., 2022). There was also evidence that cultivar influenced the number of *Agrobacterium*-contaminated explants (Table 6). However, as all significant differences were exhibited in cultivars cultured on MT1 media, this was likely an effect of media, not cultivar.

Significant differences were observed in both proportion of responding explants to total explants and the number of shoots per responding explant when analyzing for the effect of explant source. Hypocotyls had the highest response rate, as well as the highest number of shoots regenerated. This was observed in both the control and co-cultivation treatment groups (Table 5). These results were similar to those previously reported in published studies (Galán-Ávila in 2020 and 2021). Although cotyledons had lower rates of shoot regeneration, instances of true organogenesis were observed in these explants. Whereas hypocotyl shoots could have been the result of lingering meristematic tissue, some cotyledonary node explants exhibited indications of indirect organogenesis, producing multiple novel shoots (Figure 8C-E). This behavior has been shown in previous studies (Chaohua et al., 2016; Galán-Ávila et al., 2020), indicating the need for continued focus on this explant source. Additionally, cotyledons showed the highest rates of transformation, exhibiting a 6-fold rate increase over hypocotyls (Table 6).

Despite having such a positive response to tissue culture, hypocotyls produced no transformed shoots and very little GFP-expressing callus (Table 6). This contradicts the 2021

Galán-Ávila transformation study that reported the highest transformation rate in hypocotyls, recovering seven transformed plants. The difference between the studies might be due to: 1) Shoots originating from axillary buds or shoot apical meristems were mistaken for direct organogenesis. or 2) In both Galán-Ávila studies, as well as in my experiments, hypocotyl explants were excised from seedlings by making an excision just below the apical meristem to remove the shoot apical meristem (SAM) and cotyledons. In both studies, shoots from the center of the hypocotyl, as well as shoots originating from the side of the hypocotyl explant, often in an opposite arrangement were produced (Figure 7). These shoots may originate from meristems that have not been fully excised. Other studies have shown hypocotyls to be a viable explant source for *C. sativa* (Mandolino & Ranalli, 1999; Movahedi et al., 2016b, 2016a). These studies, unlike the Galán-Ávila studies, reported indirect organogenesis of shoot from hypocotyl-derived callus. 3) *Agrobacterium* contamination could explain the difference in results in my experiments and other studies. As previously mentioned, MT1 (developed and tested in the Galán-Ávila studies) showed a significantly higher rate of *Agrobacterium* contamination than in this research. This type of contamination is common and can occur in defiance of the antibiotics that are often added to the media to control it. Typically, to prevent the reporting of false positives, a PCR assay is performed, utilizing a set of primers that bind outside of the borders of the expression cassette, running inward. However, there is no indication that this PCR assay was performed in the 2021 Galán-Ávila study. As such, it is possible that the transformations reported in that study were a combination of transient expression and *Agrobacterium* contamination. Future work will need to account for the possibility of improperly excised explants, as well as account for the possibility of *Agrobacterium* contamination.

Finally, since no transformed shoots were observed or confirmed, there is no way to accurately interpret the effect of the GRFGIF chimera on shoot regeneration. However, this growth regulator has shown promising results in previous experiments, both in *C. sativa* and in other crop species (Debernardi et al., 2020; G. Luo & Palmgren, 2021; Zhang et al., 2021). As such, future experiments should continue to examine the usefulness of this growth regulator in overcoming recalcitrance in *C. sativa*.

Conclusion

The present study was unable to demonstrate the establishment of a high throughput, stable transformation system for *Cannabis sativa*. This study provided results that raised questions for future research. Some results were contradictory to published studies reporting plant transformation and regeneration so more work is needed to fully understand the nature of these in a tissue culture recalcitrant plant and its close relative. I confirmed some previously reported findings, including explant sources and genotypes that respond well to *in vitro* tissue culture and *Agrobacterium*-mediated transformation. I also identified weaknesses in previous studies. Finally, our findings provided pathways for continued experiments in the pursuit of high throughput stable *Cannabis sativa* transformation.

Chapter 3

Conclusions and recommendations

As perceptions and values surrounding the viability of *C. sativa* as a source for medicines, products, and recreation change, there will be increasing interest in the value of the plant as an economically important crop. With this interest will rise the need for study of the various cannabinoids produced by the plant. As discoveries, such as genomic mapping and biosynthesis pathways, are illuminated, there will be a continued need for *C. sativa* genetic engineering. To meet this need, a robust system for the regeneration of stably transformed plants will continue to be necessary.

Although I was unable to recover stably regenerated plants in this study, I was able to make observations that can help further the pursuit of the creation of such a system. The cultivars ‘Futura 75’ and ‘USO 31’ responded well to *in vitro* tissue culture and should be utilized in the future. Functional vectors were created, containing the GRFGIF chimera and the mGFP5-ER reporter gene. Cotyledonary node explants, although having lower rates of shoot regeneration compared with hypocotyl segment explants, were more amenable for transformation. Cotyledonary node explants appeared to develop indirect organogenesis of shoots. Utilizing this information, further experiments should be performed that could increase both shoot regeneration rate and transformation rate. Robust systems for cotyledonary node transformation have been developed for other species such as tomato and soybean. These systems could be used as a reference point to further develop the system for *C. sativa*. Utilizing inputs that have been shown to improve transformation in cotyledons, such as surfactants or sonification, could provide such improvements for a *C. sativa* transformation system. Ultimately, what may be required is a “ground up” approach to developing this system. This could include transient assays to determine which promoters and terminators work best in transformation vectors used for genetic engineering of *C. sativa*. Further experiments could include assays to

determine the best antibiotic resistance markers to be used for selection. Regardless of which future experiments are performed, it is obvious that continued work is needed to develop a high throughput system for the stable transformation of *C. sativa*.

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Appendix

(A) Test Vector



(B) Negative Control Vector



Figure 1. A list of expression constructs used in *Agrobacterium*-mediated transformation experiments. (A) The test vector used a chimeric GRF3-GIF1 protein 2xCaMV 35s promoter::GRF3GIF1::OcsT; Ocs terminator. (B) The positive control vector used a green fluorescent protein reporter LAT59 promoter::mEmerald::OcsT. All plasmids are binary vectors and harbor a green, fluorescent reporter 2xCaMV 35S::mGFP5-ER::MasT; Mas terminator. Vectors also contain a kanamycin resistance gene for bacterial selection.

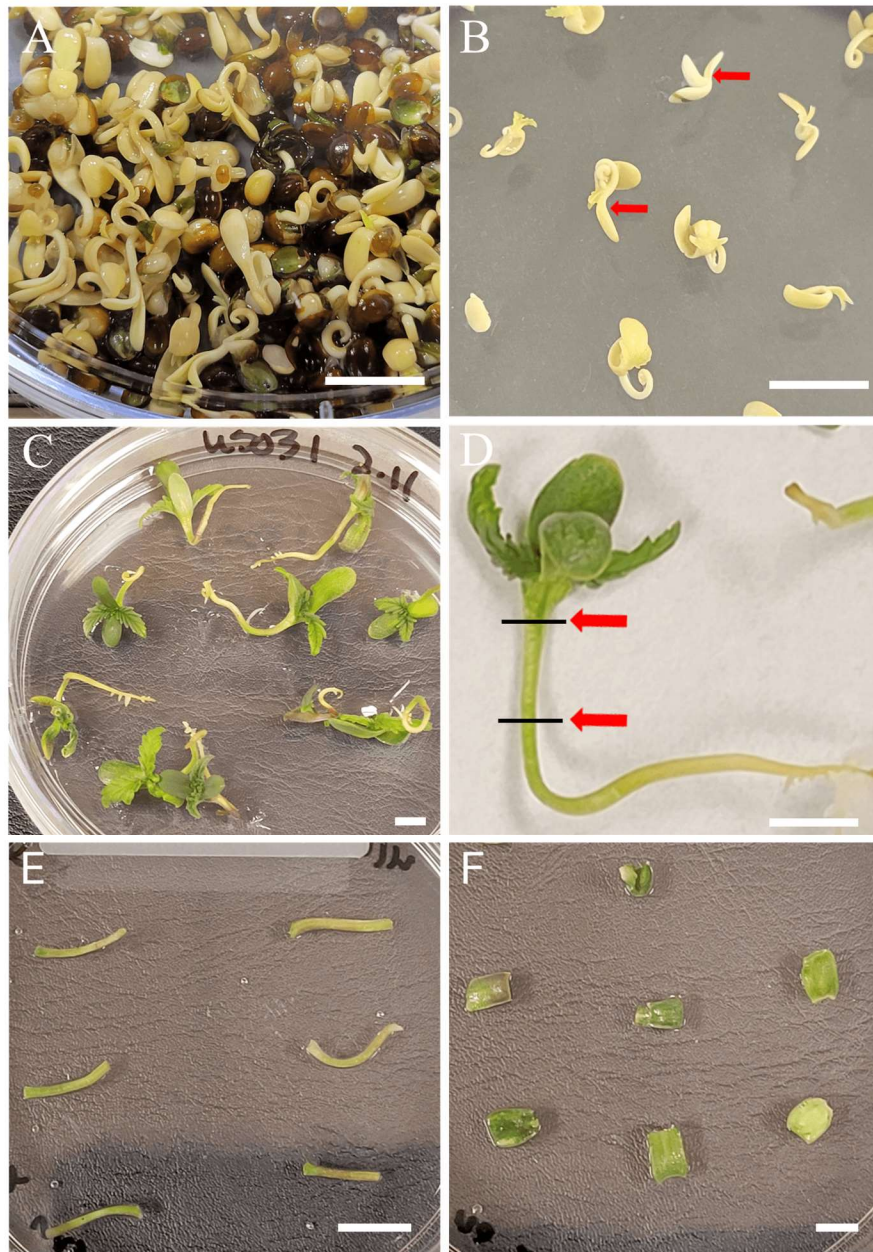


Figure 2. Sterilization, germination, and explant preparation. (A) Germinated seed 72 h. after liquid media germination. (B) Three-day-old seedlings after having seed coats removed and placed onto solid germination media, epicotyl indicated. (C) Seven-day-old seedlings with fully expanded first pair of true leaves. (D) Close-up of an individual seven-day-old seedling, arrows indicate dissection points. (E) Example of prepared hypocotyl explants, placed on co-cultivation media. (F) Example of prepared cotyledon explants, placed on co-cultivation media. Scale bars: 1 mm.

Table 1. Media tested to compare effects of improved media components. All media was pH-adjusted to 5.7 using 0.1 M sodium hydroxide (NaOH). Media used in control treatments did not contain antibiotics. Abbreviations: ACS: acetosyringone; MES: 2-(N-morpholino)ethanesulfonic acid; GA: gibberellic acid; meta-Topolin (MET), thidiazuron (TDZ), PEO-IAA: 2-(1H-Indol-3-yl)-4-oxo-4-phenyl-butyric acid; PPM: Plant Preservative Mixture; KAN: kanamycin; CFT: cefotaxime; CRB: carbenicillin; TIM: timentin; IAA: indole-3-acetic acid; ZTN RBS: zeotin riboside; NAA: 1-naphthaleneacetic acid

Media	MT1-C	MT1-H	MT2
Base	2.2 g/L MS basal medium with vitamins, and 15 g/L sucrose.		2.2 g/L MS basal medium with vitamins, and 15 g/L sucrose.
Germination Media (GM)	Additives		
	3% (v/v) H3O3 and 2% (v/v) PPM		3% (v/v) H3O3 and 2% (v/v) PPM.
Inoculation Media (CCM)	Additives		
	39.2 mg/L ACS and .5 g/L MES.		39.2 mg/L ACS and .5 g/L MES.
Media	MT1-C	MT1-H	MT2
Base	2.2 g/L MS basal medium with vitamins, 15 g/L sucrose, and 3.5 g/L Gelzan.		4.4 g/L MS basal medium with increased meso-vitamins, 30 g/L sucrose, and 3.5 g/L Gelzan
Co-Cultivation Media (CCM)	Additives		
	39.2 mg/L ACS, .4 mg/L TDZ, and .2 mg/L NAA	39.2 mg/L ACS	39.2 mg/L ACS, 0.5 g/L MES, .1 mg/L GA, .5 g/L MET, and 0.29 mg/L PEO-IAA
Shoot Induction Media (SIM)	Additives		
	.4 mg/L TDZ and .2 mg/L NAA	None	.1 mg/L GA, .5 g/L MET, and 0.29 mg/L PEO-IAA
	Agrobacterium Control		
Contains 100 mg/L KAN	250 mg/L CFT and 250 mg/L CRB		1 mL/L PPM, 200 mg/L CFT, 200 mg/L CRB, and 150 mg/L TIM
Media	MT1-C	MT1-H	MT2
Base	4.4 g/L MS basal medium with increased meso-vitamins, 30 g/L sucrose, and 3.5 g/L Gelzan		4.4 g/L MS basal medium with increased meso-vitamins, 30 g/L sucrose, and 3.5 g/L Gelzan
Root Induction Media (RIM)	Additives		
	0.2 mg/L NAA, 0.5 mg/L IAA, and 0.01 mg/L ZTN RBS		0.2 mg/L NAA, 0.5 mg/L IAA, and 0.01 mg/L ZTN RBS
	Agrobacterium Control		
Contains 50 mg/L KAN	200 mg/L CFT, 200 mg/L CRB, and 150 mg/L TIM		200 mg/L CFT, 200 mg/L CRB, and 150 mg/L TIM

Table 2. Concentrations of mesonutrients and vitamins contained in basal media formulations utilized in media treatment experiments.

<u>Media</u>	<u>MT1</u>	<u>MT2</u>
Basal Media	2.2 g/L MS basal medium with vitamins	4.4 g/L MS basal medium with increased meso-vitamins
<u>Meso-vitamins</u>	<u>Concentration (mg/L)</u>	
Calcium chloride (anhydrous)	166.1	830.5
Magnesium sulfate (anhydrous)	90.4	451.8
Potassium phosphate (monobasic)	85	425
Glycine (free base)	1	5
Myo-inositol	50	250
Nicotinic acid	0.25	1.25
Pyridoxine HCL	0.25	1.25
Thiamine HCL	0.05	0.25

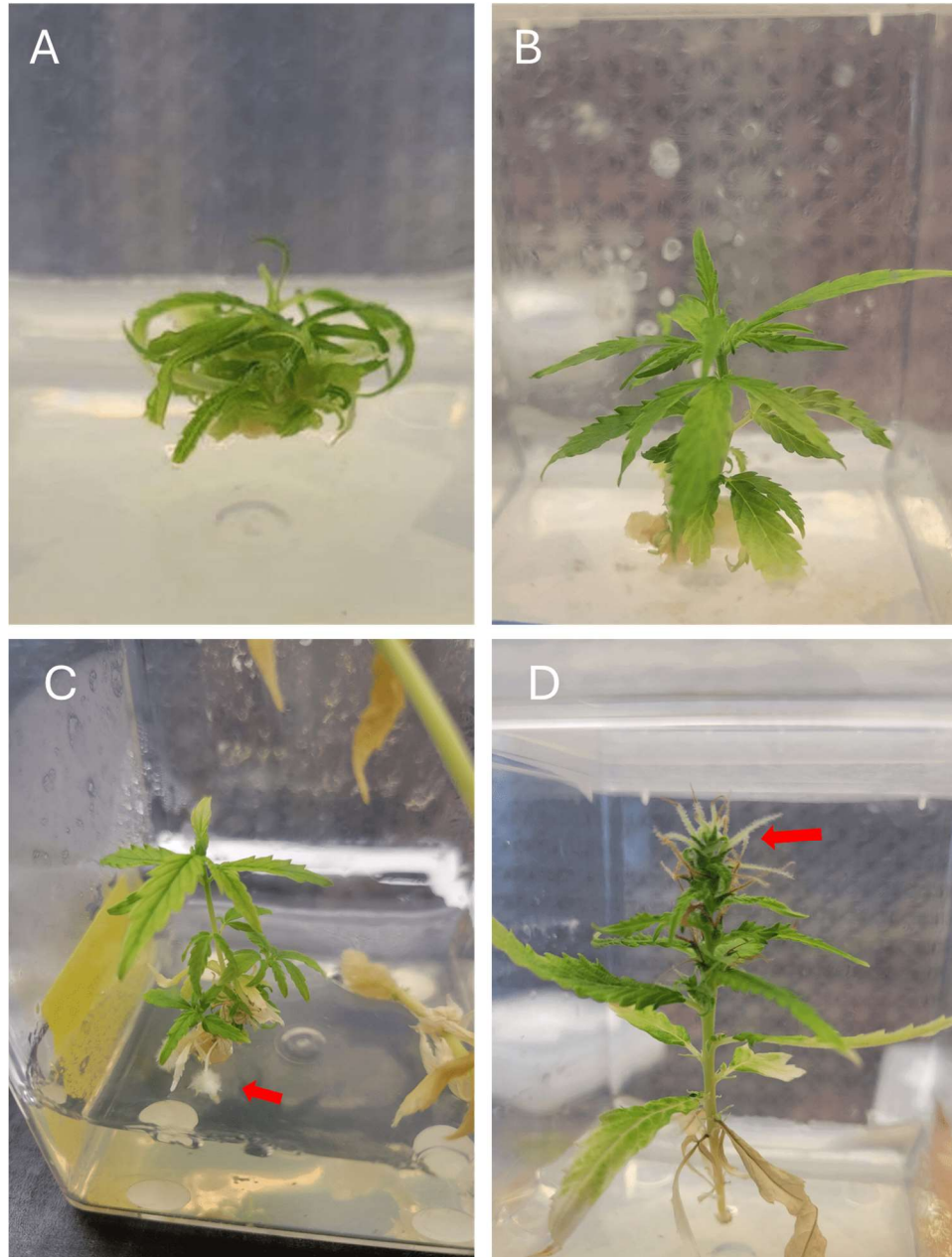


Figure 3. *Cannabis sativa* on rooting media in Magenta boxes. (A) A newly transferred cotyledonary node explant, with several novel shoots. (B) Shoots regenerated from a *C. sativa* hypocotyl explant. (C) Hypocotyl-derived *C. sativa* shoots with newly formed roots.. (D) Hypocotyl-derived *C. sativa* shoot with fully female flower structure. Note the pistils, structures with an ovule, and two protruding styles capped with stigmas extending beyond the bract.

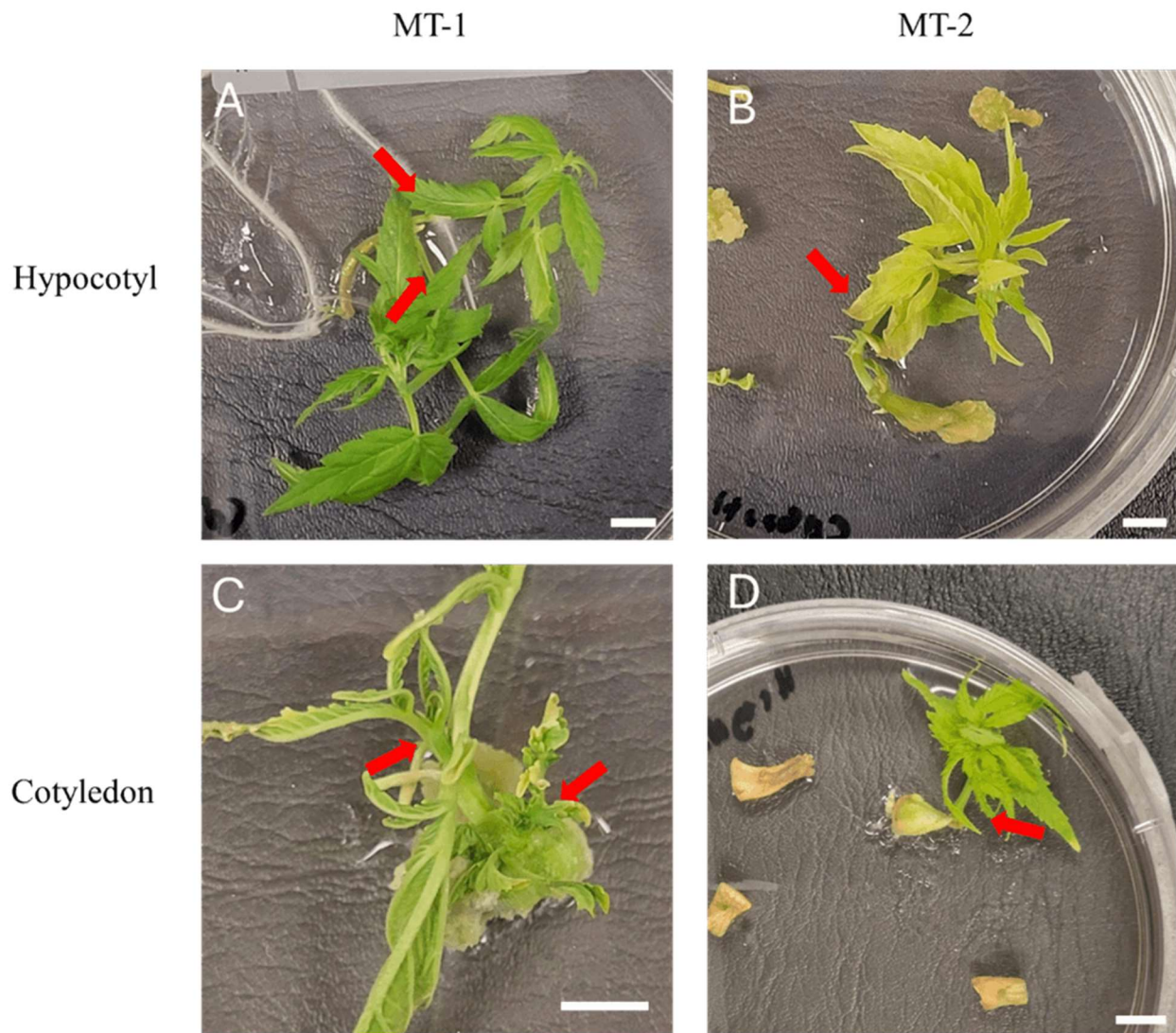


Figure 4. Different plant regeneration patterns, derived from *C. sativa* hypocotyl and cotyledon explants. All photos were taken after 28 days on their respective shoot induction media (SIM). Scale bars: 1 mm. (A) Shoots regenerated from a hypocotyl explant cultured on MT1. Note two shoots growing in opposite pattern. (B) A single shoot regenerated from a hypocotyl explant cultured on MT2. (C) Multiple shoots regenerated from a cotyledon explant cultured on MT1. (D) A single shoot regenerated from a cotyledon explant cultured on MT2.

Table 3. Effect of media on *in vitro* shoot regeneration. Displayed is the proportion of responding explants (explants with shoots) to total explants and the proportion of the number of shoots to responding explants. *In vitro* *C. sativa* hypocotyl and cotyledon explants were cultured on their respective media. Standard error is included. Non-parametric Kruskal-Wallis and pairwise Wilcoxon tests were used to determine significant differences ($p < 0.05$). Different letters indicate significant differences between factors.

Control			
Explant	Media	Responding Explants /total explants	Shoot/responding explant
Hypocotyl	MT1	0.52 ^a ± 0.03	1.43 ^a ± 0.06
	MT2	0.49 ^a ± 0.04	1.21 ^a ± 0.08
Cotyledon	MT1	0.09 ^a ± 0.03	0.56 ^a ± 0.15
	MT2	0.15 ^a ± 0.04	0.33 ^a ± 0.08
Co-cultivation and selection			
Explant	Media	Responding Explants /total explants	Shoot/responding explant
Hypocotyl	MT1	0.34 ^a ± 0.04	0.92 ^a ± 0.07
	MT2	0.35 ^a ± 0.03	0.93 ^a ± 0.07
Cotyledon	MT1	0.04 ^a ± 0.01	0.16 ^a ± 0.04
	MT2	0.04 ^a ± 0.02	0.11 ^a ± 0.04
Combined treatments			
Explant	Media	Responding Explants /total explants	Shoot/responding explant
Hypocotyl	MT1	0.44 ^a ± 0.03	1.11 ^a ± 0.05
	MT2	0.40 ^a ± 0.03	1.02 ^a ± 0.05
Cotyledon	MT1	0.04 ^a ± 0.01	0.30 ^a ± 0.06
	MT2	0.08 ^a ± 0.02	0.19 ^a ± 0.04

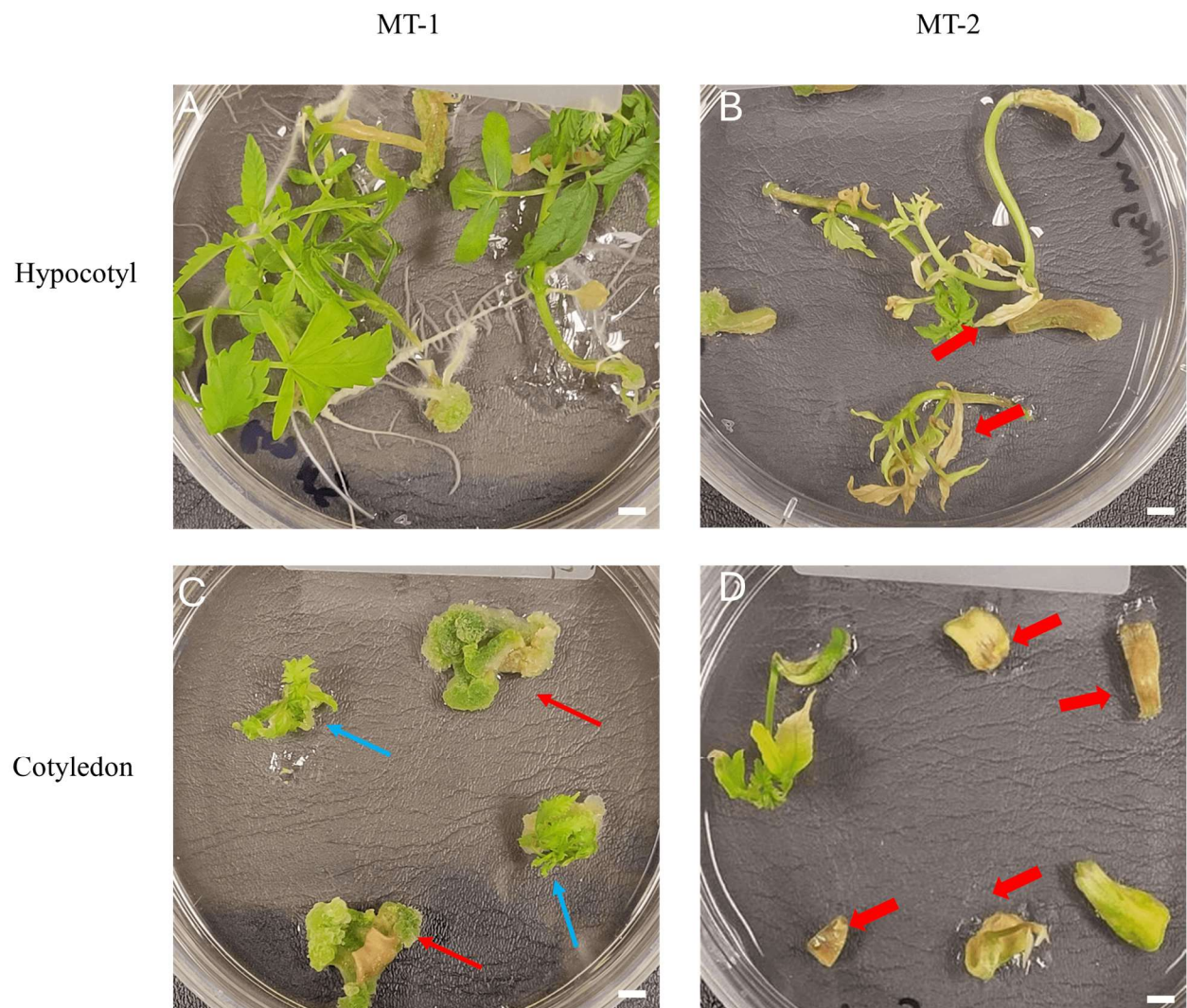


Figure 5. Different tissue culture response patterns derived from *C. sativa* hypocotyl and cotyledon explants. All photos were taken after 28 days on their respective shoot induction media (SIM). Scale bars: 1 mm. (A) Hypocotyl explants cultured on MT1. Explants cultured on MT1 were observed to be generally healthier than those cultured on MT2. (B) Hypocotyl explants cultured on MT2. Arrows indicate tissue necrosis. (C) Cotyledon explants cultured on MT1. Cotyledon explants on MT1 exhibited induced callus, indicated by red arrows. Blue arrows indicate multiple shoots originating from callus. (D) Cotyledon explants cultured on MT2. Arrows indicate explants exhibiting necrosis.

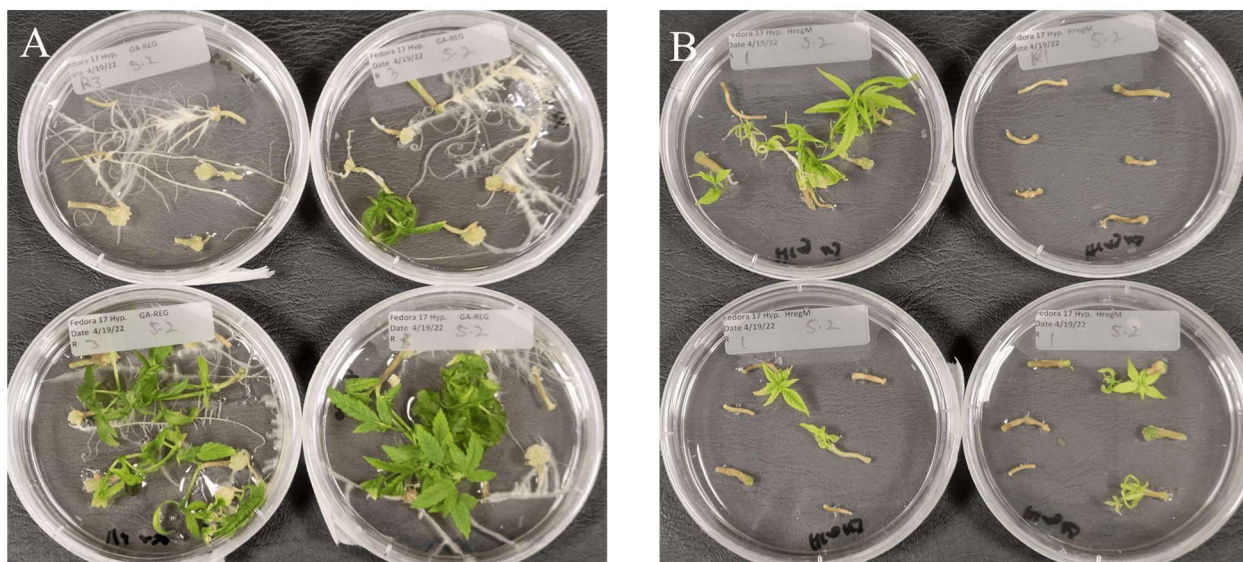


Figure 6. Spontaneous root induction on *C. sativa* hypocotyl explants. All photos were taken after 14 days on their respective shoot induction media (SIM). Scale bars: 1 mm. (A) Hypocotyl explants cultured on MT1. Hypocotyl explants cultured on MT1 were observed to show spontaneous rooting. Shoots regenerated on MT1 were also observed to show more healthy, green growth when visually compared to those cultured MT2 (B) Hypocotyl explants cultured on MT2. Explants cultured on MT2 were, in general, not observed to exhibit spontaneous rooting.

Table 4. Effect of cultivar on *in vitro* shoot regeneration. Displayed is the proportion of responding explants (explants with shoots) to total explants and the proportion of the number of shoots to responding explants. *In vitro* *C. sativa* hypocotyl and cotyledon explants were cultured on their respective media. Standard error is included. Non-parametric Kruskal-Wallis and pairwise Wilcoxon tests were used to determine significant differences ($p < 0.05$). Different letters indicate significant differences between factors. *indicates a proportion below 0.01 ± 0.01

Control			
Explant	Cultivar	Responding Explants /total explants	Shoot/responding explant
Hypocotyl	Futura 75	$0.58^a \pm .04$	$1.41^{ab} \pm .05$
	Fedora 17	$0.38^b \pm .05$	$1.02^b \pm .12$
	USO 31	$0.53^{ab} \pm .04$	$1.52^a \pm .07$
Cotyledon	Futura 75	$0.18^a \pm .05$	$0.48^a \pm .15$
	Fedora 17	$0.02^b \pm .02$	$0.33^a \pm .17$
	USO 31	$0.17^a \pm .05$	$0.44^a \pm .12$
Co-culture + selective regeneration			
Explant	Cultivar	Responding Explants /total explants	Shoot/responding explant
Hypocotyl	Futura 75	$0.40^a \pm .03$	$1.06^a \pm .06$
	Fedora 17	$0.27^b \pm .04$	$0.66^b \pm .10$
	USO 31	$0.46^a \pm .05$	$1.07^a \pm .09$
Cotyledon	Futura 75	$0.04^a \pm .01$	$0.18^a \pm .05$
	Fedora 17	$0.01^b \pm .01^*$	$0.02^b \pm 0.02$
	USO 31	$0.06^a \pm .02$	$0.17^a \pm .05$
Combined Treatments			
Explant	Cultivar	Responding Explants /total explants	Shoot/responding explant
Hypocotyl	Futura 75	$0.46^a \pm .03$	$1.17^a \pm .05$
	Fedora 17	$0.30^b \pm .03$	$0.77^b \pm .09$
	USO 31	$0.48^a \pm .03$	$1.22^a \pm .07$
Cotyledon	Futura 75	$0.05^{ab} \pm .01$	$0.29^a \pm .06$
	Fedora 17	$0.01^b \pm .01^*$	$0.13^b \pm 0.06$
	USO 31	$0.06^a \pm .02$	$0.29^a \pm .06$

Table 5. Effect of explant source on in vitro shoot regeneration. Displayed is the proportion of responding explants (explants with shoots) to total explants and the proportion of the number of shoots to responding explants. In vitro *C. sativa* hypocotyl and cotyledon explants were cultured on their respective media. Standard error is included. Non-parametric Kruskal-Wallis and pairwise Wilcoxon tests were used to determine significant differences ($p < 0.05$). Different letters indicate significant differences between factors

Control		
Explant	Responding Explants /total explants	Shoot/responding explant
Hypocotyl	$0.51^a \pm 0.03$	$1.32^a \pm 0.05$
Cotyledon	$0.09^b \pm 0.02$	$0.43^b \pm 0.08$
Co-culture + selective regeneration		
Explant	Responding Explants /total explants	Shoot/responding explant
Hypocotyl	$0.38^a \pm 0.02$	$0.94^a \pm 0.05$
Cotyledon	$0.04^b \pm 0.01$	$0.13^b \pm 0.03$
Combined Treatments		
Explant	Responding Explants /total explants	Shoot/responding explant
Hypocotyl	$0.42^a \pm 0.02$	$1.07^a \pm 0.04$
Cotyledon	$0.06^{b\pm} \pm 0.01$	$0.24^b \pm 0.05$

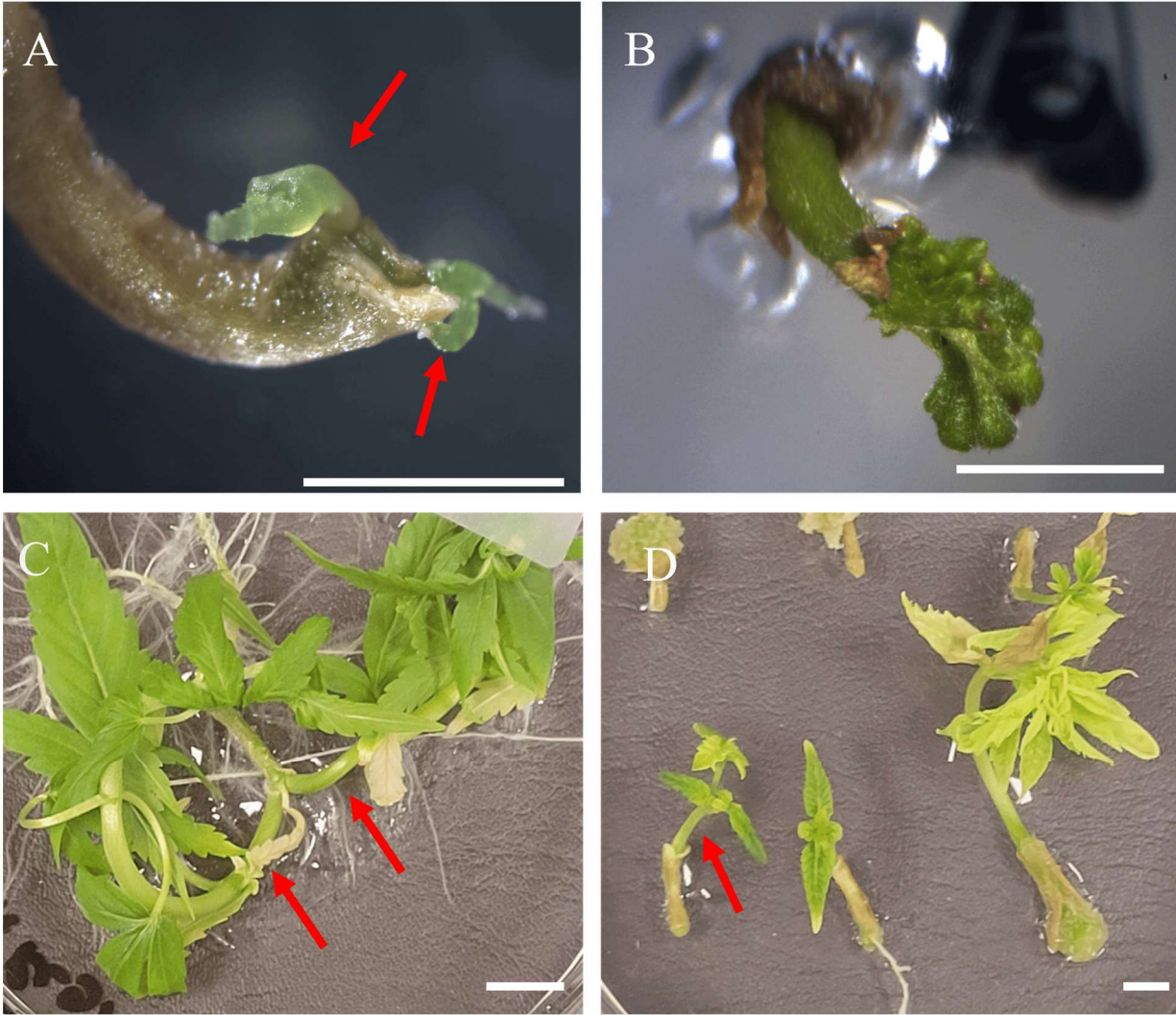


Figure 7. Shoot development patterns observed in *C. sativa* hypocotyl explants. Scale bars: 1 mm. All photos are from the control group. (A) View of shoot primordia from hypocotyl explant, displaying opposite growth pattern. Photo taken after 7 days on MT1 (B) A single shoot, derived from hypocotyl explant. Note growth originating from the pith of explant, as opposed to growth originating from the cortex, as displayed in photo A. Photo taken after 14 days on MT1 (C) two shoots shoot originating from a hypocotyl explant. Arrows indicate shoots displaying opposite growth pattern. Photo taken after 28 days on MT1 (D) Hypocotyl explants showing single shoot growth pattern. Photo taken after 28 days on MT1.

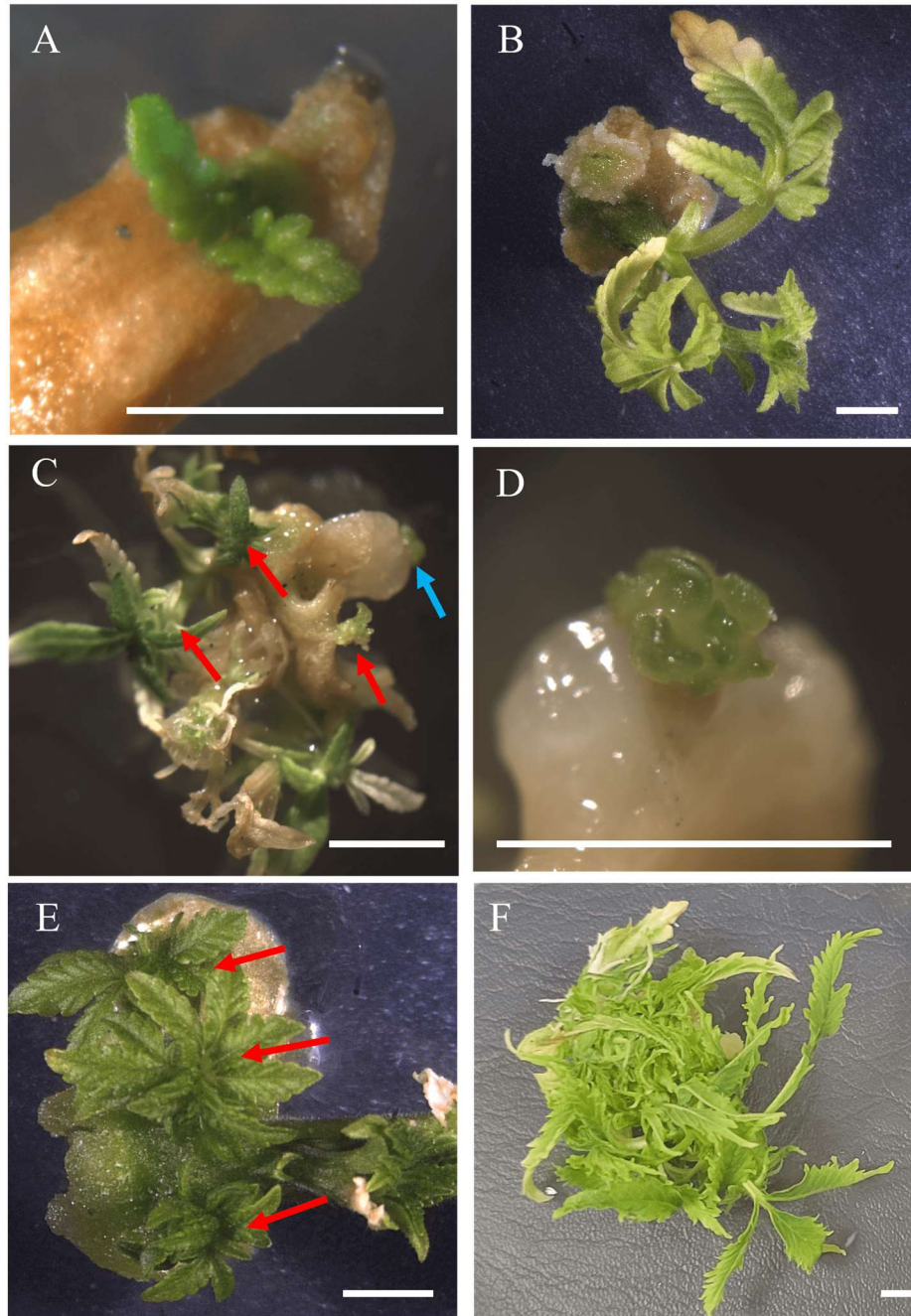


Figure 8. Shoot development patterns observed in *C. sativa* cotyledon explants. Scale bars: 1 mm. All photos are from the control group. (A) Single shoot originating from a cotyledonary node on a cotyledon explant. Photo taken after 14 days on MT2. (B) Single shoot originating from callus derived from a cotyledon explant. Photo taken after 14 days on MT2 (C) Red arrows indicate multiple shoots originating from callus derived from a cotyledon explant. Green arrow indicates meristematic tissue in D. Photo taken after 14 days on MT2. (D) Close-up of meristematic tissue originating from cotyledon-derived callus. (E) Arrows indicate several, novel shoots originating from callus derived from cotyledon explant. Photo taken after 28 days on MT1. (F) The same cotyledon explant, shown in E, exhibiting continued novel shoot production. Photo taken after 42 days on MT1.

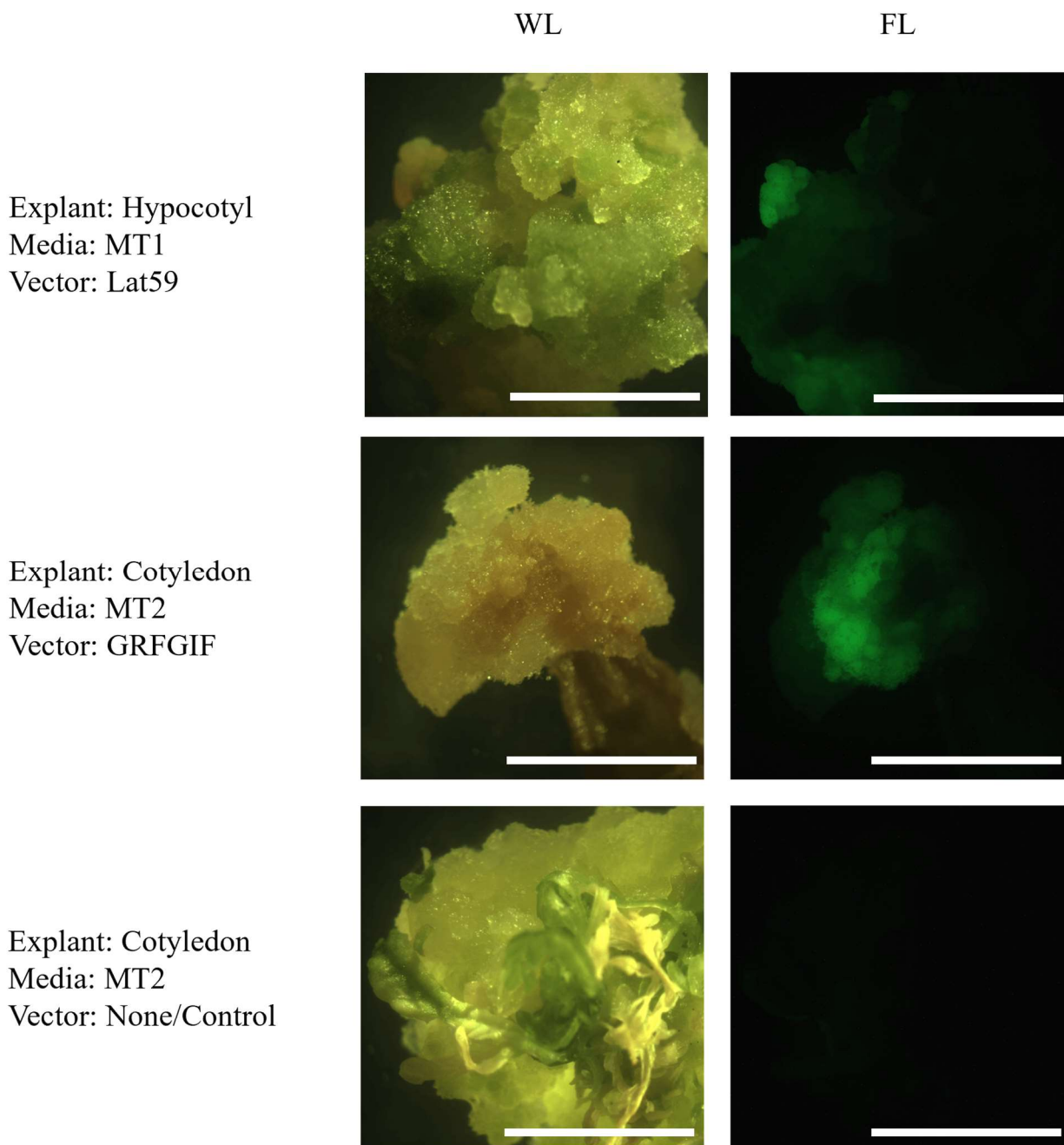


Figure 9. Green fluorescent protein (GFP) fluorescence in transformed *C. sativa* callus. Scale bars: 1 mm. Samples are labeled accordingly with explant source, media treatment and vector. Callus was photographed using an epifluorescent microscope. Column FL (fluorescence light conditions) was photographed using a GFP filter set (emission 509 nm). Column WL was photographed using the same epifluorescent under white light.

Table 6. Effect of explant source, media, cultivar, and transformation vector on *Agrobacterium* contamination (Agro-contaminated) and transformation rate. Displayed is the proportion of contaminated explants (explants with residual *Agrobacterium* overgrowth) to total explants and the proportion of the explants showing expression of mGFP5-ER to responding explants. In vitro, *C. sativa* hypocotyl and cotyledon explants were cultured on their respective media. Standard error is included. Non-parametric Kruskal-Wallis and pairwise Wilcoxon tests were used to determine significant differences ($p < 0.05$). Different letters indicate significant differences between factors. * indicates a proportion below 0.01 ± 0.01 . ** indicates that no instances were observed for that category.

A) Effect of Explant Source				
Explant	n (pooled)		Agro-contaminated explants/total explants	Explants expressing GFP/total explants
Hypocotyl	1041		0.07 ^a ± .01	0.01 ^b ± .01*
Cotyledon	1010		0.07 ^a ± .01	0.06 ^a ± .01
B) Effect of Media Type				
Explant	Media		Agro-contaminated explants/total explants	Explants expressing GFP/total explants
Hypocotyl	MT1		0.15 ^a ± .02	0.00 ^b ± .00**
	MT2		0.01 ^b ± .01	0.02 ^a ± .01
Cotyledon	MT1		0.13 ^a ± .02	0.09 ^a ± .02
	MT2		0.01 ^b ± .01	0.05 ^b ± .01
C) Effect of Cultivar				
Explant	Media	Cultivar	Agro-contaminated explants/total explants	Explants expressing GFP/total explants
Hypocotyl	MT1	Futura 75	0.10 ^b ± 0.02	0.00 ^a ± 0.00**
		Fedora 17	0.15 ^{ab} ± 0.04	0.03 ^a ± 0.02
		USO 31	0.20 ^a ± 0.03	0.00 ^a ± 0.00**
	MT2	Futura 75	0.01 ^a ± 0.01*	0.00 ^a ± 0.00**
		Fedora 17	0.01 ^a ± 0.01*	0.02 ^a ± 0.1
		USO 31	0.00 ^a ± 0.00**	0.02 ^a ± 0.1
Cotyledon	MT1	Futura 75	0.07 ^b ± 0.04	0.06 ^a ± 0.02
		Fedora 17	0.10 ^{ab} ± 0.03	0.11 ^a ± 0.03
		USO 31	0.21 ^a ± 0.04	0.09 ^a ± 0.02
	MT2	Futura 75	0.01 ^a ± 0.01*	0.03 ^a ± 0.02
		Fedora 17	0.00 ^a ± 0.00**	0.02 ^a ± 0.02
		USO 31	0.00 ^a ± 0.00**	0.08 ^a ± 0.03
D) Effect of Transformation Vector				
Explant	Media	Treatment	Agro-contaminated explants/total explants	Explants expressing GFP/total explants
Hypocotyl	MT1	GrfGif	0.15 ^a ± 0.02	0.02 ^a ± 0.01
		Lat59	0.14 ^a ± 0.03	0.00 ^a ± 0.00**
	MT2	GrfGif	0.00 ^a ± 0.00**	0.01 ^a ± 0.01*
		Lat59	0.01 ^a ± 0.01*	0.01 ^a ± 0.01*
Cotyledon	MT1	GrfGif	0.12 ^a ± 0.03	0.12 ^a ± 0.02
		Lat59	0.13 ^a ± 0.03	0.05 ^b ± 0.01
	MT2	GrfGif	0.01 ^a ± 0.01*	0.05 ^a ± 0.01
		Lat59	0.00 ^a ± 0.00**	0.04 ^a ± 0.01

Table 7. Oligonucleotide primers designed for verification of stable, *Agrobacterium*-mediated transformation of *C. sativa*. All primers were designed via Primer3 (v. 4.1.0) and verified in silico via Snapgene.

Gene	Direction	Tm	# of Base Pairs	Sequence
GRFGIF	Forward	64° C	21	GTCGGTGCCGGAGAACAGACG
	Reverse	63° C	20	GCTCCCTCCTCGGCCTTCAG
mGFP	Forward	57° C	19	TGCCATGTGTAATCCCAGC
	Reverse	58°C	22	ACTTTTCACTGGAGTTGTCCCA

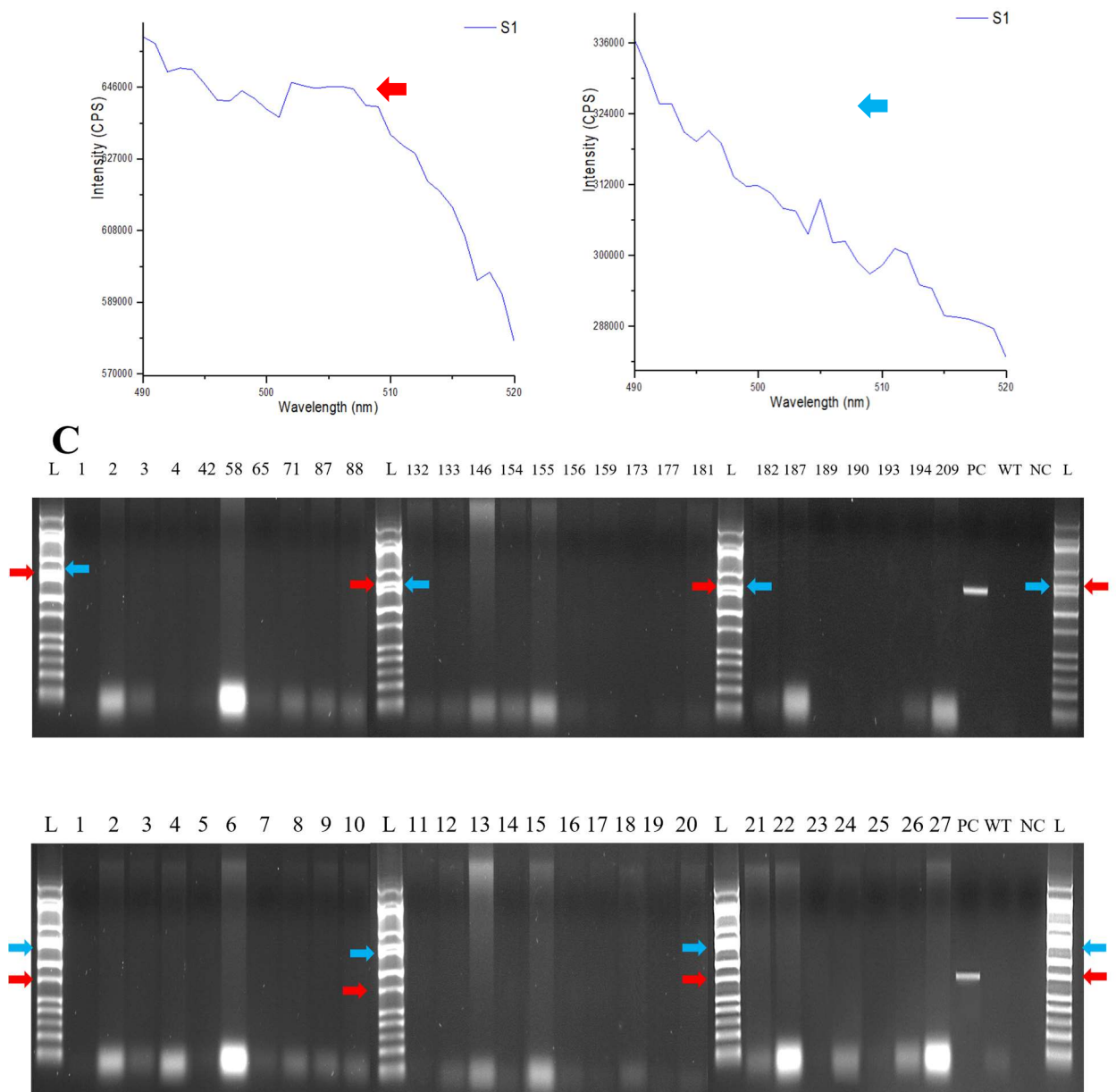


Figure 10. Verification of expression of transgene in *C. sativa* explants treated with *Agrobacterium* co-cultivation. (A) Fluorimetric measurement of agroinfiltrated leaves of *C. sativa* plant indicating expression of mGFP5-ER. Red arrow indicates expression, i.e., emission occurring at approximately 510 nm. This plant was used as a positive control. (B) Fluorimetric measurement of leaves regenerated from *C. sativa* explants treated with *Agrobacterium* co-cultivation. Blue arrow indicates where the emission should be measured. (C) Polymerase chain reaction (PCR) detection of GRFGIF chimeric gene (top) and mGFP5-ER (bottom). Red arrows indicate where amplicon is expected to appear (1421 bp and 756 bp, respectively). Blue arrows indicate the 1400 bp landmark in the ladder. Abbreviations: L: Hi-lo ladder; PC: positive control; WT: wild type; NC: negative control (water).



Figure 11. Verification of expression of transgene in *C. sativa* explants treated with *Agrobacterium* co-cultivation. (A) Hypocotyl-derived *C. sativa* shoots, cultured on MT1, exhibit tissue necrosis from kanamycin toxicity. (B) Hypocotyl-derived *C. sativa* shoots, cultured on MT2, exhibit bleaching from kanamycin toxicity. (C) Hypocotyl-derived *C. sativa* shoots, cultured on RIM, exhibit bleaching from kanamycin toxicity. (D) Abnormal growth pattern observed in hypocotyl-derived *C. sativa* shoots, cultured on MT2.

Vita

Tyler Newton was born in Douglasville, GA in 1981. He graduated in 2018 with a Bachelor of Science Degree in Plant Sciences. He is currently pursuing her master's in Plant Sciences with a concentration in Plant Molecular Genetics from the University of Tennessee, Knoxville. He is working to develop a high-throughput, stable transformation system for *Cannabis sativa*, which may be useful for genetic modification of *C. sativa*, for crop improvement. .