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Katedra agroenvironmentální chemie a výživy rostlin

Vliv kadmia na oxidační stres u rostlin

doktorská disertační práce

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Prohlášení:

Prohlašuji, že jsem doktorskou disertační práci na téma „Vliv kadmia na oxidační stres u rostlin“ vypracovala samostatně a použila jen pramenů, které cituji a uvádím v přiloženém seznamu literatury.

V Praze dne 16.7.2015 ……

Podpis

Poděkování:

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Obsah

1 ÚVOD

V životním prostředí se vyskytuje řada látek, které mají negativní vliv na živé organismy. Jednou ze skupin těchto látek jsou rizikové prvky, mezi něž řadíme většinu kovů a polokovů. Kadmium je jedním z prvků s největšími riziky, který se do životního prostředí dostává zejména antropogenní činností.

Kontaminace půdy rizikovými prvky je závažným problémem mnoha zemí, včetně některých oblastí České republiky. Pro odstranění rizikových prvků z půdy je odzkoušena řada remediačních metod. V současnosti s ohledem na stav životního prostředí je využívána skupina tzv. fytoremediačních metod, které využívají pro odstranění nebo transformaci kontaminantů zelených rostlin a s nimi asociovaných mikroorganismů.

Skupina rostlin, která se pro tyto účely nejčastěji využívá, je označována jako hyperakumulátory, které akumulují ve vysokých koncentracích rizikové prvky do kořenů a nadzemní biomasy bez projevů fytotoxicity. Nejvíce druhů s touto schopností se vyskytuje v čeledi *Brassicaceae*. Zástupci této čeledi, zejména *Noccaea caerulescens* a *Arabidopsis halleri*, se využívají jako modelové rostliny při výzkumu aspektů schopnosti hyperakumulace.

Proto, aby se mohla s maximální možnou mírou využít schopnost hyperakumulace, je zapotřebí znát způsob příjmu, vazby a mechanismy kumulace rizikových prvků v rostlině a také metabolické procesy, které probíhají po příjmu rizikových prvků rostlinou. Tyto metabolické procesy jsou stresovou reakcí rostlin na přítomnost rizikového prvku. Pro zvýšení ochrany proti rizikovým prvkům - kovům vyvinuly rostlinné buňky mechanismy, pomocí nichž jsou ionty kovů, které se dostanou do cytosolu, ihned komplexovány a inaktivovány. Sloučeniny, které se účastní komplexace kovů, zahrnují organické kyseliny, volné aminokyseliny, glutathion, fytochelatiny, metalothioneiny, metalochaperony a proteiny teplotního šoku. Přes rozsáhlý výzkum těchto metabolických procesů a schopnosti tolerance u hyperakumulátorů, nejsou dodnes známy všechny mechanismy a aspekty těchto reakcí.

2 LITERÁRNÍ PŘEHLED

2.1 Kadmium

Kadmium (Cd) se v životním prostředí vyskytuje s valencí Cd^{2+} (Traina, 1999). Kadmium je neesenciálním rizikovým prvkem, který má negativní vliv na živé organismy, především svou schopností bioakumulace do potravinového řetězce (Sanità di Toppi a Gabbrielli, 1999; Kabata-Pendias a Mukherjee, 2007; Lovy et al., 2013), do kterého dle Avezeda et al. (2012) Cd vstupuje v důsledku jeho vysoké mobility v systému rostlina-půda.

Pro svou vysokou toxicitu a dobrou rozpustnost jeho sloučenin ve vodě je Cd velmi významným polutantem prostředí, kde se chová jako kumulativní jed s doprovodnými karcinogenními a teratogenními účinky (Pinto et al., 2004; Deckert, 2005). Anjum et al. (2014) uvádějí, že bylo Cd zařazeno jako sedmý toxin z top dvaceti. Kadmium bylo Mezinárodní agenturou pro výzkum rakoviny klasifikováno jako lidský karcinogen (Deckert, 2005). V lidském těle se jeho poločas rozpadu odhaduje na 15 až 20 let, jedná se tedy o chronickou toxicitu Cd (Meyer a Verbruggen, 2012).

V životním prostředí je Cd přítomno v půdě, vodě a atmosféře (Gallego et al., 2012; Mihaličová Malčová et al., 2014), kde je jeho obsah ovlivněn zejména antropogenní činností. Touto cestou se do atmosféry dostává 3 - 10 krát více Cd než z přírodních zdrojů - vulkanickou činností, lesními požáry a prachovými částicemi (Meyer a Verbruggen, 2012). Kadmium je hlavním průmyslovým polutantem, zejména v oblastech souvisejících s tavením Zn a nadměrnou silniční dopravou, kde kontaminuje prostředí atmosférickou depozicí (Clemens 2006; Hasan et al., 2009). Zvýšený obsah Cd v půdách byl způsoben také hnojením čistírenskými kaly a fosforečnými hnojivy s vyšším obsahem Cd (Kirkham, 2006; DalCorso et al., 2008; Lovy et al., 2013). Podle Allowaye (1995) přetrvává Cd v půdě více jak 1 000 let.

2.1.1 Kadmium v půdě

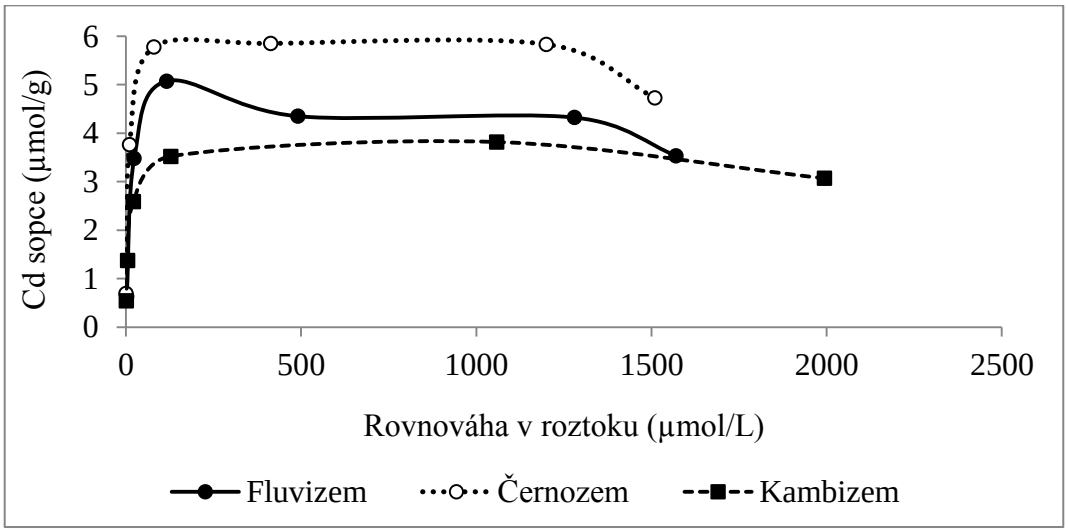
V přirozeném prostředí se Cd v půdě nevyskytuje izolovaně, ale jako komponent Pb:Zn minerálů (Adriano, 2001) nebo je sorbováno na půdní koloidy, dále v organických sloučeninách a jako součást půdního roztoku (Alloway, 1999; Kabata-Pendias a Mukherjee, 2007). V oblastech s nízkým antropogenním vlivem může docházet k jeho uvolňování během mineralizačního procesu (Sanità di Toppi a Gabbrielli, 1999). Za nekontaminovanou půdu je považována taková, která obsahuje méně než 0,5 mg Cd/kg. Obsah Cd však může dosahovat vyšších hodnot v závislosti na typu mateční horniny (Zadeh et al., 2008). Magmatické a metamorfované horniny mají nižší obsah Cd v rozmezí 0,02 až 0,2 mg/kg. Naopak

sedimenty dosahují vyšších obsahů Cd, hodnoty se pohybují od 0,1 mg/kg do 25 mg/kg (Cook a Morrow, 1995). Obecně je Cd v půdě a sedimentech přítomno v koncentracích, které jsou menší než 1 mg/kg (Das et al., 1997; Kabata-Pendias a Pendias, 2001; Clemens 2006). Tato hodnota je i maximální přípustné množství Cd v půdách spadajících do zemědělského půdního fondu ČR (Vyhláška MŽP 13/1994).

Kadmium nepodléhá mikrobiální nebo chemické degradaci a přetrvává v půdě dlouhou dobu (Bolan et al., 2003). Může se v půdě vázat na organickou hmotu, jílové minerály a hydroxidy Fe, Mn a Al (Das et al., 1997). V půdním profilu se obvykle nachází v oblasti s nejvyšším obsahem organických látek (Alloway, 1999).

Mobilita a dostupnost Cd pro rostliny v půdě závisí na mnoha půdních a rostlinných faktorech. Sorpce, respektive adsorpce a desorpce Cd v půdě je hlavním faktorem, který ovlivňuje mobilitu Cd v půdě a jeho osud v prostředí (Fontes a dos Santos, 2010). Sorpce kovů je konkurenční proces mezi ionty daného kovu v roztoku a ionty vázanými na povrchu půdy (Echeverría et al., 1998). Na sorpční kapacitu půdy má vliv řada půdních vlastností: hodnota pH, kationtová výměnná kapacita (KVK), obsah uhličitanů a fosfátů, půdní organická hmota, obsah křemičitanů, oxidy a hydroxidy. U minerální složky půd je to také obsah jílových částic a (hydr)oxidů Fe a Al (Vidal et al., 2009). Fontes a dos Santos (2010) uvádějí jako hlavní faktory mající vliv na sorpci Cd v půdě tyto dva parametry - hodnotu pH a KVK. Vliv na sorpci Cd v půdě má také přítomnost dalších kovů, jako je Cu, Cr, Pb a Zn. Markiewicz-Patkowska et al. (2005) zjistili v modelovém pokusu, že adsorpce kovů je významnější z jednoprvkového roztoku než z víceprvkového roztoku, kde dochází ke konkurenci přítomných kovů. Tyto výsledky potvrdili Zemanová et al. (2014b) v sorpčním modelovém pokusu s Cd, Cu, Pb a Zn (obr. 1).

Obr. 1 Sorpční isotermy Cd ve víceprvkovém roztoku (Zemanová et al., 2014b).



Pouze část celkového obsahu Cd v půdě je dostupná pro rostliny (Clemens, 2006). Biologická dostupnost Cd pro rostliny je ovlivněna rozsahem vazeb s organickými a anorganickými ligandy v půdním roztoku. Silně vázané kovy jsou méně toxické pro organismy, než slaběji vázané formy a nejvíce toxické jsou tedy volné ionty (Adriano, 2001). Kadmium je pro svou větší mobilitu v půdě oproti jiným těžkým kovům více dostupné rostlinám (Das et al., 1997; Zhao a Masaihiko, 2007). Nejvýznamnější vliv na dostupnost Cd mají z půdních faktorů hodnota pH a obsah organické hmoty (Clemens, 2006). Výsledky pokusů prokázaly lineární trend mezi hodnotou pH půdy a příjmem Cd. Snížením hodnoty pH dochází ke zvýšení obsahu Cd v rostlinách v důsledku jeho zvýšené rozpustnosti a mobility (Kirkham, 2006). Vliv hodnoty pH a obsahu organické hmoty ve své práci prokázali Benavides et al. (2005), kteří zjistili, že příjem Cd u kukuřice byl nižší na kyselých půdách s vysokým obsahem organické hmoty. Vliv přítomnosti dalších prvků na absorpci Cd potvrdili Cosio et al. (2004), kteří ve své práci přídavkem Ca snížili příjem Cd rostlinou. Antagonistický vztah Zn a Cd zjistili u salátu Costa a Morel (1994).

Příjem Cd a jeho biologickou dostupnost pro rostliny ovlivňuje významný faktor - rhizosféra (oblast v bezprostřední blízkosti kořenů) (Benavides et al., 2005). V rhizosféře dochází k chelataci kovů karboxylovými kyselinami vylučovanými rostlinou, zvyšuje se difusní gradient a urychluje příjem kovů (Fuksová et al., 2007; Maestri et al., 2010). Dle Lina a Aartse (2012) mohou přítomné exudáty v rhizosféře také inhibovat příjem kovů kořeny rostlin a takto ovlivňovat toleranci rostlin a akumulaci kovů v rostlině. Například odrůda ječmene „Sahara“ akumuluje více Zn než odrůda „Clipper“ v důsledku vyšší kořenové exsudace organických kyselin a aminokyselin (Rasouli-Sadaghiani et al., 2011). Lux et al. (2011) uvádějí inhibici příjmu a akumulace Cd kořeny v rhizosféře vlivem rozpuštěných iontů La^{3+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+} a Mn^{2+}.

V rhizosféře mají dále vliv přítomné mikroorganismy, které mohou ovlivňovat příjem Cd změnou jeho rozpustnosti, mobility, specifity a změnou hodnoty pH v důsledku srážení (Lin a Aarts, 2012). Významný vliv má zejména přítomnost arbuskulárních mykorhizních (AM) hub, které jsou symbionty široké řady cévnatých rostlin (Shahabivand et al., 2012). Göhre a Paszkowski (2006) prokázali u rostlin rostoucích na kontaminovaných půdách vliv AM hub na zmírnění stresu působeného přítomnými kovy. Asociace mykorhizy a hostitelských rostlin rostoucích v prostředí kontaminovaném Cd vede ke zlepšení nutričního stavu a snížení nebo změně příjmu Cd (Andrade et al., 2008). Dle Janouškové a Pavlíkové (2010) souvisí nižší toxicita Cd v mykorhizosféře *Nicotiana tabacum* (L.) s přítomností mimokořenového mycelia a alkalizací substrátu vyvolanou jeho přítomností. Zvýšenou

toleranci rostlin ke Cd a Zn v důsledku očkování mykorhizními houbami a hnojením P uvádění Shen et al. (2006). Přes řadu studií však nejsou dosud mechanismy působení AM hub na zmírnění stresu u hostitelských rostlin zcela objasněny. Výsledky pokusů jsou variabilní a závisí na konkrétní rostlině, houbě a kovu (Shahabivand et al., 2012).

2.1.2 Kadmium v rostlinách

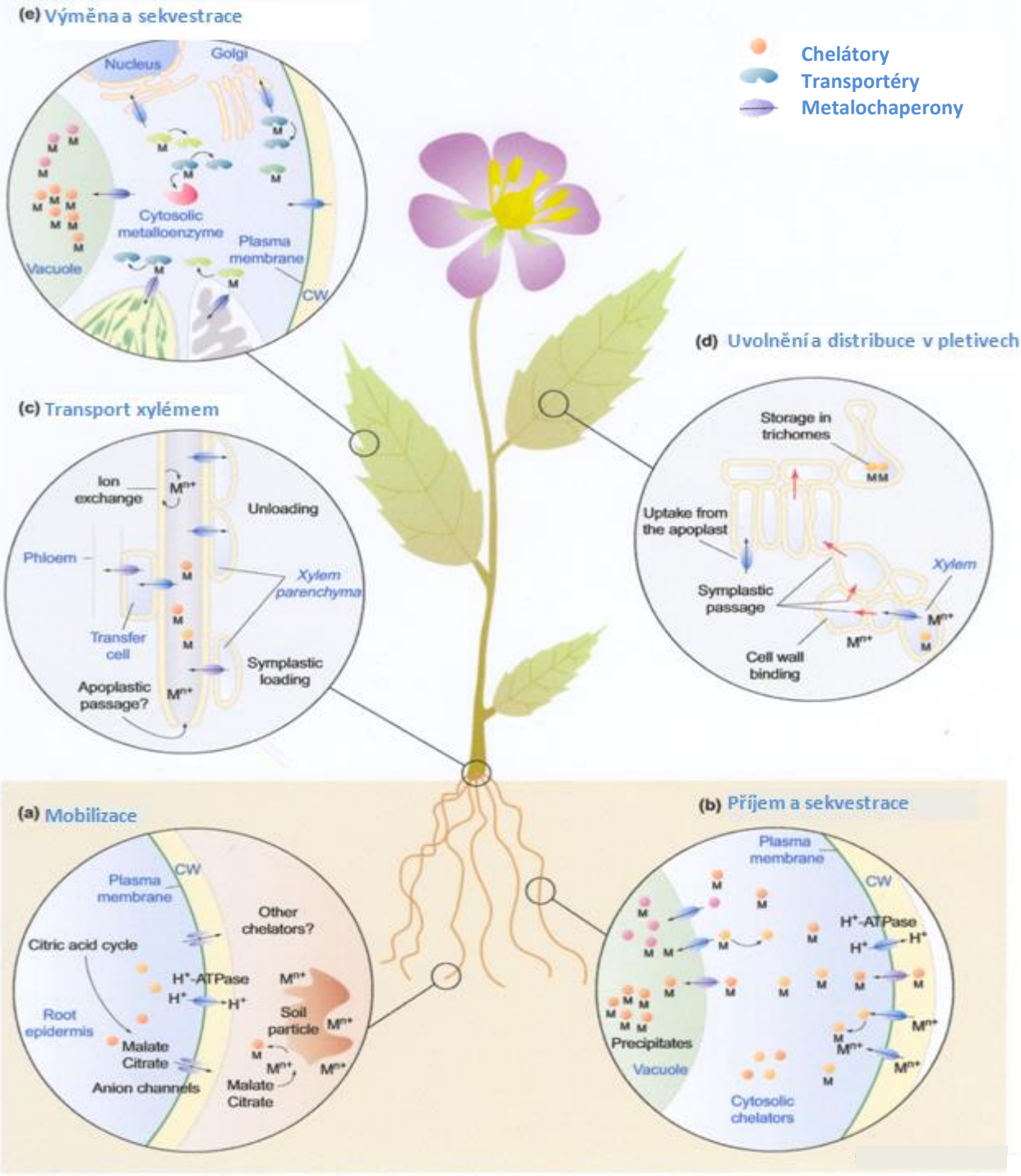
2.1.2.1 Příjem Cd rostlinou

Kadmium je rostlinami přijímáno převážně kořenem - v závislosti na dostupnosti a jeho koncentraci v půdě nebo ve vodě (DalCorso et al., 2008), v menší míře povrchem listů - kutikulou listů z atmosféry (Hovmand et al., 1983). Příjem Cd z půdy rostlinou probíhá pasivně difúzí (Das et al., 1997), pravděpodobně pomocí Fe^{2+}, Ca^{2+} a Zn^{2+} transportérů/kanálů s nízkou specifitou (Clemens, 2006). Kadmium vstupující do rostlin je v konkurenčním vztahu o transmembránové nosiče s ostatními prvky, jako jsou K, Mg, Cu, Ni a Zn (Benavides et al., 2005).

Kadmium snadno vstupuje do kořenů přes kortikální pletiva (DalCorso et al., 2008), kde je jeho absorpce řízena rozdílným elektrochemickým potenciálem mezi aktivitou Cd^{2+} iontů v cytosolu a v apoplastu kořenů (Hasan et al., 2009). U ovsa (*Avena sativa* L.) byl v kořenech prokázán přenos Cd z cytosolu do vakuoly přes tonoplast pomocí Cd^{2+}/H^{+} antiportu (Salt a Wagner, 1993). Do xylému se Cd dostává apoplastickou a/nebo symplastickou cestou (Salt et al., 1995) v komplexu s organickými kyselinami nebo fytochelatiny (Li et al., 2011). Význam při tomto transportu mají i další S, N a O ligandy, např. aminokyseliny a metalothioneny (Hasan et al., 2009). Cataldo et al. (1983) zjistili v xylému sóji komplexaci Cd^{2+} především s komponenty aminokyselin/peptidových frakcí. Clemens et al. (2013) označují translokaci přes xylém jako klíčový faktor variability akumulace Cd v obilninách. Přijaté Cd je přeneseno do prýtu rostliny pomocí cévních svazků, které regulují také jeho pohyb rostlinou (Kuppelwieser a Feller, 1991). Následně je Cd uloženo/akumulováno do rostlinných pletiv a buněčných kompartmentů (Prasad, 1995).

Tyto molekulární mechanismy zprostředkovávající přechod Cd a dalších kovů do rostlin popsali ve své práci Clemens et al. (2002) (obr. 2).

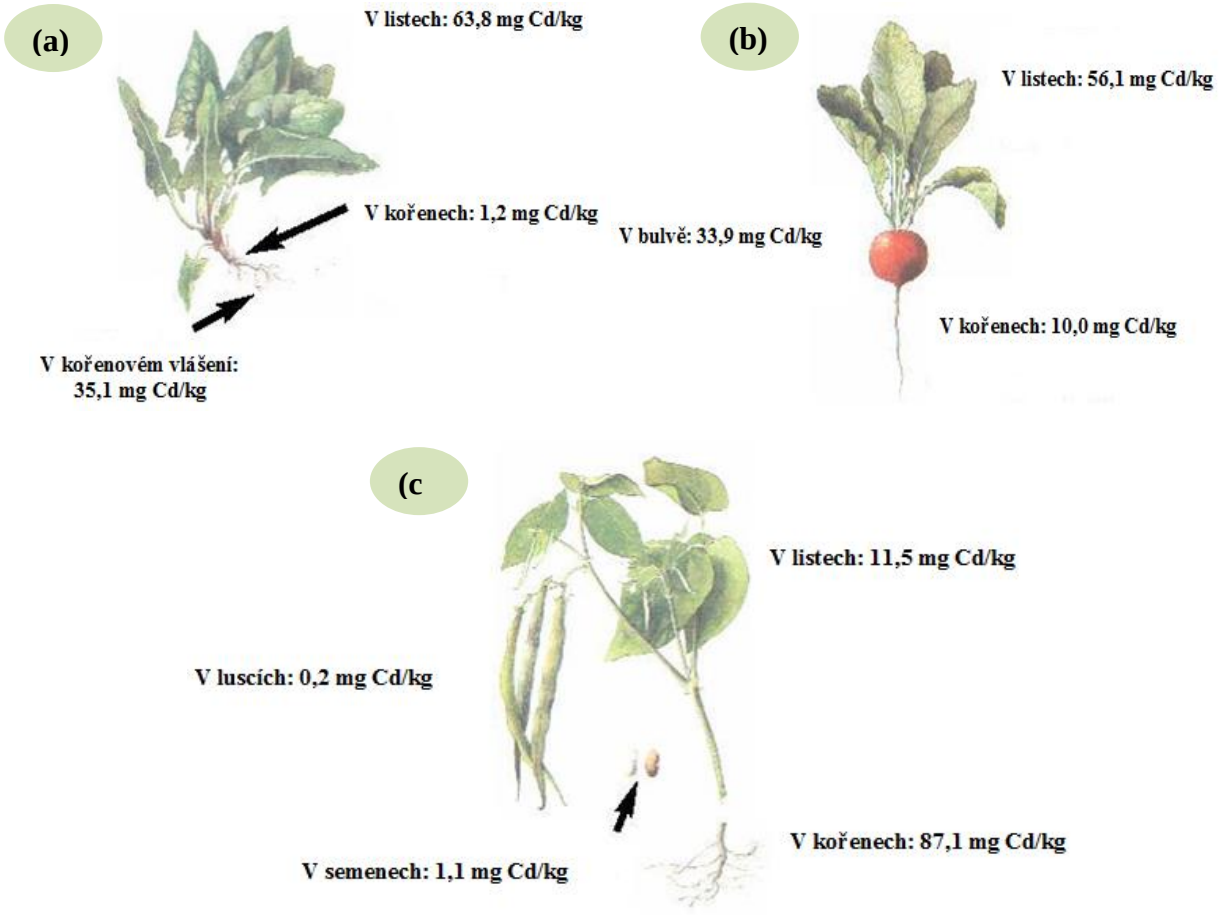
Obr. 2 Přechod kovů z půdy do rostliny (upraveno dle Clemens et al., 2002).



(a) mobilizace kovu v půdě pomocí chelátorů a okyselení rhizosféry; (b) příjem hydratovaných iontů kovu nebo komplexů kov-chelát regulovaných systémy v plasmatické membráně, uvnitř buňky jsou kovy chelátovány a přebytečné množství je uloženo do vakuoly; (c) transport kovů z kořenů do prýtu probíhá xylémem; (d) distribuce v apoplastu listových buněk, transport přes plasmodesmu; (e) intracelulární distribuce kovů specifickými transportéry a metalochaperony. (CW=buněčná stěna; M=kov).

Obsah Cd v rostlinných pletivech je v přímé závislosti na koncentraci Cd dostupného v životním prostředí, délce expozice (Cibulka, 1991) a stáří rostliny (Pál et al., 2006). Kadmium má větší tendenci hromadit se v pletivech nadzemní části rostliny než kořenech (Clemens, 2006). Řada autorů (Cibulka, 1991; Benavides et al., 2005; Hasan et al., 2009) však uvádí, že nejvíce Cd se v rostlině nachází v pletivech kořenů, dále listech, stoncích, plodech a zásobních orgánech, nejnižší obsah mají semena. Vaněk et al. (2012) potvrdili tyto výsledky u fazole. Tito autoři dále uvádějí opačný jev v obsahu Cd u špenátu a ředkvičky (obr. 3).

Obr. 3 Obsah Cd ve (a) špenátu, (b) ředkvičce a (c) fazoli (upraveno dle Vaněk et al., 2012).



Vyšší přítomnost Cd v kořenech rostlin než v jiných orgánech byla pozorována u sóji, fazole, vojtěšky, kukuřice a rajčat (Alloway, 1999). Obdobné výsledky potvrdili Tlustoš et al. (2007) ve svých nádobových pokusech s rychle rostoucími dřevinami rodu *Salix* sp.. Obsah Cd v různých částech rostlin se lišil v závislosti na úrovni kontaminace dané půdy. Rostliny pěstované na nekontaminované (obsah Cd byl 0,42 mg/kg) a mírně kontaminované půdě (obsah Cd byl 4,73 mg/kg) prokázaly vyšší akumulaci Cd v listech oproti kořenům. Opačný efekt v akumulaci Cd pozorovali výše zmínění autoři při pěstování rostlin na silně

kontaminované půdě (obsah Cd byl 30,5 mg/kg). Z těchto výsledků je zřejmé, že obsah Cd v různých částech rostlin je variabilní dle druhu rostliny.

Koncentrace Cd v nekontaminovaných rostlinách se pohybuje v rozmezí 0,05 až 2 mg/kg (Zadeh et al., 2008). Zvýšené koncentrace Cd přijatého rostlinou představují pro rostlinu stres. Pro citlivé rostliny jsou toxické hodnoty 5-10 mg/kg (White a Brown, 2010), méně citlivým nevadí ani hodnoty nad 150 mg/kg Cd (Kabata-Pendias a Pendias, 2001). Dle Hasana et al. (2009) však již relativně nízké koncentrace Cd mění metabolismus rostlin.

2.1.2.2 Toxicita Cd

Ačkoli toxické účinky Cd na biologické systémy byly popsány řadou autorů, např. Das et al. (1997); Sanità di Toppi a Gabrielli (1999); Deckert (2005); Kabata-Pendias a Mukherjee (2007); Hasan et al. (2009), mechanismy toxicity Cd nejsou dosud zcela objasněny. Dle Schützendübele a Polla (2002) může toxicita Cd vyplývat z jeho vysoké afinity pro thiolové skupiny. Vazba Cd na thiolové skupiny strukturních bílkovin a enzymů vede k inhibici aktivity a/ nebo interferenci redox-enzymové regulace (Hall, 2002). Významná skupina enzymů, které jsou dle Prasada (1995) inhibovány Cd souvisí s asimilací uhlíku v rostlinách, jedná se například o enzym Rubisco. Vzhledem ke svým chemickým vlastnostem Cd snadno reaguje s celou řadou biologicky aktivních molekul, včetně proteinů, fosfolipidů, purinů, porfyrů, nukleových kyselin a enzymů (Deckert, 2005). Kadmium může snadno v některých enzymech nahrazovat Zn. Tato záměna kovů způsobí sníženou nebo zvýšenou aktivitu takového enzymu. Všechny tyto změny a jiné vlastnosti biologicky aktivních molekul obsahujících Cd jsou důležitou podstatou nebezpečnosti Cd pro živé systémy (Cibulka, 1991).

U vyšších rostlin Cd vyvolává zakrnělost až úhyn rostliny vlivem jeho negativního působení na růst a vývoj (DalCorso et al., 2008). Cd inhibuje proces klíčení a vývoj klíčních rostlin (Pál et al., 2006). Chaffei et al. (2004) zjistili peroxisomovou senescenci v listech vlivem Cd. Při expozici Cd dochází také k inhibici růstu a tvorbě bočních kořenů (DalCorso et al., 2008). Cytotoxické působení Cd u různých rostlinných druhů pozorovali Benavides et al. (2005), kteří prokázali chromozomální aberace a inhibici mitotických procesů vedoucích ke změně buněčného cyklu a dělení.

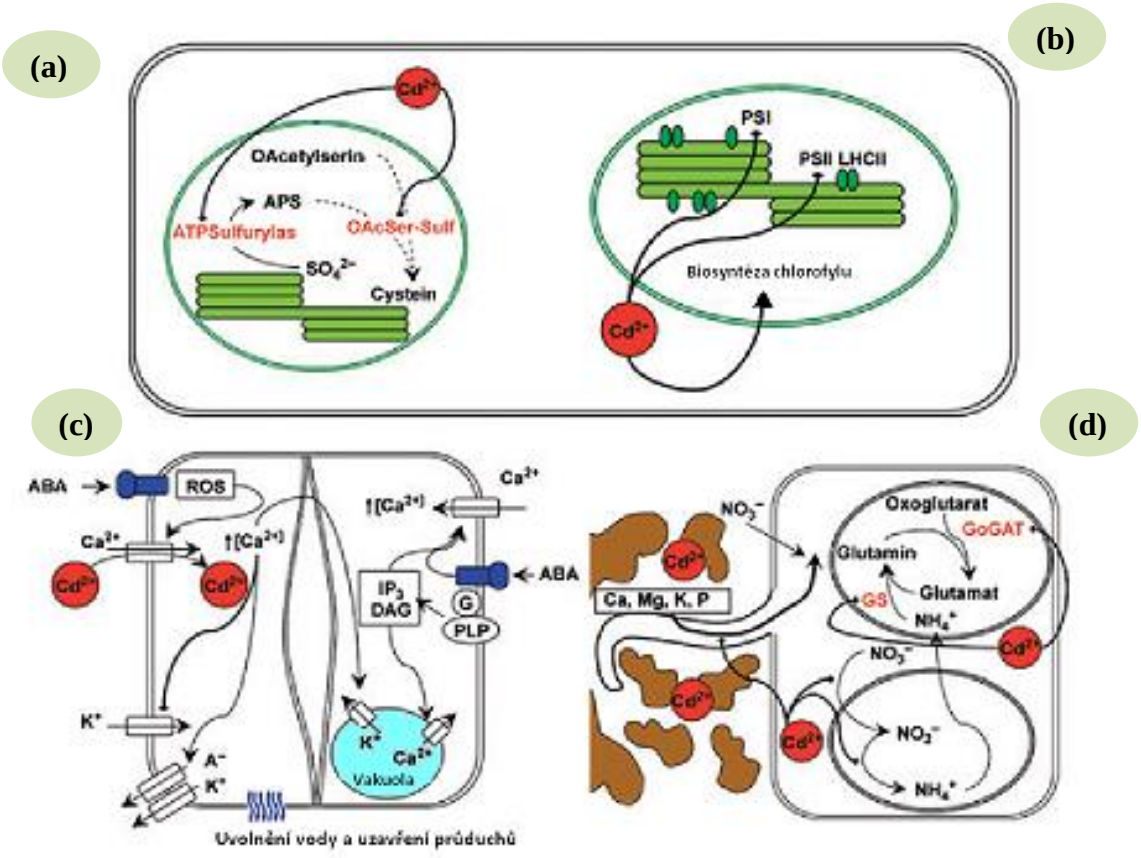
V půdě Cd ovlivňuje dostupnost a příjem minerálních látek pro rostliny a také ovlivňuje populace půdních mikroorganismů (Moreno et al., 1999). Tento kov snižuje vodní potenciál buňky (Barceló a Poschenrieder, 1990), inhibuje příjem a akumulaci esenciálních

minerálních látek (Kummerová a Brandejsová, 1994), snižuje vodivost průduchů (Barceló a Poschenrieder, 1990) a významně poškozuje fotosyntetický aparát rostlin inhibicí aktivity obou fotosystémů (Siedlecka a Krupa, 1996; Khan et al., 2006). Dle Padmaje et al. (1990) je negativní vliv Cd na fotosyntézu způsoben inhibicí biosyntézy chlorofylu. Inhibici fotosyntézy vlivem krátkodobého i dlouhodobého působení Cd prokázali Küpper et al. (2007) u *Thlaspi caerulescens* a Popova et al. (2008) u kukuřice, hrachu a ječmene. Parmar et al. (2013) zjistili, že Cd může způsobit destrukci chlorofylu v důsledku substituce Mg v chlorofylu a i b. Biosyntéza chlorofylu může být ovlivněna Cd v důsledku inhibice aminolevulátu (Mysliwa-Kurdziel a Strzalka, 2002). Naopak při zvýšené akumulaci aminolevulátu vlivem Cd dochází k tvorbě reaktivních forem kyslíku a ovlivnění redoxního stavu a homeostázy rostlin (Noriega et al., 2007; Goncalves et al., 2009). Kadmium snižuje absorpci dusičnanů a jejich transport z kořenů do nadzemních částí tím, že omezuje aktivitu nitrátreduktázy ve výhoncích rostlin (Hernández et al., 1996). Dle Chaffeiho et al. (2004) dochází také ke snížení aktivity nitritreduktázy. Podle těchto autorů Cd negativně ovlivňuje aktivitu glutaminu a glutamátsyntetázy a zároveň zvyšuje aktivitu glutamátdehydrogenázy (obr. 4). Vlivem Cd dochází ke změnám permeability buněčných membrán, kdy se přítomností Cd v rostlině zvyšuje osmotický potenciál, a tím se zvyšuje propustnost membrán pro ionty a vodu. Obecně lze říci, že Cd narušuje příjem, pohyb, využití živin (Ca, Mg, P a K) a vody rostlinami (Das et al., 1997; Kirkham, 2006). Pro růst rostlin s agronomickým využitím je zejména významný antagonistický vztah Cd a P. Příjem P rostlinami v přítomnosti Cd byl redukován více jak o 40 % oproti kontrolní variantě (Kabata-Pendias a Pendias, 2001). Metwally et al. (2005) zjistili významnou inhibici příjmu P, K, S, Ca, Zn, Mn a B v rostlinách hrachu po expozici Cd. Snížený příjem Ca a K potvrdili také Nedjimi a Daoud (2009) u hyperakumulátoru Cd *Atriplex halimus* subsp. *schweinfurthii*.

Dle Asgher et al. (2015) je signalizace při stresu vlivem Cd těsně vázána na obsah endogenních a exogenních regulátorů růstu rostlin. Tito autoři uvádějí jako hlavní skupinu regulátorů růstu rostlinné hormony (např. auxiny, cytokininy, kyselinu abscisovou atd.), které mají významnou roli ve vývoji rostlin. Hsu a Kao (2003) prokázali ochranný efekt kyseliny abscisové (ABA) proti toxicitě Cd u sazenic rýže. Exogenní aplikace ABA redukovala rychlost transpirace, snížila obsah Cd a zvýšila toleranci rostlin vůči Cd. Dle Ghorbanli et al. (2000) omezují negativní vliv Cd také fytohormony gibereliny. Tito autoři pozorovali vliv giberelinů u rostlin sóji, u kterých přídavek giberelinů způsobil částečnou eliminaci vlivu Cd na kořeny a nadzemní biomasu a zvětšil listovou plochu a délku stonku. Hu et al. (2013) zjistili narušení homeostázy auxinů v semenech rostlin *Arabidopsis* vlivem Cd. Cd snížilo

obsah kyseliny indoloctové (IAA) a zvýšilo aktivitu IAA oxidáz. Piotrowska-Niczyporuk et al. (2012) uvádějí obnovující vliv cytokininů (CK) na fotosyntézu při stresu vlivem Cd. Vlivem CK byla zvýšena kapacita fotosyntézy a obsah primárních metabolitů. Stěžejní signální molekulou v metabolismu rostlin při stresu vlivem Cd je ethylén (Asgher et al. (2015). Nicméně o jeho mechanismech, které se podílejí na zvyšení tolerance rostlin vůči Cd, je známo dosud velmi málo.

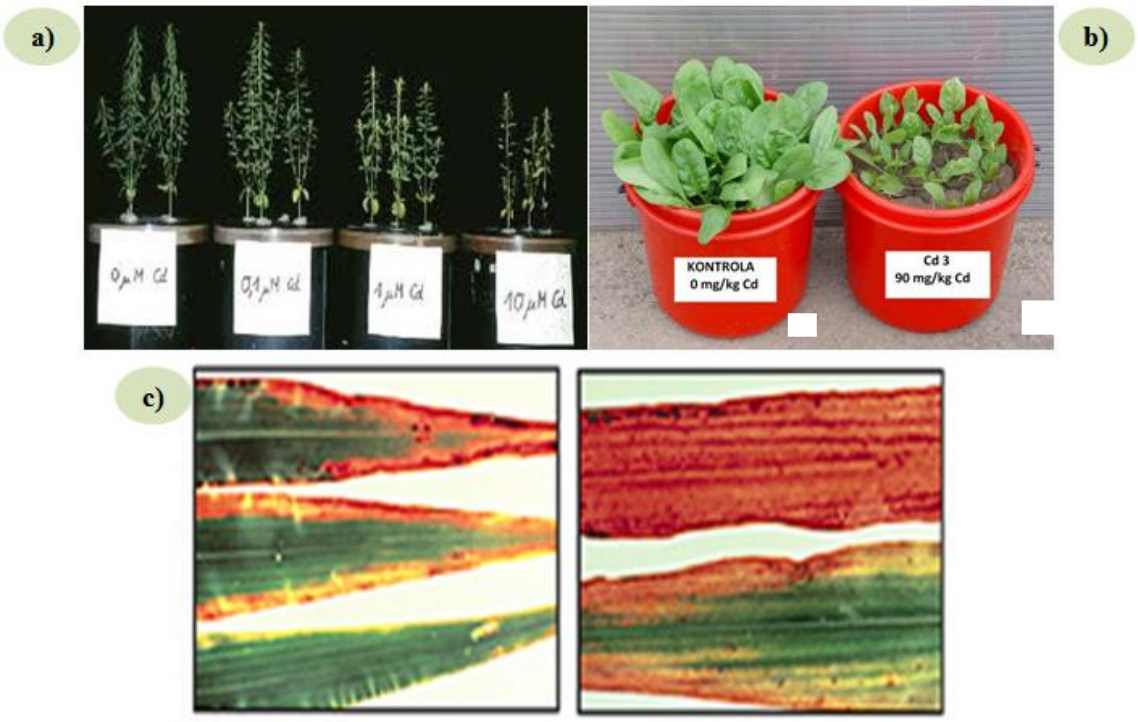
Obr. 4 Vliv Cd na rostlinné buňky (upraveni dle DalCorso et al., 2008).



Po vstupu do buňky Cd inhibuje metabolismus síry - (a) fotosyntézu a (b) biosyntézu chlorofylu; (c) záměnou za Ca^{2+} ionty Cd vstupuje do průduchů a aktivuje otvírání aniotů plasmatické membrány a K^+ kanálů, které způsobuje uzavření průduchů (aktivně je tento proces řízen kyselinou abscisovou - ABA); (d) Cd způsobuje v kořenech inhibici enzymů podílejících se na asimilaci dusíku – aktivita obou reduktáz je inhibována, Cd ovlivňuje aktivitu GS (glutaminsyntetáza) a GOGAT (glutamátsyntetáza) enzymů při asimilaci amonného iontu. ROS-reaktivní formy kyslíku; PLP-proteinfosfolipáza, podílející se na DAG (diacylglycerol) a IP_3 (inositol-3-fosfát) signalizaci; PSI-fotosystém I; LHCII-světlo sběrný systém II.

Příznaky toxicity Cd na rostlinách jsou snadno identifikovatelné, jejich příklady uvádí obr. 5. Jedním z prvních efektů působení Cd na rostliny je omezení růstu (Costa a Spitz, 1997; Tlustoš et al., 2006), jehož příčinou je dle Hasana et al. (2009) inhibice syntézy bílkovin. Rascio et al. (2008) pozorovali u klíčních rostlin rýže vystavených Cd stresu inhibici růstu kořenů a změny v jejich morfogenezi. Dalšími symptomy jsou zakrnělost a chloróza listů, hnědnutí kořenů a červenohnědé nekrózy na mladých listech. Chlorózy listů jsou způsobeny inhibicí biosyntézy chlorofylu v důsledku potlačení příjmu Fe rostlinami vlivem vysokého obsahu Cd v půdě (Das et al., 1997; Alloway, 1999). Pokles obsahu chlorofylu a karotenoidů v *Brassica napus* vlivem Cd zjistili Larsen et al. (1998). Příčinou výskytu červenohnědých skvrn jsou pravděpodobně změny v metabolismu fenolů a fenolických látek (Barceló et al., 1986). Pokud není Cd dostatečně rychle detoxikováno, může způsobit poruchy oxidačně - redukčního řízení buňky, poškození růstových reakcí, lignifikaci a nakonec smrt buňky. Clemens et al. (2006) uvádějí, že se Cd přímo nepodílí na těchto redox reakcích, protože Cd ionty nemění oxidační stav a neúčastní se Fentonovy ani Haber-Weissovy reakce. Kadmium při oxidačním stresu zvyšuje tvorbu volných radikálů a reaktivních forem kyslíku (Zengin a Munzuroglu, 2005) nebo snižuje koncentraci enzymových a neenzymových antioxidantů (Hasan et al., 2009).

Obr. 5 Příznaky toxicity Cd na rostlinách: a) lnu (*Linum usitatissimum* L.), b) špenátu setého (*Spinacia oleracea* L.) a c) čiroku cukrového (*Sorghum saccharatum* Pers.) (www.web2.mendelu.cz; foto pokus KAVR; APS Digital Image Collections, 2000).

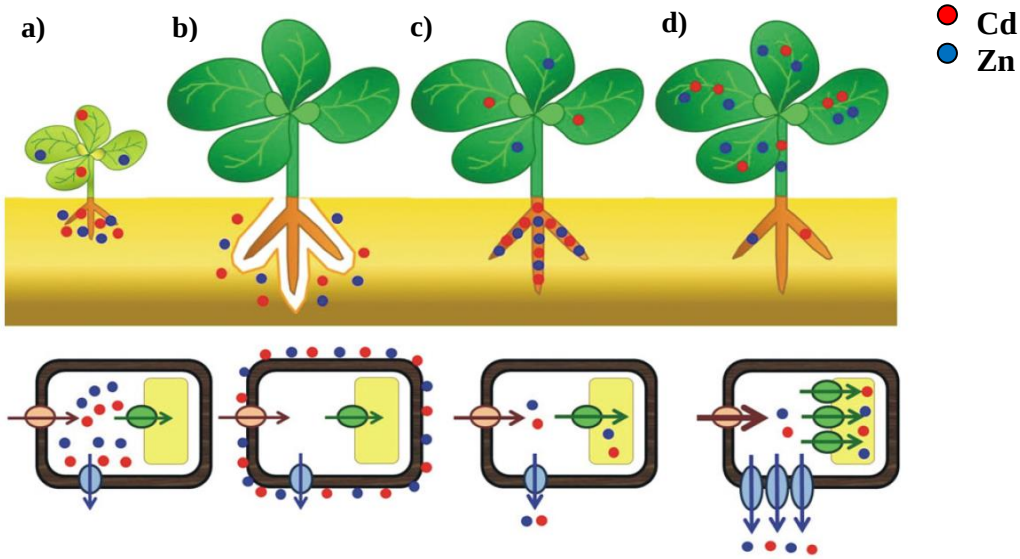


2.1.2.3 Hyperakumulátory kadmia

Rostliny lze podle přístupu ke zvýšenému obsahu rizikových prvků v prostředí rozdělit do tří skupin: na exkludory (omezující příjem), indikátory (bez obranných mechanismů) a akumulátory (se schopností akumulace) (Callahan et al., 2006). Rostliny, které jsou schopny redukovat příjem, používají mechanismy („bariéry“) omezující absorpci iontů do pletiv. V porovnání s exkludory nedisponují indikátory žádnými ochrannými mechanismy a citlivě reagují na zvýšené koncentrace rizikových prvků. Obsahy rizikových prvků v biomase těchto rostlin jsou úměrné koncentraci v prostředí, především v půdě. Rostliny se schopností akumulace (hromadění prvků) mají v pletivech zabudován mechanismus detoxikace, který jim umožňuje akumulovat ve své nadzemní biomase vysoké obsahy rizikových prvků, dokonce i při jejich nízké koncentraci v půdě (Fischerová et al., 2006; Fuksová et al., 2007). Rozdělení rostlin dle Lina a Aartse (2012) podle chování vůči Zn a Cd je uvedeno na obr. 6. Dle Pála et al. (2006) zahrnuje mechanismus tolerance rostlin vůči Cd jeho hromadění a uložení ve formě komplexu s aminokyselinami, proteiny a peptidy.

Hyperakumulátory jsou rostliny s významnou schopností akumulovat prvky v biomase, které přežívají pomocí mechanismů hypertolerance a hyperakumulace i na silně kontaminovaných půdách (Baker et al., 2000; Kabata-Pendias a Pendias, 2001; Sarma, 2011). Hyperakumulující rostliny nevykazují přes vysoký obsah rizikových prvků v biomase žádné projevy toxicity (Sarma, 2011). Obecně tyto rostliny rostou pomalu a produkují malé množství biomasy, a tím je limitováno jejich využití pro odstranění daného kovu z prostředí (Lou et al., 2013). Pro metabolismus hyperakumulátorů jsou důležité organické ligandy, zejména glutation, metalothioneiny, histidin, citrát a malát (Lovy et al., 2013).

Obr. 6 Rozdělení rostlin do skupin podle chování vůči Zn a Cd a jejich molekulární mechanismy (upraveno dle Lin a Aarts, 2012).



(a) citlivé rostliny – nemohou blokovat příjem kovu kořeny nebo transport kovu do prýtu rostliny a projeví se u nich toxický vliv v kořenech a prýtu; (b) exkludory – odolné rostliny, které zabrání příjmu kovu kořeny nebo rychle odstraní kov po vstupu do kořenových buněk; (c) tolerantní nehyperakamulující rostliny – po vstupu do kořenů je kov sekvestrován do vakuol, a tím je inhibována translokace kovu do prýtu rostliny; (d) hypertolerantní hyperakumulující rostliny – aktivní příjem kovu kořenem a následný transport xylémem do prýtu rostliny, kde je kov uložen do vakuol.

Baker et al. (2000) definovali jako hyperakumulátory Cd rostliny schopné hromadit tento prvek v nadzemních částech v koncentracích překračujících hladiny 100 mg/kg. Dosud bylo identifikováno několik druhů schopných akumulovat Cd v tak vysokých koncentracích, jedná se zejména o zástupce čeledi *Brassicaceae*. Schopnost hyperakumulace byla dokázána u těchto druhů: *Noccaea caerulescens* (Maestri et al., 2010), *Noccaea goesingense, Noccaea praecox* (Lombi et al., 2000), *Arabidopsis halleri* (Yang et al., 2004), *Sedum alfredii* (Deng et al., 2007) a *Rubia tinctorum* (Baker et al., 2000).

Z hlediska fytoremediace je zapotřebí především věnovat pozornost translokaci Cd v rostlině (rozdělení akumulovaného Cd mezi kořeny a prýt rostliny) a bazálním pochodům rostlinného metabolismu. Distribuce Cd není v hyperakumulujících rostlinách ovlivněna dobou působení kovu, avšak může záviset na druhu rostliny, ekotypu, stáří rostliny a koncentraci daného kovu (Huguet et al., 2012).

Byla publikována řada studií týkajících se mechanismu příjmu Cd v *N. caerulescens* a jeho translokace v tomto druhu rostliny. Příjem Cd u *N. caerulescens* je zprostředkován vysokoafinitními přenašeči pro Cd exprimovanými v plazmatické membráně kořenových buněk (Lombi et al., 2002). U *A. halleri* je příjem Cd pravděpodobně zprostředkováván kanály, které slouží pro vstup Zn do rostliny (Zhao et al., 2006). Akumulace Cd u *N. caerulescens* je závislá na metabolismu rostliny a není inhibována jinými dvojmocnými ionty ani La^{3+} (Zhao et al., 2002). Vliv lokality na akumulaci Cd u *N. caerulescens* prokázali Lombi et al. (2000) u dvou ekotypů *N. caerulescens*, Ganges (Francie) a Prayon (Belgie). Cd je přednostně ukládáno do vakuol epidermálních buněk, dále mohou jeho významné koncentrace obsahovat i buňky mezofylu (Vogel-Mikuš et al., 2008) a buněčné stěny kořenů (Hu et al., 2009). Ueno et al. (2008) zjistili u *A. halleri* pokles obsahu Cd v xylému v přítomnosti Zn. U *A. halleri* je Cd převážně ukládáno do buněk mezofylu (Zhao et al., 2000).

Hyperakumulující druhy jsou charakterizovány vysokou schopností translokace Cd z kořenů do nadzemních částí (Zhao et al., 2006), zatímco u nehyperakumulujících druhů je zřejmě pouze část Cd translokována do nadzemní části prostřednictvím apoplastu (Chan a Hale, 2004). Přesun Cd z kořenů do prýtu rostliny se pravděpodobně děje prostřednictvím xylému a hnací silou je transpirace listů (Hart et al., 1998), která může být při dlouhodobé expozici Cd negativně ovlivněna.

2.2 Oxidační stres u rostlin

Abiotické i biotické stresové faktory mohou vyvolat u rostlin oxidační stres, charakteristický prudkou přechodnou tvorbou velkého množství reaktivní formy kyslíku (ROS) (Gill a Tuteja, 2010). Rostlinné buňky produkují ROS nepřetržitě jako vedlejší produkt aerobního metabolismu, zejména peroxid vodíku (H_2O_2), superoxid ($O_2^{\bullet-}$), hydroxylový radikál ($OH^{\bullet}$) a singletový kyslík ($^1O_2$), jejich zdroje a účinky shrnuje tabulka 1 (Møller a Sweetlove, 2010). ROS vznikají redoxními reakcemi v chloroplastech, mitochondriích a cytoplasmatu pomocí mimobuněčných enzymů nebo enzymů vázaných na membránu. Reaktivní formy kyslíku mohou inaktivovat enzymy, oxidovat proteiny a poškozovat DNA a RNA. V důsledku uvedených reakcí nastává buněčná smrt.

Přesto, že se Cd neúčastní přímo redoxních reakcí (Stohs a Bagchi, 1995), může nepřímo aktivovat NADPHoxidázy v membránách, a tím zvýšit tvorbu superoxidového radikálu a peroxidu vodíku (Gallego et al., 2012). Nadprodukci ROS a následnou peroxidaci membránových lipidů v kořenech kukuřice (*Zea mays*) vlivem Cd zjistili ve své práci

Mihaličová Malčová et al. (2014). Dle Cuyperse et al. (2010) způsobuje Cd v rostlinách oxidační stres tím, že blokuje esenciální funkční skupiny v biomolekulách a dalšími nepřímými mechanismy, jako je narušení elektronového transportního řetězce. Romero-Puertas et al. (2002) zjistili oxidační modifikace proteinů vlivem Cd^{2+} v rostlinách hrachu, jejichž důsledkem byly oxidované proteiny účinněji degradovány, což dle těchto autorů korelovalo se zvýšenou proteolytickou aktivitou.

Tabulka 1: Přehled zdrojů a biologických účinků ROS (Piterková et al., 2005).

Forma kyslíku	**Zdroj**	**Biologický efekt**
O_2	atmosferický kyslík, PSII, různé enzymy (superoxiddismutáza, kataláza)	inhibice fotosyntézy (preference oxygenasové reakce ribulosa-1,5-bisfosfátkarboxylázy/oxygenázy), náhodná produkce volných radikálů
$O_2^{\cdot -}$	osvětlené chloroplasty, PSII a PSI, mitochondrie v přítomnosti NADH, Fe-S proteiny, cytochrom P450, elektronový transportní řetězec v endoplasmatickém retikulu, herbicidy (paraquat a nitrofen), enzymové reakce: xanthinoxidáza, NAD(P)Hoxidáza, aldehydoxidáza, urikáza	peroxidace lipidů, inaktivace enzymů, depolymerizace polysacharidů, reakce s H_2O_2 za tvorby $OH^{\cdot}$, schopnost oxidovat síru, askorbát a NADPH, redukovat cytochrom c a ionty kovů
H_2O_2	glykolátoxidáza v glyoxysomech, osvětlené chloroplasty - PSII, mitochondrie v přítomnosti NADH, β-oxidace mastných kyselin, Fe-S proteiny a enzymové reakce (SOD, glykolátoxidáza, aminoxidáza, oxalátoxidáza, peroxidázy)	inhibice fixace CO_2, inaktivace enzymů Calvinova cyklu, oxidace sulfhydrylů a flavonolů, substrát oxidační reakce
$OH^{\cdot}$	Haberova-Weissova reakce, Fentonova reakce	velmi silné oxidační činidlo, poškození DNA, peroxidace lipidů, degradace proteinů, produkce C_2H_4
$^1O_2$	excitované chlorofylové molekuly v tripletovém stavu, znečištění vzduchu (NO_2, O_3, atd.)	mutageneze, peroxidace lipidů, fotooxidace aminokyselin

Zvýšená koncentrace ROS je důležitým jevem pro vznik hypersensitivní reakce rostlin a následnou programovanou buněčnou smrt (Piterková et al., 2005). Nástup a intenzita přechodné masivní produkce ROS jsou obvykle velmi rychlé, ale jsou různé v závislosti na studované rostlině a na použitých podnětech stimulujících tento jev (Wojtaszek, 1997). ROS hrají tedy v biologických systémech dvojí roli: (i) slouží jako signální molekuly

pro expresi genů a (ii) jako toxické meziprodukty aerobního metabolismu způsobují poškození či zánik buňky (Møller a Sweetlove, 2010).

Reaktivní formy kyslíku napomáhají vzniku nekróz rostlinného pletiva nejen jako obranné látky, ale také poškozují proteiny a DNA a iniciují peroxidaci mastných kyselin membránových lipidů, čímž narušují integritu a funkčnost membrán (Bhattacharjee, 2005). Dle Grãtaa et al. (2005) je právě peroxidace lipidů indikátorem oxidačního stresu. Peroxidační poškození plazmalemy vede k úniku buněčného obsahu a k buněčné smrti. Intracelulární poškození membrány ovlivňuje respirační aktivitu mitochondrie a způsobuje také ztrátu schopnosti chloroplastu fixovat CO_2 (Piterková et al., 2005). V rostlinách funguje H_2O_2 především jako signální molekula a účastní se regulace v expresi genů při abiotickém stresu (Neill et al., 2002). H_2O_2 může také difundovat z místa produkce do celé rostliny, kde v nízkých koncentracích řídí např. oxidační zesíťování (glyko)proteinů buněčných stěn, čímž přispívá k jejich zesílení a snižuje tak jejich náchylnost k enzymové degradaci (Wojtaszek, 1997).

Ochranu před oxidačním poškozením rostlin působením ROS zajišťuje řada antioxidačních obranných systémů lokalizovaných v různých buněčných strukturách. Antioxidační obranné mechanismy zahrnují neenzymové a enzymové systémy. Schützendübel a Polle (2002), Martins et al. (2011) a Grãtao et al. (2012) řadí mezi enzymové antioxidační systémy superoxiddismutázu (SOD), katalázy (KAT), askorbátperoxidázu (APX), guajakolperoxidázu (GPOX) a glutationreduktázu (GR). Enzym SOD přeměňuje $O_2^{\bullet-}$ na H_2O_2, který může být detoxifikován APX a dalšími enzymy na H_2O (Grãtao et al., 2012). Aktivace a inhibice antioxidačních enzymů nezávisí jen na intenzitě stresového faktoru, ale také na typu pletiva a věku rostliny (Benavides et al., 2005). Zvýšenou aktivitu antioxidačních enzymů (např. KAT a SOD) vlivem Cd stresu uvádějí Lin a Aarts (2012) u hyperakumulujících druhů *N. caerulescens*, *Brassica juncea* a *Sedum alfredii*. Obdobný jev zjistili Wójcik et al. (2005) u *N. caerulescens*, kdy vysoká koncentrace Cd způsobila zvýšení APX aktivity, ale zároveň došlo ke snížení aktivity enzymu SOD.

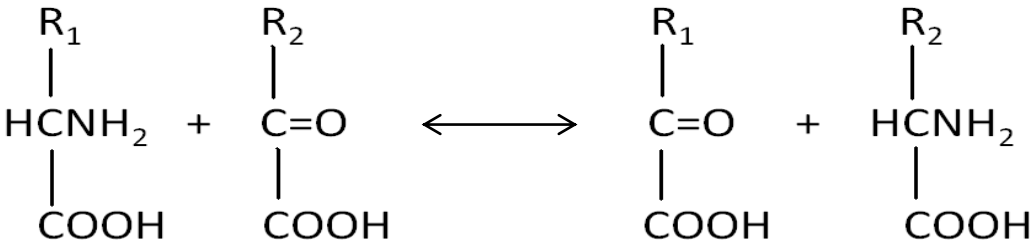
Mezi nejvýznamnější neenzymové antioxidační systémy podle výše zmíněných autorů patří aminokyseliny, glutation, askorbová kyselina a thioly. Význam aminokyselin, konkrétně prolinu v antioxidační ochraně potvrdili ve své práci Sharma a Dietz (2006). Dále Huguet et al. (2012) zdůrazňují důležitou roli fytochelatinů a jejich prekurzoru glutationu v antioxidačním systému hyperakumulujících rostlin.

2.3 Aminokyseliny

Při dlouhodobé expozici kovům rostliny často syntetizují a akumulují v nadměrných koncentracích látky, které rostlině pomáhají překonávat působený stres. Mezi tyto rostlinné metabolity patří peptidy, fytochelatiny, aminy, organické kyseliny a specifické aminokyseliny. Dle Sharmy a Dietze (2006) mají především aminokyseliny a organické kyseliny význam při hyperakumulaci a toleranci kovů v rostlinách, ačkoli dosud nejsou zcela jasné mechanismy jejich působení. Häusler et al. (2014) uvádějí význam aminokyselin jako součásti proteinů, prekurzoru pro anabolismus a v některých případech jako signální molekulu v rostlinách.

V podmínkách, kdy není rostlina ovlivněna přítomností žádného stresoru, mají aminokyseliny stěžejní význam při interakci mezi C a N metabolismem. Při této interakci jsou aminokyseliny metabolity obsahujícími N a umožňují využití C pro růst rostlin (Fritz et al., 2006). Biosyntéza proteinogenních aminokyselin v rostlinách probíhá biochemickými reakcemi, z nichž vzniklé aminokyseliny patří do glutamát-, aspartát-, pyruvát-, serin- a shikimát-biogenetické rodiny (Singh, 1999). Celé spektrum těchto aminokyselin jsou rostliny schopné syntetizovat z anorganických zdrojů N (Fritz et al., 2006). V konečné fázi biosyntézy je často amino skupina přenesena pomocí enzymů aminotransferáz (transamináz) na 2-oxokarboxylovou kyselinu (Brückner a Westhauser, 2003). Syntéza jedné aminokyseliny často vyžaduje degradaci aminokyseliny jiné. Aminotransferázy katalyzují přenos aminoskupiny z druhého uhlíku příslušné aminokyseliny na druhý uhlík příslušné oxokyseliny (obr. 7). Takto vzniká nová aminokyselina a nová oxokyselina. Aminokyseliny mohou být v určitých případech nahrazeny aminy, které poskytují příslušné aminoskupiny. Jako akceptorové molekuly aminoskupin mohou sloužit také aldehydy. Koenzym pyridoxal-5´-fosfát je přeměněn na pyridoxamin-fosfát prostřednictvím přenosu aminoskupiny ze substrátu. Tato aminokyselina se tedy stává oxokyselinou a následně je uvolněna. Pyridoxamin-fosfát přenáší nově získanou aminoskupinu na oxokyselinu a dává tak vzniknout nové aminokyselině (Singh, 1999).

Obr. 7 Schéma transaminace (upraveno dle Singh, 1999).



Aminokyselina A **Oxokyselina B** **Oxokyselina A** **Aminokyselina B**

Aminokyseliny se v rostlinách mohou hromadit v poměrně vysokém obsahu ve vyvinutých listech a kořenech. Jejich transport do tzv. „sink“ pletiv, ve kterých jsou využity pro růst nebo skladovány, zprostředkovává xylém a floém. Na rozdíl od mikroorganismů jsou rostliny schopné využít syntetizované aminokyseliny jako prekurzory pro sekundární metabolismus (Fritz et al., 2006).

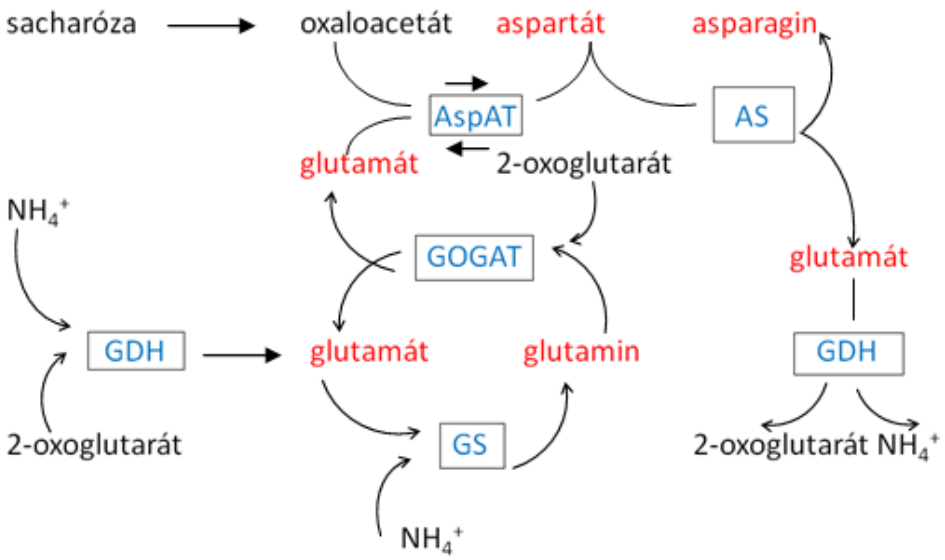
Abiotické stresy mohou měnit formy a koncentrace transportovaných aminokyselin v rostlinách (Müller Queiroz et al., 2012). Tito autoři zjistili pokles koncentrace aminokyselin v kořenech u *Glycine max*, který pravděpodobně souvisí se sníženým příjmem dusičnanů kořeny a vede ke snížení asimilace dusíku a následné syntézy aminokyselin. Podle Amaranta a Sodeka (2006) změny ve složení aminokyselin v xylému jsou dobrým indikátorem změn v metabolismu dusíku v kořenech. V rostlinách může docházet k oxidaci proteinů a aminokyselin, která je způsobena ROS nebo vedlejšími produkty oxidačního stresu. Proteinové aminokyseliny, zejména arginin, histidin, lysin, prolin, treonin a tryptofan, uvolňují při oxidaci karbonylové skupiny, které následně inhibují nebo mění svou aktivitu a zvyšují citlivost k proteolytickému útoku (Gill a Tuteja, 2010).

Costa a Spitz (1997) ve svém experimentu prokázali vliv Cd na obsah aminokyselin v *Lupinus albus*. Přítomnost Cd v médiu vyvolala změny ve složení aminokyselin. Výsledný vliv na aminokyseliny lze rozdělit do tří skupin. První skupinu reprezentují aminokyseliny prolin, hydroxylysin a asparagin, u kterých došlo k zvýšení obsahu vlivem Cd. U druhé skupiny aminokyselin (glutamát, cystein, glycin) došlo k poklesu obsahu v přítomnosti Cd. Do třetí skupiny patří zbylé aminokyseliny, u kterých nebyl prokázán vliv Cd na jejich obsah. Dle Deckerta (2005) má Cd vysokou afinitu ke glutamátu, aspartátu, cysteinu a histidinu.

2.3.1 Kyselina glutamová a glutamin

Amoniak je v rostlinách asimilován do organické formy jako kyselina glutamová (Glu) a glutamin (Gln), které jsou následně donory dusíku v biosyntéze všech esenciálních aminokyselin, nukleových kyselin a dalších komponent s obsahem dusíku, např. chlorofylu (Lea, 1993). Kyselina glutamová a glutamin mohou být následně využity pro tvorbu kyseliny asparagové (Asp) a asparaginu (Asn). Tyto aminokyseliny a aminy rostlina používá pro translokaci organického dusíku ze zdroje do pletiv (Coruzzi, 2003). Mezi enzymy vyvolávající primární asimilaci amoniaku do těchto N-transportních aminokyselin Glu/Gln a Asp/Asn patří glutaminsyntetáza (GS), glutamátsyntáza (GOGAT), aspartátaminotransferáza (AAT) a asparaginsyntáza (AS) (obr. 8). Analýzou HPLC prokázali Lam et al. (1995), že Glu a Gln spolu s Asn a Asp dosahují 60 - 64 % z celkového obsahu volných aminokyselin v listech rostlin *Arabidopsis*, kde jsou dále transportovány do cévních svazků.

Obr. 8 Asimilace dusíku v rostlině *Arabidopsis* (upraveno dle Coruzzi 2003).



Kyselina glutamová může být prekurzorem nebo vedlejším produktem degradace specifických aminokyselin, jako je arginin, prolin a histidin (Glevarec et al., 2004). Podle Pavlíka et al. (2012) jsou kyseliny glutamová a asparagová klíčové aminokyseliny pro biosyntézu nukleových kyselin, ATP, cytokininů a chlorofylu. Obě aminokyseliny hrají důležitou roli v růstu, obraně a reprodukčních procesech rostlin. Tuto skutečnost uvádějí také Bhatia et al. (2005). Kyselina glutamová byla dle těchto autorů hlavní aminokyselinou v pletivech rostlin *Stackhousia tryonii* (Bailey), které byly ošetřeny přídavkem niklu. Důležitý

význam Glu pro rostliny zjistili také Fritz et al. (2006), kteří pozorovali relativně konstantní obsah Glu v listech tabáku (*Nicotiana tabacum* L., cv. Petit Havana SR1) při růstu rostlin s rozdílným obsahem N v substrátu a variabilním termínem odběru vzorků.

2.3.2 Kyselina asparagová a asparagin

Kyselina asparagová jako „majoritní“ aminokyselina je syntetizována přesunem amino skupiny z Glu na oxalacetát a pyruvát. Asparagin je syntetizován z kyseliny asparagové v závislosti na Gln transaminací (Fritz et al., 2006). Asp je prekurzorem pro syntézu esenciálních aminokyselin lysinu, methioninu, threoninu a rozvětvených aminokyselin isoleucinu, leucinu a valinu (Karchi et al., 1993; Curien et al., 2008). Lea et al. (2007) uvádějí asparagin jako hlavní transportní sloučeninu v xylému při transportu z kořenů do listů a ve floému při transportu z listů do semen v řadě rostlin. Obdobně také podle Parsons a Baker (1996) je Asn hlavní aminokyselina v xylému a floému *Lupinus albus* (L.). Tito autoři zjistili vysoké koncentrace asparaginu zejména v hlízkách kořenů, kde byl Asn uložen jako budoucí zdroj N pro rostlinu. Dle Zhanga et al. (2013) je asparagin hlavní formou N v pletivech rostlin *Arabidopsis*.

2.3.3 Prolin

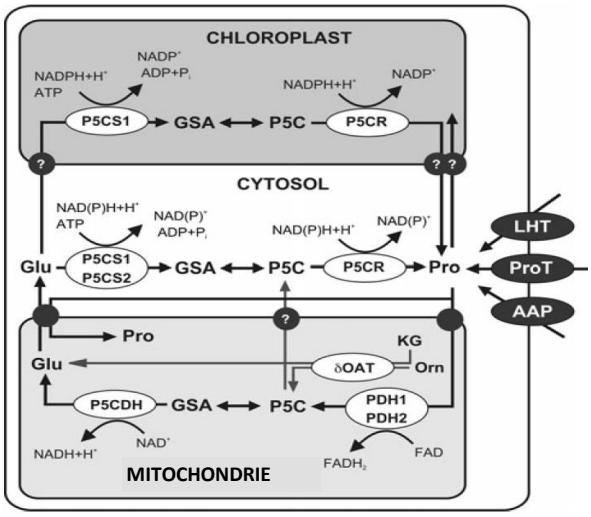
Iminokyselina prolin (Pro) obsahuje sekundární aminoskupinu a je tedy unikátní mezi proteinogenními aminokyselinami. V buněčném metabolismu má Pro důležitou roli jako součást proteinů a také jako volná aminokyselina. Vzhledem k jeho cyklické struktuře má Pro omezenou konformační flexibilitu, která udává uspořádání peptidového řetězce, a tím způsobuje stabilizaci nebo destabilizaci sekundární struktury konformace proteinů (Lehmann et al., 2010).

V rostlinách je Pro syntetizován z glutamátu přes pyrrolin-5-kaboxylát pomocí dvou po sobě jdoucích redukcí, které katalyzují P5Csyntetáza a P5Creduktáza (Hare et al., 1999) (obr. 9). Prvním enzymem, který se na biosyntéze Pro z glutamátu podílí, je glutamátkináza, která přeměňuje kyselinu glutamovou na γ-glutamylfosfát (Seddon et al., 1989; Pavlíková et al., 2007). Tato konverze je pravděpodobně dle Táborského a Vašákové (1994) odpovědí na působení abiotických stresorů. Dle Pavlíkové et al. (2008) by stanovení aktivity tohoto enzymu mohlo být vhodným nástrojem pro sledování metabolických změn vyvolaných působením stresorů ve vegetačním období rostlin. Tito autoři sledovali změny v aktivitě glutamátkinázy (GKA) u špenátu setého (*Spinacia oleracea* L., odrůda Matador) v podmínkách chronického stresu vlivem Cd a Zn. V těchto pokusech zjistili autoři regulační úlohu glutamátkinázy při stresu vlivem rizikových prvků a její potencionální využití jako

biomarkru stresu. GKA je v rostlinách regulována zpětnou vazbou podle obsahu prolinu (Štefl a Vašáková, 1984). Alosterická regulace GKA volným Pro umožňuje v rostlinách zvýšit obsah glutamátu, který je důležitý pro vytvoření peptidové vazby mezi γ-karboxylovou skupinou glutamátu a α-amino skupinou cysteinu a následné využití při syntéze glutationu a následně fytochelatinů (Pavlíková et al., 2008). Řada autorů, např. Hall (2002), Lima et al. (2006), Meyer et al. (2011), uvádějí syntézu fytochelatinů jako důležitý mechanismus při detoxifikaci kovů, avšak pokud je výrazně snížen obsah glutationu dochází k oxidačnímu stresu rostlin (Mishra et al., 2006).

Alternativním substrátem, ze kterého může být Pro v rostlinách syntetizován, je ornitin. Této metabolické cesty se účastní enzym ornitin-δ-aminotransferáza, který podporuje především degradaci argininu, a tím z něj mobilizuje dusík (Funck et al., 2008). Degradace Pro probíhá v mitochondriích a je katalyzována prolindehydrogenázou a P5Cdehydrogenazou (Hare et al., 1999 - obr. 9).

Obr. 9 Model metabolismu a transportu prolinu v buňkách *Arabidopsisu* (Lehmann et al., 2010).



Substráty jsou značeny černě: Pro - prolin, Glu - glutamová kyselina, Orn - ornitin, P5C - pyrrolin-5-karboxylát, GSA - glutamin-γ-semialdehyd, KG - α-ketoglutarát. Enzymy jsou světlé ovály: P5CS - P5Csyntetáza, P5CR - P5Creduktáza, PDH - prolindehydrogenáza, P5CDH - P5Cdehydrogenáza, δOAT - ornitin-δ-aminotransferáza. Transportéry jsou tmavé kruhy: AAP - aminokyseliny permeáza, ProT - prolintransportér, LHT - lysin-histidin transportér. Transportéry, které byly popsány pouze z fyziologického hlediska, jsou zobrazeny v tmavých kruzích s otazníkem.

Zvýšené množství Pro v rostlinách je způsobeno řadou stresových faktorů, např. zasolením, rizikovými prvky, suchem, vysokými a nízkými teplotami (Verbruggen a Hermans, 2008). Řada autorů, např. Alia et al. (2001), Kishor et al. (2005) a Pál et al. (2006), popsala různé funkce prolinu v rostlinách. Prolin má v rostlinách význam při zmírňování osmotického stresu, je zdrojem C a N (zásoba pro období po ukončení působení stresorů), stabilizuje syntézu bílkovin a funguje jako antioxidant a pH regulátor. Gill a Tuteja (2010) označují Pro za potenciální inhibitor programované buněčné smrti. Szabados a Savouré (2010) uvádějí jako jednu z funkcí Pro ochranu enzymu nitrátreduktázy při osmotickém stresu a stresu rizikovými prvky. Alia et al. (2001) prokázali efektivní reakci mezi volným prolinem a $^1O_2$, jehož obsah byl prolinem snižován. Prolin má tedy antioxidační vlastnost, v důsledku vlivu na aktivitu ROS a při kumulaci a deaktivaci superoxidu (Matysik et al., 2002). Podobné výsledky prokázali Islam et al. (2009) u tabáku (*Nicotiana tabacum* L.) vystaveného Cd stresu. Dle těchto autorů buňky tabáku akumulovaly vysoké obsahy Pro, a tím zmírnily inhibiční efekt Cd na jejich růst.

Při chelataci Cd iontů v rostlinách je tvořen netoxický komplex Cd-Pro (Sharma et al., 1998; Sun et al., 2007; Pavlíková et al., 2008). Costa a Spitz (1997) uvádějí jako jeden z projevů Cd stresu redukci vývoje rostlin v důsledku degradace proteinů přes katabolismus aminokyselin a s tím související nárůst obsahu prolinu a polyaminů v buňkách rostlin.

U nestresovaných rostlin mohou exogenní dávky prolinu působit toxicky. Příznakem intoxikace Pro jsou změny v chloroplastech a ultrastrukturách mitochondrií (Hare et al., 2002).

2.4 Mastné kyseliny

Mastné kyseliny (MK) jsou esenciální komponenty všech rostlinných buněk, jejichž biosyntéza je primární metabolickou cestou a je nezbytná pro buněčné dělení, růst a vývoj rostlinných buněk, zejména při klíčení semen (Graham, 2008; Rogalski a Carrer, 2011). V rostlinných buňkách jsou tyto látky důležitou složkou buněčných membrán, suberinu a kutinu, které tvoří strukturální bariéru pro prostředí (Beisson et al., 2007). Mastné kyseliny působí jako skladovací rezervy, které mohou být metabolizovány za účelem výroby energie; jsou stavebními kameny membránových lipidů a působí jako signalizační molekuly (Baker et al., 2006). Tyto sloučeniny přispívají k odolnosti vůči stresu remodelací membránové fluidity (Iba, 2002), kdy dochází k uvolnění α-linolenové kyseliny pomocí lipáz (Grechkin, 1998). Upchurch (2008) a Los et al. (2013) uvádějí tento jev jako vlastnost přizpůsobení se rostlin stresu, při které je změna obsahu nenasycených mastných kyselin regulována desaturázami

mastných kyselin. Desaturázy MK jsou enzymy, které zabudovávají dvojné vazby do mastných acyl řetězců a hrají klíčovou roli při udržování správné struktury a fungování biologických membrán (Los a Murata, 1998). Dle Bléea (2002) je volná α-linolenová kyselina sama o sobě stresovým signálem a prekurzorem pro fyto-oxylipin biosyntézu. Dále mohou MK působit jako modulátory obrany rostlin při expresi genů (Kachroo et al., 2001).

Mastné kyseliny jsou důležitou součástí procesu senescence listů (Yang a Ohlrogge, 2009). Dle Yanga a Ohlrogge (2009) dochází při senescenci listů ke snížení obsahu MK. Tento jev potvrdili Koiwai et al. (1981) u listů tabáku (*Nicotiana tabacum*). Mastné kyseliny uvolněné hydrolýzou lipidů mohou být při senescenci listů dále metabolizovány β-oxidací v peroxizómech listů a klíčících semen (Graham, 2008).

Při působení stresorů dochází ke změnám v zastoupení jednotlivých mastných kyselin. Toto potvrdili Hernández a Cooke (1997) v rostlinách hrachu (*Pisum sativum* cv. Argona) při ošetření rostlin 50 μM roztokem Cd. Tato koncentrace Cd zvýšila v rostlinách hrachu relativní obsah nenasycených mastných kyselin (zejména 18:1 a 16:1) a naopak snížila obsah nasycených mastných kyselin (zejména 16:0). Obdobné výsledky pozorovali Nouairi et al. (2006) v listech *Brassica juncea* při působení stresu vlivem Cd. Podle Thompsona et al. (1998) a Skórzyńska-Polit a Krupa (2006) Cd zvyšuje aktivitu lipoxygenáz, které jsou odpovědné za katalýzu při peroxidaci lipidů a jako substrát využívají komponentů membránových lipidů, zejména polynenasycené mastné kyseliny. Nenasycené mastné kyseliny (18:2 a 18:3) v membránových lipidech mohou podlehnout peroxidaci v důsledku zvýšené akumulace ROS a zvýšené aktivitě peroxidáz vyvolaných přítomností Cd, která má za následek poškození a ztrátu integrity membrán. Verdoni et al. (2001) zjistili významný pokles nenasycené mastné kyseliny 18:3 a zvýšení obsahu nenasycených mastných kyselin 18:1 a 18:2 v primárních listech rostlin rajčat. Dle Le Guédard et al. (2012) je nenasycená MK 18:3 většinou spojena s galaktolipidy, které tvoří více než 85 % thylakoidních lipidů a jsou důležité pro fotosyntetické aktivity. Pro rostliny rostoucí při oxidačním stresu je nenasycená MK 18:3 substrátem, který je rychle degradován na metabolity, jenž jsou využity dále na syntézu kyseliny jasmonové a oxylipinů pro regulaci vývoje a růstu rostlin (Savchenko et al., 2014).

Mastné kyseliny jsou nerozpustné ve vodě a mají velmi rozmanitou strukturu závislou na typu membrány, které jsou součástí (Rogalski a Carrer, 2011). Mastné kyseliny se v buňkách téměř nikdy nevyskytují volně, ale vázané v membránách převážně ve formě esterů glycerolu (glycerolipidy) (Murray et al., 2003). Všechny atomy uhlíku, které se nacházejí v MK, jsou odvozeny od acetyl-koenzymu A (acetyl-CoA) přítomného v plastidech.

Koncentrace acetyl-CoA v chloroplastech je asi 30 – 50 μmol, což je dostačující pro syntézu MK jen na několik vteřin. Nicméně asociace acetyl-CoA zůstává relativně konstantní, jak při světelné fázi, tak i při fázi temné (Ohlroggeav a Browseb, 1995). Acetyl-CoA může být tvořen v plastidech různými reakcemi, ale přesný podíl acetyl-CoA z každé reakce je stále neznámý. Předpokládá se, že velká část z acetyl-CoA je odvozena od glukózy-6-fosfátu a z pyruvátu (respektive fosfoenolpyruvátu), který je transportován z cytoplasmy do plastidů.

Biosyntéza MK probíhá především ve dvou subcelulárních komponentech - chloroplastech a endoplazmatickém retikulu. Dle Sidorova a Tsydendambaeva (2014) probíhá *de novo* syntéza MK jako palmitová nebo stearová kyselina z acetyl-CoA v plastidech, zatímco přeměna olejové kyseliny v linolovou a linolenovou kyselinu probíhá v endoplazmatickém retikulu. Dále tito autoři uvádějí, že v plastidech také probíhá desaturace stearové kyseliny na kyselinu olejovou. První reakcí biosyntézy MK je tvorba malonyl-CoA z acetyl-CoA a CO_2 za katalýzy acetyl-CoAkarboxylázy. Tato reakce probíhá ve dvou krocích, které jsou katalyzované jediným enzymovým komplexem. Acetyl-CoAkarboxyláza obsahuje dvě podjednotky, na každou z nich je kovalentně k lysinovým zbytkům proteinu přes ε-aminokyselinu navázán biotin, který má v tomto případě funkci nosiče CO_2. Tento enzym určuje rychlost biosyntézy MK a jeho aktivita je regulována. Aktivovaný CO_2 je transportován biotinem k acetyl-CoA, a tím je syntetizován malonyl-CoA (Ohlroggeav a Browseb, 1995).

K degradaci mastných kyselin ve většině organismů dochází především prostřednictvím β-oxidace. U rostlin jsou hlavním místem β-oxidace mastných kyselin peroxisomy, kde jsou snadno oxidovány mastné kyseliny s dlouhým řetězcem (Poirier et al., 2006). Peroxisomy jsou sférické organely specializované na oxidační reakce. Rovněž se podílejí na metabolismu tuků a účastní se části fotorespiračního cyklu.

3 CÍLE A HYPOTÉZY PRÁCE

Disertační práce je zaměřena na studium vlivu stupňovaných dávek Cd na metabolismus vybraných druhů rostlin, především hyperakumulátorů. Pro hodnocení vlivu Cd na rostlinu byly stanoveny následující hypotézy:

1. Při příjmu a akumulaci Cd v rostlinách dochází k fyziologickým a metabolickým změnám. Významné jsou změny zejména v obsahu a složení volných aminokyselin a mastných kyselin.

2. Změny v obsahu a složení volných aminokyselin a mastných kyselin se liší v hyperakumulujících a nehyperakumulujících rostlinách i mezi jednotlivými zástupci hyperakumulujících druhů rostlin.

3. Při působení stupňovaných koncentrací Cd dochází k odlišným změnám v obsahu volných aminokyselin v nadzemních částech rostliny a v kořenech.

K potvrzení uvedených hypotéz byly stanoveny následující cíle:

1. Stanovení vlivu stupňované koncentrace Cd v půdě na obsah tohoto prvku ve vybraných rostlinách.

2. Posouzení vlivu příjmu stupňovaných dávek Cd na změny v metabolismu hyperakumulujících a nehyperakumulujících rostlin, především hodnocení změn obsahu volných aminokyselin a mastných kyselin.

3. Hodnocení změn v obsahu makro- a mikroelementů v rostlinách, ke kterým došlo v souvislosti se stupňovanou kontaminací Cd.

4 MATERIÁL A METODY

Pro sledování vlivu Cd na metabolismus rostlin byly založeny vegetační nádobové pokusy v pokusném skleníku Katedry agroenvironmentální chemie a výživy rostlin.

Jako pokusné rostliny byly použity:

a) penízek modravý (*Noccaea caerulescens* FK Mey, dříve *Thlaspi caerulescens* J. & C. Presl) - ekotyp Ganges a Redlschlag
b) penízek časný (*Noccaea praecox* Wulfen, FK Mey)
c) huseníček Hallerův (*Arabidopsis halleri* O`Kane a Al-Shehbaz)
d) špenát setý (*Spinacia oleracea* L., odrůda Matador)

Pro pěstování rostlin byla použita černozem (CE modální) - lokalita Suchdol (pH_{KCl} = 7,2 ± 0,3; $C_{org.}$ (%) = 3,1± 0,7; KVK ($mmol_{(+)}/kg$) = 225 ± 19,5; Cd (mg/kg) = 0,42 ± 0,05), která byla hnojena N (ve formě NH_4NO_3 p. a.), P a K (ve formě K_2HPO_4 p. a.) a kontaminována Cd ve formě $Cd(NO_3)_2 \cdot 4H_2O$ (p. a.) dle schématu uvedeného v tabulce 2. Rostliny hyperakumulátorů byly pěstovány ve skleníku při těchto parametrech: teplota den/noc 24 °C/ 18 °C, půdní vlhkost 60 % maximální vodní kapacity, světelný režim den/noc 16 h/ 8 h, intenzita osvětlení 375 W/m^2. Rostliny špenátu setého byly pěstovány ve vegetační hale při těchto parametrech: teplota den/noc 20 ± 5 °C/ 15 ± 2 °C, půdní vlhkost 60 % maximální vodní kapacity, světelný režim den/noc 14 ± 2 h/ 8 ± 2 h.

Tabulka 2: Schéma nádobových pokusů.

	Varianta	Navážka zeminy (kg)	Hnojení NPK			Přídavek Cd (mg/kg)
			Dávka N (g/nádoba)	Dávka P (g/nádoba)	Dávka K (g/nádoba)	
Noccaea sp. a *Arabidopsis halleri*	Kontrola Cd1 Cd2 Cd3	3	0,3	0,1	0,24	0 30 60 90
Špenát setý	Kontrola Cd1 Cd2 Cd3	5	0,5	0,16	0,4	0 30 60 90

V rámci pokusů byly provedeny následující analýzy:

1. *Měření parametrů fotosyntézy*

Rychlost čisté fotosyntézy (P_N), rychlost transpirace (E), stomatální vodivost (g_s) a intracelulární koncentrace CO_2 (C_i) byly měřeny v listech *in situ* pomocí přenosného

infračerveného analyzátoru plynů LCpro+ (ADC BioScientific Ltd., Hoddesdon, Velká Británie).

2. *Analýza obsahu prvků v rostlinách*

Obsah Cd, vybraných makro- a mikroelementů byl stanoven metodou optické emisní spektrometrie s indukčně vázaným plazmatem (ICP-OES, VarianVistaPro, Austrálie) a obsah K, Ca a Mg metodou plamenové atomové absorpční spektrometrie (FAAS, Varian SpectrAA-280, Varian, Austrálie) po rozkladu na suché cestě (Miholová et al., 1993).

3. *Analýza volných aminokyselin*

Po homogenizaci čerstvé biomasy (~0,5 g) v kapalném N byly volné aminokyseliny extrahovány 10 ml roztoku metanolu a redestilované vody (7:3, v/v). Derivatizace byla provedena sadou EZ:faast firmy Phenomenex (Pavlík et al., 2010) a obsah byl měřen metodou GC-MS, na přístroji Hewlett-Packard 6890N/5975 MSD (Agilent Technologies, USA) s kolonou Zebron ZB-PAA-MS 10 m × 0,25 mm.

4. *Analýza mastných kyselin*

Celkový obsah mastných kyselin (volných a odvozených od lipidů) byl stanoven po jejich převedení na příslušné methylestery - transesterifikace dle Stránského a Jursíka (1996 a, b). Vzorky suché rostlinné biomasy (~0,2 g) byly extrahovány 2 ml roztoku metanolu a chloroformu (3:2, v/v). Obsah mastných kyselin byl měřen metodou GC-MS, na přístroji Thermo Scientific DSQ II Single Quadrupole GS-MS (Thermo Fisher Scientific) s nepolární kolonou Zebron ZB-5 30 m × 0,25 mm × 0,25 µm.

5. *Statistická analýza*

Statistické analýzy byly provedeny pomocí jednofaktorové a vícefaktorové analýzy rozptylu (ANOVA a MANOVA). Následně byl použit Tukeyho post-hoc test na hladině významnosti $\alpha < 0,05$ a lineární korelace a/nebo korelace s použitím funkce polynomu 3. stupně (R^2). Pro hodnocení byl použit program Statistica 9.0 a 12.0 (StatSoft, USA). U vybraných výsledků byla použita analýza hlavních komponent (PCA) v programu CANOCO 4.5 (ter Braak a Šmilauer, 2002).

5 VÝSLEDKY

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The changes of contents of selected free amino acids associated with cadmium stress in *Noccaea caerulescens* and *Arabidopsis halleri*

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ABSTRACT

Changes of free amino acid (AA) contents (glutamic acid, glutamine, aspartic acid, asparagines, proline, hydroxyproline) in *Noccaea caerulescens* and *Arabidopsis halleri* under cadmium soil contamination (Cd1 = 30, Cd2 = 60, Cd3 = 90 mg/kg soil) are reported. Results of the pot experiment confirmed different effect of Cd on *N. caerulescens* in contrast to *A. halleri* and the higher stress adaptation of *A. halleri*. Total free AA contents in both plant species were not significantly modified by Cd contamination. The glutamic acid and glutamate contents in plant biomass were decreased under Cd2 and Cd3 stress. The declines of contents of both AA can be caused by intensive syntheses of plant defense elicitors, but declines in *A. halleri* were significantly lower in contrast to *N. caerulescens*. The content of aspartic acid was increased in *N. caerulescens* under Cd stress, but in *A. halleri* its changes were not observed. The different pathways of nitrogen utilization of tested plants were confirmed: the major AA forms used for nitrogen transport are glutamate for *N. caerulescens* and asparagine for *A. halleri*. The increase of proline content was determined only in *N. caerulescens* growing under Cd stress in the beginning of growing period.

Keywords: heavy metals; nitrogen-transport amino acids; *Thlaspi caerulescens*

Hyperaccumulators of heavy metals are plants which actively take up exceedingly large amounts of heavy metals from soil. Heavy metals are not retained in the roots but they are translocated to the shoots and accumulated in aboveground organs, especially in leaves, at concentrations 100–1000-fold higher than those found in non-hyperaccumulating species. After the uptake of heavy metals by plant roots, their translocation to shoots and detoxification within the storage sites are two critical steps. This is achieved by chelation, transport, trafficking, and sequestration by organoligands at a subcellular level (Clemens et al. 2002). The potential ligands are grouped into three major classes: oxygen donor ligands (e.g. carboxylates), sulphur donor ligands (e.g. metallothioneins and phytochelatins), and nitrogen donor ligands (e.g. amino acids) (Bhatia et al. 2005).

Two Brassicaceae species, *Arabidopsis halleri* (*AH*) and *Noccaea caerulescens* (*NC*) (formerly *Thlaspi caerulescens*), have become popular models for the study of heavy metal hyperaccumulation (Hanikenne and Nouet 2011). In *AH*, Cd is mostly accumulated in mesophyll cells (Zhao et al. 2000) and high metal concentrations were also observed in the trichomes (Sarret et al. 2009). In the leaves of *NC*, Cd is more concentrated in the epidermis, but the mesophyll is still the major storage compart-

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ment due to its larger volume (Vogel-Mikuš et al. 2008). In leaf cells of hyperaccumulators, metals are generally sequestered in vacuoles (Huguet et al. 2012). This vacuolar compartmentalization is considered as the major metal detoxification pathway, limiting possible interferences between toxic elements and cell metabolism (Verbruggen et al. 2009).

Amino acids (AA) are the precursors to proteins and also their constituents and they play an important role in metabolism and development (Ježek et al. 2011). Plants that were exposed to toxic metals have also been shown to accumulate specific AA, which may have beneficial functions. The AA which are accumulated under heavy metal stress, play various roles in plants, including acting as signaling molecules, and osmolytes, regulating ion transport and facilitating detoxification (Xu et al. 2012). Reports of the role of AA in the hyperaccumulation of metals (including Cd) by plants are limited; therefore the present investigation aims to determine the changes and differences in accumulation of selected free AA in *NC* and *AH* associated with Cd soil contamination. Asparagine and glutamic acid as well aspartic acid and glutamine are involved in N-assimilation, transport and transamination processes of vascular plants, therefore the changes of these amino acids were investigated in detail. Accumulation of Cd and content of selected free AA were measured to test the ability of these plants to tolerate Cd contamination.

MATERIAL AND METHODS

The effect of Cd concentration on the levels of free amino acids was investigated in the pot experiment. Two species of Brassicaceae family were selected: *Noccaea caerulescens* (formely *Thlaspi caerulescens* J. & C. Presl, FK Mey) ecotype 'Ganges' (southern France) and *Arabidopsis halleri* (O'Kane and AL Shehbaz) (northern France). The plants were planted into plastic pots (two plants per pot) containing 3 kg of soil (Table 1). Soil was thoroughly mixed with 0.3 g N, 0.10 g P, and 0.24 g K applied in the form of ammonium nitrate and potassium hydrogen phosphate for control treatment and with the same amount of nutrients plus Cd in the form of $Cd(NO_3)_2 \cdot 4\ H_2O$ in concentrations: 0 (control), 30 (Cd1), 60 (Cd2) and 90 (Cd3) mg/kg, for treated variants. The plants were cultivated under natural light and temperature conditions at the experimental hall. Plants were harvested 30 and 90 days after Cd application. Samples were kept frozen in liquid nitrogen for transport and then at –30°C until extraction procedure.

For determination of Cd, plant samples were decomposed using the dry ashing procedure (Miholová et al. 1993). The concentrations of Cd were determined by ICP-OES with axial plasma configuration (VarianVistaPro, Varian, Mulgrave, Australia).

Total free AA compounds were determined using an EZ-faast amino acid analysis procedure (Phenomenex, Santa Clara, USA). Samples were analyzed for AA contents by the gas chromatography coupled with mass spectrometry detection using a HP 6890N/5975 instrument (Agilent Technologies, Torrance, USA; as described by Pavlík et al. (2012)).

The statistical analyses were performed using hierarchic analyses of variance (ANOVA) with inteactions at 95% ($P < 0.05$) significance level with subsequent Tukey's *HSD* test and linear correlation (R^2). All analyses were performed by using Statistica 9.1 software (StatSoft, Tulsa, USA).

RESULTS AND DISCUSSION

Results of the pot experiment revealed the different effect of Cd on *NC* and *AH*. Aboveground biomass of *NC* decreased with increasing Cd2 and Cd3 rates (Table 2). The higher Cd rates significantly reduced aboveground biomass in both sampling periods (41% and 64% decrease of yield for Cd3 treatments compared to the control). The lowest Cd rate stimulated the dry aboveground biomass yield of *AH* (42% and 52% increase compared to control). On the other hand, the Cd3 rate reduced the yield (33% and 22% decrease compared to

Table 1. Basic characteristics and total element contents in experimental soil

Soil type	pH	C_{org} (%)	CEC ($mmol_+$/kg)	Cd (mg/kg)
Modal Chernozem	7.2 ± 0.1	1.83 ± 0.01	258 ± 0.1	0.42 ± 0.05

CEC – cation exchange capacity

Table 2. Effect of Cd on aboveground biomass yield and Cd content of *Noccaea caerulescens* (*NC*) and *Arabidopsis halleri* (*AH*). I. – 30 days; II. – 120 days from planting

Sampling period	Control		Cd1		Cd2		Cd3	
	NC	*AH*	*NC*	*AH*	*NC*	*AH*	*NC*	*AH*
Yield of aboveground biomass (g dry matter per pot)								
I.	0.63 ± 0.03	0.67 ± 0.11	0.53 ± 0.10	0.95 ± 0.07	0.41 ± 0.02	0.85 ± 0.13	0.37 ± 0.03	0.45 ± 0.10
II.	2.50 ± 0.21	2.30 ± 0.09	2.54 ± 0.21	3.50 ± 0.25	2.10 ± 0.18	2.20 ± 0.20	0.90 ± 0.12	1.80 ± 0.16
Cd content (mg/kg)								
I.	41 ± 0.2^{aA}	121 ± 0.3^{aA}	1656 ± 1.8^{aB}	990 ± 0.4^{aB}	2860 ± 0.6^{aC}	1447 ± 0.9^{aC}	3773 ± 1.2^{aD}	2141 ± 0.4^{aD}
II.	26 ± 0.4^{cA}	66 ± 0.3^{bA}	726 ± 1.2^{cB}	452 ± 0.4^{cB}	1639 ± 0.6^{cC}	1194 ± 3^{cC}	2629 ± 0.9^{cD}	1022 ± 0.5^{cD}

Data are means ± S.E. (n = 3). Different letters (a) indicate significantly different values ($P < 0.05$) between samples of plant species. Different letters (A) indicate significantly different values ($P < 0.05$) between treatments of plant species. Control – 0 mg Cd/kg soil; Cd1 – 30, Cd2 – 60 and Cd3 – 90 mg Cd/kg soil

control). Visual toxicity symptoms with necrosis were observed on neither of plant species. This finding corresponded with the results of Rascio and Navari-Izzo (2011).

In comparison to *NC*, Cd contents in aboveground biomass of *AH* were lower at all Cd treatments (Table 3), but they were above the 100 mg/kg Cd threshold that defined Cd hyperaccumulation in the natural environment (Cosio et al. 2005). The opposite effects were observed in control variants – higher Cd contents in *AH* in contrast to *NC* were determined. Significant negative linear correlation between contents of Cd in the aboveground biomass and biomass yield was calculated (R^2 = 0.99–1 for *NC*; R^2 = 0.92–1 for *AH*).

Our data correspond with those by Pavlíková et al. (2002, 2008) and Procházková et al. (2012) who reported that excessive amounts of toxic elements in contaminated soil inhibited plant growth and development due to their phytotoxicity. Reduced growth observed at contaminated treatments may be partly due to lower net photosynthetic rate, but not exclusively, since it was argued that the reduced growth might be also due to increased tissue permeability. It might also result from inhibition of cell division (Redondo-Gómez et al. 2011). Reduction in growth can be linked to the high trace elements accumulation, as in this case plants have to spend extra energy to cope with the high trace element concentrations in the tissues (Israr et al. 2006).

The Cd soil contamination did not significantly modify the total contents of free AA in the aboveground biomass of both species (Figure 1). Nevertheless, contents of total free AA were decreased in the aboveground biomass of *NC* at Cd treatments in comparison with control. The total contents of free AA in aboveground biomass of *NC* were two times higher in contrast to *AH*.

The major free AA determined in *NC* and *AH* were glutamic acid (Glu), glutamine (Gln), asparagine (Asn) and aspartic acid (Asp) (Table 3). In all higher plants, inorganic N is at first reduced to ammonia prior to its incorporation into organic form. Ammonia is assimilated into organic form as Glu and Gln, which serve as the N donors in the biosynthesis of essentially all amino acids and other nitrogen-containing compounds (Sánchez-Pardo et al. 2012). The free Glu content in the aboveground biomass of *NC* was stimulated under Cd1 treatment (24% increase compared to the control), but Cd2 and Cd3 treatments decreased its content. The significant changes of free Glu were observed for Cd3 treatment (66% decrease compared with control). Our findings indicated the decrease of Glu concentration under Cd2 and Cd3 treatments also for *AH*, but declines of Glu concentrations were significantly lower compared to *NC*. Sharma and Dietz (2006) and Pavlík et al. (2010) published opposite results for plants grown under trace elements stress – increase of Glu concentration in stressed plants. The Glu decline in *AH* and *NC* biomass can be caused by intensive syntheses of plant defence elicitors. The significant relationships between Glu concentrations in both plants and biomass yield, and also between Glu concentrations and Cd content in biomass were confirmed using linear regression (R^2 = 0.99 for both sampling periods).

Table 3. The concentrations of selected free amino acids in aboveground biomass during growth of *Noccaea caerulescens* and *Arabidopsis halleri* (μmol/kg fresh weight ± S.E.; n = 3). I. – 30 days; II. – 120 days from planting

	Control		Cd1		Cd2		Cd3	
	I.	II.	I.	II.	I.	II.	I.	II.
Noccaea caerulescens								
Glu	244 ± 8^{aA}	2228 ± 4^{bA}	303 ± 9^{aB}	1476 ± 13^{bB}	182 ± 8^{aC}	1237 ± 41^{bC}	51 ± 3^{aD}	833 ± 11^{bD}
Gln	9520 ± 8^{aA}	$14\,356 \pm 5^{bA}$	6715 ± 24^{aB}	$18\,970 \pm 13^{bB}$	6641 ± 8^{aC}	4042 ± 20^{bC}	5940 ± 12^{aD}	2242 ± 19^{bD}
Pro	6944 ± 8^{aA}	6480 ± 4^{bA}	$16\,919 \pm 13^{aB}$	8566 ± 25^{bB}	$16\,449 \pm 9^{aB}$	5707 ± 8^{bC}	$10\,360 \pm 17^{aC}$	7216 ± 12^{bD}
Hyp	N.D.	23 ± 3^{A}	N.D.	55 ± 2^{B}	N.D.	18 ± 1^{C}	N.D.	6 ± 1^{D}
Asp	2632 ± 6^{aA}	7071 ± 6^{bA}	3274 ± 12^{aB}	5816 ± 13^{bB}	2708 ± 9^{aA}	3460 ± 1^{bC}	2990 ± 39^{aC}	6470 ± 12^{bD}
Asn	2840 ± 6^{aA}	3306 ± 5^{bA}	2533 ± 8^{aB}	1748 ± 13^{bB}	1781 ± 9^{aC}	1372 ± 20^{bC}	1459 ± 14^{aD}	2478 ± 12^{bD}
Arabidopsis halleri								
Glu	723 ± 4^{aA}	1535 ± 9^{bA}	736 ± 15^{aA}	3150 ± 19^{bB}	1265 ± 27^{aB}	1767 ± 31^{bC}	985 ± 5^{aC}	1447 ± 4^{bD}
Gln	5917 ± 5^{aA}	5033 ± 5^{bA}	3226 ± 8^{aB}	1172 ± 26^{bB}	1596 ± 25^{aC}	1403 ± 5^{bC}	2089 ± 5^{aD}	1357 ± 14^{bC}
Pro	258 ± 49^{aA}	429 ± 3^{bA}	224 ± 2^{aB}	278 ± 1^{bB}	389 ± 12^{aC}	232 ± 1^{bC}	196 ± 1^{aB}	360 ± 9^{bD}
Hyp	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Asp	915 ± 6^{aA}	1732 ± 10^{bA}	1122 ± 4^{aB}	6082 ± 9^{bB}	1225 ± 8^{aC}	1617 ± 9^{bC}	1593 ± 4^{aD}	1308 ± 6^{bD}
Asn	3155 ± 12^{aA}	6364 ± 14^{bA}	2324 ± 14^{aB}	$18\,170 \pm 90^{bB}$	3468 ± 12^{aC}	2582 ± 9^{bC}	3985 ± 10^{aD}	5440 ± 15^{bD}

Different letters (a) indicate significantly different values ($P < 0.05$) between samples of plant species. Different letters (A) indicate significantly different values ($P < 0.05$) between treatments of plant species. N.D. – not detected. Glu – glutamic acid; Gln – glutamine; Pro – proline; Hyp – hydroxyproline; Asp – aspartic acid; Asn – asparagine (Asn). Control – 0 mg Cd/kg soil; Cd1 – 30, Cd2 – 60 and Cd3 – 90 mg Cd/kg soil

Gln is dominant free AA in biomass of *NC*. Gln concentrations in *AH* were significantly lower in contrast to *NC*. These results showed a different pathway of nitrogen utilization of both plants. Cd treatments resulted in a decrease of Gln contents compared with control (for Cd3 treatment – by 85% for *NC* and by 73% for *AH*). Results confirmed the significant relationship between contents of Gln, biomass yield and Cd content in biomass (R^2 = 0.99 for both sampling periods). Gln is not only the major AA used for N transport in *NC*, but also a key metabolite that acts as an amino donor to other free amino acids, primarily catalyzed by glutamate synthase. This pathway interacts with carbohydrate metabolism or the energy status of the plant leaves (Hodges et al. 2003). Gln and Glu can be used to form Asp and Asn, and these four AA are used to translocate

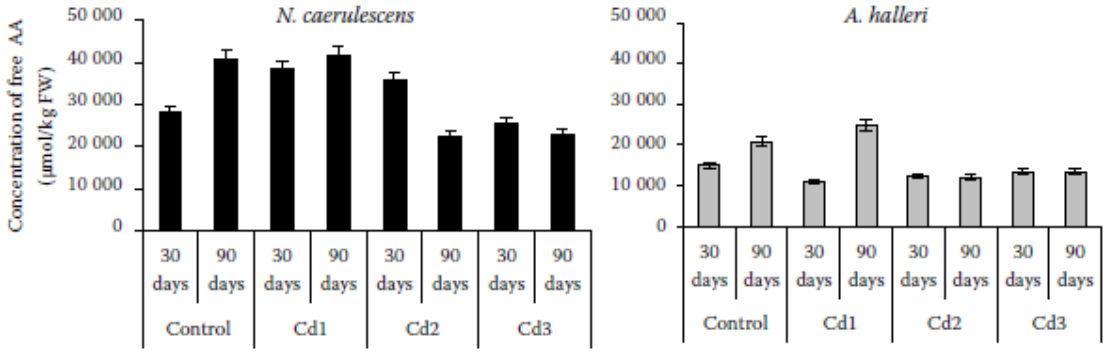

Figure 1. Total contents of free amino acids in aboveground biomass of *Noccaea caerulescens* and *Arabidopsis halleri* exposed to increasing rates of Cd in soil. Values are the means (± S.E.) of 3 replicates. AA – amino acid; Control – 0 mg Cd/kg soil; Cd1 – 30, Cd2 – 60 and Cd3 – 90 mg Cd/kg soil

organic N from sources to sinks (Mokhele et al. 2012). Our results indicated the increase of free Asp concentrations under Cd stress in *NC*. All Cd treatments increased accumulation of free Asp contents (24%, 29% and 11% increase compared to the control) in the aboveground biomass. The changes of Asp in *AH* were not significant. The strong linear relationship between Asp and Glu was calculated: for *AH* (R^2 = 0.90), and for *NC* (R^2 = 0.68). Aspartate amino-transferase plays a central role in both Asp synthesis and catabolism. According to Pandey et al. (2004), aspartate amino-transferase activity decreased considerably during stress, and the reduction was greater with increased stress.

Asparagine is an AA used to store and transport N from sources to sinks. Our determinations showed a decrease of Asn concentration in biomass of *NC* only in the 2nd sampling period (by 25–59% in contrast to control). *AH* did not shown significant changes under Cd stress. This could be associated with the remobilization of assimilated nitrogen as proteins and other substance. According to Zhang et al. (2013) Asn is a major form of N transported to sink tissues in *Arabidopsis* mutant. Our results confirmed their findings: free Asn was the dominant free AA in *AH*. Substantial changes in xylem Asn level can occur under certain stress conditions often associated with reciprocal changes in Asp levels (Antunes et al. 2008).

Proline accumulation was reported in tissues/organs of plants subjected to various abiotic stresses including risk element toxicity for many plants (Zengin and Munzuroglu 2005, Mistra and Dubey 2006 etc.). Our results did not confirm this trend. The significant increase of free Pro contents in Cd treatments of *NC* in contrast to control was determined in the 1st sampling period. Pro increases in the 2nd sampling period were lesser compared to the 1st sampling period. The free Pro accumulation in aboveground biomass was affected not only by Cd soil contamination, but also by process of plant adaptation to chronic stress and plant growing period (Pavlíková et al. 2008). *AH* showed similar Pro content in biomass in both sampling periods (Table 3). The content of free Pro in aboveground biomass of *AH* was about 15–75 times less in comparison to *NC*. According to García-Ríos et al. (1997), Pro inhibition depends on Glu concentration. This finding confirms the ability of plant chronically stressed by toxic elements to obtain adequate Glu concentration for synthesis of phytochelatins. The relationship between free Pro and Cd contents in biomass was confirmed using linear correlation and the most significant relationship was calculated for the 2nd sampling period (R^2 = 0.65–1).

Hydroxyproline (Hyp) is a major AA in plant cell wall hydrolysates (Deepak et al. 2010). Considerably more hydroxyproline is found in the protein of rapidly proliferating tissue than in proteins of slowly proliferating tissue. The free Hyp was found only in aboveground biomass of *NC* during the 2nd sampling period (Table 3). The lowest concentration of Cd increased the content of free Hyp. Opposite effect – decrease of free Hyp content was found for Cd2 and Cd3 treatments. These results showed linear relationship between contents of free Pro and contents of free Hyp (R^2 = 0.33–1) in *NC*. The Hyp content was associated with the beginning of plant senescence. Hyp in *AH* biomass was below detection limit of gas chromatography (GC).

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The significance of methionine, histidine and tryptophan in plant responses and adaptation to cadmium stress

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ABSTRACT

Noccaea caerulescens (*NC*) and *Arabidopsis halleri* (*AH*) were studied to compare cadmiunm (Cd) accumulation and resistance. After 30, 60 and 90 days of plant cultivation in Cd contaminated soil (Cd1 = 30, Cd2 = 60 and Cd3 = 90 mg Cd/kg soil) amino acids were determined in plants. The comparison between both species showed that Cd stress resulted in different changes of amino acids levels playing a significant role in plant adaptation to Cd stress. Our analyses indicated higher accumulations of amino acids in the roots of *NC* compared to *AH*. Contrasting responses of plants to Cd contamination were confirmed in methionine metabolism. Methionine was determined only in roots of *AH* after 30 and 60 days of plant cultivation. Free methionine content decreased with increasing Cd contamination (Cd3 treatment – 40% decrease compared to the control treatment). Our results also showed that *NC* contains more than 10-fold higher content of histidine than *AH*. These observations indicated that this amino acid may be involved in Cd resistance and accumulation by reducing oxidative damage. Tryptophan plays a major role in the regulation of plant development and in defense responses. Its significant increase for *NC* treatments in contrast to *AH* treatments was determined.

Keywords: abiotic stress; amino acids; heavy metals; *Thlaspi caerulescens*

Cadmium (Cd) can have detrimental effects on plant growth and development even at very low concentrations. Leaf concentrations greater than 5–10 µg Cd/g dry matter (DM) are toxic to most plants (White and Brown 2010). Nevertheless, a few plant species have evolved the ability to accumulate and tolerate Cd concentrations that exceed 0.1% dry shoot mass without showing stunted growth and/or other toxicity symptoms (Koren et al. 2013). The term 'hyperaccumulator' was coined for plants that actively take up exceedingly large amounts of one or more heavy metals from the soil. Moreover, the heavy metals are not retained in the roots but are translocated to the shoot and accumulated in the aboveground organs, especially leaves, at concentrations 100–1000-fold higher than those found in non-hyperaccumulating species (Rascio and Navari-Izzo 2011, Pollard et al. 2014). Hyperaccumulators are tolerant to metals, but hyperaccumulation and tolerance are genetically independent traits.

According to Leitenmaier and Küpper (2011) hyperaccumulator plants have to store the taken up metal in a way that it does not harm important enzymes and especially not photosynthesis. It was shown that high amounts of metals are stored specifically in the vacuoles of large epidermal cells (Küpper et al. 1999, 2001), where no chloroplasts are located, and therefore, photosynthesis cannot be inhibited. According to Cappa and Pilon-Smits (2014) hyperaccumulators have enhanced levels of transporters (as a result of gene duplication) for uptake into the root and translocation within the

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plant. Hypertolerance and resistance mechanisms that were identified include enhanced levels of chelators or of enzymes that convert the element to less toxic forms.

Noccaea caerulescens and *Noccae praecox* are considered to be the most Cd-tolerant plant species, and they show the highest Cd hyperaccumulation capacities (Cosio et al. 2004, Vogel-Mikuš et al. 2005). In addition, *Arabidopsis halleri* can accumulate significant amounts of Cd without detrimental effects on plant growth and development (Zhao et al. 2006). According to Meyer et al. (2011) concentrations of phytochelatins in Cd-treated roots were the highest in *A. thaliana*, intermediate in *A. halleri* and the lowest in *N. caerulescens*. The comparison between hyperaccumulator with non-accumulator sister species (e.g. *A. halleri* with *A. thaliana*) suggests that hyperaccumulating features could reside in sequence mutations, gene copy number and/or in different expression levels of the proteins that contribute to metal tolerance (Gallego et al. 2012). According to Maestri et al. (2010) the two plant species *N. caerulescens* and *A. halleri* have evolved different mechanisms to control hyperaccumulation. The impact of trace elements on plant metabolism means that hyperaccumulator species must possess mechanisms for more efficient protein turnover. Proteomic analysis revealed the modulation or specific induction of several proteins involved in protein metabolism (DalCorso et al. 2013). The changes of amino acid levels can play a significant role in the physiological mechanism; therefore objectives of this study were to evaluate the differences of amino acid metabolism as expression of resistance to Cd soil contamination.

MATERIAL AND METHODS

The effect of Cd concentration on the levels of free amino acids was investigated in the pot experiment (Zemanová et al. 2013). Two species *Noccaea caerulescens* (formely *Thlaspi caerulescens* J. & C. Presl, FK Mey) ecotype cv. Ganges (southern France) (*NC*) and *Arabidopsis halleri* (O'Kane and AL Shehbaz) (northern France) (*AH*) were planted into pots (two plants per pot) containing 3 kg of soil (Chernozem modal, CEC 258 $mmol_+/kg$, C_{org} 1.83 %, pH_{CaCl_2} 7.2, total Cd content 0.42 mg/kg). Soil was thoroughly mixed with 0.3 g N, 0.10 g P, and 0.24 g K applied in the form of ammonium nitrate and potassium hydrogen phosphate for control treatment and with the same amount of nutrients plus Cd in the form of $Cd(NO_3)_2 \cdot 4\,H_2O$ in concentrations: 0 (control); 30 (Cd1); 60 (Cd2) and 90 (Cd3) mg/kg, for treated variants. Plants were harvested 30, 60 and 90 days after Cd application.

The amino acids from methanol + H_2O extracts from mature leaves were determined using EZ-faast amino acid analysis procedure (Phenomenex, Santa Clara, USA). Samples were analyzed for amino acid contents by GC-MS using the Hewlett Packard 6890N/5975 MSD (Agilent Technologies, Torrance, USA) (Pavlík et al. 2010).

Plant samples were decomposed using the dry ashing procedure in a mixture of oxidizing gases (O_2 + O_3 + NO_x) in a Dry Mode Mineralizer Apion (Tessek, Prague, Czech Republic). The ash was dissolved in 1.5% HNO_3. Aliquots of the certified reference material RM NCS DC 73350 poplar leaves (purchased from Analytika, Czech Republic) were mineralized under the same conditions for quality assurance. The Cd concentrations were analyzed by ICP-OES (Varian VistaPro, Varian, Mulgrave, Australia).

RESULTS AND DISCUSSION

Results of the pot experiment revealed the different effect of Cd on *NC* and *AH*. The yield of the aboveground biomass of *AH* was higher for all treatments in contrast to *NC* (Figure 1). Growing Cd doses were associated with the inhibition of above-ground and root biomass and with the enhancement of Cd content in leaves. The higher Cd content was determined for all Cd treatments of *NC* compared to Cd treatments of *AH* (Figure 2). The opposite effects were observed in control variants – higher Cd contents in *AH* in contrast to *NC* were determined. Our data correspond with those by Zemanová et al. (2013) and Procházková et al. (2012) who reported that excessive amounts of toxic elements in contaminated soil inhibited plant growth and development due to their phytotoxicity. The Cd content of roots was determined without replication, because there was a lack of biomass. The analyses showed values similar to Cd contents in the aboveground biomass (data is not shown here). Toxic Cd levels reduced incorporation of free amino acids into proteins. It caused the decline in protein content and therefore the decline of biomass accumulation (Solanki and

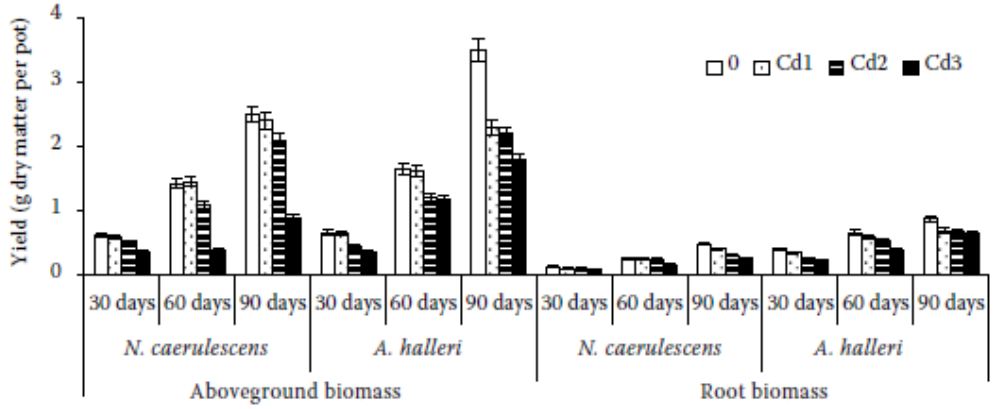

Figure 1. Effect of Cd on the aboveground biomass and root yield (g dry matter per pot) of *Noccaea caerulescens* and *Arabidopsis halleri*: 30, 60, 90 days from planting. Data are means ± S.E. (n = 3). Control – 0 mg Cd/kg soil; Cd1 – 30, Cd2 – 60 and Cd3 – 90 mg Cd/kg soil

Dhankhar 2011). This finding confirmed our results – higher Cd content in plant biomass and lower yield of *NC* in contrast to *AH*.

The changes of amino acid levels can play a significant role in mechanism of plant adaptation to Cd stress. Chaffei et al. (2004) suggested that an increase in the proportion of high N:C amino acids is a protective strategy in plants for preserving roots as a nutritional safeguard organ to ensure future recovery. Consistent with this hypothesis, our analyses indicated the accumulation of a large amount of amino acids in the roots of *NC* compared to *AC* (Figure 3). The highest accumulation of amino acids (AA) was determined on Cd2 treatment of *NC* after 90 days of cultivation. The high content of AA in roots of *NC* indicated high Cd accumulation and tolerance of this plant. The amino acid accumulation in *NC* roots also suggested that these Cd-chelating molecules are highly active in plant roots and that upon binding Cd, they may form a complex that can be translocated from the roots to the shoots (Couturier et al. 2010).

Methionine (Met) is one from AA with different content in *AH* in contrast to *NC*. Methionine originates from three convergent pathways: the carbon backbone deriving from aspartate, the sulfur atom from cysteine, and the methyl group from the β-carbon of serine. It is an amino acid that supports additional roles than simply serving as a building block for protein synthesis. This is because methionine is the immediate precursor of *S*-adenosylmethionine, which plays numerous roles of being the major methyl-group donor in

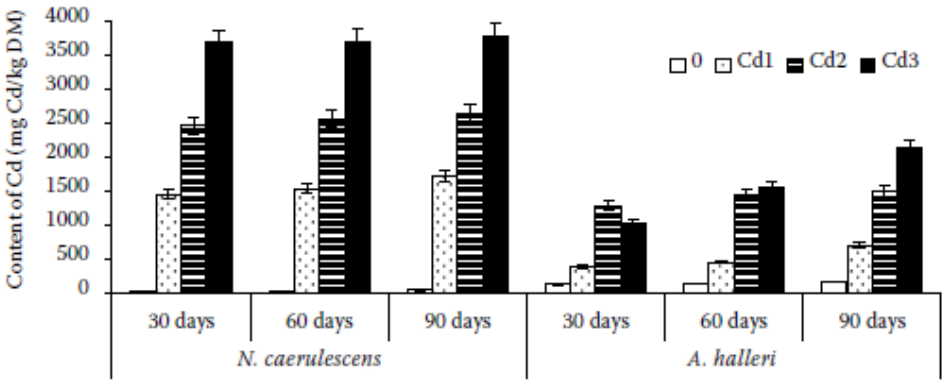

Figure 2. Total contents of Cd in the aboveground biomass of *Noccaea caerulescens* and *Arabidopsis halleri* exposed to increasing rates of Cd in soil. Data are means ± S.E. (n = 3). DM – dry matter; control – 0 mg Cd/kg soil; Cd1 – 30, Cd2 – 60 and Cd3 – 90 mg Cd/kg soil

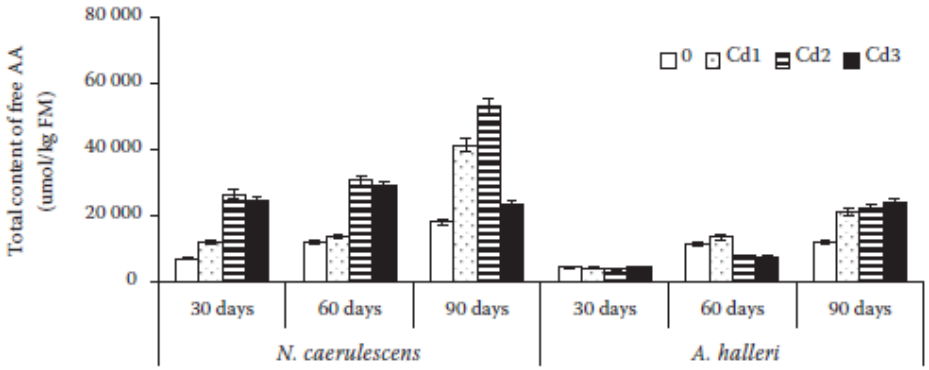

Figure 3. Total contents of free amino acids (AA) in roots of *Noccaea caerulescens* and *Arabidopsis halleri* exposed to increasing rates of Cd in soil. Data are means ± S.E. (n = 3). Control – 0 mg Cd/kg soil; Cd1 – 30, Cd2 – 60 and Cd3 – 90 mg Cd/kg soil; FM – fresh matter

transmethylation reactions and an intermediate in the biosynthesis of polyamines and of the phytohormone ethylene etc. (Ravanel et al. 1998). Met was differentially regulated between the tested plant species since its content was determined only in roots of *AH* after 30 and 60 days of plant cultivation (Figure 4), but it was not detected in *NC*. The effect of Cd contamination was confirmed after 60 days of plant cultivation, free Met content decreased with increasing Cd contamination (Cd3 treatment – 40% decrease compared to control treatment). Similar results were published for two lines of tobacco by Pavlíková et al. (2014). The results showed that *NC* regulated more effective Met compared to *AH*. This AA is quickly transformed into the required products or incorporated into a protein without increased accumulation in plant. The Met accumulation in *AH* plants in contrast to *NC* can be related to the oxidation of Met to methionine sulfoxide, which alters the activity and conformation of various proteins, can be reversed by methionine sulfoxide reductase (MSR). MSR participates in a protein repair system that is one of the defensive mechanisms that diminishes oxidative destruction (Li et al. 2012, Zagorchev et al. 2013). According to Ingle et al. (2005) Met biosynthesis is suppressed in *Alyssum lesbiacum*, indicating that thiol groups are diverted toward cysteine and glutathione biosynthesis. In contrast, methionine synthase was induced after metal treatment in *Phytolacca americana* (Zhao et al. 2011), suggesting there are diverse strategies for metal detoxification in hyperaccumulator species.

The observation of Holmes and Appling (2002) shows the possibility of a metabolic link between methionine and histidine (His) biosynthetic pathway through accumulation of 5'-amino 4-carboxamide ribonucleotide interfering with Met biosynthetic pathway. His was found to play an important role in regulation of biosynthesis of other AA, in chelation

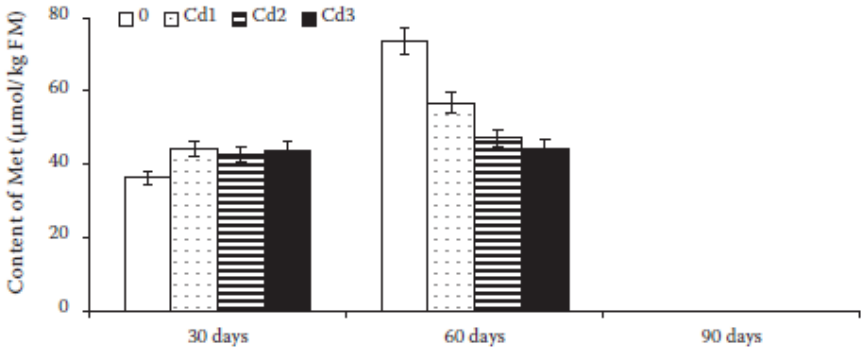

Figure 4. Total contents of free methionine (Met) in roots of *Arabidopsis halleri* exposed to increasing rates of Cd in soil. Data are means ± S.E. (n = 3). Values of Met analyzed after 90 days of plant cultivation were below detection limit of gas chromatography. Control – 0 mg Cd/kg soil; Cd1 – 30, Cd2 – 60 and Cd3 – 90 mg Cd/kg soil; FM – fresh matter

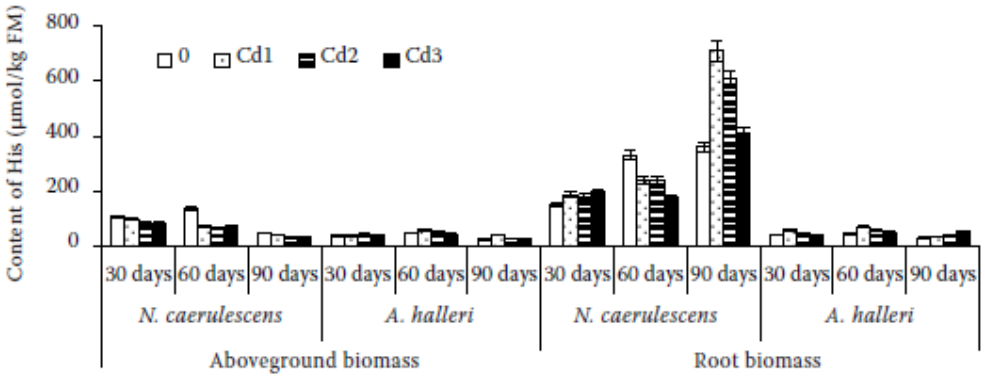

Figure 5. Total contents of free histidine (His) in the aboveground biomass and roots of *Noccaea caerulescens* and *Arabidopsis halleri* exposed to increasing rates of Cd in soil. Data are means ± S.E. (n = 3). Control – 0 mg Cd/kg soil; Cd1 – 30, Cd2 – 60 and Cd3 – 90 mg Cd/kg soil; FM – fresh matter

and transport of metal ions, and in plant reproduction and growth (Stepansky and Leustek 2006). Our result showed that content of His was accumulated during *NC* growing period, while significant changes were not observed in *AC* roots (Figure 5). The highest difference between *NC* and *AH* treatments was determined on Cd2. Accumulation of free His in *NC* roots of Cd2 treatment is more than 19-fold higher in contrast to Cd2 treatment of *AH*. These observations indicated that His may be involved in Cd resistance and accumulation by reducing oxidative damage. According to Xu et al. (2012) the high accumulation of His in plant promoted Cd uptake and improved root-to-shoot Cd transport, which thereby increased leaf Cd accumulation. Compared to other known low-molecular-weight metal chelators such as phytochelatins and nicotianamine, histidine is of relatively low metabolic cost. His biosynthesis does not involve the assimilation of sulfate as is required for the biosynthesis of phytochelatins, and it contains 6 C and 3 N atoms compared with nicotinamine (12 C and 3 N) or phytochelatins (approximately 18 or 36 C, 5 or 10 N, and 2 or 4 S) (Stepansky and Leustek 2006).

The histidine biosynthesis pathway is integrated with a number of other metabolic pathways including tryptophan (Trp). Tryptophan plays a major role in the regulation of plant development and defense responses and it is the precursor for indolacetic acid, a plant hormone necessary for cell expansion. Our results showed the significant increase of this AA for *NC* treatments (Figure 6). According to Pavlík et al. (2012) Trp biosynthesis is induced by stresses. However, the significant changes during growing period of *AH* were not confirmed. Little is known about Trp-mediated trace elements tol-

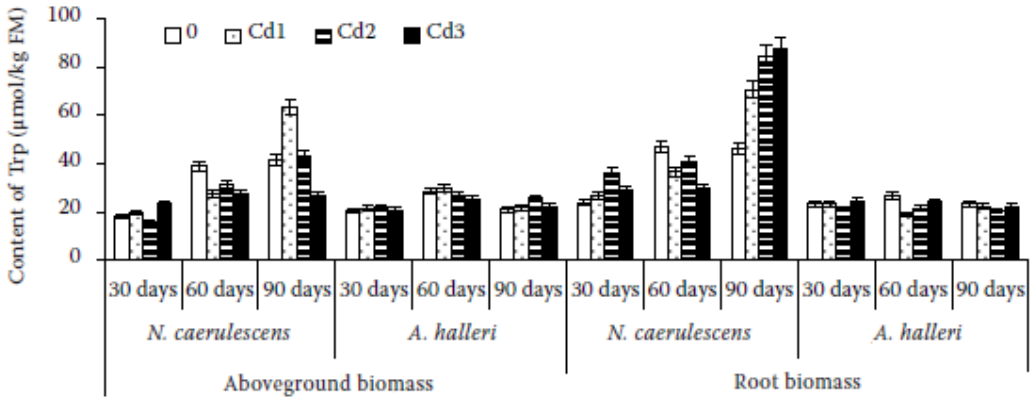

Figure 6. Total contents of free tryptophan (Trp) in the aboveground biomass and roots of *Noccaea caerulescens* and *Arabidopsis halleri* exposed to increasing rates of Cd in soil. Data are means ± S.E. (n = 3). Control – 0 mg Cd/kg soil; Cd1 – 30, Cd2 – 60 and Cd3 – 90 mg Cd/kg soil; FM – fresh matter

erance (Sanjaya et al. 2008). Sanjaya et al. (2008) reported that increased Trp levels make Cd less available to the plant, decrease Cd transport and thus reduce Cd accumulation. Metal ions and the bivalent Trp side chain indole were found to interact cooperatively (Li and Yang 2003).

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Physiology

Fatty acid profiles of ecotypes of hyperaccumulator *Noccaea caerulescens* growing under cadmium stress

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ABSTRACT

Changes in the fatty acid (FAs) composition in response to the extent of Cd contamination of soils (0, 30, 60 and 90 mg Cd kg^{-1}) differed between ecotypes of *Noccaea caerulescens* originating from France – Ganges, Slovenia – Mežica and Austria – Redlschlag. Mežica ecotype accumulated more Cd in aboveground biomass compared to Ganges and Redlschlag ecotypes. Hyperaccumulators contained saturated fatty acids (SFAs) rarely occurring in plants, as are cerotic (26:0), montanic (28:0), melissic (30:0) acids, and unusual unsaturated fatty acids (USFAs), as are 16:2, 16:3, 20:2 and 20:3. Typical USFAs occurring in the family *Brassicaceae*, such as erucic, oleic and arachidonic acids, were missing in tested plants. Our results clearly indicate a relationship between Cd accumulation and the FAs composition. The content of SFAs decreased and the content of USFAs increased in aboveground biomass of Ganges and Mežica ecotypes with increasing Cd concentration. Opposite trend of FAs content was determined in Redlschlag ecotype. Linoleic (18:2*n*−6), α-linolenic (18:3*n*−3) and palmitic (16:0) acids were found in all ecotypes. The results observed in *N. caerulescens* ecotypes, showed that mainly Mežica ecotype has an efficient defense strategies which can be related on changes in FAs composition, mainly in VLCFAs synthesis. The most significant effect of ecotype on FAs composition was confirmed using multivariate analysis of variance.

Introduction

Plant membrane structure maybe regarded as the first living structure that is a target for heavy metal toxicity (Hall, 2002). In plants, Cd can lead to many morphological, physiological, biochemical and structural alterations (Shah and Dubey, 1998) via at least five specific mechanisms. These include: (i) inactivation of proteins by binding to sulfhydryl groups; (ii) displacement of metals from metabolites or metalloenzymes; (iii) induction of detoxification of metabolites; (iv) induction of senescence and proteolysis of proteins; and (v) increasing formation of reactive oxygen species. Fatty acids (FAs) and fatty acid-derived molecules have multiple roles in cells. FAs act as storage reserves that can be metabolized to produce energy; they are the building blocks of membrane lipids and act as signaling molecules (Baker et al., 2006). FAs are crucial components of cellular membranes. According to Ben Ammar et al. (2007), the decrease in the membrane lipids and FAs content may be correlated with an inhibition of lipid-biosynthesis pathways and/or a stimulation of lipolytic and peroxidative activities. Skórzyńska-Polit and Krupa (2006) assume that when lipid peroxidation occurs via free radicals, the FAs react with other cell compounds and numerous reactions are initiated in a "cascade", causing degradation of free and membrane-bound FAs and finally degradation of the biological membranes. Maksymiec (1997) showed that membrane unsaturation was closely related to heavy metal tolerance in a number of higher plants. The biosynthesis of FAs occurs predominantly in the two subcellular compartments – chloroplasts and endoplasmic reticulum. De novo synthesis from acetyl-CoA of FAs such as palmitic or stearic and also desaturation of stearic acid to oleic one occur in plastids, whereas the further conversion of oleic acid in linoleic and further linolenic acid occurs in the endoplasmic reticulum (Sidorov and Tsydendambaev, 2014). FAs contribute to

Abbreviations: FAs, fatty acids; FAMEs, methyl esters of fatty acids; Ga, ecotype from Ganges, France; Me, ecotype from Mežica, Slovenia; SFAs, saturated fatty acids; Re, ecotype from Redlschlag, Austria; SVLCFAs, saturated very-long-chain fatty acids; USFAs, unsaturated fatty acids.

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inducible stress resistance through the remodeling of membrane fluidity, the release, through lipase activity, of α-linolenic acid, and as modulators of plant defense gene expression (Upchurch, 2008). The ability to adjust membrane lipid fluidity by changing levels of unsaturated fatty acids (USFAs) is a feature of stress acclimating plants provided mainly by the regulated activity of fatty acid desaturases (Upchurch, 2008; Los et al., 2013). Fatty acid desaturases are enzymes that introduce double bonds into fatty acyl chains and play a key role in the maintenance of the proper structure and functioning of biological membranes (Los and Murata, 1998).

A limited number of plant species, called hyperaccumulators, accumulate heavy metals to extremely high, normally severely toxic concentrations in their shoots. In comparison with normal plants, hyperaccumulators are characterized by strongly enhanced rates of uptake, tolerance and root-to-shoot transport of the metals in question (Assunção et al., 2001; Krämer, 2010). These plants are highly attractive model organisms for experiments because they have overcome major physiological problems that limit metal accumulation in biomass, and have toxic element tolerance (Verbruggen et al., 2009; Ueno et al., 2011). Selective pressure of contaminated sites, where the ecotypes of hyperaccumulator *Noccaea caerulescens* originate from, affected their metabolism (metabolism of amino acids, gas-exchange plant parameters, accumulations of macro- and microelements, etc.). Cadmium is chemically similar to certain metal elements, including Fe, Zn and Ca, and, therefore, could displace these elements from metalloproteins (Verbruggen et al., 2009). The elements, such as Fe, Zn, Cu, Mn, Ni, are cofactors of metalloenzymes and their contents related to plant defense against oxidative stress (Cakmak, 2000; Mustafiz et al., 2014). Candan and Tarhan (2005) found that the peroxidation of polyunsaturated fatty acids in membranes increased with decreasing Ca^{2+} concentrations during plant growing period.

Changes in metabolisms of ecotypes of *N. caerulescens* were detected in our previous experiments therefore we can assume significant changes of FAs profiles. We hypothesized that selective pressure of geographically distinct contaminated places, from which *N. caerulescens* ecotypes originated, reflect in different changes of FAs profiles. The aim of the present work was to examine the changes in accumulation of saturated fatty acids (SFAs) and unsaturated fatty acids (USFAs) in aboveground biomass of *N. caerulescens* ecotypes upon exposure to Cd stress.

Material and methods

Plant material and cultivation conditions

In the pot experiments, *Noccaea caerulescens* (formerly *Thlaspi caerulescens* J. & C. Presl, FK Mey) ecotypes originating from Mežica, Slovenia (Me), Ganges, France (Ga) and Redlschlag, Austria (Re) were used. The Mežica mining district source area is characterized by the presence of ore minerals of geogenic/technogenic origin (cerussite, sphalerite, smithsonite and galena). The environs of Mežica are strongly polluted with Pb and Zn. Because Cd is found as a trace element in sphalerite and smithsonite, its content correlates with that of Zn. Maximum Cd content in soil was about 265.6 mg kg^{-1} (Gosar and Miler, 2011). The mining district of Les Malines in the vicinity of Ganges (France) is very rich in Zn and Pb, associated with Fe–S (pyrite) components and according to Escarré et al. (2011) soil Cd content was 35.2–225 mg kg^{-1}. The bedrock of Redlschlag is composed of serpentine, which contains high levels of Ni and Cr and some Zn and Co. Because the soil pH is neutral (approx. pH 6.55), the main problems for plants are low concentrations and availability of micronutrients, although Mg is abundant (46,400 mg Mg kg^{-1}; Puschenreiter et al., 2005). According to Wenzel and Jockwer (1998) Cd content was 3.7 mg kg^{-1} in soils of Redlschlag.

For the cultivation of *Noccaea* plants, 3 kg of soil (from the non-polluted site Prague-Suchdol, Chernozem – pH = 7.2, CEC = 258 $mol_{(+)}$ kg^{-1}, $C_{org.}$ = 1.8%, Cd_T = 0.42 mg kg^{-1}) was thoroughly mixed with nutrients (0.3 g N, 0.10 g P, and 0.24 g K applied in the form of NH_4NO_3 and K_2HPO_4) as the control treatment and with the same amount of nutrients plus Cd ($Cd(NO_3)_2{\cdot}4H_2O$) in concentrations: 30, 60, 90 (Cd1, Cd2, Cd3) mg Cd.kg^{-1}, for treated variants. The water regime was controlled and the soil moisture was kept at 60% MWHC (maximum water-holding capacity). Each treatment was performed in five replications. Plants were harvested 90 and 120 days after Cd application.

Analyses

Determination of fatty acids

Overall content of fatty acids (free and derived from various lipids) was determined after their conversion to respective methyl esters (FAMEs). Samples of dry biomass (~0.2 g) were extracted 2 ml of methanol + chloroform (3:2, v/v) on a shaker for 24 hours. Transesterification of FAs according to method of Stránský and Jursík (1996a,b) was carried out. The content of FAMEs was measured by GC–MS (Thermo Scientific DSQ II Single Quadrupole GS–MS, Thermo Fisher Scientific) with a nonpolar column Zebron ZB-5 30 m × 0.25 mm × 0.25 μm. The injection volume was 1 μl of sample in a splitless mode. The carrier gas was helium (He, purity 5.0) with a constant flow rate of 1 ml min^{-1}. The temperature program of oven: initial temperature 50 °C (for 2 min), 8 °C min^{-1} ramp to a temperature of 320 °C (for 10 min); inlet temperature 250 °C and transfer line temperature 260 °C.

As a complementary tool, analysis on specially designed column for FAMEs analysis (BPX70: 30 m × 0.22 mm × 0.25 mm from SGE) for double bond position confirmation with authentic standards – Supelco 37 Component FAME Mix (methyl esters of: linoleic acid 18:2*n*−6, α-linolenic acid 18:3*n*−3, cis-11,14-eicosadienoic acid 20:2*n*−6 and cis-11,14,17- eicosadienoic acid 20:3*n*−3), was performed on selected samples. Oven temperature program was set from 120 °C to 260 °C at a rate of 10 °C min^{-1}, injector temperature 280 °C with split mode.

Analyses of cadmium in plant biomass

Plant samples were decomposed using the dry ashing procedure. The ash was dissolved in 20 mL of 1.5% HNO_3 (v/v) (electronic grade purity, Analytika Ltd., Czech Republic) and kept in glass tubes until analysis. Aliquots of the certified reference material RM NCS DC 73350 poplar leaves (purchased from Analytika, CZ) were mineralized under the same conditions for quality assurance. The Cd concentrations were determined by ICP-OES with axial plasma configuration (Varian VistaPro, Varian, Australia).

The statistical analyses were performed using multivariate analysis of variance (MANOVA) with multivariate F value (Wilks' lambda). A MANOVA was applied to identify the effect of treatments, sampling period, ecotypes and their interactions as independent variables, and contents of saturated and USFAs as dependent variables. A MANOVA was followed by post hoc comparison Tukey test ($P < 0.05$) and linear correlation and/or correlation using function of the polynomial 3rd degree (R^2). All analyses were performed with Statistica 12.0 software (StatSoft, Tulsa, USA).

Results

Biomass yield and Cd accumulation

Cd accumulation in aboveground biomass of ecotypes of *N. caerulescens* grown under Cd stress for 90 and 120 days are shown in Fig. 1. Cd contents of biomass showed significant differences among tested ecotypes of *Noccaea* sp. and among treatments. The

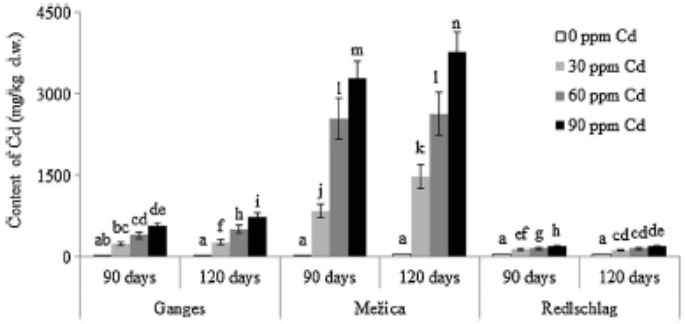

Fig. 1. Cadmium accumulation in leaves of ecotypes of *N. caerulescens* exposed to various Cd concentrations in soil for 90 days and 120 days.

highest Cd content was found in the biomass of Me plants in contrast to Ga and Re plants. The toxic effect of Cd on plant growth was confirmed only for treatment Cd3 and significant decrease of yield was found after 120 days of plant cultivation (Table 1). The highest yields of aboveground biomass were determined on Cd1 treatment of all ecotypes (30 mg Cd kg^{-1} soil) and the significantly highest yield of Me ecotype in comparison with Ga and Re was shown.

Changes in the amounts of SFAs and USFAs of aboveground biomass

Changes in the total amounts of SFAs and USFAs of aboveground biomass of all ecotypes of *N. caerulescens* under Cd stress are shown in Fig. 2. After 120 days of plant cultivation, the SFAs content in Me and Ga ecotypes declined significantly with increasing of soil Cd contamination (R^2 = 0.94–1). The higher Cd contamination reduced the content of SFAs of both ecotypes (45% and 30% decrease compared to control, respectively) compared to control. The opposite trends of the USFAs content of these plants were confirmed (R^2 = 0.94–0.97). The higher Cd rate stimulated the content of USFAs of both ecotypes (42% and 18% increase compared to control, respectively). Our findings indicated an opposite effect of Cd concentration on total SFAs and USFAs contents in ecotype Re (28% increase, R^2 = 0.99, and 29% decrease, R^2 = 0.99–1 compared to control, respectively). The changes of total FAs contents after 90 days of plant cultivation were similar as presented results.

Major SFAs contained in plants of *Brassicaceae* sp., such as lauric (12:0), myristic (14:0), palmitic (16:0), stearic (18:0), arachidic (20:0), behenic (22:0) and lignoceric (24:0) acids, were detected in all tested ecotypes (Table 2). Composition of SFAs of Re ecotype differed from Me and Ga ecotypes by higher content of 24:0 FA (average of values 8.08 wt% versus 3.36 wt%, Table 2).

Apart from these usual SFAs saturated very-long-chain fatty acids (SVLCFAs) – cerotic (26:0) and montanic (28:0) acids – have been found in all ecotypes. Melissic acid (30:0) was determined only in Me ecotype (Table 3; MS spectrum of SVLCFAs–supplementary material S1–S3). Biosynthesis of detected SFAs is described on Fig. 3. These SVLCFAs (26:0, 28:0, 30:0) were mainly presented in Me ecotype (after 90 days of cultivation–control treatment Me 4.81, Ga 1.38 and Re 1.90 wt% of total FAs contents). After 120 days, percentage of 28:0 on all control treatments increased and Cd treatments confirmed strong effect of Cd contamination on this FA level. Its decrease was determined in contrast to control treatment. 30:0 FA was measured only in ecotype Mežica. Percentage of 30:0 FA decreased with the extent of Cd contamination.

Analyses of USFAs have confirmed 7,10-hexadecadienoic (16:2*n*−6), 7,10,13-hexadecatrienoic (16:3*n*−3), 9,12-octadecadienoic (18:2*n*−6) and 9,12,15-octadecatrienoic (18:3*n*−3) acids in all ecotypes (Table 4) (Fellenberg et al., 1987). 11,14-eicosadienoic (20:2*n*−6) and 11,14,17-eicosatrienoic (20:3*n*−3) acids were detected only in Cd treatments of Me and Ga ecotypes (MS spectrum of USFAs – supplementary material S4–S9).

The content of 18:3*n*−3 FA was higher in Me and Ga ecotypes (both averages of values 35.0 wt% of total FAs content) than in Re (29.8 wt% of total FAs; Table 3). After 90 days of plant cultivation, the level of 18:3*n*−3 and 18:2*n*−6 FAs increased in Me and Ga ecotypes, while level of 16:0 FA decreased. The significant relationship between contents of 18:3*n*−3 and 18:2*n*−6 FAs and Cd content of

Table 1
Aboveground biomass yield (g dry matter per pot) of ecotypes of *N. caerulescens* grown under varying Cd concentrations (0, 30, 60, 90 mg Cd kg^{-1}).

Ecotypes of *N. caerulescens*	Treatment, mg Cd kg^{-1}	Days after Cd application	
		90	120
Redlschlag	0	1.6 ± 0.01bc	2.9 ± 0.05fg
	30	2.2 ± 0.12cdefg	3.0 ± 0.11f
	60	1.6 ± 0.07bcd	1.9 ± 0.37cdeg
	90	1.5 ± 0.03bc	1.8 ± 0.02bcde
Ganges	0	1.6 ± 0.11 cd	2.5 ± 0.41efg
	30	2.9 ± 0.21f	2.4 ± 0.37defg
	60	2.5 ± 0.15efg	2.1 ± 0.18cde
	90	1.0 ± 0.07ab	1.1 ± 0.05ab
Mežica	0	1.2 ± 0.01ab	1.4 ± 0.02bc
	30	2.2 ± 0.06cdefg	5.1 ± 0.14h
	60	1.1 ± 0.04ab	4.4 ± 0.04h
	90	1.0 ± 0.01a	1.2 ± 0.03ab

Plants were harvested 90 and 120 days after Cd application.
Data represent mean ± S.E. of three replicates. Different letters in tables indicate significantly different values (P< 0.05, in column) between FAs and treatment × sampling period × ecotype calculated by MANOVA.

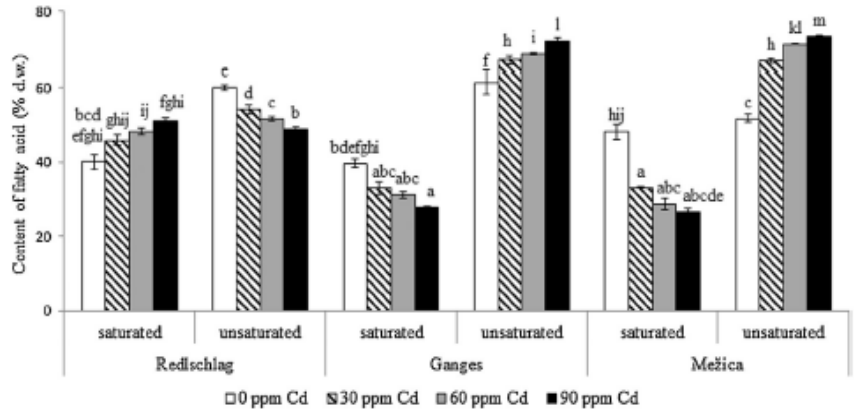

Fig. 2. Total SFAs and USFAs contents (% d.m.) of ecotypes of *N. caerulescens* grown under varying Cd concentrations for 120 days.

Me and Re ecotypes was confirmed using linear regression (Me $R^2 = 0.84$; Re $R^2 = 0.61–1$ for both FAs). This relationship is not significant for Ga ecotype ($R^2 = 0.38–1$ for both FAs). After 120 days of Cd plant stress, the levels of 18:3*n*−3 and 18:2*n*−6 FAs increased in Me and Ga ecotypes. The contents of 18:3*n*−3, 18:2*n*−6 and 16:0 confirmed strong linear correlations with Cd contents of Me and Ga ecotypes (Me $R^2 = 0.95$ for both USFAs and $R^2 = 0.67–1$ for 16:0; Ga $R^2 = 0.98$ for both USFAs and $R^2 = 0.94–1$ for 16:0). Results showed opposite trend for Re ecotype – decrease of 18:3*n*−3 and 18:2*n*−6 levels and increase of 16:0 level. The negative correlations of content of 18:3*n*−3 and 18:2*n*−6 ($R^2 = 0.98–1$ for both FAs), as well as the positive correlation of 16:0 ($R^2 = 0.70$) were calculated for Re ecotype.

Fig. 3 has shown that hyperaccumulators synthesized essential FAs with maximum efficiency with using only three desaturases.

Relationships among FAs composition and Cd contamination

Correlation coefficients (supplementary material S10–S12) confirmed relationships among different FAs and also relationships among FAs and Cd contamination. Correlations among different SFAs and VLCFAs related to their feedback regulation. From calculated correlation 12:0–14:0 and SFAs with higher number of C atoms in the aliphatic chain we can see the best adaptation of SFAs metabolism for Me ecotype. The maximum number of correlations among SFAs found for Me ecotype (S11) showed the possibility of close cooperation of SFAs metabolisms located in chloroplasts and endoplasmic reticulum.

Correlation among SFAs and USFAs (Fig. 3) showed as regulation of anabolic processes from 12:0 to 16:0, as regulation of catabolic processes contributing to the optimal quantity of free USFAs for

Table 2
Profile SFAs of ecotypes of *N. caerulescens* treated with different Cd concentrations (0, 30, 60, 90 mg Cd kg^{-1}).

Treatment		mg Cd kg^{-1}	SFA						
			12:0	14:0	16:0	18:0	20:0	22:0	24:0
Re	90 days	0	1.58 ± 0.56abcd	0.79 ± 0.15ab	25.64 ± 0.94bcde	1.29 ± 0.78a	0.40 ± 0.01a	0.71 ± 0.16abcde	7.29 ± 0.93efg
		30	1.04 ± 0.15abc	0.62 ± 0.07ab	22.73 ± 0.10bcde	1.65 ± 0.14ab	0.28 ± 0.001a	0.50 ± 0.06cde	5.91 ± 0.09hi
		60	1.18 ± 0.33abc	0.75 ± 0.11ab	21.35 ± 0.33bcde	2.16 ± 0.05ab	0.51 ± 0.04a	1.14 ± 0.07abcd	11.00 ± 0.39def
		90	1.24 ± 0.25abc	0.68 ± 0.13ab	22.76 ± 1.06abcde	1.70 ± 0.47ab	0.33 ± 0.10a	0.83 ± 0.10e	10.17 ± 0.44i
	120 days	0	1.12 ± 0.51abc	0.57 ± 0.28ab	21.84 ± 0.24bcde	1.56 ± 0.70a	0.25 ± 0.10a	0.52 ± 0.22abcd	7.32 ± 0.25efg
		30	3.23 ± 0.38 cd	1.38 ± 0.25ab	28.53 ± 0.35bcde	1.38 ± 0.10ab	0.31 ± 0.04a	0.60 ± 0.11bcde	5.41 ± 0.53fgh
		60	2.55 ± 0.96bcd	1.34 ± 0.83b	26.27 ± 0.72cde	1.85 ± 0.01ab	0.31 ± 0.01a	0.76 ± 0.01abcde	8.08 ± 0.43ghi
		90	2.06 ± 0.61d	1.65 ± 0.43ab	27.00 ± 1.30e	1.66 ± 0.12a	0.34 ± 0.12a	0.73 ± 0.24abcde	9.47 ± 1.07cde
Ga	90 days	0	0.20 ± 0.04a	0.46 ± 0.25ab	18.64 ± 1.01abc	2.37 ± 0.50abc	0.28 ± 0.25a	0.18 ± 0.005ab	3.70 ± 0.20bcd
		30	0.29 ± 0.07a	0.50 ± 0.06a	22.48 ± 2.02bcde	3.34 ± 0.39abc	0.31 ± 0.03a	0.21 ± 0.04ab	3.72 ± 0.30bcd
		60	0.31 ± 0.02a	0.55 ± 0.03ab	19.00 ± 0.58ab	3.52 ± 0.74abc	0.35 ± 0.03a	0.22 ± 0.03ab	3.71 ± 0.6bcd
		90	0.26 ± 0.1ab	0.44 ± 0.14ab	18.61 ± 0.17ab	3.73 ± 0.28abc	0.31 ± 0.31a	0.20 ± 0.03abc	3.78 ± 0.5abc
	120 days	0	1.01 ± 0.44abc	0.68 ± 0.2ab	27.01 ± 1.6de	3.22 ± 0.73abc	0.44 ± 0.18a	0.27 ± 0.11abc	4.14 ± 0.53bcd
		30	0.58 ± 0.26a	0.66 ± 0.14a	22.37 ± 2.05bcde	2.97 ± 0.63ab	0.26 ± 0.01a	0.20 ± 0.05a	3.95 ± 0.28bc
		60	0.22 ± 0.008ab	0.48 ± 0.02ab	22.53 ± 0.85bcdeb	2.30 ± 0.17abc	0.26 ± 0.06a	0.17 ± 0.01ab	3.52 ± 0.35bcd
		90	0.26 ± 0.01a	0.44 ± 0.01a	19.08 ± 0.31ab	2.48 ± 0.38ab	0.29 ± 0.005a	0.16 ± 0.01a	3.34 ± 0.21bc
Me	90 days	0	1.7 ± 0.25abcd	1.33 ± 0.10ab	27.06 ± 0.05cde	4.55 ± 0.20bc	13.16 ± 0.02c	0.75 ± 0.10de	3.29 ± 0.005abc
		30	0.81 ± 0.03abc	0.88 ± 0.01ab	24.37 ± 2.69bcde	2.18 ± 0.18ab	0.55 ± 0.08a	0.40 ± 0.07abcd	2.18 ± 0.26ab
		60	0.32 ± 0.07ab	0.45 ± 0.001ab	22.49 ± 3.15bcde	1.94 ± 0.37ab	0.27 ± 0.01a	0.13 ± 0.001a	2.01 ± 0.25ab
		90	0.45 ± 0.05ab	0.67 ± 0.05ab	13.26 ± 0.35a	5.06 ± 0.30c	0.39 ± 0.05a	0.22 ± 0.1abcde	3.56 ± 0.20abcd
	120 days	0	1.06 ± 0.20abc	0.73 ± 0.20a	22.65 ± 0.55bcde	2.47 ± 0.15abc	3.88 ± 0.20b	1.05 ± 0.10e	8.30 ± 0.20ghi
		30	0.45 ± 0.25ab	0.65 ± 0.05a	23.80 ± 1.01abcd	2.43 ± 0.55ab	0.27 ± 0.23a	0.23 ± 0.05ab	2.00 ± 0.21a
		60	0.21 ± 0.05a	0.39 ± 0.01ab	22.16 ± 0.88bcde	2.21 ± 0.99abc	0.26 ± 0.005a	0.12 ± 0.005a	1.81 ± 0.16ab
		90	0.23 ± 0.04ab	0.42 ± 0.04ab	21.20 ± 0.47bcde	1.58 ± 0.80abc	0.27 ± 0.02a	0.12 ± 0.005abc	0.84 ± 0.13ab

Plants were harvested 90 and 120 days after Cd application. Results are expressed in percentage of total fatty acid (%). Method of SFAs identification – MS/KI (MS, mass spectrum; KI, Kovats retention index).
Data represent mean ± S.E. of three replicates. Different letters in tables indicate significantly different values ($P < 0.05$, in column) between FAs and treatment × sampling period × ecotype calculated by MANOVA.

Table 3
Profile SVLCFAs of ecotypes of *N. caerulescens* treated with different Cd concentrations (0, 30, 60, 90 mg Cd kg^{-1}).

Treatment		mg Cd kg^{-1}	SVLCFA		
			26:0	28:0	30:0
Re	90 days	0	1.40 ± 0.39abcd	0.50 ± 0.16abc	n.d.**
		30	1.98 ± 0.01de	0.69 ± 0.02abcd	n.d.**
		60	3.55 ± 0.41bcd	0.82 ± 0.14abcd	n.d.**
		90	2.16 ± 0.09ef	1.49 ± 0.03abcdef	n.d.**
	120 days	0	4.07 ± 0.56f	1.95 ± 0.36def	n.d.**
		30	2.06 ± 0.14f	1.08 ± 0.02bcdef	n.d.**
		60	3.96 ± 0.16a	1.65 ± 0.17ef	n.d.**
		90	4.22 ± 0.27 cd	2.34 ± 0.17abcde	n.d.**
Ga	90 days	0	0.87 ± 0.25abc	0.51 ± 0.05abc	n.d.**
		30	1.01 ± 0.14abcd	0.50 ± 0.08ab	n.d.**
		60	0.77 ± 0.18abc	0.54 ± 0.14ab	n.d.**
		90	0.90 ± 0.09abcd	0.59 ± 0.10abcd	n.d.**
	120 days	0	1.07 ± 0.43abcd	0.80 ± 0.45abcd	n.d.**
		30	0.72 ± 0.09ab	0.35 ± 0.06a	n.d.**
		60	0.63 ± 0.08abc	0.33 ± 0.05a	n.d.**
		90	0.61 ± 0.05a	0.33 ± 0.04a	n.d.**
Me	90 days	0	1.73 ± 0.30abcd	2.24 ± 0.35f	0.84 ± 0.05e
		30	1.01 ± 0.29abcd	1.54 ± 0.49bcdef	0.67 ± 0.11d
		60	0.50 ± 0.04ab	0.46 ± 0.06abc	0.18 ± 0.06b
		90	1.17 ± 0.05abcd	1.53 ± 0.20cdef	0.36 ± 0.02c
	120 days	0	3.09 ± 0.52ef	2.92 ± 0.40f	0.53 ± 0.03d
		30	0.83 ± 0.15abc	1.09 ± 0.10abcd	0.30 ± 0.02bc
		60	0.52 ± 0.005ab	0.51 ± 0.07abc	0.26 ± 0.001a
		90	0.43 ± 0.08abcd	0.63 ± 0.03abcde	0.21 ± 0.04bc

Results are expressed in percentage of total fatty acid (%). Method of SVLCFAs identification – MS/KI.
Data represent mean ± S.E. of three replicates. Different letters in tables indicate significantly different values ($P < 0.05$, in column) between FAs and treatment × sampling period × ecotype calculated by MANOVA.
** n.d. - not detected.

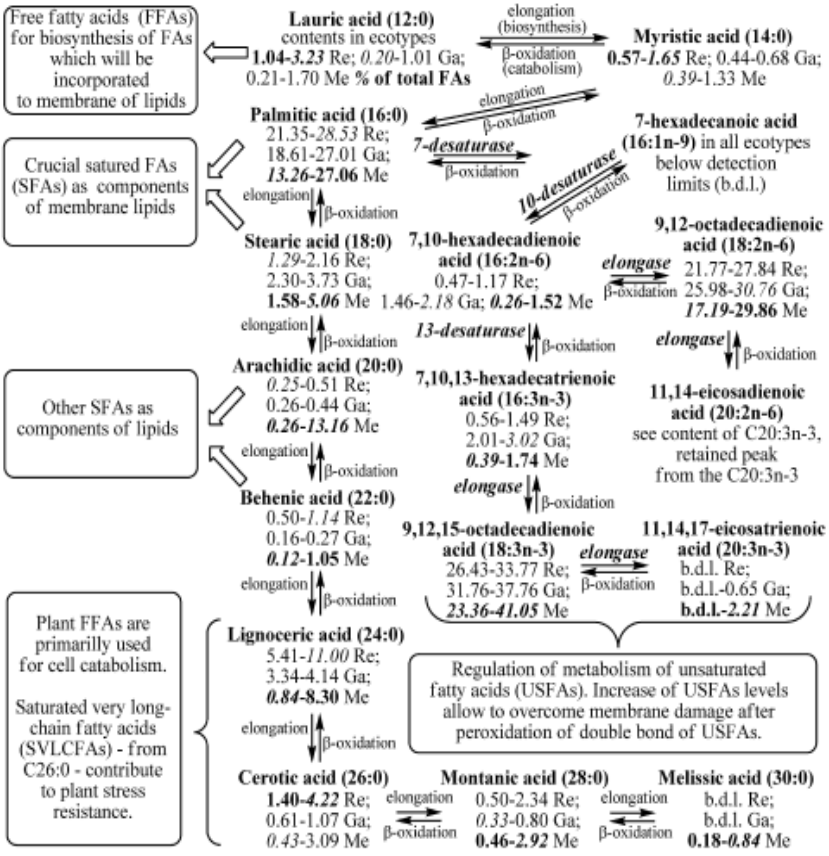

Fig. 3. Scheme of metabolic pathways of detected FAs.

Table 4
Profile USFAs of ecotypes of *N. caerulescens* treated with different Cd concentrations (0, 30, 60, 90 mg Cd kg^{-1}).

Treatment		mg Cd kg^{-1}	USFA				
			16:2	16:3	18:2	18:3	20:X*
Re	90 days	0	1.17 ± 0.02 g	1.49 ± 0.07ef	26.35 ± 0.05fg	32.35 ± 0.06e	n.d.**
		30	1.04 ± 0.05efg	1.19 ± 0.14bcde	27.84 ± 0.21hi	33.77 ± 0.01f	n.d.**
		60	1.13 ± 0.01fg	1.42 ± 0.06ef	23.77 ± 0.23e	28.67 ± 0.07 cd	n.d.**
		90	0.68 ± 0.03bc	0.90 ± 0.03b	23.94 ± 0.05e	29.58 ± 0.38d	n.d.**
	120 days	0	0.79 ± 0.005 cd	0.97 ± 0.01bc	26.09 ± 0.08fg	31.99 ± 0.008e	n.d.**
		30	0.98 ± 0.01defg	1.20 ± 0.02bcde	23.34 ± 0.13de	28.67 ± 0.32 cd	n.d.**
		60	0.87 ± 0.02cde	1.06 ± 0.03bcd	22.41 ± 0.04 cd	27.54 ± 0.15bc	n.d.**
		90	0.47 ± 0.02ab	0.56 ± 0.02a	21.77 ± 0.24bc	26.43 ± 0.12b	n.d.**
Ga	90 days	0	1.59 ± 0.01hi	2.22 ± 0.02hi	30.76 ± 0.02 h	37.76 ± 0.2jk	n.d.**
		30	2.04 ± 0.03kl	2.85 ± 0.07j	27.72 ± 0.1hi	33.89 ± 0.07f	0.34 ± 0.03b
		60	2.18 ± 0.08l	3.02 ± 0.03j	28.97 ± 0.05jkl	35.41 ± 0.1gh	0.18 ± 0.008ab
		90	1.85 ± 0.04jk	2.46 ± 0.04i	29.33 ± 0.01klm	35.84 ± 0.03hi	0.62 ± 0.05 cd
	120 days	0	1.46 ± 0.03 h	2.01 ± 0.008gh	25.98 ± 0.03f	31.76 ± 0.08e	n.d.**
		30	1.54 ± 0.04 h	2.14 ± 0.07 h	28.19 ± 0.02ij	34.51 ± 0.09fg	0.61 ± 0.04 cd
		60	1.56 ± 0.03 h	2.16 ± 0.04 h	28.96 ± 0.04jkl	35.40 ± 0.01gh	0.53 ± 0.03c
		90	1.77 ± 0.01ij	2.46 ± 0.02i	30.22 ± 0.02mn	36.96 ± 0.03ij	0.65 ± 0.04 cd
Me	90 days	0	0.26 ± 0.07a	0.39 ± 0.008a	17.19 ± 0.07a	23.36 ± 0.37a	n.d.**
		30	0.87 ± 0.03cde	1.06 ± 0.04bcd	25.62 ± 0.52f	36.97 ± 0.86ij	0.31 ± 0.01b
		60	0.89 ± 0.07cdef	1.19 ± 0.01bcde	28.36 ± 0.35ijk	39.87 ± 0.2lm	0.16 ± 0.005ab
		90	1.52 ± 0.04 h	1.74 ± 0.07fg	28.55 ± 0.47ijk	38.63 ± 0.2kl	2.21 ± 0.17e
	120 days	0	0.34 ± 0.03a	0.48 ± 0.01a	21.22 ± 0.15b	29.76 ± 0.24d	n.d.**
		30	0.96 ± 0.02defg	1.09 ± 0.06bcd	27.00 ± 0.02gh	37.29 ± 0.004j	0.31 ± 0.01b
		60	1.17 ± 0.01 g	1.37 ± 0.05de	28.52 ± 0.03ijk	39.61 ± 0.22l	0.77 ± 0.03d
		90	1.04 ± 0.02efg	1.29 ± 0.01cde	29.86 ± 0.14lmn	41.05 ± 0.01 m	0.27 ± 0.01b

Results are expressed in percentage of total fatty acid (%). Method of USFAs identification – 16:2, 16:3 – MS; 18:2, 18:3, 20:X – MS/KI.
Data represent mean ± S.E. of three replicates. Different letters in tables indicate significantly different values ($P < 0.05$, in column) between FAs and treatment × sampling period × ecotype calculated by MANOVA.
* Data represent $\sum$ unsaturated C 20= C 20:2 + C 20:3 (unseparated peak).
** n.d. - not detected.

lipid biosynthesis. Lipids are degraded by peroxidation of double bonds under oxidative stress. The highest number of correlations was found for Me ecotype in contrast to Ga and Re. During catabolic process β-oxidation of VLCFAs to palmitic acid (16:0) is induced and USFAs are formed from this FA (Fig. 3). These regulation processes create the conditions for adaptation of FAs metabolism to Cd stress.

The content of USFAs is regulated by feedback. Plant syntheses USFAs from SFAs (12:0, 14:0, 16:0) contained in chloroplasts or USFAs are obtained by catabolic process of VLCFAs from endoplasmic reticulum or from 18:0 FA supplied from chloroplasts. Correlation between USFAs (16:2*n*−6 and 16:3*n*−3) and SFAs (12:0, 14:0, 16:0) were calculated for all ecotypes. 16:2*n*−6 and 16:3*n*−3 USFAs are the products of desaturases from 16:0 and they are used as substrates for biosynthesis of different USFAs (S10–12).

Both ecotypes Me and Ga contained higher percentage of 16:2*n*−6 and 16:3*n*−3 in Cd treatments in contrast to control and 20:3*n*−3 was detected only in their Cd treatments. This finding was not confirmed for Re ecotypes. For acclimation to oxidative stress plants mainly need USFAs (18:2*n*−6, 18:3*n*−3). The percentage of these USFAs increased in Me ecotype with Cd contamination. For Ga ecotype this result was confirmed only for 120 days of plant cultivation. For syntheses of 18:2*n*−6 and 18:3*n*−3, 16:2*n*−6 and 16:3*n*−3 were elongated in chloroplasts or 20:2*n*−6 and 20:3*n*−3 are oxidized in endoplasmic reticulum.

Relationships among FAs composition and treatment × sampling period × ecotype

As it was calculated using multivariate analysis of variance MANOVA, contents of saturated and USFAs were significantly affected by treatment (Wilks' lambda 0.0000, F = 57.8, $P = 0.0000$), sampling period (Wilks' lambda 0.0171, $F = 67.2$, $P = 0.0000$), ecotype (Wilks' lambda 0.0000, $F = 645.5$, $P = 0.0000$) and treatment × sampling period × ecotype (Wilks' lambda 0.0000, $F = 25.1$, $P = 0.0000$). Results showed the most significant effect of ecotype.

Discussion

Biomass yield and Cd accumulation

According to Lombi et al. (2000) and Roosen et al. (2003) different populations of *N. caerulescens* possess different levels of Cd hyperaccumulation ability. Results of our pot experiment confirmed this finding. Accumulation of Cd in ecotypes decreased in the order Me > Ga > Re. All ecotypes of *N. caerulescens* have been identified as a Cd hyperaccumulators, which are defined by Baker et al. (2000) as plants being able to accumulate more than 100 mg Cd kg^{-1} (0.01%) in the shoot dry weight. The highest yields of biomass were determined on Cd1 treatments. Our results are consistent with the findings of other authors. According to Whiting et al. (2000) in some of the Ganges populations, local adaptation of mine ecotypes to soils highly contaminated with Zn, Pb and Cd seems to have occurred to such a degree that moderate concentrations of Cd are actually required for optimal growth, a response that may involve increased root allocation into Cd-enriched patches of soil. Roosens et al. (2003) speculated that the growth stimulation by Cd is specific to plants from the Ganges and according to these authors it is possible that Cd has acquired a biological role in some populations of *N. caerulescens*. The yields of tested ecotypes were reduced only by Cd3 contamination (90 mg Cd kg^{-1}). Our results are consistent with our previous findings (Zemanová et al., 2013, 2014) and correspond with those by Hasan et al. (2009), Pavlíková et al. (2002, 2008) and Procházková et al. (2012) who reported that excessive amounts of toxic elements in contaminated soil inhibited plant growth and development due to their phytotoxicity. All plants grew without chlorosis, leaf rolls and stunting, which are according to Benavides et al. (2005) the main and easily visible symptoms of Cd toxicity in plants.

Analysis of our results clearly showed that the degree of Cd contamination affected the tested plants. We noticed not only a quantitative biomass reduction, but also detected qualitative changes in the fatty acid composition of leaves.

Changes in the amounts of SFAs and USFAs of aboveground biomass

Under stress conditions, homeostatic regulation of the physiological fluidity of membrane lipids is regulated by a balance between the desaturation of FAs and the synthesis of membrane lipids. Oxidative stress accelerates the desaturation of membrane lipids and a result is increase in the level of unsaturation of FAs in membrane lipids. An increase in the unsaturation of FAs depends on the synthesis de novo of FA desaturases via the expression of genes for these enzymes (Mikami and Murata, 2003; Los et al., 2013). Plant tolerance under stress increases with the increased activities of desaturases (Demin et al., 2008).

The main differences between FAs composition of *N. caerulescens* ecotypes Me and Ga from control and Cd treated plants were shown by a dramatic decrease in the percentage of SFAs, and an increase in the percentage of USFAs. Our findings indicated opposite effect for Re ecotype. The percentage of different FAs was remarkably dependent on Cd rates and plant ecotypes. The results of Nouairi et al. (2006) indicated similar changes of FAs in leaves of *Brassica juncea* grown under Cd stress. According to Thompson et al. (1998) Cd enhanced lipoxygenase activity, which responsible for catalyzing lipid peroxidation by using membrane lipid components as substrates, particularly USFAs.

SFAs (12:0, 14:0) are precursors of different SFAs, mainly for 16:0. Efficient strategy of these SFAs regulation provides the ability to overcome oxidative damage.

As a consequence of Cd-induced accumulation of ROS and induced enzymatic peroxidase activities, USFAs (18:2, 18:3) in plant membrane lipids may undergo peroxidation resulting in damage and loss of membrane integrity. Verdoni et al. (2001) published significant decrease of 18:3 FA and increase of 18:1 and 18:2 FAs in primary leaves of tomato. Upchurch (2008) reviewed that in stress tolerant plants the degree of membrane lipid unsaturation, principally FA 18:3 content decreases in response to heavy metal stresses. Our results supported this finding only for Re ecotype, but they showed opposite trend mainly for Me ecotype. The percentage of FA 18:3 in Me ecotype was increased with degree of Cd contamination. FA 18:3 is mostly associated with galactolipids which account for more than 85% of thylakoid lipids and that these lipids are crucial for photosynthetic activities (Le Guédard et al., 2012). For plant growing under oxidative stress FA 18:3 is substrate rapidly degraded into metabolites, which lead to the production of jasmonic acid and oxylipins regulating growth and plant development (Savchenko et al., 2014). FA 18:1 determined in tested plants below detection limit showed higher tolerance of all ecotypes to oxidative damage. Source of three double bonds (18:3) in contrast to one double bond (16:1 and 18:1) is more effective in plant stress defense. Reduction of FA 18:1 percentage allows use of assimilated C into compounds which can be catabolized.

In plants, VLCFAs (from 26:0 to 30:0) are synthesized by successive addition of two carbon units to 16 or 18 fatty acid substrates by an elongation system (Fig. 3). The elongating enzymes are membrane-associated and organized in a complex referred to as the elongase (Moon et al., 2004). Genes of these VLCFAs have been identified in different plants, although VLCFAs were not detected. These genes known as sleeping genes were induced in tested ecotypes by phylogenetic development under selective pressure of contaminated sites. VLCFAs allow accumulation of energy supply in compounds which are rapidly catabolized into acyl-CoA. According to Xiao and Chye (2011), an acyl-CoA pool in the membrane and their recombinant proteins could bind various acyl-CoA esters such as 16:0-CoA, 18:1-CoA or 20:4-CoA *in vitro*. 18:2-CoA and 18:3-CoA are the precursors in phospholipid membrane repair.

Biosynthesis of VLCFAs decreases amount of energy necessary for plant growth and development. Catabolic processes of these FAs decrease plant sensitivity to environmental stress. Our results showed that Cd inhibited the elongation step from 28:0 to 30:0 in Re plants. This finding reflects different sensitivities of tested ecotypes to Cd stress.

The results observed in tested ecotypes, showed that mainly Me ecotype has an efficient defense strategies which can be related on changes in FAs composition, mainly in VLCFAs synthesis.

In conclusion, our hypothesis was supported by results of the pot experiment which confirmed that ecotypes of *N. caerulescens* growing under selective pressure of geographically distinct contaminated places differed in physiological performance. Our hypothesis was confirmed using multivariate analysis of variance. The results of analysis showed the most significant effect of ecotype on composition of FAs. The comparison between hyperaccumulator ecotypes showed significant differences of FA composition related to Cd chronic stress. Hyperaccumulators contained SFAs little occurring in plants, as are 26:0, 28:0, 30:0 acids, and USFAs, as are 16:2, 16:3, 20:2 and 20:3. The results showed that mainly Mežica ecotype has an efficient defense strategies which can be related to changes in FAs composition, mainly in VLCFAs synthesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2015.02.012.

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Erratum

Erratum to "Fatty acid profiles of ecotypes of hyperaccumulator *Noccaea caerulescens* growing under cadmium stress" [J. Plant Physiol. 180 (2015) 27–34]

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The authors regret the following error: An incorrect classification of plant *Noccaea caerulescens* from Mežica has been used in the above mentioned publication.

The correct classification is *Noccaea praecox* Wulfen.

Our incorrect classification was based on a botanical taxonomy and the finding of Peer et al. (2006) in paper Assessment of plants from the *Brassicaceae* family as genetic models for the study of nickel and zinc hyperaccumulation (New Phytologist (2006) 172: 248–260) defined *Noccaea* from Mežica as *N. caerulescens*. Likar et al. (2010) have shown that *N. praecox* is a closely related species to *N. caerulescens* and two *N. caerulescens* seeds obtained from material collected in Mežica in northern Slovenia (Peer et al., 2006) were classified using molecular analyses as *N. praecox*.

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Changes in the contents of amino acids and the profile of fatty acids in response to cadmium contamination in spinach

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ABSTRACT

Changes of amino acid (AAs) contents (glutamic acid – Glu, aspartic acid – Asp) and fatty acids profile (FAs) in spinach under cadmium (Cd) soil contamination (Cd1 = 30, Cd2 = 60, Cd3 = 90 mg/kg soil) are reported here. Spinach plants were sampled 25, 40, 55 and 75 days after sowing. Growing Cd soil contamination was associated with the strong inhibition of above-ground biomass (23.5–6.3 g dry matter per pot) and with the enhancement of Cd content (0.60–72.38 mg/kg dry matter) in leaves. During 55 days of plant growing the increase of Glu and Asp content was associated with the enhancement of Cd content. The highest accumulation of AAs was determined on Cd3 treatment after 55 days of cultivation. Strong decreases of both AAs were confirmed in the last sampling period for Cd treatments (reduction of Glu content of Cd3 treatment to ca. 64% and Asp content to ca. 72% in contrast to control). The content of saturated fatty acids increased (mainly palmitic acid) and the content of unsaturated fatty acids decreased in spinach aboveground biomass with increasing Cd concentration. Results of multivariate analysis of variance MANOVA showed the significant effect of Cd contamination for FAs metabolism, but the most significant effect was confirmed for plant growing period.

Keywords: abiotic stress; heavy metals; peroxidation of lipids; *Spinacia oleracea* L.

Cadmium (Cd) is a heavy metal released into the environment by thermal power and heating plants, metal industries, urban traffic, sewage sludge and phosphate fertilizers (Pavlíková et al. 2002a, Vollmann et al. 2015). Plants have no metabolic requirement for Cd, however it is relatively easy available to plants. A frequent outcome following exposure to Cd is the overproduction of reactive oxygen species, potentially causing oxidative damage in plant cells and thus requiring the intervention of antioxidant defense systems (Sandalio et al. 2001). Cadmium induces oxidative stress in plants by blocking essential functional groups in biomolecules and by indirect mechanisms such as interaction with the antioxidant defense system, disruption of the electron transport chain or induction of lipid peroxidation (Cuypers et al. 2010). It decreases water stress tolerance of plants. The accumulation of Cd in plant tissues caused damages to the photosynthetic apparatus; it inhibited photosynthesis by increasing stomatal and mesophyll resistance to carbon dioxine uptake (Gallego et al. 2012). The reduction in photosynthetic rate led to a limited supply of metabolic energy and therefore to nitrogen (N) assimilation restriction. Nitrogen flow through amino acids can change in response to Cd stress. Plants that were exposed to toxic elements have also been shown to ac-

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cumulate specific amino acid (AAs), which may have beneficial functions and play various roles in plants (Xu et al. 2012b, Pavlíková et al. 2014a).

The visual symptoms of Cd toxicity in plants are chlorosis and necrosis of leaves, browning of roots and cell apoptosis. The chlorosis may be due to Fe deficiency, because Fe binding in spinach is affected by Cd accumulation (Pavlíková et al. 2002b, Martin et al. 2012). Cadmium is chemically similar to certain metal elements, including Fe, Zn, Mn, Mg and Ca, and, therefore, can displace these elements from metalloproteins (Verbruggen et al. 2009, Lux et al. 2010). The elements, such as Fe, Zn and Mn are cofactors of metalloenzymes (for example superoxide dismutase, cytochrome P450) and their contents related to plant defense against oxidative stress (Cakmak 2000).

Cadmium stress induced leaf senescence. During senescence activity of the plant hormones are changed and chlorophyll and proteins are degraded. Declining photosynthesis and continued active export of sugars combine to make senescing tissues increasingly carbon-starved. Amino acids derived from proteolysis are an important source of carbon skeletons. Glucogenic AAs give acetyl-CoA to stress metabolism. Simultaneously lipids are degraded into glycogen and free fatty acids (FAs), from which acetyl-CoA arises by ß-oxidation. Acetyl-CoA is the initial substrate for synthesis of FAs and several AAs. For this reason the aim of this study was to characterize changes in metabolism of transfer AAs and FAs composition in spinach in relationship to plant growing period and to Cd stress.

MATERIAL AND METHODS

Adaptation of spinach (*Spinacia oleracea* L. cv. Matador) plants to excessive Cd levels in soil was investigated in pot experiment repeated for two years. For this experiment, 20 spinach seeds were sown into plastic pods containing soil mixture as specified below. The plants (10 plants per pot) were cultivated from April to June under natural light and temperature conditions at the experimental hall of the Czech University of Life Sciences Prague, Czech Republic. The water regime was controlled and the soil moisture was kept at 60% MWHC (maximum water-holding capacity).

For cultivation of spinach plants, 5 kg of Chernozem soil (pH_{KCl} = 7.2, C_{ox} = 1.83%, CEC (cation exchange capacity) = 258 $mmol_+/kg$) was thoroughly mixed with 0.5 g N, 0.16 g P, and 0.4 g K applied in the form of ammonium nitrate and potassium hydrogen phosphate for control treatment and with the same amount of nutrients plus cadmium (applied in $Cd(NO_3)_2 \cdot 4\ H_2O$) for treated variants. Three concentrations of Cd (Cd1 = 30, Cd2 = 60, Cd3 = 90 mg/kg) were applied. Each treatment was performed in three replications every year. The presented data are the average of both experimental years. Spinach plants were sampled 25, 40, 55 and 75 days after sowing.

The free amino acids from methanol + H_2O extracts from mature leaves were determined using EZ-faast amino acid analysis procedure (Phenomenex, Santa Clara, USA). Samples were analyzed for AAs contents by GC-MS using the Hewlett Packard 6890N/5975 MSD (Agilent Technologies, Torrance, USA). Samples were separated on a ZB-AAA 10 m × 0.25 mm AA analysis GC column using the constant carrier gas (He) flow (1.1 mL/min) (Pavlík et al. 2012).

For analyses of Cd contents plant samples were decomposed using the dry ashing procedure. The ash was dissolved in 1.5% HNO_3. Aliquots of the certified reference material RM NCS DC 73350 poplar leaves (purchased from Analytika, Czech Republic) were mineralized under the same conditions for quality assurance. The Cd concentrations were analyzed by ICP-OES (Varian VistaPro, Varian, Mulgrave, Australia).

Overall content of fatty acids (free and derived from various lipids) was determined after their conversion to respective methylesters (FAMEs). Samples of fresh biomass (~0.2 g) were extracted by 2 mL of CH_3OH + $CHCl_3$ (3:2, v/v) on a shaker for 24 h. Acid catalysed transesterification of FAs with acetylchloride according to the method of Stránský and Jursík (1996) was carried out. The content of FAMEs was measured by GC-MS (Thermo Scientific DSQ II Single QuadrupoleGS-MS, Thermo Fisher Scientific, Waltham, USA) with a nonpolar column Zebron ZB-5 30 m × 0.25 mm × 0.25 µm (Zemanová et al. 2015). FAs were determined in biomass sampled after 25 and 55 days of plant growing. For the lack of biomass it was not possible to determine FAs in Cd3 treatment. Individual FAMEs were identified by their mass spectra fragmentation as well as their coelution with synthetic standards (Supelco 37). The percentage of saturated fatty acids (SFAs) and unsaturated fatty acids (USFAs) was compared for control and treated plants.

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The statistical analyses were performed using multivariate analysis of variance (MANOVA) with multivariate *F*-value (Wilks' lambda). A MANOVA was applied to identify the effect of treatments and growing period and their interactions as independent variables, and contents of Cd, yield of biomass, free AAs and FAs as dependent variables. A MANOVA was followed by the post-hoc comparison Tukey's test ($P < 0.05$). All analyses were performed with Statistica 12.0 software (StatSoft, Tulsa, USA).

RESULTS AND DISCUSSION

The results of the pot experiment revealed the toxic effect of Cd on spinach plants. Plant response to excessive Cd content in soil was assessed on the basis of a decreased spinach dry matter and increased concentrations of this elements in the aboveground biomass (Figures 1 and 2). Growing Cd doses (from 30–90 mg Cd/kg soil) were associated with strong inhibition of the aboveground biomass (23.5–6.3 g per pot after 75 days) and with enhancement of Cd content (0.60–72.38 mg/kg after 75 days) in leaves. Compared to the untreated control, the biomass yield of Cd3 treatment was reduced to ca. 27% while the Cd content in aboveground biomass was enhanced up to 120-fold. No

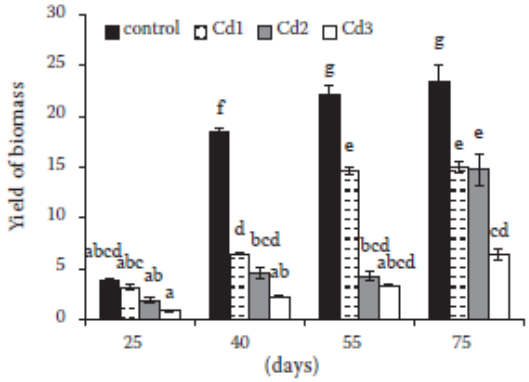

Figure 1. Aboveground biomass yield (g dry matter per pot) of spinach aboveground biomass. Explanation for Figures 1–4: Spinach was grown under varying Cd concentrations (0, 30, 60, 90 mg Cd/kg). Plants were harvested after 25, 40, 55 and 75 days of spinach cultivation. Data represent means ± standard error of three replicates every year ($n = 6$). Different letters indicate significantly different values ($P < 0.05$) between treatment × growing period calculated by MANOVA

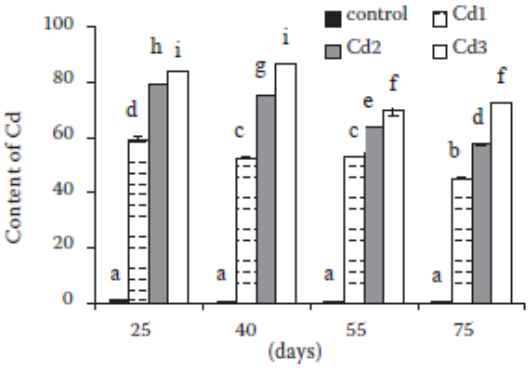

Figure 2. Cadmium (Cd) accumulations in aboveground biomass of spinach (mg/kg dry matter)

significant differences in the biomass yields and Cd contents in plants were observed between individual experimental years. Our data correspond with those by Pavlík et al. (2010), Pavlíková et al. (2014a) who reported that excessive amounts of toxic elements in contaminated soil inhibited plant growth and development due to their phytotoxicity. The damage caused by Cd led to senescence and to the bleaching of chlorophylls at Cd3 treatment. Magnesium in chlorophyll is replaced with Cd (Küpper et al. 1998). The visual symptoms of Cd toxicity – chlorosis and necrosis of leaves were confirmed for example by Pavlíková et al. (2008) and Martin et al. (2012).

Plants exposed to toxic metals accumulated specific AAs, which may have beneficial functions and play various roles (Xu et al. 2012a, 2012b, Pavlíková et al. 2014a,b). Chaffei et al. (2004) suggested that an increase in the proportion of high N:C by AAs, is a protective strategy in plants. Consistent with this hypothesis, our analyses indicated the accumulation of a large amount of glutamic acid (Glu) and aspartic acid (Asp) in Cd treatments in 55th day of plant cultivation (Figures 3a,b). The highest accumulations of both AAs were determined on Cd3 treatment after 55 days of cultivation. Glu and Asp are used to transfer N from source organs to sink tissues and to build up reserves during periods of N availability for subsequent use in growth, defense, and reproductive processes. Strong decrease of both AAs were confirmed in the last sampling period for Cd treatments (after 75 days of plant growing). Glu content of Cd3 treatment was reduced to ca. 64% of the control treatment. Asp content was decreased to ca. 72%. This decrease related to the interaction of onto-

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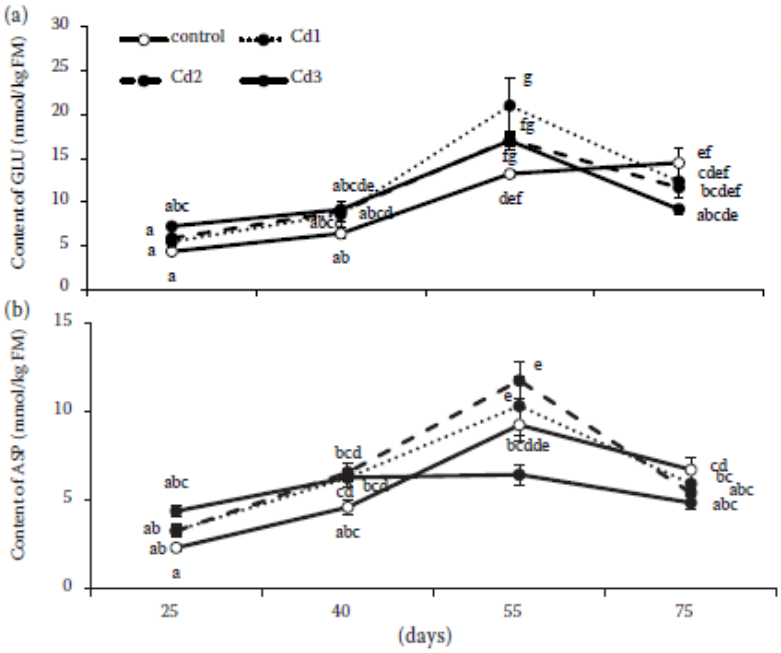

Figure 3. The concentrations of (a) free glutamic acid (GLU) and (b) free aspartic acid (ASP) in the aboveground biomass of spinach (mmol/kg fresh matter (FM))

genetic period and effect of Cd stress. Changes of Asp contents were less significant in contrast to Glu, because Glu is used in the synthesis of glutathione and phytochelatins in plant cells (Vitória et al. 2001). According to Zemanová et al. (2013) the declines of contents of both AAs can be caused by intensive syntheses of plant defense elicitors. Pavlík et al. (2012) confirmed that contamination of heavy metals caused depletion in the pools of free Glu and Asp in lettuce plants growing for 75 days.

As it was calculated using the multivariate analysis of variance MANOVA, contents of Cd, Asp, Glu and yield of biomass were significantly affected by treatments (Wilks' lambda 0.003, $F = 226.2$, $P = 0.00$*), growing period (Wilks' lambda 0.33, $F = 77.6$, $P = 0.00$*) and treatments × growing period (Wilks' lambda 0.044, $F = 17.33$, $P = 0.00$*). The results showed the most significant effect of treatments.

The comparison between treatments showed significant differences of FAs composition related to Cd stress only in 25th day of plant cultivation (Figure 4). The Cd contamination the increased the content of saturated fatty acids (by 44% for Cd1 and 94% for Cd2) compared to control. Our results from the 55th day of plant cultivation confirmed increase only by 16% for both Cd treatments. SFA contained in plants – palmitic acid (16:0) was detected in all treatments and in both sampling periods. Arachidic acid (20:0) was only detected in control treatment in day 25 of plant cultivation (Figure 5).

Analyses of unsaturated fatty acids have confirmed 7,10,13-hexadecatrienoic (16:3n-3), 9,12-octadecadienoic (linoleic acid, 18:2n-6) and 9,12,15-octadecatrienoic (α-linolenic acid, 18:3n-3)

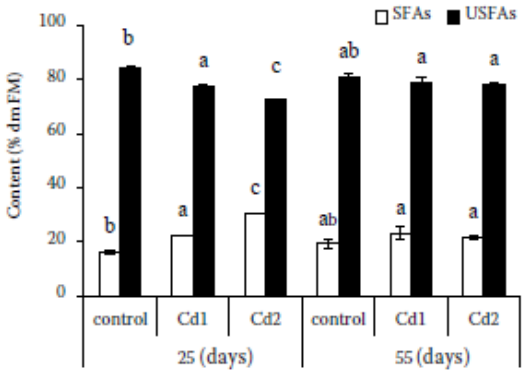

Figure 4. Total saturated fatty acids (SFAs) and unsaturated fatty acids (USFAs) contents of spinach. Explanation for Figures 4 and 5: Spinach was grown under varying Cd concentrations (0, 30, 60, 90 mg Cd/kg). Plants were harvested after 25 and 55 days of spinach cultivation. Data represent means ± standard error of three replicates every year ($n = 6$). Different letters indicate significantly different values ($P < 0.05$) between treatment × growing period calculated by MANOVA

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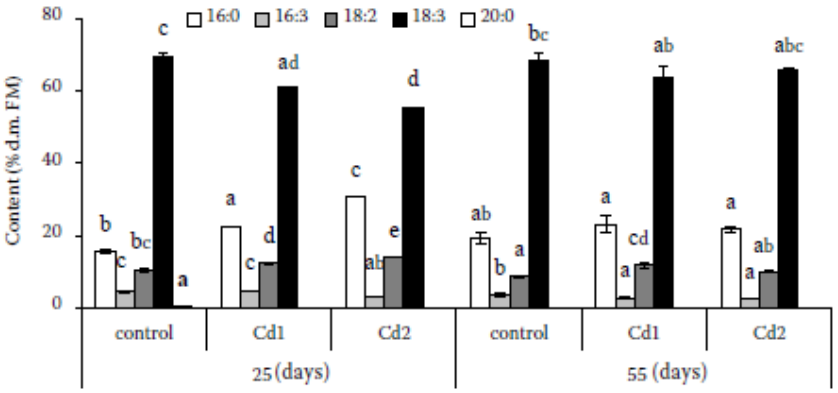

Figure 5. Profile saturated fatty acids and unsaturated fatty acids contents of spinach

acids in all treatments. Decreases of USFAs percentage were detected for 16:3n-3 and 18:3n-3 FAs in both Cd treatments. 16:3n-3 USFA is the product of desaturases from 16:0 and it is used as substrate for biosynthesis of different USFAs (Zemanová et al. 2015). For acclimation to oxidative stress plants mainly need USFAs – 18:2n-6, 18:3n-3. The percentage of 18:2n-6 FA increased in plants with Cd contamination. In accordance with our results Verdoni et al. (2001) published a significant decrease of linolenic acid (18:3) and increase of 18:1 and 18:2 FAs in primary leaves of tomato. Upchurch (2008) reviewed that in stress tolerant plants the degree of membrane lipid unsaturation, principally linolenic acid decreases in response to heavy metal stresses. Our results supported this finding. For plant growing under oxidative stress linolenic acid is substrate rapidly degraded into metabolites, which lead to the production of oxylipins, for example jasmonic acid regulating growth and plant development (Savchenko et al. 2014).

As it was calculated using the multivariate analysis of variance MANOVA, contents of SFAs and USFAs were significantly affected by variants (Wilks' lambda 0.067, $F = 35.53$, $P = 0.00$*), growing period (Wilks' lambda 0.201, $F = 49.2$, $P = 0.00$*) and variants × growing period (Wilks' lambda 0.119, $F = 23.60$, $P = 0.00$*). The most significant effect was confirmed for plant growing period.

Zemanová et al. (2015) clearly showed importance of a relationship between Cd accumulation and the FAs composition in Cd hyperaccumulator *Noccaea caerulescens*. According to these results SFAs decrease and USFAs increase in biomass of *N. caerulescens* with increasing Cd concentration is a typical feature of plants resistant to Cd stress. An opposite trend of FAs content was determined in spinach biomass – non hyperaccumulating plant. The results of Nouairi et al. (2006) indicated similar changes of FAs in leaves of *Brassica juncea* grown under Cd stress. The comparison between hyperaccumulator and spinach showed significant differences of FAs composition related to Cd chronic stress. The number of identified FAs in spinach biomass was very low compared to the hyperaccumulator. Saturated very-long-chain fatty acids (VLCFAs) were found only in hyperaccumulating plants. Biosynthesis of VLCFAs decrease the amount of energy necessary for plant growth and development. Catabolic processes of these FAs decreased plant sensitivity to environmental stress. This finding reflects that hyperaccumulator in contrast to spinach has an efficient defense strategy relating to changes in FAs composition.

The results of our experiment showed changes in transfer AAs contents (the highest accumulation of AAs on Cd3 treatment after 55 days of cultivation) and in FAs composition (significant increase of palmitic acid) in spinach in relationship to growing Cd soil contamination. Multivariate analysis of variance confirmed a significant effect of growing period of plants to these changes. For this reason investigation of changes in the plant metabolism is necessary to test in long-term conditions.

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5.6 Zemanová, V., Pavlík, M., Pavlíková, D., Hnilička, F. Responses to Cd stress in two *Noccaea caerulescens* ecotypes originating from differently contaminated sites. Archives of Environmental Contamination and Toxicology. Manuscript AECT-D-15-00128R1. (after major revision)

Archives of Environmental Contamination and Toxicology

Responses to Cd stress in two Noccaea species - Noccaea praecox and Noccaea caerulescens originating from differently contaminated sites

--Manuscript Draft--

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Article Type:	Original Research
Keywords:	Adaption to Cd stress; Amino acids; Heavy metal; Lipids; Nutrient metabolism
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Abstract:	The two Noccaea species - Noccaea praecox originating from Mežica, Slovenia (Me) (Pb, Zn, Cd pollution) and Noccaea caerulescens from Redlschlag, Austria (Re) (high levels of Ni, Cr, Mg) were studied to compare Cd accumulation and tolerance. After 120 days of plant cultivation in Cd-contaminated soil (90 mg Cd.·kg-1 soil), gas-exchange parameters (e.g., net photosynthetic rate, transpiration rate, stomatal conductance, and intercellular CO2 concentration), fatty acids, and selected macro- and microelements were determined in addition to N utilization by plants. The comparison between ecotypes showed that Cd stress resulted in similar changes in gas-exchange parameters. Contrasting responses of plants to Cd contamination were confirmed by the macro- and microelement contents and fatty acid and amino acid metabolism. Significantly higher accumulations of Cd and strong decreases in the levels of K, Ca, Na and Fe were observed in the Me plants in contrast to the Re plants. The higher Re plant ability to take in some cations is a result of selective pressure due to contamination. Different ion uptake by plants affected the activities of metalloenzymes. Significant increases in the glutamic acid/proline ratio resulted from higher adaption of the Me in contrast to the Re plants.
Response to Reviewers:	Reviewer #3: The aim and hypotheses were formulated: The aim of this study was to characterize changes in nitrogen metabolism, elements content, fatty acids and gas-exchange parameters of two Noccaea species - Noccaea praecox and Noccaea caerulescens (from serpentine group) - growing under strong Cd stress. Our objectives are: (1) to confirm differences among tested parameters for

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Responses to Cd stress in two *Noccaea* species - *Noccaea praecox* and *Noccaea caerulescens* originating from differently contaminated sites

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Abstract

The two *Noccaea* species - *Noccaea praecox* originating from Mežica, Slovenia (Me) (Pb, Zn, Cd pollution) and *Noccaea caerulescens* from Redlschlag, Austria (Re) (high levels of Ni, Cr, Mg) were studied to compare Cd accumulation and tolerance. After 120 days of plant cultivation in Cd-contaminated soil (90 mg Cd kg^{-1} soil), gas-exchange parameters (e.g., net photosynthetic rate, transpiration rate, stomatal conductance, and intercellular CO_2 concentration), fatty acids, and selected macro- and microelements were determined in addition to N utilization by plants. The comparison between ecotypes showed that Cd stress resulted in similar changes in gas-exchange parameters. Contrasting responses of plants to Cd contamination were confirmed by the macro- and microelement contents and fatty acid and amino acid metabolism. Significantly higher accumulations of Cd and strong decreases in the levels of K, Ca, Na and Fe were observed in the Me plants in contrast to the Re plants. The higher Re plant ability to take in some cations is a result of selective pressure due to contamination. Different ion uptake by plants affected the activities of metalloenzymes. Significant increases in the glutamic acid/proline ratio resulted from higher adaption of the Me in contrast to the Re plants.

Keywords: Adaption to Cd stress; Amino acids; Heavy metal; Lipids; Nutrient metabolism;

Introduction

Cadmium is a heavy metal that is released into the environment by thermal power and heating plants, metal industries, cement factories, urban traffic, sewage sludge and phosphate fertilizers (Gratão et al. 2005). High concentrations of Cd in the soil can be toxic to plants. In leaves, concentrations of Cd that are higher than 5–10 μg g^{-1} DW are toxic to most plants (White and Brown 2010; Lux et al. 2011). However, some species can hyperaccumulate Cd to concentrations in excess of 100 μg g^{-1} DW in their leaves without showing any negative symptoms (Baker et al. 2000).

Cadmium can enter the plant through nonspecific cation channels as well as through different divalent cation transporters, competing with essential mineral nutrients for absorption (Verbruggen et al. 2009; Lux et al. 2011). Therefore, the uptake and distribution of essential mineral nutrients, such as Fe, Ca, Mg and K, can be severely disturbed in the presence of high levels of cadmium (Martin et al. 2012).

Cadmium induces oxidative stress in plants by blocking essential functional groups in biomolecules and by indirect mechanisms such as interaction with the antioxidant defense system, disruption of the electron transport chain or induction of lipid peroxidation (Cuypers et al. 2010). Plants exposed to Cd had elevated levels of mitochondrial ROS production, indicating that this organelle had become dysfunctional (Heyno et al. 2008). This element has been shown to be one of the most effective inhibitors of photosynthetic activity (Gallego et al. 2012). It can enter chloroplasts and disturb chloroplast function by inhibiting the enzymatic activities involved in chlorophyll biosynthesis, pigment–protein complexes, the O_2-evolving reactions of photosystem II, electron flow around photosystem I and chloroplast structure (Ying et al. 2010; Molins et al. 2013 etc.). Cd reduced the photochemical processes more in older leaf segments than in younger ones, but the functional status of the dark phase of photosynthesis was more strongly diminished in younger ones (Drążkiewicz and Baszyński 2005). According to Perfus-Barbeoch et al. (2002), stomatal closure, damage to the photosynthetic machinery and interference with pigment synthesis cause a general depression of photosynthetic efficiency, lowering the effective quantum yield. Moreover, by inhibiting enzymes involved in CO_2 fixation, Cd decreases carbon assimilation. Cadmium at low concentrations induced net photosynthetic rate (P_N), content of chlorophyll and carotenoids in hyperaccumulator *Lonicera japonica* (Jia et al. 2015). The results of Dias et al. (2013) indicated that high Cd concentration strongly inhibited P_N. This inhibition was followed by a decrease in transpiration rate (E) and stomatal conductance (g_s). According to Deglinnocenti et al. (2014) the reduction in P_N observed in Cd-treated plants *Lycopersicon esculentum* was not linked to stomatal limitation as it was also indicated by the unchanged CO_2 intracellular concentration (C_i). Nwugo and Huerta (2008) found high Ci value for rice seedlings

(Oryza sativa) exposed to high Cd concentrations. High C_i indicated that the inhibition of photosynthesis was also due to an inhibition of Calvin cycle enzymes and/or an inhibition of the photosynthetic electron transport chain. The increase of C_i may be explained by modifications of RuBisCO activities of plant (Redondo-Gómez et al., 2011). According to Leitenmaier and Küpper (2011), hyperaccumulator plants have to store the excess metal in such a way that it does not harm important enzymes and especially not photosynthesis. It has been shown that high amounts of metals are stored specifically in the vacuoles of large epidermal cells (Küpper et al. 1999; Frey et al. 2000; Küpper et al. 2001), where no chloroplasts are located so that photosynthesis cannot be inhibited.

The comparison between hyperaccumulators and non hypeaccumulating plants showed significant differences of fatty acids (FAs) composition related to Cd chronic stress. Lipid changes in *Brassica juncea*, the well-known Cd-hyperaccumulator specie, revealed a more stability of its cellular membranes to cadmium-stress as compared to Cd-sensitive specie, *B. napus*. The levels of polyunsaturated fatty acids mainly C18:3, C16:3 and C16:1t declined in *B. napus* (Nouairi et al. 2006).The number of identified FAs in spinach biomass was very low compared to hyperaccumulator *Noccaea caerulescens*. Saturated very-long-chain fatty acids (VLCFAs) have been found only in hyperaccumulating plants. Biosynthesis of VLCFAs decreases amount of energy necessary for plant growth and development. Catabolic processes of these FAs decrease plant sensitivity to environmental stress (Zemanová et al. 2015a, 2015b).

Several studies have focused attention on the role of the amino acids in metal tolerance of plants. The amino acids that accumulate under heavy metal stress play various roles in plants, including acting as signaling molecules, acting as osmolytes, regulating ion transport and facilitating detoxification. Heavy metals in xylem sap are transported almost entirely complex with amino acids (Liao et al. 2000). Histidine, aspartic acid, glutamic acid and asparagine are related to the long-distance transport of xylem. Krammer et al. (1996) reported that histidine accumulation is responsible for nickel hyperaccumulation in *Alyssum*. Salt et al. (1999) observed the presence of a Ni-histidine complex in the xylem sap of *N. caerulescens*. Proline (Pro) played a role in the alleviation of Cd toxicity by detoxifying ROS, thereby increasing the glutathione concentration and protecting antioxidative enzyme activities in *Solanum nigrum* seedlings (Xu et al. 2009). A higher accumulation of Pro in *S. nigrum* supports the observed higher Cd tolerance in *S. nigrum* than in *S. torvum*. Hydroxyproline is an important component of the Casparian band. A high accumulation of Hyp in *S. torvum* roots may play a protective role in preventing Cd translocation from the roots to the aerial parts of the plant (Xu et al. 2012a,b).

~~The responses of plant metabolism to stress have usually been studied in experiments focused on an acute stress. This form of stress, however, does not accurately reflect environmental conditions (Sanita di Toppi and~~

~~Gabbrielli 1999). Plants usually grow and develop in conditions of long term chronic stresses. For this reason, our study focuses on the investigation of changes in the metabolism of plants growing on an environmentally relevant substrate – soil – and occurring under chronic stress caused by cadmium.~~ Hyperaccumulators of toxic elements are highly attractive model organisms because they have overcome major physiological problems that limit metal accumulation in biomass and have toxic element tolerance (Verbruggen et al. 2009; Ueno et al. 2011). In the presented study, two *Noccaea* species - *N. caerulescens* and *N. praecox* were cultivated to investigate the effect of strong Cd-polluted soil on plant metabolism. Internal transcribed spacer (ITS) rDNA sequences from *N. praecox* populations from Slovenia showed 99% similarity and formed a sister group to *N. caerulescens*. Evolutionary development of extraordinary Cd hyperaccumulation abilities in particular *N. praecox* populations may be closely related to the levels of this element in the soil (Likar et al. 2010). Similarly studies of *N. caerulescens*, which showed that ecotypes growing naturally in low Cd-containing soils have much lower hyperaccumulation capacity compared to the ecotypes growing in high Cd-containing soils (Gonneau et al. 2014).

~~The aim of this study was to characterize changes in nitrogen metabolism, elements content, fatty acids and gas-exchange parameters of *Noccaea* growing under Cd stress.~~ The aim of this study was to characterize changes in nitrogen metabolism, elements content, fatty acids and gas-exchange parameters of two *Noccaea* species - *Noccaea praecox* and *Noccaea caerulescens* (from serpentine group) - growing under strong Cd stress. Our objectives are: (1) to confirm differences among tested parameters for hyperaccumulating plants growing on Cd contaminated and non-contaminated soil; (2) to show that the regulation of Pro biosynthesis from Glu is dependent on the Glu:Pro ratio and is determined by phenotypic variability; (3) to assess phenotypic variability for tested parameters between two *Noccaea* species (with 99% genotypic similarity) growing in pot experiment under strong Cd stress.

Material and methods

Plant material and cultivation conditions

In the pot experiments, *Noccaea praecox* (formerly *Thlaspi praecox* Wulfen*)* from Mežica, Slovenia (Me) and *Noccaea caerulescens* (formerly *Thlaspi caerulescens* J. & C. Presl, FK Mey*)* from Redlschlag, Austria (Re) were used. The Mežica mining district source area is characterized by the presence of ore minerals of geogenic/technogenic origin (cerussite, sphalerite, smithsonite and galena). The environs of Mežica are strongly polluted with Pb and Zn. Because Cd is found as a trace element in sphalerite and smithsonite, its content correlates with that of Zn (Gosar and Miler 2011). The bedrock of Redlschlag is composed of serpentine, which

contains ~~high levels~~ large amouts of Ni and Cr and some Zn and Co. Because the soil pH is neutral (approx. pH 6.55), the main problems for plants are low concentrations and availability of micronutrients, although Mg is abundant (46,400 mg Mg kg^{-1} – Puschenreiter et al. 2005).

For the cultivation of *Noccaea* plants (2 plants per pot), 3 kg of soil (from the non-polluted site Prague-Suchdol, Chernozem – pH=7.2, CEC=258 mmol~~(+)~~ kg^{-1}, C_{org}=1.8%, Cd_T =0.42 mg kg^{-1}) was thoroughly mixed with nutrients (0.3 g N, 0.10 g P, and 0.24 g K applied in the form of NH_4NO_3 and K_2HPO_4) as the control treatment and with the same amount of nutrients plus Cd ($Cd(NO_3)_2.4H_2O$) at 90 ~~(Cd)~~ mg Cd kg^{-1} for treated variants. The water regime was controlled, and the soil moisture was kept at 60% MWHC (maximum water-holding capacity). Each treatment was performed in five replications. Plants were harvested 120 days after Cd application. Samples were kept frozen in liquid nitrogen for transport and then at –30 °C until extraction.

Analyses

Determination of gas-exchange parameters

The net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were measured in the leaves *in situ* using the portable gas-exchange system LCpro+ (ADC BioScientific Ltd., Hoddesdon, Great Britain) from 10:00 to 11:30 Central European summer time. The irradiance was 595 µmol m^{-2} s^{-1} photosynthetically active radiation, the temperature in the measurement chamber was 22.7 °C, and the duration of the measurement of each sample was 15 min after the establishment of steady-state conditions inside the measurement chamber (Pavlíková et al. 2014).

Analysis of free amino acids in plant biomass

The amino acids in methanol+H_2O extracts were determined using the EZ-faast amino acid analysis procedure (Phenomenex, U.S.A). Amino acid content was analyzed by GC-MS using a Hewlett Packard 6890N/5975 MSD (Agilent Technologies, USA). Samples were separated on a ZB-AAA 10 m x 0.25 mm amino acid analysis GC column using constant carrier gas (He) flow (1.1 ml min^{-1}). The oven temperature program was as follows: initial temperature 110 °C, 30 °C.min^{-1} ramp to 320 °C. The temperature of the injection port was 280 °C. A total of 1.5-2 µl sample was injected in split mode (1:15, v/v). The MS conditions were as follows: MS source 240 °C, MS quad 180 °C, auxiliary 310 °C, electron energy 70 eV, scan m/z range 45-450 and sampling rate 3.5 scan s^{-1} (Pavlík et al. 2012).

Determination of fatty acids

Samples of fresh biomass (~0.2 g) are extracted in 2 ml of methanol + chloroform (3:2, v/v) on a shaker for 24 hours. Transesterification of fatty acids was performed in the supernatant according to method of Stránský and

Jursik (1996a,b). The content of methyl esters of fatty acids was measured by GC-MS (Thermo Scientific DSQ II Single Quadrupole GS–MS, Thermo Fisher Scientific) with a nonpolar column Zebron ZB-5 30 m x 0.25 mm x 0.25 µm. The injection volume was 1 µl of sample in a splitless mode. The carrier gas was helium (He, purity 5.0) with a constant flow rate of 1 ml/min. The temperature program of oven: initial temperature 50 °C (for 2 min), 8 °C min^{-1} ramp to a temperature of 320 °C (for 10 min); inlet temperature 250 °C and transferline temperature 260 °C.

Analyses of cadmium and additional elements in plant biomass

Plant samples were decomposed using the dry ashing procedure as follows: an aliquot (~1 g) of the dried and powdered biomass was weighed in a borosilicate glass test tube and decomposed in a mixture of oxidizing gases (O_2+O_3+NO_x) at 400 °C for 10 hours in a Dry Mode Mineralizer Apion (Tessek, Czech Republic). The ash was dissolved in 20 mL of 1.5% HNO_3 (v/v) (electronic grade purity, Analytika Ltd., Czech Republic) and kept in glass tubes until analysis. Aliquots of the certified reference material RM NCS DC 73350, poplar leaves, (purchased from Analytika, CZ) were mineralized under the same conditions for quality assurance. The Cd and macro- and microelement concentrations were determined by ICP-OES with axial plasma configuration (Varian VistaPro, Varian, Australia).

Water extractable-Cd contents in leaves were measured in 0.2 g dried biomass suspended in 50 mL deionized water and shaken for 2 h at 20 °C. The suspension was filtered at 0.2 µm porosity with a cellulose nitrate filter and then acidified with HNO_3. Cd concentrations were measured by ICP-OES with axial plasma configuration (Varian VistaPro, Varian, Australia) (Perronnet et al. 2000).

Statistical analyses were performed using hierarchic analyses of variance (ANOVA) considering interactions at the 95% ($P < 0.05$) significance level with subsequent Tukey's HSD test and correlation (R^2). All analyses were performed with Statistica 9.1 software (StatSoft, USA).

A principal component analysis (PCA), in the CANOCO 4.5 (ter Braak and Šmilauer 2002) software, was used to evaluate multivariate data. We used standardisation of species data because data of different character and units were analysed together. The PCA was used to make visible correlations between all the analysed data and similarity of the different treatments. Obtained results were visualised in the form of a bi-plot ordination diagram created by CanoDraw program.

Results

Yield of aboveground biomass and Cd content

The yield of aboveground biomass, although similar in both varieties under Cd stress, indicated greater reduction of Re-Cd (50 % reduction in contrast to control) than Me-Cd (39 % reduction compared to control –Figure 1). The biomass Cd contents showed significant differences between *Noccaea sp.* The highest Cd content was found in the biomass of Me-Cd plants (257-fold greater Cd content in Me-Cd than Me). The Cd contents of Re were significantly lower than in Me, and increase in Cd content under Cd stress was only 68.5 fold (Table 1). Similar differences in water-extractable Cd were determined between treatments (Table 1). The highest proportion of water-extractable Cd was measured for Me and Me-Cd. There was a close negative relationship between the yield of the aboveground biomass of all tested plants and plant Cd content (R^2 = -0.71). There was no relationship between yield and water-extractable Cd (R^2 = -0.29). This result is caused by the different Cd accumulation in proteins or pectins isolated after hydrolyses of the plant cytoskeleton.

Plant gas-exchange parameters

To compare the Cd adaptation of the two species, the effect of soil contamination (90 mg $Cd.kg^{-1}$) on gas-exchange parameters was analyzed. As shown in Table 2, Cd treatment inhibited the photosynthetic rate (P_N), but significant differences between species were not observed. The P_N was also similar in the both control treatments. Cd soil contamination ~~produced~~ caused only 8 - 12 % P_N reduction. There was a close negative relationship between P_N and Cd content in plant biomass (R^2 = -0.69). Both the transpiration rate (E) and stomatal conductance (g_s) increased under Cd stress (Table 2). Both ~~ecotypes~~ species had 3.5-fold greater E in Cd treatment in contrast to controls. Similar but non-significant trends were found for g_s and intracellular CO_2 concentration (C_i). The water-use efficiency (WUE) was estimated from P_N and E. The WUE of Me-Cd and Re-Cd compared to the controls declined by 25.3–26.4 %.

Plant element contents

The element contents of Me and Re were different; however, the trends of the changes were similar (Table 1). The content of the tested elements in the aboveground biomass was affected by Cd supply. The Mg contents of Cd treatments were not affected by Cd contamination. Reductions in K, Ca and Na contents were observed in both Cd treatments. The highest reductions in these elements were determined for Me-Cd (45 % K, 36 % Ca and 64 % Na reduction). The most significant relationship was between Ca and Cd contents (and water-soluble Cd) (R^2 = 0.77 and 0.86, respectively). Reductions in K, Ca and Na affected WUE (R^2 = 0.52– 0.58). Cd reduced Fe in both treatments. There was a close relationship between C_i and the Fe contents (R^2 = 0.69 – 0.95).

Free amino acids

Table 3 includes amino acids related to assimilation, transport and accumulation of nitrogen in plants (Asn, Asp, Glu and Gln). Minor changes in Asp and Glu contents as a result of Cd contamination were observed in both species. Identical changes in Asn (decrease by 58 % in Me-Cd and 50 % in Re-Cd) were found for all Cd-treated plants. The opposite trend was observed for Gln, primarily for Me-Cd (increase by 90 %). These AA showed a close correlation with WUE (R^2=0.80), and an effect of Fe content on amino acids (AA) formation was observed (R^2=0.93).

The contents of proline (Pro) and serine (Ser) increased in both species grown under Cd stress, but the content of free Pro was significantly higher in both the control and Cd treated Re than in Me. The regulation of Pro biosynthesis from Glu is dependent on Glu:Pro ratio (Table 4). The results confirmed a higher Glu:Pro ratio in both Me treatments than in Re. Plants with a higher Glu:Pro ratio are better adapted to Cd stress, because Glu can be used for the formation of a peptide bond between the γ-carboxyl group of glutamate and the α-amino group of cysteine and is used in the synthesis of glutathione and phytochelatins in plant cells. A significant correlation between these AA (Pro and Ser) and Asn, Asp, Glu and Gln was found (R^2 = 0.76~~, resp.~~ and 0.93, respectively). The correlation between ~~these AA~~ Pro or Ser and water-soluble Cd (R^2 = 0.88) confirmed the direct effect of Cd on their contents.

Increased Cd contents of plants increased the content of Hyp, a major AA in plant cell wall hydrolysates (Hyp increase of 11 % in Me-Cd and 38 % in Re-Cd). The correlation between Cd and Hyp (R^2 = 0.75) confirmed the direct effect of Cd on this AA.

Fatty acids

Increased peroxidation of fatty acids is associated with Cd soil contamination and Cd detoxification processes in the plant cell (Figure 2). The content of saturated and unsaturated fatty acids in control plants did not significantly differ (saturated fatty acids = 42.7-48.3 % of total fatty acids; unsaturated fatty acids 51.7-57.3 %). A significant decline in saturated fatty acids (36 %) and increase in unsaturated fatty acids (33 %) was observed for Me-Cd. Linear correlations confirmed close relationships between saturated and unsaturated fatty acid contents and Cd content in Me-Cd biomass (R^2 = 0.99). Cd soil contamination did not significantly modify the total contents of saturated and unsaturated fatty acids in Re biomass.

Results of principal component analysis (PCA)

In the PCA performed on all the plant parameters,the first axis of the PCA analysis explained 47%, the first two axes 79% and the first four axes together, 96% of the variability of all analysed data (Figure 3). The first ordination axis divided individual pots into the *N. caerulescens* group on the left side and *N. praecox* on the right

side of the diagram. This indicates a large effect of used plant species on yield of aboveground biomass, content of elements, concentrations of selected free AA and plant gas-exchange parameters. For both plant species, marks for treatments (control, Cd) were located in the different parts of the diagram, which indicates a high effect of treatments on all the recorded data. The length and direction of the vectors of the studied parameters indicate links among themselves with respect to the treatments and plant species. The concentrations of free AA (Asn, Pro, Gln, Glu, Ser, Hyp), total concentrations of Ca and Fe were accumulated more in *N. caerulescens*. On the other hand, concentrations of Asp, total concentrations of K, Mg, and Cd_T as well as concentrations of water extractable-Cd (Cd_w) were accumulated more in *N. praecox*. The concentrations of Ser and Hyp were accumulated more in *N. caerulescens* in Cd treatment. The concentrations of Asp were accumulated more in *N. praecox* in control treatment. The yield was positively correlated with WUE as indicated by an angle between the vectors for them of less than 90° and was negatively correlated with Cd_T as the angle between vectors for yield/WUE and Cd_T was greater than 90°. Two vectors did not positively correlated, if the angle between them is larger than 90°. A long vector for particular parameters indicates a strong effect on the results of the analysis, and vice versa.

Discussion

The yields of both *Noccaea* species were reduced by Cd contamination (90 mg $Cd.kg^{-1}$), but greater reductions of Re-Cd than Me-Cd were found. We previously published similar results for *N. caerulescens* (ecotype "Ganges") (Zemanová et al. 2013, 2014). Our data agree with those of Pavlíková et al. (2002, 2008) and Procházková et al. (2012), who reported that excessive amounts of toxic elements in contaminated soil inhibited plant growth and development due to their phytotoxicity. Selective pressure of contaminated soil has affected quantitative characteristics of plants (Snustad and Simmons 2009). This pressure resulted in higher adaptation of plants from Mežica (Me).

The accumulation of Cd in plant tissues damaged the photosynthetic apparatus, which is generally protected from Cd contamination by reduction of free heavy-metal ion concentration in the cytoplasm by complexation with S- and O-ligands and sequestration in the vacuoles (Wójcik et al. 2005). Ueno et al. (2005) showed that Cd is complexed with malate and stored in vacuoles in *N. caerulescens* leaves. In our experiment, Cd supplementation inhibited P_N in plants and increased C_i, E and g_s. ~~No significant differences between ecotypes were observed~~The significant difference between species was observed only for E. An excess of Cd may decrease the activity of enzymes involved in C fixation; thus, the increase of intercellular CO_2 concentration found in plants exposed to Cd may be explained by alterations in RuBisCO activity. According to Shi and Cai

(2008), the increase in C_i suggests that the enzymatic dark reactions of photosynthesis were affected. The increase in C_i/C_a determined by Dias et al. (2013) suggests that non-stomatal limitation strongly contributes to the reduction of P_N. The increase in g_s may be related to an alteration in the K:Ca ratio in guard cells and/or alterations in the concentration of abscisic acid, which controls stomatal movement. The decreased leaf photosynthetic rate resulting from the highest decreases in K and Na supply (mainly in the Me ~~ecotype) was due to an increase in stomatal conductance and transpiration rate~~ was due to high C_i. C_i indicated that the inhibition of photosynthesis was due to an inhibition of Calvin cycle enzymes and/or an inhibition of the photosynthetic electron transport chain. The increase of C_i may be explained by modifications of RuBisCO activities of plant (Redondo-Gómez et al., 2011). Potassium supply may also affect photosynthesis through changes in leaf morphology and anatomy because the specific leaf area as well as leaf thickness and density may depend on K content (Zhang et al. 2006; Battie-Laclau et al. 2014). Eker and Uysal (2013) found that enhanced K supply played a crucial role in the protection of spinach against Cd-induced oxidative stress by decreasing lipid peroxidation and enhancing the antioxidant defense system.

One of the crucial factors affecting the influence of Cd on plant metabolism and physiological processes is its relationship with other mineral nutrients (Lux et al. 2011; Dias et al. 2013). In this study, plant exposure to Cd decreased the plant content of several elements such as Ca, K, Na, Fe. Differences in element reduction in the tested *Noccaea* plants were observed. The ability of plants to take in elements was affected by the selective pressure of the differently contaminated sites. Cadmium can enter the plant through nonspecific cation channels as well as through different divalent cation transporters, competing for absorption with mineral nutrients (Verbruggen et al. 2009; Lux et al. 2011). Therefore, the uptake and distribution of mineral nutrients, such as Fe, Ca, Mg and K, can be severely disturbed (Martin et al. 2012). Cadmium is chemically similar to certain metal elements, including Fe, Zn and Ca, and, therefore, could displace these elements from metalloproteins (Verbruggen et al. 2009). Mg in chlorophyll can also be displaced by Cd. The Cd-Chl complex is highly unstable and decays shortly after formation (Küpper et al. 2007). Soil Cd contamination did not decrease the Mg contents in plants in our experiment. Our results showed no significant increase in Mg content in plants originating from Redlschlag, a place with high Mg content in soils. Lower decline of cation content in Re in contrast to Me is in line with the results of Gonneau et al. (2014). According to their findings *N. caerulescens* from serpentine soil with Mg excess has adapted to nutritionally poor environments by increasing their cation uptake and allocating more energy to cation absorption. The energy cost of ion uptake is high; the supplementary uptake of cations could cause a trade-off with plant growth.

Cd ion uptake occurs via the same transmembrane carriers used to uptake Ca^{2+}, Fe^{2+}, and Mg^{2+} (Papoyan et al. 2007). Rivelli et al. (2012) found that Cd can compete with several essential nutrients (e.g., Ca, K), altering their concentration in tissues. The effect of Cd on osmotic potential could be ascribed to dysfunctions of membrane integrity caused by displacement of Ca from the cell surface by Cd or, as suggested by Poschenrieder and Barcelò (2004), by the increase of solutes in cells, probably in the vacuoles, that store Cd-complexes. According to Candan and Tarhan (2005), the peroxidation of polyunsaturated fatty acids in membranes increased with decreasing Ca^{2+} concentrations during the plant growth period. Our results of Me specie confirmed a decrease in the content of saturated fatty acids not only in relation to increasing Cd content but also with declining Ca content (correlation between Ca content and unsaturated fatty acids $R^2 = 0.66$). The increased content of unsaturated fatty acids in plants growing under Cd stress is caused by increased desaturase activities (Wang 2004; Upchurch 2008).

Several studies have shown that Cd toxicity led to Fe deficiency in plants (Martin et al. 2012). Our results confirmed the decrease in Fe content in the presence of high levels of Cd. Lower antioxidant defenses caused by Cd-induced Fe and Ca deficiency can also contribute to ROS production and lipid peroxidation in Cd-stressed plants (Rodríguez-Serrano et al. 2009). There was a significant correlation between Fe and water-soluble Cd content (R^2=0.80). ~~However, the molecular mechanism underlying Fe accumulation in relationship to Cd tolerance and accumulation is still not fully understood.~~According to Gonneau et al. (2014) negative correlation between Cd and Fe was found in non-metallicolous *N. caerulescens*. This suggests that under strong Cd stress, Fe transport pathway may play a major role.

The reduction in photosynthetic rate led to a limited supply of metabolic energy and therefore to N assimilation restriction (Figure 4). Nitrogen flow through amino acids can change in response to Cd stress. The decline in the free amino acid level may be a consequence of decreased nitrate reductase activity. This decrease in trace metal-treated plants may also reflect a decrease in photosynthesis because sugars are essential for nitrate reductase expression (~~Solanki and Dhankhar 2011~~, Cambell 1999). Moreover, the decline in the activities of nitrate reductase and nitrite reductase is likely to be a consequence of a direct interaction between the metal and –SH groups at the active site of the enzymes. Inhibition of photosynthesis and nitrate or nitrite reductase is reflected in the assimilation of C and N in the AA.

The Cd contents of tested plants increased the content of Hyp, a major AA in plant cell wall hydrolysates. Hydroxyproline-rich glycoproteins secreted by plant cells are believed to have a broad range of functions, ranging from providing structural integrity to mediating cell-cell interactions and communication (Wu et al.

2001), and are formed in oxidative stress conditions (De Graaf et al. 2001). Free amino acids are formed by the catabolism of these compounds, and they are transported to developing leaves (Feller et al. 2008). The highest Hyp content was determined in Re-Cd plants and significant differences between Me, Me-Cd and Re were not confirmed. This finding highlights the lower tolerance of Re to Cd stress. Our results showed that senescence was induced and destruction of proteins was higher in Re than Me. According to Schaller (2004), plant senescence increases N mobilization.

Glu, Gln, Asp and Asn are used to transfer nitrogen from source organs to sink tissues and to build up reserves during periods of nitrogen availability for subsequent use in growth, defense, and reproductive processes. According to Pi et al. (2014), an Asp decrease could affect K deficiency. Our finding confirmed a correlation between Asp and K ($R^2 = 0.57$). ~~The changes in Asp and Glu contents in tested plants showed a similar tolerance to Cd contamination by both species.~~ The changes in Asp and Glu contents in tested plants showed effect of Cd contamination to both species. A decrease in Asp and Glu contents as a result of Cd contamination was also observed in our previous work (Zemanová et al. 2013). In our experiment the significant decrease of Asp was confirmed only for Re-Cd which highlights the lower tolerance of Re to Cd in contrast to Me.

Levels of Glu, which is required for the formation of a peptide bond between the γ-carboxyl group of glutamate and the α-amino group of cysteine and is used in the synthesis of glutathione and phytochelatins in plant cells (Vitória et al. 2001), are related to the allosteric regulation of glutamate kinase activity by free Pro. Our results showed significantly lower Pro accumulation in Me in contrast to Re. Me plants are able to inhibit the formation of Pro from Glu by feedback by a relatively lower level of accumulated Pro than Re. According to García-Ríos et al. (1997), the regulation of Pro biosynthesis from Glu is dependent on the Glu:Pro ratio and is determined by genotype-environment interaction. Plants with a higher Glu:Pro ratio are better adapted to Cd stress. Our results confirmed this finding and different physiological behavior of Me was shown in contrast to Re was shown.

A significant increase in Gln content was determined for both species. Zemanová et al. (2013) confirmed this finding for the ecotype "Ganges". Gln is not only the major amino acid used for nitrogen transport but is also a key metabolite that acts as an amino donor for other free amino acids, a reaction that is primarily catalyzed by glutamate synthase. This pathway interacts with carbohydrate metabolism and the energy status of the organ (Hodges et al. 2003). A decrease in Gln is reflected in the biosynthesis of purine bases from which nucleic acids, ATP and plant hormone cytokinins are formed (Pavlík et al. 2012). Decrease of Gln content confirmed different physiological behavior of no hyperaccululumating plants in contrast to hyperaccumulators.

Asn is an AA used to store and transport N from sources to sinks. According to Zhang et al. (2013), Asn is the major form of N transported to sink tissues in *Arabidopsis* mutants. Our observations showed a decrease in Asn concentration in the biomass of all plants treated with Cd. Pavlík et al. (2010) confirmed similar results for spinach growing under arsenic stress. However, Lea et al. (2007) published opposite results; cadmium induced an almost 10-fold increase in the asparagine concentration of tomato roots but only a four-fold increase in the leaves.

Conclusion

Comparison of *N. caerulescens* and *N. praecox* showed that Cd stress resulted in similar changes in gas-exchange parameters. Contrasting responses of plants to Cd contamination were observed in element contents and fatty acid and amino acid metabolism. Significantly higher accumulation of Cd and strong declines of K, Ca, Na and Fe were determined in the Me plants in contrast to the Re plants. The increased ability of Re plants to take in some cations is a result of the selective pressure of growth in a contaminated area. The significant increase in the glutamic acid/proline ratio was determined for the hyperaccumulating ~~ecotype~~ specie from Slovenia.

Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

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Figure 1 The yield of aboveground biomass (mg.kg^{-1}). The values represent the means of data obtained in the experiment (n = 5, i.e., five replications per each treatment). Cd concentration was either 0 or 90 mg Cd.kg^{-1} soil. Letters refer to significantly different values that are significant ($P \leq 0.05$) - A, B comparison between both ~~ecotype~~species; a, b comparison between treatments in each ~~ecotype~~ specie.

Figure 2 Total content of saturated and unsaturated fatty acids in ~~both ecotypes of~~ *N. caerulescens* and *N. praecox* leaves. The values represent the means of data obtained in the experiment (n = 3, i.e., three replications per each treatment). Cd concentration was either 0 or 90 mg Cd.kg^{-1} soil. Letters refer to significantly different values that are significant ($P \leq 0.05$) - A, B comparison between both ~~ecotype~~species; a, b comparison between treatments in each ~~ecotype~~specie.

Figure 3 Ordination diagram showing the results of PCA analysis with selected parameters in *N. caerulescens* and *N. praecox*. Treatment abbreviations: Cd - treatment 90 mg Cd.kg^{-1}. Parameters abbreviations: yield - yield of aboveground biomass, sat FA - total content of saturated fatty acids, unsat FA - total content of unsaturated fatty acids, P_N - net photosynthetic rate, C_i - intercellular CO_2 concentration, E - transpiration rate, g_s - stomatal conductance, WUE - water-use efficiency, C_T - total content of Cd, C_W - water extractable-Cd, Ca - total content of Ca, Fe - total content of Fe, K - total content of K, Mg - total content of Mg, Na - total content of Na, Asp - concentration of free aspartic acid, Asn - concentration of free asparagine, Pro - concentration of free proline, Glu - concentration of free glutamic acid, Gln - concentration of free glutamine, Ser - concentration of free serine, Hyp - concentration of free hydroxyproline.

Figure 4 The effect of Cd stress on plant metabolism

Figure
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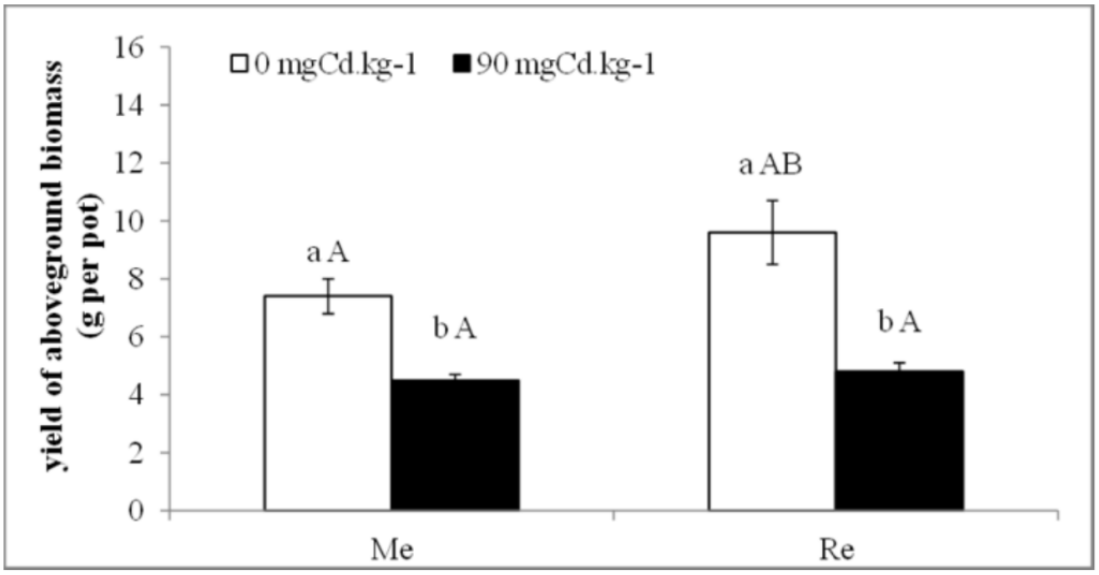

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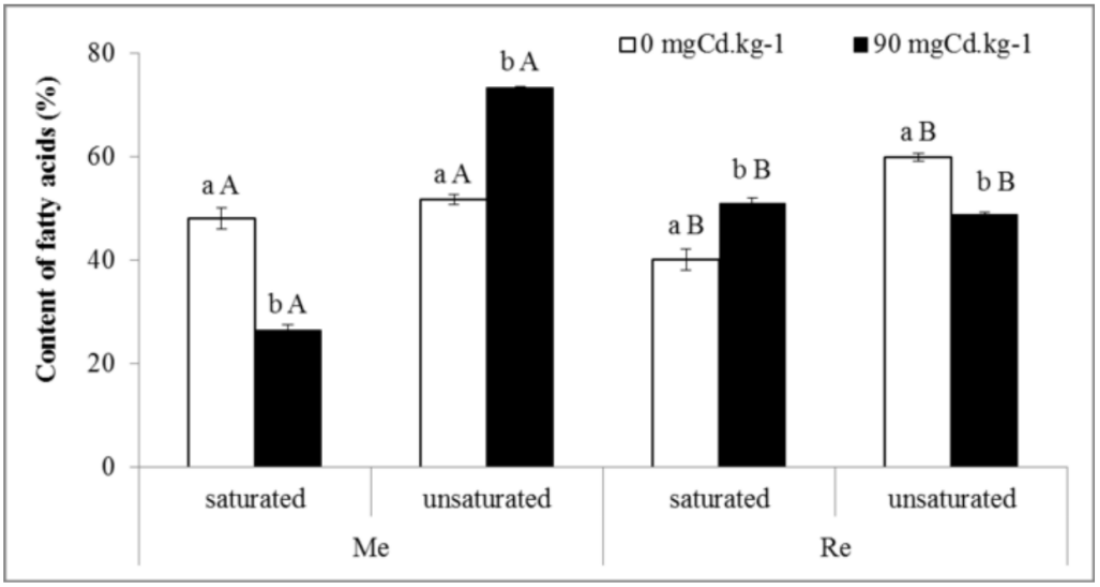

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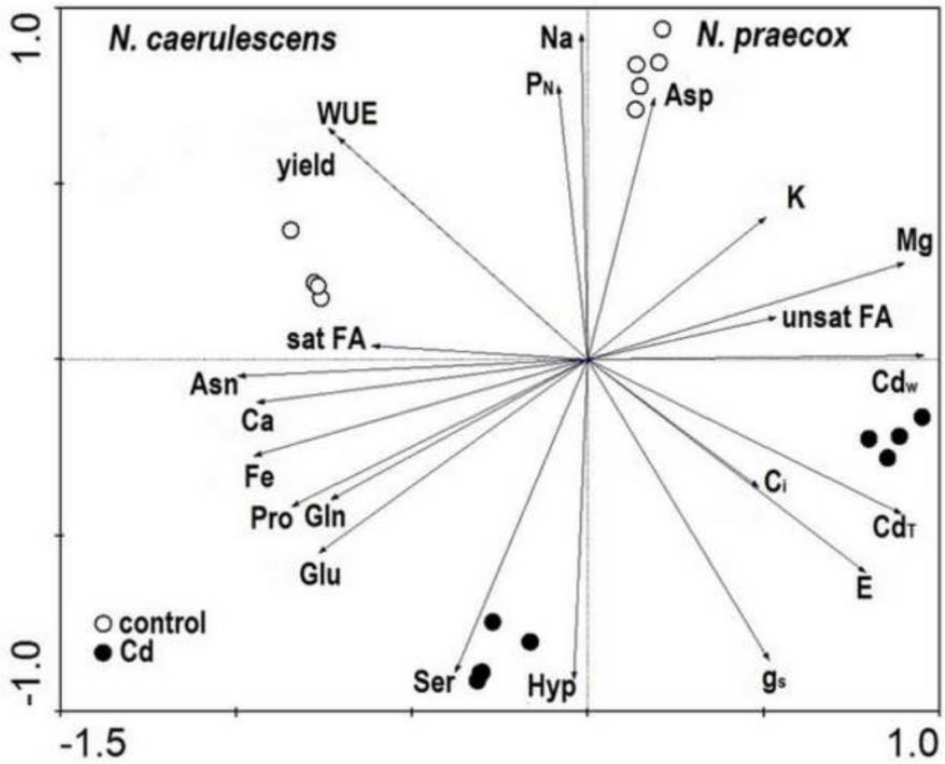

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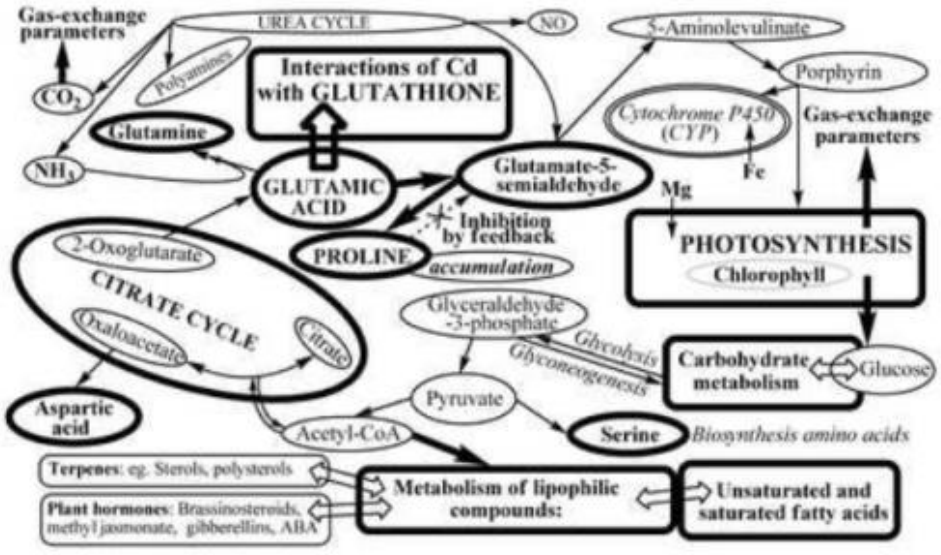

Table
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Table 1 The element contents in the aboveground biomass of plants. The values represent the means of data obtained in the experiment (n = 5, i.e., five replications per each treatment). Letters refer to significantly different values that are significant ($P \leq 0.05$) - A, B comparison between both species; a, b comparison between treatments in each specie.

Treatment	Me	Me-Cd	Re	Re-Cd
Cd_T (mg.kg^{-1})	28.0 ± 1.6^{aA}	7201 ± 15.9^{bA}	2.0 ± 0.1^{aB}	137 ± 9.6^{bB}
Cd_w (mg.kg^{-1})	12.5 ± 0.4^{aA}	1300 ± 9.7^{bA}	0.2 ± 0.0^{aB}	1.0 ± 0.1^{bB}
K (%)	5.99 ± 0.23^{aA}	3.29 ± 0.15^{bA}	4.95 ± 0.19^{aB}	4.55 ± 0.11^{bA}
Mg (%)	0.22 ± 0.03^{aA}	0.22 ± 0.04^{aA}	0.17 ± 0.02^{aB}	0.18 ± 0.01^{bB}
Ca (%)	2.18 ± 0.09^{aA}	1.40 ± 0.10^{bA}	2.77 ± 0.07^{aB}	2.47 ± 0.15^{aB}
Fe (mg.kg^{-1})	227 ± 26^{aA}	176 ± 18^{bA}	474 ± 31^{aB}	441 ± 27^{bB}
Na (mg.kg^{-1})	277.8 ± 15.0^{aA}	99.6 ± 8.9^{bA}	126.0 ± 12.1^{aB}	78.4 ± 8.7^{bB}

Table 2 The effect of Cd contamination on CO_2 intracellular concentration (C_i), net photosynthetic rate (P_N), stomatal conductance (g_s) and transpiration rate (E) of *Noccaea sp.* (n = 5). From these data, the water-use efficiency was estimated (WUE = P_N /E). The values represent the means of data obtained in the experiment (n = 5, i.e., five replications per each treatment). Cd concentration was either 0 or 90 mg Cd.kg^{-1} soil. Letters refer to significantly different values that are significant ($P \leq 0.05$) - A, B comparison between both species; a, b comparison between treatments in each specie.

Treatment	P_N (μmol CO_2.m^{-2}. s^{-1})	E (mmol H_2O. m^{-2}. s^{-1})	g_s (mol.m^{-2}.s^{-1})	C_i (vpm)	WUE
Me	8.68±0.9^{aA}	0.433±0.03^{aA}	0.322±0.015^{aA}	354±15^{aA}	20.05
Me-Cd	7.97±1.1^{aA}	1.505±0.11^{bA}	0.989±0.026^{bA}	360±21^{aA}	5.30
Re	8.50±0.6^{aA}	0.263±0.02^{aB}	0.361±0.021^{aA}	332±17^{aA}	32.30
Re-Cd	7.49±0.7^{bA}	0.917±0.04^{bB}	0.917±0.076^{bA}	367±19^{bA}	8.17

Table 3 The concentrations of selected free amino acids in the aboveground biomass of plants. The values represent the means of data obtained in the experiment (n = 5). Letters refer to significantly different values that are significant ($P \leq 0.05$) - A, B comparison between both species; a, b comparison between treatments in each specie.

Free amino acid	Me	Me-Cd	Re	Re-Cd
			μmol.kg^{-1}	
glutamic acid (Glu)	9110±109^{aA}	9037±65^{aA}	12295±98^{aB}	13990±86^{bB}
aspartic acid (Asp)	5248±87^{aA}	5323±7^{aA}	5382±115^{aA}	4244±93^{bB}
glutamine (Gln)	13398±99^{aA}	25477±49^{bA}	13176±82^{aB}	27545±79^{bB}
asparagine (Asn)	7288±95^{aA}	3051±101^{bA}	30386±84^{aB}	15179±96^{bB}
proline (Pro)	951±62^{aA}	1220±69^{bA}	3338±73^{aB}	5299±65^{bB}
serine (Ser)	2502±22^{aA}	5102±35^{bA}	6168±37^{aB}	8664±42^{bB}
hydroxyproline (Hyp)	297±38^{aA}	329±19^{aA}	302±26^{aA}	417±31^{bB}

Table 4 The Glu:Pro amino acid ratio, which affects the regulation of Pro biosynthesis.

Treatment	Me	Me-Cd	Re	Re-Cd
GLU/PRO	9.58	7.41	3.68	2.64

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Changes in amino acids metabolism and in contents of selected microelements in two Noccaea species as a result of cadmium stress

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Abstract:	The objective of this study is to determine amino acid changes in Noccaea caerulescens (Redlschlag, Austria - Re) and Noccaea praecox (Mežica, Slovenia - Me) originating from differently contaminated sites associated with Cd stress. After 120 days of plant cultivation in Cd-contaminated soil (90 mg Cd kg soil-1), free amino acids and selected microelements (Cu, Mn, Ni, Zn) were determined in addition to N utilization by plants. Cadmium contamination reduced Zn and Ni contents in plants but did not significantly affect Cu and Mn in all treatments. The Cd effect on plant stress metabolism resulted in changes in levels of selected free amino acids playing an important role in adaptation to stress and plant senescence. The result of this study indicated that two groups of amino acids (phenylalanine, tyrosine and tryptophan - necessary for protein biosynthesis; branched-chain amino acids - valine, leucine, and isoleucine - building blocks of proteins) were accumulated more in the leaves of control Me treatment than in the leaves of control Re treatment. Under Cd stress our analyses confirmed increase of the contents of these amino acids in both species, but the significant changes were determined only for Re-Cd. Contrasting responses of species to Cd contamination were confirmed for alanine, phenylalanine, threonine and sarcosine. Significant increases in the sarcosine content resulted from higher adaption of N. praecox (Me) in contrast to N. caerulescens (Re). The high Cd accumulation in Me plants is enabled by Cd chelation by sarcosine.	

Changes in amino acids metabolism and in contents of selected microelements in two *Noccaea* species as a result of cadmium stress

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Abstract

The objective of this study is to determine amino acid changes in *Noccaea caerulescens* (Redlschlag, Austria - Re) and *Noccaea praecox* (Mežica, Slovenia - Me) originating from differently contaminated sites associated with Cd stress. After 120 days of plant cultivation in Cd-contaminated soil (90 mg Cd kg soil^{-1}), free amino acids and selected microelements (Cu, Mn, Ni, Zn) were determined in addition to N utilization by plants. Cadmium contamination reduced Zn and Ni contents in plants but did not significantly affect Cu and Mn in all treatments. The Cd effect on plant stress metabolism resulted in changes in levels of selected

free amino acids playing an important role in adaptation to stress and plant senescence. The result of this study indicated that two groups of amino acids (phenylalanine, tyrosine and tryptophan - necessary for protein biosynthesis; branched-chain amino acids - valine, leucine, and isoleucine - building blocks of proteins) were accumulated more in the leaves of control Me treatment than in the leaves of control Re treatment. Under Cd stress our analyses confirmed increase of the contents of these amino acids in both species, but the significant changes were determined only for Re-Cd. Contrasting responses of species to Cd contamination were confirmed for alanine, phenylalanine, threonine and sarcosine. Significant increases in the sarcosine content resulted from higher adaption of *N. praecox* (Me) in contrast to *N. caerulescens* (Re). The high Cd accumulation in Me plants is enabled by Cd chelation by sarcosine.

Keywords: adaption to Cd stress; amino acids; diversity of hyperaccumulators; heavy metal; sarcosine; zinc

Introduction

Cadmium is considered as being one of the most toxic metals that exhibits adverse effect on all biological processes of human, animals and plants. Although Cd is toxic for plant growth, it is readily taken up by roots and translocated to the shoots. It can enter the plant through nonspecific cation channels as well as through different divalent cation transporters, competing with essential mineral nutrients for absorption (Lux et al. 2011; Verbruggen et al. 2009). Therefore, the uptake and distribution of essential mineral nutrients, such as Fe, Zn, Mn, Ca, Mg, can be severely disturbed in the presence of high levels of Cd (Martin et al.

2012). The hyperaccumulating plants usually have a greater capacity to translocate Cd from root to aboveground part. Higher rate of xylem loading is probably the reason of enhanced root-to-shoot translocation of Cd (Lu et al. 2008).

Cadmium exposure alters guard cell function, induces leaf chlorosis, perturbs the cellular redox balance and inhibits growth (Pavlíková et al. 2002; Verbruggen et al. 2009). It induces oxidative stress in plants by blocking essential functional groups in biomolecules and by indirect mechanisms such as interaction with the antioxidant defense system, disruption of the electron transport chain or induction of lipid peroxidation (Cuypers et al. 2010). Plants exposed to Cd had elevated levels of mitochondrial ROS production, indicating that this organelle had become dysfunctional (Heyno et al. 2008). This element has been shown to be one of the most effective inhibitors of photosynthetic activity (Gallego et al. 2012). It can enter chloroplasts and disturb chloroplast function by inhibiting the enzymatic activities involved in chlorophyll biosynthesis, pigment-protein complexes, the O_2-evolving reactions of photosystem II, electron flow around photosystem I and chloroplast structure (Molins et al. 2013; Ying et al. 2010; etc.). According to Leitenmaier and Küpper (2011), hyperaccumulating plants have to store the excess metal in such a way that it does not harm important enzymes and especially not photosynthesis. It has been shown that high amounts of metals are stored specifically in the vacuoles of large epidermal cells (Frey et al. 2000; Küpper et al. 1999; Küpper et al. 2001), where no chloroplasts are located so that photosynthesis cannot be inhibited. Zemanová et al. (2015) confirmed changes in the fatty acids composition in response to the extent of Cd contamination of soils differed between *N. caerulescens* and *N. praecox*.

The adaptation of plants to toxic concentrations of elements depends upon various mechanisms operating at both intra and intercellular levels. Amino acids (AA) metabolism may play an important role in plant stress resistance, by osmotic adjustment and the

accumulation of compatible osmolytes; detoxification of active oxygen species and toxic elements; and intracellular pH regulation (Singh 1999). Plants exposed to toxic metals have been shown to accumulate specific AA, which may have beneficial functions and play various roles (Pavlíková et al. 2014 a,b; Xu et al. 2013). Xu et al. (2012) found that Cd also markedly increased the production of several organic and AA in *Solanum nigrum*. This finding suggested that the compaction of heavy metals by metabolites, such as organic acids and AA, is crucial in metal detoxification, transport and accumulation. Xie et al. (2014) confirmed the differential accumulation of AA - glycine (Gly), proline (Pro), serine (Ser), threonine (Thr) and glutamic acid (Glu) could be associated with the differential Cd tolerance in two ecotypes of bermudagrass. According to Pavlíková et al. (2014b) tolerance of tobacco plants to toxic metals was associated with the maintenance of accumulation of Pro, methionine (Met) and γ-aminobutyrate (GABA). Solanki and Dhankhar (2011) reported that when trace element toxicity crosses the threshold limit, the protein level decreases and this might be due to the breakdown of protein synthesis mechanism at toxic concentration level of trace elements or due to reduced incorporation of free AA into proteins, especially to proteins or glycoproteins rich to Pro, hydroxyproline (Hyp), Gly, cysteine (Cys), Ser, alanine (Ala), histidine (His) etc., linked either with physiological processes (growth of cell wall, pollen tubes, pollen fertility), and or stress (Cassab 1998; De Graaf et al. 2001; Showalter 1993).

Our study focuses on the investigation of changes in the AA metabolism of hyperaccumulating plants growing on an environmentally relevant substrate - soil - and occurring under chronic stress caused by Cd. The aim of this study was to characterize changes in AA metabolism, that are associated with plant detoxification, and in contents of selected microelements and to relate these changes to the adaption strategies of *N. caerulescens* and *N. praecox* growing under Cd stress.

Material and methods

Plant material and cultivation conditions

Noccaea caerulescens (formerly *Thlaspi caerulescens* J. & C. Presl, FK Mey) and *Noccaea praecox* (*Thlaspi praecox* Wulfen) are pseudo-metallophytes growing both on unpolluted and trace metal enriched soils. These species are particularly known to accumulate some trace metals in its aboveground parts. In the pot experiments, *N. praecox* from Mežica, Slovenia (Me) and *N. caerulescens* from Redlschlag, Austria (Re) were used. The Mežica mining district source area is strongly polluted with Pb and Zn. Because Cd is found as a trace element in sphalerite and smithsonite, its content correlates with that of Zn (Gosar and Miler 2011). The bedrock of Redlschlag is composed of serpentine, which contains high levels of Ni and Cr and some Zn and Co. Because the soil pH is neutral (approx. pH 6.55), the main problems for plants are low concentrations and availability of micronutrients, although Mg is abundant (46,400 mg Mg kg^{-1} - Puschenreiter et al. 2005).

For the cultivation of *Noccaea* plants, 3 kg of soil from the non-polluted site Prague-Suchdol (Table 1) was thoroughly mixed with nutrients and Cd (Table 2). Plants were cultivated in a greenhouse under controlled conditions: temperature day/night 24 °C/18 °C and light intensity: day/night 16 h/8 h. The water regime was controlled, and the soil moisture was kept at 60 % MWHC (maximum water-holding capacity). Each treatment was performed in five replications. Plants were harvested 120 days after Cd application. Samples were kept frozen in liquid nitrogen for transport and then at –30 °C until extraction.

Analyses

Analysis of free amino acids in plant biomass

The content of free amino acids was determined after their derivatization by EZ-faast set (Phenomenex, U.S.A). Samples of fresh biomass (~0.5 g) were extracted 10.5 ml of methanol + redestiled H_2O (7:3, v/v) for 24 hours. Derivatization of free AA according to method of

Neuberg et al. (2010) was carried out. The content of free AA was measured by GC-MS using a Hewlett Packard 6890N/5975 MSD (Agilent Technologies, USA). Samples were separated on a ZB-AAA 10 m x 0.25 mm AA analysis GC column using constant carrier gas (He) flow (1.1 mL min^{-1}). The oven temperature program was as follows: initial temperature 110 °C, 30 °C min^{-1} ramp to 320 °C. The temperature of the injection port was 280 °C. A total of 1.5 µL sample was injected in split mode (1:15, v/v). The MS conditions were as follows: MS source 240 °C, MS quad 180 °C, auxiliary 310 °C, electron energy 70 eV, scan m/z range 45–450 and sampling rate 3.5 scan s^{-1} (Pavlík et al. 2012).

Analyses of cadmium and additional elements in plant biomass

Plant samples were decomposed using the dry ashing procedure. The ash was dissolved in 20 mL of 1.5 % HNO_3 (v/v) (electronic grade purity, Analytika Ltd., Czech Republic) and kept in glass tubes until analysis. Aliquots of the certified reference material RM NCS DC 73350, poplar leaves, (purchased from Analytika, CZ) were mineralized under the same conditions for quality assurance. The Cd and macro- and microelement concentrations were determined by ICP-OES with axial plasma configuration (Varian VistaPro, Varian, Australia).

Statistical analyses were performed using hierarchic analyses of variance (ANOVA) considering interactions at the 95% ($P < 0.05$) significance level with subsequent Tukey's HSD test and correlation (R^2). All analyses were performed with Statistica 9.0 software (StatSoft, USA).

Results

As it reported in our previous paper (Zemanová et al. 2015), the yield of aboveground biomass, although similar in the species under Cd stress, indicated greater reduction of Re-Cd than Me-Cd. The biomass Cd contents showed significant differences between the *Noccaea*

species. The highest Cd content was found in the biomass of Me-Cd plants (7040 mg kg^{-1}). The Cd content of Re (134 mg kg^{-1}) was significantly lower than in Me (Table 3).

Plant microelement contents

The microelement contents of Me and Re were different; however, the trends of the changes were similar (Table 3). The high cation contents in control treatment of serpentine population of *N. caerulescens* (Re) were presented. Manganese and Ni contents of Re control treatment were significantly higher in contrast to Me control treatment (by 58 % and by 46 % higher content). The contents of the tested elements in the aboveground biomass were affected by Cd supply. Cadmium reduced Zn and Ni contents but did not significantly affect Cu and Mn in all treatments. Zn and Ni contents was hardly affected in Re-Cd (59 % Zn reduction; 91 % Ni reduction), and Me-Cd showed only 1 % Zn reduction and 40 % Ni reduction. Our results showed different physiological behavior of tested species.

Free amino acids

The result of this study indicated that two groups of amino acids (i)aromatic amino acids - phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) - necessary for protein biosynthesis, for biosynthesis of auxin from Trp and for antioxidant metabolites from Phe and Tyr; ii) branched-chain amino acids (BCAAs) - valine (Val), leucine (Leu), and isoleucine (Ile) - building blocks of proteins) were accumulated more in the leaves of control Me treatment than in the leaves of control Re treatment (by 8 % and 16 %, respectively). Under Cd stress our analyses revealed increase of the contents of these AA in both species, but the significant change was confirmed only for Re-Cd (Table 4).

Phenylalanine is the AA for which significant difference between varieties was confirmed. Cd contamination significantly affected its content only for Re-Cd (increase by 37 % for Re-Cd in contrast to increase by 3 % for Me-Cd). The contents of Tyr and Trp were increased in Cd treatments, but significant Trp increase was determined only for Re-Cd (by 27 %). The close

correlations between Cd contents in plants and contents of these AA were calculated (R^2 = 0.86 Phe, 0.77 Trp, 0.81 Tyr).

The increases in free Leu and Ile concentrations under Cd stress were determined only for Re-Cd (by 21 % Leu, by 40 % Ile). The significant changes of these AA were not confirmed for Me treatments. A different trend was observed for Val in both Cd treatments, but the changes were not significant. The correlations between Cd contents in plants and Leu and Ile contents were calculated (R^2 = 0.65–0.67), this relationship was not confirmed for Val (R^2 = 0.17). As both threonine (Thr) and methionine (Met) serve as substrates for Ile synthesis, their syntheses and catabolism also affected isoleucine availability. A significant correlation between Thr and Ile was found (R^2 = 0.75). The Met concentrations were below detection limit of GC. Both species tended to have higher concentrations of Thr in the Cd treatments and difference between Thr contents in species was significant. Significant Thr increase was determined only in Re-Cd in contrast to control Re plants (increase by 63 %).

These AA (except for Thr) also correlated with Sar (N-methylglycine) (R^2 = 0.52–0.70). Sar (Figure 1) was found only in Me, Me-Cd and Re. Its content in Re-Cd was below the detection limit. Its physiological effect in plants is unknown. One of the major biosynthetic pathways of plant stress metabolite glycine betaine leads *via* Sar. Sar accumulation is differed for tested species, we can speculated, that Sar content is one of significant factors of adaptability of these hyperaccumulators. Significance of Sar for microorganisms and its well-described chelating properties indicate its role in plants.

The difference of glycine (Gly) content between control treatments of both species was not significant, but higher content (by 18 %) was determined in Re. The significant Gly decrease was found only for Re-Cd. Gly is crucial AA for the biosynthesis of Sar, Cys *via* Ser. Gly is involved in the biosynthesis of phytochelatines and also in the biosynthesis of antioxidant metabolites, and it is part of Gly-rich proteins that affect the growth and function of cell walls.

Therefore, the decline of Gly contents in both varieties must be evaluated as activation of adaption processes to the Cd toxic effect.

For ornithine (Orn) significant difference was determined between tested species and only between Me and Me-Cd. Ornithine is one of AA of urea cycle. These AA are metabolized in a lack of energy for the biosynthesis of metabolites by ureases. Ni cofactor is typical for ureases. Increase of Orn is a response to Cd stress and it associated with nitrogen and carbon metabolism through a transfer AA aspartate (Asp) and with the tricarboxylic acid cycle (TCA) *via* fumarate.

The concentrations of alanine (Ala) were changed in relation to species and to Cd content in plant. The Ala concentrations were decreased in plants growing on the Cd treatments (Me-Cd by 15 % and Re-Cd by 19 % in contrast to control treatments). The significant difference of Ala contents was determined between tested plants. Lower Ala content was confirmed for Me treatments (by 22 % for control and by 19 % for Cd treatment in contrast to Re plants).

The amino acid γ-aminobutyric acid (GABA) increases in response to different stresses. Our results showed no significant differences in GABA content between controls and Cd treatments and between species. GABA is an important stress metabolite originating from glutamic acid. Succinate (part of the TCA cycle) is formed by GABA catabolism *via* succinate semialdehyde.

The relationship between contents of GABA and Ala in plant biomass was confirmed by our results using linear correlation and significant relationship was calculated for both Me and Re plants (R^2 = 0.94). This correlation is very important because GABA is increased with a decrease of Ala content. As Ala, GABA is important as a chelating agent in cytosol. Increase of GABA at the expense of Ala leads to an increase of pH in cytosol, which determines as the activities of enzymes well as activity and transport of plant hormones.

Discussion

Noccaea plants originated from heavy metals contaminated soils are known as the metallicolous (Visioli et al. 2014). Tested species are differed by their adaptation to different soil contamination. *N. caerulescens* from Redlschlag, Austria has adapted to grow on serpentinite, a soil naturally rich in Ni, Co, Cr in contrast to *N. praecox* originated from Mežica, Slovinia which has adapted to growth on soil highly contaminated by Cd, Zn and Pb. According to Gonneau et al. (2014) *N. caerulescens* from serpentine soil with Mg excess has adapted to nutritionally poor environments by increasing their cation uptake and allocating more energy to cation absorption. For this reason Cu, Mn and Ni accumulation of Re control treatment was significantly higher in contrast to Me control treatment and differences in element reduction in the tested *Noccaea* plants were observed. Plant exposure to Cd decreased the content of Zn and Ni. Reductions of both contents of these elements were significant for Re-Cd in contrast to Me-Cd. The most significant Zn decline was determined in Re-Cd plants (Re-Cd by 59 % and Me-Cd 1 % in contrast to control treatment). These results confirmed higher metal tolerance and accumulation in metallicolous Me in contrast to serpentine Re (Reeves et al. 2001; Visioli et al. 2014).

Cadmium can enter the plant through nonspecific cation channels as well as through different divalent cation transporters, competing for absorption with mineral nutrients (Lux et al. 2011; Verbruggen et al., 2009). Therefore, the uptake and distribution of mineral nutrients, such as Zn and Mn can be severely disturbed (Martin et al. 2012). Cadmium is chemically similar to certain metal elements, including Fe, Zn and Ca, and, therefore, could displace these elements from metalloproteins (Verbruggen et al. 2009).

Biogenic elements, Zn, Cu, Mn, Fe and Ni are metalloenzyme cofactors (for example for superoxide dismutase), a broad class of important biomolecules forming biological metal complexes that perform a wide range of important functions, for example defense against

oxidative stress (Fernández-Ocaña et al. 2011). Ni-urease affected the regulation of N and C metabolism (Gerendás et al. 1999; Polacco et al. 2013) by linking urease cycle to TCA cycle. The formation of binding between metal and enzyme is dependent on the metal concentration as a cofactor in the cell (Egleston and Morel 2008). The decline of metal contents may be resulted to a decrease in metalloenzyme activities. We propose that the increased ability of uptake of these elements by Re control plants in contrast to Me is a result of selection pressure from the serpentine area. The decrease of elements uptake by Re-Cd plants from Cd contaminated soil in contrast to Me-Cd is related to better use of these elements in metabolism.

The increased content of unsaturated fatty acids in plants growing under Cd stress is caused by increased desaturase activities (Upchurch 2008; Wang 2004; Zemanová et al. 2015). It also appears that the activities of plant desaturases can be affected by Ni content based indirectly on a study by Cao et al. (2010). According to this study, bacterial desaturases have a Ni-binding site. Gerendás et al. (1999) described the effect of Ni on N plant metabolism, and our results confirmed a significant effect of decreased Ni on the AA levels. High plant Ni content is linked to maximum efficiency of Ni incorporation into the active center of ureases and glyoxalases. According to Gerendás et al. (1999) and Thornalley (1998), ureases and glyoxalases have a significant role in cell catabolism, and AA catabolism is associated with plant senescence. Mustafiz et al. (2010, 2014) showed a significant effect of the glyoxalase pathway and the two enzymes glyoxalase I and glyoxalase II in plant stress metabolism.

Ureases are metalloenzymes that catalyze the hydrolysis of urea, an intermediate of plant arginine catabolism involving in nitrogen remobilization from source tissues (Whitte 2011). Arginine degradation in mitochondria leads to the formation of ornithine and urea *via* arginase activity (urease-ornithine cycle). In turn, ornithine can be used in glutamate synthesis, and urea is a source of nitrogen that can be sensed by plant cells and used for amino acid synthesis

(Planchais et al. 2014). According to Planchais et al. (2014), when Ni is a limiting factor for urease activity, urea and ornithine can accumulate in *Arabidopsis*. Our results showed an increase in ornithine in both Me-Cd and Re-Cd treatments.

Urease and glyoxalase activity can be a source of energy during stress metabolism in hyperaccumulating plants (Mustafiz et al. 2010). For the above reasons, the use of Ni as a cofactor of urease and glyoxalases is important to the adaptation of hyperaccumulators to Cd contamination. A higher Ni content of Me-Cd in contrast to Re-Cd was found in our experiment. This finding may explain the high ability of *N. praecox* from Mežica (Me) to use metabolite catabolism for the formation of compounds involved in the detoxification of toxic elements.

Amino acid homeostasis is essential for plant growth, development and defense (Liu et al. 2010). This homeostasis is regulated by *de novo* biosynthesis, uptake/translocation, and protein synthesis/turnover. End product inhibition at the branching points of the biosynthesis of branched chain AAs (BCAAs) (i.e., Leu, Ile and Val) is pivotal to balance the fluxes between different AA pathways (He et al. 2013). According to Joshi et al. (2010) accumulation of free BCAAs may serve as a substrate for the synthesis of stress-induced proteins and BCAAs may act as signaling molecules to regulate gene expression. Significant increases of Leu and Ile contents were detected in our experiment only for Re-Cd. This finding confirmed that Re plants are less adapted to Cd stress and showed that stress in Re plants activated enzymes associated with degradation and with senescence.

Leu together with Zn is present in many enzymes - Leu aminopeptidases, which degrade proteins (Tailor 1993). These enzymes are related to the defense roles against stress and are connected with transport of auxin, formation of jasmonic acid and senescence of plants which is induced by stress for example by heavy metals. Higher content of Leu was determined in Cd treatment of Re plants but which have no significantly longer life span in contrast to Me

plants. This result is not consistent with finding of Pavlíková et al. (2014b) that higher Leu content was determined in tobacco plants with longer life span. Our finding can be connected with significantly lower contents of Zn (cofactor of superoxide dismutase) in Re plants than Me plants. Reduction of Zn content in Re plants in comparison with Me plants is one of the reasons for reducing of the capacity of plant antioxidant system (Cakmak 2000; Fernández-Ocaña et al. 2011).

Xu et al. (2012) found accumulation of Ile and Val in *Solanum nigrum* (high Cd accumulation and tolerance) under Cd stress. However, our result showed that under Cd stress, Ile content increased for less Cd-tolerant Re plants but unchanged for more Cd-tolerant Me plants, which suggests that two different stress defense pathway may exist in these two genotypes.

Pathways regulating Thr, Met and Ile metabolism are very efficiently interconnected in plants. As both Thr and Met serve as substrates for Ile synthesis, their synthesis and catabolism under different developmental and environmental conditions also influence Ile availability (Joshi et al. 2010).

Phenylalanine (Phe), Tyr and Trp are necessary not only for protein biosynthesis; Phe is also a substrate for the phenylpropanoid pathway that produces numerous plant secondary products, especially antioxidative metabolites - flavonoids, anthocyanins, lignins, phenylpropanoic acids, including salicylic acid, and phenolic compounds (Rice-Evans et al. 1996), some of them are growth promoters, and growth inhibitors. Tryptophan is the precursor for indolacetic acid, a plant hormone necessary for growth and development of plant cell. Trp plays a major role in the regulation of plant development and defence responses. Characteristically, Trp biosynthesis is induced by stresses. Sanjaya et al. (2008) reported that increased Trp levels make Cd less accessible to the plant, decrease Cd transport and thus reduce Cd accumulation. Metal ions and the bivalent Trp side chain indole were found to interact cooperatively (Li and

Yang 2003). This finding is corresponded with our results. The significant Trp increase was determined only in Re-Cd plants, no in more Cd tolerant Me plants.

The alanine is markedly accumulated in response to stress in plants and it especially discussed in relation to intracellular pH regulation. Results presented here demonstrate opposite trend, Ala contents in both species growing on Cd contaminated soil were decreased. Pavlík et al. (2010) confirmed our results only for no hyperaccumulating plants growing on slightly As contaminated soil. Strong As soil contamination led to increased Ala content in plants. Similar results were reported for tobacco growing on Zn contaminated soil (Pavlíková et al. 2014b). According to Hjorth et al. (2006) the increased content of free Ala might be caused by a reduction in the rate of protein syntheses and an increased synthesis of Ala due to disturbance of the alanine aminotransferase reactions. Our results showed that hyperaccumulating plants do not accumulate Ala in cytosol for pH regulation, but they can use it for biosynthesis of proline/alanine-rich protein kinase (Mori et al. 2013) or histidine- and alanine-rich protein (Komatsu et al. 2009). Both species accumulate GABA for pH regulation (Bor et al. 2009).

GABA plays different roles in plant metabolism including carbon-nitrogen metabolism, energy balance, signaling and development, stress defense (Sawaki et al. 2009). The GABA shunt plays a key role in carbon and nitrogen partitioning by linking amino acid metabolism and the tricarboxylic acid cycle, which is essential for higher plant species (Seher et al. 2013). This role is confirmed by our finding of a significant correlation between GABA content and the total content of free amino acids (R^2 = 0.99). Increases in GABA levels in response to short exposures to different abiotic and biotic environmental stressors in several plants are commonly observed (Kinnersley and Turano 2000; Pavlíková et al. 2014b). Our results are consistent with these findings, but GABA accumulation was not significant.

Sarcosine (N-methylglycine) is an intermediate compound in trimethylglycine (glycine betaine) metabolism (Figure 2), but its physiological effects in plants are unknown (Oda et al.

2005). Glycine betaine is known as metabolite accumulating during oxidative stress (Kholová et al. 2009). Therefore Sar decline (by methylation of Gly *via* SAR to betaine glycine (Niu et al. 2014) or by lack of Gly caused by reaction of Gly and choline (Ashraf and Foolad 2007)) is related with biosynthesis of glycine betaine and plant defense against oxidative stress. Our results confirmed a correlation between Gly and Sar (R^2 = 0.57), but no correlation between Met and Sar was found because Met contents were below the detection limit. We propose that Met participates in the N-methylation of Gly *via* S-adenosylmethionine, which is a methyl donor (Ibrahim et al. 1998; Roje 2006). Oxidative stress of Re-Cd in contrast to Me-Cd was significantly higher as shown in a dramatic decrease of Sar content in Re-Cd. In the Cd treatments, Sar was detected only in Me-Cd. Its content in Re-Cd was below the detection limit. The high Cd accumulation in Me-Cd may be due to chelation of Cd by Sar. According to Krishnakumar et al. (1996) and (Tewari 2012), Sar can be complexed with Cd and other metals. Low Sar decline is advantage for Me specie compared to Re. The Sar role in hyperaccumulating plants is analogous to the role of His forming metal chelates, mainly with Ni (Krämer et al. 1996), and protecting nucleid acids against oxidative stress.

Conclusion

Our results emphasize that *N. caerulescens* and *N. praecox* growing under the selective pressure of differently contaminated places can differ in physiological performance. From these results it is clearly to see complex changes in the regulation of stress metabolism of plants exposed to selection pressure during their phylogenetic development at the specific polluted areas. Contrasting responses of *Noccaea* species to Cd contamination were observed in microelement contents and AA metabolism. Manganese and Ni contents of Re control treatment were significantly higher in contrast to Me control treatment. The increased ability of Re plants to take in some cations is a result of the selective pressure of growth in a

contaminated area. The contents of the tested elements in the aboveground biomass were affected by Cd supply. Cadmium reduced Zn and Ni contents mainly in Re-Cd but did not significantly affect Cu and Mn in all treatments. Contrasting responses of species to Cd contamination were confirmed for alanine, phenylalanine, threonine and sarcosine. Significant increases in the sarcosine content resulted from higher adaption of *N. praecox* (Me) in contrast to *N. caerulescens* (Re). The high Cd accumulation in Me plants is enabled by Cd chelation by sarcosine. Plant growth is limited by these changes of stress metabolism.

Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

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Fig. 1 The concentrations of sarcosine (Sar) in the aboveground biomass of plants. The values represent the means of data obtained in the experiment (n = 5). Letters refer to significantly different values that are significant ($P \leq 0.05$) - A, B comparison between both varieties; a, b comparison between treatments in each variety.

Fig. 2 The effect of Cd on trimethylglycine metabolism.

Figure 1
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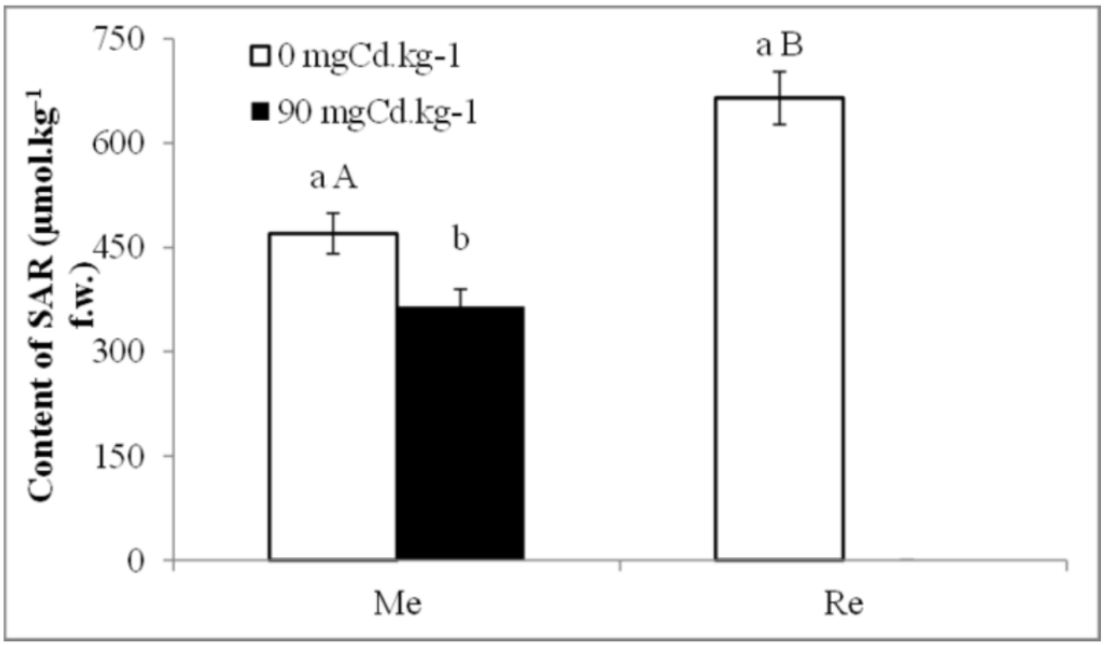

Figure 2
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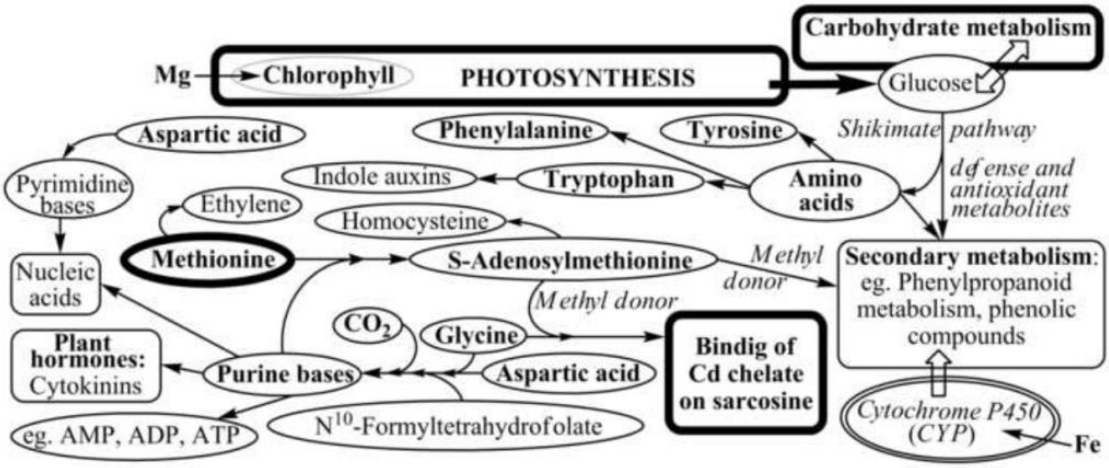

Table
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Table 1 Characteristics and total initial Cd concentration in experimental soil.

Soil type / subtype	pH_{KCl}	CEC* ($mmol_{(+)}\ kg^{-1}$)	$C_{org.}$ (%)	Cd_T ($mg\ kg^{-1}$)
Chernozem / modal	7.2±0.1	258±2.8	1.83±0.01	0.42±0.05

*CEC - cation exchange capacity

Table 2 Design of experiment and dose of elements.

Treatment	N (g per pot)	P (g per pot)	K (g per pot)	Cd ($mg\ kg^{-1}$)
	NH_4NO_3	K_2HPO_4		$(Cd(NO_3)_2\ 4H_2O)$
control	0.3	0.1	0.24	0
Cd	0.3	0.1	0.24	90

Table 3 The microelement contents in the aboveground biomass of plants. The values represent the means of data obtained in the experiment (n = 5, i.e., five replications per each treatment). Letters refer to significantly different values that are significant ($P \leq 0.05$) - A, B comparison between both varieties; a, b comparison between treatments in each variety.

Treatment	Me	Me-Cd	Re	Re-Cd
Cd ($mg\ kg^{-1}$)	26.4 ± 0.7^{aB}	7045 ± 66^{bB}	2.4 ± 0.05^{aA}	134 ± 6^{bA}
Zn ($mg\ kg^{-1}$)	543 ± 46^{aA}	539 ± 38^{aB}	495 ± 29^{bA}	201 ± 16^{aA}
Cu ($mg\ kg^{-1}$)	5.4 ± 0.5^{aA}	6.0 ± 0.3^{bA}	6.8 ± 0.7^{aB}	6.2 ± 0.6^{aA}
Mn ($mg\ kg^{-1}$)	70.1 ± 11.0^{aA}	76.2 ± 9.2^{aA}	110.8 ± 8.5^{aB}	108.4 ± 7.8^{aB}
Ni ($mg\ kg^{-1}$)	71.6 ± 6.7^{bA}	42.8 ± 3.2^{aB}	104.5 ± 6.7^{bB}	9.2 ± 0.9^{aA}

Table 4 The concentrations of selected free amino acids in the aboveground biomass of plants. The values represent the means of data obtained in the experiment (n = 5). Letters refer to significantly different values that are significant ($P \leq 0.05$) - A, B comparison between both varieties; a, b comparison between treatments in each variety.

Free amino acid	Me	Me-Cd	Re	Re-Cd
		μmol kg^{-1}		
alanine (Ala)	772 ± 2^{aA}	652 ± 65^{bA}	994 ± 39^{bB}	805 ± 59^{aB}
γ-aminobutyric acid (GABA)	368 ± 6^{aA}	382 ± 49^{aA}	333 ± 39^{aA}	392 ± 54^{aA}
glycine (Gly)	471 ± 42^{aA}	446 ± 35^{aA}	578 ± 41^{bB}	449 ± 58^{aA}
ornithine (Orn)	180 ± 1^{aA}	312 ± 23^{bA}	287 ± 25^{aB}	336 ± 31^{aA}
phenylalanine (Phe)	609 ± 31^{aB}	625 ± 38^{aA}	561 ± 39^{aA}	770 ± 41^{bB}
tryptophan (Trp)	714 ± 56^{aA}	747 ± 42^{aA}	630 ± 46^{aA}	800 ± 57^{bA}
tyrosine (Tyr)	625 ± 71^{aA}	643 ± 68^{aA}	599 ± 57^{aA}	789 ± 39^{bB}
isoleucine (Ile)	137 ± 15^{aA}	139 ± 11^{aA}	120 ± 17^{aA}	168 ± 19^{bA}
leucine (Leu)	560 ± 34^{aA}	573 ± 38^{aA}	537 ± 31^{aA}	651 ± 41^{bA}
valine (Val)	808 ± 75^{aB}	728 ± 59^{aA}	601 ± 54^{aA}	568 ± 50^{aA}
threonine (Thr)	822 ± 9^{aA}	875 ± 35^{aA}	1440 ± 50^{aB}	2348 ± 92^{bB}

Author contribution statement

All co-authors have equal contribution to this articel.

6 SUMÁRNÍ DISKUZE

6.1 Výnos biomasy, obsah kadmia a vybraných prvků

Dle Mihaličové Malčové et al. (2014) nemá Cd v rostlinách žádnou známou funkci jako živina a je více či méně pro rostliny toxické. Existují však práce popisující Cd jako kofaktor metaloenzymů karbonanhydrázy (Lane et al., 2005) a 3-deoxy-D-arabinoheptulosonat-7-fosfátsyntázy, který katalyzuje první reakci aromatické biosyntetické cesty v bakteriích, houbách a rostlinách (Shumilin et al. 1996, 2004). Z těchto prací vyplývá, že metaloenzym s kofaktorem Cd je aktivnější než s kofaktorem jiného kationtu. Proto je zajímavé zjištění, že pokusy s hyperakumulátory ukazují, že nízká dávka Cd (varianta Cd1 s 30 mg/kg) má na růst *N. caerulescens*, *N. praecox* a *A. halleri* stimulační účinky (Zemanová et al., 2013, 2014a, 2015a). Varianty Cd2 (60 mg/kg) a Cd3 (90 mg/kg) významně redukovaly růst nadzemní biomasy oproti kontrolní variantě u všech testovaných rostlin (v průměru o ≈ 50 %). Výnos nadzemní biomasy klesal v pořadí *N. praecox* >> *A. halleri* > *N. caerulescens* ekotyp Redlschlag >> *N. caerulescens* ekotyp Ganges. Z hlediska dlouhodobého působení stresoru měl největší odolnost *N. praecox* ze Slovinska, který nevykazoval žádné viditelné změny způsobené toxickou hladinou Cd v půdě. Fytotoxický vliv Cd byl zřejmý u variant Cd3 (90 mg Cd/kg) a Cd2 (60 mg Cd/kg) *N. caerulescens* ekotyp Redlschlag z Rakouska, u kterého byly pozorovány nekrózy na okrajích listů. U žádného zástupce z čeledi *Brassicaceae* nebyl pozorován výskyt chloróz na listech. Podobné výsledky zjistili ve svém pokusu s ekotypy *N. caerulescens* Cosio et al. (2005).

Z výsledků pokusů vyplývá schopnost *N. caerulescens* a *N. praecox* akumulovat více Cd v nadzemní biomase a kořenech než *A. halleri* (Zemanová et al., 2013, 2014a). Bert et al. (2003) zjistili u *A. halleri* vyšší obsah Cd v kořenech oproti nadzemní biomase. Naše výsledky však tuto skutečnost nepotvrdily. U *A. halleri*, *N. praecox* a obou ekotypů *N. caerulescens* byl zjištěn vyšší obsah Cd v nadzemní biomase než v kořenech. Dle Lovy et al. (2013) mají tyto rostlinné druhy vysoký příjem Cd kořeny, nízkou sekvestraci v kořenech a zvýšenou translokaci Cd z kořenů do buněk nadzemní biomasy, kde dochází k ukládání a detoxifikaci Cd. Při srovnání obsahu Cd v hyperakumulujících rostlinách klesala schopnost akumulace tohoto prvku v pořadí *N. praecox* > *N. caerulescens* ekotyp Ganges >> *A. halleri* > *N. caerulescens* ekotyp Redlschlag.

V porovnání s výsledky pokusů s hyperakumulujícími rostlinami má Cd negativní vliv na růst a vývoj rostlin špenátu setého (*Spinacia oleracea* L.). Tento zástupce nehyperakumulujících rostlin patří mezi méně citlivé rostliny na kontaminaci vlivem Cd,

avšak jeho výnos klesal se zvyšující se dávkou Cd v půdě. V porovnání s kontrolní variantou došlo u varianty Cd3 (90 mg/kg) ke snížení výnosu biomasy o 73 %. Obdobné výsledky zjistili Pavlíková et al. (2008) a Salaskar et al. (2011) u špenátu setého rostoucího v půdě kontaminované Cd. U varianty Cd3 (90 mg/kg) byl vizuálně prokázán fytotoxický vliv Cd výskytem chloróz listů. Salaskar et al. (2011) tento fytotoxický vliv Cd u špenátu nepotvrdili.

Akumulace Cd v rostlinách špenátu setého byla srovnatelná s akumulací Cd u *N. caerulescens* ekotyp Redlschlag, zejména u nejvyšší dávky Cd (90 mg/kg). Obsahy však nedosahovaly hranice pro zařazení mezi hyperakumulující rostliny, přesto je dle Salaskara et al. (2011) možné využít špenát setý pro fytoremediaci. Dle těchto autorů se obsah Cd v rostlinách zvyšoval s dobou vegetace. Naše pokusy prokázaly opačný vliv délky vegetace.

Jedním z faktorů majících vliv na efekt Cd v metabolismu a fyziologických procesech rostlin je jeho vztah s dalšími minerálními prvky (Lux et al. 2011; Dias et al. 2013). Nárůst obsahu Cd v nadzemní biomase testovaných rostlin penízku vedl k poklesu obsahu K, Ca, Na a Fe. Porovnání obsahu těchto prvků v *N. praecox* a *N. caerulescens* ukázalo jejich významný pokles v biomase *N. praecox*. Dle Rodríguez-Serrana et al. (2009) nedostatek Fe a Ca způsobený Cd vede k snížení antioxidační obrany a může přispět k produkci ROS a peroxidaci lipidů v kadmiem stresovaných rostlinách. Eker a Uysal (2013) zjistili u špenátu, že zvýšený obsah K má klíčovou roli v ochraně proti oxidačnímu stresu indukovanému kadmiem a snižuje peroxidaci lipidů. Z mikroprvků byl především významně snížen obsah Zn a Ni. Tyto biogenní prvky spolu s Cu, Mn a Fe jsou kofaktory metaloenzymů (např. pro superoxiddismutázu) a tvoří biologické komplexy s kovy, které mají řadu důležitých funkcí, např. obranu proti oxidačnímu stresu (Fernández-Ocaña et al. 2011).

6.2 Parametry fotosyntézy

Wójcik et al. (2005) uvádějí jako jeden z toxických efektů akumulace Cd v rostlinných pletivech poškození fotosyntetického aparátu. Dle Burzynski a Klobus (2004) je fotosyntetický aparát zvláště citlivý na stres vlivem Cd a společnou reakcí rostlin na tento stres je omezení fotosyntézy. Významnými inhibitory fotosyntetické aktivity jsou ROS (Gallego et al., 2012), jejichž produkce v rostlině se zvyšuje při expozici Cd (Heyno et al., 2008). Výsledky měření parametrů rychlosti výměny plynů a fotosyntézy u *N. praecox* a *N. caerulescens* prokázaly inhibici rychlosti čisté fotosyntézy (P_N) a zvýšenou rychlost transpirace (E), stomatální vodivost (g_s) a intracelulární koncentraci CO_2 (C_i). Kontaminace Cd však neměla významný vliv na fotosyntézu. Obdobné výsledky zjistili Zhang et al. (2014) u hybridu dochanu klasnatého (*Pennisetum americanum* × *Pennisetum purpureum*) a vetiverie

(*Vetiveria zizanioides*). Naopak Sandalio et al. (2001) stanovili v listech hrachu redukci transpirace a rychlosti fotosyntézy. Dle Shia a Caia (2008) zvýšená C_i ukazuje na ovlivnění enzymů temnostní fáze fotosyntézy. Zvýšení g_s pravděpodobně souvisí se změnou v poměru K:Ca ve svěracích buňkách a/ nebo změnami v koncentraci kyseliny abscisové, která kontroluje pohyb průduchů. Zhang et al. (2006) uvádějí možné ovlivnění fotosyntézy draslíkem prostřednictvím změn v morfologii a anatomii listů. Také naše výsledky potvrdily pokles obsahu K v biomase rostlin a možný vliv na rychlost fotosyntézy a výměny plynů.

6.3 Obsah volných aminokyselin

Aminokyseliny mají v metabolismu a vývoji rostlin důležitý význam, slouží jako prekurzory pro proteiny a jejich součásti (Ježek et al., 2011). Xu et al. (2012) uvádějí mnohostrannou roli aminokyselin v rostlinách v přítomnosti rizikových prvků. Dle Halla (2002) může řada aminokyselin v pletivech rostlin působit jako ligandy pro komplexaci kovů a tím zvýšit toleranci rostliny vůči tomuto kovu. Výsledky pokusu s hyperakumulátory *N. caerulescens* ekotyp Ganges a *A. halleri* nevykazují statisticky průkazný vliv Cd na celkový obsah volných aminokyselin v nadzemní biomase. Významné rozdíly v celkovém obsahu volných aminokyselin byly pozorovány mezi rostlinnými druhy, *N. caerulescens* dosahoval 2-krát větších obsahů v nadzemní biomase než *A. halleri*. Při dlouhodobém působení Cd se obsah celkových volných aminokyselin u obou hyperakumulátorů snížil v porovnání s kontrolní variantou (varianta Cd3 snížila obsah o ≈ 50 wt% u *N. caerulescens* a ≈ 25 wt% u *A. halleri*). Obdobné výsledky pozorovali Pavlíková et al. (2014a,b) u rostlin tabáku (*Nicotiana tabacum* L.) při stresu vlivem Zn. Vliv nejnižší dávky Cd (30 mg/kg) měl však opačný efekt na celkový obsah volných aminokyselin v nadzemní biomase *N. caerulescens*. Tato dávka Cd zvyšila akumulaci volných aminokyselin v porovnání s kontrolní variantou při krátkodobé i dlouhodobé expozici rostlin tomuto prvku. Odlišný vliv mělo Cd na obsah volných aminokyselin v kořenech hyperakumulátorů. U *N. caerulescens* došlo při krátkodobém i dlouhodobém stresu vlivem Cd ke zvýšení celkového obsahu volných aminokyselin u kontaminovaných variant, zejména střední dávky Cd (60 mg/kg) oproti kontrolní variantě (≈ 150-200 wt%). Tato akumulace souvisí dle Couturiera et al. (2010) s funkcí aminokyselin jako chelatačních molekul, kdy po vytvoření komplexu s Cd je tento komplex translokován z kořenů do nadzemní biomasy rostlin. Dle Chaffeiho et al. (2004) je zvýšení obsahu N:C aminokyselin v kořenech ochranná strategie zajištění živin pro budoucí využití. Rostliny *A. halleri* prokázaly tento trend pouze při dlouhodobém působení Cd (≈ 30

wt%). U tohoto rostlinného druhu byl v kořenech pozorován obdobný jev v celkovém obsahu volných aminokyselin jako v nadzemní biomase.

Rozdíly mezi hyperakumulátory Cd byly pozorovány zejména v profilu volných aminokyselin. Z celkového obsahu volných aminokyselin v nadzemní biomase *N. caerulescens* a *A. halleri* byly nejvíce zastoupeny kyselina glutamová (Glu; v průměru ≈ 5 wt% u *N. caerulescens* a ≈ 8 wt% u *A. halleri*), glutamin (Gln; v průměru ≈ 40 wt% u *N. caerulescens* a ≈ 20 wt% u *A. halleri*), kyselina aspragová (Asp; v průměru ≈ 20 wt% u *N. caerulescens* a ≈ 12 wt% u *A. halleri*) a asparagin (Asn; v průměru ≈ 10 wt% u *N. caerulescens* a ≈ 50 wt% u *A. halleri*). Obdobné výsledky byly zjištěny i při porovnávání vybraných ekotypů *N. caerulescens* a *N. praecox*. Dle Sánchez-Parda et al. (2013) jsou Glu a Gln organické formy přeměněného amoniaku v rostlinách a jsou zdrojem dusíku v biosyntéze esenciálních aminokyselin a dalších sloučenin obsahujících dusík. Obsahy Glu a Gln dosahovaly nižších hodnot v nadzemní biomase *A. halleri* než v *N. caerulescens* a *N. praecox*. Varianty s vyšší dávkou Cd (60 mg/kg a 90 mg/kg) snížily obsah těchto volných aminokyselin v *A. halleri* a *N. caerulescens*. Opačný efekt měla nejnižší dávka Cd (30 mg/kg). U *N. praecox* došlo vlivem nejvyšší dávky (90 mg/kg) k snížení obsahu Glu a naopak zvýšení akumulace Gln. Tyto výsledky jsou v rozporu s prací Sharmy a Dietze (2006) a Pavlíka et al. (2010), kteří pozorovali zvýšení obsahu Glu při stresu rostlin vlivem přítomnosti rizikových prvků. U ekotypu *N. caerulescens* z Rakouska byly pozorovány jen nepatrné změny v obsahu Glu. Mokhele et al. (2012) uvádějí možnost využití Glu a Gln pro syntézu Asp a Asn a jejich roli v translokaci organického dusíku ze zdroje do zásobních pletiv. Obsahy Asp a Asn v nadzemní biomase *A. halleri* byly bez významných změn, naopak u *N. caerulescens* a *N. praecox* došlo k akumulaci Asp vlivem zvyšující se kontaminace kadmiem. V obsahu Asn došlo u ekotypů *N. caerulescens* a *N. praecox* k poklesu. Podobné výsledky zjistili Pavlík et al. (2010) v listech špenátu rostoucím v prostředí kontaminovaném arsenem. Opačné výsledky publikovali Lea et al. (2007). Výsledky obsahu Asn v *A. halleri* potvrdily zjištění Zhanga et al. (2013), podle kterých je Asn hlavní formou transportu dusíku do zásobních pletiv v rostlinách *Arabidopsis*.

Řada autorů (Zengin a Munzuroglu, 2005; Mistra a Dubey, 2006 atd.) studovala akumulaci iminokyseliny prolinu (Pro) v pletivech a orgánech rostlin vystavených působení abiotických stresorů, včetně rizikových prvků. Výsledky pokusu s hyperakumulátory *A. halleri* a *N. caerulescens* ekotyp Ganges tuto akumulaci Pro vlivem Cd potvrdily pouze u *N. caerulescens* ekotyp Ganges, který dosahoval 15-75krát vyšších obsahů než *A. halleri*. Kontrolní varianta u *A. halleri* vykazovala vyšší obsah Pro v porovnání s kontaminovanými

variantami. Opačný efekt Cd byl pozorován u *N. praecox* a *N. caerulescens* ekotyp Redlschlag, u nichž došlo vlivem nejvyšší dávky Cd (90 mg/kg) k zvýšení obsahu Pro. Porovnání obsahu této aminokyseliny ukázalo nižší akumulaci Pro v *N. praecox*. Tento hyperakumulátor je pravděpodobně schopen inhibovat tvorbu Pro z Glu zpětnou vazbou. Podle García-Ríosy et al. (1997) je inhibice Pro závislá na koncentraci Glu a rostliny s vyšším poměrem Glu:Pro jsou lépe přizpůsobeny Cd stresu. Akumulace volného Pro byla ovlivněna nejen dávkou Cd, ale také adaptací rostlin na chronický stres a délkou vegetační doby. Tyto výsledky jsou v souladu s publikací Pavlíková et al. (2008).

Významný rozdíl mezi hyperakumulátory *N. caerulescens* ekotyp Ganges a *A. halleri* byl pozorován v obsahu hydxyprolinu (Hyp), který je hlavní aminokyselinou hydrolysátů buněčných stěn rostlin. Tyto sloučeniny jsou vytvářeny v rostlinách při oxidačním stresu (De Graaf et al., 2001). V ekotypech *N. caerulescens* a v *N. praecox* se obsah Hyp zvyšoval s dávkou Cd v půdě, naopak v nadzemní biomase *A. halleri* byl obsah pod mezí detekce stanovení.

Pro růst, vývoj a obranu rostlin je nezbytná rovnováha (homeostáze) aminokyselin (Liu et al., 2010). He et al. (2013) uvádějí jako stěžejní prvek pro rovnováhu toků mezi různými dráhami aminokyselin konečný produkt inhibice na větvících se místech v biosyntéze aminokyselin s rozvětveným řetězcem (tj. leucin, isoleucin a valin). U *N. caerulescens* ekotyp Redlschlag došlo k významnému zvýšení obsahu leucinu (Leu) a isoleucinu (Ile), což potvdilo, že ekotyp je méně přizpůsobený Cd stresu v porovnání s ostatními testovanými rostlinami. Tento ekotyp při stresu vlivem Cd aktivuje enzymy, které jsou spojené s degradací a senescencí rostlin. Substrátem pro syntézu Ile jsou threonin (Thr) a methionin (Met), jejichž syntéza a katabolismus v různých vývojových a přírodních podmínkách ovlivňuje dostupnost Ile (Joshi et al. 2010). Významný vztah v obsahu mezi Thr a Ile byl prokázán u *N. praecox* a *N. caerulescens* ekotyp Redlchlag. Obsah Met byl u těchto rostlin pod mezí detekce příslušné metody stanovení. Další významnou aminokyselinou, jejíž biosyntézu vyvolávají stresové podmínky je tryptofan (Trp). Dle Sanjaya et al. (2008) zvýšená hladina Trp snižuje přístupnost Cd pro rostliny, jeho transport a akumulaci v rostlinách. K zvýšení obsahu Trp došlo pouze u rostlin *N. caerulescens* ekotyp Redlschlag.

Porovnání obsahů jednotlivých volných aminokyselin mezi rostlinami *N. praecox* a *N. caerulescens* ekotyp Redlschalg prokázalo snížení obsahu alaninu (Ala) se zvyšující se dávkou Cd v půdě. Tato aminokyselina je akumulována zejména v odezvě na stres ve vztahu k regulaci intracelulárního pH. Podle Hjhortha et al. (2006) je zvýšený obsah volného Ala způsoben snížením rychlosti syntézy proteinů a zvýšením syntézy Ala

v důsledku narušení reakce alaninaminotransféráz. Naše výsledky ukazují, že hyperakumulátory Cd neakumulují Ala v cytosolu pro regulaci pH. Tyto rostliny mohou Ala použít pro biosyntézu proteinkináz obsahujících Pro/ Ala (Mori et al., 2013) nebo biosyntézu bílkovin bohatých na His a Ala (Komatsu et al., 2009). Pro regulaci pH akumulují tyto druhy γ-aminomáselnou kyselinu (GABA) (Bor et al., 2009). Tato aminokyselina má v metabolismu rostlin různou roli, je součástí C-N metabolismu, energetické bilance, vývoje rostlin, obrany proti stresu a slouží jako signální molekula (Sawaki et al. 2009). Naše výsledky neprokázaly významný vliv Cd na obsah GABA v testovaných rostlinách.

Významnou aminokyselinou, u níž však není znám fyziologický účinek na rostliny je sarkosin (Sar). Sar (N-methylglycin) je meziprodukt v metabolismu trimethylglycinu (glycin betain) (Oda et al., 2005), který je znám jako metabolit akumulující se při oxidačním stresu (Kholová et al. 2009). Z tohoto důvodu je pokles Sar spojován s biosyntézou glycinbetainu a obranou rostlin proti oxidačnímu stresu. Tento pokles je způsoben methylací glycinu *via* Sar na glycin betain (Niu et al., 2014) nebo nedostatkem glycinu vyvolaném reakcí glycinu a cholinem (Ashraf a Foolad, 2007). Obsah Sar u *N. praecox* a *N. caerulescens* ekotyp Redlschlag značí významnější vliv Cd na oxidační stres ekotypu Redlschlag, u kterého došlo k výraznému poklesu obsahu Sar v kontaminované variantě (pokles pod mez detekce). Podle Krishnakumar et al. (1996) a Tewari (2012) může Sar vytvářet komplexy s Cd a dalšími kovy. Toto může být důvod vysoké akumulace Cd v rostlinách *N. praecox*, kde dochází pravděpodobně k chelataci Cd se Sar.

V rostlinách špenátu setého byl prokázán významný vliv kontaminace Cd na celkový obsah volných aminokyselin. V porovnání s výsledky v nadzemní biomase *N. caerulescens*, *N. praecox* a *A. halleri* (Zemanová et al., 2014a ; Zemanová et al., nepublikováno) byl pozorován opačný trend v reakci na zvyšující se dávku Cd v půdě při dlouhodobém působení. Akumulace volných aminokyselin klesala se stupňující se dávkou Cd v první polovině vegetační doby, avšak v druhé polovině vegetačního cyklu došlo k nárůstu celkových obsahů volných aminokyselin vlivem dávky Cd. Dle Hirnera et al. (2006) jsou aminokyseliny důležitou součástí ve výživě dusíku při růstu rostlin v rozmanitých podmínkách, kdy slouží jako zdroj organického dusíku. V profilu volných aminokyselin byly nejvíce zastoupeny kyselina glutamová (Glu; ≈ 30 wt%) a kyselina asparagová (Asp; ≈ 20 wt%). Podobné zjištění uvádí Coruzzi (2003) v rostlinách *Arabidopsis* a Di Martino et al. (2003) v listech špenátu při stresu zasolením. Změny v obsahu Asp byly v porovnání s Glu méně významné, což je pravděpodobně způsobeno využitím Glu pro syntézu glutathionu a fytochelatinů v rostlinných buňkách (Vitória et al., 2001). Na významné rozdíly v metabolismu aminokyselin mezi

nehyperakumulujícími a hyperakumulujícími rostlinami poukazuje absence Sar v listech špenátu setého.

6.4 Obsah mastných kyselin

Vliv dlouhodobého působení Cd na obsah mastných kyselin byl zjišťován u vybraných ekotypů *N. caerulescens* a *N. praecox* po 90 a 120 dnech vegetace. Statisticky významné rozdíly byly zjištěny zejména po 120 dnech vegetace u všech pokusných rostlin. Obsah nasycených mastných kyselin u *N. praecox* a *N. caerulescens* ekotyp Ganges klesal se zvyšující se dávkou Cd v půdě. Opačný efekt varianty Cd3 byl pozorován u *N. caerulescens* ekotyp Redlschlag. Výsledky jsou v souladu s prací Nouairia et al. (2006), kteří zjistili podobné změny v obsahu nasycených mastných kyselin v listech *Brassica juncea* při stresu vlivem Cd. Celkový obsah nenasycených mastných kyselin vykazuje opačné trendy u jednotlivých pokusných rostlin. U *N. praecox* a *N. caerulescens* ekotyp Ganges došlo ke statisticky průkaznému zvýšení tohoto obsahu u varianty Cd3 v porovnání s kontrolní variantou. Pokles celkového obsahu nenasycených mastných kyselin se projevil u *N. caerulescens* ekotyp Redlschlag. Dle Thompsona et al. (1998) zvyšuje Cd aktivitu lipoxygenasy a tím ovlivňuje obsah nenasycených mastných kyselin v membránách, které slouží jako substrát při peroxidaci lipidů.

U všech pokusných jedinců byly stanoveny tyto nasycené mastné kyseliny: laurová (12:0), myristová (14:0), palmitová (16:0), stearová (18:0), arachidová (20:0), behenová (22:0) a lignocerová (24:0). Nejvyšších obsahů z těchto nasycených mastných kyselin dosahovala v obou odběrech u *N. praecox* a ekotypů *N. caerulescens* kyselina palmitová (16:0, ≈ 50% z celkového obsahu nasycených mastných kyselin). Dále byl pozorován vyšší obsah kyseliny lignocerové (24:0) u *N. caerulescens* ekotyp Redlschlag v porovnání s *N. caerulescens* ekotyp Ganges a *N. praecox*.

Při analýzách byly stanoveny obsahy nasycených mastných kyselin s ultra dlouhým řetězcem (počet C ≥ 26), které nejsou běžně přítomny v pletivech nehyperakumulujících rostlin. Jedná se o kyselinu cerotovou (26:0), montanovou (28:0) a melissovou (30:0). Kyselina melissová byla stanovena pouze u *N. praecox*, v jehož nadzemní biomase došlo ke zvýšení obsahu kyseliny se zvyšující se dávkou Cd v půdě. Tyto nasycené mastné kyseliny slouží jako zdroj energie na opravu buněčných membrán při oxidačním stresu vyvolaném přítomností Cd v prostředí.

Cd způsobuje akumulaci ROS a ovlivňuje enzymatickou aktivitu peroxidáz, přičemž dochází k peroxidaci nenasycených mastných kyselin a změně jejich zastoupení

v membránách. Tento jev potvrdili Verdoni et al. (2001) v primárních listech rajčat, kde Cd vyvolal významný pokles 18:3 kyseliny a zvýšení obsahu 18:1 a 18:2 kyseliny. Pokles mastné kyseliny 18:3 jako odpověď na stres vlivem rizikových prvků zjistil také Upchurch (2008) při studiu stupně nenasycenosti membránových lipidů ve stresu tolerantních rostlin. Nenasycené mastné kyseliny, které byly stanoveny v *N. praecox* a v ekotypech *N. caerulescens* jsou 7,10-hexadekadienová (16:2n-6), 7,10,13-hexadekatrienová (16:3n-3), 9,12-oktadekadienová (18:2n-6) a 9,12,15-oktadekatrienová (18:3n-3) kyselina. U *N. praecox* a *N. caerulescens* ekotyp Ganges byly stanoveny také nenasycené mastné kyseliny s 20 uhlíky v řetězci, a to kyselina 11,14-eikosadienová (20:2n-6) a kyselina 11,14,17-eikosatrienová (20:3n-3). Nejvíce zastoupena byla kyselina 9,12,15-oktadekatrienová (18:3n-3), jejíž obsah byl vyšší v *N. praecox* a *N. caerulescens* ekotyp Ganges (oba ≈ 35 wt% z celkového obsahu mastných kyselin) než v *N. caerulescens* ekotyp Redlschlag (≈ 30 wt% z celkového obsahu mastných kyselin). Pomocí lineární regrese byl zjištěn signifikantní vztah mezi obsahem kyseliny 9,12-oktadekadienová (18:2n-6), kyseliny 9,12,15-oktadekatrienová (18:3n-3) a obsahem Cd v nadzemní biomase *N. praecox* a *N. caerulescens* ekotyp Redlschlag. Tento vztah nebyl významný u *N. caerulescens* ekotyp Ganges.

Pro zjištění rozdílů v metabolismu mastných kyselin v odezvě na působení Cd u nehyperakumulujících rostlin byl stanoven obsah mastných kyselin v rostlinách špenátu setého. Výsledky ukazují odlišný trend v obsahu nasycených a nenasycených mastných kyselin. Obsah nasycených mastných kyselin se v rostlinách špenátu zvyšoval se stupňující se dávkou Cd v půdě a naopak obsah nenasycených mastných kyselin klesal. Podobné výsledky byly zjištěny u *N. caerulescens* ekotyp Redschlag (Zemanová et al., 2015a) a v listech pepře (*Capsicum annuum*) při stresu vlivem Cd (Jemal et al., 2000). Při porovnání našich výsledků s hyperakumulátory *N. praecox* a *N. caerulescens* dochází u rostlin špenátu setého k signifikantním změnám v profilu mastných kyselin. V listech špenátu setého byly stanoveny tyto mastné kyseliny: palmitová (16:0), 7,10,13-hexadekatrienová (16:3n-3), 9,12-oktadekadienová (18:2n-6), 9,12,15-oktadekatrienová (18:3n-3) a arachidová (20:0). Nejvíce zastoupenou mastnou kyselinou je kyselina 9,12,15-oktadekatrienová (18:3n-3) (≈70 wt% z celkového obsahu mastných kyselin), jejíž obsah klesá se zvyšující se dávkou Cd v půdě. Tento efekt Cd potvrdil Verdoni et al. (2001) v listech rajčat. Dle Upchurche (2008) má skupina nenasycených mastných kyselin s 18 C specifickou roli v udržování funkce membrán. Semipermeabilita, fluidita a integrita membrán umožňuje rostlinám efektivně překonávat oxidační stres.

7 ZÁVĚR

Všechny pokusné rostliny z čeledi *Brassicaceae* akumulovaly více jak 100 mg Cd/kg sušiny a splnily tedy kritérium pro zařazení mezi hyperakumulátory tohoto prvku. Z výsledků vyplývá, že nejvyšší dávka Cd (90 mg/kg) má výrazný negativní vliv na metabolismus *A. halleri* a *N. caerulescens* ekotyp Ganges a Relschlag. Oproti tomu nejnižší dávka Cd (30 mg/kg) měla na všechny testované rostliny stimulační efekt. Za nejtolerantnější k vlivu Cd lze na základě výnosu nadzemní biomasy, obsahu Cd, výsledků parametrů fotosyntézy a nepřítomnosti viditelných projevů kontaminace Cd považovat *N. praecox* ze Slovinska.

Změny obsahů volných aminokyselin mohou hrát významnou roli v mechanismu adaptace rostlin na stres vlivem Cd. Celkový obsah volných aminokyselin nevykazoval významné rozdíly mezi *N. praecox*, ekotypy *N. caerulescens* a *A. halleri*. Rozdíly v metabolismu aminokyselin v testovaných rostlinách je zřejmý z profilu volných aminokyselin. Dominantní volnou aminokyselinou byl v nadzemní biomase *N. praecox* a *N. caerulescens* glutamin, v nadzemní biomase *A. halleri* byl touto volnou aminokyselinou asparagin. Tyto výsledky ukazují na odlišnou cestu využití dusíku u *Noccaea* sp. a *Arabidopsis* sp.. Obsah volného prolinu v testovaných rostlinách značí různou odolnost jednotlivých hyperakumulátorů a jejich ekotypů vůči působení Cd. Významný rozdíl mezi testovanými hyperakumulátory byl v obsahu volného sarkosinu, který vytváří pravděpodobně cheláty s Cd. Dle výsledků stanovení volných aminokyselin je nejlépe adaptován na stres vlivem Cd *N. praecox* ze Slovinska.

Obsahy nasycených a nenasycených mastných kyselin a jejich změny byly obdobné u *N. praecox* a *N. caerulescens* ekotyp Ganges, opačně reagoval *N. caerulescens* ekotyp Redlschlag. U všech testovaných zástupců byla hlavní nasycenou mastnou kyselinou kyselina palmitová (16:0). Z nenasycených mastných kyselin byla u všech pokusných rostlin nejvíce zastoupena kyselina 9,12-oktadekadienová (18:2n-6) a 9,12,15-oktadekatrienová (18:3n-3). Na odlišný metabolismus mastných kyselin hyperakumulujících rostlin proti běžným nehyperakumulujícím rostlinám poukazuje stanovení přítomnosti nasycených mastných kyselin s ultra dlouhým řetězcem (počet C $\geq$ 26), které pravděpodobně slouží hyperakumulujícím rostlinám jako zdroj energie při dlouhodobém působení stresoru.

Z výsledků pokusů jsou zřejmé komplexní změny v regulaci stresového metabolismu u hyperakumulujících rostlin, které jsou výsledkem selekčního tlaku během jejich fylogenetického vývoje v konkrétních oblastech znečištění.

Pokus se špenátem setým (*Spinacia oleracea* L.) prokázal fytotoxický efekt Cd, zejména u variant s vyšší dávkou Cd (60 mg/kg a 90 mg/kg). Rostliny špenátu setého přes

negativní vliv Cd na výnos biomasy akumulovaly v pletivech vysoké obsahy Cd. V metabolismu volných aminokyselin se u tohoto nehyperakumulujícího druhu projevilo několik společných znaků s hyperakumulátory rodu *Noccaea* - celkový obsah volných aminokyselin a dominantní volná aminokyselina glutamin. Rozdíl byl zejména patrný v akumulaci volného prolinu. Výsledky v obsahu mastných kyselin ukazují odlišné chování špenátu setého oproti hyperakumulujicím rostlinám. Vlivem Cd došlo k zvýšení celkového obsahu nasycených mastných kyselin a snížení celkového obsahu nenasycených mastných kyselin. V porovnání s testovanými rostlinami rodu *Noccaea* měly rostliny špenátu setého rozdílný profil mastných kyselin, v němž hlavní mastnou kyselinou byla kyselina 9,12,15-oktadekatrienová (18:3n-3).

Přítomnost Cd v půdě vyvolala u pokusných rostlin rozmanitou reakci na jím způsobený stres. V obecné rovině byla u hyperakumulujících a nehyperakumulujících rostlin prokázána řada společných znaků chování v metabolismu volných aminokyselin a mastných kyselin, avšak při zaměření se na jednotlivé části v těchto metabolických reakcích jsou patrné rozdíly u testovaných zástupců těchto dvou skupin rostlin. Základní rozdíl spatřujeme především v poměru Glu:Pro a tvorbě sarkosinu a mastných kyselin s velmi dlouhými řetězci v hyperakumulujících rostlinách.

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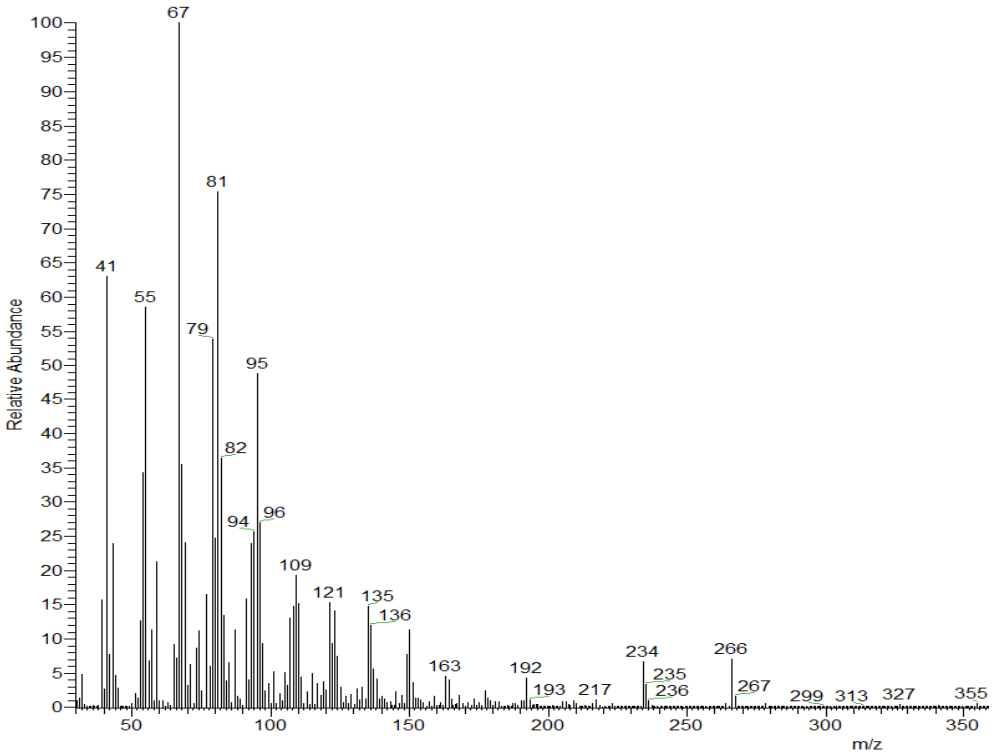
9 PŘÍLOHY

a)



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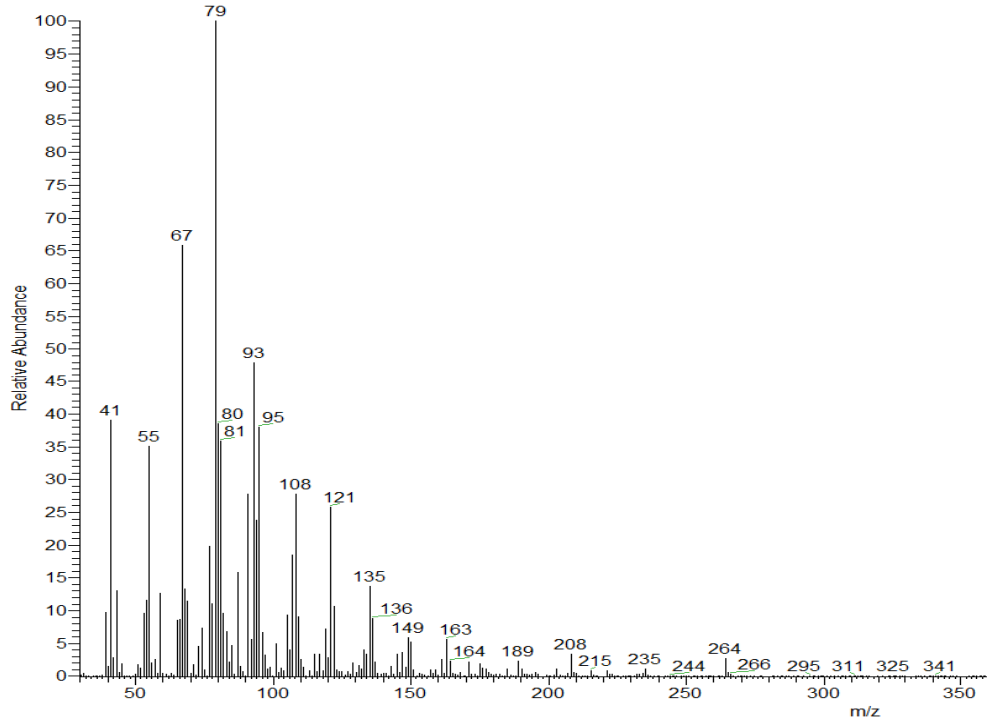
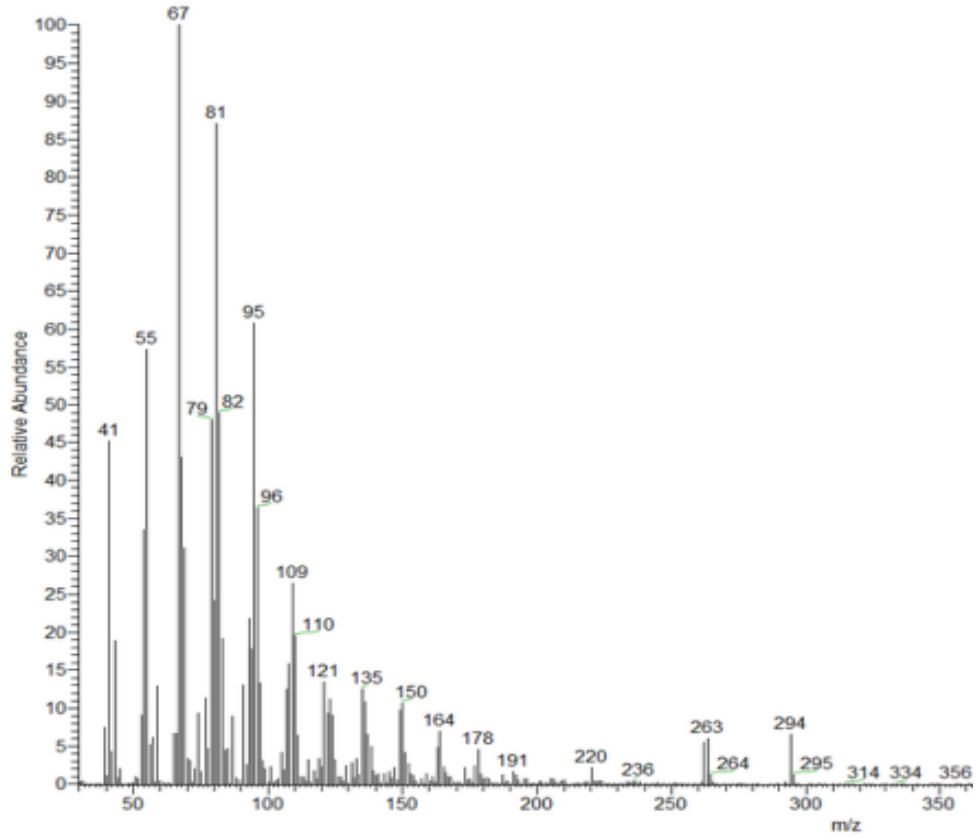


Příloha 1 Hmotnostní spektra nenasycené mastné kyseliny: (a) 7,10-hexadekadienová kyselina (16:2n-6) a (b) 7,10,13-hexadekatrienová (16:3n-3).

a)



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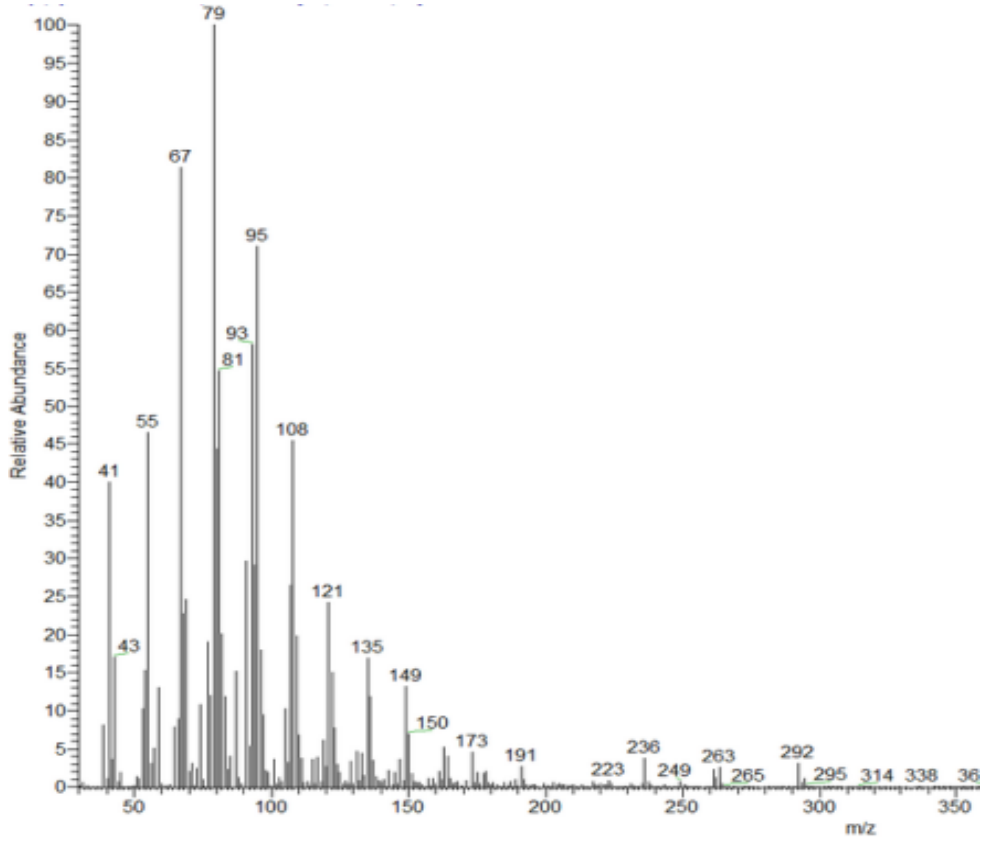
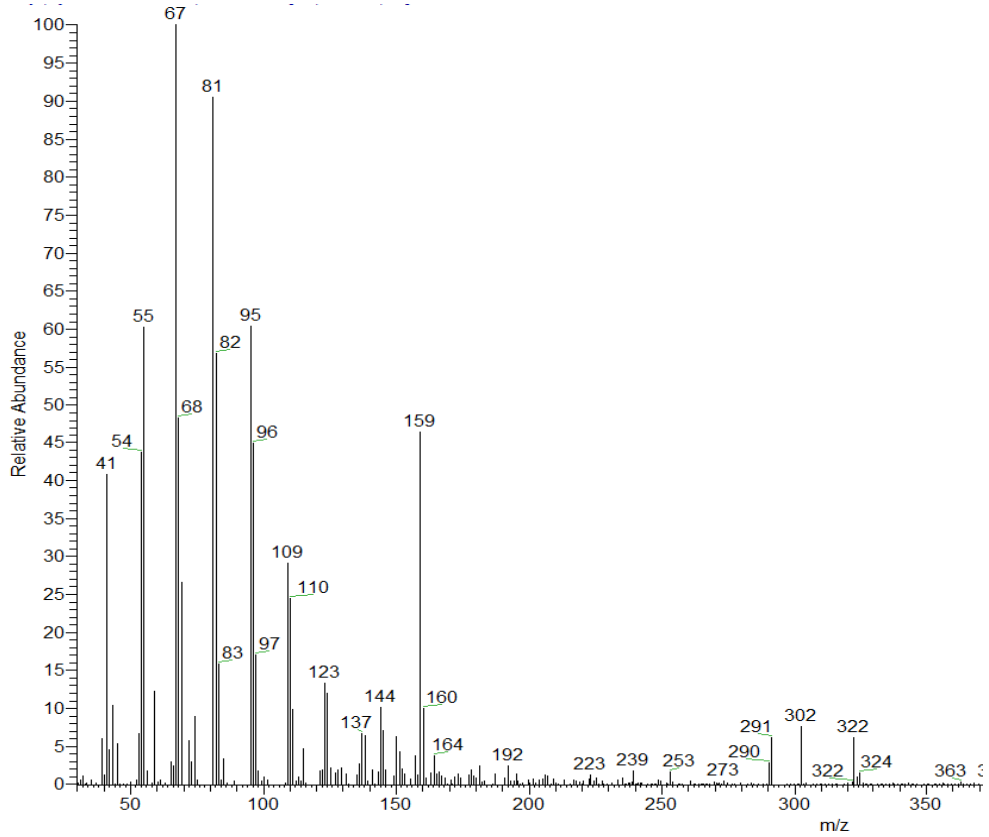


Příloha 2 Hmotnostní spektra nenasycené mastné kyseliny: (a) 9,12-oktadekadienová kyselina (18:2n-6) a (b) 9,12,15-oktadekatrienová kyselina (18:3n-3).

a)



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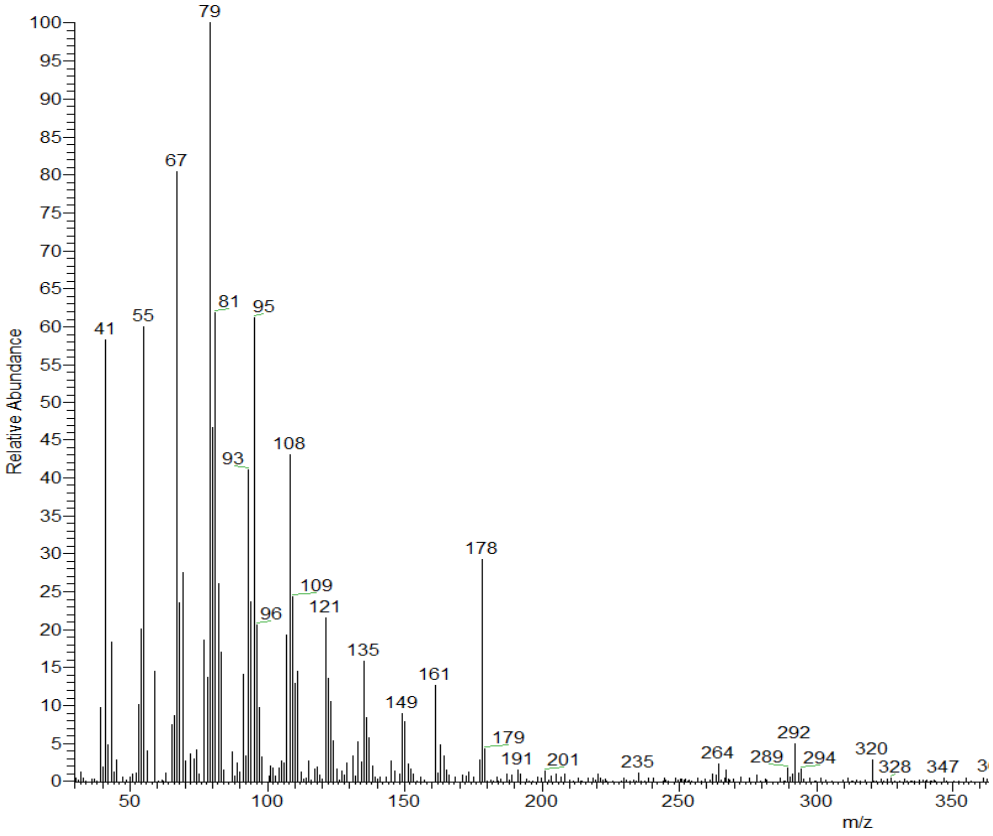
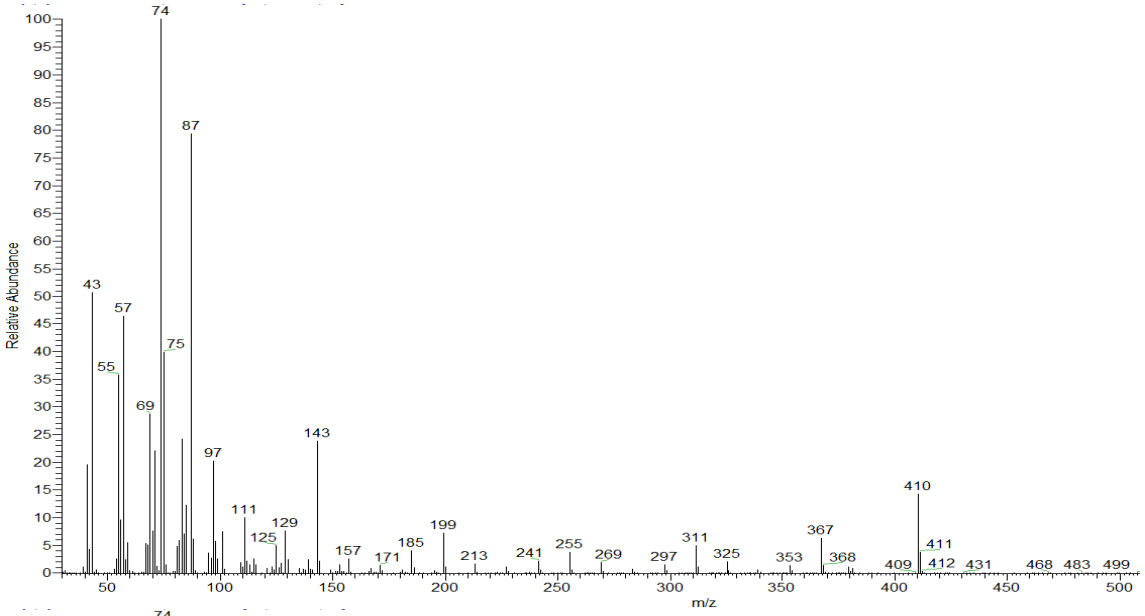


Příloha 3 Hmotnostní spektra nenasycené mastné kyseliny: (a) 11,14-eikosadienová kyselina (20:2n-6) a (b) 11,14,17-eikosatrienová kyselina (20:3n-3).

a)



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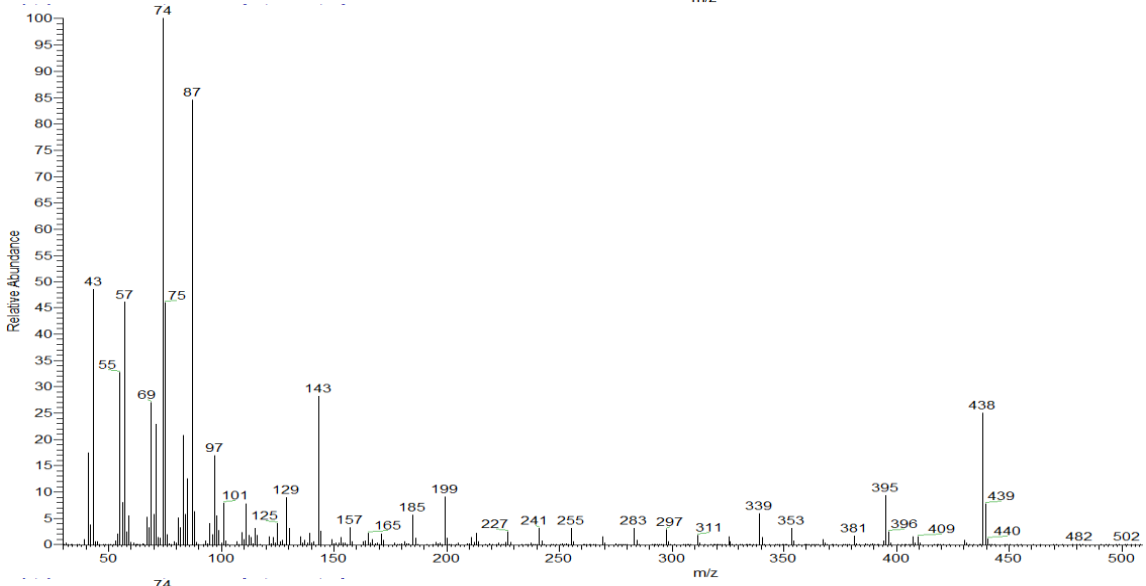

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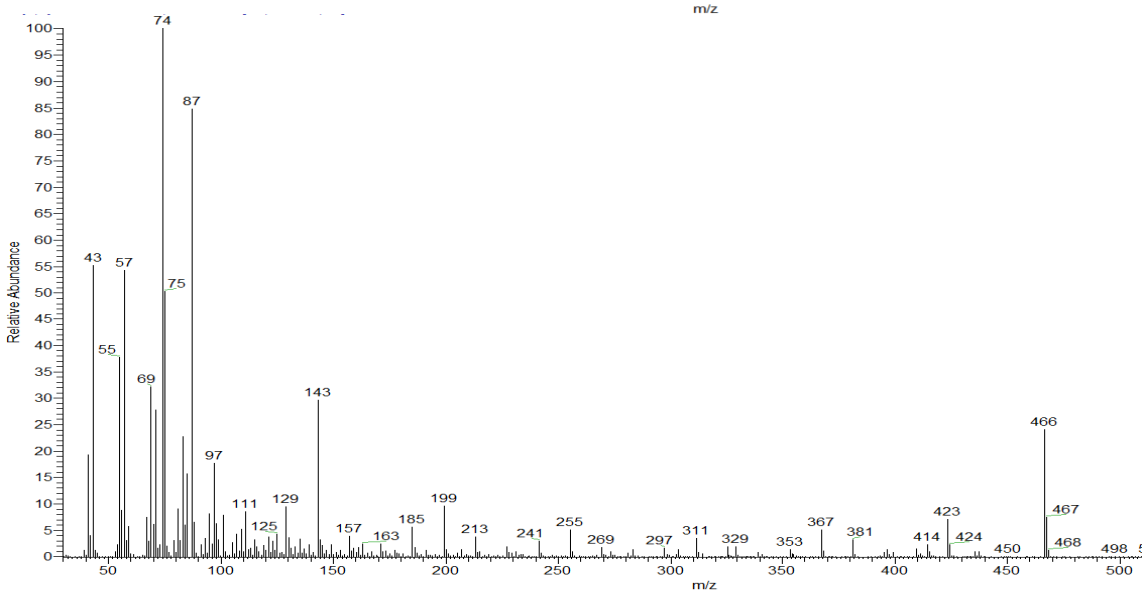


Příloha 4 Hmotnostní spektra nasycené mastné kyseliny s velmi dlouhým řetězcem: (a) cerotová kyselina (26:0), (b) montanová kyselina (28:0) a (c) melisová kyselina (30:0).

a)

b)

c)

Příloha 5 Rostliny *Noccaea* sp.: (a), (b) předpěstování ze semen a (c) po 30 dnech vegetace v kontaminovaném prostředí.

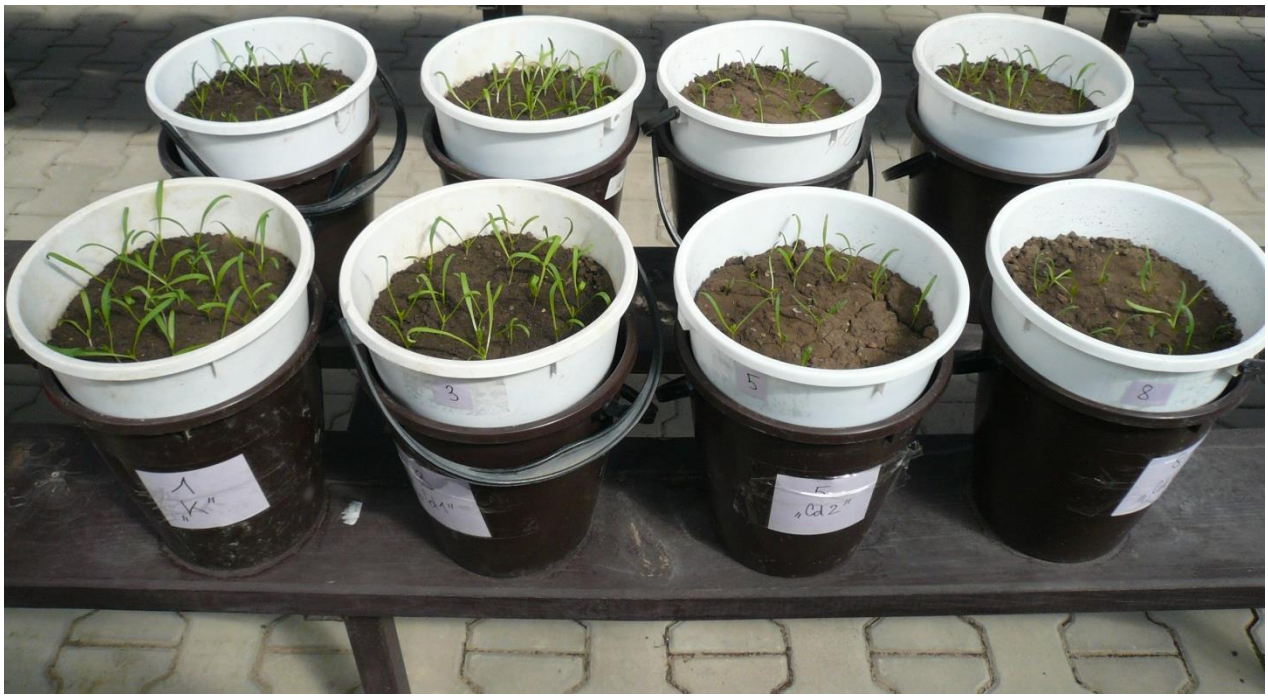
a)

b)

c)

Příloha 6 Rostliny *Noccaea* sp. po 90 dnech vegetace v kontaminovaném prostředí: (a) *N. praecox*, (b) *N. caerulescens* ekotyp Ganges, (c) *N. caerulescens* ekotyp Redlschlag.

a)



b)

Příloha 7 Rostliny špenátu setého: (a) 25 dní vegetace a (b) 55 dní vegetace.