Research article

Changes in the leaf nutrient and pigment contents of *Berberis microphylla* G. Forst. in relation to irradiance and fertilization

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ABSTRACT

Berberis microphylla G. Forst. commonly named calafate, is a Patagonian shrub that grows in humid areas of the steppe, coastal thickets, edges and gaps of *Nothofagus* forests or along streams and rivers, with small purple berries. The objective of this study was to evaluate the changes in leaf nutrient (carbon, nitrogen, carbon:nitrogen, phosphorus and potassium) and pigment contents (chlorophyll *a* and *b*, chlorophyll *a:b* ratio and carotenoids) of *B. microphylla* plants growing under different irradiances (low = 24%, medium = 57%, and high = 100% of the natural irradiance) and fertilization levels (0 = 0.00 g, 1 = 3.36 g, and 2 = 6.72 g per plant) during two growing seasons (2008–2009, 2009–2010). Also, we explored the relationships of these variables with anthocyanin, as well as with total phenol fruit contents. The fertilization has been highlighted, particularly in the content of foliar nutrients, where nitrogen, phosphorus and potassium contents were highest with fertilization level 2 (2.0%, 0.1%, and 0.6%, respectively), while carbon:nitrogen ratio (37.5) was maximum on fertilization level 0. Irradiance has greatly affected the content of foliar pigments. Thus, chlorophyll *a*, *b*, and carotenoids were highest under low irradiance (0.4, 0.1 and 0.2 mmol/m^2, respectively), while chlorophyll *a:b* ratio was maximum under medium and high irradiance conditions (3.1). In addition, the quantity of fruit secondary metabolite (anthocyanin and phenol) could be estimated using carbon and potassium leaf contents and chlorophyll *a* and *b* contents. On the other hand, the annual climatic variability between 2008-2009 and 2009–2010 mainly affected the variables on nutrient and pigment contents, likely evidencing the influence of two distinct climate periods, El Niño/La Niña phenomena, respectively. The changes observed in the leaf nutrient and pigment contents of *B. microphylla* could be related to the acclimation capacity of *B. microphylla* shrubs to changes in environmental conditions via arrangements in leaf composition.

1. Introduction

Berberis microphylla G. Forst. shrubs grow in Patagonian environments from Neuquén to Tierra del Fuego (Job, 1942), with different light, water and nutrient availability. This shrub inhabits dry and humid areas of natural grasslands as well as in the marine coast or along riversides, even coexisting with trees at the edge of *Nothofagus* forests (Moore, 1983). It is a medium size shrub, evergreen, spiny and erect belonging to the so-called group of minor fruit or underutilized woody species that are relevant for diversification of agri-food production. It is classified as a non-timber forest product, particularly interesting because of its bluish black fruits are rich in phenolic compounds. This berry can be consumed fresh or processed in marmalades, jams, non-alcoholic beverages and ice cream. *Berberis microphylla* has aesthetic characteristics that make it an excellent ornamental shrub due to its pendulous yellow flowers and the extended flowering period. In addition, its colorful trifid spines can protect orchards and gardens (Massardo et al., 2006). Recent research also indicated that rural populations appreciate it as fuelwood and that berry orchards are being planned taking advantage of their economic potential. The commercial characteristics that highlight the species are the sweet acidified taste of its fruits and its nutraceutical properties, among others (Arena et al., 2018). However, fruiting yield is related to the environmental physiology where the shrubs develop. Solar radiation is of great importance due to its role as energy source in photosynthesis (Field and Mooney, 1983), regulating the production of dry matter and consequently the production of fruit (Peri and Lasagno, 2006).

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In natural environments, plants often receive fluctuations in irradiance, from full sun to shade, caused by shading within the shrub canopy itself (Knapp and Smith, 1987), and the proximity to other plants. The variation in irradiance has been recorded within *B. microphylla* canopy, finding that the incidence of light was 30% of full intensity at half of the shrub height; however, large variation among plants was detected (9%–74%) (Peri and Lasagno, 2006).

Nutrient availability and pH of soils also strongly influence plant growth and distribution of native species (Wade et al., 2011), being crucial for the vegetative cycle, yield, and quality of fruits (Jeppsson, 2000).

The environment affects gene expression which can be evidenced through the changes in the leaf nutrient (Sardans et al., 2006) and leaf pigment contents (Valladares et al., 2000; Damesin, 2003; Larcher, 2003; Zúñiga et al., 2006; Lichtenthaler et al., 2007; Soler et al., 2011). Previous studies on evergreen shrubs showed variation in the nutrient and foliar pigment contents (Katahata et al., 2007; Muller et al., 2011) as a response to variable environmental conditions. Other studies determined several plant responses at different shading levels and with the use of different shade-mesh colors in fruit tree production (Stampar et al., 2001; Shahak et al., 2004a, 2004b; Cohen et al., 2005; Jakopic et al., 2007; Dufault and Ward, 2009), through the modification of radiation and temperature levels.

These techniques are widely used as management tools in several commercial crops and are useful to mitigate extreme environmental conditions. In this context, the inter-annual climate variations play a crucial role, and even more if extreme climatic events are considered. For example, the natural phenomenon globally known as El Niño-Southern Oscillation (ENSO) presents two extremes: a warm phase known as El Niño, and a cold phase known as La Niña (Thatje et al., 2008). Another phenomenon that can also influence fruit production at higher latitudes is called Southern Hemisphere Annular Mode (SAM) which describes variations of the westerly wind belt that affects the middle to higher latitudes of the southern hemisphere (Marshall, 2003).

Besides the considerations about microclimate and the changes of foliar nutrients and pigments, other relationships can be useful for crop management, e.g. correlations with phenological, morpho-physiological and biochemical variables, for a better understand of the vegetative and reproductive behavior of *B. microphylla* plants and its distribution. Therefore, the objective of this study was to evaluate changes in leaf nutrient (carbon, nitrogen, carbon:nitrogen ratio, phosphorus and potassium) and pigment contents (chlorophyll *a*, chlorophyll *b*, chlorophyll *a:b* ratio and carotenoids) of *B. microphylla* plants, growing under different irradiance (low = 24%, medium = 57%, and high = 100% of the natural irradiance) and different fertilization levels (0 = 0.00 g, 1 = 3.36 g, and 2 = 6.72 g per plant), during two growing seasons (2008–2009 and 2009–2010). This knowledge could be useful to define management practices of the aerial parts of this shrub, like plantation distance and pruning schemes. On the other hand, the influence of additional nutrients is crucial to define fertilization plans. Complementary, we explored the fitting of models to estimate anthocyanin and total phenol fruit contents (previously determined by Arena et al., 2017), looking to evaluate different tools to obtain fruit characteristics throughout leaf characteristics. This information could be interesting and profitable for pharmaceutical and nutraceutical industries.

2. Materials and methods

2.1. Plant material and growing conditions

Plants of *B. microphylla* were obtained during autumn in 2004 by rhizome propagation (Arena and Martínez Pastur, 1995) from a natural population near Ushuaia city, Tierra del Fuego (54° 48′ SL, 68° 19′ WL, 30 m elevation). All mother plants were collected from the same

geographic area, where they grow close each other, with their rhizomatous systems interleaved. This fact together with the ability of natural cloning (i.e. rhizomes) of this species indicates that they most likely come from the same genotype.

The climate in Ushuaia is characterized by short, cool summers and long, snowy winters with frequent occurrence of frost. The oceanic climate results in low daily temperature amplitude, while the temperatures between coldest and warmest month vary between -7 °C and 14 °C (mean annual temperature is 5.3 °C). Therefore, the climate is humid (relative humidity is 77% on average) despite rather low average annual precipitation (578 mm). Frequent and strong, mostly westerly, winds are characteristic for the region (www.en.climate-data.org).

In October 2006, two years old plants with a single shoot of 8.5 ± 0.9 cm in length were planted out into plastic pots (12 L), with a substrate (7 kg) obtained from the same area where the plants were collected. The following soil characteristics (Lázzari et al., 1994) were determined ($n = 3$): texture: loam sandy, pH: 5.2 ± 0.03, organic matter: $13.9 \pm 0.8\%$, total nitrogen: $0.30 \pm 0.03\%$, available phosphorus: 35.8 ± 0.7 ppm and available potassium: 117.0 ± 10.1 ppm. Water field capacity was $43.2 \pm 3.4\%$. The experiment was conducted in the experimental field of Centro Austral de Investigaciones Científicas at Ushuaia city (Tierra del Fuego, Argentina). Ammonium nitrate (NH_4NO_3), calcium superphosphate (Ca $(H_2PO_4)_2$) and potassium sulphate (K_2SO_4) were the fertilizers employed in a N:P:K ratio of 1.0:0.6:1.2 at three levels: (0 = 0.00 g, 1 = 3.36 g and 2 = 6.72 g per plant). Fertilizers were divided into equal parts (1.68 and 3.36 g) to be added at the beginning of flowering and during the fast shoot growth according to previous experiments (Arena and Vater, 2003).

Three levels of irradiance were tested (low = 24%, medium = 57%, and high = 100% of the natural irradiance). The low and medium levels were obtained using a micro-tunnel covered with one or two layers of commercial black shade cloth (35% shade, Sebastopol, POLYTEX, Chile). To set up this system, irradiance registered in previous studies was considered. Irradiance in the upper canopy of wild shrubs (100%) decreased to $35.4 \pm 5.5\%$ at their half height ($n = 15$). Irradiance at 0.80 m height from the soil was measured with a Luximeter (Tenmars Lux/FC Light meter TM 201, Taiwan, previously calibrated against a quantum sensor) at midday during days with a clear sky. The average value for the photon flux density (2197 ± 498 μmol m^{-2}s^{-1}, measured with a PAR/LAI AccuPAR LP-80 ceptometer, METER Group, Inc. USA) near Ushuaia city during December and middle January, was comparable to previous years reported by Martínez Pastur et al. (2007). Finally, soil moisture varied between 70% and 87% of the field capacity (Aquaterr Soil Moisture M-300 Instrument, USA). Air and soil (15 cm deep) temperatures, as well as air relative humidity, were measured with data loggers (HOBO 8K, Onset, USA) every hour during two growing seasons (2008–2009 and 2009–2010). Finally, Southern Oscillation Index (SOI) (http://www.bom.gov.au/climate/current/soiht m1.shtml) and Southern Hemisphere Annular Mode Index (SAM) (htt ps://legacy.bas.ac.uk/met/gjma/sam.html) were obtained for the same growing seasons to analyze the influence of the regional climate in our samplings and outputs.

2.2. Samplings for laboratory determinations

To analyze the variation of leaf nutrient content according to the environmental changes, we combined the three light levels (low, medium, high), and the three levels of fertilization (0, 1, 2) along two growing seasons (season 1 = 2008–2009, season 2 = 2009–2010). Moreover, the sampling month (November, December, January, February and March) was also considered to analyze variations in the leaf pigment content. 100–120 plants for each combination of irradiance and fertilization level were employed, totalizing 9 treatments in more than 900 pots. Five plants per treatment were randomly chosen every month

during each growing season to determine the leaf pigment contents. Meanwhile, five plants per treatment were randomly chosen at the beginning of March for nutrient tissue content determinations. Finally, new leaves of the middle portion of the one-year-old shoots during each growing season were selected for both pigment and nutrient tissue contents.

2.2.1. Leaf nutrient concentration

The leaves were dried in a forced draft oven at 65 °C until constant weight and ground in a mill containing 1 mm stainless steel screen to obtain the dried samples (0.5–1.0 g). Nitrogen (N) was determined by Kjeldahl technique using a Buchi K350 (Buchi, Flawil, Switzerland), while carbon, phosphorus and potassium were determined with a plasma emission spectrometry (ICPS 1000 III, Shimadzu, Kyoto, Japan).

2.2.2. Leaf pigment concentration

Foliar discs of 0.28 cm^2 were obtained from the leaves, which were immediately weighed and preserved refrigerated until the quantification of the foliar pigments. The leaf fresh weight/area ratio was also calculated. Chlorophyll *a* and *b* and carotenoids were determined using a spectrophotometric method following Sims and Gamon (2002). Two foliar disks were ground in 2 ml aqueous acetone-tris buffer solution (80% v/v, pH 7.8), and were centrifuged at 1610 g for 10 min at 20 °C. The supernatant was used to determine the absorbance at 470, 537, 647 and 663 nm in a Shimadzu 1203 UV–vis spectrophotometer (Japan), and then to calculate carotenoids, and chlorophylls *a* and *b* contents. Leaf pigment contents in mmol/m^2 were a function of the calculated solution concentrations, where the total volume of extraction solution and extracted total leaf area was previously described by Arena (2016).

2.3. Statistical analysis

The following analyses were carried out: (i) Three-way ANOVAs (irradiance, fertilization level, and growing season) and Tukey post-hoc test (p < 0.05) for tissue nutrient content. (ii) Four-way ANOVAs (irradiance, fertilization level, growing season, and month) and Tukey post-hoc test (p < 0.05) for pigment content and chlorophyll *a:b* ratio. (iii) Principal components analysis (PCA) performed with matrixes of foliar nutrient and pigment contents, employing correlation coefficients, and using levels of each treatment (light, fertilization and growing season) for the plot classification. This analysis graphically shows similarities among plots (plants) according to the evaluated characteristics (leaf nutrient and pigment contents). (iv) Permutational multivariate ANOVAs (PermANOVA, Anderson, 2001) were conducted to simultaneously evaluate the response of the set of nutrient or pigment variables, and permutational analysis of multivariate dispersions (PermDISP, Anderson, 2004). These analyses were used to compare the multivariate dispersions among groups, using for both Bray-Curtis distance, 4999 randomizations, and Monte Carlo test for pair-wise comparisons. Finally, (v) multiple regressions were conducted among leaf nutrient and pigment contents with total phenol (mg tannic acid equivalents/100 g fresh fruit weight) and anthocyanin (mg cyanidine-3-glucoside/100 g fresh fruit weight) contents of fruits in high and medium irradiance. For methodological details and main results of phenol and anthocyanin variables see Arena et al. (2017). We used a stepwise multiple regression to identify which variables better fit in models to estimate phenol and anthocyanin contents. We employed a p < 0.05 for the significance of each variable to be included into the model, analyzing the convenience of avoiding the inclusion of the constant in the model, and used 100 steps for the final model selection. The model was evaluated through r^2-adj, standard error of estimation (SE) defined as the average of the difference between predicted versus observed values, and mean absolute error (AE) defined as the average of the difference between predicted versus observed absolute values (Statgraphics Centurion software, Statpoint Technologies, US).

3. Results

3.1. Environmental parameters

Air and soil temperatures increased in accordance with irradiance treatments, with significant differences among them for most of the months (Figures 1A, 1B, 1D, and 1E). Air and soil temperatures showed maximum values in December and January during both first and second growing seasons. However, temperatures during October and November were colder in the second growing season (air: 5-6 °C; soil: 4-6 °C) than in the first growing season (air: 7-8 °C; soil: 6-8 °C). When the SOI and SAM indexes were analyzed (Table 1), differences between both growing seasons were detected. Positive SOI values were found during the first growing season, while negative SOI values were observed during the second growing season, which could indicate the presence of La Niña and El Niño events, respectively. However, SAM indices were higher during the first than the second growing season, indicating higher temperatures in the first growing season compared with the second one. As a result, in these latitudes, La Niña phenomenon produces a warmer and rainier climate, while El Niño phenomenon is associated with a cooler climate.

Relative humidity remained steady between 70-80% during the first growing season, whereas a strong decrease (60–65%) occurred in December during the second growing season (Figures 1C and 1F). This drop coincided with low precipitation registered in Ushuaia city during such month. This event, together with the increasing air temperature and precipitation events reflect the changes in the regional climate.

3.2. Leaf nutrient content

Significant differences were found in nutrient tissue contents, except for phosphorus when irradiance was considered as the main factor (Table 2). Thus, carbon and carbon:nitrogen ratio were higher under high irradiance (48.0% and 37.0%, respectively), while nitrogen and potassium concentrations were greater under low irradiance (2.0% and 5970.9 ppm, respectively). On the other hand, nitrogen, phosphorus, and potassium contents were higher with fertilization level 2 (2.0%, 1003.7 ppm, and 6087.4 ppm, respectively), while carbon:nitrogen ratio (37.5) was maximum without fertilization (Table 2). Concerning phosphorus and potassium contents, these were significantly higher during the second (911.7 ppm and 5367.5 ppm, respectively) than during the first growing season. Finally, carbon, nitrogen and carbon:nitrogen ratio were similar between both growing seasons.

Significant interactions were found for potassium nutrient content (Table 2). When the interactions between factors were analyzed, it was observed that irradiance and growing season treatments presented some fluctuations in the variation range for potassium nutrient content (Figure 2). Indeed, potassium content did not present significant differences among different irradiances during the first growing season; however, potassium content was significantly different among the irradiance treatments during the second growing season, being maximum under low irradiance.

When all nutrient contents were analyzed through a PCA, the axis one was more correlated with nitrogen and carbon:nitrogen (eigenvalue = 3.267, variation explained = 65.4%), while axis two was more correlated with carbon and phosphorus (eigenvalue = 1.029, variation explained = 20.6%) (Figure 4, left panel). Likewise, PermANOVA for nutrients only presented significant differences among fertilization levels (p = 0.012), being level 0 significantly different from level 2 (Table 4, Figure 4, left panel). Meanwhile, PermDISP only showed significant differences when the growing season was compared (p = 0.024) (Table 4, Figure 4, left panel).

3.3. Leaf pigment content

Significant differences were found in leaf pigments when irradiance was considered as the main factor (Table 3). Thus, chlorophyll *a, b,* and
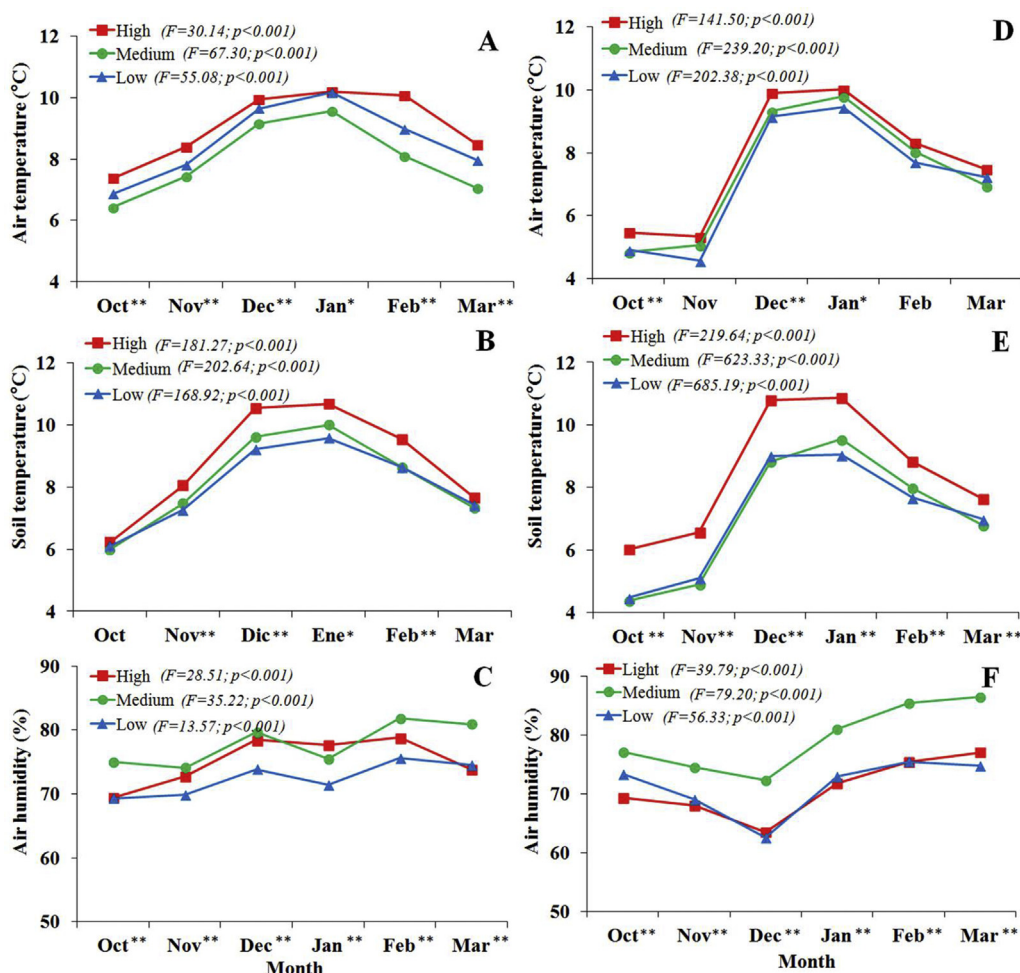
Figure 1. Microclimatic data (monthly averages of: air temperature, soil temperature and air humidity) recorded in the experimental field during the first 2008–2009 growing season (A to C) and the second 2009–2010 growing season (D to F). High, medium and low corresponded to levels of irradiance (low, medium and high) (Arena et al., 2017). F-statistic and p-value (one-way ANOVAs) in each panel indicates monthly differences for each level of irradiance. Asterisks on the x axis indicates the level of significance (** = <0.01, * = between 0.01-0.05).

carotenoids were highest under low irradiance (0.4, 0.1 and 0.2 mmol/m^2, respectively), while chlorophyll *a:b* ratio was maximum under medium and high irradiance conditions (3.1). The leaf fresh weight:area ratio increased significantly ($p < 0.001$) from low to high irradiance (0.029–0.036 g/cm^2). On the other hand, chlorophyll *a, b* and carotenoids were higher under fertilization level 2 (0.4, 0.1 and 0.2 mmol/m^2, respectively), while chlorophyll *a:b* ratio was significantly higher in level

1 (3.1) than level 0 (3.0), although non-significant differences were found with the level 2. Leaf fresh weight:area ratio significantly increased with fertilization level ($p < 0.046$) (from 0.032 g/cm^2 for level 0–0.033 g/cm^2 for level 2). Besides this, different monthly tendencies were observed according to each specific pigment. The highest chlorophyll *a* content was found from January to March (0.4 mmol/m^2), while chlorophyll *b* and carotenoids were maximum during January (0.1 and 0.2 mmol/m^2, respectively). However, chlorophyll *a:b* ratio was greater during November and December (3.0–3.1 mmol/m^2), decreased during January and February, and increased again during March (3.1 mmol/m^2). Leaf fresh weight:area ratio significantly varied ($p < 0.001$) across the months (0.030–0.034 g/cm^2, respectively), being maximum during February. Finally, chlorophyll *a, b,* and carotenoids were significantly higher during the second growing season (0.4, 0.1 and 0.2 mmol/m^2, respectively), while chlorophyll *a:b* ratio was similar in both growing seasons. Leaf fresh weight:area ratio significantly varied ($p < 0.001$) with the growing season (0.031–0.033 g/cm^2), being maximum during the second growing season.

Significant interactions among the main factors were found for pigment content, except for carotenoids (Table 3, Figure 3). When the interactions between irradiance and fertilization level were analyzed, it was observed that chlorophyll *a* content significantly varied among fertilization levels under high irradiance conditions (0.21–0.34 mmol/m^2), while such differences were lower under medium and low irradiance levels (0.39–0.42 mmol/m^2) (Figure 3A). The same trend was observed for chlorophyll *b* (Figure 3D). Also, chlorophyll *a* significantly varied among the months under high irradiance (0.21–0.29 mmol/m^2), with a peak in January (0.33 mmol/m^2), and then abruptly decreased.

Table 1. Southern Oscillation Index (SOI) and Southern Hemisphere Annular Mode Index (SAM) registered during 2008–2009 and 2009–2010 growing seasons.

Growing Season	SOI	SAM
2008–2009		
October	13.40	2.21
November	17.10	1.03
December	13.30	1.01
January	9.40	2.02
February	14.80	0.94
March	0.20	0.50
2009–2010		
October	-14.70	1.47
November	-6.00	-1.20
December	-7.00	1.09
January	-10.10	-0.40
February	-14.50	-2.12
March	-10.60	0.04

Table 2. Effects of irradiance, fertilization level and growing season on leaf nutrient content. C: organic carbon (%), N: nitrogen (%), C:N: carbon:nitrogen ratio, P: phosphorus (ppm) and K: potassium (ppm).

Main effects	C	N	C/N	P	K
A = irradiance					
Low	47.22b	2.01a	23.98c	878.24	5970.91a
Medium	47.24b	1.62b	31.41b	861.15	4194.24b
High	48.05a	1.37c	36.97a	797.94	3471.76b
F(p)	5.2(0.010)	26.3(<0.001)	31.8(<0.001)	0.6(0.550)	12.1(<0.001)
B = fertilization level					
0	47.33	1.37c	37.54a	696.15b	2947.45c
1	47.59	1.68b	29.58b	837.51ab	4602.06b
2	47.59	1.96a	25.24c	1.003.68a	6087.4a
F(p)	0.5(0.599)	22.5(<0.001)	30.7(<0.001)	8.1(0.001)	19.3(<0.001)
C = growing season					
1st	47.53	1.62	31.44	779.84b	3723.77b
2nd	47.48	1.71	30.13	911.71a	5367.50a
F(p)	0.1(0.830)	1.7(0.202)	1.0(0.324)	4.4(0.043)	15.8(<0.001)
Interactions A x B x C F(p)	0.1(0.990)	1.2(0.306)	1.3(0.283)	0.2(0.929)	3.2(0.024)

F(p) = F statistic and probability of Fisher test. Different letters in each column and for each factor indicate significant differences according to the Tukey test ($p \leq 0.05$).

However, chlorophyll *a* under medium and low irradiance levels did not greatly change after the peak of January (Figure 3B). The same performance was observed for chlorophyll *b* under low irradiance conditions (Figure 3E). Significant interactions were not observed between irradiance and growing season for chlorophyll *a*, although a significant interaction was detected for chlorophyll *b* due to differences in their foliar content under low irradiance levels (Figures 3C and 3F, respectively).

When all pigment content variables were analyzed together with a PCA, the axis one was more correlated with chlorophyll *a* and *b* (eigenvalue = 2.362, variation explained = 59.1%), while axis two was more correlated with chlorophyll *a:b* ratio and carotenoids (eigenvalue = 1.506, variation explained = 37.6%) (Figure 4, right panel). Likewise, PermANOVA found differences among different irradiance levels (p = 0.007) for pigment variables, being the low light level significantly different from the medium and high light levels. However, these differences were not detected between medium and high irradiance levels (Table 4, Figure 4, right panel). PermDISP also detected differences for the different irradiance levels (p < 0.001), which were significantly different between medium and high, and medium and low intensities (Table 4, Figure 4, right panel). Likewise, the variance among these data was also significantly different for the two studied growing seasons (p = 0.007) (Table 4, Figure 4, right panel).
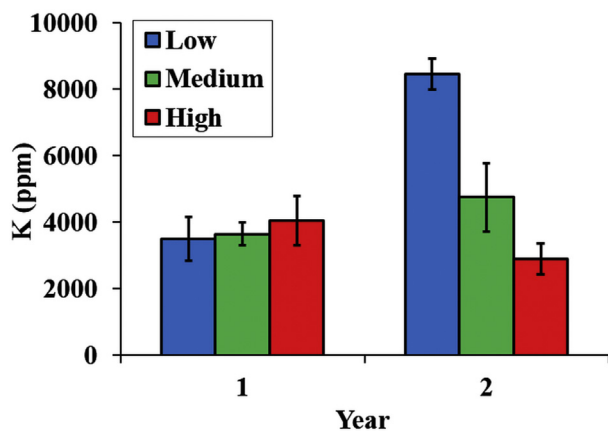
3.4. Fitting of models to estimate total anthocyanin and phenol contents using leaf nutrient and pigment contents

Anthocyanin (mg cyanidine-3-glucoside/100 g fresh fruit weight) content was estimated through a linear model using chlorophyll *a* (mg/g), carbon (%) and potassium (ppm), while phenols (mg tannic acid equivalents/100 g fresh fruit weight) were estimated with the same variables plus the inclusion of chlorophyll *b* (mg/g). The obtained models presented the following parameters and statistics:

Anthocyanins = -808.51 * chlorophyll *a* + 14.457 * carbon +0.0632 * potassium (1)

The main statistics of Eq. (1) were r^2-adj = 89.52%, ESE = 104.0 (mg cyanidine-3-glucoside/100 g fresh fruit) and MAE = 72.2 (mg cyanidine-3-glucoside/100 g fresh fruit).

Anthocyanin decreased when chlorophyll *a* increased, and increased with carbon and potassium contents (Figure 5A). Fitting showed good performance for most of the range of the nutrient variations. However, the model showed negative values at lower anthocyanin contents recorded (Figure 5B).

Phenols = -3022.84 * chlorophyll *a* + 5882.20 * chlorophyll *b* + 28.829 * carbon +0.0956 * potassium (2)

The main statistics of Eq. (2) were r^2-adj = 99.27%, ESE = 86.0 (mg tannic acid equivalents/100 g fresh fruit) and MAE = 49.3 (mg tannic acid equivalents/100 g fresh fruit).

Phenols decreased when chlorophyll *a* increased, and increased with carbon and potassium contents, but also increased with chlorophyll *b* (Figure 5C). Fitting showed good performance for most of the range of the nutrient and chlorophyll *b* variations (Figure 5D).

4. Discussion

4.1. Leaf nutrient content

The leaf nutrient contents can mostly relate to physiological processes like respiration, photosynthesis, mineral nutrient absorption and water intake (Marschner, 1995). Our results also indicate that fertilization had a higher influence over the leaf nutrient content than irradiance, although many variables presented significant correlation with both factors.

The leaf content of carbon, nitrogen, and potassium, as well as carbon:nitrogen ratio in *B. microphylla*, were dependent on the irradiance,

Figure 2. Foliar potassium content (ppm) showing the significant interactions of the ANOVAs (Table 2) between growing seasons (Year 1 = 2008–2009 and Year 2 = 2009–2010) and irradiance (low, medium and high). Bars represent ±standard deviation.

Table 3. Effects of irradiance, fertilization level, month and growing season on leaf pigment content. Chla: chlorophyll a (mmol/m^2), Chlb: chlorophyll b (mmol/m^2), CR: carotenoids (mmol/m^2) and Chla:b: chlorophyll a:b ratio.

Main effects	Chla	Chla	CR	Chla:b
A = irradiance				
Low	0.397a	0.142a	0.192a	2.856b
Medium	0.323b	0.108b	0.183b	3.087a
High	0.283c	0.092c	0.176b	3.123a
F(p)	203.9(<0.001)	241.0(<0.001)	13.0(<0.001)	105.3(<0.001)
B = fertilization level				
0	0.294c	0.101c	0.171c	2.996b
1	0.345b	0.115b	0.183b	3.052a
2	0.370a	0.126a	0.197a	3.018ab
F(p)	93.3(<0.001)	56.2(<0.001)	35.7(<0.001)	4.1(0.017)
C = month				
November	0.231c	0.076d	0.150c	3.045a
December	0.328b	0.108c	0.183b	3.078a
January	0.385a	0.133a	0.206a	2.964b
February	0.372a	0.131ab	0.186b	2.924b
March	0.366a	0.123b	0.193b	3.101a
F(p)	145.2(<0.001)	124.9(<0.001)	52.6(<0.001)	17.3(<0.001)
D = growing season				
1st	0.300b	0.099b	0.175b	3.033
2nd	0.372a	0.128a	0.192a	3.011
F(p)	242.5(<0.001)	226.3(<0.001)	48.0(<0.001)	1.9(0.170)
Interactions A x B x C x D F(p)	2.7(<0.001)	2.7(<0.001)	1.1(0.329)	3.2(<0.001)

F(p) = F statistic and probability of Fisher test. Different letters in each column and for each factor indicate significant differences according to the Tukey test (p ≤ 0.05).

and air and soil temperatures, where carbon and carbon:nitrogen ratio being higher under high irradiance levels. These results are opposite to those found for *Nothofagus pumilio* seedlings growing at different irradiances (Soler et al., 2011), where the highest carbon content was obtained in seedlings grown at low irradiance levels. In this case, the results were coincident with those obtained for *N. pumilio* seedlings that presented the highest nitrogen foliar contents under 26% of the natural light (Soler et al., 2011), and with those in *Abies balsamea* where the nitrogen content was higher (2.7%) under 40% of the irradiance, which can be explained by the luxury consumption effect (Evans et al., 2001). As was found for nitrogen, the potassium content was higher in low irradiance levels, as was also found in *N. pumilio* seedlings (Soler et al., 2011). Potassium also stimulates the fixation of CO_2, improving photosynthesis for those plants that grow in low irradiance levels (Marschner, 1995). This mineral, like other nutrients such as magnesium and calcium, is related to cell turgor, water intake, pH regulation and the building of cellular structures (Marschner, 1995), while its content decreases when the availability of light and soil water content increases. Potassium could also be related to the chlorophyll a synthesis and with its enzymatic activators (Latsague et al., 2014). Although the foliar contents of nitrogen and potassium are within the expected values, the foliar content of phosphorus could be considered lower than the expected value according to this author, and this is coincident with those values registered in the leaves of plants grown naturally in the original population (850 ± 50 ppm) (Arena, 2016).

Our data showed that leaf nitrogen, phosphorus, and potassium contents, as well as carbon:nitrogen ratio, were dependent on the level of fertilization. The contribution of increasing doses of fertilizers also increased the content of leaf nitrogen, phosphorus and potassium (Marschner, 1995). However, in our analyses of phosphorus content, we detected significant differences only between fertilization levels of 0 and 2. According to this, an intermediate dose would not be recommended if the ultimate goal is to increase the leaf nutrient contents. The highest nutrient contents at low irradiance can evidence that they would be in excess for the physiological requirements, showing then a luxury

consumption (Evans et al., 2001) due to the scarce fruit production in plants grown at this irradiance level (Arena, 2016).

It is important to note that phosphorus and potassium contents were higher during the second growing season compared to those obtained in the first one. Particularly for potassium, a higher content was found in the leaves of the treatments with low irradiance and fertilization levels during the second growing season. Because fertilization had a great influence over the whole set of leaf nutrient contents, further studies must be conducted in order to detect the optimum level of fertilization to achieve different management objectives (e.g. increasing fruit yield and leaf area of the plants).

4.2. Leaf pigment content

The synthesis of chlorophylls and carotenoids are regulated by different endogenous (e.g. enzymatic activity) and exogenous factors (e.g. availability of nutrient, water, and light) during the growth and differentiation processes (Timko, 1998; Valladares et al., 2000). Efficient acclimation to changing environmental conditions is a prerequisite for the survival and competitiveness of plants in the field. Proper acclimation to the light availability at a given habitat is essential to allow for efficient light utilization under light-limiting conditions and to avoid photo-oxidative damage under excess-light condition. Adjustments of leaf architecture, chloroplast structure, composition of the photosynthetic electron transport chain, and regulation of photosynthetic light utilization are involved during the acclimation process to light. Some of the typical characteristics of high light acclimated plants are increased thickness of leaves with more cell layers and larger cells, increased number of chloroplasts per cell with reduced grana stacking and higher chlorophyll a:b ratio with increased β-carotene and xanthophyll cycle pigment levels, among others (Schumann et al., 2017). In this context, plants acclimated to low-light appears to exhibit greater amounts of chlorophyll b and increased photosynthetic light harvesting antennae size of both PS II and PS I in order to optimize light capture relative to high-light grown plants. Furthermore, high-light grown plants appear to
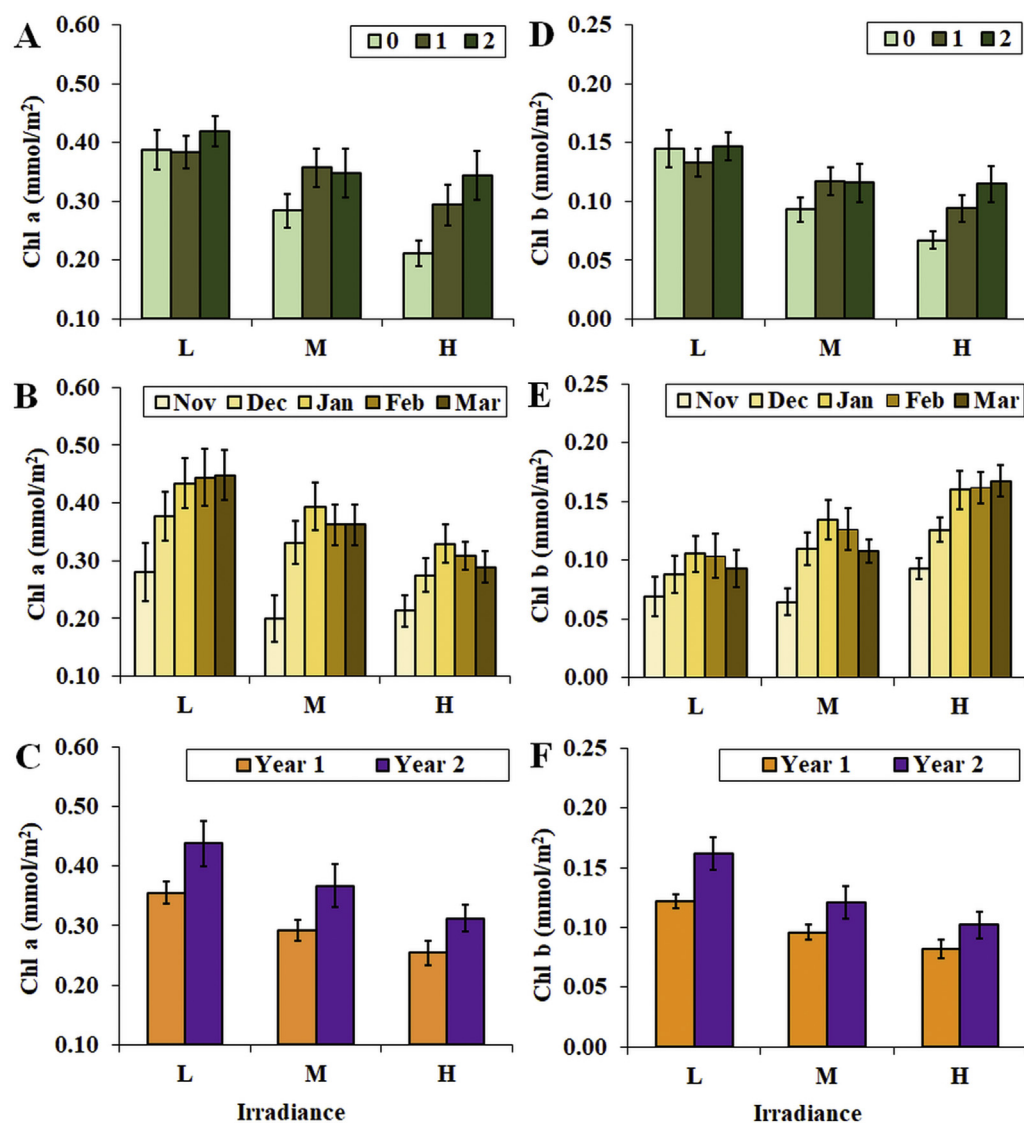
Figure 3. Leaf chlorophyll content showing significant interactions of the ANOVAs (Table 3) between A chlorophyll *a* (Chla) and D chlorophyll *b* (Chlb): irradiance (L: low, M: medium, H: high) and fertilization level (0, 1 and 2); B chlorophyll *a* (Chla) and E chlorophyll *b* (Chlb): irradiance (L: low, M: medium, H: high) and month (Nov: November, Dec: December, Jan: January, Feb: February and Mar: March) and **C** chlorophyll *a* (Chla) and F chlorophyll *b* (Chlb): irradiance (L: low, M: medium, H: high) and the growing season (Year 1 = 2008–2009 and Year 2 = 2009–2010). Bars represent ±standard deviation.

be less sensitive to photoinhibition than plants acclimated to low-light (Gray et al., 1998). However, acclimation is not a response absolute to irradiance due to these characteristics can be met by modulating either irradiance or temperature (Gray et al., 1998). As was expected pigment concentration in *B. microphylla* was stronger influenced by irradiance than by fertilization, as was observed in our multivariate analyses. Specifically, leaf pigments were dependent on leaf fresh weight per area, irradiance and air and soil temperatures. Indeed, it decreased 1.7, 1.9 and 1.3 times for chlorophyll *a*, chlorophyll *b* and carotenoids, respectively, from low to high irradiance levels. The contents of leaf pigments found in *B. microphylla* are higher than those obtained in other shrub species (Zinnert et al., 2013), and are in agreement with those reported for trees and shrubs of other temperate forests (Valladares et al., 2000; Zúñiga et al., 2006; Lichtenthaler et al., 2007; Aranda et al., 2008; Soler et al., 2011; Esteban et al., 2015). The values of the chlorophyll *a:b* under a high irradiance (2.8–3.6) and under a low irradiance (2.5–3.1) are characteristic of sunny and shady leaves respectively (Damesin, 2003), although they were somewhat lower than those reported for *Nothofagus* species (Zúñiga et al., 2006). Carotenoids are structurally diverse pigments and related derivatives, so their functions depend on the chemical structure. They serve as accessory light-gathering pigments and can also act to protect chlorophyll pigments from damaging destructive photo-oxidation reaction in high irradiation (Valladares et al., 2000;

Lichtenthaler et al., 2007). Also, mediate responses to biotic and abiotic signals, and control plant architecture (Wurtzel, 2018). The carotenoid content in *B. microphylla* follow the same trend as chlorophylls, e.g. major content in leaves exposed to low irradiance.

The leaf pigment contents are closely related to the leaf nutrient contents (Larcher, 2003), explained by the chemical composition of the pigments (Timko, 1998), as well as the involved physiological processes (Damesin, 2003). This could explain the higher contents of the leaf pigments in the fertilized compared with the unfertilized treatments (chlorophylls *a*, *b* and the carotenoids were higher in 1.3, 1.2 and 1.2 times, respectively), affecting the chloroplasts formation (Field and Mooney, 1986) and their photosynthetic efficiency (Martínez Pastur et al., 2007). However, future studies should explore deeply the interactive effect of fertilization and irradiance levels on *B. microphylla* growth. In *Olea europea* a significant and positive correlation was observed between nitrogen and foliar chlorophyll content (Boussadia et al., 2010), as well as between the addition of nitrogen, phosphorus and potassium and the chlorophyll content in several other species (Duli Zhao et al., 2003). On the other hand, a close correlation could be observed between the content of the leaf pigments and the presentation (timing) of the phenological phases in treatments with high irradiance and fertilization (Arena, 2016), where precocity in the presentation of some vegetative phases may have favored the formation of foliar
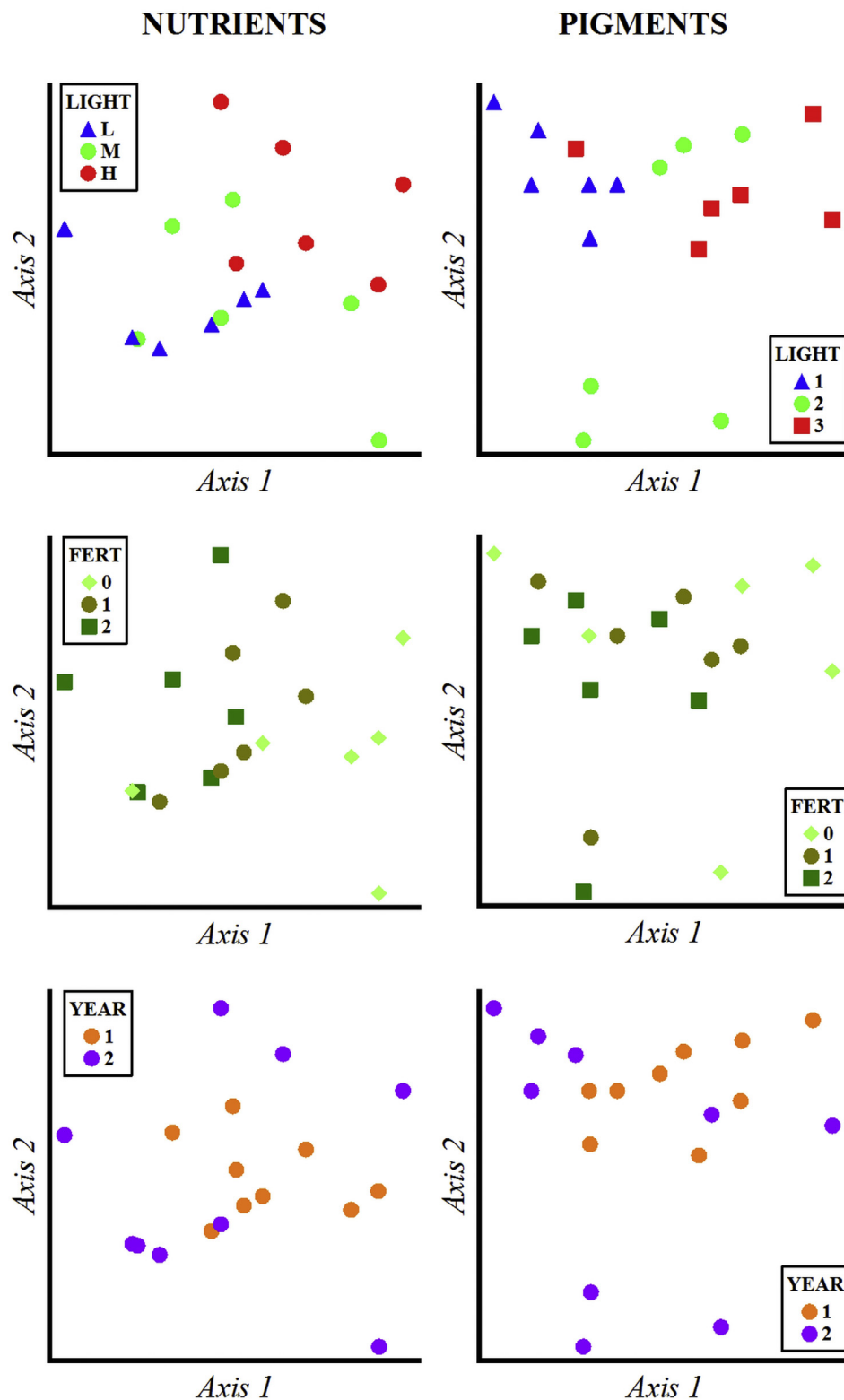
Figure 4. Principal component analyses (PCA) conducted with the leaf nutrient (organic carbon, nitrogen, carbon:nitrogen relation, phosphorus and potassium) (left panel) and pigment contents (chlorophyll *a*, chlorophyll *b*, carotenoids and chlorophyll *a:b* (right panel) during March. LIGHT = irradiance (bue: low, green: medium, red: high), FERT = fertilization levels (light green: 0, brown: 1, dark green: 2) and YEAR = growing season (orange: 2008–2009, purple: 2009–2010). The units of variables are presented in Tables 2 and 3.

pigments. In addition, a close relationship was observed between the leaf pigment content and the leaf fresh weight:area, the maximum content of pigments coinciding with the maximum values of this ratio observed in February. Similarly, the highest chlorophyll *a* concentration in *Citrus* spp. was found in those months where plants showed great demand for photo assimilates (Reyes et al., 2000), in coincidence with the results obtained in this work.

Proven benefits from nitrogen fertilizer have been recently based on the evaluation of the photosynthetic system performance (Tranavičienė et al., 2008). A positive correlation between fertilization, rate and chlorophyll content is well documented for several plant species (Jeppsson, 2000; Duli Zhao et al., 2003; Latsague et al., 2014; Boussadia et al., 2010). However, in this study fertilization had no effect on the pigment contents under low light conditions. On the other hand, because the

Table 4. PermANOVA and PermDISP statistical results for the set of variables related to leaf nutrients (organic carbon, nitrogen, carbon/nitrogen ratio, phosphorus and potassium) and leaf pigments (chlorophyll *a*, chlorophyll *b*, carotenoids and chlorophyll *a:b*) in March, according to the growing season, irradiance and fertilization levels. Variable units and levels of main factors are presented in Tables 1 and 2.

| Set of variables	Factors	Pair-wise comparisons	Statistics	PermANOVA	PermDISP
Nutrients	Irradiance		F (p)	1.002 (0.345)	0.581 (0.582)
		High vs. Medium	t (p)	0.518 (0.720)	0.916 (0.389)
		High vs. Low	t (p)	1.467 (0.159)	1.102 (0.318)
		Medium vs. Low	t (p)	0.848 (0.427)	0.038 (0.971)
	Fertilization level		F (p)	5.310 (0.012)	1.110 (0.373)
		0 vs. 1	t (p)	1.986 (0.057)	1.139 (0.305)
		0 vs. 2	t (p)	2.883 (0.016)	1.170 (0.306)
		1 vs. 2	t (p)	1.472 (0.148)	0.137 (0.898)
Pigments	Growing season		F (p)	1.308 (0.257)	5.481 (0.024)
	Irradiance		F (p)	5.941 (0.007)	18.703 (<0.001)
		High vs. Medium	t (p)	0.827 (0.438)	3.977 (0.004)
		High vs. Low	t (p)	5.148 (0.002)	4.553 (0.157)
		Medium vs. Low	t (p)	2.753 (0.002)	5.454 (0.002)
	Fertilization level		F (p)	0.498 (0.665)	1.775 (0.202)
		0 vs. 1	t (p)	0.270 (0.885)	0.648 (0.526)
		0 vs. 2	t (p)	0.972 (0.421)	2.073 (0.049)
		1 vs. 2	t (p)	0.848 (0.528)	1.273 (0.240)
	Growing season		F (p)	1.478 (0.240)	9.393 (0.007)

F (p) = probability of Fisher test.
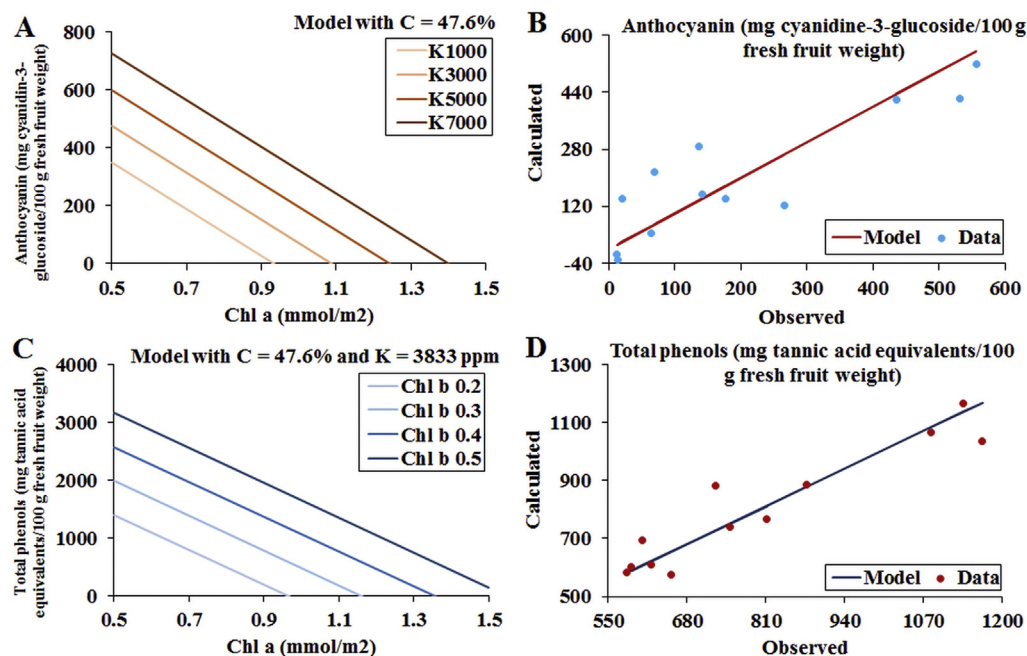
Figure 5. Graphical representation of multiple regression model responses (left panels A and C) and plotting of observed vs. calculated and modelled data (right panels B and D), to estimate anthocyanins (mg cyanidine-3-glucoside/100 g fresh fruit weight, above panels A and B) and total phenols (mg tannic acid equivalents/100 g fresh fruit weight, below panels C and D) in fruits, using nutrient contents (carbon (%), and potassium (ppm) and pigments (chlorophyll *a* (mg/g) and *b* (mg/g), as independent variables. Model details are described in Material and methods section.

metabolic process and fruit development are also regulated by the nitrogen supplementation (Arena et al., 2017), the signaling pathways of secondary metabolites and nitrogen could interact (Tranavičienė et al., 2008).

Finally, the regression models allow to estimate the fruit secondary metabolites (anthocyanin and phenol) concentrations using carbon and potassium leaf contents and chlorophylls (*a* and *b*). Under high irradiance and fertilization level 2, carbon and potassium leaf contents were maxima and chlorophyll contents were minimal, while fruits produced in this treatment had the highest anthocyanin and phenol concentrations. The proposed equations could be a useful tool for industries that need to estimate anthocyanin and phenols concentration on fruits, e.g. making estimations without destroying the source from where metabolic

compounds are extracted. Although these models could evidence some link between nutrients and foliar pigments in the regulation of metabolic processes at the fruit level, this study cannot ensure that this explicatory relation exist. One important limitation of modeling comes from the raw data we used, which include all the treatment combination of this study. On one hand, the wide range of conditions allowed us to model the full variation of nutrient and pigment contents. However, we cannot consider potential constraints among the different components.

4.3. Influence of seasonal climatic variability

Besides the punctual influence on the different growing seasons over each nutrient or pigment variables, the annual climatic variability was

crucial to understanding the differences. The outputs obtained one growing season compared with the other affected the whole set of variables of both nutrient and pigment contents, which influences the data dispersion shown on the multivariate analysis. The results indicate that seasonal and climatic variations play a key role in determining foliar nutrient and pigment content. Photosynthetic pigments are indicators of the level of plant acclimation to the environment, and for many species, there is a consistent relationship between pigment content and environmental variables (Bündchen et al., 2016). According to our results, *B. microphylla* can quickly acclimate to changes of light, nutrient input, and seasonal climate variations by shifting the foliar pigment content.

In this sub-antarctic zone of southern Patagonia, the plants are subjected to continuous stress (e.g. freezing temperatures in winter and even in spring, dry summers followed by rainy ones, or days with high evapotranspiration rates) to which plants respond by adjusting their metabolic processes (Diehl et al., 2003). It could, therefore, be inferred that the variations observed in this study are the expected for those plant species living in strongly seasonal environments and resulted in two distinct climate periods, La Niña and El Niño phenomena, respectively.

Arena et al. (2017) found higher fruit anthocyanin and phenol contents together with soluble solids and soluble solids:acidity ratio during the first growing season than the second growing season in the same experiment, although only in plants growing under high and medium irradiance levels. They related these results to the higher temperatures during the spring. It is also known that temperature is a limiting factor of the photosynthetic rate, so, at higher temperatures, the photo-assimilates are produced at a faster rate, which makes an increase in the translocation from leaves to the different possible plant structures, including fruits. If there were no significant differences in potassium content at high and medium light treatments between the first and the second seasons, the contents of chlorophyll *a* and *b* were higher during the second growing season than during the first one, confirming the negative correlation with the fruit composition.

4.4. Recommendations to management and futures studies

The observed changes in the leaf nutrient and pigment contents of *B. microphylla* could be related to the environmental conditions of the studied treatments and to the fertilization levels, evidencing the acclimation capacity of this shrub to shifting environmental conditions via leaf composition arrangements. Our results highlight the relevance of fertilization to enhance the foliar nutrient content, and the effect of irradiance on foliar pigments. Also, the relationship between fruit secondary metabolites (anthocyanin and phenol contents) with carbon and potassium leaf contents and chlorophylls (*a* and *b*) were evidenced. As a recommendation, the growing of *B. microphylla* shrubs under high irradiance (with adequate planting distance and balanced pruning in a commercial orchard) justified the addition of 6.7 g of fertilizer per plant (level 2) and allowed the highest fruit anthocyanin and phenol content. Although this shrub seems to be highly adaptable species to climatic variations, future studies should evaluate its response to other environmental factors affected by climate change (e.g., rainfall alterations).

Declarations

Author contribution statement

Miriam Arena, Guillermo Martinez Pastur, María Vanessa Lencinas, Rosina Soler, Gimena Bustamante: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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