1. Introduction

*Arachis hypogaea* Linn. (*A. hypogaea* L.), commonly called peanut, groundnut, mongbhal, earthnut, goober, and monkeynut, is an annual legume with the potential for nitrogen fixation (Dwivedi et al. 2003; Bertoli et al. 2015). Numerous uses of peanut in agriculture and industry have made it a potential cash crop for developing and developed countries (Guchi 2015). Apart from being used in producing oil the peanut is used commercially to produce peanut processors (peanut butter and peanut candy, etc.) biodiesel, peanut flour, and snacks (Arya et al. 2016). Bioactive compounds, including polyphenols, antioxidants, saponins, resveratrol, alkaloids, tocopherols, proteins, minerals, and vitamins, besides promising fatty acid profile and essential micronutrients increase the overall market of peanuts (Duncan et al. 2006; Cherif et al. 2010; Sim et al. 2012). These bioactive compounds are the target of many pharmaceutical and nutraceutical industries due to their health-promoting properties. Moreover, these compounds impart beneficial physiological and nutritional properties to peanuts (Chung et al. 2016). Among them, flavonoids hold prominent position as they are potential antioxidants in plants and protect them from oxidative stress (Andersen and Markham 2005).

Flavonoids are primarily pigments of a plant. Although no definite physiological role has been established many studies indicated their role as phytoalexins, oviposition stimulants, protectants against ultraviolet (UV) radiations, allelochemicals, disease-resistant agents, photoreceptors, and signals for nodulation and antioxidants (Adhikari et al. 2019; Nabavi et al. 2020). Flavonoids are mainly distributed in the skins of peanuts as procyanidins and epicatechins. Other flavonoids extracted from peanuts include dihydroquercetin, 5,7-dimethoxyisoflavone, Cyanidin 3-sambubioside, rutin, Luteolin methyl ether, Rhodinin A, Quercetin, Isorhamnetin-3-O-β-D-glu, 3′,5,7-trihydroxy-4′-methoxyisoflavone, etc. (Mingrou et al. 2022). Generally, *Chalcone synthase* (CHS) acts on the baseline structure of flavonoids formed by coumaroyl-CoA and three molecules of malonyl-CoA, and forms naringenin chalcone. *Chalcone isomerase* (CHI) forms naringenin from naringenin chalcone. *Flavanone-3-hydroxylase* (F3H) adds the –OH group to flavanone at position 3 to synthesize dihydroflavonol. Moreover, F3H adds the –OH group to B-ring at the 3′ or 5′ position to produce dihydroquercetin. *Flavonol synthase* (FLS) transforms dihydroflavonols flavonols, while *Dihydroflavonol 4-reductase* (DFR) catalyzes the production of proanthocyanidin and anthocyanin and competes with FLS for dihydroflavonol to synthesize leucoanthocyanidins. *Anthocyanidin synthase* (ANS) produces leucoanthocyanidins from anthocyanidin, while *Anthocyanidin reductase* (ANR) and *Leucoanthocyanidin 4-reductase* (LAR) catalyze the reduction of leucoanthocyanidin to proanthocyanidins and catechin, respectively (Falcone Ferrery et al. 2012; Petrusa et al. 2013; Saito et al. 2013).

The composition and accumulation of flavonoids are influenced by temperature fluctuations, availability of nutrients, light exposure, UV damage, and water availability (Liu et al. 2013). Water-deficient condition causes oxidative stress in plants increasing polyphenolic compounds such as flavonoids (Akula and Ravishankar 2011; Medda et al. 2021). The oxidative stress leading to the synthesis of flavonoids results...
in reactive oxygen species (ROS) scavenging during drought stress (Cruz de Carvalho 2008; Kubra et al., 2021b). Therefore, the response of flavonoid biosynthetic pathway genes during drought stress is critically important to understanding the stress response mechanism in plants. Moreover, photoperiod is one of the essential mediators of plant’s growth and development. It is also one of the keen regulators of plant flavonoid biosynthesis. A longer light period in photoperiod duration can induce flavonoid production because it increases light-related energies in plants (Koyama et al. 2012).

From this study, we understand the biosynthesis and accumulation of flavonoids under these essential mediators of flavonoids in potential crop plants i.e. peanut, which is rich in biologically active flavonoids. Moreover, we hypothesized the coordination pattern among the expression of flavonoid biosynthetic enzymes irrespective of variable stimuli to produce a specific set of flavonoids. Two peanut varieties were selected based on yield trials. Bari 2011 was superior to PG 1247 in yield potential, drought tolerance, and resistance against fungal attacks (Naeeem-ud-Din et al. 2012). The study also provides preliminary data to unravel the coordination among the expression of flavonoid biosynthetic genes and indicated the possible role of flavonoids in plant metabolism.

2. Materials and methods

2.1. Seed procurement and germination

Peanut seeds were obtained from Oilseed Research Programme, National Agricultural Research Centre (NARC), Pakistan, which maintains germplasms of oilseed crops to evaluate candidate varieties, oilseed hybrids for adaptability, resistance to insect pests/diseases, conduct and maintain yield trials and oil quality. Ten varieties collected were subjected to a germination rate test. Bari 2011, Golden, Bard-479, Pothwar, Bari 2000 and PG-1247 showed a more than 70% germination rate. Two peanut varieties were selected based on germination rate, yield trials, and color of the seed coat. Bari 2011 was superior to PG 1247 in yield potential, drought tolerance, and resistance against fungal attacks (Naeeem-ud-Din et al. 2012). The color of the seed was also included in the various inclusion criteria as it depends on flavonoids. PG 1247 has a tan seed coat, while Bari 2011 has a variegated light tan seed coat color pattern. Experiments on the selected peanut varieties were conducted after approval from the institutional review board, ASAB, NUST, Pakistan. The procured seeds were dehusked and sterilized with 70% ethanol for 2 min followed by soaking in autoclaved distilled water for 3–5 min to remove residual alcohol. Seeds were air-dried before keeping them at 4°C for 48 h to break dormancy. Seeds were germinated by placing them in a sterilized-damped cotton wool layer at 25°C in the dark.

2.2. Seeding growth and photoperiod treatment

Seeds with uniform growth were planted in an autoclaved mixture of coconut husk and soil in a 1:1 ratio. Plants of each variety were divided into four groups with five replicates. Plants of each group were grown approximately for three weeks in four growth chambers under variable light/dark conditions (8/16 h (8 h light), 12/12 h (12 h light), 16/8 h (16 h light), and 24/0 h (24 h light)) (Cox 1978; Carvalho et al. 2010). Plants with 16 h light conditions were selected as the control group. The peanut is a summer crop and usually grows in the 16 h light condition. Photosynthetic active light was provided through 32W white fluorescent tube light, Phillips, with a light intensity of 2650 lm. Growth room temperature was kept at 28°C. Plants were watered regularly according to pot capacity measured through the method explained by Lopez and Barclay (2017). Leaves of both peanut varieties were harvested at day 7 and day 15 and immediately freeze-dried in liquid nitrogen, and were kept at −80°C until further processing.

2.3. Plant growth and water-deficient treatment

Uniformly germinated seeds of two selected peanut varieties were grown using the cigar roll method (Zhu et al. 2005). Germinated seeds were wrapped between two autoclaved filter paper sheets of A5 size as cigar rolls in sterilized conditions. These cigar rolls were soaked vertically in a Hoagland solution (Phytotech Labs, US) in 1L beakers. Approximately 8–10 cigar rolls were placed in each beaker regularly refilled with the Hoagland solution. Beakers were kept in controlled environment conditions, including 16/8 h light and dark conditions and a temperature range of 23–26°C. Based on the methodology adopted by Merchuk-Ovnat et al. (2017), two-week-old seedlings were divided into well-watered seedlings or control group (ww) and water-deficient seedlings treated group (wd) with 20 plants in each group. A 1L beaker containing plants in cigar rolls was supplemented with the Hoagland solution to maintain 4 cm of water level, while wd was left to dry gradually. Plants were allowed to grow for one week in a controlled environment with a temperature of 25°C and 16/8 h light and dark conditions. Leaf samples from the control and treated groups were collected on day 2, day 4, and day 8 in plastic tubes followed by a quick freeze in liquid nitrogen and stored at −80°C. Each treatment was repeated three times.

2.4. Relative gene expression analysis

Total RNA from leaf samples was extracted through TRIzol/Tri-reagent RNA extraction, as described by Jaakola et al. (2001). RNA quality and quantity analysis was performed using NanoDrop™2000/2000c Spectrophotometers (ThermoFisher Scientific, USA) and agarose gel electrophoresis before converting it into cDNA. ThermoFisher Scientific™RevertAid First Strand cDNA Synthesis Kit was used to synthesize single-stranded cDNA, while oligo dT primer was used. RT-PCR of beta-actin (housekeeping gene) was carried out to confirm the synthesis of cDNA. The relative gene expression was performed according to manual instructions provided with Thermo-Fisher Scientific Maxima SYBR Green/ROX qPCR Master Mix using a 7500 Fast Real-Time PCR system (Applied Biosystem, USA). The primer efficiency was calculated using a standard curve analysis. Each experiment was performed in triplicate. For primer designing, sequences for each gene were retrieved from the National Center for Biotechnology Information (NCBI) and PeanutDB. Primer3 (https://bioinfo.ut.ee/primer3-0.4.0/) was used to design the primer sets used in this study. Primer
sequences and gene information have been provided in supplementary Table S1. Relative gene expression was calculated by the Livak method (2^-ΔΔCt method) (Livak and Schmittgen 2001).

### 2.5. Spectrofluorometry of leaves and taproot

The accumulation pattern of flavonoids in the leaves and taproot of all the studied groups of Bari 2011 was studied using the spectrofluorometer system (FluoroMax-4, Horiba Scientific, USA), which was operated by FluorEssence software version 3.5 (Horiba Scientific, USA) (Ali et al. 2018; Kubra et al. 2021a). At least three fully-grown leaf were freshly plucked and subjected to spectrofluorometry. Synchronous spectra with a 200–700 nm excitation range and 60 offsets were recorded to evaluate flavonoid accumulation in the leaves of treated and control groups. Emission spectra with 395–550 nm range after excitation at 380 nm were recorded for the thoroughly washed tap root to analyze flavonoid accumulation. All recorded spectra were normalized using GraphPad Prism® version 9 (GraphPad Software Inc., USA). Before calculating the mean spectra for each studied group, data were analyzed for outliers.

### 2.6. Statistical analysis

Experimental data collected from biological replicate and triplicate were used to perform statistical analysis. GraphPad Prism® version 9 (GraphPad Software Inc., USA) and Microsoft Excel 365 (Microsoft, USA) performed all the statistical analyses. To analyze inter-varietal variation, a two-way ANOVA with Bonferroni post hoc test was performed to determine the significance of variation. Pearson’s correlation coefficient was determined using GraphPad Prism® version 9 to find an association between selected variables. GraphPad Prism® version 9 (GraphPad Software Inc., USA) and CorelDRAW Graphics Suite 2020 (Corel Corp. Canada) were used to prepare graphs and figures.

### 3. Results

#### 3.1. Expression analysis of flavonoid biosynthetic genes under variable photoperiod duration

Transcript profiling of flavonoid biosynthetic genes indicated the time-dependent expression of genes except that of AhFLS in both selected varieties. On day 7, the treated plants depicted significantly higher expression of AhCHS, AhCHI, AhFLS, AhLAR, AhANS, AhF3H, and AhDFR compared to those...
treated with 16 hr light treatment in PG1247 (Figure 1). On the other hand, Bari 2011 showed upregulation in the expression of AhCHS, AhF3H, AhFLS, AhDFR, and AhLAR at all photoperiod treatments compared to the control group on day 15 (Figure 2). According to a two-way ANOVA, the F-value for interaction was highest for AhLAR on days 7 and 15, while the F-value for interaction was lowest for AhDFR on day 7 and AhCHI on day 15 (Table 1). As shown in Figure 5(b), maximum variation in the expression was observed in Bari 2011 on day 15, whereas maximum variation in expression was observed in PG1247 on day 7.

3.2. Correlation analysis of flavonoid biosynthetic genes under variable photoperiod duration

Correlation analysis between light duration and expression of the genes revealed that the expression of AhCHI and AhFLS is positively correlated with daylight except on day 15 in Bari 2011 (Table 2). On the other hand, AhANS and AhF3H showed a negative association with daylight in both varieties. While little to no association was observed between AhDFR and photoperiod. The correlation analysis also revealed the dependency of expression among flavonoid biosynthetic genes under light treatment (Figure 4). In Bari 2011, a higher degree of positive association was observed between the expression AhLAR with AhF3H \((r = 0.9392)\) and AhANS \((r = 0.9392)\), while the moderate positive association was observed between the expression AhCHS with AhF3H \((r = 0.6021)\), AhFLS \((r = 0.7992)\), AhDFR \((r = 0.682)\) and AhLAR \((r = 0.7148)\) under photoperiod treatment. On the contrary, AhCHI showed a moderate negative correlation with the expression of AhF3H \((r = −0.7448)\), AhLAR \((r = −0.783)\), and AhDFR \((r = −0.5242)\) in Bari 2011 under light treatment. In PG1247, all genes showed a moderate to strong positive association with the expression of all genes except that of AhLAR under photoperiod treatment.

3.3. Relative flavonoid biosynthetic genes expression in response to water-deficient condition

Many genes depicted a low degree of variation within varieties and water-deficient treatments (Table 1). However, AhANS showed the highest variation for stress duration and varieties. The expression of AhCHS, AhCHI, and AhF3H was upregulated in PG1247 and Bari 2011 on day 2. Whereas AhFLS and AhDFR were significantly
upregulated in Bari 2011 only. Furthermore, the prolonged water-deficient condition led to the downregulation of AhCHS, AhCHI, AhFLS, and AhDFR in PG1247 and Bari 2011. The expression of AhF3H was upregulated in PG1247 and Bari 2011, but AhANS was upregulated in PG1247 only on days 4 and 8 (Figure 3).

### 3.4. Correlation analysis of flavonoid biosynthetic genes in response to water-deficient condition

Correlation analysis showed a strong to moderate negative association between water-deficient conditions and the expressions of AhCHS, AhDFR, AhFLS, and AhCHI, while a strong to moderate positive correlation was recorded between water deficiency and the expression of AhLAR and AhANS in both varieties (Table 2). The correlation analysis showed that the expression of AhANS has a strong to moderate negative relation with the expression of all the genes in both varieties except with AhLAR ($r = 0.9997$) in PG1247 under water-deficient conditions (Figure 4). AhCHI showed a positive association with AhF3H and AhFLS, while AhLAR indicated a negative association with AhDFR in both peanut varieties. AhLAR indicated a positive association with AhCHI, AhF3H, and AhFLS in Bari 2011, while a negative association with AhCHI, AhF3H, and AhFLS in PG 1247. AhFLS indicated a positive relation with AhF3H and AhCHI, while AhCHS showed a positive strong relation with AhDFR expression in both varieties under water-deficient conditions.

### 3.5. Accumulation of flavonoid in leaves and taproot in response to water-deficient conditions

The effect of photoperiod and water-deficient conditions on flavonoid accumulation was evaluated through the spectrophotometer analysis of taproot and leaves in Bari 2011. The leaf samples of water-deficient-treated plants displayed seven prominent florescent regions, 330–350, 378, 390–425, 449–460, 463, 510–530 and 579–605 nm, which correspond to catechins (Pal et al. 2012; Holser 2014), apigenins (Park et al. 2013; Tu et al. 2016), non-acylated flavonol glucosides (Donaldson and Williams 2018), kaempferols, flavonols, flavones (Guharay et al. 2001; Voicescu et al. 2014), and anthocyanins (Drabent et al. 1999), respectively (Figure 5 (a)). Under water-deficient conditions, leaves of the control group had lower accumulation on days 2 and day 8 compared to plants that were grown in water-deficient conditions. But, on day 4, leaves of water-deficient plants showed a higher accumulation of flavonoids compared to control plants. The day 8 control and day 4 treatment showed a higher accumulation of apigenin, non-acylated

### Table 1. F-value computed through a two-way ANOVA showing variation among the expression of flavonoid biosynthetic genes under photoperiod and water-deficient treatments.

| Factors | Flavonoid Biosynthetic Genes |
|---------|-------------------------------|
| Photoperiod Day 7 | AhCHS | AhCHI | AhF3H | AhANS | AhFLS | AhDFR | AhLAR |
| Light duration | AhCHS | AhCHI | AhF3H | AhANS | AhFLS | AhDFR | AhLAR |
| Varieties | 12.43*** | 5.26*** | 5.53*** | 216.5*** | 3.94** | 16.56*** | 0.186 |
| Light duration*Varieties | AhCHS | AhCHI | AhF3H | AhANS | AhFLS | AhDFR | AhLAR |
| Correlation of expression of flavonoid biosynthetic genes with photoperiod and water-deficient conditions. | r= Pearson r that showing a correlation between two variables. p-value less than 0.05 is considered to be statistically significant.*p < 0.05; **p < 0.01; ***p < 0.001.

### Table 2. Correlation of expression of flavonoid biosynthetic genes with photoperiod and water-deficient conditions.

| Genes | Bari 2011 | Bari 2011 | PG 1247 | PG 1247 | Water Deficiency |
|-------|-----------|-----------|---------|---------|-----------------|
| Photoperiod | Day 7 | Day 15 | Day 7 | Day 15 | Day 7 | Day 15 |
| AhCHS | 0.8948 | 0.2947 | 0.8927 | 0.8691 | 0.9838 | 0.1149 | 0.1601 | 0.2005 |
| r | 0.03846 | 0.9575 | 0.9765 | 0.8253 | 0.8328 | 0.3735 | 0.0275 | 0.7777 |
| p-value | 0.753 | 0.4572 | 0.7928 | 0.3098 | 0.3177 | 0.0130 | 0.6251 | 0.4661 |
| AhCHI | 0.0927 | 0.2976 | 0.9827 | 0.8691 | 0.9683 | 0.9149 | 0.9685 | 0.2005 |
| r | 0.9765 | 0.5733 | 0.6212 | 0.938 | 0.9998 | 0.7942 | 0.5921 | 0.7777 |
| p-value | 0.9932 | 0.0742 | 0.9932 | 0.3098 | 0.938 | 0.0130 | 0.6373 | 0.4661 |
| AhF3H | 0.4692 | 0.2976 | 0.9575 | 0.8691 | 0.9683 | 0.9149 | 0.9685 | 0.2005 |
| r | 0.2709 | 0.5733 | 0.6212 | 0.938 | 0.9998 | 0.7942 | 0.5921 | 0.7777 |
| p-value | 0.5554 | 0.0742 | 0.9932 | 0.3098 | 0.938 | 0.0130 | 0.6373 | 0.4661 |
| AhANS | 0.9685 | 0.1601 | 0.9991 | 0.9827 | 0.9683 | 0.9149 | 0.9685 | 0.2005 |
| r | 0.9991 | 0.5966 | 0.5921 | 0.938 | 0.9998 | 0.7942 | 0.5921 | 0.7777 |
| p-value | 0.6373 | 0.3601 | 0.6373 | 0.3098 | 0.938 | 0.0130 | 0.6373 | 0.4661 |
| AhDFR | 0.2092 | 0.1601 | 0.1497 | 0.2005 | 0.9838 | 0.1149 | 0.1601 | 0.2202 |
| r | 0.9508 | 0.3177 | 0.3177 | 0.3177 | 0.9998 | 0.0130 | 0.3177 | 0.3177 |
| p-value | 0.9997 | 0.0130 | 0.0130 | 0.0130 | 0.9998 | 0.0130 | 0.0130 | 0.0130 |
| AhLAR | 0.5342 | 0.6412 | 0.5342 | 0.6412 | 0.5342 | 0.6412 | 0.5342 | 0.6412 |
| r | 0.5342 | 0.6412 | 0.5342 | 0.6412 | 0.5342 | 0.6412 | 0.5342 | 0.6412 |
| p-value | 0.5342 | 0.6412 | 0.5342 | 0.6412 | 0.5342 | 0.6412 | 0.5342 | 0.6412 |

AhCHI = Chalcone isomerase, AhCHS = Chalcone synthase, AhF3H = Dihydroflavonol reductase, AhF3H = Flavanone 3-hydroxylase, AhANS = Anthocyanine synthase, AhFLS = Flavanol synthase, AhLAR = Leucoanthocyanidin reductase.
Figure 3. Relative gene expression of flavonoid biosynthetic pathway enzymes in peanut varieties (Bari 2011 and PG 1247) under water-deficient conditions. Differential expression of AhCHS (a), AhCHI (b), AhF3H (c), AhFLS (c), AhANS (e), AhDFR (f), AhLAR (g) at Day 2 (D2C = control and D2 T = water deficient), Day 4 (D4C = control and D4 T = water deficient) and Day 8 (D8C = control and D8 T = water deficient). Data were collected from three independent observations and represented as mean ± SEM. Student’s t-test with Welch’s correction was used to compare internal control (a group with no sugar treatment) with treatment groups (Note: Statistical tests were performed on $\Delta\Delta\text{Ct}$). $p$-value smaller than 0.05 is considered significant. *$p$ < 0.05; **$p$ < 0.01; ***$p$ < 0.001.
flavanol glucosides, kaempferol, flavonol, flavones, and anthocyanin, whereas the day 2 control showed a higher accumulation of non-acylated flavonol glucosides, kaempferol, flavonol, and flavones. Taproot of a plant in response to water-deficient condition exhibited six prominent fluorescent regions at the wavelength of 395–425, 449–460, 490, and 510–530 nm, which correspond to non-acylated flavonol glucosides, morins, flavonol, and flavone (Figure 5 (b)). Day 2 treatment depicted a higher accumulation of non-acylated flavonol glucosides compared to its control, whereas the day 2 and day 4 control had a higher accumulation of flavonol and flavone compared to their respective treatment groups. The accumulation of morins was also observed in day 2 control.

3.6. Accumulation of flavonoid in leaves and taproot in response to photoperiod

Exposure to different light durations leads to variable accumulation pattern of flavonoid in leaves and the taproot of Bari 2011 (Figure 6). Seven prominent fluorescence regions at wavelengths 330–350, 390–425, 449–466, 463, 490, and 510–530 nm, correspond to catechins, non-acylated flavonol glucosides, kaempferol, 7-hydroxyflavone (Landström et al. 2021), flavonol, flavones, and anthocyanin, respectively. On day 7 of treatment, compared to control (16 h) the accumulation of non-acylated flavonol glucosides, kaempferol, 7-hydroxyflavone, flavonol, flavones, and anthocyanin was lower in leaves of 8 and 12 h light-treated plants, whereas a higher accumulation of non-acylated flavonol glucosides, flavonol and flavones was observed in leaves of 24 h light-treated plants. However, six prominent fluorescence regions at a wavelength of 390–425, 449–466, 490, and 510–530 nm were observed in the taproot of light-treated plants. The 24 h light-treated plants had a higher accumulation of non-acylated flavonol glucosides, kaempferol, 7-hydroxyflavone, flavonol, and flavones compared to control, whereas the accumulation was lower in 8 and 12 h light-treated plants. The prolonged exposure to light treatments resulted in an opposite accumulation pattern as observed on day 7 in leaves and roots. On day 15, the leaves of light-treated plants displayed nine prominent fluorescent regions at 330–350, 378, 390–425, 449–466, 463, 490, and 510–530 nm. Compared to control, all the light-treated groups had a higher accumulation of roots. Whereas, the accumulation trend in roots was opposite, six prominent fluorescence regions at wavelengths of 390–425, 449–466, 463, 490, and 510–530 nm were observed. A higher accumulation at all wavelengths was observed in all light-treated groups; however, only control and 8 hr treatment depicted a higher accumulation of kaempferol, morins, flavonol, and flavones.
4. Discussion

Understanding the biosynthesis of flavonoids in plants has been the target of genetic engineering to develop stress-tolerant plants, functional foods, and engineered microbial strains capable of producing biologically active flavonoids at an industrial scale (Pandey et al. 2016). Peanut is a legume with bio-active flavonoids that impart antioxidant, anti-mutagenic and anti-proliferative activity (De Villa et al. 2017). Many genes depicted a low degree of variation within varieties and water-deficient treatment in peanut and suggested that peanut has a higher threshold level of drought perception. Occasional up-regulation of flavonoid gene expression may be due to replenishing the flavonoid reservoir within a cell. A lower accumulation of flavonoids also signifies the use of stored flavonoids against oxidative stress due to water-deficient conditions. The differential expression of flavonoids within varieties suggested that drought tolerance is genotype-dependent. Furthermore, the correlational analysis suggested that water-deficient conditions induced anthocyanins and catechins. On the other hand, lower accumulation of respective peaks of anthocyanins and catechins in water-deficient seedlings also inferred the role of anthocyanins and catechins in water-deficient-induced stress.

The plant produces flavonoids in specific quantities. They may have a specific threshold for accumulating flavonoids even under specific inducers. A threshold accumulation of flavonoids is necessary for plants as flavonoids at higher accumulation act as mutagens, pro-oxidants, and inhibitors among these genes indicated a coordination pattern in their expression to produce a specific class of flavonoids. The expression of AhFLS depicted a high degree of positive correlation with the expression of AhCHS, AhCHI, and AhF3H in all treatment groups, which indicated that the plant would produce flavonol if AhCHS, AhCHI, AhF3H, and AhFLS are upregulated. On the contrary, the plant favors anthocyanins production by AhANS in coordination with AhF3H as the expression of AhANS showed a strong positive correlation only with AhF3H, while a significant negative association was shown with AhCHS and AhCHI. The expression of AhDFR depicted a positive correlation with the expression of AhCHS and AhF3H, whereas a negative association with AhCHI suggested that AhDFR, AhCHS, and AhF3H work in coordination to produce leucoanthocyanidins. The association of AhLAR with other genes remained indecisive as in some treatment groups, they showed a positive association, and in some, they showed a negative association. A strong degree of positive association has been observed between the expression of AhFLS and AhDFR in all treatment groups, which depicted that there may be similarities in their regulation.

Several reports indicated that flavonoid accumulation occurs in plant tissues in response to longer light exposure (Singh et al. 2017). The given study also demonstrated that longer light duration stimulates the biosynthesis and accumulation of flavonoids. However, the shorter period of light also triggered flavonoid production and accumulation. The enhanced production of flavonoids at a shorter period of light may be due to the role of flavonoids in nutrient acquisition from the rhizosphere (Cesco et al. 2010). As the plant cannot meet its energy requirement in a shorter light duration, it depends upon other external factors to fulfill its needs. Zoratti et al. (2014) suggested that the expression of R2R3 MYB transcription factors (TFs) primarily controls the expression of biosynthetic genes e.g. in petunia, R2R3 MYB TFs regulate the expression of CHS, F3H, DFR, ANS, and UFGT to produce anthocyanin (Naing and Kim 2018). Hence, the light is important in regulating flavonoid biosynthesis in plants. The expression of DFR and ANS in grape, CHS, CHI, and DFR in Arabidopsis, CHS, CHI, DFR, ANS, and LAR in rice is induced by sucrose treatment.

Figure 5. Synchronous fluorescence spectra of Bari 2011 leaves under water-deficient conditions, showing six distinct peaks represented as M1, M2, M3, M4, M5, M6, and M7 (a). Synchronous fluorescence spectra of leaves were recorded with an excitation range of 200–700 nm and offset of 60. Emission spectra of the taproot of Bari 2011 under water-deficient conditions, showing six distinct peaks represented as M1, M2, M3, M4, M5, and M6 (b). Emission spectra of the taproot were recorded after excitation at 380 nm with emission spectra range of 395–550 nm. Samples were collected on day 2, day 4 and day 700 nm and offset of 60.

Textures:

- Phenotypic variation
- Water availability
- Anthocyanin biosynthesis
- Genetic factors
- Environmental conditions
- Economic value
- Functional foods
- Genetic engineering
- Anthocyanins
- Catechins
- Leucoanthocyanidins
- MYB TFs
- Sucrose treatment
of other metabolic enzymes (Skibola and Smith 2000). In this study, the accumulation and expression patterns of flavonoids indicated the presence of a feedback loop between them. For example, prolonged stress condition shuts down the expression of flavonoid genes, but higher accumulation of flavonoids has been observed in such plants and vice versa. But there are some other conditions in which lower expression and lower accumulation have been observed, which depicted that those flavonoids showing patterns do not have a significant role in that stress. But, higher expression and higher accumulation conclude that such flavonoids have a specific role in that very stress.

The specificity and complexity of flavonoid biosynthesis and accumulation hinder concluding about their exact role in metabolism. They may have a more complex role other than that of being antioxidants. By compiling the chemical reactions driven by flavonoid biosynthetic enzymes, a reaction pathway has been proposed (Figure 7). Flavonoid biosynthetic pathway takes input as p-coumaroyl-CoA, malonyl-CoA, H⁺, oxoglutarate, O₂ and NADPH as reactants and gives NADP, H₂O, succinate, CO₂ along a whole class of flavonoids (Figure 11). Oxoglutarate emerges as the master regulator of metabolite and acts as a crossroad between carbon and nitrogen metabolic pathway (Huergo Luciano and Dixon 2015). Basically, oxoglutarate reflects the nutritional status of the cell, and this may be the point where the flavonoid biosynthesis is related to the nutrient source. The link of the flavonoid biosynthetic pathway with H⁺ ion cannot be ignored while studying flavonoid regulation. The first input H⁺, which mostly accumulates in higher concentration during stress, is the main trigger of the pathway (Alberti and Cuthbert 1982). Because of the involvement of H⁺, it may be concluded that the flavonoid biosynthetic pathway is sensitive to pH changes and help the plant to formulate stress response to stress. NADPH, a metabolite to induce stress resistance, also participates in the production of flavonoids, suggesting the role of the flavonoid biosynthetic pathway in stress tolerance (Larochelle et al. 2006). With metabolic regulators, the flavonoid biosynthetic pathway produces succinate, a crossroad of several metabolic pathways, at various levels along flavonoids. Succinate is also involved in epigenetics, signal transduction, and elimination and formation of ROS (Tretter et al. 2016). It means that the flavonoid biosynthetic pathway is triggered by oxidative stress, which produces antioxidants and signals in the form of succinate to devise strategies to mitigate stress. This

Figure 6. Normalized fluorescence spectra of leaves and roots of Bari 2011 (peanut variety) under photoperiod treatment. Synchronous fluorescence spectra of Bari 2011 leaves on day 7 of photoperiod treatment, showing seven distinct peaks represented as M1, M2, …, M7 along with a peak of chlorophyll a and b, while (a) synchronous fluorescence spectra of Bari 2011 leaves on day 15 of photoperiod treatment displaying two more peaks (M2 and M3) along other peaks on day 7, indicating more diversity of flavonoid on day 15 than on day 7 (b). Synchronous fluorescence spectra of leaves were recorded with an excitation range of 200–700 nm and offset of 60. Emission spectra of the taproot of Bari 2011 on day 7 (c) and day 15 (d) of photoperiod treatment showing six peaks represented as M1, M2, …,M6 with spectral deviation. Emission spectra of the taproot were recorded after excitation at 380 nm with emission spectra range of 395–550 nm. Four treatment groups were formed based on the duration of light (8, 16, 12 and 24 h). Data were collected from nine independent observations and represented as the mean.
study coordinates among the expression of flavonoid biosynthetic genes; however, it needs further exploration. Furthermore, the interaction of the flavonoid biosynthetic pathway with other pathways involved in carbon metabolism, nitrogen metabolism, and stress-responsive pathways must be carried out to establish their exact physiological role in the plant.

5. Conclusion

Photoperiod and water-deficient conditions differentially regulated the expression of structural flavonoid biosynthetic genes and the accumulation pattern of flavonoids in peanuts, indicating the presence of precise regulatory control of flavonoid biosynthesis. Furthermore, genotype-dependent expression of flavonoid biosynthetic genes in peanuts in response to photoperiod and water-deficient conditions indicated the involvement of specific genetic factors that mediate flavonoid production. This study also signified the presence of a feedback mechanism among the expression and accumulation of flavonoids. Moreover, a precise coordination pattern exists among various flavonoid genes in peanuts irrespective of external stimuli and variety. There is still a long way to understand the dynamics of flavonoid’s biosynthetic pathway completely. By studying this pathway under photoperiod variation and water-deficient conditions, the need for integrative study involving other metabolic pathways and their relation to flavonoid biosynthetic pathways has been identified.

Acknowledgements

We acknowledge the Higher Education Commission, Pakistan, and the National University of Sciences and Technology (NUST), Pakistan, for providing research facilities and funding. We sincerely appreciate Oil Seed Programme-National Agricultural Research Centre (NARC), Pakistan for providing valuable insight, while seed selection and necessary data are associated with the selected peanut variety. We are grateful to the National Institute of Laser and Optronics (NILOP), Pakistan, for assisting us in conducting fluorescence spectroscopy at their facility.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This study was supported partly by students research fund (National University of Sciences and Technology) and Higher Education Commission under National University Research Program (NRPU) 2016-17.

Notes on contributors

Maryam Khan is the part of Plant Cell Signaling Research Group led by Dr. Rabia Amir.
Saman Taufig is the part of Plant Cell Signaling Research Group led by Dr. Rabia Amir.
Irum Nauman is the part of Plant Cell Signaling Research Group led by Dr. Rabia Amir.
Norina Noor is the part of Plant Cell Signaling Research Group led by Dr. Rabia Amir.
Tooba Iqbal is the part of Plant Cell Signaling Research Group led by Dr. Rabia Amir.
Dr. Hina Ali is working as Scientific Officer at National Institute of Lasers and Optronics, Pakistan.
Dr. Rehan Zafar Paracha is currently working as Associate Professor at School of Interdisciplinary Engineering and Sciences, NUST, Pakistan.
Faiza Munir is currently working as Assistant Professor at Atta ur Rahman School of Applied Biosciences, NUST, Pakistan.
References

Adhikari B, Dhungana SK, Waqas Ali M, Adhikari A, Kim I-D, Shin D-H. 2019. Antioxidant activities, polyphenol, flavonoid, and amino acid contents in peanut shell. J Saudi Soc Agric Sci. 18(4):437–442. doi:10.1016/j.jssas.2018.02.004.

Akula R, Ravishankar GA. 2011. Influence of abiotic stress signals on secondary metabolites in plants. Plant Signal Behav. 6(11):1720–1731.

Alberti KG, Cuthbert C. 1982. The hydrogen ion in normal metabolism: a review. Ciba Found Symp. 87:1–19. doi:10.1016/S0097-9047(2)06961.5.

Ali H, Saleem M, Anser MR, Khan S, Ullah R, Bilal M. 2018. Validation of fluorescence spectroscopy to detect adulteration of edible oil in very virgin olive oil (EVOO) by applying chemometrics. Appl Spectrosc. 72(9):1371–1379. doi:10.1366/0003702817864485.

Andersen OM, Markham KR. 2005. Flavonoids: chemistry, biochemistry and applications. CRC Press.

Arya SS, Salve AR, Chauhan S. 2016. Peanuts as functional food: a review. J Food Sci Technol. 53:31–41.

Bertioli DJ, Cannon SB, Froncicke L, Huang G, Farmer AD, Cannon EK, et al. 2015. The genome sequences of Arachis duranensis and Arachis ipaensis, the diploid ancestors of cultivated peanut. Nat Genet. 47(3):438.

Carvalho IS, Cavaco T, Carvalho LM, Duque P. 2010. Effect of photo-period on flavonoid pathway activity in sweet potato (Ipomoea batatas (L.) Lam.) leaves. Food Chem. 118(2):384–390.

Cesco S, Neumann G, Tomasi N, Pinton R, Weiskopf L. 2010. Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition. Plant Soil. 329(1):1–25. doi:10.1007/s11104-009-0266-9.

Cheirif AO, Trabelsi H, Ben Messaouda M, Kâabi B, Pellerin I, Cesco S, Neumann G, Tomasi N, Pinton R, Weisskopf L. 2010. Molecular characterization of Leucoanthocyanidin reductase and Flavonol synthase gene in Arachis hypogaea. Saudi J Biol Sci. 28(4):2301–2315. doi:10.1016/j.sjbs.2010.01.024.

Chung H-H, Sung Y-C, Shyur L-F. 2016. Deciphering the biosynthetic pathway genes and transcription factors in peanut under water deficit conditions. Front Plant Sci. 12:1140.

Cruz de Carvalho MH. 2008. Drought stress and reactive oxygen species: production, scavenging and signaling. Plant Signal Behav. 3(3):156–165.

De Villa R, Rosoa J, Mine Y, Taso R. 2021. Impact of solid-state fermentation on factors and mechanisms influencing the bioactive compounds of grains and processing by-products. Crit Rev Food Sci Nutr. 1–26. doi:10.1080/10408389.2021.1898999.

Donaldson L, Williams N. 2018. Imaging and Spectroscopy of natural flavonoids in pine needles. Plants. 7(1):10.

Drabent R, Pilszka B, Obszewska T. 1999. Fluorescence properties of plant anthocyanin pigments. I. fluorescence of anthocyanins in Brassica oleracea L. extracts. Photochem Photobiol B. 50(1):53–58.

Duncan CE, Gorbet DW, Talcott ST. 2006. Physicochemical content and antioxidant capacity of water-soluble isolates from peanuts (Arachis hypogaea L.). Int Food Res J. 39(8):898–904.

Dwivedi S, Crouch J, Nigam S, Ferguson M, Paterson A. 2003. Molecular breeding of groundnut for enhanced productivity and food security in the semi-arid tropics: opportunities and challenges. Adv Agron. 80:153–221.

Falcone Ferreyra ML, Rius S, Casati P. 2012. Flavonoids: biosynthesis, biological functions, and biotechnological applications. Front Plant Sci. 3:2222.

Guchi E. 2015. Aflatoxin contamination in groundnut (Arachis hypogaea L.) caused by Aspergillus species in Ethiopia. Appl Environ Microbiol. 3(1):11–19.

Guharay J, Sengupta B, Sengupta P. 2001. Protein–flavonoid interaction: fluorescence spectroscopic study. Proteins. 43(2):75–81.

Holser RA. 2014. Near-infrared analysis of peanut seed skins for catechins. Am J Anal Chem. 5(06):378.

Huerco Luciano F, Dixon R. 2015. The emergence of 2-oxoglutarate as a master regulator metabolite. Microbiol Mol Biol Rev. 79(4):419–435. doi:10.1128/MMBR.00383-15.

Jaakola L, Piriltülä AM, Halonen M, Hohtola A. 2001. Isolation of high quality RNA from bilberry (Vaccinium myrtillus L.) fruit. Mol Biotechnol. 19(2):201–203. doi:10.1385/MBB:19:2:201.

Koyama K, Ikeda H, Poudel PR, Goto-Yamamoto N. 2012. Light quality affects flavonoid biosynthesis in young berries of Cabernet Sauvignon grape. Phytochemistry. 78:54–64.

Kubra G, Khan M, Hussain S, Iqbal T, Muhammad J, Ali H, et al. 2021a. Molecular characterization of Leucoanthocyanidin reductase and Flavonol synthase gene in Arachis hypogaea. Saudi J Biol Sci. 28(4):2301–2315. doi:10.1016/j.sjbs.2021.01.024.

Kubra G, Khan M, Munir F, Gul A, Shah T, Hussain A, et al. 2021b. Expression characterization of flavonoid biosynthetic pathway genes and transcription factors in peanut under water deficit conditions. Front Plant Sci. 12:1140.
Arabidopsis: structural and genetic diversity. Plant Physiol Biochem. 72:21–34. doi:10.1016/j.plaphy.2013.02.001.

Sim EW, Lai SY, Chang YP. 2012. Antioxidant capacity, nutritional and phytochemical content of peanut (Arachis hypogaea L.) shells and roots. Afr J Biotechnol. 11(53):11547–11551.

Singh B, Kumar A, Malik AK. 2017. Flavonoids biosynthesis in plants and its further analysis by capillary electrophoresis. Electrophoresis. 38(6):820–832.

Skibola CF, Smith MT. 2000. Potential health impacts of excessive flavonoid intake. Free Radic Biol Med. 29(3–4):375–383. doi:10.1016/s0891-5849(00)00304-x.

Tretter L, Patocs A, Chinopoulos C. 2016. Succinate, an intermediate in metabolism, signal transduction, ROS, hypoxia, and tumorigenesis. Biochim Biophys Acta Bioenerg. 1857(8):1086–1101. doi:10.1016/j.bbabio.2016.03.012.

Tu L-Y, Pi J, Jin H, Cai J-Y, Deng S-P. 2016. Synthesis, characterization and anticancer activity of kaempferol-zinc (II) complex. Bioorg Med Chem Lett. 26(11):2730–2734.

Voicescu M, Ionescu S, Gatea F. 2014. Photophysical properties of some flavones probes in homogeneous media. J. Fluoresc. 24(1):75–83.

Zhu J, Kaeppler SM, Lynch JP. 2005. Mapping of QTLs for lateral root branching and length in maize (Zea mays L.) under differential phosphorus supply. Theor Appl Genet. 111(4):688–695.

Zoratti L, Karpinnen K, Luengo Escobar A, Häggman H, Jaakola L. 2014. Light-controlled flavonoid biosynthesis in fruits. Front Plant Sci. 5:534. doi:10.3389/fpls.2014.00534.