Localization of nepetalactone and dehydronepetalactone biosyntehesis and accumulation in *Nepeta rtanjensis* leaves

Neda Aničić[1], Dragana Božić[1], Marijana Skorić[1], Slavica Dmitrović[1], Jelena Boljević[1], Jasmina Nestorović Živković[1], Danijela Mišić[1]

[1]Institute for Biological Research "Siniša Stanković", Department for Plant Physiology, University of Belgrade, Bul. despota Stefana 142, 11060 Belgrade, Serbia

ABSTRACT

Nepeta rtanjensis Diklić & Milojević (Lamiaceae) produces potent bioactive iridoid monoterpenes nepetalactone and dehydronepetalactone. However, the exact production site of these monoterpenes *in planta* has not yet been determined. Generally, glandular trichomes are known to be the main tissue where biosynthesis, accumulation and secretion of volatile secondary metabolites take place. In our work, the tissue localization of nepetalactone and dehydronepetalactone was determined by dichloromethane-dipping of freshly harvested *Nepeta rtanjensis* leaves. Extracts from leaves after organic solvent dipping and the solvent after leaf dipping, which contained trichome-specific compounds, were separately analyzed by UHPLC/DAD/+HESI-MS/MS. The efficiencies of four different dichloromethane-dipping durations (20, 40, 120 and 300 seconds) were evaluated. Relative contents of both nepetalactone and dehydronepetalactone increased in extracts of glandular trichomes concomitant to the increase in dipping duration. On the other hand, we observed a decrease in the relative contents of these compounds in extracts of leaves after dipping with the increase of dipping duration. As confirmed by SEM, the extraction procedure we used has caused a collapse of the subcuticular space of the capitate glands on the leaf surface, whereas no other damage to the leaf surface has been observed. Our data implicate that the leaf glandular trichomes are a major site of storage and accumulation of nepetalactones. This finding is of prime significance for the further elucidation of nepetalactone biosynthetic pathway. One of aims of our further research is isolation and characterization of gene for iridoid synthase. Based on homology of sequences from NCBI database degenerative, primers were designed and two partial sequences potentially representing iridoid synthase were isolated. To further substantiate one of isolated sequences as gene candidate, its specific expression in tissues selected for analysis belonging to different developmental stages was estimated. The results revealed that expression levels in tissues concur with the observed amounts of nepetalactone.

RESULTS

- **Leaf tissue localization of nepetalactone biosynthesis**

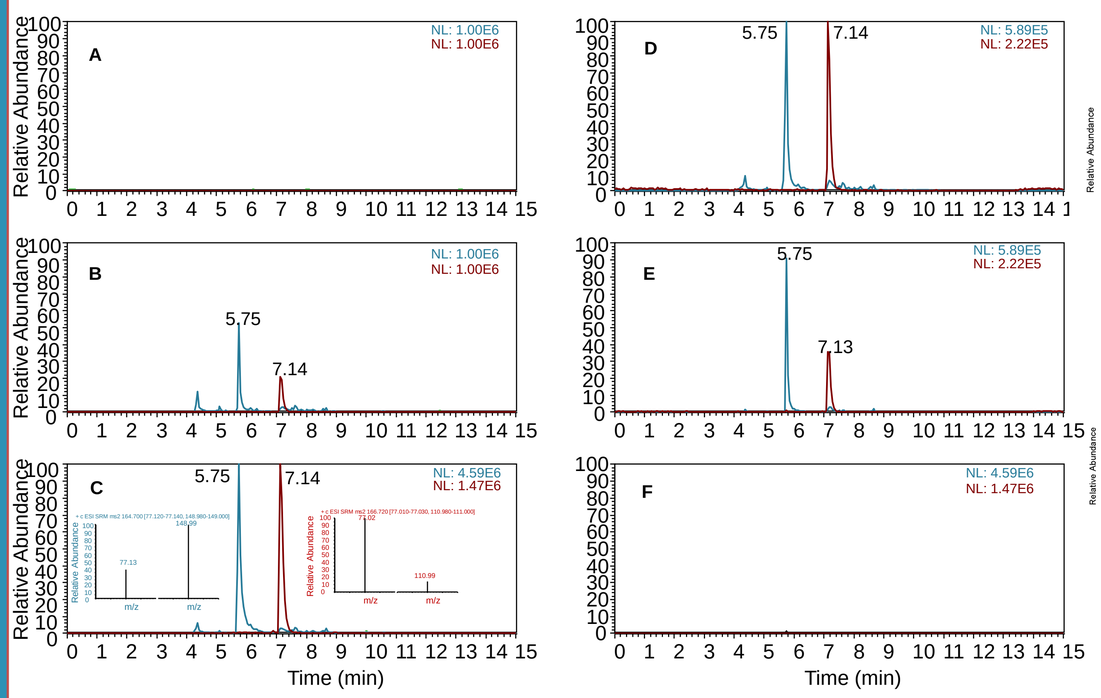

Fig. 1. UHPLC/+HESI–MS/MS single reaction monitoring (SRM) chromatograms representing dehydronepetalactone (Rt 5.75 min) and nepetalactone (Rt 7.14 min) of the following samples: (A-C) Organic solvent after 0, 20, and 120s dipping; (D-F) *Nepeta rtanjensis* leaves after 0, 20 and 120 s-dip in dichloromethane. Extracted are MS^2 spectra of dehydronepetalactone (marked in blue) and nepetalactone (marked in red), showing major fragments used in SRM experiment for the quantification.

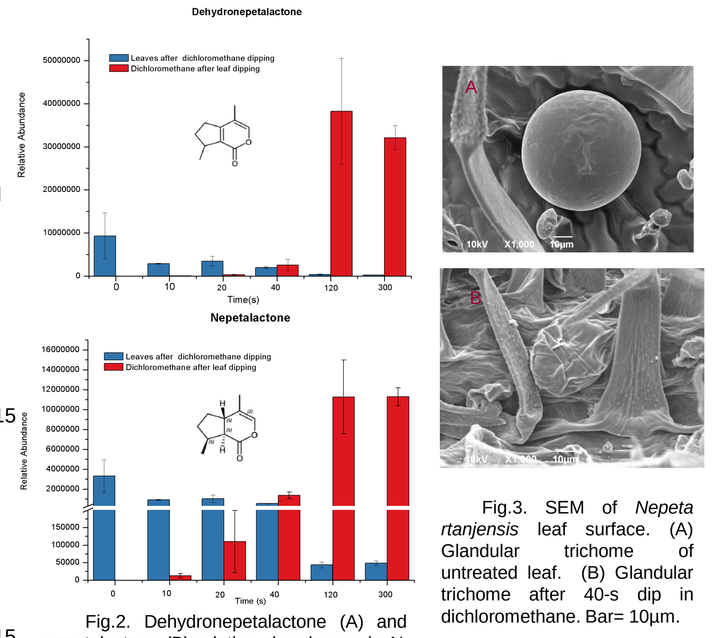

Fig.2. Dehydronepetalactone (A) and nepetalactone (B) relative abundances in *N. rtanjensis* leaves after organic solvent dipping and organic solvent after four dipping durations (0, 20, 40, 120 and 300 seconds) analyzed by UHPLC/DAD/+HESI-MS/MS. The results represent the mean ± SE of 3 replicates.

Fig.3. SEM of *Nepeta rtanjensis* leaf surface. (A) Glandular trichome of untreated leaf. (B) Glandular trichome after 40-s dip in dichloromethane. Bar= 10µm.

MATERIAL AND METHODS

Freshly harvested fully developed *Nepeta rtanjensis* leaves were extracted by immersion in dichloromethane for four different periods (20, 40, 120 and 300 seconds). After dipping, leaves were air-dried in a fume hood, grounded in LN_2 and resuspended by sonication in 96% methanol (w: v= 1:10). After being air-dried in a fume hood, the residues of dichloromethane after dipping were resuspended in 75% methanol. All extracts were analyzed by UHPLC/DAD/+HESI-MS using procedure described by *Mišić* et al. 2014. Scanning electron microscopy (SEM) was used to examine surfaces of untreated and 40s –dipped leaves. The previously developed dry-ice abrasion method (*Brückner* et al. 2014) was used for trichome isolation. Total RNA was isolated from trichomes using the commercial Spectrum™ Plant Total RNA Kit (Sigma–Aldrich), according to the manufacturers' specifications. RNA from other tissue samples was extracted using Trizol (Invitrogen, 15596-026) as described by *Bogdanović* et al. 2012. Gene for iridoid synthase in *Catharanthus roseus* (*Geu-Flores* et al. 2012) was used as base sequence for BLAST search of NCBI database and several genes of *Lamiaceae* were shown to be highly similar. Based on the conserved amino acid sequences obtained by BLAST search, a set of degenerative primers were designed and used for PCR with cDNA as template. The gained PCR product was cloned into pGEM-T easy vector (Promega). Resulting products were verified by sequencing. For gene expression analysis, primer pairs were designed to provide the best possible distinction of candidate gene. Samples were prepared using the Maxima SYBR Green/ROX qPCR Master Mix (Life technologies), and quantitative realtime PCR analysis was performed using the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems).

RESULTS

- **Iridoid synthase gene candidate expression analysis**

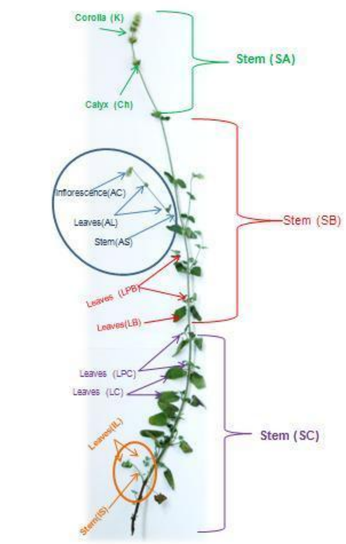

Fig.4. *Nepeta rtanjensis* tissues, belonging to different developmental stages, selected for the analysis of gene expresion of iridoid synthase candidate gene, and of the nepetalactone content. Plants used in experiments were field cultivated, and were in the flowering stage.

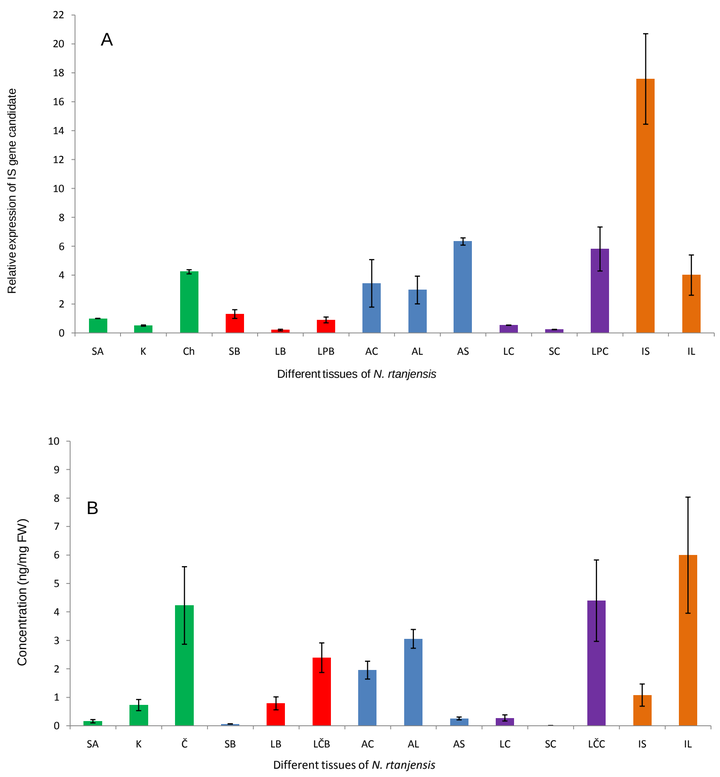

Fig.5 (A) Iridoid synthase candidate gene expression analysis in selected tissues belonging to different developmental stages; (B) *Trans-cis* nepetalactone content in *Nepeta rtanjensis* tissues selected for analysis belonging to different developmental stages. Presented values are means of three independent measurements.

Fig.6. Primer design for the expression analysis; Alignement of isolated candidate genes for iridoid synthase; Red bordered are the primers used for the expression analysis.

CONCLUSION

Accumulation and storage of nepetalactones in *Nepeta rtanjensis* leaves are primarily confined to glandular trichomes. Therefore, potential candidate gene for iridoid synthase was isolated from glandular trichomes of *N. rtanjensis* leaves. The expression levels of isolated candidate gene for iridoid synthase in *N. rtanjensis* tissues of different developmental stages are concurrent with the observed amounts of nepetalactone in these tissues. Thus, results indicate that the isolated candidate gene is potentially involved in the biosynthesis of nepetalactone. However, further investigations of isolated candidate gene functional characterization are yet to confirm its identity.

Acknowledgements: This work was supported by the Serbian Ministry of Education, Science and Technological Development (grant No. OI173024).

References

Brückner, Kathleen, et al. "Characterization of two genes for the biosynthesis of abietane-type diterpenes in rosemary (Rosmarinus officinalis) glandular trichomes." *Phytochemistry* 101 (2014): 52-64.

Bogdanović, Milica D., et al. "Reverse transcription of 18S rRNA with poly (dT) 18 and other homopolymers." *Plant Molecular Biology Reporter* 31.1 (2013): 55-63.

Geu-Flores, Fernando, et al. "An alternative route to cyclic terpenes by reductive cyclization in iridoid biosynthesis." *Nature* 492.7427 (2012): 138-142.

Mišić, Danijela, et al. "Simultaneous UHPLC/DAD/(+/−) HESI–MS/MS Analysis of Phenolic Acids and Nepetalactones in Methanol Extracts of Nepeta Species: A Possible Application in Chemotaxonomic Studies." *Phytochemical Analysis*26.1 (2015): 72-85.