

# Chlorophyll degradation effected by lichen substances

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*Received 23 June 1997, accepted 7 November 1997*

Epiphytic lichens can exert some negative actions on trees, since they penetrate to different degrees into several tissues of the phytophore. *Evernia prunastri* (L.) Ach. hyphae progress into xylem vessels of *Fagus sylvatica*, *Quercus pyrenaica* and *Betula alba*. This is on the basis of a mechanism through which lichens inject metabolic inhibitors in the xylem vessels, and, after their translocation in the xylem sap, they reach different organs of the phytophore and produce defoliation. In this report, we have noted that usnic acid is more impermeant than atranorin through the chloroplast membrane of *Quercus rotundifolia* leaves. On the other hand, we have also noted the degradation of chlorophyll in response to lichen substances.

**Keywords:** chlorophyll degradation, *Evernia prunastri*, lichen phenols, *Quercus rotundifolia*

## INTRODUCTION

Many substances synthesized by lichen thalli have damaging effects on trees. Asahina and Kurokawa (1952) described the inhibition of tea shoot-development by epiphytic lichens. Epiphytic lichens can penetrate to a different degree into phytophore tissue (Brodo 1973). Moreover, they sometimes show an extensive penetration of rhizines through the cork, cortex and cambium reaching the living wood (Ascaso *et al.* 1980). With the help of both scanning and transmission electron microscopy, Orús and Ascaso (1982) established that hyphae of *Evernia prunastri* (L.) Ach. appear inside the conducting tissues of *Quercus rotundifolia*. Ascaso *et al.* (1983) observed some ultrastructural

alterations in the chloroplast of leaves from *Q. rotundifolia* twigs when they were incubated with some natural compounds isolated from *E. prunastri*. It has been established that many lichen substances have allelopathic effects against different organisms. Orús *et al.* (1981) found that lichen substances, isolated from *E. prunastri* thalli, interfered with some live. Vicente and Estévez (1976) have studied the action of chloroatranorin on the manganese-dependent photolysis in the chloroplast of *E. prunastri* and found that the drug removes  $Mn^{2+}$  ions resulting in photolysis inhibition. The purpose of this work is to demonstrate that lichen substances were able to penetrate into the chloroplast of *Q. rotundifolia* leaves causing chlorophyll degradation.

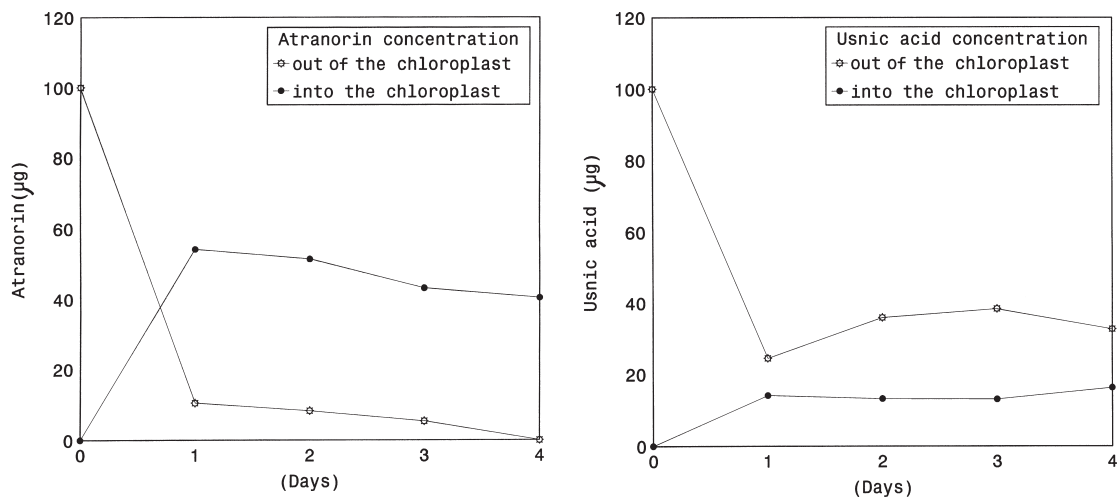


Fig. 1. Permeability of chloroplast membrane from *Quercus rotundifolia* leaves against lichen substances from *Evernia prunastri*.

## MATERIAL AND METHODS

100 µg of usnic acid and 100 µg of atranorin were dissolved in 1.0 ml of acetonitrile, and then were added to 6.0 ml of 0.2% NaCl (w/v). The mixture was shaken during a period of three days in darkness at room temperature, to promote acetonitrile evaporation. 1.0 ml of lamellar suspension was then added, and the mixture was stored 1 day in the dark at 4°C. After that time, the mixture was centrifuged at 7 000 g for 10 min at 0°C. Two phases were obtained: the supernatant, and the precipitate which contained lamellar suspension. 9.0 ml of diethylether:ethyl acetate (65:35v/v) were added in the tube which contained the supernatant and then was vortexed for 2 min. The organic phase was taken off and this extraction step was repeated. At the end, 18.0 ml of organic phase were obtained. They were evaporated to dryness and analyzed by HPLC after to be dissolved with 1.0 ml of acetonitrile. A similar process was followed with the precipitate after breaking lamellar suspension in 2.0 ml of water in a sonic oscillator (20 Hz s<sup>-1</sup>) for 5 min.

Active chloroplasts for DCIPIP photoreduction (Orús *et al.* 1981) were isolated by grinding 1.5 g oak leaves to a fine powder in some sufficient volume of 50 mM phosphate buffer, pH 6.9. The homogenate was filtered through a multilayer gauze and centrifuged at 7 000 g for 10 min.

Degradation of chlorophyll *a* and *b* was observed after their incubation with 0.1 N HCl, 0.1 N NaOH, 0.23 mg ml<sup>-1</sup> evernic acid and 0.23 mg ml<sup>-1</sup> atranorin, in different solutions. The quantity of chlorophyll in a *Quercus rotundifolia* leaf was estimated according to Stain *et al.* (1971).

HPLC analysis of lichen phenolics was performed according to Legaz and Vicente (1983) by using a Varian 5000 liquid chromatograph equipped with a vista CDS 401 computer. Chromatographic conditions were as follows: reverse phase p column (200 mm × 4 mm i.d.), packed with Micro-pack MCH-10; mobile phase, acetic acid:water (2:98 v/v);

acetonitrile (1:4 v/v); flow rate 0.7 ml min<sup>-1</sup>; pressure, 66 atm; temperature 25°C; absorbance range 0.002; detector, UV at 280 nm.

## RESULTS

HPLC analysis of both supernatant and pellet obtained after incubating chloroplasts with usnic acid or atranorin confirms that it is more difficult for usnic acid than for atranorin to traverse through the chloroplast membrane. After one day of incubation of chloroplasts with the corresponding phenol, the amount of atranorin recovered from the pellet was about 3.5 times higher than that of usnic acid, as it is shown in Fig. 1. In both cases, the phenol disappeared from the incubation medium at a rate higher than that of binding to the chloroplast structure.

Isolated chlorophylls from *Quercus rotundifolia* leaves incubated for 90 min with 0.1 N HCl, 0.23 mg ml<sup>-1</sup> evernic acid or 0.23 mg ml<sup>-1</sup> atranorin in different solutions, showed progressive decrease of their concentration in solution, whereas the concentration of chlorophyll control, incubated without additives, remained almost constant during incubation (Fig. 2).

The spectrophotometric analysis of the chlorophyll pool extracted from *Quercus rotundifolia* indicated that the chlorophyll mixture has two absorbance maxima, the main at 430 nm and a sec-

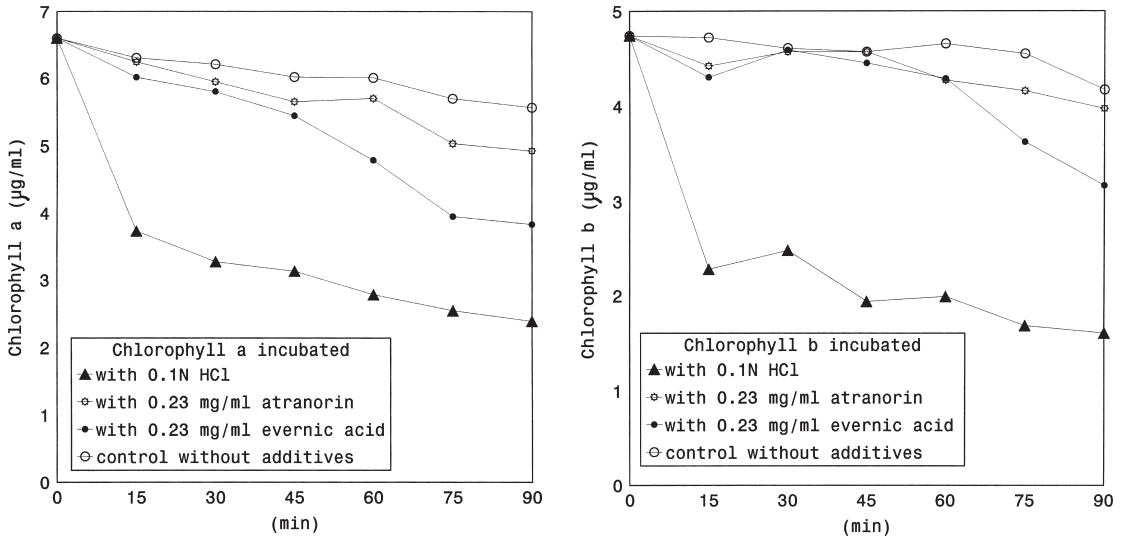


Fig. 2. Chlorophylls *a* and *b* degradation in response to lichen substances.

ondary at 665 nm. The peak of absorbance at 430 nm completely disappeared after incubation of chlorophyll with HCl or evernic acid, whereas the peak of absorbance in the red zone of spectrum was not only attenuated but also displaced to longer wavelength values (about 675 nm). Incubation of chlorophyll with NaOH and atranorin displaced the peak of absorbance in the blue zone towards wavelength values lower than 400 nm. A similar displacement was found for the red zone after NaOH treatment (absorbance maximum at 645 nm), as it is shown in Fig. 3.

## DISCUSSION

Orús *et al.* (1981) found that *Evernia prunastri* phenolics induced a loss of photosynthetic ability of *Quercus rotundifolia* leaves and leaf senescence. These experiments were performed as a chemical simulation of natural epiphytic status by inducing the uptake of mixture of the four main phenolics of this lichen species by detached oak branches. Later, Inoue *et al.* (1983, 1987), Lascève *et al.* (1990) and Vavasseur *et al.* (1991) attribute this loss to the action of usnic acid on spinach, sunflower and *Commelina communis* chloroplasts, respectively. However, usnic acid did not naturally occur inside of parenchyme leaves of *Quercus pyrenaica* (Legaz *et al.* 1988) whereas evernic

acid and possibly other lichen depsides more easily reached photosynthetic tissues (Legaz *et al.* 1988, Monsó *et al.* 1993). These last results are in agreement with those found here, since atranorin seemed to be actively taken by (or bound to) isolated chloroplasts from *Q. rotundifolia* whereas the uptake of usnic acid was severely restricted (Fig. 1).

Experiments performed using isolated chlorophylls indicated that both atranorin and evernic acid produced disappearance of the absorbance maximum at 430 nm in a similar way to that shown after HCl treatment. Since acidic treatments induced a loss of  $Mg^{2+}$  and the consequent pheophytinization, it is possible that chelation of magnesium ions by evernic acid was on the basis of this loss, in agreement with that described by Ascaso and Rapsch (1986). In addition, the absorbance maximum at 665 nm was clearly diminished by evernic acid indicating that some substituents of pyrrolic rings were modified after phenolic treatment (Fig. 3). This decrease was not followed by a shift of the absorbance maximum in a way similar to that found after alkalization of chlorophyll solution. This indicated that evernic acid did not produce transformation of chlorophyll into chlorophyllide.

However, atranorin displaced the absorbance maximum in the blue zone of the spectrum towards 440–450 nm whereas the absorbance maxi-

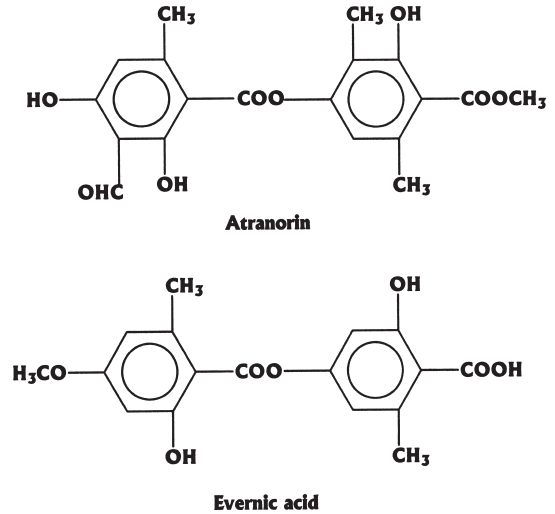
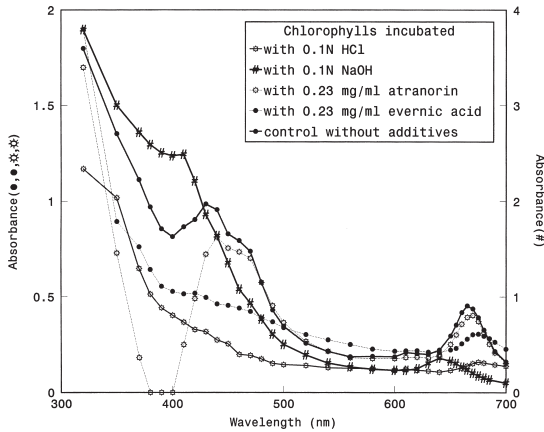


Fig. 3. Maximum of the absorbance of chlorophylls from *Quercus rotundifolia* leaves incubated with and without lichen substances from *Evernia prunastri* (A). Chemical structures of atranorin and evernic acid (B).

mum at 665 nm remained unchanged (Fig. 3). Differences in the chemical structure of both depsides, atranorin and evernic acid, concerning esterification of the carboxyl group in 1 position, possibly decreased the chelating ability of atranorin, so, evernic acid seems to be the sole phenolic able to interfere with phorophyte photosynthesis.

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