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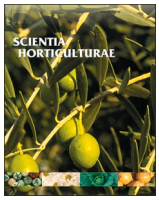

Arbuscular mycorrhizal symbiosis modulates the apocarotenoid biosynthetic pathway in saffron

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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 (flavor), and safranal (aroma), apocarotenoids deriving from zeaxanthin. In this study, an integrated biochemical and molecular analysis was carried out on fresh saffron stigmas 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 analyzed. Rare arbuscules (AMF trade structures) were found in mycorrhized plants. However, the expression of *D27, CCD7*, and *NCED* involved in the synthesis of strigolactones (SLs) and abscisic acid (ABA), which promote AM symbiosis, did not change in the stigmas. The transcription of *β-LYC* and *CCD4a/b* was not affected by AMF, whereas that of *CCD2*, which encodes the key enzyme producing major apocarotenoids, was upregulated. The crocin content was reduced in treated plants even if the expression of *ALDH, UGT74AD1*, and *UGT91P3*, involved in crocin synthesis, did not change. Conversely, *UGT709G1*, implicated in picrocrocin synthesis, was overexpressed in the inoculated plants, thus the safranal content was increased in the spice.

1. Introduction

Crocus sativus L. (*Iridaceae* family) is a geophyte commonly named saffron probably originating from ancient Greece (Kazemi-Shahandashti et al., 2022). Saffron is mainly cultivated in the Middle East and Mediterranean regions (Cardone et al., 2020). Being a sterile crop, reproduction is vegetative and depends on man, who has been propagating the plant through replacement corms for millennia. During the short autumn flowering intense and onerous manual labor is required to collect the perishable flowers and delicately separate and dehydrate the red stigmas to produce the precious spice. The fine organoleptic properties and the difficult production are the reasons for its high price and the nickname of "red gold" (Gresta et al., 2008; Bagur et al., 2017). The cultivation of saffron has declined sharply in Europe mainly because the cost of manual labor has increased and the technology has not progressed for this crop. Saffron production is also facing challenges such as labor shortages, climate change, poor soil fertility, soil diseases, rodent infestation, and viral and fungal diseases (e.g., *Fusarium oxysporum* and

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Rhizoctonia violacea), which are reducing its global production (Gresta et al., 2008; Cardone et al., 2020).

Traditionally used as a condiment, natural colorant, and in folk medicine, the recent revisiting of saffron bioactivity has renewed scientific and consumer interest in this plant (Bagur et al., 2017; Caser et al., 2020; Stelluti et al., 2023a, 2021). The inebriant sensorial profile is primarily attributed to three carotenoid derivatives, namely crocins (coloring capacity), picrocrocin (flavor), and safranal (aroma) (Fig. 1). In international trade agreements, the evaluation of the coloring, bittering, and aromatic strength (by UV−vis spectrophotometric analysis of the aqueous extracts) and of the physical characteristics of the spice serves to classify saffron in three quality categories according to ISO 3632 (2011) (García-Rodríguez et al., 2017). Crocins, also called crocetin esters, are water-soluble glycosylated crocetins, of which *trans*-crocetin di-(β-D-gentiobiosyl) ester (named *trans*-4-GG) and *trans*-crocetin di-(β-D-glucosyl) (β-D-gentiobiosyl) ester (named *trans*-3-Gg) are the main components (García-Rodríguez et al., 2017, 2014). Picrocrocin is the water-soluble glucoside of safranal, which is converted to safranal by a two-step enzymatic hydrolysis/dehydration process producing 4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (HTCC) as an intermediate or directly by chemical hydrolysis and dehydration at high temperatures and extremely acidic or basic pH (Himeno and Sano, 1987) (Fig. 1). Safranal, a volatile and mainly poorly soluble in water cyclic monoterpene aldehyde, is the major aromatic compound of saffron. The nutraceutical and therapeutic properties of saffron are mostly attributed to safranal and *trans*-isomers of crocins (García-Rodríguez et al., 2014). Altogether, these secondary metabolites are apocarotenoids derived from the oxidative cleavage of zeaxanthin and preferentially synthesized in stigma chromoplasts (Baba et al., 2015).

Apocarotenoids are generated by carotenoid dioxygenase enzymes (CCD1, CCD2, CCD4, CCD7, CCD8, and NCED), which cleave one or two double bonds of carotenoids (Ahrazem et al., 2010, 2016; Rubio-Moraga et al., 2014a). Other carotenoid derivatives are the phytohormones strigolactones (SLs) and abscisic acid (ABA) (Bruno et al., 2016; Nambara and Marion-Poll, 2005). SLs are signaling molecules known to promote the initiation of arbuscular mycorrhizal (AM) symbiosis (Fiorilli et al., 2019). Beside other functions in plant development, both SLs and ABA are positive regulators of AM symbiosis especially under phosphate ($H_2PO_4^-$, Pi) limitation, and AM fungi (AMF) can promote their production in plants (Fiorilli et al., 2019). Belonging to the subphylum Glomeromycotina (Spatafora et al., 2016), AMF are obligate biotrophs that form mutualistic symbiosis with most land plants, including relevant crops, such as *Solanum lycopersicum* L., *Vitis vinifera* L., *Hordeum vulgare* L., *Zea mays* L., and *Oryza sativa* L. (Chialva and Bonfante, 2018). They inhabit the host roots receiving sources of organic carbon (glucose and lipids) for hyphae growth while their extraradical mycelium explores the soil beyond the depletion zone around the roots and supplies the host with water and macro- and micro- nutrients, especially in soils with low nutrient availability (Chialva and Bonfante, 2018; Smith and Smith, 2011). For instance, several compositions of AMF, namely *Claroideoglomus* sp., *Funneliformis* sp., *Diversispora* sp., *Glomus* sp., and *Rhizophagus* sp.; *Glomus intraradices, G. microageregatum* and *G. Claroideum*; and *Funneliformis mosseae* enhanced the content of N, P, K, S, Ca, Cu, Fe, Mn, Mg, and Zn in the roots of *Cucumis sativus* L. seedlings (Chen et al., 2017). Exchanges between the host plant and AMF occur within plant cortical cells through highly branched hyphae called arbuscules (Lanfranco et al., 2018). AMF, considered plant biostimulants (EU Regulation, 2019/1009), respond to the need for sustainable agriculture. Indeed, they improve the biomass and quality of

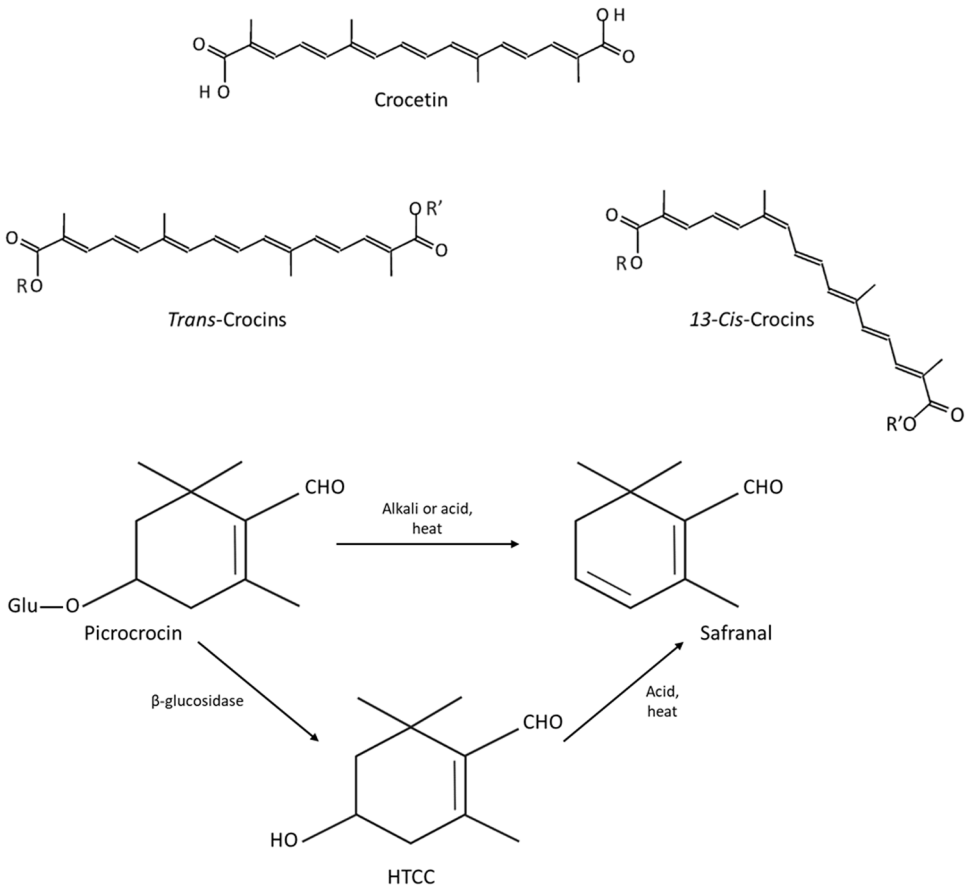

Fig. 1. Chemical structures of saffron crocins (glycoside esters of crocetin) and conversion of picrocrocin to safranal directly and via the intermediate HTCC. Readapted from Bagur et al. (2017) and Himeno and Sano (1987).

plants by acting as bio -fertilizers and -pesticides, thus limiting the application of agrochemicals (Rouphael and Colla, 2020). Colonization by AMF is usually more frequent in greenhouse conditions, where temperatures can be controlled and competition with the resident microbiota and predation by soil microfauna can be avoided (Berruti et al., 2016; Gavito et al., 2005). Among the species mainly used for the formulation of inocula, *Rhizophagus intraradices* colonized saffron well in the vegetative phase (Berruti et al., 2016; Caser et al., 2018).

Although mycorrhization is limited to the root, modulation of plant metabolism by AMF occurs both locally and systemically and can vary with different plant species or AMF (Chialva et al., 2022). Several secondary metabolic pathways are activated by AMF, such as the carotenoid, phenylpropanoid, and antioxidant pathways, which lead to the production of phytochemicals and phytohormones (Adolfsson et al., 2017; Chialva et al., 2023; Kumar et al., 2021; Venice et al., 2021). Different secondary metabolites were enhanced in AMF-inoculated medicinal and ornamental plants (Bianciotto et al., 2018), e.g., safranal in the spice of *Crocus sativus* L. (*R. intraradices*; Stelluti et al., 2023b); calendoflavoside and isorhamnetin-malonyl-glucoside in the aerial parts of *Calendula officinalis* L. (a mixture of *Claroideoglomus etunicatum, C. claroideum*, and *R. intraradices*; Engel et al., 2016); ascorbic acid, bornylacetate, 1,8-cineole, and α- and β-thujones in the essential oil of *Salvia officinalis* L. (*Glomus intraradices*; Geneva et al., 2010); hypericin and pseudohypericin in the shoots of *Hypericum perforatum* L. (a mixture of *Funneliformis constrictum, F. geosporum, F. mosseae*, and *R. intraradices*; Zubek et al., 2012); and artemisinin in leaves of *Artemisia annua* L., where a higher concentration of jasmonic acid (JA) was also found (*R. intraradices*; Mandal et al., 2015). The increase in phytochemicals has been related to the enriched nutritional status of mycorrhized plants and to changes in several metabolic pathways and phytohormone biosynthesis induced by AMF (Chialva et al., 2023; Kumar et al., 2021). However, the mechanisms by which AMF modulate plant metabolism are complex and not yet understood (Kumar et al., 2021). Venice et al. (2021), integrating mRNA sequencing and proteomics analysis, found that *Lotus japonicus* roots colonized by *Gigaspora margarita* activated important symbiotic pathways including nutrient transport and phenylpropanoid biosynthesis. Adolfsson et al. (2017) investigated the systemic changes in the leaves of mycorrhized (*R. irregularis*) *Medicago truncatula* plants, which showed improved shoot growth; they found an up-regulation of genes involved in the biosynthesis of flavonoids, terpenoids, JA, and ABA, resulting in an increased content of anthocyanins, flavonoids, ABA, and cytokinins in the leaves. Chialva et al. (2023) observed that, similarly to wheat, maize, tomato, and rice, *Coffea arabica* mycorrhized by *F. mosseae* showed a re-organization of the main metabolic pathways along their root-shoot axis, involving nutrient acquisition, carbon fixation, and primary and secondary metabolism (such as phenylpropanoid and flavonoid biosynthesis pathways). The mycorrhized coffee plants revealed a greater epigeous biomass, and a higher amount of sugars (glucose and fructose), threonate, amino acids, and flavonoid- and kaempferol- glycosides in roots; by contrast a number of phenolic acids decreased.

AMF also provide non-nutritional benefits, including resistance to biotic and abiotic stresses, such as extreme temperatures, drought, and salinity (Rouphael and Colla, 2020), which are predicted consequences of climate change and can have a negative impact on saffron production (Cardone et al., 2020; Gresta et al., 2008). AM symbiosis with *R. intraradices* and a mixture of *R. intraradices* and *F. mosseae* in potted conditions significantly reduced the wilting rate of saffron corms ascribable to *Fusarium* and *Rhyzoctonia* infections compared to the uninoculated controls (Caser et al., 2020b). Jasmonate and AMF (*F. mosseae*) increased the growth of saffron plants under conditions of salinity stress (mainly at 50, 75, and 100 mM) by improving enzymatic and non-enzymatic antioxidants and increasing osmolyte levels (Hamidian et al., 2023).

The aim of this research was to study the potential modulation of the apocarotenoid biosynthesis pathway in saffron stigmas induced by AMF (*R. intraradices*). An integrated transcriptional and biochemical analysis was carried out. Key genes for the synthesis of apocarotenoids promoting AM symbiosis and those related to spice quality were studied in the stigmas, as well as the content of major apocarotenoids was investigated in the spice. Since the increase of secondary metabolites in plants was associated with a mineral enrichment induced by AM symbiosis, the mineral nutrient content of corms at flowering was also analyzed. The hypothesis was that an AMF-mediated transcriptional modulation in the stigmas together with a potential enrichment in minerals of flowering corms may enhance quality-related apocarotenoids in the saffron spice.

2. Material and methods

2.1. 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. (2023b).

Large-sized corms (> 19 g) of *Crocus sativus* L. were sowed in pots (4 L) with sterile perlite (1.5 L and one corm per pot; Centro Evergreen Turco s.a.s., Moncalieri, Turin, Italy) at the end of August 2020. About 10 g of inoculum was placed under the corms (MycAgro Lab, Breteniére, FR). The inoculum consisted of a substrate of calcined clay, vermiculite, and zeolite with AMF spores, mycorrhizal root fragments, and hyphae fragments of *Rhizophagus intraradices*, isolate FR121 [International Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) bank], with a titration superior at 10 infectious propagules g^{-1}. Weekly irrigation was performed until the emergence of the roots, then fertigation every 2 weeks using a modified Long-Ashton solution (Hewitt, 1966) with 300 μM of Pi. The experiment consisted of three randomized replicates (blocks) per uninoculated controls (Ctr, 6 pots per block) and treated plants (Myc treatment, 12 pots per block).

2.2. Evaluation of AM colonization

At flowering, the plant roots were rid of topsoil, cleaned, and stained with 0.1% (w:v) cotton blue in 90% lactic acid overnight and then destained with water (two hours) and 90% lactic acid in deionized water 1:2 (v:v) (two hours). The staining protocol was performed twice. The roots were then stored in 90% lactic acid for further microscope analyses. Root fragments 1 cm in length were placed on microscope slides for observation under the light microscope (Trouvelot et al., 1986). The AMF colonization was assessed by observing ~180 cm of root. The absence of the fungus in Ctr was checked in ~60 cm of root.

2.3. Determination of mineral element content

The concentration of P, S, K, Ca, Mg, B, Cu, Fe, Mn, Mo, and Zn in flowering corms was determined with an ICP-AES (Thermo Scientific, USA) at the Ionomic service (CEBAS-CSIC, Murcia, Spain). The corms were 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 Ctr and Myc treatment. One corm from different plants was used in each biological replicate.

2.4. Quantitative reverse transcription PCR (qRT-PCR)

Gene expression changes were quantified on three biological replicates of saffron stigmas at red-scarlet stage by using qRT-PCR. After flowers were harvested, stigmas were gently separated, 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. To check genomic DNA contamination a PCR and agarose gel electrophoresis were performed using primers for *Tubulin*. 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).

Primer sequences, listed in Table 1, were designed by using Primer3 program (https://bioinfo.ut.ee/primer3-0.4.0/) 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 oligonucleotide pairs was tested before performing qRT-PCR analysis (Livak and Schmittgen, 2001).

2.5. Preparation of saffron extracts

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 by solving 50 mg of powdered spice into 5 mL of deionised water, as in Caser et al. (2020). The solutions were stirred using a magnetic stir bar at 1000 rpm for 1 h in the dark and at room temperature (~21 °C), 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.

2.6. 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 applying a 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.

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

Identification of compounds was carried out as previously reported by Carmona et al. (2006) and Sánchez et al. (2008). Calibration curves (García-Rodríguez et al., 2014) were established for: *trans*-5-nG, *trans*-4-GG, and *trans*-4-ng Ci=(0.00746 ± 0.00004)Ai–(0.00571 ± 0.12863), correlation coefficient (R^2)=0.9999; *trans*-3-Gg, *trans*-2-G, *trans*-2-gg, and *trans*-1-g, Ci=(0.00713 ± 0.00003)Ai–(0.00472 ± 0.05608), R^2=0.9999; *cis*-4-GG, Ci=(0.00531 ± 0.0004)Ai–(0.00571 ± 0.12863), R^2=0.9999; *cis*-3-Gg and *cis*-2-gg Ci=(0.00500 ± 0.00003)Ai–(0.00331 ± 0.05608), R^2=0.9999; picrocrocin, Ci=(0.02900 ± 0.00002)Ai+(0.51940 ± 0.02631), R^2=0.9999, and safranal, Ci=(0.03227 ± 0.00063)Ai+(0.05101 ± 0.03103), R^2=0.9989 by taking into account the limits of detection (LOD) and quantification (LOQ) reported by García-Rodríguez et al. (2014). Ci and Ai were the concentration (mg L^{-1}) and the HPLC peak area of the corresponding compound i, respectively.

HTCC was obtained by hydrolysis of picrocrocin according to Himeno and Sano (1987). The mg L^{-1} calibration line of the extract is Ci=(0.01477 ± 0.00001)Ai+(0.26448 ± 0.01339), R^2=0.9999. Safranal (purity ≥ 88%) and crocetin esters (*trans*-4-GG and *trans*-3-Gg, purity ≥ 99%) were obtained from Sigma-Aldrich (Madrid, Spain) and Phytolab GmbH & Co. KG (Vestenbergs-greuth, Bravaria, Germany), respectively and picrocrocin was isolated as described by Sánchez et al. (2008).

2.7. Statistical analysis

Significant differences were verified with the *t*-test ($p < 0.05$) after checking the data for normality (Shapiro–Wilk's test, $p \geq 0.05$) and homoscedasticity (Levene's test, $p \geq 0.05$). The R-studio software was used. Data were reported as mean and standard deviation.

3. Results and discussion

3.1. AMF colonization and mineral nutrient content in flowering corms

AMF was present in Myc plants, while it was absent in Ctr roots. For Myc plants, extraradical hyphae were identified in 18% of root fragments while intraradical hyphae, vesicles, and arbuscules were observed

Table 1
Sequences and parameters of the primers used for qRT-PCR analysis.

Primers	Sequences	Concentration (nM)	Tm (°C)	Product size (bp)
Cs18s_F	ATGTCAGCGGAACATTCAATC	300	59.95	191
Cs18s_R	TCAGTCTGCTAAGTAGCTATG	300	60.00	
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	
ALDH_F	GGACAAGCTTGCTTTTACTGG	300	59.03	100
ALDH_R	GCCACCAAGCTCCAATGTTA	300	61.03	
UGT74AD1_F	ACCTAGTCGTCGGGCCTATT	300	59.98	212
UGT74AD1_R	GGCGGTAGATTGTGTCCACT	300	60.00	
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	

in 15% of the fragments. The frequency of AMF colonization ranged from 1% to 10% in 9% of fragments and from 50% to 90% in 6% of fragments. Vesicles were seen in 10% of fragments (and were frequent-abundant in 8% of fragments), while rare arbuscules, i.e., the specialised trade structures of AMF (Lanfranco et al., 2018), were observed. AM colonization is regulated by Pi, an important plant growth-limiting and low-mobility macronutrient, by a local (arbuscule formation) and systemic way (Lanfranco et al., 2018). When the supply of Pi is high arbuscule formation is repressed (Lanfranco et al., 2018). The reason why rare arbuscules were found may be that the large corms used could already have contained sufficient reserves of nutrients, including Pi, which mainly support the early growth stages and autumn flowering of *C. sativus* after the dry summer season (Koocheki and Seyyedi, 2015). As evidence of this, there were no differences between Ctr and Myc for most of the mineral nutrients analyzed in the corms at flowering, including P (Table 2). However, in the roots of Myc plants, a biofertilizer effect was observed for some micronutrients, namely Zn, Mo, and likely Fe (the increase of which was almost significant, $p = 0.08$) (Table 2), which are important for plant physiology and enzyme cofactors, among other roles (Hänsch and Mendel, 2009). Iron (II) is a cofactor of carotenoid dioxygenases (CCD), which are involved in the biosynthesis of apocarotenoids (Rubio-Moraga et al., 2014b). Molybdenum is a cofactor of ABA-aldehyde oxidase (AAO), which catalyses the last step of ABA biosynthesis (Tuteja, 2007). Thus, the enhanced Mo and likely Fe content in the treated corms (Table 2) may contribute to a possible increase in CCD2 and AAO activity in Myc plants (Fig. 2). Regarding zinc, in Malik and Ashraf (2017) the differential expression profile between the stigma and the rest of the *Crocus* flower suggested that many zinc-finger genes were expressed in the stigma; particularly, the zinc-finger transcription factor CsSAP09 may have a possible role in regulating apocarotenoid metabolism in saffron, since it appeared to be highly expressed in the stigma during the anthesis phase, corroborating with the accumulation pattern of apocarotenoids.

3.2. Effect of arbuscular mycorrhization on apocarotenoid metabolism

Genes involved in the biosynthesis of major secondary metabolites of saffron, namely crocins, picrocrocin, and safranal, are exclusively expressed in saffron stigmas (Baba et al., 2015; Jain et al., 2016). Lycopene represents the first branch point of the carotenoid pathway (Fig. 2). It is the substrate of a chromoplast-specific lycopene β-cyclase (β-LYC), which catalyses the synthesis of β-carotene and together with lycopene ε-cyclase produces α-carotene (Baba et al., 2015). AM symbiosis did not affect the transcription level of *β-LYC* (Table 3). Both *D27* and *CCD7* (Table 3) were found highly expressed in saffron stigmas (Ahrazem et al., 2012; Jain et al., 2016; Rubio-Moraga et al., 2014a) and together with CCD8 are implicated in the biosynthesis of SLs (Rubio--Moraga et al., 2014a) (Fig. 2): D27 is a β-carotene isomerase, which converts all *trans*-β-carotene into 9-cis-β-carotene; CCD7 cleaves 9-cis-β-carotene into 10′-apo-β-carotenal and β-ionone; CCD8 then converts 10′-apo-β-carotenal in carlactone, the precursor of SLs. Under Pi limitation plants produce and exudate SLs, which act as signaling molecules in early plant–AMF interactions and promote AM symbiosis (Fiorilli et al., 2019). In this study, AM symbiosis also did not affect the expression level of *D27* and *CCD7* in stigmas, maybe because the saffron plants were not particularly deficient in Pi at flowering. However, since 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., 2014a), their transcript levels may have increased in other parts of the Myc plants.

β-carotene is also a substrate of CCD1, CCD4a, and CCD4b, producing HTCC and two β-ionone (volatile aroma compounds) (Ahrazem et al., 2010; Rubio et al., 2008) (Fig. 2); while *CCD1* is constitutively expressed, CCD4a/b react more actively with β-carotene (Ahrazem et al., 2016; Rubio et al., 2008). Gene expression of *CCD4a/b* was also not changed by AMF (Table 3).

CCD2 produces HTCC and crocetin dialdehyde by cleaving zeaxanthin derived from hydroxylation of β-carotene. HTCC is then converted into picrocrocin by the UDP-glucosyl transferase UGT709G1 (Diretto et al., 2019; Gómez-Gómez et al., 2017) (Fig. 2). In contrast to the other genes studied, *CCD2* and *UGT709G1* were overexpressed in Myc plants (Table 3). Although *CCD2* was upregulated, the HTCC content in Myc plant spice was not affected (Table 4), possibly due to the concurrent increased expression of *UGT709G1* (Table 3), which ultimately led to the enhanced content of safranal in the spice. This result agrees with Moradi et al. (2022), which found a high correlation among the expression of *UGT709G1* in saffron stigmas and picrocrocin and safranal content in the spice. However, in this study, the picrocrocin content did not change for Myc treatment (Table 4). This may be due to the conversion of picrocrocin to safranal when stigmas are dried, either directly or via HTCC. These results confirm that the expression of *UGT709G1* in stigmas can predict the production of the main aromatic compound safranal in the spice (Diretto et al., 2019; Moradi et al., 2022).

CCD2 also produces crocetin dialdehyde, which is converted to crocetin by aldehyde dehydrogenases (ALDH) (Fig. 2) (Gómez-Gómez et al., 2017). Crocetins are then glycosylated: UGT74AD1 produces crocins with one and two glucose molecules, which are substrates for UGT91P3 (López-jimenez et al., 2021) (Fig. 2). Glycosylation generates crocins, which accumulate in the vacuole (Gómez-Gómez et al., 2017). Gene expression of *ALDH, UGT74AD1*, and *UGT91P3* was not affected by AM symbiosis (Table 3) even though the content of crocins was overall reduced in Myc plant spice except for *trans*-2-gg, which was not affected (Table 4). Similarly, a lower content of *trans*-crocetin di-(β-D-gentiobiosyl) ester (also known as crocin I) in saffron treated with a mixed inoculation of *R. intraradices* and *F. mosseae* was found by Caser et al. (2019). The expression of genes involved in important steps of crocin synthesis correlated well with crocin content (Gómez-Gómez et al., 2017; López-jimenez et al., 2021). Moradi et al. (2022), investigating the effects of different B to R light ratios on saffron, observed that the content of *trans*-crocetin esters in the spice was highly correlated with the expression of *CCD2, ALDH31l* and *UGT74AD21* in the stigmas; however *cis*-crocetin esters correlated poorly with the expression of *CCD2, ALDH31l*, and *UGT74AD21*. In our study, the reduction of crocins in the spice of Myc-treated plants may be due to an unbalanced organic C cost to the plant during early association with AMF (Smith and Smith, 2011), as crocins might have been used as a glucose source for AMF. Up to 20% of total plant carbon can be used to support AMF development (Wu et al., 2011) and, in addition to lipids, plants transfer hexoses, such as glucose, to the fungal symbiont (Bago et al., 2002). A positive correlation between glucose allocation from leaves to roots was found in *Prunus persica* (L.) Batsch. seedlings colonized by AMF *G. mosseae* and *G. versiforme* (Wu et al., 2011). Interestingly, in our study, mycorrhized saffron roots exhibited many vesicles, the specialized storage structures of AMF (Azcón-Aguilar et al., 1999; Maiti and Ghosh, 2020).

Zeaxanthin, substrate of CCD2, can also be interconverted to

Table 2
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	ns (0.08)
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	* (0.04)
Zn (mg kg^{-1})	18.07 ± 0.86	24.91 ± 2.09	* (0.01)

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

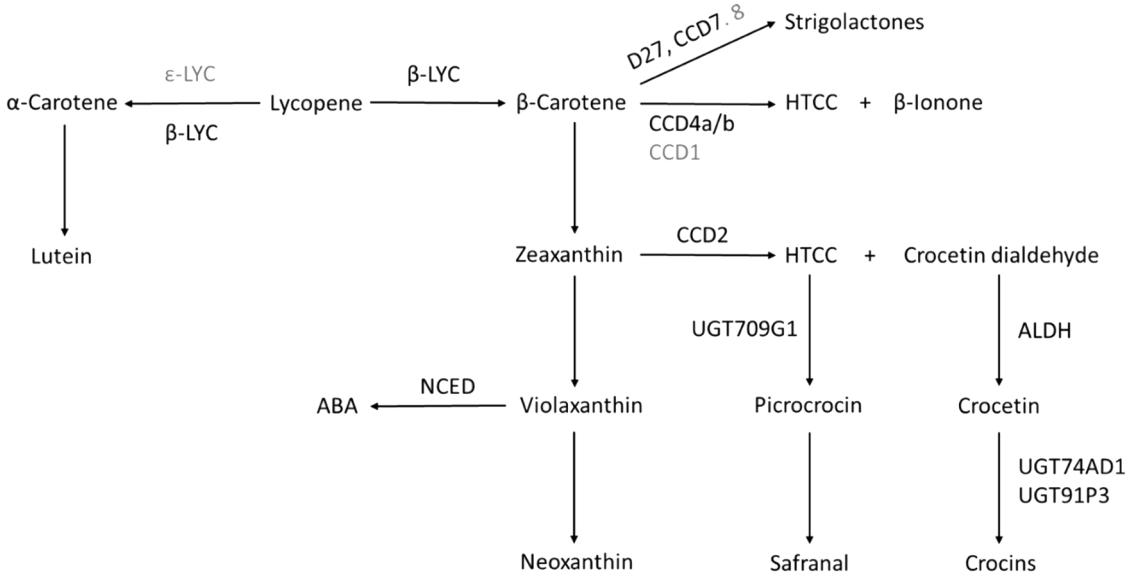

Fig. 2. Overview of the biosynthesis pathway of carotenoids/apocarotenoids in saffron.

Table 3
Expression analysis by qRT-PCR of genes involved in apocarotenoid metabolism on saffron fresh stigmas in mycorrhized plants (Myc). Non-mycorrhized plants were used as controls (Ctr). The relative expression (mean fold changes and standard deviations) was shown for each analyzed 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	*** ($3.36e^{-3}$)
UGT709G1	1.09 ± 0.58	2.94 ± 0.60	* ($1.88e^{-2}$)
ALDH	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

* $p < 0.05$.
*** $p < 0.001$; ns = not significant.

violaxanthin, which is then converted to neoxanthin. The enzyme 9-cis-epoxycarotenoid dioxygenase (NCED) cleaves violaxanthin and neoxanthin to form xanthoxin, a direct precursor of ABA (Baba et al., 2015; Jain et al., 2016) (Fig. 2). The expression level of *NCED* did not change in stigmas of Myc plants (Table 3). However, in this study, only stigmas were used for transcript generation while, similarly to the genes involved in SL biosynthesis, *NCED* was also found expressed in other saffron tissues (Jain et al., 2016).

The effect of AM symbiosis on the metabolism of apocarotenoids in saffron stigmas was investigated for the first time. Taking the results together, *R. intraradices* enhanced the safranal content and reduced the crocin content in the saffron spice. The reduction of crocins in the spice of mycorrhized plants could be further investigated to better understand the response of saffron to AMF (Smith and Smith, 2011). Plants vary greatly in their response to AMF species, and the efficacy of bio-inoculants can also be influenced by environmental conditions (Berruti et al., 2016; Li et al., 2022). AMF inoculants can be combined with other microorganisms to obtain a microbial synergy that may be more beneficial to plants (Etesami et al., 2021; Giovannini et al., 2020). In a previous work, a mixed inoculant of *R. intraradices* and the plant growth promoting bacteria *Bacillus megaterium* and *Paenibacillus durus* gave the best results regarding saffron spice quality and corm production (Stelluti et al., 2023b).

Table 4
Apocarotenoid content in the spice obtained 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})	Abbreviations for crocins	Ctr	Myc	*p*
Crocins				
trans-crocetin (β-D-neapolitanosyl)-(β-D-gentiobiosyl) ester	*trans*-5-nG	4.00 ± 0.75	1.56 ± 0.48	*** ($1.22e^{-4}$)
trans-crocetin di-(β-D-gentiobiosyl) ester	*trans*-4-GG	52.02 ± 5.76	25.06 ± 8.59	*** ($1.44e^{-4}$)
cis-crocetin di-(β-D-gentiobiosyl) ester	*cis*-4-GG	6.38 ± 0.99	1.88 ± 0.74	*** ($0.79e^{-5}$)
trans-crocetin (β-D-glucosyl) (β-D-gentiobiosyl) ester	*trans*-3-Gg	33.48 ± 5.00	22.88 ± 6.74	* ($1.24e^{-2}$)
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	*** ($0.04e^{-5}$)
trans-crocetin (β-D-neapolitanosyl)-(β-D-glucosyl) ester	*trans*-4-ng	3.83 ± 0.52	1.29 ± 0.44	*** ($0.42e^{-5}$)
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	*** ($1.89e^{-5}$)
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	*** ($2.32e^{-4}$)

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

4. Conclusions

An integrated biochemical and transcriptional analysis on the stigmas allowed for the first time to shed light on the influence exerted by *R. intraradices* on the biosynthesis of the main apocarotenoids responsible for the quality of saffron. Although *β-LYC* transcription was not affected by AMF, the *CCD2* gene, encoding the key enzyme that produce major apocarotenoids, was overexpressed. However, the crocin content was reduced in the spice derived from mycorrhized plants probably due to an unbalanced plant organic C cost at flowering. Conversely, the *UGT709G1* gene involved in picrocrocin synthesis was upregulated in fresh stigmas, consequently, the content of safranal, the main aromatic compound of saffron was increased.

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 micronutrient content.

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CRediT authorship contribution statement

Stefania Stelluti: Conceptualization, Investigation, Formal analysis, Writing – original draft. **Gianluca Grasso:** Investigation, Writing – review & editing. **Sergio G. Nebauer:** Conceptualization, Methodology, Writing – review & editing. **Gonzalo Luis Alonso:** Investigation, Writing – review & editing. **Begoña Renau-Morata:** Methodology, Writing – review & editing. **Matteo Caser:** Methodology, Investigation, Writing – review & editing. **Sonia Demasi:** Methodology, Investigation, Writing – review & editing. **Erica Lumini:** Conceptualization, Methodology, Writing – review & editing. **María Lourdes Gómez-Gómez:** Conceptualization, Methodology, Writing – review & editing. **Rosa Victoria Molina:** Conceptualization, Methodology, Formal analysis, Writing – review & editing. **Valeria Bianciotto:** Conceptualization, Methodology, Writing – review & editing. **Valentina Scariot:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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