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# Does air pollution influence the success of species translocation? Trace elements, ultrastructure and photosynthetic performances in transplants of a threatened forest macrolichen

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ABSTRACT

Species translocation can be considered as a primary conservation strategy with reference to *in situ* conservation. In the case of lichens, translocations often risk to fail due stress factors associated with unsuitable receptor sites. Considering the bioecological characteristics of lichens, air pollution is among the most limiting stress factors. In this study, the forest macrolichen *Lobaria pulmonaria* was used as a model to test the hypothesis that the translocation of sensitive lichens is effective only in unpolluted environments.

At purpose, 500 fragments or whole thalli were translocated in selected beech forests of Central Europe (the Western Carpathians, Slovakia) where the species disappeared in the past and in oak forests of Southern Europe (Tuscany, Central Italy) where native populations are present. Prior to the translocation (May 2016) and after one year, morphological and ultrastructural features, trace elements as well as chlorophyll *a* fluorescence emission were analysed. Four years later, the effectiveness of lichen translocation was further evaluated as presence of the transplants and of newly formed individuals.

After one year, the translocation ensured an effective survival of the thalli in remote oak and beech forests characterized by a negligible or low contamination by heavy metals. The transplants were considered successful and developed new lobules and rhizines, attaching by themselves to the bark of the host trees, looking overall healthy, without evident signs of alteration also at ultrastructural level. Moreover, in a few cases newly formed individuals were observed after four years. On the other hand, the results highlighted the link between the unsuccess of the translocation and air pollution in other areas of the Western Carpathians and suggested that current air quality still limits the possibility of recolonization in areas where the model species disappeared.

## 1. Introduction

Plant translocation for conservation purposes may be achieved by increasing the number of populations within their natural range, reintroducing species to their former range or introducing species to sites similar to their original habitat (e.g., Milton et al., 1999; Fenu et al., 2019). However, plant translocations often risk to fail and require specific protocols and techniques to be applied, accounting for multiple factors, such as species and habitat characteristics, selection of appropriate sites, interactions with other organisms, climatic and edaphic parameters (Brooker et al., 2011; Godefroid et al., 2011; IUCN/SSC, 2013). In the case of epiphytic lichens, an effective translocation may

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be achieved by setting up a permanent transplant of symbiotic propagules, thallus fragments or whole thalli to new sites to (re-)colonize and increase the number of conserved populations or to reinforce local populations. However, transferring of a target species from a donor to a receptor site and establishing new self-maintaining populations can be anything but easy for rare and endangered lichens (Scheidegger and Werth, 2009. Therefore, lichen translocation with reference to species conservation and habitat restoration requires specific protocols, especially in disturbed environments (Smith, 2014; Ballesteros et al., 2017). It should take into account the characteristics of the model/target species (such as morphology, reproduction strategy, habitat preference and substrate) and of the receptor site (e.g., air quality, cardinal aspect, sun irradiance, humidity), as well as potential threats given by the presence of ecological competitors (Smith, 2014).

As a model species for threatened macrolichens, *Lobaria pulmonaria*, a tripartite, nitrogen fixing foliose lichen, is considered as an "umbrella" species requiring a suitable habitat to maintain viable populations and that associates with a variety of other rare or endangered lichens (e.g. Gustafsson et al., 1999; Campbell and Fredeen, 2004; Nascimbene et al., 2007, 2010) and of red-listed wood beetles (Nilsson et al., 1995). *Lobaria pulmonaria* has suffered a general decline throughout Europe as a consequence of air pollution and intensive forest management, which can alter or destroy its habitat and whose effects are expected to be further exacerbated by climate change (Nascimbene et al., 2016). The sensitivity of the species to atmospheric pollution by $SO_2$ and heavy metals has been extensively and traditionally considered in lichen monitoring (Hawksworth and Rose, 1970). Currently, the species is red-listed in several European countries, in particular in Central Europe, where it is also protected by law (Paoli et al., 2019). In Mediterranean countries, although widely declined, the species has a lower level of inclusion in conservation policies (Paoli et al., 2019). *Lobaria pulmonaria* is also an indicator of important forest habitats for the conservation of understudied groups (e.g. bryophytes) (Scheidegger and Werth, 2009; Brunialti et al., 2015). Hence, its occurrence and abundance can be used for mapping forest sites worthy of conservation (Campbell and Fredeen, 2004; Nascimbene et al., 2010; Brunialti et al., 2015).

The translocation of symbiotic propagules, thallus fragments or whole thalli can be regarded as a method for *in situ* conservation of threatened lichen populations (Scheidegger et al., 1995). Transplants of *L. pulmonaria* were carried out in different areas, e.g., in the UK (Hawksworth, 1971; Farmer et al., 1992), Switzerland (Scheidegger, 1995; Scheidegger et al., 1995), Sweden (Hazell and Gustafsson, 1999; Gustafsson et al., 2013), Norway (Gauslaa et al., 2006, 2020), Latvia (Mežaka, 2014) and outside Europe, in the USA (Denison, 1988) and Canada (Coxson and Stevenson, 2007a,b; Bidussi et al., 2013). However, there is need of follow-up studies investigating whether transplants are effective to support recolonization in relation to current air pollution where the species has been missing since decades (such as beech forests in Central Europe) or in one of the main habitats in Mediterranean ecosystems, i.e., oak forests. In fact, beech forests are one of the main habitats for *L. pulmonaria* populations in continental Europe (e.g., Nadyeina et al., 2014) and oak-dominated forests (as well as beech forests) are among the main habitats in the Mediterranean area (e.g., Rubio-Salcedo et al., 2015; Benesperi et al., 2018).

At purpose, *L. pulmonaria* samples (fragments or whole thalli) from a logged forest stand in Central Italy were transplanted: i) in selected beech forests in Central Europe (the Western Carpathians, Slovakia) to investigate whether air pollution potentially limits the success of translocation in areas where the model species disappeared during 20th century; ii) in mediterranean mixed oak forests in Southern Europe (Tuscany, Central Italy) that host autochthonous populations to investigate whether the translocation of single individuals to remote areas may ensure their *in situ* conservation. Our working hypothesis is that for sensitive forest macrolichens the translocation is effective for the conservation of viable individuals only in unpolluted environments. Prior to the exposure and one year later, morphological and ultrastructural features, chemical elements content and photosynthetic performances were assessed. Four years later, the effectiveness of lichen translocation was assessed in terms of presence of the transplants and of newly formed individuals.

## 2. Materials and methods

### 2.1. Experimental constraints

In beech forests of Central Europe as well as in Mediterranean ecosystems, a limitation posed to the experimental use of the threatened forest macrolichen *L. pulmonaria* can be ascribed to the fact that this sensitive species is either protected (as in Central Europe) or at least rare. As a consequence, in such environments, also in consideration of the amount of samples needed to get reliable results, the species is generally not used for transplant experiments.

The background for our study was offered in 2016 by a legal logging for timber in a Mediterranean oak wood forest in Tuscany (Central Italy), that heavily damaged also sensitive cryptogams, including a large population of *L. pulmonaria* (Fačkovcová et al., 2019; Paoli et al., 2019). Hundreds of thalli fell to the ground or remained attached to the cut trunks: numerous whole thalli and thallus fragments have been saved by volounteers and researchers and about 500 of them (60% were fertile) were used for this research. The material, mostly harvested from the forest edge (where it was particularly abundant) was preserved in paper bags/boxes and stored in a climatic chamber at 16 ± 2 °C, RH 60 ± 5%, photoperiod of 12 h at about 40 $\mu$mol $m^{-2}$ $s^{-1}$ photons PAR until translocation.

### 2.2. Lobaria pulmonaria translocation

Based on the consideration that due to logging in the native site fallen thalli would die, a priority was set up to ensure their conservation: at purpose, between May and June 2016, 375 thalli/thallus fragments were transplanted to three remote areas in Tuscany that already host native *L. pulmonaria* populations (to test whether the translocation to unpolluted areas may ensure their *in situ* conservation) and 125 thalli in the Western Carpathians, Slovakia (Fig. 1) to test whether air pollution potentially limits the success of translocation in areas where the model species occurred in the past, but is currently extinct (Pišút, 1985, 1999, 2005).

Each thallus was fixed to the bark of potentially suitable trees with a vegetable glue, within 2 m from the ground, the cardinal exposure being selected avoiding the driest side of the bark illuminated by direct sunlight. To allow a better stabilization of larger thalli, a nylon-net was temporary placed along the circumference of the trunk. Within 24 h, the net was removed and the thalli could stand alone. Alternatively, they were stabilized by a stapler. Generally, 3–5 thalli were fixed to each selected tree (Table 1), simulating their natural growth conditions.

### 2.3. Study sites

The main characteristics of the sites are summarized in Table 1. Data on air quality and meteorological parameters are shown in Table 2. The localities in the Western Carpathians were carefully selected with regard to prevent the contact with local populations of *L. pulmonaria* and potential erosion of local genetic diversity due to differences between genetic structure of the regions north and south of the Alps (cf. Widmer et al., 2012; Scheidegger et al., 2012).

### 2.4. Vitality of the transplants and assessment of the translocation

The vitality of the samples was assessed through the analysis of chlorophyll (Chl) *a* fluorescence emission, measuring the potential quantum yield of primary photochemistry ($F_V/F_M$), where

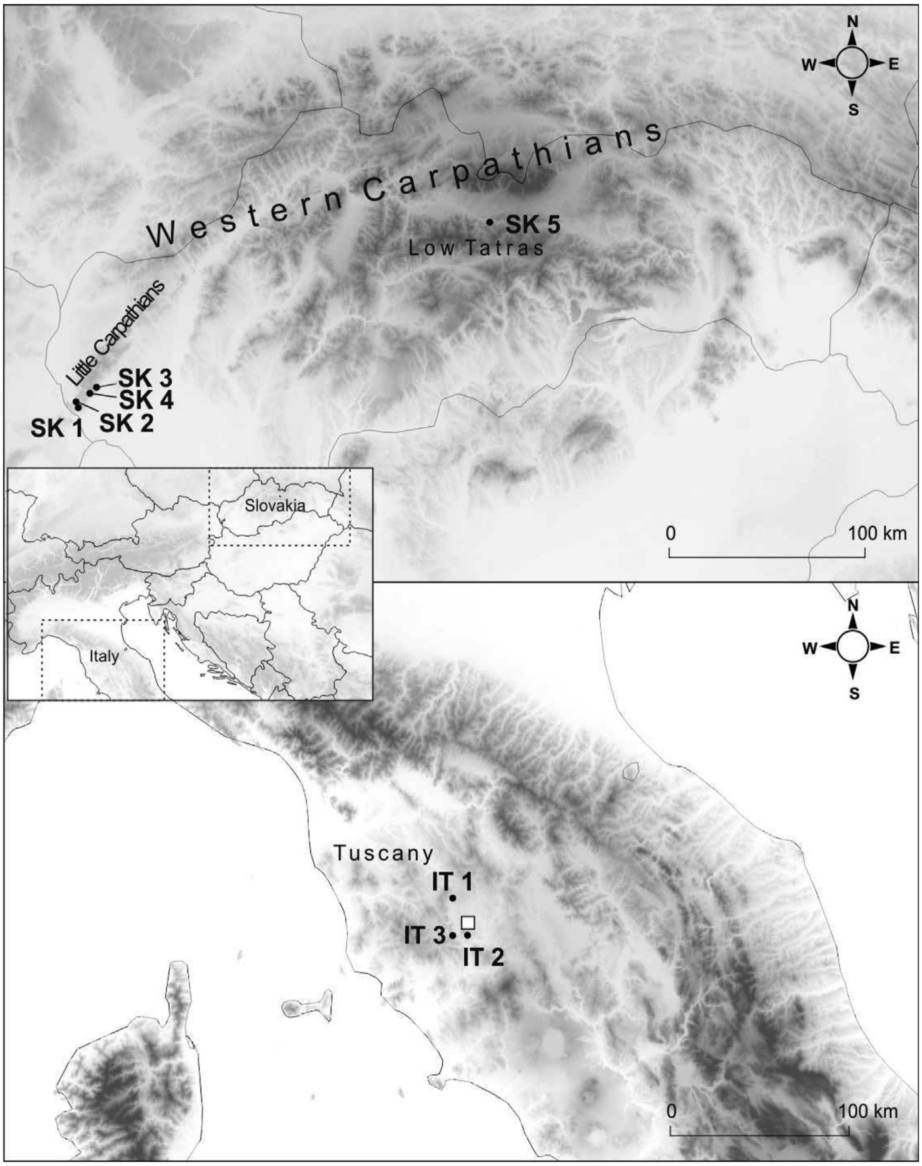

**Fig. 1.** Study sites (SK1–5) in the Western Carpathians (Slovakia) and (IT1–3) in Tuscany (Central Italy), together with the site of origin for the translocation of *Lobaria pulmonaria* (square).

**Table 1**
Characteristics of the sites selected for the translocation. IT = Italy; SK = Slovakia.

| locality (with code) | coordinates | Altitude m a.s.l. | habitat | substrate for translocation | No. trees | No. thalli | presence of native *Lobaria* |
|---|---|---|---|---|---|---|---|
| **IT1** remote area ”Montagnola Senese” oak forest in Tuscany (Italy) | 43°19′17″N 11°15′15″E | 280 | dominated by deciduous oaks | *Quercus cerris* | 40 | 80 | < 100 thalli |
| **IT2** remote area ”Bassa Val di Merse” oak forest in Tuscany (Italy) | 43°08′14″N 11°21′16″E | 290 | dominated by holm oaks | various, mostly *Quercus ilex* | 40 | 175 | > 100 thalli |
| **IT3** remote area ”Tocchi” oak forest in Tuscany (Italy) | 43°08′09″N 11°15′14″E | 410 | dominated by holm oaks | *Quercus ilex* | 40 | 120 | < 20 thalli |
| **SK1** Institute of Botany, SAS, Bratislava, *peri*-urban trees (Slovakia) | 48°10′21″N 17°04′01″E | 192 | peri-urban, scattered trees | *Populus* and *Quercus* | 5 | 25 | extinct during 20th century |
| **SK2** Little Carpathians, ”Lamač”, beech forest near Bratislava (Slovakia) | 48°11′55″N 17°03′07″E | 220 | beech forest, narrow valley | *Fagus sylvatica* | 3 | 25 | extinct during 20th century |
| **SK3** Little Carpathians, ”Svätý Jur” beech forest in the Western Carpathians (Slovakia) | 48°15′52″N 17°11′26″E | 300 | beech forest, hill slope | *Fagus sylvatica* | 5 | 25 | extinct during 20th century |
| **SK4** Little Carpathians, ”Biely kríž” beech forest in the Western Carpathians (Slovakia) | 48°14′13″N 17°08′44″E | 420 | beech forest, hill slope | *Fagus sylvatica* | 3 | 25 | extinct during 20th century |
| **SK5** remote area in the Low Tatras, beech forest in the Western Carpathians (Slovakia) | 49°00′22″N 19°51′04″E | 715 | beech forest, arrow valley | *Fagus sylvatica* | 3 | 25 | extinct during 20th century |

**Table 2**
Air quality and meteorological parameters (as intervals of yearly average temperature, precipitation, air humidity) in the study sites (years 2016–2017). Average atmospheric concentrations (as interval of $NO_x$, $SO_2$ and total suspended particles – TSP) and meteorological data in the Western Carpathians have been extracted from models of the Slovak hydrometeorological institute (2018a,b). Meteorological data from Tuscany have been extracted from the database SIR (Regione Toscana, http://www.sir.toscana.it) and air quality data from Lamma (http://www.lamma.rete.toscana.it).

| Parameters | Site of origin of the samples | Forested remote areas with mostly healthy samples (IT1–3) | Forested remote area with mostly healthy samples (SK5) | Forested area with variable samples (SK4) | Peri-urban and forested areas with mostly damaged samples (SK1–3) |
|---|---|---|---|---|---|
| $NO_x$ (μg/m$^3$) | < 5 | < 5 | < 4 | 7.5–12 | 7.5–12 |
| $SO_2$ (μg/m$^3$) | < 5 | < 5 | < 0.6 | 5–7 | 5–7 |
| TSP (μg/m$^3$) | < 10 | < 10 | 5–10 | 20–30 | 20–30 |
| Precipitation (mm) | 700–800 | 700–800 | 700–800 | 700–800 | 600–700 |
| Temperature (°C) | 14–15 | 14–15 | 4–6 | 7–8 | 7–10 |
| Air humidity (%) | 70–72 | 70–72 | 76–78 | 72–75 | 72–75 |

$F_V = (F_M - F_0)$ is the variable fluorescence, $F_0$ is the calculated basal fluorescence and $F_M$ is the maximum Chl *a* fluorescence. Thalli were kept hydrated (sprayed with mineral water) and dark adapted for at least 15 min (covered with a black velvet cloth) before the measurements. Then, using a Plant Efficiency Analyzer fluorimeter (Handy PEA, Hansatech Ltd, Norfolk, UK), they were lightened for one second with a saturating (up to 3000 μmol of photosynthetically active photons $m^{-2}·s^{-1}$) light pulse and fluorescence emission was recorded for one second. Measurements were carried out before and one year after the translocation (May–June 2017) on thalli of *L. pulmonaria* still occurring in the logged stand and for comparison in a nearby unlogged stand (Paoli et al., 2019), as well as on those transplanted to the receptor sites. If present, also native thalli in the receptor sites were measured. One hundred measurements were taken from each site (1–4 replicates were recorded on each thallus, according to the number of the thalli and their size, in order to minimize error estimates owing to intrathalline variability). Furthermore, a visual assessment of the transplants was conducted using a 10× magnifying lens accounting for: 1) percentage of transplants still attached to the trunks (year 2017); 2) development of new rhizines (root-like structures) that anchor the transplants to the substrate; 3) absence/presence of signs of discoloration (up to bleaching) and necrotic parts. Thalli were attributed to the categories of 'damaged' (presence of diffused discoloration, necroses or holes between the typical reticulated ridges with marked depressions that characterize the thallus), 'variable' (with occasional discolorations or necroses), or 'healthy' (no visual injury). Lastly, the presence of the transplants and of newly formed individuals were assessed four years later (up to February 2020).

### 2.5. Content of trace elements

For each experimental site, three independent replicates were prepared from randomly selected thallus fragments. Since the analytical procedure is destructive for the samples, only a small amount of *L. pulmonaria* was harvested (delicately with plastic tweezers). Air dried samples were milled under liquid nitrogen using ceramic mortar and pestle. For each sample, about 200 mg of lichen material cleaned from impurities (e.g., bark pieces, other lichens and mosses) were mineralized, using a mixture of 3 mL of 70% $HNO_3$, 0.2 mL of 60% HF and 0.5 mL of 30% $H_2O_2$ in a microwave digestion system (Milestone Ethos 900). Trace elements (Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, S and Zn) were quantified by inductively coupled plasma mass spectrometry (ICP-MS, Perkin- Elmer Sciex 6100) and the concentrations were expressed on dry weight basis (N = 3 for each site). The analytical quality was checked with the Standard Reference Material IAEA-336 'lichen' (recoveries in the range 92–117%), and the precision of analysis was estimated by the variation coefficient of five replicates (within 11% for all elements).

### 2.6. Ultrastructural characteristics

Lichen samples were kept on filter paper wet with deionized water for 24 h to ensure complete hydration of the thalli. Specimens were fixed with 2.5% glutaraldehyde overnight at 5 °C, post-fixed with 1% osmium tetroxide for 1.5 h at room temperature, dehydrated with ethanol to propylene oxide and embedded in Spurr's epoxy medium. Ultrathin sections (50 nm) were collected on copper grids and stained with uranyl acetate and lead citrate. A FEI EM 208S TEM (FEI, Eindhoven, The Netherlands), with an accelerating voltage of 80 kV, was used for observations. The image analysis of the cellular ultrastructural characters within a median section of algal and fungal cells was performed on electron micrographs by the software program AnalySIS (FEI, Eindhoven, NL); cytoplasmic and chloroplast droplets, altered organelles and other ultrastructures were examined. Ten ultrathin sections were examined per each experimental condition, avoiding the selection of those parts of the thallus manifestly necrotic.

### 2.7. Data interpretation and statistics

A one-way ANOVA was run to investigate differences between the maximal quantum yield of primary photochemistry in different sites ($p < 0.05$) and the Tukey test was used for post-hoc comparisons (N = 100 for each site). The average ratio between the concentration of each element after and prior to the exposure (Exposed to Control – EC ratio) was used to interpret accumulation data according to Frati et al. (2005). For a synthetic assessment of heavy metal depositions, a Pollution Load Index (PLI) inspired to Tomlinson et al. (1980) was adapted to lichens and calculated according to the formula $PLI = (PI_1 \times PI_2 \times PI_3 \times \ldots \times PI_n)^{1/n}$, where n (11) is the number of chemical elements (Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, S and Zn) and PI represents the Exposed to Control (EC) ratio of each element.

## 3. Results

### 3.1. The translocation experiment

One year after the translocation (2017), 70–90% of *L. pulmonaria* thalli were still present on the trees in Tuscany (Central Italy) and 65–85% in the Western Carpathians (Slovakia) (Table 3). Taking into consideration the development of new lobules and rhizines (to anchor the thallus to the substrate) and the appearance of the thalli, the translocation was considered successful only in remote protected areas in Central Italy (IT1–3, Tuscany) and remote sites in Slovakia (SK5, Low Tatras Mts., also known as Nízke Tatry Mts.). The thalli developed new rhizines and attached by themselves to the bark of the host trees, looking overall healthy, with a dominating green/brown colour and without evident symptoms of alteration.

On the other hand, the translocation was not considered successful in other localities of the Western Carpathians (SK1–4, the Little

**Table 3**
Potential quantum yield of primary photochemistry ($F_V/F_M$), as indicator of the vitality of the samples. Average ± standard deviation and 95% confidence interval. IT = Italy; SK = Slovakia. Values followed by a different letter are statistically different (Tukey test, $p < 0.05$).

| Samples measured | Locality | $F_V/F_M$ | | Appearance of the thalli | New lobules and rhizines | Thalli still attached | | New thalli |
|---|---|---|---|---|---|---|---|---|
| | | Mean ± SD | 95% CI | | | | | |
| *Prior to the translocation (in 2016) | Site of origin: logged area | 0.727 ± 0.042a | 0.705 – 0.750 | healthy | | | | |
| | | 2017 | 2017 | 2017 | 2017 | 2017 | 2019–2020 | 2020 |
| Transplants | IT1 forested remote area | 0.705 ± 0.034ab | 0.687–0.723 | healthy | yes | 87% | 70% | yes |
| Transplants | IT2 forested remote area | 0.713 ± 0.031ab | 0.697–0.730 | healthy | yes | 70% | 50% | yes |
| Transplants | IT3 forested remote area | 0.711 ± 0.037ab | 0.691–0.731 | healthy | yes | 90% | 65% | no |
| Transplants | SK1 peri-urban area | 0.691 ± 0.041b | 0.669–0.713 | damaged | no | 65% | 15% | no |
| Transplants | SK2 forested area | 0.668 ± 0.115b | 0.600–0.735 | damaged | no | 60% | 10% | no |
| Transplants | SK3 forested area | 0.537 ± 0.169c | 0.446–0.628 | damaged | no | 75% | 15% | no |
| Transplants | SK4 forested area | 0.721 ± 0.059a | 0.689–0.753 | variable | no | 80% | 10% | no |
| Transplants | SK5 forested remote area | 0.734 ± 0.036a | 0.714–0.753 | healthy | yes | 85% | 60% | no |
| Native *L. pulmonaria* | IT1–3 forested remote areas | 0.718 ± 0.038ab | 0.704–0.742 | healthy | | | | |
| *Native *L. pulmonaria* | Site of origin: logged area | 0.539 ± 0.117c | 0.476–0.601 | variable | | | | |
| *Native *L. pulmonaria* | Site of origin: adjacent unlogged area | 0.743 ± 0.031a | 0.726–0.760 | healthy | | | | |
| | Anova results | F = 61.99 p = 0.000 | | | | | | |

Paoli et al. (2019), for comparison.

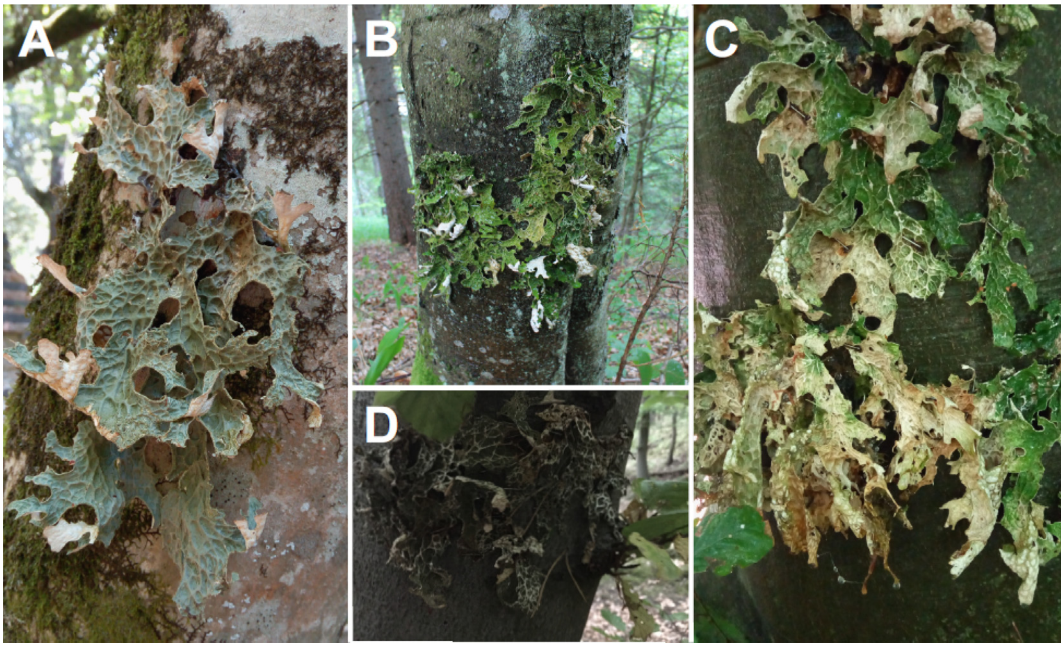

**Fig. 2.** *Lobaria pulmonaria* transplants: A – mixed oak forests, remote site in Tuscany (IT2), thalli with no visual injury; B – remote site in the Western Carpathians (beech forest in the Low Tatras, SK5), thalli with no visual injury; C – beech forest in the Little Carpathians (SK4), variable thalli with partial discolorations; D – beech forest near Bratislava, border of the Western Carpathians (SK2), damaged thalli with diffused discolorations.

Carpathians and surroundings of Bratislava): the thalli did not attach by themselves to the bark of host trees, and showed variable (in SK4) up to evident symptoms of damage (in SK1–3), consisting in discoloration and curling of the surfaces, presence of necrotic parts and holes between bridges (Fig. 2). Several thalli appeared easily detachable from the host trees. A last check completed in February 2020 revealed that only 10–15% of the thalli remained attached to the trees in SK1–4 and about 60% in the Low Tatras (SK5). On the whole, no newly formed thallus was found in the Western Carpathians (Slovakia). A share of 50–70% of the samples translocated in oak forests in Tuscany (Central Italy) was still present (February 2020) and in two (IT1, IT2) out of three areas an attempt of colonization by newly formed individuals was observed, as presence of few scattered small thalli (< 5 at both sites).

### 3.2. Vitality of the thalli

The translocation to remote sites ensured an effective survival of the thalli: those individuals transplanted within remote areas in Tuscany (IT1–3) and the Western Carpahians (SK5) showed photosynthetic performances ($F_V/F_M$) comparable to those in the native site (Table 3).

Samples in SK4 (beech forest) showed high values of $F_V/F_M$ but did not manage to attach by themselves to the substrate, while samples in SK1–SK3, showed lower performances (although they were still photosynthetically active) and signs of visual alteration, consisting in decolorations and curling of the surfaces and presence of necrotic parts.

The question whether the photosynthetic performance of the thalli relocated to the remote areas in Tuscany was similar to that of native *L.*

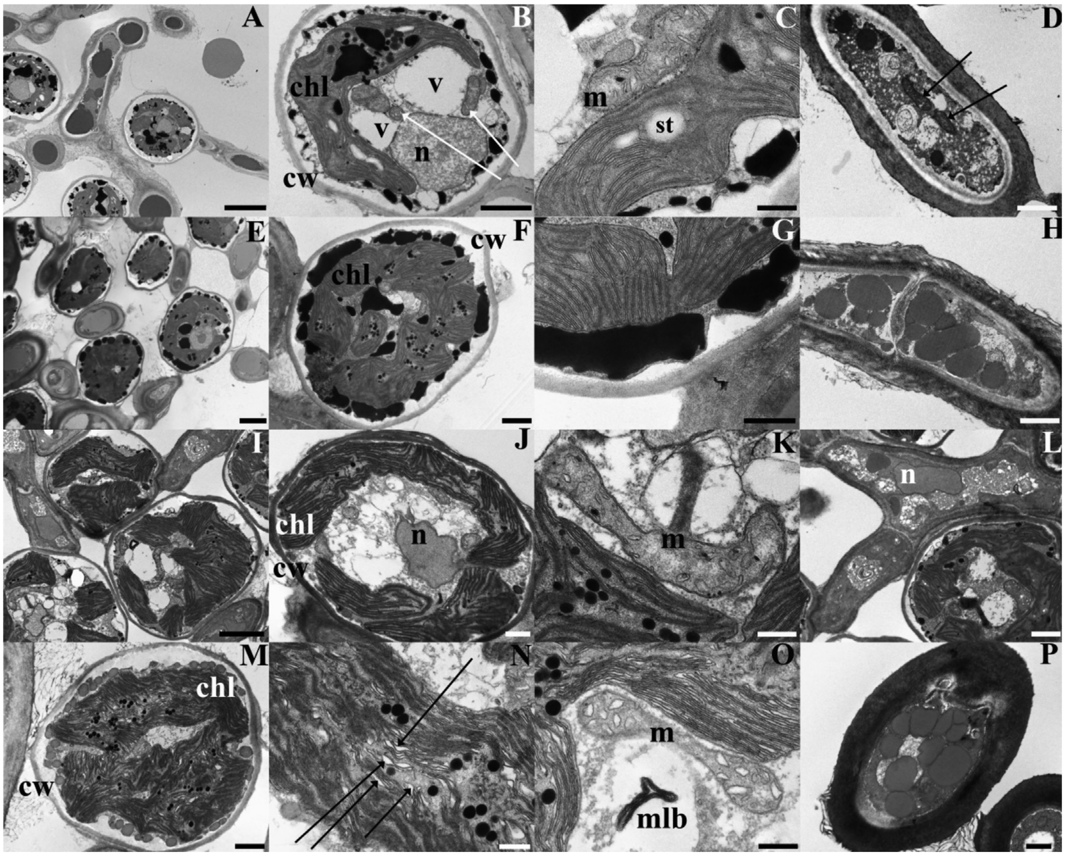

**Fig. 3.** TEM micrographs of *Lobaria pulmonaria* in a representative set of samples present in oak (Tuscany, Central Italy) and beech forests (the Western Carpathians, Slovakia) [substrate, location, condition, code]. Description of the sites in Table 1. [oak forest, remote site in Tuscany, 'healthy', IT2] (A–D). (A) The micrograph shows a magnified image of the thallus, just below the upper cortex, with algal and fungal cells. (B) A single algal cell, surrounded by the cell wall (cw) shows a large chloroplast (chl), nucleus (n), vacuoles (v), mitochondria (arrows), as well as some electron dense deposits in the cytosol. (C) A mitochondrion (m) next to a chloroplast showing thylakoids with a straight course and starch grains (st). (D) A fungal cell, surrounded by a thick cell wall, showing a cytoplasm with vacuoles, electron dense droplets and mitochondria (arrows). [beech forest, remote site in the Low Tatras (Slovakia), 'healthy', SK5] (E–H). (E) Low-magnified micrograph of the medulla and the algal layer, where algal and fungal cells are evident. (F) A single algal cell, surrounded by the cell wall, shows a large chloroplast and electron-dense deposits in the cytosol. (G) Inside the chloroplast, thylakoids are arranged with a straight course. The outer protoplasm is occupied by large electron-dense deposits. (H) Fungal cell, delimited by a thick cell wall, contain large electron-dense vacuoles. [beech forest, Little Carpathians, 'variable', SK4] (I–L). (I) Algal and fungal cells from the upper part of the thallus. (J) A single algal cell, delimited by a cell wall (cw), with a large chloroplast (chl) and a nucleus (n). Some of the thylakoids are arranged with a wavy course. (K) A mitochondrion next to a chloroplast with thylakoids and electron-dense plastoglobules. (L) A fungal cell next to an algal cell: the former, surrounded by a thick cell wall, shows a nucleus (n), vesicles and vacuoles in the cytoplasm. [beech forest, surroundings of Bratislava, 'damaged', SK2] (M–P). (M) A single algal cell surrounded by the cell wall (cw) shows a large chloroplast (chl) with scattered electron-dense plastoglobules. Thylakoids are arranged with a wavy course. (N) Details of the thylakoid system: the wavy course and dilated thylakoids (arrows) are well visible. (O) A mitochondrion (m) with well developed cristae and a multilamellar body (mlb). (P) A fungal cell, delimited by a thick cell wall, with large dense vacuoles. Scale bars: 4 μ (A), 2 μ (E, I), 1 μ (B, D, F, H, L, M), 500 nm (G, J, P), 300 nm (C, K, N, O).

*pulmonaria* in the receptor sites was addressed investigating chlorophyll *a* fluorescence emission also in autochthonous samples available within such areas. Overall, $F_V/F_M$ values were within the range 0.704–0.722 (95% confidence interval) for relocated thalli and 0.704–0.742 for native thalli ($p > 0.05$), thus confirming that the two groups were characterized by equal photobiont vitality. Due to the lack of native thalli, such comparison was not possible in the Western Carpathians.

### 3.3. Thallus ultrastructure

TEM micrographs of *L. pulmonaria* are shown in Fig. 3: for practical reasons, a selection of samples exposed in the receptor sites is reported. In particular (according to the description of Fig. 2): A) mixed oak forests, remote areas in Tuscany (thalli with no visual injury – IT2; it also represents the condition of the thalli prior to the translocation); beech forests in the Western Carpathians, namely B) remote areas of the Low Tatras (thalli with no visual injury – SK5); C) the Little Carpathians (thalli with occasional discolorations – SK4); D) the surroundings of Bratislava, at the border of the Western Carpathians (thalli with diffused discolorations – SK2).

Most of TEM observations focused on the photobiont layer located between the mycobiont's medulla and upper cortex (Fig. 3A, E). Samples before translocation or exposed to remote areas in Tuscany (IT2) and in Western Carpathians (SK5) showed similar features. *Lobaria pulmonaria* from remote areas had the typical structural organization of a thallus with upper and lower cortex made up of compacted hyphae of the mycobiont, and a looser medulla between them. Algal cells, surrounded by the cell wall, contained a large chloroplast with a well-developed thylakoid system, starch grains and plastoglobules (B, F). Thylakoid membranes were arranged with regular, straight and parallel course (C, G). The cytoplasm hosted large amounts of electron-dense osmiophilic deposits; nucleus, mitochondria and vacuoles featured their typical appearance (B, F). Fungal cells, delimited by a thick cell wall, had typical vacuoles, mitochondria, lipid droplets and other ultrastructures without alterations (D, H). In general, the ultrastructure of the samples exposed in the Little Carpathians (thalli with occasional discolorations – SK4) and of those exposed in the surroundings of Bratislava (thalli with diffused discolorations – SK2) was comparable with some alterations. In both cases, the thylakoids, even though well developed, had a wavy course (J, N) and dilated thylakoids were visible (N). The cytoplasm of the algal cells still contained lipid droplets (M), but lacked the large amount of electron dense deposits found in the thalli without discolorations (IT2 and SK5). Mitochondria of the algal cells appeared regular with well-developed cristae (K, O), while multilamellar bodies were well evident (O). Fungal cells of both SK4 and SK2 did not exhibit remarkable differences from those of healthy thalli IT2 and SK5 (L, P), thus suggesting that thephotobiont cells were, on the whole, more affected than the mycobiont ones.

### *3.4. Content of trace elements*

In order to assess whether air pollution might have influenced the (un-)success of the translocation in the Western Carpathians, the study sites were clustered according to the characteristics of the transplants based on previously reported results (macroscopic characters, photobiont vitality and ultrastructure): 1) sites where most of the thalli are without signs of morphological and ultrastructural alteration, with high photosynthetic performances and that spontaneously attached to the substrate by producing new rhizines, i.e., remote sites in Slovakia (SK5, the Low Tatras); 2) sites with variable samples – thalli with occasional signs of discolorations, high photosynthetic performances, but that failed to attach by themselves, i.e., the Little Carpathians (SK4); 3) sites with mostly damaged samples – thalli with evident symptoms of alteration, variable photobiont vitality and that did not attach by themselves to the bark of host trees, i.e., the Little Carpathians (SK3 and SK2) and the surroundings of Bratislava (SK1). The accumulation of heavy metals was also considered in comparison with that of *L. pulmonaria* successfully translocated to oak forests in remote areas of Tuscany (IT1–3). Accordingly, element concentrations in *L. pulmonaria* prior to the translocation, Exposed to Control (EC) ratios and the overall Pollution Load Index (PLI) after one year translocation were summarized in Table 4.

Element concentrations in *L. pulmonaria* prior to the exposure correspond to the background of unpolluted environments in Italy (Bargagli and Nimis, 2002; Cecconi et al., 2019). Such values also reflect the condition of native populations of *L. pulmonaria* growing in IT1–3. According to Exposed to Control (EC) ratios (*sensu* Frati et al., 2005), the samples translocated to oak forests in remote areas of Tuscany (IT1–3) were essentially unaffected and did not accumulate heavy metals or metalloids (EC ≤ 1.25 for all elements). In samples translocated to the Western Carpathians, the content of the following elements reflected an accumulation: 10 out of 11 (As, Cr, Cu, Mn, Ni, Pb, S, Sb, Zn, but Al) in sites with mostly damaged thalli (SK1–3); 7 (As, Pb, Mn, Ni, S, Sb, Zn) in sites with mostly variable thalli (SK4, Biely kríž) and 5 (Cr, Cu, Ni, Sb Zn) in remote sites with mostly healthy thalli (SK5, the Low Tatras).

The Pollution Load Index (PLI) provides a simple tool for assessing the overall level of pollution caused by the investigated elements. Hence, according to PLI (Table 4), heavy metals accumulation in translocated lichens follows this order: sites with mostly damaged thalli (SK1–3) > sites with variable thalli (SK4) > sites with mostly healthy samples (SK5) > remote areas in Tuscany (IT1–3). Such order also reflects average atmospheric pollution levels modeled from automatic monitoring stations (Table 2).

## 4. Discussion

### *4.1. Thallus ultrastructure and content of trace elements*

The discussion follows a bottom up scheme from ultrastructural to macroscopic level. TEM micrographs suggested that in the contaminated areas the photobiont was more affected than the mycobiont in this model species. On the whole, the thalli translocated to remote oak forests in Tuscany and remote beech forests in the Western Carpathians showed a regular ultrastructure; a common feature is the presence of large amounts of electron-dense deposits in the photobiont (Fig. 3).

Those electron-dense cytoplasmic deposits could be referred as osmiophilic lipids, already reported as a response to environmental conditions (Giełwanowska and Olech, 2012). Bychek-Guschina (2002) regarded lipid metabolism in lichens as a strategy for adaptation; accordingly, large amounts of lipid droplets in the peripheral parts of

**Table 4**
Concentrations of selected elements in *Lobaria pulmonaria* prior to the translocation (average ± standard deviation) and Exposed to Control (EC) ratios with Pollution Load Index (PLI) after one year from translocation. EC ratios according to Frati et al. (2005): normal 0.75 < EC ≤ 1.25, accumulation EC > 1.25 (light grey), severe accumulation EC > 1.75 (dark grey).

| **Parameters** | **Samples prior to the translocation (µg/g)** | **Transplants** – EC ratios (average) after one year from translocation (2017) | | | |
|---|---|---|---|---|---|
| | | Forested remote areas with mostly healthy samples (IT1–3) | Forested remote area with mostly healthy samples (SK5) | Forested area with variable samples (SK4) | Peri-urban and forested areas with mostly damaged samples (SK1–3) |
| Sb | 0.08 ± 0.01 | 1.14 | 1.86 | 3.57 | 5.58 |
| Zn | 19 ± 1 | 1.06 | 4.37 | 3.35 | 5.56 |
| Pb | 1.3 ± 0.1 | 1.00 | 1.13 | 3.29 | 2.54 |
| As | 0.13 ± 0.04 | 1.10 | 0.95 | 2.27 | 2.36 |
| Ni | 1.4 ± 0.3 | 1.11 | 1.96 | 1.38 | 1.76 |
| Mn | 53 ± 2 | 1.04 | 0.77 | 3.31 | 1.60 |
| Cu | 6.0 ± 1.1 | 1.19 | 1.30 | 1.25 | 1.56 |
| S | 1042 ± 46 | 1.04 | 1.23 | 1.45 | 1.41 |
| Cr | 1.7 ± 0.2 | 1.12 | 1.31 | 1.10 | 1.35 |
| Cd | 0.66 ± 0.22 | 1.09 | 0.90 | 0.76 | 1.43 |
| Al | 536 ± 120 | 1.12 | 0.86 | 1.11 | 1.10 |
| PLI | - | **1.09** | **1.34** | **1.80** | **2.04** |

the cell suggest a good adaptation to the environmental conditions. Furthermore, lipid contents were reported to show seasonal variations, with few or no lipid droplets in winter and large amounts of lipids observed in summer (Fiechter and Honegger, 1988; Giełwanowska and Olech, 2012).

In comparison, thalli from the sites enriched with heavy metals of the Western Carpathians showed altered chloroplasts with thylakoids arranged in a wavy course and local dilation, in addition to multilamellar body occurrence and a lower amount of electron dense deposits in the cytoplasm of the photobiont.

Similar observations were reported in previous studies carried out in the Western Carpathians in sites contaminated by heavy metals, where altered vitality and ultrastructural changes were observed in transplanted lichens (Paoli et al., 2015, 2016). Both in green algal lichens (*Evernia prunastri* and *Xanthoria parietina*) and in cyanolichens (*Peltigera praetextata*), ultrastructural alterations involved thylakoids degeneration, swelling of cellular components, plasmolysis and an increase of lipid droplets, which on the whole gave the photobionts an aged appearance (Paoli et al., 2015, 2016). A similar pattern was also reported and discussed for the lichen *Pseudevernia furfuracea* exposed to atmospheric pollution by heavy metals both in the field in Italy and after incubation with the same heavy metals in laboratory trials (Sorbo et al., 2011).

In our work, the content of trace elements in lichen thalli was used as a proxy of air pollution, aware that also phytotoxic air pollutants (e.g., $SO_2$, $NO_x$) not directly measured in the thalli might have influenced the responses of the transplants. According to PLI lower heavy metals (and metalloids) accumulation was found in remote areas characterized by higher photobiont vitality and vice versa a higher accumulation occurred in those sites where the thalli appeared damaged after one year of exposure. Noteworthy, a severe accumulation (*sensu* Frati et al. 2005, EC $> 1.75$) was calculated for some of the investigated elements (Table 4). In the case of Zn, possible explications are the low backgrounds (around 19 μg/g) at the site of origin. As a comparison, the lichen *E. prunastri* exposed from 30 up to 180 days in the Little Carpathians showed average Zn levels of 23 μg/g and peaks of 42 ± 4 μg/g (Paoli et al., 2015, 2017). In the case of Mn in SK4 (EC = 3.31), natural geological characteristics of the area and depositions from quarrying activities in neighbouring sites likely influenced the elemental content (also in the case of As), as evinced from previous studies in the Carpathians (Paoli et al., 2014, 2017). Furthermore, at concentrations like those occurring in SK4 (about 175 μg/g), Mn can contribute to reduce the vitality of the photobionts in Lobarion species (Hauck et al., 2006). In the case of Sb (moreover in SK1–4, with EC $> 3.00$), geochemistry and uptake from traffic emissions could have contributed to depositions, given the limited distance from the urban district of Bratislava (Guttová et al., 2011) and the ability of lichen transplants to trap this element even in low polluted environments (Loppi and Paoli, 2015). The levels of Pb, despite reflecting a severe accumulation, on the whole indicate a condition of low pollution from this element (e.g., Bargagli and Nimis, 2002; Cecconi et al., 2019). On the other hand, the accumulation of S reported in SK1–4 could well reflect higher atmospheric levels of phytotoxic S-containing compounds, to which the model species is traditionally considered very sensitive (Hawksworth and Rose, 1970).

### 4.2. Vitality of the thalli and (un-)success of translocations

Prior to the translocation, samples were homogeneous in terms of photobiont vitality, as indicated by the parameter $F_V/F_M$ (Paoli et al., 2019). The translocation to remote and protected areas ensured (after one year) an effective survival of the thalli chiefly in oak forests in Central Italy (IT1–IT3), where autochthonous *L. pulmonaria* was already present or alternatively, in beech forests of the Western Carpathians (SK5), where the environment was more humid and colonized by other sensitive foliose and fruticose species. In such environments, the thalli were characterized by a negligible (IT1–IT3) or low (SK5) accumulation of heavy metals, overall suggesting negligible or low environmental pollution of the sites (i.e., high environmental quality). The transplants were considered successful and developed new lobules and rhizines and attached by themselves to the bark of the host trees, looking overall healthy, with a dominating green/brown colour and without evident symptoms of alteration. Moreover, in a few cases (IT1, IT2), newly formed individuals were observed after four years.

On the opposite, those thalli transplanted to poorly colonized smooth barks at the border of the Western Carpathians (SK1–SK3), featured higher heavy metals accumulation and despite being still photosynthetically active, they did not attach to the substrate by themselves and showed signs of visual alteration, consisting in bleaching and curling of the surfaces as well as presence of necrotic parts. Most of them (85%) detached from the barks in the period 2016–2020, leading to an unsuccessful translocation. In fact, concerning the potential reintroduction of the species to its former range in Central Europe, for a successful translocation, receptor sites should provide optimal habitats, hosting suitable niches in terms of aspect, pH of the substrate, sun irradiance, humidity as well as air quality (e.g., low concentration of heavy metals, $NO_x$, $SO_2$ for sensitive species as *L. pulmonaria*); otherwise, sooner or later the thalli will die (Smith, 2014). In this sense, *L. pulmonaria* was widely distributed in the past all over the Western Carpathians until the beginning of the twentieth century (33 mountain areas in the actual territory of Slovakia), from oak woods in hilly elevations up to spruce forests in the mountains, with the highest colonization in beech forests (Pišút, 1985, 1999, 2005). Several findings of healthy thalli were reported even in the surroundings of the capital, Bratislava. During the twentieth century, a dramatic decline occurred (Pišút, 1985, 1999, 2005) due to intensive logging (mostly old beech trees) and air pollution, especially caused by S-containing compounds and heavy metals (e.g. Guttová et al., 2011; Lackovičová et al., 2013). After 1970, due to significant habitats deterioration, only 9 mountain areas remained colonized (Jasičová and Zahradníková, 1976; Pišút, 1985, 2005). Nowadays, the old-growth forests with optimal habitats for *L. pulmonaria* are scattered and rare and the species is confined to very few remote mountain ranges. In general, all over the Western Carpathians low levels of air pollution (by $NO_x$, $SO_2$ and total suspended particles) are mirrored by a higher share of fruticose and pollution sensitive lichens in forested areas, while higher environmental levels of pollution correspond to their decrease (Paoli et al., 2014; Guttová et al., 2017). It is therefore argued that current air quality as well as habitat characteristics (including microclimate) still play a main role for living and survival of sensitive species such as *L. pulmonaria*, as suggested by the results of our experiment. Similarly, Farmer et al. (1992) linked the damage (chlorosis and necrosis) occurred to *Lobaria* transplants (lasting about 900 days) to air pollution by acid rain and the consequent decline of sensitive lichens in the UK. Gauslaa et al. (2020) highlighted the influence of canopy throughfall in natural (*Picea*) forest stands on the elemental content of *L. pulmonaria* transplanted for one year and pointed out that in unpolluted sites the uptake was positively correlated with habitat characteristics such as soil and bark pH, especially for macronutrients (Ca, Mg, K, P). Bidussi et al. (2013) found that the growth of *L. pulmonaria* transplants along an altitudinal gradient was substantially stimulated in situations corresponding to higher pH and avoiding an excess of direct sunlight. Forest lichens, as the model species *L. pulmonaria*, are sensitive to sudden increases of solar radiation and dry conditions, which, if in excess of their ecological range, may negatively affect their photosynthetic activity and overall vitality (Gauslaa and Solhaug, 1999).

Hence, "good" photosynthetic parameters (as suggested by the measured values of $F_V/F_M$) are only a premise for a possible survival and growth of the samples, since in several cases it can be sufficient a small share of active algal cells to detect chlorophyll *a* fluorescence emission also in a chlorotic thallus of a contaminated environment. Therefore, when designing a successful translocation, we should take into account, beyond potential physiological and chemical aspects linked to quality of the receptor site and habitat suitability, also the morphological characteristics of the samples, e.g. size (small fragments may have higher chance to remain attached than larger thalli), translocation methods and substrates (e.g., Hawksworth, 1971; Hallingbäck, 1990; Bidussi et al., 2013; Mežaka, 2014; Smith, 2014). Furthermore, reproductive strategies of the target species and potential threats to the establishment should be considered: a successful translocation may create in turn niches for other organisms (including small animals), whose absence limits or even hampers the diffusion of asexual propagules and the spreading of newly formed individuals (Smith, 2014).

Scheidegger (1995) demonstrated how vegetative diaspores (isidioid soredia) of *L. pulmonaria* developed anchoring hyphae within 2–4 months from the exposure into previously uncolonised trees. Despite most of the diaspores (about 60%) were lost within the first two months, new thalli with 0.5 mm lobes were formed after 15 months (Scheidegger, 1995). Hallingbäck (1990) succeeded in an experiment of colonizing the bark of *Acer platanoides* with thallus fragments (size of about 1 mm) of *L. pulmonaria*: new thalli were established after 18 months and such colony was still present and spread to nearby trees after 18 years (Smith, 2014). On the other hand, *L. pulmonaria* thalli exposed to test their tolerance to current microclimate in a locality of the UK where it was likely common in the past, were detached after 19 months (Hawksworth, 1971). Noteworthy, another experiment with 1120 transplants of *L. pulmonaria* set up on 280 aspens in Sweden resulted in the survival of 23% of the thalli after 14 years (Gustafsson et al., 2013). As a rule of thumbs, the translocation of samples may ensure not only the conservation of single individuals and reinforce local populations, as in our case (IT1–3), but even enhance the colonization of potential suitable habitats, especially when natural dispersion is unsuccessful (Scheidegger, 1995). However, translocations should not prescind from considerations on a possible genetic contamination over wide geographical areas (Widmer et al., 2012). *In situ* conservation is widely considered as a primary conservation strategy, but it does not protect the habitat of origin. Therefore, an overall protection system should pay attention to the safeguard of the whole ecosystems and the scientific research underlines an international responsibility for the protection of habitats hosting sensitive species (e.g., Eaton and Ellis, 2014) and supports the inclusion of *Lobaria* species within European conservation policies, such as the Habitat Directive.

## 5. Conclusions

The hypothesis that for sensitive forest macrolichens the translocation is effective for the conservation of single healthy individuals only in unpolluted environments was verified. The translocation to remote unpolluted areas ensured an effective survival of the thalli after one year only where native *L. pulmonaria* was already present, or in remote areas suitable for a well-developed lichen colonization. In such situations, the transplants were successful and did not show evident morphological and ultrastructural alterations. Moreover, they developed new lobules and rhizines and in a few cases, newly formed individuals were observed after four years. Lichen translocation was not effective to support recolonization where the model species disappeared during 20th century (most of the sites in the Western Carpathians) in presence of current air pollution, as reflected by heavy metals accumulated and the damage endured by the transplants. It should be clear that the translocation of samples might ensure only the conservation of single individuals and/or enhance the colonization of potentially suitable habitats, but requires careful evaluation of niche demands of the target species.

## CRediT authorship contribution statement

**Luca Paoli:** Conceptualization, Methodology, Investigation, Validation, Writing - original draft, Writing - review & editing. **Anna Guttová:** Conceptualization, Methodology, Investigation, Validation, Writing - original draft, Writing - review & editing. **Sergio Sorbo:** Methodology, Investigation, Validation, Writing - original draft. **Anna Lackovičová:** Methodology, Investigation. **Sonia Ravera:** Investigation, Writing - review & editing. **Sara Landi:** Methodology, Investigation, Validation. **Marco Landi:** Methodology, Investigation, Validation. **Adriana Basile:** Methodology, Investigation, Validation. **Luigi Sanità di Toppi:** Methodology, Validation, Writing - review & editing. **Andrea Vannini:** Methodology, Investigation. **Stefano Loppi:** Conceptualization, Methodology, Validation, Writing - original draft. **Zuzana Fačkovcová:** Conceptualization, Methodology, Investigation, Validation, Writing - original draft, Writing - review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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