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Total Flavonoids and Phenolic Compounds of English Daisy (*Bellis Perennis* L.) Affected by Foliar Application of Nano-Phosphorus Fertilizers

Nayyereh Ghorbani [1]*, Hossein Moradi [2], Mehran Kanani [1], Mahboubeh Ashnavar [3]

1 Department of Horticulture, Faculty of Agricultural Sciences and Natural Resources, University of Mohaghegh Ardabili, Iran
2 Department of Horticulture, Faculty of Agricultural Sciences, Sari Agricultural Sciences and Natural Resources University, Sari, Iran
3 Ph.D Student of Medicinal Plants, Faculty of Plant Production, Gorgan Agricultural Sciences and Natural Resources University

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ABSTRACT

Bellis perennis is an ornamental, medicinal plant with a beautiful appearance. The current study aimed to assess the potential of daisy flowers cv. 'Habanera' to synthesize secondary metabolites after being affected by nano-phosphorus. The experiment was performed using a completely randomized design with three replications in greenhouse conditions. To achieve the research objectives, a range of parameters were observed, including total phenol content, total flavonoid content, and free radical scavenging activity in different organs (i.e. flower, leaf, and root), as well as photosynthetic pigments, anthocyanin, and quercetin contents. The results indicated that nano-phosphorus had significant effects on the said parameters ($p< 0.05$ and 0.01). Chlorophyll A, total chlorophyll, and carotenoids reached maximum content in response to the 1.5 g L^{-1} treatment. Nonetheless, the total phenol content in petals, free radical scavenging capacity, anthocyanin content and quercetin content were adversely affected by the nano-phosphorus treatment and, in fact, the said parameters showed higher contents in the control treatment. The application of nano-phosphorus fertilizer (3 g L^{-1}) caused the highest flavonoid content in the leaves. At a concentration of 0.5 g L^{-1}, it increased total flavonoid content and total phenols significantly in the roots. Phosphorus is necessary for producing primary and secondary metabolites, phospholipids, and nucleic acids in plants. Thus, its functions can vary greatly in plant organs.

Introduction

Flavonoid and its derivatives, such as flavonol quercetin, are an important part of secondary metabolites, as they have indispensable roles in plants and in pharmaceutical programs (Kim et al., 2003; Ghasemzadeh and Ghasemzadeh, 2011). A tremendous resurgence in the interest and use of medicinal plant products has been recorded throughout the past decades (Briskin, 2000). Secondary metabolites often account for less than 1% of the total carbon and occur in specific cells or organs (Bourgaud et al., 2001). A common role of secondary metabolites in plants is their defense mechanisms against herbivores, pests, and pathogens (Bennett and Wallsgrove, 1994). Combinations of plant secondary metabolites usually result in beneficial medicinal effects that are unique to particular plant species or groups, and the combinations of secondary metabolites in a particular plant are often taxonomically distinct (Wink, 1999; Briskin, 2000; Meena and Patni, 2008). Secondary

* Corresponding author's email:
nayyer.ghorbani@gmail.com

products are commonly involved in plant defense, as they cause cytotoxicity for microbial pathogens and, if not too toxic, they can prove useful as antimicrobial medicines in humans. Likewise, secondary metabolites that are involved in defense against herbivores through neurotoxin activity can have beneficial effects as antidepressants, sedatives, muscle relaxants, or anesthetics (Briskin, 2000). An enormous variety of secondary metabolites are derived from shikimic acid (phenylpropanoid pathway) or aromatic amino acids, many of which play important roles in defense mechanisms (Bennett and Wallsgrove, 1994).

Flavonoids are major floral pigments in plants and give rise to ivory and cream colors (flavonols and flavones), yellow and orange (aurones and chalcones), or red-pink-purple-blue ranges of colors (the anthocyanins) (Glover, 2007). Flavonoids usually play a range of important roles in plants, including defense against pathogens and predators, protection against UV light, and participation in pollen development and germination (Mogren et al., 2006; Glover, 2007). Flavonoids mainly occur in aboveground tissues, whereas their content in stalks and roots is usually limited (Rasmussen and Breinholt, 2003).

Quercetin is a flavonoid which is ubiquitously found in nature and could comprise chemotherapeutic drugs for the treatment of protease and skin cancer, primarily because it shows inhibitory effects on the growth of tumorigenic cells (Mu et al., 2007; Kalinova and Dadakova, 2009). Furthermore, quercetin is an important antioxidant that significantly inhibits the oxidation of HDL cholesterol (Robaszkiewicz et al., 2007).

English daisy (*Bellis perennis* L.) is a small perennial herb that belongs to the Asteraceae family (Siatka and Kašparová, 2010). While it is also known as common daisy, English daisy contains an extensive range of phenolic compounds such as flavonoids (Toki et al., 1991; Nazaruk and Gudej, 2001; Gudej and Nazaruk, 2001), anthocyanin (Toki et al., 1991), tannins (Siatka and Kašparová, 2010), and phenolic acids (Grabias et al., 1995). In particular, the flavonoid quercetin has been reported in daisy flowers (Gudej and Nazaruk, 1997).

Phosphorus (P) plays an important role in energy storage and transfer in plants. An adequate supply of P is needed for producing high-quality crops. Beyond its role in energy-transferring processes, P is a structural component of phospholipids, nucleic acids, nucleotides, coenzymes, and phosphoproteins (Barker and Pilbeam, 2015). It is required for the biosynthesis of primary and secondary metabolites for having essential functions as a constituent of phospholipids and nucleic acids (bio-membranes) and plays a key role in cellular metabolism (Nell et al., 2009; Marschner, 2011).

Excessive volumes of fertilizers have added to the tribulations of susceptible ecologies and have become troublesome as run-off (Tilman et al., 2011). There is an urgent need to tackle the excessive usage of fertilizers by finding alternatives to the current patterns of fertilizer use (Ghormade et al., 2011). Nanotechnology is one area of expertise that provides a system to deal with the issue. It employs nanoparticles that have one or more dimensions in the order of 100 nm or less (Auffan et al., 2010). Nanoparticles are highly prized for their size-dependent qualities, high surface-to-volume ratio, and unique optical properties (Ghormade et al., 2011).

The presence and concentration of secondary metabolites can depend on a range of factors, including genetics (plant species) (Heyworth et al., 1998; Estell et al., 2016) and nutrient availability (Powell and Raffa, 1999; Estell et al., 2016). Considering the beneficial effects of plant secondary metabolites in human life and the role of phosphorus in plant secondary metabolism, this study aimed to evaluate the influence of nano-P fertilizer on flavonoid and phenolic compounds in daisy flowers.

Material and Methods

The current study was performed in the greenhouse and laboratory of Sari University of Agricultural Sciences and Natural Resources. F1 seeds of the English daisy (*Bellis perennis* L. 'Habanera') (Pakan Bazr Esfahan) were disinfected by sodium hypochlorite (2%) and sown in the nursery to produce seedlings (Temp: 26±2 ˚C, RH: 70±5 %, Light (day: night: 10 and 14 hrs)). The seedlings were then transferred into 2.5 L pots at the four-leaf stage. The culture medium was a mixture of soil, peat, coco fiber, sand, and rotten manure (2, 1, 2, 1, 0.5 respectively). No other fertilizer was applied other than rotten manure (Table 1) and irrigation was carried out twice a week.

Table 1. Test analysis of soil used in the cultivation of *Bellis perennis* 'Habanera'

N (%)	P (ppm)	K (ppm)	pH	EC (dS m^{-1})
0.2	31	311	7.5	1.2

The experiment was performed in greenhouse conditions and was laid out on a completely randomized design with three replications to facilitate evaluations of how nano-P affected phenolic and flavonoid contents in daisy flowers. The nano-P was applied at concentrations of 0, 0.5, 1.5, and 3 g L^{-1}. Foliar spraying was performed on the aerial parts of plants, starting from the six-leaf stage, and was repeated three times in total, with 20-day intervals. Sampling was done at the flowering stage.

Photosynthetic pigments

Photosynthetic pigments (Chl a, Chl b, and carotenoid) were measured according to relevant procedures. Young mature leaves were selected for measuring photosynthetic pigments according to Porra (2005). In this method, the pigments were extracted in aqueous 80% methanol and determined in the same solvent at wavelengths of 470, 652.4, and 665.2 nanometers via a spectrophotometer (Lambda 25, UV/VIS, Perkin Elmer; USA). The concentrations of Chl and carotenoid were measured using the following formulae:

Chl a: (16.72 × A665.2) – (9.16 × A652.4)
Chl b: (34.09 × A652.4) – (15.28 × A665.2)
Carotenoid: (1000 × A470) – (1.63 × Chl a) – (104.9 × Chl b)/221

Determination of phytochemical parameters

To determine the content of total phenol, flavonoid content, and free radicle scavenging capacity, 0.5 g of dried samples (i.e. leaves, flowers, and roots) was homogenized in methanol (80%, 1:10 ratio). The homogenized mixture was put on a shaker for 24 hours and subsequently centrifuged for 5 min at 3000 rpm (Hermle Z 216 MK; Germany). The supernatant was used for determining the phytochemical parameters.

Determination of total flavonoid content

The colorimetric aluminum chloride method was used with some modifications for flavonoid determination (Chang et al., 2002). Briefly, 0.5 ml of crude extract was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water. The mixture was left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm by the spectrophotometer (Lambda 25, UV/VIS, Perkin Elmer, USA). Total flavonoid contents in the roots, leaves, and flowers were calculated from a calibration curve as quercetin. The calibration curve was prepared by preparing quercetin solutions at concentrations of 12.5 to 100 mg/ ml in methanol.

Determination of total phenol content

Total phenolic contents were determined by the Folin-Ciocalteau method (Kaur and Kapoor, 2002). Briefly, 200 µL of crude extract (1 mg/mL) was made to reach 3 mL with distilled water and was mixed thoroughly with 0.5 mL of Folin–Ciocalteau reagent for 3 min. Then, 2 mL of 20% (w/v) sodium carbonate was added. The mixture was kept in the dark for 60 min, and the absorbance was measured at 760 nm. The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid g^{-1} DW.

Determination of antioxidant activity (DPPH)

The antioxidant activity of the extract was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay based on a method by Villano et al. (2007) with some modifications. Briefly, 200 µL of each extract (100–500 µg/mL) was mixed with a 3.8 mL DPPH solution. The extraction was incubated in the dark at room temperature for 1 hour. Then, the absorbance of the mixture was measured at 517 nm using a spectrophotometer (Lambda 25, UV/VIS, Perkin Elmer; USA). Ascorbic acid was used as a positive control and the ability of the sample to scavenge DPPH radicals was determined according to the following:

DPPH scavenging effect = (Control OD − Sample OD)/ (Control OD) DPPH scavenging effect = (Control OD − Sample OD)/(Control OD) × 100

Determination of anthocyanin content

Anthocyanin in daisy flowers was measured based on a method by Wagner (1979) with some modifications. In brief, 0.5 g of dry tissue from the flowers was ground in methanol (1:10 ratio). The extract was incubated in the dark for 4 hours and then centrifuged (10 min, 4000 rpm). The absorbance was measured at a wavelength of 520 nm and the anthocyanin content was measured based on the A=εbc formula as follows:

A= absorbance content, ε: extinction coefficient (330 mM cm^{-1}), b; cuvette size (1 cm), and c; anthocyanin content (mol g^{-1})

Determination of quercetin

For phytochemical determination, extracts from the dry powder were prepared according to the method proposed by Samee and Vorarat (2007)

with some modifications. After extract preparation, the extract samples were treated in an ultrasonic bath for 10 min and were then centrifuged at 3500 rpm. The supernatant was passed through a microinjection filter (0.45) and injected into the HPLC. The Merck Hitachi apparatus was equipped with the Lacrom Pump Model 7100-diode array detector (285 nm) and the C18 column (with a length of 25 cm and a diameter of 4.6 mm). The standard calibration curve of quercetin was obtained and plotted from different quercetin concentrations (0, 10, 50, and 100 ppm). The correct amount of compounds was computed based on the obtained formula for each compound (Ghorbani et al., 2013).

Statistical analysis

Data analysis was performed using SAS V9.2 software. The comparison of mean values were carried out by Duncan's multiple range test at the 5 % probability level.

Results

The results from the analysis of variance (Table 2) suggested that nano-phosphorus treatments have a significant effect on the physiological, biochemical, and phytochemical parameters of *Bellis perennis*.

Photosynthetic pigments

Photosynthetic pigments, including total chlorophyll and Chl a, were significantly affected by nano-phosphorus (NP) treatments, whereas Chl b and carotenoid contents were not affected significantly (Fig. 1). The highest content of total Chl occurred as a result of applying 1.5 g L^{-1} NP, whereas the lowest occurred in the control.

The results indicated that the NP treatment (up to 1.5 g L^{-1}) enhanced total chlorophyll content. A further increase in the NP concentration adversely affected the total chlorophyll content, probably because of toxicity caused by P accumulation in the tissues.

Total flavonoid content in the roots, leaves, and petals

The flavonoid content was significantly affected by the treatments. The highest and lowest contents of flavonoid in the petals occurred as a result of NP (1.5 g L^{-1}) and NP (0.5 g L^{-1}), respectively (Table 3).

Furthermore, the application of NP (0.5 g L^{-1}) and NP (3 g L^{-1}) produced the highest content of total flavonoids in the roots and leaves, respectively (Fig. 2). However, NP (3 g L^{-1}) resulted in the lowest level of total flavonoids in the roots. With respect to the production of total flavonoids, plant organs reacted differently to the application of NP (Table 3 and Fig. 2).

Total phenol content and antioxidant activity (DPPH)

The total phenol content in the petals of *Bellis perennis* cv. 'Habanera' was negatively affected by NP treatments. The highest content of total phenol in the petals was observed in the control treatment, whereas NP (0.5 g L^{-1}) produced the lowest content (Table 2). Additionally, the highest content of total phenol in the leaves was produced in the control treatment, whereas NP (1.5 g L-1) resulted in the highest total phenol (3.98±0.35 a) in the roots (Fig. 3). Moreover, the antioxidant activity in the flowers of Bellis perennis cv. 'Habanera' indicated that NP treatments adversely affected antioxidant capacity. The control treatment produced the highest level of antioxidant activity, whereas NP (0.5 g L^{-1}) led to the lowest content (Table 3).

Anthocyanin content

The results indicated that the control treatment produced the highest content of anthocyanin in the flowers of daisy, whereas NP treatments significantly affected anthocyanin production in the flowers (Table 3). The trend of changes in anthocyanin content was almost in line with the changes in total phenol and antioxidant capacity (Fig. 3).

Quercetin content

Nano-Phosphorus treatments adversely affected the quercetin content in the daisy flowers. The highest content of quercetin in the flowers was found in the control group, whereas the lowest content occurred by applying NP (1.5 g L^{-1}) (Table 3).

Discussion

Phosphorus (P) is an essential nutrient for a variety of plant functions and is a major component of nucleic acids, sugar phosphates, ATP, and phospholipids, all of which play key roles in photosynthesis (Bieleski, 1973; Reich et al., 2009). The effectiveness of foliar P application can vary depending on the P status of the soil, soil moisture, crop type, and fertilizer formulation (Noack et al., 2010). The data suggested that NP (up to 1.5 g L^{-1}) enhanced total chlorophyll content, whereas a further increase in the NP concentration adversely affected total chlorophyll content, possibly because of P accumulation and the occurrence of toxicity in the tissues.

Table 2. Analysis of variance (sum of squares) of Nano-Phosphorus treatments on *Bellis perennis.*

Source Change	DF	Total phenol			Total flavonoid			DPPH radical scavenging			Photosynthetic pigments				Anthocyanin	Quercetin
		Flower	Leaf	Root	Flower	Leaf	Root	Flower	Leaf	Root	Chl a	Chl b	total Chl	carotenoid	Flower	Flower
Nano-Phosphorus levels	3	10.95*	0.01*	0.56*	15.4**	0.06^{ns}	0.05**	6.3^{ns}	4.2*	0.9**	0.05**	0.07**	0.17**	0.006*	0.0001*	37417**
Experimental error	8	2.15	0.003	0.1	2.5	0.02	0.005	1.8	0.7	0.06	0.002	0.01	0.01	0.003	0.00002	1024
Coefficient of Variation%	-	11	4	8	15	8	6	1	0.9	0.2	1.9	11	3	8	9	13

* & ** significant at 5 and 1% probability level and NS is not significant.

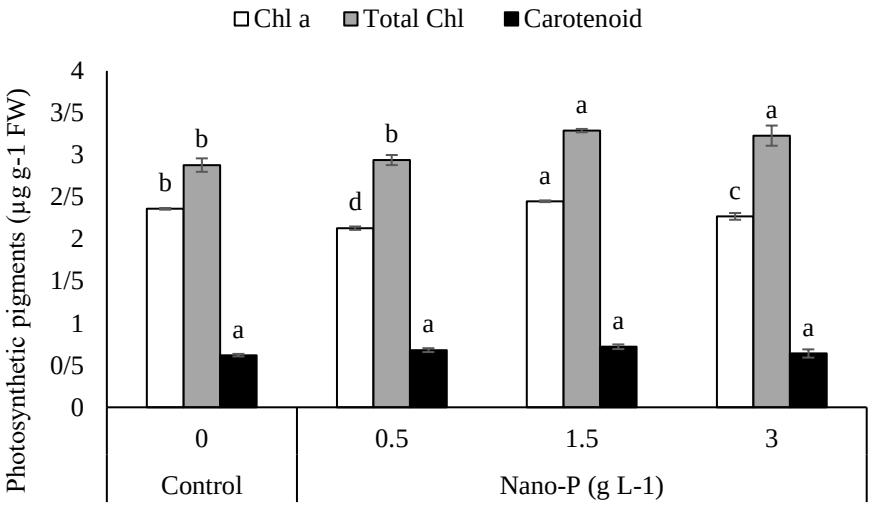

Fig. 1. The effect of foliar application of nano-phosphorus on photosynthetic pigments in English daisy 'Habanera'
Dissimilar words and error bars indicated significant differences based on Duncan's multiple range test (P<0.05) and (mean ±SE, n=3), respectively.

Table 3. The effect of different concentrations of nano-phosphorus on phenolic parameters of daisy 'Habanera'

Treatments		Total phenol in petals(mg gallic acid g^{-1} DW)	Total flavonoid in petals (mg g^{-1} DW)	Anthocyanin (mol g^{-1})	DPPH radical scavenging (µg ml^{-1})	Quercetin (µg g^{-1} DW)
Control	0	15.6†±0.78a	11.7±0.44a	0.061±0.004a	75.31±0.65a	390.04±25.12a
Nano-P	0.5	11.05±0.36b	7.5±0.05b	0.046±0.003b	68.05±0.12b	174.94±19.91bc
(g L^{-1})	1.5	13.87±1.36ab	12.5±1.8a	0.053±0.004ab	71.95±0.56ab	136.88±16.26c
	3	12.79±0.51ab	9.4±0.07ab	0.051±0.002b	71.27±1.32ab	219.93±8.59b

Values followed by the same letters in a column are not significantly different based on Duncan's test at the 5% level.

† (Mean ±SE, n=3).

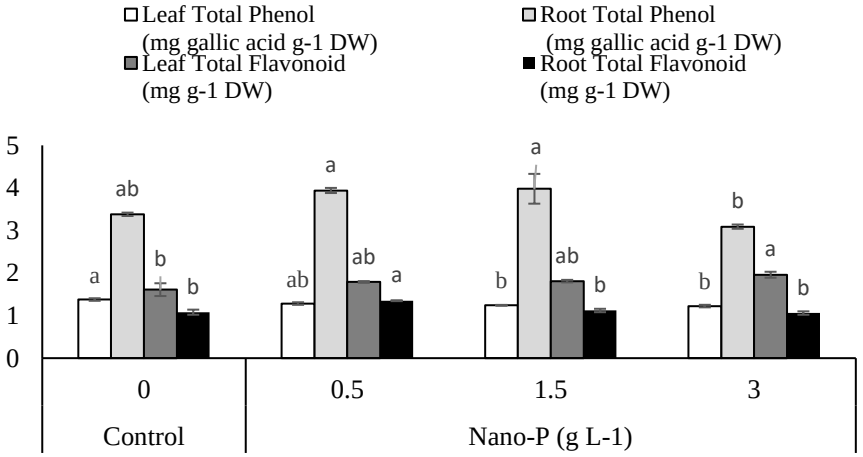

Fig. 2. Effect of foliar application of nano-phosphorus on total phenol and total flavonoid contents in the roots and leaves of English daisy 'Habanera'
Dissimilar words and error bars indicated significant differences based on Duncan's multiple range test (P<0.05) and (Mean ±SE, n=3), respectively.

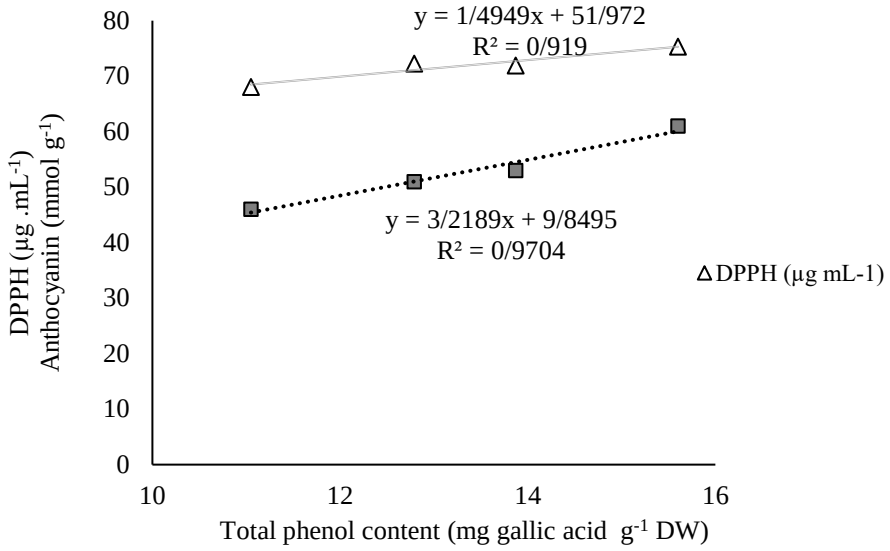

Fig. 3. Relation between total phenol content in petals, anthocyanin content, and free radical scavenging capacity in English daisy 'Habanera'

Flavonoids, as plant secondary metabolites, act as antioxidant compounds since they are immobile and, also, they have the ability to survive against harsh environments (Treutter, 2006). Flavonoids are a group of phenolics in which two rings, each having six carbons, are linked by a three-carbon unit. The antioxidant activity of flavonoids, including anthocyanins, chalcones, aurones, flavanols (e.g. quercetin, myricetin, and kaempferol), flavones, and condensed tannins, usually depends on the presence of OH groups, especially the 3-OH group (Baba and Malik, 2015). The difference between the total flavonoid contents induced by foliar application of NP treatments might be due to the synthesis of different flavonoid compounds, i.e. "performed" or "induced" compounds by applying NP at different concentrations. The "performed" flavonoids are innate compounds that are synthesized during normal plant growth, whereas "induced" compounds are synthesized during stress conditions (Treutter, 2006). The use of different NP concentrations affected the production of phenol and flavonoid compounds. Stewart et al. (2001) demonstrated that the content of flavonols, as a constituent of total flavonoids in plant tissues, is influenced by the nutritional status of the plant.

Phenolic compounds have redox properties, which allow plants to act as antioxidants (Soobrattee et al., 2005). Oxidative stress is considered as a substantial, if not crucial, trigger of the initiation and development of many diseases such as inflammation, heart diseases, neurodegenerative diseases, cataract, cancer, arteriosclerosis, and aging (Zima et al., 2001; Astley, 2003; Luk'yanova et al., 2007). Plants rich in secondary metabolites and with high free radical scavenging activity have high antioxidant activity and can survive against a variety of environments. Free radical scavenging activity was found to be positively correlated with total phenol content in daisy flowers cv. 'Habanera' (Fig.2).

Carotenoids, flavonoids, and betalains are major flower pigments in the English daisy. The pigments involved in flower color are water-soluble and are generally located in the vacuole; the most common type of such pigments is anthocyanin (Davies, 2008). As a group, anthocyanins are strong antioxidants. The stable structure and powerful antioxidant activity of anthocyanins result from their double-bond conjugated system which enables electron delocalization (Iriti et al., 2004). Additionally, the degree and position of hydroxylation and methoxylation, in the B ring of anthocyanins, can modulate their stability and reactivity (Glover, 2007). Anthocyanins are glucosidal compounds and need sugars for their synthesis (Hapkins, 1999). It is possible that the NP treatment activated the conversion of sugars into starch and resulted in a decrease of anthocyanin production. Secondary metabolites are more likely to be produced in higher amounts under stress conditions. In this study, the control treatment produced the highest level of quercetin, which might be due to the effect of phosphorus on

maintaining the favorable growth condition for plants and subsequently unfavorable condition for the production of quercetin which is produced in a higher amount under unfavorable and stress conditions. Further increases in P concentrations may cause toxic conditions, leading to increased quercetin production.

Conclusion

Different organs of daisy showed different responses to the application of nano-phosphorus concentrations. The application of NP on daisy flowers negatively affected total phenol content in petals, anthocyanin content, and free radical scavenging. Furthermore, the application of NP on daisy flowers reduced the quercetin content in flowers. The application of NP treatments increased the total phenol and total flavonoids content in the roots, as well as the total flavonoid production in the leaves and petals. The application of NP could increase total phenol and flavonoid in some organs of daisy flowers. Thus, NP can be a favorable treatment for the production of some secondary metabolites in different parts of English daisy plants.

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Conflict of Interest

The authors declared no conflict of interest in this work.

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