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Oxidative stress and usnic acid content in *Parmelia caperata* and *Parmelia soredians* (Lichenes)☆

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Abstract

High light levels together with Paraquat treatment or exposure to pollutants (e.g. SO_2) can cause oxidative stress in epiphytic lichens. In some cases, a significant increase in ascorbic acid and other antioxidant metabolites, sometimes occurring in lichens only, was observed. In this study, usnic acid was measured by HPLC in *Parmelia caperata* and *Parmelia soredians* thalli treated with Paraquat, a herbicide which transfers electrons from various transport systems to oxygen, producing $O_2^{\bullet -}$ superoxide radicals. In light, Paraquat acts by generating active oxygen species within the chloroplast, thus simulating the oxidative component of environmental stress. The significant increase in the usnic acid content measured in *P. soredians* thalli (+36.3%) is in line with the hypothesis that it has an antioxidant action, but it is in contrast to the limited increase measured in *P. caperata* thalli (+13.7%). These apparently contradictory results confirm those found in the literature, which attribute different functions to usnic acid depending on the lichen species considered and on whether they have other detoxifying substances in their thalli. These studies are of potential application-oriented interest in relation to research into new active principles to be used in the pharmaceutical, food and cosmetic fields and/or in environmental biomonitoring.© 2001 Éditions scientifiques et médicales Elsevier SAS

Keywords: Lichens; Usnic acid; Oxidative stress

1. Introduction

Lichens produce a great number of different secondary metabolites, many of which occur exclusively in these symbiotic organisms. The biological role of lichenic substances is not yet well known, although they do often have a protective function for the lichen.

Sometimes they have antibiotic action against microorganisms, as it has been demonstrated for usnic acid [1]; at times they are toxic to herbivores, mainly invertebrates, as is the case for orsellinic acid [2], atranorine and vulpinic acid [3].

Some lichenic substances have a defensive function against excessive exposure to light, like calcium oxalate, which could deposit as a whitish layer on the thallus surface, increasing the albedo [4]. Furthermore, it has been suggested that some lichenic substances may have an important role in protecting thalli from the dangerous toxic action of free radicals produced by oxidative stress exposure.

Oxidative stress in lichens may be caused by many agents, including high light levels in the habitat, SO_2 pollution and herbicides such as Paraquat, which transfer electrons from various transport systems to oxygen, producing $O_2^{\bullet -}$ superoxide radicals. In light, Paraquat acts by generating active oxygen species within the chloroplast, thus simulating the oxidative component of environmental stress [5].

In recent studies, a significant increase in the ascorbic acid content was observed in *Parmotrema reticulatum* 1 week after a single Paraquat treatment [6]. In this study, usnic acid was measured by HPLC in *Parmelia caperata* and *Parmelia soredians* thalli treated with Paraquat, to investigate whether it is involved in the mechanism of defense against oxidative stress caused by light and Paraquat.

At present, lichens have an important role in bioindication techniques that allows the mapping of pollution

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effects over wide areas, with a high sampling density. Indeed, epiphytic lichens, which are ideal bioaccumulators and biomonitors because of their specific morphology and physiology, are often used as markers in environmental pollution monitoring for the following reasons:

1. epiphytic lichen biodiversity maps can be correlated to pollution and/or human toxicosis maps in high-risk areas [7];
2. the accumulation of pollutants (e.g. heavy metals) in thalli can show the distribution pattern of each pollutant and the degree of deviation from natural background conditions [8];
3. the presence or increased production of detoxifying substances can be used as oxidative stress markers in environmental biomonitoring [6].

In this paper, the possible role of usnic acid in environmental biomonitoring studies was evaluated.

2. Materials and methods

2.1. Sample collection and treatment

P. caperata (L.) Ach. and *P. soredians* Nyl. were collected from the Belvedere Hill, maximum elevation 100 m, near Santa Margherita Ligure in eastern Liguria (north-west Italy), on the sea coast. According to previous bioindication studies the survey area has a high naturality degree [9]. Thalli growing on olive trees were collected as described elsewhere [5]. Some thalli were collected before treatment and used as controls (July and October control thalli) while others were treated with Paraquat (treated in July and collected in October). Each thallus was treated as follows: an area surrounding the sample was sprayed with 50 ml dm^{-2} of 0–2% Paraquat dissolved in deionized water. Technical grade Paraquat (Gramoxone W, ICI Solplant) was used as recommended by the manufacturer (3–5 kg ha^{-1}). The lichen thalli were sprayed only once and collected 3 months after treatment.

2.2. Usnic acid extraction and determination

Usnic acid extraction and determination were carried out according to the protocol described by Coassini-Lokar et al. [10] with minor modifications. All the samples of *P. caperata* and *P. soredians* were thoroughly cleaned of tree bark fragments and other debris in the laboratory immediately after collection. The cleaned samples were dried for 24 h above silica gel in a desiccator to standardize their water content, in the dark at room temperature (r.t.). The dry material was split up into 100 mg samples and stored at −20°C until used. The lichen material was also oven-dried at 60°C for 48 h and weighed to obtain the percentage dry weight.

For usnic acid extraction, samples of frozen thalli (100 mg) were extracted with acetone (10 ml) for 24 h at r.t. in the dark; the extracts were filtrated and evaporated until dry. The residues were dissolved in pure methanol (10 ml), divided into aliquots and stored at −20°C until the time of HPLC determination.

For usnic acid determination, a Perkin–Elmer (Norwalk, CT) HPLC apparatus with a UV detector and a Shimadzu (Kyoto, Japan) data processor were used. Sample injection system: Rheodyne valve with 20 μl sample loop; column: Nucleosil 10 C_{18} (Macherey–Nagel, Germany); mobile phase: methanol–water–acetic acid (81:14:5); flow rate: 1.0 ml min^{-1}; detector: UV 254 nm; range: 0.016 AUFS. The usnic acid was determined with reference to an authentic standard (Sigma, St. Louis, MO). The external standard method was used.

The detector response was linear in the range between 2 and 200 ng per 20 μl samples, and the chromatographic system was able to detect 0.5 ng of usnic acid in the 20 μl injected. The recovery rate of the usnic acid standard solution (100 ng/20 μl) was 91.8 ± 1.9% (n = 23); the experimental results were corrected for this value. The data obtained were statistically processed by using a standard non-parametric variance analysis method (ANOVA) for determining significant inter-group differences [11].

Table 1
Percentage dry weight (dry wt%) and usnic acid content (mg/100 g dry wt) in the thalli of *P. caperata* and *P. soredians* collected without treatment (July and October controls) and 3 months after a single Paraquat treatment (treated in July and collected in October) [a]

Samples	*P. caperata*		*P. soredians*	
	Dry wt (%)	Usnic acid (mg/100 g dry wt)	Dry wt (%)	Usnic acid (mg/100 g dry wt)
July controls	94.4	1.022 ± 0.035 (17)	96.8	1.088 ± 0.037 (25)
October controls	94.4	1.125 ± 0.026 (15)	96.9	0.557 ± 0.017 (8)
Treated -3 months	96.7	1.162 ± 0.046 (20)	96.9	1.482 ± 0.084 (25)

[a] The data are mean ± SEM values; the number of samples is indicated within brackets.

3. Results

The usnic acid content of untreated and Paraquat-treated thalli of *P. caperata* and *P. soredians* are shown in Table 1. In *P. caperata*, both the control and the treated thalli collected in October show a limited increase in usnic acid content in comparison to the controls collected in July (10.1 and 13.7%, respectively). The one-way ANOVA showed that the inter-group differences in usnic acid content (July controls vs treated and July controls vs October controls) were significant ($P < 0.02$ and $P < 0.05$, respectively); no significant difference was observed between the treated thalli and the October controls.

In *P. soredians*, the Paraquat-treated thalli showed a noticeable increase in their usnic acid content (36.2%) as compared with the July controls, while the October controls showed a strong decrease (-48.8%) in comparison to the July controls. The one-way ANOVA showed that the inter-group differences in usnic acid content (July controls vs treated and July controls vs October controls) were highly significant ($P < 0.001$); furthermore the July and October control thalli differed significantly from each other ($P < 0.001$).

4. Discussion

Usnic acid is a lichenic substance that has been found to be an effective chemotherapeutic agent for many common microorganisms [1] as well as for human tuberculosis, leprosy and certain tumors [12].

As regards its possible antioxidant and detoxifying action, the data available in the literature are contradictory [13–15]. For instance, *Cladonia subtenuis* and *Parmeliopsis ambigua* have considerably higher usnic acid levels in thalli growing in those sun-exposed habitats than in others growing in the shade [16]. These results are in line with those reported for ascorbic acid, which is a powerful antioxidant, both in lichens [6] and in higher plants [17]. In summer, water deficit and desiccation are increased by bright light and contribute towards creating a stressful environment; in this situation, the presence of an antioxidant may constitute a great advantage and therefore antioxidant metabolite production is presumably increased.

However, the seasonal variations in usnic acid content reported by Taguchi et al. [18] are quite different, indicating a minimum in summer and a maximum in early spring. In this case, usnic acid seems to have a metabolic role rather than a defensive antioxidant one.

In our experiments, the significant increase in usnic acid content measured in Paraquat-treated *P. soredians* thalli ($+36.25\%$) is in line with the hypothesis that it has an antioxidant action, while the limited increase measured in *P. caperata* thalli ($+13.7\%$) may be better explained by a different metabolic role.

To conclude, our apparently contradictory results confirm those found in the literature, which attribute different function to usnic acid, depending on the lichen species considered, which may have several different detoxifying systems in their thalli.

As regards the use of usnic acid as a marker in environmental biomonitoring, studies are in progress to evaluate its physiological seasonal fluctuations in epiphytic lichens growing in high-risk areas in Liguria. These data, together with those on the variations produced by oxidative stress (high light levels, Paraquat treatment, SO_2 pollution, etc.) may clarify the possible use of usnic acid determination for such an aim.

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