

COMPARATIVE STUDY OF POLLEN AND PISTIL IN *CROCUS SATIVUS* L. (*IRIDACEAE*) AND ALLIED SPECIES

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ABSTRACT - *Crocus sativus* L. is mainly known for the production of the drug saffron. Because of its sterility, it is propagated vegetatively by means of corms. To gain information on the reproductive biology of saffron and allied species, a comparative study on pollen and pistil of *Crocus sativus* L., *C. cartwrightianus* Herb., *C. thomasi* Ten. and *C. hadriaticus* Herb. was carried out. Pollen and pistils gathered at anthesis were examined by light (LM) and scanning electron microscopy (SEM). Pollen shape and size, anomalous pollen grain percentage, pollen viability, pollen germination *in vitro* and on self-, and cross-pollinated stigmas were examined. Pistils at different developmental stages were examined by light microscopy. *C. hadriaticus* had the smallest pollen; *C. sativus* showed a higher percentage of anomalous and aborted grains and a lower percentage of viable grains. Pollen germination *in vitro* as well as on differently pollinated stigmas was lowest in *C. sativus*. Pistil organization was similar in all the species, but ovule number and integuments varied. Embryo sacs mature early, and female gametophyte development is regular for some days after flower anthesis. Capsules with seeds were obtained from all diploid species as well as in saffron after free- and cross-pollination. Results confirm that sterility in *C. sativus* is mainly confined to pollen.

KEY WORDS - Pollen, *Crocus*, *C. sativus*, *C. cartwrightianus*, *C. thomasi*, *C. hadriaticus*

INTRODUCTION

C. sativus L. "aggregate" is a group of autumn-flowering crocuses, widespread in the Mediterranean area, (Mathew, 1982). Most of them occur wild, some others are cultivated for ornamental or officinal purposes, but the most economically important is *Crocus sativus* (saffron crocus), grown for the production of saffron.

In the last century saffron production decreased, mainly because of the reduced flower number on each plant due to a scarcity of selected corms for cultivation and high production costs (Tammara, 1990). Some attempts have been made to obtain corms and flowers *in vitro* (Igarashy *et al.*, 1993) or to hybridise *C. sativus* with *C. thomasi* (Chichiriccò and Grilli Caiola, 1986). However, because of the high sterility of saffron, the genetic amelioration of this species is still an unsolved problem.

Little is known about possible ancestors and the ways in which saffron originated (Estilai, 1978). Comparative morphological, cytological and phenological studies (Ghaffari, 1986; Brighton, 1977; Mathew, 1977, 1982; Feinbrun, 1958; Karasawa, 1933; Pathak, 1940) led to the hypothesis that the most probable ancestors of *C. sativus* are *C. cartwrightianus* (Mathew, 1982) or *C. thomasi* (Chichiriccò, 1989) from which saffron may be derived by polyploidy. However, recent quantitative and qualitative DNA analysis (Brandizzi and Grilli Caiola, 1996; 1998) indicated that DNA composition of *C. sativus* is more similar to that of *C. cartwrightianus*, from which it could be originated by polyploidy or mutation and polyploidy. In order to gain more data on the reproductive biology of *C. sativus* and its allies, a comparative study was carried out on pollen and pistil of different *C. sativus* clones, *C. cartwrightianus*, *C. thomasi* and *C. hadriaticus*, all cultivated in similar conditions.

MATERIAL AND METHODS

MATERIAL - Plants used for this research are listed in Table 1. Some *C. cartwrightianus* plants produced albin flowers, others lilac or pink flowers. Preliminary data on DNA content (Brandizzi and Grilli Caiola, 1996) indicated that both plants were diploid with the same DNA content. However, here we studied separately pollen from white (Ccw) flowers and lilac (Ccl) flowers. All the corms were cultivated at the Department of Biology of the University of Rome "Tor Vergata" (Italy) from 1994 to 1997. Pollen was gathered at anthesis and used for light microscopy (LM), and scanning electron microscopy (SEM) and for hand pollinations which were made as previously reported in Chichiriccò and Grilli Caiola (1984). Pistils were collected from flowers at different developmental stages. Some plants were used for self- and cross-pollinations. Other plants were freely pollinated and left on the ground until capsule maturation.

TABLE 1 - *CROCUS* SPECIES AND CLONES USED

taxa	chromosome number	provenance	source	label
<i>Crocus sativus</i> L.	2n=24 - sterile	Italy: L'Aquila	cultivated	Cs It
<i>Crocus sativus</i> L.	2n=24 - sterile	Spain: Albachete	cultivated	Cs Sp
<i>Crocus sativus</i> L.	2n=24- sterile	Israel	cultivated	Cs Is
<i>C. cartwrightianus</i> Herb.	2n=16- fertile	Greece	wild	Cc
<i>C. cartwrightianus</i> Herb.	2n=16- fertile	United Kingdom	cultivated	Cc w* Cc l**
<i>C. thomasi</i> Ten.	2n=16- fertile	Italy: Matera and Castel Del Monte	wild	Cth
<i>C. hadriaticus</i> Herb.	2n= 16- fertile	United Kingdom	cultivated	Cha

* with white flowers, ** with lilac flowers

METHODS *Light microscopy.* Shape and size observations were made on about 400 fresh pollen grains which were put in cedar oil or in germination medium. Measurements of the grains were made by a Leitz Laborlux light microscope (LM) equipped with ocular micrometer. Pollen nuclei were observed on fresh pollen using an interference Zeiss microscope, and by means of fluorochrome 4,6-diamidino-2-phenylindole (DAPI) in Mac Ilvain buffer according to the Vergne *et al.* (1987) method and observed by fluorescence microscopy. Pistil preparation was made by fixing in Navashine, dehydrating in tertiary butanol, embedding in parafin and stained by Eματοxylin as reported in Grilli Caiola and Chichiriccò (1991).

Pollen viability was checked by the following tests. The enzymatic activity of alcohol dehydrogenase (ADH) was tested by means of nitroblue tetrazolium (NBT) according to the Hauser and Morris (1964) procedure. The fluorescence in fluorescein diacetate (FDA) was used for the fluorochromatic reaction (FCR) test according to the Heslop-Harrison and Heslop-Harrison (1970) technique. Alexander staining was applied to distinguish the aborted and non aborted pollen grains, according to Alexander (1969).

Pollen germination *in vitro* was detected in a modified Pfahler (1967) medium containing 10% sucrose and 0.1% boric acid with the procedure described in previous papers (Chichiriccò and Grilli Caiola, 1986; Grilli Caiola *et al.*, 1993; Grilli Caiola and Brandizzi, 1997). Pollen was examined two hours after inoculation. *In vivo* germination was detected on hand pollinated stigmas by aniline blue staining according to Kho and Baër (1968). Two days after pollination the stigmas were fixed in FAA (formaldehyde, acetic acid and ethanol, 1: 1: 18) then stained with aniline blue and observed by light microscope. Percentage of germinated pollen grains was evaluated on the total number of pollen grains present on the stigma at the moment of collecting.

For scanning electron microscopy (SEM) pollen samples were placed in 3% glutaraldehyde in sodium cacodylate buffer and postfixed in osmium tetroxide. After dehydration in a graded series of acetone the samples were critical-point dried, coated with gold in a Balzers Coating System and observed with a Zeiss DSM 950. For SEM size measurements pollen grains were randomly selected from each species and clones. On the central region of the pollen surface at 10,000 magnification the spinulae were counted and the average value was reported for 100 μm^2 .

RESULTS

POLLEN MORPHOLOGY- In all taxa examined, pollen grain shape was spherical, only a few grains appearing ovoidal. Diameter measurements were made on spherical grains.

Dimensions of pollen grains of different taxa are reported in Table 2. *C. sativus* pollen has the highest dimensions, and its values are accompanied by highest deviation standard indicating that grain size is not homogenous (Fig. 1). *C. sativus* from Spain has dimensions higher than those from Italy and Israel. *C. hadriaticus* has the smallest pollen grains and the most homogenous size (Fig. 2). No significant differences occur between pollen of *C. cartwrightianus* with white [Ccw] or lilac flowers [Ccl].

Pollen of all *Crocus* examined with the SEM appears inaperturate but is pantoaperturate with exine surface covered by spinulae whose density (number /100 μm^2 pollen area) differs in each species: 9-10 in *C. sativus*, 10 and 6 respectively in *C. cartwrightianus* [Ccw] and *C. cartwrightianus* [Ccl], 14 in *C. thomasi*, and 36 in *C.*

TABLE 2 - POLLEN SIZE (μm) AT SEM, ANOMALOUS POLLEN GRAIN PERCENTAGE AND POLLEN VIABILITY TESTED BY ALCOHOL DEHYDROGENASE (ADH), FLUORESCIN DIACETATE (FDA) AND ALEXANDER⁺ OF *CROCUS* SPECIES AND CLONES

CROCUS SPECIES	SEM (μm)	ANOMALOUS grains(%)	ADH ⁺ (%)	FDA ⁺ (%)	ALEXANDER ⁺ (%)
<i>C. SATIVUS</i> [CsIt]	88.4 \pm 13.2	34	56.8	66.0	69
<i>C. SATIVUS</i> [CsSp]	89.8 \pm 15	45	49.2	55.5	67
<i>C. SATIVUS</i> [CsIs]	87.2 \pm 16	47	47.8	71.1	60
<i>C. CARTWRIGHTIANUS</i> [CCW]	60.3 \pm 1.9	14.3	66.8	85.7	98.2
<i>C. CARTWRIGHTIANUS</i> [CCL]	62.4 \pm 2.3	12.7	52	87.3	85
<i>C. THOMASII</i> [CTH]	65.9 \pm 2.11	11.4	52	88.5	94
<i>C. HADRIATICUS</i> [CHA]	58.2 \pm 1.3	1	45	N.D	99



Figure 1 - Pollen grain of *Crocus sativus*. L.M. 500X (original)

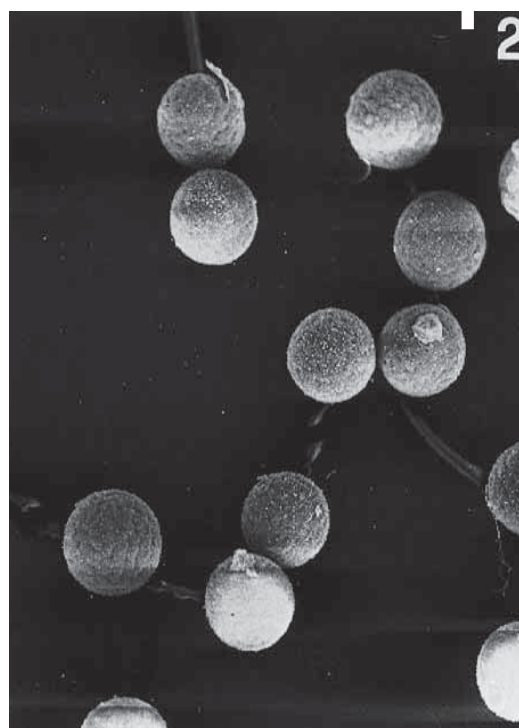


Figure 2 - SEM micrograph of *Crocus hadriaticus* pollen. 200X (original)

hadriaticus. In addition, pollen of *C. sativus* shows very large bands of broken exine. Similar aspects are less pronounced in the other species.

Anomalous grains, generally smaller, empty, collapsed and with the wall which is broken in many sites were present in the highest percentage (47%) in *C. sativus* from Israel, in lowest percentage (1%) in *C. hadriaticus* (Table 2).

POLLEN VIABILITY - In all samples, pollen treated by DAPI staining appears bicellular with a generative cell nucleus which usually is less fluorescent and amoeboid shaped. A thin elongated generative cell is also evident on fresh pollen with an interference microscope.

The ADH test used as index of pollen viability (Table 2) revealed lower values than with the other tests. In addition, high differences appeared inside the *C. cartwrightianus* with white [Ccw] or lilac flowers [Ccl], these having a high percentage of ADH⁺. Pollen viability detected by FDA test (Table 2) has given higher values than by ADH, mainly in *C. cartwrightianus* [Ccl] which appears similar to *C. thomasii*. Also homogeneous values have been obtained on the Alexander⁺ pollen grains. With this test *C. sativus* has lowest percentage of non aborted grains (Table 2), whereas *C. hadriaticus* has the highest one (1%).

IN VITRO POLLEN GERMINATION (Table 3) - Pollen germination percentage is low in all samples, but *C. sativus* has the lowest value germination percentage (31%), the highest being in *C. hadriaticus*. *In vitro* germination of *C. sativus* pollen is accompanied by numerous anomalies, such as pollen tubes which are spiral with a thin, sometimes bifurcated or elongated tip.

IN VIVO POLLEN GERMINATION (Table 3) - Pollen germination percentage *in vivo* in *C. sativus* [Cs It] after self- and cross-pollinations (21 and 22 % respectively) paralleled that *in vitro* (20 %). In *C. thomasii*, the percentage of germinating grains in self- (58 %) and in cross-pollinations (56 %) were rather similar but higher than *in vitro*. A similar result has been observed in *C. cartwrightianus* in which the pollen germination on self-pollinated stigmas is higher than *in vitro* and significantly lower in lilac flowers compared to white ones. The results of the pollen germination on stigmas after interspecific pollinations (Table 4) revealed that *C. sativus* pollen germinated in low percentage on stigmas of all the other species. *C. cartwrightianus* [Ccl] pollen germinated in a lower percentage on stigmas of *C. sativus*, but with high similar values on *C. thomasii*. *C. thomasii* showed highest values of germination on stigmas of both *C. sativus* and of *C. cartwrightianus*.

We also compared the pollen germination in *C. sativus* [Cs It] clones in different years. No significant differences were recorded among the pollen from corms cultivated from 1992 to 1994.

GYNOECIAL ORGANIZATION - At anthesis, pistil of the *Crocus* species examined have 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. Here the stylar cavity opens into three locules (Fig. 3). The ovary is trilocular and trilobular and along the axial region of the locules placentas differentiate the ovules (Fig. 4) which are obliquely attached to the ovarian axis in six longitudinal rows, two for each locule. Ovules are anatropous and bitegmic with a large hypostase (Fig. 4). The external integument extends beyond the internal one and forms a narrow micropylar canal. Megasporogenesis occurs early upon 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. About 90% of ovules develop an embryo sac which is 7-nucleate

TABLE 3 - PERCENTAGE OF GERMINATING POLLEN GRAINS OF *C. SATIVUS*, *C. CARTWRIGHTIANUS*, *C. THOMASII* AND *C. HADRIATICUS* IN VITRO AND ON SELF- AND CROSS-POLLINATED STIGMAS, CAPSULED ON SEEDS.

	<i>in vitro</i>	<i>on stigmas</i>		capsules	seeds
		self-	cross-		
<i>Crocus sativus</i> [Cs It]	20	21	22	+*	+*
<i>Crocus sativus</i> [Cs Sp]	19	17	21	-	-
<i>Crocus sativus</i> [Cs Is]	18	17	15	-	-
<i>C. cartwrightianus</i> [Cc w]	23	60	49	+	+
<i>C. cartwrightianus</i> [Ccl]	31	52	46	+	+
<i>C. thomasi</i> [Cth]	19.6	58	56	+	+
<i>C. hadriaticus</i> [Cha]	50	70.	70	+	+

* after pollination with *C. cartwrightianus*

TABLE 4 - PERCENTAGE (%) OF GERMINATED POLLEN GRAINS ON CROSS-POLLINATED STIGMAS OF DIFFERENT *CROCUS* SP

POLLEN	STIGMA			
	<i>C. sativus</i>	<i>C. cartwrightianus</i>		<i>C. thomasi</i>
	[Cs It]	[Ccw]	[Ccl]	[Cth]
<i>Crocus sativus</i> [Cs It]	21.5	19	20	22
<i>C. cartwrightianus</i> [Ccw]	40	56	50	50
<i>C. cartwrightianus</i> [Ccl]	28	46	49	50
<i>C. thomasi</i> [Cth]	59.2	60	72	57

when mature and contains a substance which stains red with Poinceau especially during the initial developmental stages. Pistils of *C. cartwrightianus* and *C. thomasi* have similar organization, but the dimensions of ovary, ovule and embryo sac are different at anthesis. The ovary of *C. cartwrightianus* contains 9-10 ovules for each locule and the inner ovule integument is formed by 4-5 layers. *C. thomasi* has 12-14 ovules for each locule and each ovule has an integument 4-5 cell layers thick at the micropyle. In all species the megagametophyte is just evident in young flower and preserves its integrity for some days after the flower wilts.

CAPSULE AND SEED FORMATION - All the *Crocus* species produced capsules and seeds from emasculated and hand cross-pollinated pistils as well as from emasculated or non emasculated and freely pollinated pistils. No capsules and seeds were obtained from emasculated and self-pollinated pistils or from emasculated and unpollinated ones, thus indicating the absence of apomictic processes. Experiments carried out in the field demonstrated that although self- cross- and unpollinated pistils of saffron did not usually produce fruits and seeds, in 1995 a capsule with seeds was obtained from a freely pollinated saffron plant grown near *C. cartwrightianus*. In nature this was possible because *Crocus sativus* and *C. cartwrightianus* were simultaneously flowering and the weather was hot (about 25°C) and sunny. These ecological conditions favoured the presence of visiting pollinators (*Bombus silvestris*) which were attracted by the *Crocus* scented flowers. Capsule and seeds obtained were larger than those of *C. cartwrightianus*, *C. thomasii* and *C. hadriaticus* but very similar in other aspects such as shape, colour and seed arrangement and capsule dehiscence.



Figure 3 - Transversal section of *C. cartwrightianus* ovary showing three locules and the axis crossed by three nectaries. LM 300x (original)

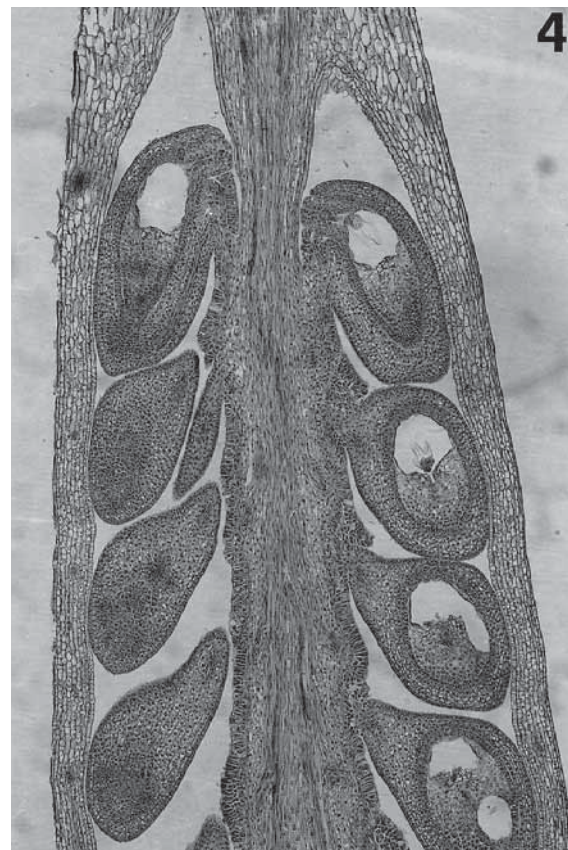


Figure 4 - Longitudinal section of *C. thomasii* ovary with the ovules containing embryo sac. LM 240x (original)

DISCUSSION

Pollen of the *Crocus* species and clones examined show differences mainly in grain size and surface structure, and in anomalous, viable and aborted grains percentage. *C. sativus* pollen has the highest dimensions but also the highest size variation and the highest percentage of anomalous and aborted grains. Variations exist among the clones because pollen of saffron from Spain has dimensions higher than the other clones. In addition, previous studies (Grilli Caiola *et al.*, 1985; Grilli Caiola, 1994; 1995) indicated that the intine of *C. sativus* pollen is thinner than that of *C. thomasii* and *C. cartwrightianus*.

Dimensions of *C. cartwrightianus* pollen are not significantly different in white and lilac flowers and are also similar to those previously reported for the same species (Grilli Caiola, 1995). *C. thomasii* pollen instead has similar dimensions to *C. cartwrightianus* but its exine is less broken and it has the highest density of spinulae (Grilli Caiola, 1994). *C. hadriaticus* pollen is the smallest, but the most regular and viable.

Data on pollen viability obtained by different techniques indicated a difference in *C. sativus* from Israel [Cs Is] which differs for its low ADH⁺ and high FDA⁺ percentage in comparison to the other clones and for its highest percentage of anomalous grains. Most of the anomalous pollen grains are aborted. Differences were also evident in ADH⁺ percentage of *C. cartwrightianus* [Ccw] from white flowers in comparison to the other viability tests, whereas the data of ADH⁺ in *C. thomasii* confirm those previously reported (Grilli Caiola, 1994).

Regarding the use of ADH test to identify viable pollen grains, it is worth mentioning that ADH is an enzyme transcribed and translated by the gametophyte. Thus its presence in the pollen depends on the developmental stage (Stinson and Mascarenhas, 1985). The use of ADH for *Crocus* appears less suitable than the FDA and Alexander test to check pollen viability mainly for *C. sativus*. In fact on comparing the data obtained with these methods, both FDA⁺ and Alexander⁺ percentages agree with the anomalous grain percentage, indicating that most of the anomalous grains are aborted, except in *C. cartwrightianus* and *C. thomasii* in which most of the grains which appear anomalous are not aborted. Data on pollen germination *in vitro* and *in vivo* confirm the low pollen germination in *C. sativus*, whereas its stigma is able to induce pollen germination in other species.

Values of germination *in vivo* are very similar in all the *C. sativus* clones and seem to be significantly influenced by the developmental stage of flower, but less by external conditions.

C. cartwrightianus pollen of white and lilac flowers does not show different *in vivo* germination. Germination in self- and cross- as well as on *C. sativus* is only slightly lower than in *C. thomasii*. No significant differences were observed between *C. cartwrightianus* white and lilac flowers in cross pollinations and fertility, suggesting that white and lilac colour flowers are different forms of the same genome. This confirms the data on their similar diploid genome and their similar DNA content when measured by cytofluorimetry (Brandizzi and Grilli Caiola, 1998) other than their fertility as verified from capsule and seed productions in the field.

C. cartwrightianus and *C. thomasii* pollen is self-incompatible but cross-compatible. All these species are also cross-compatible with *C. sativus* (Chichiriccò,

1996) indicating that both should be considered as possible ancestors of saffron. *C. hadriaticus* is the most fertile species. It produces capsules and seeds from cross-, freely- or hand-pollinated pistils. Its pollen results to be able of fertilising *C. sativus* ovules as well as self fertilization (Chichiriccò, 1996) but in the mixed cultures with *C. sativus* we never have observed the production of capsules or seeds from saffron.

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