Isolation and regeneration of cell suspension-derived Foeniculum vulgare protoplasts

Naseem Aljaramany, and *Sokrat G.* Monakhos*

Botany Department, Moscow Timiryazev Agricultural Academy, 127434, Moscow, Russian Federation

Abstract. Hybrid development incorporating lines with cytoplasmic male sterility (CMS) has become an increasingly effecting technique for utilizing heterosis in vegetable crops. Currently, somatic hybridization has become prevalent in the Apiaceae family, which includes carrots, celery, and leeks. Therefore, as a first step, a simple and efficient protocol for isolating and regenerating protoplasts is established for the commercial fennel hybrid "Dragon" as a source of CMS that will be transferred later into carrot via protoplast fusion. To this end, cell suspensions from Dragon were initiated as a source for protoplast isolation.

1 Introduction

Fennel is a cross-pollinated crop with a somatic chromosomal number of 2n=22. It belongs to the family Apiaceae, is native to Europe and the Mediterranean region, and is cultivated in temperate climates around the world.

The potential for creating high-yielding cultivars through plant breeding is practically limitless. In several crops, intentional heterosis exploitation through breeding has resulted in notable yield gains, with hybrid breeding serving as a key component [1].

Cytoplasmic male sterility systems offer significant advantages for successful hybrid breeding as they facilitate effective pollination control. Typically, CMS can be transferred into crops using conventional crossing with their relative species that are cytoplasmic sterile. Regrettably, interspecific hybridization is not always effective due to various reproductive barriers, in this case applying specialized techniques such as embryo rescue is necessitating [3].

An alternative approach to interspecific hybridization is the generation of hybrid plants via asymmetric protoplast fusion. Since somatic embryogenesis is demonstrated by protoplast fusion regard its highly responsive to *in vitro* culture conditions, this technique represents a promising tool for the genetic improvement of Apiaceae crops such as celery and carrot. Protoplast fusion allows for the combination of genetic material from different plant species, overcoming the barriers to traditional hybridization, and the ability to regenerate whole plants from fused protoplasts makes this a valuable approach for broadening the gene pool and developing novel hybrid varieties.

* Corresponding author: s.monakhos@rgau-msha.ru

Carrots (Daucus carota L. 2n = 2x = 18) have traditionally served as a model system for experiments on protoplast isolation and regeneration. Carrot protoplasts have been successfully derived from green tissue and cell suspensions. Similarly, mesophyll and cell suspensions have been utilized to isolate celery protoplasts [2].

Protoplast of fennel to our knowledge, however, no reports of isolation and regeneration from any portion of the plant exist. As a result, the decision is made to investigate cell suspension cultures as a protoplast regeneration source. The commercial fennel hybrid "Dragon" is the subject of this research, which also outlines a method for isolating, growing, and regenerating fennel plants using protoplasts, starting with cell suspension cultures.

2 Materials and Methods

2.1 Plant materials

“Dragon” commercial hybrid of Foeniculum vulgare were cultivated in vitro from seeds. Aseptic material was derived from fennel (2n = 2x = 22) seeds sterilized using the following procedures, seeds were sown on 9 cm round Petri dishes contain solid Murashige and Skoog medium [4] with vitamins, 20 g/L sucrose, 2 mg/L glycine, 25 mg/L Ferric sodium EDTA, and 6 g/L plant tissue culture agar were added to the medium and the pH was adjusted to be equal to 5.8 immediately prior to autoclaving. The plates were incubated at 24 ± 1 °C to facilitate seed germination. Seedlings that reached 15 days old, were transferred to containers with the same solid medium.

2.2 Initiation of cell suspensions and callus induction

Explants were cultivated on MS medium (30 g/L sucrose, 0.5 mg/L 2,4-D, 0.5 g/L casein hydrolysate, 0.5 mg/L kinetin, as well as 7 g/L agar) then maintained in the dark at 24 ± 1 °C. Then monthly fragile callus was selected and renewed.

To start cell suspension culture, friable callus was cultured in 3 cm transparent round Petri dishes with 6 mL of liquid suspension medium. Then incubated on the shaker 60 rpm at 24 ± 1 °C in the dark promoting optimal growth and development of the cell suspension.

Suspension was replenished once a week using the same culture volume with fresh liquid medium until it reached a stable state with consistent division and growth.

2.3 Isolation and purification of protoplasts using obtained suspension

In order to separate protoplasts, suspension cultures with 5- to 9-week-old were utilized using a modified version of Grzebelus et al.'s procedure [5]. Approximately 1 g of cell suspension was finely sliced, 10 ml of enzyme solution was added carefully (0.5% cellulase, 0.05% pectinase, 20 mM MES, 5 mM CaCl2, and 0.6 M mannitol). The digestion was held in the dark at 24±1 °C with gentle agitation overnight.

Subsequently after digestion, the protoplasts were filtered through 100μm and 40μm nylon sieves, and centrifuged at 150 g for 10 minutes. The viable protoplasts in the resulting pellet were then washed with 7 ml of W5 media [6] then twice with 5 ml of 0.6 M mannitol and centrifuged after each wash at 150 g for 8 minutes. The purified protoplasts were enumerated using Neubauer counting chamber /hemocytometer and diluted with the same W5 medium to a concentration of 1×10^5 protoplasts per ml [2].

2.4 Protoplasts cultivation and regeneration

Protoplast culture medium was CPP (carrot petiole protoplast) medium [7] with some modifications, this medium contains the same macro- and micro-nutrients as well as organic acids [8], and vitamins [9] which known as B5, in addition, 74 g/L glucose, and 250 mg/L casein hydrolysate (pH 5.6). Protoplast cultures were incubated at 24 ± 1 °C in the dark. Microcalli that were formed were placed on solid CPP medium supplemented with 23 g/L glucose, 0.1 mg 1-naphthaleneacetic acid, and 0.2 mg/L zeatin.

Shoots that are going to be gained will be transferred to hormone free medium to let them develop into plants [2]. Then the ploidy level of the regenerated plants should be analysed to ascertain whether the regenerated protoplasts exhibited somaclonal variations in ploidy from the original germplasm, since identifying heteroploids is an important step in the breeding of plant forms with altered ploidy.

3 Results

Petioles and leaf blade derived callus (Fig. 1a) has been cultivated in liquid media. After around four weeks, Cell cultures reached a stationary growth phase (Fig. 1b), with cells beginning to divide within the first week (Fig. 1c). Once per week, they were then subcultured. Characteristically, cell clusters measuring 2-3 mm in size was formed. After four cycles of subculturing, the cell suspension had stabilized and was conducive to protoplast isolation. Protoplasts were obtained in high quality and quantity, healthy with spherical shape (Fig. 1d).

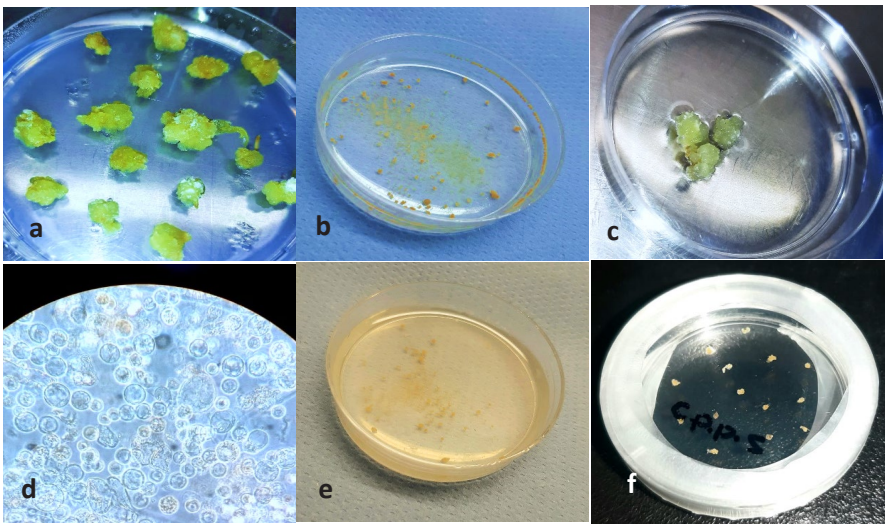

Fig. 1. a. Petioles and leaf blade derived callus, b. around four weeks after cultivation in liquid medium, c. callus subcultured, d. isolated protoplasts, e. Microcalli obtained from protoplast cultivation, f. transferred microcalli to solid medium

Based on earlier investigations, we were assured of the effectiveness of the protoplast isolation and culture techniques to generate larger regenerated plantlets.
Protoplasts from Dragon, produced microcalli (Fig. 1e) and finally callus (Fig. 1f). However, the regenerated shoots will fully develop into plants when cultivated on a hormone-free growth medium.

4 Discussion

Effective protoplast Plants are subject to a variety of factors, including the origin tissue and genotype, the technique used to isolate the protoplasts, their density, the composition of the medium, the conditions for growth, and media replacement methodology [10, 11].

This paper seeks to focus on plant regeneration of the commercial fennel hybrid "Dragon" and describes a technique for protoplasts isolating, cultivating and regenerating of fennel plants beginning from cell suspension cultures. is informed by prior research exploring the regeneration of Apium graveolens L. protoplasts, which utilizes Daucus carota as a model system for protoplast cultivation [2] This approach involves the isolation of protoplasts from cell suspensions then culture in a nutrient-rich medium, leading to the formation of cell walls, cell division, and ultimately, the regeneration of entire plantlets.

The source material used for protoplast isolation is a crucial factor in the successful regeneration of protoplasts into plants. Cell suspensions are frequently employed to isolate protoplasts that exhibit a significant high capacity for regeneration. Substantial previous investigations have demonstrated that cell suspension cultures are more amenable to protoplast regeneration relative to alternative tissue types, due to their unique characteristics, such as the ability to self-renew and differentiate into various cell types [12, 13].

5 Conclusion

In conclusion, a straightforward procedure for the culture and regeneration of fennel protoplasts is provided here. To our knowledge, this is the first investigation into the regeneration and culture of fennel protoplasts in the commercial hybrid "Dragon" fennel. The selected responsive genotype is currently being employed for somatic hybridization with the aim of introducing CMS in carrot, since carrot seed production is based on nuclear-cytoplasmic male sterility [14].

Acknowledgments

The article was made with support of the Ministry of Science and Higher Education of the Russian Federation in accordance with agreement № 075-15-2022-317 date April 20, 2022 on providing a grant in the form of subsidies from the Federal budget of Russian Federation. The grant was provided for state support for the creation and development of a World-class Scientific Center “Agrotechnologies for the Future”.

References

1. A. Akbari, K. Bahmani, M. Kazan, Ö.-F. Bilgin, J. Rahimi, A.-I. Darbandi, N.-E. Kafkas, S. Kafkas, *Analysis of Fennel Breeding Populations Based on Distinctness, Uniformity, and Stability (DUS) Testing via Morphological Descriptors and DNA Molecular Markers*, Genet Resour Crop Evol. (2024) https://doi.org/10.1007/s10722-024-01934-3
2. S. Bruznican, T. Eeckhaut, J.-V. Huylenbroeck, H.-D. Clercq, D. Geelen, *Regeneration of cell suspension derived Apium graveolens L. protoplasts,* Plant Cell Tiss Organ Cult (2017) https://doi.org/10.1007/s11240-017-1273-9
3. D. Nettancourt, *Incompatibility and Incongruity in Wild and Cultivated Plants*, (Springer-Verlag Berlin Heidelberg, 2001) https://doi.org/10.1007/978-3-662-04502-2

4. T. Murashige, F. Skoog, Physiol. Plant. **15** (1962)
5. E. Grzebelus, M. Szklarczyk, R. Baranski, Plant Cell Tiss Org., **109** (2012) https://doi.org/10.1007/s11240-011-0078-5
6. L. Menczel, F. Nagy, Z.-R. Kiss, P. Maliga, Theor Appl Genet, **59** (1981) https://doi.org/10.1007/Bf00264975
7. R. Dirks, V. Sidorov, C. Tulmans, Theor Appl Genet. **93** (1996) https://doi.org/10.1007/Bf00224080
8. K.-N. Kao, M.-R. Michayluk, Planta, 110 (1975) https://doi.org/10.1007/Bf00380613
9. O.-L. Gamborg, R.-A. Miller, K.Ojima, Exp Cell Res., **50** (1968) https://doi.org/10.1016/0014-4827(68)90403-5
10. T. Eeckhaut, P.-S. Lakshmanan, D. Deryckere, V.-E. Bockstaele, V.-J. Huylenbroeck, Progress in plant protoplast research. Planta. 1003 (2013) https://doi.org/10.1007/s00425-013-1936-7
11. J. Mottley, *Cell, Tissue and Organ Culture, Protoplast Culture*, Encyclopedia of Rose Science, 90-99 (2005) https://doi.org/10.1016/B0-12-227620-5/00104-X
12. Q. Han, H. Shen, Z. Zhang, Acta Hortic Sin. 34 (2007) https://doi.org/10.1007/s11240-017-1273-9
13. F. Tan, H. Shen, S. Wang, J. Zhou, S. Guo, Acta Hortic Sin., 36 (2009)
14. A.V. Voronina, A.V. Vishnyakova, S.G. Monakhos, A.A. Ushanov, A.A. Mironov, Journal of Water and Land Development, **55** (2022) https://doi.org/10.24425/jwld.2022.142314