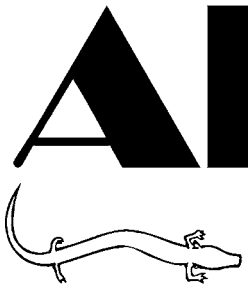

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ABS ACTA BIOLOGICA SLOVENICA
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V spomin rastlinske fiziologinje in zaslužne profesorice, dr. Nade Gogala (1937–2013)

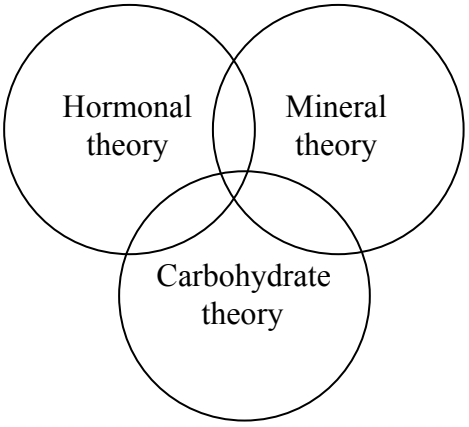
V sredini novembra je nenadejano od nas odšla priljubljena profesorica, dr. Nada Gogala, univerzitetna diplomirana biologinja. Generacijam slovenskih biologov, agronomov, biotehnologov in pedagogov se je zapisala v spomin kot prizadevna učiteljica, iskriva sogovornica in pronicljiva znanstvenica. V slovenskem in mednarodnem znanstvenem prostoru je poznana kot pionirka raziskav mikorizne simbioze in razvoja rastlinskih tkivnih kultur.

Nada Gogala se je rodila 9. maja 1937 v Ljubljani. Svoja otroška leta je preživljala na posestvu v Bistri, po opravljeni maturi pa se je vpisala na študij biologije tedanje Prirodoslovno matematično-filozofske fakultete. Z glivami se je profesionalno začela ukvarjati že v okviru svoje diplomske naloge, po diplomi, v letu 1960 pa se je zaposlila najprej kot asistentka za botaniko. Delovno mesto profesorice za fiziologijo rastlin na Oddelku za biologijo je prevzela po upokojitvi prof. dr. Mirana Vardjana v letu 1981. Njeno pestro osebno in profesionalno pot smo v reviji Acta Biologica Slovenica podrobneje predstavili že ob njenih jubilejih v letu 1997 in 2007, tokratni prispevek pa obravnava predvsem njeno znanstveno-raziskovalno dejavnost. Za svoje prepoznavne dosežke na pedagoškem, raziskovalnem in organizacijskem področju je bila večkrat nagrajena, med drugim z Redom zaslug za narod s srebrno zvezdo v letu 1987, z nagrado iz Sklada Borisa Kidriča (1989), z Jesenkovim priznanjem Biotehniške Fakultete (1994), v letu 1998 pa je bila imenovana za Zaslužno profesorico na Univerzi v Ljubljani.

Bogata bibliografska zapuščina prof. dr. Nade Gogala obsega 415 zapisov, med katerimi je 73 izvirnih znanstvenih člankov, 19 strokovnih in 10 poljudnih člankov, ter številni učbeniki. V okviru širokega področja fiziologije rastlin raziskovalno delo prof. Gogalove s sodelavci lahko razvrstimo v tri smiselno povezane celote. Med zgodnejšimi so njene raziskave regulacije razvoja rastlin in tehnik tkivnih kultur, v katerih najvidnejše mesto

pripada vlogi jasmonske kisline v razvoju rastlin. V pomembnem delu, ki ga je začrtala že z raziskavami v diplomski nalogi in doktorski disertaciji, se posveča raziskovanju mikoriznih gliv in regulaciji mikorizne simbioze. Tudi v teh raziskavah proučuje rastlinske hormone in signalne molekule, ter komunikaciji med rastlino in glivo v simbiozi. V tretji sklop pa lahko uvrstimo raziskave stresa pri rastlinah. Med temi lahko izpostavimo vpliv težkih kovin na rastline in glive ter pomen mikorize pri zmanjševanju stresa. Med njenimi publikacijami najdemo tudi raziskave vpliva magnetnega polja na rastline in glive ter raziskave vpliva UV sevanja na rastline. Po podatkih dostopnih v bazi Tomson Reuters (2013) od začetka spremljanja statistike do danes, je prof. Gogala s sodelavci objavila tudi do 10 člankov letno. Njeni izvirni znanstveni članki izkazujejo visoko odmevnost s 464 citati, tudi do 35 v enem samem letu. Med

najodmevnejšimi so njene raziskave pomena jasmonske kisline za razvoj rastlin in za razvoj mikorize, raziskave privzema kovin v mikorizne glive in raziskave pomena magnetnega polja za rast rastlin. Najpogosteje citirani sta njeni publikaciji o vplivu jasmonske kisline na protoplaste krompirja in pregledni članek v katerem poudarja pomen oglikohidratne, mineralne in hormonske hipoteze na razvoj mikorizne simbioze



Slika 1: Sinteza pomena rastlinskih hormonov in signalnih molekul v komunikaciji med rastlino in glivo (Gogala 1991).

Vizija dr. Nade Gogala o prihodnosti slovenske rastlinske fiziologije je presegala okvire znanstveno-raziskovalno dejavnosti. S svojimi študenti je intenzivno razvijala eno najobetavnejših področij, rastlinsko biotehnologijo in svoja znanja in bogate izkušnje prenašala v prakso v podjetjih Rast, Semenarna, Krka, Lek in drugih. Uspešne postopke vzgoje nekaterih okrasnih rastlin in praproti v pogojih *in vitro* so dopolnjevale tudi raziskave pridobivanja sekundarnih metabolitov za pogoje farmacevtike. Z enako mero zavzetosti se je lotevala tudi reševanja problemov okoljske problematike s pilotskimi testi pozelenitve mežiške doline, v sodelovanju z lokalnimi gospodarskimi družbami. Bazične raziskave v znanosti so neobhodno potrebne za razvoj znanstvenih področij in našega razumevanja delovanja rastlin. Vodilno vlogo znanosti v družbi pa lahko zagotovimo le z njenim poslanstvom, to je z uporabo izsledkov znanstvenih raziskav za boljši jutri. Tega se je še posebno dobro zavedala. Veliko daljnosežnih idej in raziskovalne širine je bilo potrebne za tako napredne aplikativne zamisli. In Nada je s svojo iniciativnostjo in mentorskim vodstvom znala spodbujati in usmerjati tovrstno kreativno ustvarjalnost.

Profesorica Gogala je s svojimi predavanji iz življenja rastlin vtisnila neizbrisen pečat številnim generacijam slovenskih študentov biologije, agronomije in biotehnologije na Biotehniški fakulteti ter študentom Pedagoške fakultete Univerze v Ljubljani in Univerze v Mariboru. Tudi kot mentorica je svojim doktorandom, magistrantom in diplomantom vselej znala zbuditi zanimanje do raziskovanja in predstaviti pomembnost razkrivanja skrivnosti rastlin. S svojim pedagoškim znanjem in vodstvenimi izkušnjami je pomembno prispevala tudi k oblikovanju številnih novih študijskih programov na Univerzi v Ljubljani. Tudi po upokojitvi v letu 1997 je še naprej zavzeto pomagala pri izvajanju nekaterih predmetov, se vključevala v oblikovanje Botaničnega terminološkega slovarja in pomagala pri oblikovanju nekaterih učbenikov. Zapostavila pa ni niti strokovnega in poljudnega objavljanja s številnimi zanimivostmi iz sveta rastlin.

Ohranili jo bomo v lepem in trajnem spominu.

Marjana Regvar
Vodja katedre za botanikoin fiziologijo rastlin

Izbrana bibliografija prof. dr. Nade Gogala

Peternel, Š., Gabrovšek, K., Gogala, N., Regvar, M., 2009. In vitro propagation of European aspen (*Populus tremula* L.) from axillary buds via organogenesis. Sci. Hortic., 121 (1), 109–112.

Vodnik, D., Jentschke, G., Fritz, E., Gogala, N., Godbold, D.L., 1999. Root-applied cytokinin reduces lead uptake and affects its distribution in *Norway spruce* seedlings. Physiol. Plant. 106 (1), 75–81.

Ružič, R., Jerman, I., Gogala, N., 1998. Effect of weak low-frequency magnetic fields on spruce seed germination under acid conditions. Can. J. For. Res., 28, 609–616.

Ružič, R., Jerman, I., Gogala, N., 1998. Water stress reveals effects of ELF magnetic fields on the growth of seedlings. Electro-Magnetobiol., 17 (1), 17–30.

Jentschke, G., Marschner, P., Vodnik, D., Marth, C., Bredemeier, M., Rapp, C., Fritz, E., Gogala, N., Godbold, D.L., 1998. Lead uptake by *Picea abies* seedlings: effect of nitrogen source and mycorrhizas. J. Plant Physiol., 153, 97–104.

Vodnik, D., Byrne, A.R., Gogala, N., 1998. The uptake and transport of lead in some ectomycorrhizal fungi in culture. Mycol. Res.,102 (8), 953–958.

Bavcon, J., Gogala, N., Gaberščik, A., 1998. Influence of UV-B radiation on Norway spruce seedlings (*Picea abies* (L.) Karst.). Radiol. Oncol., 32 (1), 83–87.

Ružič, R., Gogala, N., Jerman, I., 1997. Sinusoidal magnetic fields: effect on the growth and content of ergosterol in mycorrhizal fungi. Electro-Magnetobiol., 16 (2), 129–142.

Benedičič, D., Ravikar, Maja, Gogala, N., 1997. The regeneration of bean plants from meristem culture. Phyton (Horn), 37 (1), 151–160.

Regvar, M., Gogala, N., Žnidaršič, N., 1997. Jasmonic acid affects mycorrhization of spruce seedlings with *Laccaria laccata*. Trees (Berl. West), 11, 511–514.

Regvar, M., Gogala, N., Zalar, P., 1996. Effects of jasmonic acid on mycorrhizal *Allium sativum*. New Phytol., 134, 703–707.

Bavcon, J., Gogala, N., 1996. The influence of UV-B irradiation on the mitotic activity in *Picea abies* (L.) Karst. Phyton (Horn), 36 (3 Suppl.), 47–50.

Vodnik, D., Božič, M., Gogala, N., Gabrovšek, K., 1996. Growth response of ectomycorrhizal Norway spruce seedlings transplanted on lead-polluted soil. Phyton (Horn), 36 (3 Suppl.), 77–80.

Jurc, M., Jurc, D., Gogala, N., Simončič, P., 1996. Air pollution and fungal endophytes in needles of Austrian pine. Phyton (Horn), 36 (3, Suppl.), 111–114.

Regvar, M., Gogala, N., 1996. Changes in root growth patterns of (*Picea abies*) spruce roots by inoculation with an ectomycorrhizal fungus *Pisolithus tinctorius* and jasmonic acid treatment. Trees (Berl. West), 10, 410–414.

Al Sayegh-Petkovšek, S., Kraigher, H., Batič, F., Ggogala, N., Agerer, R., 1995. Mycorrhizal potential of two forest research plots in Zavodnje and Mislinja. Acta pharm. (Zagreb), 45 (2, Suppl.), 333–336.

Kugonič, N., Gogala, N., 1995. Mycorrhization of *Tanacetum vulgare* in power plant ash. Acta pharm. (Zagreb), 45 (2, Suppl.) 337–339.

Ravnikar, M., Bevc, L., Gogala, N., 1995. The influence of jasmonic acid on water, Ca and some other ion uptake of the potato (*Solanum tuberosum* L.) in vitro. Acta pharm. (Zagreb), 45 (2), 241–244.

Tavzes, Č., Brzin, J., Svetek, J., Regvar, M., Gogala, N., Schara, M.V., 1995. Membrane fluidity response of mycorrhizal fungus *Laccaria laccata* to jasmonic acid. Acta pharm. (Zagreb), 45 (2), 311–315.

Regvar, M., Gogala, N., 1995. The influence of jasmonic acid on development of mycorrhizae. Acta pharm. (Zagreb), 45 (2), 317–319.

Gabrovšek, K., Gogala, N., 1995. Cytokinins affect ectomycorrhiza formation in Norway spruce seedlings. Acta pharm. (Zagreb), 45 (2), 321–324.

Virant-Klun, I., Gogala, N., 1995. Effect of water stress on release of ethylene in germinating maize seeds (*Zea mays* L.). Acta pharm. (Zagreb), 45 (2), 391–394.

Bavcon, J., Gaberščik, A., Gogala, N., 1995. Influence of low temperatures and UV-B radiation on chlorophyll flourescence kinetic in *Picea abies* (L.) Karst. Acta pharm. (Zagreb), 45 (2, Suppl.), 359–362.

Virant-Klun, I., Gogala, N., 1995. Impact of VAM on phosphorus nutrition of maize with low soluble phosphate fertilization. J. Plant Nutr., 18 (9), 1815–1823.

Vodnik, D., Gogala, N., 1994. Seasonal fluctuations of photosynthesis and its pigments in 1-year mycorrhized spruce seedlings. Mycorrhiza (Berl.), 4 (6), 277–281.

Camloh, M., Gogala, N., Rode, J., 1994. Plant regeneration from leaf explants of the fern *Platycerium bifurcatum* in vitro. Sci. Hortic. 56, 257–266.

Žel, J., Schara, M.V., Svetek, J., Nemec, M., Gogala, N., 1993. Influence of aluminum on the membranes of mycorrhizal fungi. Water Air Soil Pollut., 71, 101–109.
Ravnikar, M., Vilhar, B., Gogala, N., 1992. Stimulatory effects of jasmonic acid on potato stem node and protoplast culture. J. Plant Growth Regul., 11, 29–33.
Camloh, M., Gogala, N., 1992. In vitro culture of *Platycerium bifurcatum* gametophytes. Sci. Hortic., 51, 343–346.
Žel, J., Blatnik, A., Gogala, N., 1992. In vitro aluminium effects on ectomycorrizal fungi. Water Air Soil Pollut., 63, 145–153.
Gogala, N., 1991. Regulation of mycorrhizal infection by hormonal factors produced by hosts and fungi. Experientia, 47, 331–340.
Vilhar, B., Ravnikar, M., Schara, M.V, Nemec, M., Gogala, N., 1991. The influence of jasmonic acid on biophysical properties of potato leaf protoplasts and roots. Plant Cell Rep., 10, 541–544.
Ravnikar, M., Gogala, N., 1990. Regulation of potato meristem development by jasmonic acid in vitro. Plant Growth Regul., 9, 233–236.
Žel, J., Gogala, N., 1989. Influence of aluminium on mycorrhizae. Agric. Ecosyst. Environ., 28, 569–573.
Križaj, D., Vodovnik, L., Pohleven, F., Gogala, N., 1987. Electrical stimulation: its effects on growth and ion accumulation in *Lactuca sativa* L. J. Bioelectr., 1 (6), 129–136.
Gogala, N., 1970. Einfluß der natürlichen Cytokinine von *Pinus sylvestris* L. und anderer Wuchsstoffe auf das Myzelwachstum von *Boletus edulis* var. pinicolis VITT. Österr. Bot. Z., 118, 321–333.

Reference

COBBISS, 2013. http://www.cobiss.si/
Gogala, N. 1991. Regulation of mycorrhizal infection by hormonal factors produced by hosts and fungi. Experientia 47 (4), 331–340.
Kovač, M. 1997. Jubilej: Šestdesetletnica prof. dr. Nade Gogala. Acta Biologica Slovenica 41 (4), 69–74.
Regvar, M. 2007. Jubilej: Rastlinska fiziologinja in zaslužna profesorica, dr. Nada Gogala. Acta Biologica Slovenica 50 (1), 65–67.
Thomson Reuters (2013) http://thomsonreuters.com/

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Za vedno je odšla zaslužna profesorica dr. Nada Gogala

V petek 15. novembra smo onemeli ob vesti, da je zaslužna profesorica prof. dr. Nada Gogala za vedno odšla. Njena znanstvena in pedagoška pot se je začela že zgodaj. Že kot študentka je bila tehnična sodelavka pri prof. dr. Ernestu Mayerju, predstojniku tedanjega Inštituta za botaniko. Po diplomi je postala asistentka in kasneje samostojna univerzitetna učiteljica in raziskovalka v skupini prof. dr. Mirana Vardjana na Katedri za fiziologijo rastlin, Oddelku za biologijo, Biotehniške fakultete, Univerze v Ljubljani. Po upokojitvi prof. dr. Mirana Vardjana je prevzela mesto predstojnice katedre. Z doktorsko disertacijo o hormonalni regulaciji mikorize je začrtala svojo raziskovalno pot in dejavnost svojih diplomantov, doktorandov in sodelavcev na Katedri za fiziologijo rastlin. V okviru različnih projektov je sodelovala s številnimi domačimi in tujimi raziskovalci. Rezultat njene srčnosti pri raziskovalnem in pedagoškem delu je bila vrsta kakovostnih znanstvenih člankov in kar je še bolj pomembno, pod njenim mentorstvom so začeli svojo raziskovalno pot odlični pedagogi in vrhunski raziskovalci, ki danes delujejo na Biotehniški fakulteti, Nacionalnem inštitutu za biologijo in še na nekaterih drugih ustanovah. Po upokojitvi je svoje obsežno znanje delila tudi s študenti Pedagoške fakultete, Univerze v Mariboru. Leta 1998 je postala zaslužna profesorica Univerze v Ljubljani. K izobraževalnem procesu je prispevala tudi s sodelovanjem pri pisanju osnovnošolskih in srednješolskih učbenikov, ter s prispevki v revijah Pionir in Proteus. Kot soavtorica je sodelovala pri nastanku Botaničnega terminološkega slovarja.

Nada ni bila le strokovnjakinja in pedagoginja na področju fiziologije rastlin, ampak tudi predana biologinja. Njeno predanost stroki kažejo različne dejavnosti. Med drugim je bila tudi predsednica Društva biologov Slovenije. Bila je prepričana, da si biologija kot stroka zasluži več pozornosti, kot jo je deležna. Njena želja je bila združiti biologe, zato je na vseh ravneh delovala povezovalno. Dolga leta je bila tudi članica uredniškega odbora slovenske biološke znanstvene revije Acta Biologica Slovenica, ki jo izdaja društvo. Člani društva smo ji za njen prispevek zelo hvaležni.

Čeprav je dala Nada neizbrisen pečat slovenski biologiji in veliko prispevala k svetovni zakladnici znanja na področju rastlinske fiziologije, se je spominjam predvsem kot profesorice z velikim srcem, polne topline in zagnanosti.

Nada hvala, ker si delček življenja delila z nami!

Alenka Gaberščik
Glavna in odgovorna urednica ABS
in predsednica Društva biologov Slovenije

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Medicinal mushrooms native to Slovenia

Zdravilne gobe rastoče v Sloveniji

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Abstract: Slovenia with its diverse environment is home to more than 2400 fungal species out of which especially many macromycetes have for millennia been used worldwide as natural remedies. These species of mushrooms were in the past picked from the nature, but today can be cultivated as fruiting bodies or fungal biomass on different substrates. They possess immunomodulating, antiviral, antibacterial and anticancer activities and can be used against allergies, dementia, Alzheimer disease and in many other diseases. They represent a vast potential as natural remedies with no or very little adverse effects and can be processed into food supplement or further developed into medicines. These mushrooms are a natural treasure, which enables us to be more self-sufficient if we cultivate them for medical and certain species for nutritional purposes as well.

Keywords: medicinal mushrooms, Slovenia, polysaccharides, cancer, natural remedies, endangered fungi

Povzetek: Slovenija s svojim raznovrstnim okoljem je dom več kot 2400 različnim vrstam gliv, med katerih se mnogo makromicet globalno uporablja v obliki naravnih zdravil. Te vrste so v preteklosti nabirali v naravi, danes pa jih lahko gojimo v umetnih pogojih, na različnih substratih, kot glivno biomaso ali trosnjake. Pripisujejo jim sposobnosti krepitve imunskega sistema, delovanje proti rakavim obolenjem, virusom, bakterijam, Alzheimerjevi bolezni, alergijam, demenci in še veliko drugim boleznim. Predstavljajo ogromen potencial kot naravna zdravila brez ali le z zanemarljivimi nezaželenimi stranskimi učinki in jih je mogoče predelati v prehrnaska dopolnila ali celo zdravila. Te gobe so naravno bogastvo, ki nam omogoča večjo samopreskrbo, če jih gojimo za medicinske, nekatere pa tudi za prehranske namene.

Ključne besede: zdravilne gobe, medicinske gobe, Slovenija, polisaharidi, rak, naravna zdravila, ogrožene glive

Introduction

For millennia mushrooms have been used not just for food, but also for other purposes, among which medicinal use was one the most prominent (Wasser et al. 2000). Although mushroom cultivation techniques were mastered in the last few decades, while in the past their use was dependent mostly on natural habitats and growing seasons. Nowadays cultivation techniques for many mushroom species are used in large-scale cultivation facilities and many of these cultivated species are

available on the market not just as food, but also as food supplements and medicines. Wild species, which were commonly used in the past, are now often neglected because of artificial cultivation. Some of the most interesting species are cultivated in liquid and solid media and their biomass is used in food supplement or even medicine production. The annual market of medicinal mushrooms and their derivative dietary supplements worldwide was estimated at 1.2 billion USD in 1991 (Chang 1996) and 6 billion USD in 1999 (Wasser et al. 2000) and is still growing. According to studies conducted so far, medicinal mushrooms have a very long tradition in Asian countries, whereas their use in the Western hemisphere has been slightly increasing only in the last decades (Lindequist et al. 2005). Many scientific research articles are proving their medicinal actions and potential use as natural medicines also in the Western hemisphere.

In Slovenia the use of medicinal mushrooms was not very common in the past and not a lot of information was available on this topic. Since the past decades research in the field of medicinal mushrooms is progressing in Slovenia, with research groups focusing on medicinal properties and different cultivation techniques of mushrooms. Recently, more than 2400 fungal species have been recorded in Slovenia (Jurc et al. 2005), not including lichen-forming fungi. Many of these species, especially saprophytic ones, possess different medicinal properties with majority of them still in need of identification.

The most important and most widely recognised medicinal mushrooms growing in Slovenia are *Ganoderma lucidum, Cordyceps militaris, Trametes versicolor, Grifola frondosa, Hericium erinaceus, Auricularia auricula, Fomes fomentarius, Fomitopsis pinicola, Piptoporus betulinus, Laricifomes officinalis, Pleurotus ostreatus* and *Schizophylum commune* with their distribution, traditional use and medicinal properties presented in this article (Vrhovec 2010).

Ganoderma lucidum (Curtis) P. Karst. (1881) (Ganodermataceae, Polyporales, Agaricomycetes, Agaricomycotina, Basidiomycota)

In Slovenia *G. lucidum* is an endangered and protected species, growing on broadleaf tree stumps, with 50 locations reported through the whole area of Slovenia (Ogris 2013). Wild growing fruiting bodies are very small in comparison to specimens cultivated on artificial sawdust-based substrates, and because of that a very poor substitute for cultivated mushrooms. Still many people pick them in nature even further endangering its natural habitats.

In the last few years this species is becoming popular in Slovenia as a food supplement. *G. lucidum* food supplements users mostly report its chemotherapy side-effects reducing, anti-allergic, immune system enhancing, anti-viral, anti-bacterial and stress reducing properties.

G. lucidum is a popular medicinal mushroom, considered inedible due to its toughness and bitterness. Traditionally it was used in Japan and China to treat nephritis, chronic hepatitis, hepatopathy, gastric ulcers, asthma, bronchitis, insomnia and arthritis (Jong et. al. 1992, Hobbs 1995, Chang and Buswell 1999, McKenna et al. 2002). *G. lucidum* contains more than 400 bioactive substances from the groups of polysaccharides, triterpenes, sterols, nucleosides, fatty acids and proteins (Mizuno 1995, Kim and Kim 1999, McKenna 2002, Gao et al. 2002, Boh 2013) from which polysaccharides and triterpenes have been researched the most. Polysaccharides comprise one of the major sources of pharmacologically active compounds in *G. lucidum*. It contains more than 100 types of polysaccharides (Wasser 2005) showing strong immunomodulating activities. The major immunomodulating effects include mitogenecity and activation of immune effector cells such as T lymphocytes, macrophages and NK cells, leading to the production of cytokines including interleukins, tumor necrosis factor alpha and interferons (Zhou et. al. 2002a). *G. lucidum* contains more than 140 triterpenes (Yue et. al. 2010), which inhibit histamine release, viral induction and cholesterol synthesis and show hepatoprotective, anti-hypertensive, anti-inflammatory, apoptosis inducing, antioxidative, anti-tumour, anti-microbial and immunomodulating activity (Boh et al. 2007, Powell 2010). Tyrosinase contained in *G. lucidum* fruiting bodies shows genoprotective effects (Shi et al. 2002). Jin and coworkers (2012) published an up to date review on *Ganoderma lucidum* and its clinical studies on cancer patients.

***Cordyceps militaris* (L.) Link (1833) (Cordycipitaceae, Hypocreales, Hypocreomycetidae, Sordariomycetes, Pezizomycotina, Ascomycota)**

C. militaris grows mostly in the north-western and central part of central Slovenia with 13 locations reported (Ogris 2013). This year new locations were found near the city of Bled, where fruiting bodies start to emerge from the soil on the pastures in the late October. *C. militaris* is not traditionally used in Slovenia as a medicinal mushroom. Its dry fruiting bodies are much smaller in size and weight and are not abundant enough to be picked for consumption. Some native *C. militaris* strains are cultivated in Slovenia by Slovenian companies and used as high-quality food supplements.

Cordyceps ascocarp originates at its base, on an insect larval host and ends in a club-like cap, including the stipe and stroma. There are more than 700 species of *Cordyceps* identified worldwide (Powell 2010) out of which only two (*C. sinensis* and *C. militaris*) are used in medicinal practice on a large scale. In nature these species grow as parasites on moth larvae (Kirk et al. 2001). The wild form of *Cordyceps sinensis* has always been one of the most expensive medicinal "herbs" and in ancient China was in the past reserved almost exclusively to members of the Emperor's court (Holliday et al. 2005). In 2006 the price for one kg was 12.000 USD (Paterson 2008) and has nearly doubled in the last few years, but the demand is still growing due to pharmacologically active substances contained in this fungus, attracting broad public interest.

In 2000 Wu and his coworkers found out that only *C. militaris*, producing cordycepin (3′-deoxyadenosine), has similar pharmacological activity to *C. sinensis*, whose biological characteristics were studied early in the 1950s. *C. militaris* was used traditionally as a natural medicine and for treating cancer (Ng and Wang 2005). Cordycepin is considered as a main bioactive metabolite of *C. militaris* (Hung et al. 2009) and is reported to exhibit anti-viral, anti-tumor, anti-fungal, anti-bacterial, anti-leucemic activities as well as anti-metastatic action and prevention of alcohol-induced hepatotoxicity (Koc et al. 1996, Kim et al. 2002, Zhou et al. 2002b, Nakamura et al. 2006, Lee et al. 2013, Cha et al. 2013).

***Pleurotus ostreatus* (Jacq.) Quél. (1871) (Pleurotaceae, Agaricales, Agaricomycetidae, Agaricomycetes, Agaricomycotina, Basidiomycota)**

Pleurotus ostreatus is one of the most popular cultivated edible mushroom species, which in 1997 accounted for 14.2% of the world total edible mushroom production (Chang 1999). Mostly it is cultivated on pasteurized straw, stumps or logs (Pavlik and Pavlik 2013) and in Asia also on supplemented sawdust (Gregori et al. 2007b). It can also be cultivated on different agricultural leftovers (spent brewery grains, oil press cakes, bran, corn cobs, seed hulls etc.) (Gregori et al. 2007b). Its cultivation on logs and stumps is considered as very easy and is widespread between amateur growers around the world.

In Slovenia this species can be found on broadleaf trees late in autumn. It is not well recognized by the broad public as edible and not at all recognized as medicinal. There are 81 locations of *P. ostreatus* reported in Slovenia (Ogris 2013).

P. ostreatus is mostly mentioned as an edible mushroom, but besides its gastronomic properties it also contains bioactive substances, especially polysaccharides, which show immunomodulating (Bauerova et al. 2009) as well as LDL lowering and blood lipid levels improving properties (Bobek et al. 1991, Bobek et al. 1997, Opletal et al. 1997, Gunde-Cimerman et al. 2001, Hossain et al. 2003). Polysaccharides from *P. ostreatus* show anti-cancer and hepatoprotective activities and increase activity of superoxide dismutase, catalase and counter the age related reduction in levels of vitamins C and E (Kurashige et al. 1997, Gu and Sivam 2006, Jayakumar et al. 2006, Jayakumar et al. 2007, Thanasekaran et al. 2010).

***Trametes versicolor* (L.) Lloyd (1921) (Polyporaceae, Polyporales, Agaricomycetes, Agaricomycotina, Basidiomycota)**

T. versicolor is one of the most researched medicinal mushrooms (Powell 2010) and an abundant species in Slovenia with 151 locations reported (Ogris 2013). Usually thousands can be found everywhere where wood debris is present. It grows on all types of dead wood, but prefers broadleaf trees. Sporocarps form mainly in the

late autumn and early spring, when snow starts to melt providing moisture to the emerging fruiting bodies. Gathering of *T. versicolor* is not very common in Slovenia, but is becoming more and more popular as a natural way of treating different health issues. People use it for the preparation of tea and tinctures for immune system enhancement (Vrhovec 2010). Because of its wide distribution and abundant quantities in Slovenia, we can consider this species as a medicinal mushroom with a big potential in natural disease treatment, especially for people with low income. Its gathering presents almost no danger of misidentification with other species.

In China and Japan they developed two medicines (PSP and PSK), composed of *T. versicolor* water-soluble polysaccharopeptides, and with many clinical trials performed. It was shown that PSP and PSK are very effective in the treatment of different cancer types including gastric, lung, nasopharyngeal, colorectal, breast, oesophageal as well as uterine cancer (Tsukagoshi et al. 1984, Ng 1998, Parris 2000, Fisher and Yang 2002, Cui and Chisti 2003, Kanazawa et al. 2005, Jimenez-Medina et al. 2008, Standish et al. 2008). *T. versicolor* also shows strong anti-viral activities proven *in vitro* (Hirose et al. 1987, Tochikura et al. 1987, Hobbs 2004, Mlinaric et al. 2005, Ng et al. 2006) and also on HIV patients (Pfeiffer 2001). *T. versicolor* polysaccharides and polysaccharopeptides besides anti-cancer possess also immune system enhancing activities (Tzianabos 2000, Standish et al. 2008).

Hericium erinaceus (Bull.) Pers. (1797) (Hericiaceae, Russulales, Agaricomycetes, Agaricomycotina, Basidiomycota)

H. erinaceus is a well-known edible medicinal mushroom, with a distinct shape, which resembles a beard or a monkey's head. It grows on dead broadleaf trees and some species from this genus (*H. abietis*) also on pine trees. There are 18 locations of *H. erinaceus* reported in Slovenia mostly in the central, northeast and southwestern part of the country (Ogris 2013). In Slovenia *H. erinaceus* is a very rare, endangered and protected species and the knowledge about its use is not known to the broader public. In the last few years it is gathering on reputation due to the products on the market produced through artificial cultivation of fruiting bodies and biomass.

In Asia they also call it "a Natures nutrient for neurons", because it contains erinacines, which stimulate the biosynthesis of the nerve growth factor and catecholamines in the central nervous system (Kenmoku et al. 2002, Shimbo et al. 2005, Mori et al. 2008, Kawagishi et al. 2011). This species shows a good potential for treating Alzheimer's disease, dementia, multiple sclerosis and even physical damages of nerves (Kolotushkina et al. 2003, Mori et al. 2009). It has been demonstrated by several studies over the last 2–3 decades that *H. erinaceus* possesses anticancer activities, strongly linked to immunomodulation (Liu et al. 2000, Lee and Hong 2010, Khan et al. 2013), acts against methicillin-resistant *Staphylococcus aureus* (Kawagishi 2005) and even gastritis caused by *Helicobacter pylori* (Xu et al. 1985, Yu et al. 1999) or ethanol ingestion (Abdulla et al. 2009).

Grifola frondosa (Dicks.) Gray (1821) (Meripilaceae, Polyporales, Agaricomycetes, Agaricomycotina, Basidiomycota)

G. frondosa usually grows on stumps or the base of hardwood trees like oak and weights up to ten kilograms (Rogers 2011). There are 44 locations reported in Slovenia (Ogris 2013). It is considered as an endangered and protected species, not often used in culinary or medicinal purposes.

G. frondosa is one of the tastiest polypores, similar to eggplant in flavor (Rogers 2011). It contains polysaccharides, which are the major active components. Several beta-glucan, heteropolysaccharide and proteoglycan fractions have been isolated with potent immunomodulatory action, including D-fraction and MD-fraction (Powell 2010). The D-fraction, the MD-fraction, and other extracts, often in combination with whole *G. frondosa* fruiting bodies powder, have shown particular promise as immunomodulating agents and as an adjunct to cancer and HIV therapy (Kodama et al. 2002, Kodama et al. 2003). They may also provide some benefit in the treatment of hyperlipidemia, hypertension, and hepatitis (Mayell 2001). Antidiabetic and cholesterol action has also been reported for fruiting bodies and extracts of *G. frondosa* (Kubo et al. 1994, Kubo and Nanba 1997).

***Schizophyllum commune* Fr. (1815) (Schizophyllaceae, Agaricales, Agaricomycetidae, Agaricomycetes, Agaricomycotina, Basidiomycota)**

113 locations of this species are officially reported in Slovenia (Ogris 2013), but in general is more widespread on logs, branches and stumps of broadleaf and coniferous trees, especially poplar, birch, spruce and pine (Evans and Kibby 2005, Rogers 2011). In Thailand it is used as a gourmet mushroom, prepared in dishes as are fried eggs or fried rice. This species was also found growing in hay bales (Webster 1991). In Slovenia the broader public because of lack of information does not yet use it. There are some reports of its use as a tea (Vrhovec 2010), but they are very rare.

S. commune contains polysaccharides with schizophyllan, having a molecular weight of 450 kD, being the most researched. It was shown to inhibit solid Sarcoma 180 tumor (Komatsu et al. 1969), prolongs survival and time to recurrence in stage II cervical cancer patients (Okamura et al. 1989, Miyazaki et al. 1995). Salahuddin (2008) tested different *S. commune* extracts and determined that they show a broad spectrum of antimicrobial, antioxidant, cytotoxicity and anti-human papilloma virus activities. In traditional Chinese medicine this fungus is recommended for general weakness and debility (Rogers 2011). It is recommended to cook the fruiting bodies before usage otherwise the fungus can spread inside the living healthy tissue of humans as well as animals (Kano et al. 2002, Rogers 2011).

***Auricularia auricula* (L.) Underw. (1902) (Auriculariaceae, Auriculariales, Agaricomycetes, Agaricomycotina, Basidiomycota)**

There are 69 locations of this species officially reported in Slovenia (Ogris 2013). In Slovenia it grows almost exclusively on dead elder (*Sambucus nigra*) branches late in autumn. Pohleven (2010) reports on its use in Europe during the Middle Ages, when a saying was used "Auricularia put on the eye, removes all the pain". As people get to know this species and its culinary and medicinal use, they start picking and using it although traditionally it is not well known and used in Slovenia.

A. auricula is a very popular mushroom in traditional Chinese medicine and especially in traditional Chinese cuisine. Cultivation of this species is very popular in Asia and *A. auricula* is frequently mentioned as the first mushroom species to be cultivated in 600 A.D. (Chang and Miles 1987).

Ying (1987) reported of *A. auricula* being activitve against Ehrlich carcinoma and Sarcoma 180. In experiments conducted by Chen et al. (2008a) and by Zeng et al. (2013) polysaccharides extracted from *A. auricula* significantly decreased the levels of total cholesterol, triglyceride, and low-density lipoprotein cholesterol in hyperlipidemic mice and rats.

***Fomes fomentarius* (L.) J.J. Kickx (1867) (Polyporaceae, Polyporales, Agaricomycetes, Agaricomycotina, Basidiomycota)**

This species grows on dead hardwood trees, in Slovenia more frequently on beech (*Fagus silvatica*) trees. *F. fomentarius* is very popular in Slovenian tradition as an ornamental item and also for other uses. Dried fruiting bodies are lit by beekeepers and smoke is used for its sedative-like action it has on the bees. In Slovenia it is used for transferring the blessed fire from churches into homes and using it as incense in religious rituals. The same ritual is known also in Siberian tribes and Ainu people in Japan (Rogers 2011). There are no records of use of this fungus for medicinal purposes in Slovenia, but because of its wide distribution through the whole area, especially the eastern parts of Slovenia (168 locations reported by Ogris, 2013), it could be more often used as a medicinal remedy or as a food supplement. This species can be cultivated on mixture of sawdust and supplements (Stamets 2005) as an alternative to wild-grown specimens.

F. fomentarius has been firstly mentioned by Hippocrates (460–377 B.C.) who mentioned its topical use for cauterizing wounds and for externally treating inflamed organs (Stamets 2005). Dissociated context of this fungus was found besides the famous Oetzi, more than 5000 years old iceman found in the Italian Alps and was supposedly used as a fire starter or as a natural medicine (Capasso 1998, Rogers 2011).

F. fomentarius shows antibacterial properties. Peintner et al. (1998), Stamets (2005) and Suay et al. (2000) reported of its activity against *Pseudomonas aeruginosa, Serratia marcescens, Staphylococcus aureus, Bacilus subtilis* and *Mycobacterium smegmatis*, a relative of the pathogenic *Mycobacterium tuberculosis.* Polysaccharides contained in *F. fomentarius* show activity against sarcoma 180 tumors in mice (Ito et al. 1976) and anticancer activity against human gastric cancer cells (Chen et al. 2008b). Using in vitro models Seniuk et al. (2011) established that glucan complexes from *F. fomentarius* completely depressed the growth of *Candida albicans,* had an antimicrobial effect on *H. pylori*, possessed simultaneously weak toxicity and high anti-HIV-1 activity in comparison with zidovudine (Retrovir) and concluded that due to the very low toxic properties on blood cells even in very high concentrations, these complexes may be used as a source of biopolymers for the creation of essentially new agents for wide applications in infectious pathology.

Fomitopsis pinicola (Sw.) P. Karst. (1881) (Fomitopsidaceae, Polyporales, Agaricomycetes, Agaricomycotina, Basidiomycota)

With 150 locations of *F. pinicola* reported in Slovenia this mushroom can be considered as an abundant and very common species. In Slovenia it most commonly inhabits dead spruce trees, but can be also found on poplar, beech, birch and other tree species. *F. pinicola* is in general considered as one of the most common polypores in the world (Rogers 2011). This author knows of no reports of its medicinal use in Slovenia.

Traditionally in Northern America this mushroom was used on wounds to stop bleeding, as a daily tonic to stop inflammation of the digestive tract, to increase general resistance and against headaches (Rogers 2011). Chemical compounds found in *F. pinicola* include steroids, sesquiterpenes, lanostane triterpenoids and triterpene glycosides (Haghi 2011). In Germany homeopathic remedies are prepared from *F. pinicola*, but without stated indications. Alkaline extracts from *F. pinicola* show anti-hyperglycemic effects in streptozotocin induced diabetes mellitus rats (Lee et al. 2008). Cheng et al. (2008) observed antiinflammation and antiangiogenic effects of *F. pinicola* ethanolic extract and polysaccharides.

Laricifomes officinalis (Vill.) Kotl. & Pouzar (1957) (Fomitopsidaceae, Polyporales, Agaricomycetes, Agaricomycotina, Basidiomycota)

In Slovenia *L. officinalis* is an endangered and protected species with only three locations reported by Ogris (2013) and nine additional locations reported by Dakskobler et al. (2011). Pietka (2004b) and Mukhin et al. (2005) reported that number of *L. officinalis* specimens is also decreasing in Poland and Russia. The fruiting bodies of Slovenian specimens grow on old, thick larch trees (*Larix decidua*) that often have a dry or broken top and grow between 1430 and 1790 meters above sea level (Dakskobler et al. 2011). There are many other locations of *L. officinalis* in Slovenia, very carefully protected by mycologists, because its growth in nature is very slow, and because this species is very sought after due to its medicinal properties. Regardles of the fact that this species is endangered and protected in Slovenia, people still gather and sell it on the black market for around 90 USD per kg (Vrhovec 2010). They use it as a tea, for strengthening the immune system or by smoking as an ailment against bronchitis.

Before it was brought to the edge of extinction, *L. officinalis* was available in European pharmacies, as a purgative, anticancer agent, antipyretic and analgesic drug, as an abortive agent or to inhibit bleeding in disorders of the teeth, as an anti-swelling agent or a sedative and to cure disorders of the digestive system (cited in: Pietka 2004b). Stamets (2005) reported of activity against orthopox viruses, caused by *L. officinalis* extracts. There were successful attempts already conducted for artificial inoculation of larch trees with *L. officinalis* mycelia (Pietka and Grzywacz 2005, Gregori et al. 2007a) and even artificial cultivation of mycelia in laboratory conditions (Pietka 2004a). There are still a few *L. officinalis* products on the EU market.

***Piptoporus betulinus* (Bull.) P. Karst. (1881) (Fomitopsidaceae, Polyporales, Agaricomycetes, Agaricomycotina, Basidiomycota)**

There are officially 144 locations of this species reported in Slovenia (Ogris 2013), but many other not reported locations also exist. It grows on dead or dying birch trees mostly in the eastern part of the country. In Slovenia this species is used as an immunomodulator for people as well as for domestic animals. For this purpose decoctions and teas are used with reported efficient immunomodulating activity.

This species was found beside the Oetzi, more than 5000 years old iceman found in the Italian Alps and was admittedly used against parasites *Trichuris trichuria* (Capasso 1998). In folk medicine this mushroom was used to stop bleeding (Stamets 2005) as an antiparasitic and antimicrobial agent in the treatment of wounds and for the treatment of rectal cancer and stomach diseases. Tea obtained from this mushroom has antibacterial, antifatiguing, immunoenhancing, and soothing properties (Lemieszek et al. 2009).

Lemieszek et al. (2009) also reported that *P. betulinus* fractions elicit anticancer effects attributed to decreased tumor cell proliferation, motility and the induction of morphological changes. Schlegel et al. (2000) isolated an antibiotic named piptamine from this species. Kanamoto et al. (2001) reported that betulinic acid derivatives, extracted from this specie show activity against HIV viruses. Stamets (2005) reports of *P. betulinus* extracts having activity against vaccinia and cowpox viruses. Six lanostane-type triterpene acids were isolated from the fruiting bodies of *Piptoporus betulinus* by Kamo et al. (2003) showing anti-inflammatory properties. Also Manez et al. (1997) reported that terpenoids from this species reduced dermal inflammations. Betulinic acid – a pentacyclic triterpene, isolated from *P. betulinus*, was identified as a melanoma–specific cytotoxic agent completely inhibiting human melanomas without toxicity (Pisha 1995).

Conclusions

Slovenia with its small but very diverse geography and environment is home to more than 2400 fungal species. Many of these species, especially macromycetes were in the past used as a natural medicine. The old knowledge about their use is very scarce in Slovenia, but is returning from other countries and scientific literature mentioning their medicinal activities.

Mushrooms of this kind represent very accessible natural medicines, with no or little side effects with a very low price. The fact that the majority of medicinal mushrooms are wood-inhabiting species, shows a big potential for Slovenia. More than 50% of its area is covered by forest and has a very active wood industry, with sawdust as the main byproduct.

Beside species mentioned above, many other species native to Slovenia also contain medicinal compounds and can be used for medicinal purposes: *Coprinus comatus, Laetiporus sulphureus*, *Ganoderma applanatum, Polyporus umbellatus, Agaricus sp., Phallus impudicus, Albatrellus confluens, Lepista inversa, Heterobasidion annosum, Pycnoporus cinnabarinus, Craterellus curnicopioides, Tremella mesenterica, Lactarius deliciosus, Flammulina velutipes, Sparassis crispa, Armillaria mellea, Trametes suaveolens, Innonotus obliquus, Agrocybe cylindracea* and others.

Zaključek

Slovenija s svojim majhnim, a raznovrstnim okoljem, je dom več kot 2400 vrstam gliv. Veliko teh vrst, še posebno makromicete so v preteklosti uporabljali kot naravna zdravila. Staro znanje o njihovi uporabi je v Sloveniji zelo redko, vendar se vrača iz drugih držav ter znanstvene literature, ki dandanes vedno bolj posveča pozornost tem vrstam gob.

Te vrste gob predstavljajo lahko dostopna naravna zdravila, brez ali z zelo redkimi nezaželenimi stranskimi učinki ter zelo nizko ceno. Dejstvo, da večina zdravilnih gob raste na lesu, kaže na velik potencial, ki ga s svojo več kot 50 % prekritostjo z gozdom in razvito lesno industrijo premore Slovenija.

Poleg zgoraj omenjenih vrst zdravilnih gob tudi sledeče vrste vsebujejo zdravilne učinkovine ter so uporabne kot potencialna naravna zdravila: *Coprinus comatus, Laetiporus sulphureus*, *Ganoderma applanatum, Polyporus umbellatus, Agaricus sp., Phallus impudicus, Albatrellus confluens, Lepista inversa, Heterobasidion annosum, Pycnoporus*

cinnabarinus, Craterellus curnicopioides, Tremella mesenterica, Lactarius deliciosus, Flammulina velutipes, Sparassis crispa, Armillaria mellea, Trametes suaveolens, Innonotus obliquus, Agrocybe cylindracea and others.

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References

Abdulla, M.A., Noor, S.M., Sabaratnam, V., Abdullah, N., Wong, K.H., Ali, H.M., 2009. Effect of Culinary-Medicinal Lion's Mane Mushroom, *Hericium erinaceus* (Bull.: Fr.) Pers. (Aphyllophoromycetideae), on Ethanol-Induced Gastric Ulcers in Rats. Int. J. Med. Mushr., 11 (3), 325–336.

Bauerova, K., Paulovicova, E., Mihalova, D., Svik, K., Ponist, S., 2009. Study of new ways of supplementary and combinatory therapy of rheumatoid arthritis with immunomodulators. Glucomannan and Imunoglukan in adjuvant arthritis. Toxicol. Ind. Health, 25 (4–5), 329–335.

Bobek, P., Giner, E., Kuniak, L., Babala, J., Jurcovicova, M., Ozdin, L., Cerven, J., 1991. Effect of mushroom *Pleurotus ostreatus* and isolated fungal polysaccharide on serum and liver lipids in Syrian hamsters with hyperlipoproteinemia. Nutrition, 7 (2), 105–108.

Bobek, P., Ozdin, L., Kajaba, I., 1997. Dose-dependent hypocholesterolaemic effect of oyster mushroom (*Pleurotus ostreatus*) in rats. Physiol. Res., 46 (4), 327–329.

Boh, B., 2013. *Ganoderma lucidum*: A Potential for Biotechnological Production of Anti-Cancer and Immunomodulatory Drugs. Recent Patents on Anti-Cancer Drug Discovery, 8, 255–287.

Boh, B., Berovič, M., Zhang, J.S., Zhi-Bin, L., 2007. Ganoderma lucidum and its pharmaceutically active compounds. Biotechnology Annual Review, 13, 265–301.

Capasso, L., 1998. 5300 years ago, the Ice man used natural laxatives and antibiotics. Lancet, 352, 1864.

Cha, J.Y., Ahn, H.Y., Cho, Y.S., Je, J.Y., 2013. Protective effect of cordycepin-enriched *Cordyceps militaris* on alcoholic hepatotoxicity in Sprague–Dawley rats. Food and Chemical Toxicology, 60, 52–57.

Chang, S.T., 1996. Mushroom research and development – equality and mutual benefits. Proceedings of the 2nd International conference on Mushroom Biology and Mushroom Products, Pennsilvanya, 581 pp.

Chang, S.T., 1999. World production of cultivated and medicinal mushrooms in 1997 with emphasis on *Lentinus edodes* (Berk) Sing. Int. J. Med. Mush., 1, 291–300.

Chang, S.T., Buswell, J.A., 1999. *Ganoderma lucidum* (Curt.: Fr.) P. Karst. (Aphyllophoromycetideae) – a mushrooming medicinal mushroom. Int. J. Med. Mushrooms, 1 (2), 139–146.

Chang, S.T., Buswell, J.A., Miles, P.G., (eds.) 1992. Genetics and breeding of edible mushrooms. 2nd ed. Gordon & Breach Science Publishers, New York, 324 pp.

Chang, S.T., Miles, P.G., 1987. Historical record of the early cultivation of *Lentinus* in China. Mushroom Journal of the Tropics, 7, 47.

Chen, G., Luo, Y.C., Li, B.P., Li, B., Guo, Y., Li, Y., Su, W., Xiao, Z.L., 2008. Effect of polysaccharide from *Auricularia auricula* on blood lipid metabolism and lipoprotein lipase activity of ICR mice fed a cholesterol-enriched diet. J. food. Sci., 73 (6), 103–108.

Chen, W., Zhao, Z., Chen, S.F., Li, Y.Q., 2008. Optimization for the production of exopolysaccharide from *Fomes fomentarius* in submerged culture and its antitumor effect in vitro. Bioresource Technology, 99, 3187–3194.

Cheng, J.J., Lin, C.Y., Lur, H.S., Chen, H.P., Lu, M.K., 2008. Properties and biological functions of polysaccharides and ethanolic extracts isolated from medicinal fungus, *Fomitopsis pinicola*. Process Biochemistry, 43, 829–834.

Cui, J., Chisti, Y., 2003. Polysaccharopeptides of *Coriolus versicolor*: physiological activity, uses, and production. Biotechnol. Adv., 21 (2), 109–122.

Dakskobler, I., Seliškar, A., Podgornik, G., 2011. Razširjenost in ekologija vrste *Laricifomes officinalis* (Vill.) Kotl. & Pouzar v Julijskih Alpah (Slovenija); Distribution and ecology of *Laricifomes officinalis* (Vill.) Kotl. & Pouzar in the Julian Alps (Slovenia). Gozd, 69, 139–153.

Evans, S., Kibby, G., 2005. Naturfuehrer, Pilze, 1st ed. Dorling Kindersley, Starnberg, 296 pp.

Fisher, M., Yang, L.X., 2002. Anticancer effects and mechanisms of polysaccharide-K (PSK): implications of cancer immunotherapy. Anticancer Res., 22 (3), 1737–1754.

Gao, Y., Zhou, Sh., Chen, G., Dai, X., Ye, J., 2002. A phase I= II study of a *Ganoderma lucidum* (Curt.:Fr.) P. Karst. extract (Ganopoly) in patients with advanced cancer. Int. J. Med. Mushrooms, 2002, 4 (3), 207–214.

Gregori, A., Piskur, B., Gregori, M., Jurc, D., 2007a: Spread of the *Fomitopsis officinalis* inoculated in stems of living larch in Slovenia, The fourth international medicinal mushroom conference, Ljubljana, 304–305.

Gregori, A., Švagelj, M., Pohleven, J., 2007b. Cultivation Techniques and Medicinal Properties of *Pleurotus* spp. Food Technol. Biotechnol., 45 (3), 236–247.

Gu, Y.H., Sivam, G., 2006. Cytotoxic effect of oyster mushroom *Pleurotus ostreatus* on human androgen-independent prostate cancer PC-3 cells. J. Med. Food., 9 (2), 196–204.

Gunde-Cimerman, N., Plemenitas, A., 2001. Hypocholesterolemic Activity of the Genus *Pleurotus* (Jacq.: Fr.) P. Kumm. (Agaricales s. I., Basidiomycetes), Int. J. Med. Mushr., 3 (4), 395–398.

Haghi, A. K., 2011. Food Science: Research and Technology. CRC Press, 131 pp.

Hirose, K., Hakozaki, M., Kakuchi, I., Matsunaga, K., Yoshikumi, C., Takakashi, M., Tochikura, T.S., Yamamoto, N., 1987. A biological response modifier, PSK, inhibits reverse transcriptase in vitro. Biochem. Biophys. Res. Commun., 149 (2), 562–567.

Hobbs, C., 2004. Medicinal Value of Turkey Tail Fungus *Trametes versicolor* (L.:Fr.) Pilat (Aphyllophoromycetideae), Int. J. Med. Mushr., 6 (3), 346–347.

Hobbs, Ch., 1995. Medicinal Mushrooms: An Exploration of Tradition, Healing, and Culture , 2nd Ed., Botanica Press, Inc.: Santa Cruz, CA, USA.

Holliday, J., Cleaver, M., 2005. *Cordyceps*, Encyclopedia of Dietary Supplements. New York: Marcel Dekker, pp. 842.

Hossain, S., Hashimoto, M., Choundhury, E.K., Alam, N., Hussain, S., Hasan, M., Choudhury, S.K., Mahmud, I., 2003. Dietary mushroom (*Pleurotus ostreatus*) ameliorates atherogenic lipid in hypercholesterolaemic rats. Clin. Exp. Pharmacol. Physiol., 30 (7), 470–475.

Hung, L.T., Keawsompong, S., Hanh, V.T., Sivichai, S., Hywel-Jones, N.L., 2009. Effect of temperature on cordycepin production in *Cordyceps militaris*. Thai journal of agricultural science, 42 (4), 219–225.

Ito, H., Sugiura, M., Miyazaki, T., 1976. Antitumor polysaccharide fraction from the culture filtrate of *Fomes fomentarius*. Chemical & pharmaceutical bulletin, 24 (10), 2575.

Jayakumar, T., Ramesh, E., Geraldine, P., 2006. Antioxidant activity of the oyster mushroom, *Pleurotus ostreatus*, on CCl(4)-induced liver injury in rats. Food Chem. Toxicol., 44 (12), 1989–1996.

Jayakumar, T., Thomas, P.A., Geraldine, P., 2007. Protective effect of an extract of the oyster mushroom, *Pleurotus ostreatus*, on antioxidants of major organs of aged rats. Epx. Gerontol., 42 (3), 183–191.

Jimenez-Medina, E., Berruguilla, E., Romero, I., Algarra, I., Collado, A., Garrido, F., Garcia-Lora, A., 2008. The immunomodulator PSK induces *in vitro* cytotoxic activity in tumour cell lines *via* arrest of cell cycle and induction of apoptosis. BMC Cancer, 8,78.

Jin, X., Ruiz, B.J., Sze, D.M.Y., Chan, G.C.F., 2012. *Ganoderma lucidum* (Reishi mushroom) for cancer treatment (Review). Cochrane database syst. Rev., 13 (6), 1–35.

Jong, S.C., Birmingham, J.M., 1992. Medicinal benefits of the mushroom *Ganoderma*. Adv. Appl. Microbiol., 37, 101–134.

Jurc, D., Piltaver, A., Ogris, N., 2005. Glive Slovenije – Fungi of Slovenia, Studia forestalica Slovenica, Ljubljana, 497 pp.

Kamo, T., Asanoma, M., Shibata, H., Hirota, M., 2003. Anti-inflammatory lanostane-type triterpene acids from *Piptoporus betulinus*. Journal of Natural Products, 66 (8), 1104–1106.

Kanamoto, T., Kashiwada, Y., Kanbara K., Gotoh, K., Yoshimori, M., Goto, T., Sano, K., 2001. Anti-human immunodeficiency virus activity of YI-FH 312 (a betulinic acid derivative), a novel compound blocking viral maturation. Antimicrobial Agents and Chemotherapy, 45 (4), 1225–1230.

Kanazawa, M., Yoshihara, K., Abe, H., Iwadate, M., Watanabe, K., Suzuki, S., Endoh, Y., Takita, K., Sekikawa, K., Takenoshita, S., Ogata, T., Ohto, H., 2005. Effects of PSK on T and dendritic cells differentiation in gastric or colorectal cancer patients. Anticancer Res., 25 (1B), 443–449.

Kano, R., Oomae, S., Nakano, Y., Minami, T., Sukikara, M., Nakayama, T., Hasegawa, A., 2002. First Report on *Schizophyllum commune* from a Dog. J. Clin. Microbiol., 40 (9), 3535–3537.

Kawagishi, H., 2005. Anti-MRSA compounds of *Hericium erinaceus*. Int. J. Med. Mushr., 7 (3), 350.

Kawagishi, H., Simada, A., Shizuki, K., Ojima, F., Mori, H., Okamoto, K., Sakamoto, H., Furukawa, S., 2011. Erinacine D, a stimulator of NGF-synthesis, from the mycelia of *Hericium erinaceum.* Heterocyclic Communications, 2 (1).

Kenmoku, H., Shimai, T., Toyomasu, T., Kato, N., Sassa, T., 2002. Erinacine Q, a new erinacine from *Hericium erinaceum*, and its biosynthetic route to erinacine C in the basidiomycete. Biosci. Biotechnol. Biochem., 66 (3), 571–575.

Khan, A., Tania, M., Liu, R., Rahman, M.M., 2013. *Hericium erinaceus*: an edible mushroom with medicinal values. J. Complement. Integr. Med., 10 (1), 1–6.

Kim, H.W., Kim, B.K., 1999. Biomedical triterpenoids of *Ganoderma lucidum* (Curt.: Fr.) P. Karst. (Aphyllophoromycetideae). Int. J. Med. Mushrooms, 1 (2), 121–138.

Kim, J.R., Yeon, S.H., Kim, H.S., Ahn, Y.J., 2002. Larvicidal acivity against *Plutella xylostella* of cordycepin from the fruiting body of *Cordyceps militaris*. Pest Manag., 58, 713–717.

Kirk, P.M., Cannon, P.F., David, J.C., Stalpers, J.A., 2001. Ainsworth and Bisby's dictionary of the fungi, 9th ed. CAB International, Walingford.

Koc, Y., Urbano, A.G., Sweeney E.B., McCaffrey, R., 1996. Induction of apoptosis by cordycepin in ADA-inhibited TdT–positive leukemia cells. Leukemia, 10, 1019–1024.

Kodama, N., Komuta, K., Nanba, H., 2002. Can maitake MD-fraction aid cancer patients? Alter. Med. Rev., 7 (3), 236–239.

Kodama, N., Komuta, K., Nanba, H., 2003. Effect of Maitake (*Grifola frondosa*) D Fraction on the activation of NK cells in cancer patients. J. Med. Food, 6 (4), 371–377.

Kolotushkina, E.V., Moldavan, M.G., Voronin, K.Y., Skibo, G.G., 2003. The influence of *Hericium erinaceus* extract on myelination process in vitro. Fiziol. Zh., 49 (1), 38–45.

Komatsu, N., Okubo, S., Kikumoto, S., Kimura, K., Saito, G., 1969. Host-mediated antitumor action of Schizophyllan, a glucan produced by *Schizophyllum commune*. Gann., 60, 137–144.

Kubo, K., Aoki, H., Nanba, H., 1994. Anti-diabetic activity present in the fruit body of *Grifola frondosa* (Maitake). Biol. Pharm. Bull., 17, 1106–1110.

Kubo, K., Nanba, H., 1997. Anti-hyperliposis effect of Maitake fruit body (*Grifola frondosa*). Biol. Pharm. Bull., 20, 781–785.

Kurashige, S., Akuzawa, Y., Endo, F., 1997. Effects of *Lentinus edodes, Grifola frondosa* and *Pleurotus ostreatus* administration on cancer outbreak, and activities of macrophages and lymphocytes in mice treated with a carcinogen, N-butyl-N-butanolnitrosoamine. Immunopharmacol. Immunotoxicol., 19 (2), 175–183.

Lee, J.S., Hong, E.K., 2010. *Hericium erinaceus* enhances doxorubicininduced apoptosis in human hepatocellular carcinoma cells. Cancer Lett., 297, 144–154.

Lee, S.I., Kim, J.S., Oh, S.H., Park, K.Y., Lee, H.G., Kim, S.D., 2008. Antihyperglycemic Effect of *Fomitopsis pinicola* Extracts in Streptozotocin-Induced Diabetic Rats, Journal of Medicinal Food. September 2008, 11 (3), 518–524.

Lee, S.Y., Debnath, T., Kim, S.K., Lim, B.O., 2013. Anti-cancer effect and apoptosis induction of cordycepin through DR3 pathway in the human colonic cancer cell HT-29. Food and chemical toxicology, 60, 439–447.

Lemieszek, M.K., Langner, E., Kaczor, J., Kandefer-Szerszen, M., Sanecka, B., Mazurkiewicz, W., Rzeski, W., 2009. Anticancer Effect of Fraction Isolated from Medicinal Birch Polypore Mushroom, *Piptoporus betulinus* (Bull.: Fr.) P. Karst. (Aphyllophoromycetideae): *In Vitro* Studies. Int. J. Med. Mushr., 11 (4) 351–364**.**

Lindequist, U., Niedermeyer, T.H.J., Juelich, W.D., 2005. The pharmacological potential of mushrooms. Evidence-based Complementary and Alternative Medicine, 2, 285–299.

Liu, C., Gao, P., Qian, J., Yan, W., 2000. Immunological study on the antitumor effects of fungus polysaccharides compounds. Wei Sheng Yan Jiu, 29, 178–180.

Manez. S., Recio, M.C., Giner, R.M., Rios, J.L., 1997. Effect of selected triterpenoids on chronic dermal inflammation. European Journal of Pharmacology, 334 (1), 103–105.

Mayell, M., 2001. Maitake extracts and their therapeutic potential. Altern. Med. Rev., 6 (1), 48–60.

McKenna, D.J., Jones, K., Hughes, K., 2002. Reishi Botanical Medicines. The Desk reference for Major Herbal Supplements, 2nd Ed., The Haworth Herbal Press: New York, London, Oxford, 825–855.

Miyazaki, K., Mizutani, H., Katabuchi, H., Fukuma, K., Fujisaki, S., Okamura, H. 1995. Activated (HLA–DR+) T-lymphocyte subsets in cervical carcinoma and effects of radiotherapy and immunotherapy with Schizophyllan on cell-mediated immunity and survival. Gynecologic oncology, 56, 412–420.

Mizuno, T., 1995. Reishi, *Ganoderma lucidum* and *Ganoderma tsugae*: bioactive substances and medicinal effects. Food Rev. Int., 11 (1), 151–166.

Mlinarič, A., Kac, J., Pohleven, F., 2005. Screening of selected wood-damaging fungi for the HIV-1 reverse transcriptase inhibitors. *Acta pharm.*, 55 (1), 69–79.

Mori, K., Inatomi, S., Ouchi, K., Azumi, Y., Tuchida, T., 2009. Improving effects of the mushroom Yamabushitake (*Hericium erinaceus*) on mild cognitive impairment: a double-blind placebo-controlled clinical trial. Phytother. Res., 23 (3), 367–372.

Mori, K., Obara, Y., Hirota, M., Azumi, Y., Kinugasa, S., Inatomi, S., Nakahata, N., 2008. Nerve growth factor-inducing activity of Hericium erinaceus in 1321N1 human astrocytoma cells. Biol. Pharm. Bull., 31 (9), 1727–1732.

Mukhin, V.A., Kotiranta, H., Knudsen, H., Ushakova, N.V., Votintseva, A.A., Corfixen, P., Chlebicki, A., 2005. Distribution, frequency and biology of *Laricifomes officinalis* in the Asian part of Russia. Russian Journal of Mycology and Phytopathology, 39 (5), 34–42.

Nakamura, K.N., Yoshikawa, Y., Yamaguchi, Y., Kagota, S., Shinuzuka, K., Kunitomo, M., 2006. Antitumour effect of cordycepin (3'-deoxiadenosine) on mouse melanoma and lung carcinoma cells involves adenosine A3 receptor stimulation. Anticancer Res., 26, 43–47.

Ng, T.B., 1998. A review of research on the protein-bound polysaccharide (polysaccharopeptide, PSP) from the mushroom *Coriolus versicolor* (Basidiomycetes: Polyporaceae). Gen. Pharmacol., 30 (1), 1–4.

Ng, T.B., Wang, H., Wan, D.C.C., 2006. Polysaccharopeptide from the Turkey Tail Fungus *Trametes versicolor* (L.:Fr.) Pilát Inhibits Human Immunodeficiency Virus Type 1 Reverse Transciptase and Protease, Int. J. Med. Mushr., 8 (1), 39–43.

Ng, T.B., Wang, H.X., 2005. Pharmacological actions of *Cordyceps*, a prized folk medicine. J. Pharm. Pharmacol., 57, 1509–1519.

Ogris N., 2013. Podatkovna zbirka gliv Slovenije *Boletus informaticus.*

Okamura, K., Suzuki, M., Chihara, T., Fujiwara, A., Fukuda, T., Goto, S., Ichinohe, K., Jimi, S., Kasamatsu, T., Kawai, N., Kizuguchi, K., Mori, S., Nakano, H., Noda, K., Sekiba, K., Suzuki, K., Suzuki, T., Takahashi, K., Takeuchi, K., Takeuchi, S., Yajima, A., Ogawa, N., 1989. Clinical evaluation of Schizophyllan combined with irradiation in patients with cervical cancer. A randomized controlled study, a five-year survival rate. Biotherapy, 1, 103–107.

Opletal, L., Jahodar, L., Chobot, V., Zdansky, P., Lukes, J., Bratova, M., Solichova, D., Bluden, G., Dacke, C.g., Patel, A.V., 1997. Evidence for the anti-hyperlipidaemic activity of the edible fungus *Pleurotus ostreatus*. Br. J. Biomed. Sci., 54 (4), 240–243.

Parris, K., 2000. The Use of Mushroom Glucans and Proteoglycans in Cancer Treatment. Alternative medicine review, 5(1).

Paterson, R.R., 2008. Cordyceps: a traditional Chinese medicine and another fungal therapeutic biofactory? Phytochemistry, 69 (7), 1469–95.

Pavlik, M., Pavlik, Š., 2013. Wood decomposition activity of oyster mushroom (*Pleurotus ostreatus*) isolate in situ. Journal of forest science, 59 (1), 28–33.

Peintner, U., Poeder, R., Pumpel, T., 1998. The iceman's fungi. Mycol. Res., 102 (10), 1153–1162.

Pfeiffer, M., 2001. The clinical use of *Coriolus versicolor* supplementation in HIV+ patients and the impact on CD4 count and viral load. 3rd International Symposium on Mushroom Nutrition.

Pietka, J., 2004a. The development of *Fomitopsis officinalis* mycelium grown on organic media and larch wood under laboratory conditions. Sylwan, 9, 34.42.

Pietka, J., 2004b. Localities of *Fomitopsis officinalis* in Poland. Acta Mycologica, 39 (1), 33–45.

Pietka, J., Grzywacz, A., 2005. In situ larch inoculation with *Fomitopsis officinalis*. Polish Botanical Journal, 50 (2), 225–231.

Pisha, E., Chai, H., Lee, I.S., Chagwedera, T.E., Farnsworth, N.R., Cordell, G.A., Beecher, C.W., Fong, H.H., Kinghorn, A.D., Brown, D.M., Wani, M.C., Wall, M.E., Hieken, T.J., Das Gupta, T.K., Pezzuto, J.M., 1995. Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. Nature Medicine, 1 (10), 1046–1051.

Pohleven, F., 2010. Judeževo uho ali bezgova goba, najstarejša gojena zdravilna goba. Les, 62 (2), 55.

Powell M., 2010. Medicinal mushrooms: A clinical guide. 1st ed. Mycology press, East Sussex. pp. 128.

Rogers, R., 2011. The Fungal pharmacy, The complete guide to medicinal mushrooms & lichens of north America, 1st ed. North Atlantic Books, Berkeley, 591pp.

Salahuddin, M.B.H.A.H., 2008. Biological Activities Of *Schizophyllum Commune* Fr., Thesis Submitted In Fulfillment Of The Requirements For The Degree Of Master Of Science, Faculty Of Science, University Of Malaya, Kuala Lumpur, 194 pp.

Schlegel, B., Luhmann, U., Haertl, A., Graefe, U., 2000. Piptamine, a new antibiotic produced by *Piptoporus betulinus* Lu 9-1. The Journal of Antibiotics, 53 (9), 973–974.

Seniuk, O.F., Gorovoj, L.F., Beketova, G.V., Savichuk, H.O., Rytik, P.G., Kucherov, I.I., Priluskay, A.B., Prilutsky A.I., 2011. Anti-infective properties of the melanin-glucan complex obtained from medicinal tinder bracket mushroom, *Fomes fomentarius* (L.: Fr.) Fr. (Aphyllophoromycetideae). Int. J. Med. Mushr. 13 (1), 7–18.

Shi, Y., James, A.E., Benzie, I.F.F., Buswell, J.A., 2002. Genoprotective effects of selected mushroom species, Mushroom Biology and Mushroom Products. Sánchez et al. (eds).

Shimbo, M., Kawagishi, H., Yokogoshi, H., 2005. Erinacine A increases catecholamine and nerve growth factor content in the central nervous system of rats. Nutrition Research, 25, 617–623.

Stamets, P., 2005. Mycelium running, 1st ed. Ten speed press, Berkeley, 339 pp.

Standish, L.J., Wenner, C.A., Sweet, E.S., Bridge, C., Nelson, A., Martzen, M., Novack, J., Torkelson, C., 2008. Trametes versicolor Mushroom Immune Therapy in Breast Cancer. J. Soc. Integr. Oncol., 6 (3), 122–128.

Suay, I., Arenal, F., Asinsio, F. J., Basilio, A., Cabello, M. A., Díez, M.T., García, J. B., González del Val, A., Gorrochtegui, J., Hernández, P., Peláez, F., and Vicente, M. F., 2000. Screening of basidiomycetes for antimicrobial activities. Antonie van Leeuwenhoek, 78, 129–139.

Thanasekaran, J., Aloysius, P.T., Mathivanan, I., Pitchairaj, G.J., 2010. An extract of the oyster mushroom, *Pleurotus ostreatus,* increases catalase gene expression and reduces protein oxidation during aging in rats. Chin. Int. Med., 8 (8), 774–780.

Tochikura, T.S., Nakashima, H., Hirose, K., Yamamoto, N., 1987. A biological response modifier, PSK, inhibits human immunodeficiency virus infection in vitro. Biochemical and Biophysical Research Communications, 148 (2), 726–733.

Tsukagoshi, S., Hashimoto, Y., Fujii, G., Kobayashi, H., Nomoto, K., Orita, K., 1984. Krestin (PSK). Cancer Treat. Rev., 11 (2), 131–155.

Tzianabos, A., 2000. Polysaccharide immunomodulators as therapeutic agents: Structural aspects and biologic function. Clin. Microbiol. Rev., 13, 523–533.

Vrhovec, B., 2010. Zdravilne gobe Slovenije in 100 okusnih gob, 1st ed. Narava, Kranj, 149 pp.

Wasser, P., 2005. Reishi or Ling Zhi (*Ganoderma lucidum*), Encyclopedia of Dietary Supplements. New York: Marcel Dekker, pp. 842.

Wasser, S.P., Sokolov, D., Reshetnikov, S.V., Timor-Tismenetsky, M., 2000. Dietary Supplements from Medicinal Mushrooms: Diversity of Types and Variety of Regulations. Int. J. Med. Mushr., 2, 1–19.

Webster, J., 1991. Schizophyllum in hay bales. Mycologist, 5 (3), 118.

Wu, W., Gao X., Cui, X., Qian, G., Chen W., 2000. Review on studies and applications of *Cordyceps militaris.* Acta Agric. Shanghai, 16, 99–104.

Xu, C.P., Liu, W.W., Liu, F.X., Chen, S.S., Liao, F.Q., Xu, Z., Jiang, L.G., Wang, C.A., Lu, X.H., 1985. A double-blind study of effectiveness of *Hericium erinaceus* pers therapy on chronic atrophic gastritis. A preliminary report. Chin. Med. J., 98 (6), 455–456.

Ying, J., 1987. Icons of medicinal fungi. Science Press, Beijing, 575 pp.

Yu, C.G., Xu, Z.M., Zhu, Q.K., 1999. Cytoprotective effects of *Hericium erinaceus* on gastric mucosa in rats. Chinese. J. Gastrent., 1999–2002.

Yue, Q.X., Song, X.Y., Ma, C., Feng, L.X., Guan, S.H., Wu, W.Y., Yang, M., Jiang, B.H., Liu, X., Cui, Y.J., Guo, D.A., 2010. Effects of triterpenes from *Ganoderma lucidum* on protein expression profile of HeLa cells. Phytomedicine: International journal of phytotherapy and phytopharmacology, 17 (8–9), 606–613.

Zeng, F., Zhao, C., Pang, J., Lin, Z., Huang, Y., Liu, B., 2013. Chemical properties of a polysaccharide purified from solid-state fermentation of auricularia auricular and its biological activity as a hypolipidemic agent. J. Food Sci., 78 (9), 1470–1475.

Zhou, Sh., Gao, Y., Chen, G., Dai, X., Ye, J., Gao, H., 2002. A phase I = II study of a *Ganoderma lucidum* (Curt.: Fr.) P. Karst. (Ling Zhi, reishi mushroom) extract in patients with chronic hepatitis B. Int. J. Med. Mushrooms, 4 (4), 321–328.

Zhou, X.X., Meyer, C.U., Schmidtke, P., Zeep, F., 2002. Effect of cordycepin on interleukin-10 production of human peripheral blood mononuclear cells. Eur. J. Pharmacol., 453, 309–317.

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Strigolaktoni – signalne molekule v arbuskularni mikorizi in regulatorji rasti in razvoja rastlin

Strigolactones – signal molecules in arbuscular mycorrhiza and regulators of plant growth and development

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Izvleček: Strigolaktoni so bili najprej odkriti kot rizosferne signalne molekule, s katerimi parazitske rastline prepoznavajo prisotnost svojih gostiteljev. Kasneje je bilo ugotovljeno, da imajo ključno vlogo pri nastanku arbuskularne mikorize (AM), ki je pomembna za mineralno prehrano več kot 80 % kopenskih rastlin. Šele nedavno pa so dognali, da strigolaktoni sodelujejo tudi pri regulaciji različnih rastno-razvojnih procesov v rastlini. Tako sodelujejo pri apikalni prevladi oz. kontroli stranskega obraščanja, razvoju korenin, nodulaciji in drugje. Članek predstavlja vlogo strigolaktonov pri razvoju AM, ter njihovo vlogo endogenih regulatorjev. Izpostavlja jih kot snovi, ki z regulacijo procesov udeleženih pri pridobivanju mineralnih hranil in razporejanju virov sodelujejo pri uravnavanju ravnotežja med koreninami in nadzemnim delom rastline.
Ključne besede: simbioza, razvoj, apikalna prevlada, transport avksina

Abstract: Strigolactones were first discovered as rhizosphere signals by which parasitic weeds detect the presence of a host plant species. It was later recognized that they play a critical role in facilitating the formation of arbuscular mycorrhiza (AM), symbiosis with fungi, crucial for the acquisition of plant nutrients in over 80% of land plant species. Recently, strigolactones have also been shown to participate in regulation of several plant developmental processes. They are involved in the control of apical dominance (shoot branching), root development, nodulation, etc.. The paper presents the role of strigolactones in development of AM and their implication in other physiological processes. It discusses a possible role of strigolactones as integrators of the root-to-shoot balance, nutrient acquisition, and resource allocation.

Keywords: symbiosis, development, apical dominance, auxin transport

Uvod

Rastline lahko zaradi pritrjenega načina življenja razpolagajo le z viri, do katerih lahko dostopajo s svojimi organi. Korenine rastlini omogočajo sprejem vode in mineralnih hranil iz tal. Ko je razpoložljivost teh virov omejena, obstajajo tri možnosti odziva: rast korenin v neizkoriščene predele tal, povečanje učinkovitosti koriščenja obstoječih virov s pomočjo lastnih kemičnih mehanizmov (npr. s koreninskim izločkom), ali pa simbioza z organizmi, ki imajo lažji dostop do virov v pomanjkanju. Pomembna oblika tovrstne simbioze je mikoriza – asociacija rastline in glive. Mikorizne glive z zunajkoreninskim micelijem izkoriščajo hranila iz dodatnega volumna tal, poleg tega pa izločajo ekstracelularne encime in druge spojine, ki mobilizirajo hranila iz za

rastlino nedostopnih oblik, npr. iz organske snovi. Te procese lahko gliva vrši v območjih, ki so tudi več metrov oddaljena od korenin rastline in z mineralnimi hranili, ki jih tam pridobi, oskrbuje rastlino. Tako si rastlina z mikorizo močno poveča razpoložljivost hranil.

Mikorizna simbioza se pojavlja v več tipičnih oblikah, ki so značilne za posamezne kombinacije simbiontov in se prevladujoče pojavljajo v različnih tipih ekosistemov. Za funkcionalno mikorizo se morajo razviti različne strukture. Posebej so pomembne tiste, ki omogočajo transportne procese in izmenjavo snovi med obema partnerjema (npr. arbuskuli pri arbuskularni mikorizi, Hartigova mreža pri ektomikorizi). Te in ostale strukture mikorize se razvijejo v rastno-razvojnih procesih, ki so pod kontrolo rastnih hormonov, sladkorjev ter mineralnih hranil (Gogala, 1991). Prvi koraki pri razvoju mikorize pa so povezani s prepoznavanjem kompatibilnih simbiontov. Pri iskanju kemičnega signala rastline, ki vzpodbuja rast hif arbuskularnih mikoriznih gliv h koreninam, so ugotovili, da v tem procesu sodelujejo strigolaktoni (Akiyama in sod., 2005). Zanimivo je, da so kmalu zatem prepoznali tudi regulatorni pomen teh molekul v nekaterih razvojnih procesih v sami rastlini (Gomez-Roldan in sod. 2008; Umehara in sod. 2008). To je strigolaktone v relativno kratkem času postavilo ob bok glavnim skupinam rastlinskih rastnih hormonov.

Članek obravnava biosintezo strigolaktonov, njihove funkcije pri vzpostavitvi arbuskularne mikorize in regulatorno vlogo, ki jo imajo v rastno-razvojnih procesih v rastlini.

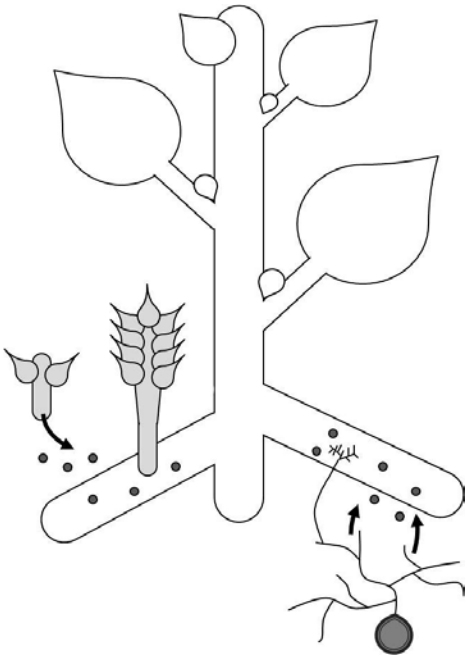
Regulatorna vloga strigolaktonov pri razvoju arbuskularne mikorize

Arbuskularna mikoriza (AM) je endotrofna oblika mikorize, pri kateri gliva, v celicah koreninske skorje rastline gostiteljice, oblikuje strukture imenovane arbuskuli, ki služijo izmenjavi snovi med simbiontskima partnerjema. Arbuskuli so povezani z zunaj-koreninskim prepletom hif glive. Po hifah se transportirajo snovi od micelija do arbuskulov in obratno.

Koreninski eksudat, ki ga izloča rastlina gostiteljica, vpliva na presnovo mikorizne glive. Zaradi vzbujene presnove se hifa glive začne podaljševati v dolžino in diferencira, ko doseže gostiteljsko rastlino. Na stiku oblikuje apresorij, strukturo, s katero se pritrja na korenino in služi penetraciji hife v celice koreninske skorje. Štiri do pet ur po nastanku apresorija se oblikuje predpenetracijski aparat, struktura, ki definira pot rasti hif skozi rastlinske celice. Sledi proces oblikovanja arbuskula (Parniske 2008).

Pred nastankom predpenetracijskega aparata potuje jedro rastlinske celice proti pričakovani točki vstopa glive. Jedro rastlinske celice se potem premika pred nastajajočim predpenetracijskim aparatom, kot da kontrolira njegovo pot nastajanja skozi celico. Mikrotubuli in mikrofilamenti skupaj z gostimi cisternami endoplazmatskega retikuluma oblikujejo votlo cev, ki poveže jedro z mestom apresorija. Šele potem lahko hifa vstopi v gostiteljsko celico. Kot rezultat koordiniranega razvoja se znotraj rastlinske celice razvije razvejana struktura hif imenovana arbuskul. Arbuskul je ločen od citoplazme gostiteljske celice. Hranila in signalne molekule se izmenjujejo preko površine, sestavljene iz periarbuskularne membrane, membrane hife in periarbuskularnega medmembranskega prostora (Parniske 2008).

Raziskovanja, povezana s strigolaktoni, so se začela, ko je bilo opaženo, da semena parazitskih rastlin rodov *Striga* in *Orobanche* ne kalijo, če niso izpostavljena koreninskemu eksudatu gostiteljske rastline. Koreninski eksudat pospešuje njihovo kalitev. Enak učinek je bilo opaziti tudi pri sporah AM gliv. Če so spore AM gliv izpostavili koreninskemu eksudatu, so kalile in se začele razraščati. Te ugotovitve so napeljevale na domnevo, da koreninski eksudat vsebuje določene signalne molekule, ki vplivajo na parazitske rastline in na AM glive, ter da AM glive in parazitske rastline reagirajo na enake signalne molekule eksudata. Dokler skupina teh molekul ni bila kemijsko identificirana, so bile molekule imenovane razvejitveni faktorji; hifa AM glive se je namreč ob stiku z rastlinskim eksudatom razvejila. Predpostavljali so, da gre za flavonoide, do leta 1995, ko so Bécard in sodelavci pri mutantih koruze (*Zea mays*) z manjkajočimi encimi za sintezo flavonoidov, dokazali normalno kolonizacijo z arbuskularnimi mikoriznimi glivami (Bécard in sod. 1995). Za tem je bilo testiranih več sekundarnih metabolitov eksudata, dokler niso leta 2005 Akiyama in sodelavci, izolirali in identificirali 5-deoksi-strigol kot razvejitveni faktor. Ta spojina pripada skupini molekul, imenovanih strigolaktoni (Slika 1).

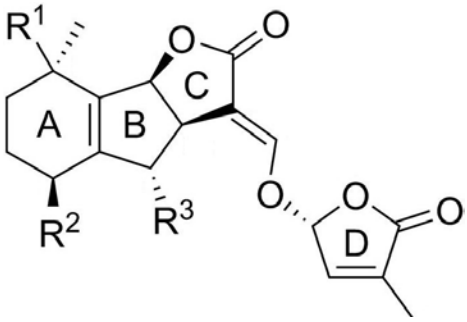


Slika 1: Strigolaktoni delujejo kot signalne molekule, s katerimi nekatere parazitske rastline prepoznavajo prisotnost gostiteljskih rastlin (levo) ter s pomočjo katerih poteka prepoznavanje partnerjev in regulacija razvoja pri arbuskularni mikorizi (desno).

Figure 1: Strigolactones act as signal molecules, by which parasitic plants detect the presence of the hosts (left) and which are involved in the recognition of arbuscular mycorrhizal symbionts and regulation of AM development (right).

Izolacijo 5-deoksi-strigola so izvedli iz metuljnice *Lotus japonicus*. Postopek je bil zahteven, ker gre za precej nestabilno spojino, zastopano v izredno majhni koncentraciji. Vedelo se je, da gre za nizkomolekularno spojino, saj je koreninski eksudat, filtriran skozi polprepustno membrano, še vedno učinkoval na AM glive. Analize topnosti so pokazale, da je molekula lipofilna. Znano je bilo tudi, da koreninski eksudat rastlin, ki rastejo v pomanjkanju fosforja, močneje učinkuje na AM glive, kot eksudat dobro prehranjenih rastlin, iz česar so predvidevali, da fosfor vpliva na biosintezo te spojine. Zato so rastline za namen izolacije gojili hidroponsko, v razmerah pomanjkanja fosfata. Lipofilne molekule, izločene v hranilno raztopino, so ekstrahirali z etil-acetatom in testirali učinkovitost ekstrakta, pri čemer so dobili pozitivne rezultate. Ekstrakt so ločili na kisle, bazične in nevtralne frakcije. Učinkovitost se je pokazala le za nevtralno frakcijo. Iz hidroponske raztopine so s pomočjo aktivnega oglja ekstrahirali nevtralne lipofilne molekule, na aktivno oglje vezane substance raztopili v acetonu in opravili kromatografsko ločbo. Pri tem so sproti preizkušali aktivnost posameznih frakcij. Kot aktivno molekulo so določili 5-deoksi-strigol, strigolakton, katerega osnovna struktura je prikazana na sliki 2 (Akiyama in sod. 2005).

Do sedaj je bilo odkritih več molekul strigolaktonov in znano je, da so te spojine široko razširjene po rastlinskem kraljestvu. Nadaljnje raziskave so potrdile, da so strigolaktoni neobhodni pri vzpostavitvi arbuskularne mikorize.



Slika 2: Strukturna (stereokemijska) formula osnovne molekule strigolaktona z označenim ABC tricikličnim obročem in nanj, z enol-etersko vezjo, vezanim, metil-butenolidnim, laktonskim D obročem (Akiyama in sod., 2005).

Figure 2: Structure (stereochemical formula) of a basic strigolactone molecule. Methylbutenolide, lactone D ring is coupled to tricyclic ABC ring *via* the enol ether double bond (Akiyama et al., 2005).

Biosinteza strigolaktonov

Kmalu po odkritju strigolaktonov so Matusova in sodelavci (2005) ugotovili, da triciklični ABC obroč strigolaktonov nastane s cepitvijo C_{40} karotenoidov, ki so po svojem izvoru tetraterpeni. Za sintezo terpenov v rastlini sta znani dve presnovni poti. Presnovna pot mevalonske kisline v citosolu in metileritrol-fofatna (MEP) presnovna

pot v plastidih. Matusova in sodelavci (2005) so ugotovili, da strigolaktoni najverjetneje izhajajo iz metileritrol-fosfatne presnovne poti.

Vhodni molekuli za metileritrol-fosfatno presnovno pot sta piruvat in gliceraldehid-3-fosfat. Ob njuni kondenzaciji nastane C_5 spojina, 1-deoksi-D-ksiluloze-5-fosfat. Reakcijo katalizira encim 1-deoksi-D-ksiluloze-5-fosfatna sintaza (DXS). Sledi izomerizacija metilne skupine in hidrogeniranje dvojne vezi med ogljikom in kisikom, kar katalizira encim 1-deoksi-D-ksiluloze-5-fosfatna reduktoizomeraza (DXR). Reakcija vodi do nastanka metileritrol-fosfata (MEP). Iz MEP nastane izopentenil-difosfat (IPP) in njegova izomera dimetilalil-difosfat (DMAPP), ki sta fosforilirana izoprena. Sledi polimerizacija izoprena do C_{40} karotenoidnega prekurzorja, fitoena (Taiz in Zeiger, 2006). Nastanek nenasičenih vezi v verigi fitoena katalizirata encima fitoen-desaturaza (PDS) in ζ-karoten-desaturaza (ZDS). Nastanejo fitofluen, ζ-karoten in končno likopen, s konjugirano dvojno vezjo, ki absorbira svetlobo. S ciklizacijo končnih delov verige likopena nastane α-karoten in njegova izomera β-karoten, ki je prekurzor za sintezo z mikorizo povezanih karotenoidov; strigolaktonov, mikoradicina in cikloheksenonskih derivatov. S hidroksilacijo cikloheksenskih obročev nastane iz α-karotena lutein, iz β-karotena pa zeaksantin (Strack in Fester 2006; Anh-Tuan in sod. 2011).

Katalizo C_{40} karotenoidnega prekurzorja vrši encim karotenoid-cepitvena-dioksigenaza (CCD). Do sedaj še ni jasno, kateri karotenoid je substrat za encim CCD. Delovanje CCD je specifično, na vez med C9-10 (C9'-10') in vez med C11-12 (C11'-12'). S cepitvijo, metilacijo ter drugimi strukturnimi spremembami nastane iz molekule karotenoida C_{15} prekurzor strigolaktonov, C_{14} dialdehid iz katerega nastane mikoradicin in C_{13} keton, cikloheksenon (Akiyama 2007). Strigolaktone rastline sintetizirajo v sledeh, mikoradicin in cikloheksenonske derivate pa, v primeru mikorize, v velikih količinah (Strack in Fester 2006).

Na koreninah mnogih rastlin je, zaradi kolonizacije z arbuskularnimi mikoriznimi glivami, opazno rumeno obarvanje. Za obarvanje je odgovoren do sedaj še neidentificiran rumen pigment, katerega kromofor je mikoradicin. Rumen pigment se v obliki hidrofobnih kapljic akumulira v vakuolah kortikalnih celic korenine, v fazi degradacije arbuskula (Akiyama 2007). Njegova vloga ni znana, je pa očitno rezultat cepitve karotenoidnega prekurzorja.

Tudi akumulacija cikloheksenonskih derivatov je močno pogojena z mikorizacijo, saj na porast ne vpliva niti biotski stres, kot je infekcija s patogeni ali endofiti, niti abiotski stres kot so vročina, mraz, velika intenziteta svetlobe, težke kovine in suša (Maier 1997). Glikoziliran cikloheksenon, blumenol C, je aglikon blumenina. Blumenin je v sledeh prisoten tudi v nemikoriziranih koreninah, vendar njegov delež znatno naraste v mikoriziranih koreninah. Blumenin inhibitorno vpliva na zgodnje faze razvoja arbuskula, iz tega sledi, da bi cikloheksenonski derivati lahko bili endogeni negativni regulatorji kolonizacije, ki služijo koordiniranemu razvoju simbioze (Akiyama 2007).

Cikloheksenski obroč β-karotena ostane po cepitvi intakten in predstavlja A obroč strigolaktona. Matusova in sodelavci (2005) so predvideli, da C_{15} aldehidnemu intermediatu sledi hidroksilacija, hidrogeniranje, oksidacija, epoksidacija, oksidativna dekarboksilacija in ciklizacija, s čimer nastane C_{14}, ABC-triciklični obroč. Za D obroč se predpostavlja, da je produkt druge presnovne poti. Z vezavo na ABC triciklični obroč nastane 5-deoksi-strigol. Ta spojina je prvi produkt biosinteze, ki učinkuje kot strigolakton (Akiyama 2007).

Posamezna rastlina verjetno vsebuje najmanj dva do pet različnih strigolaktonov (Rochange 2010). Vsi do sedaj znani strigolaktoni so sestavljeni iz tricikličnega ABC obroča. C obroč izkazuje funkcije laktona. Z enol-etersko vezjo se povezuje z metil-butenolidnim, laktonskim D obročem. 5-deoksi-strigol je verjetno najenostavnejši strigolakton in predpostavlja se, da je prekurzor za biosintezo drugih, kompleksnejših molekul strigolaktonov, saj se pojavlja v eksudatu mnogih rastlin, tako enokaličnic (Awad in sod. 2006), kot dvokaličnic (Yoneyama in sod. 2008). 5-deoksi-strigol je bil dokazan tudi v mahu vrste *Physcomitrella patens* (Proust in sod. 2011).

Vloga strigolaktonov in karotenoidnih derivatov pri nastanku arbuskularne mikorize

Strigolaktoni vplivajo na kalitev spor arbuskularnih mikoriznih gliv, poleg tega so sprožilci zaporedja molekularnih in celičnih dogodkov, ki

so potrebni, da hifa postane fiziološko infektivna (Akiyama 2007). Sprožijo ekspresijo mitohondrijskih genov in posledično vplivajo na povečano respiratorno aktivnost (Tamasloukht 2003). Aktivacija mitohondrijev povzroči oksidacijo lipidov, ki so glavni vir ogljika spor mikoriznih gliv. To omogoči pospešeno rast in podaljševanje hife do korenin gostiteljske rastline (Besserer in sod. 2006). V tleh je ta kemotaktični odziv pogojen z razdaljo, kar je možno povezati s podvrženostjo strigolaktonov razgradnji. V vodi enol-esterska vez med C in D obročem, ki predstavljata aktivno mesto molekule, hidrolizira. To pojasni upad sicer močnega vpliva strigolaktonov na arbuskularne mikorizne glive ob prisotnosti vode (Akiyama 2007). V tleh ta lastnost strigolaktonov povzroči, da se koncentracija z oddaljevanjem od korenine hitro zmanjšuje, zato je učinek na AM glive prisoten le v neposredni bližini gostiteljske rastline (Parniske 2008).

Učinek strigolaktonov na hife AM gliv so testirali s pomočjo fluorescenčnega označevanja mitohondrijev hif kalečih spor. Besserer in sodelavci (2006) so opazili povečano gostoto mitohondrijev na enoto površine pri hifah AM glive *Gigaspora rosea* tretiranih z analogom strigolaktona GR24, glede na kontrolo. Mitohondrije so fluorescenčno označili na tri različne načine in ugotavljali gostoto po eni in po petih urah. V vseh primerih je bila gostota mitohondrijev tretiranih hif večja. Učinek strigolaktonov na AM glive so potrdili tudi s štetjem razvejitev in merjenjem dolžine hif. Besserer in sodelavci (2006) so hife AM glive *Gigaspora rosea* tretirali z analogom strigolaktona GR7. Razvejanje in rast tretiranih hif je bila ves čas trajanja poskusa močnejša kot pri kontroli. Ker so omenjene raziskave potekale le na eni vrsti AM gliv v *in vitro* razmerah, je potrebno preverjanje teh učinkov tudi na drugih vrstah gliv.

Pomen karotenoidne presnovne poti, iz katere izhajajo strigolaktoni, pri vzpostavljanju arbuskularne mikorize so dokazali s proučevanjem genov za encime, ki sodelujejo pri pretvorbi nekaterih intermediatov. Raziskave genov, ki kodirajo encim 1-deoksi-D-ksiluloze-5-fosfatna sintaza (DXS), mikoriziranih korenin trnate meteljke (*Medicago truncatula*) so pokazale, da obstajata dva izogena DXS; DXS1 in DXS2. DXS1 se izraža na konstitutivnem nivoju po celi rastlini, še posebej v fotosintezno aktivem tkivu. DXS1 izoforma encima je vključena v presnovne poti primarne presnove. Ekspresija izogena DXS2 je inducirana z mikorizacijo in sicer le v koreninskem sistemu rastline, izoforma encima DXS2 pa je vključena v presnovne poti sekundarne presnove (Strack in Fester 2006). Utišanje mikorizno specifičnega izogena DXS2 s pomočjo RNAi je imelo za posledico drastičen upad prisotnosti cikloheksenonskih derivatov in mikorizno specifičnih fosfatnih transporterjev. Iz tega sledi, da imajo karotenoidni derivati pomembno vlogo pri uspešni vzpostavitvi arbuskularne mikorize in nadaljnjem razvoju arbuskulov (Akiyama 2007). Povečana transkripcijska aktivnost gena za encim fitoen-desaturazo (PDS) med vzpostavitvijo arbuskularne mikorize in povečana količina fitoena v mikoriziranih koreninah, tretiranih z inhibitorjem encima PDS, nakazuje, da je aktivacija biosinteze karotenoidov v mikoriziranih koreninah splošen pojav (Strack in Fester 2006). Pri mutantih koruze (*Zea mays*), 'pale yelow 9' (y9), z manjkajočimi geni za sintezo encimov karotenoidnih izomeraz, ki katalizirajo konverzijo ζ-karotena v likopen, je bilo opaziti občuten upad kolonizacije korenin z arbuskularnimi mikoriznimi glivami in eksudacije strigolaktonov. V koreninah ni bilo zaznati mikoradicina. Prisotni arbuskuli sicer niso izkazovali očitnih anomalij, vendar se je njihovo število močno zmanjšalo.

Metileritrol-fosfatna presnovna pot ter njeno nadaljevanje, karotenoidna presnovna pot, potekata v plastidih. Plastidi gostiteljskih celic rastline imajo pomembno vlogo pri oblikovanju arbuskula. Spremembe v presnovi plastidov, ki jih inducira mikorizacija, omogočijo rast in razmnoževanje plastidov. Njihovo število se poveča. Med seboj se povežejo s posebnimi strukturami, imenovanimi stromuli. Stromuli so s stromo plastida napolnjeni tubularni podaljški plastidov in služijo komunikaciji med plastidi. Na ta način tvorijo plastidi obsežen preplet, ki obda predpenetracijski aparat in kontrolira vstop hife in oblikovanje arbuskula (Strack in Fester 2006). V plastidih okoli arbuskula se poveča količina encima DXR, ki je udeležen v metileritrol-fosfatni presnovni poti. Posledično se poveča količina karotenoidnih derivatov. Količina DXR je posebno velika v plastidih okrog senescentnega arbuskula. Poleg biosinteze karotenoidov se v celici poveča

biosinteza maščobnih kislin in aminokislin. To je pomembno, predvsem v zgodnji fazi razvoja arbuskula, ko rastlina oblikuje periarbuskularno membrano okoli rastočega arbuskula, saj maščobne kisline sodelujejo pri tvorbi lipidnega dvosloja, aminokisline pa gradijo membranske proteine. V fazi razvoja arbuskula so plastidi majhni, lečaste oblike, v fazi degradacije arbuskula pa izdolženi, nepravilnih oblik (Strack in Fester 2006).

Razmislek o možnem poteku dogodkov povezanih z vzpostavitvijo arbuskularne mikorize je sledeč. Rastlina proizvaja strigolaktone, katerih sinteza je, kot so pokazale nedavne transkriptomske raziskave, stimulirana ob pomanjkanju mineralnih hranil (Bonneau in sod. 2013). Izloča jih v rizosfero, kjer vplivajo na energetsko presnovo spor gliv na način, da pospešijo oksidacijo njihovih založnih lipidov. To omogoči rast hif in njihovo razvejanje, s čimer gliva najde gostiteljico. Na stiku korenine in hife se oblikuje apresorij, ki služi penetraciji hife do celic gostiteljice. Rastlina odreagira na fizičen dražljaj. Jedra celic, na katere pritiska hifa s svojo rastjo, se pomaknejo v smer proti dražljaju. Odreagirajo tudi drugi organeli, posledično nastane predpenetracijski aparat. Za tem hifa vstopi v celico. Obdajo jo plastidi, v katerih se aktivirajo geni metileritrol-fosfatne presnovne poti. Oblikujejo se stromuli, s katerimi se plastidi povežejo med sabo. Tako nastala struktura kontrolira razrast hife. V plastidih se začne intenzivna biosinteza karotenoidnih derivatov; strigolaktonov v sledeh, močno pa poraste količina cikloheksenonskih derivatov – skupni prekurzor je C_{13} molekula, ki nastane s cepitvijo C_{40} karotenoida. Rastlina verjetno lahko aktivno regulira količinsko vsebnost enega in drugega produkta, ki pa imata na glivo nasprotujoč si učinek; strigolaktoni stimulativni, cikloheksenonski derivati pa inhibitorni.

Cikloheksenonski derivati in njihovi nadaljnji produkti zavirajo razrast arbuskula, s čimer verjetno lahko preprečijo, da bi hifa zavzela preobsežen del celice, sočasna povečana biosinteza maščobnih kislin in aminokislin pa omogoča nastanek periarbuskularne membrane, s katero rastlina omeji prostor glivi in izoblikuje površino preko katere poteka izmenjava snovi. Plastidi potem kontrolirajo tudi nadaljnje procese sukcesije arbuskula.

Vloga strigolaktonov pri uravnavanju rasti rastline

Spoznanje, da nekatere rastline, kot sta navadni repnjakovec (*Arabidopsis thaliana*) in beli volčji bob (*Lupinus albus*), proizvajajo in izločajo strigolaktone, kljub temu, da ne tvorijo arbuskularne mikorize, je postavljalo vprašanje, zakaj bi rastlina sintetizirala spojine, ki spodbujajo rast parazitskih rastlin in od katerih nima nobene potencialne koristi. Ali to pomeni, da strigolaktoni sodelujejo tudi pri procesih, ki se dogajajo v rastlini? Raziskave so pokazale, da so strigolaktoni udeleženi pri hormonalni regulaciji aktivacije in rasti stranskih poganjkov (Gomez-Roldan in sod. 2008; Umehara in sod. 2008; Xie in sod., 2010), ki je, kot kaže, mnogo bolj kompleksna, kot je bilo znano do sedaj. Poleg tega so kasneje ugotovili, da so strigolaktoni vpleteni tudi v regulacijo rasti korenin, elongacije koreninskih laskov, tvorbe adventivnih korenin, sekundarne rasti, fotomorfogeneze, kalitve in nodulacije (Foo in Reid 2013).

Mehanizem kontrole aktivacije stranskih brstov je že dolgo znan kot apikalna prevlada. Glavni poganjek proizvaja hormon avksin, ki potuje s polarnim transportom po steblu navzdol, in preprečuje rast stranskih poganjkov. Avksin potuje striktno bazipetalno in ne vstopa v stranske brste, kar pomeni, da je njegova vloga pri regulaciji aktivnosti brstov posredna. To dejstvo je odprlo področje intenzivnih raziskav. Večletne fiziološke, genetske in biokemijske študije so privedle do oblikovanja hipoteze glede aktivnosti stranskih brstov. Hipoteza temelji na predpostavki, da obstaja molekula, ki vpliva na koncentracijo avksina, ki potuje s polarnim transportom po steblu in preprečuje stranskemu brstu, da bi svoj lasten tok avksina priključil polarnemu transportu, zaradi česar je rast stranskega brsta zavrta. Hipotezi v prid govori odkritje hormona, ki ustreza delovanju omenjene molekule. Ta hormon je strigolakton (Domagalska in Leyser 2011).

Za aktivacijo brsta je nujna vzpostavitev odvodnega sistema za avksin iz brsta, kar je regulirano z intenziteto toka polarnega transporta avksina v steblu. Polarni transport avksina, po parenhimu ksilema, nadzirajo PIN proteini (Petrášek in sod., 2006). Povečan tok avksina je bilo opaziti pri 'more axillary growth' (MAX) mutantih navadnega

repnjakovca (*Arabidopsis thaliana*), z okvarjenimi geni za sintezo strigolaktonov, kar je povzročilo močnejše stransko obraščanje in s tem večjo koncentracijo avksina v glavnem steblu. Pri teh mutantih je bilo opaziti tudi povečanje količine PIN proteinov (Bennett in sod., 2006). Ta izid je bil glede na prejšnji koncept apikalne prevlade nepričakovan in nakazuje na vlogo strigolaktonov pri uravnavanju stranskega obraščanja, saj zaradi povečane koncentracije avksina sicer ne bi smelo priti do stranskega obraščanja.

Strigolaktoni se sintetizirajo v koreninah in v poganjkih in potujejo akropetalno, najverjetneje po parenhimu ksilema. Vlogo strigolaktonov pri stranskem obraščanju so poskušale razjasniti številne raziskave, na podlagi poskusov s cepljenjem. Uporabljalo se je MAX mutante različnih rastlin. V osnovi sta dva tipa MAX mutantov. MAX2 mutanti lahko proizvajajo strigolaktone, vendar so okvarjeni v signalni poti, odvisni od strigolaktonov. MAX1, MAX3 in MAX4 mutanti pa imajo okvarjene gene za sintezo strigolaktonov, zato strigolaktonov ne proizvajajo. Natančneje, gre za okvaro genov za encim karotenoid-cepitvena-dioksigenaza (CCD), ki vrši katalizo C_{40} karotenoidnega prekurzorja, posledično izostane sinteza strigolaktonov. Rastline, cepljene na korenine divjega tipa ali pa MAX2 mutanta, ki niso imele okvarjenih genov za signalizacijo s strigolaktoni, se niso obraščale, ker se je strigolakton transportiral iz korenin v nadzemni del in preprečeval obraščanje. MAX2 mutanti so se obrasli v vsakem primeru, ker kljub prisotnosti strigolaktonov v koreninah, le-teh niso mogli transportirati v nadzemni del. MAX1, MAX3 in MAX4 mutanti pa so se obrasli v primeru, ko so bili cepljeni na korenine, ki niso proizvajale strigolaktonov. Rastline divjega tipa cepljene na podlago, ki ni proizvajala strigolaktonov se niso obrasle, kar lahko pomeni, da se strigolaktoni proizvajajo tudi v nadzemnem delu rastline (Domagalska in Leyser 2011).

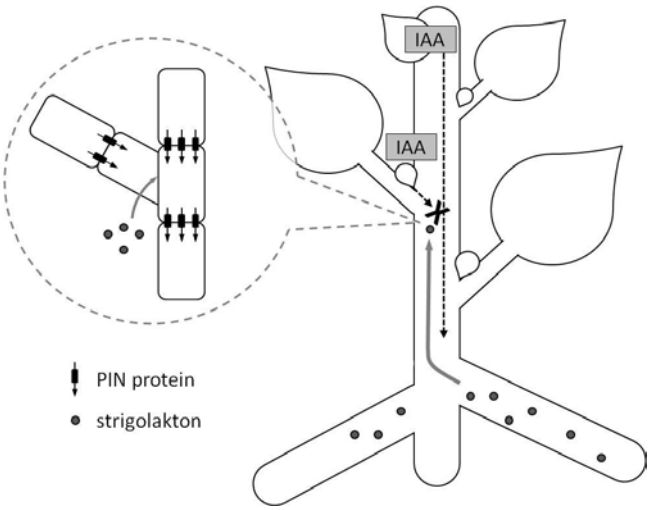
Za razumevanje mehanizma delovanja strigolaktonov pri stranskem obraščanju je bilo potrebno natančneje opredeliti mehanizem delovanja avksina. Ta mehanizem so poskušali pojasniti z modelom usmerjenega transporta avksina. Model opisuje proces, pri katerem se začetni tok avksina od vira k ponoru postopno omeji na tisto zaporedje celic, katerih avksinski transporterji so visoko aktivni. Na tak način se ustvari neprekinjen tok avksina od brsta do stebla. To pomeni, da brst, ko se aktivira, začne proizvajati avksin, ki se poskuša odvesti v steblo na način, da se poveže s polarnim transportom avksina po steblu. Proces temelji na pozitivni povratni zanki, avksin namreč usmerja in nadzoruje lasten transport v smeri toka k ponoru. Posledično nastane sloj celic z relativno visoko koncentracijo avksina, ki se odvaja od vira k ponoru. Celice se kasneje diferencirajo v sistem žile (Domagalska in Leyser 2011).

Molekularne raziskave so z odkritjem PIN proteinov potrdile pozitivno povratno zanko avksina. Avksin inducira izražanje genov za PIN proteine in preprečuje odstranjevanje že vgrajenih proteinov iz membrane celic. Model usmerjenega transporta avksina ustreza konceptu apikalne prevlade. Avksin, ki se nahaja v epidermalnem sloju brsta, se mora odvesti iz brsta. Ta faza je sestavni del iniciacije lista in njegove ekspanzije. Zato je nujna vzpostavitev polarnega transporta avksina od nastajajočega lista preko nastajajočih prevajalnih elementov brsta do glavnega stebla rastline. Vzpostavitev tega toka je mogoča le, če je koncentracija avksina v epidermalnem sloju brsta dovolj visoka, da inducira prepisovanje genov za PIN proteine, saj morajo biti ti prisotni v membrani, da avksin lahko potuje od ene celice do druge. Če je koncentracija avksina prenizka, brst ostane dormanten. Koncentracija avksina v steblu naj bi določala kapaciteto ponora in s tem možnost vzpostavitve polarnega transporta avksina iz brsta v steblo. Višja koncentracija avksina kot je v steblu, manj ima stranski brst možnosti za aktivacijo. Stranski brst, ki se aktivira, z vzpostavitvijo toka avksina v steblo, viša koncentracijo avksina v steblu in s tem preprečuje drugim stranskim brstom, da bi se aktivirali. Če je glavni brst odstranjen, se koncentracija avksina v steblu zniža, kar omogoči stranskim brstom, da se aktivirajo (Domagalska in Leyser 2011).

Do tu pa še ni jasno, kaj omogoča stransko obraščanje MAX mutantov, glede na to, da prihaja do stranskega obraščanja kljub visoki koncentraciji avksina v glavnem steblu rastline. Umehara in sodelavci (2008) ter Gomez-Roldan in sodelavci (2008) so pokazali pomen strigolaktonov kot endogenih hormonov, ki preprečujejo stransko obraščanje. Ugotovili so, da strigolaktoni zmanjšujejo akumulacijo PIN proteinov v membrani. Na ta način onemogočajo vzpostavitev polarnega

transporta avksina iz brstov in povečujejo kompeticijo med brsti (Prusinkiewicz in sod., 2009; Crawford in sod., 2010). Na spodnji del stebla, z dvema stranskima brstoma, nanešen strigolakton povzroči prevlado enega brsta nad drugim, kar pomeni, da strigolakton ne preprečuje aktivacije obeh brstov, ampak omogoča kompeticijo med njima na način, da se eden od njiju aktivira, drugi pa ostane dormanten. V primeru, da je na steblu brez glavnega poganjka prisoten le en stranski brst, aplikacija strigolaktona na bazalni del stebla ne prepreči aktivacije tega brsta. Če pa se na bazalni del stebla brez glavnega brsta, z dvema stranskima brstoma, nanese strigolakton, na mesto glavnega brsta pa avksin, je inhibitorni učinek avksina ojačan z inhibitornim učinkom strigolaktona, zato stranska brsta ne odženeta (Domagalska in Leyser 2011).

Strigolaktoni torej dopolnjuje regulacijo stranskega obraščanja z avksinom. Kadar je koncentracija strigolaktona v rastlini nizka in koncentracija avksina to dopušča, odžene več stranskih brstov, ki povišajo koncentracijo avksina z vzpostavitvijo lastnega polarnega toka avksina. Ko pa je koncentracija strigolaktonov v rastlini visoka in avksini dopustijo aktivacijo stranskih poganjkov, bo odgnal le brst, ki bo prvi vzpostavil lasten polaren tok avksina, dodatna koncentracija avksina v steblu ob sočasnem delovanju strigolaktonov, pa bo preprečila aktivacijo ostalih brstov (Slika 3).



Slika 3: Strigolaktoni sodelujejo pri kontroli stranskega obraščanja oz. pri apikalni prevladi (za razlago glej besedilo!).
Figure 3: Strigolactones participate in the regulation of lateral growth and apical dominance (see text for explanation!).

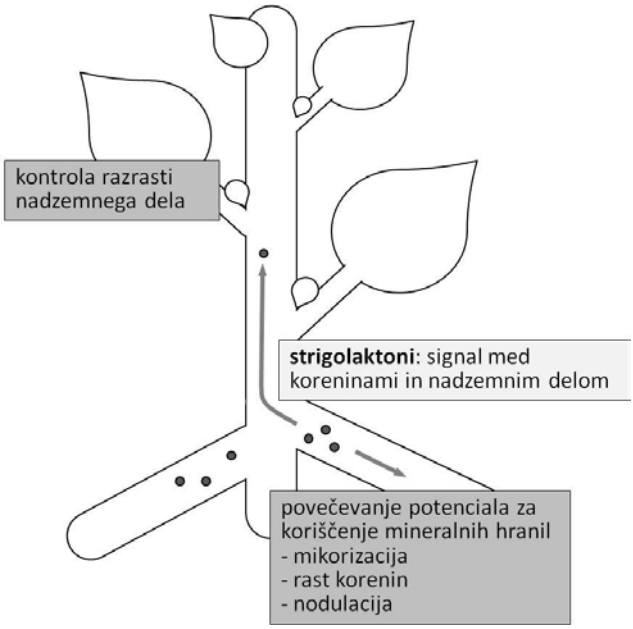
Sklepi

Strigolaktoni vplivajo na vzpostavitev mikorize. Vplivajo tudi na razrast poganjkov. Ista skupina molekul kontrolira dva procesa, ki močno vplivata na razvoj rastline ter njeno sposobnost kljubovanja razmeram v okolju in ki sta na prvi pogled popolnoma neodvisna drug od drugega. Kakšna je torej možna povezava med tema procesoma? Znano je, da pomanjkanje hranil v tleh vpliva na razmerje med nadzemnim delom rastline in koreninami. Zato bi lahko bila razlaga »dvojnega delovanja« strigolaktonov naslednja: pomanjkanje fosforja v tleh povzroči povečano tvorbo strigolaktonov v rastlini. Strigolaktoni potujejo navzgor po rastlini in omejujejo razraščanje. Zaradi povečane koncentracije v koreninah, začnejo strigolaktoni prehajati v rizosfero s koreninskim eksudatom. V rizosferi prisotne arbuskularne mikorizne glive se na strigolaktone odzovejo s povečano presnovno aktivnostjo in posledično pospešeno rastjo. Rastlina pridobi simbiontskega partnerja, ki ji priskrbi nove zaloge fosfata. Zaradi povečane količine fosforja se zmanjša sinteza strigolaktonov. Njihova koncentracija v koreninah upade, posledično se zmanjša transport v nadzemni del. Zmanjšana represija PIN proteinov zaradi manjše koncentracije strigolaktonov omogoči stranskim brstom vzpostavitev lastnega polarnega toka avksina in njihovo aktivacijo.

Ob tem pa se postavlja še eno vprašanje. Strigolaktoni preprečujejo vgradnjo PIN proteinov v membrano in s tem zavirajo tok avksina iz brsta ter preprečujejo njegovo aktivacijo. Ali to pomeni, da lahko z oviranjem toka avksina iz apikalnega meristema v glavnem brstu, upočasnjujejo njegovo rast? To bi pomenilo, da so strigolaktoni obveščevalne molekule, ki jih podzemni

del pošilja nadzemnemu, da ga obvešča o stanju fosforja v tleh ter posledično o možnosti izgrajevanja novih celičnih struktur in energijskih molekul, torej o možnosti nadaljnje rasti (Slika 4, glej tudi Foo in Reid, 2013).

Strigolaktoni so le eden od mnogih primerov kompleksnosti procesov živega sveta. Po skoraj stoletje veljavnem prepričanju, da razrast poganjkov uravnava izključno avksin, je danes jasno, da proces še zdaleč ni tako preprost, in da je morda povezan tudi z drugimi, nadceličnimi nivoji regulacije, kot so mikoriza in tudi ostale ekološke funkcije ekosistema. Regulacija mikorize in rastno-razvojnih procesov rastline s pomočjo strigolaktonov obstaja že milijone let in vprašanje je, kakšne interakcije v tej zvezi so se še razvile v milijonih let zemeljske evolucije.



Slika 4: Strigolaktoni uravnavajo rast nadzemnega dela in korenin glede na razpoložljivost mineralnih hranil.
Figure 4: Strigolactones regulate the growth of shoots and roots with respects to avaliablity of mineral nutrients.

Summary

Strigolactones are metabolites ubiquitously present in higher plants. They derive from a carotenoid-based pathway. Their basic structural unit is a tricyclic lactone that is connected to a butyrolactone by an enol ester bridge.

Strigolactones have been first identified as germination stimulants for root parasitic weeds, such as witchweeds (*Striga* spp.) and broomrapes (*Orobanche* spp.). The ability of non-host plants to germinate parasitic plant seeds suggested strigolactones may exist and may have role(s) that are independent of host-parasite interactions. The first evidence for this came from the studies of mycorrhiza, symbiosis between plants and fungi. During initial stages of formation of arbuscular mycorrhiza (AM) – a type of mycorrhiza which can be found in > 80% of land plants – strigolactone, 5-deoxy-strigol, is required as a branching factor to help arbuscular mycorrhizal fungi to interact with plant roots (Akyama et al., 2005). It was also found that at later stages of infection there are other compounds of the carotenoid-based pathway involved in the control development of arbuscules, intraradical structures of AM.

Further studies were motivated since it was known that strigolactones are produced also by non-mycorrhizal plants. These studies revealed that strigolactones participate in regulation of several plant developmental processes. In less than five years, roles for strigolactones have been defined in shoot branching, secondary growth, root growth and nodulation. Great progress has been made in understanding the mechanisms of shoot branching control, where it has been shown that enodgeneous strigolactones suppress development of axilary buds. It is known that these buds must export auxin to be activated, and they compete for the common auxin transport pathway through the main stem to the root. Strigolactones act systemically to dampen polar auxin transport stream form the buds to the stem reducing the accumulation of auxin transporters (PIN1) on cell membranes. This enhances competition between buds for the common auxin sink in the stem (Domagalska and Leyser, 2011).

Strigolactones are fascinating as signaling molecules as they can act both inside the plant as an endogenous hormone and in the soil as a rhizosphere signal (Foo et al. 2011). Being involved in regulation of the processes related to the aquisition of mineral nutrients and in the control of the growth of above ground plant parts, they could be regarded as integrators of the root-to-shoot balance and resource allocation.

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Viri

Akiyama, K., 2007. Chemical identification and functional analysis of apocarotenoids involved in the development of arbuscular mycorrhizal symbiosis. Bioscience, Biotechnology and Biochemistry, 71, 1405–1414.

Akiyama, K., Matsuzaki, K., Hayashi, H., 2005. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature, 435, 824–827.

Anh-Tuan, P., Kwang-Kim, J., Hoon-Kim, H., Young-Lee, S., Il-Park, N., Un-Park, S., 2011. Carotenoid accumulation and characterization of cDNAs encoding phytoene synthase and phytoene desaturase in garlic (*Allium sativum*). Journal of Agriculture and Food Chemistry, 59, 5412–5417.

Awad, A. A., Sato, D., Kusumoto, D., Kamioka, H., Takeuchi, Y., Yoneyama, K. 2006. Characterization of strigolactones, germination stimulants for the root parasitic plants *Striga* and *Orobanche*, produced by maize, millet and sorghum. Plant Growth Regulation, 48, 221–227.

Bécard, G., Taylor, L., Douds, D., Pfeffer, P., Doner, L. 1995. Flavovoids are not necessary plant signal compounds in arbuscular mycorrhizal symbioses. Molecular Plant-Microbe Interactions, 8, 252–258.

Bennett, T., Sieberer, T., Willett, B., Booker, J., Luschnig, C., Leyser, O. 2006. The *Arabidopsis* MAX pathway controls shoot branching by regulating auxin transport. Current Biology, 16, 553–563.

Besserer, A., Puech-Pagés, V., Kiefer, P., Gomez-Roldan, V., Jauneau, A. in sod. 2006. Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. PLoS Biology, 4, 1239–1247.

Bonneau, L., Huguet, S., Wipf, D., Pauly, N., Truong, H. N. 2013. Combined phosphate and nitrogen limitation generates a nutrient stress transcriptome favorable for arbuscular mycorrhizal symbiosis in *Medicago truncatula*. New Phytologist, 199, 188–202.

Crawford, S., Shinohara, N., Sieberer, T., Williamson, L., George, G., Hepworth, J., Müller, D., Domagalska, M. A., Leyser, O. 2010. Strigolactones enhance competition between shoot branches by dampening auxin transport. Developement, 137, 2905–2913.

Domagalska, M. A., Leyser, O., 2011. Signal integration in the control of shoot branching. Nature reviews; molecular cell biology, 12, 211–221.

Foo, E., Reid, J. B., 2013. Strigolactones: New Physiological Roles for an Ancient Signal. Journal of Plant Growth Regulation, 32, 429–442.

Gogala, N., 1991. Regulation of mycorrhizal infection by hormonal factors produced by hosts and fungi. Experientia, 47, 331–340.

Gomez-Roldan, V., Fermas, S., Brewer, P. B., Puech-Pagés, V., Dun, E. A. in sod. 2008. Strigolactone inhibition of shoot branching. Nature, 455, 189–195.

Maier, W., Hammer, K., Dammann, U., Schulz, B., Strack, D., 1996. Accumulation of sesquiterpenoid cycloheksenone derivatives induced by an arbuscular mycorrhizal fungus in members of the *Poaceae*. Planta, 202, 36–42.

Matusova, R., Rani, K., Verstappen, F., Franssen, M., Beale, M., Bouwmeester, H., 2005. The strigolactone germination stimulants of the plant-parasitic *Striga* and *Orobanche* spp. are derived from the carotenoid pathway. Plant Physiology, 139, 920–934.

Nordström, A., Tarkowski, P., Tarkowska, D., Norbaek, R., Astot, C., Dolezal, K., Sandberg, G., 2004. Auxin regulation of cytokinin biosynthesis in *Arabidopsis thaliana*: A factor of potential importance for auxin-cytokinin-regulated development. PNAS, 101, 8039–8044.

Parniske, M., 2008. Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nature reviews; microbiology, 6, 763–775.

Petrášek, J., Mravec, J., Bouchard, R., Blakeslee, J. J., Abas, M., Seifertová, D., Wiśniewska, J., Tadele, Z., Kubeš, M., Čovanová, M., Dhonukshe, P., Skůpa, P., Benková, E., Perry, L., Křeček P., Lee, O. R., Fink, G. R., Geisler, M., Murphy, A. S., Luschnig, C., Zažímalová, E., Friml, J. 2006. PIN Proteins Perform a Rate-Limiting Function in Cellular Auxin Efflux. Science, 312, 914–918.

Proust, H., Hoffmann, B., Xie, X., Yoneyama, K., Schaefer, G. D., Yoneyama Koichi, Nogue, F., Rameau, C., 2011. Strigolactones regulate protonema branching and act as a quorum sensing-like signal in the moss *Physcomitrella patens*. Developement, 138, 1531–1539.

Prusinkiewicz, P., Crawford, S., Smith, R. S., Ljung, K., Bennett, T., Ongaro, V., Leyser, O., 2009. Control of bud activation by an auxin transport switch. PNAS, 106,17431–17436.

Rochange, S., 2010. Strigolactones and their role in arbuscular mycorrhizal symbiosis. V: Arbuscular mycorrhizas: Physiology and function. Koltai, H., Kapulnik, Y., New York, Springer: 323 str.

Strack, D., Fester, T., 2006. Isoprenoid metabolism and plastid reorganization in arbuscular mycorrhizal roots. New Phytologist, 172, 22–34.

Taiz, L., Zeiger, E., 2006. Plant Physiology. 4. izdaja. Sinauer Associates, Massachusetts, 764 str.

Tamasloukht, B., Sejalon-Delmas, N., Kluever, A., Jauneau, A., Roux, C., Bécard, G., Franken, P., 2003. Root factor induce mitochondrial-related gene expression and fungal respiration during the developmental switch from asymbiosis to presymbiosis in the arbuscular mycorrhizal fungus *Gigaspora rosea*. Plant Physiology, 131, 1468–1478.

Tanaka, M., Takei, K., Kojima, M., Sakakibara, H., Mori, H., 2006. Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance. The Plant Journal, 45, 1028–1036.

Umehara, M., Hanada, A., Yoshida, S., Akiyama, K., Arite, T. in sod., 2008. Inhibition of shoot branching by new terpenoid plant hormones. Nature, 455, 195–201.

Xie, X., Yoneyama, K., Yoneyama, K. 2010. The strigolactone story. Annual Review of Phytopathology, 48, 93–117.

Yoneyama, K., Xie, X., Sekimoto, H., Takeuchi, Y.,Ogasawara, S. in sod., 2008. Strigolactones, host recognition signals for root parasitic plants and arbuscular mycorrhizal fungi, from *Fabaceae* plants. New Phytologist, 179, 484–494.

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Deciduous and evergreen tree responses to enhanced UV-B treatment during three years

Odziv listopadne in vednozelene drevesne vrste v času 3-letne izpostavljenosti povečanemu sevanju UV-B

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Abstract: This paper reports a study of the strategies in Norway spruce (*Picea abies* (L.) Karst.) and European beech (*Fagus sylvatica* L.) for coping with enhanced UV-B radiation. Trees, as plants in general, possess diverse systems which respond to UV-B radiation. Changes in physiology, biochemistry and morphology have been observed in trees under enhanced UV-B radiation. The efficiency of trees' UV-B protective systems depends on plant characteristics and state of development as well as can be correlated with the UV-B dose and the environmental conditions. The two tree species were exposed outdoors to enhanced UV-B simulating 17% ozone depletion for three years during which time, selected parameters were monitored. Selected physiological parameters were monitored three times a year on beech leaves and three needle age classes of spruce. Spruce and beech exhibited great variability in the amounts of chlorophyll, methanol-soluble UV-B and UV-A absorbing compounds, and optimum quantum yield of photosystem II. The effects of UV-B radiation also varied with needle and leaf development stage and interaction with environmental conditions. Enhanced UV-B radiation triggered responses in both trees and a reduced negative effect of UV-B radiation on spruce photochemical efficiency was observed during prolonged drought. The results show high UV-B tolerance of both tree species and indicate the complexity of plant response to UV-B, involving multilevel interactions with environmental factors and thus emphasizes the necessity of long-term investigations on trees in a natural ecosystem.

Keywords: *Picea abies, Fagus sylvatica*, UV-B radiation, long-term field experiment

Izvleček: V raziskavi smo preučevali strategije spoprijemanja s povečanim sevanje UV-B pri smreki (*Picea abies* (L.) Karst .) in bukvi (*Fagus sylvatica* L.). Sadike obeh drevesnih vrst so bile posajene na prostem in za obdobje treh let izpostavljene povečanemu sevanju UV-B. Izbrane parametre smo spremljali trikrat letno na listih oziroma treh starostnih razredih iglic. Tako pri smreki kot pri bukvi smo izmerili veliko variabilnost v vsebnostih UV-B absorbirajočih snovi, fotosinteznih barvil in fotokemični učinkovitosti. Učinek povečanega sevanja UV-B je bil odvisen od razvojne faze iglice oziroma lista ter od okoljskih razmer. Povečano sevanje UV-B je sprožilo posamezne odzive pri obeh drevesnih vrstah. Zmanjšan negativni učinek sevanja UV-B

na fotokemično učinkovitost smreke smo opazili v tretji poskusni sezoni in ga razlagali kot omilitveni učinek suše. Pri letošnjih iglicah, ne pa tudi listih ali starejših iglicah, je bila prisotna neznačilna tendenca povečane sinteze UV-B absorbirajočih snovi pod povečanim sevanjem UV-B. Rezultati so pokazali veliko strpnost obeh drevesnih vrst do povečanega sevanja UV-B, obenem pa potrdili kompleksen odziv na povečano sevanje UV-B, ki je odvisno tudi od razvojne faze rastline in okoljskih razmer.

Ključne besede: *Picea abies, Fagus sylvatica*, sevanje UV-B, večletni poskus

Introduction

Depletion of the stratospheric ozone over the past several decades has resulted in enhanced levels of UV-B radiation reaching the biosphere (Madronich et al. 1998, Ajavon et al. 2006). A United Nations report states that it is estimated that full recovery of stratospheric ozone on a global scale will not occur before 2050 – 2100 and will be depend upon continued compliance with the Montreal Protocol and addressing the interactions between ozone recovery and atmospheric changes, such as climate change. The enhanced UV-B radiation remains an issue which can affect biocenosis significantly.

Most of the studies on the effects of enhanced UV-B radiation on plants have involved agricultural species, with much fewer studies on trees, even though the importance of trees in both ecosystems and ecosystems and in economics is considerable. The knowledge of UV-B radiation effects on trees is mainly based on short-term experiments and/or controlled growth conditions. Detrimental effect of UV-B observed in those studies occurs rarely in field-grown trees, where natural light conditions and other environmental factors contribute to diverse responses of trees (Mirecki and Teramura 1984, Laakso and Huttunen 1998, Laakso et al. 2000, Sullivan at al. 2005). Long-term field UV-B effects have been studied scarcely, and various responses of trees to enhanced UV-B radiation were reported. Three-year studies on conifers reported reductions in growth (Sullivan and Teramura 1992) and a reduction of UV-B absorbing compounds Kinnunen et al. 2001) on pine trees under UV-B exposure. An increase of UV-B absorbing compounds was observed in Douglas fir and Ponderosa pine during two year UV-B irradiation (Warren et al. 2002). No reduction of growth and photosynthesis/secondary compounds was detected at Douglas fir, Norway spruce and Scots pine after two or three years of UV-B exposure (Bassman et al. 2002, Turtola et al. 2006). A five-year UV-B irradiation of Norway spruce led to a decrease of some growth parameters, but not of photosynthesis or UV-B absorbing compounds (Trošt Sedej and Gaberščik 2008). A five-year exposure to increased UV-B of deciduous trees (ash, *Fraxinus excelsior*; silver birch, *Betula pendula*; lime, *Tilia cordata*; English oak, *Quercus robur*; and sycamore maple, *Acer pseudoplatanus*) resulted in decreased photosynthesis, transpiration and stomatal density (Keiller and Holmes 2001). In a three-year study on red maple (*Acer rubrum*), tulip poplar (*Liriodendron tulipifera*) and sweetgum (*Liquidambar styraciflua*), photosynthesis generally did not decline and poplar exhibited an increase of UV-B absorbing compounds (Sullivan et al. 1994), while European beech (*Fagus sylvatica*) showed increased photosynthesis after three years of UV-B exposure (Šprtová at al. 2003). Reduced photosynthetic activity led to reduced leaf elongation, plant growth and biomass production in some cases (Warren et al. 2002, Bassman et al. 2003, Kirchgessner et al. 2003, Lavola et al. 2003, Lenk and Buschmann 2006, Trošt Sedej and Gaberščik 2008). Leaf size in deciduous trees has been variously reported to have been decreased by enhanced UV-B radiation (Newsham et al. 1999, Keiller and Holmes 2001, Sullivan at al. 2003), increased (Sullivan at al. 2003, Šprtová at al. 2003) or unchanged (Kostina et al. 2001). Increase in leaf thickness under enhanced UV-B radiation was observed in some deciduous trees (Sullivan et al. 1994, Antonelli et al. 1998, Newsham et al. 1999), where leaf thickening was due to an increase in either the thickness of the spongy parenchyma (Kostina et al. 2001) or of the palisade parenchyma (Nagel et al. 1998) .

Trees possess diverse biochemical, physiological and morphological mechanisms which respond to UV-B radiation, so that the ambient UV-B might be viewed both as a stressor and a photomorphogenic signal (Prado et al. 2012). Trees' resistance to enhanced UV-B is partially based on a high epidermal screening capacity due mainly to phenolics (Fischbach et al. 1999, Hoque and Remus 1999, Trošt Sedej and Gaberščik 2008, Rozema et al. 2002, Turtola et al. 2006). Important components of the defence systems against UV and a number of stress factors are also other secondary compounds such as terpenes, (Turtola et al. 2006, Prado et al. 2012), reflectance of UV (Hoque and Remus 1999, Láposi et al. 2009), special anatomical arrangement and increased cell wall thickness of epidermal cells (Sullivan at al. 1994, Antonelli at al. 1998, Newsham at al. 1999, Hoque and Remus 1999, Chalker-Scott and Scott 2004) and small, thick leaves (P´yankov and Kondrachuk 1998). The proportion of UV-B radiation reaching the leaf mesophyll is generally higher in deciduous broadleaf trees than in evergreen conifer trees (Day 1993), that indicate greater sensitivity of deciduous trees to enhanced UV-B radiation but lower maintenance costs. UV-B sensitivity is closely related to the development state of leaves, where the epidermis of fully grown leaves filters UV-B more efficiently than that of young leaves (Day et al. 1992, DeLucia at al.1992, Day et al. 1996, Ruhland and Day 1996, Laakso et al. 2000, Trošt and Gaberščik 2001, Neitzke and Therburg 2003, Trošt Sedej and Gaberščik 2008).

The degree of UV-B shielding in trees depends on environmental conditions (Neitzke and Therburg 2003, Julkunen-Tiitto et al. 2005, Lenk and Buschmann 2006, Trošt Sedej and Gaberščik 2008). The higher sensitivity to UV-B was due to low temperatures in spruce (Bavcon et al. 1996) and increased ozone at beech (Zeuthen et al. 1997). Drought exposure in pine and spruce (Petropoulou et al. 1995, Manetas et al. 1997, Trošt Sedej and Gaberščik 2008) and nutrient deficiency at birch (Keski-Saari et al. 2005) alleviated UV-B effect. Experiments testing elevated CO_2 and enhanced UV-B radiation indicated that increased CO_2 either amelioratied or had no effect on photosynthesis or biomass allocation (Sullivan, 1997, Caldwell at al. 1998, and Lavola at al. 2000). Some studies (Laakso et al. 2000, Sullivan 2005) proved species/population specific responses to enhanced UV-B radiation, with woody species varying widely in their responses under changing environmental conditions and also responding slowly, that is why further long-term outdoor research is necessary.

Norway spruce (*Picea abies* (L.) Karst.) and European beech (*Fagus sylvatica* L.) are the most common tree species of natural forests in Central Europe. Plants were exposed to ambient and enhanced UV-B levels at the outdoor experimental plot. We have examined physiological and growth responses to UV-B radiation (Sullivan and Teramura 1992, Bassman et al. 2002, Šprtová et al, 2003 Turtola et al. 2006, Trošt Sedej and Gaberščik 2008) taking into account the effect of concomitant environmental conditions (Petropoulou et al. 1995, Manetas et al. 1997, Neitzke and Therburg 2003, Keski-Saari et al. 2005, Trošt Sedej and Gaberščik 2008) and considering the variation of tree response according to needle/leaf development stage (Naidu et al. 1993, Latola et al. 2001). This paper offers an insight into complex response of Norway spruce and European beech to UV-B exposure during a three year study under realistic environmental conditions, thus it provides an additional view to a deciduous and an evergreen tree strategy of coping with enhanced UV-B radiation.

Materials and methods

Outdoor experimental plot

Norway spruce (*Picea abies* (L.) Karst.) and European beech (*Fagus sylvatica* L.) one-year seedlings were planted in an outdoor research plot (Botanical Garden, University of Ljubljana: 320m a.s.l., 46°35´N, 14°55´E). Fifty seedlings for each of the two treatments were planted in 5 clay pots (62x21x19 cm) in a mixture of compost and peat (1:1). Plants were transplanted to a new soil mixture every February. The pots were buried at ground level to minimise soil temperature variation and desiccation (Sullivan and Teramura 1992).

Two different treatments were applied in the experiment. A UV-B supplement system was designed, as described by Björn and Teramura (1993) and exposure resulting from 17% ozone depletion, corresponding to a 35-55% increase of ambient $UV\text{-}B_{BE}$ (UV-B+), was simulated using Q-Panel

UV-B 313 lamps (Cleveland, OH, USA), which emit from 275 nm to 400 nm with peak emission at 313 nm, filtered with cellulose diacetate filters. Ambient radiation (UV-B) was simulated using the same lamps filtered with Mylar foil. The doses were calculated and adjusted weekly (Björn and Murphy 1985) using the generalised plant action spectrum of Caldwell (1968). The system was timer controlled. Ambient UV-B (Fig. 1a). UV-A and PAR radiation were monitored at the site by a three-channel dosimeter (Häder et al. 1999). The experiment was conducted over three growing seasons from May till October.

Cumulative water balance data (Fig. 1b) calculated as the difference between total monthly precipitation and total monthly potential evapotranspiration according to Penman's equation, were obtained from Slovenian Environment Agency. Seasonal cumulative water balance (CWB) represents three seasonal periods of beech growth and development: March to June, March to August and March to October. Principal Component Analysis showed a high correlation between seasonal cumulative water balance and environmental factors air and soil temperature, insolation, precipitation and potential evaporation and other plant parameters at the outdoor experimental plot. Seasonal cumulative water balance, as a water disposability indicator, was chosen as the most characteristic environmental factor, and was used in the correlation analyses.

Physiological and biochemical measurements

Measurements were carried out three times per growth season, in May, August and October, the key phases for tree growth and development (young leaves, growth phase peak, end of growth) as determined in the preliminary year (Trošt and Gaberščik 2001). Leaves and needles, the latest of three needle-age classes: current (c), current+1 (c+1) and current+2 (c+2), were sampled from five (for biochemical measurements) and ten (for physiological measurements) of the upper

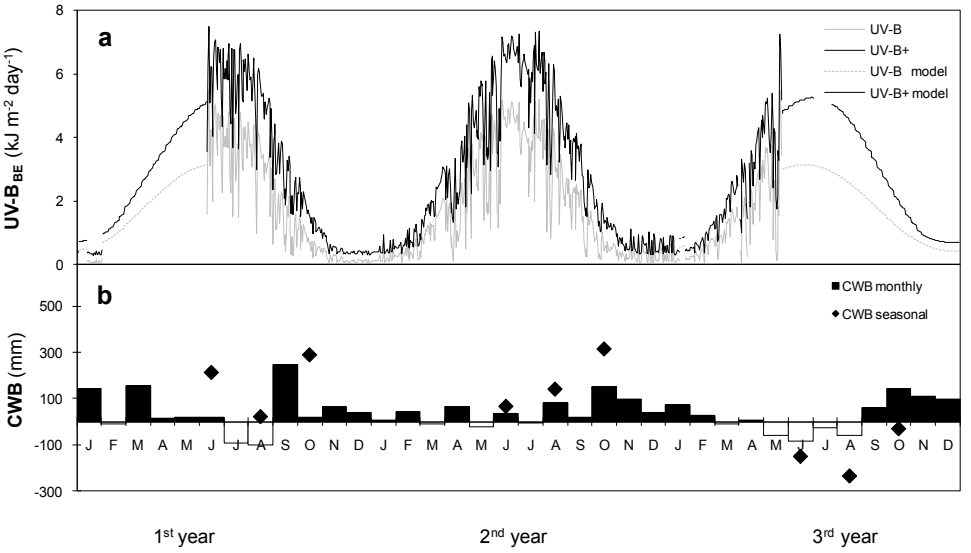

Fig. 1a: Monitored ambient (UV-B) and enhanced (UV-B+) daily dose of biologically active UV-B (UV-B_{BE}) radiation, calculated ambient (UV-B model) and enhanced (UV-B+ model) daily dose of biologically active UV-B (UV-B_{BE}) radiation (Björn and Murphy, 1993) in the two treatments established at the experimental site in Ljubljana. 1b. Monthly and seasonal cumulative water balance (CWB) in Ljubljana.

Slika 1a: Izmerjeni naravni (UV-B) in povečani (UV-B+) dnevni odmerki biološko aktivnega sevanja UV-B (UV-B_{BE}), izračunani naravni (UV-B model) in povečani (UV-B+ model) dnevni odmerki biološko aktivnega sevanja UV-B (UV-B_{BE}) po modelu Björn and Murphy (1993) na poskusni ploskvi Botaničnega vrta v

horizontal branches of randomly selected trees *per* plot.

Photochemical efficiency was estimated by measuring the chlorophyll *a* fluorescence of photosystem II using a modulated fluorometer (OS-500, Opti-Sciences, USA). Measurements were carried out *in vivo* at noon on clear days. Optimal quantum yield (F_v/F_m), defined as ($F_m - F_o)/F_m$, where F_m represents maximal and F_o minimal fluorescence of a dark adapted sample, was determined.

Total chlorophyll content (Chl *a*+*b*) was determined as described by Lichtenthaler (1987). The Chl *a*+*b* content was calculated per sample DM, from extinction coefficients at 644 and 662 nm in acetone [100% (v/v)] (UV/VIS spectrophotometer Lambda 12, Perkin-Elmer, Norwalk, CT, USA).

The total content of methanol-soluble UV-B ($A_{280-320}$) and UV-A ($A_{320-400}$) absorbing compounds was estimated according to Caldwell (1968). The extinction coefficients of the samples were measured in the UV-B and UV-A spectral range 280-400 nm (UV/VIS spectrometer), calculated per sample DM and integrated to estimate the total content of UV-B and UV-A absorbing compounds.

Morphometric measurements

At the end of growth season, the leaf length, width and thickness as well as needle total length and diameter (optical microscope Zeiss KF2, Carl Zeiss, Germany) were measured. The parameters were determined in ten randomly selected needles of three age-classes and leaves *per* plot sampled at the upper horizontal branches.

Statistical analysis

The independent-samples t-test was used to compare means of measured parameters at the two UV-B treatments. Spearman's coefficient was used to investigate bivariate correlations between the environmental factors and measured parameters. Samples in all the tests were randomly chosen without replication. Statistically significant differences were marked as: non-significant ($p < 0.05$), * ($p \leq 0.05$), ** ($p \leq 0.01$) and *** ($p \leq 0.001$). Analyses were accomplished by SPSS for Windows 13.0.0.

Results

Physiological and biochemical responses

The responses of Norway spruce and European beech varied from neutral to negative or positive during three years exposure to UV-B, and were varying with plant developmental state, growth season and environmental conditions (Fig. 2., Tab. 1.).

In the autumn of the first season Norway spruce manifested reduced F_v/F_m under enhanced UV-B in c+1 needles and in spring of the second season in c and c+1 needles. In the third season, higher F_v/F_m under enhanced UV-B was observed in c and c+1 needles in spring, while in summer of this season F_v/F_m increased in all three needle age classes. In all three spruce needle age classes, F_v/F_m exhibited a tendency to reduced values in spring and optimal values later in the season. The F_v/F_m values of the European beech were significantly reduced under enhanced UV-B in the spring of the second season. F_v/F_m values were low during the second and third season under both treatments (Fig. 2).

In the Norway spruce, the Chl *a*+*b* content responded to enhanced UV-B in all three needle age classes. The young needles manifested increased Chl *a*+*b* content twice under enhanced UV-B, while Chl *a*+*b* content in c+1 and c+2 needles was decreased three times and increased once. In the European beech Chl *a*+*b* content significantly decreased under enhanced UV-B in autumn of the second season and increased in autumn of the third season (Table 1).

The $A_{280-320}$ content was high in both tree species under both UV-B treatments during all growth seasons. Needles/leaves were poorly responsive to enhanced UV-B radiation. Under UV-B+ exposure, spruce current needles showed tendency to higher $A_{280-320}$ content, while beech leaves manifested tendency to lower $A_{280-320}$ content under UV-B+ exposure. Compared with UV-B treatment, the $A_{280-320}$ content under UV-B+ radiation decreased significantly once, in summer of the dry third season. $A_{320-400}$ content was high in both tree species and responded rarely to enhanced UV-B (Table 1).

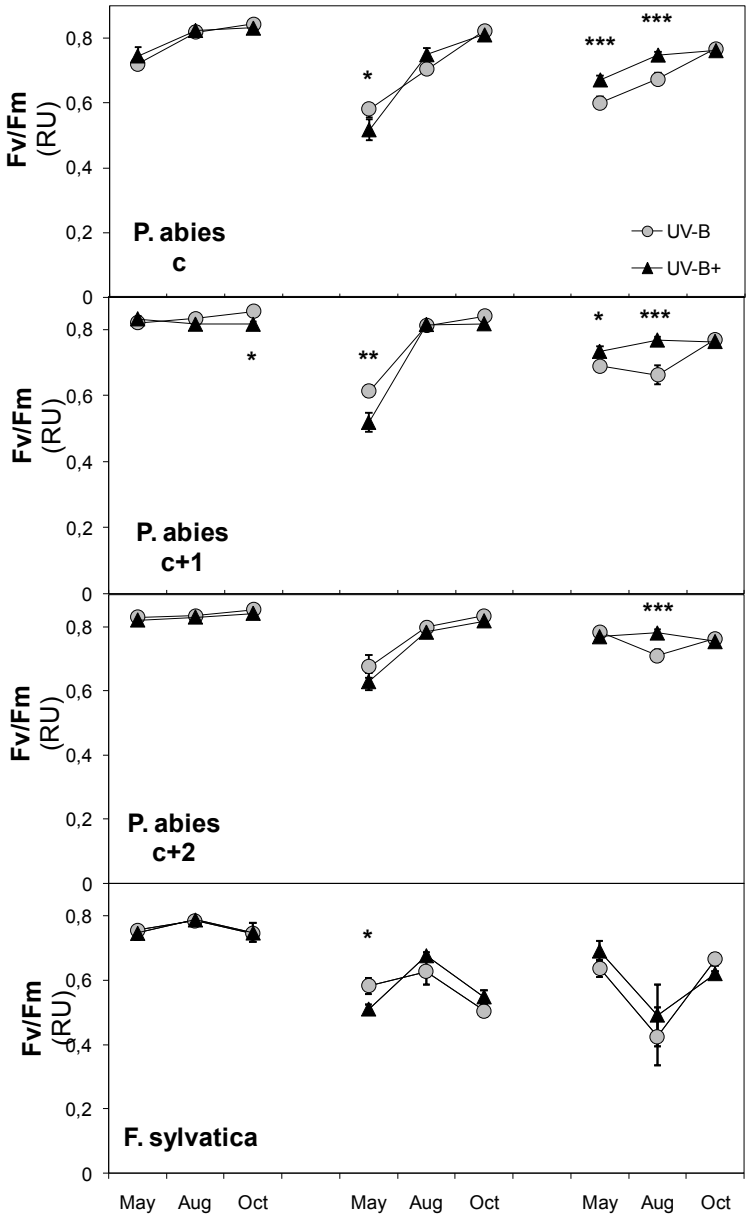

Figure 2: Optimal quantum yield (F_v/F_m) for three needle age classes of Norway spruce (c, c+1, c+2) and leaves of European beech exposed to ambient (UV-B) and enhanced (UV–B+) UV-B radiation during three growth seasons. Data are means ± SE, n = 10, significant differences (Independent-samples t-test) are marked with: * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$).

Slika 2: Potencialna fotokemična učinkovitost (F_v/F_m) treh starostnih razredov iglic smreke (c, c+1, c+2) in listov bukve, izpostavljenih naravnemu (UV-B) in povečanemu (UV-B+) sevanju UV-B tekom treh let. Podatki so srednje vrednosti ± SE, n = 10, značilna razlika (neodvisni t-test) je označena z: * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$).

Table 1: Biochemical parameters: chlorophyll content (Chl *a*+*b* [mg g^{-1} DM]), content of UV-B absorbing compounds ($A_{280-320}$ [relative units]) and content of UV-A absorbing compounds ($A_{320-400}$ [relative units]) for three needle age classes (c, c+1, c+2) of Norway spruce and leaves of European beech exposed to ambient (UV-B) and enhanced (UV-B+) UV-B radiation during three growth seasons. Data are means ± SE, n = 5, significant difference (Independent-samples t-test) is marked with: * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$).

Tabela 1: Biokemijski parametri: vsebnost klorofila (Chl *a*+*b* [mg g^{-1} DM]), vsebnost UV-B absorbirajočih snovi ($A_{280-320}$ [relativna enota]) in vsebnost UV-A absorbirajočih snovi ($A_{320-400}$ [relativna enota]) v treh starostnih razredih iglic (c, c+1, c+2) smreke in v listih bukve, izpostavljenih naravnemu (UV-B) in povečanemu (UV-B+) sevanju UV-B tekom treh let. Podatki so srednje vrednosti ± SE, n = 5, značilna razlika (neodvisni t-test) je označena z: * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$).

			1st year						2nd year						3rd year					
			May		Aug		Oct		May		Aug		Oct		May		Aug		Oct	
			UV-B	**UV-B+**	**UV-B**	**UV-B+**	**UV-B**	**UV-B+**	**UV-B**	**UV-B+**	**UV-B**	**UV-B+**	**UV-B**	**UV-B+**	**UV-B**	**UV-B+**	**UV-B**	**UV-B+**	**UV-B**	**UV-B+**
P. abies																				
Chl *a*+*b*	c	Mean	3,15	3,10	2,96	3,12	7,66	4,82	6,94	**14,36**	7,42	5,60	6,64	5,84	6,89	9,23	2,62	2,78	1,87	**2,70**
		± SE	0,76	0,35	0,11	0,34	1,07	0,31	0,81	0,59	0,98	0,76	0,70	0,53	1,16	1,56	0,15	0,34	0,12	0,27
		p		ns		ns		ns		***		ns		ns		ns		ns		*
	c+1	Mean	2,91	1,90	3,65	3,95	7,93	8,91	6,19	**10,76**	3,61	3,46	4,85	5,58	1,31	1,29	1,68	2,18	1,19	**1,96**
		± SE	0,32	0,22	0,37	0,38	0,58	1,00	0,77	1,83	0,27	0,28	0,41	0,83	0,14	0,35	0,14	0,27	0,11	0,21
		p		ns		ns		ns		*		ns		ns		ns		ns		*
	c+2	Mean	5,55	2,49	4,16	2,50	4,37	5,19	5,41	**6,45**	3,42	**4,01**	4,47	3,91	0,41	0,34	1,60	1,88	1,07	1,36
		± SE	0,42	0,51	0,45	0,39	0,43	0,40	0,73	0,54	0,50	0,14	0,48	0,27	0,27	0,06	0,14	0,17	0,16	0,27
		p		ns		ns		ns		***		*		ns		ns		ns		ns
F. sylvatica																				
Chl *a*+*b*		Mean	1,31	2,86	3,52	4,05	2,71	2,75	2,43	3,71	3,35	2,96	3,62	**2,87**	5,71	6,51	4,07	3,31	3,21	**3,92**
		± SE	0,60	0,28	0,61	0,57	0,14	0,12	0,35	0,54	0,42	0,13	0,23	0,20	0,36	0,31	0,45	0,28	0,11	0,19
		p		ns		ns		ns		ns		ns		*		ns		ns		*
P. abies																				
$A_{280-320}$	c	Mean	1820	2052	2239	2365	2395	2702	2689	2961	2410	2886	3073	3877	3790	3518	1603	1720	1683	1845
		± SE	91,8	106,6	26,7	235,7	81,4	64,3	318,6	350,9	304,2	457,4	298,7	373,2	715,1	1016,5	322,3	303,4	214,4	261,5
		p		ns		ns		ns		ns		ns		ns		ns		ns		ns
	c+1	Mean	2724	2237	2690	**2576**	3088	2937	2612	2585	2147	1898	3250	2959	1994	1356	2250	1657	2060	2887
		± SE	190,7	85,1	44,1	84,6	125,8	263,8	47,6	249,3	473,7	439,2	190,9	309,4	491,8	310,5	276,3	165,1	99,1	898,1
		p		ns		*		ns		ns		ns		ns		ns		ns		ns
	c+2	Mean	2535	2011	2984	2893	2900	2888	2473	2432	2894	2236	3172	3294	1178	1043	1884	1681	1915	1831
		± SE	361,0	81,9	168,3	86,4	108,8	75,7	182,4	169,9	550,3	262,9	214,4	193,6	308,2	120,9	235,2	171,7	173,9	129,1
		p		ns		ns		ns		ns		ns		ns		ns		ns		ns
F. sylvatica																				
$A_{280-320}$		Mean	2506	2223	1879	1898	1724	1796	2062	1927	2545	2403	1821	1705	1550	1470	1700	1467	1937	1586
		± SE	205,0	199,9	106,6	65,0	94,9	263,0	179,6	104,3	326,7	239,3	72,7	67,0	95,0	134,8	71,1	42,3	189,5	181,9
		p		ns		ns		ns		ns		ns		ns		ns		ns		ns
P. abies																				
$A_{320-400}$	c	Mean	2543	2596	1774	2118	1286	1467	2961	3060	1900	2399	2083	**3015**	2897	2998	978	1173	854	1251
		± SE	173,7	52,8	125,6	241,2	126,2	139,1	441,2	296,0	236,8	382,0	197,6	215,5	551,7	1041,1	218,2	180,7	110,9	174,1
		p		ns		ns		ns		ns		ns		*		ns		ns		ns
	c+1	Mean	2319	**1705**	1858	1784	1869	1767	1818	1685	1512	1422	2154	2096	1014	798	1204	962	1001	1267
		± SE	161,1	72,3	55,5	102,6	212,3	156,7	77,2	134,5	293,8	263,7	126,7	260,7	233,8	175,4	183,8	95,8	47,2	242,8
		p		*		ns		ns		ns		ns		ns		ns		ns		ns
	c+2	Mean	1947	1518	2057	2181	1717	1747	1664	1584	2125	1614	2142	2471	653	655	1041	1096	937	1055
		± SE	197,5	122,7	98,3	97,9	76,9	58,0	72,9	152,2	436,7	186,5	243,7	171,9	114,7	60,0	126,1	139,5	83,2	99,1
		p		ns		ns		ns		ns		ns		ns		ns		ns		ns
F. sylvatica																				
$A_{320-400}$		Mean	3053	2946	2287	2307	1945	1833	1943	2045	2710	2732	1655	1556	1480	1475	1745	**1571**	1914	1588
		± SE	366,9	290,8	249,8	68,6	180,9	275,4	219,0	137,0	386,9	354,7	119,7	86,0	103,4	265,8	83,2	43,7	213,5	254,4
		p		ns		ns		ns		ns		ns		ns		ns		*		ns

Table 2: Morphometric needle/leaf parameters of Norway spruce and European beech exposed to ambient (UV-B) and enhanced (UV-B+) UV-B radiation determined at the end of growth seasons. Parameters: needle total length (m), average diameter (mm), leaf length (mm), width (mm), thickness (µm). Data are means ± SE, n = 50, significant difference (Independent-samples t-test) is marked with: * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$).

Tabela 2: Morfometrični parametri iglic smreke in listov bukve izpostavljenih naravnemu (UV-B) in povečanemu (UV-B+) sevanju UV-B tekom treh let. Parametri: skupna dolžina iglic (m), premer iglice (mm), dolžina lista (mm), širina lista (mm), debelina lista (µm). Podatki so srednje vrednosti ± SE, n = 10, značilna razlika (neodvisni t-test) je označena z: * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$).

		1st year		2nd year		3rd year	
		UV-B	**UV-B+**	**UV-B**	**UV-B+**	**UV-B**	**UV-B+**
P. abies							
Needle length	Mean	77.82	81.19	99.03	100.2	65.86	58.73
	± *SE*	*11.16*	*12.23*	*30.07*	*30.67*	*8.98*	*3.49*
	p		ns		ns		ns
Needle diameter	Mean	0.59	0.56	0.41	0.39	0.53	0.43
	± *SE*	*0.018*	*0.016*	*0.009*	*0.012*	*0.026*	*0.014*
	p		ns		ns		ns
F. sylvatica							
Leaf length	Mean	–	–	–	–	44.0	40.8
	± *SE*					2,3	4,1
	p						ns
Leaf width	Mean	–	–	–	–	26.2	25.4
	± *SE*					1.6	2.1
	p						ns
Leaf thickness	Mean	–	–	–	–	97.5	89.9
	± *SE*					8.3	2.2
	p						ns

Morphometric modifications

Enhanced UV-B radiation did not affect needle/leaf morphology of neither of the trees after the three year observation (Table 2).

Interactions of UV-B radiation with environmental conditions and plant development

Correlations between the measured tree parameters and tree age, seasonal tree development, seasonal cumulative water balance and enhanced UV-B radiation are shown in Table 3. In Norway spruce and European beech tree age was generally correlated with decreasing parameter values, with the exception of UV-B and UV-A absorbing compounds in spruce young needles, which failed to correlate with tree age and beech chlorophyll content which increased with tree age. Seasonal tree development correlated scarcely with measured parameters, but there was a strong positive correlation with seasonal cumulative water balance. The content of UV-B absorbing compounds in spruce c needles failed to correlate with tree characteristics or environmental conditions and none of the tree responses could be correlated with the UV-B radiation dose (Table 3).

Discussion

Diverse physiological responses to enhance UV-B varying with environmental condition

The responses of spruce and beech to enhanced UV-B radiation varied according to the needle/leaf development stage, growth season and environmental conditions. In both species, most of monitored parameters did not vary with enhanced UV-B solely (Fig. 2, Tab. 1).

F_v/F_m was the parameter most sensitive to enhanced UV-B radiation. The F_v/F_m decrease under enhanced UV-B was measured in spring

Table 3: Correlations between tree parameters and tree age, seasonal tree development, seasonal cumulative water balance (CWB) and UV-B treatment (UV-B+) in Norway spruce (different needle age classes: c, c+1, c+2) and European beech. Tree parameters: optimal quantum yield (F_v/F_m), total chlorophyll content (Chl *a*+*b*), content of UV-B ($A_{280–320}$) and UV-A ($A_{320–400}$) absorbing compounds. Bivariate correlation (Spearman´s coefficient ρ) is marked with: ns ($p > 0.05$), * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$).

Tabela 3: Korelacije med merjenimi parametric dreves in starostjo bdrevesa, sezonskim razvojem, sezonsko kumulativno vodno bilanco (CWB) ter povečanim (UV-B+) sevanjem UV-B treh starostnih razredov iglic smreke (c, c+1, c+2) in listov bukve. Merjeni parametric drevesa: potencialna fotokemična učinkovitost (F_v/F_m), vsebnost klorofila (Chl *a*+*b*), vsebnost UV-B absorbirajočih snovi ($A_{280–320}$) in vsebnost UV-A absorbirajočih snovi ($A_{320–400}$). Bivariatna korelacija (Spearmanov koeficient ρ) je označen z: ns ($p > 0.05$), * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$).

		Tree age		**Seasonal development**		**CWB**		**UV-B+**	
		ρ	p	ρ	p	ρ	p	ρ	p
P. abies									
Fv/Fm	c	*–0.389*	*	*0.740*	**	*0.477*	**		ns
	c+1		ns	*0.471*	**	*0.525*	**		ns
	c+2	*–0.734*	***		ns	*0.597*	**		ns
F. sylvatica									
Fv/Fm		*–0.602*	**		ns	*0.444*	**		ns
P. abies									
Chl *a*+*b*	c	*–0.452*	*		ns		ns		ns
	c+1	*–0.767*	***		ns	*0.601*	***		ns
	c+2	*–0.856*	***		ns		ns		ns
F. sylvatica									
Chl *a*+*b*		*0.491*	**		ns	*–0.469*	**		ns
P. abies									
$A_{280–320}$	c		ns		ns		ns		ns
	c+1	*–0.452*	*	*0.396*	*	*0.725*	***		ns
	c+2	*–0.769*	***		ns	*0.495*	*		ns
F. sylvatica									
$A_{280–320}$		*–0.406*	**		ns	*0.291*	**		ns
P. abies									
$A_{320–400}$	c		ns		ns	*0.423*	*		ns
	c+1	*–0.811*	***		ns	*0.726*	***		ns
	c+2	*–0.856*	***		ns		ns		ns
F. sylvatica									
$A_{320–400}$		*–0.514*	**	*–0.228*	*	*0.310*	**		ns

of the second season in the young needles of spruce and beech leaves as well. It has been demonstrated that UV-B radiation alters the structure and function of chloroplasts, so that the potential photochemical efficiency might decrease (Björkman and Demmig-Adams 1994, Musil 1996, Adams and Barker 1998, Wu et al. 2011). Some studies on trees indeed showed a decrease of photochemical efficiency (Naidu et al. 1993, Bavcon et al. 1996), but in others no decrease was observed (Petropoulou et al. 1995, Manetas et al. 1997, Chalker-Scott and Scott 2004). A lowered F_v/F_m may be considered also as a down-regulation mechanism whose aim is lowering of the electron supply in the Calvin cycle as a result of the UV-B induced stress, which was apparently due to UV-B penetration into the mesophyll of recently emerged needles/leaves. It has been shown that in some trees the epidermis of fully grown needles and leaves filters UV-B more efficiently than the

epidermis in young ones (Neitzke and Therburg 2003, Day et al. 1992, DeLucia at al.1992, Day et al. 1996, Ruhland and Day 1996, Laakso et al. 2000, Trošt Sedej and Gaberščik 2008). The effect of UV-B on older leaves was diminished due to higher UV-B absorbing compounds content, self-shading and increased xeromorphic characteristics of needles/leaves. In the third season, the period of the most negative cumulative water balance, the photochemical efficiency of both UV-B exposed and control trees was low, but in Norway spruce the values were significantly higher in irradiated plants (Fig. 2) indicating the alleviating effect of UV-B radiation on drought. Such effect has been observed in Mediterranean conifers (Petropoulou et al. 1995, Manetas et al. 1997). Since UV-B radiation is unable to penetrate into the mesophyll of fully grown spruce needles (Fischbach et al. 1999), it can be concluded that the effect of UV-B on photochemical efficiency is indirect. UV-B radiation could benefit the water relations of plants through stomata closure (Petropoulou et al. 1995, Manetas et al. 1997), through promoted wax synthesis (Björn et al. 1997) and through cross-resistance of plants which are exposed to any oxidative stress (Turtola et al. 2006). In our study reductions of photochemical efficiency were more common in the recently emerged needles/leaves than in the later development stages. This reflects a remarkable capability for recovery, in which the disturbances of young organs are not expressed in the later stages. Similar findings have been reported in loblolly pine by Naidu and co-workers (1993).

Chlorophyll levels showed no consistent response to enhanced UV-B in any of the trees studied, the results suggesting dependence on development phase and environmental conditions. Other studies reported negative, neutral and rarely, positive effects of enhanced UV-B radiation on chlorophyll content (Šprtová et al. 1999, Bassman et al. 2003, Kirchgessner et al. 2003, Lavola et al. 2003, Trošt Sedej and Gaberščik 2008, Láposi et al. 2009). It has been shown that UV-B radiation can not only inhibit chlorophyll synthesis or cause its photo-oxidation (Bornman 1989, Middleton and Teramura 1993), but also increase the biosynthesis of photosynthetic pigments under favourable irradiation conditions (Middleton and Teramura 1993, Jordan 1996).

High tolerance to UV-B radiation

Norway spruce and European beech appear to possess an effective filter consisting of UV-B and UV-A absorbing compounds already present in young needles/leaves in May, soon after emergence from buds. The content did not change with seasonal development or enhanced UV-B radiation but it was affected by drought (Tab. 3). The total amount of methanol-soluble UV-B absorbing compounds in Norway spruce and European beech was three and two times higher, respectively (Tab. 1) than in some herbaceous species, where the same analytical method was used (Gaberščik et al. 2001, Gaberščik et al. 2002a, Gaberščik et al. 2002b, Breznik et al. 2005).

The low variability of UV-A and UV-B absorbing compounds content is probably part of a protective strategy of long-lived woody plants. Studies indicate that most conifers contain large amounts of UV-B absorbing compounds in the epidermis (Sullivan et al. 1996, Fischbach et al. 1999, Hoque and Remus 1999, Turtola et al. 2006) and the epidermis of fully grown leaves effectively filters UV-B (Day et al. 1992, Day et al. 1996, Ruhland and Day 1996, Fischbach et al. 1999, Hoque and Remus 1999). In the young needles of *Abies lasiocarpa* and *Picea engelmannii* less than 1% of UV-B radiation penetrates into the mesophyll (DeLucia et al. 1992), in European beech the UV-B penetration into young leaves was greater than in developed leaves (Neitzke and Therburg 2003). The production of UV-B absorbing compounds does not always depend on the UV-B dose (Rau and Hofmann 1996, Turtola et al. 2006), and consequently some higher plants from tropical, high-altitude and aquatic environments contain saturating amounts of UV absorbing compounds (Teramura and Sullivan 1994, Germ et al. 2002). It was hypothesized that the receptors triggering the biosynthesis of UV-B absorbing compounds are saturated in plants growing in open environments, therefore they provoke maximal synthesis over a wide range of irradiance (Sullivan et al. 1996), thus, UV absorbing compounds seem to be mainly constitutive. Meta-analysis which generalised an overall response of woody plants under two supplemental UV-B levels proved that woody plants show no significant changes in most variables under the low supplemental UV-B level (Li at al. 2010).

In the dry third season, the content of UV-B and UV-A absorbing compounds was low under both UV-B treatments. Production of UV-B absorbing compounds is an energy demanding process (Gaberščik et al. 2002a), which is why low levels of UV absorbing compounds coheres to low Chl and F_v/F_m values. The subtle changes observed in these parameters may have additional causes, such as changes in leaf histology and biochemistry (Hoque and Remus, 1999), both of which vary with the environmental conditions. The results pronounce the complex influences of UV-B and environmental condition on plants.

Tolerance to elevated UV-B in trees is, in addition to the content of the high methanol-soluble UV-B absorbing compounds, also increased by the presence of cell wall bound UV-B absorbing compounds in conifers (Fischbach et al. 1999, Hoque and Remus 1999, Rozema et al. 2002, Turtola et al. 2006). Other factors supporting this tolerance include reflectance of UV light (Hoque and Remus 1999), special anatomical arrangements and increased epidermal cell wall thickness of epidermis (Hoque and Remus 1999, Chalker-Scott and Scott 2004), small, thick leaves (P´yankov and Kondrachuk 1998), and large amounts of other secondary compounds, which are part of plants' defence systems against many stress factors (Turtola et al. 2006).

Our results show that both Norway spruce and European beech possess effective protection against the UV-B radiation. This protection depends not only on UV-B dose but generally on the state of development and environmental conditions that influence the efficiency of the UV-B protective systems.

Needle/leaf morfology

In the three year study period, it was found by comparing samples from ambient and enhanced UV-B treatment at the outdoor experimental plot (Tab. 2) that elevated UV-B radiation exerts no significant influence on the needle/leaf morphology of Norway spruce and European beech.

Earlier studies found that leaf size of deciduous trees decreases upon exposure to enhanced UV-B radiation (Antonelli at al. 1998, Newsham at al. 1999, Keiller and Holmes, 2001 and Sullivan at al.2003), other studies found that it increases (Sullivan at al.2003) or is not affected (Kostina at al. 2001). In the UV-B exposed conifers, reduced needle area (Laakso et al. 1996, Bassman et al. 2003, Zu et al. 2010) and shorter needles (Naidu et al. 1993, Sullivan et al. 1996) were reported. Compared to our study those experiments used 2-3 times higher suppplemental UV-B doses, which might be the reason for reduced leaf area in some cases. Other studies reported no effect on growth in UV-B exposed conifers (Petropoulou et al. 1995, Lavola et al. 2003). The response of deciduous trees to UV-B radiation is thickening of the leaves, which decreases the penetration of UV-B radiation to the leaf mesophyll (Sullivan et al. 1994, Antonelli et al. 1998, Newsham et al. 1999, Šprtová et al. 2003). The increase in leaf thickness is due to anatomic changes of spongy parenchyma (Kostina et al. 2001) or palisade parenchyma (Nagel at al. 1998) and corresponds to xeromorphic characteristics of plants from harsh environments, adapted to high irradiation, water stress and nutrient-poor soil (Turunen and Latola 2005). There are several studies on cross-tolerance of plants which are exposed to UV-B and other oxidative stresses (Turtola et al. 2006), uch as drought (Petropoulou et al. 1995, Manetas et al. 1997), drought and high light (Poulson et al. 2005), and cold (Mendez et al. 1999, Chalker-Scott 1999). On the other hand, there are again cases which show a decrease in cuticle thickness with increasing altitude (Turunen and Latola 2005). Diversity of results in different studies, including the current study, indicates the complexity of plant response to UV-B as a function of the whole spectrum of environmental factors and their multilevel interactions and emphasizes the necessity of long-term investigations on trees in natural ecosystems.

Conclusions

The responses of Norway spruce and European beech to enhanced UV-B radiation varied moderately according to the needle/leaf development stage, growth season and above all, environmental conditions. Most of monitored parameters did not respond only to enhanced UV-B solely in any of the studied tree species. Photochemical efficiency was the parameter most responsive to enhanced UV-B radiation and reductions of

photochemical efficiency were observed in the recently emerged needles/leaves, but not in the later development stages, suggesting a recovery capability. Chlorophyll levels showed no consistent response to enhanced UV-B radiation in any of the studied trees, results suggesting dependence on development phase and environmental conditions. Norway spruce as well as European beech manifested an effective filter consisting of UV-B and UV-A absorbing compounds already present in young needles/leaves in May, soon after emergence from buds. The content of the compounds did not change neither with seasonal development nor with enhanced UV-B radiation but was most sensitive to drought. The total amount of methanol-soluble UV-B absorbing compounds in Norway spruce and European beech was as much as three and two times higher, respectively than in tested herbaceous species. During three years' observation, elevated UV-B radiation exerted no significant influence on the needle/leaf morphology of Norway spruce and European beech. Our results prove that the evergreen Norway spruce and the deciduous European beech possess effective protection against the UV-B radiation, which depends specifically on UV-B dose levels but generally on the developmental state and environmental conditions that influence the efficiency of the UV-B protective systems. This study also indicates the complexity of plant response to UV-B, involving multilevel interactions with environmental factors and thus emphasizes the necessity of long-term investigations on trees in a natural ecosystem.

Povzetek

Drevesa se na povečano sevanje UV-B odzivajo preko različnih struktur in mehanizmov na biokemijskem, fiziološkem in morfološkem nivoju. Učinkovitost zaščite pred povečanim sevanjem UV-B je odvisna od značilnosti rastlinske vrste, razvojne faze rastline, okoljskih razmer ter odmerka UV-B sevanja. V raziskavi smo preučevali odzive na povečano sevanje UV-B pri smreki (*Picea abies* (L.) Karst .) in bukvi (*Fagus sylvatica* L.). Sadike obeh drevesnih vrst smo posadili na prosto za obdobje treh let ter jih izpostavili naravnemu in povečanemu sevanju UV-B. Izbrane parametre, optimalno fotokemično učinkovitost, vsebnost klorofilov, vsebnost UV-A in UV-B absorbirajočih snovi, smo spremljali trikrat letno na listih oziroma treh starostnih razredih iglic. Morfološke analize smo izvedli po treh letih obsevanja. Tako pri smreki kot pri bukvi smo izmerili veliko variabilnost v vsebnostih UV-B absorbirajočih snovi, fotosinteznih barvil in fotokemični učinkovitosti. Povečano sevanje UV-B je sprožilo posamezne odzive pri obeh drevesnih vrstah. Učinek povečanega sevanja UV-B se je spreminjal z razvojno fazo iglice in lista ter z okoljskimi razmerami. Zmanjšan negativni učinek sevanja UV-B na fotokemično učinkovitost smreke smo opazili v tretji poskusni sezoni in ga razlagamo kot omilitveni učinek suše. Pri letošnjih iglicah, ne pa tudi pri listih ali starejših iglicah, je bila prisotna tendenca povečane sinteze UV-B absorbirajočih snovi pod povečanim sevanjem UV-B. Rezultati so pokazali veliko strpnost obeh drevesnih vrst do povečanega sevanja UV-B in tudi kompleksen odziv na povečano sevanje UV-B, ki se spreminja tako z razvojno fazo rastline kot z okoljskimi razmerami. Dolgotrajne raziskave v naravnem okolju so zato, za dolgožive vrste kot so drevesa, nujne.

References

Adams, W.W., Barker, D.H., 1998. Seasonal changes in xanthophyll cycle-dependent energy dissipation in *Yucca glauca.* Nuttall. Plant Cell Environ., 21, 501–511.

Antonelli, F., Bussotti, F., Grifoni, D., Grossoni, P., Mori, B., Tani, C., Zipoli, G., 1998. Oak (*Quercus robur* L.) seedling responses to a realistic increase in UV-B radiation under open space conditions. Chemosphere, 36, 4–5, 841–845.

Bassman, J.H., Edwards, G.E., Robberecht, R., 2002. Long-term exposure to enhanced UV-B radiation is not detrimental to growth and photosynthesis in Douglas-fir. New Phytol., 154, 107–120.

Bassman, J.H., Edwards, G.E., Robberecht, R., 2003. Photosynthesis and growth in seedlings of five forest tree species with contrasting leaf anatomy subjected to supplemental UV-B radiation. Forest. Sci., 49, 176–187.

Bavcon, J., Gaberščik, A., Batič, F., 1996. Influence of UV-B radiation on photosynthetic activity and chlorophyll fluorescence kinetics in Norway spruce (*Picea abies* (L.) Karst.) seedlings. Trees, 10, 172–176.

Björkman, O., Demmig-Adams, B., 1994. Regulation of photosynthetic light energy capture, conversion, and dissipation in leaves of higher plants. In: Sulze E.D., Caldwell M.M., (eds.): Ecophysiology of Photosynthesis. Springer Verlag, Berlin, 17–48.

Björn, L.O., Callaghan, T.V., Johnsen, I., Lee, J.A., Manetas, Y., Paul, N.D., Sonesson, M., Wellburn, A.R., Coops, D., Heide-Jorgensen, H.S., Gehrke, C., Gwynn-Jones, D., Johanson, U., Kyparissis, A., Levizou, E., Nikolopoulos, D., Petropoulou, Y., Stephanou, M., 1997. The effects of UV-B radiation on European heathland species. Plant Ecol., 128, 252–264.

Björn, L.O., Murphy, T.M., 1985, Computer calculation of solar ultraviolet-radiation at ground-level. Physiol. Plantarum, 64, A23–A23.

Björn, L.O., Teramura, A.H., 1993 Simulation of daylight ultraviolet radiation and effects of ozone depletion. In: Young A.R., et al. (Eds.): Environmental UV Photobiology Plenum Press, New York, 41–71.

Björn. L.O., Murphy, T.M., 1993. Computer calculation of solar UV radiation at ground level. In: Young A.R., Björn L.O., Moan J., Nultsch W. (eds.): Environmental UV Photobiology, Plenum Press, New York, 63–69.

Bornman, J.F., 1989. Target sites of UV-B radiation in photosynthesis of higher plants. J. Photoch. Photobio., 4, 145–158.

Breznik, B., Germ, M., Gaberščik, A., Kreft, I., 2005. Combined effects of elevated UV-B radiation and the addition of selenium on common (*Fagopyrum esculentum* Moench) and tartary (*Fagopyrum tataricum* (L.) Gaertn.) buckwheat. Photosynthetica, 43, 583–589.

Caldwell, M.M., 1968. Solar UV radiation as an ecological factor for alpine plants. Ecol. Monog., 38, 243–268.

Caldwell, M.M., Björn, L.O., Bornman. J.F., Flint, S.D., Kulandaivelu, G., Teramura, A.H., Tevini, M., 1998. Effects of increased solar ultraviolet radiation on terrestrial ecosystems. J. Photochem. Photobiol. B: Biol., 46 (1–3), 40–52.

Chalker-Scott, L., 1999. Environmental significance of anthocyanins in plant stress responses. Photochem. Photobiol., 77 (1), 1–9.

Chalker-Scott, L., Scott, J.D., 2004. Elevated ultraviolet-B radiation induces cross-protection to cold in leaves of Rhododendron under field conditions. Photochem. Photobiol., 79, 199–204.

Day, T.A., 1993. Relating UV-B radiation screening effectiveness of foliage to absorbing-compound concentration and anatomical characteristics in a diverse group of plants. Oecologia, 95, 542–550.

Day, T.A., Howells, B.W., Ruhland, C.T., 1996. Changes in growth and pigment concentrations with leaf age in pea under modulated UV-B radiation field treatments. Plant Cell Environ., 19, 101–108.

Day, T.A., Vogelmann, T.C., DeLucia, E.H., 1992. Are some plant life forms more effective than others in screening out ultra violet-B radiation? Oecologia Heidelberg, 92, 513–519.

DeLucia, E.H., Day, T.A., Vogelman, T.C., 1992. UV-B and visible light penetration into needles of two species of subalpine conifers during foliar development. Plant Cell Environ., 15, 921–929.

Fischbach, R.J., Kossmann, B., Panten, H., Steinbrecher, R., Heller, W., Seidlitz, H.K., Sandermann, H., Hertkorn, N., Schnitzler, J.P., 1999. Seasonal accumulation of ultraviolet-B screening pigments in needles of Norway spruce (*Picea abies* (L.)Karst). Plant Cell Environ., 22, 27–37.

Gaberščik, A., Germ, M., Škof, A., Drmaž, D., Trošt-Sedej, T., 2002a. UV-B radiation screen and respiratory potential in two aquatic primary producers : *Scenedesmus quadricauda* and *Ceratophyllum demersum*. Verh. Int. Ver. Theor. Angew. Limnol., 27, 422–425.

Gaberščik, A., Novak, M., Trošt-Sedej, T., Mazej, Z., Germ, M., Bjőrn, L.O., 2001. The influence of enhanced UV-B radiation on the spring geophyte *Pulmonaria officinalis.* Plant Ecol., 154, 51–56.

Gaberščik, A., Vončina, M., Trošt-Sedej, T., Germ, M., Bjőrn, L.O., 2002b. Growth and production of buckwheat (*Fagopyrum esculentum*) treated with reduced, ambient, and enhanced UV-B radiation. J. Photochem. Photobiol. B, 66, 30–36.

Germ, M., Mazej, Z., Gaberščik, A., Häder, D.P., 2002. The influence of enhanced UV-B radiation on *Batrachium trichophyllum* and *Potamogeton alpinus* – aquatic macrophytes with amphibious character. J. Photochem. Photobiol. B., 66, 37–46.

Häder, D.P., Lebert, M., Marangoni, R., Colombetti, G., 1999. ELDONET – European light dosimeter network hardware and software. J. Photochem. Photobiol. B, 52, 51–58.

Hoque, E., Remus, G., 1999. Natural UV-screening mechanisms of Norway spruce (*Picea abies* [L.] Karst.) needles. Photochem. Photobiol. 69, 177–192.

Jordan, B.R., 1996. The effects of ultraviolet-B radiation on plants: a molecular perspective. Adv. Bot. Res., 22, 97–162.

Julkunen-Tiitto, R., Häggman, H., Aphalo, P.J., Lavola, A., Tegelberg, R., Veteli, T., 2005. Growth and defense in deciduous trees and shrubs under UV-B. Env. Pollution, 137(3), 404–414.

Keiller, D.R., Holmes M.G., 2001. Effects of long-term exposure to elevated UV-B radiation on the photosynthetic performance of five broad-leaved tree species. Photosynthesis Research, 67, 229–240

Keski-Saari, S., Pusenius, J., Julkunen-Tiitto, R., 2005. Phenolic compounds in seedlings of *Betula pubescens* and *B. pendula* are affected by enhanced UVB radiation and different nitrogen regimes during early leaf ontogeny. Global Change Biol., 11, 1180–1194.

Kinnunen, H., Huttunen, S., Laakso, K., 2001. UV-absorbing compounds and waxes of Scots pine needles during a third growing season of supplemental UV-B. Environ. Pollut., 112, 215–220.

Kirchgessner, H.D., Reichert, K., Hauff, K., Steinbrecher, R., Schnitzler, J.P., Pfundel, E.E., 2003. Light and temperature, but not UV radiation, affect chlorophylls and carotenoids in Norway spruce needles (*Picea abies* (L.) Karst.). Plant Cell Environ., 26, 1169–1179.

Kostina, E., Wulff, A., Julkunen-Tiitto,R., 2001. Growth, structure, stomatal responses and secondary metabolites of birch seedlings (*Betula pendula*) under elevated UV-B radiation in the field. Trees, 15, 483–491.

Laakso, K., Huttunen, S., 1998. Effects of the ultraviolet-B radiation (UV-B) on conifers: a review. Environ. Pollut., 99, 319–328.

Laakso, K., Kinnunen, H., Huttunen, S., 1996. Effects of ultraviolet radiation on the growth of Scots pine and Norway spruce. 5th Meeting of Finnish Plant Scientists, Kuopio, Finland. Kuopio University Publications, C45, 58–60.

Laakso, K., Sullivan, J.H., Huttunen, S., 2000. The effects of UV-B radiation on epidermal anatomy in loblolly pine (*Pinus taeda* L.) and Scots pine (*Pinus sylvestris* L.). Plant Cell Environ., 23, 461–472.

Láposi, R., Veresa, S., Lakatosb, G., Oláha, V., Fieldsendc, A., Mészárosa, I., 2009. Responses of leaf traits of European beech (*Fagus sylvatica* L.) saplings to supplemental UV-B radiation and UV-B exclusion. Agricultiral and Forest Meteorology, 149(5), 745–755.

Latola, K., Kinnunen, H., Huttunen, S., 2001. Needle ontogeny of mature Scots pines under enhanced UV-B radiation. Trees Struct. Funct., 15, 346–352.

Lavola, A., Aphalo, P.J., Lahti, M., Julkunen-Tiitto, R., 2003. Nutrient availability and the effect of increasing UV-B radiation on secondary plant compounds in Scots pine. Environ. Exp. Bot., 49, 49–60.

Lavola, A., Julkunen-Tiitto, R., de la Rosa, T.M., Lehto, T., Aphalo, P.J., 2000. Allocation of carbon to growth and secondary metabolites in birch seedlings under UV-B radiation and CO2 exposure. Physiologia Plantarum, 109, 260–267.

Lenk, S., Buschmann, C., 2006. Distribution of UV-shielding of the epidermis of sun and shade leaves of the beech (*Fagus sylvatica* L.) as monitored by multi-colour fluorescence imaging. J. of Plant Physiology, 163(12), 1273–1283.

Li, F-R., Peng , S-L., Chen, B-M., Hou, Y-p., 2010. A meta-analysis of the responses of woody and herbaceous plants to elevated ultraviolet-B radiation. Acta Oecologica, 36, 1, 1–9.

Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids – pigments of photosynthetic biomembranes. Method. Enzymol., 148, 350–382.

Manetas, Y., Petropoulou, Y., Stamatakis, K., Nikolopoulos, D., Levizou, E., Psaras, G., Karabourniotis, G., 1997. Beneficial effects of enhanced UV-B radiation under field conditions: Improvement of needle water relations and survival capacity of *Pinus pinea* L seedlings during the dry Mediterranean summer. Plant. Ecol., 128, 100–108.

Mendez, M., Gwynn-Jones, D., Manetas, Y., 1999. Enhanced UV-B radiation under field conditions increases antocyanin and reduces risk of photoinhibition but does not affect growth in the carnivorous plant *Pinguicula vulgaris*. New Phytologist, 144, 275–282.

Middleton, E.M., Teramura, A.H., 1993. The role of flavonol glycosides and carotenoids in protecting soybean from ultraviolet-B damage. Plant Physiol., 103, 741–752.

Mirecki, R.M., Teramura, A.H., 1984. Effects Of Ultraviolet-B Irradiance On Soybean 5. The Dependence Of Plant-Sensitivity On The Photosynthetic Photon Flux-Density During And After Leaf Expansion. Plant Physiol., 74, 475–480.

Musil, C.F., 1996. Accumulated effect of elevated UV-B radiation over multiple generations of the arid-environmment annual *Dimorphotheca sinuata* DC (Asteraceae). Plant Cell Environ., 19, 1017–1027.

Nagel, L.M., Bassman, J.H., Edwards, G.E., Robberecht, R., Franceshi, V., 1998. Leaf anatomical changes in *Populus trichocarpa, Quercus rubra, Pseudotsuga menziesiiand Pinus ponderosa* exposed to enhanced ultraviolet-B radiation. Physiologia Plantarum, 104, 385–396.

Naidu, S.L., Sullivan, J.H., Teramura, A.H., DeLucia, E.H., 1993. The effects of ultraviolet-B radiation on photosynthesis of different aged needles in field-grown loblolly-pine. Tree Physiol., 12, 151–162.

Neitzke, M., Therburg, A., 2003. Seasonal Changes in UV-B Absorption in Beech Leaves (Fagus sylvatica L.) along an Elevation Gradient. Blackwell Verlag, Berlin, Forstw. Cbl., 122, 1–21.

Newsham, K.K., Greenslade, P.D., McLeod, A.R., 1999. Effects of elevated ultraviolet radiation on *Quercus robur* and its insect and ectomycorrhizal associates. Global Change Biology, 5, 881–890.

Petropoulou, Y., Kyparissis, A., Nikolopoulos, D., Manetas, Y., 1995. Enhanced UV-B radiation alleviates the adverse-effects of summer drought in 2 mediterranean pines under field conditions. Physiol. Plantarum, 94, 37–44.

Poulson, M.E., Donahue, R.A., Konvalinka, J., Boeger, T., 2002. Enhanced tolerance of photosynthesis to high-light ad drought stress in *Pseudotsuga menziesii* seedlings grown in ultraviolet-B radiation. Tree physiology, 22 (12), 829–838.

Prado, F.E., Rosa, M., Prado, C., Podazza, G., Interdonato, R., González, J.A., Hilal, M., 2012. UV-B Radiation, Its Effects and Defense Mechanisms in Terrestrial Plants. In: Prasad, M.N.V. (ed.): Environmental Adaptations and Stress Tolerance of Plants in the Era of Climate Change. Springer, New York, 57–83.

P'yankov, V.I., Kondrachuk, A.V., 1998. Structure of the photosynthetic apparatus in woody plants from different ecological and altitudinal groups in Eastern Pamir. Russ. J. Plant Physiol., 45, 481–490.

Rau, W., Hofmann, H., 1996. Sensitivity to UV-B of plants growing in different altitudes in the Alps. J. Plant physiol., 148, 21–25.

Rozema, J., Bjorn, L.O., Bornman, J.F., Gaberscik, A., Hader, D.P., Trošt, T., Germ, M., Klisch, M., Groniger, A., Sinha, R.P., Lebert, M., He, Y.Y., Buffoni-Hall, R., de Bakker, N.V.J., van de Staaij, J., Meijkamp, B.B., 2002. The role of UV-B radiation in aquatic and terrestrial ecosystems – an experimental and functional analysis of the evolution of UV-absorbing compounds. J. Photoch. Photobiol. B, 66, 2–12.

Ruhland, C.T., Day, T.A., 1996. Changes in UV-B radiation screening effectiveness with leaf age in *Rhododendron maximum*. Plant Cell Environ., 19, 740–746.

Šprtová, M., Špunda, V., Kalina, J., Marek, M.V., 2003. Photosynthetic UV-B Response of Beech (*Fagus sylvatica* L.) Saplings. Photosynthetica, 41(4), 533–543.

Sullivan, H.J., 2005. Possible impact of changes in UV-B radiation on North American tress and forests. Environ. Pollut., 137, 380–389.

Sullivan, J.H., 1997. Effects of increasing UV-B radiation and atmospheric CO2 on photosynthesis and growth: implications for terrestrial ecosystems. Plant Ecology, 128, 195–206.

Sullivan, J.H., Gitz, D.C., Peek, M.S., McElrone, A.J., 2003. Response of three eastern tree species to supplemental UV-B radiation: leaf chemistry and gas exchange. Agr. and Forest. Meteor., 120, 1–4.

Sullivan, J.H., Howells, B.W., Ruhland, C.T., Day, T.A., 1996. Changes in leaf expansion and epidermal screening effectiveness in *Liquidambar syraciflua* and *Pinus taeda* in response to UV-B radiation. Physiol. Plantarum, 98, 349–357.

Sullivan, J.H., Teramura, A.H., 1992. The effects of UV-B radiation on loblolly pine. 2. Growth of field-grown seedlings. Tree-Sruct. Funct., 6, 115–120.

Sullivan, J.H., Teramura, A.H., Dillenburg, L.R., 1994. Growth and photosynthetic responses of field-grown sweetgum (*Liquidambar styraciflua*: Hamamelidaceae) seedlings to UV-B radiation. Am. J. Bot., 81, 826–832.

Šprtová, M., Špunda, V., Kalina, J., Marek M.V., 2003. Photosynthetic UV-B Response of Beech (*Fagus sylvatica* L.) Saplings. Photosynthetica, 41, 4, 533–543.

Teramura, A.H., Sullivan, J.H., 1994. Effects of UV-B radiation on photosynthesis and growth of terrestrial plants. Photosynth. Res., 39, 463–473.

Trošt, T., Gaberščik, A., 2001. The effect of enhanced UV-B radiation on Norway spruce (*Picea abies* (L.) Karst.) needles of two different age classes. Acta Biologica Slovenica, Ljubljana, 44, 13–25.

Trošt, T., Gaberščik, A., 2008. The effects of enhanced UV-B radiation on physiological activity and growth of Norway spruce planted outdoors over 5 years. Trees, 2008, 22, 4, 423–435

Turtola, S., Sallas, L., Holopainen, J.K., Jalkunen-Tiitto, R., Kainulainen, P., 2006. Long term exposure to enhanced UV-B radiation has no significant effect on growth or secondary compounds of outdoor-grown Scots pine and Norway spruce seedlings. Environ. Expl. Bot., 56, 80–86.

Turunen, M., Latola, K., 2005. UV-B radiation and acclimation in timberline plants. Environ. Pollut., 137, (3), 390–403.

Warren, J.M., Bassman, J.H., Mattinson, D.S., Fellman, J.K., Edwards, G.E., Robberecht, R., 2002. Alteration of foliar flavonoid chemistry induced by enhanced UV-B radiation in field-grown *Pinus ponderosa*, *Quercus rubra* and *Pseudotsuga menziesii*. Journal of Photochemistry and Photobiology B: Biology, 66, 125–133.

Wu, H., Abasova, L., Cheregi, O., Deak, Z., Gao, K., Vass, I., 2011. D1 protein turnover is involved in protection of Photosystem II against UV-B induced damage in the cyanobacterium *Arthrospira* (*Spirulina*) *platensis.* Journal of Photochemistry and Photobiology B: Biology, 104 (1–2), 320–325.

Zeuthen, J., Mikkelsen, T.N., Paludan-Müller, G., Ro-Poulsen, H., 1997. Effects of increased UV-B radiation and elevated levels of tropospheric ozone on physiological processes in European beech (*Fagus sylvatica* L.). Physiol. Plant., 100, 281–290.

Zu, Y-G., Pang, H-H., Yu, J-H., Li, D-W., Wei, X-X., Gao, Y-X., Tong H., 2010. Responses in the morphology, physiology and biochemistry of *Taxus chinensis* var. *mairei* grown under supplementary UV-B radiation. Journal of Photochemistry and Photobiology B: Biology, 98 (2), 152–158.

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Alizarin red S staining of the crustacean cuticle: implementation in the study of *Porcellio scaber* larvae

Histokemijska analiza kutikule rakov z barvilom alizarin rdeče S: uporaba v proučevanju ličink raka enakonožca vrste *Porcellio scaber*

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Abstract: Exoskeletal cuticle of crustaceans is a chitinous matrix, produced apically by epidermis and stiffened by sclerotization and calcification. Embryos of terrestrial isopod crustacean *Porcellio scaber* develop within the female brood pouch, marsupium, and after hatching larvae mancae continue their development in the marsupium for another week. This study was performed to reveal at the histochemical level whether the exoskeletal cuticle of marsupial mancae is already calcified. Fifteen different procedures of histochemical staining with alizarin red S (ARS), established for calcified tissue localization primarily in vertebrate histology, were evaluated on mancae and adult *P. scaber* specimens. The best differential staining of the the exoskeletal cuticle was obtained by neutral buffered formaldehyde fixation, followed by paraffin sections staining with ARS 1 (pH 9) or ARS 2 (pH 6.4) or ARS 3 (pH 4.8) solution. Clear differential staining was achieved also in cryosections of formaldehyde fixed samples, stained with ARS 1 solution (pH 9). Our results suggests that prominent calcification of exoskeletal cuticle is present during postembryonic development of *P. scaber* mancae in the marsupium. Exoskeleton hardening is likely important also for body movements, that we observed in mancae before they are released from marsupium. The proposed procedures of ARS method are presumed to be applicable for histochemical studies of other calcified chitinous matrices.

Keywords: calcification, histochemistry, larval development, terrestrial isopods, Crustacea

Izvleček: Eksoskeletna kutikula rakov je hitinski matriks na apikalni strani epidermisa. Trdnost kutikule je posledica sklerotizacije in kalcifikacije organskega matriksa. Embriji kopenskega raka enakonožca *Porcellio scaber* se razvijajo v vrečastem valilniku samice, marzupiju, kjer po izleganju nadaljujejo svoj razvoj približno en teden tudi ličinke manke. S histokemijsko metodo smo ugotavljali, ali je eksoskeletna kutikula marzupijskih mank že kalcificirana. Na vzorcih mank in odraslih rakov *P. scaber* smo ovrednotili petnajst različnih postopkov histokemijske analize z barvilom alizarin rdeče S (ARS), ki se uporabljajo za lokalizacijo kalcificiranega tkiva v histologiji vretenčarjev. Eksoskeletna kutikula se je izrazito diferencialno obarvala v primeru fiksacije z nevtralno raztopino formaldehida, ki ji je sledilo bar-

vanje parafinskih rezin z eno izmed raztopin ARS: ARS 1 (pH 9), ARS 2 (pH 6.4) ali ARS 3 (pH 4.8). Jasno lokalizacijo smo dosegli tudi z barvanjem zamrznjenih rezin vzorcev, fiksiranih v formaldehidu in barvanih z raztopino ARS 1 (pH 9). Rezultati kažejo na znatno kalcifikacijo eksoskeletne kutikule že v postembrionalnem razvoju mank *P. scaber* v marzupiju. Trdnost eksoskeleta je najverjetneje pomembna tudi za gibanje mank, ki smo ga opazili pred sprostitvijo iz valilnika. Pričakujemo, da bodo postopki, ki jih predlagamo, uporabni tudi za histokemijska proučevanja kalcifikacije drugih hitinskih matriksov.

Ključne besede: kalcifikacija, histokemija, razvoj ličink, kopenski raki enakonožci, Crustacea

Introduction

Mineralized organic matrices constitute many morphologically and functionally diverse structures in different organisms ranging from bacteria to humans. Their unique feature is a prominent mineral component, which is closely connected with the organic matrix. Mineralization increases matrix strength and hardness that provides protection against environmental pressures and support for muscle attachment. A common type of mineralization in living organisms is calcification, the deposition of different calcium minerals in the organic matrices (Bonucci 2007). Well known examples of calcified organic matrices in vertebrates are bones and teeth, whose basic organic component is collagen. Exoskeleton of crustaceans or exoskeletal cuticle is a representative example of a calcified matrix based on chitinous organic scaffold.

The exoskeletal cuticle is a complex hierarchically structured extracellular matrix, consisting of the polysaccharide chitin, proteins, lipids and also minerals. It is produced by a single-layered epidermis during embryonic development and it is renewed periodically during molting in adults. The ultrastructure of exoskeletal cuticle in adult isopod *Porcellio scaber* has been described in detail (Ziegler 1997, Hild et al. 2008, Seidl and Ziegler 2012). It comprises the outermost epicuticle, exocuticle and the inner endocuticle. Thin epicuticle is composed mainly of lipoproteins and consists of thinner 5-layered outer epicuticle and thicker inner epicuticle. Exocuticle and endocuticle are calcified and comprise sublayers of chitin–protein fibers arranged in a characteristic helicoidal pattern (Fig. 1).

Cuticle in adults is calcified due to deposition of crystalline calcium carbonate (calcite), amorphous calcium carbonate (ACC) and amorphous calcium phosphate (ACP) (Ziegler 1994, Ziegler 1997, Becker et al. 2005, Luquet 2012). Recent studies have shown defined spatial distribution of both polymorphs of calcium carbonate in different layers of adult isopod cuticle (Hild et al. 2008, Hild et al. 2009, Neues et al. 2011, Seidl et al. 2011). In *P. scaber* the exocuticle contains both calcite and ACC, whereas the endocuticle is calcified only by ACC (Hild et al. 2008). There are a few data on the structure and composition of the exoskeletal cuticle in embryonic and larval stages in crustaceans. The central focus of this study is to show at the histochemical level whether the larval cuticle in *Porcellio scaber* is calcified or not.

The specimens of *Porcellio scaber* belong to a group of terrestrial isopod crustaceans (Oniscidea). The embryonic development takes place in the fluid-filled brood pouch (marsupium) on the ventral side of female body, that has likely been of a great adaptive significance in the colonization of land by crustacean species (Hornung 2011, Warburg 2011). Intramarsupial development of *P. scaber*, from released fertilized eggs to embryos and marsupial larvae mancae lasts approximately 35 days in laboratory conditions and it was described morphologically with twenty progressive stages (Wolff 2009, Milatovič et al. 2010). After hatching of embryo from two egg envelopes (chorion and vitelline membrane), larva termed manca stays in the marsupium for a week and is subsequently released to the external environment (Supplementary fig. 1).

Our previous study shows that the cuticle in *P. scaber* marsupial mancae exhibits main ul-

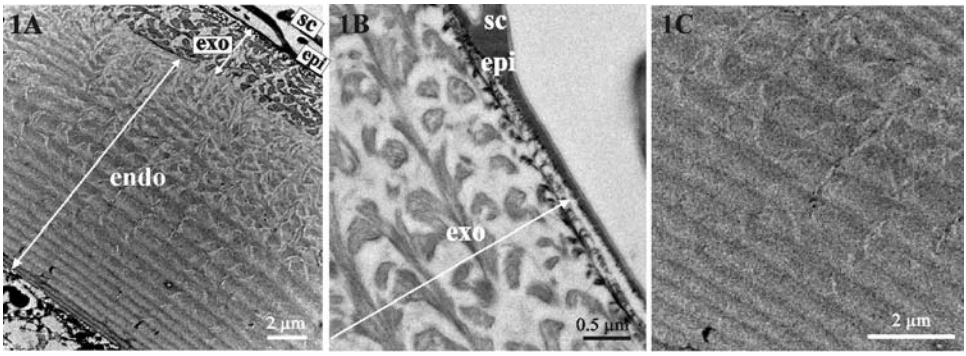

Figure 1: Ultrastructure of the exoskeletal cuticle in adult *P. scaber*. A – Cuticle is organized in distinct horizontal layers: epicuticle (epi), exocuticle (exo) and endocuticle (endo). B – Epicuticle (epi) is a thin and electron dense layer with prominent scales (sc). Exocuticle (exo) comprises chitin–protein fibers, arranged in a characteristic pattern. C – Endocuticle comprises lamellar chitin-protein sublayers.

Slika 1: Ultrastruktura eksoskeletne kutikule odraslega raka *P. scaber*. A – Kutikulo sestavljajo različni sloji: epikutikula (epi), eksokutikula (exo) in endokutikula (endo), kot si sledijo od zunanjega proti notranjemu delu. B – Epikutikula (epi) je tanek, elektronsko gost sloj z izrazitimi luskami na površini (sc). Eksokutikula (exo) vsebuje hitinsko – proteinska vlakna, ki so urejena v značilen vzorec. C – Endokutikula vsebuje lamelarne podsloje hitinsko – proteinskih vlaken.

trastructural features of the adult crustacean cuticle (Mrak et al. 2012). Cuticle of marsupial mancae is composed of three layers, the outermost thin electron dense epicuticle, the middle exocuticle and the innermost endocuticle. The characteristic pattern of chitin-protein fiber arrangement in the exocuticle is already present and sublayers are evident in the endocuticle (Fig. 2). The thickness of cuticle is up to 3 µm. Some morphological features suggesting cuticle renewal are observed occasionally in marsupial mancae, such as cuticle detachment from the epidermis, partly disintegrated inner portion of the endocuticle, assembling of the new cuticle and apical protrusions of epidermal cells with electron dense tips (Fig. 2B).

The data on mineralization of chitin matrix in mancae larvae is very limited. Mancae, released from the marsupium (postmarsupial mancae), were investigated in this respect by Hadley and Hendricks (1987) using energy dispersive X-ray spectroscopy (EDS) in the isopod *Porcellionides pruinosus*. They detected calcium in the cuticle of mancae already released to the external environment, but in much lower quantities compared to the adult cuticle levels. Concerning marsupial mancae, exoskeleton calcification is still an open question.

Localization and characterization of calcified tissues can be performed by different methods, each of them focused to address specific questions regarding tissue composition and structure. Identification of inorganic components can be achieved using different biophysical and morphological methods (Bonucci 2007). The advantage of histochemical techniques is simple and quick performance that is important when screening of many samples is needed to gain preliminary information about the presence of mineralized tissue. On the basis of these results selected samples can be further analysed in detail with advanced and highly demanding biophysical techniques, like: X-ray, neutron and electron diffraction for studying the structure of crystals; energy dispersive X-ray elemental analysis (EDS) and electron energy-loss spectroscopy (EELS) for analysing the presence of specific elements and their distribution in the tissue; infrared and Raman spectroscopy for revealing details about molecular structure and especially mineral forms in the tissue. A commonly used histochemical method to demonstrate calcified tissue, applied also in our study, is alizarin red S (ARS) staining. According to Virtanen and Isotupa (1980) and Lievremont et al. (1982), alizarin red S molecules react with calcium ions via its sulfonate and hydroxyl groups, forming brick-red precipitates (ARS-calcium salts, complexes and chelates). Other cations (like magnesium, barium,

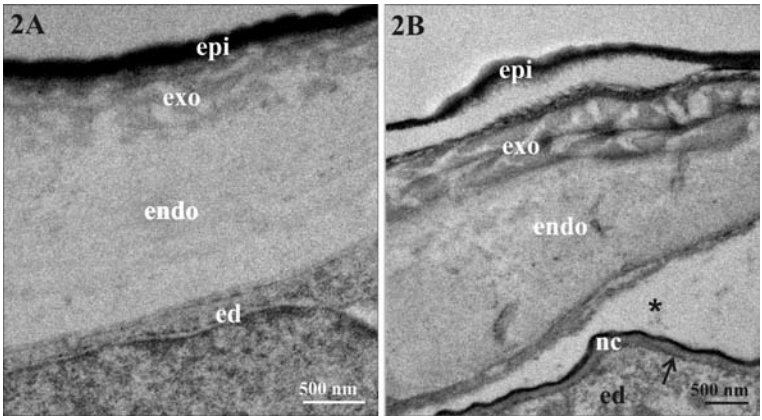

Figure 2: Ultrastructure of *P. scaber* marsupial manca cuticle. Differentiation in epicuticle (epi), exocuticle (exo) and endocuticle (endo) is evident. The micrograph B shows some features of cuticle renewal: cuticle detachment from the epidermis, ecdysal space (*) between the old and the new cuticle, partial disintegration of inner portion of endocuticle, newly assembling cuticle (nc) and protrusions with electron dense tips (arrow) on apical plasma membrane of epidermal cell (ed).

Slika 2: Ultrastruktura kutikule pri marzupijskih mankah raka *P. scaber*. Diferenciacija v epikutikulo (epi), eksokutikulo (exo) in endokutikulo (endo). Mikrografija B prikazuje nekatere značilnosti obnavljanja kutikule, kot so: odstopanje kutikule od epidermisa, levitveni prostor (*) med staro in novo kutikulo, delna razgradnja notranjega dela endokutikule, nastajanje nove kutikule (nc) in izrastki z elektronsko gostimi konicami (puščica) na apikalni plazmalemi epidermalnih celic (ed).

copper, zinc, iron, aluminium) react with ARS as well, but normally only calcium is present in biological tissues in sufficient quantities for demonstration (McGee-Russell 1958, Puchtler et al. 1969, Lievremont et al. 1982). ARS is also an anionic dye and as such causes non-specific pink background staining. According to the literature, that reports several modifications in procedure of the staining technique, the histochemical result is greatly influenced by the fixation method and pH of staining solutions. Fixation method has a significant effect on preservation of calcium in tissue, while pH of staining solution effects staining sensitivity and staining specificity. As this method has relatively low sensitivity, small differences in calcium concentrations can not be distinguished and low calcium concentrations can not be detected.

This study, based on alizarin red S staining, a histochemical approach for calcified tissue localization, was performed to show whether cuticle is calcified already during intramarsupial larval development in isopod crustacean *Porcellio scaber*. Considering many modifications of this method, particularly regarding fixation and staining pH, we have evaluated several procedures for estimating the presence of cuticle calcification in *P. scaber* marsupial mancae. This method is expected to be useful also in the studies of calcification of other chitinous matrices.

Materials and methods

In this study the histochemical localization of calcified tissues by alizarin red S (ARS) was implemented to estimate the presence of exoskeleton calcification in marsupial mancae of the species *Porcellio scaber* Latreille, 1809 (Crustacea: Isopoda). Adult animals were maintained in a laboratory culture, in soil and leaf litter, at 25°C, high relative humidity and a 12-h light/12-h dark cycle. Gravid females with mancae in the marsupium were selected from laboratory culture. Thirteen mancae were isolated from the marsupia, anesthetized and fixed. They were carefully perforated with a thin needle, enabling better infiltration of the fixative and/or the embedding medium. Adult animals that were without any signs of molting were used as positive controls for histochemical reaction. Animals were anesthetized, transversely cut in two pieces and fixed. Subsequently they were always processed simultaneously with the samples of marsupial mancae in all procedures applied. Negative controls of histochemical reaction were the sections of adult and mancae samples preincubated in decalcification solution (10% ethylenediaminetetracetic acid disodium salt (EDTA), pH 7.4, 5 minutes) before ARS staining.

Fixation was performed in five ways (Table 1): (a) three different chemical fixations, (b) chemical fixation followed by freezing or (c) freezing. In the procedure (a) – chemical fixation, three different fixatives were used, recommended by several authors: (a1) Carnoy fixative (absolute ethanol-chloroform-glacial acetic acid, 6:3:1), (a2) 3.7% formaldehyde in 0.1 M cacodylate buffer (pH 7.2) or (a3) 70% ethanol. Formaldehyde is a widely recommended fixative, also for studies of calcium localization (McGee-Russell 1958, Bancroft and Gamble 2008, Kiernan 2008), although some authors prefer Carnoy fixative and describe formaldehyde as unsuitable fixative for studies of calcium deposits as it could remove some calcium during prolonged fixation (Puchtler et al. 1969, Presnell and Schreibmann 1997). Alcoholic fixatives are widely recommended for calcified tissue localization as they preserve calcium in tissue (Puchtler et al. 1969, Presnell and Schreibmann 1997, Bancroft and Gamble 2008, Kiernan 2008), though it is a poor fixative for preservation of tissue structure. After fixation, samples were dehydrated through an ascending series of ethanol and in xylene, infiltrated with paraffin wax at 60 °C overnight and embedded afterwards. Transversal sections (10 µm) were cut with a Leica RM2265 microtome, transferred to water on microscope slides and stretched and dryed on a hot plate.

Samples prepared for paraffin sectioning were exposed to aqueous solutions several times, which could affect the tissue by removing amorphous calcium. Several authors demonstrated high solubility of ACC in aqueous solutions (Brečević and Nielsen 1989, Gal et al. 1996, Meiron et al. 2011). To avoid calcium loss from the tissue, cryosectioning was performed, which minimizes exposure to aqueous solutions. Specimens designated for cryosectioning were either fixed in 3.7% formaldehyde in 0.1 M cacodylate buffer (pH 7.2) and frozen afterwards (procedure b) or directly frozen without any pretreatment (procedure c). They were embedded in tissue freezing medium (Jung). Sections (10 µm) were cut with a Leica CM1850 cryostat at –20°C. Cryosections were transferred directly to microscope slides and dryed at room temperature.

According to the literature, ARS staining is greatly influenced by pH of staining solution. Alkaline ARS solution liberates less calcium and has lower sensitivity but enables more precise localization of calcified tissue (Puchtler et al. 1969, Kiernan 2008). Acidity of ARS solution leads to releasing of more calcium ions from tissue and consequently calcium localization appears more dispersive and sensitivity for calcified tissue demonstration appears higher. Paraffin sections and cryosections were stained by three different staining solutions of ARS, that were recommended by several authors (McGee-Russell 1958, Puchtler et al. 1969, Presnell and Schreibmann 1997, Bancroft and Gamble 2008, Kiernan 2008). ARS solution 1 (purple-red coloured) was 0.5% solution in 0.2 M Trihydroxylmethyl aminomethane (Tris-HCl) buffer, pH 9. ARS solution 2 (dark red coloured) was 1% aqueous solution, adjusted to pH 6.4 with 10% NH_4OH. ARS solution 3 (brown-red coloured) was 1% aqueous solution, adjusted to pH 4.8 with 10% ammonium hydroxide (NH_4OH). Before staining, paraffin sections were deparaffinized and rehydrated in a descending series of ethanol to distilled water and after staining, they were dehydrated and mounted in synthetic resin Pertex. Cryosections were stained directly and after staining they were mounted in glicerol jelly. Staining duration was 20-30 seconds for paraffin sections and 10 seconds for cryo sections, followed by a quick rinse in distilled water. The applied procedures are summarized in Table 1.

Sections were imaged by Zeiss AxioImager Z.1 light microscope, equipped with a HRc AxioCam camera and Axiovision software. Cryosections were inspected immediately after mounting. Intensity of the histochemical reaction with ARS was classified semiquantitatively in four categories: (i) no staining, (ii) light staining, (iii) moderate staining and (iv) intense staining.

Results

In this study the calcification of exoskeleton in marsupial mancae of *Porcellio scaber* Latreille, 1809 (Crustacea: Isopoda) was estimated at the histochemical level by alizarin red S (ARS) method for calcified tissues localization. Several methods have been suggested for ARS staining and here we tested five different fixations in combination with three different staining solutions, altogether fifteen different procedures, on *P. scaber* marsupial

Table 1: Summary of the fixation and alizarin red S staining procedures, used in this study on *Porcellio scaber* marsupial mancae.

Tabela 1: Povzetek postopkov fiksacije in histokemijske lokalizacije z barvilom alizarin rdeče S, uporabljenih v tej študiji marzupijskih mank raka enakonožca *Porcellio scaber*.

	(a) chemical fixation		**(b) chemical fixation followed by freezing**	**(c) freezing at –20 °C**
(a1) Carnoy fixative	(a2) neutral buffered 3.7% formaldehyde	(a3) 70% ethanol	neutral buffered 3.7% formaldehyde	
	↓		↓	
	dehydration		tissue freezing medium embedding and freezing	
	↓		↓	
	paraffin embedding			
	↓			
	paraffin sectioning		cryosectioning	
	↓			
	deparaffinization and rehydration			
	↓			
	staining in alizarin red S solution 1 (pH 9) or 2 (pH 6.4) or 3 (pH 4.8)			
	↓			
	quick rinse in distilled water			
	↓		↓	
	dehydration			
	↓			
	mounting in resin		mounting in glicerol jelly	

mancae. We analysed the staining intensity of mancae cuticle in comparison to other tissues (background) and regarding positive and negative controls of histochemical reaction.

In Carnoy fixed specimens (procedure a1) no differential staining of the cuticle was obtained, neither in adults nor in mancae sections (Table 2, Supplementary fig. 2). ARS staining of Carnoy fixed specimens resulted in light red staining of all tissues with basic ARS solution (ARS 1). ARS 2 (pH 6.4) and ARS 3 (pH 4.8) solutions resulted in deeper red staining, displaying intensely stained cuticle and moderately stained other tissues, i.e. connective tissue and muscles (Table 2, Supplementary fig. 2).

ARS staining of neutral buffered formaldehyde fixed specimens (procedure a2) resulted in clearly differential staining of exoskeleton (Table 2, Supplementary fig. 3). The exoskeletal cuticle in adults and mancae was intensely red, while other tissues like glands and muscles were not stained. Negligible background was visible with acid ARS staining solution (ARS 3, pH 4.8). In specimens pretreated in decalcification solution (EDTA) for negative controls, the cuticle was not red stained with ARS and only in the case of ARS 2 and ARS 3 staining solutions negligible background was observed (Supplementary fig. 3).

The overall histological structure of specimens fixed in 70 % ethanol (procedure a3) was not so well preserved in comparison to specimens fixed in formaldehyde (Supplementary fig. 4). We did not obtain any undoubtedly differential staining of the exoskeleton in ethanol fixed samples (Table 2, Supplementary fig. 4). In all mancae and adult sections cuticle and other tissues were stained nearly with the same intensity, except for the more intensely stained adult cuticle with basic ARS (ARS 1). In negative controls (pretreated in EDTA) a very faint red staining was noticeable (Supplementary fig. 4).

In cryosections of neutral buffered formaldehyde fixed and frozen samples (procedure b) a clearly differential staining of exoskeletal cuticle

Table 2: Summary of Alizarin red S staining results of *Porcellio scaber* marsupial mancae and adults: Specimens were fixed by five different methods: (a1) Carnoy fixative, (a2) 3.7% neutral buffered formaldehyde, (a3) 70% ethanol, (b) 3.7% neutral buffered formaldehyde, followed by freezing and cryosectioning or (c) freezing and cryosectioning. Staining was performed with one of the following Alizarin red S solutions: ARS 1 (pH 9), ARS 2 (pH 6.4) or ARS 3 (pH 4.8). Intensity of ARS staining is classified as: ○ – no staining; * – light staining; ** – moderate staining; *** – intense staining. 'Diffusion' marks diffusion artifacts. Where different staining intensities were obtained for mancae and adults, both results are presented separately: mancae / adults. Gray labeled fields mark the procedures that resulted in clearly differential staining of exoskeletal cuticle in comparison to other tissues, indicating their suitability for calcified exoskeleton localization.

Tabela 2: Povzetek rezultatov histokemijske reakcije z barvilom alizarin rdeče S pri marzupijskih mankah in odraslih rakih enakonožcih *P. scaber*: Vzorce smo fiksirali na pet različnih načinov: (a1) s fiksativom Carnoy, (a2) s 3.7% nevtralno raztopino formaldehida, (a3) s 70% etanolom, (b) s 3.7% nevtralno raztopino formaldehida in zamrzovanjem ali (c) samo z zamrzovanjem. Za barvanje smo uporabili eno od naslednjih raztopin barvila alizarin rdeče S: ARS 1 (pH 9), ARS 2 (pH 6.4) ali ARS 3 (pH 4.8). Intenziteta ARS barvanja je označena z naslednjimi kategorijami: ○ – brez obarvanja; * – rahlo obarvanje; ** – zmerno obarvanje; *** – intenzivno obarvanje. 'Diffusion' označuje difuzijski artefakt. Kjer je bila intenziteta obarvanja različna pri mankah in odraslih, sta navedena oba rezultata ločeno na način: manke / odrasli. Sivo obarvana polja označujejo postopke, pri katerih se je eksoskeletna kutikula izrazito diferencialno obarvala v primerjavi z drugimi tkivi, kar kaže na to, da so primerni za lokalizacijo kalcificiranega eksoskeleta.

procedure	(a) chemical fixation and paraffin sectioning					
	(a1) Carnoy fixative		(a2) 3.7% formaldehyde		(a3) 70% ethanol	
staining solution	exoskeletal cuticle	other tissues	exoskeletal cuticle	other tissues	exoskeletal cuticle	other tissues
ARS 1 (pH 9)	*	*	***	○	***	**
ARS 2 (pH 6.4)	***	**	***	○	***	***
ARS 3 (pH 4.8)	***	**	***	○	***	***

procedure	(b) 3.7% formaldehyde, freezing and cryosectioning		(c) freezing and cryosectioning	
staining solution	exoskeletal cuticle	other tissues	exoskeletal cuticle	other tissues
ARS 1 (pH 9)	***	*	***	**/*
ARS 2 (pH 6.4)	*** diffusion	*/**	**/*** diffusion	*/**
ARS 3 (pH 4.8)	*** diffusion	*/**	**/*** diffusion	*/***

in adults and mancae was obtained by ARS 1 solution (pH 9) (Table 2, Supplementary fig. 5). Staining in ARS 2 (pH 6.4) and ARS 3 (pH 4.8) resulted in intensely red exoskeleton in adults and mancae, but in the sections of adult specimens considerable staining of other tissues was also evident. In addition, a diffuse red staining in the close vicinity of exoskeletal cuticle was observed in all samples subjected to ARS 2 or ARS 3 solutions. Diffusion of stain occurs as a consequence of calcium salts solubility in staining solutions and is termed diffusion artifact. Negative controls (pretreated in EDTA) showed no red staining in ARS 1 and a very faint colouring in ARS 2 and ARS 3 (Supplementary fig. 5).

In the specimens which were not chemically fixed and were frozen only (procedure c), staining was not clearly differential, except for the basic ARS staining (ARS 1) of adult cuticle, where exoskeleton was intensely red and only a light

background was visible (Table 2, Supplementary fig. 6). In all other stainings applied to frozen only sections various difficulties regarding histochemical reaction were encountered: (i) staining of other tissues, (ii) diffusion artifacts and (iii) nonuniform staining of different slides or sections that was evident especially in mancae. Negative controls displayed no staining, only in sections of adults pretreted with EDTA and exposed to acid staining solution a faint colouring was visible (Supplementary fig. 6).

Discussion

Histological demonstration of calcified tissues is commonly performed by alizarin red S (ARS) and von Kossa's methods (Bancroft and Gamble 2008). Although a great variety of other more advanced and sophisticated methods for calcium demonstration is available, histological methods are beneficial for examination of numerous samples to gain preliminary coarse information about the calcified tissue localization. Von Kossa's method is not specific for the calcium cations, but depends on the presence of the salt anion (carbonate, phosphate, oxalate), while alizarin red S (sodium alizarin sulphonate) reacts with calcium (McGee-Russell 1958, Bancroft and Gamble 2008). It reacts also with other metallic cations like copper, magnesium, barium, zinc, iron, aluminium, etc, but generally they are not present in biological structures in sufficient quantities for histochemical demonstration (Puchtler et al. 1969, Lievremont et al. 1982). A great variety of Alizarin red S method modifications are reported in the literature. Modifications involve mainly differences in tissue fixation and in pH of staining solution. Fixation methods and pH of staining solution have an effect on calcium preservation in tissue, staining sensibility and specificity. Acidity of ARS solution leads to releasing of more calcium ions from tissues (Puchtler et al. 1969, Kiernan 2008). Consequently calcium localization appears more dispersive and sensitivity for calcified tissue demonstration appears higher. Basic ARS solution has lower sensitivity but enables more precise localization of calcified tissue. ARS usually slightly stains the background tissue as well as it is an anionic dye (Puchtler et al. 1969, Kiernan 2008). Previous studies that include method of ARS staining are particularly based on vertebrates calcified tissues, while systematic evaluations of this method for other calcified biological systems rarely occur. Here we show a comparison of fifteen different modifications of ARS method applied to mancae and adult isopod crustaceans, to establish a quick, simple and inexpensive procedure appropriate to screen a large number of samples to estimate the presence of cuticle calcification.

The best differential staining of the exoskeletal cuticle in marsupial mancae and in adults as positive control was achieved by 3.7% formaldehyde fixation (2 days) and paraffin sections staining with ARS 1 (pH 9), ARS 2 (pH 6.4) or ARS 3 (pH 4.8) solution. The exoskeletal cuticle was specifically stained in all samples and the background remained unstained. Samples kept in neutral formaldehyde solution for a month, did not give positive staining of mancae cuticle (data not shown). Although formaldehyde is a widely recommended fixative, some authors described it as unsuitable fixative for studies of calcium deposits as it could remove some calcium during prolonged fixation (Puchtler et al. 1969, Presnell and Schreibmann 1997).

Alcoholic fixatives are widely recommended for calcified tissue localization as it is said they preserve calcium in tissue (Puchtler et al. 1969, Presnell and Schreibmann 1997, Bancroft and Gamble 2008, Kiernan 2008), though less adequate preservation of tissue structure could be caused by dehydrating effect. Since ARS staining of 70% ethanol fixed specimens in our study was not clearly specific, with intense or moderate staining of the background, we consider that 70% ethanol is not a suitable fixative for differential calcified cuticle localization. Staining of Carnoy fixed specimens was also not clearly differential, as in additon to exoskeletal cuticle all other tissues were stained too. We included this fixation in our study as it was recommended for vertebrate calcified tissues by Puchtler et al. (1969) and Presnell and Schreibmann (1997). Our results also showed that all Carnoy fixed tissues were less intensely stained in basic ARS solution in comparison to other two ARS solutions. We conclude that Carnoy fixative is not suitable for calcified cuticle identification.

Next, we performed cryosectioning to minimize exposure of samples to aqueous solutions, that could cause loss of amorphous forms of calcium from tissue as it is known that amorphous calcium

carbonate has high solubility in water (Brečević and Nielsen 1989, Gal et al. 1996, Meiron et al. 2011). These methods keep most of the tissue components intact and are considered a better choice to study tissue composition, in spite of the fact that tissue structures appear poorly resolved. These methods are also less time consuming than conventional histological methods. Cryosections of samples fixed in neutral buffered formaldehyde and stained by basic ARS solution (ARS 1, pH 9) resulted in evidently differential staining of exoskeletal cuticle in marsupial mancae and in adults (a positive control). In all other procedures applied to specimens frozen after chemical fixation and to frozen only specimens, the histochemical reaction was either not differential or other imperfections were encountered, like diffusion artifacts and nonuniform staining. Diffusion artifacts, observed after staining in neutral and acid ARS solutions, were described also by Puchtler et al. (1969) in human tissues. Diffusion artifacts presumably appear due to the higher solubility of calcium salts in acid solutions in comparison to basic solutions. Nonuniform staining of sequential slides or sequential cryosections on the same slide that we observed in the specimens of frozen only mancae were possibly due to minimal variations in washing after staining.

Our results showed that cuticle of marsupial mancae was intensely and differentially stained by four different Alizarin S histochemical procedures, which also resulted in the differential staining of the exoskeletal cuticle in adults. These results suggest that prominent calcification of exoskeletal cuticle is present during postembryonic development of *P. scaber* mancae in the marsupium. Calcification provides hardness of exoskeleton that enables its protective role and mobility of the animal. Our findings show an importance of cuticle calcification for exoskeleton rigidity in mancae before they leave the marsupium. These findings support previous suggestions made by Surbida and Wright (2001) and Ouyang and Wright (2005), that do not give direct evidence of cuticle calcification since the aims of these studies were focused to other issues, like investigations of osmotic tolerance and total calcium concentration in developmental stages. Surbida and Wright (2001) presume that wide osmotic tolerance of *Armadillidium vulgare* marsupial mancae is a consequence of calcification of their cuticle. Ouyang and Wright (2005) suggest that cuticle calcification starts in the stage of marsupial manca, as they observed the increase of total calcium concentration in isopod *Armadillidium vulgare* late-stage marsupial manca. In order to address the issue of calcium forms in marsupial mancae additional analytical methods for demonstration of mineral forms should be performed.

Conclusions

Exoskeletal cuticle of marsupial mancae and adults of *P. scaber* was differentially stained by the following varieties of the histochemical Alizarin red S method:

(a) in paraffin sections of formaldehyde fixed samples, stained with Alizarin red S solutions ARS 1 (pH 9), ARS 2 (pH 6.4) or ARS 3 (pH 4.8) and
(b) in cryosections of samples fixed in formaldehyde, stained with basic ARS solution (ARS 1, pH 9).

This study suggests that prominent calcification of exoskeletal cuticle occurs already in marsupial mancae of isopod crustacean *P. scaber*. Exoskeleton hardening is likely important also for body movements, that we observed in mancae before they are released from marsupium.

Alizarin red S procedures that resulted in distint differential staining of exoskeletal cuticle in this study are expected to be applicable for localization of calcified chitinous matrices in other species.

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Povzetek

Eksoskeletna kutikula rakov je apikalni zunajcelični matriks epidermisa, osnovan na hitinskem organskem ogrodju in utrjen s sklerotizacijo in kalcifikacijo. Tvorba nove kutikule poteka v zgodnjem razvoju in ob vsaki levitvi pri odraslih

osebkih. Pomemben proces v formiranju nove kutikule je tudi kalcifikacija, nalaganje kalcijevih mineralov v organski matriks. Embriji kopenskega raka enakonožca vrste *Porcellio scaber* se razvijajo v vodnem okolju valilnika (marzupija), ki je vrečasta struktura na trebušni strani samice. Embriji se izležejo v ličinke manke, ki nadaljujejo razvoj v valilniku še približno teden dni. Predhodno smo ugotovili, da kutikula marzupijskih mank kaže osnovne ultrastrukturne značilnosti kutikule odraslih živali, kot so organizacija v glavne sloje – epikutikulo, eksokutikulo in endokutikulo ter razporeditev hitinsko – proteinskih vlaken v značilen vzorec (Mrak in sod. 2012). V tej študiji smo ugotavljali, ali je eksoskeletna kutikula pri marzupijskih mankah že kalcificirana. V ta namen smo uporabili histokemijsko tehniko za lokalizacijo kalcificiranega tkiva z barvilom alizarin rdeče S (ARS), ki omogoča preprost in hiter pregled večjega števila vzorcev in je osnova za nadaljne bolj zahtevne in natančne tehnike. Glede na to, da je v literaturi predlaganih več modifikacij te metode, ki se primarno uporablja v histologiji vretenčarjev, smo izvedli pet različnih načinov fiksacije tkiva: (a1) v fiksativu Carnoy, (a2) v 3.7% nevtralni raztopini formaldehida ali (a3) v 70% etanolu, (b) fiksacija v 3.7% nevtralni raztopini formaldehida in zamrzovanje ali (c) samo zamrzovanje. Barvali smo s tremi različnimi raztopinami barvila: ARS 1 (pH 9), ARS 2 (pH 6.4) ali ARS 3 (pH 4.8). Za pozitivno kontrolo smo uporabili barvanje eksoskeletne kutikule odraslih živali, za negativo kontrolo pa predhodno inkubacijo rezin odraslih živali in mank v dekalcifikacijski raztopini EDTA. Eksoskeletna kutikula marzupijskih mank in odraslih živali se je izrazito diferencialno obarvala pri vzorcih fiksiranih v nevtralni raztopini formaldehida, vklopljenih v parafin in barvanih z eno izmed raztopin barvila Alizarin rdeče S: ARS 1 (pH 9), ARS 2 (pH 6.4) ali ARS 3 (pH 4.8). Da bi se čimbolj izognili vodnim raztopinam, v katerih so amorfne oblike kalcijevih soli dobro topne, smo v študijo vključili fiksacijo vzorcev z zamrzovanjem in barvanje kriostatskih rezin. Pri tej tehniki se je eksoskeletna kutikula diferencialno obarvala v primeru zamrznjenih rezin vzorcev, predhodno fiksiranih v formaldehidu, ki smo jih barvali z bazično raztopino ARS 1 (pH 9). Postopki histokemijske lokalizacije z barvanjem ARS, ki so se izkazali kot primerni, bodo predvidoma uporabni tudi pri študijah kalcifikacije drugih hitinskih matriksov.

Eksoskeletna kutikula marzupijskih mank *P. scaber* se je izrazito diferencialno obarvala s štirimi različnimi postopki metode ARS, pri katerih smo enako diferencialno obarvanje dobili tudi v primerih eksoskeletne kutikule pri odraslih (pozitivne kontrole). Ti rezultati kažejo na znatno kalcifikacijo eksoskeletne kutikule že v razvojnem obdobju pred sprostitvijo v zunanje okolje. Eksoskelet se torej oblikuje in kalcificira že pri ličinkah mankah v marzupiju, kar je najverjetneje pomembno tudi za gibanje mank, ki smo ga opazili pred sprostitvijo iz valilnika. Za ugotavljanje oblik kalcijevih soli v eksoskeletu marzupijskih mank bi bilo v nadaljevanju dela potrebno uporabiti analitske metode za identifikacijo mineralnih oblik, kot sta npr. infrardeča in Raman spektroskopija.

References

Bancroft, J.D., Gamble, M., 2008. Theory and Practice of Histological Techniques, 6th ed. Churchill Livingstone Elsevier, pp. 249–250.

Becker, A., Ziegler, A., Epple, M., 2005. The mineral phase in the cuticles of two species of Crustacea consists of magnesium calcite, amorphous calcium carbonate and amorphous calcium phosphate. Dalton Trans, 1814–1820.

Bonucci, E., 2007. Methodology. In: Schreck, S. (ed.): Biological calcification: Normal and Pathological Processes in the Early Stages. Springer – Verlag, Heidelberg, pp. 23–51.

Brečević, L., Nielsen, A.E., 1989. Solubility of amorphous calcium carbonate. J Cryst Growth, 98 (3), 504–510.

Gal, J.-Y., Bollinger, J.-C., Tolosa, H., Gache, N., 1996. Calcium carbonate solubility: a reappraisal of scale formation and inhibition. Talanta, 43, 1497–1509.

Hadley, N.F., Hendricks, G.M., 1987. X-ray microanalysis of the cuticle surface of the terrestrial isopod *Porcellionides pruinosus***.** Can J Zool, 65, 1218–1223.

Hild, S., Marti, O., Ziegler, A., 2008. Spatial distribution of calcite and amorphous calcium carbonate in the cuticle of the terrestrial crustaceans *Porcellio scaber* and *Armadillidium vulgare*. Struct Biol, 163 (1), 100–108.

Hild, S., Neues, F., Žnidaršič, N., Štrus, J., Epple, M., Marti, O., Ziegler, A., 2009. Ultrastructure and mineral distribution in the tergal cuticle of the terrestrial isopod *Titanethes albus*. Adaptations to a karst cave biotope. J Struct Biol, 168, 426–436.

Hornung, E., 2011. Evolutionary adaptation of oniscidean isopods to terrestrial life: Structure, physiology and behavior. Terrestrial Arthropod Reviews, 4 (2), 95–130.

Kiernan, J.A., 2008. Histological and Histochemical Methods: Theory and Practice, 4th ed. Scion Publishing Limited, Bloxham, pp. 338–339.

Lievremont, M., Potus, J., Guillou, B., 1982. Use of alizarin red S for histochemical staining of Ca^{2+} in the mouse; some parameters of the chemical reaction in vitro. Acta anat, 114, 268–280.

Luquet, G., 2012. Biomineralization: insights and prospects from crustaceans. Zookeys, 176, 103–121.

McGee-Russell, S.M., 1958. Histochemical methods for calcium. J Histochem and Cytochem, 6, 22–42.

Meiron, O.E., Bar-David, E., Aflalo, E.D., Shechter, A., Stepensky, D., Berman, A., Sagi, A., 2011. Solubility and bioavailability of stabilized amorphous calcium carbonate. J Bone Miner Res, 26 (2), 364–372.

Milatovič, M., Kostanjšek, R., Štrus, J., 2010. Ontogenetic development of *Porcellio scaber*: Staging based on microscopic anatomy. J Crustacean Biol, 30 (2), 225–235.

Mrak, P., Žnidaršič, N., Tušek-Žnidarič, M., Klepal, W., Gruber, D., Štrus, J., 2012. Egg envelopes and cuticle renewal in *Porcellio* embryos and marsupial mancas. Zookeys, 176, 55–72.

Neues, F., Hild, S., Epple, M., Marti, O., Ziegler, A., 2011. Amorphous and crystalline calcium carbonate distribution in the tergite cuticle of moulting *Porcellio scaber* (Isopoda, Crustacea). J Struct Biol, 175 (1), 10–20.

Ouyang, D., Wright, J., 2005. Calcium accumulation in eggs and mancas of *Armadillidium vulgare* (Isopoda: Oniscidea). J Crustacean Biol, 25 (3), 420–426.

Presnell, J.K., Schreibmann, M.P., 1997. Humanson's Animal Tissue Techniqes, 5th edition. The Johns Hopkins University Press, Baltimore in London, pp. 223–224.

Puchtler, H., Meloan, S.N., Terry, M.S., 1969. On the history and mechanism of alizarin and alizarin red S stains for calcium. J Histochem and Cytochem, 17 (2), 110–124.

Seidl, B.H.M., Huemer, K., Neues, F., Hild, S., Epple, M., Ziegler, A., 2011. Ultrastructure and mineral distribution in the tergite cuticle of the beach isopod *Tylos europaeus* Arcanglei, 1938. J Struct Biol, 174, 512–526.

Seidl, B.H.M., Ziegler, A., 2012. Electron microscopic and preparative methods for the analysis of isopod cuticle. Zookeys, 176, 73–85.

Surbida, K.L., Wright, J.C., 2001. Embryo tolerance and maternal control of the marsupial environment in *Armadillidium vulgare* Brandt (Isopoda: Oniscidea). Physiol Biochem Zool, 74, 894–906.

Virtanen, P., Isotupa, K., 1980. Staining properties of alizarin red S for growing bone in vitro. Acta anat, 108, 202–207.

Warburg, M.R., 2011. The oniscid isopod female reproductive system and gestation, with a partial review. Invertebr Reprod Dev, 1–24.

Wolff, C., 2009. The embryonic development of the malacostracan crustacean *Porcellio scaber* (Isopoda, Oniscidea). Dev Genes Evol, 219, 545–564.

Ziegler, A., 1994. Ultrastructure and electron spectroscopic diffraction analysis of the sternal calcium deposits of *Porcellio scaber* Latr. (Isopoda, Crustacea). J Struct Biol, 112, 110–116.

Ziegler, A., 1997. Ultrastructural changes of the anterior and posterior sternal integument of the terrestrial isopod *Porcellio scaber* Latr. (Crustacea) during the moult cycle. Tissue Cell, 29 (1), 63–76.

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Learning the process of the cell cycle in 13- and 14 year-olds

Učenja procesa celičnega cikla pri 13 in 14 letnikih

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Abstract: The new biology curriculum introduced the teaching of mitosis to 13- and 14-year-olds students in Slovenia. Mitosis is a challenging topic for this age. In our study, we enrolled a sample group of 95 students to check if the method of teaching mitosis first described by Danieley (1990) could be effective for students of this age. Prior to the survey, the students had not yet dealt with the division of cells; most of them did not even know that all living organisms are made of cells. The results show that this method is effective; enrolled students used logical reasoning and were thus able to understand how the events in the cell cycle and the process of mitosis follow one another. The majority of students correctly arranged 15 drawings presenting the stages of the cell cycle after the lesson, and their knowledge retention was satisfactory. Incorrect placements of drawings did not show any typical mistakes in students thinking about the cell cycle that should create specific concerns for the biology teachers.

Keywords: mitosis, cell cycle, understanding, biology, 13-year-olds, 14-year-olds

Izvleček: Novi učni načrt v Sloveniji je vpeljal poučevanje mitoze za učence, stare 13 in 14 let. Mitoza je za to starost zahtevna tema. V naši raziskavi smo na vzorcu 95 učencev preverili, ali je metoda poučevanja mitoze, ki jo je prva predstavila Danieley (1990), lahko učinkovita za učence te starosti. Učenci pred raziskavo še niso obravnavali delitve celice, večina tudi ni vedela, da so vsi organizmi zgrajeni iz celic. Rezultati kažejo, da je uporabljena metoda učinkovita; učenci so z uporabo logičnega razmišljanja bili sposobni razumeti, kako si sledijo dogodki v procesu celičnega cikla in mitoze. Večina učencev je po učni uri pravilno razporedila 15 slik, ki predstavljajo faze celičnega cikla. Njihovo znanje je bilo zadovoljivo trajno. Nepravilne razporeditve niso pokazale nikakršnih značilnih napak v razmišljanju učencev glede celičnega cikla, na katere bi morali biti učitelji biologije pri poučevanju posebej pozorni.

Ključne besede: mitoza, celični cikel, razumevanje, biologija, 13-letniki, 14-letniki

Introduction

Cell biology is a wide, complex, and rapidly evolving field of biology which, as we know from school practice, causes a lot of trouble for students as well as for teachers. Highly competent teachers are needed for effective teaching of cell biology; research has shown, however, that prospective teachers of biology have a deficit of cell biology knowledge (Dikmenli, 2010). Students have difficulties understanding and integrating this knowledge (Castro, 2009; Lewis and Kattmann, 2004; Locke and McDermid, 2005; Mbajiorgu et al., 2007; Štraus et al., 2006; Venville and

Treagust 1998; Venville et al., 2005; Williams et al., 2012). Many students learn science topics as isolated facts and do not construct links between old and new knowledge. As a consequence they find it difficult to understand subsequent topics (Smith, 1988). BouJaoude (as cited in Cavallo, 1996) even says that students consistently learn by memorizing, and as a result form misconceptions about scientific concepts. In addition, the factual way of obtaining knowledge can be frustrating for students, and therefore draws them away from science in school and later in career choices (Novak, 1988).

In the modern world it is essential to understand the basic concepts of cell biology to obtain efficient scientific literacy of citizens (Venville et al., 2005). Therefore, several authors and institutions around the world have attempted to improve the teaching of cell biology in order to change students' misconceptions in the most reliable way, while at the same time helping students achieve high levels of expertise that includes understanding and the ability to apply acquired knowledge. Wyn and Stegink (2000) proposed actively involving middle, high school and college students in the learning of mitosis by role-playing. Similarly, sock and yarn modeling can engage middle, high school, and college students in the lesson of meiosis (Stavroulakis, 2005). Locke and McDermid (2005) found undergraduate students responded well when engaged and activated in the manipulation of a pool of noodles which represented chromosomes and chromatids in mitosis. Danieley (1990; see also Shields, 2006) and Lawson (1991) proposed teaching mitosis through a learning cycle. Lawson's lesson includes exploring actual plant tissues and an investigation and was developed for use in high schools. Danieley's lesson includes observing the drawings of individual cells and focuses on understanding development in the process of mitosis.

In Slovenia, elementary school provides education from grades 1 to 9. The students are generally aged between 6 and 14. We are now in the period of the introduction of the new biology curriculum for the grades 8 and 9, so we are looking for solutions that would enable the general population to understand the foundations of cell biology. Teachers are faced with problems of: (1) how to present cell biology content in the most comprehensive manner; and (2) how to provide students with what they'll need for everyday life, as well as a solid foundation for any further education. When deciding what will be taught and in what order, it is important to consider that students find mechanisms in cell biology difficult to understand because of the difficulty in presenting this topic with no specific instruments (Mbajiorgu et al., 2007). This topic also requires a certain level of abstract thinking (Banet and Ayuso, 2000; Smith and Sims, 1992). Lawson and Thompson (1988) found, that the reasoning ability of studentswas statistically and significantly related to the number of misconceptions in genetics. Students on the level of concrete operations held more misconceptions. On the other hand, Smith and Sims (1992) came to the conclusion that formal operational thought is not strictly required for the solution of the majority of classical genetics problems, and that studentspossess the cognitive skills that are needed to address the most typical problems in classical genetics. In addition, there are technics of teaching available that contribute to a greater understanding of genetics concepts.

Purpose of the Study

We started from the realization that students in general have difficulties in learning biological processes (Dikmenli, 2010; Strgar, 2010; Straus, 2006; Venville et al., 2005). Cavallo (1996) found that there were very few students who were able to connect conceptual knowledge of meiosis with procedural knowledge, so phases of the process of meiosis. She says that we should consider whether the greater amount of knowledge about the stages of meiosis is necessary for a better understanding of meiosis. She also suggested that it should be checked, whether detailed instructions on the stages of meiosis may present an obstacle or interfere with students in forming the concepts of meiosis. We found that the method of teaching/learning a similar process i.e. the process of mitosis first presented by Danieley (1990; see also Shieds, 2006) was in accord with Cavallo (1996). It focuses on the logic of the process of mitosis and avoids giving too many details. Besides that Danieley's (1990) method also employs a non-traditional sequence of learning content, which could have some impact on the level of understanding of mitosis

in students. Teachers in teaching genetics mostly stick to traditional teaching methods and traditional sequencing of learning content, and use similar learning strategies (Watts and Jofili, 1998).

In 2011 a new curriculum came into use in Slovenia, according to which students start to learn mitosis at the age of 13 (beginning in school year 2012–2013). The previous curriculum has anticipated teaching mitosis at the age of 14 (so one year later). The purpose of our study, therefore, was to establish whether Danieley's (1990) method could be an effective way of teaching mitosis for 13 year-olds.

Material and methods

Participants

The study was conducted in the autumn of 2011 on a group of 95 students. In this sample there were 39 (41.1%) students aged 13 (8th grade of elementary school in the Slovenian school system) and 56 (58.9%) students aged 14 (9th grade). The sample group consisted of 51 (53.7%) girls and 44 (46.3%) boys. None of the enrolled had learned about mitosis at school yet at the time of this study.

The students in our group were an average population according to the results of their self-evaluation: They were moderately successful in biology (M = 3.15, SD = 0.977), biology was somewhat easier to nearly as difficult for them as for their classmates (M = 3.32, SD = 0.941). They learned biology materials at a moderate speed in comparison to their classmates (M = 2.99, SD = 1.092). On these three issues the 13-year-olds responded similarly to 14-year-olds (all ps > 0.05). On two issues girls responded similarly to boys (all ps > 0.05). However, there was a difference between genders in one question; boys more often thought that biology was easier for them than for their classmates (Mann- Whitney test, U = 787.000, z = –2.540, p < 0.05).

We also checked students' attitudes toward biology. The students in our sample did not want to learn biology (M = 2.39, SD = 1.147), they did not want to be taught more biology in school (M = 2.26, SD = 1.206), they did not want a job which would use biology (M = 2.04, SD = 1.077), and were undecided whether knowledge of biology would help them in everyday life (M = 3.10, SD = 1.183). The 13-year-olds responded similarly to the 14-year-olds (all ps > 0.05), and the girls responded similarly to boys (all ps > 0.05).

Tests

Pre-test (before the lesson)

We prepared a pre-test which consists of three units. The first unit was a short knowledge test to check the students' knowledge before the lesson. We asked them one basic question concerning cells ("Which of the following organisms are made of cells: bacterium, bee, human, oak, paramecium and fungus?") and four questions concerning mitosis ("What is mitosis?", "Where does it take place?", "When does it take place?", and "Why does it take place?").

The second unit of the pre-test was a worksheet with 15 drawings of the stages of the cell cycle (Shields, 2006). The drawings in the worksheet were focused on the movement of chromosomes. Stage 1 represented a cell with a visible nucleus; stage 2 represented a cell with a chromatin. Stages 3–9 showed a logical sequence, starting from two-chromatid chromosomes which then get separated, to where eventually each chromatid moves to the opposite pole of the cell. Stages 10–13 showed another logical sequence in which the division of the cell begins, thus, the mitotic spindle is less and less visible. Stage 14 represented an almost divided cell with a chromatin, and stage 15 represented the two daughter cells with visible nuclei. The 15 drawings of the stages of the cell cycle were randomly mixed in a worksheet. Students who had not learned these subjects in school by the time of our research had to arrange drawings in a correct sequence using only logic.

The third unit of the pre-test consisted of seven questions, to which the students responded with the help of a 5-point Likert scale (1 – completely disagree, 5 – completely agree), giving their agreement with given statesments. This is how we gathered data about the performance of students in biology class (3 questions), and about their attitude towards biology (4 questions).

Post-test (immediately after the lesson)

Immediately after the lesson, all students again arranged 15 drawings of the stages of the cell cycle

in a worksheet. The purpose of this test was to establish the quality and quantity of knowledge that students mastered during the lesson.

Late post-test (four weeks after the lesson)

An identical worksheet for the classification of the 15 drawings of the stages of the cell cycle was given to the students again four weeks after the lesson. The purpose of this test was to establish the retention level of knowledge that students mastered during the lesson.

Procedure

We began the lesson with a short introduction and instructions for work, followed by pre-testing (10 minutes). Each pupil (1) answered five questions, which tested the knowledge of biology, (2) assessed seven statesments on a Likert scale, and (3) completed the worksheet with 15 drawings of stages of the cell cycle. Everyone made two identical copies of the worksheet, where one was given to the teacher and the second was needed to continue the lesson. This was followed by a lesson (30 minutes), during which students learned about the processes of the cell cycle and particularly of mitosis and its importance. We used the method first proposed by Danieley (1990); the worksheet was taken from the book Biology Inquiries (Shields, 2006).

In this phase students were put in small groups to review their completed worksheets of 15 drawings of stages of the cell cycle, and discuss the placements of all drawings. It was important that each pupil explained his placements and described them in his own words. Then the teacher led a whole-class discussion. The whole class discussed the placement of each drawing and gave reasons for that particular placement. It was essential that in this part of the lesson, the teacher did not use professional biological terms such as chromosome, DNA, gene, mitotic spindle, etc., which is a classic way of teaching mitosis (Jofili and Watts, 1998). Throughout the discussion everyone used terms that are used in everyday language; the reason why mitosis is so challenging for students is because students learn about the process, and at the same time they first encounter a lot of new expressions, of usually foreign origin (Knippels et al., 2005). After the students had understood the sequence of events in the process of the cell cycle, the teacher introduced professional terminology and expanded on new topics such as why cells divide, when do new cells generate in the living being, how do living beings grow, and that mitosis is a process that enables the precise transfer of genetic information to both daughter cells. The objectives of this lesson were two, students should: (1) understand the sequence of events in the cell cycle; and (2) understand what the cell cycle and mitosis are, as well as where, when, and why they take place.

Students arranged the 15 drawings of stages of the cell cycle once more at the end of the lesson (post-test, 5 minutes), and then again four weeks later (late post-test, 5 minutes).

Statistical analysis

The data analysis was carried out using SPSS statistical software (version 20). The Mann- Whitney test was used to identify statistically significant differences between girls and boys, and between 13- and 14 year-olds. A paired-samples *t*-test was used to identify statistically significant differences among the results of three consecutive tests (pre-test, post-test, late post-test). The effect size estimate *r* was calculated. A principal component analysis (PCA) was conducted on 15 items with orthogonal rotation (varimax) to establish in how many components the stages of mitosis will be positioned. The value of the Kaiser-Meyer-Olkin measure of sampling adequacy was 0.78, which means that our sample size was adequate for PCA. Bartlett's test of sphericity was highly significant ($\chi^2 = 1303.649$, df = 105, $p < 0.001$), indicating that correlations between items were sufficiently large for PCA.

Results

Knowledge test

Living beings are made of cells

Most students were aware of the fact that cells are the building blocks of human beings (85% correct answers). But there were fewer correct answers about other organisms: bee (67%), bacterium (53%), oak (51%), fungus (48%), and paramecium (42%). The differences between the

responses of students of different ages were only statistically significant for the bacterium (Mann Whitney test, $U = 774.000, z = -2.244, p = 0.025$), and human beings (Mann Whitney test, $U = 724.500, z = -3.750, p < 0.001$). In bacterium more knowledge was shown by 13-year-olds, while in humans more knowledge was shown by 14-year-olds. Differences between the knowledge of girls and boys were not found (all ps > 0.05).

What is mitosis, and where, when, and why it takes place

On each of the four questions on mitosis (what is mitosis, where, when, and why it takes place) only 1.1–3.2% of students responded. None of them correctly answered the first three questions, only the fourth question (why mitosis takes place) was correctly answered by 2.1% of students. These students wrote that mitosis is required for reproduction or cell division. The 13-year-olds showed similar knowledge as the 14-year-olds (all ps > 0.05). The differences between the knowledge of girls and boys were not found (all ps> 0.05).

Arranging 15 drawings of stages in the cell cycle

Arranging 15 drawings of stages in the cell cycle

Before the lesson, 78% of those enrolled correctly placed drawings of the first stage of the cell cycle and 20–33% of students correctly placed drawings of the other 14 stages (Fig. 1). Immediately after the lesson, the first stage was correctly placed by all students; other drawings (of the other 14 stages) were correctly placed by 88–98% of students. Therefore, after the lesson students were able to arrange all drawings much more accurately than before the lesson. Four weeks after the lesson the number of correct answers were slightly lower than immediately after the lesson, but still very high; the drawing of first stage of the cell cycle was correctly placed by 98% of the students and drawings of the other 14 stages of the cell cycle by 73–86% of the students.

Students placed most of the drawings not only in their correct positions, but also in almost all possible incorrect positions (Tab. 1). Before the lesson the range of positions for individual draw-

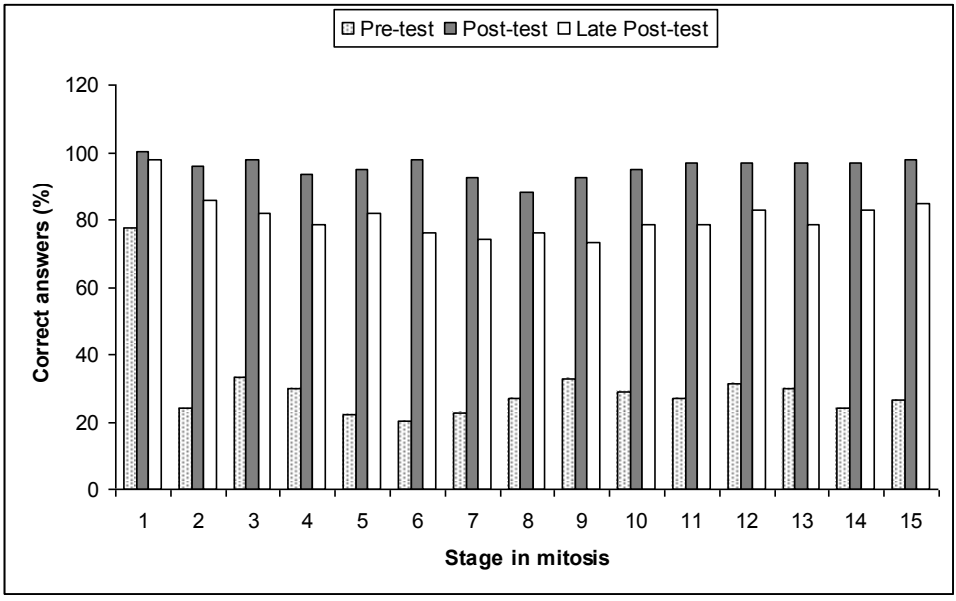

Figure 1: Share of correct placements of 15 drawings of stages in the cell cycle before the lesson, immediately after the lesson, and four weeks after the lesson (N = 95).

Slika 1: Deleži pravilnih razvrstitev 15 slik faz celičnega cikla pred poukom, takoj po njem in štiri tedne kasneje (N = 95).

ings was 10–14. Immediately after the lesson the range was much smaller than before the lesson (0–7 positions). The only exception was stage 8, with the range of 13 that did not change compared to the test before the lesson. Therefore, we see that the knowledge of students after the lesson was better. Four weeks after the lesson the range expanded again to 9–13 positions. The exception was stage 12 with a range of 6.

Principal component analisys

Four components were extracted, which explained 78.17% of the total variance (Tab. 3). The items that cluster on the same components suggest that component 1 represents two types of a cell: (1) a cell with duplicated chromosomes consisting of two sister chromatids in prophase and metaphase; and (2) a cell with daughter chromosomes that are being pulled toward the poles, while cytokinesis starts in anaphase. Component 2 represents a cell with daughter chromosomes that are being pulled toward the poles before cytokinesis starts in anaphase. Component 3 represents one cell or two cells with formed nucleus in interphase, and component 4 represents a cell with chromatin in early prophase and late telophase.

Table 1: Median and range of placements of 15 drawings of stages in the cell cycle before the lesson, immediately after the lesson, and four weeks after the lesson (N = 95).

Tabela 1: Mediana in razpon razvrstitev 15 slik faz celičnega cikla pred poukom, takoj po njem in štiri tedne kasneje (N = 95).

	Pre-test		*Post-test*		*Late Post-test*	
Stage of mitosis	*Mdn*	*Range*	*Mdn*	*Range*	*Mdn*	*Range*
1	1	14	1	0	1	12
2	3	14	2	6	2	12
3	3	14	3	7	3	12
4	6	12	4	6	4	10
5	6.5	11	5	6	5	11
6	6	13	6	4	6	12
7	8	10	7	7	7	12
8	8	13	8	13	8	11
9	8	12	9	6	9	12
10	10	11	10	6	10	9
11	11	12	11	2	11	10
12	12	13	12	3	12	6
13	12	14	13	2	13	11
14	13	14	14	7	14	13
15	4	14	15	5	15	13

The analysis showed that students placed 12 stages statistically significantly better immediately after the lesson than before the lesson. Only in stages 2, 9, and 11 the difference was not statistically significant (Tab. 2). The effect size of changes after the lesson was large in stages 10, 14, and 15, in stages 1, 3–8, 12, and 13 the effect size was medium, and in stages 2, 9, and 11 it was small or negligible. Four weeks after the lesson students' achievements decreased; this decrease was statistically significant in seven stages (stages 3, 4, 6, 9, 10, 14, 15). The effect size of the decrease four weeks after the lesson was medium in stages 3 and 10, and small in the rest of the stages.

Stages 3–6 and stages 10–13 (from total of 15 stages) were included in the first component. The first sequence of four drawings (stages 3–6) represents mitosis from prophase to metaphase, the latter sequence of four drawings (stages 10–13) represents anaphase and telophase. The two sequences represent the starting and the finishing parts of mitosis. The results therefore show that students were able to recognise the first and the

Table 2: Statistically significant differences in placement of 15 drawings of stages in the cell cycle before the lesson, immediately after the lesson, and four weeks after the lesson (Paired-samples *t*-test; N = 95). Statistically significant values are shown in bold type.
Tabela 2: Statistično pomembne razlike v razporeditvi 15 slik faz celičnega cikla pred učno uro, takoj po njej in štiri tedne kasneje (Paired-samples *t*-test; N = 95). Statistično pomembne razlike so prikazane v poudarjenem tisku.

	Pre-test/Post-test				Post-test/Late Post-test			
Stage of mitosis	*t*	*df*	*p* (2–tailed)	*r* (effect size)	*t*	*df*	*p* (2–tailed)	*r* (effect size)
1	5.169	92	**< 0.01**	0.32	–1.313	92	0.193	0.14
2	1.567	92	0.118	0.10	–0.764	92	0.447	0.08
3	7.021	92	**< 0.01**	0.42	–3.028	92	**0.03**	0.30
4	–59.000	92	**< 0.01**	0.31	2.505	92	**0.014**	0.25
5	7.474	92	**< 0.01**	0.44	–1.975	92	0.051	0.20
6	6.454	92	**< 0.01**	0.39	–2.736	92	**0.07**	0.27
7	5.031	92	**< 0.01**	0.32	–0.573	92	0.568	0.06
8	7.044	92	**< 0.01**	0.42	–1.744	92	0.084	0.18
9	–0.154	92	0.877	0.01	–2.413	92	**0.018**	0.24
10	–11.671	92	**< 0.01**	0.61	3.363	92	**0.01**	0.33
11	–0.614	92	0.540	0.04	–0.856	92	0.394	0.09
12	–3.924	92	**< 0.01**	0.25	–0.071	92	0.944	0.01
13	–6.201	92	**< 0.01**	0.38	0.660	92	0.511	0.07
14	–8.543	92	**< 0.01**	0.49	2.758	92	**0.07**	0.28
15	–10.240	92	**< 0.01**	0.56	2.687	92	**0.09**	0.27

Table 3: Summary of principal component analysis for 15 drawings of the stages in the cell cycle.
Tabela 3: Zbirnik analize glavnih komponent za 15 slik faz celičnega cikla.

	Rotated factor loadings			
Stage of mitosis	Duplicated chromosomes; daughter chromosomes are pulled toward the poles and cytokinesis starts	Daughter chromosomes are pulled toward the poles	Nucleus	Chromatin
3	**–0.915**	0.020	0.226	0.071
4	**–0.914**	0.073	0.218	0.189
12	**0.912**	–0.039	0.256	0.158
5	**–0.907**	0.149	0.196	0.165
11	**0.880**	0.015	0.249	0.256
13	**0.880**	0.012	0.148	0.085
6	**–0.823**	0.114	0.251	0.223
10	**0.754**	0.100	0.266	0.285
8	–0.015	**0.862**	–0.102	–0.091
7	–0.078	**0.699**	0.043	0.137
9	0.095	**0.641**	0.057	0.187
14	0.375	**–0.580**	–0.122	**–0.529**
1	–0.150	0.090	**–0.740**	0.016
15	0.391	–0.312	**–0.702**	0.310
2	–0.084	–0.248	0.209	**–0.894**
Eigenvalue	6.55	2.59	1.52	1.07
% of variance	43.69	17.26	10.10	7.12

latter sequence and to arrange them correctly. Stages 7–9 were included into the second component; they all represent a sequence of events in anaphase. Stages 1 and 15 were included in the third component; they both represent interphase, the first one is a parent cell, the second one two daughter cells. Stages 2 and 14 were included in the fourth component; the first represents a parent cell in early prophase, and the second developing daughter cells in late telophase. The main elements in cells in these two drawings are thin threads of uncoiled chromosomes (chromatin). So we can see that the students recognised the logical sequences of stages which helped them arranging the drawings in the correct order. Difficulties were encountered with intermediate stages or the connection of these partial sequences in the entire sequence of 15 drawings.

Discussion

One of the fundamental processes in biology which represents the biggest problems for students is also mitosis. Usually it is taught in the traditional way, starting with a presentation of the technical, biological terms such as chromosome, DNA, gene, mitotic spindle, etc. (Watts and Jofili, 1998). Danieley (1990) and Shields (2006) proposed a different instructional strategy. This one is focused on the logic of stages in the process of mitosis and the cell cycle. Teaching/learning of mitosis in the way suggested by Danieley (1990) makes the most sense if carried out on those who are only just starting to learn about mitosis. Students try to independently place the 15 images of intermixed phases of the cell cycle in a logical sequence. After students understand the course of events in the cell cycle, biological terms for structures and stages of the cell cycle are introduced to upgrade the knowledge of biology. Our study focused on students aged 13 and 14, and gave us better insight into students' capacity to understand this process, which can be used in planning activities and teaching the cell cycle.

Knowledge test

In our study we intended to address mitosis, therefore we were interested in whether students have the basic knowledge into which they can reasonably place mitosis (BaneTele and Ayuso, 2000). First of all we tested students for their knowledge of the fact that cells are the building blocks of all living beings. In the test there was a human and a bee as representatives of the animal kingdom. Most students were aware of the fact that the human (85%) and the bee (67%) are built of cells. This result suggests that students best mastered the concept of cellular structure in connection with animals. Only 42–53% of students knew that representatives of other kingdoms (bacteria, protists, fungi, and plants) are also composed of cells. There were two statistically significant differences between 13- and 14-year olds. The 13-year olds knew better than the 14-year olds that bacteria were made of cells, while the 14-year olds knew better that humans were made of cells. This difference could be explained by the biology curriculum, as the 13-year-olds were learning about the system of living beings including bacteria in the year this study was conducted, while the 14-year-olds were learning about the human body. There was also one statistically significant difference in knowledge between genders: the girls knew better than the boys that bees are made of cells. This fact could be connected to the fact that bees are insects with positive connotation, which appeals to girls more than to boys (Fakin, 2012).

The results show that majority of 13-year-olds did not acquire the concept of the cellular structure of living things, i.e. the fact that all living beings are made from cells. This suggests that teaching strategies, which enrolled students have experienced in primary school, were not successful in bringing about the understanding of this phenomenon. Banet and Ayuso (2000) who studied the biology knowledge of 15-year-olds also came to the conclusion that most students do not know that all living beings are built from cells. We also tested students for their knowledge of mitosis (what is mitosis; where, when, and why it takes place). The very low response rate and only 2.1% of correct answers show that students in our sample had not been familiar with mitosis before the lesson. Such a result was expected because our school system did not include mitosis and cell division into the curriculum for students less than 13 years old before school year 2012–2013.

Arranging 15 drawings of stages in mitosis

Students first sorted 15 pictures of the stages of the cell cycle before the lesson. The purpose of this first arranging of drawings was to find out how the students think so we could then work with that (Newton, 2004). Before the lesson, the drawing of the first phase was correctly placed by 78% of students, while the other 14 drawings were correctly placed by 20–33% of students (Fig. 1).

The first phase represented a single parent cell with a visible nucleus (interphase of a cell cycle), which was a typical cell, familiar to students. It was also the first drawing in the sequence of 15 drawings in the worksheet. Our speculation is that both these facts helped the majority of students correctly identify this drawing as the first stage in the cell cycle.

Students could potentially place each drawing in any of the 15 positions. We found that before the lesson the 14 drawings were most often placed in correct positions while one drawing was most often placed in incorrect position (stage 15; 42% of the students placed it in the position of stage 2). Drawing 15 represented two daughter cells with visible nuclei (interphase of a cell cycle). The results suggest that students recognised a typical cell as represented in drawings 1 and 15, and therefore placed them one after the other. The rests of the 13 drawings having no visible nucleus were viewed as atypical according to the knowledge that students had at the time. As Fisher (1985) stated, it is more difficult to generate new knowledge than to retrieve something one already knows. When confronted with an answer that seems right, students tend to avoid any additional solving of a problem and they choose this answer. We think that this is the reason why so many students placed drawings 1 and 15 separately from the rest of the 13 drawings.

Apart from this there were no other frequent incorrect placements of drawings. The most common mistake was placements of drawings close to the correct position, but not quite correctly: just before or just after the correct position and two places before or two places after the correct position. Students with such placements, even if they were incorrect, however, showed that they recognized the similarity of images. Therefore, these errors were not considered critical. These results indicate that students did not make any typical mistakes that could reflect a misunderstanding to which the teacher should be especially attentive when teaching mitosis and the cell cycle using this method.

The results show that students gained an understanding of the events in the cell cycle during the lesson, and that strategy implemented in the lesson was an effective way of teaching/learning this process.

We deduced this from the results, which show that immediately after the lesson students placed 12 drawings statistically significantly more correctly than before the lesson (Tab. 2). The range of incorrect placements was also highly reduced (0–7 incorrect positions) for most of the drawings (Tab. 1) compared to the test before the lesson. Only stage 2 which represents a single cell with a chromatin stood out (drawing 8). Its range immediately after the lesson was 13, and did not change in comparison to the pre-test. This can be explained by the fact that this stage does not logically fit into the sequence, so students did not know where to place it.

We found that students' achievements were lower four weeks after the lesson compared to the test immediately after the lesson. 98% of students correctly placed the drawing of the first stage in the cell cycle (Fig. 1) four weeks after the lesson. The other 14 drawings were correctly placed by 73–86% students. The range of incorrect placements was a bit higher in comparison to the test immediately after the lesson (Tab. 1), and 8 drawings were placed statistically significantly different than immediately after the lesson (Tab. 2). However, the effect size of these changes was small for 13 drawings and medium for two drawings, which suggests that the knowledge students gained by this method was sufficiently permanent.

The presented method is therefore an effective way of teaching/learning the basics of the process of the cell cycle. Students gained an understanding of the basic frame of the cell cycle. This represents a firm hierarchical concept needed for meaningful learning (Mintzes et al., 2001). Once students gain this it can be gradually upgraded. How far knowledge should be upgraded depends on the skills of students and on the curricula.

The new biology curriculum in Slovenia which was first implemented in school year 2012–2013, and introduced teaching mitosis to 13-year-olds. Most students at this age are capable of logical reasoning. The method of teaching we tested in our survey focuses on recognising and understanding the logical sequence of events in the cell cycle. Based on our results we can conclude that this method is effective and suitable for teaching both 13- and 14-year-olds equally. Students could solve the problem using logic. Similar cases where students could successfully solve problems using algorithmic methods were reported in other fields, for example in teaching meiosis by Stewart and Dale (as cited in Cavallo, 1992) and Williams et al. (2012).

In the lesson presented, the teacher let the students to describe the events and the drawings of stages of the cell cycle using colloquial language. This is because extensive genetic terminology adds to the difficulties that students experience (Knippels et al., 2005); it does not contribute to an understanding of events and puts students off the subject. During the lesson students spontaneously named structures and drawings according to what these structures reminded them of: as spaghetti (chromatin) and butterflies (duplicated chromosomes consisting of sister chromatids). Professional terminology was presented to students only after they had already understood the course of events in the cell cycle. This, perhaps not to our surprise, gained little understanding on the part of the biology teachers who were involved in the study. Most of the teachers found the use of such terminology too childish and inappropriate for a school situation.

We argue that teaching/learning should use this method to its advantage because it helps students gaining understanding of the process and is the opposite of learning by memorising. As stated in Watts and Jofili (1998), teachers should strive towards better quality of knowledge and not so much towards its quantity.

Another surprising fact concerning teachers emerged during our study. The general response concerning applying the presented method in class as a means of teaching/learning mitosis and the cell cycle was quite negative. Most teachers found it interesting only as a way of evaluating students' knowledge after the lesson. Reasons for that are yet to be thoroughly investigated. One of the possible explanations could be inertia to changes which suggests that classical way of teaching still predominates in our biology classes (Watts and Jofili, 1998).

Conclusions

The presented method proved to be effective in helping 13- and 14- year-old students understand the sequence of events in the cell cycle. The retention of students' knowledge was satisfactory. We also did not find any missconceptions in students that could impede the use of this method. Students were successful at learning and therefore not put off the subject of mitosis, as is too often likely to happen with this complex topic.

The only problem is that teachers are not yet sufficiently familiar with this method and those who are, do not favour it, comparing to the classical teaching strategy. Responsibilty is therefore on the work with in-service and pre-service teachers to make them understand alternative methods of teaching mitosis.

The method used in this study could be applied in teaching other biological topics, the most obvious being meisosis, but also such as photosinthesis, respiration, digestion, and others.

References

Banet, E., Ayuso, E., 2000. Teaching Genetics at Secondary School: A Strategy for Teaching about the Location of Inheritance Information. Journal of Science Education, 84 (3) 313–351.

Castro, J., 2009. Misconceptions in Genetics: Genes and Inheritance. Accessed 6/12/2012. Available online at: http://www.csun.edu/~jcc62330/coursework/690/Assignments/castro_misconception.pdf.

Cavallo, A. M. L., 1992. Student's Meaningful Learning Orientation and Their Meaningful Understanding of Meisosis and Genetics. (Conference Paper). (ERIC Document Reproduction Service No. ED356140).

Cavallo, A. M. L., 1996. Meaningful Learning, Reasoning Ability, and Students' Understanding and Problem Solving of Topics in Genetics. Journal of Research in Science Teaching, 33(6), 625–656.

Danieley, H., 1990. Exploring Mitosis through the Learning Cycle. The American Biology Teacher, 52(5), 295–296.

Dikmenli, M., 2010. Misconceptions of Cell Division Held by Student Teachers in Biology: a Drawing Analysis. Scientific Research and Essay, 5(2), 235–247.

Fakin, T., 2012. Znanje in odnos učencev do metuljev in komarjev [Elementary school students' knowledge and attitudes toward butterflies and mosquitoes]. Unpublished graduation thesis, University of Ljubljana, Slovenia.

Fisher, K. M., 1985. A Misconception in Biology: Amino Acids and Translation. Journal of Research in Science Teaching, 22(1), 53–62.

Lawson, A. E., 1991. Exploring Growth (and Mitosis) Through a Learning Cycle. The American biology teacher, 53(2), 107–110.

Lawson, A. E., Thompson, L.D., 1988. Formal reasoning Ability and Misconceptions Concerning Genetics and Natural Selection. Journal of Research in Science Teaching, 25(9), 733–746.

Lewis, J., Kattmann, U., 2004. Traits, Genes, Particles and Information: Re-visting Students Understandings of Genetic. Journal of Science Education, 26, 195–206.

Locke, J., McDermid, H. E., 2005. Using Pool Noodles to Teach Mitosis and Meiosis. Genetics, 170(1), 5–6. Accessed 6/12/2012. Available online at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1449698/.

Mbaijorgu, N. M., Ezechi, N. G., Idoko, E. C., 2007. Addressing Nonscientific Presuppositions in Genetics Using a Conceptual Change Strategy. Accessed 6/12/2012. Available online at: www.interscience.wiley.com.

Mintzes, J. J., Wandersee, J. H., Novak, J. D., 2001. Assessing Understanding in Biology. Journal of Biological Education, 35(3), 118–124.

Newton, D., 2004. Teaching Tricky Science Concepts. Warwickshire, UK: Scholastic Ltd.

Novak, J. D., 1988. Learning Science and the Science of Learning. *Studies in Science Education, 15,* 77–101.

Shields, M., 2006. Biology Inquiries. San Francisco, CA:Yossey-Bass.

Smith, M. U., Sims, S. O. (1992). Cognitive Development, Genetics Problem Solving, and Genetics Instruction: A Critical Review. Journal of Research in Science Teaching, 29, 701–713.

Stavroulakis, A. M., 2005. Meio-socks and other Genetic Yarns. The American Biology Teacher, 67(4), 233–238.

Strgar, J., 2010. Biological Knowledge of Slovenian Students in the Living Systems Content Area in PISA 2006. Acta Biologica Slovenica, 53(2), 99–108.

Štraus, M., Repež, M., Štigl., S. (eds.), 2006. Nacionalno poročilo PISA 2006: naravoslovi in matematični dosežki slovenskih učencev (National report PISA 2006: achievements of Slovenian students in the field of science and mathematics). Ljubljana: National centre PISA, Pedagoški inštitut.

Venville, G., Gribble, S., Donovan, J., 2005. An Exploration of Young Children's Understandings of Genetics Concepts from Ontological and Epistemological Perspectives. Science Education, 89, 614–633.

Venville, G. J., Treagust, D. F., 1998. Exploring conceptual Change in Genetics Using a Multidimensional Interpretive Framework. Journal of Research in Science Teaching, 35(9), 1031–1055.

Watts, M. and Jofili, Z., 1998. Toward Critical Constructivistic Teaching. International Journal of Science Education, 20, 159–170.

Williams, M., Montgomery, B. L., Manokore, V., 2012. From Phenotype to Genotype: Exploring Middle School Students' Understanding of Genetic Inheritance in a Web-Based Environment. The American Biology Teacher, 74(1), 35–40.

Wyn, M., Stegink, S., 2000. Role-playing Mitosis. The American Biology Teacher, 62(5), 378–381.

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Model of 3D structure of putative parasitism factor, expansin (EXPB2) from golden potato cyst nematode *Globodera rostochiensis*

Model 3D-strukture verjetnega parazitskega dejavnika ekspanzina (EXPB2) pri rumeni krompirjevi ogorčici *Globodera rostochiensis*

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Abstract: Expansins are a group of plant cell wall loosening proteins. In animals, functional expansin (EXPB1) has been discovered in the golden potato cyst nematode *Globodera rostochiensis*. In plant-parasitic nematodes expansins act as the parasitism factors or effectors. Molecular variability of another expansin (*exp*B2) gene was evaluated in diverse populations of the *G. rostochiensis*. 3D modelling of GR-EXPB2 protein sequences revealed variants with different tertiary protein structure. Superimposing PDB structures of the protein model of common type protein with two longer variants revealed difference in position of one loop in the two longer proteins. All longer GR-EXPB2 variants originated from South America.

Key words: Cell wall degradation, *Globodera rostochiensis,* effectors, expansin, parasitism factor, plant-parasitic nematode, potato cyst nematode, 3D structure.

Izvleček: Ekspanzini so skupina proteinov, ki zrahlja rastlinsko celično steno. Pri živalih so odkrili funkcionalni ekspanzin (EXP1) pri vrsti rumene krompirjeve ogorčice, *Globodera rostochiensis*. Pri rastlinsko parazitskih ogorčicah ekspanzini delujejo kot parazitski dejavniki oz. efektorji. Pri *G. rostochiensis* je bila ovrednotena molekulska raznolikost dodatnega ekspanzinskega gena (*exp*B2). Modeliranje 3D-strukture proteina GR-EXPB2 je razkrilo različke proteina z različno terciarno strukturo. Z nalaganjem strukture daljših različic proteina nad najpogostejšo krajšo različico proteina se je razkrila različna pozicija ene zanke pri obeh daljših proteinih. Vse daljše različice proteina GR-EXPB2 izvirajo iz Južne Amerike.

Ključne besede: razgradnja celične stene, *Globodera rostochiensis*, efektorji, ekspanzin, parazitski dejavnik, rastlinsko parazitske ogorčice, 3D-struktura.

Introduction

Expansins are a group of proteins that operate by loosening non-covalent interactions between components of the plant cell wall making the individual components vulnerable to attack by other cell wall degrading enzymes (Cosgrove 2000). These proteins were thought to be specific to plants; however an active expansin EXPB1 has unexpectedly been identified in the plant-parasitic nematode, golden potato cyst nematode *Globodera rostochiensis* (Woll.) Behrens (Qin et al. 2004). *G. rostochiensis* parasitize different Solanaceous plant species and affects potato production worldwide.

Therefore *G. rostochiensis* is subjected to strict quarantine regulations in many countries. In Slovenia *G. rostochiensis* has spread in the last decade (Širca et al. 2010).

When potato cyst nematodes (PCN) invade a plant, they produce a mixture of lytic enzymes and expansins in their oesophageal glands and secrete them through the stylet into the plant. These proteins assist in the migration of infective juveniles through the host plant's tissues, and in the feeding site formation. Additionally, the host plant's own expansin genes are up-regulated upon nematode infection (Fudali et al. 2008). Expansins B1 and B2 were determined in the EST analysis of *G. rostochiensis* (Popeijus et al. 2000, http://www.nematodes.org/nembase4/overview.shtml). Molecular variability of *exp*B2 gene was evaluated in the diverse populations of the *G. rostochiensis* (Gerič Stare et al. 2012). One-hundred thirty-eight determined sequences of the Gr-*exp*B2 gene (FJ705444, GQ152151 – GQ152166, GQ152168 – GQ152288) resulted in different protein sequences. A majority (126 out of 138; 91.3%) of the DNA sequences resulted in protein sequences of 154 amino acids (AA) in length. Two sequences resulted in slightly longer proteins (156 AA) due to an identical 6-bp insertion into the fourth exon. One sequence resulted in a 181 AA protein sequence due to a 1-bp insertion in the fourth exon and a subsequent frame shift which thwarted the normal stop codon. Nine sequences were determined as pseudogenes due to short deletions in exons, subsequent frameshift and premature stop codon.

The aim of this study was to determine tertiary structure in variants of GR-EXPB2 protein determined in our previous study (Gerič Stare et al. 2012).

Materials and methods

3D protein models of predicted proteins without signal peptide were constructed with the Swiss-Model Workspace (Arnold et al. 2006) while protein structures were compared by superimposing pdb files with TopMatch (Sippl and Wiederstein 2008, Sippl 2008).

Results

Three-dimensional (3D) protein structure modelling

The 3D protein structure models were built to pinpoint differences between the predicted GR-EXPB2 proteins of different clones (pseudogenes were excluded from the analysis) (Fig. 1). An automated fold recognition technique constructed two different models based on the mature protein sequences in clone 5a which represented the most common protein sequence since 88 (64%) sequences resulted in such identical proteins. For clone 5a, the first 3D model was based on the Barwin lectin, a protein from barely seed (PDB ID: 1bw3A, E value $1.40e^{-7}$ and 18% identity) and the second on pollen allergen PHL P1 from *Phleum pratense* (PDB ID: 1n10A, E value $2.0e^{-20}$ and 17.5% identity). These two models differed significantly in their arrangement of local patterns (Fig. 1). The second model was a more likely candidate for the GR-EXPB2 structure on account of its lower E value and was also previously suggested as the best template for domain 2 of the GR-EXPB1 by Kudla et al. (2005). While GR-EXPB1 is composed of a carbohydrate binding domain coupled to an expansin domain (Kudla et al. 2005), the GR-EXPB2 3D model predicted in this study was composed only of expansin domain suchlike the putative expansins from plant parasitic nematodes *Bursaphelenchus xylophilus* and *B. mucronatus* (Kikuchi et al. 2009).

Models of clones 21b and 33b were constructed to search if the longer predicted proteins have structural properties similar to a common type of protein. Both clones resulted in 3D protein models based on template 1n10A (Fig. 2). Superimposing PDB structures of common type protein with longer proteins revealed a difference in the position of one loop of the two longer proteins (Fig. 2).

Discussion

Expansins cause loosening and extension of cell walls by acid-induced disruption of non-covalent hydrogen bonds between cellulose and hemicellulose fibers, promoting slippage between the polymers and allowing the cell to absorb water, ultimately leading to cell wall expansion

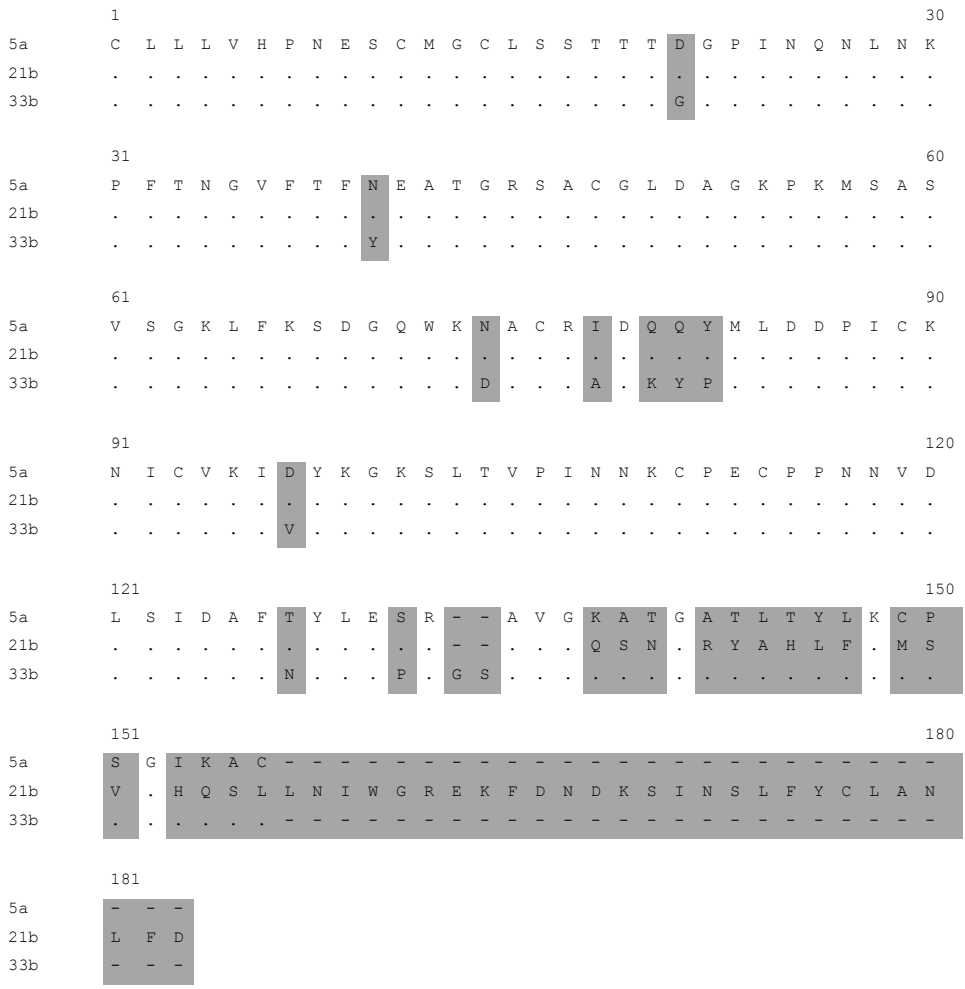
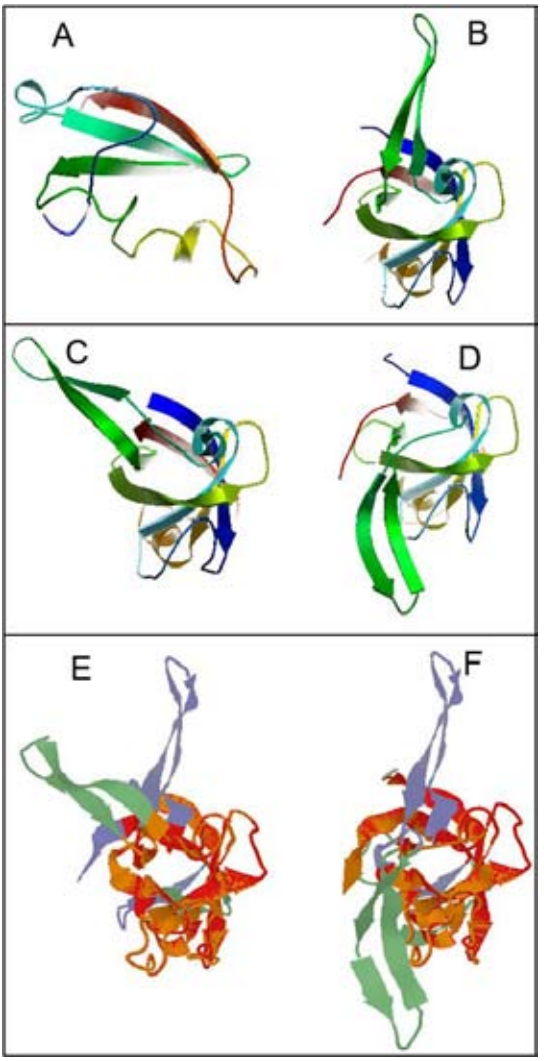


Figure 1: The alignment of the predicted variant GR-EXPB2 proteins from different clones. The sequence of clone 5a represents the most common protein, GR-EXPB2 type protein. The clones 21b and 33b represent the longer predicted proteins. Variable sites in the alignment are highlighted.

Slika 1: Poravnava predvidenih različkov proteina GR-EXPB2 iz različnih klonov. Zaporedje klona 5a predstavlja najpogostejši različek, tipični protein GR-EXPB2. Klona 21b in 33b predstavljata daljša različka proteina. Spremenljiva mesta v poravnavi so poudarjena.

(Cosgrove 2000, Whitney et al. 2000). No hydrolytic or other enzymatic activity has been found to account for expansin's unique effects on the cell wall. Functional binding target of expansin in the *Arabidopsis thaliana* cell walls was recently determined. Expansin binding to highly specific cellulose domains enriched in xyloglucan leads to loosening of the cell walls, whereas more abundant binding to pectins is unrelated to activity (Wang et al. 2013). It was proposed that the several highly-conserved tryptophans in the cellulose-binding module (CBM) may function in expansin binding to its target (Shcherban et al. 1995). The exact mechanism of action of the expansins is still not characterized in details. The shortage of functional information reflects the scarcity of



informative structural data, given that the only solved crystal structures represent one bacterial expansin and the expansin-like protein in maize (Dal Santo et al 2013). While GR-EXPB1 consists of an expansin domain and a carbohydrate-binding module, GR-EXPB2 consists solely of the expansin domain. GR-EXPB1 has considerable enzymatic activity only when both domains are present. Furthermore, CBM alone did not show expansin activity (Kudla et al. 2005). However it has been shown that also GR-EXPB2 consisting solely of the expansin domain exhibits the activity. The *in planta* expression and activity of GR- EXPB2 has been demonstrated in *Nicotiana benthamiana, Solanum lycopersicum* and *S. tuberosum* leaves (Dr. Shawkat Ali, personal communication). Further functional analysis of the GR-EXPB2 variants are planned to assess possible difference in activity between protein variants. It was suggested that the high conservation of this multigene family indicates that the mechanism by which expansins promote wall extension tolerates little variation in protein structure (Shcherban et al. 1995). Different position of the loop in the longer GR- EXPB2 variants (Fig. 2) could affect the interaction with polymers of the cell wall and the proteins function. Therefore it needs to be determined whether the longer protein varianst have the activity at all and if it is any different from the common type GR-EXPB2 protein.

Figure 2: The 3D models of the predicted GR-EXPB2 protein from different clones. A – The first model of the common type protein (clone 5a) based on the barely seed protein (1bw3A). B – The second model of the common type protein (clone 5a) based on PHL P 1, a major Timothy grass pollen allergen (1n10A). C – Models of the first longer predicted protein (clone 21b) constructed on template 1n10A. D – Models of the second longer predicted protein (clone 33b) constructed on template 1n10A. E, F – Superimposing PDB structures of the second model of common type protein with longer proteins (clones 21b and 33b, respectively) revealed differences in position of one loop in the two longer proteins (shown by the arrows).

Slika 2: 3D zgradba modelov proteina GR-EXPB2 iz različnih klonov. A – Prvi model najpogostejšega zaporedja proteina (klon 5a) na osnovi proteina iz semena ječmena (1bw3A). B – Drugi model najpogostejšega zaporedja proteina (klon 5a) na osnovi proteina PHL P 1, alergena iz trave *Phleum pratense* (1n10A). C – Model prvega daljšega različka proteina (klon 21b) na osnovi šablone 1n10A. D – Model drugega daljšega različka proteina (klon 33b) na osnovi šablone 1n10A. E, F – Z nalaganjem strukture daljših različic proteina nad najpogostejšo krajšo različico proteina se razkrije različna pozicija zanke (kažejo puščice) pri obeh daljših proteinih (klona 21b in 33b).

All longer protein variants of GR-EXPB2 originate from the Bolivian populations. South America is the origin of potatoes as well as their nematode pests like PCN. PCN originate from the Andean Highlands of South America, where they co-evolved with their plant hosts (potatoes and other members of the family Solanaceae). The range of virulence of PCN present in that area is far greater than that present in European populations as their initial introduction into Europe represented bottle neck (EFSA 2012). Several studies have showed higher variability of South American PCN populations compared to European populations including our studies of variability of cell wall degrading proteins pectate lyase 2 and expansin B2 (Gerič Stare et al. 2011, 2012).

Conclusion

Comparison of GR-EXPB2 protein variants revealed differences in position of one loop in the two longer protein variants compared to the shorter common type protein.

Povzetek

Ekspanzini so proteini, ki z zrahljanjem nekovalentnih vezi pomagajo pri razgradnji rastlinske celične stene. Pri živalih so odkrili funkcionalni ekspanzin (EXP1) pri vrsti rumene krompirjeve ogorčice *Globodera rostochiensis* (Nematoda). Pri rastlinsko parazitskih ogorčicah ekspanzini delujejo kot parazitski dejavniki oz. efektorji. V predhodni študiji smo ovrednotili molekulsko raznolikost dodatnega ekspanzinskega gena (*exp*B2) pri različnih populacijah in patotipih *G. rostochiensis*. Pri večini (126 od 138; 91,3 %) zaporedij DNA smo s pomočjo »*in silico*« prepisa pridobili proteinska zaporedja dolžine 154 AK. Dve zaporedji sta dali malenkost daljši protein (156 AK) zaradi identične 6-bp dolge insercije v četrtem eksonu. Eno zaporedje je vodilo v daljši protein (181 AK) zaradi insercije 1-bp v četrti ekson in posledičnega premika bralnega okvirja, ki je pokvaril zaključni kodon. Devet zaporedij je predstavljalo pseudogene, saj so krajše delecije v eksonih vodile v premik bralnega okvirja in prezgodnji zaključni kodon. V tej študiji smo določili modelno 3D-strukturo proteina GR-EXPB2. Modeliranje je razkrilo različno terciarno strukturo različkov proteina različne dolžine. Z nalaganjem strukture daljših različic proteina nad najpogostejšo krajšo različico proteina se je razkrila različna pozicija ene zanke pri obeh daljših proteinih. Vse daljše različice proteina GR-EXPB2 smo določili pri populacijah *G. rostochiensis* iz Bolivije. Krompirjeve ogorčice izvirajo iz Južne Amerike, kjer so se razvile v ko-evoluciji s svojimi gostitelji. V Evropo je bilo vneseno manjše število cist iz majhnega območja Južnega Peruja, zato vse krompirjeve ogorčice v Evropi kažejo le manjši delež biotske raznovrstnosti trenutno prisotne v Južni Ameriki. Z našimi raziskavami smo dokazali večjo raznolikost Južno Ameriških populaciji nasproti evropskim glede na proteine za razgradnjo celične stene, ekspanzine in pektat liaze.

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References

Arnold, K., Bordoli, L., Kopp, J., Schwede, T. 2006. The SWISS-MODEL Workspace: A web-based environment for protein structure homology modelling. Bioinformatics, 22, 195–201.

Cosgrove, D.J., 2000. Loosening of plant cell walls by expansins. Nature, 407, 321–326.

Fudali, S., Sobczak, M., Janakowski, S., Griesser, M., Grundler F.M.W., Golinowski. W. 2008. Expansins are among plant cell wall modifying agents specifically expressed during development of nematode-induced syncytia. Plant Signaling & Behavior, 3 (11), 969–971.

Dal Santo, S., Vannozzi, A., Battista Tornielli, G., Fasoli, M., Venturini L., Pezzotti, M., Zenoni S. 2013. Genome-wide analysis of the expansin gene superfamily reveals grapevine-specific structural and functional characteristics. Plos One, 8 (4), e62206.

EFSA Panel on Plant Health (PLH). 2012. Scientific Opinion on the risks to plant health posed by European versus non-European populations of the potato cyst nematodes *Globodera pallida* and *Globodera rostochiensis*. EFSA Journal 2012, 10(4):2644: 71 pp.

Gerič Stare, B., Fouville, D., Širca, S., Gallot, A., Urek, G., Grenier, E. 2011. Molecular variability and evolution of the pectate lyase (*pel*-2) parasitism gene in cyst nematodes parasitizing different Solanaceous plants. Journal of Molecular Evolution,72, 169–181.

Gerič Stare, B., Lamovšek, J., Širca, S., Urek, G., 2012. Assessment of sequence variability in putative parasitism factor, expansin (*exp*B2) from diverse populations of potato cyst nematode *Globodera rostochiensis*. Physiological and Molecular Plant Pathology, 79, 49–54.

http://www.nematodes.org/nembase4/overview.shtml

Kikuchi, T., Li, H., Karim, N., Kennedy, M.W., Moens, M., Jones, J.T. 2009. Identification of putative expansin-like genes from the pine wood nematode, *Bursaphelenchus xylophilus*, and evolution of the expansin gene family within the Nematoda. Nematology,11, 355–364.

Kudla, U., Qin, L., Milac, A., Kielak, A., Maissen, C., Overmars, H., et al. 2005. Origin, distribution and 3D-modeling of Gr-EXPB1, an expansin from the potato cyst nematode *Globodera rostochiensis*, FEBS Letters, 579, 2451–2457.

Popeijus, H., Blok, V.C., Cardle, L., Bakker, E., Phillips, M.S., Helder, J., Smant, G., Jones, J.T., 2000 Analysis of genes expressed in second stage juveniles of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida* using the expressed sequence tag approach. Nematology, 2 (5), 567–574.

Qin, L., Kudla, U., Roze E.H.A., Goverse, A., Popeijus, H., Nieuwland, J., et al., 2004. A nematode expansin acting on plants, Nature, 427, 30.

Shcherban, T.Y., Shi, J., Durachko, D.M., Guiltinan, M.J., McQueen-Mason, S.J., Shieh, M., Cosgrove D.J. 1995. Molecular cloning and sequence analysis of expansins – a highly conserved, multigene family of proteins that mediate cell wall extension in plants. Proc. Natl. Acad. Sci. USA, 92, 9245–9249.

Sippl, M.J. 2008. On distance and similarity in fold space. Bioinformatics, 24, 872–873.

Sippl, M.J., Wiederstein, M. 2008. A note on difficult structure alignment problems. Bioinformatics, 24, 426–7.

Širca, S., Geric Stare, B., Strajnar, P., Urek, G., 2010. PCR-RFLP diagnostic method for identifying *Globodera* species in Slovenia. Phytopathologia Mediterranea, 49, 361–369.

Wang, T., Park, Y.B., Caporini, M.A., Rosay, M., Zhong, L., Cosgrove D.J., Hong M. 2013. Sensitivity-enhanced solid-state NMR detection of expansin's target in plant cell walls. Proc. Natl. Acad. Sci. USA, Published online before print September 24, 2013, doi: 10.1073/pnas.1316290110.

Whitney, S.E., Gidley, M.J., McQueen-Mason, S.J. 2000. Probing expansin action using cellulose/hemicellulose composites. Plant J, 22, 327–334.

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Fax and/or e-mail of the corresponding author – (Times New Roman 12)
Keywords in English – (Times New Roman 12)
Keywords in Slovene – (Times New Roman 12)
Running title – (Times New Roman 12)
Abstract in English (Times New Roman 12, title – Times New Roman 14 bold)

Abstract in Slovene – (Times New Roman 12, title – Times New Roman 14 bold)
Introduction – (Times New Roman 12, title – Times New Roman 14 bold)
Material and methods – (Times New Roman 12, title – Times New Roman 14 bold)
Results – (Times New Roman 12, title – Times New Roman 14 bold)
Discussion – (Times New Roman 12, title – Times New Roman 14 bold)
Summary in Slovene – (Times New Roman 12, title – Times New Roman 14 bold)
Figure legends; each in English and in Slovene – (Times New Roman 12, title – Times New Roman 14 bold, figure designation and figure title – Times New Roman 12 bold)
Table legends; each in English and in Slovene – (Times New Roman 12, title – Times New Roman 14 bold, table designation and table title – Times New Roman 12 bold)
Acknowledgements – (Times New Roman 12, title – Times New Roman 14 bold)
Literature – (Times New Roman 12, title – Times New Roman 14 bold)
Figures, one per page; figure designation indicated top left – (Times New Roman 12 bold)
Tables, one per page; table designation indicated top left – (Times New Roman 12 bold)
Page numbering – bottom right – (Times New Roman 12)

15. Peer Review

All Scientific Articles shall be subject to peer review by two experts in the field (one Slovene and one foreign) and Brief Note articles by one Slovene expert in the field. With articles written in Slovene and dealing with a very local topic, both reviewers will be Slovene. In the compulsory accompanying letter to the editor the authors must nominate one foreign and one Slovene reviewer. However, the final choice of referees is at the discretion of the Editorial Board. The referees will remain anonymous to the author. The possible outcomes of the review are: 1. Fully acceptable in its present form, 2. Basically acceptable, but requires minor revision, 3. Basically acceptable, but requires important revision, 4. May be acceptable, but only after major revision, 5. Unacceptable in anything like its present form. In the case of marks 3 and 4 the reviewers that have requested revisions have to accept the suitability of the corrections made. In case of rejection the corresponding author will receive a written negative decision of the editor-in-chief. The original material will be erased from the ABS archives and can be returned to the submitting author on special request. After publication the corresponding author will receive the *.pdf version of the paper.