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Published in:
Phytochemistry

DOI:
[10.1016/j.phytochem.2020.112457](https://doi.org/10.1016/j.phytochem.2020.112457)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2020

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Dudek, B., Schneider, B., Hilger, H. H., Stavenga, D. G., & Martinez-Harms, J. (2020). Highly different flavonol content explains geographic variations in the UV reflecting properties of flowers of the corn poppy, *Papaver rhoeas* (Papaveraceae). *Phytochemistry*, 178, [112457].
<https://doi.org/10.1016/j.phytochem.2020.112457>

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Highly different flavonol content explains geographic variations in the UV reflecting properties of flowers of the corn poppy, *Papaver rhoeas* (Papaveraceae)

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ARTICLE INFO

Keywords:

Papaver rhoeas
Papaveraceae
Flavonols
Pigments
Flower color

ABSTRACT

Papaver rhoeas, the corn poppy, is a very common weed in cereal fields all over the world. Its flowers generally display a bright red coloration, but their reflectance in the ultraviolet (UV) wavelength range varies geographically. Whereas the UV reflectance of East Mediterranean flowers is minor, that of Central European ones is substantial. By comparing the pigmentation of the differently reflecting flowers, we found that only East Mediterranean flower petals contain high amounts of UV absorbing flavonol glycosides. The most abundant compounds were isolated by solid phase extraction and preparative HPLC, and their structures were elucidated by NMR and HRESI-MS, yielding seven kaempferol and quercetin glycosides, mostly unknown in *P. rhoeas* petals. Additionally, reflectance and transmittance measurements revealed that wavelength-selective scattering effects do not contribute to the flower color differences observed within this species. Possible abiotic and biotic factors influencing the UV reflecting properties of East Mediterranean and Central European poppies are discussed.

1. Introduction

The coloration of flowers is an important trait to attract pollinators and to help ensure the reproduction of plants. Flower color involves two optical phenomena, (i) the reflection and scattering of light by floral structures and (ii) wavelength-selective absorption by floral pigments in the visible and UV range (Kay et al., 1981; Kevan and Backhaus, 1998; Lee, 2007; van der Kooi et al., 2019, 2016; van der Kooi and Stavenga, 2019; Vignolini et al., 2012). The pigments accumulate and localize at different subcellular compartments of floral tissue, providing specific hues depending on their absorption spectrum (Exner and Exner, 1910; Kay et al., 1981; Kugler, 1963; van der Kooi et al., 2016).

Flavonoids are the most extensively studied class of floral pigments. They are derived from the phenylpropanoid pathway, which produces different subclasses of flavonoids, including chalcones, flavonols, and anthocyanins, among others. Flavonoids are known to participate in a wide variety of biological processes, from plant defense, protection

against UV radiation, and auxin transport regulation to male fertility regulation and enhancement of attractiveness for pollinators (Harborne, 1994; Hollósy, 2002; Mallikarjuna et al., 2004; Rozema et al., 1997; Sosa et al., 2004). Previous studies have shown that flavonoid levels can be subject to modifications in plants exposed to stress conditions (Albert et al., 2009; Havaux and Kloppstech, 2001; Jansen et al., 1998) and that their accumulation in floral tissue can vary along geo-climatic gradients (Del Valle et al., 2015; Koski and Ashman, 2015a). Changes in the composition and concentration of flavonoids can modify flower color appearance, influencing the interactions of plants with pollinators and thereby also their reproduction.

An interesting example of a species whose petals vary in color along its distribution range is the corn poppy, *Papaver rhoeas* L. (Papaveraceae) (Martínez-Harms et al., 2020). According to archeological and taxonomic evidence, this species spread about five thousand years ago from the East Mediterranean into Central Europe as an agricultural weed (Godwin, 1975; Kadereit, 1990; Willerding, 1986; Zohary and Hopf,

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<https://doi.org/10.1016/j.phytochem.2020.112457>

Received 5 February 2020; Received in revised form 24 June 2020; Accepted 5 July 2020

Available online 20 July 2020

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1994). Spectral reflectance data show that while corn poppies universally have a high reflectance in the red wavelength range, reflectance in the UV-A (ca. 315–380 nm) is substantially higher in petals of Central European (Germany) poppies (Daumer, 1958; Kugler, 1947; Lotmar, 1933; van der Kooi and Stavenga, 2019) than in their East Mediterranean (Israel) counterpart (Dafni et al., 1990; Martínez-Harms, 2012; Martínez-Harms et al., 2020). Poppies' vivid red coloration originates from the combined effects of a highly pigmented epidermis with anthocyanins (Acheson et al., 1956; Matysik and Benesz, 1991; Schmid and Huber, 1931; Schmid and Körperth, 1936; Scott-Moncrieff, 1936) and efficient light scattering by serpentine-wavy cell walls and a non-pigmented mesophyll with air cavities (van der Kooi and Stavenga, 2019). Given that insect pollinators are sensitive to light in the UV-A range of the spectrum, differences in the UV reflecting properties of poppies would modify their appearance for pollinators (Martínez-Harms et al., 2020).

The present study addresses the differences in the UV reflecting properties of flowers of *P. rhoeas* from the East Mediterranean and Central Europe. Our chemical analyses focused on the UV-A absorbing pigment content of petals of corn poppies with different UV reflectance. Additionally, the influence of wavelength-selective scattering was investigated.

2. Results

2.1. Light scattering by floral structures

P. rhoeas plants grown in a glasshouse from seeds collected in Germany developed only petals with high UV-A reflectance (Fig. 1A). In

contrast, plants grown from seeds collected in Israel developed petals with low UV-A reflectance, petals with substantial UV-A reflectance, as well as petals with intermediate patterns consisting of areas with low and substantial UV-A reflectance (Fig. 1B–D). Additionally, most flowers of both color morphs showed a black spot of variable size with low UV-A reflectance at the petal base.

To study the contribution of wavelength-selective scattering by petal components to the UV-A properties of *P. rhoeas* flowers, we compared their reflectance spectra with transmittance spectra (van der Kooi and Stavenga, 2019). To reduce light scattering by refractive index differences, we immersed petal pieces in water. We found that in flowers with low as well as substantial UV-A reflectance the transmittance spectra resembled the reflectance spectra (Fig. 1E and F). We hence conclude that the differences in UV-A reflectance observed between flowers of *P. rhoeas* do not result from wavelength-selective scattering but from differences in the pigment composition.

2.2. Identification and quantification of the pigments in petals of *P. rhoeas*

We analyzed the pigments responsible for the coloration differences by HPLC in an acidic pH regime, and focused on uniformly colored apical petal parts. The profiles of extracts of UV-A reflecting flowers from Israel and Germany closely resembled each other. In contrast, the profiles of extracts of the Israeli flowers with low UV-A reflectance were strikingly different from the highly reflecting ones (Fig. 2A).

By means of their UV/Vis spectra, several red anthocyanins with pelargonidin and cyanidin aglycones were identified (Fig. 2A and B) (Macz-Pop et al., 2006). This is consistent with the literature (Acheson

Fig. 1. RGB-colored and UV images of flowers of *P. rhoeas* and their spectral characteristics. A) Example of a *P. rhoeas* flower with high UV reflectance cultivated from seeds collected in Central Europe. B–D) *P. rhoeas* flowers cultivated from seeds collected in the East Mediterranean. B) *P. rhoeas* flower with low UV reflectance. C) *P. rhoeas* flower with substantial UV reflectance. D) *P. rhoeas* flower with an intermediate pattern of UV reflectance. Note that the flower presents areas with negligible and areas with substantial UV reflectance. Scale bars: 1 cm. E) Reflectance spectrum (R, red curve) of the adaxial side of a petal of a flower of *P. rhoeas* reflecting negligibly in the UV (UV–) and its transmittance spectrum (T, green curve) measured with a microspectrophotometer. F) Reflectance spectrum (R, red curve) of a petal of a *P. rhoeas* flower from Central Europe, reflecting well in the UV (UV+) and its transmittance spectrum (T, green curve). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

et al., 1956; Schmid and Huber, 1931; Scott-Moncrieff, 1936). The main peak was a cyanidin glycoside in case of the Israeli poppies, and a pelargonidin glycoside in case of the German ones. Altogether, the total anthocyanin concentration was higher in flowers with low UV-A reflectance than in flowers with substantial UV-A reflectance. The low light absorbance between 315 and 380 nm by anthocyanins (Fig. 2B) indicates that they are not responsible for the low UV-A reflectance of *P. rhoeas* flowers.

Our subsequent analyses focused on compounds highly abundant in extracts of flowers with low UV-A reflectance, but almost absent in extracts of flowers with substantial UV-A reflectance (Fig. 2A). Pronounced HPLC signals appeared between R_t 30 and 40 min, showing UV/Vis absorption spectra with maxima around 260 nm and 350 nm (Fig. 2B), which are typical for flavonols (Mabry et al., 1970). In particular, the peaks are assignable to two different flavonol aglycones, one with absorption maxima at 255 and 355 nm and shoulders at 266 and 298 nm and another one with maxima at 267 and 349 nm and a shoulder at 297 nm (Fig. 2A and B). In addition to the difference in the absorption spectra, the shorter R_t of peaks 1–4 indicate higher polarity and thus suggests a higher degree of oxygenation of the aglycone compared to peaks 5–7 (Mabry et al., 1970). UV/Vis absorption spectra of compounds sharing one aglycone were nearly identical (Fig. S1).

The structures of the seven main flavonols (Fig. 2C) were elucidated (^1H and ^{13}C NMR data and HR masses are summarized in Tables S1–2, and Table 1, respectively; detailed structure elucidation is described in

Supporting Information, Notes S1). NMR and HRESI-MS data confirmed the presence of glycosides of two different flavonols, quercetin and kaempferol. Four quercetin derivatives were identified, among them the monoglycosylated quercetin 3-O- β -D-glucopyranoside (isoquercitrin, 4) (Braca et al., 2003; Gutzeit et al., 2006), and three diglycosylated compounds: compound 1 was assigned as quercetin 3-O- β -D-glucopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside (Byun et al., 2010; Huang et al., 2015), compound 2 was assigned as quercetin 3-O- α -L-arabinopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside (Lee et al., 2014; Takemura et al., 2002), and compound 3 as quercetin 3-O-[3'-O-acetyl- β -D-glucopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside] (Huang et al., 2015).

Compounds 1 and 4 differed from 5 and 7 only in the additional hydroxy group attached to position 3'. Hence, compound 5 was identified as kaempferol 3-O- β -D-glucopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside (Iwashina et al., 2000; Tanaka et al., 2001) and compound 7 as kaempferol 3-O- β -D-glucopyranoside (astragalin) (Kim et al., 2004).

Finally, the derivative 6 was assigned as kaempferol 3-O-[3'-O-acetyl- β -D-glucopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside], which, to the best of our knowledge, represents an undescribed natural product (for NMR, HRESI-MS, and UV/Vis spectra see Fig. S2–S7, Tables S1–S2, Table 1). It shows ^1H NMR signals typical for *m*-coupled protons H-6 and H-8 (δ 6.18; 6.40; J = 1.5 Hz) in the ring A of flavonols, and for an AA'XX' spin system in the *p*-substituted symmetrical ring B (H-2'/6' δ 8.17 and H-3'/5' δ 6.91) of kaempferol aglycone. The compound is

Fig. 2. Flavonoids in *P. rhoeas* petal extracts. A) HPLC profiles of different color morphs recorded at 254 nm, UV- (weakly UV reflecting); UV+ (strongly UV reflecting). The peaks are labeled according to their UV/Vis absorption spectra as anthocyanins ○ or flavonols ● with colors corresponding to panel B. Intensity values of the stacked chromatograms (UV- and UV+, Israel) are enhanced by an offset of 30 and 15 mAU, respectively. Peak numbers correspond to those of compounds 1–7 shown in C. B) UV/Vis absorption spectra of anthocyanins and flavonols. C) Structures of flavonols in *P. rhoeas* petals. D) Total content of flavonols and anthocyanins in mg per g apical petal tissue, compared for different color morphs (error bars represent standard deviation). * Content in weakly UV reflecting petals is significantly higher than in strongly UV reflecting ones (ANOVA, flavonols: $F = 10.45$, $p = 0.011$; anthocyanins: $F = 23.37$, $p = 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1

UHPLC-HRESI-MS data (negative ionization mode) of seven flavonol glycosides from Mediterranean *P. rhoeas* petals (for structures compare Fig. 2C) and their relative proportion of the total flavonol content.

Compound		t _R	m/z [M-H] ⁻		References	Rel. share of total flavonol content
no.	Name	(min)	exp.	calculated (for)		(%)
1	Quercetin 3-O-[β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside]	12.43	625.1401	625.1399 (C ₂₇ H ₂₉ O ₁₇)	Byun et al., 2010; Huang et al., 2015	35.8 ± 8.6
2	Quercetin 3-O-[α-L-arabinopyranosyl-(1 → 6)-β-D-glucopyranoside]	12.70	595.1282	595.1294 (C ₂₆ H ₂₇ O ₁₆)	Lee et al., 2014; Takemura et al., 2002	4.8 ± 1.5
3	Quercetin 3-O-[3'''-O-acetyl-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside]	13.03	667.1498	667.1505 (C ₂₉ H ₃₁ O ₁₈)	Huang et al., 2015	11.8 ± 3.3
4	Quercetin 3-O-β-D-glucopyranoside	13.54	463.0873	463.0871 (C ₂₁ H ₁₉ O ₁₂)	Braca et al., 2003; Gutzeit et al., 2006	not included (peak overlapping)
5	Kaempferol 3-O-[β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside]	13.14	609.1450	609.1450 (C ₂₇ H ₂₉ O ₁₆)	Iwashina et al., 2000; Tanaka et al., 2001	25.7 ± 6.2
6	Kaempferol 3-O-[3'''-O-acetyl-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside]	13.81	651.1560	651.1556 (C ₂₉ H ₃₁ O ₁₇)		15.2 ± 3.6
7	Kaempferol 3-O-β-D-glucopyranoside	14.27	447.0924	447.0922 (C ₂₁ H ₁₉ O ₁₁)	Kim et al., 2004	6.7 ± 2.1

diglycosylated (doublets of protons at the anomeric centers H-1'' δ 5.19; H-1''' δ 4.11), whereas the sugar units are β-(1 → 6) linked to each other according to the HMBC correlations between H-6''a/b and C-1''', and a shift of the C-6'' signal to low field in comparison to a monoglycosylated compound. The diglycoside is attached to C-3 of ring C of the kaempferol, indicated by the HMBC correlation between H-1'' and C-3 (δ 135.3). Both sugars were identified as β-D-glucopyranoses by the large ³J_{H-H} coupling constants, characteristic for the axial configuration of H-1'' to H-5'' and H-1''' to H-5'''. Additionally, an O-acetyl unit attached to C-3''' was established from an HMBC correlation between the signal of H-3''' at the low field (δ 4.82) and the carbonyl C-atom of the acetyl unit (δ 172.7).

The monoglycosylated flavonols isoquercitrin (4) and astragalin (7) were previously reported as constituents of *P. rhoeas* petals (Hillenbrand et al., 2004), but compounds 1–3, 5, and 6 were found in *P. rhoeas* petals for the first time.

All flavonols identified had very similar spectrophotometric properties (Fig. S1) as the spectra of quercetin and kaempferol derived glycosides differed only by 6 nm and all compounds shared the 3-O-β-D-glucosylation. All other modifications occurred at this 3-O-glucose unit and thus didn't influence the absorption of the aromatic ring system markedly (for literature UV/Vis absorption data of the flavonol-3-O-glucosides/-sophorosides see e.g. Djoukeng (2008): data for quercetin-3-O-glucoside, quercetin-3-O-sophoroside, quercetin 3-O-(acetyl) glucoside: λ_{max} = 355 nm, kaempferol-3-O-glucoside and kaempferol-3-O-sophoroside λ_{max} = 348/351 nm). The extinction coefficients of the kaempferol and quercetin glycosides do also not differ markedly according to the literature when measured under the same conditions (e.g. Wagner (1968): data for quercetin-3-gentiobioside logε_(360nm) = 4.20, kaempferol-3-gentiobioside logε_(352nm) = 4.35). Therefore, the HPLC-DAD peak areas at 351 nm could be used to estimate the relative proportion of each compound in the total flavonol content. Compounds 1–3 and 5–7 were included into the calculation, whereas compound 4 had to be excluded because of the overlapping of the small peak with the most intense anthocyanin peak, making the integration impossible even with the smaller extinction of anthocyanins at this wavelength. The ratio between quercetin and kaempferol derived compounds was approximately 1:1, and for both aglycones the 3-O-[β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside] derivative was the main compound (1/5 ca. 36 or 26% of total flavonol content, respectively) (Table 1). The compounds 3 and 6, bearing an additional acetyl substitution, accounted for 12 and 15% of the total flavonol content, whereas the other compounds were present in minor proportions.

The total amount of flavonols and anthocyanins in *P. rhoeas* petals was estimated by a mass balance of the compound classes separated by solid phase extraction (SPE) (Table S3). Both, flavonol (F) and

anthocyanin (A) content was significantly higher in petals with low UV-A reflectance (m_F = 15.5 ± 4.2 mg/g; m_A = 17.1 ± 1.7) than in petals with substantial UV-A reflectance (Fig. 2D). Petals with substantial UV reflectance from Israel (m_F = 8.5 ± 2.1 mg/g; m_A = 10.8 ± 2.3) and Germany (m_F = 5.6 ± 0.6 mg/g; m_A = 8.1 ± 0.3) showed similar content of both compound classes (one-way analysis of variance (ANOVA), flavonols: *F* = 10.45, *p* = 0.011; anthocyanins: *F* = 23.37, *p* = 0.001). Purity of the SPE fractions was checked by HPLC-DAD. Due to the intrinsically imperfect separation capability of the SPE material, the flavonol fractions also contained anthocyanins, which were negligible in case of petals with low UV-A reflectance compared to the flavonols, but higher in case of petals with substantial UV-A reflectance because of the low amount of flavonols. Therefore, masses of flavonols in substantially UV reflecting petals is lower than reported here.

Flavonols but not anthocyanins have their main absorption maximum in the UV-A region. Therefore, we can summarize that the low UV-A reflectance of flowers of *P. rhoeas* from the East Mediterranean is caused by a set of differently substituted kaempferol and quercetin glycosides. Additionally, reflectance differences in the UV-B region can be assumed, as higher amounts of anthocyanins and flavonols, both absorbing light between 250 and 315 nm, were detected in poppy flowers with low UV-A reflectance.

3. Discussion

The UV-A reflectance of Central European poppies is substantially higher (Daumer, 1958; Kugler, 1947; Lotmar, 1933; van der Kooi and Stavenga, 2019) than that of their East Mediterranean counterpart (Dafni et al., 1990; Martínez-Harms, 2012; Martínez-Harms et al., 2020). We found that a different UV absorbing pigment content in flower petals underlies these variations. Several flavonol glycosides, identified in poppies with low UV-A reflectance, were not detected or had very low abundance in poppies with substantial UV-A reflectance. The core structures, kaempferol and quercetin, as well as their glycosidic moieties, were elucidated. While compounds 1–3, 5, and 6 are reported for the first time in *P. rhoeas*, compound 6 is an undescribed natural product. Additionally, the total content of anthocyanins was significantly higher in weakly UV-A reflecting than in strongly UV-A reflecting flowers, but as anthocyanins absorb mostly in the red and UV-B region and not in the UV-A, their importance for the UV-A reflectance will be minor.

Flavonoids are common plant specialized metabolites with various functions. In addition to their role as phytoalexins in many plants, the concentration of flavonoids can be subject to modifications in response to abiotic stress events, such as exposure to low temperatures (Albert et al., 2009; Havaux and Klopstech, 2001), salinity conditions (Agati

et al., 2011), or high UV radiation (Hectors et al., 2014; Hollósy, 2002; Jansen et al., 1998; Tevini et al., 1981). The protective effect against UV radiation (Hollósy, 2002; Rozema et al., 1997; Shirley, 1996) is attributed to their distinct UV absorption (Agati and Tattini, 2010; Rozema et al., 1997), and to their capacity to act as scavengers of radicals and reactive oxygen species (Agati et al., 2009; Yamasaki et al., 1997).

Accumulation and composition of flavonoids in plants can vary along geo-climatic gradients (Del Valle et al., 2015; Jaakola and Hohtola, 2010; Koski and Ashman, 2015a,b) influencing their coloration. Studies in the shore campion (*Silene littorea*), for example, revealed that flavonoids (especially anthocyanins) increased with temperature and UV-B radiation and decreased with precipitation (Del Valle et al., 2015). In silverweed (*Argentina anserina*) an increase in the size of the UV absorbing bullseye pattern (UV reflecting petal tips, UV absorbing bases) correlated with an increase in UV radiation towards higher altitudes (Koski and Ashman, 2015b) as well as towards the equator (Koski and Ashman, 2015a). Similarly in *P. rhoeas*, floral UV-A and probably UV-B absorption is higher in East Mediterranean populations, which are exposed to higher UV radiation than Central European ones (Seckmeyer et al., 2008), suggesting that flavonols and anthocyanins may protect those flowers against UV-induced stress. The fact that plants grown from seeds collected in the East Mediterranean developed petals with low UV reflectance as well as petals with high UV reflectance when cultivated in Central Europe indicates that in native populations of *P. rhoeas* the synthesis of flavonols could be influenced by environmental factors.

Flavonol ratios may also change in response to abiotic factors. It was reported that in flowers of *Arnica montana* L. cv. ARBO decreasing temperatures triggered an increase in the 3',4'-dihydroxylated to 4'-hydroxylated flavonols ratio (e.g. quercetin to kaempferol) (Albert et al., 2009), conferring higher protective capacity to floral tissue due to a more potent action of 3',4'-dihydroxylated compounds as radical scavengers (Rice-Evans et al., 1996). In weakly UV reflecting petals of *P. rhoeas*, quercetin and kaempferol glycosides were present in similar amounts. Considering that the plants used in our study were cultivated under the same conditions, further investigations are needed to evaluate the effect of abiotic factors on the quercetin to kaempferol ratio in flowers of *P. rhoeas*.

Differences in flower color derived from modifications in the content of pigments can influence the attractiveness of flowers for pollinators and thereby also the plant's reproductive success. Reproduction represents a key process in the invasion of new habitats by plants. It has been proposed that modifications in floral traits could help ensure the reproduction of exotic plants by facilitating their interactions with novel types of pollinators (Eckert et al., 2010). The spread of *P. rhoeas* from the East Mediterranean into Central Europe decoupled this species from its native pollinators, inducing a shift from glaphyrid (Glaphyridae: Coleoptera) to hymenopteran pollination (Dafni et al., 1990; Dobson et al., 1996; Keasar, 2010; McNaughton and Harper, 1960). Glaphyrid beetles and hymenopterans differ substantially with respect to their receptor-based color vision. On one hand, the eyes of the glaphyrid beetle *Pygopleurus israelitus* contain receptors maximally sensitive to UV-A, green, and red light (Martínez-Harms et al., 2012), a condition that would allow glaphyrids to discriminate colors of *P. rhoeas* flowers with low and with substantial UV-A reflectance from a green background (Martínez-Harms et al., 2020). On the other hand, most hymenopterans have receptors maximally sensitive to UV, blue, and green light (Peitsch et al., 1992) conferring them the capacity to easily discriminate colors of UV reflecting poppies, but not those of pure red poppies (Martínez-Harms et al., 2020). Despite being self-incompatible (Lawrence, 1975) and therefore dependent on pollinators for reproduction, *P. rhoeas* successfully established self-perpetuating populations (i.e. it became naturalized) in human made habitats across Europe (Godwin, 1975; Kadereit, 1990; Willerding, 1986; Zohary and Hopf, 1994). We hypothesize that the enhanced UV-A reflectance resulting from a reduction in the content of floral flavonols promoted the attraction of novel types of pollinators, facilitating the spread of

P. rhoeas across Europe.

4. Experimental

4.1. Plant material

Plant material was raised from seeds of *Papaver rhoeas* L. (Papaveraceae) collected in the East Mediterranean at the geographic coordinate 32°29'58.09"N, 34°53'34.68"E (Israel), and Central Europe at the geographic coordinate 50°57'6.08"N, 11°37'43.20"E (Germany) in the Berlin Botanical Garden (Freie Universität Berlin, Germany), and in the glasshouse facilities of the Max Planck Institute for Chemical Ecology (Jena, Germany), and used for optical and chemical analysis, respectively. The glasshouse chamber was equipped with Philips Son-T Agro 400 W Na lights to support an illumination period of 14 h. Temperatures ranged from 19 to 21 °C in the night and from 21 to 23 °C during daytime with a relative air humidity of 50–60%.

4.2. Photography

Images of flowers were taken using a digital camera modified for increased UV sensitivity (EOS 10D, Canon USA Inc., Lake Success, NY, USA) with a quartz lens (105 mm, UV-Nikkor, Nikon, Tokyo, Japan). For UV exposures, a narrow bandpass filter was used (Baader Venus U-Filter, Baader Planetarium, Mammendorf, Germany), which consisted of a Schott UG11 substrate with dielectric coating that totally blocked wavelengths in the visible and infrared range while transmitting between 320 and 380 nm with a half-band-width of 60 nm. For exposures in the visible spectrum, we used a broad bandpass filter that transmitted light between 400 and 700 nm.

4.3. Spectral reflectance and transmittance measurements

The reflectance and transmittance spectra of petals were measured using a microspectrophotometer (MSP). The MSP consisted of a Leitz Ortholux (Leitz, Wetzlar, Germany) microscope, an Avaspec-2048 CCD detector array spectrometer (Avantes, Apeldoorn, Netherlands) and a deuterium-halogen lamp (Avantes AvaLight-D(H)-S). The microscope objective was an Olympus LUCPlanFL N 20 × /0.45. The reflectance spectra of small epidermal cell areas (10 × 10 μm²) from the adaxial petal surface were measured with a white diffuse reflectance tile (WS-2, Avantes) as reference. The transmittance spectra were measured from petal pieces immersed in water to reduce scattering losses.

4.4. Pigment extraction, purification, and quantification

Petals from 15 plants of *P. rhoeas* cultivated in the glasshouse from seeds collected in the East Mediterranean and 15 plants cultivated from seeds collected in Central Europe were harvested and sorted according to their origin and UV reflectance properties using the camera described above. Only flowers with uniform UV patterns were used for pigment extraction. The basal area of the petals was excised, the remaining apical petal parts (ca. 10 g tissue for each sort) were pooled, and grinded under liquid nitrogen, and used for all other analyses.

For analytical HPLC-PDA comparison, always 50 mg petal material was extracted with 1 ml of a 1:1 mixture of methanol (HPLC gradient grade, VWR Chemicals, Darmstadt, Germany) and acidified water (ultrapure water Milli-Q; 0.1% (v/v) formic acid, purity > 98%, Sigma-Aldrich, Steinheim, Germany) resulting in a concentration of 50 mg petal tissue per ml solvent (three replicates each of petals with high UV reflectance from Germany, and of petals with low and high UV reflectance from Israel). After 30-min sonification in an ice bath, the sample was centrifuged (16,100 g, 4 °C, 10 min) and the supernatant was used for analytical HPLC-PDA analysis (method 1, chapter 4.5). Analysis of the chromatograms/spectra and extraction of peak areas at 351 nm were done with the Agilent LC ChemStation software.

For isolation of flavonol glycosides and their structure elucidation, a composite sample of ca. 5 g apical parts of petals with low UV reflectance from Israel was extracted (1:1 mixture of methanol and acidified water/0.1% (v/v) formic acid, two times 15 min sonification), filtered (1 μ m) and dried *in vacuo*. Afterwards, SPE was performed to separate flavonols from anthocyanins. The sample was dissolved in acidified water (0.1% formic acid (v/v)) and aliquots were loaded onto Chromabond drug cartridges (silica gel phase with bifunctional modification: RP8, octylsilyl/strong anion exchanger – quaternary amine; adsorbent weight 500 mg; Macherey Nagel, Düren, Germany). After washing with acidified water, flavonols were eluted with a 3:2 mixture of methanol and acidified water (0.1% (v/v) formic acid). Anthocyanins were obtained with acidified methanol (1% (v/v) hydrochloric acid, ACS reagent, Sigma-Aldrich). Fractions were dried, subjected to preparative HPLC (method 2, chapter 4.5), and analyzed by NMR and MS.

For quantification, 0.7–1.8 g of apical parts of petals from German plants as well as of petals with high and low UV reflectance from Israeli plants were grinded under liquid N₂ cooling, and extracted with the respective volume of solvent to reach a concentration of 150 mg ml⁻¹ (1:1 mixture of methanol and acidified water/0.1% (v/v) formic acid, two times 15 min sonification in an ice bath). Samples were centrifuged (5242 g, 10 °C, 10 min), and dried *in vacuo*. By means of SPE, anthocyanins and flavonol glycosides were separated. Each sample was dissolved in acidified water (0.1% formic acid (v/v)) and 20–60 mg of the raw extracts were trapped on a Chromabond drug cartridge (silica gel phase with bifunctional modification: RP8, octylsilyl/strong anion exchanger – quaternary amine; adsorbent weight 500 mg; Macherey Nagel, Düren, Germany). After washing with acidified water (fraction 1), flavonols were eluted with a 3:2 mixture of methanol and acidified water (0.1% (v/v) formic acid, fraction 2). Anthocyanins were obtained with acidified methanol (1% (v/v) hydrochloric acid, ACS reagent, Sigma-Aldrich, fraction 3). Fractions were dried under N₂ (g) and weighted for quantification of total anthocyanin and flavonol glycoside content (Table S3). The analysis was performed with three replicates.

Afterwards, a one-way ANOVA was applied to the quantification data, followed by a Tukey HSD test to reveal which factor levels differed from each other. The analyses were performed in R 3.3.1 (R Core Team, 2016).

4.5. Chromatography

The analytical HPLC-PDA system consisted of an Agilent HP1100 series (binary pump G1312A, auto sampler G1313A, and photodiode array detector G1315B, 200–700 nm; Agilent, Waldbronn, Germany), equipped with an EC250/4 Nucleodur C18 HTec column from Macherey-Nagel (5 μ m; injection volume 20 μ L). Method 1: binary gradient linearly increasing in 21 min from 20 to 67% methanol in acidified water (0.1% (v/v) trifluoroacetic acid, purity > 99.9%, Roth), subsequent washing step (100% methanol) and equilibration at starting conditions. The flow rate was 1 ml min⁻¹ and the detection wavelengths were 211, 254, 281, 351 and 460 nm.

For preparative HPLC, a LC-20AT chromatography system (Shimadzu, Kyoto, Japan) equipped with autosampler SIL-10AP, fraction collector FRC-10A and UV/vis detector SPD-20A was used. The separation column was a VP250/10 Nucleodur C18 HTec, 5 μ m (Macherey Nagel). Method 2: 2-min focusing step with 30% acidified methanol in acidified water (0.1% (v/v) HCOOH), followed by a gradient linearly increasing in 33 min to 50% methanol, washing, and re-equilibration of the column (flow rate 3.5 ml min⁻¹). The detector was operating at 351 and 500 nm and the seven most abundant peaks at 351 nm were collected. Therefore, collection was performed between 24 and 50 min for peaks with higher intensity levels than 8000 μ V.

4.6. Structure elucidation

Dried fractions from preparative HPLC were dissolved in MeOH-*d*₄

(99.96%, Euriso-top, St.-Aubin Cedex, France). ¹H NMR, PURGE-¹H NMR, ¹H-¹H DQF COSY, HMBC, HSQC, and selective TOCSY spectra were measured at 300 K on a Bruker Avance III HD 700 MHz NMR spectrometer (Bruker Biospin, Karlsruhe, Germany), equipped with a TCI 1.7 mm microcryoprobe. The operating frequency was 700.45 MHz for ¹H and 176.13 MHz for ¹³C. The spectra were referenced to the residual solvent signals of MeOH-*d*₄ (¹H – δ 3.31, ¹³C – δ 49.15). All NMR data were processed with Bruker Topspin software version 3.1.

Ultra-high-performance liquid chromatography-high resolution electrospray ionization-mass spectrometry (UHPLC-HRESI-MS) was performed with an Ultimate 3000 series RSLC (Dionex, Sunnyvale, CA, USA) and an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). UHPLC was used with an Acclaim C18 column (150 $\times$ 2.1 mm, 2.2 μ m, Dionex) at a flow rate of 300 μ L min⁻¹ in a binary solvent system of water (solvent A) and acetonitrile (hypergrade for LC-MS, Merck, Darmstadt, Germany) (solvent B), both containing 0.1% (v/v) formic acid (eluent additive for LC-MS, Sigma Aldrich). Sample volumes were loaded onto the column and eluted by using the following binary gradient: linear increase from 0% B to 100% B within 15 min – 100% B constant for 5 min – equilibration time at 0% B for 5 min. This system was coupled to an LTQ-Orbitrap XL mass spectrometer in which ionization was accomplished using electrospray ionization (ESI). ESI source parameters were set to 4 kV for spray voltage, 35 V for transfer capillary voltage at a capillary temperature of 275 °C. The samples were measured in positive and negative ionization mode in the mass range of *m/z* 150 to 1500 using 30,000 *m/z* Δm resolving power in the Orbitrap mass analyzer. Data was evaluated and interpreted using XCALIBUR software (Thermo Fisher Scientific, Waltham, MA, USA).

4.7. Spectroscopic data of isolated compounds

4.7.1. Kaempferol 3-O-[3'-O-acetyl- β -D-glucopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside (6)

Analytical HPLC: *R*_t 38.9 min; UV/Vis (MeOH–H₂O): $\lambda_{\max}$ 267, 349 nm;

¹H NMR (700 MHz, MeOH-*d*₄): δ 8.17, 2H, d, *J* = 8.8 Hz, H-2'/6'; 6.91, 2H, d, *J* = 8.8 Hz, H-3'/5'; 6.40, 1H, d, *J* = 1.5 Hz, H-8; 6.18, 1H, d, *J* = 1.5 Hz, H-6; 5.19, 1H, d, *J* = 7.9 Hz, H-1''; 4.82, 1H, dd, *J* = 9.3, 9.4 Hz, H-3''; 4.11, 1H, d, *J* = 7.9 Hz, H-1'''; 3.98, 1H, dd, *J* = 12.0, 2.0 Hz, H-6''b; 3.76, 1H, dd, *J* = 11.9, 2.0 Hz, H-6''b; 3.61, 1H, dd, *J* = 12.0, 5.8 Hz, H-6''a; 3.59, 1H, dd, *J* = 11.9, 5.5 Hz, H-6''a; 3.43, 1H, dd, *J* = 8.7, 9.4 Hz, H-3''; 3.40, 1H, ddd, *J* = 9.6, 5.8, 2.0 Hz, H-5''; 3.37, 1H, dd, *J* = 9.4, 9.6 Hz, H-4''; 3.33, 1H, dd, *J* = 9.4, 9.6 Hz, H-4''; 3.04, 1H, ddd, *J* = 9.6, 5.5, 2.0 Hz, H-5''; 2.10, 3H, s, acetyl-CH₃.

¹³C NMR (175 MHz, MeOH-*d*₄): δ 179.0, C-4; 172.7, acetyl-COOH; 166.6, C-7; 162.9, C-5; 161.3, C-4'; 158.7, C-2; 158.4, C-9; 135.3, C-3; 132.3, C-2'/C-6'; 122.7, C-1'; 116.1, C-3'/5'; 105.5, C-10; 104.6, C-1''; 104.0, C-1'; 100.0, C-6; 78.7, C-3''; 77.8, C-3'; 77.6, C-5'; 77.2, C-5''; 75.7, C-2''; 73.0, C-2'''; 69.6, C-6''; 69.5, C-4''; 95.1, C-8; 100.0, C-6; 62.1, C-6''; 21.0, acetyl-CH₃.

UHPLC: *R*_t 10.5 min; HRESI-MS: *m/z* 651.1560 [M-H]⁺ (calcd. for C₂₉H₃₁O₁₇, 651.1556).

4.7.2. Known compounds

For ¹H and ¹³C NMR data of compounds 1–5 and 7, see Tables S1 and S2. For HRESI-MS data, see Table 1.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We like to thank Randolph Menzel, Natalia Márquez and Eru and Luna

Martínez for their support while conducting this study. We are grateful to Riya Christina Menezes for performing the LC-MS measurements.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.phytochem.2020.112457>.

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