Impact on leaf morphology of *Vitis vinifera* L. cvs. Riesling and Cabernet Sauvignon under Free Air Carbon dioxide Enrichment (FACE)

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Abstract

Atmospheric carbon dioxide (CO_2) concentration has continuously increased since pre-industrial times and has currently reached a growth rate of approximately 2.3 ppm per year. For the majority of plant species elevated CO_2 (eCO_2) improves photosynthesis and thus plant biomass. To investigate the effects of eCO_2 on morphological leaf characteristics the two *Vitis vinifera* L. cultivars, Riesling and Cabernet Sauvignon, grown in the Geisenheim VineyardFACE (Free Air Carbon dioxide Enrichment) system were used. The VineyardFACE is located at Geisenheim University comparing future atmospheric CO_2-concentrations (eCO_2, predicted for the mid-21st century) with current ambient CO_2-conditions (aCO_2). Experiments were conducted under rain-fed conditions for two consecutive years (2015 and 2016). For both varieties six leaves per repetition of each CO_2 treatment were selected to measure epidermal flavonoid (Flav) and leaf chlorophyll (Chl) indices using a portable leaf clip. Same leaves were sampled for spectrophotometric analysis of the leaf pigments chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoid (Car). Additionally, leaf cross-sections were produced as permanent preparations to investigate thickness of the different leaf layers. Pictures were taken using a light microscope and consecutive measurements were conducted with an open source image software. Both cultivars did not differ in leaf chlorophyll meter readings or leaf pigments between the two CO_2 treatments over the two years of investigations. Differences found in leaf cross-sections were detected in palisade parenchyma and epidermis of Cabernet Sauvignon under eCO_2, whereas Riesling remained unaffected for all leaf layers. The observed results within grapevine leaf tissues provide insights to seasonal adaptation strategies of grapevines under future elevated CO_2 concentrations.

Introduction

Atmospheric carbon dioxide, one of the most relevant greenhouse gases has been increasing continuously since pre-industrial times from 280 ppm in 1750, and is predicted to exceed 700 ppm by the end of 21st century (IPCC, 2014). For most of C3 plant species elevated CO_2 improves photosynthetic apparatus resulting in an increased plant biomass production. Only few field studies on grapevines under elevated CO_2 conditions have been conducted, showing higher yield and vegetative biomass due to enhanced net assimilation rates (Bindi et al., 2001; Moutinho-Pereira et al., 2009; Edwards et al. 2017; Wohlfahrt et al., 2018).
Anatomical alteration of leaves under eCO_2 has been reported for several tree and C3 species, e.g. increase in leaf thickness and layers, extension of leaf cells and chloroplast development (Thomas and Harvey, 1983; Robertson and Leech, 1995; Saxe et al., 1998). The increase in leaf thickness of the grapevine cultivar Touriga Franca was explained by extended spongy parenchyma and partly due to an increase in palisade parenchyma under eCO_2 conditions (Moutinho-Pereira et al., 2009).
The aim of this study was to investigate the effects of eCO_2 on morphological leaf characteristics of the two *Vitis vinifera* L. cultivars Riesling and Cabernet Sauvignon grown in the Geisenheim VineyardFACE system.

Materials and methods

The experimental study was performed at the VineyardFACE described by Wohlfahrt et al. (2018), located at Hochschule Geisenheim University, Germany (49° 59′ N, 7° 57′ E, 94 m above sea level). The field trial was

implemented in 2013 as a ring-shaped system comparing future atmospheric CO_2-concentrations (eCO_2, predicted for the mid-21st century) with current ambient CO_2-conditions (aCO_2). Both CO_2 treatments were replicated three times. The vines were trained in a vertical shoot positioning system (VSP) and pruned to five nodes per m^2. The planting distance of vines was 0.9 m x 1.8 m and rows were north-south orientated. The cultivars *Vitis vinifera* L. cv. Riesling (clone 198–30 Gm) grafted on rootstock SO4 (clone 47 Gm) and cv. Cabernet Sauvignon (clone 170) grafted on rootstock 161–49 Couderc were used. Experiments were conducted under rain-fed conditions for two years, 2015 and 2016.

In both growing seasons, six leaves per FACE-ring were measured on the adaxial and abaxial side with a Dualex Scientific portable chlorophyll meter (Force A, Orsay, France) to determine epidermal flavonoid (Flav) and leaf chlorophyll (Chl) indices according to Cerovic et al. (2012). Additionally, a nitrogen balance index (NBI) was calculated as the ratio of Chl and Flav. After execution of field measurement same leaves were sampled to analyse leaf pigments. About 30 mg of freeze-dried leaf tissue was extracted on ice in 100% acetone with a total volume of 4.9 ml. The absorption at 400 to 780 nm was measured using a spectrophotometer. Chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoid (Car) content were determined according to Lichtenthaler (1987).

For morphological traits six leaves per repetition of each CO_2 treatment were sampled on the same dates in 2015 and 2016. Cut leaves were immediately fixed in a FAA solution (ethanol, H_2O, formaldehyde and glacial acetic acid). After 24 h leaf samples were transferred and stored in an ethanol solution. Firstly, leaf tissues were dehydrated using an increasing ethanol/isoporopanol series, infiltrated and embedded in paraffin under low air pressure conditions. By using a rotary microtome (Leica, RM 2155, Nussloch, Germany) sections of 5 µm were prepared and fixed on microscopic slides. Subsequently, samples were stained using consecutive staining and washing solutions according to Wacker (2006). Pictures of the leaf cross-sections were taken using a fluorescence microscope (Keyence, Biozero BZ-8000K, Neu-Isenburg, Germany). Measurements of pictures were conducted with ImageJ, an image analysis software (National Institutes of Health, Bethesda, MD, USA).

Statistical analyses were performed with the statistical software R, version 3.4.2 (R Foundation for Statistical Computing, Vienna, Austria). Data for all parameters were tested using multi-factor (treatment, block, year and interaction treatment x year) analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test for significant differences ($P \leq 0.05$ level). For all parameters, means per ring were calculated and used for statistical analyses.

Results and discussion

Chlorophyll meter indices did not differ between years or treatments for both cultivars (Table 1). Only Riesling Chl index increased under CO_2 enrichment over the two years ($P=0.0629$), even though not significantly. NBI index was higher in leaves of Cabernet Sauvignon compared to Riesling in both years. This was shown earlier by higher amino acid concentration in berries of Cabernet Sauvignon during berry development in 2015 and 2016 (Wohlfahrt et al., 2020). As shown in Table 1, leaf pigments (Chl a, Chl b, total Chl and Car) were highly stimulated by the year and not by eCO_2. This is in accordance with results of total chlorophyll and carotenoid content in beech leaves, where a varying nutrient supply caused significant differences in pigment contents, whereas eCO_2 revealed no effects on pigment concentration (Polle et al., 1997).

Total leaf thickness and spongy parenchyma of Riesling and Cabernet Sauvignon remained less affected under eCO_2 conditions (Table 2). However, significant differences found in leaf cross-sections of the two CO_2 treatments were detected for upper and lower epidermis and the palisade parenchyma of Cabernet Sauvignon. Whereas under eCO_2 the palisade parenchyma increased, the epidermal tissue decreased in thickness. In a previous study it was suggested that an increased leaf and therefore parenchyma thickness under eCO_2 was due to an enlargement of cells and not by an increase in cell division (Moutinho-Pereira et al., 2009). This could be explained by the same amount of parenchyma layers in both CO_2 treatments (data not shown). Also, palisade parenchyma in Riesling showed in tendency a small increase. However, the ratio between palisade and spongy parenchyma hardly differed by the treatment in Riesling whilst in Cabernet Sauvignon the treatment effect was more pronounced ($P=0.017$). Contrary to the leaf morphology of the red cultivar Touriga Franca, which resulted in thicker spongy parenchyma and thus lower or unchanged palisade to spongy parenchyma ratio (Moutinho-Pereira et al., 2009), the palisade to spongy parenchyma ratio increased und eCO_2 within Cabernet Sauvignon. This leads to the conclusion that different climatic effects could possibly be responsible for this circumstance. Nevertheless, these morphological alterations in leaves affect photosynthesis of plants since a higher internal leaf surface enhances the ability to absorb CO_2 to a larger extent. Such impact

on photosynthetic activity has been shown in the Geisenheim VineyardFACE system by Wohlfahrt et al. (2018).

Conclusion

The observed results within grapevine leaf tissues can provide first insights to seasonal adaptation strategies of grapevines under future elevated CO_2 concentrations. However, regardless of the CO_2 treatment the year effect and in particularly the plant water status may have a large impact on leaf morphology too. Still, both types of ground tissue, palisade and spongy parenchyma contain chloroplasts. Even though the palisade parenchyma contains a high number of chloroplasts compared to the spongy parenchyma, the latter is very prominent in terms of the intercellular air space in the lower mesophyll. Chlorenchyma and aerenchyma are both of utmost importance for the photosynthetic rate which in parts may help to explain that under eCO_2 the photosynthetic activity will be further stimulated. Both, in Riesling and Cabernet Sauvignon such higher activity has been shown in the early years of adaptation of the vines by increase in sap flow, biomass production and hence impact on cluster weight.

Table 1. Results of chlorophyll meter readings of leaf chlorophyll (Chl), flavonoid (Flav) and nitrogen balance index (NBI) as well as leaf pigments content (in dry matter, DM) for chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoid (Car). Data represent mean ± SD of the three rings and six leaves per treatment.

	Dualex indices			mg g^{-1} DM			
	Chl	Flav	NBI	Chl a	Chl b	Chl total	Car
2015							
R aCO_2	26.09±2.26	2.90±0.04	9.02±0.68	2.16±0.28	1.04±0.16	2.67±0.36	0.64±0.07
R eCO_2	29.11±2.63	2.92±0.04	10.04±0.76	2.16±0.29	1.07±0.14	2.68±0.35	0.69±0.09
2016							
R aCO_2	27.02±3.04	2.80±0.07	9.75±1.31	3.95±0.30	3.58±0.27	5.70±0.39	0.94±0.13
R eCO_2	30.31±1.06	2.86±0.11	10.65±0.57	3.81±0.31	3.43±0.18	5.48±0.40	0.93±0.10
P value							
treatment	*0.0629*	*0.3883*	*0.1152*	*0.8264*	*0.7360*	*0.7966*	*0.6282*
year	*0.4799*	*0.1030*	*0.2499*	***1.897e-05***	***5.658e-08***	***2.306e-06***	***0.0023***
treatment x year	*0.9256*	*0.7516*	*0.9143*	*0.8375*	*0.4907*	*0.7397*	*0.7439*
2015							
CS aCO_2	29.66±3.52	2.64±0.09	11.32±1.58	2.09±0.31	0.98±0.15	2.58±0.38	0.60±0.10
CS eCO_2	29.54±2.81	2.62±0.21	11.50±1.99	2.41±0.28	1.17±0.15	2.98±0.35	0.70±0.08
2016							
CS aCO_2	29.03±3.07	2.53±0.11	11.63±1.68	4.22±0.23	3.73±0.11	6.04±0.28	0.96±0.11
CS eCO_2	31.41±1.63	2.58±0.07	12.28±0.99	4.54±0.48	3.71±0.08	6.36±0.48	1.11±0.21
P value							
treatment	*0.4835*	*0.8502*	*0.6456*	*0.1576*	*0.3410*	*0.1603*	*0.1748*
year	*0.3339*	*0.3339*	*0.5484*	***1.055e-05***	***3.584e-09***	***9.703e-07***	***0.0016***
treatment x year	*0.6339*	*0.6339*	*0.7930*	*0.9500*	*0.1701*	*0.7615*	*0.8164*

Table 2. Thickness of total leaf tissue, palisade parenchyma, spongy parenchyma and ratio of palisade to spongy parenchyma. Data represent mean ± SD of the three rings and six leaves per treatment.

	Thickness [µm]				
	Total thickness	Upper/lower epidermis	Palisade parenchyma	Spongy parenchyma	Palisade / spongy parenchyma ratio
2015					
R aCO_2	171.98±5.71	34.82±3.09	51.65±4.08	86.64±1.61	0.61±0.04
R eCO_2	177.35±4.93	39.75±1.28	55.60±4.74	83.15±6.16	0.69±0.11
2016					
R aCO_2	166.89±14.30	34.40±2.71	45.79±1.97	83.48± 9.15	0.56±0.06
R eCO_2	176.22±14.62	33.98±1.90	50.37±4.09	87.35±11.98	0.58±0.04
P value					
treatment	*0.1528*	*0.0954*	*0.0937*	*0.9553*	*0.1987*
year	*0.5189*	***0.0329***	***0.0396***	*0.8780*	*0.0687*
treatment x year	*0.6794*	***0.0555***	*0.8892*	*0.2980*	*0.4998*
2015					
CS aCO_2	191.99±7.58	40.05±5.99	59.40±2.85	89.52±3.18	0.68±0.03
CS eCO_2	195.97±9.87	34.78±1.35	66.66±3.97	93.91±4.62	0.72±0.00
2016					
CS aCO_2	172.37±10.80	34.78±0.40	48.88±4.30	87.19± 7.97	0.56±0.01
CS eCO_2	175.00±11.42	31.20±3.17	56.00±0.38	85.42±10.07	0.67±0.08
P value					
treatment	*0.3301*	***0.0405***	***0.0039***	*0.6341*	***0.0170***
year	***0.0004***	***0.0403***	***0.0004***	*0.0786*	***0.0080***
treatment x year	*0.8367*	*0.6470*	*0.9689*	*0.2801*	*0.1764*

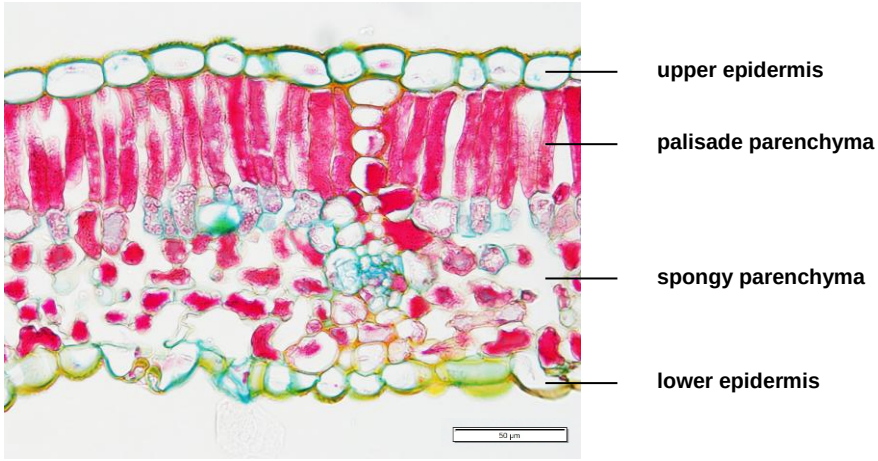

Figure 1. Histological tissue section of a *Vitis vinifera* cv. Riesling leaf as basis for measurements of epidermal and parenchymatic shares.

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