

Analysis of the virus-induced inhibition of photosynthesis in malmsey grapevines

B. Sampol, J. Bota, D. Riera, H. Medrano and J. Flexas

Laboratori de Fisiologia Vegetal, Departament de Biologia, Universitat de les Illes Balears. Carretera de Valldemossa, Km 7.5, ES-07122 Palma de Mallorca, Balears, Spain

Summary

Author for correspondence:
Jaume Flexas
Tel: +34 971 173446
Fax: +34 971 173184
Email: jaume.flexas@ps.uib.es

Received: 16 May 2003
Accepted: 3 July 2003

doi: 10.1046/j.1469-8137.2003.00882.x

- Virus infections decrease photosynthesis in plants, but the mechanistic basis is poorly understood. This was analysed in Banyalbufar malmsey, a grapevine (*Vitis vinifera*) variety of Mallorca (Spain).
- The aim of this study was to analyse the mechanisms by which virus infection affect photosynthesis. Gas exchange (limitation analysis), chlorophyll fluorescence and Rubisco activity were compared in potted virus-infected and virus-free potted plants, and in field-grown young lowly infected and older highly infected plants.
- Virus infection resulted in decreased photosynthesis (c. 50%). Stomatal limitation was unaffected in virus-infected plants, demonstrating that stomatal closure was not causing photosynthesis decreases. Chlorophyll fluorescence and limitation analysis suggested that the inhibition of primary light reactions was only a minor effect of virus infection. By contrast, mesophyll conductance to CO₂ and Rubisco activity substantially decreased in virus-infected plants, corresponding to increases in the limitations to photosynthesis imposed by mesophyll conductance and carboxylation.
- It is concluded that decreases in carboxylation and, possibly, in mesophyll conductance are the primary mechanisms by which virus infection impairs photosynthesis in Banyalbufar malmsey.

Key words: Banyalbufar malmsey (*Vitis vinifera*), biotic stress, grapevines, grapevine fan leaf virus (GFLV), grapevine leaf roll-associated virus (GLRaV), mesophyll limitations, photosynthetic metabolism, stomatal conductance.

© *New Phytologist* (2003) **160**: 403–412

Introduction

Biotic stresses, such as virus infections, cause serious economic losses to many crops world-wide (Boyer, 1982). A portion of these yield reductions induced by biotic stress may be due to reduced photosynthesis in infected plants. Several studies have demonstrated that photosynthesis is a primary target of different types of biotic stress, such as infestation of leaf-feeding mites (Lin *et al.*, 1999), inoculation with soil-borne pathogenic fungus (Nogués *et al.*, 2002) or virus infection (Balachandran *et al.*, 1997).

Virus infection is especially problematic in crops because, unlike other diseases, its impact cannot be reduced by phytosanitary treatments. In grapevines (*Vitis vinifera*), in particular, a number of viruses are known to affect grape yield and quality (Walter, 1988; Credi & Babini, 1997; González *et al.*, 1997;

Guidoni *et al.*, 1997; Cabaleiro *et al.*, 1999), with its important world-wide economic impact (Walter & Martelli, 1996). Several studies have shown that virus-infected grapevines present alterations of diverse metabolic processes, such as respiration, the activity of several enzymes, transport of assimilates, hormonal balance and photosynthesis (Pozsar *et al.*, 1969; Walter, 1988).

Despite its importance for crop yield, the mechanisms by which viruses induce the inhibition of photosynthesis in host plants are still unclear. Some studies have focused on the photosynthetic primary reactions, because virus coat protein seems to accumulate in the membranes of chloroplasts and thylakoids of the infected plants (Zaitlin, 1987; Reinero & Beachy, 1989; Rahoutei *et al.*, 2000). It has been suggested that they inhibit the electron transport of photosystem II (PSII), and this has been demonstrated in tobacco mosaic virus (TMV) (Hodgson

et al., 1989; Reiner & Beachy, 1989; Balachandran & Osmond, 1994; Balachandran *et al.*, 1994a; Rahoutei *et al.*, 1999, 2000) and cucumber mosaic virus (CMV) (Técsi *et al.*, 1994). A few studies have also shown that several enzymes involved in CO₂ assimilation, starch synthesis and photoassimilate transport, are inhibited by virus infection (Balachandran *et al.*, 1994b; Técsi *et al.*, 1994). However, as noted by Nogués *et al.* (2002), plant diseases can potentially impair the performance of the three main processes of photosynthesis: the primary light reactions (light absorbance, thylakoid electron transport and ATP synthesis), the stomatal and/or mesophyll control of CO₂ supply, and the activity of the carbon reduction cycle. Thus, examination of a single photosynthetic process might not allow a full identification of the mechanisms of disease-induced limitation.

In the present work, we focused on the effects of virus infection on photosynthetic metabolism in the 'Malvasia de Banyalbufar' (Banyalbufar malmsey), a grapevine variety of Mallorca (Balearic Islands, Spain). This variety had almost disappeared but the historically recognized high quality of its wines (Habsburg-Lorena, 1869) has brought new interest in its cultivation. However, all currently existing individuals are produced from a single survival stock, which is virus infected. Several independent laboratories have confirmed that the Banyalbufar malmsey stock is currently infected by at least three different viruses (grapevine leaf roll-associated virus, GLRaV; grapevine fan leaf virus, GFLV; and grapevine fleck virus, GFkV). These infections affect grape yield and quality in an age-dependent manner, so that the severity of the effects increases as vines become older (unpubl. obs.), forcing premature pull-out of the vineyard.

The aim of the present work was to analyse the effects of these virus on the photosynthetic metabolism of Banyalbufar malmsey, and to identify the mechanistic basis for virus-induced photosynthetic inhibition, by comparing photosynthetic parameters of virus-infected plants with virus-free plants, previously obtained with a shoot meristem culture method (Durán-Vila *et al.*, 1988).

Materials and Methods

Plant material and treatments

Two types of *V. vinifera* L. cv. Malvasia were studied: pot-grown plants (virus-free and infected) and field-grown plants of different ages (5 yr and 10 yr old), all of which were virus-infected.

Virus-free plants were obtained by *in vitro* culture of apex tissue collected from actively growing vines under field conditions. *In vitro* culture was performed according to Durán-Vila *et al.*, 1988. A total of 120 shoot sections, 0.5–1.0 cm long, obtained from proliferating shoots derived from the culture (3 months before cultivation), were used for propagation. They were transferred to a shoot tip culture medium without hormones. Two months later, 40 plants had survived (33% success). These rooted plantlets measuring 3–5 cm were transferred to

pots of 0.3 l volume, filled with vermiculite and irrigated every 3 d. The pots were enclosed in clear polyethylene bags to minimize moisture loss and kept under culture cabinet conditions (20°C and 75% relative humidity). The bags were opened and closed periodically to minimize stress and were completely removed when no wilting was observed. Afterwards, plantlets were transferred to new pots (2 l of volume), filled with vermiculite. Plantlets were irrigated every 3 d with 50% Hoagland's solution (Hoagland & Aron, 1950) and exposed to outdoors conditions. Plants having about 20 leaves were transferred to new pots (40 l volume) filled with commercial Prohumine turbe (Comercial Projar S.A., Valencia, Spain) and exposed to ambient conditions, irrigated with 50% Hoagland's solution every 3 d. Photosynthetic parameters were determined at this stage. The presence of the following viruses was tested using commercial enzyme-linked immunosorbent assay (ELISA) coating and conjugate antibodies preparations (Bioreba AG, Reinach, Switzerland): GLRaV-1, 2, 3, GFLV and GFkV. These viruses are described in the Universal Virus Database (www.ncbi.nlm.nih.gov/ICTVdb/ICTVdb/index.htm). GLRaV is a complex of about eight virions from at least three genera. The viruses assayed in this group are now identified as the Ampeloviruses GLRaV-1 (00.017.0.03.001), GLRaV-3 (00.017.0.03.003) and the Closterovirus GLRaV-2 (00.017.0.01.009). Grapevine Fan leaf virus is a Nepovirus (00.018.0.03.016) and Grapevine Fleck Virus is a Maculavirus (00.077.0.03.001). Two independent laboratories tested all forty surviving plants. As shown in Table 1, 15 of the *in vitro* obtained plants were still infected by two of the viruses, GLRaV and GFLV (Virus infected, VI), while the other 25 were completely virus-free (noninfected, NI). Therefore, the percentage of success of virus eradication was 62.5%.

Field-grown plants were studied at Can Pico and Dalt s'Era vineyards in Banyalbufar (Mallorca, Spain). At both sites plants were grafted on rootstock Paulsen 1103 and trained in bilateral cordon without irrigation. In Can Pico, 10-yr-old-plants were studied. These plants presented symptoms of severe virus infection (GLRaV, GFLV and GFkV). The presence of all these viruses was confirmed by ELISA test (Table 1). We define these plants as 'Highly infected' (HI). In Dalt s'Era, 5-yr-old plants were studied. An ELISA test demonstrated the presence of GLRaV, GFLV and GFkV in these plants, although visual symptoms were not apparent, which confirms the strong interaction between plant age and the severity of virus infection (Walter & Martelli, 1996). Parameters related to fruit quality also confirmed that the effects of the infection were stronger in Can Pico than in Dalt s'Era (B. Sampol, unpubl. data). Therefore, we refer to the latter plants as 'Less infected' (LI).

In August, nutrient solution containing 8% nitrogen (N) and 8% phosphorus (P) (Actigil; Rhône Poulenc S.A., Paris, France) was spread over leaves of highly affected plants in Can Pico to test early suggestions that high nutrient availability mitigates the effects of virus infection (Balachandran & Osmond, 1994) and to further test the mechanistic basis for

Table 1 Enzyme-linked immunosorbent assay (ELISA) results on the presence (+) or absence (–) of grapevine fan leaf virus (GFLV), grapevine fleck virus (GFkV) and grapevine leaf roll-associated virus (GLRaV) virus in potted *ex vitro* acclimatized noninfected (NI) and infected (VI) *Vitis vinifera* plants, and in field-grown young lowly infected (LI), old highly infected (HI) and HI plants sprayed with nutrient solution (HI + N)

| Treatment | GFLV | GFkV | GLRaV |
|-----------|------|------|-------|
| NI | – | – | – |
| VI | + | – | + |
| LI | + | + | + |
| HI | + | + | + |
| HI + N | + | + | + |

Of 40 *ex vitro* acclimatized plants, 25 were noninfected and 15 were infected.

photosynthesis depression in virus-infected plants. These plants are referred as 'Highly infected plus Nutrients' (HI + N).

Environmental conditions and leaf water status

Plants were grown outdoors under typical Mediterranean summer conditions with a rainfall below 30 l m⁻² from late May to August. All plants were irrigated at field capacity with a drip system twice a week in July and August. Mean temperature values from July to August were 24°C, with maximum values of c. 32°C and minimum of c. 16°C.

Leaf water status was determined in mature leaves as the midday leaf water potential, using an Scholander chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA).

Gas exchange measurements

The interaction of the effects of leaf age and virus infection on net CO₂ assimilation (A_N) and stomatal conductance (g_s) was analysed in *ex vitro*-acclimatized potted plants. The A_N and g_s values were determined by gas exchange in three different leaf stages (old basal, mature medium and young apical), three times during August, using a Li-6400 (Li-Cor Inc., Lincoln, NE, USA). Measuring conditions were saturating light (1500 μmol photons m⁻² s⁻¹), ambient temperature (between 30°C and 32°C) and CO₂ concentration (360 μmol mol⁻¹). Leaves from four different plants were measured for each treatment (VI and NI) and sampling time.

The response of A_N to substomatal CO₂ concentration (C_i) was only analysed in medium leaves, after observing that those leaves were the most affected by virus infection (see the Results section). The A_N–C_i curves were performed in leaves from three different plants according to Escalona *et al.* (1999), although in these experiments the external CO₂ range was increased to 1500 p.p.m. The C_i values were corrected for cuticular conductance according to Boyer *et al.* (1997).

In field-grown plants, light-saturated A_N and g_s were determined at mid-morning in six leaves from different plants for

each treatment (HI, LI and HI + N), using the same conditions as for pot-grown plants. A_N–C_i curves were determined in four of these six leaves.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence parameters were measured using a portable pulse amplitude modulation fluorometer (PAM-2000; Walz, Effeltrich, Germany) on attached leaves with natural saturating light around mid-morning (09 : 00 hours, solar time). Photon flux density (PPFD) incident on the leaves was always higher than 1000 μmol m⁻² s⁻¹, which is known to be above photosynthesis saturation in field-grown grapevines (Flexas *et al.*, 1998; Escalona *et al.*, 1999). A measuring light of about 0.5 μmol photon m⁻² s⁻¹ was set at a frequency of 600 Hz to determine the background fluorescence signal (F_o), at predawn (04 : 00 hours, solar time). The same light intensity was used to measure the steady state fluorescence signal (F_s) under sunlight conditions at mid-morning, although its frequency was increased to 20 kHz. To obtain predawn (F_m) and steady-state (F_m') maximum fluorescence yields saturation pulses of about 10 000 μmol photon m⁻² s⁻¹ and 0.8 s duration were applied. Maximum quantum efficiency of PSII (F_v/F_m) was calculated as described by Björkman & Demmig (1987):

$$F_v/F_m = (F_m/F_o)/F_m$$

PS II photochemical efficiency (ΔF/F_m'), was calculated as described by Genty *et al.* (1989).

$$\Delta F/F_m' = (F_m' - F_s)/F_m'$$

and used for the calculation of the linear electron transport rate (ETR) according to Krall & Edwards (1992):

$$ETR = \Delta F/F_m' \times \text{PPFD} \times 0.5 \times \text{ABS}$$

(PPFD is the photosynthetic photon flux density incident on the leaf; 0.5 is a factor which assumes equal distribution of energy between the two photosystems (the actual factor has been described to be between 0.4 and 0.6) (Laisk & Loreto, 1996; Albertsson, 2001). The leaf absorptance (ABS) was determined according to Schultz (1996), yielding values between 0.80 and 0.89 for all plants analysed. Therefore, an average value of 0.84 was used for calculations. Non-photochemical quenching of chlorophyll fluorescence (NPQ) at mid-morning was calculated according to Bilger & Björkman (1994) as:

$$\text{NPQ} = (F_m - F_m')/F_m'$$

Estimation of CO₂ concentration in the chloroplasts and mesophyll conductance

The CO₂ concentration in the chloroplasts (C_c) can be estimated from combined gas-exchange and chlorophyll fluorescence measurements (Di Marco *et al.*, 1990; Harley

et al., 1992; Loreto *et al.*, 1994; Epron *et al.*, 1995). We have used the method of Epron *et al.* (1995) for our estimations, as previously described (Flexas *et al.*, 2002). According to this method, the ETR measured by chlorophyll fluorescence, can be divided in two components:

$$\text{ETR} = \text{ETR}_A + \text{ETR}_P$$

(ETR_A is the fraction of ETR used for CO_2 assimilation; ETR_P is the fraction of ETR used for photorespiration). Both ETR_A and ETR_P can be solved from data of A_N , dark respiration (R_D) and ETR, and from the known stoichiometries of electron use in photosynthesis and photorespiration (see Epron *et al.*, 1995 for details). A R_D value of $-1 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ was used, since it was previously shown to be quite constant under varying environmental conditions in different grapevine varieties (Escalona *et al.*, 1999, and unpublished results).

Cc was calculated according to Laing *et al.* (1974) as:

$$S = (\text{ETR}_A/\text{ETR}_P)/(C_c/O)$$

(S is the specificity factor of Rubisco; O is the oxygen mole fraction at the oxygenation site, assumed to be equal to the mole fraction in the air). The S value was previously determined *in vitro* to be 100 mol mol^{-1} at 25°C in grapevines (Bota *et al.*, 2002). After estimation of Cc, the mesophyll conductance (g_{mes}) was approximated as $g_{\text{mes}} = (A_N + R_D)/(C_i - C_c)$ (Epron *et al.*, 1995), and used to construct A_N -Cc curves from data on A_N - C_i curves.

Limitation analysis

From A_N - C_i curves, the limitation to photosynthesis imposed by CO_2 diffusion through stomata (I_{g_s}) was calculated according to Farquhar & Sharkey (1982). Recently, Bernacchi *et al.* (2002) have established an analogous calculation of the limitation imposed by CO_2 diffusion through the leaf mesophyll ($I_{g_{\text{me}}}$). This was calculated by transforming A_N - C_i curves to A_N -Cc curves from the estimated values of mesophyll conductance. Limitations due to carboxylation capacity (I_{c_c}) and maximum photosynthetic capacity ($I_{A_{\text{me}}}$) were calculated according to Martin & Ruiz-Torres (1992), although in this case A_N -Cc curves were used instead of A_N - C_i curves, as the former may be more representative of the CO_2 conditions at the carboxylating site.

Briefly, the meaning of this limitation analysis is as follows. The I_{g_s} was calculated comparing the plant under study with a theoretical plant with identical carboxylation efficiency (i.e. the initial slope of the A_N - C_i curve) and A_{max} (i.e. light- and CO_2 -saturated photosynthesis) but with infinite g_s (i.e. zero stomatal limitation). The $I_{g_{\text{me}}}$ was calculated analogously, and compares the actual plant with one with infinite g_{mes} (i.e. zero mesophyll limitation). Similarly, I_{c_c} compares the plant studied with a theoretical plant having identical g_s and A_{max} but with infinite carboxylation efficiency. The $I_{A_{\text{me}}}$ compares

the studied plant with a plant having identical g_s and carboxylation efficiency but infinitely great A_{max} .

Biochemical analysis

For each treatment (both in pot-grown and field-grown plants) five leaf discs (5.3 cm^2) from different plants were freeze-clamped *in situ*. Samples were grounded to a fine powder in a mortar, previously chilled with liquid N_2 and homogenized in 1 ml ice-cold extraction medium (0.1 M Bicine, pH 8, 50 mM β -mercaptoethanol, 11 mM 6% polyethylene glycol (PEG) 4000, 11 mM Na-diethyl-dithio-carbamate (DIECA), 2 mM benzamidine, 1% (v : v) protease inhibitor cocktail (Sigma-Aldrich Co., St. Louis, MO, USA), 2.5% Tween 20). Extracts were centrifuged ($14\,500 \text{ g}$ at 4°C for 2 min) and the supernatant immediately assayed at 25°C for Rubisco activity. Initial and total activities were determined as previously described (Delgado *et al.*, 1993).

In pot-grown plants, the same discs were used to determine chlorophyll, carotenoids and total soluble protein. Determination of chlorophyll and carotenoid contents was performed according to Lichtenthaler & Wellburn (1983) and total soluble protein content was determined according to Bradford (1976).

Growth determinations

Basal shoot diameter, shoot length, lateral shoot length, total number of leaves, and length of four average leaves were measured on each plant once a week during August in four *ex vitro* acclimatized plants for each treatment (VI and NI). Shoot length was measured with a metal rule, and leaf length and basal shoot diameter were measured with a digital micrometer (Comecta S.A., Barcelona, Spain). Leaf elongation rate was calculated as the difference in leaf length between two consecutive samplings.

Statistical analyses

Statistical analyses of the data were performed with the SPSS 9.0 software package (SPSS, Chicago, IL, USA). Three-way ANOVA, including treatment, leaf stage and date of measurement as factors was performed to analyse the interactive effects of these factors on leaf photosynthesis. One-way ANOVA with treatment as the factor was also performed for all the studied parameters. Differences between means were revealed by Walter-Duncan test ($P < 0.05$).

Results

Photosynthetic differences between uninfected and virus-infected potted plants

In order to determine possible interactions between virus infection, time of the season and leaf age, A_N was measured three

Table 2 ANOVA analysis of the effects of date, stage, treatment and their interactions on light-saturated net CO₂ assimilation (A_N) in potted *ex vitro* acclimated noninfected (NI) and infected (VI) *Vitis vinifera* plants

| Parameters | df | F | P |
|----------------------|----|-------|----|
| Date | 2 | 2.62 | ns |
| Stage | 2 | 13.45 | * |
| Treatment | 1 | 16.67 | * |
| Date–stage | 4 | 0.65 | ns |
| Date–treatment | 2 | 0.12 | ns |
| Stage–treatment | 2 | 3.28 | * |
| Date–stage–treatment | 4 | 1.58 | ns |

*Statistically significant differences between treatments at $P < 0.05$; ns, nonsignificant differences.

times during August in three different leaf stages, in both VI and NI plants. The A_N value was affected by virus infection and both leaf age and position. However, time was not a significant factor on A_N (Table 2). The interaction of leaf stage and virus infection significantly affected A_N . On average, A_N of VI plants was reduced by 21% with respect to NI plants, but the medium mature leaves were the most affected by virus (45% average reduction, see Table 3). Medium mature leaves were used for further comparisons among VI and NI plants (Tables 4–6).

Virus infection had no effect on leaf water potential (Table 3) and Fv/Fm (not shown). However, virus infections had significant effects on pigment and protein contents, and all the other photosynthetic parameters analysed (A_N , g_s and ETR) were also significantly affected by infection (Table 3). Both A_N and g_s were reduced to a similar extent, so that the

intrinsic water-use efficiency (A_N/g_s) remained unaffected (not shown). The decrease of ETR was matched by an increase of NPQ (not shown). ETR : A_N and ETR : A_G^* ratios were similar for NI and VI plants. This supports the notion that most of the reducing equivalents generated in the electron transport rate are used for photosynthesis and photorespiration (Flexas *et al.*, 2002), which allows the method of Epron *et al.* (1995) to be used for the estimation of g_{mes} . The estimated g_{mes} was decreased by *c.* 50% in VI plants (Table 3).

Virus infection resulted in significant reductions of both the initial slope of A_N -Ci curves and the maximum photosynthesis at high CO₂ (Fig. 1). A limitation analysis was applied to A_N -Ci and A_N -Cc curves (Table 3). In NI plants, I_{c_e} was the highest limitation to photosynthesis and $I_{A_{max}}$ the lowest, as usually observed (Martin & Ruiz-Torres, 1992). All limitations except I_{g_s} increased significantly in VI plants. The greatest per cent increase in VI plants was that of $I_{A_{max}}$, but it was still the lowest of all limitations, the highest still being I_{c_e} (Table 3).

Age- and nutrient-dependent reductions of net photosynthesis in field-grown virus-infected plants

The comparison of HI and LI plants under field conditions yielded very similar results to those observed in potted plants, thus confirming the known interaction between plant age and virus infection (Walter & Martelli, 1996).

As in potted plants, leaf water potential and Fv/Fm were not affected by virus treatments. Leaf protein content was 65% lower in HI than in LI plants (Table 4). Interestingly, foliar treatment with Actigil resulted in complete recovery of protein content only 1 wk after the treatment was applied (HI + N, Table 4). The differences between LI and HI plants

Table 3 Leaf water potential (Ψ), chlorophyll (Chl), carotenoid and total soluble protein contents of leaves, light-saturated net CO₂ assimilation (A_N), stomatal conductance (g_s), electron transport rate (ETR), ETR to A_N ratio, ETR to gross photosynthesis (A_G^*) ratio, and mesophyll conductance (g_{mes})

| Parameter | NI | VI | % Change | P |
|---|--------------|--------------|----------|----|
| Ψ (MPa) | -0.4 ± 0.1 | -0.6 ± 0.1 | - | ns |
| [Chl] (mg m ⁻²) | 269.3 ± 16.5 | 159.6 ± 11.7 | -40.7 | * |
| [Carotenoids] (mg m ⁻²) | 441.5 ± 10.1 | 311.5 ± 21.3 | -29.4 | * |
| [soluble protein] (mg m ⁻²) | 15.1 ± 0.7 | 11.3 ± 0.6 | -25.2 | * |
| A_N ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) | 12.6 ± 0.7 | 6.9 ± 0.9 | -45.2 | * |
| g_s (mol H ₂ O m ⁻² s ⁻¹) | 0.18 ± 0.01 | 0.10 ± 0.01 | -43.5 | * |
| ETR ($\mu\text{mol e}^- \text{ m}^{-2} \text{ s}^{-1}$) | 123.1 ± 4.5 | 86.5 ± 8.0 | -29.8 | * |
| ETR : A ($\mu\text{mol e}^- \mu\text{mol CO}_2^{-1}$) | 10.4 ± 0.9 | 11.9 ± 2.6 | - | ns |
| ETR : A_G^* ($\mu\text{mol e}^- \mu\text{mol CO}_2^{-1}$) | 6.9 ± 0.7 | 7.3 ± 1.5 | - | ns |
| I_{g_s} | 0.36 ± 0.03 | 0.40 ± 0.02 | - | ns |
| $I_{g_{mes}}$ | 0.02 ± 0.02 | 0.29 ± 0.01 | +31.0 | * |
| I_{c_e} | 0.51 ± 0.02 | 0.62 ± 0.04 | +17.8 | * |
| $I_{A_{max}}$ | 0.03 ± 0.01 | 0.16 ± 0.01 | +81.2 | * |

The calculated limitations are: stomatal limitation (I_{g_s}), the limitation imposed by mesophyll conductance ($I_{g_{mes}}$), limitation due to carboxylation capacity (I_{c_e}), and limitation due to maximum photosynthetic capacity ($I_{A_{max}}$) in potted noninfected (NI) and virus-infected (VI) plants. Values are means ± SE, $n = 4-6$. % Change indicates the percentage of change (-, decrease; +, increase) of VI in respect to NI plants for each parameter. *Statistically significant differences between treatments at $P < 0.05$; ns, nonsignificant differences.

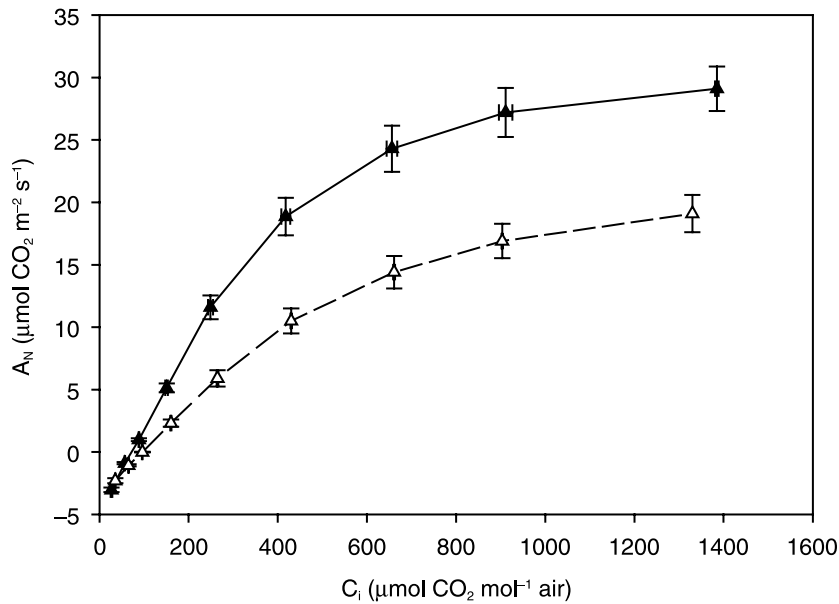


Fig. 1 The relationship between net CO₂ assimilation (A_N) and substomatal CO₂ concentration (C_i) in potted-grown Noninfected (NI, closed triangles) and virus-infected (VI, open triangles) *Vitis vinifera* plants. Values are means \pm SE, $n = 4$.

Table 4 Leaf water potential (Ψ), total soluble protein contents of leaves, light-saturated net CO₂ assimilation (A_N), stomatal conductance (g_s), electron transport rate (ETR), ETR to A_N ratio, ETR to gross photosynthesis (A_G^*) ratio, and mesophyll conductance (g_{mes}) in field-grown lowly infected (LI), highly infected (HI) and highly infected plus nutrients (HI + N) plants

| Parameter | LI | HI | % Change | HI + N | % Change |
|---|------------------|------------------|----------|-------------------|----------|
| [soluble protein] (mg m ⁻²) | 51.3 \pm 6.4a | 17.7 \pm 9.1b | -65.4 | 49.6 \pm 6.8a | ns |
| Ψ (MPa) | -1.3 \pm 0.2a | -1.4 \pm 0.1a | ns | -1.6 \pm 0.1a | ns |
| A_N (μ mol CO ₂ m ⁻² s ⁻¹) | 9.5 \pm 0.2a | 4.9 \pm 0.6b | -48.4 | 7.7 \pm 0.1c | -18.9 |
| g_s (mol H ₂ O m ⁻² s ⁻¹) | 0.14 \pm 0.01a | 0.06 \pm 0.01b | -57.1 | 0.11 \pm 0.01c | -21.4 |
| ETR (μ mol e ⁻ m ⁻² s ⁻¹) | 104.1 \pm 6.1a | 81.6 \pm 5.5b | -21.6 | 94.0 \pm 7.5a | -9.7 |
| ETR : A_N (mol e ⁻ mol ⁻¹ CO ₂) | 10.9 \pm 0.8a | 16.6 \pm 3.2a | ns | 12.1 \pm 1.2a | ns |
| ETR : A_G^* (mol e ⁻ mol ⁻¹ CO ₂) | 6.1 \pm 0.9a | 7.7 \pm 2.3a | ns | 6.5 \pm 0.8a | ns |
| I_{g_s} | 0.26 \pm 0.03a | 0.33 \pm 0.10b | +26.9 | 0.21 \pm 0.03a | ns |
| I_{g_m} | 0.30 \pm 0.03a | 0.53 \pm 0.05b | +43.4 | 0.41 \pm 0.04a | ns |
| I_{c_e} | 0.56 \pm 0.01a | 0.69 \pm 0.04b | +18.8 | 0.64 \pm 0.03ab | +12.5 |
| $I_{A_{max}}$ | 0.03 \pm 0.01a | 0.08 \pm 0.03b | +62.0 | 0.04 \pm 0.01ab | +25.0 |

The calculated limitations are: stomatal limitation (I_{g_s}), the limitation imposed by mesophyll conductance (I_{g_m}), limitation due to carboxylation capacity (I_{c_e}), and limitation due to maximum photosynthetic capacity ($I_{A_{max}}$) in potted noninfected (NI) and virus-infected (VI) plants. Values are means \pm SE, $n = 4-6$. % Change indicates the percentage of change (-, decrease; +, increase) of HI or HI + N with respect to LI plants for each parameter. Different letters indicate statistically significant differences between treatments at $P < 0.05$; ns, nonsignificant differences.

in A_N , g_s , ETR, ETR : A_N , ETR : A_G^* and g_{mes} were similar to those previously observed between NI and VI plants (Table 4). Foliar treatment with Actigil resulted in substantial, although incomplete, recovery of these parameters.

Both the initial slope of A_N - C_i curves and the maximum photosynthesis at high CO₂ were lower in HI than in LI plants (Fig. 2). The HI + N plants showed an intermediate pattern (Fig. 2). Limitation analysis revealed that in LI plants, I_{c_e} was again the most important limitation to photosynthesis (Table 4). All limitations, including I_{g_s} , were significantly greater in HI than in LI plants. The $I_{A_{max}}$ presented the highest per cent increase, but again I_{c_e} and $I_{g_{mes}}$ were the strongest limitations in HI plants (Table 4). Foliar treatment with Actigil

resulted in complete recovery of I_{g_s} and $I_{A_{max}}$, and in partial recovery of I_{c_e} and $I_{g_{mes}}$ (Table 4).

Discussion

The present results clearly show that virus infection resulted in lowered photosynthesis in Malmsey grapevines, as already observed in other grapevine varieties (Pozsar *et al.*, 1969; Abrasheva & Slavcheva, 1974; Guidoni *et al.*, 1997; Cabaleiro *et al.*, 1999) and other plant species (Hodgson *et al.*, 1989; Reiner & Beachy, 1989; Balachandran & Osmond, 1994; Balachandran *et al.*, 1994a,b, 1997; Técsi *et al.*, 1994; Rahoutei *et al.*, 2000). The results also confirm a strong effect on photosynthesis of the

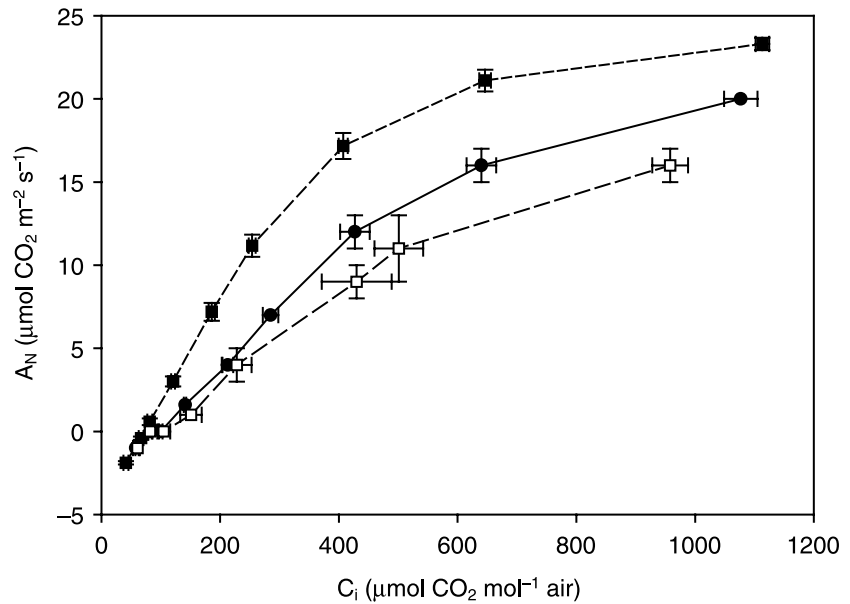


Fig. 2 The relationship between net CO₂ assimilation (A_N) and substomatal CO₂ concentration (C_i) in field-grown lowly infected (LI, closed squares), highly infected (HI, open squares) and highly infected plus nutrient (HI + N, closed circles) *Vitis vinifera* plants. Values are means \pm SE, $n = 4$.

Table 5 Initial and total Rubisco activity and activation state in noninfected (NI) and virus-infected (VI) potted plants

| Parameter | NI | VI | %Change | P |
|----------------------------|------------------|-----------------|---------|----|
| % Initial Rubisco activity | 100.0 \pm 33.9 | 73.3 \pm 21.0 | -26.7 | * |
| % Total Rubisco activity | 100.0 \pm 32.0 | 90.3 \pm 23.0 | -9.7 | * |
| % Activation state | 51.3 \pm 6.0 | 42.6 \pm 8.3 | -17.0 | ** |

% Change indicates the percentage of change (-, decrease; +, increase) of VI in respect to NI plants for each parameter.

*Statistically significant differences between treatments at $P < 0.05$; ns, nonsignificant differences.

interaction between virus infection and plant age (Walter & Martelli, 1996) and the interaction between virus infection and nutrient supply (Balachandran & Osmond, 1994; Balachandran *et al.*, 1994b).

By contrast to most previous studies, the combination of gas-exchange, chlorophyll fluorescence and photosynthesis limitation analysis used in the present study allows evaluation of the effects of virus infection on partial photosynthetic processes, such as primary light reactions, stomatal and/or mesophyll control of CO₂ supply, and the activity of the carbon reduction cycle.

Chlorophyll and carotenoid contents were strongly reduced in virus-infected plants, as previously described (Abrasheva & Slavcheva, 1974; Balachandran *et al.*, 1994b; González *et al.*, 1997; Rahoutei *et al.*, 2000). However, chlorophyll fluorescence analysis suggests that virus-induced damage to PSII was not the main factor limiting photosynthesis in infected plants. By contrast to previous suggestions by Balachandran *et al.* (1994a) it was found that Fv/Fm was unaffected by virus infection, suggesting that this did not induce photo-inhibitory damage. Moreover, the observed decreases of ETR in infected

plants were paralleled by identical increases of NPQ, which suggests enhanced photoprotection rather than photoinhibition (Rahoutei *et al.*, 2000). Balachandran *et al.* (1997) have demonstrated an increased de-epoxidation state of the xanthophyll cycle in virus-infected plants. The fact that $I_{A_{max}}$ was the lowest photosynthetic limitation in all the studied plants and that it completely recovered after foliar nutrient supply, while A_N did not, further suggests that primary light reactions were not the most limiting factor for photosynthesis in virus-infected plants. These results are in agreement with those of Técsi *et al.* (1994), who showed that, despite reduced Rubisco content, and possible reductions of net CO₂ assimilation and PSII electron transport, the photosynthetic capacity (i.e. the photosynthetic rate measured in saturating CO₂) was not affected by virus infection in *Cucurbita pepo*.

The diffusion of CO₂ through both the stomata and the mesophyll was lower in VI than in NI, and in HI than in LI plants, as indicated by reduced g_s and g_{mes} . However, the constancy of $A_N : g_s$ and I_{g_s} among treatments reveals that stomatal diffusion of CO₂ was not limiting photosynthesis in infected plants. This was also supported by the fact that foliar nutrient supply resulted in complete recovery of I_{g_s} , but not of A_N . By contrast, decreased CO₂ diffusion through the mesophyll could be an important factor limiting photosynthesis in virus-infected plants. At least in field-grown highly infected plants, $I_{g_{mes}}$ was very high, and of similar order of magnitude to I_{c_c} . Moreover, $I_{g_{mes}}$ was only partly recovered by foliar nutrient application, as it was A_N . This is, to our knowledge, the first time that decreased g_{mes} is suggested as a possible factor responsible for virus-induced decreases of photosynthesis. It has recently been shown that g_{mes} is more variable than previously thought, and that it could limit photosynthesis under drought (Flexas *et al.*, 2002), high temperature (Bernacchi *et al.*, 2002) and salinity stress (Centritto *et al.*, 2003).

Table 6 Initial and total Rubisco activity and activation state in field-grown lowly infected (LI), highly infected (HI) and highly infected plus nutrient (HI + N) plants

| Parameter | LI | HI | % Change | HI + N | % Change |
|----------------------------|--------------|--------------|----------|-------------|----------|
| % Initial Rubisco activity | 100.0 ± 5.8a | 67.4 ± 12.2b | -32.6 | 98.7 ± 6.6a | ns |
| % Total Rubisco activity | 100.0 ± 6.1a | 84.0 ± 13.6a | ns | 97.8 ± 3.7a | ns |
| % Activation state | 78.8 ± 3.0a | 60.0 ± 4.0b | -23.8 | 78.7 ± 4.0a | ns |

Values are means ± S.E, $n = 4-6$. % Change indicates the percentage of change (-, decrease; +, increase) of HI or HI + N in respect to LI plants for each parameter. Different letters indicate statistically significant differences between treatments at $P < 0.05$; ns, nonsignificant differences.

Table 7 Several parameters reflecting canopy development at harvest in potted noninfected (NI) and virus-infected (VI) plants

| Parameter | NI | VI | % Change | <i>P</i> |
|------------------------------------|--------------|------------|----------|----------|
| Shoot length (cm) | 132.5 ± 10.5 | 88.4 ± 7.9 | -33.3 | * |
| Leaves per plant | 51.3 ± 1.6 | 38.5 ± 4.4 | -25.0 | * |
| Length of the main leaf vein (mm) | 73.7 ± 7.4 | 49.9 ± 4.5 | -32.3 | * |
| Total Leaf area (mm ²) | 6754 ± 884 | 3203 ± 480 | -52.6 | * |

*Statistically significant differences between treatments at $P < 0.05$.

Nevertheless, the present analysis also suggests that decreased carboxylation capacity was the main factor limiting photosynthesis in virus-infected plants. I_c was by far the greatest limitation to photosynthesis in uninfected and all the different virus-infected plants. Moreover, it was only partly recovered by foliar nutrient application, as it was A_N . The I_c value is usually related to Rubisco activity (von Caemmerer, 2000). To confirm that this was a main limiting factor for photosynthesis in virus-infected plants, initial and total Rubisco activities were determined (Tables 5 and 6). Initial Rubisco activity of VI plants was only 73% of that of NI plants, although total Rubisco activity was still up to 90%. Similarly, initial Rubisco activity of HI plants was only 67% of that of LI plants, and total Rubisco activity was 84%. Therefore, it can be concluded that virus infection results in reduced carboxylation capacity through impairment of Rubisco activity. This is mainly due to the decreased activation state of the enzyme (Tables 5 and 6). Foliar nutrient application resulted in almost complete recovery of both initial and total Rubisco activity, although recovery of I_c was not as high. The reasons for this discrepancy remain to be analysed.

In conclusion, the present analysis suggests that the presence of GFLV and GLRaV viruses in Banyalbufar malmsey grapevines reduces their photosynthetic capacity through reductions of the carboxylation capacity and of the CO₂ diffusion capacity through the mesophyll, rather than by impairment of primary light reactions or CO₂ diffusion through the stomata. The photosynthetic reductions observed in VI plants (*c.* 50%) were reflected in similar (25–50%) reductions of several growth parameters (Table 7). Similar growth reductions have already been observed in plants subjected to virus infections (Balachandran *et al.*, 1997) and other biotic stresses (Nogués *et al.*, 2002). Furthermore, it can be concluded that

shoot tip meristem culture is an appropriate method to eliminate virus from infected plants, although it takes several years to obtain mature and acclimatized plants capable of growing and yielding under field conditions. Meanwhile, foliar application of nutrient solution substantially alleviates some of the effects of virus on photosynthesis and, thus, could be used to improve growth and yield in field-grown, virus-infected plants.

Acknowledgements

The authors gratefully acknowledge A. Canudas, J. M. Escalona, J. Gulías, S. Martínez, M. T. Moreno and J. Ramis for helping in field data collection. Dr J. Cifre is acknowledged for helpful advice in statistical analysis of the data. The authors are indebted to Dr M. Ribas-Carbó for helpful comments on the manuscript and grammar corrections. This paper is part of Project 'Recuperació de la Malvasia de Banyalbufar' (0262995). Grants to B. Sampol were from FPI02 Conselleria d'Innovació i Energia (Govern de les Illes Balears).

References

- Abrasheva P, Slavcheva T. 1974. Influence of fan leaf disease on the intensity of photosynthesis in grapevine. *Gradinar. Lozhar. Nauka (Sofia)* 11: 125–129.
- Albertsson PA. 2001. A quantitative model of the domain structure of the photosynthetic membrane. *Trends in Plant Science* 6: 349–354.
- Balachandran S, Osmond CB. 1994. Susceptibility of tobacco leaves of photoinhibition following infection with two strains of tobacco mosaic virus under two nitrogen nutrition regimes. *Plant Physiology* 104: 1051–1057.
- Balachandran S, Osmond CB, Makino A. 1994a. Effects of two strains of tobacco mosaic virus on photosynthetic characteristics and nitrogen partitioning in leaves of *Nicotiana tabacum* cv xanthi during photoacclimation under two nitrogen nutrition regimes. *Plant Physiology* 104: 1043–1050.

- Balachandran S, Osmond CB, Daley PF. 1994b. Diagnosis of the earliest strain-specific interactions between tobacco mosaic virus and chloroplasts of tobacco leaves *in vivo* by means of chlorophyll fluorescence imaging. *Plant Physiology* 104: 1059–1065.
- Balachandran S, Hurrey VM, Kelley SE, Osmond CB, Robinson SA, Rohozinski J, Seaton GGR. 1997. Concepts of plant biotic stress. Some insights into stress physiology of virus-infected plants, from the perspective of photosynthesis. *Physiologia Plantarum* 100: 203–213.
- Bernacchi CJ, Portis AR, Nakano H, von Caemmerer S, Long SP. 2002. Temperature response of mesophyll conductance. Implications for the determination of Rubisco enzyme kinetics and for limitations to photosynthesis *in vivo*. *Plant Physiology* 130: 1992–1998.
- Bilger W, Björkman O. 1994. Relationships among violaxanthin deepoxidation, thylakoid membrane conformation, and nonphotochemical chlorophyll fluorescence quenching in leaves of cotton (*Gossypium hirsutum* L.). *Planta* 193: 238–246.
- Björkman O, Demmig B. 1987. Photon yield of O₂ evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. *Planta* 170: 489–504.
- Bota J, Flexas J, Keys AJ, Loveland J, Parry MAJ, Medrano H. 2002. CO₂/O₂ specificity factor of ribulose-1,5-bisphosphate carboxylase/oxygenase in grapevines (*Vitis vinifera* L.): first *in vitro* determination and comparison to *in vivo* estimations. *Vitis* 41: 163–168.
- Boyer JS. 1982. Plant productivity and environment. *Science* 218: 443–448.
- Boyer JS, Wong SC, Farquar GD. 1997. CO₂ and water vapour exchange across leaf cuticle (epidermis) at various water potentials. *Plant Physiology* 114: 612–615.
- Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. *Analytical Biochemistry* 72: 248–254.
- Cabaleiro C, Segura A, Garcia-Berrios JJ. 1999. Effects of GLRaV3 on the physiology and must of *Vitis vinifera* L. cv. Albariño following contamination in the field. *American Journal of Enology and Viticulture* 50: 40–44.
- von Caemmerer S. 2000. *Biochemical models of leaf photosynthesis*. Collingwood, Australia: CSIRO Publishing.
- Centritto M, Loreto F, Chartzoulakis K. 2003. The use of low [CO₂] to estimate diffusional and non-diffusional limitations of photosynthetic capacity of salt-stressed olive saplings. *Plant, Cell & Environment* 26: 585–594.
- Credi R, Babini AR. 1997. Effects of virus and virus-like infections on growth, yield, and fruit quality of Albana and Trebbiano Romagnolo grapevines. *American Journal of Enology and Viticulture* 48: 7–12.
- Delgado E, Parry MAJ, Lawlor DW, Keys AJ, Medrano H. 1993. Photosynthesis, ribulose-1,5-bisphosphate carboxylase and leaf characteristics of *Nicotiana tabacum* L. genotypes selected by survival at low CO₂ concentrations. *Journal of Experimental Botany* 44: 1–7.
- Di Marco G, Manes F, Tricoli D, Vitale E. 1990. Fluorescence parameters measured concurrently with net photosynthesis to investigate chloroplastic CO₂ concentration in leaves of *Quercus ilex* L. *Journal of Plant Physiology* 136: 538–543.
- Durán-Vila N, Juárez J, Arregui JM. 1988. Production of viroid-free grapevines by shoot tip culture. *American Journal of Enology and Viticulture* 3: 217–220.
- Epron D, Godard D, Cornic G, Genty B. 1995. Limitation of net CO₂ assimilation rate by internal resistances to CO₂ transfer in the leaves of two trees species. *Plant, Cell & Environment* 18: 43–51.
- Escalona JM, Flexas J, Medrano H. 1999. Stomatal and non-stomatal limitations of photosynthesis under water stress in field-grown grapevines. *Australian Journal of Plant Physiology* 26: 421–433.
- Farquhar GD, Sharkey TD. 1982. Stomatal conductance and photosynthesis. *Annual Review of Plant Physiology* 33: 317–345.
- Flexas J, Bota J, Escalona JM, Sampol B, Medrano H. 2002. Effects of drought on photosynthesis in grapevines under field conditions: an evaluation of stomatal and mesophyll limitations. *Functional Plant Biology* 29: 461–471.
- Flexas J, Escalona JM, Medrano H. 1998. Down-regulation of photosynthesis by drought under field condition in grapevine leaves. *Australian Journal of Plant Physiology* 25: 893–900.
- Genty B, Briantais JM, Baker NR. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta* 990: 87–92.
- González E, Mosquera MV, San José MC, Díaz T. 1997. Influence of virus on the chlorophyll, carotenoid and polyamide contents in grapevine microcuttings. *Journal of Phytopathology* 145: 185–187.
- Guidoni S, Mannini F, Ferrandino A, Argamante N, Di Stefano R. 1997. The effect of GLRaV and Rugose Wood sanitation on agronomic performance and berry and leaf phenolic content of Nebbiolo clone (*Vitis vinifera* L.). *American Journal of Enology and Viticulture* 48: 438–442.
- Habsburg-Lorena LS. 1869. *Die Balaearum in Wort und Bild*. Leipzig, Germany: 1869.
- Harley PC, Loreto F, Di Marco G, Sharkey TD. 1992. Theoretical considerations when estimating the mesophyll conductance to CO₂. *Plant Physiology* 98: 1429–1436.
- Hodgson RAJ, Beachy RN, Pakrasi HB. 1989. Selective inhibition of photosystem II in spinach by tobacco mosaic virus: an effect of the viral coat protein. *FEBS Letters* 245: 267–270.
- Hoagland DR, Arnon DI. 1950. The water-culture method for growing plants without soil. Cir. 347. California Agr. Exp. Sta.
- Krall JP, Edwards GE. 1992. Relationship between photosystem II activity and CO₂ fixation in leaves. *Physiologia Plantarum* 86: 180–187.
- Laing WA, Ögren WL, Hegeman RH. 1974. Regulation of soybean net photosynthetic CO₂ fixation by the interaction of CO₂, O₂ and ribulose 1,5 diphosphate carboxylase. *Plant Physiology* 54: 678–685.
- Laik A, Loreto F. 1996. Determining photosynthetic parameters from leaf CO₂ exchange and chlorophyll fluorescence: ribulose-1,5 bisphosphate carboxylase/oxygenase specificity factor, dark respiration in the light, excitation distribution between photosystems, alternative electron transport rate, and mesophyll diffusion resistance. *Plant Physiology* 110: 903–912.
- Lichtenthaler HK, Wellburn AR. 1983. Determinations of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. *Biochemical Society Transactions* 603: 591–592.
- Lin TB, Schwartz A, Saranga Y. 1999. Photosynthesis and productivity of cotton under silverleaf whitefly stress. *Crop Science* 39: 174–184.
- Loreto F, Di Marco G, Tricoli D, Sharkey TD. 1994. Estimation of photorespiratory carbon dioxide recycling during photosynthesis. *Australian Journal of Plant Physiology* 26: 733–736.
- Martin B, Ruiz-Torres NA. 1992. Effects of water-deficit stress on photosynthesis, its components and component limitations, and on water use efficiency in wheat (*Triticum aestivum* L.). *Plant Physiology* 100: 733–739.
- Nogués S, Cotxarrera L, Alegre L, Trillas MI. 2002. Limitations to photosynthesis in tomato leaves induced by Fusarium wilt. *New Phytologist* 154: 461–470.
- Pozsar BI, Horvath L, Lehoczy J, Sarospataki GY. 1969. Effect of the grape chrome-mosaic and grape fan leaf yellow mosaic virus infection on the photosynthetic carbon dioxide fixation in vine leaves. *Vitis* 8: 206–210.
- Rahoutei J, Barón M, García-Luque I, Droppa M, Neményi A, Horváth G. 1999. Effect of tobamovirus infection on thermoluminescence characteristics of chloroplasts from infected plants. *Zeitschrift für Naturforschung* 54: 634–639.
- Rahoutei J, García-Luque I, Barón M. 2000. Inhibition of photosynthesis by viral infection: effect on PSII structure and function. *Physiologia Plantarum* 110: 286–292.
- Reinero A, Beachy RN. 1989. Reduced photosystem II activity and accumulation of viral coat protein in chloroplasts of leaves infected with Tobacco mosaic virus. *Plant Physiology* 89: 111–116.

- Schultz HR. 1996. Leaf absorbance of visible radiation in *Vitis vinifera* L. estimates of age and shade effects with a simple field method. *Scientia Horticulturae* 66: 93–102.
- Técsi CI, Maule AJ, Smith AM, Leegood RC. 1994. Metabolic alterations in cotyledons of Cucurbita pepo infected by cucumber mosaic virus. *Journal of Experimental Botany* 45: 1541–1551.
- Walter B. 1988. Some examples of the physiological reaction of the vine in the presence of virus. *Bulletin OIV* 61: 383–390.
- Walter B, Martelli GP. 1996. Clonal selection of the vine: Sanitary and pomological selection. Influence of viruses and quality. *Bulletin OIV* 69: 945–971.
- Zaitlin M. 1987. Plant virus–host interactions. *Annual Review of Plant Physiology* 38: 291–315.