



The Determination of Protoplast Culture's Efficiency with Chickpea (*Cicer arietinum* L.) and Grass Pea (*Lathyrus* sp.) Species Grown in Vitro Conditions

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Abstract

Anthraxnose (*Ascochyta rabiei* Labr.) disease, which causes significant economic damage in chickpea (*Cicer arietinum* L.) cultivation, is widespread in Türkiye, particularly in Central Anatolia. Literature studies suggest that *Lathyrus* species can serve as a valuable source of tolerance against this disease. Since conventional hybridization between chickpea and grass pea species has been unsuccessful, this study aimed to determine the success of protoplast fusion as a cellular hybridization method. Four different chickpea cultivars (Azkan, Aksu, Göktürk, and Bahadır) and three *Lathyrus* genotypes (*L. cicera*, *L. sativus* (B), and *L. sativus* (S)) were germinated under in vitro conditions. Protoplast yield was evaluated using different explant sources, including radicle, plumule, and radicle + plumule. The highest protoplast yield was obtained from *L. cicera* × Göktürk chickpea hybrid (25×10^5 protoplast g^{-1}), while the lowest yield was recorded in *L. sativus* (S) × Aksu chickpea hybrid (2.5×10^5 protoplast g^{-1}). Among explant sources, the highest protoplast yield was found in radicle + plumule combinations (23.8×10^5 protoplast g^{-1}), whereas the lowest was in radicle explants (2.5×10^5 protoplast g^{-1}). These results suggest that radicle + plumule explant sources enhance protoplast fusion efficiency and increase the probability of obtaining heterokaryons with desired characteristics.

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1. Introduction

Climate change is increasingly intensifying the stress factors that constrain agricultural productivity. Under the influence of these factors, it is becoming progressively challenging to meet the nutritional needs of the growing global population, adversely affecting the ability of people to maintain a balanced and healthy diet. For a nutritious diet, an individual requires 70 g of protein per day, of which 40 g should be derived from plant sources (Akçin, 1988). Among food legumes, chickpea (*Cicer arietinum* L.) has a relatively low protein content (approximately 19%); however, its seeds are rich in essential amino acids such as lysine and methionine. Moreover, chickpea possesses a balanced nutritional profile due to its vitamin and mineral content (Pekşen and Artık, 2005). When the agronomic characteristics of chickpeas are examined, their well-developed root system and symbiotic relationship with *Rhizobium* bacteria grant them a highly significant role in dryland farming systems. In addition, its ability to leave a clean field after harvest and its tolerance to drought and salinity have increasingly contributed to solving recently observed agricultural problems. In 2020, chickpea was cultivated over an area of 14.8 million hectares worldwide (14 841 940 ha), yielding approximately 15 million tons (15 083 871 tons) of produce (Anonymous, 2022a). In the same year, chickpea was cultivated on 511 560 ha in Türkiye, yielding 630,000 tons. Türkiye's chickpea yield, at 123 kg da⁻¹, is higher than the world average of 101.6 kg da⁻¹ (Anonymous, 2022b). However, in 2021, the area under chickpea cultivation in Türkiye declined to 487 885 ha, a decrease attributed to environmental pressure from climate change and increased diseases and pests that cause economic losses. The most significant disease limiting chickpea cultivation in Türkiye is anthracnose (*Ascochyta rabiei* (Pass.) Labr.). This disease can cause yield losses of up to 90% in chickpeas. In some years, it may result in regions, especially Central Anatolia, not

producing any chickpea harvest. As conventional control methods have not reached sufficient levels, sustainable chickpea production can only be achieved through the development of anthracnose-tolerant cultivars (Toker and Çağırğan, 1996; Azkan et al., 1999; Akdağ, 2001). Due to Türkiye being the center of diversity for the genus *Cicer*, the Turkish flora naturally comprises 10 species and numerous subspecies (Anonymous, 2022c). However, despite the existence of various types, no reports indicate that these species possess tolerance against anthracnose. Anthracnose (*Ascochyta* sp.) causes reduced yield and plant loss in chickpeas and affects other food legumes. For peas, for instance, McCutchan (2001), who worked on anthracnose tolerance, emphasized that the genus *Lathyrus* represents a valuable source of tolerance against *Ascochyta* sp. disease. Moreover, transferring the genes that confer this tolerance to chickpeas through conventional hybridization is difficult or sometimes impossible. In addition, traditional breeding methods take many years, and the acquired tolerance is quickly lost. Thus, the use of biotechnological methods, such as protoplast culture, a complementary approach to traditional breeding, is of utmost importance in shortening the process and achieving more effective results in conferring anthracnose tolerance in chickpea (*Cicer arietinum*) (Toker and Çağırğan, 1996; Azkan et al., 1999; Akdağ, 2001). Protoplast culture is defined as the culture of cells from which the cell walls have been removed by various methods, leaving the plasma membrane intact. Once the cell wall is removed, the remaining structure is called a protoplast. Under appropriate conditions, protoplasts can maintain viability, regenerate a new cell wall, continue to divide by mitosis, and eventually form new calli, tissues, and subsequently, new plantlets (Babaoğlu and Özcan, 2002; Bozdoğan et al., 2013). (See Figure 1 for a schematic representation of the steps involved in protoplast culture; Anonymous, 2022d).

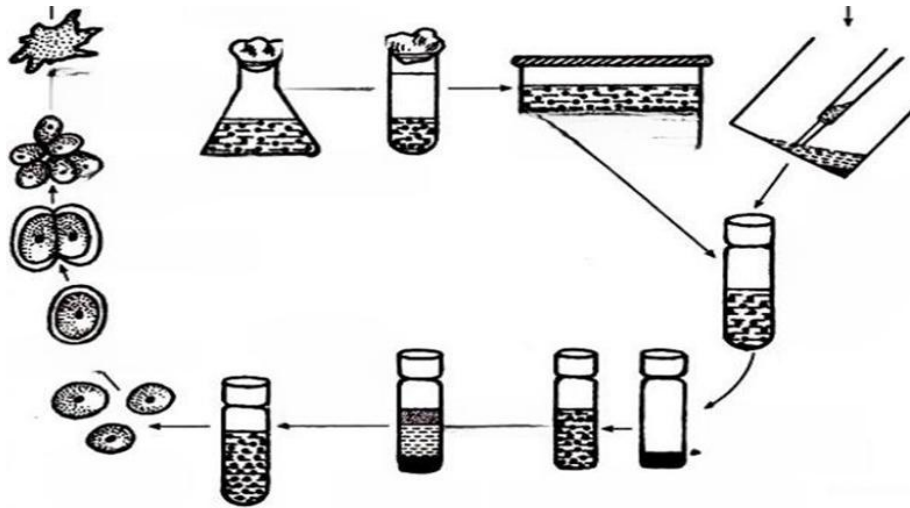


Figure 1. Schematic representation of the procedures in protoplast culture (Anonymous, 2022)

Babaoğlu (2000) stated that mature zygotic embryos, immature cotyledons, seedling cotyledons, hypocotyls, epicotyls, and seedling root tips can be used as sources for protoplast isolation. His study aimed to determine the optimum explant source for protoplast isolation from *Lupinus mutabilis*, and he reported that the shoot tip is the best protoplast source, yielding 4.81×10^6 protoplast g^{-1} (fresh weight). Gómez-Maldonado et al. (2001) indicated that various organs, such as embryos, roots, and pollen, can be used as source materials for protoplast isolation. In their study, they were the first to use seedling stems as an explant source for *Pinus pinaster*. They reported a protoplast viability of 80–90% and noted that the centrifugation step in their method damaged the protoplasts. Due to the lack of success in obtaining natural hybrids between pea and grass pea, McCutchan (2001) attempted to produce anthracnose-tolerant plants using biotechnological methods through somatic hybridization. In that study, suitable micropropagation and protoplast techniques were developed for selected *Pisum* sp. and *Lathyrus* sp. genotypes. Subsequently, protocols for organogenesis and somatic embryogenesis were refined for these species. Protoplasts from *Pisum* sp. and *Lathyrus* sp. were fused using electrofusion to generate asymmetric somatic hybrids, and molecular markers were then used to identify anthracnose-tolerant types in pea. Yan et al. (2004) reported that new bacterial blight

resistance genes for rice were transferred via somatic hybridization from wild rice (*Oryza meyeriana*) to a japonica rice cultivar (*Oryza sativa* Cv. 8411). Twenty-nine progenies were obtained from the somatic hybrids, and seven of these hybrids, along with their parental lines, were analyzed using AFLP molecular markers with eight primer pairs. The results confirmed that the hybrids contained characteristic bands from both parents. The regenerated rice plants exhibited traits of both *O. sativa* and *O. meyeriana*, and two somatic hybrids showed the highest resistance to bacterial blight. Eight plants exhibited moderate resistance in a rice breeding program to improve bacterial blight resistance. Protoplast culture is not only applied to develop disease-tolerant plants but also to enhance crop yield. Ekmekçi and Sözen (2005) conducted protoplast fusion between *Origanum onites* (*Greek oregano*) and *O. majorana* (sweet marjoram) to develop a new oregano variety with increased essential oil production. Their results confirmed that the sibs produced following protoplast fusion were indeed hybrids. Zhou et al. (2019) noted that protoplast yield varies according to plant species and methods. Their study on *Platycladus orientalis* isolated protoplasts from young leaf tissues using various techniques and achieved yields ranging from 1×10^4 to 8.2×10^4 protoplast g^{-1} (fresh weight). They also stated that the plant species selected for the method were compatible with the procedure.

2. Material and Methods

2.1. Plant material

This study was conducted at the Biotechnology Laboratory of Selçuk University, Faculty of Agriculture. The plant material included four registered chickpea cultivars (*Cicer arietinum* L. cv. Göktürk, Azkan, Bahadır, Aksu) and three *Lathyrus* genotypes (*L. cicera* Hauman, *L. sativus* (B), and *L. sativus* (S)).

2.2. Laboratory equipment and chemicals

The laboratory setup included autoclave-sterilized Eppendorf tubes, micropipettes and tips, a laminar flow cabinet, forceps, scalpels, a Bunsen burner, Petri dishes, Magenta GA7 boxes, glass jars, sterile gloves, microscope slides, and a compound light microscope. Hypochlorite acid was sterilized, while Murashige and Skoog (MS) medium, agar, sucrose, and additional chemicals were provided under the Scientific Research Projects (BAP) grant.

2.3. In vitro germination procedures

2.3.1. Equipment sterilization

All equipment was sterilized under a laminar airflow hood with a 0.22 µm HEPA filter. The workspace was disinfected with 96% ethanol, and the UV-C light was left on for 20 minutes before the experiments.

Scalpels and forceps were sterilized by flaming after being treated with 70% ethanol. Explants were dissected on autoclaved ceramic tiles wrapped in aluminum foil. Magenta GA7 boxes, Petri dishes, Eppendorf tubes, micropipette tips, and glass jars were autoclaved at 121 °C and 1.02 atm for 20 minutes.

2.3.2. Preparation of growth medium

Half-strength MS medium was used for germination. The medium was supplemented with 3% (w/v) sucrose, and the pH was adjusted to 5.8 using 0.1-1 N KOH or HCl. Agar (0.8%) was added, and the solution was autoclaved at 121 °C and 1.02 atm. Sterile culture vessels were filled with 25 mL of medium.

2.3.3. Seed surface sterilization and germination

Seeds were pre-washed under running tap water for 5 minutes. They were then surface sterilized in 70% ethanol for 1 minute and in 20% commercial sodium hypochlorite solution for 3 minutes. Seeds were rinsed three times with sterile distilled water. The sterilized seeds were placed on MS medium in culture vessels and incubated under controlled conditions: 24 ± 2 °C temperature, 65% humidity, 5000 lux light intensity, and a 16/8-hour light/dark photoperiod.



Figure 2. Germination of chickpea and *Lathyrus* seeds under in vitro conditions

2.4. Protoplast culture

2.4.1. Selection of explant source

Two-to-three-week-old seedlings were used as explant sources. Three different explant types were tested:

Radicle

Plumule

Radicle + Plumule combination

Explants were selected based on their meristematic activity to maximize protoplast yield. The protocol was adapted from Ekmekçi and Sözen (2005).



Figure 3. Image of seedlings grown under in vitro conditions

2.4.2. Preparation of CPW medium and enzyme solution

CPW medium and an enzyme solution were prepared according to Table 1. All solutions

were autoclaved at 121 °C and 1.21 atm before use.

Table 1. CPW medium composition

Macronutrients	mg l ⁻¹	Micronutrients	mg l ⁻¹	Enzym mixture	%
CaCl ₂ 2H ₂ O	1480	CuSO ₄ 5H ₂ O	0.025	Pektinaz	0.5
KH ₂ PO ₄	27	KI	0.16	Selülaz	2
KNO ₃	1012	pH	5.8	Driselaz	0.1
MgSO ₂ 7H ₂ O	246				

Explants were cut into 1 cm² pieces and incubated in 15 mL Falcon tubes containing enzyme solution (9% mannitol, 10% sucrose, cellulase, driselase, and pectinase) for 16 hours at 20-22 °C in darkness. The tubes were agitated at 200 rpm for 2-3 minutes every hour

to facilitate enzymatic digestion. After digestion, protoplast suspensions were centrifuged at 1000 rpm for 10 minutes to separate viable protoplasts. The protoplast-enzyme solution was then washed three times with CPW medium.

Table 2. Composition of mprot nutrient medium

Macronutrients	mg l ⁻¹	Micronutrients	mg l ⁻¹	Organic Nutrients	mg l ⁻¹	Organic Compounds	mg l ⁻¹
NH ₄ NO ₃	1650	H ₃ BO ₃	6.2	Sukroz	30000	Driselaz	1
KNO ₃	1800	MnSO ₄ 4H ₂ O	22.3	Glycine	2	Pektinaz	1
CaCl ₂ 2H ₂ O	440	ZnSO ₄ 4H ₂ O	8.6	Inositol	100	IAA	1
MgSO ₄ 7H ₂ O	370	KI	0.86	Nikotinik Asit	0.5	2-4, D	1
KH ₂ PO ₄	170	Na ₂ MoO ₄ 2H ₂ O	0.25	Pridoksin-HCl	0.5	Zeatin	1
Na ₂ Edta	37.5	CuSO ₄ 7H ₂ O	0.025	Thiamin-HCl	0.1	IBA	1
FeS ₄ 7H ₂ O	27.8	CoCl ₂ 6H ₂ O	0.025	Sellülaz	1	NAA	1

Table 3. Composition of MS nutrient medium

Macronutrients	mg l ⁻¹	Micronutrients	mg l ⁻¹	Organic Nutrients	mg l ⁻¹	Organic Compounds	mg l ⁻¹
NH ₄ NO ₃	1650	H ₃ BO ₃	6.2	Sukroz	30000	Kinetin	1
KNO ₃	1800	MnSO ₄ 4H ₂ O	22.3	Agar	10000	NAA	0.5
CaCl ₂ 2H ₂ O	440	ZnSO ₄ 4H ₂ O	8.6	Glycine	2	2-4, D	0.5
MgSO ₄ 7H ₂ O	370	KI	0.86	Inositol	100	IAA	0.5
KH ₂ PO ₄	170	Na ₂ MoO ₄ 2H ₂ O	0.25	Nikotinik Asit	0.5		
Na ₂ EDTA	37.5	CuSO ₄ 7H ₂ O	0.025	Pridoksin-HCl	0.5		
FeSO ₄ 7H ₂ O	27.8	CoCl ₂ 6H ₂ O	0.025	Thiamin-HCl	0.1		

2.5. Protoplast fusion

600 µL of protoplast suspension from each species was mixed in a centrifuge tube for

somatic hybridization. Polyethylene glycol (PEG) and additional enzyme components (20% cellulase, 30% disease, 15% pectinase, and 10% PEG) were added to promote fusion.

The suspension was centrifuged at 1000 rpm for 10 minutes. The fused protoplasts were transferred onto a solid MS medium supplemented with IAA, kinetin, 2,4-D, and NAA (see Table 3). The plates were incubated in the dark for 24 hours, followed by exposure to 500 lux light intensity, which was gradually increased. Table 3. MS Medium Composition for Protoplast Culture. After 60 days, callus formation was observed.

2.5.1. Protoplast yield and statistical analysis

Protoplast viability was assessed using trypan blue staining under a Novex light microscope. Protoplast counts were recorded using a hemocytometer. The following formula was used to calculate protoplast yield: Protoplast/mL = (Average cell count per square) $\times$ (Dilution factor) $\times 10^4$
Total yield = (Protoplast/mL) $\times$ (Total volume used)

Data were analyzed using JMP 7 software, and microscopy images were processed with CellProfiler 4.2.5 for enhanced visualization (Stirling et al., 2021).

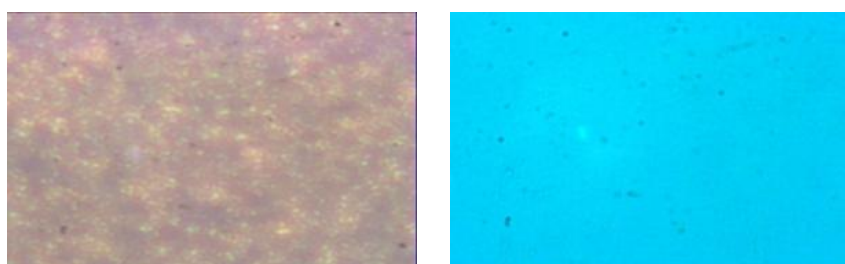


Figure 4. Light microscope image of protoplast fusion (x400 on the left; x1000 on the right)

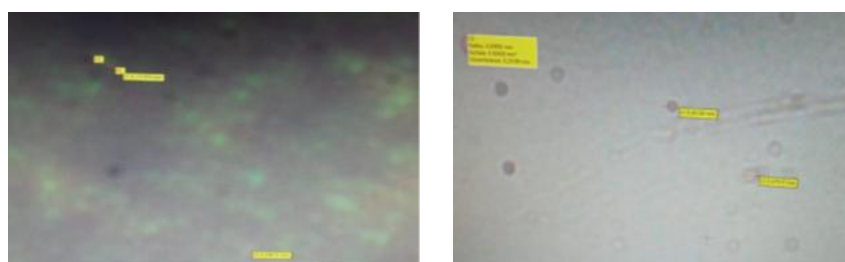


Figure 5. Light microscope image of protoplast fusion (x400 on the left; x1000 on the right)

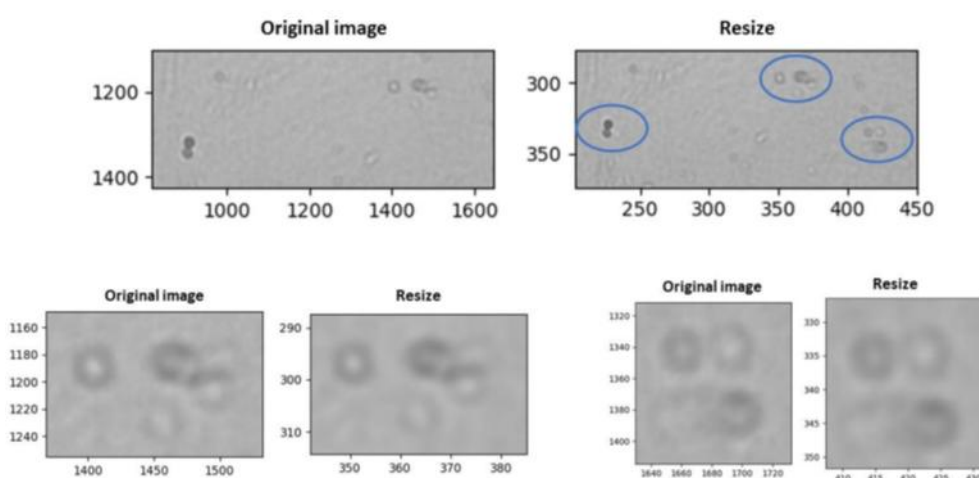


Figure 6. Original image obtained with a light microscope (x1000) (left) and the “threshold” image obtained with the CellProfiler 4.2.5 program.

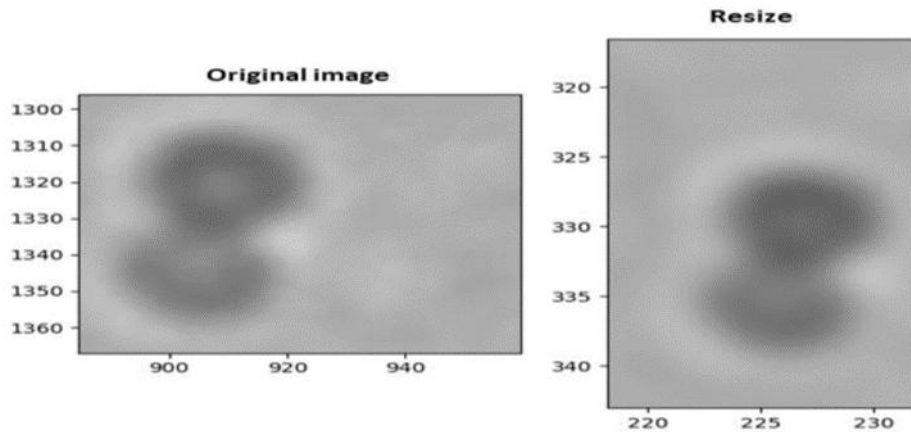


Figure 7. The “resize” image and the enlarged multiple fusion image obtained in the CellProfiler 4.2.5 program of the original image obtained with a light microscope (x1000)

3. Results

3.1 Examination of microscope images

In vitro, protoplasts on chickpeas and dandelions (Figure 4) show microscope images obtained in the culture. The obtained cells varied between 40 μm and 110 μm (Figure 5). It has been observed that these unions can be two-cell and multiple unions. As a result of the research, cellular fusion was observed in all somatic hybrid groups.

3.1 Protoplast yield

Our study employed the protoplast culture method for *Lathyrus* sp., examining the protoplasts to assess their compatibility with *Cicer* sp. The yields are given in figures. Based on the observations, the best protoplast yield was 25×10^5 in fresh weight in the hybrids *L. cicero* X Göktürk chickpea variety. The lowest protoplast yield was found in the *L. sativus* (black genotype) X Aksu chickpea variety, where a 2.5×10^5 protoplast g^{-1} count was performed in fresh weight.

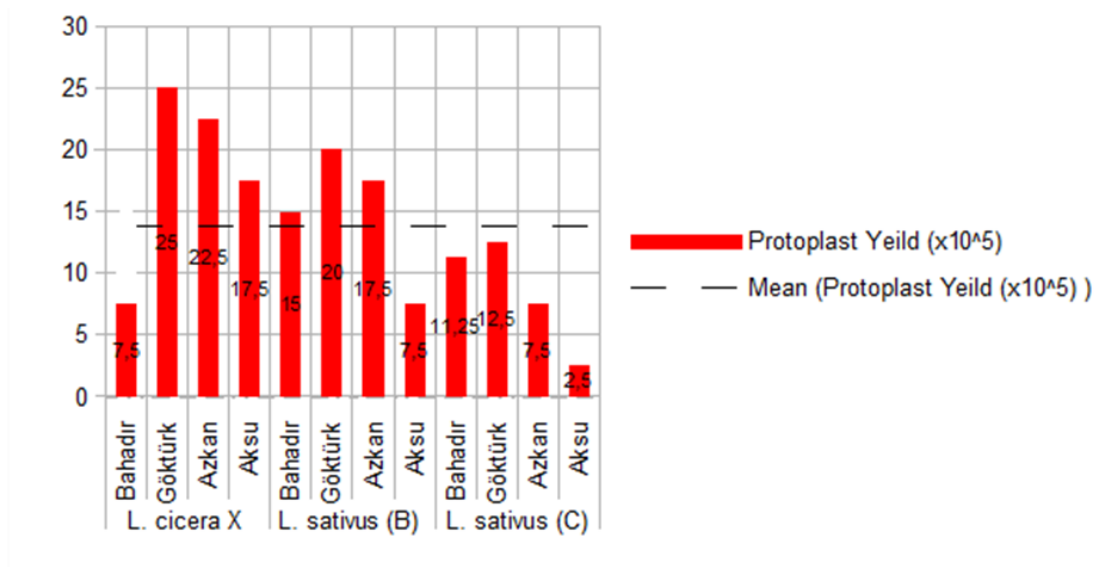


Figure 8. Protoplast yield according to species used (protoplast g^{-1})

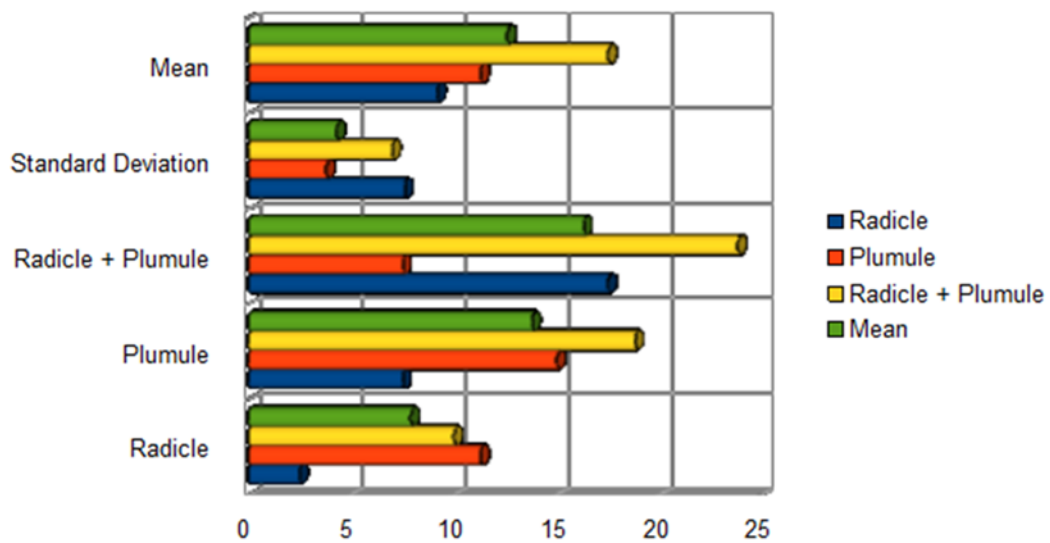


Figure 9. Comparison of Protoplast Yield Based on Explant Sources ($\times 10^5$ protoplasts g^{-1})

Observations based on explant type revealed that in both *Lathyrus* sp. and *Cicer* sp. the highest protoplast yield was obtained from materials using Radicle + Plumule, with a fresh weight yield of 23.8×10^5 protoplasts g^{-1} . The lowest yield was recorded in materials using Radicle alone, with a fresh weight yield of 2.5×10^5 protoplasts g^{-1} .

4. Discussion

Protoplasts are structures from which cell walls have been removed using various methods, and under suitable conditions, two different protoplasts can generate new plants. Somatic hybridization is a subject of multiple studies in modern plant breeding, focusing on breeding objectives such as yield, quality, or disease resistance (Bozdoğan et al., 2013). Yan et al. (2004) developed plants tolerant to bacterial blight disease in rice, McCutchan (2001) worked on obtaining plants tolerant to anthracnose disease in peas, and Ekmekçi and Sözen (2005) utilized somatic hybridization to enhance quality in thyme. Aksenik shoot cultures grown under in vitro conditions are suitable starting materials for protoplast culture because they prevent damage to the explant during surface sterilization or exposure to residues of certain substances (Babaoğlu and Özcan, 2002). Babaoğlu (2000) stated that

mature excised embryos, immature cotyledons, seedling cotyledons, hypocotyls, epicotyls, and seedling root tips could be used as protoplast sources and aimed to determine the optimal explant source for protoplast isolation from *Lupinus mutabilis*. In his study, shoot tip protoplasts provided the highest protoplast yield among the explant sources, with a fresh weight yield of 4.81×10^6 protoplasts g^{-1} . In addition to the protoplast source suggested by Babaoğlu (2000) and Gomez-Maldonado et al. (2001), who pointed out that different organs such as embryos, roots, and pollen were utilized as source material for protoplast isolation, seedling stems were used as the explant source for *Pinus pinaster* for the first time. In the method they developed, they noted that centrifugation damaged the protoplasts. Furthermore, Jia et al. (2016) investigated the effect of centrifugation at different speeds on the protoplast yields obtained from the mesophyll cells of wheat leaves. As a result of the research, it was stated that the highest protoplast yield of 7.31×10^6 protoplasts g^{-1} fresh weight was obtained in enzyme solutions containing 0.4 M Mannitol and at a centrifuge speed of 1000 rpm. Contrary to Gomez-Maldonado (2001), Jia et al. (2016) reported that increased centrifugation speed positively

affected protoplast yield and viability. In our research, the method used also included centrifugation processes, as in the study by Ekmekçi and Sözen (2005). In their work on different thyme species, they determined the protoplast yield as 2.8×10^6 protoplasts g^{-1} fresh weight and emphasized the role of centrifugation, particularly during the fusion stage. McCutchan (2001), who conducted research with a different fusion method, found a protoplast yield of 2.0×10^5 protoplasts g^{-1} fresh weight in protoplast cultures between peas and vetch using the electrofusion method. (Zhou et al., 2019), who stated that protoplast yield varies depending on plant species and methods, achieved the highest protoplast yield of 4.8×10^6 protoplasts g^{-1} fresh weight under optimal conditions in their protoplast isolation study using young leaf tissues from *Platycladus orientalis* with different methods. We successfully applied protoplast fusion techniques between *Cicer arietinum* (chickpea) and *Lathyrus* species to overcome the limitations of conventional hybridization in transferring anthracnose (*Ascochyta rabiei*) tolerance. Our results demonstrated that the highest protoplast yield was obtained from the *L. cicera* $\times$ *Göktürk* hybrid (25×10^5 protoplasts g^{-1} fresh weight). In contrast, the radicle + plumule explant combination yielded the highest average across genotypes (23.8×10^5 protoplasts g^{-1}), highlighting the importance of explant source selection. These findings are consistent with those of Babaoğlu (2000), who reported a yield of 4.81×10^6 protoplasts g^{-1} using shoot tips of *Lupinus mutabilis*, emphasizing that explant type plays a critical role in protoplast isolation efficiency. While Gómez-Maldonado et al. (2001) observed detrimental effects of centrifugation on protoplast integrity in *Pinus pinaster*, our methodology, in line with Jia et al. (2016), utilized 1000 rpm centrifugation without compromising viability and even enhancing yield, suggesting that centrifugation parameters must be optimized species-specifically. McCutchan (2001) achieved a relatively lower yield (2.0×10^5 protoplasts g^{-1}) using electrofusion in *Pisum* $\times$ *Lathyrus* hybrids, whereas our use of PEG-mediated

fusion produced markedly higher yields, indicating the potential advantage of chemical fusion techniques in particular leguminous species. Similarly, Ekmekçi and Sözen (2005) reported a protoplast yield of 2.8×10^6 g^{-1} in *Origanum* species. They underscored the significance of centrifugation particularly during the fusion process, a factor which also positively influenced our outcomes. Recent literature further supports our observations. Al-Nema et al. (2023) obtained 2×10^4 protoplasts/mL with 72% viability from *Brassica oleracea* hairy roots after 8 hours of enzymatic digestion. Rakosy et al. (2022) successfully generated somatic hybrids in *Nicotiana tabacum* via micro-electrofusion, demonstrating regeneration competency. Additionally, Kumari et al. (2021) developed *Brassica juncea* $\times$ *Sinapis alba* somatic hybrids with enhanced resistance to *Alternaria* blight and heat stress. These advances confirm the increasing value of somatic hybridization in addressing biotic and abiotic stress factors. Taken together, our findings align well with those in the literature and reinforce the notion that protoplast yield and fusion success are highly dependent on plant species, explant source, enzymatic composition, and fusion protocol. Our results provide one of the few successful case studies demonstrating the feasibility of intergeneric somatic hybridization between *Cicer* and *Lathyrus* species, laying the groundwork for the potential introgression of anthracnose resistance from wild relatives into elite chickpea. The differences between our results and those from the literature may arise from variations in plant species, fusion mechanisms, enzyme solutions used, and, in other words, the methods applied.

5. Conclusions

This study applied protoplast culture fusion methods between *Cicer arietinum* (chickpea) and *Vicia sativa* (vetch), allowing for hybridization between the species. The compatibility of the cellular hybridization technique with the species and the success of the fusion process were observed. The average protoplast yield, based on the species used, was

found to be 13.9×10^5 protoplasts g^{-1} fresh weight, while the average protoplast yield based on the explant sources used was 12.6×10^5 protoplasts g^{-1} fresh weight. Based on these values, using Radicle + Plumule as the explant source is anticipated to enhance protoplast yield, increasing the likelihood of obtaining desired heterokaryons with the required characteristics. Based on the received data, it was observed that cellular hybridization between species could be successfully achieved. Microscopic examinations included fusion tracking and protoplast yield evaluations, and the development of the resulting structures in media was followed to complete the study. Protoplast culture studies conducted between different species significantly impact agricultural matters, particularly regarding tolerance to various disease agents and resistance to environmental conditions. Transferring tolerance and resistance mechanisms from wild to non-related plant species can improve these traits in other plants.

Declaration of Author Contributions

The authors declare that they have contributed equally to the article. All authors declare that they have seen/read and approved the final version of the article ready for publication.

Declaration of Conflicts of Interest

All authors declare that there is no conflict of interest related to this article.

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