Metabolic responses of terrestrial macrolichens to nickel

Jozef Kováčık¹,a, Slawomir Dreslerb, Petr Babulac

¹ Department of Biology, University of Trnava, Priemyselná 4, 918 43 Trnava, Slovak Republic
² Department of Plant Physiology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland
³ Department of Physiology, Faculty of Medicine, Masaryk University, Kamenice 753/5, 625 00 Brno, Czech Republic

ARTICLE INFO
Keywords:
Antioxidants
Heavy metals
Organic acids
Oxidative stress
Reactive oxygen species (ROS)
Thiols

ABSTRACT
Short-term (24 h) responses of Cladonia arbuscula subsp. mitis (formerly known as Cladina and this name is used to distinguish the tested species) and Cladonia furcata to nickel (Ni²⁺) excess (10 or 100 μM) were compared. Cladonia accumulated more Ni at higher Ni dose (1.717 mg total Ni/g DW). K amount was unaffected and Ca amount decreased in Cladina only. Fluorescence microscopy detection of total/general ROS and hydrogen peroxide showed Ni-stimulated increase in both species being more pronounced in Cladonia and in mycobiont partner mainly. Nitric oxide visualization (diaminonaphthalene staining) also revealed elevation in response to Ni that could contribute to synthesis of protective metabolites: they may include ascorbic acid or reduced glutathione which increased in Ni-exposed Cladina or Cladonia, respectively. Only low content of phytochelatin 2 was detected in Ni-treated Cladonia and the role in Ni chelation is not apparent. Among aliphatic organic acids, content of citric or succinic acid was not or slightly affected by Ni, production of malic acid dropped by 50% in both species and α-ketoglutaric acid showed the opposite behavior in the tested species. Data indicate that even short-term Ni treatments induce metabolic changes and symptoms of oxidative stress in lichens, confirming that nickel is not non-toxic metal as frequently visible from standard biochemical assays of basic physiology. Ascorbic acid and GSH rather than aliphatic organic acids seem to contribute to Ni tolerance.

1. Introduction

Nickel (Ni) is an essential “ultramicronutrient” with often substantially lower toxicity compared to other divalent cations such as cadmium as observed in vascular plants (Kováčık et al., 2010) or algae (Kováčık et al., 2016). Notwithstanding this, it may affect growth, physiology and molecular mechanisms in vascular plants if present in excess (Fourati et al., 2016; Van Hoewyk et al., 2018). Ni accumulation under natural conditions in lichens has been studied in the past (Nieboer et al., 1972; Purvis et al., 2007) but physiological responses have rarely been monitored and even high doses (250–500 μM) had no or little effect on basic physiology (Bačkor et al., 2010).

Elevated accumulation of heavy metals in the cells typically evokes imbalance in the generation and removal of reactive oxygen species (ROS), leading to oxidative stress and damage of biomolecules. Ni is a non-redox active metal unable to stimulate formation of ROS directly but elevated oxidative stress (measured as malondialdehyde accumulation) has been detected in various vascular species (Gomes-Junior et al., 2006) but only slightly in lichens (Bačkor et al., 2010). To our knowledge, ROS formation by fluorescence microscopy in Ni-treated lichens has not yet been monitored and data from algae showed lower Ni toxicity at this level compared to Cd (Kováčık et al., 2016). Nitric oxide (NO) is a gaseous protective molecule generating by plant cells also under metal excess aimed at improving metabolic pathways to cope with heavy metal excess (Kováčık et al., 2014). NO donor was found to modulate Ni uptake and toxicity in vascular plants too (Rizwan et al., 2018).

Plant cells possess various mechanisms to cope with oxidative stress including non-enzymatic antioxidants. Among them, ascorbic acid (AsA) and thiols (reduced form of glutathione, GSH and phytochelatins, PCs) are the most important ROS scavengers and/or metal chelators. Ni excess was found to enhance AsA accumulation (Rizwan et al., 2018) but responses of thiols in algal-like organisms are rather negative after prolonged exposure (García-García et al., 2018) or less expressive in comparison with Cd in the green microalgae (Kováčık et al., 2016). To our knowledge, the impact of Ni on AsA and thiols in lichens is not known. Other metals such as Cd, Cr, Zn or Pb have been tested at the level of antioxidants including AsA, GSH (Sanità di Toppi et al., 2004, 2005) or PCs (Pawlik-Skrowońska and Bačkor, 2011 and the references therein). Aliphatic organic acids, mainly citric and malic acids are potential metal chelators and they are often affected by metals (Dresler et al., 2014) including Ni (Gajewska et al., 2013) but have not yet been
deeply monitored in lichens.

Toxicity of metals in lichens has been frequently monitored by simple common spectrophotometric detection of ROS or lipid peroxidation (Bačkor et al., 2010, 2011) which do not have sufficient sensitivity under low metal stress (see e.g. Kováčik and Babula, 2017 for details). The main aim of this work was to detect concentration-dependent responses to Ni in two common macrolichens at the level of oxidative stress-related parameters by several fluorescence reagents aimed at detecting both “total/general” and individual ROS (hydrogen peroxide) as well as nitric oxide. Non-enzymatic antioxidants including chelators (phytochelatin 2, aliphatic organic acids) were also monitored. Data are compared with similar studies (lichens, if available), non-vascular (algae) or vascular plants aimed at highlighting eventual common responses in various life lineages.

2. Materials and methods

2.1. Plant material, experimental design and statistics

Macrolichen samples were collected during early autumn 2017: Cladonia arbuscula subsp. mitis (formerly known as Cladina and this name is used in the whole text to distinguish the tested species) was collected from the control locality in the forest near the village Slaná dolina (Slovakia) as reported previously (Bačkor et al., 2009) and Cladonia furcata was collected from the forest adjacent to the village Krnča (Slovakia). Both localities may be considered as unpolluted in terms of heavy metals (see also Bačkor et al., 2009 for metal content in Cladonia from polluted and control/unpolluted locality) and both species are common lichens. Macroscopic foreign material was manually removed and samples were prepared and analyzed within 1 week after harvest.

Approximately 0.1 g DW (air-dried) samples were used for analyses. They were exposed to Ni in screw-cap inert plastic tubes (50 mL; Sarstedt, Nümbrecht, Germany) for 24 h in solutions prepared with HEPES buffer (pH 6.5). Nickel (Ni²⁺) was added in the form of chloride in the final concentration of 10 or 100 μM. Controls were maintained in HEPES buffer only. Samples were kept in the cultivation room (12 h/12 h, 24/19°C day/night) at PAR ~30 μmol m⁻² s⁻¹. Absolute dry mass of lichens was determined by weighing the sub-samples dried in an oven at 90°C.

After the incubation period, samples for biochemical analyses were washed thrice with deionised water, carefully dried with filter paper and extracted as mentioned below. Three individual samples were used for each individual species, treatment and parameter (n = 3) leading to 72 total samples. One-way ANOVA followed by a Tukey’s test (MINITAB Release 11, Minitab Inc., State College, Pennsylvania, USA) was used in accordance with manufacturer’s instructions. Samples were washed with fresh phosphate-buffered saline (PBS) buffer (0.05 M, pH 7.2) and incubated in PBS buffer containing 50 μM DCF-DA in darkness for 30 min at room temperature (RT; Kováčik et al., 2016). Amples® UltraRed (568Ex/681Em nm, Life Technologies, USA) is reagent for H₂O₂ detection according to manufacturer’s instructions. Samples were incubated in a working solution (50 μl of 10 mM Amplex stock solution, 100 μl of horseradish peroxidase 1 U/ml in 50 mM PBS buffer, pH 6.0, Sigma-Aldrich and 4.85 ml of reaction buffer 50 mM PBS, pH 6.0) for 30 min at RT and darkness (Kováčik and Babula, 2017). Reactive nitrogen species/nitric oxide (RNS/NO) were stained using 2,3-diaminonaphthalene (DAN, 365Ex/415Em nm, Sigma-Aldrich): Stock solution of DAN in 0.62 M HCl was diluted by PBS buffer (0.05 M, pH 6.8) to the final concentration of 250 μM (Kováčik et al., 2015a,b).

All samples were washed three times in the buffer prior to observation using fluorescence microscope and appropriate set of filters (Axioskop 40, Zeiss, Germany). Images were processed using the NIS elements software (Nikon, Japan).

2.4. Quantification of thiols, ascorbic acid and organic acids

Samples were extracted in 0.1 M HCl or deionised water with cold mortar and pestle and addition of small amount of inert so-called sea sand (Penta s.r. o., Prague, Czech Republic) to achieve complete cell disruption followed by centrifugation at 14 000 g for 15 min at 5°C (centrifuge Hettich Mikro 200R).

Reduced glutathione (GSH) and phytochelatin 2 (PC2) were quantified in 0.1 M HCl homogenates following the method described by Perez-Rama et al. (2005). The thiol peptides derivatized with monobromobimane were measured using capillary electrophoresis set coupled with diode-array detector (UV-VIS/DAD, 190–600 nm (Agilent 7100, Agilent Technologies, Santa Clara, CA, USA). Detection was performed at signal wavelengths of 390 nm (Kováčik and Dresler, 2018). Freshly prepared standard solutions were used for the identification and quantification of GSH (Sigma-Aldrich, St. Louis, MO, USA) and PC2 (Anaspec Inc., San Jose, CA, USA).

The assay of reduced ascorbic acid (AsA) was performed by bathophenanthroline method with the detection at 534 nm from 0.1 M HCl homogenates as reported earlier (Kováčik et al., 2017b).

Organic acids were monitored in extracts prepared by homogenization of fresh samples in deionised water. The homogenate was incubated for 30 min at 50°C. Quantification was done by capillary electrophoresis method as described in detail previously (Dresler et al., 2014).

3. Results and discussion

3.1. Accumulation of Ni and mineral nutrients

Total content of Ni did not differ between Cladina and Cladonia at lower dose (10 μM) but Cladonia contained higher amount in 100 μM treatment (Fig. 1). Concentration-dependent accumulation differed in species: 7.67-fold difference between 10 and 100 μM in Cladina and 9.70-fold difference between 10 and 100 μM in Cladonia were observed (Fig. 1). In the previous study, the same Cladina from other locality contained ca. 5-times more Ni in total fraction after exposure to 10 or 100 μM Ni over 24 h and Ni accumulation was higher in Cladina than in other lichen Peltigera (Bačkor et al., 2010). Absolute values of total Ni were higher in the present work than those found by Bačkor et al. (2010) who reported ca. 65 and 338 μg total Ni g⁻¹ DW in 10 and 100 μM Ni treatments. At the same time, we note unit error of metal content in the work by Bačkor et al. (2010): mmol/g DW in the cited paper should be μmol/g DW, no tissue may contain over 30 mmol Cd or Ni/g DW = over 3300 mg Cd or 1700 mg Ni/g DW. On the contrary to relatively high Ni accumulation in the present study, other lichens such
previous report from Cladonia populations or the moss Racomitrium (Bačkor et al., 2009).

Though previous study demonstrated that different Cladonia species have rather similar accumulation capacity of Zn, Cd, Pb and As and could be considered as weak accumulators under natural conditions (localities with metallic contamination; Osyczka and Rola, 2013), our results indicate considerable sorption of Ni from the treatment solution: by taking exposure volume (50 ml), lichen sample (0.1 g DW) and applied Ni concentration (100 μg M Ni/50 ml) into account, maximal theoretical amount of total Ni is 2934.5 μg Ni g −1 DW and Cladonia accumulated over 41% (1717.68 μg Ni g −1 DW; see Fig. 1) from theoretically available amount. Comparison with previous work where microalgae Scenedesmus was exposed to 10 μM Ni over 24 h under identical exposure conditions indicates similar Ni accumulation by lichen in the present work (over 100–400 μg Ni/g DW in alga depending on the age; see Kováčik et al., 2016 for details). Considerable Ni accumulation by lichen thalli is also visible from comparison with vascular plant chamomile exposed to 60 or 120 μM Ni, where roots accumulated ca. 600 or 1000 μg Ni g −1 DW (Kováčik et al., 2009).

Excessive metal uptake often negatively affects accumulation of essential mineral nutrients though the impact of Ni is less negative in comparison with e.g. Cd in vascular plants (Kováčik et al., 2010) or algae (Kováčik et al., 2016). Present data did not confirm negative impact of Ni on potassium content but calcium content decreased (~31%) in Cladina in response to higher Ni dose (Fig. 1). Higher basal Ca content in Cladonia could contribute to maintenance of Ca level in this species. Other metals such as Hg or Pb had negative impact on mineral nutrients depending on the given metal (Kováčik et al., 2017b). Further studies focused on the interaction between metals and calcium/potassium level in lichens could show their role in metal tolerance.

3.2. Stress-related responses to Ni excess

Fluorescence microscopy is fast and efficient technique for the detection of oxidative balance (alteration of reactive oxygen species formation) but, to our knowledge, it has not yet been used on lichen samples under metal excess. Detection of “general ROS” and hydrogen peroxide (Fig. 2A and B) revealed clear signal in control of both species, indicating ROS formation comparable with water moss species (Kováčik et al., 2017a) or algae (Kováčik et al., 2015a). Even lower Ni dose (10 μM) stimulated increase in the signal of general ROS and H2O2 in both species and further elevation in 100 μM Ni treatment (Fig. 2A and B), indicating that oxidative stress (meaning imbalance between ROS generation and removal) occurs also under Ni excess. Signal was more visible in Cladonia, which could be, at least partially, evoked by higher Ni accumulation. These microscopic data are in contradiction to previous reports from lichens including Cladina where Ni doses 10–500 μM over 24 h had no impact on TBARS level (an indicator of oxidative damage, Bačkor et al., 2010) and even 500 μM Cu over 24 h had rather slight impact on hydrogen peroxide and superoxide level in Cladina and Cladonia assayed by standard spectrophotometry (Bačkor et al., 2011). It was confirmed in our work that fluorescence microscopy may detect Ni-induced ROS level more precisely than common spectrophotometric techniques (see also Kováčik and Babula, 2017 for comparison of spectrophotometry and fluorescence microscopy detection of ROS). In terms of anatomy, mycobiont seems to be more sensitive compared to photobiont under low Ni concentration mainly (10 μM) because signal of ROS in mycobiont was stronger in comparison with photobiont (empty/black areas are likely algal groups). In 100 μM Ni treatment, both myco- and photobiont visibly contributed to elevated fluorescence signal (Fig. 2).

Nitric oxide (NO) signal was elevated by 100 μM Ni in both species and by 10 μM Ni in Cladonia, indicating positive impact of Ni on NO formation (Fig. 2C). In agreement with these observations, 10 μM Ni (24 h of exposure under identical conditions) elevated NO signal in the green alga Scenedesmus quadricauda (Kováčik et al., 2016) or 200 μM Ni

Fig. 1. Accumulation of total nickel (Ni) and content of potassium (K) and calcium (Ca) in Cladonia arbuscula subsp. mitis (formerly known as Cladina and this name is used in all figures to distinguish the tested species) or Cladonia furcata treated with the given nickel concentrations over 24 h. Data are means ± SDs (n = 3). Values for Cladina or Cladonia followed by the same small or capital letter are not significantly different according to Tukey’s test (P < 0.05). ** and *** indicate significant difference at 0.01 and 0.001 level of Student’s t-test between Cladina and Cladonia in the given treatment. Control contained 4.51 (Cladina) and 5.52 (Cladonia) μg Ni g −1 DW.

as common Xanthoria parietina revealed negligible accumulation of e.g. Cd and exposure to 9 μM Cd over 24 or 48 h led to only ca. 4–5 μg Cd g −1 in intracellular fraction/ca. 11 μg Cd g −1 DW in total fraction (Sanità di Topp et al., 2005). Absorbed (so-called intracellular) Ni fraction in the present samples was ca. 5–9-times lower than total Ni content and Cladonia contained significantly more Ni than Cladina at higher Ni dose too (data not shown). In terms of natural (control) content of Ni in the present study (see head of Fig. 1), it is in line with

as common Xanthoria parietina revealed negligible accumulation of e.g. Cd and exposure to 9 μM Cd over 24 or 48 h led to only ca. 4–5 μg Cd g −1 in intracellular fraction/ca. 11 μg Cd g −1 DW in total fraction (Sanità di Topp et al., 2005). Absorbed (so-called intracellular) Ni fraction in the present samples was ca. 5–9-times lower than total Ni content and Cladonia contained significantly more Ni than Cladina at higher Ni dose too (data not shown). In terms of natural (control) content of Ni in the present study (see head of Fig. 1), it is in line with

as common Xanthoria parietina revealed negligible accumulation of e.g. Cd and exposure to 9 μM Cd over 24 or 48 h led to only ca. 4–5 μg Cd g −1 in intracellular fraction/ca. 11 μg Cd g −1 DW in total fraction (Sanità di Topp et al., 2005). Absorbed (so-called intracellular) Ni fraction in the present samples was ca. 5–9-times lower than total Ni content and Cladonia contained significantly more Ni than Cladina at higher Ni dose too (data not shown). In terms of natural (control) content of Ni in the present study (see head of Fig. 1), it is in line with
after 9 days of exposure enhanced NO formation in *Oryza sativa* (Rizwan et al., 2018). NO is a vital plant molecule affecting physiological and protective responses also to Ni excess (Rizwan et al., 2018). Its impact on ROS/NO formation in the present study (stimulation) resembles the action of Cd in algae under identical concentrations and exposure time and may be seen as one of signals for subsequent metabolic changes: for example, combined action of Cd and NO donor stimulated increase in AsA content in the green microalga (Kováčik et al., 2015b). Increase in AsA amount has also been reported in Ni-exposed rice roots and shoots with NO donor co-application (Rizwan et al., 2018). We found no data related to impact of Ni on AsA content in lichens but, in line with our data, hexavalent Cr (9.6 μM) or Cd (4.5 and 9 μM) stimulated AsA accumulation in *Xanthoria parietina* after short-term treatment (Sanità di Toppi et al., 2004; Sanità di Toppi et al., 2005). Not only cadmium (more pronouncedly) but also nickel evoked increase in AsA in microalga *Scenedesmus* after 24 h of exposure to 1 and 10 μM (Kováčik et al., 2016), indicating that ascorbate level responds probably to given metal irrespective of taxonomic level. Basal AsA content in *Cladonia* or *Cladina* (41.2–42.8 μg g⁻¹ DW = 0.23–0.24 μmol g⁻¹ DW) is within the order reported in *Xanthoria* (ca. 0.5 μmol g⁻¹ DW; Sanità di Toppi et al., 2005) or *Parmelia*

### 3.3. Ascorbic acid and thiols in response to Ni excess

Ascorbic acid (AsA) is an essential plant antioxidant and its suppressed amount is often reflected in increased ROS production (Kováčik et al., 2017b). No negative impact of Ni on AsA content in *Cladonia* and strong stimulation in *Cladina* (Fig. 3) indicates various responses in these two lichens. In agreement, dose-dependent increase in AsA has also been observed in rice treated by 200 μM Ni over 9 days (Rizwan et al., 2018). We found no data related to impact of Ni on AsA content in lichens but, in line with our data, hexavalent Cr (9.6 μM) or Cd (4.5 and 9 μM) stimulated AsA accumulation in *Xanthoria parietina* after short-term treatment (Sanità di Toppi et al., 2004; Sanità di Toppi et al., 2005). Not only cadmium (more pronouncedly) but also nickel evoked increase in AsA in microalga *Scenedesmus* after 24 h of exposure to 1 and 10 μM (Kováčik et al., 2016), indicating that ascorbate level responds probably to given metal irrespective of taxonomic level. Basal AsA content in *Cladonia* or *Cladina* (41.2–42.8 μg g⁻¹ DW = 0.23–0.24 μmol g⁻¹ DW) is within the order reported in *Xanthoria* (ca. 0.5 μmol g⁻¹ DW; Sanità di Toppi et al., 2005) or *Parmelia*
Cladina and J. Kováč.

Cladonia furcata treated with the given nickel concentrations over 24 h. Data are means ± SDs (as determined from exposure (Sanità di Toppi et al., 2004; Sanità di Toppi et al., 2005). In the vascular species, Ni was found to stimulate GSH synthesis (Drzewiecka et al., 2017). Basal GSH values (ca. 260 μg g⁻¹ DW = 0.85 μmol g⁻¹ DW, Fig. 3) are higher than those detected in Cladonia furcata (ca. 0.1 μmol g⁻¹ DW; Pawlik-Skowrońska and Bačkor, 2011) but lower than in common lichen Xanthoria parietina (ca. 3.6 μmol g⁻¹ DW; Sanità di Toppi et al., 2004). Phytochelatin 2 (PC2) appeared in Cladonia only in response to Ni treatments, which could be related to higher GSH content in these samples (Fig. 3). These data, however, do not indicate eventual significance of PC2 for Ni tolerance because values (1.44–1.63 μg g⁻¹ DW) are lower compared to unicellular green algae such as Scenedesmus where Ni treatments did not differ from the control (ca. 5 μg PC2 g⁻¹ DW; Kováč et al., 2016). In agreement with our data, PC2 and PC3 were not previously detected in control thalli of Cladonia furcata and appeared only after exposure to 200 μM Zn or Pb (Pawlik-Skowrońska and Bačkor, 2011). All these data indicate that phytochelatins do not play significant role in response to Ni excess but synthesis of AsA in Cladina and GSH in Cladonia may have a protective impact and requires further studies.

3.4. Responses of organic acids to Ni excess

Malic acid was more abundant than citric acid in control thalli of both species, followed by α-ketoglutaric and succinic acids and Cladina usually contained more acids than Cladonia (Fig. 4). To our knowledge, these acids were not frequently monitored in lichens. Molar amounts are similar to those observed in the lichen Lecanora polytrpota containing 0.83 μmol g⁻¹ of citric acid and 0.45 μmol g⁻¹ of malic acid (Pawlik-Skowrońska et al., 2006) and our control values are ca. 0.79–1.01 μmol g⁻¹ DW (citric acid) and 1.26–1.83 μmol g⁻¹ DW (malic acid). Amount of acids in microalgae (as some of them are photobionts in lichens) may also be briefly compared: control Cocco(myxa contained ca. 113 and 30 μg g⁻¹ FW of citric and malic acid, respectively (and ca. 10-times more per g DW considering water content 90%, Kováč et al., 2015b).

Citic acid accumulation was not affected by Ni excess in any species and succinic acid slightly increased in Cladonia (Fig. 4). On the contrary, malic acid decreased by ca. 2-fold in both species and at both Ni doses while α-ketoglutaric acid showed the opposite behavior in the tested species, including sharp increase in Cladonia (Fig. 4). These data are partially in agreement with earlier observations from green microalgae, where elevation of succinic and malic acids but depletion of citric acid in response to 10 μM Ni was observed (Kováč et al., 2016). In vascular plants such as wheat shoots, Ni excess (50 or 100 μM over 7 days) evoked strong increase in citric and malic acids (Gajewska et al., 2013) and it was suggested that Ni stress redirects the carbon metabolism to provide carbon skeletons for synthesis of organic acids (among others) to support defense mechanisms against oxidative stress (including Ni chelation). No or negative impact of Ni on the main acids in the present lichen species does not support their role in Ni chelation but the significance of α-ketoglutaric acid should be investigated in the future.

4. Conclusions

Present study has shown, to our knowledge for the first time, formation of ROS and NO in Ni-exposed lichens as proven by fluorescence microscopy. Unlike data from other lichen species where standard spectrophotometry did not show Ni-enhanced oxidative stress, ROS and NO changes observed here were considerable and occurred likely in fungal partner mainly. Owing to positive impact of NO on the metabolism under metallic excess, its generation may contribute, at least partially, to Ni-induced enhancement of ascorbic acid or glutathione in the studies species. Negative impact of nickel on malic acid

Fig. 3. Quantitative changes of reduced ascorbic acid (AsA), reduced glutathione (GSH) and phytochelatin 2 (PC2) in Cladonia arbuscula subsp. miitls (marked as Cladina) or Cladonia furcata treated with the given nickel concentrations over 24 h. Data are means ± SDs (n = 3). Values for Cladina or Cladonia followed by the same small or capital letter(s) are not significantly different according to Tukey’s test (P < 0.05). * and *** indicate significant difference at 0.05 and 0.001 level of Student’s t-test between Cladina and Cladonia in the given treatment. nd – not detectable.

control thalli (80 μg g⁻¹ DW = 0.46 μmol g⁻¹ DW; Calatayud et al., 1999).

On the contrary to AsA, reduced glutathione (GSH) content was depleted in Cladina exposed to 100 μM Ni but stimulated in Cladonia at both Ni doses (Fig. 3). We found no data related to impact of Ni on GSH in lichens but other metals such as Cr(VI) and mainly Cd evoked depletion even in response to lower doses (4.5–9 μM) after 48 h of exposure (Sanità di Toppi et al., 2004; Sanità di Toppi et al., 2005). In the green microalgae, Ni had concentration-dependent impact on GSH but less negative compared to Cd (Kováč et al., 2016). In Euglena gracilis, the impact of Ni (50–1000 μM) on thiosls including GSH was negligible after 24 h but rather negative after 72 h of exposure (García-García et al., 2018). In the vascular species, Ni was found to stimulate GSH synthesis (Drzewiecka et al., 2017). Basal GSH values (ca. 260 μg g⁻¹ DW = 0.85 μmol g⁻¹ DW, Fig. 3) are higher than those detected in Cladonia furcata (ca. 0.1 μmol g⁻¹ DW; Pawlik-Skowrońska and Bačkor, 2011) but lower than in common lichen Xanthoria parietina (ca. 3.6 μmol g⁻¹ DW; Sanità di Toppi et al., 2004). Phytochelatin 2 (PC2) appeared in Cladonia only in response to Ni treatments, which could be related to higher GSH content in these samples (Fig. 3). These data, however, do not indicate eventual significance of PC2 for Ni tolerance because values (1.44–1.63 μg g⁻¹ DW) are lower compared to unicellular green algae such as Scenedesmus where Ni treatments did not differ from the control (ca. 5 μg PC2 g⁻¹ DW; Kováč et al., 2016). In agreement with our data, PC2 and PC3 were not previously detected in control thalli of Cladonia furcata and appeared only after exposure to 200 μM Zn or Pb (Pawlik-Skowrońska and Bačkor, 2011). All these data indicate that phytochelatins do not play significant role in response to Ni excess but synthesis of AsA in Cladina and GSH in Cladonia may have a protective impact and requires further studies.

3.4. Responses of organic acids to Ni excess

Malic acid was more abundant than citric acid in control thalli of both species, followed by α-ketoglutaric and succinic acids and Cladina usually contained more acids than Cladonia (Fig. 4). To our knowledge, these acids were not frequently monitored in lichens. Molar amounts are similar to those observed in the lichen Lecanora polytrpota containing 0.83 μmol g⁻¹ of citric acid and 0.45 μmol g⁻¹ of malic acid (Pawlik-Skowrońska et al., 2006) and our control values are ca. 0.79–1.01 μmol g⁻¹ DW (citric acid) and 1.26–1.83 μmol g⁻¹ DW (malic acid). Amount of acids in microalgae (as some of them are photobionts in lichens) may also be briefly compared: control Cocco(myxa contained ca. 113 and 30 μg g⁻¹ FW of citric and malic acid, respectively (and ca. 10-times more per g DW considering water content 90%, Kováč et al., 2015b).

Citic acid accumulation was not affected by Ni excess in any species and succinic acid slightly increased in Cladonia (Fig. 4). On the contrary, malic acid decreased by ca. 2-fold in both species and at both Ni doses while α-ketoglutaric acid showed the opposite behavior in the tested species, including sharp increase in Cladonia (Fig. 4). These data are partially in agreement with earlier observations from green microalgae, where elevation of succinic and malic acids but depletion of citric acid in response to 10 μM Ni was observed (Kováč et al., 2016). In vascular plants such as wheat shoots, Ni excess (50 or 100 μM over 7 days) evoked strong increase in citric and malic acids (Gajewska et al., 2013) and it was suggested that Ni stress redirects the carbon metabolism to provide carbon skeletons for synthesis of organic acids (among others) to support defense mechanisms against oxidative stress (including Ni chelation). No or negative impact of Ni on the main acids in the present lichen species does not support their role in Ni chelation but the significance of α-ketoglutaric acid should be investigated in the future.

4. Conclusions

Present study has shown, to our knowledge for the first time, formation of ROS and NO in Ni-exposed lichens as proven by fluorescence microscopy. Unlike data from other lichen species where standard spectrophotometry did not show Ni-enhanced oxidative stress, ROS and NO changes observed here were considerable and occurred likely in fungal partner mainly. Owing to positive impact of NO on the metabolism under metallic excess, its generation may contribute, at least partially, to Ni-induced enhancement of ascorbic acid or glutathione in the studies species. Negative impact of nickel on malic acid
accumulation or no impact on citric acid does not support their eventual role as Ni chelators and low content of phytochelatin 2 indicates the same. Further studies should focus on the role of individual lichen partners (mycobiont vs. photobiont) in responses to Ni along with the role of lichen specific metabolites.

Authors' dedication

The authors dedicate this work to the memory of the Slovak investigative journalist Jan Kuciak and his fiancée Martina Kusnirova (both 1990–2018).

Disclosure statement

The authors declare that there are no conflicts of interest.

Authors contribution

JK: lichen collection, spectrophotometry and statistics/manuscript preparation.
SD: other analytical methods.
PB: microscopy.

Acknowledgement

Analyses were supported by internal sources of individual workplaces. Sponsors had no involvement in the present study.

References


