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Eterična olja s potencialom za zatiranje varoje (*Varroa destructor*): mehanizmi toksičnosti in negativen vpliv na medonosno čebelo (*Apis mellifera*)

Essential oils with the potential for varroa mite control (*Varroa destructor*): mechanisms of toxicity and negative impact on honey bee (*Apis mellifera*)

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Izvleček: Varoja (*Varroa destructor*) je pomemben dejavnik umiranja čebel, zato njeno učinkovito zatiranje predstavlja enega izmed osrednjih problemov čebelarjenja. Trenutno čebelarji za zatiranje varoje največ uporabljajo sintetične akaricide. Zaradi njihovih negativnih učinkov na čebele ter kemičnih ostankov v čebeljih pridelkih je velik trend k vzpodbujanju uporabe naravnih akaricidov. Med naravne snovi s potencialnim akaricidnim delovanjem sodijo eterična olja in njihove aktivne učinkovine. Med njimi se nekatere, kot je timol, že dlje časa uporabljajo v čebelarstvu. V tem prispevku povzemamo dosedanje znanje o mehanizmih toksičnosti eteričnih olj, s poudarkom na delovanju na živčni in imunski sistem, ter o ostalih vplivih na čebele. Ugotavljamo, da bi lahko bila nekatera eterična olja glede na ugotovljene mehanizme toksičnosti uporabna za zatiranje varoje, vendar pa so njihovi negativni učinki na čebele zelo slabo raziskani. Še posebej so pomembna znanja o njihovem vplivu na imunski odziv, saj so spremembe le tega navedene kot eden izmed potencialnih možnih vzrokov za upad čebeljih družin. Med eteričnimi olji prevladujejo podatki za timol ter njegove pripravke (Apiguard®, Api Life VAR®), precej manj pa je podatkov o drugih pogostih aktivnih učinkovinah eteričnih olj. Zaključujemo, da obstaja potreba po sistematičnem testiranju vplivu akaricidnih eteričnih olj na čebele, s poudarkom na dolgotrajnih izpostavitvah izvedenih po principu aktualnih smernic testiranja strupenosti. Velik izziv za prihodnje raziskave predstavlja optimizacija nanosa in standardizacija uporabe eteričnih olj in njihovih učinkovin v čebelarstvu.

Ključne besede: akaricidi, eterična olja, *Apis mellifera*, živčni sistem, imunski sistem, varoja

Abstract: The parasitic bee mite varroa (*Varroa destructor*) is among the most serious honey bee pests. Beekeepers utilize a wide range of different synthetic acaricides to keep mite populations under control. However, due to documented adverse impact of synthetic substances, the use of naturally derived acaricides, among these essential oils, is greatly being promoted. Thymol is already used in beekeeping. We present a review of the existing knowledge regarding the effects of essential oils on honey bees *Apis mellifera*. We focus only on those that have potential acaricide action.

We discuss their mechanisms of toxic action on the immune and nervous systems. We conclude that due to their mechanisms of toxicity several essential oils could be used for varroa mite control, still very little data regarding the negative effects of essential oils on honey bees are known. In particular, knowing their interferences with the immune response is important to be able to predict the potential effect on the colony health. The majority of toxicity data currently exist for thymol and its commercial preparations under acute exposure (Apiguard®, Api Life VAR®), but the data for a number of other potential acaricide-related essential oils are missing. We recognize the need for systematic screening of potential toxicity and sublethal effects of essential oils with acaricide action on honey bees. Standardised application of essential oils in honey bee keeping remains a challenging task for the future.

Keywords: acaricides, essential oils, *Apis mellifera*, nervous system, immune system, varroa mite

Uvod

Medonosne čebele (*Apis mellifera*) so eden ključnih opraševalcev in so zato pomembne ne samo za okolje, temveč tudi ekonomsko. V zadnjih 10-15 letih se med čebelarji in v širši skupnosti pojavlja pereč problem nenadzorovanega propadanja čebeljih družin. Dosedanje raziskave so pokazale, da je za propad čebeljih družin krivih več dejavnikov, kot so: intenzivno kmetijstvo, uporaba pesticidov, stradanje in slaba prehranjenost čebel ter predvsem pojavnost virusov in invazivnih vrst kot so pršice varoja (*Varroa destructor*), azijski sršen (*Vespa velutina*) in mali panjski hrošč (*Aethina tumida*) (Sánchez-Bayo in sod. 2016). Bolezni pri čebelah niso nič novega, vendar pa je njihovo širjenje pospešil človek zaradi nenamernega vnosa patogenov v nova okolja, v katerih čebele še niso razvile odpornosti nanje. Zunanji zajedalec varoja (*Varroa destructor*) je prvotno zajedal vzhodno medonosno čebelo (*Apis cerana*), po prenosu na novega gostitelja medonosno čebelo *Apis mellifera* pa se je varoja razširila skoraj po celotnem svetu in danes predstavlja grožnjo medonosni čebeli (Rosenkranz in sod. 2010). Domnevajo, da je varoja glavni dejavnik pri umiranju čebeljih družin. Pršica *V. destructor* je namreč relativno novi parazit medonosne čebele, zato odnos gostitelja in parazita še ni uravnotežen, poleg tega čebelarji nimajo dolgotrajnih izkušenj z zatiranjem varoje. Varoja se je v kratkem razširila skoraj po vsem svetu. Dandanes je praktično okužena že vsaka čebelja družina, z izjemo Avstralije. Brez rednega zdravljenja lahko večina čebeljih družin v zmernem podnebju propade v obdobju 2-3 let. Redna zdravljenja povečujejo stroške čebelarstva in tveganje za kemične ostanke v čebeljih izdelkih. Varoja velja za ključnega povzročitelja upada števila čebelarjev in zmanjšanega števila čebeljih družin v Evropi; skupaj s svetovnim zmanjšanjem naravnih opraševalcev, lahko ta pršica še dodatno zmanjša uspešnost opraševanja (Rosenkranz in sod. 2010).

Učinkovito zatiranje varoje je zato nedvomno eden izmed osrednjih problemov čebelarjenja. Čebelarji za zatiranje varoje uporabljajo različne sintetične pripravke, različne načine nanosa kemikalij in tudi biotehnološke metode (Dietemann in sod. 2015). Trenutno so za zatiranje škodljivcev in zunanjih parazitov največ v uporabi sintetične kemične spojine, ki hitro zagotavljajo svoj učinek. Večja uporaba sintetičnih akaricidov s strani čebelarjev temelji na večji in hitrejši učinkovitosti, vendar se le-ti kopičijo in so dolgo obstojni v čebeljem vosku, na njih pršice razvijejo odpornost, dokazani pa so tudi negativni učinki na čebele. Te učinkovine so lahko škodljive tudi za ljudi. Posledično je na področju raziskav s čebelami velik trend k vzpodbujanju testiranja naravnih snovi z akaridicnim delovanjem, t.i. naravnih akaricidov. V Sloveniji je bil leta 2008 uveden enotni operativni program za zatiranje varoze čebel, saj je bil v zimskem obdobju 2006/2007 opažen 16 % upad čebeljih družin. V omenjenem programu je bilo priporočeno, da se vsako leto poveča uporaba naravnih zdravilnih učinkovin, ki

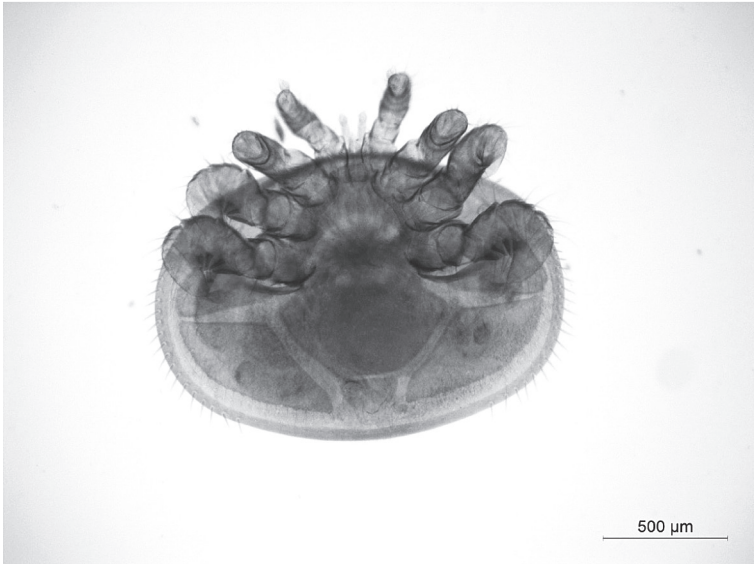
vsebujejo organske kisline in eterična olja (Rejski program za kranjsko čebelo 2010).

Trajnostni princip zatiranja varoze naj bi temeljil na uporabi akaricidov, ki predstavljajo visoko učinkovitost v boju proti pršicam in nizko tveganje za čebele, pa tudi za človeka (Imdorf in sod. 1999). Zato se kot nadomestilo sintetičnim akaricidom vse bolj vzpodbuja uporaba akaricidov z naravnim izvorom oz. naravnih akaricidov. V nasprotju s sintetičnimi akaricidi, v čebeljih pridelkih ostaja manj naravnih pripravkov in pršice nanje ne razvijejo odpornosti. Med naravne akaricide sodijo predvsem nekatera eterična olja ter organske kisline. Vendar pa imajo lahko tudi naravni akaricidi v določenih primerih neželene učinke na zdravje čebel, kar se zgodi predvsem pri neustrezni uporabi, pri kateri lahko pride do lokalno presežene za čebele škodljive koncentracije uporabljene snovi.

Ugotavljamo, da so podatki o potencialnih negativnih vplivih naravnih akaricidov na čebele pomanjkljivi. Zato smo pripravili pregled obstoječih znanj na tem področju z namenom spodbude tovrstnih raziskav v prihodnosti. Osredotočili smo se predvsem na tista eterična olja, ki imajo dokazan toksični učinek na varojo. Povzeli smo podatke o strupenosti teh snovi za čebele, posebej pa smo se osredotočili na mehanizme toksičnosti, s poudarkom na živčni in imunski sistem. Primerjalno podajamo tudi mehanizme delovanja toksičnosti najbolj uporabljenih sintetičnih akaricidov.

Vpliv varoje (*Varroa destructor*) na čebele

Varoja *(V. destructor)* (Sl. 1) za preživetje potrebuje gostitelja. V razvojnem ciklu parazita samic obstajata dve ločeni fazi. Prva je foretična faza, ki poteka na odrasli čebeli, druga je reprodukcijska faza, ki poteka v pokritih satnih celicah trotov, delavk in matic. Samci in nimfe pršice *V. destructor* živijo kratek čas in njihov razvoj poteka izključno v pokritih satnih celicah. Varoja pije hemolimfo ličink in bub znotraj pokritih celic satovja in odraslih čebel (Rosenkrantz in sod. 2009). Simptome pri čebelah kot posledica okuženosti imenujemo varoza. Čebele varoja prizadene na več načinov. Najbolj občutljive so ličinke v razvoju in bube, saj se zaradi izgube hemolimfe med ontogenetskim



Slika 1: Pršica varoja *Varroa destructor* (ventralno). Sliko smo posneli na trajnem preparatu na Oddelku za biologijo (foto A. Jemec Kokalj).

Figure 1: Varroa mite *Varroa destructor* (ventral view). Foto was taken by A. Jemec Kokalj at the Department of Biology (permanent slide preparation).

razvojem bistveno zmanjša njihova teža. Izguba teže je odvisna od števila pršic. V povprečju ličinke in bube delavk izgubijo 7% telesne teže, odrasli troti pa 11–19%, odvisno od stopnje okužbe. Zaradi okužbe se skrajša življenjska doba delavk, ki prej začnejo s pašno aktivnostjo, zmanjšana je tudi sposobnost učenja in navigacije, kar posledično zmanjša uspešno vrnitev pašnih čebel v panj. Troti, ki so bili okuženi med njihovim razvojem, imajo zmanjšano sposobnost parjenja z maticami (Duay in sod. 2002) in v okuženih družinah je rojenje okrnjeno (Fries in sod. 2003, Villa in sod. 2008). Varoja je prenašalka različnih čebeljih virusov, kot so virus deformiranih kril, virus akutne paralize, kašmirski virus čebel in virus mešičkaste zalege (Rosenkrantz in sod. 2009). Domnevajo, da umiranje čebeljih družin predvsem posledica okužb z virusi, ki jih prenaša varoja, in ne toliko posledica neposrednega vpliva varoje na čebele.

Vrste akaricidov

Akaricidi, ki se uporabljajo v čebelarstvu, so bodisi sintetični ali pa naravni. Najpogosteje uporabljeni sintetični akaricidi in njihovi pripravki za zatiranje varoje v zadnjih 15 letih so: organofosfat kumafos (CheckMite®, Asuntol®, Perizin®), piretroid tau-fluvalinat (Apistan®, Klartan®, Mavrik®), flumetrin (Bayvarol®) in formamidin amitraz (Rosenkranz in sod. 2010). Slovenski čebelarji uporabljajo predvsem flumetrin (Bayvarol®), amitraz (Varidol®) in kumafos (CheckMite$^{+®}$).

Med naravne učinkovine z akaricidnim učinkom sodijo predvsem eterična rastlinska olja ter organske kisline. Rastlinska eterična olja so kompleksne mešanice dišavnih snovi, pridobljene iz rastlin z ekstrakcijo vodne pare, suho destilacijo ali mehansko obdelavo brez ogrevanja (Vigan in sod. 2010). Glavne sestavine eteričnih olj so monoterpeni in seskviterpeni, ki predstavljajo približno 90 % vseh sestavin, v manjšem deležu pa so prisotne še druge sorodne aromatske spojine (Blenau in sod. 2012). V rastlinah imajo eterična olja različne fiziološke vloge, kot so privabljanje/odganjanje insektov, zaščita pred vročino/mrazom in kot obramba pred paraziti (Koul in sod. 2008). Imajo tudi fungicidno in baktericidno aktivnost. Zaradi teh lastnosti imajo eterična olja dokazano toksično delovanje na različne vrste organizmov, tudi na ljudi (Vigan 2010). Celoten seznam potencialnih eteričnih olj, ki imajo potencialne akaricidne lastnosti (povzročijo smrtnost različnih vrst pršic), je zelo obširen (Imdorf in sod. 1999, Koul in sod. 2008, Blenau in sod. 2012). Med najpogostejše omenjena imena rastlin, iz katerih so bila proizvedena eterična olja z dokazanim vplivom na varojo, sodijo: *Thymus vulgaris*, *Thymus kotschyanus*, *Eucalyptus camaldulensis*, *Salvia officinalis*, *Origanum vulgare*, *Azadirachta indica*, *Citrullus colocynthis*, *Cymbopogon citratus*, *Satureia hortensis*, *Rosemarinus officinalis, Lavandula officinalis* in *Tagetes minuta* (Ghasemi in sod. 2011). Aktivne učinkovine teh eteričnih olj, s katerimi največkrat povezujejo akaricidno aktivnost, so timol, kafra, mentol, karvakrol, idr. (Imdorf in sod. 1995, Sammataro in sod. 2009, Blenau in sod. 2012). Imdorf in sod. (1999) povzemajo testiranja učinkovitosti različnih učinkovin eteričnih olj na zmanjšanje okuženosti čebel z varojo. Kar 150 različnih eteričnih olj in njihovih učinkovin je bilo testiranih v laboratorijskih pogojih in le malo se jih je izkazalo za učinkovite pri testiranju v panjih, z izjemo timola. Na učinkovitost zdravljenja čebel z eteričnimi olji namreč močno vplivajo precejšnje spremembe v lokalnih razmerah v okolju in znotraj čebeljih družin, predvsem temperatura. Eterična olja so namreč hlapljiva, zato je regulacija koncentracij učinkovin pri tretiranju v panju otežena. Poleg tega so težave tudi pri standardizaciji vsebnosti aktivnih učinkovin eteričnih olj, saj je znano, da je po ekstrakciji eteričnega olja iz iste vrste rastlin vsebnost aktivne učinkovine lahko različna.

Glede na mrežo predstojnikov agencij za zdravila (*angl.* Heads of Medicines Agencies; HMA 2015), ki deluje v okviru Evropske komisije, se v državah članicah EU uporabljajo naslednja eterična olja, njihove sestavine in komercialni pripravki: timol (Api Var®, Apiguard® in Thymovar®), evkaliptusovo olje, kafra, levomentol (Apilife Var®) ter zmesi 3-*p*-cimenola, 2,4 heksandiojske kisline in drugih eteričnih olj (Mehpatika®). V Sloveniji čebelarji uporabljajo pretežno pripravke, ki vsebujejo velik delež timola (Apiguard® in Thymovar®).

Glavne tarče akaricidov pri čebeli

V tem poglavju želimo najprej na kratko predstaviti dva glavna fiziološka sistema čebel,

t.j. živčni in imunski sistem, na katera prvenstveno delujejo sintetični akaricidi in eterična olja z akaricidnim delovanjem. V nadaljevanju pa smo pripravili pregled obstoječih podatkov o vplivu določenih akaricidov na komponente omenjenih sistemov. Poleg vplivov eteričnih olj na čebele primerjalno podajamo tudi podatke o vplivu nekaterih najbolj pogosto uporabljenih sintetičnih akaricidov.

Živčni sistem čebel

Holinergični sistem je eden izmed najpomembnejših ekscitatornih sistemov živčnega sistema čebel (Bicker 1999, Thany in sod. 2010). Holinergični nevroni sintetizirajo ter sproščajo živčni prenašalec acetilholin, ki so ga dokazali bolj ali manj po celotnem živčnem sistemu. Acetilholin deluje na dva tipa receptorjev: muskarinske in nikotinske, muskarinski so pretežno presinaptični, nikotinski pa postsinaptični (Thany in sod. 2010). Muskarinski receptorji so pomembni za uravnavanje sproščanja acetilholina, saj z vezavo na muskarinske receptorje, zavira nadaljnjo sproščanje acetilholina, aktivacija postsinaptičnih nikotinskih receptorjev pa povzroči vzburjenje postsinaptičnega nevrona. Acetilholinesteraza (AChE), na katero inhibitorno delujejo nekateri akaricidi, je encim, ki povzroča inaktivacijo acetilholina s hitro hidrolizo v sinapsah (Thany in sod. 2010). Večino encima AChE v glavi čebel najdemo predvsem v sestavljenem očesu in očescih (Kral 1980, Kral in Schneider 1981). V možganih pa je prisotna v področju antenalnih lobusov ter kaliksov gobastih teles, optičnih lobusih, v nevropilu, ki povezuje obe hemisferi ter področjih znotraj protocerebruma (sprednjih možganov) (Kreissl in Bicker 1989, Scheidler in sod. 1990). Nikotinskih receptorji se v možganih čebel nahajajo predvsem na Kenyonovih celicah gobastih teles ter nevronih antenalnih lobusov (Bicker 1999, Nauen in sod. 2000, Deglise in sod. 2002, Wustenberg in Grunewald 2004, Barbara in sod. 2005, Thany in sod. 2010). Pokazali so, da so nikotinski receptorji vpleteni v nastanek olfaktornega (vonjalnega) spomina, ki je zelo pomemben za uspešno usmerjanje čebel na pašo (Cano Lozano in sod. 1996, 2001). Možganske strukture pri čebeli, ki so pomembne za olfaktorno učenje, so antenalni lobusi, gobasta telesa, lateralni deli protocerebruma (sprednjih možganov) ter subezofagealni ganglij (Mobbs 1985). Prvo procesiranje olfaktornih informacij poteka v antenalnih lobusih, zato le-ti po analogiji predstavljajo olfaktorne bulbuse pri vretenčarjih (Hildebrand in Shepherd 1997). Vonjalna informacija se nato prevaja v višje integracijske centre kot so gobasta telesa ter po poteh, ki vodijo do subezofagealnega ganglija. Gobasta telesa so osnovni možganski center, ki nadzoruje kompleksa vedenja in so bistvena za nastanek olfaktornega spomina (Heisenberg 1998, Menzel 2001). Čebelje gobasto telo je sestavljeno iz parnih kaliksov (klobukov), pedunkla (peclja) ter dveh lobusov (režnjev), alfa in beta. Najpomembnejši vhodni informacijski del gobastih teles predstavljajo kaliksi, kamor prihajajo informacije iz drugih senzoričnih področij čebeljih možganov (Schurmann 1973, Mobbs 1982, Kenyon 1896). Kenyonove celice, nevroni gobastih teles, hkrati obdelujejo različne senzorične informacije ter jih nato prevajajo v alfa ter beta lobusa, ki predstavljata glavna izhodna dela gobastih teles (Schurmann 1970, Mobbs 1982, Rybak in Menzel 1993, 1998).

Gama-aminomaslena kislina (GABA) je osnovni inhibitorni živčni prenašalec centralnega živčnega sistema ter živčno-mišičnih stikov (Chapman 1998). Pri čebelah je GABA prisotna v nevropilu, v strukturah, povezane z učenjem in spominom (antenalni lobus – tipalnični reženj, gobasto telo ter optični lobusi – vidni reženj) (Schäfer in Bicker 1986, El Hassani in sod. 2009). Z uporabo protiteles proti GABA so pokazali njeno prisotnost v lokalnih internevronih, v projekcijskih nevronih pa je prisotnost živčnega prenašalca GABA precej okrnjena (Bicker in sod. 1985, Meyer in sod. 1986, Schäfer in Bicker 1986). GABA se v možganih čebele nahaja v približno 5% nevronov. Inhibitorni GABA internevroni v gobastih telesih vplivajo v nastanek olfaktornega spomina pri čebeli, tako da inhibirajo delovanje Kenyonovih celic (Grunewald 1999). Živčni prenašalec GABA so dokazali tudi v vidnih projekcijah, predvsem v optičnih lobusih (Schäfer in Bicker 1986). Pri medonosni čebeli verjetno obstajata vsaj dve vrsti GABAergičnih receptorjev, na pikrotoksin (antagonist GABA receptorjev) občutljivi ter na picrotoksin neobčutljivi receptorji (Sachse in Galizia 2002).

Oktopamin spada v skupino biogenih aminov (Farooqui 2011). Pri žuželkah oktopamin

deluje kot nevrotransmiter, nevromodulator in nevrohormon in ima zato pomembno vlogo v različnih fizioloških procesih. V perifernem živčnem sistemu modulira aktivnost letalnih mišic, perifernih organov in večine senzoričnih organov. V osrednjem živčnem sistemu je vpleten v regulacijo motivacije, desenzibilizacije senzoričnih informacij, vzburjenja, iniciacije in vzdrževanja različnih ritmičnih vedenj, higienskega vedenja in kompleksnega socialnega vedenja, skupaj z učenjem in spominom. Pri čebelah so pokazali predvsem vpletenost oktopamina v plesno komunikacijo ter pašno aktivnost, uravnavanje iztegovanja žela ter olfaktorno učenje (Burrell in sod. 1995, Hammer in Menzel 1998, Farooqui in sod. 2003, Schwaerzel in sod. 2003, Barron in sod. 2007). V možganih čebel je oktopamin prisoten predvsem v gobastih telesih in v nevropilu optičnih lobusov, predvsem v meduli ter v nevrosekretornih celicah, ki inervirajo žlezo corpora cardiaca (Mercer in sod. 1983, Kreissl in sod. 1994). Čebele imajo samo eno vrsto oktopaminskega receptorja AmOA1 (Grohmann in sod. 2003). Pri nevrohormonalnem delovanju oktopamina se le-ta sprošča v hemolimfo, transportira v ciljna tkiva in sproži mobilizacijo lipidov in ogljikovih hidratov, ter tako omogoči žuželkam podaljšano aktivnost ali pa okrevanje iz obdobja povečanega povpraševanja po energiji. Vpliva tudi na mobilizacijo hemocit po bakterijskih okužbah. Domnevajo, da posreduje pri celičnem imunskem odzivu, kot so hemocitna fagocitoza in nodulacija med bakterijsko okužbo (Farooqui 2011).

Imunski sistem čebel

Imunski sistem čebel je prirojen in pretežno nespecifičen. Imunski sistem žuželk predstavlja: kutikula kot fizikalno-kemijska bariera, humoralna imunost, celična imunost in vedenjske strategije za zmanjšanje prenosa bolezni na nivoju družine. Humoralno imunost predstavljajo različne molekule, kot so antimikrobni proteini (AMP), proteini podobni komplementu in encimske kaskade, ki uravnavajo tvorbo melanina (Hillyer 2016). Humoralni odgovor se sproži s strani vzorčno prepoznavnih receptorjev ali receptorjev PRR, ki so odgovorni za prepoznavanje mikroorganizmov v telesu, kar sproži sintezo različnih AMP (James in Xu 2012). Sinteza AMP je uravnavana preko številnih signalnih poti, štiri so našli tudi pri čebelah: Toll (pomembna za imunost ter razvoj), Imd, JNK in JAK/STAT (Evans in sod. 2006). Humoralni odgovor nastaja lokalno v prebavilih na mestu vdora mikroorganizmov, nastaja pa tudi sistemsko v hemocelu, kjer receptorji PRR prepoznavajo patogene ter inducirajo sintezo AMP v maščobnih telescih ter hemolimfi. Nekateri lični encimi, kot so esteraze, karboksilesteraze ter lizocimi lahko delujejo kot AMP (Hillyer 2016). Celično imunost predstavljajo odgovori različnih vrst hemocit po prepoznavanju patogenov, kar sproži fagocitozo (bakterije, virusi), nodulacijo (glive, skupki bakterij) ali enkapsulacijo (mnogocelični paraziti). Celična imunost omeji infekcije s strani patogenih organizmov (Evans 2006, Hillyer 2016). Fagocitozo spremlja sinteza melanina ter melanizacija nodulov ter kapsul. Značilna je hitra sinteza in nalaganje melanina na mesto okužbe in poškodbe. Ključni encim za sintezo melanina je fenoloksidaza. Akaricidi lahko vplivajo na čebelji imunski sistem na vseh nivojih z oslabitvijo humoralnega in celičnega odziva, pa tudi vedenjske imunosti. Maščobno telesce je pri žuželkah organ s številnimi metabolnimi funkcijami in je med drugim glavni organ udeležen v imunskem odzivu organizma. Celice maščobnega telesca, imenovane oenociti, so odgovorne za produkcijo proteinov, ki se sprostijo v hemolimfo in sodelujejo pri prepoznavanju patogena in pri mehanizmih imunske obrambe (Hillyer 2016). Tkivo maščobnega telesca je urejeno v tanke sloje celic ob integumentu, v direktnem kontaktu s hemocelom, bolj pogosto pa se ga najde v zadku žuželke (Roma in sod. 2010).

Mehanizmi delovanja sintetičnih akaricidov in eteričnih olj na čebele

Vpliv na živčni sistem

Pogosto uporabljen sintetični akaricid kumafos je organofosfat. Organofosfosfati inhibirajo acetilholinesterazo (AChE) (Fukuto 1990). Ker je AChE glavna sestavina večine sinaptičnih prenosov v žuželkah, lahko njeno zaviranje povzroči splošne motnje v delovanju vseh organskih sistemov (Kreissl in Bicker 1989, Desneaux in sod. 2007). Amitraz spada med formamidne akaricide. Formamidi delujejo toksično preko

agonističnega delovanja na receptor za nevromodulator oktopamin (Evans in Gee 1980, Dudai in sod. 1987). Naslednji pogosto uporabljen akaricid tau-fluvalinat je piretroid (Davies in sod. 2007). Sintetični piretroidi blokirajo napetostno odvisne natrijeve kanalčke žuželk v membranah živčnih celic in s tem blokirajo delovanje živčnih celic (Davies in sod. 2007).

Tudi eterična olja delujejo toksično predvsem preko vpliva na živčni sistem (Blenau in sod. 2012). Ena izmed pomembni tarč eteričnih olj v živčnem sistemu so receptorji za biogena amina oktopamin in tiramin. To so pokazali za eterična olja, ki vsebujejo eugenol, α-terpineol in cimetni alkohol (Enan 2001). Toksičnost *p*-cimena, timola, karvakrola, α-terpineola in karvona pri vinski mušici *Drosophila melanogaster* je sorazmerna z afiniteto vezave na tiraminski receptor, kar pomeni, da vse te snovi delujejo preko tega receptorja (Enan in sod. 2005). Potrjena je tudi delovanje eugenola, cimetnega alkohola, transanetola na oktopaminski receptor ter timola na receptor za tiramin (Blenau in sod. 2012). Pokazano je bilo, da nekatere snovi eteričnih olj delujejo tudi na GABAergični živčni sistem. Karvakrol, pulegon in timol so pozitivni alosterični modulatorji žuželčjih $GABA_A$ receptorjev in s tem dodatno ojačajo učinek delovanja živčnega prenašalca GABA (Blenau in sod. 2012). Tarče nekaterih monoterpenov so tudi TRP ionski kanalčki, ki jih najdemo v senzoričnih sistemih žuželk, kot je vidni sistem, pa tudi na termo-, mehano- ter proprioreceptorjih (Fowler in Montell 2013). Pokazano je bilo, da karvakrol, timol, eugenol, cimetni aldehid, mentol in karveol delujejo inhibitorno na TRPL ionske kanalčke, ki so del družine TRP kanalčkov in za katere je znano, da so pomembni pri fototransdukciji, medtem ko pa kafra in borneol stimulirata te kanalčke (Blenau in sod. 2012).

Vpliv na imunski sistem

Delovanje sintetičnih akaricidov na imunski sistem čebel je slabo raziskano. Naša prejšnja študija ter tudi študije tujih avtorjev so pokazale, da kronična izpostavitev akaricidom tau-fluvalinat, kumafos, amitraz in flumetrin spremeni izražanje nekaterih genov pri čebelah, ki so povezani z imunskim sistemom (Boncristiani in sod. 2012, Garrido in sod. 2013, Cizelj in sod. 2016).

Boncristiani in sod. (2012) so pokazali vpliv kumafosa na izražanje genov, ki so vpleteni v celični imunski odziv. Kumafos pri čebelah inhibira gen *Dscam* odgovoren za izražanje higienskega vedenja čebel, ki služi za obrambo proti varoji. Ta gen se izraža v hemocitah, kjer ima vlogo pri prepoznavanju in odstranjevanju patogenov. Kumafos je zmanjšal tudi izražanje gena *basket*, katerega izražanje aktivira melanizacijo ter protimikrobne in apoptotične obrambne mehanizme. Isti avtorji so pokazali tudi inhibicijo gena za vitelogenin, ki med drugim stimulira normalno delovanje hemocit pri imunskem odzivu (Amdam in sod. 2004, Boncristiani in sod. 2012). Garrido in sod. (2013) so pri čebelah, tretiranih s kumafosom in flumentrinom, ugotovili spremenjeno izražanje genov za antimikrobne peptide (AMP), ki so del humuralne imunosti. V raziskavi Cizelj in sod. (2016) smo pokazali, da je vpliv kronične vzpostavitve kumafosa na imunski sistem kompleksen ter odvisen od razvojne stopnje čebele. Največje zmanjšanje smo opazili pri izražanju genov ličink in sicer za AMP defensin-1, za signalni peptid Spaetzle in gen za PGRP-SC protein, odgovoren za prepoznavanje patogenov. Pri bubah z belimi očmi je kumafos vplival predvsem na gene za AMP: *abaecin, lysozyme-2 in defensin-1*. Pri odraslih čebelah smo po izpostavitvi kumafosu pokazali povečano izražanje genov, ki kodirajo AMP in beljakovine, ki so vključene v JAK / STAT in JNK signalne poti (Cizelj in sod. 2016). Vse te rezultate vplivov akaricidov je zaradi kompleksnosti imunskega sistema težko natančno pojasniti. Ugotovljeno je bilo tudi, da akaricida kumafos in tau-fluvalinat ter fungicid klorotalonil spremenijo sestavo bakterijske flore v prebavilih čebel in na tak način morebiti vplivajo na imunski sistem čebel (Kakumanu in sod. 2016).

Vplivi eteričnih olj na imunski sistem čebel so praktično neraziskani. Boncristiani in sod. (2012) so ugotovili, da ima kronično tretiranje s pripravkom Apiguard®, ki vsebuje timol, podobne učinke na izražanje genov imunskega odgovora (*Dscam* in *basket*) kot kumafos**.** Zaradi podobnih mehanizmov delovanja sestavin eteričnih olj na organizme lahko pričakujemo, da tudi drugi naravni akaricidi vplivajo na imunski sistem, za potrditev pa so potrebne nadaljnje raziskave.

Tabela 1: Pregled obstoječih podatkov o strupenosti eteričnih olj in njihovih aktivnih učinkovin za *čebele* (*Apis mellifera*). Viri so razporejeni kronološko.
Table 1: An overview of existing data on the toxicity of essential oils and their active ingredients for bees (*Apis mellifera*). References are arranged chronologically.

Razvojna stopnja čebele	Način izpostavitve	Testiran parameter strupenosti	Testirana snov	Opisan učinek	Vir
Odrasle čebele, različne starosti	Izpostavitev v panjih, 15 g timola zavitega v gazo/panj, 4 x vnos v panj vsake 4 dni	Smrtnost	Timol (kristali)	Niso opazili vpliva na odrasle čebele in zalego	Marchetti in Barbattini 1984
Odrasla čebela	Inhalacija, zrakotesna posoda z vmesnim obdobjem prezračevanja.	Smrtnost	a.) timol b.) kafra c.) mentol d.) evkaliptusovo olje*	a.) 72 h LD50 = 3 µg/L zraka b.) 72 h LD50 = 30-40 µg/L zraka c.) 72 h LD50 = 10-15 µg/L zraka d.) 72 h LD50 = 350 µg/L zraka	Imdorf in sod. 1995
Odrasla čebela	Inhalacija; nanos raztopin (10 µL) na dno 3.4 L zaprte posode, v kateri je kletka s čebelami	Smrtnost	24 različnih eteričnih olj	15 eteričnih olj je imelo > 10 % učinek na čebele po 72 h. Največji učinek (100 % smrtnost) so imela eterična olja iz česna, čebule, in pelina. Sledijo timijan (92 %), origano (87 %), evkaliptus (67 %), poprova meta (48 %), koriander (40 %), kumina (17 %) idr.	Imdorf in sod. 1995
Odrasla čebela	Preko voska, laboratorij	Smrtnost	Eterična olja majarona, cimet, nageljnove žbice, in sivka	Po 3 dneh 10 % olja iz nageljnovih žbic povzroči 100 % smrtnost, 10 % olja iz majarona pa 20 % smrtnost čebel.	Imdorf in sod. 1995
Odrasle čebele, delavke, okužene z pršico *Acarapis woodi*	Nanos raztopine na dno steklene posode, čebele v kletki.	Smrtnost	a.) karvakrol b.) citral c.) *d*-limonen d.) mentol e.) pulegon f.) timol g.) a-terpineol	a.) 24 h LC50 = 11 µg/mL b.) 24 h LC50 = 10 c.) 24 h LC50 =10 d.) 24 h LC50 =5.3 e.) 24 h LC50 =6.6 f.) 24 h LC50 =1.7 g.) 24 h LC50 =17.1	Ellis in Baxendale 1997
Odrasle čebele, delavke, okužene z varojo	Nanos raztopine na dno petrijevke. Čebele izpostavljene v petrijevki.	Smrtnost	a.) timol b.) karvakrol c.) cimetovo olje d.) mentol e.) citronellal f.) a-terpineol	a.) 24 h; 5 mg/kletko ni učinka b.) 24 h; 2 mg/kletko:11 % smrtnost c.) 24 h; 5 mg/kletko, 44 % smrtnost d.) 24 h; 5 mg/kletko; 56 % smrtnost e.) 24 h; 15 mg/kletko; 94 % smrtnost f.) 24 h; 5 mg/kletko; 78 % smrtnost	Lindberg in sod. 2000
Odrasla čebela, delavka	Oralna izpostavitev, raztopina saharoze, laboratorij	Smrtnost	a.) Limonska trava[1] b.) *Šetrajevo* olje[2] c.) Timijanovo olje[3] d.) Origanovo olje[4] e.) različne mešanice olj	24 h in 48 h; ni natančnih podatkov za LC50; smrtnost pri: a.) 2 µg/čebelo b.) 5 µg/čebelo c.) 8 µg/čebelo d.) 3 µg/čebelo e.) 24 h LD50 = 16-122 µg/čebelo in 48 h LD50 = 19-357 µg/čebelo	Albo in sod. 2003

Odrasla čebela	Izpostavitev v panjih	Smrtnost, razmnoževanje	a.) Apiguard®[5] b.) Apilife Var®[6]	Po 4 tednih ni bilo učinka na preživetje odraslih čebel po tretiranju z 2,5 palice Api Life VAR ali 1 gelom Apiguarda/ panj. V obeh primerih pride do značilnega zmanjšanja velikosti zalege, kar pomeni vpliv na reprodukcijo.	Floris in sod. 2004
Odrasle čebele, delavke	Nanos raztopine na dno petrijevke. Čebele v petrijevki.	Smrtnost	a.) timijan[8] b.) navadni lovor[9] c.) sivka[10]	a.) 24 h LC50 = 22; 48 h LC50 = 12 in 72 h LC50 = 8 µL/petrijevko b,c) 72 h LC50 > 20 µL/petrijevko	Damiani in sod. 2009.
Odrasla čebela, delavka	Topična izpostavitev, 5 µL na dorzalno stran trupa, laboratorij	Smrtnost	a.) Timol b.) Origanovo olje* c.) Olje nageljnovih žbic[11] * d.) Mentol	a.) 4 h LC50 = 210.3 µg/čebelo b.) 4 h LC50 = 331,3 c.) 4 h LC50 = 238,6 d.) 4 h LC50 = 523,5	Gashout in Guzman-Novoa 2009
Larva	Nanos raztopine na dno testne posode, 5 µL; v panju	Smrtnost	a.) Timol b.) Origanovo olje* c.) Olje nageljnovih *žbic** d.) Mentol	a.) 4 h LC50 = 150,7 µg/larvo b.) 4 h LC50 = 236,4 c.) 4 h LC50 = 281,4 d.) 4 h LC50 = 382,8	Gashout in Guzman-Novoa 2009
Odrasle čebele, 2-dni stare	Izpostavitev v panjih po navodilih proizvajalca[7] (28 dnevna izpostavitev)	Vedenje	Apiguard®	*Čebele delavke se izogibajo stiku z* Apiguardom. Ob stiku s pripravkom odrasle čebele ventilirajo (hitro zamahujejo s krili), mlajše (2- in 4-dneve čebele) ne ventilirajo.	Mondet in sod. 2011
Odrasle čebele, različne starosti	Izpostavitev v panjih po navodilih proizvajalca[7] (30 dnevna izpostavitev)	Subletalni vplivi na izražanje genov	Apiguard®[5]	Značilna sprememba izražanja genov, ki so udeleženi v detoksifikaciji in imunskem odzivu.	Boncristiani in sod. 2012
Odrasle čebele, različne starosti	Izpostavitev v panjih, 3 tablete vsakih 7 dni/panj (vzorčenje čebel 1 teden po vnosu pripravka, vzorčenje vsak mesec 1x (skupno 3 jeseni in 3 spomladi)	Vedenje	Apilife Var®[6]	Opažen upad fototaktičnega vedenja (zmanjšan odziv na svetlobo).	Carayon in sod. 2014, Alayrangues in sod. 2016

* sestava ni znana

1 *Cymbopogon citratus,* 67 % citral, 33 % neznano

2 *Satureia hortensis*, 35 % karvakrol, 40 % ϒ-terpinene, 5 % *p*-cimene

3 *Thymus vulgaris*, 40 % timol, 18 % *p*-cimene; 13 % ϒ-terpinene; 17 % neznano

4 *Origanum vulgare*, 25 % timol, 14 % borneol, 7 % ϒ-terpinene, 40 % neznano

5 25 % timol v želatini

6 74 % timol, 3,7 % mentol, 3,7 % kafra, 16 % evkaliptovo olje

7 http://www.vita-europe.com/products/apiguard/#HowtouseApiguard

8 *Thymus vulgaris*, 65 % timol, 5,4% karvakrol

9 *Laurus nobilis*

10 *Lavandula officinalis, Lavandula hybrid*

11 *Syzygium aromaticum*

Drugi negativni vplivi akaricidov na čebele

Zaradi načina aplikacije akaricidov v čebelarstvu so glede učinkov najbolj relevantni rezultati kroničnih tretiranj čebel s subletalnimi koncentracijami. Neželeni učinki subletalnih koncentracij kumafosa na čebele so zelo dobro dokumentirani. Tuji avtorji so poročali tudi o spremembah izražanja genov za detoksifikacijo in hormonov, ki sodelujejo pri nastanku različnih vedenj čebel, kot je juvenilni hormon III (Boncristiani in sod. 2012, Garrido in sod. 2013, Schmehl in sod. 2014, Chaimanee in sod. 2016). Subletalni odmerki kumafosa negativno vplivajo na vitelogenin in hexamerin 70B v čebeljih maticah, kar verjetno zmanjšuje njihovo življenjsko dobo, reproduktivno zmogljivost in povečuje oksidativni stres (Chaimanee in sod. 2016). Nivo izražanja proteina hexamerin Hsp70 po tretiranju s kumafosom je bil spremenjen tudi pri ličinkah (Gregorc in sod. 2012). Kumafos vpliva tudi na premer končnega, mešičkasto razširjenega dela (acinusa) krmilne žleze in sproži povečani nivo programirane celične smrti (Smodiš Škerl in Gregorc 2010). Ugotovljenih je bilo tudi veliko učinkov kumafosa na čebele na nivoju celotne družine. Kumafos zmanjša pašno aktivnost čebel (Schneider in sod. 2009) hkrati pa negativno vpliva na prenos hrane med pašnimi čebelami in delavkami ter med čebelami, ki skrbijo za ličinke, kar posledično negativno vpliva na razvoj zalege (Bevk in sod. 2012). Prav tako se lahko zmanjša prenos hrane med pašnimi čebelami plesalkami ter spremljevalkami, kar pomembno vpliva na usmerjanje čebel na pašo (Farina in Wainselboim 2005). Matice družin, ki so bile tretirane s kumafosom, imajo lahko zmanjšano telesno težo, zmanjšano težo ovarijev, poleg tega se matice nenavadno vedejo, njihove ličinke pa imajo visoko stopnjo umiranja (Haarmann in sod. 2002, Collins in sod. 2004, Pettis in sod. 2004). Po tretiranju s kumafosom je lahko zmanjšana tudi viabilnost spermijev trotov (Burley in sod. 2008). Akutna letalna doza kumafosa je odvisna od starosti čebel in znaša 3 to 6 µg na čebelo in je manjša za stare čebele (van Buren in sod. 1992).

Amitraz je formamidni akaricid, ki stimulira receptor za nevromodulator oktopamin (Evans in Gee 1980, Dudai in sod. 1987), zato bi lahko vplival na pašno aktivnost čebel, vendar to ni raziskano. Pokazana pa je bila njegova akutna toksičnost na celice srednjega črevesja ličink čebel (Gregorc in Bowen 2000). Akaricid tau-fluvalinat deluje na napetostno odvisne natrijeve kanalčke in tako učinkovito odstranjuje varojo, vendar je relativno varen za čebele, saj ga čebelji detoksifikacijski encimi citokrom-P450-monooksigenaze učinkovito in hitro metabolizirajo (Johnson in sod. 2010). Vendar tau-fluvalinat verjetno ni neškodljiv za čebele in vpliva na zdravje matic in trotov. Matice, izpostavljene visokim odmerkom tau-fluvalinata, so manjše od neizpostavljenih. Troti, izpostavljeni temu akaricidu tekom razvoja, bodisi spolno ne dozorijo in so neplodni ali pa imajo manjšo količino spolnih celic, vendar pa to naj ne bi imelo vpliva na število njihovih potomcev (Johnson in sod. 2010). Ne glede na nedvoumno dokazane negativne učinke kumafosa in tau-fluvalinata na čebele, pa raziskovalci Berry in sod. (2013) po aplikaciji v subletalnih koncentracij komercialnih pripravkov Apistan® (tau-fluvalinat) in Check Mite® (kumafos) v panje niso zaznali negativnih učinkov obeh akaricidov na čebelje družine. Apistan® ali Check Mite® tako nista imela vpliva na količino zalege, medu, stopnje pašne aktivnosti, na čas vračanja pašnih čebel nazaj v panj in stopno okuženosti z nosemo.

Če primerjamo akutne letalne doze najbolj pogostih sintetičnih akaricidov, lahko ugotovimo, da je izmed zgoraj opisanih akaricidov amitraz najmanj toksičen za čebele, tau-fluvalinat in flumentrin pa sta najbolj toksična (Oruc in sod. 2012, Dai in sod. 2017). Namreč, akutna letalna doza amitraza je 14.83 µg na ličinko čebel, kumafosa 2.7 µg na ličinko in tau-fluvalinata 0.83 µg na ličinko, flumentrina pa 0.527 µg na odraslo čebelo.

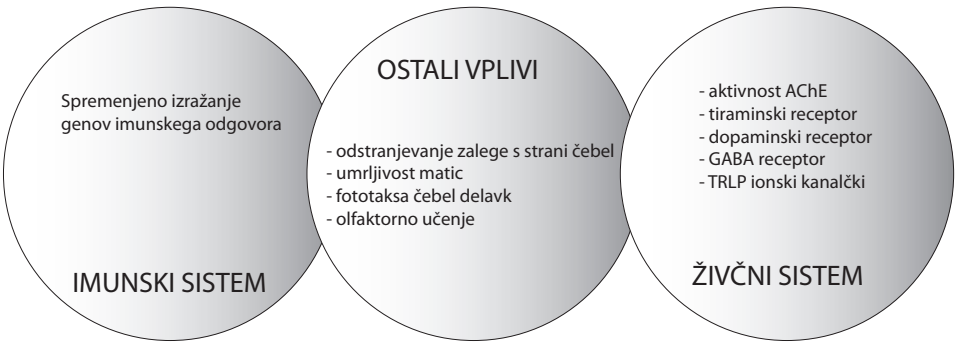
V primerjavi s sintetičnimi akaricidi je za naravne akaracide znanih bistveno manj podatkov glede njihove varnosti za čebele, sploh kar se tiče kroničnih izpostavitev oziroma izpostavitev subletalnim koncentracijam. V Tabeli1 smo zbrali obstoječe podatke o strupenosti različnih eteričnih olj z akaricidnim delovanjem za čebele *A. mellifera*. Ugotavljamo, da so bile čebele v večini dosedanjih študijah izpostavljene akutno (4-72 ur) zelo visokim koncentracijam izbranih testnih snovi. Slednje je vodilo v visoko smrtnost čebel. Čebele so bile eteričnim oljem izpostavljene pod zelo različnimi pogoji. Eterična olja so bila

bodisi nanesena direktno v petrijevko ali steklено posodo, v vosek, direktno na trup čebele, v nekaterih študijah pa so bile čebele izpostavljene v panju. Podajanje izpostavitvenih doz je zato neenotna. Čebele so bile pogosto okužene, saj so avtorji vzporedno proučevali tudi vpliv na zmanjšanje pojavnosti varoje, zato ti rezultati ne podajo informacije, kako bi se na testirano snov odzvale zdrave čebele. Zaključujemo, da obstaja potreba po sistematičnem testiranju vplivu naravnih akaricidov na čebele, kjer bi bile čebele le-tem izpostavljene pod enakimi pogoji in bi bili poskusi izvedeni po principu aktualnih smernic testiranja strupenosti, npr. OECD TG 245.

Na voljo je le malo podatkov o subletalnih učinkih akaricidnih eteričnih olj na čebele (Boncristiani in sod. 2012). Poleg že zgoraj omenjenih učinkov na imunski sistem (Boncristiani in sod. 2012) je bilo pokazano, da timol vpliva tudi na odstranjevanje zalege s strani čebel (Marchetti in sod. 1984, Floris in sod. 2004), povečuje umrljivost matic (Whittington in sod. 2000), zmanjša fototakso čebel delavk (Bergougnoux in sod. 2013) ter preprečuje olfaktorno učenje: pogojevanje refleksa iztegovanja proboscisa pri čebelah delavkah (Bonnafé in sod. 2016). Učinke eteričnih olj z akaricidnim delovanjem na čebelo smo povzeli na Sliki 2.

Zaključki in nadaljnje smernice

V prispevku smo predstavili obstoječa znanja o vplivu eteričnih olj s potencialnim akaricidnim delovanjem na medonosno čebelo *A. mellifera*. Ugotavljamo, da je v nasprotju s sintetičnimi akaricidi, področje vpliva akaricidov z naravnim izvorom precej manj raziskano. Kratkotrajne študije so pokazale, da so v določenih visokih odmerkih nekatera eterična olja strupena za čebele. Vendar uporaba v čebelarstvu temelji na nižjih, subletalnih koncentracijah s podaljšanim tretiranjem. Testiranja subletalnih koncentracij eteričnih olj za zatiranje varoje v laboratoriju so pokazale visoko akaricidno učinkovitost velikega števila eteričnih olj ter relativno nizko toksičnost za čebele, zato so nadaljnje takšne raziskave perspektivne. Kot glavni tarči delovanja eteričnih olj na čebele smo izpostavili živčni ter imunski sistem. Še posebej so pomembna znanja o njihovem vplivu na imunski odziv, saj so spremembe le tega navedene kot eden izmed potencialnih možnih vzrokov za slabo zdravje čebel. Med eteričnimi olji prevladujejo podatki za timol ter njegove pripravke (Apiguard®, Apilife Var®), precej manj pa je podatkov o drugih pogostih aktivnih učinkovinah eteričnih olj. Zaključujemo, da obstaja potreba po sistematičnem testiranju vplivu akaricidnih eteričnih olj na čebele, s poudarkom na dolgotrajnih izpostavitvah izvedenih po principu aktualnih smernic testiranja strupenosti, npr. OECD TG 245.



Slika 2: Povzetek učinkov nekaterih eteričnih olj, ki se uporabljajo za zatiranje varoje, na medonosno čebelo *Apis mellifera.* (AChE:acetilholinesteraza; GABA: Gama-aminomaslena kislina).

Figure 2: Summary of the effects of some acaricide essential oils on the honey bee *Apis mellifera*. (AChE:acetylcholinesterase; GABA: gamma-aminobutyric acid).

Velik izziv za prihodnje raziskave predstavlja optimizacija aplikacije in standardizacija uporabe eteričnih olj in njihovih učinkovin v čebelarstvu.

Summary

Beekeeping has been recognized as an essential part of food production in countries with intensive agriculture. Honey bees (*Apis mellifera*) are important pollinators and have great environmental, agronomic and economic importance. The parasitic bee varroa mite (*Varroa destructor*) is among the most serious honey bee pests and cause substantial economic losses in beekeeping industry. Effective varroa mite treatment is therefore a pressing issue worldwide. Beekeepers utilize a wide range of different synthetic chemical substances (called acaricides), and application techniques to keep mite populations under control. However synthetic acaricides cause mite resistance, they are persistent in the wax and have documented adverse effects on honey bees. Therefore the use of naturally derived substances is being promoted. One of these substances with known varroa mite toxic actions are essential oils. Among them, thymol is the one with the most frequent use. In this paper we review the existing knowledge regarding the effects of essential oils with known acaricide actions on honey bee *A. mellifera*. In particularly, we focus on the mechanisms of toxic action on the immune and nervous system. Also, other effects on honey bees are presented. We conclude that some essential oils could be effectively used to treat varroa mite but currently very little data regarding their sublethal chronic effects on honey bees are known. In particular, their interference in the immune response is important to be able to predict the potential effect on the colony health. The majority of toxicity data currently exist for thymol and its commercial preparations (Apiguard®, Apilife Var®), but the data for a number of other essential oils with the acaricidal potential are missing. We recognize the need for systematic screening of potential toxicity and sublethal effects of essential oils with acaricide action on honey bees. Existing honey bee toxicity testing guidelines should be employed, e.g. OECD TG 245. Standardized application of essential oils in beekeeping remains a challenging task for the future.

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Evaluation of cyanobacteria biomass derived from upgrade of phycocyanin fluorescence estimation

Vrednotenje biomase cianobakterij na osnovi nadgradnje ocene fluorescence fikocianina

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Abstract: The number of harmful cyanobacterial blooms has increased significantly at the global level in recent years. One of the characteristics of cyanobacteria that gives them advantage over other phytoplankton organisms are auxiliary photosynthetic pigments, such as phycocyanin. This fluorescent pigment emits light at a different wavelength as chlorophyll and can therefore be used for detection of cyanobacteria *in situ*. In this study we used submersible phycocyanin fluorescence sensors and compare their voltage output to concentration of extracted phycocyanin, cell counts and biovolume. The relation was linear in all three cases; however, the variability of regression line slopes between different cyanobacteria strains was high in the case of PC extract concentration and cell count. The highest uniformity in the linear fits was between fluorescence signal and biovolume therefore making it the best candidate for fluorescence sensor voltage output conversion. In the context of this work we also compared different methods for PC extraction. Modifying the equations by subtracting the absorption at 750 nm almost entirely reduces the false PC concentration estimation due to sample turbidity.

Keywords: fluorescence measurements, phycocyanin, cyanobacteria

Izvleček: V zadnjih letih se število škodljivih cvetenj cianobakterij na globalni ravni močno povečuje. Ena od značilnosti cianobakterij, ki jim prinaša prednost pred ostalimi fitoplanktonskimi organizmi, so pomožna fotosintezna barvila, med katerimi prevladuje fikocianin. Fikocianin fluorescira pri drugi valovni dolžini kot klorofil, zato lahko s pomočjo meritve fluorescence ugotavljamo prisotnost cianobakterij v vodnem okolju *in situ*. S potopnim senzorjem fluorescence fikocianina smo opravili meritve dveh sojev cianobakterije *Microcystis aeruginosa* in nitaste cianobakterije vrste *Arthrospira platensis*. Odnos med koncentracijo ekstrahiranega fikocianina, številom celic in njihovim biovolumnom ter fluorescenco fikocianina je bil v vseh treh primerih linearen, vendar pa je bila variabilnost naklonov regresijske premice v

primeru koncentracije fikocianinskega ekstrakta in števila celic med različnimi vrstami cianobakterij visoka. Najvišje ujemanje naklonov linearnih regresij je bilo med signalom senzorja in biovolumnom, zaradi česar je najboljši kandidat za pretvorbo izhodne napetosti fluorescenčnega senzorja v limnološko pomembno količino. V okviru tega dela smo primerjali tudi različne protokole za ekstrakcijo fikocianina. Obstoječe enačbe za pretvarjanje absorpcije v koncentracijo fikocianina smo dopolnili z odštevanjem absorpcije vzorca pri 750 nm in s tem zmanjšali zavajujočo oceno koncentracije.

Ključne besede: meritve fluorescence, fikocianin, cianobakterije

Introduction

Cyanobacteria are a part of the phytoplankton community in each water body. Problems occur if their concentration increases. Most cyanobacterial genera produce cyanotoxins – a very diverse group of toxic substances, which pose a threat to the environment, animals and people. The number of blooms at the global level has significantly increased (Paerl et al. 2011) also due to nutrient load from anthropogenic sources.

Reliable and accurate information on cyanobacteria concentration in water bodies enables us to respond appropriately and prevent risks to animal and human health. Traditional methods of phytoplankton monitoring have been available for many years, as part of national legislations in many countries, and from 2000 a part of official monitoring procedures within the EU Water Framework Directive (Directive 2000/60/EC). Precise determination of phytoplankton species composition can be achieved with microscopic examination. The method is time-consuming and requires specialized knowledge. Moreover, the overall apprehension on spatial and temporal distribution of phytoplankton in water body is very limited.

One of the characteristics of cyanobacteria that gives them advantage over other phytoplankton organisms are auxiliary photosynthetic pigments such as phycocyanin (PC), alophycocyanin, and phycoerythrin. They allow cyanobacteria to use the available light more efficiently (Raps et al. 1983). PC is a fluorescent pigment, which emits light at a different wavelength as chlorophyll so the PC fluorescence can be used to detect the presence of cyanobacteria in the aquatic environment *in situ* and discriminate them from other phytoplankton.

Field sensors measuring *in vivo* fluorescence have been successfully applied in various oceanographical and limnological studies, giving real-time results on a detailed spatial and temporal scale. Detection limits and correlation between PC signal and biovolume have also been determined (Kong et al. 2013, Kasinak et al. 2015). Despite the advantages, limitations in the estimation of cyanobacterial abundance with PC fluorescence sensors have been reported (Gregor et al. 2007, Chang et al. 2012).

In this study we used submersible phycocyanin fluorescence sensors to measure concentration of three different cyanobacterial strains. We compared the results with cell counts, biovolume and concentration of extracted PC in order to find the most suitable parameter for converting the voltage output of the sensors into limnological values. In the context of this work we also compared different methods for PC extraction. In contrast to chlorophyll the extraction of phycocyanin is not standardized.

Materials and Methods

Laboratory cultures

Three different axenic cell lines of cyanobacteria and one of green algae were used: two different strains of unicellular *Microcystis aeruginosa* (microcystin-producing strain PCC 7806 and non-producing strain PCC 7005) from the Institute Pasteur (Paris, France), *Arthrospira platensis* SAG 85.79, a filamentous representative of cyanobacteria, and green algae *Desmodesmus communis* 276-4b from the SAG collection (Goettingen, Germany). The cyanobacteria and green algae were grown and maintained under

sterile conditions in 100 mL flasks with 50 mL Jaworski medium at room temperature exposed to natural daylight.

Fluorescence measurements

For fluorescence measurements a portable KM 245 (Arhel, Slovenia) system equipped with submersible PC fluorometer Cyclops 7 (Turner, USA) was used. A magnetic stirrer (C-MAG MS4, IKA, Germany) was used to prevent settling. The PC sensor excites the cyanobacterial PC at 590 nm (FWHM 13 nm) and measures fluorescence emission above 630 nm. The volume of measured sample was 800 mL. Each sample was measured for 5 minutes with 4.5 Hz sampling frequency and then the average signal was calculated. The results are presented in relative units [r.u.] that correspond to the voltage output of the sensor.

Cell counts and biovolume

The cell counts were determined with a Bürker-Türk haemocytometer (Brand, Germany) under an inverted Eclipse TE300 microscope (Nikon, Japan). The biovolume was calculated from the average biovolume of individual cells estimated by shape assimilation to known geometric forms and measurement of the main dimensions in more than100 randomly selected cells of each species (Hillebrand et al. 1999).

Determination of PC concentrations

We tested three different methods, using two different saline buffers and two methods for cell lysis. All tests were done on *M. aeruginosa* PCC 7806. Cell concentration was 5 x 10^6 cells/mL.

Method A - modified protocol by Horváth et al. (2013)

We centrifuged *M. aeruginosa* PCC 7806 culture with LC-321, (Tehtnica Železniki, Slovenia) at 4000 rpm for 15 min, removed the supernatant and substituted it with phosphate buffer (concentration of KH_2PO_4 was 0.1 mol/L, pH 6.8). We sonicated the sample with CV 18 ultrasonic homogenizer (tip diameter 6 mm, power 22 W, frequency 20 kHz, sonication volume 10 mL; Sonics and Materials, USA) for 10 minutes. After centrifugation (4000 rpm, 5 min), absorbance was measured with Nanocolor VIS (Macherey-Nagel, Germany) spectrophotometer.

Method B - protocol by Meriluonto et al. (2017)

Samples with different concentration of cells were filtered through a GF/C glass-fibre filter (Sartorius Stedim Biotech, Germany). The maximum vacuum during filtration did not exceed 31 kPa in order to avoid cell lysis. The filter with the cells was freeze-thawed to induce lysis. A saline buffer was added to glass tubes with filters and shaken until the filters broke down. The buffer was prepared by dissolving 8.77 g (0.15 mol/L) NaCl, 2.01 g (27 mmol/L) KCl, 11.36 g (80 mmol/L) Na_2HPO_4, 2.72 g (20 mmol/L) KH_2PO_4, 3.73 g (10 mmol/L) Na_2EDTA in distilled water to a final volume of 1 L. pH was adjusted using 5 M NaOH to 7,45 - 7,50. After dissolving the sample in the buffer, the tube was kept at 4 °C for 16 hours, after which the sample was shaken and sonicated for 20 min in an ultrasonic bath DC200H (mrc, Israel) with tap water and ice. After centrifugation (4000 rpm, 20 min), absorbance of supernatant was measured.

Method C

Method C was the same as method A, except that we used the buffer described in the method B.

Extractions were done under dim light to avoid photochemical degradation of PC. The concentrations of PC (in mg per L of water sample) were calculated according to de Marsac and Houmard (1988), Bennet and Bogorad (1973) and Fujita (1979), respectively:

$$PC\ [mg/L] = \frac{(A_{620} - 0.7 \times A_{650}) \times Ve}{7.38 \times Vs \times l} \quad \ldots 1$$

$$PC\ [mg/L] = \frac{(A_{615} - 0.474 \times A_{652}) \times Ve}{5.34 \times Vs \times l} \quad \ldots 2$$

$$PC\ [mg/L] = \frac{(198 \times A_{620} - 133 \times A_{650} - 0.190 \times A_{565}) \times Ve}{Vs \times l} \quad \ldots 3$$

Ve is the volume of buffer extract (mL), *Vs* volume of water sample (L), *l* optical path length (cm) and *A* absorbance at different wavelengths. Molar extinction coefficients and molecular weight of the phycocyanin are incorporated in the equations.

Results

PC extraction method

The difference between the minimum and maximum PC concentration obtained from the three methods using the same equation was between 17 and 18 % (depending on the equation) and was not statistically significant ($p > 0.1$). On the other hand, the choice of equation had larger impact on the estimation of PC concentration. The difference between concentration estimations from equation 1 and equations 2 and 3 was approximately 60% and was statistically significant ($p < 0.05$), whereas the estimations from equations 2 and 3 were not significantly different ($p > 0.05$). PC extraction from similar biovolume of green algae *Desmodesmus communis*, which does not contain PC, showed values ranging from 17 to 33 % of PC concentration in *M. aeruginosa*. To reduce the overestimation of PC concentration due to the nonspecific turbidity of the sample we have modified the equations similarly as for the chlorophyll extraction (ISO 10260, 1992) and subtracted absorbance at 750 nm which is caused by suspended solids. These are present in spite of centrifugation and are composed mainly of residues of the glass-fibre filter. Absorption spectra showed that the photosynthetic pigments absorb very little light at this wavelength.

$$PC\ [mg/L] = \frac{((A_{620}-A_{750})-0.7\times(A_{650}-A_{750}))\times Ve}{7.38\times Vs\times l} \quad \ldots 1m$$

$$PC\ [mg/L] = \frac{((A_{615}-A_{750})-0.474\times(A_{652}-A_{750}))\times Ve}{5.34\times Vs\times l} \quad \ldots 2m$$

$$PC\ [mg/L] = \frac{(198\times(A_{620}-A_{750})-133\times(A_{650}-A_{750})-0.190\times(A_{565}-A_{750}))\times Ve}{Vs\times l} \quad \ldots 3m$$

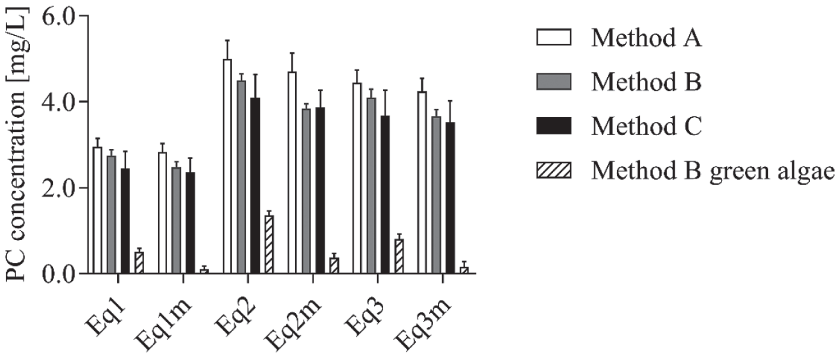

Figure 1: Comparison of PC extraction using three different methods and three different equations. Standard equations (Eq1, Eq2 and Eq3) were compared with modified equations (marked with m), where we subtracted the absorbance at 750 nm, which is indicative for nonspecific turbidity. Cyanobacteria *Microcystis aeruginosa* PCC 7806 and green algae *Desmodesmus communis* were used.

Slika 1: Primerjava treh različnih metod ekstrakcije PC in treh različnih enačb. Obstoječe enačbe (Eq1, Eq2 and Eq3) smo primerjali z dopolnjenimi, kjer smo odšteli absorpcijo vzorca pri 750 nm. Uporabili smo cianobakterije *Microcystis aeruginosa* PCC 7806 in zelene alge *Desmodesmus communis.*

Modification of equations resulted in 3.6 to 5-fold decrease of virtual PC concentration estimation in green algae sample. The decrease was statistically significant ($p < 0.01$). The decrease in PC concentration estimation in *M. aeruginosa* was around 10 %. The decrease in PC concentration using the modified equations was not statistically significant except in the case of method B, using equations 2 and 3.

Laboratory cultures

Three axenic lines of cyanobacteria, differing in shape, size and structure were used. Both strains of *M. aeruginosa* are unicellular and spherical. Average cell size of PCC 7806 was 28.81 ± 12.39 µm^3 and of PCC 7005 22.49 ± 7.66 µm^3. Cyanobacteria *A. platensis* is filamentous, the cell size was 163 ± 63 µm^3, filament length was 212 ± 96 µm. Concentration of extracted PC per cell was the lowest in PCC 7806 (0.55 ± 0.03 pg/cell). Concentration of extracted PC in PCC 7005 was 0.05 ± 0.01 pg/cell and 3.3 ± 0.3 pg/cell in *A. platensis*. PC was extracted according to method B and equation 1.

PC fluorescence of different laboratory strains

The same sample measured with fluorescence sensor was used for PC extraction, cell count and biovolume determination. The PC fluorescence intensity was in positive linear correlation with the concentration of extracted PC, cell count or biovolume (Fig. 2 A, B, C). The slopes of the linear fit were the most diverse when comparing PC fluorescence to concentration of extracted PC (Fig. 2 A). The increase in PC fluorescence was greater in *M. aeruginosa* PCC 7005 (k was 171 ± 16) than in PCC 7806 (k was 10.4 ± 0.4), despite the smaller size and 10-fold lower average PC

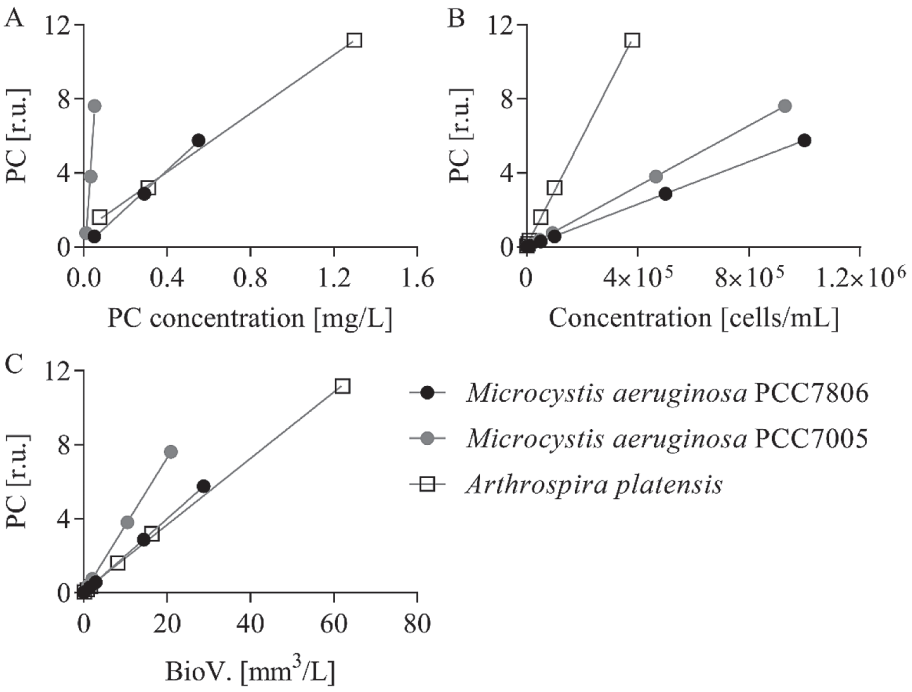

Figure 2: Relationship between phycocyanin (PC) fluorescence of three laboratory cultures and (A) extracted phycocyanin, (B) cell count and (C) total biovolume.

Slika 2: Odnos med fluorescenco fikocianina treh laboratorijskih kultur ter (A) koncentracijo ekstrahiranega fikocianina, (B) številom celic ter (C) biovolumnom.

content per cell. The slope of the linear fit was similar in filamentous *A. platensis* (k was 7.9 ± 0.2) and spherical *M. aeruginosa* PCC 7806. The average content of PC per cell in *A. platensis* was 6 fold higher than in PCC 7806.

The order of the linear slopes was different when we compared PC fluorescence to cell count. The increase in cell count resulted in the highest increase of PC fluorescence in *A. platensis* (k was $2.93 \times 10^{-5} \pm 3 \times 10^{-7}$). Slopes for the *M. aeruginosa* PCC 7806 and PCC 7005 were similar ($5.75 \times 10^{-6} \pm 8 \times 10^{-9}$ and $8.18 \times 10^{-6} \pm 1 \times 10^{-8}$ respectively).

Differences in the slopes of the linear fit were most similar when we compared PC fluorescence to biovolume. The highest slope (k was 0.3641 ± 0.0005) was calculated for *M. aeruginosa* PCC 7005, *M. aeruginosa* PCC 7806 was in the middle (k was 0.1996 ± 0.0003) and *A. platensis* had the lowest slope (k was 0.179 ± 0.002).

Discussion

We compared three different PC extraction methods and three different equations for assessing PC concentration. The difference between the minimum and maximum PC concentration obtained from the three methods was around 17 % and was not statistically significant. The largest difference in concentration was between method A and C that only differ in the buffer composition. Although there is no significant difference in the estimation of the concentration, method B has some advantages over method A. A combination of sonication with one freeze–thaw cycle enables shorter extraction time than sonication alone, and freezing the samples allows them to be stored.

The largest differences in estimation of PC concentration were due to different equations - 60%. By subtracting the absorbance at 750 nm from the absorbance at other wavelengths we almost entirely reduced the false PC concentration estimation. This was the most effective when we used equation after de Marsac and Houmard (1983). The modification did not statistically significantly influence the estimation of PC concentration in samples with cyanobacteria but reduced the virtual PC concentration estimation in green algae sample. This enables us to evaluate and minimize the disturbances that arise from the extraction of mixed samples from water bodies without any additional procedures. Nevertheless, additional studies should be made to confirm the modification and show influence of substances present in natural water samples.

PC can also be detected through the measurement of its fluorescence *in situ* as has been shown in the field and the laboratory. To translate the signal into limnological language we have to calibrate the sensor. Methods for calibration are different and may compromise the utility of these tools. In some studies, manufacturer settings are used without additional calibrations (Bowling et al. 2012) or the output of the sensor is calibrated in respect to the PC concentration (Song et al. 2013). Purchased solutions with defined PC concentration are usually used. Impurity of the solutions can reduce the precision of calibration. We have tested the relation between sensor output and cell count, biovolume and concentration of PC extracted from the same samples. The slopes of the linear fit were the most diverse when comparing PC fluorescence to the concentration of extracted PC. There was no obvious order in the slopes from different cyanobacteria species: *M. aeruginosa* PCC 7005 had the steepest slope when we compared PC fluorescence to extracted PC despite 10 fold lower PC cell content. Difference between slopes was almost 22 times. The differences were smaller (5 fold) when we compared PC fluorescence to cell count.

The highest uniformity in the linear fits was achieved when we compared PC signal with the cell biovolume. Similar results have been demonstrated in other studies (Kasinak et al. 2015). Our results show that the PC fluorescent signal is species dependent. Calibration of the PC fluorescent sensors with only one species, typically *M. aeruginosa* (Bastien et al. 2011), is inadequate for reliable conversion between PC fluorescence and biovolume of cyanobacteria.

Conclusions

- A combination of sonication with one freeze–thaw cycle is the most effective PC extraction method

- The largest differences in estimation of PC concentration are due to different equations used for calculating PC concentration from absorbance
- Modifying the equations by subtracting the absorption at 750 nm almost entirely reduces the false PC concentration estimation
- Relation between PC fluorescence signal and cell count, concentration of extracted PC and biovolume is linear
- The highest uniformity in the linear fits is between PC fluorescence signal and biovolume

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Impact of UV radiation and selenium on two buckwheat species

Vpliv UV sevanja in tretiranja s Se na dve vrsti ajde

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Abstract: The impact of selenium (Se) addition and UV radiation on Tartary buckwheat and hybrid buckwheat were studied. Both buckwheat species grew outdoors at the experimental field of the Biotechnical Faculty in Ljubljana. They were exposed to four different treatments regarding the UV radiation (ambient or reduced) and added Se (naturally accessible or foliary treated with Na selenate in concentration 10 mg Se L^{-1}). The content of pigments (chlorophyll *a* and *b*, carotenoids, anthocyanins) and UV absorbing compounds, transpiration rate, photochemical efficiency of photosystem II (PS) II and respiratory potential were measured. At the end of experiment we determined the biomass of different plant parts. The results showed that irrespective of the buckwheat species the added Se lowered the content of chlorophyll *a* and carotenoids, while it increased the effective quantum yield of PS II and transpiration rate. UV radiation reduced the content of anthocyanins only. Se and UV-B radiation as independent factors exerted no impact on buckwheat yield. Hybrid buckwheat had a higher physiological activity than the Tartary buckwheat yet a smaller biomass of plant parts, including reduced yield. Ambient UV radiation had a slightly negative impact on hybrid buckwheat while it had no noticeable negative impact on Tartary buckwheat. The Se treated Tartary and hybrid buckwheat were suitable for human and animal diet regarding to Se concentrations in leaves and grains.

Keywords: Tartary buckwheat, hybrid buckwheat, selenium, selenate, UV radiation

Izvleček: Namen dela je bil ugotoviti, kako dodatek selena (Se) in izpostavljenost naravnemu in zmanjšanemu UV sevanju vplivata na tatarsko in hibridno ajdo. Na polju Biotehniške fakultete smo gojili obe vrsti ajde in ju izpostavili štirim različnim obravnavanjem glede na izpostavljenost UV sevanju ter dodani Se (naravno dostopen ali foliarno dodan kot natrijev selenat v koncentraciji 10 mg Se L^{-1}). Merili smo vsebnost barvil (klorofila *a* in *b*, karotenoidov in antocianov) in UV absorbirajočih snovi, stopnjo transpiracije, fotokemično učinkovitost fotosistema II (FS II) in dihalni potencial. Ob koncu poskusa smo določili biomaso posameznih rastlinskih delov. Rezultati so pokazali, da je dodani Se ne glede na vrsto ajde znižal vsebnosti klorofila *a* in karotenoidov, povečal pa je dejansko fotokemično učinkovitost FS II in stopnjo transpiracije. UV sevanje je povečalo vsebnost antocianov. Se in UV sevanje

kot samostojna dejavnika nista imela vpliva na pridelek ajde. Hibridna ajda je imela večjo fiziološko aktivnost od tatarske, a manjšo biomaso rastlinskih delov, vključno z manjšim pridelkom. Naravno UV je sevanje na hibridno ajdo delovalo nekoliko negativno, na tatarsko ajdo pa ni imelo opaznega negativnega vpliva. S selenom tretirani tatarska in hibridna ajda sta bili, kar se tiče vsebnosti Se v listih in zrnih, primerni za uporabo v prehrani ljudi in živali.

Ključne besede: Tatarska ajda, hibridna ajda, selen, selenat, UV sevanje

Introduction

Selenium (Se) is essential micronutrient for human and animals. The lack of selenium in human diet can cause severe health problems, while in high concentrations it is toxic (White 2016). In Slovenia Se level in the soil is low (Pirc and Šajn 1997, Kolenc 2013) and consequently, there is a lack of Se in crops. Therefore, an alternative is the addition of Se to the eatable plants that are capable to incorporate anorganic forms of Se in their biomass (Germ et al. 2007). Essentiality of Se for plants has not been proven, but several studies show positive effect of Se addition on plant growth and production (Xue et al. 2001).

Se has been reported to play important protective roles for plants exposed to different environmental constraints, such as drought, salt, low or high temperatures and UV radiation (Kuznetsov et al. 2003, Germ et al. 2007, Djanaguiraman et al. 2010, Yao et al. 2010, Nawaz et al. 2015).

Buckwheat is a plant which can successfully grow in environmental conditions (high UV radiation, drought) which are less suitable for growth of many other crops (Bonafaccia et al. 2003). Researchers believe that, in the face of rapid climate change, especially the increase in UV radiation, it could become an alternative crop, as it is an important source of antioxidants in human nutrition (Fabjan et al. 2003, Kreft et al. 2006). Tartary buckwheat (*Fagopyrum tataricum* Gaertn.) is a nutrient rich plant and has a lot of positive effects on human health (Wieslander et al. 2012). Tartary buckwheat grain is a good source of vitamins B1, B2 and B6 and proteins with high biological value (Bonafaccia et al. 2003). It also has relatively high crude fiber content, and even more rutin and other phenolic compounds than common buckwheat (Fabjan et al. 2003). Hybrid buckwheat (*Fagopyrum hybridum*) is a new buckwheat taxon that was recently obtained by the interspecific crossing of *Fagopyrum tataricum* (4x = 32) × *Fagopyrum giganteum* (Fesenko and Fesenko 2010), although little is known about its properties (Golob et al. 2016, Golob et al. 2018).

The present study aimed to investigate the influence of Se addition, UV radiation and combination of Se treatment and UV radiation on selected biochemical and physiological parameters, biomass and accumulation of Se in Tartary and hybrid buckwheat.

Materials and methods

Tartary buckwheat (*Fagopyrum tataricum*) and hybrid buckwheat (*F. hybridum*) were grown outdoors in an experimental field in Ljubljana. Experiment was designed in four blocks. Each block was divided into eight plots (each, 0.75 m × 1.0 m), one for each treatment and for each buckwheat species. Each block was covered with two different types of panels. The first panel was transparent to UV and visible radiation, thus transmitting wavelengths from 290 nm and above (UV_{amb}), and the second panel was transparent only to the visible region of the spectra, and not for the UV region (UV–), with transmission of wavelengths >380 nm. At the beginning of flowering, half of the experimental plants under each tip of panels, had the foliage treatment with a solution of sodium selenate in concentration 10 mg Se L^{-1} (Se+), with the other half of the plants were treated only with water (Se0). Two weeks after the Se treatments, three plant specimen from each plot (subsamples) out of the four plots for each treatment were used for morphological, anatomical, biochemical and physiological analyses. At the end of the experi-

ment, the plants were harvested, weighed and the plant parts were air dried and lyophilised (Christ Alpha freeze dryer), then homogenised in an agate planar micromill, and used for analysis of the Se contents.

The contents of chlorophyll (Chl *a*, *b*) and carotenoid were determined according to Lichtentaler and Buschman (2001a, b) and measured with a UV/Vis spectrometer. The anthocyanin contents were determined according to Drumm and Mohr (1978). The contents of UV-A and UV-B absorbing compounds were evaluated according to Caldwell (1968).

The potential and effective photochemical efficiency of photosystem (PS) II were evaluated according to Schreiber et al. (1996) using a fluorometer (PAM 2500 Portable Chlorophyll Fluorometer; Heinz Walz GmbH, Germany). The transpiration rate was measured using a steady-state leaf porometer (Decagon Devices, Inc. Pullman, WA, USA). The respiratory potential of the mitochondria was determined as described by Kenner and Ahmed (1975). Preparation of leaf tissue and extraction process is described by Germ et al. (2005).

The total Se content was determined using hydride generation atomic fluorescence spectrometry. Here, 0.2 g of sample was weighed out in a Teflon tube. Digestion of the samples was carried out in the closed tubes, with a mixture of H_2SO_4, HNO_3, H_2O_2 and V_2O_5. HF was added only to the samples that contained fibres. Afterwards, reduction of Se(VI) to Se(IV) was carried out by the addition of concentrated HCl and with heating to 90 ºC for 10 min. After digestion and reduction of the samples, they were diluted with Milli-Q water, and Se was determined using hydride generation atomic fluorescence spectrometry. Each sample was analysed as two replicates. Details of the method of digestion and optimal measurement conditions were described by Smrkolj and Stibilj (2004). The accuracy of the method was validated with the use of certified reference material 'Spinach Leaves' (NIST 1570a).

The normal distribution of the data was tested using Shapiro-Wilk tests and the homogeneity of variance was assessed using Levene's test. For statistical analysis of the data, multivariate analysis of variance was used. The dependent variable was compared with three independent variables: selenium (Se) treatment (Se0 and Se+), UV radiation (UV- and UV_{amb}), species (S)of buckwheat (T and H) and combinations Se×UV, Se×S, UV×S. Differences between treatments were tested using one-way analysis of variance followed by Duncan post-hoc tests. The level of significance was accepted at $p < 0.05$. The SPSS Statistics software, version 20.0 (IBM) was used for the calculations.

Results

Results of multivariate analysis of variance showed that Se addition influenced effective photochemical efficiency of PS II, transpiration rate and content of chlorophyll *a* and carotenoids. UV radiation influenced only content of anthocyanins in leaves. Content of protective substances (anthocyanins, UVA-absorbing compounds and UVB-absorbing compounds), effective photochemical efficiency of PS II and transpiration rate differed between both species (Tab. 1).

Results showed that addition of Se decreased content of chlorophyll *a* and carotenoids content in leaves. On the other hands, Se addition increased effective photochemical efficiency of PS II and transpiration rate (Fig.1).

The plants grown under reduced UV-B radiation had a significantly lower content of anthocyanins than those who were grown in conditions of ambient UV radiation (Fig. 2).

Content of UV-B and UV-A absorbing compounds and content of anthocyanins were higher in Tartary buckwheat comparing to hybrid buckwheat. Hybrid buckwheat had higher transpiration rate and effective photochemical efficiency of PS II than Tartary buckwheat (Fig. 3).

The interaction of buckwheat species and UV radiation conditions was significant for the content of anthocyanins and grain biomass per plant. Tartary buckwheat plants produced a significantly higher amount of anthocyanins under ambient UV radiation than under the reduced UV radiation, while for hybrid buckwheat the anthocyanin content did not differ between UV treatments (Fig. 4a). Similarly Tartary buckwheat plants produced higher grain biomass when grew under ambient UV radiation comparing to reduced UV radiation, while in hybrid buckwheat we observed oposite trend (Fig. 4b).

Table 1: Results of multivariate analysis of variance for evaluation of impact of selenium treatment (Se), UV radiation (UV), buckwheat species (species) and interaction between the observed impacts (Se×UV, Se×species and UV×species) on measured parameters.

Tabela 1: Rezultati multivariatne analize variance za ovrednotenje vpliva dodajanja selena (Se), UV sevanja (UV), vrste ajde (vrsta) ter interakcije med posameznimi vplivi (Se×UV, Se×vrsta in UV×vrsta) na merjene lastnosti

	Independent variable			Combinations		
Parameter	**Se**	**UV**	**species**	**Se×UV**	**Se×species**	**UV×species**
Chlorophyll *a*	**0.0293***	0.2943	0.3908	0.8870	0.6799	0.6281
Chlorophyll *b*	0.7830	0.9930	0.9218	0.3005	0.6261	0.5769
Carotenoids	**0.0058***	0.5964	0.3672	0.7003	0.9320	0.5419
Antocyanins	0.6690	**0.0399***	**0.0133***	0.9324	0.9662	**0.0483***
UV-B abs. compounds	0.2559	0.1101	**0.0172***	0.3221	0.7394	0.1302
UV-A abs. compounds	0.2433	0.1189	**0.0010***	0.7014	0.5997	0.1999
ETS activity	0.2493	0.2614	0.7035	**0.0021***	0.3468	0.3288
F_v/F_m	0.3228	0.0914	0.3855	0.9541	0.6923	0.8736
$\Delta F/F_m'$	**0.0411***	0.4742	**0.0013***	0.2449	0.1356	0.4074
Transpiration	**0.0015***	0.1231	**0.0011***	0.7636	0.2682	0.9102
Biomass of grains	0.4965	0.1275	**0.0014***	0.8641	0.1904	**0.0401***

* Statistically significant ($p<0.05$) influence of factor on selected variable is shown in bold.

* Statistično značilen ($p<0,05$) vpliv dejavnika na izbrano lastnost je poudarjen.

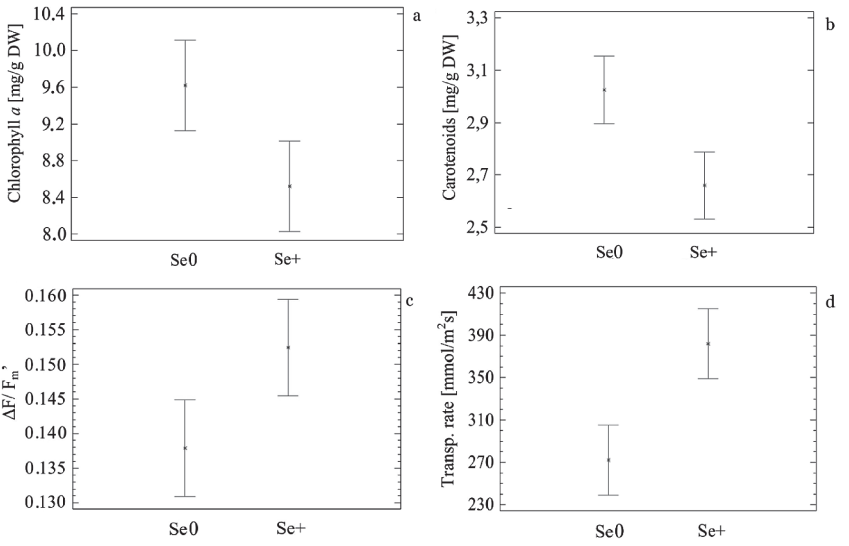

Figure 1: Impact of Se treatment on (a) content of chlorophyll *a*, (b) content of carotenoids, (c) effective photochemical efficiency of PS II ($\Delta F/F_m'$) and (d) transpiration rate (transp. rate). Data are means ± standard deviation (n = 4 for each treatment).

Slika 1: Vpliv dodajanja Se na (a) vsebnost klorofila *a,* (b) količino karotenoidov, (c) dejansko fotokemično učinkovitost FS II ($\Delta F/F_m'$) in (d) transpiracijo (transp. rate). Podatki so predstavljeni kot povprečja ± standardni odklon (n = 4 za vsak tretma).

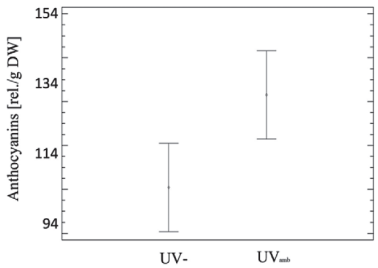

Figure 2: Impact of UV radiation on content of anthocyanins in buckwheat leaves. Data are means ± standard error (n = 4 for each treatment).

Slika 2: Vpliv UV sevanja na vsebnost antocianov v listih obeh vrst ajde. Podatki so predstavljeni kot povprečja ± standardna napaka (n = 4 za vsak tretma).

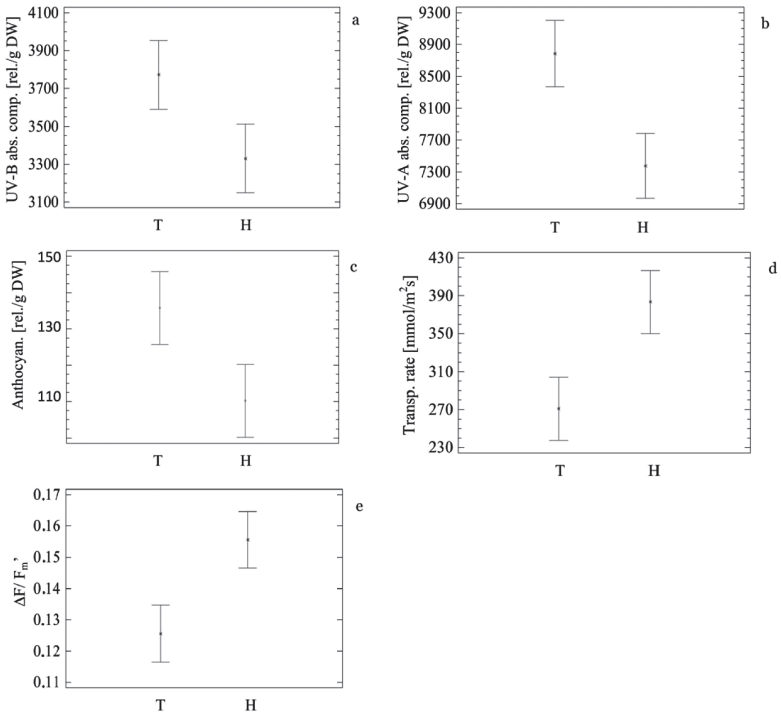

Figure 3: Significant difference in content of (a) UV-B and (b) UV-A absorbing compounds, (c) content of anthocyanins, (d) transpiration rate (transp. rate) and (e) effective photochemical efficiency of PS II ($\Delta F/F_m'$) between Tartary and hybrid buckwheat. Data are means ± standard deviation (n = 4 for each treatment).

Slika 3: Značilne razlike v vsebnosti (a) UV-B in (b) UV-A absorbirajočih snovi, (c) v vsebnosti antocianov , (d) transpiraciji in (e) dejanski fotokemični učinkovitosti FS II med tatarsko in hibridno ajdo. Podatki so predstavljeni kot povprečja ± standardni odklon (n = 4 za vsak tretma).

If we compared all treated groups (Se0UV-, Se0UV_{amb}, Se+UV-, Se+UV_{amb}), we observed that Se+UV_{amb} treated plants had significantly higher biomass of grains per plant than plant from Se+UV- and Se0UV-. There were no statistically significant differences in grain biomass in different treatments of hybrid buckwheat. On the contrary to Tartary buckwheat, Se+UV_{amb} treated hybrid buckwheat had lower grain biomass (but not significantly) than hybrid buckwheat from other treatments (Fig. 5).

The interaction of Se treatment and UV radiation was significant for the respiratory potential measured as electron transport system (ETS) activity. In plants, grown under ambient UV radiation, Se treatment significantly increased ETS activity, while in plants, grown under reduced UV radiation, Se treatment decreased ETS activity (Fig. 6).

Analysis of Se content in stems, leaves and seeds showed that foliar spraying with Se significantly increase contents of Se in all plant parts. Concentrations of Se were the highest in leaves and grains. There were no significant differences in Se content between buckwheat's species (Tab. 2).

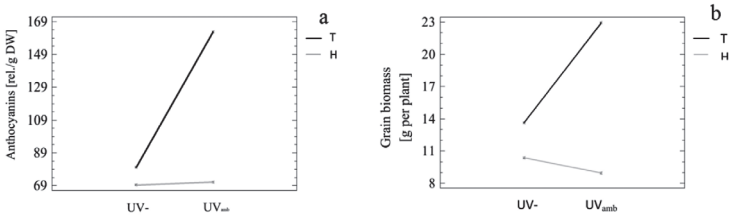

Figure 4: Effect of combination of buckwheat species and UV radiation conditions to (a) anthocyanin content and (b) grain biomass per plant.

Slika 4: Vpliv interakcije med vrsto ajde in UV sevanjem na (a) količino antocianov in (b) biomaso semen na rastlino.

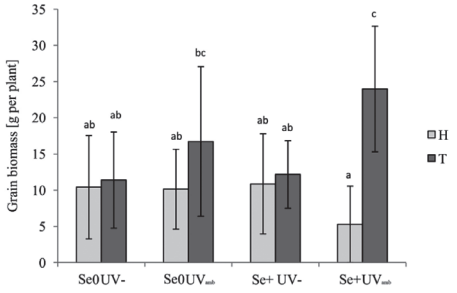

Figure 5: Grain biomass of Tartary buckwheat (T) and hybrid buckwheat (H) grown under different treatments. Data are means ± standard deviation (n = 4 for each treatment). Different letters indicate statistically significant differences.

Slika 5: Biomasa zrn tatarske (T) in hibridne ajde (H), gojenih v različnih razmerah. Podatki so predstavljeni kot povprečja ± standardni odklon (n = 4 za vsak tretma). Različne črke prikazujejo statistično značilne razlike.

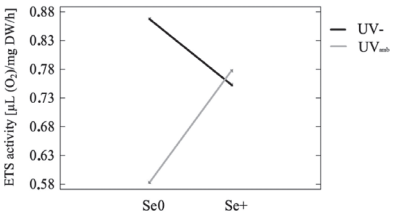

Figure 6: Effect of combination of Se treatment (Se0, Se+) and UV radiation (UV-, UV_{amb}) on the activity of electron transport system (ETS).

Slika 6: Vpliv interakcije med tretiranjem s Se (Se0, Se+) in UV sevanjem (UV-, UV_{amb}) na aktivnost elektronskega transportnega sistema (ETS).

Table 2: Content of Se (ng/g DW) in leaves, seeds and stems of Tartary buckwheat and hybrid buckwheat from different treatments.

Tabela 2: Vsebnost Se (ng/g SM) v listih, semenih in steblih tatarske in hibridne ajde, gojene v različnih razmerah.

	Tartary buckwheat			Hybrid buckwheat		
	seeds	**leaves**	**stems**	**seeds**	**leaves**	**stems**
Se0 UV_{amb}	28 ± 3^{a}	57 ± 4^{a}	14 ± 3^{a}	33 ± 1^{a}	67 ± 8^{a}	15 ± 3^{a}
Se0 UV-	37 ± 8^{a}	58 ± 7^{a}	18 ± 1^{a}	20 ± 2^{a}	59 ± 4^{a}	20 ± 3^{a}
Se+ UV_{amb}	335 ± 86^{b}	466 ± 69^{b}	144 ± 36^{b}	553 ± 109^{b}	389 ± 58^{b}	152 ± 30^{b}
Se+ UV-	616 ± 140^{b}	678 ± 159^{b}	245 ± 61^{b}	616 ± 157^{b}	475 ± 99^{b}	207 ± 43^{b}

Data are means ± standard error (n = 4 for each treatment). Different letters indicate statistically significant differences.

Podatki so predstavljeni kot povprečja ± standardna napaka (n = 4 za vsak tretma). Različne črke predstavljajo statistično značilne razlike.

There was a trend of decreased Se content in Se treated Tartary and hybrid buckwheat grown under ambient UV radiation comparing to plant grown under reduced UV radiation, but due to high variability of results the differences were not statistically significant (Tab. 2).

Discussion

In the present study foliar treatment with Se in concentration of 10 mg L^{-1} in plants significantly decreased content of chlorophyll *a* and carotenoids and increased effective photochemical efficiency of PS II and transpiration rate regardless of buckwheat species and UV radiation condition (Fig. 1). Similarly Xue et al. (2001) report about decreased concentration of chlorophyll in lettuce grown in Se enriched soils. On the other hand Nawaz et al. (2016), in the study with maize, observe increase in total chlorophyll content in plant, foliarly treated with Se. As reported by Breznik et al. (2005), the addition of selenate reduce the chlorophyll *a* content and increase the effective photochemical efficiency of the PS II in Tartary buckwheat. In the study of Padmaja et al. (1989) Se inhibited porphobilinogen synthase activity and decreased total chlorophyll content in light grown mung bean seedlings. The dose dependent response of porphobilinogen synthase activity and chlorophyll content to selenium suggested the possible role of this enzyme in chlorophyll biosynthesis. In buckwheat sprouts, grown from seeds previously soaked in solution of sodium selenate, Se treatment did not influenced content of chlorophyll *a* and carotenoids (Germ et al 2015). Increased

effective photochemical efficiency of the PS II as well as transpiration rate in plants indicated increased photosynthetic activity of Se treated buckwheat despite slight decrease of chlorophyll *a* in the present study.

The influence of ambient UV radiation compared to reduced UV radiation on the biochemical and physiological parameters of buckwheat species was small. Ambient UV radiation increased only anthocyanins content in buckwheat leaves (Fig. 2). That was expected, since absorption of the excess photons at high radiation is one of the important functions of anthocyanins in plants (Gould 2004).

Hybrid and Tartary buckwheat significantly differed in some biochemical and physiological parameters. Hybrid buckwheat compared to Tartary buckwheat had a higher content of chlorophyll *a* and *b* and carotenoids as well as higher respiratory potential measured with electron transport system (ETS) activity, transpiration rate and effective photochemical efficacy of PS II regardless Se treatment and UV radiation (Fig. 3). All that indicated that hybrid buckwheat had higher photosynthetic activity. Higher ETS activity in Se treated hybrid in comparison to Se treated Tartary buckwheat observed also Golob et al. 2016. On the other hand, Tartary buckwheat had higher content of anthocyanins and UV-B and UV-A absorbing substances comparing to hybrid buckwheat (Fig. 3). Anthocyanins and UV absorbing compounds are protective substances with antioxidative effect. Higher content of protective substances is probably a consequence of adaptation to unfavourable environmental conditions, as Tartary buckwheat originates from cooler areas of the eastern Qing Zang Plateau, Chuan Xi Plateau and Yun Gui Plateau at high altitude, often > 1500m above sea level (Chen 2001).

The interaction between the buckwheat species and UV radiation conditions significantly influenced biomass of grains and anthocyanins content in leaves (Fig. 4). UV radiation did not play an essential role in grain biomass and anthocyanins content in hybrid buckwheat plants. We observed that ambient UV radiation slightly decreased grain biomass compared to reduced UV radiation in hybrid buckwheat. On the contrary, in Tartary buckwheat a significantly larger grain yield and higher content of anthocyanins was recorded in plants, grown under ambient UV radiation in comparison to plants, grown under reduced UV radiation. The biggest difference in grain biomass between Tartary and hybrid buckwheat was in plants, growing in the conditions of ambient UV radiation and fertilized with selenium. Under this treatment, Tartary buckwheat reached significantly higher grain yield than hybrid buckwheat. There was a trend of decreased biomass of grains in $Se+UV_{amb}$ treated plants in comparison to other treatments (Fig. 5). Golob et al. (2018) grown hybrid buckwheat in similar conditions (with and without Se treatment and under reduced or ambient UV radiation conditions) and also found out that plants grown under ambient UV radiation and treated with Se reached lower biomass of grains and leaves. On the other hand, present study showed that Tartary buckwheat grown under ambient UV radiation had higher biomass of grains, especially when was treated with Se. This indicated better adaptation of Tartary to UV radiation, possibly due to its place of origin (Chen 2001).

The interaction between Se addition and UV radiation conditions significantly influenced ETS activity in both buckwheat species. Addition of Se increased ETS activity in plants grown under ambient UV radiation and decreased it when plants grew under reduced UV radiation (Fig. 6). Increased respiratory potential could be a sign that Se treatment caused slight stress for plants and increased demand for energy devoted for protection (Germ and Gaberščik 2003). Similarly, as it was observed in our study, found Germ et al. (2005) for pumpkins. Interaction between the added Se and the UV-B radiation did not significantly influence ETS activity, however, there was a tendency that addition of Se increased ETS activity in pumpkins grown under ambient UV-B radiation and lowered it in plants grown under reduced UV-B radiation. Our results were opposite to those obtained by Germ (2006). In this study, Se added to common buckwheat grown under ambient UV radiation reduced respiratory potential and increased it in plants grown under reduced UV radiation.

Se treated Tartary and hybrid buckwheat showed a great ability to accumulate high concentrations of Se in grains and leaves with no visible signs of toxic effect. Se accumulated mostly in edible parts of buckwheat plants which is very important, while grains and leaves are often used for human and animal consumption. There were

no differences in Se content in plants between Tartary and hybrid buckwheat (Tab. 2), which is not in agreement with foundlings of Golob et al. (2016), who reported about one third lower concentration of Se in Se treated hybrid buckwheat comparing to Tartary buckwheat. However, they used two-fold higher concentration of Se (20 mg Se L^{-1}) in spraying solution. This study showed that UV radiation did not significantly affected Se accumulation in plant parts of Tartary and hybrid buckwheat. However, we observed a tendency that ambient UV radiation decreased Se accumulation in all plant parts of Se treated Tartary and hybrid buckwheat (Tab. 2). Results are in agreement with Golob et al. (2018), who observed statistically significant decrease of Se accumulation in Se treated hybrid buckwheat grown under ambient UV radiation comparing to hybrid buckwheat grown under reduced UV radiation. However, in hybrid buckwheat plants not treated with Se, they observed opposite effect of UV radiation. UV radiation did not influenced accumulation of Se in grains of foliarly treated wheat (Golob et al. 2017).

Se treatment had positive effect on Tartary and hybrid buckwheat, since it increased photosynthetic activity but did not have significant effect on biomass. Ambient UV radiation had slightly negative effect on hybrid buckwheat. Se treatment increased respiratory potential in plants, grown under ambient radiation conditions, which indicated increased potential for protection against environmental constraints. Results showed that UV radiation exerted no negative effect in Tartary buckwheat and had slightly negative effect on hybrid buckwheat. Se treated Tartary and hybrid buckwheat were safe for human and animal consumption regarding to Se concentrations.

Povzetek

V Sloveniji je vsebnost Se v tleh nizka in posledično je Se malo tudi v kulturnih rastlinah in v prehrani ljudi in živali. Dodajanje Se rastlinam, ki ga v procesu presnove vgradijo v svojo biomaso v organski obliki, je zato primerna alternativa. Številne študije dokazujejo, da ima Se tudi pomembno vlogo pri zmanjševanju negativnih učinkov pri rastlinah zaradi delovanja različnih okoljskih dejavnikov, tudi UV sevanja. Ajda je rastlina, ki lahko akumulira relativno velike količine Se, če ji ga dodajamo. Poleg tega ima ajda visoko biološko vrednost, saj vsebuje tudi velike količine rutina, ki je antioksidant, kakovostne beljakovine, vlaknine, nenasičene maščobne kisline ter vitamine B1, B2 in B6.

Cilj raziskave je bil ugotoviti, kakšen vpliv imata sevanje UV in Se na tatarsko in hibridno ajdo. Na polju Biotehniške fakultete smo po parcelah posejali obe vrsti ajde. Rastline smo izpostavili naravnemu UV sevanju in zmanjšanemu UV sevanju. Polovico rastlin smo foliarno gnojili z raztopino natrijevega selenata (10 mg Se/L), ostala polovica je ostala negnojena. Merili smo vsebnost klorofila *a*, klorofila *b*, karotenoidov, antocianov ter UV-A in UV-B absorbirajočih snovi. Poleg tega smo merili tudi transpiracijo, fotokemično učinkovitost FS II in dihalni potencial s pomočjo meritev aktivnosti ETS. Ob koncu poskusa smo stehtali svežo in suho biomaso rastlin.

Rezultati so pokazali, da je dodajanje Se značilno vplivalo na povišanje dejanske fotokemične učinkovitosti FS II in transpiracije ter znižanje vsebnosti klorofila *a* in karotenoidov. Rezultati so pokazali večjo fotosintezno aktivnost s Se obravnavanih rastlin, medtem ko na biomaso gnojenje ni imelo vpliva. Naravno UV sevanje je značilno vplivalo le na povečanje vsebnosti antocianov. Se in UV sevanje kot samostojna dejavnika nista vplivala na pridelek ajde. Hibridna ajda je imela večjo fiziološko aktivnost od tatarske, a manjšo biomaso rastlinskih delov, vključno z manjšim pridelkom. Naravno UV Sevanje je na hibridno ajdo delovalo nekoliko negativno, na tatarsko ajdo pa ni imelo opaznega negativnega vpliva. S selenom obravnavana tatarska in hibridna ajda sta bili, kar se tiče vsebnosti Se v listih in zrnih, primerni za uporabo v prehrani ljudi in živali.

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Response of macrophyte *Berula erecta* to low concentrations of NaCl *in vitro*

Odziv vrste *Berula erecta* na nizke koncentracije NaCl *in vitro*

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Abstract: Macrophyte *Berula erecta,* grown in tissue culture, was exposed to various low concentrations of NaCl in the water (1–100 mg L^{-1}). Added NaCl had a positive effect on plant's growth and development. The number of shoots increased, as well as the length of the roots. The lowest concentration (1 mg L^{-1}) increased photochemical efficiency of photosystem II (Fv/Fm) while the highest (100 mg L^{-1}) slightly decreased it. Chlorophyll content was negatively affected by NaCl addition after 3 weeks. Carotenoid and anthocyanin levels were firstly raised and later lowered in NaCl treatment comparing to control. Overall, added NaCl had no negative effect on plants morphology, while decreased amount of pigments was observed.

Keywords: NaCl, *Berula erecta*, photochemical efficiency, growth parameters, pigments

Izvleček: Makrofit ozkolistni koščec (*Berula erecta*), gojen v tkivni kulturi, smo izpostavili različnim koncentracijam NaCl v vodi (1–100 mg L^{-1}). Dodani NaCl je pozitivno vplival na rast in razvoj rastline. Število poganjkov se je povečalo v primerjavi s kontrolo, prav tako so bile korenine rastlin, izpostavljene NaCl, daljše kot pri kontrolnih rastlinah. Nizka koncentracija NaCl (1 mg L^{-1}) je povišala vrednost fotokemične učinkovitosti, medtem ko je visoka koncetracija (100 mg L^{-1}) rahlo znižala vrednost tega parametra. Vsebnost klorofilov se je ob koncu poskusa občutno znižala pri rastlinah, izpostavljenim NaCl. Vsebnost karotenoidov in antocianinov se je na začetku povišala in nato nižala proti koncu poskusa. Zaključimo lahko, da dodan NaCl ni negativno vplival na morfologijo rastlin, medtem ko je znižal vsebnost barvil v rastlinah.

Ključne besede: NaCl, *Berula erecta*, fotokemična učinkovitost, rastni parametri, barvila

Introduction

The evolution of a physiology of plants that utilized K^+/H^+ rather than Na^+ was a vital step in the colonization of fresh water, and provided the basis for colonization of the land (Willey 2016). Therefore K^+ and not Na^+ (as in animals), is the primary osmoticum and the electrochemical gradients of H^+ the primary energizer of ion transport in plants (Willey 2016).

The role of Na^+ in plants is not fully understood, but trace amounts are required for the growth of plant species with C_4 and CAM photosynthetic pathways (Willey 2016). Small amounts of Na^+ are also essential, and they benefit the growth of some terrestrial plants, but in contrast to animals, the majority of them have no enzymatic requirements for Na^+ (Willey 2016).

For most plants even mild salinity is highly toxic. Salinity induces osmotic, ionic and oxidative stresses, inhibits plant growth, and disturbs photosynthesis and metabolism (Shabala and Munns 2017). Na^+ is physico-chemically similar to K^+ and Na^+ competes with K^+ in cell metabolism. It can be used as a partial replacement for K^+ and aids in the opening and closing of stomata, which helps regulate internal water balance. The chloride is a component of the water-splitting system of photosystem II and is involved in the stomatal regulation of many species and is therefore an essential micronutrient. Sodium competes with cations potassium, calcium, magnesium and ammonium for its uptake by the plant (Shabala and Munns 2017). Chloride can compete with anions nitrate, phosphate and sulfate uptake. Therefore, if concentration of sodium or chloride is high in the growing medium, while other beneficial elements are at low or normal levels, the plant increase acceptance of what is in excess, and this can lead to a lack of another elements. Therefore, the plant may not acquire sufficient levels of a required beneficial elements and this can lead to its deficiency in the tissue (Shabala and Munns 2017).

In the studies of the effects of various elements on plants, the elements are very often added in the form of salts. Therefore, the purpose of our study was to investigate the plant response to sodium salt in the form of NaCl. The objective of our study was to investigate the effect of salts, below the concentrations featured for saline soil, which can be promote and not inhibitory for plants. We tested the influence of various low concentrations of NaCl on species *Berula erecta*. To reach these, we measured several parameters describing growth and development, physiology and biochemistry of the plants.

Materials and methods

Plants and growth conditions

Detached shoots of *Berula erecta* L. were placed on 20 mL of solid Murashige and Skoog (1962) medium (MS) without growth regulators. The MS medium was supplemented with 0.8% Difco Bacto agar, with 3% sucrose, and adjusted to pH 5.7–5.8 before autoclaving. Two shoots were placed on the surface of the MS medium in a culture vessel for root induction and after three weeks the vessels were filled with an additional 20 mL of sodium chloride (NaCl) (98%, Sigma–Aldrich®, Taufkirchen, Germany) aqueous solutions at concentrations of 1, 10, and 100 mg L^{-1} for another three weeks. Controls consisted of plants that were treated with water. The vessels were incubated under controlled conditions at 23 ± 2 °C, with a photoperiod of 16 h at 38–50 mol $m^{-2}s^{-1}$ (Osram L 58W/77 – Fluora) and at 50% relative humidity. All experiments were repeated twice.

Measurements of selected parameters

In order to monitor the growth and developmental parameters, the dry and fresh weight, the length of plants and roots and number of shoots were monitored weekly.

Photochemical efficiency (maximum quantum yield; Fv/Fm) was measured weekly on 10-12 plants from each concentration treatment, using a fluorometer (Handy PEA, Hansatech, Kings Lynn, UK). The measurements of chlorophyll *a* fluorescence were made after 10 min of darkness, pro-vided by dark-adaptation clips. Fluorescence was excited with a saturating beam of 'white light' (PPFD = 8000 mol $m^{-2}s^{-1}$, 0.8 s) (Schreiber et al. 1995).

For content of chlorophylls *a* and *b,* carotenoids and anthocyanins, leaves of 4–9 plants from each treatment were selected. The amounts of pigments were determined as described by Lichtenthaler and Buschmann (2001). The total anthocyanin content was measured as described by Drumm and Mohr (1978).

Statistical analysis

The statistical package SPSS® 24.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. The level of statistical significance (p) among different treatments was determined by the analysis of variance (ANOVA) using the post hoc Duncan test. Differences at $p < 0.05$ were considered statistically significant. Different letters indicate significant differences. The number of replicates was from 10-24 for growth and developmental parameters, 10-12 for photochemical efficiency and 4-9 for pigments.

Results and discussion

The fresh weight of *B. erecta* was slightly increased in NaCl exposed plants compared to control at the beginning. More pronounced positive effect of NaCl was observed in the end of an experiment, the average values being 2.45, 5.26, 3.31 and 3.76 g for control, 1, 10 and 100 mg L^{-1} respectively. Dry weight was statistically increased in the end of an experiment, the average values ranged from 0.14 to 0.20 g for treated plants and 0.12 g for control. Many studies have shown that the fresh and dry weights of the shoot system are affected, either negatively or positively, by changes in salinity concentration, type of salt present, or type of plant species (Amira and Qados 2011, Al-Karaki 2000). Beneficial effects of salts usually occurs at concentrations around 50 mg L^{-1} (Willey 2016) which is also evident from our study.

Plant height was statistically positively affected by 100 mg L^{-1} in all weeks of an experiment, average values being between 4.77 and 6.45 cm while to control height reached 4.29 to 5.80 cm. The other two concentrations only increased plant height in week 3 compared to the control. The general trend of increasing the length of the bean plants exposed to 2 mg L^{-1} NaCl was observed (Amira and Qados 2011). Generally speaking, the elongation of the stem when treated with low concentrations of salts may induce osmotic adjustment activity in the plants which may improve growth. On the other hand, plant height of tomato and *Atriplex lentiformis* decreased with increasing NaCl in the nutrient solution (up to 5 mg L^{-1}) (Al-Karaki 2000, Smit et al. 2017).

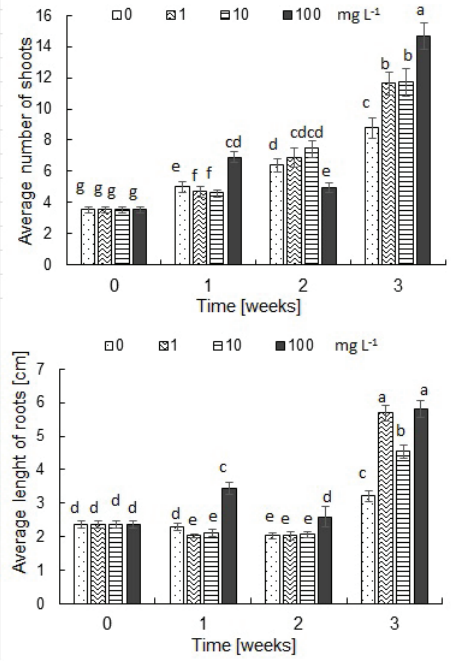

Figure 1: The number of shoots and length of roots in *Berula erecta* at low concentrations of NaCl *in vitro* (means ± SD, n=10-24).

Slika 1: Število poganjkov in dolžina korenin pri vrsti *Berula erecta*, izpostavljeni nizkim koncentracijam NaCl *in vitro* (povprečne vrednosti ± SD, n=10-24).

The promotion effect of low concentrations of NaCl was demonstrated also after determination of the average length of roots and the average number of shoots (Fig. 1). The highest concentration positively affected root length from the beginning, while the other two NaCl concentrations increased the length of roots towards the end of an experiment compared to the control (Fig. 1). The shoots were positively affected by 100 mg L^{-1} in week 1 and 3, while in week 2 this concentration decreased the number of shoots. In week 3 concentrations 1 and 10 mg L^{-1} also increased the number of shoots (Fig. 1). In *Atriplex lentiformis,* concentration of 5 mg L^{-1} NaCl reduced the number of shoots (Al-Karaki, 2000).

It looks that take up of salts, which can reduce the growth of plants (Shabala and Munns 2017), in our experimental system did not reduce the ability of plants to grow.

Photochemical efficiency (Fv/Fm) in all three treatments ranged from 0.81 to 0.83, which shows, that added NaCl did not affect the process of photosynthesis, since these values indicate that plants are in good condition (Schreiber et al. 1995). However, there were some statistical significant differences between treatments. The concentration of 1 mg L^{-1} increased photochemical efficiency towards the end of an experiment, while 100 mg L^{-1} decreased it (data not shown). In concentration of 10 mg L^{-1} the differences were observed between weeks, with the highest overall value in week 2 (data not shown). NaCl an especially Na^+ can negatively affects photosynthesis processes, photosystems or pH homeostasis metabolism due to H^+-coupled Na^+ efflux mechanisms (Shabala and Munns 2017).

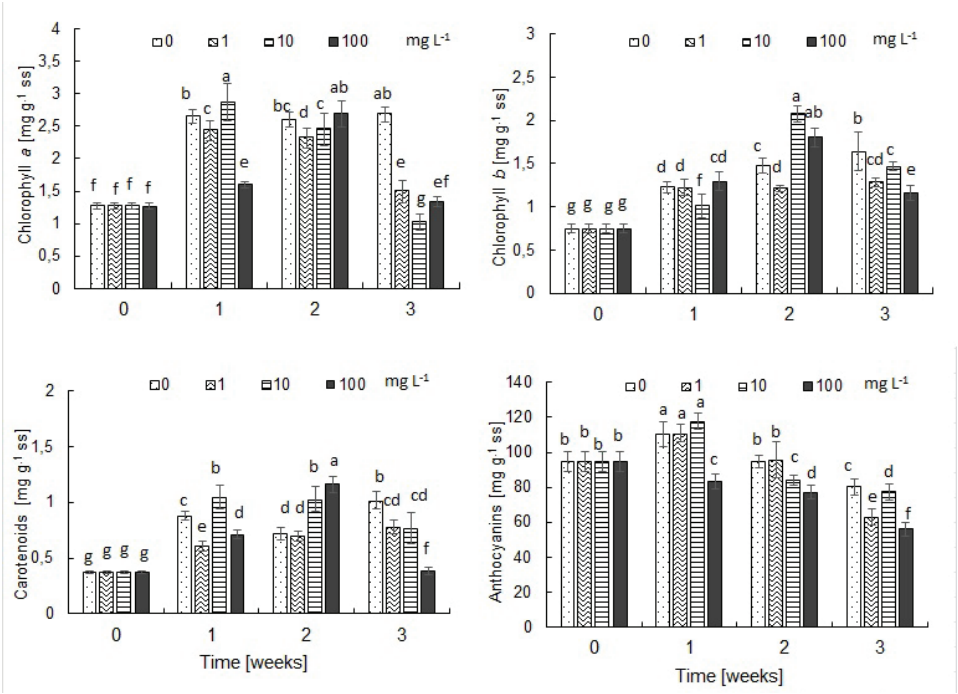

Figure 2: The content of pigments in leaves of NaCl treated *Berula erecta* (means ± SD, n=4-9).
Slika 2: Vsebnost pigmentov v listih vrste *Berula erecta*, izpostavljene nizkim koncentracijam NaCl *in vitro* (povprečne vrednosti ± SD, n=4-9).

The average content of chlorophyll *a* and *b* and carotenoids in *B. erecta* increased in weeks after the NaCl addition comparing to the content before the addition (Fig. 2). The highest NaCl addition decreased the content of chlorophyll *a* in week 1, while in week 3 the addition of NaCl decreased the amount of this pigment regardless concentration (Fig. 2). The similar decrease was observed with NaCl treatment in *Chrysanthemum* species (Lee and van Iersel 2008, Chen et al. 2003), *Atriplex lentiformis* (Smit et al. 2017) and onion (Hanci et al. 2016).

Significant decrease in chlorophyll *b* was observed in 1 and 100 mg L^{-1} in week 3, when all treatments were lower as control (Fig. 2). In week 2 the highest two concentrations increased the content of this pigment. Tort and Turkyilmaz (2004) reported that the exposure of barley to 7 and 14 mg L^{-1} NaCl led to the decrease in chlorophyll *a* and *b*. Also in *Atriplex lentiformis* exposed to 2-5 mg L^{-1} the content of chlorophylls decreased (Smit et al. 2017).

Reduction in chlorophyll content is commonly observed phenomena as salinity increases and plants are subjected to salt stress. The determination of chlorophylls is therefore usual way to determine salt tolerance.

The peak concentration of carotenoids was measured in week 2. The amount of carotenoids then lowered towards the end of an experiment, with the lowest carotenoids content in 100 mg L^{-1} treatment. The same was observed in bean plants to salt stress where the formation of carotenoids was inhibited and a decrease was observed as well (Amira and Qados 2011). The amount of anthocyanins first increased but towards the end decreased. Since the content of carotenoids and anthocyanins was lower we presume that plants were not under stress because these pigments start to accumulate in less favourable conditions (Fargašová 1998, Winkel-Shirley 2002).

Conclusions

Sodium chloride in low concentrations that were used in our experiments, had no negative effect on plant morphology and no severe effect on the process of photosynthesis although it lowered chlorophyll *a* content in the end. The stress was absent since the protective pigments (carotenoids, anthocyanins) were not increased and values of photochemical efficiency showed that plants are in good condition at all treatments. We can conclude that chosen concentrations had not yet triggered stress in the selected species.

Povzetek

V različnih študijih vpliva elementov na rastline so elementi pogosto dodani v obliki soli. Cilj našega poskusa je bil ugotoviti, kako se rastline odzivajo na natrijeve soli v obliki NaCl, v koncentracijah nižjih od tistih, ki so značilne za slana tla. Makrofit ozkolistni koščec (*Berula erecta*) smo v tkivni kulturi izpostavili različnim nizkim koncentracijam NaCl v vodni raztopini (1–100 mg L^{-1}). Dodane nizke koncentracije NaCl, uporabljene v poskusu, so pozitivno vplivale na rast in razvoj rastlin. Povečalo se je število poganjkov in dolžina korenin. Najnižja koncentracija NaCl (1 mg L^{-1}) je povečala, največja (100 mg L^{-1}) pa rahlo zmanjšala maksimalno fotokemično učinkovitost fotosistema II (Fv/Fm). Dodani NaCl je znižal vsebnost klorofilov na koncu poskusa. Vsebnost karotenoidov in antocianinov se je najprej povečala, nato nekoliko zmanjšala proti koncu poskusa pri rastlinah tretiranih z NaCl v primerjavi s kontrolo. Zaključimo lahko, da izbrane nizke koncentracije NaCl niso negativno vplivale na morfologijo rastlin in fotosintezo. Pri rastlinah pri nobeni obravnavi nismo opazili stresa, saj se zaščitni pigmenti niso akumulirali, izmerjene vrednosti fotokemične učinkovitosti pa so pokazale na dober fitnes rastlin. To pomeni, da izbrane koncentracije NaCl še niso sprožile stresa pri izbrani rastlinski vrsti.

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Identification of alien *Fallopia* taxa using molecular methods

Določanje tujerodnih dresnikov (*Fallopia* spp.) z molekulskimi metodami

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Abstract: The non-native species of knotweeds (*Fallopia* sect. *Reynoutria*) are morphologically very similar and it is often difficult to distinguish between the hybrid *F.* ×*bohemica* and parental taxa, *F. japonica* and *F. sachalinensis*. To distinguish 30 samples of knotweeds, collected in Slovenia, we used PCR RFLP analysis of the *trn*K intron of plastid DNA in combination with the amplification of microsatellite nuclear locus KW6, which is a specific diagnostic marker for *F. sachalinensis*. We established that the combination of both markers unambiguously identifies the following samples: *F. japonica* (var. *japonica*), *F. sachalinensis* and *F.* ×*bohemica.* Based on described molecular markers we confirmed that the maternal parent of the taxon *F.* ×*bohemica* was *F. japonica* for all analysed hybrids. In addition, two species from *Fallopia* sect. *Sarmentosae* (*F. baldschuanica* and *F. multiflora*) were also analysed. Both could be distinguished from species of *Fallopia* sect. *Reynoutria,* but for the discrimination between them, some other markers should be used.

Keywords: *Fallopia*, invasive species, Slovenia, hybridization, plastid DNA, *trn*K, microsatellite locus, KW6, RFLP

Izvleček: Tujerodni dresniki (*Fallopia* sect. *Reynoutria*) so si morfološko zelo podobni. Še posebej težko je razlikovati med križancem *F.* ×*bohemica* in starševskima vrstama *F. japonica* in *F. sachalinensis*. Z uporabo dveh različnih molekulskih označevalcev, plastidnega zaporedja DNA *trn*K v kombinaciji z analizo RFLP in mikrosatelita KW6, ki je potencialno specifičen za vrsto *F. sachalinensis*, smo analizirali 30 vzorcev dresnikov, ki smo jih nabrali na območju Slovenije. Ugotovili smo, da s kombinacijo obeh označevalcev nedvoumno določimo naslednje taksone: *F. japonica* (var. *japonica*), *F. sachalinensis* in *F.* ×*bohemica.* S pomočjo molekulskih označevalcev smo kot materinsko vrsto vseh vzorcev križancev določili japonski dresnik. V analizo smo vključili tudi predstavnika slakovcev, *F. baldschuanica* in *F. multiflora*. Ta se s pomočjo uporabljenih molekulskih označevalcev zanesljivo razlikujeta od dresnikov, za razlikovanje med vrstama slakovcev pa bi bilo treba poiskati nove označevalce.

Ključne besede: *Fallopia*, invazivna vrsta, Slovenija, križanci, plastidna DNA, *trn*K, mikrosatelitni lokus, KW6, RFLP

Introduction

Invasive alien taxa from genus *Fallopia* origin in East-Asia and are one of the most troublesome invaders worldwide, especially in Europe and North America (Bailey et al. 2009, Tiebre et al. 2007, Forman and Kesseli 2003). The influence of invasive alien species is known as the second greatest threat to biodiversity after the loss of habitats (Wilson 1991) and this is probably one of the main reasons for numerous studies of invasive species in last decades.

In Slovenia, three alien taxa from *Fallopia* sect. *Reynoutria* known also as knotweeds, thrive: *F. japonica* (Houtt.) Ronse Decr. var. *japonica*, *F. sachalinensis* (F. Schmidt) Ronse Decr., and their hybrid *F. ×bohemica* (Chrtek & Chrtková) J. P. Bailey (Strgulc Krajšek and Jogan 2011). Additionally, two deciduous vining woody perennials from *Fallopia* sect. *Sarmentosae* have been reported, *F. baldschuanica* (Regel) Holub (Strgulc Krajšek and Jogan 2011) and *F. multiflora* (Thunb.) Haraldson (Balant et al. 2015).

In Europe, *F. japonica* is a male sterile clone (Bailey et al. 2009) and is reproducing vegetatively. Hollingsworth and Bailey (2000) analysed 150 British and 16 other European populations of *F. japonica* and determined identical RAPD profiles for all samples, thus proving clonal growth. In native range of East Asia, *F. japonica* occurs also as male (rarely) and hermaphroditic plants, but they were not introduced to Europe (Bailey 2003). *Fallopia japonica* in Europe is octoploid (2n=8x=88) (Bailey et al. 2007), as was proved also by measuring the genome size of samples in Czech Republic (Suda et al. 2010) and in Slovenia (Strgulc Krajšek and Dolenc Koce 2015). In Slovenia the species is common in lowlands but present also in higher regions up to 1150 m a. s. l. in Julian Alps (Strgulc Krajšek and Jogan 2011).

Fallopia sachalinensis is a tetraploid (2n=4x=44), represented in Europe with hermaphroditic and female plants (Bailey et al. 2009). Hexaploid and octoploid specimens can be found in introduced range (Czech Republic) too, but they are very rare (Mandak et al. 2003). In Slovenia it is known from less than 20 localities scattered across the country (Strgulc Krajšek and Jogan 2011).

The hermaphroditic plants of *F. sachalinensis* are the source of pollen, and in localities where they grow close to *F. japonica*, hybrids (*F. ×bohemica*) may occur. The hybrids have been known from English gardens since at least 1872 (Bailey and Conolly 2000). They have the highest genetic variation of all taxa belonging to *Fallopia* sect. *Reynoutria* because hybrids originated multiple times (Mandak et al. 2005) and they can backcross to their parents (Bailey et al. 2009). Hybrids of F1 generation are mostly hexaploids (2n=6x=66) but many other chromosome numbers have been reported, mostly among different backcrosses (Bailey et al. 2009). The flowers of *F. ×bohemica* in Slovenia are male or hermaphroditic, both types on the same plant (Strgulc Krajšek and Jogan 2011).

The reliable determination of the taxa from the *Fallopia* sect. *Reynoutria* can be difficult, especially when completely developed leaves or flowers are not available. Two specific molecular markers were developed for *F. japonica* and *F. sachalinensis.*

The first is the *trn*K intron of plastid DNA, which has been used extensively as a phylogenetic marker for classification of plants. In identification of *Fallopia* taxa it has been used in combination with restriction endonuclease HhaI that cuts the intron in two segments of different size in *F. japonica* var. *japonica* (Hollingsworth et al. 1999), whereas in *F. sachalinensis* this specific restriction site is lacking. Plastids within the study group are inherited maternally and unidirectional hybridisation between *F. japonica* var. *japonica* and *F. sachalinensis* has been proved for specimens collected in Great Britain, always with *F. japonica* var. *japonica* as the maternal parent (ibid.).

The second marker is nuclear, simple sequence repeat (SSR) or microsatellite locus, KW6, which is potentially diagnostic and specific marker for *F. sachalinensis* (Grimsby et al. 2007).

Plant material of different *Fallopia* taxa, collected in West and Central Europe (Tiebre et al. 2007, Hollingsworth et al. 1999, Hollingsworth and Bailey 2000, Suda et al. 2010) and North America (Gammon et al. 2007, Grimsby et al. 2007, Forman and Kesseli 2003, Grimsby and Kesseli 2010) have been already examined in studies dealing with sexual reproduction of knotweeds, but there is a lack of the knowledge about the South European populations. We used PCR

RFLP analysis of the *trn*K intron in combination with amplification of nuclear, microsatellite locus KW6. The combination of both markers could be used for the recognition of hybrids between *F. japonica* and *F. sachalinensis* (Grimsby et al. 2007, Hollingsworth et al. 1999) and for the determination of female parent species of *F. ×bohemica* specimens. We have included also the samples of *F. baldschuanica* and *F. multiflora* to inspect if these two species can be distinguished from taxa of *Fallopia* sect. *Reynoutria* using the same molecular markers.

Material and methods

Plant material and DNA extraction

A total of 32 samples of *Fallopia* taxa were collected in different sites across Slovenia (Appendix): 8 samples of *F. japonica*, 2 samples of *F. sachalinensis,* 20 hybrid plants (*F. ×bohemica*), 1 specimen of *F. baldschuanica* and 1 of *F. multiflora*.

In previous study (Bímová et al. 2003) was shown that regeneration from rhizomes was the major mode of vegetative reproduction in the complex of *Fallopia* sect. *Reynoutria*, therefore rhizome segments with some winter buds were collected in the field. They were further grown in the laboratory in plastic pots (12 L) filled with garden substrate, watered, and only slightly pressed into the soil. After the development of young shoots, some intact leaves were removed and 100 mg of fresh young intact leaf material was used for DNA extraction with a Plant Genomic DNA Miniprep Kit according to recommended protocol (Sigma-Aldrich).

RFLP analysis of the trnK intron

The *trn*K intron was amplified using the universal primers described by Demesure et al. (1995). All PCRs were done in 25 µL with 2 µL diluted genomic DNA (approximately 20 ng), 1 µL of each primer (10 µM), 2.5 µL of 10X reaction buffer, 2.5 µL of 25 mM $MgCl_2$, 0.6 µL of 10 mM combined dNTPs, and 0.2 µL of Taq DNA polymerase (5 units/µL). The remaining volume was filled up with water. Amplification was performed with the following cycles: 5 min denaturation at 94 °C; 30 cycles of 92 °C for 45 s, 53 °C for 45 s, 72 °C for 3 min; followed by a final extension at 72 °C for 10 min. The PCR products were then digested with restriction endonuclease HhaI as follows: 5 µL of PCR product was digested with 1 µL HhaI (10 units/µL) for 1 hour at 37 °C. Results of restriction were visualized on a 1 % agarose gels (0.5X TBE buffer) stained with ethidium bromide under the ultra violet light.

Amplification of the KW6 SSR

The nuclear, simple sequence repeat (SSR) marker KW6 was amplified using primers as described in Grimsby et al. (2007). All PCRs were done in 25 µL with 2 µL diluted genomic DNA, 1 µL of each primer (10 µM), 2.5 µL of 10X reaction buffer, 2.5 µL of 25 mM $MgCl_2$, 0.6 µL of 10 mM combined dNTPs, and 0.2 µL of Taq DNA polymerase (5 units/µL). The remaining volume was filled up with water. The PCR profile was: 1 cycle of 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 54 °C, and 30 s at 72 °C and finally 1 cycle at 72 °C for 10 min. PCR products were separated on 1.7 % agarose gels (0.5X TBE buffer) and visualized with ethidium bromide under the ultra violet light.

Results and discussion

The combination of all results of the molecular analysis of *Fallopia* samples is shown in Table 1 and on the photo of electrophorese gel (Fig. 1). The result for *F. multiflora* sample was the same as for *F. baldschuanica* and is not shown.

Table 1: Results of the RFLP analysis of the *trn*K intron and amplification of microsatellite locus KW6 in *Fallopia* samples from Slovenia.
Tabela 1: Rezultati analize RFLP introna *trn*K in pomnožitve mikrosatelitskega lokusa KW6 pri slovenskih vzorcih iz rodu *Fallopia*.

	RFLP analysis of the *trn*K intron		KW6 amplification
Taxon	**Amplification of plastid (trnK) DNA**	**Digestion with the restriction enzime HhaI**	
F. japonica	2 700 bp long fragment	1 600 in 1 100 bp long restriction fragments	not amplified
F. sachalinensis	2 700 bp long fragment	undigested 2 700 bp long fragment	338 bp long fragment
F. ×bohemica	2 700 bp long fragment	1 600 in 1 100 bp long restriction fragments	338 bp long fragment
F. baldschuanica	2 700 bp long fragment	undigested 2 700 bp long fragment	not amplified
F. multiflora	2 700 bp long fragment	undigested 2 700 bp long fragment	not amplified

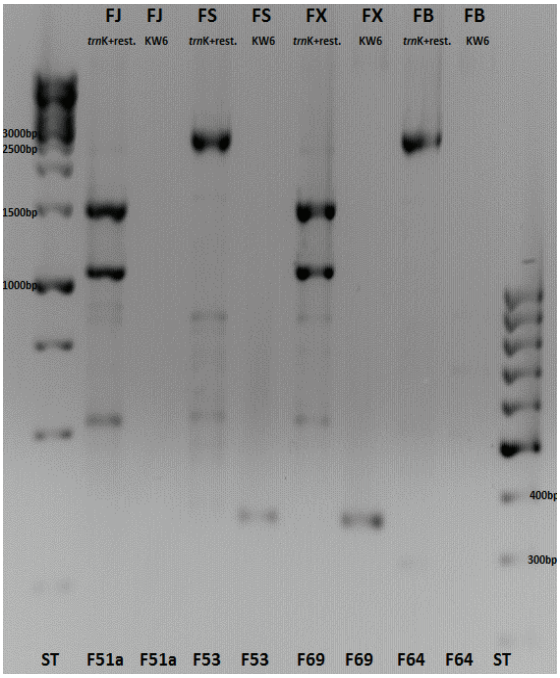

Figure 1: PCR RFLP profiles of *trn*K intron of cpDNA (*trn*K + restriction) and profiles of PCR products of microsatellite locus KW6 nDNA (KW6) of knotweeds *F. japonica*, *F. sachalinensis*, hybrid *F. ×bohemica* and Russian vine (*F. baldschuanica*).
Legend: ST – 1 kbp size marker (left) and 100bp size marker (right); FJ – *F. japonica*; FS – *F. sachalinensis*; FX – *F. ×bohemica*; FB – *F. baldschuanica*. Marks at the bottom of the gel represent sample ID.
Slika 1: PCR RFLP profili introna *trn*K cpDNA (*trn*K + restrikcija) in profili produktov pomnoževanja mikrosatelitskega lokusa KW6 nDNA (KW6) dresnikov *F. japonica*, *F. sachalinensis,* križanca *F. ×bohemica* ter grmastega slakovca (*F. baldschuanica*).
Legenda: ST – DNA standard z lestvico 1.000 bp (skrajno levo) oz. 100 bp (skrajno desno); FJ – *F. japonica*; FS – *F. sachalinensis*; FX – *F. ×bohemica*; FB – *F. baldschuanica*. Oznake v spodnjem delu slike so oznake vzorcev.

The plastid sequence *trn*K is 2700 bp long and has been amplified in all analyzed specimens of *F. japonica, F. sachalinensis*, *F. ×bohemica*, *F. baldschuanica,* and *F. multiflora*. After the digestion with the enzyme HhaI, it remained intact in *F. sachalinensis, F. baldschuanica* and *F. multiflora.* In *F. japonica* and *F. ×bohemica,* it was cut in two restriction fragments, 1600 and 1100 base pairs long; all analysed hybrid specimens *F. ×bohemica* had plastid haplotype of *F. japonica.* Given the maternal inheritance of plastid DNA in *Fallopia* (Hollingsworth et al. 1999) and the apparent male sterility of *F. japonica* in Slovenia (Strgulc Krajšek and Jogan 2011) the present results provide strong evidence that hybridization between *F. japonica* and *F. sachalinensis* in investigated samples from Slovenia was unidirectional, with *F. japonica* as the maternal parent. The unidirectional hybridisation has been previously demonstrated in Great Britain (Hollingsworth et al. 1999).

The nuclear, simple sequence repeat (SSR) marker, KW6 was amplified in all samples of *F. sachalinensis* and *F. ×bohemica.* It is a potentially diagnostic *F. sachalinensis*-specific marker (Grimsby et al. 2007). Its presence in hybrid specimens together with restricted sequence *trn*K confirms the morphological identification of analysed specimens.

Since the hybrid *F. ×bohemica* is highly fertile (Tiebre et al. 2007, Strgulc Krajšek and Dolenc Koce 2015) and the plants mostly have male flowers (Grimsby et al. 2007, Strgulc Krajšek and Dolenc Koce 2015), backcrosses may occur (Bailey et al. 2009). Among such backcrosses the genomic marker KW6 for *F. sachalinensis* can be lost and hybrid can no longer be identified (Grimsby and Kesseli 2010). In our study there were no such specimens.

We have tested the selected markers also for *F. baldschuanica* and *F. multiflora*. The combination of these results was different than for taxa from *Fallopia* sect. *Reynoutria*. The plastid *trn*K segment was not digested by enzyme HhaI (as in *F. sachalinenis*) and there was no amplification of KW6 marker (as in *F. japonica*). Consequentely the combination of these markers could be used for the differentiation between the sections, but not between the species within *Fallopia* sect. *Sarmentosae* (*F. baldschuanica* and *F. multiflora*).

Another hybrid between *F. japonica* and *F. baldschuanica*, *F. ×conolyana* J. P. Bailey, is also expected in Slovenia, as it was reported from many European countries (Bailey 2001). In Great Britain it is common along railways (ibid.). In the costal part of Slovenia where *F. baldschuanica* is invasive (Strgulc Krajšek and Jogan 2011) and in some localities in other parts of the country where it is grown as an ornamental plant in gardens (Balant et al. 2015), the hybrid *F. ×conolyana* could occur. This hybrid could not be distinguished from *F. japonica* with molecular markers we have used.

In conclusion, (1) we established that the combination of both markers (PCR RFLP analysis of the *trn*K intron of plastid DNA and the amplification of microsatellite nuclear locus KW6) unambiguously identifies *F. japonica* (var. *japonica*), *F. sachalinensis* and *F. ×bohemica*, (2) we confirmed that *F. japonica* was the maternal parent of all analysed hybrid *F. ×bohemica*, and (3) *F. baldschuanica* and *F. multiflora* can be distinguished from species of *Fallopia* sect. *Reynoutria* using the combination of both markers, but for the discrimination between these two species other markers are needed.

Povzetek

V Sloveniji so prisotni trije taksoni invazivnih tujerodnih dresnikov (*Fallopia* sect. *Reynoutria*) in sicer *Fallopia japonica* (Houtt.) Ronse Decr. var. *japonica*, F. *sachalinensis* (F. Schmidt) Ronse Decr. in njun križanec *F. ×bohemica* (Chrtek & Chrtková) J. P. Bailey. Taksoni so si med seboj morfološko zelo podobni, še posebej težko pa je razlikovati med križancem *F. ×bohemica* ter starševskima vrstama *F. japonica* in *F. sachalinensis*.

Z uporabo dveh različnih molekulskih označevalcev, plastidnega zaporedja DNA *trn*K v kombinaciji z analizo RFLP z uporabo restrikcijske endonukleaze HhaI ter mikrosatelita KW6, ki je potencialno specifičen za vrsto *F. sachalinensis*, smo analizirali 30 vzorcev dresnikov, ki smo jih nabrali na območju Slovenije. Med njimi je bilo 8 vzorcev vrste *F. japonica*, 2 vzorca *F. sachalinensis* in 20 vzorcev križanca *F. ×bohemica*.

Ugotovili smo, da lahko s kombinacijo obeh označevalcev nedvoumno določimo vse tri taksone (tab. 1, sl. 1). Pri vrsti *F. japonica* (var. *japonica*) pomnoženi 2700 bp dolg fragment *trn*K endonuk-

leaza HhaI razreže na dva dela velikosti 1600 bp in 1100 bp, mikrosatelit KW6 pa se ne pomnoži. Pri vrsti *F. sachalinensis* pomnoženi 2700 bp dolg fragment *trn*K po uporabi endonukleaze HhaI ostane cel, pomnoži pa se mikrosatelit KW6. Pri križancu *F. ×bohemica* pomnoženi 2700 bp dolg fragment *trn*K endonukleaza HhaI razreže na dva dela velikosti 1600 bp in 1100 bp, kot je značilno za japonski dresnik, pomnoži pa se mikrosatelit KW6, kar je značilno za sahalinski dresnik. S tem smo tudi potrdili, da je japonski dresnik materinska vrsta vseh analiziranih vzorcev križancev.

V analizo smo vključili tudi dva predstavnika slakovcev, *F. baldschuanica* (Regel) Holub in *F. multiflora* (Thunb.) Haraldson. Oba se s pomočjo uporabljenih molekulskih označevalcev zanesljivo razlikujeta od dresnikov (tab. 1, sl. 1), saj pomnoženi 2700 bp dolg fragment *trn*K po uporabi endonukleaze HhaI ostane cel, mikrosatelit KW6 pa se ne pomnoži. Za razlikovanje med vrstama slakovcev pa bi bilo treba poiskati nove označevalce.

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Appendix: Sampling localities

Japanese knotweed (*Fallopia japonica*)

F10–KAMNIK; Slovenia, Gorenjska, Kamnik, Zgornje Perovo, beside petrol station, road branch to Zg. Perovo; N 46°12'34.0'', E 14°36'27.8''; ruderal place beside road; MTB: 9753/4; leg: S. Strgulc Krajšek and S. Anžlovar, 15.4.2010.

F22–KRESNICE; Slovenia, Zasavje, Kresnice, by River Sava; N 46°06'20.3'', E 14°46'44.6''; meadow beside River Sava; MTB: 9854/4; leg: N. Jogan, 27.9.2010.

F27–BF-MOST; Slovenia, Ljubljana, beside Večna pot, by Glinščica brook, beside bridge on eastern side of Biotechnical Faculty; N 46°02'58.3'', E 14°28'33.0''; bank of regulated brook; MTB: 9952/4; leg: S. Strgulc Krajšek and S. Anžlovar, 15.4.2010.

F51–CELJE; Slovenia, Štajerska, Celje, Selce, between brooks Voglajna in Ležnica before the confluence, by the road underpass under railway line, N 46°13'58,68'', E 15°16'42,58''; ruderal place beside road; MTB 9757/4; leg: S. Strgulc Krajšek and T. Pogačnik Lipovec, 22.9.2010.

F59–CELJE-POLULE; Slovenia, Štajerska, Celje, Polule, left bank of river Savinja by road bridge; N 46°13'0,49'', E 15°15'51,02''; river bank; MTB 9757/4; leg: S. Strgulc Krajšek and M. Bjelić, 26.1.2012.

F60–DEBRO-SAVINJA; Slovenia, Štajerska, by the road Celje–Laško, Debro; N 46°10›39,65», E 15°14'19,39'' river bank, MTB 9857/1; leg: S. Strgulc Krajšek and M. Bjelić, 26.1.2012.

F61–MARNO; Slovenia, Štajerska, by the road Rimske Toplice–Hrastnik, E from Marno; N 46°8›18,4», E 15°8'37,67''; forest edge, 9866/4; leg: S. Strgulc Krajšek and M. Bjelić, 26.1.2012.

F65–ZAGORJE; Slovenia, Zasavje, Zagorje, Toplice, left bank of regulated brook Medija; N 46°8›19,98», E 14°59'26,11''; brook bank, MTB 9955/1; leg: M. Bjelić, 25.2.2012.

Giant knotweed (*Fallopia sachalinensis*)

F50–CELJE-POLULE; Slovenia, Štajerska, Celje, Polule, around the bus station opposite the school; N 46°12'58.1'', E 15°15'45.3'', ruderal site beside road; MTB 9757/4; leg.: M. Škornik, 20.6.2010.

F53–CELJE-MEDLOG; Slovenia, Štajerska, Celje, Medlog, beside connecting road from Medlog to highway A1; N 46°14'45.2'', E 15°13'46.0''; road bank; MTB 9757/3; leg: S. Strgulc Krajšek and T. Pogačnik Lipovec, 22.9.2010.

Bohemian knotweed (*Fallopia × bohemica*)

F01–VIŽMARJE; Slovenia, Ljubljana, Vižmarje, by the Tacenska street, N 46°6'22,61", E 14°27'45,31"; ruderal site by the street; MTB: 9852/4, leg: S. Strgulc Krajšek, 11.11.2009.

F02–KOKRICA; Slovenia, Gorenjska, Kranj, Naklo, crossroad on the Kranj to Naklo road to highway A2 (Kranj Zahod); N 46°15'45.3'', E 14°19'60.0''; ruderal place beside road; MTB: 9752/1; leg: S. Strgulc Krajšek, 20.4.2010.

F05–KRANJ; Slovenia, Gorenjska, Kranj, Savski otok, left riverbank of Sava; N 46°14'37.3'', E 14°21'01.0''; river bank; MTB: 9752/3; leg: S. Strgulc Krajšek, 20.4.2010.

F07–MEDVODE; Slovenia, Gorenjska, Medvode, Jeprca, unpaved parking place by Jeprca to Medvode road; N 46°09'15.6'', E 14°23'54.8''; ruderal place beside road resting place; MTB: 9852/1; leg: S. Strgulc Krajšek, 20.4.2010.

F09–ŠENTVID; Slovenia, Ljubljana, Šentvid, Poljane; N 46°05'44.6''; E 14°28'19.9''; ruderal place beside road; MTB: 9952/2; leg: S. Strgulc Krajšek, 20.4.2010.

F24–BF; Slovenia, Ljubljana, beside Večna pot, by Glinščica brook, by fence of Biotechnical Faculty; N 46°03'02.7'', E 14°28'19.5''; bank of regulated brook; MTB: 9952/2; leg: S. Strgulc Krajšek and S. Anžlovar, 15.4.2010.

F34–FUŽINE; Slovenia, Ljubljana, Studenec, by PST trail, 100 m E from the bridge over Ljubljanica river; N 46°3'1,06", E 14°33'57,59"; river bank; MTB: 9953/1; leg: S. Strgulc Krajšek, 15.6.2010.

F35–CHENGDUYSKA; Slovenia, Ljubljana, Fužine, by the bus station Chengdujska; N 46°3'17,21", E 14°34'1,14"; ruderal place beside the road; MTB: 9953/1; leg: S. Strgulc Krajšek, 15.6.2010.

F38–BOKALCE; Slovenia, Ljubljana, Bokalce; N 46°3'1,25", E 14°26'32,7"; by the unpaved field road; MTB: 9952/2; leg: S. Strgulc Krajšek and B. Dolinar, 29.7.2010.

F49–CELJE-BREG; Slovenia, Štajerska, Celje, Breg, by Dornov studenec; N 46°13'20,75", E 15°16'12,74"; ruderal place beside road; MTB: 9757/4; leg: S. Strgulc Krajšek and T. Pogačnik Lipovec, 22.9.2010.

F51–CELJE; Slovenia, Štajerska, Celje, Selce, between brooks Voglajna and Ležnica before the confluence, by the road underpass under railway line, N 46°13'58,68", E 15°16'42,58"; ruderal place beside road; MTB 9757/4; leg: S. Strgulc Krajšek and T. Pogačnik Lipovec, 22.9.2010.

F56–PIRNIČE; Slovenia, central Slovenia, Zgornje Pirniče, by the road from Zg. Pirniče to graveyard; N 46°8'33,98", E 14°25'58,25"; ruderal place beside the road; MTB: 9852/4, leg: S. Strgulc Krajšek, 23.1.2012.

F57–VERJE; Slovenia, central Slovenia, Verje, by the bridge over Sava under the hydroelectric power plant Medvode; N 46°8'38,25", E 14°24'53,34"; ruderal place beside the road; MTB: 9852/4, leg: S. Strgulc Krajšek, 23.1.2012.

F58–MEDVODE-KROŽIŠČE; Slovenija, Gorenjska, Medvode, by regional road Jeprca–Medvode, bus station near roundabout; N 46°8'52,69", E 14°24'30,23"; ruderal place beside the road; MTB: 9852/3; leg: S. Strgulc Krajšek, 23.1.2012.

F66–BRITOF; Slovenia, Gorenjska, Kranj, by the road to Šenčur, 200 m SE from the Britof; N 46°15'37,95", E 14°23'41,18"; ruderal place beside the road; MTB: 9752/1; leg: S. Strgulc Krajšek, 2.3.2012.

F68–BLED-CESTA SVOBODE; Slovenia, Gorenjska, Bled, road resting place by the road to Bohinj, SW from Bled; N 46°21'24,16", E 14°5'26,23"; ruderal place beside road resting place; MTB: 9650/2; leg: S. Strgulc Krajšek, 2.3.2012.

F69–KRANJ-BRDO-AC; Slovenia, Gorenjska, Kranj, by the road Kranj–Kokrica by overpass over highway; N 46°15'45,65", E 14°21'13,47"; ruderal place beside the road; MTB: 9752/1; leg: S. Strgulc Krajšek, 2.3.2012.

F70–NOMENJ(V); Slovenia, Gorenjska, Sava Bohinjka valley, Nomenj, road branch from the main road on the E edge of the settlement; N 46°17'19,41", E 14°0'47,44"; ruderal place beside the road; MTB: 9750/1; leg: S. Strgulc Krajšek, 2.3.2012.

F71–NOMENJ(Z); Slovenia, Gorenjska, Sava Bohinjka valley, Nomenj, branch from the main road on the W edge of the settlement; N 46°17'20,95", E 13°59'51,46"; ruderal place beside the road; MTB: 9750/1; leg: S. Strgulc Krajšek, 2.3.2012.

F72–LJ-BRDO-AC; Slovenia, Ljubljana, Brdo, highway exit Brdo, under the highway overpass; N 46°3'18,41", E 14°27'9,68"; ruderal place beside the road, MTB: 9952/2; leg: S. Strgulc Krajšek and M. Bjelić, 30.3.2012.

Russian vine (*Fallopia baldschuanica*)

F64–NOVA GORICA; Slovenia, Vipava valley, Nova Gorica, by the gas station in Grčna; N 45°57'8,98", E 13°39'8,46"; bushes by the road, MTB: 0047/2; leg: N. Jogan, 3.11.2010.

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Herbarij v izobraževanju učiteljev razrednega pouka

Herbarium in primary teacher education

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Izvleček: V članku opredeljujemo pomen herbarija v izobraževanju učiteljev razrednega pouka za dvig interesa za rastline in znanja o rastlinah med osnovnošolci. Slovenske študente, bodoče učitelje razrednega pouka, smo usposabljali v izdelovanju herbarija. V raziskavi je sodelovalo 86 študentov. Študenti so po izdelavi herbarijev izpolnili anketni vprašalnik, s katerim smo preverjali njihovo znanje in izkušnje. Študenti poročajo, da so med izdelovanjem herbarijev v povprečju spoznali tri nove rastlinske vrste. 74% študentov je imelo ob tem težave, predvsem z določanjem vrst in postopki herbariziranja. Več kot dve tretjini študentov je herbarij izdelovalo že v osnovni ali srednji šoli, predvsem pri predmetih naravoslovje in biologija. Vsi, z izjemo enega študenta, nameravajo herbarij v bodoče vključiti v pouk. V aktivnosti vidijo predvsem potencial za poučevanje učencev o anatomiji in vrstni pestrosti rastlin, za razvijanje njihovih naravoslovnih spretnosti ter odnosa do dela in biologije. Sklenemo lahko, da so sodelujoči študenti prikazali ustrezen nivo poznavanja vsebine ter znanja za poučevanje za uspešno implementacijo herbarija v šolo, kar lahko vodi do dviga interesa za rastline in znanja o rastlinah med osnovnošolci.

Ključne besede: herbarij, študent, kompetence, botanika, šola

Abstract: In this article, we define the importance of herbarium in primary teacher education to enhance primary school student's interest in, and knowledge of plants. Slovene pre-service primary school teachers were trained to make their own herbarium. The study involved 86 undergraduate students. After making their own herbarium they completed a written questionnaire about gained knowledge and experiences. The results show that students, while making herbarium, learned on average three new plant species. 74 % of students reported having some difficulties in determination of plant species and the herbarization procedures. More than two thirds of students reported that they experienced making herbarium in primary or secondary school. Most of them in science and biology classes in primary school. All, except one, plan to use herbarium in their teaching practice. They see the potential of the activity in teaching primary school students about plant anatomy and species diversity, science skills, work attitudes and attitudes towards biology. To conclude, participating students demonstrated desired level of content knowledge and pedagogical content knowledge to successfully implement the herbarium into primary education, which could be beneficial in enhancing primary school student's interest in, and knowledge of plants.

Keywords: herbarium, student, competences, botany, school

Uvod

Herbarij je urejena zbirka posušenega, stisnjenega in reprezentativnega rastlinskega materiala opremljena z etiketami. Namenjena je znanstvenemu raziskovanju, učenju in dokumentiranju (Botanični terminološki slovar 2011). Tehnika stiskanja in sušenja rastlin med polama papirja prepreči zvijanje in gubanje rastlinskih delov. Tehnika se uporablja že od 16. stoletja, ter omogoča ohranjanje večine značilnosti rastlin, izvzemši na primer barvo cvetov in vonjave, ki jih izdelovalec herbarija lahko zapiše v etiketo (Flannery 2013, Royal Botanic Garden Edinburg 2017). Odsotnost vode v posušenih rastlinah zavre delovanje bakterij in gliv, ki razkrajajo rastlinska tkiva. Nekaterih rastlinskih delov, kot so na primer sočni plodovi in velika semena, ne moremo ohranjati po opisanem postopku. Shranjujemo jih v alkoholu ali v škatlicah (Flannery 2013).

V zadnjem času herbarij ponovno pridobiva na veljavi. Ni več obravnavan le kot knjižnica rastlinskih vrst. Med razlogi za porast zanimanja za herbarijske zbirke velja izpostaviti pomen herbarija pri dokumentiranju biotske pestrosti rastlin na določenem območju (Joppa in sod. 2011), za dokumentiranje spreminjanja rastlinskih združb (Kohler 2006) in za dokumentiranje okoljskih sprememb (na primer spremembe v času cvetenja rastlin) (Neil 2009). Herbarizirane rastline so tudi genetski »posnetek« iz preteklosti, ko je bila rastlina nabrana in herbarizirana. Molekularni biologi iz herbariziranih primerkov lahko pridobivajo genetski material – tudi iz primerkov starih dvesto let (Andreasen in sod. 2009).

Herbarij ima pomembno vlogo tudi v izobraževanju mladih. Številne raziskave so pokazale, da ljudje v povprečju izkazujejo več zanimanja za živali kot za rastline (Bebbington 2005, Darley 1990, Gatt in sod. 2007; Strgar 2007, Wandersee in Schussler 1999, 2001). Nezanimanje za rastline sta Wandersee in Schussler (2001) poimenovala s terminom »rastlinska slepota«. Znaki zanjo so nezmožnost zaznave oziroma fokusa na rastlinske vrste v okolju, nezmožnost prepoznavanja pomembnosti rastlin v okolju, pomanjkanje znanja o rastlinah, pomanjkanje osebne izkušnje pri opazovanju rasti in razvoja ter določanju vrste, nezmožnost prepoznave estetske in unikatne biološke značilnosti rastline ter nagnjenje k rangiranju rastlin kot podrejene živalskim vrstam (Strgar 2007, Wandersee in Schussler, 2001). Mihičinac (2013) v svoji diplomski nalogi ugotavlja, da slovenski osnovnošolci in bodoči učitelji biologije ocenjujejo pouk botanike kot zahteven in dolgočasen.

Da bi pri učečih zbudili večji interes za rastline, Hershey (1992) predlaga, da bi pri pouku biologije manj uporabljali že vnaprej pripravljena učna gradiva o rastlinah, kot so trajni preparati, plastični modeli in herbarijski primerki, ter več pozornosti posvečali praktičnemu pouku z rastlinami, kot na primer gojitvam rastlin, izdelovanju herbarijev itn. Izpostavlja pomen neposrednih, konkretnih učnih dejavnosti in izkušenj z rastlinskim materialom. Raziskave, ki bi obravnavale vključevanje herbarija v pouk biologije oziroma botanike, niso pogoste (npr. Almeida in sod. 2006, Flannery 2013, Neil 2009, Ohkawa 2000). Opisujejo postopke izdelave in uporabe herbarija pri pouku, analizirajo vrstno pestrost rastlin v izdelanih herbarijih, uporabljajo herbarij kot eno od metod za spremljanje biotske pestrosti lokalnega okolja in okoljskih sprememb. Strgar (2007) poudarja vlogo učitelja pri dvigu interesa za rastline pri učencih. Izpostavlja, da morajo biti učiteljeva strokovnost, entuziazem in interes za predmet prepoznani že pri usposabljanju bodočih učiteljev.

V članku izpostavljamo pomen izobraževanja bodočih učiteljev razrednega pouka v Sloveniji za zgodnje vključevanje herbarija v pouk o rastlinah. V prvem triletju osnovne šole učitelji razrednega pouka poučujejo predmet spoznavanje okolja (Program Osnovna šola. Spoznavanje okolja. Učni načrt 2011), kjer učenci spoznavajo različne vrste organizmov v različnih okoljih (Tab. 1). V četrtem in petem razredu osnovne šole pa poučujejo predmet naravoslovje in tehnika (Program Osnovna šola. Naravoslovje in tehnika. Učni načrt 2011). V četrtem razredu učenci razvrščajo rastline po skupnih značilnostih, v večje skupine rastlin (npr. cvetnice, mahovi, praprotnice), spoznavajo zgradbo rastlin ter najpogostejše vrste v neposredni okolici. Poudarek je tudi na razvoju naravoslovnih spretnosti in postopkov, kot so opazovanje, razvrščanje, uvrščanje in urejanje. Nikjer ni eksplicitno navedeno, da naj učitelji pri pouku naučijo učence izdelovati herbarij, vendar pa so v tabeli 1 predstavljeni učni cilji priložnost, da se učenci ob izdelovanju herbarija podrobneje spoznajo z zgradbo rastlin in njihovo raznolikostjo, ter razvijajo svoje naravoslovne spretnosti opazovanja, razvrščanja itn.

Tabela 1: Naravoslovni predmeti ter učni cilji, ki učitelju razrednega pouka omogočajo implementacijo herbarija.
Table 1: Science subjects and learning goals that enable primary school teacher's implementation of herbarium as class activity.

Predmet	Razred	Učni cilj
Spoznavanje okolja	1.	Učenci prepoznajo, poimenujejo in primerjajo različna živa bitja in okolja.
	3.	Učenci razlikujejo in opišejo živa bitja in okolja, v katerih živijo, ter kako ponavljajoče se spremembe vplivajo nanje (noč – dan, letni časi).
Naravoslovje in tehnika	4.	Učenec zna razvrstiti živa bitja v skupine po skupnih značilnostih. Učenec zna opredeliti vrsto kot osnovno enoto za razvrščanje in da so glavne skupine živih bitij kraljestva. Učenec zna prepoznati najpogostejše vrste rastlin, živali in gliv v neposrednem okolju. Učenec zna razložiti zunanjo zgradbo rastlin. Učenec zna razlikovati med rastlinami s cvetovi in rastlinami brez cvetov.
	5.	Učenec zna opisati najbolj značilne kulturne rastline. Učenec zna razložiti, zakaj je manjša pestrost življenja na obdelovalnih površinah kot v prosti naravi.

Za namen raziskave smo postavili naslednja raziskovalna vprašanja:

1. Ali izdelovanje herbarija pripomore k boljšemu poznavanju vrstne pestrosti rastlin?
2. S kakšnimi težavami se študentje soočajo med postopki priprave herbarija?
3. Koliko študentov je izdelalo herbarij že v šoli in pri katerih predmetih?
4. Kaj se lahko osnovnošolci, po mnenju študentov, naučijo ob izdelovanju herbarija?

Metode dela

Vzorec in opis dejavnosti

V raziskavi je sodelovalo 86 študentov prvega letnika dodiplomskega študijskega programa Razredni pouk na Pedagoški fakulteti Univerze v Ljubljani. Študenti so na vajah predmeta Naravoslovje - biološke vsebine prejeli podrobna navodila o izdelavi herbarija. Rastline so študenti sami določili in nabrali, ustrezno posušili ter izdelali herbarijske pole. Sledila je priprava herbarijske etikete, kjer so navedli znanstveno in slovensko ime vrste, rastišče ter časovne in krajevne podatke nabiranja. Herbarij vsakega študenta je vseboval deset različnih vrst rastlin. Študenti so se morali izogniti lesnatim rastlinam in zavarovanim rastlinskim vrstam. Za določanje rastlin so bili študentom v pomoč tiskani in spletni določevalni ključi ter druga literatura. Delo je potekalo v mesecu aprilu in maju 2017. Herbariji so bili naknadno strokovno pregledani. Študenti so prejeli povratno informacijo o svojem izdelku.

Zbiranje podatkov in instrument

Ob oddaji herbarija so študentje izpolnili anonimni anketni vprašalnik o izvedeni dejavnosti. Izpolnjevanje anketnih vprašalnikov ni bilo časovno omejeno. Študenti so v povprečju anketni vprašalnik izpolnjevali 10 minut. Anketni vprašalnik je vključeval osnovna demografska vprašanja ter vprašanja, vezana na raziskovalna vprašanja. Vprašanja so bila odprtega in zaprtega tipa.

Obdelava podatkov

Za vprašanja odprtega tipa smo uporabili tehniko kodiranja podatkov, in sicer odprt način kodiranja, kjer smo podatke organizirali po njihovi vsebinski sorodnosti. Uporabili smo nivo deskriptivne statistike, in sicer frekvenčno porazdelitev (*f, f*%) spremenljivk. Rezultati so prikazani tabelarično.

Rezultati

Uvodoma nas je zanimalo, katere vrste rastlin so študenti uvrstili v svoj herbarij ter koliko je to pripomoglo k spoznavanju novih vrst. V tabeli 2 in 3 so rastline navedene z rodovnimi imeni, saj so študenti pogosto v odgovoru pomanjkljivo navedli ime vrste (npr. zlatica in ne ripeča zlatica). V kolikor so študenti navedli natančno določeno vrsto rastline, smo to izpostavili. Iz tabele 2 je razvidno, da so najpogosteje izbrane zlatice (12,1 %). V herbarij jih je uvrstilo kar 82,6 % študentov. Med pogosteje izbranimi rodovi so tudi detelja, marjetica, ivanjščica in regrat. Študentje so že pred izvedbo dejavnosti poznali dobri dve tretjini herbariziranih rastlin (69 %). Iz tabele 3 lahko razberemo, da med novo spoznanimi rastlinami izstopata plazeči skrečnik (*Ajuga reptans*) (15,1 % študentov prvič spoznalo med izdelavo herbarija) in vrednikov jetičnik (*Veronica chamaedrys)* (14 % študentov prvič spoznalo med izdelavo herbarija).

Tabela 2: Seznam najpogostejših rastlinskih rodov, ki so jih študenti predstavili v herbarijih.
Table 2: A list of most commonly named genera students presented in their herbariums.

Rodovi rastlin v herbarijih študentov	Delež rodu med vsemi rodovi *f*(%)	Delež študentov, ki je rastlino tega rodu herbariziral *f*(%)
Ranunculus	12,1	82,6
Trifolium	10,2	69,8
Bellis	9,6	65,1
Leucanthemum	8,0	54,6
Taraxacum	7,3	50,0
Ajuga	6,3	43,0
Fragaria	5,1	33,7
Viola	4,9	32,6
Anemone	4,6	31,4
Lamium	4,3	29,1
Salvia	4,3	29,1
Knautia	4,1	27,9
Veronica	3,9	26,7

Tabela 3: Seznam najpogostejših rastlinskih rodov oziroma vrst, s katerimi so se študentje prvič srečali med izdelavo herbarija.
Table 3: A list of most commonly named genera of species students came across for the first time while making herbarium.

Rodovi oziroma vrste rastlin, ki so jih študenti prvič spoznali	Delež rodov/vrst med vsemi herbariziranimi *f*(%)	Delež študentov *f*(%)
Ajuga reptans	9,1	15,1
Veronica chamaedrys	8,4	14,0
Anemone nemorosa	4,9	8,1
Trifolium	4,9	8,1
Salvia pratensis	3,5	5,8
Glechoma	2,8	4,6
Hepatica	2,8	4,6
Knautia	2,8	4,6
Ranunculus ficaria	2,8	4,6
Capsella	2,1	3,5
Corydalis	2,1	3,5
Isopyrum	2,1	3,5
Polygala	2,1	3,5

Zanimalo nas je, ali so se študentje med postopki priprave herbarija imeli kakršnekoli težave (Tab. 4). Kar 74 % študentov je odgovorilo pritrdilno. Največ težav so imeli z določanjem rastlin (66,7 %).

Tabela 4: Vrste težav, s katerimi so se študentje srečevali med izdelavo herbarija.
Table 4: Types of difficulties students had to deal with while making herbarium.

Vrsta težave	*f*	*f*(%)	Primeri odgovorov
Nabiranje	3	5,26	Še največ težav mi je predstavljajo najti rastlino. Pri nekaterih rastlinah nisem našla cveta.
Določanje	38	66,7	Nekaj težav sem imela pri določanju rastlin. Težave sem imela pri prepoznavanju plazečega skrečnika. Imela sem težave pri določanju rastlin, ker sem jih določevala, ko so bile že posušene.
Herbariziranje	12	21,0	Nekatere rastline so se po sušenju spremenile. Nekatere rastline so zgnile med postopkom sušenja. Imela sem težave pri sušenju debelih čebulic, zaradi gnitja rastlin in večkratnega ponovnega nabiranja.
Drugo	3	5,26	Nikoli še nisem tega počel, zato je bilo veliko težav. Imela sem težave le na začetku zaradi zavarovanih rastlih.

Vsi študenti, razen enega, nameravajo herbarij uporabljati pri svojem pedagoškem delu. Zanimalo nas je, koliko študentov je v preteklosti že izdelovalo herbarij v šoli ter pri katerem predmetu oziroma v katerem obdobju šolanja. 59 % študentov se je srečalo z izdelovanjem herbarija v času šolanja. Med študenti, ki so na vprašanje odgovorili pritrdilno, se jih je 36,4 % z izdelavo herbarija srečalo pri predmetu biologija v osnovni šoli, kar 56,8 % vprašanih pa se je z izdelavo herbarija srečalo v zgodnejših obdobjih šolanja, največ pri predmetu naravoslovje (31,8 %) (Tab. 5).

Tabela 5: Šolski predmeti, pri katerih so študentje, bodoči učitelji razrednega pouka, izdelovali svoje herbarije.
Table 5: School subjects where students of primary teacher education were making their own herbariums.

Ime predmeta	Obdobje šolanja	Število študentov (f)	Delež študentov f(%)
Spoznavanje okolja	1., 2. in 3. razred	6	13,6
Naravoslovje in tehnika	4. in 5. razred	5	11,4
Naravoslovje	6. in 7. razred	14	31,8
Biologija	8. in 9. razred	16	36,4
Biologija	srednja šola	3	6,8
Skupaj		44	100

Bodoče učitelje razrednega pouka smo spraševali, kaj se lahko učenci naučijo ob izdelovanju herbarija pri pouku. Odgovore študentov smo kategorizirali v tri kategorije kompetenc: znanje in razumevanje, spretnosti ter odnos do dela in narave. Največ študentov (52,7 %) je odgovorilo, da se učenci z aktivnostjo lahko naučijo prepoznavati različne vrste rastlin, izpostavljajo tudi pridobljene naravoslovne spretnosti ter odnos do dela in narave (Tab. 6).

Tabela 6: Kompetence, ki jih učenci dobijo ob izdelavi herbarija.
Table 6: Competences primary school students can achieve by making herbarium.

Kategorije	Delež študentov		
Podkategorije	**f**	**f(%)**	**Primeri odgovorov**
Znanje in razumevanje			
Spoznavanje in določanje rastlinskih vrst	77	52,7	Učenci se naučijo prepoznati različne vrste rastlin in njihove značilnosti. Učenci z izdelavo herbarija poglobijo znanje o rastlinah in se naučijo postopka razvrščanja ter sistematičnosti.
Zgradba rastlin (rastlinskih organov)	9	6,2	Učenci spoznajo sestavne dele rastlin. Učenci se naučijo kakšne so rastline pod zemljo.
Spretnosti			
Herbariziranje	17	11,6	Učenci usvojijo postopek herbariziranja ter kako se ravna z rastlinami. Učenci se naučijo, da lahko rastline tudi trajno shranjujemo.
Praktično delo	9	6,2	Učenci rokujejo z rastlinami. Z izdelavo herbarija iščeš in preučuješ rastline in je to bolj zabavno kot se jih samo učiti.
Odnos do dela in narave	16	11	Učenci se naučijo samostojnega dela, odgovornosti do časa in dela. Razvijajo natančnost in skrbnost. Učenci se naučijo potrpežljivosti in discipline. Učenci se približajo naravi.

Diskusija in zaključki

Izobraževanje bodočih učiteljev je ena od ključnih priložnosti za spreminjanje šolske prakse. V raziskavah, ki obravnavajo različne vidike poučevanja botanike, je izpostavljen pomen praktičnega pouka z rastlinami (npr. Hershey 1992) ter vloga učitelja pri dvigu interesa za rastline pri učencih (npr. Strgar 2007). Raziskave tudi izpostavljajo, da imajo otroci že v rani mladosti manjše zanimanje za rastline kot živali (npr. Wandersee in Schussler 1999, 2001). Navedeno argumentira pomen zgodnjega vključevanja herbariziranja v pouk, zato se vsi bodoči učitelji razrednega pouka na Pedagoški fakulteti Univerze v Ljubljani praktično spoznajo s herbariziranjem rastlin.

Iz rezultatov pričujoče raziskave lahko ugotovimo, da je izdelava herbarija študentom omogočila spoznavanje novih vrst rastlin. V povprečju je vsak študent spoznal najmanj tri nove vrste rastlin. Predvsem je pomembno, da so se naučili samostojno izdelovati herbarij. Kar tri četrtine vprašanih študentov je poročalo, da so imeli težave pri izvedbi dela; največ težav so imeli s samim določanjem rastlinskih vrst ter s postopkom herbariziranja rastlin. Na njihove izkušnje in sposobnosti herbariziranja rastlin lahko pomembno vplivajo pretekle izkušnje s herbariziranjem. Ugotavljamo, da se je več kot polovica študentov spoznala s herbariziranjem rastlin v osnovni ali srednji šoli. Od tega jih je četrtina izdelovala herbarij v obdobju od 1. do 5. razreda osnovne šole, ko učence poučujejo učitelji razrednega pouka.

Za bodoče učitelje razrednega pouka je pomembno, da se zavedajo, katere kompetence lahko učenec pridobi ob izdelovanju herbarija pri pouku. S tem, po našem mnenju, obstaja tudi večja verjetnost implementacije herbarija v pouk. Razen enega študenta, so vsi izrazili namero, da bodo uporabljali herbarij v svoji pedagoški praksi. Z izdelavo herbarija pri pouku lahko, po mnenju bodočih učiteljev, dosegamo tudi kognitivne učne cilje. Študenti najpogosteje izpostavljajo, da se učenci lahko naučijo prepoznavati različne vrste rastlin in njihove zgradbe. Študenti izpostavljajo tudi naravoslovne spretnosti. Učni načrti osnovnošolskih predmetov spoznavanje okolja ter naravoslovje in tehnika zelo izpostavljajo pomen razvijanja naravoslovnih spretnosti (Program Osnovna šola. Naravoslovje in tehnika. Učni načrt 2011, Program Osnovna šola. Spoznavanje okolja. Učni načrt 2011). Med nekaterimi študenti obstaja tudi zavedanje, da lahko z opisano dejavnostjo učenci razvijajo svoj odnos do dela in narave. Naučijo se lahko vrlin, kot sta skrbnost in odgovornost pri delu. Po mnenju nekaterih študentov prispeva tudi k razvijanju odnosa do narave. Slednje je še kako pomembno za zbujanje večjega interesa za rastline med mladimi.

Neposredno delo z organizmi je pomembno za učinkovito poučevanje biologije (Lock 1994, Strgar 2007), zato je še toliko bolj pomembno, da bodoči učitelji bioloških vsebin razumejo pomen izkustvenega učenja z organizmi pri pouku. Raziskava je pokazala, da herbariziranje rastlin dosega svoj namen v programu biološkega izobraževanja bodočih učiteljev razrednega pouka. Iz analize odgovorov študentov je namreč razvidno, da herbariziranje prispeva k njihovemu boljšemu poznavanju vrstne pestrosti rastlin ter razumevanju temeljnih kompetenc, ki jih učenec pridobi s herbariziranjem rastlin pri pouku. V prihodnje načrtujemo optimizirati opisano dejavnost s povečanjem števila nabranih vrst v herbariju, kar bo zelo verjetno pozitivno vplivalo na njihovo poznavanje vrstne pestrosti rastlin. Dopolniti želimo tudi zahtevane informacije o nabranih rastlinah na etiketi. Študente namreč želimo spodbuditi k razvijanju spretnosti opazovanja raznolikosti in funkcij posameznih rastlinskih organov ter k izboljšanju poznavanja botanične terminologije.

Summary

In this article, we define the importance of herbarium in primary teacher education to enhance primary school student's interest in, and knowledge of plants. There is a wider consensus among researchers that students show less interest in plants than animals. Therefore, it was recommended to implement more practical work with living organisms and to improve training of future teachers to increase the interest in botany among students. The article presents how Slovene students, future primary school teachers, were trained to make their own herbarium. The study involved 86 undergraduate students in their second

semester of four years long study programme at the University of Ljubljana, Faculty of Education.

After making their own herbarium, consisting of ten plant species, they completed a questionnaire. We were asking them about plant names of the species they included into herbarium, which of those were new for them, which difficulties students had to deal with while making herbarium, if they already experienced making herbarium in primary or secondary school and which competences, in their opinion, primary school students can develop while making herbarium. Open questions were categorized. Descriptive statistics was used to analyze the data.

The results show that students learned new plant species as a result of making their own herbariums. On average, they learned three new species. 74% of students reported having some difficulties in determination of species and the herbarization procedures. All of them, except one, plan to implement this activity into their teaching practice. 69% of students report that they experienced making herbarium in primary or secondary school. Most of them in science (31.8%) and biology (36.4%) classes in primary school. Lastly, future teachers see the potential of the described activity in teaching primary school students about plant anatomy and species diversity, and developing science skills. In addition, some future teachers think primary school students can develop their work attitudes and attitudes towards nature.

To conclude, the present study showed that future primary school teachers, who were trained to make their own herbarium, demonstrated desired content knowledge and pedagogical content knowledge to successfully implement the herbarium into primary education, which could be beneficial in enhancing primary school student's interest in, and knowledge of plants.

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ABS ACTA BIOLOGICA SLOVENICA
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Z rastlinami povezani že 35 let

Connected with plants for last 35 years

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Izvleček: V letu 2017 člani Slovenskega društva za biologijo rastlin (http://www.plantslo.org/) praznujemo kar 35 let neprekinjenega delovanja, čeprav ne v enaki zasedbi in tudi ne pod enakim imenom. V tem času se je veliko spremenilo, vendar pa je ostala nespremenjena naša strast do odkrivanja skrivnega življenja rastlin.

Ključne besede: biologija rastlin, fiziologija rastlin, društvo

Abstract: In 2017 we, the members of the Slovenian Society of Plant Biology, celebrate 35 years of continuous action although not under the same name and not with the same members. Many things have changed during those years, but our true passion to uncover the secret life of plants has remained the same.

Keywords: plant biology, plant physiology, society

V nekih drugih časih…

Vse se je uradno začelo leta 1969, ko je bilo ustanovljeno *Jugoslavensko društvo za fiziologijo biljaka*, v okviru katerega so delovali tudi slovenski raziskovalci, vključno z enim od ustanoviteljev društva – prof. dr. Miranom Vardjanom (1919-2005) (Gogala 2008). Konec sedemdesetih let je prišlo do ustavne preobrazbe društev. Jugoslovanska društva so prenehala z delovanjem in ustanavljati so začeli republiška društva, ki so bila povezana v zveze jugoslovanskih društev. Zveze so koordinirale skupne akcije v jugoslovanskem in mednarodnem prostoru. Jugoslovanski rastlinski fiziologi so bili že takrat povsem v stiku z evropsko rastlinsko fiziologijo, na kar kaže tudi dejstvo, da je bilo Evropsko združenje društev za rastlinsko fiziologijo (FESPP) ustanovljeno šele leta 1978 v Edinburghu na Škotskem. Že leta 1984 je eden od ustanovnih članov jugoslovanskega društva, prof. dr. Miloje Šarić iz Novega Sada, predlagal, da bi enega od kongresov FESPP-a organizirali v Jugoslaviji, saj bi se ga tako lahko udeležilo več predstavnikov iz vzhodnoevropskih društev, katerim je bila pot na zahod takrat še bolj ali manj zaprta. Ideja je bila dobro sprejeta in tako je 6. kongres FESPP potekal v Splitu z veliko člani vzhodnoevropskih društev, ki so prvič sodelovali kot polnopravni člani FESPP-a (Lichtenthaler 2004). Član organizacijskega odbora je bil tudi dr. Franci Pohleven, danes priznani profesor na Oddelku za lesarstvo Biotehniške fakultete.

Leta 1982 je bilo vse pripravljeno tudi za registracijo Slovenskega društva za rastlinsko fiziologijo. Po svojih najboljših močeh je starosta slovenskih in jugoslovanskih rastlinskih fiziologov – prof. dr. Nada Gogala (1937-2013), katere znanstveno-raziskovalnega delovanja niso nikoli omejevale državne meje, pripravila statut društva. V njem je v prvi vrsti opozorila na pomen rastlinske

fiziologije. Ker je bila vizionarka, je že v osnutek statuta vnesla pomen aplikacij s področja rastlinske fiziologije za gospodarstvo kot zametka sodobne biotehnologije in pomen vzgoje kadrov. To pa za takratne čase ni bilo dovolj in urad za registracijo društev statuta ni hotel potrditi. Prof. Gogala si je nato natančno ogledala statute že delujočih društev in v slovenskega vnesla vse manjkajoče člene: o krepitvi bratstva in edinstva, delovanja v duhu socialistične in samoupravne miselnosti in predvsem o boju proti razdiralcem Jugoslavije, prepoznavanju notranjih sovražnikov med člani društva in vse kar je še sodilo zraven. A tudi tako izpopolnjen statut ni prešel faze potrditve. Prof. Gogala je obupano vse skupaj prepustila svojemu mlademu asistentu Franciju Pohlevnu (osebni razgovor s prof. Pohlevnom).

Kot se spominja prof. Pohleven, je imel on več sreče. Še istega dne se je na poti domov ustavil v marketu, kjer ga je na blagajni pozdravil znanec iz sosednjega bloka in ga vprašal zakaj je tako zamišljen. Prof. Pohleven mu je zaupal, da ima težave z registracijo društva. Znanec se je takoj ponudil, da lahko zadevo uredi, saj da v službi dela v isti pisarni kot referentka za registracije društev. Že naslednjega dne se je pokazalo, da se z referentko celo poznata, saj sta se kot članica folklorne skupine France Marolt, on pa pevec akademskega pevskega zbora Tone Tomšič, srečevala na vajah v skupnih prostorih. Vse papirje so že drugi dan uredili in nato ob steklenici vina potrdili statut ter registracijo Slovenskega društva za rastlinsko fiziologijo, katerega prvi predsednik je postal prof. dr. Miran Vardjan, nato pa je bila do leta 1992 predsednica prof. Nada Gogala.

Slovensko društvo za fiziologijo rastlin je bilo vključeno v skupno Jugoslovansko društvo za fiziologijo rastlin, katerega predsedstvo se je na dve leti selilo iz ene republike v drugo. V okviru jugoslovanskega društva so Slovenci organizirali tri simpozije, ki so potekali vsaka tri leta, vedno v drugi republiki. Prvemu je predsedoval prof. dr. Vardjan, ostalima dvema pa prof. dr. Gogala (Gogala 2008). 3. simpozij Jugoslovanskega društva za fiziologijo rastlin smo leta 1977 organizirali slovenski rastlinski fiziologi v Simonovem zalivu pri Izoli. Člani slovenskega društva so se redno udeleževali tudi simpozijev v drugih republikah. Že hiter pregled zbornika 8. simpozija leta 1987 v Tuheljskih Toplicah na Hrvaškem (Jelaska 1987) pokaže, da so bili takrat že dejavni številni, še danes zelo pomembni in dejavni člani društva.

Boj za neodvisnost

Leta 1990, že v politično zelo napetih časih, je bilo slovensko društvo na vrsti za predsedovanje jugoslovanskemu društvu. V okviru predsedovanja je slovensko društvo organiziralo tudi simpozij v Gozd Martuljku. Glede na potek dogodkov se je kasneje pokazalo, da je bil to v resnici zadnji skupni jugoslovanski kongres. Organizatorji simpozija so dobili kar precej odpovedi predvsem s strani srbskega društva. No, nekaj pa jih je le prišlo, predvsem iz Novega Sada in eden iz Beograda. Akademik prof. dr. Rudolf Kastori iz Novega Sada se je v Gozd Martuljek pripeljal z avtom lado. Takoj ob prihodu je vprašal prof. Pohlevna, kam naj parkira avto, saj srbski mediji poročajo, da Slovenci besno razbijajo srbske avtomobile. Prof. Pohleven se je nasmehnil in mu zagotovil, da se ne bo nič zgodilo. V primeru, da bi se, mu bo povrnil škodo iz lastnega žepa. Seveda je imel prof. Pohleven prav in simpozij je mirno minil; prof. Kastori pa se je ob odhodu Pohlevnu opravičil (osebni razgovor s prof. Pohlevnom).

Sledil je junij leta 1991 – le nekaj dni pred razglasitvijo slovenske samostojnosti je svojo doktorsko disertacijo zagovarjala ena od članic društva z najdaljšim statusom – danes izr. prof. dr. Maja Ravnikar ter vodja Oddelka za rastlinsko biotehnologijo in sistemsko biologijo na Nacionalnem inštitutu za biologijo. V komisiji za zagovor doktorata je bila tudi prof. dr. Mirjana Nešković z Biološke fakultete Univerze v Beogradu. Politični položaj je bil skrajno napet in do zadnjega ni bilo jasno, ali bo prof. Nešković sploh prišla. Prišla je in zagovor je bil uspešno opravljen (osebna zabeležka).

Leto 1992 in 8. kongres FESPP-a v Antwerpnu v Belgiji. Prvič na kongres potujemo kot priznani slovenski državljani. Pod vplivom domoljubnega navdušenja smo zelo prizadeti, ko na kongresnih priponkah zagledamo napis Jugoslavija. Družno napise počrnimo in ponosno kot državo napišemo Slovenija. Na kongresu, razen zelo močne srbske delegacije, ni nikogar iz nekdanje države. Na dnevnem redu kongresa je bila, tako kot vedno, tudi generalna skupščina FESPP-a. Ta naj bi dokončno

odločila o statusu našega društva znotraj evropske organizacije. Nam se je seveda zdelo samo po sebi umevno, da bodo republiška društva avtomatično preoblikovana v državna društva in bila kot taka sprejeta v FESPP. Kot mnogokrat v tistih časih, smo bili razočarani. V FESPP-u realnosti o nastanku novih držav na območju Jugoslavije sploh niso zaznali. Srbi so si močno prizadevali, da bi jih priznali za edine naslednike jugoslovanskega društva. Glede na zaplete in nejasnost položaja, je bil pred generalno skupščino nato sklican dodaten sestanek, na katerem naj bi vse prizadete strani predstavile svoje argumente. S slovenske strani se ga je udeležila takratna predstavnica društva v FESPP-u, v času od 1993 njegova predsednica in danes njegova častna članica prof. dr. Maja Kovač z Nacionalnega inštituta za biologijo. Vsi, ki smo bili tam, smo držali pesti. Profesorica Kovač se je hrabro borila in izbojevala je izjemno pomembno zmago, društvo je bilo priznano kot samostojno nacionalno društvo za fiziologijo rastlin (osebna zabeležka).

Končno plujemo v bolj mirnih vodah in na široko odpiramo naša vrata

Z osamosvojitvijo društva to dobi tudi prvi logotip, ki ga je oblikovala prof. Gogala. Ob premišljevanju o ustreznem znaku, je naletela na star učbenik obrtne šole. V njem so bili predstavljeni različni narodni motivi, ki se lahko uporabijo v različnih aplikacijah. Med njimi je zagledala rožico, katere spodnji del lahko predstavlja tako cvetni pecelj kot tudi epruveto iz katere izrašča nova rastlina (osebni razgovor s prof. Gogalo). Znak je več kot desetletje predstavljal naše društvo.

Ena pomembnejših dejavnosti društva je bila in ostaja organizacija simpozijev. Prvega smo zelo uspešno pripravili leta 1993. Imel je močno mednarodno udeležbo in ta postane stalnica naših srečanj. Kot tuji predavatelji se simpozijev udeležujejo predvsem znanstveniki, ki so na kakršenkoli način povezani z raziskavami članov društva. Ni pa zanemarljiva tudi vzpostavitev novih sodelovanj med vabljenimi tujimi gosti in udeleženci simpozija. Kot na primer vedno znova pove stalni udeleženec teh simpozijev prof. dr. Hrvoje Fulgosi z Inštituta Ruđer Bošković v Zagrebu na Hrvaškem, je bila za njegovo znanstveno kariero ključna udeležba na 1. simpoziju Slovenskega društva za fiziologijo rastlin v Gozd Martuljku, kjer se je spoznal z vabljenim predavateljem prof. dr. Reinholdom G. Herrmannom z Univerze v Münchenu (osebni razgovor s prof. Fulgosijem).

S svojimi vabljenimi predavanji so naše simpozije obogatili tudi nekateri »zvezdniški« znanstveniki. Z navdušenjem smo se v mislih skupaj s prof. dr. Christianom Körnerjem z Univerze v Baslu v Švici (https://plantecology.unibas.ch/koerner/index.shtml) povzpeli z njegovimi slavnimi žerjavi v vrh krošenj dreves, da bi sledili vplivom povečane koncentracije ogljikovega dioksida v ozračju na naravno vegetacijo. Prepolna predavalnica je pričakala tudi prof. dr. Douga Soltisa iz Floridskega prirodoslovnega muzeja na Floridski univerzi v Gainesvillu na Floridi in člana Ameriške nacionalne akademije znanosti. Profesor Soltis je skupaj z ženo Pam (https://www.floridamuseum.ufl.edu/museum-voices/soltis-lab/) idejni vodja neformalne mednarodne filogenetske skupine za kritosemenke (Angiosperm Phylogeny Group, APG). Ta želi doseči soglasje o taksonomiji kritosemenk, na podlagi rastlinskih sorodstvenih odnosov, odkritih s filogenetskimi raziskavami. Na naših simpozijih smo gostili še enega člana Ameriške nacionalne akademije znanosti – prof. dr. Michaela R. Freelinga z Univerze Berkeley v Kaliforniji (http://plantandmicrobiology.berkeley.edu/profile/freeling), ki je s svojim prepoznavnim slogom navdihoval poslušalce, kako uporabljati rastlinsko primerjalno genomiko za testiranje evolucijskih hipotez.

Zadnjemu 6. simpoziju je predsedoval dr. Tine Grebenc z Gozdarskega inštituta Slovenije, izveden pa je bil v Hočah pri Mariboru ob sodelovanju doc. dr. Andreje Urbanek Krajnc s Fakultete za kmetijstvo in biosistemske vede Univerze v Mariboru.

Po uspehu 3. simpozija, ki smo ga pripravili ob 20. obletnici neprekinjenega organiziranega delovanja članov društva (Dolenc Koce in sod. 2002), smo se odločili, da začnemo bolj dejavno izpolnjevati zavezo, ki je ostala zapisana še prvi različici statuta – da tudi v okviru društva skrbimo za razvoj kadrov. Od takrat razpisujemo denarne pomoči za naše mlajše člane, s katerimi lahko sofinancirajo svoje raziskovalne obiske v tujih laboratorijih ali se udeležijo pomembnih konferenc v tujini. O svojih izkušnjah nato poročajo ostalim članom na srečanjih društva.

V okviru simpozijev smo izvedli tudi dve izjemno dobro obiskani satelitski delavnici: leta 2002 o določanju genoma in leta 2010 o uporabi PCR v realnem času v rastlinski biologiji. Čas med simpoziji zapolnjujemo z organizacijo različnih predavanj z domačimi in tujimi predavatelji.

Novi časi – novo ime

Člani društva pokrivajo najrazličnejša področja povezana z raziskavami rastlin. Dejavnost članov je že davno presegla fiziologijo v tistem najožjem pomenu, saj se ukvarjamo tudi z genetiko, molekularno biologijo, biokemijo, sistemsko biologijo rastlin, če se omejim le na nekatere discipline, med katerimi danes le težko potegnemo jasno črto. S povsem enakimi težavami so se soočila sorodna stanovska društva in združenja po vsem svetu. Na prelomu tisočletja sta se tako dve največji združenji, American Society of Plant Physiologists in Federation of European Societies of Plant Physiology preimenovali v American Society of Plant Biologists (ASPB) in Federation of European Societies of Plant Biologists (FESPB). Ob 20-letnici delovanja leta 2002 je takratna predsednica (1998–2006) prof. dr. Marina Dermastia, ki danes deluje na Nacionalnem inštitutu za biologijo, predlagala podobno preimenovanje tudi našega društva. Do uradne zamenjave imena v Slovensko društvo za biologijo rastlin (SDBR) je nato prišlo v času predsedovanja (2007 – 2011) prof. dr. Dominika Vodnika leta 2009.

V času priprav na 4. simpozij o rastlinski fiziologiji leta 2006 (Dolenc Koce in sod. 2006) smo zamenjali tudi logotip društva, ki bolj poudarja raznolikost raziskav članov od molekularnih osnov procesov v rastlinah, povezanosti rastlin z zunanjo in notranjo mikrofloro do vključenosti v okolje.

V času od 2012 do 2016 je društvo mirno vodila skozi viharne vode današnjega časa doc. dr. Jasna Dolenc Koce z Oddelka za biologijo Biotehniške fakultete Univerze v Ljubljani. Leta 2017 je mesto predsednice SDBR zasedla dr. Špela Baebler z Nacionalnega inštituta za biologijo.

Društvo se odziva na sodobne izzive časa

Društvo po svojem statutu deluje v javnem interesu. Zaradi tega so njegovi člani obvezani, da se odzivajo na izzive časa. Tako smo leta 1998 v okviru 2. simpozija organizirali medijsko zelo podprto okroglo mizo o biotehnologiji. Prof. dr. Dominik Vodnik z Oddelka za agronomijo Biotehniške fakultete je kot član in predsednik društva sodeloval v Komisiji za delo z gensko spremenjenimi organizmi.

Društvo je vključeno v našo stanovsko krovno organizacijo FESPB (https://www.fespb.org/); zelo dejavno pa smo povezani tudi z Evropsko organizacijo za rastlinsko znanost (EPSO) (http://www.epsoweb.org/), v kateri je slovenska predstavnica, tudi članica društva, izr. prof. dr. Maruša Pompe Novak z Nacionalnega inštituta za biologijo. EPSO zastopa več kot 220 raziskovalnih inštitutov, oddelkov in univerz iz 31 evropskih držav. Cilj EPSO je s političnim delovanjem usmerjati nadaljnji razvoj in vlaganja v raziskave rastlin in povečati vpliv teh raziskav na vsa področja našega življenja.

Naše delo je zelo očarljivo

EPSO je dal leta 2012 pobudo za organizacijo prvega Dneva očarljivih rastlin (DOR) (http://www.plantslo.org/dan_rastlin/index.php), kot smo Fascination of Plants Day (http://www.epsoweb.org/fascination-plants-day) poimenovali pri nas. Cilj DOR je čim večje število ljudi po vsem svetu navdušiti za rastline in jim predstaviti njihov pomen za kmetijstvo, za trajnostno proizvodnjo hrane, za hortikulturo, gozdarstvo in ohranjanje zdravega okolja. Poudarja tudi pomen industrij, ki so povezane z rastlinami, na primer papirne, lesne, kemične, farmacevtske. V organizacijo dogodkov so vključene tako raziskovalne in izobraževalne ustanove, kot tudi botanični vrtovi, muzeji in galerije. Število sodelujočih držav, se vsako leto povečuje, tako da je pri DOR-u leta 2017 sodelovalo že 52 držav po Evropi, Severni in Južni Ameriki, Aziji, Afriki in Avstraliji s 710 dogodki.

SDBR je z navdušenjem sprejelo pobudo in se takoj zelo dejavno vključilo v organizacijo pod vodstvom dr. Pompe Novak. K sodelovanju

je pritegnilo med drugim Nacionalni inštitut za biologijo, Oddelke za agronomijo, biologijo, krajinsko arhitekturo, ter lesarstvo Biotehniške fakultete Univerze v Ljubljani, Botanični vrt Univerze v Ljubljani, Kmetijski inštitut Slovenije, Gozdarski inštitut Slovenije, Prirodoslovni muzej Slovenije, Društvo študentov biologije, Biotehniški izobraževalni center Ljubljana, Zavod Parnas, Triglavski narodni park, revijo PIL, National Geographic Slovenia, National Geographic Junior. Čeprav je svetovni dogodek organiziran vsako drugo leto, smo se v Sloveniji odločili, da DOR praznujemo vsako leto, tako da ima zdaj že zelo lepo tradicijo. Življenje rastlin obiskovalcem predstavljamo na priložnostnih stojnicah, pa tudi vodenih ogledih, bralnih uricah ter fotografskih, likovnih in literarnih natečajih (Dermastia 2012, 2015, 2016, Dolenc Koce in Pompe Novak 2017).

…in za konec

Glede na viharno zgodovino društva se na prvi pogled zdi, da se, poleg simpozijev in DOR, v našem SDBR ne dogaja veliko. V divjem času v katerem živimo in v katerem imamo vse manj časa zase, se tudi zdi, da številni člani, ki so v društvu od začetka, več časa namenjajo delovanju v drugih društvih (na primer v Slovenskem biokemijskem društvu, Slovenskem genetskem društvu, Slovenskem mikrobiološkem društvu, Društvu za varstvo rastlin), ki so bolj tesno povezana z njihovim trenutnim raziskovalnim delom. Prav zaradi tega je prav, da se ob tem visokem jubileju spomnimo, od kod SDBR izhaja, kakšen je bil njegov vpliv na razvoj rastlinske znanosti v Sloveniji in da je ta še vedno znaten.

Še na mnoga uspešna leta, Slovensko društvo za biologijo rastlin.

Zahvala

Zahvaljujem se številnim članom društva, ki so me oskrbeli z informacijami iz polpretekle zgodovine našega društva. Zahvaljujem se tudi Tomažu Sajovcu za lektoriranje prispevka.

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INSTRUCTIONS FOR AUTHORS

1. Types of Articles

SCIENTIFIC ARTICLES are comprehensive descriptions of original research and include a theoretical survey of the topic, a detailed presentation of results with discussion and conclusion, and a bibliography according to the IMRAD outline (Introduction, Methods, Results, and Discussion). In this category ABS also publishes methodological articles, in so far as they present an original method, which was not previously published elsewhere, or they present a new and original usage of an established method. The originality is judged by the editorial board if necessary after a consultation with the referees. The recommended length of an article including tables, graphs, and illustrations is up to fifteen (15) pages; lines must be double-spaced. Scientific articles shall be subject to peer review by two experts in the field.

REVIEW ARTICLES will be published in the journal after consultation between the editorial board and the author. Review articles may be longer than fifteen (15) pages.

BRIEF NOTES are original articles from various biological fields (systematics, biochemistry, genetics, physiology, microbiology, ecology, etc.) that do not include a detailed theoretical discussion. Their aim is to acquaint readers with preliminary or partial results of research. They should not be longer than five (5) pages. Brief note articles shall be subject to peer review by one expert in the field.

CONGRESS NEWS acquaints readers with the content and conclusions of important congresses and seminars at home and abroad.

ASSOCIATION NEWS reports on the work of Slovene biology associations.

2. Originality of Articles

Manuscripts submitted for publication in *Acta Biologica Slovenica* should not contain previously published material and should not be under consideration for publication elsewhere.

3. Language

Articles and notes should be submitted in English, or as an exception in Slovene if the topic is very local. As a rule, congress and association news will appear in Slovene.

4. Titles of Articles

Title must be short, informative, and understandable. It must be written in English and in Slovene language. The title should be followed by the name and full address of the authors (and if possible, fax number and/or e-mail address). The affiliation and address of each author should be clearly marked as well as who is the corresponding author.

5. Abstract

The abstract must give concise information about the objective, the methods used, the results obtained, and the conclusions. The suitable length for scientific articles is up to 250 words, and for brief note articles, 100 words. Article must have an abstract in both English and Slovene.

6. Keywords

There should be no more than ten (10) keywords; they must reflect the field of research covered in the article. Authors must add keywords in English to articles written in Slovene.

7. Running title

This is a shorter version of the title that should contain no more than 60 characters with spaces.

8. Introduction
The introduction must refer only to topics presented in the article or brief note.

9. Illustrations and Tables
Articles should not contain more than ten (10) illustrations (graphs, dendrograms, pictures, photos etc.) and tables, and their positions in the article should be clearly indicated. All illustrative material should be provided in electronic form. Tables should be submitted on separate pages (only horizontal lines should be used in tables). Titles of tables and illustrations and their legends should be in both Slovene and English. Tables and illustrations should be cited shortly in the text (Tab. 1 or Tabs. 1-2, Fig. 1 or Figs. 1-2; Tab. 1 and Sl. 1). A full name is used in the legend title (e.g. Figure 1, Table 2 etc.), written bold, followed by a short title of the figure or table, also in bold. Subpanels of a figure have to be unambiguously indicated with capital letters (A, B, …). Explanations associated with subpanels are given alphabetically, each starting with bold capital letter (**A**), a hyphen and followed by the text.

10. The quality of graphic material
All the figures have to be submitted in the electronic form. The ABS publishes figures either in pure black and white or in halftones. Authors are kindly asked to prepare their figures in the correct form to avoid unnecessary delays in preparation for print, especially due to problems with insufficient contrast and resolution. Clarity and resolution of the information presented in graphical form is the responsibility of the author. Editors reserve the right to reject unclear and poorly readable pictures and graphical depictions. The resolution should be 300 d.p.i. minimum for halftones and 600 d.p.i. for pure black and white. The smallest numbers and lettering on the figure should not be smaller than 8 points (2 mm height). The thickness of lines should not be smaller than 0.5 points. The permitted font families are Times, Times New Roman, Helvetica and Arial, whereby all figures in the same article should have the same font type. The figures should be prepared in TIFF, EPS or PDF format, whereby TIFF (ending *.tif) is the preferred type. When saving figures in TIFF format we recommend the use of LZW or ZIP compression in order to reduce the file sizes. The photographs can be submitted in JPEG format (ending *.jpg) with low compression ratio. Editors reserve the right to reject the photos of poor quality. Before submitting a figure in EPS format make sure first, that all the characters are rendered correctly (e.g. by opening the file first in the programs Ghostview or GSview – depending on the operation system or in Adobe Photoshop). With PDF format make sure that lossless compression (LZW or ZIP) was used in the creation of the *.pdf file (JPEG, the default setting, is not suitable). Figures created in Microsoft Word, Excel, PowerPoint etc. will not be accepted without the conversion into one of the before mentioned formats. The same goes for graphics from other graphical programs (CorelDraw, Adobe Illustrator, etc.). The figures should be prepared in final size, published in the magazine. The dimensions are 12.5 cm maximum width and 19 cm maximum height (width and height of the text on a page).

11. Conclusions
Articles shall end with a summary of the main findings which may be written in point form.

12. Summary
Articles written in Slovene must contain a more extensive English summary. The reverse also applies.

13. Literature
References shall be cited in the text. If a reference work by one author is cited, we write Allan (1995) or (Allan 1995); if a work by two authors is cited, (Trinajstić and Franjić 1994); if a work by three or more authors is cited, (Pullin et al. 1995); and if the reference appears in several works, (Honsig-Erlenburg et al. 1992, Ward 1994a, Allan 1995, Pullin et al. 1995). If several works by the same author published in the same year are cited, the individual works are indicated with the added letters a, b, c,

etc.: (Ward 1994a,b). If direct quotations are used, the page numbers should be included: Toman (1992: 5) or (Toman 1992: 5–6).The bibliography shall be arranged in alphabetical order beginning with the surname of the first author, comma, the initials of the name(s) and continued in the same way with the rest of the authors, separated by commas. The names are followed by the year of publication, the title of the article, the full name of the journal (periodical), the volume, the number in parenthesis (optional), and the pages. Example:

Mielke, M.S., Almeida, A.A.F., Gomes, F.P., Aguilar, M.A.G., Mangabeira, P.A.O., 2003. Leaf gas exchange, chlorophyll fluorescence and growth responses of Genipa americana seedlings to soil flooding. Experimental Botany, 50(1), 221–231.

Books, chapters from books, reports, and congress anthologies use the following forms:

Allan, J.D., 1995. Stream Ecology. Structure and Function of Running Waters, 1st ed. Chapman & Hall, London, 388 pp.

Pullin, A.S., McLean, I.F.G., Webb, M.R., 1995. Ecology and Conservation of *Lycaena dispar*: British and European Perspectives. In: Pullin A. S. (ed.): Ecology and Conservation of Butterflies, 1st ed. Chapman & Hall, London, pp. 150-164.

Toman, M.J., 1992. Mikrobiološke značilnosti bioloških čistilnih naprav. Zbornik referatov s posvetovanja DZVS, Gozd Martuljek, pp. 1-7.

14. Format and Form of Articles

The manuscripts should be sent exclusively in electronic form. The format should be Microsoft Word (*.doc) or Rich text format (*.rtf) using Times New Roman 12 font with double spacing, align left only and margins of 3 cm on all sides on A4 pages. Paragraphs should be separated by an empty line. The title and chapters should be written bold in font size 14, also Times New Roman. Possible sub-chapter titles should be written in italic. All scientific names must be properly italicized. Used nomenclature source should be cited in the Methods section. The text and graphic material should be sent to the editor-in-chief as an e-mail attachment. For the purpose of review the main *.doc or *.rtf file should contain figures and tables included (each on its own page). However, when submitting the manuscript the figures also have to be sent as separate attached files in the form described under paragraph 10. All the pages (including tables and figures) have to be numbered. All articles must be proofread for professional and language errors before submission.

A manuscript element checklist (For a manuscript in Slovene language the same checklist is appropriately applied with a mirroring sequence of Slovene and English parts):
English title – (Times New Roman 14, bold)
Slovene title – (Times New Roman 14, bold)

Names of authors with clearly indicated addresses, affiliations and the name of the corresponding author – (Times New Roman 12)
Author(s) address(es) / institutional addresses – (Times New Roman 12)
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.

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REVIEW PAPER - PREGLEDNI ČLANEK :

ČLANKI – ARTICLES:

OBLETNICA - ANNIVERSARY: