**IRANIAN JOURNAL OF BOTANY 28 (1), 2022**
**DOI: 10.22092/ijb.2022.126955**

# CYTOGENETICS OF SAFFRON (CROCUS SATIVUS), A NATURAL AUTOTRIPLOID SPECIES; AN INVESTIGATION OF THE REASONS FOR ITS STERILITY

**S. M. Ghaffari**

Received 2022. 01. 08; accepted for publication 2022. 06. 07

Ghaffari, S. M. 2022. 06. 30: Cytogenetics of saffron (*Crocus sativus*), a natural autotriploid species; an investigation on the reasons for its sterility. *Iran. J. bot. 28 (1): 57-63.* Tehran.

The red-orange-colored stigmas of saffron are one of the most expensive spices in the world. Meiosis processes in saffron showed that *Crocus sativus* L. is an autotriploid species with 2*n*=3x=8+8+8=24 chromosomes. The results obtained from the meiosis behavior indicated that the major role in the sterility of saffron is autotriploidy in this crop. Arrangement of chromosomes as trivalents in meiosis and formation of these trivalents in the forms of frying pan, chain type, Y type, V type, and triangle type at metaphase I, causes their imbalance junction at anaphase I. Unbalance disjunction of chromosomes at anaphase I and II with the occurrence of the lagging chromosomes in these phases, causes sterile gametes. Also, the formation of chromatid bridges at anaphase I and II with the occurrence of micronuclei at telophase II and tetrad stage are the other reasons for the sterility.

*Seyed Mahmood Ghaffari (correspondence<ghaffari@ibb.ut.ac.ir>), Institute of Biochemistry and Biophysics, University of Tehran, P.O. Box 13145-1384, Tehran, Iran.*

**Keywords**: Saffron; *Crocus sativus*; cytogenetic**;** autotriploid; Iran

**سیتوژنتیک زعفران (*Crocus sativus L.*)، یک گونه اتوتریپلوییدِ طبیعی و بررسی دلایل عقیمی آن**

**سید محمود غفاری**: استاد مؤسسه تحقیقات بیوشیمی و بیوفیزیک دانشگاه تهران، تهران، ایران

یکی از گرانترین ادویه‌های جهان خامه و کلاله قرمز نارنجی خشک شده زعفران است. فرآیند میوز در زعفران نشان داد که Crocus sativus یک گونه اتوتریپلوییدِ می‌باشد که واجد تعداد کروموزوم 2n=3x=8+8+8= 24 می‌باشد. نتایج بدست آمده از رفتار کروموزومها در تقسیم میوز نشان داد که نقش اصلی عقیمی در زعفران مربوط به همین اتوتریپلوییدِ بودن آن است. چینش کروموزومها بصورت تری‌والان و تشکیل این تری‌والان‌ها به شکل ماهیتابه‌ای، زنجیری، Y شکل، V شکل و نوع مثلثی در مرحله متافاز اول باعث تفرق نامتعادل آنها در آنافاز اول می‌شود. تفکیک نامتعادل کروموزومها در آنافاز اول و دوم با بروز کروموزومهای عقب مانده در این مراحل ایجاد گامتهای عقیم می‌کند. همچنین تشکیل پلهای کروماتیدی در آنافاز اول و دوم و بروز هسته‌های کوچک در مراحل تلوفاز دوم و تتراد دلایل دیگری برای عقیمی هستند.

## INTRODUCTION

Archeological and historical sources indicate that the cultivation of saffron (*Crocus sativus* L., Iridaceae) is very old dating back to 1500-2500 B.C., probably originated in Iran, Asia Minor, or Greece (Tammaro 1987; Negbi 1999; Grilli Caiola & al. 2004). The genus *Crocus* currently consists of about 230 taxa according to Rukšāns, (2017). It is distributed from Western Europe and Northwestern Africa to Western China, with the center of the species diversity being on the Balkan Peninsula and west Turkey (Mathew 1982; Ranđelović & al. 2012; Harpke & al. 2013, 2014; Rukšāns 2017). The dry style and red-orange-colored stigmas of the saffron are one of the world's most expensive spices. In Iran, saffron is grown in the eastern part of the country, primarily in Khorasan province. Saffron is used mainly as a dye in industry, as a spice in cooking, as a food colorant, and as a component of

drugs and perfumes (Mathew 1982; Humphries 1996; Behnia & al. 1999; Abdullaev 2007; Cardone & al. 2020; Fallahi & al. 2021; Husaini & al. 2021). Himmerbaur (1926) showed that the *Crocus sativus* has 2n=24 chromosomes for the first time. Karasawa (1932, 1933, 1943) for the first time reported that cultivated *C. sativus* was an autotriploid with 2n=24 chromosomes which formed up to eight trivalents during meiosis. Estilai & Aghamohammadi (1977) have reported that Iranian saffron has 2n=24 chromosomes, and on basis of their finding they assumed that the sterility of saffron is due to its inviable pollen produced by its irregular meiosis. Brighton (1977) showed the karyotype of cultivated *C. sativus* from the central part of Iran, Turkey, France, and England and confirmed that they are triploid. Ghaffari (1986) indicated that the sterility of saffron is due to its triploidy, and phenomena such as laggard chromosomes, non-disjunction, and occurrence inversions will complete this sterility.

According to Mathew (1982), *Crocus cartwrightianus* is the species most similar to *C. sativus* from morphological and cytological points of view. He considers it more probable that *C. sativus* originated from *C. cartwrightianus* by autotriploidy and then it was selected by man for its stigmas which are used to produce saffron. Recent quantitative and qualitative DNA analyses (Brandizzi & Grilli Caiola 1996, 1998) indicated that DNA composition of *C. sativus* is more similar to that of *C. cartwrightianus*. Grilli Caiola & al. (2004) also indicated that *C. sativus* is very closely related to *C. carrtwrightianus* by RAPD analysis. Nemati, & al. (2019) believe that 99.3% of saffron GBS(Genotyping-By-Sequencing) alleles are present in Crocus cartwrightianus and this species is the only producer of saffron.

The diversity of variants of saffron with an increased number of stigmas has been reported by Aghamohammadi (1977) and Estilai (1978). Estilai assumed that this variability could be due to developmental abnormalities, chromosomal variation, and or a rare gene mutation. Although he did determine the chromosome number of four of the rare plants by examining root tips and found that the chromosome number was 2n = 24, as for normal saffron. Ghaffari & Bagheri (2009) indicated that these flowers occur by the fusion of two or more flowering buds on one corm. Cytological and morphological studies showed that this characteristic is unstable and is not genetically controlled. Behdani & Izanloo (2019) indicated that there was no statistically significant correlation between microsatellite marker alleles and the number of stigmas. In this study, all stages of mitotic cell division and meiosis behavior, and pollen stainability are presented.

## MATERIALS AND METHODS

The corms of cultivated *Crocus sativus* studied in the present investigation were collected from five different agricultural areas of Iran (Table 1). The somatic chromosomes were observed in meristematic cells of root tips. The root tips were severed and pretreated in a saturated solution of α-bromonaphthalene for 7 hours at 4°C. They were then fixed in Pienaar's solution (6 parts absolute alcohol, 3 parts chloroform, and 2 parts propionic acid) for 24 hours and stored in 70% ethanol. Staining was carried out by the Feulgen reaction enhanced by squashing in two percent acetocarmine.

Meiotic chromosomes were observed in pollen mother cells. Immature flower buds were fixed in Pienaar's solution for 24 hours and stored in 70% ethanol. The anthers were then stained and squashed in 2% acetocarmine and mounted in Venetian Turpentine (Wilson 1945). The staining technique was used to estimate pollen viability (Dafni & Firmage 2000) with cotton blue.

Table 1. Different areas of saffron cultivation have been sampled in this study.

| Agricultural areas | Coordinate | Altitude (m) |
|---|---|---|
| Khorasan: Birjand | 34° 52' 34.8" N<br>59° 9' 12.6" E | 1426 |
| Khorasan: Qaen | 33° 44' 32.9" N<br>59° 11' 18.7" E | 1430 |
| Khorasan: Gonabad | 34° 20' 54.9" N<br>58° 42' 46.6" E | 1086 |
| Khorasan: Bajestan | 34° 31' 50.3" N<br>58° 9' 2.7" E | 1200 |
| Karaj to Shahdasht | 35° 55' 53" N<br>50° 55' 53" E | 1257 |

## RESULTS AND DISCUSSIONS

In this study, the corms of *Crocus sativus* from 5 localities in Iran were chromosomally examined using their root tip meristematic cells (Table 1.). The results of mitotic studies were the same in all localities. Various phases of mitosis are shown in Fig. 1. In the prometaphase, the chromosomes were observed as discrete structures, and each chromosome was including two chromatids and a constricted centromere where the two chromatids were joined. (Fig. 1B). Metaphase plate showed 2n= 24 chromosomes (Fig.1C). These chromosomes have been shown in eight triplets according to previous reports (Ghaffari 1986; Ghaffari & Bagheri 2009). At the beginning of anaphase separation of sister, chromatids occurred normal (Fig.1D). In general, the autotriploidity of saffron is confirmed in this study as mentioned in the previous investigations (Karasawa 1933; Brighton 1977; Chichiricco 1984; Ghaffari 1986; Ghaffari & Bagheri 2009).

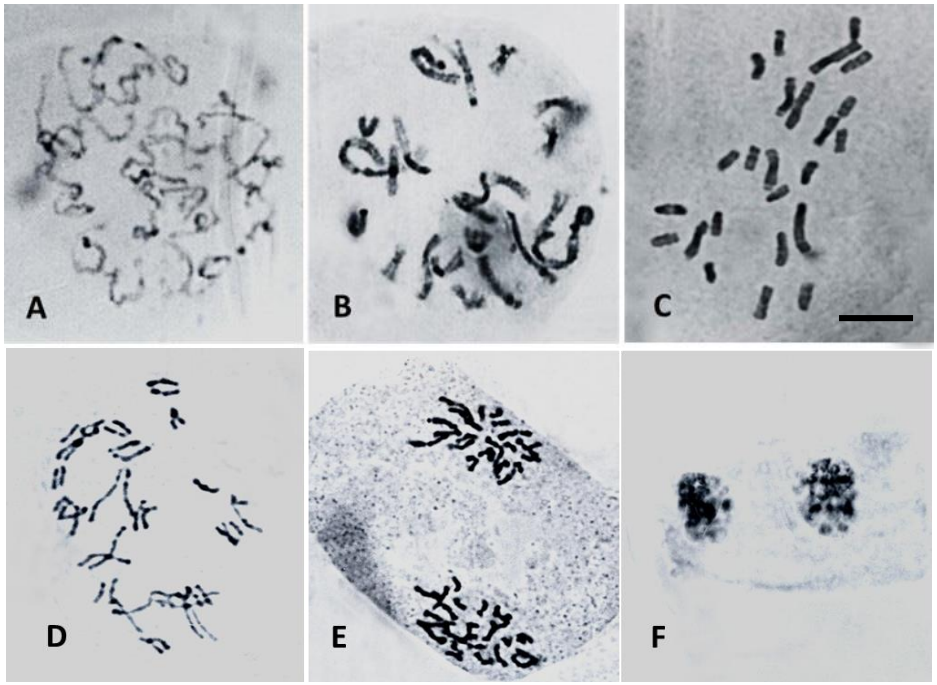


Fig.1. Mitotic phases of saffron (A-F). A, prophase; B, prometaphase; C, metaphase, 2n= 24; D, start of anaphase (chromatids begin to separate); E, anaphase; F, telophase. Scale bar = 5µm.

The meiosis process in five agricultural areas of saffron farms showed that *C. sativus* is an autotriploid with 2n=3x= 8+8+8=24 as reported previously (Karasawa 1933; Brighton 1977; Chichiricco 1984; Ghaffari 1986; Ghaffari & Bagheri 2009). Meiosis behavior in all samples and in all stages of meiosis was similar. Here the results of the Birjand sample (table 1) are presented. A study of chromosome pairing at diakinesis and metaphase I revealed the occurrence of trivalents, bivalents, and univalent (Figs. 2A, B). Analysis of 120 pollen Mother Cells (PMCs) at metaphase I indicated that the 8 trivalents occurred in a higher frequency (58.33%) than the other associations (Table 2). The different shapes of the trivalents were ring- rod (frying pan), chain type, Y type, V type, and triangle type (Figs. 2A, B). The frying pan-shaped trivalents were more than the other types of trivalents, ranging from 3 to 8, followed by V type, chain type, Y type, and triangle type regarding the number of associations. The least frequent shape was the triangle type. Out of a total of 924 trivalents observed in 120 PMCs at metaphase I only 56 PMCs showed triangle type trivalents, ranging from 0-2 and the modal frequency is 0. Since the different shapes of trivalents are of particular interest, the distribution frequency for these is given in Table 3.

Table 2. Frequency of chromosome associations at metaphase I

| No. of PMCs observed | Association of chromosomes at metaphase I | | |
|---|---|---|---|
| | 8III | 7III+1II+1I | 6III+2II+2I |
| 120 | 70 | 38 | 12 |
| % | 58.33 | 31.66 | 10 |

Table 3. Shapes of chromosome associations and their frequency in the saffron at metaphase I.

| No. of the | Shape | No. of PMCs observed | No. of associations observed | Range | Mode |
|---|---|---|---|---|---|
| 1 | Ring-rod (frying pan) | 120 | 600 | 3-8 | 4 |
| 2 | V type | 70 | 108 | 0-3 | 1 |
| 3 | Chain type | 72 | 90 | 0-3 | 1 |
| 4 | Y type | 62 | 66 | 0-2 | 0 |
| 5 | Triangle type | 56 | 60 | 0-2 | 0 |
| ∑ Trivalents | | 120 | 924 | 1-8 | 8 |
| ∑ Bivalents | | 120 | 56 | 0-2 | 0 |
| ∑ Univalents | | 120 | 58 | 0-2 | 0 |

Data on chromosome disjunction at anaphase I is given in Table 4. It showed the abnormal behavior of chromosomes at anaphase I. (Figs. 2C, D, E, F). In maximum cases, 11-13 type of disjunction was observed. Only four PMCs showed 9-15 disjunction, sixteen PMCs showed 10-14 disjunction, and twelve PMCs showed equal (12-12) disjunction (Table 4). Occasionally in some cells laggard chromosomes at anaphase I, telophase I, and anaphase II were observed (Figs. 2G, H, I). Lagging chromosomes were ranging from 1 to 2. Due to the abnormal behavior of chromosomes at anaphase I, a few chromosomes lagged behind on the metaphasic plate and did not include in either pole. The laggards may lead to the formation of micronuclei. Also, single and double chromatin bridges were found at anaphase I and anaphase II (Figs. 2J, k, L). Two binucleated at telophase II and two binucleated microspores in the tetrad stage were observed (Figs. 2M, N). This deviation from normal microsporogenesis and abnormal meiotic behavior results in sterile pollen grains and hence reduced pollen viability.

The pollen stainability obtained from 1830 grains in saffron was 83.49% (table 5, Fig. 2O). This high stainability is inverse of the other autotriploid plants. Low pollen stainability in autotriploid plants is widely reported in the literature (Park & al. 2002; Tyagi & Dubey 1989; Chen & al. 2007; Diao & al. 2009). Thus, it may be concluded that pollen fertility estimated by stainability in saffron does not exactly reflect the fertility of pollen. These results suggested that all unbalanced pollen grains are nonfunctional. Sterility is a key challenge that hinders the crossbreeding in saffron. Sterility has precluded traditional plant breeding methods to increase saffron yield. Other technics such breeding by induced Gamma rye (Rastegar & al. 2007), production of autohexaploids by induced Colchicine (Aghamohammadi 1977), and selection of rare saffron specimens with more than three stigmas in flowers (Ghaffari & Bagheri 2009) have not been successes. However, researchers have shown cultural practices and the application of fertilizers improve the productivity of saffron (Behzad & al. 1992; Behnia & al. 1999; Jahan & Jahani 2007; Rezaian & Paseban 2007; Rezvani & al. 2007).

Table 4. Chromosome disjunction at anaphase I.

| Total number of cells | Number of chromosomes moving to each pole | | | |
|---|---|---|---|---|
| | 9-15 | 12-12 | 13-11 | 10-14 |
| 50 | 4 | 12 | 18 | 16 |

Table 5. Pollen stainability

| No. of pollen | No. of grains stained | No. of grains unstained |
|---|---|---|
| 1830 | 1528 | 302 |
| % | 83.49 | 16.51 |

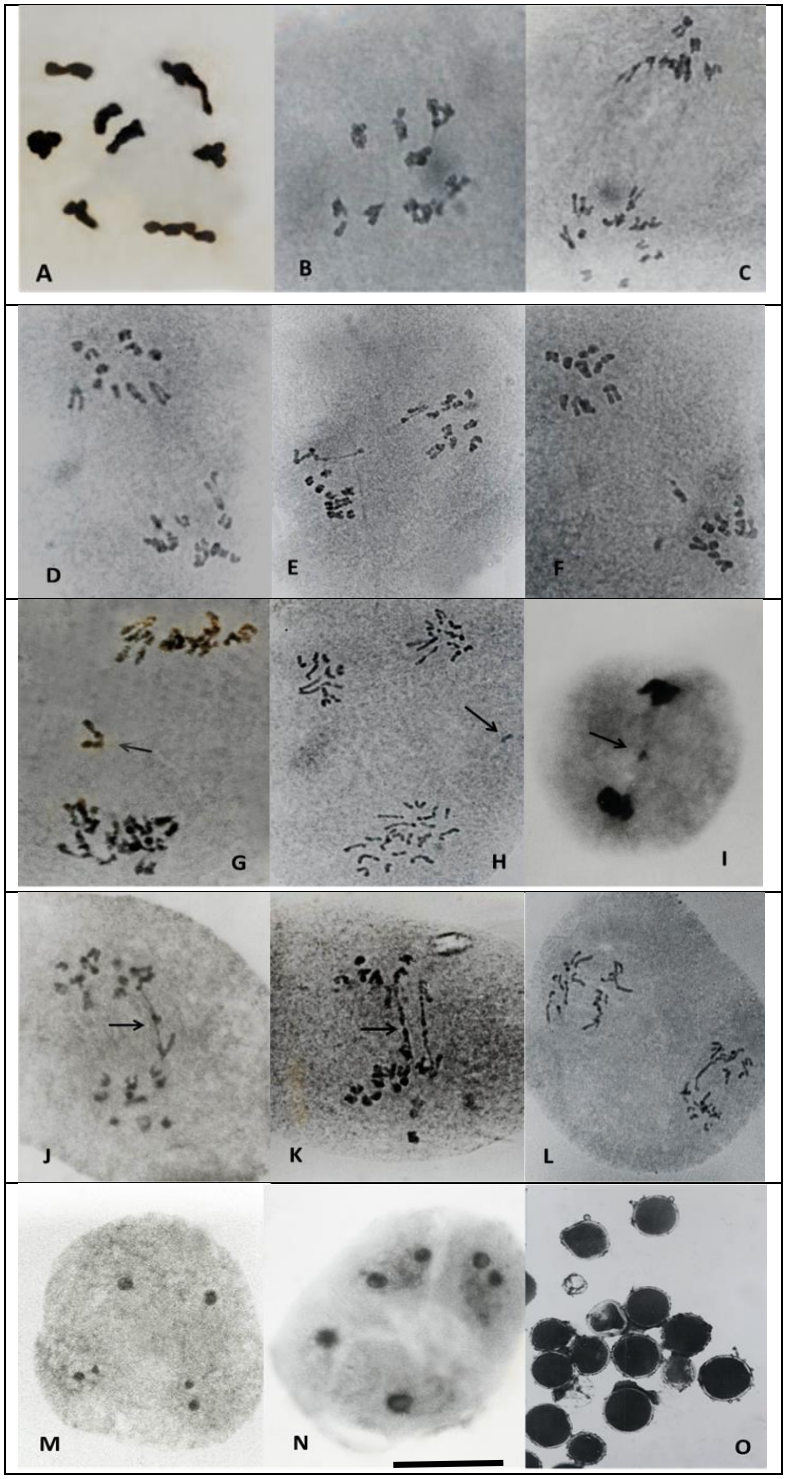


Fig. 2. Meiosis in *Crocus sativus* (A-O): A, metaphase I, showing 8 trivalent in the form of frying-pan, chain type, Y type, V type, and triangle type; B, metaphase I, showing 7 trivalents, one bivalent and one univalent; C-F, anaphase I, showing (10-14), (11-13), (12-12) and (15-9) chromosomes segregation respectively; G-I, showing laggard chromosome (arrows); J-K, anaphase I, showing one and two chromatid bridges (arrows); L, anaphase II, showing chromatid bridge; M, telophase II, showing two binucleated; N; tetrad stage, showing two binucleated microspores; O, stained and unstained pollen grains. Scale bar = 10 µm.

## REFERENCES


Abdullaev, F. 2007: Biological properties and medical use of saffron (*Crocus sativus*). -Acta Horticulturae 739: 339-345.

Aghamohammadi, Z. 1977: An investigation of asexual reproduction, induction of variability of mutagens and sexual sterility of saffron (*Crocus sativus*) as measured by pollen stainability and pollen germination in relation to chromosome pairing. M. Sc. Thesis, University of Tehran. Tehran, Iran.

Behdani, M. A. & Izanloo, A. 2019: Evaluation of genetic variations in saffron with more than three stigmas using SSR and ISSR molecular markers. -Saffron Agronomy & Technology 7: 347-357.

Behnia, M. R., Estilai, A. & Ehdaie, B. 1999: Application of fertilizers for increased saffron yield. -Journal of Agronomy and Crop Science 182: 9-15.

Behzad, S., Razavi, M. & Mahajeri, M. 1992: The effect of various amounts of ammonium phosphate and urea on saffron production. -Acta Horticulturae 306: 337-339.

Brighton, C. A. 1977: Cytology of *Crocus sativus* and its allies (Iridaceae). -Plant Systematics and Evolution 128: 137-157.

Brandizzi, F. & Grilli Caiola, M. 1998: Flow cytometric analysis of nuclear DNA in *Crocus sativus* and allies (Iridaceae). -Plant Systematics and Evolution 211 (3-4): 149-154.

Brandizzi, F. & Grilli Caiola, M. 1996: Quantitative DNA analysis in different Crocus species (Iridaceae) by mean of flow cytometry. -Giornale Botanico Italiano 130: 643-645.

Cadone, L., Castronuovo, D., Perniola, M., Cicco, N. & Candido, V. 2020: Saffron (*Crocus sativus* L.), the king of species: An overview. -Scientia Horticulturae 272:109560.

Chen, Q. F., Hsam Sai, L. K. & Zeller, F. J. 2007: Cytogenetic studies on diploid and autotetraploid common buckwheat and their autotriploid and trisomics. -Crop Science 47: 2340-2345.

Chichiricco, G. 1984: Karyotype and meiotic behavior of the triploid *Crocus sativus* L. -Caryologia 37: 233-239.

Dafni, A. & Firmage, D. 2000: Pollen viability and longevity: ecological and evolutionary implications. -Plant Systematics and Evolution 222: 113- 132.

Diaom, W. P., Bao, SY., Jiang, B., Cui, L., Qian, CT. & Chen, JF. 2009: Cytogenetic studies on microsporogenesis and male gametophyte development in autotriploid cucumber (Cucumis sativus L.): implication for fertility and production of trisomics. -Plant Systematics and Evolution 279:87-92.

Estilai, A. 1978: Variability in saffron (*Crocus sativus*). -Experientia 34: 725

Estilai, A. & Aghamohammadi, Z. 1977: Pollen stainability and pollen germination in relation sterility of saffron (*Crocus sativus*). Bulletin Faculty of Science Tehran University 9: 10-15.

Fallahi, H. R., Abbasi Avval Bohlooli, S., Pahlavan, Z., Hosseini, S. M., Hosseini, SAH. & Ghohestani-Bojd, P. 2021: Saffron vegetative growth as affected by transplanting and direct corm planting under field conditions. Journal of Horticulture and Postharvest Research 4 (Special issue: Recent advances in saffron):1-10.

Ghaffari, S. M. 1986: Cytogenetic studies of cultivated *Crocus sativus* (Iridaceae). -Plant Systematics and Evolution 153:199-204.

Ghaffari1, S. M. & Bagheri, A. 2009: Stigma variability in saffron (*Crocus sativus* L.). -African Journal of Biotechnology 8: 601-604,

Grilli Caiola, M., Caputo, P. & Zaier, R. 2004: RAPD analysis in *Crocus sativus* L. accession and related Crocus species. -Biolgia Plantarum. 48: 375-380.

Harpke, D., Meng, S., Rutten, T., Kerndor, H. & Blattner, FR. 2013: Phylogeny of *Crocus* (Iridaceae) based on one chloroplast and two nuclear loci: ancient hybridization and chromosome number evolution. -Molecular Phylogenetics and Evolution 66: 617-627.

Harpke, D., Peruzzi, L., Kerndorff, H., Karamplianis, T., Constantinidis, T., Ranđelović, V., Ranđelović, N., Jušković, M., Pasche, E. & Blattner, FR. 2014: Phylogeny, geographic distribution and new taxonomic circumscription of the *Crocus reticulatus* species group (Iridaceae). -Turkish Journal of Botany 38: 1182-1198.

Himmerbaur, W. 1926: *Zur Entwicklungsgeschichte von Crocus sativus* L. Festschrift, Tschirch Taunitz, Leipzig. pp.335-350

Humphries, J. 1996: The essential saffron companion. Grub Street, London

Husaini, A. M., Nadiya Jan, K. & Wani, GA. 2021: Saffron: A potential drug-supplement for severe acute respiratory syndrome coronavirus (COVID) management. -Heliyon 7: e07068.

Jahan, M. & Jahani, M. 2007: The effect of chemical and organic fertilizers on saffron flowering. -Acta Horticulturae 739: 81-86.

Karasawa, K. 1932: Kariological studies of some flowering *Crocus*. -Botanical Magazine (Tokyo) 46: 800-802.

Karasawa, K. 1933: The triploidy of *Crocus sativus* L. and its high sterility. -The Japanese Journal of Genetics. 9: 6-8.

Karasawa, K.1943: Karyological studies in Crocus. III. -Journal of Japanese Botany 12: 475-503.

Mathew, B. 1982: The Crocus. A revision of the genus Crocus (Iridaceae). Batsford, B.T. Ltd., London.

Negbi, M. 1999: Saffron cultivation: past, present and future prospects. In: Negbi, M. (ed.). Saffron *Crocus sativus* L. Harwood Academy Publication, pp. 19-30. Amsterdam.

Nemati, Z., Harpke, D., Gemicioglu, A., Kerndorff, H. & Blattner, F. R. 2019: Saffron (*Crocus sativus*) is an autotriploid that evolved in Attica (Greece) from wild *Crocus cartwrightianus*. -Molecular Phylogenetics and Evolution 136:14-20.

Park, S. M., Wakana, A., Kim, J. H. & Jeong, C. S. 2002: Male and female fertility in triploid grapes (*Vitis* complex) with special reference to the production of aneuploid plants. -Vitis 41: 11-19.

Randelovic, N., Randelovic, V. & Hristovski, N. 2012: *Crocus jablanicensis* (Iridaceae), a new species from the Republic of Macedonia, Balkan Peninsula. -Annales Botanici Fennici 49: 99-102.

Rastegari, J., Vedadi, S, & Ghaffari S. M. 2007: Causing mutagenesis through the Gamma rays upon the saffron immature corm buds. -Journal of Nuclear Science and Technology (JonSat) 40: 41-46.

Rezaian, S. & Paseban, M. 2007: The effect of micronutrients and manure fertilizers on the quantity and quality of Khorasan saffron. -Acta Horticulturae 739: 155-158.

Rezvani-Moghaddam, P., Mohammad-Abadi, A. A. & Sabori, A. 2007: Effect of different animal manure on flower yield and qualitative and quantitative characteristic of forage production of saffron (*Crocus sativus*) in Mashhadconditions. -Acta Horticulturae 739: 159-163.

Rukša-ns, J. 2017: The world of crocuses. The Latvian Academy of Sciences.

Tammaro, F. 1987: Notizie storico-colturali sullo zafferano (*Crocus sativus* L., Iridaceae) nell′s area mediterranea. -Micologia e Vegetazione Mediterranea 2: 44-59.

Tyagi, B. R. & Dubey, R. 1989: Meiotic chromosome behavior and fertility in autotriploids of Egyptian henbane, *Hyoscyamus muticus* L. -Caryologia 42: 153-163.

Wilson, G. B. 1945: The Venetian turpentine mounting medium. -Stain Technology 20: 133-135.