10.2478/v10279-012-0012-8

VERSITA

BOTANICA LITHUANICA
ISSN 2029-932X

2012, 18(2): 101–108

EFFECT OF EXTERNAL AND INTERNAL FACTORS ON SECONDARY METABOLITES ACCUMULATION IN ST. JOHN'S WORTH

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Abstract

Radušienė J., Karpavičienė B., Stanius Ž., 2012: Effect of external and internal factors on secondary metabolites accumulation in St. John's worth [Vidinių ir išorinių faktorių įtaka antrinių metabolitų kaupimuisi paprastojoje jonažolėje]. – Bot. Lith., 18(2): 101–108.

The effect of modified external factors such as temperature and light intensity, and plant internal factors – phenological phase and their possible interaction was investigated on accumulation of bioactive secondary metabolites in *Hypericum perforatum* L. The plants were grown under different temperature and light intensity conditions in a greenhouse. The results suggested that accumulation of secondary metabolites highly depends on temperature and light intensity conditions and phenological cycle, though the influence of both variables differed for the particular compounds. Phenolic compound quantities changed greatly during plant development, and the highest levels were reached at flowering phase under both experiment conditions. Changes in naphthodianthrones content at higher temperature and light intensity followed the same increasing tendency as that for phenolic compounds. The significant decrease of temperature and light intensity were found to be crucial negative factors for accumulation of naphthodianthrones. The highest level of hyperforin was accumulated at vegetative phase that fall down during phenological development of plants under both experiment conditions.

Keywords: HPLC analyses, naphthodianthrones, phenolic compounds, environment conditions, phenological development.

INTRODUCTION

Environmental factors such as light intensity, temperature, water availability, type and composition of soil and several other have a substantial influence on the quality and productivity of medicinal plants. They are not stable in habitats and the consequences of their variation are difficult to predict. Plants of the same species occurring in different environments may differ significantly in their content of particular secondary metabolites (Szakiel et al., 2010). Main function of plant secondary metabolites is thought to be the adaptation of plants to their environment (Kliebenstein, 2004). The evaluation of secondary metabolites accumulation under modified environment presents more correct information about effect of environment factors on metabolic pathways. Considerable research work in the last period addresses to various aspects of modelling these factors using new numerical methods (Mihailovic & Eitzinger, 2007). The factors that can be easy to control in greenhouse are light intensity and temperature. On the other hand, they are known as the crucial environment factors strongly related to the phenological development and secondary metabolism of medicinal plants (Hornok, 1992; Zobayed et al., 2005). It is possible to achieve high production of secondary metabolites within a very short period of cultivation under optimized conditions in a controlled environment system (Afreen, 2005). The stimulating of biosynthesis on secondary metabolites in medicinal crops by control and optimization of external and internal factors may be applied to develop the biotechnologies of high quality drug production (Poutaraud & Girardin, 2005).

Hypericum perforatum L. is one of the most famous medicinal plants which proven health effects of major secondary metabolites and which market value is growing up (Butterweck et al., 1998; Nathan, 2001; Shelton, 2009). Previous research has provided a large volume of information on bioactive compounds changes in respect to environment and phenological development of *Hypericum* species wild populations (Couceiro et al., 2006; Çirak et al., 2007, 2008). Such kind of research may be of poor scientific value, because difficult to define, which internal or external factor affects the changes of secondary metabolites. The objective of this research was to examine the influence of modified external factors such as temperature and light intensity, and plant internal factors – phenological phase, on accumulation of bioactive secondary metabolites in *H. perforatum*.

MATERIALS AND METHODS

Plant material. Seeds of *Hypericum perforatum* were germinated in a float system under a 16/8 h light/dark, 22/18°C day/night growth chamber conditions in February. A total of three seedlings 5–8 cm in length were transplanted into one pot of 30 cm in diameter, filled with the commercial growing substrate and then transferred to greenhouse. Two experiments of different temperature and light intensity growth conditions were arranged in greenhouse by using 50 % transparent polyethylene cover. For each experiment 40 pots with plants were treated, thus a total of 80 pots were used. Temperature was measured using a Sato Keiryoki MFG R-704 thermo hydrograph; light intensity was measured at one meter high above the plants by a Delta-T Sun Scan Canopy Analyser. Average temperature and light intensity throughout the growth period of experiment-1 was 32 °C and 1618.6 $\mu molm^{-2}s^{-1}$, while of experiment-2 – 24 °C and 803.4 $\mu molm^{-2}s^{-1}$, respectively.

Whole plants from two greenhouse experiments were harvested between May and September in four different growth phases: vegetative, budding, flowering and fruiting, from ten randomly selected pots for each phase. Harvested plants were air-dried and then mechanically ground to obtain a homogenous drug powder.

Extraction. Samples of about 0.5 g (weighed with 0.0001 g precision) were extracted in 50 ml of 100 % methanol by ultrasonication at 40 ºC for 30 min. in a Sonorex Super model RK 225H ultrasonic bath. The prepared extracts were filtered through a membrane filter with pore size of 0.22 µm (Carl Roth GmbH, Karlsruhe, Germany) and kept in a refrigerator until analysis no longer than three hours. The extracts for naphthodianthrones analysis after ultrasonication were exposed to light for 30 min. for photoconversion of protohypericin into hypericin and protopseudohypericin into pseudohypericin.

HPLC analysis. A Shimadzu Prominence LC (Shimadzu Europa GmbH, Duisburg, Germany) chromatographic system equipped with two LC-20-AD model pumps, a SIL-20AC auto-injector, a thermostat CTO-20AC and a SPD-M20A detector was used for HPLC-PAD analysis of extracts. Separation of all compounds was carried out using a YMC Pack Pro-C18 (**YMC Europe GmbH, Dinslaken, Germany**) column (150 mm × 4 mm i.d.; 3 µm particle sizes) with 10 mm guard-precolumn. Separation of phenolics and hyperforin was performed using a binary mobile phase system of solvent A (water containing 0.1 % trifluoroacetic acid (TFA)) and solvent B (acetonitrile containing 0.1% TFA) at a flow rate of 1.0 mL min^{-1}. Injection volume was 10 µL. The following gradient elution programme was used: 0–1 min (A 95→95 %, B 5→5 %), 1–14 min (A 95→80 %, B 5→20 %), 39–39.5 min (A 0→95 %, B 100→5 %), 39.5–45 min (A 95→95 %, B 5–5 %). The detection was monitored at 210–790 nm wave length with constant column temperature at 40 °C.

The hypericin and pseudohypericin elution programme was isocratic. The mobile phase consists of acetonitrile containing 0.1 % TFA. Flow rate of mobile phase was 1.1 mL·min^{-1}. Injection volume of extract was 10 µL. The maximal absorption on the UV spectra of hypericin and pseudohypericin was at 580 nm wavelengths. Identity of constituents was also confirmed by UV spectra reference standards. The eluted compounds were identified by comparison of the retention time and UV spectra with those of the reference standards. The maximal absorption on the UV spectra of compounds were obtained as follows: hyperforin – 270 nm, chlorogenic acid – 325 nm, amentoflavone – 332 nm, kaempferol – 346 nm, quercitrin – 347 nm, apigenin-7-O-glucoside, ru-

tin and hyperoside – 353 nm, quercetin – 368 nm, hypericin and pseudohypericin – 580 nm wavelength.

The stock solutions of reference standards were prepared by dissolving them in methanol. Calibration curves were established by diluting the stock solutions with methanol in appropriate quantities. The quantities of compounds were calculated from an external standard calibration curves established on six concentrations in the following range: 6.1–310 μg/ mL for chlorogenic acid and rutin, 3.4–304 μg/mL for hyperoside, 3.6–610 μg/mL for isoquercetin, 2–200 μg/mL for quercitrin and quercetin, 0.75–75 μg/ml for amentoflavone, 0.8–80 μg/ml for kaempferol, 11–1060 μg/mL for hyperforin, 0.5–105 μg/mL hypericin and 10.4–110 μg/mL for pseudohypericin. All calibration curves showed good linear regression ($r^2 \geq 0.999$) within the test range. Each sample was analysed twice and the mean value was used for calculation. The concentration of compounds was expressed as mg/g dry mass (DM).

Chemicals. The reference substances apigenin-7-O-glucoside (purity ≥ 98 %), chlorogenic acid (purity 98.03 %), rutin trihidrate (purity 99.02 %), isoquercetrin (purity 99.0 %), kaempferol (purity ≥ 96 %), quercitrin (purity 99.0 %), hypericin (purity 99.79 %) and pseudohypericin (98.03 %) were purchased from Karl Roth (Germany). Amentoflavone (purity 98 %), hyperoside (purity 98.6 %), quercetin (purity 95.4 %), and hyperforin reference material (42.6 % hyperforin and 14.1 % adhyperforin, HPLC) were obtained by ChromaDex (USA). Acetonitrile and methanol of HPLC grade were supplied by Karl Roth (Germany).

Data analysis. The results were performed for the statistical analysis using SPSS 16.0 software computer package. The data were subjected to the analysis of variance (ANOVA). The Student's *t* statistics (?) was performed for testing the difference of each compound quantity between experiments. Post-hoc comparisons of mean quantities were made using the Duncan Multiple Range Test. A value of $p \leq 0.05$ was considered to be significant.

RESULTS

A greenhouse experiments were conducted to investigate the effects of growth conditions (temperature and light intensity) together with phenological stage on accumulation of secondary metabolites in *Hypericum perforatum*. Two-way ANOVA described the complex relationship between the content of compounds as dependent continuous variable with both nominal variables – 'experiment' (growth conditions) and 'phase' (phenological) as well as their possible interaction term. According to the data of statistical analysis ($p < 0.05$) the 'experiment × phase' interaction was not significant for all compounds what indicated that the effect of 'experiment' did not depend on the level of 'phase' and vice versa (Table 1). The two variables, i.e. 'experiment' and 'phase', jointly did not affect the content of compounds. Therefore, the tests for the individual effects were valid and showed a significant 'experiment' effect for amentoflavone ($F = 69.00$; $p < 0.001$), apigenin-7-O-glucoside ($F = 29.69$; $p < 0.001$), chlorogenic acid ($F = 34.30$; $p < 0.001$), hyperoside ($F = 71.20$; $p < 0.001$), kaempferol ($F = 27.90$; $p < 0.001$), rutin ($F = 8.28$; $p < 0.01$), quercetin ($F = 70.86$; $p < 0.001$), quercitrin ($F = 62.36$; $p < 0.001$), and total phenolics ($F = 60.53$; $p < 0.001$) quantities, but no significant for hyperforin and hypericins. On the other hand, the 'phase' effect was significant for chlorogenic acid ($F = 3.41$; $p < 0.029$), hyperoside ($F = 3.13$; $p < 0.039$), quercetin ($F = 4.00$; $p < 0.016$), total phenolics ($F = 3.12$; $p < 0.04$), and hyperforin ($F = 11.41$; $p < 0.001$), but no significant for other detected constituents (Table 1).

While the two-way ANOVA allowed to get a general picture of relationships, for detailed analysis one must refer to the more concrete methods of *t*-test and one-way analysis of variance.

The *t*-test comparison of mean quantities of bioactive compounds between two experiments showed highly significant differences ($p < 0.05$) in amentoflavone, apigenin-7-O-glucoside, chlorogenic acid, hyperoside, kaemferol, quercetin, quercitrin and total phenolics during entire phenological cycle (Table 2).

Higher temperature and light intensity of experiment-1 determined the considerably higher values of corresponding compounds (Fig. 1). The content of rutin did not differ significantly in budding and fruiting plants from both greenhouse experiments, while the highest difference ($t = 10.89$, $p < 0.001$) of this compound was detected in flowering plants. The significant difference in accumulation of hyperforin

Table 1. Two-way analysis of variance (ANOVA) of relationship between bioactive compounds accumulation and growth conditions of experiments together with phenological phases and their possible interaction

Compounds	Experiments		Phenological phases		Interaction experiment × phase	
	F	p	F	p	F	p
Amentoflavone	69.00	0.001*	0.54	0.661	0.27	0.845
Apigenin-7-O-glucoside	29.69	0.001*	2.77	0.058	1.40	0.261
Chlorogenic acid	34.30	0.001*	3.41	0.029*	1.70	0.187
Hyperoside	71.20	0.001*	3.13	0.039*	1.01	0.399
Kaemferol	27.90	0.001*	1.99	0.135	0.47	0.706
Rutin	8.28	0.01*	1.02	0.396	0.89	0.455
Quercetin	70.86	0.001*	4.00	0.016*	0.49	0.689
Quercitrin	62.36	0.001*	2.35	0.091	0.28	0.841
Total phenolics	60.53	0.001*	3.12	0.04*	1.05	0.384
Hyperforin	1.07	0.31	11.41	0.001*	0.64	0.594
Hypericin	0.12	0.729	0.99	0.409	2.34	0.092
Pseudohypericin	0.11	0739	1.73	0.181	1.36	0.272

* – significant differences at $p < 0.05$.

Table 2. The *t*-test comparison results of paired mean quantities of bioactive compounds in *Hypericum perforatum* between different growth conditions of experiments during phenological cycle

Compounds	Vegetative		Budding		Flowering		Fruiting	
	t	*p*	*t*	*p*	*t*	*p*	*t*	*p*
Amentoflavone	4.69	0.001*	3.97	0.015*	4.37	0.005*	4.58	0.010*
Apigenin-7-O-glucoside	3.16	0.01*	7.38	0.001*	3.33	0.016*	3.49	0.013*
Chlorogenic acid	3.12	0.01*	3.57	0.014*	4.36	0.01*	3.17	0.019*
Hyperoside	4.66	0.001*	4.12	0.019*	4.81	0.003*	8.49	0.002*
Kaemferol	2.97	0.01*	4.09	0.012*	9.53	0.001*	4.49	0.019*
Rutin	4.64	0.004*	1.92	0.076	10.89	0.001*	1.72	0.136
Quercetin	4.58	0.001*	4.98	0.003*	4.72	0.013*	8.04	0.001*
Quercitrin	4.46	0.002*	6.59	0.004*	3.28	0.040*	16.36	0.001*
Total phenolics	4.30	0.001*	4.71	0.010*	4.29	0.005*	7.90	0.002*
Hyperforin	10.02	0.001*	1.63	0.183	0.97	0.376	1.23	0.265
Hypericin	0.31	0.758	0.05	0.966	3.05	0.033*	3.92	0.008*
Pseudohypericin	0.24	0.813	0.39	0.708	4.74	0.013*	2.80	0.031*

* – significant differences at $p < 0.05$.

between different conditions was estimated only in plants of vegetative phase ($t = 10.02$; $p < 0.001$). In contrast to the phenolic compounds examined, the quantities of naphthodianthrones were found to be of lower dependence to changes in temperature and light intensity conditions before plant flowering phase. Significant differences of hypericin and pseudohypericin accumulation were detected in flowering ($t = 3.05$, $p < 0.033$ and $t = 4.74$, $p < 0.013$, respectively) and fruiting ($t = 3.92$, $p < 0.008$ and $t = 2.80$, $p < 0.031$, respectively) phases between experiment-1 and experiment-2.

One-way analysis of variance was performed for further statistical analyses of the results. A post-hoc Duncan's Multiple Range test enabled us to specify differences in quantities of compounds among phenological phases of two experiments. The multiple comparison indicated that significantly highest level of phenolic compounds, namely apigenin-7-O-glucoside (0.699 mg/g), chlorogenic acid (0.836 mg/g), hyperoside (5.632 mg/g), rutin (0.233 mg/g), quercetin (0.426 mg/g), quercetrin (1.179 mg/g), and total phenolics (9.029 mg/g) was reached at flowering phase of plants grown under experiment-1 conditions (Fig. 1). Plants from experiment-2 accumulated significantly higher levels of apigenin-7-O-glucoside (0.162 mg/g), hyperoside (1.985 mg/g), rutin (0.19 mg/g), quercetin (0.172 mg/g), quercetrin

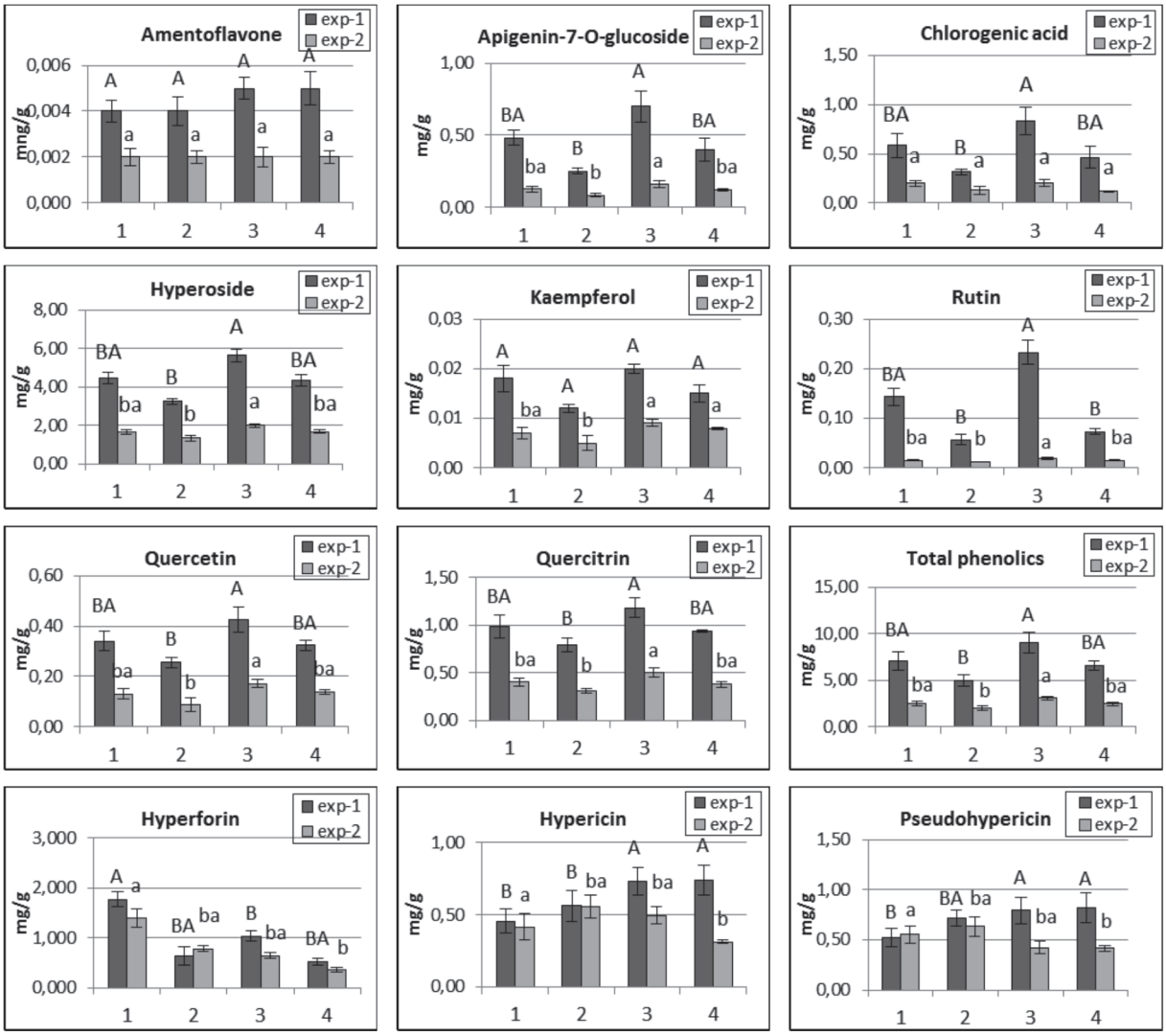

Fig. 1. Accumulation trends of secondary metabolites in *Hypericum perforatum* grown under different greenhouse conditions during phenological cycle. Mean values ± SE quantities (mg/g DM) marked with the same letter are not significantly different at $p < 0.05$ using Duncan Multiple Range test. Phenological phases: 1 – vegetative, 2 – budding, 3 – flowering, 4 – fruiting. Experiment-1 – average temperature 32 °C, light intensity 1618.6 $\mu molm^{-2}s^{-1}$; experiment-2 – average temperature 24 °C, light intensity 803.4 $\mu molm^{-2}s^{-1}$

(0.499 mg/g), and total phenolics (3.052 mg/g) in flowering phase, too as from experiment-1. Meanwhile, the accumulation of chlorogenic acid did not expose difference among phases of plants in experiment-2. In terms of amentoflavone, no significant differences were observed among phenological phases in both experiments, meanwhile the content of kaemferol exposed differences among phases under lower temperature and light intensity conditions of experiment-2. The highest amount of hyperforin was detected in plants of vegetative phase from experiment-1 and experiment-2 (1.773 and 1.389 mg/g, respectively) and decreased linearly with further plant growth phases in both experiments, too. The accumulation of hypericin and pseudohypericin increased in flowering (0.729 and 0.794 mg/g, respectively) and fruiting (0.738 and 0.817 mg/g, respectively) plants only under higher temperature and light intensity conditions of experiment-1. On the contrary, lower temperature and light intensity of experiment-2 influenced significant decrease of hypericin and pseudohypericin from 0.646 to

0.311 mg/g and from 0.817 to 0.414 mg/g, respectively, during phenological changes.

DISCUSSION

Many internal and external factors influence the chemical composition of plants and it is difficult to define, which factor affected particular changes in chemical compounds. In our study, we evaluated the influence of external factors such as temperature and light intensity together with internal factor – phenological development phases on accumulation of bioactive compounds in *Hypericum perforatum* plants. The present study revealed that high temperature and light intensity positively influence the accumulation of naphthodianthrones, acylphloroglucinol hyperforin and phenolic compounds in *H. perforatum*. The previously developed statistical models described the similar tendency for quantitative effects of temperature and light on phytochemical composition of *H. perforatum* plants (Odabas et al., 2009, 2010). On the other hand, evaluation of bioactive compound quantities during phenological cycle helps to obtain the plant developing phase producing the highest value of desirable compounds and their changes under a modified environment system. Taken together, the results suggested that accumulation of secondary metabolites highly depends on abiotic environment and phenological cycle though the influence of both variables differed for the particular compounds. Chlorogenic acid, rutin, hyperoside, apigenin-7-O-glucoside, quercitrin, quercetin and total phenolics changed greatly during plant development and the highest levels reached at flowering phase in both experiments of growth conditions. The results are in accordance with previous literature data (Couceiro et al., 2006; Çirak et al., 2007) reporting the highest accumulation of phenolic compounds during plant flowering from various populations. Standardization of *H. perforatum* products has been based mainly on quantification of hypericin, pseudohypericin and hyperforin (Poutaraud & Girardin, 2005). Changes in hypericin and pseudohypericin content during plant development under higher temperature and light intensity conditions followed the same increasing trend as that for phenolic compounds. Higher light intensity and temperature stimulate the photosynthetic efficiency and accumulation of naphthodianthrones. An increase of hypericins under higher light intensity and temperature linked to higher abundance of dark glands, which are considered as synthesis sites of corresponding compounds (Briskin & Gawienowski, 2001; Zobayed et al., 2006). Similarly, the highest content of hypericin in *H. perforatum* during flowering has been reported by other authors (Büter & Büter, 2002; Couceiro et al., 2006; Çirak et al., 2007; Bagdonaite et al., 2010). On the contrary, lower temperature and light intensity produced significant decrease of hypericins to the lowest level at flowering and fruiting phases. The significant decrease of temperature and light intensity during phenological development of plants were found to be crucial negative factors for corresponding compounds accumulation.

The present results indicated that the highest level of hyperforin was accumulated at vegetative phase that fall down during phenological development of plants in both experiments. However, our data for hyperforin decrease during phenological cycle did not match to previously reported results (Büter & Büter, 2002; Çirak et al., 2008). On the other hand, it is in a partial agreement with the results obtained by Couceiro et al. (2006), who detected the decrease of hyperforin content under higher temperature. The discrepancy between results on the accumulation of hyperforin reported in different sources seems to be caused by low stability and rapid oxidation of this compound at different stages of plant processing and analysis. The additional research of external and internal factors effect on accumulation of hyperforin would be of high priority on understanding the pathway and degradation of this important secondary metabolite. However, the observed differences in plant bioactive compound content are often difficult to interpret as many abiotic conditions usually interfere with complex internal factors.

ACKNOWLEDGEMENTS

The research was funded by the Research Council of Lithuania, grant No. MIP-57/2010.

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VIDINIŲ IR IŠORINIŲ FAKTORIŲ ĮTAKA ANTRINIŲ METABOLITŲ KAUPIMUISI PAPRASTOJOJE JONAŽOLĖJE

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Santrauka

Buvo tirta augimo aplinkos šviesos ir temperatūros sąlygų bei augalų fenologinio vystymosi įtaka bioaktyviųjų junginių kaupimuisi paprastojoje jonažolėje. Augalai buvo auginami šiltnamyje dviejose skirtingose temperatūros ir apšvietimo sąlygose. Tyrimų rezultatai parodė, kad antrinių metabolitų kaupimasis priklauso tiek nuo aplinkos sąlygų, tiek nuo fenologinio vystymosi fazės, tačiau abiejų šių faktorių įtaka atskirų junginių pokyčiams yra skirtinga. Fenolinių junginių kiekiai reikšmingai pakito augalų fenologinio vystymosi eigoje ir didžiausius kiekius pasiekė žydėjimo fazėje skirtingose augimo sąlygose. Naftodiantronų kiekio didėjimo tendencija nustatyta fenologinio vystymosi eigoje prie aukštesnės temperatūros ir intensyvesnio apšvietimo, tačiau žemesnės temperatūros ir silpnesnio apšvietimo sąlygose šių junginių kiekis reikšmingai sumažėjo augalų vystymosi eigoje. Vegetatyvinėje fazėje augalai sukaupė didžiausius hiperforino kiekius, kurie reikšmingai sumažėjo tolesnio augalų vystymosi eigoje nepriklausomai nuo auginimo sąlygų.