Alteration of Photosynthesis in Grapevines Affected by Esca

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ABSTRACT

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To further understand the development of esca disease in grapevine, its physiological impact on plants grown in the vineyard was characterized, focusing mainly on photosynthesis. For this purpose, the state of the photosynthetic apparatus was evaluated in symptomatic and asymptomatic leaves in esca-infected grape plants, and wood carbohydrates stored in annual canes were assayed. In symptomatic leaves, esca greatly affected grapevine physiology. Foliar symptoms were associated with both stomatal closure and alteration of the photosynthetic apparatus as revealed by (i) a decrease in CO₂ assimilation, transpiration, and a signifi-

cant increase in intercellular CO₂ concentration; (ii) a strong drop in both the maximum fluorescence yield and the effective Photosystem II quantum yield; and (iii) a reduction of total chlorophyll but a stable carotenoid content. On symptomatic canes, all these variables were affected more on leaves with symptoms than without symptoms, suggesting a gradation in photosynthesis disruptions in the plant according to the degree of symptom severity. In addition, canes of symptomatic plants had reduced carbohydrate reserves during the winter rest, whether they exhibit symptoms or not. The following year, the lower pool of reserves may contribute to a significant decrease in plant development, as well as a global loss in plant vigor.

Additional keywords: *Vitis vinifera*.

Esca is a destructive grapevine disease affecting vineyards all over the world. Several fungi, including mainly *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum* (11,12,27,38), and *Fomitiporia mediterranea* (10,15,16), are found to be associated with the development of the esca syndrome. Furthermore, external factors such as climate (14,18) or training system (20,55) may favor symptom appearance (23). However, physiological aspects of the disease are not fully elucidated, and esca appears to be a very complex disease (37). The banning of sodium arsenite, which was the only chemical control treatment against esca in vineyards, means that new strategies have to be developed. Thus, characterizing the impact of esca on grapevine physiology represents a key step to point out a target that could enable control of this disease.

Esca primarily affects mature grapevine and is caused by a sequence of several fungi in different parts of the wood, which has been attributed recently to specific symptoms and damages (13,27,54). Esca symptoms appear in severe or chronic forms, although both can occur alone or together on the same plant (13,27). The year-to-year unpredictable discontinuity in symptom expression is a characteristic trait of esca disease (37,54). The severe form, called apoplexy, is a sudden wilting of the entire plant or of part of it, and can occur from June to September in Italy (54). The chronic form is characterized by both external and internal symptoms (37). External symptoms consist of light green to chlorotic areas turning into necrotic spots between the veins and/or along the leaf margins. Leaf chlorosis caused by esca suggests that photosynthesis and thus carbohydrate metabolism

may be perturbed (4,33). It has been described that changes in carbohydrate physiology of grapevine may be induced by various stress types such as low temperatures (25), drought (17), or chemicals (32,45,46), which can lead to disruptions of grapevine carbon nutrition. Similarly, grapevine pests and pathogens such as bacteria (4,22), viruses (47), fungi (5), or insects (42) may generate similar perturbations.

Most studies carried out to date on the effects of esca on grapevine physiology dealt with responses of in vitro-grown plants or calluses. Their growth is reduced when infected with some esca-associated fungi (48,53), and both leaf chlorophyll content and fluorescence are affected (48). However, no information is available on the physiological impact of esca on vineyard-grown plants.

The aim of this work was to study carbon metabolism modifications following esca foliar symptoms on vineyard-grown plants. Carbon metabolism is the key factor for grapevine yield and vigor and is defined by both photosynthetic rate and mobilization of carbohydrate reserves. Moreover, any factor affecting the photosynthesis in woody plants may disrupt the amount of synthesized sugars (30) and consequently the export of reserves to storage organs. Since carbohydrates accumulated in perennial organs are used for the early development of annual organs in spring (50,59), a decrease of sugar storage might have consequences for these organs.

The objective of this work was to analyze the response of net CO₂ assimilation to intercellular CO₂ concentration in diseased grapevines having esca leaf discoloration. In addition, chlorophyll fluorescence measurements allowed the evaluation of the relative limitation of leaf photosynthesis imposed by changes in stomatal conductance and PSII electron transport efficiency (40). Also, photosynthetic pigments were examined, and sugar contents were measured in winter on canes that showed symptoms during the previous summer to evaluate the carbohydrate reserves in esca-infected vines.

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MATERIALS AND METHODS

Plant material. Experiments were performed on Chardonnay grapevines (*Vitis vinifera* L.) planted in 1986, grafted on 41B rootstock, and trained according to the Chablis method. The experimental site was located in the Moët et Chandon vineyard in Avize (France) with an average annual temperature of 10.8°C and 480.7 mm annual precipitation. From 2001 to 2005, vines with esca foliar symptoms were located, and two groups were defined: (i) vines that did not show any esca symptoms; these were presumed to be healthy and considered controls; and (ii) vines with visible symptoms in August, which were considered diseased plants. At our experimental site, *Phaeomoniella chlamydospora* and *Fomitiporia mediterranea* were identified in diseased plants

according to the protocol of Larignon and Dubos (27), although the former was present in higher proportion.

Since esca symptoms appeared early in August, all gas exchange and fluorescence measurements were performed on leaves in August and September 2004. Pigment contents were assayed in August 2003 and 2004. During winter dormancy in January 2004 and 2005, carbohydrate reserves were measured as described below.

Gas exchange. Net photosynthesis (P_n), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and transpiration rate (E) were determined simultaneously on leaves with a portable infrared gas analysis system (Li-Cor Model 6400, Lincoln, NE). Measurements were conducted between 9 a.m. and 11 a.m. before the “midday depression” (9). The infrared gas analysis system

Fig. 1. Changes in **A**, net photosynthesis (P_n), **B**, stomatal conductance (g_s), **C**, intercellular CO_2 concentration (C_i), and **D**, transpiration rate (E) in leaves of control and esca-infected grape plants. Significant differences at $P < 0.05$ between leaves of control and diseased plants are marked by an asterisk. Values are means and standard errors ($n = 5$).

Fig. 2. Effective PSII quantum yield images of light-adapted leaves from **A**, control and **B**, **C**, and **E**, esca-infected grape plants measured in September. The artificial color code ranges from black (0.000) to pink (1.000), as shown at the bottom of the images. **D**, The visible light photograph of a symptomatic leaf corresponding to the fluorescence image in **E**. Scale bars = 0.8 cm.

was equipped with a clamp-on leaf cuvette that exposed 6 cm² of leaf area. Environmental conditions during photosynthetic measurements were the following: photosynthetically active radiation $\geq 1,500 \mu\text{mol m}^{-2} \text{s}^{-1}$, relative humidity around 30%, and chamber temperature at 20°C. CO₂ was maintained at a constant level of 400 $\mu\text{mol liter}^{-1}$ using a LI-6400-01 CO₂ injector with a high-pressure liquid CO₂ cartridge source. Gas exchange was measured on the following five leaf types: (i) leaves of asymptomatic control plants; (ii) leaves from asymptomatic canes on affected plants (aC); (iii) leaves without symptoms from symptomatic canes (sC) on diseased plants; (iv) green areas (gA) of leaves with symptoms; and (v) yellow areas (yA) of leaves with symptoms. Gas exchange measurements were repeated three times on five leaves from five different plants of each group both in August and September.

Chlorophyll fluorescence. Chlorophyll fluorescence reflects leaf photosynthesis intensity since it results from absorbed light energy that was not used for photosynthetic reactions and heat dissipation (41). Leaf chlorophyll fluorescence was quantified on leaves with an IMAGING-PAM chlorophyll fluorometer (Walz, Effeltrich, Germany). The measuring system applies an array of blue light-emitting diodes (peak wavelength 470 nm) for saturating light pulses, the frequency of which was adjusted to 10 Hz. Measurements were carried out for a 25 × 34 mm leaf area. During a saturation pulse application (5,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$), the fluorescence yield (F) and the maximum fluorescence yield (F_m') were assessed in leaves exposed to sunlight. The efficiency of PSII (ΦPSII) represented the number of electrons transported by a PSII reaction center per mole of quanta absorbed by PSII. ΦPSII was calculated according to Genty et al. (19): $\Phi\text{PSII} = (F_m' - F)/F_m'$. On each image, the values of the selected fluorescence parameters were averaged. Chlorophyll fluorescence and gas exchange measurements were carried out sequentially on the same five leaves of the five leaf types.

Photosynthetic pigments. Leaf slices were dissected and pigments were extracted overnight at 4°C under continuous agitation in 80% (vol/vol) acetone amended with 0.5% (wt/vol) MgCO₃ to prevent chlorophyll acidification. Crude extract was centrifuged at 10,000 × g for 10 min at 4°C, and the supernatant was used to estimate pigment concentrations spectrophotometrically according to the absorbance coefficients determined by Lichtenthaler (29). Results were expressed in milligrams per gram fresh weight (FW). Pigments were analyzed on the previously defined five groups of leaves. Pigment contents were determined on 20 leaves of each group in August 2003 and 2004.

Carbohydrates. Carbohydrate measurements were performed on three types of canes during two winter dormancy periods, 2004 and 2005: (i) control plant canes; (ii) asymptomatic canes of diseased plants; and (iii) symptomatic canes of diseased plants. Measurements were carried out in triplicate on six canes of each type.

Canes were frozen in liquid nitrogen and stored at -80°C. They were freeze-dried for 72 h and then ground with an SM2000 blender (Retsch, Haan, Germany). The extraction was performed using 100 mg of extract in the presence of 0.1 M phosphate buffer (pH 7.5) at 4°C, and the homogenate was centrifuged at 10,000 × g for 15 min at 4°C. The pellet was kept for starch analysis, whereas the supernatant was used for soluble sugar determination.

The pellet was resuspended in dimethylsulfoxide/8 N hydrochloric acid (4:1, vol/vol). Then, starch was dissolved for 30 min under continuous agitation at 60°C. After centrifugation at 20°C for 15 min at 13,000 × g, 100 μl of the supernatant was mixed with 100 μl of iodine-HCl solution (6 mg of KI and 0.3 mg of I₂ in 0.05 N HCl) and 1 ml of distilled water. The absorbance was read at 600 nm after 15 min at room temperature. Results were expressed in milligrams of starch per gram dry weight (DW).

One-hundred microliters of supernatant was used to determine soluble carbohydrate concentration with an enzymatic kit (Diag-

nostics GmbH, Martinsried, Germany) according to the manufacturer's protocol. Sugar concentrations were determined by 340 nm absorbance of NADPH, and results were expressed in milligrams per gram of DW.

Statistical analysis. To determine whether physiological parameters of esca-affected plants were significantly different from healthy plants, data were subjected to analysis of variance followed by a least significant difference test. Differences at $P < 0.05$ were considered significant.

RESULTS

Gas exchange. Significant differences in P_n, g_s, C_i, and E were observed between control and diseased leaves (Fig. 1). P_n of control leaves was similar in August and September, at 10.34 ± 2.02 and $11.26 \pm 2.07 \mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$, respectively (Fig. 1A). In diseased plants, P_n of asymptomatic cane (aC) leaves did not show significant differences relative to control leaves. Conversely, P_n significantly decreased in leaves of symptomatic canes (sC) whether they exhibited symptoms (August and September) or not (August). In leaves without symptoms, the decline of photosynthesis represented $36.5 \pm 10.5\%$ (SE) in August. In symptomatic leaves, P_n alteration was more drastic for both green (gA) and yellow areas (yA). P_n was reduced, respectively, by $82.5 \pm 8.3\%$ and $94.7 \pm 4.1\%$ in August and by $79.9 \pm 8.5\%$ and $90.1 \pm 7.4\%$ in September.

In control leaves, g_s values were similar in August and September, corresponding to 0.13 ± 0.04 and $0.16 \pm 0.03 \text{ mol H}_2\text{O m}^{-2} \text{s}^{-1}$, respectively (Fig. 1B). On diseased plants, g_s was not affected in leaves of aC. No differences were registered in g_s for leaves without symptoms of sC in August, but a significant decrease of $42.1 \pm 14.3\%$ was noticed in September. In gA and yA of affected leaves, g_s was reduced throughout the assessment period.

In control leaves, C_i increased between August and September (Fig. 1C). During the assessment period, C_i was not significantly different between control leaves, aC leaves, and leaves without symptoms from sC. But gA and yA of symptomatic leaves had significantly higher C_i values than the control. In August, the higher CO₂ concentration represented $63.2 \pm 11.8\%$ and $82.6 \pm 13.2\%$ in gA and yA, respectively.

E values of control leaves remained stable in August and September ranging from 3.05 ± 0.79 to $2.88 \pm 0.42 \text{ mmol H}_2\text{O m}^{-2} \text{s}^{-1}$ (Fig. 1D). On diseased plants, aC leaves and leaves without symptoms from sC showed no differences in E compared with control leaves. However, gA and yA of symptomatic leaves had lower E values than those measured in control leaves. In August, the reduction represented $47.6 \pm 21.3\%$ and $54.2 \pm 8.2\%$ in gA and yA, respectively.

Chlorophyll fluorescence. In control leaves, ΦPSII was 0.69 ± 0.05 (Figs. 2A and 3A). No significant differences were observed in aC leaves (Fig. 2B). In asymptomatic leaves from sC, ΦPSII values were not significantly different from the control, although the ΦPSII image revealed a slow decrease, especially around the leaf veins (Fig. 2C). Similarly, in gA of symptomatic leaves, ΦPSII values were not significantly lower than in control leaves. In contrast, the ΦPSII decrease in yA corresponded to $82.0 \pm 25.1\%$ as revealed by the artificial color from green to black of the ΦPSII image (Figs. 2D and E and 3A).

The maximum fluorescence (F_m') showed the same trend as ΦPSII (Fig. 3B). In control leaves, F_m' was 0.59 ± 0.22 . No significant differences were noted between control leaves, aC leaves, leaves without symptoms from sC, and gA of symptomatic leaves. But in yA, F_m' values were significantly lower than in the control, the corresponding decrease being $91.7 \pm 4.9\%$ (Fig. 3B). Therefore, chlorophyll fluorescence was affected only in the discolored area of symptomatic leaves since both ΦPSII and F_m' were near zero.

Photosynthetic pigments. Esca had a significant effect on the pigment content of leaves of diseased plants (Fig. 4). In control plants, total leaf chlorophyll concentration was $2.50 \pm 0.21 \text{ mg g}^{-1} \text{ FW}$ (Fig. 4A). No differences were found in diseased plant leaves that did not develop symptoms. However, on symptomatic canes, a significant chlorophyll content reduction was registered in leaves with symptoms, reaching 30% in the gA and 90% in the yA. Interestingly, the chlorophyll *a/b* ratio was 2.22 ± 0.10 in control leaves and was similar in leaves of both asymptomatic and symptomatic diseased plants, as well as in both green and yellow areas (Fig. 4B). Carotenoid content in control leaves was $0.12 \pm 0.09 \text{ mg g}^{-1} \text{ FW}$ (Fig. 4C). On diseased plants, no differences in carotenoids were recorded relative to control leaves.

Carbohydrates. As photosynthesis was lower on symptomatic leaves of esca-infected plants, we investigated whether carbohydrate storage changed in canes of diseased plants during the winter dormancy period (Fig. 5). In 2004 and in control canes, starch and soluble carbohydrate concentrations were $8.23 \pm 1.26 \text{ mg g}^{-1} \text{ DW}$ (Fig. 5A) and $49.37 \pm 12.66 \text{ mg g}^{-1} \text{ DW}$ (Fig. 5C), respectively. However, in both asymptomatic and symptomatic canes of diseased plants, starch amount decreased drastically by 53.56 ± 9.14 and $55.53 \pm 3.89\%$, respectively (Fig. 5A), whereas soluble carbohydrate content was not statistically modified (Fig. 5C). Similar results were obtained in 2005 (Fig. 5B and D).

DISCUSSION

Photosynthesis was greatly affected in leaves with esca symptoms, leading to perturbations in wood perennial reserve storage by reducing the amount of accumulated carbohydrates. Net photosynthesis, stomatal conductance, and transpiration declined in symptomatic leaves, whereas intercellular CO_2 concentration increased. The decrease of CO_2 assimilation may reduce carbohydrate synthesis and also export to sink organs as grapes develop, especially sugar accumulation in berries (7).

The simultaneous reduction of P_n and g_s in symptomatic leaves might suggest a global water stress in the plant. Indeed, similar perturbations of these parameters have been described before in grapes affected by Pierce's disease (22) or by vascular wilt diseases caused by pathogenic fungi and bacteria in other plants

(21,35,40,43). Pathogens responsible for the vascular wilt disease enter the xylem vessels and then proliferate within the vessels causing water stress in their hosts by increasing resistance to water flow (56). Reduced water flow results in leaf water deficits that can lead to stomatal closure and lower transpiration (44). However, in the case of esca, observations differ from vascular wilt diseases in that foliar symptoms are unlikely due to water stress caused by blockage of the xylem vessels (57). Indeed, only a part of a trunk is affected by the vascular occlusion and mainly the nonfunctional woody rings are affected (40).

Fig. 4. A and C, Photosynthetic pigment concentration and B, chlorophyll *a*/chlorophyll *b* ratio (Chl *a/b*) in leaves of control and esca-infected grape plants in August 2004. Significant differences between leaves of control and diseased plants at $P < 0.05$ are marked by an asterisk. FW = fresh weight. Values are means and standard errors ($n = 20$).

Fig. 3. Data showing A, effective PSII quantum yield (ΦPSII) and B, maximum fluorescence (F_m') of light-adapted leaves in control and esca-infected grape plants measured in September. Significant differences between leaves of control and diseased plants at $P < 0.05$ are marked by an asterisk. Values are means and standard errors ($n = 5$).

Another hypothesis is that leaf chlorosis and stomatal closure may be caused by extracellular fungal toxin(s) segregated into the vessels of the trunk and branches, then transported upward by the transpiration stream. In fact, toxins produced by the esca fungi *Phaeoemoniella chlamydospora*, *Phaeoacremonium aleophilum*, and *Fomitiporia mediterranea* and absorbed at very low doses by detached leaves of grapevine or injected into the woody tissue of shoots produced foliar symptoms of esca (51,52). Moreover, the involvement of fungal toxins in symptom development has been described previously in grapevine affected by *Eutypa lata*, the causal agent of Eutypa dieback, which infects xylem tissue. Eutypine, the toxin secreted by *E. lata* present in the trunk, is believed to be transported by the sap towards the herbaceous parts of the vine and trigger the expression of symptoms in leaves and other organs (55).

However, the stomatal limitation does not appear to be the main factor leading to the reduction in P_n . In parallel to the decrease of net CO_2 assimilation and stomatal closure, C_i was higher in both green and yellow areas on symptomatic leaves. This indicates that CO_2 fixation was limited by internal processes within these leaves. The reduced photosynthesis could be due to lower activity of photosynthetic enzymes, such as Rubisco (24,44,49), or carbonic anhydrase, which catalyzes the conversion of CO_2 to HCO_3^- or enzymes used in the utilization of the photoassimilates (2).

Moreover, reductions in ΦPSII and F_m' may have been involved in the decrease of P_n . A strong decrease in both ΦPSII and F_m' was observed in yellow areas of symptomatic leaves. The quantum yield of electron transfer to PSII (ΦPSII) provides information on the overall efficiency of PSII reaction centers under light and indicates the proportion of light absorbed by the chlorophyll associated with PSII that is used in photochemistry. Therefore, the decline of ΦPSII may reflect a down-regulation of electron transport (31,40) caused by a reduction in the fraction of open reaction centers.

Contents of total chlorophyll were significantly reduced in diseased plants, correlated with the development of yA on leaves.

Fig. 5. Starch and soluble carbohydrate concentrations in canes of control and esca-infected grape plants in **A** and **C**, January 2004 and **B** and **D**, January 2005. Significant differences between leaves of control and diseased plants at $P < 0.05$ are marked by an asterisk. DW = dry weight. Values are means and standard errors ($n = 6$).

Since the chlorophyll *a/b* ratio was unchanged for symptomatic plants, both chlorophyll *a* and *b* were apparently damaged at the same level. Similar reductions in pigment levels have been reported in plants infected with various pathogens, caused by the degradation of pigments or the inhibition of photosynthesis (1,3,4,26). In contrast, carotenoid content was not affected by esca since their concentration was similar in asymptomatic and symptomatic leaves. Therefore, carotenoids in asymptomatic leaves maintain a protective reaction of the plant membranes (29) against fungi as in control leaves.

Our results suggest a gradation in photosynthesis disruptions caused by esca disease. Indeed, the intensity of gas exchange alteration was correlated with disease development stage. On diseased plants, no alteration of photosynthetic parameters was detected in asymptomatic canes. Conversely, within symptomatic ones, a slight decline in P_n and g_s was observed in leaves without symptoms, whereas leaves with discoloration symptoms were more strongly affected.

Photosynthetic apparatus alteration in symptomatic leaves was accompanied by a decrease of carbohydrate storage. In grapevine, starch represents a source of carbohydrate storage in perennial wood (39,59). These amylaceous reserves accumulate during summer when source leaves are fully expanded (58). During winter dormancy, starch reserves are reduced in canes bearing leaf foliar symptoms in summer, confirming the reduced photosynthesis in the corresponding leaves.

In grapevine, the development of reproductive (inflorescences) and vegetative (annual stems, roots, and leaves) organs is initiated during the previous year (6,34). In early spring, young developing leaves cannot perform photosynthesis and thus represent carbohydrate sinks until they reach about one-third of their final size (58). The leaf sink/source transition occurs at the onset of flowering (59), suggesting that the early development of annual organs from winter bud through flowering is supported by carbohydrates accumulated in perennial organs during the previous year. Moreover, it has been shown that carbohydrates mobilized from reserves in canes and roots are used by the rapid growth of inflorescences and vegetative structures in early spring (50). Carbon reserves are thus progressively mobilized from roots or perennial stems and sustain the expansion of new young roots, annual stems, and leaves, as well as emerging inflorescences (59). This pool of carbon is restored in perennial organs by the photosynthetic activity in mature leaves, extending from flowering to leaf senescence, especially during berry ripening (8,9,36,59). These data suggest that the perturbation of reserve restoration in the wood of annual canes, due to esca disease, may affect the development of annual organs the year after, especially inflorescences that are sensitive to the amount of available sugars (28).

The perturbation of starch storage was similar in symptomatic and asymptomatic canes, although the latter does not exhibit alterations of the photosynthesis parameters. This suggests that carbohydrates synthesized in asymptomatic canes of diseased plants are equally distributed among all the canes and likely in the perennial wood. This would indicate that the management of carbohydrate metabolism in the whole plant takes into account the global situation and that asymptomatic canes contribute to overcome the damage due to symptomatic ones. However, the lower content of starch reserves in all the canes may affect plant vigor the following year.

Our results indicate that the carbohydrate physiology is widely affected in vines that develop esca foliar symptoms. Stomatal but mainly nonstomatal limitation with pigment degradation clearly plays a role in the intensity of photosynthesis decrease. Carbon metabolism of a grapevine showing foliar symptoms for several successive years is affected in terms of reserve accumulation. Thus, foliar symptoms could lead to a deprivation of yield and vigor affecting more or less rapidly plant longevity, demonstrating that the chronic form of esca is also deleterious for grapevine.

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