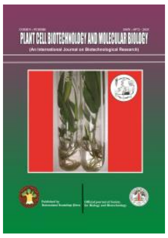

Plant Cell Biotechnology and Molecular Biology

Volume 24, Issue 7-8, Page 17-21, 2023; Article no.PCBMB.11776
ISSN: 0972-2025

Protoplasts Isolation from Sardinian Germoplasm of Saffron (*Crocus sativus* L.)

G. M. Scarpa [a*]

[a] *Dipartimento Di Agraria, University of Sassari, Sassari, Italy.*

Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

Article Information

DOI: 10.56557/PCBMB/2023/v24i7-88450

Editor(s):
(1) Prof. Hon H. Ho, State University of New York, USA.
Reviewers:
(1) Warji, Universitas Lampung, Indonesia.
(2) Ahmed Moufti, Regional Center for Education and Training Professions. Morocco.
(3) Somnath Das, The University of Burdwan, India.

Short Research Article

Received: 16/09/2023
Accepted: 20/11/2023
Published: 29/11/2023

ABSTRACT

Genetic studies of saffron indicate low heritability and strong environmental effects for most of the traits examined, suggesting local variability that has been selected over time. In vitro culture can be of support for genetic improvement programs, multiplication, or production of secondary metabolites. This is particularly relevant for saffron due to its triploidy. In the present work, the in vitro culture of Sardinian genotypes of saffron, using protoplasts, is described. Protoplasts were obtained from young leaves using a KAO enzyme mixture and cultured on KM8P substrate. The use of Evans et al. enzymes did not result in the production of protoplasts. The results presented in this work should be considered as the initial phase in the in vitro production of secondary metabolites obtained from local saffron genotypes.

Keywords: Crocus; In vitro culture; triploidy; secondary metabolites.

**Corresponding author: E-mail: grazia@uniss.it;*

ABBREVIATIONS AND ACRONYMS

CPW *: solution as described by Baghalian et al. [1]*
KAO *: enzyme solution as described by Bayat et al. [2]*
KM8P *: substrate as described by Ghanbari et al. [3]*
KM8 *: substrate as described by Ghanbari et al. [3]*
g *: acceleration due to gravity, g ≃ 9,81 m/s²*
MES- 2 *: (N-morpholino)ethanesulfonic acid*

1. INTRODUCTION

The production of saffron (*Crocus sativus* L.) in Italy can be estimated between 450 kg and 600 kg per year, covering about 50/55 hectares. The majority of companies involved in saffron cultivation are located in Sardinia. Based on local traditional agriculture, the corms are usually exchanged among farmers within the island, without any selection. Saffron is commonly grown for edible purpose, though it has long been known also for its medical and colouring qualities. Both in vitro and *in vivo* pharmacological efficacy of isolated compounds and extracts has been highlighted. Moreover, the bioactive compounds of *Crocus sativus* L. have proven their effectiveness in modern pharmacological research [4,5]. Moreover, extracts obtained from the discarded part of the flower have shown antioxidant and antimicrobial activity [6]. The potential use of tepal extracts for glycemic control has also been studied, compared to the more well-known use of stigmas: in vitro assays showed that tepal extracts had a greater inhibitory action than stigma extracts on α-amylase activity [7]. The propagation of saffron takes places exclusively by agamic way, due to its triploidy. Despite this, it is possible to observe morphological and biochemical variances in the different cultivation environments, between and within populations [1-3] that indicates low heritability and strong environmental effects for most of the traits they examined. In this context, the use of in vitro culture could be of support both for genetic improvement programs of the species, and production of secondary metabolites [8,9]. With the aim of evaluating an innovative approach to genetic improvement of local germplasm, our study team decided to begin a protoplast culture, starting from young leaves and roots explants.

2. MATERIALS AND METHODS

Corms of saffron, <2 cm in diameter, collected by private growers working in Sardinia, were used for the test. The corms were grown in a climatic cell, 23+1° C, 2000 lux, photoperiod 18 hours, using agricultural perlite as a substrate, irrigated every 3 days, to supply a constant humidity of the substrate. Fifteen days after planting, the first leaves were detected, and the samples collected.

Sterilization of plant material was carried out on the whole corms, by immersion in a 10% solution of a commercial bleach (8% hypochlorite), followed by 3 rinses in sterile water. The substrate, antibiotics and enzyme solutions were sterilized by 0.22 µm filters.

Leaves and roots were removed from corms and cut into small parts to increase the contact surface with the enzymes. The fragments were placed in 90 cm diameter petri dishes in a 10 M CPW solution [10] including ampicillin (400 mg l 1), gentamycin (10 mg l 1) and tetracyclin (10 mg l 1) antibiotics. Subsequently, the isotonic solution was replaced with an enzymatic solution composed of: KM8P substrate [11], antibiotics, as described above, and enzymes. Two mixtures of enzymes were evaluated: Kao [12] and the mixture proposed by Evans et al. [13] for cereal leaves.

The presence of protoplasts was observed after 30 min. The protoplast suspension was filtered (63 µm pore size) and protoplasts separated from the enzymatic mixture by centrifugation, 30 x g, 5 min. The protoplasts were resuspended in KM8P liquid medium, washed 3 times by centrifugation (30 x g, 5 min) and finally cultured at a density of 50 protoplast per µl, in Petri dishes (diameter = 3.5 cm) containing 2 ml of KM8P media. The Petri plates were incubated at 37° C in the dark. Analysis for wall formation and subsequent divisions began 24 hours after the start of incubation, under a light microscope. One day after plating, protoplasts were centrifuged (60 x g, 5 min) and the supernatant was removed and replaced with 1 ml KM8P and 1 ml KM8. After 48 hours, the substrate KM8P was completely removed, and protoplasts resuspended in KM8.

3. RESULTS AND DISCUSSION

Isolation of protoplasts took place when leaves were incubated in KAO enzyme solution, as described in Evans et al. [12]. No protoplasts were obtained when Evans [13] enzymes were used. No protoplast production was obtained starting from the roots. Despite the fact that this solution [13] has been applied for cereals, in monocotyledonous species such as saffron, as in our case, did not give the expected results. Instead, when KAO enzyme mixture was used [12], protoplasts were obtained from the leaves in a total number of 103 per ml. After 90 min of incubation the first protoplasts were detected, while the maximum value was reached after two hours. Longer time of contact with enzymes, caused complete cell digestion. Leaves that released protoplasts underwent mitotic divisions, forming small clusters (Fig. 1a, b), but did not continue dividing after 8 days of culture.

Darvishi [14] obtained viable protoplasts from *Crocus sativus* L, when an enzyme solution of MS medium, comprising 0.1% (w/v) Pectolyase Y-23, 1% Cellulose R-10, 1% Driselase, 0.1% MES and 0.3 M mannitol at pH 5.7, was used. Their results indicated that after enzyme treatment for 3 h the number of protoplasts and their viability were the highest. Increase of enzyme treatment duration led to a decreasing number of viable protoplasts. Similarly, Takashi [15] isolated protoplast starting with callus and isolated cell culture. In their experience, division occurred when protoplasts were cultured included in alginate and in copresence of nurse beads, in MS medium supplemented with 2,4-D and Zeatin. A similar protocol was used by Ebrahimzadeh [16], who regenerated shoots 6-7 months after protoplast isolation, when protoplasts were included in alginate, cultured in copresence of nurse cells on MS medium, supplemented with 2.4D and 6BA. According to their studies, the use of alginate and nurse cells increased the survival of C. sativus protoplasts.

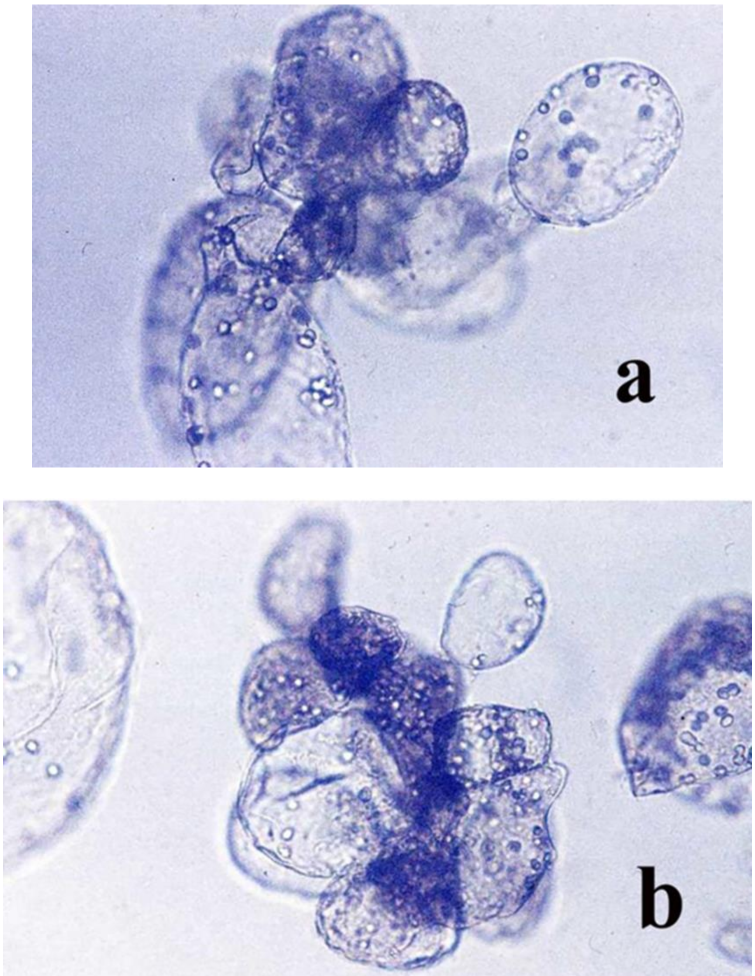

Fig. 1. Clusters obtained from the division of saffron protoplasts after 5 days (a) and 8 days (b) of culture

4. CONCLUSION

Although several studies have been conducted on saffron, concerning protoplast isolation and culture with positive results, more steps seem to be necessary for the definition of a protocol that ensures stabile division of cells or development of callus, to produce secondary metabolites in bioreactors. Our results are to be considered as a first phase for in vitro production of viable protoplasts obtained from local genotypes of saffron.

ACKNOWLEDGEMENTS

The present work is co-financed by the RAS (Autonomous Region of Sardinia), Advanced Technologies for LANds management and Tools for Innovative Development of an Eco Sustainable agriculture, and by the University of Sassari, Italy (Fondo di Ateneo per la Ricerca 2020).

COMPETING INTERESTS

Author has declared that no competing interests exist.

REFERENCES

1. Baghalian K, Shabani Sheshtamand M. Jamshidi AH. Genetic variation and heritability of agro-morphological and phytochemical traits in Iranian saffron (*Crocus sativus* L.) populations. Industrial Crops and Products 2010;31(2):401-406.
2. Ghanbari J, Khajoei-Nejad G, van Ruth S M. Effect of saffron (*Crocus sativus* L.) corm provenance on its agro-morphological traits and bioactive compounds, Scientia Horticulturae. 2019;256., 108605, ISSN 0304-4238, Available:https://doi.org/10.1016/j.scienta.2019.108605.
3. Bayat M, Rahimi M, Ramezani M. Determining the most effective traits to improve saffron (*Crocus sativus* L.) yield. Physiol Mol Biol Plants. 2016;22(1):153-161. DOI:10.1007/s12298-016-0347-1.
4. Butnariu M, Quispe C, Herrera-Bravo J, Sharifi-Rad J, Singh L, Aborehab NM, Bouyahya A, Venditti A, Sen S, Acharya K, Bashiry M, Ezzat S.M, Setzer WN, Martorell M, Mileski KS, Bagiu IC, Docea AO, Calina D, Cho WC. The Pharmacological Activities of *Crocus sativus* L.: A Review Based on the Mechanisms and Therapeutic Opportunities of its Phytoconstituents, Oxidative Medicine and Cellular Longevity. 2022; Article ID 8214821:29. Available:https://doi.org/10.1155/2022/8214821.
5. Slimani C, El Goumi Y, Rais C, El Ghadraoui L, Benjelloun M, Lazraq A. Micropropagation and potential of bioactive compounds of saffron (*Crocus sativus* L.) for nutrition and health. Notulae Scientia Biologicae. 2022;14:3(Sep. 2022), 11278. DOI: https://doi.org/10.55779/nsb14311278
6. Lachguer K, El Merzougui S, Boudadi I. et al. Major Phytochemical Compounds, In Vitro Antioxidant, Antibacterial, and Antifungal Activities of Six Aqueous and Organic Extracts of *Crocus sativus* L. Flower Waste. Waste Biomass Valor. 2023;14:1571–1587 . Available:https://doi.org/10.1007/s12649-022-01964-x
7. Bellachioma L, Morresi C, Albacete A, Martínez-Melgarejo PA, Ferretti G, Giorgini G, Galeazzi R, Damiani E, Bacchetti T. Insights on the Hypoglycemic Potential of *Crocus sativus* Tepal Polyphenols: An In Vitro and In Silico Study. International Journal of Molecular Sciences. 2023; 24(11):9213. Available:https://doi.org/10.3390/ijms24119213
8. Chib S, Thangaraj A, Kaul S, Dhar MK, Kaul T. Development of a system for efficient callus production, somatic embryogenesis and gene editing using CRISPR/Cas9 in Saffron (*Crocus sativus* L.). Plant Methods. 2020;16:1–10
9. Ozyigit II, Dogan I, Hocaoglu-Ozyigit A, Yalcin B, Erdogan A, Yalcin IE, Cabi E, Kaya Y. Production of secondary metabolites using tissue culture-based biotechnological applications. Front Plant Sci. 2023 Jun 29;14:1132555. DOI: 10.3389/fpls.2023.1132555. PMID: 37457343; PMCID: PMC10339834.
10. Frearson EM, Power JB, Cocking EC. Isolation, culture and regeneration of Petunia leaf protoplasts. Dev. Biol. 1973; 33:130-137.
11. Kao KN, Michayluk MR. Nutritional requirements for growth of Vicia hajastana

cells and protoplasts at very low population density in liquid media. Planta. 1975;126: 105–110.
12. Kao KN, Michayluk MR. Plant Regeneration from Mesophyll Protoplasts of Alfalfa, Zeitschrift für Pflanzenphysiologie. 1980;96(2):135-141, ISSN 0044-328X, Available:https://doi.org/10.1016/S0044-328X(80)80051-1.
13. Evans PK, Keates AG, Cocking EC. Isolation of Protoplasts from Cereal Leaves. Planta. 1972;104(2):178–181. Available:http://www.jstor.org/stable/23369679.
14. Darvishi E, Zarghami R, Mishani CA, Omidi M, Sarkhosh A. In vitro production of pathogen-free plantlets via meristem culture in saffron (*Crocus sativus* L.). Biotechnology. 2006;5(3):292-295.
15. Takashi I, Takeshi O, Hiroko K. Regeneration of Saffron Protoplasts Immobilized in Ca-alginate Beads, Japanese Journal of Breeding. 1990;40(2): 153-157, Released on J-STAGE April 18, 2008, Online ISSN 2185-291X, Print ISSN 0536-3683, Available:https://doi.org/10.1270/jsbbs1951.40.153.
16. Ebrahimzadeh H, Karamian R, Nouri Dalouei MR. Shoot Regeneration from Saffron Protoplasts Immobilized in Ca-Alginate Beads. Journal of Sciences Islamic Republic Of Iran. 2000;11(2):69-72. Available:https://www.sid.ir/en/journal/ViewPaper.aspx?id=33783