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Photosynthetic functioning of individual grapevine leaves (*Vitis vinifera* L. cv. Pinot noir) during ontogeny in the field

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Summary

Field studies were conducted to investigate ontogenic changes in photosynthesis of a single grapevine leaf (*Vitis vinifera* L. cv. Pinot noir) subtending the fruit. A 40-day-old leaf was physiologically most active with regard to net photosynthetic (*Pn*) and electron transport rates. Variable to maximum fluorescence ratios of dark-adapted leaves (F_v/F_m = 0.77) were higher in mature leaves than in expanding (0.66) or senescent ones (0.65). Lower F_v/F_m values in these stages seemed to be caused not by photoinhibition but by a low photochemical capacity as suggested from the chlorophyll *a/b* ratios. In isolated thylakoids, lower rates of whole chain and PSII activity were observed in expanding and senescent leaves, while higher rates were observed in mature leaves. A similar trend was noticed for Rubisco and total soluble proteins. The artificial exogenous electron donors Mn^{2+} failed to restore the loss of PSII activity in senescent leaves, while DPC and NH_2OH significantly restored the loss of PSII activity. The marked loss of PSII activity in senescent leaves was primarily due to the loss of 33, 28-25, 23 and 17 kDa polypepides. A marked loss of Rubisco activity in senescent leaves is mainly due to the loss of 15 (SSU) and 55 (LSU) kDa polypeptides.

K e y w o r d s *:* chlorophyll fluorescence, donor side, electron transport, photosystem.

A b b r e v i a t i o n s : Car = carotenoids, Chl = chlorophyll, DCBQ = 2,6-dichloro-p-benzoquinone, DCPIP = 2,6-dichlorophenol indophenol, DPC = diphenyl carbazide, F_o = minimal fluorescence, F_m = maximum fluorescence, LSU = large subunit, MV = methyl viologen, PS = photosystem; Rubisco = ribulose-1,5-bisphosphate carboxylase, SDS-PAGE = sodium dodecylsulphate-polyacrylamide gel electrophoresis, SSU = small subunit.

Introduction

Ontogenetic changes in photosynthetic properties of grape leaves have been studied by Kriedemann *et al*. (1970) and Intrieri *et al*. (1992). Photosynthetic rates typically increase with leaf expansion and the maximum rate of photosynthesis is achieved prior to full expansion with rates often declining when the leaves become senescent (Constable and Rawson 1980, Roper and Kennedy 1986). Several reports indicate that the rate of *Pn* changes with individual leaf age (Davis and McCree 1978, Kennedy and Johnson 1981) as well as on a whole canopy basis during the growing season (Christy and Porter 1983, Wells 1988). Maximum photosynthetic activity under optimal conditions and ambient CO_2 concentration is typically reached at, or slightly before the time when leaves reach full expansion (Alleweldt *et al*. 1982). During further leaf development, photosynthetic capacity, stomatal conductance (Schultz *et al*. 1996), leaf dry mass per area, nitrogen (Poni *et al*. 1994), protein (Bettner *et al*. 1986) and photosynthetic enzymes including Rubisco (Hunter *et al*. 1994) decrease.

During ontogeny of photosynthetically active leaves, *i.e.* from their unfolding to senescence, the ultrastructure of chloroplasts in the mesophyll cells changes substantially (Hudak 1997, Kutik 1998). The main features of this development are increase of chloroplast size in maturing leaves and decline of their number during leaf senescence, accumulation of starch in the chloroplasts of just mature leaves, accumulation of plastoglobuli during leaf senescence, and quantitative changes of the thylakoid system and in the thylakoid stacking degree during whole leaf ontogeny.

During leaf development studies on several woody perennials showed that a high CO_2 assimilation rate was observed in mature leaves (full-leaf expansion), which then declined (Kennedy and Johnson 1981, Roper and Kennedy 1986). However, patterns of leaf photosynthesis as a function of leaf age vary among fruit species. In apple, mature well-exposed leaves showed little variation in assimilation for about 4 months (Kennedy and Fujii 1986). In sour cherry leaf photosynthesis increased 4 to 5-fold during the period of rapid lamina expansion, was stable for 4 weeks and then decreased gradually (Sams and Flore 1982). In grape leaf photosynthesis showed a peak approximately 35-40 d after unfolding and a decline thereafter (Kriedemann *et al*. 1970, Kriedemann 1977). In this paper, we report the concurrent changes of leaf pigments, electron transport activities, Chl fluorescence, total soluble proteins, Rubisco and nitrate reductase activities in grapevine leaves (cv. Pinot noir) during their ontogeny.

Material and Methods

P l a n t m a t e r i a l a n d e x p e r i m e n t a l d e s i g n : Leaves of *Vitis vinifera* L. cv. Pinot noir were collected from selected 10-year-old plants grafted to 3309 C and grown under field conditions with upright growing shoots (Cordon Royat) in the Istituto Agrario di San Michele all'Adige, Italy. The leaf age classes were: expanding leaf (stage 1;

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5-10 d), just fully expanded leaf (stage 2; 15-20 d), mature leaf (stage 3; 35-40 d), old mature leaf having very small black spots (stage 4; 65-70 d), and marked yellowish senescent leaf (stage 5; 100-120 d).

Pigment determination: Chl was extracted with 100 % acetone from liquid N_2-frozen leaves and stored at -20 °C. Chl and Car were analyzed spectrophotometrically according to LICHTENTHALER (1987).

Gas exchange: Gas exchange was measured using a portable gas analyzer system, model LCA-2 (Analytical Development Co., Hartford, UK). These measurements were taken on 15-20 leaves at >1000 µmol quanta m^{-2} s^{-1}, about 33 °C leaf temperature and at about 34 Pa ambient partial pressure of CO_2.

Modulated Chl fluorescence: Chl fluorescence was measured on leaf discs using a PAM 2000 fluorometer (H. Walz, Effeltrich, Germany). Before the measurements, the leaves were dark-adapted for 30 min. F_o was measured by switching on the modulated light (0.6 kHz); PPFD was < 0.1 µmol m^{-2} s^{-1} at the leaf surface. F_m was measured at 20 kHz with a 1 s pulse of 6,000 µmol m^{-2} s^{-1} of white light.

Electron transport: Thylakoid membranes were isolated from the leaves as described by BERTHHOLD *et al.* (1981). Whole chain electron transport ($H_2O \rightarrow$ MV) and partial reactions of photosynthetic electron transport mediated by PSII ($H_2O \rightarrow$ DCBQ) and PSI ($DCPIPH_2 \rightarrow$ MV) were measured as described by NEDUNCHEZHIAN *et al.* (1997). Thylakoids were suspended at 10 µg Chl ml^{-1} in the 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 500 µM DCBQ.

DCPIP photoreduction: The rate of DCPIP photoreduction was determined as the decrease in absorbance at 590 nm using a Hitachi 557 spectrophotometer. The reaction mixture (3 ml) 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. Where mentioned, the concentrations of $MnCl_2$, DPC and NH_2OH were 5, 0.5 and 5 mM, respectively.

Total soluble proteins: Total soluble proteins were extracted by grinding two leaves (0.3-0.5 g fresh weight) in a mortar with 6 ml of 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 11,000 *g* for 10 min. The clear supernatant was decanted slowly and used as the soluble proteins. The concentration of soluble proteins was determined by the method of BRADFORD (1976). Bovine serum albumin was used as the standard.

Extracts and assay of Rubisco activity: Fully expanded 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 DTT and 0.25 mM EDTA. The extract was clarified by centrifugation at 10,000 *g* for 10 min. The clear supernatant was decanted slowly and used for Rubisco analysis. The assay for Rubisco activity was carried out as described by NEDUNCHEZHIAN and KULANDAIVELU (1991).

Nitrate reductase activity: Leaves (100 mg) were suspended in a glass vial containing 5 ml of the 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 at 27 °C for 60 min. Suitable aliquots of the assay medium were removed for nitrate analysis. The amount of nitrate formed was expressed as µmol NO_2^- formed g^{-1} tissue h^{-1} (JAWORSKI 1971).

SDS-PAGE: Thylakoid membranes and crude leaf extracts 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 and 60 mM DTT and 8 % sucrose using a SDS-Chl ratio of 20:1. The final chlorophyll concentration of the membrane sample was adjusted to 0.5 mg Chl ml^{-1}. Before loading onto the gel, the membrane samples were heated at 100 °C for 3 min and the insoluble material was removed by centrifugation at 15,000 *g* for 5 min. Electrophoresis was performed at 20 °C with constant current (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).

Results and Discussion

The contents of Chl and Car per unit of leaf area, and the Chl *a*/*b* ratio increased with leaf development and then declined (Tab. 1). Similar changes were observed in cotyledons whose area and total Chl contents increased during the 15-40 d of their metabolic activity, the result being an increase and decline in the Chl amount per cotyledon (MILLERD *et al.* 1971, HONG and SCHOPER 1981). The low content of Chl *a* in expanding and senescent leaves was manifested by low Chl *a*/*b* ratios. Our observations are in agreement with earlier reports (FEDTKE 1973, DIEPENBROCK and GEISLER 1978). The reduction of Chl content in senescent leaves was probably related to an enhanced activity of chlorophyllase (REDDY and VORA 1986). At early developmental stages, the higher Chl concentration in mature leaves confirms the findings of other investigators (MARINI and MARINI 1983, HUNTER and VISSER 1989, PETRIE *et al.* 2000).

The Chl *a*/*b* ratio was markedly higher in mature leaves than in expanding and senescent leaves (Tab. 1). The decrease in Chl *a*/*b* ratio in senescent leaves is mainly due to a decrease in Chl *a* with leaf aging (HUNTER and VISSER 1989). This is in agreement with findings of KRIEDEMANN *et al.* (1970) for grapevine leaves of various ages. Since Chl *a* is considered to reflect a more exact characteristic of photosynthetic activity (ŠESTÁK 1966), the tendency towards a higher content might partially explain the higher photosynthetic rates found in mature leaves. Chl/Car ratios varied from >5 in young and adult leaves to <4 in senescent leaves (Tab. 1). The Car breakdown between maturity and senescence was 29 % compared to 54 % for Chl. The Chl/Car ratio decrease in senescent leaves reflected the relatively high retention of Cars. The changes of photosynthetic pigments during leaf development and senescence in grapevine was similar to

Table 1

Chlorophyll (Chl) [μmol m^{-2}] and carotenoid (Car) [mg m^{-2}] contents and their ratios, values of ground (F_o) and variable fluorescence (F_v), ratio of F_v and maximum fluorescence (F_v/F_m), net photosynthetic rate (*Pn*) [μmol m^{-2} s^{-1}], electron transport activities [whole chain ($H_2O \rightarrow$ MV), PSII ($H_2O \rightarrow$ DCBQ; $H_2O \rightarrow$ DCPIP), and PSI ($DCPIPH_2 \rightarrow$ MV) [μmol(O_2) mg^{-1}(Chl) h^{-1}], total soluble proteins [g kg^{-1} (fr.m.)], Rubisco [mmol(CO_2) mg^{-1}(protein) h^{-1}] and nitrate reductase [mmol(NO_2^{-1}) mg^{-1}(fr.m.) h^{-1}] as a function of leaf age. Pinot noir leaves were expanding (stage 1), fully expanded (2), mature (3), old mature (4), and senescent (5). Each value is the mean of 10 (pigments), 10-15 (fluorescence) or 5 (electron transport, Rubisco, nitrate reductase) measurements for each leaf stage

	Stages of leaf age				
	Expanding	Expanded	Mature	Old mature	Senescent
Chl *a*+*b*	225 ± 11	304 ± 15	420 ± 21	318 ± 15	196 ± 9
Chl *a*/*b*	2.4 ± 0.1	3.2 ± 0.2	4.6 ± 0.2	3.4 ± 0.1	2.7 ± 0.1
Car	42.3 ± 2.0	56.4 ± 2.4	73.6 ± 3.1	68.2 ± 2.9	52.1 ± 2.4
Chl/Car	5.3 ± 0.2	5.4 ± 0.2	5.7 ± 0.2	4.7 ± 0.2	3.8 ± 0.1
F_o	0.5 ± 0	0.7 ± 0	0.5 ± 0	0.5 ± 0	0.5 ± 0
F_v	1.1 ± 0.1	1.6 ± 0.1	1.8 ± 0.1	1.3 ± 0.1	1.0 ± 0
F_v/F_m	0.7 ± 0	0.7 ± 0	0.8 ± 0	0.7 ± 0	0.7 ± 0
Pn	2.4 ± 0.1	7.2 ± 0.3	11.8 ± 0.5	5.1 ± 0.2	2.7 ± 0.1
Whole chain [$H_2O \rightarrow$ MV]	104.8 ± 4.9	132.5 ± 6.3	164.2 ± 7.6	100.4 ± 5.1	48.5 ± 2.2
PSII [$H_2O \rightarrow$ DCBQ]	114.0 ± 5.6	122.2 ± 5.9	156.0 ± 7.2	104.8 ± 4.8	59.3 ± 2.9
PSII [$H_2O \rightarrow$ DCPIP]	129.8 ± 6.1	134.3 ± 5.4	172.8 ± 8.1	110.6 ± 5.4	54.3 ± 2.6
PSI [$DCPIPH_2 \rightarrow$ MV]	234.4 ± 12.1	288.2 ± 13.2	358.6 ± 16.2	315.5 ± 15.0	290.4 ± 13.8
Total soluble proteins	28.4 ± 1.3	32.7 ± 1.2	43.7 ± 1.9	31.6 ± 1.5	19.9 ± 0.9
Rubisco	24.2 ± 1.0	38.9 ± 1.4	47.7 ± 1.9	32.8 ± 1.6	20.3 ± 1.1
Nitrate reductase	31.8 ± 1.4	49.7 ± 2.2	71.2 ± 3.2	51.9 ± 2.0	29.5 ± 1.3

that found in other species (SESTAK 1985, SIFFEL *etal* . 1993). Ground fluorescence (F_o) reflecting the size of antenna Chl of PSII (KRAUSE and WEISS 1984) did not change consistently with leaf age (Tab. 1). By contrast variable fluorescence (F_v) and variable to maximum fluorescence ratios (F_v/F_m) of dark-adapted leaves reached peaks in mature leaves (stage 3) while lower values were obtained in growing and senescent leaves (Tab. 1). Hence, photons absorbed by the photosynthetic apparatus were used more efficiently by mature leaves than by young or senescent leaves. High F_v/F_m values obtained at stage 3 are typical for non-photoinhibited mature leaves (DEMMIG and BJORKMAN 1987). High F_v/F_m is a result of a high photochemical capacity of PSII reaction centers and is independent from Chl concentration. Lower F_v/F_m values in expanding and senescent leaves in comparison with mature ones are probably not due to photoinhibition but to a low photochemical capacity as suggested from the Chl *a*/*b* ratios. During leaf ontogeny, a rapid increase in the capacity of PSII photochemistry (increasing F_v/F_m) to leaf maturity and a decline with senescence has been reported (LICHTENTHALER 1987, SIFFEL *et al*. 1993).

However, studies with isolated thylakoids from different stages indicated that all photosynthetic electron transport activities increased with leaf development and then declined (Tab. 1). The PSII-mediated electron transport $H_2O \rightarrow$ DCBQ and $H_2O \rightarrow$ DCPIP increased from young to mature leaves and then declined (Tab. 1). A similar trend was noticed for whole chain electron transport ($H_2O \rightarrow$ MV) activity. The high PSII rate in mature leaves, found in our experiments, agrees with earlier reports (STRNADOVA and SESTAK 1974, SESTAK *et al*. 1978).

DCPIP collects electrons after PQ (LIEN and BANNISTER 1971, OUITRAKUL and IZAWA 1973) but benzoquinone at the reducing side of PQ (LIEN and BANNISTER 1971) in PSII. In the presence of the above PSII electron acceptors, the loss of PSII activity in senescent leaves was approximately the same. Thus, senescence-induced changes must be prior to PQ in the electron transport. Among the artificial electron donors tested DPC and NH_2OH donates electrons directly to the PSII reaction center (WYDRZYNSKI and GOVINDJEE 1975). In senescent leaves the PSII activity was reduced to about 69 % when water or $MnCl_2$ served as electron donor (Tab. 2). In contrast, a significant restoration of PSII-mediated DCPIP reduction was observed when NH_2OH and DPC were used as electron donors (Tab. 2). Thus the inhibition of PSII may be ascribed to an alteration of the water splitting system, since the addition of DPC and NH_2OH restored significantly

Table 2

Effect of exogenous electron donors on PSII activity ($H_2O \rightarrow$ DCPIP) in thylakoids (μmol(DCPIP red.) mg^{-1}(Chl) h^{-1}) isolated from mature and senescent leaves. Each value is the mean of 5 measurements for each leaf stage

Exogenous donors	Mature leaf	Senescent leaf
$H_2O \rightarrow$ DCPIP	172.8 ± 8.5	54.3 ± 2.6
DPC $\rightarrow$ DCPIP	180.2 ± 7.9	158.3 ± 7.4
$NH_2OH \rightarrow$ DCPIP	178.4 ± 8.1	156.1 ± 7.1
$MnCl_2 \rightarrow$ DCPIP	173.6 ± 8.5	62.4 ± 2.9

its activity. This is in good agreement with findings that the water-oxidizing system is sensitive to ageing (BISWAL and BISWAL 1988, NEDUNCHEZHIAN *et al.* 1995).

The inactivation of PSII electron transport activity in senescent leaves is supported by the fact that the related protein(s) is (are) exposed at the thylakoid surface (SEIDLER 1994). A comparison of thylakoids from senescent leaves with those of mature leaves showed specific losses of 33, 28-25, 23 and 17 kDa polypeptides (Figure). The three 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 *etal* . 1984, ENAMI *et al.* 1994). Our results indicate that the significant losses of 33, 23 and 17 kDa extrinsic polypeptides and 28-25 kDa LHCP2 polypeptides could be the reason for marked losses of O_2 evolution in senescent leaves. Similar observations were made with in dark-adapted *Vigna* seedlings during senescence (NEDUNCHEZHIAN *et al.* 1995).

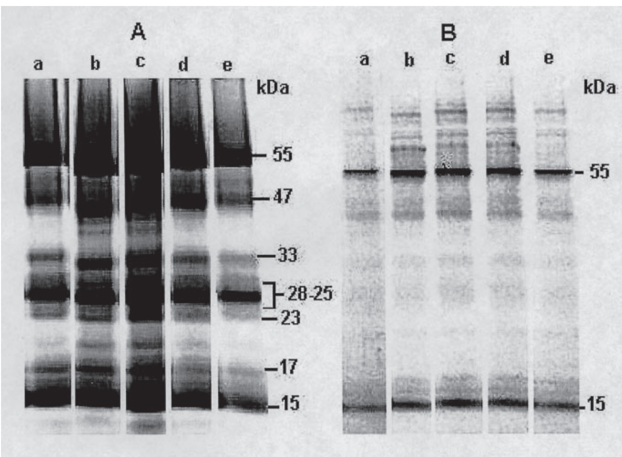

Figure: Coomassie Brilliant stained polypeptide profiles of thylakoid membranes (**A**) and crude leaf extracts (**B**) isolated from leaves at different phenological stages. Lane a, expanding (stage 1); lane b, fully expanded (2); lane c, mature (3); lane d, old mature (4) and lane e, senescent (5) leaves. Gel lanes were loaded with equal amount of protein (100 µg) for Rubisco and Chl (70 µg) for thylakoid membranes.

The amount of total soluble proteins gradually increased during leaf development and then declined. The soluble protein content was lower (55 %) in senescent leaves than mature leaves (Tab. 1). This relatively low level of soluble proteins in senescent leaves might have been due to a decrease of the synthesis of Rubisco, the major soluble protein in leaves. The reduction in the overall photosynthetic rates correlates well with the decrease of Rubisco activity in senescent leaves. If the Rubisco activity was expressed on a protein basis, a low activity in young leaves was followed by an increase to the maximum and a final decrease. Our observations agree with earlier reports (DALEY *et al.* 1978, ZIMA *et al.* 1981, HUNTER *et al.* 1994). A higher amount of Rubisco activity was observed in mature leaves, while a significant reduction was observed in senescent leaves. A reduction of 57 % was noticed when compared to mature leaves (Tab. 1). The loss of Rubisco activity is also supported by SDS-PAGE analysis of crude leaf extracts, a marked loss of LSU (nuclear encoded protein - 55 kDa) and marginal losses of SSU (chloroplast encoded protein - 15 kDa) polypeptides were observed in senescent leaves (Figure). The loss of LSU and SSU is one of the reasons for marked losses of Rubisco activity in senescent leaves. Similar results were also found in dark-adapted *Vigna* seedlings during senescence (NEDUNCHEZHIAN *et al.* 1995).

In vivo, a marked reduction of nitrate reductase activity was noticed in senescent leaves. This may reflect a balance between the synthesis of the active nitrate reductase enzyme or its activation on the one hand and degradation or inactivation on the other. The decreased nitrate reductase activity might reflect the reduction in nitrate uptake by the roots. This reduced uptake might be due to the feed back inhibition of amino acids formed in leaf blades and transported from there to the shoot (CLARKSON 1986).

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