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REVIEW

Latent virus infections in *Crocus sativus* and *Crocus cartwrightianus*

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Summary. In over two decades, while studying saffron reproductive biology, we frequently found ultrastructural alterations typical of potyvirus infection in stigmas, styles and leaves of *Crocus sativus* (saffron) and *C. cartwrightianus* (wild and ornamental species, a putative ancestor of saffron) from different provenance. This suggests that these viruses are widely diffused in cultivated *Crocus* spp., possibly causing latent infections. The few data found in literature, while highlighting the general lack of attention given by plant virologists to *Crocus* spp., nevertheless confirm that potyviruses, particularly *Bean yellow mosaic virus* (BYMV), can cause asymptomatic infections in these host species. The reasons and possible implications of widely distributed potyvirus latent infections in *Crocus* spp. are reported and discussed, with the aim of increasing general awareness of these viruses, and of encouraging sanitary selection programs focused on saffron, that could improve the quantity and quality of yields of the most expensive spice commodity grown.

Key words: potyvirus, saffron, virus infection, cytopathology, pinwheels.

Introduction

Crocus sativus L. has been cultivated since ancient times as the source of saffron. This is possibly the most expensive spice on earth, which consists of the dried stigmas of *C. sativus*, and is well-known for medicinal and flavoring properties. *Crocus sativus* is a triploid species (Mathew, 1977; Ghaffari, 1986), which is self- and out-sterile (Grilli Caiola, 2005). The plant is therefore unable to produce seeds and must necessarily be propagated via corms. Sterility depends on an irregular triploid meiosis, resulting in many anomalies in sporogenesis and gametophyte development (Chichiriccò, 1999; Grilli Caiola, 2004) which lead to the production of abnormal pollen and self-sterile pollination. Though the occurrence of seeds in the field has been reported only once (Piccioli, 1932; Grilli Caiola, 2004), the in vitro cross-pollination (fertilisation) of the ovaries of *C. sativus* with pollen of *C. cartwrightianus* (Grilli Caiola, 2005; Grilli Caiola *et al.*, 2010) and *C. thomasii* Ten. (a self-incompatible, but cross-fertile species) (Chichiriccò, 1999), has resulted in the production of capsules and viable seeds. *Crocus hadriaticus* is also able to fertilise *C. sativus* (Chichiriccò, 1996), while the pollination of other *Crocus species* with pollen of *C. sativus* did not result in the production of any seeds (Grilli Caiola, 2005).

The genetic origin of *C. sativus* is yet to be ascertained: it may have occurred by autotriploidy from a wild *Crocus*, probably by fertilisation of diploid unreduced egg cells by haploid sperm cells or from haploid egg cells, each fertilised by two haploid

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sperms (Grilli Caiola, 2004, 2005), or by allopolyploidy through the hybridisation of *C. cartwrightianus* and *C. hadriaticus* (Castillo *et al.*, 2005; Grilli Caiola and Canini, 2010). Regarding saffron ancestors, Brighton (1977) in a karyological study suggested *C. cartwrightianus* or *C. thomasii* as possible candidates, and our studies indicate *C. cartwrightianus* as the most likely ancestor (Brandizzi and Grilli Caiola, 1998; Grilli Caiola *et al.*, 2004, Grilli Caiola and Canini, 2010). Amplified Fragment Length Polymorphism analysis confirmed that the quantitative and qualitative traits of DNA from these species are compatible with *C. sativus* (Zubor *et al.*, 2004). Furthermore, flowering in *C. cartwrightianus* very likely resembles that observed in *C. sativus*. Brighton (1977) asserted that saffron shows homogenous and stable biological characters all over the world, slightly diverging only in some morphological and biochemical features (Tammaro, 1990). Indeed, Random Amplified Polymorphic DNA) investigations on *C. sativus* DNA from five diverse locations (in Europe and Israel) did not identify any genomic differences, though the respective plants showed distinct morphological properties (Grilli Caiola *et al.*, 2004).

Evidence of latent potyvirus infections in *Crocus* spp.

In the last decades we have ultrastructurally examined stigmas, styles and leaves of more than fifty plants of *C. sativus, C. cartwrightianus* and *C. thomasii* from different provenance. Though all the collected plants were apparently healthy, in about 60% of them we found cytoplasmic inclusions typical of potyvirus infection in both *C. sativus* and *C. cartwrightianus*, but never in the wild species *C. thomasii* (Grilli Caiola 1982; Grilli Caiola, unpublished) (Table 1, Figure 1). In particular, the most frequent inclusions observed by transmission electron microscopy, after conventional fixation in 3% glutaraldehyde followed by 1% osmium tetroxide and embedding in Spurr or Araldite (Grilli Caiola, 1982), were pinwheel bundles associated with laminated aggregates and tubular structures. These are the typical features of type-2 potyviruses, according to Edwardson and Christie's (1978) classification. Considering that a representative member of this potyvirus group is *Bean yellow mosaic virus* (BYMV) (Hull, 2002), and that this virus has been frequently found in *C. sativus* by different authors (Table 2), it is very likely that BYMV was one of the main viruses responsible of the observed latent virus infections also in our samples. It is noteworthy that inclusions typical of BYMV in symptomless *C. sativus* had already been reported by Russo *et al.* (1979), confirming that this, and possibly other potyviruses, may cause latent infections in this species. Nevertheless, the presence of other potyviruses, at least in our samples of *C. cartwrightianus*, is suggested by the presence of scrolls, which are typical inclusions induced by type I, III and IV potyviruses according to Edwardson and Christie's (1978) classification.

A similar multiple potyvirus infection in *C. sativus* was reported by Pisi and Bellardi (1990), who found, besides BYMV inclusions, also short and

Table 1. Presence of potyvirus inclusions in tissues of different *Crocus* species from diverse origins. *Crocus sativus* and *C. cartwrightianus* are cultivated species, while *C. thomasii* is a wild species.

Crocus species	Provenance	Tissue examined	Potyvirus inclusions
C. sativus	L'Aquila province, Italy	Stigmas and leaves	Pinwheels laminated aggregates crystalline bodies
C. cartwrightianus (white flowers)	Botanical Garden, Amsterdam, Netherlands	Stigmas	Pinwheels laminated aggregates crystalline bodies scrolls
C. cartwrightianus (violet flowers)	England, UK	Stigmas	Pinwheels laminated aggregates
C. thomasii	Castel del Monte (Bari), Italy	Gynoecia and leaves	No inclusions

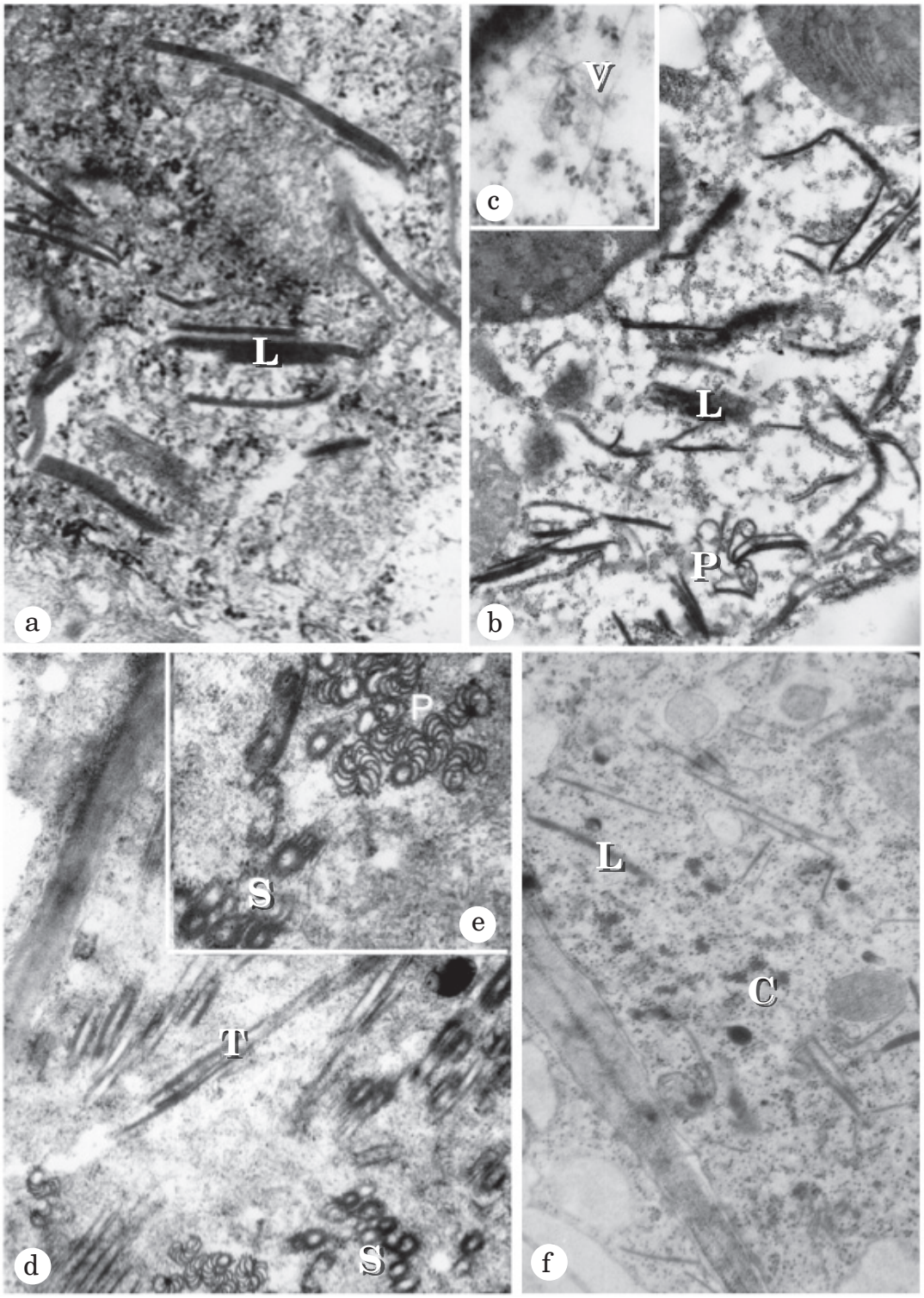

Figure 1. Potyvirus cytoplasmic inclusions in stigmas of *Crocus sativus* (a-c) and *C. cartwrightianus* (d-f) from symptomless plants. L, laminated structures; P, pinwheels; S, scrolls, C, crystalline bodies; T, tubular structures; V, virus-like particles.

Table 2. Presence of virus infections in *Crocus* spp. reported in literature. ArMV, *Arabis mosaic virus* (genus *Nepovirus*); BYMV, *Bean yellow mosaic virus* (genus *Potyvirus*); CMV, *Cucumber mosaic virus* (genus *Cucumovirus*); IMMV, *Iris mild mosaic virus* (genus *Potyvirus*); ISMV, *Iris severe mosaic virus* (genus *Potyvirus*); NMV, *Narcissus mosaic virus* (genus *Potexvirus*); TNV, *Tobacco necrosis virus* (genus *Necrovirus*); TRV, *Tobacco rattle virus* (genus *Tobravirus*); TSWV, *Tomato spotted wilt virus* (genus *Tospovirus*); TuMV, *Turnip mosaic virus* (genus *Potyvirus*); Y-C, unidentified potyvirus.

Crocus species	Origin	Virus	Symptom	Examined organs/tissues	Cytopathology	Reference
Not specified	Denmark	TRV	Yellow/brown oval necrotic spots on leaves, small flowers with color breaking	Not specified	Not examined	Van Slogtern, 1958
C. sativus	Siena province, Italy	BYMV	Symptomless	Leaves, roots	Pinwheels, laminated structures, crystalline bodies	Russo *et al.,* 1979
C. tomasinianus	Netherlands	ISMV	Stunting, leaf chlorosis, petal color breaking	Flowers, leaves	Not examined	Brunt and Phillips, 1979
C. sativus	L'Aquila province, Italy	Potyvirus (BYMV?)	Symptomless	Stigmas	Pinwheels, laminated aggregates, crystalline bodies	Grilli Caiola, 1982
Not specified	Lisse, Netherlands	BYMV	Not specified	Not specified	Not examined	Anonymous 1981,1983
Not specified	North Europe	CMV, Y-C	Mosaic, retarded growth	Leaves, flowers	Not examined	Bellardi and Pisi, 1987
Not specified	North Europe	BYMV	Not specified	Leaves	Pinwheels, laminated aggregates, crystalline bodies	Pisi and Bellardi, 1990
C. sativus	East China	TuMV	Mosaic	Not specified	Laminated aggregates, scrolls	Chen and Chen, 2000; Jishuang, 2000; Chen *et al.*, 2004
Not specified	Nagoya, Japan	BYMV	Not specified	Leaves	Pinwheels, laminated aggregates, crystalline bodies	Kaneshige *et al.*, 1991
Not specified	Lithuania	TRV	Yellow/brown oval necrotic spots on leaves, small flowers with color breaking	Leaves and flowers	Not examined	Navalinskiene and Samuitiene, 2001
Not specified	Lithuania	IMMV	Narrowed, twisted with greenish/ necrotic spots and streaks; petals color breaking and crinkled	Leaves and flowers	Not examined	Navalinskiene and Samuitiene, 2001
C. flavus	Breezeland and Lisse districts, Netherlands	ISMV, BYMV TNV, TRV NMV	Color breaking, stunting, yellowing, necrosis	Flowers, leaves	Not examined	Miglino *et al.*, 2005
C. sativus	New Zealand	TuMV	Chlorosis, mild to severe mosaic, necrosis	Leaves	Potyvirus-like particles	Ochoa Corona *et al.*, 2007
C. sativus	Porchères, France	Potyviruses	Symptomless	Stigmas	Not examined	D'Agostino *et al.*, 2007 (Bouvier *et al.*, 2003)
Not specified	Lithuania	CMV	Not specified	Flowers	Not examined	Samuitiene and Navalinskiene, 2008
C. chrisanthus *C. vernus*	Lithuania	ArMV + TRV, + TSWV + TNV (mixed)	Dwarfing, chlorotic streaks, necrotic spots	Leaves, flowers	Not examined	Samuitiene *et al.*, 2008

curved laminated aggregates characteristic of potyviruses belonging to subdivision IV of Edwardson and Christie (1983, 1984). Unfortunately, the authors did not specify if the plants were symptomless or not, though in a previous paper (Bellardi and Pisi, 1987), possibly referring to the same plants, they reported that the four plants containing potyviruses, out of the 50 examined from an imported stock of corms, were symptomless.

D'Agostino *et al.* (2007), in an Expressed Sequence Tags database from a saffron stigma cDNA library coming from a symptomless plant collected in France (Bouvier *et al.*, 2003) identified four tentative consensus sequences, Cl000209:1 (61 ESTs), Cl000582:1 (18 ESTs), Cl001827:1 (5 ESTs) and Cl000731 (2 ESTs) showing similarity to potyviral sequences. This finding, besides indicating that the sequenced library likely derived from a plant with latent potyvirus infection, confirms the wide spread occurrence of such viruses in cultivated saffron.

The presence of potyvirus latent infections in *C. cartwrightianus*, a species probably progenitor of saffron (Grilli Caiola and Canini, 2010), deserves particular attention. This could mean that these viruses have been infecting *Crocus* spp. since ancient times, and that mild virus strains have been selected during host-pathogen co-evolution. Furthermore, the transmission through reproductive organs from *C. cartwrightianus* to *C. sativus* cannot be excluded, as in the *Potyviridae* family virus transmission by pollen to pollinated plants can occur (Mink, 1993). On the other hand, potyviruses such as BYMV are widely diffused in plants in the Iridaceae (Tsuji *et al.*, 1996) and they are aphid transmitted (Hull, 2002). Infections could, therefore, also occur through aphid vectors. However, the fact that we have never found signs of potyvirus infection in the wild species *C. thomasii* weakens this hypothesis.

The exclusive vegetative propagation of *C sativus* through corms, besides being the main likely way of spreading viruses, has also possibly determined the selection of potyvirus strains with low aggressiveness, responsible for mild or latent infections. Moreover, the morphological traits of *Crocus* flowers make it difficult to visually ascertain mild symptoms of stunting or petal colour breaking, often associated to potyvirus infection, and this may also favour virus spread in *C. sativus* crops.

Other plant viruses infecting *Crocus* spp.

Besides BYMV, other potyviruses have sometimes been found in cultivated *Crocus*, particularly *Turnip mosaic virus* (TuMV), *Iris severe mosaic virus* (ISMV) and *Iris mild mosaic virus* (IMMV) (Novalinskiene and Samuitiene, 2001; Miglino *et al.*, 2005). However, in these cases the examined plants showed symptoms of mosaic and chlorosis of the leaves and petal colour breaking (Table 2). As outlined in Table 2, potyvirus infected plants have been found worldwide, though in some cases these plants were originally derived from corms imported from North Europe. The summary of reports of viruses in *Crocus* spp. (Table 2) also indicates that two soilborne viruses, *Tobacco rattle virus* (TRV), which is transmitted by nematodes (Hull, 2002), and the almost ubiquitous *Tobacco necrosis virus* (TNV), transmitted by the fungus *Olpidium brassicae (*Hull, 2002) have also been found in these plants. A third virus sometimes found in *Crocus* spp., is the aphid transmitted *Cucumber mosaic virus* (CMV) (Bellardi and Pisi, 1987; Samuitiene and Novalinskiene, 2008), possibly the most successful plant pathogenic virus with the widest host range (more than 1,000 plant species, in 365 genera from 85 families) and with worldwide distribution (Gallitelli, 2000). In any case, it should be noted that all the above viruses have been identified in plants with symptoms (Table 2), thus it is not known whether they may be responsible for latent infections as well.

In view of the possible widespread occurrence of potyviruses causing latent infections in *Crocus* spp. a question to be addressed is the influence of single or multiple viral infections on the productivity and quality of saffron. This is important, considering the positive economic impact that may result from even modest improvements in the production and the profile of important secondary metabolites from virus-free plants. Though no data are available for saffron, it was shown that latent virus infections significantly decrease crop production (Hull, 2002). It is also possible that yield losses often experienced by saffron growers may be in part attributed to latent virus infections, besides other pathogens such as bacteria and fungi listed as main cause of saffron diseases in Italy (Cappelli, 2006) and other parts of the word (Kafi *et al.*, 2006). Certainly, the difficulty in detecting virus symptoms such as colour breaking in *Cro-*

cus fields, and the fact that saffron is considered a minor crop, at least in Europe, possibly account for the few reports on presence and distribution of viruses in these species in scientific literature. Another reason for the scanty reports probably is the fact that in the last 30 years saffron cultivation area has changed dramatically, decreasing to less than 2000 ha in European countries (Greece, Spain and Italy) and increasing to over 50,000 ha in East Asia (mainly Iran and India) (Fernandez, 2004; Gresta *et al.*, 2008). This is due to the lower manual labour costs in these countries. In these new areas of production phytosanitary controls are possibly not carried out.

The European and Mediterranean Plant Protection Organization (EPPO) has published "Guidelines for inspection of *Crocus* for symptoms of viruses and nematodes", which state that BYMV symptoms can be best detected after flowering and tend to disappear at the end of the growing season (EPPO Bulletin, 2002). This possible symptom disappearance, and that growers, after flower harvest, usually reduce field inspections, would further explain the paucity of reports about virus infections in the crops. Indeed, until now published papers on saffron almost exclusively outline cultural practices to improve production (reviewed in Gresta *et al.*, 2008), and enhance the biosynthetic pathways involved in the synthesis of the secondary metabolites of major interest (crocetin, picrocrocin and safranal; Bouvier *et al.*, 2003; Carmona *et al.*, 2007; Rubio Moraga *et al.*, 2009) and their natural properties.

Saffron has been considered for many years as a secondary crop, relegated to poor soils and adverse climate conditions, thus not deserving particular attention from the scientific viewpoint (Negbi, 2009). Nevertheless, the current rapid expansion of interest in nutraceuticals, and the discovery or confirmation of interesting properties of saffron, in particular as a cancer chemopreventive and curative (Abdullaev, 2004; Aung *et al.*, 2007), an antidepressant (Wang *et al.*, 2010), an antiamylogenic in Alzheimer's disease (Papandreou *et al.*, 2006) and an inhibitor of age-related macular degeneration (Falsini *et al.*, 2010), should increase plant scientists' interest in this species. In particular, an important goal is the improvement both of quantity and quality of the production, although saffron sterility hampers conventional plant breeding approaches to this target. Phytosanitary selection of virus-free corms, besides the cultural practices, could be of great help to improve production, as it has been for other Iridaceae such as iris and gladiolus, infected by the same set of viruses which affect saffron (Van der Vlugt, 1994; Katoch *et al.*, 2003). Though potyvirus detection in corms is generally more difficult that in leaves, at least in gladiolus and with the ELISA technique (reviewed in Katoch *et al.*, 2003), the multiplex RT-PCR approach has proved to be successful to concurrently detect up to four potyviruses in calla lily plants (Hu *et al.*, 2010), a species which is also affected by latent potyvirus infection (Chen *et al.*, 2004).

Sanitation procedures for saffron corms should also be investigated. Up to now, the only sanitation practices suggested have been for fungal pathogens, and have included fungicide treatments before planting (Tammaro, 1999; Capelli, 2006; Akbari *et al.*, 2010). However, there is the possibility that thermotherapy could be used for eradicating viruses as for other bulbous plants (Conci and Nome, 1991). At this regard Chen *et al.* (2006) reported successful eradication of TuMV, CMV and TRV from callus and differentiating shoots of *C. sativus*.

Concluding remarks and perspectives

The presence of latent potyvirus infections need to be investigated in large scale phytosanitary programs, being as these viruses are possibly responsible for the low productivity and decay of many saffron crops worldwide. Unfortunately the previously mentioned EPPO certification scheme for the production of virus free saffron mother stocks (EPPO Bulletin, 2002) includes only visual inspection for symptoms, which obviously will not detect potyvirus latent infections. Thus, this protocol should be implemented, adding at least ELISA tests for the detection of BYMV. Such tests should also be performed by growers' organizations in each cultivation region, as growers tend to plant their own propagated corms, both for economic reasons and for the rules imposed by the Protected Denomination of Origin (DOP) certification. This protects and safeguards the provenance and origin of product in Europe. In this way, latent virus infections will be maintained indefinitely, unless these latent infections

do not degenerated into more severe infections causing visible symptoms. There is also the possibility that the presence of these viruses contributes to the sterility of saffron and to the quality of the production, which is fundamental in order to obtain a constant set and quantity of secondary metabolites required by nutraceutical and pharmacological applications. The knowledge acquired on these topics is particularly important in Europe where high crop performance, both in terms of quantity and quality, is necessary to for growers to stay competitive in a global market. Furthermore, the possibility of significantly ameliorating crop performance would persuade farmers to cultivate saffron in low-fertility marginal areas where adverse climate prevails and soils are poor, and where saffron can be easily adapted (Negbi, 1999). This expanded cultivation of a valuable crop would improve income and benefit farmers in low income regions.

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