A Comparative Study on Protoplast Isolation Protocol from Different Explants of Strawberry

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This study explored the efficient isolation of protoplasts from various strawberry explants, including leaf mesophyll and leaf callus. A comparative analysis of protoplast yield and viability was conducted across these tissue types. Various concentrations of mannitol, enzymes, infiltration times, and incubation periods were evaluated to optimize protoplast isolation. Leaves were harvested from *in vitro* grown strawberry plants for mesophyll protoplasts. Leaf disks of four different cultivars were cultured on three different media to induce callus: T1 (MS +2 mg L-1 NAA, 1 mg L-1 Kinetin , 3% sucrose, 0.7% agar, pH 5.7), T2 (MS + mg L-1 NAA, 1 mg L-1 Kinetin + 2 mg L-1 AgNO3, 3% sucrose, 0.7% agar, pH 5.7), and T3 (MS + 2 mg L-1 NAA + 0.5 mg L-1 Kinetin, 3% sucrose, 0.7% agar, pH 5.7. The Akara cultivar exhibited the highest callus formation across all media (T1, T2, and T3). However, friable callus was formed only in T2 for all cultivars. The results demonstrated the successful isolation of protoplasts from all explants, with potential implications for further strawberry breeding and genetic research applications

Keywords: Breeding, callus, cell walls, mesophyll, protocol

1. INTRODUCTION

The strawberry (*Fragaria x ananassa* Duch.) is one of the most consumed berry fruit species worldwide (Silva et. al., 2024). It is recognized as a functional food that contributes positively to human health due to its abundance of antioxidants, polyphenols, fiber, vitamins, and various essential nutrients (Aaby et al., 2018; Andrianjaka-Camps et al., 2017; Michalska et al., 2017Its consumption, both in fresh and processed forms (such as ice cream, jam, marmalade, fruit juice, etc.), along with its aromatic properties, establishes its prominent position in both domestic and international markets (Witter et al., 2012). Global strawberry production exceeds 9 million tons (FAO, 2024), with Turkey ranking fourth, producing 800,000 tons, following China (3.5 million tons), the USA (1.5 million tons), and Mexico (1 million tons).

The primary objectives in strawberry breeding programs are to develop varieties that are high-yielding, early-maturing, resistant to diseases and pests, tolerant to environmental stresses, with high fruit quality and extended shelf life (Faedi, 2016; Alam et al., 2024; Mazzoni et al., 2021; Turci et al., 2021). However, biotic stress factors such as *Botrytis cinerea* (gray mold) significantly limit yield and fruit quality in strawberries (Yousef et al., 2024; Kahramanoğlu et al., 2022). Numerous studies in the literature report that *Botrytis* negatively impacts key yield and quality traits, such as fruit size, fruit water, and dry matter content, and reduces the nutrient element content of the plant (Terry et al., 2007; Evenhuis et al., 2006).

Cultivated crops have lower resistance to biotic stress factors compared to wild species. Therefore, there is an urgent need to develop resistant varieties to ensure both crop health and productivity. Traditional breeding methods can be long and costly, and achieving high levels of resistance to diseases and pests in strawberries can be challenging (Silva Linge et al., 2024; Barth et al., 2022). Furthermore, strawberries' high ploidy and heterozygosity introduce additional challenges in classical breeding approaches (Feldmann et al., 2024; Edger et al., 2019).

Advancements in plant biotechnology enable the generation of novel variations that cannot be achieved through traditional methods. The successful regeneration capacity of strawberries through *in vitro* techniques such as micropropagation (Naing et al., 2019), somaclonal variation (Nehra et al., 1990), protoplast culture (Barcelo et al., 2019; Nyman and Wallin, 1988), and somatic hybridization (Geerts et al., 2009) provides new opportunities for gene transfer and other cellular-

molecular breeding approaches (Yoo et al., 2022). Among these techniques, somatic hybridization and genetic transformation stand out. However, efficient protoplast culture and transformation are critically important for the success of these applications (Ranaware et al., 2023; Shulaev et al., 2008).

Protoplast isolation and culture provide a unique platform for somatic hybridization, genetic transformation, and gene editing, thereby accelerating crop improvement programs (Davey et al., 2005). By removing the cell wall, protoplasts enable direct access to the plasma membrane, facilitating the uptake of DNA, RNA, or other molecules (Bock, 2015). Even though protoplasts have a very high potential for genetic modification, their use can still be challenging due to factors such as enzyme composition, osmotic stabilizers, tissue source, and culture conditions (Yang et al., 2024; Reed and Bargmann, 2021; Guan et al., 2010).

This study investigated an efficient method for protoplast isolation from multiple strawberry explant types (leaf mesophyll and leaf callus). The main goals were to (i) compare protoplast yields and viability across these explant sources and (ii) optimize the isolation parameters, including enzyme composition, mannitol concentration, and incubation time. By refining these protocols, we aim to contribute to advanced breeding and biotechnological applications in strawberries.

2. MATERIALS AND METHODS

2.1. Plant Material and Explant Preparation

This study used both local and commercial strawberry cultivars known for their tolerance to Botrytis (Gray mold). Plantlets (stolons) were obtained from mother plants sourced from the Çukurova University and YALEX production fields. Shoot tips were selected as the starting material and, after surface sterilization, were cultured *in vitro*. The explants obtained from the production fields were cultured on MS medium containing 1.5 mg L^{-1} IBA and IAA to generate *in vitro* plantlets. Young leaves collected from the shoots subcultured at 4-week intervals, were used as the protoplast source. The young leaf tissues of these plants, which were subcultured four times, were processed according to the protocol described by Barcelo et al. (2019).

The leaves were finely cut into 5–10 mm strips for leaf mesophyll to facilitate enzymatic digestion.

Callus induction was induced from leaf explants. Explants were placed on three different media (T1, T2, T3) in a growth chamber (25 ± 2°C) under a 16/8 h (light/dark) photoperiod.

T1: MS +2 mg L^{-1} NAA, 1 mg L^{-1} Kinetin , 3% sucrose, 0.7% agar, pH 5.7

T2: MS + mg L^{-1} NAA, 1 mg L^{-1} Kinetin + 2 mg L^{-1} $AgNO_3$, 3% sucrose, 0.7% agar, pH 5.7

T3: MS + 2 mg L^{-1} NAA + 0.5 mg L^{-1} Kinetin, 3% sucrose, 0.7% agar, pH 5.7

Explants were sub-cultured every 3–4 weeks to maintain and promote callus proliferation. Morphological changes and callus formation rates were monitored. The fresh weight of calli was measured to determine growth efficiency.

2.2. Protoplast Isolation

Protoplasts were isolated using a modified protocol based on previously reported methods (Barcelo et al., 2019; Kuzminsky et al., 2016; Mii and Shiota, 1990). Briefly:

a) **Enzyme Solution Preparation**: An enzyme solution containing cellulase R10 (1–2% w/v) and macerozyme R10 (0.5–1% w/v) was prepared in a mannitol-based osmoticum (0.5–0.7 M), supplemented with 1.0 mM KH_2PO_4, 5.00 mM NH_4NO_3, and 5.00 mM citric acid to stabilize the protoplast membranes (Kuzminsky, 2016).

b) **Enzymatic Digestion**: Leaf or callus tissues (~0.2 g) were incubated in the enzyme solution in Petri dishes. To facilitate enzymatic digestion, leaves were finely cut into ~0.5-1.0 mm strips for leaf mesophyll protoplast isolation,

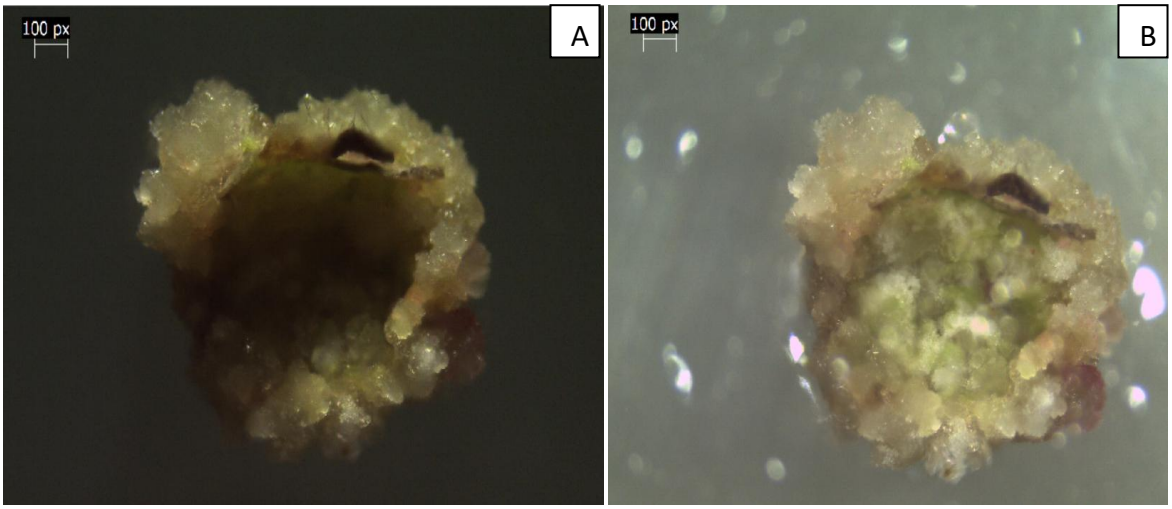
c) **Incubation**: The samples were incubated for 14-16 hours in the dark at 25 ± 2°C with gentle shaking at 60 rpm.

2.3. Protoplast Collection: The digested mixture was filtered through a 0.70 µm nylon mesh to remove undigested debris. Protoplasts were collected by centrifugation at 500 rpm for 3 minutes, washed once with the W5 osmoticum solution (5 mM Glucose, 154 mM NaCl, 5 mM KCl, 125 mM $CaCl_2$; pH 5.7), and resuspended in a minimal volume of the same solution (Menczel et al., 1981). After centrifugation at 500 rpm for 3 minutes, the supernatant was discarded and the pellet was resuspended in 3 mL of W5 solution. A 21% sucrose solution was used to purify the protoplast.

2.4. Viability Assessment: Protoplast viability was determined using 0.4% Trypan Blue staining. Stained samples were examined under a microscope, and the percentage of viable cells was recorded.

3. RESULTS

3.1. Callus Induction and Growth: The callus induction ratio varied among different cultivars and media types (Fig.2). The Akara cultivar exhibited the highest callus induction (100%) across all media (T1, T2, and T3). Similarly, Osmanlı cultivated on T2 exhibited a high callus induction rate of approximately 90%. In contrast, the Kara cultivar showed the lowest callus induction ratios, particularly in T1, where the induction was nearly absent. Tüylü demonstrated moderate callus induction across the media, with the highest percentage recorded in T1 (around 60%) and lower values in T3. Overall, Akara consistently exhibited the highest callus formation, while Kara had the lowest response across all tested conditions. Friable callus was formed in T2 for all cultivars, indicating that T2 is a suitable medium for successful protoplast isolation (Fig.1).



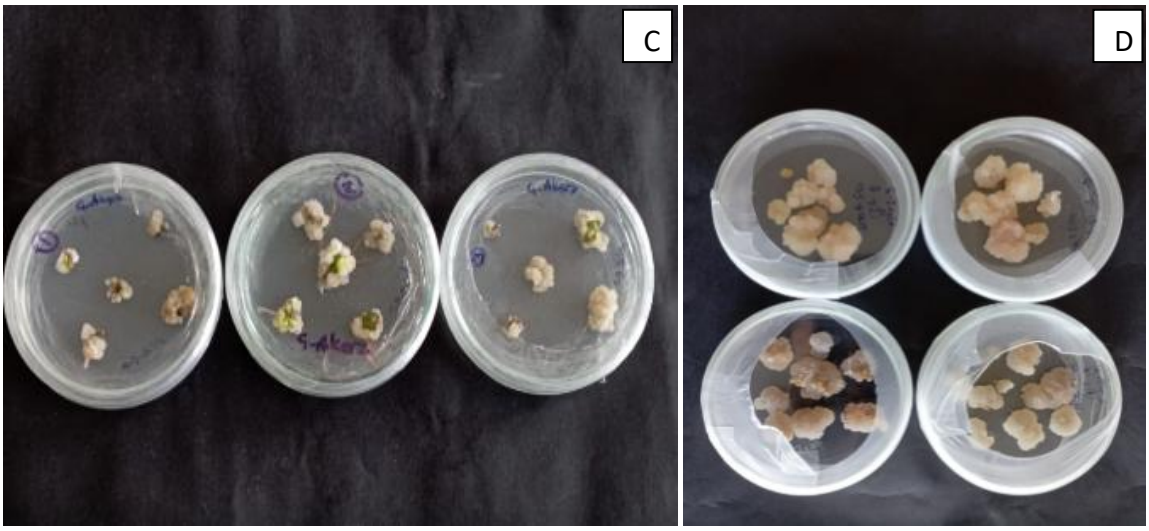


Fig.1 A and B) Callus development in T2 medium, C) Callus development on T1, T2 and T3 media, and D) Callus development after subculture

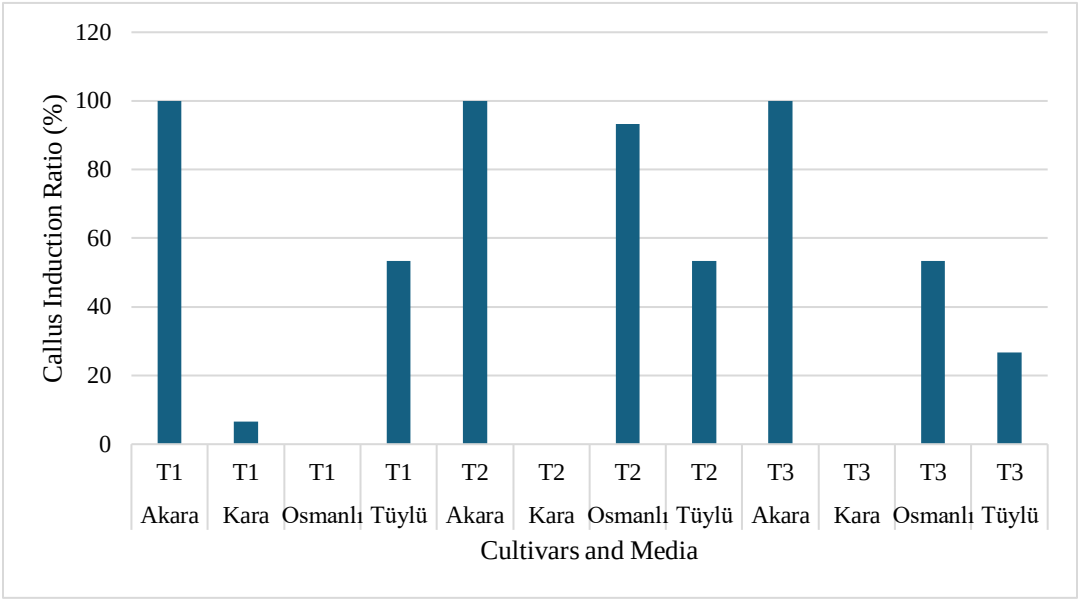


Fig.2. Callus induction ratio of different cultivars in various media

3.2. Optimization of Protoplast Isolation

The enzyme concentration played a critical role in protoplast isolation, with a combination of 2 % cellulose R10 and 1 % macerozyme R10 in 0.6 M mannitol providing the highest yield without compromising cell viability. Regarding the incubation period, the optimal digestion time for leaf mesophyll tissue was 14 hours.

3.3 Protoplast Yield and Viability

Protoplast yield and viability varied across different tissue types. Leaf mesophyll exhibited high viability at approximately 85 %. Similarly, leaf callus showed about 85% viability but had a slightly higher yield of 3–4 × 10^5 protoplasts g^{-1} FW

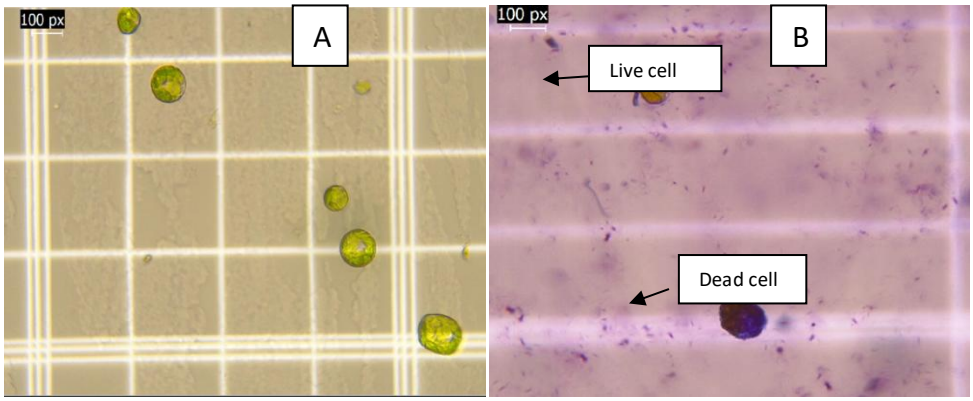


Fig.3. A) Isolated protoplasts B) Live and dead protoplast cells.

This study demonstrates that an optimized protocol efficiently isolate protoplasts from multiple strawberry explant types, with each tissue exhibiting unique advantages in terms of yield and viability. The successful isolation of protoplasts from different explants underscores the versatility of the protocol in strawberry biotechnology. Callus-derived protoplasts, yielded a higher number of cells, possibly due to the friable nature of this tissue and its active cell division status. However, leaf mesophyll protoplasts showed superior viability, indicating that younger, photosynthetically active cells might endure enzymatic stress better. The choice of culture medium (T1, T2, or T3) played a crucial role in obtaining high-quality callus. Notably, the T2 medium stimulated robust callus formation from explant sources, providing ample tissue for protoplast isolation. Optimizing enzyme concentration and osmoticum is critical for maintaining protoplast membrane integrity. While increasing enzyme concentration can speed up cell wall degradation, it may also compromise cell viability. In this study, an intermediate enzyme concentration yielded the best balance between yield and viability.

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