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Kinetin Induced Changes in Rutin content in *Knautia sarajevensis* (G. Beck) Szabó Shoot Cultures

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Abstract: *Knautia sarajevensis*, Dipsacaceae, is an endemic species found at wood margins and meadows only on mountains of Dinaric Alps. Members of this family are widely used in traditional medicine as rich sources of pharmacologically important substances. Since it is well known that flavonoid compounds are one of the main carriers of biological activities of plant extracts, the aim of this study was to investigate cytokinin effects in concentration changes of flavonoid constituents. Presence of four different flavonoid constituents was noticed: quercetin, naringenin, hesperetin and rutin in extracts of *K. sarajevenis* shoots cultivated on three *in vitro* treatments (control, 1.0 mg L^{-1} kinetin and 10.0 mg L^{-1} kinetin), but only rutin content was quantified. All extracts were prepared using dried material and 80% methanol HPLC grade. Analysis of rutin indicated that high cytokinin concentrations did induce improvement of rutin content, but these concentrations are still lower than those recorded for control treatment. Further analysis using different types and concentrations of cytokinins are necessary to establish a pattern of cytokinin induced concentration changes in content of these four investigated flavonoids in *Knautia sarajevensis*.

INTRODUCTION

Knautia sarajevensis (G. Beck) Szabó is a member of Dipsacaceae family, which is consisted from 10 genera and around 270 species, distributed through Europe, East Asia, south and central Africa (Mabberley, 2008). This is an endemic species of Bosnian Mountains (Ehrendorfer, 1980). Research regarding secondary metabolites of Dipsacaceae family included identification of active components (Rezanova *et* Naidakova, 1974; Alimbaeva *et al*., 1986; Movsumov *et al*., 2011), and only a few papers reported analysis of their bioactive properties (Hung *et al*., 2005; Chunmei *et al*., 2010). Some of the Dipsacaceae species are used in traditional Chinese medicine (Zhang *et al*., 2003), but in Europe this family is still not well investigated. From six, in Bosnia recorded Dipsacaceae genera (*Cephalaria* Schrad. Ex Roem. & Schult; *Dipsacus* L.; *Knautia* L.; *Scabiosa* L.; *Succisa* Heller; *Succisella* Beck), literature data regarding bioactive compounds are available only for a few species. Identified secondary metabolites from Dipsacaceae species beside medicinal importance are also used in chemotaxonomy. It is considered that Dipsacaceae family is characterized by loganin, loganic acid and swertosid (Jensen *et al*., 1979; Perdetzoglou *et al*., 2000; Horn *et al*., 2001).

Plant metabolism is consisted from primary (necessary for plant cell survival) and secondary metabolism (necessary for plant survival under changing environmental conditions). Production of secondary metabolites is usually very low (around 1% of dry mass), and it is dependent upon physiological and

developmental stage of the plant (Hartmann, 1996). All secondary metabolites can be classified into tri major groups: terpenes (cardiac glycosides, carotenes, sterols etc.); phenols (phenolic acids, coumarins, lignans, stilbenes, tannins and lignin) and components containing sulphur or nitrogen (alkaloids and glucosinolates) (Verpoorte, 2000). Phenols can be further grouped into simple phenols, phenolic acids, flavonoids, stilbenes, lignans and hydrolysed and condensed tannins (Shahidi *et* Ho, 2005). Flavonoids are diverse group of phenolic compounds and include flavones, flavanones, flavonols, flavanonols, isoflavons, flavanols (catechin) and anthocyanidins (Shahidi *et* Ho, 2005). Importance of secondary metabolites for humans is evident, since at least one quarter of all drugs in developed countries contain components that are directly or indirectly of plant origin. Furthermore, 11% out of 252 essential medicines are considered to be produced exclusively from flowering plants (Verpoorte, 2000). During photosynthesis and stress conditions reactive oxygen species (ROS) are formed in plant tissues. In normal conditions plant antioxidative system (AOX) scavenges radicals and protects the plant. Any kind of stress puts ratio between ROS and AOX out of equilibrium. To, stress induced, ROS accumulation plants enzymatic AOX is activated (including radical scavengers like SOD-superoxide dismutase, APX-ascorbate peroxidase, GPX-guaiacol peroxidases, GST-glutathione S-transferase etc.) and nonenzymatic antioxidative system (carotenoids and flavonoids) (Gill *et* Tuteja, 2010). Protective role of flavonoids in different biological systems is often attributed to their ability to scavenge radical electrons and chelate metal ions (Hernández *et al*., 2009). The aim of this study was to investigate effect of kinetin on rutin concentration as a bioactive compound.

EXPERIMENTAL

Plant material and *in vitro* culture: Seeds of *Knautia sarajevensis* were collected at Mt. Igman, Veliko polje, during august, 2012. Seeds were cultivated on Murashige *et* Skoog basal medium (1962; MS) containing 3% sucrose and without plant growth regulators (no PGR). Prior to cultivation seed coat and elaiosome was removed. After 30 days, seedlings were collected and cultivated on three different treatments (control – MS basal media without PGR; 1KIN – MS basal media containing 1 mg L^{-1} kinetin and 10KIN – MS basal media containing 10 mg L^{-1} kinetin). Before culture, root and apical meristem from shoots were removed in aseptic conditions. All media were autoclaved after pH adjustment to 5.7, and addition of 0.8% agar (HiMedia). All cultures were kept in growth chamber (light 2,000 lux, 70% humidity and temperature of 23 ±2°C).

Extract preparation: 80 mg of air dried plant material from each treatment was separately homogenized with addition of 80% HPLC grade methanol (SIGMA) and incubated 24 hours at 4°C, after which supernatant was collected for analysis.

Solution of standards: Flavonoids stock solutions of rutin, quercetin, naringenin and hesperitin were prepared in methanol. Solutions were kept in the dark at +4°C. Prior to injection into the HPLC system, all solutions were filtered through 0.45 μm syringe filter.

HPLC analysis: Qualitative analysis of the flavonoids rutin, quercetin, naringenin and hesperetin were done with DAD detector using wavelenght 290 nm for rutin and quercetin and at 370 nm for naringenin and hesperetin. Quantitative analysis of the rutin was done with DAD detector at 290 nm wavelennght. Analysis was performed on a column Eclipse XDB – C18 RP (4.6 mm x 250 μm, 5 μm particle size) by quaternary pump. Flow rate of mobile phase was 1 mL min^{-1}, injection volume 20 μL and the temperature of column was 35°C. Calibration curve with 5 points in the concentration range from 2.5 μg mL^{-1} to 100 μg mL^{-1} (start of the curve was set to zero) was established for rutin standard. Coefficients of correlation for all target analyses were $r^2 \geq 0.999$. The mobile phases were acetonitrile (MF - A) and 5% aqueous solution of acetic acid (MF - B) in gradient separation (0 min 5% B; 15 min 15% B; 25 min 15% B; 40 min 22% B; 70min 22% B; 80 min 25% B; 90 min 5% B; stop).

RESULTS AND DISCUSION

Analysis of rutin content in kinetin treated and control samples was performed (Figure 1). Kinetin was applied on shoot cultures of *K. sarajevensis* using solid culture. Analysis was preformed after 4 week of cultivation and presence of rutin was confirmed in all tested shoots independent upon treatment. Application of 1 mg L^{-1} kinetin induced decrease of rutin content, but application of 10 mg L^{-1} kinetin induced increase in rutin content, which was lower than in the control treatment (Figure 1). Qualitative analysis of *K. sarajevensis* shoot extracts showed the presence of selected flavonoids (quercetin, naringenin and hesperetin) in kinetin treated and control samples. Detected flavonoids were not quantified and were used only as qualitative parameters of shoot extracts.

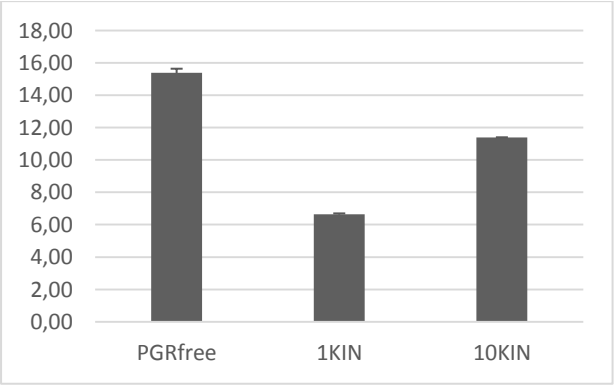

Figure 1. Content of analysed flavonoids (ng mL^{-1}) in shoot cultures of *K. sarajevensis* after kinetin treatment

PGRfree – MS basal media without PGR; 1KIN – MS basal media containing 1 mg L^{-1} kinetin and 10KIN – MS basal media containing 10 mg L^{-1} kinetin

Application of plant growth regulators can affect secondary metabolite production through effect of enzymes on phenylpropanoid synthesis (Sangwan *et al*., 2001). Plant growth regulators can stimulate flavonoid production, as recorded for BA application on *Arnica montana*, where stimulation of apigenin and luteolin production was noticed (Indu *et al*., 2013), stimulation of production of quercetin, catechin and myricetin was noticed for *Cyperus rotundus* (Krishna *et* Renu, 2013), or increased production of rutin, luteolin, quercetin and kaempferol in *Hypericum* (Pasqua *et al*., 2003; Shilpashree *et* Rai, 2009) was also detected.

Biosynthetic pathway and simulative mechanisms of flavonoid production is still not clear. The question remains does the plant growth regulators induce formation of different biosynthetic enzymes responsible for flavonoid biosynthesis (Shilpashree *et* Rai, 2009) or other mechanisms are responsible for such effect of plant growth regulators. Our research showed that application of kinetin induces changes in individual flavonoids, and this change is usually treatment dependent, but complexity of metabolic pathways that lead for such changes remain unclear.

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Summary/Sažetak

Knautia sarajevensis, Dipsacaceae, je endemična biljka koja naseljava rubna područja šuma dinarskih alpa. Pripadnici ove porodice su široko zastupljeni u tradicionalnoj medicine kao bogat izvori farmaceutski značajnih supstanici. S obzirom da je poznata činjenica da flavonoidne komponente su jedan od glavnih nositelja biološke aktivnosti biljnih ekstrakata, cilj ove studije nio je ispitati uticaj citokinina na promjene koncentracije flavonoidnih konstituenata. Utvršeno je prisustvo svačetiri ispitivana flavonoida: kvercetin, naringenin, hesperitin i rutin, u ektraktima izdanaka K. sarajevensis koji su kultivisani na tri in vitro tretmana (kontrola, 1.0 mg L-1 kinetina i 10.0 mg L-1 kinetina), ali je samo kvantifikacija izvršena za rutin. Svi ekstrakti su pripremljeni ekstrakcijom iz suhog materijala 80% metanolom HPLC čistoće. Analiza rutina pokazala je da visoke koncentracije citokinina povećavaju koncentraciju rutina, al ove koncentracije su i dalje niže nego one zabilježene za kontrolne biljke. Daljnja analiza sa većim rasponom koncentracija i različitim tipovima citokinina je neophodna za uspostavljanje obrascacitokinin induciranih promjena sadraja testitanih favonoida kod vrste K. sarajevensis.