Epicuticular waxes from caatinga and cerrado species and their efficiency against water loss

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ABSTRACT

The effects of the contents and chemical composition of the foliar epicuticular waxes of species from the caatinga (*Aspidosperma pyrifolium*, *Capparis yco*, *Maytenus rigida* and *Ziziphus joazeiro*) and cerrado (*Aristolochia esperanzae*, *Didymopanax vinosum*, *Strychnos pseudoquina* and *Tocoyena formosa*) were evaluated as to the resistance to water loss by means of an experimental device constructed for this purpose. In general, the waxes of the caatinga species investigated were more efficient against water loss than cerrado species. Increase of the thickness of the waxy deposits from 40 to 90μ g.cm^{-2} had no significant effect on the resistance to water loss. The chemistry of the wax constituents was shown to be an important factor to determine the degree of resistance to evaporation. *n*-Alkanes and alcoholic triterpenes were the most efficient barriers, while hentriacontan-16-one (a ketone) and ursolic acid (an acid triterpene) revealed low efficiency. The higher efficiency of the waxes of the leaves from caatinga species (mainly those of *C. yco* and *Z. joazeiro*) is probably accounted for the predominance of *n*-alkanes in their composition. The lower efficiency of the waxes of *A. pyrifolium* (caatinga), *T. formosa* and *A. esperanzae* (both species from the cerrado) is probably a consequence of the predominance of triterpenoids in the waxes of the two former species and hentriacontan-16-one in the latter.

Key words: caatinga, cerrado, epicuticular waxes, *n*-alkanes, triterpenoids, water diffusion.

INTRODUCTION

The caatinga is a semi-arid ecosystem of northeast Brazil, characterized by a dry, spiny and predominantly leaf-shading forest vegetation. Annual mean rainfalls in the caatinga range from 500 to 750 mm, reaching in drier years amounts as low as 300 mm (Sampaio 1995). In turn, the cerrado is a savanna ecosystem with acidic soils and low amounts of mineral nutrients, but high amounts of aluminum and iron. Annual rainfall in cerrados may come up to 2,000 mm (Sampaio 1995). The typical woody cerrado vegetation is xeromorphic and the physiognomy of the ecosystem, in many areas, depends on the occurrence of periodic fires, in the absence of which a savanna community may turn out a dry forest (Coutinho 1983). It has been assumed that cerrado xeromorphism is not associated with hydric conditions, but rather that it is a consequence of a chronic low availability of edaphic mineral nutrients (Arens 1958a, b) and aluminum toxicity (Goodland 1971).

The maintenance of a hydric balance is a problem affecting all organisms, chiefly those from terrestrial habitats, where water availability is more or

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less restricted (Hadley 1981). Pioneering studies about water loss by caatinga and/or cerrado plants were carried out by Ferri (1944, 1955), Rachid (1947), Rawitscher (1948), Ferri and Labouriau (1952) and Ferri and Coutinho (1958). Thick cuticles not always correspond to high resistance to cuticular transpiration. Ferri (1944, 1955), Rachid (1947) and Rawitscher (1948) observed that cerrado species with thick foliar cuticles may have high rates of cuticular transpiration. In addition, thick cuticles sometimes contain low amounts of waxy epicuticular deposits (Amaral et al. 1985). Lack of correlation between cuticle thickness, xeromorphism and anti-transpiring efficiency has been frequently shown (Schieferstein and Loomis 1956, Kurtz 1958, Sitte and Rennier 1963, Hull and Baker 1979, Lyshede 1982, Jordaan and Kruger 1998). In plants, water loss occurs not only through the stomata, but also across the cuticle. Cuticular transpiration is controlled mainly by deposits of wax on the foliar surface. Reduction of water permeability across the cuticle is the primary function of epicuticular waxes (Schönherr 1982).

The efficiency of cuticles to restrain water permeability has been evaluated either with intact leaves (Hall and Jones 1961), reconstituted waxes (Grncarevic and Radler 1967), isolated cuticles (Schönherr and Riederer 1989) or foliar discs (Hoad et al. 1996). Experimental devices in such studies enable the evaluation of the influence on water permeability of various factors, such as thickness of waxy films, wax architecture and chemical composition. At the same time, they avoid the influence of factors such as stomata and trichomes, which disturb measurements of cuticular transpiration. A comparative study using different methods for measuring cuticular water permeability was provided by Kirsch et al. (1997).

So far no studies are available regarding the efficiency on the reduction of water permeability of foliar epicuticular waxes of plants native to Brazil. Recently, a comparative analysis about the chemistry of epicuticular waxes between caatinga and cerrado plants was published (Oliveira and Salatino

2000), involving four species of each habitat. According to the authors, *n*-alkanes are important constituents of the waxes from all species studied, and are predominant in the caatinga species, while waxes of the cerrado species contain triterpenoids as main constituents. *Aristolochia speranzae* (other cerrado species) stand out for containing hentriacontan-16-one (an aliphatic ketone) as predominant constituent. The present investigation aims to evaluate the efficiency of crude waxes and their main constituents from caatinga and cerrado species against water loss, using a simple experimental device.

MATERIALS AND METHODS

Material – Adult and intact leaves of *Aspidosperma pyrifolium* Mart. (Apocynaceae), *Capparis yco* Mart. (Capparaceae), *Maytenus rigida* Mart. (Celastraceae) and *Ziziphus joazeiro* Mart. (Rhamnaceae) were collected in a caatinga area of Alagoinha (Pernambuco State, northeast Brazil). Leaves of *Aristolochia speranzae* O. Ktze. (Aristolochiaceae), *Didymopanax vinosum* (Cham. & Schltd.) Marchal (Araliaceae), *Strychnos pseudoquina* A. St.-Hil. (Loganiaceae) and *Tocoyena formosa* (Cham. & Schltd.) K. Schum. (Rubiaceae) were collected in a cerrado area of Pirassununga (State of São Paulo, southeast Brazil). Voucher specimens from caatinga and cerrado were deposited in the herbaria of the Federal University of Pernambuco (UFP) and of the University of São Paulo (SPF), respectively.

Waxes extraction and analyses – Epicuticular waxes of each species were extracted from 500 g leaves previously dried in the shade by rapid surface washing with chloroform during 60 sec (Silva Fernandes et al. 1964). The waxes were fractionated by silicagel column chromatography with solvents with increasing polarity. Aliphatic compounds and alcohol triterpenoids were obtained by preparative thin layer chromatography, using silicagel impregnated with sodium fluoresceine 0.02% and *n*-hexane: chloroform (7:3) as mobile phase. Chloroform: ethyl acetate: ethyl ether (4:3:3) were used for

acid triterpenoids (Salatino and Silva 1988). Chromatogram spots were visualized under long wave UV light. The wax constituents thus obtained were analyzed in a gas chromatograph HP 5890 ser. II Plus interfaced with a mass spectrometer HP 5989B using the eletronic impact method at 70 eV. A capillary column HP-5MS of fused silica (30 m × 0.25 mm) and helium at a flow of 1 cm.min^{-1} were used. Runs were programmed to start at 150°C for 2 min, raising the temperatures at a rate of 10°C.min^{-1} until 300°C. The temperature of the injector and detector was 300°C. Identification of the compounds was achieved by comparison of retention times with authentic samples and of the corresponding mass spectra with data of the library Wiley275-pc (Hewlett Packard).

Reconstitution of epicuticular waxes – Chloroform solutions at 10, 15, 20 and 25 mg.mL^{-1} of the individual crude waxes were prepared and used to impregnate discs of Whatman paper (grade AA, cat. 2017013, diameter 13 mm) to achieve 40, 60, 70 and 90 μg.cm^{-2}, respectively, which are comparable with contents ranging from 40.4 to 82.3 μg.cm^{-2}, reported by Oliveira and Salatino (2000) for the foliar epicuticular waxes of the species studied in the present work. Similar procedures for Whatman paper impregnation were followed with the *n*-alkane fraction, hentriacontan-16-one, ursolic acid, lupeol and epifriedelinol, which correspond to the main constituents of the foliar epicuticular waxes of *Z. joazeiro*, *A. esperanzae*, *T. formosa*, *D. vinosum* and *M. rigida*, respectively (Oliveira and Salatino 2000). The efficiency of wax recrystallization on the paper discs was monitored by scanning electronic microscopy.

Experimental model – Ten "U" shaped glass tubes with 250 mm long and internal diameter 13 mm were prepared to evaluate the influence of epicuticular waxes as anti-transpiring barriers (Figure 1). The ends "a" and "b" have diameters of 1 and 10 mm, respectively, but the "a" end is a little longer than "b", so that the water may exert some pressure on the disk at the "b" end. The Whatman paper discs

impregnated with epicuticular waxes or their constituents were placed in the "b" end of the tubes, and a rubber ring and a plastic cover were used to fix the paper disc on place and prevent water leakage. The plastic cover has an opening of 5 mm in diameter, corresponding to the free evaporating surface. Distilled water (25°C, pH 6.9) was introduced into the device with a syringe through the "a" end until it reached the inner surface of the disk in the other end. The "a" end was then sealed with histologic paraffin. The tubes were examined to make sure they contained no air bubbles inside. Control tubes were mounted similarly, but with paper discs free from wax material.
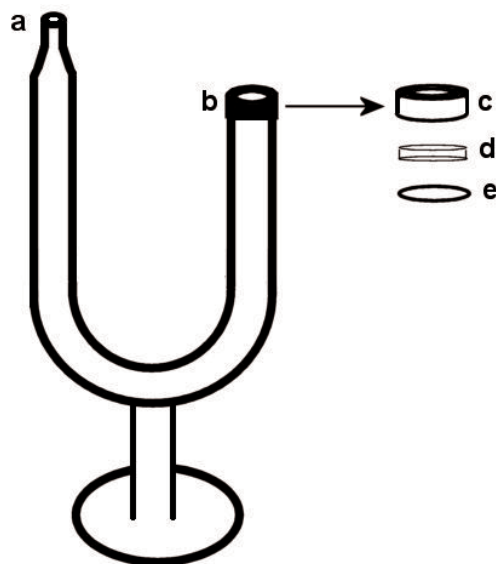

Fig. 1 – Schematic representation of the device to evaluate the water permeability of epicuticular waxes from caatinga and cerrado species. a = water inlet (diameter 1 mm); b = water vapour outlet (diameter 5 mm); c = plastic cover; d = Whatman paper disc (diameter 13 mm); e = rubber ring. Refer to section Methods for further details.

Measurements of water permeability – The loss of water (*E*) through the paper discs was evaluated by weighing each experimental device with an analytical balance (Mettler Toledo AG204) three times at intervals of 1h under conditions of controlled air humidity and temperature. Relative air humidity

and temperature during measurements were 65% and $25 \pm 0.5°C$, respectively. The difference of vapor pressure was estimated to be 11.14 mbar (1.1 kPa) at the mentioned conditions of air humidity and temperature (Unwin 1980). The values obtained were plotted as water loss *versus* wax contents on the discs (μg.cm^{-2}) and represent means of 30 measurements. The results were statistically evaluated by means of ANOVA, using the software SPSS for Windows, V. 5.0 (SPSS Inc.).

RESULTS AND DISCUSSION

WATER PERMEABILITY, SOURCES OF WAXES AND THICKNESS OF WAX DEPOSITS

According to the observed water permeability, the samples of waxes studied in the present work may be classified in three categories: 1) waxes with very low permeability ($E \leq 0.20$ mmol.m^{-2}.s^{-1}), such as *Z. joazeiro* and *C. yco*; 2) waxes with low permeability (E ranging from 0.20 mmol.m^{-2}.s^{-1} to 0.40 mmol.m^{-2}.s^{-1}), such as *M. rigida*, *D. vinosum* and *S. pseudoquina*; 3) waxes with relatively permeability ($E > 0.40$ mmol.m^{-2}.s^{-1}), such as *T. formosa*, *A. pyrifolium* and *A. esperanzae*. A value of $E = 0.40$ mmol.m^{-2}.s^{-1} at 25°C is equivalent to a cuticular permeability (P) of approximately 100×10^{-5}m.s^{-1} (Pearcy et al. 1989). The values of water permeability observed in the present work lie in the range of values of P obtained with isolated cuticles, namely $3, 6 \times 10^{-7} - 1, 4 \times 10^{-4}$m.s^{-1} (Lendzian and Kerstiens 1991, Kerstiens 1996a,b, Riederer and Schreiber 2001).

Many investigations evidenced that the thickness of leaf wax deposits have little influence on cuticular transpiration (Riederer and Schreiber 2001), although increases of epicuticular waxes on leaves of plants under hydric stress have been observed (Bondada et al. 1996). Data of Figure 2 show that the increase in the wax contents from 40 to 90 μg.cm^{-2} did not reduce significantly the water permeability ($P > 0.05$). Is has been claimed that, at least in some species, thick cuticles are not linked to hydric stress. For example, it has been suggested that

the oligotrophic edaphic conditions of the cerrado is an important factor inducing the diversion of excess photosynthate to alternative metabolic routes that lead, for example, to cuticular components (Arens 1958a, b). In addition, high irradiance conditions prevail both in the caatinga and cerrado, which could be another factor selecting for thick wax deposits. It has been shown that epicuticular waxes are crucial for plant photoprotection (Barnes and Cardoso-Vilhena 1996). However, highly significant difference exists in the comparison between species ($P < 0.001$). Differences in chemical composition are the most likely factor accounting for the results reported in this study, which is in agreement with observations previously reported (Grncarevic and Radler 1967, Schönherr 1982, Schreiber and Riederer 1996).

The waxes of *C. yco* and *Z. joazeiro* gave similar values of water permeability (Figure 2, $P = 0.058$). Also similar values were observed for the waxes of *S. pseudoquina* and *D. vinosum* ($P = 0.375$). This is not surprising, because similar chemical compositions have been observed between members of both species pairs (Oliveira and Salatino 2000). Among the four species from the caatinga, the waxes of three of them (*Z. joazeiro*, *C. yco* and *M. rigida*) promoted a reduction in water permeability three or four times higher than the waxes of the cerrado species (Figure 2). In contrast, the water permeability observed for the latter waxes is approximately 10 times lower than the controls (wax free paper discs). According to Schönherr and Riederer (1989) removal of the epicuticular waxes leads to an increase in water permeability of 10 to 10.000 times.

Higher restriction of water loss is expected for leaves of plants from the caatinga rather than the cerrado, because hydric stresses are by far higher in the former than in the latter habitat. Accordingly, Ferri (1944, 1955) observed transpiration rates much lower by leaves of caatinga species than cerrado species, the latter showing no signs of hydric deficit. Lower transpiration rates were observed by Schreiber and Riederer (1996) with cuticles of leaves from tropical species, which are often
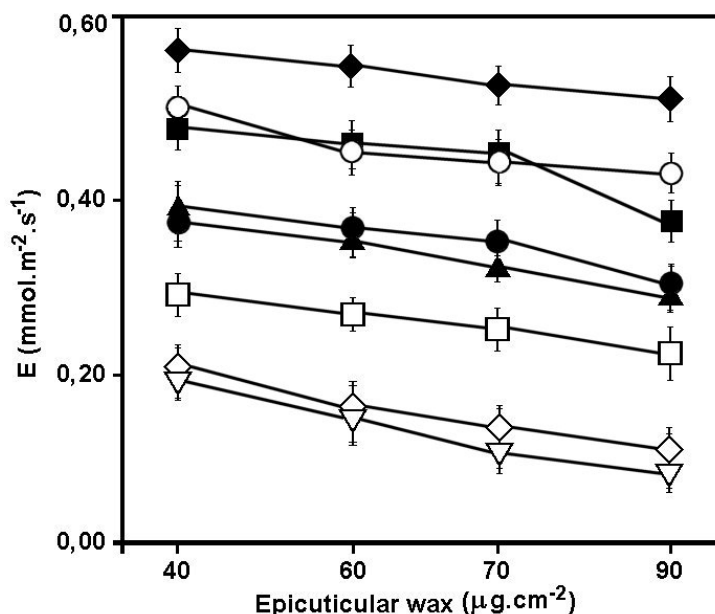
Fig. 2 – Evaporation rates (E) in Whatman paper discs impregnated with foliar epicuticular waxes of species from caatinga (empty symbols) and cerrado (full symbols). (○) *Aspidosperma pyrifolium*, (◇) *Capparis yco*, (□) *Maytenus rigida* (▽) *Ziziphus joazeiro*, (■) *Aristolochia esperanzae*, (▲) *Didymopanax vinosum*, (●) *Strychnos pseudoquina*, (♦) *Tocoyena formosa*. Values correspond to means ± sd (n = 30), obtained at 25°C and 65% relative humidity.

exposed to low water availability, high irradiance and low pressures of air water vapor, such as is particularly the case of the species from the caatinga. The mentioned authors observed higher transpiration rates for Mediterranean species, although they bear evident xeromorphic features, such as thick cuticular layers.

WATER PERMEABILITY × EPICUTICULAR CONSTITUENTS

As was observed in the essays with crude waxes, increases in the amounts of the isolated wax constituents did not significantly reduce water permeability (Figure 3). Rather, highly significant differences ($P < 0,001$) were observed comparing the efficiency of the different classes of wax constituents. *n*-Alkane fractions, formed mainly by homologues with 27–33 carbon atoms (foliar waxes of *C. yco* and *Z. joazeiro*, Oliveira and Salatino 2000),

were the most effective constituents as promoters of water permeability (Figure 3). The least efficient constituents were hentriacontan-16-one (ketone) and the ursolic acid (acid triterpenoid). The alcohols triterpenoid epifriedelinol, lupeol and the mixture lupeol/β-amyrin behaved as moderately efficient (Figure 3).

The low rates of evaporation observed with paper discs impregnated with *n*-alkanes are coherent with the high water permeability observed for the crude waxes of *Z. joazeiro* and *C. yco* (Figure 2), in which such constituents prevail (Oliveira and Salatino 2000). The higher efficiency of *n*-alkanes over other wax constituents has been observed also by other authors, such as Grncarevic and Radler (1967). In addition, these authors observed that oleanolic acid, a triterpenoid chemically very similar to ursolic acid, has low efficiency as a barrier against water loss. Again the relatively high permeability of the
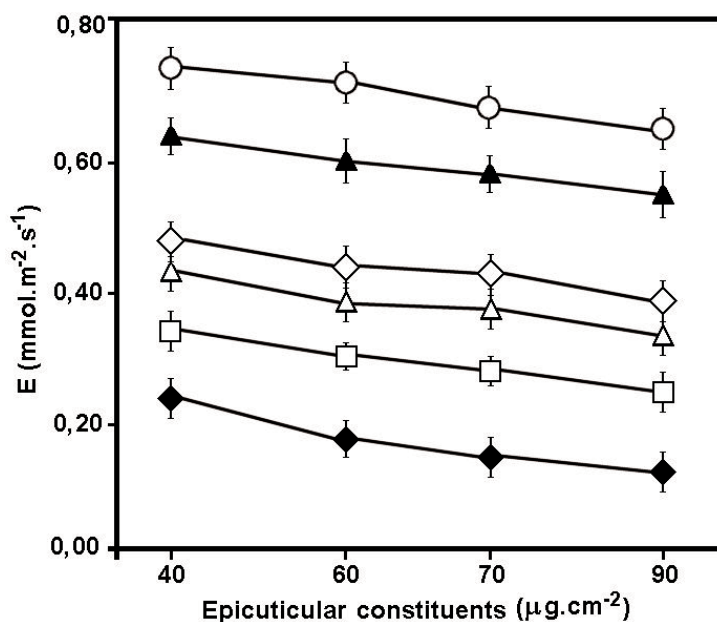
Fig. 3 – Evaporation rates (E) in Whatman paper discs impregnated with constituents separated from foliar epicuticular waxes of species from caatinga and cerrado. Empty symbols correspond to triterpenoids. (◯) ursolic acid, (▲) hentriacontan-16-one, (◇) lupeol, (△) lupeol + β-amyrin, (□) epifriedelinol, (◆) n-alkanes. Values correspond to means ± sd (n = 30), obtained at 25°C and 65% relative humidity.

crude wax of *T. formosa* (Figure 2) is accounted for its high content (54.6%) of ursolic acid (Oliveira and Salatino 2000).

The least efficient foliar wax from the caatinga studied in the present work is that of *A. pyrifolium*. It is worth noting that it is also the only caatinga wax here studied that has high ursolic acid content (46.4%, Oliveira and Salatino 2000). It is known that, in spite of an exceptional content of wax (1317 $\mu g.cm^{-2}$), apple skins have water permeability tens of times higher than 23 other fruit cuticles with lower wax contents (Schreiber and Riederer 1996). The epicuticular wax of apple skins has ursolic acid as major constituent (Belding et al. 1998). According to Schönherr (1976), the carboxylic group (-COOH) has high affinity for water molecules. In fact, free fatty acids are also relatively inefficient anti-transpiring agents, whose efficiency significantly increases upon esterification (Grncarevic and Radler 1967). These observations indicate that water permeability of waxes is dependent on the polarity of their constituents (Holloway 1969). Coherently, the alcoholic triterpenoids yielded results of water permeability much lower than the more polar ursolic acid (Figure 3). It is also worth observing that substances with the same functional group but exhibiting different polarities behave also differently as anti-transpiring barriers. This is the case of lupeol and epifriedelinol, both alcohols triterpenic, but the latter having higher mobility on silicagel chromatographic plates (results not shown), which is indicative of lower polarity. Coherently, epifriedelinol was shown to be more efficient than lupeol against water evaporation (Figure 3).

However, factors other than the mere polarity probably play a role as determinants of efficiency against water permeability. For instance, based on chromatographic behavior, hentriacontan-16-one surpasses only *n*-alkanes in polarity, and yet its water permeability is second only to that of ursolic acid

(Figure 3). A possible factor influencing water permeability, which, however, was not investigated in the present work is the detailed wax architecture, as seen through scanning electron microscopy (Baker 1982).

ECOLOGICAL CONSIDERATIONS

The most obvious role of epicuticular waxes is the restriction of water loss by transpiration. In caatinga habitats, apparently epicuticular waxes alone do not suffice to warrant survival along the most severe periods of drought. Leaf shading is almost always the rule among caatinga plants, the leaves thus escaping the harshest pressures of hydric shortage. *Z. joazeiro* is an exception, being a woody species that only rarely loses its leaves. Survival of *Z. joazeiro* is guaranteed by an efficient stomatal mechanism, combined with extensive root systems capable of taking up moisture from deep layers under the soil surface (Ferri 1955, Andrade-Lima 1981, Sampaio 1995). Results of the present work suggest that an additional factor of drought resistance of *Z. joazeiro* is the composition of its foliar epicuticular wax, characterized by a high proportion of *n*-alkanes (78.6%), the most efficient wax constituents against water permeability. High proportions of *n*-alkanes (76.5%) also characterize the composition of the foliar epicuticular wax of *C. yco*, although in this species leaf persistence is not observed as *Z. joazeiro*. According to Ferri (1955), specimens of *C. yco* grow in the shade of other species (*Bumelia sartorum* Mart. Sapotaceae, *Caesalpinia pyramidalis* Tul. Leguminosae and *Spondias tuberosa* Mart, Anacardiaceae) as a means of adaptation to drought conditions. However, after the leaves of these species have been shed, those of *C. yco* still persist for some time, but not as long as the leaves of *Z. joazeiro* (Andrade-Lima 1981, Sampaio 1995, Machado 1997). A hypothesis that could be speculated at this point is the possibility that the prevalence of *n*-alkanes in the foliar waxes could be a factor contributing to enable a lengthier rate of leaf-shade by *C. yco*. Higher rates of cuticular transpiration by *A. pyrifolium* than *M. rigida* have been observed (Ferri 1955). In the present investigation, the foliar wax of the former species was ranked as more water permeable than that of the latter, which is in agreement with Ferri's report.

With regard to the cerrado species, it has been claimed that woody species often show no signs of water deficiency, some of them having underground organs searching water at depths reaching sometimes 18 m under the surface, as is the case of *Andira humilis* Mart. Ex. Benth., Leguminosae (Ferri 1944). Thick cuticles, sclerophyly and other peculiar features of the woody species of cerrado have been assigned to the effect of the acid and nutrient deficient soils of the cerrado, in many cases combined with the effects of periodic fires (Coutinho 1983, Salatino 1993). It has been speculated that the frequent prevalence of triterpenoids in the foliar waxes of cerrado plants may be a feature selected for its value as protection against the pressure of biotic agents (insect herbivory, for example) rather than as anti-transpiring agents (Salatino et al. 1998, Oliveira and Salatino 2000). Evidence that wax triterpenoids from cerrado species may act as protection against herbivore has been shown (Varanda et al. 1992, Salatino et al. 1998).

RESUMO

A influência do teor e da composição química das ceras epicuticulares foliares de espécies da caatinga (*Aspidosperma pyrifolium*, *Capparis yco*, *Maytenus rigida* e *Ziziphus joazeiro*) e do cerrado (*Aristolochia esperanzae*, *Didymopanax vinosum*, *Strychnos pseudoquina* e *Tocoyena formosa*) foram avaliadas em relação à resistência à perda de água, através de um dispositivo experimental construído para essa finalidade. Em geral, as ceras das espécies da caatinga foram mais eficientes contra a perda

de água que as das espécies do cerrado. O aumento da espessura dos depósitos cerosos de 40 até 90 $\mu g.cm^{-2}$ não alterou significativamente a resistência à evaporação. A natureza química dos componentes das ceras revelou-se um fator importante na determinação do grau de resistência à evaporação. n-Alcanos e triterpenos alcoólicos foram os constituintes mais eficientes como barreiras à evaporação, enquanto hentriacontan-16-ona (uma cetona) e ácido ursólico (triterpeno com função carboxílica) foram menos eficazes. A maior eficiência das ceras epicuticulares de duas espécies da caatinga (*C. yco* e *Z. joazeiro*) provavelmente se deve à predominância de *n*-alcanos em sua composição. A menor eficiência das ceras epicuticulares de *A. pyrifolium* (outra espécie da caatinga), *T. formosa* e *A. esperanzae* (ambas espécies do cerrado), à predominância em suas ceras de ácido ursólico para as duas primeiras e hentriacontan-16-para a última.

Palavras-chave: caatinga, cerrado, ceras epicuticulares, *n*-alcanos, triterpenóides, evaporação.

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