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Temperature-Associated Effects on Flavonol Content in Field-Grown *Phaseolus vulgaris* L. Zolfino del Pratomagno

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Abstract: Combining the need to increase local *Phaseolus vulgaris* L. production, with the objective of identifying which abiotic inductors (irrigation and temperature) and/or elicitors (nitrogen fertilizer treatment) can be used as potential strategies to improve flavonol content, open-field experiments were conducted in Pisa and Bologna (Italy) over two years, using the local landrace of interest "Zolfino del Pratomagno", and a commercial variety, Verdone. Full-irrigation and nitrogen treatment, individually, and in combination, increased yield and decreased flavonols, respectively, in both genotypes and locations. Yield, under all treatments, was significantly higher in Pisa (17.5–21.9 °C) than Bologna (21.4–24.8 °C) for Verdone, but was the equivalent for Zolfino. An inverse correlation between average mean temperature and flavonol content was evident only in Zolfino. The feasibility of increasing dietary flavonol production using decreasing temperature as a strategy with some degree of control was then tested by cultivating Zolfino at different altitudes (108 (24 °C) to 800 m a.s.l. (18 °C)) in Pratomagno. Increasing the altitude induced a significant 3-fold increase in seed-coat kaempferol glycosides, comprising more than 95% of the total flavonols. Temperature-associated effects on flavonol synthesis warrants consideration when selecting a cultivation environment to augment the kaempferol-based, anticarcinogenic benefits of Zolfino.

Keywords: *Phaseolus vulgaris* L.; Zolfino; flavonols; kaempferol glycosides; abiotic inductors; temperature

1. Introduction

The Food and Agricultural Organization (FAO) of the United Nations and the 68th United Nations General Assembly declared 2016 "The Year of the Pulse". The objective was to raise global awareness of the many benefits of pulses (including the common bean), and to promote their cultivation, not only to meet the demand of an increasing human population but to obtain quality food.

The common bean (*Phaseolus vulgaris* L.) is an important nutritional source of protein, carbohydrates, minerals, and vitamins, particularly in developing countries. In addition to the nutritional value, the common bean has increasingly gained attention as a functional or nutraceutical food. This is attributable to the presence of secondary metabolite phytochemicals (including flavonoids), which are integral to the link between food and functional benefits, including antioxidant, antimutagenic, and anticarcinogenic biological activities [1,2]. With the new impetus in

functional food research, initial studies (which are still ongoing) were primarily focused on examining secondary metabolite profiles and additional functional compounds in different bean genotypes, with less focus directed towards the effect of environmental factors on accumulation [1–6].

Adaptive responses to environmental factors that induce phytochemical synthesis in crops can be triggered by abiotic inductors (drought, temperature, UV irradiation, and salinity) and chemical/biochemical elicitors, both abiotic (mineral element nutrition) and biotic [7]. Therefore, the requisite to study the impact of environmental inductors and agronomic management practices on phytochemical content, with the objective of identifying which factors can be used as suitable strategies for inducing physiological increases in phytochemicals, has become an increasingly important research focus over recent years [7]. For the common bean, given that more than 60% of global production is cultivated under non-irrigated conditions [8], particular attention has been paid to the agronomic practice of deficit or restricted irrigation, ensuring that field capacity is maintained at 40%–60%. Deficit irrigation or moderate water stress was shown to induce phytochemical content in seeds (flavonoids) with variable effects on yield [9,10] and is currently utilized in identifying more drought-resistant genotypes [9–11].

In the Italian context, legume consumption has decreased significantly and consistently from 1961 to 2015 and, to commemorate the "The Year of the Pulse", an appeal was made to increase local production [12]. Combining the need to increase local production together with improving food quality, the aim of the present study is to investigate polyphenol/flavonol content in response to abiotic inducers (irrigation and temperature) and elicitors (nitrogen fertilizer treatment) in the Italian *P. vulgaris* L. landrace "Zolfino del Pratomagno", compared to a commercial variety, Verdone. To this end, open-field experiments were conducted in both Pisa and Bologna over two consecutive years. Of interest was the inverse correlation between decreasing temperature and increasing flavonol content, evident only in Zolfino. The feasibility of improving flavonol composition in Zolfino by adopting temperature as a strategy with some degree of control in the form of altitudinal variation was then investigated in Pratomagno (Tuscany).

2. Materials and Methods

2.1. Materials

The phenolic acids, kaempferol aglycone and kaempferol glycosidic standards, as well as the reagents used for the high-performance liquid chromatography (HPLC) separation of polyphenols, were as reported previously [4]. Acetonitrile was obtained from Merck (Darmstadt, Germany). All other reagents used were of analytical grade.

2.2. Germplasm, Open-Field Experimental Locations, and Treatments

Open-field experimental trials were performed over two consecutive years (2004 and 2005) in Bologna, Italy (Ozzano, 44°25′ N, 11°28′ E, 67 m asl) and Pisa, Italy (San Piero a Grado, 43°40′ N, 10°19′ E, 4 m a.s.l.), respectively. Meteorological data (temperature and precipitation) over the cultivation period was obtained from ARPA (*Agenzia Regionale Prevenzione ed Ambiente* of the province of Emilia Romagna) and ARSIA (*Agenzia Regionale per lo Sviluppo e Innovazione nel Settore Agricolo Forestale* of Tuscany) for Bologna and Pisa, respectively. In Bologna, the average daily temperature range from sowing to harvest was 20.0 to 24.3 °C in Year 1 and 21.4 to 24.8 °C in Year 2. For Pisa, the average daily temperature range was 17.5 to 21.8 °C in Year 1 and 17.9 to 21.9 °C in Year 2. The soil at the experimental farm of Ozzano was classified as a fine, mixed, mesic, Udertic Ustochrept, with 36%, 28%, and 36% of sand, silt, and clay, respectively, and a pH (1:2.5 soil to water) of 7.7. The soil type at the experimental farm of San Piero a Grado was classified as a Typic Xerofluvent, with 67%, 16%, and 17 % of sand, silt, and clay, respectively, and a pH (1:2.5 soil to water) of 8.1.

Two *Phaseolus vulgaris* L. genotypes were sown, namely, the local landrace "Zolfino del Pratomagno" and a commercial variety, Verdone. Both accessions were grown under either non-

irrigated or full irrigation conditions during the growth cycle. Soil moisture content was measured with conventional tensiometers placed 30 cm below the soil surface in the respective plots. Irrigation was performed in irrigated plots with drip-system hoses along the rows, which were set to be activated when the water potential descended below −50 kPa. Irrigated plots were maintained at full capacity whereas non-irrigated plots, subject to natural precipitation were as follows: Bologna, *ca* 50% and 35% of field capacity for the first and second year, respectively; Pisa, *ca* 46% and 65% of field capacity for the first and second year, respectively. Both non-irrigated and irrigated treatments for both genotypes were either without fertilizer treatment or subjected to fertilizer treatment in the form of urea (46% N), of which 50 units were, respectively, distributed at sowing and after ground cover.

A strip split plot experimental design was adopted, with 3 replicate blocks. Each experimental block was comprised of the irrigation factor (non-irrigated; irrigated) in 2 principle large-scale plots (vertical factor), the genotype factor (Verdone, Zolfino) in 2 plots within each large scale plot (horizontal factor), and in turn, the nitrogen fertilization factor (no fertilizer, fertilizer) in 2 sub-plots. Each single experimental block was 12 × 12 m with 8 elemental subplots, each with a surface area of 6 × 3 m, respectively. Seeds were sown in rows at a distance of 10 cm along the row and 45 cm between rows to attain a plant density of 20 plants m^{-2}. In both years, manual sowing was performed in late March and late April for Pisa and Bologna, respectively. Seeds were harvested after 1096 ± 18 and 1146 ± 29 growing degree days for Verdone and Zolfino, respectively, corresponding to the period ranging from early-September to mid-October.

To test the effect of temperature as a strategy to improve phytochemical content in the ecotype landrace "Zolfino del Pratomagno", open-field experimental trials in the Pratomagno region (Tuscany, 43°44′34′′ N, 11°43′22′′ E) were undertaken on farm locations at 108, 150, 280, 350, 500, and 800 m a.s.l., all with a south to south-east facing orientation. In each location, a surface area of approximately 0.25 ha, seed was manually sown in lines around mid-April 2007. The soil was comprised of 61%, 24%, and 15 % of sand, silt, and clay, respectively, with a pH (1:2.5 soil to water) of 7.55. In each farm, the same agronomic practices for the main productive factors (soil tillage, seed density, fertilization, irrigation) were adopted. The pods were harvested at seed maturity in September. The overall mean daily temperatures, coinciding with seed filling, were calculated at 23.1, 22.8, 21.9, 21.4, 20.2, and 18.0 °C at 108, 150, 280, 350, 500, and 800 m a.s.l., respectively. From the pooled bean harvest in each farm, three randomized 1-kg samples were collected and used for subsequent analyses.

2.3. Polyphenol Extraction and Measurement

Total polyphenol content was extracted according to Xu and Chang [13] and measured according to the Folin Ciocalteau method [14]. Flavonoid content was measured according to the procedure reported below.

For the experiment examining the effect of altitude on the polyphenol content, phenolic acids and flavonoids were extracted from each of the constituent parts, namely, seed coat, endosperm, and embryo, according to Dinelli et al. [4].

Phenolic acids were extracted and determined according to the method of Mattila and Kumpulainen [15]. Free phenolic acids were extracted with a mixture of methanol and 10% acetic acid, whilst bound phenolic acids were liberated firstly with alkaline and then acid hydrolysis, and respectively extracted with diethyl ether/ethyl acetate (1:1). All fractions were quantified separately by HPLC–DAD (Beckman liquid chromatograph consisting of a Gold 126 multisolvent pump, a photodiode array detector Beckman 168, and a Spark Holland autosampler), as reported previously [4], and the identification of individual single phenolic acids was based on the comparison of their retention times and diode array scans with those of the authentic standards. For each detected phenolic acid, quantitative data in both the free and bound fraction were combined for the expression of the total content.

Extraction and HPLC–DAD analysis of kaempferol and the glycosidic derivatives were performed according to Dinelli et al. [4]. Identification of kaempferol aglycone, kaempferol 3-*O*-glucoside, and kaempferol 3-*O*-rutinoside was based on the comparison of their retention times and

diode array scans with those of authentic commercial standards (Indofine Co., Hillsborough, USA). The same approach was employed for the identification of kaempferol 3-*O*-diglucoside (3-*O* xylosylglucoside) and kaempferol 3-*O*-acetylglucoside by using authentic standards isolated previously [4,16].

2.4. Seed Color Intensity

For each altitude, five replicates of 20 seeds were randomly selected, and the color determined according to the procedure proposed by Sako et al. [17]. The seed images with a resolution of 720 dpi were processed using the image analysis software Assess (American Phytopathological Society Press). The color intensity of each seed was extracted by averaging, independently, the red, green, and blue components of the pixels representing the seed. The color intensity was measured as an arbitrary value ranging from 0 to 255.

2.5. Statistical Analysis

Statistical analyses were performed on each variable measured (total polyphenols, individual polyphenol constituents). A mixed model was adopted to investigate the effect of abiotic inductors and elicitors on each variable. The factors, irrigation, fertilizer treatment, and genotype were considered fixed factors, whilst year and location were considered casual effect factors. Statistical significance was assigned to differences and interactions using the SPSS General Linear Model (GLM) (SPSS Inc. Chicago, IL, USA - 1998). To investigate the effect of altitude on each variable, an analysis of variance was conducted using SYSTAT 12. A GLM and each variable were entered as the "dependent" against the "independent" factor altitude. The source of variation was considered significant at a *p* level of <0.01 or <0.05.

3. Results and Discussion

The purpose of this study is to assess which abiotic inductors (water and temperature) and elicitors (nitrogen fertilization) affect total polyphenol and flavonol content in two *Phaseolus vulgaris* L. genotypes. Thereafter, a single inductor/elicitor could be selected with the objective of choosing a plausible strategy with some degree of control for implementation in the field to augment phytochemical content. In the framework of local production, the ecotype "Zolfino del Pratomagno" is an important landrace with a dietary source of kaempferol glycosides [4,18]. Hence, Zolfino was selected, along with a commercial variety, Verdone, which was also reported to contain kaempferol glycoside flavonols [18], for comparative purposes.

3.1. Effects of Genotype, Location (Temperature), Irrigation and Fertilizer Treatment on Polyphenol and Flavonol Expression

Given that yield is an important determinant, yield measurements were also included when assessing the effect of inductors on the two genotypes under open-field conditions. Significantly higher yield, total polyphenols, and flavonols were evident in the commercial variety, Verdone, compared to the local landrace, Zolfino (Table 1). Total polyphenol content in Verdone was *ca* six times greater than that of Zolfino (Table 1), invariably attributable to the higher presence of condensed tannins and proanthocyanidins which are characteristic of more pigmented seeds [2]. Overall yield was significantly higher in Pisa (distinguishable as a cooler environment than Bologna), whereas secondary metabolite levels were significantly higher in Bologna (Table 1). Irrigation (full field capacity) resulted in a significant improvement in overall yield compared to the non-irrigated condition (*ca* 45%–50% of field capacity), similar to work reported previously [8,10,11,19] Total polyphenol and flavonol contents in both genotypes were significantly higher in non-irrigated compared to irrigated conditions, corroborating previous findings [9,10,20].

Nitrogen fertilizer treatment predictably induced an overall significant increase in yield but reduced flavonol content [20] (Table 1). Besides the effect of exogenous nitrogen fertilizer on yield and flavonol content, the soil mineral element (nutritional) composition, reported to influence

polyphenol and flavonoid accumulation [20], was not investigated in the present study. According to the Soil Survey Division Staff, USDA [21], the pH of the soils of Bologna and Pisa were classified as slightly alkaline and moderately alkaline, respectively. Interestingly, common bean genotypes cultivated in both slightly and moderately alkaline soils contained significantly higher polyphenol contents compared to genotypes cultivated in strongly to moderately acidic soils (pH 5.1–6.0), irrespective of the color [22]. The polyphenol contents reported for genotypes in the study of Chávez-Mendoza et al. [22] on slight-to-moderate alkaline conditions were equivalent to ranges of the present study. There is scarce literature on the effect of soil composition (sand, silt, clay) on the polyphenol/flavonoid contents in the areal parts of crop plants. A recent study [23] reported that a clay loam soil (36%:30%:34% for sand:silt:clay, respectively) analogous to that for Bologna was associated with a higher polyphenol and flavonoid contents in the areal parts of *Amaranthus caudatus* than other soil types including sandy loam (66%:13%:21% for sand:silt:clay, respectively) similar to that of Pisa. However, the interaction analyses performed in the present study showed an additional factor associated with specific changes in flavonol content (namely, temperature, as will be demonstrated).

Table 1. Principle factor effects (year, genotype, location, irrigation, and fertilizer treatment) on yield, and polyphenol and flavonol contents. * p significant at $p < 0.05$, ** p significant at $p < 0.01$, and ns = not significant ($n = 48$). FC = field capacity.

Principle Factors	Polyphenols (mg g^{-1})	Flavonols (µg g^{-1})	Yield (t ha^{-1})
Year 1	3.79 *	409.0 *	2.00 ns
Year 2	3.49 *	349.7 *	1.90 ns
Genotype Zolfino	1.18 **	353.9 *	1.77 **
Genotype Verdone	6.10 **	404.8 *	2.13 **
Pisa (17.5 to 21.9 °C)	3.45 **	354.8 *	2.28 *
Bologna (21.4 to 24.8 °C)	3.84 **	403.9 *	1.63 *
No Irrigation (*ca* 50% FC)	3.72 *	416.2 **	1.58 **
Full Irrigation	3.56 *	342.2 **	2.33 **
No Fertilizer	3.72 ns	409.8 **	1.61 **
Fertilizer	3.56 ns	348.9 **	2.29 **

Interactions for the principle factors (year, genotype, location, irrigation, and fertilizer treatment) on yield, polyphenols, and flavonols were then investigated. The following interactions proved significant ($p < 0.05$): Location x Irrigation, Irrigation × Fertilizer, and Location × Genotype. For the interaction Location × Irrigation, the yield was higher in Pisa than Bologna for non-irrigated (respectively, 2.0 and 1.2 t ha^{-1}) and irrigated conditions (respectively, 2.6 and 2.1 t ha^{-1}), potentially indicating that the lower average daily temperatures impacted on increasing yield in addition to the effect of irrigation. Total polyphenol content was shown to be significantly higher in non-irrigated conditions in Bologna (4.0 mg g^{-1}) than Pisa (3.5 mg g^{-1}) and similar under irrigation in both locations (3.4 mg g^{-1}). For the interaction Irrigation x Fertilizer treatment, fertilizer application resulted in a 2-fold increase in yield only under irrigated conditions (3.0 t ha^{-1}), with no effect under non-irrigated conditions (1.5 t ha^{-1}). In contrast, fertilizer application resulted in a significant decline in flavonol content with irrigation (285 µg g^{-1}) and no change under non-irrigated conditions (400 µg g^{-1}), respectively.

The interaction Location × Genotype was of particular interest and is illustrated in Table 2 for both yield and flavonol content. The higher-yielding commercial variety Verdone was more sensitive to location, whereas the local ecotype Zolfino produced similar yields in both Pisa and Bologna (Table 2), thereby proving a more resistant genotype. By examining the condition of irrigation within the interaction Genotype × Location for yield, the yield for both varieties in Bologna under non-irrigated conditions was *ca* 52.5% of that at full-irrigation, whereas for Pisa, the yield under non-irrigated conditions was *ca* 76.5% of that at full-irrigation, showing that the yield losses were minimized under

moderate water stress in the cooler environment of Pisa (Table 2). Given that increasing temperatures, drought and uneven rainfall are becoming increasingly common due to climate change [8,20], the cultivation of more resistant genotypes (such as Zolfino) and the implementation of deficit irrigation (*ca* 50% of field capacity analogous to the non-irrigated conditions within the present study) could be employed as strategies to minimize yield loss whilst sustaining increased flavonol content [9].

Table 2. The interaction Location × Genotype on yield and flavonol content. Values are the means ± SD. The interaction Location × Genotype is significant at $p < 0.05$ for yield and flavonol content. For all treatments $n = 24$, for non-irrigated $n = 12$, and for irrigated $n = 12$.

Location × Genotype	Pisa Zolfino	Bologna Zolfino	Pisa Verdone	Bologna Verdone
Yield (t ha^{-1})				
All Treatments	1.83 ± 0.63	1.71 ± 0.87	2.72 ± 0.85	1.55 ± 0.81
Non-Irrigated	1.55 ± 0.51	1.22 ± 0.38	2.43 ± 0.65	1.10 ± 0.40
Irrigated	2.09 ± 0.66	2.20 ± 0.97	3.08 ± 0.94	1.97 ± 0.87
Flavonols (µg g^{-1})				
All Treatments	388.5 ± 84.5	319.3 ± 61.0	321.0 ± 68.9	488.9 ± 136.0
Non-Irrigated	434.3 ± 85.0	350.9 ± 49.0	342.5 ± 76.0	545.0 ± 118.0
Irrigated	351.6 ± 72.0	287.6 ± 49.0	299.0 ± 55.8	431.2 ± 132.0

The interaction Location × Genotype showed higher flavonol contents for Zolfino in Pisa than Bologna, with the reverse trend evident for Verdone (Table 2). The predominant flavonol was kaempferol 3-*O*-glucoside, consistently accounting for 78.80% ± 0.53% and 78.40% ± 6.10% of the total kaempferol constituents in Zofino and Verdone, respectively. Hence, changes in flavonol content were reflected primarily by changes in kaempferol 3-*O*-glucoside. Although flavonol content for both varieties was higher in non-irrigated conditions within each respective environment, the overall higher levels found in Zolfino and Verdone for the locations, Pisa and Bologna, respectively (Table 2), suggested that an additional factor was impacting in an inverse manner on the two accessions.

Given that temperature variation was a distinguishing factor between the two locations, the temperature was then analyzed. The average mean temperatures throughout the crop cycle (from sowing to harvest) were negatively correlated to flavonol content in Zolfino for the respective treatment combinations, with non-irrigated + fertilizer ($p < 0.05$) and irrigated + no fertilizer ($p < 0.01$) attaining significance. Lower temperatures were, similarly, shown to produce the highest isoflavone content in soybean [24]. In contrast, for Verdone, a positive correlation between increasing temperature and flavonol content was evident throughout the crop cycle, but was particularly significant during the seed filling period with all treatments attaining significance at non-irrigated + no fertilizer ($p < 0.05$), non-irrigated + fertilizer ($p < 0.01$), irrigated + no fertilizer ($p < 0.05$), and irrigated + fertilizer ($p < 0.01$), respectively. Interestingly, flavonol content in both genotypes is distinguished by the kaempferol chemotype, yet synthesis of the respective kaempferol derivatives appeared to be regulated differently with regard to temperature. Future studies are warranted to further investigate this phenomenon.

3.2. Effects of Altitude (Temperature) on Flavonol Constituent Expression and Distribution within the Ecoptype "Zolfino del Pratomagno"

Given that yield in the local landrace of interest, "Zolfino del Pratomagno", was unaffected by location (temperature) and that increasing flavonol content was correlated to lower temperature over a two-year period, the feasibility of increasing flavonol production, using temperature as a strategy with some degree of control, was tested by cultivating Zolfino at different altitudes in Pratomagno. Since there is interest in promoting local ecotype production, and "Zolfino del Pratomagno" is most often cultivated in hilly and mountainous regions, this genotype was investigated. Moreover, increases in bioactive kaempferol glycosides are of particular interest as the latter shown to be specifically important in reducing the risk of chronic diseases, particularly cancer growth, by acting

on a range of intracellular as well as extracellular targets involved in the cell signaling pathways linked to apoptosis, angiogenesis, and inflammation [25]. In order to examine the effect of decreasing temperature on flavonol content, without confounding factors attributable to differences in soil water content and mineral nutrition, all experimental sites at the different altitudes were subject to the same agronomic management practices. The analysis was expanded to investigate flavonol and phenolic constituents, as well as the distribution within the seed with increasing altitude (decreasing temperature).

The flavonols in Zolfino were composed of the flavonol aglycone kaempferol, as well as kaempferol glycosides, including kaempferol 3-*O*-glucoside, kaempferol 3-*O*-xylosylglucoside, kaempferol 3-*O*-acetylglucoside, and kaempferol 3-*O*-rutinoside. The phenolic acids included ferulic acid and benzoic acids (hydroxybenzoic and vanillic acids). Total flavonol content increased significantly ($p < 0.01$) from 150 ± 40 to 444 ± 25 µg g^{-1} from 108 (23.1 °C) to 800 m a.s.l (18.0 °C), respectively. No significant differences were obtained in total phenolic acid content along the altitude gradient. Kaempferol, the substrate for the glycosidic derivatives, decreased significantly with increasing altitude, whereas a significant increase was evident in the monoglucosidic product kaempferol 3-*O*-glucoside (Figure 1A). The diglycosidic product, kaempferol 3-*O*-xylosylglucoside (synthesized by the addition of second sugar moiety onto kaempferol 3-*O*-glucoside), was the second-largest kaempferol constituent and was similarly shown to increase significantly with altitude (Figure 1B). Kaempferol 3-*O*-acetylglucoside (an alternative compound synthesized from monoglucosidic kaempferol) increased between 500 and 800 m a.s.l., whereas kaempferol 3-*O*-rutinoside decreased between 108 and 350 m a.s.l. (Figure 1B,C). Similar to the Mexican, Peruvian, and Brazilian landraces [3,5], two classes of phenolic acids were distinguished in Zolfino, namely, the benzoic acid and *p*-coumaric acid derivatives. The benzoic acids included *p*-hydroxybenzoic and vanillic acid, whereas the only *p*-coumaric acid derivative detected was ferulic acid. A significant incremental increase in ferulic acid accumulation was evident between 108 and 500 m a.s.l., whereas the inverse trend was evident for the benzoic acids between 108 and 350 m a.s.l. (Figure 1D), resulting in the consistent level of phenolic acids for the different altitudes investigated.
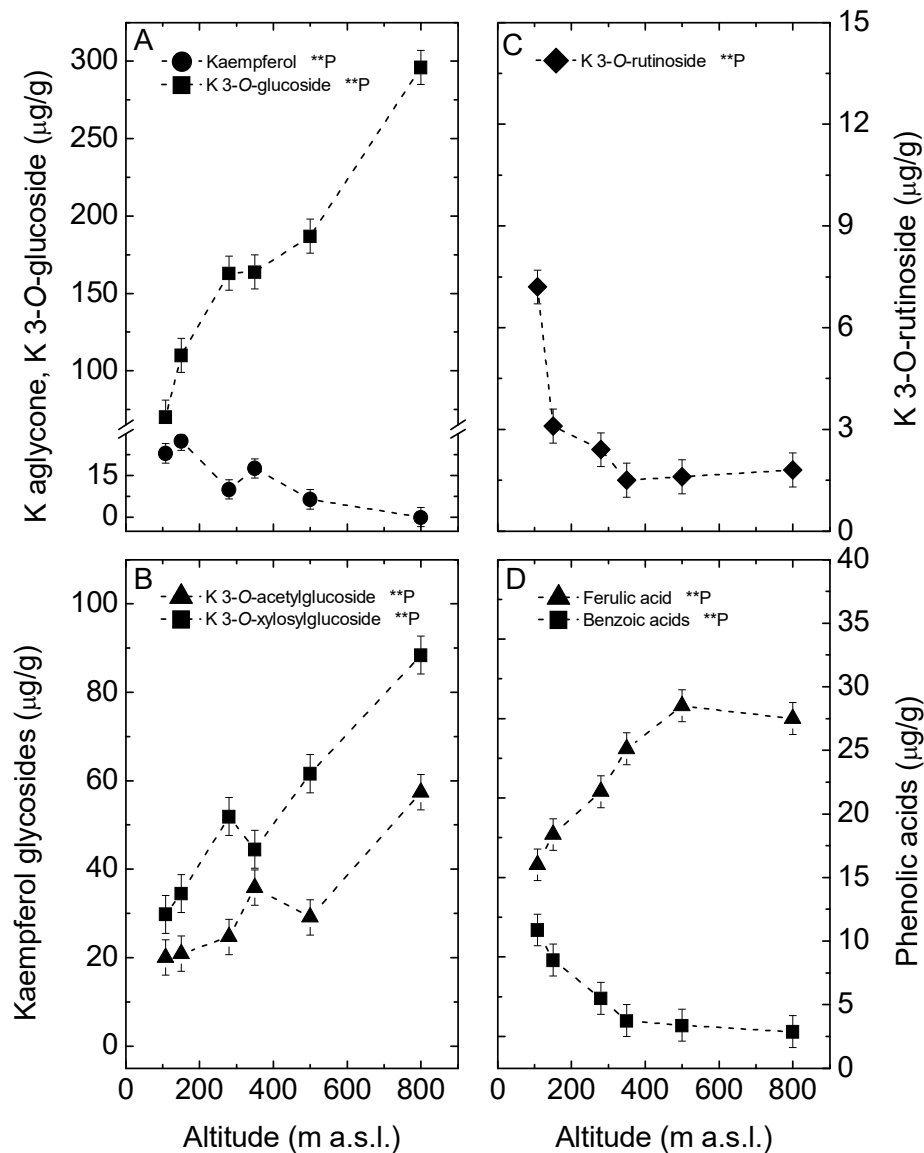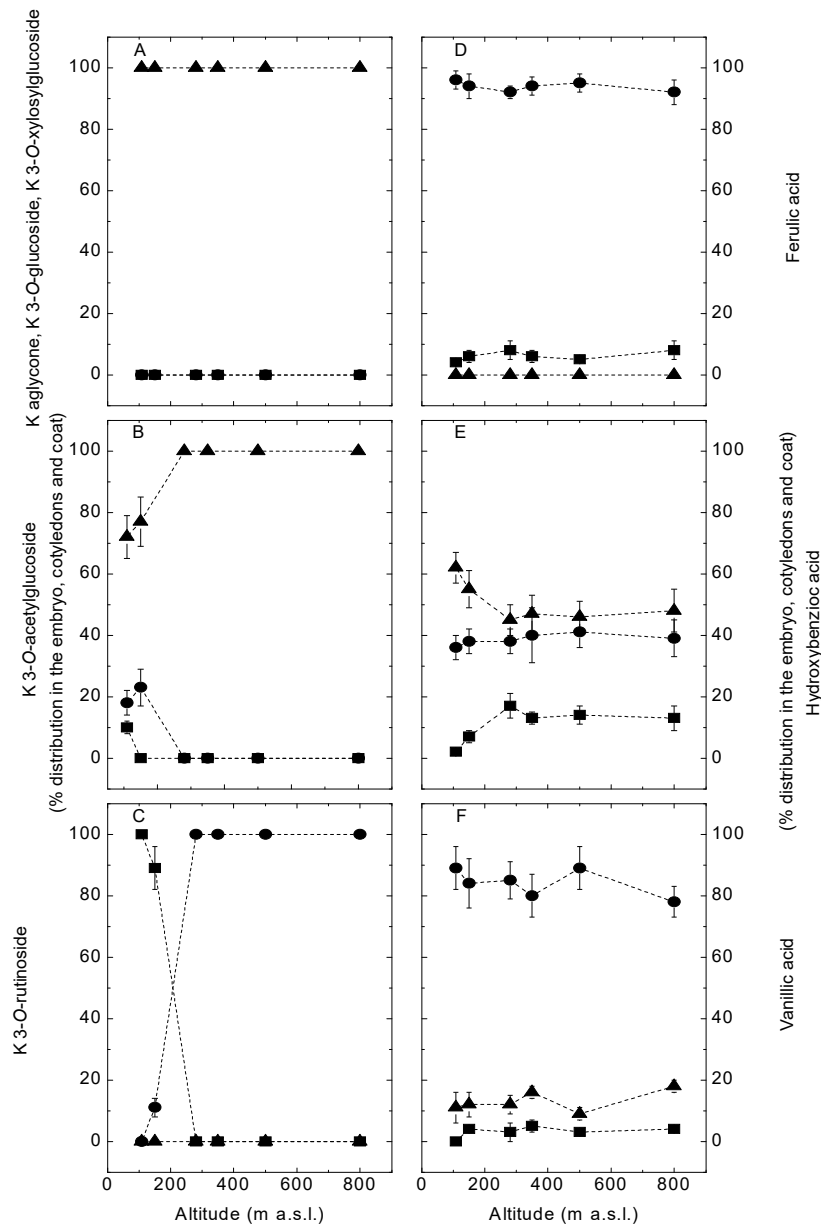
Figure 1. Altitudinal variation in the levels of (**A**) kaempferol aglycone and kaempferol 3-*O*-glucoside,
(**B**) kaempferol 3-*O*-diglucoside and kaempferol 3-*O* acetylglucoside, (**C**) kaempferol 3-*O*-rutinoside,
and (**D**) ferulic acid and benzoic acids in "Zolfino del Pratomagno". Superscripts were significant at
** $p < 0.01$. For each altitude, $n = 6$.

To our knowledge, the present results show for the first time the effect of lower temperatures
inducing increased kaempferol flavonols in a common bean genotype, both at altitudes inferior to
100 m a.s.l. (Pisa) as well as in altitudes varying from 108 to 800 m a.s.l. Increasing altitude resulted
in a 3.0-fold increase in the kaempferol glycosides. Soil composition and pH were constant, as were
management practices along the altitude gradient. Increased flavonoid accumulation has been shown
to be associated with increased UV radiation and colder temperatures, evident at higher altitudes
and northern latitudes [26]. In developing seeds not directly exposed to direct UV-B radiation, the
temperature may likely be the principal environmental factor, although UV-B radiation-mediated
stimulation of phenylpropanoid metabolism via signal transduction cannot be excluded. Previous

research has shown an increased quercetin-to -kaempferol type flavonol ratio, strongly correlated to a decrease in temperature for field-grown *Arnica montana* and *Brassica oleracea* [27,28]. The increase in the quercetin to kaempferol ratio was suggested to be more dependent on temperature than on UV-B radiation [26]. Water deficit was similarly shown to the lead to an increased quercetin-to-kaempferol glycoside ratio in white clover [29], similarly suggesting that abiotic stress promotes the synthesis of quercetin glycosides. However, common bean genotypes vary in flavonol chemotype [7,30]. Flavonol glycosides were previously quantified in the seed coats, with pinto beans, beige/brown in color, possessing kaempferol glycosides, light and dark red kidney beans possessing quercetin glycosides, and black beans possessing kaempferol, quercetin, and myricetin glycosides [30]. Hence, quercetin production is not evident for all common bean genotypes, including Zolfino [4,18]. To date, a decreasing temperature has been shown to induce an increase in the quercetin-to-kaempferol ratio in various leafy vegetables, rich in quercetin glycosides [27,28,30]. Whether lower temperature would favor the production of quercetin glycosides over that of kaempferol glycosides in common bean varieties that possess both flavonol chemotypes remains to be investigated. Nonetheless, there is a temperature associated effect on kaempferol glycoside production in Zolfino, which is interesting in terms of maximizing the synthesis of these components, given their specific importance in the role of reducing cancer growth [25].

Although it is well-reported that the seed coat of the common bean has the highest proportion of polyphenols, [4,7,22,30], the distribution of individual constituents in the different seed fractions (seed coat, cotyledons and embryonic axis) has not been reported for a temperatures gradient in the form of altitudinal variation. In Zolfino, the seed coat, cotyledons and embryonic axis represented 8.86% ± 0.45%, 89.65% ± 0.53%, and 1.49% ± 0.18% of the seed (n = 30). Kaempferol aglycone, kaempferol 3-*O*-glucoside, and kaempferol 3-*O*-xylosylglucoside were present exclusively within the seed coat at all altitudes investigated (Figure 2A). Kaempferol 3-*O*-acetylglucoside was present exclusively in the seed coat between 280 and 800 m a.s.l., with 10% present in the embryo only at 108 m a.s.l. and *ca* 20% in the cotyledons only at 108–150 m a.s.l. (Figure 2B). Kaempferol 3-*O*-rutinoside was present exclusively in the embryo at 108 m a.s.l. and in the cotyledons between 280 and 800 m a.s.l. (Figure 2C). Kaempferol compounds have been shown to be present in cream and yellow-colored common beans [3,5]. Present results corroborated previous findings showing that the dominant polyphenol measured in yellow bean seed coats was kaempferol 3-*O*-glucoside [31]. Given that the kaempferol derivatives imparted the characteristic pale yellow sulfur ("zolfo" in Italian) color to Zolfino [4], color intensity was measured. Color intensity increased significantly ($p < 0.01$) with increasing altitude as follows: 150 ± 1.89, 151 ± 1.02, 154 ± 0.89, 153 ± 1.1, 156 ± 0.59, and 160.30 ± 0.68 μg/g at 108, 150, 280, 350, 500, and 800 m a.s.l, respectively. Total flavonoid content was significantly correlated to color intensity (r = 0.424, $p < 0.05$, n = 36). The use of grain color indexes may serve to provide an easy indirect measure of flavonol accumulation.

Figure 2. Distribution (%) in the seed coat (▲), cotyledons (●) and embryonic axis (■) of (**A**) kaempferol aglycone, kaempferol 3-*O*-glucoside and kaempferol 3-*O*-diglucoside, (**B**) kaempferol 3-*O* acetylglucoside, (**C**) kaempferol 3-*O*-rutinoside, (**D**) ferulic acid, (**E**) *p*-hydroxybenzoic acid, and (**F**) vanillic acid of "Zolfino del Pratomagno" at different altitudes. For each altitude, *n* = 6.

Ferulic acid was present predominantly in the cotyledons, with less than 10% existing in the embryo at all altitudes. Both *p*-hydroxybenzoic and vanillic acid (Figure 2E,F) were present in all seed fractions, with the lowest percentage distribution in the embryo. Within the embryo, the *p*-hydroxybenzoic acid percentage at 108–150 m a.s.l. was significantly lower and was mirrored by a significantly higher percentage in the seed coat (Figure 2E), whilst distribution in the cotyledons remained consistent. Vanillic acid was present predominantly in the cotyledons at all altitudes

(Figure 2F). Total phenolic acid content remained consistent with altitudinal variation and was shown to be located primarily in the cotyledons, as has been reported previously for other common bean varieties [1,5].

The present work shows, for the first time, a temperature-associated accumulation in kaempferol derivatives. From the literature, it is shown that cells of the seed coat, cotyledons, and embryonic axis tissues have been shown to be independently equipped with phenylpropanoid metabolic activity [32]. Whether phenolic acids and flavonoids measured in the different seed components of the present study were a result of either synthesis in the respective cells or a result of transport is not known. Nonetheless, flavonol accumulation of Zolfino evidently commenced with an increased carbon flux into secondary (phenylpropanoid) metabolism via phenolic acid and flavanone (naringenin) intermediates, respectively, to the first detectable primary flavonol substrate, kaempferol aglycone. Despite the incremental reduction in the aglycone content with decreasing temperature, flux through this compound was increased, evident from the accumulation kaempferol 3-*O*-glucoside, kaempferol 3-*O*-xylosylglucoside and kaempferol 3-*O*-acetylglucoside within the seed-coat cells (Figure 3A), which collectively contributed to more than 95% of the flavonol content. Though flavonoids are found in most compartments of the cell, most conjugated flavonols, synthesized and glycosylated in the cytoplasm, are transported either via vesicles or membrane transporters to the vacuole [32]. The decline in the seed-coat benzoic acids likely reflected the preferential directional flux of phenolic acids towards flavonol biosynthesis with increasing altitude (Figure 3A). In the cotyledons of Zolfino (Figure 3B), the concomitant increase and decrease in ferulic acid and benzoic acids, respectively, suggested a preferential flux from trans-cinnamic acid towards *p*-coumaric acid with increasing altitude. The simultaneous decrease in the flux of phenolic acid substrates towards flavonol biosynthesis was reflected by the decrease in kaempferol 3-*O*-rutinoside (the only kaempferol glycoside detectable in the cotyledons between 150 and 800 m a.s.l.), and the disappearance of kaempferol 3-*O*-acetylglucoside after 150 m (Figure 3B). The latter two components were also detected in the embryo, but only at the lowest altitudes. In the embryo, increasing altitude was associated with unchanging trace quantities of phenolic acids. The reason for the presence of trace levels of kaempferol derivatives in the cotyledons and embryos at the lower altitudes is unknown.

Figure 3. Simplified diagram showing polyphenol composition in relation to increasing altitude in the seed coat (**A**) and cotyledons (**B**). Only the constituents detectable in the respective seed fractions were illustrated in circles with the accompanying arrows indicating either an increased or decreased content with increasing altitude. The width of the arrow next to each component is a relative indication of the pool size with increasing altitude.

4. Conclusions

The present study is aimed at identifying which abiotic inductors (irrigation and temperature) and/or elicitors (nitrogen fertilizer treatment) could potentially be used as strategies to augment kaempferol flavonols [4] in the local landrace of interest "Zolfino del Pratomagno". Total polyphenol and flavonol content in both Zolfino and a commercial variety, Verdone (similarly containing kaempferol flavonols [18] for comparative purposes), were significantly higher under non-irrigated conditions (*ca* 45%–50% of field capacity) compared to full irrigation, corroborating previous findings [9,10]. Moreover, nitrogen fertilizer treatment induced a reduction in flavonol content in both

genotypes. Of great interest was the inverse correlation between average mean temperature throughout the crop cycle over the two-year period and flavonol content, evident only in Zolfino. Lower temperatures, either in cooler areas at lower altitudes or with altitudinal variation (108 (24 °C) to 800 m a.s.l. (18 °C)), promoted flavonol synthesis in the local landrace Zolfino. The most predominant kaempferol glycoside present was kaempferol 3-*O*-glucoside. Increasing the altitude induced a significant 3-fold increase in seed-coat kaempferol glycosides, comprising more than 95% of the total flavonol content. To the best of our knowledge, the present study is the first to show a temperature-associated effect on kaempferol glycoside expression. Previous findings have shown that decreased temperature induced an increase in the quercetin to kaempferol ratio in other crops [26–28]. Unlike many common bean genotypes, Zolfino is not shown to produce quercetin glycoside biosynthesis [4,18]. Temperature-associated effects on kaempferol biosynthesis warrant consideration when selecting a cultivation environment to augment the kaempferol-based anticarcinogenic benefits [25] present in this genotype. Future research worthy of investigation would be to introduce the factor of no-irrigation (and/or deficit irrigation), shown both by this article and previous studies [9–11] to increase flavonol content, to investigate a possible synergy or combined effect with temperatures at different altitudes. A recent study showed that the Manteca yellow bean (distinctive for the accumulation of seed-coat kaempferol 3-*O*-glucoside) does not impair the absorption of iron, unlike black and red seed types that inhibit iron absorption [31]. Future studies could be similarly aimed at investigating ion bioavailability in Zolfino in order to further promote and support local production for additional functional aspects.

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