# Physiological responses to soil lime in wild grapevine (*Vitis vinifera* ssp. *sylvestris*)

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***Abbreviations:*** A, net photosynthetic rate; BAP, 6-benzylaminopurine; Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; Ci, intercellular $CO_2$ concentration; C*x*+*c*, carotenoids; $F_0$, minimal fluorescence level in the dark-adapted state; $F_m$, maximal fluorescence level in the dark-adapted state; $F_s$, steady state fluorescence yield; $F_v$, variable fluorescence level in the dark-adapted state; $F_v/F_m$, maximum quantum efficiency of PSII photochemistry; ΦPSII, quantum efficiency of PSII; Gs, stomatal conductance; NAA, naphthaleneacetic acid; RGR, relative growth rate.

**Abstract:**

Lime-induced chlorosis is a widespread nutritional disorder affecting grapevines cultivated in calcareous soils. A greenhouse experiment was conducted to investigate the response of *Vitis vinifera* ssp. *sylvestris* to soil lime by evaluating the effects of a range of soil $CaCO_3$ contents (0 to 60%) on plant growth, nutrient content (iron, potassium, nitrogen and phosphorus) and photosynthetic performance (gas exchange, chlorophyll fluorescence parameters and photosynthetic pigments). The highest soil $CaCO_3$ concentration induced nutrient imbalances and significantly inhibited photosynthetic function, causing a reduction in carbon gain and consequently, a drastic growth reduction and high mortality. However, all the plants survived external $CaCO_3$ contents of up to 40%, and reduction in growth at 20% $CaCO_3$ was slightly lower than that recorded in several previously studied lime-tolerant varieties of grapevine. Plants grown at 20% $CaCO_3$ maintained net photosynthesis values of around 6 μmol $m^{-2}$ $s^{-1}$, a similar chlorophyll content to that of the control plants and dawn $F_v/F_m$ values close to the optimal values for unstressed plants. Up to the 40% $CaCO_3$ treatment, the study species was capable of maintaining Fe uptake by the roots and translocation to leaves, while controlling the nutritional status of N and P. Our study indicates that the studied population of *V. vinifera* ssp. *sylvestris* could provide a source of genetic diversity for lime tolerance improvement in grapevine.

**Keywords:** Calcareous soil; *Vitis vinifera*; photosynthesis; tolerance.

## 1. Introduction

Calcareous soils are common in some of the most important European viticultural areas and grapevines growing on such soils often suffer lime-induced Fe chlorosis (Bavaresco et al. 2005), although the response varies according to grapevine variety (Tangolar et al. 2008). The response of susceptible grapevines to lime-stress includes reduction in shoot and root growth, lower yield and a characteristic leaf interveinal yellowing. Lower biomass in susceptible plants is related to reduced root growth due to soil bicarbonate and to a lower photosynthetic rate that also depends on a decreased leaf chlorophyll under Fe stress conditions (Bavaresco et al. 2003). At low Fe-availability in soils, the grapevine responds as a typical "Strategy I" plant, increasing iron uptake, proton extrusion, and reducing capacity (Varanini and Maggioni 1982; Brancadoro et al. 1995; Nikolic and Kastori 2000). Different grapevine rootstocks show considerable quantitative differences in terms of the root response reactions involved in "Strategy I", particularly with regard to rhizosphere acidification, root $Fe^{III}$ reducing capacity and rates of Fe uptake (Brancadoro et al. 1995; Maggioni 1980).

Lime-induced chlorosis has a strong impact on the fruit industry because it affects both the yield and quality of the fruit, and on the other hand the fertilizers used for its control and prevention are often expensive, not very efficient in the long term, and some are considered not environmentally friendly (Abadia et al. 2011; Álvarez-Fernández et al. 2011). The most useful method in viticulture to overcome this form of stress is to graft grape varieties onto lime-tolerant rootstocks. Grapevine rootstocks can be pure *Vitis* species or hybrids, mainly between *Vitis riparia*, *Vitis rupestris* and *Vitis berlandieri*, which differ in response when grown in calcareous soils (Bavaresco et al. 1994). Lime-tolerant grapevine rootstocks such as 140 Ru and 41B are used by

viticulturists on calcareous soils worldwide; however, the ideal rootstock has yet to be obtained.

*Vitis vinifera* (L.) ssp. *sylvestris* (Gmelin) Hegi, the wild subspecies of *Vitis vinifera* L., is the only native Eurasian subspecies and constitutes a valuable genetic resource for cultivated grapevines (Negrul 1938). The distribution of the wild grapevine has been dramatically reduced in its major sites of diffusion, initially by the spread of pathogens from North America over the last 150 years and, more recently, as a result of habitat fragmentation and disbranching by humans (Grassi et al. 2006; Ocete et al. 2011a; 2012). Wild grapevine populations maintain considerable genetic polymorphism and manifest wide variability (McGovern et al. 1996); the disappearance of these populations from their natural habitat would constitute an irreversible loss for the environment and for breeding programs (Grassi et al. 2006; Ocete et al. 2011b). To our knowledge, the effects of soil lime on the wild subspecies of *Vitis vinifera* have not been explored to date.

The main objective of this research was to analyze the physiological response of *Vitis vinifera* ssp. *sylvestris* to soil lime. Specifically: (1) to analyze the growth response of plants in a range of soil $CaCO_3$ contents, from 0 to 60%; (2) to examine the effects of $CaCO_3$ on plant tissue concentrations of Fe, N, P and K; and (3) to ascertain the extent to which $CaCO_3$ levels determine plant performance, in terms of influence on the photosynthetic apparatus (PSII photochemistry), gas exchange characteristics and photosynthetic pigments.

## 2. Materials and Methods

### *2.1. Plant material and calcium carbonate treatments*

Plant material was collected from the wild grapevine population "14/Rute/1", which is located on the banks of the Anzur river in Córdoba province, in the Subbetic mountain range of southern Spain (38º 02' 40''N – 05º 07' 17'' W). The soil at the site is a hypercalcic calcisol (FAO 1999), composed of 41% sand, 34% silt and 25% clay, of pH 7.6-8.1 and 62-67% calcium carbonate (Ocete et al. 2007).

Axillary buds were taken from individuals of *Vitis vinifera* ssp. *sylvestris* belonging to the population described above and washed with water and household detergent before gently rinsing with distilled water. The buds were then sterilized by immersion in absolute ethanol (1 m) immediately followed by immersion in a 20% solution of sodium hypochlorite (5% active chloride), with a few drops of Tween-20, for 20 m and finally rinsed three times with sterilized water (5 minutes per rinse). The buds were then placed individually into sterile test tubes (21 x 150 mm) with 8 ml of the nutritive medium described by Troncoso et al. (1990), modified to include 0.32 μM of BAP and 0.13 μM of NAA as growth regulators. The tubes were sealed with polypropylene caps and parafilm and placed in a culture chamber at 24 ºC, with a light intensity of 30 $\mu Em^{-2}s^{-1}$ and a photoperiod of 16 hours of light. Buds from the resulting plantlets were subcultured for 45 days in the same medium to obtain an extremely homogeneous group of plants. The resulting plants were adapted according to Cantos et al. (1993), transferred to individual plastic pots (diameter 11 cm) filled with perlite and placed in a glasshouse under minimum-maximum temperatures of 21-25ºC, at 40-60% relative humidity and natural daylight (minimum and maximum light flux: 200 and

1000 μmol $m^{-2}$ $s^{-1}$, respectively). Pots were carefully irrigated with 20% Hoagland's solution (Hoagland and Arnon 1938), as required.

When the plantlets were around 20 cm in height, they were transferred to four different calcium carbonate soil treatments: 0, 20, 40 and 60% $CaCO_3$ (fifteen replicate pots per treatment). The different soil treatments were prepared by mixing sterilized fine siliceous sand (Quality Chemicals, Ref. 7631-86-9) with finely divided $CaCO_3$ (particle size < 5 μm in diameter) (Panreac Ref. 141212.0416) in the appropriate proportion. The fine, clay-sized fraction of $CaCO_3$, or active lime (Drouineau 1942), is able to generate and maintain high levels of $HCO_3$ in the soil solution (Inskeep and Bloom 1986) and is therefore a reliable indicator to predict the development of lime-induced chlorosis (Tagliavini and Rombolà 2001).

*2.2. Growth*

From each treatment, three complete plants (roots and shoots) were harvested at the beginning, and the remaining twelve at the end of the experiment (i.e. following 30 days of treatment). These plants were dried at 80ºC for 48 h and then weighed.

Relative growth rate (RGR) of whole plants was calculated using the formula:

$$RGR = (\ln Bf - \ln Bi) \cdot D^{-1} \quad (g\ g^{-1} day^{-1})$$

where Bf = final dry mass, Bi = initial dry mass (average of the three plants from each treatment dried at the beginning of the experiment) and D = duration of experiment (days).

Leaf area was determined from the projected area by scanning and digitalising the leaves (Epson V30, Seiko Epson Corp., Nagano, Japan), and using appropriate software (MideBMP v. 4.2.; Ordiales-Plaza 2000) for processing and analysis.

*2.3. Mineral analysis*

At the end of the experimental period, leaf and root samples were carefully washed with distilled water and then dried at 80ºC for 48 h and ground. Samples of 0.5 g each were then digested by wet oxidation with concentrated $HNO_3$, under pressure in a microwave oven to obtain the extract. Concentrations of Fe, K, and P in the extracts were determined by optical spectroscopy inductively coupled plasma (ICP-OES) (ARL-Fison 3410, USA). Total N concentration was determined by Kjeldahl digestion using an elemental analyzer (Leco CHNS-932, Spain).

*2.4. Gas exchange*

Gas exchange measurements were taken from randomly selected, fully expanded leaves (n = 20, one measurement per plant plus eight extra measurements taken randomly), following 30 days of treatment, using an infrared gas analyzer in an open system (LI-6400, LI-COR Inc., Neb., USA). Net photosynthetic rate (A), intercellular $CO_2$ concentration ($C_i$) and stomatal conductance to $CO_2$ ($G_s$) were determined at an ambient $CO_2$ concentration of 400 µmol $mol^{-1}$ at 20 - 25ºC, 50 ± 5% relative humidity and a photon flux density of 1600 µmol $m^{-2}$ $s^{-1}$. Values of the parameters A, $C_i$ and $G_s$ were calculated using the standard formulae of Von Caemmerer and Farquhar (1981).

199 *2.5. Chlorophyll fluorescence*

200

201 Chlorophyll fluorescence was measured in randomly selected, fully developed
202 leaves (n = 20) using a portable modulated fluorimeter (FMS-2, Hansatech Instruments
203 Ltd., England), following 30 days of treatment. Light- and dark-adapted fluorescence
204 parameters were measured at dawn (stable, 50 μmol $m^{-2}$ $s^{-1}$ ambient light) and midday
205 (1600 μmol $m^{-2}$ $s^{-1}$) in order to investigate the effect of soil $CaCO_3$ content on the
206 sensitivity of plants to photoinhibition.

207 Plants were dark-adapted for 30 minutes, using purpose-designed leaf–clips.
208 The minimal fluorescence level in the dark-adapted state ($F_0$) was measured using a
209 modulated pulse (<0.05 μmol $m^{-2}$ $s^{-1}$ for 1.8 μs) which was too small to induce
210 significant physiological changes in the plant. The data recorded represented an average
211 taken over a 1.6 second period. Maximal fluorescence in this state ($F_m$) was measured
212 after applying a saturating actinic light pulse of 15,000 μmol $m^{-2}$ $s^{-1}$ for 0.7s. The value
213 of $F_m$ was recorded as the highest average of two consecutive points. Values of variable
214 fluorescence ($F_v = F_m - F_0$) and maximum quantum efficiency of PSII photochemistry
215 ($F_v/F_m$) were calculated from $F_0$ and $F_m$. This ratio of variable to maximal fluorescence
216 is related to the potential photochemical efficiency of PSII, and dark-adapted values of
217 $F_v/F_m$ can be used to quantify photoinhibition (Krivosheeva et al. 1996).

218 The same leaf section of each plant was used to measure light-adapted
219 parameters. Steady state fluorescence yield ($F_s$) was recorded following adaptation of
220 the plants to ambient light conditions for 30 minutes. A saturating actinic light pulse of
221 15,000 μmol $m^{-2}$ $s^{-1}$ for 0.7 s was then used to produce the maximum fluorescence yield
222 ($F_m'$) by temporarily inhibiting PSII photochemistry.

223 Using fluorescence parameters determined in both light- and dark-adapted states,

the following were calculated: quantum efficiency of PSII ($\Phi_{PSII} = (F_m' - F_s)/ F_m'$), which measures the proportion of light absorbed by the chlorophyll associated with PSII that is used in photochemistry (Maxwell and Johnson, 2000); and non-photochemical quenching ($NPQ = (F_m - F_m') / F_m'$), which is linearly related to heat dissipation (Maxwell and Johnson, 2000).

*2.6. Photosynthetic pigments*

At the end of the experimental period, photosynthetic pigments were extracted from fully expanded leaves of plants grown under each treatment, using 0.05 g of fresh plant material in 10 ml of 80% aqueous acetone (n = 12). After filtering, 1 ml of the suspension was diluted with a further 2 ml of acetone, and chlorophyll a (Chl *a*), chlorophyll b (Chl *b*) and carotenoid (C*x*+*c*) contents were determined with a Hitachi U-2001 spectrophotometer (Hitachi Ltd, Japan), using three wavelengths (663.2, 646.8 and 470.0 nm). Pigment concentrations (μg $g^{-1}$ fwt) were calculated following the method of Lichtenthaler (1987).

*2.7. Statistical analysis*

Statistical analysis was carried out using Statistica v. 6.0 (Statsoft Inc.). Pearson coefficients were calculated to assess the correlation between different variables. Data were analyzed using one- and two-way analyses of variance (*F*-test). Data were tested for normality with the Kolmogorov-Smirnov test and for homogeneity of variance with the Brown-Forsythe test. Tukey tests were applied to significant test results for identification of important contrasts. Measured differences between fluorescence at dawn and midday were compared using the Student test (*t*-test).

## 3. Results

### *3.1. Growth*

All the plants survived up to the 40% $CaCO_3$ treatment, while fifty percent of the plants treated with 60% $CaCO_3$ did not survive until the end of the study period.

Relative growth rate (RGR) showed a significant reduction with increasing external $CaCO_3$ content ($r = -0.71$, $p < 0.0001$; Fig. 1A). Similarly, total leaf area was inversely correlated with external $CaCO_3$ ($r = -0.75$, $p < 0.0005$; Fig. 1B). RGR and total leaf area maintained similar values in plants exposed to $CaCO_3$ contents of 20 and 40% (ANOVA, Tukey test, $p > 0.05$). Relative to the control, the reduction in both the 20 and 40% $CaCO_3$ treatments was around 28% for RGR, and 39% for total leaf area. Plants grown in the 60% $CaCO_3$ treatment presented marked chlorosis from around the second week of the experiment.

### *3.2. Chemical analysis of plant samples*

Leaf Fe concentration showed little variation until the 40% $CaCO_3$ treatment, and then decreased, reaching its lowest value at 60% $CaCO_3$. There were no significant differences in root Fe up to 40% $CaCO_3$ (ANOVA, Tukey test, $p > 0.05$), but a sharp increase was recorded at 60% $CaCO_3$ (Fig. 2A). Leaf K showed similar values in the control and 20% $CaCO_3$ treatment (ANOVA, Tukey test, $p > 0.05$) but decreased under exposure to 40 and 60% external $CaCO_3$. Root K showed no clear trend in relation to $CaCO_3$ content (Fig. 2B).

Tissue N concentrations were similar in the roots and in the leaves (two-way ANOVA, $p > 0.05$). Leaf N slightly decreased on exposure to 20% $CaCO_3$, but showed no clear response to further increases in external $CaCO_3$ content; root N showed a

similar trend, with the decrease found at 20% $CaCO_3$ sharper than that recorded in leaves (Fig. 2C). In contrast, leaf P concentration did not show a clear relationship with increasing lime content, with a marked increase occurring at 20% $CaCO_3$, whereas root P showed a slightly increasing trend with increased soil $CaCO_3$ content (Fig. 2D).

*3.3. Gas exchange*

Net photosynthesis rate (A) decreased significantly with increasing external $CaCO_3$ level ($r = -0.87$, $p < 0.0001$; Fig. 3A), with the most drastic decline occurring from 40 to 60% soil $CaCO_3$. A was directly correlated with RGR ($r = 0.65$, $p < 0.0005$). Stomatal conductance (Gs) showed a similar trend to A (Fig. 3B). In contrast, intercellular $CO_2$ concentration (Ci) showed a slight increase up to 40% $CaCO_3$, and then increased markedly at the highest external $CaCO_3$ content (Fig. 3C).

*3.4. Chlorophyll fluorescence*

Maximum quantum efficiency of PSII ($F_v/F_m$), measured at both dawn and midday, decreased slightly with increasing soil lime content up to the 40% $CaCO_3$ treatment, with a sharp decline observed at the highest lime content, reaching significantly lower values than those of the control (ANOVA, Tukey test, $p < 0.005$, in both cases). Values of $F_v/F_m$ measured at dawn remained at around 0.80 up to the soil lime content of 40% but declined substantially on exposure to the highest $CaCO_3$ level, reaching values of around 0.50. These reductions were mainly the result of higher $F_0$ values (data not presented). Values of $F_v/F_m$ were always lower at midday than at dawn (*t*-test, $p < 0.05$; Fig. 4A).

Quantum efficiency of PSII (ΦPSII), measured at dawn, showed a similar pattern to that of $F_v/F_m$, reaching minimum values in the 60% $CaCO_3$ treatment; midday

values decreased significantly on exposure to 20% $CaCO_3$ (ANOVA, Tukey test, $p < 0.005$) but showed no response to further increases in external $CaCO_3$ content. ΦPSII values were significantly lower at midday than at dawn (*t*-test, $p < 0.0001$; Fig. 4B). Finally, non-photochemical quenching (NPQ) did not show a clear relationship with $CaCO_3$ content at dawn, whereas it increased significantly on exposure to 60% $CaCO_3$ at midday (Fig. 4C).

### *3.5. Photosynthetic pigments*

Pigment concentrations decreased significantly with the increase in $CaCO_3$ content (Chl *a*: $r = -0.81$, $p < 0.0001$; Chl *b*: $r = -0.77$, $p < 0.0001$; C*x*+*c*: $r = -0.81$, $p < 0.0001$); however, pigment concentrations in control plants and those exposed to 20% $CaCO_3$ showed no significant differences (ANOVA, Tukey test, $p > 0.05$, in all cases; Fig. 5).

## 4. Discussion

To rigorously evaluate the degree of lime tolerance/susceptibility of *Vitis vinifera* ssp. *sylvestris*, the physiological response of the experimental plants was compared primarily with the existing information about lime-tolerant rootstocks and species of the genus *Vitis* that are used worldwide by viticulturists on calcareous soils. *V. vinifera* ssp. *sylvestris* proved to be highly tolerant to high lime conditions, since all experimental plants survived exposure to levels of up to 40% $CaCO_3$. In our study, the reduction in relative growth rate of plants exposed to 20% $CaCO_3$ was around 28%. Bavaresco et al. (1995) reported that shoot biomass reduction of the lime-tolerant species *V. cinerea* and *V. berlandieri* was around 41% and 35%, respectively, under exposure to 19% active lime. Moreover, calcareous soil (19.3% active lime) reduced the total dry mass of *V. vinifera* L. cv. "Pinot blanc" grafted on the lime-tolerant "41B" rootstock to around 44% of the control value (Bavaresco et al. 2003). To our knowledge, there are no studies to date evaluating the effects of lime soil contents above 20% in the genus *Vitis*. In this regard, it must be emphasized that relative growth rate and total leaf area in our study maintained similar values in the 20% and 40% $CaCO_3$ treatments, thus reinforcing the argument regarding the tolerance of the study species.

Evaluation of iron-efficiency in grapes is of great importance in order to successfully select lime-tolerant plant material, since the bioavailability of iron for plant requirement is strongly impaired under calcareous soil conditions (Bert et al. 2013; Zancan et al. 2008). While our results reflect the fact that Fe uptake and translocation mechanisms were disrupted at 60% $CaCO_3$, leaf and root Fe concentrations were maintained up to the 40% $CaCO_3$ treatment, indicating that *V. vinifera* ssp. *sylvestris* is capable of maintaining Fe uptake by the roots and translocation to leaves, even under

350 extremely high lime conditions. Bavaresco et al. (2003) reported that *V. vinifera* L. cv.
351 "Pinot blanc" grafted on the lime-tolerant "41B" rootstock experienced a reduction in
352 leaf Fe concentration of around 23% when growing on a calcareous soil (19.3% active
353 lime). It is well known that lime-tolerant grapevine rootstocks feature certain specific
354 physiological mechanisms to overcome lime-induced iron chlorosis, including improved
355 root Fe uptake and reducing capacity (Nikolic et al. 2000).

356 Besides the previously described disturbance of Fe metabolism, lime-stress is
357 often associated with other nutritional disorders. For example, calcareous soil
358 conditions (17% active lime) affect the P and K plant tissue concentrations of
359 *V. vinifera* L. cv Aurora grafted on the medium lime-tolerant rootstock "S.O.4"
360 (Bavaresco and Poni 2003). Similarly, Bavaresco et al. (2003) reported a reduction in
361 leaf P and K in *V. vinifera* grafted on the lime-tolerant "41B" rootstock, when grown on
362 a calcareous soil, as well as an important decrease in the total N content of the plants (of
363 around 50% relative to the non-calcareous control). In our study, plants exposed to 20%
364 soil $CaCO_3$ presented no reduction in P and K concentrations, and leaf N content only
365 showed a slight reduction of around 12% relative to the control. Our results indicate that
366 calcareous soil conditions do not severely alter the nutritional status of the study
367 species.

368 The study of growth and mineral status may not be sufficient to evaluate the
369 degree of lime tolerance/susceptibility of a certain species. Evaluation of the
370 photosynthetic performance of *V. vinifera* ssp. *sylvestris* could therefore provide
371 valuable information by which to rank this species according to its tolerance to high
372 lime conditions. In our study, increasing external $CaCO_3$ contents reduced net
373 photosynthesis rate (A) and stomatal conductance (Gs). More specifically, A was
374 reduced by 25% and 40% relative to the control in plants exposed to 20% and 40%

375 $CaCO_3$, respectively. Bavaresco et al. (2006) reported an approximate 50% reduction in
376 A in three-year-old *V. vinifera* L. cv "Pinot Blanc" vines grafted on the lime-susceptible
377 rootstock "3309 C", under exposure to 16% lime, whereas the reduction in A found in
378 *V. vinifera* grafted on the medium lime-tolerant rootstock "S.O.4" growing in a
379 calcareous soil (17% active lime) was around 20%, relative to the control (Bavaresco
380 and Poni 2003). Our data reflect that there was no direct relationship between the effects
381 on A and Gs, since there was no reduction in Ci. Covarrubias and Rombolà (2013)
382 recorded an important decrease in Phosphoenolpyruvate carboxylase (PEPC) activity in
383 the Fe-chlorosis tolerant "140 Ruggeri" grapevine rootstock, caused by the presence of
384 bicarbonate in the nutrient solution. In this regard, the impairment of photosynthetic
385 function detected in our study could be partly related to a decrease in PEPC activity.
386 Additionally, the reported decline of Gs in the absence of a decrease in $C_i$ indicates that
387 the reduction in photosynthetic activity of *V. vinifera* ssp. *sylvestris* could be partially
388 due to the effects of high external lime conditions on the photosynthetic apparatus.
389 In our study, the maximum quantum efficiency of PSII ($F_v/F_m$) and the quantum
390 efficiency of PSII (ΦPSII) were affected by external $CaCO_3$ contents from 20% $CaCO_3$
391 upwards, suggesting that lime-stress enhances the photoinhibition induced by light
392 stress. The midday values of $F_v/F_m$ in plants exposed to 20% and 40% $CaCO_3$ were
393 considerably recovered at dawn; however, in plants exposed to 60% $CaCO_3$, dawn
394 $F_v/F_m$ values remained considerably lower than the control parameters for unstressed
395 plants (around 0.8; Björkman and Demmig 1987), indicating the occurrence of chronic
396 photoinhibition or photodamage (Werner et al. 2002). The increase in NPQ at the
397 highest external $CaCO_3$ treatment indicates that the plants dissipated light as heat,
398 thereby protecting the leaf from light-induced damage (Maxwell and Johnson, 2000).
399 Bavaresco et al. (2006) recorded a substantial increase in $F_0$ level and significantly

lower values of $F_v/F_m$ in *V. vinifera* plants grafted onto the rootstock "3309 C", when grown in a calcareous soil. Similarly, our data reflected that the decline in $F_v/F_m$ with increasing external $CaCO_3$ content was caused by higher values of $F_0$. This increase can be attributed to a reduction in the energy transfer from the PSII antennae to the reaction centres (Maxwell and Johnson 2000), possibly due to the recorded decrease in chlorophyll concentration with increasing external lime. The notable decrease in the concentration of chlorophyll recorded at 60% $CaCO_3$ may be partially related with the lower availability of certain nutrients involved in chlorophyll synthesis recorded in this treatment (i.e. Fe and N; see Lawlor, 2002).

Despite these effects of high lime content on plant performance, it must be emphasized that the photosynthesis rate and pigment concentration of plants grown at 20% $CaCO_3$ did not show a drastic reduction in comparison to the control treatment (these plants maintained A values around 6 μmol $m^{-2}$ $s^{-1}$ and similar chlorophyll content to that of the control plants), and that dawn $F_v/F_m$ values in these plants remained around the optimal values for unstressed plants. Moreover, it should be noted that, while our results revealed more negative effects on the photosynthetic function in plants exposed to 40% $CaCO_3$ than in those exposed to 20% $CaCO_3$ (i.e., lower photosynthetic rates and pigment concentrations), this did not lead to a more marked reduction in plant growth. Taken together, these results seem to indicate that plants exposed to 20% and 40% external $CaCO_3$ experienced similar overall effects on photosynthetic function over most of the experimental period.

## 5. Conclusions

This is the first study to analyze the physiological effects of soil $CaCO_3$ content above 20% on the genus *Vitis*. Our study revealed that the highest $CaCO_3$ level tested (60%) induced nutrient imbalances and significantly inhibited photosynthetic function, which caused an overall reduction in carbon gain and consequently, high mortality and a drastic reduction in the growth of the surviving plants. Interestingly, at 20% external CaCO3, the concentrations of Fe, N, P and K in plant tissues were virtually unaffected; moreover, plant growth and photosynthetic function were also not drastically affected in this treatment. Following comparison of these results with the existing literature on several lime tolerant grapevine varieties, and considering that the overall physiological response of plants grown at 40% external $CaCO_3$ did not differ considerably from that of plants exposed to 20% $CaCO_3$, we can affirm that the study species can be considered to be highly tolerant to lime stress.

Recent success in grapevine genetic research raises hope for an improved and efficient use of the genetic resources of wild species within breeding programs, offering new possibilities for introducing resistant characteristics from these species into the gene pool of high-quality grapevines in a very efficient way as well as in a manageable time frame (Eibach et al. 2010; Bert et al. 2013). Our study indicates that plants of *Vitis vinifera* ssp. *sylvestris* from the "14/Rute/1" population could provide a valuable source of genetic diversity for improving grapevine tolerance to calcareous soil conditions, a common challenge for viticulture at present.

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**Fig 1.** Relative growth rate (A) and total leaf area (B) of *Vitis vinifera* ssp. *sylvestris*, in response to treatment with a range of external $CaCO_3$ contents for 30 days. Values represent the mean ± SE, n = 12.

**Fig 2.** Total iron (A), potassium (B), nitrogen (C) and phosphorus (D) concentrations in the leaves (○) and roots (●) of *Vitis vinifera* ssp. *sylvestris*, in response to treatment with a range of external $CaCO_3$ contents for 30 days. Values represent the mean ± SE, n = 3.

**Fig 3.** Net photosynthetic rate, A (A), stomatal conductance, $G_s$ (B), and intercellular $CO_2$ concentration, $C_i$ (C) in randomly selected, fully developed leaves of *Vitis vinifera* ssp. *sylvestris*, in response to treatment with a range of external $CaCO_3$ contents for 30 days. Values represent the mean ± SE, n = 20.

**Fig 4.** Maximum quantum efficiency of PSII photochemistry, $F_v/F_m$ (A), quantum efficiency of PSII, ΦPSII (B), and non-photochemical quenching, NPQ (C), at midday (●) and at dawn (○) in randomly selected, fully developed leaves of *Vitis vinifera* ssp. *sylvestris*, in response to treatment with a range of external $CaCO_3$ contents for 30 days. Values represent the mean ± SE, n = 20.

**Fig 5.** Chlorophyll *a* (chl *a*) (A), Chlorophyll *b* (chl *b*) (B), and carotenoid (C*x*+*c*) (C) concentrations in randomly selected, fully developed leaves of *Vitis vinifera* ssp. *sylvestris*, in response to treatment with a range of external $CaCO_3$ contents for 30 days. Values represent the mean ± SE, n =12.