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Effect of soil moistureon the percolation of lichen substances from *Cladonia verticillaris* (Raddi) Fr. in a quarzarenic neosol from Brazil

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ABSTRACT

Cladonia verticillaris (Raddi) Fr. is a lichen species that grows on sandy soil of tablelands coexisting with savannah-like vegetation in Brazil. This species produces different lichen phenolics that are leached to the soil by the rainwater and exhibit several biological activities that could affect the habitat. The effect of soil moisture in the retention and percolation of proteins, protocetraric acid (PRO) and fumarprotocetraric acid (FUM), the main phenolics produced from *C. verticillaris*, was analyzed in this work. Proteins were retained in dry soil but they percolated when the soil was water-saturated. FUM was strongly retained in this kind of acidic soils and transformed into PRO by spontaneous hydrolysis when the soil moisture increased. This study demonstrates that proteins, PRO and FUM that were effectively leached to the soil, retained, transformed or percolated to deeper soil layers depending on the amount of previous rainfall. This fact must be taken into account in order to consider the biological effects of these substances on the vegetation of coastal tableland Brazil.

KEYWORDS

Coastal tableland;
Fumarprotocetraric acid;
HPLC;
Lichen phenolics;
Protocetraric acid.

INTRODUCTION

Theso-called "Tabuleiros Costeiros" (coastal tableland) are one among thetwenty majorlandscape unitsestablished in Brazil,determined from the morphostructural, geomorphological and geographical characteristics of the areain which they appear[1]. Tableland is composed by sedimentary soils where the most commonly occurring are latosols and urtisols[2]. These soils generally have low content of organic matter, low capacity to retain water and nutrients, low cation exchange capacity, low base saturation, acidity increasing with the depth, sandy texture, predominance of kaolinite in the clay fraction and a fragile, physical structure[3,4]. Quartzarenic neosols can appear in some areas; these soils, without lithic contact within 50 cm deep, present a sandy texture in all the horizons to a depth of 150 cm[5]. Quartzarenic neosols are very homogeneous 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 (savannah-like vegetation).

In these soils, several lichens species of the *Cladoniaceae* family, including *C. verticillaris*, commonly appear[6]. This species produces bioactive secondary metabolites, mainly fumarprotocetraric (FUM) and protocetraric (PRO) acids.*C. verticillaris* is an endemic species in the east coast of Brazil and also occurs in some habitats at the interior of Pernambuco state, Brazil[7].

Lichens produce a great variety of secondary metabolites and most of those are unique in the plant kingdom. Their biological activity concerns to algal photoprotection against intense radiation, as well as allelochemical, antiviral, antitumor, antibacterial, antiherbivore, antioxidant, antipyretic and analgesic actions[8]. Many studies demonstrated the close relationship between lichens and their habitats in the NE of Brazil. Recently Silva et al[9]. showed that FUM from *C. verticillaris*, interacts with the rocky or soil substrate forming chelates and modifying them by chemical action. Silva[10] demonstrated that lichen substances from *C. salzmannii*, leached out to the soil of these regions, influence on soil microbial activity as well as on the occurrence of arbuscular mycorrhizal fungi. But the evidence of the antimicrobial activity of lichen secondary substances seems to be weak in comparison with other suggested functions, such as light filtering screen and protection again herbivore[11]. Silva and Pereira[12] reported that phenols from *C. verticillaris*,leached out by rainwater, promote changes of the chemical composition of the soil, such as a pH decrease and alteration of the levels of hydrogen and aluminum. This fact suggests a direct connection between the lichen, the soil and the plants. Since lichen substances affect soil chemical composition and microbial population, they play an ecological role in the distribution of vegetation.

The aim of this work was to study the percolation dynamics of PRO and FUM from *C. verticillaris* in a typical quartzarenic neosol of the coastal tableland of the NE of Brazil depending on the soil moisture in order to study the influence of the lichens occurrence in the ecosystem they inhabit.

TABLE 1 : Some characteristics of soils used in the experiments

pH	4.85
Cation exchange capacity (cmol/dm3)	6.8
Base saturation (%)	12
Field moisture capacity (g H2O/g soil)	0.25
Coarse sand (%)	57
Fine sand (%)	28
Silt (%)	15.8
Clay (%)	1.2
Organic matter g/Kg soil	9.8

MATERIAL AND METHODS

Experimental soil

A quartzarenic neosol[5] from the "Cerrado" region was sampled in the upper horizon (0-20 cm) from an area in which *C. verticillaris* was growing at Mamanguape region in the NE of Brazil (6º 50´ 19´´south latitude and 35º 8´ 11´´west longitude) Soil samples were dried in air at 25ºC until required. The main physical and chemical properties of the soil were determined and listed in TABLE 1.

X-ray diffractometric (XRD) analysis of soils

Analysis of soil samples by energy dispersive X-ray spectrometry (XRD) was realized in the Laboratório de Tecnología Mineral eAmbiental, Center of Technology and Geociências, UFPE. The sampleswere sieved through sieves of 45 μm pore, equivalent to 345 meshes,and after this, softened in mortar for mechanical disintegration. Assayswere realized in a Bruker D2 Phaser diffractometer to 30 kV and10 mA, with irradiation of Cu-Kα1 = 1.54060 Å using a detectorBruker-Lynxeye. The band of reading was 2 θ, consisting of the emissionof a bundle of X-rays through a sample sheet of 0,4 mmof thickness, inangles that change between 4° and 80°, with intervals of 0.0202°s–1, to20 rpm and a time of counting for step of 0.5 s for sample. The obtainedpeakswill be related to the chemical elements contained in the samplesof analyzed material[13]. The information was graphically representedusing the software Bruker Diffrac. suite Eva, identifying and quantifyingthe samples by means

of a bank of information for standards, COD —Crystallography Open Date back.

Scanning electron microscopy (SEM)

Samplesof sandy soils, were washed with distilled water and fixed in 3% (v/v)glutaraldehyde-0.1 M buffer phosphate, pH 6.7, during 6 h, washed inthe same buffer, post-fixed with 1% (w/v) osmium tetroxide for 2 hand dried in a series of acetone solutions. Finally, cells were driedat the critical point, covered with gold/palladium and scanned at20 kV by using a JEOL JSM 6400 SEM (Japan). The digital images were obtained using an INCA camera and software (Oxford) joined the system[13].

Preparation of lichen extracts

C. verticillaris was collected at the same region in which the soil was sampled. Thalli were dried in air flow at 25°C and stored in the dark until required. Lichen substances were obtained by washing 6 g of thalli with 50 mL pure acetone for 15 min at 30°C withcontinuous shaking. The acetone extract was saved to use later. For the extraction of proteins, the same thalli were dried and macerated with 50 mL distilled water and the homogenate centrifuged at 10,000 ×*g* for 15 min at 4 °C. The pellet was discarded and the supernatant mixed with the acetone extract saved before and evaporated to dryness in a speedvac concentrator. To the dry residue, 50 mL of distilled water were added and the homogenate was centrifuged at 10,000 ×*g* for 15 min at 4°C. The pellet was discarded and the supernatant (hydrosoluble extract) was used for soil percolation assays. When indicated, hydrosoluble extracts from *C. rangiferina* (L.) F. H.Wigg collected in Puerto de San Glorio, Spain (UTM 30TUN5769) was used. These extracts were prepared as described for *C. verticillaris*. Quantification of proteins in the hydrosoluble extract was performed before to be mixed with the acetone extract, by the method of Lowry et al[14]. after precipitation of proteins with trichloroacetic acid[15]. Quantification of PRO and FUM were performed after separation by HPLC.

HPLC analysis of phenolics

HPLC separation was carried out as described by Santiago et al[16]. but using an isocratic elution A:B, 70:30 v/v instead of a gradient; mobile phase, solvent A: ACN (100%) and solvent B: acetic acid/water (2/98, v/v) at a flow of 1 mL/min. 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 phenolsaginst their corresponding area counts. FUM standard was isolated and purified from *C. verticilliaris* as described by Pereira[17]. PRO standard was isolated and purified from *C. verticillaris* as described by Tigre et al[7]. Atraronin (ATR) was purchased from Sigma-Aldrich® Chem. Co (St. Louis, USA). In order to analyse the possible interconversion of some phenolics, one in other, an hydrosoluble extract from *C. rangiferina* containing 0.42 mg/mL (0.89 μmol/mL) of FUM, and 0.41 mg/mL (1.09 μmol/mL) of ATR was prepared. This preparation, as well as three successive dilutions in distilled water, 1:1 v/v; 1:3 v/v and 1:7 v/v were analyse by HPLC in order to quantify PRO, FUM and ATR

Soil percolation experiments

Plexiglas columns (10 cm × 4 cm i.d) were filled with 90 g of the experimental soil and two different experiments were performed. In the first case, distilled water was added to the soil in order to maintain the field moisture capacity (FC) at 100% (wet soil experiments). In the other case, no water was added to the column (dry soil experiments). To both classes of soils, 10 mL of a hydrosoluble extract from *C. verticillaris* containing 15.1 μg of proteins, 275 μg (0.68 μmol) of PRO and 491 μg (1.04 μmol) of FUM was added on the top of both columns. Later, successive volumes of 10 mL distilled waterwere added to a total of 70 mL. Percolated extract was collected as fractions of 10 mL. Quantitation of total proteins, PRO, and FUM was done for each collected fraction. Experiments were repeated three times with different soil samples.

RESULTS AND DISCUSSION

Soils from Manguape mainly contained quartz, SiO_2, in the horizon C (from 15–20 cm of depth) and relatively low concentrations of other materials in

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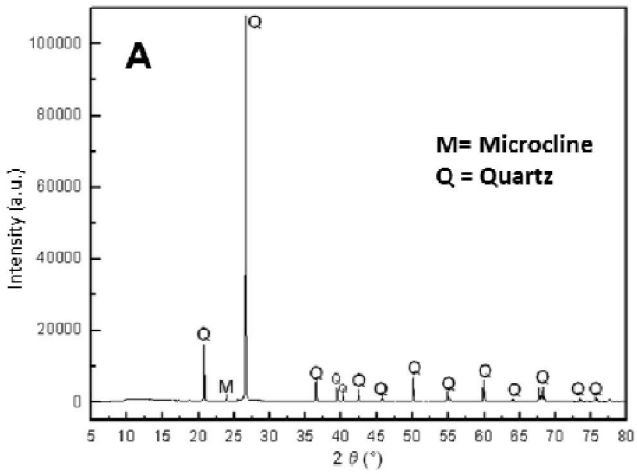

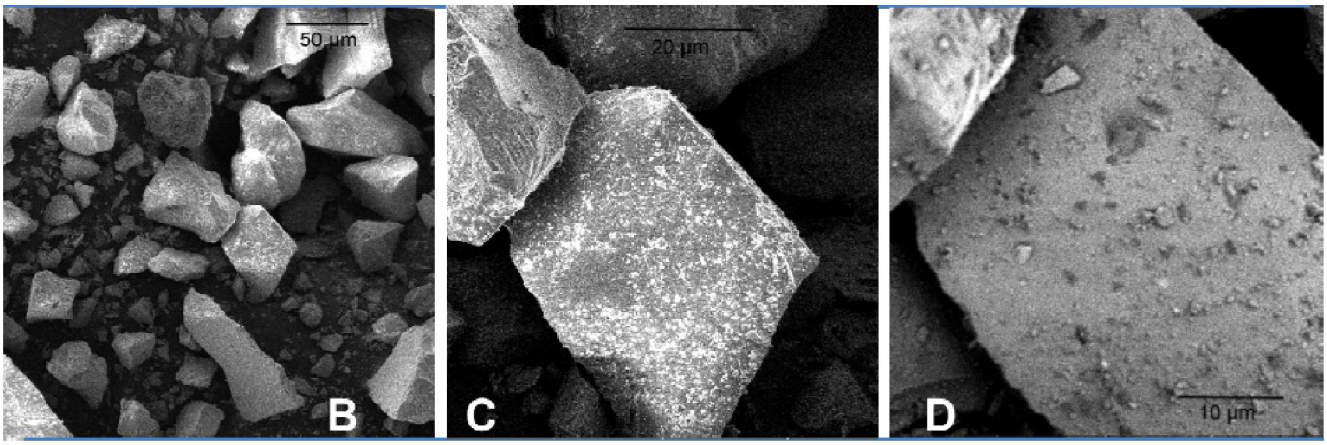

Figure 1 : A) XRD analysis of Mamanguape soil samples collected in the upper horizon (0-20 cm) from the A horizon showing that silica is the main component of these soils. Q= quartz; M= microcline. B-D) Scanning electron microscopy ofMamanguape soil samples, SiO_2 particlesm collected in the upper horizon (0-20 cm) at three different degrees of magnification

the horizon a (0 to 15 cm of depth), such as microcline, $KAlSi_3O_8$ (Figure 1A). This predominance of quartz in the Mamanguape soils is characteristic of quartzarenic neosols, as described in the literature[13,18]. The intensity of XRDresponse for the different components confirmed the predominance ofquartz as the main mineral present in these samples. Quartz crystallized in the trigonal trapezohedric system (Figure 1B-D) whereas microcline does it in the triclinic, pinacoidal system.

Substances leached from *C. verticillaris* differently interacted with soil particles depending on the hydration degree of the quarzarenic neosol. Figure 2 shows the profiles of percolated proteins (A and B), PRO and FUM (C and D) when an hydrosoluble extract of *C. verticillaris* was added to the columns packed with both kinds of samples, at 100% FC as well as dry soils. The total amount of proteins recovered in the seven fractions (70 mL volume) eluted from wet soil was 26.69 µg, this is 77% more than the amount added to the column. However, the amount of proteins recovered in the first four fractions was almost the same that was added (15.68 µg) (Figure 2A).

Dry soil needs 30 mL of distilled water to begin the percolation process. Just in the first eluted fraction, the same amount of proteins that was obtained in the experiment with wet soil (4.06 µg), was recovered (Figure 2B). In this case, it was necessary to percolate 70 mL of water to recover almost the same amount of protein added to the column (15.83 µg recovered compared to 15.1 µg added). The percolation profiles of FUM and PRO were very different in both experimental conditions (Figure 2C

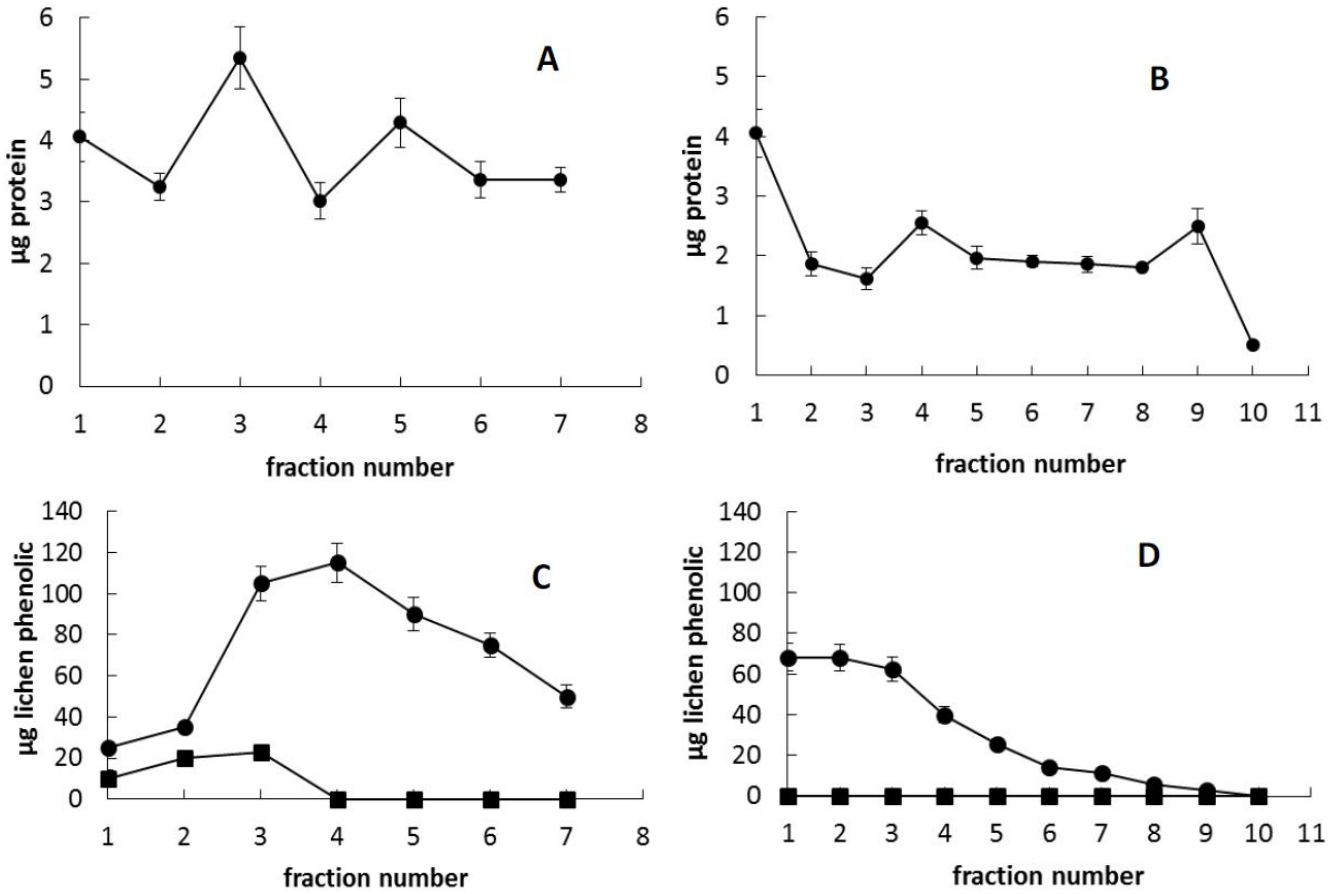

Figure 2 : Elution profiles of proteins (A and B) and PRO and FUM (C and D) in wet, 100% FC (A and C) and dry (B and D) soils

and D). From the wet soil, the amount of recovered PRO was increasing until fraction 4 to decrease later. These four fractions contained 280 µgof PRO, about 100% of thee total added to the column, whereas 215 µg of PRO were recovered in the three next fractions. Total amount of PRO recovered in the seven fractions (70 mL) was 495 µg, 80% more than that added to the column (Figure 2C).

Percolation behavior of FUM was different to that observed for PRO, since the first depsidone was only detected in the first three fractions of eluate (Figure 2C). Total amount of recovered FUM was 53 µg that represent 10.8% of the initial added amount. Only PRO was percolated through dry soil and recovered in the seven fractions. In total, 289.7 µg were percolated, this is, 5.1 % more than the amount charged onto column. With independence on the soil moisture, FUM seemed to be retained whereas PRO percolated, even in an amount higher than that added to the beginning of the experiment.

Bearing this in mind, it was analyzed if the increase in soil water content could produce any transformation of a phenolic into another. To verify this hypothesis, an hydrosoluble extract obtained from *C. rangiferina* containing 0.89 µmol/mL of FUM and 1.09 µmol/mL of ATR and their corresponding serial dilutions 1:1 v/v; 1:3 v/v and 1:7 v/v were prepared. These different solutions were analyzed by HPLC and the results are shown in Figure 3. A peak with a retention time of 7.7 min (PRO) represented less than 1% of the total area counts in the chromatogram corresponding to the first dilution (Figure 3A). The second serial dilution (1:1 v/v) produced a decrease in the area counts of the peaks of both FUM and ATR as well as an increase of area counts of PRO (Figure 3B). Successive dilutions 1:3, v/v and 1:7, v/v reproduce this behaviour where it can be observed that the decrease in the concentration of FUM and ATR was concomitant to the increase in concentration of PRO (Figure 2C and D)as the dilu-

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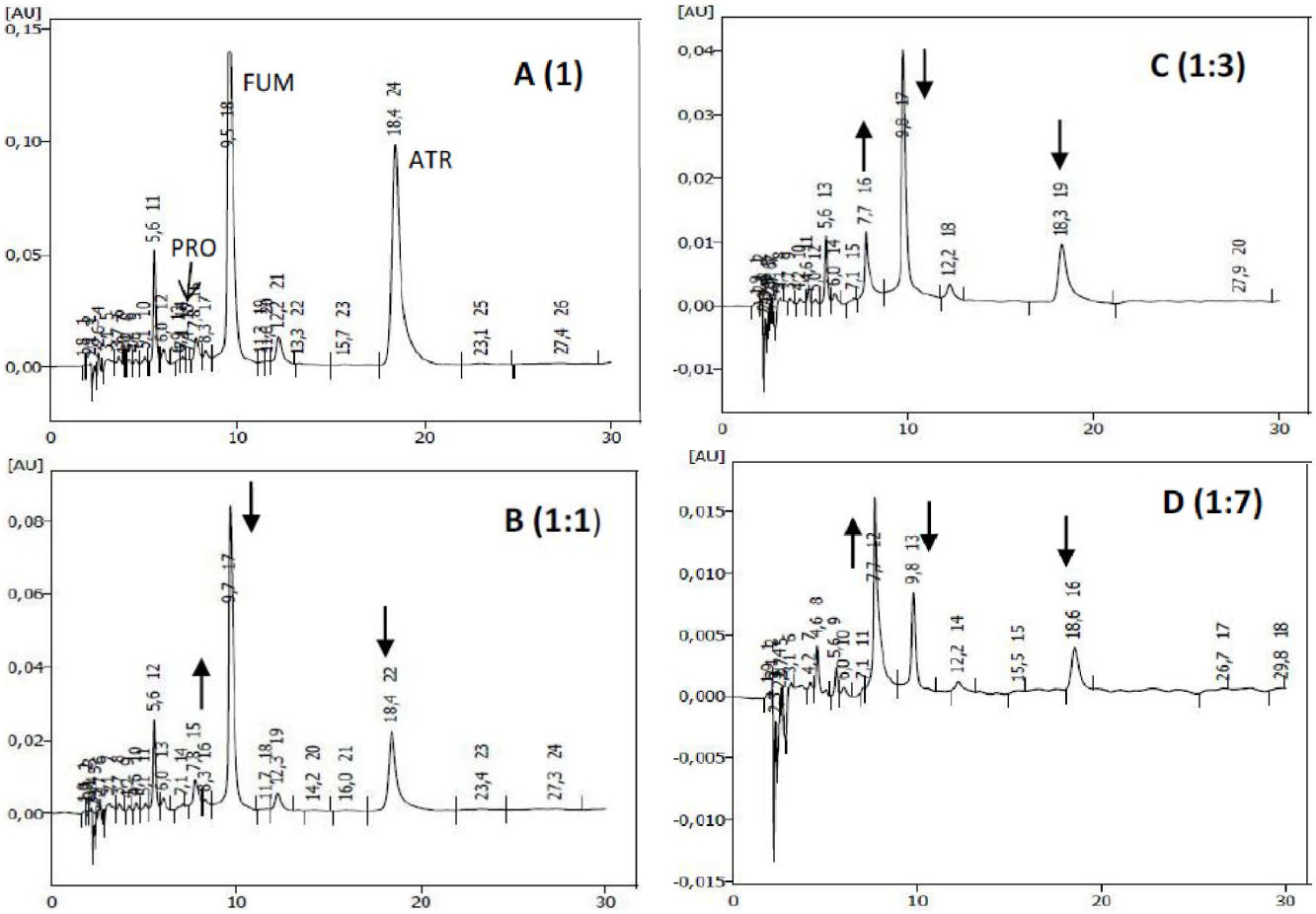

Figure 3 : HPLC separation of ATR, PRO and FUM in serial water dilutions of a lichen extract from *C. rangiferina*; 1; 1:1; 1:3 and 1:7, (v/v) represent the degree of dilution

tion degree increases. Quantitation of this process is shown in TABLE 2. Decrease of FUM concentration related to increase of PRO concentration follows a lineal equation, y=0.033-0.0302x; r^2= 0.82, where y are µmol/mL of PRO and x are µmol/mL FUM.

Assuming that the concentration of PRO increases with the lichen extract dilution as a consequence of a spontaneous hydrolysis of FUM, it was possible to say that the 1:1, v/v dilution produced a transformation of 2.3% FUM, 4.9% for a dilution 1:3, v/v and 11.5% for 1:7, v/v dilution. Figure 4 summarizes biosynthesis of FUM from ATR through PRO. At high water content, an spontaneous hydrolysis of ester bound in FUM molecule could be occur which would implicate the loss of the 4C side chain and the consequent conversion to PRO.

The lichen *C. verticillaris* has a high affinity for sedimentary soils, so often it is founded in the tableland in the NE of Brazil such as Mamanguape. In this area quartzarenic neosols are dominant. They have a sandy texture at least to 2 meters deep[5]. Contact of lichen acids with the mineral soil greatly influences ecosystems. Bjelland et al.[19] said that the interface between the lichen and the substrate is a place of great chemical activity where lichens are involved in the production of acids and complexing metal compounds[20,21]. This interaction depends on environmental factors including the rainfall distribution.

Mamanguape has an annual rainfall average of 1634.2 mm, mainly concentrated from April to July. Rain can remove lichen acids and proteins of *C. verticillaris* depending on their distribution and intensity. Profile of percolation of these substances had depended on the moisture of the soil (Figure 2). Our results show that the dry soil had retained proteins from other sources that easy percolate when it is completely water saturated (Figure 2A).

FUM was highly retained by both treated soils

TABLE 2 : Quantification of FUM, PRO and ATR in water serial dilutions from *C. rangiferina*.extracts

Dilution v/v	PRO		FUM		ATR	
	µmol/mL	% of inicial	µmol/mL	% of inicial	µmol/mL	% of inicial
1	0,0093	100	0,89	100	1,09	100
1:1	0,015	161	0,42	47	0,57	52
1:3	0,019	204	0,21	23,5	0,25	23
1:7	0,039	419	0,10	11,2	0,13	12

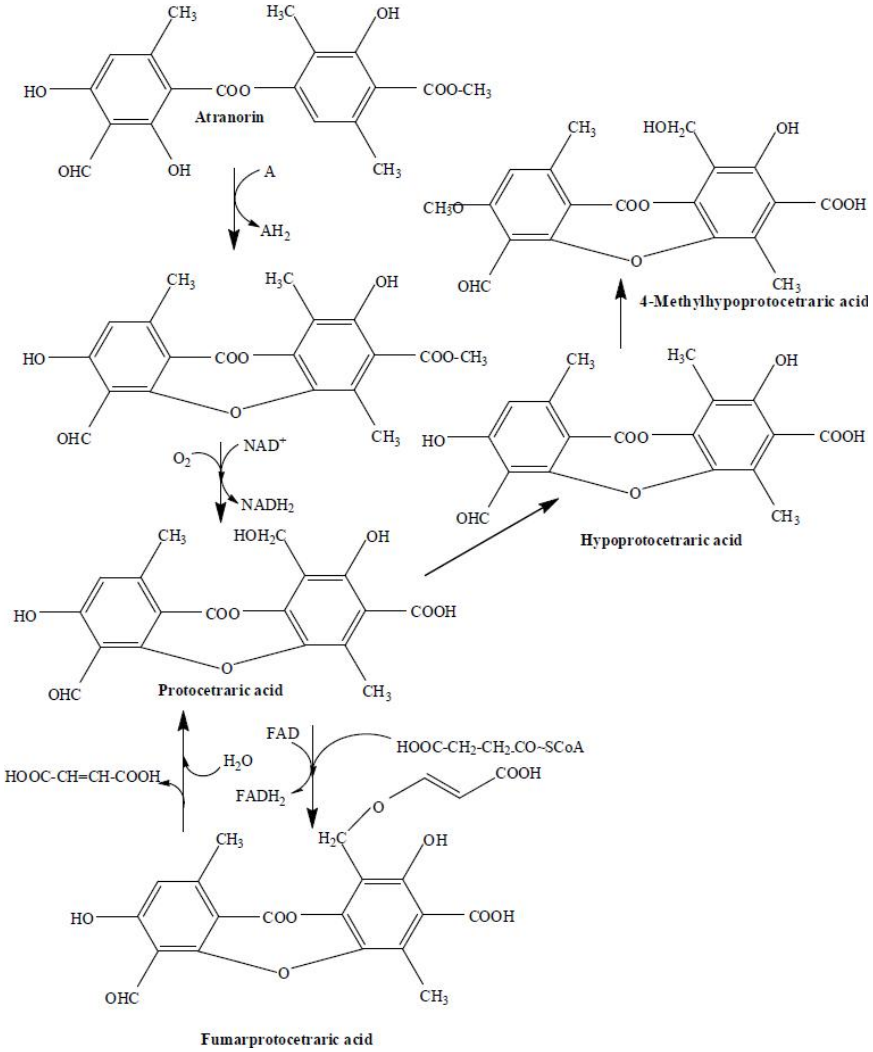

Figure 4 : Biosynthetic pathway of fumarprotocetraric acid from atranorin.

(Figure 2). In contrast, the amount of PRO recovered was higher than that added (80% more in the wet soil and 5.1% in the dry one). It has been proved that spontaneous hydrolysis of FUM into PRO is possible due to a dilution effect (Figure 3 and TABLE 2). For this reason the recovery of PRO percolated was higher in the wet soil than in the dry one.

From these results, we can conclude that the amount of proteins, PRO and FUM from *C. verticillaris* that leached to the soil, would be retained, transformed or percolated to deeper soil layers, depending on the amount of rainfall. During high rainfall episodes, PRO could rapidly percolate developing poor biochemical actions on the soil. During the period of low rainfall PRO would be retained by the soil and percolated slowly as a function of the soil moisture. Then, its ability to modify

the chemical nature of the soil would increase. FUM shows a different dynamic. It is highly retained by the soil and can be hydrolyzed to form PRO as a consequence of the soil moisture. These results show that the effects of PRO and FUM would be maintained in the superficial layers of the quartzarenic neosols that characterize this geographic region. In addition, Tigre et al.[7] found that the changes induced by PRO and FUM in tableland soils mainly depend on the relative amount of both compounds rather than the amount of each one in separate. In addition, Vasconcelos et al[13]. found that the thalli of *C. verticillaris* can entrap small mineral particles,detached from the soil by the mechanical action exerted throughhydration–dehydration cycles of the lichen thalli and retained duringtheir growth. These mineral particles are probably original soil components chemicallymodified by exocellular lichen metabolites

Lichen acids such PRO and FUR, also exert an influence on the ecosystem through indirect effects mediated by changes in metabolic activity of soil microorganisms and mycorrhizae[22]. Legaz et al.[23]propose the use of lichen phenolics as herbicides. These phenols may enter the xylem fluid, reaching the leaves and cause defoliation of the plant in to a greater or lesser degree. Silva[10] shows the positive effect of *C. salzmannii* on the *Genipa americana* development. Tigre et al[7]. showed that PRO of *Parmotrema dilatatum* and FUM of *C. verticillaris* stimulates leaf growth in *Lactuca sativa* while PRO stimulates roots and hypocotyls growth. Other allelopathic effects of FUM in *Alliumcepa* L. have been reported by Yano-Melo et al.[25]. All these results help to understand the biological effect of FUM and PRO upon the growth and the rain-dependent development of "Cerrado" vegetation.

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