Compendium of Plant Genomes
Series Editor: Chittaranjan Kole

Jyoti Vakhlu
Sheetal Ambardar
Seyed Alireza Salami
Chittaranjan Kole *Editors*

The Saffron Genome

Springer

Compendium of Plant Genomes

Series Editor

Chittaranjan Kole, President, International Climate Resilient Crop Genomics Consortium (ICRCGC), President, International Phytomedomics & Nutriomics Consortium (IPNC) and President, Genome India International (GII), Kolkata, India

Whole-genome sequencing is at the cutting edge of life sciences in the new millennium. Since the first genome sequencing of the model plant *Arabidopsis thaliana* in 2000, whole genomes of about 100 plant species have been sequenced and genome sequences of several other plants are in the pipeline. Research publications on these genome initiatives are scattered on dedicated web sites and in journals with all too brief descriptions. The individual volumes elucidate the background history of the national and international genome initiatives; public and private partners involved; strategies and genomic resources and tools utilized; enumeration on the sequences and their assembly; repetitive sequences; gene annotation and genome duplication. In addition, synteny with other sequences, comparison of gene families and most importantly potential of the genome sequence information for gene pool characterization and genetic improvement of crop plants are described.

Jyoti Vakhlu • Sheetal Ambardar •
Seyed Alireza Salami •
Chittaranjan Kole
Editors

The Saffron Genome

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Editors
Jyoti Vakhlu
School of Biotechnology
University of Jammu
Jammu, India

Sheetal Ambardar
School of Biotechnology
University of Jammu
Jammu, India

Seyed Alireza Salami
Department of Horticultural Sciences
University of Tehran
Keraj, Iran

Chittaranjan Kole
Raja Ramanna Fellow, DAE
ICAR—National Institute for Plant Biotechnology
Government of India
New Delhi, India

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This book series is dedicated to my wife Phullara and our children Sourav and Devleena

Chittaranjan Kole

Preface to the Series

Genome sequencing has emerged as the leading discipline in the plant sciences coinciding with the start of the new century. For much of the twentieth century, plant geneticists were only successful in delineating putative chromosomal location, function, and changes in genes indirectly through the use of a number of "markers" physically linked to them. These included visible or morphological, cytological, protein, and molecular or DNA markers. Among them, the first DNA marker, the RFLPs, introduced a revolutionary change in plant genetics and breeding in the mid-1980s, mainly because of their infinite number and thus potential to cover maximum chromosomal regions, phenotypic neutrality, absence of epistasis, and codominant nature. An array of other hybridization-based markers, PCR-based markers, and markers based on both facilitated construction of genetic linkage maps, mapping of genes controlling simply inherited traits, and even gene clusters (QTLs) controlling polygenic traits in a large number of model and crop plants. During this period, a number of new mapping populations beyond F_2 were utilized and a number of computer programs were developed for map construction, mapping of genes, and for mapping of polygenic clusters or QTLs. Molecular markers were also used in the studies of evolution and phylogenetic relationship, genetic diversity, DNA fingerprinting, and map-based cloning. Markers tightly linked to the genes were used in crop improvement employing the so-called marker-assisted selection. These strategies of molecular genetic mapping and molecular breeding made a spectacular impact during the last one and a half decades of the twentieth century. But still they remained "indirect" approaches for elucidation and utilization of plant genomes since much of the chromosomes remained unknown and the complete chemical depiction of them was yet to be unraveled.

Physical mapping of genomes was the obvious consequence that facilitated the development of the "genomic resources" including BAC and YAC libraries to develop physical maps in some plant genomes. Subsequently, integrated genetic–physical maps were also developed in many plants. This led to the concept of structural genomics. Later on, emphasis was laid on EST and transcriptome analysis to decipher the function of the active gene sequences leading to another concept defined as functional genomics. The advent of techniques of bacteriophage gene and DNA sequencing in the 1970s was extended to facilitate sequencing of these genomic resources in the last decade of the twentieth century.

As expected, sequencing of chromosomal regions would have led to too much data to store, characterize, and utilize with the-then available computer software could handle. But the development of information technology made the life of biologists easier by leading to a swift and sweet marriage of biology and informatics, and a new subject was born—bioinformatics.

Thus, the evolution of the concepts, strategies, and tools of sequencing and bioinformatics reinforced the subject of genomics—structural and functional. Today, genome sequencing has traveled much beyond biology and involves biophysics, biochemistry, and bioinformatics!

Thanks to the efforts of both public and private agencies, genome sequencing strategies are evolving very fast, leading to cheaper, quicker, and automated techniques right from clone-by-clone and whole-genome shotgun approaches to a succession of second-generation sequencing methods. The development of software of different generations facilitated this genome sequencing. At the same time, newer concepts and strategies were emerging to handle sequencing of the complex genomes, particularly the polyploids.

It became a reality to chemically—and so directly—define plant genomes, popularly called whole-genome sequencing or simply genome sequencing.

The history of plant genome sequencing will always cite the sequencing of the genome of the model plant Arabidopsis thaliana in 2000 that was followed by sequencing the genome of the crop and model plant rice in 2002. Since then, the number of sequenced genomes of higher plants has been increasing exponentially, mainly due to the development of cheaper and quicker genomic techniques and, most importantly, the development of collaborative platforms such as national and international consortia involving partners from public and/or private agencies.

As I write this preface for the first volume of the new series "Compendium of Plant Genomes," a net search tells me that complete or nearly complete whole-genome sequencing of 45 crop plants, eight crop and model plants, eight model plants, 15 crop progenitors and relatives, and 3 basal plants is accomplished, the majority of which are in the public domain. This means that we nowadays know many of our model and crop plants chemically, i.e., directly, and we may depict them and utilize them precisely better than ever. Genome sequencing has covered all groups of crop plants. Hence, information on the precise depiction of plant genomes and the scope of their utilization are growing rapidly every day. However, the information is scattered in research articles and review papers in journals and dedicated Web pages of the consortia and databases. There is no compilation of plant genomes and the opportunity of using the information in sequence-assisted breeding or further genomic studies. This is the underlying rationale for starting this book series, with each volume dedicated to a particular plant.

Plant genome science has emerged as an important subject in academia, and the present compendium of plant genomes will be highly useful both to students and teaching faculties. Most importantly, research scientists involved in genomics research will have access to systematic deliberations on the plant genomes of their interest. Elucidation of plant genomes is of interest not only for the geneticists and breeders, but also for practitioners of an array of plant science disciplines, such as taxonomy, evolution, cytology,

physiology, pathology, entomology, nematology, crop production, biochemistry, and obviously bioinformatics. It must be mentioned that information regarding each plant genome is ever-growing. The contents of the volumes of this compendium are, therefore, focusing on the basic aspects of the genomes and their utility. They include information on the academic and/or economic importance of the plants, description of their genomes from a molecular genetic and cytogenetic point of view, and the genomic resources developed. Detailed deliberations focus on the background history of the national and international genome initiatives, public and private partners involved, strategies and genomic resources and tools utilized, enumeration on the sequences and their assembly, repetitive sequences, gene annotation, and genome duplication. In addition, synteny with other sequences, comparison of gene families, and, most importantly, the potential of the genome sequence information for gene pool characterization through genotyping by sequencing (GBS) and genetic improvement of crop plants have been described. As expected, there is a lot of variation of these topics in the volumes based on the information available on the crop, model, or reference plants.

I must confess that as the series editor, it has been a daunting task for me to work on such a huge and broad knowledge base that spans so many diverse plant species. However, pioneering scientists with lifetime experience and expertise on the particular crops did excellent jobs editing the respective volumes. I myself have been a small science worker on plant genomes since the mid-1980s and that provided me the opportunity to personally know several stalwarts of plant genomics from all over the globe. Most, if not all, of the volume editors are my longtime friends and colleagues. It has been highly comfortable and enriching for me to work with them on this book series. To be honest, while working on this series I have been and will remain a student first, a science worker second, and a series editor last. And I must express my gratitude to the volume editors and the chapter authors for providing me the opportunity to work with them on this compendium.

I also wish to mention here my thanks and gratitude to the Springer staff, Dr. Christina Eckey and Dr. Jutta Lindenborn in particular, for all their constant and cordial support right from the inception of the idea.

I always had to set aside additional hours to edit books beside my professional and personal commitments—hours I could and should have given to my wife, Phullara, and our kids, Sourav, and Devleena. I must mention that they not only allowed me the freedom to take away those hours from them but also offered their support in the editing job itself. I am really not sure whether my dedication of this compendium to them will suffice to do justice to their sacrifices for the interest of science and the science community.

Kalyani, India Chittaranjan Kole

Contents

Contributors

Aelaei Mitra Department of Horticulture, Faculty of Agriculture, University of Zanjan, Zanjan, Iran

Ambardar Sheetal Metagenomic Laboratory, School of Biotechnology, University of Jammu, Jammu, Jammu and Kashmir, India;
National Center for Biological Sciences, Bengaluru, India

Anand Rythem School of Biotechnology, University of Jammu, Jammu, India

Bhagat Madhulika School of Biotechnology, University of Jammu, Jammu, India

Bhagat Nancy Metagenomic Laboratory, School of Biotechnology, University of Jammu, Jammu, J&K, India

Chesti M. H. Faculty of Agriculture, SKUAST Kashmir, Wadoora, Sopore, Jammu and Kashmir, India

Fayaz Suheeba Division of Soil Sciences, Faculty of Horticulture, SKUAST Kashmir, Shalimar Campus, Jammu and Kashmir, India

Gupta Vishal Biocontrol Laboratory, Advanced Centre for Horticultural Research, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Udheywalla, India

Haq Syed Anam Ul Genome Engineering and Societal Biotechnology Lab, Division of Plant Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Srinagar, Jammu and Kashmir, India

Husaini Amjad M. Genome Engineering and Societal Biotechnology Lab, Division of Plant Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Srinagar, Jammu and Kashmir, India

Irshad Insha Division of Soil Sciences, Faculty of Horticulture, SKUAST Kashmir, Shalimar Campus, Jammu and Kashmir, India

Karbasi Alireza Department of Agricultural Economics, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

Kirmani Nayar Afaq Division of Soil Sciences, Faculty of Horticulture, SKUAST Kashmir, Shalimar Campus, Jammu and Kashmir, India

Lambarraa-Lehnhardt Fatima Farm Economics and Ecosystem Services Group, Leibniz Centre for Agricultural Landscape Research (ZALF), Müncheberg, Germany

Lmouden Assem United Nations Development Programme (UNDP), Rabat, Morocco

Mansotra Ritika Metagenomic Laboratory, School of Biotechnology, University of Jammu, Jammu, J&K, India

Mir Ikhlaq A. Centre for Climate Change and Mountain Agriculture, Division of Environmental Science, FOH, SKUAST Kashmir, Shalimar Campus, Jammu and Kashmir, India

Mir Waseema Jehaan Division of Soil Sciences, Faculty of Horticulture, SKUAST Kashmir, Shalimar Campus, Jammu and Kashmir, India

Moradi Shirin Department of Horticultural Sciences, Faculty of Agricultural Science and Engineering, University of Tehran, Karaj, Iran

Razdan V. K. Division of Plant Pathology, Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Chatha, India

Salami Seyed Alireza Department of Horticultural Sciences, Faculty of Agricultural Science and Engineering, University of Tehran, Karaj, Iran; Department of Horticultural Science, Faculty of Engineering and Agricultural Science, University of Tehran, Tehran, Iran

Salehi Fahimeh Department of Horticulture, Faculty of Agriculture, University of Zanjan, Zanjan, Iran

Sbabou Laila Center of Research Plants and Microbial Biotechnologies, Biodiversity and Environment, Team of Microbiology and Molecular Biology, Faculty of Sciences, Mohammed V University in Rabat, Rabat, Morocco

Sharma Pooja School of Biotechnology, University of Jammu, Jammu, India

Sharma Satish Kumar Regional Horticulture Research Sub-Station, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Bhaderwah, India

Sofi J. A. Division of Soil Sciences, Faculty of Horticulture, SKUAST Kashmir, Shalimar Campus, Jammu and Kashmir, India

Sowdhamini Ramanathan National Center for Biological Sciences, Bangalore, India

Vakhlu Jyoti Metagenomic Laboratory, School of Biotechnology, University of Jammu, Jammu, Jammu and Kashmir, India

Wani Zahoor Ahmed Department of Botany, Government Degree College Anantnag, Anantnag, Jammu & Kashmir, India

Zandi Dareh Gharibi Bahareh Department of Agricultural Economics, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

Zouagui Rahma Center of Research Plants and Microbial Biotechnologies, Biodiversity and Environment, Team of Microbiology and Molecular Biology, Faculty of Sciences, Mohammed V University in Rabat, Rabat, Morocco

Abbreviations

%	Per cent
˚C	Degree Celsius
ANOVA	Analysis of variance
BC	Backcross
BLAST	Basic local alignment search tool
bp	Base pair
CDS	Coding sequence
CFU	Colony Forming Unit
cp	Chloroplast
CTAB	Cetyl trimethylammonium bromide
DEG	Differentially expressed gene
DNA	Deoxyribose nucleic acid
dNTP's	Deoxynucleotide triphosphate
dsDNA	Double stranded DNA
E.coli	*Escherichia coli*
EC	Enzyme Commission
EDTA	Ethylene diamaine tetra acetic acid
EST	Expressed sequence tag
F1	First filial generation
FACS	Fluorescence-activated cell sorting
GA	Gibberellic acid
GO	Gene ontology
Hcl	Hydrochloric acid
IAA	Indole 3-Acetic Acid
IR	Inverted repeat
ITS	Internal transcribed sequence
KEGG	Kyoto Encyclopedia of Genes and Genome
KO	KEGG orthology
LA	Luria-Bertani agar
LB	Luria Bertani
LINE	Long interspersed nuclear elements
LTR	Long terminal repeats
ME	Mate-pair
MEGA	Molecular evolutionary genetic analysis
MISA	MIcroSAtellite tool
mM	Milimolar
MS	Murashige and Skoog

N50	Minimum contig length
NaOH	Sodium hydroxide
NB(A)	Nutrient broth (Agar)
NCBI	National Center for Biotechnology Information
NF	Not found
NGS	Next generation sequencing
nt	Nucleotide
Orf/ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PE	Pair end/Paired-end
PGPB	Plant Growth Promoting Bacteria
pH	Negative logarithm of hydrogen ion concentration
pmole	Picomole
QC	Quality Check
RAPD	Random amplified polymorphic DNA
RE	Repetitive element
RFLP	Restriction fragment length polymorphism
RNA	Ribose nucleic acid
RNase	Ribonuclease enzyme
RPKM	Reads per kilo base per million
rpm	Rotations per minute
rRNA	Ribosomal RNA
SAM	Sequence alignment map
SINE	Short interspersed nuclear element
SNP	Single nucleotide polymorphism
SRAP	Sequence-related amplification polymorphism
SSAP	Sequence-specific amplification polymorphism
SSR	Simple sequence repeat
STMS	Sequence tagged microsatellite site
STS	Sequence tagged site
TE	Transposable Element
TF	Transcription factor
UV	Ultraviolet
v/v	Volume/Volume
viz.	Namely
w/v	Weight/Volume
ZFN	Zinc finger nuclease
ZIP	Iron-regulated transporter-like

Part I

Introduction

Crocus Sativus Saffron: A 360-Degree Overview

1

Ritika Mansotra and Jyoti Vakhlu

Abstract

Crocus sativus L., an autumnal herbaceous flowering plant, is known for being the most valuable spice in the world. Because of its three main biological active compounds crocin, picrocrocin and safranal, it is highly beneficial to human health. The global demand for the spice is increasing due to its major role in the medicinal, cosmetics, perfumery and textile dye-producing industries. The thorough study of the geophyte is the need of the hour, as its production is declining year after year. The chapter discusses the origin, history, dissemination, production, economic value, physio-morphological traits, cytogenetics, penological stages, climatic adaption, agronomical practices, phytochemistry and adulteration of saffron. Additionally, an overview of the current knowledge of the biotechnological interventions and saffron'omics' is mentioned.

1.1 Introduction

Genus *Crocus* (pl. crocuses) is a flowering plant comprising around 90 species, belonging to the iridaceae family. Among all, *Crocus* is one of the most enthralling and fascinating genus (Mohtashami et al. 2021). The beauties of the geophyte have been acknowledged for more than 4000 years back, and exploited as a tonic agent in traditional medicines, as a spice in food, dye agent, and in cosmetic and perfume preparations. In recent times, saffron is attracting more attention from the consumers mainly because of its advantageous properties for human health, and thereby its demand gets increased year after year (Roshanravan and Ghaffari 2021; Ghaffari and Roshanravan 2019). However, saffron yield has radically decreased due to different reasons, and consequently new investigations have paid attention to the improvement of yield and quality of stigma by applying the appropriate agronomic practices, for instance, use of biostimulants, and fertilizers that would increase the nutrient retention capacity and water availability (Ahmad et al. 2021a; Magotra et al. 2021). In order to safeguard the future of the saffron crop, it is obligatory to understand the complete aspects of this small geophyte. Although enormous literature reviews are available on different facets of saffron, such as distribution and production, chemical composition, reproductive biology, cultivation, harvesting and traditional as well as modern uses (Gupta

JV and RM, together, coordinated the study, and drafted the manuscript.

R. Mansotra · J. Vakhlu (✉)
Metagenomic Laboratory, School of Biotechnology, University of Jammu, Jammu, India
e-mail: jyotivakhlu@jammuuniversity.ac.in

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et al. 2021; Kothari et al. 2021b; Cardone et al. 2020). However, this chapter aims to summarize most of the recent studies documented for various aspects of saffron.

1.2 Origin and History of Saffron

There is uncertainty in the knowledge of the origin and domestication of saffron. However, various authors differ in their opinion about the site of the first appearance of saffron plants. Vavilov (1951), designated its origin in the Middle East whereas other authors propose Asia Minor or the islands of southwest Greece as its possible area of origin. From these preliminary regions, it was probably spread to India, China and the Middle Eastern countries; then the Arabs escalated its spread throughout the Mediterranean basin that covers a portion of Europe, Africa and Asia (Mohtashami et al. 2021; Schmidt et al. 2019). It was believed that the Romans brought saffron into Great Britain, whereas the Arabs introduced saffron to Spain (Cardone et al. 2020). Some of the archeological and detailed historical studies document that the domestication of saffron dates back to 1600–1700 BC (José Bagur et al. 2018) and, it was also evidenced by frescoes in the Minoan Palace of Knossos, Crete, that represent maiden plucking saffron flowers (Cardone et al. 2020). Nonetheless, various contributions in research (Negbi 1999) also approves that the process of domestication of saffron has been started during the late Bronze Age in Crete. According to Negbi (1999), the wild ancestor of domesticated *C. sativus* was *Crocus cartwrightianus* Herb. However, *Crocus thomasii* Ten., or *Crocus pallasii* Herb, also has been considered as probable saffron ancestor. However, detailed information on saffron ancestors is still in ambiguity (Schmidt et al. 2019).

Owing to its history of great antiquity, authors, mainly Greeks and Romans, from ancient times mentioned saffron as divine in their texts and Old Testament, such as Virgil (Georgian, IV 182), Homer (Iliad, Book IX and XII), Ovid (Metamorphosis) and Hippocrates and Pliny (Naturalis Historia, XXI, VI). The first knowledge and use of saffron for the treatment of dyspnea, menstruation, head and painful urination in Egypt date back from 3100 B.C. to 476 A.D., which was revealed by the historical document 'Papyrus Ebers' (Cardone et al. 2020; José Bagur et al. 2018). However, in Iran, the first cultivation of saffron was reported during the Kingdom of Media, 708–550 B.C. in the Zagros and Alvand mountains (Bahrami et al. 2020). Likewise, in ancient Rome (753 B.C. to 364 A.D.) saffron was documented on its use in perfume, temples and in medicinal preparation. Saffron was later spread to Europe and Italy in the tenth century and fourteenth century A.D., respectively. Dominican Friar Santucci of Navelli introduced saffron in Italy, who actually stole it from Spain and, thereby planted it in Abruzzo (L'Aquila) (Cardone et al. 2020). There are no proper historical records available that estimate saffron cultivation in India, particularly Kashmir; however Wan Zhen, the medical Chinese writer, reported its presence in India in the third century A.D. Many experts also suppose that saffron propagation in Kashmir originated from Persia (Bahrami et al. 2020; Cardone et al. 2020). It was established by Persian rulers to gardens and parks of Kashmir that were newly built by them. According to one of the theorie, it was considered that in prehistoric times when Persia conquered Kashmir, Persian corms of saffron were sowed in the soil of Kashmir around 500 B.C. (Bahrami et al. 2020).

1.3 Dissemination, Production and Economic Value

For a long period of time, saffron has been majorly cultivated in Iran, and it was the main trader of saffron to other countries (Kothari et al. 2021b). However, with the exploration of its distinctive properties over centuries, its cultivation in other parts of the world increased intensively. Currently, it is largely cultivated in Iran, India, Afghanistan, Greece, Morocco, Spain, China, Italy, Turkey, France, Pakistan, Switzerland, Israel, Azerbaijan, the United Arab Emirates, Japan and Egypt (Mohammadi and Reed 2020; Shahnoushi et al. 2020); and attempts have

been implemented to introduce saffron cultivation in non-traditional countries also such as Australia, New Zealand, Chile, the USA and Argentina (Kothari et al. 2021a). The largest producer of saffron is Iran which accounts for 90% of the worldwide production, 418 t/year (Cardone et al. 2020; Mohammadi and Reed 2020). The main regions of Iran under saffron cultivation are Khorasan Razavi (84,738 ha), Southern Khorasan (15,754 ha), Yazd, Fars, Isfahan, Kerman and Northern Khorasan (5260 ha) as well as other small-scale cultivating provinces (2248 ha) (Kothari et al. 2021b; Cardone et al. 2020). Moreover, production of saffron in Iran has tremendously increased which can be concluded from the data of 2007 to 2017, i.e. from 59,000 ha and 230 t to 108,000 ha and 376 t respectively with an average possible yield of 3.53 kg/ha (Koocheki et al. 2019). Heart province of Afghanistan has a total area of 7557 ha under cultivation which is then followed by 3674 ha in the Jammu and Kashmir region of India (Ganaie and Singh 2019; Kumar and Sharma 2018), and Krokos Kozani, Ano Komi, Kato Komi, Karyditsa, Agia Paraskevi, Pefkopigi and Petrana areas of Greece with 1000 ha. Morocco wherein the major saffron cultivating area is Taliouine (730 ha) and minor is Taznekht (120 ha) is contributing a total of 850 ha. Spain has 150 ha under cultivation; Italy has 70 ha followed by France with 37 ha. In addition, Castilla-La Mancha, Toledo, Albacete, Cuenca and Ciudad Real are major cultivating areas of Spain, whereas in Italy, it is more widespread in Sardinia, Abruzzo and Sicily and, small-scale cultivation occurs in Umbria, Tuscany as well as other regions of South Italy (Cardone et al. 2020).

According to the various reports from European countries, production of saffron has been severely decreased during particular years in countries like Spain, Greece and Italy. Spain has faced a drastic drop in 1971 from 6000 to 150 ha, whereas in 2006, 116 ha were cultivated for saffron, which then reached 165 ha in 2016. In 2016, the average yields of saffron for irrigated lands and dry lands in Spain were 14 and 9 kg ha^{-1}, respectively. In Greece, it was reduced from 1600 to 860 ha in 1982; however, saffron cultivation was tremendously increased between 2010 and 2017 in Greece. Italy reported a tremendous loss in 1910 from 300 to 6 ha (Cardone et al. 2020). Additionally, in Asian countries like India, a rapid decline in the production was observed from 1997 to 2015, i.e. from 5707 to 3674 ha with an annual production loss of 15.95 to 9.6 t, respectively (Ganaie and Singh 2019). Notwithstanding, yield per unit area in Iran has also significantly fallen in the time period 1982–2017 from 5.1 to 3.5 kg/ha (Shahnoushi et al. 2020).

Globally, top saffron exporting countries in 2019 are Iran, 44.5%; Spain, 21.8%; Afghanistan, 12.6%; China, 2.5% followed by Hong Kong, 2.4%; Greece, 2.08%; France, 1.84% and Portugal, 1.52% whereas topmost saffron importing countries are Spain, 14.9%; Hong Kong, 14.7%; Saudi Arabia, 8.65%; the United Arab Emirates, 8.12%; India, 8.01%; the United States, 7.11%; Italy, 4.66% followed by France, 3.7%; Sweden, 3.13%; China, 1.75% (%; out of total trade of $229 M) (https://oec.world/) (Table 1.1a and b). On the whole, the global import of saffron has gradually increased between the years 2012 and 2016, at an annual rate of 7% (Cardone et al. 2020). India stands as the fifth-largest saffron importer in 2019; however, Afghanistan and Hong Kong (China) turn out to be large as well as fast exporters of saffron in the time period 2012–2016. Between 2018 and 2019, the exports of saffron decreased by −41%, from $388 to $229 M (https://oec.world/).

1.4 Physio-Morphological Traits

Saffron, *C. sativus*, is a herbaceous perennial geophyte that reaches a height of 10–25 cm from the upper surface of the ground. It is a low-growing plant that develops from its underground stem, i.e. corms (Magotra et al. 2021). Thereby, the plant is also considered as subhysteranthous, as the flowers directly arise from corms before or after the appearance of leaves (Fig. 1.1). However, this particular trend proves as a good strategy to conquer the effect of periodic drought. This could also help in mechanized harvesting of flowers in such a manner that it

Table 1.1 **a** Percentage of world's leading saffron exporting countries in the year 2019 (%; out of total trade of $229 M); available from https://oec.world/. **b** Percentage of world's leading saffron importing countries in the year 2019 (%; out of total trade of $229 M); available from https://oec.world/

Country	Export value (M)	Percentage (%)
a		
Iran	$102	44.5
Spain	$49.8	21.8
Afghanistan	$28.9	12.6
China	$5.73	2.5
Hong Kong	$5.48	2.4
Greece	$4.77	2.08
France	$4.22	1.84
Portugal	$3.47	1.52
b		
Country	Import value (M)	Percentage (%)
Spain	$34.1	14.9
Hong Kong	$33.6	14.7
Saudi Arabia	$19.8	8.65
The United Arab Emirates	$18.6	8.12
India	$18.3	8.01
The United States	$16.3	7.11
Italy	$10.7	4.66
France	$8.47	3.7
Sweden	$7.17	3.13
China	$4	1.75

only incises the flowers without upsetting the leaves (Koocheki and Seyyedi 2019). Corm is a massive starch structure that is generally 0.5–6.5 cm in diameter (horizontally) (Cardone et al. 2020), and is wrapped up by a reticulated fibrous sheath, known as tunics (Fig. 1.1). It consists of parenchyma cells with a circular basal node through which roots arise. The corm is of variable shape and weight, such as ovoid or subglobose depressed to flattened and from 0.5 to 50 g, respectively (Cardone et al. 2020; Koocheki and Seyyedi 2020). The tuberous-bulb, i.e. corm, of saffron is similar to the gladiolus corm. The minimum size of corm that is able to flower is 2.5 cm in diameter and above 8 g in weight (Serghini et al. 2016). However, in the apex position, an average size corm of 3–3.5 cm produces 1–2 apical buds and 4–7 secondary buds that are unevenly arranged in a spiral form. Apical buds eventually give rise to new leaves, floral axis and 1–3 large daughter corms, whereas lateral buds (secondary buds) produce several small daughter corms that too depend on the size of the mother corm. Additionally, a cauline axis and tuft of leaves are produced from the secondary buds which thereby help in the drawing of nutrients through photosynthesis. Many studies have reported the composition of corm, elucidated as glucose, glutamic and aspartic acids, glycine, threonine, cysteine, serine, tyrosine, histidine, alanine, arginine, lysine, proline, leucine, valine, phenylalanine, methionine and two saponins (one triterpenic and another steroidic) along with high molecular weight protein (Cardone et al. 2020; Koocheki and Seyyedi 2020, 2019; Koocheki et al. 2019).

The root system of saffron comprises two major types: fibrous and contractile roots (Fig. 1.1). They are adventitious in nature and grow from the bottom of the corm; however, they differ in structure as well as function. The fibrous roots are absorbing roots that are formed from the

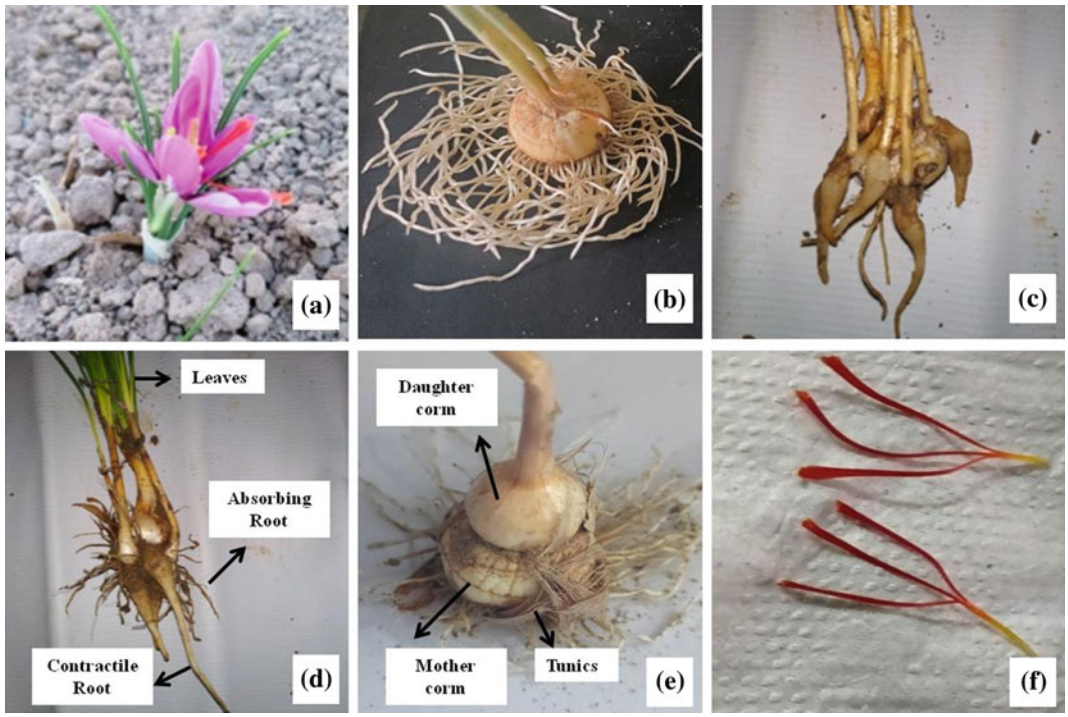

Fig. 1.1 *Crocus sativus* plant morphology **a** Saffron flower **b** Absorbing Roots **c** Contractile Roots **d** Saffron plant **e** Corm types and **f** Saffron stigma

base of the mother corm and appear as the shoots grow. These roots are thin (1 mm), straight and 15–20 cm long and play a major role in the absorption of water and nutrients. In contrast, the second type called the contractile roots emerge from the base of lateral buds. The latter are thick, large, and whitish in color and have the tuber organ-like appearance. Their major function is pulling the corm deeper inside the soil by pulling and pushing activity, so that corm would be positioned at optimum depth inside the ground. These roots are generally produced in response to variable soil conditions such as temperature and light and hence, phenolic compounds, particularly p-coumaric acid, have a major positive effect on their contraction, formation and growth (Koocheki and Seyyedi 2020; Rashed-Mohassel 2020).

Saffron leaves are grass-like slender, linear, long and channeled, with curved margins and a pointed apex. They are dark green in color having dimensions of around 2–3 cm broad and 40–70 cm long. On the upper side, they have a white central strip and two lateral strips that are even more intensely colored. The emergence of leaves overlaps with a flowering or may occur shortly after it. However, photosynthetic activity conducted by saffron leaves is essential for the multiplication of daughter corms. Each corm may produce 6–15 leaves, though the number is entirely dependent on the weight, horizontal diameter of the corm as well as on vitality of buds (Cardone et al. 2020).

Each corm of saffron produces 1–3 flowers that begin to flourish in the early month of autumn, i.e. toward the end of September, and can proceed up to early winter, i.e. beginning of November. The flower is usually composed of a large perianth that is slender funnel-shaped and tube-like in appearance, having 6 violet color tepals with darker veins (three are external whereas three are internal, with dimensions 20 and 47 mm long and 11 and 23 mm in width) (Mohtashami et al. 2021). Initially, the saffron flowers are protected by whitish membranous

bracts. The gynoecium or pistil part of the flower is composed of an inferior ovary that extends into a yellow-green slender stylus (9–10 cm long), which crosses the perigonium tube and, therefore culminated into 3 dark red filaments, 30–40 mm long, drooping over of the perianth segments. These filaments are named stigma, a commercial part of saffron whose length exceeds the anthers as well as of tepals. The ovary is egg-shaped, 3-celled and is hidden in between the leaf's base. Three stamens (androecium), about 15 mm long, consist of filaments (short and free) and anthers (long, basifixed and yellow in color) attached to the throat of the perianth. Each fresh flower weighs 300–500 mg approximately with fresh and dry stigmas weight around 25–47 mg and 6–7 mg, respectively, therefore around 160,000–110,000 saffron flowers are needed to get 1 kg of spice (Rashed-Mohassel 2020).

1.5 Cytogenetics and Penological Stages of Saffron

Cytological studies unraveled that saffron is triploid, i.e. $2n = 3x = 24$; $x = 8$. It causes an atypical chromosomal pairing at the prophase stage of meiosis and asymmetrical allocation of chromosomes at metaphase, thereby resulting in many anomalies in the sporogenesis process and gametophyte development. Furthermore, owing to its triploidy, saffron is considered as self-sterile and mostly male-sterile, because it produces a low number of viable pollen grains that allows it to propagate vegetatively via corms, not by regular means of sexual reproduction. When in in vitro conditions, the ovary of *C. sativus* is cross-fertilized with pollens of *C. cartwrightianus* and *C. thomasii* Ten (a self-incompatible species), which resulted in the production of capsules with viable seeds. Additionally, *Crocus hadriaticus* is also able to fertilize *C. sativus*. However, other species of *Crocus* are not suitable for pollination with the pollen of *C. sativus*, as they do not produce any seeds. Although the origin of saffron polyploidy is obscure, many authors have hypothesized about it (IA et al. 2021; Shokrpour 2019). The more probable origin of autotriploidy of saffron may be from wild *Crocus* by the fertilization of diploid unreduced egg cell with haploid sperm cell or two haploid sperms with a haploid egg cell, or by allopolyploid through the hybridization of *C. hadriaticus* and *C. cartwrightianus*. On the contrary, there is less probability of saffron origin by a cross between diploids and tetraploids because there is no occurrence of tetraploids among allies of *C. sativus*. In addition, Nemati et al. (2019) reported that it is evolved in Attica (Greece) by the two different genotypes combination of *C. cartwrightianus*.

Moreover, investigating the saffron samples from different countries, that showed appropriate morphological differences, resulted in no notable genetic differences. Therefore, the most recognized karyotype of saffron consists of 8 triplets detailed as subacrocentric (1, 2); metacentric (3, 4, and 8); submetacentric (6, 7) and, on the other hand, triplet 5 has two subtypes such as metacentric 5(1) and subacrocentric and smaller 5(2, 3) (IA et al. 2021).

Phenological studies of the saffron plant depict it as a summer dormant and winter active perennial crop (Behdani et al. 2016). The biological life cycle of the saffron plant in its main cultivating areas exhibits similar stages, i.e. from flowering and above-ground vegetative growth in autumn to the production of replacement corms in summer. Based on the observation of field records; there are six phenological growth stages of saffron described below.

1. Dormant stage: It generally begins from late May till late October, and is divided into two phases (primary and secondary dormancy). However, the real dormant period continues up to the first fortnight of July during which corms seem to show neither morphological changes nor any external growth, though internal physiological as well as morphogenetic changes do exist. In the secondary dormancy phase (as known as pseudo-dormancy phase), initial leaf genesis that

usually starts from mid-July to early-August and, and flower initiation as well as differentiation occur which start from 1 to 15 August may continue up to the month of October.

2. Flowering stage: It marks its presence from the second fortnight of October to the second fortnight of November. During this period, flower emergences previously to or coincides with the leaf appearance depending upon the environmental conditions, as saffron is a hysteranthous plant. The growth and development of the root system also take place along with the emergence of flowers and leaves.
3. The third stage starts from late November to late December. Throughout this phase of vegetative growth, roots and leaves continue to develop, however, the base of shoots starts to swell, which results in the formation of replacement corms on the buds of the mother corm, due to the photosynthetic activity of leaves.
4. Mid-level growth of daughter corms (late December until late January). During winters, reserves of mother corm start to deplete for the growth of replacement corms.
5. From late January to late March, there is a reduction in root system absorption capacity, though daughter corms reach their final growth.
6. During the final stage of growth (from late March to late May), mother corm roots cease and break away because of the increase in temperature that resulted in a decrease in soil moisture. Photoassimilates progress toward the corms from the leaves; thereby leaves begin to senescence from the apex to the base. Daughter corms are fully developed and ready for latency. This stage initiates the period of dormancy (Rashed-Mohassel 2020; Behdani et al. 2016).

On the whole, these phenological stages can be affected by the change in environmental conditions or agronomic practices.

1.6 Climatic Adaption

Temperature and rainfall are the key climatic factors that affect the timing of phenological stages of saffron, mainly, flower initiation and emergence (Wang et al. 2021; Dastranj et al. 2019; Bidad 2016). Saffron crop follows a constant temperature regime, i.e. 25–27 °C in the summer requires for the initiation of the flowering, whereas 15–17 °C in the winter is required for flower emergence. So, the optimum temperature required for flower initiation should be higher than for flower emergence. According to various literature studies, there is a positive correlation between the early emergence of flowering and low temperature (Chourak et al. 2021). However, an unusually low temperature during the short period of flower emergence would adversely affect flower development. It has been reported that incubating conditions of 30 °C for 120 days or 25 °C for 150 days resulted in the inhibition of bud growth as well as flower formation. In addition, there is complete flower abortion at incubating conditions of 25 and 30 °C for 180 days (Wang et al. 2021). According to the recent study by Wang et al. (2021), the full flowering of saffron can be promoted by firstly incubating corms at a slightly high temperature (30 °C) for approximate 2–3 weeks, then corms are needed to be transferred to the ambient temperature of about 25 °C for around 1–2 months and, at last bringing them to a lower temperature of 15–20 °C in early October. Thus, the flowering of saffron follows a pattern of warm-intermediate and cool temperatures.

Saffron is entirely a strategic rain-fed crop; therefore, rainfall in autumn (majorly September) is crucial for the flowering of saffron, as it will meet the supply of water requirement during the period of flower emergence (Dastranj et al. 2019). Although saffron's water requirement is low, in case of decreased or late rainfall, water stress ultimately reduces the yield, growth as well as the development of crop (Cardone et al. 2020). Various studies indicate that if the rainfall

event occurs when the temperature is higher than 15–17 °C (optimum range for flower emergence), the vegetative growth will coincide with a flowering period, hence would interfere with the harvesting practices of saffron. This may ultimately increase the overall cost of saffron along with a reduction in yield. It is thereby concluded that first rainfall timing is crucial, as rainfall in summer has a negative effect on saffron yield.

Kashmir growing saffron areas receive 100–150 mm annual (optimum requirement) rainfall with snow in winter, whereas Spain reported annual rainfall of around 400 mm in dry temperate conditions. In Greece, 500 mm annual rainfall is accounted for the good flower yield (Kumar et al. 2008).

1.7 Agronomical Practices

1.7.1 Major Planting Parameters

Generally, the soil type, planting time, depth of planting, pattern and density are considered to play a crucial role in the flowering and growth of daughter corms. The preferable type of soil for the growth of saffron is supposed to be loose, well-drained clayey calcareous soil with high organic content and slightly alkaline (Kothari et al. 2021b; Cardone et al. 2020; Koocheki and Seyyedi 2020). It can be justified by explaining the soil type of different saffron growing areas, for instance, saffron growing soil of Kashmir, India, has markedly higher alkalinity as compared to its adjacent areas. Additionally, the medium humus-clayey or sandy soil in the Abruzzo (Navelli, Italy) has good limestone content, high organic matter and low phosphates, whereas in Sardinia, soil with alluvial deposits and uniform sandy clay-like texture has been reported. In Spain, corms are planted in friable soil that is lightly calciferous; however, Greece's soil texture is known for its sandy clay loam to clay loam, with a high content of calcium carbonate and pH of 7.4. Nonetheless, Moroccan fairly loose saffron soil is also considered as sandy or calcareous clay soil. It is believed that all these soil factors are meant to prevent it from corm rot because corm rot generally takes place in water-logged and humid soil (Cardone et al. 2020).

Several researchers have also conducted the soilless cultivation of saffron, but results have been in contradiction with each other. A few studies that investigated the development of saffron in culture systems such as aeroponics and hydroponics have reported the reduction of root length yet no considerable differences regarding the saffron yield and crocin as well as crocetin concentration. Even though these results predicted that cultivation of saffron under soilless controlled conditions could lead to an alternative method of outdoor farming, in order to conquer the negative impact of climate change such as increased temperature and the effect of pests and pathogens. It might be useful in accelerating the spice yield and quality too (Fallahi et al. 2020; Salas et al. 2020; Caser et al. 2019). In contrast, some studies have accounted for the negative consequences of soilless cultivation in saffron (Cardone et al. 2020).

An appropriate planting time of saffron corm is an important parameter; however, it usually varies from area to area depending upon the climatic conditions. The planting of saffron corm is performed in the beginning or middle of its dormancy period in order to induce flower formation (Kothari et al. 2021b; Koocheki and Seyyedi 2020; Shokrpour 2019). Several researchers demonstrated the middle of July as the best time period for planting because during this period corms are in the middle of true dormancy. However, in Italy corm planting is frequently executed in the second fortnight of August, whereas in Spain it is 15–30 June, in Greece in between the September month, and in India from mid-July to August. Moreover, various reports on the corms planting in Iran suggest mid-May to early June as the appropriate time for the displacement as well as the planting of corms on new farms (Cardone et al. 2020). It is concluded that during the period of true mid dormancy, corm growth is reduced by inhibitor hormones, which thereby help farmers to dig out the corms for future re-plantation. After this period, tissue differentiation occurs that makes

the corm more sensitive to translocation, though waiting for the termination of the dormancy period would also lead to a reduction in flower induction and yield (Kothari et al. 2021b; Cardone et al. 2020; Koocheki and Seyyedi 2020).

Planting depth depends on the weight of the mother corm; however, the recommended depth is around 12–15 cm for 8.1–12 g corms. It is proposed that deeper planting would cause a significant reduction in the flowering, particularly from buds that are located at the base of corms (Kothari et al. 2021b; Koocheki and Seyyedi 2020). In addition, the number of daughter corms that are usually produced from lateral buds of mother corms would be reduced. On the contrary, surface planting may cause serious injury to planted corms because of cold and heat stress in winter and summer, respectively (Gerdakaneh et al. 2017).

The pattern of plantation is the key factor that keeps a check on the density of saffron corms and spacing, which is thereby crucial for the appropriate flower yield (Sharifi et al. 2021). The row planting method is advocated for the ease in irrigation and hoeing; preferably, the beds are raised to 15–20 cm height with 1.2–1.5 m width, and in between the beds, a 30 cm wide path is recommended that acts as a water drainage system (Kothari et al. 2021b; Koocheki and Seyyedi 2020). This pattern of planting prevents the adverse moisture content in the raised beds. In Abruzzo (Navelli, Italy), the common pattern that follows is four rows per patch with planting depth of 10–15 cm, 20–25 cm row spacing and 10–15 cm spacing between the corms. However, in Greece, corms are planted in furrows at 15–17 cm depth, with a 20–25 cm gap between the rows and 11–13 cm corms spacing. The suggested corm density for the saffron plantation is 75–100 corms m^{-2} with 25 cm distance; to mark up to this planting density, approximately, 8–13 tons/ha corms are needed (that too depends on the size of corm). A few studies reported the planting density in Spain, i.e. 60 corms m^{-2}, while it varies from 10 to 50 corms m^{-2} in Sardinia (Kothari et al. 2021b; Cardone et al. 2020).

Nevertheless, following the above-mentioned planting density and pattern, saffron yield during the first year of plantation is quite low. Therefore, a higher planting density is considered as a substitute approach to compensate the loss of yield during the early years.

1.7.2 Corm Characteristics: Size, Weight and Storage

Saffron corm acts as a source of food reserves (Koocheki and Seyyedi 2020). These nutrient reserves in mother corm determine the saffron growth mainly during the early stages of its growth. In general, there is a positive correlation between mother corm size, daughter corm formation and flowering in saffron (Sahabi et al. 2017). It has been proven that heavier corms provide more energy to daughter corms, accordingly enhancing the daughter corm growth until they become independent. Consequently, the greater quantity of food reserves in mother corms augments flower yield by enhancing the plant growth and fibrous and contractile root expansion (Ebrahimi et al. 2021; Koocheki and Seyyedi 2020). Additionally, corm size has a direct impact on the leaf area, as large-size corms produce more leaf area, whereas small corms result in smaller leaf area. A large-sized corm has an average weight of 10 g, and is reflected as a potential size for the maximum flower number and yield (Sharifi et al. 2021). A few studies suggested that an increase in the concentration of elements, particularly, Nitrogen and phosphorous in large-sized daughter corms, also leads to an improved yield. The optimum-sized corm is a basic requirement for balanced nutrition in the growth of daughter corms and ultimately saffron production (Koocheki and Seyyedi 2020).

The criterion for the classification of corm size is not uniform, but a few researchers categorized 5–9 g as small-sized and 10–14 g as large-sized mothr corms, whereas others divided it into four groups such as small ≤ 4 g, medium 4.1–8 g, large 8.1–12 g and very large ≥ 12 g in their studies. However, only a small number of total corms produced in fields are greater than 12 g. The weight determination is considered as a more appropriate and precise way of sorting the

corms rather than diameter measurement because diameter value is not a practical approach to sorting. Saffron corms can't be stored for a longer period of time in cold rooms because moisture, temperature, gases, light, pests and diseases are considered to play an important factor during saffron corm storage. Hence, the optimum storage condition from harvesting to planting is established to be 30 °C for 20 days. Additionally, poor storage practices yield corms more sensitive to pathogens, especially fungal pathogens such as *Fusarium oxysporum*, *Penicillium digitatum*, *Aspergillus niger* and *Rhizopus stolonifera* (Koocheki and Seyyedi 2020).

1.7.3 Fertilizers and Biostimulants

Soil amendment is a general practice in saffron as in other crops, which enhances the physical and chemical properties of soil, for the enhancement of the yield. Unlike chemical fertilizers, organic fertilizers have significant application as they increase the cation exchange capability and uptake of N, K and Ca without having any adverse effect on soil biology and texture. The positive effects of organic fertilizers have been reported by various researchers, and they propose multiple reasons behind the use of organic fertilizers, some of which have been explained here (Aalizadeh et al. 2021; Ebrahimi et al. 2021; Koocheki and Seyyedi 2020).

Soil organic matter is one of the major factors that guides the use of organic fertilizers for the cultivation of saffron. The shortage of rainfall, repeated harvesting and poor vegetation generally lead to the reduction of organic matter content. In addition to this, the perennial life cycle of plants is also a reason for the decrease in the amount of organic matter, mainly afterwards the third year of cultivation (Aalizadeh et al. 2021; Madahi et al. 2017). This is because of the fact that multiyear production is the outcome of the first year cultivation of plants, and after that usually the corms are left undisturbed.

The effects of foliar fertilizer application on the saffron yield have also been investigated. Application of foliar fertilizers is recommended during February and March, as there is a reduction of radical activity, and as a result plant growth is dependent on leaf photosynthesis (Cardone et al. 2020). In addition to fertilizers, manures also play an important role in saffron cultivation. Cow-, chicken- or sheep-based manure is frequently used for enhancing the soil fertility in Italy (Ebrahimi et al. 2021; Cardone et al. 2020), whereas in Morocco farm manure is generally used during the first, third or fourth year of cultivation. However, in Iran vermicompost is significantly adapted.

On the other hand, biostimulants also have encouraging effects on saffron yield as well as enhance the content of bioactive compounds. Caser et al. (2019) estimated the effect of inoculum consisting of *Rhizophagus intraradices* and *Funneliformis mosseae*, and concluded that it increased the yield. Despite this, they resulted that *Rhizophagus intraradices* standalone increased the spice antioxidant activity and picrocrocin, crocin II and quercetin contents. Additionally, Ghanbari et al. (2019) proposed that inoculum of a mycorrhizal fungus with fertilizer augments saffron productivity, quality of stigma and phenolic as well as flavonoid contents in tepals. Ambardar et al. (2014), and Ambardar and Vakhlu (2013) (our group) reported bacterial associations with the saffron rhizosphere and cormosphere by cultivation-dependent as well as cultivation-independent metagenomics approaches. They evaluated the plant growth promotion activities of all the bacteria associated, but the major emphasis was given to *Bacillus*. Magotra et al. (2021) furthermore highlighted the importance of the most promising plant growth-promoting bacterium *Bacillus* sp. strain D5 isolated from saffron cormosphere as a biocontrol-biofertilizer in saffron cultivation fields.

1.7.4 Crop Rotation and Intercropping

To avoid the immense use of fertilizers, crop rotation has been appreciably adopted in saffron plants (Cardone et al. 2020; Koocheki and Seyyedi 2020). Maize, oat or linseed is

commonly cultivated in rotation with saffron in Kashmir. Saffron is cultivated for around 15 years in the same field continuously and the fields are then either left bare or planted with other crops for 2–3 years to avoid the building up of insects, pests and diseases as well as for the restoration of soil fertility (Kothari et al. 2021b). Gresta et al. (2016) conducted the experiment in Sicily, Southern Italy, and concluded that fava bean can be implemented for crop rotation as it promotes replacement corm yield and stigmas.

Furthermore, the saffron intercropping system, particularly with aromatic and medicinal plants, has been established, as it helps to avoid loss of nutrients and water. However, the candidate species should have the same ecophysiological necessity as saffron. For example, *Cuminum cyminum* L. that belongs to the Apiaceae family is cultivated as a medicinal plant in arid and semiarid regions of Iran. It is drought-tolerant and grows up to 22–30 cm tall, therefore can be intercropped along with saffron. Additionally, watermelon and pumpkin are frequently intercropped with saffron as there is no lifecycle overlap issue. They also neutralize the adverse environmental effects, and hence are economically and environmentally feasible. Moreover, they make outstanding groundcover and keep the soil cool by holding on to the moisture throughout warm seasons. Besides these, saffron-chamomile, saffron-black seed and saffron-ajwain rotation have also shown considerable results (Koocheki and Seyyedi 2020).

1.7.5 Irrigation

The practice of irrigation is completely absent in most of the saffron cultivated areas such as Greece, Sardinia and Abruzzo because the arrival of rain itself coincides with the most important time of irrigation, i.e. end of summer. On the contrary, in a semi-arid region of Spain, Castilla-La Mancha, 70% area is under irrigation of the total cultivated area, whereas in Morocco, irrigation is usually done with volumes of 350–500 m^3 of water ha^{-1} weekly from September to November, and at 15-day intervals from December to March (Cardone et al. 2020). However, during the period of deep corm dormancy, no irrigation is required (April–August). Low or late rainfall along with inadequate irrigation systems lead to the decline in saffron production in the Kashmir valley of India (Kafi et al. 2018). Koocheki and Seyyedi (2016) concluded that in western Macedonia, irrigation enhanced the leaf development whereas it reduced the qualitative traits of spice, flower number and bioactive compounds (crocin and picrocrocin) content. The three major irrigation methods employed are sprinkler, surface and drip/micro, but they are preferred according to the type of soil and climatic conditions. Irrigation management consists of applications such as providing moisture during early spring (March) that is required for the corm development and complete growth of daughter corms, light irrigation during autumn with appropriate time interval perhaps needed for the flower emergence and acceleration of blooming (Cardone et al. 2020). Therefore, critical periods of saffron irrigation are March and April (in which corms grow), followed by September (for the improvement of flower yield) (Kafi et al. 2018).

1.7.6 Harvesting of Flowers

Harvesting of flowers is the most crucial step in agronomical practices. It is laborious, time-consuming and generally a manual process, which formulates saffron as the costliest spice in the world (Kafi et al. 2018). The hand-picking of flowers is usually done during the early hours of the morning, so as to ensure its high qualitative traits with greater resistance to degeneracy. An average of 45–55 min are required for the manual hand-picking of 1000 flowers, however, more than 100–130 min are needed for the removal of stigmas for drying. So, to produce 1 kg of dried saffron, a total of 370–470 h is required (Cardone et al. 2020). The flowers are picked right after they are fully bloomed, and their stigmas are at utmost red in color. However, the task must be

done soon after sunrise as further exposure to sunlight leads to color and flavor loss (Kothari et al. 2021b). It may even get withered under the sunlight. Moreover, harvesting of flowers can also be carried out mechanically by using a machine system that performs the function in two parts: the first is the detachment of corolla from the stem, whereas the second gathers the detached flower through a vacuum collector. Soon after harvesting, the flowers are carried indoors for separation of stigmas from the petals and stamens with great precision (Cardone et al. 2020). During this phase, the stigmas plus the uppermost 2 mm of style are separated by opening up the flowers. However, if the style portion exceeds 2 mm, saffron is then regarded as inferior quality.

1.7.7 Post-Harvest Treatment

Drying is the crucial post-harvest step, which is aimed at maintaining the physical and chemical properties of spice and decreasing the moisture content that extends the spice shelf life. During drying, stigmas lose approximately 80% of the initial weight, and it critically influences the flavor and aroma of the spice (Kothari et al. 2021b). Different countries follow different ways of drying. For instance, Spain prefers artificial drying 'toasting', where stigmas are positioned in silk sieve layers 2–3 cm thick and are then exposed to 70 °C for half an hour using a butane gas fire; in Greece, stigmas are dried for 12–24 h while placing in silk sieve layers in a dark room and are exposed to 20 °C temperature initially which is then raised to 30–35 °C. In Morocco, stigmas are dried in the sun for a time period of about 2 h while placing on a thin layer of cloth or for 7–10 days in the shade (Cardone et al. 2020). In Kashmir, India, the traditional drying practice followed by farmers for saffron takes 27–53 h in shade. Immediately after fully drying, spice with a moisture content of 8–10% should be stored in sealed containers so as to protect it from light to avoid bleaching (Kothari et al. 2021b).

1.8 Phytochemistry of Saffron

Saffron spice (dried stigma) is majorly composed of volatile, non-volatile and aroma-yielding compounds. They are around 150 in number, but approximately 50 have been identified so far. The main biological active chemical compounds that constitute saffron are crocin, crocetin, picrocrocin and safranal (Mzabri et al. 2019). They are all found naturally in stigma, and are responsible for the various properties such as color, taste, aroma and smell. Safranal is the major volatile constituent whereas, among the non-volatile compounds, the major ones are crocin, crocetin, picrocrocin, anthocyanins, isophorones and flavonoids (for instance, kaempferol and quercetin). The various natural compounds identified in saffron spice (stigmas) are listed in Table 1.2 (Mohtashami et al. 2021). In addition to all these, saffron also contains vitamins, proteins, amino acids, mineral matter, starch and gums. The low levels of other non-volatile compounds mostly carotenoids that include lycopene, zeaxanthin and various α- and β-carotenes are also present (Finley and Gao 2017).

The essential bioactive compounds that are obtained from saffron are further discussed in brief:

(a) **Crocin and crocetin**

Crocin is a rare carotenoid pigment, which is responsible for the coloration of the spice (yellow-orange). Structurally, it has a chain of mono or diglycosyl polyene esters of crocetin. Crocetin (also known as α-crocetin or crocetin I) is lipophilic in nature having a conjugated polyene structure with a function group of two carboxylic acids. However, the glycosidic forms of crocetin such as digentiobioside (crocin), gentiobioside, gentioglucoside, glucoside, and diglucoside are hydrophilic in nature, and are produced by the esterification of hydrophilic gentiobiose (s) or other precursors of sugar with crocetin (IA et al. 2021; Finley and Gao 2017). Acid or enzymatic hydrolysis of crocin thereby produces crocetin as

Table 1.2 Natural compounds identified in saffron spice (stigmas) (Mohtashami et al. 2021)

Flavonoids	Monoterpenoids	Carotenoids	α-hydroxy acids
Kaempferol-7-O-sophoroside	(4R)-4-hydroxy-2,6,6-tri methylcyclohex-1-enecarbaldehyde 4-O-[β-D-glucopyranosyl(1 → 3)-β-D-glucopyranoside]: R = β-D-glucopyranosyl (1 → 3)-β-D-glucopyranoside	Zeaxanthin	Lactic acid
Isorhamnetin-3-O-glucoside	Picrocrocin: R = β-D-glucopyranosyl	β-carotene	Glycolic acid
Sophoraflavonoloside		Crocetin: R1 = R2 = H	Malic acid
		Crocin-1: R1 = R2 = β-gentiobiosyl	
		Crocin-2 (crocetin (β-D-glucosyl)-(β-gentiobiosyl)-ester): R1 = β-Dglucopyranosyl, R2 = β-gentiobiosyl	
		Crocin-3 (crocetin mono (β-gentiobiosyl) ester): R1 = H, R2 = β-gentiobiosyl	
		Dimethyl crocetin: R1 = R2 = methyl	
		Trans-crocetin-1-al-1-O-β-gentiobiosyl ester (R = β-gentiobiosyl	

one of the end products. It is estimated that 30% of the total metabolites in saffron is contributed by crocin; however, its total content in saffron also depends on the geographical location where it is grown, and the type of method that is administrated for the extraction. The common existing isoforms of crocin and crocetin are 'trans' forms, but few are cis-crocetin and their glycosides (Jose Bagur et al. 2018). Crocin (trans crocetin di-β-D-gentiobiosyl ester) has been accountable for the highest coloring ability. Comparatively, lipophilic metabolites contribute less toward the color of extracts. Crocin has been reported for a wide range of applications in medicines because of its various properties, such as hypolipidemic effect, antioxidant and vasodilation to the retina and choroid (Jose Bagur et al. 2018; Liu et al. 2018). It also inhibits the antigen expression of adenovirus in the infected cells at the early tumor stage. It also increases the level of enzymes such as glutathione reductase and glutathione-transferase. Moreover, crocetin also has antidiabetic and anti-tumor characteristics along with a great potential of acting as a cardiac protectant (Mykhailenko et al. 2019).

(b) **Picrocrocin**

Picrocrocin is the key metabolite that influences the flavor and is responsible for the bitter taste of saffron stigmas. It accounts for 5–15% of the total metabolites of saffron. Its formula is $C_{16}H_{26}O_7$ having a molar weight of 330.37 g/mol. Structurally, it is a monoterpene glycoside of constituent safranal. Aglycone (4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde ($C_{10}H_{16}O_2$) is released when picrocrocin is digested with β-glucosidase, which is therefore converted to safranal during the process of drying of plant material. It is generally known for its antiproliferative activity in cancer cells (Mzabri et al. 2019).

(c) **Safranal**

The main volatile compound, safranal, is primarily accountable for the aroma of the saffron stigmas. Its chemical formula is $C_{10}H_{14}O$, and has a molar mass of 150.21 g/mol. Safranal is formed during the degradation pathway of zeaxanthin, in which picrocrocin is an intermediate product (Abu-Izneid et al. 2020). It estimates 70% of the volatile fraction; however, its concentration depends on the drying as well as preservation process followed.

On account of its medicinal attributes, safranal shows excellent antioxidant as well as free radical scavenging properties. It also shows cytotoxic activity against cancer cells in vitro (Lambrianidou et al. 2021). In addition to these, it is known for its anticonvulsant as well as antidepressant activities in model animals and pilot-scale studies of humans. Moreover, it performs as an agonist at γ-aminobutyric acid type A (GABAA) receptors (major inhibitory neurotransmitter receptors) (Singletary 2020).

1.9 Saffron Adulteration

The common adulterants of saffron are oils, molasses or mineral substances in order to increase the weight of stigmas, and adding up the various artificial colorants or dyes with the aim of improving the appearance of stigmas. However, this practice is against the ISO 3632 standards that are aimed to implement for the quality check of stigmas worldwide (Cardone et al. 2020). The quality of the saffron is determined by analyzing the amount of major biological active compounds such as crocin, picrocrocin and safranal (responsible for color, flavor and aroma) present in the stigmas. It is believed that the higher the amount of these phytochemicals, the higher will be the quality of stigmas. The adulteration practices are most common in saffron due to its high cost and demand. These fraudulent practices have been noticed as far back as 600 years ago, and some serious punishments were also enforced for fraud mainly in European countries in order to maintain the originality of the spice (Koocheki and Milani 2020).

The commonly used adulterants of saffron are

- Old saffron
- Various other parts of the saffron plant (stamens or perigone)
- Other plant parts such as flowers from *Carthamus tinctorius* and *Calendula officinalis*, stigmas from other *Crocus* sp. that have no dye properties (*Crocus vernus*, *Crocus speciosus* and many more), ground red pepper, perianths from certain spices such as carnations, *Allium porrum* small roots, *Curcuma longa*, sandalwood and campeche wood powder, cut pieces of herbaceous plants colored with azoic dye, and flower slices of *Papaver rhoeas* L., *Punica granatum*, *Arnica montana*, *Scolymus hispanicus*
- Both beetroot (rich in betanine) and safflower, which contains carthamidin and carthamin, are also reported as saffron adulterants
- Animal substances, e.g. dried meat fibers
- Colored gelatin fibers are also accounted for as artificial adulterants
- Organic dye adulterants are Martins yellow, fucsina, tropeolina, picric acid, tartrazine, naphtanol yellow, azorubine, erythrocine, Cochineal A red, orange-yellow and many more
- Madder plant whose roots are characterized by polyphenolic reddish hydroxyanthraquinones.

However, a few adulterants are accounted for increasing the weight of stigmas, for instance, syrup, glycerin, honey and olive oil; and chemicals like sodium, calcium, barium sulfate, calcium carbonate, potassium nitrate, potassium hydroxide, monopotassium tartrate and sodium borate are also in practice for the purpose of the same (Koocheki and Milani 2020).

Considering, the preservation of quality and authenticity of the most valuable spice, high-throughput analytical methods are employed for the detection of potential adulterations. Spectrophotometric as well as chromatographic techniques that are used for the determination of

saffron adulteration are somehow incapable as they can't detect the adulterants like safflower, turmeric or marigold. Infrared spectroscopy techniques also detect the saffron adulteration that is caused by the addition of other plant parts, but it evaluates only one adulterant at a time (Koocheki and Milani 2020). However, NMR spectrometry (with high-field magnets) is considered as a precise and accurate method for the analysis, as it detects the compounds even at low concentrations. This method is more reliable for dealing with widespread saffron fraud practices (Petrakis et al. 2017). Currently, the Loop-mediated isothermal amplification (LAMP) technique is also in use as it is simple, quick and highly specific for the detection of saffron adulteration (Koocheki and Milani 2020). Moreover, an electronic nose is another probable method that determines fraudulent saffron by identifying the specific constituents responsible for saffron odor and, thereby investigates its chemical makeup (Kiani et al. 2017).

An alternative economical way that could be used in order to get rid of saffron adulteration is to produce bioactive compounds, which are present in low amounts in plants by using various biotechnological approaches. The important ones are listed as in vitro organ or tissue culturing and genetic manipulations (Nielsen et al. 2019). Furthermore, it is discussed in the topic of genetic improvement of saffron mentioned below.

1.10 Biotechnological Interventions

1.10.1 Tissue Culture (in Vitro Culturing)

Saffron is propagated by the conventional vegetative method using the renewal corms usually called daughter corms, yearly. The rate multiplication is slow, as 3–4 cormlets/corms are produced from each mother corm (Koocheki and Seyyedi 2020; Rashed-Mohassel 2020). Another limiting factor in conventional propagation is the fungal infestation that corresponds to the loss of corms during storage (Kumar et al. 2008). In vitro multiplication has therefore been advocated as the best alternative from the perspective of ensuring rapid multiplication and pathogen-free corm production (Cardone et al. 2020). Consequently, tissue culturing presents a significant potential for the large-scale production of disease-free corms using different mediums with varying concentrations of auxin and cytokinin. Some technical data regarding the somatic embryogenesis, and direct and indirect organogenesis of saffron corm/plant has been published (Sharma et al. 2021; Sargazi Moghadam et al. 2019). In spite of various protocols by many researchers, there is still a low rate of successful response during the in vitro culturing of saffron. The major reason for the lack of responsiveness of saffron tissues is contamination, a serious problem during the culturing of cormous monocotyledons, where corms are used as explants in most of the cases (Moshtaghi 2020). The two-step surface sterilization, involving the treatment of mercuric chloride and sodium hypochlorite, has definitely resolved the issue to some extent; still, endogenous microorganisms are difficult to cease during in vitro culturing. Ting et al. (1979) presented the first successful report on preliminary studies on tissue culture of saffron, followed by another description of callus induction and plantlet regeneration in the year 1981. Among all the explants used for the callus induction in saffron, corms and leaves have been majorly emphasized for the frequent selection of embryogenic calluses. Consequently, different explants produced different types of response, which is mainly based on the composition of medium and plant growth regulators. The most commonly used medium for saffron in vitro culturing is the Murashige and Skoog medium (MS medium) in which 6-Benzylaminopurine (BAP) is used as a source of cytokinin. However, auxin that are generally reported are 2,4-D (2,4 Dichlorophenoxyacetic acid), NAA (Naphthalene acetic acid) and IAA (indole-3-acetic acid) for the direct organogenesis of saffron, while 2,4-D is widely used for the purpose of indirect organogenesis. Along with auxin and cytokinin, 2% coconut milk has also been reported to be used for the callus induction in saffron. Additionally, a high concentration of sucrose was reported for the

microcorm induction in vitro (Sharma et al. 2021; Ziaratnia and Amini 2021; Moshtaghi 2020). The utilization of thidiazuron (TDZ) and picloram in varied concentrations were approved as highly effective for the induction and proliferation of somatic embryos (Chib et al. 2020). Furthermore, for the efficient induction of somatic embryos from saffron explants, some studies suggested the supplementation of jasmonic acid (0.5 mg/l) in combination with sucrose in an MS medium (Moshtaghi 2020).

Stigma-like structures (SLS) produced during in vitro culturing were accounted to be induced from most of the floral explants, including half ovaries, stigmas, petals, anthers and stamens. However, the frequencies of induction of stigma-like structures and secondary metabolite concentration mainly depend upon the wide range of basal medium supplemented with varying concentrations of plant growth regulators (Moshtaghi 2020). Among the floral explants, the best response of SLS was induced from ovary explants on MS medium, supplemented with NAA and BA at the concentration of 5.4 and 44.4 μM, respectively. However, crocin and picrocrocin contents were comparatively lower (6 and 11 times) in the stigmas produced by in vivo saffron growth. On the other hand, some reports suggested that crocin, picrocrocin and safranal contents could be enhanced in SLS by adding the appropriate precursors such as sodium acetate, glycine, L-alanine and serine. The rare earth elements such as La^{3+} (60 μM) and Ce^{3+} (20 μM) together can promote the *C. sativus* cell growth and crocin content to 4.4 mg g^{-1} (Cardone et al. 2020). Mir et al. (2014) perhaps explained the synthesis of a considerable quantity of apocarotenoids in stigmas that were produced from ovary explants under in vitro environment. They claimed that alternative ways of production of SLS and stigmas, rich in apocarotenoids, hold enormous potential for the commercialization of spice in foreseeable future. On the contrary, Kareem et al. (2019) considered stigmas, anthers and tepals as explants, and achieved the best response regarding the induction of stigma and SLS from stigma explants on MS medium having 19.8 μM BAP and 14.6 μM NAA.

In vitro tissue culturing of saffron, therefore, holds a great promise to meet the worldwide demand for saffron spice. The biotechnological approach may overcome the major hindrance regarding the cultivation of saffron such as the limited availability of daughter corms. Alternatively, secondary metabolite contents could be further augmented using this approach. On contrary, tissue culturing of saffron leads to the production of small-size corms that may not be able to survive under in vivo field conditions. Hence, the protocols reported so far are needed to be better refined, so that technology could be utilized at the commercial scale (Moshtaghi 2020).

1.10.2 Role of Genome Editing in Saffron

The environmental biotic and abiotic factors are critical for the germination, growth and reproduction of any crops, and saffron is no exception. These include the availability of water, sunlight, nutrients, carbon dioxide, humidity, rainfall and essential soil microbes. However, continuous change in global climate leads to inconsistent alteration in the factors significant for saffron productivity (Kothari et al. 2021a; Kouzegaran et al. 2020). In this context, saffron can manage to stay alive and productive only by adapting the important traits that can tolerate the climate change effects such as tolerance to heat, waterlogging, salinity, drought and resistance to pests and pathogens. These requirements call for the use of interventional biology, one of which is synthetic biology. Synthetic biology involves the basic knowledge of biological sciences and bioengineering principles for the reconstruction or redesign of existing biological systems (Saurabh 2021). The literature has reported an array of genes that can be used for the improvement of saffron which may provide resilience to the vagaries of climate. Over the past 30 years, explorations have been made regarding the genetic modification strategies for the improvement of crop traits, primarily based on gene silencing and genome editing. However, research

findings revealed that genome editing gradually to be becoming more feasible tool that is being implemented in most of the organisms and/or cells (Stephens and Barakate 2017).

Genome editing (GE) is an advanced molecular technique that is used for the making of deliberate changes at the specific target DNA sequences through programmable nucleases. These GE tools have been acknowledged for their precision, specificity and efficiency (Kim et al. 2021). They have the potential to eliminate the undesirable trait(s) and insert the desirable trait(s) by producing the site-specific double-strand breaks (DSBs) using engineered nucleases (molecular scissors), and finally repair the DSBs. The two mechanisms used for the repairing of DSBs are homology-directed recombination (HDR) and non-homologous end joining (NHEJ). HDR is less efficient than NHEJ, but it is less error-prone and hence more accurate than the NHEJ (Bhattacharya et al. 2021; Saurabh 2021). In the beginning, the customized zinc finger nucleases have been addressed for the purpose of gene editing of plants by conventional *Agrobacterium*-mediated genetic transformation. However, they have a few limitations such as being time-consuming and less cost-effective. Therefore, another tool named as Transcription Activator-Like Effector Nucleases (TALEN) has been subsequently discovered for the successful engineering of plants. Nevertheless, the discovery of CRISPR/Cas9 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) associated nucleases (Cas) completely revolutionized the gene-editing previous platforms that usually have protein-based DNA targeting specificity (Campa et al. 2019). However, CRISPR/Cas9 systems guide RNA directed gene-editing tools and therefore considered as RNA-mediated most effective strategy for the genome targeting and editing (Shan et al. 2020). The various genome-edited mutant plants that have been reported so far include monocots such as wheat, rice, barley, maize and banana, and dicots for instance *Arabidopsis*, tomato, soybean, potato, cotton, citrus, cucumber and grapes. To date, the successful application of CRISPR has been documented in a few model plants such as *Arabidopsis*, tobacco and rice (Nadakuduti and Enciso-Rodríguez 2021). Although these gene-editing tools have been proved as a major breakthrough for the development of successful biotic and abiotic stress-tolerant crop varieties (Modrzejewski et al. 2019), researchers are still working to create the edited saffron plant that could effectively tolerate the abiotic as well as biotic stresses such as deadly fungal diseases that cause major loss of saffron productivity. Sharma et al. 2021 mentioned a simple and efficient *Agrobacterium rhizogenes* strain ARqua1-mediated transformation protocol for *Crocus sativus* L. Chib et al. 2020 also reported stable integration of Cas9 in the *Crocus* calli.

CRISPR/Cas9 systems have been investigated by many research groups for the successful creation of stress-tolerant traits in most of the crops (Modrzejewski et al. 2019). Hence, they are considered as incredible powerful stress-tolerant tools which could be employed for the development of improved and tolerant saffron plant that can tackle several stresses efficiently. The reprogramming of the saffron genome could be done in order to confer the resistance against stresses by editing the gene associated with it (Sharma et al. 2021). Although the greenhouse studies to field trails can provide enough information for the successful management of stresses in the light of GE tools, a few basic concerns are needed to be addressed before trails in future research. The probable concerns related with the GE strategies of saffron are poor understanding of various biochemical pathways and their associated complexity, and insufficient knowledge of the genes associated with valuable agronomic traits. Hence, these major concerns are needed to be explained rigorously before commencing the gene-editing approach to saffron (Taheri-Dehkordi et al. 2020).

1.11 Saffron'omics'

1.11.1 Genomics

Although the karyotype of saffron has been studied by a number of authors on different ecotypes from various countries, it has always

been found to be $2n = 3x = 24$, with no noteworthy karyological variation (IA et al. 2021). However, various reports have identified the variations in the phenotypic as well as phytochemical traits of saffron mainly because of the epigenetic changes, which thereby correspond to the immediate requirement of developing molecular markers that can identify these particular variations at the molecular level (Busconi et al. 2021). With this regard, 27 SSRs markers were estimated in 8 Iranian saffron ecotypes and 29 wild alleles to determine the molecular variability. Additionally, the effectiveness of these markers regarding their prediction of genetic variability in these saffron ecotypes was also evaluated (IA et al. 2021). Moreover, a few studies examined variation within *C. sativus* L. at genetic and epigenetic levels using the Factorial Correspondence Analysis of AFLP (Amplified Fragment Length Polymorphism) and methyl-sensitive AFLP (Busconi et al. 2015).

Nevertheless, different approaches such as inter simple sequence repeats (ISSR), random amplified polymorphic DNA (RAPD) and microsatellite analysis were employed to access the variableness of saffron from various different geographic regions, but they all concluded *C. sativus* as a monomorphic species (Mir et al. 2021). Conversely, researchers are still performing their efforts for a better understanding of the genomic profile of *C. sativus*.

1.11.2 Transcriptomics

Transcriptomic studies on saffron have not gained much attention, and the probable reason for less consideration is because of the low or null genetic variability attributed to its triploid sterility and vegetative propagation (Panchangam et al. 2016). *Crocus* species are mainly recognized for their valued metabolites that follow a specific pattern in the developmental stages, and are usually synthesized in the stigma tissues. Several non-volatile and volatile metabolites are also present in other tissues of *Crocus* (Mykhailenko et al. 2021). The knowledge of the expression of numerous candidate genes throughout the development stages provides a brief understanding of metabolite production pattern. Therefore, cataloging the transcriptomic of saffron stigmas is necessary for highlighting the molecular basis of color, flavor, biogenesis and genomic organization as well as the biology of the spices specifically. Presently, there is a total of 6768 saffron Expressed Sequence Tags (ESTs) available at (http://www.ncbi.nlm.nih.gov/nucest/?term=), while the initial set of 6603 high-quality ESTs from the cDNA library of saffron stigmas was provided by D'Agostino et al. (2007). Similar other transcriptomic studies on saffron have dissected the biosynthetic pathways of carotenoids and flavonoids. Moreover, detailed transcriptomics analysis has also identified the carotenoids cleavage dioxygenase (CCD2), which is actually a novel dioxygenase that catalyzes the initial step of crocin biosynthesis obtained from carotenoid zeaxanthin (Gao et al. 2021; Panchangam et al. 2016).

1.11.3 Microbiome

Microbial communities are known to present in diverse environmental niches. These communities have their own diversity, interaction, complexities, cooperation and competition. These microbial communities are generally host-associated microbiomes that help in determining the healthy condition of the host (Sharma et al. 2019). Microbiomes of plants play an important role in the uptake of nutrients and protection against various stresses (biotic as well as abiotic). Environmental microorganisms from the soil and endophytic microorganisms of saffron greatly affect the healthy growth as well as the blossoming of saffron corms. Generally, the corms are mostly influenced by microorganisms present in the soil. However, saffron corm endophytic microbiome also plays a major role in the growth of the plant and has been extensively studied by many researchers (Ahmad et al. 2021b). Research has also been conducted for the microbiome of saffron corm planting area soil that includes both the bacterial 16S ribosomal

RNA and fungal ITS ribosomal RNA for the comparison of the differences in microbial diversity between soils that show good saffron growth (Shuwen et al. 2019). Our group has also reported microbiome associations (bacterial as well as fungal) with the saffron rhizosphere and cormosphere by both cultivation-dependent as well as cultivation-independent metagenomics approaches (Ambardar et al. 2014; Ambardar and Vakhlu 2013).

1.11.4 Metabolomics

Metabolome is a unique compilation of cellular functioning parts which are associated with the expression of sequenced genomes in all living organisms such as bacteria, plants and animals. Currently, metabolomic studies have been proved as an incipient tool for functional gene annotation as well as characterization, predominantly for those genes that are involved in regulatory pathways. Additionally, metabolomic studies provide a brief identification of substrates and products of enzymes, without any need of investing in the heterologous expression systems (Ashrafian et al. 2021; Sen et al. 2021). Likewise, saffron metabolomics has also provided a comprehensive, unprejudiced, quantitative and qualitative synopsis of its metabolites, for instance, crocetin esters, picrocrocin and safranal, revealing their therapeutic and aesthetic functions (IA et al. 2021). Different analytical approaches have been proposed by various authors for the detection of saffron adulterants, such as UV spectroscopy, infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (NMR), capillary electrophoresis, high-performance liquid chromatography (HPLC) and mass spectroscopy (MS) (Koocheki and Milani 2020; Dowlatabadi et al. 2017; Petrakis et al. 2017). However, these methods are primarily based on the target compound analysis, meant for the detection of adulterants and therefore have a major limitation related to the fact that only a limited number of adulterations could be identified. Moreover, NMR and MS are the most widespread analytical techniques used in the field of metabolomic studies (IA et al. 2021; Senizza et al. 2019). Metabolite fingerprinting using ^{1}H NMR spectra and chemometrics for the validation of both Iranian and Italian saffron has been reported (Dowlatabadi et al. 2017). The insights into the structural variability of saffron metabolites and differentiation of sugars associated with them using ^{1}H NMR were well documented. Therefore, ^{1}H NMR-based metabolomic studies serve as an effective tool to control the deterioration of saffron quality. Overall, brief quantification of metabolites is essential in order to understand the dynamics of the saffron metabolome, which thereby results in analyzing the pathways associated with saffron.

1.11.5 Proteomics

A comprehensive understanding of the biological role of proteins mandates the pre-knowledge of their structure and function. However, researchers from different biological disciplines, still face challenges in the identification of proteins and thereby prediction of their structure by analyzing the amino acid sequences. Although with the advent of proteomics studies, the characterization of proteins (either known or unknown) has been feasible to some extent (Dupree et al. 2020), proteomic investigation that was carried out previously in saffron recognized differentially accumulated proteins, which recommend insights into the underlying molecular mechanisms (Chen et al. 2021). Besides, a dearth of validated structural information for most of the plant proteins represents a major obstacle to functional annotation, evolutionary analyses and building interaction networks (IA et al. 2021). Even though a plethora of tools are accessible for the prediction as well as visualization of protein secondary and tertiary structures, still, complete analyses were available for only a few plant gene families. Therefore, a gap in the knowledge of saffron proteomics has frequently highlighted the requirement for refining the bioinformatics tools.

1.12 Conclusion and Future Research Recommendations

Saffron is one of the most valuable spices as far as its exploitation for medicinal purposes is concerned. However, still little effort have been done so far in the direction of exploring its potential for the subsequent improvement of traits. With its growing demand, the need of the hour is the integration of the current biotechnological approaches for gathering the data so as to get the utmost output toward its production. Moreover, future research should be conducted on the use of appropriate biostimulants that could enhance its productivity by improving the efficiency of saffron metabolic pathways, facilitating nutrients assimilation and augmenting quality attributes (color and aroma). Overall, researchers need to investigate the saffron plant more precisely so as to get a holistic view of its biology, which will further pave the way to construct or design the strategy for the up-gradation of this crop in a variety of aspects.

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Part II
Omics Studies on Saffron

Reference Genome of Saffron "The Golden Condiment"

2

Sheetal Ambardar, Jyoti Vakhlu, and Ramanathan Sowdhamini

Abstract

Saffron (*Crocus sativus*) is often referred to as the golden condiment and is known for its economical and medicinal value. *C. sativus* is a sterile triploid plant being propagated by vegetative propagation and therefore is reported to lack genetic variation based on the available molecular markers. Despite its economical importance, the omics information of this plant is scarce with few transcriptome and molecular markers studies. Whole genome of *C. sativus* was recently sequenced and assembled using Illumina sequencing technology. The genome size was estimated to be 3.5 Gb using flow cytometry and the de-novo draft genome was 3.0 Gb long covering 84.24% of the genome with an N50 value of 1860. The genome was annotated and 53,546 functional genes were identified from the *C. sativus* genome. In addition, repetitive regions (862,275), SSR (964,231), transcription factors (5726), and metabolic pathways (395) were identified from the *C. sativus* genome. Orthology analysis of *C. sativus* was performed against four plant species wherein the *C. sativus* was found phylogenetically closer to *Asparagus officinalis*. The draft genome can be used as the reference genome for future studies and can provide a valuable genomic resource for the research community.

2.1 Introduction

Identification and exploitation of genetic variation is the basis of plant breeding. Before the era of genomics, conventional tool and methodologies were used to develop improved varieties by plant breeding. Traditional selection based on phenotype is tedious and time-consuming. With the availability of genomic tools and resources, a new revolution of plant breeding has been initiated that facilitated the study of the genotype and its relationship with the phenotype.

The genome sequencing of the first plant *Arabidopsis thaliana*, sequenced using Sanger sequencing technologies in 2000, was a cutting-edge achievement in the field of plant genomics that boosted the demand for genomic information but was time-consuming, laborious, and expensive (Weigel and Mott 2009). Second-generation sequencing technology that came around 5 years after the *Arabidopsis* genome was sequenced in 2005 and revolutionized sequencing technology with its high speed and low cost. Subsequently, third-generation sequencing technologies lead to the development of contiguous, chromosome-

S. Ambardar (✉) · J. Vakhlu
School of Biotechnology, University of Jammu, Jammu, Jammu and Kashmir 180006, India
e-mail: sheetalambardar@gmail.com

S. Ambardar · R. Sowdhamini
National Center for Biological Sciences, Bellary Road, Bangalore 560065, India

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scale genome assemblies in many plants (Li et al. 2021; Xia et al. 2021; Song et al. 2021). Next-generation sequencing (NGS), which includes both second- and third-generation sequencing technologies, has substantially expanded the ability to analyze and understand plant genomes and reduce the gap existing between genotype and phenotype. Since the genome of *A. thaliana* has been sequenced, approx 1935 plants' genome has been sequenced and assembled using second- and third-generation sequencing and are publically available [NCBI Genome database (https://www.ncbi.nlm.nih.gov/genome/browse#!/eukaryotes/plants)]. Genomic sequence availability enabled researchers to identify the genes of agronomical importance and genetic markers, improve knowledge of breeding, develop genetic mapping, and establish evolutionary relationships between the sequenced species via comparative genomic analysis (Harkess et al. 2017; Alonge et al. 2020; Liu et al. 2020; Busconi et al. 2021).

Recently, the ginger (*Zingiber officinale*) diploid genome was sequenced, assembled, and phased into two haplotypes using hybrid NGS sequencing (Illumina, PacBio, and Hi-C reads), and its unique gingerol biosynthetic pathway was identified (Li et al. 2021). In addition, in passion fruit (Passiflora edulis Sims), chromosome-scale genome assembly has deciphered the evolutionary information and mechanism of flavor synthesis (Xia et al. 2021). With the genome sequence of celery (*Apium graveolens*), researchers were able to study the sequential polyploidizations and karyotype evolution (Song et al. 2021). In addition, advancement in sequencing technologies allows for the survey of genome-wide epigenetic variation responsible for heritable traits at high resolution using bisulfite sequencing (Bi-seq), methylated DNA immunoprecipitation sequencing, i.e. MeDIP-seq, and methylation-sensitive restriction enzyme sequencing, i.e MRE-seq (Busconi et al. 2021).

In recent years, the focus of the plant genomics has been shifted from a single reference genome to using a "pan-genome". Pan-genome is a representation of all genomic content in a particular plant species individuals which has been made possible due to the reduction in the cost of high-throughput sequencing and advances in sequence assembly algorithms. A consensus genome can be drawn from the multiple reference genomes of an entire population that provide multiple advantages over a single, linear reference genome sequence in evolutionary studies, functional genomics, and breeding of cultivated plants (Marschall et al. 2018; Jayakodi et al. 2021). Pan-genome analysis of plants was initiated in 2009 with the model plant *A. thaliana* (Weigel and Mott 2009) followed by various plants' pan-genome reported subsequently like rice, maize, tomato, sorghum, wild cabbage, rape Seed, and pigeon pea (Huang et al. 2012; Jiao et al. 2012; Mace et al. 2013; Aflitos et al. 2014; Golicz et al. 2016; Dolatabadian et al. 2020; Zhao et al. 2020).

2.2 Saffron, *Crocus sativus* L.

Saffron referred to as "Golden Condiment" is the world's most expensive spice costing about 70,000 INR/pound. About 130,000 flowers (42.38 kg) are required to obtain 1 kg of saffron spice in Kashmir. Saffron has two years plant cycle with three different stages as dormant, flowering, and vegetative (Magotra et al. 2021). The stigmas of flowers which when dried form the commercial spice "Saffron" (Yasmin and Nehvi 2018) Saffron, a member of the family Iridaceae, is being grown in only Jammu & Kashmir (UT) in India which ranks second in Saffron's global market with a total production of 16 Metric Tonns (Yasmin and Nehvi 2018; Mzabri et al. 2019; Ganaie and Singh 2019; Magotra et al. 2021).

Medicinal properties: More than 150 volatile and aroma-yielding compounds have been reported from saffron that contribute to the flavor, color, aroma, and cosmetic and medicinal properties. However, the main chemical constituents present in the stigma that makes the spice are crocin, crocetin, picrocrocin, and safranal (Xing et al. 2021; Maggi et al. 2020; Rahmani et al. 2017; Samarghandian and Borji 2014). Its use for therapeutics has been well

documented in Ayurveda and modern medicine as well (Rahmani et al. 2017). Saffron and its components have been reported to have medicinal properties such as it is used as antidepression, antinociceptive, anti-inflammatory, anticonvulsant, antigastric, antiparkinsonian, antigenotoxic, tumoricidal, hypolipidaemic, and tumoricidal effects and may also be useful in preventing atherosclerosis (Xing et al. 2021; Ghaffari and Roshanravan 2019; Rahmani et al. 2017). The components of saffron like Crocin are reported to be most effective in cancer therapy (Chryssanthi et al. 2011). It is also used as flavoring and coloring agents in food and promotes satiety (Gout et al. 2010).

2.3 *Crocus sativus*, Triploidy and Sterility

C. sativus is an perennial sterile triploid plant ($2n = 24$) that flowers in autumn with ~3.5 Gb hap-loid genome (Brandizzi and Grilli Caiola 1996, 1998; Ambardar, et al. 2022). The cultivated *C. sativus* was first time reported to be an autotriploid by Karasawa in 1932 with $2n = 24$ chromosomes which formed up to eight trivalents during meiosis (Fig. 2.1) (Karasawa 1932, 1933, 1942). Aghamohammadi (1977) and Estilai (1978) confirmed the autotriploidy of saffron and further reported that the sterility of saffron is due to its pollen that is inviable due to the irregular meiosis. Brighton, in (1977), further confirmed that the *C. sativus* cultivated in the central part of Iran, Turkey, France, and England is also triploid (Brighton 1977; Brighton et al. 1973; Ghaffari 1986). Recently, Schmidt and coworkers established a high-resolution FISH karyotype of *C. sativus* using a survey sequencing of the saffron genome to develop cytogenetic landmark probes. These probes were localized on 92 chromosomal positions and established the chromosomal composition of saffron as autotriploid (Schmidt et al. 2019).

C. sativus is reported to be originated from its wild precursor *C. cartwrightianus* that has played a major role in saffron evolution, and it has been suggested that changes in its ploidy resulted in autotriploid saffron (Karasawa 1932, 1933, 1942). Previously, reports considered saffron *Crocus sativus* as an allotriploid species involving diploid progenitors with $2n = 2x = 16$ chromosomes, such as *Crocus cartwrightianus*, *Crocus hadriaticus Herb., C. oreocreticus Burtt., Cro-cus pallasii Goldb. ssp. Pallasii, Crocus thomasii Ten,* and *C. asumaniae Mathew.* (Jacobsen and Ørgaard 2004; Agayev et al. 2010; Harpke et al. 2013). In another report, saffron was reported to be an allotriploid species using IRAP analysis, with *C. cartwrightianus* and *C. pallasii* subsp. *pallasii* (or close relatives) as ancestors (Alsayied et al. 2015). However, in 2019, *C. sativus* was been reported to be autotriploid with only *C. cartwrightianus* as its ancestor using single nucleotide polymorphism analyses of an intron of the nuclear topoisomerase gene, and genotyping-by-sequencing, and found that saffron and *C. cartwrightianus* show very high allele similarity, concluding that *C. cartwrightianus* genotypes formed the autotriploid saffron (Nemati et al. 2019). Schmidt and coworkers demonstrated that *C. sativus* is an autotriploid hybrid derived from heterogeneous *C. cartwrightianus* cytotypes by comparative FISH of six Crocus species from 11 accessions (Schmidt et al. 2019).

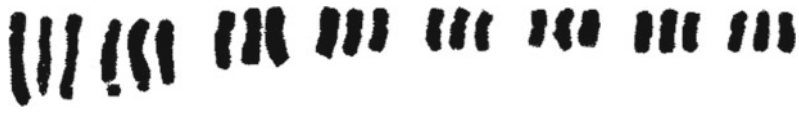

Fig. 2.1 Karyotype of cultivated *Crocus sativus*, showing eight triplets. *Source* Ghaffari (1986)

2.4 Available Genomic Resources of Saffron

2.4.1 Genetic Diversity

The existence of genetic diversity in *Crocus sativus* has globally been debated. Ample studies suggested that saffron lacks genetic diversity, which has been evaluated using various molecular markers such as sequence-specific amplified polymorphism (SSAP) and retrotransposon microsatellite amplified polymorphism (REMAP) (Bukhari et al. 2014), Random amplified polymorphic DNA—RAPD (Ali et al. 2013; Zheng et al. 2013; Keify and Beiki 2012; Qadri et al. 2012; Caiola and Canini 2010; Imran et al. 2010), Inter Simple Sequence Repeat—ISSR (Zheng et al. 2013; Rubio-Moraga et al. 2009), SSR—Simple Sequence Repeat (Zheng et al. 2013), Amplified Fragment Length Polymorphism—AFLP (Siracusa et al. 2013; Fernández et al. 2011; Nazzal et al. 2011; Caiola and Canini 2010), interretroelement amplified polymorphism (IRAP) (Alsayied et al. 2015), Retrotransposons (Alavi-Kia et al. 2008), single nucleotide polymorphism—SNP (Fernández et al. 2011; D'Agostino et al. 2007), and expression sequence Tags—EST (D'Agostino et al. 2007). As per these molecular markers used, all the studied accession appeared identical clones not only because of morphological characters but also at a molecular level suggesting that *C. sativus* is a monomorphic species.

However, recently limited genetic variation has been reported in this plant using D2 and epigenetic analysis (Qadri et al. 2012; Busconi et al. 2018, 2021; Mir et al. 2021). Qadri et al. (2012) compared the diversity of saffron genotypes estimated through D2 analysis and molecular markers among a set of 200 saffron genotypes from different saffron growing areas of Kashmir using DNA fingerprinting using RAPD markers that showed considerable genetic variability among different saffron genotypes. In addition, Busconi et al. (2018) have reported the high epigenetic variations among 17 Saffron accessions of different geographic origins, during four consecutive years of vegetative propagation under open field conditions using Methylation-Sensitive Amplified Fragment Length Polymorphism (MS-AFLP) analysis (Busconi et al. 2018). Busconi et al. (2021) reported the epigenetic variation in the five phenotypically different accessions of saffron, showing differences in tepal pigmentation, the yield of saffron, and flowering time, but genetically similar by AFLP analysis (Busconi et al. 2015), using a methylation-sensitive restriction enzyme-sequencing (MRE-seq) approach (Busconi et al. 2021). A high number of SNPs were detected by comparing the sequences of the different accessions, demonstrating high genetic variability that has arisen as a consequence of the prolonged vegetative propagation (Busconi et al. 2021). In addition, Mir et al. (2021) have reported the existence of limited genetic variation in different saffron accessions using RAPD and ISSR-based primers. With an average percentage of ISSR polymorphism as 52.48% and the average percentage of RAPD polymorphism as 66.44% resulting in 9% of the variance among populations and 91% of the variance within populations based on AMOVA, the existence of genetic differences, though limited, was reported in saffron.

2.4.2 Saffron Transcriptomics

Many transcriptome analyses of *C. sativus* have been performed to understand the pathways related to apocarotenoid biosynthesis and flowering regulatory in *C. sativus*. The molecular basis of apocarotenoid biosynthesis pathway in *C. sativus* was studied in-depth using various transcriptomics studies (Baba et al. 2015; Jain et al. 2016; Tan et al. 2019; Yue et al. 2020). Initially, Baba et al. (2015) performed the transcriptome sequencing of *C. sativus* stigma and flower using Illumina Genome Analyzer IIx platform to understand the apocarotenoid biosynthesis and other aspects of stigma development in *C. sativus*. *De-novo* transcriptome assembly resulted in 64,438 transcripts (32,204 unigenes) using trinity software. Zeta-carotene

isomerase, desaturase, carotenoid isomerase, and lycopene epsilon-cyclase genes involved in the carotenoid/apocarotenoid pathway were identified for the first time, in this study. Apocarotenoid biosynthesis was reported to be localized in the stigma as carotenoid/apocarotenoid pathway genes including phytoene synthase, phytoene desaturase, and carotenoid cleavage dioxygenase 2 are highly expressed in stigma. In addition, 2601 transcription factors belonging to 76 families were identified with the relative abundance of Myb family (Baba et al. 2015).

In another study, transcriptome sequencing was performed from corm, tepal, leaf, stigma, and stamen of *C. sativus* using the Illumina platform wherein a total of 105,269 unique transcripts (average length of 1047 bp and N50 length of 1404 bp) were identified. Differential expression of many transcripts involved in apocarotenoid biosynthesis was observed in stigma. More transcription factors (3819) belonging to 87 families were identified than in the previous study (Baba et al. 2015) with MYB, MYB-related, WRKY, C2C2-YABBY, and bHLH Tfs reported to be involved in secondary metabolism (Jain et al. 2016).

Further, in order to discover the potential key catalytic steps involved in apocarotenoid biosynthesis in saffron, in-depth transcriptome and metabolomic analysis of stigmas at different developmental stages was performed and a total of 61,202 unigenes were obtained. Co-expression network analysis identified 28 regulators and 32 putative carotenogenic genes and 15 candidate genes closely related to safranal and crocin production. In addition, oxidation of crocetin dialdehyde into crocetin by the aldehyde dehydrogenase (*Cs*ALDH3) gene was validated by creating a crocetin-producing yeast strain. A new branch in the apocarotenoid biosynthesis pathway was investigated for the first time wherein geranyl-geranyl pyrophosphate converts into copalol and *ent*-kaurene by class I and II diterpene synthases (*Cs*EKL1/2/3 and *Cs*CPS1) (Tan et al. 2019).

Recently, the evolution of apocarotenoid biosynthesis in *Crocus sativus* was studied using full-length transcriptome PacBio sequencing wherein 31,755 protein-coding genes, with 50.1% forming paralogous gene pairs were identified. Authors have reported that the *C. sativus* genome has evolved due to two rounds of whole-genome duplication events that occurred ~28 and ~114 million years ago, respectively. Further, the recent β WGD event was reported to confer a major impact on family expansion of secondary metabolite genes that were necessary for the biosynthesis of apocarotenoids in

C. sativus. CCD2 enzyme that was highly expressed in the stigma was reported to be evolved from the CCD1 family via the β WGD event (Yue et al. 2020).

Researchers have been focusing on the factors that affect floral development using transcriptomics, since increasing the flower number of saffron crocus is a viable way to produce more saffron to meet the ever-increasing demand in the market (Qian et al. 2019; Hu et al. 2020; Renau-Morata et al. 2012). Qian et al. (2019) performed full-length transcriptome analysis of flowering and non-flowering saffron using the single-molecule real-time (SMRT) sequencing method and identified 75,351 full-length saffron crocus unigenes, 79,028 SSRs, 72,603 lncRNAs, and 25,400 alternative splicing (AS) events. The schematic of the flowering gene regulatory network in saffron was reported for the first time and 62 putative flower-related genes were identified that played important roles in vernalization (VRNs), gibberellins (G3OX, G2OX), photoperiod (PHYB, TEM1, PIF4), age (SPLs), and autonomous (FCA) pathways. Tissue-wide and time course expression analysis revealed that the transcripts increasingly accumulated in flowers during the flower development period (Qian et al. 2019).

Another study was conducted to understand the genetic regulation of flowering in *C. sativus* by Hu et al. (2020) by morphological, physiological, and transcriptomic analyses using apical bud samples from *C. sativus* during the floral transition process. The flowering transition process was divided into three stages: an undifferentiated period, the early flower bud differentiation period, and the late flower bud differentiation period based on morphological

results. Transcriptomic analysis identified 60,203 unigenes, among which 19,490 were differentially expressed significantly. 165 unigenes were associated with flowering. In addition, these unigenes were significantly enriched in the sugar metabolism, hormone signal transduction, cell cycle regulatory, photoperiod, and autonomous pathways. Differential expression analysis showed that the levels of sugar, gibberellin (GA3), auxin (IAA), and zeatin (ZT) were steadily upregulated whereas the levels of starch and abscisic acid (ABA) were gradually downregulated. Based on these results, the authors have proposed a hypothetical model for the regulatory networks of the flowering transition in saffron (Hu et al. 2020).

Transcriptomic analysis of saffron main buds in different stages of development was conducted in order to gain insight into the floral induction regulatory networks in *C. Sativus* (Renau-Morata et al. 2012). In this study, various genes have been identified that could be used as molecular markers for example FT that is associated with flowering time signals and LFY and TFL1 that are meristem identity genes. Authors have reported that the auxins induce the release of corm dormancy which is in turn inhibited by abscisic acid. Similarly, flower initiation is promoted by auxins and inhibited by gibberellins. In addition, working models for hormone signaling pathways mediating flowering have been proposed based on transcriptome analysis (Renau-Morata et al. 2012).

2.4.3 Saffron Genome

Whole-genome sequencing is reported to identify the great variety of genetic diversity in a population thereby gaining an understanding of the relationship between the inherited genome and observed heritable traits. Various molecular markers (RAPD, ISSR, AFLP, and SSR microsatellites) based and epigenetic approaches discussed earlier have suggested the existence of limited genetic variability. However, to discover authentic genetic markers, mining genes for secondary metabolites pathways, and improvement of breeding, sequencing of its genome is the only alternative. In addition, it's ancestry is also controversial that could be also settled if its complete genome sequence is available. The draft genome of Saffron has been reported by our group for the first time (Ambardar et al. 2022). *C. sativus* corms were collected from Kishtwar, J&K (33.3116° N, 75.7662° E) in 2019. Genome size of the plant was confirmed to be 3.5 Gb by flow cytometric and k-mer-based method using Jellyfish that was comparable to earlier reports on saffron being grown in Italy, Spain, and Israel using flow cytometry (Brandizzi and Grilli Caiola 1996, 1998). The *C. sativus* de-novo assembly and annotation pipeline has been well represented in Fig 2.2.

The draft genome of Saffron was sequenced using Illumina sequencing with two different insert DNA libraries (550 and 800 bp), and a total raw data of 321 Gb was generated having an overall coverage of ~92X based on the genome size. The raw data was analyzed for quality, and the sequences having Phred score greater than 30 were retained for better quality assembly. The genome was assembled using two *de-novo* genome assemblers namely Soapdenovo2 (Luo et al. 2012) and MaSuRCA (Zimin et al. 2013), and the genome assembly with MaSuRCA (Cs_Assembly_2) with an N50 value of 1860 and 84.24% genome coverage was found comparatively better than genome assembly with Soapdenovo2 (Cs_Assembly_1) with an N50 value of 1596 and 77.9% genome coverage (Table 2.1). The quality of genome assemblies was accessed using BUSCO (Simão et al., 2015) against Viridiplantae lineage from the OrthoDB database, and the BUSCO genome completeness was more in Cs_Assembly_2 (44.46%) than Cs_Assembly_1 (7.32%) (Table 2.1). Genome assembly using Illumina sequencing is reported to have a low N50 value due to the short reads that are difficult to assemble in repeat regions. Using this sequencing technology, de-novo genome assembly in *Polygonum cuspidatum* and *Linum usitatissimum* was having N50 values of 3215 and 694 with 98.5% and 81% genome coverage, respectively

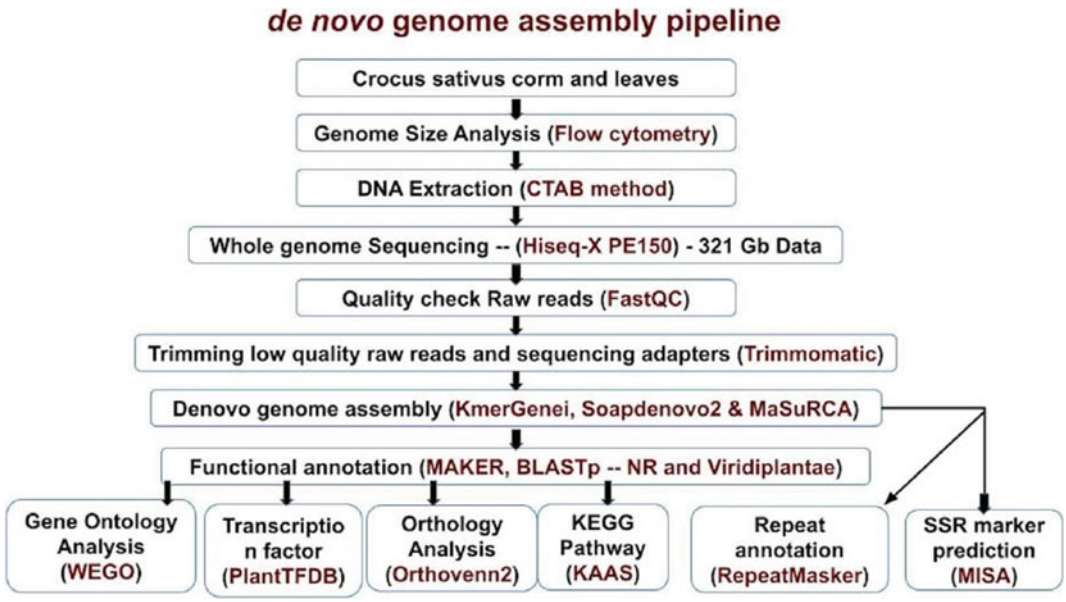

Fig. 2.2 De-novo genome assembly and annotation pipeline of *Crocus sativus*. Black color text represents the analytical processes and Red color text represents the software/instrument used to perform the processes. *Source* Ambardar et al. (2022)

(Zhang et al. 2019; Wang et al. 2012). Whole-genome sequencing raw reads and draft genome of *Crocus sativus* have been submitted to NCBI SRA under bioproject PRJNA734464 and PRJNA739096, respectively. All the processed data including draft genome, annotated proteins, and supplementary tables can be accessed at CAPS_NCBS server http://caps.ncbs.res.in/download/csat.

Most of the raw data was utilized for genome assembly as evident by the high mapping percentage (87.28%) of raw reads that mapped back to the saffron genome. We also have compared previously published transcriptome reports on saffron (Jain et al. 2016; Baba et al. 2015) with the draft genome by mapping the transcripts to the genome, and high mapping percentages of 99.92% and 92.02% were observed against Cs_Assembly_2. It was interesting to note that even though the genome assembly was fragmented, most of the reported CDS/exons are present in the draft genome as evident by a high

Table 2.1 Comparative assembly statistics of *C. sativus* genome using soapdenovo2 and MaSuRCA *de-novo* assemblers

Assemblies	Soapdenovo2	MaSuRCA
	Cs_Assembly_1	Cs_Assembly_2
Kmers	71	99
N50 scaffold (bases)	1596	**1863**
Number of scaffolds	1,505,129	**2,564,042**
Largest scaffold (bases)	45,973	**46,734**
Total sequence length	2,787,926,280	**3,014,612,563**
GC%	43.2	**43.2**
Genome coverage (%)	77.90%	**84.24%**
BUSCO (%)	7.81%	**44.46%**

Bold signifies comparatively better values of assembly statistics

mapping percentage (Jain et al. 2016; Baba et al. 2015).

2.4.3.1 Repetitive Region in *Crocus Sativus* Genome and SSR Marker Prediction

Repetitive regions in the draft genome were identified using Repeatmasker and GenomeScope v2 (Ranallo-Benavidez et al. 2020; Smit et al. 2010), and SSR markers were identified using MISA (Beier et al. 2017). Total repeats length in *C. sativus* genome (Cs_assembly_2) was 1,460,908,750 bp (40.8%) as predicted by GenomeScope version 2. A total of 8,62,275 repeats were identified in Cs_assembly_2 wherein simple repeat (48.41%) and LTR (30.34%) were the most abundant in the genome. Specifically, Copia and Gypsy were the most abundant LTR repeats (Table 2.2).

A total of 964,231 SSR markers were identified in Cs_assembly_2 wherein monomeric SSR repeats (486,140–50.4%) were more abundant as compared to dinucleotide (294,819–30.5%) and trinucleotide repeats (146,991–15.2%) with "A", "TA", and"TTG" most abundant SSRs in each group. The abundance of tetranucleotide (15,375–1.59%), pentanucleotide (8596-0.9%), and hexanucleotides (12,310-1.27%) repeats each was less than 2% of total SSRs with "AAAT", "TATAT", and "TAACCC" most abundant in respective SSRs (Table 2.3). SSR markers are reported to be multi-allelic, relatively abundant, and widely dispersed across the genome and have been used in genetic diversity analysis, parentage assessment, species identification, and mapping genetic linkage (Feng et al. 2016). These markers can be further evaluated for their application in *C. sativus*. Earlier transcriptomics studies on *C. sativus* transcriptome have reported the presence of comparatively less number of SSRs [16,721 and 79,028 (Jain et al. 2016; Qian et al. 2019)] as compared to the present study (964,231 SSRs) based on genome sequence

2.4.3.2 Gene Prediction and Annotation

De-novo assembled draft genome (Cs_Assembly_2) was further analyzed for gene prediction using a well-reported annotation pipeline MAKER which has genome annotation software like AUGUSTUS and SNAP (Campbell et al. 2014). Annotation by MAKER requires EST evidence that was provided by previously published *C. sativus* assembled transcripts data (Jain et al. 2016) and protein evidence as Viridiplantae

Table 2.2 Classification of repetitive sequences in *C. sativus* genome representing the abundance of Simple repeats and LTRs

Repetitive region	Numbers
Simple repeats	415,561
LTR	260,472
Low_complexity	64,624
DNA	64,205
LINE	45,739
Satellite	3946
RC_Helitron	3072
rRNA	2749
SINE	848
tRNA	650
Other	340
snRNA	54
Retroposon	15
Total	862,275

Source Ambardar et al. (2022)

Table 2.3 SSR markers from *Crocus sativus* draft genome (Cs_assembly_2) depicting the more relative abundance of monomeric repeat microsatellites

SSR types	Count	Relative % age	Most abundant	% age
Monomeric repeat microsatellite	486,140	50.4	"A"	44.6
Dinucleotide repeat microsatellite	294,819	30.5	"TA"	16.5
Trinucleotide repeat microsatellite	146,991	15.2	"TTG"	5.72
Tetranucleotide repeat microsat-elite	15,375	1.59	"AAAT"	7.7
Pentanucleotide repeat microsat-elite	8596	0.9	"TATAT"	3.2
Hexanucleotide repeat microsat-elite	12,310	1.27	"TAACCC"	5.3
Total	964,231			

Source Ambardar et al. (2022)

database (UNIPROT). For AUGUSTUS, the gene prediction model was selected to be maize and for SNAP, hmm library as of *Oryza sativa*. A total of 254,038 proteins were predicted using the MAKER pipeline and were further annotated using BLASTp against NR (NCBI) and viridiplanteae (UNIPROT) database with modified parameters (E-value-1e^{-3}, sequence identity >40%, and query coverage >70%). A total of 52,435 proteins were annotated in saffron genome based on BLASTp against the NR database. BUSCO analysis revealed the presence of 75.7% of the plant-conserved genes/orthologues in the *C. sativus* genome. Based on Genome annotation results, *C. sativus* was found phylogenetically closer to *A. officinalis* as *the* maximum number of proteins were annotated against *Asparagus officinalis* (9213) as both the plants belong to the same plant order Asparagales (Fig 2.3).

The Association of these annotated proteins with GO annotation was studied in detail, and 85% of total proteins (43,649) were found associated with gene ontology (GO) ids. 22,092 proteins were associated with biological processes abundant in cellular and metabolic processes, 24,399 proteins with cellular components localized in cell and organelle parts and 34,442 proteins were associated with molecular functions most abundant in catalytic and transporter activities (Ambardar et al. 2022).

2.4.3.3 Transcription Factors

Transcription factors (TFs) play crucial roles in plant development, cell cycling, cell signaling,

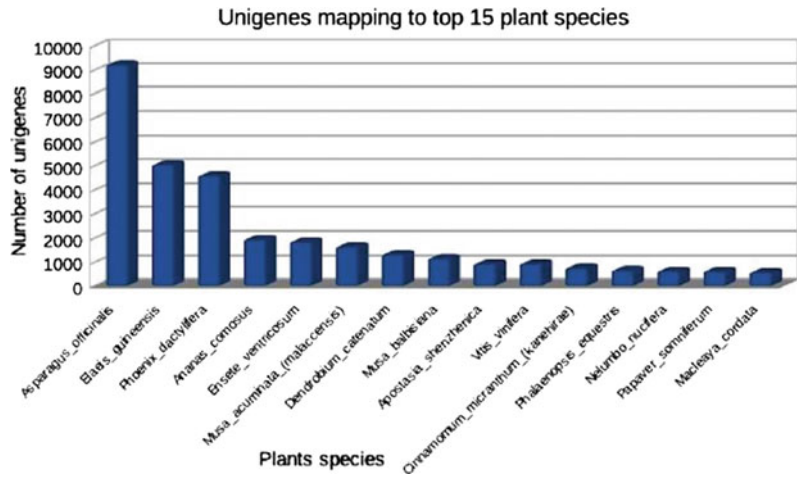

Fig. 2.3 Maximum number of *Crocus sativus* unigenes mapping to *Asparagus officinalis*

and stress response, to name a few, DREB, bZIP, MYB, NAC, Zinc-finger, HSF, Dof, WRKY, NF-Y, etc. In *C. sativus*, 5726 unique proteins were identified as transcription factors (TFs) belonging to 57 TFs families. MYB & MYB-related family proteins (11.86%) were the more abundant Tfs, followed by bHLH, C2H2, NAC, FAR1, C3H, ERF, bZIP, WRKY, B3, etc. (Fig. 2.4). TFs like MYB & MYB-related, bHLH, and WRKY are reported to regulate secondary metabolite (apocarotenoid) biosynthesis in *C. sativus* (Jain et al. 2016). Earlier reports on *C. sativus* transcriptome have identified less number of TFs (3819, 2601), whereas the most abundant Tfs family remains the same (Jain et al. 2016; Baba et al. 2015).

2.4.3.4 Orthology Analysis

Our group has also compared the *C. sativus* with other plants using Orthology analysis for the first time. As *C. sativus* is the only member from the Iridaceae family whose genome has been sequenced, therefore plants from the same order like *A. officinalis*, *P. equestris*, and *A. shenzhenica* were selected for comparative analysis along with *O. sativus* (Rice) as a model plant. Orthology analysis was performed by comparing *C. sativus* annotated proteins with annotated proteins of *A. officinalis*, *P. equestris*, *A. shenzhenica,* and *O. sativus* (Rice) using Orthovenn2. We identified a total of 23,744 protein clusters out of which 7328 protein clusters were common in all the plant species whereas 2510 proteins cluster were unique to *C. sativus* only (Fig. 2.5a). Conserved clusters among these five plant species comprised of 51,803 proteins, wherein the maximum number of proteins belonged to *O. sativa* (14,668) followed by *C. sativus* (10,001), *A. officinalis* (9552), *P. equestris* (9012), and *A. shenzhenica* (8570) and were enriched in defense response, RNA modification, DNA integration, regulation of transcription, rRNA processing, and protein phosphorylation (Fig. 2.5b). The protein clusters specific to *C. sativus* (2510) comprised of 7914 proteins that were associated with nucleic acid binding, transferase, hydrolase, oxidoreductase activity, and protein- and DNA-binding activity but do not have orthology with any of these four species and need further study. In addition, *C. sativus* and *A. officinalis* shared maximum number of protein clusters as compared to other plants thereby indicating that these two plant species are phylogenetically closer as compared to other plants compared in the study (Fig. 2.5c) (Ambardar et al. 2022).

2.4.3.5 Metabolic Pathway Analysis

In order to study various metabolic pathways for the growth and development of *C. sativus*, the annotated protein were mapped against the KEGG database of monocot using an online webserver KAAS [Kyoto Encyclopedia of Genes and Genomes Automatic Annotation Server) (Moriya et al. 2007)]. A total of 395 metabolic pathways were identified in *C. sativus* and most of the pathways and genes involved in the pathways were found in the draft genome, to name a few, carbohydrate metabolism; energy metabolism; lipid metabolism; nucleotide metabolism; amino acid metabolism; glycan

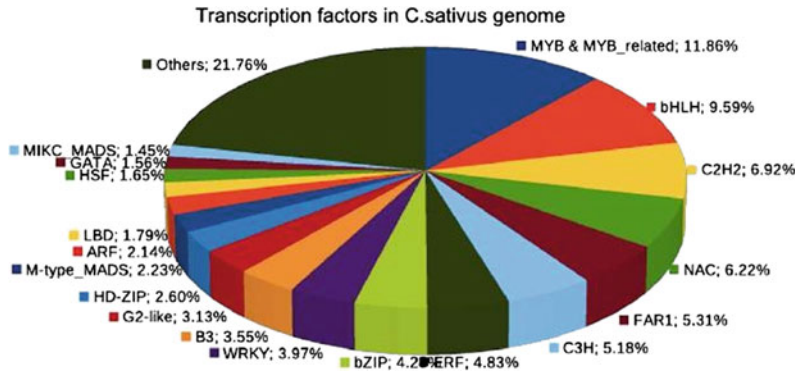

Fig. 2.4 Transcription factors identified in *Crocus sativus* genome wherein MYB and MYB-related Tfs were most abundant. *Source* Ambardar et al. (2022)

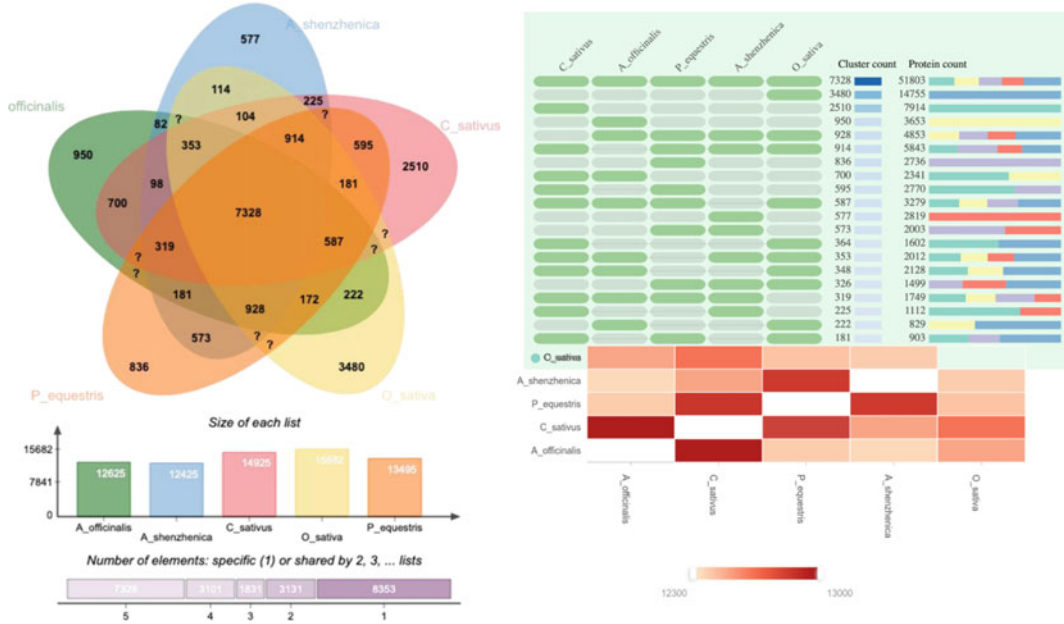

Fig. 2.5 **a** Orthology analysis of *C. sativus* with neighboring plants from the same order along with Rice. **b** Conserved and unique protein clusters and proteins. **c** Heatmap of overlapping cluster numbers between each pair of plant species representing more number of overlapping clusters between *C. sativus* and *A. officinalis* *Source* Ambardar et al. (2022)

metabolism; biosynthesis of terpenoids, polyketides, and other secondary metabolites (Ambardar et al. 2022). Since, Saffron (dried stigma) is enriched with apocaritenoids like crocins, picrocrocin, and safranal, the biosynthesis of apocarotenoid pathway and associated genes was studied in detail. All the genes encoding the enzymes involved in the carotene biosynthesis pathway, regulating the apocarotenoids synthesis, were present in the *C. sativus* genome, despite the draft genome was fragmented.

2.5 Conclusion

The present study was conducted to establish a de-novo reference genome of *C. sativus* for the first time. De-novo assembly of *C. sativus* has been constructed using only Illumina short read, thus, has a large number of scaffolds and assembly gaps thereby indicating that our assembly should be referred to as a draft genome sequence. The draft genome of *C. sativus* has been assembled using Illumina sequencing and is 3.01 Gb long with an N50 value of 1860, covering 84.24% of the genome. A total of 53,546 functional genes were identified from *C. sativus* genome including 5726 transcription factors. In addition, 862,275 repeats and 964,231 SSR markers were also identified from the *C. sativus* genome. Many metabolic pathways along with the associated genes were identified in the *C. sativus* genome assembly including the apocarotenoids biosynthesis pathway that is responsible for the production of crocin, crocetin, picrocrocin, and safranal. In addition, evolutionary relationships of *C. sativus* with other plants of the same order have been revealed via comparative genomic analysis. Nevertheless, this study represents the first attempt to assemble the *C. sativus* genome, providing a valuable resource for the community to facilitate future research. The draft genome can be further used to identify the genes of agronomical importance and regulatory mechanisms behind different genes, discover and develop genetic markers, and improve knowledge of breeding and genetic mapping.

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SaffronOMICS: Novel Approaches Toward Putting Saffron Data at Work

3

Seyed Alireza Salami and Amjad M. Husaini

Abstract

Saffron genome is very complex; therefore, usual tools to survey its genome and transcriptome have not been able to answer many questions yet. Recently, several new approaches have been developed based on OMICS methodologies and led to a better understanding of genes and related networks and biological processes in saffron. Consequently, the enormous amount of data generated should be processed and analyzed. High-throughput OMICS approaches enabled us to access a comprehensive list of genes and their predicted products and have changed our insights deeply toward a better understanding of different biological pathways, genes structure, expression and function, gene and genome editing, and regulatory engineering. Many efforts have been performed to increase our knowledge about the saffron genome, transcriptome, proteome, and metabolome which revealed several genes responsible for flavor, aroma, and color, especially in carotenoid and flavonoid pathways. Sequencing the whole genome of saffron just recently has provided a spark of light to answer the questions and ambiguities in saffron more accurately. These genomic sequences could be integrated with transcriptome, proteome, and metabolome data to unravel the mysteries of the biological process, origin, and ancestral parentage of red gold.

3.1 Introduction

Saffron (*Crocus sativus* L.) belongs to the Iridaceae family. Due to its widespread use, limited area of cultivation, and high demands in coloring, perfumery, flavoring, and pharmaceutical industries, saffron is known as the highest-value spice in the world (Ahmad et al. 2014; Vahedi et al. 2014, 2018; Moradi et al. 2021, 2022). Saffron is an autumn flowering plant with purple flowers while other *Crocus* species emerge their magnificent colorful flowers (yellow, orange, pink, white, violet, etc.) also in spring. More than 235 *Crocus* species have been reported so far (Rukšāns 2017). Wild *Crocus* species are also of particular importance due to their relevance to *C. sativus* and theories of saffron origin and parentage, as an alternative source for apocarotenoids, and ornamental and pharmaceutical

S. A. Salami (✉)
Department of Horticultural Sciences,
Faculty of Agricultural Science and Engineering,
University of Tehran, Karaj, Iran
e-mail: asalami@ut.ac.ir

A. M. Husaini
Genome Engineering and Societal Biotechnology Lab., Division of Plant Biotechnology, University of Agricultural Sciences and Technology of Kashmir, Shalimar Campus, Srinagar, Jammu and Kashmir, India

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properties (Fernández 2004; Ordoudi et al. 2019; Zengin et al. 2020; Taheri-Dehkordi et al. 2020).

Saffron stigma contains several hundred aromatic and non-volatile compounds, many of which are carotenoids such as Zeaxanthin, Lycopene, α-carotene, and β-carotene (Gomez-Gomez et al. 2010). It also contains remarkable amounts of apocarotenoids such as crocins, picrocrocin, and safranal which are responsible for its color, bitter aroma, and taste, respectively (Singla and Bhat 2011; Rezaee and Hosseinzadeh 2013). These compounds have several therapeutic effects against cancers, inflammation, respiratory infections, scarlet fever, smallpox, hypoxia, asthma, blood disorders, insomnia, paralysis, heart diseases, flatulence, stomach upsets and disorders, gout, chronic uterine hemorrhage, dysmenorrhea, amenorrhea, baby colic, and eye disorders. Saffron is also an aphrodisiac, a digestive stimulant, and a tonic for dysentery and measles (Abdullaev and Frenkel 1999; Rezaee-Khorasany et al. 2019). It also contains camphorols (Carmona et al. 2007), which encrypt the core genes of the carotenoid pathway enzymes (Cuningham and Gantt 1998). With such a wide range of nutrient and pharmacological activities, these compounds are of huge interest in the food, beverage, and pharmaceutical industries.

A unique gene network is underlying apocarotenoid production in saffron and a few other wild *Crocus* species. Revealing the regulatory mechanisms of genes in the apocarotenoid pathway is likely to help in its metabolic engineering to produce a higher amount of bioactive compounds by either up-regulation and down-regulation or knockout of the key genes (Caldentey and Inze 2004). Rapid advances in OMICS sciences and biotechnological approaches in recent years facilitate the breeding programs in *Crocuses*. Using integrative OMICS approaches toward characterization of the saffron genome, transcriptome, proteome, and metabolome in parallel with the development of bioinformatics tools to analyze and integrate the data is likely to shed light on the molecular basis of flavor and color biogenesis in saffron toward its breeding (Husaini and Asharaf 2010). OMICS-based studies of saffron can pave the way for its sustainable production in many regions of the world, far from its origin or major area under cultivation (Haq et al. 2022). The present chapter focuses on the recent developments in OMICS-based research conducted in *C. sativus* toward putting carotenoids in food and health basket.

3.2 How OMICS-Based Approaches Benefit Saffron Trade

Major indices for successful saffron business in the global markets are quality and yield. Saffron is mainly used as a spice and as a dye in food. It has been also used as textile handicrafts and medieval Islamic miniatures, as a perfume, and as a folk herbal medicine. Saffron is extremely expensive due to the manual labor required for its cultivation, harvesting, and handling. Global saffron producers are Iran with very large differences in production followed by India, Greece, Afghanistan, Morocco, Spain, Italy, China, and Azerbaijan. In a globalized and technologically advanced saffron market, the winner is who adapts with rapid innovation and evolution of technologies mainly those that improve the added value to saffron quantity and quality.

OMICS tools appear as promising high-throughput approaches to authenticate the quality, safety, and origin of any food commodities including saffron. On the other hand, such technologies may also answer major questions regarding saffron origin and evolution, and different phytochemical profiles of saffron populations belong to different geographical regions. The main scopes and aims to coordinate SaffronOMICS studies are crop improvement, adulteration and origin of saffron, traceability of the product, and determination of authenticity. These technologies included genomics, transcriptomics, proteomics, metabolomics, and many other "omics", if integrated together can not only attribute to a deeper understanding of the biology of saffron but also allow us to put saffron data at work toward saffron breeding and maximum efficiency.

Current concerns in commercial saffron production include genetic erosion due to sterility as

a triploid plant and fraud. One of the major crucial questions right now is where saffron originated and how it evolved? Fraud is the other main issue in the saffron market that must be referred. The sites where the first saffron plants appeared differ according to the opinion of various authors and might be somehow strategic and political. However, from scientific points of view, new emerging OMICS technologies and the new generation of sequencing platforms could be able to decode the genome of the red gold to answer this question. Saffron fraud involves two issues: mixing other substances with authentic saffron and selling saffron from one country as a product of another region. In this regard, OMICS-based approaches not only could help us in the deep investigation of saffron codes but also could help us to add a value to its trade through removing such issues.

3.3 OMICS Approaches in Saffron

A phenotype of an organism is a consequence of a set of functionally integrated processes that occur at various levels of DNA, RNAs, proteins, and metabolites within cells. Genetic background and its basic information can be transcribed into transcriptome which later translates the proteins and generates the proteome. These proteins then synthesize primary and secondary metabolites (metabolome). OMICS approaches mainly explore the genome, transcriptome, proteome, and metabolome. These technologies reveal the relationships among genes and their regulatory networks underlying biological processes based on specific genetic and evolutionary contexts. Such studies create a comprehensive view of the genome and its components and regulatory elements and pave the way for the genetic engineering of plants to improve the desired traits (Urano et al. 2010; Fleury et al. 2010).

SaffronOMICS, an initiative of the European Cooperation in Science and Technology (COST), and CrocOMICS, funded by Dr. Salami in 2017 at the Center for Genetic Resources of *Crocuses* and Saffron, Iran (CGRCS), aim to extend and strengthen collaborative joint research projects globally on developing "omics" approaches to decode the saffron and *Crocus* species genomes and survey and annotate the genomes of genus *Crocus*, molecular phylogeny and ancestry relationships of *Crocuses*, DNA fingerprinting, transcriptomics, phytochemical profiling and metabolomics, proteomics, and breeding of these valuable crops. These initiatives besides other frameworks such as OSEC (Organization of the Saffron Producing and Exporting Countries) aim to develop a sustainable economy based on High Value Agricultural Products (HVAPs). Achieving this goal is not possible except with crop improvement, traceability of the product, determination of authenticity, and approval of adulteration and origin of saffron.

Tools have been steadily developed particularly during the last two decades to investigate gene structure, gene expression, and function. These are included new cloning methods (GateWay and TOPO cloning), microarray, mass spectroscopy (MS), gene silencing (VIGS and RNAi), and novel gene and genome editing tools (CRISPR-Cas9). Among these approaches, high-throughput Next-Generation Sequencing (NGS) coupled with bioinformatics tools and pipelines have made the sequencing of complex and huge genomes possible. Genomics alongside other omics approaches facilitate breeding programs. Several NGS applications have been developed toward plant breeding such as de novo genome sequencing, whole-genome re-sequencing, investigation of genetic diversity and population structure using Genotyping-by-Sequencing (GBS), studying of the domestication and evolution, Transcriptome profiling, small RNA sequencing and post-transcriptome studies, epigenetics and methylation studies, DNA–protein interaction using Chip-Seq., and mapping (Ray and Satya 2014) (Fig. 3.1). Consequently, huge amounts of data have been produced by high-throughput sequencing approaches leading to the development of various bioinformatics algorithms, and it has made the computational biology and big data more prominent. On the other hand, the wealth of data generated by high-throughput methodologies will advance our understanding of gene structure and functions

and biological pathways such as apocarotenoids biosynthesis network. As in many other species, bioinformatics plays a crucial role in the structural characterization of the saffron genome, transcriptome, and proteome. Characterization of the saffron genome will reveal the possible genetic diversity of various geographical distinct saffron accessions. Characterization of stigmas and petals transcriptome reveals the molecular basis of aroma, flavor, and color in saffron and wild *Crocus* species, toward a better understanding of the pathways involved in the biosynthesis of secondary metabolites mainly crocins, crocetin, safranal, and picrocrocin.

3.4 Genomics of Saffron and Allied Species

A great revolution has emerged by publishing genome and genomic data of many crops including saffron (Ambardar et al. 2021). Today we are witnessing a data tsunami as a result of sequencing projects. Data generated through whole-genome sequencing (WGS) projects unveiled the mysteries behind the plants' genome and have changed our insights deeply. Among many genome projects, the saffron de novo genome sequencing project has been one of the projects of interest to researchers due to many reasons (Ambardar et al. 2021).

Historically, many efforts have been performed before saffron genome sequencing for a better understanding of the genome structure, genome variation, genomic organization, and gene expression networks in *Crocus* species. The unique biosynthesis pathway of apocarotenoids in the stigma as well as the biosynthesis of other secondary metabolites have been studied by several authors (Bouvier et al. 2003; Moraga et al. 2004, 2009; Castillo et al. 2005; Rubio et al. 2008; Ahrazem et al. 2010a, b; Trapero et al. 2012).

Various molecular markers (RAPDs, ISSRs, AFLPs, SSRs, GBS, etc.), different genetic and

Fig. 3.1 Next-generation sequencing (NGS) applications toward saffron breeding

phylogenic barcodes (*rpoC1*, *matK*, *tmH-psbA*, etc.), and epigenetic approaches were used and most of them have suggested the existence of limited genetic variability in saffron which strengthens the theory that saffron is monomorphic species (Chase et al. 2007; Moraga et al. 2009; Seberg and Peterson 2009; Siracusa et al. 2013; Nemati et al. 2014; Mir et al. 2015, 2021; Busconi et al. 2015). Although, there are evidence which show that different saffron populations harbor some degree of polymorphism.

The preliminary analysis of DNA methylation level using the methyl-sensitive AFLP technique has revealed differences in DNA methylation among saffron accessions (Busconi et al. 2015). Later, an epigenetic approach using methylation-sensitive restriction enzyme-sequencing (MRE-seq) revealed high epigenetic variability among saffron accessions due to methylated regions in transcription factors responsible for flowering (MADS-box and TFL) and biosynthesis of pigments (MYB). Results also supported a possible signature of selection among accessions collected from different geographical regions (Busconi et al. 2021).

Seberg and Peterson (2009) verified the robustness of *rpoC1*, *matK,* and *tmH-psbA* proposed by Chase et al. (2007), plus *rps8-rpl36*, *accD,* and *ndhF* on 86 *Crocus* species. Results showed that although DNA barcoding still has some limitations to be ideal, it can represent a promising system to survey standardized portions of the genome. Gismondi et al. (2013) analyzed different *Crocus* genomes using DNA barcodes toward extending the molecular phylogeny of this genus. They argued that independent events due to several geographical pressures might be the major reasons for evolving different *Crocus* species. They also confirmed the potential of DNA barcodes for interspecific and intraspecific identification, population studies, and discriminating and certificate saffron authenticity.

Among various molecular markers, GBS could provide a good coverage of a genome to screen nucleotide polymorphisms (SNPs) without any prior sequence information (Elshire et al. 2011). GBS markers should be able to infer the species history, parent, and area of origin of saffron (Nemati et al. 2019). Although data needs to be validated using more samples from a wider geographical area, especially possible areas of saffron origin, however, results showed that saffron is an autotriploid, and *Crocus cartwrightianus* from Greek might be the sole progenitor of the saffron (Nemati et al. 2019).

Although saffron may have been at the forefront of the list of genome projects, there were limitations that have made the genome of this valuable plant just recently sequenced (Ambardar et al. 2021). The Saffron genome is relatively large, complex, and poorly characterized. Saffron is a perennial triploid sterile plant ($2n = 3x = 24$) with a rare genetic variation between populations due to its vegetative reproduction nature and also rare somatic mutations, segregation distortions, and transversions (Brandizzi and Grilli Caiola 1998; Agayev et al. 2009). The haploid genome size of *C. sativus* was estimated to be 3.5 Gb and the draft genome is 3.01 Gb long with 84.24% genome coverage (Ambardar et al. 2021). The presence of repetitive sequences in the structure of saffron DNA has severely limited the genomic studies on this plant (Brandizzi and Grilli Caiola 1998; Fernández 2004; Qian et al. 2019).

Therefore, to discover authentic genetic markers and single nucleotide polymorphisms (SNPs), mine functional genes related to secondary metabolites, unlock the secret of saffron origin, and improve of saffron breeding, sequencing of its genome was the only alternative that has been just recently achieved (Alsayied et al. 2015; Nemati et al. 2019; Ambardar et al. 2021).

3.5 Transcriptomics

Several techniques are available to discover the gene expression profile at various developmental stages and/or in response to biotic or abiotic stresses or elicitors. These include traditional Northern blot or more novel approaches such as Q-RT-PCR, microarray, EST sequencing, Serial Analysis of Gene Expression (SAGE), Massively Parallel Signature Sequencing (MPSS™), and RNA-Seq (Velculescu et al. 1995; Enjuto et al.

1995; Cushman and Bohnert 2000; Brenner et al. 2000; Wang et al. 2009; Ali et al. 2011). Among different approaches, the development of high-throughput sequencing methods (ESTs sequencing, SAGE, MPSS™, and RNA-Seq) has changed our insights into gene expression and gene regulatory networks underlying biological processes such as the biosynthesis of secondary metabolites.

One of the major resources used to screen mRNAs and discover their structure, diversity, and expression is ESTs (Dong et al. 2005). The first set of 6603 high-quality ESTs from cDNA library of a saffron stigma is accessible through the *Saffron Genes* database http://www.saffrongenes.org (D'Agostino et al. 2007).

Many challenges of traditional transcriptome analysis were resolved by emerging of NGS (Garg and Jain 2013). RNA-Seq made it possible to accurately identify and study the expression of genes involved in regulatory and metabolic networks (Wang et al. 2009; Marguerat and Bähler 2010). The first report on high-throughput transcriptome sequencing of *Crocus* stigma and flower using the Illumina platform has been presented by Baba et al. (2015). 64,604,402 flower and 51,350,714 stigma-generated reads provided a rich source for understanding the carotenoid/apocarotenoid metabolism in *Crocus*. Among 64,438 transcripts, 41.5% were specific to *Crocus,* and among them, transcription factors mainly Myb played a significant role. Jain et al. (2016) sequenced the transcriptome of five different tissues/organs of *C. sativus* and revealed 16,721 SSRs and 3819 transcription factors (MYB, WRKY, C2C2-YABBY, and bHLH) might be involved in apocarotenoids biosynthesis (Jain et al. 2016).

As mentioned above, previous reports proposed the gene expression patterns involved in apocarotenoid biosynthesis in saffron; however, the mechanism(s) that regulates the tissue or developmental stage-specific biosynthesis of apocarotenoids remained unknown. Toward this goal, transcriptome-wide identification revealed that C2H2, C3H, and AN20/AN1 transcription factors may regulate apocarotenoid biosynthesis in saffron. CsSAP09 was highly expressed in stigma at the anthesis stage and was highly induced in response to abiotic stresses (Malik and Asharaf 2017).

Identifying flowering regulatory genes and possible molecular transition mechanism(s) underlying flowering leads to high saffron yield and quality by increasing the number of flowers and stigma properties. Toward these goals, high-throughput differential expression of genes in flowering versus non-flowering saffron plants revealed 62 putative flower-related genes involved in vernalization (PB.20221.2, PB.38952.1), gibberellins (G3OX, G2OX), photoperiod (PHYB, TEM1, and PIF4), autonomous (FCA), and age (SPLs) pathways. Patterns matched the special and temporal dynamics of gene expression essential for flowering in saffron (Fig. 3.2) (Qian et al. 2019). Hu et al. (2020) proposed another hypothetical model for the regulatory networks of flowering transition in saffron based on transcriptome data obtained from apical buds during an undifferentiated stage, the early flower bud differentiation, and the late flower bud differentiation (Fig. 3.3). 165 differentially expressed flowering-related unigenes were enriched in the hormone signal transduction pathways (GA3, auxin, ABA, and zeatin), sugar metabolism, photoperiod, and autonomous pathways. Merging two models propose a central core of genes that may be involved in flowering and flowering transition in saffron including *FD*, *FT*, *SPL1*, *SOC1*, *LFY*, *AP1*, and *AP2*.

A gene regulatory network is also responsible for crocins biosynthesis and accumulation in saffron which is developmentally controlled. RNA-Seq analysis of stigmas of *C. sativus*, *C. cartwrightianus*, and *C. ancyrensis* revealed potential regulatory transcription factors involved in crocins production over time. Among up- and down-regulated transcription factors, 11 of them (ARF, bHLH, C2H2, HB, CBF/DREB1, ALFIN, and NF-YC families) are expressed in the stigma and seven of them were directly related to apocarotenoid biosynthesis (Ahrazem et al. 2019).

Apocarotenoids biosynthesis and accumulation and flower initiation and formation regulatory networks have not been the only targets of

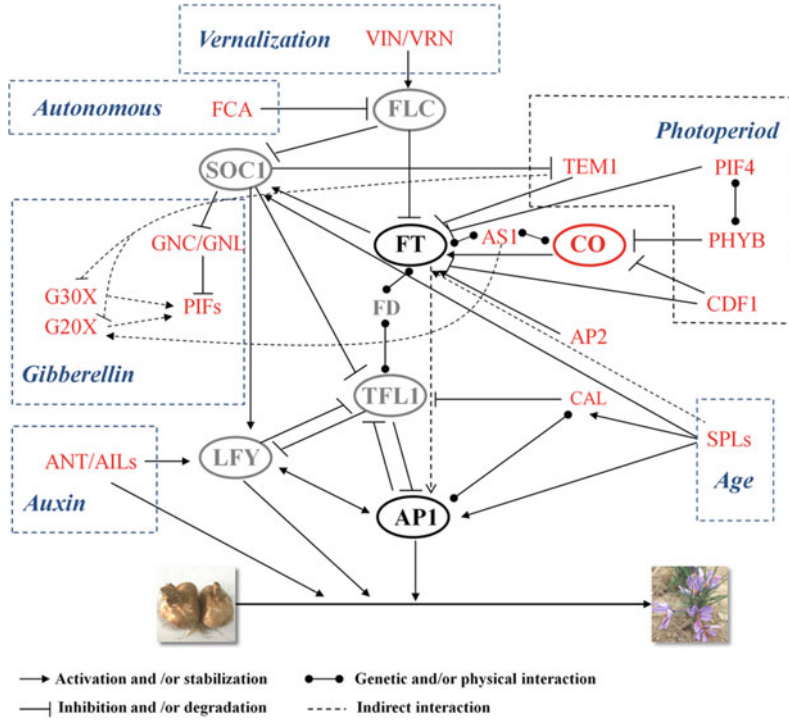

Fig. 3.2 Schematic diagram of gene regulatory network involved in flowering of saffron (Qian et al. 2019)

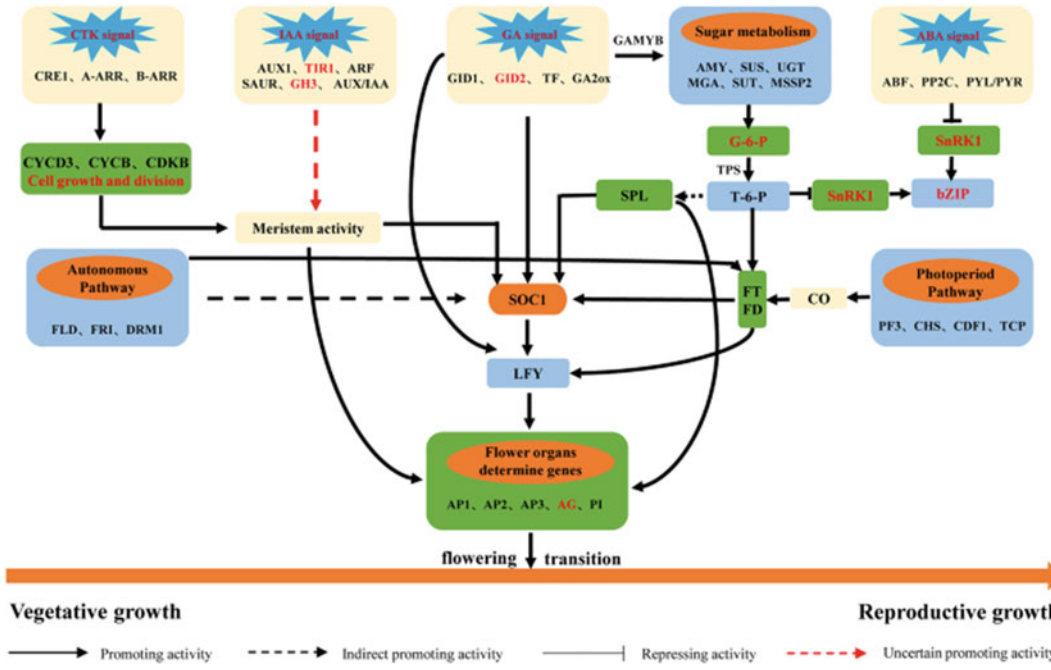

Fig. 3.3 Hypothetical model for the molecular regulatory networks of flowering transition in the saffron (Hu et al. 2020)

transcriptome studies. Transcript profiling of carotenoid/apocarotenoid biosynthesis genes was also performed during corm development toward a better understanding of molecular mechanisms underlying bud dormancy/growth in saffron which eventually affect its yield and quality. Expression profiles of related genes during eight developmental stages showed the lowest expression of *CsPSY*, *CsPDS*, *CsZDS*, *CsCRTISO*, *CsLYC-β1*, *CsLYC-ε*, *CsBCH2*, and *CsNCED* genes during the dormant period, while *CsZCO*, *CsCCD2*, *CsUGT*, and *CsALDH* expression was increased during reproductive period and floral differentiation (Sharma et al. 2019).

Yue et al. (2020) captured about 32,000 non-redundant transcripts which may unveil the evolutionary secret of saffron and its secondary metabolite biosynthetic pathways. The results suggested that the genome may have been subjected to at least two rounds of whole-genome duplication (WGD) events. Possible divergence of CCD2 from the CCD1 family and enhanced accumulation of three major distinct compounds, i.e. crocins, picrocrocin, and safranal in the stigma are the consequence of the recent β WGD event. These findings provide clues for further genetic studies, breeding programs, and crop improvement for saffron in Iran and other saffron producing countries.

Whole transcriptome data revealed that cytochrome P450 (*CYP* genes) are involved in crocins biosynthesis in saffron. Among 487 up-regulated and 1021 down-regulated genes, 12 major *CYP* genes were differentially expressed. Results showed that *CYP72A219*, *CYP72A15*, *CYP97B2*, *CYP71A1*, and *CYP86A8* were all expressed in the pistils at a high level and may be possible candidates involved in crocins biosynthesis (Gao et al. 2021).

Salami et al. (Unpublished data) studied the de novo transcriptome assembly of "Red Gold" stigmas and embryogenic and non-embryogenic calli using Illumina RNA-Seq. A total of 262,024 transcripts with a mean size of 607 bp and N50 of 884 bp were obtained from filtered reads by Bridger as the best assembler with k-mer 25. DGE analysis showed 22,579 and 34,768 transcripts as co-expressed transcripts specific to stigma and calli. GO analysis revealed that the cellular component was most represented while TF analysis showed that bHLH was the most highly represented family. A total of 33,093 SSRs from 262,024 cDNA sequences were obtained. Whole transcriptome analysis and RT-PCR confirmed that apocarotenoid biosynthesis genes are highly up-regulated in the stigma tissue as compared to calli, providing molecular basis for the accumulation of apocarotenoids in stigma. This study will provide a basis to better understand the saffron biology and will reinforce further research endeavors and get new insights into multiple stigma formation, apocarotenoid biosynthesis, and somatic embryogenesis in saffron (Salami et al. Unpublished data).

Data processing, data analysis, and interpretation are the major bottlenecks while working with sequencing reads regardless of the platforms used. Complete reconstruction of the transcripts from a large number of short reads brings great computational challenges (Clarke et al. 2013; Zhao et al. 2011). The sequencing error rate and data validation should also be considered as other main issues. So far, many software packages such as Abyss (Simpson et al. 2009), Trinity (Grabherr et al. 2011), Velvet/Oase (Schulz et al. 2012), SOAPdenovo-Trans (Xie et al. 2014), Bridger (Chang et al. 2015), BinPacker (Liu et al. 2016) have been developed for assembly readings. Graph construction Overlap graph and De Bruijn are the two main strategies used by the above software for assembly (Grabherr et al. 2011; Chang et al. 2015). The performance of de novo assembly tools including BinPacker, Bridger, Oases-Velvet, and Trinity can be tested by considering quality metrics such as N50 length, the total number of contigs, and alignment scores. Vahedi et al. (2019) reported better performance of Bridger than other assemblers in saffron. Oases suffered from relatively high chimera rates and redundancies which cause genes families with high similarity assembled into one transcript. Although Trinity showed good performance, however, it created more false positives than Bridger.

Among the various methods used to study the differential expression of transcripts, Q-RT-PCR

is one of the most accurate methods and considered as the gold standard to assess changes in gene expression profile (Gutierrez et al. 2008). Real-time PCR is still widely used to confirm information from comprehensive transcriptome analysis including RNA-Seq (Ahrazem et al. 2018; Qian et al. 2019). As a gold standard, data normalization is important which is accomplished using internal control genes called housekeeping genes (Bustin 2000). Among several housekeeping genes that were applied for relative gene expression analysis, *tubulin*, *18S rRNA*, *actin*, *EF-Iα*, *Ubiquitin*, and *rbcl* are more popular (Gomez-Gomez et al. 2018; Ahrazem et al. 2018; Qian et al. 2019; Hu et al. 2020; Taheri-Dehkordi et al. 2020). The expression of an internal control gene must be constant between different tissues, different treatments, and during all developmental stages (Bustin 2000; Wang et al. 2019). Therefore, in each study, the stability of reference genes should be evaluated to select the most appropriate reference gene in order to normalize the data (Jain et al. 2006; Jaiswal et al. 2019).

At the post-transcriptional level, small RNAs and other non-coding RNAs play important regulatory roles in secondary metabolic biosynthesis and many other cellular processes (Ng et al. 2011; Boke et al. 2015; Taheri-Dehkordi et al. 2021). These negative regulators repress target genes through mRNA cleavage or translational inhibition (Bartel 2004; Voinnet 2009). Despite important functions, their regulatory roles in saffron have not been thoroughly investigated. Deep transcriptome sequencing of different *Crocus* tissues using specific Library Preparation Kits enables us to capture other types of RNA molecules than mRNAs such as miRNAs and lncRNAs which led to the identification of transcriptional regulators and their targets involved in the biosynthesis of apocarotenoids. So far, little is known about the regulatory roles of miRNAs in saffron stigma and other tissues.

ESTs can be considered as one of the major resources for miRNAs prediction particularly when there is a lack of genomics and transcriptome data. Three novel miRNAs, namely csa-miR1, csa-miR2, and csa-miR3, were forecasted in saffron by in silico ESTs computational analysis which targeted the genes involved in plant growth, biotic and abiotic stresses, senescence, translation, and post-translational modifications (Guleria et al. 2012). Two other novel miRNAs and their targets were also predicted in silico in mature stigmas. miR414 and miR837-5p targeted six transcription factors and one protein kinase probably involved in the carotenoid/apocarotenoid biosynthetic pathway (Zinati et al. 2016).

A robust workflow was developed for in silico computationally identifying miRNAs and their targets in a saffron based on transcriptome data (Fig. 3.4) (Taheri-Dehkordi et al. 2021). The efficiency of several assembly tools such as Trans-ABySS, Trinity, Bridger, SPAdes (3.14.1), and Evidentialgene was evaluated based on both reference-based and non-reference-based metrics. The Evidentialgene was the best de novo transcriptome assembler for saffron, followed by Trinity. A total of 66 miRNAs targeted 2880 genes including several transcription factors. Interestingly, *CCD* and *UGT* genes were among the candidate targets of csa-miR156g and csa-miR156b-3p, respectively. Whereas, previous data represented no specific apocarotenoid genes targeted by miRNAs (Yue et al. 2020). This could be due to the power of integrated analysis and the existence of an innate specific regulation dynamic of the post-transcriptional process in saffron (Taheri-Dehkordi et al. 2021).

To extend the unigene collections and provide a comprehensive atlas of *Crocus* transcriptome toward integrated OMICS studies, more detailed transcriptome data should be generated with a focus on different floral tissues at different developmental stages, wild *Crocuses* mainly ancestral species, and various growing conditions. In parallel, due to the large genome size, developments of targeted sequencing approaches to capture a certain number of saffron genes will allow the study of functional SNPs and epigenetic modifications for a much lower price compared to the WGS and re-sequencing.

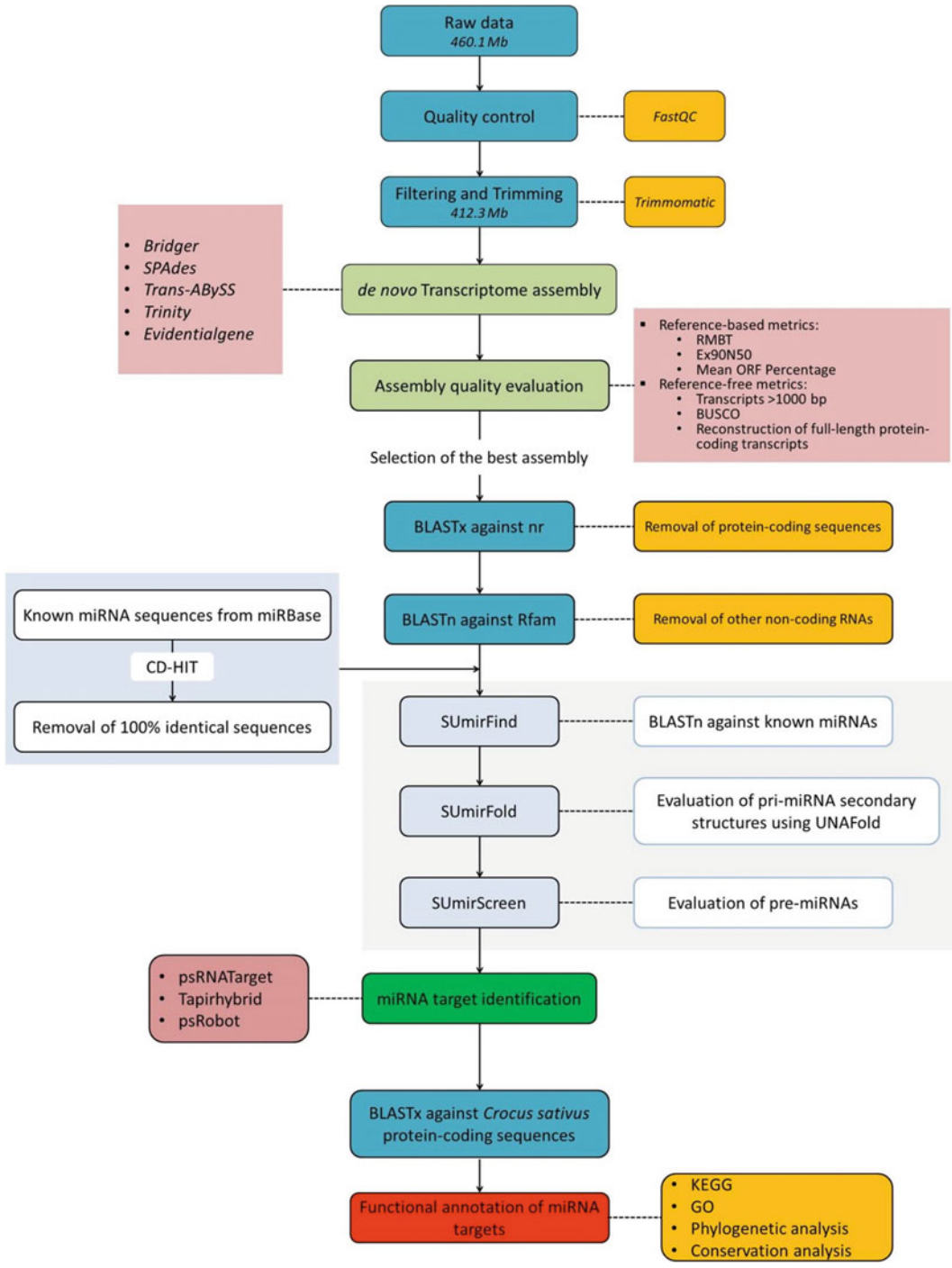

Fig. 3.4 Workflow of in silico miRNA identification in the transcriptome of saffron (Taheri-Dehkordi et al. 2021)

3.6 Metabolomics

Metabolome is a collection of cellular chemical species with very diverse physico-chemical properties associated to a specific genomic background. Metabolomics is defined as the comprehensive study and analyses of metabolomes. It includes tools and approaches for the separation, characterization, and quantification of a wide variety of compounds such as metabolites that are involved in regulatory pathways (Beale and Sussman 2011). Plant metabolomics helps to improve our deep understanding of plant biochemistry and metabolism toward plant functional genomics and make a link between genotypes and phenotypes. In addition, it will provide unique insights into the growth and development, physiology, stress resistance, biodiversity, etc. (Fiehn et al. 2000; Fiehn 2002).

Technically, mass spectrometry (MS) alone or coupled with liquid chromatography–mass spectrometry (LC–MS), capillary electrophoresis–mass spectrometry (CE-MS) and gas chromatography–mass spectrometry (GC–MS), ultra-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UPLC-QTof-MS/MS), ultra-high-resolution Fourier transform ion cyclotron MS (FT-MS), and nuclear magnetic resonance (NMR) are the most commonly used tools in quantitative analysis of hundreds of plant metabolites (Aharoni et al. 2002; Jorge et al. 2016a, b; Xu et al. 2019).

Saffron metabolomics is a very interesting topic and provides a comprehensive insight into the compounds responsible for its color, aroma, and taste. Moreover, metabolomics data can serve as a marker for authentication and quality deterioration in saffron (Ordoudi et al. 2015). 1H NMR-based metabolomics not only revealed structural variations in crocetin esters and picrocrocin but also offered certain benefits for describing secondary metabolites and determining the storage threshold (4 years) for the freshness of saffron (Ordoudi et al. 2015). Metabolite fingerprinting using 1H NMR spectra was also reported for the authentication of Iranian and Italian saffron (Cagliani et al. 2015). Toward the same goal, an effort has been made to identify novel markers for saffron authenticity and quality assurance using LC/MS. A number of 34 metabolic authenticity markers were identified including kaempferol 3-O-glucoside, kaempferol 3-O-sophoroside, kaempferol 3,7-O-diglucoside, kaempferol 3,7,4'-O-triglucoside, kaempferol 3-O-sophoroside-7-O-glucoside, and geranyl-O-glucoside (Guijarro-Díez et al. 2015).

Targeted metabolomics approaches based on HPLC were performed to screen 53 saffron populations from Iran for crocins, picrocrocin, and safranal content. Results revealed a significant chemical diversity among populations (Vahedi et al. 2018).

An untargeted UHPLC-ESI/QTOF-MS approach was used to investigate the authenticity and traceability of saffron. According to the results, anthocyanins and glycosidic flavonols were the strongest markers of adulteration; whereas, flavonoids and hydroxybenzoic acids were the best markers of origin (Senizza et al. 2019). A total of 54 compounds were identified in floral parts of saffron including stigmas, tepals, and stamens by UPLC-QTof-MS/MS. Metabolite profiling distinguished coniferin and crocin-2 as two specific components in stigmas but not tepals and stamens. However, tepals are a rich source of flavonoids (Xu et al. 2019).

Toward functional characterization of sequenced genomes in the "postgenomics" era, metabolomics is expected to be a novel approach. Achieving the broadest overview of metabolic composition is very complex and entails establishing a fully integrated strategy for optimal sample extraction, separation, detection, identification, automated data gathering, analysis, and ultimately quantification. Therefore, both analytical and computational developments are essential to achieve these goals.

Simultaneous identification and quantification of metabolites in saffron are necessary to better understand its metabolome. In the future, metabolomics will play a key role in complementing datasets obtained from the existing "omics" technologies in saffron. However, the major challenges still remain in finding variations in

biochemical pathways and metabolic networks that might correlate with the physiological and developmental phenotype of a cell and tissue, lack of reference compounds, lack of a comprehensive available database, need for appropriate, and dedicated bioinformatics tools (Fukusaki and Kobayashi 2005).

Although different metabolomics approaches have been developed in saffron, however, still very limited information on *C. sativus* and particularly wild *Crocuses*' metabolome is available. From this point of view, a more extensive investigation of polar/non-polar metabolites involved in primary and secondary metabolism is needed, with a particular emphasis on volatile compounds presumably involved in the generation of all the organoleptic characteristics of saffron. Only in this way, there will be the chance to reach a better elucidation of the various biological processes that make saffron such a peculiar and attractive spice.

3.7 Proteomics

Proteomics has been defined as the study and analysis of total proteins expressed differentially at any time in any organ, tissue, and cell under various treatments and conditions (Wilkins 2009; Job et al. 2011). To better understand the biological role of proteins, identification and prediction of their three-dimensional (3D) structure and function are necessary (Pieper et al. 2006). A comprehensive archive of structural information for saffron strengthens our ability for functional annotation, evolutionary analyses, and building interaction networks (Pentony et al. 2012). Although proteomics studies hold promise in the characterization of both known and unknown proteins of saffron and a multitude of tools are available to predict and visualize secondary and tertiary structures of proteins to date, a limited number of protein sequence entries that belong to a few selected gene families in *Crocus* are reported in databases compared to the genomic and transcriptome data (http://www.ncbi.nlm.nih.gov/protein/?term=Saffron). Only a few of these inputs have been reviewed.

Somatic embryogenesis involves complex cellular and molecular changes and is considered as a very important process in many plant species including saffron. Proteomics data in saffron will definitely shed light on a better understanding of the expression networks underlying this phenomenon. Such data suggested a possible key role of ascorbate–glutathione in somatic embryogenesis (Sharifi et al. 2012). The proteome of *C. sativus* was also studied under cadmium (Cd) toxicity. Twenty-six proteins involved in different biological pathways were up- and down-regulated under Cd stress (Rao et al. 2017). Differential proteome data of flowering and non-flowering saffron buds under cold stress using isobaric tags for relative or absolute quantitation (iTRAQ) revealed a possible "reactive oxygen species–antioxidant system–starch/sucrose interconversion flowering pathway" which triggers the floral initiation under a crucial temperature (Chen et al. 2021).

Advances in proteomics approaches, modeling algorithms to predict 3D structures, and incorporation and integration of data would help to address various biological questions about saffron. Toward the construction of a comprehensive *Crocus* protein atlas, identification of proteins expresses in different *Crocus* tissues during different developmental stages and treatments, integrates those data with other omics datasets, and creates data integration and building networks as suggested.

3.8 History of Saffron Cultivation and Domestication

Saffron (*C. sativus* L.) is one of the most important plants in the Iridaceae family. Rate of ploidy, chromosome number ($2n = 2x = 6$ to 64), and genome size are variable in different *Crocus* species, whereas *C. sativus* is a sterile triploid ($2n = 3x = 24$). Due to vegetative propagation, there is very little genetic variation among saffron accessions. The origin of saffron is still a mystery. One of the saffron ancestors is very likely *C. cartwrightianus* ($2n = 2x = 16$) native to Greece which might be the sole

progenitor of the saffron (Nemati et al. 2019). Although, the origin of saffron and its ancestral species are still controversial. It is not completely clear if another species was involved as ancestor (s) of *C. sativus*. It is supposed that some *Crocus* species grown in Iran could be also a possible ancestor of *C. sativus* but further investigation at the genomic level needs to prove it. Eight wild *Crocus* species are growing in Iran (Fig. 3.5). Nuclear genome sequencing and surveying the chloroplast genome of wild species besides *C. sativus* would be a key point for revealing the phylogenetic structure and relationships of wild species and saffron.

The oldest evidence related to saffron cultivation and use is found in the Greek and dates back to more than 3500 years ago (Nemati et al. 2019). The origin of saffron appeared to differ in various reports and might be somehow strategic and political. According to Vavilov, the origin of saffron is the Middle East. However, saffron has been used in different formats in ancient Egypt, Greece, and Rome. Saffron was introduced in the Iberian Peninsula by Arabs and spread to the rest of Europe during the Middle Age. Saffron was first exported to China in the thirteenth century and then reached Japan at the beginning of the seventeenth century (Fernandez 2004). It seems that wild *C. cartwrightianus* which is quite close to saffron morphologically was used for different purposes, and then its mutant was domesticated (Negbi 1999). On the other hand, *C. sativus* is assumed to be an allopolyploid hybrid. Recent molecular investigations hypothesize the involvement of *C. cartwrightianus* and *Crocus thomasii* as parental species (Brandizzi and Grilli Caiola 1998; Tsaftaris et al. 2011) or *C. cartwrightianus* and probably *Crocus pallasi* (Harpke et al. 2013). Moreover, *C. cartwrightianus*, *C. thomasii*, *C. pallasii,* and *Crocus mathewii* from Turkey and *Crocus hausknechtii*, *Crocus almehensis*, *Crocus serotinus*, and *Crocus michelsonii* from Iran were reported as possible parents (Frello and Heslop-Harrison 2000; Grilli Caiola et al. 2011; Alavi-Kia et al. 2008; Petersen et al. 2008; Tsaftaris et al. 2011; Gismondi et al. 2013; Erol et al. 2014).

3.9 Saffron Genome Structure and Essential Key Points to Decode the Genome of Red Gold

The first plant genomes to be sequenced about twenty years ago were *Arabidopsis* 125 Mb/1C (The *Arabidopsis* Genome Initiative 2000) and rice 420 Mb/1C (Goff et al. 2002). However,

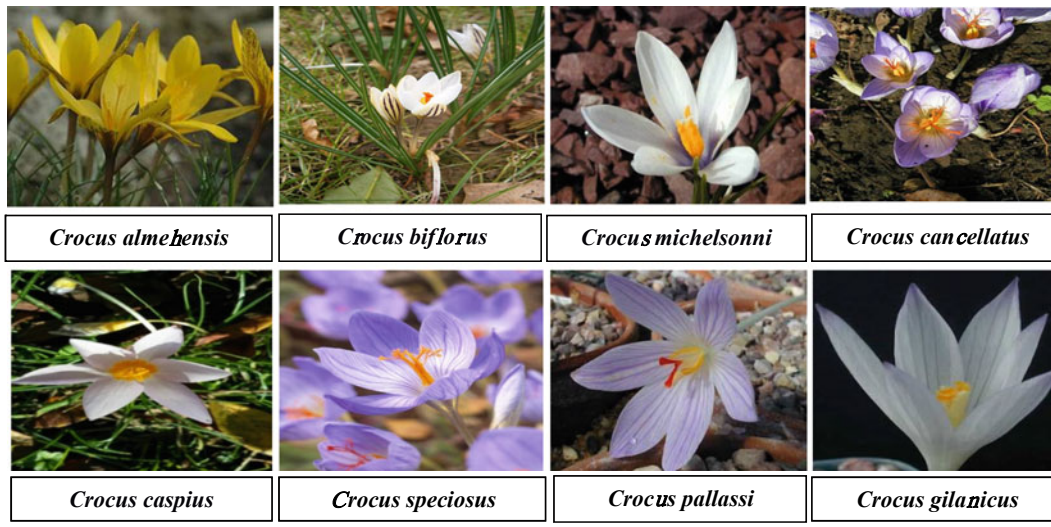

Fig. 3.5 Eight *Crocus* species are growing wild in Iran

current NGS platforms have revolutionized WGS in recent years. Until a few years ago, genome projects were limited to *Arabidopsis*, rice, maize, grapevine, and wheat; however, by emerging the NGS and different sequencing platforms, several hundred of plant genomes have been sequenced so far and more genome projects are in the waiting list (NCBI: https://www.ncbi.nlm.nih.gov/assembly) (Chen et al. 2019).

Different studies have demonstrated the karyotype of *C. sativus* ($3n = 3x = 24$) (Mather 1932; Karasawa 1933; Brighton 1977; Chichiriccò 1984; Ghaffari 1986; Agayev 2002). The genome size has been calculated as about 10.5 Gb by flow cytometry (Brandizzi and Grilli Caiola 1998) and hence the size of the 1C genome is about 3.55 Gb. Nevertheless, saffron de novo genome sequencing has been a big challenge due to the triploid nature of its genome and a large number of repetitive DNAs including retrotransposons, ribosomal DNA, and satellite DNAs (Schmidt and Heslop-Harrison 1998; Frello and Heslop-Harrison 2000; Alavi-Kia et al. 2008; Ambardar et al. 2021). Discriminating and sorting the triplets by flow cytometry face challenges. Although multi-color fluorescent in situ hybridization (FISH) of satellite DNA enabled the unequivocal identification of each individual chromosome. However, it seems that the only applicable strategy is a combination of short- and long-read WGS using Illumina and PacBio platforms. In brief, the major concerns regarding saffron genome sequencing include big genome size, different classes of repetitive DNA sequences, polyploidy, GC-rich genome, costs of the sequencing, and eventually data analysis. Therefore, to generate a high-resolution draft of the saffron genome and its putative ancestor(s), the following roadmap will be proposed:

- An Illumina-based WGS assembly using paired-end sequences and mate pair reads plus long-read PacBio-based WGS assembly.
- Single chromosome sequencing.
- Whole transcriptome sequencing (RNA-Seq) using different tissues.
- The annotation of the whole genome.
- Optical mapping.
- De novo identification of repetitive DNAs and physical mapping along chromosomes using FISH.
- WGS of putative saffron parental species alongside the Whole-genome re-sequencing of *C. sativus* from different regions of the world.

3.10 Decode the Genome of Red Gold

Saffron is mostly used as spice and food colorant and as a dye in textile handicrafts and medieval Islamic miniatures, as a perfume, and as a folk herbal medicine. Saffron is extremely expensive, due to its luxurious nature and the manual labor required for its cultivation, harvesting, and handling. The countless attractions and properties of saffron have made its genome sequencing project as one of the most controversial genome projects among many other genome projects. Emerge of second- and third-generation sequencing technologies, coupled with bioinformatics tools/pipelines, has made the sequencing of complex and huge genomes such as saffron easier.

The first draft of the saffron genome was recently published in June 2021 using the HiSeqX platform (150-bp paired-end reads) to generate 321 Gb data ($\sim 92\times$ coverage) (Ambardar et al. 2021). The draft genome was 3.01 Gb long, and totally, 862,275 repeats, 964,231 SSRs, and 5726 transcription factors have been identified. Although, it was a progressive research, however, as proposed above, short- and long-read sequencing combining Illumina and PacBio platforms alongside optical mapping will provide a higher resolution draft of the saffron genome. A comprehensive package of bioinformatics tools and pipelines was used to analyze and interpret the data including FastQC tool (Andrews 2010), trimmomatic software (Bolger et al. 2014), Soapdenovo2 (Luo et al. 2012), MaSuRCA (Zimin et al. 2013), BUSCO (Simão et al. 2015), Bowtie2 (Langmead 2010), BWA (Li and Durbin 2010), Epeatmasker and GenomeScope (Ranallo-Benavidez et al. 2020; Smit et al. 2015), etc. Raw reads and the draft

genome have been submitted in SRA under bioprojects PRJNA734464 and PRJNA739096, respectively. All the processed data is accessible at http://caps.ncb-s.res.in/download/csat.

Finally, the annotated saffron genome will facilitate the discovery of putative authentic genetic markers, genes related to secondary metabolites, transcription factors, and orthologous genes; resolve ambiguities related to the saffron origin and ancestry; and improve the breeding programs.

3.11 Conclusions

The genome has a level of biological organization. However, the gene context and expression must be also considered regarding the overall phenotype of an organism. Several tools along with omics approaches (genomics, transcriptomics, proteomics, metabolomics, etc.), have been developed rapidly for investigating gene structure and function over the past 40 years. Consequently, the enormous amount of data generated needs to be processed. Integration of multi-OMICS data not only advances our understanding of gene expression, gene structure, and function but also makes gene and genome editing and regulatory engineering possible. Nevertheless, the bottleneck is how to put saffron data at work which involves proper efficient strategies for data generation, data storage, data processing, data analysis, and visualization. To date, many efforts have been performed to increase our knowledge about the saffron genome, transcriptome, proteome, and metabolome, and several genes responsible for color, flavor, and aroma in saffron have been cloned. In the last decade, whole-genome sequences have been obtained for representatives of most major plant groups; however, the entire genome of saffron has been sequenced just recently that could be integrated with transcriptome, proteome, and metabolome data to unravel the mysteries of biological process and origin and ancestral parentage of red gold. A high resolution draft of saffron genome and eventually the annotated genome sequence also provides fundamental information toward its breeding.

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Bioinformatics for Saffron-Omics and Crop Improvement

4

Syed Anam Ul Haq, Seyed Alireza Salami, and Amjad M. Husaini

Abstract

Bioinformatics is the bedrock of modern studies using omics-based approaches like genomics, transcriptomics, proteomics, metabalomics, ionomics, etc. The complex web of molecular and genetic interactions that connect individual components of an organelle or a cell with the overall scheme of organismal behaviour cannot be elucidated without bioinformatics. There are hundreds of bioinformatics tools available to the researchers for conducting the studies involving the processing of high-throughput data. In this chapter, we highlight some of the major bioinformatics tools that are popularly used by plant biologists, and some of which have been used in saffron research for the analysis of complex data generated by modern omics-technologies. There is a huge scope for such molecular studies in saffron owing to its peculiar nature and importance as a spice, and medicinal plant. Bioinformatics is therefore indispensible for understanding the biology of saffron.

4.1 Introduction

Saffron (*Crocus sativus* L.) is a sterile triploid plant with a genome size of $1C = 3.45$ Gbp (Pandita 2021). Stigma part of saffron flower constitutes the saffron spice and to acquire 1 kg of saffron, 150,000–200,000 flowers must be gathered by hand. Each flower has three stigmas, which are separated and dried manually. The saffron spice is made up of these stigma threads. Saffron encompasses a number of therapeutic effects, including anticancer, antimutagenic, antioxidant, antiviral and even anti-COVID capabilities (Husaini et al. 2021; Premkumar and Ramesh 2010; Husaini and Wani 2020). Bioactive compounds in saffron have a wide range of medicinal applications, including coronary artery disease, neurological disorders, respiratory disorders, diabetes, fever and colds. A thorough examination of its medicinal characteristics has shown its enormous untapped medicinal potential, which might aid in the treatment of COVID-19 patients and post-COVID-19 complications (Husaini et al. 2021; Ahmed and Husaini 2021).

S. A. U. Haq · A. M. Husaini (✉)
Genome Engineering and Societal Biotechnology Lab, Division of Plant Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar Campus, Srinagar, Jammu and Kashmir 190025, India
e-mail: amjadhusaini@skuastkashmir.ac.in

S. A. Salami
Department of Horticultural Science, Faculty of Engineering and Agricultural Science, University of Tehran, Tehran, Iran

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4.2 Why Is Bioinformatics Important?

The researchers and scientists working in molecular biology face an immense challenge today owing to the generation of huge amount of data through high-throughput omics-based technologies. The scale at which data is generated has necessitated the advancement of technologies for 'processing' it at a faster pace for generating useful information. These have led to integration of biology with complicated algorithms, and modelling approaches, and have evolved into an advanced discipline called 'Bioinformatics', which is actually a short form of 'biological informatics'.

Bioinformatics is the bedrock for molecular studies on genome, transcriptome, proteome, metabalome, etc. in saffron and other plants (Husaini and Ashraf 2010). The complex web of molecular and genetic interactions that connect individual components of an organelle or a cell with the overall scheme of organismal behaviour can be elucidated only through an integrated approach involving advanced molecular biology, biostatistics, and information technology (Fig. 4.1). This systems biology approach depends upon the advances in bioinformatics applications. The disciplines of biological sciences, computer sciences and information technology merge in bioinformatics discipline (Husaini et al. 2009). It involves data management, algorithm development and data mining in the field of biology. Initially, bioinformatics was pushed by the need to create databases of biological sequences. The first database was created shortly after the insulin protein sequence was made available in 1956 (Jhala et al. 2011). The challenges faced by the bioinformatics community today include the efficient storage of data and providing easy access to it (Untergasser et al. 2007; Singh et al. 2011). Some of the major bioinformatics tools and databases which are useful for omics-based studies in plants are briefly discussed hereunder.

4.3 Bioinformatics for Saffron-Omics

In the first International Symposium on Saffron Biology and Biotechnology (Spain), it was stressed that germplasm bank and gene bank for

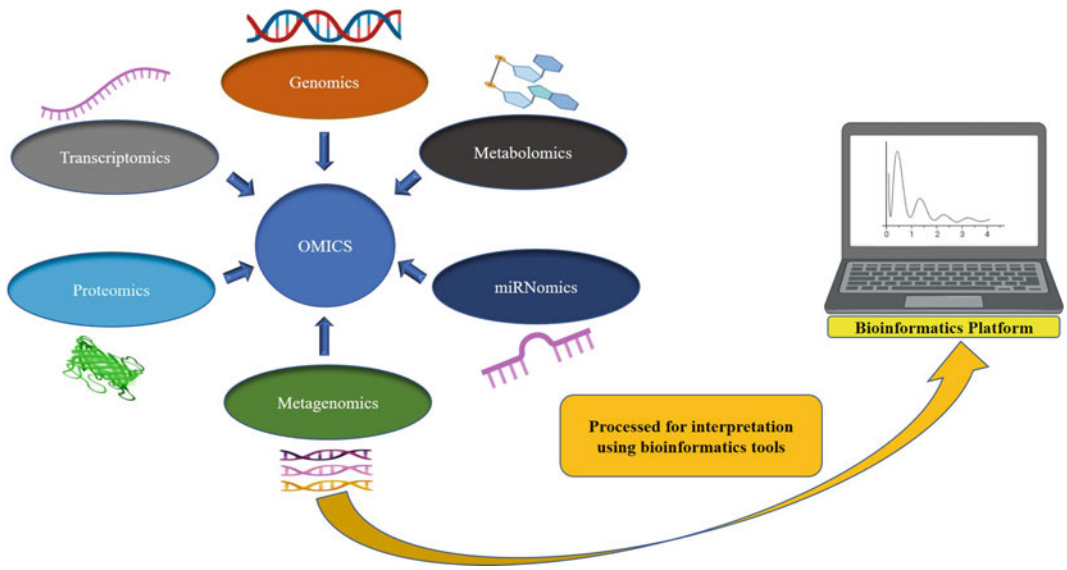

Fig. 4.1 Bioinformatics is central to deciphering meaningful 'information' from the high throughput data derived through the use of modern -omics studies

saffron should be created through the joint effort of saffron growing countries. A consortium named as CROCUSBANK was created with two major objectives. First objective was to collect and multiply saffron corms from all the countries that cultivate saffron; and the second was to collect the saffron allies for developing a better understanding about the evolution, taxonomy, physiology and ecology of Crocus sp. Bioinformatics tools have helped a lot in developing a better understanding of the peculiar saffron biology (Table 4.1).

Bioinformatics has played a critical role in the structural characterisation of saffron genomic DNA at the sequencing level. It has aided in determining the amount of cultivated saffron's genetic diversity, as predicted a decade ago in (Husaini et al. 2009). The *Arabidopsis thaliana* (L.) Heynh. genome was previously the sole significant information resource for modern genotyping and sequence characterisation available to plant biologists, having been disclosed in 2000 (Kaul et al. 2000). This information base was strengthened even more with the completion of the rice (*Oryza sativa* L.) genome project and the publishing of the rice genome sequence (Sasaki 2005). Despite the fact that many crop genome sequencing studies are rapidly producing a wide and diverse bank of knowledge on plant DNA sequences, (D'Agostino et al. 2007) just built a key database for saffron stigmas to manage and analyse expressed sequence tags (ESTs). D'Agostino et al. (2007) describe the database as the first reference collection for Iridaceae genetics, molecular biology of stigma formation, and metabolic pathways governing saffron secondary metabolism. Some saffron studies (Fernández 2004; Husaini et al. 2009; Gómez-Gómez et al. 2010) indicate the need for improved techniques for bioinformatics analysis of transcriptome and genomic data.

4.3.1 Saffron Flowering and Stigma Development

Large-scale saffron expression profiling studies have created massive amounts of data, and bioinformatics has proven useful in 'deriving insight' from this data. As expected, omics investigations and bioinformatics approaches used to characterise saffron stigmas have produced a significant new understanding about the molecular basis of taste, colour biogenesis, genomic organisation and the biology of the saffron gynoecium (Husaini et al. 2009). The function of the C-class MADS-box gene is critical for the production of saffron flowers (Wafai et al. 2019). Tsaftaris et al. (2004) used molecular techniques and bioinformatics tools to investigate the expression of MADS-box genes in saffron flowers. To better understand the process of saffron flower development, the relative transcript changes of the CsAP3 and CsNAP genes have been investigated across different phases of flower development. However, no direct link in the expression of these genes has been established (Wafai et al. 2015, 2019). Furthermore, (Wafai et al. 2019) examined how nuclear factors interact with the B class gene CsAP3 via the CArG1 promoter region. Following the extraction of nuclear proteins, a CArG1 sequence was synthesised artificially. Using the Electrophoretic Mobility Shift Assay (EMSA), the binding association of the CArG1 region with pure nuclear protein was investigated, and the complex was used for protein identification using LCMS and bioinformatics tools. NAC-like protein (NAP) was found to interact with the CArG1 region of AP3 promoter.

Activity-based protein profiling (ABPP) is an emerging technology in chemical proteomics, and utilises a combination of chemistry, proteomics and bioinformatics. Besides its use in drug selectivity and diagnostics, it is finding increased application in plant science (Serim et al. 2012; Willems et al. 2014; Morimoto and Van der Hoorn 2016). The first attempt to use ABPP in saffron was done for understanding stigma development at different stages, and it involved the use of multiple molecular probes (multiplexing) (Husaini et al. 2018). The study effectively discovered and quantified 67 active glycosidases that are differently active throughout stigma development using bioinformatics analysis, suggesting that glycosidase activity

Table 4.1 Bioinformatic tools and databases used by various researchers for saffron data analysis

S. No.	Bioinformatic tools/databases	URL	Remarks	Reference(s)
1	MEGA	http://www.megasoftware.net/	• It was useful for the phylogenetic analysis in saffron • Phylogenetic analysis of carotenoid cleavage dioxygenase (CCD) protein • Phylogenetic analysis for identification and characterisation of aldehyde dehydrogenase (ALDH) candidate transcripts • Phylogenetic analysis in twenty-five saffron genotypes for polymorphism and genetic diversity • Phylogenetic analysis in fifty saffron genotypes collected from different regions of Iran and other countries	Anabat et al. (2020), Zarini et al. (2019), Demurtas et al. (2018), Frusciante et al. (2014)
2	GeneAlex	https://biology-assets.anu.edu.au/GenAlEx/Welcome.html	• It was useful in genetic diversity analysis in saffron • AMOVA analysis using GenAlex for genetic variation within and among saffron species	Larsen et al. (2015), Zarini et al. (2019)
3	DnaSP	http://www.ub.edu/dnasp	• DnaSP has been used to study DNA sequence polymorphism among different haplotype groups in saffron	Anabat et al. (2020)
4	Pln TFDB	http://plntfdb.bio.uni-potsdam.de/v3.0/	• It has been used for functional and evolutionary study of plant transcription factors • In *Crocus sativus*, different genes encoding transcription factors have been identified using PlnTFDB	Zinati et al. (2016)
5	KEGG	http://www.kegg.jp/ http://www.genome.jp/kegg/	• It is highly useful in the biological interpretation of genome sequences, pathway maps and pathway modules • In saffron, 8251 unigenes have been mapped into 130 standard pathways • 14,671 genes have been annotated in saffron using KEGG database	Busconi et al. (2015)
6	WEGO	http://wego.genomics.org.cn/	• It is an important web tool for plotting GO annotations • Functional classification of unigenes has been done in saffron using WEGO software	Hu et al. (2020)

(continued)

Table 4.1 (continued)

S. No.	Bioinformatic tools/databases	URL	Remarks	Reference(s)
7	Trinity	https://github.com/trinityrnaseq/trinityrnaseq/releases/tag/v2.8.6	• Trinity is used for assembly of RNAseq data in plants • Has been used for de novo assembly of saffron transcriptome data	Yue et al. (2020), Mahmodi et al. (2014), Tan et al. (2019), Hu et al. (2020)
8	TransDecoder	https://github.com/TransDecoder/TransDecoder	• TransDecoder has been used to find putative coding areas within transcript sequences • It is a very useful algorithm to detect putative genes and their protein-coding regions • 67 active glycosidases, differentially active during stigma development have been identified in saffron, signifying the role of glycosidase activity in saffron maturation	Yue et al. (2020), Husaini et al. (2018)
9	Prodigal	https://github.com/hyattpd/Prodigal	• It is a quick, lightweight, open-source gene prediction program • It was used for detection of open-reading frames in the de novo assembled transcripts of saffron	Husaini et al. (2018)
10	GeneMarkS-T	http://topaz.gatech.edu/GeneMark/license_download.cgi	• GeneMarkS-T is a programme that identifies protein-coding regions in RNA transcripts • Using it in conjunction with two other algorithms (prodigal, TransDecoder), open reading frames of fifty or more amino acids were identified from de novo constructed transcripts of *Crocus sativus* L.	Husaini et al. (2018)
11	PlantTFcat	https://www.zhaolab.org/PlantTFcat/	• Identification and categorisation of plant transcription factors and transcriptional regulators involved in crocin biosynthesis in saffron	Ahrazem et al. (2018)
12	RepeatMasker package	https://www.repeatmasker.org/ https://github.com/rmhubley/RepeatMasker	• It is used to summarise repetitive elements found in the genomic DNA sequences • Identification of simple sequence repeats (SSRs)/ microsatellites has been done in saffron using RepeatMasker package (version 2.6.0)	Yue et al. (2020)
13	GenoType and GenoDive	http://www.patrickmeirmans.com/software	• These help in the identification of different genotypes • Estimation of genetic differentiation between different Iranian saffron accessions	Busconi et al. (2015)

(continued)

Table 4.1 (continued)

S. No.	Bioinformatic tools/databases	URL	Remarks	Reference(s)
14	Agilent mass profiler professional (MPP) software	www.agilent.com	• For metabolomics and proteomics research, it offers comprehensive compound identification/annotation as well as integrated pathway analysis • Has been used in saffron for normalisation of metabolomics-based data	Senizza et al. (2019)
15	psRNATarget	https://www.zhaolab.org/psRNATarget/	• It generates a detailed list of short RNA-target pairings and is utilised for high-throughput analysis of next-generation data • Zinc-finger transcription factors could be the targets of miRNAs in saffron • Apocarotenoid biosynthesis genes are not targeted by miRNAs in saffron	Yue et al. (2020)
16	Bowtie	http://bowtie.cbcb.umd.edu/	• It's a memory-efficient, rapid alignment programme for aligning small DNA sequence reads to large genomes • A study while performing the transcriptome expression analysis in saffron, mapped the sequenced filtered libraries to the reference transcriptome using Bowtie	Ahrazem et al. (2018)
17	DESeq 2 package	http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html	• It is used for the identification of differentially expressed genes (DEGs) in plants • Has been used in identification of differentially expressed genes in different saffron tissues	Yue et al. (2020)
18	Cufflinks	http://cole-trapnell-lab.github.io/cufflinks	• It is used in the estimation of expression levels of transcript sequences • Expression levels of genes responsible for saffron crocin biosynthesis in different stigma developmental stages have been studied using Cufflinks • Abundance of aligned reads in saffron transcriptomics data has been estimated using Cufflinks v.2.1.1	Demurtas et al. (2018) Ahrazem et al. (2018)

(continued)

Table 4.1 (continued)

S. No.	Bioinformatic tools/databases	URL	Remarks	Reference(s)
19	PIECE	https://probes.pw.usda.gov/piece/index.php	• It has been used for the comparative analysis of gene structures for intron and exon comparison in saffron	Ahrazem et al. (2020)
20	Mascot	http://www.matrixscience.com	• Identification of different upregulated and downregulated proteins under cadmium toxicity in saffron	Rao et al. (2017)
21	MaxQuant	http://www.maxquant.org	• It is used in mass-spectrometry (MS)-based proteomics data analysis • Peptide and protein identification in different developmental stages of saffron stigma has been done using MaxQuant (version 1.5.0.25 or 1.5.3.30)	Husaini et al. (2018)
22	Perseus	https://maxquant.net/perseus/	• It is used for interpreting protein quantification, interaction, normalisation, cross-omics comparisons and multiple-hypothesis testing • Saffron stigma spectra files submitted to an Andromeda search in MaxQuant were finally analysed and filtering of the results was done for post-translational modification, pattern recognition, time-series analysis in Perseus version 1.5.5.3	Husaini et al. (2018)
23	MISA-Web	http://misaweb.ipk-gatersleben.de/	• It is used for microsatellite prediction and counting • Some simple sequence repeats (SSRs) have been counted using MIcroSAtellite (MISA) Perl script in saffron	Yue et al. (2020)

plays a significant role in the maturation of saffron stigma. Open Reading Frame Detection and Domain Annotation Softwares like Gene-MarkS-T (Tang et al. 2015), TransDecoder (Haas et al. 2013) and Prodigal (Hyatt et al. 2010) were used. The experiment shows how ABPP combined with bioinformatic predictive algorithms can be used to profile quantitative glycosidase activity in non-model plant species like saffron.

The mechanical behaviour of saffron flower could have an important role in the development of machinery for post-harvest processing. Zeraatkar et al. (2015) have developed a 3-D geometrical model of saffron flower by reverse engineering and laser scanning technology.

4.3.2 Saffron Corm Rot

Fusarium oxysporum-caused saffron corm rot is a severe disease that causes significant losses in saffron-producing countries (Cappelli 1994;

Husaini 2014). ABPP was employed to take an exhaustive snapshot of the active proteome by using a combination of probes specific for serine hydrolases, α-glycosidases, β-glycosidases and cysteine proteases. Bioinformatics was used to detect the decreased activity of an α-glycosidase during *F. oxysporum* infection, supporting the notion that *F. oxysporum* suppresses AGLU1 in the apoplast to overcome its antifungal action (Xiao et al. 1994; Monroe et al. 1999) (Table 4.1). While putative α-glycosidase (100 kD) and ß-glucosidases (50–70 kD) activities increased after infection, serine hydrolases (50, 60 kD) activities decreased (Husaini et al. 2018). Furthermore, numerous ß-glucosidases (45–60 kD) appeared, whereas others (65–70 kD) vanished. The activity profile of cysteine proteases, particularly papain-like Cys proteases and vacuolar processing enzymes, was dramatically altered in the ABPP-based chemical proteomics investigation. Bioinformatics tools can be of immense importance in the characterisation of the target signals in such ABPP studies involving multiple probes.

4.3.3 Molecular Modelling

Successful application of both fine-scale and network-scale informatics for understanding signalling pathways is quite important (Kahlem and Newfeld 2009). Molecular dynamics and docking approaches are used to understand the interactions between saffron metabolites and transport proteins like β-lactoglobulin (Sahihi 2016). The prediction of metabolic functions is connected with the construction of protein interaction networks (Guan and Kiss-Toth 2008; Wetie et al. 2014). Some *C. sativus* studies have used target deconvolution, reverse screening, protein modelling and docking criteria so that they are able to detect molecular targets and functional domains (Nithya and Sakthisekaran 2015; Bhattacharjee et al. 2012; Ganai and Husaini 2021). T and B-cell epitopes have been predicted in Iranian saffron using bioinformatics (Saffari et al. 2008). The predicted peptides could be useful for vaccine development. Recently bioinformatics analysis involving flexible molecular docking followed by atomic level interaction showed the possibility of using saffron-based remedy for novel coronavirus (Ganai and Husaini 2021). In-depth analysis showed that the interactions between saffron bioactive molecule picrocrocin and the residues of ACE2 could be crucial for receptor-binding domain (RBD) binding and, therefore, can disrupt the interaction between RBD and ACE2. The bioinformatics analysis provided a basis for further studies through animal models or clinical studies for COVID 19 management.

4.4 Bioinformatics Resources for Crop Improvement and Omics Studies

4.4.1 SAM and BCF Tools

SAM and BCFtools are a one-of-a-kind set of tools for processing and analysing sequencing data. The SAM and BCF programs are widely used to process and analyse high-throughput sequencing data. They contain, among other things, file format conversion and manipulation tools, as well as sorting, querying, statistics, variant calling and effect analysis tools (Danecek et al. 2021). SAMtools is a software package and library for parsing and altering SAM/BAM alignments. It can convert between alignment formats, sort and combine alignments, remove PCR duplicates, create pileup per-position information, call SNPs and short indel variations, and display alignments in a text-based viewer (Li et al. 2009).

4.4.2 MEGA

Many analytical methodologies and tools for phylogenomics and phylomedicine are included in the Molecular Evolutionary Genetics Analysis (MEGA) programme (Kumar et al. 2018). MEGA has a lot of tools for putting together sequence alignments, inferring evolutionary trees,

measuring genetic distances and diversities, inferring ancestral sequences, computing time trees and evaluating selection (Kumar et al. 2016).

4.4.3 Trinity

Despite the growing availability of tools and defined workflows for constructing transcriptome assemblies, de novo transcriptome assembly using relatively short Illumina paired-end reads remains a difficult task. When dealing with hundreds of millions of reads, early genome assemblers employed pairwise overlaps between lengthy reads to extend contigs. This strategy is no longer possible. TRINITY is a software tool for conducting de novo (as well as the genome-guided version of) transcriptome assembly using RNA-seq data. A number of perl scripts are included in the Trinity package for generating statistics to measure assembly quality and encapsulating external tools for undertaking downstream analysis (Freedman 2016).

Trinity, created at the Broad Institute and the Hebrew University of Jerusalem, is a unique approach for de novo transcriptome reconstruction from RNA-seq data that is both efficient and reliable. Trinity is made up of three separate software modules: Inchworm, Chrysalis, and Butterfly, which are used in order to handle huge amounts of RNA-seq reads. Trinity divides the sequence data into multiple de Bruijn graphs, each representing the transcriptional complexity at a single gene or locus, and then processes each graph separately to extract full-length splicing isoforms and separate transcripts produced from paralogous genes.

Because *C. sativus* lacks whole-genome sequencing, de novo transcriptome analysis is a good and required platform for expanding molecular research on this plant (Tan et al. 2019). Many studies have used Trinity for De Novo assembly of saffron transcriptomics data (Mahmodi et al. 2014; Hu et al. 2020; Tan et al. 2019).

4.4.4 SMART 9

SMART (Simple Modular Architecture Research Tool) is a web-based resource for protein domain identification and annotation, as well as protein domain architecture analysis (Letunic et al. 2021). Protein domain databases are still useful for annotation and research. The SMART database (Schultz et al. 1998) combines a powerful web-based interface with manually curated hidden Markov models (Krogh et al. 1994; Eddy 2011) for numerous domains. It has been popular and widely utilised by scientists all around the world for nearly 25 years (Letunic et al. 2021). The backend of SMART is a PostgreSQL-powered relational database management system (RDBMS) that holds all SMART domain annotations, protein annotations and sequences, taxonomic information, and pre-calculated protein analyses for the entire Uniprot, Ensembl and STRING proteomes (Letunic et al. 2021).

4.4.5 MPI Bioinformatics Toolkit

The MPI Bioinformatics Toolkit is an open, interactive web tool for protein bioinformatics analysis that is both comprehensive and collaborative. It provides both specialists and non-experts with a wide range of integrated, cutting-edge bioinformatics tools, developed both outside (e.g. BLAST+, HMMER3, MUSCLE) and internally (e.g. HHpred, HHblits, PCOILS) (Alva et al. 2016). The Toolkit now has 35 external and internal tools that cover functions including sequence similarity searching, sequence feature prediction and sequence categorization. The Toolkit has become a significant resource for experimental biology, biomedical research and teaching protein sequence analysis to students in the life sciences due to its range of capability, close interconnection of its constituent modules and ease of use (Gabler et al. 2020; Zimmermann et al. 2018).

4.4.6 BiGGEsTS

BiGGEsTS is a free open-source graphical software application for revealing local gene coexpression in particular time intervals while incorporating important gene annotation information. Gonçalves et al. (2009) made it publicly available at (http://kdbio.inesc-id.pt/software/biggests). BiGGEsTS also includes well-known preprocessing tools for filtering genes, treating missing values, and smoothing, normalising, and discretizing expression data. The analysis of both data and results is aided by a visualisation module. Coloured matrices (heatmaps), expression and pattern charts, and dendrograms are examples of graphical representations. Gene Ontology (GO) annotations can be used to investigate biclusters. BiGGEsTS can also build ontology graphs for enriched GO words, as well as filter and/or sort biclusters based on a variety of numerical and statistical criteria (Gonçalves et al. 2009).

4.4.7 PlantGDB

PlantGDB is a genomics database with information on green plant sequencing (Viridiplantae). PlantGDB provides annotated transcript assemblies for 100 plant species, with transcripts mapped to their genomic contexts when feasible, and is integrated with a variety of sequence analysis tools and online services. PlantGDB's genome browsers (xGDB) offer a graphical user interface for viewing, assessing and annotating transcript and protein alignments to chromosomal or BAC-based genome assemblies (Duvick et al. 2007). PlantGDB periodically uploads and parses all Viridiplantae sequences from GenBank (Benson et al. 2007) and Uniprot (Consortium 2019) into ~70,000 individual data sets according to species or subspecies origin. PlantGDB also offers online access to the GeneSeqer alignment programme, which allows users to calculate spliced alignments of expressed transcripts to a target genomic sequence, as previously mentioned (Dong et al. 2004). PlantGDB provides genome browsers (xGDB) for 14 plant species with fully or partially sequenced genomes (Schlueter et al. 2006). Modularity and expandability are key design features of the xGDB browsers, allowing a browser for a newly emerging genome to be quickly deployed and stocked with computed alignments. xGDB offers a user-friendly interface with access to internal and external data sources, zoomable and customizable transcript alignment views, BLAST, search tools, and the ability to evaluate alignments and add new annotations online (Duvick et al. 2007).

4.4.8 KEGG

KEGG is an integrated database resource for biological interpretation of genome sequences and other high-throughput data (Kanehisa et al. 2016). It is a benchmark knowledge base that brings together existing knowledge on molecular interaction network systems such as pathways and complexes (PATHWAY database), information on genes and proteins generated by genome projects (GENES/SSDB/KO databases) and knowledge on biochemical compounds and reactions (COMPOUND/GLYCAN/REACTION databases) (Kanehisa et al. 2004). The KEGG Orthology (KO) database stores gene and protein molecular activities related with ortholog groupings. The KEGG pathway maps, BRITE hierarchies and KEGG modules are created as networks of KO nodes that represent the cell's and organism's high-level functions. The KEGG GENES database now contains over 4000 whole genomes that have been annotated with KOs and can be utilised as a reference data set for KO assignment and subsequent reconstruction of KEGG pathways and other molecular networks (Kanehisa et al. 2016). In contrast to most other databases, KEGG uses a unique approach to genome annotation. To begin, molecular functions are kept in the KO database and linked to ortholog groups, allowing experimental evidence from one organism to be extended to other organisms. Individual gene annotation in the GENES database is as simple as assigning K numbers to KO entry identifiers to generate

linkages to the KO database. Original data, such as gene names and descriptions in the GENES database, are not updated, even if they conflict with the KO assignment. Second, ortholog groups are constructed in the context of KEGG pathway maps and other molecular networks, which are all built as *K*-number node networks. As a result of the genome annotation technique, which converts a gene set in the genome to a K number set, KEGG pathways and other networks are automatically reconstructed, allowing high-level functions to be interpreted (Kanehisa et al. 2016). Using the KEGG database in Saffron, (Hu et al. 2020) performed KEGG pathway analysis of differentially expressed genes (DEGs) and mapped 8251 unigenes into 130 conventional pathways (*C. sativus* L.). In addition, the KEGG database was used to annotate 14,671 genes in Saffron (Hu et al. 2020).

4.4.9 TrichOME

TrichOME is a "omics" database that makes studying plant trichomes more easier. The database contains a substantial amount of functional omics data, such as expressed sequence tag/unigene sequences, microarray hybridizations from trichome and control tissues, mass spectrometry-based trichome metabolite profiles, and trichome-related genes selected from the literature. Sequence similarity with common databases was used to annotate the expressed sequence tag/unigene sequences (e.g. Gene Ontology, Kyoto Encyclopedia of Genes and Genomes, and Transporter Classification Database). To facilitate comparative study, the unigenes, metabolites, curated genes and probe sets have been mapped against each other. Bioinformatics tools, with an emphasis on the mining of trichome-specific genes in unigenes and microarray-based gene expression profiles, are also integrated into the database. Because the genes and metabolites expressed in trichomes are often underrepresented in non-tissue-targeted cDNA libraries, TrichOME is a valuable and unique resource for plant trichome research (Dai et al. 2010). The trichome research community is very interested in transcription factors and transporters, as the former can govern secondary metabolic pathways and the latter can transfer natural products across the plasma membrane and tonoplast. TrichOME uses the PlantTFDB and TCDB databases to annotate unigenes (Guo et al. 2007; Saier Jr et al. 2006). These comprehensive annotations help to study the genes involved in secondary metabolism.

4.4.10 PlantTFcat

PlantTFcat is a high-performance web-based analytic tool that uses InterProScan domain patterns in protein sequences to identify and categorise plant Transcription factor (TF)/Transcriptional regulator (TR)/Chromatin regulator (CR) genes from genome-scale protein and nucleic acid sequences. PlantTFcat's complete prediction logics are based on 108 reported plant TF/TR/CR families' linkages between gene families and conserved domains. These logics efficiently separate TF/TR/CR families that have conserved domains in common. PlantTFcat is made up of an intuitive web interface that lets users to submit huge numbers of sequences and get analysis results, as well as a complex back-end high-performance prediction module that searches possible domains from the user-submitted sequences using InterProScan. This back-end module screens potential TFs/TRs/CRs by referring to the featured conserved domain patterns of each family (Dai et al. 2013). Ahrazem et al. (2018) validated the identified candidate transcription factors in saffron transcriptomics data using PlantTFcat (http://plantgrn.noble.org/PlantTFcat/).

4.4.11 PlnTFDB

The Plant Transcription Factor Database (Pln TFDB) is an integrated database that contains

putatively comprehensive collections of transcription factors (TFs) and other transcriptional regulators (TRs) in plant species with fully sequenced and annotated genomes. Pln TFDB contains complete sets of 84 TFs and TRs from 19 species ranging from unicellular red and green algae to angiosperms, indicating >1.6 billion years of gene regulation network evolution (Pérez-Rodríguez et al. 2010). Pln TFDB is a web interface that allows to search through huge (almost complete) sets of transcription factors from a variety of plant species, including *A. thaliana*, *Populus trichocarpa*, *O. sativa*, *Chlamydomonas reinhardtii* and *Ostreococcus tauri* (Riaño-Pachón et al. 2007). Zinati et al. (2016) performed homology-based search against the PlnTFDB in order to identify genes encoding transcription factors in the network in saffron (*C. sativus*).

4.4.12 Ensembl Plants

Ensembl Plants is a comprehensive database that provides genome-scale information for 39 plant species that have been sequenced. Genome sequence, gene models, functional annotation and polymorphic loci are among the data sets accessible; for the latter, additional data such as population structure, individual genotypes, linkage and phenotype data is available for some species (Bolser et al. 2017). Ensembl Plants is one of a variety of services that use the Ensembl software architecture to analyse, store and disseminate genomic data (each focusing on a different sector of the taxonomic space). To enable basic and translational biological research, Ensembl uses genome sequences as a framework to integrate variant, functional, expression, marker and comparative data and make it available through a standard set of interactive and programmatic interfaces. Ensembl gives easy access to catalogues of genetic diversity and information about the functional importance of individual variants in the context of plant breeding (e.g. population structure, individual genotypes, linkage and phenotype data) (Bolser et al. 2017).

4.4.13 WEGO

WEGO (Web Gene Ontology Annotation Plot) is a simple yet effective tool for visualising, comparing and graphing the results of GO annotation. Unlike other charting applications, WEGO is designed to work with the directed acyclic graph structure of GO to make histogram production of GO annotation results easier. Many prominent biological research programs, such as the rice genome project and the silkworm genome project, have employed WEGO extensively. It has become one of the most commonly used techniques for downstream gene annotation analysis, particularly in comparative genomics (Ye et al. 2006). In saffron (*C. sativus*), (Hu et al. 2020) performed functional classification of unigenes using WEGO software.

4.4.14 edgeR

edgeR (Empirical Analysis of Digital Gene Expression in R) is a tool for RNA-seq, ChIP-seq, CAGE, and SAGE data differential expression (DE) analysis with biological replicates. The edgeR approach gathers input from all of the genes and applies weighted likelihood and F-test techniques to calculate dispersion. It can employ the trimmed mean of M-values, upper-quartile (UQ) technique, Relative Log Expression (RLE) and DESeq for normalisation. It can compare paired and unpaired groups or utilise a Generalised Linear Model (GLM). Single-cell RNA-seq can also benefit from the upper-quartile (UQ) technique (scRNA-seq) (Robinson et al. 2010; McCarthy et al. 2012).

4.4.15 Bowtie

The Bowtie (Langmead et al. 2009) software allows huge collections of sequencing reads to be aligned to a reference sequence, such as the human genome, in an ultrafast and memory-efficient manner. The package includes tools for creating reference genome indexes as well as aligning short

reads using the index as a guide. Many comparative genomics operations start here, including variant discovery and digital gene expression analysis. Bowtie is best for aligning short reads to huge genomes, but it can also handle arbitrary tiny reference sequences (e.g. amplicons) and reads up to 1024 bases. Bowtie is optimised for short read sets in which (a) many of the reads have at least one excellent, valid alignment, (b) many of the reads are of high quality and (c) the number of alignments reported per read is low (close to 1) (Langmead 2010). Ahrazem et al. (2018) while performing the transcriptome expression analysis in saffron, mapped the sequenced filtered libraries to the reference transcriptome using Bowtie with default parameters.

4.4.16 KaPPA-View

One of the most difficult aspects of determining gene functions is interpreting omics data. Plant genomes have a much higher prevalence of multigene families than animal genomes (Fraser 2000). As a result, numerous homologous gene products are frequently ascribed to a single enzymatic activity in plants, which makes comprehending the specific contributions of gene functions in plant metabolism more difficult. Metabolic pathway databases and tools are thus critical for deciphering individual gene activities from omics data and comprehending their functions. An analytical tool called KaPPA-View was created for this purpose, allowing users to view their transcript and/or metabolite data on a collection of comprehensive plant metabolic pathway maps (Tokimatsu et al. 2005). Users can easily find variations in samples by viewing transcripts and metabolites on metabolic pathway maps at the same time. The KaPPA-View tool is simple to use and straightforward, making the potentially time-consuming chore of evaluating and comparing the complicated data correlations that are typical of metabolic investigations much easier. The visualisation tool is more beneficial for developing theories than for drawing firm conclusions about the functions of certain genes (Tokimatsu et al. 2006).

4.4.17 Transcriptogramer

The transcriptogram (Rybarczyk-Filho et al. 2011), a systems biology-based method to analyse transcriptomes, uses protein–protein interaction (PPI) to build an ordered gene list (Morais et al. 2019). In a nutshell, this technique groups genes based on the likelihood that their products would interact with one another, and then orders them in one dimension. In other words, on the ordered gene list, interacting genes should move closer to each other. The average expression of functionally linked genes in a particular window with a settable radius is then calculated using the ordered gene list. This method lowers noise in gene expression data and increases data measurement consistency (Da Silva et al. 2014). The transcriptogramer R programme can analyse RNA-Seq and Microarray expression data by applying the limma package's procedure to transcriptograms, which is known to function effectively and quickly in a variety of situations (Conesa et al. 2016). Miotto et al. (2019) performed protein ordering process using Transcriptogramer v1.0 software (Rybarczyk-Filho et al. 2011) in *A. thaliana.*

4.4.18 Cufflinks

In RNA-Seq samples, it assembles transcripts and determines their abundances. It takes aligned RNA-Seq reads and condenses them into a manageable number of transcripts. The amount of reads supporting each transcript is then used to calculate their relative abundance. Cuffdiff, which is part of the Cufflinks package, takes aligned data from two or more conditions and uses a rigorous statistical analysis to identify genes and transcripts that are differentially expressed (Trapnell et al. 2010, 2012). In order to accurately quantify the expression of a gene, it is essential to identify the splice variants (isoforms) accurately and find which isoform produced each read of the RNA-Seq reads. Incomplete or incorrect transcriptome annotation can lead to inaccurate expression values (Trapnell et al. 2010). Since a sample may contain reads from many splice variants of a gene,

Cufflinks is able to infer the splicing structure of such genes. However, certain genes possess multiple alternative splicing sites, and there can be multiple reconstructions of the gene model which explain the sequencing data. Hence, Cufflinks reports a parsimonious transcriptome assembly of the data (Trapnell et al. 2012). Sahoo et al. (2016) performed transcriptome assembly using Cufflinks-2.2.1 software in *Curcuma longa* L. cv. Kedaram. Ahrazem et al. (2018) estimated abundance of aligned reads in saffron transcriptomics data using Cufflinks v.2.1.1.

4.4.19 Paintomics

Paintomics is a cross-platform web application based on Perl and Python scripts that operate on an Apache web server. Users must upload gene expression and metabolite concentration files, as well as offer lists of relevant attributes and specify the organism under research, using a simple web form. It supports over 100 top species from several biological kingdoms and allows users to request any other organism from the KEGG database (García-Alcalde et al. 2011). Paintomics is a simple but powerful approach for combining transcriptomics and metabolomics data from the same set of samples in genomics research. The programme basically takes gene expression and metabolite quantifications and shows them on KEGG maps. Paintomics' key features include painted KEGG maps for a variety of organisms; joint visualisation of various types of omics data, showcasing both significant and non-significant changes; pathway enrichment computation based on both transcriptomics and metabolomics data; interactive images with link-outs to KEGG info and experimental values; and easy download of mapped data for further analysis (García-Alcalde et al. 2011).

4.4.20 PIECE

PIECE is a plant gene comparison and evolution database containing annotated genes from 25 plant species having sequenced genomes. The database contains information on 17 eudicots, 5 monocots, 2 green algae and the moss Physcomitrella patens. PIECE features a graphical viewer for seeing a gene structure pattern diagram together with the resultant bootstrapped dendrogram for each gene family, as well as a user-friendly interface for doing various sorts of searches. Also accessible is GSDraw, a web server version of PIECE's programme for drawing schematic diagrams of gene structures. PIECE is a powerful tool for comparing gene sequences that gives crucial information on how plant genomes developed (Wang et al. 2013). For example, in saffron comparative analysis of gene structures was done with the comparative genomics database PIECE for Plant Intron and Exon Comparison (Ahrazem et al. 2020).

4.4.21 MISA-Web

The MISA microsatellite finder is a programme that searches nucleotide sequences for microsatellites. MISA can locate ideal compound microsatellites, which are made up of numerous occurrences of more than one simple sequence motif, in addition to perfect microsatellites (Beier et al. 2017). Yue et al. (2020) counted simple sequence repeats (SSRs), also known as microsatellites using MIcroSAtellite (MISA) Perl script in saffron.

4.4.22 Prodigal

Prodigal is an open-source gene prediction software that is quick and light. It may be used in processes for automated microbiological annotation. It's now part of the Swiss Institute of Bioinformatics' microbial genomics browser and is used on a regular basis at NCBI (Hyatt et al. 2010).

4.4.23 GeneMarkS-T

It's used to find protein-coding regions in RNA transcripts from scratch. It's a good technique for predicting genes in metatranscriptomes. It offers

conventional strand-specific tools for analysing transcripts generated by assembly of stranded RNA-Seq reads (Tang et al. 2015).

4.4.24 MaxQuant

It is a proteomics software for analysing large mass-spectrometric data sets. It specifically aims at the high-resolution mass-spectrometric data (Tyanova et al. 2016a). A search engine 'andromeda' and a viewer application are integrated into MaxQuant.

4.4.25 Perseus

Researchers may use this software system to evaluate protein quantitation, interactions, and post-translational modifications. It's used to analyse MaxQuant's output statistically. It uses tools for the analysis of omics-data, including cross-omics comparisons, normalisation, pattern recognition, time-series analysis and multiple-hypothesis testing (Tyanova et al. 2016b). It is easy to use interactive workflow environment available for free use.

4.4.26 GenAlex

It is a cross-platform package and runs within Microsoft Excel. It enables population genetic analyses, including heterozygosity, F statistics, Nei's genetic distance, pairwise relatedness, etc. It can conduct AMOVA, Mantel tests, principal coordinates analysis, multivariate and 2D spatial autocorrelation and TWOGENER (Peakall and Smouse 2006).

4.4.27 DnaSP

It is a tool for analysing DNA sequence data variation and employs a Graphic User Interface that is easy to use. It provides a detailed characterisation of DNA sequence variation at different time scales, using intraspecific, inter-population or both (Rozas et al. 2017).

4.4.28 TransDecoder

TransDecoder finds putative coding areas within transcript sequences, such as those created by Trinity's de novo RNA-Seq transcript assembly or those built using Tophat and Cufflinks' RNA-Seq genome alignments (Haas et al. 2013; Haas and Papanicolaou 2019).

4.4.29 RepeatMasker Package

It is used to screen DNA sequences for interspersed repeats. The program's output is a detailed annotation of the repeats performed by the program cross-match, using the Smith-Waterman-Gotoh algorithm or WU-Blast (Smit et al. 2013).

4.4.30 GenoType and GenoDive

These two programs are used to analyse the genotypic diversity in clonal/asexual organisms. GenoType uses input data from microsatellites, allozymes, AFLP's or RAPD's, and can handle haploid and polyploid data. GenoDive performs bootstrap test to check if the indices of genotypic diversity are different for pairs of populations (Meirmans and Van Tienderen 2004).

4.4.31 PsRNATarget

It's a web server for analysing small RNA targets in plants. It combines two critical functions: (a) reverse complementary matching of short RNA and target transcript, and (b) target-site accessibility assessment. It blends three user-friendly interfaces and aids in high-throughput analysis of next-generation data. It takes short RNAs and transcript sequences given by users. Its result offers a thorough list of short RNAs as

well as target locations on potential transcripts that match the small RNAs (Dai and Zhao 2011).

4.4.32 DESeq2 Package

It utilises sophisticated features such as shrinkage estimators for dispersion and fold change to perform quantitative analysis of comparative RNA-seq data. It uses the negative binomial distribution to estimate variance-mean dependency in count data and to check for differential expression (Love et al. 2014).

4.5 Future Perspective

Bioinformatics research to elucidate the biochemical roles of saffron proteins and bioactive chemicals have a lot of potential (Husaini et al. 2018, 2021; Ganai and Husaini 2021). Omics research has aided in a better understanding of the molecular mechanics of saffron flower growth, which might lead to the production of saffron flowers with carpels instead of stamens, doubling the yield. This may be a lofty goal, but it is surely attainable. Bioinformatics and DNA microarray technologies might be effective in discovering sources of resistance and agronomically interesting characteristics for biotechnological transfer to saffron. Such methods can also aid in recognising the variety of diverse regional or genetic groupings of farmed saffron in order to infer links between them. The information gathered can be used to build biological pathways involved in the biosynthesis of saffron's main components (Husaini and Ashraf 2010). Although bioinformatics tools can be used for the prediction of useful molecular information, however that needs to be validated using in vitro experiments too.

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Genetic Mapping and Molecular Markers in Saffron

5

Seyed Alireza Salami and Amjad M. Husaini

Abstract

Saffron (*Crocus sativus* L.) is a sterile triploid ($2n = 3x = 24$) plant with a big complex genome and rare and limited breeding history. Breeding and genetic improvement of saffron is not easy due to its male sterility caused by triploidy and lack of enough diversity, whereas several hybrids are introduced by crossing the wild *Crocus* species. Poor breeding background in saffron led to genetic erosion and lack of superior cultivar (s) adapted to diverse geographical conditions, and different biotic and abiotic stresses. Success in saffron breeding depends on the selection of the best elite genotypes/and clones. As a result, collecting, screening, acquiring reliable information about genetic diversity and population structure, selection, and protection of genetic resources of saffron are essential. To achieve these goals different morphological, molecular, biochemical, and cytological markers are considered as the major tools to study the genetic diversity of clones, similarities and differences within and among populations, assess the genetic structure and phylogeny of saffron and *Crocus* species. New advances based on OMICS approaches have enabled genetic improvements in saffron through the molecular breeding programs which has encouraged breeders to adopt precision breeding approaches in *C. sativus*. Followed by identification of diversity among saffron populations, it is possible to preserve such a valuable saffron gene pool for initiating a comprehensive breeding program.

5.1 Introduction

Saffron (*Crocus sativus* L.) is a sterile triploid ($2n = 3x = 24$) monocot that belongs to Iridaceae. It is the most precious spice in the world known as red gold, and Iran is known as the world's largest producer of saffron (Vahedi et al. 2014, 2018; Taheri-Dehkordi et al. 2020, 2021; Moradi et al. 2021, 2022). Sterile saffron plant has a big complex genome and rare breeding history (Vahedi et al. 2019). Nevertheless, its wild allies are diploid and produce fertile seeds. Breeding and genetic improvement of saffron is not easy, because of its male sterility caused by triploidy (Schmidt et al. 2019), whereas several hybrids are introduced by crossing wild *Crocus* species. Regardless of how saffron emerged as an

S. A. Salami (✉)
Department of Horticultural Sciences, Faculty of Agricultural Science and Engineering, University of Tehran, Karaj, Iran
e-mail: asalami@ut.ac.ir

A. M. Husaini
Genome Engineering and Societal Biotechnology Lab, Division of Plant Biotechnology, University of Agricultural Sciences and Technology of Kashmir, Shalimar Campus, Srinagar, Jammu and Kashmir, India

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auto triploid or allotriploid species, limited success has been achieved using conventional breeding methods in saffron and hence new approaches such as protoplast fusion, mutagenesis using different mutagen agents, chromosome doubling and polyploidy may facilitate its breeding programs (Ahooran et al. 2009). Probable wild allies could be considered as potential candidates to alter saffron traits by cross-pollination. Breeding mainly requires diversity which has been neglected in saffron so far. Such a poor breeding background in saffron led to genetic erosion and lack of superior cultivar (s) adapted to diverse geographical conditions, and biotic and abiotic stresses mainly viral, fungal and bacterial diseases, salinity and moisture stress. Restoration of fertility will enable seed propagation, introduction of genetic variability by recombination, and selection for saffron breeding (Schmidt et al. 2019).

New advances in genomic technologies, transcriptomics, proteomics, and metabolomics, have enabled genetic improvements in saffron through the molecular breeding programs. The application of genomic tools and techniques has encouraged *C. sativus* breeders to adopt precision breeding approaches. Using OMICS approaches and data integration help us to better characterize the genome, transcriptome, proteome, and metabolome of saffron and other *Crocus* species towards a deeper insight into molecular basis of flavor and color biogenesis in saffron which definitely pave its breeding programs (Husaini and Asharaf 2010; Haq et al. 2022).

Success in saffron breeding depends on the selection of the best elite genotypes/and clones, but without genetic diversity, all efforts to introduce new cultivars will be failed. As a result, collecting, screening, selection, and protection of genetic resources of saffron are essential. Plant genetic resources are potentially used for future breeding programs. Acquiring reliable information about genetic diversity and population structure is a prerequisite for plant selection and consequently breeding a superior variety (Karp et al. 1997). To achieve these goals different morphological, molecular, biochemical, and cytological markers are considered as the major tools to study the genetic diversity of clones, similarities and differences within and among populations, assess the genetic structure and phylogeny of *Crocus* species. Therefore, to discover authentic genetic markers, distinguish single nucleotide polymorphisms (SNPs), functional genes related to secondary metabolites, unlock the secret of saffron origin, and improvement of saffron breeding, we will present an overview of molecular approaches towards saffron breeding in this chapter. Breeding and genetic improvement of saffron, possibilities and challenges, resources and tools are considered in the present report. Identification of saffron germplasms and wild species, screening and estimating their potential will lead to preserve genetic resources and select superior clones/ecotypes to breed new saffron varieties adapted for specific geographical regions.

5.2 An Overview of Breeding in Saffron and Wild Allies

One of the inevitable consequences of modern agriculture and using modified varieties with maximum yield and acceptable quality is the reduction of genetic diversity which leads to loss of many useful genes and genetic resources. This issue is more complicated in a plant such as saffron which naturally is sterile triploid. Poor genetic and breeding background in saffron led to genetic erosion which is a great threat to this plant against various biotic and abiotic stresses. In this regard, it is necessary to first estimate the level of genetic diversity in germplasm assemblages and plant populations that are used to improve plants and genetic studies. Genetic diversity is the process of expressing differences or similarities between species, populations, or individuals using specific statistical methods or models. Towards this goal, various approaches can be used, one of which is the application of molecular tools (Mohammadi and Prasanna 2003). Genetic relationships can be used as a guide for selection of useful genes and traits (Etminan et al. 2013).

Although, the most important plant in the Iridaceae and in the genus *Crocus* is *C. sativus*, however, wild *Crocus* species are of considerable importance because of their relevance to *C. sativus* (Fernandez 2004), theories of saffron origin and being considered as the saffron parental species (Schmidt et al. 2019), high potential alternative sources for extraction of apocarotenoids (Ordoudi et al. 2019), use as a ornamental bulb (Taheri-Dehkordi et al. 2020), and their potential for pharmaceutical uses (Zengin et al. 2020). The evolutionary history of wild *Crocus* species defiantly is longer than the cultivated saffron variety. *C. sativus* L. has been cultivated for its spice for at least 3500 years. The history of saffron appearance, its origin and domestication are not clear but perhaps the natural breeding was happened long time ago in this plant. Saffron wild allies are diploid and produce fertile seeds which are easy to breed. One of the ancestors of saffron is very likely the diploid *Crocus cartwrightianus* ($2n = 2x = 16$) native to Greece (Aegean islands), while its triploid mutant "*C. sativus*" was selected and domesticated (Fernández 2004; Nemati et al. 2019). This species has floral morphology that is quite close to that of *C. sativus*. In many studies, *C. sativus* L. has been considered as a result of triploid mutation from wild species of autotriploid (Mathew 1983; Chichiriccò 1984; Negbi 1999; Negbi and Negbi 2002; Nemati et al. 2019). In some studies, saffron is a hybrid result of allotriploidy between two wild diploid species (Agayev 2002; Maggi et al. 2011). Molecular data support the involvement of *C. cartwrightianus* and *Crocus thomasii* as parental species (Brandizzi and Caiola 1998; Tsaftaris et al. 2011) or *C. cartwrightianus* and probably *Crocus pallasi* (Harpke et al. 2013). Moreover, *Crocus mathewii* from Turkey, *Crocus serotinus*, *Crocus hausknechtii*, *Crocus michelsonii*, and *Crocus almehensis* from Iran were mentioned as possible parents (Frello and Heslop-Harrison 2000; Alavi-Kia et al. 2008; Petersen et al. 2008; Tsaftaris et al. 2011; Gismondi et al. 2013; Erol et al. 2014). The allotriploidy hypothesis is also supported by heterosis, expressed in more vigorous perianth parts and style branches compared to those of *C. cartwrightianus*, and by amphiplasty, where in a hybrid the formation of a satellite in one of the satellite chromosomes is inhibited by the other(s) (Agayev 2002). By comparative FISH study of different *Crocus* species including *C. sativus*, *C. cartwrightianus*, *C. thomasii*, *Crocus oreocreticus*, *Crocus asumaniae*, *Crocus hadriaticus*, *Crocus pallasii* ssp. *pallasii*, and *Crocus cancellatus*, this hypothesis took hold that saffron (*C. sativus*) is an autotriploid hybrid derived from heterogeneous *C. cartwrightianus* cytotypes. It seems that heterogeneous *C. cartwrightianus* cytotypes encompass most of the chromosomal diversity required for the saffron formation. According to these findings, an evolutionary scenario for the formation of the autotriploid saffron was proposed (Fig. 5.1).

Cross-pollination of *C. sativus* with *C. thomasii* has been reported to produce some viable seeds (Chichiriccò 1989; Caiola 1999), which is promising to start breeding programs based on hybridization. However, being triploid, saffron never bears seed naturally and it is propagated extensively by daughter corms annually. As a result, saffron has a rare breeding history and limited success has been achieved using traditional breeding methods. Although, saffron as a clone naturally can be scarcely and slowly changed genetically and its improvement is hardly possible through clonal selection (Dhar et al. 1988; Piqueras et al. 1999), however, a long history of cultivation in different geographical regions with various climatic conditions and selection by humans defiantly had evolutionary impact on the genetic background of saffron clones through different sorts of mutations, deletions, inversions, translocations, transversions, transitions, polyploidy, incomplete segregation, somatic recombination, and segregation distortion. Several documents have reported that local saffron specimens/ecotypes were selected based on clonal selection in different countries, i.e., "Ghayenat" in Iran, "Lacha" in India, Abruzzo and Sardinia in Italy, Castilla-La Mancha in Spain, and Kozani in Greece. These ecotypes were completely differing in their yield and quality.

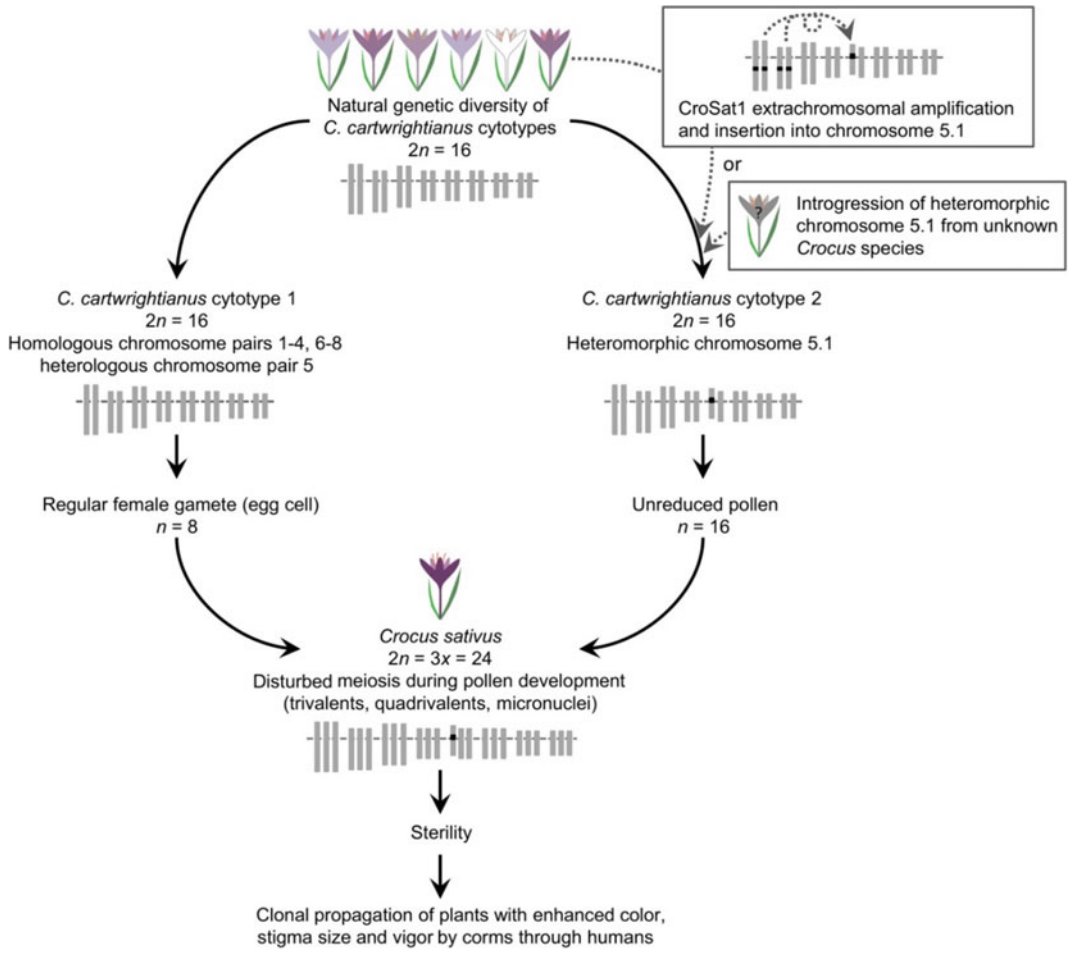

Fig. 5.1 Evolutionary scenario revealed by FISH illustrates the emergence of saffron (*C. sativus* L.) from a cross between heterogeneous *Crocus cartwrightianus* cytotypes with phenotypically different ranges of flower colors from white to purple and chromosomal variation. Cytotype 1 contained homologous chromosome pairs 1–4 and 6–8, all traceable to at least one of the five *C. cartwrightianus* cytotypes. This cytotype 1 plant was assumed that fertilized by unreduced pollen (n = 16) of cytotype 2, as unreduced gametes. Cytotype 2 provided two chromosomes to each triplet. The heteromorphic chromosome 5.1 might result from rearrangement by extra chromosomal amplification of CroSat1, or has been introgressed from an as yet unknown *Crocus* species. The offspring are today's triploid saffron with severe disturbances of pollen meiosis, including trivalents, univalents, and laggard and chromatin bridges. Consequently, these saffron plants are sterile and can only be propagated vegetatively by corms. From Schmidt et al. (2019)

Success in saffron breeding depends on the selection of the best elite genotypes/clones, but without genetic diversity, all efforts to introduce new cultivars will be failed. Acquiring reliable information about genetic diversity in an existing germplasm is a prerequisite for selection, breeding, and conservation programs (Karp et al. 1997). Several characteristics could be considered as breeding traits to introduce new "superior" clones of saffron such as large numbers of flowers and large corms, daughters corm bearing attributes, length and thickness of the stigmas,

dry stigma weight, higher appocarotenoids content particularly crocin content, and tolerant to biotic and abiotic stresses.

Clonal selection independently and in combination with polyploidy and hybridization with wild close relatives of *C. sativus* is mostly promising (Mir et al. 2015). Methods of in vitro technique and molecular approaches should be also applied if necessary. Many attempts have been conducted using tissue culture approaches which have been reviewed comprehensively in Chap. 12. Other approaches such as molecular, morphological, biochemical, and cytological markers have also been used to assess diversity in saffron.

5.3 Molecular Markers in Saffron and Its Wild Allies

Before designing a breeding program for saffron, one should have enough information about the genetic diversity of the relevant germplasm and quantitative and qualitative improvement in saffron requires accurate identification of the genetic structure as a prerequisite of breeding programs. In this regard, information about morphological, biochemical, and molecular diversity is crucial (Baghalian et al. 2010; Gresta et al. 2008).

Compared to other types of markers, genetic molecular markers have very few limitations. These markers cover much of the genome, affected neither by environmental conditions nor developmental stages, require less analysis time (Mammadov et al. 2012). DNA markers have been shown to be valuable in plant breeding, study of genetic diversity and gene mapping. Molecular markers have been widely used to reveal the level of polymorphism in different crops. These markers have potential to identify the variation at DNA level which is not observable in plant morphology. Molecular markers are divided into two categories: protein markers and DNA-based markers. DNA-based markers are used to determine genetic diversity and phenotypic association in different species. In these markers, a specific sequence of DNA molecules is easily detected and their inheritance can be seen. These markers are divided into two general categories of DNA markers based on hybridization and DNA markers based on polymerase chain reaction (PCR) depending on how they show polymorphisms. PCR-based approaches are in demand because of their simplicity and also because they can be carried out with only small quantities of sample DNA. Genetic diversity and relationships among species or populations is an important topic in genetics and plant breeding. Some of molecular markers such as RAPDs and ISSRs are simple, cheap and fast and require little amount of DNA with no need for prior genomic information. Some other markers such as SSRs and AFLPs are more complicated but more informative (Sarikamiş et al. 2010). Nevertheless, all of them consider as useful tool for assessing the genetic diversity, population structure and for map-based cloning approaches towards breeding.

Different types of molecular markers have been used in saffron to assess the genetic diversity and polymorphism, heritability of agro-morphological and phytochemical traits, distinction and variability of *C. sativus* from several geographic areas, molecular phylogeny and taxonomic analysis of different species of genus *Crocus*, genetic variations within and between species, variability among different saffron clones, saffron origin, and authenticity (Pardo et al. 2004; Zubor et al. 2004; Caiola et al. 2004; Alavi-Kia et al. 2008; Moraga et al. 2009; Baghalian et al. 2010). These included RAPDs (Moraga et al. 2009; Ali et al. 2013; Beiki et al. 2010; Caiola and Canini 2010; Imran et al. 2010; Keify and Beiki 2012; Qadri et al. 2012; Zheng et al. 2013), ISSRs (Moraga et al. 2009; Zheng et al. 2013), SSRs (Moraga et al. 2009; Namayandeh et al. 2013; Nemati et al. 2012; Zheng et al. 2013), AFLPs (Caiola and Canini 2010; Fernández et al. 2011; Nazzal et al. 2011; Siracusa et al. 2013; Zubor et al. 2004), Retrotransposons (Alavi-Kia et al. 2008), SNPs (D'Agostino et al. 2007; Fernández et al. 2011), and ESTs (D'Agostino et al. 2007).

Saffron breeders are suspicious about the existence of genetic variability in this plant, although, deep efforts need to assert or reject this.

Assay with RAPDs has revealed limited genetic differences among saffron samples from Italy, Iran, India, Greece, and Spain. Nevertheless, analysis of phenotypes revealed differences in flower size, tepal shape, and color intensity in plants from Israel and Italy. Some researchers revealed that saffron is a monomorphic species in nature due to its triploidy and vegetative reproduction, and therefore there is no significant variation among saffron ecotypes (Alavi-Kia et al. 2008; Moraga et al. 2009; Fluch et al. 2010). As a result, phenotypic differences in saffron have been created only depending on the environmental conditions (Siracusa et al. 2010; Maggi et al. 2011), hence, only one saffron cultivar is cultivated worldwide. Other researchers have shown limited genetic differences in DNA level and found some variations among different saffron clones (Álvarez-Ortí et al. 2004; Sik et al. 2008; Nemati et al. 2012; Keify and Beiki 2012; Siracusa et al. 2013; Nemati et al. 2014). As a result, by selecting the best clones in saffron, high-yielding corms with higher flowering rate and other commercial attributes can be achieved.

In saffron, RAPD markers have been used for investigating the variability and distinction of *C. sativus* from several geographical areas (Pardo et al. 2004). Caiola et al. (2004) evaluated the genetic diversity of 24 wild *Crocus* species alongside six saffron ecotypes using RAPD markers. A total of 233 bands were observed in saffron ecotypes, 14 of which showed polymorphism. In wild species, out of 227 amplified bands, 53 showed polymorphisms. RAPD and SRAP markers have also been used for identifying molecular variation among different saffron genotypes from Iran (Beiki et al. 2010). RAPD markers were found to show promise in identifying variation among different saffron genotypes from Kashmir region (Imran et al. 2010; Qadri et al. 2012). Keify and Beiki (2012) using 26 RAPD markers showed that clones collected from Iran are genetically diverse. RAPD and ISSR markers have been used in many studies to study the molecular diversity of saffron and the result has been significant (Shokrpour et al. 2017). Izadpanah et al. (2015) showed morphological and molecular differences among 36 samples of saffron were collected from Khorasan Razavi and South Khorasan. Moraga et al. (2009) studied domestic saffron ecotypes from different countries along with *Crocus kotschyanus* by ISSR, RAPD, SSR markers, however, no polymorphic band was observed among plants. They reported that domesticated saffron is cloned and that the specimens are not only morphologically identical but also molecularly similar. While in *C. kotschyanus* bands with different sizes and sequences were observed.

Molecular markers like sequence-related amplified polymorphisms (SRAPs) have also been promising to access the genetic diversity of saffron (Keify and Beiki 2012). The SRAP markers are simple, reliable, and highly functional (Li and Quiros 2001). Due to its obviousness and production of high-resolution bands, it is suitable for gel extraction and sequencing subsequently (Sun et al. 2006).

Diversity and relationships within and between species of *C. sativus* and its relatives analyzed by inter-retroelement amplified polymorphism (IRAP). High polymorphisms were identified between accessions of wild relatives with further variation between the species. In contrast, no polymorphisms were seen among 17 *C. sativus* accessions obtained from different geographical regions from Kashmir through Iran to Spain. IRAPs did not generate a tree position suggesting origination from one diploid species, and autopolyploidy was not supported (Alsayied et al. 2015).

Micro-satellite markers are also robust discriminative tools to study genetic diversity of different species. SSR markers were successfully used in saffron, and DNA polymorphisms were reported using this set of molecular markers (Nemati et al. 2012, 2014). SSRs can be isolated from different sources. The fiasco technique is one of the fastest and most effective methods of separating microsatellite markers that have been used in various plant species (Aibin et al. 2008). Twenty-seven SSR markers were evaluated on eight saffron ecotypes in Iran and 29 wild allies to evaluate the molecular diversity and capacity of these markers according to their effectiveness in establishing genetic relationships in *Crocus*

ecotypes (Nemati et al. 2014). Genetic diversity of twenty-two saffron ecotypes was studied using 25 SSR and 5 SNP markers. Filthy alleles were amplified using SSR primers, among them 33 alleles were polymorphic. Five polymorphic SNP markers were classified into transitions (C/T and A/G) and transversions (A/T and C/G) according to their substitution types, and the transitions were more common than the transversions (Javan and Gharari 2018).

AFLP markers have also been used for the study of genetic diversity among different saffron species (Zubor et al. 2004) and for identifying genetic variability in saffron from different origins (Siracusa et al. 2013). Recent reports have analyzed 112 cases using methyl-sensitive-AFLP to look for changes at the genetic and epigenetic levels. These studies show the presence of high epigenetic diversity (35.57% of polymorphic peaks and 28 types of effective epigenotypes (Busconi et al. 2015).

Retero-transposons have also been used for studying the genetic diversity among different saffron species (Alavi-Kia et al. 2008). Expression Sequence Tags-ESTs and EST-SSRs also have the potential to identify the molecular variations at functional/transcriptional levels (D'Agostino et al. 2007).

Molecular markers can also be used for taxonomic studies. The taxonomy of *Crocus* is extremely complicated due to the lack of clear distinctive characters, wide range of habitats and heterogeneity of the morphological traits, and cytological data (Moraga et al. 2009). Whether saffron has undergone modifications along its millenarian cultivation and whether it has one or more ancestors is still uncertain (Caiola et al. 2004). Genetic relatedness has been studied using molecular markers and it was found that *C. sativus* is very closely related to *C. cartwrightianus* and is also similar to *C. thomasii* (Caiola et al. 2004). Wild *Crocuses* are valuable considering their ornamental and pharmaceutical properties (Taheri-Dehkordi et al. 2020). Wild species are also highly valued for their resistance to pests, diseases, and non-living stresses such as salinity, cold, and heat. Beiki et al. (2013) investigated the genetic diversity of *C. sativus*, *Crocus speciosus*, *C. cancellatus*, *Crocus caspius*, and *Crocus haussknechii* by ISSR markers. The results showed that *C. sativus* with a similarity coefficient of 0.48 is very similar to *C. cancellatus* from Fars province. SSR markers have been successfully adopted for the analysis of genetic diversity in different types of plants, especially in saffron (Sarikamiş et al. 2010). Study of genetic diversity and phylogenetic relationships using inter-retrotransposon markers among *C. almehensis*, *C. michelsonii*, *C. cancellatus*, *C. speciosus*, *C. caspius*, *Crocus gilanicus*, and *Crocus haussknechti* in Iran showed that *C. almehensis* and *C. michelsonii* are most similar to *C. sativus* and might be possible ancestors of saffron. While Alavi-Kia et al. (2008) previously reported that *C. cartwrightianus*, which originated in Greece, is very similar to the saffron species and therefore can be considered as the possible ancestor of saffron (Caiola et al. 2004; Moraga et al. 2009; Nemati et al. 2019).

5.4 Approaches Towards Saffron Breeding

Genetic diversity is crucial in all breeding programs. Crop improvement relies on new gene combinations and their consequent expression in a new genetic context. Ancestral and wild species are a major source of genetic diversity (Vaughan et al. 2007; Heslop-Harrison and Schwarzacher 2012). With a basic chromosome number of $x = 8$, the saffron is a sterile species propagated exclusively by vegetative corms (Petersen et al. 2008; Agayev et al. 2009). Low but existing variations in saffron clones can be due to environmental effects and clonal variations (Siracusa et al. 2013; Babaei et al. 2014). Therefore, its breeding is applicable through clonal selection, mutagenesis, polyploidy, tissue culture, and gene/genome editing approaches such as CRISPR-Cas9. During long history of saffron cultivation worldwide, saffron lines have been selected clonally for better quality and higher yield, Multi-flowering, larger flowers and corms, flowers with stronger color and more aroma

stigmas, early or late flowering, simultaneously flowering, lack of leaves at flowering time, number of leaves, their vigor, length, thickness and weight of the stigmas, higher appocarotenoids content, better adaptation and tolerant to biotic and abiotic stresses. However, there has been little success due to saffron triploid sterile nature. Two major difficulties of saffron breeding through clonal selection include proper clone recognition and difficulty in bringing clones to cultivars. Clonal selection in saffron involves screening and identification and of superior clones and consequently creating new valuable forms experimentally. Corms and flowers mainly stigmas are the main targets for saffron breeding. Breeding through mutations or polyploidy induction can be exploited by breeders for generating variability in corms and flowers (Zaffar et al. 2008).

Although mutation induction using physical and or chemical mutagens (irradiation, colchicines, etc.) is considered a useful method for increasing the genetic variability and crop improvement in vegetative propagated crops, it has not been effective enough in saffron yet. There are a few reports concerning successful mutation/polyploidy induction in saffron but they never ended up with a new saffron cultivar. Irradiation of saffron corms with 0.5 Kr Gamma rays resulted in plants which produced higher number of corms and flowers and heavier stigmas. On the other hand, Gamma irradiation may result in chimera plants, plants with slower growth rate, increase in size of stomata reduction in its number (Akhund-Zade and Muzaferova 1975). Evaluation data of C3 generation along with chromosomal (root tip/PMC) studies will further confirm the role of mutation/polyploidy in inducing variability in saffron (Zaffar et al. 2004). Clonal selection independently and in combination with the polyploidy and hybridization involving wild saffron allies is mostly promising. To detect variability molecular markers are the best offer.

The main purposes to coordinate OMICS studies in saffron are crop improvement, adulteration and origin of saffron, traceability of the product and determination of authenticity. New advances in OMICS technologies may enable genetic improvements in saffron through the molecular breeding programs. Molecular markers lead to answer a plenty of questions that exist in saffron. Today, rely on the advance in PCR, sequencing, and OMICS technologies; a new path has been taken in determining plant species relationships (Nazzal et al. 2011). Conventional and novel molecular markers such as Genotyping-by-Sequencing (GBS) have been developed which are able to deeply survey the differences at DNA level. Using the GBS, it is possible to find and introduce molecular markers and determine the simultaneous genotype in a wide range of species including saffron and its wild allies (Elshire et al. 2011; Poland and Rife 2012; Narum et al. 2013; Nemati et al. 2019). GBS has been used as a promising molecular marker for analyzing plant genetic diversity and population structure analysis (Soorni et al. 2017). Moreover, GBS is used for studying genetic structure and molecular phylogeny of *Crocus* species and populations (Nemati et al. 2019). GBS is a fast, high-throuput and robust approach based on the sequencing of fragments produced by special restriction enzyme(s). By selecting the appropriate restriction enzyme(s), duplicate areas of the genome are not considered, and areas with low copy number can be targeted with two or three times more efficiency (Gore et al. 2007). The GBS data provide information about millions of potential single nucleotide polymorphisms (SNPs), and small INDELs across the genomes as the basis for high-performance genotyping (Soorni et al. 2017). GBS was used to unravel the saffron origin and its relationships to examine parents. According to these data, 99.3% of saffron alleles are similar to *C. cartwrightianus* (Nemati et al. 2019). GBS has been also used to determine the genetic diversity and population structure of different saffron ecotypes were collected from different regions of Iran alongside discovering authentic genetic markers and single nucleotide polymorphisms (SNPs), mining functional genes related to secondary metabolites and improvement of saffron breeding. GBS data confirmed the existence of genetic variability among Iranian

saffron populations collected from different geographical regions (Unpublished data). Altogether, these results provide useful information on genetic diversity of saffron that can be used for further genetic studies and to preserve and utilize such valuable genetic resources. These findings will help breeders to improve saffron production. Also, GBS would be considered to generate genetic linkage maps, performing genome-wide association studies, and genomic selection for traits of interest towards saffron improvement. Due to the identification of diversity among saffron populations, it is possible to preserve this valuable saffron gene pool for initiating a comprehensive breeding program.

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Part III
Microbiomics

6 Soil Classification, Nutrient Management and Land Cover of Saffron Growing Areas of Kashmir Valley

Nayar Afaq Kirmani, J. A. Sofi, M. H. Chesti, Ikhlaq A. Mir, Insha Irshad, Suheeba Fayaz, and Waseema Jehaan Mir

Abstract

Soils are the important natural resources and their classification is essential for their sustainable development. The declining yield and area under saffron cultivation during the last decade accentuated the reasons behind the cause in this region. Non-availability of scientific nutrient management and non-application of essential nutrients followed by the blanket application had further deteriorated soil health. Standardization of integrated nutrient management (INM) project was formulated which was funded under horticultural mini-mission program during 2005–2010 followed by the mega-project under National Agricultural Innovation Program where all the loop holes from the production to consumption were addressed and revival of the saffron production was possible at large scale. The diagnostic horizons were by and large found to be A_p–B_1–B_{2t}–C_k with thick clay cutans observed in the sub-surface horizons and are classified as Typic Hapludalfs associated with Ruptic-Alfic-Eutrudepts. Textural variation in soils from loam to clay loam observed were neutral in reaction and normal in electric conductivity. Illite dominated the clay minerals followed by mixed layer, vermiculite and chlorite. The highest yield and corm production were observed under INM project and the results obtained were validated in the field through On Farm Trails (OFT's) under NAIP mega project on saffron, which confirmed the average yield of 6.37 kg/ha under 4-year cycle where achievable. The landcover mapping was carried out using Linear Imaging Self Scanning (LISS-IV) data of Oct–Nov 2013, with a spatial resolution of 5.8 M at 1:20,000 scale. The study revealed that the saffron fields cover a total area of 3161.06 ha comprising of 49.65% and saffron-horticulture (dual crop) covers a total area of 254.36 ha which is 3.99% of the total geographic area.

6.1 Introduction

To organize the knowledge based on properties soils are classified in a systematic manner. Based on their characteristic similarities or difference

N. A. Kirmani (✉) · J. A. Sofi · I. Irshad · S. Fayaz · W. J. Mir
Division of Soil Sciences, Faculty of Horticulture, SKUAST Kashmir, Shalimar Campus, Jammu and Kashmir, India
e-mail: nakimani@skuastkashmir.ac.in

M. H. Chesti
Faculty of Agriculture, SKUAST Kashmir, Wadoora, Sopore, Jammu and Kashmir, India

I. A. Mir
Centre for Climate Change and Mountain Agriculture, Division of Environmental Science, FOH, SKUAST Kashmir, Shalimar Campus, Jammu and Kashmir, India

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they are grouped in well-defined taxa which help us in transfer of technologies at international level by different users of the land for the benefit of mankind (Kirmani et al. 2018a). The soil taxonomic system of classification was designed in 1975 by United States Department of Agriculture (USDA) to meet the soil survey needs worldwide. The second edition of *Soil Taxonomy* was publication in 1999 (Soil Survey Staff 1999), followed by the several editions of *Keys to Soil Taxonomy* (Soil Survey Staff 2014) which served two purposes. Firstly, it acquaints users of the taxonomic system with recent changes; secondly, it provides the taxonomic keys necessary for the classification of soils.

With an annual decline of 7.5% in area under saffron in Kashmir as compared to annual growth of 11.5% in Iran in last decade there has been decrease of 215% in production, 83% in area and 72% in productivity in this decade (Husaini et al. 2010), due to acute drought from 1999–2003 the input/output (I/O) ratio has fallen to the level of 1:0.69 with the lowest productivity of 1.57 kg ha^{-1} during 2003–2004 (Annonymous 2008, 2009). The losses of the order of 30% on cultivation by saffron cultivators as suggested by the ratio cannot be sustained by them for long (Nehvi et al. 2004).

The fifth-degree polynomial trend can be fitted with R^2 value of 0.5912, 0.4022 and 0.537, for area, production and productivity, respectively, the data since 1982–2018 can predicts the dynamic changes in these parameters under saffron cultivation in future (Fig. 6.1). The sharp fall in the production during 2014–2015 and 2017–2018 is because of floods in 2014 and prolonged dry spell during 2017 indicates the high dependence of the crop on the climate of the region. Declining soil health has resulted in non-standardized nutrient management and application of essential nutrients (Munshi et al. 2002).

Long history of cultivation commonly practiced by saffron farmers in the valley without any nutritional support has made soils deficient in nutrients and almost unproductive (Ganai 2002; Kirmani et al. 2018d). The maintenance of soil health by the blanket application of fertilizers may not keep pace for sustaining its productivity (Sofi et al. 2018) but the balanced nutrition in terms of chemical fertilizers, bio-fertilizers and organic manures (Kirmani et al. 2010) and had led to renewed interest in integrated nutrient management (Kirmani et al. 2014). Organic manure incorporation along with fertilizers affects the amount and distribution of organic *N* fraction in soil (Santhy et al. 1998; Amiri 2008).

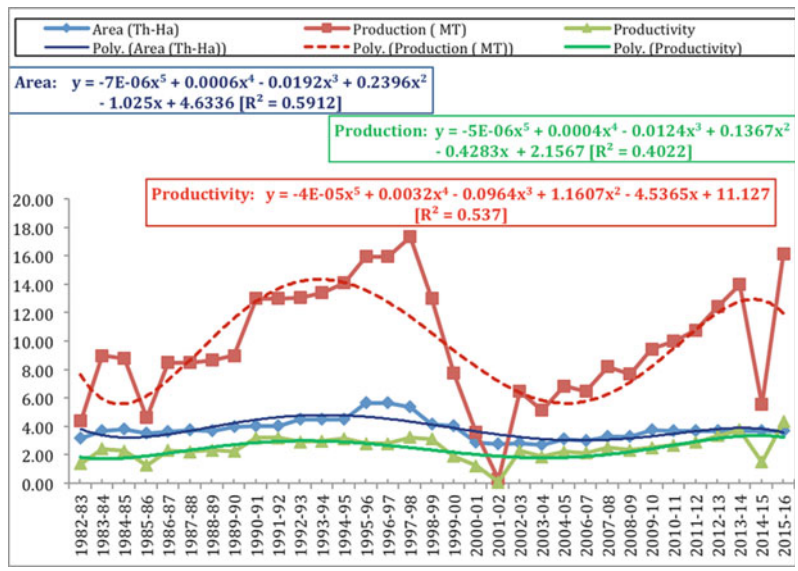

Fig. 6.1 Area, production and productivity trends of the saffron cultivation in Kashmir with 5th degree polynomial curves and R^2 (Anonymous 2014, 2020)

6.2 Location and Climatic Description

The state of Jammu and Kashmir, India, is geographically located at 33° 30′ N to 34° 30′ N and 74° 20′ E to 75° 03′ E, occupies central position in the Asian continent and lies between the southern flank of Greater Himalayan range and North flank of Pir Panjal range, with an altitude of 1500–2050 m (amsl) (Kirmani et al. 2013). The table lands which are also known as *karewa* soils are the pleistocene and post-pleistocene deposits, comprising of 450–500 m thick pile of sediments, with an altitude of 1600–1800 m (amsl) and show lacustrine pedogenetic environments (Pal and Srivastava 1982) (Fig. 6.2). The location map of saffron growing area of Kashmir valley (District Pulwama) along with the vertical section of the Karewa soils show three distinct layers.

The first is the Soil pedon layer up to 150 or 200 cm followed by the hard clay pans layer 200–400 cm approximately which is followed below by the third silty and sandy deposit layers 10–20 cm each indicating the Lacustrine deposits of early period (Kirmani 2005) (Fig. 6.2). Having mid to high-altitude temperate agro-climatic zone, a growing period of 150–210 days, Kashmir is a broad valley with arable land use and moist, sub-humid, agro-ecological zone (Rana et al. 2000).

The maximum precipitation is received in the form of snow during winter and annual precipitation varies from 600 to 900 mm. Sub-zero temperatures are recorded from November to January. In the month of July, the mean maximum temperature reaches 24.5 °C and mean minimum temperature reaches minus 2.0 °C in January. The moisture temperature regimes are udic with mesic, respectively (Kirmani 2005).

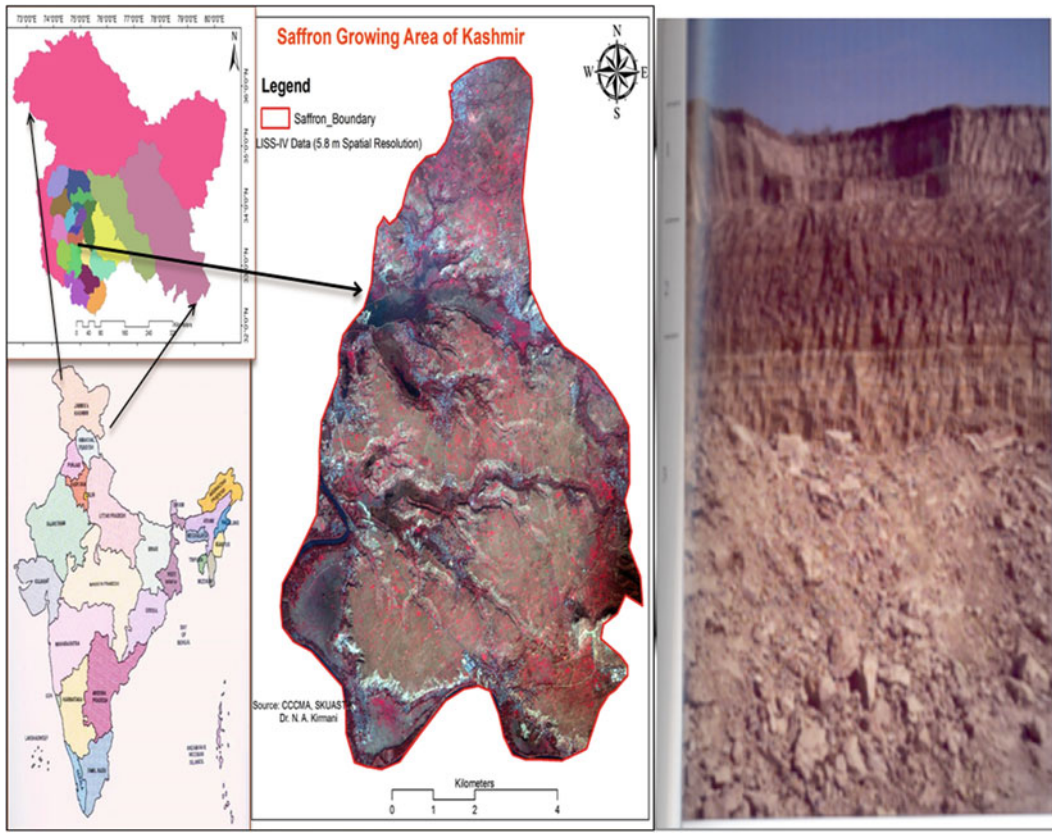

Fig. 6.2 Location Map of saffron growing area of Kashmir Valley (District Pulwama) and vertical section of the Karewa soils showing three layers: **a** Pedon layer, **b** hard clay pans, **c** silty deposit layer (Kirmani 2005)

6.3 Methodology of Soil Classification and Nutrient Management

The Soil Survey Manual methods (Soil Survey Staff 2003) and as per the "Proforma for soil-site description and soil characteristics" (Sehgal 1994) were used for collection of soil profile data in the field. Munsell's soil colour notation was used to observe soil colour. The texture, structure, consistency and other morphological characteristics were all described and noted horizon-wise for each profile. Physical, chemical and mineralogical laboratory analysis was done on the soil samples of each soil profile collected horizon-wise. The soils were identified and characterized by epi-pedons (surface) and endo-pedons (sub-surface) and then classified by following "Keys to Soil Taxonomy" (Soil Survey Staff 1998, 2014). The X-ray diffractometer was used for the mineralogical analyses for sand, silt and clay fractions in order to authenticate the primary and secondary minerals present in these soils.

6.4 Site and Morphological Characteristics

The site characteristics of some profiles under saffron cultivation are mentioned in Table 6.1. These sites are distributed in different areas of the saffron growing area in Pulwama district of Kashmir valley. The elevation varied from 1625 to 1700 m amsl with almost flat topography with slope gradient from 0 to 3%. Very slow erosion and runoff will be observed as the slope length of 0–50 m and 50–150 m was found with good drainage and groundwater depth > 10 m. Stoniness of < 3.0% surface coverage and < 2.5 cm Stone size were found in these soils. The significant bearing of altitude and relief on the soil properties of the valley has been observed (Mahapatra et al. 2000).

Moist soil surface and sub-surface colour varied from 10YR 4/3 (brown) to 10YR 5/4 (yellowish brown) and 10YR 3/4 (dark yellowish brown), respectively. The change in colour in the

Table 6.1 Soil-site characteristics of saffron growing areas of Kashmir valley (Kirmani 2005)

Location	Elevation (amsl) (m)	Topography	Slope gradient (%)	Slope length (m)	Erosion/runoff	Drainage	Ground water depth (m)	Flooding	Stone size diameter (cm)
Lethpora	1625	Flat	0–1	0–50	None/very slow	Well drained	>10	No	<2.5
Schandlora	1625	Flat	0–1	50–150	None/very slow	Well drained	>10	No	<2.5
Konibal	1625	Flat	0–1	50–150	None/very slow	Well drained	>10	No	<2.5
Wuyan	1600	Flat	0–1	0–50	None/very slow	Well drained	>10	No	<2.5
Shar	1700	Terraced	1–3	0–50	Very slow/very slow	Well drained	>10	No	<2.5
Balhama	1650	Flat	1–3	0–50	Very slow/very slow	Well drained	>10	No	<2.5

lower horizons was observed to be 10YR 6/4 (light yellowish brown) and 10YR 7/4 (very pale brown). The dark colour in the sub-surface horizons indicates the illuviation of clay. Shinde et al. (1984) while studying the soils of the state reported similar colour variation below the depth and colour hue of 10YR, with 4–6 value and 2–4 chroma has been observed (Verma et al. 1990; Mushki 1994; Katoo 2001) in the *Karewa* soils of the valley.

Loamy texture was observed in the surface *A* and Sub-surface *C* horizons, while as clay loam texture was observed in the B_2 horizon (Table 6.2a, b). The diagnostic horizons were found to be A_p–B_1–B_{2t}–C_k in first three profiles of Pampore area (P_1–P_3) and A_p–B_1–B_{2t}–B_3–C_k in P_4. While as C_k was not found in P_5, P_6 showed horizonization of A_p–BA–C_1–C_k. Sub-angular blocky with weak grade to angular blocky with moderate grade of soil structure was observed in surface and B_{2t} horizon, respectively (Table 6.2a, b). Similar observations in soil structure have been observed (Shinde et al. 1984; Katoo 2001; Kirmani et al. 2013).

The consistencies of soils were hard when dry, friable when moist, sticky and plastic to very sticky and plastic when wet on the surface and lower horizons, respectively. Thick clay cutans indicating illuviation of clay can be seen clearly in the middle layers of the profiles. With depth there has been an increase in the fineness of the roots and root zone up to 120 cm was detected (Kirmani 2005). Shinde and Talib (1984) had reported the rooting zone of 20–75 cm.

6.5 Physico-chemical Properties and Particle Size Distribution

Slight acidic (6.99 ± 0.36) to neutral (7.15 ± 1.70) pH was observed in surface horizons and sub-surface horizons, respectively, of these profiles (Table 6.3). Electrical Conductivity was normal and average organic carbon content was observed to be 8.26 ± 3.07 g kg^{-1} in surface layers and 3.96 ± 2.41 g kg^{-1} in sub-surface layers. Slight effervescence with dilute HCl indicating low calcium carbonate content and strong effervescence indicating very high calcium carbonate in the lower horizons was observed. The CEC ranged from 13.29 ± 2.38 $cmol_c$ kg^{-1} on surface and 14.28 ± 3.89 $cmol_c$ kg^{-1} in sub-surface layers with calcium being the dominant cation followed by magnesium and potassium. The base saturation was found to be 77.30 ± 3.25 and 72.44 ± 17.14% in these layers, respectively (Kirmani 2005).

Average coarse sand varied from 1.02 ± 1.09 to 1.58 ± 1.31%, while as fine sand varied from 32.33 ± 2.34 to 24.7 ± 6.39%. The mean silt content varied from 47.08 ± 2.68 to 42.49 ± 10.36% in these layers, respectively. The average clay content increases and then decreased with depth and varied from 18.68 ± 2.37 to 26.08 ± 8.73%, while as the silt showed reverse trend, which resulting in clay loam texture of middle horizon and loam in the above and below horizons of it.

6.6 Mineralogical Make Up of These Soils

X-ray diffractograms of sand and silt fractions gave strong and sharp reflections of Primary quartz, Plagioclase, mica and orthoclase. Dominance of illite in clay fraction was observed by the strong peeks between 9 and 10 A°. Identification of smectites between 17 and 18 A° peaks was identified by salvation with glycerol which allows their separation. Illite was the dominant clay minerals followed by mixed layer, vermiculite and chlorite (Kirmani 2005; Kirmani et al. 2013) (Table 6.4).

6.7 Soil Classification

The soils were classified as per USDA "Soil Taxonomy", and the diagnostic horizons were found to be A_p–B_1–B_{2t}–C in four soil profiles, A_p–B_1–B_{2t}–B_3–C_k and A_p–BA–C_1–C_k in one profile each, Ochric epipedon was dominant in the surface horizon of Pampore (P_1–P_5) with dominant Argillic endopedon and these soils are classified as Typic Hapludalfs associated with

Table 6.2a Morphological characteristics of some soil profiles of saffron growing areas of Kashmir Valley (Kirmani 2005)

Location	Horizon	Depth (cm)	Boundary		Diagnostic horizon	Matrix colour	Mottle colour	Texture	Coarse fragments
			Dist.	Topo.					
P_1 Lethpora (Pul.)	A	0–20	c	s	A_p	10YR 5/4 (D), yellowish brown: 10YR 4/4 (M) dark yellowish brown	Nil	l	Nil
	B	20–55	g	i	B_1	10YR 4/3 brown	Nil	cl	Nil
	B	55–85	c	w	B_{2t}	10YR 3/4 dark yellowish brown	Nil	cl	Nil
	C	85–120+			C_k	10YR 6/4 light yellowish brown	Nil	l	Nil
P_2 Schandlora (Pul.)	A	0–20	c	s	A_p	10YR 5/4 (D), yellowish brown: 10YR 4/4 (M) dark yellowish brown	Nil	l	Nil
	B	20–52	g	i	B_1	10YR 4/3 brown	Nil	cl	Nil
	B	52–88	c	w	B_{2t}	10YR 3/4 dark yellowish brown	Nil	cl	Nil
	C	88–120+			C_k	10YR 6/4 light Yellowish Brown	Nil	l	Nil
P_3 Konibal (Pul.)	A	0–17	c	s	A_p	10YR 5/4 (D), yellowish brown: 10YR 4/4 (M) dark yellowish brown	Nil	l	Nil
	B	17–34	g	i	B_1	10YR 3/4 dark yellowish brown	Nil	cl	Nil
	B	34–88	c	w	B_{2t}	10YR 3/2 very dark grayidh brown	Nil	cl	Nil
	C	88–102+			C_k	10YR 6/4 light yellowish brown	Nil	l	Nil
P_4 Wuyan (Pul.)	A	0–20	c	s	A_p	10YR 6/4 (D) light yellowish brown: 10YR 5/4 (M) yellowish brown	Nil	sil	Nil
	B	20–40	g	i	B_1	10YR 5/4 yellowish brown	Nil	l	Nil
	B	40–70	g	i	B_{2t}	10YR 3/4 dark yellowish brown	Nil	cl	Nil
	B	70–100	c	w	B_3	10YR 5/3 brown	Nil	cl	Nil
	C	100–120 +			C_k	10YR 7/4 very pale brown	Nil	l	Nil
P_5 Shar (Pul.)	A	0–20	c	s	A_p	10YR 6/3 (D) pale brown: 10YR 4/3 (M) brown	Nil	l	Nil
	BA	20–55	g	i	B_1	10YR 4/3 brown	Nil	cl	Nil
	B	55–90	g	i	B_{2t}	10YR 3/3 dark brown	Nil	cl	Nil
	C	90–120+			C	10YR 3/3 dark brown	Nil	cl	Nil

(continued)

Table 6.2a (continued)

Location	Horizon	Depth (cm)	Boundary		Diagnostic horizon	Matrix colour	Mottle colour	Texture	Coarse fragments
			Dist.	Topo.					
P_6 Balhama (Pul.)	A	0–15	c	s	A_p	10YR 5/4 (D), yellowish brown: 10YR 4/4 (M) dark yellowish brown	Nil	l	Nil
	B	15–40	g	i	B_A	10YR 3/4 dark yellowish brown	Nil	cl	Nil
	B	40–90	d	b	C_l	10YR 5/4 yellowish brown	Nil	sil	fg 10–20%
	C	90–120+			C_k	10YR 5/4 yellowish brown	Nil	sil	fg 20–30%

c clear; *g* gradual; *s* smooth; *i* irregular; *w* wavy; *d* diffused: *l* loam; *cl* clay loam; *sil* silty loam

Table 6.2b Morphological characteristics of some soil profiles of saffron growing areas of Kashmir Valley (Kirmani 2005)

Location	Diagnostic horizon	Depth (cm)	Structure			Consistence				Porosity		Cutans			Nodules	Roots		Effervescence (with dil. HCl)
			Size	Grade	Type	Dry	Moist	Wet		Size	Qty.	Type	Th.ness	Qty.		Size	Qty.	
Lethpora (Pul.)	A_p	0–20	m	1	sbk	dsh	mfr	ws	wp	f-m	c	Nil			Nil	m-f	m	Nil
	B_1	20–55	c	2	abk	dh	mfi	ws	wp	f-m	c	T	Thick	Cont.	Nil	f	m	Nil
	B_{2t}	55–85	c	2	abk	dh	mfi	wvs	wvp	vf	m	T	Thick	Cont.	Nil	vf	c	Nil
	C_k	85–120+	m	1	sbk	dsh	mfr	ws	wp	f	f	Nil			Nil	vf	f	ev
Schandlora (Pul.)	A_p	0–20	m	1	sbk	dsh	mfr	ws	wp	f-m	c	Nil			Nil	m-f	m	Nil
	B_1	20–52	c	2	abk	dh	mfi	ws	wp	f-m	c	T	Thick	Cont.	Nil	f	m	Nil
	B_{2t}	52–88	c	2	abk	dh	mfi	wvs	wvp	vf	m	T	Thick	Cont.	Nil	vf	c	Nil
	C_k	88–120+	m	1	sbk	dsh	mfr	ws	wp	f	f	Nil			Nil	vf	f	ev
Konibal (Pul.)	A_p	0–17	m	1	sbk	dsh	mfr	ws	wp	f-m	c	Nil			Nil	m-f	m	Nil
	B_1	17–34	c	2	abk	dh	mfi	ws	wvp	f-m	c	T	Thick	Cont.	Nil	f	m	Nil
	B_{2t}	34–88	c	2	abk	dh	mfi	wvs	wp	vf	m	T	Thick	Cont.	Nil	vf	c	Nil
	C_k	88–120+	m	1	sbk	dsh	mfr	ws	wp	f	f	Nil			Nil	vf	f	ev
Wuyan (Pul.)	A_p	0–20	m	1	sbk	dsh	mvfr	wss	wsp	f-m	c	Nil			Nil	m-f	m	es
	B_1	20–40	m	2	sbk	dsh	mfr	ws	wp	f-m	c	Nil			Nil	f	m	es
	B_{2t}	40–70	m	2	abk	dh	mfi	ws	wp	vf	m	T	Thick	Cont.	Nil	vf	c	e
	B_3	70–100	c	2	abk	dh	mfi	wvs	wvp	f	f	T	Thick	Cont.	Nil	vf	f	e
	C_k	100–120+	m	1	sbk	dsh	mfr	ws	wp	f	f	Nil			Nil	vf	f	ev
Shar (Pul.)	A_p	0–20	m	1	sbk	dsh	mfr	ws	wp	f-m	c	Nil			Nil	m-f	m	Nil
	B_1	20–55	m	2	abk	dh	mfi	ws	wp	f-m	c	T	Thick	Patchy	Nil	f	m	Nil
	B_{2t}	55–90	c	2	abk	dh	mfi	wvs	wvp	vf	m	T	Thick	Cont.	Nil	vf	c	e
	C	90–120+	c	2	abk	dh	mfi	wvs	wvp	vf	m	T	Thick	Cont.	Nil	vf	f	e
Balhama (Pul.)	A_p	0–15	m	1	sbk	dsh	mfr	ws	wp	f-m	c	Nil			Nil	m-f	m	Nil
	BA	15–40	m	2	sbk	dh	mfi	ws	wp	f-m	c	T	Thin	Broken	Nil	f	m	ev
	C_1	40–90	m	2	sbk	dh	mfr	ws	wp	vf	m	Nil			Nil	vf	c	ev
	C_k	90–120+	m	2	sbk	dh	mfr	ws	wp	f	f	Nil			Nil	vf	f	ev

m medium; *c* coarse; 1 = weak; 2 = moderate:: *d* dry; *m* moist; *w* wet; *h* hard; *sh* slighty hard; *fr* friable; *fi* firm; *s* sticky; *p* plastic:: *s* size, *f* fine; *m* medium; *c* coarse:: Qty. *f* few; *m* many; *c* common:: *T* Argillans:: *e* slight; *es* strong; *ev* voilent

Table 6.3 Physico-chemical properties, particle size distribution and texture of some soils under saffron cultivation (Kirmani 2005)

Location	Depth (cm)	pH (1:2)	EC ds m^{-1}	OC g kg^{-1}	$CaCO_3$ %	CEC $cmol_c$ kg^{-1}	Exchangeable cations				% base	C. sand %	Fine sand %	Silt %	Clay %	Texture
							Ca^{++}	Mg^{++}	K^+	Na^+	Saturation					
Lethpora	0–20	6.70	0.05	7.80	0.00	11.35	6.50	1.92	0.78	Tr	81.07	0.2	33.5	48.0	17.8	l
	20–55	7.20	0.12	5.07	0.00	16.50	9.53	2.50	0.70	Tr	77.13	0.1	26.0	44.1	29.2	cl
	55–85	7.40	0.21	3.90	0.00	17.16	9.78	2.75	0.78	Tr	77.56	0.7	23.0	43.3	32.2	cl
	85–120+	7.65	0.10	3.12	17.80	12.54	7.20	2.10	0.46	Tr	77.83	2.3	27.5	48.7	20.6	l
Schandlora	0–20	6.50	0.10	9.75	0.00	15.31	8.73	2.50	0.70	Tr	77.87	0.3	32.0	49.1	17.8	l
	20–52	7.20	0.04	5.85	0.00	16.24	9.20	2.60	0.78	Tr	77.49	0.5	27.0	44.9	27.5	cl
	52–88	7.39	0.07	1.95	0.00	17.29	9.86	2.77	0.70	Tr	77.03	0.9	23.3	43.0	32.5	cl
	88–120+	7.82	0.10	1.17	15.70	12.14	6.70	1.94	0.70	Tr	76.90	3.0	28.3	48.2	19.8	l
Konibal	0–17	7.13	0.06	6.24	0.00	12.54	7.10	2.10	0.87	Tr	80.30	1.0	31.6	48.9	17.5	l
	17–34	7.30	0.13	1.95	0.00	15.58	8.88	2.49	0.78	Tr	78.03	0.7	27.5	42.3	28.5	cl
	34–88	7.10	0.05	3.51	0.00	17.82	9.85	2.85	0.78	Tr	75.67	1.0	23.0	41.9	34.1	cl
	88–120+	7.80	0.09	1.95	16.50	12.28	7.00	1.96	0.70	Tr	78.69	2.6	25.7	48.0	22.4	l
Wuyan	0–20	7.42	0.13	8.97	1.40	9.90	5.12	1.58	0.57	Tr	73.47	0.2	34.7	49.0	16.0	l
	20–40	7.70	0.10	6.63	1.20	13.33	7.30	2.23	0.70	Tr	76.70	0.9	34.1	48.7	15.6	l
	40–70	7.28	0.08	2.73	2.40	15.18	8.12	2.43	0.70	Tr	74.08	0.7	26.5	45.1	27.2	cl
	70–100	7.90	0.10	6.24	5.70	16.63	8.98	2.66	0.61	Tr	73.65	0.8	23.1	42.8	32.8	cl
	100–120+	7.44	0.13	1.95	10.80	13.99	7.98	2.24	0.61	Tr	77.35	5.0	26.0	48.0	20.4	l
Shar	0–20	6.89	0.80	12.87	0.00	14.92	8.23	2.39	0.96	Tr	77.59	3.0	28.2	44.7	22.5	l
	20–55	7.43	0.04	8.97	0.00	15.31	8.33	2.45	1.04	Tr	77.22	1.3	26.5	39.0	32.0	cl
	55–90	7.64	0.10	7.80	1.40	17.03	9.35	2.72	0.78	Tr	75.51	1.0	21.3	40.1	36.9	cl
	90–120+	7.60	0.09	4.68	1.80	16.50	9.25	2.64	0.78	Tr	76.81	1.8	21.7	39.5	37.0	cl
Balhama	0–15	7.31	0.09	3.90	1.10	15.71	8.32	2.53	0.70	Tr	73.50	1.4	34.0	42.8	20.5	
	15–40	7.78	0.10	6.63	3.40	15.71	8.45	2.51	0.61	Tr	73.67	1.7	26.0	41.5	30.2	cl

(continued)

Table 6.3 (continued)

Location	Depth (cm)	pH (1:2)	EC ds m^{-1}	OC g kg^{-1}	$CaCO_3$ %	CEC $cmol_c$ kg^{-1}	Exchangeable cations				% base Saturation	C. sand %	Fine sand %	Silt %	Clay %	Texture
							Ca^{++}	Mg^{++}	K^+	Na^+						
	40–90	7.84	0.10	2.80	5.60	12.80	6.85	2.10	0.70	Tr	75.34	2.5	25.9	49.0	22.6	Sil
	90–120+	7.60	0.11	2.30	6.20	11.62	6.23	1.86	0.29	Tr	72.13	4.0	24.0	51.7	20.1	Sil
Surface	Mean	*6.99*	*0.21*	*8.26*	*0.42*	*13.29*	*7.33*	*2.17*	*0.76*		*77.30*	*1.02*	*32.33*	*47.08*	*18.68*	l: loam d: clay
	Sd	*0.36*	*0.29*	*3.07*	*0.65*	*2.38*	*1.37*	*0.37*	*0.14*		*3.25*	*1.09*	*2.34*	*2.68*	*2.37*	Sil: silty loam
Sub-surface	Mean	*7.15*	*0.09*	*3.96*	*4.43*	*14.28*	*7.94*	*2.29*	*0.66*		*72.44*	*1.58*	*24.32*	*42.49*	*26.08*	
	Sd	*1.70*	*0.04*	*2.41*	*6.01*	*3.89*	*2.19*	*0.62*	*0.21*		*17.14*	*1.31*	*6.39*	*10.63*	*8.73*	

Italics represents Mean and Sd values of parameters in Surface and Sub surface horizons

soils of Balahama (P_6) having Ochric epipedon at surface and Cambic horizon in lower layer. Patchy clay cutans known as Argillans were witnessed in the endopedon with calcium carbonate deposition and were classified as Ruptic-Alfic-Eutrudepts. The classification and development of lacustrine soils under Kashmir valley have been studied (Kirmani 2005; Kirmani et al. 2013). Some karewa soils were classified earlier as Vertic Hapludalfs and Typic Eutrochrepts, with adjoining Typic Hapludalfs and Fluventic Eutrochrepts (Shinde et al. 1984; Kirmani et al. 2018a). Spatial distribution of various soil taxonomical classes is shown in Fig. 6.3.

While studying two benchmark soils of Kashmir valley Pal and Deshpande (1987) reported the Lithological discontinuity after 91 cm with increase in clay content up to 71 cm, they classified these soils as Mollic Hapludalf (Gogji Pather) and Mollic Haplaquepts (Wathora). Gupta et al. (1988) classified the soils of valley as Agriudolls, Hapludalfs and Ochraqualf (Jalali et al. 1989) and forest soils as Typic Hapludoll, Lithic Hapludoll and Typic Argiudolls (Verma et al. 1990). The Lethpora command area has been classified as Hapludalfs and Eutrochrepts (Katoo 2001). Najar (2002) classified the *Karewa* soils under orchards as Hapludalfs and Eutrochrepts (Najar et al. 2009). The area covered under Inceptisoils is much more higher than the alfisoils and other soil taxonomic classes (Table 6.5).

6.8 Nutrient Management for Sustainable Saffron Production

Integrated nutrient management (INM) on saffron yield, corm production and soil health was studied during 2006–2010, in one of the Mini Mission projects funded by ICAR, at SKUAST Kashmir. Different levels of inorganic fertilizers (Nitrogen, Phosphorous and Potassium) were applied along with farm yard manure (FYM), Vermicompost and bio-fertilizers following the standard application procedures. The highest yield of 3.64 and 3.51 was observed when

Table 6.4 Relative abundance of minerals in sand, silt and clay fractions (qualitative) of these soils (Kirmani 2005)

Pedons with depth (cm)	Minerals >	Illite	Vermiculite	Chlorite	Mixed layer
Lethpora (P_1)	0–20	++++	++	+	+++
	20–55	++++	++	+	+++
	55–85	++++	++	+	+++
	85–120+	++++	++	+	+++
Balhama (P_6)	0–15	++++	++	+	+++
	15–40	++++	+	++	+++
	40–90	++++	+	++	+++
	90–120+	++++	+	++	+++

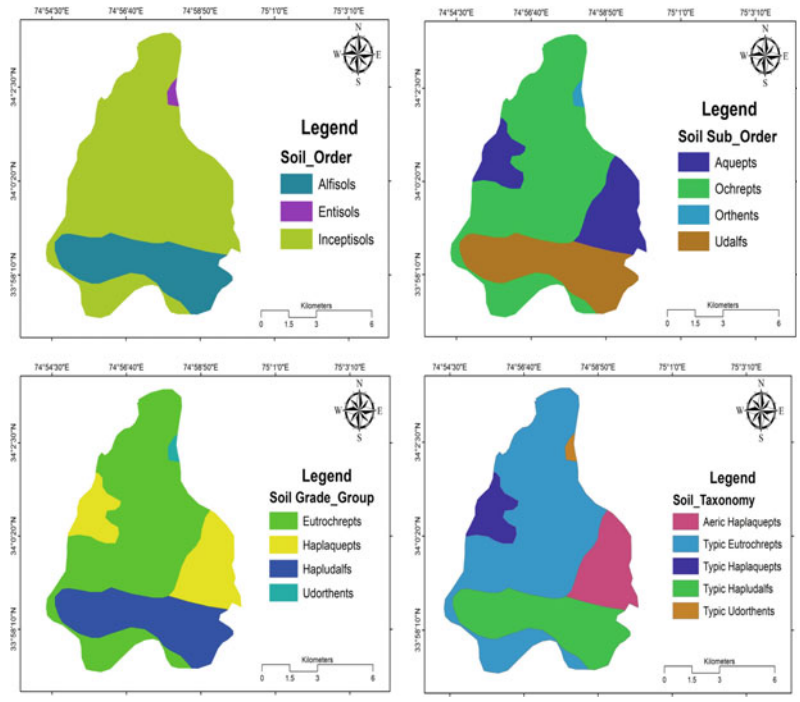

Fig. 6.3 Spatial distribution of soil taxonomical classes: soil orders, sub-orders, great groups and sub groups of saffron growing area

Nitrogen and FYM were applied at 90 kg ha^{-1} and 60 t ha^{-1}, respectively (Fig. 6.3). The increase over their respective controls (2.31 and 2.45 kg ha^{-1}) was calculated as 57.57 and 43.26%, respectively (Kirmani et al. 2014).

The results obtained were recommended for validation in the field through On Farm Trails (OFT's) under NAIP mega project on saffron, which confirmed the average yield of 6.37 kg/ha under 4 year cycle (Nehvi et al. 2010; Nehvi 2014). Maximum corm production of 102.60 q ha^{-1} and 131 q ha^{-1} was observed when nitrogen and FYM were incorporated in soils at 90 kg ha^{-1} and 60 t ha^{-1}, respectively (Fig. 6.4). 79.62 and 260.97% increase over control (57.12 q ha^{-1}) was observed (Kirmani et al. 2018b, c).

The new chapters in organic saffron production can be laid down as the higher levels of organic and inorganic application were found at par. The beneficial effect of integrated nutrient management not only on nutrient availability

Table 6.5 The overall classification with area statistics of the saffron growing soils

Soils	Order	Sub-order	Great-group	Sub-group	Area (ha)
Karewas (table land)	Alfisols	Udalfs	Hapludalfs	Typic Hapludalfs	1470.62
Associated soils (on side slopes and valleys)	Inceptisols (4811.24 ha)	Ochrepts Aquepts Udepts	Eutrochrepts (3649.42 ha) Haplaquepts (1161.81 ha) Eutrudepts	Typic Eutrochrepts Typic Haplaquepts Aeric Haplaquepts Ruptic-Alfic-Eutrudepts	3649.42 376.17 785.64 NA
Hilly area	Entisols	Orthents	Udorthents	Typic Udorthents	39.59
Total area (*as per satellite boundary and may vary with actual statistical area)*					6321.45

Source Rana et al. (2000), Ahmad (2003), Kirmani (2005), Najar et al. (2009), Kirmani et al. (2013)

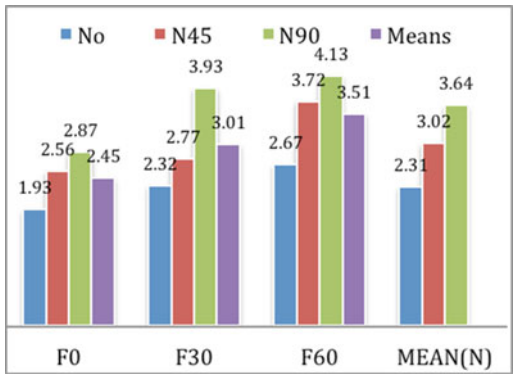

Fig. 6.4 Yield of saffron (kg ha^{-1}) as effected by FYM and nitrogen

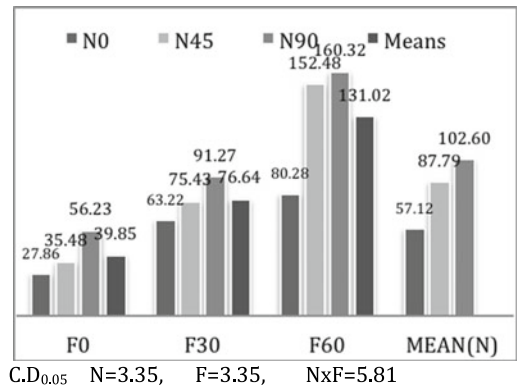

Fig. 6.5 Corm production (q ha^{-1}) as effected by FYM and nitrogen (Kirmani et al. 2018b, c)

(Sofi et al. 2008) but also on physico-chemical properties of soil (Dutta et al. 2003) and enhanced microbial activity (Goyal et al. 1999) have already been documented. The conversion to available forms of nutrients and build-up of organic carbon along with N, P, K and micronutrient in soils (Kirmani et al. 2010a) could be the reasons behind yield enhancement (Kirmani et al. 2010b). Superiority of manures in saffron production has also been documented in other parts of the world under saffron cultivation (Koocheki 2003; Mollafilabi 2003; Nehvi et al. 2010) (Fig. 6.5).

6.9 Land Use–Land Cover and Contours of Saffron Growing Area

The landcover mapping and assessment of saffron growing area of district Pulwama was carried out at the Centre for Climate Change and Mountain Agriculture, SKUAST—Kashmir, India during 2014–2015. The study formed the part of the RKVY funded project on "Resource mapping of Kashmir valley using RS and GIS approach". Indian Remote Sensing satellite data "IRS P6, Linear Imaging Self Scanning

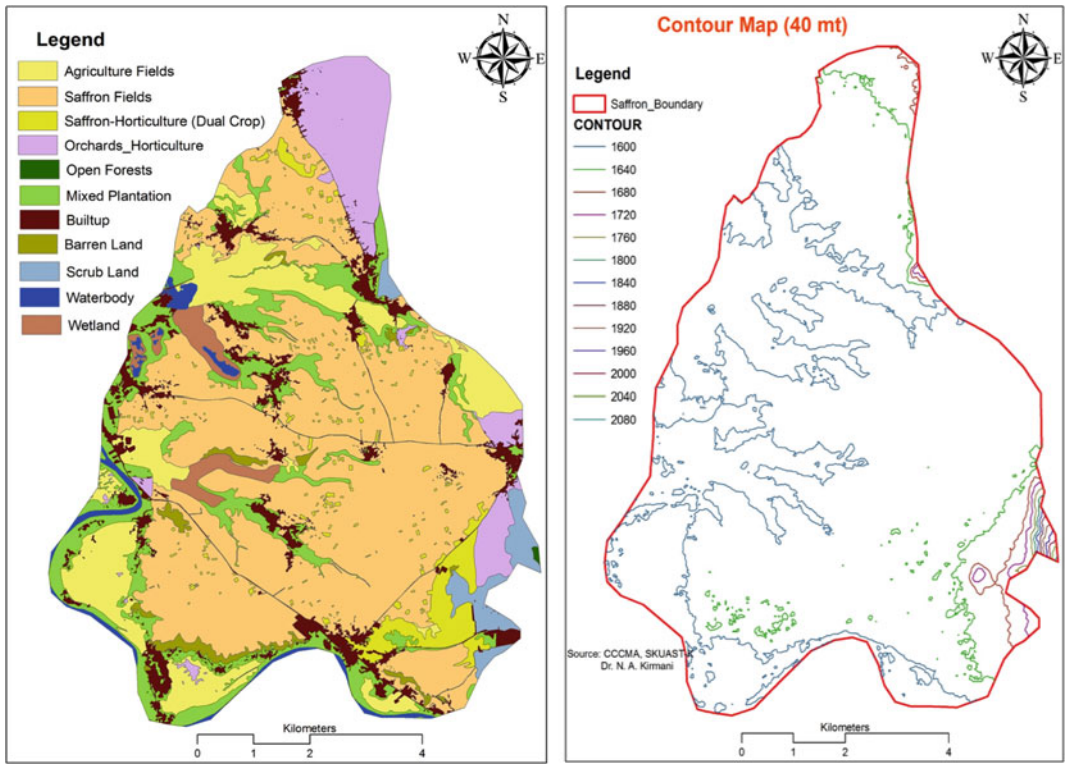

Fig. 6.6 Land use land cover and contour (40 m) map of saffron growing area of Kashmir Valley (Kirmani et al. 2018b, c)

(LISS-IV) data of Oct–Nov 2013", with a spatial resolution of 5.8 M was used to generate the landcover map at 1:20,000 scale. The boundary was selected outside the saffron growing area based on the tone and textural variation in the satellite data. The study revealed that the total study area covered an area of 6366.61 ha which include saffron fields and saffron with horticulture (dual crop). Out of which saffron fields covers a total area of 3161.06 ha comprising of 49.65% and saffron-horticulture (dual crop) covers a total area of 254.36 ha which is 3.99% of the total geographic area (Figs. 6.6 and 6.7).

Similarly, agriculture fields cover a total area of 754.65 ha (11.85%), orchards cover an area of 511.32 ha (8.0%). Likewise, the other land covers present in the area are mixed plantation (755.65 ha), builtup (462.38 ha), scrub land (141.88 ha), wetland (134.82 ha), waterbody (94.91 ha) and barren land (92.74 ha) comprised the 11.86%, 7.26%, 2.22%, 2.11%, 1.49% and 1.45%, respectively.

The contours generated from the Advanced Space borne Thermal Emission and Reflection Radiometer (ASTER) 30 m Digital Elevation Model (DEM) revealed that the area covers between 1600 and above 2080 m and has been classified into 13 classes of 40 m interval. A contour shows the points of equal altitude and is illustrated by contour lines, the essence of the contour map lies in the fact that it depicts the cliffs, steep and gently sloping regions. The middle portion of the area shows a large flat table land with some gentle slopes towards the North and the western part of the region and is best suitable for the saffron cultivation. The close interval in the contour lines in the southeastern region depicts that the area is having steep slopes and is mountainous region and is not suitable for the cultivation of saffron.

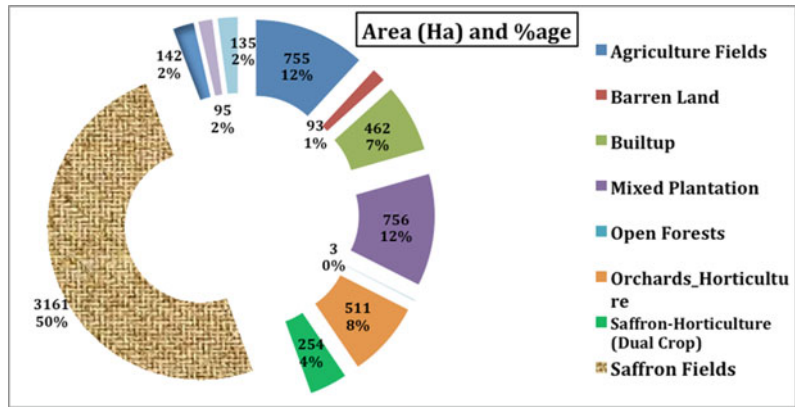

Fig. 6.7 Land use/land cover statistics from LISS IV (5.8 m) satellite data, area (ha) along with their percentage of total area covered

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Cultromic and Metabarcodic Insights into Saffron-Microbiome Associations

7

Nancy Bhagat, Ritika Mansotra, Sheetal Ambardar, and Jyoti Vakhlu

Abstract

Saffron associated microbiome has been studied by both cultivation-dependent, i.e. culturomic, and cultivation-independent, i.e. metabarcodic approaches. The microbiome has been mainly studied from the root to corm, but few reports are related to the microbiome associated with leaves, stamen, petal, stigma, etc. Researchers have primarily studied diversity and simultaneously tried to isolate Plant Growth Promoting Microbes associated with saffron by culturomics technique. Culturomics has also been used to isolate and characterize the endophytes and the pathogens of saffron. The metabarcodic study on saffron has been initiated and majorly done in our laboratory, with only one report from china. Bacteriome and mycobiome of below ground parts of saffron have been studied by directly analyzing the nucleic acid extracted from the rhizosphere and cormosphere during different growth stages and from different geographical locations.

7.1 Introduction

Microbial communities are associated with all plants and have specific interaction with specific plant parts (Berg et al. 2014; Liu et al. 2019). The plant microbiome in the rhizosphere (a thin layer of soil surrounding the roots), phyllosphere (above ground surface of the plant), endosphere (internal tissues of the plant) influence the plant growth and development. These spheres are enriched with diverse microbial community (Compant et al. 2019; Liu et al. 2020; Gupta et al. 2021). The rhizosphere is the hotspot of active microbial diversity and activity that benefits plants by various means (Pathan et al. 2018). The rhizosphere microbial diversity is known to be recruited from the surrounding soil, and it is transferred horizontally from the soil environment to the roots (Frank et al. 2017). However, the seeds of the plants also act as a reservoir of microorganisms; hence, these can be vertically transmitted through seeds as well (Barret et al. 2016; Newcombe et al. 2018). Soil microflora is not constant, as it keeps changing with the

Nancy Bhagat and Ritika Mansotra have equal contribution.

N. Bhagat · R. Mansotra · S. Ambardar · J. Vakhlu (✉)
Metagenomic Laboratory, School of Biotechnology, University of Jammu, Jammu, J&K 180006, India
e-mail: jyotivakhlu@jammuuniversity.ac.in

S. Ambardar
National Center for Biological Sciences, GKVK Post, Bellary Road, Bengaluru 560065, India

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change in soil pH, availability of nutrients, moisture content, temperature etc. (Fierer 2017). The microbial composition of rhizosphere is influenced by the release of root exudates which includes phenolic compounds, organic compounds, fatty acids, plant growth regulators etc. (Mimmo et al. 2014; Hassan et al. 2019; Qu et al. 2020). The change in the microbial composition under the influence of these factors is known as the rhizosphere effect (Dotaniya and Meena 2015; Gan et al. 2021). Plant–microbe interaction in the rhizosphere of various plants has been studied in rice (Zhang et al. 2021b), wheat (Simonin et al. 2020), banana (Kaushal et al. 2020), watermelon (Meng et al. 2019), *Arabidopsis* (Schneijderberg et al. 2020), and saffron (Ambardar and Vakhlu 2013; Ambardar et al. 2014, 2016, 2021). The endophytes colonize roots internally mainly through cracks present on the root surface and from the growing tip of the roots and vertically as well from seed and corm (Lata et al. 2019; Yadav et al. 2020). Endophytes spread from one part of the plant to other systematically through xylem tissue and then colonize in specific tissue and also from seed to other aerial parts (Kumar et al. 2020; Krishnamoorthy et al. 2020; Taulé et al. 2021).

Some of the microorganisms form a close association with their host plant irrespective of the environmental conditions, niches and soil type that forms the core microbiome of the plant. The core microbiome comprises of keystone species that are evolutionary evolved with time and contain important functional genes for the fitness of the host plant (Compant et al. 2019). The core microbiome has been reported in various plants such as rice (Eyre et al. 2019), wheat (Kuźniar et al. 2020), *Salvia miltiorrhiza* (Chen et al. 2018), blueberry (Saati-Santamaría et al. 2021), saffron (Bhagat et al. 2021; Ambardar et al. 2021) etc.

The plant microbiome consists of both beneficial and pathogenic species (Pascale et al. 2020; Zhang et al. 2021a). The beneficial bacteria are referred to as plant growth promoting bacteria (PGPB) that promote the growth of the host plant by direct and indirect mechanisms (Baoune et al. 2021). The direct mechanism involves phosphate solubilization, siderophore production, and production of phytohormones such as indole acetic acid, cytokinins and gibberellins, whereas the indirect mechanism involves induced systemic resistance (ISR) wherein PGPB induce the defense system of the plant by inducing defense related genes (Enebe and Babalola 2019; Bhattacharyya et al. 2020). These PGPB can also show antagonism against the phyto-pathogens through the production of siderophores, antibiotics, lytic enzymes, and volatile compounds (hydrogen cyanide) hence act as biological control agents (BCA) (Liu et al. 2020). Various strains of the genera *Bacillus*, *Pseudomonas* and *Arthrobacter* are widely used as PGPB as well as bio control agents (Shafi et al. 2017; Timmusk et al. 2017). The PGPB are widely used nowadays as part of a sustainable approach for limiting the use of chemical fertilizers (Chandra et al. 2018; Mokrani et al. 2020). The microbial inoculation typically starts with the screening of a single strain with multiple PGP traits followed by pot trials, green house trials and then field trials. Most of the PGPB fails to modulate its effect in field trials, but there are success stories such as in soya bean (Xiang et al. 2017), tobacco (Guo et al. 2020), rice (Naher et al. 2021), and wheat (Yadav et al. 2021). Recently, in saffron as well, a PGPB *Bacillus* sp. strain D5 with multiple PGP properties gave positive results in field trials (Magotra et al. 2021).

In order to study the complete microbial diversity of a niche, cultivation-dependent studies need to be complemented by cultivation-independent studies (Hatzenpichler et al. 2020). Cultivation-independent approaches can be further classified into metabarcoding and metagenomic approaches. Cultivation-independent metabarcoding is further done using two different approaches, i.e. cloning-dependent and cloning-independent direct sequencing of targeted genes. Cloning-dependent metabarcoding approaches generally underestimate microbial diversity as it suffers from an inherent cloning bias. The direct sequencing of any sample eliminates bias resulting from cloning and enables extensive sequencing of microbial populations resulting in a better representation of microbial diversity in

various ecological niches (Abdelfattah et al. 2018; Rossum et al. 2020). Gene targeted, i.e. 16S rDNA, ITS and whole genome-based metabarcoding has been used extensively to study microbial diversity and for the study of interactions occurs among the microbes (Brumfield et al. 2020; Regalado et al. 2020). Different sequencing techniques such as Sanger's method, ion torrent, illumina, and Pacbio are being used to explore the microbial diversity (Nkongolo and Narendrula-Kotha 2020). The sequencing of the same sample using different sequencing platforms can give a complete insight of the structure and function of the microbial diversity. This chapter highlights the microbial diversity associated with the saffron plant, an important cash crop both by culuromics and metabarcoding. Over view of the work done in saffron, microbiomics has been given in Fig. 7.1. Culturomics has been used primarily for the isolation and characterization of PGPB and pathogenic fungi, and metabarcodic approach has been used to get a complete picture of microbial diversity associated with below ground parts of saffron during different growth stages.

7.2 Microbial Cultivation-Based, Culturomic Approach

For decades, the most common approach for the studies of the microbiome is the conventional cultivation-dependent method (Nemr et al. 2020). Culturomics has been commonly employed in the in vitro growth and isolation of the microorganisms from different environmental samples. This method represented one of the major pillars for the expansion of microbiology. Even though high throughput technologies explore microbial diversity at high speed, still culturable-dependent approaches are considered indispensable and have been extensively used for

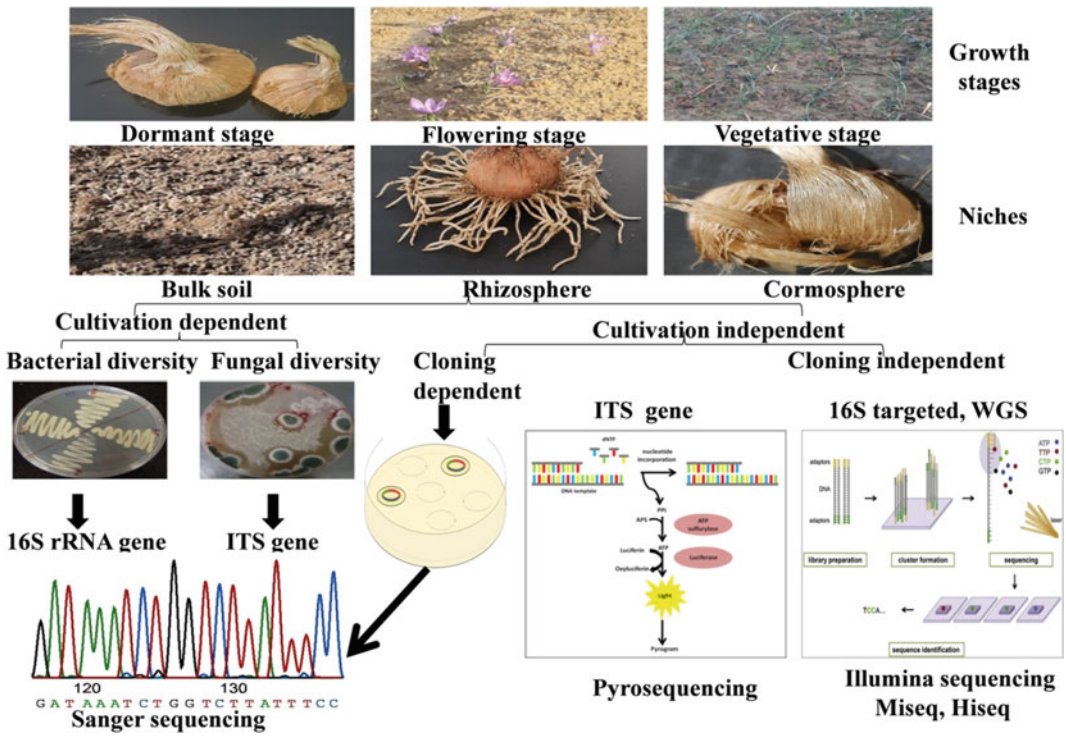

Fig. 7.1 Overview of the work done in saffron microbiome using different techniques in Metagenomic Laboratory, School of Biotechnology, University of Jammu

the unlocking of the environment microbiomes and further exploration of these microorganisms in the biotechnological applications (Martellacci et al. 2019; Sarhan et al. 2018). From time to time, several attempts have been made for the improvement of the culturability of different environmental microbiota that is primarily based on the modifications of traditional culturable methods and growth conditions (Sarhan et al. 2020). The optimization studies of the growth media composition and concentration, incubation time, addition of signaling molecules, solidifying agents and cell density are conducted by various researchers, for the effortless in vitro culturing of rarely grown microorganisms (Sarhan et al. 2019). In this regard, the diversity of culturable community (bacteria and fungi) associated with saffron corms, roots, leaves, anthers and stigmas have been investigated comprehensively by using the culture-based approach (Ambardar and Vakhlu 2013; Gupta and Vakhlu 2015; Kour et al. 2018; Magotra et al. 2021; Hu et al. 2021; Rasool et al. 2021).

7.2.1 Culturable Rhizosphere, Cormosphere and Bulk Soil Microbiome

7.2.1.1 Isolation and Characterization of Pathogens by Culturomics

The plant pathogens have been reported to be isolated and characterized by cultivation based method and by using the same approach, Gupta and Vakhlu (2015) isolated three fungi, namely *Fusarium oxysporum* R1 (Fox R1), *Fusarium solani* and *Penicillium* sp., from the infected corms showing typical symptoms of rot, collected from the saffron fields of Kashmir, India. Apart from these, *F. solani*, *Penicillium citreosulfuratum*, *Penicillium citrinum*, *Stromatinia gladioli* and *Rhizopus oryzae* are also reported as fungal pathogens of saffron (Hu et al. 2021; Belfiori et al. 2021). Amongst all these fungal pathogens, *F. oxysporum* is reported as one of the most destructive pathogens in saffron (Gupta and Vakhlu 2015; Hu et al. 2021; Belfiori et al. 2021).

7.2.1.2 Isolation and Characterization of Bacterial Diversity and PGPB by Culturomics

The isolation and characterization of cultivable saffron microbiome/microbes from the rhizosphere, cormosphere and bulk soil was initiated by our group (Ambardar and Vakhlu 2013; Gupta and Vakhlu 2015; Kour et al. 2018; Magotra et al. 2021) followed by other research groups (Sharma et al. 2015; Chamkhi et al. 2019; El Aymani et al. 2019; Hu et al. 2021; Rasool et al. 2021). The major emphasis has been put on the cultivation-based bacterial diversity analysis as the bacterial strains isolated from diverse environmental sources have shown a positive influence on many parameters of plant yield and growth and thus are known as plant-growth promoting bacteria, PGPB (Magotra et al. 2021). Our group at Metagenomic Laboratory Group, SBT, JU, India has isolated cultivable bacteria from the rhizosphere, and bulk soil of saffron (Ambardar and Vakhlu 2013; Gupta and Vakhlu 2015), cormosphere (mother corm and daughter corm sheath) (Kour et al. 2018; Magotra et al. 2021). The cultivation of microbes associated with saffron was initiated way back in 2010 wherein Ambardar and colleagues collected samples from the Pulwama district (Jammu and Kashmir, India) during its flowering period (October–November 2010) of saffron. The cultivable bacteria have been isolated from the bulk soil and rhizosphere and have further been examined for various plant growth promoting (PGP) activities such as solubilization of phosphate, production of indole acetic acid (IAA) and siderophore. The bacterial isolates were further identified by the analysis of amplified V1–V3 region of 16S rRNA gene sequence. The rhizobacteria have been catalogued into six different species namely *Acinetobacter calcoaceticus DSM 30006(T)*, *Pseudomonas tremae CFBP 6111(T)*, *Pseudomonas kilonensis 520-20(T)*, *Chryseobacterium elymi RHA3-1(T)*, *Bacillus aryabhattai B8W22(T)* and *Pseudomonas koreensis Ps 9-14(T)* belonging to four different genera such as *Acinetobacter*, *Bacillus*, *Chryseobacterium* and *Pseudomonas*; whereas bulk soil bacterial isolates represented ten different

bacterial species such as *Arthrobacter globiformis DSM 20124(T)*, *Bacillus methylotrophicus CBMB205(T)*, *B. aryabhattai B8W22(T)*, *B. aryabhattai B8W22(T)*, *B. aryabhattai B8W22 (T)*, *Brevibacterium halotolerans LMG 21660 (T)*, *B. halotolerans LMG 21660(T)*, *Brevibacterium frigoritolerans DSM 8801(T)*, *Pseudomonas parafulva AJ 2129(T)* and *B. halotolerans LMG 21660(T)* belonged to four different genera namely *Arthobacter*, *Bacillus*, *Brevibacterium* and *Pseudomonads*. The bacterial isolates that showed maximum in vitro PGP traits further subjected to in vivo screening via pot assay and showed the positive effect on the growth of Saffron (Ambardar and Vakhlu 2013).

Initially, bacteria were isolated randomly and screened for PGP and biocontrol activity but based on the reported advantages of *Bacilli* species, subsequently, isolation procedure was made specifically for the *Bacilli*. *Bacilli* are reported to have direct and indirect growth promoting activities in most of the plants and its spores have long shelf life. Subsequently, the cultivable *Bacilli* were isolated from cormosphere of saffron during three distinct stages (Vegetative-April, Dormant-August and Flowering-November) from the saffron fields of Wuyan village in 2012 (Pulwama district of Kashmir, J&K, India). Isolates were further examined for various plant growth promoting (PGP) activities such as solubilization of phosphate, production of indole acetic acid (IAA), siderophore and ammonia as well as various extracellular enzyme activities such as cellulase, amylase and protease. To restrict the number of bacteria, only protease producing cormosphere bacteria (1000 bacteria) were further screened for the additional PGP properties. 35 bacteria strains, showing maximum number of PGP properties and biocontrol property against the *F. oxysporum* R1 (pathogenic fungi of saffron recovered from the infected corm tissue by Gupta and Vakhlu 2015), were identified using 16S RNA sequence analysis. *Bacilli* namely *Bacillus thuringiensis DC1*, *Bacillus megaterium VC3* and *Bacillus amyloliquefaciens DC8* having multiple PGP traits along with corm rot pathogen antagonism were found effective in saffron in vivo conditions (Kour et al. 2018) (Fig. 7.2).

Gupta and Vakhlu (2015) have isolated bacteria from the bulk and rhizosphere soil sample of saffron fields. Among the 400 bacterial isolates isolated from rhizosphere and bulk soil of saffron, two isolates namely *Bacillus mojavensis* W1 and *B. amyloliquefaciens* W2 significantly inhibited the growth of pathogenic fungal isolates namely *F. oxysporum*, *F. solani* and *Penicillium* sp. Moreover, along with antagonistic activity, *B. amyloliquefaciens* W2 produced protease and siderophore. Phylogenetically, *B. amyloliquefaciens* W2 is related to the plant associated group of *Bacillus—B. amyloliquefaciens* strain FZB42, which is commercially available biocontrol agent. The biocontrol activity of the *B. amyloliquefaciens* W2 was evaluated in in vivo conditions (pot assays) and has been reported to reduce incidence of corm rot disease caused by *F. oxysporum* R1 (Gupta and Vakhlu 2015).

Magotra et al. (2021) focused on *Bacillus* species specifically from the cormosphere using three different media viz. Nutrient Agar, Luria–Bertani and Minimal Media agar. The corm samples were collected in 2015 from Kishtwar region of J&K, India, during three different life stages of saffron plant. The isolated species of *Bacillus* (181 isolates) were evaluated for plant growth promoting and biocontrol properties by both in vitro qualitative as well as quantitative assays. Out of 181 *Bacilli* isolates, only 13 *Bacillus* sp. namely (*B. thuringiensis* DC1, *B. megaterium* DC2, *B. amyloliquefaciens* DC8, *Bacillus mycoides* DC7, *B. thuringiensis* FC6, *B. megaterium* VC2, *B. amyloliquefaciens* VC5, ***Bacillus* sp. strain D5** (Bar D5), *Paenibacillus polymxya* D7, *Bacillus subtilis* FR1O, *B. aryabhattai* LB9, *B. aryabhattai* LB17 and *B. megaterium* FR9) have shown multiple PGP activities, whereas 4 strains from 13 shortlisted isolates inhibited the growth of *F. oxysporum* R1. *Bacillus* sp. strain D5 (Accession number *KT228251*) showed the best in vitro PGP activities as well as antifungal activity towards the *F. oxysporum* R1.

Fig. 7.2 Effect of *Bacilli* consortia on different growth parameters of *Crocus sativus* in vivo. Corms inoculated with the cormosphere bacteria (T1–T5) representing increase in number and length of roots and shoots, cormlets number and not as much of disease incident as compared to control (C). C, control represents corms grown in normal soil with no bacterial consortia. T1–T5, test represents corms treated with bacterial consortia; T1–T5 are five repetition of the same experiment. *Source* Kour et al. (2018)

The particular strain *Bacillus* sp. strain D5 furthermore evaluated in in vivo conditions (pot assays and in traditional as well as non-traditional saffron fields) for its antifungal property against Fox R1. The in vivo evaluation was performed using Bar D5 based bio-formulation prepared using the calcium carbonate (ratio-1:2). The significant increase in the important parameters that contribute to saffron plant growth promotion was observed in the pot trails; moreover shoot number, root number and daughter cormlets number have been enhanced by 4.9 folds, 17.7 folds and 6.6 folds respectively (Fig. 7.3). It was observed that corm rot disease incidence was decreased by 3.6 folds in the pot trails using Bar D5 bio-formulation. The field estimation of Bar D5 based bio-formulation in the traditional area revealed increase in stigma length by 1.2 folds, flower number by 2 folds and decrease in the disease incidence by 2.3 folds, when compared to the control corms (without any bio-formulation treatment). Additionally, number of daughter corms also increased by 2.83 folds during vegetative phase in traditional area. Moreover, Bar D5 bio-formulation showed noteworthy increased in all the growth parameters in vegetative and flowering stage in the non-traditional area as well. The flower number and stigma length also increased by 1.7 folds and 1.1 folds respectively in the flowering stage (non-traditional area). The disease incidence also reduced by 1.9 folds in the non-traditional area during flowering stage (Magotra et al. 2021).

Apart from our group, microbial community associated with the rhizophere, cormosphere and soil of the saffron have also been reported by other contemporary groups. In 2021, Rasool and coworkers reported a total of 13 bacterial strains from rhizospheric soil of saffron fields (Pampore area of Pulwama district, Jammu and Kashmir, India) during the flowering stage, having different PGP characteristics and antagonistic activity against *Sclerotium rolfsii* and *F. oxysporum* under in vitro conditions. Based on morphology, microscopy, biochemical characterization and 16S rRNA sequencing, the isolates were identified as *B. aryabhattai*, *B. frigoritolerans*, *Alcaligenes faecalis* subsp. *Phenolicus*, *Alcaligenes* spp., *Bacillus* spp., *Pseudomonas* spp., and *Pantoea* spp. In an another study, *Pseudomonas aeruginosa* strain YY322, isolated from the saffron rhizospheric soil of Shanghai, China, and was reported to show antagonistic activity against the saffron fungal pathogens such as *F. oxysporum*, *P. citreosulfuratum*, *F. solani* and *S. gladioli* in vitro (Hu et al. 2021).

On comparing the bacterial community from rhizosphere of saffron from different geographical regions, it is observed that *Bacillus* spp. and *Pseudomonas* spp. have been commonly shared irrespective of geographic locations and time period of study by different groups (Ambardar

Fig. 7.3 Comparison of plants samples taken from the fields in Kishtwar in 2018. **a** Represents control samples and; **b** represents Bar D5 bio-formulation treated plant samples before sowing. The treated plant samples illustrated better growth in regard to number and size of shoots and roots as well as for daughter corms production. *Source* Magotra et al. (2021)

and Vakhlu 2013; Rasool et al. 2021; Hu et al. 2021). However, *Acinetobacter* and *Chryseobacterium* reported in the rhizosphere of saffron fields in Pulwama district of Jammu and Kashmir by Ambardar and Vakhlu (2013) have not been observed by Rasool et al. (2021). The reason of their absence from Rasool's report despite sample taken from the same region could be many, including climate change, which is quite evident in Kashmir.

El Aymani and coworkers (2019) reported fungal species isolated from the soils, corms and roots of main saffron producing area Taliouine (Morocco). The samples for the mycological studies were collected in September (corms) and in December (soil and roots of plants), 2018. *F. solani*, *Fusarium roseum*, *F. oxysporum*, *Fusarium culmorum*, *Fusarium* sp., *Aspergillus niger*, *Aspergillus fumigates*, *R. oryzae*, *Trichoderma* sp. and *Penicillium* sp. were mainly identified in the corms, roots and soils of saffron plants.

7.2.1.3 Isolation and Characterization of Endophytes by Culturomics

Endophytes are generally phylogenetically as well as functionally diverse and are reported to elicit host defensive response (Compant et al. 2021; Ghasemnezhad et al. 2021). Studies on endophytes have unravel their function in host plant growth promotion, resistance to abiotic stresses, disease resistance, plant functional trait expression, chemical profile structuring of particular host plants, and many other related facets (Adeleke and Babalola 2021; Mukherjee et al. 2021). Saffron production has declined worldwide due to corm rot disease due to pathogenic fungi, e.g. *Fusarium*, *Rhizoctonia*, *Penicillium* etc. (Magotra et al. 2021; Castro et al. 2021; Belfiori et al. 2021). Endophytic species produce antifungal compounds and antibiotics that guard plants against pathogenic bacteria, fungi, nematodes and insects and cause the development of systematic resistance in plants (Ghasemnezhad et al. 2021; Tufail et al. 2021; Hernandez-Pacheco et al. 2021; Pérez-Equihua and Santoyo 2021). Exploration of the plant-endophyte interactions (fungal as well as bacterial) in saffron by culture-dependent method corresponds to a good approach to start implementing scientific practices to cultivate saffron sustainably.

I. **Endophytic bacterial community**: Bacterial endophytes have been extensively studied as they exhibit plant growth-promoting traits such as production of indole-3-acetic acid (IAA) and maintain the levels of ethylene by 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Sharma et al. 2015; Ahmad et al. 2021; Jan et al. 2021). Moreover endophytic bacteria offer nutrients to the plants, like phosphorous, nitrogen and iron through phosphate solubilization,

nitrogen fixation and siderophores production, respectively (Adeleke et al. 2021; Husseiny et al. 2021; Patel et al. 2021). The bacterial endophytes also guard the plants from various abiotic stress conditions such as moisture, temperature, soil type and other environmental as well as climatic factors (Tufail et al. 2021).

Six genera of bacterial endophytes *Paenibacillus*, *Staphylococcus*, *Pseudomonas*, *Bacillus*, *Enterobacter* and *Brevibacterium* have been reported from saffron corm and leaf tissue collected from saffron fields of Pampore, Kashmir, India, in August 2013 (Sharma et al. 2015). They have great potential of producing amylase, cellulase, protease and lipase enzyme which is related to host plant colonization ability and nutrition. Moreover, chitinase, siderophore, phosphate solubilization and phytohormone production potential of these microbes is related to plant growth promotion (Sharma et al. 2015). Isolation rate (IR) of bacterial endophytes was found to be higher in leaf tissue as compared to the corm tissue bacterial endophytes isolation rate. An isolation rate value was represented by percent number of bacterial endophytes isolated corresponding to total number of tissue fragments incubated. In 2021, Ahmad and colleagues have recovered a total of 306 cultures of bacterial endophytes, from 2408 fragments of shoot (103 isolates) and corms (203 isolates) collected from four different locations: Srinagar, Budgam, Pulwama and Kishtwar (Jammu and Kashmir, India). The frequency of colonization and species richness has been observed higher in the corm tissues (54.6%) as compared to the shoot tissues (30.9%). Bacterial endophytes colonize corm tissues more abundantly than shoot tissues because corms are nutrient-rich, fleshy and the conditions are in favor for the colonization of endophytes. Conversely, shoot supports fewer endophytes growth because shoot development occurs at the vegetative phase which is quite short phase of the saffron life cycle. Moreover, shoots are directly exposed to the strong physiochemical variations that usually reduce the entophytes colonization (Ahmad et al. 2021). Based on 16S rRNA gene sequence analyses, bacterial isolates were identified into 47 OTUs and 28 bacterial genera. *Proteobacteria* has been the most dominant phylum, consisting of approximately 51% of the OTUs; followed by *Firmicutes* (36.1%) comprised the second-largest phylum, then *Actinobacteria* (10.6%) and *Bacteroidetes* (2.0% OTUs). Among all, *Bacillus* (66 isolates) has been the most dominant genus, followed by 57 isolates of *Burkholderia*, and *Pantoea* (20 isolates). *Bacillus* and *Burkholderia* were present in both the niches (corms and shoots) and all four different locations, occupying about 40.1% of all the bacterial genera. Bacterial endophytes specific to each niche were also identified and 13 bacterial genera were specific to the corms (*Xanthomonas*, *Streptomyces*, *Obesumbacterium*, *Enterobacter*, *Microbacterium*, *Paraburkholderia*, *Sphingomonas*, *Alcaligenes*, *Lysinibacillus*, *Paenibacillus*, *Methylobacterium*, *Micrococcus* and *Flavobacterium*), while only four bacterial genera specific to the shoot tissues (*Pseudomonas*, *Klebsiella*, *Erwinia*, and *Kocuria*) (Ahmad et al. 2021).

Additionally, these bacterial endophytes exhibited different plant growth promotion activities including phosphate solubilization, nitrogen fixation, and the production of ammonia, siderophores, IAA and ACC deaminase. Nine strains namely *Pantoea eucalypti* E62CS3, *Pantoea conspicua* E66CS3, *Ba. megaterium* E67CS3, *Klebsiella oxytoca* E105CS6, *Obesumbacterium proteus* E92CS4, *Rahnella aquatilis* E48CS3, *Paraburkholderia phenazinium* CSEB, *Achromobacter xylosoxidans* CSEB4, and *Bacillus stratosphericus* E96CS5 have produced greater than 100 mg L^{-1} of IAA. Additionally, three isolates produced the maximum amounts of ACC deaminase such as *Erwinia persicina* E127CS7 (60.2 µmol α-KB mg^{-1} h^{-1}), *Bu. gladioli* E39CS3 (53.4 µmol α-KB mg^{-1} h^{-1}), and *Ba. megaterium* E67CS3 (50.0 µmol α-KB mg^{-1} h^{-1}). *R. aquatilis* E48CS3, *O. proteus* E92CS4 and *Bu. gladioli* E39CS3 efficiently solubilized the inorganic phosphate. *Bu. gladioli* E39CS, *Phyllobacterium ifriqiyense* B2B8, *P. conspicua* E66CS3 and *Paraburkholderia soli*

CSEB14 have been the potential siderophores producers. Among the bacterial endophytes, six strains *Ba. mojavensis* CS4EB32, *Ba. amyloliquefaciens* E87CS4, *Ba. siamensis* E80CS4, *Ba. halotolerans* E79CS3, *Streptomyces achromogenes* E91CS4 and *Bu. gladioli* E39CS3, displayed a wide range of antifungal activity against fungal pathogen of the saffron plant such as *F. oxysporum* strains CSE15 and R1 (Ahmad et al. 2021).

Jan and colleagues recovered 704 bacterial endophytes from vegetative and reproductive organs of *Crocus sativus* (such as corms, roots, leaves, anthers and stigmas), collected from the multiple sites (Konibal, Letpur, Androosa, Baroosa, Chandhar and Lodhu; Jammu and Kashmir India) and based on 16S ribosomal gene sequencing and BLASTn analysis, 52 bacterial OTUs were resolved from the 182 bacterial morphotypes (Jan et al. 2021). *B. frigoritolerans* (23 isolates), *Bacillus fexus* and *Bacillus simplex* (20 isolates each) have been most the widespread whereas *Enterobacter tabaci*, *Pseudomonas reinekei* and *B. thuringiensis* observed as the least abundant (8 isolates each). Among the endophytic bacteria, five strains, listed as, *Bacillus cereus*, *Staphylococcus felis*, *B. halotolerans*, *Bacillus proteolyticus* and *Pseudomonas graminis* were represented by all the study sites (Konibal, Letpur, Androosa, Baroosa, Chandhar and Lodhu; Jammu and Kashmir India), whereas *Bacillus albus* and *Bacillus wiedmannii* were exclusively present in Konibal and Letpur respectively. In addition, *E. tabaci* and *Staphylococcus sciuri* were confined to (Baroosa). *Bacillus lentus* (15) has been reported as the most abundant species in stigma; *Pseudomonas putida* (14) and *Serratia grimesii* (14) in leaf and corm tissues, respectively; *Enterobacter cloacae* (13) showed the greatest abundance in anther and *Sphingomonas aerolata* (12) represented as the most abundant bacterial species in roots (Jan et al. 2021).

Jan and colleagues (2021) reported highest colonization rate for roots, followed by stigma, corm, anther and least for leaves. These results are in contrast to previous study conducted by Sharma et al. (2015) which observed higher in leaves than corms (bacterial endophyte colonization frequency). However, many studies have reported higher endophytic colonization of roots because of the release of signalling molecules with the aim of initiation of early communication between the host plants and endophytes, and accordingly steer the colonization process (Ahmad et al. 2021).

II. **Endophytic fungal community**: Endophytic fungi are defined as fungal groups that colonize the intercellular spaces of living and healthy plant tissues without triggering any disease symptoms (Gakuubi et al. 2021). They are mutualists that provide their hosts resistance to various biotic and abiotic stresses and in exchange receive protection and nutrients from the plant (Adeleke and Babalola 2021; Suryanarayanan and Shaanker 2021). Plant-endophytic fungi are a common source of secondary metabolites, in addition to those which are produced by the plant itself. The anticancer drug paclitaxel (Taxol) have been discovered from the endophytic fungus *Taxomyces andreanae* associated with the host species *Taxus brevifolia* (Belfiori et al. 2021; Gakuubi et al. 2021). Many studies have been conducted that explore the diversity as well as biotechnological potential of endophytic fungi across tissues in different ecological niches of the most diverse plant species (Adeleke and Babalola 2021; Ghasemnezhad et al. 2021). The use of these endophytic fungi as biocontrol agents has a very low impact on the environment, as they reduce the implementation of agrochemicals and fertilizers hazardous to the environment (Bian et al. 2021).

The species composition of endophytic community varies across different tissues, depending on the capability of the endophytic fungal species to use specific substrates. Various study identifying the endophytic fungal communities in different tissues of saffron plants such as tepals, corms, stigmas, etc. has been reported so far by culture-dependent approach (Wani et al. 2016; Belfiori et al. 2021; Jan et al. 2021).

Wani and colleagues comprehensively investigated the endophytic fungi as well as their properties in corms of saffron, cultivated in Jammu and Kashmir, the union territory of India (Wani et al. 2016, 2018). These authors have identified 36 OTUs. Among these 36 OTUs, only three have been commonly shared with the study conducted by Belfiori et al. (2021), i.e., *Cadophora malorum*, *F. oxysporum* and *Talaromyces pinophilus* identified in the corms. Some other OTUs observed by Wani and colleagues occurred in the other plant tissues examined: *Alternaria alternata* (in tepals, stems, leaves and stigmas), *Botrytis cinerea* (leaves), *Aspergillus flavipes* (stigmas) and *Epicoccum nigrum* (leaves). They observed *Phialophora mustea* as the dominant species which is followed by *C. malorum* and *Talaromyces cellulolyticus* (Wani et al. 2016, 2018).

The diversity of the fungal endophytes that are associated with different tissues (such as corms, leaves, stems, tepals, and stigmas) of *C. sativus* cultivated in the different sites of the Sicily (south Italy) and in the Umbria region (central Italy) has been estimated by Belfiori et al. (2021). They adopted an isolation-based approach (culture-dependent approach) to build a collection of fungal strains that could be used in the future screening for the classification of biologically active molecules as well as biocontrol agents against plant pathogens. A total of 135 endophytic—fungal isolates have been recovered from corms (72), leaves (18), stems (10), tepals (22), and stigmas (13) of saffron healthy plants. These isolates have been identified by the analysis of full ITS ribosomal gene sequence. 34 OTUs at 97% identity have been detected by clustering of the sequences. Out of 34 OTUs, 28 OTUs belonged to division *Ascomycota*, 5 belonged to *Basidiomycota*, and rest 1 to *Mucoromycota*. Most of the OTUs of the division *Ascomycota* belonged to subdivision *Pezizomycotina* that has been clustered in four classes; *Dothideomycetes* has been the most represented, followed by *Eurotiomycetes*, *Sordariomycetes*, and *Leotiomycetes*. In addition to subdivision *Pezizomycotina*, *Saccharomycotina* has also been represented (1 OTU only), in the class *Saccharomycetes*. Among *Ascomycota*, the highest number of OTUs have represented by order *Eurotiales* and *Pleosporales*, followed by *Hypocreales*, *Helotiales*, *Dothideales*, *Sordariales*, *Leotiomycetes incertae sedis*, *Capnodiales*, *Xylariales* and *Saccharomycetales*. Most of the OTUs of the *Basidiomycota* belonged to the subdivision *Agaricomycotina*, clustered in the classes of *Tremellomycetes* (2 OTUs) and *Agaricomycetes* (2 OTUs) and, while only one OTU has been clustered in the class *Microbotryomycetes* belonged to the subdivision *Pucciniomycotina*. Among *Basidiomycota*, mainly four orders were represented, that is, *Sporidiobolales*, *Russulales*, *Agaricales* and *Filobasidiales* (1 OTU each). Finally, *Mucoromycota* (1 OTU) belonged to the *Mucoromycetes* class and order *Mucorales*. Species names have been assigned to few OTUs using the BLASTn and phylogenetic analysis, corresponding to the strong dominance of *Cadophora luteo-olivacea* in the corm tissue, followed by *C. malorum* and *T. pinophilus*. *Cadophora* spp. have been detected in corms only whereas *T. pinophilus* has been recovered from corm as well as stem tissues. *Epicoccum* and *Stemphylium vesicarium* dominated in leaves and *A. alternata* in tepals. A slight dominance of *A. alternata* and *Mucor fragilis* have been observed in stigmas. *A. alternata* have been occurred in all tissues excluding corm tissues. In addition to *A. alternata*, few other species have been shared between different tissues of corms, stems, leaves, tepals and stigmas such as *Aureobasidium pullulans*, *F. oxysporum* and *Talaromyces* spp. (Belfiori et al. 2021).

Interestingly, the study by Jan and colleagues unraveled *Aspergillus ustus* and *T. pinophilus* as the dominant species (Jan et al. 2021). They recovered a sum total of 1170 culturable endophytic isolates from numerous tissue segments (6480) of various vegetative organs (corm, root and leaf) as well as reproductive organs (anther and stigma) of saffron plant across 6 different sites that have been separated by a distance of no less than 1 km in the saffron growing area of Pampore, district Pulwama (Jammu and Kashmir, India), lies within the geographical

coordinates (34° 0.02′ latitude and 74° 0.93′ longitude). Out of 1170 culturable endophytic isolates, 440 belong to the fungal endophytic isolates [roots (142 isolates), corms (114 isolates), leaves (44 isolates), anther (51 isolates) and stigmas (89 isolates)]. Among all fungal endophytic OTUs explained: *A. ustus*, 47 isolates; *T. pinophilus*, 46 isolates and *Talaromyces favus*, 44 isolates have been reported as the most abundant species, followed up by various other species of *Fusarium* and *Aspergillus*, whereas *Talaromyces verruculosus*, *Fusarium equiseta* and *Aspergillus dimorphicus* as the least abundant species (Jan et al. 2021).

Several endophytic fungi are known for certain bioactivities. For example, some of the *Talaromyces* spp. that have been detected in corms, stems, and leaves (Kazerooni et al. 2019), *T. pinophilus* (known an interesting species because it inhibits fungal phytopathogenic species) promises interesting biotechnological potential due to its useful reservoir of various biomass-degrading enzymes, for instance α-amylase, endoglucanase, cellulase etc. (Li et al. 2017) and secondary metabolites with the insecticidal activity (Vinale et al. 2017). Likewise, *Talaromyces assiutensis* has previously been reported as an effective nematicidal species (Hamza et al. 2017) in olive nurseries as well as a source of antimicrobial metabolites (Deka and Jha 2020). The occurrence of *T. assiutensis* in saffron might be allied to these properties because nematodes are one of the worst saffron enemies.

7.3 Cultivation-Independent Metabarcoding Approach for Microbiome Studies

The majority of the species present on this earth are of microorganisms that play a central role in regulating the various processes of ecosystem (Jia et al. 2018). The standard laboratory techniques available for the cultivation of microorganisms can cultivate only 1% of the bacteria and up to 5% of the fungus (Martiny 2019; Ambardar et al. 2016). With the advent of high throughput sequencing technologies, the uncultivable diversity is being easily explored from any environmental sample. The different techniques used are metagenomics (for community diversity), metatranscriptomics and metaproteomics (for functional analysis). The metagenomics has been widely explored for the study of microbial diversity associated with different plants such as rice (Hong et al. 2016; Sengupta et al. 2017), wheat (Wang et al. 2021), maize (Enebe and Babalola 2020), sweet potato (Puri et al. 2019), tomato (Ottesen et al. 2019), *Arabidopsis* (Bai et al. 2015; Regalado 2019) and saffron as well (Ambardar et al. 2014, 2021; Bhagat et al. 2021). The microbiome of saffron has been extensively studied in different growth stages, i.e. flowering phase, dormant phase and vegetative phase; different niches i.e. bulk soil, rhizosphere, and cormosphere; and also across different geographical locations i.e. Kashmir, Kishtwar, and Bengaluru in India and Taliouine in Morocco using Metagenomic approach (Ambardar et al. 2014, 2016, 2021; Bhagat et al. 2021). The knowledge gap regarding the information about the microbiome associated with saffron was identified by our team first and we have been adding to this data and knowledge generated incrementally.

7.3.1 Microbiome Across Saffron Niches During Different Growth Stages

In the year 1957, Hutchinson (1957) gave the definition of fundamental niche, as the needs of a species for maintaining positive population growth rate exclusive of inter specific interactions. The different parts such as stem, roots, leaf, seed, fruit, flower etc. of the plant; hosts different microbial community, hence each part serves as different the ecological niches for the microbes (Zheng and Gong 2019). The microbial diversity present in the soil represents the common reservoir and niche specific microbes are supposed to be recruited from the soil with the passage of evolution and also from seed through vertical transmission (Taule et al. 2021). The microbial

diversity associated with saffron in different niches rhizosphere and cormosphere with bulk soil as reference has been studied during the different growth stages, i.e. flowering stage and dormant stage. The bacterial diversity associated with three niches were studies using cultivation-independent cloning dependent and direct high throughput sequencing approach using next generation sequencing technologies.

I. **Cultivation-Independent Cloning Dependent Metabarcoding—Bacterial Diversity**
In this study a full length 16S rRNA gene was amplified from the metagenomic DNA isolated from bulk soil, rhizosphere and cormosphere during flowering and dormant stages. Than 16S rRNA metagene clone library was constructed using TA cloning kit (Fermentas). Clones obtained were randomly screened by colony PCR using M13 primer and selected based on Amplified Ribosomal DNA Restriction analysis (ARDRA). Further, 16S rRNA gene of selected clones were sequenced using Sanger method, cataloguing the bacterial diversity up to species level. Bacterial diversity in the bulk soil was found more as compared to rhizosphere and cormosphere using cloning dependent approach in both the growth stages (Ambardar et al. 2014).

(a) **Flowering stage**: During flowering stage, a total of 5 phyla were reported in bulk soil, 3 in rhizosphere and 2 in cormosphere with two phyla, i.e. *Proteobacteria* and *Firmicutes* found common in all the niches. In cormosphere, 98% of the bacterial diversity belonged to phylum *Proteobacteria* and 2% to *Firmicutes*. In bulk soil 13 genera were catalogued with *Acidobacteria* being dominant. In rhizosphere, *Pseudomonas* was the most dominant genus among 8 catalogued bacterial genera. Cormosphere bacteria comprised of 6 different genera, dominated by the genus *Pantoea*. At species level, maximum number of bacterial species was reported from rhizosphere (21) followed by cormosphere (12) and bulk soil only 2 species. The bacterial diversity associated with different niches showed significant variation ($p < 0.05$) and none of the bacterial genus was found common in the all the three niches, however few common bacteria were found while comparing two niches like *Pantoea* (*Pantoea vagans*, *Pantoea agglomerans* and *Pantoea eucrina*) and *Enterobacter (E. ludwigi)* common in rhizosphere and cormosphere; *Pseudomonas (P. frederiksbergensis)* and *Acidobacteria GP6* common in rhizosphere and bulk soil and *Staphylococcus epidermidis* common in cormosphere and bulk soil (Fig. 7.4).

(b) **Dormant stage**: In dormant stage, bacterial diversity was catalogued only in bulk soil and cormosphere since corms lack roots during dormant stage. 4 phyla were reported from bulk soil and 3 from cormosphere, *Proteobacteria* being the common in both. In bulk soil, *Arthrobacter* was the dominant genera among the total 16 genera catalogued whereas *Chryseobacterium* was the dominant among the 12 bacterial genera catalogued in cormosphere. 5 genera (*Rhizobium*, *Burkholderia*, *Stenotrophomonas*, *Chryseobacterium*, *GP6*) were found common in both the niches. 11 bacterial species were catalogued from bulk soil, 12 from cormosphere were identified and 2 species (*Rhizobium lusitanum* and *Stenotrophomonas maltophilia*).

On comparison, of bulk soil sample during two different growth stages, 3 genera (*GP4*, *GP6* and *Ralastonia*) were found common whereas *Stenotrophomonas* and *Enterobacter* were common in cormosphere in both the growth stages. This was the first report for the identification of bacteria using cultivation-independent 16S metabarcoding approach associated with rhizosphere, cormosphere and bulk soil of saffron (Ambardar et al. 2014). Although in cloning dependent approach the bacteria were

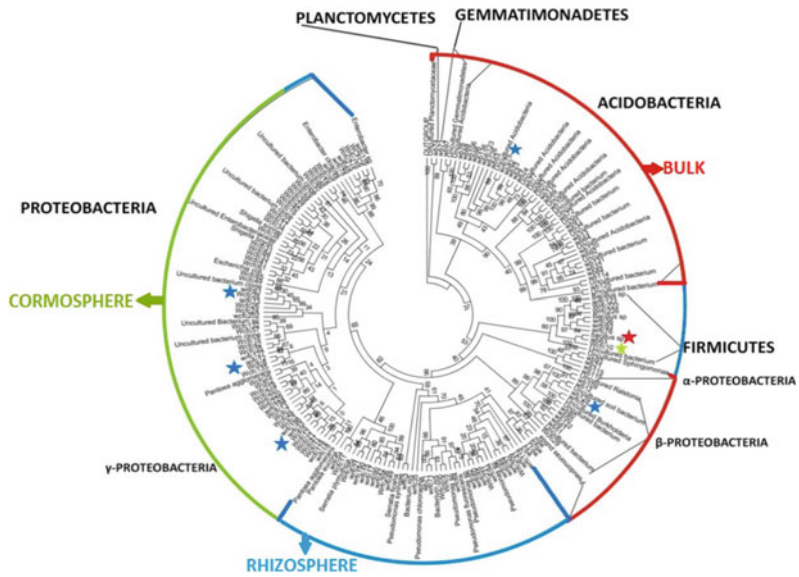

Fig. 7.4 Phylogenetic tree representing all the sequences of bulk soil (red), cormosphere (green) and rhizosphere (blue) which are clustered in separate clads represented by different colours. Star in the clad depicts the common bacteria between the two samples, i.e. blue stars in green clad depicts the bacteria common in rhizosphere and cormosphere whereas red and green stars in blue clad represent the cormosphere and rhizosphere bacteria common to bulk soil. *Source* Ambardar et al. (2014)

characterized up to species level but the limitation is that only dominant bacteria are captured as a result of inherent biases in the methodologies such as lysis, extraction, PCR amplification, cloning and sequencing bias (Ambardar et al. 2014).

On comparing bacterial diversity catalogued by culture dependent and culture independent (metabarcoding) approach, at phylum level, all the phyla captured by culture dependent approach were also present in metabarcoding data. 4 such phyla (*Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteriodetes*) were found common. At genus level, 5 genera (*Acinetobacter*, *Arthobacter*, *Bacillus*, *Chryseobacterium*, and *Pseudomonas*) were catalogued by both the approaches. The genus *Brevibacterium* was captured by cultivation-dependent approach but metabarcoding technique fails to capture this genus. In addition, 38 genera were catalogued by only metabarcodic approach.

II. **Cloning Independent Metabarcodic Approach–Fungal Diversity**

As cloning dependent metabarcodic approach suffers from cloning bias so direct sequencing of DNA from any sample eliminates this bias. Cloning independent metabarcodic approach involved the high throughput sequencing of phylogenetic genes (ITS and 16S rRNA) amplified from metagenomic DNA. Ambardar and coworkers (2016) catalogued the fungal diversity associated with bulk soil, rhizosphere and cormosphere during two growth stages using high throughput sequencing of Inter transcribed spacer (ITS) region. The phylogenetic gene was amplified from extracted metagenomic DNA using universal ITS primers and sequenced using the pyrosequencing at Roche Diagnostics India Pvt. Ltd., New Delhi (Ambardar et al. 2016).

(a) **Flowering stage**: During this stage, a total of 6 fungal phyla were catalogued from all the niches. Phylum *Zygomycota* was found dominant in rhizosphere and *Basidiomycota* cormosphere and phylum *Ascomycota* was dominant in bulk soil. *Pseudogymnoascus* was the dominant fungus among 19 fungal genera catalogued from bulk soil, *Cryptococcus* fungus was dominant among total 35 fungi in cormosphere and in rhizosphere, *Mucor* was the most dominant fungi among the total 45 genera. On comparison, 13 fungi were found common in all the niches.

(b) **Dormant stage**: During this stage also like flowering stage, *Zygomycota* was dominant in cormosphere and *Ascomycota* in bulk soil. 44 fungal genera were catalogued from bulk soil and 5 genera from cormosphere were reported. In both the niches, genus *Rhizopus* was found to be the dominant genera. 4 genera were common in cormosphere and bulk soil. It has been reported that the fungal diversity was niche as well as growth stage specific.

Niche specific associations were observed during both growth stages as specific fungi were found to be associated with each niche. In flowering stage, 32 fungal genera were specific to rhizosphere, 22 to cormosphere and 6 to bulk soil whereas in dormant stage, 40 fungi were specific to bulk soil and only one to cormosphere. On comparing, cormosphere niche during both the growth stages, phylum *Basidiomycota* was dominant during flowering stage but *Zygomycota* was dominant during dormant stage. The phyla which was dominant at dormant stage was rare at flowering stage and vice-versa (*Basidiomycota*: Flowering = 93.2% Dormant = 0.05% and *Zygomycota*: Flowering = 0.8% Dormant = 99.7%). However, bulk soil fungi were dominated by *Ascomycota* during both the stages. This study was the first report on the fungal diversity associated with the root of *C. sativus* and first report on the fungi associated with corm of any plant with the temporal and spatial variation in the fungal community structure (Ambardar et al. 2016) (Fig. 7.5).

Shuwen and coworkers (2021) have studied the fungal diversity associated with the surface of corms having different growth characteristics. Corms with different characteristics divided in to 5 groups such as infected corms (group-A), unable to germinate (group-B), able to germinate but not bloom (group-C), able to germinate and produce one flower per corm (group-D), and able to germinate and produce two flowers per corm (group-E) were collected. DNA was isolated from the surface of corms and then PCR was done using the ITS specific primers. The amplified product from each sample was pooled and then the double-terminal library was constructed. The amplicon library was sequenced on Miseq Illumina platform. The results revealed that the 65 OTUs were common among all the groups and lowest rich and diversity of fungi was reported in infected corms. At family level, in group-A, three fungal families, Trichocomaceae, Helitiales, Leotiomycetes were reported. In group-B (Aspergillaceae, Eurotiomycetes, Eurotiales), group-C (Cladosporiaceae, Capnodiales, Pseudeurotiaceae), group-D (Rutstroemiaceae, Plectosphaerellaceae, Glomerellales, Filobasidiales, Tremellomycetes) and in group-E (Mycosphaerellaceae, Didymellaceae, Pleosporales, Dothideomycetes, Hypocreales, Sordariomycetes) were reported. At genus level difference in the abundance of 5 genera *Cladosporium*, *Lambertella*, *Passalora*, *Penicillium*, and *Talaromyces* among the different group. Three of these genera *Cladosporium*, *Penicillium*, and *Talaromyces* have also been also reported by Ambardar et al. (2016).

7.3.2 Microbiome Across Different Geographical Location

The cormosphere and rhizosphere bacteriome has been studied across different locations in India and Morocco. The microbial diversity associated with cormosphere (corm sheath) of saffron has been studied in two regions in India, Kishtwar

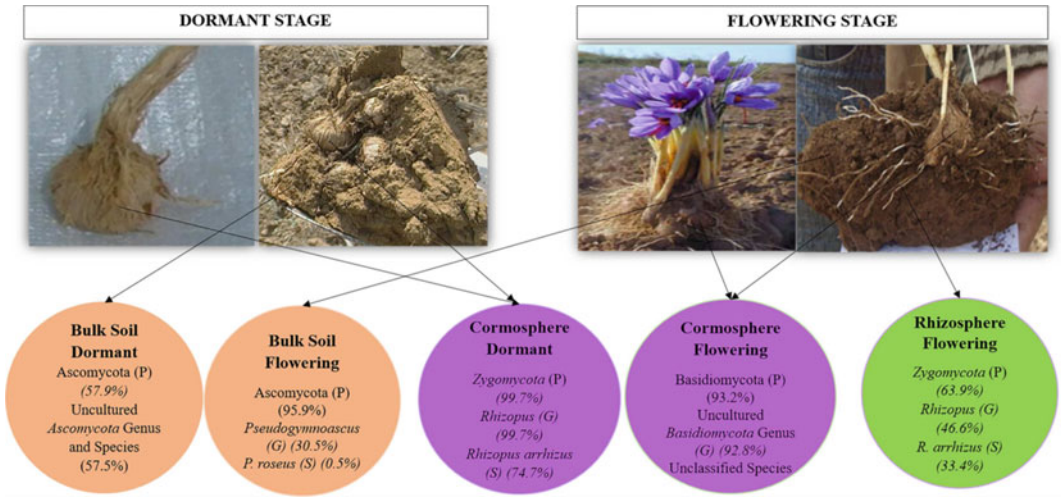

Fig. 7.5 Dominance pattern of fungal community in each of niche during two growth stages. During flowering stage, dominance of *Rhizopus arrhizus* (*Zygomycota* phylum) in rhizosphere, *Pseudogymnoascus roseus* (*Ascomycota* phylum) in bulk soil was observed whereas in the cormosphere, the sequences belonging to dominant *Basidiomycota* phylum could not be classified upto genera or species level. During dormant stage, *Rhizopus arrhizus* (*Zygomycota* phylum) was dominant in cormosphere whereas in the bulk soil the sequences belonging to dominant *Ascomycota* phylum could not be classified upto genera or species level. In the figure, P represents phylum, G represents genus and S represents species of fungi. *Source* Ambardar et al. (2016)

(2011, 2012, 2013) and Kashmir (2013) and from Taliouine (2016), Morocco during the vegetative phase of the saffron life cycle (Bhagat et al. 2021). The metagenomic DNA was extracted from all the samples, libraries were prepared using Truseq Nano DNA Library preparation kit (Catalog No. 20015964, Illumina, CA, USA) and whole genome shotgun sequencing was done using Illumina NextSeq 500 platform with 2 × 150 base paired-end configuration from Xcelris Labs Limited, Ahmadabad, Gujarat, India. The raw reads obtained were de-novo assembled using CLC Genomics Workbench 6.0 (CLC bio, Aarhus, Denmark), the assembled contigs obtained were analyzed for taxonomic classification using online web server using MG-RAST i.e. Metagenomic Rapid annotation using Subsystem Technology server version 4.0.3 (Meyer et al. 2008).

A total of 16 microbial phyla were present in all the samples, consisted of 13 bacterial phyla (*Proteobacteria*, *Firmicutes*, *Acidobacteria*, *Chlorobi*, *Actinobacteria*, *Planctomycetes*, *Gemmatimonadetes*, *Verrucomicrobia*, *Chloroflxi*, *Deinococcus-Thermus*, *Cyanobacteria* and *Bacteroidetes*) bacterial, 2 fungal (*Ascomycota* and *Basidiomycota*), 1 archaeal (*Euryarchaeota*). These phyla represented the core phyla associated with the cormosphere of saffron across different geographical location and among all *Proteobacteria* was found to be the most abundant phylum. A total of 73 bacterial genera were reported from all the locations and 24 genera were found common represented the core genera associated with saffron across different geographical location in Asian and African subcontinent. *Pseudomonas* (12.78 and 8.66%) was most abundant in Kashmir and Morocco whereas *Streptomyces* (14.3%) was the dominant in Kishtwar. Although the Kashmir and Kishtwar are geographically closer to each other, PCA plot analysis clustered the saffron's microbial diversity in Morocco and Kashmir with each other and Kishtwar clustered away (Bhagat et al. 2021). In addition to 24 core genera, unique genera specific to each locations were identified as 5 unique genera (*Microscilla*, *Planctomyces*, *Marivigra*, *Candidatus koribacter*, and *Plesiocystis*) in Kashmir, 5 unique genera in Kishtwar

(*Kribbella*, *Saccharopolyspora*, *Rhodococcus*, *Amycolatopsis* and *Streptosporangium*) and 7 unique genera in Morocco (*Aeromicrobium*, *Methylovorus*, *Bdellovibrio*, *Algoriphagus*, *Chryseobacterium*, *Geodermatophillus* and *Rahnella*) (Fig. 7.6). It has been concluded that the saffron cormosphere share common microbiome across different geographical locations, but the unique genera specific to each location can be used as biomarker and the cormosphere bacteriome is location specific.

Similar to cormosphere, the microbial diversity associated with rhizosphere of saffron grown across different regions in India was studied (Ambardar et al. 2021). Saffron rhizosphere diversity were studied among different 8 regions in Kashmir (Wuyan, Patalbagh_upper range, Patalbagh_lower range, Alchibagh, Galandar, Chandhara, Hatiwara, Samboora), 1 region in Kishtwar (Berwar) and one laboratory grown sample in Bengaluru, India. The rhizosphere soil samples were collected during the flowering stage of saffron life cycle in the year 2016. The metagenomic DNA was extracted and 16S rDNA sequencing library was constructed targetting the V3 and V4 hyper-variable regions of the 16S rDNA gene (Illumina, San Diego, CA, USA). The sequencing of the constructed libraries was done using the MiSeq sequencing platform (Illumina) using a 2 × 250 cycle V3 kit.

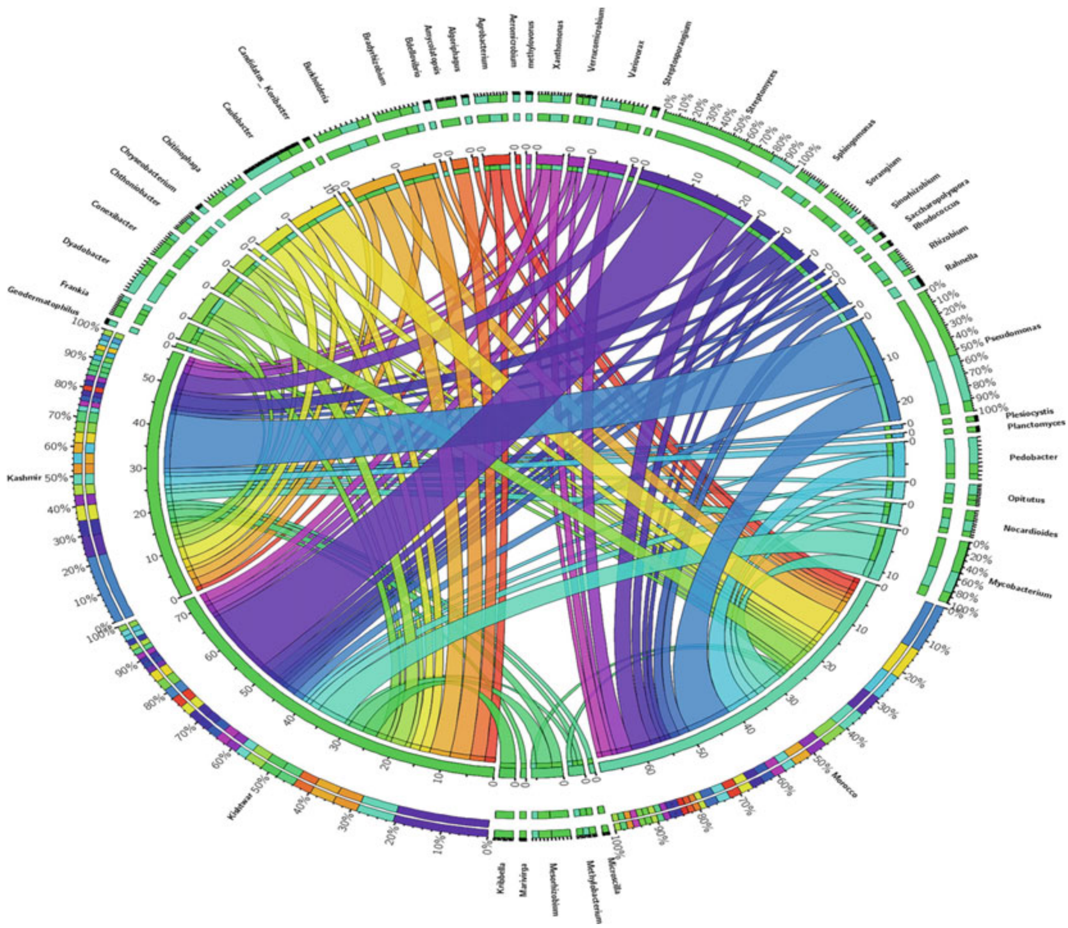

Fig. 7.6 Abundance of core genera and unique genera in three geographical locations. Central line (ribbons) represents the abundance of each genus and connects genera to samples. The outer three rings are the stacked bars representing the relative contribution of particular genera in every sample. *Source* Bhagat et al. (2021)

The QIIME 1 pipeline (Quantitative Insights into Microbial Ecology version 1.9.1) was used for the data analysis and the taxonomic affiliation was assigned using GreenGenes taxonomy database (Caporaso et al. 2010). The bacterial diversity associated with the rhizosphere among all the 10 samples were catalogued at phylum, genus and species level. A total of 16 phyla, 273 genera and 72 species were catalogued from all the 10 samples. 11 phyla, 53 genera and 8 species were found common in all the 10 samples that constituted the core rhizo-bacteriome associated with saffron grown in all the regions (Ambardar et al. 2021) (Fig. 7.7). At phylum level, *Proteobacteria* was the most abundant in all the 10 samples. *Bacillus* was the most abundant bacterial genus in Kishtwar, Bengaluru and three fields in Kashmir whereas *Lysobacter* was abundant in rest of 5 fields. Dominance of 3 bacterial species, *Bacillus flexus*, *Lysobacter brunescens* and *Janthiobacterium lividium* were observed in the rhizo-bacteriome of saffron grown in all the 10 locations. *B. flexus* was found abundant in Kashmir, Bengaluru and 5 fields in Kashmir (Wuyan, Galandar, Hatiwara, Patalbagh_upper range and Patalbagh_lower range) whereas *L. brunescens* was dominant in Chandhara and Samboora; and *J. lividium* in Alchibagh. The laboratory grown sample in Bengaluru showed more similarity with Kashmir sample because the seeds sown in Bengaluru were from Kashmir origin; it indicated that the bacterial diversity associated with saffron rhizosphere is plant driven. In addition to core bacteriome, each location harbored some unique genera and species as well. We were able to catalogue a significant number of plant growth promoting bacterial genera (110 out of 273) and species (21 out of 73) in the rhizosphere of saffron based on the reported literature in other plant and saffron earlier.

Our recent reports identified the core microbiome in cormosphere (Bhagat et al. 2021) and rhizosphere (Ambardar et al. 2021) which persisted in the specific niche irrespective of geographical locations. Unique cormosphere bacteria specific to each location could be used as molecular markers. In addition, rhizo-bacteriome in saffron was reported to be plant driven.

As Kashmir and Kishtwar regions were similar in both the above studies, we wanted to identify the core-bacteriome between cormosphere and rhizosphere of saffron by comparing the bacterial diversity associated with them. However, both the niches (cormosphere and rhizosphere) were sequenced using two different sequencing techniques, platforms and different bioinformatics analysis pipeline used were also different. Cormosphere samples have been sequenced using Illumina NextSeq 500 platform whereas rhizosphere samples using Illumina Miseq platform. Both the sequencing platform vary in data output and read length wherein data generation in NextSeq is more but the read length is less in contrast to Miseq where less data is generated with long paired ends are generated. The probable reason for the change in diversity can only be concluded with certainty after sequencing the samples using same sequencing platform.

In Kishtwar, a total of 56 genera were identified in cormosphere and 138 genera in the rhizosphere of saffron. A core bacteriome, identified across these two niches, consisted of 28 genera (*Agrobacterium*, *Amycolatopsis*, *Anaeromyxobacter*, *Arthrobacter*, *Bacillus*, *Bradyrhizobium*, *Chitinophaga*, *Conexibacter*, *Dyadobacter*, *Gemmata*, *Gemmatimonas*, *Hypomicrobium*, *Kibbella*, *Mesorhizobium*, *Methylobacterium*, *Mycobacterium*, *Nocardioides*, *Novosphingobium*, *Opitutus*, *Phenylobacterium*, *Rhizobium*, *Rhodococcus*, *Sinorhizobium*, *Sphingobium*, *Stenotrophomonas*, *Streptomyces* and *Variovorax*). In addition, 26 bacteria genera were unique to cormosphere and 110 genera unique to rhizosphere of saffron in Kishtwar (Fig. 7.8).

In a similar way, core microbiome between cormosphere and rhizosphere of saffron grown in Kashmir was identified and consisted of 16 genera (*Agrobacterium*, *Bacillus*, *Burkholderia*, *Chitinophaga*, *Dyadobacter*, *Flavobacterium*, *Hyphomicrobium*, *Mesorhizobium*, *Nocardiodies*, *Opitutus*, *Planctomyces*, *Rhizobium*, *Sphingomonas*, *Streptomyces* and *Variovorax*)

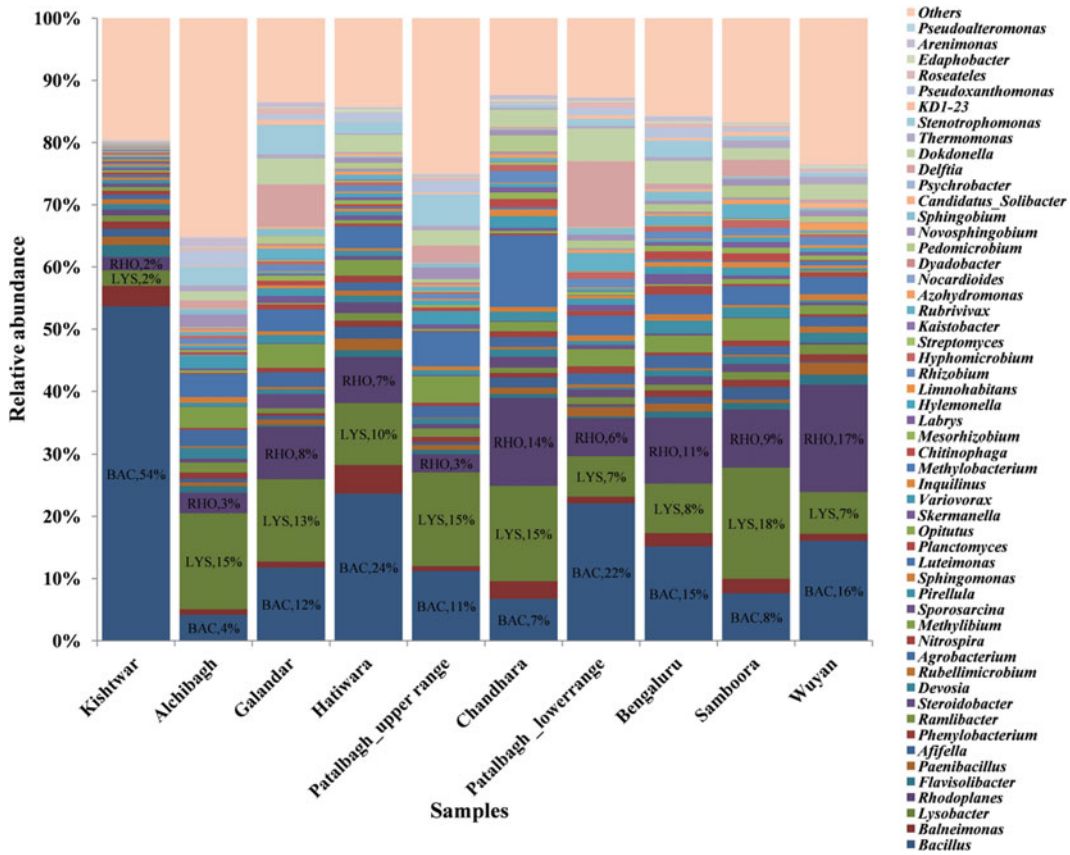

Fig. 7.7 Comparison of bacterial genera in saffron rhizosphere from different geographical locations. The OTU catalogued up to genus level were normalized to 10,000. BAC, *Bacillus*; LYS, *Lysobacter*; RHO, *Rhodoplane*; JAN, *Janthiobacterium* were dominant genera in these samples. In this figure, Kishtwar sample is distinct from the Kashmir samples. The microbial communities of Kashmir and Bengaluru samples are similar as compared to Kishtwar sample. *Source* Ambardar et al. (2021)

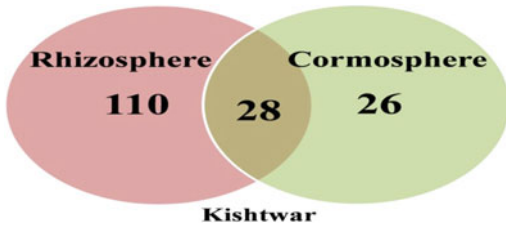

Fig. 7.8 Venn diagram showing the common and unique bacterial genera in the rhizosphere and cormosphere of Kishtwar, India

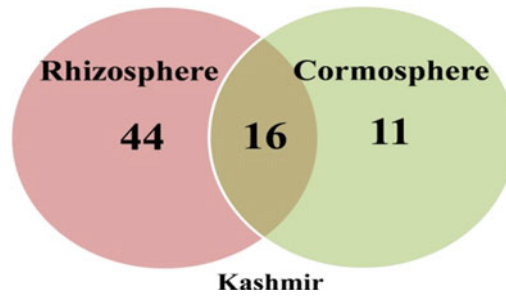

Fig. 7.9 Venn diagram showing the common and unique bacterial genera in the rhizosphere and cormosphere of Kashmir, India

that have been reported common in the cormosphere and rhizosphere of saffron grown in Kashmir. In addition to core-bacteriome, 11 genera were present specifically in the cormosphere of Kashmir and 44 genera in all the region of Kashmir (Fig. 7.9).

7.4 Conclusions

Though microbiomic work on saffron was initiated by our group a decade back, but recently many researchers have shown interest in this study. The microbiome associated with any plant has both negative and positive effect and so is true for saffron. Saffron is grown in many regions of the globe but microbiomics studies are few and lot needs to be done in this area. The interest in saffron microbiome is also on account of it being mono genetic crop lacking genetic diversity. The diversity in the microbiome on account of variations in climate and soil type can be used as molecular markers. For this microbiome of the saffron across the geographical regions it is being cultivated needs to catalogues and needs international collaboration. This study can identify the core microbiome common to saffron grown across geographical regions, location specific unique microbiome, that can be developed into barcode for saffron grown in specific region.

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Saffron, Bacteria and Mycorrhiza

8

Mitra Aelaei and Fahimeh Salehi

Abstract

Proper management in protecting and improving soil fertility in the modern agricultural systems provides the nutrients needed by the plant and eventually increases the yield and quality of crops. Organic farming is recommended today due to its huge impact on human life and health. The use of chemical fertilizers causes a crisis of environmental pollution, especially pollution of soil and water resources, which enter the human food resources in a chain and threaten the human society. Also, chemical fertilizers in the long time destroy the physicochemical properties of the soil and make it difficult for plant roots to penetrate, and ultimately lead to loss of yield. Addressing this challenge requires the use of modern farming approaches including the use of biological fertilizers. Running towards this purpose, researchers evaluate the living and active soil community and identify beneficial soil microorganisms to further use them to increase soil fertility and increase plant growth and crop yield. As a result, these bio-fertilizers can be a suitable and desirable alternative to chemical fertilizers and one of their significant effects is to create the highest efficiency and yield in order to produce plant growth stimulants to provide absorbable nutrient. In other words, these microorganisms are able to prepare nutrients from their non-absorbable forms to the absorbable forms during the biological process. The use of these biological fertilizers as suitable nutritional elements plays an essential role towards improving the vegetative and reproductive properties of saffron. As saffron is usually grown in arid and semi-arid climates, which is poor in soil organic matter, the availability of such fertilizers during the cultivation of this plant would be very effective.

8.1 Introduction

8.1.1 Sustainable and Organic Agriculture

Due to the increasing population in the world and the limits of food production on the other hand, different efficient strategies and approaches should be considered to guarantee supplying adequate food globally. According to forecasts, in the next 50 years, the world's population will increase to 10 billion people, and in order to provide enough food for such population, productivity and yield must be increased significantly. However, since food production is not easy enough, it requires

M. Aelaei (✉) · F. Salehi
Department of Horticulture, Faculty of Agriculture, University of Zanjan, 45371-38791 Zanjan, Iran
e-mail: maelaei@znu.ac.ir

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planning and the application of practical ideas. In order to produce more food, cultivation and high quality production should be increased (Glick 2012), in this regard factors that increase the quantity and quality of the crops should be considered carefully to ultimately improves crop performance (Koocheki et al. 2011). In fact, today, the use of low-input systems and the development of new management methods, including fertilizer management, are crucial to create sustainable agriculture (Yang et al. 2021). Biodegradable chemical fertilizers, especially phosphate fertilizers, seem to be a proper approach to improve the quality of soil and eventually crops, especially in poor soils, where nutrients are widely used with the aim of significantly reducing or eliminating inputs (Kader et al. 2002; Sharma 2002).

Undoubtedly, the use of bio-fertilizers, in addition to the positive effects on soil properties and soil fertility (Piromyou et al. 2011) has several other benefits economically, environmentally and socially and can be used as a suitable and desirable alternative of chemical fertilizers (Saleh-Rastin 2001). These fertilizers are prepared on the basis of natural selection of soil beneficial organisms to achieve different goals (Asgharzadeh 2005) and have a special importance in increasing yield and maintaining sustainable soil fertility (Sharma 2003). One of the most effective impacts of such fertilizers is their higher efficiency and effectiveness to produce plant growth stimulants in order to provide nutrients in an absorbable manner (Asgharzadeh 2005).

Due to the cheaper price of chemical fertilizers, easier access and use, and faster return on investment, they are strong competitors for bio-fertilizers (Lucy et al. 2004; Barea et al. 2005). However, the use of chemical fertilizers in recent years has caused a crisis of environmental pollution, especially pollution of soil and water resources, which has reached human food sources in chains and has threatened the health of human society (Singh and Kapoor 1998). However, the daily increase in chemical fertilizers price, necessity of sustainable use of water and soil resources alongside the production of healthy crops led to more attention to bio-fertilizers (Glick 1995). Towards these goals, modern approaches such as evaluation of dynamic population of soil microorganisms to develop bio-fertilizers will be required (Singh and Kapoor 1998). It is necessary to identify beneficial microorganism in the soil to use them as bio-fertilizer (Singh and Kapoor 1998). In case of medicinal plants, most consumers pay attention to the quality of the crops. As a result, sustainable agriculture is also reliable in order to create the desired quality, therefore seems necessary moving towards sustainable agricultural systems and producing healthy products. In fact, the use of these fertilizers significantly improves soil structure, organic matter content and soil fertility, which has numerous benefits for plants and soil (Patra et al. 2000). The appropriate quantitative and qualitative utilization of agricultural crops not only depend on different genetic factors but also are affected by environmental features such as type and quantity of fertilizers (Jami et al. 2020).

8.1.2 Bio-Fertilizers

Bio-fertilizers are not exclusively organic materials from animal manures, plant residues, green manure, etc., but also include bacteria as well as beneficial fungi, each with a specific purpose (such as nitrogen fixation and release of phosphate, potassium and iron ions from insoluble compounds) (Manaffee and Kloepper 1994; Koocheki 2004; Kucey 1988; Han et al. 2006). Fungi and bacteria cause changes in the chemical properties of the soil, usually in the upper horizons of the soil that contain degradable materials (Calvaruso et al. 2009). It also occurs in a few millimeters around the root (rhizosphere) where the activity of microorganisms is much higher than in the surrounding soil due to the presence of root secretions and abundant nutrients in the rhizosphere (Dokora et al. 2003; Calvaruso et al. 2009). The rhizosphere refers to a thin layer (usually 1–3 mm) of soil around the root in which living organisms are qualitatively and quantitatively affected by root activity (Benizri et al. 2001; Bowen and Rovira 1999). The

secretion of compounds such as high amounts of sugar, amino acids and organic acids causes bacteria to accumulate in the rhizosphere, which is about 10–1000 times the soil surface (Penrose et al. 2001). Also, minerals in this area are mostly affected by weathering due to lowering the pH by root secretion, root coexistence with fungi and bacteria and community of free bacteria. The results of several studies indicate that the aeration of minerals by fungi and bacteria has a great impact on the cycle of elements and plant nutrition (Wallander 2000; Calvaruso et al. 2006).

Different groups of microorganisms in the rhizosphere may be beneficial, harmful, or harmless to the host plant (Benizri et al. 2001). Plant growth promoting rhizobacteria (PGPRs) are one of the most important of these microorganisms which play an important role in promoting plant growth (Lynch 1990; Manaffe and Kloepper 1994; Vessey 2003; Sharma 2003). In general, the use of PGPRs has become very widespread today (Reed and Glick 2004; Glick 2012). These growth-promoting bacteria, which are considered to be beneficial bacteria, are usually actively present around plant roots that cooperate in absorbing elements and stimulating plant growth (Boer and Copeman 1974; Vessey 2003; Figueiredo et al. 2011).

8.1.3 Types of Soil Bacteria and the Role of Reinforcing Them

Usually soils contain a huge variety of microorganisms, including bacteria, actinomycetes, fungi, algae, and protozoa. One gram of soil contains approximately 9×10^7 bacteria, 4×10^6 actinomycetes, 2×10^5 fungi, 5×10^3 protozoa and 3×10^1 nematodes (Alexander 1990). The amount of these microorganisms varies in different soils. Bacteria in the soil are able to grow rapidly and cause chemical changes in the environment around the plant. In fact, these microorganisms often attach to the soil particles or are present around the plant roots (Lynch 1990). Most of the bacteria around the roots are probably present due to the large amounts of nutrients, including amino acids, sugars, and organic acids secreted by the plant roots, which are used by bacteria for growth and development and increase the yield of crops (Whipps 1990). Interactions between soil bacteria and plant roots can be beneficial, harmful or neutral. For example nitrogen fixation by bacteria usually results in higher yield (Glick et al. 2007).

These bacteria have been commercially used as biological tools to enhance plant growth (Reed and Glick 2004) and known as PGPB bacteria (Fig. 8.1) (Bashan and Holguin 1998). Many different soil bacteria are considered plant-enhancing bacteria, and different strains of a species do not have the same genetic makeup and metabolic ability. The use of microorganisms in agriculture is necessary to improve the absorption of nutrients by plants. In this regard, uses of rhizosphere bacteria that promote plant growth based on sustainable agriculture are extremely important in different parts of the world and have increased over the past two decades (Khayamim 2009; Norouzi and Khademi 2010).

Azotobacter (*Azotobacter* spp.), *Azospirillum* (*Azospirillum* spp.), *Pseudomonas* spp., *Bacillus* (*Bacillus* spp.) and *Rhizobium* are among the most important active bacteria around plant roots (Saleh-Rastin 2001; Zahir et al. 2004). These microorganisms have a cooperative relationship with the host plants (Vessey 2003). There are also some reports on plant growth promoting properties of *Flavobacterium*, *Enterobacter*, *Arthrobace*, *Clostridium*, *Acinetobacter*, *Burkholderia*, *Enterobacter*, *Rhizobium*, *Serratia*, *Erwinia*, *Alcaligenese* (Bowen and Rovira 1999; Antoun and Kloepper 2001). These bacteria play an important role in increasing plant growth. In fact, in addition to bio-stabilization of nitrogen and solubilization of soil phosphorus, they produce a variety of growth hormones such as auxin, cytokinin, gibberellin, which stimulate plant growth and development (Zahir et al. 2004). In the interaction of specific bacteria with legume plants, it has been found that they cause an increase in fresh and dry weight due to better nitrogen uptake and stabilization (Dobbelaere et al. 2003). *Pseudomonas* are aerobic Gram-negative bacteria, rod-shaped, straight or slightly

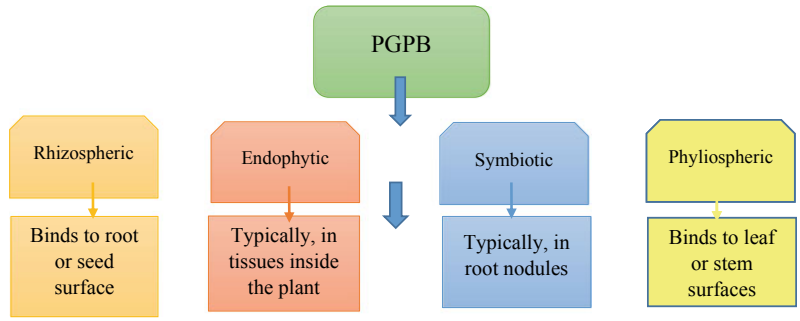

Fig. 8.1 Plant growth-promoting bacteria (PGPB) includes bacteria that interact with plants in different ways (Glick 2014)

curved, 2–4 μm long and 1–8 μm in diameter, a polar flagellum and several lateral flagella, chemo-organotrophic (Bashan and Holguin 1997). These bacteria are found in the root canals and capillaries, and in addition to the root surface, they live and grow inside the cells of the latex layer, the space between these cells, the endoderm, and the phloem vessels of the root (Bashan and Holguin 1998). Of course, some strains of *Pseudomonas fluorescence* actively enhance plant growth, but some species do not have a significant effect on plants (Huang et al. 2013). These bacteria produce metabolites such as growth regulators and a variety of vitamins. They also directly increase plant growth by affecting nutrient uptake (Kloepper et al. 1991). These bacteria have also been reported to improve enzymatic activity, microbial biomass, soil respiration, reduction of microbiological properties, and active microflora in the decomposition of organic matter (Rejili et al. 2012). *Azotobacter* and *Pseudomonas* have an effective interaction with important crops like corn, sorghum, wheat, etc. They increase the efficiency of nutrient absorption (Saleh-Rastin 2001). Inoculation of canola seedlings and corn seeds with *Pseudomonas putida* strain GR12-2 and *Pseudomonas aurantiaca* strain SRI increased the roots' length (Xie et al. 1996; Rosas et al. 1998). Such an increase in growth was associated with the production of Indole-3-acetic acid (IAA), the dissolution of phosphate, and the production of antifungal agents by bacteria (Rosas et al. 1998). Khan (2005) observed that inoculation of plant roots with *Pseudomonas* and *Acinetobacter* increased the absorption of iron, zinc, magnesium, calcium, potassium and phosphorus (Khan 2005). Dordipour et al. (2010) showed that inoculation of soil with *Azospirillum lipoferum* and *Azotobacter chrococoum* increased dry weight and potassium uptake by soybean plants (Dordipour et al. 2010). According to the reports by Leithy et al. (2006), there is a positive effect of *Azotobacter* towards increasing the amount of essential oil and some of the major components of essential oil in rosemary (Leithy et al. 2006). Since the main constituents in essential oils are terpenoids which are made of isoprenoid units (C_5H_8), its biosynthesis is highly correlated with acetyl CoA, ATP, NADPH, which itself depends on the concentration of phosphorus. Growth stimulants play an important role in the absorption of phosphorus (Loomis and Corteau 1972). *Azospirillum* bacteria have also been effective on plant root morphology through the production of auxin, gibberellic acid and kinetin (Tien et al. 1979). Marcelo et al. (2000) showed that beans inoculated with *Azospirillum brasilense*, a growth-promoting and nitrogen-fixing bacteria, increased root length and area compared to control treatment and root system with thinner and longer roots are obtained (Marcelo et al. 2000) which secrete some biologically active substances like B vitamins, nicotinic acids, pantothenic acid, biotin, auxins, gibberellins, etc. cause such a change in performance (Kader et al. 2002). According to the report, the use of bio-fertilizers (containing microorganisms such as *Azotobacter* and *Azospirillum*) and their replacement with artificial growth regulators are highly effective in improving the growth characteristics and

essential oil compositions of sage (Youssef et al. 2004). *Rhizobium*s that coexist with plants to stabilize nitrogen in legumes can increase their growth and yield. Like other growth-promoting rhizosphere bacteria, they stimulate other non-legume plants (Chabot et al. 1996). They can also dissolve organic and mineral phosphates (Antoun et al. 1998) and have a dual positive effect on plant nutrition in terms of nitrogen fixation and phosphate solubility (Peix et al. 2001). These bacteria produce IAA, a common metabolite of L-tryptophan metabolism (Ghosh and Basu 2006; Mandal et al. 2007). Inoculation of radish with rhizobium, a free-living bacterium, increased auxin production and root growth (Besharati et al. 2016). Synergistic (synergistic) interactions with arbuscular mycorrhiza have also been reported (Barea et al. 2005). Rai et al. (2004) also found that the use of rhizobium increased honeysuckle height and biomass. Consumption of growth-promoting bacteria improved the growth and yield of wheat, corn and sorghum (Kumar et al. 2001; Lin et al. 1983). Phosphate-solubilizing bacteria are a group of microorganisms that are able to convert insoluble phosphorus in soil into an available form for the plant. One of the most important species of this family is *Bacillus* (Tilak et al. 2005). *Bacillus subtilis* is a gram-positive bacterium which showed beneficial properties even in food industries to enhance the quality of soybean meal nutritional value and antioxidant properties of black soybean (Yang et al. 2021). *Bacillus* growth-promoting bacteria are of particular importance due to their widespread distribution in the soil, the ability to colonize the rhizosphere of many plants, and the production of a diverse range of metabolites (Bashan and Holguin 1997). Inoculation of plants with *B. subtilis* bacteria increased the percentage of root length in rice (Preeti et al. 2002). *Bacillus cereus* was also used as a solvent for mineral silicates in research by Badr (2006). Studies by Dordipour et al. (2010) also showed increased growth of soybean on *B. subtilis* (Barbedo 2013). Han and Lee (2005) reported increased plant uptake of potassium and phosphorus in soils containing phosphate rock and potassium-containing minerals inoculated with phosphorus-soluble bacteria (*Bacillus mucilaginosus*) and potassium (*Bacillus megaterium*) (Han and Lee 2005). The results of Sheng (2005) showed that inoculation of *Bacillus edaphicus* into cotton and rapeseed plants in greenhouse conditions increased the root dry weight of both plants by 19% and shoot dry weight by 24% and 21%, respectively. In addition, the concentration of potassium in cotton was about 31–34% and in rapeseed was 28–31% and the concentration of nitrogen and phosphorus was increased. The absorption of more nutrients by plants inoculated with bacteria can be attributed to the development of root growth due to auxin production in the rhizosphere and eventually better absorption of water and nutrients from the soil (Sheng 2005). Inoculation of the plant with growth-promoting bacteria *Paenibacillus polymyxa*, which is a nitrogen-fixing agent, increased the induction of ERD15 gene expression in *Arabidopsis*, which is a drought tolerant gene (Timmusk and Wagner 1999). In recent years, many experiments have been performed on the microbial role to the coexistence of mycorrhizal fungi and growth-promoting bacteria under various conditions like drought stress, salinity, etc. in crops and alkaloid plants (Ghorbanpour et al. 2011; Wu et al. 2008). Inoculation of bean seeds with *Rhizobium leguminosarum* in Phaseoli reduced root rot and increased the fresh and dry weight of plant roots (Buonassisi et al. 1986). The presence of bacteria around soybean roots also increases root growth. This increase in growth may be due to the competitive ability of bacteria to be located in the rhizosphere of the plant, which causes the production of the hormones auxin and cytokinin, and due to the role of auxin and cytokinin, which increases cell division and expansion and elongation of plant tissue, followed by weight gain and becomes dry roots (Gray and Smith 2005; Dobbelaere et al. 2003).

Bacteria directly and indirectly strengthen the plant (Glick 2012). Direct mechanisms include molecular nitrogen fixation, dissolving phosphate compounds through the secretion of organic

acids and phosphatases, and better absorption of potassium (Tilak et al. 2006), degradation by the production of organic acids (citric, oxalic, and tartaric) (Song and Huang 1988) and the chelation of silicon to remove potassium, silica, and aluminum from insoluble potassium-bearing minerals (Barker et al. 1997; Bennett et al. 1998), production of iron (siderophore) and increasing the usability of iron that occurs in conditions of iron deficiency and better absorption (Besharati et al. 2016). Also sulfur oxidation (Tilak et al. 2006), pH reduction by secretion of organic acids (acetate, lactate, oxalate, tartrate and succinate, citrate, gluconate, ketogluconate, glycolate, etc.) or proton secretion (Chabot et al. 1996) increases the ability to absorb nutrients (Tilak et al. 2006), improvement of soil structure, production of plant growth regulators and production of enzymes such as ACC-deaminase (Rodrıguez and Fraga 1999), which further stimulate plant growth and increases the quantity and quality of the crop (Glick 1995; Omidi et al. 2009; Sturz and Christie 2003; Vessey 2003). Meanwhile, PGPB sometimes has a high effect on both nitrogen fixation and phosphate solubility (Rai 2006), while the resources of phosphate fertilizers in the world are limited and their production is costly (Gyaneshwar et al. 2002) and on the other hand, the efficiency of phosphorus fertilizers in calcareous soils is very low, which in order to solve these problems, microorganisms such as bacteria are used as phosphate solvent (Chabot et al. 1996). In indirect mechanisms, these bacteria modulate or neutralize the adverse effects of one or a number of plant pathogens by promoting induced or acquired systemic resistance (Schippers et al. 1987) and reducing soil toxicity (Singh et al. 2011). Expansion of the plant root system and the modification of root morphology by the production of various secondary metabolites, such as auxin, increase the number of capillary and secondary roots which are features of rhizosphere bacteria to enhance the plant growth (Glick 1995; Aloni et al. 2006; Besharati et al. 2016). Indeed, a higher germination rate was observed in mustard and marshmallow during exposure to PGPRs (Golpayeghani et al. 2010). Interestingly, an increase in leaf area, protein content and chlorophyll content, resistance to abiotic stresses, and delay in the aging process (Adesemoye and Kloeppe 2009), production of secondary metabolites (Sangwan et al. 2001; Ping and Boland 2004) and plant growth regulators (Esitken et al. 2010) are transparent impacts of bacterium-based bio-fertilizers (Adesemoye and Kloeppe 2009). Since secondary metabolites are derived from primary metabolites, a plant can only produce more secondary metabolites if it is well developed (Farooqi and Shukla 1990).

Rhizosphere bacteria elucidated the synthesis of theosopanal alkaloids in *Hyoscymus niger* and subsequently increased the content of hyoscyamine and scopolamine (Ghorbanpour et al. 2011). Such bacteria are also promoting the production of indole acetic acid and cytokinin using the amino acids tryptophan and adenine (Zahir et al. 2004). Ethylene modulates the growth and development of plants and is also produced in response to stress plants. Ethylene synthesis is influenced by various factors such as temperature, gravity, nutrition, plant hormone levels and types of biological stress (Abeles et al. 1992). Generally, researchers today consider the synthesis of auxin and the regulation of ethylene production in young seedlings as the most important mechanism of rhizosphere bacteria in stimulating plant growth (Glick 1998) (Fig. 8.2).

8.1.4 ACC Deaminase

One of the enzymes in bacteria is 1-aminocyclopropane-1-carboxylate (ACC) deaminase. This enzyme is responsible for the breakdown of ethylene precursors into ACC, ammonia, alpha-ketobutrate (Honma and Shimomura 1978). By decreasing the level of ACC in plants, ACC deaminases reduce the level of ethylene, which inhibits plant growth at high concentrations of this hormone (Glick et al. 2007). Due to the presence of tryptophan and other small molecules secreted by plants, bacteria in the soil cause the synthesis and secretion of Indole acetic acid, some of which is absorbed by plants, which can increase cell proliferation.

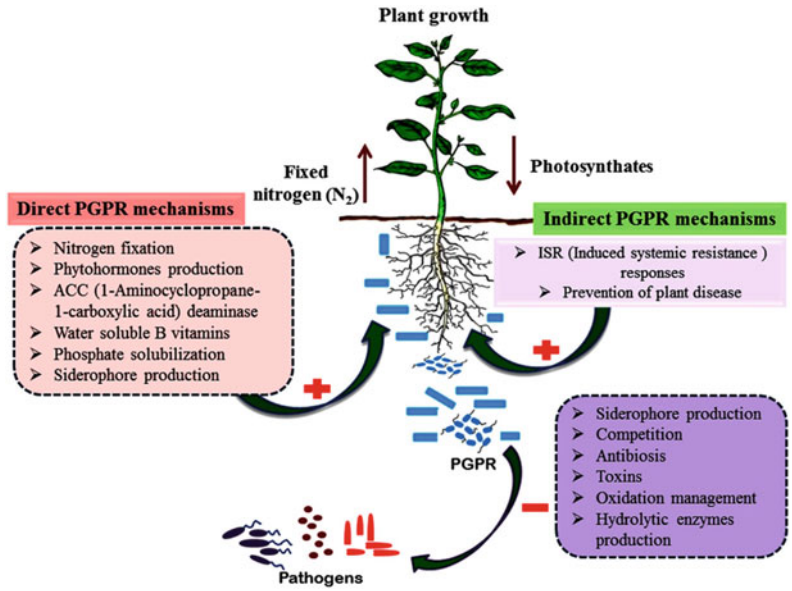

Fig. 8.2 Mechanism of plant growth promoting rhizobacteria (PGPR) (Nazir et al. 2018)

They also transcribe the enzyme ACC synthase, which is involved in the formation of ACC (a non-ribosomal amino acid) and thus reduces ethylene in plants (Penrose et al. 2001). As a result, plants that have growth-promoting bacteria containing ACC deaminase grow longer in their roots and have longer roots and branches under different ethylene-induced stresses to inhibit growth (Glick 2014).

One theory is that IAA-producing bacteria interact with plants by activating the production of IAA, which activates ACC synthase transcription, which ultimately results in the release of relatively high concentrations of ACC and subsequent ethylene inhibitory levels. As a result, these bacteria must inhibit plant growth, but since ethylene inhibits IAA signal transduction, transcription of ACC synthase is limited (Prayitno et al. 2006; Stearns et al. 2001; Glick 2014).

8.1.5 Enzymes and Biochemical Properties

The ACC deaminase was first purified from *Pseudomonas* sp. (Honma and Shimomura 1978). Most of the information on the biochemical properties of ACC deaminase has been provided by Honma et al. (Honma and Shimomura 1978; Honma 1993). The amino acids 1-alanine, dl-alanine, and dl-ralinecan limit the activity of the ACC deaminase. While α-aminoisobutyric amino acid can induce the activity of this enzyme. ACC deaminase is a member of a large group of enzymes that use Pyridoxal-5-phosphate (vitamin B6) as a major factor in enzymatic activity (Christen and Metzler 1985). *Rhizobium* strain which express ACC deaminase, is more efficient than strains which induces formation of nodules in plant roots (Ma et al. 2004).

8.1.6 Mycorrhiza Fungi

Mycorrhiza symbiosis is one of the most widely known symbiotic relationships between plants and microorganisms that exist in all ecosystems (Shiranirad 1998). Mycorrhiza fungi are essential elements in the stable soil systems, symbiosis with the roots of more than 97% of plants (Smith and Read 2008). These fungi date back more than 460 million years in the terrestrial ecosystem (Rillig 2004). The importance of Mycorrhiza

in agriculture is based on their special role as a link between soil and plant. Mycorrhizal fungi increase the absorption of water and nutrients by plants due to the effective increase in root uptake by hyphae. It also improves the absorption of nitrogen, potassium, magnesium, copper and zinc in poor soils.

Applying the mycorrhiza fungi increases plant dry matter due to higher absorption of water and nutrients. Eventually, mycorrhiza increases photosynthetic activity and stabilizes carbon dioxide and produces more leaf area index, which ultimately increases carbon dioxide stabilization and shoots biomass production (Smith and Read 2008). In this regard, it has been reported that inoculation with *Glomus intraradices* increased the dry matter of the shoots and roots of the Acacia plants (Duponnois et al. 2005).

Kariminejhad and Nadeian (2003) reported that mycorrhiza treatments increased clover root dry weight due to the spread of VAM mycelium in the soil and improving absorption of moisture and nutrients and therefore increase the synthesis of various assimilates such as glucosacral fructose and amino acids in the plant and root protection against environmental stress (Schellenbaum et al. 1998).

Mycorrhiza fungi have a wide network of hyphae that increase the level and speed of root absorption, which is effective in absorbing water and nutrients, especially sedentary elements of phosphorus, zinc, and copper and improves plant growth conditions (Marschner and Dell 1994). The symbiosis of fennel root with several species of mycorrhiza fungi significantly improved the amount and quality of essential oil and anethole (Kapoor et al. 2004). Among different types of biofertilizers, mycorrhiza fungi have a very positive effect on the qualitative and quantitative characteristics of their coexisting plants (Harrier and Watson 2004). These fungi increase the photosynthesis rate due to the increase in leaf area and stabilization of carbon dioxide per unit weight of leaves. Mycorrhizae also increase the uptake of manganese, calcium, iron, potassium and nitrogen (Jeffries 2001).

8.1.7 Positive Effects of Bio-Fertilizers on Saffron

Saffron (*Crocus sativus* L.) is one of the most valuable crops, and its dried stigma is used as food additives for flavoring and coloring and as a drug in medicine (Vahedi et al. 2014, 2018; Taheri-Dehkordi et al. 2020, 2021; Moradi et al. 2021, 2022). With an area under cultivation of about 92,822 ha, an annual production of 351.7 tons and an average yield of 3.79 kg/ha, Iran is the largest producer of saffron in the world (Vahedi et al. 2018; Taheri-Dehkordi et al. 2020). The difference in saffron yield per hectares in Iran compare to other major producing countries is due to inadequate feeding methods and also differences in physical and chemical properties of soils in the cultivated areas (Koocheki et al. 2011).

Saffron plants are influenced by climatic factors, weeds, pests and diseases, irrigation, storage and planting date, texture and soil structure during growth period (Aghhavani Shajari et al. 2015; Koocheki et al. 2015). Proper agricultural managements such as the use of fertilizers play an important role towards better quantity and quality of saffron (Rezvani Moghaddam et al. 2013). The use of bio-fertilizers as a suitable nutritional factor plays critical role in improving the vegetative properties of saffron, increasing the fresh weight and dry weight of coriander, and increasing the amount of roots (Nehvi et al. 2010). These changes are affected by the activity of microorganisms (Behdani 2005). In fact, since saffron is mainly grown in regions with arid and semi-arid climates, the soil of these areas has little organic matter (Shiranirad 1998). Therefore, in order to improve saffron production, organic and biological fertilizers that contain one or more types of beneficial microorganisms need to improve the soil organic matter. These microorganisms increase the growth of plants by increasing access

to phosphorus, nitrogen and other nutrients, increasing water uptake and production of plant hormones, creating resistance to pathogens and environmental stresses (Sabzevari et al. 2010).

Significant changes in saffron flower yield depend on soil-related variables such as organic matter, mineral nitrogen, available phosphorus and exchangeable potassium (Temperini et al. 2009). The type and source of nutrients could also affect the flowers' yield (Behdani 2005). Studies have also shown that bio-fertilizers increased the fresh weight of flowers and arbuscular fungi provided 25% of its nitrogen requirement. Due to the importance of saffron and its widespread use in various industries, organic production of saffron is in high demand and it is one of the major goals in saffron sustainable agriculture (Jami et al. 2020).

One of the major concerns to obtain high yield and high quality saffron is nutrition requirements, which could be achieved through a proper soil management using bio-fertilizers. Growth-promoting bacteria can improve plant growth by providing plant nutrients, secreting plant growth hormones and organic acids, and increasing plant growth, biological activity, changes in secondary metabolites, and environmental health. To investigate the effects of bio-fertilizers including bacteria on saffron, different concentrations of *B. subtilis* (10^2, 10^5, 10^6, 10^8 cfu/ml) were co-cultured with two ecotypes of saffron, Natanz and Zanjan and dried flower yield, dried stigma yield, flower length, stigma length, Nitrogen, Phosphorous and potassium, and secondary metabolites were analyzed. Results showed significant positive effects of bacteria on saffron yield and quality (Salehi et al., unpublished). The highest stigma fresh and dry weight (41.44 mg and 7.13 mg, respectively) and the highest amount of nitrogen, phosphorus and potassium obtained while plants were treated with 10^8 cfu/ml *B. subtilis* (Salehi et al. 2022). Also, the length of the flowers and stigmas were influenced by bacteria. Gutierrez-Manero et al. (2001) found that gibberellins are involved in cell longitudinal growth as well as auxin and cytokine in cell division. Therefore, it seems that the increase in the length of flowers and stigmas might be affected by these hormones. In other words, the effect of bacteria on growth can be generalized to the production of auxin and gibberellin (Figs. 8.3 and 8.4).

Crocin, picrocrocin and safranal were also significantly affected by bacteria. The taste and

Fig. 8.3 The effect of different concentrations of *Bacillus subtilis* on saffron flower length (left to right: 0, 10^2, 10^5, 10^6, 10^8 cfu/ml) (Salehi et al., unpublished)

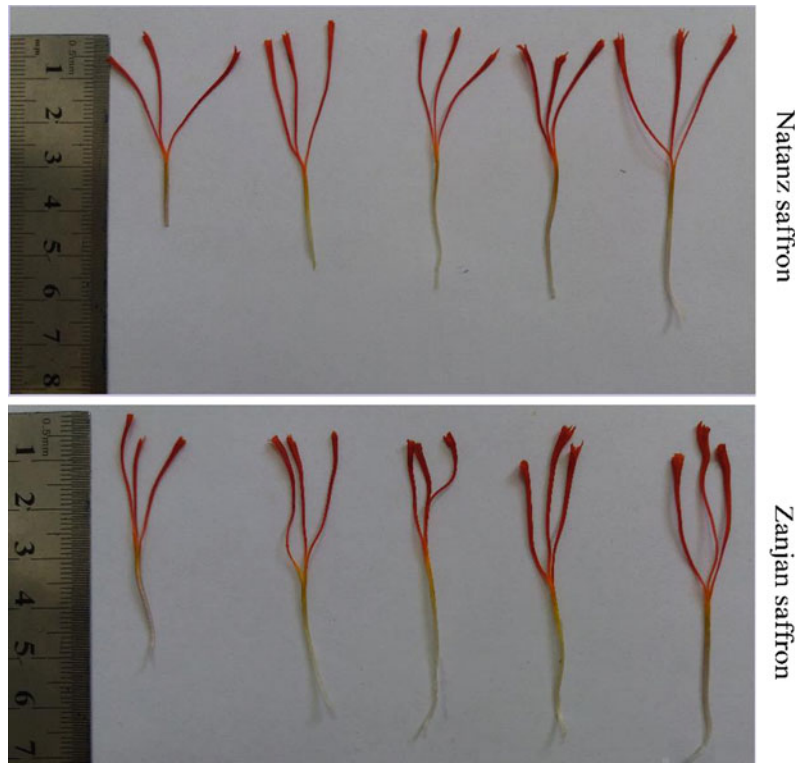

Fig. 8.4 The effect of different concentrations of *Bacillus subtilis* on length of saffron stigmas (left to right: 0, 10^2, 10^5, 10^6, 10^8 cfu/ml) (Salehi et al., unpublished)

smell of saffron were increased by using growth-promoting bacteria. Therefore, treatments with *B. subtilis* as a bio-fertilizer seem to be beneficial in saffron fields (Salehi et al. 2022).

8.2 Conclusion

One of the most important concerns in modern agriculture to achieve higher yield and quality is the performance of nutrition systems, and bio-fertilizers play crucial roles in this regard. These fertilizers have a more positive effect on the biological performance of plants than chemical fertilizers. The use of biological resources in agriculture has a long history. Mycorrhiza fungi and coexisting bacteria are among the most beneficial biological components of fertile soils. Growth-promoting bacteria are able to improve plant growth by providing plant nutrients, secreting plant growth hormones and organic acids, and increasing plant growth, biological activity, and changes in secondary metabolites. Mycorrhiza fungi also increase the efficiency of absorption of macro and micro elements through their symbiotic relationship with plant roots. In parallel with their role in nutrient uptake, mycorrhiza filaments allow the saffron plants to have better access to water through reactions such as regulating stomata movement, increasing water hydraulic conductivity, osmotic regulation, and stabilizing cell water potential.

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9 Diversity and Bioprospection of the Fungal Endophytic Microbiome of *Crocus sativus* L. (Saffron)

Zahoor Ahmed Wani

Abstract

Crocus sativus L. commonly known as saffron is a small geophyte comprising of a subterranean corm, leafy vegetative shoot and purple-colored flowers. The early evidence of cultivation and utilization of saffron dates back to 2500–1500 BC in Mediterranean regions. *C. sativus* has a triploid genotype which results in abnormal gamete formation and hence it is propagated vegetatively. The medicinal and aromatic property of saffron is due to the apocarotenoids: Crocin and safranal, present in the stigma of the flower. The cultivation and production of *C. sativus* is constantly declining worldwide for the last few decades due to various biotic and abiotic factors. One of the most important factors that influences plant health is the endophytic community harbored by the host plant. *C. sativus* harbors a huge diversity of fungal endophytes with significant bioactive potential. The application of microbes of endophytic origin for sustainable cultivation and crop management of saffron are also reported. The endophytic microbes of saffron also yield bioactive natural products for pharmacological and industrial applications.

Z. A. Wani (✉)
Department of Botany, Government Degree College Anantnag, Anantnag, Jammu & Kashmir 192101, India
e-mail: zawani1986@gmail.com

9.1 Introduction

Crocus sativus L. belongs to the family Iridaceae of magnoliophyta class of monocots. *C. sativus* has a triploid genotype ($2n = 3x = 24$); there is no proper segregation of chromosomes in meiosis resulting in abnormal gamete formation. Therefore, the propagation of *C. sativus* is by vegetative multiplication via manual "divide-and-set" of the underground corms/bulbs or by interspecific hybridization (Negbi et al. 1989). *C. sativus* is a small geophyte, comprising a subterranean part known as corm/bulb, leafy vegetative shoot and purple-colored flowers (Fig. 9.1). *C. sativus* is a perennial plant with its life cycle divided into three distinct phases. The life cycle of saffron is adapted to the climate of the Mediterranean region and is quite similar in all countries, but they differ in only the timing of events. The life cycle of saffron cultivated in J&K has the dormant phase extending from March to October, followed by the generative phase extending for less than a month, which is followed by the vegetative phase extending from November to February (Fig. 9.2). Corm development and bud sprouting occur during the dormant phase, flowering takes place during the generative phase and leaf development is the main activity during the vegetative phase (Wani et al. 2016). Saffron shows summer dormancy, which is related to superior survival and high persistence under severe drought conditions. This

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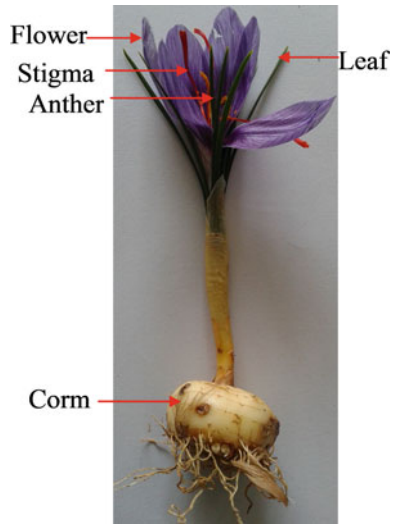

Fig. 9.1 Morphology of *C. sativus* L.

strategy of saffron is of great ecological significance, particularly in view of the climate change resulting in increased temperature and drought conditions.

The most important part of this plant is the dried red stigma known as Saffron,[1] which has been used as a medicinal herb and spice since time immemorial (Javadi et al. 2013; Baba et al. 2015a; Ghaffari and Roshanravan 2019). *Crocus* synthesizes a unique set of compounds known as apocarotenoids,[2] which are synthesized in the stigma part of the plant (Ashraf et al. 2015). The apocarotenoids of saffron are crocin, picrocrocin and safranal which are responsible for the color, flavor and aroma of saffron, respectively (Kumar et al. 2009). Owing to its high demand in dye, perfumery and flavoring industries, it is one of the most expensive spices in the world and is recognized as *Red gold*. The diverse compositions of *C. sativus* metabolites contribute an important role in plant development and adaptation to various stress conditions. Apart from this, saffron metabolites are reported to have tremendous therapeutic properties and its pharmacological importance has been appreciated both by the traditional, Avicenna's Canon of Medicine (al-Qanun fi al-tib) as well as modern scientific reports (Bhargava 2011; Hosseinzadeh and Nassiri-Asl 2013; Baba et al. 2015a; Ghaffari and Roshanravan 2019).

Saffron is primarily cultivated in Iran, Spain, India, Greece, Morocco, Italy, Turkey and France (Fernández 2004). In India, commercial cultivation of saffron occurs mainly in Kashmir.[3] Saffron has been cultivated in Kashmir since 750 AD. However, the cultivation of *Crocus* is observing a constant decline worldwide including J&K state due to various factors (Gresta et al. 2008). It is reported that there was a decrease of 83% in the area and 72% in the productivity of saffron in a single decade in Kashmir (Wani et al. 2016). This decline in the cultivation of saffron worldwide, due to poor agronomic practices and disease management together with a lack of breeding approaches, is a matter of concern to the saffron growers as well as agricultural scientists. Efforts are being made to understand the biology of the crop and replace the traditional practices of saffron cultivation with modern technology-driven cultivation approaches. The sterile nature of *Crocus* is hampering the conventional breeding approach, the modern biotechnological approach has also failed to deliver due to a lack of established transformation protocols in *Crocus* and now plant–microbe relationships are being explored as an alternative for sustainable cultivation of *Crocus* (Wani et al. 2016, 2017). The cultivation of *Crocus* is restricted to specific agro-climatic regions with a temperate climate, and also the propagation of *Crocus* occurs by means of underground corms. It is expected that the microbiome associated with *Crocus* might have a significant influence on the adaptation and functioning of the plant.

[1] In Kashmir, Himalayas saffron is locally known as "Kong", and "Zafran" in Urdu.

[2] Apocarotenoids are the degradation products of the carotenoids.

[3] Kashmir is a Himalayan valley in the state of Jammu and Kashmir (J&K) in India.

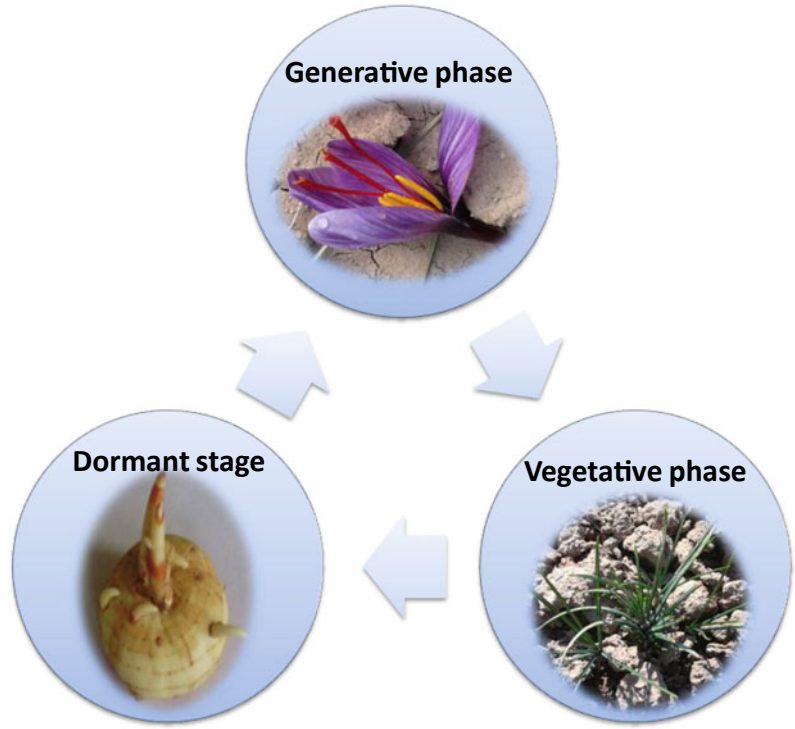

Fig. 9.2 Phenology of *C. sativus* L.

9.2 Decline in Saffron Productivity: Causes and Concerns

C. sativus is an important high-value crop plant. The cultivation and production of *Crocus* is constantly declining worldwide since the last few decades. The cultivation of saffron is restricted to specific agro-climatic regions with a temperate climate. The replacement of corms, breaking dormancy, transition from vegetative to reproductive stage, development of floral bud, floral emergence, etc. are all tightly regulated by various environmental factors like temperature, irrigation, sunlight, etc. It is reported that temperature regulates the growth and flowering of *Crocus* by affecting the enzyme activity in plant metabolism. Saffron production does not require much water, but it is reported that first irrigation is very important for flower emergence and the length of the flowering period of saffron. However, the declining trend in saffron production and quality is mainly attributed to poor agronomic practices and disease management together with a lack of breeding approaches. *C. sativus* has remained outside the realm of genetic improvement because of its sterile nature. Moreover, biotechnological approaches have failed to deliver, because transformation protocol has not been established so far. There are a few reports where genes involved in the flowering and apocarotenoid biosynthetic pathway have been cloned and characterized (Rubio-Moraga et al. 2004; Frusciante et al. 2014; Baba et al. 2015b). Also, a few transcription factors regulating the biosynthesis of these compounds have been identified and cloned (Ashraf et al. 2015). However, none of these genes have been taken forward for transforming *Crocus* for enhanced production of apocarotenoids.

An important aspect of the sustainable cultivation of saffron is the adequate production of healthy corms which is extremely important to guarantee flower production. However, the corms in their natural environment are constantly under siege from a multitude of disease-causing organisms including viruses, bacteria, nematodes and especially fungi. Several fungal species

belonging to genera *Fusarium*, *Rhizoctonia*, *Penicillium*, *Macrophomina*, *Aspergillus*, *Sclerotium*, *Phoma*, *Stromatinia*, *Cochliobolus*, *Sclerotium*, *Rhizopus*, *Porostereum*, *Talaromyces*, *Epicocum*, etc. are reported to be associated with saffron diseases (Ahrazem et al. 2010; Wani et al. 2016, 2018). Considerable work has been done on pathogens causing diseases in *C. sativus* worldwide (Cappelli and Di Minco 1999; Palmero et al. 2014; Gupta and Vakhlu 2015; Wani et al. 2016, 2017). Corm rot caused by *Fusarium oxysporum* is the most destructive disease in saffron, causing severe performance losses in most saffron fields (Cappelli 1994). The symptoms of corm rot include pigmentation, and in later stages of the disease tissue desiccation takes place. Infected plants die off early which results in the reduction of corm yield, flowering and stigma production. The corm rot disease was first detected in Japan (Yamamoto et al. 1954). Corm rot disease is currently widespread throughout the saffron-producing countries, causing substantial yield losses. With a disease incidence of 100% and severity ranging from 6 to 46%, corm rot disease results in reduced plant growth and yield of saffron in Kashmir as well (Husaini et al. 2010).

To avoid pathogen attacks, *Crocus* corm has developed several physical and chemical barriers, as well as a system of active defense reactions. Recently a new chitinase, SafchiA, isolated from corms of *C. sativus* has been reported to play an important role in saffron defense response induced by fungal (*Fusarium oxysporum* f. sp. *tuberose*) infection, and mediates inhibition of fungal growth under in vitro condition (López and Gómez-Gómez 2009). *C. sativus* is characterized by the presence of saponins in stigma and corm tissues, where they seem to play an antifungal role. The ability of a plant to resist diseases is also dependent on soil conditions such as structure, compaction, drainage, temperature and level of biological activity, along with cultural practices such as planting date and application of fertilizers or herbicides (Ahrazem et al. 2010). The corm rot caused by *Fusarium* and other fungi in saffron grown in Kashmir is being managed by using chemical fungicides such as Carbendazim (broad-spectrum benzimidazole fungicide), Myclobutanil (triazole chemical), Mancozeb (subclass of carbamate), Bavistin (50% WP Carbendazim) and Tecto (benzimidazole fungicide). However, the deleterious impact of these chemicals on the environment as well as human beings is well established. These chemicals also affect the beneficial microflora associated with the plant and put selection pressure on the evolution of resistant pathotypes. Therefore, biological control is gaining importance for integrated pest/disease management. There is a diverse community of microorganisms (endophytes) which interact positively with plants in agricultural systems in relation to their nutrition and ability to resist biotic and abiotic stress. The endophytes have the potential to be manipulated such that the benefits of their positive effects are harnessed.

9.3 Plant Endophyte Interactions: A Way Forward for Sustainable Agriculture

The association of plants with microbes dates back to more than 400 million years ago. This prolonged and close association of two or more different organisms is a major driving force for the expansion of biological diversity from genes to ecosystems (Kuo 2015). The complex interplay of the diverse array of microbial communities with the host plant affects its ecophysiology such as plant nutrition, growth rate, resistance to biotic and abiotic stress conditions, as well as plant survival and distribution. The advancement of endophytism as a discipline of science began in 1886 when De Barry put forth the concept of "endophyte". The term endophyte (Gr. *endon*, within; *phyton*, plant) was first coined by De Bary and was applied to "any organism occurring within plant tissues". However, this discipline of science did not receive much attention until the recent recognition of its pharmaceutical and ecological significance (Gunatilaka 2006). Since then, endophytes have created immense scientific curiosity pertaining to their biology, evolution, ecology and applications.

Endophytic biology is pursued in research with a multitude of objectives that can be broadly classified into two categories—bioprospecting and plant–microbe symbiosis (Wani et al. 2015). Under bioprospecting, the endophytes are studied for genuine microbial metabolites, potential host metabolites and VOCs for agriculture, food and aroma industry and alternate fuels. While in plant–microbe interactions, we study the effect of symbiosis on plant adaptation as well as plant metabolism and microbial symbiont metabolism, the complexity of the association of endophytes with their host plant is of great ecological significance owing to their compatibility, ease of re-infection and pattern of colonization (Sikora et al. 2010; Wani et al. 2017). Many of the fungal endophytes have been found to produce antimycotic volatile organic compounds (VOCs). VOCs produced by microorganisms are regarded as important info-chemicals in the biosphere which influence the dynamics of the ecosystem and vice versa (Wheatley 2002). It seems reasonable that the VOC-producing microorganisms may be preferentially establishing symbiotic associations with higher plants as they contribute to the host defense mechanism by inhibiting the plant pathogens. Production of VOCs may also help them to compete with other microbes for space, nutrients and making associations with plants. Greater utilization of microorganisms of endophytic origin in agricultural systems could possibly allow reductions in the use of inorganic fertilizers, herbicides and pesticides with no impact on crop vigor and yield. Thus, in future, endophyte technology holds the key to a potential gateway to sustainable agriculture development.

9.4 Plant–Microbe Association in *C. sativus*

A lot of work is being done on *C. sativus* to understand the biology of the plant. However, work on plant–microbe interaction in *Crocus* is gaining momentum over the last few years. There are various reports of the application of microbes with established plant growth-promoting properties on the production of saffron. The antagonistic potential of *Trichoderma viride* isolates collected from soil was investigated against *Crocus* corm rot pathogen, *F. oxysporum* (Mir et al. 2011). In Spain, the application of *Bacillus subtilis* FZB24 spore solution to saffron corms significantly increased leaf length, flower per corm and total stigma biomass and decreased the time required for corms to sprout. Moreover, a significant increase in the quantity of picrocrocin, crocetin and safranal compounds is reported, when the plants are soil drenched with *B. subtilis* FZB24 spore solution 14 weeks after the sowing (Sharaf-Eldin et al. 2008). Aytekin and Acikgoz (2008) reported that the production of saffron can be increased by treatment of corms with a synthetic hormone (polystimulin A6 and K) and microorganism-based material like bio-humus.

Recently, some studies on bacterial endophytes and rhizospheric bacterial associates of *C. sativus* are reported culture-dependent and culture-independent approaches (Ambardar and Vakhlu 2013; Ambardar et al. 2014; Sharma et al. 2015). A *Bacillus amyloliquefaciens* strain W2 collected from rhizospheric soil was found effective against corm rot caused by *F. oxysporum* (Gupta and Vakhlu 2015). Sharma and colleagues isolated cultivable bacterial endophytes from saffron plants and assessed them for plant growth-promoting activities. Molecular and phylogenetic analysis grouped the fifty-four bacterial isolates into eleven different taxa, viz. *Bacillus licheniformis*, *B. subtilis*, *Bacillus cereus*, *Bacillus humi*, *Bacillus pumilus*, *Paenibacillus elgii*, *Bacillus safensis*, *Brevibacillus* sp., *Pseudomonas putida*, *Staphylococcus hominis* and *Enterobacter cloacae*. *B. licheniformis* was the dominant endophyte in both leaves and corms of saffron. Ambardar and colleagues reported the bacteria associated with rhizosphere, cormosphere and bulk soil of Saffron, using the cultivation-independent 16S rRNA gene-targeted metagenomic approach. Saffron during the flowering stage revealed the presence of 22 genera, but none of the genera was common in all three samples. The bulk soil bacterial community was represented by 13 genera with *Acidobacteria* being the dominant genus, while as a

rhizospheric bacterial community was represented by eight different genera with *Pseudomonas* being the dominant genus, and Cormospheric bacterial community comprised of six different genera, dominated by the genus *Pantoea* (Ambardar et al. 2014). Recently, *Streptomyces achromogenes*, an endophyte of *C. sativus*, was reported to produce the antibacterial compound Juglomycin A (Ahmad et al. 2020).

Although the accessions of *C. sativus* cultivated in different regions show little genetic variability, the yield and productivity of saffron vary considerably. This could be attributed to variations in agricultural practices, and various biotic and abiotic factors. One of the most important factors that influences plant health is the endophytic community harbored by the host plant. The cultivation of *Crocus* is restricted to specific agro-climatic regions with a temperate climate, and also the propagation of *Crocus* is by means of underground corms. Therefore, the microbiome associated with *Crocus* might have a significant influence on the adaptation and functioning of the plant. Thus, it is imperative to understand the patterns of distribution and community structure of endophytes of *C. sativus*, as well as their interactions with the host plant, for sustainable agriculture and crop management of this high-value medicinal and aromatic plant.

9.5 Fungal Endophytic Microbiome of *C. sativus*

C. sativus harbors a huge diversity of fungal endophytes. Wani et al. reported the fungal endophytic community of *C. sativus* cultivated in J&K, India. A total of 294 fungal endophytes were isolated from *Crocus* corms which were grouped into 100 morphotypes based on phenotypic characters like growth pattern, colony texture and colony color, as well as the morphology of conidia and conidiophores. Molecular phylogenetic studies based on ITS1-5.8S-ITS2 ribosomal gene sequence analyses assigned these endophytes into 36 distinct species, spreading over 19 genera (Table 9.1). The diversity and composition of the endophytic community were almost similar across different sites in J&K state. However, the diversity and composition of the endophytic community varied temporally at the two different phonological stages of the *Crocus* lifecycle. It was higher at the dormant than at the vegetative stage, indicating an influence of host/corm health status on the endophytic diversity (Fig. 9.3). This may be explained by the fact that during the vegetative stage, the corms get flaccid, nutrient deficient and relatively inactive, thus supporting the growth of fewer endophytes inside the corm tissues. In addition, the corms remain in the vegetative stage during the winter season which is marked by snowfall and low temperatures, thus creating conditions that are less favorable for the growth of the endophytes (Wani et al. 2016).

The Saffron microbiome was dominated by dark septate endophytes (DSEs) with an isolation frequency of more than 30%, particularly *Phialophora mustea* and *Cadophora malorum* being the most dominant endophytes (Table 9.1). Interestingly, molecular phylogeny assigned these DSEs to a single clad, indicating a strong effect of the host genotype on the selective recruitment of endophytes (Fig. 9.4). This indicates host–endophyte specificity in the *Crocus* plant vis-à-vis *P. mustea* and *C. malorum*, and these species being the most preferred endophytes of the host. It is suggested that these associations might have developed over centuries of cultivation of saffron and transmitted vertically as the host is propagated only by vegetative means using corms (Wani et al. 2016). The higher colonization of DSEs in the corms of *Crocus* indicates an ecological significance, as it is reported that the melanized hyphae[4] are considered to be of importance for the host to survive stress conditions. The cell wall melanin can trap and eliminate oxygen radicals generated during abiotic stress, particularly drought stress. Also, the DSEs associated with *Crocus* produce a significant amount of Indole Acetic Acid (IAA), and it is reported that IAA increases colonization

[4] Melanized hyphae are a characteristic feature of dark septate endophytes, as they have melanin pigment present in their hyphae.

Table 9.1 The table presents the thirty six different fungal endophytes (their GenBank accession numbers and isolation frequency) isolated from *C. sativus*

ITS genotype	Molecular identification (GenBank accession no.)	Isolation frequency (%)
ITS1	*Aspergillus flavipes* (KR135119)	3.7
ITS2	*Trichoderma harzianum* (KR135120)	3.4
ITS3	*Cadophora malorum* (KR135121)	12.9
ITS4	*Fusarium oxysporum* (KR135122)	4.1
ITS5	*Alternaria alternata* (KR135123)	4.4
ITS6	*Penicillium pinophilum* (KR135124)	3.7
ITS7	*Paecilomyces tenuis* (KR135125)	1.7
ITS8	*Porostereum* sp. (KR135126)	1
ITS9	*Talaromyces pinophilus* (KR135127)	0.3
ITS10	*Aspergillus dimorphicus* (KR135128)	1
ITS11	*Aspergillus terreus* (KR135129)	1.4
ITS12	*Aspergillus iizukae* (KR135130)	1.4
ITS13	*Aspergillus pseudodeflectus* (KR135131)	3.7
ITS14	*Fusarium incarnatum* (KR135132)	0.3
ITS15	*Alternaria* sp. (KR135133)	0.3
ITS16	*Fusarium solani* (KR135134)	1.4
ITS17	*Talaromyces verruculosus* (KR135135)	2.4
ITS18	*Eucasphaeria* sp. (KR135136)	1.7
ITS19	*Penicillium canescens* (KR135137)	2
ITS20	*Talaromyces cellulolyticus* (KR135138)	9.5
ITS21	*Penicillium* sp. (KR135139)	0.7
ITS22	*Penicillium chrysogenum* (KR135140)	0.7
ITS23	*Epicoccum nigrum* (KR135141)	1
ITS24	*Phialophora mustea* (KR135142)	15
ITS25	*Penicillium griseofulvum* (KR135143)	9.2
ITS26	*Ilyonectria robusta* (KR135144)	0.3
ITS27	*Alternaria brassicae* (KR135145)	0.3
ITS28	*Mortierella alpina* (KR135146)	2
ITS29	*Penicillium* sp. (KR135147)	1
ITS30	*Acremonium* sp. (KR135148)	2.4
ITS31	*Cladosporium silenes* (KR135149)	1
ITS32	*Fusarium tricinctum* (KR135150)	1.7
ITS33	*Leptodontidium orchidicola* (KR135151)	2.4
ITS34	*Botrytis fabiopsis* (KR135152)	0.3
ITS35	*Paecilomyces marquandii* (KR135153)	0.3
ITS36	*Gloeosporium* sp. (KR135154)	1

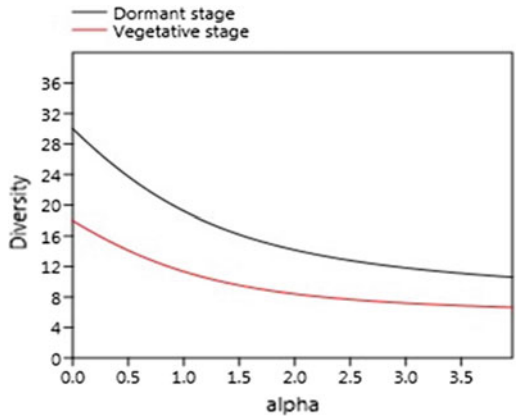

Fig. 9.3 Diversity profile graph of fungal endophytes at two stages of *Crocus* life cycle. Black line indicates diversity profile at dormant stage, while red line indicates the diversity profile at vegetative stage. The plot indicates clearly that the diversity of endophytes associated with *C. sativus* is higher during the dormant stage of its life cycle

efficiency of the endophytes, possibly via interference with the host defense system (Navarro et al. 2006). The production of IAA or related compounds may be an important property for plant colonization by endophytes. Therefore, the endophytes, *P. mustea* and *C. malorum* are efficient colonizers in *C. sativus* and may confer tolerance to the host against a variety of environmental stress factors. Also, *P. mustea* and *C. malorum* isolates showed intra-specific strain variations, indicating that these symbiotic associations are species-specific rather than strain-specific (Wani et al. 2016).

Some endophytic strains recovered from the *Crocus* corm were identified as being members of commonly observed genera of soil fungi, e.g., *Fusarium*, *Penicillium*, *Talaromyces*, *Trichoderma* and *Paecilomyces*. These fungi are characteristically free-living saprophytes that can also be opportunistic root endophytes or latent pathogens.[5] Pathogenicity assay indicated some of the endophytes of *Crocus* as latent pathogens, as they displayed virulence with varying levels of severity under both in vitro and in vivo conditions (Fig. 9.5). For instance, endophytes like *Alternaria alternata*, *Epicoccum nigrum*, *F. oxysporum*, *Acremonium* sp. *Penicillium pinophilum* and *Talaromyces cellulolyticus* displayed moderate to high virulence under both in vitro and in vivo conditions. However, *Aspergillus pseudodeflectus*, *Botrytis fabiopsis*, *Penicillium canescens*, *Porostereum* sp., *Paecilomyces marquandii*, *Talaromyces pinophilus* and *Talaromyces verruculosus* displayed low virulence under in vivo condition and therefore considered as low-risk pathogens (Wani et al. 2016).

Another important study on the fugal endophytes of saffron cultivated in Taliouine (Morocco) was conducted by Chamkhi et al. (2018). A total of 60 fungal isolates were recovered from segments of *C. sativus* corms, and it was observed that *Rhizopus oryzae* was the most dominant fungal endophyte with an isolation frequency of 93.4%, followed by *Aspergillus fumigatiaffinis* and *Aspergillus niger*, with isolation frequencies of 4.83% and 1.61%, respectively (Chamkhi et al. 2018). Interestingly, the dominant fungal endophytes associated with saffron in both the studies were not similar, and it is suggested that differences in the environment of the studied areas might have influenced the endophytic colonization (Chamkhi et al. 2018). Plants growing in different geographical regions are confronted with different environmental challenges. These environmental cues in combinatorial effect with host genotype shape the endophytic diversity harbored by the host plants (Arnold 2007; Wani et al. 2015).

9.6 Bioactive Potential of Fungal Endophytes of *C. sativus*

Endophytes are proficient producers of bioactive metabolites and drug-like molecules. Thus, they represent a huge bio-resource for the isolation of novel bioactive molecules for applications in medicine, agriculture and industry (Porras-Alfaro and Bayman 2011; Jalgaonwala et al. 2017). This is not surprising in the light of their evolution over millions of years in diverse ecological niches and natural habitats. Extracts from several

[5] They live as normal endophytes in the host plant, but can turn pathogenic under stress condition or produce disease symptom in the host plant upon re-infection.

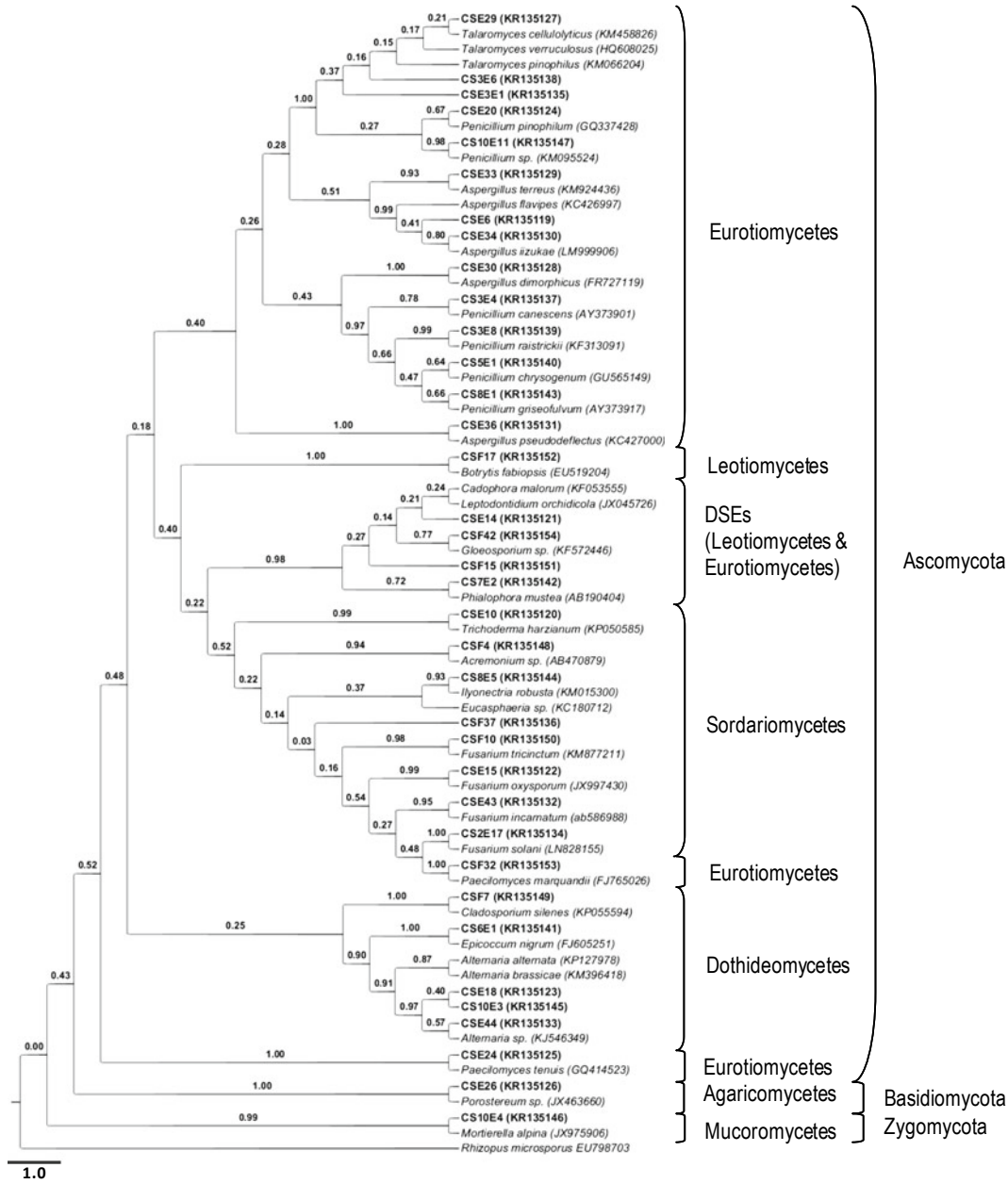

Fig. 9.4 Phylogeny of endophytes of *Crocus sativus* using maximum parsimony analysis based on ITS1-5.8S-ITS2 sequence. Only strain names with accession numbers are provided in the phylogenetic tree for the endophytes isolated in this study. The tree is rooted with *Rhizopus microsporus* (a zygomycete, EU798703)

endophytes of *Crocus* showed promising antimicrobial activities. Four new metabolites, Phialomustin A–D isolated and characterized from an endophyte (*P. mustea* CS7E2) of *C. sativus*, are reported to have potential antimicrobial and anticancer activities (Nalli et al. 2015). Further, a unique quinazoline alkaloid with cytotoxic and antifungal activities is isolated from *Penicillium vinaceum*, an endophyte of *C. sativus* (Zheng et al. 2012). The ethyl

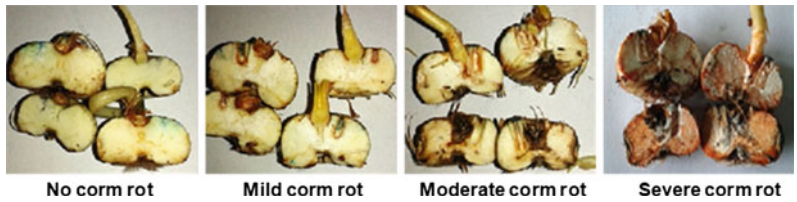

Fig. 9.5 Corms of *C. sativus* re-infected with endophytes produced rotting symptoms with different levels of severity

acetate extracts of *R. oryzae* and *A. fumigati-affinis* and *A. niger* showed significant antimicrobial and antioxidant properties (Chamkhi et al. 2018).

Several endophytes are reported to inhibit the growth of plant pathogenic fungi, thereby indicating a strong bio-control potential, which can be harnessed to control corm rot and other microbial diseases after carrying out further studies, particularly under field conditions. By virtue of the antimicrobial activities, the endophytes may be imparting resistance to the host plant against microbial diseases. Also, these properties may be helping them to dominate the microbial populations in the corresponding ecological niches leading to their efficient colonization in the plants. Another benefit, which these endophytes provide to the host plant, is the production of the plant growth hormones. Phytohormone production by endophytes is probably the best-studied mechanism of plant growth promotion, leading to morphological and architectural changes in plant hosts, thus contributing to the overall growth and development of the plant.

An oleaginous fungal endophyte, *Mortierella alpina* CS10E4 isolated from *C. sativus* produces polyunsaturated fatty acids (PUFAs) including arachidonic acid (AA). *M. alpina* CS10E4 shifts the metabolic flux of *Crocus* toward enhanced production of apocarotenoids by modulating the expression of key genes of the apocarotenoid pathway. Further, *M. alpina* CS10E4 enhanced tolerance to corm rot disease by releasing arachidonic acid, which acts as a conserved defense signal and induces jasmonic acid production in endophyte-treated *Crocus* corms (Wani et al. 2017). A basidiomycete, *Porostereum* sp. CSE26 produces chlorinated aromatic compounds (CAMs), i.e., 3-Chloro-4-methoxybenzaldehyde and 2, 3-Dichlorophenyl isothiocyanate, having phytotoxic activity against *Arabidopsis* plants. It is presumed that these compounds may be acting as pathogenic determinants of *Porostereum* sp. CSE26 (Wani et al. 2018).

The endophytic communities associated with *C. sativus* produce a diverse array of biomolecules like phytohormone, enzymes, anticancer, antimicrobial, antioxidant, phytotoxic compounds, etc. Therefore, the endophytes associated with *C. sativus* can be harnessed to develop agro-technologies for sustainable cultivation of Saffron and also yield bioactive natural products for pharmacological and industrial applications.

9.7 Conclusion

C. sativus is an important medicinal and aromatic plant. It is the only plant species which produces apocarotenoids like crocin, picrocrocin and safranal in significant amounts. These compounds impart organoleptic properties to saffron making it the world's costliest spice. This plant has remained outside the realm of genetic improvement because of its sterile nature. Poor agronomic practices and disease management together with a lack of breeding approaches have led to a declining trend in saffron production and quality. This advocates the need to explore other possibilities for enhancing the production of *Crocus* apocarotenoids. The plant–endophyte interface provides an important ecological marketplace for harnessing the potential of

endophytes to produce compounds of therapeutic potential or exert their positive influence on plants to enhance the production of specialized metabolites of plant origin. *C. sativus* harbors a great diversity of fungal and bacterial endophytes. These endophytes produce a diverse array of bioactive molecules which can be harnessed for pharmacological and industrial applications.

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Epidemiology and Management of Corm Rot of Saffron

10

Vishal Gupta, V. K. Razdan, and Satish Kumar Sharma

Abstract

Saffron, a low volume high value crop, is obtained from the dried stigmas of *Crocus sativus* flowers. The major constituents of saffron are phytochemicals like crocin, picrocrocin, safranal and crocetin, which attribute to its broad spectrum medicinal properties, because of which it has attained the status of being one of the costliest dietary spices. Saffron is mainly being cultivated in Iran, India, Afghanistan, Greece, Italy, Spain, etc. Though limited to only four districts of the Union Territory of Jammu and Kashmir, India has the second largest area under saffron cultivation, after Iran. However, in India saffron productivity is very low (2.0–2.5 kg/ha) as compared to other countries. In the recent past its production has declined in several countries, due to the vegetatively propagated and labour intensive nature of the crop, genetic erosion, lack of modern cultivation practices and mechanization, urbanization of traditional saffron cultivation land, and prevalence of corm rot of saffron. Saffron has antioxidant, radical scavenging and anti-inflammatory properties, and acts as anti-arthritic agents reducing cholesterol and triglycerides. It acts against stress and anxiety and also helps in decreasing depressive conditions. Saffron has immunological effects and is effective against neurological diseases including Alzheimer and Parkinson-like behaviour. It also shows anti-cancer properties, and its extract reduces dental caries. Saffron has also been used to dye textiles and in human and animal histological staining. It is as well used in various cuisines, confectionaries and non-alcoholic beverages. Corm rot, prevalent in almost all the saffron growing areas, has emerged as one of the major limitations for the successful cultivation of saffron, globally. The disease is primarily noticed during flowering (October–November) and grubbing stages (May–July) and is manifested as symptoms like drooping, damping-off, yellowing, wilting of shoots, basal stem rot and corm rot. In Kashmir, 46% corm rot incidence has been reported with none of the saffron fields being free from the disease. The disease is also widespread in Kishtwar (up to 100%), with maximum incidence and severity in Lower Poochal (59.33

V. Gupta (✉)
Biocontrol Laboratory, Advanced Centre for Horticultural Research, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Udheywalla, India
e-mail: vishal94gupta@rediffmail.com

V. K. Razdan
Division of Plant Pathology, Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Chatha, India

S. K. Sharma
Regional Horticulture Research Sub-Station, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Bhaderwah, India

J. Vakhlu et al. (eds.), *The Saffron Genome*, Compendium of Plant Genomes,
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and 35.00%, respectively). Cultivation of saffron, a perennial crop with planting cycle of 5–17 years, is carried out without proper phyto-sanitary inspection; hence the infected and/or infested corms act as an important source of primary inoculum of corm rot. Plant pathogens such as *Fusarium oxysporum* f. sp. *gladioli*, *Fusarium oxysporum*, *Rhizoctonia* sp., *Aspergillus* sp., *Penicillium* sp. and *Macrophomina* sp. have been found associated with corm rot complex disease of saffron. Most of them survive in infected corms and soil as dormant structures. The association of multiple soil-borne pathogens shows synergism in the incidence of corm rot. Human mediated cultural operations, field implements, bullock carts or tractors used for field preparation and use of infected corms help in the dispersal of pathogens. Even the air currents carrying dust particles along with plant debris containing the inoculum assist in the dispersal of plant pathogens. Heavy rainfall also helps in the short-distance dispersal of plant pathogen. The presence of primary inoculum in the field, infestation of soil by plant parasitic nematodes/rodents, faulty agronomic practices, erratic rainfall and poorly drained fields are the pre-disposing factors for corm rot. The disease drastically reduces the saffron yield, corm development and production of cormlets. Thereby, contributing towards direct economic losses and fluctuations in saffron prices. Although chemical fungicides are very effective, yet growing concern about their deleterious impacts on the environment and human beings, along with pesticide residues in the produce, has resulted in exploring alternative eco-friendly and sustainable methods, for the management of corm rot. Beneficial microbes have proved effective in managing the disease because they compete for energy, food and ecological niche or substrate with the pathogen, and produce inhibitory allelo-chemicals and induce of systemic resistance in the plant. *Bacillus* spp., due to the advantage of it producing heat and desiccation resistant spores, significantly reduces the incidence of corm rot. Though carbendazim causes maximum disease suppression, yet *Pseudomonas fluorescens*, *Trichoderma viride* and *Trichoderma harzianum* are greatly effective against corm rot. Soil solarization for 4 and 6 weeks significantly reduces the corm rot incidence. Corm dip in mancozeb + carbendazim results in 85.49% reduction in corm rot. Field sanitation and proper drainage are equally important for the management of corm rot. Application of plant growth promoting fungi (PGPF) and plant growth promoting bacteria (PGPB) are also widely advocated in different crops to amelioration biotic and abiotic stresses and promote plant growth.

Saffron is one of the costliest dietary spices in the world. It is obtained from the dried stigmas of purple flowers of *Crocus sativus* plant and has unique colour, taste and aroma. Saffron plant belongs to the family Iridaceae and has perennial herbaceous characteristics. Due to its unique medicinal properties against several human diseases considered as natural drug (Moraga et al. 2009). Due to this proven medicinal properties against human diseases saffron consumption and demand are increasing day by day (Abdullaev 2002). The main constituents of saffron are carotenoids, anthocyanins, glycosides, aldehydes, monoterpenes, flavonoids, riboflavin, thiamine, amino acids, proteins, starch, and gum along with many other chemical compounds (Abdullaev 1993; Liakopoulou-Kyriakides and Kyriakidis 2002; Winterhalter and Straubinger 2000; Fernandez 2004).

Saffron is presently being cultivated in Iran, Greece, Afghanistan, Morocco, India, Italy, Spain, Germany, Switzerland and Azerbaijan. However, Australia (Tasmania), Canada, Central Africa, China, Egypt, parts of England, France, Israel, Mexico, New Zealand, Sweden (Gotland), Turkey (Safranbolu), and the United States (California and Pennsylvania) have also explored the possibilities of saffron cultivation though, on a small scale. New areas are being explored by the public and private sectors, as well as the national and international development agencies, to enhance the saffron production. Due to the minimal requirements of irrigation, synthetic

fertilizers and pesticides, it is considered as low-input crop (Ghorbani and Koocheki 2017; Gresta et al. 2008).

Saffron is mainly cultivated in Khorasan Razavi (82,712 ha), South Khorasan (15,754 ha), North Khorasan province (5260 ha) and other provinces (1544 ha) of Iran which contributes about 76, 15, 7 and 2% in the production of saffron, respectively (Anonymous 2017). In Castile-La Mancha (Spain), predominate saffron areas are Albacete, Toledo, Cuenca, Ciudad Real and Guadalajara, which contribute 97% of saffron production in the country. India has the second largest area under saffron cultivation, which is mainly limited to the four districts of the Union Territory of Jammu and Kashmir, Pulwama (Khrew, Ladoo, Dussu, Lathipora, Sambora, Awantioora and Koil), Budgam (Nagam, Sarwin, Hapatnar, Gopalpora, Hyathpora, Chrawani and Chirar-i-sharief), Srinagar (Zeewan, Khunmoh, Balhama, Sampora and Yachnambal) and Kishtwar (Lower Poochal, Upper Poochal, Nagin, Hatta, Laynyal, Tund, Matta, Cherrhar, Bhera-bhata and Hullar), with 3200, 300, 165 and 120 ha, under its cultivation, respectively. Whereas, saffron production at a small scale has also been initiated in adjoining Himalayan regions of Punjab, Himachal Pradesh, Ladakh and Uttarakhand. However, in India the saffron productivity is very low (2.0–2.5 kg/ha) as compared to the other saffron producing countries (Gupta et al. 2020, 2021). In Afghanistan, saffron cultivation was initiated, in the areas of Ghor, Nangarhar, Noristan, Bamyan, Sar-e-Pul, Uruzgan, Farah, Badakhshan, Herat, Kabul and Kunduz. In Greece, with an area of 1800 ha, the major area under saffron cultivation is Kozani in Macedonia (Fernandez 2004). Saffron is a neglected crop in Italy and is mainly cultivated on L'Aquila (Piana di Navelli and Abruzzo), Sardinia (Province of Medio Campidano), Tuscany (San Gimignano, Florence Hills and Maremma) and Umbria (Cascia and CittàdellaPieve) having an area of 500 ha. Saffron cultivation is limited to an area of 200 ha in Taliouine and Taznakht regions, in Morocco (Figs. 10.1 and 10.2).

C. sativus grows in a specific environment and climatic condition. It flourishes well in Mediterranean agro-eco-condition, which is characterized by warm, dry summers, meagre autumn–winter–spring rainfall (400–500 mm per annum) and cool to cold winters (Deo 2003). It can withstand considerable frosts (−10 °C) and can tolerate snow in the winter. In recent years, saffron production has declined drastically in the various saffron producing countries, which is mainly due to the vegetatively propagated nature of the plant (Nematia et al. 2019), high labour demand (Agayev et al. 2009), genetic erosion (Fernandez 2007), lack of mechanization, urbanization of traditional saffron cultivation

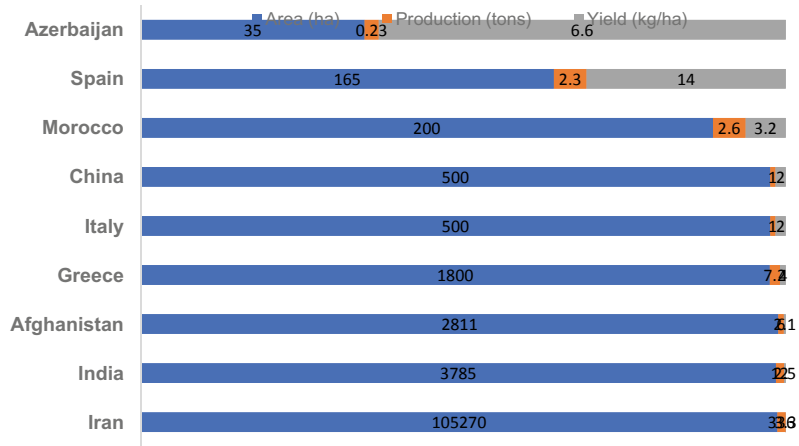

Fig. 10.1 Main saffron-producing countries of the world

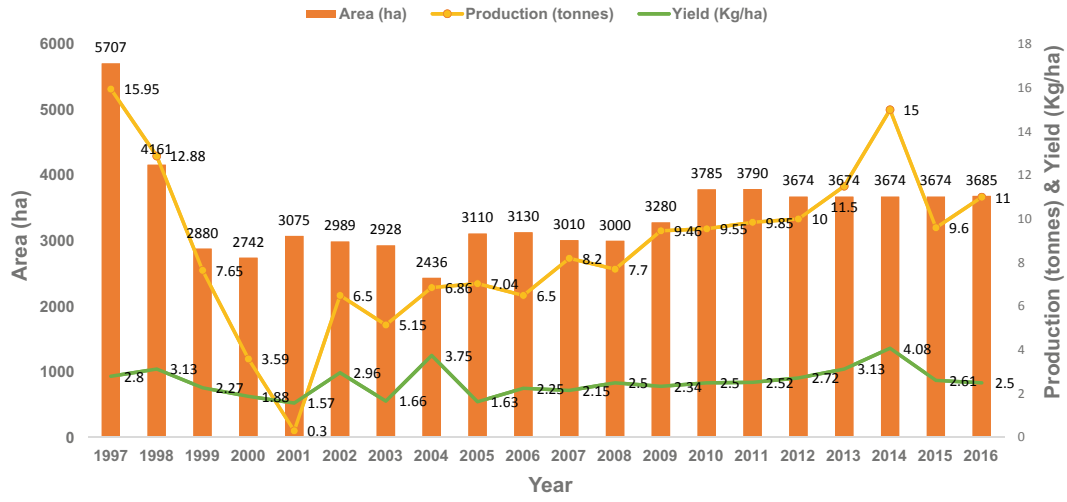

Fig. 10.2 Area, production and yield of saffron in India

land, continuation of traditional cultivation practices and prevalence of corm rot of saffron (Kalha et al. 2008).

Economic Importance

Saffron, one of the most expensive dietary spices, is obtained from the golden coloured dried stigmas of *C. sativus* flowers. Since ancient times, saffron has immense impact on several spheres of life. The major constituents of saffron stigma are crocin ($C_{44}H_{64}O_{24}$), which is responsible for its colour, picrocrocin ($C_{16}H_{26}O_7$) attributing its bitter taste, safranal ($C_{10}H_{14}O$) its aroma and crocetin ($C_{20}H_{24}O_4$), which gets converted into crocin upon glycosylated. Crocin and crocetin (carotenoids) which are formed after the oxidative degradation of zeaxanthine, possess the antioxidant properties (Kosar and Baser 2020). The broad spectrum of medicinal properties of saffron is due to the presence of these phytochemicals.

Under the clinical trials it has been observed that saffron extract reduces plasma cholesterol and triglyceride (Lahmass et al. 2017). Crocetin and crocins both have strong antioxidant and radical scavenging properties, are responsible for the anti-inflammatory activity, and act as anti-arthritic agents (Poma et al. 2012). Safranal is known to decrease the duration of seizure, delay the onset of convulsions and reduce the mortality rate due to convulsion (Hosseinzadeh and Talebzadeh 2005). Saffron also helps in decreasing depressive conditions (Asrari et al. 2019). Saffron extracts have inhibitory effects on the *Streptococcus mutans*, *Lactobacillus* and *Candida albicans*, responsible for dental caries (Karbasaki et al. 2016).

Saffron has shown antiproliferative effect of malignant carcinomic human alveolar basal epithelial cells (Al-Snafi 2016). It also delimits the cell proliferation and reduces the number and incidence of hepatic dyschromatic nodules (Amin et al. 2011). Reduction in telomerase activity, cancer cells apoptosis, inhibition of cell proliferation, enhancement of cell differentiation, modulation of cell cycle progression and cell growth, modulation of tumour metabolism, stimulation of cell-to-cell communication and immune modulation are the mechanisms exhibited in the chemo-preventive activity by saffron (Colapietro et al. 2019).

Saffron acts against stress and anxiety (Boskabady and Farkhondeh 2016), and regular consumption of saffron ameliorates anxiety and depression (Ghajar et al. 2017). Saffron consumption also generate preterm delivery due to increased uterine contractions in female mice

(Zeinali et al. 2009; Revuelta et al. 2000). Several studies have shown that, due to their potent antioxidant effects, saffron and its constituents have immunological effects (Forchetti 2005), and are effective against neurological diseases including Alzheimer (Liu et al. 2010; Geromichalos et al. 2010; Cirmi et al. 2016; Costa et al. 2016). Crocin has protective effect against Parkinson-like behaviour (Mohammadzadeh et al. 2018). The saffron suspension effectively stimulates humoral and cell-mediated immunity (Vijayabhargava and Asad 2011).

Saffron has been used to dye silk, cotton or wool textiles (Takaoka et al. 1992; Tsatsaroni and Eleftheriadis 1994; Liakopoulou-Kyriakides et al. 1998; Tsatsaroni et al. 1998). In combination with hematoxylin, erythrosine and other chemicals it is also utilized as a dye for human and animal histological staining (Desmettre et al. 2001; Rostoker et al. 2001; Alyahya et al. 2002; Edston et al. 2002).

In Kashmir, saffron has a long history of being used in various Kashmiri cuisines and tea (*kehwa*) (Salwee et al. 2013). It is widely used in confectionery, alcoholic and non-alcoholic beverages. It is also used in sausages, oleomargarines, dairy products such as butter, cheese and ice cream for colouring and flavour improvement. Saffron petals are used in animal feeding, and due to the presence of carotenoidcrocins and flavonol kaempferol, they have antidepressant (Moshiri et al. 2006), antioxidant (Sánchez-Vioque et al. 2012), anti-proliferative (Sánchez-Vioque et al. 2016), antinociceptive and anti-inflammatory effects (Hosseinzadeh et al. 2005). Therefore, this important source can be explored for developing health enhancing products (Zeka et al. 2015). Anthocyanins and other phenolic pigments extracted from the petals of saffron are used as natural food additives (Lotfi et al. 2015). Saffron leaves are used as a source of forage for sheep and goats, but because of its low protein and mineral contents and low digestibility due to high fibrous contents, its utility is reduced as a feed (Valizadeh 1988).

10.1 Epidemiology of Corm Rot Complex

Corm rot of saffron has emerged as one of the major limitations for successful cultivation of saffron, globally. The disease has been referred by various names, including dry rot, brown rot, violet root rot, copper web, basal rot, corm rot, death blight and yellows (Table 10.1).

The disease is prevalent in almost all the saffron growing areas and is primarily noticed during flowering (October–November) and grubbing periods (May–July). The infected plants exhibit varied symptoms like drooping, damping-off, yellowing, wilting of shoots, basal stem rot and corm rot (Di Primo et al. 2002). Frequent out-breaks of corm rot complex is responsible for the abandoning of saffron cultivation in France and Spain (Zadoks 1981). The disease was reported to occur in 65 out of the 80 fields in the L'Aquila area (Spain), and was the major limiting factor of stigma yield of saffron (Cappelli and Minco 1998). In Kashmir, none of the saffron fields has been free from the incidence of corm rot (Dhar 1992), and 46% disease incidence has been reported in traditional saffron growing areas. Similarly, in the District Kishtwar of Jammu region also, the disease was widespread and prevalent in all the saffron fields, causing substantial losses in the saffron yield. Maximum disease incidence and severity was recorded in Lower Poochal (59.33 and 35.00%, respectively) (Gupta et al. 2011). In Kishtwar the disease incidence has ranged from 15 to 100%, and has been aggravating year after year (Gupta and Vakhlu 2015). The disease incidence and severity varied across the locations, primarily due to the variations in the presence of primary inoculum in the field, infestation of soil by plant parasitic nematodes and agronomic practices adopted by the farmers. Moreover, with large acreages planted with the sole crop (saffron) with minimal genetic variability, the likelihood of pathogen infection becomes greater, resulting in a high disease

Table 10.1 Occurrence of corm rot of saffron in the world

Country	References
France in 1728	Duttamel
Fuchu Tokyo-Fu and Kozu, Kanagawa-Ken in Japan in 1909	Abe (1933), Yamamoto et al. (1954)
Kanagawa-Ken Agricultural Experiment Station, Japan in 1918	
Okazaki in 1919	
Spain and France	Madan et al. (1967)
Italy	Francesconi (1973), Carta et al. (1982), Cappelli et al. (1991), Cappelli (1994), Fiori et al. (2011)
Greece	Goliaris (1999)
Morocco	Aymani et al. (2019)
Scotland	Sutton and Wale (1985)
Iran	Ghorbani and Koocheki (2017)
Netherlands	Schenk (1969)
China	Xu and Ge (1990)
India	Shah and Srivastava (1984)

incidence and even leading to an epiphytotic condition (Coakley 1995). Erratic rainfall and poorly drained saffron fields pre-disposes the crop to corm rot that drastically reduces the saffron yield and corm development and production of cormlets (Table 10.2).

Numerous plant pathogens such as *Fusarium oxysporum* f. sp. *gladioli*, *Fusarium oxysporum*, *Rhizoctonia* sp., *Aspergillus* sp., *Penicillium* sp. and *Macrophomina* sp. have been found associated with corm rot complex disease of saffron. *F. oxysporum* f. sp. *gladioli* survives in

Table 10.2 Pathogens associated with corm rot of saffron

Pathogen associated	References
Bacillus croci	Mizusawa (1923)
Fusarium bulbigenum var. *Blasticola*	Abe (1933)
Sclerotinia gladioli and *Fusarium oxysporum* f. sp. *gladioli*	Yamamoto et al. (1954)
Penicillium cyclopium	Francesconi (1973)
Fusarium oxysporum f. sp. *gladioli*	Di Primo et al. (2002)
Burkholderia gladioli	Fiori et al. (2011)
Penicillium corymbiferum	Schenk (1969), Gu and Zhi (1997)
Sclerotinia bulborum and *Rhizoctonia crocorum*	Alarcon and Sanchez (1968)
F. oxysporum f. sp. *gladioli*, *Macrophomina phaseolina*, *F. oxysporum*, *F. solani* and *Sclerotium rolfsii*, *Rhizoctonia crocorum* and *Phoma crocophi*	Shah and Srivastava (1984), Thakur et al. (1992), Sud et al. (1999), Kalha et al. (2007), Gupta et al. (2011), Hassan and Devi (2003)
Pseudomonas gladioli	Xu and Ge (1990)
Aspergillus terreus, *A. flavus*, *A. flavipes* and *A. Niger*	Aymani et al. (2019)
Rhizoctonia violacea	Bentata et al. (2017)

infected corms and soil as mycelium, chlamydospore, microconidia and macroconidia (Brayford 1996) and is readily disseminated to uninfested saffron growing areas along with diseased seed corms (Cappelli and Di Minco 1999). Due to the vegetative propagation sequential infection initiates through the corm by germinating spores or by mycelium which gain entry directly through roots or through wounds caused by insects, nematodes, rodents or mechanical injury. Later, resting structures of the pathogens are formed in the infected host tissues. Crop residues containing the resting structures are incorporated into saffron field soils. Then, intensive microbial activity occurs and successions of microorganisms develop in the decomposing plant tissues (Katan 2017). Survival of soil-borne pathogens responsible for corm rot can be active or passive for various years in the absence of host. Further, the pathogens also possess the autonomous and passive mode of dispersal which is helpful in the spatial–temporal distribution of corm rot disease in saffron. Tammaro (1999) found that temperatures above 10–12 °C along with rainy weather was favourable for the establishment of fungal infection in saffron corms. Entombment of infected plant parts, corms and infested soil are the major sources of initiation and spread of the disease. The association of multiple soil-borne pathogens in diseased corms of saffron shows synergism, for increased corm rot incidence.

Saffron crop is cultivated in various ways world over, in Kashmir it is planted in raised beds (Nehvi et al. 2018), whereas, in Kishtwar it is in furrows (Kalha et al. 2008) and in China it is in two-stage cultivation (Zhou et al. 2020). Being a perennial crop the planting cycle of saffron varies from 5 to 17 years. Corms are mainly harvested during June–July and planted in the field during August. Planting is carried out without proper phyto-sanitary inspection, hence the infected and/or infested corms act as source of primary inoculum of corm rot. All the pathogens responsible for the disease are either necrotrophic or hemibiotrophic, and need living host to complete their life cycle. Pathogens are stimulated and drawn towards the host roots in response to root secretions/exudates, or they may come in direct contact with the host tissue. Although host surface can be a source of nutrients for germinating spores and colonization of the pathogens, but it can as well act as a barrier by triggering host defence (Cantu et al. 2008) or presence of saponins (Rubio-Moraga et al. 2013) to their survival. Saffron plants are infected in the field by the germinating spores or by the mycelium, which may gain direct entry into the roots or through wounds induced by insects, nematodes, rodents or intercultural operations. Presence of plant parasitic nematodes are known to aggravate the soil borne diseases caused by the *Fusarium* spp. They act as bio-predisposing agent in encouraging infection of plants by creating wounds (Khan 2008), modify host (localized and systemic modifications), influence root exudations and alter host immunity (Khan and Sharma 2020). Fusarium wilt is reported to intensify the presence of nematodes, as they cause lesions on the root, subsequently weakening the plant immunity and easing the penetration of the pathogen (Dinesh et al. 2014), which ultimately lead to the increased in population of *F. oxysporum* (Almeida 2017).

Corms are collected from the field by digging with small hoe or breast plough and then collected by hand. During digging, injury with the farm tools predisposes the corms to the disease. Further, mingling of infected and healthy corms without removing adhering soil during storage, facilitates the infection of corm rot. Many a time, to clean the corms for planting, all the plant elements (outer tunic) are removed and left over the field which play an important role in the initiation of the disease in the successive season. Bullock carts or hired tractors used for ploughing and field preparation, also help in the dispersal of pathogens from one field to another. Therefore, saffron being a perennial crop, field sanitation gains all the more importance in the cultivation. Heavy rainfall also helps in the short-distance dispersal of plant pathogen.

Stagnation of water and poor drainage in the fields result in reduced soil aeration, thereby causing rotting to the saffron corms. Sometimes runoff flow of water also carries infested soil and

infected plant parts along with it to contaminate disease-free areas, drainage canals, irrigation reservoirs and rivers. Once water coming from contaminated sources is used for irrigation or naturally reaches disease-free areas, the pathogen spreads quickly and efficiently into and between saffron growing fields. Several soil-borne diseases have been reported to have dispersed through soil and water into the new areas (Peterson et al. 2014; Dita et al. 2018). Even the air currents carry the dust particles along with plant debris containing the inoculum and assisting in the dispersal of plant pathogens. Grazing of animals is also a possible means for the pathogen dissemination. Fungal propagules attached within fur, hoofs or feathers of animals are easily dispersed from one field to other.

Human mediated cultural operations, picking and grading of saffron also contribute in the dispersal of pathogens. Farm tools and equipment, clothes, footwear, etc., used in the infested areas can disseminate and spread the pathogens into disease-free areas (Dita et al. 2018). Seed trade is another potential means of disseminating the plant pathogens to far flung areas, even across the continents. Exchange of contaminated farm tools and planting diseased materials are also the means for the dispersal of corm rot pathogens.

10.2 Management

Historically, saffron was cultivated on vast areas in certain parts of the world, but due to lack of scientific approach, inadequate farmers' attention and regional conflicts, the growing areas either declined or were abandoned altogether. This low volume high value crop, preferred by the consumers for its functional food traits, has redrawn the attention of scientists, policymakers, extension workers and farming community. It is now again being rediscovered and recognized as the legendary crop and is gaining its due status in the world food, spice and pharmaceutical market. Traditionally, saffron was considered as a neglected, minor and low input demanding crop, which would flourish by their own destiny. The prevalence of various abiotic stresses and wide spread of corm rot complex significantly reduces the production and productivity of saffron. It also adversely affects the production of daughter corms, the only source for seed material for the subsequent cropping season. Thereby, contributing towards direct economic losses, and fluctuations in its prices.

Under field experiments, various approaches like soil solarization, biocontrol and chemical methods have been evaluated for the management of corm rot of saffron in naturally infested sick field. Maximum suppression in disease incidence (70.33%) has been recorded when the corms were treated with carbendazim, followed by the treatment with *Trichoderma viride* and *Trichoderma harzianum*, which was at par with soil solarization, resulting in the reduction in corm rot incidence by 61.55, 57.15, 59.90%, respectively, over control (Kumar 2018). Kamili et al. (2007) recorded 85.49% reduction in corm rot of saffron in Kashmir by dipping corm in mancozeb 75 WP @ 0.3% + carbendazim 50 WP @ 0.1%. Benzimidazole derivatives (carbendazim) play an important role in the management of soil and seed borne diseases by inhibition of DNA synthesis and blockage of nuclear division in pathogenic fungi (Zhou et al. 2016). Several other workers have also demonstrated that the chemical fungicides were very effective in managing corm rot of saffron under pot and field conditions (Sud et al. 1999; Wani 2004; Ahmad and Sagar 2007; Ahmad et al. 2018; Gupta et al. 2011). But growing concern about the deleterious impacts of synthetic chemicals on the environment as well as human beings, along with pesticide residues in the produce (Bourguet and Guillemaud 2016), has caused the awareness to explore the alternative eco-friendly and sustainable methods, for the management of soil-borne diseases. Use of beneficial microbes and soil disinfestation are the widely applied approaches for the management of soil borne diseases in various agricultural systems (Stapleton and Devay 1986; Stapleton 2000; Pal and Gardener 2006; Gill et al. 2017). *Bacillus* spp., due to the advantage of it producing heat and desiccation resistant spores, has

significantly reduced the incidence of corm rot (40%) of saffron under pot conditions (Gupta and Vakhlu 2015). This may be due to competition for energy, food and ecological niche or substrate, production of inhibitory allelo-chemicals and induction of systemic resistance (Choudhary and Varma 2016). Soil solarization for 4 and 6 weeks significantly reduces the corm rot incidence (47.9 and 52.2% during 2000 and 34.7 and 41.4% in 2001, respectively) over non-solarized check (Wani 2004). Fusarium wilt, root rot and damping off diseases are economically and successfully managed by the soil solarisation (McGovern and McSorley 1997; Chellemi and Mirusso 2006; Gelsomino et al. 2006; Katan 2017).

Besides suppression in the incidence of corm rot of saffron, population of *F. oxysporum* f. sp. *gladioli* was drastically reduced after 30, 60 and 90 days of sowing of saffron crop with maximum percent reduction of 58.33, 66.66 and 72.50%, respectively, by soil solarisation, which was at par with 48.18, 57.27 and 66.36%; 44.88, 55.12 and 62.99%; 39.25, 48.32 and 54.66%; 47.50, 58.33 and 64.16%; 40.65, 53.65, and 56.91% and 56.91, 65.04 and 67.47% in *T. viride* @ 1×10^7 cfu/ml, corm treatment with *T. harzianum* @ 1×10^7 cfu/ml, corm treatment *Pseudomonas fluorescens* @ 1×10^9 cfu/ml, corm treatment with *Bacillus subtilis* @ 1×10^9 cfu/ml, soil application with Vesicular Arbuscular Mycorrhizae @ 1 kg/acre and corm treatment with carbendazim 50WP @ 0.2%, respectively as compared to before sowing of corms in the respective treatment (Kumar 2018). Applications of *T. harzianum*, *Trichoderma hamatum*, and *T. viride* repressed the populations of *F. oxysporum* and *Rhizoctonia solani* by 46–56% and 31–44%, respectively, after two months of planting chickpea, whereas, 36–54% and 15–27% decrease was observed after four months, which might be due to the presence of mycoparasites that grow and multiply rapidly (Jeffries and Young 1994).

Application of plant growth promoting fungi (PGPF) and plant growth promoting bacteria (PGPB) are widely advocated against an array of different crops for amelioration of biotic and abiotic stresses for sustainable agriculture. Being inhabitants of outer and internal tissues of host, these beneficial microbes promote plant growth by having diverse mechanisms such as production of useful metabolites and signals, phytohormones, bio-fertilization, phyto-stimulation, antibiotic resistance, bio-remediation, rhizosphere engineering, lytic enzymes, increased resistance to osmotic stress and other abiotic factors (Ambardar and Vakhlu 2013; Gupta et al. 2011; Husaini 2014).

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11 Pathogenicity and Genetic Diversity of Microbes in *Crocus Sativus* L. and Various Strategies Combating Diseases

Rythem Anand, Pooja Sharma, and Madhulika Bhagat

Abstract

Saffron, one of the most expensive spices, is constantly in demand by the consumers. Due to its complex nature, the cultivation of saffron is limited to its special geographical region. Being a subterranean crop, it is highly susceptible to a number of diseases caused by several pathogens leading to severe loss in flower quality, stigma production, and overall yield. There is a vast genetic diversity of the microbes affecting saffron including both the disease-causing as well as the friendly microbiota present in the soil. Several strategies have been employed to protect the crop from pathogenesis, including commercial methods, intrinsic and extrinsic defense responses, nanoformulated agrochemicals for targeted delivery, and developing sensors for early diagnosis of the pathogen or diseases, including integrated "omic" approaches. This chapter aims to provide an overview of saffron and its cultivation practices, various pathogens associated, their genetic diversity, management strategies, and other new technologies that can be involved in order to improve the saffron quality and productivity.

11.1 Introduction

The dry stigma of *Crocus sativus* L. is an expensive spice of the family Iridaceae and is commonly known as Kesar, Safran, Zafran, Kum Kum, etc. Being the costliest spice by weight, its price varies from 1500 to 2200 Euro/kg and has gained the designation of "golden condiment, golden zest, or red gold". The low yield per acre and extensive labor to harvest the flowers and separate the stigma from the petals make them very expensive (Skinner et al. 2017; Mykhailenko et al. 2020). As reported that more than 418 tons/annum of saffron are produced worldwide, Kashmir valley in India occupies the second position in its production having 3674 ha of land under cultivation whereas first place is secured by Iran in production with an area of 108,000 ha followed by 7557 ha in Afghanistan, 1000 ha in Greece, 850 ha in Morocco, 150 ha in Spain, 70 ha in Italy, and 37 ha in France (Farooq and Meraj 2016; Cardone et al. 2020). Due to their exotic taste, aroma, coloring, flavoring agent, and pharmaceutical properties, the saffron cultivation is gaining a lot of interest from various industries such as dye, textile, culinary adjunct, flavoring, food and coloring, and drug industries (Melnyk et al. 2010; Kothari et al. 2021) (Fig. 11.1).

R. Anand · P. Sharma · M. Bhagat (✉)
School of Biotechnology, University of Jammu, Jammu 180006, India
e-mail: madhulikasbt@gmail.com; madhulikabhagat@jammuuniversity.com

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Scientific Classification

Kingdom : Plantae

Division : Magnoliophyta

Class : Liliopsida

Order : Asparagales

Family : Iridaceae

Genus : Crocus

Species : *C. sativus*

(a) **(b)**

Fig. 11.1 **a** Flower of saffron (*Crocus sativus* L.) **b** Classification of *Crocus sativus* L.

11.2 Phytochemistry and Therapeutic Uses

The potential quality of saffron entirely depends on the content, composition, and environmental conditions of secondary metabolites. It contains more than 150 volatile aromatic compounds and a large number of non-volatile substances such as carotenoids including zeaxanthin, lycopene, α- and β-carotenes, glycosides, monoterpenes, aldehydes, flavonoids, anthocyanins, vitamins (especially riboflavin and thiamine), and amino acids. Moreover, *C. sativus* is the only plant species that produces apocarotenoids mainly crocin ($C_{44}H_{64}O_{24}$, molecular weight of 976.972 g/mol), picrocrocin ($C_{16}H_{26}O_7$, molecular weight 330.37 g/mol), safranal ($C_{10}H_{14}O$, molecular weight of 150.21 g/mol), and crocetin ($C_{20}H_{24}O_4$, molecular weight of 328.4 g/mol), which when glycosylated gets converted into crocin, in significant amounts. These phytochemicals are responsible for the distinguishing properties of the saffron such as crocin is responsible for the color, while picrocrocin (monoterpene glycoside) attributes toward the bitterness, and safranal (monoterpene aldehyde) provides a distinctive aroma (Gupta et al. 2021). Due to the presence of these diverse ranges of phytochemicals, saffron exhibits a broad spectrum of medicinal properties and has been used for the treatment of several human ailments since ancient times (Christodoulou et al. 2015). As a traditional medicine, it is used in the treatment of inflammations, suppression of muscular spasms, relieving depression and cramps, stimulation of menstrual flow, control of appetite, treatment of coughs, respiratory problems, and flatulence (Hosseinzadeh et al. 2008) and as a cosmetic remedy for skin problems (acne), wounds, rashes, etc. (Mzabri et al. 2019). Several scientific studies have shown saffron to possess therapeutic value with multiple pharmacological effects, including antioxidant, anti-inflammatory, diabetes mellitus, cardiovascular diseases, asthma, neuronal injury, Parkinson's disease, anti-arthritic, depression, bronchitis, asthma, sedative effect, cancer, etc. (Saleem et al. 2006; Zheng et al. 2007; Del-Angel et al. 2006; Ahmad et al. 2005; Abu-Izneid et al. 2020; Husaini et al. 2021).

11.3 Diversity and Cultivation

C. sativus L. (Saffron) is a perennial sterile triploid ($2n = 24$), an autumn blooming crop with ~3.5 Gb haploid genome and has been cultivated since 3500 years ago. The origin of the native cultivable *C. sativus* is suggested to be derived from its wild variety, *C. cartwrightianus* (Srivastava et al. 2010), which gives its complex nature and is constantly been subjected to exploration for their phenotypic, cytological, molecular, and biochemical aspects. Traditionally, the cultivation has always been through vegetative propagation; considering its sterile nature thus new corms production lacks genome variations but still the phenotypic variations are observed in the stigma, stamens, styles, petals, the appearance of tepals, corms that are generally not inherited but may be considered due to the spontaneous mutations. Additionally, the vegetative propagation and cultivation of saffron land for other purposes have also contributed toward the lack of genetic variations. Currently, different molecular markers are explored in different

studies to assess the genetic variations such as RAPD, ISSR (Inter Simple Sequence Repeat), SSR (Simple Sequence Repeat), AFLP (Amplified Fragment Length Polymorphism), and SNP (single nucleotide polymorphism) (Mir et al. 2021). The high levels of alternative phenotypic variations related to phenology, floral morphology, and saffron production observed suggested epigenetics as a possible reason given that gene expression can be influenced by both genetic and epigenetic changes (Busconi et al. 2021). Overall the techniques of the production have not changed since ancient times, which involve intensive labor for flower picking and stigma separation.

Cultivation of saffron requires a temperate and dry climate; however, its vegetative growth coincides with cold weather. It grows at an elevation of 1500–2000 m amsl. In India, it is being cultivated mainly in Srinagar (mostly Pampore) and the adjoining areas of Srinagar such as Bhaderwah, Anantnag, Shopian, and Budgam (Ganaie and Singh 2019). Methods of its cultivation may vary with different soil conditions, climate, depth of bulbs, and the distance among the plants. The optimum conditions required by saffron are fertile loamy, sandy, loose, less dense, well-irrigated, well-drained calcareous or gravelly soil, having less moisture with an optimum pH range of 6.8–7.8 and electrical conductivity (E.C.) below 2 dS m^{-1}. Plants grow better in sunlight than in shady conditions. The temperature requirement ranges between 4 and 23 °C with 0.1–1.1 m precipitation. Raised beds lead to well-drained soil, and 20–30 tons of manure is reported to enhance the soil's organic content. Mathew (1999) reported that some species of *Crocus* flower first followed by sprouting of leaves in warm weather, while some species grow leaves and flowers together. But mainly photoperiod and temperature exert a profound influence on the flowering of saffron. It is noticed that rain preceding flowering boosts saffron yields, and rainy or cold weather during flowering promotes disease. Also, persistently damp and hot conditions harm the crops. In order to understand the influence of climate dynamics on vegetative growth, flowering, and fruiting of plants, the phenological understanding of the saffron crop plays a crucial role.

Within the life cycle of saffron, there are five stages viz., Sprouting, flowering, vegetative growth, formation of daughter corms followed by a dormant stage that predominates in summer. All these stages further depend on the geographical, climatic, and other environmental conditions. The corms are sown in the late August or early September followed by flowering that initiates after 30–40 days and continues for a few months. Later leaves and other vegetative growth takes place, followed by the development of daughter or secondary corms along with the mother corm, and it is also observed that the color of leaves changes from green to yellow on the completion of the development of daughter corms and thereafter the leaves wither off (Fig. 11.2) followed by corms entering the dormant phase that could be later dug out for further cultivation (Skinner et al. 2017). The dormant corms show no morphological changes, neither internal nor external growth takes place. Once the dormancy breaks, the fibrous roots form a plate at the base of the root from which emerges the shoots followed by leaves and flowers wrapped in cataphylls (Corcoles et al. 2015).

Post-harvest activities such as drying methods and storage duration can also affect the quality of saffron. The stigma is traditionally dried in shade, under ambient temperature, which in addition to prolonging the drying period also increases the risk of contamination (Fallahi et al. 2021). Additionally, dormant corms are usually stored at 25 °C until required. Alternatively, harvested corms are subjected to chemical disinfestation; these treated corms are dried and stored in the dark at room temperature until the next planting season. Still, as a subterranean organ, the corm is susceptible to many diseases that are caused by various pathogens. Also, environmental factors such as soil chemistry, irrigation, and usage of agrochemicals also have major effects on the production of saffron and corm quality (López and Gómez-Gómez 2009).

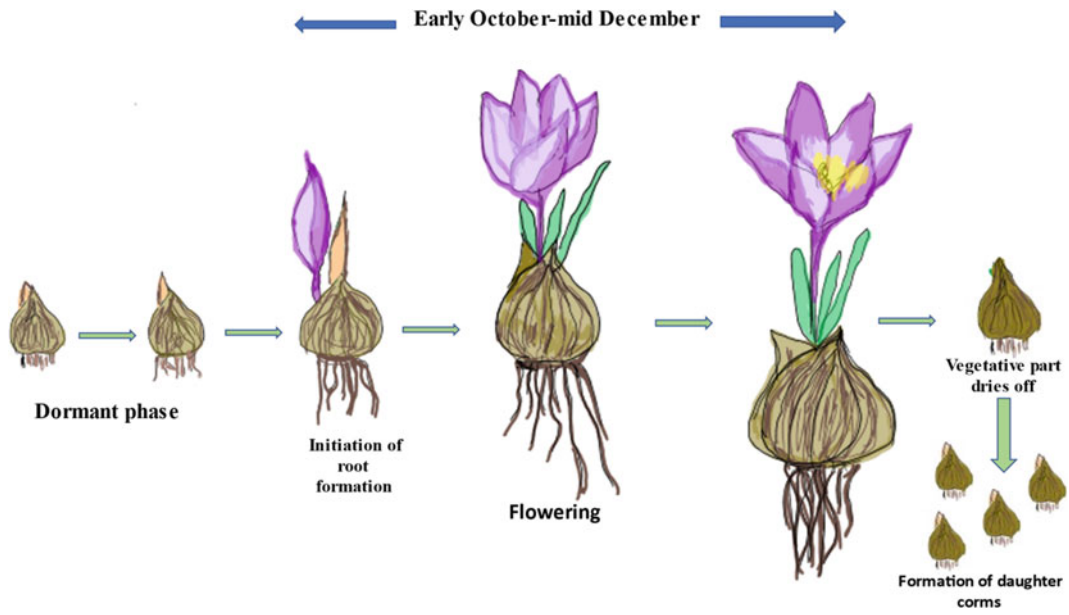

Fig. 11.2 Life cycle of *Crocus sativus* L.

11.4 Pathogenicity and Genetic Diversity of Microbes

A number of studies indicate that the decline in saffron cultivation is acknowledged for many reasons and one of them being the continual use of diseased material for planting (Magrotra et al. 2021; Ganaie and Singh 2019; Taufique et al. 2017). Once these infected corms are used as planting material, either their growth is very poor or they do not germinate at all. Since crocus is a subsurface growing plant thus their corms are susceptible to a number of pathogenic attacks via. microbes, nematodes, viruses, etc. (Rubio-Moraga et al. 2009). Once attacked, various essential functions of a plant are disrupted as they interfere, alter, or inhibit the basic functioning of the cells leading to the disease condition, poor plant growth, or can further kill the plant if infected by a necrotrophic pathogen. In the initial stages, the infection is present in a few cells, and later, it spreads to a wider area leading to the visible symptoms in plants. Apart from these biotic factors (fungal, bacterial, viral pathogens, nematodes, etc.), abiotic factors (climatic, nutritional disbalance, fertilizers, pesticides, soil problems, etc.) also contribute toward the manifestation of the diseases. In India, the disease occurs during the flowering season or during the dormant phase. This affects the saffron cultivation area of about 30–40% in Kishtwar and about 4–40% in the Kashmir division (Wani 2004; Gupta et al. 2011; Nehvi et al. 2012). Among diseases, corm rot is considered the most destructive one, as cited by various literature, and it is caused by the combined effect of several pathogens and saprophytes (Gupta et al. 2021). Corm rot is also known as dry rot, basal rot, and brown rot and is caused mostly by *Fusarium*, and its major symptoms include damping-off, wilting of shoots, yellowing of shoots and leaves followed by withering off of its vegetative portion leading to death. The pathogen is known to survive in the plant and its soil as chlamydospores, microconidia, and mycelia. They enter the new crop area by contaminated corms through mycelia or spores leftover in surrounding soil from the previous cultivation. Once in contact, they infect the healthy plants either through wounds (cuts) or roots (Cappelli and Di Minco 1999). Similarly, *Uromyces croci* infects

leaves causing the "rust disease", and here also, the association of its mycelia with the host leads to the spread of the disease and infecting new corms (Ahrazem et al. 2010a, b). Several other fungal species have also been reported to be associated with saffron viz., *Fusarium* spp, *Rhizoctonia*, *Penicillium*, *Aspergillus*, *Sclerotium*, *Phoma* spp., *Macrophomina phaseolina*, *Pythium* spp., *Phoma crocophi*, *Alternaria alternata*, *Talaromyces cellulolyticus*, *Talaromyces pinophilus*, *Porostereum* sp. *Penicillium pinophilum*, *Acremonium*. *Rhizopus*, etc. (Gupta and Vakhlu 2015; Gupta et al. 2021). The pathogenic *Aspergillus niger* species causes black mould infection at the rooting stage and transmits through infected corms and soil; *Penicillium* species causes "blue mould rot", infection shows dark brown patches in the scales of the corms; and *Penicillium crocicota* and *Penicillium chrysogenum* are responsible for damping-off, wilting of shoots, and formation of dark lesions beneath the sheath (Ahrazem et al. 2010a, b; Cappelli et al. 1991). *Rhizoctonia* infects the corm region rather than the stem, and infected corms show chlorosis that spreads to the whole of the corm and later to other adjoining corms. Moreover, there is an appearance of white mycelial growth on the infected and rotten corms, and the penetration into the host takes place through protective sheaths (also known as tunic) leading to the rotting of the whole corm (Madan et al. 1966). Other pathogens such as *Bacillus croci*, *Trichoderma*, *Phytophthora*, *Fusarium bulbigenum var. blasticola*, *Sclerotinia gladioli*, and *Alternaria* affect both stems and corms of *C. sativus* (Raj et al. 2013). Among the bacterial pathogens, *Burkholderia gladioli* was reported to cause bacterial soft rot in Saffron (Fiori et al. 2011). Similarly, plant viruses are transferred through vectors and feed on plants or also can be introduced through the wound, and vectors like aphids transmit the viruses of different genera, like *Potyvirus* viz., *Turnip mosaic virus* (TuMV), *Iris severe mosaic virus* (ISMV), *Narcissus mosaic virus* (NMV), and *Cucumovirus* like *Cucumber mosaic virus* (CMV) that infect *Crocus*. Viral infections include yellowing (chlorosis) or browning (necrosis), mosaic patterns, and plant stunting. In addition to pathogenic infection, the presence of plant-parasitic nematodes aggravates these diseases. They encourage infection in plants by creating wounds, modifying the host response, altering its immunity, and influencing root exudates (Gupta et al. 2021). Considerably the soil microbiota plays an important role in saffron cultivation as they affect the soil texture, corm development, environment, etc. The diversified microflora living in association with plants known as endophytes are important for the plant's survival, development, and immune system (Liu et al. 2017). Although possessing phylogenetic relationships with the soil-borne microbes, they usually exhibit an eco-friendly role in enhancing agricultural production (Le Cocq et al. 2017). Understanding the effect of their interactions among themself and within the saffron rhizosphere and cormosphere at various plant growth stages, along with knowledge attained with the associated changes in the microbial community due to their interaction, may help to relate the effect on plant health. Among the endophytes, *B. gladioli* E39CS3 is the main microbe found in the Kashmir valley that is reported to have a pronounced effect on the growth of the plants. They are the potent antifungal agents having an antagonistic effect on *Fusarium* rot as well. However, some endophytic strains of *A. alternata*, *Aspergillus pseudodeflectus*, *P. pinophilum*, and *B. gladioli* have been reported to cause extensive rotting in saffron corms (Wani et al. 2016, 2017; Ahmad et al. 2021). Rhizobacteria are also the growth-promoting bacteria associated with the roots of *Crocus* (Ambardar et al. 2016). Another species of bacteria showing a positive interaction with saffron is the plant growth-promoting bacteria, *Bacillus amyloliquefaciens W2* (KF663600), used as a biocontrol in disease management. Other beneficial microbes observed to be good for corm germination and blooming are *Nectriaceae*, *Penicillium*, *Aspergillus*, and *Saccharomycetales*. However, *Fusarium oxysporum* causing corm rot in *C. sativus* has been found to be present during flowering and dormant stages with a slightly higher abundance in roots (Ambardar et al. 2016).

11.5 Plant Defense Response

In order to fight against the pathogenic attacks, plants have developed certain intrinsic defense responses and they detect pathogens either by the non-self recognition of certain molecules (PAMPS) or by detecting intact or degraded self cells (Hemati Kakhki 2009). The interaction with the host generates two types of responses; non-host responses and specific host resistance responses (Fig. 11.3). The first category of defense response includes cell wall components, waxes, resins, and several secondary metabolites. In the case of saffron, the fibrous sheath provides protection against pathogens and water loss. In addition to this, a large number of secondary metabolites such as saponins, phenolics, cyclic hydroxamic acids, cyanogenic glycosides, isoflavonoids, and sesquiterpenes are present in the plant that act as a defense mechanism against a wide range of pathogens (Dixon 2001). These secondary metabolites are either present in active or inactive forms that get activated during pathogen invasion. Among these, saponins are known for high antifungal activity, and in saffron, the saponins are largely produced in stigma and corm tissue that may act on pathogen infection (Cardone et al. 2020). The presence of phenolic compounds along with high levels of peroxidases, catalase, and superoxide dismutase that have been detected at different developmental stages of corms helps in counteracting reactive oxygen species (Keyhani 2006). Usually infected and/or infested corms act as a source of disease in the other corms. The synergistic activity of the pathogens along with the abiotic factors helps in the initiation of disease and its further spread. The second kind of response generated on detection of pathogens is the hypersensitive response (localized cell death at the site of infection), expression of defense genes (PR genes), and oxidative response (Moraga et al. 2004). The hypersensitive response (HR) results in the accumulation of antimicrobial compounds, such as phytoalexins, peptides, phenolics, peroxidases, and polyphenol oxidase enzymes that are involved in defense response (Agrios 2005; Ortega and Pearson 2005; Kortekamp and Zyprian 2003). Additionally, transcriptional activation of defense-related genes, opening–closing of ion channels, modifications of protein phosphorylation status, and activation of several antioxidant enzymes work as responses against pathogen infection.

The defense mechanisms generated in different parts of saffron involve the increased levels of salicylic acid and enhanced expressions of pathogenesis-related genes (PR). These proteins, present in a low concentration in healthy tissue, get accumulated on perception of a pathogen. There are around 17 families of PR genes viz., the PR-1 and PR-17 proteins, 1,3-glucanases (PR-2),

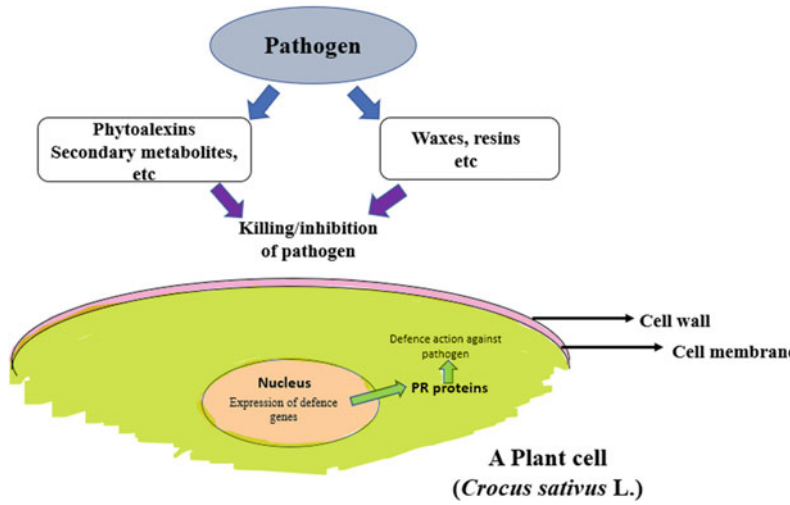

Fig. 11.3 Defense mechanism of plant (*Crocus sativus* L.) against pathogen attack

plant chitinases (PR-4, PR-4, PR-8, PR-11), thaumatin-like proteins (PR-5), proteinase-inhibitors (PR-6), endoproteases (PR-7), peroxidases (PR-9), ribonuclease-like proteins (PR-10), defensins (PR-12), thionins (PR-13), lipid transfer proteins (PR-14), and oxalate oxidases (PR-15; PR-16) (Ahrazem et al. 2010a, b). All these proteins exhibit potent antimicrobial activities, and their mode of action involves the disruption of the fungal cell wall, channel and pore formation, and inhibition of DNA synthesis and cell cycle (Sels et al. 2008; Theis and Stahl 2004). The expression of PR and other defense genes are regulated by several transcription factor (TF) families including zinc-finger-type WRKY factors having one conserved DNA-binding region (WRKY domain), highly conserved region (WRKYGQK peptide), and zinc-finger motif (CX4–7CX22–23HXH/C) (Ahrazem et al. 2010a, b). WRKY genes respond well to the pathogen attack and their homologs found in *Crocus* are WRKY-2 and WRKY. Chitinase proteins are yet another set of pathogenesis-related proteins that are activated on the interaction of the pathogen with the host (Grover 2012).

11.6 Strategies to Combat the Diseases of *Crocus Sativus*

In order to combat saffron yield losses, certain strategies can be explored to prevent microbial infection in saffron. To begin with, certain culturing practices such as preparation of raised beds for the proper drainage; sorting and grading of the corms (for example, appropriate size of corms should be >8 to 6 g) before sowing; standardizing of the methods for the cultivation of crop plant including proper corm rate, density, depth, spacing, and avoiding wound or injuries during packaging, transportation, and storage; better long-term storage facilities; crop rotation; and weed control are followed (Negbi 1999; Gresta et al. 2008; Husaini et al. 2010; Gupta et al. 2021).

Another strategy to get pathogen-free cultivation material is to use tissue culture-derived plantlets considered as the most reliable source of pathogen-free planting material for the fields (Leifert et al. 1994). Additionally using exclusion techniques in practice can also help to sort out any disease-causing agents in uncontaminated regions. In order to carry out exclusion, there should be a proper screening of the planting material that can be done by vegetative compatibility grouping (Fernández et al. 2011). Moreover, for identification, detection of the target pathogens, and quantification, some DNA-based techniques can also be explored (DeShields et al. 2018). Popular commercial ways to combat diseases of saffron include the use of chemical fertilizers and pesticides. Studies have shown that the growth of various pathogens, especially fungi, is inhibited by Carbendazim 50% WP (wettable powder) followed by Triadimefon 25% WP, Bitertanoi 25% WP, Dithianon 75% WP, Zineb 75% WP or benomyl, or copper-based solution (Mir et al. 2012). These chemicals have shown good antimicrobial activity against *Phytophthora*, *Fusarium*, *Rhizoctonia*, etc. They either help in reducing infestation or inhibit them. However, these suffer certain drawbacks such as non-biodegradability and the chemical residues left in the environment for a long period of time that may affect the commercial value of saffron (Ali and Bhat 2018). Another popular approach to combat inconsistent performance is the use of biological control agents instead of chemical management viz., *Gliocladium virens*, *Trichoderma viride*, *Trichoderma harzianum*, etc. that prevents the growth of phytopathogens to a certain level (Srivastava et al. 2013). Studies showed the effectiveness of *B. amyloliquefaciens* strain W2 in controlling corm rot disease caused by *Fusarium* species (Gupta and Vakhlu 2015). Efficacy of these biological controls is mostly affected by various abiotic factors; thus, an integrated disease management (IDM) module is being advocated against the management of diseases which is based on harmonized integration of compatible production and protection methods. IDM technology demonstrations have been conducted under farmers' participatory program (FPP) mode where scientific guidance from sowing to harvesting along with usage of chemicals were provided to the beneficiaries (Razdan et al. 2012).

The involvement of new technologies like nanotechnology may be a great source of innovation to improve yields, in the detection and identification of pathogens or diseases. In this category, different nanoparticles with favorable characteristics, like pore size, shape, and surface properties, can be effectively used in crop protection having a precise and targeted delivery. Nanoparticles can be directly used as a plant protectant or as a carrier for existing fungicides, pesticides, herbicides, and RNAi-mediated coupled components. For example, silver nanoparticles are most explored in plant defense, which disturb the cell DNA, metabolic activity, electron transport chain, and nutrient uptake of microorganisms leading to the death of the pathogens. Various pathogenic fungi such as *Colletotrichum gloeosporioides*, *Alternaria solani*, *F. oxysporum*, *Fusarium solani*, *Macrophomina phaseolina*, *Rhizoctonia solani*, and *A. niger* are reported to be controlled by silver nanoparticles. Silica-silver-based nanoparticles are reported to inhibit the growth of bacteria *Pseudomonas syringae* and *Xanthomonas campestris* pv. *vesicatoria* up to 100%. Copper-based nanoparticles were found to be effective against bacteria *X. campestris* pv. *phaseoli* and fungi *F. solani*, *A. solani*, *Aspergillus flavus*, etc. Nanoparticle carriers for fungicidal use are silica, chitosan, polymer mixes, etc. These nanoparticles can be used also as carriers for double-stranded RNA (dsRNA) or for agrochemicals and can be applied by spraying or soaking seeds, corms, roots, etc. Nanoparticles for carrier-based fungicides are very popular as they can reduce the loss through volatilization, increase solubility, and release chemicals at target sites slowly. The process of nanoencapsulation of the agrochemicals could be formulated to have controlled and sustainable delivery of the active ingredient against the target pathogens. Moreover, the delivery of dsRNA via. nanoparticle complexes is expected to be the future of RNAi-mediated control of pests/pathogens without genetic modification of crops (Gebremichael et al. 2021). Additionally, nanodiagnostic tools can be developed for the identification and detection of the pathogen load using nanosensors such as quantum dots, gold nanoparticles, and array-based sensors. Other than these, nanopore sequencing and microneedle tools are other such sensing tools (Li et al. 2020). Other technological advances over the last few decades have enabled more comprehensive approaches to understand and identify pathogenesis and its underlying mechanisms. Among these technologies, the omics tools allow detailed examination of plant and microbial characteristics along the genotype–phenotype spectrum and help in the development of more effective management strategies for improvements in plant breeding to field practices curtailing the spread of plant pathogens. Understanding the genetic basis for plant pathogen susceptibility and how the plant microbial community influences expression, further cascading metabolomic and volatilomic (screening of plants for phenotypic traits) pathways resulting in infection, present numerous opportunities for intervention that can be tuned and optimized for plant disease management situations. The application "of omic" technologies, while still in its early stages, has generated a huge database by exploring the physiological, agronomic, transcriptomics, proteomics, and metabolomics-based studies that are then analyzed by computational biology approach to find target genes for improving saffron production. Corm and stigma libraries from Crocus via. bioinformatics analysis have identified several genes associated with defense responses. For example, Demurtas et al. (2018) identified genes expressed in *C. sativus* stigmas and encoding putative ALDH enzymes. Gene CsALDH5F1, which belongs to family 5, comprises succinic semialdehyde dehydrogenases (EC 1.2.1.24) and is a close homolog of AtALDH5F1, involved in plant defense against reactive oxygen species, and a few proteins identified are PR-1, PR-2, PR-5, PR-10, WRKY-2, WRKY-4, PAL, SOD, peroxidase, etc. Transcriptomic studies revealed the identification of pathways such as carotenoids, whereas saffron metabolomics provided the qualitative and quantitative approach to evaluate the enormous array of putative metabolites among the saffron (Ordoudi et al. 2015; Gikas et al. 2021). The global sequencing of a cDNA library formed

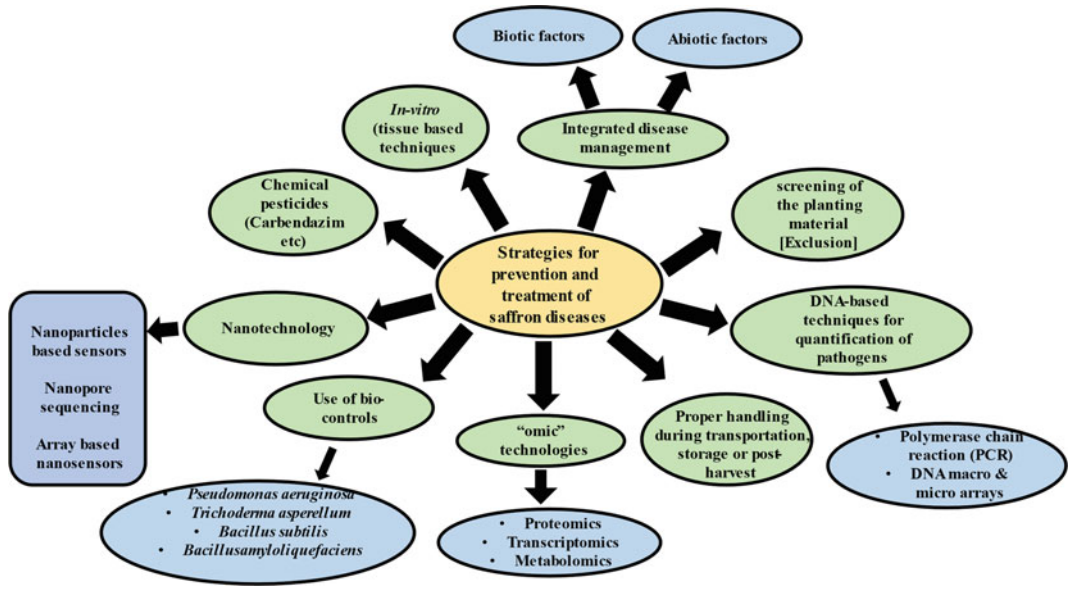

Fig. 11.4 Strategies for the prevention and treatment of diseases in *Crocus sativus* L

from mature stigmas of *C. sativus* was approved as the first saffron database. Saffron genes are a freely available resource for the research community concerned in saffron genomics (D'Agostino et al. 2007). Moreover, genetic modification techniques are becoming increasingly feasible due to improved techniques and demystification of the various side effects of transgenic technology. Clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated (Cas) systems may be also employed to introduce useful genome modifications through genome engineering of saffron (Belhaj et al. 2013; Husaini 2014; Chib et al. 2020). Additionally, a complete genome sequence may help to discover authentic genetic markers, and secondary metabolites genes to produce an improved quality of saffron (Fig. 11.4).

11.7 Conclusion and Prospects

Saffron cultivation and production are constantly under siege by a multitude of disease-causing pathogens and climatic changes. These pathogens (bacteria, fungi, viruses, nematodes, etc.) infecting saffron affect the development of plants and the quality of saffron and reduce the yield of the crop. Among all, the disease caused by *Fusarium* (corm rot) is the most devastating infection of *C. sativus* L. Improvement and standardization of the existing cultural practices in a specific agro-climatic condition are urgently required for the better management of the disease. Moreover, certain biosecurity measures should also be taken in order to have disease-free planting materials in the field that helps in preventing the spread of disease. Exploring plant defense systems, defense-pathogenesis-related genes, and their interactions would help in combating the disease along with the involvement of new technologies.

Eco-friendly biological controls are a preferred alternative to the chemical ones being employed in the fields of *C. sativus*. These have non-toxic properties and leave no harmful chemical residues after use. Popular biocontrols explored are *T. viride*, *T. harzianum*, *G. virens*, *B. amyloliquefaciens*, etc. Additionally, nanotechnology is applied to address commercial agrochemical problems. Using biodegradable material for nanoencapsulation, pesticides, herbicides, and fungicides prove to be a potent

alternative in plant disease management. These are reported to have a targeted delivery having low volatility, slow rate and sustained delivery, and generation of no chemical residues with increased stability. These could prove to be eco-friendly and economical formulations in the in-vivo field trials. Nano-based sensors/diagnostic tools can be also explored to detect the initial symptoms in saffron corms and soil. The nanomaterial-based clustered regularly interspersed palindromic repeats CRISPR-Cas9 technology could help in the management of several plant diseases. Integrating multi-omic approaches can help us understand the microbial players in the system, and how they disperse and are distributed across time and space including understanding their individual and combined functions. Data may provide us with a multidimensional view of potential diseases, plant defense, stress response, and understanding of their mechanism. Pitfalls and limitations toward integrating these approaches are to develop the hypothesis-based studies that test the ecological theory in order to understand how microbial diversity, pathogenicity, and dynamics shift under a changing environment. Another drawback is the availability of a handful of databases that are required for conducting multi-omics. Taxonomic or chemical compound databases are currently in their nascent phases as more microbes are sequenced and discovered worldwide. Moreover, information obtained from omics comes in different formats and with different means of preprocessing, analyzing, and interpreting the final results. To apply these results with the issues could be challenging.

To exert sustainable control over saffron diseases, it requires an understanding of their development, defence mechanisms and combinations of various modern culturing practices, tissue culture, use of biocontrols, biodegradable nanopesticides/nanofertilizers, along with integration of multi-omics techniques.

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Part IV
In-vitro Micropropagation in Saffron

Tissue Culture Techniques for Saffron Improvement

12

Seyed Alireza Salami

Abstract

Several clonal propagation methods are able to produce true-to-type clones of saffron plants under aseptic controlled conditions. Saffron in vitro culture included organogenesis, embryogenesis, Stigma-like structure generation, cormogenesis, cell suspension culture, and protoplast culture is being widely accepted today not only as a robust approach for large-scale propagation of uniform plants in relatively short time, but also as an important tools to produce pathogen-free plants, mass selection of superior clones, production of secondary metabolites (crocin, safranal, picrocrocin, and crocetin), gene transformation and genetic modification, crop improvement through somaclonal variations, conservation of endangered and rare species, and gene and genome editing using CRISPR/Cas9. Saffron as an economic sterile triploid plant only reproduced through asexual reproduction but the rate of conventional propagation through the daughter corms is low. Due to global increasing demands for expanding saffron cultivation areas and its high commercial value, the saffron industry is completely dependent on the development of efficient in vitro culture methods to guarantee the mass production of uniform and disease-free cormlets and corms.

S. A. Salami (✉)
Department of Horticultural Sciences, Faculty of Agricultural Science and Engineering, University of Tehran, Karaj, Iran
e-mail: asalami@ut.ac.ir

12.1 Introduction

Among several clonal propagation methods which resulted in true-to-type clones of a mother plant, tissue culture is the continuous process of in vitro culture of different cells, protoplasts, tissues, or organs under aseptic controlled conditions which include optimized concentration of macro and microelements and vitamins, pH, and adequate light, humidity, and temperature. Nowadays, plant tissue culture is being widely accepted not only as a commercial approach for large-scale propagation of plants in relatively short time period and space, but also as an important technology and tool to produce pathogen-free plants, mass selection of superior clones, and production of secondary metabolites of many plant species including geophytes such as saffron (Plessner and Ziv 1999). Moreover, its potential in genetic modification, crop improvement through somaclonal variations and conservation of endangered and rare species could not be ignored (Taheri-Dehkordi et al. 2020).

Many plants like saffron have no choice, but to propagate vegetatively. Saffron (*Crocus sativus* L., Iridaceae), a male sterile ($2n = 3X = 24$), self-incompatible geophyte with no seed set, is

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propagated through corms. It means that corms are indispensable for saffron propagation. For many countries such as Iran which accounts for over 90% of the world's saffron production (Vahedi et al. 2018; Taheri-Dehkordi et al. 2020), the obtaining of clean propagules for subsequent cultivation of a wide area of lands may thus be considered as one of the most important objectives in itself. There are many issues that limit the large-scale production of pathogen-free corms. Large-scale production of daughter corms is a long-drawn process on its own. Typically, a few cormlets per mother corm per season are produced in fields through natural breeding and the mother corm perishes after forming less than 4–10 daughter corms (Deo 2003; Husaini et al. 2010; Fernandez 2004). Besides, the latent endogenous infection in mother corms infects daughter corms and thereby affects the productivity and flowering. Therefore, tissue culture approaches offer great potential in overcoming these challenges and to modernize the cultivation of the saffron through the selection and mass vegetative propagation of pathogen-free corms or genetic improvement.

The chapter focuses on in vitro culture of saffron (*C. sativus* L.) and its wild allies. The main objective is generally to describe the tissue culture techniques and approaches towards saffron improvement, large-scale production of pathogen-free corms, genetic transformation and genetic improvement using gene and genome editing techniques such as CRISPR-Cas9, somatic hybrids production, and attempts will be discussed towards better understanding the various developments and components of in vitro culture, present and future scopes and challenges encountered during in vitro multiplication of saffron and wild *Crocus* species.

12.2 In Vitro Micropropagation of *Crocus* Species

Many *Crocuses* are known as ornamental bulbs; among them *C. sativus* L. (saffron), the most valuable edible spice in the world is the most renowned member of this genus (Negbi 1999). Saffron is a unique source of apocarotenoids, including crocin, picrocrocin, and safranal, which contribute to its color, flavor, and aroma, respectively (Tarantilis et al. 1995; Taheri-Dehkordi et al. 2020, 2021; Moradi et al. 2021, 2022). These secondary metabolites have several therapeutic effects against cancers, respiratory infections, scarlet fever, smallpox, hypoxia, asthma, blood disorders, insomnia, paralysis, heart diseases, flatulence, stomach upsets and disorders, gout, chronic uterine hemorrhage, dysmenorrhea, amenorrhea, baby colic, and eye disorders. Saffron is also an aphrodisiac, a digestive stimulant, and a tonic for dysentery and measles and therefore more than just one spice has been noticed in recent years in many countries (Abdullaev and Frenkel 1999; Rezaee-Khorasany et al. 2019).

Due to global increasing demands for saffron cultivation and commercial production of the most expensive spices, high throughput cost-effective and large-scale approaches need to be developed such as organogenesis and somatic embryogenesis in order to mass propagate the clean corms and microcorms and increase the yield of saffron (Nehvi and Yasmin 2016).

Saffron is propagated vegetatively only through annual renewal of mother corms due to sterile triploid nature of the species. Moreover, it is grown slowly as a geophyte and its natural multiplication rate is relatively low (Deo 2003; Zeybek et al. 2011; Taheri-Dehkordi et al. 2020). Corms development even takes 2–3 seasons for achieving the size and weight for flowering (Sharma et al. 2019). Commercial cultivation of saffron is conventionally labor working and human dependent. Annual cultivation requires digging up, breaking apart, and replacing the microcorms. However, low multiplication rates of cormlets compounded with problems of fungal, bacterial, and viral infestation in fields restrain the availability of adequate planting material and hence drastically reduce the productivity of saffron (Husaini et al. 2010). Generally, low corm production is due to poor breeding background and lack of superior cultivars adopted to diverse geographical conditions, low naturally occurring cormlet bearing, biotic

and abiotic stresses mainly viral, fungal and bacterial diseases, salinity and moisture stress, inadequate public knowledge, input and technical support, poor infrastructure and inefficient technology delivery system, poor crop management, and low multiplication rates of conventional propagation methods (Mir et al. 2010).

Besides conventional methods of propagation which are very slow and pose a high risk of survival and expansion of pathogens, in vitro culture approaches contribute importantly to the commercial propagation of saffron and its wild allies. However, contamination is the biggest problem in saffron micropropagation especially through its underground corms which are used as explants. Most of the times, different pathogens such as Fusarium corm rot and latent diseases attack the saffron corms and reduce growth and flowering, which consequently cause a great commercial loss (Plessner et al. 1990; Plessner and Ziv 1999; Sharma et al. 2019). They usually stay alive after replanting that cause further expansion of pathogens in the next season cultivation. Hence, low multiplication rates and pathogen infestation of corms are the bottlenecks to availability of sufficient quality planting material (Kiran et al. 2011). Many of these limitations have been overcome with the development and improvement of in vitro micropropagation and tissue culture infrastructure. In vitro micropropagation of saffron holds promise and represents an important potential to effectively propagate clean, pathogen-free, and uniform propagules for subsequent cultivation of a wide area of lands which guarantee production of high-quality flowers on a commercial scale.

ccDifferent tissue culture methods such as organogenesis, callogenesis, cell suspension culture, and somatic embryogenesis have been shown to be effective for mass and free-pathogen reproduction of *Crocus* species including saffron (Moshtaghi 2020). The use of tissue culture provides the requisite platform for the improvement of saffron traits through molecular breeding (Taheri-Dehkordi et al. 2020). *Crocuses* are considered as recalcitrant plants for genetic transformation and genetic improvement (Chib et al. 2020); however, tissue culture techniques can be very useful for *Crocus* genetic modification of saffron using genome/gene editing tools such as CRISPR-Cas9 and chromosome doubling, production of improved new varieties, or produce somatic hybrids through protoplast fusion or even in vitro culture to produce apocarotenoids using stigma-like structures (Chib et al. 2020; Moshtaghi 2020, Taheri-Dehkordi et al. 2020). Recently, a successful CRISPR/Cas9 system was reported for gene editing in saffron (Chib et al. 2020).

Moreover, such techniques can be used for *Crocus* germplasm conservation. Besides *C. sativus* L. which has been widely distributed almost in all provinces in Iran, eight other wild *Crocus* species are growing in Iran including *Crocus caspius*, *Crocus speciosus*, *Crocus cancellatus*, *Crocus pallasii*, *Crocus almehensis*, *Crocus gilanicus*, *Crocus biflorus*, and *Crocus michelsonii* which have been illustrated in Fig. 12.1 (Taheri-Dehkordi et al. 2020). This number accounts for a small portion of more than 235 species of crocuses known and have been described in The Word of *Crocuses* (Rukšāns 2017). Wild *Crocuses* are of particular importance due to their relevance to *C. sativus* and theories of saffron origin and parentage (Fernández 2004; Schmidt et al. 2019), as an alternative source for apocarotenoids extraction (Ordoudi et al. 2019), and ornamental and pharmaceutical properties (Zengin et al. 2020). On the other hand, some of these species are on the verge of extinction in different countries (Fernández 2007; Taheri-Dehkordi et al. 2020). Systematic conservation of plant genetic resources is one of the primary objectives to prevent extinction and reduce the biodiversity loss (Fernández et al. 2011), and different approaches of plant tissue culture have a great impact on ex situ plant conservation (Niazian 2019; Taheri-Dehkordi et al. 2020).

12.3 Tissue Culture Techniques and Workflow

Apart from the plant species and the workflows, in vitro micropropagation starts from an explant (leaf, petiole, stem, meristem, buds and nodes,

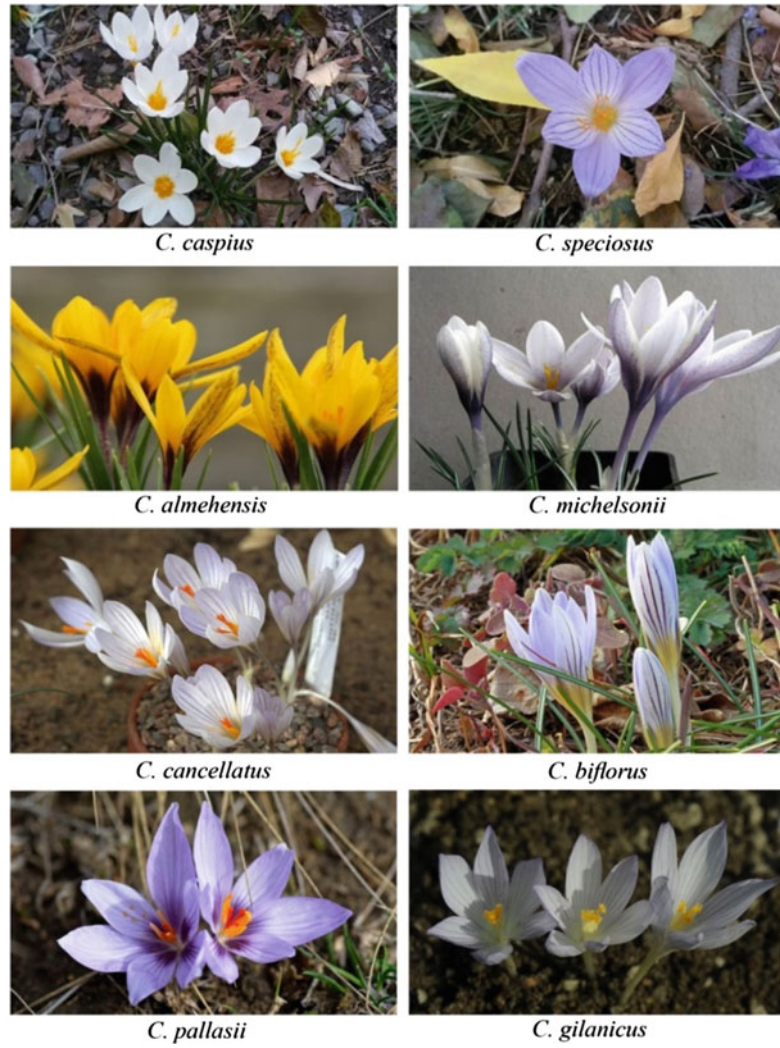

Fig. 12.1 *Crocus* species grown wild in Iran (Taheri-Dehkordi et al. 2020)

roots, floral segments, corm, corm segments, ovary, ovule, stamen, petal, stigma, style, perianth, pistil, corolla) excised from a vigorous mother plant. Sterilized explants can be later differentiated directly or indirectly into independent plants (clones) during rounds of initiation, multiplication, rooting, and acclimatization stages. A standard flowchart summarizing tissue culture processes has been shown in Fig. 12.2.

Like many other plants that grow from corms, direct and indirect organogenesis and embryogenesis through in vitro culture is the most effective method for mass production of saffron healthy plant materials and its wild allies concerning the limited rate of regeneration from isolated protoplasts (Plessner and Ziv 1999; Sharma et al. 2008; Darvishi et al. 2007; Isa et al. 1990; Blazquez et al. 2009; Moshtaghi 2020). In vitro propagation of saffron either through somatic embryogenesis or cormogenesis is considered to be a robust method for large-scale propagation of pathogen-free corms (Moshtaghi 2020; Taheri-Dehkordi et al. 2020). Cell suspension culture systems are also used for large-scale culturing of plant cells derived from callus, from which secondary metabolites such as apocarotenoids could be extracted (Moshtaghi 2020). As mentioned different explants are used

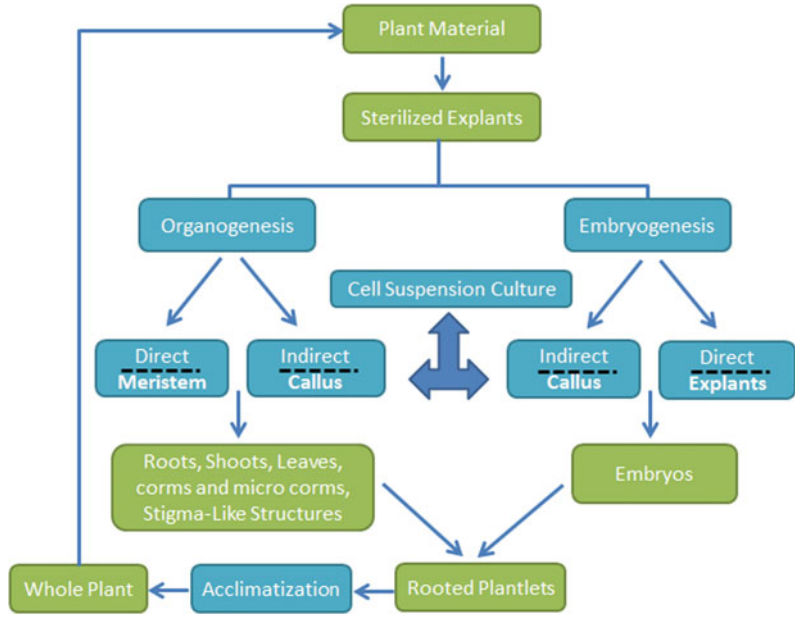

Fig. 12.2 In vitro culture flow chart in *Crocus* species including saffron

for in vitro establishment of saffron (Plessner and Ziv 1999). But still contamination is the biggest issue in saffron micropropagation.

12.3.1 Explants and Surface Sterilization

Different types of explants such as corm and corm segments, leaf and leaf mesophyll, shoots, shoot meristem, apical and lateral buds, inflorescence, ovary, ovule, pistil, petal, style, perianth, stamen, anthers, stigma, and callus have been used for somatic embryogenesis, protoplast culture, direct shoot regeneration, microcorm formation, callus production, stigma-like structures formation and cell culture metabolite synthesis of *Crocus* species mainly *C. sativus*, among them corm and corm segments which have eye buds, usually respond better than other explants for producing shoots (Plessner and Ziv 1999; Karaoglu et al. 2007; Sharma and Piqueras 2010). Corms usually collect from the field and are contaminated with soil and different contaminants. Therefore, the surface sterilization of explants is a crucial step to remove a wide variety of contaminants (bacteria, fungi, etc.) which may contaminate the in vitro media and the explants during initiation and proliferation further declines the production (Altan et al. 2010; Taheri-Dehkordi et al. 2020). However, viruses which negatively affect the corm growth vigor and yield can only be eliminated by thermotherapy for a specific period of time at high temperature and/or meristem tip culture (Nesi et al. 2009; Majourhay et al. 2007). Hence, successful disinfection procedure guarantees the establishment and maintenance of geophytes explants.

Most of the times, it is difficult to completely disinfect the explants due to latent endogenic infection and sometimes the contamination rate of corms has been reported to be very high (Yasmin et al. 2013; Taheri-Dehkordi et al. 2020). However, using an efficient disinfection protocol may result in 100% clean explants in *Crocus* species thereby guarantees the in vitro productivity (Taheri-Dehkordi et al. 2020). A review of four decades of investigation on saffron tissue culture revealed that surface sterilization of explants (corms, leaves, shoots, whole flower buds or parts of the inflorescence, including the stigma, style, ovary, anthers, and petals) using different combination of fungicides, bactericides

and antibiotics, EtOH, NaOCl, and $HgCl_2$ and Ag, Au, Cu nanocolloids has been an integral crucial part of the procedure as shown in Table 12.1 (Teixeira da Silva et al. 2016; Taheri-Dehkordi et al. 2020). They concluded that contaminants mainly fungal agents (species of *Fusarium* causing corm rot disease) are the main limiting factors which affect the availability of clean plant material (Ahrazem et al. 2010; Husaini et al. 2010; Teixeira da Silva et al. 2016). Moreover, despite epiphytic microorganisms, endophytic microorganisms may remain cryptic and not be detected until a more advanced culture phase (Curvetto et al. 2006; Teixeira da Silva et al. 2016).

Usually, the percentages of contamination and explant survival rate are recorded during a certain period of time to evaluate the efficiency of disinfection protocols. Generally, a balance between eliminating infection and having receptive tissue must be considered. In one of the most recent attempts, Taheri-Dehkordi et al. (2020) compared six different sterilization procedures to develop an efficient low-cost and easy-to-use protocol for disinfection of corms towards in vitro mass production of cormlets in saffron and its wild allies *C. caspius* and *C. speciosus*. Taheri-Dehkordi et al. (2020) showed that thoroughly washing tunics removed corms with tap water combined with EtOH, Rovral-TS (fungicide), $HgCl_2$ and Domestos® treatments resulted in 100% clean explants with over 85% survival rate (Table 12.1).

12.3.2 Tissue Culture Media

Under in vitro aseptic controlled conditions on artificial media which composed optimized concentration of macronutrients, micronutrients, vitamins, plant growth regulators (auxins, cytokinins, gibberlic acid, etc.), carbon source and some gelling agents (agar, phytagel) in solid media, totipotent cells are able to regenerate the entire plant. Different media such as MS, SH, GB5, LS, CHE, MMS, and White's media were used for in vitro culture, among them Murashige and Skoog medium (MS) is most extensively used (Bhagyalakshmi 1999; Verma et al. 2016; Moshtaghi 2020). Sterilized explants of saffron can be differentiated directly or indirectly into independent rooted plants under such controlled conditions.

12.3.3 Tissue Culture Approaches in Saffron

12.3.3.1 Organogenesis

Organogenesis in saffron refers to the development of adventitious organs (roots, shoots, leaves, corms and microcorms, stigma-like structures) or primordia directly from the meristem or indirectly from callus (Fig. 12.1). Organogenesis usually involves altering the balance of cytokinins and auxins to stimulate shoots, roots or corm/cormlet and stigma-like structures regeneration. Indirect organogenesis involves differentiation of unorganized cells called callus from different types of explants. Callus is also considered as the primary starter material for cell suspension culture towards metabolite production, indirect embryogenesis, and stigma-like structures in saffron and wild *Crocuses* (Hori et al. 1988; Zeng et al. 2003; Mir et al. 2010; Taheri-Dehkordi et al. 2020).

Indirect Organogenesis and Callugenesis

Ding and colleagues reported the first successful in vitro callus induction in saffron about 40 years ago (Ding et al. 1979, 1981). Although, it was a magnificent achievement at that time, callus production is considered as a routine job in saffron industry nowadays. Thereafter, calli can either enter the organogenesis, cell suspension culture, or embryogenesis (Fig. 12.1).

Many factors affect in vitro callugenesis in *Crocus* species among them hormonal combination, their concentration and balance, types of explants and species considered as the major components along with other factors such as developmental stage of corms, time of corms lifting, type of culture media and percentage of sucrose, nitrogen source (Igarashi and Yuasa 1994; Mir et al. 2010; Vatankhah et al. 2012; Vahedi et al. 2014; Verma et al. 2016; Moshtaghi

Table 12.1 A review of disinfection protocols for saffron and *Crocus* tissue culture in the last 40 years

TW → 70% EtOH 10–20 s → 0.1% $HgCl_2$ 15 min → 3–4 × SW	Ding et al. (1979, 1981) (China)	Corms
TW → NaOCl or 0.1% $HgCl_2$ 30 min → SW	Chen and Huang (1980) (China)	Corms
0.1% $HgCl_2$ 3 min → DW (10 min)	Homes et al. (1987) (Belgium)	Corms
RTW → 1% $HgCl_2$ 3–4 min → 75% alcohol + Tween-80 30 min → 3 × SW	Ilahi et al. (1987) (Pakistan)	1–2 cm corms
0.1% NaOCl 5 min → 1% NaOCl 5 min → 70% EtOH 2–3 min → 3 × SW	Koyama et al. (1988), Namera et al. (1987) (Japan)	Flower buds
1% NaOCl 10 min → 3 × SW	Sano and Himeno (1987) (Japan)	Flower buds
TW → 0.1% $HgCl_2$ 8 min → 10% NaOCl 8 min	Gui et al. (1988) (China)	Corms
RTW 2 h → excised flower buds 3–4 cm long in 7% Domestos 30 min → 5 × SW	Fakhrai and Evans (1990) (UK)	Descaled corms
RTW 30 min → dip in 70% EtOH → 0.25% $HgCl_2$ 30 min → 3 × SW	Plessner et al. (1990) (India)	Corms, apical buds
70% EtOH 1 min → 2.5% NaOCl 8 min → 3 × SW	Sarma et al. (1990, 1991) (Japan)	Flower buds
0.15% $HgCl_2$ 4 min → SW	George et al. (1992) (India)	Sprouted corms
0.1% $HgCl_2$ 2 min → SW	Dhar and Sapru (1993) (India)	Corms
TW → 0.1% $HgCl_2$ 10 min → 3 × SDW	Ahuja et al. (1994) (India)	Corms
70% EtOH 1 min → 0.1% $HgCl_2$ 15 min → SW	Guang and Shi (1995) (China)	Corms
TW 40 min → 70% EtOH 10 s → 0.1% $HgCl_2$ 15 min → 4–5 × SW	Liu et al. (1995) (China)	Corms
TW → 70% EtOH 1–2 min → 0.1% $HgCl_2$ 7–10 min → 4–5 × SW	Yang et al. (1996) (China)	Corms
4 °C for 21 d → soapy water → 70% EtOH 30 s → 0.1% $HgCl_2$ 8–10 min → 3 × SW	Jia et al. (1996) (China)	Floral buds, style, stigma, anthers, ovaries, and corms
RTW → excised flower buds in 70% EtOH 5 min → 2.5% NaOCl 15 min → 3 × SDW	Ebrahimzadeh et al. (1996) (Iran)	Descaled corms
EtOH 5 min → 10% Domestos 30 min → 3 × SDW	Castellar and Iborra (1997) (Spain)	Corms with developed leaves and unsheathed flowers just prior to flowering
0.15% $HgCl_2$ 4 min → several rinses in SDW	Bhagyalakshmi (1999) (India)	Ovaries (2.5–5 cm)
RTW 1–2 h → DW → 80% EtOH 25 s → 3 × SDW → 0.8% NaOCl 20 min with sonication → 3 × SDW	Escribano et al. (1999), Piqueras et al. (1999), Blázquez et al. (2004a; b) (Spain)	Corms
RTW → floral buds excised → soapy water 10 min → DW → 70% EtOH 1 min → 5.25% NaOCl + 3–4 drops Tween–80/500 mL 15 min → 4 × SDDW	Loskutov et al. (1999) (USA)	Corms with floral buds
TW → 0.1% $HgCl_2$ 10 min → 3 × SDW	Ebrahimzadeh et al. (2000a) (Iran)	Descaled corms
RTW → 70% EtOH 5 min → 1% NaOCl 10 min → 3 × SDW	Ebrahimzadeh et al. (2000b) (Iran)	5–10 cm long flower buds

(continued)

Table 12.1 (continued)

75% EtOH 3 × → 0.1% $HgCl_2$ 10 min → 3 × SW	Wang and Chen (2000) (China)	Corms
TW → 70% EtOH 10 min → 0.1% $HgCl_2$ 10 min → 4–5 × SW	Zhao et al. (2001a) (China)	Corms
TW 40 min → 70% EtOH 8 min → 0.2% $HgCl_2$ 8 min → 4–5 × SW. Apical buds → TW → 70% EtOH 10 min → 0.1% $HgCl_2$ 10 min → 4–5 × SW	Zhao et al. (2001b) (China)	Corms
TW → 70% EtOH 30 s → 0.1% $HgCl_2$ 8 min → 4 × SW	He et al. (2002) (China)	Corms
TW → 70% EtOH 30 s → NaOCl 15 min → 5 × SW	Chen et al. (2003a, b) (China)	Corms
Soapy water → DW → 70% EtOH 1 min → 0.1% $HgCl_2$ 8 min → 5 × SDW	Zeng et al. (2003) (China)	Floral buds
TW 1–2 h → DW → 70% EtOH 20 s → NaOCl 20 min → 5 × SDW	Chen et al. (2004) (China)	Shoots, leaves, flowers
TW → 0.15% $HgCl_2$ 10 min → 3 × SW	Karamian (2004) (Iran)	Cormlets
70% EtOH time NR → 0.1% HgCl2 10 min → 5 × SW	Sharma et al. (2005) (India)	Corms
TW → 70% EtOH 30 s → 0.1% $HgCl_2$ 8 min → 0.05% $HgCl_2$ 5 min → 3–4 × SW	Zhao et al. (2005a, b) (China)	Corms
RTW 30 min → dip in 70% EtOH → 2% $HgCl_2$ 30 min → 3 × SDW → 1% ascorbic acid 10 min	Darvishi et al. (2006) (Iran)	Descaled corms
RTW 30 min → 70% EtOH 15 s → 0.1% $HgCl_2$ 8 min → 4–5 × SDW	Wang et al. (2006) (China)	Shoots derived from sprouting corms
0.5–1.0 mg/L GA3 → TW 40 min → 75% EtOH 1 min → 0.1% $HgCl_2$ 15 min → 5 × SW → 0.1% $HgCl_2$ 5 min → 6 × SW	Zhang et al. (2007) (China)	Stored corms
70% EtOH 1 min → 0.1% $HgCl_2$ 8 min → 3 × DW	Jun et al. (2007) (China)	Styles, perianths of closed flower buds
(1) H_2SO_4 1 min → 80% bleach (ACE) 20 min; (2) H_2SO_4 2.5 min → 80% bleach (ACE) 20 min; (3) H_2SO_4 1 min → 0.7% $AgNO_3$ 10 min; (4) H_2SO_4 2.5 min → 0.7% $AgNO_3$ 10 min; (5) 3% dry or liquid fungicide; (6) 1, 2, 3, 4, 5 or 6% PPMTM 1 h; (7) hot water (40, 42.5, 45 and 47.5 °C) → 50% bleach (Axion) 20 min. All treatments → 5 × SDW	Karaoğlu et al. (2007) (Turkey)	Descaled corms
70% EtOH 45 s → 0.2% $HgCl_2$ 20 min → 3 × SDW 15 min	Sheibani et al. (2007) (Iran)	Descaled corms
TW → 70% EtOH 10 s → 0.1% $HgCl_2$ 10 min → 5 × SDW	Sharma et al. (2008) (India)	Stored corms
RTW 2 h → dip in DW→ 80% EtOH 30 s→ 3 × SDW→ 0.8% NaOCl 20 min with sonication → 3 × SDW	Blázquez et al. (2009) (Spain)	Dormant corms

(continued)

Table 12.1 (continued)

RTW 30 min → 0.5% benzalconium chloride 15 min → 70% EtOH 2 min → 1% NaOCl + Tween-80 20 min → 3 × SDW	Namin et al. (2009, 2010) (Iran)	Flower buds
RTW + 0.5% Cedepol + Tween-20 time NR → DDW → 70% EtOH 1 min → 0.1% $HgCl_2$ 10 min → 5 × DW	Quadri et al. (2010) (India)	Corms
RTW → 70% EtOH 3 min → NaOCl (% NR) 10 min → 4 × SDW	Mir et al. (2010) (India)	Ovaries from flower buds
RTW 30 min → dishwashing liquid → 1% benzalconium chloride 10 min → TW → 70% EtOH 2 min → 1% NaOCl 15 min → 3 × SDW	Sharifi et al. (2010, 2012) (Iran)	Flower buds
RTW → 70% EtOH 2 min → 0.1% $HgCl_2$ 5 min → 20% bleach + Tween-20 10 min → 3 × SDW	Vatankhah et al. (2010, 2014) (Iran)	Corms
RTW 1 h → brushed with Tween-20 → 0.1% streptomycin sulfate + 0.1% Bavistin 30 min → DW → 70% EtOH 30–45 s → 0.1% $HgCl_2$ 10–12 min → 5–6 × SDW	Devi et al. (2011, 2014) (India)	Descaled corms
RTW 2 h → DW → 80% EtOH 30 s → 6 × SDW → 0.8% NaOCl 20 min with sonication → 3 × SDW	Diaz-Vivancos et al. (2011) (Spain)	Shoots derived from sprouting corms
TW 0.5–1 h → 2 × SW→ 70% EtOH 30 s → 0.1% $HgCl_2$ 8 min → 4–5 × SW	Wang et al. (2011) (China)	Stored corms
RTW 30 min → 70% EtOH 60 s → 2% NaOCl 10 min + 0.01% $HgCl_2$ 15 min → 3–4 × SDW	Sivanesan et al. (2012) (Korea)	
RTW + 0.5% Extran + Tween-20 → DDW → 70% EtOH 1 min → 0.5% $HgCl_2$ 6 min → 5 × DW	Parray et al. (2012) (India)	Corms
RTW 20 min → 5.25% NaOCl + Tween-80 10 min → 3 × DW	Sharafzadeh (2012) (Iran)	Leaves
RTW 30 min → tunics removed → 70% EtOH 30 s → 0.15% $HgCl_2$ 20 min → 3 × DW	Zeybek et al. (2012) (Turkey)	Corms with tunics removed
Immersed in water with dishwashing liquid for 5 min → TW 30 min → 0.05% $HgCl_2$ 30 min → 2 × SW → 0.02% $HgCl_2$ 2 min → 4 × SW for 10 min	Wang and Xiao (2012) (China)	Corms
(1) 70% EtOH 5 min → 50% NaOCl 15 min; (2) 5% Tween-20 in DW 60 min → 70% EtOH 20 min → 50% NaOCl 5 min → 7% H_2O_2 10 min; (3) 5% Tween-20 in DW 75 min → 70% EtOH 20 min → 7% H_2O_2 20 min; (4) 5% Tween-20 in DW 90 min → 70% EtOH 15 min → 7% H_2O_2 15 min; 5) 5% Tween-20 in 10% EtOH 10 min → 0.15% H_2O_2 10 min. (1)–(5) → 3 × SDW. Explants 5–10 mm^3 with apical and axillary buds	Cavusoglu et al. (2013) (Turkey)	Corms

(continued)

Table 12.1 (continued)

TW → 70% EtOH 2 min → 5% NaOCl + 0.1% Tween-20 10 min → corms cut into segments → 70% EtOH 10 s → 0.1% $HgCl_2$ 3 min → 4 × SDW	Simona et al. (2013) (Romania)	Corms
70% EtOH 30 s → 3 × SW → 0.8% NaOCl 20 min → sonication → 3 × SDW	Renau-Morata et al. (2013) (Spain)	Corms
70% EtOH 1 min → 35% NaOCl 7 min → 35% Nanosilver (precise specifications NR) 10 min → several washes in SDW	Shahabzadeh et al. (2013) (Iran)	Corms
RTW 10 min → 0.1% carbendizime + 0.2% mancozeb (fungicides) + Tween-20 10 min → 50% NaOCl 10 min → 1.6% $HgCl_2$ 5 min → SDW	Yasmin et al. (2013), Yasmin and Nehvi (2014) (India)	Corms
RTW → 70% EtOH 3–4 min → 0.1% $HgCl_2$ 10 min → 5 × DW	Mir et al. (2014) (India)	Apical buds of tunics removed flower buds
TW 1 h → 70% EtOH 30 s → 0.1% $HgCl_2$ 15 min → 4 × SW	Pen and Hu (2014) (China)	Corms
RTW 30 min → detergent → 1% benzalconium chloride 10 min → TW → 70% EtOH 4 min → 1% NaOCl 15 min → 3 × SDW	Vahedi et al. (2014, 2015) (Iran)	Corms
5% NaOCl 8 min (shaking) → 80% EtOH 1 min → 37% Nanosilver 17 min (shaking) → 3 × DW	Mirjalili and Poorazizi (2015) (Iran)	Corms
RTW → 70% EtOH 3 min → NaOCl 10 min → 4 × SDW	Mir et al. (2015) (India)	Corms
RTW 10 min → Dipping in antiseptic detergent 5 min (shaking) → RTW → 70% EtOH 1 min→ 0.2% $HgCl_2$ 10 min → 5–6 × SDW	Jalal et al. (2015) (India)	Buds excised from corms
Immersed in SW 20 min → 70% EtOH 2 min → 0.5% NaOCl + one drop Tween-20 for 10 min → 4 × SW	Sajjadi-fard and Pazhouhandeh (2015) (Iran)	Corms
TW → 75% EtOH 60 s → 3–4 × SW → 0.1% $HgCl_2$ 12–15 min → 3–4 × SW	Yang et al. (2015) (China)	Corms
RTW 1 h → detergent (Tween-20, hair brush) → 0.6 ml/100 ml Savlon antiseptic solution 30 min → Rinsing with DW → 70% EtOH 30–45 s → 0.1% $HgCl_2$ 10–20 min → SDW	Verma et al. (2016) (Turkey)	Corms, leaf, stem
TW 30 min → 70% EtOH 45 s → 0.2% $HgCl_2$ 20 min → 4 × SDW	Lagram et al. (2016) (Morocco)	Corms with tunics removed
RTW 30 min → 0.1% $HgCl_2$ 30 min → 6–7 × SDW → 70% EtOH 1 min → 3–4 × SDW → 20% NaOCl 20 min → 6–7 × SDW	Sevindik and Mendi (2016) (Turkey)	Corms with tunics removed
RTW → 1% Labolene (v/v) + 2–3 drops Tween-20 → 0.1% $HgCl_2$ 9–10 min	Kashtwari et al. (2018) (India)	Daughter corms
RTW 30 min → 70% EtOH 90 s → 1 × SDW → 5% NaOCl 5 min → + one drop of Tween-20 for 15 min → 3–4 × SDW	Firoozi et al. (2019) (Iran)	Corms with tunics removed

(continued)

Table 12.1 (continued)

TW → tunics removed → Tween-20 5 min → 0.1% $HgCl_2$ 5 min → 4% NaOCl 5 min → 5 × DW	Chib et al. (2020) (India)	Corms with tunics removed
(1) RTW 30 min → Heat treatment (45 °C) 15 min → 70% EtOH 1 min → 1.5% NaOCl 15 min → 3 × SDDW; (2) RTW 30 min → 70% EtOH 1 min → 1.5% NaOCl 15 min → 3 × SDDW; (3) RTW 30 min → Brushing and cleaning withTween-20 → 0.6% (V/V) 2,4-Dichloro Meta Xylenol (DCMX) 30 min → 1 × SDDW → 70% EtOH 45 s → 3 × SDDW; (4) Preliminary washing with TW → Soaking in TW containing a few drop washing detergent (shaking) 15 min → RTW 20 min→ 70% EtOH 30 s → 2 mg/mL Benomyl (fungicide) 15 min → 2.5% NaOCl 20 min → 3 × SDDW; (5) Preliminary washing with TW → Soaking in TW containing a few drop washing detergent + Tween-20 for 15 min → RTW 30 min → 70% EtOH 30 s → 0.3% (W/V) Rovral-TS (fungicide) 30 min → 10% (V/V) Domestos ® 30 min → 3 × SDDW; (6) Preliminary washing with TW → Soaking in TW containing a few drop washing detergent + Tween-20 for 15 min→ RTW 30 min → 70% EtOH 30 s → 0.3% (W/V) Rovral-TS (fungicide) 30 min → 0.1% $HgCl_2$ 15 min → 0.05% $HgCl_2$ 5 min → 10% (V/V) Domestos ® 30 min → 3 × SDDW	Taheri-Dehkordi et al. (2020) (Iran)	Corms with tunics removed

AgNO3 silver nitrate; *s* second(s); *DDW* double distilled water; *DW* distilled water; *EtOH* ethyl alcohol (ethanol); *GA3* Gibberellic acid; H_2O_2 hydrogen peroxide; $HgCl_2$ mercury chloride; H_2SO_4 sulphuric acid; min minute(s); *NaOCl* sodium hypochlorite (values presented are assumed to represent the % of active chlorine); *NR* not reported in the study; *PPMTM* plant preservation mixture (plant cell technology); *RTW* running tap water; *SDW* sterilized (by autoclaving) distilled water; *SDDW* sterilized (by autoclaving) double distilled water; *SW* sterile water; *TW* tap water; *h*hour(s). An update of Teixeira da Silva et al. (2016)

2020). Corms of *C. sativus* showed higher nodal callus production while harvested in June compared to October and grown on B5 medium supplemented with 1 mg/L 2,4-D + 4 mg/L kinetin compared to MS and LS media (Vatankhah et al. 2012). Among five wild *Crocus* species, a frequency of callus induction up to 100% was recorded in *Crocus oliveri* ssp. *Oliveri* using the corms (versus leaf and stem) were grown on MS medium containing 5% (w/v) sucrose supplemented with 4 mg/L NAA + 4 mg/L TDZ compared to GB5, LS, and CHE media containing 2% (w/v) sucrose (Verma et al. 2016). Ziaratnia et al. (2012) also reported that B5 medium supplemented with 2 mg/L 2,4-D + 8 mg/L kinetin better promoted compared callugenesis compared to MS. Brittle calli induced in MS medium containing 1 mg/L 2,4-D and 0.2 mg/L kinetin (Yasini et al. 2013). Among different explants including corm, leaf, leaf end, and leaf scale, only corms responded favorably to callugenesis on MS medium supplemented with 1 mg/L IBA and 2 mg/L BAP which consequently regenerated to microcorms on MS containing 0.3 mg/L TDZ, 1 mg/L BAP, 2 mg/L IBA, and 0.01 mg/L GA3 in 4 °C (Sajjadifar and Pazhoohande 2015). Increasing the concentration of 2,4-D to 5 mg/L resulted in larger calli. In contrast, increasing the concentration of NAA to 5 mg/L reduced the

embryogenesis (Moghbeli et al. 2015). They reported MS containing 3 mg/L BAP without NAA as the best medium for embryogenesis. No callus was initiated in the media without Plant Growth Regulators (PGRs). However, 1 mg/L 2,4-D combined with 1 mg/L increased callugenesis and consequent growth of calli (Lagram et al. 2016). Nitrogen sources have different impacts on shoot regeneration from non-embryogenic calli. Nitrogen in the form of nitrate ($NO3^{-}$) was more effective than ammonia ($NH4^{+}$) for induction of shoots indirectly from callus (Igarashi and Yuasa 1994).

Willow extract as an alternative source of phyto hormones was also shown to be initiated callus and organogenesis in saffron (Jalal et al. 2015). Callus initiation was observed almost in all concentrations of willow extract. However, direct organogenesis was properly initiated in 20% plant extract while higher concentrations resulted in dormant organogenesis or arrested callus formation and organogenesis (Jalal et al. 2015).

On the other hand, in vitro shoot and root organogenesis in saffron is associated with histological and biochemical changes during different developmental stages and the content of saccharides as well. The highest content of MDA, proline, and phenolic compounds in nodular calli could possibly explain that the onset of organogenesis and differentiation requires a certain level of oxidative stress (Vatankhah et al. 2014).

Several studies reported callus induction and indirect/direct organogenesis in saffron. The first successful in vitro callus induction in saffron went back to four decades ago (Ding et al. 1979, 1981). Ilahi et al. (1987) then described indirect organogenesis in saffron and differentiation of callus into buds. Calli and buds were respectively initiated and differentiated in half-strength MS medium containing 0.5 mg/L 2,4-D and 0.5 mg/L BAP plus 2% coconut milk. They reported that balance of auxins and cytokinins enhances or suppresses callus formation, shoot-bud formation, and rooting.

In vitro microcorm production has been obtained through indirect organogenesis from leaf segments. Successful callugenesis was obtained on MS medium supplemented with 1.0 mg/L BA and 1.0 mg/L 2,4-D. Consequently, maximum proliferation towards microcorm production was achieved with 2.0 mg/L BAP and 0.50 mg/L NAA (Raja et al. 2007). Adventitious shoots initiated from callus in MS medium containing 1.5 mg/L BAP or 8 mg/L BAP and 2 mg/L NAA after 2–4 months. However, higher number of shoots (about 3x) formed while 8 mg/L BAP was used (Lagram et al. 2016).

Verma et al. (2016) regenerated shoots from non-embryogenic calli. A decrease in TDZ concentration with the addition of IAA progressively enhanced shoot formation in the k. A maximum number of shoots per callus was observed on MS supplemented with 2 mg/L IAA, 2 mg/L TDZ, and 2 mg/L BAP.

Parray et al. (2012) reported that TDZ found to be effective for induction and production of cormlets from non-embryogenic callus due to its role in biosynthesis of cytokinins as also were reported before (Thomas and Katterman 1986; Plessner et al. 1990). Several reports expressed the positive effect of TDZ on multiplication of corms (Blazquez et al. 2001; Parray et al. 2012). TDZ was also found to be effective for shoot regeneration. High-quality calli were induced on MS medium supplemented with 2 mg/L 2,4-D and 1 mg/L BA. Treatments containing 5 mg/L NAA and 5 mg/L TDZ proved to be the best growth regulator combination for shoot regeneration from those calli (Vahedi et al. 2014). Although TDZ was found to be effective in many tissue culture studies in saffron, however, to develop a more robust cost-effective commercial protocol, in vitro culture of saffron was performed successfully on a culture medium free of TDZ (Taheri-Dehkordi et al. 2020). Corm multiplication rate increased using higher concentration of sucrose (4%) as carbon source due to its effect on increasing osmotic pressure, inhibiting the vacuolation and shrinkage of cytoplasm and thereby increasing the amount of biomass accumulation (Sharma et al. 2008; Parray et al. 2012). Using 5% sucrose instead of 3% sucrose also resulted in larger cormlets containing higher fresh weight (Lagram et al. 2016).

Direct Organogenesis

Direct shoots have been generated from small corms, apical bud, lateral buds, and ovaries (Plessner et al. 1990; Bhagyalakshmi 1999; Sharma et al. 2008; Simona et al. 2013; Sivansan et al. 2014). Direct shoots have been generated from apical buds (Plessner et al. 1990). Corms were produced in vitro on MS medium. They reported that cytokinins particularly zeatin and the auxin 2,4-D were essential for bud development. Plessner and colleagues (1990) also reported that ethylene and ethaphon pretreatments inhibited leaf development and rooting, but induced corm production as well as dormancy (Plessner et al. 1990). Shoots and leaves regenerated from apical and lateral buds of corms through direct organogenesis on MS medium containing 6 mg/L BAP. Microcorms are best initiated on MS medium containing 1 mg/L IAA (Karaoglu et al. 2007). In other studies, shoots with a low frequency are induced at 14 mg/L BA, 13 mg/L IBA1 and 50 g/L sucrose (Sharma et al. 2008) and on SH medium supplemented by 3 mg/L BAP and 1 mg/L of NAA (Hagizade 2016).

Direct adventitious shoot were also generated from ovaries of saffron on full strength MS medium supplemented with 0.54 μM NAA and 2.22 μM BA. Combination of growth regulators, developmental stage, incubation temperature, and light/darkness conditions has a major impact on regeneration (Bhagyalakshmi 1999). Combination of 0.1 mg/L 2,4-D and 2 mg/L BAP initiated high percentage of shoot formation but using 1 mg/L BAP alone resulted in more shoots per explant. Also, cormogenesis was initiated in 1 mg/L IBA and 5% sucrose (Zeybek et al. 2012). Simona et al. (2013) reported adventitious shoots formation and later microcorms production from lateral and apical buds (direct organogenesis) on MS medium containing 1 mg/L 2,4-D and 1 mg/L BAP and through indirect organogenesis from corms on 0.25 mg/L 2,4-D and 1 mg/L BAP.

The highest number and the longest shoots were obtained in MS medium containing 21.6 μM NAA and 22.2 μM BAP, whereas more than 10 microcroms per explant were generated on MS medium containing 2 mg/L BAP and 0.5 mg/L NAA. Light had no significant positive effects on microcorm formation and their growth (Mir et al. 2014). Direct shoots and microcorms were regenerated from corm explants on SH medium containing 2 mg/L BAP and 0.5 mg/L NAA. Frequency of microcorm production was affected by sucrose concentration and the average of six microcorms per explant were induced in a medium supplemented with 6% sucrose (Sivansan et al. 2014).

As reviewed in many of the above reports, during organogenesis a combination of one auxin and one cytokinin with proper concentrations and balance is usually crucial. Also, direct organogenesis and consequently shoots formation without passing through the intermediate callus phase is preferred over indirect shoots for saffron propagation. Less time is usually required for generating direct shoots compared to indirect ones. Direct shoots have low or no variety and are genetically uniform. The rate of regeneration using indirect organogenesis is limited.

Stigma-Like Structure and Callus Culture Towards Apocarotenoids Production

Tissue culture can be used as an alternative biochemical tool to produce major apocarotenoids in saffron through the formation of stigma-like structure (SLS) and or callus culture (Namin et al. 2010). Organogenesis can be achieved through production of stigma-like structures of *C. sativus* L. towards crocin, picrocrocin, and safranal production. Type of explants, type of culture medium, and type and concentration of hormones determine the frequency of induction and formation of stigma-like structures in saffron in vitro. Several patterns of stigma-like primordium formation were reported from ovaries (Sano and Himeno 1987; Loskutov et al. 1999; Namin et al. 2010), stigmas (Koyama et al. 1988; Sarma et al. 1990), petals (Lu et al. 1992; Jia et al. 1996), anthers (Fakhrai and Evans 1990), stamens (Zhao et al. 2001a), and style (Namin et al. 2010).

Sano and Himeno (1987) were the first who reported Stigma-like structures (SLS) production

towards production of apocarotenoids in vitro using young stigma plus ovaries, single stigmas, and half ovaries, among them half ovaries were the best explant. Stigma-like structures were formed directly on LS supplemented with 10 ppm NAA and 1 ppm kinetin or on Nitsch medium supplemented with 1 ppm NAA and 1 ppm BA. An average concentration of crocin and picrocrocin in the stigma-like structures grown on Nitsch medium were about threefold higher than those grown on LS medium (Sano and Himeno 1987).

Sarma et al. (1990) reported in vitro production of stigma-like structures from stigma explants of *C. sativus* L. on MS medium supplemented with 10 mg dm^{-3} NAA and BA (1 mg dm^{-3}). The NAA was found to be an important additive to promote stigma-like structure formation. Crocin, picrocrocin, safranal, and crocetin metabolites were successfully produced in SLS but in a lower concentration than natural stigmas (Sarma et al. 1991). However, the same amounts of crocin, crocetin, picrocrocin, and safranal were reported in stigma-like structure compared to natural stigmas (Loskutov et al. 1999). Half-ovary explants of *C. sativus* L. were proliferated into stigma-like structures on Gamborg B5 medium containing 5.4 μM NAA, 44.4 μM BA, MS organics, 0.05% casein hydrolysate, and 11.2 μM L-alanine (Loskutov et al. 1999). Casein hydrolysate and L-alanine led to an increased in apocarotenoids content and promotion of stigma-like structures from floral organs in saffron on MS medium supplemented with 5 mg/L kinetin and 4 mg/L 14 NAA (Zeng et al. 2003). L-Alanine and sodium carbonate can increase the synthesis of Acetyl CoA in plant which used for terpenoid production such as crocin and crocetin (Otsuka et al. 1992; Zeng et al. 2003). Therefore, precursor feeding enables us to increase apocarotenoids content in SLS.

Namin et al. (2010) reported the origin and induction of stigma-like structures from ovary and style explants. Stigma-like structures on MS supplemented with 10 mg/L NAA and 10 mg/L BAP were directly originated through meristematic cells or indirectly in the form of colorless globular structures from parenchyma tissue. Conical structures and then long stigma-like structures were observed mostly inside the calli in injured ovaries after 5–8 months. A few leaf-like structures and incomplete bud flowers were noted on the outer layer of the calli.

Direct and indirect induction of SLS and the amount of apocarotenoids in vitro depend on hormonal combination and their concentration and balance, types of explants, precursor feeding, light condition, and temperature (Loskutov et al. 1999; Ebrahimzadeh and Karamian 2000; Zeng et al. 2003). Low concentrations of NAA and BA induce direct generation, while high concentrations of NAA and BA induce indirect SLS generation (Loskutov et al. 1999; Ebrahimzadeh and Karamian 2000). More than double amount of crocin was produced in SLS under light condition compared to dark condition (Zeng et al. 2003). Room temperature (25 °C) was reported to be the best optimum temperature for SLS induction (Loskutov et al. 1999; Zeng et al. 2003).

Callus cultures could be considered as a potent source for induction of crocin, crocetin, picrocrocin, and safranal (Visvanath et al. 1990). Callus cultures were obtained from floral buds on MS medium supplemented with 2 mg/L 2,4-D and 0.5 mg/L kinetin induced to produce red globular callus and red filamentous structures (Visvanath et al. 1990).

Crocin production using *C. sativus* callus by two-stage culture system was reported by Chen et al. (2003a). Saffron callus was grown in a two-stage culture on B5 medium supplemented with casein hydrolysate (300 mg/L) at 22 °C in dark with 2 mg/L NAA and 1 mg/L BA to give maximum biomass (16 g dry wt/L), and with 2 mg/L IAA (indole-3-acetic acid) and 0.5 mg/L BA for crocin formation. The maximum crocin production (0.43 g/L) was achieved by this two-stage culture method.

Rare elements are reported to promote crocin production in cell culture of *C. sativus* L. (Chen et al. 2004). La^{3+} and Ce^{3+}, either individually or as a mixture, promoted crocin production of *C. sativus* L. callus but Nd^{3+} had little effect and all metal ions were toxic above 100 μM. La^{3+} (60 μM) promoted growth of callus

significantly but increased crocin only slightly. Ce^{3+} (40 μM) significantly promoted crocin production but had little effect on cell growth. They showed that La^{3+} (60 μM) and Ce^{3+} (20 μM) together gave the highest dry weight biomass (20.4 g/L), crocin content (4.4 mg/g), and crocin production (90 mg/L).

12.3.3.2 Somatic Embryogenesis

In vitro propagation of saffron and wild *Crocus* species could be also achieved through somatic embryogenesis directly from a cell or a tissue or indirectly from callus (Fig. 12.1). Somatic embryogenesis (SE) and subsequent regeneration into plantlets is a striking alternative for robust cormlet production and high throughput large-scale multiplication of saffron which offers a strategic tool for genetics improvement (Chugh and Khurana 2002; Devi et al. 2014; Taheri-Dehkordi et al. 2020). Somatic embryos can be also used for the production of synthetic seeds, for genetic transformation, and other biotechnological applications (Sevindik and Mendi 2016).

Somatic embryogenesis includes induction, differentiation and maturation of spherical, scutellum (horn-shaped), and cotyledon structures in saffron as a monocot species (Denis et al. 1991; Thrope et al. 1995). Somatic embryos are usually produced by somatic cells. Despite generative embryos, somatic embryos have no endosperm, suspensor, and shell and therefore are dependent on nutrients and hormones provided by culture medium. Somatic embryos have no dormancy and embryogenesis from basic cells to the formation of seedling is started immediately after being placed on culture medium and continues without any break (Kumar 2003). Somatic embryos have small cotyledons, compressed cytoplasmic content, big nucleus, small vacuole, and low starch grains. Direct embryogenesis involves embryo generation directly from a cell or a tissue without intermediate phase of callus formation and through the cells which are called preembryonic or embryonic. These specific cells just need proper concentration of plant growth regulators to begin cellular division under proper conditions and induce embryogenesis from hypocotyl epidermis, epidermis tissue, and symmetrical nuclei. In the indirect embryogenesis, somatic embryos are induced after the callus induction phase.

In saffron vegetative apex (George et al. 1992), shoot meristem (Ebrahimzadeh et al. 2000), apical or terminal buds (Blázquez et al. 2004; Sheibani et al. 2007), shoot meristem along with a pair of leaf primordia (Ahuja et al. 1994; Karamian 2004), axillary buds (Sheibani et al. 2007), rectangular section from central meristematic region (Sharifi et al. 2010), true leaves which were still inside the leaf sheath or leaves alone (Devi et al. 2014; Halim et al. 2018; Firoozi et al. 2019), mature and immature flower parts (Halim et al. 2018), corms (Sheibani et al. 2007; Sivansan et al. 2012; Halim et al. 2018; Firoozi et al. 2019; Taheri-Dehkordi et al. 2020) were used as explant for somatic embryogenesis.

Callus induction and subsequently somatic embryogenesis indirectly or direct embryogenesis and their efficiency are influenced by many factors such as *Crocus* species, type of culture media, plant growth regulators combination and their concentration, explant types, TDZ concentration, jasmonic acid and ABA treatments, sucrose content, oxidative stress followed by reactive oxygen species (ROS) production, solid or immersion system, etc. (Ahuja et al. 1994; Ebrahimzadeh et al. 2000; Karamian 2004; Blazquez et al. 2004a, b, 2009; Sheibani et al. 2007; Verma et al. 2016; Taheri-Dehkordi et al. 2020). Some studies reported that embryo maturation, germination, and differentiation are GA3-dependent (Ahuja et al. 1993; Ebrahimzadeh et al. 2000; Karamian 2004; Sivansan et al. 2012).

Aerial organ meristems of saffron grown on LS medium containing 2×10^5 M NAA and 2×10^5 M BAP were successfully differentiated into somatic embryos. Subsequently, mature embryos germinated in half MS medium containing 20 mg/L GA3. Germinated embryos were differentiated into microcorms when transferred to half MS containing 2% activated charcoal and 5×10^5 M of NAA and BAP (Ahuja et al. 1993). Similarly, somatic embryos were successfully developed from aerial organ meristem on LS medium containing 2×10^5 M NAA and

2×10^5 M BAP. Mature embryos germinated in 1/2 MS medium containing 25 mg/L GA3 (Ebrahimzadeh et al. 2000).

Karamian (2004) reported that a combination of 2,4-D with kinetin or BA is essential for high frequency embryo induction. ABA (1 mg/L) led to the maturation of embryos and mature embryos were consequently germinated on 25 mg/L GA3 (Karamian 2004). Sheibani et al. (2007) reported that TDZ (0.5 mg/L) induced embryogenic calli after eight weeks. Embryos matured in hormone-free MS medium containing 6% sucrose. Matured embryos then turned to microcorm after 3 months in 1/2 MS medium.

Sivansan et al. (2012) studied somatic embryogenesis in *Crocus vernus* using different culture media (SH, B5, N6, AM, and MS) and different concentrations of 2,4-D in combination with 0.5 mg/L TDZ. The highest rate of embryogenesis and somatic embryo induction was obtained in SH medium supplemented with 1 mg/L 2,4-D + 0.5 mg/L TDZ under 10 μmol m^{-2} s^{-1} PPFD. Mature embryos germinated in SH medium containing 1 mg/L GA3. Germinated embryos are converted to seedling in SH medium supplemented with 1 mg/L GA3 and 6% sucrose.

Verma et al. (2016) studied callus induction, somatic embryogenesis, and plant regeneration in five *Crocus* species. Cotyledonary somatic embryos were induced in three of five species from different explants (leaf, stem, and corm) cultured in four different media (MS, GB5, LS, and CHE). Among five wild *Crocus* species, the highest average number of embryos formed in *C. oliveri* ssp. *Oliveri* followed by *Crocus specious* ssp. *Specious* and *Crocus pestalozzae* using leaf explant on MS medium containing 5% (w/v) sucrose supplemented with 2 mg/L NAA + 2 mg/L TDZ + 100 mg/L ABA (Verma et al. 2016).

Direct somatic embryogenesis of saffron was performed on SH medium containing 2,4-D and TDZ. Somatic embryos converted into cormlets after shooting on SH medium supplemented with 2 mg/L BAP and 1 mg/L GA3 and 6% sucrose (Hagizade 2016).

Taheri-Dehkordi et al. (2020) developed a robust workflow for indirect somatic embryogenesis and cormlet production in saffron (*C. sativus* L.) and its wild allies. They reported successful somatic embryo germination and cormlet production indirectly through embryogenic calli in *C. sativus* L. and two wild C*rocus* species included *C. caspius* and *C. speciosus* (Fig. 12.3). Corms cultured on MS medium containing different combinations of 2,4-D, Picloram, BAP, and Kin. Explants neither induced callus nor calli-like structures in MS medium free of PGRs and turned necrotic after four weeks in all three species. The frequency of callus induction (100%), embryogenic callus induction (80%), and the number of embryos formed per explant (6.45) was higher in saffron compared to the wild *Crocus* species. The best result was obtained in MS medium containing 10.18 μM 2,4-D + 4.44 μM BAP in all three species. Calli were subsequently developed into somatic embryos in *C. sativus* within 4 weeks versus 8 and 10 weeks in *C. caspius* and *C. speciosus*, respectively. While calli cultured in a PGR-free medium, somatic embryos were further developed. Globular embryos were prevalent; embryos, individually or as clusters, were white or pale yellow, small, and globular in shape. Globular somatic embryos with a soft and shiny appearance have further developed into somatic embryos. Somatic embryos were later germinated and converted into cormlets after shooting. Well-developed cormlet tunics were observed after 10–12 weeks. The highest conversion frequency and maximum cormlet weight were achieved in MS containing 5.37 μM NAA + 8.88 μM BAP.

A higher fresh weight embryogenic calli which indicate high quality of calli were produced in temporary immersion system versus solid media (Blazquez et al. 2004b). Jasmonic acid (Blazquez et al. 2004b), TDZ (Sheibani et al. 2007) and ABA (Verma et al. 2016) also improve efficiency of somatic embryogenesis. In addition, oxidative stress followed by production of reactive oxygen species (ROS) induces totipotency to gain embryogenic competence by the somatic cells (Blazquez et al. 2004a, 2009).

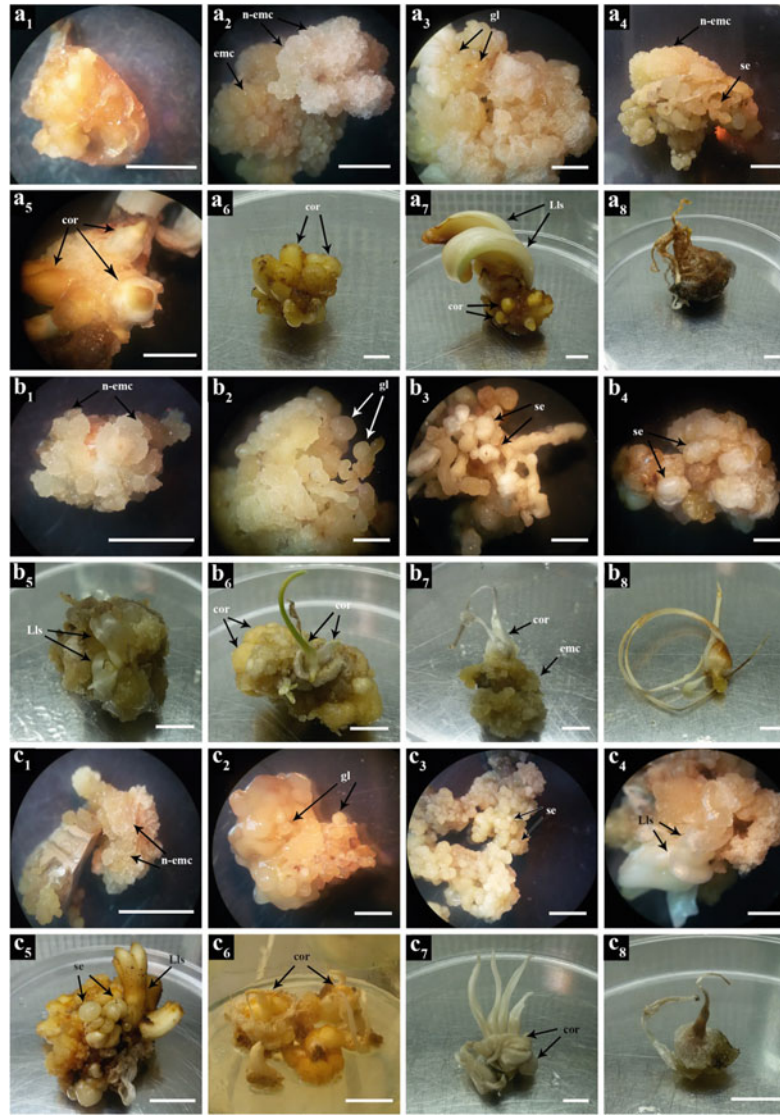

Fig. 12.3 Plant regeneration through somatic embryogenesis and cormlet production in *Crocus sativus* L. (a1–a8), *Crocus caspius* (b1–b8), and *Crocus speciosus* (c1–c8). emc; embryogenic callus, n-emc; non-embryogenic callus, gl; globular embryo, se; somatic embryo, cor; cormlet, Lls; leaf-like structure (Bar ¼ 10 mm). From Taheri-Dehkordi et al. (2020)

Several developmental and biochemical changes take happen during saffron somatic embryogenesis (Blazquez et al. 2009). The embryogenic callus underwent internal segmented divisions with the formation of globular embryos followed by further development of the embryoids by the emergence of a shoot apical meristem and cotyledon (monopolar stage) with the subsequent differentiation of a minicorm (dipolar stage). Changes in an antioxidant capacity related to superoxide dismutase, catalase, ascorbate peroxidase, dehydroascorbic acid reductase, and glutathione reductase activities were also reported (Blazquez et al. 2009).

Data also suggested that many genes are differently expressed during embryogenesis (Kumar and Van Staden 2019). A group of receptor-like kinases (RLKs) which contain leucine-rich repeats (LRRs) in their extracellular domain regulates the signal transduction leading to the development of somatic embryos. The main group of RLKs includes leucine-rich-repeat RLKs (LRR-RLKs) (Tichtinsky et al. 2003). Somatic Embryogenesis Receptor-like Kinase

(SERK) gene belongs to the group of highly conserved leucine-rich receptor-like kinase II (LRRII-RLK) (Hecht et al. 2001). SERK plays a significant role among the genes engaged in plant embryogenesis and the early expression of the SERK gene is closely correlated to the formation of embryogenic cells in many plant species (Kumar and Van Staden 2019; Taheri-Dehkordi et al. 2020). SERK gene expression is considered a molecular marker of competent somatic embryogenic cells (Taheri-Dehkordi et al. 2020).

Cost effectiveness of in vitro culture mass production is very crucial and the cost of shoots, corms and cormlets production is high due to chemical components especially PGR and hand works. Therefore, alternative methods are required among them using robotic automated systems, using liquid or semi-liquid media, TDZ-free protocols and photoautotrphic micropropagation under high light and CO_2 intensity are the main alternatives to reduce the costs (Sharma and Piqueras 2010; Taheri-Dehkordi et al. 2020). To develop an efficient protocol with lower cost for in vitro propagation of *Crocus* species, 2,4-D, α-naphthalene acetic acid (NAA), and Picloram were used as auxin sources and cytokinins other than TDZ, i.e., N6-benzylaminopurine (BAP) and Kinetin (Kin) were used. It is noteworthy that BAP and Kin are around 30 times and 15 times cheaper than TDZ, respectively (Gantait and Vahedi 2015; Taheri-Dehkordi et al. 2020).

12.3.3.3 Gene Transformation and Genetic Improvement in Saffron

The main economically and medicinally important pigments of saffron are crocin, safranal, picrocrocin, and crocetin. Despite the high value of these compounds, there has been limited success in enhancing the content of these bioactive molecules commercially. Several efforts for genetic improvement through biotechnological approaches have been made in the past; however, limited success has been achieved with many reasons. In recent years, however, there was an increasing demand for tissue culture and genetic engineering for propagation and genetic improvement of saffron. Tissue culture is useful commercial approach for large-scale production of pathogen-free corms in relatively short period and space, mass selection of superior clones, and production of secondary metabolites (Plessner and Ziv 1999). Furthermore, tissue culture approaches can be used towards genetic modification by transferring genes, produce new plant species through fusing somatic cells (protoplast culture), crop improvement through somaclonal variations, and conservation of endangered and rare species (Vasil and Vasil 1979; Taheri-Dehkordi et al. 2020).

Plant protoplast (cells without cell wall) cultivation and regeneration is one of the effective approaches of cell culture that can be used to produce new somatic hybrid plant through fusing cells. Genetic modification using protoplast fusion can be performed in order to overcome challenges such as sexual incompatibility, multiembryo, and male and female sterility for intraspecific, interspecific, intrageneric, and intergeneric crosses (Crosser and Ollitrault Olivares-Fuster 2000). Limited success has been achieved using conventional breeding methods in a sterile plant-like saffron and hence protoplast fusion or mutagnesis may facilitate its breeding programs to improve its agricultural characteristics (Ahooran et al. 2009).

Isa et al. (1990) reported the first successful protoplast isolation from calli derived from apical bud and corms. A digestion mixture containing low concentration of pectinase, pectolyase, mannitol and cellulase R-10 and driselase derived from *Trichoderma viridae* and *Irpex lactes,* respectively, was used under dark condition. Protoplast immobilization in Ca-alginate beads, nurse culture, and plating density (5×10^4 protoplasts per mL) affected the growth and division of protoplasts (Isa et al. 1990). MS liquid medium containing pectolyase, cellulase, deriselase, and mannitol was used to separate saffron protoplast from callus (Noori-Daloii et al. 2000).

Karamian and Ebrahimzadeh (2001) designed a robust workflow of protoplast isolation to plantlet regeneration from protoplasts-derived embryogenic calli of *C. cancellatus*. A digestion mixture containing cellulase R-10, driselase,

pectinase, pectolyase, and mannitol was used to isolate protoplasts directly from embryogenic calli of *C. cancellatus* obtained from shoot meristem culture on LS medium containing 4 mg/L kinetin and 1 mg/L 2,4-D. Highly efficient growth of protoplasts was achieved when those embedded in Ca-alginate beads and cultured with nurse cells in MS medium containing 2 mg/L kinetin, 1 mg/L 2,4-D, and 100 mg/L ascorbic acid at 25 °C in darkness. Somatic embryos developed either on ½ MS free hormone or with 1 mg/L abscisic acid, germinated on ½ MS supplemented with 25 mg/L of GA3 and formed plantlets on ½ MS containing 1 mg/L BA and 1 mg/L NAA at 20 °C in a 16/8 h light/dark cycle (Ebrahimzadeh et al. 2000; Karamian and Ebrahimzadeh 2001).

Hasrak and Zarghami (2013) isolated high viable saffron protoplast from embryogenic calli were treated in the enzymatic mixture containing 0.1% pectolyase, 1% cellulase, 1% deriselase, 0.1% MES, and 0.3 M mannitol for 3 h. Langari-Sfezar (2017) used cellulase, pectinase, mannitol, and MES as enzyme extraction mixture for isolating protoplasts from leaf mesophyll and flowers of saffron. The petal protoplasts were then fused using 40% PEG.

Moradi et al. (2020) developed an in vitro system for metabolite production in saffron. Crocin was obtained in a cell suspension culture established from style-derived calli following the Salicylic acid elicitation. Despite cell growth inhibition, the highest SA concentration (1 mM) led to a sevenfold enhanced production of crocin and a fourfold increase of phenolics compared to mock cells. A robust transient gene transformation was developed to express the heterologous vacuolar markers RFP-SYP51, GFPgl133Chi, and AleuRFP in protoplasts derived from the saffron cell suspensions. SA application caused a rapid stress effect, leading to cell death (Fig. 12.4).

Development of a robust system for efficient callus production and somatic embryogenesis and development of an efficient transformation system are pre-requisite crucial steps towards gene transformation, gene and genome editing in saffron. Although there have been several successful reports of different tissue culture applications in saffron, however, there have been few successful reports of gene transformation in this plant. Saffron and perhaps other *Crocus* species are recalcitrant plants for genetic transformation and genetic improvement, largely due to difficulties in Agrobacterium-mediated transformation and vegetative reproduction (Chib et al. 2020). Enter into post-transformation generation, recently, a successful CRISPR/Cas9 system was developed as an attractive alternative for precise gene editing in saffron (Chib et al. 2020). Agrobacterium-mediated transformation resulted in the successful integration of the binary vector pYLCRISPR/Cas9 (modified pCAMBIA1300 backbone) into the somatic embryos of saffron with a transformation efficiency of 4%. This effort was an introduction to efficient gene knockout or edits of saffron in future.

12.4 Conclusion

Plant tissue culture is being widely used as a commercial tool for large-scale propagation of pathogen-free plant materials in short time period and space. These technologies included organogenesis, embryogenesis, SLS generation, cormogenesis, cell suspension culture, and protoplast culture, etc., also guarantee mass selection of superior clones, production of secondary metabolites (crocin, safranal, picrocrocin, and crocetin), genetic modification, gene and genome editing, crop improvement through somaclonal variations and conservation of endangered and rare species. Saffron is an economic sterile triploid plant that is only reproduced through vegetative methods but the rate of conventional propagation through the daughter corms is low. Due to global increasing demands for expanding saffron cultivation areas, the saffron industry is completely dependent on the development of efficient tissue culture methods to guarantee the mass production of uniform and disease-free cormlets and corms which leads to better flowering, higher yield, and adaptation to mechanization. Along with the development of efficient methods in saffron propagation and robust gene

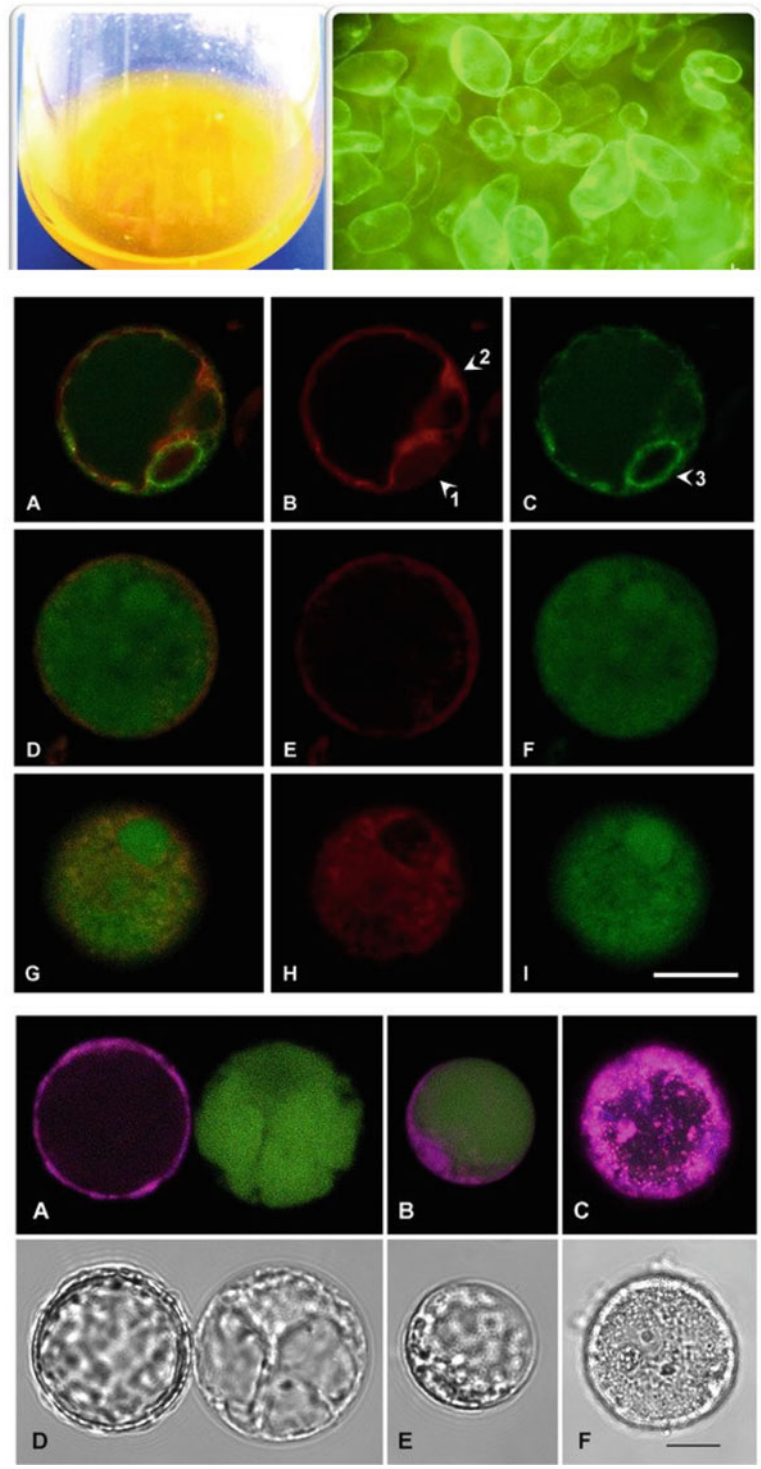

Fig. 12.4 In vitro system for metabolite production in saffron and a robust transient transformation of protoplasts with vacuolar markers. Saffron cell suspension cultures of style explants (**a**). **A–C** Protoplast expressing RFP-SYP51 and GFPgl133Chi. **D–F** Equatorial optical section of a protoplast expressing AleuRFP and GFPgl133Chi; **G–I** Polar optical section of the same protoplast shown in **D–F** and confirming the same distribution of fluorophores. Scale bar 20 μm (**b**). Autofluorescent protoplasts from saffron calli. **A** Two protoplasts showing opposite fluorescent patterns, on the left one with only orange-to-red emission (magenta color) in the cytosolic organelles (and little fluorescence in the central vacuole), the other on the right with only green emission in the central vacuole. **B** Protoplast from the same population with both emission patterns. **C** The most common fluorescent pattern showed by protoplasts treated with SA 1 mM. **D**, **E** Cells in control conditions do not show signs of stress. **F** SA treatment causes visible stress in a protoplast. Scale bar 10 μm (**c**). From Moradi et al. (2020)

transformation and gene/genome editing technologies such as CRISPR/Cas9, a promising prospect has been created for the genetic improvement of saffron in the near future.

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Saffron Production Under Controlled Conditions

13

Shirin Moradi

Abstract

Red gold is an appellation given to the red stigma of saffron, due to the importance and high value of this plant. This plant is not only the most expensive spice in the world, but also very effective in modern medicine for the treatment of diseases such as Alzheimer's, liver disease, and most importantly cancer. The quality of saffron stigma depends on environmental factors, nutrition, and post-harvest processes such as drying and storage conditions. Despite its value, saffron cultivation has not significantly changed since thousands of years ago. Today, saffron cultivation is facing challenges such as climate change, soil diseases, and labor shortages which reduce saffron production in the world. This chapter reviews studies on new methods of saffron production under controlled conditions to find solutions and meet challenges and help producers to increase healthy and disease-free products with the highest quality by using new cultivation methods.

13.1 Introduction

Saffron is an inestimable plant in the *Iridaceae* family, for thousands of years due to its red stigma is known as the most expensive spice in the world. Not only in cooking, but also in the pharmaceutical sector it is very valuable (Vahedi et al. 2014, 2018; Taheri-Dehkordi et al. 2020, 2021; Moradi et al. 2021, 2022). From 3600 years ago, the Greeks discovered the antibacterial properties of saffron and used it to treat eye inflammation and toothache. Ancient Romans also due to these properties used them to treat dry cough. In ancient Egypt, for the first time, saffron was used as a cosmetic product, Cleopatra used saffron and milk as a cosmetic combination to beautify and rejuvenate the skin. Saffron is known as a valuable plant not only in traditional medicine, but also in modern medicine since the great effect of saffron as an antidepressant and anti-inflammatory has been confirmed. In the last years, several pharmacological experiments and preclinic and clinical data have proven the anti-cancer properties of saffron (Lambrianidou et al. 2021).

Due to the high importance of saffron in cooking, industry, and medicine, the cultivation of this plant is always considered. This plant needs a dry and warm climate, to have the highest quality of flowers and corm, it is necessary to have hot summers and cold winters.

S. Moradi (✉)
Department of Horticultural Sciences, Faculty of Agricultural Science and Engineering, University of Tehran, Karaj, Iran
e-mail: shirinmoradi@alumni.ut.ac.ir

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Saffron is mainly cultivated in Iran, Spain, India, Greece, China, and Afghanistan.

Saffron is a self and out the sterile plant, several reports confirm that saffron is an autotriploid plant with gametophytic male sterility. That's why the only way saffron propagates is by vegetative propagation through the underground organs (Grilli Caiola 2004).

Saffron cultivation is facing a lot of challenges. First, the plant has a low multiplication rate, which is about 3–4 corms per year. The second problem is various diseases, including fungal diseases in quails and viral diseases that have been reported recently. Lack of soil nutrition and water competition are other problems in saffron cultivation (Chib et al. 2020). As a result, producers and researchers are looking for a way to increase plant growth rate and increase the quality of saffron flowers and corms by controlling environmental conditions.

13.2 In Vitro Culture

Micropropagation through in vitro culture is a good solution especially for slow-growing plants, in addition to increasing the production rate; it also increases the quantity and quality of production. Tissue culture also provides a way to perform modern plant breeding with the help of biotechnology, thereby increasing the speed of the breeding process.

13.2.1 Disinfection

There are many studies on in vitro cultivation of saffron, which are described below. But the most important and the first challenge in in vitro cultivation of monocotyledonous plants, especially plants that are propagated by underground parts, is contamination.

Corm, as the main producing organ in saffron, has main and lateral buds and has high reproductive power. As this plant is located under the soil, it is internally infected with different types of fungi, nematodes, and bacteria. As a result, eliminating this internal contamination and disinfection is one of the biggest problems in saffron in vitro production. For a successful tissue culture protocol, having proper disinfection instruction with the highest survival rate and the lowest contamination percentage is essential (Teixeira da Silva1a et al. 2016). There are many disinfectants with different instructions. Selecting the proper instructions with high effectiveness is dependent on the plant species, explant's tissue, its size and age, and even the environmental conditions from which the mother plant was obtained. In saffron tissue culture, different organs have been used as explants, including leaves, lateral buds, inflorescences, different parts of flowers, and corm, and for each organ, there are different disinfection procedures. The contamination and disinfection challenges of corms are higher than in other samples which is because the corms stay under the soil for years and the microorganisms easily move cell to cell by absorbing water and nutrients and enter the internal layer of corms. Many bacterial, fungal, and nematode contaminants and diseases have been reported in saffron, but the elimination of fungi contamination is the biggest problem in saffron tissue culture. Fifteen different species of fungi including *Aspergillus, Beauveria*, *Botrytis*, *Burkholderia*, *Gladioli*, *Cladosporium*, *Colchiobolus*, *Fusarium*, *Penicillium*, and *Rhizoctonia* have been extracted from saffron quails, which have been collected from different Asian and European regions (Ahrazem and Trapero 2010).

Many chemicals are used to disinfect the explants, among them sodium hypochlorite is the most common substance used for disinfection. In saffron, this substance has been effective in disinfecting various organs such as flowers and leaves, but in the corm explant, the use of sodium hypochlorite alone has not had an effect on eliminating endogenous contamination (Taheri-Dehkordi et al. 2020). Mercury chloride ($HgCl_2$) is known as the strongest disinfectant in plant tissue culture. There are many reports about its positive effect on eliminating corm's endogenous contamination in different species of saffron from 01 to 2% (w/v). High concentration of sodium hypochlorite (>50%) and mercuric chloride

(>1.6%) have a negative effect on the survival rate of plants and causes yellowing (Salwee and Nehvi 2014). However, due to its high toxicity and environmental pollution, the use of this substance is not recommended. Benzalkonium chloride is another substance that has been reported to disinfect corms in combination with ethanol and sodium hypochlorite, which has had a positive effect on removing contamination (Sharifi et al. 2012). Sulfuric acid was even used to disinfect the corms, but this reduced the plant's viability (Karaoglu et al. 2007). Some reports have suggested that the positive effects of fungicides, especially systemic fungicides, are appropriate to eliminate endogenous corm contamination in combination with other substances (Yasmin and Nehvi 2014; Taheri-Dehkordi et al. 2020).

There are a variety of antibiotics used to eliminate endogenous bacteria and fungi in culture media. So far, no study has been reported on the use of antibiotics to eliminate endogenous contamination.

Other methods have been utilized to reduce the usage of hazardous toxic substances, including the use of high and low temperatures, keeping quails for some days at 4 °C had a positive effect on the elimination of endogenous contaminants, in contrast to the use of high temperatures did not have a positive effect on reducing contamination in the in vitro culture. However, still, the use of fungicides and Mercury for the removal of endogenous infection is very common. It is hoped that in further researches, the use of low-toxicity and effective substances will replace these dangerous substances to disinfect corms.

13.2.2 Somatic Embryogenesis

Somatic embryogenesis is the process in which the embryos are formed from an origin other than the zygote (somatic cells). Somatic embryogenesis as an efficient tool has facilitated various processes such as large-scale cloning, biotechnology and modern breeding, gene transfer, and the production of synthetic seeds. Various factors such as explant type, age, orientation, growth regulators, additive components, environmental conditions, and induced stress affect somatic embryogenesis (Gantait and Vahedi 2015). In the case of saffron and other *Crocus* species, various explants have been used for obtaining somatic embryogenesis including flower parts, leave segments, and corms. Among the explants, corm has the highest potential for somatic embryogenesis compared to other explants due to having main and many lateral buds as well as the high amount of starch as a source of energy and availability throughout the year. After corm, the second suitable explant for somatic embryogenesis is the leaf's basal part. This explant has up to 70% callus formation per explant, however, its potential of production is less than the corm, but leaf callus formation is faster than that of corm (Verma et al. 2016). The use of a suitable culture medium with different concentrations of salt and vitamins is another important factor that affects embryogenesis. Different culture media have been used to induce direct and indirect embryogenesis in saffron such as MS,1/2 MS, LS, B5, CHE, GB5, Nitsch, and Nich (Verma et al. 2016; Raja et al. 2007). Among them, the MS culture medium has been reported as the most efficient culture medium in most studies. One of the important factors in inducing embryogenesis is the use of a combination of cytokines and auxins in different ratios (Raja et al. 2007; Gantait and Vahedi 2015; Verma et al. 2016). Cytokinins are very important because of their direct effect on cell division and cell development. Thidiazuron (TDZ) has a positive effect on cell development due to its effect on increasing the production and collection of high levels of purine in the cell. It also converts adenine to adenosine, a process that is very effective in cell division and increased protein synthesis. As a result, this cytokinin is the strongest hormone for inducing direct and indirect embryogenesis in a variety of microsamples (Parray et al. 2012; Gantait and Vahedi 2015; Verma et al. 2016; Chib et al. 2020). The use of this chemical in MS medium induces direct embryogenesis from corm explants, also with the help of various concentrations of TDZ embryogenesis from the leaf in saffron is possible. But this material is very expensive, and therefore, for

industrial production and low-cost culture medium should be looking for a suitable alternative. The second strongest cytokine is benzyl adenine (BA), and many reports have suggested that the use of this hormone, along with auxins such as 2,4-Dichlorophenoxyacetic acid (2,4-D), is very effective in producing saffron by direct somatic embryogenesis (Taheri-Dehkordi et al. 2020). In addition to various types of auxin and cytokinin, other plant growth regulators are effective in the process of somatic embryogenesis. An example is abscisic acid (ABA), some studies have proven that it is effective on somatic embryogenesis in two ways, firstly, by inducing auxin production, it induces embryogenesis, and secondly, by increasing the osmotic potential, it affects the development of somatic embryos. The positive effect of ABA on the development of saffron somatic embryos has been reported (Vahedi et al. 2015). GA is another regulator that is used for the germination of somatic embryos in saffron. The next stage after embryo production is the development and germination of the embryo (Ebrahimzadeh et al. 2000a; Karamian 2004). Induced stress is one of the methods used for embryo development. One of these techniques is osmotic stress which by increasing the osmotic pressure increases the accumulation of osmolytes in embryonic cells and causes maturation in embryos. Various osmotic are used for this stress, for example, growth regulators such as ABA, Paclobutrazol (PBZ), and Ancymidol which are often used as growth retardants, have a positive effect on the maturity of the embryo (Devi et al. 2011). Polyethylene glycol (PEG) is an osmotic agent that induces maturity in the somatic embryo and promotes the germination of somatic embryos (Vahedi et al. 2015). Carbohydrates, especially sugars, are the main source of energy. The use of sugars not only increases the induction of embryogenic callus production, but also increases the accumulation of substances in the cells, causing osmotic pressure and inducing maturity in embryos. Numerous studies have reported the positive effect of high sucrose concentration in culture medium on embryonic development and embryo germination (Karaoglu et al. 2007; Parray et al. 2012).

Additive compounds is another factor that affects embryogenesis. For example, the use of trace elements such as La^{3+}, Ce^{3+}, Nd^{3+} and their positive effects of different concentrations on callus formation and production of crocin were studied (Chen et al. 2004). They indicated that the highest amount of crocin production was observed in callus and the highest amount of callus production was observed in the treatment that contained La^{3+} (60 μM), Ce^{3+} (20 μM).

Other factors also affect embryogenesis, such as light and temperature. One of the reasons for the success of in vitro cultivation and increasing the growth rate of plants in this method is the production of plants in completely controlled conditions. Conditions from the culture medium to environmental conditions such as temperature and light are under control. One of the important factors in embryogenesis is light. Many studies have been done on this factor. Most reports suggest the use of darkness to induce callus formation. But light is essential for embryo induction and organogenesis. Most studies have suggested the use of 50–70 μmol m^2 s^{-1} light intensity and photoperiod of 16 h/8 h light/dark for induction of embryos and organ production (Devi et al. 2011; Verma et al. 2016; Taheri-Dehkordi et al. 2020).

13.2.3 In Vitro Corm Acclimatization

There are several ways to produce corm in both direct and indirect methods in the in vitro culture. In the indirect method, corm is produced through embryogenic callus our somatic embryos. One of the most important problems of this method is to create mutations during the callus formation processes.

But in the direct method, cormlets directly were produced from the corm buds. For this purpose, the producers culture the buds in multiplication culture media that mostly contain high levels of cytokinin and low concentration of auxins, then after the production of some shoots from one bud, they try to increase the concentration of carbohydrates to make the big-size corms. There are some successful protocols from

bud culture to acclimatization and flowering in the greenhouse (Parray et al. 2012).

The first study on the growth quality of in vitro cormlet in the greenhouse condition was done by Devi et al. (2011). They used the soil culture system in 20–30 °C temperature and 70% relative humidity condition, they reported up to 90% successful sprouting of corms.

In another study, in vitro corms that were obtained from somatic embryos were transferred to greenhouse conditions (20 °C temperature and 70% relative humidity). They reported 25% flowering and 55% vegetative growth of corms with a 2.5 g mean weight. They also reported high quality of stigma derived from these corms which reveals the success of in vitro production of saffron in this study (Parray et al. 2012).

13.2.4 Interspecific Hybridization

As mentioned earlier, saffron is propagated vegetatively due to problems such as triploid and male infertility; as a result, it has a very low diversity. Interspecific hybridization is a way to generate variability and transfer good traits to intended types.

For these purposes, first study on in vitro interspecific hybridization was done by Chichiricco et al. (1985). They reported the low pollen germination and abnormality of pollen as the reason that caused sterility. However, further studies reported the successful in vitro crossing and seed production of *Crocus sativus* with *Crocus cartwrightianus*, *Crocus hadriaticus, C. cartwrightianus,* and *Crocus Thomasii* (Chichirico 1996; Grilli Caiola et al. 2001, 2004).

13.2.5 Production of Saffron Metabolites in Vitro

Stigma is the harvestable part of saffron that is used as the spice and contains high levels of secondary metabolites compared to other flower organs, it yields 80 mg per flower, which means, for 1 kg of dry saffron, 150,000 flowers are needed. As a result, due to the low amount of stigma production, the researchers looked for a way to produce secondary metabolites in vitro. Many studies have been performed on the production of stigma-like structures since 1987. Different culture media were used in different studies, including LS, N6, B5, MS, and G5. Among them, the highest percentage of production was observed in the LS and G5 culture media (Mir et al. 2010).

Various flower organs have been used to produce a stigma-like structure in various studies, among which stigma and ovary explants have shown the best results, however, Zhao et al. (2001) reported that stigma-like structures obtained from the stamen explants could produce more secondary metabolites than stigma-like structures obtained from other flower organs.

Various hormonal compounds have also been used to produce this structure. Most reports have suggested a positive effect of the combination of BA as cytokinin and NAA as auxin. However, Ebrahimzadeh et al. (2000b) reported the use of Kinetin and NAA as the best treatment to produce a stigma-like structure in MS culture medium with 17% frequency.

The highest percentage of stigma-like structure formation per sample was reported by Mir et al. (2010) with 60% SLS production from ovary explants.

Recently, Moradi et al. (2020) reported the positive effect of salicylic acid on increasing saffron secondary metabolites in suspension-cultured cells.

In the last study, researchers were able to co-express the genes that have an important role in the production of saffron apocarotenoids in *Nicotina benthamiana* plant, using viral clones derived method. With this method, they were able to increase the accumulation of crocin (0.2%) and picrocrocin (0.8%) in the dry leaves weight of this plant just in 2 weeks. They suggested this method may be an attractive and fast way to produce saffron apocarotenoid in a cheap and sustainable way compared to the traditional methods (Marti et al. 2020).

13.3 Saffron Production Under Control Condition Ex Vitro (Greenhouse)

While there are many successful reports of saffron in vitro cultivation, this method still faces problems such as endogenous contamination of samples and the use of highly toxic substances to disinfect and eliminate this contamination. As a result, due to the importance of the environment, this method faces the challenge of reducing the use of toxic substances. Of course, a lot of research and studies are being done to solve this problem and improve production protocols.

Greenhouse cultivation has long been the focus of attention due to its many benefits such as increased yield, increased flowering period, and continuous and sustainable production. In recent years, saffron cultivation in greenhouses has been very popular. This method not only increases the cultivation per unit area and increases the yield, but also increases the quality of saffron produced in the greenhouse due to control of environmental conditions.

In addition, this method can be an effective solution to the negative effects of climate change such as water deficit, reducing the number of fertile lands, and high temperatures.

13.3.1 Cultivation Methods

Saffron production in the greenhouse is done in both soil and soilless methods, each of which is explained below.

- **Soil cultivation**

The production of saffron in the soil is very similar to its traditional field cultivation. But it has some benefits including controlling environmental conditions, controlling diseases and weeds, as well as better water management, and reducing water wastage. Some use only loamy soil as a substrate. Using a combination of sand, soil, and farm yard manure with a ratio of 1:1:1, with chemical fertilizer, was proposed as a suitable method for flowering and production of corm in the greenhouse (Devi et al. 2011). In general, the production of soil-based greenhouse saffron is more expensive than field cultivation, but this method makes it possible to control salinity, irrigation, and disease in this system.

- **Soilless cultivation**

Due to many problems caused by climate change, it has reduced the quality of saffron produced on farms. It has also become impossible to mechanize the production method due to the traditional cultivation system. For this reason, a lot of money is spent on human hand labor for picking up the flowers and separating stigmas. A lot of research has been done to shift cultivation from farm to greenhouse and create conditions for mechanization of saffron production (Molina et al. 2004, 2005a, b; Maggio et al. 2006; Gresta et al. 2017).

- **Hydroponic**

In the last 15 years, there are many reports on saffron production in a hydroponic system with different substrates (Molina et al. 2004, 2005a, b; Maggio et al. 2006; Gresta et al. 2017; Mollafilabi et al. 2017).

The first study on saffron production was done after 90 days of corms incubation during the dormant time (mid-Jun–mid-September) in dark with high-temperature conditions. Incubated corms were then transferred to the trays with rock wool as substrate. They reported the successful protocol for forcing the corms and increasing the flowering period time for 1–2 months (Molina et al. 2004, 2005a). In another study, Maggio et al. (2006) followed the same protocol of incubation and forcing of corms but with different substrates. They tried to investigate the effect of perlite compared with a 1:1 (v/v) mix of peat and perlite and vermiculite on the saffron plants' characteristics grown under greenhouse conditions. The results indicated that the flower number and the stigma quality in vermiculate were more than in other treatments. They mentioned

that choosing a suitable substrate is very important to get high-quality stigma.

The highest saffron production in the study that compare the saffron production in rock wool and peat moss as substrates in hydroponic system, was observed in peat moss treatment by 5.28 g^2 that was 11 times more than field production (Mollafilabi et al. 2017).

- **Aeroponic**

The aeroponic system is another soilless culture used in saffron greenhouse production. In the first study, saffron production and quality were investigated by 3 different methods: soil-based culture, hydroponic, and aeroponic systems. They reported the highest biomass and root development in the aeroponic system compared to other methods. But the results didn't show a significant difference in the quality of stigma in all treatments.

In the last years, the specific method of saffron culture is very common that some studies named this method kind of Aeroponic but some named that method a fogoponic system. In the new study, the flowering and corm production of the hydroponic system with the substrate and this kind of method without substrate was investigated. For this purpose, they chose 2 different corm weights and incubate corm in the growth chamber according to Molina et al. (2004), then they transferred the corms and investigated in with and without substrate system at 17 °C temperature and 67% relative humidity condition. They reported that the number of flowers didn't show a significant difference but the quality of stigma was more in non-substrate treatment compared to other treatments (Poggi et al. 2017).

Although there are many reports that confirm high-quality saffron production in soilless systems, especially the aeroponic system, the production of corms in this system was not successful and the weight of corms produced in the greenhouse was less than the corms in the field (Maggio et al. 2006; Molina et al. 2010; Renau-Morata et al. 2012; Poggi et al. 2017).

Accordingly, some studies suggested the handing combination system between the greenhouse and field (Fig. 13.1). They proposed that it is better to incubate the dormant corm in the greenhouse until the end of flowering and after flowering, the corms were transferred to field conditions for corm production. In this method, both high-quality saffron and big-size replacement corm were obtained (Renau-Morata et al. 2012; Poggi et al. 2017).

13.3.2 Nutrition Management

The soil-based greenhouse cultivation, as mentioned earlier, has many similarities with the traditional system. As a result, the proposed fertilizers in the traditional system can also be used in this method. Organic fertilizers not only improve the quality of saffron stigma, but also increase the corm and stigma yield, therefore, most of the studies suggested their use in the saffron production process. Usage of 40–60 tons of cow manure per hectare, depending on soil characteristics, was proposed to improve soil quality with high yield in the flowering stage (Askari-Khorasgani and Pessarakli 2019).

- **Fertigation**

In soilless systems, the use of a nutrient solution called fertigation is common. Most studies on the production of saffron in a soilless system have suggested a 1/2 Hoagland solution to obtain high-quality flowers and corm (Molina et al. 2004; 2005b; Maggio et al. 2006; Gresta et al. 2017; Mollafilabi et al. 2017).

In one study, they used the half-strength Otsuka-A prescription as the main nutrition solution in saffron production in the greenhouse condition (Kajikawa et al. 2018).

In a system without a culture medium, the plant is not given nutrient solution during the flowering period and the plant uses only the nutrients stored in the corm. After flowering and soil transfer, fertilization is done like soil-based systems.

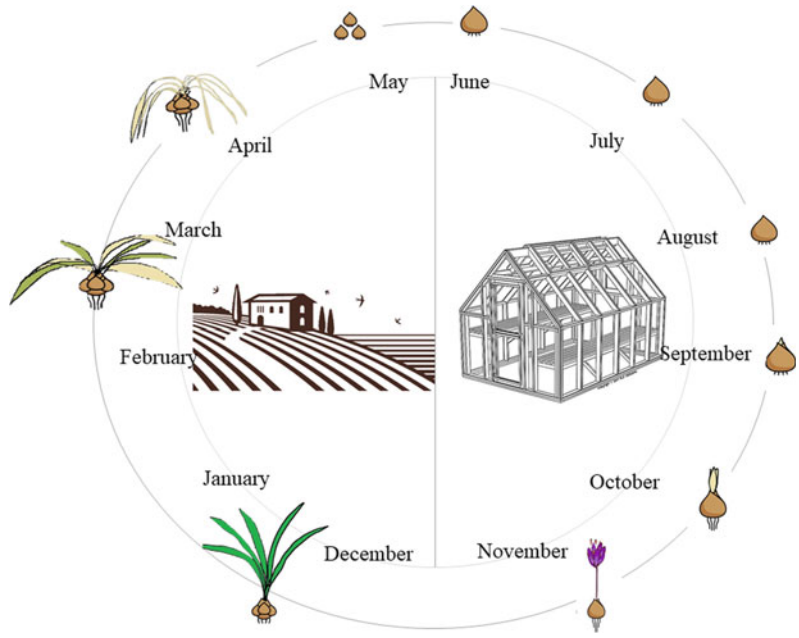

Fig. 13.1 Saffron life cycle in handing combination system between the greenhouse and field, Saffron growth stages which growing under control condition (right), saffron growth stages growing in the field (left)

Spraying saffron leaves with complete fertilizer and some promoters like amino acid, fulvic acid, and humic acid in March and late winter was proposed by previous studies due to a positive effect on saffron yield and quality (Askari-Khorasgani and Pessarakli 2019).

- **Bio-fertigation**

There is a high diversity of microorganisms in the soil, using the materials containing living microorganisms that make the useful relationship with their host that named Bio-fertigation. Rhizobacteria are a group of these useful microbes that improve the growth quality of their host and live in rhizospheres. In one study, 100 rhizobacteria were isolated from the rhizosphere of saffron at the flowering time. After isolating, they screened these rhizobacteria, and finally, they tested the promoting effect of these on plant growth. They reported six different plant growth-promoting rhizobacteria (PGPR) that could be used as biofertilizers (Ambardar and Vakhlu 2013). Using *Curtobacterium herbarum* Cs10 as a biofertilizer, increased the number of flowers and length of stigma in the saffron plant (Díez-Méndez and Rivas 2017).

Increasing the amount of saffron secondary metabolites (safranal, crocin, and picrocrocin) by more than 70% compared to the control was caused by using the combination of manure and vermicompost with the *Azotobacter sp. PTCC 1658* + *B. subtilis* + *P. aeruginosa* as biofertilizers (Alizadeh et al. 2019).

13.3.3 Water Availability

With a water requirement of less than 400 mm rainfalls in the year, saffron is grown in an arid or semi-arid area. After leaf drying, dormancy begins in saffron. During the dormancy period, saffron corm needs dry condition and doesn't need any irrigation. The first irrigation is done in the mid of September. Water availability is very important in photosynthetic performance. The water stress in vegetative stage of saffron plant decreased photosynthetic rate (Renau-Morata et al. 2012).

In the last study, investigating the effect of different irrigation method on saffron yield and quality, indicate that drip system by producing maximum stigma dry weight in all treatment and high-water efficiency was the best method for

saffron irrigation. However, a high percentage of crocin was observed in the sprinkler method (Mollafilabi et al. 2017).

13.3.4 Drought and Salt Stress Under Greenhouse Culture

Drought stress in the vegetative and flowering stages of the saffron plant affects all morphological and physiological characteristics. It makes a difference in foliar shape, decreases the chlorophyll content, and decreases saffron stigma yield (Mzabri et al. 2017).

Because saffron is produced in arid and semi-arid regions, it must face salinity. Therefore, in a study, the effect of different salinity concentrations on the growth characteristics of saffron was investigated. They reported the significant effect of salinity on all saffron characteristics. The results showed that salinity decreases the plant height and flower number, but increases the flower's fresh weight. The threshold concentration of salinity on stigma yield was 50 Mm NaCl (Eskandari Torbaghan et al. 2011).

13.3.5 Environmental Condition Control

Saffron is a triploid geophyte plant that can only be propagated vegetatively. All developmental stages, especially the stage of corm production that is critical for the next yield production, are strongly dependent on environmental factors such as temperature, light, humidity, etc.

Saffron life cycle has six different stages phonologically contain sprouting, cataphylls and flowers appearance, leave appearance and development, replacement corms development, plant senescence and dormancy, and three crop management periods: dormancy, flowering, and vegetative growth (leaf and replacement corm development). All of these stages and periods need specific environmental condition.

- **Temperature**

  Temperature is an important environmental factor that has a key role in geophyte growth stages, most geophytes follow an alternation between cold and warm temperatures for vegetative, reproductive, and flowering stages. Saffron also is one of them that needs a warm-cold-warm sequence for a successful life cycle. As mentioned previously, all saffron stages need specific environmental conditions.

  – Effect of temperature on corm storage and dormancy

  For dormancy period, saffron requires high temperature, but it is important to know that the extremely high temperature or long duration delays flowering in the saffron plant (Molina et al. 2004). The optimal temperature for breaking dormancy and flower initiation is 24–27 °C for 90–115 days. The temperature higher than this range or longer duration delay flowering (Molina et al. 2004, 2005a, b, 2010). Using low temperature for breaking dormancy of promoting flower formation decreases the plant growth and delay flowering and storage at cold weather for long time reduce the quality of stigma (Molina et al. 2004).

  – Temperature and flower induction and development

  Flower initiation that occurs inside the corm during the dormancy period needs a high temperature (24–27 °C), but for flower emergence, it needs a low temperature of about 16–17 °C, normally the flowering time is from mid-October to mid-November depending on climate. Cold storage requires the optimal condition such as atmosphere, the stage of flower initiation, temperature degree, and duration of storage, if the optimal conditions don't properly well, the flower abortion and increase in flower size are accrued (Molina et al. 2005a, b).

  By using different techniques and control conditions, the saffron flower period could be

extended from early September to the end of March with high quality of stigma production. For this purpose, it would be possible to lift the corm from the field two months before the leaf withering and then incubate at 25 °C for 60–150 days in dark conditions. For flower emergence, it just needed to transfer the batch of corms to 17 °C temperature under light conditions. The 60–150 days corm incubation doesn't show a significant difference in stigma yield and quality. So, it has been suggested to put the corm batch in flower emergence condition gradually. With this protocol, it is possible to extend the flowering period and also the transferring of the batch could be done by Machin (Molina et al. 2004).

– Saffron vegetative growth and daughter corm development on the effect of temperature
  After flowering, the leaf appears on corms and vegetative growth starts. Vegetative growth it's a key stage for saffron production because corm replacement development occurs in this stage. The weight and size of the daughter corm (replacement corm) are very critical because they guarantee next year's saffron yield. A variety of air temperatures in day and night conditions were investigated to find the best temperature for daughter corm development. The highest weight of daughter corm was reported at 15/6 °C day/night temperature (Kajikawa et al. 2018).
– Carotenoid biosynthesis and temperature
  Saffron has three important secondary metabolites crocin (color), picrocrocin (test), and safranal (aroma) all of these are apocarotenoid compounds and determine the quality and price of saffron dry stigma. The temperature has a critical role in the production, harvest, and post-harvest period. As mentioned previously long tome incubation at high temperature our storage the corms at low temperature affect the amount of secondary metabolites in saffron stigma. For dry temperature, it has proposed the fast and high temperature, using the oven at 40–60 °C for 30–50 min showed increasing in crocin content (ISO 36-32, Askari-Khorasgani and Pessarakli 2019).

- Light
  Light is another environmental factor that plays an important role in whole plant growth stages. Not only it is the main source of energy for photosynthesis, but also acts as a key environmental signal. Light influences different processes in plant growth such as germination, seasonal detecting, growth habits, and transition between vegetative and flowering. The light could affect plant growth in three aspects: intensity, duration, and quality.

– Light intensity and saffron life cycle
  In the first study on saffron production in the greenhouse, it was suggested to use artificial light intensity in the range of 1000–1500 l× to get well-developed plants (Jerzy 1981). In another research, the results didn't show a significant difference between 1000 and 200 l× intensity for saffron forcing, due to these decreasing the energy cost they proposed use of low intensity for saffron forcing (Jerzy 1981). In the soilless production of saffron, the 20–50 μmol m^{-2} s^{-1} light intensity was used for all stages after dormancy which needs dark conditions (Molina et al. 2004, 2005a, b; Maggio et al. 2006; Gresta et al. 2017).
  The plant grown in a greenhouse in this light intensity exhibited low irradiance characteristics and low photosynthetic performance, accordingly, the daughter corm produced in this condition didn't have high quality compared to field product corms (Renau-Morata et al. 2012).
  In the new research, the 250 μmol m^{-2} s^{-1} light intensity was suggested for the daughter corm development stage in the hydroponic culture system (Kajikawa et al. 2018).
– Light duration in different stages of saffron growth
  Saffron is a short-day plant for the flowering stage, it needs 8/16 h day/night for flower emergence, also in this photoperiod, the aerial biomass is significantly higher than in shorter

photoperiod hours. But for daughter corm formation and development, the 11/13 h of day and night were suggested (Kajikawa et al. 2018).

- Light quality and its effects on saffron production

  Light quality is spectral energy distribution, refers to the composition of light as to wavelengths, it contains the big range of spectrum with low frequency (radio wave) to high frequency (gamma ray), the visible light is the range of spectrum between 400 and 700 nm that is important for plant photosynthesis and called 'Photosynthetic active radiation (PAR)'. The different light spectrum has a specific photoreceptor.

In the last years, the study of different effects of light spectra on plant growth processes has become an interesting issue, especially for vertical farming.

The effect of different Red (R)/Far-red (FR) ratios on vegetative and flowering stages in the soilless saffron production was investigated. The results showed that a low ratio of R/FR promotes the translocation of photosynthetic products from source to sink (Kajikawa et al. 2018).

In the last research, the effect of the different light spectrum (different ratios of blue (B) to red (R) light) on photosynthetic performance, morphological, biochemical, and molecular characteristics of the saffron plant in both flowering and vegetative stages were investigated (Moradi et al. 2021). The results showed a significant difference in the effect of R and B light on vegetative growth, especially on morphological characteristics. Compact plants with good shape leaves were obtained from B light treatment, in contrast, R light made plants with narrow leaves and close cataphylls (Fig. 13.2). They suggested this compactness in plants, grown under blue light, maybe due to the result of cryptochromes activities that inhibit hypocotyl cell elongation and increase cell enlargement by regulating the biosynthesis and transport of endogenous auxins and their analogs.

In the daughter corm developmental stage, light spectra showed a significant difference. Plants that grown under B light made big daughter corms with good quality and round shape but in low number (1–2 daughter corm per corm), in contrast, plant grown in R light condition made a high number of small daughter corm, the results showed that by increasing the B to R light ratio, the size of daughter corms was increased, but the number of them was decreased (Fig. 13.3). The results of the biomass partitioning of their study indicated that biomass was mostly partitioned to the underground parts such as root and daughter corms in the plants grown under high proportion B light. It seems that B light promotes apical dominance in corm buds and inhibits the growth of lateral buds by regulating auxin, cytokinin, and strigolactone level in the sink and source parts (Moradi et al. 2021).

In the secondary metabolite aspect, the highest quality of stigma was recorded in monochromatic light treatments (R and B light). R light showed the highest concentration of crocin, picrocrocin, and safranal compared to different ratios of R and B light treatments (Moradi et al. 2021, 2022).

13.3.6 CO_2 and Saffron Production

The CO_2 concentration in the atmosphere is about 400 μmol mol^{-1}, the plant can absorb CO_2 from the air but due to its critical role in photosynthesis, CO_2 fertilization is widely used for enhancing photosynthesis and increasing the yield.

In saffron production also CO_2 enrichment was suggested in many studies. Using CO_2 at 600 ppm showed the best photosynthetic performance and high quality of saffron production in the greenhouse (Molina et al. 2004, 2005a, b; Maggio et al. 2006; Gresta et al. 2017).

13.3.7 Technology Progress and Mechanization of Saffron Production

Saffron is a very expensive and valuable spice and its production frequency is very low (on

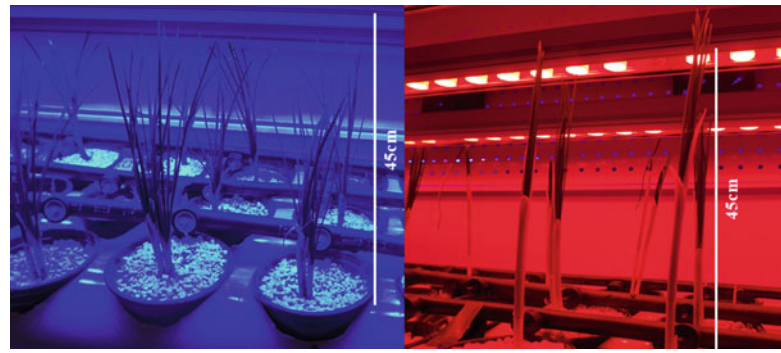

Fig. 13.2 Leaf shape of saffron plant grown under Red (R) and Blue (B) lights 40 days after flowering. The white bars show the distance between the pot and LED lamps (Moradi et al. 2021)

Fig. 13.3 Daughter corm of saffron plant grown under different ratios of Blue (B) to Red (R) lights 120 days after flowering. In each treatment, the new corms are arranged based on their weight from largest to smallest together with the last one, which is the remaining of the mother corm (Moradi et al. 2021)

average about ten kilograms of dried saffron per hectare after the second or third year of planting). The process of picking up saffron flowers and separating the stigma from the flowers is the most difficult part of the harvest, which unfortunately is still done manually with a lot of hand labors. This not only greatly increases the cost of production, but also reduces the quality of saffron due to the elimination of volatiles during the process of time harvesting by hand. Unfortunately, due to the traditional production system, the mechanization of the production and harvesting pathway has not yet been successfully completed. In recent years, due to efforts for shifting saffron cultivation from farm to greenhouse, there is hope for the possibility of creating a fully mechanized route from production to harvest in this system. In recent years, however, a number of machines have been produced separately on the farm. These include the following machines.

Corm digging machine: After 5–6 years of saffron production in the field due to the high density of corm and weak performance of the soil, the yield is decreased. For this reason, the corm should be lifted from the soil and transferred to a new field. This machine lifts the corm from the underground and separates the corm and soil.

Corm sorting machine: The size of the corm is critical for determining the yield for next year. So the specific machine like this, that grade and sort the corms according to weight or diameter is required.

Planting machine: There are three different types of planting machines including traditional or old planting machine that just make the hole or farrow for planting and hand labor needed for planting the corm. Semi-automatic machine that has the set for hand labor make the hole or farrow for planting the human part plants the corm and the other part of machine covers the corms. Automatic type in this machine all stages of planning process are done automatically.

Harvesting machine: There are also two types of harvesting machines. The first machine has a sit and flowers are picked up manually along the rows. The second one is a portable saffron harvesting device which the operator holds in a standing position and picks flowers using cutting fingers and then transfers them into the back-carried container by a suction mechanism.

Separator machine: Also, there are traditional and automatic types. In traditional type machine contain of different part such as container- shaker- cylindrical picker and vacuum pump. This machine separates the stigma from the flower by shaking and different weight of flower and stigma. The automatic type works based on image processing that first finds the right spot for cutting then cuts the stigmas and separate different part of flower (Saeidirad 2020).

In the last research, the computer-based greenhouse with automatic production processes from planting to packaging was proposed. But for this idea, some physiological and biological researches on saffron plant are required for synchronizing flowering time, obtaining uniform plants with the same height, and extending flowering time around the year (Perez-Vidal and Gracia 2020).

13.4 Conclusion

Red gold is the name of the saffron dry stigma that represents the importance of this plant in cooking, medicine cosmetic, and industry. It's the sterile geophyte that propagates by corm. The quantity and quality of production of this plant are strongly dependent on environmental factors. The production of this plant in completely controlled conditions to reduce contamination, increase production per area, increase product quality, and mechanization of the production route has been considered in recent years. Saffron production in controlled conditions is done in different ways, which are described in this chapter.

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Part V
Saffron Metabolomics

Saffron: Metabolomics and Quality Point of View

14

Mitra Aelaei and Fahimeh Salehi

Abstract

Plant secondary metabolites are absolutely diverse categories of organic compounds that possessed low molecular weight, display regulatory functions, and are synthesized within specific cells. A growing body of evidence suggests that environmental, physiological, and genetic factors can affect the biosynthesis of secondary metabolites. A considerable proportion of the global markets of medicinal plants deals with secondary metabolites, so plant metabolites have a remarkable economical value. Active ingredients in medicinal plants and spices can be used as medicines, and flavoring, fragrance, and pigment ingredients in different industries. Secondary metabolites not only protect saffron plant under biotic and abiotic stresses, but also consider as valuable natural chemical compounds which affect human health as food or medicine. Secondary metabolites of saffron stigma are mainly included crocin (color), picrocrocin (flavor) and safranal (odor/aroma). In addition to use in food and cosmetic industries, saffron also has several pharmacological effects and it is considered as an excellent medical cure for some disorders and diseases. These secondary metabolites determine the quality of saffron products from stigma to any other finished products. Saffron is used as a facilitator, appetite suppressant, sedative, diaphoretic, and expectorant and for the treatment of liver and gallbladder disorders, toothache, cardiovascular disorders, and cancer. However, one of the major challenges in the relevant industries is achieving maximum extraction efficiency of compounds. Metabolomics defines as comprehensive high-throughput analyses of metabolomes and includes tools and approaches for the separation, characterization and quantification of metabolites. There are different methods for extracting and purifying metabolites of saffron, classified into two categories, traditional and novel approaches. Novel method such as supercritical fluid is widely used due to applying low temperature and short time as well as higher extraction efficiency. Different High-performance liquid chromatography (HPLC) methods are used as a fast and robust approach to determine the secondary metabolites of saffron.

14.1 Chemical Compounds in Saffron

Saffron (*Crocus sativus* L.), the highest-value spice, has widespread use in coloring, perfumery, flavoring, and pharmaceutical industries (Vahedi

M. Aelaei (✉) · F. Salehi
Department of Horticulture, Faculty of Agriculture, University of Zanjan, 45371-38791 Zanjan, Iran
e-mail: maelaei@znu.ac.ir

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et al. 2018; Taheri-Dehkordi et al. 2020, 2021; Moradi et al. 2021, 2022). During the last decade, studies on saffron identified more than 150 volatile and non-volatile compounds in this plant. Among them the most important secondary metabolites are crocins, a family of yellow pigments, picrocrocin, a colorless, bitter glycoside, and safranal, a volatile oil responsible for the smell and aroma of saffron. Most of these metabolites are present in saffron stigma (Fig. 14.1) (Haghighi et al. 2007; Amin and Hosseinzadeh 2012). This plant also contains ten to twelve percent water, five to seven percent minerals, a small amount of carbohydrates, five to eight percent fat and wax (Nair et al. 1995), proteins, sugars, vitamins, flavonoids and phenolics with antioxidant activity, amino acids, minerals, gums, and other chemical compounds. On the other hand, this plant contains active and inactive substances from carotenoid compounds including xanthine, lycopene, and various types of alpha and beta carotenes (Haghighi et al. 2007; Goli et al. 2012).

The highest levels of compounds in saffron include sis and trans apocrocinoids, crosin, picrocrocine-beta-diclucopyranoside-hydroxyl-beta-cyclositral (Kanakis et al. 2004). In fact, apocarotenoids are derived from oxidative breakdowns of C40 carotenoids and occur in saffron flowers, especially in stigma chromoplasts (Gomez-Gomez et al. 2010). During recent studies, variouscompounds of volatile substances have been identified in stigma tissues (Rubio et al. 2008), shown that destructive processes on apocartenoids cause the creation of aromatic characteristics and finally the presence of safranal in saffron. (Dauria et al. 2006). Carotenoids have different characteristics that attract the attention of metabolic engineers, including the fact that carotenoids are responsible for color in flowers and fruits and also have antioxidant properties (Gomez-Gomez et al. 2010).

14.2 Color in Saffron

Crocin is the main reason of specific color of saffron stigma (Caballero-Ortega et al. 2007), which is a glycoside compound that contains two gentobiose molecules (Fatemi 2001) and its derivative, which is an aglycone called crustin (Figs. 14.2 and 14.3). Crocin is one of the 20 naturally occurring dicarboxylic carotenoids in nature that is readily soluble in water and is found as estroglucosyl in the stigma of saffron and the fruits of gardenia (Rajabi et al. 2015; Sedaghat 2000; Jaymand et al. 2007). There are different types of crocin, the highest concentration of which is Di-Gentiobiosyl Crocetin ester with the formula $C_{24}H_{64}O_{26}$. In order to produce this secondary metabolite, oxidation was performed on a carotenoid called protocrocin, which produces pigments (Momeni 2000), and finally, crocin is obtained during its hydrolysis. However, due to the separation of two gentobiose sugar molecules from crocin, conventional substances remain ascrocetin, the structure of which is similar to the aliphatic chain of carotenes

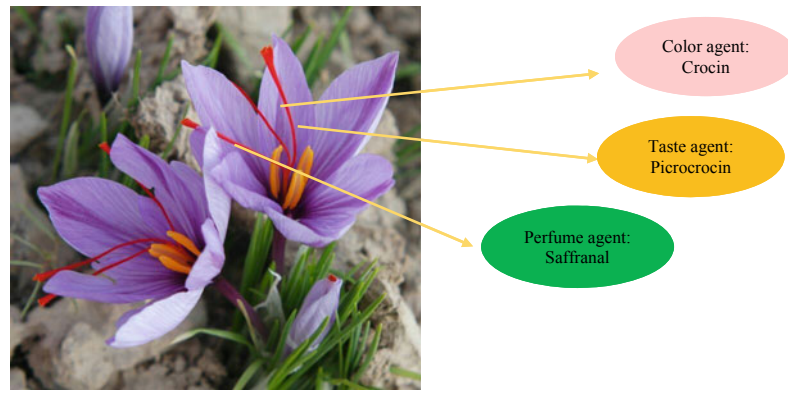

Fig. 14.1 The main secondary metabolites of saffron stigma

(Fatemi 2001). Crocin is known as a precursor of vitamin A. As a result, administration of this substance in higher doses is possible due to its lower toxicity than vitamin A derivative (Abdullah 2002). Due to this property, it is used as a colorant in food and medicine. This trait is important in saffron as an important trait in determining the quality of saffron, which can be evaluated by spectrograph at a wavelength of 440 nm (Rajabi et al. 2015).

1. Crocetin (only all-trans)
2. Trans-2-G, trans-crocetin (β-D-glucosyl)-ester
3. Trans/cis-3-Gg, trans/cis-crocetin (β-D-glucosyl)- (β-D-gentiobiosyl) ester
4. Trans/cis-4-GG, trans/cis-crocetin di-(β-D-glucosyl)- (β-D- gentiobiosyl) ester.

14.3 Flavor in Saffron

The cause of the bitter flavor in saffron is a colorless glycoside called picrocrocin with the chemical formula $C_{16}H_{26}O_7$ as a monoterpene aldehyde (Fig. 14.4). Picrocrocin is bitter and crystalline, which is converted into an aromatic aldehyde called safranal through thermal or enzymatic decomposition (Behnia 1991; Nair et al. 1995). Picrocrocin is actually a glycoside that has two halves: its sugar half contains D-glucose and its aglycone half contains 2, 6, 6-trimethyl-4-hydroxy-1-carboxyaldehyde-1-cyclohexane (Iborra et al. 1992). In other words, safranal product is produced during the hydrolysis of picrocrocin in saffron stigma (Haghighi et al. 2007). Despite its bitter taste, this compound has the potential to be present as a food additive and is one of the most important indicators of the quality and value of spices and medicines in saffron (Li et al. 1999).

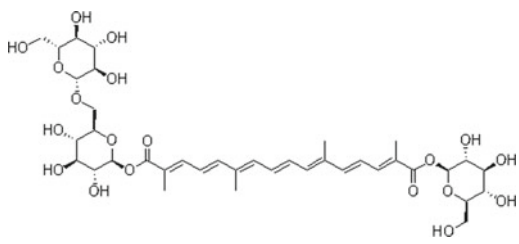

Fig. 14.2 Structure of crocin (Heidari and Khalili 2014)

14.4 Aromatic Compounds in Saffron

Once dried stigma releases a special and pleasant aroma described by Aristophanes as a sensual smell (Li and Wu 2005). In ancient Greece, saffron is known and used as a royal color and is used as a fragrance in various circles (Giaccio 2004; Abrishami and Jamalzadeh 1987). The aroma of saffron is due to the presence of volatile oils in it, which is a very fluid and colorless liquid from the category of terpenes ($C_{10}H_{16}$). The extracted essential oils simply absorb oxygen and are converted into a thick, brown liquid called safranal $C_{10}H_{14}O_2$ (Fig. 14.5). Due to the high instability of saffron essential oils, it cannot be marketed, but the saffron alcohol solution is used for flavoring in food and perfumery (Behnia 1991). When fresh saffron is harvested, the amount of safranal is very low, and during the process of drying and storing the stigma, picrocrocin breaks down into safranal and its amount will be increased. In fact, safranal is obtained by separating sugar from picrocrocin. During this process, a two-step enzymatic/dehydration reaction occurs and picrocrocin is converted to safranal either directly by high-temperature dehydration at a high pH (6–10) or first to the intermediate compound 4-R hydroxy-β cyclocytral, which is converted to safranal (Gregory et al. 2005).

14.5 Medicinal and Therapeutic Properties of Saffron

From ancient times, the saffron plant had many uses as a medicinal plant and spice. In ancient Greece, it was used as a regulator and enhancer of vision to treat many diseases, including gynecological diseases (Abrishami and Jamalzadeh 1987). Saffron is used as a cough reliever for chronic cough and bronchitis, toothache relief,

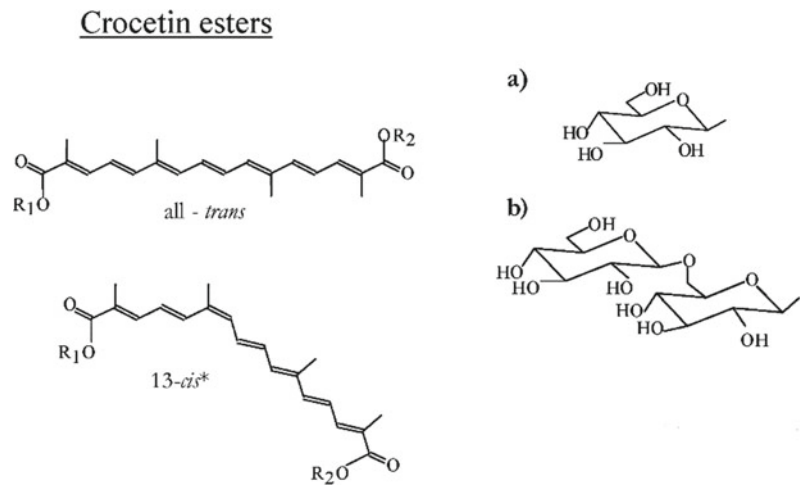

Fig. 14.3 Crocetin esters (Moratalla-López et al. 2019)

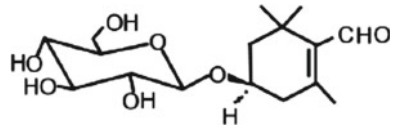

Fig. 14.4 Structure of picrocrocin (Moratalla-López et al. 2019)

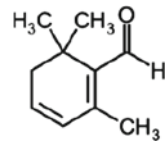

Fig. 14.5 Structure of Safranal (Moratalla-López et al. 2019)

seizure relief, insomnia treatment, excretion of kidney stones and gallstones, anticonvulsant, laxative, and sexual desire enhancer. In some countries, it is used as a sedative and treatment for abdominal pain (Melnyk et al. 2010; Shahi et al. 2016). Given that today, the issue of depression is one of the most important problems in the world with many direct and indirect consequences; about 11.6% of the world's population is composed of depressed people, and their numbers are increasing day by day. As a result, saffron has long been used as an antidepressant (Suganya et al. 2016). In fact, this plant has an antidepressant effect by modulating some substances such as serotonin in the brain (Hill 2004). Saffron extract is involved in the treatment of vasodilation and lowering blood pressure. Also, the dry stigma of this plant has strong antioxidant properties due to the presence of quercetin, which is an anti-inflammatory agent (Mousavi and Bathaie 2009; Verma and Bordia 1998). Quercetin increases antibiotic characteristics and lowers blood cholesterol levels, thus reducing the risk of heart attacks and causing the use of this plant in the treatment of cardiovascular disease (Baker and Negbi 1983; Kamalipour and Akhondzadeh 2011). Crocin inhibits carcinogenesis induced by 12-o-tetra decanoyl-13-acetate (Molnár et al. 2000). Crocin also protects brain cells by blocking the expression of alpha-NTF factor, a gene which is responsible for apoptosis of cells and breaks down the hereditary material of cells (Fatemi 2001). Crocetin, like crocin, has sedative properties and prevents the development of cancer cells (Jaymand et al. 2007). Safranal also has comutagenic properties by acting on the GABAA-benzodiazepine receptor complex (Abdullah et al. 2003). The medicinal and therapeutic properties of saffron are summarized in Fig. 14.6.

14.6 Extraction of Compounds from Saffron Stigma

Various methods have been used to extract saffron compounds (Fig. 14.7). The most common method is soaking. The best solvent in this method is a mixture of 50:50, water: methanol. Various researchers have evaluated the effect of

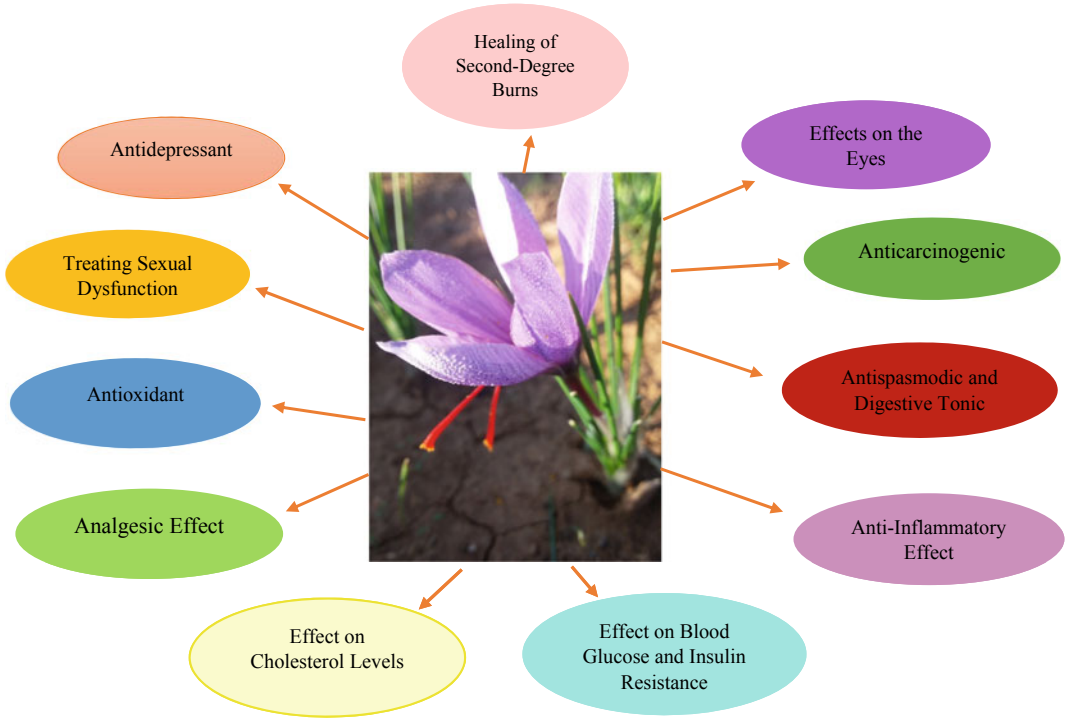

Fig. 14.6 Medicinal and therapeutic properties of saffron

factors involved in the extraction interval including temperature, time, solvent type, particle size, filtration type, and so on. Extract preparation has a very significant effect on the accuracy and precision of data. It has been reported that the type of solvent, time, and method of extraction not only affect the penetration rate of the compounds across the cell wall, but can also alter their stability (Orfanou and Tsimidou 1996).

14.7 Traditional Methods

Methanol, ethanol, and water are usually used as a solventto extract bioactive compounds. Extraction and purification of crocin have been widely reported in the literatures (Zareena et al. 2001; Pfister et al. 1996). After removing the fat with diethyl ether, the stigma is extracted 2–3 times using 90–70% ethanol or methanol. Extraction time, refining stage of the extract, type of filters, and solvents affect the color strength of the saffron extract. The highest extraction period of the saffron extract is related to 50% methanol, followed by 50% ethanol, 25% ethanol, and water. With increasing extraction time up to 24 h, color strength decreases. It has been found that the best size for smooth pores to achieve the highest color strength is 5–10 μm. The extraction step affects the color strength of the extract. There are two situations: if smoothing is done after dilution, the color strength will decrease, and if smoothing is done before dilution, it will increases the color strength (Orfanou and Tsimidou 1996). Zarghami and Heinz (1971) lipid compounds of saffron were extracted by ethyl ether method in dry ice bath. These compounds include isofuran, 2,6,6-trimethyl-4-hydroxy-1-cyclohexane-1-carboxyaldehyde and 2,4,4-trimethyl-3-formyl-hydroxy-52-cyclohexidine-1-one. After centrifugation and filtration, the extract was inoculated and the volatiles were separated and recovered using gas chromatography (Zarghami and Heinz 1971).

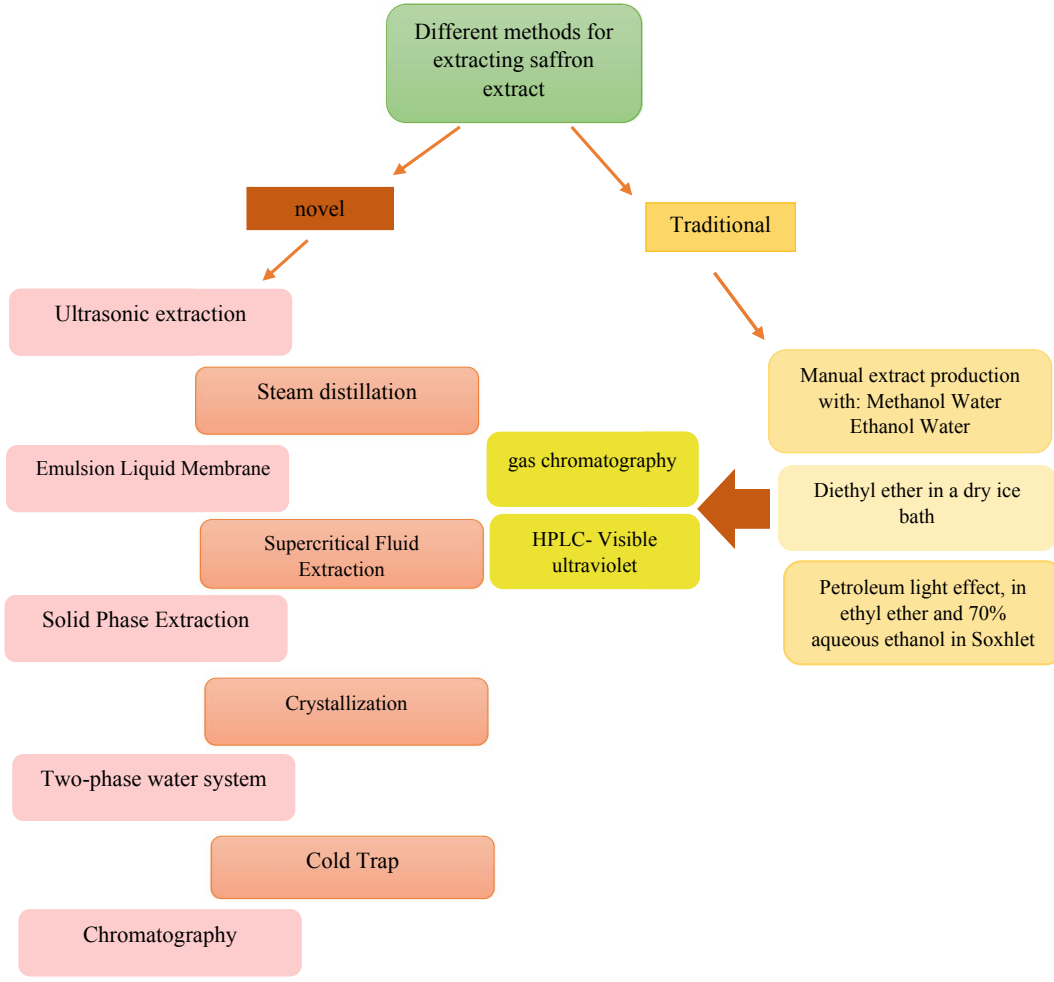

Fig. 14.7 Extraction of saffron compounds

In another study, petroleum in ethyl ether and 70% ethanol in Soxhlet were used to extract compounds from saffron stigma. Initial separation of crocin was performed in a column containing packing materials with a particle size of 57–76 μm and a pressure of 2×10^5 Pascals. Saffron extract was injected into the column and washed with a mobile phase stream (methanol and water) at a flow rate of 0.5 ml/min. To obtain high purity crocin components, the extract was first concentrated at 40 °C in the dark using a rotary evaporator and then cultured from a column containing an aqueous methanol binder as the vaporizing phase. The purity of crocin was assessed by visible ultraviolet High-performance liquid chromatography (HPLC). The results showed that when silica gel was used, the purity of crocin 1 was less than 95%, because crocin was highly polarized. In contrast, a mixture of styrene-vinyl benzene binder and modified starch polymers as a fixed phase with aqueous ethanol solution as the mobile phase increased the purity of crocin by 96% (Zhang et al. 2004).

14.8 New Methods and Approaches

14.8.1 Ultrasonic Extraction

Ultrasound is a mechanical wave that requires an elastic medium to scatter sounds with different wave frequencies. Today, ultrasound is a

promising method in the food industry. The main mechanism of ultrasonic extraction is related to the phenomenon of inactivation of enzymes in the preservation of food products and elimination of contamination from food through the propagation of ultrasonic waves (Cavitation). As sound wave passes through an elastic medium, it causes the particles to move longitudinally, acting as a piston at the surface, resulting in a sequence of contraction and expansion phases (McClements 1995). Ultrasound improves the process of extraction of plant compounds, i.e., swelling of the tissue in order to absorb the solvent, as well as the exit of the compounds from the tissue to the solvent by creating porosity and pores in the cell wall (Vinatoru 2001).

The most common ultrasonic equipment for plant extraction purposes is the ultrasonic purification bath and the probe system, which are applicable on an industrial and laboratory scale (Lagha et al. 1999). This method can be widely used in various food processes such as mixing materials, crystallization, degassing, material extraction, refining, freezing, drying, mass transfer, facilitation of oxidation, and other food processes. Extraction of various compounds with ultrasonic technology has comparative advantages over traditional methods:

1. Penetration of solvent into cells
2. Improve mass transfer
3. Biodegradation of cells on the surface and inside plants to accelerate the spread and release of intracellular materials.

Solutions used to extract bioactive compounds of saffron in ultrasound should be selected based on the number of compounds and their maximum area in total ion Chromatograms. Therefore, solvent polarity, viscosity, and molecular weight are important factors in this field (Jalali-Heravi et al. 2009). In an experiment, the active compounds in saffron were extracted using the ultrasound method, and the effect of various factors such as sound intensity, time, and sonication method at 20 °C on the extraction efficiency was investigated and it was concluded that pulsed ultrasound method with Shorter periods of time leads to higher extraction rates than continuous periods. As a result, it was observed that the use of ultrasound technology significantly improves the efficiency of extraction of active ingredients of saffron compared to traditional methods such as extraction with cold water (Kadkhodaee and Hemmati-Kakhki 2006). This technology can be used to extract volatile compounds of saffron as well as crustin (Kyriakoudi and Tsimidou 2015). Kadkhodaee and Hemmati-Kakhki (2006) investigated the extraction of saffron active compounds using high-power ultrasound and constant frequency of 30 kHz during the experiment followed by cold water extraction. According to the results, ultrasound significantly increased the extraction speed and thus reduced the processing time. Extraction efficiencies increased with increasing sonication time and amplitude (Kadkhodaee and Hemmati-Kakhki 2006).

14.8.2 Steam Distillation

Steam distillation is one of the valuable methods for separating volatile compounds such as essential oils from plants (Fig. 14.8), which is based on the distribution of components between the two phases. In this method, the plant does not come into contact with water, but is placed in a separate container in the vicinity of water vapor. So far, steam distillation, Microsimultaneous Steam Distillation-Solvent Extraction and Vacuum Head Space methods have been used and compounds including 6,6,2-trimethyl-3,1-cyclohexidine-1-carboxaldehyde (safranal), 5,5,3-Trimethyl-2-cyclohexane-1-one (isophorine), -trimethyl-3-cyclohexane-1-one (isoform isomer) and 6,6,2-trimethyl-2-cyclohexane 04, 1-DNA and 6,6,2-trimethyl-4,1-cyclohexidine-1-carboxaldehyde (safranal isomer) has been identified (Sugiura et al. 1994; Tarantilis and Polissiou 1997).

14.8.3 Emulsion Liquid Membrane

Emulsion liquid membrane is one of the best separation methods for the extraction of metal contaminants and molecular species and is

known for its properties such as high mass transfer rate, high sensitivity, low solvent requirement, and low investment (Fig. 14.9). In this type of membrane, surfactants are used to produce emulsions. The ELM process consists of a membrane phase, a dispersed or internal phase, and an external phase. Both phases are aqueous solutions, while the membrane phase is an organic liquid and somewhat hydrophobic, forming a thin boundary layer with the dispersed phase. The external aqueous phase usually contains the compound to be extracted. The membrane phase is usually not miscible with either the external phase or the dispersed phase and usually contains additives and surfactants that are usually optional to increase the selectivity, stability, and permeability of the membrane layer (Mokhtari and Pourabdollah 2013).

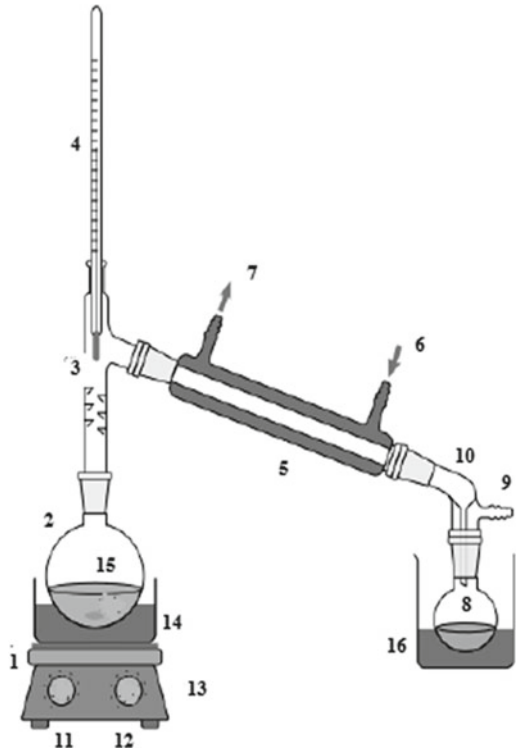

Fig. 14.8 Steam distillation extractor: 1. Heater, 2. Distillation balloons 3. Distillation tower. 4. Thermometer (to determine the boiling temperature), 5. Coolant, 6. Cold water inlet, 7. Cold water outlet, 8. Product collection balloon, 9. Vacuum gas inlet, 10. Vapor collector, 11. Heat regulator, 12. Stirrer speed regulator, 13. Heater plate, 14. Oil or sand bath. 15. Stirrer, 16. Cold water bath (Heydari and Haghayegh 2014)

14.8.4 Supercritical Fluid Extraction

Any substance that is at a temperature and pressure higher than its critical temperature and pressure is called a supercritical fluid. This fluid has the properties of a gas and a liquid, in other words, in terms of thermodynamics, it is similar to gases (with high penetration coefficient) and similar to liquids, and has a wide range of solubility for compounds (Fadavi et al. 2005). This phase has the same solvency as liquid. But on the other hand, it has the characteristics of transmission in gases. The low viscosity of the supercritical fluid, together with its high penetration power, along with the high buoyancy force results in better mass transfer properties of this fluid compared to conventional solvents. Fuzzy extraction and separation speeds can be significantly faster than the normal extraction process (Fig. 14.10) (Ashraf-Khorassan et al. 1997).

An ideal fluid should have the following characteristics:

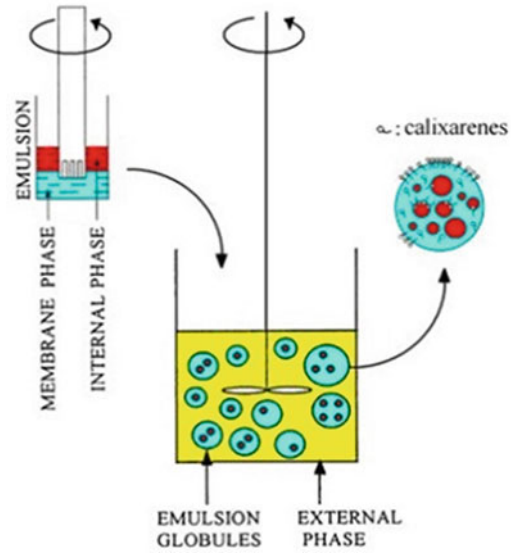

Fig. 14.9 View of the ELM) system (Mokhtari and Pourabdollah 2013)

1. Low supercritical temperature and pressure.
2. It should not be dangerous to human health. It should not be flammable and toxic.
3. It should be chemically inert with high purity and low cost (Fadavi et al. 2005).

This method can be used to extract saffron bioactive compounds, but most of the research has focused on safranal (Liang et al. 2014; Zougagh et al. 2006). This method reduces the supercritical fluid density. This is due to the increased penetration of supercritical solvent into the cell (Lozano et al. 2000). The extraction of saffron compounds was carried out using supercritical carbon dioxide and methanol at 40–80 °C, a pressure of 20 and 40 MPa and carbon dioxide flow rate of 3 ml/min. After time intervals of 15, 30, 60, 90, 120, 180, and 240 min, sampling was performed and immediately analyzed by HPLC. The results showed that the extraction rate of all compounds increased by increasing the temperature. This can be due to the increase in water vapor pressure and consequently increasing the polarity of carbon dioxide. The vapor pressure of coated compounds also increases with increasing temperature, which leads to solubility at higher temperatures (Nerome et al. 2016).

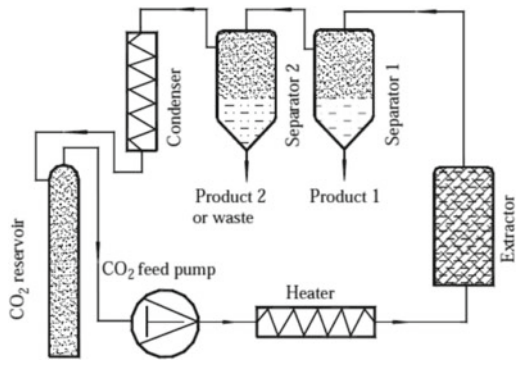

Fig. 14.10 Extra critical fluid extraction system (Heydari and Haghayegh 2014)

14.8.5 Solid Phase Extraction (SPE)

Today, the use of solid phase extraction has replaced the previous methods (liquid–liquid extraction and soxhlet). This method provides an acceptable amount of recycling efficiency. Conventional extraction techniques include solid, liquid, and gas phase extraction. One of the things to consider is the reduction in the amount of organic solvent used. SPE involves the adsorption of analyte on a solid adsorbent (fixed phase of aminopropyl or silica gel-bonded lectadacyl) followed by washing with a suitable solvent (Poole 2003). During the SPE process the cartridge is first treated with a solvent. The solution containing the analyte in the solid phase is then activated (Hennion 1999) (Fig. 14.11). The SPE technique has many applications today, especially in the separation of proteins and peptides (Schmerr et al. 1998; Visser et al. 2005). Non-polar fixed phases are used in SPE (Schmerr et al. 1998) and are commonly used in conjunction with HPLC and electrophoresis (Naylor and Tomlinson 1998; Tomlinson et al. 1997).

The SPE technique has been used to isolate saffron compounds, such as picrocrocin (Sánchez et al. 2009). In another study, Bolhasani et al. (2005) investigated the separation of picrocrocin and crocin by the SPE method.

14.8.6 Crystallization

In an experiment to extract crocin from saffron stigma, crystallization method was used, in which 80% ethanol was recognized as the best solvent. The crystallization process was performed in two stages at different temperatures. In the first stage, the purity of crocin crystals was low, therefore, the second stage of crystallization was performed to increase its purity. At this stage, purer crystals were obtained at 5 °C. Using UV–Visible spectroscopy and HPLC, the purity of crocin crystals was compared with its purity obtained by Fluka. According to the results, the purity of these crystals was about 13 times higher than Fluka. The total purity of crystalline

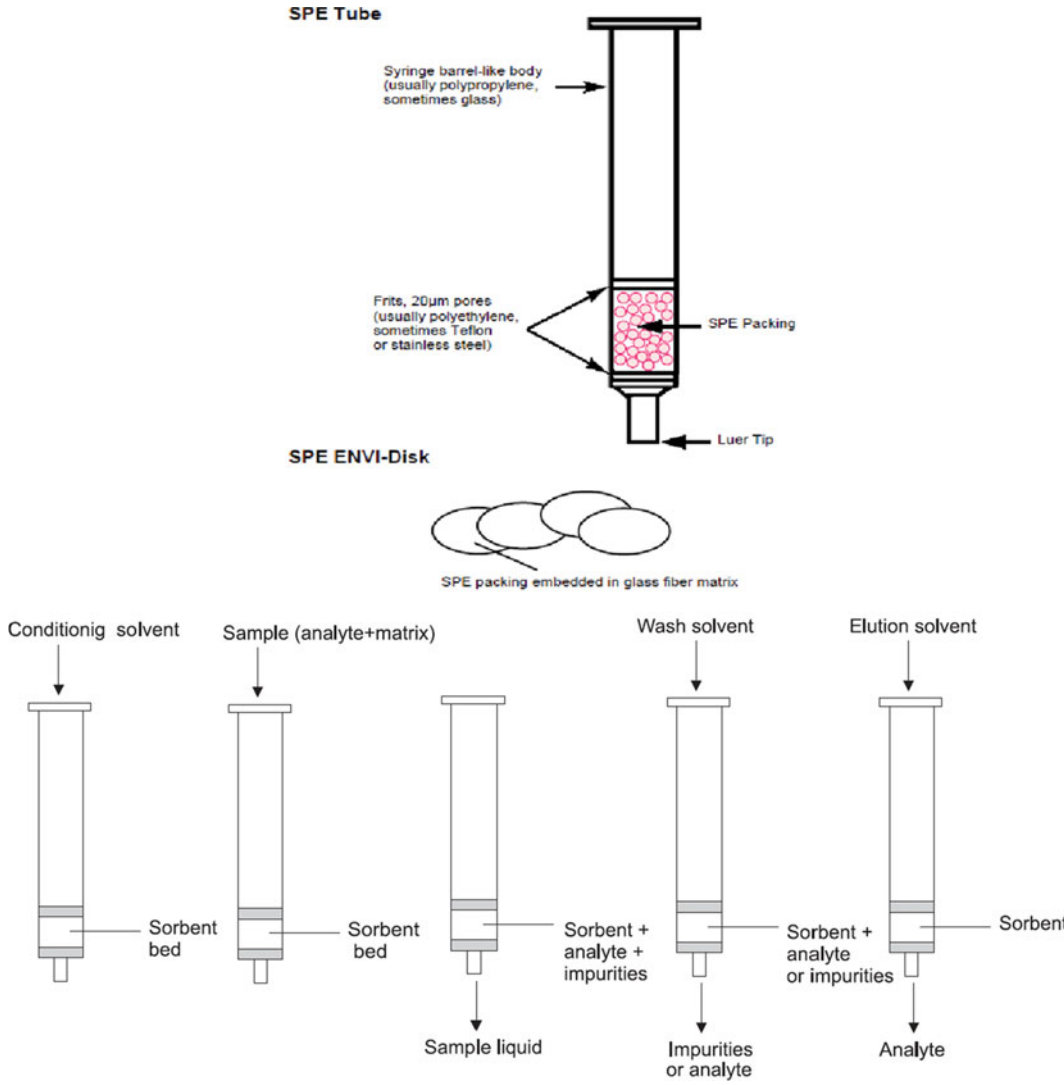

Fig. 14.11 A type of solid phase extraction pipe (top). Solid phase extraction steps (bottom)

crocin in this study was more than 97% (Hadizadeh, et al. 2010).

14.8.7 Two-Phase Water System

Montalvo-Hernández et al. (2012) investigated crocin recycling from saffron stigma in two-phase aqueous system. In this study, different mixtures of polyethylene glycol (PEG) with potassium phosphate, PEG with dextran (DEX), ionic solutions of potassium phosphate with sodium phosphate, and ethanol with potassium phosphate were used. Crocin was recycled effectively with two-phase PEG-potassium phosphate system (Montalvo-Hernández et al. 2012).

14.8.8 Cold Trap

Since safranal is a volatile oil, cold trap must be used to separate it. To separate safranal, 3 g of saffron was extracted twice using 70 °C water

and placed in a centrifuge (4000 rpm for 20 min). The surface pH was adjusted to 1 by HCl and incubated at 70 °C for 1 h under stirring conditions. Concentration was then performed using a rotary evaporator. Volatile compounds (mainly safranal) were isolated in a cold trap (−78 °C) (Iborra et al. 1992).

14.8.9 Chromatography

Chromatography methods such as thin-layer chromatography (Jin et al. 1986) and gas chromatography (Casas-Catalán and Doménech-Carbó 2005; Carmona et al. 2006) are recommended as the most efficient methods to analyze saffron compounds.

HPLC is now widely used for the separation and purification in a variety of fields, including pharmaceuticals, biotechnology, the environment, polymers, and food industry. HPLC has become the method of choice for analyzing a wide range of compounds over the past decade. In this regard, in order to quantify crocins, measure picrocrocin, safranal in different saffron samples, this type of chromatography equipped with optical (Vis) and fluorescence (UV) detectors is used (Caballero-Ortega et al. 2007; Lozano et al. 1999).

In 1999, Lozano et al. used HPLC to investigate the components and purity of commercial saffron through a diode array detector. In this method, 10 types of saffron metabolites that were responsible for taste, aroma, and color were identified and measured with high accuracy, precision, and selectivity. Some synthetic dyes that were considered counterfeit were also identified (Lozano et al. 1999). Also, during an experiment, for the first time, a HPLC method equipped with an ultraviolet–visible detector was used to determine the types of biologically active crocins. This method was successfully used to measure four types of crocins in three samples of saffron and plants containing crocin. The results showed that HPLC can be used as a quality control method for detecting crocin (Li et al. 1999). In the studies performed by Abdullah (2002) different types of saffron from 11 countries, including Iran, were evaluated using the HPLC method to evaluate the quantity and quality of this plant (Caballero-Ortega et al. 2007).

14.9 Conclusion

Saffron is one of the most expensive spices in the world with several applications in food industry. Due to present of unique bioactive compounds in saffron stigma, it is also used as a valuable medicinal plant in pharmaceutical industry. One of the major challenges in the relevant industries is achieving maximum extraction efficiency of saffron compounds. For extracting and purifying bioactive compounds of saffron, there are different methods, which are classified into two categories, traditional and novel high-tech approaches. During the extraction process, the lower the temperature and the shorter the time, cause less damage to the composition and quality of the extract produced. Therefore, new methods such as supercritical fluid is widely used due to applying low temperature and short time as well as higher extraction efficiency. HPLC-based approaches are also used as a fast and robust method to determine the secondary metabolites of saffron today.

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Saffron in Phytotherapy: Pharmacological Properties and Medicinal Uses

15

Rahma Zouagui and Laila Sbabou

Abstract

Crocus sativus L. or Saffron is the most expensive spice in the world and is also used as a coloring and flavoring agent. In several cultures and civilizations, saffron is known for its various therapeutic properties. Research in ethnobotany and ethnopharmacology focuses on the medicinal potential of plants in several populations and a better understanding of the use of plants by humans. The exploitation of these data can help in the development of new modern drugs and alternative therapeutic approaches. The healing and culinary peculiarities of saffron are related to the presence of four major bioactive compounds, which are crocin, crocetin, picrocrocin, and safranal. In this current chapter, we discuss therapeutic applications of saffron mainly linked to the abundant metabolites. A large number of studies reported the antioxidant potential of saffron, which involves its antitumor, anti-inflammatory, and neuroprotective effects. Moreover, a neuroprotective activity of saffron was widely assessed in neurodegenerative diseases like depression, Alzheimer, and schizophrenia, and is particularly attributed to safranal and crocin. We have also noted the therapeutic potential of crocin in neuro-inflammatory diseases and the antibacterial activity of saffron against a few clinical strains. However, the use of saffron must be done with great care to avoid any possible toxicity.

15.1 Saffron Through History

Saffron is a spice derived from the dried stigmas of *Crocus sativus* Linné, and due to its distinctive organoleptic attributes and its cumbersome harvesting process, it has long been considered the world's costlier spice. Nowadays, saffron is mainly used as a culinary condiment for its golden hue and unique aroma, but in the past, it was widely employed as a dye, perfume, and notably as a healing herb (Basker and Negbi 1983; Mousavi and Bathaie 2011). Saffron cultivation reaches back more than 3000 years and has been reported in many cultures and civilizations (Ferrence and Bendersky 2004). For almost three millennia, saffron held a special rank among all medicinal plants and has been listed in Greek, Chinese, Indian, and Arabian pharmacopoeias (Day 2011; Ferrence and Bendersky 2004). In fact, generations of scholars have reported the various healing properties of saffron in relation to the treatment of 90 medicinal

R. Zouagui · L. Sbabou (✉)
Center of Research Plants and Microbial Biotechnologies, Biodiversity and Environment, Team of Microbiology and Molecular Biology, Faculty of Sciences, Mohammed V University in Rabat, Rabat, Morocco
e-mail: lailasbabou@gmail.com; l.sbabou@um5r.ac.ma

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purposes (Basker and Negbi 1983; Pandita 2021). Those therapeutic aspects have been elucidated by means of ethnobotanical investigations, as well as ancient medicinal sources that overview the range of medicinal uses of saffron in the past.

The earliest historical reference related to saffron cultivation goes back to about 2300 BCE during the era of the Akkadian empire (actual Iraq) (Gadd and Edwards 1971), while the first report on saffron's medical use was found in an Assyrian dictionary of botany written during the reign of Ashurbanipal (668–633 BCE) (Pritchard 1969; Thompson 1949). The Assyrians used saffron to treat dyspnea, menstrual disorders, disease of the head, delivery, and painful urination (Thompson 1908). In the Greco-Roman classical period (eighth century BC to the third-century CE), the therapeutic aspect of saffron was testified through multiple frescoes found in the archeological sites of Knossos and Akrotiri in Greece (Ferrence and Bendersky 2004). One of these frescoes portrays the whole line of saffron production where a Greek goddess supervises the picking of stigma to fabricate a phytotherapeutic drug. The lower part of the same frescoes shows a woman using saffron to treat her bleeding foot (Ferrence and Bendersky 2004; Marinatos 1976). These bronze age frescoes are the first pictorial illustration of saffron's use as a medicinal remedy (Basker and Negbi 1983).

In ancient Greece (2000–146 BCE), saffron was used to treat numerous health issues. Indeed, saffron was cited among 257 drugs in the *Hippocratic Corpus* (fifth–fourth century BCE), and it was used to treat eye infections, skin ulcers, open wounds, and as a styptic (Hippocrates and Adams 1939; Hirschberg et al. 1982). *Erasistratus* (fourth–third century BCE) suggested saffron to treat throat inflammation, earache, mouth, and genital ulcers (Hirschberg et al. 1982). *Diokles* (third century BCE) and *Dioscorides* (first century CE) used saffron as a treatment for corneal disease, cataracts, toothache, erysipelas, and as a soothing substance (Giaccio 2004; Mousavi and Bathaie 2011).

In ancient Egypt (3100 BCE–476 CE), saffron was cited in The *Ebers Papyrus* and linked to gastrointestinal and ocular diseases treatment, menstruation disorder, urinary system regulation, and labor induction (Alonso et al. 2012; Bryan 1930; Woenig 1886). In ancient Rome (753 BCE–364 CE), it was employed to refresh facial skin, treat coughs, and reduce eye inflammation (Mousavi and Bathaie 2011). Saffron has also been reported in many ancient Indian herbal formulations and in Ayurvedic medicine. Indeed, it has been employed in a large variety of medical conditions like asthma, neurasthenia, cough, vomiting, dyspepsia, general debility, insect stings, skin diseases, and kidney infection (Rios et al. 1996). In China, the therapeutic use of saffron dates back to at least 3000 years BCE and this spice was highly treasured for its health beneficial properties (Mzabri et al. 2019). Traditional Chinese medicine uses saffron as a heart stimulant, analgesic, diuretic, immune stimulant, diaphoretic, and emmenagogue. It was also used to protect the brain from oxygen deprivation, treat rheumatoid arthritis, and aid in healing broken bones (Mousavi and Bathaie 2011).

In the Middle East, multiple therapeutic aspects of saffron have been reported in numerous major Islamic traditional medicine books. Among them: *Al-Qanun fi'l-Tibb* (Canon of Medicine) by Avicenna (980–1037 CE), *Ferdows al-Hekmah fi'l-Tibb* (The Paradise of Wisdom in Medicine), by Ali Ibn Raban Tabari (838–870 CE), *Al-Hawi fi'l-Tibb* (Comprehensive Book of Medicine) by Abu Bakr Razi (865–925 CE) and *Kamel alSannat al-Tibbyah* (Complete Book of the Medical Art) by Ali ibn al'-Abbas al-Majusi (930–994 CE). Those scholars described various therapeutic applications of saffron including its use as hypnotic, aphrodisiac, hepatoprotective, antidepressant, anti-inflammatory, bronchodilatory, and labor inducer (Hosseinzadeh and Nassiri-Asl 2013; Javadi et al. 2013).

During the first century CE, Pliny the Elder (23–79 CE) indicates a saffron-based collyrium for blurred vision, and also mentions saffron to treat pleurisy, erysipelas, insomnia, and promote hair growth (Jones 1951; Nielson 1974). In the second century CE, Soranus of Ephesus used saffron for obstetrical indications (Temkin 1956).

Subsequently, in the seventh century, the Fundamentals of Medicine by Paulus of Aegina described a saffron-containing eyewash designed to treat conjunctivitis and corneal ulcers, lid swelling, eye injuries, and eye abscess (Hirschberg et al. 1982). In the eighth century, the Syriac Book of Medicines reports the use of saffron for hemicranial headaches, delirium, abortion, contraception, palpitations, earaches, diarrhea, and vomiting (Budge 1976; Riddle 1992).

Many centuries later, the English botanist and surgeon John Gerard (1545–1612) underlined the antidepressant properties of saffron and suggested it to treat jaundice, constipation, smallpox, and pestilence (Gerard 2015). In the seventeenth century, this spice was used as an appetite stimulant and in the eighteenth century, it was recommended to relieve nausea and vomiting (Basker and Negbi 1983; Estes and Kuhnke 1984). During the mid-twentieth century in North Africa, Arabs and Berbers used saffron to induce abortions, prevent miscarriages, and treat diabetes, melancholia, splenomegaly, hepatomegaly, bruises, asthma, and pertussis (Boulos 1983). It was also used for snake bites, headaches, hematoma, and dysmenorrhea (Basker and Negbi 1983; Duke 2002; Rios et al. 1996; Tang and Eisenbrand 1992).

In recent decades, the therapeutic use of saffron knew a decline and it was mainly used as culinary spice (Basker and Negbi 1983). However, the historic research in ethnobotany and ethnopharmacology gives an insight on how ancient nations had been conscious toward medicinal potential of the plants that surround them (Day 2011), and a better understanding of the dynamics and evolution of human plant use (Heinrich et al. 2006). Those findings can be exploited to parse the way for developing novel modern drugs and alternative therapeutic approaches in the era of re-interest in herbal medicine.

15.2 Saffron Phytochemistry

Phytochemical analysis of saffron revealed the presence of more than 150 volatile and several nonvolatile active compounds (Samarghandian and Borji 2014). The volatile fraction contains more than 34 compounds that are terpenes and their esters among which safranal is the major chemical (Alonso et al. 1998; Lage and Cantrell 2009; Semiond et al. 1996). The nonvolatile compounds include carotenoids, lipophilic and hydrophilic carbohydrates, proteins, amino acids, vitamins (especially riboflavin and thiamine), mucilage, starch, gums, pigments (crocin, α, and β-carotenes, mangicrocin, anthocyanin, lycopene, flavonoids and zeaxanthin), alkaloids, saponins, and many other chemicals (Abdullaev 2002; Carmona et al. 2006; Rios et al. 1996; Samarghandian and Borji 2014; Winterhalter and Straubinger 2000). In addition, saffron stigma contains various mineral elements such as calcium (863–1070 μg/g), potassium (10.70–11.70 mg/g), sodium (42–100 μg/g), magnesium (1300–1470 μg/g), and iron (92–135 μg/g) (D'Archivio et al. 2014). A study on free amino acids indicated that alanine, proline, and aspartic acid are the major amino acids in saffron spice (Del Campo et al. 2009). The concentration of the above constituents may fluctuate depending on the origin of cultivation, soil fertility, and growing and processing conditions (D'Archivio et al. 2014; Gregory et al. 2005; Rios et al. 1996; Tong et al. 2015). Indeed, it has been reported that several physical and chemical modifications take place during the dehydration stage. These modifications are mainly controlled by post-harvest treatment (Razak et al. 2017). This is likely the reason behind the variance in the spice content reported in different producer countries (Bathaie et al. 2014). The proximate chemical composition of saffron spice is shown in Table 15.1.

The major determinant of saffron quality in terms of its healing attributes and culinary uses is dependent on the presence of four metabolites, which are crocin, crocetin, picrocrocin, and safranal (Giaccio 2004). These compounds belong to the carotenoids family and are the most abundant bioactive compounds in *C. sativus* stigmas. They include fat-soluble constituents, such as α and β-carotene, lycopene, zeaxanthin, and water-soluble ones like the apocarotenoid crocetin, and crocins, the polyene esters of the mono- and di-glycoside crocetin (Maggi et al.

Table 15.1 Proximate composition of saffron spice (Kumar et al. 2008; Rios et al. 1996; Sampathu et al. 1984; Srivastava et al. 2010)

Component	Mass percentage
Moisture	6–12
Mineral matter	4–8.5
Raw fiber	4–5
Proteins	10–14
Total sugar	26–27
Gentiobiosides	2–3
Glucose	7–8
Fructose	1–2
Xylose and rhamnose	Traces
Pentosanes	6–7
alfa-crocin	2
Carotenoids	1
Starch	6–7
Pectins	6
Gums and dextrins	9–10.5
Fats	5–8
Total oils	5–9
Volatile	0.3–0.8
Vitamins[a]	0.3–138[b]

[a]Riboflavin and Thiamine
[b]in μg/g

2020). Known as powerful antioxidants, these metabolites have been reported to be responsible for the yellowish color, bitter taste, and unique aroma ascribed to this spice (Rios et al. 1996; Winterhalter and Straubinger 2000). Saffron active compounds are yielded after the bio-oxidative cleavage of carotenoids in the apocarotenoids biosynthesis pathway (Baba et al. 2015). Indeed, Zeaxanthin is symmetrically cleaved at the 7,8 and 7',8' positions in the presence of a specific carotenoid cleavage oxygenase (CCD). The crocetin dialdehyde is converted to crocetin by aldehyde dehydrogenase and then gives rise to crocin by at least two UDPG-glucosyltransferase. The C10 product, 3-OH-β-cyclocitral is dehydrogenated to yield picrocrocin and then converted to safranal (Frusciante et al. 2014). Saffron stigmas accumulate a large amount of crocetin and its digentiobiosyl ester, crocin, and they both present approximately 6–16% of dry weight (Gregory et al. 2005). Picrocrocin, precursor of safranal, counts up to 13% of spice mass (Caiola and Canini 2010; Samarghandian and Borji 2014), while safranal presents up to 2% of dry matter and about 70% of volatile fraction (Alonso et al. 1996; Maggi et al. 2009; Masi et al. 2016).

Crocetin ($C_{20}H_{24}O_4$; MW: 328,402 g/mol; IUPAC name: (2*E*,4*E*,6*E*,8*E*,10*E*,12*E*,14*E*)-2,6,11,15-tetramethylhexadeca-2,4,6,8,10,12,14-heptaenedioic acid), is a 20-carbon carotenoid that after glycosylation receive the name of crocin. Both crocetin and crocin are reported as the most abundant compound in saffron stigma and are responsible for the unique color of the spice (Bathaie et al. 2014; José Bagur et al. 2018). Crocetin is characterized by a symmetrical structure with seven double bonds and a carboxylic group at each end of the polyene chain (Giaccio 2004). It is insoluble in water and most organic solvents, however, its anionic species

show a high water solubility (Bathaie et al. 2014). Crocetin is present mostly as its *trans*-isomer, while the *cis*-crocetin exists as minor compound (Bathaie et al. 2014).

Crocin ($C_{44}H_{64}O_{24}$; MW: 977 g/mol; IUPAC name: bis[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-[[(2*R*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2yl]oxymethyl]oxan-2-yl](2*E*,4*E*,6*E*,8*E*,10*E*,12*E*,14*E*)-2,6,11,15 tetramethylhexadeca-2,4,6,8,10,12,14-heptaenedioate), is a glycoside carotenoid derived from crocetin and the main responsible for dyeing property of saffron (Giaccio 2004). It is a crocetin digentiobiose ester ($C_{20}H_{24}O_4$), characterized by a 20- carbon atom chain and two carboxylic groups at the extremities (Giaccio 2004; Leone et al. 2018). The crocin family includes various glycosyl esters such as mono- and di-glycosylated esters of the dicarboxylic acid crocetin (Kamel et al. 2009). These glycosyl esters contain a sugar moiety which makes crocin an unusually hydrophilic carotenoid in nature (Bathaie et al. 2014). Chemical analysis of saffron revealed various derivatives of crocin that occur as pair of *cis–trans*-isomers among which, crocin 1 or α-crocin is the most abundant and present about 10% of saffron's dry matter (Caballero-Ortega et al. 2007).

Picrocrocin ($C_{16}H_{26}O_7$; MW: 330.37 g/mol; IUPAC name: (4*R*)-2,6,6-trimethyl-4-[(2*R*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxycyclohexene-1-carbaldehyde), is a colorless monoterpene glycoside which is responsible for the saffron's bitter taste and flavor (Buchecker and Eugster 1973). It is the second most abundant metabolite in saffron and has only been identified in the species *C. sativus* L.; therefore, it is considered the saffron authenticity biomarker (Caiola and Canini 2010; Lage and Cantrell 2009; Maggi et al. 2020; Pitsikas 2016; Samarghandian and Borji 2014). During the drying process, the picrocrocin cracks into an aglycon and into a molecule of glucose. The aglycon loses a molecule of water and gives rise to the volatile compounds safranal (Giaccio 2004).

Safranal ($C_{10}H_{14}O$; MW: 15021 g/mol; IUPAC name: 2,6,6-trimethylcyclohexa-1,3-diene-1-carbaldehyde), is a cyclic terpenic aldehyde that is the major compound of volatile fraction and the main responsible for the aroma of saffron (Giaccio 2004; Lage and Cantrell 2009). The fresh stigma is odorless, the characteristic smell of saffron spice appears during the post-harvesting stages. Indeed, safranal is produced after the hydrolysis and dehydration of its precursor picrocrocin by the action of the *beta*-glycosidase enzyme (Giaccio 2004). Thereby, the ratio between bitterness and aroma of the final product is mainly influenced by the drying process (Leone et al. 2018). The structure of saffron's major compounds are illustrated in Fig. 15.1.

15.3 Pharmacokinetics of Saffron Active Components

A growing literature is devoted to assess the health beneficial properties of saffron bioactive molecule, in particular, carotenoids. However, the form of administration, absorption, and metabolism of this class of molecule needs to be discovered (Chryssanthi et al. 2011). The current knowledge about the carotenoid's absorption and uptake shows that they are released from the foodstuff matrix in the gastrointestinal tract, and due to their lipophilic character, they are absorbed throught the intestinal cells by passive diffusion and incorporated in its intact form into chylomicrons before secretion in the blood circulation system (Erdman Jr et al. 1993; Parker 1996). The major carotenoids in saffron are known to be particularly soluble in water because they are glycoside esters, but like the other carotenoids, their pharmacokinetic proprieties remain poorly understood and need more investigations (Reboul 2019).

Bioaccessibility is defined as the potential or fraction that is released from the matrix in the gastrointestinal tract to be absorbed by an organism (Saura-Calixto et al. 2007), while bioavailability refers to the rate of an active constituent that reaches the blood circulation (Granado et al. 2006). From the pharmacokinetic point of view, crocins could not act as a bioactive element when they are introduced orally, but

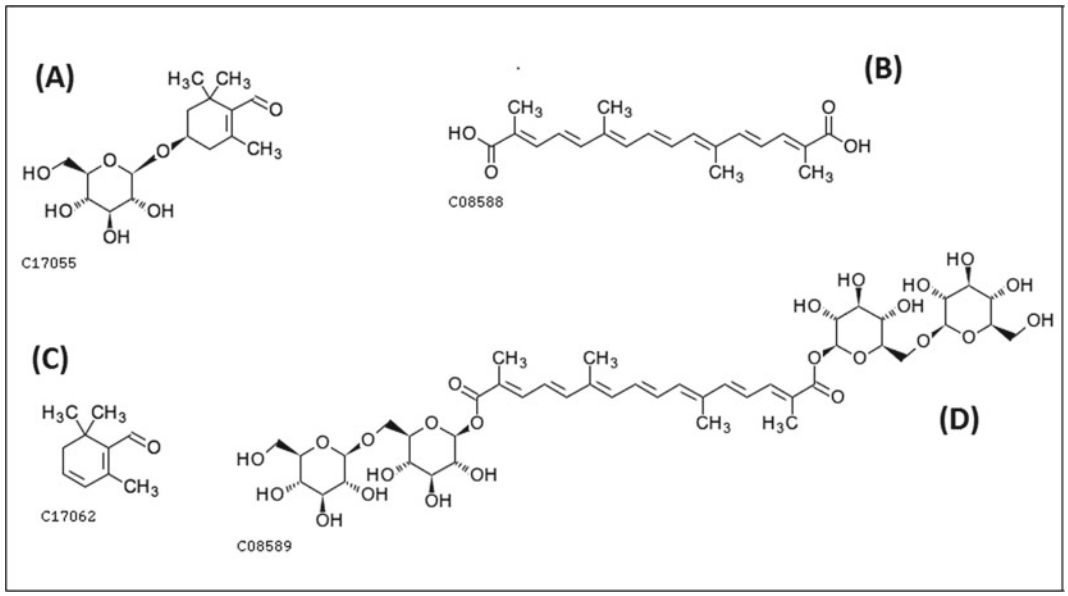

Fig. 15.1 The structures of saffron major compounds. **a** Picrocrocin, **b** Crocetin, **c** Safranal, **d** Crocin

hydrolyzed into crocetin in the intestinal tract (Asai et al. 2005). In this line, several studies have shown that oral consumption of crocins is not bioavailable in the cellular system, and recently, a rat bioassay study reported that oral introduction of crocin resulted in 56–81 times more in plasma content of crocetin (Zhang et al. 2017). However, when crocin is given intravenously, the level of crocetin is low in plasma, suggesting that crocin is mainly converted to crocetin in the gastrointestinal tract (Hosseini et al. 2018). Indeed, crocins are hydrolyzed to trans-crocetin under the action of intestinal epithelial enzymes, absorbed via the intestinal mucosal layer, and then reach the circulatory system through passive diffusion (Lautenschläger et al. 2015).

Studies have reported that multiple oral doses of crocin do not increase the crocetin concentration in plasma compared to a single dose (Xi et al. 2007). This showed that crocetin is eliminated rapidly and its mean elimination half-live ($T_{1/2}$) was estimated as 6.1–7.5 h (Umigai et al. 2011). A clinical study consisted of the consumption of tea saffron (200 mg of saffron at 80 °C for 5 min) showed that crocetin was detected in the bloodstream after 2 h at high concentration (1.24–3.67 uM) (Chryssanthi et al. 2011). Mohammadpour and coworkers reported that the crocetin concentration range was 0.09–0.35 g/ml after the administration of a 16 mg crocetin capsule to healthy volunteers, at different sampling intervals (Mohammadpour et al. 2013). Those results showed that crocetin was rapidly absorbed, with a mean time of 4.4 h, to reach the maximum concentration (Umigai et al. 2011).

Pharmacokinetic studies showed that C40 carotenoids need more time to reach their maximum concentration (C_{max}) compared to crocetin. The T_{max} value of β-carotene was more than 30 h (Kostic et al. 1995; Zhi et al. 1996) and ranged from 15 to 33 h for lycopene (Gustin et al. 2004). While crocetin is absorbed considerably more rapidly, detected in plasma 1 h after the introduction, and reached the peak concentration around 4 h later (José Bagur et al. 2018). The bioaccessibility of esters of crocin and picrocrocin has been estimated at 50% and 70%, respectively. However, they were transported in low quantities (0.5% and 0.2%, respectively), which is ten times lower than crocetin (Kyriakoudi et al. 2015).

Once it reaches blood circulation, crocetin can distribute in different tissues because of its weak interaction with albumin protein (Kanakis et al. 2007). Also, it can cross the blood–brain barrier and reach the central nervous system CNS by passive transcellular diffusion (José Bagur et al. 2018). Studies have reported that nearly 59.507 ± 13.56% of crocin was excreted through feces 8 h after a single oral dose (40 mg/kg). The remaining portion in the intestinal contents was estimated as 20.437 ± 9.41%, while no trace of crocin was detected in urine during 24 h (Zhang et al. 2017).

15.4 Therapeutic Applications of Saffron

15.4.1 Neuroprotective Activity

15.4.1.1 Depression

Depression is one of the top five most prevalent diseases worldwide (Bauer et al. 2013) and the most commonly diagnosed psychological disorder (Hirschfeld 2012). It presents a global public health concern with a heavy burden and high economic cost (Ferrari et al. 2013). The psychological symptoms of depression include sadness, sleep disorder, losing appetite, feeling tired, having guilty feelings, and feeling anxious and worried (Kessler and Bromet 2013). In extreme cases, it can even lead to suicide (Mathers and Loncar 2006). Synthetic antidepressants such as selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressants (TCAs), and selective serotonin noradrenaline inhibitors (SSNRIs) are effective in reducing depression symptoms by increasing the availability of serotonin and certain other neurotransmitters (Nelson et al. 2008). However, numerous side effects have been observed, like nausea, constipation, hypotension, dry mouth, insomnia, and tachycardia (Cascade et al. 2009; Ferguson 2001; Sarkar et al. 2013). Herbal remedies can be a good alternative to synthetic drugs. Recently, several studies have reported promising outcomes on herbal agents with favorable tolerability and safety profile. Many of them are related to saffron and its bioactive compounds.

In a preclinical study, Hosseinzadeh et al. reported that the extracts of saffron stigma (0.2–0.8 g/kg), safranal (0.15–0.5 mg/kg), and crocin (50–600 mg/kg) could reduce the immobility time in a forced swimming test conducted on mice. The shown effects were comparable to that obtained with fluoxetine and imipramine. This may suggest the role of crocin and safranal in regulating the serotonergic, dopaminergic, and noradrenergic systems (Hosseinzadeh et al. 2003). In this context, several reports mentioned the modulatory effect of saffron on BDNF, CREB, and VGF pathways (Asrari et al. 2018; Dorri et al. 2015; Ghasemi et al. 2015; Razavi et al. 2017).

Due to the promising results obtained in many preclinical essays, several clinical studies have been conducted to assess the antidepressant effect of saffron. In numerous recent investigations, saffron was found to be safe and effective on depression and anxiety when compared to controls (Dai et al. 2020; Jam et al. 2017; Lopresti and Drummond 2014; Marx et al. 2019; Talaei et al. 2015; Tóth et al. 2019; Yang et al. 2018). In a randomized, double-blind, placebo-controlled study, Lopresti and colleagues showed that various doses of curcumin and combined curcumin/saffron treatment can reduce depressive and anxiolytic symptoms in people with major depressive disorder (Lopresti and Drummond 2017). Additionally, a randomized, double-blind, placebo-controlled trial was conducted on 60 women with postpartum depression. The patients were treated either with saffron (30 mg/day, 15 mg twice per day) or placebo for 6 weeks. The outcomes, according to the Hamilton Depression Rating Scale (HDRS), show that 96% of patients in the treated group experienced relief from depressive symptoms compared to 43% in the placebo group (Kashani et al. 2018).

The antidepressant effect of saffron has also been compared to that of clinical antidepressants. Khaksarian et al. compared saffron and fluoxetine efficiency in the treatment of depression. They conclude that saffron can improve the

symptoms of depressed patients in a comparable manner to fluoxetine (Khaksarian et al. 2019). In the same line, Shahmansouri et al. and Noorbala et al. reported the efficacy of saffron in the treatment of mild to moderate depression. In both studies, saffron shows a similar antidepressant effect to that of fluoxetine (Noorbala et al. 2005; Shahmansouri et al. 2014). In another trial, saffron was found to be as effective as fluoxetine with no side effects (Basti et al. 2007).

The antidepressant effects of saffron are attributed to safranal and crocin, especially crocin 1 and crocin 2 (Wang et al. 2010). It has been shown that crocin influenced the serotonergic system by having an antagonist action on the 5-HT receptor, thus increasing the serotonin uptake (Kang et al. 2012). In addition, crocin was found to be a noncompetitive inhibitor of Monoamine oxidase (MAO-A and MAO-B) by binding to the allosteric sites of the enzyme (De Monte et al. 2014). Moreover, it has been suggested that crocin can act as an antidepressant by increasing the nerve growth factor inducible (VGF) and the Cyclic adenosine monophosphate (cAMP) response element binding, brain-derived neurotrophic factor levels in rat hippocampus (Hassani et al. 2014). Although the exact mechanisms of action are still under investigation, a synergy of the serotonergic, neuroprotective, anti-inflammatory, and antioxidant activities of saffron has been hypothesized (Lopresti and Drummond 2014).

15.4.1.2 Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurologic disorder associated with gradual losses in thinking, learning, reasoning, and memory function. It is one of the most common causes of dementia, especially in the geriatric population (Finley and Gao 2017). The global prevalence of AD is estimated to be 27 million and is expected to increase and reach 86 million by 2050 (Doecke et al. 2012). The disease is characterized by the accumulation of amyloid plaques in the extracellular environment and neurofibrillary masses inside the neurons. The main component of the plaques is amyloid-ß (Aß), their deposition results in synaptic destruction and neuronal damage (Nisbet et al. 2015).

There is no effective treatment for AD and current drugs present several limitations in relation to safety and efficiency (Razak et al. 2017). In recent years, some clinical trials have studied the effect of saffron on AD management. In a 16-week, randomized, and placebo-controlled trial, 46 patients were randomly assigned to receive either saffron (15 mg twice per day) or a placebo capsule. The outcomes show that saffron induced an improvement of cognitive functions compared to placebo (Akhondzadeh et al. 2010b). In another study, saffron (15 mg twice per day) was found to be as effective as donepezil (5 mg twice per day) with no side effects (Akhondzadeh et al. 2010a). In the same context, Farokhnia et al. compared the therapeutic effect of saffron with memantine in the treatment of moderate to severe AD patients. They observed that saffron was safe and comparable to memantine in reducing cognitive decline (Farokhnia et al. 2014). Additionally, it has been reported that 2 months of treatment with a combination of nutraceuticals comprising saffron, Bacopa monnieri, L-theanine, folate, and vitamins (B and D group) significantly improved the cognitive functions compared to placebo (Cicero et al. 2017). Those findings suggest the neuroprotective action of saffron, alone or in synergy with other agents.

Crocin was found to exert an inhibitory effect on acetylcholinesterase activity and on beta-amyloid-induced apoptosis in neuronal cells (Asadi et al. 2015; Geromichalos et al. 2012). Other mechanisms are also involved in the neuroprotective action of saffron, including the reduction of phosphorylated tau formation, oxidant stress, endoplasmic reticulum stress, synaptic loss, and neuronal cell apoptosis (Deslauriers et al. 2011; Ghahghaei et al. 2013; Papandreou et al. 2006; Rashedinia et al. 2015). In addition, crocin acts as a potential anti-Alzheimer agent by reducing the glutamatergic synaptic transmission, and the proinflammatory and neurotoxic factor levels (Hatziagapiou et al. 2019). Recently, it has been reported that the protective effect of saffron on toxicity and

neuronal damage may involve the Mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K) pathways (Rafieipour et al. 2019).

15.4.1.3 Schizophrenia

Schizophrenia is a chronic brain disorder that affects up to 1% of the world's population (Pitsikas 2021). It is a complex, long-term medical illness that impair social, occupational, and individual capabilities and negatively affect the life quality of the patient. The major schizophrenia symptoms can be classified into three categories: positive symptoms (e.g., hallucinations, thought disorder, delusions, catatonic behavior), negative symptoms [e.g., asociality, anhedonia, apathy, avolition, neglect of hygiene (Adida et al. 2015)], and cognitive disturbance (e.g., confused thinking, abnormal movements, memory defect) (Freedman et al. 2003).

Schizophrenia is described as a neurodevelopmental disease and its causes are still not fully elucidated. Nevertheless, it is widely believed that numerous genetic and environmental factors contribute to psychosis causation (Freedman et al. 2003; Mullin et al. 2013). In recent years, several reports suggest that dysfunction of different neurotransmitter systems, such as dopamine (DA), glutamate, serotonin, and γ-aminobutyric acid (GABA) is associated with the appearance of this psychosis (Steeds et al. 2015). Additionally, it has been reported that positive symptoms of schizophrenia are related to the overactivation of dopaminergic neurotransmission in the striatum, while negative symptoms and cognitive impairments are associated with abnormal GABA concentration in the medial prefrontal lobe (Goto et al. 2009; Pitsikas 2021).

To assess the effect of saffron and its constituents in schizophrenia treatment, some preclinical studies have been conducted on rats. The outcomes show that acute administration of crocins (15–30 mg/kg) can counteract disruption of nonspatial recognition memory caused by the administration of the NMDA (N-Methyl-D-aspartic acid) receptor antagonist Ketamine (3 mg/kg, acutely) in rats. Further, crocins (50 mg/kg, acutely) were found effective to attenuate Ketamine (25 mg/kg, acutely) psychotomimetic induced effects (hypermotility and ataxia) in the rat. Additionally, an acute administration of crocin (50 mg/kg) could reduce the social isolation caused by treatment with Ketamine (8 mg/kg, sub-chronically) in rats (Georgiadou et al. 2014). In agreement with the above, it has been reported that crocin (25 and 50 mg/kg, acutely) reduced spatial impairments and motor disturbances induced by the introduction of the NMDA receptor antagonist MK-801 (1 mg/kg, acutely) in rats (Sun et al. 2020).

To date, a single clinical study was conducted to assess the safety and tolerability of saffron aqueous extract (SAE) and crocin in the treatment of schizophrenia. A double-blind, placebo-controlled study was performed on 61 patients with schizophrenia. In addition to their normal treatment, the patients received (15 mg twice daily) of SAE and (15 mg twice daily) of crocin or placebo for 12 consecutive weeks. The results show that SAE and crocin were safe and tolerated in patients with schizophrenia (Mousavi et al. 2015). It has also been reported that crocin and SAE can prevent insulin resistance and olanzapine-induced metabolic syndrome (MetS), a well-known side effect of this neuroleptic agent (Fadai et al. 2014).

There are poor evidence concerning the mechanisms by which saffron and its bioactive molecule exert their beneficial effect in schizophrenia. However, it has been observed that saffron and crocin partly counteract the NMDA receptor by binding to its phencyclidine (PCP) bending site (Lechtenberg et al. 2008). Moreover, it has been reported that saffron extracts and crocin normalized excessive glutamatergic synaptic transmission in rats (Berger et al. 2011). Additionally, saffron extracts and crocetin were found to have a strong affinity to the sigma (σ) 1 receptor (Lechtenberg et al. 2008).

Although schizophrenia's causes are not yet fully understood, some suggestions about possible associations with oxidative stress (Bitanihirwe and Woo 2011), and inflammation (Khandaker et al. 2015) have been advanced. The antioxidant propreties of saffron extracts can

offer some explanation for its positive effect in preclinical model of schizophrenia (Naghizadeh et al. 2013; Zheng et al. 2007). Recently, it has been reported that the neuroprotective activity of crocins was related to their ability to upregulate the expression of the silent information regulator-1 (SIRT-1), thus relieving oxidative stress. However, the potential molecular mechanisms remains unclair (Sun et al. 2020).

15.4.1.4 Antioxidant Activity

Oxidative stress is defined as an imbalance between the production of oxygen reactive species (ROS) and antioxidant defenses in cells and tissues (Pizzino et al. 2017). Oxidative stress is one of the essential elements in the development of numerous diseases such as heart failure, cancer, hepatic inflammation, hepatic cirrhosis, neurodegenerative disease, diabetes, pulmonary diseases, and so on (Al-Gubory 2014; Tsantarliotou et al. 2013). Recently, a large number of studies have been conducted on saffron's antioxidant effect. The antioxidant potential of saffron involves its antitumoral, anti-inflammatory, and neuroprotective activities (Alavizadeh and Hosseinzadeh 2014), and it is mainly due to its bioactive compounds such as crocin, crocetin, and safranal as well as its phenolic content (Karimi et al. 2010).

Assimoupoulou and colleagues reported that crocin showed high radical scavenging activity (50% and 65% for 500 and 1000 ppm in methanol solution, respectively), followed by safranal (34% for 500 ppm solution) (Assimopoulou et al. 2005). Hosseinzadeh et al. evaluated the antioxidant action of saffron on liver microsomal lipid peroxidation. They noted that saffron reduced the extent of MDA, while the effect of crocin at 1.2 mM and ethanolic extract at 500 and 1000 μg/ml were similar to that of BHT 100 μM (Hosseinzadeh et al. 2009). In the same line, it has been observed that the oral administration of crocetin and crocin for six weeks results in enhancing the activity of superoxide dismutase (SOD) in liver and kidney, glutathione peroxidase (GSH-Px) in liver, and total antioxidant capacity (TAOC) of heart and kidney and MDA in mice serum (Chen et al. 2010).

Another study by Bandegi et al., evaluated the protective effect of saffron extract (30 mg/kg) against chronic-stress-induced oxidative damage to brain, liver, and kidneys in rats. The plant extract effectively increased the level of glutathione reductase, MDA, GSH-Px, and SOD, and significantly decreased the total antioxidant capacity in the stressed animals (Bandegi et al. 2014). This antioxidant property was found to be mainly linked to glutathione reductase (GHS)-dependent inhibitory mechanism that prevents the activation of neutral sphingomyelinase (N-SMase) (Soeda et al. 2007). Santhosh et al., noted the action of crocin in reducing in vivo oxidative damage induced by snake venom. The results showed an amelioration of the snakebite complications by decreasing the oxidative stress markers and the proinflammatory cytokine levels including IL-1β, TNF-α, and IL-6 (Sebastin Santhosh et al. 2013).

Another study reported that the administration of crocin (200 mg/kg) significantly ameliorated the toxicity signs in rats pretreated with Beryllium chloride ($BeCl_2$). The glutathione hepatic contents were significantly decreased, and initiation of mRNA expression of antioxidant genes including CAT and SOD were noted (El-Beshbishy et al. 2012). The neuroprotective action of saffron extract and its active compound crocin and γ-glutamylcysteinylglycine (GSH) has been evaluated in glucose-induced neurotoxicity, using PC12 cells as a model of diabetic neuropathy. The outcomes show that saffron extract (5 and 25 mg/ml), crocin (10 and 50 μM), and GSH (10 μM) could decrease the glucose toxicity by inhibiting glucose-induced intracellular ROS production (Mousavi et al. 2010).

Additionally, it was found that saffron extract ameliorated oxidative damage by decreasing lipid peroxidation and regulating mitochondrial dysfunction in the rat brain (Del-Angel et al. 2006). Zeng and colleagues studied the effects of crocetin on vascular cell adhesion molecule-1 (VCAM-1) expression in human umbilical vein endothelia cells and monocyte-endothelial cell adhesion. The results demonstrated that crocetin could significantly enhance the cellular

antioxidant capacity and inhibited angiotensin II-induced VCAM-1 expression and monocyte-endothelial cell adhesion by suppression of nuclear factor kappa B (NF-kB) activation (Zheng et al. 2015). It was indicated that graded oral administration of crocin doses (5, 10, or 20 mg/kg/day) can prevent ovariectomy-induced osteoporosis in rats. This antioxidant effect was linked to a reduction of ROS production and improvement of SOD antioxidant activity in both serum and bone tissue (Cao et al. 2014). In a study by Rodriguez-Ruiz et al., saffron was found to act as a modulator of inflammatory micro-RNA (Mir21 and Mir142-3P) in human vascular endothelial cells (HUVECs) under stress oxidative conditions. The antioxidant mechanisms involved the modulatory effect on PTEN/AKT pathway (Rodriguez-Ruiz et al. 2016).

Furthermore, it has been reported that intraperitoneal administration of crocin (15, 30, and 60 mg/kg) for 6 weeks results in improving hyperglycaemia, spatial memory deficits, and cerebral oxidative damage in streptozotocin-induced diabetic rats by reducing lipid peroxidation levels in the cerebral cortex (Ahmadi et al. 2017). Dianat et al. studied the protective potential of crocin against cigarette smoke exposure (CSE)-mediated oxidative stress, Nrf2 modifications, and impairment of cardiac function. They concluded that crocin could protect against lung injury and cardiac dysfunction caused by cigarette smoke. The protective effect was due to crocin antioxidant activity which is probably related to both upstream and downstream regulation of the Nrf2 pathway (Dianat et al. 2018).

15.4.1.5 Anti-Inflammatory Activity

Inflammation is a biological response of the immune system to harmful stimuli such as pathogens, toxic compounds, damaged cells or irradiation (Medzhitov 2010). Inflammation is a vital process of defense that is characterized by important vascular permeability changes, inflammatory pathways activation, leukocyte recruitment and accumulation, and inflammatory markers release (Chertov et al. 2000; Ferrero-Miliani et al. 2007). However, an excess response can be fatal in diseases such as rheumatoid arthritis, Crohn's disease, rheumatoid arthritis, metabolic syndrome-associated disorders, and cancers (Choy and Panayi 2001; Eiró and Vizoso 2012).

In an in vitro study by Xu et al., crocin was found to modulate the inflammatory process by exerting a dual inhibitory effect against the cyclooxygenase 1 and 2 enzymes and prostaglandin E2 production. This anti-inflammatory effect was reported with parallel prevention of nuclear translocation of the NF-κB p50 and p65 subunits (Xu et al. 2009). Deslauriers and colleagues pointed out the therapeutic potential of crocin in neuro-inflammatory disease. They conclude that treatment with crocin results in suppressing endoplasmic reticulum stress and inflammatory gene expression in spinal cords as well as reducing T cell infiltration and macrophage activation (Deslauriers et al. 2011). The therapeutic effects of crocin were studied in arthritis-induced cartilage and bone inflammation. It was reported that co-incubation with crocin could inhibit the expression of matrix metalloproteinases (MMP-1, -2, and -13) in rabbit chondrocytes in a dose-dependent manner. In addition, crocin ameliorated cartilage degeneration and alleviated inflammation by inhibiting the interleukin-1β-induced activation of the nuclear factor kappa B pathway (Ding et al. 2013). Chen and co-workers investigated the effect of crocetin on methylcholanthrene-induced uterine cervical cancer in mice. They observed an increase in plasma levels of polymorphonuclear cells (PMN), maleic dialdehyde (MDA), inflammatory cytokine interleukin 1ß (IL-ß), and tumor necrosis factor-a (TNF-a). Moreover, crocetin exerted an inhibitory effect on nitric oxide synthase (iNOS) and COX-2 expression, which are involved in inflammatory responses leading to suppression of cancerous cell growth (Chen et al. 2015). A recent study investigated the anti-inflammatory potential effect of saffron's stigma ethanolic extract (SEE) using carrageenan-induced paw edema. The stigma extract exhibited a significant anti-edematous action by regulating the acute inflammatory response induced

by carrageenan. The maximum percentage of edema inhibition was equal to 77.33% in comparison to 88.87% obtained with the standard drug Diclofenac potassium. This nutraceutical effect was attributed to the inactivation of inflammatory response, post-carrageenan injection. However, the anti-inflammatory mechanism of action needs to be elucidated (Khan et al. 2020).

There are studies showing the immunomodulatory effect of saffron and its major bioactive compounds on cell signaling pathways. Many of them have studied the MAPK pathway and its related members (such as c-Jun *N*-terminal kinase (JNK) and nuclear factor NF-B). Those proteins are known to modulate the transcription of several genes involved in the inflammatory process of the immune system (Zeinali et al. 2017). Thus, they are considered as potential targets for anti-inflammatory therapeutic agents (Cai et al. 2009). In a study by Li et al., crocin was found to significantly inhibit the LPS-induced overexpression of MMP-1, MMP-3, and MMP-13, a disintegrin-like and metalloprotease with thrombospondin motifs (ADAMTS-4, ADAMTS-5), IL-1β, TNF-α, IL-6, iNOS, and Toll-like receptor (TLR)-2 in rat intervertebral disks. In addition, crocin suppressed the LPS-induced activation of the MAPK pathway by inhibiting the phosphorylation of JNK (Li et al. 2015). Moreover, it has been reported that crocin exerted an anti-inflammatory action by inducing the heme oxygenase-1 (HO-1) expression via Ca^{2+}/calmodulin-CAMK4-PI3K/Akt-Nrf2 signaling cascades (Kim et al. 2014).

15.4.1.6 Antibacterial Effect

Bagherzade and colleagues used an aqueous extract of saffron wastages to synthesize a green silver nanoparticle. The biosynthesized nanoparticle showed significant antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Shigella flexneri,* and *Bacillus subtilis,* and was suggested for biomedical applications (Bagherzade et al. 2017). The possible antibacterial effect of saffron against clinical isolates belonging to five different serovars of *Salmonella* was examined. The saffron samples from Iran, Greece, and Spain were artificially contaminated with the clinical isolates and incubated for 32 days at room temperature. The results show a loss of viability during room-temperature storage which suggested the role of saffron in reducing the risk of food contamination by *Salmonella* (Pintado et al. 2011). Bagherzade and colleagues used an aqueous extract of saffron wastages to synthesize a green silver nanoparticle. The biosynthesized nanoparticle showed significant antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Shigella flexneri,* and *Bacillus subtilis,* and was suggested for biomedical applications (Bagherzade et al. 2017).

In another study, the reciprocal pharmaceutical effects and antibacterial action of methanolic extract of saffron and silver nanoparticles (Ag-NPs) were evaluated against methicillin-resistant *Staphylococcus aureus*, *S. pyogenes,* and *S. epidermidis*. The results reported that saffron extract, Ag-NPs, and their combined form exerted an antibacterial action, and it was found that the combination of medium concentrations of Ag-NPs (500 ug/ml) and saffron extract (50 mg/ml) was the most effective to inhibit *S. epidermidis and S. pyogenes* growth (SAMIEE et al. 2017). The antibacterial activity of saffron was also been tested against *Helicobacter pylori* (HP). The percolated methanol and aqueous extracts of saffron were tested against 45 clinical isolates of HP in comparison with amoxicillin and metronidazole. The minimum inhibitory concentration (MIC) of the methanol extract was equal to 677 µg/ml, while a significant difference between crocin and safranal antibacterial activity against 9 clinical strains was observed. The MIC average of crocin and safranal were 263 and 16.6 µg/ml, respectively (Moghaddam 2010).

15.4.1.7 Toxicity

Regarding the wide use of saffron and its bioactive compounds in therapy, the study of its eventual toxic effect is needed. Indeed, therapeutic plants, similarly to synthetic drugs may cause undesirable effects which have to be precisely evaluated in both animal and human

studies. In a study by Hosseinzadeh et al., the acute and sub-acute toxicity of safranal was studied in rat mice. The intraperitoneal LD_{50} values of safranal in male mice, female mice, and male rats were 1.48, 1.88, and 1.50 ml/kg, respectively. Histological studies did not indicate any toxic effect on the heart, liver, and spleen. However, a low pathological effect has been observed in the kidneys and lungs. Conclusively, they found that safranal was low-toxic in intraperitoneal administration, and practically non-toxic when orally administrated in both mice and rats (Hosseinzadeh et al. 2013). The sub-acute ethanolic extract of *C. sativus* L. has been investigated by Mohajeri and colleagues. The outcomes show that intraperitoneal administration of stigma ethanolic extract could significantly reduce the hemoglobin (Hb), hematocrit (HCT) levels, and total red blood cell (RBC) count. In addition, a significant dose-dependent elevation of aspartate aminotransferase (AST), alanine transaminase (ALT), urea, uric acid, and creatinine levels were seen. Those findings indicated the toxic effects of stigma extract on hepatic and renal tissues when administrated at high doses (Mohajeri et al. 2007).

In another study, the liver toxicity of crocin was investigated after intraperitoneal administration of crocin at 200 mg/kg once a week for 4 weeks. No significant changes were observed in serum parameters, GSH, Methylenedioxyamphetamine (MDA), protein carbonyls, and activities of CAT and SOD, except for the higher dose (200 mg/kg) which decreased the GSH-Px activity (Taheri et al. 2014). It has been shown that oral administration of 200 mg/kg of saffron for 28 days could significantly decrease the spermatogenesis index which includes: repopulation index (RI), spermatogenesis (SI), and tubular differentiation (TDI) in rats. The inhibitory effect was hypothetically attributed to saffron-induced-reduction of blood testosterone levels (Khayatnouri et al. 2011). Zeynali et al., studied the abortifacient effects of non-toxic doses of aqueous saffron decoction (0.8, 0.2, and 0.4%) in mice. According to their findings, placental weight and diameter, mean fetal weight, body and tail length, and biparietal diameter in all treated groups were smaller than those in control group. Moreover, saffron extracts increased the mean numbers of resorbed and dead fetuses in a dose-dependent manner, while no severe morphological abnormalities were reported in mice that received the saffron extracts on 3rd trimester. Those findings underlined the teratogenic and abortifacient effects of saffron extracts which were found to be not safe for use in the gestational period, especially in high doses (Zeynali et al. 2009).

15.5 Conclusion

Saffron has long beeng used in indigenous medicine throughout the world. Saffron exerted numerous pharmacological effect and could act as anti-inflammtory, antidepressant, antioxidant, immunomodulatory, neuroprotective and cardio-protective. Those effects convergence result in preventing, alleviating or treating various health-related conditions. The therapeutic property of saffron is largely ascribed to its bioactive compounds. Among them, crocin is the most frequently evaluated in many in vitro and in vivo studies. However, there is a need to dissect the exact mechanism of action of saffron and its major components. Several clinical investigations have been conducted in case of depression and cancer with positive outcomes. However, more studies on other disease management need to be realized. In the same line, additional clinical trials on humans should be conducted to verify the in vitro findings, and parallelly, more safety assessments should be realized to determine the possible toxic effects of saffron in long-term administration in humans.

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Part VI
Saffron Market, Trade and Production

Economic Aspects of Saffron in the World

16

Alireza Karbasi and Bahareh Zandi Dareh Gharibi

Abstract

Saffron is one of the important and valuable agricultural and medicinal products. Specific features of saffron, such as family labor requirements, low water requirements, Irrigation in non-critical times Water needs of other plants, ability to grow in clay and sandy soils, minimal machinery needs, Ease of product transportation, and the potential acceptable income for farmers have led to an increase in the cultivation and production of saffron continuously in countries. Because of the role of saffron in generating significant foreign exchange earnings for countries, its importance as a valuable export product in the economies of countries as well as the global economy is becoming more and more apparent. Accordingly, in this chapter, topics such as production, trade, price, marketing, and processing of this product have been studied.

A. Karbasi (✉) · B. Zandi Dareh Gharibi
Department of Agricultural Economics, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran
e-mail: karbasi@um.ac.ir

B. Zandi Dareh Gharibi
e-mail: b.zandi67@mail.um.ac.ir

16.1 Introduction

We start this chapter with an Introducing of saffron, production, and global trade of this product, and then we will examine the exporters of this product in the world, the description of saffron pricing, saffron value chain and its uses, issues related to the marketing of this product. Finally, we suggestions for future research on this product.

16.2 Introducing of Saffron

Saffron, scientifically known as *Crocus Sativus* is a perennial spice plant of the lily family and is known as red gold in producing countries. This plant is the most expensive cultivated plant in the world (Gohari et al. 2013). The height of the saffron plant reaches 20–30 cm. This plant has a fleshy bulb known as corm or onion. This onion is about 5 cm in diameter and weighs a maximum of 8 g. The plant has narrow leaves which are around 6–10 cm long and 2–3 mm wide. The petals of the saffron flower are light purple with red or white stripes. The flower of the saffron plant has three stigmas and these are often collected and dried to make the saffron spice. The Saffron plant has a flashy bulb called corm or onion, about 3 cm in diameter, and weighs approximately 8 g (maximum) (Hikmat et al. 2018). Its coloring properties, pleasant bitter

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taste, and attractive aroma are very valuable in the cooking and food industry. Recently, biomedical research has focused on saffron as a powerful antioxidant as well as anti-cancer, anti-tumor, anti-depressant, anti-Alzheimer's agent, and enhancer of learning ability (Bakhtiari et al. 2015). Crocin, picrocrocin, and safranal are responsible for the color, taste, and aroma of saffron, respectively. In addition, saffron petals are a good source of anthocyanins and flavonols, and therefore, can be used as dietary supplements (Behdani and Fallahi 2016). Saffron has high export importance in the agricultural economies of Iran and India. This plant grows in the Mediterranean and West Asia climates, and in very low rainfall areas of Iran that have cold winters and hot summers, especially in areas with lower humidity (Gohari et al. 2013; Moayedishahraki et al. 2010). It is believed that the origin of saffron is located in a vast area of earth-like, Greece, Turkey, Iran, and Central Asia. Saffron is currently cultivated in countries such as Iran, India, and Greece and the evidence suggests that the saffron was brought to India by the Persian rulers around 500 B.C. (Mukhter et al. 2020).

There are different types of saffron in the market that have different prices. The International Organization for Standardization (ISO) 3632 specifies specifications for the physicochemical classification of saffron into filaments, cut filaments, and powders, which is a guide to international transactions by national standard organizations in the European Union (e.g., AFNOR, ELOT) accepted (ISO 3632-1 2011). Various physicochemical characteristics classify the Saffron stigmas based on color strength, appearance, style length, and diameter of stigma. Numerous companies offering this product offer saffron to their customers in various categories. As shown in Fig. 16.1, stigma consists of a red section containing all three bioactive components of saffron and a white section called the style.

Saffron is classified with characteristics such as style length, attachment to the stigma as well as morphological characteristics of the red section. For example in Iran, stigmas that are both thicker in diameter and flatter in length, are called “strong stigma”. If a stigma is instead a thin and wavy structure, the stigma is called a “weak stigma”. Iran, as the biggest producer of saffron, has set national specifications for the characterization of saffron in addition to the international specifications. Based on Iranian standards, the different types of traded saffron are Sargol, Pooshal, and Daste (Jafari et al. 2020).

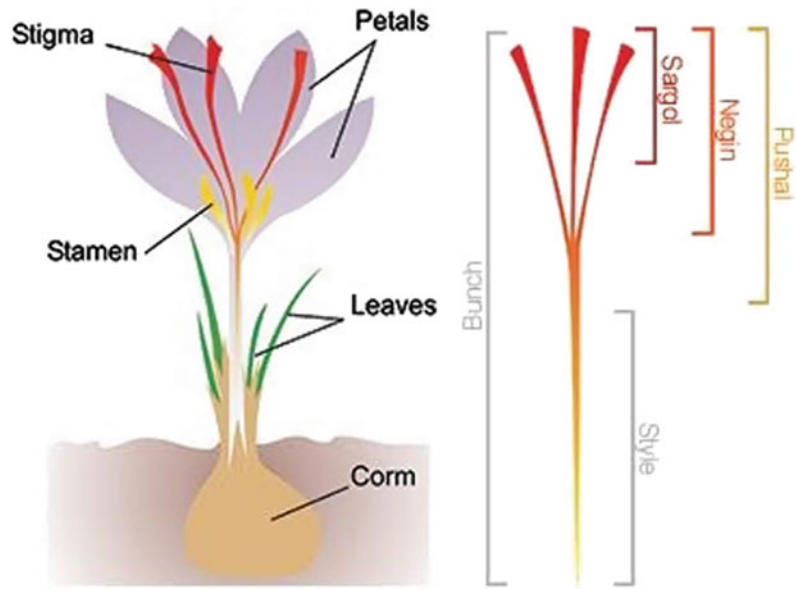

Fig. 16.1 Different parts of saffron flowers

16.3 Socio-economic Aspects of Saffron Production

Saffron is important in many ways. These include economy, medicine, foodstuff, and producing employment opportunities aspects (Golmohammadi 2019). Given that the high price of saffron adds value, this product is economically important. In addition to its economic importance, its importance is also in the agronomic, environmental, and social domains (Mzabri et al. 2019). This product is important compared to other agricultural products for many reasons. Among these reasons, we can mention the high value of this product both economically and pharmacologically, creating a lot of jobs, easy storage and transportation, and less water consumption (Asghari Lafamjani et al. 2015). Since it is possible to produce saffron on a small scale, this product can be easily produced under land restrictions. In this regard, Bouzarjomheri et al. (2016) in a study showed that in the study area due to the low water conditions of the region and the low need for water for saffron, the environmental conditions of the region are suitable for saffron cultivation. One of the important economic advantages of this product is the possibility of planting on a small scale. Therefore, farmers can cultivate saffron in the spaces between the trees using water and land. Another benefit of saffron cultivation is job creation. A lot of labor is used in the production stages of saffron. Women have a great role in all stages of the production of this product (Monazzam Esmaeilpour and Kardavani 2010). More than 80% of the labor force used in the production process of this product, including flower collection, separating of stigmas, drying of saffron, and classification and grading of the final product, are women (Azimy et al. 2020). There are also many job opportunities in the agricultural sector and beyond the harvest to the final sale and even export of saffron. Product processing and production of quality packaging are one of the most important issues that should be considered if the agricultural industry is developed for saffron. Given the importance of saffron in the global market and the need to create jobs in rural Iran, improving the production and marketing of saffron can create many job opportunities. Another advantage of saffron cultivation is the lack of need for complex machinery and equipment. Therefore, most farmers can produce saffron even with low income and low financial capital, and due to the high income of this product compared to other agricultural products and less need for investment, expanding saffron production has the potential to reduce poverty.

16.4 Saffron Production Around the World

Because saffron grows in certain climatic conditions, few countries produce it. Table 16.1 shows the average production of saffron and its share in the main saffron-producing countries of the world. Iran, Afghanistan, India, Greece, Azerbaijan, Morocco, and Spain are the major saffron-producing countries. The Islamic Republic of Iran, with 400 tons of saffron production in 2018, has an 88% share in the production of this product in the world. Afghanistan and India are second and third, respectively.

Iran, Afghanistan, India, Greece, Morocco, and Spain have traditionally been major saffron producers. Table 16.1 shows the share of each country in global saffron production. The market share of countries has changed significantly over the years, especially since 2010, due to the increasing demand for saffron. For example, Afghanistan, which started producing saffron in the late 1990s, accounted for 4.4% of global saffron production in 2018.

16.5 Trade

The position of foreign trade in development economics in different countries of the world has become so important that it is often referred to as the most important factor in the development of the domestic economy (Hendizadeh et al. 2018). Based on this, the export and import of saffron in

Table 16.1 Saffron production around the world in 2018

Country	Average annual production 2018 (tons)	Cultivation area 2018 (hectare)	Share in the world (production) 2018 (%)
Islamic Republic of Iran	400	115,000	88
Afghanistan	20	7557	4.4
India	17	5707	3.7
Greece	7	1800	1.5
Morocco	7	300	1.5
Spain	1.6	178	0.4
China	1	500	0.2
Italy	0.6	500	0.2

Resource Diverse

the world have been studied. Figure 16.2 shows the list of saffron exporting countries in the world in 2020. In the form of countries whose export value is over 45 million dollars are marked in red, including Iran, Spain, and Afghanistan, which account for 86% of the total value of global saffron exports (ITC statistics 2020) (Table 16.2).

16.5.1 Export of Saffron

For decades, Iran, Spain, and Greece have been major exporters of saffron in the world. Since 2000, the entry of countries such as Afghanistan, China, the Netherlands, and Portugal into the world market has increased and affected the value of saffron exports. The table below shows the value of saffron exports of major exporting countries during the years 2016–2020. According to the International Trade Center, the value of saffron exports in 2020 was 214 million dollars worldwide, with Iran and Spain ranking first and second in saffron exports with a share of 42% and 22%, respectively. Dedicated to themselves this year, Afghanistan and Greece also ranked third and fourth in 2020, respectively. Although currency fluctuations in recent years have affected the value of the total dollar of exports, the Islamic Republic of Iran is still the world leader in terms of volume and export value of saffron. Iran's biggest export competitor is Spain, which has been able to gain a share of the global saffron market by importing about $ 30 million (Table 16.3) worth of saffron from Iran and processing, packaging, and branding saffron.

16.5.2 Import of Saffron

The International Trade Center has announced that the number of saffron imports in the world in 2020 was worth 218 million dollars. India, the fourth largest importer of saffron and a major producer, is in high demand in South Asia. In India, it is expected to remain dependent on imports to meet domestic demand, as continued economic growth leads to more saffron being bought by households. Global markets are expected to absorb rapidly increasing saffron production in the future due to high demand (ITC Trade Map).

16.6 Price of Saffron

The most important factors affecting the GDP of trade are the price of export goods in world markets, the real exchange rate, and the export and import prices of goods. The average price of saffron (kg) in Germany, Italy, and France is between 2800 and 3500 dollars. The average price per kilogram of saffron in the UAE and Saudi Arabia is between 1700 and 2050 dollars.

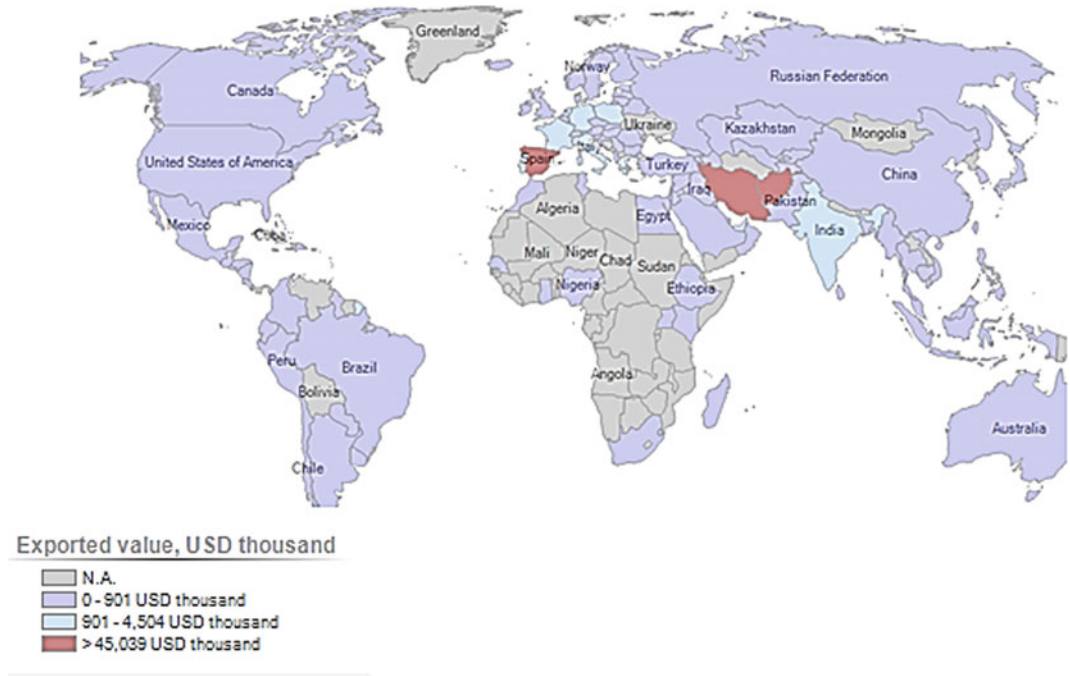

Fig. 16.2 Saffron exporting countries in the world in 2020

Table 16.2 World saffron exporters (2016–2020, USD thousand)

Exporters	Exported value in 2016	Exported value in 2017	Exported value in 2018	Exported value in 2019	Exported value in 2020
World	403,561	446,327	471,555	213,898	214,164
Islamic Republic of Iran	286,046	325,650	351,127	103,119	90,078
Spain	65,675	60,114	55,755	47,734	48,464
Afghanistan	3869	9797	21,235	26,416	45,637
Greece	3187	4185	4343	3131	4212
France	6584	7421	4732	4487	3744
Portugal	10,905	7300	4355	3780	3181
Poland	46	966	3108	2417	2477
Hong Kong, China	5208	5487	6203	5444	2406
United Arab Emirates	2143	7839	2340	2062	1838
Netherlands	4311	4558	3454	4145	1774
India	1386	1547	835	997	1650
Germany	2053	1975	2878	1992	1359
Italy	911	996	1267	1029	1021

Sources ITC calculations based on UN COMTRADE and ITC statistics

Table 16.3 World saffron importer (2016–2020, USD thousand)

Importer	Importer value in 2016	Importer value in 2017	Importer value in 2018	Importer value in 2019	Importer value in 2020
World	229,832	261,746	301,023	222,461	218,226
Hong Kong, China	8419	20,581	99,683	33,990	35,951
Spain	52,954	54,370	35,783	32,626	29,916
Saudi Arabia	9555	14,682	16,751	19,933	28,827
India	14,912	16,576	18,418	18,354	23,019
United States of America	14,944	16,417	16,560	15,761	14,846
China	3526	14,516	3680	2468	11,153
Italy	17,441	16,769	12,958	11,104	10,553
France	13,323	11,495	9850	7699	7452
Sweden	11,088	9090	7022	6545	7139
Portugal	3929	6326	3505	4902	4721
United Arab Emirates	16,665	23,547	21,505	18,247	4302
Germany	4485	4873	5391	4830	3756
Switzerland	5292	4606	3786	3473	3426

Sources ITC calculations based on UN COMTRADE and ITC statistics

This number is between 1900 and 2400 dollars for countries such as China, India, and Hong Kong (Ministry of Industry and Commerce Islamic Republic of Afghanistan 2018).

Hendizadeh et al. (2018) stated that the variable of import price per gram in importing countries increases the cost of trade and also causes the countries that have re-exports to increase their trade value after processing and final export. They showed that for a one-unit increase in the import price, the value of transactions increases by 2.5 units.

The price of saffron in the country and most likely in the international market will be affected by its final consumption. Fluctuations in saffron prices not only affect producers, but also the local economies where saffron is grown (Koocheki and Khajeh-Hosseini 2020). According to a study by Filipski et al. (2017) in a region of Morocco, a 77% increase in saffron prices during 2007–2009 improved production technology such as drip irrigation, which increased saffron flower productivity. This study showed that a 100% increase in the price of saffron causes a 133% increase in the salary of an ordinary woman who is hired for the harvest stage and a 36% increase in the salary of a man working in the cultivation stage.

16.7 Economic Performance of Saffron

The main purpose of estimating production performance is to determine whether goods and services are produced most efficiently and economically. In other words, Production performance is the relationship between the physical output of a production process and the factors of production. By comparing the economic value of the last unit of production with the cost we incur to produce it, we can determine whether the use of more production inputs leads to greater economic benefits. Therefore, the greatest economic gain is achieved somewhere. The economic profit of the last unit of production is equal to its

production cost. In many studies, different economic indicators have been used to analyze the economic performance of saffron production. These indicators are.

16.7.1 Return on Investment (ROI)

Return on investment is the percentage change in the value of an investment over a period of time.

16.7.2 Ratio of Return to Cost (RRC)

The ratio of return to cost (RRC) is another economic indicator used to evaluate the efficiency of economic activity and investment. This index is calculated using annual income from an investment divided by the cost of the investment (Salehi and Karimiyan 2017).

There are many factors influencing saffron production yield and quality before the flowering stage including the environmental conditions of the production site and agronomic conditions, etc. (Mollafilabi 2003; Negbi 2003). The quality of saffron is strongly dependent on post-harvest parameters such as improper harvesting conditions, improper drying conditions, and poor storage conditions, as well as crop management in the field. Koocheki et al. (2017) in a study examining the yield of saffron during the last 30 years in Iran stated that the decrease in saffron yield has occurred in various aspects and is mainly affected by factors such as mismanagement in agriculture, economy, post-harvest processing, and droughts.

16.8 Uses of Saffron

Saffron is used in an increasing number of applications and industries, and most likely the full potential of saffron is not yet understood. Currently, there are four main uses of saffron that should be mentioned here (more details are provided below). Saffron is used as a cooking spice and herbal medicine and in textile dyeing and perfumery. Saffron is mainly used as a condiment that provides color, smell, and taste. However, saffron can also become an element in innovative food products that adds distinctive value (Sanju an-Lopez et al. 2011). Historically, saffron has also been used for health (Kyriakoudi et al. 2015) and aesthetic purposes (Bathaie and Mousavi 2010). However, the pharmaceutical industry is still in its infancy and its use in cosmetics on an industrial scale is only partially developed. There are four main uses of saffron: cooking spices, herbal medicine, Textile and perfumery (UNIDO 2014; Ministry of Industry and Commerce Islamic Republic of Afghanistan 2018).

16.8.1 Food Industry

Saffron has a desirable and valuable place in the culinary world. Widely used as a condiment in European/Mediterranean cuisine, Middle East, North Africa, and Asia. Saffron is used as a plant and spice as well as as a flavoring. It is also increasingly used as an alternative to chemical additives, especially in Western markets (Ministry of Industry and Trade of the Islamic Republic of Afghanistan 2018).

From ancient times until today and all over the world, most of the saffron produced has been used in cooking and is still used (Basker and Negbi 1983). Saffron is widely used in the food industry due to its apocarotoid compounds crocin (color agent), picrocrocin (flavor agent), and safranal (specific aroma agent). It is used as a rice seasoning in various countries such as India, Iran, and Spain. Saffron is also used in French BouillaBaisse, Spicy Fish Soup, Italian Milano Risotto, and Cornish Saffron Cake. In Iran, saffron is used in their national food, namely, Chelo Kebab. Indian cuisine uses saffron in Biryanis, a traditional dish made from rice (Tsatsaroni et al. 1998). In Morocco, saffron is used in tea instead of mint, it is also used as a spice in various traditional dishes such as Tajine (meatballs and tomatoes) or marjoram (a sweet and salty dish made from mutton or dill). Saffron is also a key ingredient in the composition of Chermula plants, which flavors many Moroccan dishes (Modaghegh et al. 2008).

16.8.2 Medicine

In addition to being used in cooking, saffron has many medicinal benefits and is used in the treatment of various diseases such as bronchitis and cough, as an oral disinfectant, and as a sexual poison for the treatment of colds. This product has received a lot of attention due to its vitamins B1 and B2 as well as high levels of beta-carotene (Mukhter et al. 2020). Saffron has been used in traditional medicine in Iran, Egypt, and Europe for thousands of years. This product has very high potential and in the age of modern medicine, pharmaceutical companies are exploring this potential as a health supplement. In medicine, saffron is used for fever, melancholy, and enlargement of the liver and spleen. In Ayurvedic medicine, it is used to treat arthritis, impotence, and infertility. It has wide applications in Chinese and Tibetan medicines.

16.8.3 Textile Dye

In addition to all the applications mentioned for saffron, the use of saffron in the textile industry has long maintained its position and is still used today as a source for dyeing fabrics. Saffron is used in the industry for dyeing silk and yarn, etc. Saffron is used as a fabric dye, mainly in Asian countries such as India and China. The harmful effect of artificial colors has attracted the attention and use of natural colors. The use of saffron as much as an alternative dye is advantageous in the field of agro-food thanks to the high solubility of crocin in water (Ramadan et al. 2012).

16.8.4 Perfume

In the distant past, saffron was used directly to freshen the space, but now the tight perfume market has moved manufacturing companies to specific raw materials, each of which has achieved specific formulas. One of the most important components that cause the aroma of saffron is called safranal. Perfume companies have achieved specific aromas by combining safranal with other essential oils. Indeed, saffron is among the first Tanacetum balsamitas and plants used to produce oily and watery perfumes and cosmetic oils (Abrishami 1997). In Iran, saffron was recognized as an aromatic substance and it was believed that burning it as well as applying its incense to fill spaces with fragrance (Abrishami 2004).

16.9 Marketing

Marketing can be defined as activities, a set of institutions and processes of creating, communicating, delivering, and exchanging that create value for the consumer, the client, partners, and the community (Schiffman and Wisenblit 2019). In a broad definition, marketing focuses on the process by which products are delivered from the producer to the end consumer through the food system. Accordingly, marketing management includes understanding the needs of consumers and locating and selling products and services in the market (Barnard et al. 2016). Consumers are complex people whose needs and preferences are significantly different. For example, the weight of saffron supplied to the market and the type of packaging are among the important features that influence the purchasing preferences of consumers (Tohidi et al. 2021).Therefore, to design products and formulate marketing strategies that meet the needs of consumers, company managers must study consumer behavior in-depth and carefully (Schiffman and Wisenblit 2019).

As the saffron growers have no direct contact with the customer the saffron market remains unorganized as there is a long chain of intermediaries. The majority of the saffron is sold to brokers accounting for 70.86%, while 16% is sold through sub-firms and only 13% is sold through other agencies (Aga et al. 2008). Due to the lack of government intervention in the marketing of saffron, farmers are not fully aware of the price of this product and there is no price transparency in the market (Dass and Deshpande 2017; Taufique et al. 2017). Marketing and distribution of saffron are majorly dealt with intermediaries, especially brokers, local traders,

retailers, wholesalers, and firms who make huge profits leaving no scope for the farmers to progress (Qadri 2018). As there is the little role of government agencies in the marketing of saffron, farmers had to sell their local produce to intermediaries who take huge margins out of the profit and affect the growers economically.

Aminizadeh et al. (2020) in a study in Iran suggested concluding long-term contracts with Iranian saffron customers to adopt and implement production and marketing policies by further adapting Iranian saffron production and exports to the consumer culture of markets. In another study in Iran, Mohammadzadeh et al. (2020) by analyzing the competitiveness of Iranian saffron exporters in global markets, observed export standards in terms of quantity and quality, preventing saffron price fluctuations, reducing exchange rate fluctuations in accordance with government policies, reducing production fluctuations due to climate change by providing technical solutions and adequate attention of companies to short-term and long-term marketing strategies as a proposal to improve the competitive situation of the companies under study. Karbasi and Mohammadzadeh (2016) by identifying the strengths, weaknesses, opportunities, and threats in the saffron market in Iran, proposed protection strategies and location, the strategy for the recognition of saffron as an Iranian brand; product quality improvement; a database of knowledge and experience in the field of the saffron market; innovation to suit the taste of customers as suggested strategies for marketing this product.

16.10 Saffron Processing

All post-harvest operations of any product are called processing of that product, the purpose of which is to provide a market-friendly product with appropriate quality to consumers. Drying, classification, and sales packaging (Aghaei et al. 2018; Carmona et al. 2006; Ordoudi and Tsimidou 2004). Harvested saffron flowers are stored for a short time in cool and shady places and then sent to processing centers to separate the flower stigma. In the next step, the stigmas are dried traditionally by air or by using electronic dryers and dryers. In the traditional drying method, it may take up to a week, so machine-assisted drying is preferred due to its higher operating speed. In the last step, the dried stigmas are classified and graded based on color, odor, length, and shape. Fully processed saffron samples are sent to laboratories for quality testing. Finally, saffron that has a certain quality standard is considered for branding and marketing (Ministry of Industry and Commerce Islamic Republic of Afghanistan 2018). Accordingly, the use of proper and comprehensive education, holding public organizations, and promotional activities can play an effective role in encouraging farmers to conduct quality tests (Zandi Dareh Gharibi et al. 2021).

16.11 Saffron Value Chain

A commodity is compared in terms of the difference in price and real value, and in principle, the difference between the cash value of a commodity and the price that a person pays for it is the value that a person places on a commodity (Shaghaghi and Naghshineh 2006). The value chain as a strategic tool represents the set of activities that take place to create value. The value chain approach was also introduced by Michael Porter in a book entitled Competitive Advantages, and the value chain model was first proposed by Michael Porter. In this approach, the series of activities that each organization performs to present its product to its customers are systematically examined. In other words, the sets of organizational activities that cause added value in the product production process are called value chains (Hunger and Wheelen 1993). Chain management is the coordination of production, inventory, location, and transportation between participants in a chain to achieve the best combination of accountability and efficiency for market success. The purpose of the value chain is to identify systemic factors and the conditions by which value frameworks and firms can achieve higher levels of efficiency (Ruud

2010). On the other hand, the value chain of saffron is from the production of raw materials necessary for planting crops to sales and after-sales service to customers. Coordination of different parts in the value chain process is necessary to produce products with the necessary quality and with the greatest benefit for activists throughout the value chain.

Enjili et al. (2020) by prioritizing the different value chains of saffron in four areas of food, industry, cosmetics and medicine based on 6 criteria related to processing (planting, harvesting and harvesting, drying, sorting, processing, quality control and packaging operations) showed that among the food value chains of saffron, the type of encapsulation, the medicinal value chain of saffron, the type of syrup, the industrial value chain of saffron, its liquid form and the cosmetic value chains of saffron, the encapsulated form has the highest priority.

Hussaini (2016) has studied the value chain of saffron from producer to consumer. This study focuses mainly on agricultural activities and generally examines the prices and costs of other chains to identify the strengths and weaknesses of this chain for appropriate improvement. A study on the saffron value chain was conducted by Hussaini (2016) and the study of the saffron value chain is designed to interpret the entire chain from producer to consumer. This study focused mainly on agricultural activities and generally examined the prices and costs of other chains to identify the strengths and weaknesses of this chain for appropriate improvement. The value chain of saffron in this research is shown in Fig. 16.3.

Hussaini (2016) has reported that corm and labor are the most significant inputs for the cultivation of saffron. Jointly all significant inputs of production show a constant return to scale. Hence, among the four chains as a farmer, local collector, local trader, and exporter, the farmer and the exporter create high value chain.

16.12 Future Research

Hosseini and Karbasi (2020) by examining the economic and social effects of three decades of saffron research in Khorasan Razavi and South Khorasan stated that the evaluation of saffron growers from the implementation of research and transfer of results and findings has been moderate to low. It seems that the training classes are not

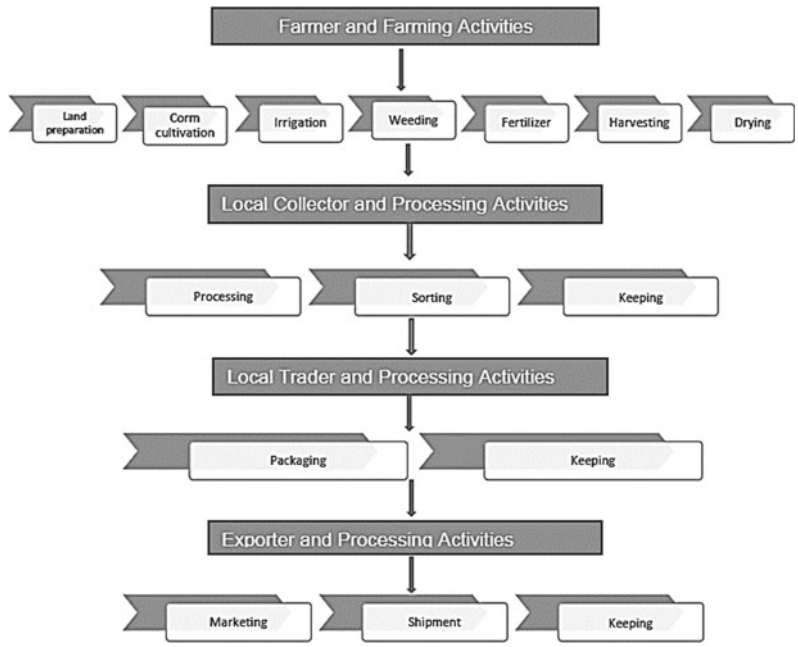

Fig. 16.3 Saffron value chain starting from producer to consumer. *Resource* Hussaini (2016)

enough for farmers and also the in-person referral of saffron promoters to farmers' farms has either not been done or has not been effective. On the other hand, according to the age and education of the studied farmers, the construction of a model farm can show farmers in practice how significantly the application of research findings in saffron cultivation increases the yield of saffron. According to three decades of saffron research, the need for research in the following areas is still felt:

Research in the field of agriculture: Integrated control of weeds, pests and diseases, the types of herbicides used by farmers, specialized saffron tools, the preparation of large onions and delivery to farmers for planting, applied research to adapt the saffron plant to changing environmental conditions, the production of real saffron seeds to improve the quality and quantity of the product.

Research in the field of economics: Fixing the real and up-to-date prices of saffron suitable for inflation and for the benefit of the farmer, product quality determination research and standardization, product market research and export development, and new farming methods.

Research in the processing department: studies examining different methods of extracting active ingredients, drying technologies, application of active ingredients in new products, principles of packaging considering international markets, studying different parts of saffron including petals, flags, and leaves.

Research in the field of promotion and education: Holding continuous educational and extension classes related to saffron issues, comprehensive and continuous research and not just for the whole farmer.

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17 Marketing Prospects for Saffron in Domestic Market: The Case of Moroccan PDO "*Saffron of Taliouine*"

Fatima Lambarraa-Lehnhardt
and Assem Lmouden

Abstract

Morocco is the world's fourth largest producer of saffron, and 95% of this production is located in the southern regions of the country in Taliouine area. The Moroccan saffron sector plays a primordial economic and ecological role. However, the lack of information on the marketing prospects of this product does raise questions on the potentialities of its market and its consumers' expectations and preferences. The purpose of this study is the analysis of the perceptions and preferences of Moroccan consumers towards this product. Particularly, the specific objectives aim firstly, the development of a strategic analysis of the Moroccan saffron sector. Secondly, the determination of consumer's preferences and attitudes and their willing to pay for this product. To achieve these objectives, we used a participatory approach with the different stakeholders in order to create a strategic SWOT matrix followed by a cluster analysis to segment saffron market and then the estimation of willingness to pay for saffron. The main results suggest that the Moroccan saffron sector is characterized by low productivity and informal marketing channels. The saffron market is organized around two consumer segments. Besides quality seekers, there is other group that paid attention to the product appearance and packaging. Finally, the willingness to pay for the label Protected Designation of Origin (PDO) "*Saffron of Talouine*" depends on the product intrinsic as well as extrinsic quality attributes.

17.1 Introduction

Morocco is the world's fourth largest producer[1] of saffron, with a production of 6.8 tons in 2018 for an area of approximately 1800 ha (ORMVAO 2019). Most of this production (95%) is located at Talouine region. The Saffron sector plays a significant role in improving income and then living conditions of small farmers in the southern region of Morocco. It is the major agriculture product at Taliouine region (MAPM 2019).[2] This agriculture activity is very profitable for the small producer located in this area. The concentration of the saffron production in the

F. Lambarraa-Lehnhardt (✉)
Farm Economics and Ecosystem Services Group, Leibniz Centre for Agricultural Landscape Research (ZALF), Müncheberg, Germany
e-mail: fatima.lehnhardt@zalf.de

A. Lmouden
United Nations Development Programme (UNDP), Rabat, Morocco

[1] Behind Iran, India, and Greece.

[2] Almost all Moroccan Saffron production (95%) come from the region of Taliouine Taznakht, the rest of the production is located in Ourika, Chefchaouen or Taza, (MAPM 2019).

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less-developed areas provides an important source of income of small household farms. The saffron picking and cutting creates seasonal employment in winter, which complement seasonal jobs provided by other agricultural activities for the rest of the year. Originally, the saffron was cultivated in a limited area of Taliouine. However, since the early 80s, the rainfall in Morocco dropped sharply and the drought has become a characteristic of the Moroccan climate. Thus, the cultivation of saffron starts to replace the less profitable cereal culture that was rain-fed oriented. This situation is very profitable for the expansion of a sustainable farming system based on low-input and crop diversification. Currently the production area is located in Souss-Massa-Drâa region and spread over the province of Taroudant (Taliouine area) and the province of Ouarzazate (Taznakht area). This area is located at the junction of the High Atlas in the north and the Anti-Atlas in the south. It is a very mountainous area with difficult access. Only the road from Ouarzazate to Agadir (via Taliouine and Taznakht) is paved (see Fig. 17.1).

Despite the expansion of the saffron production area in the last decades, the Moroccan saffron domestic market remains controlled by a traditional marketing channel, characterized by bad product reputation and deficiency of quality standards. The product is mostly sold at traditional markets (souks[3]) or herbalists (Dubois 2010). Some cooperatives working in the sector adopt the e-commerce but this circuit still limited at the international market.

Saffron is consumed in the world in various forms (condiments, medical use, aromatherapy and coloring…). In Morocco, the Saffron is mainly consumed as a condiment in filaments form. Such a form ensures the product quality for the Moroccan consumers in return for its high price (MAPM 2019). To promote the Saffron sector, the Moroccan Agriculture Policy "Plan Maroc Vert, PMV" devotes special attention to this sector. It is considered as a Terroir[4] interactive cultivated ecosystem product with high added-value and market potentialities. Such strategy was reinforced by the establishment in 2010 of a new Protected Designation of Origin (PDO) "*Saffron of Taliouine*" which guarantee the product quality and traceability from the producer to the consumer, avoid the fraud related the traditional marketing channel and promote the product at domestic market.

Even though this product promotion is beneficial for the consumer because it offers a guaranteed quality product, the lack of information on the habits and preferences of consumers towards this product imposes practical difficulties to make this process successful. The quality of saffron can contributes much more to its price determination and then influence the buying decision of the consumer at the domestic market. We suggest that the designation of origin (PDO) label "*Saffron of Taliouine*" can be a decisive attributes affecting consumers decision to buy the Saffron at domestic market. Consequently, the main objective of this study is the revelation of the Moroccan consumer preferences, attitudes and expectation towards PDO "*Saffron of Taliouine*". To reach this objective, we start first with a strategic analysis of the Moroccan saffron market through identifying the strength, weakness, opportunities and threats (SWOT matrix). Then, we use a multivariate analysis to segment the actual saffron market and to identify the potential market. Finally, we use a hedonic price model to estimate the willingness to pay for the certified product PDO "*Saffron of Taliouine*" and determine the most important attributes that affect its price. We expect that the results of this study can help the policy makers to better state the different criteria to reach the quality standards in the new established denomination of origin. Such a policy design can be beneficial for consumers through a clear positioning of high quality product at the domestic market as well as for the

[3] Souk is an open-air farmer market.

[4] Terroir can be defined as an interactive cultivated ecosystem in a given place influenced by physical environment (including climate, soil and water) and farmers know-how.

Fig. 17.1 Saffron production area in Morocco. *Source* ORMVAO (2019)

farmers by adding value to their product, which contributes to the development of local economy at marginal areas (Gresta et al. 2008).

This paper is organized as follows: in the next section, we present the methodological framework. Then, we introduce the empirical application. Finally, we expose the results and we finish with the conclusions.

17.2 Methodological Framework

The use of the marketing research enables us to evaluate the consumers' reaction to the saffron as a product in the marketplace. The objective is to know what makes the saffron attractive and which price are consumers willing to pay for this product or for a specific attribute of this product. The evaluation of the consumer preferences can be done by a panorama of traditional empirical methods using different data forms and sources. The method of hypothetical questionnaires provides great flexibility and allows to study of a multitude of market or non-market goods (Kuhfeld 2000).

This section is organized as follows, first we expose the strategic analysis methodology using the participatory approach then we explain the factorial and cluster analysis and we finish by the hedonic price modeling.

17.2.1 Strategic Analysis of Moroccan Saffron Sector

In the first stage, we conduct a strategic analysis of the Moroccan saffron sector. This analysis consists of the diagnostic of the entire system generated by the saffron product (external and internal analysis) which allows the elaboration of SWOT matrix (Weaknesses-Threats-Strengths-Opportunities) (Lambarraa 2003). The SWOT matrix gives an overview of the set of opportunities and threats that the environment of this product presents, as well as the set of strengths and weaknesses in relation to the competitive factors that define the field of activity of the (PDO) label "Saffron of Talouine" (Lambarraa and Gómez-Limón 2004). The result of the SWOT analysis is used to formulate the

strategies and policies regarding this sector. Different sorts of data are used for this analysis; first, we conducted a participatory approach with the different stakeholders (farmers, processors, retailers, consumer, scientist and expert). Then, we used additional data from the Agricultural Development Regional Office (ORMV) and the Ministry of Agriculture and Fisheries.

17.2.2 Factorial and Cluster Analysis

Other questions we want to examine is the determination of the consumer segments of saffron market. First, factor analysis is an exploratory technique applied to a collection of intercorrelated metric variables with the objective of data reduction and interpretation. The objective is to reduce and condense the information of the original variables into a smaller number of factors. The factor analysis reduces data by seeking underlying unobservable (latent) variables that are reflected in the observed variables (manifest variables). To determine the number of factors to extract, we are guided by the theory but also informed by running the analysis extracting different numbers of factors and seeing which number of factors yields the most interpretable results. Second, we used the Cluster analysis. The Cluster Analysis is an explorative analysis that tries to identify structures within the data. Cluster analysis is also called segmentation analysis or taxonomy analysis. It tries to identify homogenous groups of cases, i.e., observations, participants, respondents. This technique is used in order to classify the objects based on measured characteristics into a series of segments. In the way, that is homogeneity within segments and heterogeneity between segments. The consumer segments may be identified based on their perceptions of products, the benefits they seek from products or their lifestyles (Lambarraa-Lehnhardt et al. 2020; Saunders 1980). The goal is the identification of homogeneous consumer segments regarding "*Saffron of Taliouine*" preferences. The cluster analysis was conducted on the factor scores and employed a hierarchical method, which produced two main groups.

17.2.3 Willing to Pay for the PDO "*Saffron of Talouine*"

Finally, we estimate the hedonic regression model. It is a relatively new marketing tool. The first work dates back to the mid-50s with the psychological study of consumers' behavior. Then in the late 60s, the relationship between mathematics, statistics and psychology bring evident supplements in the development of this analysis (Lancaster 1966). In the early 70s, with Green and Rao (1972), this method becomes a tool in marketing research to assess the willing to pay of consumers. According to Saporta (1998), the model of hedonic prices is a regression that predicts ordinal variable by using qualitative variables. The hedonic regression is therefore an empirical practice to measure the contribution of product characteristics to its price (Terra 2005). It explains statistically a product price by its characteristics (Rosen 1974). The hedonic price function determines as well the implicit price of each characteristic. The model of hedonic prices has large applications in the consumer research topics (Cuevas et al. 2016; Wang et al. 2009; Suwannaporn and Linnemann 2008; Stanley and Tschirhart 1991; Abansi et al. 1990).

The model specification is

$$P_i = \alpha x_i + \beta z_i + \varepsilon_i + c \qquad (17.1)$$

where P_i is the willing to pay (WTP) for PDO "Saffron of Taliouine" by consumer i, x is a vector of physical attributes characterizing "Saffron of Taliouine" purchased by consumer i, z is a vector of socioeconomic characteristics describing consumer i, ε is the error term of the model and c is the constant of the model. The hedonic model expressed in Eq. (17.1) is estimated in semi-log functional form using Ordinary Least Squares (OLS). This regression is possible only if the price is following the normal distribution. To assure that, we use a logarithm transformation of variable price. Traditionally, the estimated coefficients from the hedonic regression are interpreted as consumers' WTP for a given attribute of the product. A positive sign indicates that consumers are willing to pay a

price premium for the attribute, while a negative sign reveals that consumers discount the attribute.

17.3 The Empirical Application

To realize the exploratory study of consumers' preferences, the data used in this analysis were obtained from face-to-face questionnaires with Moroccan consumers. We started first by conducting a pilot survey with different stakeholders (consumers, Herbalists, cooperatives and retailers) in order to prepare the final survey and setting up the attributes of the product (Lambarraa and Elyoubi 2018; Kallas et al. 2011). The questionnaire collects information on consumer's socioeconomic characteristics, their attitudes and perceptions toward "*Saffron of Taliouine*", questions on Saffron consumption and purchasing, saffron quality attributes and willing to pay for the product. The final sample consists of 106 consumers. Our data set is completed with other additional data sources from Moroccan Ministry of Agriculture (MAPM 2019) as well as interviews with professionals of the sector (cooperatives, herbalists and experts at ORMVAO).[5]

Based on the results of our pilot questionnaire, the expert's interviews, the established PDO[6] "saffron of Taliouine" and prior research[7], we define the following saffron attributes that strongly affect the buying decisions of Moroccan consumers:

- Quality of the product (flavour, aroma, taste and colour)
- Product form: filament or powder
- Type of packaging: bag or glass bottle
- Certification: Certified organic or not
- Origin of produce: Taliouine or not
- Control the drying method.

[5] Experts from the Ouarzazate Agricultural Development Regional Office (Office Régional de Mise en Valeur Agricole d'Ouarzazate), located in the main production area of saffron.

[6] As defined by the Moroccan Agriculture Ministry.

[7] Research performed on Saffron Quality attributes (e.g., Ehsanzadeh et al. 2004; Lage 2009).

17.4 Results

17.4.1 Strategic Analysis of the Moroccan Saffron Sector

The results of the strategic analysis of the Moroccan Saffron sector are summarized in Table 17.1.

The strategic analysis shows that the Moroccan Saffron sector in general, and the "*Saffron of Taliouine*" in particular, has a good potential to grow and expand further, particularly in terms of potentiality at the Moroccan and international market. The production needs to be expanded further in order to meet the increasing domestic market and to profit from the opportunities exiting at the international export market (particularly the USA and EU). Moreover, the region of Taliouine—Tazenakht is located in a less favoured area suffring since the last decades from biodiversity loss (Birouk 2009). The saffron farming system is a good alternative to enhance the biodiversity and improve the ecosystems services at these marginal areas. The "Saffron of Taliouine" positioning at the domestic and international market can be reinforced by increasing the quality of the product granted by the PDO "*Saffron of Taliouine*". Those strategic actions can stimulate the economic development and fight against poverty in the production area. However, a main constraint to promote this sector is the dominance of the traditional Farming techniques, which leads to low productivity and affects the quality of the product. Additionally, the marketing channels is mainly informal and controlled by a high numbers of intermediaries. Those constraints affect the positioning of the PDO at the domestic market and limit the access to the highly competitive international market.

17.4.2 Reduction by Factor Analysis

The Factor Analysis (FA) allows the reduction of a large number of variables to a smaller number

Table 17.1 SWOT matrix of Moroccan saffron sector

Strengths	*Weaknesses*
• Product with high added value • PDO that preserve product quality, identity and geographical origin • Farming type adapted to the climatic condition of the production area • Sustainable agriculture with low input farming system • Important source of income for the population located in less favoured mountains area • Promotion of the product by the Moroccan Agricultural policy "PMV" as a Terroir product	• Traditional Farming techniques, low productivity and high production costs • Reduced offer on high quality bulbs • Drought and water shortage problem • Unskilled (insufficient training programs) and scarce labour • Excessive Land fragmentation • Low potential of cooperation and organisations between farmers • Bad storage (in plastic bags) and conservation conditions (harvest during the day) which affects the product quality • Sales mostly at traditional markets with Non-competitive prices • Informal and undeveloped Marketing channel controlled by intermediaries (wholesalers, retailers...)
Opportunities	*Threats*
• Increase of the saffron demand at the domestic market • Increase of Moroccan saffron exportation and the opportunities at the international market after the signature of several free trade agreements (e.g. with EU and USA market) • Increasing the world spices demand • High potential demand at the tourism market in the production area • New technologies to increase productivity & adopt organic techniques • Comparative advantage of product quality compared to Iran (main rival) • High labour cost at competing countries (mainly in EU: Spain, Italy, Greece & France)	• Strong competition at international market accelerated by the liberalization of agricultural markets between countries • More than 80% of Saffron world trade is controlled by EU • Increase of Spanish exports (using 90% of Iranian production) • High prices at the world market which increase the demand for substitutes • Strong rural emigration which affect labour offer at the production area • Fraud problem affecting the quality and reputation of the Moroccan Saffron • Saffron Pest and Diseases

by grouping the variables that measure the same dimension based in shared variance (lambarraa et al. 2002). Then, we obtain factors that are representing the variables of interest. In our survey, we had many variables that characterize the preferences of the Moroccan consumers toward saffron, and we want to regroup those variables in the main factors to facilitate their interpretation. To construct the components matrix we removed variables having a value less than 0.3. Furthermore, we keep only the variables having a value greater than 0.5. This allowed a total explanation of 74% of the information for the two selected axes.

Table 17.2 shows the selected factors using a component matrix, which describes the correlation of each variable with the corresponding axis. From Table 17.2, we can restrict the initial variables into two main factors; Packaging/appearance and saffron quality.

a. ***Packing and Appearance***

The main variables summarized in this component are dry Saffron, bag and glass Packaging, fresh Saffron (less than a year of harvest), Saffron mixed with other spices, Filament-Saffron and finally organic certification of the product. Thus, the negative sign of the coefficient of saffron mixed with other spices shows that this attribute adversely affects the appearance of the product. Thus, Moroccan consumers avoid buying this type in fear of falsification. The coefficients of fresh filament Saffron are high (>0.75). The filament form is preferable to prevent any attempt of fraud. The fresh Saffron (less than a year of

Table 17.2 Factor analysis results

Variables	Factors	
	Packing and appearance	Saffron quality
Dry Saffron	0.562	–
Bag packaging	0.527	–
Glass packaging	0.565	–
Fresh Saffron (<1 year of harvest)	0.759	–
Saffron mixed with other spices	−0.519	–
Filament-Saffron	0.755	–
Certified Saffron	0.506	–
Fast drying	–	0.609
Talouine origin	–	0.633
Red color	–	0.651
Bitterness and powerful aroma	–	0.667
Taste	–	0.535
No impurity (including pistil zone)	–	0.567
Ground Saffron	–	−0.574
Pure Saffron	–	0.762

Source Own Elaboration (2019)

harvest) is characterized by strong fragrance, flavor and aroma. In light of these results, we can say that the consumption of saffron in the Moroccan market is heavily dependent on the method of packaging, appearance (filament, dry, and fresh) and the presence of labelling information especially the organic certification.

b. ***Saffron quality***

This second factor is explained mainly by the variables red color, powerful aroma, bitterness, taste, ground saffron, drying method, purity and the origin of Taliouine. The majority of coefficients has positive signs, which means that good quality saffron should be from Taliouine, red color, bitter (spicy taste) with powerful aroma and without any impurities. In this sense a fast drying method is required for the product to keep its aroma (Safranal). The ground Safran has a negative value which shows that the saffron powder is poorly appreciated by the Moroccan consumers and it is considered as a sign of low quality.

17.4.3 Cluster Analysis

Using the result from the factorial analysis, the next step is the classification of the "saffron of Talouine" consumers into a series of homogenizes groups. The classification of the consumers into different segments is based on their perceptions and preferences towards the product attributes. In this study, we used extrinsic variables (e.g. packaging, certification) and intrinsic variables (e.g. quality of the product) as well as socioeconomic variables. The results of this analysis show two main groups of "Saffron of Taliouine" consumers. The first group represents 65% and the second one 35% of the total sample.

Overall, it is clear that the whole sample gives more importance to the intrinsic quality attributes comparing to the appearance. In the first market segment, consumers prefer "Saffron of Taliouine", pure, dark red, with a bitter taste, strong aroma and without any impurity. While in the second segment, consumers tend to prefer the product's appearance and packaging. For this class, the saffron must be packed, certified, dry,

Table 17.3 Characteristics of the consumers groups using socioeconomic variables and willing to pay for the product

Variable form		Groups (%)		Total (%)
		G1	*G2*	
Sex	Male	55.6	0	55.7
	Female	10.4	34	44.3
Occupation	Free occupation, entrepreneur	**35.8**	0	35.8
	Executive	24.5	27.4	51.9
	Worker	4.7	0	4.7
	Retired	0	3.8	3.8
	Housewife/jobless	0	3.8	3.8
Studies	Not finish primary school	2.8	0.9	3.8
	Primary school	2.8	0	2.8
	Secondary school	15.1	1.9	17
	University studies	45.3	31.1	76.4
Income ($)	<150	1.9	0	1.9
	150–300	10.4	0.9	11.3
	300–450	13.2	4.7	17.9
	450–600	23.6	15.1	38.7
	>600	17	13.2	30.2
Preferred place of purchase	Hypermarket	0	4.7	4.7
	Souks	11.3	1.9	13.2
	Herbalist	20.7	13.2	34
	Cooperative	23.6	12.3	35.8
	Farmers	8.5	3.8	12.3
Age	Year	35	38	–
Willing to pay	$	3.52	4.1	–

Variable form are socioeconomic indicators for the consumers
Groups are the different groups of consumers
Total are all the consumers' sample
Bold value represents a consumers are entrepreneur or selfemployed

fresh and not mixed with other spices. To better describe those segments, we use the socioeconomic characteristics (see Table 17.3).

According to the results in Table 17.3, we can characterize each class according to the parameters taken into consideration:

- *Quality seekers Consumers* (G1): The majority are men, entrepreneurs or with free Occupation. The income level is more than $ 300. An average age of 35 years and willing to pay of 3.52 $/g. This segment buys Pure "*Saffron of Taliouine*" in bulk form mostly from Souks and herbalists. At the purchasing time, those consumers consider important the following attributes: colour, aroma, flavour, taste, purity and the filament form.
- *Packaging-labeling oriented Consumers* (G2): are employed women with high university studies. Their income exceeds $ 450. This group of consumers consider the form of appearance (packaging), organic certification, the product quality attributes (dry product, filament form and not mixed with other spices) when buying the saffron. The main place of purchasing is Herbalist, cooperative and

hypermarket. Indeed, this type of consumer has a higher willing to pay with 4.1 $/g.

Figure 17.2 summarizes domestic market Segmentation of the "*Saffron of Taliouine*".

17.4.4 Willing to Pay Model

The hedonic price method allows the determination of the implicit price of each attribute of a product that consumers can pay.

The explanatory variables retained in the estimated hedonic price model are

- Product origin: having Taliouine origin or not
- Organic certification: yes or not
- Packing form: glass bottle or Bag
- Saffron quality attributes factor (issue from factorial analysis which involved a series of quality attributes)
- Humidity degree of Saffron (dry or not)
- Form of Saffron: Filament or powder
- Consumer Income

Results derived from estimating the semi-log model are presented in Table 17.4.

All explanatory variables are significant at 1% and 5%. The signs of the different parameters are positive which means that the price of a gram of saffron can be explained by its quality attributes, the presence of organic certification, packaging, the origin of Taliouine, the form and the degree of the humidity. The results also suggest that the consumers' willingness to pay for saffron increased with income.

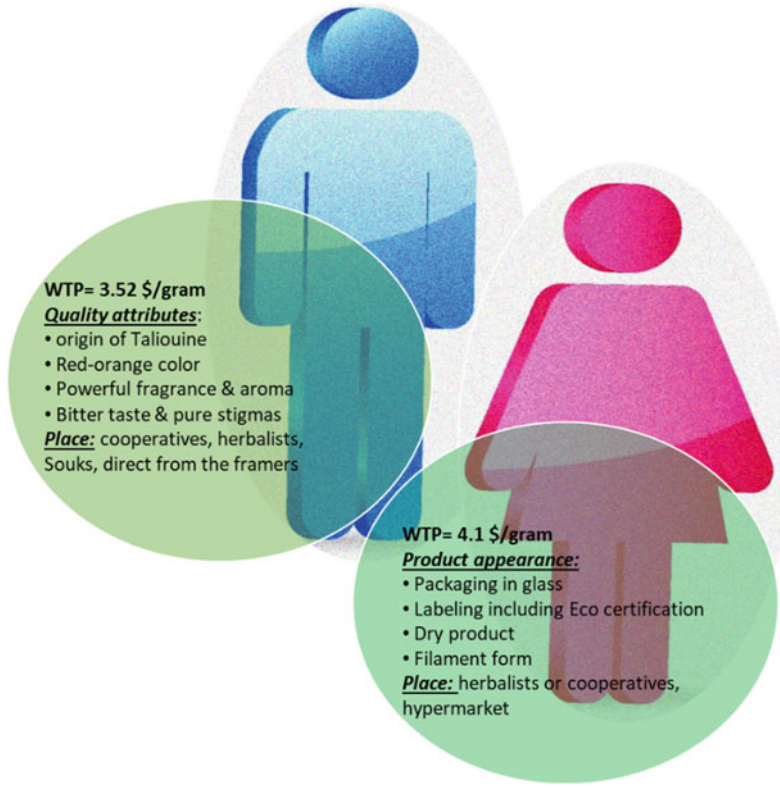

Fig. 17.2 Market segmentation of the "*Saffron of Taliouine*". *Source* Own Elaboration (2019)

Table 17.4 Estimation results of Hedonic price model

Variable	Estimate	Standard error
Taliouine origin	0.105	(0.024)***
Certification	0.516	(0.120)***
Packaging	0.336	(0.124)**
Form of saffron	0.094	(0.018)***
Quality attributes	0.790	(0.034)**
Humidity	0.052	(0.023)**
Income	0.102	(0.047)**
R^2	0.787	
F	50.290	

Note *** and ** indicate that the parameter is significant at the 1% and 5% respectively

Using the estimated model, we can decompose the willingness to pay off the consumer with the objective of calculating the implicit price of each attribute. Figure 17.3 shows the results of this decomposition.

Finally, we can conclude that Moroccan consumers willingness to pay (WTP) for quality improvement increased by 6.17 $/10 g of saffron. Improvement in the quality means an improvement on colouring (red), bitterness, aroma, taste purity, fast drying technique (to better conserve organoleptic characteristics) and an organic certification. The impact of the quality attributes is important on the implicit price of Saffron in comparison to other attributes. The WTP for an organic certified Saffron is 3.29 $/10 g which means that it is important to use an appropriate organic certification logo on the label of saffron produced in an organic way. The glass packaging is more preferred than the bag, so consumers are willing to pay 2.17 $ more for 10 g of Saffron packed in a glass bottle compared to a plastic bag. The Moroccan consumers are willing to pay $ 1.28 more for a 10 g of Saffron originally produced at Taliouine area. The filament form and dry saffron have WTP of 1.24 $/10 g and 1.13 $/10 g respectively. Ultimately, once the consumer's income increases by $ 150, its WTP for 10 g of saffron increases by 1.27 $.

17.5 Conclusion

The main objectives of this study are the strategic analysis of the Moroccan saffron sector, the analysis of the saffron market and the determination of the Moroccan consumer's preferences

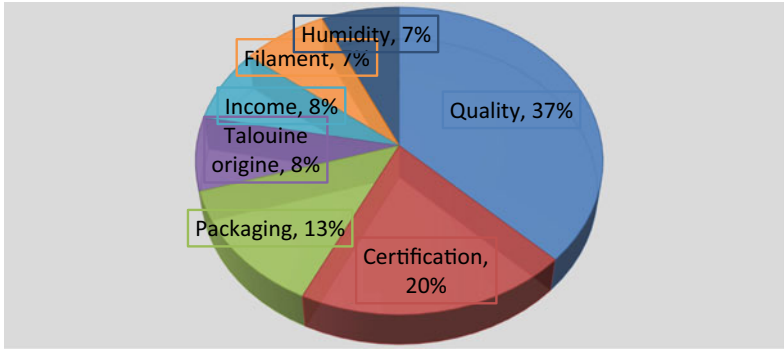

Fig. 17.3 Implicit price weight of each attribute of saffron

and expectations towards "*Saffron of Taliouine*". To reach those objectives, we established a strategic analysis of saffron sector followed by a market segmentation and then estimate the variables affecting the willing to pay for saffron.

The results from the strategic analysis show that the Moroccan Saffron sector can benefit from several strengths and opportunities that make it a key for a local development of marginal area at the atlas mountain and a medium to increase small farms income and to fight against poverty. The saffron cultivation is considered a good alternative for a more sustainable farming system than can enhance biodiversity and ecosystems services at less favoured production area. However, many factors handicap the development of this sector in Morocco such as traditional farming techniques and the informality of marketing channels, which affect the quality and then the valorisation of the final product. The organization of the sector, the modernization of the farming technique and the instauration of formal marketing channel seems to be urgent actions to take into consideration in order to increase product competitiveness at the national and the international market.

The segmentation of the Moroccan saffron market report two types of consumers. Besides the quality seekers, exist other group more focused in the product appearance, packaging and organic certification. The quality component remains an important attribute for Moroccan consumers. It is appreciated for its intrinsic characteristics (e.g. red colour, aroma, flavour, bitterness, purity of the product) and extrinsic characteristics (e.g. Taliouine Origin, organic label, and packaging). The filament remains the most preferred consumed way because it guarantee the quality of the product. Consequently, it seems important the consideration of the different quality attributes, the packaging and the organic certification in the new established PDO "*Saffron of Taliouine*".

Finally, more action should be taken regarding saffron quality, packaging and organic certification in the new established PDO label "*Saffron of Taliouine*" in order to improve its visibility and positioning at the national market.

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