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BRIEF COMMUNICATION

Effects of phytoplasma [stolbur-subgroup (Bois noir-BN)] on photosynthetic pigments, saccharides, ribulose 1,5-bisphosphate carboxylase, nitrate and nitrite reductases, and photosynthetic activities in field-grown grapevine (*Vitis vinifera* L. cv. Chardonnay) leaves

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Abstract

In leaves of field-grown grapevine, the contents of chlorophyll, carotenoids, and soluble proteins and the activities of ribulose-1,5-bisphosphate carboxylase (RuBPC) and nitrate (NR) and nitrite (NiR) reductases were decreased in phytoplasma-infected leaves, but the contents of soluble sugars and total saccharides were markedly increased. In isolated thylakoids, phytoplasma caused marked inhibition of whole chain and photosystem 2 (PS2) activities. The artificial exogenous electron donor, diphenyl carbazide, significantly restored the loss of PS2 activity in infected leaves.

Additional key words: carotenoids; chlorophyll; electron transport; donor side; photosystem 1 and 2 activities.

Phytoplasmas are wall-less, non-culturable prokaryotes associated with diseases of several hundreds of plant species (McCoy *et al.* 1989). While molecular methods have improved identification of these pathogens and their phylogenetic and taxonomic relationships, their mechanisms of introducing disease are still not understood. Possible factors most often investigated are the effects of phytoplasma infection on phloem function and on saccharide translocation and concentrations in various tissues. Analysis of pear decline-affected trees showed that starch content in the roots of diseased trees is only about one half to one third of that of healthy trees (Batjer and Schneider 1960).

Grapevine yellows (GY) diseases are a group of disorders of *Vitis vinifera* with similar symptoms. GY are characterised by yellowing and downward curling of leaves on stunted shoots. The progress of knowledge of phytoplasma diversity and origin has been dependent on tools for their characterisation, because they are non-cultivable and indistinguishable organisms, when observed by electron microscopy. Currently, DNA-based techniques are being used extensively for both detection and identification of phytoplasmas (Maixner *et al.* 1997). However, woody host such as grapevine is difficult for phytoplasma diagnosis, whatever the technique used, because of the low titre of the pathogens and the presence of high contents of phenols in the infected tissues which hinder the isolation of phytoplasma DNA.

Phytoplasmas of the stolbur-subgroup (Bois noir – BN, Vergilbungskrankheit – VK) and the elm-yellows-group (Flavescence doree – FD) are widespread in Europe and of great economic importance. The leafhopper *Scaphoideus titanus* (vector of FD) and the planthopper *Hyalesthes obsoletus* (vector of BN and VK) are vectors of GY. The significance of GY is emphasised by regulations such as the EPPO certification scheme for grapevine propagation material or the EEC plant health directive, which require freedom of grape material from phytoplasmas. Most phytoplasmas are transmitted from plant to plant by leafhoppers, but some are transmitted by

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Abbreviations: BQ, p-benzoquinone; Car, carotenoids; Chl, chlorophyll; DCPIP, 2,6-dichlorophenol indophenol; DPC, diphenyl carbazide; DTT, dithiothreitol; GY, grapevine yellows; MV, methyl viologen; NiR, nitrite reductase; NR, nitrate reductase; PS, photosystem; RuBPC, ribulose-1,5-bisphosphate carboxylase.

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psyllids (Frisinghelli *et al.* 2000) and planthoppers. To our knowledge the physiology of interactions between phytoplasmas and their plant hosts has not yet been evaluated. The aim of this work was to investigate the effect of phytoplasma infection on contents of photosynthetic pigments, soluble proteins, soluble sugar, and starch, on activities of RuBPC, NiR, NR, and PS2 in field-grown grapevine leaves.

The grapevine (*Vitis vinifera* L. cv. Chardonnay) leaves were taken from naturally yellows-infected field-grown plants located in vineyards of the Istituto Agrario di San Michele all'Adige. We classified the leaf samples into two groups according to their Chl content: infected [below 200 μmol(Chl) m^{-2}] and unaffected, control [above 350 μmol(Chl) m^{-2}] leaves. Chl concentration was estimated using the *SPAD-502, Minolta* system, which was calibrated against Chl (*a*+*b*) content measured by extraction. Chl was extracted with 100 % acetone from liquid N_2 frozen leaf discs and stored at –20 °C. Amounts of Chl, Car, and total soluble proteins were determined spectrophotometrically by the methods of Lichtenthaler (1987) and Lowry *et al.* (1951), respectively. Saccharides (soluble sugars and starch) were measured using the phenol sulphuric acid method according to Hellubust and Craigie (1978). Leaves were cut into small pieces and homogenised 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 as the RuBPC. The assay of RuBPC activity was measured as described by Bowes and Ogren (1972). Nitrate and nitrite reductase activities and the rate of $^{14}CO_2$ fixation were estimated according to Muthuchelian *et al.* (1993). Thylakoid membranes were isolated from the leaves as described by Berthold *et al.* (1981). Whole chain electron transport (H_2O→MV) and partial reactions of photosynthetic electron transport mediated by PS2 (H_2O→BQ) and PS1 (DCPIPH_2→MV) were measured as described by Nedunchezhian *et al.* (1997). The rate of DCPIP photoreduction was determined by following the decrease in absorbance at 590 nm using a *Hitachi 557* spectrophotometer (Nedunchezhian and Kulandaivelu 1991). As a donor for PS2, 1 mM DPC was added.

The contents of Chl and Car were significantly decreased in phytoplasma-infected leaves (Table 1). Both Chl *a* and Chl *b* contents were decreased and phytoplasma probably also enhanced the chlorophyllase activity in leaves. We found an increase of Car/Chl ratio and a decrease of Chl *a*/*b* ratio in phytoplasma-infected leaves. This was due to the relatively faster decrease of Chl than Car.

The content of total soluble proteins was reduced markedly in phytoplasma-infected leaves (Table 1). This relatively low content of soluble proteins might have been due to the decrease in synthesis of RuBPC, the major soluble protein of leaf. A loss of leaf protein in infected leaves would be partially accounted for damaged chloroplasts or would be the result of inhibition of protein synthesis. The reduction in the overall photosynthetic rate correlated well with the decrease of RuBPC activity in phytoplasma-infected leaves. A marked reduction of RuBPC activity was observed in severely infected leaves (Table 1). Such reduction was due to inhibition of protein synthesis induced by phytoplasma. The reduction in RuBPC activity in infected leaves correlated well with the $^{14}CO_2$ fixation (Table 1) that was an indirect effect due to the inhibition or destruction of photosynthetic pigments.

Phytoplasma-infected leaves had lower NR and NiR activities than the control leaves (Table 1), but the decline in NiR activity was much less than in NR activity. These reductions might reflect a balance between synthesis and activation on one hand and degradation or inactivation on the other. The changes in intercellular pH values due to phytoplasma infection might decrease the transfer of nitrate (substrate) from a vacuolar pool to active cytoplasmic pool accessible to the enzyme. The inhibition of NR and NiR activities might be also due to the inhibition of protein synthesis or it might have stemmed out from decreased rate of photosynthate supply in the phytoplasma-infected leaves.

Phytoplasma increased the contents of both soluble sugars and total saccharides in the leaf tissues (Table 1) which is in accord with the results of Lepka *et al.* (1999). The accumulation of saccharides could explain some of the symptoms observed after phytoplasma infection. Phytoplasma-infected plants show a marked bleaching of leaves. This bleaching of leaves might be a consequence of accumulated saccharides in the source leaves. A similar bleaching is found in transgenic tobacco plants when pholem transport is inhibited by over-expression of a heterologous invertase in the cell wall (Schaewen *et al.* 1991, Sonnewald *et al.* 1991) or by over-expression of pyrophosphatase in the phloem (Lerchl *et al.* 1996). The loss of Chl is accompanied by a general sugar mediated repression of genes involved in photosynthesis (Krapp *et al.* 1993, Lerchl *et al.* 1996).

The whole chain electron transport was inhibited to 65 % in phytoplasma-infected leaves (Table 1). However, the PS1 activity was much less diminished. In contrast to PS1, the PS2 activity mediated by both benzoquinone (BQ) and DCPIP was significantly inhibited (Table 1). DCPIP collects electrons after plastoquinone, PQ (Lien and Bannister 1971, Ouitrakul and Izawa 1973) but BQ at the reducing side of PQ (Lien and Bannister 1971). As the PS2 activity loss due to phytoplasma was similar in the systems H_2O→BQ and H_2O→DCPIP, the site of phytoplasma action must be prior to PQ in the electron transport. DPC, as artificial electron donor for PS2, donates electrons close to the PS2 reaction centre (Packham *et al.* 1982). Thus the inhibition of PS2 could be as-

Table 1. Differences in contents and ratios of chlorophyll (Chl) and carotenoids (Car), soluble proteins, soluble sugars and starch, and total saccharides, in rate of $^{14}CO_2$ fixation, in activities of ribulose-1,5-bisphosphate carboxylase (RuBPC), nitrite reductase (NiR) and nitrate reductase (NR), and in the rates of whole chain, photosystem (PS) 1 and 2 activities in thylakoids of control and phytoplasma-infected leaves. Values in parentheses show percent reduction relative to control. Means±SE of three replicates of each experiment.

Parameter		Control	Phytoplasma-infected
Chl *a*	[g kg^{-1}(fr.m.)]	2.45 ± 0.63	0.85 ± 0.10 (–65)
Chl *b*	[g kg^{-1}(fr.m.)]	0.90 ± 0.25	0.42 ± 0.09 (–53)
Chl *(a+b)*	[g kg^{-1}(fr.m.)]	3.35 ±0.96	1.27 ± 0.12 (–60)
Car	[g kg^{-1}(fr.m.)]	0.80 ± 0.05	0.38 ± 0.04 (–52)
Chl *a/b*		2.72	2.00
Car/Chl		0.23	0.30
Soluble proteins	[g kg^{-1}(fr.m.)]	44.80 ± 1.80	23.30 ± 1.30 (–48)
Soluble sugars	[g kg^{-1}(fr.m.)]	32.80 ± 1.54	47.20 ± 2.21 (+44)
Soluble starch	[g kg^{-1}(fr.m.)]	16.10 ± 0.09	29.30 ± 1.10 (+82)
Saccharides	[g kg^{-1}(fr.m.)]	48.90 ± 2.21	76.50 ± 2.95 (+56)
$^{14}CO_2$ fixation	[nmol(CO_2) kg^{-1}(prot.) s^{-1}]	61.50 ± 3.10	23.40 ± 1.12 (–62)
RuBPC	[nmol(CO_2) kg^{-1}(prot.) s^{-1}]	55.40 ± 3.80	27.10 ± 2.40 (–51)
NiR activity	[nmol(NO_2) kg^{-1} s^{-1}]	1482.00 ± 64.20	829.00 ± 42.60 (–44)
NR activity	[nmol(NO_2) kg^{-1} s^{-1}]	1124.00 ± 52.50	427.00 ± 16.10 (–62)
H_2O→MV	[mmol(O_2) kg^{-1}(Chl) s^{-1}]	132.40 ± 5.40	46.30 ± 2.22 (–65)
H_2O→BQ	[mmol(O_2) kg^{-1}(Chl) s^{-1}]	168.20 ± 6.61	72.30 ± 2.53 (–57)
H_2O→DCPIP	[mmol(DCPIP) kg^{-1}(Chl) s^{-1}]	184.50 ± 10.21	71.90 ± 4.30 (–61)
DPC→DCPIP	[mmol(O_2) kg^{-1}(Chl) s^{-1}]	186.20 ± 9.82	158.20 ± 5.22 (–15)
$DCPIPH_2$→MV	[mmol(O_2) kg^{-1}(Chl) s^{-1}]	356.60 ± 18.21	303.10 ±1 6.20 (–15)

cribed to an alteration of the water-splitting system, since the addition of DPC restored significantly its activity. So phytoplasma probably acts on the donor side of PS2.

These results allow the following general conclusion: phytoplasma infection causes non-specific, general stress responses in grapevine leaves. The found changes in contents and activities connected with photosynthesis of the infected leaf tissues are similar to those of induced senescence or ageing. Also a complicated interaction of damage to and degradation of the photosynthetic apparatus brings about the phytoplasma-induced yellowing.

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