Seasonal changes in photosynthesis, phenolic content, antioxidant activity and anatomy in apical and basal leaves from *Aristotelia chilensis* (Mol.) Stuntz

Abstract

Aristotelia chilensis (Mol.) Stuntz is an evergreen antioxidant species endemic from Chile. It grows in open areas or under the tree canopy, and its leaves emerge in early spring and summer. This study's objective was to determine annual station influence (winter, spring, and summer) on photosynthetic activity, total phenol content (TPC), antioxidant activity, and anatomy of apical and basal leaves of *A. chilensis*. We determined photosynthesis performance by measuring electron transport rate (ETR), the quantum efficiency of photosystem II (Fv/Fm), photochemical quenching (qP), and non-photochemical quenching (NPQ) with a fluorimeter. We analysed leaf extracts to determine total phenol content (TPC) and antioxidant activity by DPPH and ABTS methods. The maximum ETR and qP were recorded in spring and summer when the photosynthetically active radiation (PAR) at midday was higher (1901 µmol $m^{-2}s^{-1}$, 1968 µmol $m^{-2}s^{-1}$, respectively). Fv/Fm recorded typical physiological values in both types of leaves, around 0.8 in all seasons. The behaviour of NPQ was not influenced by the kind of leaves and season of the year. In concordance, the basal spring leaves (42.8 mg GAE g^{-1}dw) was higher TPC values.In contrast, the higher values of antioxidant activity were recorded in basal winter leaves followed by basal spring leaves. The results suggested an increase in light intensity (spring) affected positively the antioxidant activity and TPC, which correlated with higher ETR and qP values. In main anatomy results, apical leaves display morphological adaptations as area intercellular spaces and parenchyma palisade areas were bigger than basal leaves.

Keywords:A. chilensis, total phenols content, antioxidant activity, chlorophyll fluorescence.

Introduction

Some plant species, mainly of woody type, grow under adverse environmental conditions as it was the case of *Aristotelia chilensis* (Mol.) Stuntz, a dioic evergreen tree native of Chile, commonly known as *"maqui"*. These plants develop preferably in humid and drained soils of the central valley, in the slopes of both mountain ranges, streams and margins of forests, from near sea level to 2500 meters of altitude (Fredes *et al.* 2012). *A. chilensis* grows up until 4-5 meters higher, has a soft and smooth bark, and plentiful, thin and flexible ramifications. Leaves are simple, oval-lanceolate form with dentate edges and size from 4 to 9 cm. The leaves veins are marked with a long reddish petiole and stems are characterized by an intense red colour. Flowering occurs in the beginning of spring, the fruit is harvested once per year, from December to February (Turchetti and Paz 2019). In addition, *A.*

chilensis plants tolerate drought periods of less than one month. This species appears in succession as a colonizer of newly burned or exploited soils, forming dense and monospecific groups known as "*macales"* which fulfil the function of reducing erosion and generating the conditions for establishing other species forming secondary shrubs (Benedetti 2012).

Variations in environmental factors such as temperature, light radiation, water availability, among others, can cause stress and therefore, changes in plant metabolism. In fact, the photosynthetic rate decreases due to an alteration in the electron transport mechanism and CO_2 assimilation, which finally is reflected in a decrease in carbohydrate production(Sáez *et al.* 2012). Plants, in their interaction with the environment, produce a high number of secondary metabolites, which normally are not essential for the primary metabolism. The synthesis of these compounds is enhanced under stress conditionsand many of them have biological activity which are beneficial for human health, being used as biologically active compounds(Scossa and Fernie 2020).

A. chilensis fruits have been intensively studied(Masoodi *et al.* 2019) and they are used for pharmacological (Céspedes *et al.*2017, Fuentealba *et al.* 2012) and nutraceuticals purposes(Rubilar *et al.* 2011,Fredes *et al.* 2018). Their fruits are bright black edible berries, with a high presence of anthocyanin so they have up to four times more antioxidants properties than other berries(Fredes *et al.* 2014, 2018, Fuentes *et al.* 2019). However, the national and international demand for these fruits is growing and so affects the genetic heritage and biodiversity of this species. Faced with the drawbacks of the unregulated collection of fruits, the proposal to use leaves as a source of phenolic compounds arises, constituting an alternative that can be sustainable, permanent and that does not affect the state of conservation of the species.

A. chilensis leaves emerge in two periods of the year, one more abundant in early spring, and another, in summer. As evergreen species, *A. chilensis* plants retain their leaves during the winter, and they remain photosynthetically active, both in the days of moderate temperature in autumn and winter, and during the early spring(Damascos and Prado 2001). However, there are scarce studies that report the behaviour of phenolic compounds in leaves in relation to leaf ontogeny and seasonal period. The present work relates the ontogenic age of the leaves of *A. chilensis* (Mol.) Stuntz with the photosynthetic capacity and the accumulation of phenolic compounds as well as their antioxidant capacity under different natural growth conditions.

Materials and methods

The research was conducted between August 2017 and January 2018, the period in which the leaves of *A. chilensis* were collected, at the Universidad de Concepción, Biobío Region, Chile (36° 50'02.6 "S, 73° 01'54.3" W). The influence of climatic conditions on annual seasons in which this study was carried out is shown in Table 1 Suppl. (data from Dirección Meteorológica de Chile, 2018). In addition,

in the Table 1 present the photosynthetically active radiation (PAR) measurements realized at midday in the study seasons.

Adult male *A. chilensis* plants were used, which grow in a natural environment and reach a uniform height of 3 m. Six plants were selected within the university campus. The study is carried out with fully expanded leaves, where adult and young leaves are distinguished. The samples were collected near the base of the tree where the ontogeny less. Leaves were selected from the upper third of the branch corresponding to young leaves (apical leaves) and leaves from the lower third of the branch or adult leaves (basal leaves).

Light response curves

The chlorophyll fluorescence was evaluated through light responses. The leaves were previously put in the darkness for 30 min and then,exposed to different light intensities of 12.13, 25.62, 51.39, 86.64, 241.50, 479.08, 878.08 and 1280.83 μmol photons m^{-2} s^{-1}. Fluorescent signals were measured with a pulse amplitude fluorimeter (FMS 2, Hansatech Instrument, U.K). According to the terminology of Murchie and Lawson (2013), the minimum value for chlorophyll fluorescence (Fo) in the dark-adapted statewas determined by applying a weak pulse of modulated light, and the maximum fluorescence (Fm) was induced by a short pulse (0.8 s) of saturating light. The fluorescence signals were followed until they reached a steady state (Fs). To determine the maximal fluorescence in light (Fm'), various pulses of saturating light were applied. The minimal value for chlorophyll fluorescence (Fo') in the light-adapted state, was determined by turning off the actinic light, and immediately applying a 2 s far-red pulse.

The maximum photochemical efficiency of photosystem II (ΦPSII), Fv/Fm (variable fluorescence/maximal fluorescence) was calculated considering Fv = Fm - Fo. Once data fromfluorimeterwere obtained, the efficiency of photosystem II (ΦPSII= (Fm'- Fs)/Fm'), and the electron transport rate (ETR = 0.8 × ΦPSII × PAR × 0.5) were calculated. The factor 0.8 is the average value of the absorbance for the green leaves, and the factor 0.5 assumes that the efficiency of both photosystems is equal, and that the radiation is distributed equally among them. In addition, photochemical quenching (qP= (Fm'- Fs)/(Fm- Fo')) and non-photochemical quenching (NPQ = (Fm - Fm')/Fm') were calculated(Sáez *et al.* 2012).

Branches of *A. chilensis* were collected, identifying apical and basal leaves, in the different seasons of the year (winter, spring, summer). The samples were collected at midday where the light intensity is higher. At this time, the photosynthetically active radiation (PAR) was measured, the light condition of the exposed environment of the study plants was established based on the criteria described byZhen and Bugbee (2020). The PAR data was shown in Table 1.

Preparation of *A. chilensis* samples for anatomical studies

For anatomical studies, the tissue was selected from the central portion of the leaf, cut quickly, and fixed in 37% formalin, acetic acid, and 70% ethanol (FAA_{70}). The samples were dehydrated through serial solutions in ethanol and *n*-butyl acetate and embedded in paraplast. The cuts were made with a Jung Biocut 2035 microtome and stuck in glass sheets with Hatsup and Bissmut adhesives. After removing the paraplast by dipping in butyl acetate and washing with ethanol, samples were coloured with safranin and Astra blue. Subsequently they were re-dehydrated in a series of dilutions of ethanol and finally in butyl acetate. Glass sheets were visualized in a Leica ICC50 HP optical microscope(dos Santos Isaias *et al.* 2011).Apical and basal leaves selected from the three seasons of the year

Preparation of *A. chilensis*samples for chemical study

Fresh basal and apical leaves (40 g) were dried at 37°C for two days. Dried leaveswere crushed to get powder and then, maceration was performed by exhaustion in methanol-HCl 0.1%. The total extract was concentrated in a rotavaporat 37°C and lyophilized for 24 h. The extraction yield was defined as the amount of extract (mg) recovered by leaf dry weight (mg),for each sample.

Determination of total phenolic content (TPC)

The total phenolic content(TPC) in each extract/sample was determined using TPC method described byMongkolsilp *et al.* (2004), with minor modifications. The dried extract/sample was dissolved in distilled water to a concentration of 200 µg mL^{-1}. The calibration curve was established using gallic acid (10 to 200 µg mL^{-1}). The reaction mixture contained: distilled water (400 µL), sample or gallic acid solution for the standard curve (20 µL), Folin-Ciocalteu reagent (40 µL) and 15% sodium carbonate (200 µL). The reaction mixture was incubated at room temperature for 60 min in darkness, with intermittent shaking for favouring colour development. Absorbance was measured at 750 nm using UV-Vis spectrophotometer (BioTeK ELx800, Winooski, USA). TPC was expressed in mg of gallic acid equivalents per gram of dry weigh (GAE mg g^{-1} dw).

Determination of the antioxidant activity

DPPH radical scavenging assay

The free radical scavenging activity of *A. chilensis* leaf extracts and standard solution Trolox (±-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were analysed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method as reported byMorales *et al.* (2012). The assay mixture contained 270 µL of 0.06 mM DPPH radical solution, prepared in methanol, and 30 µL of Trolox at different concentrations (10-200 µg mL^{-1}) or *A. chilensis*leaf extracts. The reaction mixtureswerequickly mixed and incubated in darkness at 37 °C for 20 min. The decrease in

absorbance of each sample was measured at 515 nm using UV/Vis spectrophotometer. Trolox, a well-known antioxidant, was used as positive control, while DPPH radical solution with 1 mL methanol was taken as blank. All determinations were performed in triplicate (n=3).

ABTS radical scavenging assay

For ABTS assay, the procedure followed was that described byKuskoski *et al.* (2004)with some modifications. The stock solutions included 3.5 mM ABTS *(2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonate)* and 1.22 mM potassium persulfate. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 16 h at room temperature in darkness. Once the $ABTS^{*+}$ radical was formed, the solution was then diluted by mixing 1 mL $ABTS^{*+}$ solution with 14 mL distilled water to obtain an absorbance value of 0.70 ± 0.01 units at 750 nm. $ABTS^{*+}$ solution was freshly prepared for each assay. The reaction (60 min, in dark conditions) contained $ABTS^{*+}$radical (180 µL) and 20 µL of the samples at different concentrations. For standard Trolox and samples, dilutions of 10 to 200 µg mL^{-1}were prepared. All determinations were performed in triplicate (n=3).The antioxidant activity for both methodologies was expressed as percentage of inhibition, which corresponds to the amount of radical (DPPH and ABTS) neutralized by the extract at a certain concentration,as described in following equation

$$\% \text{ inhibition} = ((A_s - A_c)/A_c) \times 100$$

where A_c is the absorbance of control and A_s the absorbance of the samples. The antioxidant activity was expressed as IC_{50}, which was defined as the final concentration (µg extract mL^{-1}) of the tested sample required for the inhibition of radical by 50% (Kulisic *et al.* 2004, Rubilar *et al.* 2011).

Statistical Analysis

For the light response curves, eight samples for six plants were used for each type of leaf in relation to the season of the year studied. For the determination of TPC, six plants samples were used for each type of leaf in relation to the season of the year studied. For the foliar anatomy measurements, five samples with fifteen repetitions were used. The analyses were carried out with the AxioVision LE 4.8.2.0 software. The assays for the determination of antioxidant activity were carried out in triplicate. The data obtained were analysed statistically with an analysis of variance (ANOVA), and the differences between the means were determined through the Tukey test ($P \leq 0.05$). Statistical analyses were performed using the InfoStat/L software (FCAUNC, Argentina) and the graphic representations were made using SigmaPlot software version 10.0 (SPSS; Chicago, IL, USA).

Results

Effect of seasonal conditions on fluorescence parameters of both basal and apical leaves

According to chlorophyll fluorescence data in *A.chilensis*, we observed that they were affected by year's season. Indeed, the season of the year had a significant effect on ETR (Table 3 Suppl.). On the other hand, quantum efficiency of the PSII (Fv/Fm), was affected by the leaf type but not by the environmental conditions or their interactions. However, qP and NPQ was significantly affected by the leaf type and the season, and an interaction betweenboth variables (season and leaf type) was observed (Table 3 Suppl.).

Light response showed that the maximum values of ETR were found in the seasons of the year with greater luminosity (spring and summer) in both types of leaves (basal and apical, Table 2). In fact, the minimum values were observed in winter, regardless of the type of leaf, showing a strong correlation ETR/PAR during this season, as it is described this last parameter in M&M section. The parameter Fv/Fm remained with values around 0.80 in all seasons of the year analysed, without significant differences among them. In the same way, Fv/Fm values showed no significant differences between basal and apical leaves (Tables 2 and Table 3 Suppl.).

Photochemical quenching (qP) is the ratio of excitation energy trapped by open reaction centers that has been used for electron transport(Moreno *et al.* 2008). We observed that the highest qP values were recorded in spring and summer, in both types of leaves, and they all showed significance differences with respect to the data collected during the winter (Table 2).However, there were no significant differences in qP values in both apical and basal leaves during winter, where the lowest values of qP were found.

Non-photochemical quenching (NPQ) represents the influence of non-photochemical processes on the fluorescence emission of chlorophyll from a darkness to light state (Moreno *et al.*2008). The type of leaf used and the seasons of the year did not influence the behaviour of NPQ, although a trend to rise the NPQ values with the increase in the light intensity during spring and summer, especially in apical leaveswas observed (Table 2 and Table 3 Suppl.).

Foliar Anatomy

Fig. 1Suppl. shows cross sections of basal (Fig.1A) and apical (Fig. 1B) fully expanded leaves. Important anatomical differences were observed, mainly in the conformation of palisade and spongy parenchyma of both types of leaves. In apical leaves (Fig.1B), the area of parenchyma palisadewas bigger than in the basal leaves. Also, the basal leaves had smaller intercellular spaces than the apical leaves. However, no significant changes were recorded in the epidermis in both types of leaves (Table 4).

Yields of the extraction of phenolic compounds

Fig. 2Suppl. shows the yield of the extraction process of phenolics, obtained from basal and apical leaves of *A. chilensis,* in the different seasons of the year (mg extract mg^{-1} dw). When comparing the

yields, both apical and basal leaves in winter were lower than in spring and summer.Basal and apical leaves in spring and summer, respectively, showed the highest yield levels.

Determination of Total Phenol Content

TPCvaluesshowed significant differences in both basal and apical leaves, and in the different seasons (Fig.3). The highest values were recorded for extracts obtained from basal leaves in spring, followed by those of apical leaves in winter and summer. The lowest value of TPC was detected in extracts of basal leaves in winter and in those obtained from apical leaves in spring. According to TPC data from leaves of *A. chilensis*, they were not affected by the type of leaf and interaction between both factors were observed (Table 5 Suppl.).

Determination of the Antioxidant Activity

The *F*-values from two-way ANOVA for the antioxidant activities obtained by both methodologies (ABTS and DPPH), were influenced by the season of the year, the type of leaf and by the interaction between both factors as can be observed in Table 5 Suppl. In addition, the antioxidant activity for ABTS assay expressed as a function of IC_{50} is shown in Table 6.Data described in this Table strongly support significant differences in extracts of basal leaves sampled in winter, with respect to the extracts obtained in the other two seasons, as well as significant differences between both types of leavesas it can be seen by their F values. The best result of this ABTS antioxidant activity was found in basal winter leaves,which corresponds to the lower value of IC_{50}, followed to spring and summer basal leaves. Thelowest antioxidant capacity which corresponds to the higher value of IC_{50} was found in apical spring leaves by ABTS assay.

On the other hand, IC_{50} values obtained from DPPH assay are shown in Table 6.Data obtained from this assay were significantly different in both types of leaves as confirmed by their *F* values. Although the differences in antioxidant activity were small and significant, basal leaves during winter had a higher antioxidant capacity than those leaves in spring and summer. Regarding apical leaves, the extracts in all the seasons presented values statistically different, being lower in summer, which means a higher antioxidant capacity, and higher levels in both winter and spring, which is related to a lower antioxidant capacity.

Discussion

Weather conditions that characterize the different seasons of the year affect both the morphology and the physiology of the plant, and therefore, modify the growth and development of its organs, including the leaves. Consequently, the objective of this work was to analyse the effect of climatic conditions in *A. chilensis* leaves. As maquileaves emerge in two periods of the year, one more abundant in spring,

and another, in summer, we selected these two seasons to carry out this study. In addition, we also selected a third season, winter because *A. chilensis* plants retain their leaves during this period, and so, they remain photosynthetically active. Another factor analysed was the level of growth of *A. chilensis* leaves, in both apical and basal leaves, due to these leaves have different morphological and physiological characteristics, as well as apical leaves are continuously growing leaveswhich act as consumptive sinks while basal leaves, being adult leaves, are sources of photosynthetic products.

In fact, Damascos and Prado (2001)indicated that adult leaves of *A. chilensis*in winter, remain photosynthetically active during the spring and 15 days before the senescence, subjected to a low photonic flow density (150 mmol m^{-2} s^{-1}) and showed higher average values of photosynthesis. Being an evergreen tree, its leaves emerge in two seasons of the year, spring, and summer. These authors indicated that, the new leaves in the spring constitute sinks of mass and energy. However, the formation and growth of the reproductive structures of the plant are processes with high energy demand. In other studies, conducted in evergreen species, it was found that the conservation of leaves from winter to spring was not associated with the translocation of foliar nutrients before the formation of new leaves but to maintain a positive carbon balance in less favourable periods (Mendoza *et al.* 2014).

Murchie and Lawson(2013)described a revision about how fluorescence parameters can be used to evaluate changes in photosystem II photochemistry, linear electron flux, and CO_2 assimilation *in vivo*, and described the theoretical bases for the use of specific fluorescence parameters.In relation to these parameters, apical leaves showed lowerFv/Fm values than basal leaves during spring and summer,although the data recording made was always within the normal physiological values (around 0.8) throughout the year. This agrees with our results (Table 2) and so, the decrease of Fv/Fm values cannot be interpreted as a photoinhibition of the photosynthetic apparatus. This could be due to two reasons: either*A. chilensis* is tolerant to high light conditions(Lusk 2004), or this plant is not under stress, allowing it to achieve the optimum physiological performance in any season of the year (Molina-Montenegro *et al.* 2012). Other studies confirm that this species has a great phenotypic plasticity in traits associated with carbon gain and water economy, which allows it to survive both under habitats with low light and water availability (continuous forest), and with high light conditions and water scarcity(Repetto-Giavelli *et al.* 2007).

In the present study, ETR and qP values were well-correlated and appear to be dependent on light intensity. However, in *A. chilensis* basal leaves both ETR and qP values were slightly higher in spring and summer. Acosta-Motos *et al.* (2015a)studied the effect of high light intensity on chlorophyll fluorescence parameters in apical and basal leaves in myrtle plants. Under high light irradiation, basal leaves from myrtle plants displayed higher values for qP, Fv/Fm and NPQ than apical leaves (Acosta-Motos *et al.* 2015a). These results partially agreed with our results, due that Fv/Fm values of *A. chilensis* leaves were influencedby leaf type, with higher Fv/Fm values in basal leaves than apical leaves under high light conditions (spring and summer). This result can be due to a down-

regulation mechanism of PSII in high light conditions in apical leaves, which were more exposed to sunlight than basal leaves. A similar response in Fv/Fm values has been observed in pea leaves subjected to high light irradiation, being the response dependent on the exposure time to this high light intensity (Hernández *et al.* 2004). On the other hand, higher NPQ means that much of the light energy absorbed is dissipated by the protective mechanisms. However, in our results NPQ behaved differently depending on the leaf type, but no significant changes were recorded for NPQ values during the different seasons. These stable NPQ values suggested the capacity of *A. chilensis* leaves to use the excess of light energy into photosynthesis process. In contrast, in basal myrtle leaves, high NPQ values were observed during periods of high light intensity, which could facilitate the safe removal of excess light energy and minimize the generation of ROS (Acosta-Motos *et al.* 2015a).

Furthermore, in this study some differences in foliar anatomy can be found between apical and basal leaves. One of the more evident is the number of chloroplasts,more abundant in the palisade parenchyma in basal leaves. That means that photosynthetic activity must be higher in basal than in apical leaves, which must still function as a sink organ. These differences can be reflected in Fv/Fm values, being higher basal leaves during spring and summer.Interestingly, apical leaves had a higher percentage of area occupied by palisade parenchyma, an adaption that could favour the photosynthetic process. Another interesting difference was the greater intercellular spaces observed in apical leaves in relation to basal leaves. This anatomical modification in leaves, can improve the CO_2 diffusion, and facilitate its entry to the chloroplast, especially under stress conditions. A similar modification was previously described in myrtle and Eugenia plants under salinity conditions (Acosta-Motos *et al.* 2015a,b). These authors observed a decrease in the percentage of spongy parenchyma, and an increased percentage of intercellular spaces under NaCl-stress. These authors concluded that these anatomical changes may serve to facilitate CO_2 diffusion in a situation of reduced stomatal aperture(Acosta-Motos *et al.* 2015a,b).

A. chilensis is characterized by high phenols content compared to other berries, and nutritional and pharmacological effects are attributed to them. The TPC observed in this study were lower to that reported by Vidal *et al.* (2013) for *A. chilensis* leaves collected in Región del Biobío. These authors carried out theextraction in ethanol-water 50% v/v, obtained TPC values of 40 ± 0.57 mmol L^{-1}GAE, which is equivalent to 136 mg of phenols g^{-1} dw values, higher compared with samples of apical leaves of spring. A similar response was reported by Rubilar *et al.* (2011)where TPC of *A. chilensis* leaves was higher than *A. chilensis* leaves on this study (69.0 ± 0.9 mg GAE g^{-1} dw). These results could indicatethat the extraction method used influenced on theTPC quantification.

In addition, phenolic compounds exhibit a wide range of biological effects. Some of them are powerful free radical scavengers (and so, have antioxidant activity). For this reason, they are useful in the prevention of arteriosclerosis, cancer, diabetes, neurodegenerative diseases and arthritis (Gonçalves and Romano 2017). Depending on the extraction procedure,the antioxidant activity can vary, being in some cases, higher in leaves than in fruits (IC_{50} values, leaves: 8.0 ± 0.1 mg extractL^{-1},

fruit: 399.8 ± 17.5 mg extract L^{-1})(Rubilar *et al.* 2011). In our case, the antioxidant activity recorded in leaves was similar to that observed by Rubilar *et al.* (2011)in stems (IC_{50} value: 43.1 ± 1.7 mg extractL^{-1}).

The mechanism by which an antioxidant compound interacts with a radical molecule depends on the structural conformation of the antioxidant (Mishra *et al.* 2012). Granato *et al.* (2018) indicate that no single antioxidant activity assay will reflect the total antioxidant capacity. The antioxidant activity methods have particularities depending on mechanisms of action, types of radical, pH, time of exposition, and temperature. Another important factor is the use of standards to build analytical curves, which generate the sample's quantitative results (Granato *et al.* 2018).One of the tests used in this study was the DPPH assay, which is simply due to its stable nitrogen radical, but has problems with many antioxidants by reacting with different kinetics or not reacting at all(Mishra *et al.* 2012). DPPH assay is reversible due to the reaction's low reading of antioxidant capacity of some antioxidants. It was also indicated that the DPPH assay is pH-dependent, and the final result could be influenced by the deprotonation of the phenolic group (Mishra *et al.* 2012, Tirzitis and Bartosz 2010). The other method used in this study was the ABTS assay, which consists of an oxidation reaction of the coloured cation $ABTS^{\bullet+}$.The ABTS assay can be applied to lipophilic and hydrophilic compounds(Huyut *et al.* 2017). Thereby, our results determine in the DPPH assay an increase in antioxidant activity in summer in both types of leaves, coinciding with the greater luminosity, without the same trend in ABTS assay.

On the another hand, Harnly (2017)described how the measurement of *in vitro* antioxidant activity and total phenolic content using the Folin-Ciocalteu reagent are not suitable. The author indicated no standard mechanism or method to quantify antioxidant activity, and scientific research will only use advanced techniques to identify antioxidants. In this regard, chromatography techniques used to identify and quantify phenolic compounds in foods, beverages, and herbal extracts have sufficient accuracy or precision. It is also necessary to highlight what the author indicates about the method's results *a* are (usually) not comparable with the data of method *b* or even between laboratories (Harnly 2017). Therefore, as indicated by Granato *et al.* (2018),it is evident that "antioxidant activity" involves complex interactions. However, screening spectrophotometric methods to characterize the samples and give an idea of total phenolic content in the matrix.

As a general conclusion, in this study it was determined that the *A.chilensis*basal leaves showed a better photosynthetic performance as observed by higher Fv/Fm values, thatcorrelated with higher total phenol contentin high light conditions. In both types of leaves, the increase in light intensity was accompanied by a rise in NPQ values, reflecting a safe mechanism to dissipate excess light energy. In addition, apical leaves display some morphological adaptions, such as the increase in intercellular spaces in order to facilitate the entry of CO_2 inside the chloroplasts, as a mechanism to protect the photosynthetic process.Finally, in order to take advantage of the research it is necessary to know in the future the performance of the activity of the different antioxidant enzymes and compare these antioxidants mechanisms in both types of leaves.

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Table 1.Climatic conditions of Región del Biobío, Concepción, Chile, where *A. chilensis* is growing. Each parameter corresponds to the monthly average for the three seasons of the year.

Season	**Winter (August)**	**Spring (November)**	**Summer (January)**

Minimum temperature (°C)	0	8	9
Maximum temperature (°C)	14	22	29
Average temperature (°C)	9	14	17
Average rainfall (mm)	586.73	849.72	1.45
Humidity (%)	85	81	75
Average wind speed (km h^{-1})	13.7	13.6	14.8
Maximum wind speed (km h^{-1})	85.2	50	50
PAR at midday (µmol photons m^{-2} s^{-1})	435.98 ± 10.4	1901.7 ± 3.6	1968.7 ± 7.2

*Source: Dirección Meteorológica de Chile, 2018.

Table 2. Effect of the season of the year on fluorescence parameters in basal and apical leaves of *A. chilensis*.

Parameters	Winter	Spring	Summer	*F*
Basal leaf				
ETR	64.02 ± 6.47a	129.71 ± 13.69b	108.83 ± 4.83b	13.38**
qP	0.25 ± 0.03b	0.47 ± 0.04a	0.42 ± 0.02a	13.81**
Fv/Fm	0.83 ± 0.01a	0.84 ± 0.005a	0.84 ± 0.01a	0.97ns
NPQ	3.42 ± 0.11a	3.79 ± 0.15a	3.84 ± 0.22a	1.85ns
Apical leaf				
ETR	85.75 ± 5.25a	123.08 ± 12.58b	122.19 ± 3.64b	6.84*
qP	0.35 ± 0.02b	0.48 ± 0.04a	0.51 ± 0.01a	13.35**
Fv/Fm	0.83 ± 0.004a	0.82 ± 0.01a	0.82 ± 0.004a	1.16ns
NPQ	3.85 ± 0.14a	3.89 ± 0.14a	4.27 ± 0.07a	3.44ns

Data represent the mean ± SE from eight samples from each season of the year. Different letters in the same row indicate significant differences according to Tukey´s test ($P \leq 0.05$). *F* values from ANOVA for the different season of the year and type of leaf.

**F* values were significant at 95% levels probability.

***F* values were significant at 99% levels probability.

****F* values were significant at 99.9% levels probability.

Non-significant values are indicated by "ns".

Table 3. Relationship between type of leaf (A) and season of the year (B) as well as interaction of both (AXB) on fluorescence parameters in *A. chilensis*.

Source of variation	ETR	qp	Fv/Fm	NPQ
Type of leaf (A)	1.76ns	8.01*	7.19*	7.08*
Season (B)	19.22**	25.07**	0.27ns	4.06*

AXB	13.4**	19.38**	2.58ns	5.06*

F-values from two-way ANOVA for ETR, qP, Fv/Fm and NPQ. *F*-values significant at 99.9% (***), 99% (**), or 95% (*) levels of probability. Non-significant values are indicated by "ns".

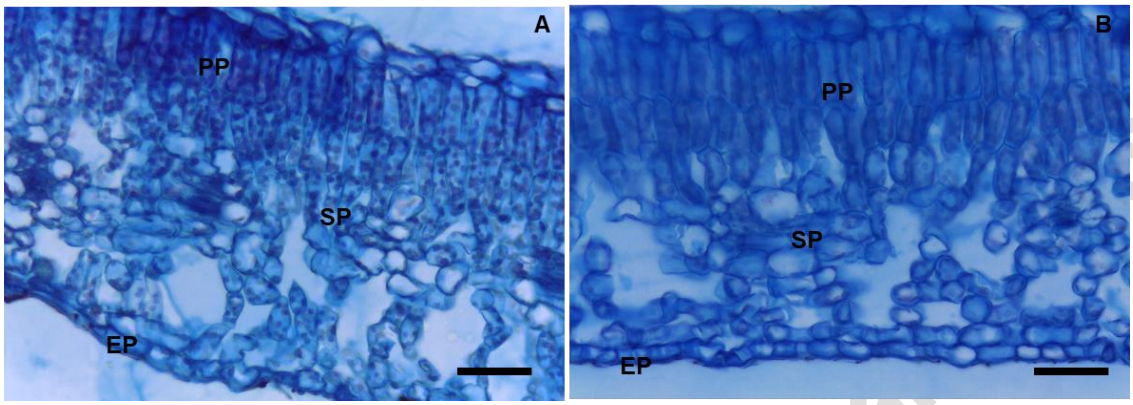

Fig. 1. Cross section of basal (A) and apical (B) leaves of *A. chilensis*. Black bars: 50µm. EP: epidermis. PP: palisade parenchyma. SP: spongy parenchyma.

Table 4. Measurements of areas (µm^2)in cross sections of basal and apical leaves of *A. chilensis.*

	Area (µm^2)		
	Basal leaf	**Apical leaf**	***F***
Adaxial epidermis	243.27 ± 8.24a	257.25 ± 7.51a	1.57ns
Palisade parenchyma	266.78 ± 7.16a	336.98 ± 5.33b	61.81**
Spongy parenchyma	203.5 ± 7.97a	214.33 ± 4.91a	2.52ns
Abaxial epidermis	135.29 ± 5.28a	140.85 ± 3.38a	0.79ns
Intercellular space	888.71 ± 23.26a	1165.04 ± 31.42b	49.97**

Data represent the mean ± SE from 75 measurements. Different letters in the same row indicate significant differences according to Tukey´s test ($P \leq 0.05$). *F* values from one-way ANOVA for the different type of leaf.

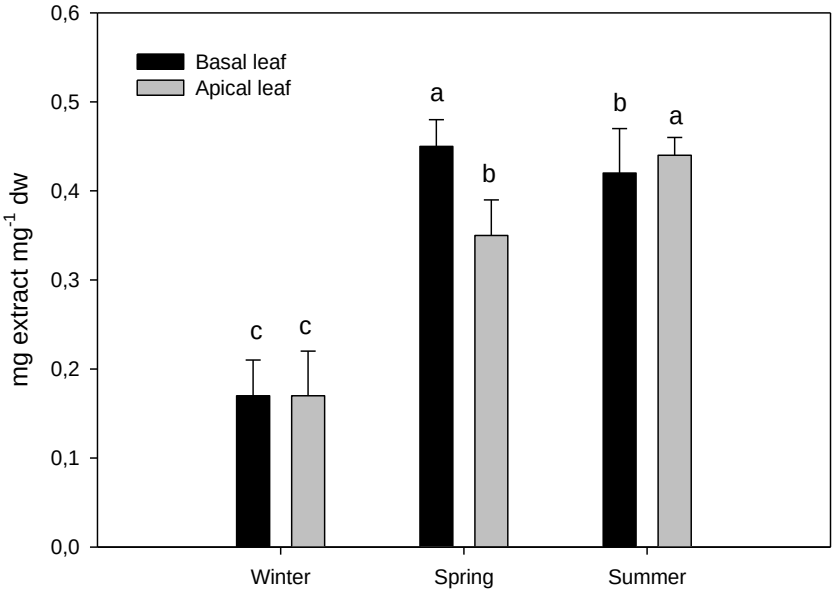

Fig. 2. Yield of extracts of basal and apical leaves of *A. chilensis* to determine phenolic content in three seasons of the year (mg extract mg $^{-1}$ dw).Data represent the mean ± SE from three samples from each season of the year. Different letters in the same row indicate significant differences according to Tukey´s test (one-way ANOVA, $P \leq 0.05$).

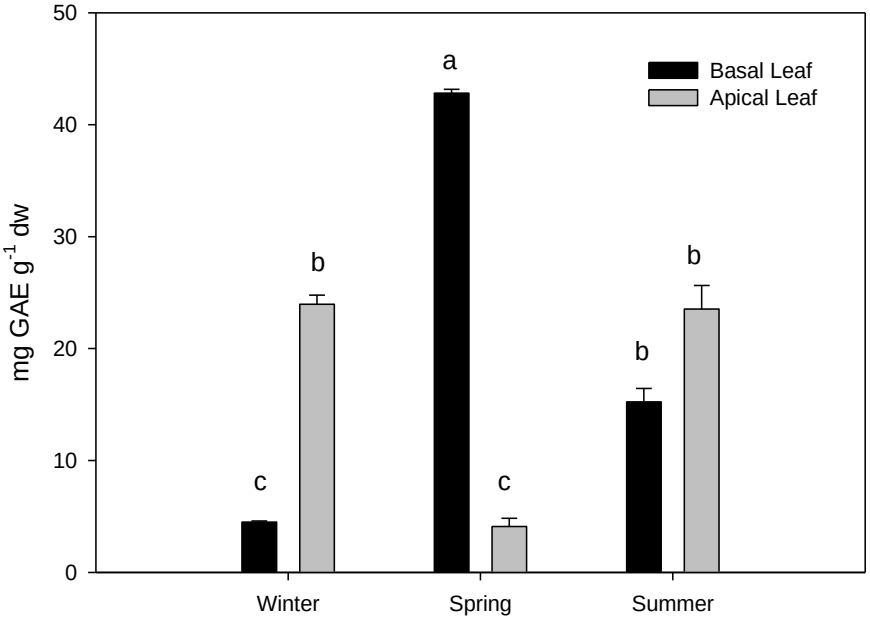

Fig. 3. TPC (expressed as mg GAE g^{-1} dw) in basal and apical leaves of *A. chilensis* in the different seasons of the year.Data represent the mean ± SE from six samples from each season of the year. Different letters indicate significant differences according to Tukey´s test (one-way ANOVA, $P \leq 0.05$).

Table 5. Relationship between type of leaf (A) and season of the year (B), as well as the interaction of both (AXB), expressed as *F*-values from two-way ANOVA, for TPC and antioxidant activity (ABTS and DPPH assays) in *A. chilensis*.

Source of variation	TPC	ABTS	DPPH
Type of leaf (A)	0.65ns	25.15**	11.04*
Season (B)	1.39*	9.15*	3.97*
AXB	1.14ns	14.48*	6.32*

F-values from two-way ANOVA for TPC, ABTS and DDPH. *F*-values significant at 99.9% (***), 99% (**), or 95% (*) levels of probability. Non-significant values are indicated by "ns".

Table 6. Antioxidant activity (ABTS assay and DPPH assay) expressed as a function of the IC_{50} obtained for each type of leaf and season of the year (µg extract mL^{-1}).

Parameters	Winter	Spring	Summer	*F*
Basal leaf				
ABTS	45.05 ± 0.30a	49.97 ± 2.24b	49.91 ± 0.97b	11.88*
DPPH	34.68 ± 0.58a	35.92 ± 0.90ab	38.08 ± 1.65b	6.93*
Apical leaf				
ABTS	51.81 ± 4.59a	69.02 ± 4.21b	56.05 ± 4.21a	12.81*
DPPH	59.49 ± 1.87c	47.37 ± 0.63b	33.89 ± 1.53a	236.03***

Data represent the mean ± SE three samples from each season of the year. Different letters in the same row indicate significant differences according to Tukey´s test ($P \leq 0.05$). *F* values from one-way ANOVA for the different type of leaf.