Saffron Reproductive Biology

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Abstract

Studies on saffron reproduction are recent and scarce compared to millenary experience of its cultivation and vegetative multiplication. Sexual reproduction concerns numerous steps: micro- and mega-sporogenesis, gamete differentiation, pollen and pistil organization and compatibility, fertilization, embryo development, seed set and fruit development. Reproductive research on saffron is difficult because the triploid genome of the plant and the still controversial opinion about where, when, how saffron originated. Saffron cultivations from different countries do not show significant variations. When nuclear DNA of *Crocus sativus* L. is compared to that of diploid allies species, it indicates that the most probable ancestor of saffron is *C. cartwrightianus* Herb., although *C. thomasii* Ten and *C. pallasii* Goldb. have been considered other possible parents of saffron. Saffron is male sterile, self-incompatible and if crossed with pollen of *C. cartwrightianus* or C. *thomasii* it seed sets and matures capsules. Self-incompatibility is not mediated by RNase, peroxidase or calcium. Moreover, experiments on stigmatic and intraovarian pollination between *C. sativus* and *C. cartwrightianus, C. thomasii* or *C. hadriaticus* indicate that embryo development is not related to apomixis or somatic embryogenesis. This has been verified both in vitro as well as in field. So in spite of numerous informations until now available on saffron sexual reproduction, many more researches are necessary before of undertaking breeding programme with the aim of ameliorating yield and quality of saffron spice.

INTRODUCTION

Among the 85 species belonging to the *Crocus* genus (Iridaceae), *Crocus sativus* L. (Saffron) is the most fascinating and intriguing species. This is not only because it produces the well known Saffron spice, but for the numerous mysteries surrounding its origins questions as: when it originated; the native area or areas; the ancestor species and the mechanisms of origin; the wild or naturalized plants; the infertility and consequent absence of fruit and seeds.

Some archaeological and historical studies indicate that domestication of saffron dates back to 2.000–1500 years BC (Negbi, 1999; Tammaro, 1987). This is derived from documents reproducing the plant or showing people collecting the crop. However the sites where the first saffron plants appeared differs according to the opinion of various authors. Vavilov (1951) placed saffron into IV plant origin centre of Middle East (Minor Asia, Transcaucasia, Turkestan), whereas more recent contributions indicate that the process of saffron domestication has to be identified on Crete during the Late Bronze Age (Negbi, 1999). According to Negbi (1999) wild *C. cartwrightianus* Herb. was harvested and used, then its mutant *C. sativus*, was observed, selected and domesticated. On the Crete *C. cartwrightianus* grows on a volcanic ash near Akrotiri, Santorini, and is harvested for local consumption by the villagers (Mathew, 1999). However *C. thomasii* Ten., or *C. pallasii* Herb., have also been indicated as possible saffron ancestor (Chichiriccò, 1989; Brighton, 1977; Tammaro, 1990). If its domestication occurred at more sites simultaneously or at different times is still not resolved. This is because saffron is not known to be wild or spontaneous and can only be propagated by human help (Mathew, 1999). Some authors e.g. Herbert (1847) believed that saffron was once naturalized in

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small areas from where it was lost as a consequence of a change in land use. This hypothesis corresponds with the finding showing that corms left in the soil for many years would eventually degenerate (Di Crecchio, 1960). Botanical descriptions of saffron are rather similar along the centuries and the Linnean scientific name, *C. sativus* L., was used to indicate only saffron. However, a few synonyms (Mathew, 1999) such as *C. orsinii* Parl, *C. autumnalis* Smith, *C. thomasii* Ten. (Di Crecchio, 1960) and a significant number of varieties of *C. sativus* occur in the literature.

There is much information on the use of saffron as dye, medicinal herb, aromatic spice (Negbi, 1999), as well as on its agronomic cultivation. Compared to this the studies on saffron biology are very few and quite very recent so that in spite of a millennia of cultivation many aspects of saffron life and its sexual reproduction has still lacking. There is need for further research into increasing saffron production and quality to cope with an increasing demand. This will be achieved by means of plants with more flowers per plant, flowers with a higher number of stigmas, increasing stigmas size or stigmas with an increased amount of dye and aroma. Traditional plant ameliorating techniques are based on a massive selection of the best samples among natural or cultivated populations; genetic breeding with wild ancestral species; and spontaneous or induced mutations. More recent is the biotechnological technique introduction (Fernández and Escribano, 2000). Each of the techniques needs the knowledge of the wild ancestors and reproductive biology of saffron. But this information has been lacking in with respect to saffron, mainly because the plant is an unfertile triploid (Mathew, 1977; Brighton, 1977; Grilli Caiola et al., 2001).

SAFFRON MORPHOLOGY AND BIOLOGY

Saffron, *C. sativus*, is an autumnal flowering geophyte with corms that are covered by a tunic, dormant during summer, sprouting in autumn, and producing 1-4 flowers in a cataphyll with linear leaves. The flower has an underground ovary, a style cm 9-10 long, dividing at the top in three red trumpet-like stigmas (2.5 cm long) that once dried form the commercial spice saffron. Flowering spans from late autumn until December according to climatic conditions. Cytological studies have indicate that saffron is a triploid species which genome shows 2n=24, x=8 chromosomes (Karasawa, 1933; Brighton, 1977; Mathew, 1982). Its triploid stage allows for vegetative multiplication, but not for regular sexual reproduction. This is because in triploids meiosis and gamete development are irregular resulting into many anomalies in sporogenesis and gametophyte development. In the literature only Piccioli (1932) referred of occurrence saffron capsules in field and Di Crecchio (1960) describes trilocular capsules containing numerous small globule like seeds. Seedless partenocarpic fruits have been obtained in vitro after pollination with pollen of *C. thomasii* (Chichiriccò 1989a and 1990) and capsules with seeds in field after pollination with *C. cartwrightianus* (Grilli Caiola, 1999).

There have been many authors hypothesizing the origin of saffron autotriploidy (Mather, 1932; Karasawa, 1933, 1940; Pathak, 1940; Brighton, 1977; Mathew, 1977; Ghaffari, 1986; Tammaro, 1990). It seems that saffron originating by a cross between tetraploids and diploids to be less probable because tetraploids are not occurring among allies of *C. sativus* (Brighton, 1977; Mathew, 1977). The more probable origin is a cross between diploids derived from the fertilization of an unreduced egg cell by haploid sperm or a haploid egg by two haploid sperms. The selfing can be excluded because of the presence of the unique chromosome in saffron genotype (Chichiriccò, 1984). Since *C. sativus* is sterile, the presence of this chromosome might be explained by the existence of chromosomal polymorphism at diploid level in the progenitors, most probably *C. thomasii* or *C. cartwrighianus* (Brighton, 1977; Mathew, 1999). Postulating the mechanism involved in the autotriploidy of *C. sativus* (Mather, 1932; Karasawa, 1933) is puzzling because this species does not spontaneously occur as suggested by its progenitors. Moreover this fact cannot be attributed to the drawback of its sterility because several autotriploid can vegetatively reproduce spontaneously and often occur in nature considered as weed of crop fields (Khoshoo and Sharma 1959; Carputo et al.,

1995).

VEGETATIVE REPRODUCTION AND CONSEQUENCES

Saffron usually multiplies year by year by means of corms. Because corm multiplication does not induce genome variations with the exception of some mutation that in a triploid saffron population are not easily detectable, all saffron should be similar one to the other. However new variants of saffron with an increased number of stigmas, maintaining 2n=24 have been reported by Estilai (1978) with a frequency of $1.2x10^{-6}$ of the rare type flowers. Morphological differences with flowers having higher number of style branches and stamens have been already described by Piccioli (1932) in saffron cultivation at L'Aquila (Italy). In addition different commercial products are known suggesting the existence of different saffron ecotypes (Tammaro, 1987) or commercial varieties (Di Crecchio, 1960).

Brighton (1977) who studied cytology of saffron reported that *C. sativus* karyotype from different countries (Iran, Turkey, France, England) was always 2n=3x=24. However in the literature other karyotypes appeared (Mather, 1932; Karasawa, 1933, 1940; Pogliani and Del Grosso, 1971)

In order to verify the possible phenotype and genotype variations in saffron, corms from different countries of cultivation (Italy, Israel, Spain, Holland) have been examined. Analysis of phenotype revealed differences in aspect flower size, tepal shape and colour intensity (Grilli Caiola et al., 2001) with lobed tepal in plants from Israel and more intense colour of tepals in plants from Sardinia (Italy). On these accessions cytofluorimetric analysis on nuclear DNA was carried out to detect genome size and base pairs composition. Results of this study revealed no differences in DNA content and composition in saffron corms from the different countries (Brandizzi and Grilli Caiola, 1998). In addition the triploid content and quality in DNA of *C. sativus* compared to that of other diploid allied and not allied *Crocus* species was confirmed. Nuclear DNA has been also analysed by the RAPD (Random Amplified Polymorphic DNA) technique (Zanier, 2000) on corms of the above listed accessions grown in the same site utilizing DNA extract from leaves and 21 (10-mer) primers. The DNA's of saffron corms from different cultivation areas produced using the RAPD technique did not identify genomic redundant differences. There were no differences in DNA found in the ten corms from saffron in L'Aquila. These RAPD data are in accordance with the DNA content and base pair composition among different *C. sativus* cultivations as revealed by the previous cytofluorimetric study.

SEXUAL REPRODUCTION

In flowering plants the sexual reproduction requires that a series of steps have to be accomplished in order to form seed and fruit. The basic steps are: micro- and macro-sporogenesis in anther and ovule respectively; pollen and embryo sac development and gamete differentiation; pollination; fertilisation; embryo development and seed set and fruit maturation. In a diploid plant the mating between female and male gamete that bring base chromosome (n) gives rise to a zygote with 2n chromosome, similar to the parents, but not identical because the gene pool during meiosis can undergo changes resulting in a gamete with different gene composition.

The morphology and biology of saffron pollen and pistil including micro- and mega-sporogenesis, have been studied in vitro and in vivo. During meiosis (Chichiriccò, 1984) many abnormalities can occur which are also commonly found in other triploid species. Microspores often display cytoplasmic degeneration or cellular deformation, consequently they do not complete meiosis or produce anomalous microspores. Afterwards many spores are aborted or pollen grains were different size and also often malformed (Chichiriccò, 1989b). Mature pollen morphology was examined with LM (Light Microscope), SEM (Scanning Electron Microscope) and TEM (Electron Transmission Microscope) (Grilli Caiola et al., 1985 and 2001). Saffron pollen grains are elliptical shape with variable size (65-140 μm) and lacking germinative pores or furrows.

About 40% of pollen grains can be anomalously shaped, collapsed or broken. Exine surface shows many spinulae randomly disposed and lipid droplets as well as numerous perforations of varying size and form (Grilli Caiola et al., 1985). Viability of saffron pollen is over 60% but germination in vitro was only 20% after 5 days in Pfahler germination medium (Pfahler, 1967). Only 0.4% of germinating pollen grains showed regular pollen tubes. Most pollen tubes were accompanied by numerous morphological anomalies such as forked tube, lister-shaped tube, swelling at the base and apex, spiralled pollen tube and thinning at their end (Chichiriccò and Grilli Caiola, 1982). Significant differences were observed in viability and germination of pollen collected from flowers in different developmental stages such as cathaphyllic flowers, young flowers, and open flowers (Grilli Caiola et al., 2001). A higher germination percentage resulted from pollen taken from mature flowers.

At anthesis pistil of *C. sativus* has stigmas 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 (Grilli Caiola and Chichiriccò, 1991). The ovary is tricarpellar and trilocular and along the axial region of the locules on placentas 10-15 ovules differentiated which are in six longitudinal rows, two rows for each locule for a total of 30-45 ovules per ovary (Grilli Caiola et al., 2001). The ovule has two integuments, the outer integument extending beyond the internal one and forms a narrow micropilar channel. Megasporogenesis occurs early within flower bud on corm sprouting and the embryo sac appears in the ovule of flower bud when flower is still enclosed within the tunic beneath the soil surface. During meiotic division irregular assortment of chromosomes was observed, and the resulted megaspores were numerically variable (4-6) and genetically unbalanced. About 90% of ovules develop an embryo sac, which is broad and 7-nucleate (*Polygonum* type). Frequently ovules do not reach a fertilizable stage due to unsuccessful megasporogenesis or megaspore development. About 12% of them develop a small embryo sac generally comprising cellular nuclei, which may be numerically variable, often no more than four nuclei are observed. Around 18% of ovules are lacking an embryo sac and these show proliferation of nucellar tissue that increases up to the micropyle. Degeneration at embryo sac occurs infrequently; in this case, the embryo sac contains abundant granular material and sometimes also micronuclei (Chichiriccò, 1987). Megasporogenesis in *Crocus sativus* is very similar to that reported in other *Crocus* species (Rudall et al., 1984).

No differences have been observed on pollen size, anomalous pollen grains percentage, viability and germinability in vitro among pollen from different saffron accessions. No significant differences have been obtained on pollen morphology and viability essayed using different tests and on germinability of pollen and female gametophyte of saffron from different accessions.

SELF-INCOMPATIBILITY

Saffron ovules are fertilized infrequently after self or out pollination. Within four days of pollination one sperm nucleus was observed close to the egg nucleus, the other being close to the polar nuclei. Developing embryo and endosperm are observed 12 days after pollination (Chichiriccò, 1987). Similar behaviour has also been observed in the embryo sac of other *Crocus* species (Rudall et al., 1984).

Triploids capable of seed set and fruit maturation are very rarely reported in literature (Khoshoo and Sharma, 1959). In saffron self-incompatibility reduces the possibility of embryo formation as it results from experiments on self- and out-cross pollination carried out in vitro and in planta (Zanier and Grilli Caiola, 2001). Emasculated flowers were pollinated in vitro and in planta by self and out pollen and the germinated pollen grains were counted on stigma after different periods from pollination. In the pistil, other than germinated pollen on the stigma the pollen tubes present along the style, the ovary and the penetrated ovule were counted. The outcome was that a low percentage of

saffron pollen germinated in vitro as well as on the stigma either after self- and out-pollination. Similar results have been obtained with pollen of plants from different countries (Italy, Israel, Spain and Holland). In all the observed samples pollen tube growth was accompanied by many anomalies of pollen tube behaviour from the tip stigma and in the style. On observing at LM the length of pollen tubes in the style it has been noted that pollen tubes arrested 1-2 days after pollination in the upper part of style.

In spite of a high number of anomalous gametophytes about 17-20% of pollen germinated on self stigma and about the same percentage has been found after out cross pollination. These values are very close to those detected in vitro. However of the ovules that were penetrated, no seed and fruit were formed from these hand pollinations (Chichiriccò and Grilli Caiola, 1984; Grilli Caiola et al., 2001). These data suggest the existence of barriers against pollen germination at the stigma and against pollen tube growth in the style and ovary.

In many plants pollen tube growth in the style is controlled by genes of SI (Self Incompatibility) and the mechanisms of which have been identified in some instances shown to be regulated by Ca^{2+} (Franklin-Tong et al., 1995), peroxidase (Herrero and Dickinson, 1979), or RNase (McClure et al., 1990). In saffron no evident relation has been found between the growth of pollen tube in the style and the RNase or peroxidase activity (Zanier and Grilli Caiola, 2001). Peroxidase activity indicates a relationship between pollen tube presence in the style and stylar peroxidase activity that increases in the presence of pollen tube but does not stop tube growth. Incompatible pollen tubes grow along the style and their discrimination occurs in another region of the gynoecium. For what concerns the role of Ca^{2+} researches have been made by using calcium selective microelectrodes to check Ca^{2+} concentrations in emasculated and hand pollinated pistils 1, 3, 7 days after pollination. Results indicated that there is a general decrease of calcium in all parts of pistil either pollinated or unpollinated (Brandizzi and Grilli Caiola, 1996).

Self- and out- intraspecific pollination using self-pollen as well pollen from other clones did not raise any consistent variations on pollen germination on the stigma. However *C. sativus* are mainly male sterile and therefore unable to seed set and fruit and this occurs either if it is grown as single clone or grown in a mixed clones community (Grilli Caiola and at., 2001). Ovule observations suggest that the embryo sac maintains viable for long time after pollen dispersion. So saffron sterility is mainly due to pollen sterility.

HYBRIDISATION

In order to improve experiments of hybridisation of saffron with diploid *Crocus* species investigations have been carried out to ascertain the possible ancestor species used as of pollen donor.

Saffron Parents

Diploid autumnal flowering *Crocus* species are distributed across a wide area from East Europe to Asia, Iran, Iraq and South Africa. The most probable ancestor of modern saffron is considered to be the diploid autumnal flowering *C. cartwrightianus* but also *C. thomasii* and *C. pallasii* have been considered possible candidates.

Studies on saffron ancestors origins have been carried out by analysing the nuclear DNA composition by means of flowcytofluorimetry and the RAPD technique (Martins et al. 1993). Cytofluorimetric analysis of nuclear DNA amount and basis composition of *C. sativus, C. cartwrightianus*, and *C. thomasii* compared to the spring diploid *C. biflorus* (Brandizzi and Grilli Caiola, 1996) has revealed that *C. sativus* has a genome close to that of *C. cartwrightianus* from which it could of originated by mutation or outcross processes. The RAPD technique (Zanier 2000) was used to discriminate nuclear DNA composition in the autumnal flowering *Crocus* belonging to *C. sativus* group: *C. sativus* L. (from L'Aquila), C. *cartwrightianus* Herb. (from wild and cultivated corms), *C. thomasii* Ten. (from wild and cultivated corms), *C. pallasii* Goldb., *C. oreocreticus* Burt., *C. hadriaticus* Herb., and *C. asumaniae* Mathew (supplied from nurseries specialised in

bulbs). The 25 primers with arbitrary 10-nucleotide sequences used had a GC content ranging from 60% to 70%. Only 21 primers gave easily interpretable amplification bands and a total of 217 PCR products were scored and included in the statistical analysis. The DNA of the individual investigated for each species invariably reproduced the same banding pattern for each random primer used in the PCR reaction. The UPGMA phenogram obtained from Nei and Li's distance matrix clearly indicated that *C. cartwrightianus* and *C. thomasii* are the two species that share the largest amount of RAPD fragments with *C. sativus*. In particular *C. cartwrightianus* is most closely related to saffron; *C. hadriaticus*, albeit not exceedingly distant from *C. sativus,* is clearly more related to *C. oreocreticus* and to *C. thomasii, C. asumanieae* and *C. pallasii* are the most divergent species.

Interspecific Stigmatic Pollination

On the basis of the above results a set of studies have been prompted on pollen morphology, viability and germination in vitro and in vivo, after self- and out-intraspecific and interspecific pollination of *C. sativus, C. cartwrightianus*, *C. thomasii*, *C. hadriaticus*. For breeding experiments *C. sativus* and *C. cartwrightianus* were grown close proximity to each other; wild *C. thomasii* was grown in a pot distant from the others; *C. hadriaticus* was grown in field in a separate bed. At the anthesis a set of plants of each species were treated as follows: 1) untreated flowers; 2) close flowers were emasculated before anther opening and isolated in a plastic bag; 3) emasculated flowers were hand pollinated with pollen of same species; and 4) emasculated flowers were pollinated with pollen of the other species. The main results of these experiments and observations carried out on pollen and pistil after the different treatment are reported in previous papers (Brandizzi and Grilli Caiola, 1996, 1998; Chichiriccò and Grilli Caiola, 1984, 1986, Grilli Caiola, 1994, 1995, 1999, Grilli Caiola et al. 2001; Zanier and Grilli Caiola, 2001) and summarized in tables 1 and 2. Pollen morphometry, viability, germination in vitro and in vivo on stigmas indicate that saffron is mainly male sterile species and does not produce seed and capsule after self and out pollination, but if pollinated with pollen of *C. cartwrightianus* or *C. thomasii* or *C. hadriaticus* it forms seed and capsule (Figure 1). *C. cartwrightianus* and *C. thomasii* were self-sterile, but allofertile and produced capsules with seeds after out-cross pollination, but not after pollination with pollen of saffron. *C. hadriaticus* is self-fertile and allo-fertile and produced many fruits and seeds.

Hybridisation in field between *C. sativus* and pollen of *C. thomasii* or *C. cartwrightianus* has indicated that 80% of the hybrid saffron gave rise to capsules with seeds when: 1) saffron was grown in rows sided by *C. cartwrightianus*; 2) when during flowering the temperature was over 22°C; 3) cultivation was massive, 4) *Bombus* were flying on the cultivations.

Intraovarian Pollination

A set of saffron gynoecia was collected at the anthesis, hand intraovarian pollinated and grown in vitro. A higher percentage of pollen tubes was detected into ovaries after intraovarian pollination: 22% after self-, 38% after outcross. No seed or capsule formation was observed. As a control the intraovarian pollinations have carried out with pollen of the other *Crocus* species. After pollination with *C. cartwrightianus* saffron ovules underwent swelling until embryo and endosperm development (Brandizzi and Grilli Caiola, 1996). However, full seed maturation was not reached in vitro.

APOMIXIS

Flowering plants other than zygotic embryos can realize apomictic embryos formed in the ovule via sporophytic or gameophytic pathways (Koltunow, 1995). Apomixis is an emerging research field aimed to identify the genes responsible for this process that could reveal a powerful tool for producing seed from hybrid or sterile plants. (Czapik, 1997; van Dijk and Damme, 2000). Agamospermy is less widespread than

vegetative reproduction, and it is especially common in polyploids species derived from hybridisation between reproductive incompatible progenitors. Studies on apomictic *Crocus* carried out by Rudall et al. (1984) lead to the conclusion that, although facultative diplospory cannot be entirely ruled out, apomixis is an unlikely explanation from the unusual amount of chromosomal variation found in some species. Embryological studies were carried out by Brighton (1977) and Chichiriccò (1989a) on *C. sativus, C. thomasii* and *C. cartwrightianus.* Preliminary analysis in vitro of ovules from pollinated flowers, revealed the presence of embryo and endosperm. However, the absence of seed in the different types of pollination induces us to consider the analysed *Crocus* species as lacking apomictic seed. Somatic embryoids have been obtained by callus formed from cell cultures of partenocarpic fruit of *C. sativus* (Chichiriccò and Grilli Caiola, 1987). Fruit were turgid and sometimes contained some ovules greatly increased and seed-like due to growth mainly in volume of the integment cell or the nucellar tissue. However no capsules and seeds were obtained from emasculated, self pollinated pistils or from emasculated unpollinated pistils thus indicating the absence of apomictic processes (Grilli Caiola et al., 2001).

POLLINATION

Saffron pollen grains have dimensions too large to be airborne, but its conspicuous flowers and nectar are useful tools for attracting insects. Among these *Apis mellifica* is considered a collector of pollen of saffron (Negbi 1999). However, other insects have been reported active in transport pollen in *Crocus* species, such as bumble-bees (Mathew, 1982), syrphids (Ferrazzi, 1991), owerwintering bees (McKee and Richardson, 1998), or wild moths in *C. cartwrightianus* (Mathew, 1982). However is difficult to establish the role of the cited insects in pollination of saffron because when flowers open saffron stigmas bend downward making it difficult to come into contact with the insects. Also in saffron the nectar origins are in the ovary canals, and it is improbable that it get up along the flower tube, which is over 10 cm long making it impossible for bees, bumble-bees or wild moths to collect it. There are no specific studies appearing in literature on insect saffron pollination being the plant considered unable of seed and fruit maturation. Our direct observations of saffron and allied *Crocus* species have evidenced that during flowering many *Bombus silvestris* fly from flower-to-flower collecting pollen. Usually *Bombus* appears at noon of sunny days when the temperature rises facilitating the opening of the anther and the optimum amount of pollen is available and intense perfume. Similar behaviour has been reported for other pollinators of *Crocus* (Ferrazzi, 1991; McKee and Richardson, 1998). In these environmental conditions both intraspecific and interspecific pollination could occur but only scarce seeds production was detected by free pollination, compared to the high production of seed from saffron after a massive hand pollination with pollen of *C. cartwrightianus or C. thomasii*. *Crocus*es are cited as "flowers for bees" because only bees collect pollen from *Crocus* flowers. However we have never observed bees in our saffron cultivation. This could depend on the particularly restricted area of cultivation or temperature, or there was a distinct absence of bee in the research area.

SAFFRON CHROMOPLASTS

Saffron is the most expensive spice known and is also a valuable herbal medicine. The three major carotenoid derivatives, crocetin glycosides, picrocrocin and safranal, are in large part responsible for the unique colour, bitter taste, and intense aroma of saffron. The possible pathway of biosynthesis of carotenoid metabolites in *Crocus* style branches derives from the postulate zeaxanthin. In fact it is synthesized in chromoplast where then it is cleaved and modified by the CsZCCD (zeaxanthin cleavage dioxygenase) and CsCCD (carotenoid cleavage dioxygenase) enzymes (Bouvier et al., 2003). The red colour of the stigmas is due to the presence of chromoplasts, the plastids typical of non-green pigmented parts of plants. In a recent research chromoplast ultrastructure of saffron stigmas was compared to that of stigmas of the supposed ancestors, *C. cartwrightianus* and *C. thomasii*. Chromoplast of *C. sativus* (Figure 2A) has tubular structure, numerous

plastoglobules and vesicles, and originates by modifications of proplastids or amyloplasts in a very early stage of stigma development (Grilli Caiola and Caprilli 1983). *C. thomasii* chromoplast (Figure 2C) basically recalls the structure of saffron chromoplast, with tubules, plastoglobules and vesicles. *C. cartwrightianus* chromoplast (Figure 2B), on the contrary, shows carotin crystals similar to those occurring in carrot roots (Trabucchi, 1964), in tomato fruit (Grilli, 1965; Rosso, 1967), and in red tomato mutant (Harris and Spurr, 1969) with the arrest of carotenoid biosynthesis at the step of carotene and lycopene, that are the precursors of saffron picrocrocin and crocetin (Camara et al, 1995). This would suggest that *C. cartwrightianus* stigmas are more rich in lycopene than those of saffron. At present we have not information about different pigment and aroma content in the diploid species of *Crocus*, which would be interesting for breeding of saffron.

CONCLUSION AND PERSPECTIVES

The above reported data suggest some considerations:

- cytological, ultrastructural and molecular studies confirm that saffron is a sterile triploid which infertility is mainly related to the male gametophyte;
- pistil can be fertilized by pollen of diploid *Crocus* species such as *C. cartwrightianus* or *C. thomasii* and can produce fruit and seeds;
- both diploid *C. cartwrightianus* and *C. thomasii* could be ancestor of saffron, but nuclear DNA amount and composition indicate a more close relation of saffron to *C. cartwrightianus*;
- saffron self-incompatibility is not related to Ca^{2+}, peroxidase or RNase activity;
- apomictic seed seem to be not present in saffron.
- chromoplast ultrastructure of saffron is similar to that of *C. thomasii*, whereas *C. cartwrightianus* chromoplasts contain carotin crystals;
- amelioration of saffron by means of hybrids at present appears a way to obtain plants with new genetic combinations;
- but vegetative multiplication, in vitro cultures, and biotechnological tools are more promising techniques for saffron ameliorating production.

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Tables

Table 1. Pollen dimension, anomalous pollen grain percentage, viability and germination in vitro on self and outcrossed stigma *C. sativus, C. cartwrightianus* and *C. thomasii*.

Crocus species	Pollen dimension	Anomalous grains	Viability (%)		Germination in vitro	Germination on stigma	
						self	autocross
	µm	%	FDA$^+$	Alexander$^+$	%	%	%
C. sativus	88,4 ± 13,2	34	66,0	69,0	19	21	22
C. cartwrightianus	60,3 ± 1,9	14,3	85,7	98,2	63	60	49
C. thomasii	65,9 ± 2,11	11,4	88,5	94,0	74	58	56

Viability has been tested by FDA (Heslop-Harrison and Heslop-Harrison, 1970) and Alexander (1969) methods.

Table 2. Number (n) of ovules, seeds per capsule, and dimensions of capsule and seed of *C. sativus*, *C. cartwrightianus* and *C. thomasii*.

	Ovules per ovary	Seed per capsule	Capsule dimension (x)	Seed dimension (x)
Crocus species	n	n	cm	mm
C. sativus X	30-45	21-20	1,97 ± 0,4	4,47 ± 0,4
C. cartwrightianus			x 1,40 ± 0,2	x 3,2 ± 0,3
C. cartwrightianus	20-30	15-30	1,6 ± 0,3	3,4 ± 0,5
(outcross)			x 1,02 ± 0,2	x 2,3 ± 0,5
C. thomasii	24-36	9-22	1,16 ± 0,2	3 ± 0,3
(outcross)			0,87 ± 0,4	x 1,88 ± 0,02

(x) length per width

Figures

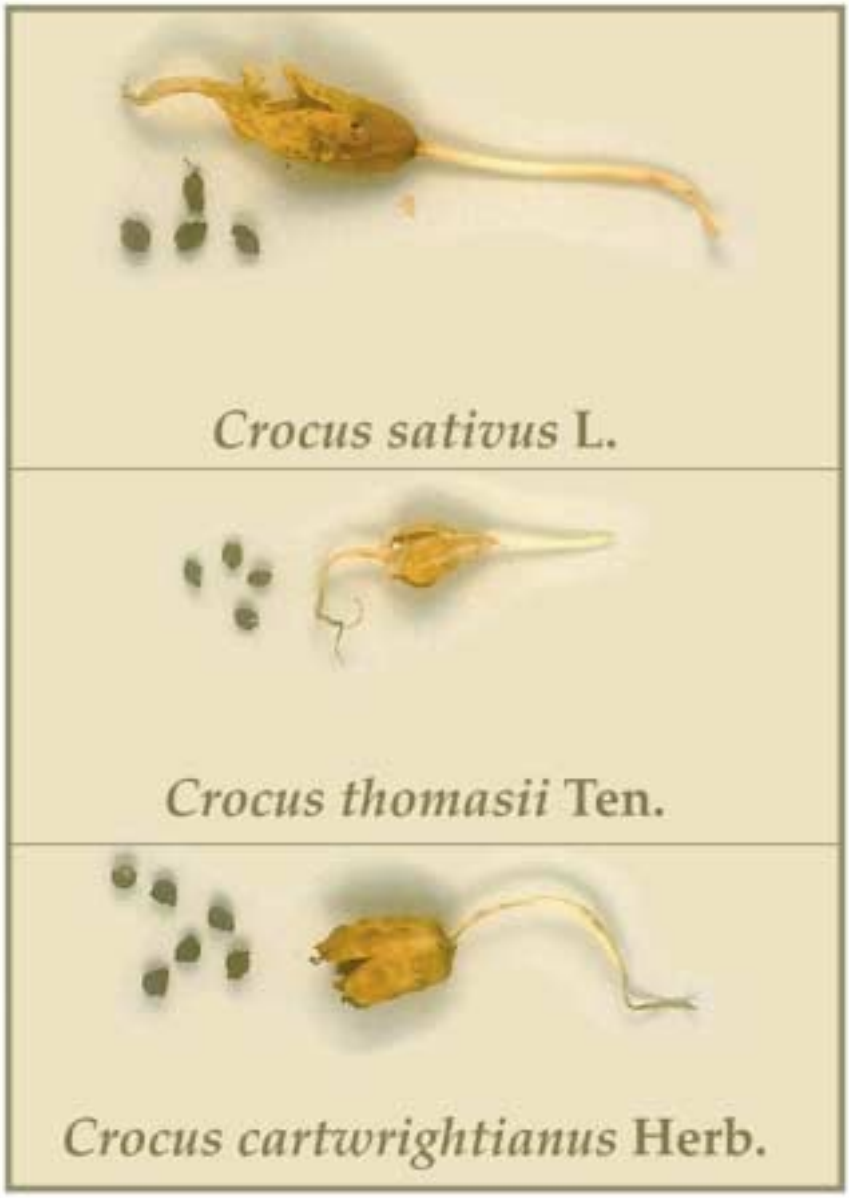

Fig. 1. Capsules with seeds of *C. sativus* hand pollinated with *C. thomasii*; *C. thomasii* and *C. cartwrightianus* after hand out pollinated.

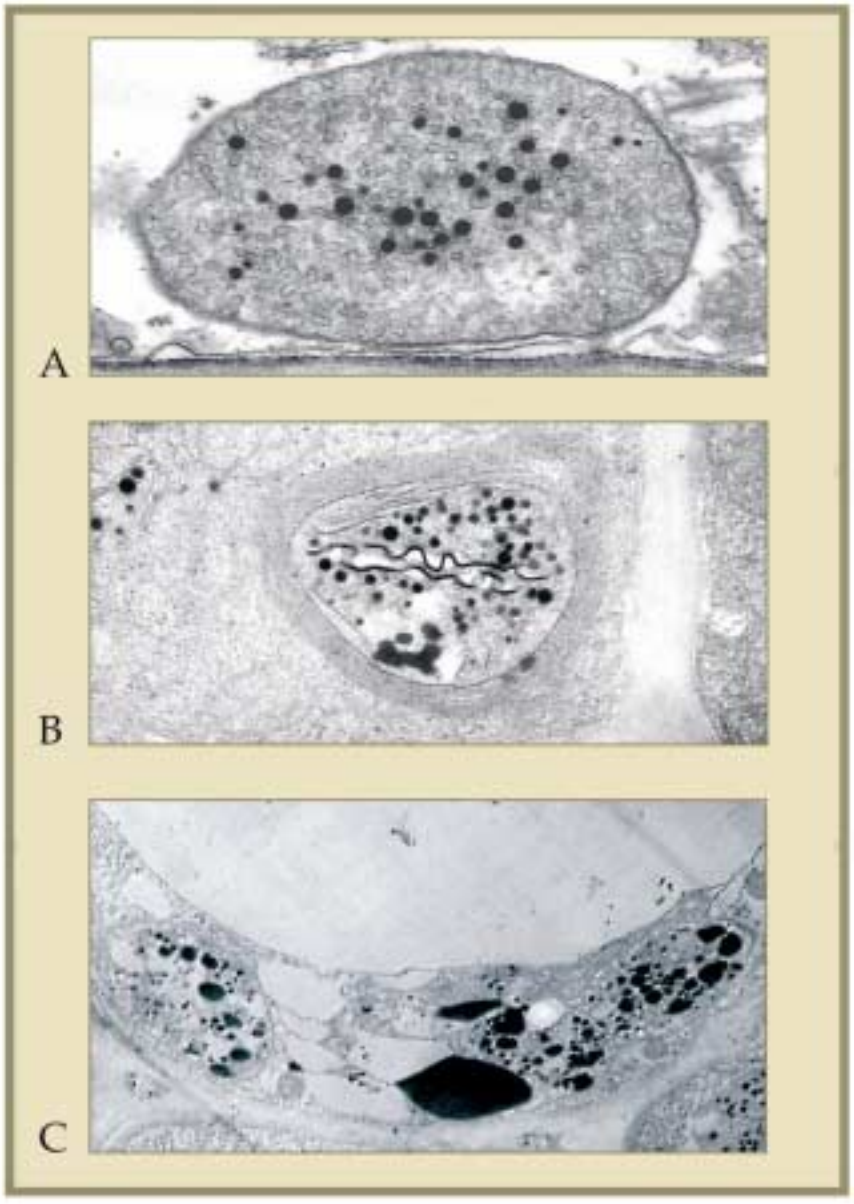

Fig. 2. Ultrastructure of chromoplasts of *Crocus sativus* (A), *C. cartwrightianus* (B), and *C. thomasii* (C).