Article

Crocus sativus L. Ecotypes from Mediterranean Countries: Phenological, Morpho-Productive, Qualitative and Genetic Traits

Loriana Cardone [1,*] , **Donato Castronuovo** [1] , **Michele Perniola** [2] , **Nunzia Cicco** [3] , **Rosa V. Molina** [4] ,
Begoña Renau-Morata [4] , **Sergio G. Nebauer** [4] and **Vincenzo Candido** [2,*]

1   School of Agricultural, Forest, Food and Environmental Sciences, University of Basilicata, Viale dell'Ateneo Lucano, 10, 85100 Potenza, Italy; donato.castronuovo@unibas.it

2   Department of European and Mediterranean Cultures, Environment, and Cultural Heritage, University of Basilicata, Via Lanera, 20, 75100 Matera, Italy; michele.perniola@unibas.it

3   Institute of Methodologies for Environmental Analysis, National Research Council, C.da S. Loja, 85050 Tito Scalo, Italy; nunzia.cicco@imaa.cnr.it

4   Departamento de Biologia Vegetal, Universitat Politècnica de València, 46071 Valencia, Spain; rvmolina@bvg.upv.es (R.V.M.); begoremo@gmail.com (B.R.-M.); sergonne@bvg.upv.es (S.G.N.)

*   Correspondence: loriana.cardone@unibas.it (L.C.); vincenzo.candido@unibas.it (V.C.)

Abstract: The characterization of *C. sativus* ecotypes is of great interest for preserving them from a possible genetic erosion due to the decrease of European cultivation surface. In this study, we evaluated four ecotypes from Italy (Sardinia and Abruzzo), Spain (Castilla-La Mancha), and Greece (Kozani) in order to detect the existence of variability and promote the biodiversity of this crop. Thirty-one traits related to saffron flowering, flower morphology, production of spice and daughter corms, vegetative development (leaf and corm traits), and spice quality, were evaluated. In addition, a genetic analysis through three PCR-based approaches, SSRs, RAPD, and SRAP was assessed. Results highlighted a phenotypic variation among ecotypes during two consecutive years. All the studied parameters were influenced by the ecotype except for the stamen length, color coordinates of tepals, leaf length, and leaf number per plant. Sardinia had a longer flowering interval, earlier flowering, and higher spice yield and quality than the other corm origins. The maximum values of morphological traits, such as stigma length, dry weight of stigmas, tepals, flowers and leaves, leaf area, and daughter corm weight were observed in the Abruzzo ecotype. Principal component analysis (PCA) showed a clear separation among ecotypes, in which Sardinia and Spain showed more similarities than Abruzzo and Kozani. Significant negative correlation was found between days to flower with stigma yield and quality. However, we could not find molecular markers discriminating among corm origins. In conclusion, this study suggests the importance of *C. sativus* ecotypes as precious source of biodiversity and bioactive compounds, and of their enhancement as fundamental prerequisite for a sustainable development strategy and as an agricultural diversification opportunity for growers.

Keywords: saffron; flowering earliness; stigma yield; corm growth; crocin; molecular markers

1. Introduction

Crocus sativus L. is a geophyte cultivated mainly for its red stigmas, a dried part of the flower is used as spice, colorant, and medicinal due to its anti-inflammatory, antidepressant, anticancer, and antioxidant properties [1]. It is known for four millennia and originated probably in the Middle East or Greece (southern Aegean islands Crete and Santorini) [2]. *C. sativus* is a sterile triploid (x = 8; 2n = 3x = 24) species which is vegetatively propagated by means of corms and evolved probably by combining two different genotypes of *C. cartwrightianus* [3]. *C. sativus* is cultivated in many areas, such as Iran, India, Afghanistan,

Morocco, and Euro-Mediterranean countries, such as Greece, Spain, and Italy with a world production of 418 t y^{-1} [4].

Recently, it occurred a loss of cultivation surface, particularly in the Mediterranean Basin countries, due to high manual work requirements in a short period (flower harvesting and processing of spice including the separation of stigmas from flower and their dehydration), lack of modernization of the cultivation methods, poor soil fertility, increased emergence of diseases (fungi, bacteria, virus, nematodes, mites), weed infestation, climate change (i.e., droughts during the phase of daughter corm development), spice adulteration, conflicts in market sector, and social factors (migration from rural sites). All these causes may have resulted in a strong genetic erosion of *C. sativus* [5–7].

Although in the European Union the production is decreased or even disappeared in some areas, such as Germany, Austria, and England (Essex and Cambridgeshire), the protected designation of origin has been attributed to the "Krokos Kozanis" (EC Reg. 378/1999) in Greece, the "Azafrán de la Mancha" (EC Reg. 464/2001) in Spain, the "Zafferano dell'Aquila" and "Zafferano di San Gimignano" (EC Reg. 205/2005), and "Zafferano di Sardegna" (EC Reg. 98/2009) in Italy [8–10].

Each traditional production country follows specific agronomic and post-harvesting techniques and obtains different qualitative grades of spice with unique features, differentiating *C. sativus* ecotypes [11].

Phenotypic variation among *C. sativus* ecotypes cultivated under different climatic conditions (Iran, Italy, and Morocco) in terms of flower morphology and productive traits is well documented in the literature. It is concluded that the parameters, such as length and dry weight of stigmas, stigma yield, flower number, number and fresh weight of daughter corms, and leaf dry weight could be influenced by corm geographical origin [12–15].

However, although morphological markers are cheap and easy, they are influenced by environmental conditions and are less reliable than molecular markers which identify the variation at DNA level [16]. Thus, other authors conducted some comparative phenotypic and genetic studies on *C. sativus* corm origins and obtained contrasting results. Siracusa et al. [17] evaluated the variability of six *C. sativus* accessions from Iran, India, Australia, Spain, and Italy, grown in the same experimental field (Sicily region). These authors found that the Italian origin (Abruzzo and Sardinia) yielded bigger corms and longer leaves compared to other origins revealing genetic differences among the investigated origins using amplified fragment-length polymorphism (AFLP) analysis. Similarly, Torricelli et al. [18] showed that Italian accessions from the Abruzzo region (Barisciano and Città della Pieve) produced more flowers than the Iranian ones and detected limited genetic differences among them, using AFLP analysis. In contrast, Grilli Caiola et al. [19] found some phenotypic differences related to flower traits with no genetic variability. Despite several specific studies have been done on the genetic characterization of *C. sativus* ecotypes based on different types of molecular markers, such as sequence-related amplified polymorphism (SRAP) [20], random amplified polymorphic DNA (RAPD), microsatellite (SSRs) [21,22], and inter simple sequence repeats (ISSR) [21], the *C. sativus* genetic diversity is not clear. Some researchers concluded that *C. sativus* is a monomorphic species [19,21,23], while others stated the presence of genetic differences among the *C. sativus* accessions [24–26].

Recently, saffron becomes attractive as functional food for its potential beneficial effects on different human diseases [1]. In particular, the presence of bioactive compounds including crocetin esters, picrocrocin, and safranal, contributes to the quality of spice with coloring, bittering, and aromatic properties, respectively [4]. Differences of chemical composition among ecotypes were detected by various techniques, such as mid-infrared spectroscopy [27,28], gas chromatography [29], electronic nose [30], and UV–visible spectrophotometry according to the International Organization for Standardization (ISO) procedure [14,31]. Crocetin esters, picrocrocin, and safranal are considered as interesting markers for the differentiation of spice obtained from corm geographical origins [14,17,32].

Preservation and characterization of the *C. sativus* ecotypes are important strategies to defend and promote the biodiversity of this crop [5,9]. In this regard, the project

entitled "CROCUSBANK" supported by the European Commission was the first to create, characterize with different descriptors, and exploit a germplasm collection of *Crocus* species, including 220 *C. sativus* accessions [9]. With the recent and alarming climate change, the biodiversity becomes an important resource, which allows to choose climate resilient ecotypes with high suitability potential [33].

The efforts to prevent the loss of *C. sativus* ecotypes in European countries, due to all causes mentioned above, require a multi-analysis approach to test the existence of their variability. Furthermore, considering the low water requirement of *C. sativus* [4] and low environmental impacts of its cultivation [34–36], this study could stimulate farmers to cultivate it, contributing positively to the agrobiodiversity.

The aim of this work was to characterize four ecotypes, namely Abruzzo (Italy), Sardinia (Italy), Castilla-La Mancha (Spain), and Kozani (Greece), cultivated in the same experimental field (Matera, Basilicata region, Southern Italy), by evaluating phenological, morphological, agronomical, qualitative, and molecular traits.

2. Materials and Methods

2.1. Study Site and Experimental Design

A two-year study (from September to May of 2016–2017 and 2017–2018) was carried out at the experimental field of Matera (40°42′, N 16°42′, E; 385 m asl). The town is localized in the eastern part of the Basilicata region (Southern Italy) and falls in the *Csa* Köppen–Geiger climate classification characterized by a warm temperate climate with dry summers [37]. The site was characterized by a Typic Calcixerepts fine loamy soil [38], texture: 35% sand, 37% silt, and 28% clay and by the following chemical properties: pH 8.3; $EC_{1:5}$ 0.28 dSm^{-1}; organic carbon 0.75%; CEC 14.4 cmol (+) kg^{-1}; N 1.14 g kg^{-1} and available P 20 mg kg^{-1}. All soil analyses were performed according to the Italian regulation [39].

Prior to the experimental trial, corms from four different geographical origins, Abruzzo and Sardinia (Italy), Castilla-La Mancha (Spain), and Kozani (Greece) (Table 1), were dipped in a 1% fungicide water solution of copper oxychloride (Sumitomo Chemical Italia S.r.l., Milano, Italy) to minimize fungal diseases caused mainly by *Fusarium* spp. and cultivated in the same field (Matera) for at least three consecutive years.

Table 1. Origin of the selected saffron ecotypes.

Ecotype	City (Province)-Region	Country	Altitude	Coordinates	Climate [1]
Abruzzo	Navelli (L'Aquila)-Abruzzo	Italy	805 m asl	42°14′ N–13°43′ E	*Cfb*
Sardinia	San Gavino Monreale (Medio Campidano)-Sardinia	Italy	100 m asl	39°33′ N–8°48′ E	*Csa*
Castilla-La Mancha	Albacete-Castilla-La Mancha	Spain	686 m asl	38°59′ N–1°51′ W	*BSk*
Kozani	Krokos-Kozani	Greece	720 m asl	40°18′ N–21°47′ E	*Cfa*

[1] Climate classification according to Köppen–Geiger [37].

The selected *C. sativus* corms with a mean horizontal diameter of 4 cm were planted by a randomized block experimental design, replicated three times, in rows 30 cm apart with a distance of 5 cm between corms and with a depth of 15 cm, achieving a density of 66.7 plants per square meter. Weed control was carried out by hand and no irrigation was applied during each crop cycle. During the flowering period of each year, between October and November, flowers were collected by hand in the early hours of each day. Successively, the harvested flowers were taken to the laboratory, where stigmas were separated manually from the rest of the flower, were dried, stored in closed glass jars in suitable condition (room temperature of 19 ± 3 °C), and kept in the dark until qualitative analysis which was carried out at the biochemical laboratory.

For each crop cycle, meteorological data were collected by using a weather station equipped with temperature and relative humidity probes (CS500-L-modified version of Vaisala's 50Y Humitter, Campbell Scientific Inc., Logan, UT, USA) and with a TE525

precipitation sensor (Texas Electronics, Texas, USA) to measure the rainfall. Collected data were recorded by a CR 10x data-logger (Campbell Scientific Inc., Logan, UT, USA) and were elaborated to have monthly rainfall, mean, maximum and minimum air temperature during the crop cycle of the two experimental years (2016–2017 and 2017–2018).

2.2. Phenological and Morpho-Productive Traits

The following parameters were recorded on 10 plants per replication: stigma and stamen length using a meter scale (mm); fresh and dry weight of stigmas, stamens, tepals and flowers (g); days to flower, flowering interval (days), number of flowers harvested (n corm^{-1}; n m^{-2}), stigma yield (kg ha^{-1}), leaf number (n plant^{-1}), leaf length (cm), fresh and dry leaf weight (g), leaf area (cm^2 plant^{-1}) and shoot number (n plant^{-1}). At the end of each crop cycle, during the senescence phase, the corms were lifted from the soil and the number of daughter corm per mother corm, fresh weight (g), horizontal diameter (cm), and yield (t ha^{-1}) of daughter corms were recorded.

The fresh weight and dry weight of stigmas, stamens, tepals, whole flowers (including stigmas, stamens and tepals), leaves, and daughter corms were measured using a digital weighing balance. Dry weight of all flower parts was measured by drying samples at low temperature (40 ± 3 °C for 24 h) in a forced air oven. Leaf dry weight was obtained by drying samples in a ventilated oven set at 65 °C for 72 h [40]. Leaf area was determined by using an area meter LI–Cor Model 3100 (LI–Cor, Inc., Lincoln, NE, USA). The number of flowers, leaves, shoots, and daughter corms per plant was counted manually. Leaves were washed with distilled water and their length was measured using a meter scale [41]. The diameter of daughter corms was measured using digital vernier calipers (LTF SpA, Cremona, Italy) [42].

Color of fresh tepal was measured on the center of the upper tepal surface according to Cardone et al. [43] and measured by means of a Minolta CR–400 Chroma Meter [44]. The colorimeter was calibrated using the standard white plate. Fifteen tepal samples were used for each corm origin and five readings were made on each set of samples. Color coordinates were expressed as L* describing lightness (L* = 0 for black, L* = 100 for white), a* describing intensity in green–red (a < 0 for green, a > 0 for red), b* describing intensity in blue–yellow (b < 0 for blue, b > 0 for yellow) according to the CIELAB color system.

2.3. Qualitative Analysis

The qualitative analysis of spice produced in 2016 and 2017 was conducted according to the ISO 3632 standard, which is the internationally accepted reference specification used at commercial level [45,46]. Five-hundred mg of powdered samples were passed through a 0.5 mm sieve, transferred into a 1000 mL volumetric flask with 900 mL of distilled water. The obtained aqueous solution was stirred for 1 h in the dark and then brought to 1000 mL with distilled water. This extract was diluted (1:10 *v/v*) with deionized water and filtrated with polytetrafluoroethylene (PTFE) filters (25 mm diameter and 0.45 μm pore size). The results of the ISO 3632:2011 parameters were expressed as values of 440 nm or coloring strength, values of 330 or aroma strength, and values of 257 or flavor strength, using an UV-Vis spectrophotometer (Ultrospec 4000, Amersham Pharmacia Biotech, Milan, Italy) according to the following equation:

$$A_{1\ \text{cm}}^{1\%}(\lambda \text{max}) = \frac{(D * 20,000)}{(100 - H)} \qquad (1)$$

where *D* is the specific absorbance; 20,000 is dilution factor of the total extract considering the amount of saffron sample; *H* is the moisture and volatile matter content, expressed as a percentage mass fraction. *H* was determined by placing 2.5 ± 0.001 g of each saffron sample in an oven from 103 ± 2 °C for 16 h and it was calculated as the percentage of the initial weight of the sample according to the formula:

$$H(\%) = (m0 - m1) * (100/m0) \qquad (2)$$

where m_0 is the mass, in grams, of the saffron portion before drying and m_1 is the mass, in grams, of the dry residue. All the analyses were performed in triplicate, and two measurements were taken for each replicate.

2.4. Molecular Analysis

For each ecotype and repetition, leaves of three individuals were collected and preserved in refrigeration at −80 °C until the moment of the extraction. Genomic DNA (gDNA) extraction was performed from about 100 mg of young leaf material. The plant material was ground into a fine powder in liquid nitrogen and was collected in a collection tube of 1.5 mL. The extraction was carried out using the E.Z.N.A. (Eazy Nucleic Acid Isolation) Plant DNA Miniprep KIT following the manufacturer's protocol and the DNA was quantified using a spectrophotometer JENWAY 6305 (Jenway). DNA was amplificated with three different types of molecular markers, microsatellites (SSRs), RAPD, and SRAP in a thermal cycler iCycler (Biorad).

2.4.1. SSRs Analysis

SSRs-PCR reactions were conducted according to Nemati et al. [22] using five selective SSRs primer combinations (ABRII/Cs2, ABRII/Cs8, ABRII/Cs10, ABRII/Cs11, and ABRII/Cs20), which are listed in Table S1. The total reaction volume was 20 μL containing 6 μL of DNA extract (30 ng), 5 μM primers, 2.0 μL 10× PCR buffer, 5 mM dNTPs, 50 mM $MgCl_2$ and one unit *Taq* DNA polymerase. The PCR program involved initial denaturation at 95 °C for 5 min; followed by 35 cycles at 94 °C for 30 s, at the annealing temperature (50–55 °C) for 30 s, and at the extension temperature (72 °C) for 2 min; followed by a final extension of 72 °C for 7 min.

2.4.2. RAPD Analysis

RAPD-PCR reactions were conducted according to Rubio Moraga et al. [21] using eleven RAPD primers (OPL07, OPL16, OPK08, OPK09, OPK15, OPJ13, OPR05, OPR06, OPR07, OPA04, and OPA09), which are listed in Table S1. The total reaction volume was 20 μL containing 6 μL of DNA extract (30 ng), 5 μM primers, 2.0 μL 10× PCR buffer, 5 mM dNTPs, 50 mM $MgCl_2$ and one unit *Taq* DNA polymerase. The cycling program began with an initial 2 min at 94 °C followed by 45 cycles at 94 °C for 30 s, 35 °C for 30 s and 72 °C for 2 min plus a final 10 min at 72 °C and storage at 4 °C.

2.4.3. SRAP Analysis

SRAP-PCR reactions were conducted according to Babaei et al. [20] using three SRAP primer combinations (M1-E18, M2-E18 and M3-E18), which arere listed in Table S1. The total reaction volume was 20 μL containing 6 μL of DNA extract (30 ng), 5 μM primers, 2.0 μL 10× PCR buffer, 5 mM dNTPs, 50 mM $MgCl_2$ and one unit *Taq* DNA polymerase. DNA amplifications were performed in 5 cycles of 94 °C for 1 min, 35 °C for 1 min, and 72 °C for 1 min for denaturing, annealing, and extension, respectively.

For all three analyses, a negative control, consisting of all reagents except for DNA, was added to test contamination. The amplified DNA obtained from SSRs, RAPD, and SRAP analysis, respectively was separated by electrophoresis in a 2% agarose gel in TBE 0.5%. The molecular weight marker used was Gene Ruler DNA Ladder Mix, 0.1 μg/μL. Finally, agarose gels were stained in an ethidium bromide solution (0.5 μg/mL). Ten microliters of amplified DNA were mixed with 2 μL sample buffer and 10 μL was applied in each well of the gel. The bands were visualized under UV light and photographed. DNA extracts obtained using the E.Z.N.A. Plant DNA Miniprep KIT from leaf saffron were characterized by yields ranging from 35 to 65 ng/μL and purity ranging from 1.8 to 2.0. All the amplified DNA fragments were scored as present (1) and absent (0). The binary data generated were used to estimate levels of polymorphism by dividing the polymorphic bands by the total number of scored bands (Table S1).

2.5. Statistical Analysis

Data on plant growth, yield, and spice quality were analyzed using two-way ANOVA, considering "ecotype" and "year" as sources of variation. Student–Newman–Keuls (SNK) test at $p \leq 0.05$ was used to test statistical differences between means. Principal component analysis (PCA) was performed to express the relationship between phenological, morpho-productive, and qualitative traits. All statistical analyses were performed using the software RStudio: Integrated Development for R, version 1.0.136 (RStudio, Inc., Boston, MA, USA).

3. Results

3.1. Climatic Data

Table 2 shows the climatic data (monthly minimum, mean and maximum temperatures, and total rainfall) recorded during the two experimental years (2016–2017 and 2017–2018) in Matera. The meteorological trend was very similar for the two years. On average, during flowering period, between October and November, the Matera site was characterized by an annual minimum, mean and maximum air temperature of 11.0, 13.8, 17.9 °C, respectively. The annual average rainfall was of 366.6 mm (Table 2). The first experimental year was rainier than the second one, in particular, greater rainfall was recorded during the phases of pre-flowering (September and October). Meanwhile, the second year was distinguished for the increased rainfall (110.2 mm) during the period of maximum daughter corm multiplication (February–March) compared to the first one (64.8 mm).

Table 2. Monthly minimum, mean and maximum air temperature and total rainfall recorded during two experimental years (2016–2017 and 2017–2018) in Matera.

	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Mean
					2016–2017					
T.min (°C)	16.0	12.9	9.8	5.3	1.8	6.4	7.9	9.0	13.6	9.2
T.mean (°C)	19.5	15.8	12.1	8.1	4.0	9.3	11.6	12.5	17.9	12.3
T.max (°C)	24.8	20.5	15.3	11.6	7.1	13.4	16.6	17.3	23.8	16.7
Rainfall (mm)	80.2	37.0	34.8	25.4	68.0	49.0	15.8	40.0	41.8	395.0
					2017–2018					
T.min (°C)	15.8	12.8	8.6	4.8	6.3	3.8	6.8	12.1	14.8	9.5
T.mean (°C)	19.6	16.2	11.1	7.9	9.2	6.7	9.9	16.1	18.9	12.9
T.max (°C)	25.2	21.3	14.6	13.8	15.2	12.2	15.9	23.2	25.0	18.5
Rainfall (mm)	52.4	14.0	62.6	26.4	14.4	62.4	47.8	10.8	47.4	338.2

3.2. Flower Morphological Traits and Colorimetric Coordinates of Tepals

Flower morphological traits varied significantly with corm geographical origin, except for the stamen length and colorimetric coordinate of tepal (Table 3). The average data of the two experimental years indicated that all flower traits were significantly higher in the Abruzzo ecotype followed by Sardinia, Kozani, and Spain. No significant effect of ecotype on L*, a* and b* parameters of tepals was reported (Table 3). On average 153.8 flowers were needed to obtain 1 g of dried stigma, and in detail 126.6, 156.3, 163.9, and 178.6 flowers were needed for the Abruzzo, Sardinia, Kozani, and Spain ecotypes, respectively (Table 3).

Some flower morphological traits were significantly influenced by the year, except for the length of stigmas and stamens, fresh weight of stigmas, tepals and flowers, dry weight of stigmas and stamens, and b*. In general, all values decreased in the second year (Table 3). The significant interactions "ecotype × year" highlighted the best performance of Abruzzo ecotype when cultivated in 2017 in terms of stigma length (41.8 mm), stigma fresh (0.0460 g), and dry weight (0.0084 g) (data not shown). In contrast, the lowest values of dry weight of all flower traits, stigmas (0.0048 g), stamens (0.0073), tepals (0.0244 g), and flowers (0.0365 g) were reached by Kozani in 2017 (data not shown).

Table 3. Flower morphological and colorimetric traits related to ecotype (E), year (Y) and their interaction.

Experimental Factors [1]	Stigma Length (mm)	Stamen Length (mm)	Stigma Fresh Weight (g)	Stigma Dry Weight (g)	Stamen Fresh Weight (g)	Stamen Dry Weight (g)	Tepal Fresh Weight (g)	Tepal Dry Weight (g)	Flower Fresh Weight (g)	Flower Dry Weight (g)	L*	a*	b*
Ecotype (E)													
Abruzzo	40.57 a	23.92	0.0438 a	0.0078 a	0.0270 a	0.0091 a	0.2878 a	0.0359 a	0.3586 a	0.0528 a	49.31	25.13	−20.92
Sardinia	36.80 b	22.75	0.0360 b	0.0065 b	0.0269 a	0.0082 b	0.2829 a	0.0324 ab	0.3430 a	0.0471 ab	52.95	27.94	−24.79
Kozani	36.63 bc	22.65	0.0302 bc	0.0055 c	0.0241 a	0.0075 bc	0.2395 b	0.0292 b	0.2968 b	0.0422 b	50.07	26.37	−20.64
Spain	34.50 c	23.00	0.0260 c	0.0061 bc	0.0168 b	0.0069 c	0.2542 b	0.0318 ab	0.2970 b	0.0448 b	48.51	28.58	−22.17
Significance [2]	**	ns	**	**	*	**	**	*	**	**	ns	ns	ns
Year (Y)													
2016	37.14	23.27	0.0352	0.0067	0.0265	0.0082	0.275	0.0348	0.3366	0.0497	47.54	28.27	−22.22
2017	37.11	22.89	0.0328	0.0062	0.0209	0.0076	0.257	0.0299	0.3111	0.0437	52.87	25.74	−22.04
Significance	ns	ns	ns	ns	*	ns	ns	*	ns	*	**	**	ns
E × Y													
Significance	**	ns	**	**	**	**	ns	**	ns	**	**	**	ns

[1] Mean values followed by a different letter are significantly different at $p \leq 0.05$, according to the Student–Newman–Keuls (SNK) test. [2] *, Significance at $p \leq 0.05$; **, significance at $p \leq 0.01$; ns, no significant difference.

3.3. Phenological, Productive and Vegetative Development Traits

All phenological and productive traits were significantly influenced by the saffron ecotype. No significant effect of the year was observed on all traits except for the daughter corm yield (Table 4). Corm origin was significantly related to the earliness and flowering interval (days). In general, 40.4 days were needed to flower from corm planting and the flowering lasted about 20 days. Sardinia bloomed about six days earlier than Kozani and showed a wider flowering period (Table 4). A greater flower number per corm, flower number per m^2, and stigma yield were also observed in the Sardinia ecotype. In contrast, Kozani obtained the lowest value in terms of flower and stigma production (Table 4).

Table 4. Phenological and productive traits related to ecotype (E), year (Y) and their interaction.

Experimental Factors [1]	Days to Flower (d)	Flowering Interval (d)	Flower Number Corm^{-1}	Flower Number (n m^{-2})	Stigma Yield (kg ha^{-1})	Daughter Corm Yield (t ha^{-1})
Ecotype (E)						
Abruzzo	41.0 ab	21.7 a	2.08 c	83.12 c	6.58 b	18.68 a
Sardinia	37.0 b	23.0 a	3.61 a	144.23 a	9.26 a	18.77 a
Kozani	43.0 a	16.0 b	2.10 c	82.68 c	4.59 c	15.13 b
Spain	40.5 ab	16.0 b	2.83 b	113.27 b	6.89 b	19.03 a
Significance [2]	*	*	**	**	**	*
Year (Y)						
2016	38.75	20.3	2.69	105.86	7.11	16.18
2017	42.00	18.1	2.64	105.79	6.54	19.63
Significance	ns	ns	ns	ns	ns	*
E × Y						
Significance	ns	ns	ns	ns	ns	**

[1] Mean values followed by a different letter are significantly different at $p \leq 0.05$, according to SNK test. [2] *, Significance at $p \leq 0.05$; **, significance at $p \leq 0.01$; ns, no significant difference.

All vegetative development traits were significantly influenced by saffron ecotype except for the leaf number and leaf length. A significant effect of the year was observed for the leaf number, leaf length, leaf area, daughter corm weight, and diameter (Table 5). Regarding to the leaf development and daughter corm multiplication, Abruzzo showed the highest fresh and dry leaf weight, leaf area, daughter corm weight, and diameter (Table 5). On the contrary, this ecotype showed the lowest number of daughter corms, in such a way that the total production was not higher than that of Sardinia or Spain, which were distinguished for the daughter corm number for plant (Table 5). Only Kozani showed significantly lower corm production according to its smaller leaf area (Table 5).

Table 5. Leaf development and daughter corm multiplication traits related to ecotype (E), year (Y) and their interaction.

Experimental Factors [1]	Leaf Number (n Plant^{-1})	Leaf Length (cm)	Leaf Fresh Weight (g)	Leaf Dry Weight (g)	Leaf Area (cm^2 Plant^{-1})	Shoot Number (n Plant^{-1})	Daughter Corm Weight (g)	Daughter Corm Number (n Plant^{-1})	Daughter Corm Diameter (cm)
Ecotype (E)									
Abruzzo	40.45	33.90	14.48 a	5.14 a	240.58 a	6.28 b	7.63 a	6.37 b	2.31 a
Sardinia	42.25	30.37	9.36 b	3.21 b	197.63 b	8.63 a	6.76 ab	9.12 a	2.22 a
Kozani	41.38	30.80	7.15 c	2.30 c	149.82 d	6.22 b	4.17 b	6.57 b	1.82 b
Spain	41.17	31.65	10.11 b	3.29 b	185.34 c	8.25 a	4.72 b	8.58 a	2.18 a
Significance [2]	ns	ns	**	**	**	*	*	*	*
Year (Y)									
2016	34.93	27.51	9.70	3.42	183.62	7.67	4.14	8.08	1.96
2017	47.69	35.85	10.85	3.55	203.07	7.03	7.50	7.24	2.30
Significance	**	**	ns	ns	**	ns	**	ns	*
E × Y									
Significance	**	**	*	ns	**	*	**	*	*

[1] Mean values followed by a different letter are significantly different at $p \leq 0.05$, according to SNK test. [2] *, Significance at $p \leq 0.05$; **, significance at $p \leq 0.01$; ns, no significant difference.

Significant interactive effects of experimental factors were recorded in all morphological traits of leaf and daughter corm, except for the leaf dry weight. This interactive effect could be explained by the greater differences presented by Abruzzo in the second year in relation to the other ecotypes. Particularly, Abruzzo obtained the maximum values of leaf area (279.06 cm^2 plant^{-1}), daughter corm weight (10.02 g), and diameter (2.60 cm) in 2017 (data not shown).

3.4. Qualitative Traits

Results of moisture and volatile matter (%), coloring power ($A^{1\%}_{1cm}$ 440 nm), bittering power ($A^{1\%}_{1cm}$ 257 nm), and aromatic power ($A^{1\%}_{1cm}$ 330 nm) of spice obtained from all ecotypes are reported in Table 6. All the values of moisture content were lower than 12%, the maximum limits established by ISO 3632 references [46]. Although all spice samples belonged to the first qualitative category according to ISO 3632, the qualitative traits were significantly influenced by corm ecotype except for the moisture and volatile matter (Table 6). The highest coloring, bittering, and aromatic powers were reached in spice obtained from Sardinia, followed by Spain, Abruzzo, and Kozani ecotypes (Table 6). No significant effects of year and "ecotype × year" interaction was found for all qualitative parameters (Table 6).

Table 6. Moisture and volatile matter, coloring, bittering, and aromatic powers related to ecotype (E), year (Y), and their interaction.

Experimental Factors [1]	Moisture and Volatile Matter (%) [3]	$A^{1\%}_{1cm}$ [4] (440 nm)	ISO [5] Reference (Crocetin Esters)	$A^{1\%}_{1cm}$ [6] (257 nm)	ISO [7] Reference (Picrocrocin)	$A^{1\%}_{1cm}$ [8] (330 nm)	ISO [9] Reference (Safranal)
Ecotype (E)							
Abruzzo	4.58	242.53 c	I	88.61 b	I	26.06 b	I
Sardinia	4.67	266.03 a	I	96.95 a	I	30.19 a	I
Kozani	4.04	223.83 d	I	80.38 c	I	24.94 b	I
Spain	4.04	251.07 b	I	89.48 b	I	28.14 ab	I
Significance [2]	ns	**		**		*	
Year (Y)							
2016	4.29	244.01	I	87.74	I	25.88	I
2017	4.35	247.73	I	89.99	I	28.66	I
Significance	ns	ns		ns			
E × Y							
Significance	ns	ns		ns		ns	

[1] Mean values followed by a different letter are significantly different at $p \leq 0.05$, according to SNK test. [2] *, Significance at $p \leq 0.05$; **, significance at $p \leq 0.01$; ns, no significant difference. [3] ISO reference: maximum 12% for all category. [4] Absorbance of 1% aqueous saffron extract at 440 nm. [5] ISO reference for crocetin esters: I category $A^{1\%}_{1cm} \geq 200$, II category $A^{1\%}_{1cm} \geq 170$, III category $A^{1\%}_{1cm} \geq 120$. [6] Absorbance of 1% aqueous saffron extract at 257 nm. [7] ISO reference for picrocrocin: I category $A^{1\%}_{1cm} \geq 70$, II category $A^{1\%}_{1cm} \geq 55$, III category $A^{1\%}_{1cm} \geq 40$. [8] Absorbance of 1% aqueous saffron extract at 330 nm. [9] ISO reference for safranal: I, II and III category $A^{1\%}_{1cm}$ minimum 20 and maximum 50.

3.5. Molecular Analysis

gDNAs obtained from individual ecotypes were used as a template to perform the molecular analyses using SSRs, RAPD, and SRAP markers. As shown in Table S1, a total of 5 SSRs primer combinations were initially screened and all were used in the analysis; 11 RAPD primers were screened and 10 were able to yield intense bands; 3 SRAP primer combinations were screened and 2 yielded reproducible bands in all samples. The number of monomorphic and polymorphic bands, and the percentage of monomorphism and polymorphism produced by different primers, and primer combinations are shown in Table S1. The number of bands varied from 3 to 4 within SRAP, from 7 to 11 bands within RAPD, and there were 4 bands for all SSRs. All the used primers and primer combinations yielded a total of 108 bands, 100.0% of which were considered monomorphic (Table S1). A representative result of SSRs, RAPD, and SRAP profiles from each saffron ecotype is shown

in Figure 1A–C, respectively. Although the bands obtained were those expected for the three types of molecular markers, all molecular markers used did not detect polymorphisms in saffron ecotypes (Figure 1).
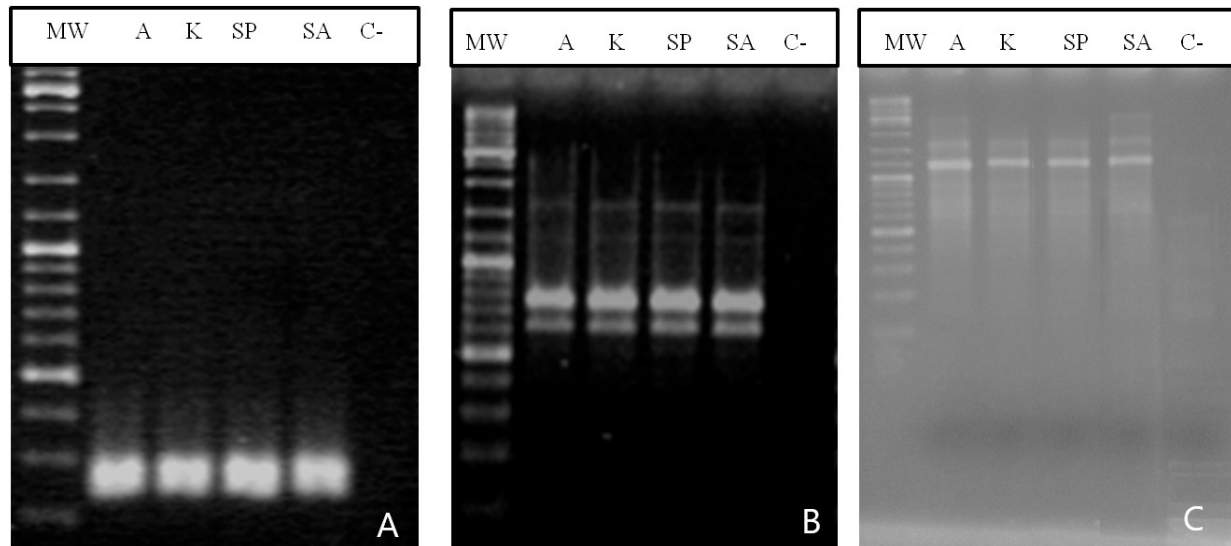

Figure 1. Bands patterns of individual gDNA representing different geographic areas. (**A**) SSR profile, (**B**) RAPD profile and (**C**) SRAP profiles. MW: Molecular weight (1 kb, Promega); A: *C. sativus* from Abruzzo (Italy); K: *C. sativus* from Kozani (Greece); SP: *C. sativus* from Spain; SA: *C. sativus* from Sardinia (Italy).

3.6. Principal Component Analysis

PCA analysis was employed to show the relationship between the phenological, flower, leaves and daughter corm morphological traits, yield, and qualitative parameters. Nineteen quantitative and qualitative variables were reduced to two principal components, which represent 68.8% of the total variability. The first component (PC1) accounted for the 39.3% of the total variability, and the second one (PC2) accounted for the 29.5%. The most important contributors to the PC1 are flower number per corm, flower number per m^2, stigma yield, coloring, bittering, and aromatic powers. The most important contributors to PC2 are stigma dry weight, leaf dry weight, leaf area, flower dry weight, tepal dry weight, and stigma length (Figure 2). Productive (flower number and stigma yield) and qualitative parameters (color, bitterness, and aroma) were positioned close indicating high positive correlation between them, as shown in the loading plot (Figure 2). The same relationship was found between the leaf area with leaf dry weight, stigma length, stigma dry weight, daughter corm weight, and diameter.

The score plot (Figure 3) shows a good separation between the *C. sativus* ecotypes: Abruzzo is characterized by high values for morphological traits as stigma length, dry weigh of stigmas, stamens, tepals and whole flower, fresh and dry leaf weight, leaf area, daughter corm weight, and diameter. Meanwhile, Sardinia and Spain are defined by high values for flower number per m^2, flower number per corm, stigma yield, coloring, bittering and aromatic powers, shoot, and daughter corm number. Kozani is characterized by late flowering time. This latter trait, expressed as days to flower, is negatively correlated with flower and stigma production, shoot number, daughter corm number per mother corm, and all qualitative parameters. Stigma length is negatively correlated with flower number, stigma yield, shoot number, daughter corm number, and aromatic power.
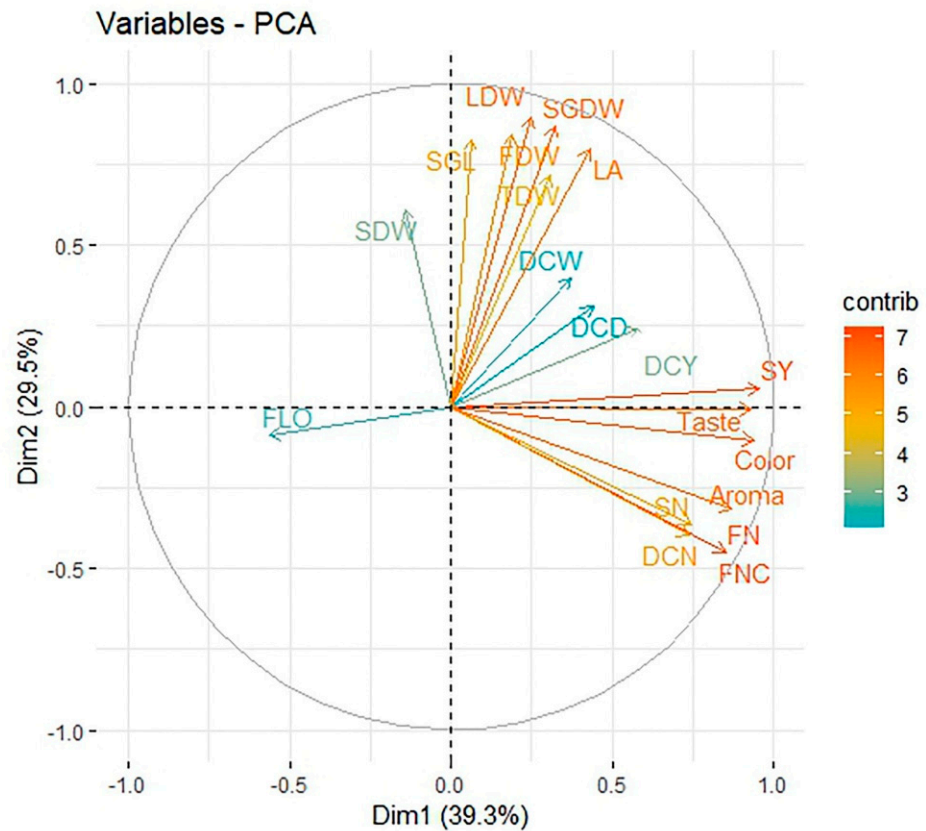
Figure 2. Correlation biplot of the two first principal component variables in the two-dimensional space. FLO = Day to Flower; SGL = Stigma Length; SGDW = Stigma Dry Weight; SDW = Stamen Dry Weight; TDW = Tepal Dry Weight; FDW = Flower Dry Weight; FNC = Flower Number per Corm; FN = Flower Number; SY = Stigma Yield; DCN = Daughter Corm Number; DCW = Daughter Corm Weight; DCD = Daughter Corm Diameter; DCY = Daughter Corm Yield; LDW = Leaf Dry Weight; LA = Leaf Area; SN = Shoot Number.
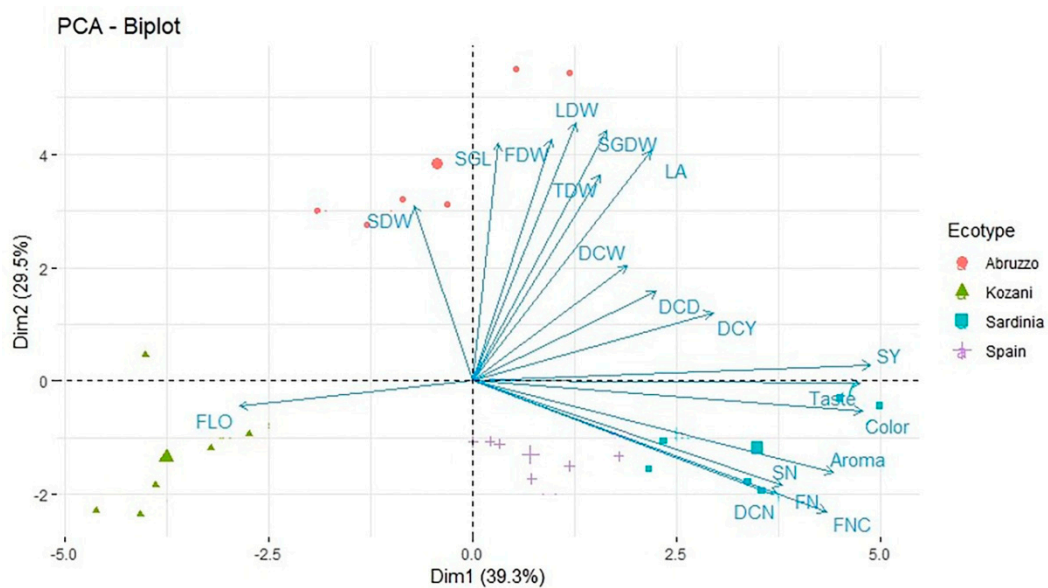

Figure 3. Biplot showing the projection from the analysis of phenological, morpho-productive, and qualitative traits.

4. Discussion

There is a risk of extinction of *C. sativus* cultivation in Europe mainly due to a high labor requirement in a short period, to the lack of modernization of the cultivation methods, and to the conflicts in marketing system [7]. This risk also implies the loss of ecotypes adapted to European areas representing a threat also to other organisms and ecosystems, including the welfare of human beings [47].

In this context, the characterization of Italian ecotypes and their comparison with other ones from European countries with saffron production, such as Spain or Greece, is of particular interest. This study evaluated four corm geographical origins of *C. sativus*: Abruzzo (Navelli, L'Aquila, Italy), Sardinia (San Gavino Monreale, Medio Campidano, Italy), Spain (Castilla-La Mancha, Albacete, Spain), and Kozani (Krokos Kozani, Greece), cultivated in the Matera site, using a multidisciplinary approach by evaluating phenological, morphological, agronomic, qualitative, and genetic aspects. These ecotypes are the most known in the Mediterranean area for food, medicinal, and cosmetic purposes [4]. Significant differences were observed among the *C. sativus* ecotypes for almost all investigated traits, particularly for the stigma length, fresh and dry stigma weight, flower number per m^2, stigma yield, fresh and dry leaf weight, leaf area, coloring, and bittering powers (Tables 3–6). The stamen length, leaf length, and number of leaves per plant were the traits identical in all the ecotypes, in agreement with results reported by De-Los-Mozos-Pascual et al. [5] and Baghalian et al. [12]. In addition to these traits, also the color intensity of tepals determined by CIELAB system was not different among ecotypes but it was significantly influenced by the year. Indeed, in the second year the flowers were clearer (higher L* and lower a* values) than in the first one. This variation in tepal color could be linked to the interaction between the presence of biologically active compounds, such as flavonoids (kampferol, rutin, quercetin, luteolin), tannins, and anthocyanins in tepal of *C. sativus* flower with the environmental conditions [48].

The best performance of the Abruzzo ecotype in terms of flower, leaf, and daughter corm dimension is in accord with the results reported by other authors, who associated the traditional annual crop cycle carried out in Abruzzo (Italy) as possible cause for a greater selection of corms with high morphological traits [17,18].

The different reaction of ecotypes in the two-year research is explained by the significant "ecotype × year" interactive effect for some traits. In particular, the obtained results showed minimum differences for the phenological traits, flower number, stigma yield, and quality in saffron compared to the flower, leaf, and daughter corm morphological traits among the two experimental years (Tables 3–5). This finding is probably due to the same summer annual temperature used for the corm storage that is responsible for the flower formation [49], and to the different climatic conditions recorded during the two growing seasons responsible for the development of flowers, leaves and corms. Indeed, the lower rainfall registered during pre-flowering period of 2017, allowed to obtain smaller flowers than those obtained in 2016. In this regard, Kozani was the most sensitive ecotype showing a significant reduction of morphological traits, as the stigma length, stigma fresh weight, fresh and dry tepal weight, fresh and dry flower weight during the second year.

Flowering is an important phase in *C. sativus* and is controlled by the environmental conditions, such as temperature and humidity [14]. The optimal temperature range for flower emergence is 15–17 °C [49]. "Earliness" or "early flowering" presented by Sardinia ecotype could be a strategy to overcome abiotic stresses, i.e., the decreased temperature during November. Although it is known that it is advisable to plant species with a gradual flowering to ease the harvesting phase and to optimize the human resources in large farms, few studies on the earliness of *C. sativus* ecotypes are available in the literature [19].

High flower production and stigma yield are the results of early flowering. This significant and negative correlation between phenological and productive traits is observed also in other crops, such as *Salvia hispanica* L. [50], *Cucumis sativus* [51], *Carthamus tinctorius* L. [52], and *Digitaria exilis* [53].

In accordance with the results of Amirnia et al. [13] and Bayat et al. [54], the corm origin has a significant impact on the flower number per m^2 and stigma yield. Similarly, Soheilivand et al. [55] investigated the diversity in flowering rate of two populations of Iran and found that the Ghaen population has higher flower production compared to the Gonabad one.

The daughter corms are the primary sinks of *C. sativus* and their growth is influenced by environmental conditions and supported by the photosynthesis activity in the leaves that contribute 90% of the biomass accumulation in the organs of the plant [56]. This characteristic explains the significant and positive correlation between leaf area with daughter corm weight and diameter. The greater rainfall recorded during leaf development and daughter corm multiplication of 2017 ensured a rise in leaf and corm traits (Table 4). In particular, Abruzzo contributed to this result showing an increase in leaf number, leaf length, leaf fresh weight, leaf area, daughter corm yield, daughter corm weight during the second year.

The importance of saffron consists also in its nutraceutical properties, due to the presence of an antioxidant substances, such as carotenoids, phenolic compounds, flavonoids, and vitamins [1,4]. Indeed, saffron is considered as a functional spice thanks to the presence of bioactive compounds, such as crocin, picrocrocin, and safranal, responsible for the qualitative traits [1].

Recently, the most suggested method to determine the saffron quality at commercial level is that specified by ISO 3632-2 which classifies the spice into three commercial categories [45,46]. In this study, all ecotypes produced a spice with specific characteristics belonging to the best quality category, according to the ISO 3632 reference [46]. This result can be explained by the good suitability of *C. sativus* in Basilicata region, as already reported in other studies [14,39].

In accordance with that indicated in our previous study [14], the coloring and bittering power of saffron stigmas, determined by UV-Vis spectrophotometry, showed a greater influence on the discrimination of *C. sativus* ecotype. Meanwhile, less variability in aromatic power compared to the coloring and bittering powers was observed. This latter result could be due to the chemical technique used for spice quality determination, which could be further investigated in combination with the high-performance liquid chromatography with diode array detector (HPLC-DAD) to obtain a more accurate value of safranal [57]. Other authors found that picrocrocin and safranal were the most significant compounds to discriminate Italian samples from Iranian ones using high-performance liquid chromatography (HPLC) and proton transfer reaction time-of-flight mass spectrometer (PTR-TOF-MS) techniques [58]. Anastasaki et al. [29] found that safranal showed significant differences between samples from Greece, Iran, Italy, and Spain using ultrasound-assisted extraction, gas chromatography followed by mass spectrometry and flame ionization.

The qualitative parameters are significantly influenced by many factors, such as the environmental conditions, soil chemical properties, and dehydration procedures [38,59,60]. Particularly, safranal is produced during thermal treatment from picrocrocin or intermediate compounds, 4-Hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (HTCC) [61]. In this study, the aromatic power varied less probably because of the same pedologic conditions, spice drying, and storing processes used in this two-year research.

The Sardinia ecotype, in addition of being distinguished for the earliness of flowering and stigma yield, is also differentiated for the quality. In particular, the significant and negative correlation between days to flower with yield and spice quality (color, taste, and aroma) explains how the early flowering was coupled with an increase in number of flowers and stigma production, and in qualitative traits of spice. This finding could be due to better suitability of Sardinia to the pedoclimatic conditions which contributed to stimulate the metabolic pathways in plants. The best qualitative performance of Sardinia was also found in studies conducted under climatic conditions of Sicily region (Southern Italy) [62] and of other cultivation sites in Basilicata region [14]. From these studies it is possible to note that the ecotype from Sardinia region shows a wide adaptation both in

environments characterized by an equal climate to the its origin site, such as in Matera (40°42′ N, 16°42′ E; 385 m asl, Basilicata region, Southern Italy) and in Geracello (37°27′ N, 14°14′ E, 450 m asl, Sicily region, Southern Italy), that fall in the Csa Köppen–Geiger climate classification (hot-summer Mediterranean climate) [59] as well as in environments with a different climate, such as Genzano di Lucania (40°50′ N, 16°08′ E, 344 m asl, Basilicata region, Southern Italy) that falls in Cfb Köppen–Geiger climate classification (temperate oceanic climate) [14].

Although significant phenotypic differences were found in most of the traits, the molecular analysis does not detect genetic variability associated to these phenotypes. This result is supported by the literature which observed that phenotypic features, such as the size and the shape of the pollen grain, were characteristic of some accessions of *C. sativus* (Israel and Sardinia), without being corroborated by genetic data obtained using RAPD molecular markers [19,63]. The monomorphism of *C. sativus* was also detected by other authors who used different genetic analysis [21,23].

The scarce of genetic variation in *C. sativus* could be associated to its sterility due to its triploid condition or it could be obtained by saffron farmers after continuous selection procedures (generation after generation) for the most productive corms [17,19]. Currently, the epigenetics, a study of heritable phenotype changes that are not associated with DNA sequence alterations [64], is considered as the probable cause of the large variability found among 17 *C. sativus* accessions from different geographic origins, grown under open field conditions [65].

5. Conclusions

PCA analysis based on phenological, morpho-productive, and qualitative traits showed a clear separation among ecotypes, in which Sardinia and Spain showed more similarities than Abruzzo and Kozani.

From phenological, quantitative and qualitative points of view, the Sardinia ecotype was early flowering, produced more flowers, and obtained a spice with high coloring, bittering, and aromatic powers. Morphologically, the Abruzzo exhibited the highest flower traits, fresh and dry leaf weight, leaf area, and daughter corm weight. Flower morphological traits are useful for a discrimination of the *C. sativus* ecotype even if they resulted more susceptible to phenotypic plasticity and variable during the two experimental years compared to the agronomic and qualitative ones.

Although wide phenotypic differences among *C. sativus* ecotypes were observed, no genetic variability was found. The molecular markers used in this study could not discriminate between the selected corm geographical origins. Further studies are needed to find possible genetic differences using other types of molecular markers and evaluating genes involved in controlling *C. sativus* flowering.

C. sativus ecotypes constitute a precious source of biodiversity and secondary metabolites with healthy benefits, and their enhancement is a fundamental prerequisite for a sustainable development strategy and agricultural diversification opportunity for growers. The saffron production is arousing particular interest for the added value due to the low use of chemical fertilizers, optimum use of water resources, and valorization of local economy in poorer rural communities.

Author Contributions: Conceptualization, L.C. and V.C.; methodology and investigation, L.C., D.C., N.C., R.V.M., B.R.-M., S.G.N. and V.C.; data curation, L.C., D.C., N.C., R.V.M., B.R.-M., S.G.N. and V.C.; writing—original draft preparation and writing—review and editing, L.C., D.C., N.C., R.V.M., B.R.-M., S.G.N. and V.C.; supervision, V.C., M.P. and R.V.M. All authors have read and agreed to the published version of the manuscript.

References

1. Bagur, M.J.; Salinas, G.L.A.; Jiménez-Monreal, A.M.; Chaouqi, S.; Llorens, S.; Martínez-Tomé, M.; Alonso, G.L. Saffron: An old medicinal plant and a potential novel functional food. *Molecules* **2018**, *23*, 30. [CrossRef]
2. Mousavi, S.Z.; Bathaie, S.Z. Historical uses of saffron: Identifying potential new avenues for modern research. *Avicenna J. Phytomed.* **2011**, *1*, 57–66.
3. Nemati, Z.; Harpke, D.; Gemicioglu, A.; Kerndorff, H.; Blattner, F.R. Saffron (*Crocus sativus*) is an autotriploid that evolved in Attica (Greece) from wild *Crocus cartwrightianus*. *Mol. Phylogenet. Evol.* **2019**, *136*, 14–20. [CrossRef]
4. Cardone, L.; Castronuovo, D.; Perniola, M.; Cicco, N.; Candido, V. Saffron (*Crocus sativus* L.), the king of spices: An overview. *Sci. Hortic.* **2020**, *272*, 109560. [CrossRef]
5. De-Los-Mozos-Pascual, M.; Fernández, J.A.; Roldán, M. Preserving biodiversity in saffron: The Crocusbank project and the world saffron and Crocus collection. *Acta Hortic.* **2010**, *850*, 23–28. [CrossRef]
6. Fernandez, J.A. Genetic resources of saffron and allies (*Crocus* spp.). *Acta Hortic.* **2007**, *739*, 167–185. [CrossRef]
7. Husaini, A.M. Challenges of climate change: Omics-based biology of saffron plants and organic agricultural biotechnology for sustainable saffron production. *GM Crops Food* **2014**, *5*, 97–105. [CrossRef]
8. Mitsopoulou, T.; Tsimidou, M.Z. Morphological characteristics of greek saffron stigmas from kozani region. *Acta Hortic.* **2004**, *650*, 189–193. [CrossRef]
9. Fernández, J.A.; Santana, O.; Guardiola, J.L.; Molina, R.V.; Heslop-Harrison, P.; Borbely, G.; Branca, F.; Argento, S.; Maloupa, E.; Talou, T.; et al. The world saffron and Crocus collection: Strategies for establishment, management, characterisation and utilisation. *Genet. Resour. Crop. Evol.* **2011**, *58*, 125–137. [CrossRef]
10. CROCUSBANK: Genetic Resources of Saffron and Allies. Available online: http://www.crocusbank.org (accessed on 18 November 2020).
11. Fallahi, H.R.; Aghhavani-Shajari, M.; Sahabi, H.; Behdani, M.A.; Sayyari-Zohan, M.H.; Vatandoost, S. Influence of some pre and post-harvest practices on quality of saffron stigmata. *Sci. Hortic.* **2020**, *278*, 109846. [CrossRef]
12. Baghalian, K.; Sheshtamand, M.S.; Jamshidi, A.H. Genetic variation and heritability of agro-morphological and phytochemical traits in Iranian saffron (*Crocus sativus* L.) populations. *Ind. Crops Prod.* **2010**, *31*, 401–406. [CrossRef]
13. Amirnia, R.; Bayat, M.; Gholamian, A. Influence of corm provenance and sowing dates on stigma yield and yield components in saffron (*Crocus sativus* L.). *Turkish J. Field Crops* **2013**, *18*, 198–204.
14. Cardone, L.; Castonuovo, D.; Perniola, M.; Cicco, N.; Candido, V. Evaluation of corm origin and climatic conditions on saffron (*Crocus sativus* L.) yield and quality. *J. Sci. Food Agric.* **2019**, *99*, 5858–5869. [CrossRef] [PubMed]
15. Soukrat, S.; Metougui, M.L.; Gabone, F.; Nehvi, F.; Abousalim, S.; Benlahabib, O. Study of diversity in some Moroccan population of saffron (*Crocus sativus* L.). *Afr. J. Agric. Res.* **2019**, *14*, 759–769.
16. Mondini, L.; Noorani, A.; Pagnotta, M.A. Assessing plant genetic diversity by molecular tools. *Diversity* **2009**, *1*, 19–35. [CrossRef]
17. Siracusa, L.; Gresta, F.; Avola, G.; Albertini, E.; Raggi, L.; Marconi, G.; Lombardo, G.M.; Ruberto, G. Agronomic, chemical and genetic variability of saffron (*Crocus sativus* L.) of different origin by LC-UV-vis-DAD and AFLP analyses. *Genet. Resour. Crop. Evol.* **2013**, *60*, 711–721. [CrossRef]
18. Torricelli, R.; Javan, I.Y.; Albertini, E.; Venanzoni, R.; Hosseinzadeh, Y.G. Morphological and molecular characterization of Italian, Iranian and Spanish saffron (*Crocus sativus* L.) accessions. *Appl. Ecol. Environ. Res.* **2019**, *17*, 1875–1887. [CrossRef]
19. Grilli-Caiola, M.; Caputo, P.; Zanier, R. RAPD analysis in *Crocus sativus* L. accensions and related *Crocus* species. *Bio. Plant.* **2004**, *48*, 375–380. [CrossRef]
20. Babaei, S.; Talebia, M.; Bahara, M.; Zeinali, H. Analysis of genetic diversity among saffron (*Crocus sativus*) accession from different regions of Iran as revealed by SRAP markers. *Sci. Hortic.* **2014**, *171*, 27–31. [CrossRef]
21. Rubio-Moraga, A.; Castillo-López, R.; Gómez-Gómez, L.; Ahrazem, O. Saffron is a monomorphic species as revealed by RAPD, ISSR and microsatellite analyses. *BMC Res. Notes* **2009**, *2*, 189–193. [CrossRef] [PubMed]
22. Nemati, Z.; Zeinalabedini, M.; Mardi, M.; Pirseyediand, S.M.; Marashi, S.H.; Khayam Nekoui, S.M. Isolation and characterization of a first set of polymorphic microsatellite markers in saffron, *Crocus sativus* (Iridaceae). *Am. J. Bot.* **2012**, *99*, e340–e343. [CrossRef] [PubMed]

23. Fluch, S.; Hohl, K.; Stierschneider, M.; Kopecky, D.; Kaar, B. *Crocus sativus* L.—Molecular evidence on its clonal origin. *Acta Hortic.* **2010**, *850*, 41–46. [CrossRef]

24. Keify, F.; Beiki, A.H. Exploitation of random amplified polymorphic DNA (RAPD) and sequence-related amplified polymorphism (SRAP) markers for genetic diversity of saffron collection. *J. Med. Plants Res.* **2012**, *6*, 2761–2768.

25. Anabat, M.M.; Riahi, H.; Sheidai, M.; Koohdar, F. Population genetic study and barcoding in Iran saffron (*Crocus sativus* L.). *Ind. Crops Prod.* **2020**, *143*, 111915. [CrossRef]

26. Mir, M.A.; Mansoor, S.; Sugapriya, M.; Alyemeni, M.N.; Wijaya, L.; Ahmad, P. Deciphering genetic diversity analysis of saffron (*Crocus sativus* L.) using RAPD and ISSR markers. *Saudi J. Biol. Sci.* **2020**, *28*, 1308–1317. [CrossRef]

27. Zalacain, A.; Ordoudi, S.A.; Díaz-Plaza, E.M.; Carmona, M.; Blázquez, I.; Tsimidou, M.Z.; Alonso, G.L. Near-infrared spectroscopy in saffron quality control: Determination of chemical composition and geographical origin. *J. Agric. Food Chem.* **2005**, *53*, 9337–9341. [CrossRef]

28. Anastasaki, E.; Kanakis, C.; Pappas, C.; Maggi, L.; del Campo, C.P.; Carmona, M.; Polissiou, M.G. Differentiation of saffron from four countries by mid-infrared spectroscopy and multivariate analysis. *Eur. Food Res. Technol.* **2010**, *230*, 571–577. [CrossRef]

29. Anastasaki, E.; Kanakis, C.; Pappas, C.; Maggi, L.; Del Campo, C.P.; Carmona, M.; Alonso, G.L.; Polissiou, M.G. Geographical differentiation of saffron by GC–MS/FID and chemometrics. *Eur. Food Res. Technol.* **2009**, *229*, 899–905. [CrossRef]

30. Carmona, M.; Martinez, J.; Zalacain, A.; Rodriguez-Mendez, M.L.; de Saja, J.A.; Alonso, G.L. Analysis of saffron volatile fraction by TD-GC-MS and e-nose. *Eur. Food Res. Technol.* **2006**, *223*, 96–101. [CrossRef]

31. Ghanbari, J.; Khajoei-Nejad, G.; van Ruth, S.M. Effect of saffron (*Crocus sativus* L.) corm provenance on its agro-morphological traits and bioactive compounds. *Sci. Hortic.* **2019**, *256*, 108605. [CrossRef]

32. D'Archivio, A.A.; Di Pietro, L.; Maggi, M.A.; Rossi, L. Optimization using chemometrics of HS-SPME/GC-MS profiling of saffron aroma and identification of geographical volatile markers. *Eur. Food Res. Technol.* **2018**, *244*, 1605–1613. [CrossRef]

33. Bhandari, H.R.; Bhanu, A.N.; Srivastava, K.; Singh, M.N.; Shreya, H.A. Assessment of genetic diversity in crop plants—An overview. *Adv. Plants Agric. Res.* **2017**, *7*, 00255.

34. Small, E. Saffron (*Crocus sativus*) the eco-friendly spice. *Biodiversity* **2016**, *17*, 162–170. [CrossRef]

35. Khanali, M.; Farahani, S.S.; Shojaei, H.; Elhami, B. Life cycle environmental impacts of saffron production in Iran. *Environ. Sci. Pollut. Res.* **2017**, *24*, 4812–4821. [CrossRef]

36. Abolhassani, L.; Khorramdel, S.; Reed, M.; Saghaian, S. Environmental Economic Analysis of Saffron Production. In *Saffron: Science, Technology and Health*; Koocheki, A., Khajeh-Hosseini, M., Eds.; Woodhead Publishing: Sawston, UK, 2020; pp. 367–390.

37. Beck, H.E.; Zimmermann, N.E.; McVicar, T.R.; Vergopolan, N.; Berg, A.; Wood, E.F. Present and future Köppen-Geiger climate classification maps at 1-km resolution. *Sci. Data* **2018**, *5*, 1–12. [CrossRef]

38. Soil Survery Staff. *Keys to Soil Taxonomy*; United States Department of Agriculture, Soil Conservation Service: Washington, DC, USA, 2010.

39. Ministero per le Politiche Agricole. *Metodi Ufficiali di Analisi Chimica del Suolo*; D.M. del 13/09/99, Gazzetta Ufficiale n. 248 del 21/10/99; Ministero per le Politiche Agricole: Rome, Italy, 1999.

40. Saleem, M.H.; Ali, S.; Rehman, M.; Rana, M.S.; Rizwan, M.; Kamran, M.; Imran, M.; Riaz, M.; Soliman, M.H.; Elkelish, A.; et al. Influence of phosphorus on copper phytoextraction via modulating cellular organelles in two jute (*Corchorus capsularis* L.) varieties grown in a copper mining soil of Hubei Province, China. *Chemosphere* **2020**, *248*, 126032. [CrossRef] [PubMed]

41. Saleem, M.H.; Fahad, S.; Khan, S.U.; Ahmar, S.; Khan, M.H.U.; Rehman, M.; Maqbool, Z.; Liu, L. Morpho-physiological traits, gaseous exchange attributes, and phytoremediation potential of jute (*Corchorus capsularis* L.) grown in different concentrations of copper-contaminated soil. *Ecotoxicol. Environ. Safe.* **2020**, *189*, 109915. [CrossRef]

42. Hameed, A.; Akram, N.A.; Saleem, M.H.; Ashraf, M.; Ahmed, S.; Ali, S.; Alsahli, A.A.; Alyemeni, M.N. Seed Treatment with α-Tocopherol Regulates Growth and Key Physio-Biochemical Attributes in Carrot (*Daucus carota* L.) Plants under Water Limited Regimes. *Agronomy* **2021**, *11*, 469. [CrossRef]

43. Cardone, L.; Castronuovo, D.; Perniola, M.; Scrano, L.; Cicco, N.; Candido, V. The Influence of Soil Physical and Chemical Properties on Saffron (*Crocus sativus* L.) Growth, Yield and Quality. *Agronomy* **2020**, *10*, 1154. [CrossRef]

44. Konica Minolta. 2003. Available online: https://www.konicaminolta.eu/fileadmin/content/eu/MeasuringInstruments/4LearningCentre/CA/PRECISECOLORCOMMUNICATION/pccenglish13.pdf (accessed on 3 December 2020).

45. International Organization for Standardization. *ISO 3632-1. Spices—Saffron (Crocus sativus L.). Part 1: Specification, 2nd ed*; International Organization for Standardization: Geneva, Switzerland, 2011; p. 6.

46. International Organization for Standardization. *ISO 3632-2. Spices—Saffron (Crocus sativus L.). Part 2: Test Methods, 1st ed*; International Organization for Standardization: Geneva, Switzerland, 2010; p. 42.

47. Van de Wouw, M.; Kik, C.; van Hintum, T.; van Treuren, R.; Visser, B. Genetic erosion in crops: Concept, research results and challenges. *Plant Genet. Resour.* **2010**, *8*, 1. [CrossRef]

48. Edreva, A.; Velikova, V.; Tsonev, T.; Dagnon, S.; Gürel, A.; Aktaş, L.; Gesheva, E. Stress-protective role of secondary metabolites: Diversity of functions and mechanisms. *Gen. Appl. Plant Physiol.* **2008**, *34*, 67–78.

49. Molina, R.V.; Valero, M.; Navarro, Y.; Guardiola, J.L.; Garcia-Luis, A. Temperature effects on flower formation in saffron (*Crocus sativus* L.). *Sci. Hortic.* **2005**, *103*, 361–379. [CrossRef]

50. Grimes, S.J.; Phillips, T.D.; Hahn, V.; Capezzone, F.; Graeff-Hönninger, S. Growth, Yield Performance and Quality Parameters of Three Early Flowering Chia (*Salvia hispanica* L.) Genotypes Cultivated in Southwestern Germany. *Agriculture* **2018**, *8*, 154. [CrossRef]

51. Wehner, T.C.; Guner, N. Growth stage, flowering pattern, yield, and harvest date prediction of four types of cucumber tested at 10 planting dates. *Acta Hortic.* **2004**, *637*, 223–230. [CrossRef]

52. Ojaq, S.M.; Mozafari, H.; Jabbari, H.; Sani, B. Evaluation of yield of safflower (*Carthamus tinctorius* L.) genotypes under semi-arid conditions. *Plant Genet. Resour.* **2020**, *18*, 270–277. [CrossRef]

53. Yerima, A.R.I.B.; Achigan-Dako, E.G.; Aissata, M.; Sekloka, E.; Billot, C.; Adje, C.O.; Barnaud, A.; Bakasso, Y. Agromorphological Characterization Revealed Three Phenotypic Groups in a Region-Wide Germplasm of Fonio (*Digitaria exilis* (Kippist) Stapf) from West Africa. *Agronomy* **2020**, *10*, 1653. [CrossRef]

54. Bayat, M.; Amirnia, R.; Tajbakhsh, M.; Ramezani, M. Evaluation of Saffron Ecotypes for Stigma Yield and Yield Components Using Different Maternal Corm Weights. *J. Plant Physiol. Breed.* **2016**, *6*, 53–64.

55. Soheilivand, S.; Agayev, Y.; Shakib, A.M.; Fathi, M.; Rezvani, E. Comparison of diversity in flowering rate of two saffron (*Crocus sativus*) populations of Iran. *Acta Hortic.* **2007**, *739*, 303–307. [CrossRef]

56. Renau-Morata, B.; Nebauer, S.G.; Sánchez, M.; Molina, R.V. Effect of corm size, water stress and cultivation conditions on photosynthesis and biomass partitioning during the vegetative growth of saffron (*Crocus sativus* L.). *Ind. Crop. Prod.* **2012**, *39*, 40–46. [CrossRef]

57. García-Rodríguez, M.V.; López-Córcoles, H.; Alonso, G.L.; Pappas, C.S.; Polissiou, M.G.; Tarantilis, P.A. Comparative evaluation of an ISO 3632 method and an HPLC-DAD method for safranal quantity determination in saffron. *Food Chem.* **2017**, *221*, 838–843. [CrossRef] [PubMed]

58. Masi, E.; Taiti, C.; Heimler, D.; Vignolini, P.; Romani, A.; Mancuso, S. PTR-TOF-MS and HPLC analysis in the characterization of saffron (*Crocus sativus* L.) from Italy and Iran. *Food Chem.* **2016**, *192*, 75–81. [CrossRef] [PubMed]

59. Lage, M.; Cantrell, C.L. Quantification of saffron (*Crocus sativus* L.) metabolites crocins, picrocrocin and safranal for quality determination of the spice grown under different environmental Moroccan conditions. *Sci. Hortic.* **2009**, *121*, 366–373. [CrossRef]

60. Cossignani, L.; Urbani, E.; Simonetti, M.S.; Maurizi, A.; Chiesi, C.; Blasi, F. Characterisation of secondary metabolites in saffron from central Italy (Cascia, Umbria). *Food Chem.* **2014**, *143*, 446–451. [CrossRef] [PubMed]

61. Kanakis, C.D.; Daferera, D.J.; Tarantilis, P.A.; Polissiou, M.G. Qualitative determination of volatile compounds and quantitative evaluation of safranal and 4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (HTCC) in Greek saffron. *J. Agric. Food Chem.* **2004**, *52*, 4515–4521. [CrossRef]

62. Gresta, F.; Avola, G.; Lombardo, G.M.; Siracusa, L.; Ruberto, G. Analysis of flowering, stigmas yield and qualitative traits of saffron (*Crocus sativus* L.) as affected by environmental conditions. *Sci. Hortic.* **2009**, *119*, 320–324. [CrossRef]

63. Grilli Caiola, M.; Di Somma, D.; Lauretti, P. Comparative study of pollen and pistil of *Crocus sativus* L. (Iridaceae) and its allied species. *Ann. Bot.* **2001**, *1*, 73–82.

64. Casadesús, J.; Noyer-Weidner, M. Epigenetics. In *Brenner's Encyclopedia of Genetics*, 2nd ed.; Maloy, S., Hughes, K., Eds.; Academic Press: Cambridge, MA, USA, 2013; pp. 500–503. ISBN 9780080961569. [CrossRef]

65. Busconi, M.; Soffritti, G.; Stagnati, L.; Marocco, A.; Martínez, J.M.; Pascual, M.D.L.M.; Fernandez, J.A. Epigenetic stability in Saffron (*Crocus sativus* L.) accessions during four consecutive years of cultivation and vegetative propagation under open field conditions. *Plant Sci.* **2018**, *277*, 1–10. [CrossRef]