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Effects of both urea and light on the ability of accumulation and secretion of proteins and phenolics by *Cladonia verticillaris*

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ABSTRACT

The lichen *Cladonia verticillaris* lives on quartzarenic neosols of Pernambuco (Brazil) in which sugar cane cultures are often established and fertilized by urea. The thalli of the above mentioned lichen are experimentally maintained on different concentrations of urea in light or in darkness, conditions that simulate agricultural uses. The capture of the external urea by the thalli was higher in light than in the dark depending on the urea concentration. Urea inside the thallus is hydrolyzed by induced urease to produce both carbon dioxide and ammonia. This implies an increase of the protein production, a part of which is secreted to the media whereas another part is retained by the thallus. Both secretion and retention of proteins are increased by light. Production of lichen phenolics, protocetraric and fumarprotocetraric acids, increases in light and fumarprotocetraric acid mainly migrates to the cortex. Both production and migration are slightly effected by urea.

KEYWORDS

Cladonia verticillaris;
Fumarprotocetraric acid;
High performance liquid chromatography;
Protocetraric acid;
Urea;
Secreted proteins.

INTRODUCTION

The terricolous lichen *Cladonia verticillaris* is an endemic species of the east coast of Brazil and also occurs in some habitats at the interior of Pernambuco state[1]. This lichen colonized the so-called "Tabuleiros costeiros" (coastal tableland) in which quartzarenic neosols are the main component of the soil. In these soils, several species of the *Cladoniaceae* family, including *C. verticillaris*, commonly appear[2]. This species produces bioactive secondary metabolites, mainly the depsidones fumarprotocetraric (FUM) and protocetraric (PRO) acids[1].

Quartzarenic neosols are very homogeneous soils and the only differences between horizons are due to the accumulation of organic matter in the uppermost 10-15 cm. They cover about 15% of the

"Cerrado" area supporting savannah-like vegetation[3]. In some part of this area sugarcane is cultured. The intensive culture of sugar cane requires a continuous contribution of nitrogen fertilizers and urea is commonly used for this purpose.

Many studies demonstrated a close relationship between lichens and their habitats in the NE of Brazil[4,5], Some species of lichens spontaneously grow on soils enriched with urea in the limits of fertilized crops or in the vicinity of farms where urea appears in the excrement and urine of animals[6]. Since lichens are one of the most sensitive groups of living organisms to nitrogen pollution[7], those growing close to a source of organic nitrogen must necessarily be nitrogen-tolerant species. The influence of an excess of urea on the lichen population growing on the periphery of crops and farms has been usually associated to the release of ammonium produced by bacterial hydrolysis of urea. Since lichens lack roots and take up water, solutes and gases through the entire thallus surface, these organisms respond more sensitively than vascular plants to changes of the atmospheric purity. The tolerance to high levels of atmospheric ammonia depends, among other factors, on the capability of the photobiont to provide sufficient amounts of carbon skeletons for ammonia assimilation[8].

However, many lichen species are able to capture directly urea that is hydrolyzed by thalline urease[9-11]. The hydrolysis of urea produces carbon dioxide which is mainly used to synthesize phenolics[12]. In *Evernia prunasti*, phenolics produce a feed-back inhibition of urease activity by blocking their active thiol groups[13]. When these phenolics are secreted to cortex, urease activity increases again and a greater amount of urea can be metabolized[14]. Since discrete amounts of these cortical phenols can be washed by rainfall and accumulated in the soil, their biological activities as bioherbicides[1] can alter the growth and occurrence of many plant species in the affected geographical area.

The aim of this work was to study the ability of absorption and accumulation of urea and the production and mobilization of PRO and FUM by *C. verticillarias* grown near sugarcane plantations, when lichen thalli directly floated on urea are maintained in darkness or white light conditions as an experimental approximation to the agraecological actions of lichens.

MATERIALS AND METHODS

Biological material

Cladonia verticillaris (Raddi) Fr., was collected from sandy soils in the microarea of the "tabuleiro" (broad) of Mamanguape (Paraíba, Brazil). Thalli were dried in air flow and stored in the dark, at room temperature, no more than two weeks. Voucher specimen has been deposited in the Herbário-UFP-Geraldo Mariz, Universidade Federal de Pernambuco (Brazil), register number 65726.

Quantation of urea retained by lichen thallus or remaining in the media

C. verticillaris thalli (1.0 g dry weight) were washed twice with distilled water and floated on 20 mL of different urea solutions (20, 40 and 60 mM) in 75 mM sodium phosphate buffer, pH 6.9, in the dark or under white light (100 μmol m^{-2} s^{-1}) from 0 to 24 h at 25 °C. As a control, 1.0 g dry thalli were incubated in the same buffer and conditions in absence of urea. At the indicated times, thalli were dried with filter paper and macerated in a mortar with liquid nitrogen. Powders were dispersed in 10 mL distilled water and centrifuged at 12,000 × g for 15 min at 4 °C. To each supernatant, 5 mL 10% (w/v) TCA were added and newly centrifuged in the same conditions. Acidified supernatants were neutralized with 10 mM NaOH and used to quantify urea with diacetyl monoxime according to the method described by Rahmatullah and Boyde [15] with modifications. Media after incubation were also treated with TCA and remaining urea was quantified as above. Experiments were achieved in triplicate.

Quantitation of proteins, PRO and FUM in thalli

Similar samples of *C. verticillaris* thalli (1.0 g dry weight) were incubated in the same experimental conditions as above. At the indicated times, thalli were dried with filter paper and secreted PRO and FUM were quantified in the media containing urea. Then, the thalli were extracted with 10 mL distilled water for 3 h at 35 °C. The aqueous extract was centrifuged at 12,000 × *g* for 15 min at 4 °C, the

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precipitate discarded and the supernatant used to determine secreted proteins and water-soluble PRO and FUM. Then, the thalli were macerated in a mortar with liquid nitrogen. Powder was extracted with 10 mL of distilled water for 20 min, centrifuged as above, and the supernatant was used to estimate soluble proteins in the thallus. The solid precipitate was then extracted with 20 mL pure acetone for 15 min at 30°C and continuous shaking, and centrifuged as above. Then, the precipitate was discarded and the acetonic extract was evaporated to dryness in a speedvac concentrator. To the dry residue, 5 mL of distillate water were added and the homogenate was centrifuged in the same conditions. The supernatant was used for quantitation of both PRO and FUM. Quantification of proteins in the extracts was performed by the method of Lowry *et al*.[16] after precipitation with trichloroacetic acid[17]. Quantification of PRO and FUM was performed after separation by HPLC. Experiments were achieved in triplicate.

HPLC analysis of PRO and FUM

High performance liquid chromatography separation of lichen phenolics was carried out using a liquid chromatograph (Spectra Physics 8810) as described by Santiago *et al*.,[18] but using an isocratic, instead of a gradient, elution profile Analytical conditions were as follows: column, Tracer Excel 120 ODSB (25 cm×4.6mm internal diameter); injection, 10 µL; mobile phase, solvent A: acetonitrile (100%) and solvent B: acetic acid/water (2 : 98, v/v), A:B, 70:30 v/v, at a flow of 1 mL·min^{-1}, temperature, 25°C; absorbance unit full scale, 0.005; detector, UV-Vis SP8490 (λ=270 nm); internal standard, salicylic acid, 0.5 mg/mL. Quantitation of each phenol was done by using the slope of the straight line obtained by linear regression from different injected mass of the standard phenol and their corresponding area counts. FUM standard was isolated and purified from *C. verticilliaris* as was described by Pereira[19]. PRO standard was isolated and purified from *C. verticillaris* as described by Tigre *et al*.,[1].

Statistical analysis.

Statistical analysis was performed using the multiple ANOVA test followed by post hoc analysis with Tukey's honest significant differences test. Differences were considered to be significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Urea uptake by lichen thalli

Thalli of *C. verticillaris* used in this work initially contained an amount of urea of about 36.54 µg·g^{-1} fresh weight (0.61 nmoles·g^{-1}). The incubation of the thalli on different urea concentrations was followed by the disappearance of this urea from the media. When incubation was carried out in the dark, the rate of urea consumption diminished with the increase of the concentration of the supplied compound. This is, the rate of urea consumption from an external solution of 20 mM was 1.02 mg h^{-1}, 0.96 for 40 mM urea and 0.56 mg h^{-1} for 60 mM urea (Figure 1A and TABLE 1). Nevertheless, light accelerated the uptake of urea, from 0.76 mg h^{-1} for 20 mM urea up to 1.34 mg h^{-1} for 60 mM urea (Figure 1B and TABLE 1). This compound completely disappeared from the media only during the incubation of lichen thalli in the dark on 20 mM urea for 24 h, whereas the remaining urea represented a considerable amount in the other treatments.

It is not easy to explain the action of light on the uptake of urea by the lichen thalli. In free living algae, urea uptake seemed to be independent on the availability of energy provided by light[20]. On this basis, it has been proposed the occurrence of aquaporins to regulate the permeability of plant membranes to water and small, uncharged molecules[21]. However, Prado et al.[22] found that the conductivity of the aquaporin water channels increased in the dark and decreased in light due to the phosphorylation of

TABLE 1 : Uptake and accumulation of external urea by *Cladonia verticillaris* thalli

Condition	Dark		Light	
[Urea] mM	Uptake (mg h^{-1})	Accumulation (µg h^{-1})	Uptake (mg h^{-1})	Accumulation (µg h^{-1})
20	1.02 ± 0.08	1.66 ± 0.11	0.76 ± 0.06	0.51 ± 0.04
40	0.96 ± 0.10	7.3 ± 0.55	1.06 ± 0.13	1.34 ± 0.15
60	0.56 ± 0.04	57.0 ± 3.17	1.34 ± 0.11	28.31 ± 3.02

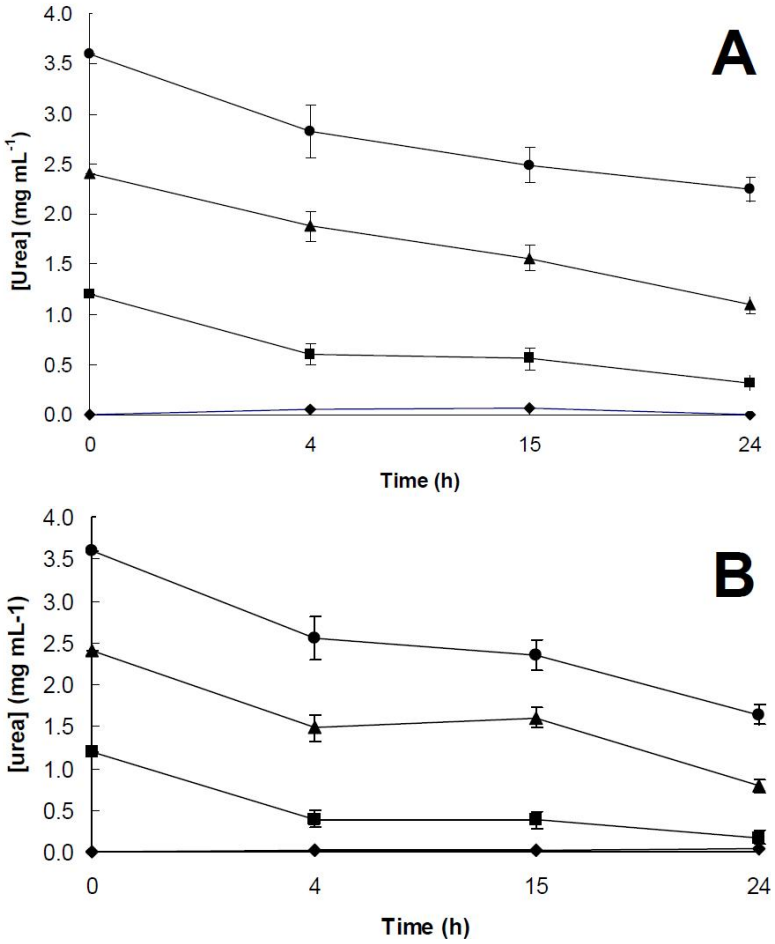

Figure 1 : Remaining urea in the media after incubation of *C. verticillaris* thalli in the dark (A) or in light (B). (◆) 0 mM urea; (■) 20 mM urea, y = -0.051x + 23.32; r^2 = 0.92 in the dark and y = -0.038x + 0.91; r^2 = 0.84 in light; (▲) 40 mM urea, y = -0.048x + 2.26; r^2 = 0.97 in the dark and y = -0.053x + 2.13; r^2 = 0.87 in light; (●) 60 mM urea, y = -0.028x + 0.99; r^2 = 0.85 in the dark and y = -0.067x + 3.27; r^2 = 0.91 in light. Values are the mean of three replicates ± standard error and adjusted to a straight line by linear regression

two serine residues in one of the membrane intrinsic proteins. In fact, *Cladonia sandstedei* thalli efficiently internalized urea in the dark[23], but, according to the results showed herein, the uptake of urea by *C. verticillaris* was positively effected by light. This seemed to indicate a passive diffusion process rather than a facilitated transport. Light could simply increase the disorder degree of the bilayer membranes as a consequence of warming to facilitate the entry of this compound into the lichen cells.

C. verticillaris thalli metabolized or accumulated urea following its disappearance from the incubation media, depending on its concentration and on the darkness or light conditions. In the dark, the kinetic of urea accumulation in thalli was very similar to the kinetic of urea disappearance from the media (Figure 2A) during the first 4 h of incubation. In light, a different behaviour was observed (Figure 2B). When lichen thalli were floated on 20 and 40 mM urea, total urea internalized by the thalli was almost metabolized since the remaining urea in thalli was similar to that found for the control in absence of exogenous urea. Only those thalli floated on 60 mM urea accumulated this compound in a similar way to that found during darkness incubation, although at lower values.

Accumulation and secretion of proteins

C. verticillaris synthesized an induced urease that hydrolyzed urea to produce NH_3 and CO_2. The enzyme was partially secreted to the media although this secretion was nullified after 24 h incubation on urea. Enzyme induction by urea was nullified at this time value in the dark but continuously increased in light[14]. Thus, it could be concluded that the hydrolysis of urea was mainly carried out inside the thallus.

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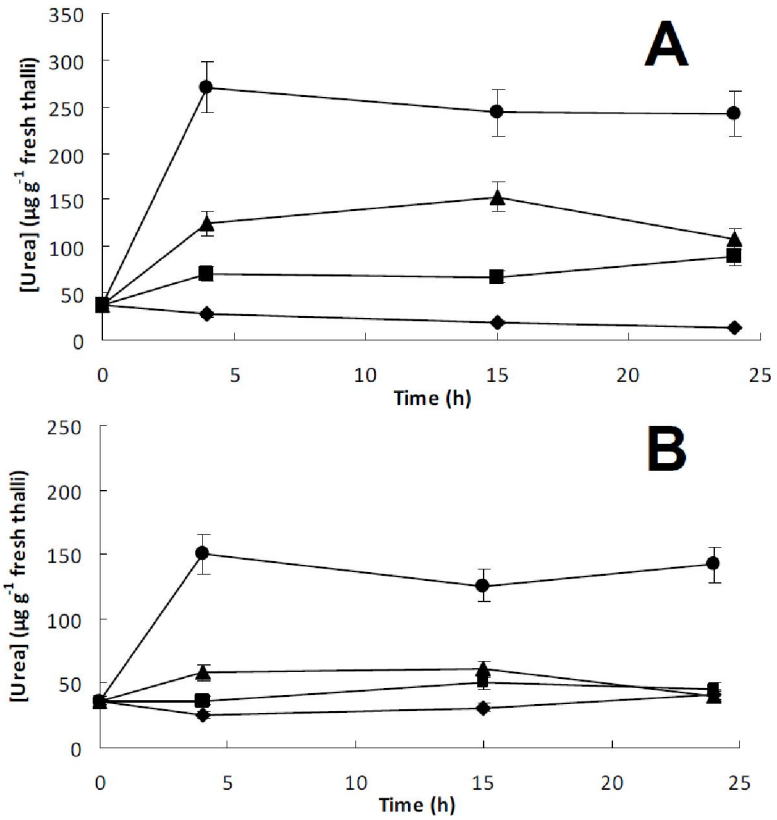

Figure 2 : Accumulated urea in the thallus after incubation of *C. verticillaris* thalli in the dark (A) or in light (B). (◆) 0 mM urea; (■) 20 mM urea, y = 1.66x + 50.0; r^2 = 0.84 in the dark and y = 0.51x + 36.68; r2 = 0.81 in light; (▲) 40 mM urea y = -0.60x2 + 16.81x + 48.24; r^2 = 0.91 in the dark and y = 1.34x + 43.30; r^2 = 0.78 in light; (●) 60 mM urea, y = 58.54x + 36.54; r^2 = 1.0 (initial segment from 0 to 4h in the dark) and y = 28.31x + 36.54; r2 = 1.0 (initial segment from 0 to 4h in light). Values are the mean of three replicates ± standard error and adjusted to a straight line by linear regression

Carbon dioxide and ammonia would be used for the synthesis of nitrogen compounds (mainly amino acids and proteins) and to produce the carbon skeletons of secondary lichen metabolites[12].

The incubation of the thalli on urea always increased the amount of proteins secreted to the media in the dark, though only the incubation on 20 mM urea promoted a level of protein secretion higher than that observed in the controls incubated without urea (Figure 3A). However, the light raised the levels of secreted protein and, in this case, all the concentrations of urea assayed herein induced a level of secretion higher than that observed in the controls without urea. Nevertheless, the highest level of secretion of protein in light was induced by 20 mM urea (Figure 3B). The amount of protein retained by the thalli remained more or less constant during 24 h incubation but 60 mM urea slightly diminished the accumulation of protein in the lichen thallus (Figure 4A). On the contrary, the amount of proteins retained by the thalli in light became maximum for 40-60 mM urea, being lower those reached by thalli incubated in 20 mM urea (Figure 4B). It can then concluded that the liberation of ammonia by the hydrolysis of urea could be used to increase the amount of protein, mainly in light conditions.

Production and distribution of phenolic acids

When urea is hydrolyzed inside the thallus, the lichen also produced phenolic substances which migrates in part from the medulla to the cortex to protect the lichen thallus against the excessive radiation[24, 25] and sometime they are leached by rainfall[26]. The main phenolics produced by *C. veticillaris* are PRO and FUM[5]. The accumulation of these phenolics in the thallus, secreted to the cortex and the amount of those dissolved in the media for each experimental condition are shown in Figures 5-7.

Fresh thalli initially contained a small amount

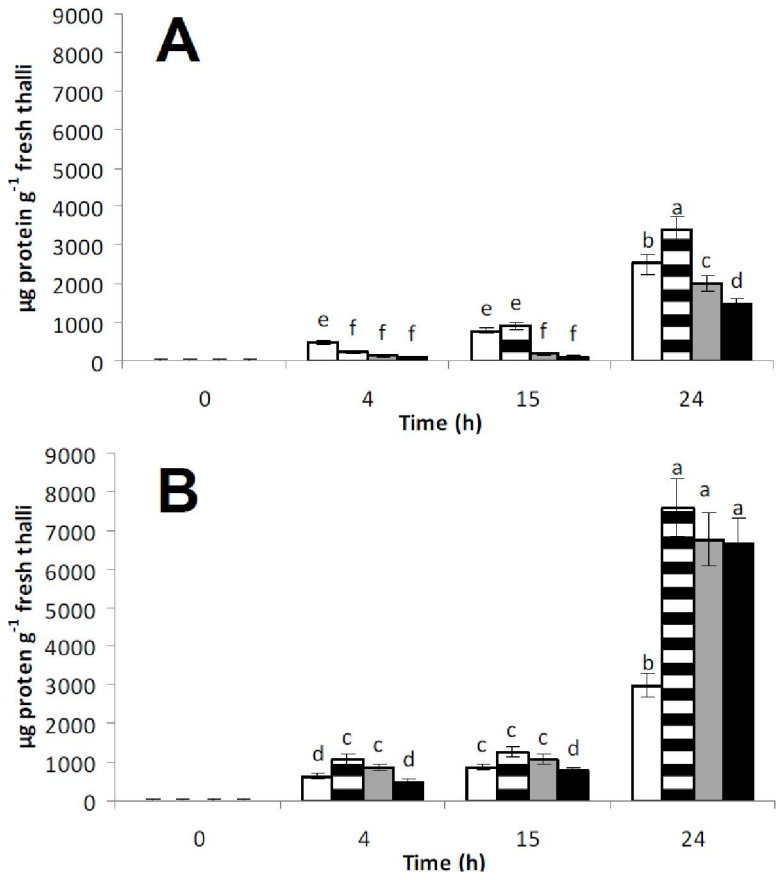

Figure 3 : Secreted protein to the media after incubation of *C. verticillaris* thalli in the dark (A) or in light (B). (white) 0 mM urea; (ruled) 20 mM urea; (grey) 40 mM urea, and (black) 60 mM urea. Values are the mean of three replicates ± standard error Different letters indicate significant differences (P < 0.05)

of PRO (6,7 µg·g^{-1} dry weight, 0.018 µmol·g^{-1}), and a high amount of FUM (0.66 mg·g^{-1} dry weight, 1.38 µmol·g^{-1}), approximately 100 times more FUM than PRO. During the first 4 h of incubation on phosphate buffer, pH 6.9, in the dark, the metabolism of lichen phenolics was activated and the amount of intrathalline PRO increased to decrease later (Figure 5 A and B). This decrease was more rapid in light than in the dark. In contrast, the accumulation of FUM in the dark decreased in absence of urea (Figure 5C) but it was efficiently recovered after 24h of thalli incubation of the thalli on 40 mM. In light, the levels of FUM accumulation were kept more or less constant with independence on the time of incubation and on the concentration of urea used (Figure 5D).

The amount of PRO adhered to the cortex and released after a water wash was higher in light (Figure 6B) than in darkness (Figure 6A), without appreciable differences in the amounts of this phenol obtained for different urea concentrations. The highest values of cortical PRO were obtained at 24 h incubation (Figure 5B). This is in agree with that described by Legaz et al.[27] and Stark et al.[25] in relation to the superficial deposition of lichen phenolic in order to produce a protective screen against irradiation. In the dark, the titer of FUM adhered to the cortex increased after 4h incubation on urea. From 4 to 15 h, the concentration of FUM in the cortex increased without significant differences for the different concentrations of urea. The highest amount of FUM superficially deposited on the cortex was obtained at 24 h of incubation on the buffer alone or 20 mM urea (Figure 6C). In light, the amount of cortical FUM increased with the incubation time on buffer. Nevertheless, urea always diminished the deposition of this depsidone on the cortex with respect to the values obtained in absence of urea (Figure 6D).

Lichen thalli incubated on phosphate buffer in the dark leached small amounts of PRO at any time (Figure 7A). Urea extensively impeded this leachate since only a small amount of this depsidone was

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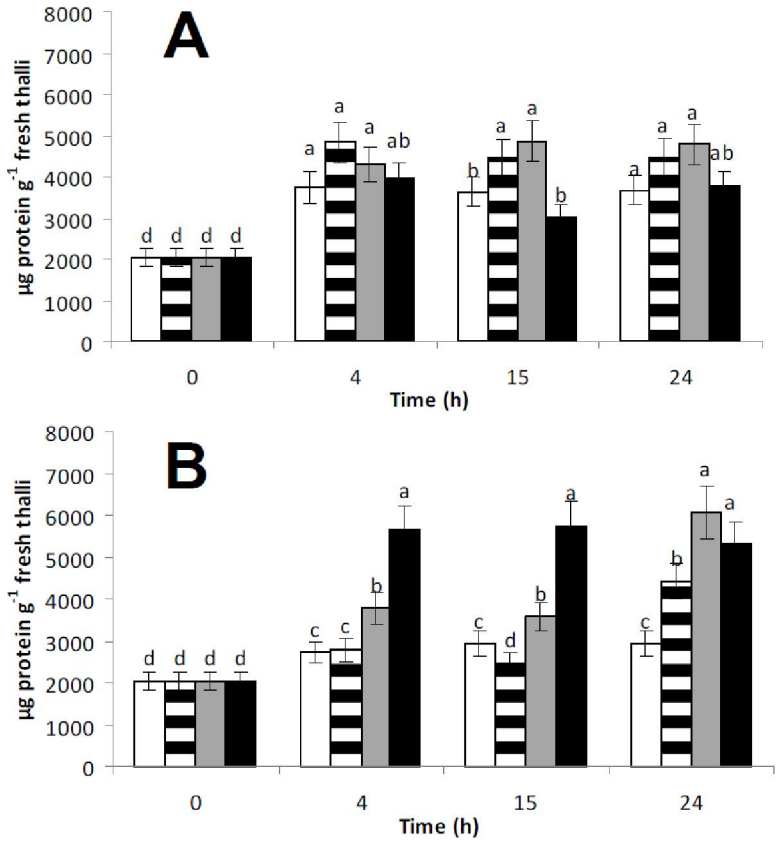

Figure 4 : Retained protein by the thallus after incubation of *C. verticillaris* thalli in the dark (A) or in light (B). (White) 0 mM urea; (ruled) 20 mM urea; (grey) 40 mM urea, and (black) 60 mM urea. Values are the mean of three replicates ± standard error. Different letters indicate significant differences (P < 0.05)

released to the medium containing 60 mM urea at 24 h incubation. In the same condition, small amounts of FUM were found in the buffer and very similar amounts of this depsidone were found in media containing urea from 15 to 24 h incubation (Figure 7C). Light accelerated the release of both PRO and FUM to the media in light but the recovery of both depsidones from the incubation media in these conditions was an inverse function of the concentration of urea supplied. Control treatment in light leached less PRO than in the dark but incubations on urea significantly increased the amount of PRO leached to the media after 24h incubation (Figure 7B and D) but FUM decreased when urea concentration was increased. The highest amounts of FUM leached to the media were found during thalli incubation on the buffer alone (Figure 7D).

The results showed in the Figure 5 are in agree to that found by Blanco et al.[12] (1984), which affirm that the carbon dioxide produced by the hydrolysis of the urea inside the lichen thalli is preferably used for the synthesis of phenols in the dark whereas in light, the use of this CO_2 must be distributed between the synthesis of phenols and its photosynthetic assimilation by the alga partner. This effect of the light is more visible for FUM than for PRO, since this latter depsidone is transformed into FUM by the combined action of two enzymatic systems that use succinyl-CoA as a precursor of the 4C adduct to the FUM[28]. Comparing the results showed in the Figures 5 and 6, it can be deduced that the PRO is preferably retained by the lichen thallus whereas the FUM migrates easier to the cortex, in which it settles. This deposition seems to be independent from the concentration of urea supplied but positively affected by light, as previously found by Mateos et al.[29] (1993). The low concentration of both depsidones found in the water leachates is the consequence of the scanty solubility of these phenols in water[30].

The agroecological consequences of the facts found here are clear. The use of urea as a fertilizer in cultures of sugar cane involves the partial use of this nitrogenous compound by terricolous lichens growin who grow in the boundaries of the cultures.

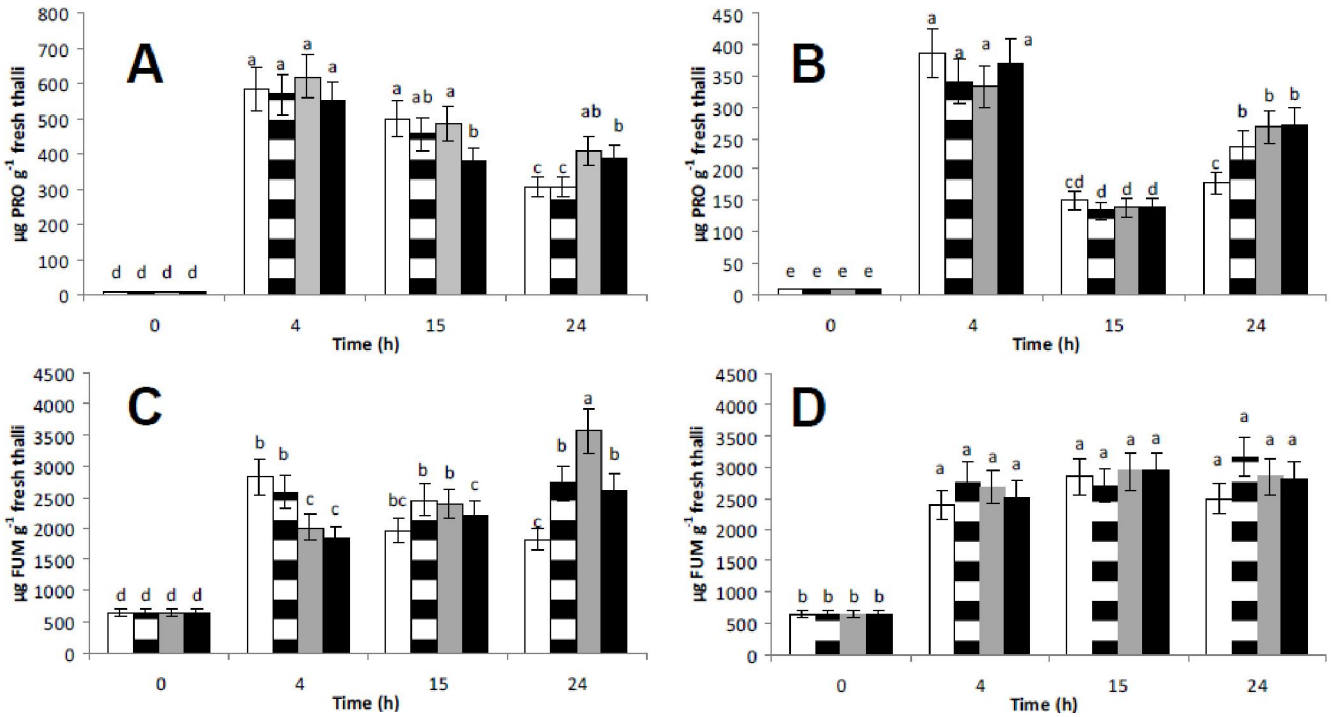

Figure 5 : Retained protocetraric acid (A,B) and fumarprotocetraric acid (C,D) by the thallus after incubation of *C, verticillaris* thalli in the dark (A,C) or in light (B,D), (White) 0 mM urea; (ruled) 20 mM urea; (grey) 40 mM urea, and (black) 60 mM urea, Values are the mean of three replicates ± standard error, Different letters indicate significant differences (P < 0.05)

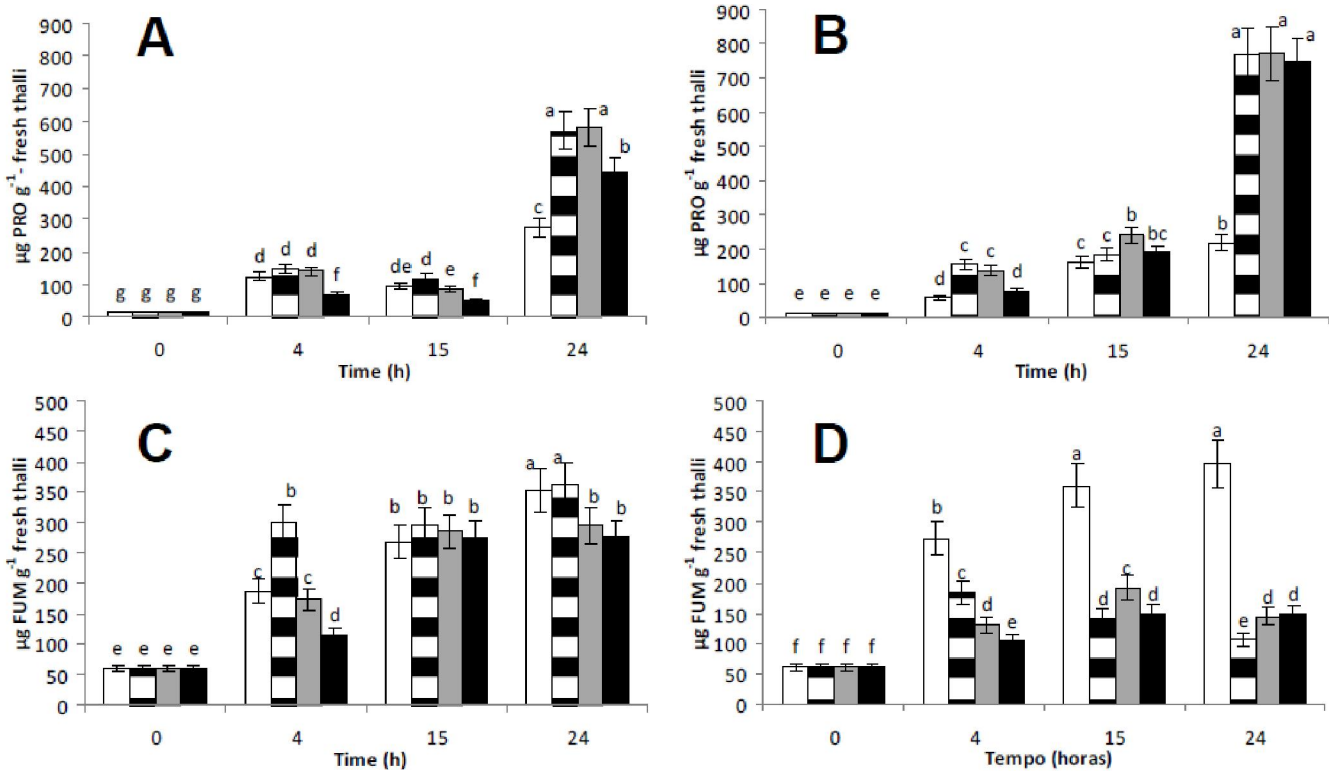

Figure 6 : Cortical protocetraric acid (A,B) and fumarprotocetraric acid (C,D) in the thallus after incubation of *C, verticillaris* thalli in the dark (A,C) or in light (B,D), (White) 0 mM urea; (ruled) 20 mM urea, (grey) 40 mM urea, and (black) 60 mM urea, Values are the mean of three replicates ± standard error, Different letters indicate significant differences (P < 0.05)

Urea then can be used by these lichens increasing the production lichen phenolics that are washed by

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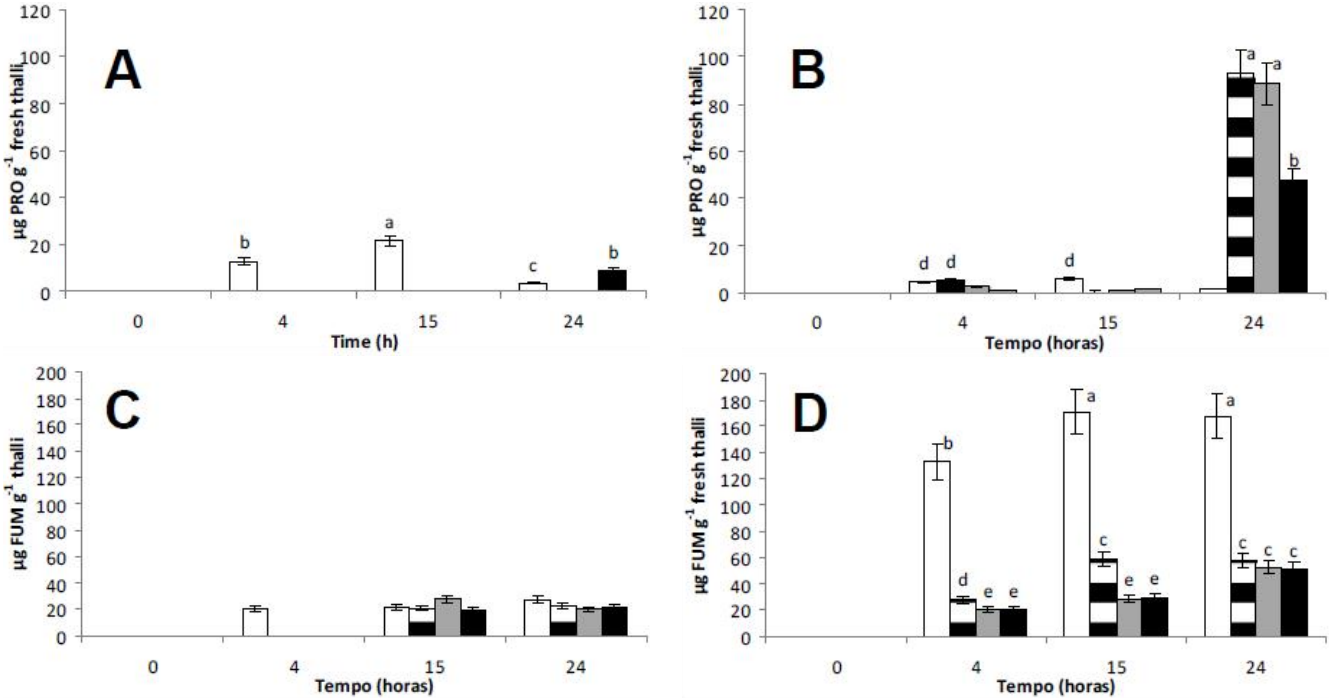

Figure 7 : Protocetraric acid (A,B) and fumarprotocetraric acid (C,D) leachated from the thallus to the media after incubation of *C, verticillaris* thalli in the dark (A,C) or in light (B,D), (White) 0 mM urea; (ruled) 20 mM urea; (grey) 40 mM urea, and (black) 60 mM urea, Values are the mean of three replicates ± standard error, Different letters indicate significant differences (P < 0.05)

rain or irrigation and retained in the soil. The chemical stability of these phenols in the soil, joined to their potential activity as bioherbicides could interfere with the growth of the cultured plants or prevents the growth of bad grasses.

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