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Investigation of gene expression diversity in *Hypericum* spp. before and after flowering under different nitrogen fertilization levels

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Abstract: The traditional medicinal herb, *Hypericum perforatum* L. has been popular for its pharmaceutical and coloring wealth since the ancient era. A secondary metabolite from the group of naphthodianthrones in *Hypericum* spp. named hypericin is responsible for the antidepression, anticancer, and antiviral characteristics of this herb. It has been found that several genes are involved in the biosynthesis pathway of hypericin. The *hyp-1* gene is participating in this biosynthesis path through the conversion of emodin to hypericin. The naphthodianthrones (hypericin and pseudohypericin) in *Hypericum* are synthesized through the polyketide pathway. In the plants, the enzyme complexes named polyketide synthase (PKS) catalyzes the reactions of polyketide pathways. The genes *HpPKS1* and *HpPKS2* are encoding PKS enzyme complexes. In this research, the relative expression of *hyp-1*, *HpPKS1,* and *HpPKS2* genes was compared in root and leaves of *Hypericum perforatum* and *H. androsaemum* L., before and after flowering under urea fertilization at 24, 48 and 72 hours after irrigation. The highest expression level of all three genes was observed after flowering in the samples of *H. perforatum* that were fertilized 72 hours after irrigation by 1 g l^{-1} urea (*hyp-1* in roots; *HpPKS1* and *HpPKS2* in leaves). The relative expression of *hyp-1* in the root was greater than in the leaves, but *HpPKS1* and *HpPKS2* expression in leaves was higher than in root. The relative expression of all three genes in *H. perforatum* was higher than in *H. androsaemum*. By increasing the interval between urea fertilization and irrigation, the relative expression of genes had an increasing trend, also by increasing the amount of urea fertilizer, relative gene expression was increased.

Key words: *Hypericum;* hypericin; *hyp-1 HpPKS1*; *HpPKS2*; transcriptional diversity

Preučevanje raznolikosti izražanja genov pri dveh vrstah krčnic (*Hypericum* spp.) pred in po cvetenju v razmerah različnega gnojenja z dušikom

Izvleček: Šentjanževka (*Hypericum perforatum* L.) je že od nekdaj popularna kot tradicionalno zdravilno zelišče zaradi svojih farmacevtskih in barvilnih lastnosti. Sekundarni metabolit iz skupine naftodiantronov v vrstah iz rodu *Hypericum* imenovan hipericin je odgovoren za antidepresivne, antikancerogene in antivirusne lastnosti tega zelišča. Ugotovljeno je bilo, da so pri biosintezi hipericina udeleženi številni geni. Gen *hyp-1* sodeluje pri tej biosintezi preko pretvorbe emodina v hipericin. Naftodiantrona (hipericin in pseudohipericin) se v šentjanževki sintetizirata po poliketidni poti presnove. V rastlinah katalizira reakcije poliketidne presnovne poti encimski kompleks poliketid sintaza (PKS). Gena *HpPKS1* in *HpPKS2* kodirata PKS encimski kompleks. V tej raziskavi je bila primerjana relativna ekspresija genov *hyp-1*, *HpPKS1,* in *HpPKS2* v koreninah in listih vrst *Hypericum perforatum* in *H. androsaemum* L.*,* pred in po cvetenju, pri gnojenju z ureo 24, 48 in 72 ur po zalivanju. Največja ekspresija vseh treh genov je bila opažena po cvetenju v vzorcih vrste *H. perforatum*, 72 ur po tem, ko je bila rastlina fertirigirana z 1 g l^{-1} uree (*hyp-1* v koreninah; *HpPKS1* in *HpPK* (v listih). Relativna ekspresija *hyp-1* v koreninah je bila večja kot v listih, a ekspresija genov *HpPKS1* in *HpPKS2* v listih je bila večja kot v koreninah. Relativna ekspresija vseh treh genov je bila v vrsti *H. perforatum* večja kot v vrsti *H. androsaemum.* S povečevanjem intervala med gnojenjem z ureo in zalivanjem se je pokazal trend naraščajoče ekspresije genov, kar se je pokazalo tudi s povečevanjem odmerka uree.

Ključne besede: *Hypericum*; hipericin; *hyp-1 HpPKS1*; *HpPKS2*; transkripcijska raznolikost

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Abbreviations: AF: Sampling after flowering; BF: Sampling before flowering; 0: Non-fertilized; 0.5-24: 0.5 g l^{-1} urea fertilizer was used 24 hours after irrigation; 1-48: 1 g l^{-1} urea fertilizer was used 48 hours after irrigation. [Hp]: *Hypericum perforatum*; [Ha]: *Hypericum androsaemum*. cDNA: complementary DNA; DNA: Deoxyribonucleic acid; ds/m: deciSiemens per meter; g l^{-1}: grams per liter; *HpPKS1*: Polyketide synthase I; *HpPKS2*: Polyketide synthase II; ppm: parts per million; RNA: Ribonucleic acid; rRNA: Ribosomal ribonucleic acid; SAS: Statistical Analysis System

Okrajšave: AF: vzorčenje po cvetenju; BF: vzročenje pred cvetenjem; 0: negnojeno; pognojeno s 0,5-24: 0,5 g l^{-1} uree 24 ur po zalivanju; 1-48:pognojeno z 1 g l^{-1} uree 48 ur po zalivanju. [Hp]: *Hypericum perforatum*; [Ha]: *Hypericum androsaemum*. cDNK: komplementarna DNK; DNA: Deoksiribonukleinska kislina; ds m^{-1}: deci Siemens na meter; g l^{-1}: grami nar liter; *HpPKS1*: Poliketidna sintaza I; *HpPKS2*: Poliketidna sintaza II; ppm: delci na miljon; RNA: Ribonukleinska kislina; rRNA: Ribosomalna ribonukleinska kislina; SAS: Sistem statistične analize

1 INTRODUCTION

Medicinal plants are one of the most important potential sources of drugs that have been used since ancient times. The World Health Organization estimates that more than 80 percent of people use medicinal herbs through traditional or modern ways. Also, many chemical drugs are also made by modeling plant compounds (Samuelsson & Bohlin, 2017). In recent years, the increasing tendency to use medicinal herbs and their compounds have been observed, and currently, more than 40 percent of the drugs used in the advanced western countries are herbaceous origin (Khosh-Khui et al., 1984). Identifying and investigating the chemical compounds of medicinal plants, not only helps to treat diseases more easily and cheaply, but also leads to a decline in drug imports. Iran, with its special climatic conditions, has a vast abundance of local medicinal herbs and is a source of rich potential for these plants.

The genus *Hypericum* (Hypericaceae family) consists of 496 worldwide species (Nürk et al., 2013; Ruhfel et al., 2013), of which 17 species have been reported from Iran and most of them have antioxidants (Silva et al., 2005), antiviral (Birt et al., 2009), an antidepressant (Butterweck, 2003) and anticancer compounds (Agostinis et al., 2002). Most of these medicinal properties are due to the presence of a group of hydrosoluble secondary metabolites, which are often present in the flowers of this perennial herb (Lazzara et al., 2015). *Hypericum perforatum* L. is one of the traditional medicinal herbs that has been used since ancient times because of its secondary metabolites and coloring properties. *Hypericum* is one of the most important sources of dye in dyeing industries and is used for coloring silk and wool in red. The boiling of the vegetative body of *Hypericum* with greenery leads to the formation of yellow color for the dyeing industry (Duke, 2002; Russo et al., 2014).

Hypericin is the key biochemical compound used to determine the quality of the extract of *Hypericum* (Duppong et al., 2004), and it has been shown that the number of dark glands in the leaf is correlated with hypericin content (Briskin & Gawienowski, 2001). Hypericin is probably the strongest natural light-sensitive substance (photosensitizer) that has been identified so far, the photosensitizers are used in photodynamic therapy of cancers (Agostinis at al., 2002; Karioti & Bilia, 2010). Although the function of hypericin in *Hypericum* species is not yet fully understood, it has been suggested that this compound plays a defensive role against herbivorous organisms and plant pathogens (Sirvent et al., 2003; Bruni & Sacchetti, 2009). Through a chemical defense system, the phytophagous insects feeding on *H. perforatum* leaves produced more antioxidant enzymes under oxidative stress caused by hypericin photosensitization. Hypericin in sunlight interact with oxygen molecules to produce oxygen free radicals and create oxidative stress, which causes phytophagous insects' behavioral avoidance feeding on the plant tissue (Guillet et al., 2000). To determine the possible role of hypericin in the defensive response of *H. perforatum*, the effects of elicitors such as salicylic acid (SA) and methyl jasmonate (MeJA) on the production of secondary metabolites in *H. perforatum* were evaluated, which resulted in increased levels of hypericin in certain concentrations of SA and MeJA (Sirvent & Gibson, 2002). Hypericin provides the energy and health for human consumers and considerably enhances the human body's defensive ability through increasing cellular oxygen absorption and vascular respiration (Miskovsky, 2002: Agostinis at al., 2002). Regarding the photodynamic properties of hypericin, this metabolite has therapeutic potential as an antiviral, antiretroviral, and anticancer agent (Zhang et al., 2016, Jendželovská et al., 2016; Kubin et al., 2005). Hypericin, a valuable polycyclic dianthroquinone, is responsible for the antidepression and antiviral characteristics (Rahnavrd, 2017).

Studies have shown that the *hyp-1* gene is one of the most important genes involved in the pathway of hypericin biosynthesis, which encodes for phenolic coupling protein, that is catalyzed *in vitro* and specifically convert emodin to hypericin (Bais et al., 2003; Zhang et al., 2018; Michalska et al., 2010). The production of emodin anthrone, an anthraquinone which is a precursor for hypericins biosynthesis (Falk, 1999), proceeds through a polyketide pathway that is catalyzed by *HpPKS1* and *Hp-*

PKS2 (Zobayed et al., 2006; Michalska et al., 2010). Recently, it was proposed that probably the function of the Hyp-1 protein is more likely associated with the reserving or carrying of hypericin in the cell than in the real biosynthesis of hypericin (Michalska et al., 2010).

In detail, the octa-β-ketoacyl chain is made from acetyl-CoA plus malonyl-Coa that catalyzed by polyketide synthase (PKS). After several steps emodin is made, phenolic oxidative coupling protein (Hyp-1) catalyzes the emodin anthrone production from emodin, then Hyp-1 catalyzes the emodin anthrone conversion to protohypericin, and finally, Hyp-1 catalyzes the formation of hypericin from protohypericin. Therefore the genes *hyp-1*, *HpPKS1,* and *HpPKS2* are playing important roles in hypericin biosynthesis (Rahnavrd, 2017).

Through enhancing some components of a plant, such as increasing the plant secondary metabolites producing parts consequently will rise the accumulation of active ingredients and bioactive compounds (Jabbari et al., 2011). Some environmental factors like nitrogen fertilization play an important role in improving secondary metabolites content. Some nutrients (nitrogen and iron) can increase the amount and quality of essential oils in thyme (Jabbari et al., 2011); similarly nitrogen and sulfur have the same role in lemongrass (Zheljazkov et al., 2011). Nitrogen in plants is used to build amino acids and enzymes, which are utilized in the biosynthesis of various bioactive compounds (Nurzyńska-Wierdak, 2013). The degree of nitrogen fertilization is the main element for plant biomass accumulation and influences the quantity and proportion of different chemicals of secondary metabolites (Daneshian et al., 2009; Politycka & Golcz, 2004), also at *in vitro* plant cell or tissue cultures (Murthy et al., 2014a; Murthy et al., 2014b). Some researches indicated that nitrogen concentration and $NH4^{+}/NO3^{-}$ ratio affect the hypericins in *Hypericum* spp. (Cui et al., 2010; Murthy et al., 2014a). Also, hypericin content significantly varied depending on the developmental stage. Different harvest stages influence the hypericin content, whereas leaf hypericin content at earlier growth stages was higher than the fruit set stage (Sun et al., 2019). Also, *Hypericum triqutrifolium* Turra showed a higher accumulation of total hypericin at the vegetative stage than the flowering and fruiting stages of herb (Azeez et al., 2017). According to the results of research to investigate hypericins variation in *Hypericum triquetrifolium* during plant growth it was observed that hypericin content in leaves was higher at the reproductive stages (flowering), but in stems was higher at the vegetative stages (Ayan & Cirak, 2008). Another research about the ontogenetical variation of hypericin and hyperphorin in *H. perforatum* revealed that the total hypericin contents decreased as developmental stages progressed (Büter & Büter, 2002).

To produce plant secondary metabolites through *in vitro* culture, acquiring information about the biosynthesis of the secondary metabolites is essential to improve the production of chemical compounds such as hypericin and hyperphorin (Vattikuti & Ciddi, 2005). Moreover, regarding that the function of a major part of plant genome is unknown so far, the relationship between enzymes, signaling substances, and small molecules is still relatively unknown. To fully understand the regulation of metabolites biosynthesis and purposeful changes in gene expression more research is required (Deepak et al., 2007). The effect of various factors on the expression of the genes involved in the biosynthesis of the pharmaceutical compounds can be investigated by a quantitative real-time PCR (qPCR) approach (Pfaffl, 2001). The production of active ingredients in medicinal plants is controlled by genetic factors. However, limited information exists about the effect of fertilizer treatments on the relative expression profiling of the genes involved in the biosynthesis pathway of active ingredients of medicinal plants.

In the present study, the effects of nitrogen fertilizer at different times after irrigation were investigated on the expression of *hyp-1*, *HpPKS1* (polyketide synthase), and *HpPKS2* genes in leaf and root of *Hypericum* spp., before and after flowering using qPCR. This is the first report about the relative expression of some genes of *Hypericum* spp. under different levels of root nitrogen supply at different time intervals after irrigation.

2 METHODS AND MATERIALS

2.1 PLANT MATERIALS

The experiments were carried out with *H. perforatum* L. and *H. androsaemum* L:. *Hypericum* seeds were provided by the Iranian Biological Resource Center (IBRC), Tehran, Iran. The seeds were planted in pots containing farm soil (5 seeds / 4 l pot). The physicochemical properties of soil were: saturation percentage (32 %), electricity conductivity (1.2 103 ds m^{-1}), pH of paste (7.7), total neutralizing value (8 %), organic carbon (0.22 %), K available (212 ppm), P available (7.6 ppm) and soil texture (sandy clay loam).

2.2 PLANT CULTURE AND FERTILIZERS TREATMENTS

A factorial design based on completely randomized design (CRD) with three replications was used. Every pot with 5 plants was considered as a replication, namely,

each treatment included three pots. The urea granular fertilizer was fertilized at 0, 0.5, and 1 mg l^{-1} in intervals at 24, 48, and 72 hours after irrigating. For RNA extraction, 0.3 g from pooled leaves or roots of three plants of each pot were used. Plant material sampling was accomplished before flowering (200 days after seed planting) and after flowering (300 days after seed planting) stage. Roots/leaves of 5 plants formed the plant material of an experimental unit (a replication of a treatment).

2.3 RNA EXTRACTION, PURIFICATION AND CDNA SYNTHESIS

Total RNA was extracted from leaf and root samples using the GeneAll RiboEx kit (BioFrontier, Korea) based on the manufacturer's protocol. Agarose gel electrophoresis and NanoDrop 1000 spectrophotometer (U.S.A) were used to determining RNA quality and integrity and checking the RNA quantity respectively. Samples of RNA were treated with RNase-free DNase Kit (Fermentas, Hanover, MD) to remove any contaminating genomic DNA, before cDNA synthesis. First-strand cDNA was synthesized from 5 µg of total RNA treated with DNase I using 200U MMLV reverse transcriptase (GREEN BIO GENE) according to the manufacturers' instructions.

2.4 PRIMER DESIGN AND REAL-TIME PCR

Primer design was carried out using online primer Quest software, the primer sequences for *hyp-1*, *HpPKS1,* and *HpPKS2* genes are listed in Table 1. According to scientific reports, the 18s rRNA gene was used as the reference gene for data normalization. In the qPCR method, increasing or decreasing of expression quantity of genes is important, which calculated by the delta-delta method (Pfaffl, 2001). The qPCR was performed in a total volume of 20 µl containing 2 µl of cDNA, 10 µl SYBR Green master mix, and 4 pmol of each primer. The amplification reactions were carried out in a Step-One qPCR System (Applied Biosystem, ABI, USA) under the following conditions: 5 min at 95 °C, 35 cycles of "95 °C for 30 s, annealing temperature (Table. 1) for 30 s and 72 °C for 40 s", and 72°C for 10 s. All amplification reactions were repeated three times under identical conditions and included a negative control. To confirm that the PCR products were produced from cDNA and not genomic DNA, appropriate control reactions (without the existence of reverse transcriptase) were carried out. This experiment was carried out with two biological and three technical repeats.

2.5 STATISTICAL ANALYSIS

The data normality was confirmed through the Kolmogorov-Smirnov test, then the relative expression levels of the genes were calculated by ΔΔCT and the Relative Expression Software Tool (REST) software (Pfaffl, 2001) was used to data analysis. One-way ANOVA and mean comparisons (Duncan's test) was accomplished using SAS software.

3 RESULTS

3.1 RELATIVE EXPRESSION OF *HYP-1* GENE

Mean comparisons of *hyp-1* gene expression revealed significant differences among treatments. The mean expression of *hyp-1* gene was lower in control treatments (1.3-fold) compared to the nitrogen supporting treatments. Three the highest relative expressions of *hyp-1* belonged to the root of *H. perforatum*: AF1-72 (13.6-fold), BF1-72 (11.3-fold), AF0.5-72 (10.4-fold). In

Table 1: Primer sequences for *hyp-1, HpPKS1,* and *HpPKS2* genes used in the quantitative real-time PCR for gene expression analysis of *Hypericum* spp.

Gene		Primer sequence (5'-3')	Annealing temperature	Fragment size
hyp-1	Forward	TCCTGAGAGGAAGTACAGTGTC	58	125
	Reverse	AGACGGCCCAGATTCATCA		
HpPKS1	Forward	GACAGAGGTGTGGAAGAAG	63	112
	Reverse	GTACATCAACTGCGCCAT		
HpPKS2	Forward	CCTTCCCTTCCGTGTGTATATG	63	184
	Reverse	CAGAGGAGAAACTGGAGGAAG		
18srRNA	Forward	TGTCTGCGATAATGGAACTG	58	108
	Reverse	ATTCATCATACTCCGCCTTAGC		

all cases, increasing the interval between urea fertilization and irrigation caused an increasing trend in gene expression level ($p < 0.01$). Furthermore, in all cases, by increasing the amount of urea fertilizer, the relative gene expression was increased ($p < 0.01$). In overall, it can be concluded that *hyp-1* relative expression in root tissue was greater than in leaf tissues ($p < 0.01$) (Figure 1).

3.1.1 Leaf

Among the leaf samples, the highest relative expression of the *hyp-1* gene belonged to the treatment of [Hp] AF1-72 (10-fold), the second to fourth highest *hyp-1* expression was observed in [Hp]AF1-48 (7.6-fold), [Hp] AF0.5-72 (7.5-fold) and [Ha]AF1-72 (7.4-fold) treatments respectively, and the next-highest gene expression was assigned to the treatment [Ha]BF1-72 (4.5-fold). In general, the mean expression level of *hyp-1* in *H. androsaemum* leaves (3.3-fold) was lower than in *H. perforatum* leaves (5.1-fold; $p < 0.01$). The lowest average expression of *hyp-1* was observed at before flowering sampled leaves of *H. androsaemum* (2.3-fold). The mean expression level in after flowering samples (5.1-fold) was higher than in before flowering (3.3-fold), so the effect of time on *hyp-1* expression in *Hypericum* leaves was significant ($p < 0.01$) (Figure 1).

3.1.2 Root

Among the root samples, the top three *hyp-1* relative expressions were [Hp]AF1-72 (13.6-fold), [Hp]BF1-72 (11.3-fold), and [Hp]AF0.5-72 (10.4-fold). Fourth to sixth the highest *hyp-1* expression belonged to [Ha] AF1-72 (9.7-fold) and [Hp]AF1-48 (9.5-fold), [Hp]BF1-48 (8.9-fold) and [Ha]BF1-72 (8.9-fold), respectively. In general, *hyp-1* mean expression in *H. androsaemum* roots (5.5-fold) was lower than in *H. perforatum* (7.1-fold), and the lowest mean expression level was observed in before flowering samples of *H. androsaemum* root tissues (5.1-fold). The relative expression of *hyp-1* in after flowering samples (6.8-fold) was higher than in before flowering samples (5.8-fold), so the effect of time on *hyp-1* expression in both *Hypericum* species roots is significant ($p < 0.01$) (Figure 1).

3.2 RELATIVE EXPRESSION OF *HPPKS1* GENE

Duncan's test for *HpPKS1* expression level revealed significant differences among means of treatments. The mean expression of *HpPKS1* gene in control treatments (0.7-fold) was lower than in the nitrogen fertilized treatments. The two highest relative expressions of *HpPKS1* belonged to leaves of *H. perforatum*: AF1-72 (9.1-fold) and BF1-72 (8-fold). The third to the seventh mean expressions of *HpPKS1* belonged to the following treatments of leaf samples, respectively: [Hp]AF1-48 (6.2-fold), [Hp]BF1-48 (6-fold), [Hp]AF0.5-72 (5.6-fold), [Ha]AF1-72 (5.2-fold) and [Hp]BF0.5-72 (5-fold). In all cases, with increasing the interval between urea fertilization and irrigation, the relative expression of *HpPKS1* had an increasing trend ($p < 0.01$). Furthermore, in all cases, by increasing the amount of urea fertilizer, *HpPKS1* expression was increased ($p < 0.01$). In overall, it can be concluded that *HpPKS1* mean expression in leaf was very greater than in root ($p < 0.01$) (Figure 2).

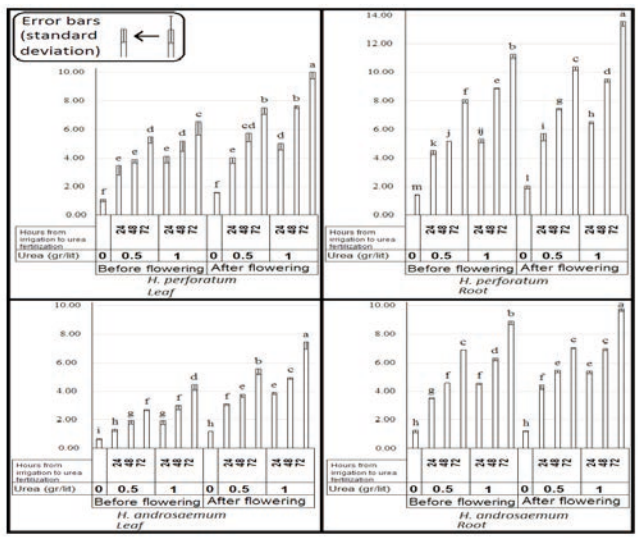

Figure 1: *hyp-1* expression level in different parts of *Hypericum* leaf and root (r = 3; $p < 0.05$)

3.2.1 Leaf

Among the leaf samples, the top four *HpPKS1* expression were belonged to *H. perforatum*: AF1-72 (9.1-fold), BF1-72 (8-fold), AF1-48 (6.2-fold) and BF1-48 (6-fold). In general, *HpPKS1* expression in *H. androsaemum* leaves (2.9-fold) was lower than in *H. perforatum* (4.6-fold; $p < 0.01$).,The lowest mean expression of *HpPKS1* was observed at before flowering samples of *H. androsaemum* leaf tissues (2.5-fold). *HpPKS1* mean expression in after flowering samples (4-fold) was higher than in before flowering (3.4-fold; $p < 0.01$), thus the developmental stage significantly affected *HpPKS1* expression level in both *Hypericum* species leaves (Figure 2).

3.2.2 Root

Among the root samples, the two highest *HpPKS1* expressions have belonged to [Hp]AF1-72 (3.3-fold) and [Ha]AF1-72 (2.6-fold). *HpPKS1* mean expression level was approximately equal in two *Hypericum* species root tissues (1.6-fold). The relative gene expression in after flowering samples (1.8-fold) was higher than in before flowering samples (1.5-fold; $p < 0.01$) (Figure 2).

3.3 RELATIVE EXPRESSION OF *HPPKS2* GENE

Based on Duncan's mean comparisons for *HpPKS2* expression level discovered significant differences among various treatments. The mean expression of *HpPKS2* gene in control treatments (0.8-fold) was lesser than in the nitogen fertilized treatments. Among all treatments, the top seven *HpPKS2* relative expressions belonged to the *H. perforatum* leaves: AF1-72 (8.4-fold), AF0.5-72 (7.8-fold), BF1-72 (7.6-fold), AF1-48 (6.4-fold), BF1-48 (6.3-fold), AF1-24 (5.3-fold), and BF0.5-72 (5.3-fold). In all cases, with increasing the interval between urea fertilization and irrigation, *HpPKS2* expression had an increasing trend ($p < 0.01$). Similarly, in all cases except two treatment, by increasing the amount of urea fertilizer, *HpPKS2* expression was increased ($p < 0.01$).

In overall, it can be concluded that the relative expression of the *HpPKS2* in leaves(3.9-fold) was very greater than in root (1.6-fold; $p < 0.01$) (Figure 3).

3.3.1 Leaf

Among the leaf samples, the top three *HpPKS2* relative expression treatments were [Hp]AF1-72 (8.4-fold), [Hp]AF0.5-72 (7.8-fold), and [Hp]BF1-72 (7.6-fold). In general, *HpPKS2* expression in *H. androsaemum* leaves (2.9-fold) was lower than in *H. perforatum* leaves (4.8-fold; $p < 0.01$). The lowest *HpPKS2* mean expression was observed at the before flowering samples of *H. androsaemum* (2.5-fold). *HpPKS2* mean expression in after flowering samples (4.3-fold) was higher than in before flowering (3.4-fold; $p < 0.01$), therefore the effect of developmental stage on *HpPKS2* expression in both *Hypericum* species leaves was significant (Figure 3).

3.3.2 Root

Among the root samples, the highest *HpPKS2* expression belonged to the treatment of [Hp]AF1-72 (3.1-

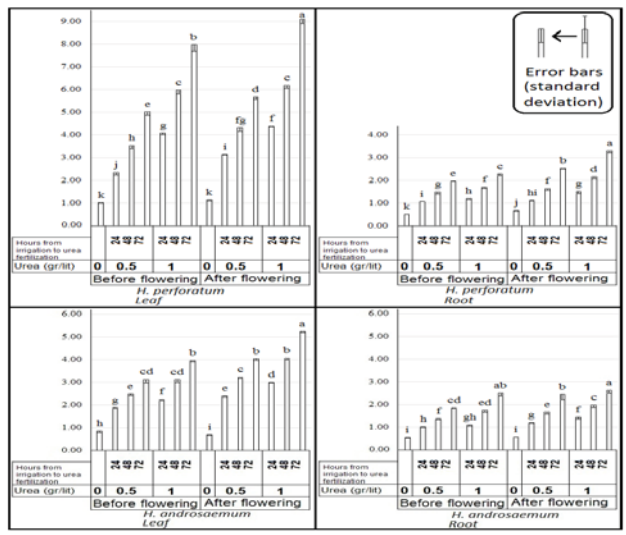

Figure 2: *HpPKS1* expression level in different parts of *Hypericum* leaf and root (r = 3; $p < 0.05$)

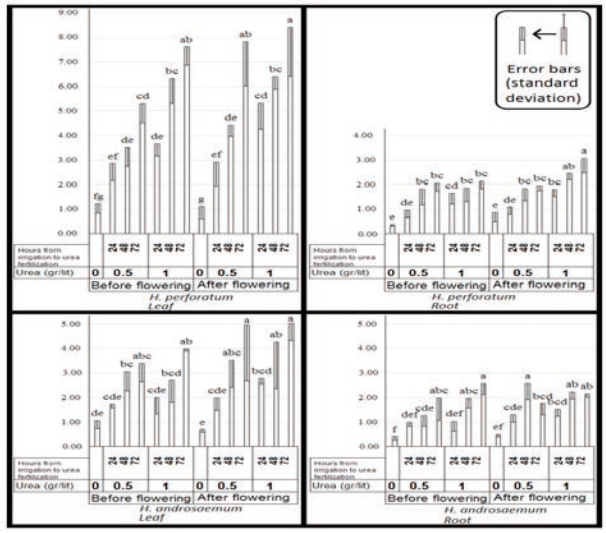

Figure 3: *HpPKS2* expression level in different parts of *Hypericum* leaf and root (r = 3; $p < 0.05$)

fold), then the treatment [Ha]BF1-72 (2.6-fold). The relative expression of the *HpPKS2* gene was approximately equal in two *Hypericum* species root tissues (1.6-fold). *HpPKS2* expression in after flowering samples (1.8-fold) was slightly higher than in before flowering (1.5-fold; $p < 0.01$) (Figure 3).

4 DISCUSSION

Production of secondary metabolites is controlled by the level of genes expression and is significantly influenced by the degree of cell differentiation, environmental conditions and the elements that are present in the plant (Vom Endt et al., 2002; Afrin et al., 2015). Lazzara et al. (2017) with the investigation of the effect of arbuscular mycorrhizal fungi (AFM) and phosphorus availability on the content of some secondary metabolites in *Hypericum perforatum*, reported that flower hyperforin concentration in mycorrhizal treatment was 17.5 % lower than non-mycorrhizal treatment. In contrast, with the use of AFM under low phosphorus availability, hypericin and pseudochypericin concentrations increased by 166.8 % and 279.2 %, respectively, while no effect of the use of AFM was found under high phosphorus availability. Based on the *H. perforatum* field study to determine the influence of N and P fertilizers on the flavonoids content, it was concluded that 125kg N ha^{-1} and 50kg P_2O_5 ha^{-1} proved optimal for high dry herb yield and flavonoids content compared to unfertilized plants (Azizi & Dias, 2004). Also, in the present study, by increasing the amount of nitrogen fertilizer, the relative expression of the genes involved in the hypericin biosynthesis pathway was increased, which was statistically significant in most cases.

The ultimate steps of hypericin biosynthesis have been proposed to be accompanied by Hyp-1, a phenolic coupling protein, a member of genes encoding plant pathogenesis-related class 10 protein family (PR-10) (Bais et al., 2003; Radauer et al., 2008; Michalska et al., 2010). Like other PR-10 family proteins, *hyp-1* looks to be developmentally regulated (Pinto et al., 2005, Liu & Ekramoddoullah, 2006). Also in other research, leaf and intact roots showed the highest expression of *hyp-1*, while stems and old parts of roots presented significantly lower expression levels, hence, there is variable existence of Hyp-1 protein in leaves at different phases of leaf development. (Karppinen, 2010). In the present study, the relative expression of all three genes studied in most cases in the after-flowering stage was higher than in the before-flowering stage.

Diverse expression levels of *hyp-1* in different tissues and organs of *Hypericum* have been reported. Košuth et al. (2007) reported absence of differences in *hyp-1* expression between leaf margins and leaf inner parts. According to their findings from *ex vitro* plants, fairly high expression of *hyp-1* gene was measured in roots. In the present study, the relative expression difference between root and leaf in the case of the *hyp-1* gene was smaller-than that of *HpPKS1* and *HpPKS2* genes. According to some studies, the *HpPKS2* expression and the hypericins content were the highest in the parts containing the dark glands, such as the flower buds, and were low in the parts free of dark glands, such as stem and root. In the case of the *hyp-1* gene, the relative expression in the root tissue was higher than the corresponding treatments in the leaf tissue ($p < 0.01$), but the relative expression of *HpPKS1*

and *HpPKS2* genes, in the root tissue, was lower than the corresponding treatments in the leaf tissue. It can be because of that the *hyp-1* gene, apart from contributing to the hypericins biosynthesis pathway, has other functions, and probably these biological roles are related to root tissue in *Hypericum* species. Because of the high expression of *HpPKS2* in the reproductive organs and as well in leaf borders, this gene may play a role in the agglomeration of defensive chemicals, such as hypericins (Agostinis et al., 2002; Onelli et al., 2002; Sirvent & Gibson, 2002; Sirvent et al., 2003).

Also, a research was conducted to find suitable sites for the synthesis of hypericin based on the level of *hyp-1* gene expression in the early stages of seedling development, and comparing the level of *hyp-1* gene expression in different parts of the plant regarding the presence or absence of dark glands. It has been found that *hyp-1* copies are found throughout the leaf tissue, but there is no significant difference in the level of gene expression in the margins and the inner parts of the leaf (Karppinen, 2010).

These results showed that *hyp-1*, *HpPKS1,* and *HpPKS2* expression in *Hypericum* significantly affected by the plant tissues, plant genus, amount and time of urea granular fertilization, and developmental phase of sampling.

5 CONCLUSIONS

In general, the results showed that using the urea fertilizer significantly affect the relative expression of studied genes, with increasing the urea fertilizer, relative expression of the studied genes increased. Gene expression values after flowering were higher than before flowering. Enhancing the time interval between irrigation and urea fertilizer application, gene expression increases. The gene *hyp-1* expression in root was higher than the leaf, but the relative expression of *HpPKS1* and *HpPKS2* genes in leaf was higher than in roots.

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