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Optimization of protoplast isolation from *Cannabis sativa* L. 'Hang Kra Rog'

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Abstract This study investigated the effects of enzyme type and concentration, osmotic environment, and digestion time on protoplast yield and viability. The results indicated that both the type and concentration of cellulase significantly influenced protoplast yield and viability. Specifically, Cellulase R-10 outperformed Cellulase RS, with a 2.0 % Cellulase R-10 yielding the highest protoplast density (4.51 $\times 10^6$ cells/g fresh weight) and a viability of 95.80%. For pectinolytic enzyme, macerozyme at a concentration of 0.3% was optimal, yielding a higher protoplast yield (4.38 $\times 10^6$ cells/g fresh weight) compared to higher concentrations. The optimal osmotic environment was achieved with 0.4 M mannitol, which maintained a viability rate above 94% and produced a maximum yield of 4.38 $\times$ 10^6 cells/g fresh weight and a viability of 95.93%. Higher mannitol concentrations led to a continuous decline in both yield and viability. Furthermore, an enzymatic digestion time of 16 hours was found to be the optimal, resulting in a maximum yield of 4.42 $\times 10^6$ cells/g fresh weight without reducing cell viability. In conclusion, the combination of 2.0% Cellulase R-10, 0.3% macerozyme, and 0.4 M mannitol, and 16 hours provides a robust and reproducible protocol for cannabis protoplast isolation. This is providing a foundation for advanced cellular and molecular studies in cannabis biotechnology.

Keywords: *Cannabis sativa* L., Cellulase, Macerozyme, Mannitol, Protoplast isolation

Introduction

Cannabis sativa L. (Cannabaceae) has long been cultivated for fiber and seed production. In recent years, however, interest in this species has expanded because of its medicinal and economic potential, particularly its non-psychoactive compounds such as cannabidiol (CBD) (Farinon *et al*., 2020; Freeman *et al*., 2019; Vandepitte *et al*., 2020). The species displays extensive

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phenotypic and chemical diversity, which underpins its classification into “drug-type” and “industrial hemp” varieties (de Meijer and Keizer, 1996). In addition, industrial cannabis varieties containing less than 0.3% THC are increasingly being used in sustainable agriculture, nutraceutical applications, and plant biotechnology research (Fike, 2016; Thomas and ElSohly, 2015).

Despite its growing importance, stable genetic transformation in cannabis remains difficult because of several biological limitations. These challenges still limit the use of functional genes and genomes in this species (Hesami *et al*., 2020). As an alternative approach, protoplast-based systems have gained attention because it doesn't have to create all new plants. Due to protoplasts consist of single living cells without cell walls. They are widely used in transient expression assays, localization studies, and genome editing experiments (Lin *et al*., 2018).

Protoplasts serve as a robust platform for exploring gene function and cellular signaling pathways. This efficiency may be affected by the removal of the cell wall significantly increasing membrane permeability, facilitating the direct introduction of nucleic acids and protein complexes (Yoo *et al*., 2007). Among the various plant tissues available, mesophyll cells from leaves are commonly used for protoplast isolation due to their high cell density and active photosynthetic pathway (Sheen, 2001). In cannabis, leaf tissue is a material for protoplast extraction. However, the enzymatic release of protoplasts from cannabis leaves is challenging due to the structural complexity and robustness of the cell wall.

The cannabis cell wall consists of cellulose microfibrils that are notably resistant to enzymatic breakdown (Lee *et al*., 2011; Zhao *et al*., 2021). Successful isolation requires a balance of multiple factors, including the concentration and combination of enzymes (cellulase and pectinase or macerozyme), incubation duration, pH, and osmotic stabilizers such as mannitol (Wu *et al*., 2009; Bai *et al*., 2020).

Morimoto *et al*. (2007) were reporting the work in protoplast isolation from suspension cultures of *C. sativa* L. while Beard *et al*. (2021) successfully isolated viable protoplasts from the high-CBD cannabis cultivar ‘Cherry × Otto II: Sweetened’ and demonstrated transient gene expression using polyethylene glycol (PEG)-mediated transformation. However, their work did not extend to cell division or regeneration. Piunno *et al*. (2019) and Galán-Ávila *et al*. (2020) refined enzyme formulations and incubation protocols to improve protoplast yield and viability. These optimized protocols employed cellulase R-10 and macerozyme R-10 in combination with osmotic and membrane-stabilizing buffers such as mannitol, MES, and calcium chloride ($CaCl_2$) to enhance cell viability and prevent lysis during digestion.

Consequently, this study aimed to optimize leaf digestion by analyzing three parameters critical to protoplast yield and viability: enzyme combination, incubation length, and osmotic stability.

Materials and methods

Plant materials

All research was performed under the Expert Center of Innovative Agriculture, Thailand Institute of Scientific and Technological Research, Pathum Thani, Thailand. *C. sativa* L. 'Hang Kra Rog' was used in this study for protoplast isolation optimization. Seeds were germinated and maintained in a growth chamber (16 hours light/8 hours dark, 27 °C day/25 °C night, and 80-100 μE). Expanded leaves from 14 day old plants were used as the source material for protoplast isolation.

Protoplast isolation, purification and quantification

An enzyme solution (15 mL) was freshly prepared prior to tissue digestion. These enzyme solutions were used in protoplast isolation, adapted from Li (2011). Protoplasts were isolated from 14-day-old plantlets, young leaves (0.3 g) were cut into thin strips (0.5–1.0 mm) (Fig. 1A) and incubated in a petri dish containing enzyme solution of 1.5, 2.0 and 2.5% (w/v) Cellulase Onozuka RS or R-10 (Yakult Honsha Co., Ltd., Tokyo, Japan); 0.3, 0.4 and 0.5% (w/v) Macerozyme R-10 (Yakult Honsha Co., Ltd., Tokyo, Japan); 0.4, 0.5, 0.6, 0.7 and 0.8 M mannitol; 20 mM MES (pH 5.7); 20 mM KCl; 0.1% Fetal bovine serum (FBS) (Sigma-Aldrich); 10 mM $CaCl_2$ and 5 mM β-mercaptoethanol, and filter-sterilized using a 0.22 μm filter (Sigma-Aldrich). Leaf strips were placed in a vacuum desiccator, and a vacuum was applied for 30 minutes to facilitate enzyme infiltration. The samples were then incubated in the dark at 25 ± 2 °C on a rotary shaker set at 40 rpm for 12, 16, or 24 hours, with each time point conducted in triplicate. Post-digestion, the cell mixture was filtered through a sterile 75 μm nylon mesh into 15 mL sterile polypropylene centrifuge tubes. The filtrate was centrifuged at 800 rpm for 3 minutes to pellet the protoplasts. The supernatant was carefully removed, and the pellet was resuspended in 3 mL of W5 solution (containing 5 mM glucose, 5 mM KCl, 2 mM MES-K buffer at pH 5.7, 125 mM $CaCl_2$, and 154 mM NaCl). After that, the suspension was transferred to a new 15 mL tube. A 5 mL aliquot of 20% (w/v) sucrose solution was carefully added and then carefully separate them into layers before being centrifuged. The protoplasts were then collected from the interphase between the

solution layers. It was then transferred to a new tube containing 3 mL of W5 solution and centrifuged as the final step. After pouring out the supernatant, the pellet is suspended again in a 1 mL volume of W5 solution and the yield of the protoplast is counted using a hemocytometer.

Protoplast density was determined using a hemocytometer. A 10 µL protoplast suspension was carefully loaded onto the hemocytometer beneath a coverslip and then examined under a light microscope at 40× magnification (Fig. 2A). Cell counts were recorded from multiple squares to calculate an average. The concentration of protoplasts per milliliter per gram of leaf tissue was calculated using the following formula:

Protoplasts/mL = Average number of cells per square × dilution factor × 10^4.

The viability of isolated protoplasts was assessed by trypan blue staining: the trypan blue solution was prepared by mixing 20 µL 0.4% trypan blue with 180 µL protoplast suspension at a 1:10 dilution, with a final concentration of trypan blue staining of 0.04%. The samples were incubated in darkness at 25 °C for 5 minutes and protoplast viability was assessed using a microscope (Fig. 2B). The protoplast viability was calculated using the following equation:

Protoplast viability (%) = (viable protoplasts/total number of protoplasts) × 100%.

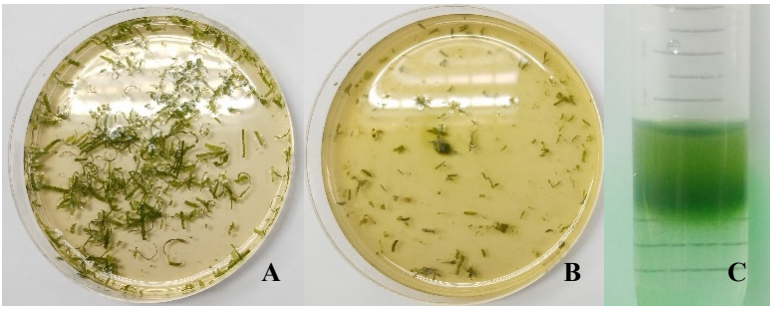

Figure 1. Protoplast isolation from leaves of *Cannabis sativa* L. 'Hang Kra Rog': (A) Leaf segments (0.5–1.0 mm wide) were immersed in enzymatic digestion medium. (B) Samples were incubated in the dark at 25 °C for 16 h. (C) Protoplasts collected at the interphase following density gradient centrifugation

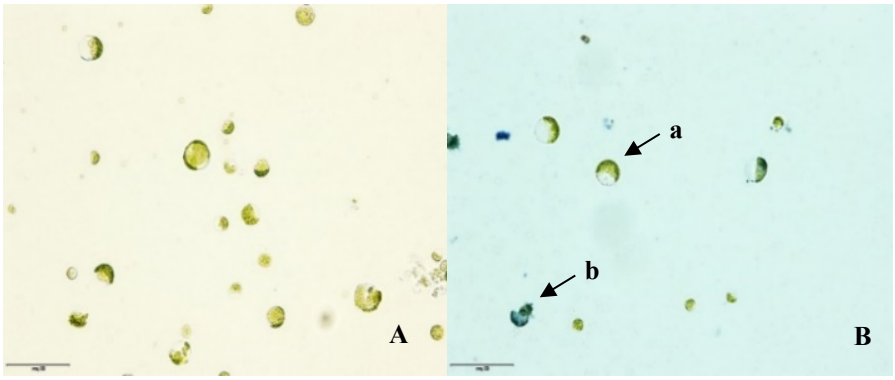

Figure 2. Isolation and viability of protoplasts from leaves of *Cannabis sativa* L. 'Hang Kra Rog'. The freshly isolated protoplasts of cannabis leaves (A) and viable protoplasts were not stained (a), while the only non-viable protoplasts were stained with trypan blue (b), scale bar = 50 µm.

Statistical analysis

Mean values were analyzed using one-way analysis of variance (ANOVA) with SPSS version 25 software (SPSS Inc., Chicago, USA). Significant differences among treatments were determined using Duncan's Multiple Range Test (DMRT) at the 0.05 probability level.

Results

Effect of enzyme concentrations on protoplast isolation

Cellulase type (Cellulase RS and R-10) and concentration (1.5%, 2.0%, and 2.5%) significantly affected both protoplast yield and viability ($p \leq 0.05$). Among the tested treatments, 2.0% Cellulase R-10 yielded the highest number of viable protoplasts ($4.51 \pm 0.28 \times 10^6$ cells/g fresh weight) with a viability of $95.80 \pm 0.92\%$. However, the yield and viability at 2.0% Cellulase R-10 were not significantly different from those obtained with 1.5% and 2.5% Cellulase R-10. In contrast, all concentrations of RS cellulase resulted in significantly lower protoplast yields and viabilities (Table 1, Figure 3).

Table 1. Effect of cellulase types and concentrations on protoplast yield and viability of protoplast isolation from cannabis (*Cannabis sativa* L. 'Hang Kra Rog') leaves

Type of celluase	Cellulase concentrations (%)	Protoplast	
		Yield (x10^6 cells/g FW)	Viability (%)
RS	1.5	4.01±0.18^{bc}	82.20±0.80^{b}
	2.0	3.82±0.08^{c}	82.47±1.29^{b}
	2.5	2.94±0.43^{d}	82.13±0.99^{b}
R-10	1.5	4.37±0.26^{ab}	96.20±0.72^{a}
	2.0	4.51±0.28^{a}	95.80±0.92^{a}
	2.5	4.23±0.23^{abc}	95.00±0.35^{a}
C.V. (%)		6.59	1.00

Note: The data are expressed as mean±SD. Numbers followed by the same letter in the same column are not significantly different at a 95% confidence level, as determined by DMRT.

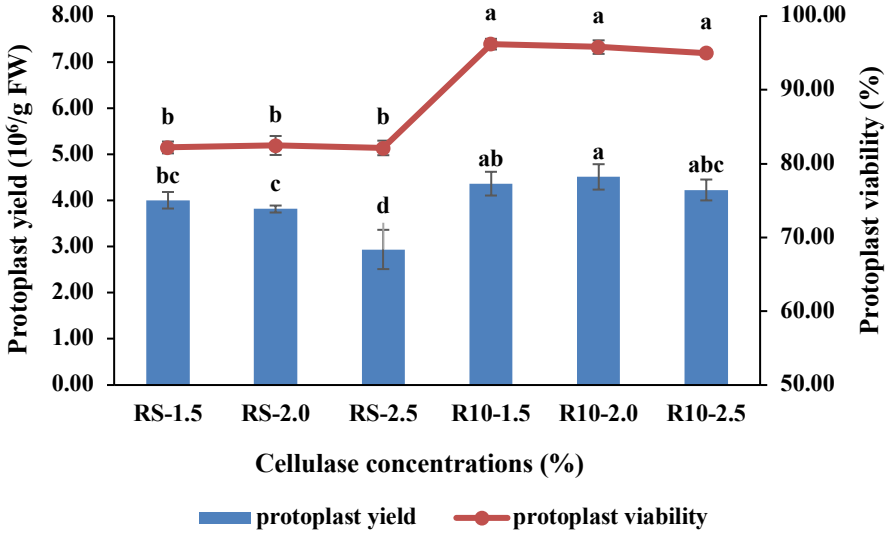

Figure 3. Effect of cellulase types and concentrations on protoplast yield and viability of protoplast isolation from cannabis (*Cannabis sativa* L. 'Hang Kra Rog') leaves

Effect of macerozyme concentrations on protoplast isolation

Macerozyme concentration (0.3%, 0.4%, and 0.5%) also influenced protoplast isolation efficiency. The data indicated that 0.3% macerozyme provided the highest yield (4.38 ± 0.11 ×10^6 cells/g fresh weight), which was significantly higher ($p \leq 0.05$) than yields obtained with 0.4% and 0.5%

concentrations. However, there were no statistically significant differences in protoplast viability across the treatments, all of which remained above 94% (Table 2, Figure 4).

Table 2. Effect of macerozyme concentrations on protoplast yield and viability of protoplast isolation from cannabis (*Cannabis sativa* L. 'Hang Kra Rog') leaves

Macerozyme concentrations (%)	Protoplasts	
	Yield (x10^6 cells/g FW)	Viability (%)
0.3	4.38±0.11^a	95.53±0.46
0.4	3.15±0.13^b	94.67±0.64
0.5	1.66±0.12^c	94.73±0.42
C.V. (%)	3.86	0.54

Note: The data are expressed as mean±SD. Numbers followed by the same letter in the same column are not significantly different at a 95% confidence level, as determined by DMRT.

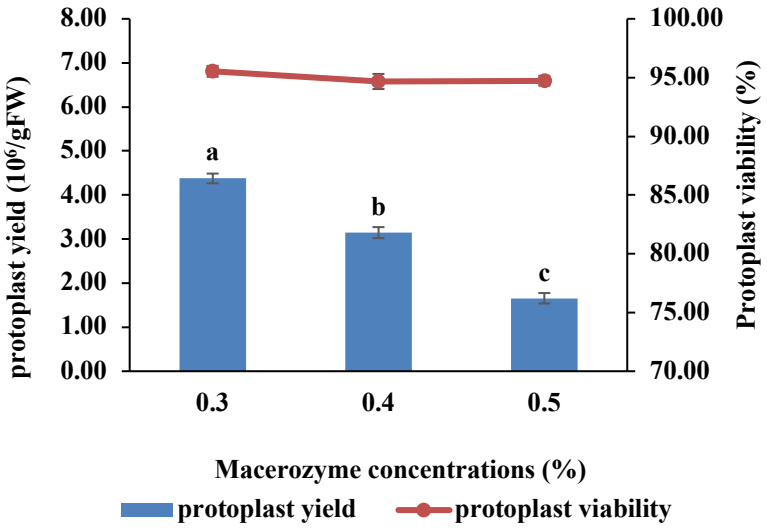

Figure 4. Effect of macerozyme concentrations on protoplast yield and viability of protoplast isolation from cannabis (*Cannabis sativa* L. 'Hang Kra Rog') leaves

Effect of mannitol concentrations on protoplast isolation

Changes in mannitol concentration (0.4–0.8 M) had a marked effect on both protoplast yield and viability. The best results were obtained at 0.4 M mannitol, which resulted in the highest protoplast yields (4.38 ± 0.06 ×10^6 cells/g fresh weight) and viability (95.93 ± 0.58). Increasing mannitol concentration above 0.4 M progressively reduced both yield and viability, with the lowest

values recorded at 0.8 M mannitol (Table 3, Figure 5). Statistical analysis confirmed significant differences among treatments ($p \leq 0.05$).

Table 3. Effect of mannitol concentrations on protoplast yield and viability of protoplast isolation from cannabis (*Cannabis sativa* L. 'Hang Kra Rog') leaves

Mannitol concentrations (M)	Protoplasts	
	Yield (x10^6 cells/g FW)	Viability (%)
0.4	4.38±0.06^a	95.93±0.58^a
0.5	4.19±0.01^b	90.93±0.50^b
0.6	3.75±0.04^c	88.60±0.20^c
0.7	1.55±0.13^d	82.73±0.12^d
0.8	1.22±0.06^e	82.60±0.40^d
C.V. (%)	2.44	0.45

Note: The data are expressed as mean±SD. Numbers followed by the same letter in the same column are not significantly different at a 95% confidence level, as determined by DMRT.

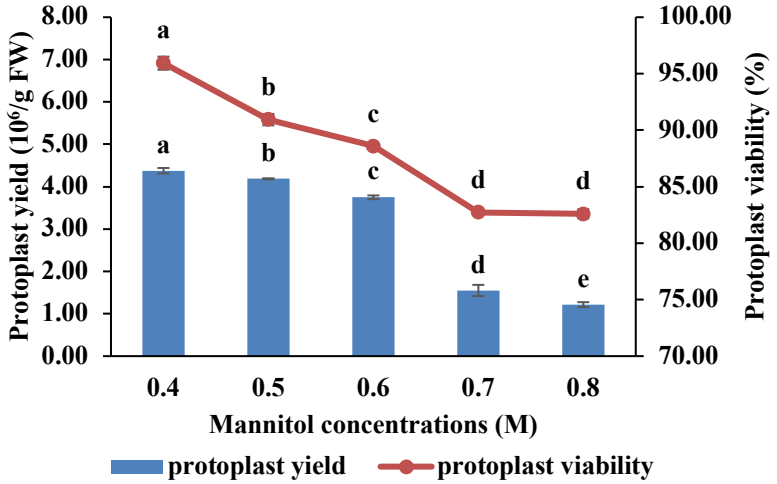

Figure 5. Effect of mannitol concentrations on protoplast yield and viability of protoplast isolation from cannabis (*Cannabis sativa* L. 'Hang Kra Rog') leaves

Effect of enzymatic incubation time on protoplast isolation

Enzymatic digestion time (12, 16, and 20 hours) also affected protoplast isolation efficiency. A 16-hour incubation period produced the highest yield of protoplasts (4.42 ± 0.02 ×10^6 cells/g fresh weight), which was significantly higher than the yields obtained at 12 and 20 hours of digestion ($p \leq 0.05$). In contrast, protoplast viability was not significantly influenced by incubation duration, remaining within the range of 93.33–94.13% across all treatments (Table 4, Figure 6).

Table 4. Effect of incubate time on protoplast yield and viability of protoplast isolation from cannabis (*Cannabis sativa* L. 'Hang Kra Rog') leaves

Incubate time (hrs.)	Protoplasts	
	Yield (x10^6 cells/g FW)	Viability (%)
12	3.63±0.05^b	93.33±0.64
16	4.42±0.02^a	94.13±0.61
20	3.70±0.04^b	94.00±0.69
C.V. (%)	0.98	0.69

Note: The data are expressed as mean±SD. Numbers followed by the same letter in the same column are not significantly different at a 95% confidence level, as determined by DMRT.

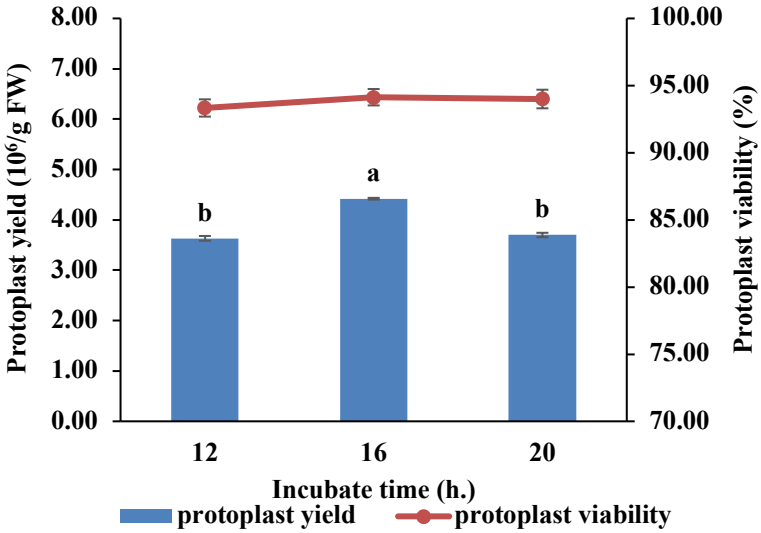

Figure 6. Effect of incubate time on protoplast yield and viability of protoplast isolation from cannabis (*Cannabis sativa* L. 'Hang Kra Rog') leaves

Discussion

The successful isolation of protoplasts from *C. sativa* L. leaves is highly influenced by multiple enzymatic. Our results clearly demonstrate that the type and concentration of cellulase are critical factors in determining both protoplast yield and viability. Specifically, Cellulase R-10 consistently produced higher yields and viability compared to Cellulase RS. This observation is consistent with findings in tobacco and tomato, where Cellulase R-10 was proven to be more efficient in degrading mesophyll tissues (Sheen, 2001; Yoo *et al*., 2007). However, during cell wall digestion, a distinct physiological challenge exists in this species: cannabis leaf tissue is rich in secondary substances such as

cannabinoids and terpenoids, which actively interfere with enzyme activity and membrane stability (Lata *et al*., 2010).

To determining the optimal pectinolytic conditions, the macerozyme concentration for protoplast isolation was optimized at 0.3%, as higher concentrations led to significantly reduced protoplast yield. The decreased protoplast yield at higher enzyme concentrations is a critical saturation point and highlights the importance of optimizing enzymatic concentrations. This balance is very important in *C. sativa* L., where the structural characteristics of the leaf and cell wall can vary dynamically depending on the cultivar and developmental stage. Similarly, studies in *Arabidopsis thaliana* (Wu *et al*., 2009) and rice (Lin *et al*., 2018) reported that elevated macerozyme levels caused excessive degradation of the cell wall and compromised membrane integrity.

The study showed that 0.4 M mannitol provided the optimal osmotic balance, which is consistent with protoplast protocols in various plant species (Xu *et al*., 2021). Furthermore, studies on cannabis indicate that elevated osmotic environments can affect both the synthesis of phytocannabinoids and the stability of intracellular components during tissue culture applications (Lata *et al*., 2010). Therefore, maintaining the optimal mannitol concentration not only preserves osmotic pressure to prevent physical collapse but may also indirectly reduce stress level and possible to induced metabolic responses during protoplast isolation.

Our findings indicated that a 16-hour incubation period resulted in the highest number of protoplasts without compromising cell viability compared to shorter or longer digestion times. Although extended exposure (e.g., beyond 20 hours) may heighten membrane permeability or trigger detrimental oxidative stress responses (Barnes *et al*., 2019), the data indicate that cannabis protoplasts can withstand overnight digestion effectively when optimal enzyme concentrations and osmotic conditions are maintained.

Recently, global interest in cannabis biotechnology has surged, particularly in genome editing, secondary metabolite engineering, and *in vitro* breeding (Zhang *et al*., 2019). However, the development of protoplast-based systems for cannabis remains severely limited. Only a few studies have explored transient gene expression and genome editing in cannabis tissues, reporting low efficiency and poor reproducibility (Galán-Ávila *et al*., 2020). Our study presents a reproducible and highly efficient method for isolating viable protoplasts from cannabis leaves. By ensuring high viability and yield, this protocol can be applied to molecular studies in cannabis, including genome editing, and metabolic manipulation.

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Conflicts of interest

The authors declare no conflict of interest.

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