UNIVERSITÀ DEGLI STUDI DI TORINO

SCUOLA DI DOTTORATO

DOTTORATO IN
SCIENZE AGRARIE, FORESTALI E ALIMENTARI

CICLO: 35

Sustainable Horticultural Solutions to Improve Saffron (*Crocus sativus* L.) Production

Stefania Stelluti

Docente guida:
Prof. Valentina Scariot

Coordinatore del Ciclo:
Prof. Domenico Bosco

ANNI
(2020; 2021; 2022)

Preface

The Ph.D. project presented in this thesis has been carried out at the Department of Agricultural, Forest, and Food Sciences of the University of Turin, from 1 November 2019 to 31 January 2023, under the supervision of Prof. Valentina Scariot and in collaboration with Dr. Valeria Bianciotto and Dr. Erica Lumini of the Institute for Sustainable Plant Protection (National Research Council – Turin, Italy). A research stage abroad of seven months, from June 2022 to January 2023, was spent at the Departamento de Producción Vegetal of the Universitat Politècnica de València (Valencia, Spain) under the supervision of Prof. Rosa Victoria Molina Romero and in collaboration with Prof. Sergio Gonzalez Nebauer (Departamento de Producción Vegetal of the Universitat Politècnica de València, Valencia, Spain), Prof. Begoña Renau-Morata (Institut de Biotecnologia i Biomedicina of the Universitat de València, Burjassot, Spain), Prof. Gonzalo Luis Alonso (Catedra de Química Agrícola, E.T.S.I. Agronomos y de Montes of the Universidad de Castilla-La Mancha, Albacete, Spain), and Prof. María Lourdes Gómez-Gómez (Botanical Institute of the Department of Science Technology, Agroforestry, and Genetics of the University of Castilla-La Mancha, Albacete, Spain).

The research was funded by the program Interreg V-A Francia Italia Alcotra (Grant No. 1139 "ANTEA – Attività innovative per lo sviluppo della filiera transfrontaliera del fiore edule"; and grant no. 8336 "ANTES - Fiori eduli e piante aromatiche: attività capitalizzazione dei progetti ANTEA ed ESSICA") and by the project titled "SaffronALP—Lo zafferano di montagna: tecniche sostenibili per una produzione di qualità" —Fondazione Cassa di Risparmio di Torino (RF = 2017.1966).

Index

Introduction

***Crocus sativus* L.**

Saffron (Fam. Iridaceae) is a cormous geophyte wordly known for the most precious saffron spice, named "red gold" (ca. 15,000 - 20,000 € kg^{-1}), which is obtained by dehydrating the red stigmas of its flowers. Saffron is a subhysteranthous plant as the flowers can appear before or after the appearance of the leaves. One or several flowers (usually three and up to 12) are produced per corm and more than 110,000 flowers are needed to obtain 1 kg of spice. The price, the highest among spices, is due to the intense manual labor required for this crop, especially to harvest the very perishable flowers and gently separate the stigmas to produce the spice during the short flowering (two to three weeks), which in Mediterranean climates occurs in autumn (Caser et al., 2019; 2020; Gresta et al., 2008). A vegetative phase follows, during which the leaves are photosynthetically active, providing nutrients for the formation of underground replacement corms, which are completely formed and enlarged in spring, when leaves are senescent. Root growth also occurs from autumn to spring. Corms then enter dormancy to overcome the dry summer season. The plant is sterile autotriploid (2n=3x=24) and reproduction is only vegetative through clonal replacement corms, which form annually and replace the mother corm. Thus, saffron is known as one cultivar, descended from a sterile triploid plant that once spontaneously arose in nature (Mir et al., 2015). Flower formation is tightly related to corm size, as it increases with larger corms, which contain sufficient nutrient reserves to support the early growth stages and ensure blooming (Renau-Morata et al., 2012; Stelluti et al., 2023). Commercially, corms of 2.5 - 3.5 cm in diameter and 10 - 20 g in weight are usually used (Caser et al., 2019; Kumar et al., 2008).

Probably originated from ancient Greece, saffron has a long history and strong tradition in the Middle East and Mediterranean regions. The saffron spice has been traditionally used as seasoning, natural dye, and in folk medicine and recent

revisiting of its bioactivity has renewed consumer and scientific interest for this plant (Cardone et al., 2020; Kazemi-Shahandashti et al., 2022). The organoleptic and therapeutic properties of the spice mainly depend on the content of apocarotenoids deriving from zeaxanthin, namely crocins (dyeing capacity), picrocrocin (bitter flavour), and safranal (inebriant aroma) (Caser et al., 2018; 2019; 2020; García-Rodríguez et al., 2014; 2017). The tepals of the saffron flower are used in Persian traditional medicine as they have bioactive properties as well, which are mainly ascribed to the presence of phenolic compounds, such as anthocyanins (Caser et al., 2020; Stelluti et al., 2021; 2023b).

The global production of saffron was 418 t y^{-1} in 2018. The main producer country is Iran, holding ca. 90% of world production (108,000 ha and 376 t in 2017, mostly in the Khorasan province), followed by Afghanistan (Herat Province, 7,557 ha), India (Jammu and Kashmir regions, 3,674 ha), Greece (mainly Kozani, 1000 ha), Morocco (mainly Taliouine, 850 ha), Spain (Castilla-La Mancha, Albacete, Toledo, Cuenca and Ciudad Real, 150 ha), and Italy (70 ha) (Cardone et al., 2020). In Italy, saffron is mainly produced in Sardinia (about 40 ha in S. Gavino, Cagliari province), Abruzzo (6 ha in Altopiano di Navelli, L'Aquila), and Sicily (5 ha in Enna); small cultivations are situated in other regions, such as Umbria (Perugia, Cascia), Tuscany (San Gimignano, Arezzo and Maremma Grossetana), and in South Italy (Cardone et al., 2020; Gresta et al., 2008). More recently, its cultivation has started in the north western Italian Alps as an alternative crop to divesify agricultural production and as a new source of income (Caser et al., 2018, 2020).

Saffron cultivation, changelles and perspectives

Saffron thrives best in a Mediterranean climate (hot and dry summers and mild winters), but it can tolerate wide ranges of climate (surviving frosts as cold as –10°C and short periods of snow cover) (Khan et al., 2020; Kumar et al., 2008).

Cultivation is generally carried out in open fields as a perennial cycle for 3 - 5 years. Generally after 4 - 5 years, spice yield decreases mainly due to competition for water and nutrients, disease and reduced reproductive capacity of the corms

(Gresta et al., 2008; Shokrpour, 2019). Sowing is commonly carried out between May and September depending on the place (Gresta et al., 2008). Early autumnal rain/irrigation is crucial to awake the corms and appears to enhance flower yield and accelerate blooming (Gresta et al., 2008; Kumar et al., 2008).

Thermoperiodism and soil characteristics are among the main environmental factors influencing saffron production (Caser et al., 2018). Optimal flower formation can be achieved by incubating corms at warm temperature (i.e., 23 - 27°C) for more than 50 days and less than 150 days for flower induction and at mid-low temperature (i.e., 15 - 17°C) for flower emergence (Molina et al., 2005). Saffron grows well in loose, low density, well irrigated and drained soils with optimum pH range between 6.8 and 7.8, and electrical conductivity (EC) below 2 dS m^{-1} (Salas et al., 2020; Gresta et al., 2008). Irrigation is not necessary as its water requirements can be satisfied by the rainfall in semi-arid conditions (Gresta et al., 2008). Nutrient availability, especially N and P, mainly affects the growth of saffron corms during the vegetative phase (Koocheki and Seyyedi, 2015; Kumar et al. 2008). Fertilisation generally consists in the application of about 20 - 30 tons ha^{-1} of organic manure (Gresta et al., 2008).

In the last decades, saffron produced in European countries has seriously decreased, e.g., about -98% in Spain (where the area of saffron cultivation was 6,000 ha in 1971), -38% in Greece (1,600 ha in 1982), and -98% in central Italy (Abruzzo) (300 ha in 1910). Conversely, the production has increased in Iran althought the yield per unit area has significantly fallen (from 5.1 kg ha^{-1} in 1982 to 3.5 kg ha^{-1} in 2017) (Cardone et al., 2020). The reason is mainly that technology has not progressed for this plant cultivation and manual labour cost has increased. In fact, most crop management techniques (sowing, weeding, flower picking, and stigma separation) are still performed manually all over the world (Gresta et al., 2008; Shokrpour, 2019). If hysteranthy is induced (by incubating the corms at 15 °C for 35 days), the harvesting of the flowers can be mechanized with mowers or grass cutters without damaging the leaves, while machines for harvesting bulbs and tubers can be adapted for lifting the corms (Cardone et al., 2020; Gresta et al., 2008). Furthermore, the plant sterility limits

the application of conventional breeding approaches for the improvement of this crop (Mir et al., 2015). Saffron cultivation is also facing challenges such as labor shortages, climate change (water deficit, salinity, and higher temperatures), poor soil fertility, soil diseases, infestation by rodents, and virus and fungal diseases (*Fusarium oxysporum*, *Penicillium* spp, *Rhizoctonia violacea*), which are reducing its world production (Gresta et al., 2008; Cardone et al., 2020).

To make saffron more profitable, shifting its cultivation to controlled environments has been proposed (Askari-Khorasgani and Pessarakli, 2019; Avarseji et al., 2013; Caser et al., 2019; Molina et al., 2005b; Salas et al., 2020). Flowering period could be extended, yield and spice quality increased, and crop management facilitated and improved with proper nutrient solutions and growing plants without pests or pathogens (Cardone et al., 2020; Caser et al., 2019; Molina et al., 2005b; Salas et al., 2020). Further, controlled conditions may counteract the negative impact of global soil degradation and climate change and increase land-use efficiency (Askari-Khorasgani and Pessarakli, 2019). The interest in the use of bioinoculants has recently increased as they are considered a sustainable agricultural solution to enhance plant quality and productivity, cope with the effects of climate change, and reduce environmental costs (Backer et al., 2018; De Pascale et al., 2017; Genre et al., 2020; Rouphael and Colla, 2020). So far, the use of bioinoculants for saffron cultivation has been still poorly investigated in controlled conditions, with generally positive results especially as regards quantitative-productive traits (Ambardar and Vakhlu, 2013; Caser et al., 2019; Kour et al., 2018; Magotra et al., 2021).

Bioinoculants: state of the art and challenges

The agricultural sector is recently facing the challenge of increasing productivity to feed a growing population while reducing the use agrochemicals like pesticides and fertilizers, thus the impacts on ecosystems and human health. In this contest, sustainable food systems is the heart of the European Green Deal and the Farm to Fork Strategy. A promising and sustainable innovation would be the use of microbial inoculants, such as plant growth promoting rhizobacteria (PGPR) and

arbuscular mycorrhizal fungi (AMF), which could complement agrochemicals, thus reducing their application. The use of microbial inoculants in horticulture has been increasing over the last two decades (Rouphael and Colla, 2020; De Pascale et al., 2017). PGPR have relevant properties, such as N-fixation, P-solubilization, siderophores and phytohormones production, and biological control (Backer et al., 2018; Lobo et al., 2019). The most effective PGPR and abundant genera reported in the rhizosphere were species from *Pseudomonas* and *Bacillus* genera. Many formulations have been applied to different crops (e.g., legumes and cereals) in the last decade (Backer et al., 2018; Lobo et al., 2019) and N-fixing bacteria of the genera *Rhizobium*, *Azotobacter*, and *Azospirillum* have been recently defined as "plant biostimulants" (Regulation EU, 2019/1009). The AMF are also included in this category. Generally, only a few AMF species are used for inocula formulation, which are generalist, widely distributed, and easily propagated (mainly *Rhizophagus intraradices*, *Funneliformis mosseae*, *Rhizophagus irregularis*, and *Claroideoglomus etunicatum*) (Basiru et al., 2021; Berruti et al., 2016). Belonging to the subphylum *Glomeromycotina*, AMF are obligate biotrophs that establish mutualistic symbiosis with most plants including relevant crops (mainly Fabaceae, Asteraceae, Poaceae, and Solanaceae) (Berruti et al., 2016; Genre et al., 2020; Lanfranco et al., 2018). They supply the plant with water and both AMF and PGPR can improve the mineral nutrient uptake by plants, crop yield, production of secondary metabolites, and tolerance to biotic and abiotic stressors, such as extreme temperatures, drought, and salinity (Rouphael and Colla, 2020; De Pascale et al., 2017).

Major players in the mycorrhizal industry are in the United States, Canada, Germany, Italy, Czech Republic, United Kingdom, and Spain. Reports indicated Asia-Pacific as the third largest player (after North America and Europe) in the global bioinoculants market with increasing demand in India, China and Taiwan. Production and application of AMF inocula are still very low in developing countries especially Africa, where South Africa and Kenya are among the major players (Basiru et al., 2021).

Producing large quantities of AMF-based inoculum is an expensive and demanding process. In fact, AMF, being obligate symbionts, cannot be cultivated in pure cultures and, most commonly, an AMF inoculum is obtained after a known AMF isolate and a host trap plant (i.e., a plant that can be massively colonized by many AMF species) are grown together in an inert medium (crude inoculum). Carrying out an extensive inoculation treatment in open-field could seem economically prohibitive. However, the application represents an initial cost as the AMF fungi will persist and give economic profits over time. In fact, AMF, as well as PGPR, can mantain long-term soil health and fertility, improve crop quality and productivity, and the tollerance to abiotic stress, which have tremendous negative impacts on crop performance and are predicted consequences of climate change and environmental degradation. Both AMF and PGPR also perform the roles of fertilizers and pesticides, and are more cost-effective than conventional fertilizers, especially in regions where phosphorus depletion in soils is a serious plant nutrition problem (Basiru et al., 2021; Berruti et al., 2016; Ceballos et al., 2013). Results from a recently conducted meta-analysis suggested that reducing PGPR-mediated plant stress led to increased gains than nutrient improvement on crop performance, suggesting that the use of chemical pesticides can be reduced in combination with PGPR inoculants (Li et al., 2022). However, to make the use of AMF inoculants more affordable for farmers, they could be autonomously produced starting from native soils; also, if a transplant stage is involved, smaller amounts of inoculum are needed (Berruti et al., 2016; Ceballos et al., 2013).

Positive effects of microbial inoculants were seen in both greenhouse and open-field conditions. Nevertheless, AMF radical colonization and PGPR rhizosphere colonization is usually more frequent in the greenhouse conditions, especially when they are applied in insufficient quantities and PGPR are inoculated in an unprotected form. This could be due to competition with better adapted resident microbiota and predation by soil microfauna, while in greenhouses sterilized substrates are usually used; in addition, the use of biocidal fumigants may challenge colonization in the field (Becker et al., 2018; Berruti et al., 2016).

To promote stable use of bioinoculants in agriculture cost-benefit analyses should be conducted. The inoculants offered on the international market have often low quality, which cause an inconsistency in their beneficial effect. Therefore, it is necessary to create bioinoculants of high quality, survival, stability, and reproducibility (by standardizing the production protocols), develop strategies for effective inoculation methods, avoid the presence of pathogens and weeds, and ensure the purity of the microbial composition and the concentration of propagules, which is a fundamental technical information for the end-users (Backer et al., 2018; Basiru et al., 2021; Berruti et al., 2016; Lobo et al., 2019). Furthermore, plant species vary greatly in their responsiveness to AMF and PGPR and the efficacy of inoculants is also affected by the different environmental conditions, indigenous microbial communities, and soil management practices (Basiru et al., 2021; Berruti et al., 2016). Therefore, a careful selection of the favorable host/niche/microbs combinations is essential to obtain optimal benefits (Berruti et al., 2016; Li et al., 2022). Finally, training end-users to efficiently apply bioinoculants and utilize microbial-friendly crop management is very important in the promotion and deployment of bioinoculants (Backer et al., 2018).

Aim of the Ph.D. project

The project of the present thesis, titled "Sustainable Horticultural Solutions to Improve Saffron (*Crocus sativus* L.) Production", aimed to improve saffron profitability especially in Europe by using sustainable practices to enhance the quality, productivity, and value of saffron products. Cultivation in controlled conditions, in hydroponics, has been proposed as a solution to facilitate and improve saffron crop management, with adequate nutrient solutions and by avoiding pests or pathogens. Furthermore, it can counteract the negative impacts of global soil degradation and climate change, such as salinity (to which saffron is very sensitive) and higher temperatures. Inoculants of beneficial microorganisms, i.e., PGPR and AMF, were evaluated to improve plant health and productivity in agreement with the forthcoming "microbial revolution". The

effects of AMF and PGPR (single type/species or mixed inoculants) on saffron have been still poorly studied in hydroponics. In particular, the effects on saffron photosynthesis, which provides nutrients for corm growth, and on the transcription of genes involved in the carotenoid/apocarotenoid biosynthetic pathway have never been investigated so far. Finally, since the violet tepals also contain phytochemicals and constitute the most abundant bioresidue (about 80% of the total flower mass), their industrial application has been considered, e.g., as a source of natural dyes, to generate a new source of income in a circular bioeconomy perspective. Therefore, processing and extractions have been studied considering sustainable techniques and solvents.

The chapters of the present thesis:

In **Chapter 1** – "**Beneficial microorganisms: a sustainable horticultural solution to improve the quality of saffron in hydroponics**" – saffron was grown in controlled conditions, in hydroponics, with different bioinoculants. The inoculants were species of the saffron rhizomicrobiota found in the literature with beneficial properties, namely, the PGPR *Bacillus megaterium* CB97032 and *Paenibacillus durus* CB1806, and the AMF *Rhizophagus intraradices*, which were used alone or in combination. The influence exerted by the beneficial microorganisms during all the crop phases on flowering trend, yields, quality, and ecophysiology of saffron was investigated, with the hypothesis that microbial synergy in mixed formulation (PGPR + AMF) would have more positively affected these parameters. During flowering, AMF colonization and the presence and activity of PGPR were assessed; the quality of the saffron spice was evaluated according to the ISO 3632 (2011) protocol; main apocarotenoids of the spice were identified and quantified with HPLC analysis; and the total phenolic content (TPC) was analysed by the Folin-Ciocalteu method. During the vegetative phase, the net CO_2 assimilation rate, stomatal conductance, and leaf transpiration rate were measured with an InfraRed Gas Analyzer; and leaf area and leaf content of chlorophylls and carotenoids were analysed. At the end of the vegetative phase,

the yield of the replacement corms was evaluated, and the starch content of the corms was analised with an assay kit. While flower and spice yield were not positively affected by the bioinoculants, the content of safranal, i.e., the principal aroma compound of saffron, was enhanced for all treatments and the TPC was improved for the mixed inoculated plants. The bioinoculants also increased the corm yield. Overall, the beneficial microorganisms improved quality and productive traits of hydroponic saffron especially when applied in mixed formulations. These results led to further investigate saffron responses at the molecular level. This work has been carried out in collaboration with the Institute for Sustainable Plant Protection (National Research Council – Turin, Italy) and Ceres Biotics Tech S.L. (Madrid, Spain). The paper has been published by Scientia Horticulturae, Elsevier (Stelluti et al., 2023).

Chapter 2 – "**Arbuscular mycorrhizal symbiosis modulates apocarotenoid biosynthesis pathway in saffron**" – investigated for the first time the modulation by the AMF *Rhizophagus intraradices* of apocarotenoid metabolism through an integrated biochemical and molecular analysis in the saffron stigmas, together with a mineral nutrient content analysis of corms at flowering. Main crocins, namely, *trans*-5-nG, *trans*-4-GG, *cis*-4-GG, *trans*-3-Gg, *cis*-3-Gg, *trans*-2-G, *trans*-4-ng, *trans*-2-gg, and *trans*-1-g; picrocrocin and HTCC (both precursors of safranal); and safranal were identified and quantified by HPLC analysis. The expression of the genes for β-LYC producing α- and β-carotene; D27 and CCD7 involved in strigolactones (SLs) biosynthesis; CCD4a and CCD4b producing HTCC and β-ionone; CCD2 producing HTCC and crocetin dialdehyde; UGT709G1 producing picrocrocin; ALDH producing crocetin; UGT74AD1 and UGT91P3 producing crocins; and NCED implicated in ABA biosynthesis was investigated by qRT-PCR analysis. In mycorrhized plant roots, a biofertilizer effect was seen for the micronutrients Fe, Zn, and Mo, which are important for plant physiology and enzyme cofactors, among other roles. For instance, Iron is cofactor of carotenoid dioxygenases (CCD), molybdenum is cofactor of ABA-aldehyde oxidase (AAO), and the zinc-finger transcription factor CsSAP09 has a

possible role in regulating apocarotenoid metabolism in saffron. In mycorrhized plants, the expression of *D27*, *CCD7*, and *NCED* genes for SLs and ABA, which promote AM symbiosis, did not change, as well as the transcription of *β-LYC* and *CCD4a/b*. Conversely, the gene encoding for CCD2, the key enzyme producing major apocarotenoids, was upregulated. The content of crocins was reduced in treated plants even if the expression of *ALDH*, *UGT74AD1*, and *UGT91P3* genes involved in crocins synthesis did not change. The gene for picrocrocin synthesis, *UGT709G1*, was also overexpressed in inoculated plants, thus the content of safranal was increased in the spice. This work has been done in collaboration with the Institute for Sustainable Plant Protection (National Research Council – Turin, Italy), with the research group of the Departamento de Producción Vegetal of the Universitat Politècnica de València (Valencia, Spain), and with researchers from the Universitat de València and from the Universidad de Castilla-La Mancha (Albacete, Spain). The article has been submitted to Scientia Horticulturae, Elsevier (Stelluti et al., submitted).

Chapter 3 – "**Comparison of different inoculants of arbuscular mycorrhizal fungi on *Crocus sativus* L. cultivated in soilless conditions**" – reported the effects of different AMF-based inoculants on plant production, spice yield and tepal quality of saffron soilless grown in open air. Both single species (*Funneliformis mosseae* BEG12 and *Rhizoglomus irregulare* BEG140) or multi-species mixture (*Funneliformis geosporum* BEG199, *Funneliformis caledonium* BEG97, *and Claroidoglomus claroideum* BEG96), were applied. Morphological analysis of saffron roots revealed an overall low mycorrhization, which was ascribed to saffron-AMF compatibility and/or growing conditions, particularly low temperatures. The antioxidant capacity of tepal extracts was influenced by the inoculants, although opposite results were observed between assays (FRAP, ABTS, and DPPH). This work has been carried out in collaboration with the Institute for Sustainable Plant Protection (National Research Council – Turin, Italy). This study has been presented at the XXXI International Horticultural

Congress (IHC2022, Anger, France) and accepted by Acta Horticulturae, ISHS (Stelluti et al., in press).

Since saffron tepals, an abundant floral bio-residue, contain polyphenols with antioxidant properties, they could become a new source of income while reducing bio-waste. In **Chapter 4** – "**Sustainable processing of floral bio-residues of saffron (*Crocus sativus* L.) for valuable biorefinery products**" – was deepened the knowledge on the phytochemical composition of extracts of saffron dried tepals and investigated whether it was impoved by greener extraction techniques and solvents. Dehydration was considered, as it is widely used in the herb sector, especially for highly perishable harvested flowers. Conventional maceration was compared with the modern Ultrasound Assisted Extraction (UAE) technique, that allows to use safer solvents, shorten the time, and reduce the energy cost of the extraction process. Different solvents were evaluated, i.e., water and three water:methanol concentrations (80:20, 50:50, and 20:80, v:v). The TPC, TAC, and antioxidant activity (by the FRAP, ABTS, and DPPH assays) were analysed. Vitamin C and phenolic compounds, namely, ferulic acid, ellagic acid, hyperoside, isoquercitrin, quercetin, rutin, catechin, and epicatechin, were identified and quantified by HPLC analysis. Vitamin C, ferulic acid, isoquercitrin, and quercitrin were detected in saffron dried tepals for the first time. In most cases, UAE with safer solvents (water or lower percentage of methanol) showed a yield of phenolic compounds and vitamin C similar to maceration, allowing to improve the extraction by halving the time. This study demonstrated that dried saffron tepals contain phytochemicals that can be obtained with green extractions, meeting the requirement for a sustainable biorefining. The article has been published on Plants, MDPI (Stelluti et al., 2021).

Chapter 5 – "**The natural colorants of the *Crocus sativus* L. flower**" – focused on the natural pigments of the red stigmas and violet tepals. Crocins are the main yellow-red pigments of the spice, which has been traditionally used as dye in food, medicines, and textiles, and their content can be influenced by horticultural

practices. The anthocyanins of the tepals are responsible for the purple color; the potential use of tepals as a source of natural dyes, which are safe and have potential health benefits over their man-made counterparts, has been proposed to promote their use as a byproduct. By analysing data from previous works, this study demonstrated for the first time how the total anthocyanin content (TAC) in tepal extracts can be affected by post-harvest practices together with the extraction solvents. Drying is a common practice for preserving harvested flowers, as they are very perishable. The TAC was significantly lower in dried tepals than in the fresh ones; the percentage of TAC reduction was of ~50% when the solvents were water and water:methanol 50:50, and of ~80% when the solvents were water:methanol 80:20 and 20:80. Therefore, water was proved to be the best greener solvent for dried tepals. This work has been presented at the XXXI International Horticultural Congress (IHC2022, Anger, France) and published on Acta Horticulturae, ISHS (Stelluti et al., 2023 - DOI: 10.17660/ActaHortic.2023.1361.13).

In the **Appendix 1**, a summary of the activities carried out during the Ph.D. is presented.

In the **Appendix 2**, education is reported.

References of the introduction

Ambardar, S., Vakhlu, J., 2013. Plant growth promoting bacteria from *Crocus sativus* rhizosphere. World J. Microbiol. Biotechnol. 29, 2271–2279. https://doi.org/10.1007/s11274-013-1393-2

Askari-Khorasgani, O., Pessarakli, M., 2019. Shifting saffron (*Crocus sativus* L.) culture from traditional farmland to controlled environment (greenhouse) condition to avoid the negative impact of climate changes and increase its productivity. J. Plant Nutr. 42, 2642–2665. https://doi.org/10.1080/01904167.2019.1659348

Avarseji, Z., Kafi, M., Sabet Teimouri, M., Orooji, K., 2013. INVESTIGATION OF SALINITY STRESS AND POTASSIUM LEVELS ON MORPHOPHYSIOLOGICAL CHARACTERISTICS OF SAFFRON. J. Plant Nutr. 36, 299–310. https://doi.org/10.1080/01904167.2012.739249

Backer, R., Rokem, J.S., Ilangumaran, G., Lamont, J., Praslickova, D., Ricci, E., Subramanian, S., Smith, D.L., 2018. Plant growth-promoting rhizobacteria: Context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. Front. Plant Sci. 871, 1–17. https://doi.org/10.3389/fpls.2018.01473

Basiru, S.; Mwanza, H.P.; Hijri, M. Analysis of Arbuscular Mycorrhizal Fungal Inoculant Benchmarks. Microorganisms 2021, 9, 81. https://doi.org/10.3390/microorganisms9010081

Berruti, A., Lumini, E., Balestrini, R., and Bianciotto, V. (2016). Arbuscular Mycorrhizal Fungi as Natural Biofertilizers: Let's Benefit from Past Successes. Front. Microbiol. 6, 1–13

Cardone, L., Castronuovo, D., Perniola, M., Cicco, N., and Candido, V. (2020). Saffron (*Crocus sativus* L.), the king of spices: An overview. Sci. Hortic. (Amsterdam). *272*.

Caser, M., Demasi, S., Victorino, Í.M.M., Donno, D., Faccio, A., Lumini, E., Bianciotto, V., and Scariot, V. (2019). Arbuscular Mycorrhizal Fungi Modulate the Crop Performance and Metabolic Profile of Saffron in Soilless Cultivation. Agronomy *9*, 232.

Caser, M., Victorino, Í.M.M., Demasi, S., Berruti, A., Donno, D., Lumini, E., Bianciotto, V., and Scariot, V. (2018). Saffron cultivation in marginal alpine environments: How AMF inoculation modulates yield and bioactive compounds. Agronomy *9*.

Caser, M., Demasi, S., Stelluti, S., Donno, D., and Scariot, V. 2020. *Crocus sativus* L.

Cultivation in alpine environments: Stigmas and tepals as source of Bioactive Compounds. Agronomy *10*.

Ceballos I, Ruiz M, Fernández C, Peña R, Rodríguez A, Sanders I. R., 2013. The In Vitro Mass-Produced Model Mycorrhizal Fungus, Rhizophagus irregularis, Significantly Increases Yields of the Globally Important Food Security Crop Cassava. PLOS ONE 8(8): e70633. https://doi.org/10.1371/journal.pone.0070633

De Pascale, S., Rouphael, Y., Colla, G., 2017. Plant biostimulants: innovative tool for enhancing plant nutrition in organic farming. Eur. J. Hortic. Sci 82, 277–285. https://doi.org/10.17660/eJHS.2017/82.6.2

García-Rodríguez, M.V., López-Córcoles, H., Alonso, G.L., Pappas, C.S., Polissiou, M.G., Tarantilis, P.A., 2017. Comparative evaluation of an ISO 3632 method and an HPLC-DAD method for safranal quantity determination in saffron. Food Chem. 221, 838–843. https://doi.org/10.1016/j.foodchem.2016.11.089

García-Rodríguez, M., Serrano-Díaz, J., Tarantilis, P.A., López-Córcoles, H., Carmona, M., Alonso, G.L., 2014. Determination of saffron quality by high-performance liquid chromatography. J Agric Food Chem 62, 8068–8074. https://doi.org/10.1021/jf5019356

Genre, A., Lanfranco, L., Perotto, S., Bonfante, P., 2020. Unique and common traits in mycorrhizal symbioses. Nat. Rev. Microbiol. 18, 649–660. https://doi.org/10.1038/s41579-020-0402-3

Gresta, F., Lombardo, M., Siracusa, L., and Ruberto, G. 2008. Saffron, an alternative crop for sustainable agricultural systems. A review. Agron. Sustain. Dev. *28*, 95–112

Kazemi-Shahandashti, S.S., Mann, L., El-nagish, A., Harpke, D., Nemati, Z., Usadel, B., Heitkam, T., 2022. Ancient Artworks and Crocus Genetics Both Support Saffron's Origin in Early Greece. Front Plant Sci 13. https://doi.org/10.3389/fpls.2022.834416

Khan, M., Hanif, M.A., Ayub, M.A., Jilani, M.I., and Shahid Chatha, S.A. 2020. Saffron. In Medicinal Plants of South Asia, (Elsevier), pp. 587–600

Koocheki, A., Seyyedi, S.M., 2015. Relationship between nitrogenand phosphorus use efficiency in saffron (*Crocus sativus* L.) as affected by mother corm size and fertilization. Ind. Crops Prod. 71, 128–137. https://doi.org/10.1016/j.indcrop.2015.03.085

Kour, R., Ambardar, S., Vakhlu, J., 2018. Plant growth promoting bacteria associated with corm of *Crocus sativus* during three growth stages. Lett. Appl. Microbiol. 67, 458–464. https://doi.org/10.1111/lam.13042

Kumar, R., Singh, V., Devi, K., Sharma, M., Singh, M.K., Ahuja, P.S., 2008. State of Art of Saffron (*Crocus sativus* L.) Agronomy: A Comprehensive Review. Food Rev. Int. 25, 44–85. https://doi.org/10.1080/87559120802458503

Lanfranco, L., Fiorilli, V., Gutjahr, C., 2018. Partner communication and role of nutrients in the arbuscular mycorrhizal symbiosis. New Phytol. 220, 1031–1046. https://doi.org/10.1111/nph.15230

Li, J, Wang, J, Liu, H, Macdonald, CA, Singh, BK. 2022. Application of microbial inoculants significantly enhances crop productivity: a meta-analysis of studies from 2010 to 2020. J Sustain Agric Environ. 1: 216– 225. https://doi.org/10.1002/sae2.12028

Lobo, C.B., Juárez Tomás, M.S., Viruel, E., Ferrero, M.A., Lucca, M.E., 2019. Development of low-cost formulations of plant growth-promoting bacteria to be used as inoculants in beneficial agricultural technologies. Microbiol. Res. 219, 12–25. https://doi.org/10.1016/j.micres.2018.10.012

Magotra, S., Bhagat, N., Ambardar, S., Ali, T., Hurek, B.R., Hurek, T., Verma, P.K., Vakhlu, J., 2021. Field evaluation of PGP *Bacillus* sp. strain D5 native to *Crocus sativus*, in traditional and non traditional areas, and mining of PGP genes from its genome. Sci. Rep. 11, 1–16. https://doi.org/10.1038/s41598-021-84585-z

Mir, J. I., Ahmed, N., Singh, D. B., Khan, M. H., Zaffer, S., & Shafi, W. (2015). Breeding and biotechnological opportunities in saffron crop improvement. African Journal of Agricultural Research, 10(9), 970-974.

Molina, R.V., Valero, M., Navarro, Y., Guardiola, J.L., García-Luis, A., 2005. Temperature effects on flower formation in saffron (*Crocus sativus* L.). Sci. Hortic. (Amsterdam). 103, 361–379. https://doi.org/10.1016/j.scienta.2004.06.005

Molina, R. V., Valero, M., Navarro, Y., García-Luis, A., Guardiola, J.L., 2005b. Low temperature storage of corms extends the flowering season of saffron (*Crocus sativus* L.). J. Hortic. Sci. Biotechnol. 80, 319–326. https://doi.org/10.1080/14620316.2005.11511937

Renau-Morata, B., Nebauer, S.G., Sánchez, M., Molina, R. V., 2012. Effect of corm size, water stress and cultivation conditions on photosynthesis and biomass partitioning during the vegetative growth of saffron (*Crocus sativus* L.). Ind. Crops Prod. 39, 40–46. https://doi.org/10.1016/j.indcrop.2012.02.009

Rouphael, Y., Colla, G., 2020. Editorial: Biostimulants in Agriculture. Front. Plant Sci. 11, 1–7. https://doi.org/10.3389/fpls.2020.00040

Salas, M. del C., Montero, J.L., Diaz, J.G., Berti, F., Quintero, M.F., Guzmán, M., Orsini, F., 2020. Defining Optimal Strength of the Nutrient Solution for Soilless Cultivation of Saffron in the Mediterranean. Agronomy 10, 1311. https://doi.org/10.3390/agronomy10091311

Shokrpour, M.. 2019. Saffron (*Crocus sativus* L.) Breeding: Opportunities and Challenges. In: Al-Khayri, J., Jain, S., Johnson, D. (eds) Advances in Plant Breeding Strategies: Industrial and Food Crops. Springer, Cham. https://doi.org/10.1007/978-3-030-23265-8_17

Stelluti, S., Caser, M., Demasi, S., Rodriguez Herrero, E., García-González, I., Lumini, E., Bianciotto, V., Scariot, V., 2023. Beneficial microorganisms: a sustainable horticultural solution to improve the quality of saffron in hydroponics. Scientia Horticulturae, 319, 112155. https://doi.org/10.1016/j.scienta.2023.112155

Stelluti, S.; Caser, M.; Demasi, S.; Scariot, V. 2021. Sustainable Processing of Floral Bio-Residues of Saffron (*Crocus sativus* L.) for Valuable Biorefinery Products. Plants, 10, 523. https://doi.org/10.3390/plants10030523

Stelluti, S., Caser, M., Demasi, S. and Scariot, V. 2023b. The natural colorants of the *Crocus sativus* L. flower. Acta Hortic. 1361, 111-118. DOI: 10.17660/ActaHortic.2023.1361.13
https://doi.org/10.17660/ActaHortic.2023.1361.13

1. Beneficial microorganisms: a sustainable horticultural solution to improve the quality of saffron in hydroponics

Scientia Horticulturae 319 (2023) 112155

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Beneficial microorganisms: a sustainable horticultural solution to improve the quality of saffron in hydroponics

Stefania Stelluti [a], Matteo Caser [a], Sonia Demasi [a], Esteban Rodriguez Herrero [b], Irene García-González [b], Erica Lumini [c], Valeria Bianciotto [c], Valentina Scariot [a,*]

[a] Department of Agricultural, Forest and Food Sciences, University of Torino. Largo Paolo Braccini 2, Grugliasco, TO 10095, Italy
[b] Ceres Biotics Tech S.L., Mar Tirreno 8, Naves C27-C29, San Fernando de Henares 28830, Madrid, Spain
[c] Institute for Sustainable Plant Protection, National Research Council, c/o Department of Life Science and System Biology, University of Turin. Viale Mattioli 25, Torino 10125, Italy

1.1. Abstract

Saffron (*Crocus sativus* L.) is the most expensive spice in the world. Its organoleptic properties are mainly dictated by the apocarotenoids crocins (dyeing capacity), picrocrocin (pleasant taste), and safranal (scent). Cultivation of saffron in controlled conditions with beneficial microorganisms may increase its profitability. In this study saffron was grown in hydroponics under a greenhouse with the PGPR *Bacillus megaterium* CB97032 and *Paenibacillus durus* CB1806 (Pgpr treatment), and the AMF *Rhizophagus intraradices* (Myc treatment), alone or mixed (Mix treatment). The influence exerted during all crop phases on flowering trend, flower and spice yield, secondary metabolites, ecophysiological traits, and corm production was investigated, with the hypothesis that microbial synergy would have more positively affected these parameters. All the bioinoculants did not positively influence flower and spice yield, but enhanced the content of safranal (up to +96% in all the treated plants). When mixed, the PGPR and AMF improved the total phenolic content (+19%) of the saffron spice. Even if no differences emerged from the ecophysiological analysis, the corm yield was improved for the inoculated plants. The single-type bioinoculants allowed to obtain a higher number of replacement corms (up to +13% for the Myc

plants) without reducing their weight, but lowering their size. When mixed, the size of the corms was restored. Together, the PGPR and AMF also increased the corm weight (+24%) of the largest corm fraction (> 1.5 cm). Thus, the bioinoculants may have stimulated the secondary metabolism of the plants by improving quality traits, rather than having acted as biofertilizers by increasing yield, at least during flowering. Overall, AMF and PGPR were proved to be a sustainable horticultural solution in hydroponics to improve the quality of saffron, especially when applied in mixed formulations.

1.2. Keywords

Crocus sativus L., AMF, PGPR, Safranal, Photosynthetic system, Corm starch

1.3. Introduction

Saffron (*Crocus sativus* L.) is a subhysteranthous geophyte of the Iridaceae family. This sterile herbaceous plant multiplies through underground corms, which storage nutrient reserves and usually bear 1–3 flowers (Kumar et al., 2008; Stelluti et al., 2021). In Mediterranean climates, saffron blooms for two to three weeks between early and late autumn. After flowering saffron enters a vegetative stage during which the leaves are photosynthetically active, providing nutrients for the formation of new corms (Renau-Morata et al., 2012). Flowering is mainly regulated by thermoperiodicity and corm size (Gresta et al., 2008). Optimal flower formation can be achieved incubating corms at warm temperature (23 - 27 °C) for more that 50 days and less than 150 days for flower induction and at mid-low temperature (15 - 17 °C) for flower emergence (Molina et al., 2005a). Flower yield increases with larger corms; commercially, corms of 2.5 - 3.5 cm in diameter and 10 - 20 g in weight are usually selected (Caser et al., 2019). The spice of saffron is obtained by dehydrating the red stigmas of its ephemeral flowers. The daily manual harvest of flowers and separation of stigmas causes it to have the highest cost among spices (Caser et al., 2020). Saffron has earned the nickname "red gold" and unsurprisingly, its price ($40 - 50 g^{-1} Khan et al., 2020) approximates that of gold ($55 g^{-1}, goldprice.org, November 2022). The spice has

been used to flavor and color food for centuries. Its pleasant bitter taste, inebriating scent, and dyeing capacity are mainly dictated by three apocarotenoids derived from zeaxanthin: picrocrocin, a glucoside of safranal (taste); safranal, a volatile monoterpene aldehyde (scent); and crocetin glycosides named crocins (dyeing capacity). *Trans*-crocetin di-(β-D-gentiobiosyl) ester and t*rans*-crocetin di-(β-D-glucosyl)-(β-D-gentiobiosyl) ester are among the main crocins found in saffron (Carmona et al., 2006; Chen et al., 2020; García-Rodríguez et al., 2017; Tarantilis et al., 1994). These compounds are used to classify the spice into categories of quality (I, II, and III) according to ISO 3632 (2011) (Caser et al., 2018, 2019, 2020). Saffron is mainly cultivated in the Middle East and Mediterranean regions. About 90% of total world production (418 t y^{-1} in 2018) comes from Iran and the remainder mainly from India, Afghanistan, Greece, Morocco, Spain, and Italy (Cardone et al., 2020). In the last century the saffron produced in Spain, Italy, and Greece has seriously decreased, mostly because the technology has not progressed for this plant's cultivation and the manual labor cost has increased (Cardone et al., 2020; Molina et al., 2005a). To make saffron more profitable in European areas, shifting its cultivation to controlled environments has been proposed (Askari-Khorasgani and Pessarakli, 2019; Avarseji et al., 2013; Caser et al., 2019; Molina et al., 2005b; Salas et al., 2020). The flowering period could be extended and crop management facilitated and improved with suitable nutrient solutions and by growing plants without pests or pathogens (Caser et al., 2019; Molina et al., 2005b; Salas et al., 2020). Further, controlled conditions may counteract the negative impact of global soil degradation and climate change (i.e., salinity and higher temperatures) and increase land-use efficiency (Askari-Khorasgani and Pessarakli, 2019). The search for sustainable practices that can provide yields comparable to those of high-intensity agriculture continues to cope with the effects of climate change and reduce environmental costs (Rouphael and Colla, 2020). After the green-revolution, there is a need for the so-called microbial revolution, based on the utilization/manipulation of plant microbiota as a sustainable tool to enhance plant productivity (Backer et al., 2018; De Pascale et al., 2017; Genre et al., 2020). The

use of beneficial microbes in agriculture begins in the early 20th century with the rhizobia (plant growth promoting rhizobacteria, PGPR) and several species of the Fabaceae family (Backer et al., 2018). PGPR comprise various genera, such as *Bacillus, Paenibacillus, Azospirillum,* and *Azotobacter,* with relevant properties, primarily N-fixation and P-solubilization, but also siderophore and phytohormone production and biological control. In the last decade, many formulations have been applied to different crops (Backer et al., 2018; Lobo et al., 2019). However, only N-fixing bacteria of the genera *Rhizobium, Azotobacter,* and *Azospirillum* are currently considered "plant biostimulants" (Regulation EU 2019/1009). This category includes also the arbuscular mycorrhizal fungi (AMF), subphylum Glomeromycotina, which establish mutualistic symbiosis with most land plants. The fungus receives photosynthesis-derived carbon and, in exchange, increases the uptake of water and mineral nutrients (such as P and N) by plants and enhances their tolerance to biotic and abiotic stresses, positively affecting plant productivity (Genre et al., 2020; Lanfranco et al., 2018). AMF are largely used in horticulture, particularly species of the genera Rhizophagus and Funneliformis, as they are generalist, widely distributed, and can be extensively propagated (Berruti et al., 2016; Giovannini et al., 2020). The upgraded plant nutritional status induced by beneficial microorganisms has been associated with increased content of secondary metabolites of interest and/or plant production in several crops, such as *Zea mays* L., *Solanum lycopersicum* L., *Capsicum annuum* L., *Ocimum basilicum* L., *Mentha* spp., *Echinacea purpurea* (L.) Moench., *Artemisia annua* L., *Stevia rebaudiana* (Bertoni) Bertoni, *Allium sativum* L., *Hypericum perforatum* L., and also in *Crocus sativus* L. (Backer et al., 2018; Bianciotto et al., 2018; Kour et al., 2018; Kumar et al., 2021; Pandey et al., 2018; Rouphael et al., 2015). Nutrient availability, especially of N and P, mainly affects the growth of saffron corms during the vegetative phase (Koocheki and Seyyedi, 2015). AMF and P-solubilizing bacteria can work synergistically improving P availability and P uptake by plants (Etesami et al., 2021; Giovannini et al., 2020). Particularly, in soilless cultivation system where low P nutrient solutions are usually used to

avoid inhibition of AM symbiosis, e.g., in experiments with *Solanum lycopersicum*, 300 μM in Volpe et al. (2018) or 3.2 μM in Chialva et al. (2020), Mannino et al. (2020), and Zouari et al. (2014). The growth promoting effects may be more beneficial when diazotrophic bacteria are added to the microbial mix, which could also further reduce the use of chemicals (Lobo et al., 2019). Moreover, *Paenibacillus* spp. and *Bacillus* spp. can stimulate the growth of AMF and potentially promote the establishment of symbiosis (Rouphael et al., 2015). The rhizomicrobiota composition is strictly controlled by plants, which select the most beneficial microbes by releasing root exudates and signal compounds (Backer et al., 2018; Genre et al., 2020; Victorino et al., 2021). Out of various PGPR recently isolated from saffron rhizosphere, *Bacillus megaterium* and *Paenibacillus* sp. presented multiple growth promoting traits and positively affected the plant production (Jami Al-Ahmadi et al., 2017; Kour et al., 2018). Chamkhi et al. (2018) characterized the AMF associated with saffron, most frequently finding the genus *Rhizophagus* (Walker et al., 2021) within the roots. So far, the responses of saffron to PGPR or AMF have been still poorly investigated in hydroponics, focusing mainly on quantitative productive traits (Ambardar and Vakhlu, 2013; Caser et al., 2019; Kour et al., 2018; Magotra et al., 2021) and only a few studies (Caser et al., 2019; Sharaf-Eldin et al., 2008) on secondary metabolites of the spice. As regards the productive traits, the spice yield was not influenced by AMF (*Rizophagus intraradices* and *Funneliformis mosseae*) (Caser et al., 2019), but it was increased by PGPR inoculants, e.g. *Pseudomonas* spp. and *Bacillus* spp. (Díez-M´endez and Rivas, 2017; Magotra et al., 2021; Sharaf-Eldin et al., 2008). The corm yield (size, number of replacement corms, and/or weight) was improved by both AMF and PGPR formulations (Ambardar and Vakhlu, 2013). Regarding the phytochemicals in saffron, the content of crocins was reduced by *Bacillus subtilis* FZB24® (Sharaf-Eldin et al., 2008), which conversely increased the content of picrocrocin, crocetin, and safranal. To our knowledge, no studies have investigated the effects of beneficial microorganisms on saffron photosynthesis, which provides nutrients for corm growth. The aim of this study was to deepen the knowledge on the influence

exerted during all crop phases by different beneficial microorganisms, investigating saffron yield and secondary metabolites, ecophysiological traits, and corm production in hydroponics. Saffron was grown in a greenhouse using PGPR with different principal capacity of plant growth promoting, i.e., *Bacillus megaterium* CB97032 (P-solubilizer) and *Paenibacillus durus* CB1806 (N-fixing), not yet tested on saffron, and the AMF *Rhizophagus intraradices*, alone or mixed. The bipartite (i.e., a plant interacting with a single type of microbe, saffron-PGPR/AMF) and tripartite (saffron-PGPR-AMF) interactions were investigated. The hypothesis was that the microbial synergy in the mixed formulation would affect saffron production, quality-related compounds, and the photosynthesis process more positively than single-type inoculum.

1.4. Material and methods

1.4.1. Plant materials and cultivation conditions

The experiment took place in an unheated greenhouse at the Department of Agricultural, Forest, and Food Sciences (DISAFA) of the University of Turin (Italy, 45°06′23.21″N Lat, 7°57′82.83″E Long; 300 m a.s.l.). Saffron grows well in drained soils with pH 6.8- 7.8, and electrical conductivity (EC) <2 dS m^{-1} (Salas et al., 2020; Gresta et al., 2008). Large-sized corms (≥19 g) were sowed on 31 August 2020 in pots (4 L, 14 × 14 cm side, and 17 cm height; one corm per pot) filled with sterile expanded perlite (compacted density of 120 ± 25 kg m^{-3}; granulometry of 2 - 6 mm; 1.5 L per pot; Centro Evergreen Turco s.a.s., Moncalieri, Turin, Italy). Out-of-trial pots allowed to visualize phenological changes of the corms. Irrigation water (pH 7.4, EC 505 μS cm^{-1}; SMAT, Grugliasco, Turin) was added weekly from corm planting to root emergence (200 mL per pot). Subsequently, fertigation with a modified Long-Ashton solution (Hewitt, 1952) as in Chitarra et al. (2016) (Table 1) was carried out every 2 weeks until leaf senescence in spring (200 mL per pot). The solution had a low P concentration (300 μM) to avoid inhibition of AM symbiosis (Chitarra et al., 2016). The pH was adjusted by adding H_2SO_4 0.1 N (pH 7, EC 979 μS cm^{-1} at 22

°C). A randomised block design was used with three replicates (consisting of three blocks) per treatment. Corms were inoculated with AMF (Myc treatment), PGPR (Pgpr treatment), and a mixture of AMF and PGPR (Mix treatment); not-inoculated corms were the controls (Ctr). Each block was composed of 12 pots per each treatment and 6 pots for the controls, for a total of 126 pots on a greenhouse bench. The AMF inoculum (MycAgro Lab, Breteniére, FR) consisted of *Rhizophagus intraradices* spores and a substrate of calcined clay, vermiculite, and zeolite and ~10 g of inoculum was put under each corm. The formulations of the two PGPR species (Ceres Biotics Tech S.L., Madrid, Spain) *Bacillus megaterium* and *Paenibacillus durus* were mixed in the nutrient solution and applied three times during fertigation: at root emergence around the end of September (73.6 mg L^{-1}); two weeks afterwards (booster dose of 7.36 g L^{-1}); after two months from the first application (73.6 mg L^{-1}). The air temperature and relative humidity in the greenhouse were daily monitored by a datalogger (Fig. 1). The daily mean temperatures during flowering are showed in Fig. 2. During flowering, the daily mean values of temperature and relative humidity (RH%) were 17 ± 3 °C and 65 ± 10%. During the two main flowering peaks, the daily mean values were 19 ± 3 °C and 67 ± 10% (first peak), and 18 ± 3 °C and 63 ± 11% (second peak).

Growing degree days (GDD) were calculated as follows:

$$GDD = \Sigma[(T\text{max} + T\text{min})/2 - Tbase]$$

Where Tmax and Tmin are the maximum and minimum daily air temperature, respectively, and Tbase is the base temperature (McMaster and Wilhelm, 1997). GDD were referred to flowering and Tbase was identified at 10 °C.

1.4.2. Evaluation of AM colonization, bacterial presence, and PGP activities

During flowering, the roots of two plants per replicate were harvested, rid of topsoil, and cleaned. For each sample, part of the roots was used for biomass measurement and the remainder was stained to evaluate AM colonization. Briefly, saffron roots were stained with 0.1% (w/ v) cotton blue in 90% lactic acid overnight and de-stained two times, with water (2 h) and 90% lactic acid diluted

in deionized water 1:1 (v/v) (2 h). The roots were then left in 90% lactic acid. The protocol was performed twice to ensure a better staining. The roots were cut into fragments of ~1 cm and placed on microscope slides (20 fragments per slide) for further analysis under a light microscope (Trouvelot et al., 1986). For Myc and Mix plants, three slides per biological replicate were observed for a total of ~180 cm of root per treatment. For both Ctr and Pgpr plants, the absence of AMF was checked in one slide per biological replicate for a total of ~60 cm of root. Two weeks after the end of flowering, the presence of the PGPR species and main PGP activities (i.e., fixed N_2 potential, P and K solubilization, and siderophore production) were analysed on a minimum of three rhizosphere samples of Ctr, Pgpr, and Mix plants. The bacterial concentration was observed in both general (TSA) and free-nitrogen (A6) media (Qaisrani et al., 2019).

Table 1. Composition of the modified Long-Ashton nutrient solution

Elements	Concentration (mM)
$MgSO_4 \cdot 7H_2O$	0.75
$NaNO_3$	1
K_2SO_4	1
$CaCl_2 \cdot 2H_2O$	2
Na_2HPO_4	0.3
FeNa-EDTA	0.025
$MnSO_4 \cdot 12H_2O$	0.005
$CuSO_4 \cdot 5H_2O$	0.00025
$ZnSO_4 \cdot 7H_2O$	0.0005
H_3BO_3	0.025
$Na_2MoO_4 \cdot 2H_2O$	0.0001

1.4.3. Yields and root biomass

At flowering (31 October – 17 November 2020), the daily number of flowers per corm and the yield of the spice were measured. The spice was obtained by dehydrating the stigmas in the shade for 48 - 72 h and then in a cold-dryer (Northwest Technologies NWT100 dryer, Boves, Italy) at 20 °C for 48 h (Vallino et al., 2021). The roots of each sample collected for AM colonization assessment

were weighed fresh. The part of the roots used for the biomass measurement was weighed fresh and then dried in an oven at 60 °C for one week to record the dry biomass. The dry weight of the total roots was calculated by comparing the dry weight of the partial roots with the fresh weights of both the total and partial roots. During the vegetative phase (end of January 2021), the number of leaves per corm and leaf length were measured on two to three plants per biological replicate, for a total of eight to nine plants per treatment. At the end of the vegetative phase (June 2021), the corms of four Ctr plants per biological replicate (for a total of 12 plants) and of seven to nine inoculated plants per biological replicate (for a total of 21 to 27 plants) were detunicated and the number, fresh weight, and size of the replacement corms were taken. For corm size, the average diameter was calculated after measuring the major and minor diameter of each corm.

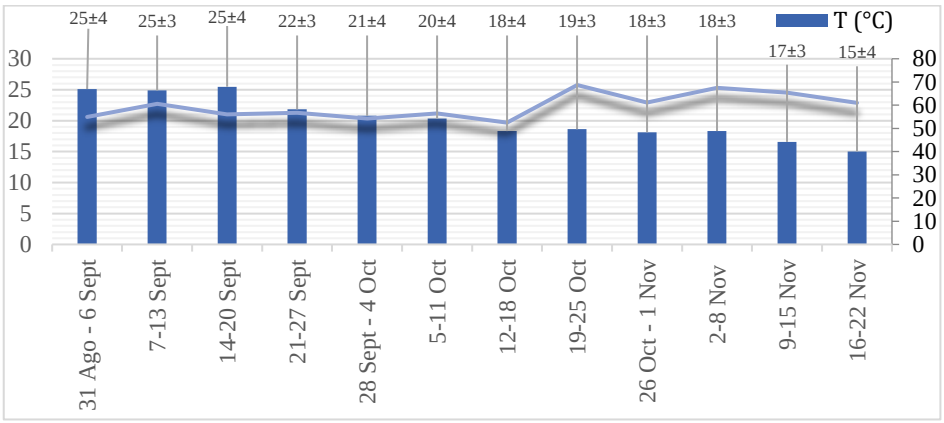


Figure 1. Weekly means and standard deviations of temperature (T, bars) and relative humidity (line) in the greenhouse from 31 August till the end of November.

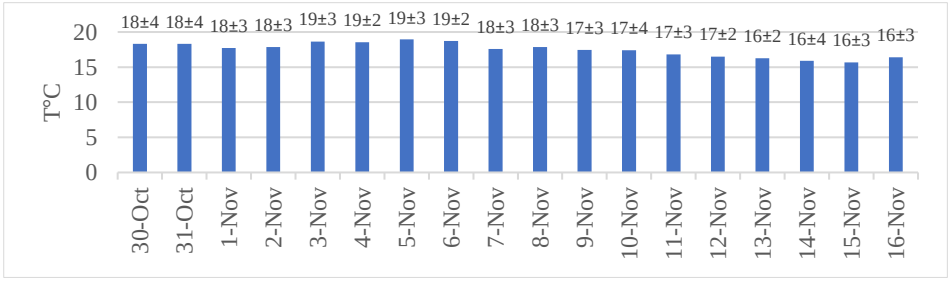


Figure 2. Daily means and standard deviations of temperature (T) in the greenhouse during flowering (30 October – 16 November).

1.5. Quality analyses on saffron spice

1.5.1. Spice extract preparation

Aqueous extracts of the spice were prepared as in Caser et al. (2020). Briefly, 50 mg of ground spice was suspended into 5 mL of deionised water. The solution was stirred (1000 rpm) for 1 h in the dark at room temperature (~21 °C), centrifuged (4 °C and 10,000 rpm), and filtered with PVDF syringe filters (25 mm diameter and 0.45 μm pore size - CPS Analitica, Milan, Italy). Two technical replicates per biological replicate were prepared.

1.5.2. Saffron quality according to ISO 3632 (2011)

The International Standard Organization (ISO 3632) sets the quality standards for the saffron spice. It requires the principal metabolites to be expressed as absorbance readings of 1% (w/v) saffron aqueous extract at 257 (picrocrocin), 330 (safranal), and 440 nm (crocins) using UV–vis spectrophotometry (García-Rodríguez et al., 2017). The saffron extracts were diluted 80x with deionised water and analysed with a spectrophotometer UV–Vis (Cary 60 UV–Vis, Agilent Technologies, Santa Clara, California, USA). The data were related to the dry matter percentage and expressed as the absorbance of a 1% (w/v) spice aqueous solution using 1 cm pathlength cells (A1% 1 cm λ max). The following formula (readapted from Giupponi et al., 2019) was used:

$$A1\%1cm(\lambda max) = (D \times dilxV)/m \times (100 - wMV)$$

where “D” is the specific absorbance, “dil” is the dilution of the extracts, “V” is the volume of the extraction solvent, “m” is the mass in grams of the extracted spice, and “wMV” is the moisture determined using the following formula: $wMV = [(m0 - m1)/m0] \times 100$ where m0 is the mass (g) of the spice before drying and m1 is the mass (g) after drying in an oven for 16 h at 103 ± 2 °C. The analysis was conducted in the dark. The extracts were stored at -20 °C for further analyses.

1.5.3. Identification and quantification of apocarotenoids by HPLC

According to García-Rodríguez et al. (2014) water extracts prepared according to ISO 3632 (1:2011) are suitable for determining the safranal content. The concentrations of crocins I and II, i.e., *trans*-crocetin di-(β-D-gentiobiosyl) ester and t*rans*-crocetin di-(β-D-glucosyl)-(β-D-gentiobiosyl) ester, and safranal in saffron extracts were detected using an Agilent 1200 High-Performance Liquid Chromatography coupled with an Agilent UV–Vis diode array detector (Agilent Technologies, Santa Clara, CA, USA). Each compound was determined by comparing retention times and UV spectra with those of the standards under the same chromatographic conditions. The standards were crocin I, crocin II, and safranal, purchased from Sigma-Aldrich (Saint Louis, MO, USA). The results were expressed as mg 100 g^{-1} dry weight (DW). The chromatographic separation was made with a Kinetex C18 column (4.6 × 150 mm^2, 5 µm, Phenomenex, Torrance, CA, USA) and acetonitrile in water as the mobile phase. The chromatographic conditions were 5% to 95% (v/v) acetonitrile in 30 min and 95% to 5% (v/v) acetonitrile in 5 min (10 min conditioning time); flow: 0.6 mL min^{-1}. The detection of crocins and safranal was assessed at 310 nm (Fig. 3).

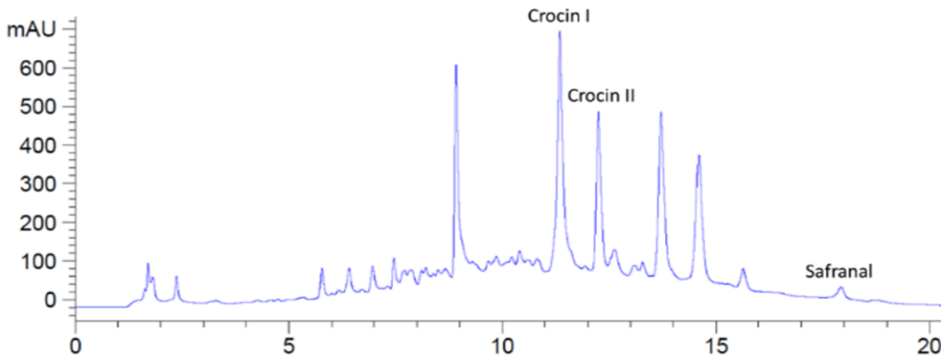


Figure 3. Chromatogram of saffron water extracts recorded at 310 nm. The following compounds are present: crocin I, crocin II, and safranal.

1.5.4. Total phenolic content

The total phenolic content was evaluated with the Folin-Ciocalteu method (Caser et al., 2020). In each tube, 200 µL of spice extract was added to 1000 µL of Folin-

Ciocalteu reagent diluted 1:10 with deionised water (v/v). After the solutions were left in the dark at room temperature for 10 min, 800 µL of Na_2CO_3 7.5% (w/v) was put in. After incubation in the dark at room temperature for 30 min, the absorbance at 765 nm was measured by means of a UV–Vis spectrophotometer (Cary 60 UV–Vis, Agilent Technologies, Santa Clara, CA, USA). The data were plotted against a gallic acid calibration curve and the results were expressed as mg of gallic acid equivalents (GAE) per 100 g of dry weight (mg GAE 100 g^{-1} DW).

1.6. Ecophysiological measurements and determination of pigment content

During the vegetative phase (end of January 2021) the net CO_2 assimilation rate (A_N, µmol CO_2 $m^{-2}s^{-1}$), stomatal conductance (*gs*, mmol H_2O $m^{-2}s^{-1}$), and leaf transpiration rate (*E*, mmol H_2O $m^{-2}s^{-1}$) were measured on six plants per treatment with an InfraRed Gas Analyzer (IRGA, ADC, model LCi-Pro; Hoddesdon, UK) (Patono et al., 2022). The leaf chamber of the instrument had a square (6.25 cm^2) aperture sealed around the edge. Saffron has peculiar leaves, which grow from the corm buds and are narrow, long, and pointed. The middle parts in length of three intact, green, healthy leaves per plant were placed for about one minute in the leaf chamber for reading. Measurements were taken between 1 and 3 pm, the concentration of CO_2 was 344 ± 58.5 ppm and the air pressure 97.2 ± 0.0 kPa. The temperature in the greenhouse ranged from 17 °C to 20 °C. The leaf area (LA, cm^2) was calculated as in Kumar (2009) using the equation [LA = 191.33e(L)0.0037], where "L" is the leaf length (mm) (Kumar, 2009).

Fifty mg of fresh leaves from six samples per treatment were then analysed for the content of chlorophylls (chl) and carotenoids according to Lichtenthaler (1987). The leaves were ground in 5 ml of 90% (v/v) methanol in water and, after an over-night extraction at 4 °C in the dark, the pigment concentration was spectrophotometrically (Cary 60 UV–Vis, Agilent Technologies, Santa Clara, CA, USA) determined at 665.2 (chl a), 652.4 (chl b), and 470 (car) nm (Caser et al., 2017; Lichtenthaler, 1987).

1.7. Starch content of the replacement corms

The starch content was analysed in three completely formed replacement corms per treatment using the Megazyme total starch assay kit (Megazyme International Ireland Ltd, Wicklow, Ireland). The analysis was based on the procedures for the determination of starch in samples containing resistant starch, D-glucose, and/or maltodextrins and the removal of D-glucose and maltodextrins by alcohol wash (Blandino et al., 2010; Fernandes et al., 2012). The corm moisture content (%) was calculated after drying in an oven at 105 °C for 24 h. The starch content was expressed as % w/w (dry weight basis).

1.8. Statistical analysis

Data were checked for normality (Shapiro–Wilk's test, $p > 0.05$) and homoscedasticity (Levene's test, $p > 0.05$). Significant differences were verified with one-way ANOVA ($p < 0.05$) and Tukey's test. When the ANOVA assumptions were not met, the data were analysed with Kruskal–Wallis test ($p < 0.05$) and Dunn's comparison test with Bonferroni adjustment. The R-studio software was used.

1.9. Results

1.9.1. Mycorrhization, bacteria presence, and PGP activities

A low intraradical presence of the AMF was found in the root fragments, with hyphae and vesicles. Regarding the Myc and Mix treatments, extraradical hyphae of *R. intraradices* were found in 18% (Myc) and 12% (Mix) of root fragments, and intraradical colonization in 15% (Myc) and 10% (Mix) of fragments. For a few fragments of both Myc and Mix treatments, AM colonization ranged from >10% to >90%. AMF were not seen for the Pgpr treatment and uninoculated Ctr. Main PGP activities and the presence of *Bacillus megaterium* CB97032 and *Paenibacillus durus* CB1806 in the rhizosphere of Ctr, Pgpr, and Mix plants were verified. A differential medium allowed the probable identification of the two

bacteria. The bacterial concentration observed in both general (TSA) and free-nitrogen (A6) media for Pgpr and Mix samples was significantly higher than for Ctr samples and not different between the two treatments (Fig. 4). The bacterial concentration in TSA medium was 3.88E+06 ± 6.80E+05 CFU mL^{-1} for Ctr, 2.54E+07 ± 1.07E+07 CFU mL^{-1} for Pgpr, and 3.30E+07 ± 1.80E+07 CFU mL^{-1} for Mix; in A6 medium it was 6.10E+06 ± 1.19E+06 CFU mL^{-1} for Ctr, 3.70E+07 ± 1.94E+07 CFU mL^{-1} for Pgpr, and 3.10E+07 ± 2.00E+06 CFU mL^{-1} for Mix. All PGP activities analysed were significantly higher in samples deriving from Pgpr and Mix treatments than in samples from Ctr, with no differences between the two treatments (Fig. 5). The ammonium (NH_4+) measured and related to the fixed N_2 potential was 380.73 ± 57.65 mg L^{-1} for Ctr, 726.18 ± 114.11 mg L^{-1} for Pgpr, and 684.36 ± 71.54 mg L^{-1} for Mix. The average radius of the P solubilization halo measured after 7 days was 3.25 ± 0.50 mm for Ctr, 5.75 ± 0.50 mm for Pgpr, and 5.00 ± 0.00 mm for Mix. The average radius of the K solubilization halo measured after 7 days was 8.00 ± 0.00 mm for Ctr, 11.00 ± 1.00 mm for Pgpr, and 11.67 ± 0.58 mm for Mix. The average radius of the Fe^{2+} mobilization halo measured after 7 days and related to siderophore production was 2.75 ± 0.50 mm for Ctr, 4.75 ± 0.50 mm for Pgpr, and 6.00 ± 1.15 mm for Mix.

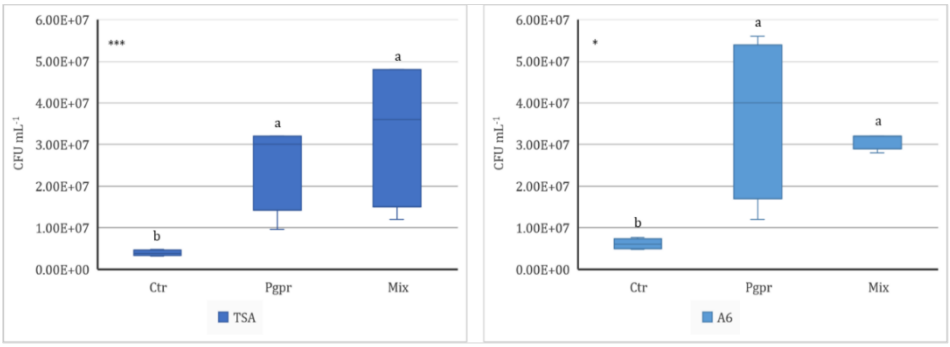


Figure 4. Bacterial quantification in general (TSA) and free-nitrogen (A6) media of rhizosphere samples from Ctr, Pgpr, and Mix plants. Values with the same letter are not statistically different at *$p < 0.05$ and ***$p < 0.001$.

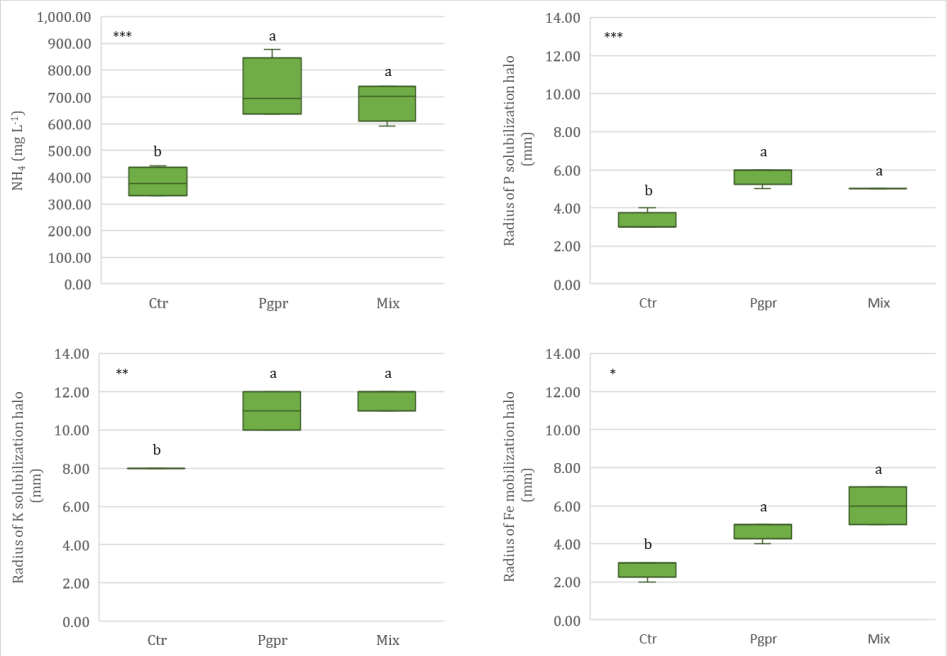


Figure 5. Ammonium measured with a colorimetric method and related to the fixed N_2 potential; average radius of the P solubilization halo measured after 7 days; average radius of the K solubilization halo measured after 7 days; average radius of the Fe^{2+} mobilization halo measured after 7 days and related to siderophore production. The analyses were performed on rhizosphere samples of Ctr, Pgpr, and Mix plants. Values with the same letter are not statistically different at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

1.9.2. Flowering trend, growing degree days, and yields

Flowering lasted from 30 October to 16 November. The anthesis of *C. sativus* began without major differences between the treatments, i.e. 63 (Ctr, Myc, and Mix) or 64 (Pgpr) days after corm sowing (Fig. 6). Flowering lasted 13 (Ctr), 15 (Mix), 17 (Pgpr), or 18 (Myc) days. Plants generated two main flowering peaks on the same days, 69 (19 ± 3 °C) and 72 (18 ± 3 °C) days after sowing. The flower percentages were 25% (Mix), 26% (Ctr), 29% (Pgpr), and 30% (Myc) on November 5th (first flowering peak); 25% (Myc), 30% (Pgpr), 31% (Mix), and 32% (Ctr) on November 8th (second flowering peak).

GDD and flower yield (flowers $corm^{-1}$) were not significantly affected by the treatments (Table 2). Conversely, mg of spice per flower was significantly

reduced in plants treated with the AM fungus (Myc) (6.7 ± 1.8 mg) compared with not-inoculated controls (7.6 ± 1.5 mg) and the other inoculated plants, i.e., Pgpr and Mix (Table 2). Regarding the corm yield (Table 2), Myc treatment gave the highest number of corms (6.9 ± 0.1) and both Myc and Pgpr corms showed lower size than Ctr, but similar weight. Mix plants performed better than Myc for corm weight and both Myc and Pgpr for corm size. When larger corms were considered (> 15 mm), the number of corms was different from Ctr only in the case of Pgpr plants, that produced less corms. Mix corms presented a similar number and were the only ones to have a higher weight (4.1 ± 0.6 g) than Ctr.

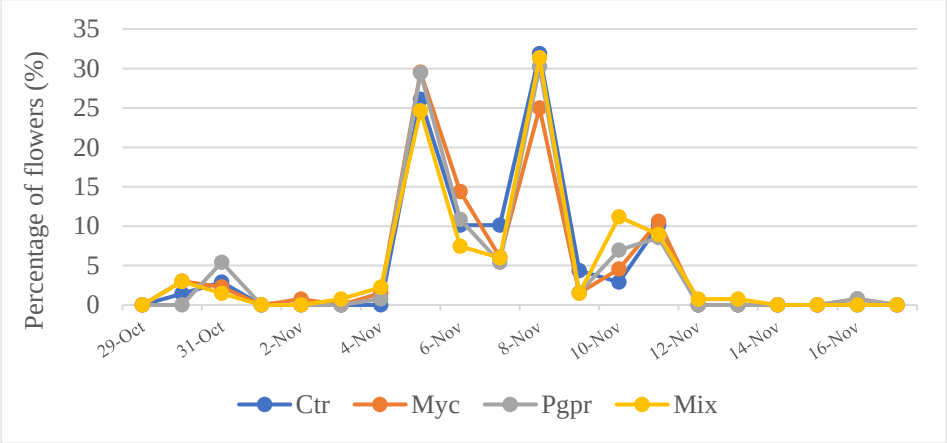


Figure 6. Percentage of daily harvested flowers of controls (Ctr) and inoculated (Myc, Pgpr, and Mix) saffron plants.

1.9.3. Quality analysis on the spice

The spice obtained from all plants had a moisture content below 12% and belonged to the quality category I (ISO 3632, 2011) (Table 3). No differences were seen between treatments for coloring strength (crocins). The flavoring strength (picrocrocin) was significantly reduced in Myc samples compared with Mix. The aromatic strength (safranal) resulted higher in Ctr than in Mix. When the safranal content was analysed by HPLC analysis (Table 3), it was significantly improved in all treated plants compared with controls, which presented 1.3 ± 0.6 mg 100 g^{-1} of safranal. The total phenolic content (TPC) was

significantly higher in the case of Mix plants (3396.4 ± 415.9 mg GAE 100 g^{-1}) compared with controls (2756.4 ± 155.6 mg GAE 100 g^{-1}), which gave a result not statistically different from the other treated plants (Table 3).

Table 2. Growing degree days (GDD), yield of flowers and spice, root biomass (dry weight), and corm yield of controls (Ctr) and inoculated saffron plants (Myc, Pgpr, and Mix).

	Ctr	Myc	Pgpr	Mix	*p*
Flowering					
GDD (°C-days)	877.0 ± 22.9	874.4 ± 21.7	874.6 ± 19.5	875.3 ± 26.4	ns
Flowers $corm^{-1}$ (n)	3.8 ± 0.9	3.7 ± 1.0	3.6 ± 1.2	3.7 ± 1.4	ns
Weight of spice $flower^{-1}$ (mg)	7.6 ± 1.5 a	6.7 ± 1.8 b	7.4 ± 1.8 a	7.5 ± 1.6 a	**
Root biomass (g)	0.49 ± 0.12	0.49 ± 0.07	0.56 ± 0.10	0.62 ± 0.16	ns
Corm yield					
Corms $plant^{-1}$	6.0 ± 0.0 c	6.9 ± 0.1 a	6.4 ± 0.1 b	6.3 ± 0.1 b	***
Corm weight $plant^{-1}$ (g)	3.4 ± 0.1 ab	3.1 ± 0.1 b	3.4 ± 0.1 a	3.6 ± 0.3 a	*
Corm size $plant^{-1}$ (mm)	19.2 ± 0.7 a	17.7 ± 1.0 b	18.0 ± 0.6 b	18.6 ± 1.3 a	***
Yield of corms with major diameter > 15 mm					
Corms $plant^{-1}$	6.2 ± 0.3 a	5.8 ± 0.1 a	5.3 ± 0.1 b	5.7 ± 0.1 ab	**
Corm weight $plant^{-1}$ (g)	3.1 ± 0.4 b	3.3 ± 0.1 ab	3.8 ± 0.1 ab	4.1 ± 0.6 a	*
Corm size $plant^{-1}$ (mm)	19.3 ± 0.1 a	18.4 ± 0.1 b	19.5 ± 0.2 a	19.5 ± 0.7 a	*

Values of mean ± standard deviation are reported. Letters indicate statistical differences. Values with the same letter are not statistically different at *p < 0.05; **p < 0.01; ***p < 0.001; ns = not significant.

1.9.4. Ecophysiological analysis, epigean development, and starch content of corms

Looking at the ecophysiological parameters (Table 4), no significant differences were found for the photosynthetic rate (A_N). Ctr plants had a higher stomatal CO_2 conductance (*gs*, 174.2 ± 23.5 mmol H_2O $m^{-2}s^{-1}$) and a transpiration rate (*E*, 3.2

± 0.4 mmol H_2O $m^{-2}s^{-1}$) not significantly different from those inoculated. Between the treated plants, *E* was greater in Myc plants (4.0 ± 0.6 mmol H_2O $m^{-2}s^{-1}$) than Pgpr, and there were no differences regarding *gs*. Finally, leaf production and leaf area were not affected by the treatments (Table 4). The content of chl a in Mix plants (1.2 ± 0.1 μg mg^{-1}) was significantly higher than in Myc, but not different than in Pgpr and Ctr. Regarding the content of chl b, no differences were found between inoculated plants and Ctr. Consequently, the sum of chl a and b was significantly greater for the Mix treatment (1.6 ± 0.1 μg mg^{-1}) than for Pgpr (1.2 ± 0.0 μg mg^{-1}) and Myc (1.1 ± 0.2 μg mg^{-1}), but not different form Ctr. Concerning the content of carotenoids, there were no differences between all treatments. The starch content (Table 4), measured in corms of similar weight for all treatments, resulted not significantly different. The moisture content of the corms was significantly higher for the Myc inoculation (7.0%) than for Ctr.

Table 3. Results of the ISO (3632, 1:2011), total phenolic content (TPC), and HPLC analyses of aqueous extracts of the spice obtained from control (Ctr) and inoculated plants (Myc, Pgpr, and Mix).

ISO (3632, 1:2011)	Ctr	Myc	Pgpr	Mix	*p*
Colour/Crocins $A^{1\%}_{1cm}$ (λ 440)	237.0 ± 17.4 (I)	236.1 ± 17.4 (I)	217.0 ± 33.8 (I)	227.3 ± 6.6 (I)	ns
Flavour/Picrocrocin $A^{1\%}_{1cm}$ (λ 257)	97.7 ± 6.3 (I) ab	89.9 ± 5.0 (I) b	95.1 ± 4.5 (I) ab	98.0 ± 3.2 (I) a	*
Aroma /Safranal $A^{1\%}_{1cm}$ (λ 330)	36.9 ± 1.8 (I) a	36.7 ± 1.8 (I) ab	31.7 ± 8.7 (I) ab	31.6 ± 1.0 (I) b	*
HPLC (λ 310)					
Safranal (mg 100 g^{-1})	1.3 ± 0.6 b	52.1 ± 15.7 a	36.7 ± 11.6 a	40.7 ± 13.2 a	**
TPC					
Folin-Ciocalteu (mg GAE 100 g^{-1})	2756.4 ± 155.6 b	3133.9 ± 392.9 ab	3241.5 ± 240.7 ab	3396.4 ± 415.9 a	*

The quality category (ISO 3632, 2011) is indicated in brackets. The limits for the quality category I are: picrocrocin >70; safranal 20 - 50; crocins >200. Values of mean ± standard deviation are reported. Letters indicate statistical differences. Values with the same letter are not statistically different at *p <0.05; **p <0.01.

Table 4. Results of ecophysiological analysis (AN = net CO2 assimilation rate; gs = stomatal conductance; E = leaf transpiration rat), leaf production, leaf area, content of chlorophylls (chl) and carotenoids of leaves, and starch analysis of corms for controls (Ctr) and inoculated plants (Myc, Pgpr, and Mix).

	Ctr	Myc	Pgpr	Mix	*p*
Ecophysiological analysis					
A_N (µmol CO_2 m^{-2} s^{-1})	6.7 ± 4.2	8.3 ± 2.5	6.4 ± 1.5	9.1 ± 4.3	ns
g_s (mmol H_2O m^{-2} s^{-1})	174.2 ± 23.5 a	112.3 ± 21.3 b	128.2 ± 30.1 b	121.7 ± 17.2 b	***
E (mmol H_2O m^{-2} s^{-1})	3.2 ± 0.4 ab	4.0 ± 0.6 a	3.1 ± 0.7 b	3.9 ± 0.3 ab	*
Leaf production and area					
Leaves $plant^{-1}$	43.1 ± 16.7	47.7 ± 11.1	46.0 ± 12.2	44.6 ± 14.2	ns
Leaves length $plant^{-1}$ (cm)	37.8 ± 4.8	36.9 ± 4.6	37.9 ± 5.4	36.1 ± 4.5	ns
Leaf area $plant^{-1}$ (cm^{-2})	92.0 ± 16.5	106.7 ± 28.4	101.0 ± 31.3	97.2 ± 30.0	ns
Chlorophylls and Carotenoids in leaves					
Chl a (µg/mg)	1.0 ± 0.0 ab	0.8 ± 0.2 b	0.9 ± 0.0 ab	1.2 ± 0.1 a	*
Chl b (µg/mg)	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	ns
Chl a+b (µg/mg)	1.4 ± 0.0 ab	1.1 ± 0.2 b	1.2 ± 0.0 b	1.6 ± 0.1 a	**
Carotenoids (µg/mg)	25.4 ± 0.3	21.5 ± 3.4	23.4 ± 0.2	27.0 ± 2.8	ns
Starch analysis					
Starch (g 100^{-1} g)	64.5 ± 4.4	50.9 ± 17.1	61.8 ± 9.5	66.8 ± 14.7	ns
Corms weight $plant^{-1}$ (g)	5.5 ± 0.5	5.7 ± 0.1	5.8 ± 0.1	5.9 ± 0.5	ns
Moisture content (%)	6.4 ± 0.2 b	7.0 ± 0.9 a	6.5 ± 0.2 ab	6.9 ± 0.2 ab	*

Values of mean ± standard deviation are reported. Letters indicate statistical differences. Values with the same letter are not statistically different at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; ns = not significant.

1.10. Discussion

1.10.1. How beneficial microorganisms affected flowering and saffron yield

Having a very short life, saffron flowers are collected immediately and more than 100,000 flowers are needed to produce 1 kg of spice (Caser et al., 2020; Mottaghipisheh et al., 2020). Microorganisms can affect flowering time (Aimo et al., 2010; Caser et al., 2019; Rouphael et al., 2015; Sharaf-Eldin et al., 2008). In Caser et al. (2019), AMF treatments (*R. intraradices* alone and with *F. mosseae*) anticipated the anthesis of soilless saffron in a greenhouse by one week. Application of *B. subtilis* significantly advanced saffron flowering in a greenhouse pot experiment (Sharaf-Eldin et al., 2008). In this study, the anthesis was not affected by the microorganisms and began at the end of October, 63 - 64 days after corm sowing, when the growing degree days (GDD) were about 875.3. Two main flowering peaks occurred about seventy days after sowing, with differences in the percentage distributions of flowers especially for Ctr and Myc (values of 26% and 30% respectively for the first peak and 32% and 25% for the second peak).

Beneficial microorganisms did not affect also the yield of flowers per corm as in Caser et al. (2019). In the present study, the AM colonization evaluated during flowering was low. Overall, little extraradical mycelium and intraradical hyphae with vesicles (specialized storage structures) and very rare arbuscules (functional trade structures) were seen under optical microscope, suggesting that *R. intraradices* might have exhibited a saprophytic behavior during flowering (Azcón-Aguilar et al., 1999; Maiti and Ghosh, 2020). According to Smith and Smith (2011), a negative mycorrhizal growth response can be caused by an imbalanced organic C cost to the plant. This might explain the reduction in spice yield (mg of spice per flower) observed for Myc plants (-12%). In a previous work, *R. intraradices* led to a decrease in spice production in the field (-20% mg of spice per flower; Caser et al., 2019). The low AM colonization and the consequent neutral mycorrhizal growth response might be explained by the

biological cycle of *C. sativus* L., that flowers in autumn sixty - ninety days after sowing the corms. Large-sized corms already contain nutrient reserves needed for early growth; for example, the concentration of P and N in corms weighting more than 8 g ranged from 2.41 g kg^{-1} to 2.82 g kg^{-1} (P) and from 12.04 g kg^{-1} to 14.88 g kg^{-1} (N) depending on the mother corm size (Koocheki and Seyyedi, 2015). Thus, the large corms used in this study may have had enough reserve nutrients to support flower development. Well mycorrhized saffron roots were observed after flowering by Aimo et al. (2010), Chamkhi et al. (2018), Lone et al. (2016), and Caser et al. (2019). Accordingly, Lone et al. (2016), monthly estimating the frequency of several AMF species in saffron grown in soil (Kashmir, India) with the Biermann and Lindermann (1981) method, observed that the frequency of colonization in the roots increased from 14.86% in September to 90.24% in March and then decreased. Caser et al. (2019) also found that the intensity of AM colonization (*R. intraradices*) in the whole hydroponic saffron root system analysed with the Trouvelot et al. (1986) method was 71.4% with an arbuscule abundance of 58.9% at the end of the vegetative phase of the first year of cultivation.

The bacteria *Bacillus megaterium* and *Paenibacillus durus*, which occur naturally in saffron rhizosphere, have multiple growth promoting traits (Jami Al-Ahmadi et al., 2017; and Kour et al., 2018). In this study they showed important functional PGP traits, such as N_2 fixation, P solubilization, siderophore production, and K solubilization (Backer et al., 2018; Lobo et al., 2019). Compared with Ctr, the fixed N_2 potential by the microbiota improved by +48% and +44% in the Pgpr and Mix samples, respectively; the P solubilization by +43% (Pgpr) and +35% (Mix); the K solubilization by +27% (Pgpr) and +31% (Mix) times; and the siderophores production by +42% (Pgpr) and +54% (Mix) (Fig. 5). When the bacteria were added to the AMF (Mix inoculum), the frequency of root colonization in some fragments was 90% with abundant vesicles and in more cases an abundant extraradical mycelium was seen, in agreement with Rouphael et al. (2015) reporting that various gram-positive bacteria, such as *Paenibacillus* spp. and *Bacillus* spp., can stimulate AMF branching and colonization. Moreover,

in Mix plants the bacteria may have mitigated the initial imbalanced C cost to the plants due to the probable saprophytic behavior of the AMF, resulting in a restored spice yield. A promoting effect on the yield of saffron spice was reported for PGPR inoculants in pot trials, i.e. *Curtobacterium herbarum* Cs10 (Díez-M´endez and Rivas, 2017), *Bacillus* sp. strain D5 (Magotra et al., 2021), and *Bacillus subtilis* FZB24® (Sharaf-Eldin et al., 2008). An increment in the corm size in greenhouses was obtained by treating saffron with *Bacillus* sp. strain D5 (Magotra et al., 2021) or AMF species (*R. intraradices* alone or mixed with *F. mosseae*; Caser et al., 2019b). Since corm size is an important parameter for flowering, the effect of bioinoculants on saffron yield could be evaluated over time in a second-year experiment in a greenhouse, as has already been done in the field (Aimo et al., 2010; Magotra et al., 2021).

1.10.2. The beneficial microorganisms enhanced the aroma of the spice

AM symbiosis is known to induce changes in plant metabolism (Bianciotto et al., 2018; Kumar et al., 2021; Rouphael et al., 2015), increasing the content of health-promoting compounds such as carotenoids and polyphenols (Bianciotto et al., 2018). The organoleptic properties of the spice produced by both inoculated and uninoculated plants belonged to the quality category I of the ISO 3632 (2011) (the highest), as in Caser et al. (2019). However, the aromatic strength (A1% 1 cm at λ 330; ISO 3632 – 2011), which is related to the safranal content, was significantly lower for Mix plants. The aromatic strength of saffron, measured with UV–vis spectrophotometry, can be altered by other compounds that can absorb at 330 nm, such as crocins (Fig. 3), interfering with the analysis (García-Rodríguez et al., 2017). García-Rodríguez et al. (2017) observed that the determination of safranal by the UV–vis method gave an overestimation compared with the determination by HPLC, with a range from 3.69 to 8.65 mg 100 mg^{-1} of saffron depending on the area of production. Similarly, in our study the HPLC analysis showed a safranal content not in line with the aroma strength measured with the UV–vis method, being higher in the Myc, Pgpr, and Mix samples (up to +96%) than in the Ctr ones. These results are in agreement with

Sharaf-Eldin et al. (2008), where the spice derived from corms drenched with a spore solution of *Bacillus subtilis* FZB24® 14 weeks after sowing had a higher content of picrocrocin (+38.9%), crocetin (+75.3%), and safranal (+8.4%) but a lower level of crocin (-60%) than that derived from uninoculated control corms. The spice produced from Mix plants also showed a total phenolic content (TPC) significantly higher (+19%) than that obtained from controls. A more positive trend of TPC was also visible for both Myc and Pgpr treatments. Probably the synergy created between the AMF and PGPR led to a more beneficial effect for the plant in agreement with Etesami et al. (2021) and Giovannini et al. (2020). Similarly, in Begum et al. (2022) a mixed inoculant of AMF and PGPR considerably increased tobacco (*Nicotiana tabacum* L.) secondary metabolites such as carotenoids and phenols under drought stress conditions. Thus, the AMF and PGPR influenced saffron secondary metabolism enhancing the production of bioactive compounds even though the roots were little mycorrhized, especially when mixed.

1.10.3. How beneficial microorganisms affected eco-physiological parameters and corm production

After flowering, growth is mainly supported by leaf photosynthesis, which is maintained high during vegetative development (Renau-Morata et al., 2012). Investigation of saffron ecophysiology during the vegetative phase is limited. Some authors (Moradi et al., 2021; Renau-Morata et al., 2012; Yarami and Sepaskhah, 2015; Zhou et al., 2022) studied the photosynthetic activity of saffron analysing the influence of different factors, such as light intensity and spectra, water stress, planting methods, corm size, and salinity and fertilizer levels, but the influence exerted by beneficial microorganisms has never been evaluated so far. Renau-Morata et al. (2012) obtained values of the net CO_2 assimilation rate (A_N) of plants grown in a greenhouse similar to our study. In the field, the plants showed an A_N similar (Yarami & Sepaskhah, 2015) or higher (20–26 μmol $m^{-2}s^{-1}$ - Renau-Morata et al., 2012) than ours, perhaps thanks to the more favorable irradiation conditions present in the field than in the greenhouse, especially

during winter (Zhou et al., 2022). In other plant species, such as *Solanum lycopersicum* L., *Nicotiana tabacum* L., *Prunus maritima* Marshall, and *Phoenix dactylifera* L., the effects of microbial treatments, i.e., AMF, PGPR, and a mix of both microbial types, on ecophysiological parameters have been recently investigated (Begum et al., 2022; Mannino et al., 2020; Raho et al., 2022; Zai et al., 2021). When treated with bioinoculants (the AMF *Rhizoglomus irregulare*; a combination of the AMF *Claroideoglomus claroideum*, *Funneliformis caledonium* and *F. geosporum*; a combination of two PGPB strains and a commercial inoculum formed by *Glomus* spp. and bacteria) *Solanum lycopersicum* L. did not significantly change A_N, stomatal conductance (*gs*), and evapotranspiration rate (*E*), but when inoculated with the commercial mixed formulation it reduced the total content of chlorophylls (Mannino et al., 2020). In this study, the total chlorophyll content in inoculated plants did not differ from Ctr, even if a tendency to be higher in Mix plants was observed; among inoculated plants it was higher in Mix plants (1.6 ± 0.1 μg mg^{-1}) than in Myc (1.1 ± 0.2 μg mg^{-1}) and Pgpr (1.2 ± 0.0 μg mg^{-1}) plants. As total chlorophyll content is strongly related to leaf N content (Mannino et al., 2020; Padilla et al., 2018), it can be hypothesized that the mycelium may have ameliorated the substrate structure and retained more N-fixing bacteria in the rhizosphere, leading to an improvement in the mineral nutrient uptake, especially N, by the plants. Also regarding leaf production, leaf area, evapotranspiration rate (*E*), and A_N, no differences were found between treated and uninoculated plants. Only the stomatal conductance (*gs*) differed between treated plants and Ctr (174.2 ± 23.5 mmol H2O $m^{-2}s^{-1}$) being lower in treated plants. This might be because the ecophysiological parameters were analysed ten days after the last fertigation. Bioinoculated plants usually decrease the *gs* when are under water limitation allowing for water preservation (Sati et al., 2022), e.g., *Arabidopsis thaliana* (L.) Heynh. with *Azospirillum brasilense* in Cohen et al. (2015) and *Solanum lycopersicum* L. in Mannino et al. (2020) and in Chitarra et al. (2016), which used AMF inocula of *R. intraradices* and *F. mosseae*. Among the inoculated plants, *E* was higher in Myc plants (4.0 ± 0.6 mmol H_2O $m^{-2}s^{-1}$) than in Pgpr plants (3.1 ± 0.7 mmol H_2O

$m^{-2}s^{-1}$) and not different from Mix plants (3.9 ± 0.3 mmol H_2O $m^{-2}s^{-1}$). This result is in line with the well-known ability of AMF to improve water status of plants, in agreement with the result of corm moisture content, which tended to be higher for treated corms and was significantly superior for Myc plants (7.0 ± 0.9%) than for Ctr plants (6.4 ± 0.2%). Microbial inoculants improved A_N and *E*, and chlorophyll content in *Nicotiana tabacum* L. (the AMF *Glomus versiforme* and the PGPR *Bacillus methylotrophicus*, alone and together - Begum et al., 2022) and *Prunus maritima* Marshall, which also presented a higher *gs* only in AMF and Mix treated plants (the AMF *Funneliformis mosseae* and the phosphate-solubilizing fungus *Apophysomyces spartima* - Zai et al., 2021); and in *Phoenix dactylifera* L. they improved *gs* and pigment content (an AMF consortium of 26 species and a mixture of the PGPR *Bacillus megaterium*, *Arthrobacter globiformis*, and *Enterobacter ludwigii*, alone and together - Raho et al., 2022).

Similarly to the physiological parameters, no differences emerged also regarding the starch content. However, corm yield resulted overall improved for the inoculated plants. In particular, the number of replacement corms was higher for treated plants than for Ctr, especially for the Myc treatment (+13%), without the weight of the corms being decreased. Corm weight is an important attribute for saffron production as corms need to be above a critical size (1 cm of diameter, ~1.1 g) to flower (Douglas et al., 2014). When only larger corms were considered (> 1.5 cm), Mix plants showed a number of corms similar to Ctr but with an increased weight (+24%). These results are in agreement with previous studies on saffron inoculated with beneficial microorganisms. The number of replacement corms was increased by a consortium of six rhizosphere-isolated bacteria (*Acinetobacteria calcoaceticus*, *Pseudomonas tremae*, *Pseudomonas kilonensis*, *Chryseobacteria elymi*, *Bacillus aryabhattai*, *Pseudomonas koreensis*) compared with uninoculated controls (without differences for corm weight) in Ambardar and Vakhlu (2013). Average weight of daughter corms was enhanced by *Bacillus megaterium* in Kour et al. (2018) and by *Bacillus* sp. strain D5 in Magotra et al. (2021).

1.11. Conclusions

Bioinoculants are considered modern agricultural tools able to reduce chemical application, promote plant defense system and enhance phytochemicals, thus increasing the quality of products. They can attract consumer interest in high-quality and sustainable saffron production. The effects of bipartite (plant–PGPR/AMF) and tripartite (plant–PGPR–AMF) interactions on saffron plants were investigated. Simultaneous inoculations of different beneficial microbes in a controlled environment allowed the responses of saffron to be evaluated under reproducible conditions. The organoleptic profile of the spice produced in hydroponics belonged to the first ISO (2011) category. Terpenes and phenols have nutraceutical properties and demonstrated pharmacological effects. Their quantification is fundamental to evaluate the quality level of the saffron spice for its use in food and in the pharmaceutical sector and to state the potential efficacy of selected bioinoculants. All beneficial microorganisms led to an increased content of the main aromatic metabolite safranal. Inoculation of the AMF *R. intraradices* decreased spice yield, probably due to a saprophytic behavior exhibited by the fungus during flowering. When the PGPR *B. megaterium* and *P. durus* were added, the yield was restored. Thus, the PGPR and AMF appeared to work in synergy. This cooperation was also seen to improve the total phenolic content of the saffron spice and corm production; indeed, Mix plants produced corms with the same size and weight as the Ctr but with a higher number, differently than single-type inoculant applications. Taking the results together, we may say that *R. intraradices* and the two bacteria *B. megaterium* and *P. durus* may have stimulated the secondary metabolism of the plants improving quality traits, rather than having acted as biofertilizers enhancing the yield, at least during flowering. This study on the effects of different beneficial microorganisms on saffron during its growth phases can lay the basis for further deepen saffron responses, e.g., at the molecular level.

Acknowledgments

The authors thank Dario Donno for the HPLC analysis, Davide Lucien Patono for the help with the IRGA instrument, Francesca Vanara for the suggestions for the starch analysis, Walter Gaino for technical support, and Francesco Berruto for the help in plant maintenance and laboratory analyses.

Funding

This research was funded by the program Interreg V-A Francia Italia Alcotra (Grant No. 1139 "ANTEA – Attività innovative per lo sviluppo della filiera transfrontaliera del fiore edule"; and grant no. 8336 "ANTES - Fiori eduli e piante aromatiche: attività capitalizzazione dei progetti ANTEA ed ESSICA").

1.12. References

Adolfsson, L., Nziengui, H., Abreu, I.N., Šimura, J., Beebo, A., Herdean, A., Aboalizadeh, J., Široká, J., Moritz, T., Novák, O., Ljung, K., Schoefs, B., Spetea, C., 2017. Enhanced secondary- and hormone metabolism in leaves of arbuscular mycorrhizal medicago truncatula. Plant Physiol. 175, 392–411. https://doi.org/10.1104/pp.16.01509

Aimo, S., Gosetti, F., D'Agostino, G., Gamalero, E., Gianotti, V., Bottaro, M., Gennaro, M.C., Berta, G., 2010. USE OF ARBUSCULAR MYCORRHIZAL FUNGI AND BENEFICIAL SOIL BACTERIA TO IMPROVE YIELD AND QUALITY OF SAFFRON (CROCUS SATIVUS L.). Acta Hortic. 850, 159–164. https://doi.org/10.17660/ActaHortic.2010.850.25

Ambardar, S., Vakhlu, J., 2013. Plant growth promoting bacteria from Crocus sativus rhizosphere. World J. Microbiol. Biotechnol. 29, 2271–2279. https://doi.org/10.1007/s11274-013-1393-2

Askari-Khorasgani, O., Pessarakli, M., 2019. Shifting saffron (Crocus sativus L.) culture from traditional farmland to controlled environment (greenhouse) condition to avoid the negative impact of climate changes and increase its productivity. J. Plant Nutr. 42, 2642–2665. https://doi.org/10.1080/01904167.2019.1659348

Avarseji, Z., Kafi, M., Sabet Teimouri, M., Orooji, K., 2013. INVESTIGATION OF SALINITY STRESS AND POTASSIUM LEVELS ON

MORPHOPHYSIOLOGICAL CHARACTERISTICS OF SAFFRON. J. Plant Nutr. 36, 299–310. https://doi.org/10.1080/01904167.2012.739249

Azcón-Aguilar, C., Bago, B., & Barea, J. M. (1999). Saprophytic Growth of Arbuscular Mycorrhizal Fungi. In A. Varma & B. Hock (Eds.), Mycorrhiza: Structure, Function, Molecular Biology and Biotechnology (pp. 391–408). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-662-03779-9_16

Backer, R., Rokem, J.S., Ilangumaran, G., Lamont, J., Praslickova, D., Ricci, E., Subramanian, S., Smith, D.L., 2018. Plant growth-promoting rhizobacteria: Context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. Front. Plant Sci. 871, 1–17. https://doi.org/10.3389/fpls.2018.01473

Barros-Rodríguez, A., Rangseekaew, P., Lasudee, K., Pathom-aree, W., & Manzanera, M. (2020). Regulatory risks associated with bacteria as biostimulants and biofertilizers in the frame of the European Regulation (EU) 2019/1009. Science of The Total Environment, 740, 140239. https://doi.org/10.1016/J.SCITOTENV.2020.140239

Begum, N., Wang, L., Ahmad, H., Akhtar, K., Roy, R., Khan, M.I., Zhao, T., 2022. Co-inoculation of Arbuscular Mycorrhizal Fungi and the Plant Growth-Promoting Rhizobacteria Improve Growth and Photosynthesis in Tobacco Under Drought Stress by Up-Regulating Antioxidant and Mineral Nutrition Metabolism. Microb. Ecol. 83, 971–988. https://doi.org/10.1007/s00248-021-01815-7

Berruti, A., Lumini, E., Balestrini, R., Bianciotto, V., 2016. Arbuscular Mycorrhizal Fungi as Natural Biofertilizers: Let's Benefit from Past Successes. Front. Microbiol. 6, 1–13. https://doi.org/10.3389/fmicb.2015.01559

Bianciotto, V., Victorino, I., Scariot, V., Berruti, A., 2018. Arbuscular mycorrhizal fungi as natural biofertilizers: current role and potential for the horticulture industry. Acta Hortic. 1191, 207–216. https://doi.org/10.17660/ActaHortic.2018.1191.29

Blandino, M., Mancini, M.C., Peila, A., Rolle, L., Vanara, F., Reyneri, A., 2010. Determination ofmaize kernel hardness: Comparison of different laboratory tests to predict dry-milling performance. J. Sci. Food Agric. 90, 1870–1878. https://doi.org/10.1002/jsfa.4027

Bonfante, P., Genre, A., 2010. Mechanisms underlying beneficial plant - Fungus interactions in mycorrhizal symbiosis. Nat. Commun. 1, 1–11. https://doi.org/10.1038/ncomms1046

Cardone, L., Castronuovo, D., Perniola, M., Cicco, N., Candido, V., 2020. Saffron (Crocus sativus L.), the king of spices: An overview. Sci. Hortic. (Amsterdam). 272. https://doi.org/10.1016/j.scienta.2020.109560

Carmona, M., Zalacain, A., Sánchez, A.M., Novella, J.L., Alonso, G.L., 2006. Crocetin esters, picrocrocin and its related compounds present in Crocus sativus stigmas and Gardenia jasminoides fruits. Tentative identification of seven new compounds by LC-ESI-MS. J. Agric. Food Chem. 54, 973–979. https://doi.org/10.1021/jf052297w

Caser, M., Demasi, S., Stelluti, S., Donno, D., Scariot, V., 2020. Crocus sativus L. Cultivation in Alpine Environments: Stigmas and Tepals as Source of Bioactive Compounds. Agronomy 10, 1473. https://doi.org/10.3390/agronomy10101473

Caser, M., Demasi, S., Victorino, Í.M.M., Donno, D., Faccio, A., Lumini, E., Bianciotto, V., Scariot, V., 2019. Arbuscular Mycorrhizal Fungi Modulate the Crop Performance and Metabolic Profile of Saffron in Soilless Cultivation. Agronomy 9, 232. https://doi.org/10.3390/agronomy9050232

Caser, M., Lovisolo, C., Scariot, V., 2017. The influence of water stress on growth, ecophysiology and ornamental quality of potted Primula vulgaris 'Heidy' plants. New insights to increase water use efficiency in plant production. Plant Growth Regul. 83, 361–373. https://doi.org/10.1007/s10725-017-0301-4

Caser, M., Victorino, Í.M.M., Demasi, S., Berruti, A., Donno, D., Lumini, E., Bianciotto, V., Scariot, V., 2018. Saffron Cultivation in Marginal Alpine Environments: How AMF Inoculation Modulates Yield and Bioactive Compounds. Agronomy 9, 12. https://doi.org/10.3390/agronomy9010012

Chamkhi, I., Abbas, Y., Tarmoun, K., Aurag, J., Sbabou, L., 2018. Morphological and molecular characterization of arbuscular mycorrhizal fungal communities inhabiting the roots and the soil of saffron (Crocus sativus L.) under different agricultural management practices. Arch. Agron. Soil Sci. 65, 1035–1048. https://doi.org/10.1080/03650340.2018.1548012

Chen, D., Xing, B., Yi, H., Li, Y., Zheng, B., Wang, Y., Shao, Q., 2020. Effects of different drying methods on appearance, microstructure, bioactive compounds and aroma compounds of saffron (Crocus sativus L.). Lwt 120, 108913. https://doi.org/10.1016/j.lwt.2019.108913

Chialva, M., Lanfranco, L., Guazzotti, G., Santoro, V., Novero, M., Bonfante, P., 2020. Gigaspora margarita and its endobacterium modulate symbiotic marker genes in tomato roots under combined water and nutrient stress. Plants 9, 1–14.

https://doi.org/10.3390/plants9070886

Chitarra, W., Pagliarani, C., Maserti, B., Lumini, E., Siciliano, I., Cascone, P., Schubert, A., Gambino, G., Balestrini, R., Guerrieri, E., 2016. Insights on the impact of arbuscular mycorrhizal symbiosis on tomato tolerance to water stress. Plant Physiol. 171, 1009–1023. https://doi.org/10.1104/pp.16.00307

De Pascale, S., Rouphael, Y., Colla, G., 2017. Plant biostimulants: innovative tool for enhancing plant nutrition in organic farming. Eur. J. Hortic. Sci 82, 277–285. https://doi.org/10.17660/eJHS.2017/82.6.2

Díez-Méndez, A., Rivas, R., 2017. Improvement of saffron production using Curtobacterium herbarum as a bioinoculant under greenhouse conditions. AIMS Microbiol. 3, 354–364. https://doi.org/10.3934/microbiol.2017.3.354

Douglas, M.H., Smallfield, B.M., Wallace, A.R., Mcgimpsey, J.A., 2014. Saffron (Crocus sativus L.): The effect of mother corm size on progeny multiplication, flower and stigma production. Sci. Hortic. (Amsterdam). 166, 50–58. https://doi.org/10.1016/j.scienta.2013.12.007

Etesami, H., Jeong, B.R., Glick, B.R., 2021. Contribution of Arbuscular Mycorrhizal Fungi, Phosphate–Solubilizing Bacteria, and Silicon to P Uptake by Plant. Front. Plant Sci. https://doi.org/10.3389/fpls.2021.699618

Fernandes, B., Dragone, G., Abreu, A.P., Geada, P., Teixeira, J., Vicente, A., 2012. Starch determination in Chlorella vulgaris-a comparison between acid and enzymatic methods. J. Appl. Phycol. 24, 1203–1208. https://doi.org/10.1007/s10811-011-9761-5

García-Rodríguez, M.V., López-Córcoles, H., Alonso, G.L., Pappas, C.S., Polissiou, M.G., Tarantilis, P.A., 2017. Comparative evaluation of an ISO 3632 method and an HPLC-DAD method for safranal quantity determination in saffron. Food Chem. 221, 838–843. https://doi.org/10.1016/j.foodchem.2016.11.089

Genre, A., Lanfranco, L., Perotto, S., Bonfante, P., 2020. Unique and common traits in mycorrhizal symbioses. Nat. Rev. Microbiol. 18, 649–660. https://doi.org/10.1038/s41579-020-0402-3

Giovannini, L., Palla, M., Agnolucci, M., Avio, L., Sbrana, C., Turrini, A., Giovannetti, M., 2020. Arbuscular Mycorrhizal Fungi and Associated Microbiota as Plant Biostimulants: Research Strategies for the Selection of the Best Performing Inocula. Agronomy 10, 106. https://doi.org/10.3390/agronomy10010106

Giupponi, L., Ceciliani, G., Leoni, V., Panseri, S., Pavlovic, R., Lingua, G., Di Filippo,

A., Giorgi, A., 2019. Quality traits of saffron produced in Italy: Geographical area effect and good practices. J. Appl. Bot. Food Qual. 92, 336–342. https://doi.org/10.5073/JABFQ.2019.092.045

Gregory, M.J., Menary, R.C., Davies, N.W., 2005. Effect of drying temperature and air flow on the production and retention of secondary metabolites in saffron. J. Agric. Food Chem. 53, 5969–5975. https://doi.org/10.1021/jf047989j

Gresta, F., Lombardo, G.M., Siracusa, L., Ruberto, G., 2008. Saffron, an alternative crop for sustainable agricultural systems. A review. Agron. Sustain. Dev. 28, 95–112. https://doi.org/10.1051/agro:2007030

Jami Al-Ahmadi, M., Mohammadi, A., Kohabadi, E.S., 2017. Characterization of Bacteria Isolated from the Saffron (Crocus sativus L.) Rhizosphere. J. Hortic. Res. 25, 5–14. https://doi.org/10.1515/johr-2017-0017

Khan, M., Hanif, M.A., Ayub, M.A., Jilani, M.I., Shahid Chatha, S.A., 2020. Saffron, in: Medicinal Plants of South Asia. Elsevier, pp. 587–600. https://doi.org/10.1016/B978-0-08-102659-5.00043-4

Koocheki, A., Seyyedi, S.M., 2015. Relationship between nitrogenand phosphorus use efficiency in saffron (Crocus sativus L.) as affected by mother corm size and fertilization. Ind. Crops Prod. 71, 128–137. https://doi.org/10.1016/j.indcrop.2015.03.085

Kour, R., Ambardar, S., Vakhlu, J., 2018. Plant growth promoting bacteria associated with corm of Crocus sativus during three growth stages. Lett. Appl. Microbiol. 67, 458–464. https://doi.org/10.1111/lam.13042

Kumar, R., 2009. Calibration and validation of regression model for non-destructive leaf area estimation of saffron (Crocus sativus L.). Sci. Hortic. (Amsterdam). 122, 142–145. https://doi.org/10.1016/j.scienta.2009.03.019

Kumar, R., Singh, V., Devi, K., Sharma, M., Singh, M.K., Ahuja, P.S., 2008. State of Art of Saffron (Crocus sativus L.) Agronomy: A Comprehensive Review. Food Rev. Int. 25, 44–85. https://doi.org/10.1080/87559120802458503

Kumar, S., Arora, N., Upadhyay, H., 2021. Arbuscular mycorrhizal fungi: Source of secondary metabolite production in medicinal plants, in: New and Future Developments in Microbial Biotechnology and Bioengineering. Elsevier, pp. 155–164. https://doi.org/10.1016/B978-0-12-821005-5.00011-9

Lanfranco, L., Fiorilli, V., Gutjahr, C., 2018. Partner communication and role of nutrients in the arbuscular mycorrhizal symbiosis. New Phytol. 220, 1031–1046.

https://doi.org/10.1111/nph.15230

Lichtenthaler, H.K., 1987. Chlorophylls and Carotenoids: Pigments of Photosynthetic Biomembranes. Methods Enzymol. 148, 350–382. https://doi.org/10.1016/0076-6879(87)48036-1

Lobo, C.B., Juárez Tomás, M.S., Viruel, E., Ferrero, M.A., Lucca, M.E., 2019. Development of low-cost formulations of plant growth-promoting bacteria to be used as inoculants in beneficial agricultural technologies. Microbiol. Res. 219, 12–25. https://doi.org/10.1016/j.micres.2018.10.012

Lone, R., Shuab, R., Koul, K.K., 2016. AMF association and their effect on metabolite mobilization, mineral nutrition and nitrogen assimilating enzymes in saffron (Crocus sativus) plant. J. Plant Nutr. 39, 1852–1862. https://doi.org/10.1080/01904167.2016.1170850

Magotra, S., Bhagat, N., Ambardar, S., Ali, T., Hurek, B.R., Hurek, T., Verma, P.K., Vakhlu, J., 2021. Field evaluation of PGP Bacillus sp. strain D5 native to Crocus sativus, in traditional and non traditional areas, and mining of PGP genes from its genome. Sci. Rep. 11, 1–16. https://doi.org/10.1038/s41598-021-84585-z

Maiti, S.K., Ghosh, D., 2020. Plant–soil interactions as a restoration tool, Climate Change and Soil Interactions. LTD. https://doi.org/10.1016/b978-0-12-818032-7.00024-2

Mannino, G., Nerva, L., Gritli, T., Novero, M., Fiorilli, V., Bacem, M., Bertea, C.M., Lumini, E., Chitarra, W., Balestrini, R., 2020. Effects of different microbial inocula on tomato tolerance to water deficit. Agronomy 10, 1–18. https://doi.org/10.3390/agronomy10020170

McMaster, G.S., Wilhelm, W.W., 1997. Growing degree-days: one equation, two interpretations. Agric. For. Meteorol. 87, 291–300. https://doi.org/10.1016/S0168-1923(97)00027-0

Molina, R.V., Valero, M., Navarro, Y., Guardiola, J.L., García-Luis, A., 2005. Temperature effects on flower formation in saffron (Crocus sativus L.). Sci. Hortic. (Amsterdam). 103, 361–379. https://doi.org/10.1016/j.scienta.2004.06.005

Molina, R. V., Valero, M., Navarro, Y., García-Luis, A., Guardiola, J.L., 2005. Low temperature storage of corms extends the flowering season of saffron (Crocus sativus L.). J. Hortic. Sci. Biotechnol. 80, 319–326. https://doi.org/10.1080/14620316.2005.11511937

Mottaghipisheh, J., Mahmoodi Sourestani, M., Kiss, T., Horváth, A., Tóth, B., Ayanmanesh, M., Khamushi, A., Csupor, D., 2020. Comprehensive chemotaxonomic

analysis of saffron crocus tepal and stamen samples, as raw materials with potential antidepressant activity. J. Pharm. Biomed. Anal. 184, 113183. https://doi.org/10.1016/j.jpba.2020.113183

Pandey, D.K., Kaur, P., Dey, A., 2018. Arbuscular Mycorrhizal Fungi: Effects on Secondary Metabolite Production in Medicinal Plants, in: Fungi and Their Role in Sustainable Development: Current Perspectives. Springer Singapore, Singapore, pp. 507–538. https://doi.org/10.1007/978-981-13-0393-7_28

Patono, D.L., Eloi Alcatrão, L., Dicembrini, E., Ivaldi, G., Ricauda Aimonino, D., Lovisolo, C., 2022. Technical advances for measurement of gas exchange at the whole plant level: Design solutions and prototype tests to carry out shoot and rootzone analyses in plants of different sizes. Plant Sci. 326, 111505. https://doi.org/10.1016/j.plantsci.2022.111505

Qaisrani, M.M., Zaheer, A., Mirza, M.S., Naqqash, T., Qaisrani, T.B., Hanif, M.K., Rasool, G., Malik, K.A., Ullah, S., Jamal, M.S., Mirza, Z., Karim, S., Rasool, M., 2019. A comparative study of bacterial diversity based on culturable and culture-independent techniques in the rhizosphere of maize (Zea mays L.). Saudi J. Biol. Sci. 26, 1344–1351. https://doi.org/10.1016/j.sjbs.2019.03.010

Raho, O., Boutasknit, A., Anli, M., Ben-Laouane, R., Rahou, Y.A., Ouhaddou, R., Duponnois, R., Douira, A., Modafar, C. El, Meddich, A., 2022. Impact of Native Biostimulants/Biofertilizers and Their Synergistic Interactions On the Agro-physiological and Biochemical Responses of Date Palm Seedlings. Gesunde Pflanz. 1053–1069. https://doi.org/10.1007/s10343-022-00668-5

Renau-Morata, B., Nebauer, S.G., Sánchez, M., Molina, R. V., 2012. Effect of corm size, water stress and cultivation conditions on photosynthesis and biomass partitioning during the vegetative growth of saffron (Crocus sativus L.). Ind. Crops Prod. 39, 40–46. https://doi.org/10.1016/j.indcrop.2012.02.009

Rouphael, Y., Colla, G., 2020. Editorial: Biostimulants in Agriculture. Front. Plant Sci. 11, 1–7. https://doi.org/10.3389/fpls.2020.00040

Rouphael, Y., Franken, P., Schneider, C., Schwarz, D., Giovannetti, M., Agnolucci, M., Pascale, S. De, Bonini, P., Colla, G., 2015. Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. Sci. Hortic. (Amsterdam). 196, 91–108. https://doi.org/10.1016/j.scienta.2015.09.002

Salas, M. del C., Montero, J.L., Diaz, J.G., Berti, F., Quintero, M.F., Guzmán, M., Orsini, F., 2020. Defining Optimal Strength of the Nutrient Solution for Soilless Cultivation

of Saffron in the Mediterranean. Agronomy 10, 1311. https://doi.org/10.3390/agronomy10091311

Sharaf-Eldin, M., Elkholy, S., Fernández, J.-A., Junge, H., Cheetham, R., Guardiola, J., Weathers, P., 2008. Bacillus subtilis FZB24® Affects Flower Quantity and Quality of Saffron (Crocus sativus). Planta Med. 74, 1316–1320. https://doi.org/10.1055/s-2008-1081293

Stelluti, S., Caser, M., Demasi, S., Scariot, V., 2021. Sustainable Processing of Floral Bio-Residues of Saffron (Crocus sativus L.) for Valuable Biorefinery Products. Plants 10, 523. https://doi.org/10.3390/plants10030523

Tarantilis, P.A., Polissiou, M., Manfait, M., 1994. Separation of picrocrocin, cis-trans-crocins and safranal of saffron using high-performance liquid chromatography with photodiode-array detection. J. Chromatogr. A 664, 55–61. https://doi.org/10.1016/0021-9673(94)80628-4

Tong, Y., Zhu, X., Yan, Y., Liu, R., Gong, F., Zhang, L., Hu, J., Fang, L., Wang, R., Wang, P., 2015. The influence of different drying methods on constituents and antioxidant activity of saffron from China. Int. J. Anal. Chem. 2015. https://doi.org/10.1155/2015/953164

Trouvelot, A., Kough, J., Gianinazzi-Pearson, V., 1986. Mesure du taux de mycorhization VA d'un système radiculaire. Recherche de méthodes d'estimation ayant une signification fonctionnelle. Mycorrhizae Physiol. Genet. Gianinazzi-Pearson, V., Gianinazzi, S., Eds.; INRA Press Paris, Fr. 217–221.

Valle García-Rodríguez, M., Serrano-Díaz, J., Tarantilis, P.A., López-Córcoles, H., Carmona, M., Alonso, G.L., 2014. Determination of saffron quality by high-performance liquid chromatography. J. Agric. Food Chem. 62, 8068–8074. https://doi.org/10.1021/jf5019356

Vallino, M., Faccio, A., Zeppa, G., Dolci, P., Cerutti, E., Zaquini, L., Faoro, F., Balestrini, R., 2021. Impact of drying temperature on tissue anatomy and cellular ultrastructure of different aromatic plant leaves. Plant Biosyst. 0, 000. https://doi.org/10.1080/11263504.2021.1922535

Victorino, Í.M.M., Voyron, S., Caser, M., Orgiazzi, A., Demasi, S., Berruti, A., Scariot, V., Bianciotto, V., Lumini, E., 2021. Metabarcoding of soil fungal communities associated with alpine field-grown saffron (Crocus sativus l.) inoculated with am fungi. J. Fungi 7, 1–15. https://doi.org/10.3390/jof7010045

Volpe, V., Chitarra, W., Cascone, P., Volpe, M.G., Bartolini, P., Moneti, G., Pieraccini,

G., Di Serio, C., Maserti, B., Guerrieri, E., Balestrini, R., 2018. The Association With Two Different Arbuscular Mycorrhizal Fungi Differently Affects Water Stress Tolerance in Tomato. Front. Plant Sci. 9. https://doi.org/10.3389/fpls.2018.01480

Walker, C., Schüßler, A., Vincent, B., Cranenbrouck, S., Declerck, S., 2021. Anchoring the species Rhizophagus intraradices (formerly Glomus intraradices). Fungal Syst. Evol. 8, 179–201. https://doi.org/10.3114/fuse.2021.08.14

Yilmaz, A., Karik, Ü., 2022. AMF and PGPR enhance yield and secondary metabolite profile of basil (Ocimum basilicum L.). Ind. Crops Prod. 176, 114327. https://doi.org/10.1016/j.indcrop.2021.114327

Zai, X.M., Fan, J.J., Hao, Z.P., Liu, X.M., Zhang, W.X., 2021. Effect of co-inoculation with arbuscular mycorrhizal fungi and phosphate solubilizing fungi on nutrient uptake and photosynthesis of beach palm under salt stress environment. Sci. Rep. 11, 1–11. https://doi.org/10.1038/s41598-021-84284-9

Zouari, I., Salvioli, A., Chialva, M., Novero, M., Miozzi, L., Tenore, G.C., Bagnaresi, P., Bonfante, P., 2014. From root to fruit: RNA-Seq analysis shows that arbuscular mycorrhizal symbiosis may affect tomato fruit metabolism. BMC Genomics 15, 221. https://doi.org/10.1186/1471-2164-15-221

2. Arbuscular mycorrhizal symbiosis modulates apocarotenoid biosynthesis pathway in saffron

Stefania Stelluti[a], Gianluca Grasso[b,f], Sergio Gonzalez Nebauer[c], Gonzalo Luis Alonso[d], Begoña Renau-Morata[e], Matteo Caser[a], Sonia Demasi[a], Erica Lumini[f], María Lourdes Gómez-Gómez[g], Rosa Victoria Molina[c], Valeria Bianciotto[f], Valentina Scariot[a]

[a]Department of Agricultural, Forest and Food Sciences, University of Torino. Largo Paolo Braccini 2, 10095, Grugliasco (TO), Italy; stefania.stelluti@unito.it; matteo.caser@unito.it; sonia.demasi@unito.it; valentina.scariot@unito.it
[b]Institut de Systématique, Evolution, Biodiversité (ISYEB), Muséum National d'Histoire Naturelle, CNRS, Sorbonne Université, EPHE, Université Des Antilles, 75005 Paris, France
[c]Departamento de Producción Vegetal, Universitat Politècnica de València, Camino de vera s.n., 46022 Valencia, Spain; sergonne@bvg.upv.es; rvmolina@bvg.upv.es
[d]Catedra de Química Agrícola, E.T.S.I. Agronomos y de Montes, Universidad de Castilla-La Mancha, Campus Universitario, 02071 Albacete, Spain; gonzalo.alonso@uclm.es
[e]Institut de Biotecnologia i Biomedicina (BIOTECMED), Universitat de València, Avda. Dr. Moliner, 50, 46100 Burjassot, Spain; begonya.renau@uv.es
[f]Institute for Sustainable Plant Protection, National Research Council, c/o Department of Life Science and System Biology - University of Turin. Viale Mattioli 25, 10125 Torino, Italy; gianluca.grasso@unito.it; erica.lumini@ipsp.cnr.it; valeria.bianciotto@ipsp.cnr.it
[g]Botanical Institute, Department of Science Technology, Agroforestry and Genetics, University of Castilla-La Mancha, Campus Universitario s/n, 02071 Albacete, Spain; marialourdes.gomez@uclm.es
*Corresponding author: valentina.scariot@unito.it

2.1. Abstract

Crocus sativus L. (saffron) has been propagated for millennia to produce the precious spice saffron, from the red stigmas. The inebriant organoleptic and bioactive properties mainly depend on the content of crocins (dyeing capacity), picrocrocin (taste), and safranal (aroma), apocarotenoids deriving from zeaxanthin. In this study, an integrated biochemical and molecular analysis on the fresh stigmas, allowed to investigate the influence exerted by the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on the production of the main saffron apocarotenoids responsible for the properties of the spice. Since mineral enrichment due to AM symbiosis has been related to changes in the secondary metabolism of plants, the mineral content of saffron corms at flowering was also analysed. In mycorrhized plants, rare arbuscules (AMF trade structures) were found. However, the expression of *D27*, *CCD7*, and *NCED* genes for strigolactones (SLs) and abscisic acid (ABA), which promote AM symbiosis, did

not change in the stigmas. Transcription of *β-LYC* was not affected by AMF, as well as *CCD4a/b* transcription, whereas that of CCD2, the key enzyme producing major apocarotenoids, was upregulated. Enrichment of Fe^{2+} by AMF (cofactor of CCD enzymes) may have contributed to this result. The content of crocins was reduced in treated plants even if the expression of *ALDH*, *UGT74AD1*, and *UGT91P3* genes involved in crocins synthesis did not change. Conversely, the *UGT709G1* gene for picrocrocin synthesis was overexpressed in inoculated plants, thus the content of safranal was increased in the spice.

2.2. Keywords

Crocus sativus L., *Rhizophagus intraradices*, safranal, crocins, *CCD2*

2.3. Introduction

Crocus sativus L. (*Iridaceae* family) is a cormous geophyte commonly named saffron, and probably originated from ancient Greece (Kazemi-Shahandashti et al., 2022). Saffron is mainly cultivated in the Middle East and Mediterranean regions, with Iran being the principal producer and exporter (Cardone et al., 2020). As a triploid sterile crop, reproduction is vegetative and depends on humans, who have been propagating the plant for millennia through replacement corms. During the short autumn flowering an intense and onerous manual labor is required to collect the perishable flowers and gently separate and dehydrate the red stigmas to produce the valuable spice. The valued organoleptic properties and difficult production are the reasons for its high price and the nickname "red gold" (Gresta et al., 2008; Bagur et al., 2017). Traditionally used as seasoning, natural dye, and in folk medicine, recent revisiting of saffron bioactivity has renewed consumer and scientific interest for this plant (Bagur et al., 2017; Mzabri et al., 2019). The inebriant sensorial profile is primarily ascribed to three carotenoid-derivatives, namely crocins (dyeing capacity), picrocrocin (flavour), and safranal (aroma) (Figure 1). In international commercial agreements, the evaluation of the colouring, bittering and aromatic strength through UV−vis spectrophotometric

analysis of aqueous extracts ($E^{1\%}_{1cm}$) and of the physical characteristics of the spice serves to classify saffron in three quality categories according to ISO 3632 (2011) (Caser et al., 2020; García-Rodríguez et al., 2014a). Crocins, also called crocetin esters, are water-soluble glycosylated crocetins among which *trans*-crocetin di-(β-D-gentiobiosyl) ester (*trans*-4-GG) and *trans*-crocetin di-(β-D-glucosyl) (β-D-gentiobiosyl) ester (*trans*-3-Gg) are the main components (Chen et al., 2020; García-Rodríguez et al., 2017; García-Rodríguez et al., 2014a). Picrocrocin is the water-soluble glucoside of safranal and is known to be present only in the *Buddleja davidii* flowers (Diretto et al., 2021) and in the genus *Crocus*, of which the only edible species is *C. sativus* (García-Rodríguez et al., 2014a). Picrocrocin is converted to safranal either by a two-step enzymatic hydrolysis/dehydration process producing 4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (HTCC) as intermediate or directly by chemical hydrolysis and dehydration at high temperatures and extreme acid or base pH (Gregory et al. 2005; Himeno and Sano 1987) (Figure 1). Safranal, a volatile and mainly poorly soluble in water cyclic monoterpene aldehyde, is the principal compound of saffron essential oil (Gregory et al., 2005). Saffron nutraceutical and therapeutic properties are mostly attributed to safranal and the *trans*-isomers of crocins (Bagur et al., 2017; García-Rodríguez et al., 2014a). Altogether, these secondary metabolites are apocarotenoids derived from the oxidative cleavage of zeaxanthin and preferentially synthesized in the stigma chromoplasts (Baba et al., 2015; Rubio-Moraga et al., 2014b).

Other derivatives from carotenoids are the hormones strigolactones (SLs) and abscisic acid (ABA) (Fiorilli et al., 2019). Besides other functions in plant development, SLs and ABA are positive regulators of arbuscular mycorrhizal (AM) symbiosis especially under phosphate ($H_2PO_4^-$, Pi) limitation and AM fungi (AMF) can promote their production by the plant (Cameron et al., 2013; Fiorilli et al., 2019; López-Ráez et al., 2015). AMF, especially belonging to the phylum *Glomeromycota*, are obligate biotrophs that live in symbiosis with most land plants including relevant crops. They inhabit the host roots receiving organic carbon sources (lipids and glucose) for the hyphal growth while their extensive

extraradical mycelium supplies the host with water and mineral nutrients, i.e., P, N, S, K, Ca, Fe, Cu, Mn, and Zn, especially in low-available nutrient soils.

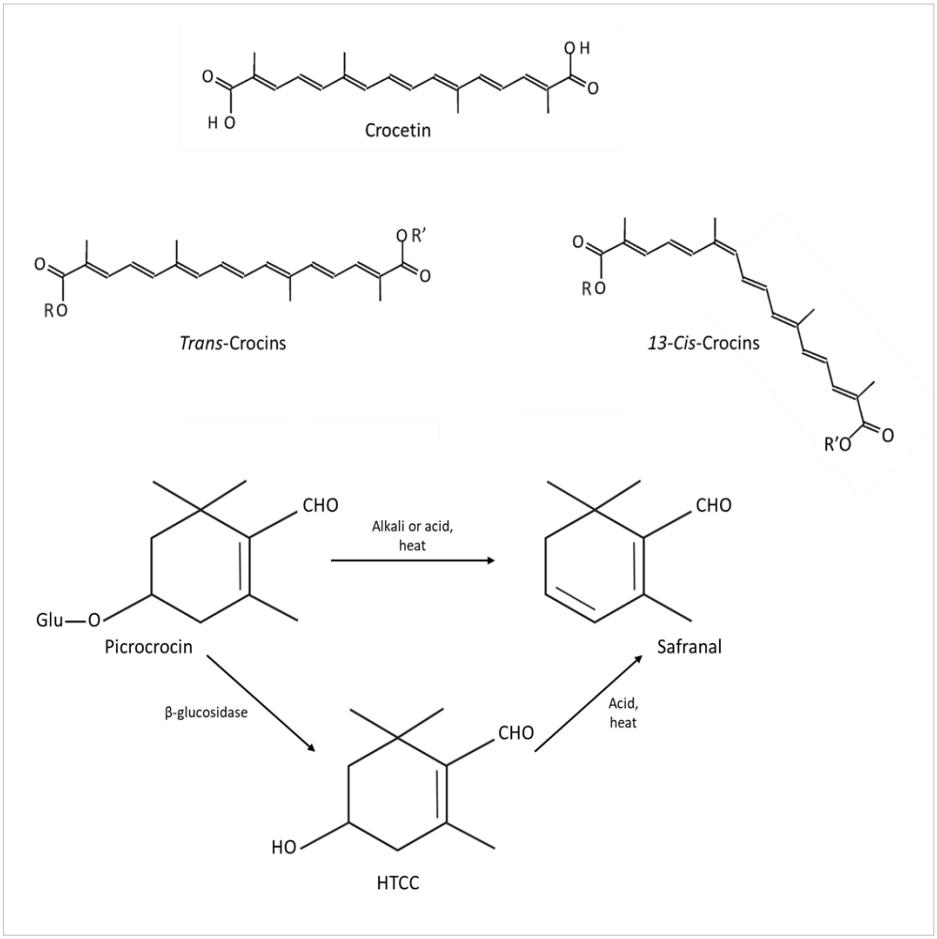


Figure 1. Chemical structures of saffron crocins (glycoside esters of crocetin) and conversion of picrocrocin to safranal directly and through the intermediate HTCC. Readapted from Bagur et al. (2017) and Himeno and Sano (1987).

AMF can positively affect plant biomass and quality by enriching the host with macro- and micronutrients, conferring resistance to abiotic stresses, and reprogramming plant primary and secondary metabolism, e.g., increasing the content of bioactive compounds such as carotenoids (Berruti et al., 2016; Bianciotto et al., 2018; Caser et al., 2019, 2018; Chialva and Bonfante, 2018;

Kaur and Suseela, 2020). AMF inoculants have been reported to improve the production of secondary metabolites of officinal plants, such as *Mentha spicata* L., *Valeriana officinalis* L., *Passiflora alata* Curtis, *Origanum onites* L., *Salvia officinalis* L, and *Calendula officinalis* L. (Bianciotto et al., 2018; Kumar et al., 2018).

Apocarotenoids are generated by carotenoid dioxygenases (CCD1, CCD2, CCD4, CCD7, CCD8, and NCED) enzymes, which cleave one or two double bonds of carotenoids (Ahrazem et al., 2010; Rubio-Moraga et al., 2014b). The content of carotenoids and apocarotenoids is positively correlated with the transcription level of the genes involved in their biosynthesis (Ahrazem et al., 2018; López-Ráez et al., 2015); e.g., in mycorrhized roots of the plant model tomato the increase of SLs was correlated with an induction of the gene *SlCCD7* in early and later stages of AM colonization (López-Ráez et al., 2015). Although mycorrhization is limited to the root, modulation of plant metabolism by AM fungi occurs both locally and systemically (Adolfsson et al., 2017; Chialva et al., 2022). In a previous work, plants inoculated with the AMF *Rhizophagus intraradices* in hydroponic improved the content of safranal (Stelluti et al., submitted). In this research we have carried out an integrated analysis of the biochemical and transcriptional modulation of apocarotenoid biosynthesis induced by AM symbiosis in saffron stigma. This is the first report on the transcriptional reprogramming of *CCD2* and *UGT709G1* inducted by AMF. Since the improved plant nutritional status by AM symbiosis have been associated to an increment of secondary metabolites (Kaur and Suseela, 2020; Kumar et al., 2021), the mineral nutrient content of corms at flowering was also analysed. We hypothesized that nutrient enrichment in mycorrhized saffron plants, together with the transcriptional activation of carotenoid dioxygenase genes, may be responsible of an enhanced content of quality-related apocarotenoids.

2.4. Material and Methods

2.4.1. Plant growth and AMF inoculation

Plants were grown on a bench in a greenhouse at the Department of Agricultural, Forest, and Food Sciences (DISAFA) of the University of Turin (Italy, 45°06′23.21″N Lat, 7°57′82.83″E Long; 300 m a.s.l.) as in Stelluti et al. (submitted). Large-sized (> 19 g) of *Crocus sativus* corms were sowed in pots (4 L; one corm per pot) with sterile perlite (1.5 L per pot; Centro Evergreen Turco s.a.s., Moncalieri, Turin, Italy) at the end of August 2020. For the AMF treatment, 10 g of inoculum was placed under the corms (MycAgro Lab, Breteniére, FR). The inoculum consisted of *Rhizophagus intraradices* spores and a substrate of calcined clay, vermiculite, and zeolite. Weekly irrigation was performed until the emergence of the roots, then fertigation every 2 weeks with a modified Long-Ashton solution (Hewitt, 1952) as in Stelluti et al. (submitted). The experiment consisted of three randomized replicates (blocks) per uninoculated controls (Ctr, 6 pots per block) and AMF treatment (Myc, 12 pots per block). During flowering (31 October – 17 November 2020), the daily mean values of temperature and relative humidity (RH%) were 17 ± 3°C and 65 ± 10%, respectively.

2.4.2. Evaluation of AM colonization

At flowering, plant roots were harvested and rid of topsoil. Part of the roots used for AMF colonization analysis were stained with 0.1% (w:v) cotton blue in 90% lactic acid overnight and then de-stained for two hours with water and two hours with 90% lactic acid in deionized water 1:2 (v:v) for microscope analyses. The protocol was performed twice and the roots were stored in 90% lactic acid. Roots were cut in 1 cm root fragments, which were placed on microscope slides (twenty fragments per slide) for observation under the light microscope (Trouvelot et al., 1986). The AM colonization was evaluated by observing three slides per biological replicate for the Myc treatment, for a total of 180 cm of root, while for

Ctr the absence of the fungus was checked in one slide per biological replicate, for a total of 60 cm of root.

2.4.3. Determination of Mineral Elements Content

P, S, K, Ca, Mg, B, Cu, Fe, Mn, Mo, and Zn were determined in flowering corms with an ICP-AES (Thermo Scientific, USA) at the Ionomic service (CEBAS-CSIC, Murcia, Spain). For each treatment, plant material was dried for 48 h at 60°C and finely ground in a Pulverisette mill (Fristch, Germany). Acid digestion of samples was done by the method explained in Nebauer et al. (2011). Three determinations in three independent biological replicates were performed for each treatment. One corm from different plants was used in each biological replicate.

2.4.4. Quantitative Reverse Transcription PCR (qRT-PCR)

Gene expression changes of key genes involved in the carotenoid and apocarotenoid metabolism were quantified on stigmas at red-scarlet stage of three biological replicates by qRT-PCR. Flowers were harvested and stigmas were gently separated and immediately frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted from grinded lyophilised stigmas using NucleoSpin® RNA Plant and Fungi kit (Macherey-Nagel, Düren, Germany). RNA concentration was measured using a NanoDrop 1000 instrument (Thermo Scientific, Waltham, MA, USA). RNA samples were treated with the TURBO DNA-free™ Kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. Genomic DNA contamination was checked by a PCR (using primers for the *Tubulin* gene) and agarose gel electrophoresis. Complementary DNA was synthesized by adding 625 ng of RNA in 20 μL of reaction volume using the SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, California, USA). Primers sequences, listed in Table 1, were designed by using the Primer3 program (https://bioinfo.ut.ee/primer3-0.4.0/) (in bold) or previously described in the literature, namely *Cs18s*, β-*LYC*, *CCD2*, *ALDH*, *UGT74AD1* (Gómez-Gómez et al., 2017), *CCD7* (Rubio-Moraga et al., 2014a), and *UGT709G1* (Diretto et al.,

2019). The amplification efficacy of the oligonucleotides pairs was tested before performing qRT-PCR analysis (Livak and Schmittgen, 2001).

Table 1. Sequences and parameters of the primers used for qRT-PCR analysis. in bold the primers designed in this study.

Primers	Sequences	Concentration (nM)	Tm (°C)	Product size (bp)
Cs18s_F	ATGTCAGCGGAACATTCAATC	300	59.95	191
Cs18s_R	TCAGTCTGCTAAGTAGCTATG	300	60	
β-LYC_F	ACGAGTGACGAGGAAGGAGA	300	59.99	202
β-LYC_R	GTCCCGTGGTTTGTCGTACT	300	59.89	
CCD7_F	ACCTCCCCGTCATCCAAT	300	60.14	111
CCD7_R	ATGACGGTTTCGGTCTCG	300	59.63	
D27_F	**GCACCAATTGTGTTGGTCTG**	**300**	**60.01**	**156**
D27_R	**CGTCTTCAGGTTCAGGTGGT**	**300**	**60.15**	
CCD4a_F	**GTCATCCTCCTCCCTCTTCC**	**300**	**60.01**	**87**
CCD4a_R	**GGCTGGTTCTGGAATGCTAA**	**300**	**60.21**	
CCD4b_F	**GAATCTTCCTTAAACACTACCCATCTC**	**300**	**60.88**	**156**
CCD4b_R	**CGAAGGAGTGTCGGGTGA**	**300**	**60.84**	
CCD2_F	TACCAAAGTGGATCCGAAGC	300	60.07	165
CCD2_R	GCATAATTGCCGGAGAGGTA	300	60.06	
UGT709G1_F	ACACCGAACGCAACTACCGT	300	62.84	333
UGT709G1_R	TCGAATAACTCGTCAATATACT	300	51.15	
ADH_F	GGACAAGCTTGCTTTTACTGG	300	59.03	100
ADH_R	GCCACCAAGCTCCAATGTTA	300	61.03	
UGT74AD1_F	ACCTAGTCGTCGGGCCTATT	300	59.98	212
UGT74AD1_R	GGCGGTAGATTGTGTCCACT	300	60	
UGT91P3_F	**TTCTGCAGCACTGGATACCA**	**300**	**60.41**	**212**
UGT91P3_R	**CGCCATCACAATCAACTCGT**	**300**	**62.07**	
NCED_F	**ATGATCGGCCATCAGTCTTC**	**300**	**60.04**	**194**
NCED_R	**CGGAGGGGTTCTTCTCTTTC**	**300**	**60.18**	

2.4.5. Saffron Extract Preparation

Saffron stigmas were dehydrated in the shade for 48 - 72 h and then in a cold-dryer (Northwest Technologies NWT100 dryer, Boves, Italy) at 20°C for 48 h (Vallino et al., 2021). Aqueous extracts of the spice were prepared according to the ISO 3632 protocol (Caser et al.,2020) by solving 50 mg of powdered spice into 5 mL of deionised water. The solution was stirred using a magnetic stir bar at 1000 rpm for 1 h in the dark and at room temperature (~21°C). The solution

was then centrifuged at 10,000 rpm and 4°C and filtered with PVDF syringe filters with a pore size of 0.45 μm (CPS Analitica, Milan, Italy). Two technical replicates for each of the three biological replicates were prepared.

2.4.6. HPLC−DAD analysis

For the HPLC analysis, 20 μL of each extract was injected into an Agilent 1200 HPLC chromatograph (Palo Alto, CA) equipped with a 150 mm × 4.6 mm, 5 μm Phenomenex Luna C18 column (Le Pecq Cedex, France) equilibrated at 30 °C. Acetonitrile (Panreac, Barcelona, Spain) in milliQ water was used as the mobile phase in HPLC applying an acetonitrile gradient starting with 20% 0–5 min; 20–80% 5–15 min; and 80% 15–20 min, at a flow rate of 0.8 mL/min. The DAD detector (Hewlett Packard, Waldbronn, Germany) was set at the wavelength 250, 330, and 440 nm for picrocrocin, safranal, and crocetin esters detection, respectively. All analyses were performed in duplicate with two measurements taken for each replicate.

Identification and quantification of crocetin esters, picrocrocin, HTCC, and safranal

For the HPLC analysis, 20 μL of each extract was injected into an Agilent 1200 HPLC chromatograph (Palo Alto, CA) equipped with a 150 mm × 4.6 mm, 5 μm Phenomenex Luna C18 column (Le Pecq Cedex, France) equilibrated at 30 °C. Acetonitrile (Panreac, Barcelona, Spain) in milliQ water was used as the mobile phase in HPLC applying an acetonitrile gradient starting with 20% 0–5 min; 20–80% 5–15 min; and 80% 15–20 min, at a flow rate of 0.8 mL/min. The DAD detector (Hewlett Packard, Waldbronn, Germany) was set at the wavelength 250, 330, and 440 nm for picrocrocin, safranal, and crocetin esters detection, respectively. All analyses were performed in duplicate with two measurements taken for each replicate.

2.4.7. Statistical analysis

Significant differences were verified with the t-test ($p < 0.05$) after checking the data for normality (Shapiro–Wilk's test, $p > 0.05$) and homoscedasticity

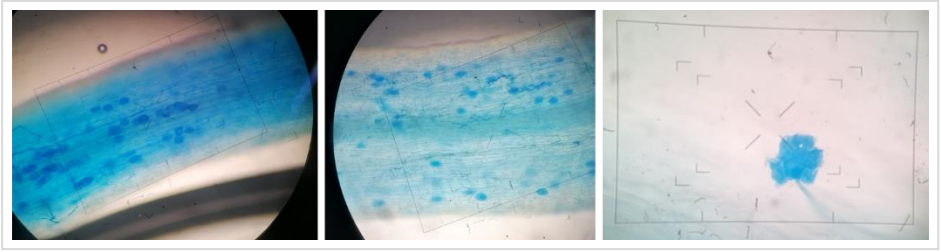
(Levene's test, $p > 0.05$). The R-studio software was used. Data were reported as mean and standard deviation.

2.5. Results and Discussion

2.5.1. AMF colonization and mineral nutrients in flowering corms

AMF presence was found for the Myc treatment, while was absent in uninoculated Ctr roots (Supplementary Figure 1). For the Myc plants, extraradical hyphae were identified in 18% of the root fragments while intraradical hyphae, vesicles, and arbuscules in 15% of the fragments. The frequency of intraradical AMF colonization ranged from 1% to 10% in 9% of the fragments and from 50% to 90% in 6% of the fragments. Vesicles were seen in 10% of the fragments (and were frequent-abundant in 8% of the fragments), while rare arbuscules were observed. The reason why rare arbuscules, the specialised trade structures of AMF (Lanfranco et al., 2018), were found in the roots could be that during flowering plants did not need a supply of mineral nutrients by the AM fungus. The large corms used could already have contained sufficient nutrient reserves, which mainly support the early growth stages and autumn flowering of *C. sativus* after the dry summer season (Koocheki & Seyyedi, 2015). As evidence of this, there were no differences between Ctr and Myc for the majority of mineral nutrients analysed in flowering corms, including P (Table 2). Indeed, AM colonization is regulated by Pi, a major plant growth-limiting and low-mobility macronutrient, by a local (arbuscule formation) and systemic way (Lanfranco et al., 2018). When Pi supply is high arbuscules formation is repressed (Lanfranco et al., 2018). However, in Myc plant roots, a biofertilizer effect was seen for some micronutrients, namely Fe, Zn, and Mo (Table 2), which are important for plant physiology and enzyme cofactors, among other roles. Iron (II) is cofactor of carotenoid dioxygenases (CCD), which are involved in the biosynthesis of apocarotenoids (Ahrazem et al., 2010; Rubio-Moraga et al., 2014b). Molybdenum is cofactor of ABA-aldehyde oxidase (AAO), which catalyses the last step of ABA biosynthesis (Hänsch and Mendel, 2009; Tuteja, 2007). Thus,

the enhanced content of Fe and Mo found in treated corms (Table 2) may contribute to an increased activity of CCD2 and AAO in mycorrhized plants (Figure 2). Regarding zinc, differential expression profiling between stigma and rest of the *Crocus* flower suggested that many zinc-finger genes were expressed in stigma. Particularly, the zinc-finger transcription factor CsSAP09, found to be highly expressed in stigma at anthesis stage corroborating with the accumulation pattern of apocarotenoids, has a possible role in regulating apocarotenoid metabolism in saffron (Malik and Ashraf, 2017).



Supplementary Figure 1. Saffron root fragments colonized by AMF *Rhizophagus intraradices*. Intraradical hyphae, vesicles, and a detail of an arbuscule (last photo on the right) are shown.

Table 2. Means and standard deviations of mineral element content of saffron corms during flowering for controls (Ctr) and mycorrhized (Myc) plants.

Mineral content	Ctr	Myc	*p*
P (g 100 g^{-1})	0.15 ± 0.03	0.19 ± 0.01	ns
S (g 100 g^{-1})	0.09 ± 0.01	0.13 ± 0.01	ns
K (g 100 g^{-1})	0.52 ± 0.09	0.58 ± 0.03	ns
Ca (g 100 g^{-1})	0.26 ± 0.04	0.30 ± 0.02	ns
Mg (g 100 g^{-1})	0.07 ± 0.00	0.08 ± 0.01	ns
B (mg kg^{-1})	3.24 ± 0.52	3.99 ± 0.10	ns
Cu (mg kg^{-1})	2.65 ± 0.11	2.51 ± 0.12	ns
Fe (mg kg^{-1})	45.41 ± 7.66	67.54 ± 12.31	*
Mn (mg kg^{-1})	10.10 ± 0.41	11.18 ± 2.39	ns
Mo (mg kg^{-1})	0.31 ± 0.04	0.81 ± 0.27	*
Zn (mg kg^{-1})	18.07 ± 0.86	24.91 ± 2.09	*

Values of mean ± standard deviation are reported. *$p < 0.05$; ns = not significant.

2.5.2. Effect of arbuscular mycorrhization on apocarotenoid metabolism

In this study we integrated biochemical and transcriptional analyses to investigate the effect of AM symbiosis on apocarotenoid metabolism in saffron stigmas. In fact, genes involved in the biosynthesis of crocins, picrocrocin, and safranal are exclusively expressed in stigmas (Jain et al., 2016).

Lycopene represents the first branch point of the carotenoid pathway (Figure 2). The gene encoding for a chromoplast-specific lycopene β-cyclase (β-LYC), highly expressed in the saffron stigmas (Baba et al., 2015), that catalyses the synthesis of β-carotene and together with lycopene ε-cyclase produces α-carotene, was analysed. AMF symbiosis resulted not to affect its transcription level (Table 3). Similarly, AM symbiosis did not affect also the expression level of *D27* and *CCD7* (Table 3). D27, a β-carotene isomerase, converts all *trans*-β-carotene into 9-cis-β-carotene, which is cleaved into 10′-apo-β-carotenal and β-ionone by CCD7 (Figure 2). These genes were found highly expressed in the saffron stigmas (Ahrazem et al., 2012; Jain et al., 2016; Rubio-Moraga et al., 2014) and, together with CCD8, which converts 10′-apo-β-carotenal in carlactone (the precursor of SLs) are implicated in the SLs biosynthesis (Rubio-Moraga et al., 2014a). SLs act as signaling molecules in early plant–AMF interaction and promote AM symbiosis (Fiorilli et al., 2019). Since plants produce and exude SLs mostly under Pi limitation, in this study they may not have been particularly deficient in Pi during flowering. Genes for SLs biosynthesis were found to be expressed in other tissues as well, such as corms (Jain et al. 2016; Rubio-Moraga et al., 2014); thus, it could be that their transcript levels were increased in other parts of Myc plants. β-carotene is also a substrate of CCD1, CCD4a, and CCD4b, which produce HTCC and two β-ionone (volatile aroma compounds) (Ahrazem et al., 2010; Rubio et al., 2008) (Figure 2); whereas CCD1 is constitutively expressed, CCD4a/b reacts more actively with β-carotene (Ahrazem et al., 2016; Rubio et al., 2008), thus the transcriptions of the latter were evaluated. The gene expression of *CCD4a/b* were also not modified by the AM fungus (Table 3). CCD2 also produces HTCC together with crocetin dialdehyde, by cleaving zeaxanthin derived from hydroxylation of β-carotene. HTCC is then converted

into picrocrocin by the UDP-glucosyl transferase (UGT) UGT709G1 (Diretto et al., 2019; Gómez-Gómez et al., 2017) (Figure 2).

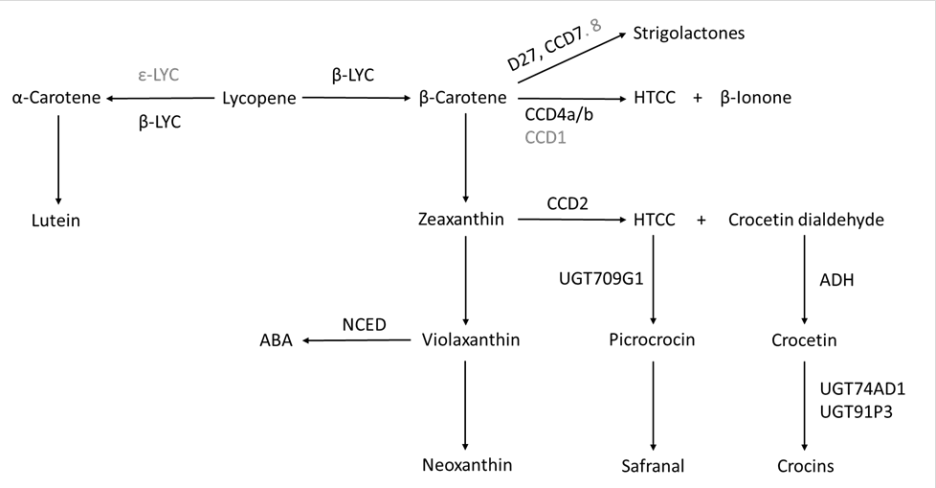


Figure 2. Overview of the biosynthesis pathway of carotenoids/apocarotenoids in saffron.

Conversely to the other genes investigated, the *CCD2* and *UGT709G1* genes, encoding for the key enzyme for the synthesis of saffron main apocarotenoids (Ahrazem et al., 2016), were overexpressed in Myc plants (Table 3). Although *CCD2* was up-regulated, the content of HTCC in the Myc plant spice was not affected (Table 4). This lower than expected HTCC levels may be due to the contemporary increased expression of *UGT709G1* (Table 3), which finally led to the increased content of safranal in the spice, a result in agreement with Stelluti et al. (submitted). At the same time, although *UGT709G1* was upregulated, the content of picrocrocin found was also lower than expected (Table 4). This could be due to the conversion of picrocrocin to safranal when the stigmas are dried, either directly or via HTCC. It might be probable that in fresh stigmas of Myc plants the content of picrocrocin (and HTCC) was higher. CCD2 also produces crocetin dialdehyde, which is converted into crocetin by aldehyde dehydrogenases (ALDH) (Figure 2). Crocetin dialdehyde is converted into crocetin by aldehyde dehydrogenases (ALDH) (Figure 2). The expression of the *ALDH* gene did not change for the Myc treatment (Table 3).

Table 3. Expression analysis by qRT-PCR of genes involved in apocarotenoid metabolism on saffron fresh stigmas in AMF mycorrhized plants (Myc). Non-mycorrhized plants were used as controls (Ctr). The relative expression (mean fold change and standard deviations) was shown for each analysed gene.

Gene	Ctr	Myc	*p*
β-LYC	1.03 ± 0.31	1.97 ± 0.78	ns
CCD7	1.02 ± 0.23	1.23 ± 0.27	ns
D27	1.01 ± 0.17	1.21 ± 0.25	ns
CCD4a	1.00 ± 0.12	1.21 ± 0.31	ns
CCD4b	1.04 ± 0.39	1.28 ± 0.32	ns
CCD2	1.10 ± 0.54	3.72 ± 0.43	***
UGT709G1	1.09 ± 0.58	2.94 ± 0.60	*
ADH	1.00 ± 0.05	1.18 ± 0.24	ns
UGT74AD1	1.08 ± 0.51	1.73 ± 0.34	ns
UGT91P3	1.00 ± 0.09	1.04 ± 0.40	ns
NCED	1.03 ± 0.29	1.09 ± 0.14	ns

Values of mean ± standard deviation are reported. *$p < 0.05$; ***$p < 0.001$; ns = not significant.

Crocetins are then glycosylated by UGT enzymes: UGT74AD1 produces crocins with one and two glucose molecules, which are substrates for UGT91P3 (López-jimenez et al., 2021) (Figure 2). Glycosylation generates crocins, soluble apocarotenoids that accumulate in the vacuole, which occupies most of the cell volume in the stigmas during anthesis (Gómez-Gómez et al., 2017). The gene expression of *UGT74AD1* and *UGT91P3* also did not change (Table 3). Even though the expression of the genes involved in important steps of crocins synthesis did not change, the content of crocins was reduced in the spice of Myc plants, except for *trans*-2-gg that was not affected (Table 4). A lower content of crocin I, known as *trans*-crocetin di-(β-D-gentiobiosyl) ester, in saffron treated with a mixed AMF inoculation of *R. intraradices* and *Funneliformis mosseae* was also found by Caser et al. (2019). As crocins are glycoside esters of crocetin, their reduction might be due to an unbalanced organic C costs for the plant during early association with the AMF. This could have led to a lowered level of glucose molecules that are substrates for crocins production. In fact, during flowering *R.*

intraradices may have shown a saprophytic behaviour as suggested by the presence mainly of vesicles, i.e. specialized storage structures (Azcón-Aguilar et al., 1999; Maiti and Ghosh, 2020).

Table 4. Apocarotenoids content in the spice from controls (Ctr) and mycorrhized (Myc) plants. Abbreviations for crocins (crocetin esters) were adopted from a previous study (Carmona et al., 2006).

Apocarotenoids (mg g^{-1})	Crocins abbreviation	Ctr	Myc	*p*
Crocins				
trans-crocetin (β-D-neapolitanosyl)-(β-D-gentiobiosyl) ester	*trans*-5-nG	4.00 ± 0.75	1.56 ± 0.48	***
trans-crocetin di-(β-D-gentiobiosyl) ester	*trans*-4-GG	52.02 ± 5.76	25.06 ± 8.59	***
cis-crocetin di-(β-D-gentiobiosyl) ester	*cis*-4-GG	6.38 ± 0.99	1.88 ± 0.74	***
trans-crocetin (β-D-glucosyl) (β-D-gentiobiosyl) ester	*trans*-3-Gg	33.48 ± 5.00	22.88 ± 6.74	*
cis-crocetin (β-D-glucosyl)-(β-D-gentiobiosyl) ester + *trans*-crocetin (β-D-gentiobiosyl) ester	*cis*-3-Gg + *trans*-2-G	17.61 ± 2.06	2.70 ± 1.12	***
trans-crocetin (β-D-neapolitanosyl)-(β-D-glucosyl) ester	*trans*-4-ng	3.83 ± 0.52	1.29 ± 0.44	***
trans-crocetin di-(β-D-glucosyl) ester	*trans*-2-gg	3.52 ± 0.64	5.05 ± 2.13	ns
trans-crocetin (β-D-glucosyl) ester	*trans*-1-g	2.23 ± 0.73	0.26 ± 0.17	***
Picrocrocin		108.41 ± 6.91	116.02 ± 12.19	ns
HTCC		14.42 ± 2.23	11.41 ± 2.77	ns
Safranal		0.37 ± 0.06	1.15 ± 0.40	***

Values of mean ± standard deviation are reported. *$p < 0.05$; ***$p < 0.001$; ns = not significant.

Zeaxanthin, substrate of CCD2, can also be inter-converted in violaxanthin, which is then converted into neoxanthin. The 9-cis-epoxycarotenoid dioxygenase (NCED) enzyme cleaves violaxanthin and neoxanthin to form xanthoxin, a direct precursor for ABA (Baba et al., 2015; Jain et al., 2016) (Figure 2). The expression level of *NCED* did not change in the stigmas of Myc plants (Table 3). However, in this study, only stigmas were used for generation of transcripts and hence does not include the genes expressed in other plant parts. Indeed, similarly to the genes involved in SLs biosynthesis, *NCED* is expressed also in other saffron tissues (Jain et al., 2016).

2.6. Conclusions

An integrated biochemical and transcriptional analysis on the stigmas allowed for the first time to shed light on the influence exerted by the AMF *R. intraradices* on the biosynthesis of the main apocarotenoids responsible for the spice quality of *C. sativus*. Although transcription of β-*LYC* was not affected by AMF, the *CCD2* gene, encoding for the key enzyme producing the main apocarotenoids, was overexpressed. However, the content of crocins was reduced in the spice derived from mycorrhized plants probably due to the organic C cost. Conversely, the *UGT709G1* gene involved in picrocrocin synthesis was upregulated in fresh stigmas, consequently, the content of safranal was increased in the spice. It could be interesting to further deepen the knowledge on the reprogramming of apocarotenoid metabolism in *C. sativus* analysing other plant tissues and the catalytic activity of enzymes in relation to the content of micronutrients.

Acknowledgments

The authors thank Francesco Berruto for the help in plant maintenance and laboratory analyses.

Funding

This research was funded by the program Interreg V-A Francia Italia Alcotra (Grant No. 1139 “ANTEA – Attività innovative per lo sviluppo della filiera

transfrontaliera del fiore edule"; and grant no. 8336 "ANTES - Fiori eduli e piante aromatiche: attività capitalizzazione dei progetti ANTEA ed ESSICA").

2.7. References

Adolfsson, L., Nziengui, H., Abreu, I.N., Šimura, J., Beebo, A., Herdean, A., Aboalizadeh, J., Široká, J., Moritz, T., Novák, O., Ljung, K., Schoefs, B., Spetea, C., 2017. Enhanced secondary- and hormone metabolism in leaves of arbuscular mycorrhizal medicago truncatula. Plant Physiol 175, 392–411. https://doi.org/10.1104/pp.16.01509

Ahrazem, O., Argandoña, J., Fiore, A., Aguado, C., Luján, R., Rubio-Moraga, Á., Marro, M., Araujo-Andrade, C., Loza-Alvarez, P., Diretto, G., Gómez-Gómez, L., 2018. Transcriptome analysis in tissue sectors with contrasting crocins accumulation provides novel insights into apocarotenoid biosynthesis and regulation during chromoplast biogenesis. Sci Rep 8, 1–17. https://doi.org/10.1038/s41598-018-21225-z

Ahrazem, O., Rubio-Moraga, A., Berman, J., Capell, T., Christou, P., Zhu, C., Gómez-Gómez, L., 2016. The carotenoid cleavage dioxygenase CCD2 catalysing the synthesis of crocetin in spring crocuses and saffron is a plastidial enzyme. New Phytologist 209, 650–663. https://doi.org/10.1111/nph.13609

Ahrazem, O., Trapero, A., Gómez, M.D., Rubio-Moraga, A., Gómez-Gómez, L., 2010. Genomic analysis and gene structure of the plant carotenoid dioxygenase 4 family: A deeper study in Crocus sativus and its allies. Genomics 96, 239–250. https://doi.org/10.1016/j.ygeno.2010.07.003

Baba, S.A., Mohiuddin, T., Basu, S., Swarnkar, M.K., Malik, A.H., Wani, Z.A., Abbas, N., Singh, A.K., Ashraf, N., 2015. Comprehensive transcriptome analysis of Crocus sativus for discovery and expression of genes involved in apocarotenoid biosynthesis. BMC Genomics 16, 698. https://doi.org/10.1186/s12864-015-1894-5

Bagur, M.J., Alonso Salinas, G., Jiménez-Monreal, A., Chaouqi, S., Llorens, S., Martínez-Tomé, M., Alonso, G.L., 2017. Saffron: An Old Medicinal Plant and a Potential Novel Functional Food. Molecules 23, 30. https://doi.org/10.3390/molecules23010030

Berruti, A., Lumini, E., Balestrini, R., Bianciotto, V., 2016. Arbuscular Mycorrhizal Fungi as Natural Biofertilizers: Let's Benefit from Past Successes. Front Microbiol 6, 1–13. https://doi.org/10.3389/fmicb.2015.01559

Bianciotto, V., Victorino, I., Scariot, V., Berruti, A., 2018. Arbuscular mycorrhizal fungi as natural biofertilizers: current role and potential for the horticulture industry. Acta Hortic 1191, 207–216. https://doi.org/10.17660/ActaHortic.2018.1191.29

Cameron, D.D., Neal, A.L., van Wees, S.C.M., Ton, J., 2013. Mycorrhiza-induced resistance: More than the sum of its parts? Trends Plant Sci 18, 539–545. https://doi.org/10.1016/j.tplants.2013.06.004

Cardone, L., Castronuovo, D., Perniola, M., Cicco, N., Candido, V., 2020. Saffron (Crocus sativus L.), the king of spices: An overview. Sci Hortic 272. https://doi.org/10.1016/j.scienta.2020.109560

Carmona, M., Zalacain, A., Sánchez, A.M., Novella, J.L., Alonso, G.L., 2006. Crocetin esters, picrocrocin and its related compounds present in Crocus sativus stigmas and Gardenia jasminoides fruits. Tentative identification of seven new compounds by LC-ESI-MS. J Agric Food Chem 54, 973–979. https://doi.org/10.1021/jf052297w

Caser, M., Demasi, S., Stelluti, S., Donno, D., Scariot, V., 2020. Crocus sativus L. Cultivation in Alpine Environments: Stigmas and Tepals as Source of Bioactive Compounds. Agronomy 10, 1473. https://doi.org/10.3390/agronomy10101473

Caser, M., Demasi, S., Victorino, Í.M.M., Donno, D., Faccio, A., Lumini, E., Bianciotto, V., Scariot, V., 2019. Arbuscular Mycorrhizal Fungi Modulate the Crop Performance and Metabolic Profile of Saffron in Soilless Cultivation. Agronomy 9, 232. https://doi.org/10.3390/agronomy9050232

Caser, M., Victorino, Í.M.M., Demasi, S., Berruti, A., Donno, D., Lumini, E., Bianciotto, V., Scariot, V., 2018. Saffron Cultivation in Marginal Alpine Environments: How AMF Inoculation Modulates Yield and Bioactive Compounds. Agronomy 9, 12. https://doi.org/10.3390/agronomy9010012

Chen, D., Xing, B., Yi, H., Li, Y., Zheng, B., Wang, Y., Shao, Q., 2020. Effects of different drying methods on appearance, microstructure, bioactive compounds and aroma compounds of saffron (Crocus sativus L.). Lwt 120, 108913. https://doi.org/10.1016/j.lwt.2019.108913

Chialva, M., Bonfante, P., 2018. Plant microbiota: From model plants to Mediterranean crops. Phytopathol Mediterr 57, 123−145. https://doi.org/10.14601/Phytopathol_Mediterr-22052

Chialva, M., Lanfranco, L., Bonfante, P., 2022. The plant microbiota: composition, functions, and engineering. Curr Opin Biotechnol 73, 135–142. https://doi.org/10.1016/j.copbio.2021.07.003

Diretto, G., Ahrazem, O., Rubio-Moraga, Á., Fiore, A., Sevi, F., Argandoña, J., Gómez-Gómez, L., 2019. UGT709G1: a novel uridine diphosphate glycosyltransferase involved in the biosynthesis of picrocrocin, the precursor of safranal in saffron (Crocus sativus). New Phytologist 224, 725–740. https://doi.org/10.1111/nph.16079

Fiorilli, V., Wang, J.Y., Bonfante, P., Lanfranco, L., Al-Babili, S., 2019. Apocarotenoids: Old and New Mediators of the Arbuscular Mycorrhizal Symbiosis. Front Plant Sci 10, 1–9. https://doi.org/10.3389/fpls.2019.01186

García-Rodríguez, M.V., López-Córcoles, H., Alonso, G.L., Pappas, C.S., Polissiou, M.G., Tarantilis, P.A., 2017. Comparative evaluation of an ISO 3632 method and an HPLC-DAD method for safranal quantity determination in saffron. Food Chem 221, 838–843. https://doi.org/10.1016/j.foodchem.2016.11.089

García-Rodríguez, M., Serrano-Díaz, J., Tarantilis, P.A., López-Córcoles, H., Carmona, M., Alonso, G.L., 2014a. Determination of saffron quality by high-performance liquid chromatography. J Agric Food Chem 62, 8068–8074. https://doi.org/10.1021/jf5019356

Gómez-Gómez, L., Parra-Vega, V., Rivas-Sendra, A., Seguí-Simarro, J., Molina, R., Pallotti, C., Rubio-Moraga, Á., Diretto, G., Prieto, A., Ahrazem, O., 2017. Unraveling Massive Crocins Transport and Accumulation through Proteome and Microscopy Tools during the Development of Saffron Stigma. Int J Mol Sci 18, 76. https://doi.org/10.3390/ijms18010076

Gregory, M.J., Menary, R.C., Davies, N.W., 2005. Effect of drying temperature and air flow on the production and retention of secondary metabolites in saffron. J Agric Food Chem 53, 5969–5975. https://doi.org/10.1021/jf047989j

Gresta, F., Lombardo, G.M., Siracusa, L., Ruberto, G., 2008. Saffron, an alternative crop for sustainable agricultural systems. A review. Agron Sustain Dev 28, 95–112. https://doi.org/10.1051/agro:2007030

Hänsch, R., Mendel R.R., 2009. Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl). Current Opinion in Plant Biology, Volume 12, Issue 3, 259-266. https://doi.org/10.1016/j.pbi.2009.05.006

Himeno, H., Sano, K., 1987. Synthesis of Crocin, Picrocrocin and Safranal by Saffron Stigma-like Structures Proliferated in Vitro. Agric Biol Chem 51, 2395–2400. https://doi.org/10.1271/bbb1961.51.2395

Jain, M., Srivastava, P.L., Verma, M., Ghangal, R., Garg, R., 2016. De novo transcriptome assembly and comprehensive expression profiling in Crocus sativus to gain insights into apocarotenoid biosynthesis. Sci Rep 6, 1–13. https://doi.org/10.1038/srep22456

Kaur, S., Suseela, V., 2020. Unraveling arbuscular mycorrhiza-induced changes in plant primary and secondary metabolome. Metabolites 10, 1–30. https://doi.org/10.3390/metabo10080335

Kazemi-Shahandashti, S.S., Mann, L., El-nagish, A., Harpke, D., Nemati, Z., Usadel, B., Heitkam, T., 2022. Ancient Artworks and Crocus Genetics Both Support Saffron's Origin in Early Greece. Front Plant Sci 13. https://doi.org/10.3389/fpls.2022.834416

Koocheki, A., Seyyedi, S.M., 2015. Relationship between nitrogen and phosphorus use efficiency in saffron (Crocus sativus L.) as affected by mother corm size and fertilization. Ind Crops Prod 71, 128–137. https://doi.org/10.1016/J.INDCROP.2015.03.085

Kumar, S., Arora, N., Upadhyay, H., 2021. Arbuscular mycorrhizal fungi: Source of secondary metabolite production in medicinal plants, in: New and Future Developments in Microbial Biotechnology and Bioengineering. Elsevier, pp. 155–164. https://doi.org/10.1016/B978-0-12-821005-5.00011-9

Lanfranco, L., Fiorilli, V., Gutjahr, C., 2018. Partner communication and role of nutrients in the arbuscular mycorrhizal symbiosis. New Phytologist. https://doi.org/10.1111/nph.15230

Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262

López-jimenez, A.J., Frusciante, S., Niza, E., Ahrazem, O., Rubio-Moraga, Á., Diretto, G., Gómez-Gómez, L., 2021. A New Glycosyltransferase Enzyme from Family 91, UGT91P3, Is Responsible for the Final Glucosylation Step of Crocins in Saffron (Crocus sativus L.). Int J Mol Sci 22, 8815. https://doi.org/10.3390/ijms22168815

López-Ráez, J.A., Fernández, I., García, J.M., Berrio, E., Bonfante, P., Walter, M.H., Pozo, M.J., 2015. Differential spatio-temporal expression of carotenoid cleavage

dioxygenases regulates apocarotenoid fluxes during AM symbiosis. Plant Science 230, 59–69. https://doi.org/10.1016/j.plantsci.2014.10.010

Mzabri, Addi, Berrichi, 2019. Traditional and Modern Uses of Saffron (Crocus Sativus). Cosmetics 6, 63. https://doi.org/10.3390/cosmetics6040063

Nebauer, S.G., Renau-Morata, B., Guardiola, J.L., Molina R.V., 2011. Photosynthesis down-regulation precedes carbohydrate accumulation under sink limitation in *Citrus*. Tree Physiology, Volume 31, Issue 2, 169–177. https://doi.org/10.1093/treephys/tpq103

Rouphael, Y., Franken, P., Schneider, C., Schwarz, D., Giovannetti, M., Agnolucci, M., Pascale, S. De, Bonini, P., Colla, G., 2015. Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. Scientia Horticulturae 196, 91–108. https://doi.org/10.1016/j.scienta.2015.09.002

Rubio, A., Rambla, J.L., Santaella, M., Gómez, M.D., Orzaez, D., Granell, A., Gómez-Gómez, L., 2008. Cytosolic and plastoglobule-targeted carotenoid dioxygenases from Crocus sativus are both involved in β-ionone release. Journal of Biological Chemistry 283, 24816–24825. https://doi.org/10.1074/jbc.M804000200

Rubio-Moraga, A., Ahrazem, O., Pérez-Clemente, R.M., Gómez-Cadenas, A., Yoneyama, K., López-Ráez, J.A., Molina, R. V., Gómez-Gómez, L., 2014a. Apical dominance in saffron and the involvement of the branching enzymes CCD7 and CCD8 in the control of bud sprouting. BMC Plant Biol 14. https://doi.org/10.1186/1471-2229-14-171

Rubio-Moraga, A., Rambla, J.L., Fernández-De-Carmen, A., Trapero-Mozos, A., Ahrazem, O., Orzáez, D., Granell, A., Gómez-Gómez, L., 2014b. New target carotenoids for CCD4 enzymes are revealed with the characterization of a novel stress-induced carotenoid cleavage dioxygenase gene from Crocus sativus. Plant Mol Biol 86, 555–569. https://doi.org/10.1007/s11103-014-0250-5

Sánchez, A.M., Carmona, M., A. Ordoudi, S., Z. Tsimidou, M., Alonso, G.L., 2008. Kinetics of Individual Crocetin Ester Degradation in Aqueous Extracts of Saffron (Crocus sativus L.) upon Thermal Treatment in the Dark. J Agric Food Chem 56, 1627–1637. https://doi.org/10.1021/jf0730993

Smith, S.E., Smith, F.A., 2011. Roles of arbuscular mycorrhizas in plant nutrition and growth: New paradigms from cellular to ecosystem scales. Annu Rev Plant Biol 62, 227–250. https://doi.org/10.1146/annurev-arplant-042110-103846

Trouvelot, A., Kough, J., Gianinazzi-Pearson, V., 1986. Mesure du taux de mycorhization VA d'un système radiculaire. Recherche de méthodes d'estimation ayant une signification fonctionnelle. Mycorrhizae Physiol. Genet. Gianinazzi-Pearson, V., Gianinazzi, S., Eds.; INRA Press Paris, Fr. 217–221.

Tuteja, N., 2007. Abscisic Acid and Abiotic Stress Signaling, Plant Signaling & Behavior, 2:3, 135-138, DOI: 10.4161/psb.2.3.4156

Vallino, M., Faccio, A., Zeppa, G., Dolci, P., Cerutti, E., Zaquini, L., Faoro, F., Balestrini, R., 2021. Impact of drying temperature on tissue anatomy and cellular ultrastructure of different aromatic plant leaves. Plant Biosyst 0, 000. https://doi.org/10.1080/11263504.2021.1922535

3. Comparison of different inoculants of arbuscular mycorrhizal fungi on *Crocus sativus* L. cultivated in soilless conditions

S. Stelluti[1], E. Lumini[2], M. Caser[1], S. Demasi[1], N. M. Falla[1], V. Bianciotto[2], V. Scariot[1a]

[1]Department of Agricultural, Forest and Food Sciences, University of Torino, Largo Paolo Braccini 2, 10095 Grugliasco, Italy

[2]Institute for Sustainable Plant Protection, National Research Council (CNR), Viale Mattioli 25, 10125 Torino, Italy

[a]Corresponding author: valentina.scariot@unito.it

3.1. Abstract

Saffron (*Crocus sativus* L.) is an autumn-flowering geophyte widely cultivated for the most precious spice, consisting of the dried red stigmas of the flower. The violet tepals are considered a valuable by-product having a high content of phenolic compounds and antioxidant capacity. The use of biostimulants may improve the yield of the plant and quality of the flower. In the present study, different arbuscular mycorrhizal (AM) fungal inoculants, i.e., a single species of *Funneliformis mosseae* (Myc+ Fm) and *Rhizoglomus irregulare* (Myc+ Ri) or a multi-species mixture of *Funneliformis geosporum*, *Funneliformis caledonium*, and *Claroidoglomus claroideum* (Myc+ Mix), were tested on saffron plants grown in soilless conditions to highlight their efficacy in improving saffron production and tepals quality. The experiment took place in the Botanic Garden of Turin (Italy). Saffron plants were grown outdoors in a containment chamber in pots containing sterilized quartz sand. The yield of flowers, stigmas, and corms and the biomass of leaves and roots were measured. Tepal extracts were analysed with the Folin-Ciocalteu method and the FRAP, ABTS, and DPPH assays. Morphological analysis of saffron roots revealed low AMF colonization, which

could be due to saffron-AMF compatibility and/or growing conditions, particularly low temperatures. Agronomic measurements showed significant differences regarding the dried weight of leaves per corm (+29.6% for Myc+ Mix compared to Myc-). The antioxidant capacity analysed in tepal extracts resulted significantly increased with the FRAP method for the treatments Myc+ Fm (+11.2%) and Myc+ Ri (+10.9%). An opposite result was seen for both ABTS and DPPH assays. Further studies could provide more information on the real influence exerted by AMF on saffron metabolism with the aim of improving flower secondary metabolites.

3.2. Keywords

saffron, AMF, biostimulants, tepals, by-products, antioxidant activity

3.3. Introduction

Saffron (*Crocus sativus* L.) is an autumnal flowering geophyte belonging to the Iridaceae family. It is widely cultivated for the most precious spice consisting of the dried red stigmas of the flower (Khan et al., 2020). More than 100,000 flowers are needed to produce 1 kg of spice (Mottaghipisheh et al., 2020; Shahi et al., 2016). The purple tepals are the most abundant bio-residues of the flower and, along with the spice, they have a high content of phenolic compounds and antioxidant capacity. Recently, saffron tepals have gained interest as a valuable by-product with potential industrial applications, also in the perspective of a circular bioeconomy (Caser et al., 2020a; Stelluti et al., 2021).

Saffron grows best in the Mediterranean climate (Khan et al., 2020). Being sterile, it is vegetatively propagated through underground organs called corms. In Mediterranean climates saffron blooms for two to three weeks from late October to early November, when the plant needs rain or irrigation. One or several flowers are produced per corm (Gresta et al., 2008; Khan et al., 2020). Seasonal and daily thermoperiodism is the main environmental factor affecting flowering (Halevy, 1990). The vegetative stage follows flowering in autumn and ends by leaves senescence in the spring (around late May). At this stage, the leaves reach

maturity and are photosynthetically active allowing the formation of replacement corms, which are completely formed and enlarged around May. The larger the corms, the smaller the number. Root growth occurs from autumn to the spring period. In the summer (around July) corms finally enter into a dormant period (Gresta et al., 2008; Rezvani-Moghaddam, 2020). Corm size has a significant effect on flowering and spice production (below 10 g, corms may not give flowers) (Kumar et al., 2009). Saffron productivity could be improved by using sustainable horticultural practices, such as the use of plant biostimulants (Caser et al., 2018, 2019).

Saffron profitability could be increased by using plant biostimulants (EU Regulation 2019/1009), such as arbuscular mycorrhizal fungi (AMF), that could improve plant nutrient use efficiency, tolerance to abiotic stress, and quality traits, as well as the availability of confined nutrients in the soil or rhizosphere (De Pascale et al., 2017; Rouphael and Colla, 2020). The final effect of the plant-AMF symbiosis is usually an improvement of the plant yield and content of secondary metabolites such as carotenoids and phenolic compounds (Bianciotto et al., 2018). However, the use of AMF-based inoculants for saffron in soilless system has been poorly studied. Saffron inoculated with the AMF *Rhizophagus intraradices* and *Funneliformis mosseae* improved the content of carbohydrates, protein, and phenolic compounds in corms (Lone et al., 2016); plants treated with the AMF *R. intraradices* alone or mixed with *F. mosseae* increased the corm size and those inoculated with the *R. intraradices* alone improved the crocin I content and antioxidant activity of the spice (Caser et al., 2019).

In the present study, several AMF based inoculants, i.e., a single species of *Funneliformis mosseae* and *Rhizoglomus irregulare* or a multispecies mixture of *Funneliformis geosporum*, *Funneliformis caledonium* and *Claroidoglomus claroideum*, were tested in potted conditions to compare their efficacy in improving saffron yield and flower quality.

3.4. Material and Methods

3.4.1. Cultivation

The experiment took place in the Botanic Garden of Turin (Viale Mattioli 25, Italy, 45°05'50.29''N Lat, 7°68'65.08''E Long). Saffron plants were grown in a greenhouse until the emergence of the spate, then outdoors in a containment chamber where they were protected from wild gray squirrels but received rain. Cultivation lasted for one cycle (July 2019–June 2020).

Saffron corms (calibre of 10/+, Fratelli Ingegnoli, Milano, Italy) were sowed on 25 July 2019 in pots (10x10x12, 1L volume) containing sterilized quartz sand as substrate (one corm per pot). Each pot with mycorrhizal treatments (Myc+) received 15 g of inoculum consisting of colonized root fragments, hyphae, and spores in a mixture of zeolite and expanded clay. The inoculum was placed 2 cm below the corm to guarantee the contact with the roots, thus favouring the symbiosis between AMF and saffron plants. Every pot used as non-mycorrhizal (Myc-) control received 15 g of the same zeolite and expanded clay substrate as mock inoculum.

A randomized block design was used with a total of 36 pots displayed in two experimental plot units (18 pots per unit) and three treatments (12 pots per treatment): (i) not-inoculated control (Myc-); (ii) an AMF mono fungal inoculum of *Rhizoglomus irregulare* BEG140 (Myc+ Ri); and (iii) an AMF multi fungal inoculum of *Funneliformis geosporum* BEG199, *Funneliformis caledonium* BEG97, and *Claroidoglomus claroideum* BEG96 (Myc+ Mix). The AMF inocula were provided by Symbiom Ltd., (Lanškroun, Czech Republic). Four pots inoculated with *Funneliformis mosseae* BEG12 (provided by MycAgro Lab, Breteniére, FR), already tested in a previous experiment, were also included (Myc+ Fm). Pots of different treatments were periodically rotated in different positions of the bench to minimize the differences due to their position in the greenhouse or outdoors in the containment chamber. Corms were not treated against fungal pathogens.

3.4.2. AMF morphological evaluation

On 30 January 2020 saffron plant roots from 2 pots (Myc- and Myc+ Ri) were quickly observed for evaluating AMF external mycelia presence under stereo microscope.

On 19 February 2020 saffron plants from 4 pots for each treatment were sampled for morphological observation of AMF colonization. Briefly, saffron roots were harvested, rid of topsoil, cleaned and stained with 0.1% (w:v) cotton blue in 90% lactic acid overnight. The roots were then de-stained two times (by a distance of 2 h), with water and 90% lactic acid in water (50:50, v:v), and stored in 90% lactic acid. The staining protocol was performed twice to ensure a better staining. The roots were finally cut into 1-cm-long fragments and placed on microscope slides for further morphological analysis (Trouvelot et al., 1986). Under light microscope up to 220 root fragments were observed for each treatment.

3.4.3. Agronomic analysis

At flowering (25 October – 12 November 2019), the number of flowers produced daily per corm and the fresh (FW) and dried (DW) weight of the flowers, stigmas, and spice (i.e., stigmas dried at 40 °C for 8 h in an oven) were measured. During the vegetative – reproductive stage, the biomass of leaves and roots was registered (19 February 2020). After the leaves withered (11 June 2020), corms were lifted, rid of topsoil, cleaned, and detunicated to determine the number, size, and weight of the replacement corms.

3.4.4. Extraction of Saffron Tepals

The extractions of dried saffron tepals (air-dried in the dark) were performed similarly to Stelluti et al. (2021). Briefly, a powdered sample:solvent ratio of 1:50 g mL^{-1} was extracted using ultrasound assisted extraction (UAE) and deionized water as solvent. The sample tubes were inserted into an ultrasonic extractor (Sarl Reus, Drap, France) using a frequency of 23 kHz for 15 min. The extracted solutions were filtered with one-layer of filter paper (Whatman No. 1, Maidstone,

UK) and centrifugated at 3500 rpm per 10 minutes at 4°C. The extractions were carried out at room temperature (ca. 21 °C) in triplicate. The extracts were stored at −20 °C for further analyses.

3.4.5. Total Phenolic Content and Antioxidant Activity of Tepal Extracts

Total phenolic content (TPC) was estimated on dried tepals using the Folin-Ciocalteu method (Stelluti et al., 2021) and the results were expressed as mg of gallic acid equivalents (GAE) per 100 g of dry weight (mg GAE 100 g^{-1} DW).
The antioxidant activity was determined with three assays (Stelluti et al., 2021): the ferric ion reducing antioxidant power (FRAP), the 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging methods. Results were expressed as millimoles of ferrous iron equivalents per kilogram of dry weight (mmol Fe^{2+} Kg^{-1} DW) for the FRAP assay and µmol of Trolox equivalents per gram of dry weight (µmol TE g^{-1} DW) for both ABTS and DPPH assays

3.4.6. Statistical Analysis

Data were checked for normality (Shapiro–Wilk's test, $p > 0.05$) and homoscedasticity (Levene's test, $p > 0.05$) and eventually transformed (log or square-root transformation) before performing a one-way ANOVA test and Tukey's *post-hoc* test. The "N° flowers $corm^{-1}$" data did not respect the ANOVA assumptions and were analysed with the Kruskal–Wallis non-parametric test. The R-studio software was used for the statistical analysis

3.5. Results and Discussion

3.5.1. Assessment of Root Mycorrhization

Very low AMF colonization was found in saffron roots. For plants inoculated with *F. mosseae* extraradical mycelium was present in about 10% of root fragments; intraradical hyphae with arbuscules and vesicles were seen in 15.5%

of fragments (frequency of colonization, F%), that were colonized between 30% and 100%. Little extraradical mycelium was found for the treatment with *R. irregulare* (in 1% of root fragments) and the mixed inoculant (in 2.6% of root fragments); for the latter, intraradical vesicles were also observed in 2.6% of root fragments (F%). No AMF structures were present in uninoculated controls.

The fungal colonization can be affected by environmental conditions, such as temperature, and AMF-plant species compatibility (Berruti et al., 2016; Caser et al., 2018; Victorino et al., 2021). According to Gavito et al. (2005), in *vitro* (root organ cultures) the AMF growth decreased at temperatures <18°C depending on the fungal species. Normally, AMF colonization is greater in controlled pot cultivation compared with open field, probably due to the reduced presence of other microorganisms and environmental extremes (Berruti et al., 2016). Accordingly, in previous works on saffron grown in pots under greenhouse and treated with AMF, Caser and colleagues (2019, 2020b) found higher intensity of colonization of the whole root system by a mixed formulation of *R. intraradices* and *F. mosseae* (93.3%), with 100.0% of arbuscules abundance. In this work where potted plants were grown outdoor, with average temperatures in autumn 2019 and winter 2019/2020 of around 14°C and 4°C (Arpa Piemonte, 2019, 2020), temperature extremes might have negatively affected the root colonization. Moreover, the AMF species used in this study might not be very compatible with saffron.

3.5.2. Agronomic analysis

Regarding the agronomic measurements (Table 1), no statistical differences between treatments were found, except for dried weight of leaves corm^{-1} that was improved in Myc+ Mix treatment in comparison to both Myc- and Myc+ Fm. A positive tendency was observed for Myc+ Ri.

For soilless saffron cultivation with AMF inoculants (*R. intraradices* alone or mixed with *F. mosseae*), similar results for the number of flower per corm and spice yield were obtained by Caser et al. (2019a). Plant growth response to AMF depends on several factors, such as genotypes and environmental conditions;

positive growth response is usually correlated to an increased mineral nutrient uptake (mostly P), while neutral or negative response to imbalanced C costs for the plant (Smith and Smith, 2011). The fungal genotypes, cultivation conditions, and poor colonization and presence of arbuscules (AMF functional trade structures) might be the reasons for the neutral response regarding plant yield.

Table 1. Agronomic measurements taken during different phases of the saffron plant lifecycle: flower and stigmas yield (flowering); biomass of leaves and roots (vegetative – reproductive phase); replacement corms yield (end of the reproductive phase). Myc+ Fm: AMF mono fungal inoculum of *Funneliformis mosseae* BEG12; Myc+ Ri: AMF mono fungal inoculum of *Rhizoglomus irregulare* BEG140; Myc+ Mix: AMF multi fungal inoculum of *Funneliformis geosporum* BEG199, *Funneliformis caledonium* BEG97, and *Claroidoglomus claroideum* BEG96; Myc-: not-inoculated control.

	Myc+ Fm	Myc+ Ri	Myc+ Mix	Myc-	*p*
Flowering phase					
N° flowers corm^{-1}	1 ± 1	2 ± 1	2 ± 1	2 ± 1	ns
FW flowers (mg)	669.2 ± 146.7	633.4 ± 119.6	626.2 ± 167.0	662.3 ± 134.8	ns
DW flowers (mg)	73.2 ± 16.8	67.7 ± 10.6	68.1 ± 17.2	72.8 ± 13.8	ns
FW stigmas (mg flower^{-1})	51.8 ± 16.0	54.9 ± 11.9	54.9 ± 18.3	54.8 ± 11.2	ns
DW stigmas (mg flower^{-1})	6.4 ± 3.5	7.2 ± 1.5	7.4 ± 2.2	7.7 ± 2.4	ns
DW stigmas (mg corm^{-1})	10.1 ± 1.1	15.1 ± 4.9	14.1 ± 3.2	17.3 ± 5.9	ns
Vegetative - Reproductive phase					
N° leaves corm^{-1}	4 ± 1	4 ± 1	6 ± 1	4 ± 1	ns
FW leaves corm^{-1} (g)	5.345 ± 0.964	5.345 ± 0.964	6.948 ± 0.756	5.199 ± 0.236	ns
DW leaves corm^{-1} (g)	1.271 ± 0.262 b	1.420 ± 0.205 ab	1.707 ± 0.178 a	1.202 ± 0.071 b	*
Length leaves corm^{-1} (cm)	-	21 ± 4	21 ± 3	20 ± 4	ns
FW fibrous roots corm^{-1} (g)	4.186 ± 1.037	3.320 ±1.329	2.902 ± 0.578	3.153 ± 0.839	ns
DW fibrous roots corm^{-1} (g)	0.992 ± 0.358	0.736 ± 0.381	0.550 ± 0.146	0.411 ± 0.091	ns
FW conctractile roots corm^{-1} (g)	3.820 ± 1.131	3.190 ± 2.918	4.446 ± 2.492	3.173 ± 1.679	ns
DW conctractile roots corm^{-1} (g)	0.584 ± 0.152	0.562 ± 0.527	0.706 ± 0.409	0.347 ± 0.148	ns

End of the Reproductive phase					
N° corms plant^{-1}	-	5 ± 2	4 ± 2	3 ± 1	ns
FW corms (g)	-	2.444 ± 1.995	2.711 ± 1.803	3.371 ± 3.131	ns
FW corms plant^{-1} (g)	-	2.237 ± 1.492	2.783 ± 0.974	3.834 ± 2.534	ns
Diameter corms (cm)	-	1.7 ± 0.4	1.9 ± 0.6	1.9 ± 0.7	ns
Diameter corms plant^{-1} (cm)	-	1.7 ± 0.2	2.0 ± 0.3	2.0 ± 0.5	ns

Values of mean and standard deviation are reported. Statistical comparisons were performed with the one-way ANOVA test or Kruskal-Wallis test; * $p < 0.05$; ns = not significant.

3.5.3. TPC and Antioxidant Activity of Tepal Extracts

The total phenolic content (TPC) was not significantly different between the AMF treatments and uninoculated controls (Table 2). This result agrees with that obtained for saffron spice derived by AMF treatments both in soilless (*R. intraradices* mixed with *F. mosseae*) (Caser et al., 2019) and soil cultivation conditions (*R. intraradices* alone or mixed with *F. mosseae*) (Caser et al., 2018). The antioxidant activity of phytoextracts is widely measured with assays based on single electron transfer (SET) reactions, such as FRAP, ABTS, and DPPH, which measure radical scavenging/reducing capacity of a sample (Arslan Burnaz et al., 2017; Stelluti et al., 2021). Regarding the FRAP method, the values of Myc+ Fm and Myc+ Ri inoculated plants were significantly higher than Myc- controls (+11.2 ± 2.0% and +10.9 ± 3.0%, respectively) (Table 2). An opposite result was seen for both ABTS and DPPH methods; the values of the Myc+ Fm and Myc+ Ri treatments were significantly lower than Myc- (-29.2 ± 3.3% and -25.4 ± 4.2%, respectively, and -31.4 ± 12.0% and -24.6 ± 2.4%, respectively) (Table 2). No significant differences were found between Myc+ Fm and Myc+ Ri treatments, neither for Myc+ Mix and Myc- controls.

Non-converging results among these methods, already highlighted by other authors (Arslan Burnaz et al., 2017; Caser et al., 2019; Stelluti et al., 2021), may be caused by several factors, such as different behaviours or synergistic/antagonistic interactions among the antioxidants, redox potential

differences, and not-free radical scavenger molecules reacting with the reagents (Huang et al., 2014; Stelluti et al., 2021). Interestingly, even though root colonization was very low, the bioinoculants somewhat affected the plant's secondary metabolism. In Caser et al. (2019b) saffron plants poorly mycorrhized in soil conditions (treatment with *R. intraradices*) produced a spice richer in crocin I, responsible for the colorant capacity of the spice, and with a higher antioxidant activity measured with the FRAP assay. We speculated that plants may have noticed the presence of AMF and responded by adjusting the antioxidants level (Bianciotto et al., 2018).

Table 2. Total phenolic content (TPC) and antioxidant activity evaluated using three methods (FRAP, ABTS, and DPPH) in extracts of dried saffron tepals. Myc+ Fm: AMF mono fungal inoculum of *Funneliformis mosseae* BEG12; Myc+ Ri: AMF mono fungal inoculum of *Rhizoglomus irregulare* BEG140; Myc+ Mix: AMF multi fungal inoculum of *Funneliformis geosporum* BEG199, *Funneliformis caledonium* BEG97, and *Claroidoglomus claroideum* BEG96; Myc-: not-inoculated control.

	Myc+ Fm	**Myc+ Ri**	**Myc+ Mix**	**Myc-**	***p***
TPC (mg GAE 100 g^{-1} DW)	2929.70 ± 157.23	2885.52 ± 87.47	2880.61 ± 40.18	3031.82 ± 45.41	ns
FRAP (mmol Fe^{2+} Kg^{-1} DW)	362.73 ± 8.33 a	361.93 ± 12.38 a	302.82 ± 20.15 b	322.13 ± 13.16 b	**
ABTS (µmol TE g^{-1} DW)	70.90 ± 1.85 b	73.05 ± 2.52 b	93.76 ± 1.65 a	91.57 ± 4.38 a	***
DPPH (µmol TE g^{-1} DW)	22.61 ± 2.16 b	23.70 ± 0.46 b	29.97 ± 1.29 a	29.53 ± 0.35 a	***

Values of mean and standard deviation are reported. Statistical comparisons were performed using the one-way ANOVA test and Tukey *post-hoc* test; ** $p < 0.01$; *** $p < 0.001$; ns = not significant.

3.6. Conclusions

The efficacy of different AMF-based inoculants on saffron in potted system was tested. The low level of mycorrhization found for all treated plants could be due

to saffron-AMF species compatibility and/or growing conditions, particularly low temperatures. The dried weight of leaves per corm was higher in plants inoculated with the mixed AMF inoculant. The antioxidant capacity of tepal extracts measured with the FRAP method resulted significantly increased by *Funneliformis mosseae* and *Rhizoglomus irregulare*, but opposite results were obtained with the ABTS and DPPH methods.

Further studies on saffron cultivated with different types of bioinoculants and/or biostimulants, could provide further information on the real influence exerted by AMF on secondary metabolism of plants, with the aim of improving healthy phytochemicals in saffron by-products and spice.

Acknowledgments

The authors thank Maria Teresa Della Beffa for the help in plant preparation and maintenance, and Miroslav Vosatka and Aleš Látr for providing the two AM fungal inocula produced by Symbiom.

3.7. References

Arpa Piemonte (2019). Il Clima in Piemonte, autunno 2019. 1–36.

Arpa Piemonte (2020). Il Clima in Piemonte, inverno 2019/2010. 1–28.

Arslan Burnaz, N., Küçük, M., and Akar, Z. (2017). An on-line HPLC system for detection of antioxidant compounds in some plant extracts by comparing three different methods. J. Chromatogr. B 1052, 66–72.

Berruti, A., Lumini, E., Balestrini, R., and Bianciotto, V. (2016). Arbuscular Mycorrhizal Fungi as Natural Biofertilizers: Let's Benefit from Past Successes. Front. Microbiol. 6, 1–13.

Bianciotto, V., Victorino, I., Scariot, V., and Berruti, A. (2018). Arbuscular mycorrhizal fungi as natural biofertilizers: current role and potential for the horticulture industry. Acta Hortic. 1191, 207–216.

Caser, M., Victorino, Í.M.M., Demasi, S., Berruti, A., Donno, D., Lumini, E., Bianciotto, V., and Scariot, V. (2018). Saffron Cultivation in Marginal Alpine Environments: How AMF Inoculation Modulates Yield and Bioactive Compounds. Agronomy 9, 12.

Caser, M., Demasi, S., Victorino, Í.M.M., Donno, D., Faccio, A., Lumini, E., Bianciotto,

V., and Scariot, V. (2019). Arbuscular Mycorrhizal Fungi Modulate the Crop Performance and Metabolic Profile of Saffron in Soilless Cultivation. Agronomy 9, 232.

Caser, M., Demasi, S., Stelluti, S., Donno, D., and Scariot, V. (2020a). Crocus sativus L. Cultivation in Alpine Environments: Stigmas and Tepals as Source of Bioactive Compounds. Agronomy 10, 1473.

Caser, M., Victorino, I.M.M., Demasi, S., Berruti, A., Lumini, E., Bianciotto, V., and Scariot, V. (2020b). Arbuscular mycorrhizal fungi association promotes corm multiplication in potted saffron (Crocus sativus L.) plants. Acta Hortic. 1287, 441–446.

Gavito, M.E., Olsson, P.A., Rouhier, H., Medina-Peñafiel, A., Jakobsen, I., Bago, A., and Azcón-Aguilar, C. (2005). Temperature constraints on the growth and functioning of root organ cultures with arbuscular mycorrhizal fungi. New Phytol. 168, 179–188.

Gresta, F., Lombardo, G.M., Siracusa, L., and Ruberto, G. (2008). Saffron, an alternative crop for sustainable agricultural systems. A review. Agron. Sustain. Dev. 28, 95–112.

Halevy, A.H. (1990). RECENT ADVANCES IN CONTROL OF FLOWERING AND GROWTH HABIT OF GEOPHYTES. Acta Hortic. 35–42.

Huang, H., Sun, Y., Lou, S., and Ye, X. (2014). In vitro digestion combined with cellular assay to determine the antioxidant activity in Chinese bayberry (Myrica rubra Sieb. et Zucc.) fruits: A comparison with traditional methods. Food Chem. 146, 363–370.

Khan, M., Hanif, M.A., Ayub, M.A., Jilani, M.I., and Shahid Chatha, S.A. (2020). Saffron. In Medicinal Plants of South Asia, (Elsevier), pp. 587–600.

Kumar, R., Singh, V., Devi, K., Sharma, M., Singh, M.K., and Ahuja, P.S. (2009). State of art of saffron (Crocus sativus L.) agronomy: A comprehensive review.

Lone, R., Shuab, R., and Koul, K.K. (2016). AMF association and their effect on metabolite mobilization, mineral nutrition and nitrogen assimilating enzymes in saffron (Crocus sativus) plant. J. Plant Nutr. 39, 1852–1862.

Mottaghipisheh, J., Mahmoodi Sourestani, M., Kiss, T., Horváth, A., Tóth, B., Ayanmanesh, M., Khamushi, A., and Csupor, D. (2020). Comprehensive chemotaxonomic analysis of saffron crocus tepal and stamen samples, as raw materials with potential antidepressant activity. J. Pharm. Biomed. Anal. 184, 113183.

De Pascale, S., Rouphael, Y., and Colla, G. (2017). Plant biostimulants: innovative tool for enhancing plant nutrition in organic farming. Eur. J. Hortic. Sci 82, 277–285.

Rezvani-Moghaddam, P. (2020). Ecophysiology of saffron. In Saffron, (Elsevier), pp.

119–137.

Rouphael, Y., and Colla, G. (2020). Editorial: Biostimulants in Agriculture. Front. Plant Sci. 11, 1–7.

Shahi, T., Assadpour, E., and Jafari, S.M. (2016). Main chemical compounds and pharmacological activities of stigmas and tepals of 'red gold'; saffron. Trends Food Sci. Technol. 58, 69–78.

Smith, S.E., and Smith, F.A. (2011). Roles of arbuscular mycorrhizas in plant nutrition and growth: New paradigms from cellular to ecosystem scales. Annu. Rev. Plant Biol. 62, 227–250.

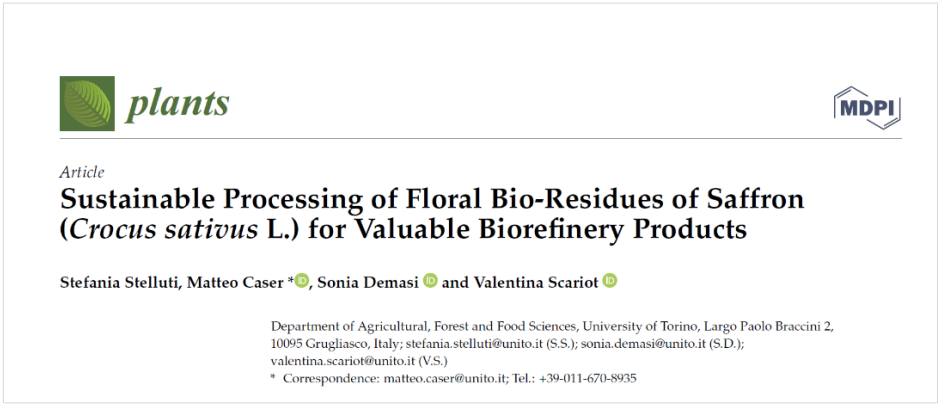
Stelluti, S., Caser, M., Demasi, S., and Scariot, V. (2021). Sustainable Processing of Floral Bio-Residues of Saffron (Crocus sativus L.) for Valuable Biorefinery Products. Plants 10, 523.

The European Parliament and the Council of the European Union. (2019). Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019 laying down rules on the making available on the market of EU fertilising products and amending Regulation (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulat. Off. J. Eur. Union 2019, 1–114.

Trouvelot, A., Kough, J., and Gianinazzi-Pearson, V. (1986). Mesure du taux de mycorhization VA d'un système radiculaire. Recherche de méthodes d'estimation ayant une signification fonctionnelle. Mycorrhizae Physiol. Genet. Gianinazzi-Pearson, V., Gianinazzi, S., Eds.; INRA Press Paris, Fr. 217–221.

Victorino, Í.M.M., Voyron, S., Caser, M., Orgiazzi, A., Demasi, S., Berruti, A., Scariot, V., Bianciotto, V., and Lumini, E. (2021). Metabarcoding of soil fungal communities associated with alpine field-grown saffron (Crocus sativus l.) inoculated with am fungi. J. Fungi 7, 1–15.

4. Sustainable Processing of Floral Bio-Residues of Saffron (*Crocus sativus* L.) for Valuable Biorefinery Products



plants

MDPI

Article

Sustainable Processing of Floral Bio-Residues of Saffron (*Crocus sativus* L.) for Valuable Biorefinery Products

Stefania Stelluti, Matteo Caser *, Sonia Demasi and Valentina Scariot

Department of Agricultural, Forest and Food Sciences, University of Torino, Largo Paolo Braccini 2, 10095 Grugliasco, Italy; stefania.stelluti@unito.it (S.S.); sonia.demasi@unito.it (S.D.); valentina.scariot@unito.it (V.S.)
* Correspondence: matteo.caser@unito.it; Tel.: +39-011-670-8935

4.1. Abstract

Tepals constitute the most abundant bio-residues of saffron (*Crocus sativus* L.). As they are a natural source of polyphenols with antioxidant properties, they could be processed to generate valuable biorefinery products for applications in the pharmaceutical, cosmetic, and food industries, becoming a new source of income while reducing bio-waste. Proper storage of by-products is important in biorefining and dehydration is widely used in the herb sector, especially for highly perishable harvested flowers. This study aimed to deepen the phytochemical composition of dried saffron tepals and to investigate whether this was influenced by the extraction technique. In particular, the conventional maceration was compared with the Ultrasound Assisted Extraction (UAE), using different solvents (water and three methanol concentrations, i.e., 20%, 50%, and 80%). Compared to the spice, the dried saffron tepals showed a lower content of total phenolics (average value 1127.94 ± 32.34 mg GAE 100 g^{-1}DW) and anthocyanins (up to 413.30 ± 137.16 mg G3G 100 g^{-1}DW), but a higher antioxidant activity, which was measured through the FRAP, ABTS, and DPPH assays. The HPLC-DAD analysis detected some phenolic compounds (i.e., ferulic acid, isoquercitrin, and quercitrin) not previously found in fresh saffron

tepals. Vitamin C, already discovered in the spice, was interestingly detected also in dried tepals. Regarding the extraction technique, in most cases, UAE with safer solvents (i.e., water or low percentage of methanol) showed results of phenolic compounds and vitamin C similar to maceration, allowing an improvement in extractions by halving the time. Thus, this study demonstrated that saffron tepals can be dried maintaining their quality and that green extractions can be adopted to obtain high yields of valuable antioxidant phytochemicals, meeting the requirement for a sustainable biorefining.

4.2. Keywords

dried tepals; total phenolic content; total anthocyanin content; antioxidant activity; vitamin C; ultrasound assisted extraction; biorefining

4.3. Introduction

Saffron (*Crocus sativus* L.), of the Iridaceae family, is a geophyte widely cultivated for its red-scarlet stigmas that, once dried, form the most expensive spice in the world ($40–50 g^{-1}, [1]). Saffron spice has nutritional and phytochemical compounds such as terpenes, phenols, and vitamin C, with antioxidant [2,3] and therapeutic [4,5] properties.

Saffron, being a triploid ($2n = 3x = 24$) sterile species, is propagated through underground clonal corms [6]. In Mediterranean climate regions flowering occurs for few weeks from early to late autumn and is mainly controlled by seasonal thermoperiodicity, soil water content, and corm size [7–9]. There are one or several flowers per saffron plant, even up to 12 [6]. Flowers have a perianth of six violet tepals, three stamens, and a style culminating in three stigmas [10]. About 110 to 300 flowers are needed to obtain 1g of spice [11,12] and tepals constitute the most abundant bio-residue (~80% of total flower mass [13]).

In Persian traditional medicine, saffron tepals are considered antispasmodic, stomachic, antitumor, antidepressant, and curative of anxiety [14]. They contain proteins, fibers, fats, ashes, minerals, polyphenols, and present high antioxidant

activity [14,15]. To our knowledge, vitamin C has never been detected in saffron tepals so far.

Phenolic compounds are a class of secondary metabolites commonly found in plants in which play various biological activities, such as defense against biotic and abiotic stresses, UV filters, attraction for pollinators, fruit dispersion, plant growth, and allelopathy [16–20]. These molecules are the main source of plant antioxidants that, by quenching ROS and reactive nitrogen species (RNS) [21], control oxidative stress, which is associated with several age-related diseases such as cancers, inflammations, and neurodegenerative disorders. This class include phenolic acids and flavonoids [22].

Phenolic acids include hydroxybenzoic and hydroxycinnamic acids and are the main phenolic compounds produced by plants [17]. They can enhance the organoleptic, nutritional, and antioxidant properties of food and are ancestors for the bioactive compounds used in pharmaceutic-cosmetic and food industries [17].

Flavonoids comprise flavonols, flavan-3-ols (such as catechins), and anthocyanins, which are the water-soluble pigments responsible for the pink-orange, red, and blue color range of flowers and fruits [22,23]. Flavonoids have antioxidant, antifungal, antibacterial, and antiviral properties and are used in the agricultural, food, and pharmaceutical-cosmetic industries [23]. In particular, anthocyanins are authorized natural food colorants in Europe with the code E-163 regardless the plant source, being considered a group of harmless substances [24].

Vitamin C is ubiquitous in plants [25]. It regulates several physiological functions, such as photosynthesis, seed germination, floral inductions, and senescence. Ascorbate is the reduced and physiologically active form, while dehydro-ascorbate is the oxidized form. The regenerative nature makes ascorbate a powerful antioxidant molecule, involved in the enzymatic and non-enzymatic defense system against oxidative stress [26]. Vitamin C is an essential dietary element since it cannot be synthetized by humans and a severe lack of its intake leads to scurvy disease [25].

The selection of suitable solvents and techniques is a basic factor to get a high extraction yield of phytochemicals from plant material [27]. Water, organic solvents, and their combination are often used to extract phenolics and vitamin C [2,3,27–29]. Methanol is commonly chosen for phenolic compounds due to its similar polarity, small dimension, and low density [30]. In a previous work, Caser et al. [15] compared conventional maceration with ultrasound assisted extraction (UAE) on fresh saffron tepals using water and different concentrations of methanol (20%, 50%, and 80%) as solvents. Because harvested flowers are very perishable, dehydration is a common practice to storage and preserve their quality since it inhibits enzymatic activity and limits microbial contamination [31].

This study aimed to investigate the phytochemical composition and antioxidant activity of dried saffron tepals and select the more effective and sustainable extractions. Conventional maceration was compared to the modern UAE technique that, allowing the use of safer solvents and shorter time, can reduce the energy cost of the extraction process [31,32]. A comparison of the results with a previous work performed on fresh saffron bio-residues [15] was also possible. According to the Directive 2008/122/EC, avoid waste production and use it as a resource is encouraged in the food waste management and an eco-sustainable processing is required in biorefining. As dried saffron tepals can have food, pharmaceutical, and cosmetic applications [13], investing in this by-product may be a promising approach to minimize losses [13] and potentially increase further the economic value of this crop [32].

4.4. Material and Methods

4.4.1. Plant Material

Saffron tepals were provided by the company “Lo Zafferano del Monviso” located in Martiniana Po, CN (Italy—44°23′ N 7°33′ E). Saffron plants were cultivated in open field selecting corms with horizontal diameters of 2.5 to 3.5 cm, which were planted in August 2018. The flowers were harvested in October—November 2018 and immediately air-dried. In the laboratory of the

Department of Agricultural, Forest, and Food Sciences (DISAFA) of the University of Turin (Italy), the dried tepals were grinded in liquid nitrogen and stored at −80 °C until use.

4.4.2. Extraction Methods

Two extraction procedures, i.e., maceration and UAE, with deionized water or 20%, 50%, and 80% methanol in deionized water (v:v) as solvents were applied. The extractions were conducted at room temperature (ca. 21 °C) and using a powdered sample–solvent ratio of 1:50 g ml^{-1}.

As regards maceration, the samples were soaked in each solvent type and kept into a glass tube under stirring (1000 rpm) in the dark for 30 min, whereas for UAE the sample tubes were inserted into an ultrasonic extractor (Sarl Reus, Drap, France) using a frequency of 23 kHz for 15 min. The extracted solutions were all filtered with one-layer of filter paper (Whatman No. 1, Maidstone, UK) and then with a 0.45 μm PVDF syringe filter (CPS Analitica, Milano, Italy). The extracts were then stored at −20 °C for analyses. All the extractions were carried out in triplicate for each solvent and method used.

4.4.3. Spectrophotometric Analysis

All the analysis were conducted in three replicates and performed as reported by Caser et al. [15].

Total Phenolic Content

Total phenolic content was estimated using the Folin-Ciocalteu method. In each plastic tube 200 μL of each phytoextract were mixed with 1000 μL of diluted (1:10) Folin–Ciocalteu reagent. After 10 min of incubation at dark and room temperature, 800 μL of Na_2CO_3 7.5% (*w/v*) were added to each tube. The samples were incubated at dark and room temperature for 30 min. The absorbance at 765 nm was measured by means of a UV–Vis spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, Santa Clara, CA, USA). The results were expressed as mg of gallic acid equivalents (GAE) per 100 g of dry weight (mg GAE 100 g^{-1} DW).

Total Anthocyanin Content

Total anthocyanins content was measured using the pH-differential method. Buffer solution at pH 1 (4.026 g KCl + 12.45 mL HCl 37% in a 1 L water volume) was added to 500 µL of phytoextract reaching 5 mL in each flask. The same was made in a second flask using a buffer solution at pH 4.5 (32.82 g $C_2H_3NaO_2$ + 18 mL $C_2H_4O_2$ in a 1 L water volume). The samples were left in the dark at room temperature for 20 min. The absorbance of both flasks was read at 515 and 700 nm at a UV–Vis spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, Santa Clara, CA, USA). The total anthocyanin content was calculated using the following formula:

$$[(A \times \text{sample dilution factor} \times 1000)/(\text{molar absorptivity} \times 1)]$$

where *A* is [(Absorbance 515 nm—Absorbance 700 nm) at pH 1]—[(Absorbance 515 nm—Absorbance 700 nm) at pH 4.5]. The results were expressed as milligrams of cyanidin 3-O-glucoside (C3G) per 100 g of dry weight (mg C3G 100 g^{-1} DW).

Antioxidant Activity

The antioxidant activity was determined using the following methods:

- Ferric ion reducing antioxidant power (FRAP) method. The FRAP solution was produced by mixing a buffer solution at pH 3.6 ($C_2H_3NaO_2$ + $C_2H_4O_2$ in water), 2,4,6-tripyridyltriazine (TPTZ, 10 mM in HCl 40 mM), and $FeCl_3 \cdot 6H_2O$ (20 mM). Then, 90 µL of deionized water and 900 µL of the FRAP reagent were added to 30 µL of phytoextract in each plastic tube. The samples were left at 37 °C for 30 min and the absorbance was read at 595 nm at a spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, Santa Clara, CA, USA). Results were expressed as millimoles of ferrous iron equivalents per kilogram of dry weight (mmol Fe^{2+} Kg^{-1} DW).
- The 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method. The ABTS radical cation (ABTS·) was obtained by the reaction

of 7.0 mM ABTS solution with 2.45 mM $K_2S_2O_8$ solution. The solution was incubated for 12–16 h in the dark at room temperature and then diluted with distilled water up to read an absorbance of 0.70 (±0.02) at 734 nm. 500 µL of diluted ABTS· was added to 15 µL of phytoextract and, after incubation in the dark at room temperature for 10 min, the absorbance was measured at 734 nm by means of a spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, Santa Clara, CA, USA). The ABTS radical-scavenging activity was calculated as

[(Abs0 – Abs1/Abs0) x 100]

where *Abs0* is the absorbance of the control (solution without phytoextract) and *Abs1* is the absorbance of the sample. The results were expressed as µmol of Trolox equivalents per gram of dry weight (µmol TE g^{-1} DW).

- The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method. To obtain 100 µM of DPPH radical cation (DPPH·) 2 mg of DPPH were mixed up with 50 mL of MeOH, up to have an absorbance of 1.000 (±0.005) at 515 nm. Then, 1.5 mL of diluted DPPH· was added to 20 µL of phytoextract and the reaction was left in the dark at room temperature for 30 min. The absorbance was read at 515 nm at a spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, Santa Clara, CA, USA). The DPPH radical-scavenging activity was calculated as

  [(Abs0 – Abs1/Abs0) x 100]

  where *Abs0* is the absorbance of the control (solution without phytoextract) and *Abs1* is the absorbance of the sample. Results were expressed as µmol of Trolox equivalents per gram of dry weight (µmol TE g^{-1} DW).

4.4.4. HPLC Analysis

Qualitative and quantitative analyses of the phytoextracts were carried out using an Agilent 1200 High-Performance Liquid Chromatography coupled with an Agilent UV–Vis diode array detector (Agilent Technologies, Santa Clara, CA,

USA). The chromatographic separation was made with a Kinetex C18 column (4.6 × 150 mm^2, 5 µm, Phenomenex, Torrance, CA, USA) using several mobile phases and recording UV spectra at different wavelengths [65], as described in Table 5.

Table 5. HPLC methods and conditions.

Methods	Classes of Interest	Stationary Phase	Mobile Phase	Wavelength (nm)
A	Cinnamic acids, Flavonols	KINETEX—C18 column (4.6 × 150 mm, 5 µm)	A: 10 mM KH_2PO_4/H_3PO_4, pH = 2.8 B: CH_3CN	330
B	Benzoic acids, catechins	KINETEX—C18 column (4.6 × 150 mm, 5 µm)	A: $H_2O/CH_3OH/HCOOH$ (5:95:0.1 *v/v/v*), pH = 2.5 B: $CH_3OH/HCOOH$ (100:0.1 *v/v*)	280
C	Vitamin C	KINETEX—C18 column (4.6 × 150 mm, 5 µm)	A: 5 mM $C_{16}H_{33}N(CH_3Br$/50 mM KH_2PO_4, pH = 2.5 B: CH_3OH	261, 348

Elution conditions. Method A, gradient analysis: 5% B to 21% B in 17 min + 21% B in 3 min (2 min conditioning time); flow: 1.5 mL min^{-1}; Method B, gradient analysis: 3% B to 85% B in 22 min + 85% B in 1 min (2 min conditioning time); flow: 0.6 mL min^{-1}; Method C, isocratic analysis: ratio of phase A and B: 95:5 in 10 min (5 min conditioning time); flow: 0.9 mL min^{-1}.

Each compound was determined comparing the retention times and UV spectra with the standards under the same chromatographic conditions as reported by Donno et al. [57]. The standards, purchased from Sigma-Aldrich (Saint Louis, MO, USA), were the following: flavonols (hyperoside, isoquercitrin, quercetin, quercitrin, and rutin), catechins (catechin and epicatechin), benzoic acids (ellagic and gallic acids), cinnamic acids (caffeic, chlorogenic, coumaric, and ferulic acids), and vitamin C (ascorbic and dehydroascorbic acids). All the analyses were performed in three replicates.

4.4.5. Statistical Analysis

All data were log transformed before the statistical analysis.

As regards the HPLC-DAD analysis, the limit of quantitation (LOQ) [57] was added when the treatments had data for only one or two replicates out of three, while the value 0 was added for the treatments without data.

The data were analyzed with the R-studio software to identify the statistically supported differences between the different extractions. Significant mean differences were verified with one-way ANOVA ($p < 0.05$) and Tukey's post-hoc test after checking the data for normality and homoscedasticity through Shapiro–Wilk's test ($p > 0.05$) and Levene's test ($p < 0.05$), respectively. The compounds non respecting the ANOVA assumptions were analyzed with Kruskal–Wallis non-parametric test ($p < 0.05$) and the post-hoc Dunn's comparison test.

As regards the data resulting from the spectrophotometric analysis, statistical comparisons were performed using ANOVA for TPC, TAC, and FRAP and Kruskal–Wallis test for DPPH and ABTS ($p > 0.05$ in Levene's test). Regarding the data resulting from the HPLC analysis, ANOVA was made for ellagic acid, hyperoside, and rutin, while Kruskal–Wallis test for the other compounds ($p < 0.05$ in Shapiro–Wilk's test).

A correlation analysis between the content of total phenolics (TPC) and anthocyanins (TAC), and the assays FRAP, ABTS, and DPPH used to measure the antioxidant activity of the extracts was made with the R-studio software. The Pearson correlation was adopted when the variables were normally distributed according to the Shapiro–Wilk's test ($p > 0.05$), otherwise the non-parametric Kendall correlation.

4.5. Results

4.5.1. Qualitative and Antioxidant Properties of Dried Tepals Extracts

Total phenolic content (TPC), total anthocyanin content (TAC), and antioxidant activity by means of three assays, i.e., FRAP, ABTS, and DPPH, were evaluated

for all phytoextracts (Table 1). Statistical comparisons between solvents for both extraction techniques separately were provided in Supplementary Table S1.

TAC and FRAP were significantly affected by the extractions. TPC showed an average value of 1127.94 ± 32.34 mg GAE 100 g^{-1} DW. TAC varied significantly among the different extractions, ranging from 178.39 ± 34.03 mg G3G 100 g^{-1} DW using UAE with 20% methanol to 413.30 ± 137.16 mg G3G 100 g^{-1} DW using UAE with water, with an average value of 282.90 ± 71.82 mg G3G 100 g^{-1} DW.

The assays revealed that all extracts exhibited antioxidant activity. FRAP showed significant differences ranging from 460.05 ± 35.55 mmol Fe^{2+} Kg^{-1} DW for UAE with 20% methanol to 571.54 ± 3.21 mmol Fe^{2+} Kg^{-1} DW for maceration with water. ABTS and DPPH had an average value of 14.07 ± 0.73 μmol TE g^{-1} DW and 21.32 ± 3.45 μmol TE g^{-1} DW, respectively.

4.5.2. Correlation Analysis

The correlation between TPC, TAC, and the antioxidant activity of the phytoextracts assessed with the FRAP, ABTS, and DPPH assays were statistically evaluated (Table 2). Significant correlations were only found in the case of UAE, between TPC and both ABTS (−0.69) and DPPH (−0.45), and between TAC and FRAP (0.86).

4.5.3. Bioactive Compounds from HPLC Analysis

The bioactive compounds extracted were as follows: ferulic acid (cinnamic acid); el-lagic acid (benzoic acid); hyperoside, isoquercitrin, quercitrin, and rutin (flavonols); epicatechin (catechin); and vitamin C (Table 3). Overall, the yield of phytochemicals was significantly affected by the different extractions ($p < 0.05$). As regards ferulic acid, 9.65 ± 2.62 mg 100 g^{-1} DW was extracted through maceration with methanol at 50%, showing no significant differences to 20%. It was not obtained by the other extractions.

Table 1. Total phenolic content (TPC), total anthocyanin content (TAC), and antioxidant activity measured with the FRAP, ABTS, and DPPH assays, in dried tepal extracts obtained through maceration (M) and Ultrasound Assisted Extraction (UAE) techniques, and the solvents water or methanol at three concentrations (20%—Met20, 50%—Met50, and 80%—Met80).

Extraction		**TPC (mgGAE 100 g^{-1} DW)**	**TAC (mgG3G 100 g^{-1} DW)**	**FRAP (mmolFe^{2+} kg^{-1} DW)**	**ABTS (µmolTE g^{-1} DW)**	**DPPH (µmolTE g^{-1} DW)**
M	Water	1142.27±43.52	345.04±132.47 a,b	571.54±3.21a	13.82±0.72	15.56±2.29
M	Met20	1123.53±59.86	268.13±26.76 a,b	506.73±13.85 b,c	14.20±0.60	17.83±2.46
M	Met50	1106.45±9.17	300.39±15.02 a,b	535.83±10.30 a,b	14.62±0.29	24.52±2.55
M	Met80	1166.96±33.15	249.13±11.97 a,b	511.72±22.49 a,b,c	14.29±0.32	24.17±1.53
UAE	Water	1150.63±11.23	413.30±137.16 a	556.90±11.91 a,b	12.76±0.81	23.55±3.60
UAE	Met20	1113.27±46.11	178.39±34.03 b	460.05±35.55 c	13.39±1.46	19.35±4.83
UAE	Met50	1066.89±26.36	277.09±49.06 a,b	506.68±21.80 b,c	15.10±0.38	24.58±1.46
UAE	Met80	1153.49± 22.74	231.70±30.19 a,b	513.67±21.12 a,b,c	14.34±0.81	21.03±1.81
p		ns	0.01413*	0.0002608***	ns	ns

Values of mean and standard deviation are reported for each variable. Statistical comparisons were performed using ANOVA for TPC, TAC, and FRAP or the non-parametric Kruskal–Wallis test for DPPH and ABTS ($p > 0.05$ in Levene's test). Values with the same letter are not statistically different at $p < 0.05$, according to Tukey's post-hoc test or Dunn's post-hoc test; * $p < 0.05$; *** $p < 0.001$; ns = not significant.

The yield of ellagic acid ranged from 1.32 ± 0.33 mg 100 g^{-1} DW for maceration with 80% methanol to 28.39 ± 4.32 mg 100 g^{-1} DW for UAE with 50% methanol, which was not significantly different to maceration with water and UAE with both 20% and 80% methanol. No extraction was performed by maceration with 50% methanol.

Table 2. Correlation analysis between total phenolic content (TPC), total anthocyanin content (TAC), and antioxidant activity measured with the assays FRAP, ABTS, and DPPH, as regards maceration (M) and UAE. Pearson or Kendall correlation coefficients are reported for *p*-values < 0.05.

	TPC	TAC	FRAP	ABTS
M				
FRAP	ns	ns	/	
ABTS	ns	ns	ns	/
DPPH	ns	ns	ns	ns
UAE				
FRAP	ns	0.86 ***	/	ns
ABTS	−0.69 *	ns	ns	/
DPPH	−0.45 *	ns	ns	ns

p-values < 0.05 show statistically significant correlations (* $p < 0.05$; *** $p < 0.001$; ns = not significant).

Hyperoside was obtained by all extractions. Its values varied from 4.35 ± 1.04 mg 100 g^{-1} DW regarding maceration with water to 27.26 ± 4.29 mg 100 g^{-1} DW for UAE with 50% methanol, which was not significantly different to the other methanol concentrations and maceration with both 20% and 80% methanol.
Isoquercitrin ranged from 0.22 ± 0.12 mg 100 g^{-1} DW for maceration with 20% methanol to 7.82 ± 3.09 mg 100 g^{-1} DW as regards maceration with 80% methanol, that not significantly differed to maceration with 80% methanol and UAE with both 20% and 50% methanol. It was not extracted by UAE with both water and 80% methanol.

Table 3. Extraction yield (mg 100 g^{-1} DW) of the compounds obtained from dried saffron tepals expressed as mg 100 g^{-1} of dried weight (DW), using the maceration (M) and Ultrasound Assisted Extraction (UAE) techniques and the solvents water or methanol at three concentrations (20%, Met20; 50%, Met50; 80%, Met80; v:v). Quantifications were obtained through the HPLC-DAD analysis.

Extractions		Cinnamic Acids	Benzoic Acids	Flavonols			Catechins		Vitamin
		Ferulic Acid	Ellagic Acid	Hyperoside	Isoquercitrin	Quercitrin	Rutin	Epicatechin	C
M	Water	0.00±0.00 b	7.67 ± 3.69 a,b,c	4.35 ± 1.04 c	0.31 ± 0.22 c,d	0.00 ± 0.00 b	8.52 ± 3.91 c	0.00 ± 0.00 b	29.61 ± 6.05 a
M	Met20	1.83 ± 0.31 a	4.43 ± 4.15 c,d	5.61 ± 0.52 a,b,c	0.22 ± 0.12 c,d	6.33 ± 5.27 a	0.32 ± 0.31 d	0.00 ± 0.00 b	33.72 ± 0.89 a
M	Met50	9.65 ± 2.62 a	0.00 ± 0.00 e	5.85 ± 4.31 b,c	4.36 ± 3.49 a,b,c	9.27 ± 3.47 a	0.00 ± 0.00 d	0.00 ± 0.00 b	0.00 ± 0.00 b
M	Met80	0.00 ± 0.00 b	1.32 ± 0.33 d,e	23.93 ± 15.51 a,b,c	7.82 ± 3.09 a	6.53 ± 0.29 a	37.61 ± 2.22 a	0.00 ± 0.00 b	0.00 ± 0.00 b
UAE	Water	0.00 ± 0.00 b	8.53 ± 8.45 b,c,d	11.58 ± 4.09 a,b,c	0.00 ± 0.00 d	0.00 ± 0.00 b	28.24 ± 4.83 a,b	0.00 ± 0.00 b	26.68 ± 4.71 a
UAE	Met20	0.00 ± 0.00 b	26.74 ±10.80 a,b	9.68 ± 6.77 a,b,c	6.46 ± 5.03 a,b	0.00 ± 0.00 b	13.46 ± 10.25 b,c	16.62 ± 15.89 a	29.17 ± 2.31 a
UAE	Met50	0.00 ± 0.00 b	28.39 ± 4.32 a	27.26 ± 4.29 a	5.57 ± 1.90 a,b,c	7.07 ± 5.12 a	7.24 ± 1.35 c	0.00 ± 0.00 b	0.00 ± 0.00 b
UAE	Met80	0.00 ± 0.00 b	23.51 ± 5.11 a,b	24.77 ± 2.25 a,b	0.00 ± 0.00 d	0.00 ± 0.00 b	9.10 ± 2.17 c	4.22 ± 2.90 a	0.00 ± 0.00 b
p		**	***	**	**	**	***	**	**

Values of mean ± standard deviation are reported. Statistical comparisons were performed using ANOVA (for ellagic acid, hyperoside, and rutin) or Kruskal–Wallis test (for the

other compounds, $p < 0.05$ in Shapiro–Wilk's test). Letters indicate statistical differences between the different extractions for each extracted compound. Values with the same letter are not statistically different at $p < 0.05$, according to Tukey's or Dunn's post-hoc test. ** $p < 0.01$; *** $p < 0.001$; ns = not significant.

Quercitrin was only achieved by means of maceration with all methanol levels and UAE with 50% methanol. Maceration with 50% methanol gave 9.27 ± 3.47 mg 100 g^{-1} DW, without significant differences with the other extractions.

The yield of rutin varied from 0.32 ± 0.31 mg 100 g^{-1} DW as regards maceration with 20% methanol to 37.61 ± 2.22 mg 100 g^{-1} DW for maceration with 80% methanol, which was not significantly different to UAE with water.

The value of epicatechin resulted 16.62 ± 15.89 for UAE with 20% methanol, without significant differences to UAE with 80% methanol. It was not attained by all other extractions.

Regarding vitamin C, 33.72 ± 0.89 mg 100 g^{-1} DW was gain through maceration with 20% methanol without significant differences to maceration with water and UAE with both water and 20% methanol. No extraction was achieved by all other extractions.

The phytochemical profile of the extracts depended on the extractions (Table 2). For example, all the molecules except epicatechin were obtained using maceration with 20% methanol, while UAE with 20% methanol extracted all the chemicals except ferulic acid and quercitrin. However, no specific method–solvent combination performed a significant higher yield of these compounds altogether (Figure 1).

4.6. Discussion

Conventional maceration and UAE are the most common extraction methods employed to analyze the antioxidant molecules of saffron tepals (Table 4). Maceration requires a high consumption of organic solvents and long extraction times [32]. UAE is a modern and green method based on ultrasonic waves that,

by increasing the permeability of plant cells through acoustic cavitation of the wall, improve the penetration of the solvent and the release of phytocompounds.

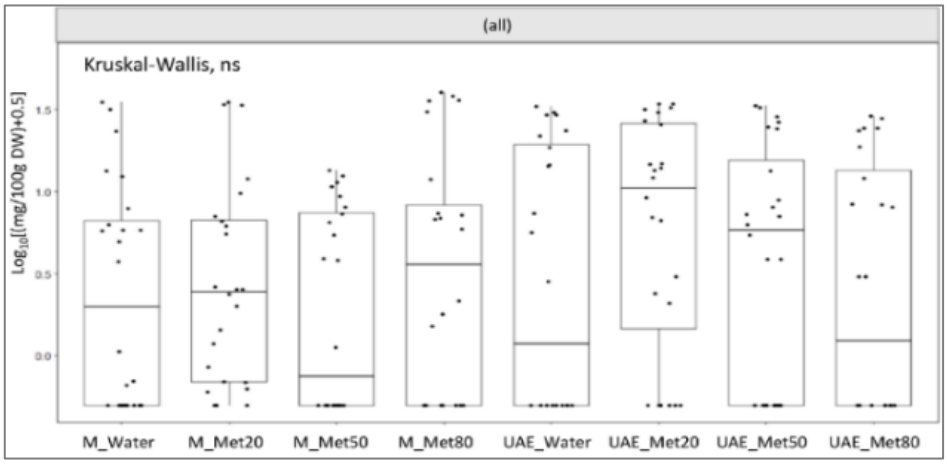


Figure 1. Effects of the extraction methods maceration (M) and Ultrasound Assisted Extraction (UAE) with the solvents water (M Water; UAE Water) and three concentrations of methanol in water (20%, Met20; 50%, Met50; 80% Met80; v:v) on the yield of all the compounds extracted and analyzed with HPLC-DAD. The extraction yield (mg 100 g^{-1} of dried weight, DW) is expressed as log transformation. Statistical comparisons were performed using the Kruskal–Wallis test; ns = not significant.

UAE can allow the use of non-toxic solvents, to work at room temperature, shorter extraction times compared to maceration [31,34–36], which needs the addition of heat to reduce the times [32], and a lower energy cost [32].

All the extracts of dried saffron tepals presented phenolic compounds (Table 1). As showed in Table 4, other authors reported their presence in dried and fresh tepals.

The total phenolic content (TPC) was not affected by the extractions (Table 1), meaning that UAE had similar performances to maceration in half the time. This result was consistent with the absence of a better extraction to obtain all the phytochemicals analyzed with the HPLC (Figure 1). Using extraction conditions similar to this study Caser et al. [15] found that for fresh saffron tepals 80% methanol gave higher TPC. Thus, dehydrated tepals may allow the use of more

ecological solvents for the extraction of phenolic compounds. UAE with water could be selected to obtain non-toxic phytoextracts to be added to foods [13].

Table 4. Some representative studies of extraction methods and solvents to extract bioactive compounds from saffron tepals in different states.

Tepals State	Extraction Methods	Solvents	Bioactive Compounds	References
Fresh	Maceration, UAE	Water and methanol at 20%, 50% and 80%	Phenolic content (ellagic acid; hyperoside; rutin; epicatechin); Anthocyanin content	[15]
Dried	Soxhlet extraction	Hexane; dichloromethane; ethanol	Phenolic and Flavonoid content	[37]
Dried	Maceration	Ethanol 25%, 50%, 75%	Anthocyanin content	[38]
Dried	Maceration	Ethyl acetate; methanol	Phenolic and Flavonoid content	[39]
Dried	Maceration	Ethanol 70%	Phenolic content (kaempferol 3-O-sophoroside-7-O-glycoside, quercetin 3,4-di-O-glycoside, kaempferol di-glycoside, kaempferol 3-O-glycoside)	[40]
Dried	Maceration	Methanol	Phenolic and Flavonoid content	[41]
Dried	Maceration	Acidified (HCl) ethanol; sulfur water solution	Phenolic and Anthocyanin content (cyanidin 3,5-diglucosides; pelargonidin 3- and 5-glucosides; delphinidin di-glucosides; pelargonidin 3-glucosides; petunidin)	[42]
Dried	Maceration, Enzyme-assisted extraction	Acidified (HCl) ethanol; Enzymatic water (Pectinex)	Antocyanin content (cyanidin 3,5-diglycosides; pelargonidin 3- and 3,5-glycosides; delphinidin 3-glycosides; petunidin)	[43]
Freeze-dried	Maceration, UAE	Acidified (HCl) methanol; Acidified (HCl) ethanol 70%; Methanol 98% in formic acid	Phenolic, Flavonoid, Anthocyanin, and Crocetin esters content	[44]
Dried	Maceration, UAE	Acidified (HCl) deuterated methanol; Trifluoroacetic acid in acetonitrile 50%	Flavonoids (kaempferol di-hexoside; kaempferol 3-O-glucoside; kaempferol 3,4′-di-O-glucoside; kaempferol 3-O-β-sophoroside; kaempferol tri-hexoside; kaempferol di-hexosides; quercetin 3,4′-di-O-glucoside; isorhamnetin 3,4′-di-O-glucoside); Anthocyanins (delphinidin 3,5-di-O-β-glucoside; delphinidin 3-O-glucoside; petunidin 3,5-di-O-β-glucoside; petunidin 3-O-glucoside)	[45]
Dried	UAE	Ethanol 50%	Flavonoids (quercetin 3-O-sophoroside; kaempferol 3-O-sophoroside; kaempferol 3-O-glucoside)	[46]
Freeze-dried	Maceration, UAE	Methanol 50%; n-hexane; Methanol/KOH 20%	Flavonoid content (kaempferol 3-O-sophoroside-7-O-glucoside; kaempferol 3,7-O-diglucoside; quercetin 3,7-O-diglucoside; isorhamnetin 3,7-O-	[32]

The technique and the solvent used can influence the extraction performance, but other factors can also affect the concentration of phytocompounds for saffron, such as provenance [47], conditions of growth [2,3], and storage [48]. Compared to the average value of TPC (1127.94 ± 32.34 mg GAE 100 g^{-1} DW), Lahmass et al. [39] attained higher TPC from saffron dried tepals using maceration with both pure methanol (~9400 mg GAE 100 g^{-1} DW) and ethyl acetate (~4200 mg GAE 100 g^{-1} DW), while a lower TPC (~342 mg GAE 100 g^{-1} DW) was found by Amir et al. [49] using UAE with pure methanol. The values of TPC were lower compared to those found in saffron spice (1819.5 mg GAE 100 g^{-1} DW) by Caser et al. [2], but higher than those obtained from some traditional Chinese medicinal plants [50], such as *Lycopus lucidus* (~930 mg GAE 100 g^{-1} DW for methanolic extract of aerial parts), *Smilax glabra* (~740 mg GAE 100 g^{-1} DW for methanolic extract of root), *Plantago asiatica* (~850 mg GAE 100 g^{-1} DW for aqueous extract of seeds), *Lobelia chinensis* (~600 mg GAE 100 g^{-1} DW for aqueous extract of whole plant), *Lithospermum erythrorhizon* (~920 mg GAE 100 g^{-1} DW for root extract), *Dianthus superbus* (~690 mg GAE 100 g^{-1} DW for aqueous extract of aerial parts), *Curcuma longa* (~800 mg GAE 100 g^{-1} DW for rhizome methanolic extract), and *Zingiber officinale* (~740 mg GAE 100 g^{-1} DW for rhizome methanolic extract), as well as from some common vegetables and fruits [50], such as spinach (*Spinacia oleracea*, ~900 mg GAE 100 g^{-1} DW for methanolic extract) and lettuce (*Lactuca sativa*, ~780 mg GAE 100 g^{-1} DW for methanolic extract).

The total anthocyanins content (TAC) had an average value of 282.90 ± 71.82 mg G3G 100 g^{-1} DW (Table 1). Even in the case of anthocyanins, UAE achieved a yield similar to maceration in half the extraction time. A higher result was seen for UAE with water (413.30 ± 137.16 mg G3G 100 g^{-1} DW), showing a percentage variation from the average yield of +46%. This is in contrast with the results obtained from fresh saffron tepals [15], for which 80% methanol performed better meaning that even in the case of anthocyanins dehydration may allow the use of greener solvents. The values of TAC were lower than those obtained from saffron tepals by Lotfi et al. [43], which get 507.5 mg G3G 100

g^{-1} DW using maceration with acidified ethanol. TAC was also lower than that found in saffron spice (1867.3 mg G3G 100 g^{-1} DW) by Caser et al. [2], but greater compared to the contents of some highly pigmented vegetables [51], such as purple cauliflower (*Brassica oleracea* var. botrytis, 201 mg G3G 100g^{-1} DW) and red cabbage (*Brassica oleracea* var. capitata f. rubra, 199 mg G3G 100 g^{-1} DW), achieved through maceration with 0.1% HCl (*v*/*v*) in 80% methanol. Grape (*Vitis vinifera*) is one of the most common sources of these molecules [52] having a TAC ranging from 75.64 to 414.95 mg G3G 100 g^{-1} DW depending on the cultivars [53], which is comparable in its maximum values to that of our study. Since plant waste are usually used for the commercial preparation of these pigments [54], saffron tepals in both dried and fresh [15] forms could be considered a good candidate for their extraction.

All assays evaluated, namely, FRAP, ABTS, and DPPH, revealed the presence of antioxidant activity in the extracts with average values of 520.39 ± 34.48 mmol Fe^{2+} Kg^{-1} DW for FRAP, 14.07 ± 0.73 μmol TE g^{-1} DW for ABTS, and 21.32 ± 3.45 μmol TE g^{-1} DW for DPPH (Table 1). Maceration with water obtained higher antioxidant activity measured with the FRAP method, which was not significantly different to UAE with water. This was in tune with that found by Caser et al. [2] for fresh saffron tepals. Interestingly, the antioxidant activity was higher compared to that found in saffron spice by Caser et al. [2] (338.2 mmol Fe^{2+} Kg^{-1} DW for FRAP and 4.6 μmol TE g^{-1} DW for ABTS) and Caser et al. [15] (4.64 ± 0.50 μmol TE g^{-1} DW for ABTS). The ABTS values were greater than those achieved from some traditional Chinese medicinal plants [50], such as *Chrysanthemum indicum* (3.03 μmol TE g^{-1} DW for methanolic extract of inflorescence), *Artemisia annua* (6.29 μmol TE g^{-1} DW for aqueous extract of aerial parts), *Campsis radicans* (4.462 μmol TE g^{-1} DW for methanolic extract of flower), and also compared to extracts obtained from some common vegetables and fruits [50], such as wild cabbage (*Brassica oleracea*, 1.01 μmol TE g^{-1} DW) and tomato (*Solanum lycopersicum*, 1.49 μmol TE g^{-1} DW). The DPPH values were similar to those found in mengkudu (*Morinda citrifolia*, up to ~23 μmol TE g^{-1} DW) by Thoo et al. [55].

The correlations among TPC, TAC, and the antioxidant activity were assessed (Table 2). Significant correlations were only found in the case of UAE. Even though the FRAP, ABTS, and DPPH assays have been extensively applied, they do have some limitations which can cause an imprecise estimation [56]. Since the antioxidant activity of plant extracts mainly depends on the content of phenolic compounds, it was expected a strong positive correlation among TPC and the assays [50,56–58]. However, TPC was either not correlated (FRAP) or negatively correlated (−0.69 ABTS and −0.45 DPPH) with the antioxidant capacity. No significant correlations among TPC and antioxidant activity was already found in extracts [59]. The negative correlation, already reported by other authors [59,60], suggests they might have opposite behavior when subjected to different solvents. The antioxidant capacity of samples may be influenced by extraction technique, solvent, pH, and metal ions [60]. Furthermore, synergistic and antagonistic interactions among the antioxidants in the extracts may interfere with the correlation, or there can be some non-phenolic molecules which can react with the Folin–Ciocalteu reagent without being free radical scavengers [59].

TAC was positively correlated with FRAP (0.86) (Table 2), indicating that anthocyanins contributed more to antioxidant capacity than TPC.

Positive correlations among the antioxidant assays were assumed [56,58], since they are all based on the same principle of electron transfer [56]. However, other authors reported the absence of significant correlation [55,59]. This might be explained by the fact that plants extracts can contain antioxidants which may react in different ways [59].

The heterogeneity of the yields achieved for each molecule (Table 3) would explain the absence of a single better extraction for these compounds altogether (Figure 1). Overall, except for ferulic acid and quercitrin, UAE get results higher (for ellagic acid and epicatechin) or comparable (for vitamin C) to maceration using greener solvents, i.e., water (for hyperoside and rutin) or lower methanol concentrations (20% methanol for isoquercitrin) in half the extraction time (Table 3). A unique ideal extraction does not exist, but the choice should fall on that most environmentally and economically convenient.

The bioactive compounds here identified and quantified (Table 3) deepen the knowledge on the molecular composition of dried saffron tepals. The extracts showed the same molecules (ellagic acid, hyperoside, rutin, and epicatechin) as those found in fresh tepals by Caser et al. [15] using an analysis protocol similar to this study. In addition, in the dried tepals were also detected ferulic acid, isoquercitrin, quercitrin, and vitamin C. In Caser et al. [15] the absence in fresh tepals of the chemicals here obtained might be attributed to the drying procedure [61,62], which can facilitate extractions by disrupting cell walls and causing the formation of cavities and intercellular spaces [63]. Vitamin C and rutin (yield up to 37.61 ± 2.22 mg 100 g^{-1} DW) showed the highest yields among all the compounds analyzed with HPLC. Vitamin C (up to 33.72 ± 0.89 mg 100 g^{-1} DW) was lower than in the spice (73.2 mg 100 g^{-1} ; Caser et al. [15]), but higher or comparable than in some freeze-dried vegetables and fruits [64], such as cabbage (*Brassica oleracea* var. capitata, up to 28.3 mg 100 g^{-1}), tomato (*Solanum lycopersicum*, up to 10.1 mg 100 g^{-1}), apple Fuji (*Malus domestica* 'Fuji', up to 3.5 mg 100 g^{-1}), mango (*Mangifera indica*, up to 18.6 mg 100 g^{-1}), pineapple (*Ananas comosus*, 5.0 mg 100 g^{-1}), and pomelo (*Citrus maxima*, up to 31.7 mg 100 g^{-1}).

4.7. Conclusions

Processing food wastes and by-products to generate high-value products for industrial application has been attractive great economic and scientific interest [66,67]. The sustainable processing of biomass, including that resulting from agriculture, into valuable and safe biobased products falls within the definition of biorefining [68]. The use of renewable resources can help farmers to expand their sources of income and create new job opportunities while reducing bio-waste and the environmental footprint in a circular bioeconomy perspective [69].

The economic use of a by-product is related to its intrinsic, nutritional, or utility value. The dried saffron tepals are largely produced after stigma are separated from the flowers and are a natural source of antioxidant compounds. This study demonstrated that the phytochemical composition and the antioxidant capacity of

extracts of dried saffron tepals is influenced by the extraction technique. In most cases, UAE with water or low percentage of methanol showed results of phenolic compounds and vitamin C similar to maceration, allowing an improvement in extractions by halving the time. Compared to the spice, the dried saffron tepals showed a lower content of total phenolics and anthocyanins, but a higher antioxidant activity.

To produce waste-derived products, bio-waste shall either be separated and recycled at source or collected separately before undergoing treatments, which gives storage an important role (Directive 2008/122/EC). The cost of biomass and its storage are some essential factors for the economic viability of biorefineries [67]. The drying technique has always been widely used in the herbs sector to stabilize and preserve plant material, and in particular flowers, which have high perishability due to the elevated moisture content [31]. The drying treatment can preserve tepals until their possible use in food or pharmacological-cosmetic industries, for example in the preparation of soaps and cosmetic products where they can act as antioxidants or colorants in place of synthetic excipients, meeting the demand of the cosmetic sector to use products of natural origin [70].

This study allowed to compare the results with those previously attained for fresh tepals by Caser et al. [15], which performed a similar extraction protocol. This work has highlighted that, after drying, saffron tepals retain bioactive phenolics. The dehydration procedure may improve extractions as safer solvents gave higher yield of phenolic compounds. Furthermore, the HPLC-DAD analysis detected some phenolic compounds (i.e., ferulic acid, isoquercitrin, and quercitrin) not previously found in fresh tepals. Vitamin C, already discovered in the spice, was interestingly detected also in dried tepals.

Taken together, these results assess that floral bio-residues of saffron can be sustainably processed to obtain high yields of valuable phytochemicals with potential applications in the pharmaceutical, cosmetic, and food industries, meeting the requirement for a sustainable biorefining and opening new possibilities for this by-product to become an important income source.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Sustainable Processing of Floral Bio-Residues of Saffron (*Crocus sativus* L.) for Valuable Biorefinery Products.

Author Contributions: Conceptualization, V.S.; methodology, M.C. and S.D.; formal analysis, M.C. and S.D.; investigation, S.S.; data curation, S.S.; writing—original draft preparation, S.S.; writing—review and editing, M.C., S.D., and V.S.; supervision, V.S.; funding acquisition, V.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the project titled "SaffronALP—Lo zafferano di montagna: tecniche sostenibili per una produzione di qualità"—Fondazione Cassa di Risparmio di Torino (RF = 2017.1966) and by the program Interreg V-A Francia Italia Alcotra "Attività innovative per lo sviluppo della filiera transfrontaliera del fiore edule–Antea" n. 1139.

Data Availability Statement: : *Crocus sativus* L. plants were kindly provided by the company "Lo Zafferano del Monviso" located in Martiniana Po, CN (Italy—44°23′ N 7°33′ E).

Acknowledgments: The authors acknowledge the company "Lo Zafferano del Monviso" for providing dried saffron tepals, Michele Lonati for statistical assistance, Marie-Jeanne Welter for helping in spectrophotometric analyses, and Dario Donno for chromatographic analyses.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

4.8. References

1. Khan, M.; Hanif, M.A.; Ayub, M.A.; Jilani, M.I.; Chatha, S.A.S. Saffron. Med. Plants South Asia 2020, 587–600, doi:10.1016/b978-0-08-102659-5.00043-4.
2. Caser, M.; Demasi, S.; Victorino, Íris, M.M.; Donno, D.; Faccio, A.; Lumini, E.; Bianciotto, V.; Scariot, V. Arbuscular Mycorrhizal Fungi Modulate the Crop Performance and Metabolic Profile of Saffron in Soilless Cultivation. Agronomy 2019, 9, 232, doi:10.3390/agronomy9050232.

3. Caser, M.; Victorino, Íris, M.M.; Demasi, S.; Berruti, A.; Donno, D.; Lumini, E.; Bianciotto, V.; Scariot, V. Saffron Cul-tivation in Marginal Alpine Environments: How AMF Inoculation Modulates Yield and Bioactive Compounds. Agronomy 2018, 9, 12, doi:10.3390/agronomy9010012.
4. Melnyk, J.P.; Wang, S.; Marcone, M.F. Chemical and biological properties of the world's most expensive spice: Saffron. Food Res. Int. 2010, 43, 1981–1989, doi:10.1016/j.foodres.2010.07.033.
5. Bagur, M.J.; Salinas, G.L.A.; Jiménez-Monreal, A.M.; Chaouqi, S.; Llorens, S.; Martínez-Tomé, M.; Alonso, G.L. Saffron: An Old Medicinal Plant and a Potential Novel Functional Food. Molecules 2017, 23, 30, doi:10.3390/molecules23010030.
6. Gresta, F.; Lombardo, G.M.; Siracusa, L.; Ruberto, G. Saffron, an alternative crop for sustainable agricultural systems. A review. Agron. Sustain. Dev. 2008, 28, 95–112, doi:10.1051/agro:2007030.
7. Halevy, A.H. recent advances in control of flowering and growth habit of geophytes. Acta Hortic. 1990, 35–42, doi:10.17660/actahortic.1990.266.3.
8. Kumar, R.; Singh, V.; Devi, K.; Sharma, M.; Singh, M.; Ahuja, P. State of Art of Saffron (Crocus sativusL.) Agronomy: A Comprehensive Review. Food Rev. Int. 2008, 25, 44–85, doi:10.1080/87559120802458503.
9. Molina, R.; Valero, M.; Navarro, Y.; Guardiola, J.; García-Luis, A. Temperature effects on flower formation in saffron (Crocus sativus L.). Sci. Hortic. 2005, 103, 361–379, doi:10.1016/j.scienta.2004.06.005.
10. Peter, K.V. Handbook of Herbs and Spices Vol. 1: Series in Food Science; Woodhead Publishing, 1518 Walnut Street, Suite 1100, Philadelphia, PA 19102-3406, USA, 2012; ISBN 978-0-85709-567-1.
11. Mottaghipisheh, J.; Sourestani, M.M.; Kiss, T.; Horváth, A.; Tóth, B.; Ayanmanesh, M.; Khamushi, A.; Csupor, D. Comprehensive chemotaxonomic analysis of saffron crocus tepal and stamen samples, as raw materials with potential antidepressant activity. J. Pharm. Biomed. Anal. 2020, 184, 113183, doi:10.1016/j.jpba.2020.113183.
12. Ashktorab, H.; Soleimani, A.; Singh, G.; Amr, A.; Tabtabaei, S.; Latella, G.; Stein, U.; Akhondzadeh, S.; Solanki, N.; Gondré-Lewis, M.C.; et al. Saffron: The Golden Spice with Therapeutic Properties on Digestive Diseases. Nutrients 2019, 11, 943, doi:10.3390/nu11050943.
13. Moratalla-López, N.; Bagur, M.J.; Lorenzo, C.; Salinas, M.E.M.-N.R.; Alonso, G.L.

Bioactivity and Bioavailability of the Major Metabolites of Crocus sativus L. Flower. Molecules 2019, 24, 2827, doi:10.3390/molecules24152827.

14. Hosseini, A.; Razavi, B.M.; Hosseinzadeh, H. Saffron (Crocus sativus) petal as a new pharmacological target: A review. Iran. J. Basic Med. Sci 2018, 21, 1091–1099.
15. Caser, M.; Demasi, S.; Stelluti, S.; Donno, D.; Scariot, V. Crocus sativus L. Cultivation in Alpine Environments: Stigmas and Tepals as Source of Bioactive Compounds. Agronomy 2020, 10, 1473, doi:10.3390/agronomy10101473.
16. Panche, A.N.; Diwan, A.D.; Chandra, S.R. Flavonoids: An overview. J. Nutr. Sci. 2016, 5, e47, doi:10.1017/jns.2016.41.
17. Kumar, N.; Goel, N. Phenolic acids: Natural versatile molecules with promising therapeutic applications. Biotechnol. Rep. 2019, 24, e00370, doi:10.1016/j.btre.2019.e00370.
18. Caser, M.; Demasi, S.; Caldera, F.; Dhakar, N.K.; Trotta, F.; Scariot, V. Activity of Ailanthus altissima (Mill.) Swingle Extract as a Potential Bioherbicide for Sustainable Weed Management in Horticulture. Agronomy 2020, 10, 965, doi:10.3390/agronomy10070965.
19. Demasi, S.; Caser, M.; Vanara, F.; Fogliatto, S.; Vidotto, F.; Negre, M.; Trotta, F.; Scariot, V. Ailanthone from Ailanthus altissima (Mill.) Swingle as potential natural herbicide. Sci. Hortic. 2019, 257, 108702,, doi:10.1016/j.scienta.2019.108702.
20. Caser, M.; Chitarra, W.; D'Angiolillo, F.; Perrone, I.; Demasi, S.; Lovisolo, C.; Pistelli, L.; Pistelli, L.; Scariot, V. Drought stress adaptation modulates plant secondary metabolite production in Salvia dolomitica Codd. Ind. Crop. Prod. 2019, 129, 85–96, doi:10.1016/j.indcrop.2018.11.068.
21. Aryee, A.N.; Agyei, D.; Akanbi, T.O. Food for Oxidative Stress Relief: Polyphenols. In Encyclopedia of Food Chemistry; Elsevier, 2018; pp. 392–398.
22. Pires, T.C.P.; Barros, L.; Santos-Buelga, C.; Ferreira, I.C. Edible flowers: Emerging components in the diet. Trends Food Sci. Technol. 2019, 93, 244–258, doi:10.1016/j.tifs.2019.09.020.
23. Brodowska, K.M. European Journal of Biological Research Natural flavonoids: Classification, potential role, and application of flavonoid analogues. Eur. J. Biol. Res. 2017, 7, 108–123, doi:http://dx.doi.org/10.5281/zenodo.545778.
24. Santos-Buelga, C.; González-Paramás, A.M. Anthocyanins. In Encyclopedia of Food Chemistry; Elsevier, 2019; pp. 10–21.
25. Granger, M.; Eck, P. Dietary Vitamin C in Human Health. Adv. Food Nutr. Res. 2018,

83, 281–310, doi:10.1016/bs.afnr.2017.11.006.

26. Akram, N.A.; Shafiq, F.; Ashraf, M. Ascorbic acid-a potential oxidant scavenger and its role in plant development and abiotic stress tolerance. Front. Plant. Sci. 2017, 8, 613, doi:10.3389/fpls.2017.00613.
27. Garavand, F.; Rahaee, S.; Vahedikia, N.; Jafari, S.M. Different techniques for extraction and micro/nanoencapsulation of saffron bioactive ingredients. Trends Food Sci. Technol. 2019, 89, 26–44, doi:10.1016/j.tifs.2019.05.005.
28. Donno, D.; Mellano, M.G.; Prgomet, Ž.; Cerutti, A.K.; Beccaro, G.L. Phytochemical characterization and antioxidant activity evaluation of mediterranean medlar fruit (Crataegus azarolus L.): Preliminary study of underutilized genetic resources as a potential source of health-promoting compound for food supplements. J. Food Nutr. Res. 2017, 56, 18–31.
29. Khoddami, A.; Wilkes, M.A.; Roberts, T.H. Techniques for Analysis of Plant Phenolic Compounds. Molecules 2013, 18, 2328–2375, doi:10.3390/molecules18022328.
30. Aliaño-González, M.J.; Espada-Bellido, E.; Ferreiro-González, M.; Carrera, C.; Palma, M.; Ayuso, J.; Álvarez, J. Ángel; Barbero, G.F. Extraction of Anthocyanins and Total Phenolic Compounds from Açai (Euterpe oleracea Mart.) Using an Experimental Design Methodology. Part 2: Ultrasound-Assisted Extraction. Agronomy 2020, 10, 326, doi:10.3390/agronomy10030326.
31. Zhao, L.; Fan, H.; Zhang, M.; Chitrakar, B.; Bhandari, B.; Wang, B. Edible flowers: Review of flower processing and extraction of bioactive compounds by novel technologies. Food Res. Int. 2019, 126, 108660, doi:10.1016/j.foodres.2019.108660.
32. Medina-Torres, N.; Ayora-Talavera, T.; Espinosa-Andrews, H.; Sánchez-Contreras, A.; Pacheco, N. Ultrasound Assisted Extraction for the Recovery of Phenolic Compounds from Vegetable Sources. Agronomy 2017, 7, 47, doi:10.3390/agronomy7030047.
33. Goupy, P.; Vian, M.A.; Chemat, F.; Caris-Veyrat, C. Identification and quantification of flavonols, anthocyanins and lutein diesters in tepals of Crocus sativus by ultra performance liquid chromatography coupled to diode array and ion trap mass spectrometry detections. Ind. Crop. Prod. 2013, 44, 496–510, doi:10.1016/j.indcrop.2012.10.004.
34. A Review on the Extraction Methods Use in Medicinal Plants, Principle, Strength and Limitation. Med. Aromat. Plants 2015, 4, 196, doi:10.4172/2167-0412.1000196.

35. Da Porto, C.; Natolino, A. Extraction kinetic modelling of total polyphenols and total anthocyanins from saffron floral bio-residues: Comparison of extraction methods. Food Chem. 2018, 258, 137–143, doi:10.1016/j.foodchem.2018.03.059.
36. Ameer, K.; Shahbaz, H.M.; Kwon, J.-H. Green Extraction Methods for Polyphenols from Plant Matrices and Their By-products: A Review. Compr. Rev. Food Sci. Food Saf. 2017, 16, 295–315, doi:10.1111/1541-4337.12253.
37. Wali, A.F.; Alchamat, H.A.A.; Hariri, H.K.; Hariri, B.K.; Menezes, G.A.; Zehra, U.; Rehman, M.U.; Ahmad, P. Antioxidant, Antimicrobial, Antidiabetic and Cytotoxic Activity of Crocus sativus L. Petals. Appl. Sci. 2020, 10, 1519, doi:10.3390/app10041519.
38. Khazaei, K.M.; Jafari, S.M.; Ghorbani, M.; Kakhki, A.H.; Sarfarazi, M. Optimization of Anthocyanin Extraction from Saffron Petals with Response Surface Methodology. Food Anal. Methods 2015, 9, 1993–2001, doi:10.1007/s12161-015-0375-4.
39. Lahmass, I.; Lamkami, T.; Delporte, C.; Sikdar, S.; Van Antwerpen, P.; Saalaoui, E.; Megalizzi, V. The waste of saffron crop, a cheap source of bioactive compounds. J. Funct. Foods 2017, 35, 341–351, doi:10.1016/j.jff.2017.05.057.
40. Sánchez-Vioque, R.; Santana-Méridas, O.; Polissiou, M.; Vioque, J.; Astraka, K.; Alaiz, M.; Herraiz-Peñalver, D.; Tarantilis, P.A.; Girón-Calle, J. Polyphenol composition and in vitro antiproliferative effect of corm, tepal and leaf from Crocus sativus L. on human colon adenocarcinoma cells (Caco-2). J. Funct. Foods 2016, 24, 18–25, doi:10.1016/j.jff.2016.03.032.
41. Jadouali, S.M.; Atifi, H.; Mamouni, R.; Majourhat, K.; Bouzoubaâ, Z.; Laknifli, A.; Faouzi, A. Chemical characterization and antioxidant compounds of flower parts of Moroccan crocus sativus L. J. Saudi Soc. Agric. Sci. 2019, 18, 476–480, doi:10.1016/j.jssas.2018.03.007.
42. Lotfi, L.; Kalbasi-Ashtari, A.; Hamedi, M.; Ghorbani, F. Effects of sulfur water extraction on anthocyanins properties of tepals in flower of saffron (Crocus sativus L). J. Food Sci. Technol. 2013, 52, 813–821, doi:10.1007/s13197-013-1058-z.
43. Lotfi, L.; Kalbasi-Ashtari, A.; Hamedi, M.; Ghorbani, F. Effects of enzymatic extraction on anthocyanins yield of saffron tepals (Crocos sativus) along with its color properties and structural stability. J. Food Drug Anal. 2015, 23, 210–218, doi:10.1016/j.jfda.2014.10.011.
44. Serrano-Díaz, J.; Sanchez, A.M.; Maggi, L.; Martínez-Tomé, M.; García-Diz, L.; Murcia, M.A.; Alonso, G.L. Increasing the Applications ofCrocus sativusFlowers as

Natural Antioxidants. J. Food Sci. 2012, 77, C1162–C1168, doi:10.1111/j.1750-3841.2012.02926.x.

45. Cusano, E.; Consonni, R.; Petrakis, E.A.; Astraka, K.; Cagliani, L.R.; Polissiou, M.G. Integrated analytical methodology to investigate bioactive compounds in Crocus sativus L. flowers. Phytochem. Anal. 2018, 29, 476–486, doi:10.1002/pca.2753.
46. Jafari, S.M.; Khazaei, K.M.; Assadpour, E. Production of a natural color through microwave-assisted extraction of saffron tepal's anthocyanins. Food Sci. Nutr. 2019, 7, 1438–1445, doi:10.1002/fsn3.978.
47. Ghanbari, J.; Khajoei-Nejad, G.; Van Ruth, S.M. Effect of saffron (Crocus sativus L.) corm provenance on its agro-morphological traits and bioactive compounds. Sci. Hortic. 2019, 256, 108605, doi:10.1016/j.scienta.2019.108605.
48. Moratalla-López, N.; Lorenzo, C.; Chaouqi, S.; Sánchez, A.M.; Alonso, G.L. Kinetics of polyphenol content of dry flowers and floral bio-residues of saffron at different temperatures and relative humidity conditions. Food Chem. 2019, 290, 87–94, doi:10.1016/j.foodchem.2019.03.129.
49. Goli, S.A.H.; Mokhtari, F.; Rahimmalek, M. Phenolic Compounds and Antioxidant Activity from Saffron (Crocus sativus L.) Petal. J. Agric. Sci. 2012, 4, 4, doi:10.5539/jas.v4n10p175.
50. Cai, Y.; Luo, Q.; Sun, M.; Corke, H. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci. 2004, 74, 2157–2184, doi:10.1016/j.lfs.2003.09.047.
51. Li, H.; Deng, Z.; Zhu, H.; Hu, C.; Liu, R.; Young, J.C.; Tsao, R. Highly pigmented vegetables: Anthocyanin compositions and their role in antioxidant activities. Food Res. Int. 2012, 46, 250–259, doi:10.1016/j.foodres.2011.12.014.
52. Solymosi, K.; Latruffe, N.; Morantmanceau, A.; Schoefs, B. Food colour additives of natural origin. In Colour Additives for Foods and Beverages; Elsevier, 2015; pp. 3–34.
53. Ribeiro, L.; Ribani, R.; Francisco, T.; Soares, A.; Pontarolo, R.; Haminiuk, C. Profile of bioactive compounds from grape pomace (Vitis vinifera and Vitis labrusca) by spectrophotometric, chromatographic and spectral analyses. J. Chromatogr. B 2015, 1007, 72–80, doi:10.1016/j.jchromb.2015.11.005.
54. Markakis, P. Anthocyanins as Food Additives. In Anthocyanins As Food Colors; Elsevier, 1982; pp. 245–253.
55. Thoo, Y.Y.; Ho, S.K.; Liang, J.Y.; Ho, C.W.; Tan, C.P. Effects of binary solvent

extraction system, extraction time and extraction temperature on phenolic antioxidants and antioxidant capacity from mengkudu (Morinda citrifolia). Food Chem. 2010, 120, 290–295, doi:10.1016/j.foodchem.2009.09.064.

56. Huang, H.; Sun, Y.; Lou, S.; Li, H.; Ye, X. In vitro digestion combined with cellular assay to determine the antioxidant activity in Chinese bayberry (Myrica rubra Sieb. et Zucc.) fruits: A comparison with traditional methods. Food Chem. 2014, 146, 363–370, doi:10.1016/j.foodchem.2013.09.071.
57. Donno, D.; Beccaro, G.; Mellano, M.; Cerutti, A.; Bounous, G. Goji berry fruit (Lycium spp.): Antioxidant compound fingerprint and bioactivity evaluation. J. Funct. Foods 2015, 18, 1070–1085, doi:10.1016/j.jff.2014.05.020.
58. Babbar, N.; Oberoi, H.S.; Uppal, D.S.; Patil, R.T. Total phenolic content and antioxidant capacity of extracts obtained from six important fruit residues. Food Res. Int. 2011, 44, 391–396, doi:10.1016/j.foodres.2010.10.001.
59. Terpinc, P.; Čeh, B.; Ulrih, N.P.; Abramovič, H. Studies of the correlation between antioxidant properties and the total phenolic content of different oil cake extracts. Ind. Crop. Prod. 2012, 39, 210–217, doi:10.1016/j.indcrop.2012.02.023.
60. Kalinowska, M.; Gryko, K.; Wróblewska, A.M.; Jabłońska-Trypuć, A.; Karpowicz, D. Phenolic content, chemical com-position and anti-/pro-oxidant activity of Gold Milenium and Papierowka apple peel extracts. Sci. Rep. 2020, 10, 1–15, doi:10.1038/s41598-020-71351-w.
61. Bilek, S.E. The effects of time, temperature, solvent: Solid ratio and solvent composition on extraction of total phenolic compound from dried olive (Olea europaea L.) leaves. GIDA, 2010, 35, 411–416.
62. Safaiee, P.; Taghipour, A.; Vahdatkhoram, F.; Movagharnejad, K. Extraction of phenolic compounds from Mentha aquatica: The effects of sonication time, temperature and drying method. Chem. Pap. 2019, 73, 3067–3073, doi:10.1007/s11696-019-00843-5.
63. Liu, Z.; Esveld, E.; Vincken, J.-P.; Bruins, M.E. Pulsed Electric Field as an Alternative Pre-treatment for Drying to Enhance Polyphenol Extraction from Fresh Tea Leaves. Food Bioprocess. Technol. 2019, 12, 183–192, doi:10.1007/s11947-018-2199-x.
64. Srivastava, R.; Ahmed, H.; Dixit, R.; Dharamveer; Saraf, S. Crocus sativus L.: A comprehensive review. Pharmacogn. Rev. 2010, 4, 200–208, doi:10.4103/0973-7847.70919.

65. Donno, D.; Mellano, M.; Prgomet, Z.; Beccaro, G. Advances in Ribes x nidigrolaria Rud. Bauer & A. Bauer fruits as potential source of natural molecules: A preliminary study on physico-chemical traits of an underutilized berry. Sci. Hortic. 2018, 237, 20–27, doi:10.1016/j.scienta.2018.03.065.
66. Laufenberg, G.; Kunz, B.; Nystroem, M. Transformation of vegetable waste into value added products:. Bioresour. Technol. 2003, 87, 167–198, doi:10.1016/s0960-8524(02)00167-0.
67. Philippini, R.R.; Martiniano, S.E.; Ingle, A.P.; Marcelino, P.R.F.; Silva, G.M.; Barbosa, F.G.; Dos Santos, J.C.; Silva, S.S. Agroindustrial Byproducts for the Generation of Biobased Products: Alternatives for Sustainable Biorefineries. Front. Energy Res. 2020, 8, 1–23, doi:10.3389/fenrg.2020.00152.
68. Motola, V.; De Bari, I.; Pierro, N.; Giocoli, A. Bioeconomy and Biorefining Strategies in the EU Member States and Be-yond—Reference Year 2018; IEA Bioenergy, 2018; ISBN 978-1-910154-60-1.
69. European Commission. A Sustainable Bioeconomy for Europe: Strengthening the Connection between Economy, Society and the Environment; 2018; European Commission; ISBN 9789279941450.
70. Bruusgaard-Mouritsen, M.A.; Johansen, J.D.; Zachariae, C.; Kirkeby, C.S.; Garvey, L.H. Natural ingredients in cosmetic products—A suggestion for a screening series for skin allergy. Contact Dermat. 2020, 83, 251–270, doi:10.1111/cod.13550.

5. The natural colorants of the *Crocus sativus* L. flower


ISHS Acta Horticulturae 1361: XXXI International Horticultural Congress (IHC2022): International Symposium on Natural Colorants from Plants
The natural colorants of the *Crocus sativus* L. flower
Authors: S. Stelluti, M. Caser, S. Demasi, V. Scariot
Keywords: saffron, spice, tepals, by-products, apocarotenoids, crocins, anthocyanins
DOI: 10.17660/ActaHortic.2023.1361.13

5.1. Abstract

Natural colorants (e.g., carotenoids and anthocyanins extracted from plants) have sparked considerable interest in recent years due to consumer concern for the safety of artificial dyes and reinforced by their potential health benefits. A rich natural source of carotenoids is saffron, the expensive spice obtained from the dried stigmas of *Crocus sativus* L. Crocins, i.e. water-soluble crocetin esters, are the principal yellow-red pigments of the spice. The content of these apocarotenoids can be affected by horticultural practices, such as the cultivation in open field or a greenhouse and the use of microbial biostimulants, such as arbuscular mycorrhizal fungi (AMF). More than 100,000 flowers are harvested to produce 1 kg of spice and the violet tepals are the main floral bio-residue. As saffron tepals contain anthocyanin pigments, they can be considered as a valuable by-product. The extraction performance of anthocyanins from saffron tepals depends on processing methods (e.g., drying) and the selection of suitable solvents.

5.2. Keywords

saffron, spice, tepals, by-products, apocarotenoids, crocins, anthocyanins

5.3. Introduction

Colors improve the aesthetic value of products (e.g., foods, clothes, cosmetics, and medications) and have a substantial impact on people's psychology. As colors can positively influence consumers' purchasing decision, they play a significant role in marketing (Prajapati and Jadeja, 2022; Räisänen et al., 2020). Synthetic

dyes are largely used in the food industry for their higher stability, coloring potential, and lower cost compared to natural counterparts. However, they are potentially harmful to health and are known to cause environmental disadvantages (Manzoor et al., 2021; Räisänen et al., 2020). In recent years natural dyes have drawn considerable interest due to consumer concern for the health and environmental safety of artificial colors. Moreover, there is a growing attention in bioactive compounds (e.g., carotenoids and anthocyanins) of natural origin that may contribute in enhancing the health-promoting qualities of foods (Manzoor et al., 2021; Rodriguez-Amaya, 2016). Thus, natural dyes are of great interest to the food coloring industry (Brglez Mojzer et al., 2016). In this context, the colourful flower of saffron is a treasure of natural pigments.

Saffron (*Crocus sativus* L.), of the *Iridaceae* family, is a geophyte propagated through underground corms. In Mediterranean climates, it blooms in autumn producing one or several flowers per corm (Gresta et al., 2008). The flower has a perianth of six purple tepals, three stamens, and a style with three stigmas (Peter, 2012). Saffron is widely cultivated for the valuable spice (\$40–50 g^{-1}), obtained by dehydrating the red stigmas (Khan et al., 2020).

The spice has been traditionally used as seasoning and yellow-orange dye in food, medicines, and textiles (Bolhassani et al., 2014; Mzabri et al., 2019). Probably for its antioxidant and possible anti-aging effects, the spice has also been used in cosmetics (Cleopatra, 30–69 BC, used to bathe in a mixture of saffron and milk) (Bathaie et al., 2014).

The dyeing capacity is mainly due to water-soluble crocetin glycosides named crocins, derived from the oxidative cleavage of carotenoid zeaxanthin (García-Rodríguez et al., 2017; Jain et al., 2016; Tarantilis et al., 1994). Crocin I and crocin II are some of the main crocins of saffron (Carmona et al., 2006; García-Rodríguez et al., 2017; Tarantilis et al., 1994). Compared to other carotenoids, the use of saffron as an alternative dye is advantageous in the agro-food sector for the high-water solubility of crocins. Saffron has been used for a long time to color butter, pasta, cheeses, and oleomargarines. A number of well-known dishes are prepared with the spice, e.g., pulao rice in India, khoreshes in Iran, paella rice

in Spain, Milanese risotto in Italy (Bathaie et al., 2014; Cardone et al., 2020). Recently, saffron has gained a renewal interest for its use in cosmetics. Beside the well-known antioxidant properties, saffron presents anti-solar, anti-pigmentation and anti-aging activities, and could also be used as a pigment or in perfumes (Mzabri et al., 2019). It has been used as a substitute for turmeric and the synthetic yellow-dye tartrazine (Mzabri et al., 2019). However, its use is reduced due to its excessive cost (Mzabri et al., 2019). Since antiquity saffron aqueous extracts have been used as dyes for wool and cotton (Bathaie et al., 2014); it continues to dye the clothes of Buddhist monks, silk, wool, and Oriental carpets (Mzabri et al., 2019).

Both spice and tepals contain anthocyanins (Caser et al., 2020), water-soluble pigments responsible for the pink-orange, red, and blue color range of the flower (Santos-Buelga and González-Paramás, 2019). More than 100,000 flowers are picked to yield 1 kg of spice (Caser et al., 2020; Mottaghipisheh et al., 2020), thus tepals constitute an abundant by-product (Moratalla-López et al., 2019). The violet tepals contain pelargonidin, from the anthocyanidin family, which gives the purple color. This compound, after reduction and oxidation reactions, can be converted to kaempferol, a yellowish flavonol (Bathaie et al., 2014). Extracted anthocyanins of saffron tepals can have industrial applications as natural dyes (Jafari et al., 2019).

The content of natural pigments in saffron flower can be affected by horticultural practices, such as the cultivation in open field or controlled conditions (greenhouses) and the use of microbial biostimulants, such as arbuscular mycorrhizal fungi (AMF) (Caser et al., 2019a, 2019b, 2020). AMF (subphylum *Glomeromycotina*, Spatafora et al., 2016) are obligate biotrophs and root endosymbionts that form mutualistic symbiosis with most land plants. As AMF can induce changes in secondary metabolism of host plants (Kaur and Suseela, 2020), they could increase the concentration of saffron dyes.

The yield of saffron colorants can also be influenced by dehydration and extraction methods (Stelluti et al., 2021). Ultrasound assisted extraction (UAE) is an effective green technique that, compared to classical maceration, allows to

reduce the use of solvents and to obtain high yields in a shorter time (Zhao et al., 2019). The selection of a suitable solvent is important to improve the extraction performance. Water, organic solvents, and their combination are often used to extract carotenoids/apocarotenoids and anthocyanins (Caser et al., 2020; Stelluti et al., 2021). Recently, research has been conducted to select effective and safer alternatives to toxic organic solvents (Ameer et al., 2017; Medina-Torres et al., 2017; Stelluti et al., 2021).

In this study recent data (i.e., Caser et al., 2019a, 2019b, 2020; Stelluti et al., 2021) on the production of natural pigments by saffron were analysed to select the more effective horticultural techniques among open field and soilless cultivation in the greenhouse, with or without the use of AMF inoculants. Similarly, for tepals the best post-harvest practice (fresh *vs* air-dried tepals) and solvent/extraction methods were assessed.

5.4. Material and Methods

In this study, data deriving from previous studies on saffron (Caser et al., 2019a, 2019b, 2020; Stelluti et al., 2021) were compared. Particularly, saffron plants were grown in open fields or a greenhouse in North Western Italy with or without inoculants of AMF, as listed in Table 1.

5.4.1. Cultivation

Briefly, corms were sowed in open fields (Caser et al., 2019b, 2020) in Alpine sites characterized by semicontinental climate and sandy-loam soil. The inter-row planting distance was 7 cm, while the between-row distance was 25 cm. Manual irrigation was provided when needed. Before planting, 10 g of AMF inoculum was placed under the corms (Caser et al., 2019b).

In greenhouse, corms were sowed in pots (4 L, 14 cm diameter and 17 cm height; two corms per pot). The pots were filled with sterile quartz sand on a layer of sterilised expanded clay. Ten grams of AMF inoculum were inserted into each vase (Caser et al. 2019a). Irrigation water was added weekly while fertigation was performed every 2 weeks starting from the emergence of the spate.

5.4.2. AM Fungi

Saffron corms were treated with two inocula of AMF (MycAgro Lab, Breteniére, FR) (Caser et al., 2019a, 2019b): one composed of a single species (*Rhizophagus intraradices*, Ri) and one of a mixture of two species (*R. intraradices* and *Funneliformis mosseae*, Ri + Fm).

Table 1. Saffron plant materials: cultivation conditions, AMF treatments, and analysis performed in the cited studies.

Saffron Plant Materials	Cultivation Conditions	Sites and Years of Corms Sowing	Treatment with AMF	Analysis Performed	Bibliography
Spice	Greenhouse	Piedmont (Grugliasco, University of Torino, DISAFA - Italy), August 2017	Ri, Ri+Fm, AMF-	HPLC on aqueous extracts (Crocin I and II)	(Caser et al., 2019a)
	Open Field	Valle d'Aosta (Chambave, Saint Cristophe, and Morgex - Italy), August 2016	Ri, Ri+Fm, AMF-	HPLC on aqueous extracts (Crocin I and II)	(Caser et al., 2019b, 2020)
Fresh tepals	Open Field	Valle d'Aosta (Chambave, Saint Cristophe, and Morgex - Italy), August 2016	/	TAC with the pH differential method	(Caser et al., 2020)
Dried tepals	Open Field	Piedmont (Martiniana Po, CN - Italy), August 2018	/	TAC with the pH differential method	(Stelluti et al., 2021)

Both inocula consisted of AMF spores and inorganic substrate (calcined clay, vermiculite and zeolite). Treatments were compared to not inoculated controls (AMF-). Corms were not treated against fungal pathogens.

Air-dried saffron tepals came from plants cultivated in open field by the company "Lo Zafferano del Monviso" (Stelluti et al., 2021).

5.4.3. Extraction Methods

Aqueous extracts from spice were prepared with a powdered sample–solvent ratio of 1:100 g ml^{-1} using maceration (Caser et al., 2019a, 2019b, 2020).

For fresh (Caser et al., 2020) or air-dried (Stelluti et al., 2021) saffron tepals, extractions were performed with a powdered sample–solvent ratio of 1:50 g ml^{-1} using ultrasound assisted extraction (UAE) and four solvents, namely, deionized water, 20%, 50%, and 80% methanol in deionized water (v:v). The sample tubes were inserted into an ultrasonic extractor (Sarl Reus, Drap, France) using a frequency of 23 kHz for 15 min. The extracted solutions were filtered with one-layer of filter paper (Whatman No. 1, Maidstone, UK) and then with a 0.45 μm PVDF syringe filter (CPS Analitica, Milano, Italy). The extracts were then stored at −20 °C for analyses. All the extractions were carried out in triplicate for each solvent and method used. Extractions of both spice and tepals were conducted at room temperature (ca. 21 °C).

5.4.4. Pigment analyses

Quantification of crocin I and crocin II was carried out using an Agilent 1200 High-Performance Liquid Chromatography coupled with an Agilent UV–Vis diode array detector (Agilent Technologies, Santa Clara, CA, USA) (Caser et al. 2019a, 2019b, 2020).

Total anthocyanins content (TAC) was measured on fresh (Caser et al., 2020) and dried (Stelluti et al., 2021) tepals using the pH-differential method. The results were expressed as milligrams of cyanidin 3-O-glucoside (C3G) per 100 g of dry/fresh weight (mg C3G 100 g^{-1}).

5.4.5. Statistical Analysis

Statistical analyses were performed on data deriving from previous studies on saffron (Caser et al., 2019a, 2019b, 2020; Stelluti et al., 2021). All data were checked for normality (Shapiro–Wilk's test, $p > 0.05$) and homoscedasticity (Levene's test, $p > 0.05$) and eventually transformed (log or square-root

transformation) before performing two-way ANOVA test end t-test. The analyses were made with the R-studio software.

Regarding saffron spice, a two-way ANOVA test was performed to examine the influence of two factors, namely cultivation conditions (i.e., open field and greenhouse) and treatments (Ri, Ri+Fm, and AMF-), on the content of crocin I + II (values obtained by Caser et al. 2019a, 2019b, 2020) and to evaluate interaction effects between these factors.

Regarding saffron tepals, in the previous studies, the TAC found in fresh (Caser et al., 2020) and dried (Stelluti et al., 2021) tepals extracts was expressed as milligrams of cyanidin 3-O-glucoside (C3G) per 100 g of fresh/dry weight of tepals. In this study, to allow comparisons, TAC results were divided for the number of flowers needed to obtain 100 g of tepals, thus getting a TAC value per flower. Mean comparisons between TAC data were then made using an independent samples t-test. A type of extraction solvent (i.e., deionised water, 20%, 50%, and 80% methanol in deionised water, v:v) was considered at a time.

5.5. Results and Discussion

5.5.1. Saffron Spice

The effects of two cultivation conditions (greenhouse and open field) and three treatments (Ri, Ri+Fm, and AMF-) and the interaction effects between these factors on the content of crocin I + II were evaluated (Figure 1).

Considering all treatments together (Ri, Ri+Fm, and AMF-), the content of both crocins (I + II) in the dried stigmas was significantly higher for the plants grown in the greenhouse (+32%) than for those cultivated in open field (Figure 1a).

Significant differences between treatments were only found in the greenhouse (Figure 1b). The application of Ri significantly increased the crocins content compared to AMF- (+36%) and Ri+Fm (+74%); the spice of the plants treated with the Ri+Fm inoculum contained a significant lower content than AMF- (-144%) and Ri (-281%). Comparing the two cultivation conditions (greenhouse

and open field) for each treatment, significant differences were only induced by Ri, which significantly incremented the pigments content for the plants in the greenhouse (+59%).

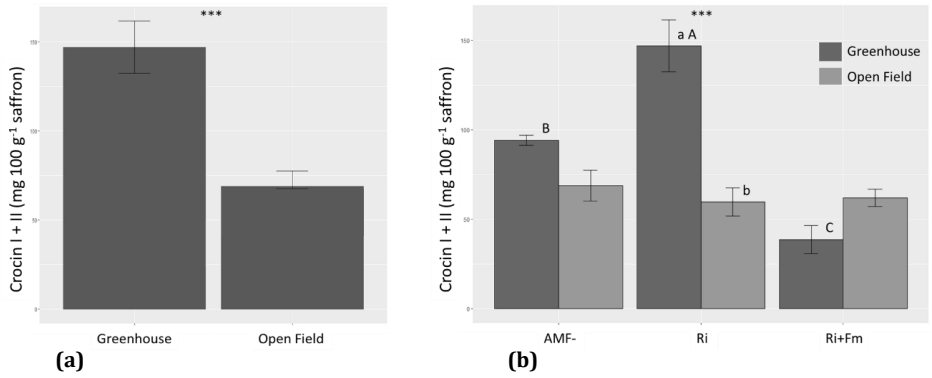


Figure 1. (a) Effects of the two cultivation conditions, i.e. greenhouse ad open field, on crocins I+II content (*** $p < 0.001$). (b) Interaction effects between the cultivation conditions (*** $p < 0.001$) and treatments (*** $p < 0.001$), i.e. AMF inocula consisting of *Rhizophagus intraradices* (Ri), *R. intraradices* and *Funneliformis mosseae* (Ri+Fm) or a control (AMF-), on crocins I+II content. Significant differences between treatments are described by capital letters, between cultivation conditions by lowercase letters. Mean comparisons were performed using two-way ANOVA test.

As previously reported (Caser et al., 2019a, 2019b), the Ri inoculum was confirmed to have a positive effect on the crocins content, which is particularly evident in greenhouse conditions.

5.5.2. Saffron Tepals

Tepals of one flower weighed 0.40 ± 0.06 (fresh tepals) and 0.05 ± 0.01 (dried tepals). Thus, 100 g of fresh tepals corresponded to 250 (= 100 g /0.40) flowers and 100 g of dried tepals to 2000 (= 100 g /0.05) flowers. Thus, to obtain a value of mg C3G flower^{-1}, the TAC of previous studies, expressed as mg C3G 100 g^{-1} tepals, was divided by 250 (fresh tepals) or 2000 (dried tepals).

In saffron dried tepals the TAC was significantly lower than in the fresh ones. The percentage of TAC reduction in dried tepals compared to fresh tepals was of

~50% when the solvents were water (-47%, * $p < 0.05$) and 50% methanol (-46%, * $p < 0.05$), and of ~80% when the solvents were 20% methanol (-79%, *** $p < 0.001$) and 80% methanol (-81%, ** $p < 0.01$).

Anthocyanins are unstable and easily degradable compounds. Heat-drying, a post-harvest process commonly used to storage plant materials (Zhao et al., 2019), can contribute to losses in their content (Brglez Mojzer et al., 2016; Qiu et al., 2018). Since harvested flowers are very perishable, freezer storage could be a good post-harvest practice to maintain the quality of fresh tepals.

5.6. Conclusions

Natural dyes can be extracted from both saffron spice and by-products for industrial applications, thus generating a new source of income in a circular bioeconomy perspective.

Further studies could be carried out to evaluate the effects of horticultural practices, such as the use of supplemental light in the greenhouse and bioinoculants (other AMF species and/or PGPR), on the content of natural pigments in the saffron flower.

5.7. References

Ameer, K., Shahbaz, H.M., and Kwon, J.-H. (2017). Green Extraction Methods for Polyphenols from Plant Matrices and Their Byproducts: A Review. Compr. Rev. Food Sci. Food Saf. *16*, 295–315.

Bathaie, S., Farajzade, A., and Hoshyar, R. (2014). A review of the chemistry and uses of crocins and crocetin, the carotenoid natural dyes in saffron, with particular emphasis on applications as colorants including their use as biological stains. Biotech. Histochem. *89*, 401–411.

Bolhassani, A., Khavari, A., and Bathaie, S.Z. (2014). Saffron and natural carotenoids: Biochemical activities and anti-tumor effects. Biochim. Biophys. Acta - Rev. Cancer *1845*, 20–30.

Brglez Mojzer, E., Knez Hrnčič, M., Škerget, M., Knez, Ž., and Bren, U. (2016). Polyphenols: Extraction Methods, Antioxidative Action, Bioavailability and

Anticarcinogenic Effects. Molecules *21*.

Cardone, L., Castronuovo, D., Perniola, M., Cicco, N., and Candido, V. (2020). Saffron (Crocus sativus L.), the king of spices: An overview. Sci. Hortic. (Amsterdam). *272*.

Carmona, M., Zalacain, A., Sánchez, A.M., Novella, J.L., and Alonso, G.L. (2006). Crocetin esters, picrocrocin and its related compounds present in Crocus sativus stigmas and Gardenia jasminoides fruits. Tentative identification of seven new compounds by LC-ESI-MS. J. Agric. Food Chem. *54*, 973–979.

Caser, M., Demasi, S., Victorino, Í.M.M., Donno, D., Faccio, A., Lumini, E., Bianciotto, V., and Scariot, V. (2019a). Arbuscular Mycorrhizal Fungi Modulate the Crop Performance and Metabolic Profile of Saffron in Soilless Cultivation. Agronomy *9*, 232.

Caser, M., Victorino, Í.M.M., Demasi, S., Berruti, A., Donno, D., Lumini, E., Bianciotto, V., and Scariot, V. (2019b). Saffron cultivation in marginal alpine environments: How AMF inoculation modulates yield and bioactive compounds. Agronomy *9*.

Caser, M., Demasi, S., Stelluti, S., Donno, D., and Scariot, V. (2020). Crocus sativus L. Cultivation in alpine environments: Stigmas and tepals as source of Bioactive Compounds. Agronomy *10*.

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

Gresta, F., Lombardo, M., Siracusa, L., and Ruberto, G. (2008). Saffron, an alternative crop for sustainable agricultural systems. A review. Agron. Sustain. Dev. *28*, 95–112.

Jafari, S.M., Mahdavee Khazaei, K., and Assadpour, E. (2019). Production of a natural color through microwave-assisted extraction of saffron tepal's anthocyanins. Food Sci. Nutr. *7*, 1438–1445.

Jain, M., Srivastava, P.L., Verma, M., Ghangal, R., and Garg, R. (2016). De novo transcriptome assembly and comprehensive expression profiling in Crocus sativus to gain insights into apocarotenoid biosynthesis. Sci. Rep. *6*, 1–13.

Kaur, S., and Suseela, V. (2020). Unraveling Arbuscular Mycorrhiza-Induced Changes in Plant Primary and Secondary Metabolome. Metabolites *10*, 335.

Khan, M., Hanif, M.A., Ayub, M.A., Jilani, M.I., and Shahid Chatha, S.A. (2020). Saffron. In Medicinal Plants of South Asia, (Elsevier), pp. 587–600.

Manzoor, M., Singh, J., Gani, A., and Noor, N. (2021). Valorization of natural colors as

health-promoting bioactive compounds: Phytochemical profile, extraction techniques, and pharmacological perspectives. Food Chem. *362*, 130141.

Medina-Torres, N., Ayora-Talavera, T., Espinosa-Andrews, H., Sánchez-Contreras, A., and Pacheco, N. (2017). Ultrasound assisted extraction for the recovery of phenolic compounds from vegetable sources. Agronomy *7*.

Moratalla-López, N., Lorenzo, C., Chaouqi, S., Sánchez, A.M., and Alonso, G.L. (2019). Kinetics of polyphenol content of dry flowers and floral bio-residues of saffron at different temperatures and relative humidity conditions. Food Chem. *290*, 87–94.

Mottaghipisheh, J., Mahmoodi Sourestani, M., Kiss, T., Horváth, A., Tóth, B., Ayanmanesh, M., Khamushi, A., and Csupor, D. (2020). Comprehensive chemotaxonomic analysis of saffron crocus tepal and stamen samples, as raw materials with potential antidepressant activity. J. Pharm. Biomed. Anal. *184*.

Mzabri, Addi, and Berrichi (2019). Traditional and Modern Uses of Saffron (Crocus Sativus). Cosmetics *6*, 63.

Peter, K. V. (2012). Handbook of Herbs and Spices Vol. 1: Series in Food Science, Technology and Nutrition.

Prajapati, R.A., and Jadeja, G.C. (2022). Natural food colorants: Extraction and stability study. Mater. Today Proc.

Qiu, G., Wang, D., Song, X., Deng, Y., and Zhao, Y. (2018). Degradation kinetics and antioxidant capacity of anthocyanins in air-impingement jet dried purple potato slices. Food Res. Int. *105*, 121–128.

Räisänen, R., Primetta, A., Nikunen, S., Honkalampi, U., Nygren, H., Pihlava, J.-M., Vanden Berghe, I., and von Wright, A. (2020). Examining Safety of Biocolourants from Fungal and Plant Sources-Examples from Cortinarius and Tapinella, Salix and Tanacetum spp. and Dyed Woollen Fabrics. Antibiotics *9*, 266.

Rodriguez-Amaya, D.B. (2016). Natural food pigments and colorants. Curr. Opin. Food Sci. *7*, 20–26.

Santos-Buelga, C., and González-Paramás, A.M. (2019). Anthocyanins. In Encyclopedia of Food Chemistry, (Elsevier), pp. 10–21.

Spatafora, J.W., Chang, Y., Benny, G.L., Lazarus, K., Smith, M.E., Berbee, M.L., Bonito, G., Corradi, N., Grigoriev, I., Gryganskyi, A., et al. (2016). A phylum-level phylogenetic classification of zygomycete fungi based on genome-scale data. Mycologia *108*, 1028–1046.

Stelluti, S., Caser, M., Demasi, S., and Scariot, V. (2021). Sustainable processing of floral

bio-residues of saffron (Crocus sativus l.) for valuable biorefinery products. Plants *10*, 1–15.

Tarantilis, P.A., Polissiou, M., and Manfait, M. (1994). Separation of picrocrocin, cis-trans-crocins and safranal of saffron using high-performance liquid chromatography with photodiode-array detection. J. Chromatogr. A *664*, 55–61.

Zhao, L., Fan, H., Zhang, M., Chitrakar, B., Bhandari, B., and Wang, B. (2019). Edible flowers: Review of flower processing and extraction of bioactive compounds by novel technologies. Food Res. Int. *126*.

General findings and Discussion

Crocus sativus L. is a precious crop that has been traditionally used for millennia, especially in the the Middle East and Mediterranean regions. The cultivation of saffron is recently facing challenges and has strongly declined in Europe, although in recent decades the interest in this crop has increased.

The Ph.D. project has been focusing on sustainable horticultural practices to improve saffron profitability, such as hydroponic cultivation and application of bioinoculants. Inoculations of different beneficial microbes in a controlled environment allowed to evaluate saffron responses under reproducible conditions. Particularly, the influence (also synergistic) exerted by the PGPR *Bacillus megaterium* CB97032 and *Paenibacillus durus* CB1806, and the AMF *Rhizophagus intraradices*, *Funneliformis mosseae*, *Rhizoglomus irregulare*, *Funneliformis geosporum*, *Funneliformis caledonium*, and *Claroidoglomus claroideum* on saffron production and quality in hydroponics was investigated. The plant responses to treatments were studied by using updated agronomic, biochemical, ecophysiological, and molecular methodologies. In particular, the effects of the bioinoculants on ecophysiological parameters and the apocarotenoid metabolism have been demonstrated for the first time.

As hypothesized, the mixed formulation of PGPR and AMF performed better than the single-type inoculants compared to the uninoculated controls, especially for the production of phytochemicals (Chapter 1). Specifically, the PGPR and the AMF *R. intraradices*, alone or in combination, led to an increased content of the main aroma metabolite safranal and the microbial cooperation in the mixed inoculant also improved the total phenolic content of the saffron spice (Chapter 1). Regarding the quantitative parameters, the yield of the spice decreased for plants treated with *Rhizophagus intraradices*, probably for a saprophytic behavior exhibited by the fungus during flowering, but restored once the PGPR were added: the bacteria may have mitigated the initial organic C cost for the plant (Chapter 1). The number of replacement corms increased in all inoculated plants,

especially for the treatment with the AMF alone, without a reduction of the corm weight (Chapter 1), an important parameter for the saffron flowering. The corm diameter was reduced by the single-type inoculants, but restored in the mixed formulation (Chapter 1). Moreover, a more positive trend was observed for the mixed inoculant regarding the total chlorophill content of the leaves, which is related to the nutritional status of plants (Chapter 1).

The saffron apocarotenoid biosinthesis pathway resulted modulated by the AMF *R. intraradices* (Chapter 2). The expression of the genes involved in the biosinthesis of strigolactones, ABA, and crocins did not change in mycorrhized plants. Conversely, the enhanced content of safranal was related to the upregulation of genes involved in picrocrocin biosynthesis (Chapter 2). AMF also increased the plant uptake of Fe, Mo, and Zn, which are involved in saffron apocarotenoid metabolism (in particular, Fe is cofactor of carotenoid cleavage dioxygenases enzymes) (Chapter 2). The crocin (color) content was overall reduced in mycorrhized saffron plants (Chapter 2), although the spice was of ISO quality 1st category also in color strength (Chapter 1). Since crocins are glucosyde esters of crocetin, the result could be linked again to an organic C cost of the AMF for the plant during flowering.

In addition to have affected quality traits of the spice (Chapter 1 and 2), AMF inoculants also influenced the antioxidant capacity of the tepals (Chapter 3). The phytochemical composition of dried saffron tepals (Chapter 4 and 5) pointed them as a valuable source of bioactive molecules, namely, vitamin C and phenolic compounds, such as the natural colorants anthocyanins, which can be obtained with sustainable extractions, i.e., by using ultrasound assisted extraction (UAE) and green solvent.

Conclusions

Research is increasingly investing in the development of modern and sustainable horticultural methods for high-quality plant-based products. In Europe, saffron is a crop intended for a niche market. In Italy, in the last decade, numerous companies have arisen, particularly in the Alpine area. Cultivation of saffron plants under controlled conditions with bioinoculants can facilitate crop management and meet consumer demands for high-quality, healthy and environmentally friendly saffron products for use in the food, cosmetic and medicinal sectors. The results of this thesis could be useful to improve the quality of the spice and the income deriving from this crop also by biorefining the tepals. Bioinoculants are considered modern agricultural tools able to reduce chemicals application, promote the plant defence system, improve plant productivity, and enhance the content of phytochemicals, thus increasing the quality of the products. As demonstrated also in this research, they have great potential to increase saffron profitability. In fact, even if their use represents an initial cost for farmers, over time it gives economic profits as they can limit the application of pesticides and fertilizers and improve the production of bioactive compounds, the organoleptic characteristics of the spice, and the tolerance to abiotic stresses, thus improving the yield an quality of saffron. To promote the application of microbial inoculants for saffron cultivation, a selection of favorable microbes species for saffron, appropriate substrates for microbial colonization, suitable inoculation protocols and crop management (e.g., fertirrigation), and optimal environmental conditions for the plant-microorganism interactions is necessary. Further studies on the modulation of saffron metabolism exerted by AMF and PGPR should be conducted, with the aim of creating products with a high market value. The influence of bioinoculants on the quality of saffron tepals should also be further deepened to improve their valor as a by-product in a circular bioeconomy perspective.

Appendix

1. Publications

- **Stelluti S.**, Grasso G., Nebauer G. S., Alonso L. G., Renau-Morata B., Caser M., Demasi S., Lumini E., Gómez-Gómez L., Molina V. R., Bianciotto V., Scariot V. Arbuscular mycorrhizal symbiosis modulates apocarotenoid biosynthesis pathway in saffron. (Submitted).
- **Stelluti S.**, Caser M., Demasi S., Rodriguez Herrero E., García-González I., Lumini E., Bianciotto V., Scariot V.. Beneficial microorganisms: a sustainable horticultural solution to improve the quality of saffron in hydroponics. Scientia Horticulturae (in press).
- **Stelluti S.**, Caser M., Demasi S., Scariot V., 2023. The natural colorants of the *Crocus sativus* L. flower. Acta Hortic. 1361, 111-118. doi: 10.17660/ActaHortic.2023.1361.13.
- **Stelluti S.**, Lumini E., Caser M., Demasi S., Falla N. M., Bianciotto V., Scariot V. Comparison of different inoculants of arbuscular mycorrhizal fungi on *Crocus sativus* L. cultivated in soilless conditions. Acta Hortic (in press).
- **Stelluti S.**, Caser M., Demasi S., Scariot V., 2021. Sustainable Processing of Floral Bio-Residues of Saffron (*Crocus sativus* L.) for Valuable Biorefinery Products. Plants (Basel). 10(3):523. doi: 10.3390/plants10030523.
- Caser M., Demasi S., **Stelluti S.**, Donno D., Scariot V., 2020. *Crocus sativus* L. cultivation in alpine environments: stigmas and tepals as source of bioactive compounds. Agronomy, 10(10):1473. https://doi.org/10.3390/agronomy10101473.

2. Oral presentations

- **Stelluti S.**, Grasso G., Nebauer G. S., Alonso L. G., Caser M., Demasi S., Lumini E., Molina Romero V. R., Bianciotto V., Scariot V. 2023. Bio-

inoculi: una pratica sostenibile per migliorare la produzione in fuori suolo di *Crocus sativus* L. – XIV Giornate scientifiche SOI - L'ortoflorofrutticoltura per la transizione ecologica. Torino, Italy, 21-23/06/2023

- **Stelluti S.**, Caser M., Demasi S., Scariot V., 2021. The colourful flower of *Crocus sativus* L.: a treasure trove of antioxidants - International Online Workshop SOI: THE COLOURS AND ANTIOXIDANTS OF FRUIT AND VEGETABLES. Online.

3. Posters

- **Stelluti S.**, Caser M., Demasi S., Scariot V, 2022. The natural colorants of the *Crocus sativus* L. flower – XXXI International Horticultural Congress IHC2022. ISHS. Anger, France, 14-20/08/2022.
- **Stelluti S.**, Lumini E., Caser M., Demasi S., Falla N. M., Bianciotto V., Scariot V., 2022. Comparison of different inoculants of arbuscular mycorrhizal fungi on *Crocus sativus* L. cultivated in soilless conditions – XXXI International Horticultural Congress IHC2022. ISHS. Anger, France, 14-20/08/2022.
- **Stelluti S.**, Caser M., Demasi S., Lumini E., Bianciotto V., Scariot V., 2022. La simbiosi micorrizica con *Rhizophagus intraradices* può essere influenzata dalla tecnica colturale in *Crocus sativus* L. - I Convegno Nazionale Orticoltura e Floricoltura (SOI). Pisa, Italy, 14-16/06/2022.
- **Stelluti S.**, Caser M., Demasi S., Scariot V., 2021. Coltivazione sostenibile dello zafferano (*Crocus sativus* L.) in campo *vs* fuori suolo, usando inoculi di biostimolanti microbici. - XIII Giornate Scientifiche della Società di Ortoflorofrutticoltura Italiana. Online, 22-23/06/2020.
- **Stelluti S.**, Demasi S., Caser M., Scariot V., 2020. Improvement of the yield and quality of saffron (*Crocus sativus* L.) using biostimulants for sustainable agricultural systems. Agricultural Chemistry Winter School (ACWS) - Interactions between biogeochemical cycles of elements in plant soil microbe systems. Torino, Italy, 10-13/02/2020.

- **Stelluti S.**, Caser M., Demasi S., Victorino I.M.M., Lumini E., Bianciotto V., Scariot V., 2020. Improving the performances and bioactive compounds of saffron (*Crocus sativus* L.) in Alpine environments using AMF inocula. - Italian Botanical Society 115th Congress. Online, 9-11/09/2020.
- Chialva M., **Stelluti S.**, Novero M., Bonfante P., Lanfranco L., 2020. Comparative transcriptomics between *Solanum lycopersicum* and *S. pennellii* sheds light into adaptation to arbuscular mycorrhizal symbiosis and combined stress resilience. - Italian Botanical Society (SBI) 115th Congress. Online, 9-11/09/2020.

4. Congresses and Seminars attended

CONGRESSES

- XXXI International Horticultural Congress - IHC2022. ISHS. Anger, France, 14-20/08/2022.
- XIII Giornate Scientifiche SOI 'I traguardi di Agenda 2030 per l'ortoflorofrutticoltura italiana'. Online, 22-23/06/2021.
- IV International Symposium on Woody Ornamentals of the Temperate Zone. ISHS. Online, 3-4/03/2021.
- Italian Botanical Society (SBI) 115th Congress (SBI, 09/2020). Online, 9-11/09/2020.
- Agricultural Chemistry Winter School (ACWS) Interactions between biogeochemical cycles of elements in plant soil microbe systems. Turin, Italy, 10-13/02/2020.

SEMINARS

- Fiori eduli: la biodiversità delle valli Piemontesi. SBI. Turin, Italy, 19/02/2019.
- Piante esotiche invasive: applicazioni del Regolamento europeo e ricadute sulla gestione del verde ornamentale. Department of Life Sciences and Systems Biology, University of Turin, Italy, 8/11/2019.

- Innovative solutions for sustainable urban farming: vertical farming, hydroponics, and landscape horticulture. Department of Agricultural, Forestry, and Food sciences, University of Turin, Italy, 13/12/2019.
- Bioproducts for organic and integrated management of horticultural crops: opportunities and challenges. SOI. Online, 26/05/2020.
- Terza Edizione della Scuola di Spettrometria di Massa accoppiata alla Gas Cromatografia Bidimensionale “Comprehensive” (GC×GC-MS) - Sic et simpliciter. Online, 06/2020.
- Career paths for PhDs working as climate change expert at the United Nations. Online, 4/03/2021.
- La tecnologia LED per le coltivazioni avanzate. In Horto Sanitas. Department of Agricultural, Forestry, and Food sciences, University of Turin, Italy, 04/2021.
- IECAG 2021, Live Session 3 - Agronomy: Biostimulants for sustainable crop production. MDPI. Online, 05/2021.
- Are vertical farms sustainable for the environment. ISHS. Online, 06/2021.
- International Online Workshop. The colours and antioxidants of fruit and vegetables. SOI. Online, 09/2021.
- Biostimolanti in ortoflorofrutticoltura. SOI. Online, 11/2021.
- Coltivazione, raccolta e prima trasformazione delle piante officinali: il Testo Unico D.Lgs n. 75/2018. Unimont. Online, 11/2021.
- English scientific writing for Italians. Department of Agricultural, Forestry, and Food sciences, University of Turin, Italy, 01/2022.
- Writing a manuscript. Department of Agricultural, Forestry, and Food sciences, University of Turin, Italy, 01/2022.

5. Ph.D. courses

- Bibliography and Bibliometry | Prof. Domenico Bosco
- Statistics: Mixed Models, Prof. Dario Sacco | Correspondence analysis, Prof. Luigi Bollani | Multivariate statistics of ecological data, Prof.

Matteo Garbarino | Cluster analysis, Proff. Giampiero Lombardi and Michele Lonati | Time series and prediction in R, Dr. Davide Ascoli

- Academic English | Jemma Faye Clair Robinson
- Drafting images for scientific purposes | Dr. Francesco Negro
- Ethics in publication | Prof. Giorgio Borreani
- Effects of climate change on crops and weed management | Prof. Francesco Vidotto

6. Teaching support activities

WORK AS TEACHING ASSISTANT (20 H) | 03-08/2022

- Course 'Analisi del Sistema Biologico (Esercitazioni)', AGR/02 and FIS/01. Degree Course in Agricultural Sciences and Technologies. Department of Agricultural, Forest, and Food Sciences of the University of Turin. Proff. Francesco Vidotto and Maria Margherita Obertino.

SEMINAR GIVEN (2 H) | 04/2022

- Microrganismi benefici del suolo (AMF e PGPR). ll caso studio dello zafferano. Floriculture and ornamental nursery course of the Degree Course in Agricultural Sciences and Technologies. Department of Agricultural, Forest, and Food Sciences of the University of Turin. Prof. Valentina Scariot.

CO-SUPERVISOR OF A MASTER DEGREE STUDENT | MASTER DEGREE COURSE IN AGRICULTURAL SCIENCES | A.A. 2020-2021.

- Title of the thesis: 'Gestione della luce e della soluzione nutritiva per aumentare la resa e la qualità dello zafferano (*Crocus sativus* L.) in serra'. Student: Francesco Berruto. Supervisor: Prof. Valentina Scariot. Department of Agricultural, Forest, and Food Sciences of the University of Turin.

Appendix 2

- Education

MASTER DEGREE IN ENVIRONMENTAL BIOLOGY (VOTE 110/110 CUM LAUDE) | UNIVERSITY OF TURIN | 10/04/2019

· Thesis entitled "Tomato M82 *vs Solanum pennellii*: the impact of water stress and mycorrhization in a tomato commercial line *vs* a wild relative" | Relator: Prof. Paola Bonfante, Co-relator: Dr. Matteo Chialva

BACHELOR IN BIOLOGICAL SCIENCES (VOTE 108/110) | UNIVERSITY OF BARI ALDO MORO | 03/10/2016

· Thesis entitled "Lichens as bioindicators of air pollution" | Relator: Prof. Linda Mastropasqua

5-YEAR SINGLE-CYCLE DEGREE PROGRAM CHEMISTRY AND PHARMACEUTICAL TECHNOLOGIES | UNIVERSITY OF BARI ALDO MORO | 2007 – 2012

(Enrolled without completing the studies)

CLASSICAL HIGH SCHOOL DIPLOMA | LICEO CLASSICO QUINTO ORAZIO FLACCO, BARI (BA) – ITALY | 2002 – 2007