



# Development of a robust transient expression screening system in protoplasts of *Cannabis*

L. Matchett-Oates<sup>1,2</sup> · Ehab Mohamaden<sup>1</sup> · G. C. Spangenberg<sup>1,2</sup> · N. O. I. Cogan<sup>1,2</sup>

Received: 8 November 2020 / Accepted: 23 March 2021 / Published online: 17 May 2021 / Editor: Ted Klein  
© The Author(s) 2021

## Abstract

Transient expression systems in mesophyll protoplasts have been utilised in many plant species as an indispensable tool for gene function analysis and efficacious genome editing constructs. However, such a system has not been developed in *Cannabis* due to the recalcitrant nature of the plant to tissue culture as well as its illegal status for many years. In this study, young expanding leaves from aseptic *in vitro Cannabis* explants were used for protoplast isolation. Factorial designs were used to optimise variables in viable protoplast isolation and transient expression of GFP, with a range analyses performed to determine, and quantify, significantly impacting variables. Viable protoplast yields as high as  $5.7 \times 10^6$  were achieved with 2.5% (w/v) Cellulase R-10, 0.3% (w/v) Macerozyme R-10 and 0.7 M mannitol, incubated for 16 h. As indicated by the transient expression of GFP, efficiency reached 23.2% with 30 µg plasmid, 50% PEG,  $1 \times 10^6$  protoplasts and a transfection duration of 20 min. Application of the optimised protocol for protoplast isolation was successfully evaluated on three subsequent unrelated genotypes to highlight the robustness and broad applicability of the developed technique.

**Keywords** Cannabis · Protoplasts · Transient · Transformation

## Introduction

*Cannabis sativa* L. (*Cannabis*) is a highly polymorphic, wind pollinated herb originating in China where evidence of its cultivation dates back to 4000 BC (Zuardi 2006). Recent interest in the medicinal properties of phytocannabinoids produced by *Cannabis* has led to increased legalisation around the world, along with a growing medicinal industry (ProCon.org 2021). Recently, chromosomal resolution of the *Cannabis* genome was published along with other genetic resources for identification of important cannabinoid biosynthesis genes (Grassa *et al.* 2018; Laverty *et al.* 2019). Next generation sequencing has also started to unravel the complexity of the *Cannabis* genome and transcriptome atlas (Braich *et al.* 2019). Progress in the genetic tools for manipulation and

analysis of genes have prompted research into *Cannabis*, but currently, *Cannabis* remains a recalcitrant species to deliver biotechnology tools to. Transgenic hairy root cultures of *Cannabis* have been performed previously using agroinfiltration of vectors with the GUS reporter gene (Wahby *et al.* 2013). However, hairy root cultures only offer the accumulation of metabolites within the root structures (Gurunani *et al.* 2015) making this transformational technique unsuitable for biotechnological applications seeking phytocannabinoids, which accumulate in the female floral tissues. Transformation of hemp callus cultures with a foreign *Escherichia coli* gene, *manA*, has previously been successful using *Agrobacterium tumefaciens* with a success rate of 31% (Feeney and Punja 2003). PCR analysis of callus cultures confirmed stable gene integration with up to four T-DNA copies being integrated into the genome; however, plantlet regeneration was unsuccessful.

With the recent release of the chromosomal assembly and high-density linkage map of the *Cannabis* genome (Grassa *et al.* 2018; Laverty *et al.* 2019), identification of genes involved in cannabinoid biosynthesis is now relatively straightforward, with benchmark standards being developed for genetic engineering of these genes (Matchett-Oates *et al.* 2020). However, to date, no procedures for the evaluation of genes

✉ L. Matchett-Oates  
lennon.matchett-oates@agriculture.vic.gov.au

<sup>1</sup> Agriculture Victoria, the Centre for AgriBioscience, AgriBio, 5 Ring Road, Bundoora, Victoria 3083, Australia

<sup>2</sup> School of Applied Systems Biology, La Trobe University, Bundoora, Victoria 3086, Australia



through functional screening exist, due to the recalcitrant nature of *Cannabis in vitro* and the difficulty in obtaining transgenic explants (Feeney and Punja 2017). In addition, the illegal status of cannabis during the time in which biotechnology has emerged and has caused *Cannabis* to fall behind in genetic improvement studies. Genetic transformation is used for the study of gene function and genetic improvement in plants. Protocols for many non-model species, in which regeneration of transgenic plants has not been achieved, have been developed for screening genome editing constructs. To date, only brief protocol outlines for protoplast isolation have been reported (Jones 1979; Morimoto *et al.* 2007), with no protoplast transient expression protocols existing for *Cannabis*. Creating stable transformants is expensive and time consuming making this approach for large scale evaluation of cannabinoid biosynthesis genes limiting. Utilising protoplasts ability to transiently express DNA constructs has allowed for high-throughput transient screening of genes in *Arabidopsis* (Marion *et al.* 2008), gene-silencing in barley (Douchkov *et al.* 2005) and functional analysis of newly isolated genes in tobacco (Fischer and Hain 1995). Such studies are yet to be conducted on *Cannabis* making the need for an efficient transient expression system in protoplasts of high importance.

Protoplasts are osmotically fragile due to the lack of a cell wall from enzymatic digestion, allowing for the transfer of DNA constructs through the plasma membrane using common methods such as PEG-mediated transfection (Yoo *et al.* 2007), *Agrobacterium* infiltration (Clough and Bent 1998) and biolistic bombardment (Vain *et al.* 1993). Transient gene expression from plant protoplasts has widely been used to study cell death related processes (Chen *et al.* 2015), developmental studies (Sheen 2001), subcellular localisation of proteins (Su *et al.* 2010) and expression of foreign genes (Zhang *et al.* 2011). During isolation, optimum conditions such as osmotic balance, enzyme concentration and digestion time need to be established by optimising all variables contributing to yield and viability. The use of reporter genes in transient assays is often used due to the non-toxic nature, stability and ease of detection allowing for protein localisation and interactions studies (Leffel *et al.* 1997) making protoplasts an important tool in gene analysis.

The evaluation of transiently expressed shared terpene biosynthesis genes between *Cannabis*, and other species has been reported (Reed and Osbourn 2018; Smirnov 2019). However, *Cannabis* contains a unique set of enzymes in the cannabinoid biosynthesis pathway, which synthesise phytocannabinoids that are only produced in this species. The elucidation of the cannabinoid biosynthesis genes in the draft *Cannabis* genome provided the first complete look into the multiple pathways involved (Van Bakel *et al.* 2011). Identification and characterisation of the first unique enzyme in the cannabinoid pathway, OLS, have evolved from identification of an unknown polyketide synthase (Raharjo *et al.* 2004). Initially

characterised as a novel polyketide synthase-olivetol synthase (Taura *et al.* 2009), later, the mechanism for olivetol production requiring olivetol acid cyclase, a DABB protein (Gagne *et al.* 2012), was identified and correctly characterised. Similarly, identification and functional studies on the oxidocyclases, tetrahydrocannabinolic acid synthase (THCAS) and cannabidiolic acid synthase (CBDAS) have evolved as technological advancements into molecular cloning techniques, and DNA sequencing has improved. Tetrahydrocannabinol (THC), one of the major constituents in *Cannabis*, was originally presumed to be formed by the isomerisation of cannabidiolic acid (CBDA) (Shoyama *et al.* 1975). Protein sequencing and PCR cloning of tetrahydrocannabinolic acid (THCA) confirmed that THC was produced from cannabigerolic acid (CBGA) by THCAS (Sirikantaramas *et al.* 2004). Identification of CBDAS (Taura *et al.* 1996) occurred shortly after with a similar strategy used for gene identification (Taura *et al.* 2007).

Genetic engineering of *Cannabis* offers the opportunity to produce higher levels of cannabinoids with tailor-made chemical profiles for medicinal applications. However, the recalcitrant nature of *Cannabis* and the absence of methods to efficiently generate transgenic plants currently makes transient expression systems important to understand the molecular regulatory mechanisms responsible for cannabinoid biosynthesis, which remain largely unknown. Prior to this report, a transient expression system in *Cannabis* protoplasts did not exist, making the characterisation of unique gene function speculative. In this present study, an efficient protoplast isolation and transient expression system using *Cannabis* leaf mesophyll protoplasts are reported. Optimisation to obtain a high yield of viable protoplasts and PEG-mediated transfection of the protoplasts using fluorescent reporter genes is also reported. Furthermore, due to the outbreeding highly heterozygous nature of *Cannabis*, strain-dependent response variation is expected; this protoplast isolation and transient expression systems is shown to be suitable to many strains, highlighting the applicability for future protoplast and transient expression studies.

## Materials and Methods

**Plant Material and Expression Vectors** All research was performed under Medicinal Cannabis Research Licence (RL011/18) and Permit (RL01118P4) issued through the Department of Health (DoH), Office of Drug Control (ODC) Australia. A specific genotype (*C. sativa*) with high THC content (25%) was used for optimisation within this study. Aseptic plantlets were derived from apical meristems of mature vegetative mother plants, which were sterilised by a 1-min 80% ethanol wash, washed three times with sterile water, disinfected with 10% (v/v) sodium hypochlorite (White King, Melbourne,

Australia) solution containing 4.5% active chlorine for 15 min and finally washed three times with sterile water. Apical meristems were cultured in root induction media containing  $\frac{1}{2}$  Murashige and Skoog salts and vitamins (MS; Murashige and Skoog 1962) (Duchefa Biochemie, Haarlem, The Netherlands), 1% sucrose (w/v) (Sigma-Aldrich, St. Louis, MO), 1% agar (w/v) (Duchefa Biochemie) and 1 mg L<sup>-1</sup> Indole-3-butyric acid (IBA) (Sigma-Aldrich) adjusted to pH 5.7 prior to autoclaving at 121°C for 15 min. The apical meristems were cultured at 26°C under lighting of 74  $\mu\text{mol m}^{-2} \text{s}^{-1}$  supplied by fluorescent lamps for 18 h per day. The green fluorescent protein (GFP) expression vector pDONR221-GFP was constructed from pDONR221 (Invitrogen, Carlsbad, CA) through BP Clonase reaction by the insertion of the attB1-CaMV35S-D-p\_turboGFP(D)\_AtuNos-t-attB2 cassette (Supplementary Information).

### Protoplast Isolation, Purification and Quantification

Protoplasts were isolated from well rooted, 1- to 2 mo-old plantlets with young leaves cut into 0.5- to 1.0-mm-thin strips (Fig. 1a–d) and incubated in a Petri dish containing digestion media comprising of 1, 2 and 2.5% (w/v) Cellulase Onozuka R-10 (Yakult Honsha Co., Ltd., Tokyo, Japan); 0.3, 0.4 and 0.5% (w/v) Macerozyme R-10 (Yakult Honsha Co.); 0.3, 0.5 and 0.7 M mannitol (Sigma-Aldrich, St Louis, MO); 20 mM MES (Sigma-Aldrich, St Louis, MO); 20 mM KCl (Sigma-Aldrich, St Louis, MO); and 10 mM CaCl<sub>2</sub> (Sigma-Aldrich), pH adjusted to 5.8 and filter-sterilised using a 0.22- $\mu\text{m}$  filter (Sigma-Aldrich). Leaf strips were incubated in the dark at 28°C without agitation for between 8 and 24 h with each digestion replicated three times. Following digestion, the cellular suspension was mechanically filtrated through a 70- $\mu\text{m}$  mesh (Corning, NY) into a sterile 50-mL polypropylene centrifuge tube (Corning, NY) and centrifuged at 700×g for 10 min. Following centrifugation, the resulting supernatant was discarded, and the pellet resuspended with 3 mL of W5 (5 mM glucose, 5 mM KCl, 10 mM MES, 125 mM CaCl<sub>2</sub>, 154 mM NaCl, pH 5.8), transferred to a 15-mL round bottomed tube and 3 mL 20% (w/v) sucrose added and centrifuged again. Protoplasts were collected from the interphase (Fig. 1e) into a fresh 15-mL round bottom tube and 3 mL W5 added and centrifuged again with the supernatant removed. Finally, the pellet was resuspended in 1 mL W5, and 100  $\mu\text{L}$  of the resuspended protoplasts were diluted with 0.5 M Evans Blue, and approximately 50 to 200 protoplasts were counted using a haemocytometer under a light microscope. The viability of the protoplasts was calculated by (viable protoplasts/total number of protoplasts) × 100%.

**PEG-Mediated Transfection** Isolated protoplasts were divided into aliquots of  $5 \times 10^5$  or  $1 \times 10^6$  and centrifuged at 700×g for 10 min with the supernatant removed. PEG4000 solutions

(20%, 30%, 40% and 50% (w/v)) (Sigma-Aldrich) were prepared by dissolving in ddH<sub>2</sub>O containing 0.1 M Ca(NO<sub>3</sub>)<sub>2</sub> 4H<sub>2</sub>O and 0.4 M Mannitol (Sigma-Aldrich). A volume of 100  $\mu\text{L}$  transformation buffer (15 mM MgCl<sub>2</sub>, 0.5 M Mannitol, 0.1% (w/v) MES, pH 5.7) was added to the protoplasts and gently mixed. Plasmid concentrations (5, 10 and 30  $\mu\text{g}$ ) in 60  $\mu\text{L}$  of ddH<sub>2</sub>O were added to the protoplast solution and gently mixed followed by addition of 150  $\mu\text{L}$  of warmed (42°C) PEG4000 solutions. The protoplast solution was mixed gently and incubated in the dark at room temperature (22 ± 1°C) for between 10 and 60 min. The transfection reaction was stopped by the addition of 5 mL W5 dropwise, followed by a further 5 mL in a gentle stream. The protoplast mixture was centrifuged once more at 700×g for 10 min, removing the supernatant and the addition of 150  $\mu\text{L}$  W5 followed by incubation in the dark at room temperature for 48 h. The expression of GFP was observed under a fluorescence microscope (OLYMPUS CKX53, Tokyo, Japan) (excitation emission wavelengths 470 to 490 nm, 510 nm).

**FACS Analysis** The protoplasts were analysed using Influx™ FACS instrument (BD Biosciences, Franklin Lakes, NJ) fitted with a 200- $\mu\text{m}$  nozzle using W5 buffer as a sheath fluid. The sheath pressure was set at 4 psi, and the sample pressure was set at 5 psi. A 466-nm Coherent Sapphire Solid state laser was used for excitation, and emission was measured using a 517/18 nm band-pass filter for GFP. The photomultiplier tube voltage was set at 16.41 V for forward scatter, 20.59 V for side scatter and 40.37 V for GFP. The threshold value for event detection was set at 0.3 on side scattering. For analysis, a gate was set using PEG-transfected control protoplasts. Frequency of GFP expressing cells was recorded, and data was processed using BD FACSTM Software v1.0.0.650 (BD Biosciences).

**Statistical Analysis** Experimental data was statistically analysed using Minitab 19 Statistical Software (Version 19, State College, PA) and R Studio (Version 1.1.453, RStudio, Inc., Boston, MA).

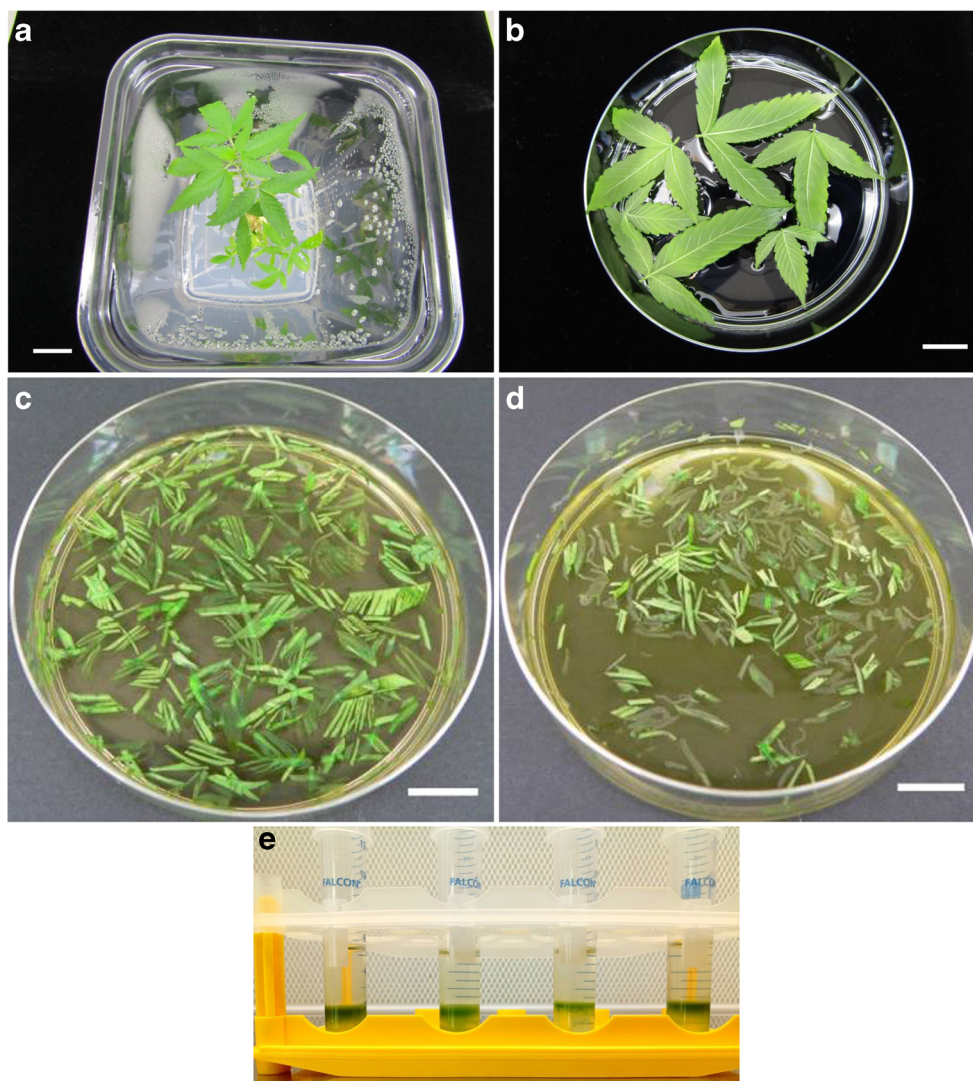
## Results

### Mesophyll Protoplast Isolation From *Cannabis* Leaf Tissue

Initially, protoplasts were generated from rapidly expanding leaves from 1- to 2-mo-old *in vitro* plantlets that were used as the source material (Fig. 1a, b). Briefly, plantlets cultured at 26°C in  $\frac{1}{2}$  MS medium with 5  $\mu\text{M}$  IBA were used for mesophyll protoplast isolation. One gram of young leaf material was cut into 0.5- to 1-mm strips and immediately transferred into digestion medium in the dark without agitation at 28°C for 8 to 24 h (Fig. 1a–d). Protoplasts were collected through a



**Figure 1.** (a) *In vitro* 1- to 2-month rooted explants grown on Murashige and Skoog medium containing  $1 \text{ mg L}^{-1}$  indole-3-butyric acid suitable for protoplast isolation. Scale bar = 2 cm. (b) Healthy *Cannabis sativa* L. leaves suitable for protoplast isolation. Scale bar = 2 cm. (c) *Cannabis sativa* L. leaves sliced into 0.5- to 1-mm-thin strips with a fresh razor blade and placed in media. Scale bar = 2 cm. (d) *Cannabis sativa* L. 16 h post digestion in the dark at  $28^{\circ}\text{C}$  without shaking. Scale bar = 2 cm. (e) Purified *Cannabis sativa* L. protoplast interphase after density gradient centrifugation.



70- $\mu\text{m}$  nylon mesh, centrifuged and washed as previously described.

**Single Factor Effect on Mesophyll Protoplast Isolation** To optimise mesophyll protoplast isolation, enzyme and mannitol concentrations and enzymatic digestion time were adjusted. Mesophyll protoplasts were isolated from young, expanding leaves with variable digestion parameters, outlined in Table 1. Total protoplast yields ranged from  $1.2 \times 10^6$  to  $9.2 \times 10^6$  per gram of fresh leaf weight with the highest yield obtained from treatment 7. Protoplast viability ranged from 39 to 79%, with the highest viability obtained from treatment 9. An L9 ( $3^4$ ) orthogonal test (Table 1) was designed to identify statistically significant variables to optimise protoplast isolation, with a range analysis performed to predict the optimal combination of the variables. The range analysis of the means between the factors and levels (Table 2), calculated Delta scores (mean score of the highest range minus the lowest mean range) for enzymolysis time (R 2.21) and Cellulase R-10 (R 1.56)

significantly influencing protoplast isolation, with Macerozyme R-10 (R 1.44) and mannitol (R 1.23) proving less significant. Analysis of each level calculated response (k1–3) from the range analysis (Table 2), the Duncans multiple range test (Duncans) assigned the best possible combination of each factor as A3:B1:C3:D2. The calculated theoretical best combination was already performed in treatment 7, with an average yield of  $5.7 \times 10^6$  viable protoplasts.

Increasing Cellulase-R10 concentration significantly increased protoplast yield within multilevel factors (Fig. 3), from  $1.31$  (1%) to  $2.86$  (2.5%)  $\times 10^6$  viable protoplasts. This trend, of increasing concentrations greatly increasing yields, was not observed for the other variables, where applicable. Macerozyme R-10 effected protoplast yields significantly at 0.3% producing  $2.93 \times 10^6$  viable protoplasts, whereas at 0.4% and 0.5%, this decreased to  $1.50 \times 10^6$  and  $2.14 \times 10^6$  viable protoplasts, respectively. A concentration of 0.7 M mannitol was more substantial in producing  $2.89 \times 10^6$  viable protoplasts, whereas 0.3 M produced  $2.01 \times 10^6$ , which

**Table 1.** Results from the orthogonal L9 (3<sup>4</sup>) array for *Cannabis sativa* L. protoplast isolation optimization

Treatment combination	Factors				Total yield	Yield of viable	Viability
	A	B	C	D	× 10 <sup>6</sup> (g FW)	× 10 <sup>6</sup> (g FW)	(%)
T1	1 (1.0%)	1 (0.3%)	1 (0.3 M)	1 (8 h)	1.8 ± 0.5	1.3 ± 0.3	71 ± 3.5
T2	1 (1.0%)	2 (0.4%)	2 (0.5 M)	2 (16 h)	2.5 ± 0.5	1.5 ± 0.2	63 ± 10.4
T3	1 (1.0%)	3 (0.5%)	3 (0.7 M)	3 (24 h)	2.0 ± 0.1	1.2 ± 0.1	59 ± 3.8
T4	2 (2.0%)	1 (0.3%)	2 (0.5 M)	3 (24 h)	3.8 ± 0.3	1.8 ± 0.1	47 ± 1.5
T5	2 (2.0%)	2 (0.4%)	3 (0.7 M)	1 (8 h)	2.6 ± 0.1	1.8 ± 0.2	68 ± 6.4
T6	2 (2.0%)	3 (0.5%)	1 (0.3 M)	2 (16 h)	5.3 ± 0.3	3.6 ± 0.2	68 ± 4.0
T7	3 (2.5%)	1 (0.3%)	3 (0.7 M)	2 (16 h)	7.8 ± 1.0	5.7 ± 1.0	72 ± 5.9
T8	3 (2.5%)	2 (0.4%)	1 (0.3 M)	3 (24 h)	3.1 ± 0.2	1.2 ± 0.01	39 ± 1.8
T9	3 (2.50%)	3 (0.5%)	2 (0.5 M)	1 (8 h)	2.2 ± 0.2	1.7 ± 0.1	79 ± 2.7

(A) Cellulase R-10 concentration (w/v), (B) Macerozyme R-10 concentration (w/v), (C) Mannitol concentration, (D) enzymolysis time, T1-9 different treatment combinations, 1, 2, 3 three factor levels

performed greater than 0.5 M, which produced  $1.67 \times 10^6$  viable protoplasts (Table 3). Enzymolysis time of 16 h produced a two-fold increase in viable protoplasts, with  $3.61 \times 10^6$  compared to a shorter digestion time of 8 h ( $1.58 \times 10^6$ ) or a longer digestion time of 24 h ( $1.39 \times 10^6$ ).

**Transient Transfection of *Cannabis* Mesophyll Protoplasts** To establish the first transient expression system, pDONR221-GFP was used to study the effects of plasmid, PEG and protoplast concentration, including incubation time on transfection efficiency. The transfection efficiency reached 23.2% in treatment 12 (30 µg plasmid, 50% PEG (w/v), 20 min of incubation and  $1 \times 10^6$  protoplasts), with the lowest recorded transfection rate of 5.78% in treatment 5 (10 µg plasmid, 20% PEG (w/v), 20 min incubation time and  $1 \times 10^6$  protoplasts).

**Single Factor Effect on Mesophyll Protoplast Transfection** Factors with multiple levels across variables (Table 3) were designed to optimise plasmid concentration, PEG

concentration (w/v), transfection time and protoplast concentration variables in transfection efficiency. Mesophyll protoplasts were transfected using the PEG-mediated transfection protocol described previously (Fig. 2) with transfection efficiency ranging from 5.78 to 23.20% with the highest average transfection rate, 23.20%, obtained from treatment 12. Within multilevel factors, increasing plasmid concentration greatly increased protoplast transfection within efficiencies between 10 and 30 µg (11.11 to 17.29%). Increasing PEG concentration from 20 to 50% saw increases in transfection efficiency, with 50% achieving 16.23% on average, compared to 20% PEG (w/v), which saw 10.93% transfection efficiency. Incubation time of 30 min gave the highest average transient efficiency of 15.82%. Similarly, a 20-min incubation resulted in 16.61% transfection efficiency. Incubation of 10 and 60 min saw a reduction of transfection efficiency, 12.02% and 13.25%, respectively. Protoplast density of  $5 \times 10^5$  achieved higher transient efficiencies on average compared to  $1 \times 10^6$ , with 14.63% and 11.72% efficiency, respectively. From the range analysis of the means (Table 4), the calculated Delta values of plasmid concentration (R 6.18%) and PEG concentration (R 5.3%) greatly influence transfection efficiency, with transfection time (R 3.8%) and protoplasts density (R 2.91%) proving less significant.

## Discussion

Protoplasts offer a versatile experimental system, with transient expression systems widely applied in *Arabidopsis* (Yoo *et al.* 2007), rice (Wang *et al.* 1988) and tobacco (Töpfer *et al.* 1988) to analyse gene expression and function and to deliver genetic improvements in plants. Crucial for the development of an efficient transient expression system for genome editing constructs is the routine generation of high-quality protoplasts

**Table 2.** Range analysis of L9 *Cannabis sativa* L. protoplast isolation orthogonal array

	A	B	C	D
k1	1.31	2.93	2.01	1.58
k2	2.40	1.50	1.67	3.61
k3	2.86	2.14	2.89	1.39
Range	1.56	1.44	1.23	2.22
Optimum combination	A3	B1	C3	D2

k1, k2, and k3 indicate mean viable protoplast  $\times 10^6$  (g FW) at the 1, 2, and 3 levels. The larger the range value, the greater the influence of the factors on test results. All values expressed as  $\times 10^6$

(A) cellulase R-10 concentration (w/v), (B) Macerozyme R-10 concentration (w/v), (C) Mannitol concentration, (D) enzymolysis time





**Table 3.** Results from the factorial array for *Cannabis sativa* L. protoplast transfection efficiency optimization

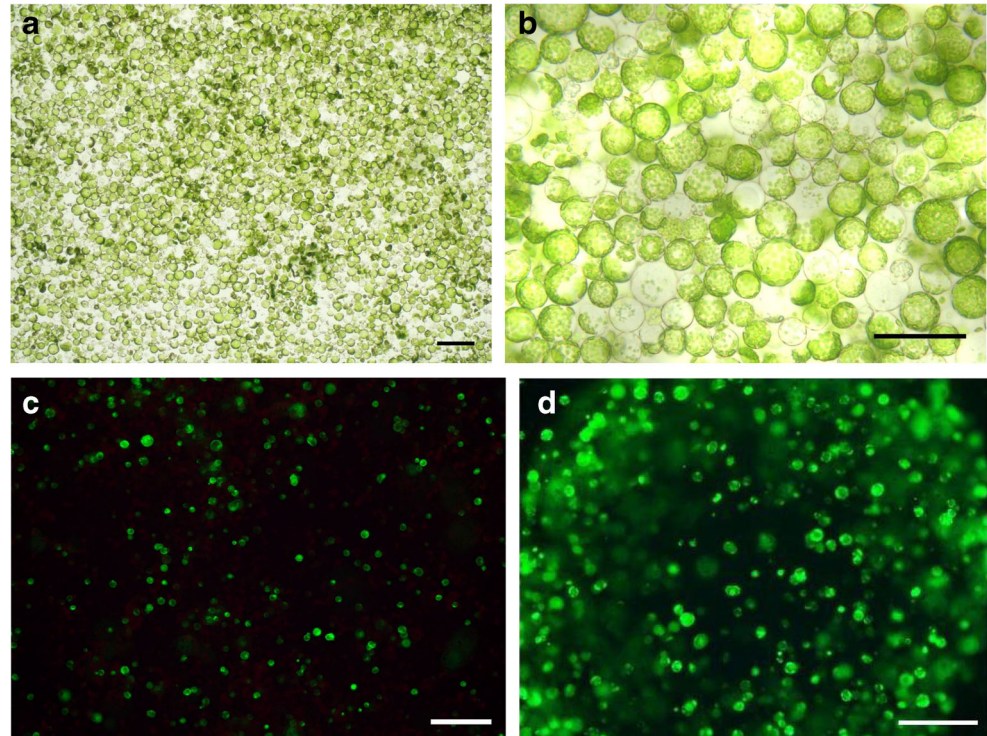
Treatment combination	Factors				Transfection efficiency (%)
	A	B	C	D	
T1	1 (5 µg)	1 (20%)	1 (10 m)	1 (0.5)	9.78 ± 2.59
T2	1 (5 µg)	2 (30%)	2 (20 m)	1 (0.5)	14.35 ± 2.48
T3	1 (5 µg)	3 (40%)	3 (30 m)	2 (1)	10.90 ± 0.35
T4	1 (5 µg)	4 (50%)	4 (60 m)	2 (1)	9.41 ± 0.48
T5	2 (10 µg)	1 (20%)	2 (20 m)	2 (1)	5.78 ± 1.22
T6	2 (10 µg)	2 (30%)	1 (10 m)	2 (1)	6.20 ± 0.47
T7	2 (10 µg)	3 (40%)	4 (60 m)	1 (0.5)	16.44 ± 2.09
T8	2 (10 µg)	4 (50%)	3 (30 m)	1 (0.5)	16.08 ± 1.35
T9	3 (30 µg)	1 (20%)	3 (30 m)	1 (0.5)	17.24 ± 1.99
T10	3 (30 µg)	2 (30%)	4 (60 m)	1 (0.5)	13.88 ± 0.33
T11	3 (30 µg)	3 (40%)	1 (10 m)	2 (1)	14.83 ± 0.66
T12	3 (30 µg)	4 (50%)	2 (20 m)	2 (1)	23.20 ± 0.61

(A) plasmid concentration, (B) PEG concentration (w/v), (C) experiment time, (D) protoplast density ( $\times 10^6$ ), T1–T12 different treatment combinations, 1, 2, 3, 4 four factor levels

and robust transfection protocols. Only two reports of isolated *Cannabis* protoplasts exist; however, the reports briefly discuss the methods for protoplast isolation with no data on protoplast yield and viability, or the data collection was not described in detail. Morimoto *et al.* (2007) digested *Cannabis* leaves with 1% Cellulase R-10, 0.2% Macerozyme R-10, 0.1% pectolyase Y-23 and 0.4 M mannitol at 30°C for 4 h with gentle agitation; however, no yield or viability data was given. Jones (1979) explored a range of enzyme combinations

to produce protoplast from young and old leaf tissue, as well as callus, with concentrations between  $1 \times 10^3$  and  $1 \times 10^5$  reported. Protoplast isolation from the closely related species *Humulus lupulus* (hops) has previously been reported from cell suspension cultures. Several cell wall digesting enzyme mixtures were trialled with varying Cellulysin and Driselase concentrations in 0.4 M mannitol osmoticum, with the highest yield of protoplasts ( $9.3$  to  $9.9 \times 10^6$  per gFW) obtained from 2% Cellulysin and 1% Driselase with 99 to 100% viability

**Figure 2.** (a) Visualisation of *Cannabis sativa* L. protoplasts under brightfield microscopy. Scale bar = 200 µm. (b) Visualisation of *Cannabis sativa* L. protoplasts under fluorescence microscopy with GFP filter set. Scale bar = 50 µm. (c) Isolated protoplasts under fluorescence microscopy with GFP filter set. Scale bar = 200 µm. (d) Isolated *Cannabis sativa* L. protoplasts under fluorescence microscopy with GFP filter set. Scale bar = 100 µm.



**Table 4.** Range analysis of factorial design for *Cannabis sativa* L. protoplast transfection efficiency

	A	B	C	D
k1	11.11	10.93	12.02	14.63
k2	11.13	11.48	15.61	11.72
k3	17.29	14.06	15.82	
k4		16.23	13.25	
Range	6.18	5.30	3.80	2.91

k1, k2, k3, and k4 indicate mean transfection efficiency (%) at the 1, 2, 3, and 4 levels. All values expressed as percentages

(A) plasmid concentration, (B) PEG concentration (w/v), (C) experiment time, (D) protoplast density ( $\times 10^6$ )

reported (Furze *et al.* 1987). Heale *et al.* (Heale *et al.* 1989) reported leaf mesophyll isolation from the hop cultivar, Challenger, by firstly removing the epidermis followed by incubating in 4% Cellulase, 0.3% Macerozyme and 2% hemicellulose for 7 h on an orbital shaker protoplast yields between 4 and  $7 \times 10^5 \text{ mL}^{-1}$  were achieved with viability ranging from 80 to 90% under optimal conditions. An efficient protocol for transient expression of LUC activity in rose (another member of the Order Rosales with *Cannabis*) leaves using *Agrobacterium* has also been reported (Lu *et al.* 2017). Recently, transient expression of  $\beta$ -glucuronidase (GUS) and GFP in a range of agroinfiltrated organs and tissues has been reported in *Cannabis*, with the protocol being optimised for hemp cultivars (Deguchi *et al.* 2020).

The development of an efficient protoplast transient expression system to screen genome editing constructs requires established protocols for the isolation of viable protoplasts and a competent transfection workflow, which until now have not been reported for *Cannabis*. With several factors affecting yield and viability of protoplasts and transfection efficiencies, this development of a routine system allows for significant advances to be made towards understanding and improving *Cannabis* cultivars. The method described here investigates the effect each variable has on protoplast isolation and transfection, with each being analysed for their significance.

To optimise protoplast isolation, Cellulase-R10, Macerozyme-R10, mannitol concentrations and digestion times were adjusted (Table 1). Under the optimum conditions, viable *Cannabis* leaf mesophyll protoplasts reached  $5.7 \times 10^6$  protoplasts g FW<sup>-1</sup> (Table 1). The increase in Cellulase concentration to 2.5% (w/v) showed significantly increased yields,  $2.88 \times 10^6$ , from the range analysis. Although 2.5% (w/v) Cellulase concentration was the highest tested, increasing levels past a saturation point has been shown to decrease protoplast yields in tobacco (Kuriakose *et al.* 2012) and *Magnolia* (Shen *et al.* 2017). The optimal Macerozyme concentration was determined to be 0.3% (w/v), whilst an increase to 0.4% saw more than a 50% drop in protoplast recovery to

$1.50 \times 10^6$  calculated from the range analysis. For both enzymes, the increase in concentration at which viable protoplast yield decreases is presumably due to the influence those enzymes have on the membrane integrity. The application of the optimised protoplast isolation protocol to verify robustness on Cannbio-2, the alternative high THC strain and a high CBD strain produced a significant yield of viable protoplasts for subsequent transient expression experiments, demonstrating the robustness and versatility of the protocol described. The protocol produced similar protoplast viability, with Cannbio-2 averaging 79%, equalling to the best performing treatment 9, and the high CBD strain producing higher levels of viability with 82% (Table 5). Although viable protoplast yield from these cultivars is proportionately fewer, the protocol has been optimised for a specific high yielding THC strain, with the calculated optimal conditions proven to be an advisory starting point for further optimisation on any chosen cultivar (Table 5).

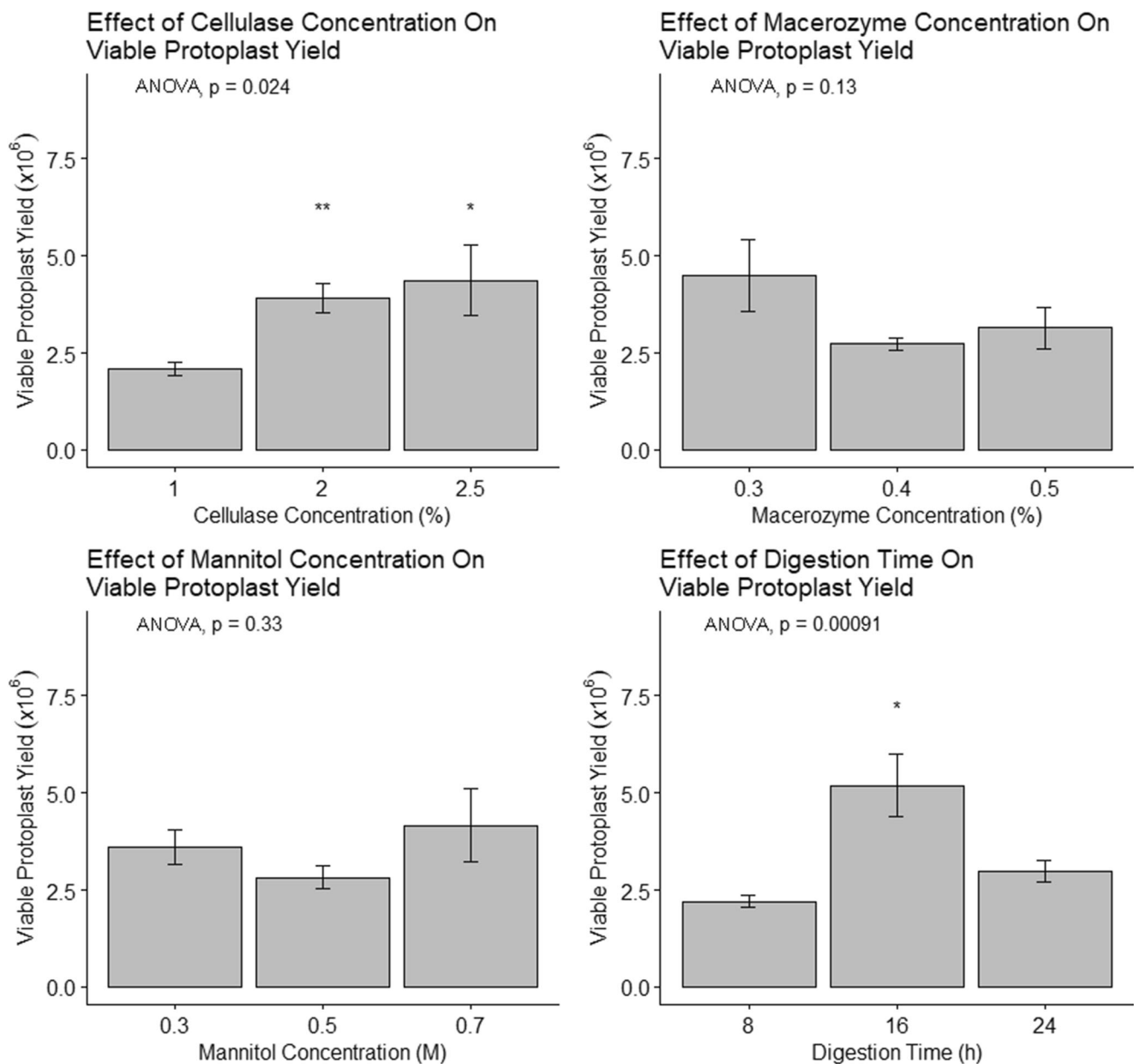
Protoplasts lack of cell walls that require a stabilized environment, which is controlled by the osmotic gradient for proper osmolarity to sustain viable protoplasts (Sain *et al.* 2017). Mannitol is frequently used for it is inert metabolically and slowly diffuses through the cellular membrane (Chawla 2011). Mannitol concentration of 0.7 M resulted in the highest yield, calculated by the range analysis, of  $2.91 \times 10^6$ , with lower concentrations of 0.3 M and 0.5 M yielding  $2.02 \times 10^6$  and  $1.67 \times 10^6$ , respectively. Similar to enzyme concentration, increasing mannitol concentration, thus causing an imbalance in the osmoticum, decreases protoplast yield, as is seen with wheat (Jia *et al.* 2016) and pineapple (Priyadarshani *et al.* 2018). Enzymolysis time significantly affected viable protoplast yield, with digestions of 24-h yielding as low as 39% viability in treatment 8, which most certainly caused by over digestion of the cell walls. Comparatively, 8 and 16 h were determined to yield significantly higher levels of viability, with 16 h shown to result in a larger concentration of viable protoplasts (Fig. 3) and the best digestion time for the release of viable protoplasts without over digestion.

The uptake of DNA through the plasma membrane of protoplasts for transient expression studies requires considerable concentrations of PEG acting in tandem with divalent cations (Maas and Werr 1989). With increasing concentrations of PEG, DNA hydration reduces causing structural changes

**Table 5.** Results of the mean *Cannabis sativa* L. protoplast isolation on multiple cultivars using optimised variable conditions

Cultivar	Yield of viable protoplasts ( $\times 10^6$ )	Viability (%)
Cannbio-2	1.97	79
High THC	1.82	72
High CBD	2.46	82



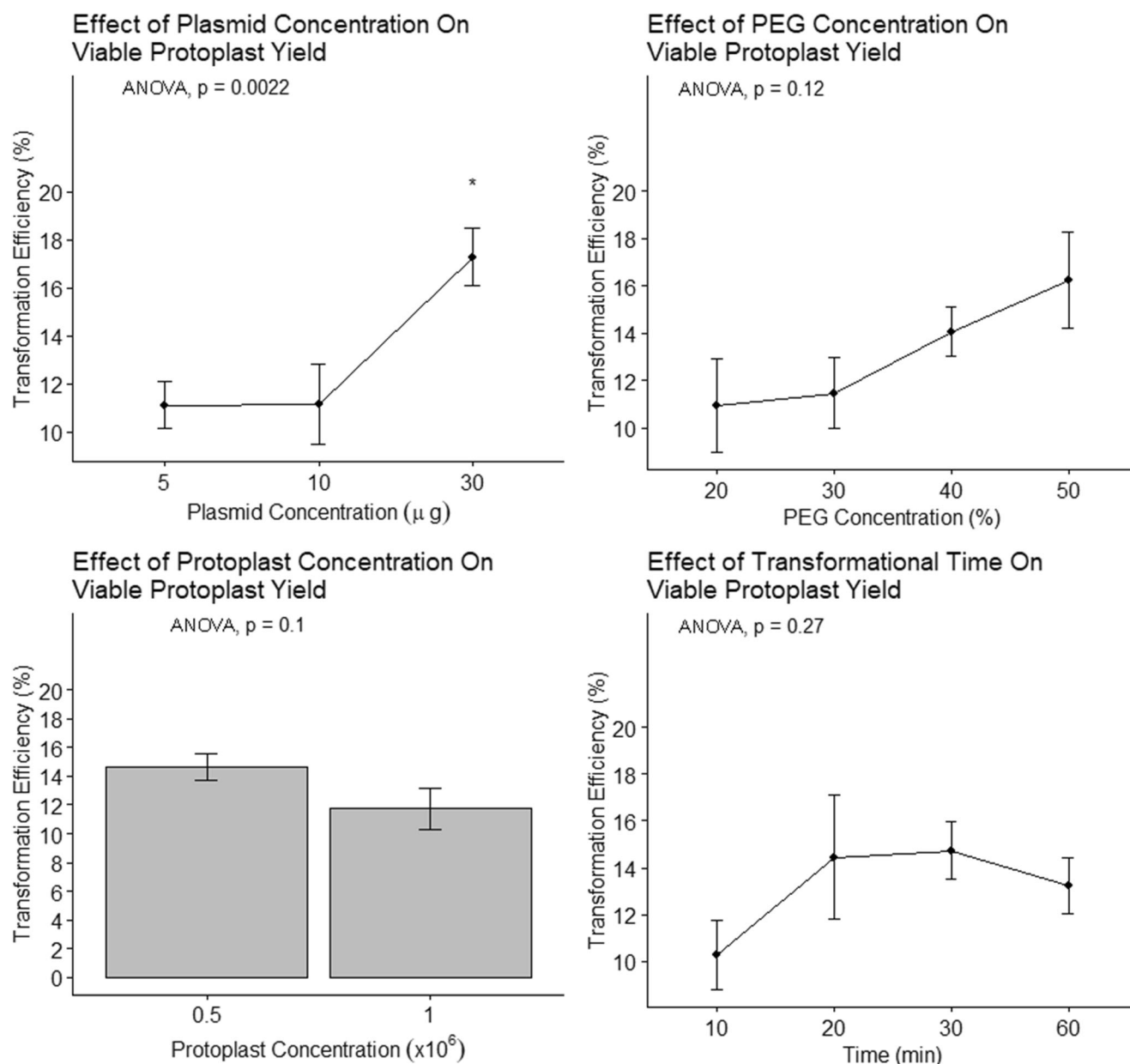


**Figure 3.** Effects of different factors on viable *Cannabis sativa* L. protoplast isolation yield. Bars represent standard errors (SE). Statistical significance was determined using a one-way ANOVA test (\* $p < 0.05$ , \*\* $p < 0.01$ ).

and thus reducing the transfection efficiency (Saenger *et al.* 1986). In *Cannabis* protoplasts, transient expression efficiency at the highest investigated PEG concentration (50%) achieved 16.23% (Fig. 4), which is a significantly higher concentration of PEG than has been previously reported in rice (Page *et al.* 2019) or pea (Nicolaisen and Poulsen 1993). Results show that increasing plasmid concentration from 5 to 10  $\mu\text{g}$  achieved similar transfection efficiencies, 11.11% and 11.13%, respectively (Table 3). These results are inconsistent with findings in pepper (Jeon *et al.* 2007), pineapple (Priyadarshani *et al.* 2018) and *Phaseolus* (Nanjareddy *et al.* 2016) in which there is an approximate doubling in relevant transient expression efficiencies between these two

concentrations. This suggests that *Cannabis* protoplasts require higher concentrations of plasmid to achieve increased levels of expression. Intermediate exposure time to high concentrations on PEG was shown to increase transfection efficiencies (Fig. 4), with the optimal exposure time determined to be 30 min, achieving 15.82%. Increasing the incubation time to 60 min saw a sharp decrease in transfection efficiency, falling to 12.02%. The increased exposure to the high concentrations of PEG resulting in lower transfection efficiencies is expected due to DNA becoming less hydrated, with similar results found in carrot, rapeseed and soybean (Rasmussen and Rasmussen 1993). This protocol has been optimised for this particular cultivar (Fig. 4). This protocol provides a relevant





**Figure 4.** Effects of different factors on *Cannabis sativa* L. protoplast transfection efficiency. Bars represent standard errors (SE). Statistical significance was determined using a one-way ANOVA test (\* $p < 0.05$ ).

starting point for optimisation regardless of genetics. This level of efficiency in transfection allows for cellular studies, including genome editing using CRISPR and ZFNs.

## Conclusion

The method described here is the first reported for the transient expression of heterologous genes in *Cannabis* protoplasts. The variables involved in protoplast isolation were verified on three cultivars with varying cannabinoid content for

protocol robustness. The transfection protocol was optimised for a high THC yielding strain within mesophyll protoplasts. This method can be easily adapted for transient expression studies using CRISPR/Cas-9, protein-protein interaction or other investigations in *Cannabis* where a transient gene expression system is desired.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s11627-021-10178-0>.

**Acknowledgements** Authors acknowledge Canopy Growth Corporation for access to plant material to evaluate the protocols, where applicable.

The authors also wish to acknowledge Navdeep Kaur for FACS operation and optimisation.

**Author contribution** LMO and EM in protocol development and optimisation. LMO assembled the manuscript.

**Funding** Funding was provided by Agriculture Victoria Services and Agriculture Victoria Research.

## Declarations

**Conflict of interest** The authors declare no competing interests.

**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

## References

- Braich S, Ballie R, Jewell L, Spangenberg G, Cogan N (2019) Generation of a comprehensive transcriptome atlas and transcriptome dynamics in medicinal cannabis. *Sci Rep* 9:1–12
- Chawla HS (2011) Introduction to plant biotechnology, vol 3. Science Publishers, Enfield New Hampshire
- Chen J, Yi Q, Song Q, Gu Y, Zhang J, Hu Y, Liu H, Liu Y, Yu G, Hang Y (2015) A highly efficient maize nucellus protoplast system for transient gene expression and studying programmed cell death-related processes. *Plant Cell Rep* 34:1239–1251
- Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16:735–743
- Deguchi M, Bogush D, Weeden H, Spuhler Z, Potlakayala S, Kondo T, Zhang Z, Rudrabhatla S (2020) Establishment and optimization of a hemp (*Cannabis sativa* L.) agroinfiltration system for gene expression and silencing studies. *Sci Rep* 10:1–11
- Douchkov D, Nowara D, Zierold U, Schweizer P (2005) A high-throughput gene-silencing system for the functional assessment of defense-related genes in barley epidermal cells. *Mol Plant Microbe Interact* 18:755–761
- Feeney M, Punja ZK (2003) Tissue culture and *Agrobacterium*-mediated transformation of hemp (*Cannabis sativa* L.). *In Vitro Cell Dev Biol - Plant* 39:578–585
- Feeney M, Punja ZK (2017) The role of *Agrobacterium*-mediated and other gene-transfer technologies in Cannabis research and product development, Chandra, S., Lata, H., ElSohly, M. (eds), *Cannabis sativa* L.-Botany and Biotechnology, vol 1, Springer, Cham. pp. 343–363
- Fischer R, Hain R (1995) Tobacco protoplast transformation and use for functional analysis of newly isolated genes and gene constructs. In: Galbraith DW, Bourque DP, Bohnert HJ (eds) *Methods in Cell Biology*, vol 50. Elsevier, pp 401–410
- Furze JM, Rhodes MJC, Robins RJ (1987) The use of agarose bead culture for the regeneration of single cell-derived colonies from protoplasts isolated from suspension cultures of *Humulus lupulus*. *Plant Cell Tiss Org Cult* 8:17–25
- Gagne SJ, Stout J, Liu E, Boubakir Z, Clark S, Page J (2012) Identification of olivetolic acid cyclase from *Cannabis sativa* reveals a unique catalytic route to plant polyketides. *Proc Natl Acad Sci* 109:12811–12816
- Grassa JB, Wenger JP, Dabney C, Poplawski SG, Motley ST, Michael TP, Schwartz CJ, Weiblen GD (2018) A complete Cannabis chromosome assembly and adaptive admixture for elevated cannabidiol (CBD) content. *bioRxiv*:458083. <https://doi.org/10.1101/458083>
- Gurunani SG, Gholse YN, Yeole MP, Chaple DR (2015) Hairy root culture: an experimental system for secondary metabolite production. *Res J Pharm Tech* 8:728–730
- Heale JB, Legg T, Connell S (1989) *Humulus lupulus* L.(Hop): *In vitro* culture; Attempted production of bittering components and novel disease resistance. In: Bajaj YPS (ed) *Medicinal and Aromatic Plants II, Biotechnology in Agriculture and Forestry*, vol 7. Springer, Berlin, Heidelberg, pp 264–285
- Jeon JM, Ahn NY, Son BH, Kim CY, Han C, Kim G, Gal SW, Lee S (2007) Efficient transient expression and transformation of PEG-mediated gene uptake into mesophyll protoplasts of pepper (*Capsicum annuum* L.). *Plant Cell Tiss Org Cult* 88:225–232
- Jia X, Zhang X, Qu J, Han R (2016) Optimization conditions of wheat mesophyll protoplast isolation. *Agri Sci* 7:850–858
- Jones RL (1979) Cell culture, protoplast isolation, and cell fusion of *Cannabis Sativa* L.: evaluation of chilling preventative chemicals and quality control of bananas in the tropics, University of Houston Central Campus, M. Sc. Thesis, DOI: 10.13140/RG.2.2.19104.43527
- Kuriakose B, Du Toit ES, Jordaan A (2012) Transient gene expression assays in rose tissues using a Bio-Rad Helios hand-held gene gun. *S Afr J Bot* 78:307–311
- Lavery KU, Stout J, Sullivan MJ, Shah H, Gill N, Holbrook L, Deikus G, Sebra R, Hughes TR, Page JE, van Bakel H (2019) A physical and genetic map of *Cannabis sativa* identifies extensive rearrangements at the THC/CBD acid synthase loci. *Genome Res* 29:146–156
- Leffell SM, Mabon SA, Stewart CN Jr (1997) Applications of green fluorescent protein in plants. *Biotechniques* 23:912–918
- Lu J, Bai M, Ren H, Liu J, Wang C (2017) An efficient transient expression system for gene function analysis in rose. *Plant Method* 13:116
- Maas C, Werr W (1989) Mechanism and optimized conditions for PEG mediated DNA transfection into plant protoplasts. *Plant Cell Rep* 8:148–151
- Marion J, Bach L, Bellec Y, Meyer C, Gissot L, Faure J (2008) Systematic analysis of protein subcellular localization and interaction using high-throughput transient transformation of *Arabidopsis* seedlings. *Plant J* 56:169–179
- Matchett-Oates L, Braich S, Spangenberg G, Rochfort S, Cogan N (2020) *In silico* enabling informed design for genome editing in medicinal cannabis, gene families and variant characterisation. *Plant Methods*. IN REVIEW
- Morimoto S, Tanaka Y, Sasaki K, Tanaka H, Fukamizu T, Shpyama Y, Shoyama Y, Taura F (2007) Identification and characterization of cannabinoids that induce cell death through mitochondrial permeability transition in Cannabis leaf cells. *J Biol Chem* 282:20739–20751
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15:473–497
- Nanjareddy K, Arthikala M, Blanco L, Arellano E, Lara M (2016) Protoplast isolation, transient transformation of leaf mesophyll protoplasts and improved *Agrobacterium*-mediated leaf disc infiltration of *Phaseolus vulgaris*: tools for rapid gene expression analysis. *BMC Biotechnol* 16:53

- Nicolaisen M, Poulsen GB (1993) Optimization of polyethylene glycol mediated transient gene expression in pea protoplasts. *Plant Cell Tiss Org Cult* 35:93–97
- Page MT, Parry MAJ, Carmo-Silva E (2019) A high-throughput transient expression system for rice. *Plant Cell Environ* 42:2057–2064
- Priyadarshani S, Hu B, Li W, Ali H, Jia H, Zhao L, Ojolo SP, Azam SM, Xiong J, Yan M, Rahman ZU, Wu Q, Qin Y (2018) Simple protoplast isolation system for gene expression and protein interaction studies in pineapple (*Ananas comosus* L.). *Plant Method* 14:95
- ProCon.org (2021) Legal Medical Marijuana States and DC. Available at: <https://medicalmarijuana.procon.org/legal-medical-marijuana-states-and-dc/> (Accessed: 24 March 2021).
- Raharjo TJ, Cheng W, Verberne MC, Peltenburg-Looman AMG, Linthorst HJM, Verpoorte R (2004) Cloning and over-expression of a cDNA encoding a polyketide synthase from *Cannabis sativa*. *Plant Physiol and Biochem* 42:291–297
- Rasmussen JO, Rasmussen OS (1993) PEG mediated DNA uptake and transient GUS expression in carrot, rapeseed and soybean protoplasts. *Plant Sci* 89:199–207
- Reed J, Osbourn A (2018) Engineering terpenoid production through transient expression in *Nicotiana benthamiana*. *Plant Cell Rep* 37:1431–1441
- Saenger W, Hunter WN, Kennard O (1986) DNA conformation is determined by economics in the hydration of phosphate groups. *Nature* 324:385–388
- Sain PM, Rodríguez S, Disalvo EA (2017) Effect of osmotic stress on the surface properties of protoplasts as measured by merocyanine 540. *Soc Plant Res* 30:75–80
- Sheen J (2001) Signal transduction in maize and *Arabidopsis* mesophyll protoplasts. *Plant Physiol* 127:1466–1475
- Shen Y, Meng D, McGrouther K, Zhang J, Cheng L (2017) Efficient isolation of Magnolia protoplasts and the application to subcellular localization of MdeHSF1. *Plant Method* 13:44
- Shoyama Y, Yagi M, Nishioka I, Yamauchi T (1975) Biosynthesis of cannabinoid acids. *Phytochem* 14:2189–2192
- Sirikantaramas S, Morimoto S, Shoyama Y, Ishikawa Y, Wada Y, Shoyama Y, Taura F (2004) The gene controlling marijuana psychoactivity molecular cloning and heterologous expression of Delta1-tetrahydrocannabinolic-acid synthase from *Cannabis sativa*. *J Biol Chem* 279:39767–39774
- Smimoff N (2019) Engineering of metabolic pathways using synthetic enzyme complexes. *Plant Physiol* 179:918–928
- Su C, Wang Y, Hsieh T, Lu C, Tseng T, Yu S (2010) A novel MYBS3-dependent pathway confers cold tolerance in rice. *Plant Physiol* 153:145–158
- Taura F, Morimoto S, Shoyama Y (1996) Purification and characterization of cannabidiolic-acid synthase from *Cannabis sativa* L. Biochemical analysis of a novel enzyme that catalyzes the oxidocyclization of cannabigerolic acid to cannabidiolic acid. *J Bio Chem* 271:17411–17416
- Taura F, Sirikantaramas S, Shoyama Y, Yoshikai K, Shoyama Y, Morimoto S (2007) Cannabidiolic-acid synthase, the chemotype-determining enzyme in the fiber-type *Cannabis sativa*. *FEBS Lett* 581:2929–2934
- Taura F, Tanaka S, Taguchi C, Fukamizu T, Tanaka H, Shoyama Y, Morimoto S (2009) Characterization of olivetol synthase, a polyketide synthase putatively involved in cannabinoid biosynthetic pathway. *FEBS Lett* 583:2061–2066
- Töpfer R, Pröls M, Schell J, Steinbüß HH (1988) Transient gene expression in tobacco protoplasts: II. Comparison of the reporter gene systems for CAT, NPT II, and GUS. *Plant Cell Rep* 7:225–228
- Vain P, McMullen MD, Finer JJ (1993) Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. *Plant Cell Rep* 12:84–88
- Van Bakel H, Stout J, Cote A, Tallon C, Sharpe A, Hughes T, Page J (2011) The draft genome and transcriptome of *Cannabis sativa*. *Genome Biol* 12:R102
- Wahby I, Caba JM, Ligerio F (2013) *Agrobacterium* infection of hemp (*Cannabis sativa* L.): establishment of hairy root cultures. *J Plant Interact* 8:312–320
- Wang YC, Klein TM, Fromm M, Cao J, Sanford JC, Wu R (1988) Transient expression of foreign genes in rice, wheat and soybean cells following particle bombardment. *Plant Mol Biol* 11:433–439
- Yoo SD, Cho YH, Sheen J (2007) *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat Prot* 2:1565–1572
- Zhang Y, Su J, Duan S, Ao Y, Dai J, Liu J, Wang P, Li Y, Liu B, Feng D, Wang J, Wang H (2011) A highly efficient rice green tissue protoplast system for transient gene expression and studying light/chloroplast-related processes. *Plant Method* 7:30–38
- Zuardi AW (2006) History of cannabis as a medicine: a review. *Braz J Psychiatry* 28:153–157