

Looking for Saffron's (*Crocus sativus* L.) Parents

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ABSTRACT

The authors analyze the archeological, historical, botanical, cytological, geographic, molecular and reproductive biology of saffron and allied species in order to establish its site and parent origin. The authors have studied saffron, *Crocus sativus* and the diploid species *C. cartwrightianus*, *C. thomasii*, *C. hadriaticus* and compared them with what was previously known from the literature. When saffron originated is still open to dispute. It has been widely known since the pre-Hellenic and Hellenic periods, but it is impossible to detect if was *C. sativus* or other *Crocus* species such as *C. cartwrightianus*. Concerning the site origin the research indicates two possible sites: one in Greece in the Mediterranean area, the other at East in Turkey-Iran-India. In both areas, records and place names connected with various species of *Crocus* constitute an important information source for the presence of saffron. Cytological, DNA, and reproductive studies on the allied species of *C. sativus* such as *C. cartwrightianus*, *C. thomasii*, *C. hadriaticus*, indicate a more likely parent of saffron may be *C. cartwrightianus* or *C. thomasii*. Both these species are diploid with a karyotype similar to saffron. In addition, their pollen can fertilize the egg cell of saffron, giving rise to seeds which are viable, germinate and form new corms. Thus, saffron can originate through fertilization of a normal reduced egg cell with an unreduced male gamete of the same *Crocus* species or by crossing between an egg cell and the male unreduced gamete of another species. The origin of Saffron by allopolyploidy seems more probable considering the recent data on its karyotype and molecular biology.

Keywords: *Crocus cartwrightianus*, *C. thomasii*, *C. hadriaticus*, progenitor/s of saffron

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INTRODUCTION

Crocus sativus L. (Iridaceae) is a small geophyte, cultivated worldwide and known as a source of the spice saffron that is used for cooking, staining, medicine, cosmetics and some other purposes. Saffron is obtained from the dried stigmas of flowers and marketed as saffron filaments or as powder from milled stigmas. It is the most expensive spice. Saffron cultivation areas as well as the produced amount have decreased worldwide, particularly last century, due to high costs for the production of the spice. By contrast the use of and demand for saffron have increased in recent years due to the promotion of new products containing saffron and to improvement in the spice's quality and its conditions of cultivation (Winterhalter and Straubinger 2000; Fernández 2004, 2007).

The interest in saffron studies and application has been reported in three symposia on Saffron Biology and Tech-

nology held respectively in Spain (2003), in Iran (2006), in Greece (2009). The research presented and discussed in these symposia forms a basis for the guidelines for the management of saffron plant and for amelioration programmes based on vegetative or crossing reproduction. The increase in grown saffron is related to the possibility of obtaining higher yields per hectare and the high quality of commercial saffron. The solution to these two problems needs genetic amelioration of the plant, the amelioration of cultivation practices, as well as controlled conditions during the preparation, storage and marketing of the spice. Successful experiments based on the clonal selection of the more productive corms of saffron (Agayev *et al.* 2009) have recently been carried out. Extensive labour will result in higher yield production in future years with the consequence that saffron growth and production will increase. The genetic amelioration of saffron by crossing requires knowledge of the original species or wild relatives as well

as of the ways and site of origin of the cultivated plant. Many studies have been dedicated to solving this puzzle although with disputed results, the exact parents of saffron remaining an open question.

Most of these studies centre round the question as to whether saffron is a true species or whether it should be considered a triploid mutation derived from a wild species by autotriploidy (Chichiriccò 1984; Mathew 1982, 1999; Negbi and Negbi 2002). According to Negbi and Negbi (2002) saffron parent should be *C. cartwrightianus* and the Aegean islands site origin where this species occurs and is still used today by inhabitants. Alternatively it is the result of allotriploidy obtained by crossing process between two wild species (Feinbrun 1958; Brighton 1977; Agayev 2002; Castillo *et al.* 2005). In the first case research is engaged in detecting the living or remote wild saffron progenitor. Alternatively we may have to search for more than one possible progenitor (Grilli Caiola 2006). In all these cases we have to establish the possible site of origin of the progenitor by comparing saffron to species which are genetically and geographically close to *Crocus sativus*. Such work needs the cooperation of many disciplines such as archeology, classic literature, botany, cytology, genetics, phytogeography, biochemistry, molecular biology.

Thanks to the work produced in various saffron-growing countries, a large amount of information has been accumulated on saffron biology and cultivation. Here we will review these studies on saffron parentage and origin by considering studies on archaeological and historical records as well those on cytological, systematics, geographic distribution and utilization of saffron and allied *Crocus* species.

HISTORICAL BACKGROUND

The archaeological and historical records are important for establishing the presence of saffron in an area as well for tracking its spread elsewhere and, finally, for arriving at a conclusion about the possible progenitor of saffron present in the same areas.

The presence of saffron is very ancient in the Mediterranean area as is testified by records, pictures, written throughout the region.

Saffron is a very charming fascinating plant, the long history of which fluctuates between myth, legend and history. According to the mythology Krokos was a beautiful boy in love with the nymph Smilax. At least until Hera, out of jealousy, transformed Krokos into the flower *Crocus* and Smilax into the climbing plant *Smilax* (Cattabiani 1996).

According to a legend the Greek god Zeus slept on a bed of saffron. Another legend relates how Alexander The Great, during the campaign to the East, when he reached Kashmir, settled his army on a plain. The following morning he saw an ocean of violet flowers around his tents and under the hooves of his horses. The flowers could only have been saffron (Aucante 2000).

Archaeological studies carried out on Crete, Cyclads Islands, and mainland Greece have revealed the presence of saffron in all these areas. *Crocus* was known in the Minoan period in Crete three thousand years ago. On the wall of the Minos Palace, at Knossos (Crete), (1700-1600 B.C.) are frescoes depicting crocus-gatherers. *Crocus* flowers are also observable on the skirts and belts of small statues and other objects found in the same Palace (Chirassi 1968). Over 150 bosom bowls were widely distributed in both settlements and tombs throughout Crete, the Aegean and Byblos and Troy. These vessels were more popular during the Neopalatial period (1700-1425 BC) and were made of serpentine carved with a decoration of six broad petals. Such a design suggests a flower of saffron *Crocus* commonly depicted in Cretan wall paintings (Bevan 2007). Other important records are found in the Palace of Akrotiri in Thera (now Santorini) where frescoes represent young women collecting crocuses (**Fig. 1**) and offering them to a divinity (Douskos 1980; Marinatos 1984; Amigues 1988; Nugent 2009). Archeologists interpret the depicted flowers as crocus saffron



Fig. 1 Gatherers of Crocuses on Wall paintings from Xeste 3 building, Akrotiri, Thera. With kind permission from the Thera Foundation – Petros M. Nomikos.

used in ritual ceremonies in the pre-Hellenic and Hellenic ages, in medicine (Forsyth 2000; Ferrence and Bendersky 2004) and as fine dye (Sarpaki 2000). Interesting is the contribution by Sarpaki (2000) on the way of collecting, preparing and using wild *C. cartwrightianus* in Thera in the past. They are similar to those adopted today in the same Island. In addition any other *Crocus* was grown during Minoan and Cycladic era. Miniature frescos of the seventeenth century B.C. representing saffron flowers were found in Syria-Palestina, suggesting that this plant was known and used in the Near East (Niemeier and Niemeier 2000).

Saffron-based pigments have been found in 50,000 year-old depiction of prehistoric beasts in the region that now is Iraq. Later Sumerians used wild growing saffron in their remedies and magical potions (Willard 2001). Ancient Persian cultivated Persian saffron *C. sativus hausknechtii* in Derbena, Khorasan by the Xth century B.C.

Toponymy suggests the presence of saffron in sites such as Krocis at Kozani in Greece and in Safranbolu (Turkey), the town chosen as a World Heritage City by United National Educational Scientific and Cultural Organization (UNESCO) in 1994 due to its well-preserved Ottoman houses and architecture. This is a site where saffron is grown and every year around the city the Festival of Harvest Saffron is celebrated. Another site is the unknown town Azupirano, “The City of Saffron”, on the banks of river Euphrates, Iran. It is the city where Sargon, founder of Akkadian Empire, is born about 2300 BC. By 1000 B.C. saffron was being widely used in Iran where it was a symbol of love and luxury (Basker and Negbi 1983). In the 4th century B.C. a main cultivation area of saffron was Corycos in Cilicia, the Mediterranean coast of Turkey. It is probable that the name of Corycos derives from *Crocus*. Other toponymy is Saffron-Walden in England.

Many Greek, Roman, and Egyptian historians, reported the use of saffron as a precious component to stain cloth, to give special colour and taste to food and drink, and for use in medical therapy (Basker and Negbi 1983; Tammaro 1987; Porter 2000). Authors such as Aesylus (525-456 B.C.), Sophocles (497-406), Hippocrates (460-370), Aristophanes (445-384 B.C.), Theophrastus (371-287 B.C.), Plautus (255-184 B.C.), Varro (116-27 B.C.), Celsus (14-37 A.C.), Pliny the Elder (23-79 A.C.), Galen (129-200 A.C.), and Petronius (?-66 A.C.) cited the use of crocus in their works. In “Oedipus at Colonus” Sophocles wrote that “the bodies of Demetra and Core were embellished by a crown of Narcissus and Crocus”. Aeschylus in “Perseus” describes

the Queen invoking her husband King Dario with “shoes dyed with Crocos”. Plautus (255-184 B.C.) in “Aulularia” mentions “the dyers of crocos”.

From the above reported information it turns out that use of crocus was widespread and perhaps grown in the past. Greece is today the homeland of around 40% of the world’s wild *Crocus* diversity (Tsoktouridis *et al.* 2009). It is possible that among the numerous species, saffron also originated. However the word “Crocus,” or “Krocos” does not guarantee the identification of the crocus flower with saffron, the plant nowadays used and cultivated. In fact it is possible that other *Crocus* species could be used for similar purposes. At Santorini, in the Cyclads Isles (Greece) *Crocus cartwrightianus* occurs as a wild plant which the local habitants use as saffron.

Theophrastus (*Historia plantarum*) and Pliny (*Naturalis Historia*) described many types of crocus. Theophrastus said “In autumn bloom the crocus, both the scentless mountain form and the cultivated one (saffron-crocus); for these bloom directly the first rains come; crocus is reproduced by roots”. Pliny wrote: “Among the crocum types the wild is the best but it is not convenient for growing in Italy in that it reduces its growth. Crocum cultivated is larger, and more fine but more delicate; it easily degenerates and does not produce much. The most appreciated crocum is from Cilicia, mainly that from Mount Corico, then that from Licia of Mount Olympus, and after that of Sicily. According to some authors second place for saffron quality goes to crocus from Thera”. Varro (*De re rustica*) suggests “sowing lilium and crocum” during the period of Pleiads, around October”. Similar suggestions occur in “Res Rustica” of Columella (second century A.C.).

From the above reported citations it is possible to deduce that the Mediterranean region is one of the probable sites of saffron origin; another site could be in the East, in Turkey-Iran- India, where saffron cultivation is reported to be thousands of years old. According to some Authors (Alberini 1990; Winterhalter and Straubinger 2000) saffron originated at first in Iran and Kashmir from where Phoenicians introduced it to the Greek and Roman world. Later (about 960 A.C.) it was brought by the Arabs and Moors to Spain. In effect the term in ancient Greek is “koricos”; the Roman’s used the term “crocum”; by contrast saffron probably originates from the Arabic word Zafaran, zaafar (Gerarde 1636). The Arabic “safran” is quite similar in various other languages: English, saffron; Italian, zafferano; French, safran; Spanish, azafran; German, saffran; Russian, shafran; Turkish, zaferen. This consideration suggests how ancient is its use worldwide.

In Europe, the diffusion of saffron was thanks to the Arabs who brought it to Spain and other territories such as Sicily. In France, it appears during the Crusades at the end of the 1300s A. D. In Provence, the major development of saffron occurred in XI and XVII centuries. In Great Britain, it was introduced in XIV century from Kashmir and then from Tripoli in 1582. It was grown in Saffron-Walden in Essex for colouring and medicine, but others sites of saffron cultivation such as Cambridge are reported (Gerarde 1636).

In Italy, saffron cultivation was introduced between 800 and 900 A.D., at first in Sicily, then in Calabria, Umbria, Tuscany and Abruzzo (about 1400 A.D.). Navelli, L’Aquila, for many centuries has been the main saffron cultivation centre in Europe, till at least to the 1960s and 70s when the production cost caused a decrease in the plant’s cultivation. Recently, after appropriate cooperative organization the saffron grown has increased in Abruzzo and been extended to many other Italian regions including Tuscany, Liguria, Umbria, Sicily, Calabria, and Sardinia (Gresta *et al.* 2007).

MORPHOLOGY AND SYSTEMATICS OF *CROCUS SATIVUS* AND ALLIED SPECIES

Until Linnaeus saffron was referred to as “cultivated crocus”. It was known for its morphology, infertility and uses, being distinct from wild crocus mainly on the basis of mor-

phological, flowering period and growing characteristics. The name Saffron was used by Gerarde in 1597, when the first edition of his “The Herball or Generall Histories of Plants” appeared. This Author in the edition of 1636 described cultivated saffron, *C. sativus* and other wild spring flowering saffron afterwards identified as *C. flavus*, *C. vernus*, *C. versicolor*, *C. nudiflorum*.

The scientific name of saffron, is due to Linneaus who in 1762 named *Crocus sativus* var. *officinalis*, a cultivated *Crocus* of Family Iridaceae. The species *Crocus sativus* L. is now recognized by Mathew (1982, 1999; Frello *et al.* 2004). The systematics and taxonomy of *C. sativus* has been complicated by synonyms appeared in the literature as *C. sativus* var. *cashmirianus* Royle (1836); *C. orsini* Parl. (1858); *C. sativus* var. *orsini* (Parl.) Maw (1886). Until to arrive to Paradies (1957) who considered *C. thomasii* a geographical subspecies of *C. sativus* L. Then, Tutin *et al.* (1968) in *Flora europaea* described the species *C. sativus* as *C. cartwrightianus*, whereas Pignatti (1982) in *Flora d’Italia* reports *C. sativus* in *C. thomasii* Ten. and as *C. medius* Balbis.

However, many other *Crocus* have been found in the wild and cultivated for ornamental purposes. This induced some authors to give a systematic order to the numerous recognized species and intraspecific taxa within the *Crocus* genus, taking into account the flowering period and the plant’s morphological and geographical features (Herbert 1847; Maw 1886).

In the “*Crocus*” 1982 edition Mathew enumerated 80 species, of which 6 were identified in 1700, 54 in 1800, 20 in 1900. Recently Petérsen *et al.* (2008) listed 88 species, 8 new species being added to the previous ones from 1983 to 2007. According to Nørbaek *et al.* (2002) more than 100 cultivars of *Crocus* are known today, these being selected by means of hybridisation between relatively few species. Probably the list of *Crocus* species is far from closed and new taxa will be added in the future.

Mathew (1982, 1999) distinguished the *Crocus* genus into subgenus *Crocus* and *Crociris*. In subgenus *Crocus* are the sections: A) *Crocus*; B) *Nudiscapus*. The morphology of the corms, tunics, bracts, bracteols, leaves, flowers and seeds, the flowering period, cytological and ecological features have been used by Mathew to divide the *Crocus* genus into a hierarchy of sub-genus, sections and series and to define the species within those infrageneric groupings.

Anthers with extrorse dehiscence characterize the subgenus *Crocus*. Scapes subtended by a membranous prophyll, enclosed and hidden within the sheathing leaves or cataphylls distinguish the section *Crocus*. Corm tunics finely fibrous usually reticulatae; flowers autumnal, leaves rather numerous usually 5-30, appearing with the flowers or shortly after; bracts flaccid, usually not closely sheathing the perianth tube membranous, white or transparent with no markings; anthers yellow; style branches 3, usually and often expanded at the apex, entire or not at most fimbriatae; seed coats covered with a dense mat of papillae. All these features characterize the series *Crocus*. To series *Crocus* belong: *C. sativus*, *C. asumaniae*, *C. oreocreticus*, *C. moabiticus*, *C. cartwrightianus*, *C. mathewii*, *C. hadriaticus*, *C. thomasii* and *C. naqabensis*.

C. sativus L. (1762) according to Mathew 1982, 1999, (Fig. 2A) is “a geophyte with depressed-globose corms, flattened at the base, with fibrous tunics, finely reticulate, extending at the apex of the corm into a neck up 5 cm long (Table 1). Cataphylls are membranous, the leaves are usually synanthous, erect, green, glabrous or ciliate. Flowers are fragrant, deep-lilac purple, with darker veins and a darker violet stain in the throat which is white or lilac, and pubescent. Perigonium tube is with segments subequal. Stamens are purplish, glabrous and anthers exceeding at least half the length of the perianth. Perigonium segments arise at a point below the base of the anthers in the throat of the flower. Capsules and seeds have been only rarely reported, saffron being considered a sterile species. It is known only as a cultivated plant”.

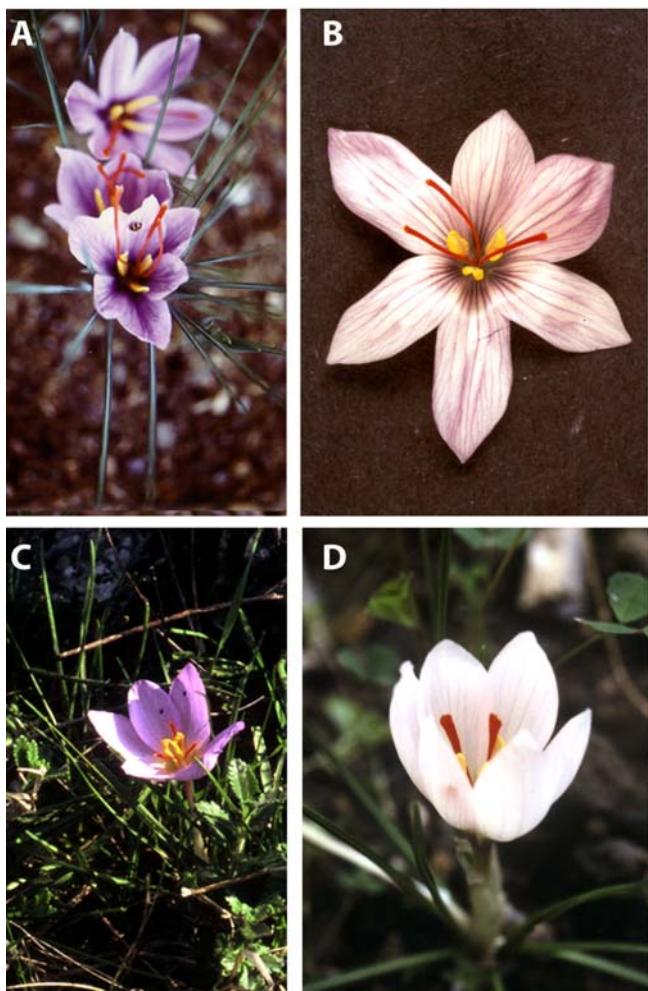


Fig. 2 Flowers of *Crocus* (**A**) *C. sativus*; (**B**) *C. cartwrightianus*; (**C**) *C. thomasii*; (**D**) *C. hadriaticus*.

C. cartwrightianus Herbert (1843) (Herbert 1847), (**Fig. 2B**). Description by Mathew (1982, 1999) is: “depressed-globose corms, with tunics fibrous, are finely reticulated, extended at the apex of the corms. Fragrant flowers are strongly veined darker, sometimes stained darker at the base of the segments and on the tube, sometimes pure white with no veining in the albinois. Throat white or lilac, pubescent. Perigonium tube is oblanceolate or obovate. Stamens are glabrous or slightly papillose at the base and anthers are yellow. Style is equaling or exceeding the anthers and at least half the length of the perigonium segments, arising at a point well below the base of the anthers and usually in the throat of the flower. Ellipsoid capsule raised on a pedicel

above the ground level at maturity. It produces seeds (**Table 1**). It is known wild and cultivated plant”.

C. thomasii Tenore (1826) (**Fig. 2C**). Description by Mathew (1982, 1999) is: “depressed globose corms with fibrous finely reticulated tunics extended at the apex of the corms into a neck up to 1 cm long. Synanthous leaves, usually equaling the flower at the anthesis, but sometimes only the tips showing, are green, glabrous or papillose at the margins. Fragrant flowers, generally not strongly veined are darker but sometimes veined or, stained violet towards the base of the segments; throat pale yellow, pubescent. Perigonium is elliptic, obovate or oblanceolate, acute or obtuse. Filaments usually pale yellow are glabrous or finely pubescent at the base; anthers yellow. Style divided at a variable point, usually ranging from just below or level with the base of the anthers to about a quarter of the way up the anthers, into 3 bright red branches, half or less than the length of the perigonium segments, expanded gradually to the apex. Capsules ellipsoid raised on a pedicel above ground level at maturity; seeds are globose, with poorly developed raphe and pointed caruncle (**Table 1**). It is known wild and cultivated plant”.

C. hadriaticus Herbert (1845) (Herbert 1847) (**Fig. 2D**). Description by Mathew (1982, 1999) is: “Corms depressed – globose with tunics fibrous finely reticulated extended at the apex of the corm into a short neck. Cataphylls white, membranous. Leaves are normally synanthous, sometimes equaling the flower at the anthesis but sometimes very short and occasionally absent, but then appearing immediately after the flowers, grey-green, ciliate. Fragrant flowers are often stained externally brownish, yellowish or violet at the base of the segments; throat yellow or rarely white, pubescent. Perigonium tube is white, yellow, brownish or violet; segments equal or the inner slightly smaller, elliptic-oblanceolate, obtuse. Filaments yellow or white, glabrous or sparsely and minutely pubescent just at the base; anthers, yellow. Style divided into 3 slender branches, each branch slightly shorter than or exceeding the anthers, less than half the length of the perigonium segments, arising at a point above the throat of the flower. Capsule are ellipsoid and raised on a pedicel above ground level at maturity; seeds are reddish-brown, sub-globose” (**Table 1**).

C. moabiticus Bornmüller & Dinsmore ex Bornmüller (1912) (Kerndorff 1988). Description by Mathew (1982, 1999) is: “corms sub-globose with fibrous tunics parallel at the base and weakly reticulate at the apex extending into a neck. Cataphylls are white, membranous. Leaves are usually present but short at flowering time, grey-green, sparsely papillose on the margin of the keel. Flowers are fragrant, veined purple to varying degrees on all six segments on a white ground colour, sometimes so heavily as to appear purple, sometimes stained darker at the base of the segments and on the tube; throat white or purple, pubescent. Perigonium tube white or purple; segments sub-equal,

Table 1 Features of *Crocus sativus* and allied species.

	Cs ^a	Cc ^b	Ct ^c	Ch ^d	Cm ^e	Co ^f	Ca ^g	Cma ^h
corm (mm)	50 ± 1.0	12 ± 2.0	10 ± 2.0	12 ± 4.0	23 ± 8.0	12 ± 2.0	12 ± 2.0	16 ± 1.0
neck (mm)	50 ± 1.2	27 ± 3.0	15 ± 3.0	8.0 ± 1.0	70 ± 10	-	12 ± 1.0	19 ± 2.0
leaves (number)	8.0 ± 1.0	9.0 ± 1.0	8.0 ± 2.0	7.0 ± 2.0	19 ± 6.0	12 ± 2.0	5.0 ± 1.0	7.0 ± 1.0
flowers (number)	3.0 ± 1.0	3.0 ± 0.5	2.0 ± 1.0	2.0 ± 1.0	5.0 ± 2.0	2.0 ± 1.0	2.0 ± 1.0	2.0 ± 0.5
perigonium l. (cm)	4.0 ± 1.0	3.0 ± 1.0	4.0 ± 2.0	6.0 ± 3.0	4.0 ± 2.0	4.5 ± 1.0	6.0 ± 0.5	7.0 ± 1.0
p. segments l. (cm)	4.0 ± 1.0	1.4 ± 0.5	2.0 ± 1.0	2.0 ± 1.0	1.5 ± 1.0	1.4 ± 1.0	2.5 ± 1.0 x	1.9 ± 1.0 x
	x 1.5 ± 1.0	x 3.2 ± 1.0	x 4.5 ± 2.0	x 4.5 ± 1.0	x 3.2 ± 2.0	x 3.3 ± 0.5	3.0 ± 1.0	3.0 ± 1.0
stamens: filaments length (mm)	1.0 ± 0.5	5.0 ± 1.0	6.0 ± 2.0	7.0 ± 4.0	2.5 ± 2.0	6.0 ± 2.0	4.0 ± 1.0	3.5 ± 1.0
stamens: anthers length (mm)	20 ± 5.0	10 ± 1.0	12 ± 2.0	9.0 ± 2.0	13 ± 4.0	13 ± 2.0	15 ± 3.0	11 ± 1.0
stigmas length (mm)	24 ± 2.0	16 ± 5.0	13 ± 2.0	13 ± 3.0	18 ± 7.0	17 ± 3.0	16 ± 2.0	-
capsule length (cm)	1.90 ± 1.0	2.0 ± 1.0	1.5 ± 0.5	1.5 ± 0.5	1.5 ± 1.0	1.5 ± 2.0	1.9 ± 1.0	2.0 ± 0.5
	x 1.40 ± 0.2	x 1.2 ± 1.0	x 0.6 ± 0.2	x 0.7 ± 0.1	x 2.5 ± 1.0	x 7.0 ± 0.5		
pedicel length	4.0 ± 1.0	4.0 ± 1.0	3.5 ± 2.0	4.0 ± 1.0	very short	at ground level	n.d.	short
seed diameter (mm)	4.4 ± 0.2	3.4 ± 1.0	3.0 ± 1.0	2.0 ± 1.0	3.5 ± 1.0	3.0 ± 2.0	2.5 ± 1.0	4.0 ± 1.0
	x 3.2 ± 0.6	x 2.3 ± 0.5	x 1.8 ± 1.0	x 3.0 ± 2.0	x 3.0 ± 1.0			
flowering	Oct-Nov	Oct-Dec	Oct-Nov	Sept-Nov	Nov-Dec	Oct-Dec	Oct-Nov	Oct-Nov

^a*Crocus sativus*; ^b*C. cartwrightianus*; ^c*C. thomasii*; ^d*C. hadriaticus*; ^e*C. moabiticus*; ^f*C. oreocreticus*; ^g*C. asumaniae*; ^h*C. mathewii*; n.d.=not detected

narrowly elliptic to oblanceolate or obovate, acute to obtuse. Filaments are white ageing to purple, glabrous; anthers are yellow. Style divided into 3 deep red clavate branches, equaling to/or much exceeding the anthers and at least half the length perigonium segments, arising at a point well below the base of the anthers in the throat of the flower. Capsule are ellipsoid, carried on a very short pedicel at maturity, sometimes not exceeding the ground level; seeds dark brown, irregularly sub globose" (**Table 1**).

Crocus oreocreticus B. L. Burtt (1949) (Burtt 1948). Description by Mathew (1982, 1999) is: "corms are ovoid, depressed-globose with fibrous tunic finely reticulated. Cataphylls are membranous. Leaves, subhysteranthous or synanthous but if absent at anthesis then developing after the flowering, are green or slightly grayish and glabrous. Flowers are mid-lilac to purple with darker veining, the external pale silvery or buff coloured throat lilac, glabrous. Perigonium tube, white or lilac; segments sub-equal, oblanceolate, obtuse, the inner usually slightly smaller than the outer. Filaments, glabrous; anthers yellow. Style divided into 3 red thickened branches, and about equaling the tips of the anthers, arising at a point at or just above the throat of the flower, below the base of the anthers. Capsule oblong, on a short pedicel just above ground level; seeds reddish-purple, sub globose" (**Table 1**).

Crocus pallasii Gold. (1817). It is rather a variable species with pale lilac flowers and rather short, inconspicuous style branches, less than half as long as the perigonium segments. It occurs from the Balkans to Iran and from the Crimea to S. Jordan. Over this large area, it varies considerably, four subspecies being recognized.

Crocus asumaniae B. Mathew *et al.* (1979). Corms are ovoid with tunic fibrous extending at the apex of the corm into a neck. Cataphylls are membranous. Leaves are hysteranthous or with the tips just showing at anthesis, slightly greyish-green, glabrous. Flowers are white, occasionally with dark veins near the base of the segments, rarely very pale lilac; throat whitish or pale yellow, glabrous. Perigonium tube white; segments sub-equal, oblanceolate or narrowly elliptic, obtuse to acute, the inner slightly smaller than the outer. Filaments white or pale yellow, glabrous; anthers yellow. Style divided into reddish-orange clavate branches, each considerably exceeding the anthers and at least half the length of the perigonium segments, arising at a point well above the base of the anthers. Capsules are ellipsoid; seed reddish-purple, subglobose.

Crocus matthewii Kerndorff and Pasche (1994). Corm 16-mm in diameter depressed globose, with tunics fibrous extended into a neck. Cataphylls are silver-white, membranous. Leaves are hysteranthous dark green, sparsely ciliate. Fragrant flowers are white or rarely pale lilac-blue, often stained deep violet at the base of the segments inside and outside; throat violet, pubescent. Prophyll, bract and bracteole present. Perigonium tube, usually violet in the upper part, paler to almost white lower down; segments sub-equal, ovate to obovate, obtuse to slightly acuminate, the inner slightly smaller than the outer. Filaments, white, glabrous; anthers, usually clearly exceeding but sometimes equaling or rarely shorter than the anthers and less than half as long as the length of the perigonium segments, arising at a point well above the base of the anthers. Ellipsoid capsule raised on a short pedicel above ground level at maturity; seeds purplish-brown, globose.

C. naqabensis Al-Eisawi and Kisawi (2001). Species nova for Jordan Flora (Al-Eisawi 1985, 2001) has features similar to *C. pallasii* but its corms have reduced tunics that do not form a neck. Moreover, flowers have a globous throat. *C. naqabensis* is also closely related to the endemic *C. moabiticus* and *C. cartwrightianus* from which it differs for the style branches which are not more than half as long as the perianth segments, for the absence of tunic necks and the glabrous throat.

According to the above reported systematic criteria *C. sativus* belongs to Family Iridaceae, genus *Crocus*, subgenus *Crocus*, section A, Series *Crocus*, Series type: *Crocus*

sativus. Section of *C. cartwrightianus*. The comparison of the autumnal flowering *Crocus* spp. indicates a strong similarity of *C. sativus* to *C. thomasii* and *C. cartwrightianus* (**Table 1**). Their morphology and dimensions are smaller than in triploid *C. sativus*. However comparison of the species on the basis of morphological characters does not allow a precise distinction of the taxa. In polyploids in fact each member of a gene pair do not contributes equally to expression level in the new phenotype (Osborn *et al.* 2003; Otto 2003). And the analysis of flower pigment composition of *Crocus* spp. and cultivars used as chemotaxonomy (Nørbaek *et al.* 2002) generally supports the classifications of Mathew.

CYTOTOLOGY AND CYTOGENETICS

The first cytological studies aimed at investigating relationships among *C. sativus* and related wild species date back to 1931 (**Table 2**). In a list of chromosome number Sugiura (1931) reported *Crocus sativus* with $2n=24$ chromosomes; the same number of 24 somatic chromosomes was detected by Morinaga and Fukushima (1931), in the root-tip cells of *C. sativus*. Subsequently, Mather (1932) found in saffron $2n=15$ and $2n=14$. Karasawa (1933, 1940) for chromosome number of *Crocus* including *C. sativus* and related species, reported $2n=24$ for *C. sativus* which proved to be autotriploid $2n=3x$, $x=8$. Pathak (1940), Feinbrun (1958), Brighton (1977), Mathew (1977), Chichiriccò (1984), Ghaffari (1986), Ebrahimzadeh *et al.* (1998) carried out numerous studies on chromosome number and karyotype of *C. sativus* and on the allied species chromosome behaviour at meiosis as well as on chromosome morphology and composition. The results of these studies confirmed *C. sativus* as triploid with $2n=3x=24$, $x=8$. A similar basic genome $x=8$ but with $2n=16$ has been found in *C. cartwrightianus*, *C. thomasii*, *C. hadriaticus* (**Table 3**). The karyotype construction on the basis of chromosome morphology and their DNA content made it possible to interpret the genome structure as that of an autotriploid (Chichiriccò 1984). However, more recent contributions on the chromosome structure of *C. sativus* accessions from different cultivation areas combined with the use of markers and fluorochromes to prove the chromosome base composition concluded with the hypothesis that saffron may be an allopolyploid (Agayev 2002; Nørbaek *et al.* 2002; Fernández 2004). In addition, Agayev *et al.* (2010) comparing karyograms structure evidenced differences between *C. sativus* L. "Kashmirianus" $2n=24$ and usual *C. sativus* $2n=24$, assuming that the first is a cultivar genetically not identical to the latter.

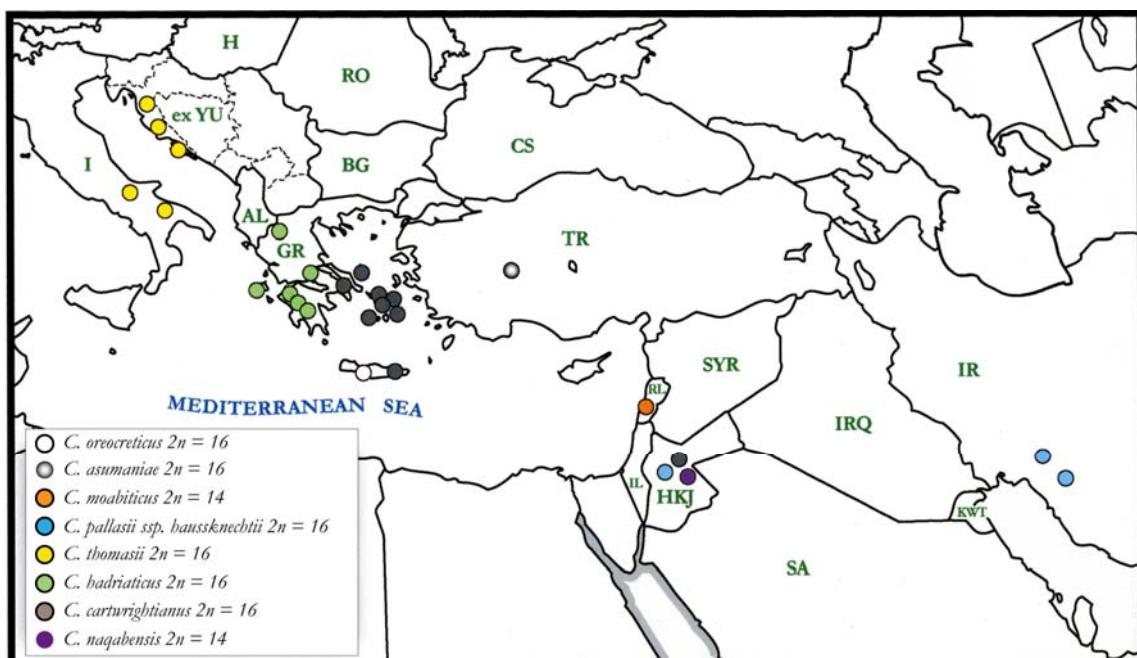
All these studies were important in discriminating the diploid *Crocus* species autumnal flowering from the diploid spring flowering. To autumnal flowering *Crocus* are diploid species with genome $2n=16$, $x=8$, as *C. cartwrightianus*, *C. thomasii*, *C. hadriaticus*, *C. oreocreticus*, *C. moabiticus*, *C.*

Table 2 Chromosome number in *Crocus sativus* L.

Chromosome	References
$2n = 24$	Morinaga and Fukushima 1931
$2n = 24$	Sugiura 1931
$2n = 14, 15$	Mather 1932
$2n = 3x = 24$	Karasawa 1940
$2n = 3x = 24$	Pathak 1940
$2n = 16, 20, 24, 40$	Karasawa 1943
$2n = 20, 22, 28$	Pogliani and del Grosso 1971
$2n = 3x = 24; x = 8$	Mathew 1977, 1999
$2n = 24$	Estilai 1978
$2n = 3x = 24; x = 8$	Chichiriccò 1984
$2n = 3x = 24; x = 8$	Ghaffari 1986
$2n = 24$	Dhar <i>et al.</i> 1988
$2n = 24$	Khan 1996
$2n = 16, 24$	Ebrahimzadeh <i>et al.</i> 1988
$2n = 3x = 24; x = 8$	Agayev 2002

Table 3 Cytology of the species in *Crocus sativus* series.

<i>Crocus</i> species	Author's name	year	Chromosome number	Reference
<i>C. asumaniae</i>	B. Mathew & T. Baytop	1979	2n = 26	Mathew 1999
<i>C. cartwrightianus</i>	W. Herbert	1843	2n = 16	Brighton 1977
<i>C. hadriaticus</i>	W. Herbert	1845	2n = 16	Brighton <i>et al.</i> 1973
<i>C. mathewii</i>	H. Kerndorff & E. Pasche	1994	2n = 16	Mathew 1999
<i>C. moabiticus</i>	F. Bornmuller & J.E. Dinsmore	1912	2n = 14	Kerndorff 1988
<i>C. naqabensis</i>	D. Al-Eisawi	2001	2n = 14	Al-Eisawi 2001
<i>C. oreocreticus</i>	B. L. Burtt	1949	2n = 16	Brighton <i>et al.</i> 1973
<i>C. pallasii</i> subsp. <i>pallasii</i>	K. L. Goldbach	1817	2n = 14	Mathew 1999
<i>dispataceus</i>	E.A. Bowles	1982	2n = 14	Mathew 1999
<i>haussknechtii</i>	(E. Boissier & Reut. x Maw) B. Mathew	1977	2n = 16	Jacobsen and Ørgaard 2004
<i>turcicus</i>	B. Mathew	1977	2n = 12	Mathew 1999
<i>C. sativus</i>	C. Linnaeus	1753	2n = 3x= 24	Agayev 2002
<i>C. thomasi</i>	M. Tenore	1826	2n = 16	Brighton <i>et al.</i> 1973

**Fig. 3** Geographical distribution of diploid autumnal flowering *Crocus* species to *C. sativus* allied.

pallasii var. *haussknechtii*, *C. mathewii*, others as *C. pallasii* with 2n= 12, 14, 16, *C. naqabensis* with 2n=14, *C. asumaniae* with 2n=26. Comparison of the karyotypes of the allied *C. sativus* species demonstrated that *C. cartwrightianus* is one progenitor only in the case of autotriploid (Mathew 1982, 1999; Grilli Caiola *et al.* 2004; Zubor *et al.* 2004; Frizzi *et al.* 2007), or *C. thomasi* (Chichiricò 1984) or more than one species. In the hypothesis of allopolyploidy e.g. *C. cartwrightianus*, *C. hadriaticus*, *C. oreocreticus* (Jacobsen and Orgaard 2004; Agayev *et al.* 2010); or *C. thomasi* and *C. pallasii* or *C. cartwrightianus* and *C. pallasii* (Tammaro 1990) are the candidates as progenitor by cross processes.

GEOGRAPHIC DISTRIBUTION

Further insights into saffron's origin arise by comparing the geographic distribution of the allied species of *C. sativus*. As shown in **Fig. 3** many diploid species of *Crocus* occur in the Mediterranean area (Baytop *et al.* 1975; Burtt 1948; Feinbrun and Shimida 1977; Jacobsen and Orgaard 2004; Kerndorff 1988; Mathew and Baytop 1976; Mathew 1982, 1999). Among them are *C. thomasi* Ten., *C. cartwrightianus* Herb., *C. hadriaticus* Herb., *C. oreocreticus* B. L. Burtt, *C. pallasii* Gold, *C. naqabensis* Al-Eisawi. *C. cartwrightianus* is distributed in south eastern Greece, *C. hadriaticus* is western Greece; *C. oreocreticus* is endemic to Crete in limestone formations up to 200 m high; *C. thomasi* occurs in Italy and in mountains of the Adriatic coast; *C.*

pallasii has a wide distribution in South Turkey and Nord Syria, as do the four subspecies; *C. asumaniae*, Turkey. Included among the Mediterranean *Crocus* is also *C. mathewii*, recently identified and distributed in a small region in southwest Turkey.

Greece and Turkey (Baytop *et al.* 1975; Mathew and Baytop 1976) are the richest countries with the highest number of *Crocus* taxa. Among the 80 species listed in Mathew (1982) 40% of total *Crocus* diversity is in Greece (Tsokdouridis *et al.* 2009; Valamoti and Sarpaki 2009). The other centre rich in *Crocus* is Turkey whose flora comprises 32 *Crocus* species; 18 of them are endemic while 30 are species cultivated as ornamental plants (Arslan *et al.* 2007).

Combining the historical records with systematic, cytogenetic and geographic distribution data (species with 2n=16, x=8) it is possible to identify the saffron cited by ancient historians as most probably being *C. cartwrightianus* or *C. thomasi* or *C. hadriaticus*.

BIOCHEMICAL AND MOLECULAR DNA ANALYSIS

The systematics of *Crocus* genus based on morphological, geographical, cytological data does not allow a precise separation of some taxa and phylogenetic relationships. This is complicated by hybridization and mutation processes resulting from different karyotypes obtained in *C. sativus* and other species from different countries. Since 2000, molecular studies have been undertaken both on *C. sativus* aggregate and on phylogeny of *Crocus* genus (Frello

et al. 2004). The above division has undergone revision, by means of DNA analysis using the cytofluorimetric method (Brandizzi and Grilli 1998), and by sequence data from five plastid regions (Petersen *et al.* 2008), Amplified Fragment Length Polymorphism (AFLP) (Zubor *et al.* 2004), and Random Amplification of Polymorphic DNA (RAPD) (Grilli *et al.* 2004).

Cytofluorimetric analyses of nuclear DNA of different *C. sativus* accessions, from Italy, Spain, Israel, and Holland have revealed some morphological differences but no differences in DNA content and base composition (Brandizzi and Grilli Caiola 1998). In addition triploid content and DNA quality of *C. sativus* was compared to that of diploid *C. cartwrightianus*, *C. thomasi*, *C. hadriaticus*. The analyses performed by flow cytometry and by zymograms of SOD and peroxidases have indicated that there is a scarce intra-specific variability among the analysed species and that *C. sativus* could originate from cross-phenomena in *C. cartwrightianus* with another fertile species. Nuclear DNA analyses by RAPD technique on leaves of the above-listed *C. sativus* accessions and diploid *Crocus* species in plants grown in the same site and utilizing 21 (10-mer) primers did not identify any genomic redundant differences. No differences in corms of saffron from l'Aquila were detected. DNA polymorphism-based taxonomy with the use of AFLP method has provided further results to confirm that the closest relative among the allied of *C. sativus* is *C. cartwrightianus*. However *C. thomasi* also shows similarity to *C. sativus* and *C. cartwrightianus*. Thus, the AFLP method proves that out of six species from series *Crocus*, it was *C. cartwrightianus* and *C. thomasi* which showed an over 70% similarity to *C. sativus* and to each other. This value suggests that among the members of the *Crocus* genus, the closest relationship is between these three species.

Studies by RAPD and microsatellite analysis of DNA in forty three isolates of *C. sativus* from 11 different countries have confirmed that *C. sativus* accessions result identical clones at molecular level (Rubio-Moraga *et al.* 2009). The tandemly repeated DNA sequence family (Frello *et al.* 2004), the internal transcribed nuclear ribosomal regions (ITS1 and ITS2), internal trnH and psbA genes of cpDNA, as well as the 5.8S and 16S ribosomal genes (Tsoktouridis *et al.* 2009) were all used to investigate the phylogeny of the genus *Crocus*. Frello *et al.* (2004) have based their phylogenetic studies on sequences from five plastid regions. They examined 86 of 88 recognized species of *Crocus* and the analysis of a total of 222 phylogenetically informative characters. A clonal origin has recently evidenced by Fluch *et al.* (2010) showing a same alleles by Iranian and Spanish whereas samples from Germany, Austria, Italy and France shared an other allelic combination. Most of these studies have been carried out to establish the possible genetic variations among different *C. sativus* accessions as well as to establish both the phylogenetic relationships of *C. sativus* to other *Crocus* species of the same group, the autumnal flowering diploid with a base genotype $x=8$, similar to that of *C. sativus*.

Results of the DNA studies have revealed small or no differences between *C. sativus* DNA and that of the allied species *C. cartwrightianus*, *C. thomasi* and *C. hadriaticus*. However *C. cartwrightianus* is considered the most probable parent of the triploid *C. sativus* (Frello *et al.* 2004). Similar results have been obtained by Frizzi *et al.* (2007) on analyzing the allozyme differentiation in *Crocus* species genome. Phylogenetically *C. sativus* appears closer to *C. cartwrightianus*, although definitive conclusions are lacking.

REPRODUCTIVE BIOLOGY

Vegetative multiplication

Many studies have been dedicated to the biology of saffron reproduction. Due to its triploidy saffron is usually multiplied by means of selected corms. This process is carried out by man. Only rarely have seeds in *Crocus sativus* been

reported in nature (Piccioli 1932; Di Crecchio 1960), despite the abundant production of sexual reproductive structures such as pollen and ovules.

Saffron has a life cycle characterized by a long summer break and vegetative activity from autumn to spring. The plant, after the loss of leaves, survives the summer as an underground corm. During this period the corm prepares the buds that will originate the new leaves and flowers. The leaves sprout from apical buds on a short stem and are embedded by whitish bracts. From other apical buds originate the flowers, which are frequently hysteranous or appear just after the leaves do. At the base of mother corms smaller new corms also occur, creating a new plant which normally produces only leaves. The number of new leaves, flowers and daughter corms depend on the dimensions and age of the mother corms, on cultivation methods, and on environmental conditions. The number and quality of buds originating leaves, flowers or young corms depends on the amount of resources that the mother corm is able to accumulate during the vegetative period. Recent research (Agayev *et al.* 2009) considers the saffron as a bulk of clones accumulated during the millennial cultivation of the plant by man. The selection of the bigger corms is proposed as a system for improving cultivated saffron (Agayev *et al.* 2009). Although the selection of the bigger corms is a common practice carried out by saffron farmers over many countries, a clonal selection of corms with high yield capacity is a programme with great potential. In fact, the corm after some years of cultivation in the same field does not produce flowers (Tammare 1987, 1990). The number of flowers depends on various factors including the age of the corms. Such considerations date back to the law according to which, in a plant, the allocation of resources between vegetative and reproductive phase are correlated. If the plant uses more resources in producing flowers, in the following years it will have fewer resources for vegetative production such as corm, stem, leaves, flowers, and fruits. More recently Schnittler *et al.* (2009) described the bulbils versus seed production in the liliaceous *Gagea*. These observations shed light on the very difficult conditions that saffron has to overcome in surviving as a wild plant in the natural state. Observations on corms left in field for 10 years without cultivation practices (Grilli Caiola 2005) demonstrated that the corms decreased their dimensions and flower production over the years. The smaller corms had only one leaf, whilst larger corms had 2-8 leaves. Thus, after ten years without cultural intervention, saffron corm loses its vigor and degenerates (Grilli Caiola 2005), giving rise to smaller corms unable to flower and produce new corms. As far back as Herbert (1847) hypothesized that wild saffron disappeared as a consequence of changes occurring in its natural habitat.

Sexual reproduction

The triploid condition of saffron causes an anomalous pairing of the chromosomes at the prophase of meiosis, and an irregular distribution of chromosomes at metaphase with a consequent infertile gametes production (Chichiriccò 1987). However, often in the triploids, pollen and ovules do not exhibit the same behaviour. Generally infertility in uneven polyploids is much higher in the pollen when compared to the ovules. This aspect has been studied in saffron and compared to the behaviour of the allied diploid species by focusing on the structural organization of the reproductive structure as well as on the process of compatibility and incompatibility among species of the *Crocus* group.

a) Microsporogenesis and pollen. Studies on pollen have demonstrated the high number of anomalous pollen grains of *C. sativus* in terms of both grain dimensions and shape. During meiotic division in saffron many abnormalities occur which are also commonly found in other triploid species (Chichiriccò 1984). Microspores often display cytoplasmic degeneration or cellular deformation; consequently they do not complete meiosis or produce anomalous micro-

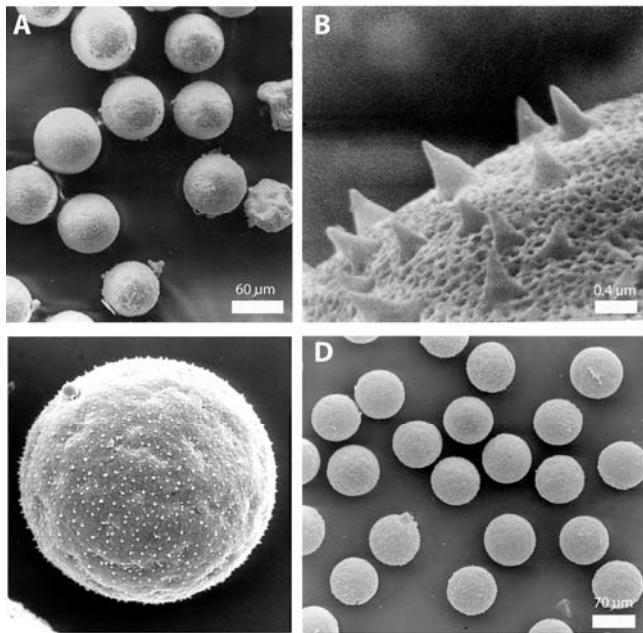


Fig. 4 Pollen grains at SEM (A) *C. sativus*; (B) spinulae on pollen of *C. sativus*; (C) *C. cartwrightianus*; (D) *C. thomasi*.

spores. Afterwards many spores are aborted or the pollen grains are of different size and also often malformed (Chichiriccò 1987). Observed at SEM (Grilli Caiola *et al.* 1985; Grilli Caiola 1999; Grilli Caiola *et al.* 2001; Grilli Caiola 2004), saffron pollen is an elliptical shape with variable size (65-140 µm) and lacking germinative pores or furrows (**Fig. 4A**). About 40% of pollen grains are anomalously shaped, collapsed or broken. The exine surface shows many randomly-disposed spinulae (**Fig. 4B**) and lipid droplets as well numerous perforations of varying size and form. Microsporogenesis and pollen organization in the diploid allied species are generally regular and shape and structure of pollen is similar. Inaperturate pollen is also common in other *Crocus* species (Grilli Caiola *et al.* 1993; Grilli Caiola 1994, 1995).

By comparing generative cell structure, viability and germination *in vitro* and on stigma (**Table 1**), it turns out that saffron has a higher percentage of anomalous pollen grains as well as a lower percentage of viable and germinating ones, both *in vitro* as well *in vivo*, when compared to the diploid species *C. cartwrightianus* (**Fig. 4C**), *C. thomasi* (**Fig. 4D**) and *C. hadriaticus*. The percentage of germination pollen *in vitro* of *C. sativus* proves to be 20% (Grilli Caiola 2005) higher as opposed to the 10% reported in Karasawa (1933). Only 0.4% of *in vitro* germinating pollen grains showed regular pollen tubes, most of the others being accompanied by numerous morphological anomalies such as forked tube, sister-shaped tube, swelling at their base and apex, spiraled pollen tubes and thinning at their ends (Grilli Caiola and Chichiriccò 1982). Significant differences were observed in the viability and germination of pollen collected from flowers at different developmental stages such as in cataphyllic flowers, young flowers and open flowers (Grilli Caiola *et al.* 2001). The highest germination percentage turned out to be from pollen taken from anthers of mature flowers, at the anthesis.

b) Megasporogenesis and embryo sac. At anthesis gynoecium of the above considered *Crocus* species have stigma of dry type, with papillae covered by a thick continuous cuticle. Stigmas are erect until anthesis but as the flower opens they bend downwards. The style is internally made up of three separate channels, forming a single cavity which in the main tract is lined with a layer of secretory cells extending to the ovary where the stylar cavity opens into three locules, being the ovary tricarpellar and trilocular with

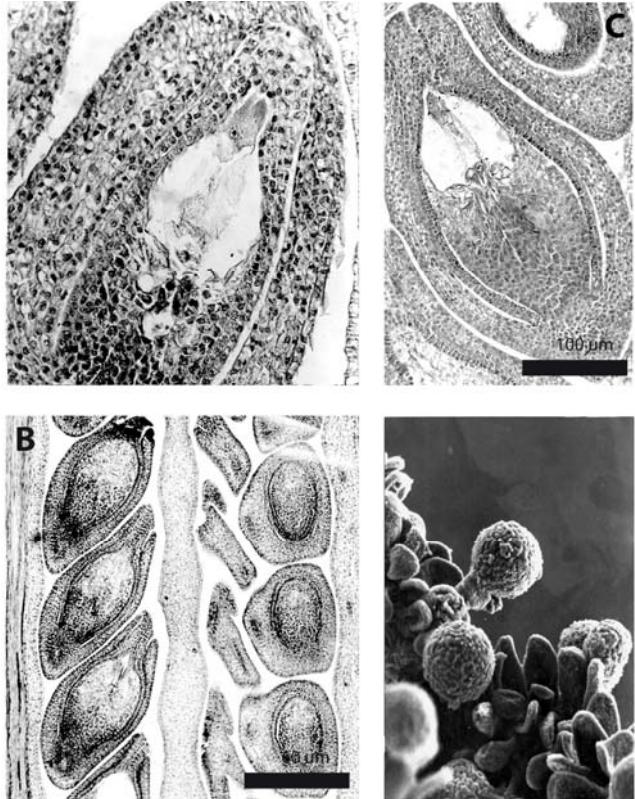


Fig. 5 Ovary and ovules at LM (A) Ovule in longitudinal section of *C. sativus*; (B) Ovary with ovules and (D) a single ovule in *C. cartwrightianus*; (C) stigma of *C. sativus* with autopolllen.

introvarian nectaries. Along the axial region of the locules are placentae on which the ovules are obliquely attached, two rows of 9 ovules for each locule. Ovules are anatropous and bitegmic with a large hypostase. The external integument extends beyond the internal one and forms a narrow micropilar canal. Megasporogenesis occurs during early upper sprouting and an embryo sac appears in the ovules of young flowers still enveloped by cataphylls, so there are no differences between embryo sacs of young and open flowers (Grilli Caiola *et al.* 2001). In *C. sativus* during meiotic division an irregular assortment of chromosomes was observed and the resulted megasporangia were numerically variable (4-6) and genetically unbalanced. About 90% of ovules developed an embryo sac which is broad and 7-nucleate (*Polygonum* type) (**Fig. 5A**). Frequently the ovules do not reach a fertilizable stage due to unsuccessful megasporogenesis or megasporangium development. About 12% of ovules develop a small embryo sac with variable number of cellular nuclei, often no more than four; around 18% of ovules are lacking an embryo sac, and show proliferation of nucellar tissue which increases up to the micropyle. Degeneration of the embryo sac is not frequent and in this case the embryo sac contains abundant granular material and sometimes micronuclei also (Chichiriccò 1987). Megasporogenesis in *C. sativus* is similar to that reported in other *Crocus* species (Rudall *et al.* 1984). The embryo sac is 7-nucleate when mature and contains a substance which stains red with Poinceau especially during the initial developmental stages. The ovary of *C. cartwrightianus* contains two rows of 9-10 ovules for each locule and the inner integument is made up of 4-5 layers (**Figs. 5B, 5C**); *C. thomasi* ovary contains two rows of 12-14 ovules for each locule and each ovule has an integument 4-5 cell layers thick at the micropyle; *C. hadriaticus* ovary has 6-9 ovules for each locule (Grilli Caiola and Chichiriccò 1991; Grilli Caiola *et al.* 2001). In all the three diploid species the megasporogenesis and embryo sac development is regular with a normal development of a female gametophyte including an egg cell, synergids, a central cell, and an antipodal apparatus (Chi-

chiriccò 1989a, 1999; Grilli Caiola *et al.* 2001).

Our recent SEM observations of the ovule surface of *C. sativus*, *C. cartwrightianus* and *C. thomasii* (unpublished data) have shown that the ovule has smooth epidermal. In *C. sativus* ovule has dimensions similar to *C. cartwrightianus* but longer and larger than in *C. thomasi*. In addition in these two species the ovule surface is formed of larger cells, swollen or sunken. In *C. thomasii* (Grilli Caiola, pers. comm.) epidermal cells are smaller, with parallel transversal walls, and a rather flat surface.

Pollination

At anthesis the saffron flower shows adhering tepals, closed anthers and initially erect red stigmas. When the flower opens the stigmas downland and the anthers dehisce. During anthesis the tube of perigonium up to the throat is filled with a liquid nectar originating from intraovarian secretory canals. The secretion rises to the upper part of the ovary and accumulates inside the perigonium tube during the night. During the morning, at increasing temperature, the tepals separate and expose the style with the stigmas downlanding. Our observations from 2000 to 2006 confirmed that insects visiting the flowers of *C. sativus* can be *Bombus sylvestris* and *Apis mellifera*. These pronubus seem to work in various ways according to the environment. Initially we studied the activity of *Bombus* in a small area where *Crocus sativus* and *C. cartwrightianus*, *C. hadriaticus*, *C. oreocreticus* were grown in soil and *C. thomasii* in pots. Usually a *Bombus* group of ten or more individuals appeared at noon of sunny days when the temperature had risen, so facilitating the opening of the flowers and the anthers and the emission of an intense perfume. In these conditions *Bombus* flew from flower-to-flower collecting pollen. On visiting flowers of different *Crocus*, *Bombus* collected mainly pollen, not nectar. From this point of view it seems a good pollinator. *Apis* have been observed on balconies of a private home in the city-center where a series of pots with saffron and allied *Crocus* species were cultivated. Bees arrived in the early morning when flowers were still closed. They were able to separate the adhering tepals and get down into the style to collect the secreted liquid. The bee's main interest was in collecting nectar. They are quick and very active on visiting numerous flowers, when the anthers are still closed. We followed the activity of these insects for many days, ascertaining that bees are able to overcome heavy difficulties represented by flower-covers applied to prevent free pollination. No fruit was obtained from saffron plants visited by bees. These observations seem to indicate that bees are not as good pollinators for *Crocus* as *Bombus* are. It is impossible to establish if these observations could indicate a different territory occupation by different pronubus or other mechanisms as those indicated by Dudareva *et al.* (2006). It is noteworthy that the same insects prefer saffron and *C. cartwrightianus* to other allied species. The absence of pollen transport from bees is revealed by the absence of pollen in honeys from regions where saffron grows such as Aquila in Italy. In Italy only a few *Crocus* pollen have been found in the honey from some Alpine, North and Central regions (Ferrazzi 1991). This contradicts what Columella reports in *Res Rustica* according to which "The cultivation of *Crocus* from *Corycius* and Sicily is useful in dying and perfuming the honey". In open fields it may be possible that bees or bumble-bees carry out pollination in saffron with consequent formation of capsules and seeds as reported by Piccioli (1932) in saffron cultivations of L'Aquila. According to our observations free fertilizations could have occurred in saffron grown near *C. cartwrightianus*, as well in *C. cartwrightianus* and *C. thomasii* when visited by *Bombus*.

Compatibility and incompatibility

Spermatophyta have evolved a genetic system of self-incompatibility (SI) in order to prevent inbreeding so favour-

ing out-crossing. However out-crossing is limited by incompatibility between different species. The mechanisms of intra- and inter-specific breeding are regulated in different ways in different plants. Sometimes structural mechanisms regulate autogamy as in plants with different floral morphs. In other cases self-incompatibility is based on signals at the level of the pro- or postgamic phase. The responsibilities for the inhibition of pollen germination or pollen growth inside the stigma or style vary according to the group of angiosperms. In some cases the cause is the Ca^{2+} ions, in others RNase, and in still others compounds secreted by cells of the stylar canal. Inside *Crocus* genus SI systems have been investigated in species of the *C. sativus* group, such as *C. sativus*, *C. thomasii*, *C. cartwrightianus*, *C. hadriaticus*, *C. oreocreticus*, the goal being to ascertain the mechanism of self-incompatibility (Chichiriccò 1989b, 1996) and establish the relationships within the different species of the group. Overall the results of this research are important for verifying the possibility of establishing the genetic compatibility of *C. sativus* with one or more diploid species. In addition the analysis of seeds obtained by means of crossing saffron with pollen of diploid allied makes it possible to compare the possible similarities to or differences from the maternal plant (Grilli Caiola *et al.* 2010). Results of these experiments have indicated that *C. sativus* is self-and allosterile (**Fig. 5D**); *C. thomasii* is self-incompatible but in outcrossing produces 86% fertilization and 84.5% seed set; *C. cartwrightianus* is self-sterile but out-fertile with a high percentage of fertilization; *C. hadriaticus* shows the highest percentage of ovules with embryo sac and 87% fertilisation after outcrossing, but 0% fertilization after self-pollination. *C. oreocreticus* has a low percentage of ovules lacking embryo sac and has 70% fertilization in out-cross and 40% in self-cross, the highest value observed in the tested species. As regards the interspecific crossing it turns out that pollen of *C. sativus* does not germinate or grow in any of the tested stigmas, but is fertilized by pollen of *C. thomasii* (16.8%), *C. hadriaticus* (8.5%), *C. oreocreticus* (11.0%), *C. cartwrightianus* (10.2%). *C. sativus* seed sets after crossing pollination with *C. thomasii* or *C. cartwrightianus*. The seeds of *C. sativus* x *C. cartwrightianus* were grown for 4 years and their germination and development was studied and compared to seeds obtained from *C. cartwrightianus* outpollinated by hand (Grilli Caiola 2005). Regarding germination, seedling, and young corms formation, the behaviour of the seeds of both the species is very similar.

On the whole the results of the numerous experiments performed on the species of *C. sativus* group and on species of other *Crocus* group indicate that the ovarian self-incompatibility (SI) is widespread within the genus *Crocus*, this resulting in a partial or complete suppression of self-fertilization. Moreover the post-zygotic SI mechanism as well as post-zygotic mechanisms of unknown nature seem to be recurrent and both are responsible for seed abortion. The interspecific ovarian incompatibility concerns only unrelated crosses. Crosses between related fertile species succeed both in fertilization and seed-set (Grilli Caiola and Zanier 2005).

Regarding the mechanisms of incompatibility, studies on RNase activity indicate that in *Crocus* RNases are not responsible for mechanisms of rejection of incompatible pollen tubes. Similar results have been obtained in studies on stylar peroxidase activity against the incompatible pollen tubes (Zanier and Grilli Caiola 2001). The Ca^{2+} also seems not to be responsible for stylar incompatibility (Brandizzi and Grilli Caiola 1996). Investigations using various methods seem to confirm that the cause of arrest of pollen tubes in the examined *Crocus* species is localized at the level of lower part of stylar and upper part of the ovary. Concerning the composition of this mechanism, it seems most probable that it is a glycosilate compound.

Pollen grains of *C. sativus* germinate in a low percentage on the stigma, and pollen tubes do not reach the ovary. These results could be proved for the triploid condition of this species which would negatively condition the correct

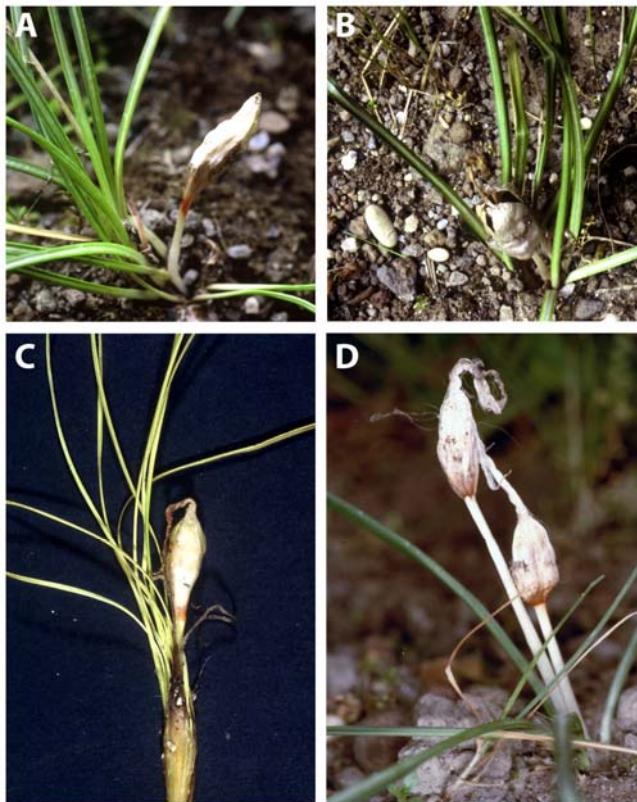


Fig. 6 Capsules (A) *C. sativus* x *C. cartwrightianus*; (B) *C. cartwrightianus*; (C) *C. thomasi*; (D) *C. hadriaticus*.

pollen germination and pollen tube development. *C. cartwrightianus* proves to be mostly an out cross-fertile. This is because, firstly, the pollen germinates on the stigma in a higher percentage after out cross-pollination in respect to self-pollination. Secondly the self-pollen tubes are blocked in the basal part of the style. Finally, the capsules are produced only by out cross-pollinated flowers.

Saffron, although sterile, produces a very high amount of pollen and floral structures which are ineffective for the purposes of seed production. This is unintelligible if we consider the high amount of energy allocated to produce floral organs and function (Cruden 2000). Production of bulbils versus seeds has been detected in *Gagea* (Liliaceae) (Schnittler *et al.* 2009).

Fruit set, seed set and seed coat microstructure

The successful pollination in *Crocus* produces a capsule maturing at soil level. *Crocus sativus* hand-pollinated with pollen of *C. cartwrightianus* originated a large capsule maturing in May and visible above ground. The capsule is erect and provided with a peduncle which connects the fruit to the basal underground corm (**Fig. 6A**). When mature the capsule opens by means of apical breakings and the seeds are dispersed on the soil after the capsule has dried. Similar behaviour is detected in the capsules of the diploid *C. cartwrightianus* (**Fig. 6B**), *C. thomasi* (**Fig. 6C**), *C. hadriaticus* (**Fig. 6D**). Seed surface microstructure has been used in many angiosperms to discriminate critical taxa (Barthlott 1981; Karcz *et al.* 2000; Zeng *et al.* 2004). Seeds from *C. sativus* pollinated with *C. cartwrightianus* were analysed under a light and scanning electronmicroscope in order to establish the similarities to or differences from the seeds of diploid *C. thomasi*, *C. cartwrightianus*, *C. hadriaticus* grown in a similar site and hand out-pollinated. The seed surface morphology, the inner structure and the dimensions of the compared species are all reported in Grilli Caiola *et al.* (2010). The seeds differ in dimension, colour, and raphe development. However, the basic morphology and structure organization are similar in all the considered species. Seed



Fig. 7 *C. sativus* x *C. cartwrightianus*: (A) 1) small roundish corm from germinated seed; 2) Long corms from seed after three years grown. (B) *C. cartwrightianus*: 1) small roundish corm from germinated seed; 2) Long corm form from seed after three years grown; 3) Cormlets formed at the basis of the corm in 2 B).

derives from an anatropous ovule which is curved and the micropyle occurs near the funicle (Grilli Caiola and Chichiricò 1991; Grilli Caiola *et al.* 2001). Due to this position, the seed carries a linear lateral wing-like protrusion known as raphe that appears as a double sheet open along the side length revealing a small opening. The raphe departs from the basal funicle and extends longitudinally along a side of the seed until the apex. The ovule is bitegmic and its inner integument protrudes as a multilayered structure. At this point in the mature seed, a caruncle is more or less evident in different *Crocus* species. The outer tegument proves to consist of seven to ten cell layers. The seed epidermis is covered by a mass of hairs of cylindrical shape, erect or curved or elongated. Endosperm has thin epidermal layer with small cells and the parenchyma with large cells radially elongated, with walls thickened by pectic, giving the seed a hard structure. Storage reserves consist of lipid drops, rare starch granules and abundant proteic granules. Seeds of *C. sativus* have the higher dimensions respect to the diploid allied species. This is in accordance with the triploid condition of the saffron. The seed coat surface has hairs which are similar in *C. sativus* and *C. cartwrightianus* but different from those of *C. thomasi* and *C. hadriaticus* (Grilli Caiola *et al.* 2010). The seeds of *C. sativus* obtained as reported above were germinated and grown in pots for four years. The same treatment was used to germinate and grow the seeds of *C. cartwrightianus* and *C. thomasi* (Grilli Caiola 2005). Results of the observations indicated that saffron seeds were viable and germinated in as high percentage as those of diploid species. In addition, the morphology and growth behaviour of the germling and seedling were similar in *C. sativus* and *C. cartwrightianus*. Seedlings of the first year had a short white prophyl from which a green leaf emerged. During growth a small roundish corm, without tunic, formed at the base of the green leaf. This corm after a dormant period produced a new green leaf and at the end of growth formed a larger new corm with reticulate-fibrous tunic. After three years of growth the corm formed three new cormlets at the base. The corms from the second year were small, ovoid in shape, and protected by reticulate tunics (**Figs. 7A, 7B**).

CHROMOPLAST STRUCTURE AND PIGMENT COMPOSITION

The commercial product and use of saffron comes from the dried stigmas, appreciated for a special colour, taste and aroma (Carmona Delgado *et al.* 2006). Saffron spice gives dishes and other material the yellow colour, bitter taste and intense aroma. Although the main use of saffron is culinary, also important are its pharmacological properties and role in medicine (Abdullaev 2003). The characteristic of colouring, flavouring, and aromatising are related to the presence in the red stigmas of glycosyl compounds, esters of crocetin carotenoids, picrocrocin, and an extensive group of ketones and terpenic aldehydes, safranal being the most outstanding.

Saffron's colour is due mainly to carotenoids, molecules generally insoluble in water but capable of generating an intense colour when in hot water. Carotenoids form a group of pigments very common in plants. They are formed of a long acyclic chain with double conjugate bonds such as lycopene, or, more frequently, of a chain terminated by six or five rings on one or both ends. Due to numerous double bonds carotenoids present various geometric isomers. They can exist as apocarotenoids in a short chain after elimination of some terminal rings and reduction in size of the molecule. In addition acid molecules of crocetin can be esterified by glucose, gentibiose and neapolitanose, giving rise to numerous esters of crocetin present in saffron. According to a hypothesis (Bouvier *et al.* 2003) crocetin in saffron derives from zeaxanthin precursor which breaks at both ends to generate crocetalinaldehyde which is oxidized to give rise to crocetin. Subsequently the glycosylation occurs through the action of a glycosyltransferase.

Saffron's characteristic bitter flavour is due to the presence of picrocrocin, a flavonoid compound. It originates through oxidative degradation of zeaxanthin and is in turn the precursor of safranal, the main compound of the saffron aroma. Picrocrocin is present in saffron in high amounts, about 13% of the dry weight.

Saffron's aroma was for a long time attributed to safranal generated from picrocrocin, but recently many other minor compounds are considered responsible for the saffron aroma. The aroma is the most appreciated component of the saffron spice, although the methods to reveal the presence of such compounds are still ongoing.

The above reported compounds are sited in organelles, the chromoplasts, occurring in the epidermal and parenchyma cells of stigma and the coloured upper part of the style. The structure of chromoplast have been examined in *C. sativus* (**Fig. 8A**) (Grilli Caiola and Caprilli 1983; Grilli Caiola 2004; Grilli Caiola and Canini 2004) as well as in the related species *C. cartwrightianus* and *C. thomasi* (Grilli Caiola and Canini 2005). In saffron the chromoplasts occur only in the red-coloured parts of stigmas and style. They appear in the red stigma of very young floral buds, and originate from amyloplasts, the only plastids present in the colourless basal portion of the style as well as in the parenchyma of ovary and resting corm. Transitional forms of amylo-chromoplasts occur in the yellow parts of the stigma and style. Saffron chromoplasts in red stigma of open flowers are roundish or elongated with tubules forming an electron-opaque reticulum. Some tubules are dilated and form vesicles at the periphery of the plastid. Among the network of numerous tubules are plastoglobules. All plastids contain one or more electron-transparent regions not positive to PATAg staining. Stigmas at anthesis show chromoplasts resulting in mainly vesicles and plastoglobules and are found close to small or large vacuoles. A few mature chromoplasts show bundles of long parallel membranes crossing or encircling the plastidial area. In red stigmas from flowers open for two days, the chromoplasts have only peripheral vesicles and plastoglobules scattered in an electron-transparent stroma. In these cells small or large vesicles in central vacuoles near to the tonoplast membrane are also detected. Chromoplasts in dried stigmas show mainly plastoglobules and empty vesicles spread in plastid, whereas in decoloured stigmas only small plastoglobules, a few vesicles and short thin membranes are observed. On the stigma surface are yellow-coloured papillae, different from the red colour of the stigma. Papillae showed a very large central vacuole and scarce peripheral cytoplasm in which numerous chromoplasts with tubulous reticulum and plastoglobules occur. In the cytoplasm surrounding these chromoplasts there are always vesicles apparently empty except for small vacuoles adhering to the plastids. The chromoplast structure of papillae is similar to that of the plastid present in the yellow part of style. In this part of the style there are plastids with some small starch granules, large plastoglobules and extended system of tubules which at the periphery of the plastid are dilated and form enlarged

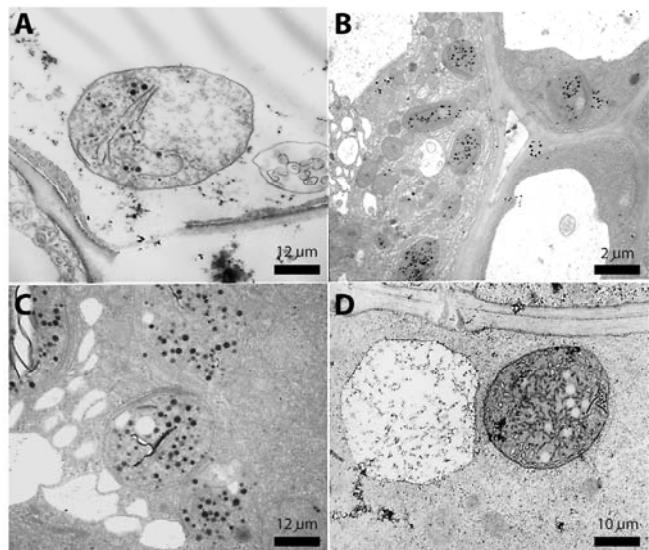


Fig. 8 Chromoplasts (**A**) from stigma of *C. sativus*; (**B**) Cells with chromoplasts from stigma of *C. cartwrightianus*; (**C**) *C. cartwrightianus*; (**D**) *C. thomasi*.

vesicles. In the stroma of these plastids one or more electrontransparent areas are always present. A comparison of plastids from the white part of style, ovary, corm and leaf indicated that plastids with a structure similar to that in chromoplasts or red and yellow part of stigma and style are never present. In the leaf plastids only some plastoglobules occur together with starch granules and thylakoids. Thus the particular structure of saffron red stigma is the presence of tubules, plastoglobules and vesicles. Comparing the ultrastructure of chromoplast of fresh to dried and untreated stigmas to those after extraction of water soluble pigment, we can conclude that apocarotenoids are localized in the tubules and plastoglobules whereas the vesicles can be the site of glycosylate derivatives of crocetin. Tubules in fact disappear in plastids of decoloured stigmas. By contrast plastoglobules, which contain mainly neutral lipids and carotenoids (Deruère *et al.* 1994), persist in all these organelles as well in chloroplasts. They are also widespread in the green and yellow leaves of saffron. In addition, the chromoplast membranes have a high permeability to allow the diffusion of overproduced pigment into cytoplasm (Emes and Tobin 1993) or in autophagic vacuoles (Mesquita 1972). Finally, the water soluble pigment crocetin can be accumulated in the central vacuole of the cell as already hypothesized by Bouvier *et al.* (2003) and Dufesne *et al.* (1999).

C. cartwrightianus chromoplasts (**Fig. 8C**) show many plastoglobules, tubules and one or more electron dense body, spiral like, often crossing the plastid similar to crystal body occurring in carrot roots (Trabucchi 1964) and in tomato fruits (Grilli 1965). *C. thomasi* chromoplasts (**Fig. 8D**) have mainly plastoglobule electrontransparent vesicles encircled in a thin envelope, some thin fibrils and characteristic tubules heavy twisted.

The comparison of chromoplasts of the *Crocus* species related to *C. sativus* suggests that despite the similarity is the basic organization of these plastids, however each species has some different elements and on the whole chromoplasts of *C. sativus* prove to be similar to those of *C. cartwrightianus* and *C. thomasi*.

Parallel to the study of chromoplast structure an analysis of pigment of stigmas of *C. sativus*, *C. cartwrightianus*, *C. thomasi* was carried out in order to detect possible differences in pigment quality and composition amount. Among the pigments separate from crocin, crocetin, picrocrocin and safranal we also analysed the presence of lycopene. This pigment is usually considered to be localized within the chromoplast as tubules due to the thickening of the intraplastidial membranes (Grilli 1965). The preliminary

data indicate that pigment composition and amount are very similar in *C. sativus* and *C. cartwrightianus*. Not lycopene has been detected in stigmas of flowers at anthesis.

CONCLUSION

The data obtained to date from archeology, systematic, cytology, molecular biology, physiology and biochemistry are still insufficient to establish a precise place and moment for the origin of saffron or its parents. However, the information accumulated in recent years offers further indications about the probability that one of the diploid *Crocus* is the parent of *C. sativus*. All data agree that the most probable parent of saffron is *C. cartwrightianus*, only if the origin of saffron is considered by autotriploidy or together with *C. thomasii*, if the origin is due to allotriploidy. In fact:

- remote archeologist and historical records suggest that a *Crocus* has been known and widely used since pre-Hellenistic and Hellenistic times. All authors agree on considering *C. cartwrightianus* as the crocus used in therapy and other fields; wild and cultivated forms are already known;
- saffron grown and used today corresponds to *C. sativus* which morphologically is very similar to *C. thomasii* with which it has been confused in the past;
- *C. sativus* is a triploid species with a karyotype similar to diploid *C. thomasii* and *C. cartwrightianus*;
- geographic distribution indicates that *C. sativus* and the wild diploid *C. cartwrightianus* and *C. thomasii* occupy common Mediterranean areas;
- biochemical and molecular biology show that DNA of *C. sativus* is most similar to that of *C. cartwrightianus* and not so distant from that of *C. thomasii*;
- reproductive biology makes it possible to ascertain that saffron is self- and allo sterile, although its ovules can be fertilized by pollen of *C. cartwrightianus* as well of *C. thomasii*. The crossing leads to seed set and fruits;
- seeds from crossing saffron with *C. cartwrightianus* are viable and capable of germinating, giving rise to a seedling and then a plant from which new corms originate. The germination and growth behaviour of saffron seeds are very similar to that of *C. cartwrightianus*;
- the chromoplast structure and pigment composition place saffron very close to *C. thomasii* and *C. cartwrightianus*;
- although the path of origin of saffron is still an open question, important new contributions could be reached by new research methods on chromosome structure and molecular genetics. The need now is to research saffron's biological aspects, mainly on crossing with the diploid allied species and then examined the progenies with improved molecular methods. It will be a very long way but we think that a young research team could meet this goal and reach interesting results in order to solve the puzzle of parents of saffron. The results could be useful to obtain genetic amelioration of the cultivated saffron.

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