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# Optimization of Isolation and Culture of Protoplasts in Alfalfa (*Medicago sativa*) Cultivar Regen-SY

**Ankush Sangra[1], Lubana Shahin[1], Sarwan K. Dhir[2*]**

[1]College of Agriculture, Family Sciences and Technology, Fort Valley State University, Fort Valley, GA, USA
[2]Department of Plant Sciences, Center for Biotechnology, Fort Valley State University, Fort Valley, GA, USA
Email: *dhirs0@fvsu.edu

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## Abstract

Alfalfa (*Medicago sativa*) is an important forage crop belonging to the Fabaceae family. It is cultivated across the world for fodder and originated in Asia. Alfalfa cultivar Regen-SY was used in this study which is a hybrid of first-generation self-parents from Regen-S (*M. sativa*) and Regen-Y (*Medicago falcata*) research cultivars. The main objective of the study was to optimize conditions for the isolation and liquid culture of alfalfa Regen-SY protoplasts. Several factors like enzyme combination, incubation time, plant age, centrifugation speed and shaker speed affecting protoplast isolation and culture were optimized in the study. The yield and viability of the protoplasts was determined by using hemocytometer and Fluorescein diacetate (FDA) staining respectively. Results showed that factors like enzyme combination, incubation time, plant age, centrifugation speed and Mannitol concentration significantly ($p \leq 0.05$) affect protoplast yield and viability whereas shaker speed didn't result in any significant difference in the yield and viability of protoplasts. Using optimum conditions protoplasts were cultured in the liquid medium and microcalli formation was achieved after five weeks of the culture. The protocol established in this study will assist researchers in the isolation and culture of protoplasts in alfalfa and will accelerate the research processes like protoplast fusion and genetic engineering.

## Keywords

Alfalfa, Protoplast, Protoplast Isolation, Protoplast Culture, Optimization

## 1. Introduction

Alfalfa (*Medicago sativa*) is one of the important leguminous forage crops be-

longing to the Fabaceae family. It originated in Asia and is cultivated across the world for fodder [1]. Alfalfa is highly nutritious containing protein (15.2%), calcium (1.5%) and phosphorous (0.2%), vitamin A, B and D. As alfalfa is a legume, it forms a symbiotic association with the bacterium *Sinorhizobium meliloti* which fixes atmospheric nitrogen [2]. A single stand of alfalfa can fix about 300 pounds of nitrogen each year. This results in the increase nitrogen availability for the plants and increase in soil nitrogen fertility for subsequent crops in rotation [3]. Alfalfa is genetically classified as autotetraploid and grows under more diverse conditions than other perennial species [4]. Alfalfa hybrid Regen-SY was released in 1989 and it was produced using first generation self-parents from Regen-S (*M. sativa*) and Regen-Y (*Medicago falcata*) research cultivars [5].

The protoplasts are the living material of the plant or bacterial cell after the removal of cell wall. Cell wall is a major hindrance towards the direct DNA transfer to the cell and is therefore required to be removed. Cocking (1960) isolated tobacco protoplast and since then it has been recorded in many crops [6]. Protoplast technology has become one of the important tools of the genetic engineering and crop breeding [7]. Dovzhenko *et al.* (2003) developed protocol for the regeneration of plants from cotyledon-based protoplast system for *Arabidopsis thaliana* for molecular studies [8].

There are many factors that influence protoplast isolation, yield, viability and culture. Seedling leaves have been reported to be one of the most convenient sources of the protoplasts [9]. In apricot, the number of the protoplast obtained increased significantly when leaves were subjected to plasmolysis for 90 minutes in 13% sorbitol solution [10]. Using mixture of enzymes pectinase and cellulase would simultaneously separate cells and degrade their cell wall [11]. Powchgee *et al.* (2006) reported that the time of incubation significantly affects the yield and viability of the protoplasts in *Anubia nana* Engler [12]. The type of the enzyme and the concentration of enzyme are two important factors that influence isolation of protoplasts [13].

Protoplasts are known to rupture in hypertonic solution and collapse in hypotonic solution [14]. Therefore, it is important to optimize the concentration of the osmoticum to be used in buffer to increase the yield of viable protoplasts. Glucose, sucrose, mannitol and sorbitol are some of the inert sugars that can be used as osmoticum in protoplast isolation [15]. Other factors that affect protoplast isolation are environmental conditions, shaking, and agitation [16]. High temperature during the protoplast isolation can cause agglutination of cell organelles in the protoplasts and can affect the stability of the plasma membrane [17].

Alfalfa is highly genotype dependent. In addition, it also shows intervarietal and intravarietal variations and form heterogenous and heterozygous populations. Therefore, it is very difficult to develop protocols for protoplast isolation, culture and regeneration accommodating all cultivars. In addition, there are many factors that influence protoplast isolation, therefore, there is need to optimize conditions for the protoplast isolation. Through this study, we are publishing first report on the optimization of conditions for the protoplast isolation and culture of

the alfalfa cultivar Regen-SY.

## 2. Materials and Methods

### 2.1. Plant Material

Alfalfa cultivar Regen-SY germplasm (PI 537440) was obtained in the form of seeds from Western Regional PI Station through U.S. National Plant Germplasm System.

### 2.2. Seed Surface Sterilization and Germination

Seeds were surface sterilized using 70% ethyl alcohol for 30 s followed by 20% bleach (Clorox®) treatment for 10 min. Seeds were rinsed with sterile distilled water for three times and then germinated on Murashige and Skoog (MS) basal medium (PhytoTechnology Laboratories, KS, USA) containing 3% sucrose and 0.7% agar (PhytoTechnology Laboratories).

### 2.3. Optimization of Factors Affecting Protoplast Isolation

Fully expanded dark leaves from plants of different age (2, 4, 6 and 8 weeks of subculture) were excised and 1 g of leaf tissue was weighed. Leaf tissues were provided incisions using sterile scalpel. Plant material was immediately transferred to deep petri dish (60 × 20 mm, Nunc Lab-Tek®) containing 10 mL of enzyme (PhytoTechnology Laboratories) solution (Table 1). Plant material was incubated with enzyme solution for 2 - 8 h in the dark with gentle shaking (50 - 70 rpm) on a shaker (Brunswick C2 Platform shaker) for enzymatic digestion. Similarly, the enzyme solution consisting of different concentrations of cellulase (PhytoTechnology Laboratories) and macerozyme (PhytoTechnology Laboratories) along with various concentration of mannitol were tested to determine their

Table 1. Enzyme mixtures used for protoplast isolation.

| Enzyme Mixture | Cellulase Onozuka R-10 (% w/v) | Macerozyme R-10 (% w/v) |
|---|---|---|
| 1 | 1.0 | 0.5 |
| 2 | 1.5 | 0.5 |
| 3 | 2.0 | 0.5 |
| 4 | 2.5 | 0.5 |
| 5 | 1.0 | 1.0 |
| 6 | 1.5 | 1.0 |
| 7 | 2.0 | 1.0 |
| 8 | 2.5 | 1.0 |
| 9 | 1.0 | 1.5 |
| 10 | 1.5 | 1.5 |
| 11 | 2.0 | 1.5 |
| 12 | 2.5 | 1.5 |

effect on protoplast isolation. Dark conditions were created by wrapping aluminum foil around the petri dish.

## 2.4. Protoplast Purification

- After enzymatic incubation the digestion solution was passed through the nylon mesh of appropriate size (50 µm).
- Protoplasts were then washed three times with cell and protoplast washing solution (CPW) containing 0.7 M mannitol and centrifuged (HN-SII centrifuge, IEC, USA) at 500 - 2000 rpm for 10 min after each washing. The composition of CPW is given in Table 2.
- Purified protoplast suspension was then checked for protoplast yield and viability.

## 2.5. Protoplast Viability and Quantification

Protoplasts were quantified using hemocytometer. Viability of the protoplasts was estimated by Fluorescein diacetate (FDA) staining assay. About 10 µL of protoplast mix was pipetted on to a Neubauer Hemocytometer (Reichert, USA) with cover slips. Entire chamber was filled with protoplast suspension and filled hemocytometer slide was viewed under microscope of 40× magnification. FDA stains living (viable) protoplasts resulting in green fluorescence.

Percentage viability was determined by the formula given below:

$$\text{Percentage viability} = \frac{\text{Number of viable cells counted}}{\text{Total cellls counted}\left(\text{Viable and dead}\right)} \times 100$$

The concentration of protoplasts per mL per gram of leaves was determined by formula:

$$\text{Concentration} = \frac{\text{Average number of cells in one large square}}{\text{weight of leaves material used} \times \text{dilution factor} \times 10^4}$$

## 2.6. Fluorescein Diacetate (FDA) Staining Assay

Fluorescein diacetate stock solution was prepared by dissolving 5 mg/mL FDA (Sigma) in acetone. Fluorescein diacetate working solution was prepared by taking

Table 2. Composition of cell and protoplast washing solution (CPW).

| S. No. | Component | mg/L |
|---|---|---|
| 1 | Calcium chloride | 148 |
| 2 | Cupric sulfate | 0.025 |
| 3 | Magnesium sulfate | 246 |
| 4 | 2-(N-morpholino)ethanesulfonic acid (MES) | 976 |
| 5 | Potassium nitrate | 101 |
| 6 | Potassium iodide | 0.160 |
| 7 | Potassium phosphate monobasic | 27.2 |
| 8 | Mannitol | 130,000 |

20 µL of working solution and mixing it with 1 mL of CPW solution. For staining, equal volume of FDA working solution and protoplast suspension was used.

### 2.7. Protoplast Culture

- Protoplasts were cultured at different densities. *i.e.* $1 \times 10^4$, $2 \times 10^4$, $1 \times 10^5$, $2 \times 10^5$ per mL of liquid KP8 medium (modified from [18], **Table 3**).
- The cultures were maintained in the dark for 5 d and then transferred to 25˚C ± 2˚C with 16 h light regime of 25 $\mu E \cdot m^{-2} \cdot s^{-1}$ light radiation.
- A mixture of KP8:K8 (modified from [19]) was used for the progressive replacement of the bathing medium after 7 (3:1), 14 (2:1), 21 (1:1) and 28 (0:1) days.
- Microscopic observations of the protoplast experiments were carried out using Olympus IX70 inverted fluorescent phase contrast microscope (Olympus, Japan).

### 2.8. Statistical Analysis

Experimental data were statistically analyzed using analysis of variance (ANOVA). Treatment means were separated by using Tukey Kramer honestly significance difference (HSD) test at $p \leq 0.05$. Data were analyzed using Shiny application (Web based software for ANOVA) [20].

## 3. Results

### 3.1. Effect of the Enzyme Concentration and Combination on the Protoplast Yield and Viability

All the enzyme treatments shown in **Table 2** were tried and out of these treatment, the treatments shown in the graph (**Figure 1**) showed considerable Digestion

**Table 3.** Composition of KP8 (KM8P) medium and K8 medium used in the study.

| S. No | Component | KP8 (mg/L) | K8 (mg/L) |
|---|---|---|---|
| 1 | Kao & Michayluk Basal Salt Mixture (PhytoTechnology Laboratories) | 3900 | 3900 |
| 2 | Benzyl aminopurine (BAP) | 0.5 | 0.5 |
| 3 | 2,4-Dichlorophenoxyacetic acid | 1 | 1 |
| 4 | Sucrose | 250 | 125 |
| 5 | Glucose | 100,000 | 10,000 |
| 6 | Mannitol | 250 | 250 |
| 7 | Iron chelate sequestrene | 26 | 26 |
| 8 | Ribose | 250 | 125 |
| 9 | Xylose | 250 | 125 |
| 10 | Fructose | 250 | 125 |
| 11 | Sorbitol | 250 | 125 |
| 12 | Coconut milk | 20 ml/L | 20 ml/L |
| 13 | Kao & Michayluk vitamin solution (PhytoTechnology Laboratories) | 10 ml/L | 10 ml/L |

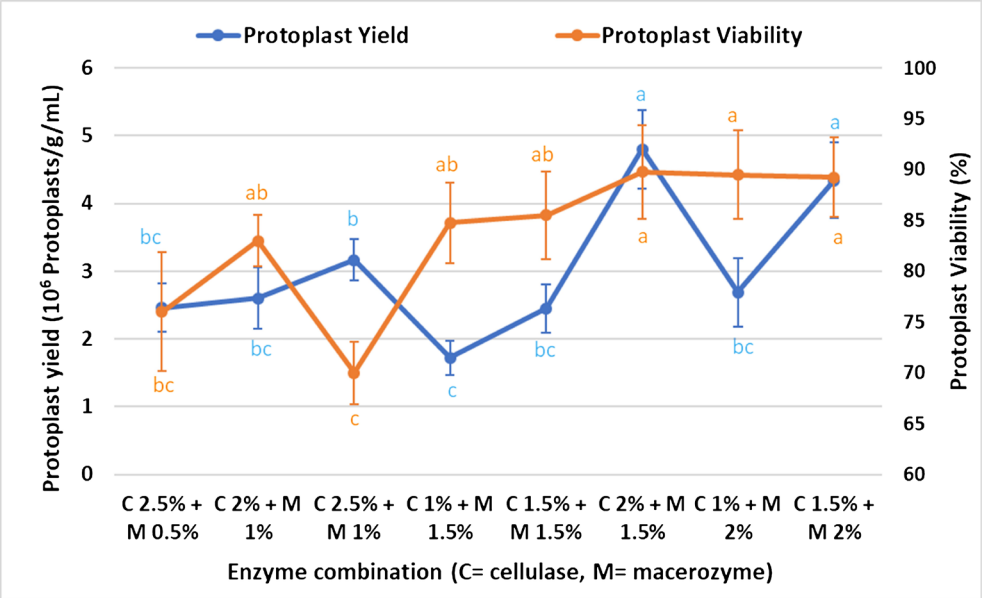


**Figure 1.** Effect of the enzyme combinations on protoplast yield and viability in alfalfa Regen-SY. Values represent mean ± SD (n = 3). The different letters indicate significant differences ($p \leq 0.05$) according to the HSD test.

of tissue within 8 h. One gram of leaf tissue was weighed and incisions were provided on the leaf surface with the help of sterile scalpel. Leaf tissue was incubated with the enzyme solution on C2 Platform shaker (New Brusnwick Scientific, Edison, NJ, USA) at 50 rpm. Some treatments showed completed digestion within 6 hours and some treatments didn't result in complete tissue digestion even after 10 hours. All treatments were observed under microscope and yield and viability was recorded for only those treatments which digested more than 50% of tissue. The maximum yield and viability was shown by treatment consissting of 2% Cellulase + 1.5% macerozyme.

### 3.2. Effect of Incubation (Enzymolysis) Time on the Protoplast Yield and Viability

The effect of emzymolyisis time was determined by using best enzyme treatment and taking leaf tissue from the 4-week old plant. One gram of the leaf tissue was incubated with best enzyme treatment for the 2, 4, 6 and 8 h. Two-hour enzymolysis treatment resulted in the minimum protoplast yield and viabilty (**Figure 2**). The tissue was not completey digested and therefore protoplast yield was low after 2 h of enzymolysis treatment. Six-hour enzyme incubation resulted in the maximum yield ($5.49 \times 10^6$) and viability (89.5%) of protoplasts. After 6 h leaf tissue was completely digested and this treatment, spherical shaped green protoplasts were obtained. The digestion of the cell wall was confirmed by absence of fluorescence after staining with calcoflour white. Two types of protoplasts were obtained, *i.e.* small (20 - 30 µm) and large (30 - 40 µm). The protoplasts had dense cytoplasm with chloroplasts arranged in peripheral area. Eight hour incubation treatment also caused complete digestion of the tissue but due to the

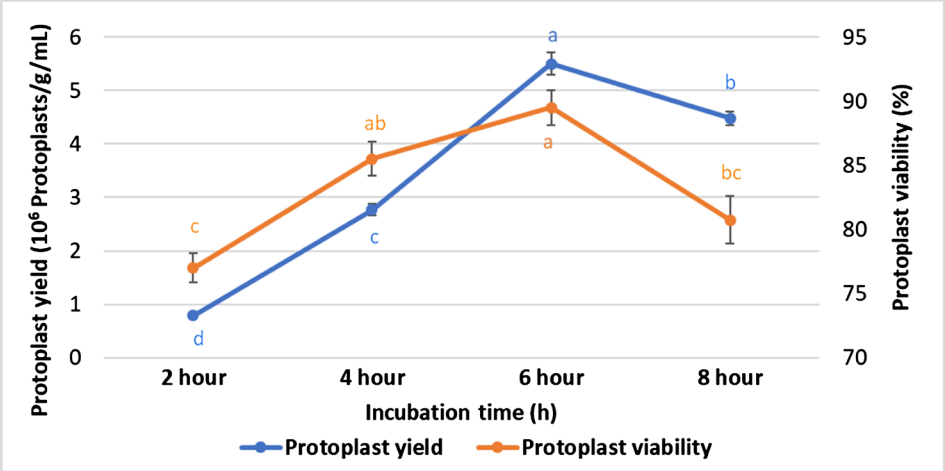


**Figure 2.** Effect of incubation time on protoplast yield and viability in alfalfa Regen-SY. Values represent mean ± SD (n = 3). The different letters indicate significant differences ($p \leq 0.05$) according to the HSD test.

prolonged exposure of the portoplasts to enzyme, there was shrinking and bursting of the protoplasts resulting in reduced yield and viability as compared to the 6 h treatment. Therefore through this experiment it was observed, exposing protoplasts after tissue digestion results in toxicity which reduce yield and viability of protoplasts.

### 3.3. Effect of Plant Age on Protoplast Yield and Viability

The effect of plant age was determined by taking 1 g leaf tissue from 2, 4, 6 and 8-week old plants. Tissue was incubated under optimum conditions of enzymatic degradtion (6 h). Protoplast yield obtained was lowest for the 2-week old plant whereas protoplast yield was highest for the 4-week old plant and protoplast viability was highest for 4-week old plant followed by 2 and 6-week old plant. Eight week old plant resulted in lowest protoplast viability (**Figure 3**). This experiment shows that age of plant has a significant effect on the protoplast yield. In case of alfalfa Regen-SY, 4-week old plant resulted in optimum results. The low protoplasts yield of 2-week old plant could be because of the fact that alfalfa Regen-SY plants at the age of 2-week does not have fully expanded leaves and leaves are often folded towards inside due to which they are not able to make optimum contact with the enzyme solution. As 4-week old plant has fully expanded leaves and less enzyme resistant content as compared to the 6 and 8-week old plant, therefore it resulted in maximum yield and viabilty.

### 3.4. Effect of Mannitol Concentration Protoplast Yield and Viability

The effect of mannitol (osmoticum) concentration was determined by using best enzyme treatment on 4-week old tissue for 6 h in CPW solution containing different concentrations of mannitol (0.2 M, 0.5 M, 0.7 M, 1 M). As protoplast can rupture in hypertonic solution and collapse in hypotonic solution, optimizing osmoticum concentration becomes very important. Protoplast yield and viability

was lowest at 0.2 M mannitol concentration causing protoplast to burst and rendering them inviable. The protoplast yield and viability was highest with 0.7 M mannitol concentraion. At this concentration, protoplast retained their shape and orientation. Using 1 M concentration of mannitol caused many protoplasts to fuse which resulted in the reduced yield and viability (Figure 4).

### 3.5. Effect of Centrifugation Speed on Protoplast Yield and Viability

To determine the effect of the centrifugation speed, 1 g of leaf tissue was excised from the 4-week old plant and incubated with best enzyme treatment for 6 h in

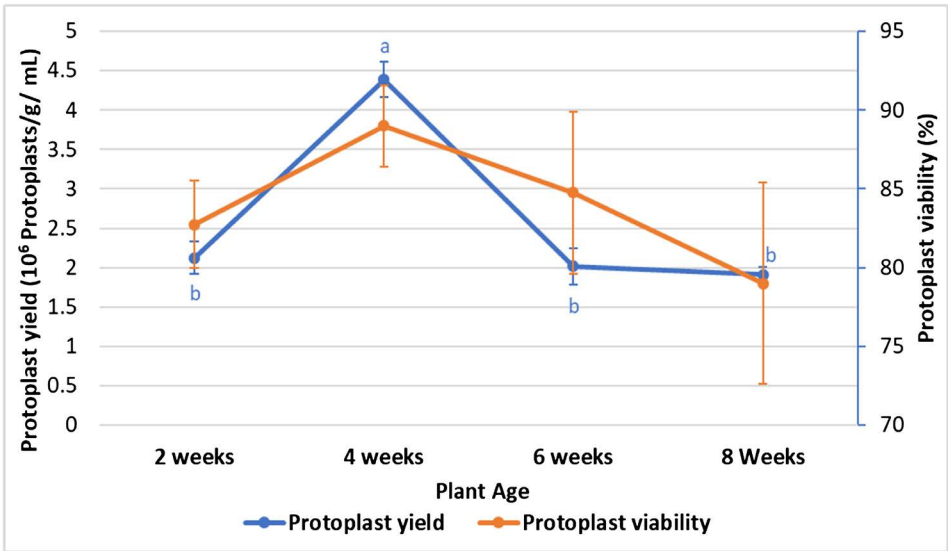


Figure 3. Effect of plant age on protoplast yield and viability in alfalfa Regen-SY. Values represent mean ± SD (n = 3). The different letters indicate significant differences ($p \leq 0.05$) according to the HSD test.

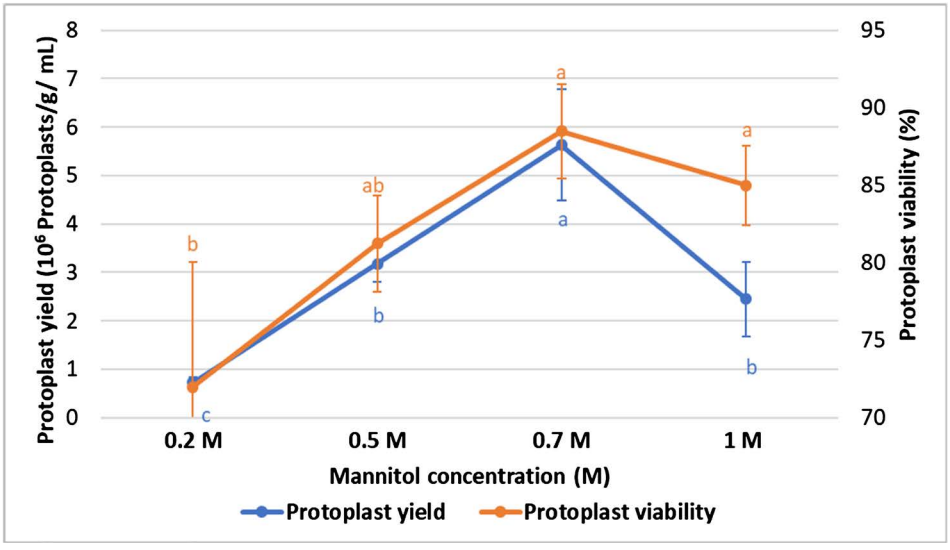


Figure 4. Effect of mannitol on protoplast yield and viability in alfalfa Regen-SY. Values represent mean ± SD (n = 3). The different letters indicate significant differences ($p \leq 0.05$) according to the HSD test.

CPW containing 0.7 M mannitol. After incubation protoplasts were purified by passing through 50 μm mesh. The protoplasts were then centirifuged at varying centrifugation speed (500, 1000, 1500 and 2000 rpm) in 15 mL centrifuge tubes for 10 min. It was observed that pellet was not completely formed at the 500 rpm and lot of plant material kept floating in the supernatant. It resulted in the lowest protoplast yield and viability (Figure 5). Optimum centrifugation speed was found to be 1000 rpm for 10 min which resulted in highest yield and viability. The reduction in yield and viability was observed at centrifugation speed over 1000 rpm. The protoplast obtained where spherical and retained their shape at 1000 rpm. Centrifugation speed of 2000 rpm resulted in rupturing of the protoplasts and loss of conformation as they were exposed to because of high centrifugation speed.

### 3.6. Effect of Shaker Speed on Protoplast Age and Viability

To study the effect of the shaker speed on the protoplast isolation, leaves from four week old plant were incubated with the best enzyme treatment at 6 hours of enzymolysis was used. Four experiments were set up at different shaker speed (50, 55, 60 and 65 rpm). The protoplast yield and viability was highest at 55 rpm speed and the protoplast yield and viability decreased over 55 rpm due to the more enzyme solution interaction with the leaf tissue (Figure 6). Shaker speed is one of the conditions that need to be optimized for the protoplast isolation. Too less shaker speed can result in increase in the incubation time which can cause enzyme toxicity making protoplasts inviable. Too high Shaker speed can cause disruption of chloroplast orientation in the protoplast as well as bursting of protoplasts. There was no significant difference in the protoplast yield and viability at different shaker speeds. Since 55 rpm resulted in maximum yield it was selected as optimum condition for the further experiment.

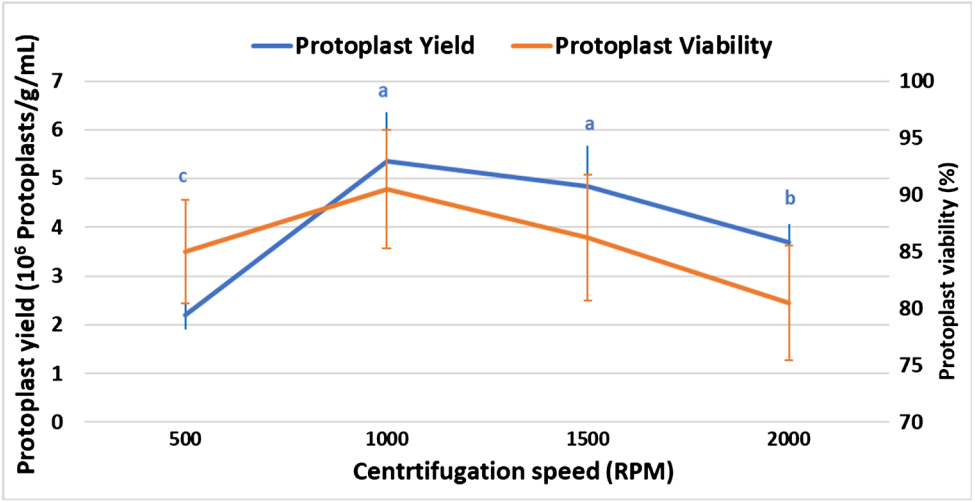


Figure 5. Effect of centrifugation speed on protoplast yield and viability in alfalfa Regen-SY. Values represent mean ± SD (n = 3). The different letters indicate significant differences ($p \leq 0.05$) according to the HSD test.

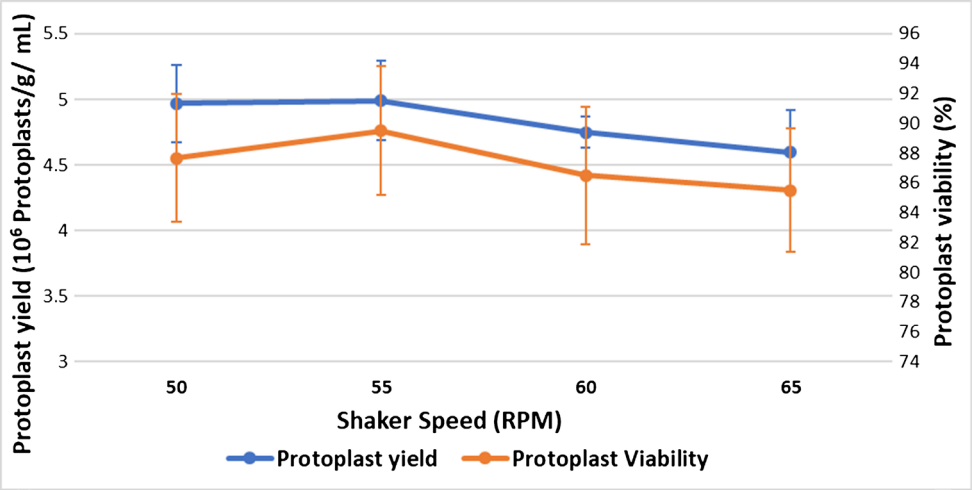


**Figure 6.** Effect of Shaker speed on the protoplast yield and viability in alfalfa Regen-SY. Values represent mean ± SD (n = 3).

### 3.7. Optimum Conditions for the Protoplast Culture of Alfalfa Regen-SY

Based on the optimization experiments, the optimum conditions for the protoplast culture of alfalfa cultivar Regen-SY are given in **Table 4**.

### 3.8. Plating Density and Efficiency

Freshly isolated protoplasts using optimum conditions (**Table 4**) were cultured at four different densities $1 \times 10^4$, $2 \times 10^4$, $1 \times 10^5$, $2 \times 10^5$ in the liquid medium. Protoplasts only survived at the densities $1 \times 10^4$ and $2 \times 10^4$ and protoplasts at the remaining plating densities died. The plating efficiency was found to be 78.25% ± 5.40% and 75% ± 5.24% for $1 \times 10^4$ and $2 \times 10^4$ culture densities respectively.

## 4. Discussion

Cocking (1960) was the first to isolate protoplasts and since then it has been reported in many plants because of its many applications [6]. Since protoplasts lack cell wall, they have been widely used for the genetic transformation, protoplast fusion and somatic mutation to generate new varieties of plants [21] [22] [23]. One of the many applications of the protoplasts technology is to establish a transient expression system which can be used to study high-throughput analysis and functional characterization of genes. To establish, efficient expression system through protoplasts there is requirement of high-quality protoplasts [24]. Enzymatic digestion is the most commonly used method for the protoplast isolation and factors like enzyme combination, osmoticum concentration and centrifugation speed significantly affect the quality of protoplast. Therefore, to isolate high-quality protoplasts there is need to optimize conditions for the isolation of the protoplasts. Leaves are the most commonly used plant material for the protoplast isolation because of their loose arrangement of mesophyll cells [25].

Incubation time is one of the main factors that affect the quality of protop-

lasts. If the incubation time is too long, it can cause damage to the plasma membrane resulting in the bursting of protoplasts whereas too less incubation time can cause reduction in number of protoplasts released [26]. In our study, incubation time of 6 h was found to be optimum. Protoplasts are known to burst in hypertonic solution and collapse in hypotonic solution [14]. Therefore, osmoticum (mannitol) concentration had a significant effect on the protoplast yield and viability in our study and 0.7 M mannitol was found to be the optimum concentration resulting in maximum yield and viability of protoplasts.

Freshly isolated protoplasts were green and spherical with clearly visible chloroplasts in them (**Figure 7(B)**). Viability of the protoplasts was determined by

**Table 4.** Optimum conditions determined based on various optimization treatments for the protoplast culture.

| **Condition** | Optimum parameter |
|---|---|
| Plant age | 4 weeks after subculture |
| Enzyme treatment | 2% Cellulase + 1.5% Macerozyme |
| Incubation time | 6 h |
| Centrifugation speed | 1000 rpm for 10 min |
| Shaker (rotator) speed | 55 rpm |
| Osmoticum (mannitol) concentration | 0.7 M |
| Temprature | Room Temprature (25˚C ± 2˚C) |
| pH | 5.8 |

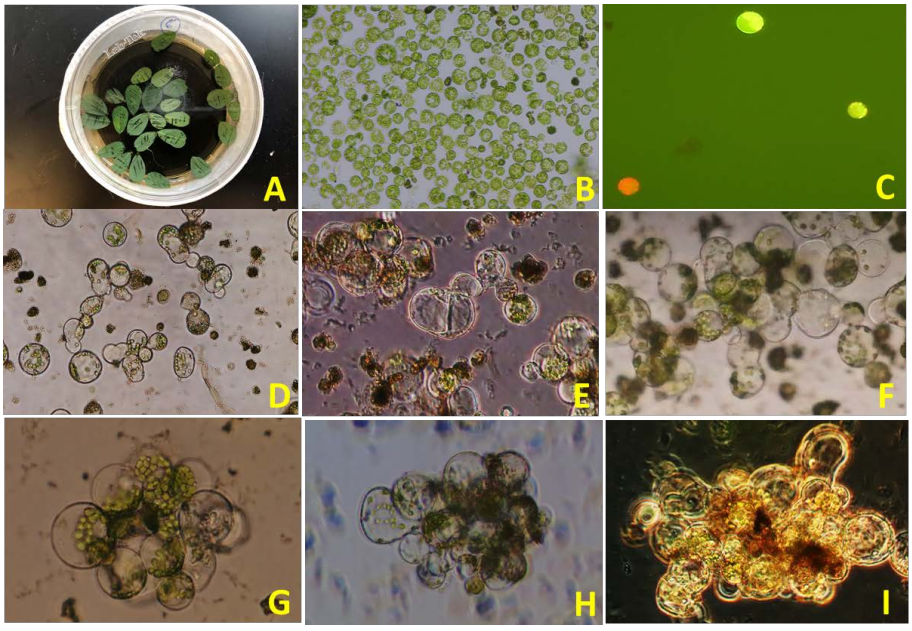


**Figure 7.** Isolation and culture of protoplasts (A) Leaf tissue in enzyme solution before enzymatic digestion; (B) Freshly isolated protoplasts using optimum conditions at 10X magnification; (C) Viability of protoplasts using FDA, green fluorescence shows viable protoplasts and red protoplasts are non-viable; (D) 4-8 cell colonies observed after 1 week of culture; (E-G) Colonies gradually increasing in number and size to form micro calli; (I) Micro-calli observed after 5 weeks of culturing. All images at 40× magnification except mentioned otherwise.

using FDA staining assay. Fluorescein diacetate is a non-polar and fluorescing substance that can penetrate through the plasma membrane. Once it enters the living cell, the esterase activity causes release of fluorescein resulting in green fluorescence when cells are observed under UV light (**Figure 7(C)**) [27]. Protoplasts produced through the enzymatic digestion were viable and started dividing after 48 hours. Protoplast kept dividing and colonies gradually increased in size (**Figures 7(D)-(H)**) and micro calli formation was achieved in 5 weeks of culturing (**Figure 7(I)**). Micro-calli formed in this study can be used for the regeneration of the whole plants via somatic embryogenesis which is preferred mode of in vitro plant regeneration in alfalfa [4].

## 5. Conclusion

The study showed that low concentration of cellulase (2%) and macerozyme (1.5%) are sufficient for the release of protoplasts in short incubation period (6 h). Results showed that factors like enzyme combination, incubation time, plant age, centrifugation speed and Mannitol concentration significantly affected the quality of the protoplasts obtained. The success achieved in the determination of optimum conditions for the isolation of viable protoplasts from alfalfa Regen-SY will provide a basis for future work on the development of a protoplast-to-plant regeneration system as well as genetic transformation of protoplasts via electroporation and other direct DNA transfer techniques. It can also be used to develop a gene expression system and to create cDNA libraries for the gene function and regulation studies.

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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