Extra and Intercellular Concentrations of Water Soluble Cations from *Xanthoria parietina* and *Phaeophyscia orbicularis* Lichenized Fungi Species

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The aim of the present study was to determine concentrations of the water soluble content of sodium (Na^+), potassium (K^+), ammonium (NH_4^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) cations accumulated in the extra and intercellular locations of Xanthoria parietina and Phaeophyscia orbicularis lichenized fungi thallus collected in the north-eastern Romania. The concentrations of the interest cations were determined by ion chromatography. An increase of electrolyte leakage, especially K^+, was observed in both Xanthoria parietina and Phaeophyscia orbicularis species, which might be a result of the cell membranes disruption. The identified and quantified cations did not exhibit well defined trends, but rather showed fluctuations between the investigated species and within the investigated time period. Multivariate statistical tools were used in order to investigate the obtained results. The most significant correlations were observed for the (K^+,Mg^{2+}) pair in both Xanthoria parietina and Phaeophyscia orbicularis species, an observation which might suggest either similarities in their locations or possible common physiological role.

Keywords: lichenised fungi, Xanthoria parietina, Phaeophyscia orbicularis, macronutrients, ionic forms, ion chromatography

Lichenised fungi, dominating presently about 8% of the land's surface [1], are ectohydric organisms lacking of specialised structures for water and gas exchange, allowing many chemicals (*e.g.* pollutants and contaminants) to be absorbed over the whole thallus surface [2]. Various morphological characteristics (*i.e.* rich thallus branching, favourable intercellular spaces within thalli) facilitate trapping of particulate pollutants onto the lichenised fungi thallus surface [3]. Since lichenised fungi accumulate pollutants in their thallus they often have been used as biomonitors in air quality assessment studies [4-9]. There are suggestions that the chemical composition of lichenised fungi may largely reflect the availability of elements in the environment [3] and, therefore, effects of air pollution on lichenized fungi chemistry and on lichenized fungi communities are often monitored in order to determine depositional patterns from various emission sources [10]. It is also documented that macronutrients (*i.e.*, sulphur, nitrogen, potassium, magnesium, calcium) are relatively mobile and their concentrations in lichenised fungi can change seasonally [11]. However, trace metal, (*e.g.*, cadmium, lead, zinc) accumulate in lichenised fungi over time and they are less mobile than macronutrients [12]. Usually, in order to evaluate the role of lichenised fungi in mineral cycling it is necessary to understand their ability to acquire, retain, internally redistribute, and release mineral elements [13]. Moreover, the interpretation of such data has to take into account in addition the fact that the uptake of one element can be directly influenced by the levels of others in specific tissues [14].

Major ways, especially of the inorganic contaminants impact on the lichenised fungi status, are accounted by the accumulated particles onto the lichenised fungi thallus surface or within intercellular spaces, ions bound to extra- or intercellular exchange sites and soluble intracellular ions [15]. The major effect induced by the contaminants/ pollutants presence onto the surface of the lichenised fungi thallus is the disturbance in the internal organisation of the cells by membranes where ions and molecules play a very important role. Once the disturbance is occurring the membrane permeability to other ions is changed, a process which is accompanied by loss of electrolytes as potassium (K) and magnesium (Mg). The K^+ leakage from the lichens is reported as the most sensitive indicator of membrane disturbance [16]. However, it is already documented that various ions may exist in different compartments within lichenised fungi and also that they are not evenly distributed throughout lichen thalli. For example, distribution of cations appears to be into the intercellular and surface fraction, ion exchange site fraction, intracellular fraction and residual fraction [15]. There is also suggestion that in such organism potassium is mainly soluble within the cells, calcium is bound, exchangeable, to sites in the cell wall and is insoluble within the cell and magnesium is present in all three locations. Usually, however, leakage of intracellular ions can be used as a measure of membrane damage [17].

However, elements accumulation mechanism in lichenised fungi thallus is directly linked to their removable degree. Elements deposited to the thallus surface can be immediately removed by rinsing rapidly the sample (few seconds for example), those accumulated in the intercellular and extracellular spaces are removed by soaking the sample for 30-60 min in distilled water or in 20 mM nickel chloride ($NiCl_2$) solution, while those bound to the cell wall or accumulated at intracellular level are removed by more complicated mechanisms (elution with 0.01 M of sulphuric acid (H_2SO_4) for 2 h or digestion with concentrated nitric acid, $HÑO_3$) [3].

The aim of the present paper is to estimate the water soluble content of sodium (Na^+), potassium (K^+), ammonium (NH_4^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) cations accumulated in the extra and intercellular locations of *Xanthoria parietina* and *Phaeophyscia orbicularis* lichenized fungi thallus collected in the north-eastern Romania. Statistical analysis of the obtained results

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is used in order to identify either similarities in cations locations or possible common physiological role.

Experimental part

Sample collection

Samples of *Xanthoria parietina* and *Phaeophyscia orbicularis* lichenised fungi thallus were collected in Iasi region, north-eastern Romania, from Tudor Vladimirescu (Lat N 47°152 , Long E 27°602 , 83 m above sea level, a.s.l.), Bucium (Lat N 47°082 , Long E 27°642 , 408 m a.s.l.) and Red Bridge (Lat N 47°152 , Long E 27°602 , 81 m a.s.l) locations. Sampling was carried out from May 2011 to December 2011.

Samples, collected with the minimum of adhering bark, were stored in paper bags and transported to the laboratory for further analysis. In the laboratory, the samples were carefully cleaned in order to remove senescent tissue and as much as possible extraneous material (adhering bark, soil and/or other particles). Samples were rinsed five times, for 5 s, with 5 mL of ultrapure water (18.2 MΩ.cm resistivity) in order to remove potential contaminants from the lichenised fungi thallus surface. Samples were air-dried to constant weight and than weighed portions were homogenised with an agate mortar and pestle. The homogenised sample was quantitatively transferred in Falcon tubes with 10 mL of ultrapure water and then further subject to ultrasonication (*Elmasonic S 70 H* ultrasonic bath) for 45 min. Since high variability and non-normal distributions can affect the elemental content analyzed from different matrices, this issue was partially controlled by carefully samples preparation and sampling, and an adequate number of replicates for each sample. Within the present work each collected samples was subject to three replicate analyses.

The experimental approach

Recent studies on the lichenised fungi potential to be used in biomonitoring studies reports that they were generally aimed 1) either in determining the deposition of contaminants by measuring the total concentration of the elements in unwashed samples [18] or 2) in determining the contaminants that are bioconcentrated by the lichenised fungi, and the samples were therefore washed prior to the determination [19]. For the present work the second approach has been used for the investigation of the water soluble content of sodium (Na^+), potassium (K^+), ammonium (NH_4^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) cations accumulated in the extra and intercellular locations of *Xanthoria parietina* and *Phaeophyscia orbicularis* lichenized fungi thallus collected in the north-eastern Romania. Part of the sequential elution technique (SET), which is of high interest in order to answer the need to determine the cellular locations of various elements in both lichens and in aquatic and terrestrial mosses [20], was used within the present work. It has been already found that in lichens, metal cations bind to extracellular anionic exchange sites located in the cell wall and plasma membrane surface [21]. Because cell wall-bound elements are readily exchangeable, extracellular amounts and proportions are thought to reflect recent environmental input [13]. For the present work the intercellular and extracellular fractions of the water soluble cations were of high interest since these fractions may contain soluble elements that externally bath the cell wall and external wall of the plasma membrane without binding to cell or to particles deposited on the surface [20].

Determination of cationic content

The water soluble content of sodium (Na^+), potassium (K^+), ammonium (NH_4^+), and calcium (Ca^{2+}) and magnesium (Mg^{2+}) cations accumulated in the intercellular spaces of *Xanthoria parietina* and *Phaeophyscia orbicularis* lichenized fungi thallus was determined by ion chromatography. A *DIONEX ICS 3000* ion chromatograph equipped with an IonPac CS17 (4×250 mm) analytical column and a CSRS 300 (4 mm) auto-suppressor were used for the analysis. Calibration curves for the interest cations (sodium, Na^+, ammonium, NH_4^+, potassium, K^+, magnesium, Mg^{2+}, calcium, Ca^{2+}) were performed once a week and one or two standards were daily analysed in order to check for instrument stability. Before the injection of each sample replicate one blank solution (ultrapure water) has been analysed.

During the analysis operators usually have to deal with measurement uncertainty, and therefore take appropriate actions to address measurement errors [22]. In the present work limit of detection (LOD) and limit of quantification (LOQ) were estimated for each analyzed cation in agreement with literature information [23]. For each cation of interest the calibration curve showed a very good correlation coefficient ($R^2 > 0.995$) which may represent a condition fulfilled for the quality assurance criteria requested when various variables are quantified.

Results and discussions

Table 1 and 2 report the final concentrations expressed in μg/g dry weight (dw) for the water soluble cations (Na^+, NH_4^+, K^+, Mg^{2+}, Ca^{2+}) identified in *Xanthoria parietina* (table 1) and *Phaeophyscia orbicularis* (table 2) lichenized fungi thallus. For each sample three replicates were subject both to preparative and analysis steps in order to verify sample homogeneity and reproducibility of sample analysis. Data presented in table 1 and 2 suggest the existence of some variability in the cations concentrations from location to location or from month to month. According to information from the literature the distribution in the concentrations of the interest chemical species might be highly variable especially in areas where damage by air pollutants may occur [24].

From the data presented in table 1 and 2 potassium ion seems to be among the most abundant water soluble cation in both *Xanthoria parietina* and *Phaeophyscia orbicularis* species, an observation which might be in good agreement with the suggestion from the literature that in such systems potassium ion is mainly soluble within the cells in the cell walls [19]. There are also evidences that a proportion of the total potassium is bound ionically in the cell wall while another part may be present as freely diffusible material retained within the permeability barriers of the intact cell [25].

The data presented in both table 1 and 2 clearly show that K^+ abundance is immediately followed by that of NH_4^+ and Na^+ cations. However, while K^+ concentration in the lichenised fungi might be considered as a measure of membrane damage [26], Na^+ losses might be of different origin or nature than the losses of K^+. Moreover, there are suggestion that while the majority of Na^+ ions is considered to be mostly associated to surface and intercellular sites, Mg^{2+} ion is considered to be mostly associated to exchange groups of the cell wall [27].

As the data in table 1 and 2 show the concentrations of both Mg^{2+} and Ca^{2+} ions are much lowered compared to all other investigated cations. Lower concentrations for these two cations were also reported in *Cladonia portentosa* [28]. Mat forming lichens usually produce a basal

Location, month	Cation (µg/g dw)				
	Na^+	NH_4^+	K^+	Ca^{2+}	Mg^{2+}
TV 05.2011	734.6 ± 25.2	1183.8 ± 80.2	4200 ± 195	181.6 ± 20.3	274 ± 37.1
TV 06.2011	348.4 ± 32.9	1544 ± 161.3	2600 ± 281.6	246.4 ± 9.3	135.3 ± 8.7
TV 07.2011	531 ± 28.2	1138.4 ± 137.3	4125.4 ± 360.8	311.3 ± 51	244 ± 36.6
TV 09.2011	425.8 ± 7.2	1695.2 ± 221.3	4110 ± 489.9	450.6 ± 65.8	380.4 ± 50.1
TV 10.2011	415.5 ± 148.6	1409.7 ± 64.2	3600 ± 440.5	61.2 ± 21.6	267.4 ± 61.9
TV 11.2011	1221.7 ± 166.9	1651.5 ± 7.8	7516.6 ± 796.5	35.3 ± 16.1	660 ± 43.6
TV 12.2011	941.8 ± 279.4	1685.1 ± 134.6	6548.9 ± 296.3	69.3 ± 16.8	537 ± 69.2
B 05.2011	707.2 ± 163	1353.9 ± 60.9	4364 ± 188.8	580.4 ± 84.1	325.3 ± 62
B 06.2011	442.5 ± 11	1844.7 ± 42.4	3976.7 ± 305.2	249.2 ± 18.9	403.8 ± 78.4
B 07.2011	695.7 ± 100.2	1554.6 ± 85.8	4942.8 ± 489	481.7 ± 62.9	463.1 ± 52.7
B 09.2011	934.7 ± 95.1	1499 ± 124.5	4800 ± 325	543.3 ± 65.1	307.6 ± 47.8
B 10.2011	727.7 ± 132	1736.1 ± 188.7	5108.2 ± 159.7	301.5 ± 34	471.2 ± 47
B 11.2011	778.1 ± 125.2	1594.4 ± 80.6	5674.9 ± 300.9	386.7 ± 30.8	500 ± 31.4
B 12.2011	859.4 ± 84.7	1523 ± 46.7	5419.8 ± 274.9	314.5 ± 35.6	507 ± 8.8
RB 06.2011	450.4 ± 33.4	1283.4 ± 64.7	3663.9 ± 437.4	500.1 ± 47.8	338.7 ± 11.7
RB 07.2011	500.4 ± 61.2	1898 ± 50.5	3600 ± 104.3	435.8 ± 49.6	292.6 ± 50
RB 09.2011	464.8 ± 48.5	1712.9 ± 130.8	3426.2 ± 119.4	848.5 ± 54	314.6 ± 17.8
RB 10.2011	1105.2 ± 433	1770.5 ± 72.4	5200 ± 140.1	365.2 ± 24.6	360 ± 5.3
RB 11.2011	663.9 ± 190.4	1830.6 ± 221.1	5428.8 ± 172.1	622.7 ± 31.7	410.4 ± 16.7
RB 12.2011	700.5 ± 112.2	1710.6 ± 48.5	4796.3 ± 213.7	574.4 ± 21.2	467.5 ± 23.7

TV- Tudor Vladimirescu, B- Bucium, RB- Red Bridge

Table 1
CONCENTRATIONS (AVERAGE ACCOMPANIED BY STANDARD DEVIATION) OF Na^+, NH_4^+, K^+, Ca^{2+}, Mg^{2+} WATER SOLUBLE CATIONS IDENTIFIED IN *XANTHORIA PARIETINA* LICHENISED FUNGI THALLUS

Location, month	Cation (µg/g dw)				
	Na^+	NH_4^+	K^+	Ca^{2+}	Mg^{2+}
TV 06.2011	2104.3 ± 64.1	911.2 ± 107.7	4042.7 ± 78	420.6 ± 20.7	146.8 ± 13.4
TV 07.2011	1054.5 ± 98.1	651.6 ± 30.3	4430.4 ± 159	613.3 ± 23.7	231.7 ± 12.4
TV 09.2011	1105.6 ± 59.3	1669.5 ± 56	7409.1 ± 263.7	988.9 ± 40.3	457.1 ± 8.4
TV 10.2011	905.6 ± 36	1114.9 ± 57.5	3314 ± 179.9	729.7 ± 115.1	123.9 ± 18.6
TV 11.2011	1329.9 ± 190.5	2058.6 ± 223.4	6615.5 ± 745	675.6 ± 79.3	235.4 ± 51.3
TV 12.2011	779.7 ± 11.1	1637 ± 68.5	6960.1 ± 282.3	483.6 ± 418.2	390.6 ± 8.3
B 06.2011	981.2 ± 141.3	1123.7 ± 69.2	3338.3 ± 159.7	734.4 ± 107.1	124.7 ± 17.3
B 07.2011	668.2 ± 84.5	1154 ± 33.6	3915.1 ± 460.5	399.1 ± 38.9	132 ± 18.8
B 09.2011	574.2 ± 24	1436.6 ± 82.1	5101.4 ± 213.4	742.3 ± 49	337.2 ± 14.8
B 10.2011	1114.5 ± 155.5	724.8 ± 78.5	2185.7 ± 206.2	362.1 ± 42.9	73 ± 9.4
B 11.2011	1072.3 ± 50.2	756.5 ± 49.7	4981.3 ± 595.9	461.7 ± 96.4	128.4 ± 34.6
B 12.2011	1532.9 ± 77.5	877 ± 55	2045.8 ± 413.7	241.8 ± 44.8	64.5 ± 20.4
RB 06.2011	1363.6 ± 65.1	2482.7 ± 370	8808 ± 798.4	1090.4 ± 265.4	439.3 ± 92.3
RB 07.2011	1277.4 ± 132.4	1951.2 ± 142	8436.2 ± 428.9	1248 ± 267.9	512.6 ± 43.9
RB 09.2011	843.4 ± 21.2	1268.3 ± 77.3	7500.7 ± 455.8	1092.6 ± 105.3	421.4 ± 23.2
RB 10.2011	2760.8 ± 295.7	874 ± 112.3	2618.4 ± 312.9	911.8 ± 51.7	110.8 ± 5.4
RB 11.2011	794 ± 118.1	1293.8 ± 109.8	4396 ± 160.2	838.2 ± 85.8	155.8 ± 23.6
RB 12.2011	898.1 ± 73.2	1289.8 ± 203.7	8173.8 ± 248	966.9 ± 59.5	495.2 ± 17.9

TV- Tudor Vladimirescu, B- Bucium, RB- Red Bridge

Table 2
CONCENTRATIONS (AVERAGE ACCOMPANIED BY STANDARD DEVIATION) OF Na^+, NH_4^+, K^+, Ca^{2+}, Mg^{2+} WATER SOLUBLE CATIONS IDENTIFIED IN *PHAEOPHYSCIA ORBICULARIS* LICHENISED FUNGI THALLUS

layer of intact dead lichen litter with a marked vertical gradient in concentrations of N, P and K [29] which have a cation exchange capacity equivalent to the living thallus [30] and might function as an effective trap for Ca^{2+}. While Ca^{2+} is located predominantly in the extracellular spaces, Mg^{2+} is more evenly distributed between extra- and intracellular space, and intracellular pools might buffer short-term changes in extracellular Mg^{2+} loadings [28]. However, it is believed that major changes in cellular physiology are caused by metal accumulated in cellular protoplasm (intracellular metal), whereas metal that is bound as exchangeable forms to binding sites in the plasma membrane or in the cell wall (extracellular metal) and intercellular metal do not have immediate effects on cellular metabolism [31].

The data presented in table 1 and 2 show that the concentration of the NH_4^+ ion is higher than that of Ca^{2+} and Mg^{2+} ion. The relatively high concentration of ammonium ion compared with Ca^{2+} and Mg^{2+} in both *Xanthoria parietina* and *Phaeophyscia orbicularis* species may indicate either the influence of nitrogen pollution or its role at metabolically level. Primary toxicity of NH_4^+ ion may arise immediately after the uptake exceeds the assimilation capacity and in this case, possible consequences are uncoupling of electron transport and membrane disfunction [32]. Actually, cell membrane transport of ammonium ion is crucial for the acquisition and metabolism of N in these organisms, the negative membrane potential favouring NH_4^+ entry into the cell along its electrochemical gradient [19]. Moreover, high levels of this ion in the cytosol may be toxic for the cells and the cellular levels of NH_4^+ tend to remain stable by a passive mechanism of NH_4^+ efflux serving as a way to protect cells from accumulation of cytotoxic ammonium levels [33]. Actually, at high concentration of this ion, its accumulation inside the cells is partially balanced by a concomitant loss of K^+ and Na^+ [34]. This mechanism might also explain the high K^+ concentrations measured in the present study. Especially in damaged cell membranes, permeability is altered and electrolyte leakage occurs, mainly in the form of K^+ ions which are the most abundant [35]. Most probable cell membrane damage (as indicated by K^+ leakage) was favoured by the accumulation of NH_4^+ concentration above a threshold level, from where it became toxic and it could not be anymore metabolised. This suggestion is highly sustained by the fact that in the present work it was observed that the effect is more pronounced in *Phaeophyscia orbicularis*

Variables	Experimental values		Theoretical values at p =0.05
	Xanthoria parietina	*Phaeophyscia orbicularis*	
(Na^+,K^+)	0.866	-	0.444
(Na^+,Mg^{2+})	0.651	-	
(NH_4^+,K^+)	-	0.777	
(NH_4^+,Mg^{2+})	-	0.685	
(NH_4^+,Ca^{2+})	-	0.585	
(K^+,Mg^{2+})	0.887	0.939	
(Ca^{2+},Mg^{2+})	-	0.730	

Table 3
CORRELATIONS BEWEEN VARIOUS CATION PAIRS IN THE INVESTIGATED LICHENIZED FUNGI SAMPLES

than in *Xanthoria parietina* species, *Xanthoria parietina* being a more tolerant organism at high N concentrations. Ammonium concentration seems to be higher in *Xanthoria parietina* species (1581 ± 216 μg/g dw, average ± stdev of all measurements) than in *Phaeophyscia orbicularis* (1293 ± 503 μg/g dw) while potassium concentration shows an opposite behaviour (4655 ± 305 μg/g dw in *Xanthoria parietina* and 5198 ± 298 μg/g dw in *Phaeophyscia orbicularis*). However, since the plasma membrane is the first site of biological interaction with toxic substances, and exogenous N is primarily transferred to the mycobiont and then to the photobiont via the hyphal cortex [36], it is reasonable to assume that ion leakage mostly occurred from fungal cells, which represent the large majority of a lichen biomass.

Within the present work, multiple linear regression analysis was also applied in order to investigate the organization and correlation of the data. Table 3 presents values for the Pearson coefficient estimated from the multiple linear regression analysis.

In table 3 are presented only the values which indicated the existence of a significant correlation at a p level of 0.05. The greatest correlation has been found between K^+ and Mg^{2+} ions in both *Xanthoria parietina* and *Phaeophyscia orbicularis* species (fig. 1a,b) which is thought to provide evidence of the role of these species at cellular level,

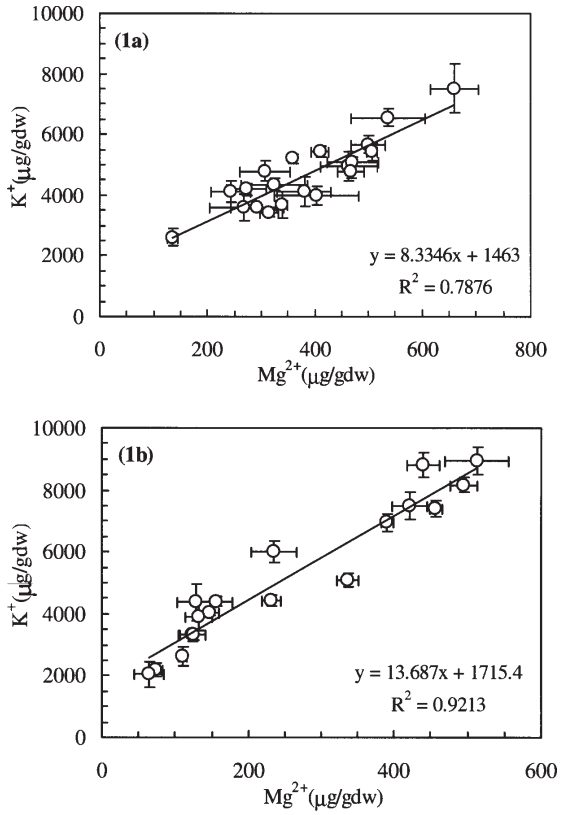

Fig. 1.a,b. K^+ concentration *vs.* Mg^{2+} concentrations in both *Xanthoria parietina* (1a) and *Phaeophyscia orbicularis* (1b) lichenized fungi samples.

especially when degradation might occur in cell membranes.

The hierarchical cluster analysis performed on the on-thallus water soluble cation concentrations are obtained from the three sampling locations based on two different lichen species, *i.e. Xanthoria parietina* and *Phaeophyscia orbicularis*. This cluster analysis allows grouping of species based on their similarities in on-thallus cation concentration. The dendrograms built from the hierarchical cluster analysis are illustrated in figure 2 for *Xanthoria parietina* and in figure 3 for *Phaeophyscia orbicularis* species.

The dendograms were generated by using as agglomeration rule the single linkage and euclidian distance as a distance measure. Usually, the shorter the "linkage distance" the stronger is the underlying similarity. For *Xanthoria parietina* species major clusters which are mainly observed are those between K^+, Mg^{2+} and Na^+ cations. These clusters are probably built based on cations similarities in the investigated lichenised fungi thallus. Sodium ion (Na^+) seems to have similar effect as the K^+/

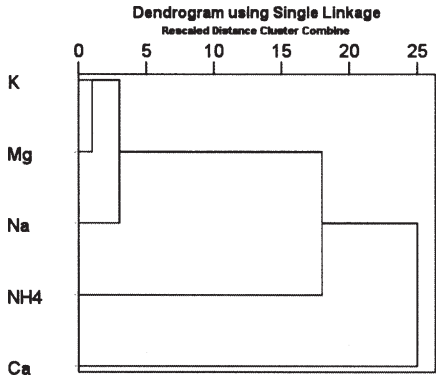

Fig. 2. Dendrograms (agglomeration rule: single linkage and distance measure: euclidian distance) generated from seasonal (May – December 2011) on-thallus water soluble cations concentrations determined in *Xanthoria parietina* species.

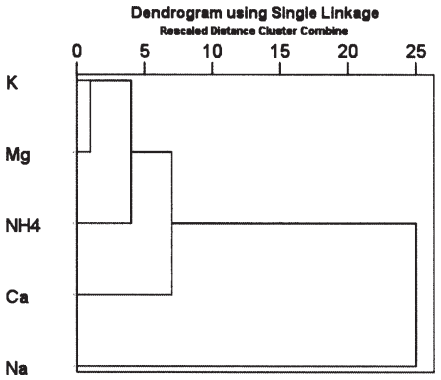

Fig. 3. Dendrograms (agglomeration rule: single linkage and distance measure: euclidian distance) generated from seasonal (May – December 2011) on-thallus water soluble cations concentrations determined in *Phaeophyscia orbicularis* species.

Mg^{2+} pair contributing beside this pair to another cluster formation. For *Phaeophyscia orbicularis* species the obtained dendogram shows also a major cluster between K^+/Mg^{2+} cation pair with subsequent contributions from NH_4^+, Ca^{2+}, and Na^+ cations. In *Phaeophyscia orbicularis* especially Na^+ cation is linked to the other clusters by a long distance branch which might suggest a strong dissimilarity between these cations. Since *Phaeophyscia orbicularis* is not a N tolerant species the strong similarity observed between K^+, Mg^{2+} and NH_4^+ cations might suggest the impact of the NH_4^+ uptake on the cells membranes physiology.

Conclusions

Concentrations of the water soluble sodium (Na^+), potassium (K^+), ammonium (NH_4^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) cations accumulated in the extra and intercellular locations of *Xanthoria parietina* and *Phaeophyscia orbicularis* lichenized fungi thallus collected in the north-eastern Romania was investigated for the first time to our knowledge. Indications were obtained for possible cells membranes disruption mechanism in both *Xanthoria parietina* and *Phaeophyscia orbicularis* species as suggested by the electrolyte leakage, especially K. The identified and quantified cations did not exhibit well defined trends, but rather showed fluctuations between the investigated species and within the investigated time period probably due to the greater capacity of the lichenised fungi thallus to intercept elements from atmospheric deposition. Significant correlations were observed for the (K^+,Mg^{2+}) pair in both *Xanthoria parietina* and *Phaeophyscia orbicularis* species, an observation which might suggest either similarities in their locations or possible common physiological role.

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