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Physiological response of field grown grapevine (*Vitis vinifera* L. cv. Marzemino) to grapevine leafroll-associated virus (GLRaV-1)

Massimo Bertamini[1], Umberto Malossini[1], Krishnasamy Muthuchelian[2] and Namachevayam Nedunchezhian[1,3]

[1]Istituto Agrario di San Michele all'Adige, 38010 San Michele all'Adige, Italy
[2]School of Energy Sciences, Madurai Kamaraj University, Madurai 625 021, India
[3]Government Higher Secondary School, Vellimedupettai, 604207, India

Summary. The physiological response of field grown grapevine (*Vitis vinifera* L. cv. Marzemino) plants to grapevine leafroll-associated virus (GLRaV-1) was studied. Changes in photosynthetic pigments and in photosynthetic activity were investigated. GLRaV-1 considerably decreased the leaf net photosynthetic rate (P_n), stomatal conductance (g_s) and the transpiration rate (E) in grapevine leaves, and also strongly reduced pigments, soluble proteins, ribulose-1,5-bisphosphate carboxylase (RuBPC) and nitrate reductase activity. In isolated thylakoids, the virus strongly inhibited whole-chain and photosystem (PS) II activity, while PSI activity was only marginally inhibited. The artificial exogenous electron donors diphenyl carbazide, manganese chloride ($MnCl_2$) and hydroxylamine (NH_2OH) did not restore lost PSII activity to virus-infected leaves. Chlorophyll fluorescence suggested that the inhibition of primary light reactions was a major effect of virus infection. Immunological studies showed that D1 protein levels of the PSII reaction centre were significantly lower in virus-infected leaves. It is concluded that the decreases in photosynthetic pigments and photosynthetic activities caused by the virus strongly impair photosynthesis in Marzemino grapevine plants.

Key words: acceptor side, electron transport, fluorescence, nitrate reductase.

Introduction

Virus diseases in grapevine are widespread. Besides having detrimental effects on the agronomic and enological performance of vines (Walter and Martelli, 1996; Guidoni *et al*., 1997), viruses cause morphological changes in the leaves and clusters, affecting phenotypical expression. On systemically infected leaves of susceptible plants virus infection frequently causes mosaic, chlorotic or mottling symptoms. All these pathological changes are closely related to reduced rates of photosynthesis and to negative effects on chloroplast number, ultrastructure and chlorophyll metabolism (Hunter and Peat, 1973; Almasi *et al*., 2000). The deterioration of chloroplast structure, pigment composition and electron transport can be attributed to damage caused mostly in photosystem (PS) II by infection stress (Reinero and Beachy, 1989; Koiwa *et al*., 1992; Balachandran *et al*., 1997).

Grapevine leafroll is a serious disease of grapevine worldwide, and several viruses are associated with it. To control this disease, certification schemes in which virus-free material is propagated and distributed are widely applied. In vines in-

Corresponding author: N. Nedunchezhian
Fax: + 91 4147 222512
E-mail: nedu1963@yahoo.com

fected with leafroll, the effects of the virus vary according to the particular virus or association of viruses involved. It is reported that in the early stage of vineyard life, grapevine leafroll-associated virus (GLRaV-1) is not detrimental to 'Dolcetto' clones whereas that same virus, especially when in association with GLRaV-3 and GVA, reduces vine vigour and berry skin phenolic content in 'Nebbiolo' clones (Mannini *et al*., 1994).

Little is known about the physiological effects of leafroll viruses on vines. In most studies, only vines with evident symptoms are examined. Photosynthesis is the process most sensitive to biotic and abiotic stresses (Balachandran *et al*., 1997). In a natural environment, grapevines are subject to attack from a wide variety of pathogens, and this makes it difficult to distinguish between the effects of single pathogens on photosynthetic processes. The aim of the present work was to examine physiological changes in field grown grapevine (*Vitis vinifera* L.) cv. Marzemino plants in response to infection with GLRaV-1.

Materials and methods

GLRaV-1-infected clones of Marzemino were heat-treated (HT) in a thermotherapy chamber with artificial lighting at approximately 34°C for 40 days, followed by *in vitro* culturing of 0.5 cm terminal shoot tip explants. Established daughter vines of mother plant (MP) and HT clones were tested with a commercial enzyme-linked immunosorbent assay (ELISA) for grapevine fan leaf virus (GFLV), grapevine fleck virus (GFkV), grapevine leafroll associated virus 1 and 3 (GLRaV-1, GLRaV-3), using a commercial kit (Agritest, Valenzano, Bari, Italy). ELISA tests were carried out on leaves and dormant cane samples of both MP and HT vines for 5 years (1998–2002). The presence of GLRaV-1 was confirmed by ELISA: some vines were infected with GLRaV-1 (virus infected, VI), while others were completely virus-free (noninfected, NI). Both VI and NI *ex-vitro* material was propagated on rootstocks (Kober 5BB). An experimental vineyard with two blocks was established in the year 2000 at Ala (South Trentino, Italy). The vines were trained to the simple pergola system.

Net photosynthesis (P_n), stomatal conductance (g_s) and the transpiration rate (E) were measured with an open gas exchange system; the cuvette in which leaves were enclosed had a 30 cm^2 glass window at the top (Central Unit CMS 400; H. Walz, Effeltrich, Germany). Data were recorded as soon as cuvette conditions reached a steady state at 1500 μmol photons m^{-2} s^{-1} (PPFD), 25°C air temperature and 60% relative humidity. The equations of Von Caemmerer and Farquhar (1981) were used to calculate the photosynthetic parameters.

Chlorophyll (Chl) concentration was estimated using the SPAD-502, Minolta (Osaka, Japan) system, which was calibrated against total Chl measured by extraction. Chl was extracted with 100% acetone from liquid N_2-frozen leaf discs and stored at -20°C. Chl, and carotenoids (Car) were analyzed spectrophotometrically following the method of Lichtenthaler (1987).

All measurements of chlorophyll fluorescence were performed with a portable pulse amplitude modulation fluorometer (PAM-2000; Walz). Before each measurement, the sample leaf was dark-adapted for 30 min with leaf-clips provided by Walz. The angle and distance from the leaf surface to the end of the optic fiber cable were kept constant during the experiments. To determine initial fluorescence (F_o) in NI and VI leaves, the weak measuring light was turned on and F_o was recorded. Then the leaf sample was exposed to a 0.1 s saturated flash of approximately 6000 μmol m^{-2} s^{-1} to obtain maximum fluorescence, F_m. The ratio of variable (F_v) to maximum fluorescence, F_v/F_m, was calculated automatically on the F_o and F_m measured [$F_v/F_m = (F_m-F_o)/Fm$]. All measurements of F_m were performed with the measuring beam set to a frequency of 600 Hz, whereas all measurements of F_m were performed with a saturating flash automatically switching to 20 kHz.

Thylakoid membranes were isolated from the leaves as described by Berthhold *et al*. (1981). Oxygen evolution [H_2O →2,6-dichloro-p-benzoquinone (DCBQ); H_2O →silicomolybdate (SiMo); PSII activity)] or uptake [$DCPIPH_2$ →methyl viologen (MV); PS1 activity)] was measured following the method of Nedunchezhian *et al*. (1997) with a Clark-type electrode (Hansatech, Kings Lynn, UK) fitted with a circulating water jacket at 27°C. Actinic light from a slide projector placed on the side of the electrode chamber was filtered through 9.5 cm of water. The light intensity was 1100 μE m^{-2} s^{-1} at the surface of the water bath cell. Thylakoid membranes were suspended at 10 μg Chl ml^{-1} in an as-

say medium containing 20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$, 5 mM NH_4Cl and 100 mM sucrose supplemented with 500 μM DCBQ and 200 μM SiMo for oxygen evolution (PSII), and 1 mM MV, 2 mM ascorbate, 5 μM DCMU, 1 mM sodium azide and 100 μM 2,6-dichlorophenol indophenol (DCPIP) for uptake (PSI). The rate of whole-chain electron transport ($H_2O \rightarrow MV$) in isolated thylakoids was measured as described by Armond *et al*. (1978). Thylakoid membranes were suspended at 10 μg Chl ml^{-1} in an assay medium containing 20 mM Tris-HCl (pH 7.5), 10 mM NaCl, 5 mM $MgCl_2$, 5 mM NH_4Cl and 100 mM sucrose supplemented with 1 mM MV and 1 mM sodium azide.

The rate of DCPIP photoreduction was determined by measuring the decrease in absorbance at 590 nm using a Hitachi 557 spectrophotometer. The reaction mixture contained 20 mM Tris-HCl, pH 7.5, 5 mm $MgCl_2$, 10 mM NaCl, 100 mM sucrose, 100 μM DCPIP and thylakoid membranes equivalent to 20 μg of Chl. Electron donation to the oxidizing side of PSII was measured in the presence of 5 mM $MnCl_2$, 0.5 mM DPC and 5 mM NH_2OH as electron donors.

Leaves were cut into small pieces and homogenized in a grinding medium of 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT) and 0.25 mM ethylene diamine tetraacetate (EDTA). The extract was clarified by centrifugation at 10,000$\times$ *g* for 10 min. The clear supernatant was then decanted slowly and used as the source for ribulose-1,5-bisphosphate carboxylase (RuBPC). All these steps were from the method of Nedunchezhian and Kulandaivelu (1991). The incubation mixture contained 50 mM DTT and 10 mM $NaH^{14}CO_2$ (9.25 kBq μmol^{-1}) in a total volume of 2.0 ml. The reaction mixture was placed in pyrex tubes and flushed with N_2 for 3 min, the tubes were sealed with serum caps and gently shaken in a water bath at 32°C for 3 min. Aliquots of 0.2 ml of the enzyme extract were then injected through the serum cap into the mixture to initiate the reaction. After 3 min at 32°C the reaction was stopped by injecting 0.2 ml 6 M glacial acetic acid. The known aliquots were transferred to Whatman No. 3 filter discs, dried under an infrared lamp, and the radioactivity was determined using a Packard model 2425 liquid scintillation counter.

Leaf tissue was suspended in a glass vial containing 5 ml of an assay medium consisting of 100 mM KH_2PO_4-KOH, pH 7.0, 100 mM KNO_3, 1% (v:v) n-propanol. The vial was sealed and incubated in the dark at room temperature (27°C) for 60 min. Suitable aliquots of the assay medium were removed for nitrate analysis. The amount of nitrate formed was expressed as $\mu mol\ NO_2^-$ formed g^{-1} protein h^{-1} (Jaworski, 1971).

Total soluble proteins were extracted by grinding leaf tissue in a mortar with 100 mM Tris-HCl, pH 7.8, containing 15 mM $MgCl_2$, 1 mM EDTA, 10 mM 2-mercaptoethanol, 10 mM PMSF in the presence of liquid nitrogen. Homogenates were filtered through nylon cloth. The extract was clarified by centrifugation at 11000$\times$ *g* for 10 min. The clear supernatant was decanted slowly and used as the soluble proteins. The Bio-rad protein assay (Bio-Rad Laboratories, Richmond, CA, USA) based on coomassie blue was used to determine total soluble protein (Bradford, 1976) using bovine serum albumin as the standard.

Thylakoid membrane proteins were separated using the polyacrylamide gel system of Laemmli (1970), with the following modifications. Gels consisted of a 12–18% gradient of polyacrylamide containing 4 M urea. Samples were solubilized at 20°C for 5 min in 2% (w:v) SDS, 60 mM DTT and 8% sucrose with a SDS-Chl ratio of 20:1. Electrophoresis was performed at 20°C with a constant current of 5 mA. Gels were stained in methanol/acetic acid/water (4:1:5, v:v:v) containing 0.1% (w:v) coomassie brilliant blue R and destained in methanol/acetic acid/water (4:1:5, v:v:v). Thylakoid membrane protein was estimated according to the method of Lowry *et al*. (1951).

The relative contents of certain thylakoid proteins per mg Chl was determined immunologically by western blotting. Thylakoids were solubilized in 5% SDS, 15% glycerine, 50 mM Tris-HCl (pH 6.8) and 2% mercaptoethanol at room temperature for 30 min. The polypeptides were separated by SDS-PAGE as above and proteins were then transferred to nitrocellulose by electroblotting at 0.4A for 3h. After saturation with 10% milk powder in Tris-buffered saline (TBS) buffer (pH 7.5), the first antibody in 1% gelatine was allowed to react overnight at room temperature. After being washed with TBS containing 0.05% Tween-20, the secondary antibody (anti-rabbit IgG [whole molecule] Biotin Conjugate, Sigma, St. Louis, MO, USA] was allowed to react in 1% gelatine for 2 h. For detec-

tion of D1 protein a polyclonal antiserum against spinach D1 protein (kindly provided by I. Ohad, Jerusalem, Israel) was used; the antibody against the 33 kDa protein of the water-splitting system was a gift from R. Barbato, Padova, Italy. Densitometry analysis of western blots was performed with a Bio-Image analyser (Millipore Corporation, MI, USA).

The experiment was performed thrice under the same environmental conditions. Statistical analysis (ANOVA) indicated that there were no differences in most measurements between the three repetitions of the experiment. The data were therefore pooled and five averages of these three repetitions. The experiments are arranged in a completely randomized design with five replications.

Results

Changes in the level of total Chl and Car in NI and VI leaves are shown in Table 1. When determined on the basis of unit fresh weight, total Chl and Car concentrations in VI leaves were reduced significantly, by 26 and 15% respectively. The Chl a/b ratio was markedly decreased in VI leaves. In contrast, the Car/Chl ratio in VI leaves increased (Table 1). The net photosynthetic rate (P_n), stomatal conductance (g_s) and the transpiration rate (E) were also significantly lower in VI leaves, with reductions of up to 54, 48 and 40% respectively (Table 1).

Photosynthesis in VI leaves was examined *in vivo* on chlorophyll fluorescence. Photochemical efficiency, measured as the F_v/F_m ratio, was 0.799 in NI leaves and 0.709 in VI leaves (Fig. 1). The level of F_v and the F_v/F_m ratio decreased significantly in VI leaves without change in the level of F_o (Fig. 1). A decrease in the F_v/F_m ratio of leaves usually reflects a change in the rate of electron transport through PSII and/or PSI. To confirm such a change, the electron transport in these thylakoid membranes was analysed *in vitro*.

Photosynthetic electron transport from $DCPIPH_2 \rightarrow MV$ (PSI) was reduced by 4% in VI leaves (Fig. 2). PSII-mediated electron transport activity was measured in VI leaves: photosynthetic electron transport from $H_2O \rightarrow DCBQ$ and from $H_2O \rightarrow SiMo$ was reduced by about 42 and 8% respectively (Fig. 2). A similar trend was seen with whole-chain electron transport ($H_2O \rightarrow MV$) activity (Fig. 2).

To locate the possible inhibition site in the PSII reaction, we examined the DCPIP reduction supported with various exogenous electron donors in the thylakoid membranes of NI and VI leaves. Wydrzynski and Govindjee (1975) showed that $MnCl_2$, DPC, NH_2OH and HQ donate electrons to the PSII reaction. Figure 3 shows the electron transport activity of PSII in the presence and absence of these compounds. PSII activity in VI leaves was reduced to about 40% when water served as the electron donor (Fig. 3). PSII activity was not

Table 1. Photosynthetic pigments, net photosynthetic rate (P_n), stomatal conductance (g_s), transpiration rate (E), soluble proteins, RuBPC and nitrate reductase activity in noninfected (NI) and virus-infected (VI) leaves. Values are means ±SE, (n=5). Percent reduction of VI compared to NI plants (mean ±SE; n=5) for each parameter is also reported.

Parameter	NI	VI	VI/NI reduction (%)
Chl a (mg g^{-1} f wt)	1.534±0.06	1.114±0.05	27
Chl b (mg g^{-1} f wt)	0.601±0.03	0.465±0.02	23
Total Chl (mg g^{-1} f wt)	2.135±0.14	1.579±0.07	26
Car (mg g^{-1} f wt)	0.806±0.04	0.685±0.02	15
Chl a/b ratio	2.55±0.12	2.39±0.10	
Car/Chl ratio	0.37±0.01	0.43±0.02	
Total soluble proteins (mg g^{-1} f wt)	35.16±1.6	25.66±1.2	27
RuBPC activity [μmol (CO_2) mg^{-1} (proteins) h^{-1}]	40.64±2.0	28.44±1.4	30
Nitrate reductase [μmol (NO_2) mg^{-1} (proteins) h^{-1}]	102.1±5.3	44.90±2.1	56
Stomatal conductance (g_s) [mmol m^{-2} s^{-1}]	56.20±2.8	29.22±1.3	48
Net photosynthetic rate (P_n) [μmol m^{-2} s^{-1}]	19.53±0.91	8.91±0.40	54
Transpiration rate (E) [mmol m^{-2} s^{-1}]	13.1±0.64	7.81±0.38	40

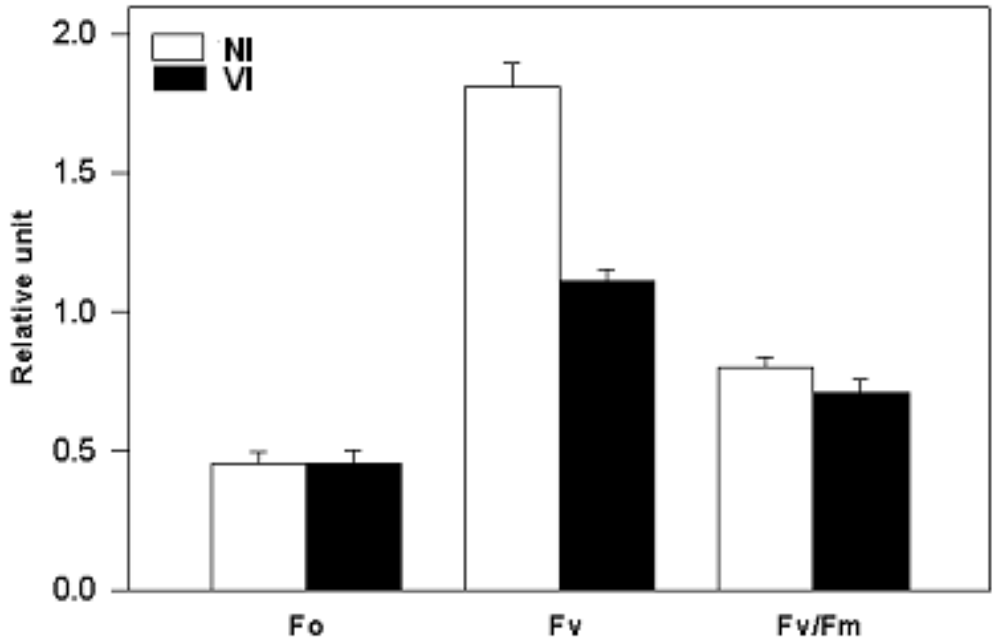

Fig. 1. Changes in the relative levels of fluorescence emitted as minimal fluorescence (F_o), variable fluorescence (F_v) and the ratio of variable to maximum fluorescence (F_v/F_m) in noninfected (NI) and virus-infected (VI) *Vitis vinifera* leaves (means ±SE; n=5).

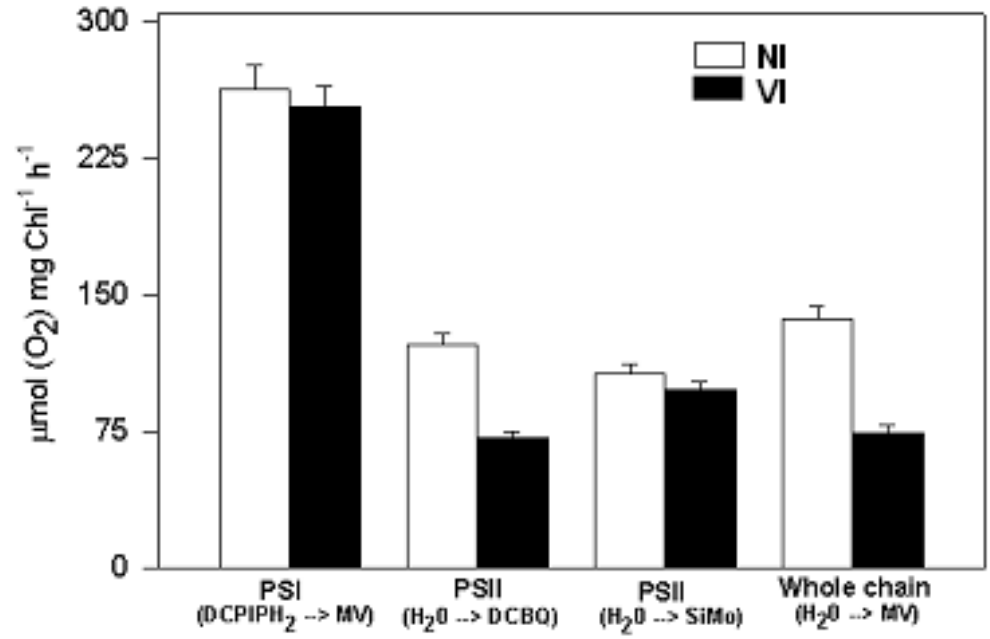

Fig. 2 Changes in the rates of whole chain (H_2O →MV), PSII (H_2O →DCBQ; H_2O →SiMo) and PSI ($DCPIPH_2$ →MV) electron transport activities in thylakoids isolated from noninfected (NI) and virus-infected (VI) *Vitis vinifera* leaves (means ±SE; n=5).

restored with DPC, NH_2OH and $MnCl_2$ as electron donors in VI leaves (Fig. 3).

Since changes in photosynthetic electron transport activity could be caused primarily by changes or a reorganization of the thylakoid components, the thylakoid polypeptide profiles of NI and VI leaves were analyzed by SDS-PAGE. A comparison of thylakoid polypeptides of VI leaves with those of NI plants indicated a marginal decrease in 33 kDa polypeptide (Fig. 4).

Virus-induced inhibition of PSII activity in thylakoids was compared with changes in the levels of D1 and 33 kDa proteins as determined by western blotting (Fig. 5) followed by quantification with Bio-Image apparatus (Fig. 5). While the decrease in the level of D1 protein in VI leaves was significant (47%), that of 33 kDa protein was negligible (4%).

The effect of grapevine leafroll virus on RuBPC and nitrate reductase activities in NI and VI leaves is shown in Table 1. When enzyme activity was expressed on a protein basis, marked reductions in RuBPC and nitrate reductase activities (30 and 56% respectively) occurred in VI leaves (Table 1). Polypeptide profiles of crude leaf extracts of RuBPC isolated from NI and VI leaves are shown in Fig. 4. The 55 large subunits (LSUs) and 15 kDa small subunits (SSUs) of RuBPC proteins marginally decreased in VI leaves. A similar trend was found with total soluble proteins (Table 1).

Discussion

In grapevines in particular, a number of viruses are known to affect grape yield and quality (Walter and Martelli, 1996; Credi and Babini, 1997; Cabaleiro *et al*., 1999), with considerable economic impact worldwide (Walter and Martelli, 1996). The results of the study clearly show that virus infection lowered photosynthesis in Marzemino grapevines, as already noted in other grapevine varieties (Guidoni *et al*., 1997; Cabaleiro *et al*., 1999), and in other plant species (Reinero and Beachy, 1989; Balachandran and Osmond, 1994; Rahoutei *et al*., 2000). Levels of Chl and Car were greatly reduced in VI plants, as already reported elsewhere (Balachandran and Osmond, 1994; Gonzalez *et al*., 1997; Rahoutei *et al*., 2000, Bertamini *et al.,* 2004). The decrease in both Chl a

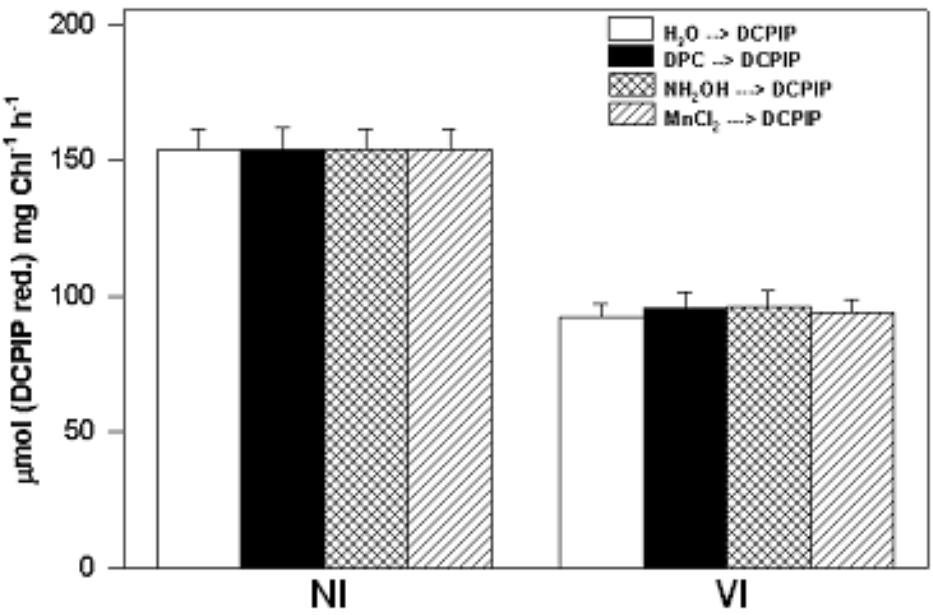

Fig. 3. Effect of various exogenous electron donors on PSII activity (H_2O →DCPIP) in thylakoid membranes isolated from noninfected (NI) and virus-infected (VI) *Vitis vinifera* leaves (means ±SE; n=5).

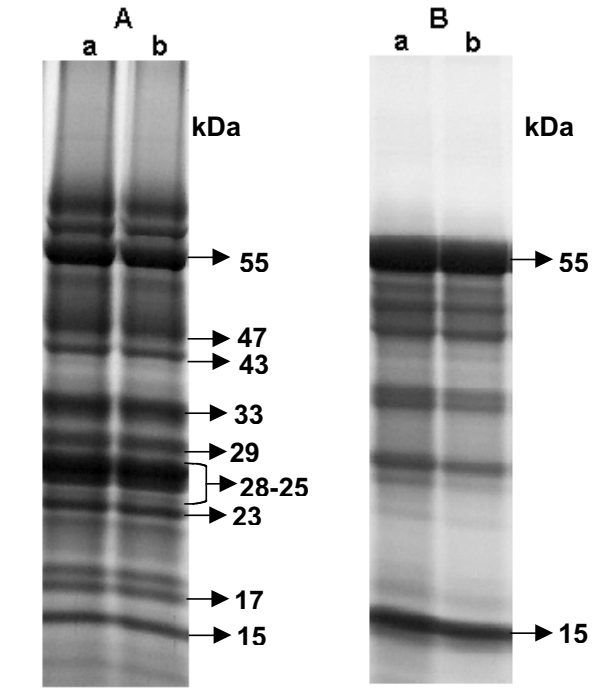

Fig. 4. Coomassie blue stained polypeptide profiles of thylakoid membranes (A) and crude leaf extracts (B) isolated from noninfected (NI) and virus-infected (VI) *Vitis vinifera* leaves. Gel lanes were loaded with equal amounts of protein (100 μg). a, NI; b, VI.

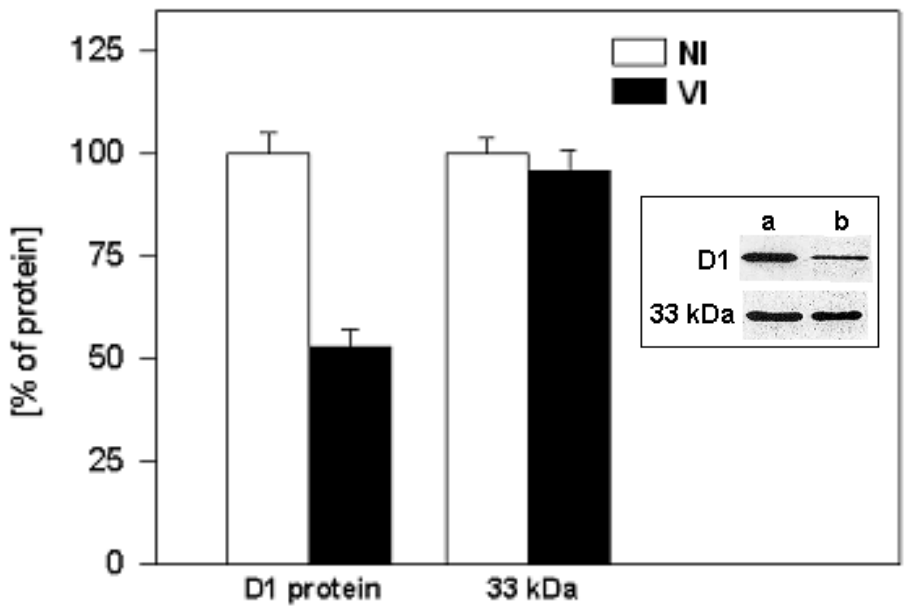

Fig. 5. Degradation of the D1 and 33 kDa proteins in thylakoids of noninfected (NI) and virus-infected (VI) *Vitis vinifera* leaves. Lane a, NI; lane b, VI. Each lane was loaded with equal amounts of Chl (5 μg). Histogram: Bio-Image densitometrical evaluation. Inset: Western-blot.

and Chl b levels of VI leaves was probably caused by the virus infection enhancing chlorophyllase activity in grapevine leaves. The Chl a/b ratio was higher in NI leaves. This was mainly due to an increase in Chl a (Bertamini *et al*., 2002a). Since Chl a is considered a more exact indicator of photosynthetic activity (Bertamini *et al*., 2002b, 2004), the higher Chl a/b ratio may partly explain the higher photosynthetic rates in NI leaves. The increase in the Chl/Car ratio and the decrease in the Chl a/b ratio of VI leaves could be due to a relatively faster decrease of Chl than Car. Similar results were reported by Bertamini *et al*., (2004) in grapevine leafroll virus infected grapevine leaves.

Lower levels of P_n due to the virus were closely related to lower g_s and E because in response to virus infection P_n, g_s and E levels decreased simultaneously. The results indicated that virus infection strongly inhibited the physiological process related to CO_2 fixation. Similar changes were reported by Guidoni *et al*. (1997) and Cabalerio *et al*. (1999) in VI plants.

The NI leaves showed good PSII activity, measured as the F_v/F_m ratio. The lower F_v/F_m ratio in VI leaves was mainly due to a decrease in variable fluorescence (F_v) without an accompanying increase in the F_o level. Similar results were reported by Reinero and Beachy (1989) in TMV-infected tobacco leaves. Analysis of the electron transport in thylakoids isolated from VI leaves showed that O_2 evolution was markedly inhibited when the electron acceptor DCBQ was used but not when the electron acceptor used was SiMo. This indicated that the acceptor side of PSII was more impaired than the donor side of PSII. Similar changes in grapevine leafroll virus and phytoplasma infection inactivated PSII in grapevine plants (Bertamini *et al*., 2002b, 2004).

The PSII-mediated reduction in DCPIP was measured in the presence of various artificial exogenous electron donors acting at the oxidizing side of PSII to locate the possible site where virus infection was inhibited. Among the various electron donors, $MnCl_2$, DPC and NH_2OH did not restore PSII activity in infected leaves. These results indicated that the changes caused by GLRaV-1 were on the acceptor side of PSII.

The most likely explanation for the inactivation of electron transport PSII activity is that the related protein(s) is (are) exposed at the thylakoid surface (Seilder, 1994). Inactivation was associated with a major decrease in the level of 33 kDa polypeptide. The extrinsic proteins of 33, 23 and 17 kDa associated with the lumenal surface of the thylakoid membranes are required for optimal functioning of the oxygen evolving machinery (Murata *et al*., 1984, Millner *et al*., 1987). The results indicate that the marginal loss of the 33 kDa polypeptide could be one of the reasons for the loss of O_2 evolution capacity in VI leaves.

The corresponding western blots revealed that there was a marked decrease in the D1 protein of VI leaves. This decrease was accompanied by a marginal decrease in the amount of 33 kDa protein of the water-splitting system, showing that the whole PSII rapidly degraded when virus infection was prolonged. A similar phenomenon was found by Bertamini *et al*. (2004) in grapevine leafroll virus infected grapevine leaves.

Levels of soluble proteins were strongly reduced in VI leaves. This may have been due to a decrease in the synthesis of RuBPC, the major soluble protein of the leaf. The loss of leaf soluble protein in VI leaves could be due in part to damaged chloroplasts, or it could be the result of protein synthesis inhibition.

The reduction in the overall photosynthetic rate correlated well with the considerable decrease in RuBPC activity that occurred in VI leaves. This reduction was due to protein synthesis inhibition induced by the virus. Such an explanation was supported by SDS-PAGE analysis of crude leaf extracts of RuBPC proteins, which showed a loss of both LSU and SSU polypeptides in VI leaves: the loss of LSU and SSU polypeptides is one of the reasons for the loss of RuBPC activity in VI leaves.

VI leaves had relatively low nitrate reductase activity. This may have reflected a balance between synthesis and activation on one hand and degradation or inactivation on the other. The change in intercellular pH levels due to virus infection may have decreased the transfer of nitrate (substrate) from the vacuolar pool to the active cytoplasmic pool accessible to the enzyme. The lower nitrate reductase activity may also have been due to the inhibition of protein synthesis, or to a lower rate of photosynthate supply in the VI leaves.

The results suggest that GLRaV-1 enhanced senescence or ageing in grapevine leaves due to lower levels of photosynthetic pigments and photosynthetic activity. In addition, GLRaV-1 inhibition occurred on the acceptor side of PSII .

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