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ANALYSIS OF THREE DIFFERENT LICHEN SPECIES AS BIOINDICATOR OF ACID RAIN. AN EXPERIMENTAL PROOF

Analyse von drei verschiedenen Flechtenarten als Bioindikatoren für sauren Regen. Ein experimenteller Beweis

by

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Key words: Lichen, bioindicator, chlorophyll, phenolic acid.
Schlagwörter: Flechten, Bioindikation, Chlorophyll, phenolische Säuren.

Summary: Lichens are known to be sensitive to many types of pollutants and they are considered as good biological monitors of air quality. Recent studies demonstrated that lichen are very sensitive to SO_2, fluorides, ozone, nitrogen oxides, peroxyacetyl nitrate (PAN) and heavy metals. One of the most important problems to get a consistent analysis is to get rid of phenolic acids which change chlorophyll to phaeophytin. On the other hand, we analyzed some different biochemical parameters (chlorophyll concentration, absorbance and fluorescence emission spectra) in three species: *Xanthoria parietina, Evernia prunastri* and *Ramalina farinacea* treated with several concentrations of H_2SO_4 quite similar to those detected in the environment of Madrid city (Spain).

The results are discussed on the base if is a possible enviromental application of these specific lichens as bioindicators of air pollution in Madrid area. We conclude that *R. farinacea* and *E. prunastri* are more useful than *X. parietina* as bioindicators of acid rain in our experimental conditions. On the other hand, the method used for chlorophyll extraction in very simple and effective to remove phenolic acids.

Zusammenfassung: Flechten sind bekanntermaßen empfindlich gegenüber vielen Arten von Luftverunreinigungen und sie werden als verläßliche biologische Zeigerpflanzen für die Luftqualität angesehen. Jüngere Untersuchungen zeigten, daß Flechten sehr empfindlich gegenüber SO_2, Fluoriden, Ozon, Stickoxiden, Peroxyacetylnitrat (PAN) und Schwermetallen sind. Eines der wichtigsten Probleme, eine einheitliche Analyse zu erreichen, ist die phenolischen Säuren loszuwerden, die Chlorophyll in Phaeophytin umwandeln. Auf der anderen Seite analysierten wir einige verschiedene biochemische Parameter (Chlorophyll-Konzentration, Absorbance und Fluoreszenz-Emmissions-Spektren) in drei Arten: *Xanthoria parietina, Evernia prunastri* und *Ramalina farinacea* wurden mit verschiedenen konzentrierten Lösungen von H_2SO_4 behandelt, wie sie auch in der Umwelt der Stadt Madrid (Spanien) gefunden wurden.

Die Resultate werden vor dem Hintergrund diskutiert, ob eine umweltbezogene Anwendung dieser drei Flechten als Bioindikatoren für Luftverunreinigungen im Stadtgebiet von Madrid möglich ist. Wir schließen aus den Resultaten, das *R. farinacea* und *E. prunastri* als Bioindikatoren für sauren Regen unter den experimentellen Bedingungen besser geeignet sind als *X. parietina*. Die Methode für die Chlorophyllextraktion ist einfach und sehr effektiv, um die phenolischen Säuren zu entfernen.

Introduction

Lichens are known to be sensitive to many types of pollutants: sulphur dioxide (MAHANEY et al. 1995; GARTY et al. 1995); heavy metals (DILLMAN 1996); O_3 (SCHEIDEGGER & SCHROETER 1995); and in general, air pollutions from traffic and industries (GONZALEZ et al. 1996). Several lichen species have different degrees of sensitivity to air pollution. Lichens may be assigned to three categories in terms of their responce to air pollution (GARTY et al. 1992): sensitive, tolerant and replacement species.

Acid rain in industrialized countries possibly accounts for the disappearance of certain lichen species. It seems to be that acidic solutions provoke degradation of chlorophyll as a consequence of the loss of Mg^{2+}. Different researchers studied the effect of simulated acid rain on lichens (SCOTT & HUTCHINSON 1987) analyzing several parameters such as growth rate of the lichens, percentage of coverage and net photosynthesis (SCOTT et al. 1989). One of the most important problems to get a consistent analysis is to get rid of phenolic acids which change chlorophyll to phaeophytin. In this work, we propuse a method to remove these substances.

On the other hand, we analyze some different biochemical parameters in three lichen species with different sensibility againts air pollutants: *Xanthoria parietina, Evernia prunastri* and *Ramalina farinacea* treated with several concentrations of H_2SO_4 quite similar to those detected in the enviroment of Madrid city (Spain).

Materials and Methods

Biological Material:

Xanthoria parietina (L.) TH. FR., *Evernia prunastri* (L.) ACH. and *Ramalina farinacea* (L.) ACH. growing on *Quercus pyrenaica* L. was collected in El Escorial (Madrid). Thalli were used inmediatly after collection or air-dried and stored during 3 months.

Extraction Chlorophyll Methods:

Aproximately 60 mg of these lichens were maintained in dryness for 5 days under a photoperiodical timing of 12 h light-12 h darkness. Only one time per day were incubated on different acidic solutions: 0, 3, 6, 9, 12 nM with pH 6.60, 6.38, 6.28, 6.23, 6.17, respectively, for 1 h at 26° C. This step was repeated along five days. After this, thalli are treated with 10 ml DMSO at room temperature in darkness during 24 h. Chlorophylls were retained in DMSO solvent. Then the photosynthetic pigments were extrated with 10 ml diethyl ether by shaking for 1 min. Both solvents were cleaved by freezing. Ethyl ether phase was dryed and then chlorophyll concentration, chlorophyll absorbance and fluorescence spectra and phenolic acids content were analyzed. DMSO phase was cromatographed in TLC to reveal the presence of lichenic substances.

Chlorophyll Analysis

Amount chlorophyll by dry weight was estimated according to BARNES et al. (1992). Chlorophyll absorbance spectrum was carried out in a DMS-90 Varian from 400-450 nm. Moreover, the emision fluorescence spectrum was realized used a excitation wavelength to 277 nm. We used a SMF-25 Kontron Spectrofluorimeter.

Phenolic Acids Determination

DMSO extracts was analysis in TLC to know if phenolic compounds were retained in this polar phase. Stationary phase was silicagel, 100 μm thickness, and mobile phase was benzene: dioxane: acetic acid (90:25:4, v/v). Spots were revealed with 35 % H_2SO_4 at 110° C.

Moreover, lichenic substances was studied in chlorophyll extracts by HPLC according to PEDROSA & LEGAZ (1993).

Results and discussion

Figure 1 shows the amount of total chlorophyll from lichen thalli treated as above indicated. The amount of chlorophyll of *E. prunastri* recently collected (Fig. 1A) decreased from 6 nM H_2SO_4. After storage, it was observed an apposite effect after 3 months. However, *X. parietina* modified the amount of chlorphyll with independence of H_2SO_4 concentration (Fig. 1B). Fresh *R.*

farinacea thalli loss chlorophyll from 3 nM to 12 nM of H_2SO_4 (Fig. 1C).

X. parietina is a very tolerant lichen against H_2SO_4 pollutant, even it is possible to see it in urban areas and cities (DÉRUELLE 1983; WIRTH & TÜRK 1974). However, *R. farinacea* seems to be a more sensitive lichen to this kind of pollution, being *E. prunastri* a lichen with middle sensibility (HAWKSWORTH & ROSE 1970).

The effect of H_2SO_4 upon chlorophyll concentration in stressed environmental conditions has been described for *Ramalina* species than *R. farinacea* (GARTY et al. 1992). However, pH values of the floating media were extremely low in such studies (pH 1.0-2.0). These values can be close to that observed in certain industrial sites (LEVIN et al. 1990) but they are not frequent in urban areas, such as Madrid, where the minimum estimated value is was pH 5.8 (Information from Servicio de Medio Ambiente Ayuntamiento de Madrid, Spain). Despite, *R. farinacea* and *E. prunastri* are sensitive to 3 and 6 nM H_2SO_4 (pH 6.38 and 6.28) respectively, in the experimental conditions above indicated (Fig. 1 A and 1 B).

According to GARTY et al. (1993), symptoms of damage of cell membranes are detected in *Ramalina duriaei* long before any indication of damage becomes apparent in the photobiont chlorophyll. These membrane alterations could be due to peroxidation of unsaturated fatty acids (MEAD 1987). Moreover, a positive correlation between accumulation of certain metals (Br, Pb, Fe, Ti) and decrease of chlorophyll content has been observed when lichens was transplated from unpolluted area to biomonitoring stations (GARTY et al. 1985).

In our study, the main cause of chlorophyll degradation could be the production of phaeophytin (BROWN & HOOKER 1977; BALAGUER & MANRIQUE 1995) since the absorbance spectrum of pigments extracted from sensitive species (Fig. 2A and 2C) showed a displacement of absorbance maximum from 430 nm (chlorophyll a maximum) to 410 nm (phaeophytin a maximum). Meanwhile, *X. parietina* did not follow this behaviour (Fig. 2B). These results are agree with those obtained from fluorescence emission spectra (Fig. 3). We can see that from 3nM to 12 nM H_2SO_4 a new peak of orange fluorescence emission aproximately at 623 nm appeared as a consequence of the presence of phaeophytin (HOLOPAINEN & KAUPPI, 1989). Such event was observed for fresh *E. prunastri* and *R. farinacea* thalli but not for *X. parietina* extracts in which the maximum of emission fluorescence appeared at 515 nm (yellow fluorescence). This could be explained because parietina from *X. parietina* thalli is not completely removed (only 75,8 % total parietin) and the phenolic antraquinone has a maximum of fluorescence emission at 515 nm (Fig. 3D). However, lichen substances were removed from *R. farinacea* and *E. prunastri* thalli (98,7 % and 92,5 % respectively). This is the reason maybe that a peak around 500 nm does not appear in the fluorescence spectrum of pigment extracts from sensitive lichens (Fig. 3). In this sense, Table 1 indicates the Rf of different phenolic acids

that are extracted by DMSO. Last, Fig. 4 shows the phenolic composition of *R. farinacea* from cortex (Fig. 4A) and medullar (Fig. 4B) and the absence of phenolics in the chlorophyll fraction extrated from the same thalli (Fig. 4C).

We conclude that fresh *R. farinacea* and *E. prunastri* thalli are more useful that *X. parietina* as a bioindicator of acid rain in our experimental conditions. This agrees with the studies in other areas (HAMADA et al. 1995). On the other hand, the methods used for chlorophyll extraction is very simple and effective to avoid endogenous phaeophytinization by phenolic acids.

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Table 1: Phenolic acids retained in DMSO and chromatographied by TLC from *E. prunastri*, *X. parietina* and *R. farinacea*.

Lichen	Phenolic composition	Rf
E. prunastri	Atranorin	92.35
	Usnic acid	88.24
	Evernic acid	77.06
X. parietina	Parietin	89.4
		81.7
R. farinacea		97.5
	Usnic acid	85.3
	Protocetraric acid	78.8

-- undeterminated

Fig. 1: Amount of total chlorophyll from fresh (●) and stored (▲) *E. prunastri* (A), *X. parietina* (B) and *R. farinacea* (C) thalli.

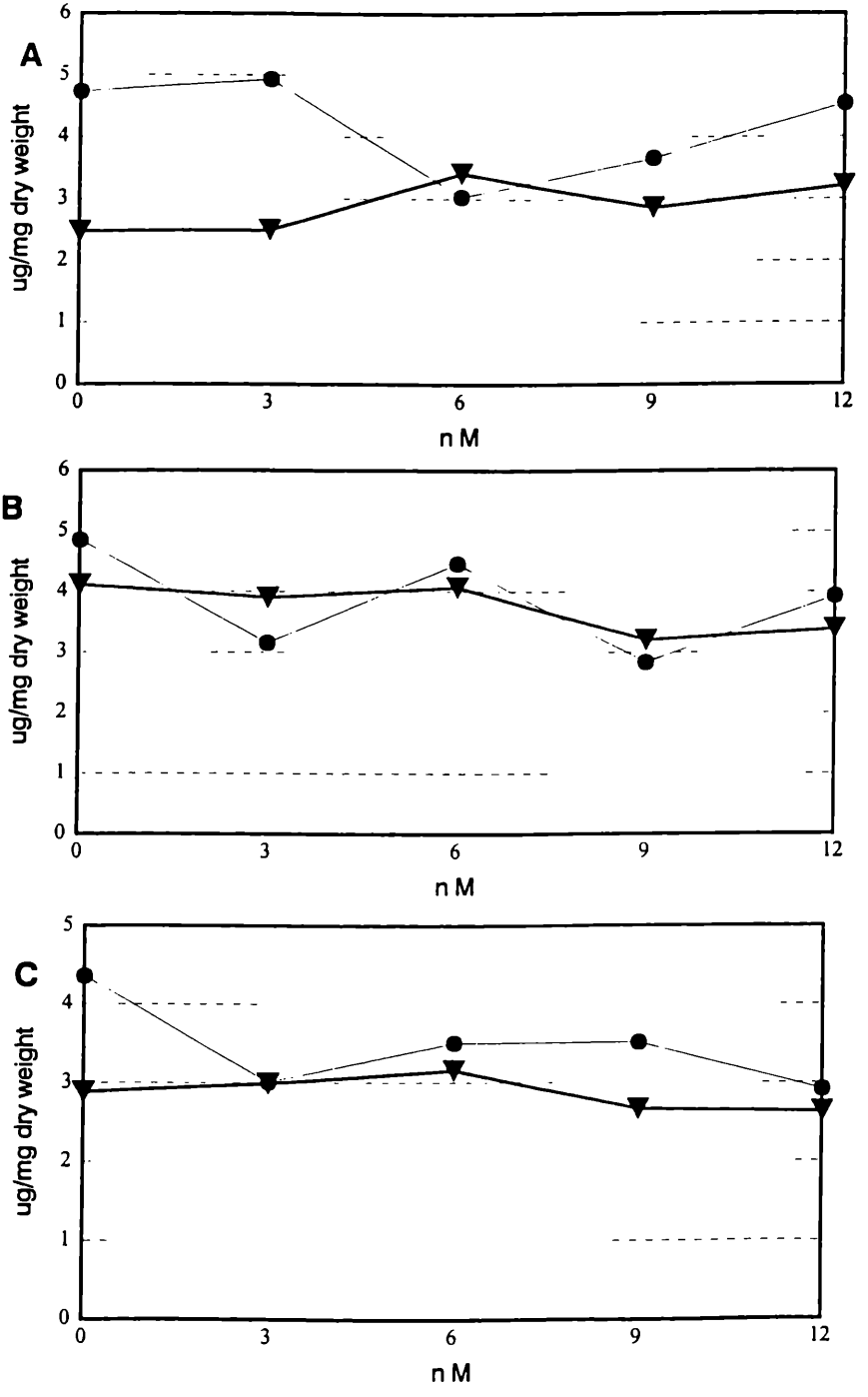

Fig. 2: Absorbance spectra of *E. prunastri* (1), *X. parietina* (2) and *R. farinacea* (3) thalli floated in 0 nM (■), 3 nM (+), 6 nM (*), 9 nM (.) and 12 nM (x) H_2SO_4.

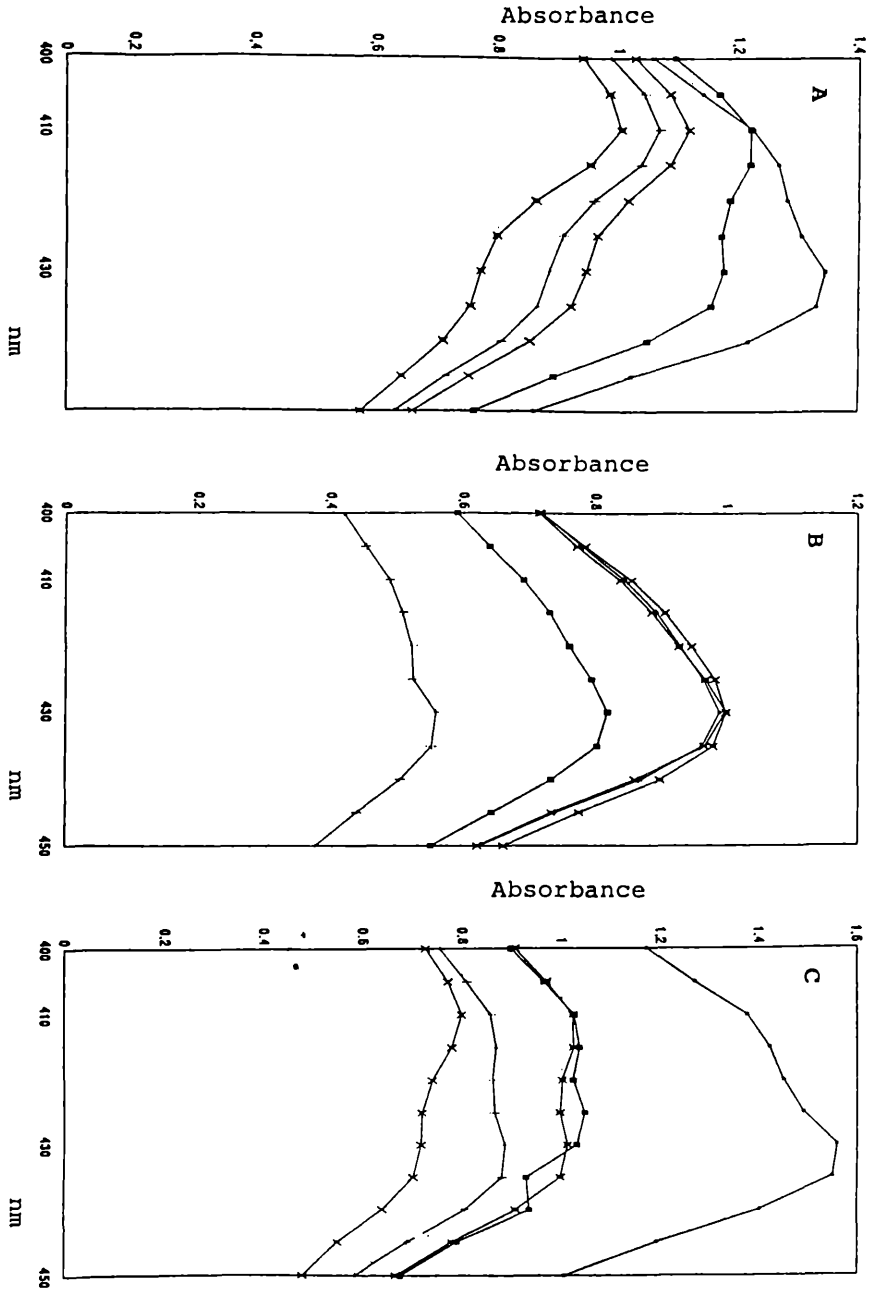

Fig. 3: Fluorescence spectra of photosynthetic pigments from (1) *R. farinacea* and (2) *E. prunastri* and (3) *X. parietina* incubated in 0 nM (A), 6 nM (B) and 12 nM (C). Fluorescence spectrum from parietin (D).

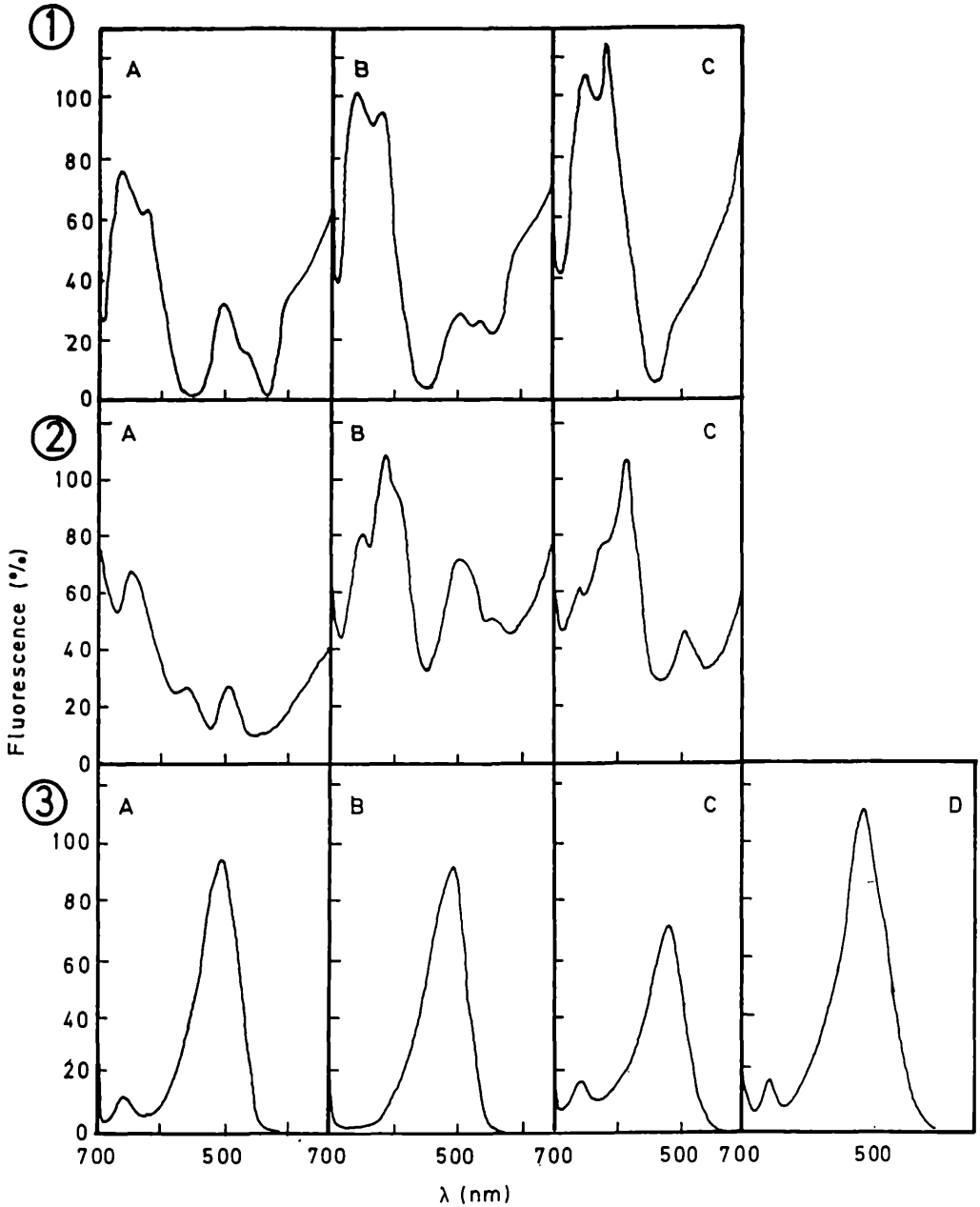

Fig. 4: Cortex (A) and medullar (B) phenolic acids from *R. farinacea* and abscence of these lichen substances in photosynthetic pigments after different extraction steps (C).