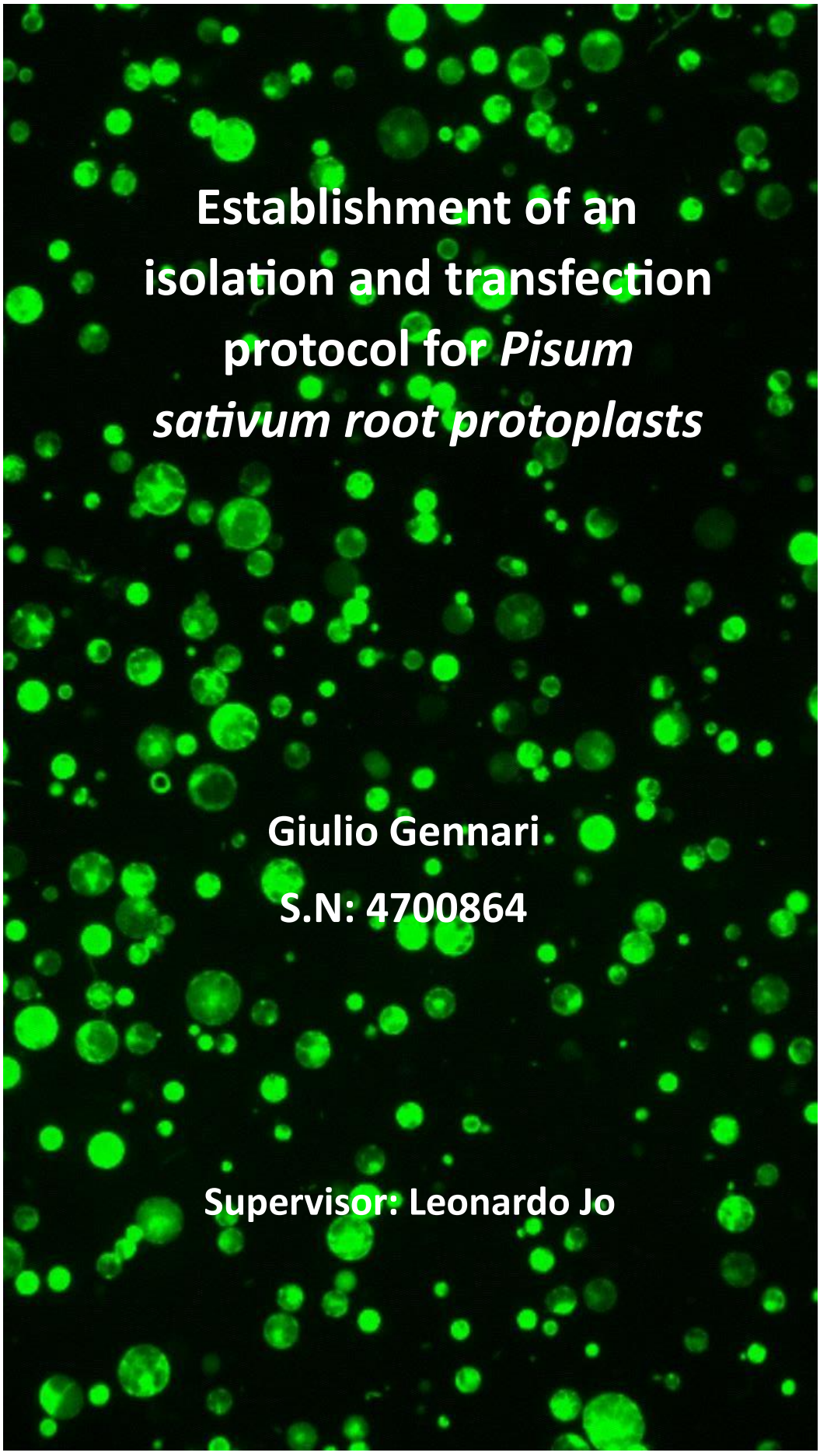

Establishment of an isolation and transfection protocol for *Pisum sativum root protoplasts*

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Science and Business Management Major Internship Report

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Acknowledgement

At the culmination of this internship project, I want to express my profound gratitude to my supervisor, Dr. L. (Leonardo) Jo. His unwavering responsibility and prioritization of students have left an indelible mark on my professional growth. I am genuinely thankful for his guidance and mentorship, which have significantly contributed to my development.

As I stand at the midpoint of my master's course, I extend my heartfelt thanks to my parents, who provided me with the opportunity to embark on this transformative journey. Gratitude is also extended to those who have been unwavering in their support, accompanying me through every step of my life.

This marks the conclusion of my master's journey, signaling the commencement of another exciting chapter in my life. I am eager and ready for the challenges and opportunities that lie ahead.

Table of content

List of abbreviation

B:

BSA: Bovine Serum Albumin.

E:

ES: Enzymatic Solution.

F:

FDA: Fluorescein Diacetate.

G:

GFP: Green Fluorescence Protein.

GOI: Genes of Interest.

P:

PEG: Polyethylene Glycol.

R:

RT: Room Temperature.

RCF: Relative centrifugal force.

RPM: Rotation per minute.

S:

SMG: Sorbitol Magnesium Solution.

V:

v/v: volume/volume.

W:

w/v: weight/volume.

Abstract

Pea (*Pisum sativum*) is a versatile and valuable crop with a wide array of applications, including its role as a food source, forage, and even a potential biofuel crop. Given the increasing interest in pea research, the development of an efficient method for transient transformation holds paramount importance. In this study, we introduce a comprehensive protocol for the isolation and transfection of pea primary root protoplasts.

Through exploration of the protoplast isolation technique, enzyme compositions and concentrations, we have successfully achieved the isolation of pea root protoplasts, yielding 7.169E+03 protoplasts · root $^{-1}$ after 6 hours of digestion. Moreover, we have identified conditions that sustain high protoplast viability for up to 16 hours.

Furthermore, by optimizing the polyethylene glycol (PEG) calcium-mediated transformation process, we have achieved an average transfection efficiency of 15.48% by subjecting protoplasts to a 30% (w/v) PEG4000 solution for 5 minutes.

In conclusion, we are confident that this protocol will find wide-ranging applications and prove highly beneficial for future research focused on the potential of pea crops for various purposes, enabling investigations such as gene expression and protein subcellular localization, and ultimately advancing our knowledge and utilization of this invaluable plant species.

Introduction

Protoplasts, plant cells with their cell walls removed through enzymatic digestion of structural components like pectin, cellulose, and hemicellulose, play a pivotal role in plant research. The typical components of a plant cell wall not only provide structural support but also contribute significantly to shaping and sizing, regulating water intake, defense against pathogens, facilitating cell-to-cell interactions, and directing cell growth (Cosgrove, 2023). Despite the absence of a protective wall, protoplasts retain most features and systems of plant cells, making them reliable models for observing and evaluating various cellular events. Notably, somatic hybridization (Ranaware et al., 2023) and plant regeneration (Jeong et al., 2021) are valuable tools facilitated by the use of protoplasts. Additionally, they prove instrumental in exploring subcellular protein localization (Vögeli-Lange et al., 1990; Zeng et al., 2021), protein-protein interactions (Chen et al., 2006; Priyadarshani et al., 2018), and investigating live cell gene promoter activity (Dron et al., 1988; Wang et al., 2021).

Protoplast isolation is the starting point for most pivotal techniques in plant biology, with the first isolation being documented in 1960 (Cocking 1960). Since then, protocols have undergone continuous development and optimization. When it comes to standard procedures for isolating protoplasts from plant organs, such as roots, a meticulous process unfolds, with the precise slicing of roots, followed by enzymatic digestion, and the subsequent separation of released protoplasts from non-digested tissue debris (Zhang et al., 2023). Finding the delicate equilibrium in enzyme composition is of capital importance. It ensures efficient cell wall degradation while minimizing damage to the fragile protoplasts, leading to successful isolation and subsequent transformation (Michael et al., 2005). Although root systems are relevant for studies about nutrient intake and response to biotic or abiotic environment, most of the efficient isolation and transfection protoplast systems predominantly target mesophyll protoplasts within protoplast research (Wang X et al., 2021).

Particularly, protoplasts have been widely employed for plant transformation (Lörz et al., 1985; Wu et al., 2020) since cell walls would otherwise block the passage of DNA into the cell. Transformation strategies encompass various techniques, including electroporation (Subburaj et al., 2023), microinjection (Masani et al., 2014; Holm et al., 2000), and method based on polyethylene glycol (PEG) and calcium-mediated (Li et al.,2022; Liu et al., 2011). For instance, through transformation, the functions of aromatic amino acid aminotransferase involved in 2-phenylethanol biosynthesis have been characterized in isolated rose petal protoplasts (Hirata et al., 2012). Moreover, some legume species have been reported successful for root protoplasts isolation and transfection, including Lotus japonicus, *Medicago truncatula* (Jia et al., 2018), *Cicer arietinum* (Cheng et al., 2020), and *Glycine max* (Wu et al., 2018).

Pisum sativum, commonly known as pea, holds significant importance as a protein-rich crop. This annual plant belongs to the Leguminosae family and plays a pivotal role in serving as a vital source of food, forage, and biofuel (Kreplak et al., 2019). Despite the many advantages offered by peas, molecular research on this crop has been somewhat restricted, primarily due to the absence of well-optimized transformation methods (Ludvíková and Griga 2022). Nevertheless, it's worth noting that existing methods primarily target pea leaf mesophyll protoplasts.

In this project, we explored distinct isolation methods, cell-wall digestion conditions, protoplast incubation solutions, and transformation conditions to refine a highly efficient protocol for isolating and transforming pea root protoplasts (Figure 1). The successful development of this protocol promises to provide significant advantages and convenience for future research involving pea studies.

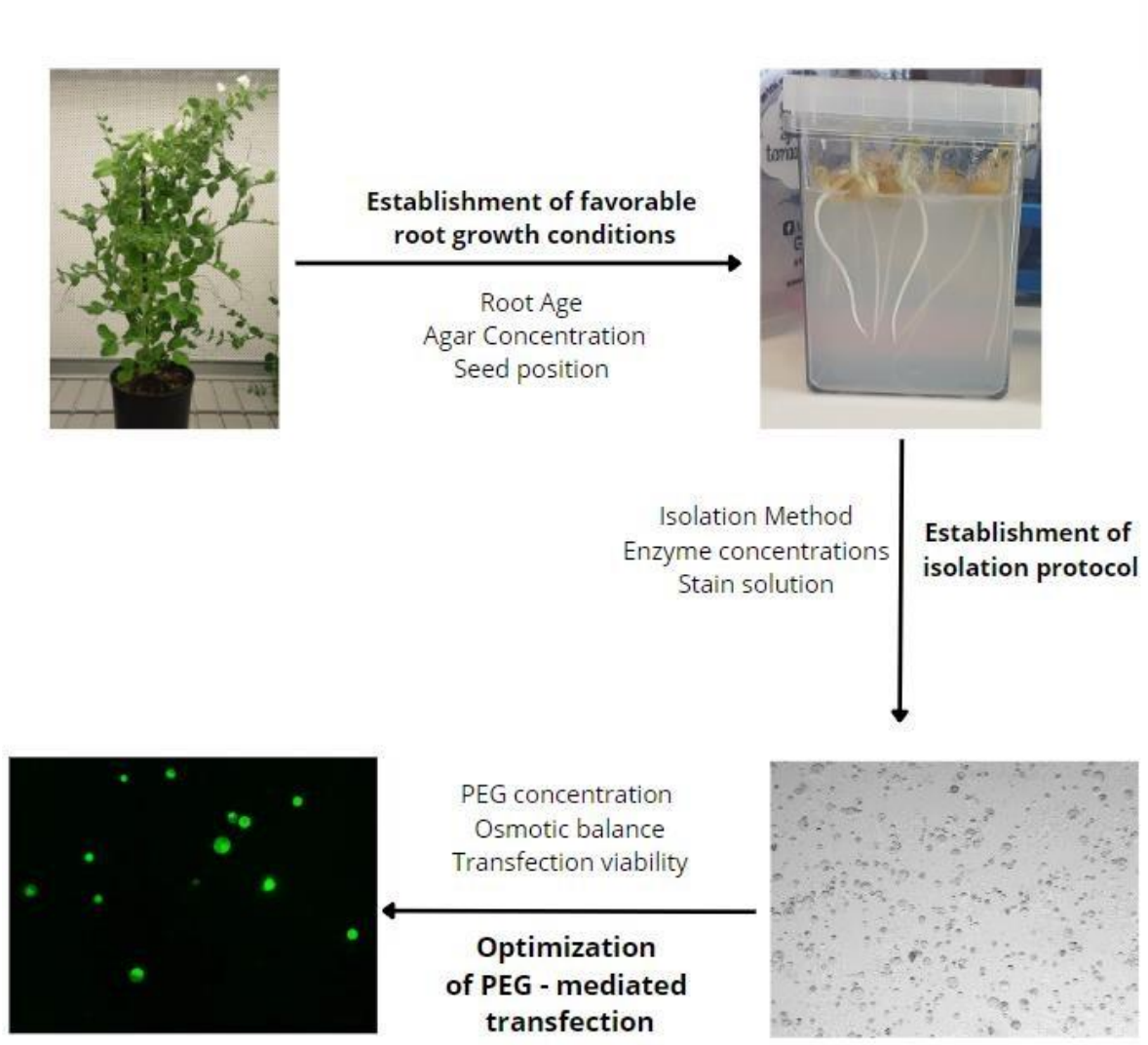


Figure 1. The process of establishing an isolation and transfection protocol for pea root protoplasts.

Scope in research and aims.

Methods for the isolation and transformation of pea root protoplasts cells has not been reported yet. In this project, the isolation and transfection protocol for pea protoplasts will be established by improving the protoplast yield, optimizing transfection efficiency, and testing the feasibility of transient gene expression. Two main goals of this project are as follow I) to establish an efficient method to isolate a high number of viable pea root protoplasts; II) to establish the conditions to highly acquire transfection efficiency of pea root protoplasts.

Results

Optimizing Protoplast Isolation: Influence of Root Age.

In our research protocol, we observed the development of roots from sown seeds to determine an appropriate stage for isolating protoplasts. It is noteworthy that the development of robust and high-quality primary roots plays a pivotal role in determining the quality of protoplasts obtained (Jia Ning et al., 2018).

During the germination process, we noted the emergence of the primary root from the seed coat, initiating primary growth. We also noted the absence of lateral roots in the first 2/3 days of growth (Figure 2A).

In our observations, roots typically reached a length over 3 cm by the fourth day (Figure 2B). This length was chosen as a criterion for subsequent protoplast isolation within the digestion enzymatic solution. At this stage, the roots predominantly consisted of primary roots, with lateral roots typically not yet emerged. We observed that 5 and, 6-day-old roots often exhibited the emergence of lateral roots and changes in the primary root's structure, as shown in our recorded observations (Figure 2C & 2D).

We selected 4-day-old roots for protoplast isolation based on their visual and morphological characteristics, aiming for straight and long roots that are most suitable for our experimental needs. Although we did not empirically establish a quantitative relationship between root age and protoplast yield or quality, our decision was guided by the observation that 4-dayold roots exhibit the desired traits.

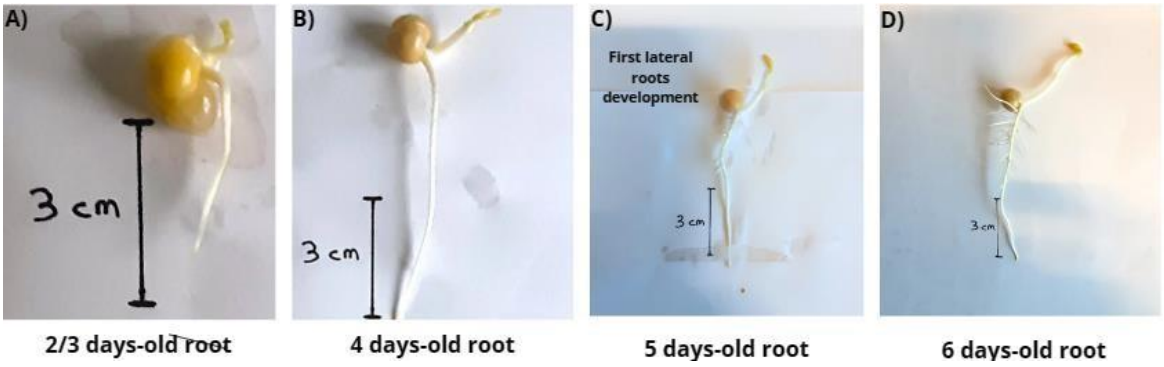


Figure 2. Visual representation of the morphological changes observed during the growth and development of roots at different ages. (A) 3 days old root. (B) 4 days old root. (C) 5 days old root. (D) 6 days old root.

Exploring Vyscozyme Unveiling the Relationship Between Protoplast Release and Digestion Time.

We identified as one of the primary goals of our study, the achievement of a high isolation efficiency of protoplasts from *P. sativum* primary roots. Therefore, we meticulously moved our focus to the optimization of the digestion solution. The enzymes that compose the digestion mix play a crucial role in breaking down the cell walls and tissues, allowing us to extract cells suitable for numerous transfection processes (Eeckhaut et al., 2013).

An enzymatic solution (ES) was initially formulated using 1% (w/v) Cellulase and 0.25% (w/v) Macerozyme, chosen for their effectiveness in achieving high-quality protoplast yields from *Arabidopsis* mesophyll tissue (Yoo, et al., 2007). This ES facilitated in our analyzed tissue, the isolation of an average of 1.386E+03 cells · $root^{-1}$ after 2 hours, 5.160E+03 cells · $root^{-1}$ after 4 hours, and 7.979E+03 cells · $root^{-1}$ after 6 hours of digestion process (Figure 3A). To quantify these protoplast concentrations, samples were taken at 2, 4, and 6 hours and their concentrations were assessed using a hemocytometer. The protoplasts were counted under a microscope in the hemocytometer grid-lined chamber, allowing for accurate determination of cell concentration at each time point. Given that in our protocol, we recommend using 4E+05 protoplasts for each transformation trial, the results obtained with the current enzymatic solution (ES) reveal a significant challenge: approximately 50 roots are required to obtain enough protoplasts for a single transfection trial. Therefore, this high root-to-protoplast ratio indicates a relatively low efficiency in protoplast isolation, leading to a considerable waste of seeds, which is a concern in terms of both resource utilization and practicality. To address this, optimizing the ES composition or the isolation process is necessary to improve the yield and efficiency of protoplast extraction.

Inspired by previous reports that utilized Viscozyme (Jeong et al., 2021), we included a novel enzyme combination in the digestion mixture, labeled as “ES + Viscozyme”. This mixture comprised 1.5% (w/v) Cellulase, 1% (w/v) Macerozyme, and 5% (v/v) Viscozyme. The inclusion of Viscozyme, coupled with an elevated concentration of the other enzymes, was a crucial modification. This enhancement improved protoplast yield, contributing to the production of well-rounded and uniformly shaped protoplasts (Figure 3B). In quantitative terms, we observed the extraction of 2.498E+04 cells · $root^{-1}$ after 2 hours of incubation, followed by 4.752E+04 cells · $root^{-1}$ after 4 hours, and a substantial increase to 7.169E+04 cells · $root^{-1}$ after 6 hours (Figure 3A). Our findings demonstrated that, with a 6-hour digestion period, only 4 roots were required to yield enough protoplasts for a single transfection process.

In conclusion, our research successfully achieved a high isolation efficiency of protoplasts from *Pisum sativum* primary roots through the optimization of our digestion solution. The introduction of Viscozyme, alongside increased enzyme concentrations, significantly improved the yield and uniformity of protoplasts.

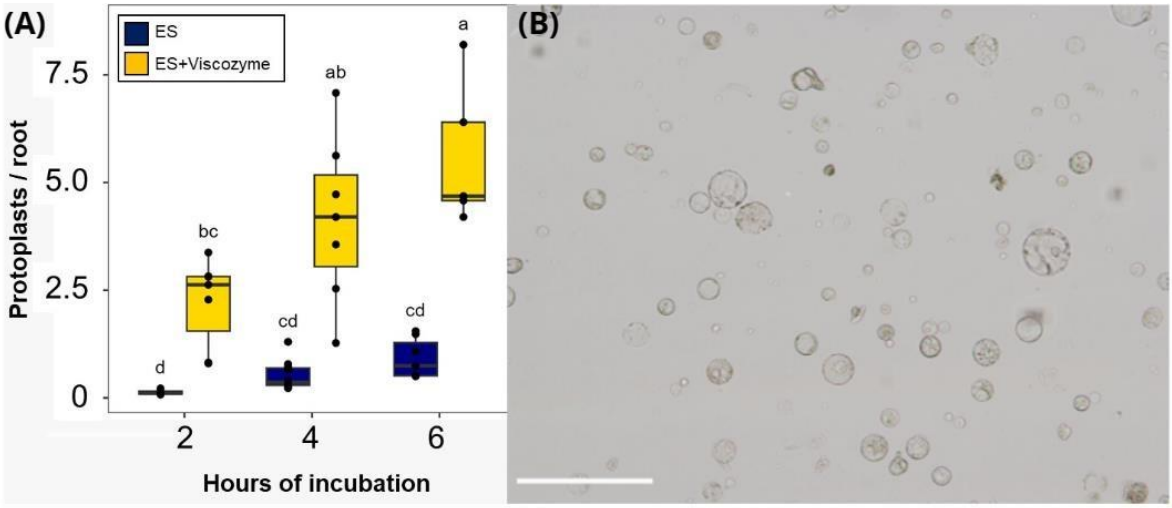


Figure 3. Introduction of viscozyme. (A) Relationship between incubation time and protoplast released per root, comparing the two different digestion solutions: ES, and ES + Vyscozyme. "a, ab, bc, cd, d" indicates groups determined by multiple comparisons tests after ANOVA analyses for protoplast isolation efficiency at different time points "2h, 4h and, 6h". (B) Well-rounded isolated protoplasts through ES + Viscozyme under light microscope 6 hours of digestion time. Scale = 100 µm.

Immersion cutting method generates higher protoplast quality and less debris contamination.

In the pursuit of optimizing both protoplast yield and quality, we explored two distinct methodologies for root cutting. In both approaches, roots were harvested after a 4-day growth period in Agar 0.4% (w/v) within magenta boxes. In the first approach, named "standard cutting", roots were meticulously segmented into 3 cm sections using a doubleedged blade, ensuring precision through snapping, subsequently roots were sectioned in slices of 1 mm each. It is noteworthy that the initial cut and subsequent sectioning were occurred outside the digestion solution. This method exposed the roots to ambient conditions for 2 minutes before immersion. On the other approach, "immersion cutting" involved the immediate transfer of a single root from the magenta box to the digestion solution, followed by the cutting step (Figure 4A). This method was designed by us, to prevent any exposure of the roots to air, minimizing the risk of drying the roots out.

To maintain methodological consistency and enable result validation, we employed identical enzyme concentrations 1.5% (w/v) cellulase, 1% (w/v) macerozyme, and 5% (v/v) viscozyme within the digestion solution. In both methods, to enhance the penetration of the digestion solution into the root tissues, the digestion broth containing the roots was subjected to a vacuum infiltration step. This involved placing the broth in a vacuum desiccator connected to a vacuum pump for 10 minutes. Following this infiltration step, the isolation process was conducted with an overnight incubation under gentle shaking for a duration of 6 hours.

Using the standard method, an average of 7.56E+04 protoplasts · $root^{-1}$ were isolated after 6 hours, while the immersion cutting method yielded 8.33E+04 protoplasts · $root^{-1}$ (Figure 4B). These results confirmed that, using the digestion solution developed, only four roots are required to generate enough protoplasts suitable for subsequent transfection processes.

Furthermore, for evaluating the protoplast viability, we conducted an FDA test (Fluorescein Diacetate). This assay serves as a crucial method for determining the viability of cells by measuring the activity of intracellular esterase. Upon hydrolysis by this esterase, FDA is converted into a fluorescent product, indicating cellular viability. Thus, the fluorescence intensity directly correlates with the metabolic activity and health of the protoplasts. We observed that the viability of protoplasts after 6 hours of digestion was comparable, with percentages of 64.22% and 65.50% for the standard and immersion methods, respectively (Figure 4C).

Although, in terms of protoplast concentration and viability, we didn't find any valuable differences, the adoption of the immersion cutting method presented advantage, especially in terms of reducing debris contamination in comparison to the conventional cutting method (Figure 4D & 4E). While we did not empirically quantify the exact variation in debris levels within the solution, our assessment was based on visual evaluations of the samples under a bright-field microscope. This qualitative observation revealed an apparent decrease in debris, which is crucial for maintaining the purity of isolated protoplasts. The reduction in debris contamination underscores the efficacy of the immersion cutting method, making it a

preferred technique for our experimental procedures due to its contribution to the integrity and cleanliness of the protoplast samples.

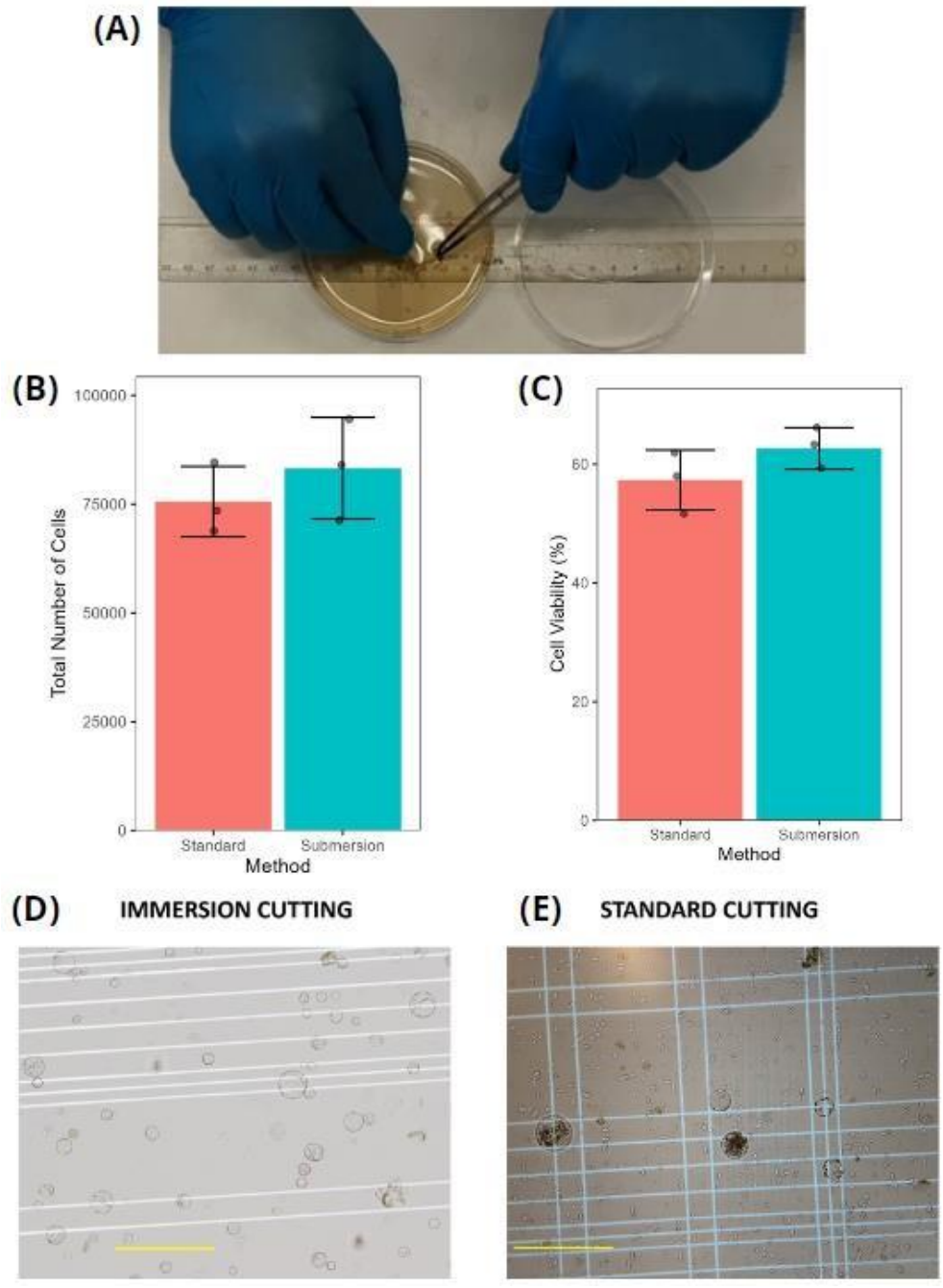


Figure 4. Comparison between standard cutting and immersion cutting procedure. (A) we illustrate the process of sectioning a Pisum Sativum root within a digestion solution. The root was meticulously positioned using a ruler, and it was sliced into 1 mm sections while being immediately immersed in the digestion solution. (B) Yield per root of protoplasts after 6 hours of digestion time. (C) Availability of protoplasts for standard and immersion methods respectively after 6 hours of digestion time. (D) Pea root protoplasts were analyzed through a hemocytometer after 6 hours of digestion time. Notably, the non-immersion cutting method resulted in a higher level of debris contamination. Scale = 100 µm.

Overnight protoplast incubation solution.

After optimizing the isolation process for *P. sativum* root protoplasts, we directed our attention to enhancing the conditions for the prolonged overnight incubation that follows the transformation process. In PEG-mediated transformation protocols, transfected cells undergo hours of incubation to facilitate the transient expression of transgenes (Jia et al., 2018). Our aim was to refine this incubation stage to ensure the viability and health of the protoplasts.

To identify the optimal incubation buffer following the digestion process, we centrifuged the isolated protoplasts at 400 RCF for 2 minutes at room temperature. Post centrifugation, we carefully removed the supernatant and resuspended the protoplast pellets in various solutions: W5, W5 with added glucose (W5 + glucose), WI, and WI with glucose (WI + glucose). The detailed compositions of these buffers are outlined on the material and methods section. We chose these solutions based on their prevalent use in protoplast research. Specifically, the WI buffer has demonstrated effectiveness with *Arabidopsis* mesophyll protoplasts (Yoo et al., 2007). Similarly, the W5 buffer was used for overnight incubation of protoplasts extracted from *Saccharum spontaneum L* leaves (Qiongli et al., 2021).

Firstly, we performed an FDA test after resuspension (0h or same day) to evaluate the immediate effect of each buffer on protoplast viability. Additionally, we repeated the viability assessment after a 16-hour incubation period (or day after) to analyze the long-term impacts of these buffers on protoplast health (Figure 5A). Protoplasts resuspended in WI solutions demonstrated average viability of 64.39% for WI with glucose and, 57.23% for WI less glucose. However, it was surprising to observe that all protoplasts incubated in WI solutions did not survive beyond 16 hours, exhibiting irregular or exploded shapes.

In contrast, cells resuspended in W5 solutions, both with and without glucose, showed promising results. Immediately after the isolation process, W5 with glucose reported an average viability of 71.65%, while W5 without glucose yielded 68.90% viable cells. However, after 16 hours of incubation, the viability decreased to 64.92% for W5 with glucose and 48.72% respectively for the same solution.

Therefore, based on our findings reported in the graph (Figure 5B), we identified W5 with glucose as the most suitable solution for maintaining high protoplast viability overnight.

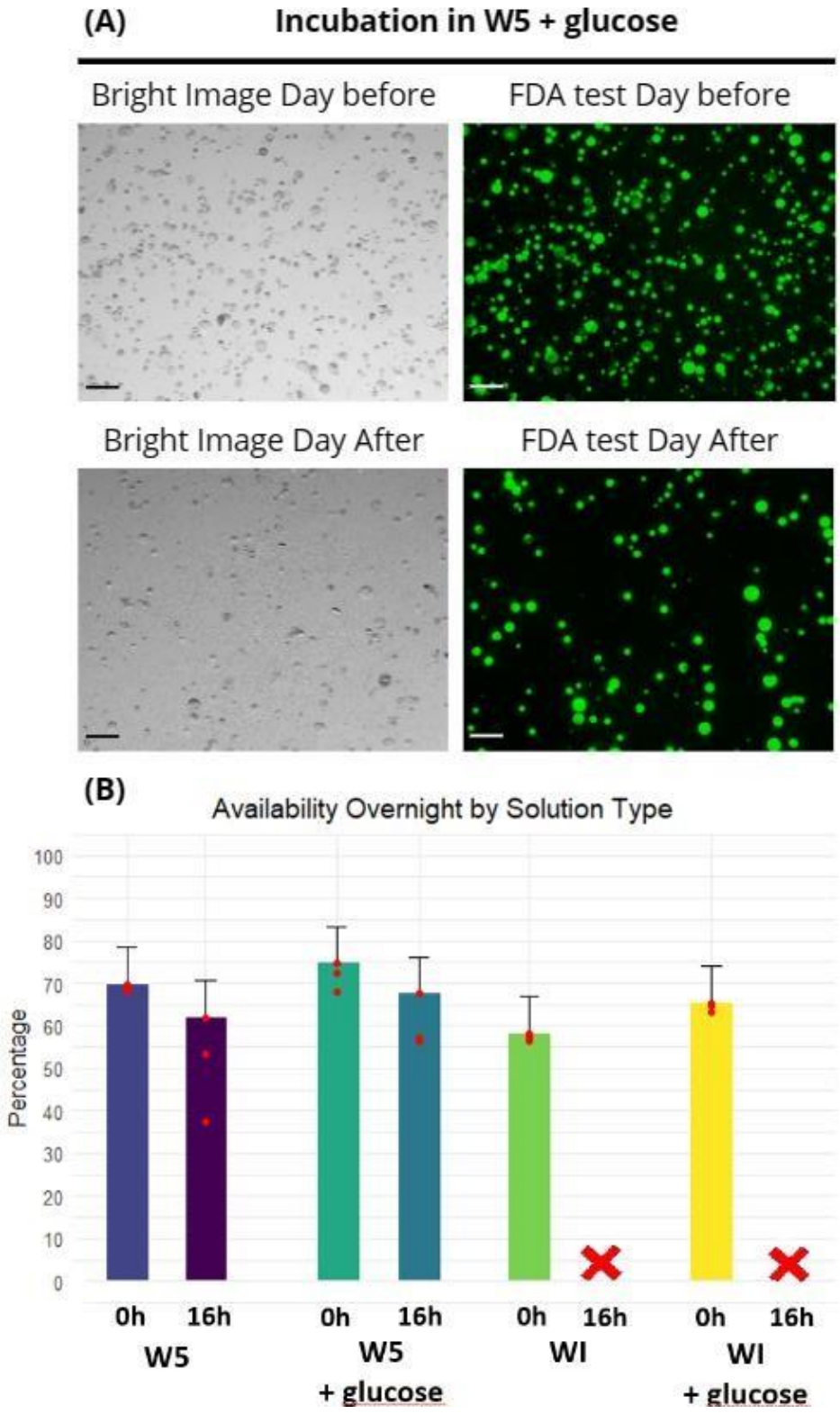


Figure 5. Exploration of different solutions for staining protoplasts overnight. (A) Images in brightness and fluorescence spectrum of protoplasts incubated in W5 + glucose solution. The protoplasts viability was compared between the day before and the day after incubation by FDA test (B) Plot comparing protoplasts availability after isolation process (0h), and subsequent 16 hours within the 4 different solutions. Scale = 100 µm.

Optimizing the solutions for the transformation of pea root protoplasts.

After identified the most sustainable solutions for keeping *P. sativum* root protoplasts alive following an incubation of 16 hours, we moved our focus to optimizing the conditions for the transformation process.

The PEG calcium-mediated transformation technique relies on the use of polyethylene glycol (PEG) in conjunction with a sorbitol-magnesium (SMG) solution. This combination is crucial for enabling the successful uptake of DNA into cells. However, the osmotic shock induced by this method, while effective for facilitating DNA entry, can also impose significant stress on the protoplasts (Schapire et al., 2016). This stress factor is an important consideration in the transformation process, as it can impact the overall health and viability of the protoplasts.

To assess the impact of osmotic shock on protoplast viability, we conducted a PEG transfection simulation experiment, without the introduction of a DNA plasmid. We evaluated the viability of cells transfected with four distinct solutions, each varying in the concentrations of MgCl2 in the SMG solution and the concentration of PEG 4000 within PEG solution. These solutions included SMG25 (MgCl2 25 mM) + PEG 20% (w/v), SMG125 (MgCl2 125 mM) + PEG 20% (w/v), SMG25 (MgCl2 25 mM) + PEG 40% (w/v), and SMG125 (MgCl2 125 mM) + PEG 40% (w/v).

Following the transformation simulation process, protoplasts were incubated for 16 hours in the incubation solution (W5 + glucose), followed by an FDA viability test (Figure 6A). Among the tested solutions, the one composed of 125 mM MgCl2 and 40% PEG exhibited the lowest protoplast viability, with an average of only 13.93% after the incubation period. Meanwhile, the solutions containing 25 mM MgCl2 + 40% PEG and 125 mM MgCl2 + 20% PEG produced similar results, with protoplast viabilities of 39.77% and 37.72%, respectively. Strikingly, the solution comprising 25 mM MgCl2 and 20% PEG recorded the highest protoplast viability at 61.26% (Figure 6B) on the following day.

As previously mentioned, the concentration of the PEG solution plays a pivotal role, impacting the stress levels experienced by the protoplast cells. However, it is also crucial for facilitating the passage of DNA plasmids through the cell membrane. Consequently, we have identified 25 mM MgCl2 and PEG 20% as the optimal concentrations within transformation solutions to minimize protoplast deterioration through protoplast transfection.

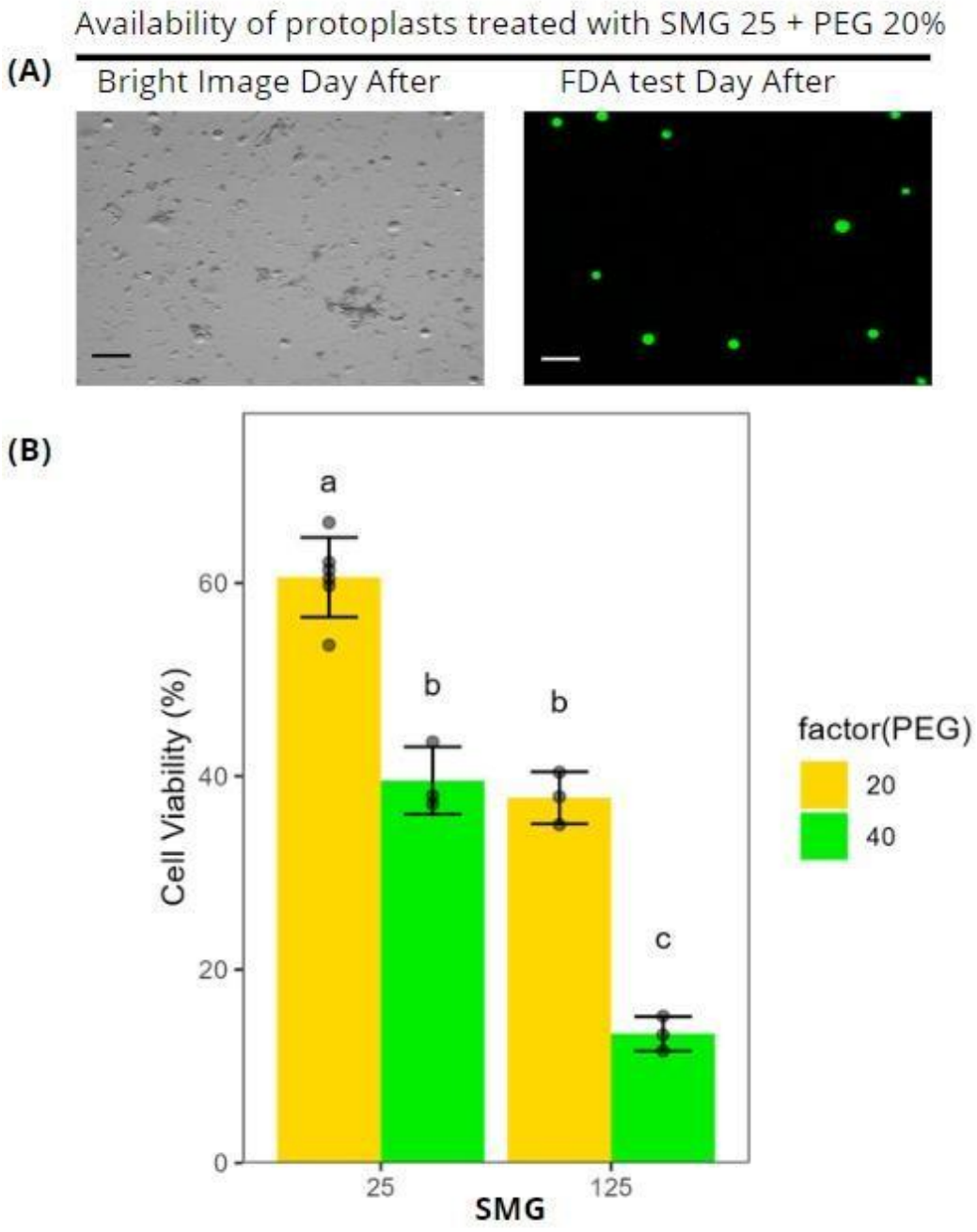


Figure 6. Osmotic balance availability, balance between PEG and SMG solution. (A) Image in bright of protoplast cells transfected with 25 mM $MgCl_2$ (mmG solution) and PEG 20%. (B) Data analysis of 4 different transfection balance solutions. "a, b, c" indicates groups determined by multiple comparisons tests after ANOVA analyses. Scale = 100µm.

PEG concentration and transformation efficiency variation.

Polyethylene glycol (PEG) serves as a fundamental component in PEG-calcium-mediated transformation. Given the inherent sensitivity of protoplasts to external conditions and the inherent cellular stress incurred during transformation, achieving an ideal transformation balance where cells maintain their viability while effectively undergoing transformation is of paramount importance (Hayashimoto et al., 1990).

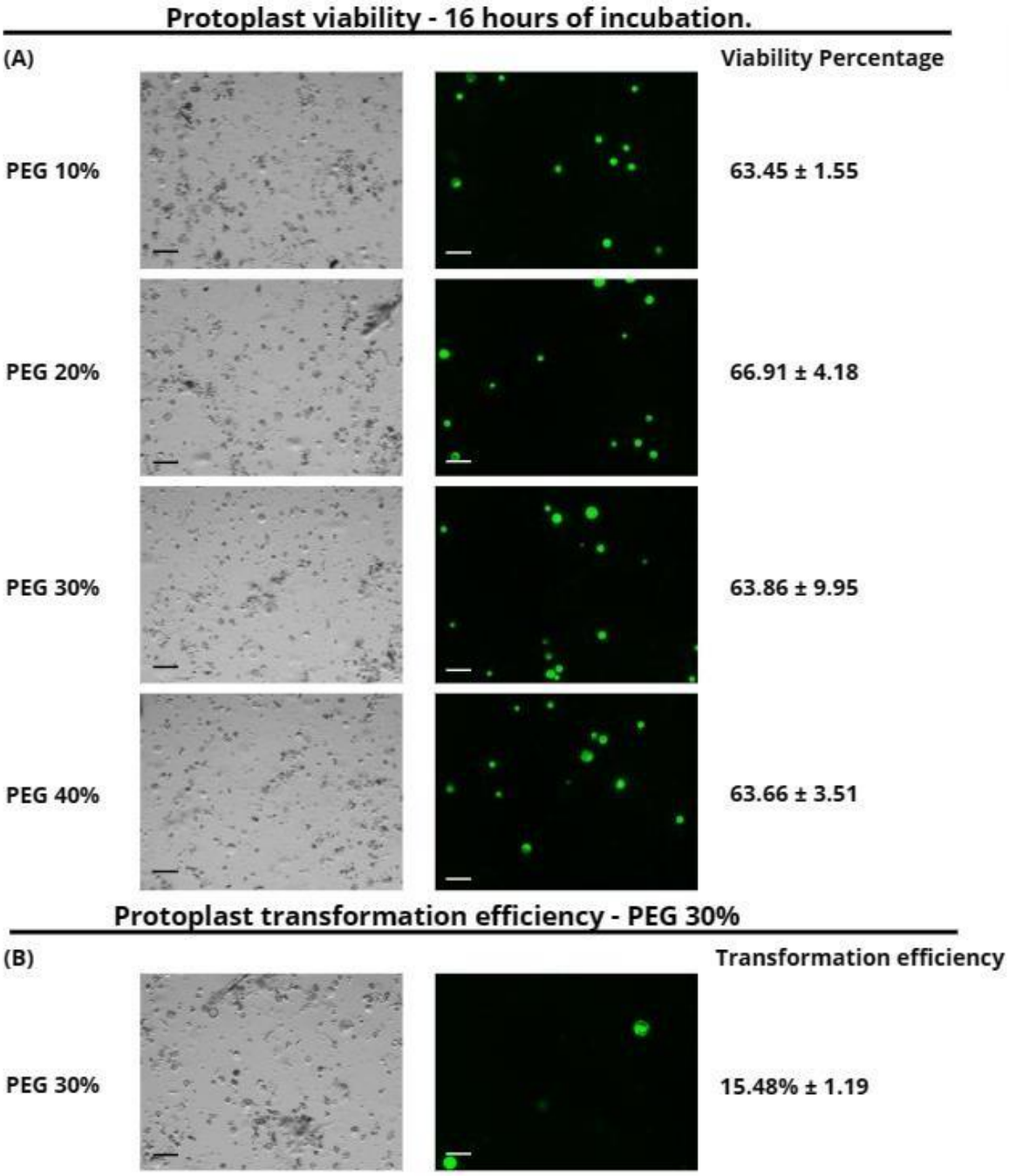
Firstly, to assess the effect of various PEG concentrations on the viability of *P. sativum* root protoplasts, we simulated a 5-minute transformation experiment using PEG at concentrations of 10% (w/v), 20% (w/v), 30% (w/v), and 40% (w/v). Following a 16-hour overnight incubation, the viability of the protoplasts was determined using an FDA assay. Interestingly, the results showed a consistent pattern of viability across the different PEG concentrations, with minimal variance observed. Protoplasts treated with 20% PEG displayed the highest viability, averaging 66.91% viable cells. This outcome aligns closely with the results from a previous experiment, underscoring the effectiveness of the 20% PEG concentration. Meanwhile, protoplasts incubated with 30% and 40% PEG showed slightly lower viability rates, at 63.86% and 63.66% respectively. The lowest concentration tested, 10% PEG, resulted in the smallest proportion of viable cells, averaging 63.45% (Figure 7A). These findings suggest that while PEG concentration does influence protoplast viability, the variance is relatively small within the tested range, particularly noting the consistent performance of the 20% PEG solution.

Subsequently, we designed an experiment encompassing multiple transformation processes, each employing distinct PEG concentrations (w/v): PEG 10%, PEG 20%, PEG 30%, and PEG 40%. Notably, in contrast to our previous experiment, we employed 20µg of a plasmid carrying a *GREEN FLUORESCENT PROTEIN* (*GFP*) gene driven by the constitutive 35S promoter (35s : GFP) as the foreign DNA. This plasmid serves as a visual marker, since upon successful integration into the cells, emits a detectable fluorescence signal following a 16-hour incubation period in W5 + glucose solution (Figure 7B).

In our study examining transformation efficiencies with varying concentrations of PEG, clear differences were observed. At a 10% (w/v) PEG concentration, there was no evidence of cell transfection, indicated by the absence of fluorescence signals. The most effective transformation occurred at a 30% PEG concentration, with an average efficiency rate of 15.48%. With 20% PEG, the transformation efficiency stood at 7.22%, and at 40% PEG, it slightly decreased to an average of 11.15% (Figure 7C). These findings suggest a specific concentration range of PEG is more conducive to successful protoplast transformation.

This experiment, focusing on the effect of different PEG concentrations on the viability of *P. sativum* root protoplasts, didn't reveal a definitive trend in how cell viability varies with PEG concentration. However, it did establish that a 30% PEG concentration is the most effective for introducing plasmid DNA into the protoplasts. Although the achieved transformation efficiency of 15.48% indicates successful transfection, there is potential for further enhancement. Recognizing this, we are now exploring other variables that could significantly impact transformation efficiency. Key factors under consideration include the concentration of the plasmid and the duration of the transfection process. Adjusting these parameters could potentially lead to more effective and efficient transformation of *Pisum*

sativum root protoplasts, further advancing our understanding and application of this technique.



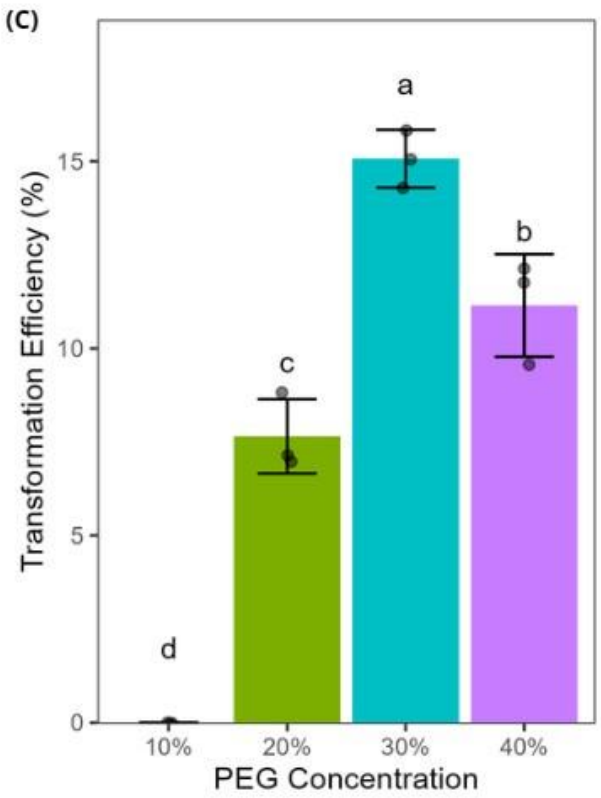


Figure 7. (A) FDA images of pea protoplasts transfected in 10% (w/v), 20% (w/v), 30% (w/v), and 40% (w/v) PEG4000 solutions. The FDA fluorescence was observed and imaged after 16 hours transfection with water. (B) Image expressing GFP signal after transfected protoplasts with PEG 30% (w/v) after 16 hours of incubation. (C) Protoplast transfection efficiency with different concentrations of PEG4000 solutions (10%,20%,30%,40% w/v). Respectively. "a, b, c, d" indicates groups determined by multiple comparisons tests after ANOVA analyses for protoplast transfection efficiency, respectively. Scale = 100 μm.

Discussions and conclusions

In this report, we have rigorously examined and enhanced a protocol for root protoplast isolation and transformation in Pea. It is noteworthy that this report represents the first documentation of such a protocol for pea roots, showcasing the innovation and significance of our work. The established and optimized protocol consistently yields a substantial number of isolated protoplasts with an acceptable level of transfection efficiency.

To account for inherent variability in the isolation and transfection process, adjustments in certain steps may enhance both protoplast yield and transfection efficiency. Researchers are encouraged to engage in additional practice and refine the protocol to suit their unique conditions and requirements, given the diverse experimental setups and pea plant varieties. While this system proves highly valuable for examining immediate regulatory and biochemical events within plant cells, it is essential to note its limitations.

Our research has demonstrated that, on average, 7.169E+04 cells · root^{-1} protoplasts are yielded after 6 hours of incubation within the digestion solution. While many factors may influence the viability of isolated protoplasts, the primary crucial factors are the enzyme compositions and their related concentrations. However, we also believe that the quality of the root plays a vital role. Unfortunately, there is a lack of established protocols for isolating protoplasts from pea roots, making it difficult to directly compare our isolation efficiency with other studies. Nevertheless, we can draw some satisfaction from our results when we consider a study on another legume species, *L. japonicus*, where an average of 1·10E+05 cells · root^{-1} was isolated (Jia et al. in 2018).

Moreover, the study has investigated into the selection of an optimal incubation solution for *P. sativum* root protoplasts. Through empirical observations, we have determined that the W5 solution, which includes glucose at a concentration of 5mM, serves as the preferred overnight solution for maintaining cell viability post-transfection. Notably, the literature reveals different choices regarding the incubation solutions. For example, in the cases of Soybean (Wu et al., 2018) and *L. japonicus* and *M. truncatula* roots (Jia et al., 2018), WI solution has been reported, differing in the presence of sorbitol or mannitol. It is noteworthy that W5, which lacks sorbitol or mannitol, is hypertonic with a smaller osmotic potential compared to WI. The balance of osmotic potential in the solution may emerges as a critical factor in influencing the efficacy of the incubation process.

Our transfection protocol demonstrates an average transfection efficiency of 15.48% for GFP, while the highest efficiency, achieved with 20 µg of GFP, reaches up to 21.5%. In contrast, other legume species, such as soybean, *L. japonicus*, and *M. truncatula* root protoplasts, have reported significantly higher transfection efficiencies, ranging from 60 to 70% (Jia et al., 2018). This discrepancy underscores the importance of further studies to explore strategies for enhancing transformation efficiency in our system. Parameters such as plasmid concentrations, transformation time, and osmotic conditions during transformation may play pivotal roles in improving the overall transformation efficiency. A comprehensive investigation into these factors is crucial for advancing the efficacy of the transformation process.

With the implementation of this refined protocol, we have achieved promising results in terms of both yields and the feasibility of transfection. This protocol stands as a versatile and convenient approach for transient gene expression, enabling the study of protein subcellular localization and various cellular events. We are confident that this comprehensive protocol will find wide-ranging applications and prove invaluable for future research focused on this significant crop.

Material and Methods.

Plant, seed harvesting, and root growth.

The growth journey of Cameor Pea plants embarked with the meticulous germination of ten seeds immersed in water within a 50 ml tube, undergoing a 14 to 16-hour soaking period on a rotator at 20 RPM. Subsequently, seeds were sown in 1 L pots filled with commercial soil, fostering growth in a Modular Climate Chamber under a long-day photoperiod (16/8) at 20°C. After approximately 14 days, germinated plants were transplanted into 3 L pots for enhanced root development. Vigilant monitoring and maintenance supported their healthy progression, culminating in the emergence of blooms around 20 days post-transplantation. A strategic cessation of irrigation initiated seed maturation, leading to harvest ready Cameor pea seeds after two and a half months.

The critical stage of seed sterilization involved a meticulous process within a desiccator using chlorine gas, ensuring the highest standards of cleanliness. Subsequent steps included bleach treatment, washing under laminar flow conditions, and overnight soaking, crucial for water absorption and metabolic activation essential for germination.

Root growth took center stage in agar-filled magenta boxes, optimizing conditions for robust primary root development. The gelation process was meticulously managed, with 200 ml of 0.4% Phytoagar (w/v) in MilliQ water in each box. Precision in sowing, directing the embryonic axis into the agar gel, facilitated vertical stem growth and root development.

After a meticulous four-day cultivation period inside the Modular Climate Chamber in the dark at 25°C, the roots exhibited impressive growth characteristics. Approximately 70-80% of the seeds sown, after 4 days, were expressing roots displayed a linear and well-defined shape, and lengths exceeding 3 cm, aligning with the desired length specified in our protocol for optimal use in the isolation process.

Agar solution: 0.4% (w/v) Phyto Agar. Autoclave and then wait for the complete jellification inside magenta boxes.

Protoplast isolation.

In this project, our focus was on optimizing and comparing isolation methods to identify the most efficient approach for isolating pea root protoplasts. Our investigation delved into different enzymatic digestion solutions, specifically comparing the conventional enzymatic solution for pea mesophyll protoplasts "ES" (Yoo et al., 2007) with a more advanced formulation incorporating Viscozyme (designated as ES + Viscozyme). This comparison aimed to enhance the efficacy of protoplast isolation for pea roots, taking a step beyond established methods and exploring the potential benefits introduced by the inclusion of Viscozyme in the enzymatic solution.

Commencing with the harvest of primary roots after a 4-day growth period, the roots were sectioned into 3 cm segments using a ruler and an edge blade. Individual root sections were then submerged in 5 ml of the respective digestion solution and cut into 1 mm slices.

The roots underwent a strategic vacuum infiltration within a desiccator for a duration of 10 minutes. Following this, a meticulous incubation in the digestion solution for 6 hours in the dark at RT, accompanied by gentle shaking, fostered the release of abundant and high-quality protoplasts. The dynamic progression of the digestion process was meticulously monitored under a microscope, revealing liberated protoplasts with a distinct rounded morphology. To quantitatively assess the concentration and kinetics of protoplast release, a comprehensive analysis was conducted using a hemocytometer at distinct time points—namely, 2, 4, and 6 hours from the initiation of the digestion process. This integrated approach allowed for realtime observation of protoplast dynamics and provided valuable insights into the efficiencies of the two enzymatic solutions.

The digestion process was concluded by introducing W5 solution, followed by filtration through 100 µm and 40 µm strainers to eliminate root pieces and minimize debris. Centrifugation at 400 x g for 2 minutes allowed for the separation of protoplasts from the supernatant. After resuspension in chilled solution and centrifugation, the protoplast pellet was delicately resuspended in W5. The digested protoplasts were then transferred to a 5 ml tube, kept on ice for 60 minutes.

Enzymatic solution "ES": Prepare 10 mM MES (pH 6.0) containing 1% (w/v) cellulase R-10, 0.5% (w/v) macerozyme R-10, 0.4 M d-Sorbitol, 10 mM CaCl2, 20 mM KCl. Warm the solution at 55 °C for 10 min to inactivate DNase and proteases, and then cool it to room temperature. Subsequently, include 0.1% (v/v) BSA, 5 mM B-mercaptoethanol.

Enzymatic solution "ES + Viscozyme": Prepare 10 mM MES (pH 6.0) containing 1,5% (w/v) cellulase R-10, 1% (w/v) macerozyme R-10, 0.4 M d-Sorbitol, 10 mM CaCl2, 20 mM KCl. Warm the solution at 55 °C for 10 min to inactivate DNase and proteases, and then cool it to room temperature. Subsequently, include Vyscozyme 5% (v/v), 0.1% (w/v) BSA, 5 mM B-mercaptoethanol.

W5 solution: 10 mM MES (pH 5.8), 154 mM NaCl, 125 mM CaCl2, and 5 mM KCl. The prepared W5 solution can be stored at 4 °C.

Optimization of protoplasts long-term incubation conditions.

In the pursuit of determining the optimal solution for long-term incubation, freshly isolated pea protoplasts underwent a centrifugation step at 400 RCF for 2 minutes at room temperature (RT). Following this, the protoplasts were delicately resuspended and subjected to incubation in distinct solutions: W5, WI, W5 + glucose, and WI + glucose. After a 16-hour incubation period, the viability of protoplasts within each solution was systematically quantified and compared. The assessment of viability, meticulously conducted through the FDA test, yielded valuable insights into the effectiveness of different incubation solutions in sustaining the health and viability of pea protoplasts.

W5 solution: 10 mM MES (pH 5.8), 154 mM NaCl, 125 mM CaCl2, and 5 mM KCl. The prepared W5 solution can be stored at 4 °C.

W5 + glucose solution: 10 mM MES (pH 5.8), 154 mM NaCl, 125 mM CaCl2, 5 mM KCl, and 5mM Glucose. The prepared W5 solution can be stored at 4 °C.

WI + glucose solution: 4 mM MES (pH 5.8), 20 mM KCl, 0.5 M D-Sorbitol, 5 mM Glucose. The prepared WI solution with glucose can be stored at 4 °C.

WI solution: 4 mM MES (pH 5.8), 20 mM KCl, 0.5 M D-Sorbitol. The prepared WI solution with glucose can be stored at 4 °C.

PEG mediated transformation.

After protoplast isolation, washing and yield measurement, the cells were centrifuged for 2 minutes at 400 RCF, and the supernatant was carefully discarded. Protoplasts were resuspended in SMG solution to a final concentration of 2 x 106 protoplasts per ml (4 x 105 per 200 µl). In total 20 µg of the GFP reporter plasmid 35S-sGFP(S65T)-NOS3′ (Chiu et al., 1996), or water was added into resuspended protoplasts, and then they were mixed well by gently invert the tube. The PEG solution was then added into protoplasts culture, and the tube was carefully inverted to ensure homogenization of the mixture. Cells were kept in PEG for 5 minutes at RT and the transfections were stopped immediately by adding 900 µl of W5 solution and followed by centrifugation for 2 minutes at 400 RCF. Cells were then washed with the W5 solution. Washed protoplasts were then resuspended in W5 solution with 5mM glucose for 16 hours. After 16 hours, the fluorescence was inspected by Leica fluorescence microscopy.

SMG solution: 5 mM MES (pH 5.8), 0.4 M d-Sorbitol, and 25 mM MgCl2. Storing at RT.

Peg-calcium transformation solution: 30% (w/v) PEG4000, 0.2 M d-Sorbitol, and 100 mM CaCl2. PEG solution should always be freshly prepared.

Verification of suitable transfection PEG4000 concentration for pea mesophyll protoplasts.

In order to test the best method for PEG transformation, we tested different concentration of PEG4000 in the PEG solution in terms of transformation efficiency and protoplast availability. To evaluate the efficiency of the transformation, cells were transfected using 20 ug of the GFP reporter plasmid 35S-sGFP(S65T)-NOS3′ (Chiu et al., 1996), while for evaluating protoplast viability, we simulated the transformation process avoiding employing the plasmid. To test which PEG concentration is suitable for the transfections of pea protoplasts, we tried PEG solutions with four different PEG concentrations (10%, 20%, 30%, 40% (w/v) PEG). The transfection occurred as described previously. The cells were washed in W5 and resuspended in W5 + glucose solution. The protoplasts were then incubated in the dark at room temperature for 16 hours. In both experiments, the viability of cells (FDA test) and the transfection efficiency was determined to assess the optimal conditions for PEG transformation of pea protoplasts cells.

Peg-calcium transformation solution: 10%, 20%, 30%, 40% (w/v) PEG4000, 0.2 M dSorbitol, and 100 mM CaCl2. PEG solution should always be freshly prepared.

Measurements.

The yield of protoplasts was determined by hemocytometer under a light microscope. A 12 µL aliquot of recovered protoplasts was applied to the edge of the coverslip to be sucked into the void by capillary action. The number of protoplasts in four counting areas of chamber was quantified under microscope. The concentration of isolated protoplast culture was determined by hemocytometer. Viability of protoplasts was determined by fluorescein diacetate assay (FDA) (Huang et al., 1986). In total 40 µl protoplast culture was mixed with 1 µl 0.2% FDA solution (dissolved in acetone). Viable protoplasts were then visualized and photographed with Leica fluorescence microscope under 488 nm laser and a GFP filter. Three pictures were taken for each treatment. Protoplast viability was then measured with formular “Protoplasts with green fluorescence divided by the total number of protoplasts in the picture × 100%”.

Data Analysis.

All statistical analyses were conducted in R version (RStudio Team 2020). Independent T test and ANOVA were applied in comparison between experiments with a P value threshold of $P < 0.05$.

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Simplified and optimized protocol for isolation and transfection of *Pisum sativum* root protoplasts.

Protoplast isolation.

(1) high-quality primary roots grew in magenta boxes with Agar concentration of 0,4% (w/v) were harvested after 4 days.

(2) Using a ruler, the roots were sectioned into 3 cm segments with an edge blade (snapped in two). The roots were transferred individually to a petri dish, where they were submersed in 5 ml of freshly prepared digestion solution and cut into sections of approximately 1mm each (20-30 cm slices from a singular root).

(3) The petri dish containing the root slices was placed in the vacuum desiccator, connected to a vacuum pump, and vacuumed infiltrated for 10 minutes at room temperature.

NOTE: Infiltration of the root tissues is critical for the quick release and yield of good protoplasts.

(4) Roots were incubated in the digestion solution at room temperature with gentle shaking overnight for 6 h.

(5) The digestion process was completed by introducing 5 ml of W5 solution into the root digestion mixture. Subsequently, the solution was passed through 100 µm tissue strainer into a 50 ml tube, in order to eliminating root pieces. An additional 5 ml of W5 was used for cleaning the petri dish.

(6) The solution was then filtrated through finer 40 µm strainer. This double-filtration process allowed us to obtain fewer debris particles while ensuring protoplasts passed through the mesh.

(7) The tube containing the digested protoplasts was centrifugated in a swinging bucket rotor at 400 x g for 2 min at room temperature.

NOTE: Decelerate the centrifuge at its lowest setting to minimize potential damage to protoplasts during centrifugation. It is highly recommended to utilize round-bottom tubes and swinging-bucket rotors for this process.

(8) The supernatant was gently removed without direct contact with the pellet. Subsequently, the protoplast pellet underwent careful resuspension in 15 ml of W5 chilled solution. The centrifugation was repeated using the settings previously mentioned.

(9) The supernatant was then delicately aspirated, and the pallet was gently resuspended in 5 ml of W5.

(10) Lastly, the digested protoplasts were transferred to a 5 ml tube using a wide-end tip pipet and kept on ice for 60 minutes.

Protoplast PEG – transfection

(1) After the cooling period. the supernatant was removed from the 5 mL tube.

(2) The protoplasts settled on the bottom were gently resuspended with the appropriate volume of SMG solution to reach a concentration of 1.8 x 10^6 protoplasts mL^{-1}.

$$x\ (\mu L) : 1.8 \times 10^6 = 200\ \mu L : 4 \times 10^5$$

NOTE: Cameor Pea root protoplasts have shown to be sensitive thoroughly the protocol, with a notable susceptibility to SMG solution. It is highly recommended to transition to the PEG – calcium mediated transfection at the earliest.

(3) For each transfection, a volume of x µL containing 20 µg of plasmid DNA was aliquoted into a 2 ml round-bottom tube, with the calculations based on the concentration of the 35s : GFP vector. Additionally, 25 µL of SMG solution was initially mixed with the plasmid.

(4) 200 µL of protoplasts was gently added to the tube.

(5) x µL of PEG solution (equal volume of protoplasts + mmG + plasmid DNA) was slowly added down the side of the tube and gently tapped to mix well.

(6) The transfection reaction was incubated at RT for 5 minutes.

(7) To culminate the transfection, 900 µL of cold W5 was added to the tube, ensuring to be well mixed.

(8) The protoplasts were collected by centrifugation at 400 x g for 2 min at room temperature. After centrifugation the supernatant was removed.

(9) Protoplasts were gently resuspended in 800 µL of W5 + glucose solution.

(10) Incubate the protoplasts overnight, positioning the tubes at a slight incline, this inclination facilitates optimal cellular distribution.

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