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Antiherbivory defense mechanisms along an environmental gradient in *restinga*

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ABSTRACT

Plant-herbivore interactions have directed the evolution of defense mechanisms in plants, which can vary intraspecifically according to environmental quality. This study evaluated variation in the production of antiherbivory defenses in plant species distributed along an environmental gradient of *restinga*. The study was performed in Parque Estadual Acaraí, state of Santa Catarina, Brazil. Three species of four plant formations with different abiotic conditions were selected for study. Specific leaf area; water, nitrogen and carbon content; and presence of phenols, tannins, lignin and alkaloids, were evaluated in leaves of ten plants per species per plant formation. The results indicated that *Symphyopappus casarettoi* possesses Low Nutritional Quality Syndrome, characterized by low specific leaf area, presence of secondary metabolites and low nitrogen content. *Dodonaea viscosa* and *Varronia curassavica* possess Nutrition and Defense Syndrome, characterized by a balance between nutritional quality (high nitrogen content and lower C:N ratio) and protection (greater thickness of leaves and higher density of trichomes). The results did not confirm the Resource Availability Hypothesis. The absence of a pattern in antiherbivory response due to the environmental gradient may indicate that the investment in defense by plants is not unidirectional and that certain morpho-anatomical attributes are typically related to conservation of resources.

Keywords: coastal plain, herbivore-plant interaction, histochemistry, Parque Estadual Acaraí, physical defense, secondary metabolites

Introduction

Plant-herbivore interactions have directed the evolution of a diversity of antiherbivory strategies, including morphological, phenological and chemical defenses that protect plants from their natural enemies (Agrawal 2006). Furthermore, chemical and structural defenses frequently co-occur in the same plant since they often serve complementary functions (Koricheva *et al*. 2004; Turley 2013) in providing protection from the great diversity of herbivores and forms of herbivory that occur in nature (Carmona & Fornoni 2013).

Plant species differ in their antiherbivory defenses, and can even vary intraspecifically in the types of defense, number of strategies and the degree of efficiency of each defense (Coley 1987; Endara *et al*. 2015). This intraspecific variation is attributed to the set of biotic and abiotic factors that govern the structure and functioning of the community in which the plants are inserted (Cipollini & Bergelson 2001; Boege & Dirzo 2004). Therefore, the evolution of antiherbivory defenses in plants is not only a result of

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plant-herbivore coevolution, but also their interaction with the environment (Coley *et al.* 1985; Fine *et al.* 2006).

Besides preventing the consumption of plant tissues by herbivores, antiherbivory defenses may play secondary roles in plants, such as the control of transpiration by the coverage of the cuticle and/or trichomes (Traw & Dawson 2002; Peeters 2002; Yeats & Rose 2013); protection of organs in development from solar radiation by means of phenolic compounds (Tattini *et al.* 2000; Close & Mcarthur 2002; Treutter 2005); regulation of leaf calcium by the production of calcium oxalate crystals (Nakata 2003; Franceschi & Nakata 2005); and increasing leaf resistance by means of growth of mechanical tissue (Heerdt & Melo Jr. 2016).

The production of antiherbivory defenses represents a cost for plants and limits their ability to respond to local abiotic factors (Quezada & Gianoli 2006; Gianoli *et al.* 2007). The opposite effect can also occur, in which response to herbivory can be limited by the environment (McGuire & Agrawal 2005). Resources are allocated to growth and the production of defenses, so it could be expected that plants that occur in areas with low nutrient availability will have less capacity to compensate for the loss of photosynthetic tissue by herbivory, and should invest more resources in antiherbivory defenses (Resource Availability Hypothesis, Coley *et al.* 1985). These defenses are composed mainly with carbon, such as terpens and phenolic compounds (Carbon-nutrient balance Hypothesis, Bryant *et al.* 1983). On the other hand, individuals that grow in fertile soil and/or in shaded site may have the ability to replace leaves lost by herbivory, due to the increased availability of nutrients in the soil. The defenses composed with carbon tend to decrease while nitrogen defenses become more important (Bryant *et al.* 1983; Coley *et al.* 1985).

Thus, unrelated species, that develop in the same community, share biological interactions and they are subject to the same selective forces that may or may not differ in defenses (through syndromes) favoring the coexistence of these species (Defenses Syndrome Hypothesis, Agrawal & Fishbein 2006).Therefore, it is expected that there would be a negative correlation between the availability of nutrition in the soil and the production of antiherbivory defenses that alter herbivore-plant relationships and that species of the same community can present similar antiherbivore defenses.

In order to test the "Resource Availability Hypothesis", "Carbon-Nutrient Balance Hypothesis" and "Syndrome Defense Hypothesis", we studied plant species from *Restinga*. *Restinga* is the vegetation that grows on sand plains of Brazilian coast. Its soils are typically characterized as being low in fertility. In addition to deal with low availability of nutrients and water in the soil, plant species of the *restinga* are subjected to high content of sodium in the soil, high light and strong winds (Melo Jr. & Boeger 2015). These conditions can affect resource allocation (Craine *et al.* 2003; Fine *et al.* 2006).

These environmental factors of *restinga* presents a great variation in small spacial scale (Melo Jr. & Boeger 2015). The plants that live near the sea (herbaceous *restinga*) have a greater amount of carbon-based defenses compared to plants inhabiting the most distant sea formations (transition forest) (Bryant *et al.* 1983; Coley *et al.* 1985). As the *restinga* environment is generally limiting to the establishment and development of plants, the characteristics of antiherbivory defenses can converge between unrelated species and present "combinations" of physiologically functional characteristics mediated mainly by the scarcity of resources (Agrawal & Fishbein 2006).

Three hundred and nineteen plant species are distributed throughout the *restinga* of Parque Estadual Acaraí (PEA) in São Francisco do Sul, with 244 being exclusive to one of four vegetation formations of the *restinga* (Melo Jr. & Boeger 2015). The high diversity of species in the *restinga* is evidence that there is abiotic variations that acts as environmental filters, which restrict the set of species that can inhabit each plant formation, resulting in turnover among the plant communities (Melo Jr. & Boeger 2015). On the other hand, there are three *restinga* species that are more plastic and can adjust their morphological and anatomical features according to their needs for survival throughout the edaphic gradient of the *restinga* in this park: *Dodonaea viscosa* (Sapindaceae), *Symphyopappus casarettoi* (Asteraceae) and *Varronia curassavica* (Boraginaceae) (Melo Jr. & Boeger 2016).

Therefore, these species provide a good model for testing the influence of hydric, edaphic and light gradients on the production of antiherbiviory defenses. This study aims to answer the following questions: (1) Is there any intraspecific variation in the production of antiherbiviory defenses along the edaphic, hydric and luminous gradient of the *restinga*? (2) How do antiherbivory defense mechanisms vary across environmental gradient in *restinga*? (3) Do the three species that occur in all of four vegetation types possess the same antiherbivory strategies? If so, (4) which kinds of syndromes defenses do the studied species show?

Materials and methods

Study site

The study was performed in the *restinga* of Parque Estadual Acaraí (PEA), a Integral Protection Conservation Unit located in the municipality of São Francisco do Sul on the northeast coast of the state of Santa Catarina (26º30'S 40º37'W). The climate is influenced by maritime humidity, with an average annual temperature of 21.3°C, and characterized as a humid mesothermic climate with hot summers (Cfa), according to Köppen, and average annual precipitation of 2,372 mm/year (Epagri 2014). The *restinga* of the park is estimated to be continuous for 6.7 ha and distributed along the coast for 17 km, and is characterized by the presence of *restinga*, dense ombrophilous forests, floodplains and mangroves (Fatma 2008).

The vegetation of PEA (Parque Estadual Acaraí) is predominantly characterized as *restinga*, which is structured into four distinct communities and distributed along an edaphic gradient. The herbaceous *restinga* (Rh), composed of psammophiles and halophytes, occurs on Quartzarenic Neosol with low water retention capacity, low nutrient content and low fertility. The shrub *restinga* (Ra) is formed by a mosaic of dense shrubs between 0.5 and 1.5 m on Ferrihumiluvic Spodosols of a non-hydromorphic nature with low fertility. The shrub-tree *restinga* (Raa) is composed of shrubs and trees between 2 and 5 m in height and occurs on edaphic conditions similar to Ra. The transition forest (Ft) is characterized by a continuous tree stratum between 15 and 20 m in height over Ferrihumiluvic Spodosols + Halic Organosols of a hydromorphic nature (Embrapa 2013; Melo Jr. & Boeger 2015). The edaphic gradient possesses low organic matter content and low water retention in the formations closer to the sea, with the levels of these factors increasing in the direction of the forest. The availability of light decreases with distance away from the sea due to the increasing density of the forest canopy, which causes increasing levels of shade (Melo Jr. & Boeger 2015).

Environmental variables

For analysis of the nutritional condition of the soil, ten measurements of the thickness of the litter, using a millimeter ruler, and ten soils samples (approximately 50 g per sample), to a depth of 15 cm, were taken in each of the four *restinga* communities.

In the laboratory, a fraction of each soil sample was weighed with an analytical balance, dried for 72 hours in an oven at 105 ºC, and then weighed again to estimate the amount of water in the soil by the method of gravimetric moisture (the difference between fresh and dry mass of the soil) (Embrapa 2013).

For chemical evaluation of the soil, the ten samples were homogenized to produce a composite sample for each community. The following variables were evaluated in the Analysis Laboratory Chemistry Soil of EPAGRI/SC according to the methods described in Tedesco *et al.* (1995): pH, phosphorus (P), potassium (K), sodium (Na), magnesium (Mg), potential acidity (H + Al, ions H+ and Al^3+), sum of bases (SB), cation exchange capacity (CEC), base saturation (V) and organic material (OM). Photosynthetically active radiation (PAR) was estimated on the individual plants with the aid of a "light meter" (LI-250A, LI-COR, Nebraska, USA).

Morphological attributes

For each species, 10 individuals were selected in each *restinga* community. From each individual, 40 fully expanded leaves were collected between the 3rd and 6th node, in apex-base direction.

Twenty leaves were separated to the morphological analyzes, ten for the anatomical analyzes, five to histochemical analyzes and five to the nutritional analysis.

Twenty fresh leaves were weighed in a precision scale in order to obtain the fresh mass (g). These leaves also were used to estimate leaf thickness (mm) with digital caliper. After that, these same leaves were dehydrated in an oven at 60 ºC until constant weight to obtain dry mass in at precision scale.

Leaf area was estimated, using a digital images made with a flatbed *scanner* using Software Sigma Pro 5.0 (version 5.0, SPSS Inc., Chicago IL, USA), which was called the remaining leaf area. To estimate the complete leaf area, the partially consumed leaves were glued to a white paper sheet and the area of the injury left by the herbivore was drawn-in with a black marker and the total leaf was re-scanned. The consumed leaf area was calculated: complete leaf area - remaining leaf area (cm^2) (García-Robledo 2005).

Leaf water content [(fresh mass – dry mass)/ fresh mass x 100); Specific Leaf Area (SLA) (leaf area/leaf dry mass ($cm^2.g^{-1}$)] were calculated (Witkowski & Lamont 1991).

Trichome density was estimated for each species by modeling with colorless nail polish on both epidermal surfaces of previously dried leaves (Segatto *et al.* 2004). Using camera lucida coupled to a light microscope, trichomes were counted in twenty 1.0 mm^2-fields of five leaves of each individual of each species.

Anatomical attributes

For the preparation of histological slides, ten leaves were fixed in FAA 70 solution (formaldehyde, acetic acid and 70 % ethanol) and subsequently stored in 70 % ethanol (Kraus & Arduin 1997).

The anatomical description of the leaves is based on permanent slides obtained by conventional methods of embedment in 2-hydroxyethylmethacrylate (Leica®), sectioned with a rotary microtome (Leica® RM2145), stained with 1 % toluidine blue (Feder & O'Brien 1968) and mounted with stained-glass colorless varnish (Acrilex®) (Paiva *et al.* 2006).

The quantitative analyses of tissue thickness were done observing transversal sections of leaves. Transverse sections of the middle-third of the leaf blade were made by hands with a disposable razor blade and clarified in 10 % sodium hypochlorite, stained with 1 % toluidine blue and mounted in 30 % glycerinated water (O'Brien & McCully 1981). The thickness of palisade and spongy parenchyma (µm) was measured using Dino Capture 2.0 software with an attached Dino Eye AM423X camera mounted on an Olympus CX31 photomicroscope.

Analysis with scanning electron microscopy (SEM) was performed with material preserved in 70 % ethanol. The material was dehydrated in an increasing ethanol series and dried to critical point with CO_2 using Critical Point Bal-Tec, CPD030, Critical Point Dryer. The samples were mounted on "stubs" and metalized in a vacuum with a gold film using Sputter Bal-Tec, SCD050, Sputter Coater. Observations and microphotographs were performed using the Tescan Vega3 LMU Scanning Electron Microscope of the Electron Microscopy Center of UFPR.

Histochemical attributes

Using five fresh leaves of five individuals of each species in each *restinga* community, (25 leaves per species per *restinga* community), transversal sections were taken by hand with a disposable razor blade from the middle-third of the leaf blade. The sections were tested by applying ferric chloride 2 % to test the presence of phenolic compounds, Reagent of Dragendorff for alkaloid compounds (Johansen 1940), Phoroglucine for lignin 2 % (Johansen 1940), Vanillin 1 % for tannin (Burns 1971) and Sudan III 1 % (Sass 1951) for lipids.

Nutritional quality of leaves

Leaf nitrogen and carbon content were used as parameters of nutritional quality of plant tissues (Silva & Batalha 2011). To estimate leaf nitrogen and carbon content, five leaves from five individuals per specie from each *restinga* community (25 leaves total per specie per *restinga* community) were dehydrated at 30 °C, ground in a Ball-type mill (Oberstein, Fritsch Germany) and sieved to a particle size of 0.25 mm. Using 20 mg of this material, capsules were mounted and placed in a Plant Tissue Analyzer (Vario El III, Elementar). The analysis was performed by the combustion method (Nelson & Sommers 1996). C:N ratio was obtained by carbon content divided by nitrogen content (Silva & Batalha 2011).

Estimates of herbivory

To estimate rates of herbivory, the Herbivory Rate (HR, %) was calculated by the following formula: $HR = CA/TA*100$ (García-Robledo 2005; Corrêa 2007), where CA = consumed leaf area and TA = total leaf area.

Statistical analysis

Means and standard deviations were calculated for all of the quantitative attributes studied. Manova premises were tested by Shapiro-Wilk and Levene test, after the means were submitted to a Multivariate Analysis of Variance (MANOVA) and *post hoc* by Tukey test ($p = 0.05$) for comparison of the evaluated attributes of individuals of the same species along the environmental gradient and among populations of the tree species.

Principal component analysis (PCA) was used to evaluate the greater variance between the defense and nutrition attributes, in order to identify the occurrence of defense syndromes among the species. All the tests were performed with the package Rcmdr (2, 1-7) and complementary packages, in the R environment (version 3.1.2) (Borcard *et al.* 2011).

Results

Fertility and water availability of the soil and light intensity

The soil of Ft had a chemical composition markedly different from those of the other vegetation communities of the *restinga* (Tab. 1). The pH was neutral, whereas the soils of the other plant communities were acidic. The concentrations of calcium and phosphorus were higher at Ft, whereas the levels of magnesium and aluminum varied somewhat among the four communities. The values of sum of bases, cation exchange capacity and base saturation were similar among herbaceous, shrub and shrub-tree *restinga*s, but were higher in Ft. The supply of potassium, sodium, organic matter and the hydric availability increased gradually along the edaphic gradient (Tab. 1).

Light intensity was greatest in Rh and Ft, followed by Ra. The lowest light intensity was in Raa due to the density of the canopy over the border vegetation (Tab. 1).

Leaf attributes

Dodonaea viscosa

The individuals of Raa presented higher values of dry mass, leaf area and specific leaf area and lower values of leaf thickness. The smaller leaf area and greater leaf thickness were observed in individuals of Rh and Ft. The density of trichomes was higher in individuals of Rh. Higher water content and greater leaf area consumed were recorded in Ra (Tab. 2) individuals.

The mesophyll is dorsiventrall, the trichomes are sparse. Non-glandular trichomes on the abaxial surface over the veins and glandular trichomes throughout the leaf blade were observed. The palisade parenchyma is composed by two layers and there were no differences among populations, while spongy parenchyma was thicker in Ft individuals. The sclerenchyma cells were cap-shaped on both sides of the central vascular bundle (Figs. 1, 2, 3).

Secondary metabolites showed variation in distribution among populations. The individuals of Raa had a higher distribution of phenolic compounds, lignin and alkaloids, followed by individuals of Ft. Tannins were present in more leaf tissues of Raa and Ft and followed by Rh (Tab. 3).

The nutritional quality was higher in Raa individuals, represented by higher nitrogen content and lower C: N ratio. The highest rate of herbivory occurred in Ra (Tab. 2).

Symphyopappus casarettoi

The dry leaf mass was lower in Raa individuals and did not

Table 1. Average values for the nutritional attributes of the soil, salinity (n=10) and photosynthetically active radiation (n=25), in the four vegetation formations of the *restinga* of Parque Estadual Acaraí, São Francisco do Sul, SC. Legend: herbaceous *restinga* (Rh), shrub *restinga* (Ra), shrub-tree *restinga* (Raa), forest transiton (Ft), SB= sum of bases, CEC= cation exchange capacity, V= base saturation, OM= organic matter, PAR = photosynthetically active radiation.

Environmental attributes	Restinga formations			
	Rh	Ra	Raa	Ft
pH	5.5	5.3	5.3	7.6
P (mg/dm^3)	15.8	11.2	1.1	195.6
K (cmolc/dm^3)	12.0	26.00	28.00	44.00
Ca (cmolc/dm^3)	0.3	0.3	0.2	6.7
Mg (cmolc/dm^3)	0.1	0.4	0.2	0.2
H + Al (cmolc/dm^3)	1.1	1.5	1.1	0.8
SB (cmolc/dm^3)	0.43	0.95	0.46	6.99
CEC (cmolc/dm^3)	1.53	2.45	1.56	7.79
V (%)	28.09	38.61	29.81	89.74
OM (g/dm^3)	0.1	0.6	0.5	3.6
Na (mg/dm^3)	12	20	18	100
Gravimetric humidity (%)	62.0	246.1	290.7	507.0
Litter (cm)	0.1	2.4	3.7	7.9
PAR (μmol.s^{-1}.m^{-2})	1506.94 ± 15.89	1068.28 ± 55.30	841.7 ± 26.66	1451.86 ± 21.75

differ among the other populations. The leaf area was higher in Raa and Ft individuals, while the specific leaf area was larger in Raa individuals followed by Ft individuals. Greater leaf thickness was recorded in Ra individuals. In Rh and Ra, the highest leaf water content was observed. The largest leaf area consumed was observed in the populations of Rh and Ft (Tab. 4).

The mesophyll is isobilateral and the palisade parenchyma of both sides of the leaf is comprised of two cell layers, where the palisade parenchyma was more thicker in Rh and Ra individuals. The spongy parenchyma was thicker in Ft individuals. The sclerenchyma cells are cap-shaped on both sides of the central vascular bundle. The glandular trichomes are sparse and equally distributed on the two sides of the leaf blade. The greatest trichome density was observed in Ft individuals (Tab. 4, Figs. 1, 2, 3).

Phenolic compounds are well distributed in leaf tissues in Ra, Raa and Ft individuals. Lignin and alkaloids were present in several leaf tissues of Ra. Ft individuals also had a wide distribution of alkaloids. The tannins were also more distributed in the mesophyll in Ra and Raa leaves than other vegetation types (Tab. 5). Nitrogen content and C: N ratio did not vary among populations. However, the C: N ratio was high compared to the other species (> 25). The highest rates of herbivory were observed in the two extreme gradient vegetation types f (Rh and Ft) (Tab. 4).

Varronia curassavica

The highest values of dry mass, leaf area and leaf thickness were observed in Raa individuals. The specific leaf

Table 2. Means and standard deviations of leaves attributes of *Dodonaea viscosa* in the *restinga* formations of Parque Estadual Acaraí, São Francisco do Sul, SC. Legend: herbaceous *restinga* (Rh), shrub *restinga* (Ra), shrub-tree *restinga* (Raa), forest transition (Ft), SLA= Specific Leaf Area (leaf area/dry mass). * represents statistically significant difference ($p \leq 0.05$)

Leaf attributes	Rh	Ra	Raa	Ft	F
Dry mass (g)	0.09 ± 0.03b	0.10 ± 0.03b	0.11 ± 0.05a	0.09 ± 0.03b	* 12.53
Leaf area (cm^2)	10.26 ± 2.65c	11.27 ± 4.43b	14.20 ± 3.22a	9.71 ± 2.80c	* 71.03
SLA (cm^2/g)	109.90 ± 71.84b	104 ± 20.94b	133.17 ± 65.76a	106.47 ± 16.62b	* 13.99
Consumed area (cm^2)	0.31 ± 0.52b	0.95 ± 1.58a	0.25 ± 0.62b	0.13 ± 0.33b	* 30.91
Total leaf thickness (mm)	0.12 ± 0.02ab	0.12 ± 0.02b	0.10 ± 0.05c	0.13 ± 0.02a	* 22.87
Palisade parenchyma thickness (μm)	106.36 ± 13.59a	103.13 ± 23.71a	105.14 ± 22.63a	134.88 ± 16.98a	*58.6
Spongy parenchyma thickness (μm)	118.58 ±15.42b	98.28 ± 26.11c	96.90 ± 18.60c	126.04 ± 13.48a	* 58.91
Trichome density (adaxial) (n/cm^2)	154.4 ± 46.73a	129.6 ± 27.93b	107.2 ± 39.94bc	90.04 ± 28.93c	* 77.69
Trichome density (abaxial) (n/cm^2)	179.2 ± 62.04a	140.8 ± 18.69b	120.00 ± 22.36bc	96.00 ± 46.18c	* 12.81
Water content (%)	66.92 ± 6.28c	74.12 ± 4.44a	67.65 ± 7.02c	70.55 ± 6.31b	* 33.89
Leaf nitrogen content (g.kg^{-1})	22.94 ± 2.47b	23.42 ± 2.43ab	29.03 ± 4.92a	21.63 ± 2.61b	* 4.967
C:N ratio	21.34 ± 1.36a	20.30 ± 2.40ab	16.66 ± 2.95b	21.99 ± 3.07a	* 4.387
Herbivory rate (%)	2.90 ± 4.69b	6.69 ± 9.18a	1.69 ± 3.84bc	1.34 ± 3.21c	* 36.52

Table 3. Histochemical tests on leaves of the *Dodonaea viscosa* in four *restinga* formations. Legend: Test results: positive (+), negative (-). Formation: herbaceous *restinga* (Rh), shrub *restinga* (Ra), shrub-tree *restinga* (Raa), transition forest (Ft).

Compound	Restinga formations	Tissue/Structure							
		Epidermis			Parenchyma			Vascular Bundle	
		Adaxial	Abaxial	Collenchyma	Palisade	Spongy	Phloem	Xylem	Fibers
Phenois	Rh	-	-	-	-	-	+	+	-
	Ra	-	-	-	-	-	+	+	-
	Raa	+	+	-	-	-	+	+	-
	Ft	-	-	+	-	-	+	+	-
Lignin	Rh	-	-	-	-	-	-	+	+
	Ra	-	-	-	-	-	-	+	+
	Raa	-	-	+	-	-	-	+	+
	Ft	-	-	-	-	-	-	+	+
Tannins	Rh	-	-	+	-	-	+	-	-
	Ra	-	-	-	-	-	+	-	-
	Raa	-	-	+	-	-	+	+	-
	Ft	-	-	+	-	-	+	+	-
Alkaloids	Rh	-	-	+	-	-	+	-	-
	Ra	-	-	+	-	-	+	-	-
	Raa	-	-	+	-	-	+	+	-
	Ft	-	-	+	-	-	+	-	-

area was higher in Ra and Ft individuals. The largest areas consumed by herbivores were in Ra and Raa individuals. Rh and Ra leaves contained a higher water content (Tab. 6).

The mesophyll is dorsiventrall, with one very thick layer of palisade parenchyma that covers more than half of the mesophyll. In Rh and Ft individuals, the lowest thickness of palisade parenchyma occurred, while the spongy parenchyma was thicker in Rh individuals. There is a high density of glandular trichomes on both surfaces of the epidermis, short non-glandular trichomes on the adaxial surface and long non-glandular trichomes on the abaxial surface. The density of glandular trichomes was higher in Rh (Tab. 6, Figs. 1, 2, 3).

The phenolic compounds showed a higher distribution in foliar tissues of Rh individuals and were similar among the other populations. Lignin, alkaloids and tannins showed a similar distribution in the leaves of the studied populations (Tab. 7). The highest N content was observed in the Rh, Raa and Ft populations, but the C: N ratio did not differ between the vegetation types. In Ra and Raa individuals, the highest rates of herbivory were observed (Tab. 6).

Defenses syndromes

The results of Manova showed that *S. casarettoi* presented

Table 4. Means and standard deviations of leaves attributes of *Symphyoppapus casarettoi* in the *restinga* formations of Parque Estadual Acaraí, São Francisco do Sul, SC. Legend: herbaceous *restinga* (Rh), shrub *restinga* (Ra), shrub-tree *restinga* (Raa), forest transition (Ft), SLA= Specific Leaf Area (leaf area/dry mass). * represents statistically significant difference ($p \leq 0.05$).

Leaf attributes	Rh	Ra	Raa	Ft	F
Dry mass (g)	0.055 ± 0.011 ab	0.051 ± 0.01b	0.058 ± 0.02a	0.056 ± 0.02ab	* 3.437
Leaf area (cm^2)	4.90 ± 1.21b	4.30 ± 1.29c	5.70 ± 1.34a	5.41 ± 1.51a	* 41.42
SLA (cm^2/g)	85.41 ± 19.06bc	79.47 ± 13.08c	106.29 ± 66.46a	90.05 ± 18.83b	* 19.95
Consumed area (cm^2)	0.32 ± 0.52a	0.19 ± 0.14b	0.18 ± 0.30b	0.36 ± 0.57a	* 9.75
Total leaf thickness (mm)	0.08 ± 0.03b	0.11 ± 0.02a	0.08 ± 0.02b	0.09 ± 0.03b	* 42.73
Thickness of upper palisade parenchyma (mm)	200.10 ± 38.44a	198.8 ± 31.54a	180.32 ± 18.64b	183.15 ± 31.15b	* 10.64
Thickness of lower palisade parenchyma (mm)	81.17 ± 30.68c	92.78 ± 30.68b	101.21 ± 15.77ab	106.30 ± 19.90a	* 12.22
Spongy parenchyma thickness (mm)	116.83 ± 19.16a	110.15 ± 18.52a	100.49 ± 18.24b	114.39 ± 25.51a	* 12.23
Trichome density (adaxial) (n/cm^2)	120.8 ± 46.36c	172.8 ± 43.54b	196.0 ± 58.87ab	227.2 ± 60.51a	*18.01
Trichome density (abaxial) (n/cm^2)	176.00 ± 61.91b	235.2 ± 109.12a	170.4 ± 63.01b	205.80 ± 59.52ab	* 3.824
Water content (%)	78.76 ± 4.17a	80.12 ± 4.15a	73.69 ± 10.13b	75.53 ± 9.34 b	*30.74
Leaf nitrogen content ($g.kg^{-1}$)	17.14 ± 0.78b	16.61 ± 1.92b	19.36 ± 1.58a	16.82 ± 2.72b	* 68.08
C:N ratio	26.08 ± 0.95a	26.99 ± 2.95a	24.42 ± 1.58a	28.58 ± 4.40a	1.923
Herbivory rate (%)	6.09 ± 7.73ab	4.56 ± 2.91bc	3.25 ± 5.14c	6.68 ± 9.38a	* 20.427

Table 5. Histochemical tests on leaves of the *Symphyopappus casarettoi* in four *restinga* formations. Legend: Test results: positive (+), negative (-). Formation: herbaceous *restinga* (Rh), shrub *restinga* (Ra), shrub-tree *restinga* (Raa), transition forest (Ft).

Compound	Restinga formations	Tissue/Structure							
		Epidermis			Parenchyma			Vascular Bundle	
		Adaxial	Abaxial	Collenchyma	Palisade	Spongy	Phloem	Xylem	Fibers
Phenois	Rh	+	+	-	-	-	+	+	-
	Ra	+	+	-	-	+	+	+	-
	Raa	+	+	-	+	-	+	+	-
	Ft	+	+	-	+	-	+	+	-
Lignin	Rh	-	-	-	-	-	-	+	+
	Ra	-	-	-	-	+	-	+	+
	Raa	-	-	+	-	-	-	+	+
	Ft	-	-	-	-	-	-	+	+
Tannins	Rh	-	-	-	-	-	+	-	-
	Ra	-	-	-	+	-	+	-	-
	Raa	+	-	-	+	-	+	-	-
	Ft	-	-	-	-	-	+	-	-
Alkaloids	Rh	-	-	-	-	-	+	-	-
	Ra	-	-	-	+	-	+	-	-
	Raa	+	+	+	+	-	+	-	-
	Ft	+	+	-	-	-	+	-	-

high C: N ratio and low nitrogen content when purchased from the other species. The highest water content may be related to the high chemical protection observed in the mesophyll of this species. *Varronia curassavica* and *D. viscosa* presented lower C: N ratio and higher nitrogen content as nutritional attributes. However, *D. viscosa* presented higher leaf thickness, lower water content and higher chemical protection. *Varonia curassavica* showed a smaller specific leaf area, greater leaf thickness, high density of trichomes and chemical protection (Tabs. 3, 5, 7, 8).

Principal component analysis (PCA) showed that components 1 and 2 accounted for 71 % of variation among species. Component 1 explained 48 % of this variation and component 2 explained 23 % of this variation. *Dodonaea viscosa* is influenced by nitrogen content in component 1 and by water content in component 2. *Varronia curassavica* differed from the other species due to the higher leaf thickness and density of trichomes and lower SLA in component 2. *Symphyopappus casarettoi* showed high C: N ratio, high water content and low N content, explained by component 1 (Fig. 4).

Discussion

The types of defenses did not vary intraspecifically. However, there were quantitative variations in the

Table 6. Means and standard deviations of leaves attributes of *Varronia curassavica* in the *restinga* formations of Parque Estadual Acaraí, São Francisco do Sul, SC. Legend: herbaceous *restinga* (Rh), shrub *restinga* (Ra), shrub-tree *restinga* (Raa), forest transition (Ft), SLA= Specific Leaf Area (leaf area/dry mass). * represents statistically significant difference ($p \leq 0.05$). ** Abaxial surface completely covered by glandular and non-glandular trichomes with no possibility of counting (Fig. 3 C, F, I, L).

Leaves attributes	Rh	Ra	Raa	Ft	F
Dry mass (g)	0.12 ± 0.07c	0.16 ± 0.04b	0.21 ± 0.06 a	0.15 ± 0.06b	* 67.17
Leaf area (cm^2)	7.57 ± 2.66c	13.48 ± 3.05b	14.95 ± 4.53a	13.27 ± 4.56b	* 146.12
SLA (cm^2/g)	67.19 ± 44.35b	78.53 ± 13.63a	66.98 ± 16.34b	84.05 ± 25.85a	* 19.05
Consumed area (cm^2)	0.16 ± 0.33c	0.71 ± 1.10ab	0.95 ± 1.17a	0.51 ± 1.10b	* 22.85
Total leaf thickness (mm)	0.13 ± 0.07bc	0.12 ± 0.06c	0.17 ± 0.06a	0.14 ± 0.04b	* 30.58
Palisade parenchyma thickness (mm)	121.26 ± 24.78a	103.08 ± 32.66b	104.21 ± 18.26b	115.14 ± 23.89a	* 11.98
Spongy parenchyma thickness (mm)	88.63 ± 24.54a	80.78 ± 23.36b	60.31 ± 17.23d	68.69 ± 21.29c	* 33.38
Glandular trichome (adaxial) (n/cm^2)	1096.00 ± 105.25a	629.6 ± 325.88b	485.6 ± 219.81b	578.4 ± 373.62b	* 16.18
Non-glandular trichome density (adaxial) (n/cm^2)	296.00 ± 85.44a	356.8 ± 154.77a	376.80 ± 156.59a	196.80 ± 104.51b	* 9.803
Trichome density (abaxial) (n/cm^2)	**	**	**	**	**
Water content (%)	76.07 ± 11.00a	78.97 ± 3.84a	69.96 ± 6.28b	69.95 ± 9.07b	* 63.58
Leaf nitrogen content (g.kg^{-1})	20.00 ± 0.75 ab	18.91 ± 0.66b	21.88 ± 1.03ab	22.07 ± 0.84a	* 82.107
C:N ratio	22.72 ± 2.40a	23.03 ± 1.16a	20.46 ± 2.57a	19.75 ±2. 19a	2.866
Herbivory rate (%)	2.35 ± 4.31b	5.88 ± 9.85a	6.18 ± 6.42a	3.16 ± 5.60b	* 15.659

Table 7. Histochemical tests on leaves of the *Varronia curassavica* in four *restinga* formations. Legend: Test results: positive (+), negative (-). Formation: herbaceous *restinga* (Rh), shrub *restinga* (Ra), shrub-tree *restinga* (Raa), transition forest (Ft).

Compound	Restinga formations	Epidermis			Parenchyma			Vascular Bundle	
		Adaxial	Abaxial	Collenchyma	Palisade	Spongy	Phloem	Xylem	Trichome
Phenois	Rh	+	+	+	-	-	+	+	-
	Ra	-	-	+	-	-	+	+	+
	Raa	+	-	+	-	-	+	+	-
	Ft	-	-	+	-	-	+	+	+
Lignin	Rh	+	-	-	-	-	-	+	-
	Ra	+	-	-	-	-	-	+	-
	Raa	-	-	-	-	-	-	+	-
	Ft	+	-	-	-	-	-	+	-
Tannins	Rh	+	-	+	-	-	+	+	-
	Ra	+	-	+	-	-	+	+	-
	Raa	+	-	+	-	-	+	+	-
	Ft	+	-	+	-	-	+	+	-
Alkaloids	Rh	-	+	+	+	-	-	+	-
	Ra	+	-	+	-	-	-	+	+
	Raa	+	-	+	-	-	-	+	-
	Ft	+	-	+	-	-	+	+	-

morphological traits and the distribution of the secondary compounds in the mesophyll, which influenced in herbivory rates among the populations in the restinga communities. Some of the attributes presented an interspecific variation pattern: water content was lower in Raa and Ft; AEF was higher in Raa for *D. viscosa and S. casarettoi*; density of trichomes was higher in Rh for *V. curassavica* and *D. viscosa*, while chemical compounds had a lower distribution in Rh.

The species of this study presented different combinations of defense attributes, which must be functionally equivalent. According to Agrawal & Fishbein (2006), species can be grouped within defensive syndromes, which are characterized by plant nutritional and leaf attributes. Evaluating Cerrado species, Silva & Batalha (2011) identified two groups of species that present high C: N ratio and other attributes of defenses, being these attributes related to low edaphic quality.

Among species of this study, *S. casarettoi* presents the lower nutritional quality (low nitrogen content and high C:N ratio) as main attribute to herbivore defense and can be classified in the low nutritional quality syndrome. The herbivory rate observed in this species is related to high water content. Differently, *D. viscosa* and *V. curassavica* presented highly nutritive tissues (high water content, high N content and low C: N ratio) but protected by chemical compounds and high leaf thickness (*D. viscosa*), high density of trichomes, low SLA and foliar thickness (*V. curassavica*). Then, both species can be classified as "nutritious and protected".

Instead, the defenses did not change in proportion to resources availability and this do not corroborates the Resource Availability Hypothesis (Coley *et al.* 1985). This hypothesis assumes that lower rates of herbivory should occur in Rh, because the low availability of nutrients in the soil of this environment influences the production of defenses based on higher carbon content. However, these attributes often presented similar values between the Rh and Ft populations (SLA, C:N ratio and chemical

Table 8. Means and standard deviations of defense and nutrition leaves attributes of *Dodonaea viscosa*, *Symphyopappus casarettoi* and *Varronia curassavica* and correlation between leaf attribute represented by components 1 and 2 obtained by PCA, in the *restinga* of Parque Estadual Acaraí, São Francisco do Sul, SC.

Defense/nutrition attributes	*D. viscosa*	*S. casarettoi*	*V. curassavica*	P	PC1	PC2
C:N ratio (g.kg^{-1})	20.03 ± 3.13b	26.52 ± 2.96a	21.43 ± 2.42b	< 0.0001	0.561	0.192
Leaf nitrogen content (g.kg^{-1})	24.32 ± 4.16a	17.48 ± 2.04c	20.77 ± 2.46b	< 0.0001	-0.583	0
SLA (cm^2/g)	118.71 ± 42.50a	90.28 ± 18.10b	74.23 ± 13.62c	< 0.0001	-0.301	0.489
Total leaf thickness (mm)	0.12 ± 0.01b	0.09 ± 0.027c	0.14 ± 0.02a	< 0.0001	-0.167	-0.599
Trichome density (adaxial) (n/cm^2)	122.08 ± 32.83b	176.25 ± 44.02b	710.25 ± 285.75a	< 0.0001	0.129	-0.604
Water content (%)	68.75 ± 4.82c	77.00 ± 4.084a	74.17 ± 4.90b	< 0.0001	0.458	0
				Variance explained by the components	2.536	1,69
				Percentage of total variance explained (%)	48	23

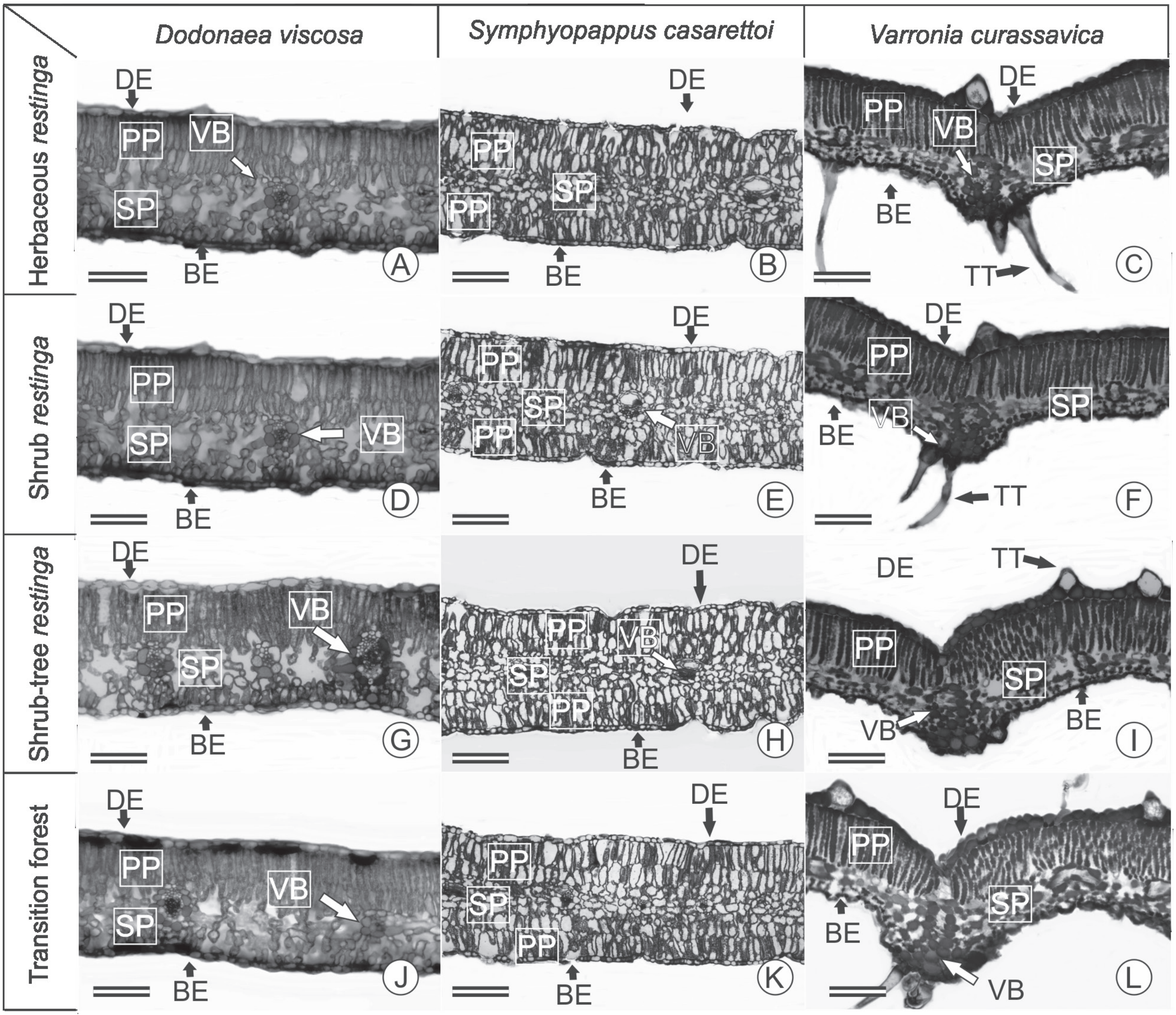

Figure 1. Transverse section of leaf blades of *Dodonaea viscosa, Symphyopappus casarettoi* and *Varonia curassavica* from four *restinga* formations in Parque Estadual Acaraí, São Francisco do Sul, SC. Legend: DE – adaxial epidermis, BE – abaxial epidermis, PP – palisade parenchyma, SP – spongy parenchyma, VB – vascular bundle, TT – non-glandular trichome. Scale bar = 120 µm.

compounds) and the rate of herbivory was not increased in the environmental gradient. Intraspecific variation in antiherbivory defenses has been demonstrated under experimental conditions (Mutikainen *et al.* 2000), but studies in natural environments remain scarce (Boege & Dirzo 2004). Studies investigating which structural attributes and chemical compounds may be antiherbivory defenses have been performed with species of the *restinga* (Corrêa 2007).

Dodonaea viscosa showed the highest rate of herbivory in Ra, where the individuals presented a high nutritional quality (nitrogen content and foliar water), lower density of trichomes and lower distribution of secondary metabolites. Conversely, in Raa, although the individuals presented high nitrogen content and low C:N ratio influenced by the higher availability of nutrients in the soil, the lower rate of herbivory was observed due to the greater protection by phenols, alkaloids and tannins. Alkaloids have a secondary action in herbivores, which is to interfere with the nervous system and reduce the absorption of nutrients and decreased the action of microorganism of digestive tract (Schardl 2002) and tannins possess immediate action of the astringent taste of tannins (Salminen & Karonen 2011).

The alkaloids presence in Raa and Ft is predicted by the Carbon-Nitrogen Balance Hypothesis (HBCN) because the greater amount of nutrient in the soil favors the allocation of nitrogen-based metabolites (Bryant *et al.* 1983). On the other hand, the presence of carbon compounds (phenols) was expected in populations of Rh and Ra due to nutrient shortage in soil, however, the reduced phenols presence in these populations is due to the high maintenance cost of the metabolites.

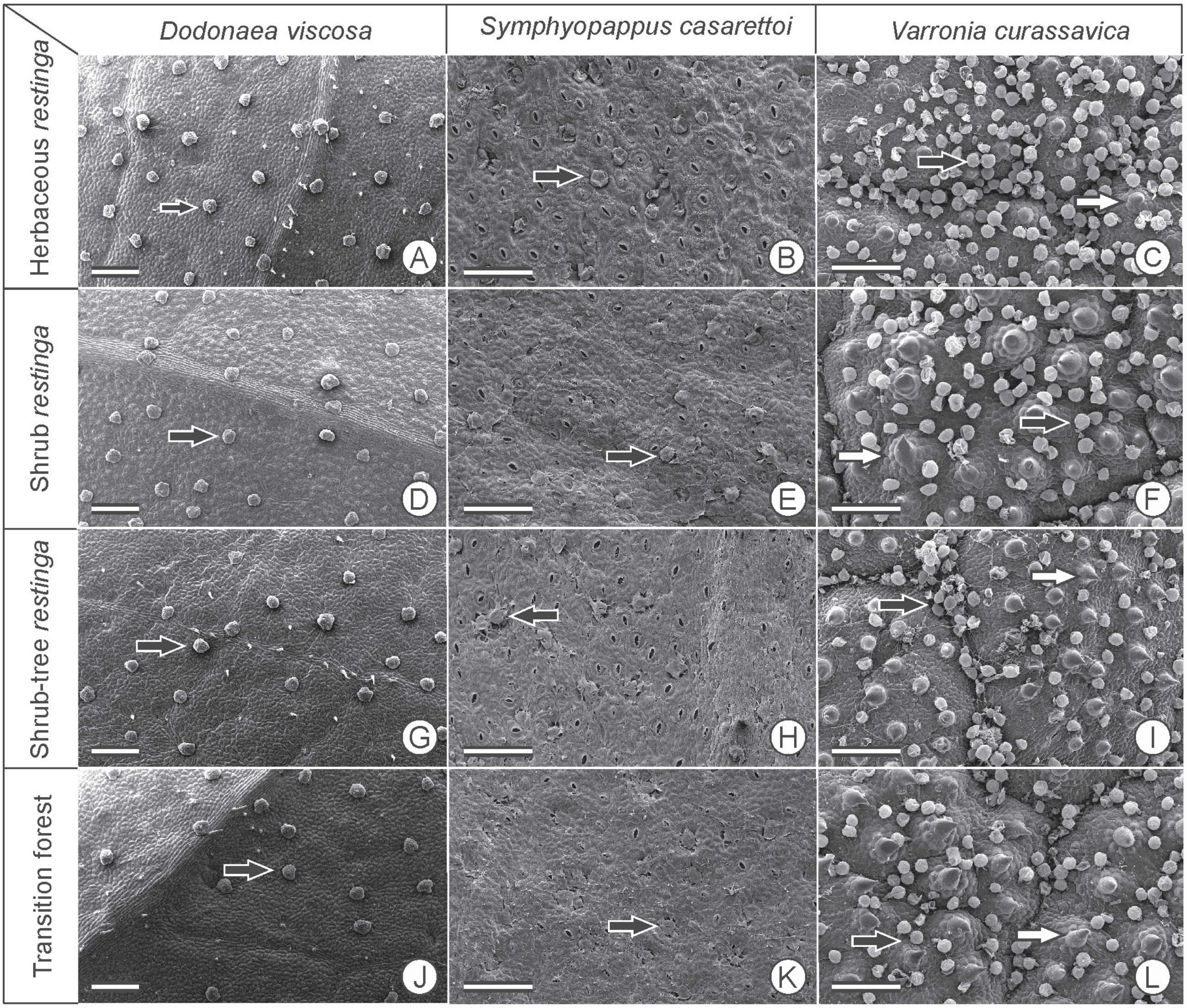

Figure 2. Coverage of glandular trichomes (black arrow) and non-glandular trichomes (white arrow) of the adaxial surface of leaf blades of the study species from four *restinga* formations in Parque Estadual Acaraí, São Francisco do Sul, SC. Scale bar = 200 µm.

Thicker leaves can hinder the actions of insect herbivores (Corrêa 2007; Hanley *et al.* 2007; Guerra *et al.* 2010) and it was observed in *D. viscosa* present in forest edges, under higher light intensity. The trade-off between thickness and leaf area was a response to high light incidence in these vegetation types, in order to reduce the transpiration surface (Melo Jr. & Boeger 2016).

The low soil fertility and high light incidence influenced the leaf production, with higher amount of mechanical tissue at the expenses of photosynthetic tissue, resulting in leaves with lower AEF at three species, especially in *V. curassavica*. This is a resource saving strategy, since leaves with lower AEF present greater longevity (Vendramini *et al.* 2002; Agrawal & Fishbein 2006, Hanley et al. 2007) and secondarily, acts as a barrier against herbivores, due to tougher leaves (Silva *et al.* 2015; Silva & Batalha 2011).

Symphyopappus casarettoi experienced less herbivory in populations of Ra, which presented lower SLA. In general, the low rates of herbivory in *V. curassavica* can be attributed to the low values of SLA. The similar situation was observed for *Inga edulis* Mart. (Fabaceae) in a forest environment (Heerdt & Melo Jr. 2016).

Symphyopappus casarettoi presented low nutritional quality (low N content and high C: N ratio) and high protection by phenols when compared to the other species. The reduction of leaf nutritional quality is a physiological defense to reduce herbivore preference (Mattson 1980). In Ra, although the individuals present high water content, the low N content associated with the low SLA values influenced negatively the herbivory (Silva & Batalha 2011).

Inversely, in Raa, *S. casarettoi* had higher SLA, influenced mainly by the lower light incidence in this environment (Melo Jr. & Boeger 2016), and experienced only minor damage from herbivores due to the low nutritional quality of the leaves of

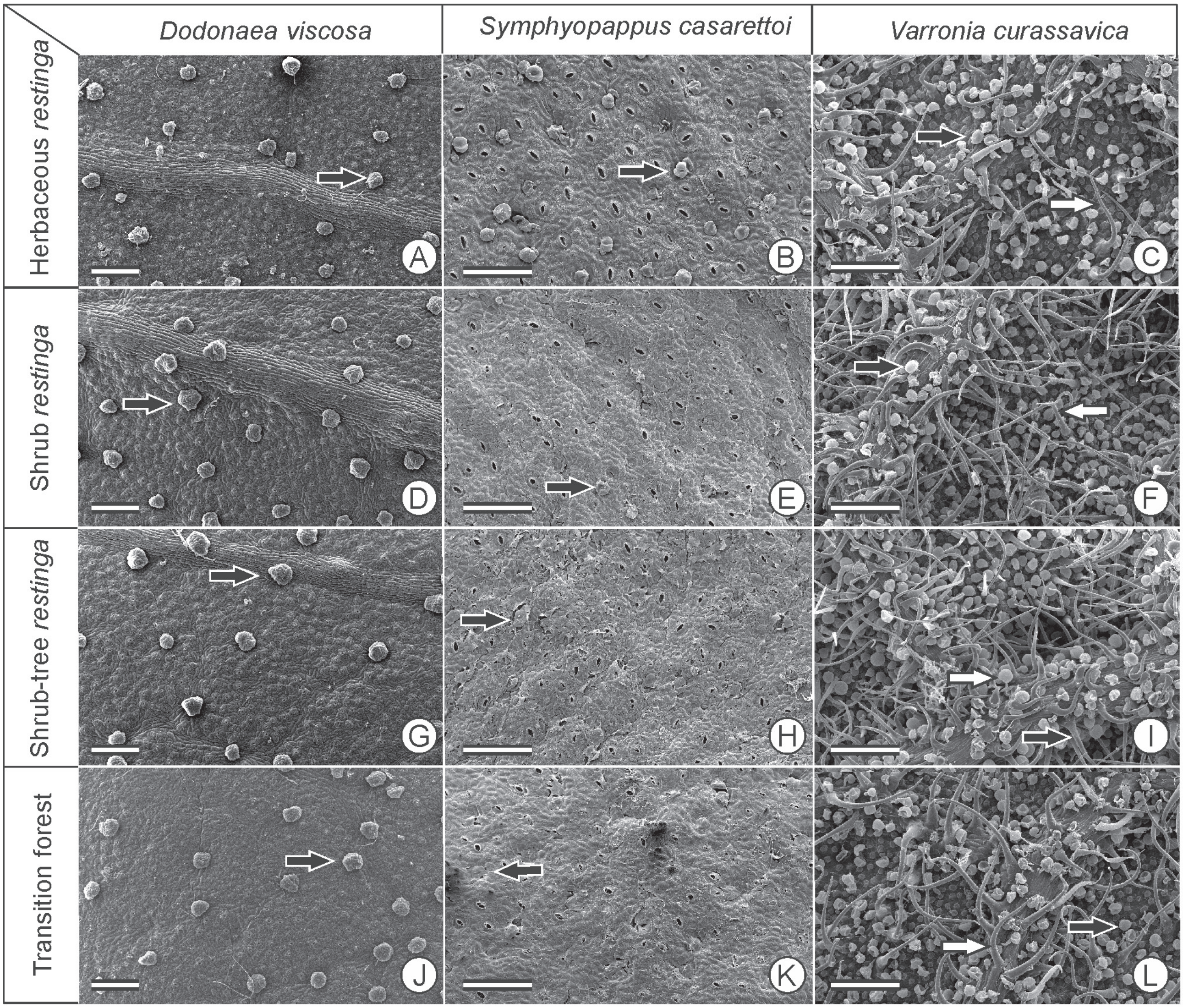

Figure 3. Coverage of glandular trichomes (black arrow) and non-glandular trichomes (white arrow) of the abaxial surface of leaf blades of the study species from four *restinga* formations in Parque Estadual Acaraí, São Francisco do Sul, SC. Scale bar = 200 µm.

these individuals (lower water content and high C:N ratio) and high protection by phenols, tannins and alkaloids. The presence of phenolic compounds in plants of the *restinga* was found to be negatively related to herbivory (Silva & Medeiros 2005) and tannins have high anti-nutritional potential and reduce palatability after binding to salivary and mucous proteins (Salminen & Karonen 2011).

Phenols presence in various tissues of mesophyll in all *S. casarettoi* populations occurs due to low resources in the restinga that results in the production of carbon metabolites (Bryant *et al.* 1983). On the other hand, the populations of Raa and Ft presented greater investment in alkaloids (nitrogen compounds) as expected by HBCN.

Symphyopappus casarettoi had the highest rates of herbivory at the two extremes of the edaphic gradient of the *restinga*. Such a pattern of herbivory may be related to the high population density of this species in Rh, which facilitates herbivore access to plants (Feeny 1976), while in the forest the high rate of herbivory may be related to the greater abundance of herbivores due to the environment being more favorable for the occurrence of insects (Basset *et al.* 2003), beyond of absence of tannins in Rh and Ft, associated to high water content of this species.

The gradual change in the rate of herbivory on *V. curassavica* among the *restinga* communities seems to result from lower values of SLA associated with a high density of glandular trichomes, as evidenced by the lower rate of herbivory in Rh, despite having high nitrogen and water content in the leaves of this population. The importance of trichomes in antiherbivory defense was demonstrated in experiments with *Aristolochia californica* (Aristolochiaceae) and *Liabum mandonii* (Asteraceae), in which predation and oviposition were negatively correlated with trichome density (Fordyce & Agrawal 2001; Molina-Montenegro *et al.* 2006).

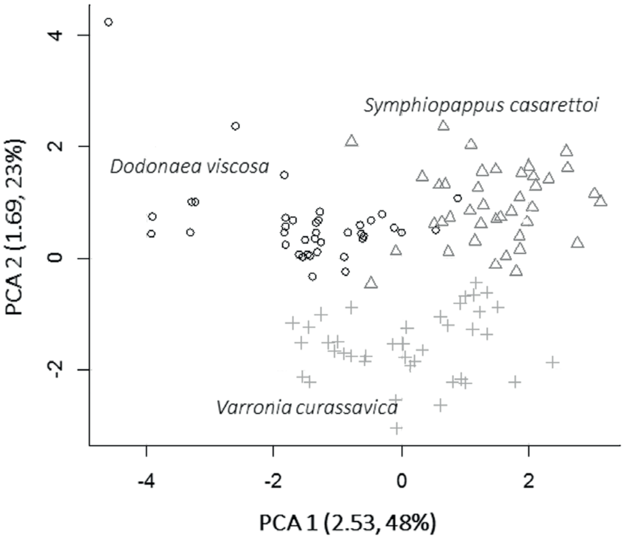

Figure 4. Principal components analysis (PCA) showing the difference between the three species of study in the *restinga* formations of the Parque Estadual Acaraí, São Francisco do Sul, SC, Brazil, in relation to the foliar attributes of defense and nutrition. The explanation and eigenvalues of the first two main components are shown in the figure.

Other studies have shown that herbivory is an inducing agent, causing an increase in the density of trichomes after defoliation (Traw & Dawson 2002; Abdala-Roberts & Parra-Tabla 2005).

In Ra, *V. curassavica* is an abundant species, which means a lower cost for locomotion to herbivores (Schuldt *et al.* 2010). A lower level of nitrogen may have also contributed to the high rate of herbivory in this population, because herbivores consume the amount of leaves necessary to acquire sufficient nitrogen for their development (Lavoie & Oberhauser 2004; Gonçalves-Alvim *et al.* 2011). On the other hand, in Raa and Ft, the high level of nitrogen, lower C:N ratio and low density of trichomes favor the action of herbivores (Silva & Batalha 2011).

The lower SLA did not prevent the herbivory in Raa and Ft, due the higher nutritional quality of these populations. These results corroborate the study of fifteen tropical species that showed that individuals with greater water content experienced higher rates of herbivory, even with low SLA, indicating that the nutritional quality of plant tissue can be more important than leaf toughness to certain species of herbivores (Poorter *et al.* 2004).

In *V. curassavica,* the uniform distribution of secondary metabolites showed that secondary metabolites are defenses less important than the high density of trichomes. Moreover, it is possible to conclude that the insects that prey on this species have high resistances to secondary metabolites (Cornell & Hawkins 2003). Although little variation, phenols and tannins present differences in distribution among populations, being present in more mesophyll tissues in Rh individuals corroborating HBCB (Bryant *et al.* 1983).

Observing the anatomical characteristics of *V. curassavica,* it is important to note that the lower rates of herbivory occurred in populations with thicker palisade parenchyma. Due to compaction of this tissue, leaves becomes more resistant to breakage as compared to leaves with more developed spongy parenchyma, which has more intercellular spaces that facilitate the breakage of the leaf blade by herbivores (Silva *et al.* 2005). Therefore, the isobilateral organization of the mesophyll in *S. casarettoi* may explain the lower rates of herbivory in this specie.

In general, the high leaf nitrogen concentrations, in this species, may occur as a result of the stressful abiotic conditions of the *restinga*, corroborating the Plant Stress Hypothesis (PSH, White 1984) (Oliveira & Cortez 2015). However, the nutritional quality is balanced by the amount of carbon and the presence of secondary metabolites in the mesophyll. For this reason *S. casarettoi* presents lower nutritional quality.

The rates of herbivory observed among the tree species of the *restinga* of PEA - Parque Estadual Acaraí are, in general, low in comparison to 20.86 % and 18.23 % for *Myrcia bergiana* (Myrtaceae) and *Tetracera breyniana* (Dilleniaceae), respectively in Ipojuca, Pernambuco (Corrêa 2007), which are two to three times the rates found in the present study (Tab. 4). The low rates of herbivory may be a result of the higher plant diversity at PEA (Melo Jr. & Boeger 2015), because greater diversity reflects a greater availability of food resources for leaf herbivores (Unsicker *et al.* 2006). Another factor to consider is the low density of the species studied along the environmental gradient at the PEA - Parque Estadual Acaraí (except *V. curassavica* in shrub *restinga* and *S. casarettoi* in herbaceous *restinga*) (Melo Jr. & Boeger 2015), which, according to the appearance hypothesis, decreases the possibility of a species being encountered by herbivores (Feeny 1976).

Conclusions

Together, the results obtained in this study suggest that *S. casarettoi* shows characteristic of the Low Nutritional Quality Syndrome, which is characterized by low nutritional value and harder leaves (reduced SLA). On the other hand, *D. viscosa* and *V. curassavica* have the characteristics of the Nutrition and Protection Syndrome, which is characterized by higher nutritional quality (water and nitrogen) with investment in defenses (thick leaves and high density of trichomes, respectively).

The broad standard deviations for the mean consumed areas of the three studied species suggests that insect herbivores have generalist feeding habits, and resist the different defense strategies presented by each plant species. The absence of a pattern in the production of antiherbivory defenses among the studied species may indicate that these defense mechanisms are associated with attributes that are related to conserving resources.

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