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Symbiotic interactions in the lichen *Ramalina farinacea* dramatically modify NO biosynthetic source in *Trebouxia* microalgae

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ABSTRACT

NO is a multifaceted molecule, key in functions such as abiotic stress tolerance and symbioses establishment and permanence. Lichens are complex symbiotic associations of microalgae, fungi and prokaryotes that release NO under stress conditions such as dessication-rehydration cycles and the presence of xenobiotics. NO synthase (NOS) oxidises L-arginine to produce NO in animals and some aquatic microalgae, while nitrate reductase (NR) reduces nitrate to NO in plants and fungi. Inhibition studies suggest that both activities might be present in thalli. Due to its multipartner compotition, our hypothesis is that *Ramalina farinacea* biosynthesises NO through both oxidative (NOS) and reductive (NR) enzymatic pathways. NR activity was quantified with a method optimised for lichens using NADH or/and NADPH, and NOS with a commercial kit in *R. farinacea* thalli and cultures of the isolated main symbionts: *R. farinacea* mycobiont, and *Trebouxia jamesii* and *Trebouxia lynnae* phycobionts. Inhibition studies in vitro were performed with L-NAME and tungstate. Immunodetection was carried out with specific polyclonal antibodies (anti-plant NADH-NR and anti-iNOS animal isoform). NADH-NR specific activity of *R. farinacea* is an order of magnitude higher than *Arabidopsis thaliana*'s and in the range of the chlorophyte *Ulva intestinalis*. *R. farinacea* mycobiont possesses a canonical plant-like Moco-NR, while *Trebouxia* phycobionts' NR activity presents interesting peculiarities. NOS has not been immunodetected and NOS-like activity is inhibited by L-NAME only partially in *T. jamesii.* Despite NOS-like activity is very high in the isolated microalgae and fungus, it is strongly depressed in the holobiont. In summary, NR activity seems to be the main source of NO biosynthesis for the holobiont *R. farinacea* but it presents intriguing features that deserve further study.

1. Introduction

Species are disappearing at a dizzying rate due to human activity, pollution and climate change. However, lichens can survive in inhospitable areas or in unfavourable conditions, i.e. deserts and tundra, and still metabolize and photosynthesize. Consequently, these impressive organisms must possess adaptations such as powerful antioxidant mechanisms to photosynthesize and metabolize even under extreme conditions. Given that our agriculture strongly relies in rhizobial or mycorrhizal symbioses, and corals are determinant for oceanic equilibrium [1,2], beside intrinsic scientific interest, knowledge of lichen intimate mechanisms can also be useful regarding biotechnological applications, alimentary and sanitary resources.

Lichens are composite symbiotic organisms consisting of fungi, a photosynthetic part, which can be either green algae or cyanobacteria, and bacterial communities [3–5]. They are poikilohydric and cannot regulate their water content so that are subject to continuous dehydration/rehydration cycles. Lichens even withstand total desiccation; they are adapted to anhydrobiosis and can resume their function quickly after rehydration [6]. This trait is key to survival in extreme environments since they can remain metabolically inactive for long periods and restore cellular processes in the presence of water [7]. During rehydration, lichens release massive amounts of ROS, that are further increased during exposure to pollutants or solar UV, especially in photosynthetic partners as they are produced in the electron chains of photosynthesis besides oxidative phosphorylation [8–10]. Thus, it is essential that they possess a powerful ROS scavenging or buffering machinery and diverse additional protection strategies against oxidative stress induced by such continuous desiccation/rehydration cycles [11]. Furthermore, desiccation tolerance is intrinsically linked to the lichen symbiosis itself as effective ROS control and common regulation of antioxidant mechanisms are crucial and eases the transition of free-living fungi, green algae

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and cyanobacteria to a lichenised state [7,9,11].

Nitrogen monoxide (NO) is a multifaceted mediator difficult to detect due to its complex chemistry, but its production in lichens was demonstrated to be critical during rehydration [12]. NO has been postulated as one of the earliest antioxidant mechanisms to have evolved in aerobic cells and in symbiotic relationships involving neutralization of free radicals and ozone [13].

As several studies have reported, NO is involved in plant physiological processes such as metabolism, stress defence (biotic and abiotic), symbiosis and plant–pathogen interactions. NO has been shown to participate in symbiotic interactions, acting as a regulatory signal of mutualistic relationships establishment or as an intermediate involved in energy metabolism [14]. NO has also been shown to be produced during defence and stress responses such as drought resistance and oxidative stress in plants [15–17], algae [18–22] and fungi [23–25]. In fungi and yeasts, NO has been linked with regulation of cell development, spore germination, reproduction, apoptosis and control of oxidative stress but the metabolic pathways are not yet well characterized [26–29].

Ramalina farinacea is a lichen consisting of a mycobiont and at least two species of phycobionts known as *Trebouxia jamesii* and a recently described species, *Trebouxia lynnae* [30] (formerly known as *T.* sp. TR9), and other associated microorganisms such as bacterial communities [31,32]. Endogenous NO has been shown to decrease ROS production and lipid peroxidation in *R. farinacea,* playing an important role in the regulation of oxidative stress and photooxidative protection of phycobionts [33]. This molecule is also involved in the regulation of the oxidative stress triggered by air pollution [34] toxic metals such as Pb and Cd [35,36]. The mitigation of Hg toxicity has also been studied in other lichen genera [37]. Recently, a possible function in the regulation of caspase activity has linked NO and active cell death in *R. farinacea* during rehydration [38].

NO production has been confirmed in *Ramalina* genus and their phycobionts, but NO biosynthesis in lichens has not been elucidated yet. NO can be produced non-enzymatically, but the main sources are enzymatic. Oxidative NO metabolism is controlled by NO Synthase (NOS), using L-arginine and three isoforms are known in animals [39–42]. The source of NO generation in plants has been and continues to be a matter of discussion [43] as it has been shown that canonical (animal) NOS is not present in plants [44,45]. A NOS was recently identified for the first time in the plant kingdom, specifically in the green algae *Ostreococcus tauri* [46], and later in other microalgae such as *Bathycoccus prasinos* and *Ostreococcus lucimarinus* [47–51]. Weisslocker-Schaetzel and colleagues [52] performed the first functional and structural analysis of NOS in algae, in which unusual features such as very high rates of NO generation.

On the other hand, probably the most important source for bacteria, algae and plants is the reductive production of NO and is mediated by nitrite reductase (Nir) and nitrate reductase (NR) [29,53]. NR requires cofactors such as NADH and/or NADPH to carry out the reduction of nitrate to result in NO. Three isoforms are known using either or both two cofactors: NADH (EC 1.7.1.1), NAD(*P*)H (EC 1.7.1.2) and NADPH (EC 1.7.1.3) [54]. In eukaryotic algae and vascular plants isoforms 1 and 2 have been described [54–56]. Isoform 3 is only found in fungi where isoform 2 has also been characterized [54,57,58]. In the 80s, NR activity was reported to be induced in darkness by nitrate in the lichen *Evernia prunastri* [59]. However, to our knowledge, NR has not been characterized in lichens or their symbionts yet.

NR and NOS enzymes appear to be involved in NO synthesis in *R. farinacea* based on results obtained in a previous paper [60]. When tungstate (NR inhibitor) was added during rehydration, the level of NO decreased which caused an increase in malondialdehyde, suggesting that a plant-like NR activity is involved in the synthesis of NO in *R. farinacea*. This result is highly correlated with NO scavenging by c-PTIO, which leads to increased ROS and lipid peroxidation in lichens [33]. A first approximation of NR specific activity using a slightly modified plant method was performed, giving a result of 91 μU/mg protein. L-NAME (NOS inhibitor) did not reduce the level of NO in lichens but the NADPH-diaphorase activity could suggest the possibility of NOS-like activity in microalgae. Furthermore, it induced increases in lipid peroxidation and cytochemical assays pointed to the chlorophyllic layer as the most affected part [60].

Therefore, we hypothesise that *R. farinacea* and its isolated phycobionts produce NO via NR and NOS-like activities. To confirm this, we will focus on the following objectives: a) to optimize the method of quantification of NR activities in *R. farinacea*; b) to quantify NR and NOS-like enzyme activities in *R. farinacea* thalli and its symbionts and c) to immunodetect NR and NOS enzymes in *R. farinacea* thalli and its symbionts.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), L-cysteine, sulfanilamide ($C_6H_8N_2O_2S$), N-1-(naphthyl) ethylenediamine dihydrochloride ($C_{12}H_{16}Cl_2N_2$), protease inhibitor cocktail, nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and NADPH were provided by Sigma Aldrich Química S.A (Tres Cantos, Spain); NG-Nitro-L-arginine methyl ester (L-NAME) and sodium tungstate dihydrate (Na_2WO_4) was purchased from Sigma Aldrich (China); ethylenediaminetetraacetic acid (EDTA) was from Merck (Germany); dithiothreitol (DTT) and NADH were from Roche Custom Biotech; inorganics such as NaCl and $MgCl_2$, Tris, polysorbate 20 (Tween 20) and ethanol (etOH) were purchased from Panreac Quimica S.A.U (Spain). NO synthase activity assay kit (colorimetric), bicinchoninic acid (BCA) assay kit, goat anti-rabbit IgG H&L (alkaline phosphatase) secondary antibody (ab6722) and protein ladder/marker (ab116028) from Abcam. Rabbit polyclonal antibody, anti-NR (AS08310) of Agrisera provided by Abyntek. Rabbit polyclonal antibody, anti-iNOS (SAB5700636) and immobilon-P polyvinylidene difluoride (PVDF) membrane of Merck. Dehydrated milk of Nestle. Sodium dodecyl sulfate (SDS), methanol, tetramethylethylenediamine (TEMED) and ammonium persulfate (APS) were purchased from Fisher.

2.2. Biological material

Ramalina farinacea (L.) Ach. lichen thalli were collected in the air-dry state from *Quercus pyrenaica* in San Lorenzo de El Escorial at 969 m altitude (Ermita Virgen de Gracia, Madrid, Spain). Thalli were dried at room temperature for 24–48 h and preserved frozen at −80 °C.

For studies with *R. farinacea* phycobionts, axenic cultures of *T. jamesii* (TR1) and *T. lynnae* (TR9) were used. For their cultivation a procedure based on [61] was followed: cultures were grown on petri dishes on semisolid bold 3 N medium with casein and glucose and incubated at 19 °C, under a 14 h/10 h light/dark cycle (25 μmol m^{-2} s^{-1}). After 21 days the cultures of each isolated phycobiont were collected.

Mycobiont isolation from *R. farinacea* was performed under sterile conditions, cultured on fungal-specific YPD (yeast, potato and dextrose) medium with ampicillin to avoid bacterial contamination and stored under the same conditions as the isolated phycobionts. Reseeding was carried out every 2–4 weeks to generate biomass and avoid degradation of the cultures.

2.3. Enzymatic specific activities

NO biosynthesis was studied by determining the stable end products such as nitrite with the Griess method (nitrate reductase reaction) and by quantifying the specific activity of NO synthase (conversion of L-arginine to L-citrulline producing NO) using a colorimetric kit [62]. For the homogenization of *R. farinacea* thalli a conical homogenizer was

used and for the phycobionts a mortar with liquid nitrogen. In all cases 100 mg of biological material per millilitre of cold lysis buffer were used. The homogenized samples were sonicated for 5 min in ice and centrifuged at 17000*g* for 15 min at 4 °C. Supernatants were collected and proteins were quantified with Bradford assay.

2.3.1. NR specific activity quantification

The extraction buffer used for NR contained: dithiothreitol 1 mM, cysteine 1 mM, EDTA 5 mM and phosphate buffer 0.2 M (pH 7.5). Immediately after homogenization protein concentration was assayed by the Bradford method.

To quantify NR specific activity, the reaction mixture contained: 100 μl of lichen sample homogenized in NR extraction buffer, 20 μl of KNO_3 (at various concentrations), 20 μl of $MgSO_4$ 9.5 mM and 50 μl of cofactors (NADH or/and NADPH at various concentrations). After 10 min of reaction, it was terminated with 20 μl of $ZnSO_4$ 26.6 mM and 20 μl of ethanol 96 % at room temperature. Then, 190 μl of the supernatant were taken and mixed with 95 μl sulphanilamide 1 % with HCl 1.5 M and 95 μl N-(1-naphthyl) ethylenediamine dihydrochloride 0.2 % (Griess reagents). After 5 min the Griess reagents produces a colored compound and the amount of nitrite produced was measured at 540 nm. Protein precipitation was checked in the reaction mixture so that it was always clear before absorbance reading to avoid unspecific optical dispersion. Blanks without substrate (KNO_3) were assayed for each sample to find out the natural nitrite present in the sample. This value was subtracted from the total activity to account for non-enzymatic nitrite. Different concentrations of tungstate were used to study the inhibition of NR activity.

2.3.2. NOS-like specific activity quantification

A commercial kit (Abcam, ab211083) based on the reaction of NO end products with the Griess reagent to produce a colored product (540 nm) was used to quantify specific NOS activity. Homogenization of the sample was carried out using the NOS buffer provided with the kit. Proteins were analysed with the BCA method and used to refer to specific activity. L-NAME was used to inhibit NOS activity.

2.4. Western blot

Protein extractions were performed with lysis buffer (NR or NOS extraction buffer) to which protease inhibitor cocktail was added (10 μl of protease inhibitor cocktail per 1 ml of lysis buffer). The homogenized samples were sonicated for 5 min in ice and centrifuged at 17000*g* for 15 min at 4 °C. Supernatants were collected and proteins were quantified with Bradford assay. For electrophoresis, 10 % acrylamide gels were prepared, 40 μg of protein were loaded per lane and were run under 100–120 V until the band of the weight of interest was centred (a colored molecular weight marker was used for correct identification of the bands). For protein transfer a PVDF membrane was used which was left at 100 V for 1 h.

Plant anti-NR (AS08310 of Agrisera provided by Abyntek) and animal anti-iNOS (SAB5700636 of Merck), both rabbit polyclonal antibodies, were used for identification of NR and NOS proteins. Rabbit secondary antibody (ab6722 of Abcam) with alkaline phosphatase substrate was employed for the development of the membrane with this substrate.

For membrane blotting a chromogenic method was used, 300 μl of NBT 10 mg/ml and 75 μl of BCIP 20 mg/ml were used in a final volume of 10 ml of alkaline phosphatase (ap) buffer (pH 7.5, 100 Mm Tris, 100 mM NaCl, 5 Mm $MgCl_2$ and 0.5 ml Tween/l ap buffer). The membrane was incubated with the mixture for 10–30 min in the dark and after that time the bands of each antibody were stained purple in the presence of ap.

2.5. Statistics

For the quantification assay of NR and NOS specific activities, 4–8 replicates were used. The results are expressed as means ± standard error. ANOVA, one-way or two-way as appropriate, was performed with the R stats package [63], a $p < 0.05$ was considered for statical significance. *Post hoc* Tukey was performed to find out the significant differences between the groups.

3. Results and discussion

As stated above, NO is key to several functions in the lichen *R. farinacea* and its production has been observed during stressful conditions [33,34]. This molecule can be biosynthesised by nitrate reductase and NO synthase activities being the first the main source in plants and the second in animals. In lichens and symbiotic microalgae, these activities have never been assessed except for our recently reported estimation in *R. farinacea* using a protocol for plants [60].

3.1. NR characterisation in Ramalina farinacea and its symbionts

3.1.1. Optimisation of NR quantification method for Ramalina farinacea

To optimize a method for lichens, the optimal concentrations of the thallus biomass, the substrate (KNO_3) and the cofactors (NADH and NADPH) were studied. Fig. 1.a shows the specific activity of NR in *R. farinacea* as a function of lichen extract concentration using the conditions of plant NR activity determination (pH 7.5, KNO_3 0.5 M, NADH 380 μM) as defined in our previous study [60]. The maximal NR-NADH specific activity is obtained with 100 mg *R. farinacea*/ml, yielding 1.90 ± 0.21 nmol NO_2 min^{-1} · mg $protein^{-1}$ (mU·mg $protein^{-1}$). Therefore, this concentration is chosen for the next optimization step.

It should be noted that in Fig. 1.a the X axis does not refer to soluble protein, but to thalli biomass, including insoluble components like cell walls polysaccharides and proteoglycans which can obstruct soluble protein extraction. Thus, although the total amount of biomass is higher, soluble proteins like enzymes may be trapped within insoluble cell debris. This effect is especially serious with lichen thalli, whose structures are designed by natural selection to act as sponges absorbing and retaining lysis buffer that cannot be recovered even after intense centrifuging. Therefore, the amount of protein varies unexpectedly with the concentration of biomass used.

In order to find the optimal concentration of KNO_3 an assay was carried out using different concentrations of KNO_3 under the following conditions: pH 7.5, 100 mg *R. farinacea*/ml and 380 μM NADH. As can be seen in Fig. 1.b, nitrate 1 M renders the highest specific activity, 1.27 ± 0.11 mU·mg $protein^{-1}$.

Some differences are observed between 0.5 M KNO_3 concentration with 100 mg of *R. farinacea* between Figs. 1.a and 1.b. These differences may be attributed to natural variability among lichen thalli. The peculiarities of the study of symbiotic associations instead of pluricellular organisms in which all the cells are clones must be highlighted. Lichens, in contrast, are cyclical associations of cells belonging to very different kingdoms that are revealing themselves as real multi-specific micro-ecosystems. It has been recently reported that each thallus may house variable proportions of each symbiont organisms depending on micro-environmental conditions not yet well characterized [32,64]. That means that, despite the use of apparently homogeneous populations of *R. farinacea*, important intrinsic variability may still appear among thalli.

The optimal concentrations of the cofactors were determined using pH 7.5, KNO_3 1 M and 100 mg lichen/ml and were found to be 380 μM NADH and 760 μM NADPH (Fig. 1.c). Indistinctively of nitrate concentration, NADH is the most effective cofactor, yielding specific activities around 3 mU · mg $protein^{-1}$ at 1 M nitrate.

When both cofactors are used simultaneously enzymatic activity remains at levels like those of NADPH alone or even lower (Fig. 2). This

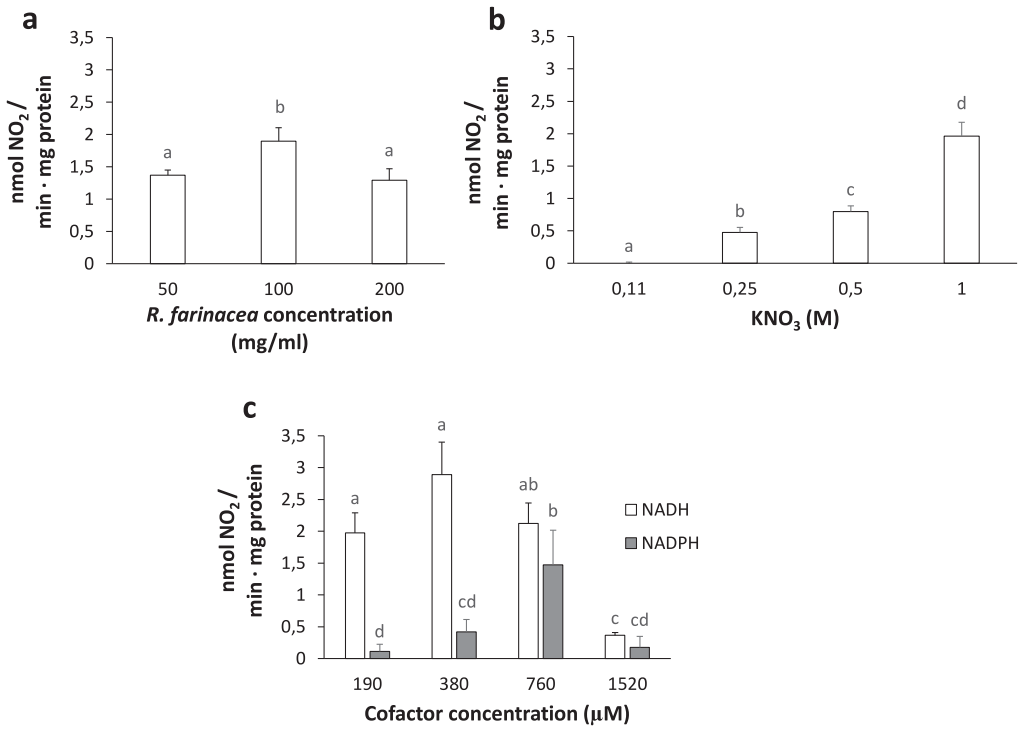

Fig. 1. *NR specific activity in* Ramalina farinacea *under different conditions. Lichen specific NR enzyme activity as a function of: a) sample concentration (KNO_3 0.5 M and NADH 380* µM*); b) nitrate concentration (100 mg* R. farinacea*/ml and NADH 380* µM*) and c) cofactor concentration (KNO_3 1 M and 100 mg* R. farinacea*/ml). Results are expressed as the average of four independent replicates ± standard error. One-way ANOVA for figures a and b and two-way ANOVA for figure c, $p < 0.05$.*

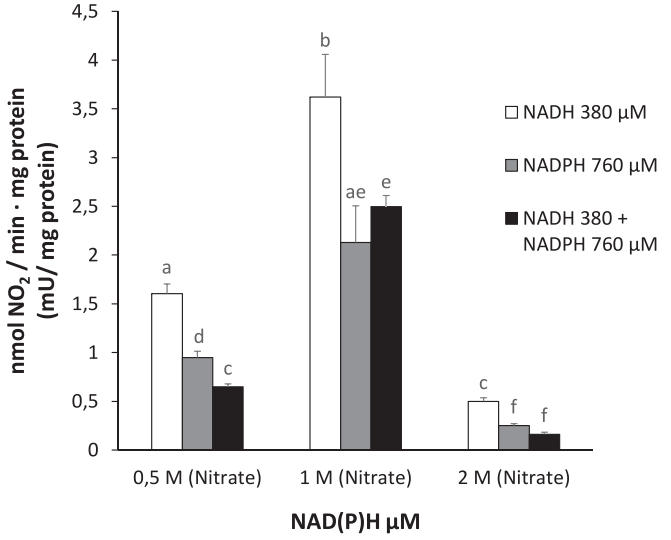

Fig. 2. *NR specific activity of* Ramalina farinacea *as a function of nitrate concentration and co-factor concentration under the following conditions: pH 7.5, 100 mg* R. farinacea*/ml, 9.5 mM $MgSO_4$, 0.5–1-2 M KNO_3. Results are shown as the mean of four independent replicates ± standard error. Two-way ANOVA, $p < 0.05$.*

could indicate that NADH-NR activity is inhibited in the presence of NADPH. There are significant differences among substrate concentrations, with nitrate 1 M providing the highest NR activity.

A slightly modified plant NADH-NR specific activity method yielded 0.09 ± 0.01 mU·mg protein^{-1} for the lichen *R. farinacea* [60], after optimization, a value of 3.62 ± 0.43 mU·mg protein^{-1} is obtained, improving the yield over 40-fold. Therefore, NR specific activity of the lichen *R. farinacea* was underestimated using plant conditions. This value is one order of magnitude higher compared with NR specific activity of *Arabidopsis thaliana*, 0.25 mU·mg protein^{-1} [65].

The comparison of NR activities of total extracts among different species is risky and must be considered with caution. Enzyme activity quantification methods must be optimised in order to find the highest apparent specific activity that can be recorded for each system or species. So, for each organism different concentrations of KNO_3 are needed, while 1 M KNO_3 results optimal for *R. farinacea*, around 5–20 mM is used for *A. thaliana* [65]. This large difference may be explained because natural selection has modelled each biological system according to different conditions.

In contrast to plants and bacteria, lichens lack waxes and cuticles to protect them from environmental stress or roots for water absorption. Even so, lichens and aeroterrestrial algae are extremophiles adapted to anhydrobiosis inhabiting very harsh habitats that have even survived outer space experiments [66]. Although little is known yet, the data point to very different cellular and molecular mechanisms to cope with abiotic stress compared to plants including special features in the physico-chemistry of photosynthesis [67]. This may explain the higher apparent Km of NR in lichens compared to plants and bacteria.

3.1.2. Quantification of NR specific activity

After optimisation of this method for lichens, NR specific activities of *Ramalina farinacea* and its mycobiont and its isolated phycobionts, *Trebouxia jamesii* and *T. lynnae* were quantified. Fig. 3. summarizes the NR specific activities of *R. farinacea* and its cultured main symbionts: *R. farinacea* mycobiont, and *T. jamesii* and *T. lynnae* microalgae. The NADH-NR specific activity of *R. farinacea* holobiont is significantly higher than its NADPH-NR or NADH+NADPH-NR specific activities.

In the case of *R. farinacea* mycobiont, NADH-NR and NADPH-NR specific activities are similar to each other and to holobiont's NADH-NR specific activity, whereas NADH+NADPH-NR specific activity is lower. However, this difference does not achieve statistical significance due to data variability.

An isoform using exclusively NADH (EC 1.7.1.1) has been reported in vascular plants but also in some microalgae (e.g. *Chlorella* spp.) and

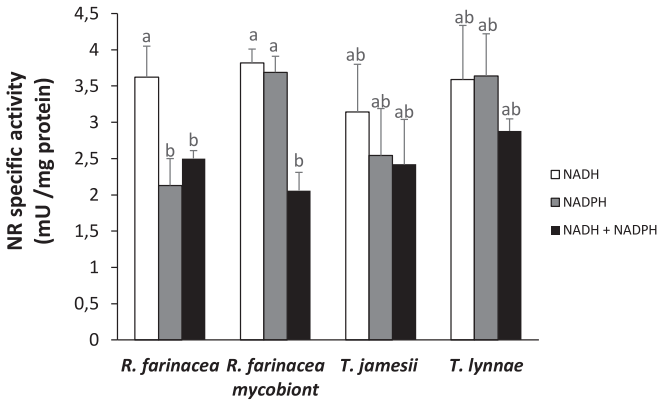

Fig. 3. *NR specific activities of* Ramalina farinacea *and its symbionts with different cofactors. Conditions: pH 7.5, 100 mg biological material/ml, $MgSO_4$ 9.5 mM, KNO_3 1 M, NADH 380* μM *and NADPH 760* μM*. Results are shown as the mean of 8 independent replicates ± standard error. Two-way ANOVA, $p < 0.05$ *.*

fungi (e.g. *Fusarium* spp., *Aspergillus niger*, *Saccharomyces cerevisiae*) [54]. The EC 1.7.1.2 isoform is described to use both NADH and NADPH cofactors, being more active with the first. It has been found, among others, in yeasts such as *Candida nitratophila* and in the pyrenoid of green microalgae (e.g. *Chlorella variegata*, *Chlamydomonas reinhardtii*, *Chlorolobion braunii* and *Ankistrodesmus braunii*) [54]. Finally, the EC 1.7.1.3 isoform requires NADPH and is specific to fungi [54,57].

R. farinacea holobiont seem to preferentially use NADH as a substrate, and NADPH when NADH is not available, but the mycobiont shows the same activity regardless of the cofactor. In both cases, the presence of NADPH seems to inhibit NADH specific activity, which could suggest the existence of an EC 1.7.1.2. Nonetheless, the isoform which employs the cofactor NADPH (EC 1.7.1.3) has been found in fungi, so the option that this isoform is also present should not be ruled out.

As primary producers within the lichen holobiont, phycobionts could have a similar dependency on NR activity as plants. NR activities in *T. jamesii* appear to be lower than in *T. lynnae* but no significant differences were found between both cofactors or across different species, including the holobiont and the mycobiont. Whereas research has shown that NR is responsible of NO biosynthesis in green microalgae such as *Chlamydomonas reinhardtii* and *Chlorella vulgaris* [53,68,69].

Optimised NADH-NR specific activity obtained in *R. farinacea* (3.62 mU·mg protein^{-1}) and its symbionts (same order) can be compared with that obtained in the chlorophyte *Ulva intestinalis* 2.70 mU·mg protein^{-1} [70]. We note that NR specific activity in this lichen and its symbionts is higher than in the model plant *A. thaliana* (0.25 mU · mg protein^{-1}) and in the Bryophyta *Physcomitrella patens* (0.04 mU·mg protein^{-1}) and lower than in Rhodophyta such as *Kappaphycus alvarezii* (0.16 · 10^3 mU·mg protein^{-1}) and *Gracilaria tenuistipitata* (1.60–3.00·10^3 mU·mg protein^{-1}) [70–72]. In addition, NR specific activities for other species can be found in our previous work [60]. It should be noted that these comparisons are general for ecophysiological studies and should be considered with caution. As mentioned above, each species has its own adaptation mechanisms depending on the taxonomic group to which they belong. As a matter of fact, green algae are considered by some taxonomists to be a subkingdom within Plantae due to notable biological differences [73].

As seen in a previous article of our group [60], the addition of tungstate, a well-known Moco-NR inhibitor, to water during rehydration causes changes in NO production in the lichen *R. farinacea*. Therefore, this enzyme seems to be responsible for biosynthesising NO in this species. The effects of tungstate on NR activities of the different symbionts measured in vitro can help characterize the enzyme(s) involved.

3.1.3. Inhibition of NR specific activity

As can be observed in Fig. 4, NADH-NR specific activity of *R. farinacea* decreases with the addition of tungstate in a dose dependent way up to 89 % with 100 μM. NADH-NR specific activity is completely abolished with tungstate 100 μM in *R. farinacea* mycobiont. Due to the difficulty of generating mycobiont biomass because of a very low growth rate, inhibition was carried out only at this concentration. Therefore, NADH-NR specific activity seems to be inhibited/decreased by tungstate in *R. farinacea* and *R. farinacea* mycobiont (Fig. 4).

In the case of the phycobionts, NR activity of *T. jamesii* decreases with increasing tungstate concentration but is not completely inhibited since, with 100 μM, it only decreases by 56 %. However, in *T. lynnae* no significant differences with the control were found, indicating an important divergence with the classical molybdenum co-factor

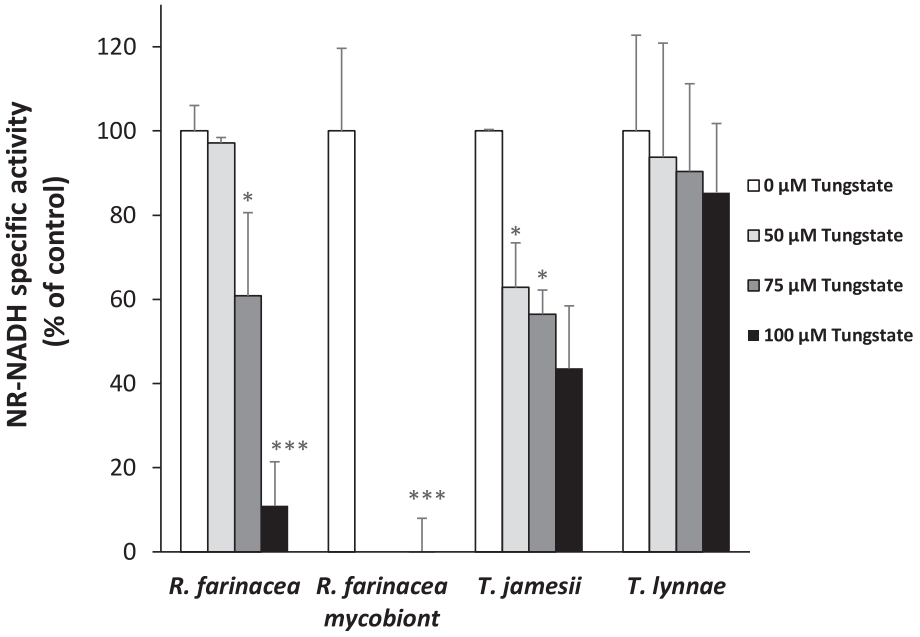

Fig. 4. *NR specific activity of* Ramalina farinacea *and its symbionts under inhibition with tungstate. Results are shown as the mean of four independent replicates in percentage respect to control (100 %) ± standard error. 100 % corresponds to the specific activity without inhibition (0* μM *tungstate). Two-way ANOVA, $p < 0.05$* and $p > 0.001$***.*

containing NR described for plants (Moco-NR, EC 1.7.1.1).

Tungstate is known to inhibit NR and other molybdate-dependent enzymes [25,60,74]. Our results demonstrate that, while the mycobiont's NR activity is sensitive to doses regularly used in the literature, the phycobionts present peculiarities. The main phycobiont of San Lorenzo de El Escorial population of *R. farinacea* has been reported to be *T. jamesii,* remaining *T. lynnae* as a secondary species cohabiting within the thalli. Both phycobionts possess very different, but complementary, physiological performances [35,75–78]. The insensibility to tungstate of the microalgae may explain the incomplete inhibition of NR activity in the holobiont by the highest dose, 100 μM, which fully inhibits the mycobiont's. The use of another NR inhibitor such as azide, well established in algae and plants [79,80], could provide interesting information about this peculiar NR phycobiont activity both in vitro and also in vivo regarding NO production and effects.

3.1.4. NR immunoblot

A Western blot using an antibody specific for plant NADH-NR was performed to immunodetect the enzyme both in the holobiont and the cultivated symbionts (Fig. 5). A band of approximately 110 kDa is present in *R. farinacea* thallus and its mycobiont, identical to that of fresh *A. thaliana*, which was used as a positive control. This agrees with the presence of the isoform EC 1.7.1.1, characteristic of plants, that uses exclusively NADH and whose activity is completely abolished by tungstate. The NR gene seems to belong to a cluster of genes that were horizontally transferred from a basidiomycete to an ascomycete ancestor [81]. Our data support the existence of a functional NR enzyme also in ascomycetes as a plausible hypothesis given that the mycobiont of *R. farinacea* is known to belong to the ascomycete group. On the other hand, the NADPH-NR activity presented above, especially in the mycobiont, should be provided by an additional tungstate-sensitive NR, either isoform EC 1.7.1.2 or EC 1.7.1.3.

In the isolated phycobionts a very dim band at 110 kD is hardly visible, instead, a lighter band at 93 kD is detected for both phycobionts, which seems also present in the mycobiont. This indicates that, unlike the thallus and the mycobiont, phycobionts' NR activity is not attributable to the same protein as in plants. Several other bands are intensely immunostained. *T. jamesii* presents a series of heavy bands above 130 kDa which are not so strong in *T. lynnae*. Also, an intense band below 93 kDa (around 70 kDa) is shared by both phycobionts and the holobiont. According to the literature and western blot troubleshooting guide from different commercial companies these bands could be precursors or degradation products of NR or else, a different isoform of NR, containing the antibody's epitope [82].

3.2. NOS characterisation in Ramalina farinacea and its isolated symbionts

Previously, the presence of NADPH diaphorase activity and the induction of lipid peroxidation in the phycobiont layer of *R. farinacea* upon rehydration with L-NAME, a known reversible inhibitor of NOS activity, raised the question of the relevance of NOS-like activity in lichen abiotic tolerance. Thus, our aim is to test whether NOS activity could also be responsible for NO production in *R. farinacea* and its symbionts.

3.2.1. Quantification of NOS-like specific activity

A commercial kit was used to determine the specific NOS activity in *R. farinacea* and its symbionts (Fig. 6): 49.93 ± 1.13 μU·mg protein^{-1} for *R. farinacea* holobiont, 447.81 ± 7.09 μU·mg protein^{-1} in *R. farinacea* mycobiont, 1031.31 ± 168.05 μU·mg protein^{-1} in *Trebouxia jamesii* and 1423.26 ± 391.83 μU·mg protein^{-1} in *Trebouxia lynnae*. NOS-like specific activity of *Chlorella vulgaris* (data not shown) was included in the analysis and gave a value of 1922.31 ± 353.34 μU·mg protein^{-1} with no significant difference compared to phycobionts. The results show that NOS-like activity of phycobionts is significantly higher than that of *R. farinacea* and its mycobiont but in the same order of magnitude of the aquatic green alga *Chlorella vulgaris*. The presence of high NOS activity in photosynthetic organisms has been related to increased ROS generation in photosystems electron chains [7–9] given the importance of NO in the stabilization of chlorophyll and the protection from photobleaching [33].

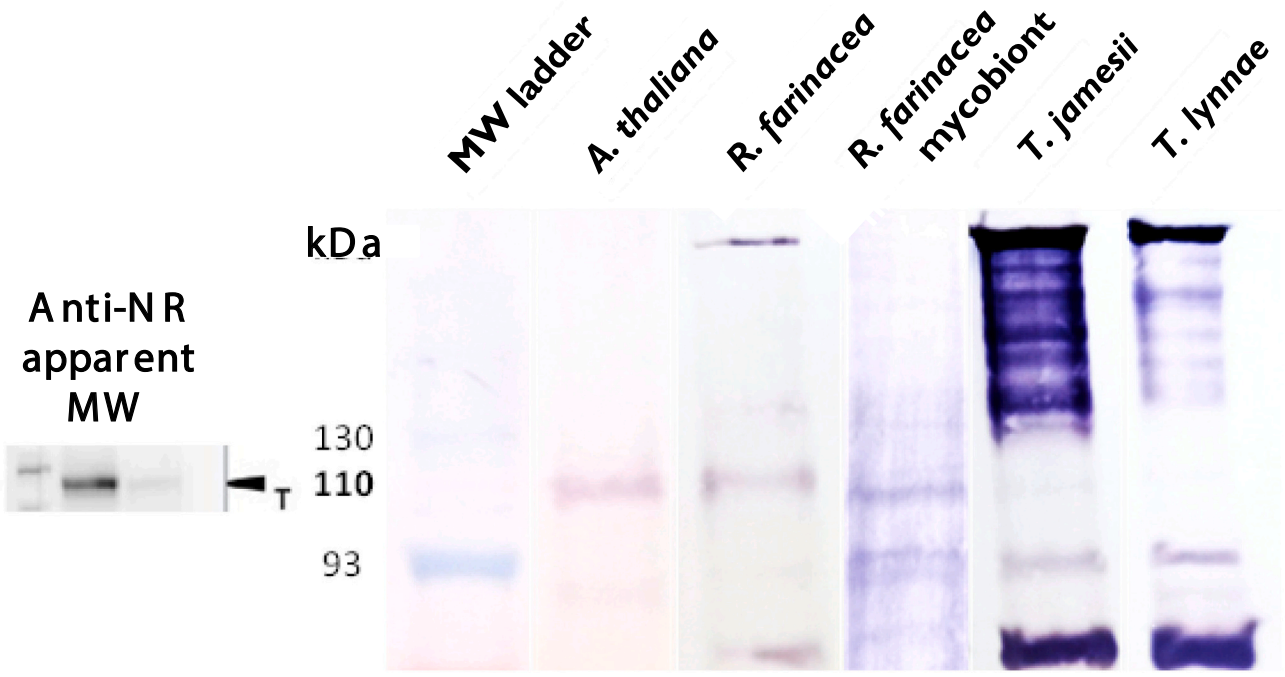

Fig. 5. *Immunodetection with anti-plant NR antibody in* Arabidopsis thaliana, Ramalina farinacea, R. farinacea *mycobiont,* Trebouxia jamesii *and* Trebouxia lynnae. *Approximately 40* μg *of protein per lane was introduced and the membrane was developed with a NBT/BCIP chromogenic method. A figure from the data sheet of the antibody is shown on the left to report the apparent weight of the protein sought in* A. thaliana *(about 110 kDa) using 20* μg *of protein and a chemiluminescent developing method.*

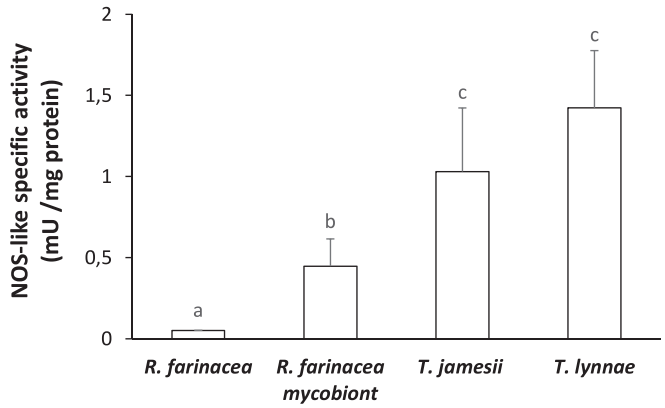

Fig. 6. *NOS-like specific activities of* Ramalina farinacea *and its symbionts. Results are shown as the mean of 8 independent replicates ± standard error. One-way ANOVA, $p < 0.05$.*

Mycobiont NOS specific activity is one order of magnitude lower than phycobionts', yet significantly higher than that of the holobiont. This is unexpected, since at least 80 % of the thallus biomass corresponds to mycobiont cells and points to a strong mutual inhibition of NOS-like activities of the symbionts when they associate to build the holobiont. The biological meaning of this feature for symbiosis deserves further investigation. The performance of bi-partite (mycobiont with each phycobiont) and tri-partite co-cultures would shed very interesting information on the mechanisms regarding NOS-like activity role and behaviour during symbiogenesis. However, new approaches must be done to improve *R. farinacea* isolation in axenic conditions and, more importantly, to optimize culture conditions in order to obtain enough biomass for extensive experimentation. Basidiomycetes are common fungi associated with lichens, but only a few species have been isolated axenically and characterized morphologically [83]. In addition, these co-culture experiments are very complex and their success rate is low. Co-cultivation studies of fungus and algae have been done and it has been observed that the lichen thallus presents additional unidentified agents and are more complex than the simple union between fungus and alga [84–86].

3.2.2. Inhibition of NOS-like specific activity

Fig. 7 shows NOS-like specific activity of *R. farinacea* and its symbionts when the inhibitor L-NAME is added in a range in line with that found in the literature [60,74]. Data from the *R. farinacea* mycobiont are presented only for L-NAME 300 μM because of biomass limitations. Though L-NAME causes slight reductions in the mean values of NOS-like activity in *R. farinacea* thalli and the mycobiont, this is not significant due to an increased dispersion of the data.

An important inhibition of enzymatic activity (over 50 %) is achieved with the higher doses of L-NAME assayed in *T. jamesii,* whereas minor, inconsistent effects (under 25 %) are observed in *T. lynnae*. In *T. jamesii* it appears that inhibition is somewhat dose-dependent but full inhibition is not observed even at the highest dose applied of this well-known inhibitor of plant NOS-like activity.

A concentration of 300 μM L-NAME was shown to increase lipid peroxidation in both the mycobiont and the phycobionts of *R. farinacea* thalli during rehydration, while NOx endproducts were also increased especially at longer times of treatment [60], a paradoxical effect of L-NAME has been frequently reported in vivo (reviewed by Kopincová et al. [87]). This could suggest that L-NAME inhibited especially critical activities since it triggered an upregulation of other NO sources which, in turn, seemed not to be efficient in lipid peroxidation control in certain areas of the chlorophyllic layer [60]. L-NAME is a nonselective inhibitor of NO synthases [87] that is hydrolysed by intracellular esterases to NG-nitro-L-arginine which is the active species that competes for the active centre with L-arginine [88]. However, its specificity out of the animal kingdom has been put into question since it was also demonstrated to inhibit NR activities in *Arabidopsis* leaf discs [89]. The use of NG-nitro-L-arginine, or other more specific, inhibitors would help better understand NOS-like activity in lichens in vivo. Recently, Prof. Lamattina's group demonstrated that L-Arg can be a possible source of N in the green microalga *Ostreococcus tauri* and thus an alternative pathway for N supply and metabolism in a photosynthetic microorganism [90].

3.2.3. NOS immunoblot

A Western blot analysis using antibodies against animal iNOS (135 kDa) did not allow to identify bands in the holobiont nor any of the cultured symbionts (data not shown). The mycobiont and the holobiont both showed some cross reactive bands above 130 and around 100 kDa.

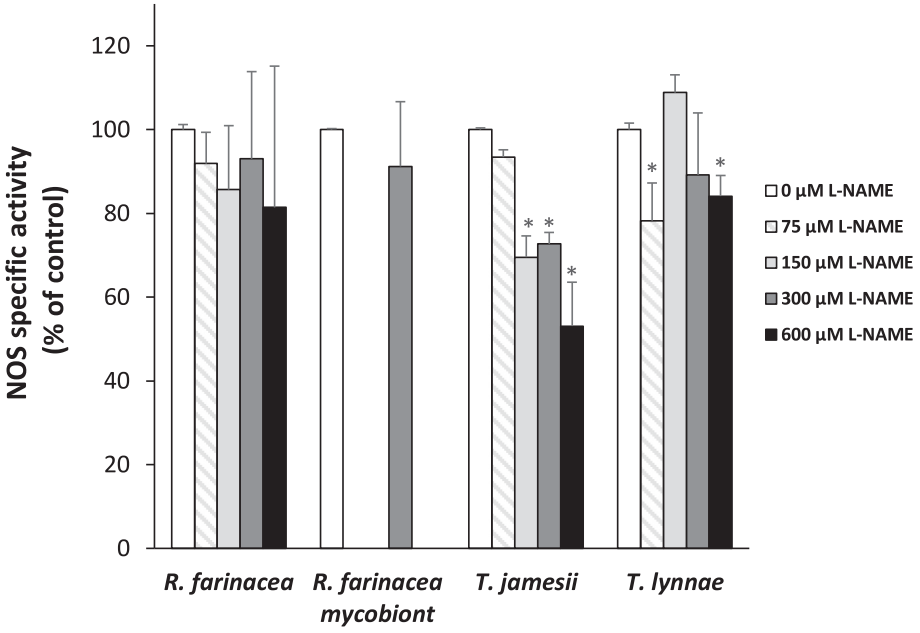

Fig. 7. *NOS-like specific activity of* Ramalina farinacea *and its symbionts under inhibition by L-NAME. Results are shown as the mean of four independent replicates in percentage respect to control (100 %) ± standard error. 100 % corresponds to the specific activity without inhibition (0 μM L-NAME). Two-way ANOVA, $p < 0.05$*.*

While *T. jamesii* showed a cross-reactive band near 110 kDa, no bands at all were revealed for *T. lynnae* nor *A. thaliana* as expected.

NO synthesis in plants is carried out by both NR and NOS-like enzymatic activities [16,49,91–93]. NOS-like activities in plants are sensitive to animal NOS inhibitors but NOS gene homologues were not found in the sequences of thousands of plants checked [50]. NOS-like proteins have been found in prokaryotes like eubacteria and archaea and some non-metazoan eukaryotes [51]. Recently, the presence of this enzyme has been observed in some species of the green lineage and in fungi [49,51,94,95]. In particular, they have been found in Trebouxiophyceae, class to which the phycobionts of *R. farinacea* belong, specifically in the chlorophyta *Leptosira obovata* [49,51]. Interestingly, NOS sequences were found in aquatic algae, the first NOS characterized in algae was the OtNOS found in the aquatic microalga *Ostreococcus tauri*, that shows a 30 % homology to iNOS [46].

Astier and co-workers propose that NOS gene could have been present in a common ancestor before the formation of the eukaryotic supergroup but was lost in the evolution of land plants which created a different mechanism than animals to produce NO [50]. On the other hand, it was observed that the some of the algae that presented NOS homologue sequences also contain NR [50]. Finally, a fungal NOS has been described in *Aspergillus niger* (Ascomycota) [51]. Another biosynthetic pathway has been found in *Aspergillus nidulans* involving L-Arginine [95].

3.3. Conclusions

To our knowledge this is the first comprehensive study of the main biosynthetic sources of NO in such a complex organism as a lichen and its isolated symbionts. Lichens are symbiotic associations that improve the biological fitness and stress tolerance of the participating organisms, allowing them to colonize almost every habitat on the planet, with a remarkable presence in the most inhospitable. The unveiling of NO role in symbiotic associations is key both for the sustainable conservation of natural ecosystems such as corals or agroecosystems that rely on rhizobial or mycorrhizae.

While many intriguing questions have been raised, our results show that:

1. The optimization of a specific method shows that *Ramalina farinacea* thalli specific NADH-NR activity is one order of magnitude higher than *A. thaliana*'s, in the range of the chlorophyte *Ulva intestinalis* and lower than some Rhodophyta. The plant method underestimated lichen NR around 40-fold.
2. *R. farinacea* mycobiont NR specific activity is in the same order as the whole thalli regardless of the cofactor added. The addition of both cofactors results in an inhibition of both NADH and NADPH-NR specific activities. Tungstate inhibition assays and Western blot show that *R. farinacea* mycobiont possess a canonical plant-like Moco-NR. Since plant NR have been described to use NADH, the results point to the presence of a supplementary isoform using NADPH.
3. NR specific activities of *Trebouxia* phycobionts are slightly lower than the mycobiont's. There are no significant differences among species or cofactors, nor a clear inhibition when both are added simultaneously. Whereas *T. jamesii* NADH-NR activity is partially inhibited by tungstate, *T. lynnae* is not. These results, taken together with Western blot analysis, demonstrate that phycobionts' NR is not a canonical plant Moco-isoform. Further studies are needed to characterize this peculiar microalgal enzyme (or enzymes).
4. NADH-NR specific activity in the holobiont (*R. farinacea* thalli) seems dominated by the mycobiont. Unexpectedly, despite the contribution of all the symbionts, NADPH-NR activity is significantly lower, almost half of the former. The addition of both cofactors results in an inhibition of NADH-NR activity.
5. Surprisingly, NOS-like activity in the *R. farinacea* holobiont is significantly lower than in any of the isolated symbionts. This points to a mutual inhibition in the symbiotic interaction whose biological meaning is intriguing and deserves further investigation.
6. In agreement with the findings in other photosynthetic organisms, phycobionts' NOS-like activity is very high and in the same range as the aquatic free microalga *Chlorella vulgaris*.
7. While NOS protein has not been immunodetected either in the thallus or in the isolated symbionts, L-NAME only partially inhibits NOS-like activity in *T. jamesii*.

In summary, NR activity seems participated by several different NR isoforms, including canonical Moco-NRs and non-canonical, their purification and characterisation remain an interesting biotechnological target. NOS-like activity is relatively important in the isolated symbionts but results strongly depressed in the lichen holobiont, by reasons that need to be further investigated. Future studies on the relevance of this findings on NO biosyntheis in vivo are also needed (i.e inhibition with different substances).

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CRediT authorship contribution statement

Joana R. Expósito: Methodology, Conceptualization, Investigation, Validation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Eva Barreno:** Conceptualization, Resources, Writing – review & editing, Visualization, Funding acquisition, Project administration. **Myriam Catalá:** Methodology, Conceptualization, Investigation, Validation, Resources, Writing – review & editing, Visualization, Supervision, Funding acquisition, Project administration.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Myriam Catala reports financial support, equipment, drugs, or supplies, and travel were provided by Government of Valencia Ministry of Innovation Universities Science and Society. Eva Barreno reports financial support, equipment, drugs, or supplies, and travel were provided by Government of Valencia Ministry of Innovation Universities Science and Society.

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Data availability

Data will be made available on request.

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