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Evaluation of the Foliar Damage That Threatens a Millennial-Age Tree, *Araucaria araucana* (Molina) K. Koch, Using Leaf Waxes

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Received: 27 October 2019; Accepted: 30 December 2019; Published: 3 January 2020

Abstract: *A. araucana* is an endemic species of the temperate forests from Chile and Argentina; protected in both countries and categorized as in danger of extinction. Individuals of this species have begun to show foliar damage (i.e., discoloration) in branches and upper parts. The discoloration begins from the base to the top and from the trunk to the branches with necrotic rings appearing; in some cases causing death; and is currently attributed to an as yet unknown disease. This study focuses on the first protective layer of plants against environmental stress and pathogens; known as leaf waxes. The abundance and distribution of three classes of leaf waxes (long chain fatty acids; alkanes and alcohols) were measured in healthy individuals of *A. araucana* from different sites and individuals that present foliar damage (sick individuals). In the case of sick individuals; their leaf waxes were measured considering the level of leaf damage; that is; leaves without; medium and full foliar damage. The most abundant class of leaf wax in both sick and healthy individuals was fatty acids; followed by alkanes and then alcohols; with common dominant chains; C₂₈ fatty acid; C₂₉ alkane and C₂₄ alcohol. Sick individuals have higher abundances of alkanes and alcohols than healthy individuals. The leaves of sick individuals have lower values of distribution indices (the carbon preference index of fatty acids and average chain length of alkanes) as foliar damage increases that are interpreted as a reduction of *in vivo* biosynthesis of waxes. This is the first evidence of *A. araucana* response to a still unknown disease that is killing individuals of this endemic species.

Keywords: alkanes; alcohols; fatty acids; unknown disease; foliar damage; ACL; CPI

1. Introduction

The *Araucaria araucana* forest is found in the temperate forests of South America [1], where species like *A. araucana* and *Nothofagus* dominate, forming mixed stands with a dense understory dominated by *Chusquea* bamboos [2]. The temperate forests have high endemism, and over time, the natural distribution of *A. araucana* forest has been reduced despite significant interest in conservation. Today, *A. araucana* forests are distributed between the Chilean and Argentinian slopes of the Andean Cordillera and two unconnected populations in the Coastal Cordillera of Chile. The main causes of the reduction of *A. araucana* forests and specifically of the *A. araucana* species, has been human- induced fires [3,4], utilization of seeds as a food supply [5], and introduction of exotic species [6–9]. The above are among the reasons why *A. araucana* is considered of high conservation value and protected on both sides of the Andean Mountains [10]. Indeed, it has been declared a natural monument since 1990 in Chile [11], with the population of the Andean Cordillera categorized as vulnerable and the population of Nahuelbuta Coastal ridge in danger of extinction [12].

A. araucana is an ancient, slow-growing and long-lived gymnosperm tree from the Pinophyta division [13], reaching ages greater than a thousand years [14,15]. Their trunks, which hold symmetrical cups, stand out, straight, long and devoid of branches at their adult stage [13]. This endemic species also has cultural importance to the native Mapuche people from Chile and Argentina, who identify with this tree when carrying out cultural, social and spiritual practices that continue to the present day [16,17].

Recently, *A. araucana* individuals started to show increasing foliar damage (i.e., discoloration) in the branches and crowns. The discoloration begins from the base of the trunk, and spreads to the top of the tree, and from the trunk to branches, with symptoms including necrotic rings on branches, in some cases causing death. These symptoms and related deaths have been attributed to an as yet unknown disease [18]. This problem stimulated a comprehensive survey to be carried out cooperatively by Chile's National Forest Corporation (Corporación Nacional Forestal—CONAF), Agricultural and Livestock Service (Servicio Agrícola y Ganadero—SAG) and experts spanning 400 sites in Chile (Regions of Biobío, Araucanía and de los Ríos) in 2016–2017. The survey determined that 93.3% of individuals are affected by this condition. A follow-up survey in 2017–2018 indicated that the percent of affected individuals increased to 98.3% and mortality has reached almost 11% of the population [19,20].

Because of this worrying situation, the SAG and CONAF in collaboration with the Ministry of Agriculture in Chile are leading a multidisciplinary public-private, national/international team to investigate this situation through a variety of different research lines, considering several theories of what might be causing this phenomenon, for example pathogens such as American bark beetles, borers and fungi [20]. Other researchers attribute the observed foliar damage to climate change, affecting the environment, forcing *A. araucana* to try to overcome changes in temperature and water balance in a short period time (e.g., 10 years). Such environmental stress could result in vulnerability to pathogenic organisms that are normally present in the environment [18–20]. These investigations are ongoing research, though the causes of the disease have not yet been determined.

A response to the foliar damage, whatever the cause, must be found in one of the main adaptations of vascular plants to the terrestrial environment, the cuticle [21]. The cuticle is a lipid coating that covers the leaves of plants and is composed primarily of two lipophilic materials: cutin and waxes [22,23]. Waxes are of vital importance since they fulfill the function of limiting the loss of water through the cuticle, minimizing the exchange (and loss) of gases (e.g., CO₂, O₂) and water (H₂O) and acting as a thermoregulatory barrier [23,24]. The leaf waxes also minimize wettability and the retention of dust particles and spores [23,25], act as a protective barrier against insects and UV-B radiation, prevent the proliferation of pathogenic microbes, and the uncontrolled adhesion of the epidermal cells of organs in the early stages of development (i.e., correct organ formation) [26–29].

Chemically, leaf waxes are a mixture of organic compounds in series of homologous chains (typically dominated by chain lengths containing 23–33 carbon atoms) with a terminal hydroxyl, carbonyl or carboxyl, including alcohols, fatty acids, alkanes, esters, aldehydes and ketones [21,22,30–32]. The long chain alcohols, fatty acids and alkanes are waxes that can be characterized by their length (>C₂₃), abundance or total concentration, and their distribution (e.g., relative abundance) [30,31,33]. The most commonly used indices of leaf waxes distribution are the average chain length (ACL) index, which describes the average number of carbon atoms per molecule weighted by the abundance of the compound and the carbon preference index (CPI), measures the relative abundance of leaf waxes with even vs. odd numbers of carbon atoms (typically even chain lengths dominate over odd in alcohols and fatty acids, and odd over even in alkanes) [34,35].

Several studies have shown that the concentration and distribution of waxes (e.g., alkanes and fatty acids) can vary within the same species, annually or seasonally, and even in organs of the same individual (i.e., between leaves) through their ontogeny. There is also environmental effect related to stress that can alter leaf waxes, including water deficit [36–38], high temperatures [39], and pollution [40,41]. These environmental effects can change the abundance and distribution of leaf waxes through biosynthesis processes or mechanical removal (i.e., erosion) [42–45], and are possible factors

associated with the diseased *A. araucana*. The alteration of lipid homeostasis has also been associated with various diseases [46]. Therefore, a study of the cuticular waxes of *A. araucana* is timely considering the current situation.

There are very few investigations of cuticle waxes from *A. araucana* [30,47] and only in healthy individuals; there is no information on leaf waxes from sick or leaf-damaged individuals to date. This study quantifies and compares the abundance and distribution (ACL and CPI) of leaf waxes among healthy individuals of *A. araucana* collected from four riparian sites of *A. araucana* forest in the Región de la Araucanía (Chile). The main compounds avoiding leaf water loss are very long chain of alkanes and alcohols derivate from fatty acids [24]. Then, we expect an increase in abundance and distribution of leaf waxes in sick individuals of *A. araucana*, with high variability among healthy individuals located in different sites. The study also compares waxes between healthy and sick individuals found on a specific site of the Parque Nacional Tolhuaca (Tolhuaca National Park—PNT), evaluating three levels of visual foliar damage (without, medium, and full foliar damage [or discoloration]) in sick individuals.

2. Materials and Methods

2.1. Study Site

The study was carried out in the Región de la Araucanía in south-central Chile, specifically in protected zones in the riparian zones of Laguna Verde Tolhuaca (Site 1; 38°12′48.3″ S; 71°44′10.5″ W; 1385 masl) in PNT, Laguna Captren (Site 2; 38°38′23.4″ S; 71°41′58.7″ W; 1282 masl), Laguna Galletué (Site 3; 38°40′33.9″ S; 71°18′58.5″ W; 1350 masl) and Laguna El Toro (Site 4; 38°42′33.0″ S; 71°20′57.4″ W; 1350 masl). The vegetation of this zone is dominated by *A. araucana* trees. The climate in the region is rainy with annual precipitation in the range of 2500 to 3000 mm, snow in winter and moderate temperatures with some relative summer drought [48].

2.2. Sampling Protocol

Leaves of *A. araucana* individuals were collected in two sampling campaigns. Sampling sites were chosen according to their accessibility, avoiding trails and easy human access to minimize anthropogenic disturbances [49]. In January 2017, leaves from only healthy individuals at four sites were collected because no sick individuals were observed at the sites (Figure 1). In December 2017, sick individuals were found only in Site 1 (PNT) and therefore leaves were collected.

Figure 1. Sampling sites in the Región de la Araucanía, Chile.

Leaves of 3 healthy individuals were collected per site ($n = 4$; total $n = 12$) and leaves of 5 sick individuals were collected at Site 1, considering 3 levels of visual foliar damage: leaves without damage (green), leaves with medium damage (discolored with green and brown) and full foliar damage (totally discolored) (see Figures 2 and 3).

Figure 2. Individuals of *A. araucana*: (a) healthy, (b) sick, and images of 3 levels of foliar damage: (c) without, (d) medium and (e) full foliar damage.

Figure 3. Leaves of sick individuals: (a) brown or with full foliar damage, (b) leaves with medium foliar damage (green with brown parts) and (c) leaves without foliar damage (green). (d) Comparison with the length in centimeters of the leaves in their different degrees of foliar damage.

To ensure similar environmental conditions at the same site (i.e., light regime, vertical location in the dossal), trees were preferably chosen far away from other trees, with leaves exposed to the sun but under the dossal, collecting those leaves without obvious herbivory damage [50]. All the individuals were adults ca. 10 meters tall, to avoid the different states of ontogeny [51]. The samples were taken at

a greater height than 5 meters with a tree pruner. Finally, leaves were collected and stored in kraft paper bags during the field sampling. Once in the laboratory they were kept at 50 °C in an oven for 15 days (model Schutzart, Memmert GmbH, Schwabach, Germany) until dryness.

2.3. Total Lipid Extraction

Dry leaves with minimal damage (i.e., herbivory) were selected and cut with solvent rinsed (methanol, acetone and hexane successively) stainless steel scissors. Total lipids of 0.5 grams of leaf per individual were extracted with 20 mL of dichloromethane:methanol (DCM:MeOH) (9:1 *v/v*) following (e.g., [50,52,53]) using microwave assisted extraction (MAE) with a Milestone Ethos Easy (Milestone, Sorisole (BG), Italy) instrument with a ramp of 100 °C at 10 min, a waiting time of 15 min at 100 °C and 30 min cooling, repeating the extraction three times. Twenty five µL of internal standard (1000 ng per µL of *cis*-10-nonadecenoic acid, 5 α -cholestane and nonadecanol) was added. The total lipid extract (TLE) was dried under gaseous N₂ using a Flexi-vap (Model 109A-YH-1, Glas-Col, Terre Haute, IN, USA).

2.4. Total Lipid Separation

The TLE was separated in two fractions of different polarity (fatty acids and neutral) via solid phase extraction using aminopropyl cartridges (Clean-up®) following published methods [54]. The neutral fraction, containing alkanes and alcohols, was eluted with 10 mL of DCM:2-propanol (2:1 *v/v*), and the fatty acids fraction was eluted with 10 mL of glacial acetic acid:diethyl-ether (1:24 *v/v*). Both fractions were concentrated under a flow of gaseous N₂ by Flexi-vap. The neutral fraction was further separated via silica gel 60 (0.063–0.200 mm, Merck KGaA®, Kenilworth, NJ, USA, activated at 450 °C) column chromatography. Relevant fractions were eluted with 4 mL of hexane (alkanes), DCM: ethyl acetate (8:2 *v/v*; alcohols). Finally, the alkane fraction was separated into saturated and unsaturated fraction via silver silica column (Pcode: 248762-2506, Sigma-Aldrich, St. Louis, MO, USA), using hexane (saturated) and DCM:MeOH (1:1 *v/v*; unsaturated).

2.5. Derivatization

The fatty acid and alcohol fractions were derivatized prior to instrumental analysis. Fatty acids were methylated with 500 µL of boron trifluoride in methanol, generating fatty acid methyl esters (FAMES). The alcohols were silylated, generating trimethylsilyl esters, using 25 µL of pyridine and 25 µL of bis-(trimethylsilyl)-trifluoroacetamide.

2.6. Leaf Wax Identification and Quantification

The leaf waxes were identified using a capillary gas chromatograph (GC17A-Shimadzu, Kyoto, Japan, column 30 m HP5, 0.25 mm id, film thickness = 0.25 µm) with helium-carrier gas at constant flow of 1.4 mL min with mass spectrometry detector (QP-5050A; Shimadzu). The GC program started at 50 °C for 1 min, increased by 10 °C/min to 130 °C, maintained for 0.1 min at 130 °C, then increased by 4 °C/min to 325 °C, and was maintained for 15 min at 325 °C. The individual wax components were identified by comparing their mass spectra and retention times with those of authentic standards.

Leaf waxes were quantified by GC with flame ionization detection (7890A, Agilent, Santa Clara, CA, USA) with a quantification standard (5 α -androstane), under the same conditions as above, but with H₂ as carrier gas. Coelution of compounds of interest was occasionally observed in the alcohol fraction, therefore, the quantification was estimated in all samples by peak integration in the mass chromatograms using characteristics *m/z* values and correcting for response factor. Then, all leaf wax abundances were normalized by the mass of the extracted dry leaf material expressed as µg/g dry leaf. To compare with other studies, abundance (µg) was also normalized to area of dry leaf of *A. araucana* (42.2 cm²) [50] (Supplementary Materials [SM]).

The average chain length was calculated following Equation (1):

$$\text{ACL (fatty acids) or (alkanes)} = \Sigma C_n \times n / \Sigma C_n \quad (1)$$

where $n > 22$ and C_n is the concentration of a fatty acids or alkanes with n carbons [35].

The carbon preference index was calculated following Equations (2) and (3) [34]:

$$\text{CPI (fatty acids)} = 0.5 \times [(\Sigma \text{ odd} / \Sigma \text{ even}) + (\Sigma \text{ odd} / \Sigma \text{ even})] \quad (2)$$

$$\text{CPI (alkanes)} = 0.5 \times [(\Sigma \text{ even} / \Sigma \text{ odd}) + (\Sigma \text{ even} / \Sigma \text{ odd})] \quad (3)$$

where the concentration of carbon chains ranges from 24 to 34 for fatty acids and from 23 to 33 for alkanes.

2.7. Statistical Analysis

A variance analysis (ANOVA) was applied to determine significant differences in abundance and distribution indices (ACL and CPI) of leaf waxes between healthy individuals at the different sites as well as between states of foliar damage of sick individuals (without, medium and full foliar damage), followed by a post-hoc Tuckey analysis to determine pairwise differences. In the case of healthy and sick individuals at Site 1, a T-test was applied to determine significant differences we assume for significant effects $p < 0.05$. Statistical analysis was performed with Sigma Plot 12.2 software (Systat Software, San José, CA, USA). All data available as Supplementary data (Data S1).

3. Results

3.1. Leaf Waxes of Healthy *A. araucana*

In all healthy individuals sampled ($n = 12$), all three identified classes of leaf wax displayed similar relative abundance patterns. Fatty acids were greatest in abundance (from 445.8 ± 308.3 to 548.4 ± 82.6 $\mu\text{g/g}$ of dry leaf), followed by alkanes (from 26.1 ± 18.8 to 95.6 ± 10.8 $\mu\text{g/g}$ of dry leaf) and then alcohols (from 0.1 ± 0.1 to 2.2 ± 3.5 $\mu\text{g/g}$ of dry leaf) (Figure 4, Supplementary Materials Table S1). The only class of leaf wax that show significant abundance differences among sites was alkanes ($F = 6.925$; $p < 0.05$).

Figure 4. Abundances of fatty acids, alkanes and alcohols ($\mu\text{g/g}$ dry leaf) in healthy *A. araucana* individuals at the four sample sites ($n = 12 \pm \text{SD}$). Error bars indicate standard deviation (SD).

The distributions of fatty acids and alkanes were slightly different among sites (Figure 5). The difference observed in the ACL index was no more than two units but the CPI difference reaches almost 10 units. Specifically, the ACL of fatty acids of healthy *A. araucana* varied from 26.9 (Site 1) to 28.0 (Site 4) and the CPI varied from 10.4 (Site 4) to 19.9 (Site 1). The ACL of alkanes varied from 28.0 (Site 4) to 29.9 (Site 3) and the CPI of alkanes varied from 4.2 (Site 4) to 8.4 (Site 2). The differences in

both distribution indices for alkanes were significant between sites ($F = 4.315$; $p < 0.05$ and $F = 21.932$; $p < 0.05$ respectively). The post-hoc analysis show significant differences in CPI of alkanes where individuals from Site 1 and Site 4 are different from Site 2 and Site 3 (Tukey $p < 0.05$) (Figure 5).

Figure 5. ACL and CPI box plot of distribution indices of fatty acids (a,c) and alkanes (b,d) from the four sites. Letters above boxes represent significance in Tukey pair-wise comparisons ($p < 0.05$); groups with the same letter are not significantly different.

3.2. Individuals of Site 1 (Sick and Healthy)

Considering only samples of *A. araucana* at Site 1, where leaves without leaf damage were obtained from sick individuals in December 2017, and comparing with individuals in healthy condition sampled previously (January 2017), we observe that the total leaf wax abundance as well as individual abundances of all three classes of were higher in sick individuals than in healthy ones (Figure 6; Table 1). The average abundance of fatty acids in sick individuals was 22.2% higher than in healthy ones (544.8 vs. 445.8 $\mu\text{g/g}$ dry leaf). The relative difference in alkanes was even greater, with a mean abundance in sick individuals that was 233.8% greater than in healthy individuals (77.1 vs. 28.9 $\mu\text{g/g}$ dry leaf). Finally, alcohol relative abundance had the greatest difference between sick and healthy individuals, with 1000% higher abundance in sick individuals than healthy individuals (23.1 vs. 2.2 $\mu\text{g/g}$ dry leaf). The differences found were significant in alkanes ($t = 2667$; $p < 0.05$) and alcohols ($t = 2584$; $p < 0.05$).

The ACL of fatty acids varied from 27.5 in healthy individuals to 28.1 in sick individuals; and the CPI varies from 14.3 in healthy individuals and 18.8 in sick individuals. In alkanes, the ACL varies from 28.6 (healthy) to 29.4 (sick) and the CPI has a very similar range between sick and healthy individuals. Although the distribution index values were higher in sick individuals, the differences were not statistically significant.

Other important aspects of leaf wax distribution are the most abundant chain length and abundance of each chain. The most abundant fatty acid chain length in sick and healthy individuals was octacosanoic acid (C_{28} ; Table 1; Figure 7), with similar abundance in sick (185.7 $\mu\text{g/g}$) and healthy individuals (192.9 $\mu\text{g/g}$).

Table 1. Average abundance of each chain of fatty acids, alkanes and alcohols, distribution index (ACL and CPI) and the total waxes with their respective standard deviation of *A. araucana* individuals.

Fatty Acids	Site	C ₂₃	C ₂₄	C ₂₅	C ₂₆	C ₂₇	C ₂₈	C ₂₉	C ₃₀	C ₃₁	C ₃₂	C ₃₃	C ₃₄	ACL	CPI	Total
Healthy	1	3.8	37.5	6.1	97.6	9.5	185.7	6.1	82.3	2.3	16.5	0.4	2.2	27.5	14.1	450.0 ± 308.6
Healthy	2	6.5	45.5	10.8	89.0	13.1	208.5	9.3	62.9	2.3	10.2	0.2	2.7	27.5	10.2	461.0 ± 281.4
Healthy	3	5.3	52.4	9.1	119.4	10.5	241.7	8.0	84.8	2.5	12.7	0.7	1.4	27.6	14.3	548.4 ± 82.6
Healthy	4	4.0	32.9	5.9	95.1	9.2	219.5	7.1	94.0	2.8	17.9	0.4	2.5	27.8	15.5	491.2 ± 324.4
Sick: without foliar damage	1	2.8	32.6	4.4	88.1	7.8	223.0	8.0	125.8	3.5	30.2	0.0	3.4	28.1	19.8	529.6 ± 178.2
Sick: medium foliar damage	1	7.0	65.6	11.8	109.1	12.2	208.0	11.8	171.2	5.5	40.5	0.0	3.6	28.1	13.5	646.3 ± 44.1
Sick: full foliar damage	1	8.3	65.6	10.3	89.9	8.6	147.6	7.4	110.5	3.4	23.4	0.0	1.8	27.7	13.0	476.7 ± 128.6
Alkanes	Site	C ₂₃	C ₂₄	C ₂₅	C ₂₆	C ₂₇	C ₂₈	C ₂₉	C ₃₀	C ₃₁	C ₃₂	C ₃₃	C ₃₄	ACL	CPI	Total
Healthy	1	1.1	0.8	2.3	1.2	5.1	1.2	8.3	0.8	5.0	0.5	2.7	0.0	28.6	5.3	28.9 ± 18.8
Healthy	2	0.9	0.6	3.1	1.6	16.9	3.7	33.8	2.9	18.6	1.8	11.7	0.4	29.4	7.7	96.1 ± 10.7
Healthy	3	1.0	0.7	3.4	1.5	13.2	3.1	26.4	2.4	15.6	1.6	9.5	0.1	29.4	7.2	78.4 ± 38.2
Healthy	4	1.2	1.0	2.8	1.4	5.2	1.1	6.1	0.7	3.7	0.4	2.6	0.1	28.4	4.7	26.2 ± 14.0
Sick: without foliar damage	1	1.3	1.0	3.6	2.1	13.4	6.0	28.2	2.6	18.2	1.8	13.0	0.4	29.4	5.6	91.5 ± 36.9
Sick: medium foliar damage	1	2.4	1.7	5.6	2.6	12.7	6.9	21.0	1.8	11.3	0.8	5.6	0.2	28.7	4.3	72.4 ± 19.0
Sick: full foliar damage	1	2.6	1.8	7.6	2.8	12.1	5.3	19.1	1.7	9.9	0.7	4.5	0.2	28.4	4.5	68.1 ± 13.5
Alcohols	Site	C ₂₃	C ₂₄	C ₂₅	C ₂₆	C ₂₇	C ₂₈	C ₂₉	C ₃₀	C ₃₁	C ₃₂	C ₃₃	C ₃₄			Total
Healthy	1	0.0	2.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	-	2.2 ± 3.5
Healthy	2	0.0	0.3	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	-	0.4 ± 0.5
Healthy	3	0.0	0.4	0.1	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	-	-	0.7 ± 0.1
Healthy	4	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	-	0.2 ± 0.2
Sick: without foliar damage	1	0.0	11.3	1.6	6.5	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	-	19.8 ± 11.1
Sick: medium foliar damage	1	0.0	14.6	1.9	5.4	0.5	1.3	0.0	0.0	0.0	0.0	0.0	0.0	-	-	23.6 ± 13.2
Sick: full foliar damage	1	0.0	12.8	1.1	9.1	0.5	2.3	0.0	0.0	0.0	0.0	0.0	0.0	-	-	25.8 ± 21.9

Figure 6. Average abundances of fatty acids, alkanes and alcohols and total wax ($\mu\text{g/g}$ dry leaf) of sick ($n = 5$) and healthy ($n = 3$) individuals of *A. araucana* from Site 1. Error bars indicate standard deviation (SD).

Figure 7. Average abundances of fatty acids, alkanes and alcohols ($\mu\text{g/g}$ dry leaf) of each chain length in sick ($n = 5$) and healthy ($n = 3$) *A. araucana* individuals. Error bars indicate standard deviation (SD).

Each specific alkane chain length is more abundant in sick individuals, but in both healthy and sick individuals the dominant homologue C_{29} , with three-fold greater abundance in sick individuals than healthy ones. Finally, the dominant chain length in alcohols was C_{24} with six-fold greater abundance in sick compared to healthy individuals. It is also important to mention that, the range of alcohol

chain lengths for sick individuals was C_{24} to C_{28} and in the healthy individuals only the C_{24} chain was identified, another possible indicator of the presence of this disease.

3.3. Sick Individuals At Site 1: Three Levels of Foliar Damage

Leaf waxes were identified in three states in sick individuals: without foliar damage, medium foliar damage and full foliar damage. We observe a decreasing trend of total alkane abundance with increasing damage, and the opposite trend in alcohols (Table 1).

The ACL and CPI values were higher in leaves of sick individuals without leaf damage than in leaves with some degree of foliar damage (Figure 8). The mean values of ACL of alkanes and CPI of fatty acids show a trend of decreasing values as damage increases. The differences in the trends observed between levels of damage were significant in the CPI of fatty acids ($F = 11,224$; $p < 0.05$) and in the ACL of alkanes ($F = 5879$; $p < 0.05$), differentiating the leaves without damage from those with medium and full foliar damage (Tukey $p < 0.05$).

Figure 8. ACL and CPI box plots of fatty acids (a,c) and alkanes (b,d) of leaf waxes from three levels of foliar damage of sick individuals. Letters above boxes represent significance in Tukey pair-wise comparisons ($p < 0.05$); groups with the same letter are not significantly different.

4. Discussion

Data on abundance and distribution of leaf waxes of this millennial tree is scarce and what is available is based on very few individuals. This study reports three different classes of leaf wax in individuals of *A. araucana* from different sites living in their natural habitat, and compares healthy and sick individuals from the same site to evaluate the influence of different leaf damage states on leaf wax distribution and abundance. The Araucariaceae family has been present since the Triassic, with the first appearance of *A. araucana* in the Cretaceous [55]. Since then, this family has been diminished in number of species, with only two species remaining in the *Araucaria* genus and only *A. araucana* living and adapted in temperate forests with a decreasing trend in the distribution range [56]. Today this species vulnerability and current climate change context increase the management and restoration initiatives in South America [57]. Therefore, to increase the success in management and restoration initiatives is important to gather any ecological information about tree species like chemical properties in leaf

waxes that might respond to environmental changes affecting interactions between organisms [58]. The following discussion section was conducted from an ecological perspective considering different levels of organization from chemical characteristics considering species, then different populations to finally end discussing at the individual level.

4.1. *A. Araucana Leaf Wax Composition*

Leaf wax compounds have a long alkyl chain that is apolar (hydrophobic) and a primary functionality such as hydroxyl, carbonyl or carboxyl group (e.g., [21]), which gives alcohols and fatty acids a polar region (hydrophilic) [59]. Thus, from an ecophysiological point of view a high percentage of fatty acids in the leaf waxes of individuals of *A. araucana* (more than 85% of total waxes analyzed) would increase the polarity of the waxy layer, which could be associated with the need for the cuticular layer to have more affinity with the water available in the environment for survival reasons. In contrast, a greater percentage of alkanes would confer a more hydrophobic layer, which would help to avoid the loss of water from inside the leaves.

Respect to the comparison between the three leaf wax classes in *A. araucana* other study report results with a dominance of alkanes (91.7%) over fatty acids (5.4%) and alcohols (2.9%) [30]. The difference in the dominance of the leaf wax classes with these study may be due to differences in the methodology of extraction (e.g., [52,53,60–62]), the ontogeny stage of the plants [30] or the inclusion of just one part of the leaf waxes ([32,63,64]). Studies of other Araucarias from the same family was been conducted and found higher abundance of alkanes, followed by fatty acid and alcohol in two species (*Araucaria angustifolia* and *Agathis australis*) [53]. It is important to highlight that in all of the above studies of species from the family Araucariaceae, the leaf samples were taken from young individuals (ca. 2 to 6 years) maintained in greenhouses for years [30,53,60]. In contrast, in the present study we selected adult individuals in their native natural environment, where the species has lived for millions of years.

4.2. *Dominant Homologues of Leaf Waxes*

This study reports for the first time the dominant leaf wax fatty acid and alcohol where C₂₈, C₂₆ and C₃₀ homologues (decreasing order) dominate in fatty acids and C₂₄ in alcohols. We observed C₂₉ to be the alkane dominant homologue in all sick and healthy *A. araucana* individuals. Other studies report C₂₉ [47] and C₃₃ [30] as dominant alkane homologues in *A. araucana*, but as already mentioned Dragota and Riederer analyzed leaves in other ontogenic stages [30]. In contrast, Raffi and Dodd reported leaf wax data from *A. araucana* adults located in four native zones of this species (The Andes and the Nahuelbuta coastal mountains in Chile) [47], and their observations are consistent with this study.

4.3. *Leaf Waxes of A. araucana from the Four Sites*

Differences in leaf wax abundances in the same species from different sites such as observed in this study has been reported previously, mainly in alkanes [65–68] and specifically in *Araucaria araucana* populations [47]. These differences have been attributed to several causes, being the environmental variables one of the main causes pointing out [69]. Variations in climatic conditions such as temperature or precipitation affect the abundance or chain length distribution of individual plants [43,66,67,70]. The change in the composition of leaf waxes has been proposed to be by genetic adaptation [47,68] or genetic expression [71]. Indeed, there are more than 100 genes implicated in the process of biosynthesis of leaf waxes [72]. In either case, adaptation or genetic expression, the ultimate cause producing the observed differences is environmental variables that would affect the water content of the leaf [37,73,74]. Cuticular leaf waxes play pivotal physiological and ecological roles. It is advantageous for trees to change their composition and properties in order to adapt to fluctuating environmental conditions [43,52,73].

4.4. Comparison of Sick and Healthy Individuals at Site 1

Sick individuals from site 1 had a greater abundance of total waxes, as well as a greater abundance of each class of wax (fatty acids, alkanes and alcohols) compared with healthy individuals at the same site, although this difference was only significant in alkanes and alcohols. ACL values of fatty acids and alkanes were higher in sick individuals (though not significantly; Table 1), most notably in the longer chains of fatty acids (*i.e.*, C₂₈, C₃₀ and C₃₂; Figure 7) and the highest abundance weighting of alkanes chains in these individuals. These results reflect a change in the biosynthesis process (see [21,25,32]) where a greater abundance of long chain alkanes and alcohols in sick individuals from *A. araucana* could indicate that the disease is intensifying the decarboxylation and reduction of the long chain length fatty acids.

Consequently, measuring both alkanes and alcohols in a population of *A. araucana* without visual foliar damage might help to identify sick individuals, in which high abundances of alkanes and presence of long chain alcohols (>C₂₄) would indicate the presence of disease. Interestingly, we do not observe differences in the dominant chain length between sick and healthy *A. araucana* individuals in all analyzed waxes (C₂₈ in fatty acids, C₂₉ in alkanes and C₂₄ in alcohols). However, sick *A. araucana* trees showed longer chain lengths of alcohols, which could be another potential early indicator of this unknown disease.

4.5. Potential Causes: Evidence of Leaf Wax Changes from Plant Diseases or Environmental Conditions

Several studies of the role of the cuticle in sick plants have been reported (e.g., [31,75,76]) with most describing a pathogenic disease because cuticle waxes help to prevent attack from bacteria, fungi and insects [75–77]. Belding et al. studied fungal infection, specifically from *Peltaster fruticola* and *Leptodontidium elatius*, in epicuticular waxes of apple cultivars, although the authors found differences in the relative proportions of alkanes, the differences were not related to the severity of fungal infection [75]. Marcell and Beatti evaluated the influence of foliar cuticle waxes on bacterial colonization in leaves from four bright corn mutants (*Zea mays* L.), determining that bacterial colonization was facilitated by a lower density of the crystalline waxes on the leaves [78]. It has also been proposed that thickness and three-dimensional structure of leaf wax crystalloids protect the leaves and fruits of fungal pathogens (*Uncinula necator*) in grape berries [79]. These and other studies mention how important cuticle waxes are for the growth and infection of pathogens in the leaves (or aerial parts of plants), suggesting that a higher density (cuticle thickness) of waxes leads to better protection [80–82]. This change in wax density must increase cuticular permeability, minimizing the exudation of nutrients that favor the growth of pathogens [80–83]. Based on this information, we expect that changes in the chemical composition of leaf waxes observed here alter their permeability by increasing the compounds derived from long chains of fatty acids as was suggested by Jetter and Riederer [24]. This is the first study showing evidence of changes in the chemical composition of leaf waxes associated with disease, however, more studies are needed to determine the specific influence of pathogens on waxes.

In addition, combined action of drought or water stress with pathogen infection cannot be ruled out, since as plants produce a thicker and more complex cuticular wax layer in response to drought stress it would confer increased tolerance to pathogen infection [84]. Although our results show leaf wax changes in sick individuals of *A. araucana*, unfortunately, the origin and causes of the disease are still unknown.

4.6. Sick Individuals: Three Levels of Foliar Damage

The evaluation of leaf waxes considering different degrees of foliar damage in sick *A. araucana* individuals showed significant differences in distribution (index of ACL alkane and CPI fatty acid), but not in abundance. The CPI of fatty acids was higher in leaves without foliar damage than leaves with foliar damage. Low CPI values of leaf waxes have been associated with bacterial degradation (e.g., [85]). However, leaf waxes are considered difficult to degrade with slow degradation rates

(>2 years) (e.g., [86]). An alternative and more plausible explanation of low CPI values is a cessation of biosynthesis of fatty acids when foliar damage occurs, with fatty acids biosynthesis continuing only in leaves of sick individuals without foliar damage. The ACL of alkanes was significantly higher in leaves of sick individuals without foliar damage indicating a greater abundance of odd long chain alkanes in leaves without foliar damage. This result also agrees with our previous interpretation of a cessation of *in vivo* biosynthesis of waxes in leaves with foliar damage. The alkanes due to their high conservation in terrestrial and aquatic environments have been the most studied leaf wax so far (e.g., [52,53,60]). Even so, ACL or CPI values of alkanes in many species, including *A. araucana*, have not been reported, this work shows differences between levels of damage in sick individuals.

5. Conclusions

This study reports a high abundance of long chain fatty acids compared to alkanes and alcohols in leaf waxes of *A. araucana*. *A. araucana* individuals have similar chemical leaf wax characteristics, where the most abundant chain in fatty acids is C₂₈, in alkanes is C₂₉ and in alcohols C₂₄. Leaf waxes are potential indicators of sick individuals in the *A. araucana* population, where a greater abundance of long chain length alkanes and alcohols observed even present in leaves without a visual foliar damage. A decrease in leaf wax distribution indices (CPI of fatty acids and ACL of alkanes) in leaves with foliar damage is interpreted as biochemical response where individuals stop *in vivo* biosynthesis of waxes.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1999-4907/11/1/59/s1>, Data S1: Supplementary data of abundance of chains in micro grams per area and weight of dry leaf; Table S1: Average abundance of each chain of fatty acids, alkanes and alcohols, distribution index (ACL and CPI) and the total waxes with their respective standard deviation of *A. araucana* individuals.

Author Contributions: S.C. planned research; S.C. and C.C.-P. carry on the sampling, G.C. and C.C.-P. did laboratory analysis; all authors contributed to interpretation, writing and editing of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Comisión Nacional de Ciencia y Tecnología (CONICYT), Fondecyt 1160719.

Acknowledgments: We thank Josef P. Werne (University of Pittsburgh) for help in the field work, comments and suggestions of the final version of the manuscript, Eduardo Tejos for laboratory training and assistance, Julio Moscoso CEO Ecogestión Limitada for help in sampling and logistics. Corporación Nacional Forestal (CONAF) is thanked for providing access to National Parks.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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