BIOLOGIA PLANTARUM 47 (2): 237-242, 2003/4

Effects of phytoplasma infection on pigments, chlorophyll-protein complex and photosynthetic activities in field grown apple leaves

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Abstract

Changes in contents of pigments, chlorophyll-protein complex, and photosynthetic activities were investigated in field grown apple (*Malus pumila* Mill.) leaves infected by Apple Proliferation phytoplasma. The contents of chlorophyll *a+b* (Chl) and carotenoids (Car) markedly decreased in infected leaves. Similar results were also observed for content of total soluble proteins and ribulose-1,5-bisphosphate carboxylase activity. When various photosynthetic activities were followed in isolated thylakoids, phytoplasma infection caused a marked inhibition of whole chain and photosystem 2 (PS2) activity. Smaller inhibition of photosystem 1 (PS1) activity was observed even in severely infected leaves. The artificial exogenous electron donors, $MnCl_2$, diphenyl carbazide, and NH_2OH, did not restore the loss of PS2 activity in both mildly and severely infected leaves. Similar results were obtained by Chl fluorescence measurements. The marked loss of PS2 activity in infected leaves was due to the reduction of contents of chlorophyll and light-harvesting chlorophyll-protein 2 complexes.

Additional key words: carotenoids; electron transport, photosystem, proteins, ribulose-1,5-bisphosphate carboxylase.

Introduction

Mycoplasmas are prokaryotic organisms. The phytoplasmas resemble the mycoplasmas of the genera *Mycoplasma* in all morphological aspects. They lack cell wall, they are bound by a triple-layered "unit" membrane, and have cytoplasm, ribosomes and strands of nuclear material. Their shape is usually sphaeroidal to ovoid or irregular tubular to filamentous and their sizes are comparable to those of the typical mycoplasmas (Frisinghelli *et al.* 2000). Phytoplasmas are associated with several hundred-plant diseases, of which many are of considerable economic importance (Seemuller *et al.* 1998). Phytoplasmas causing serious diseases in fruit trees have frequently been reported throughout the peninsula and characterized to some extent at the molecular level (McCoy *et al.* 1989, Lee *et al.* 1995, Poggi-Pollini *et al.* 1996, Marzachi *et al.* 1999).

Apple Proliferation (AP) is a serious disease of apple trees in Europe associated with several genetically slightly different phytoplasmas (Kison *et al.* 1994, Frisinghelli *et al.* 2000, Jarausch *et al.* 2000). Phytoplasma-diseased woody plants usually contain very low phytoplasma titres that are difficult to purify from infected tissues. Therefore, the inability to culture phytoplasmas has limited the knowledge of their physiology, biochemistry, and molecular biology. It is largely unknown how phytoplasmas interact with their host plants or insects.

Phytoplasmas are introduced directly into plant phloem sieve tube cells during vector feeding and translocate with phloem sap in the direction of photosynthetic "sinks". Phytoplasmal infection severely damages the physiological and biochemical processes of plants (Catlin *et al.* 1975, Kartte and Seemuller 1991, Lepka *et al.* 1999, Bertamini and Nedunchezhian 2001).

Received 15 July 2002, *accepted* 30 October 2002.

Abbreviations: AP - Apple Proliferation; Car - carotenoids; Chl - chlorophyll; CP - chlorophyll protein; DCBQ - 2,6-dichloro-*p*-benzoquinone; DCPIP - 2,6-dichlorophenol indophenol; DPC - diphenyl carbazide; DTT - dithiothreitol; F_0 - minimal fluorescence; F_v - variable fluorescence; LHCP - light-harvesting chlorophyll protein; MV - methyl viologen; PS - photosystem; RuBPC - ribulose-1,5-bisphosphate carboxylase; SDS - sodium dodecyl sulphate; SiMo - silicomolybdate.

Acknowledgements: This work was in part supported by a grant from Provincia Autonoma of Trento and National Council of Research (CNR): project "Analisi e Ricerche per il Sistema Agri-Industriale" sub-project "Prometavit".

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One of the main effects of phytoplasmal infection is a decrease in plant productivity caused by inhibition of photosynthesis (Bertamini and Nedunchezhian 2001). This decline of photosynthesis can be a result of the direct effect of infection on photosynthetic electron transport and enzymatic activities.

To our knowledge, the importance of phytoplasma infection in apple leaves has not been evaluated to date in the field. We investigated the effects of AP infection on pigments, chlorophyll-protein complexes, and photosynthetic activities in field-grown apple leaves.

Materials and methods

Plant: The apple (*Malus pumila* Mill. cv. Golden Delicious) leaves used in this study were collected from field-grown phytoplasma infected plants located in Istituto Agrario di San Michele all' Adige orchards, San Michele all' Adige, Italy. We classified the leaf samples into three groups according to their Chl content: unaffected, control leaves [above 350 μmol(Chl) m^{-2}]; mildly infected leaves [200 μmol(Chl) m^{-2}], and severely infected leaves [below 100 μmol(Chl) m^{-2}].

Pigment analysis: 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 Car were analyzed spectrophotometrically (*Hitachi 557* spectrophotometer, Tokyo, Japan) according to the method of Lichtenthaler (1987).

Chl *a* fluorescence transients: Chl *a* fluorescence induction was followed in thylakoid suspensions after excitation with broadband blue radiation (400 - 520 nm, *Corning CS 4-96,* Schott, Germany). The photomultiplier *(Hamamatzu R 376,* Tokyo, Japan) placed at 90 ° to the excitation beam, was protected an interference filter (λ_{max} 690 nm, half band width 12 nm, *Schott*, Germany). The signal from the photomultiplier was displayed on a *Iwatsu* model *SS 5802* digital oscilloscope (*Iwatsu* Corp., Tokyo, Japan). The Chl concentration of thylakoid suspension was kept as 2 g m^{-3}. DCMU was used at a concentration of 5 μM.

Activities of electron transport: Thylakoid membranes were isolated from the leaves as described Berthhold *et al.* (1981). Whole chain electron transport [H_2O → methyl viologen (MV)] and partial reactions of photosynthetic electron transport mediated by PS2 [H_2O → 2,6-dichloro-*p*-benzoquinone (DCBQ); H_2O → silicomolybdate (SiMo)] and PS1 [2,6-dichlorophenol indophenol ($DCPIPH_2$) → MV)] were measured as described by Nedunchezhian *et al.* (1997). Thylakoids were suspended at 10 g(Chl) m^{-3} 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 and 200 μM SiMo.

DCPIP photoreduction: The rate of DCPIP photoreduction was determined by following 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. Where mentioned, the concentration of $MnCl_2$, DPC, and NH_2OH were 5, 0.5, and 5 mM, respectively.

Content total soluble proteins: Total soluble proteins were extracted by grinding two leaves (0.3 - 0.5 g fresh mass) in a mortar with 6 cm^3 of 100 mM Tris-HCl, pH 7.8 containing 15 mM $MgCl_2$, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 10 mM PMSF in the presence of liquid nitrogen. Homogenates were filtered through nylon cloth. After centrifugation at 11 000 *g* for 10 min, the concentration of soluble proteins was determined in the supernatant by method of Bradford (1976).

Extracts and assay of RuBPC 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 dithiothreitol (DTT), and 0.25 mM ethylenediaminetetraacetate (EDTA). The extract was clarified by centri-fugation at 11 000 *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 Nedunchezhian and Kulandaivelu (1991).

Separation of Chl-protein complexes: Chl-protein complexes were separated according to the method of Anderson *et al.* (1978). Thylakoids were suspended in an isolation medium and homogenized in a prechilled teflon homogenizer with sodium dodecyl sulphate (SDS) (Chl to SDS ratio 1:7) at 4 °C. The homogenate was loaded onto a 10 % linear polyacrylamide gel. The samples were concentrated by applying a current of 1 mA for 30 min. Subsequently current was increased to 5 mA and the gel was run at 4 °C till the complete separation of bands was achieved. The green gels were scanner by an attachment to a *Shimadzu UV-3000* double beam spectrophotometer.

Results

Changes in pigment contents: When determined per unit fresh mass, total Chl content was drastically reduced in severely infected leaves. As much as 30 and 52 % reduction was observed in mildly and severely infected leaves, respectively. Similar trend was also noticed for Car content (Table 1). The Chl *a/b* ratio was markedly lower in infected leaves. In contrast to this, the Car/Chl ratio was increased in infected leaves (Table 1).

Table. 1. Changes of chlorophyll (Chl) and carotenoid (Car) contents [g kg^{-1}(f.m.)] and their ratios in control and phytoplasma infected leaves. Values in parentheses are percent reduction with reference to control. Means ± standard errors of 5 replicates.

Pigments	Control	Mildly infected	Severely infected
Chl *a*	2.187±0.040	1.480±0.080 (32)	0.979±0.060 (55)
Chl *b*	0.832±0.050	0.622±0.050 (25)	0.470±0.020 (44)
Chl (*a*+*b*)	3.019±0.100	2.109±0.090 (30)	1.449±0.070 (52)
Car	0.801±0.050	0.620±0.050 (22)	0.416±0.020 (52)
Chl *a/b*	2.620±0.030	2.390±0.030	2.080±0.040
Car/Chl	0.260±0.040	0.290±0.050	0.320±0.080

Changes in Chl *a* fluorescence kinetics and photosynthetic activities: The effect of phytoplasma was prominent on the level of minimal fluorescence F_0 and markedly increased in severely infected thylakoids (Table 2). The ratio of variable to maximal fluorescence F_v/F_m decreased from 0.71 to 0.64 and 0.50 in mildly and severely infected thylakoids, respectively (Table 2). Upon addition of 5 μM DCMU to the infected thylakoids, F_v was insignificantly increased (Table 2).

Table 2. Changes in chlorophyll (Chl) *a* fluorescence kinetics (F_v/F_m) in thylakoids isolated from control and phytoplasma infected leaves. Means ± standard errors of 5 replicates.

Treatment		F_0	F_v	F_v/F_m
Control	-DCMU	2.0 ± 0.11	4.9 ± 0.24	0.71 ± 0.04
	+DCMU	2.0 ± 0.12	5.2 ± 0.28	0.72 ± 0.03
Mildly	-DCMU	2.5 ± 0.13	4.5 ± 0.21	0.64 ± 0.03
	+DCMU	2.5 ± 0.13	4.7 ± 0.23	0.65 ± 0.04
Severely	-DCMU	3.5 ± 0.17	3.5 ± 0.16	0.50 ± 0.02
	+DCMU	3.5 ± 0.18	4.0 ± 0.19	0.53 ± 0.03

Photosynthetic electron transport from $DCPIPH_2$ → MV (PS1) was reduced by about 6 and 11 % in mildly and severely infected leaves, respectively (Fig. 1). The PS2 activity in systems H_2O → DCBQ and H_2O → SiMo was reduced by about 26 and 5 % in mildly and 44 and 11 % in severely infected leaves, respectively (Fig. 1). A similar trend was noticed for whole chain electron transport (H_2O → MV) activity (Fig. 1).

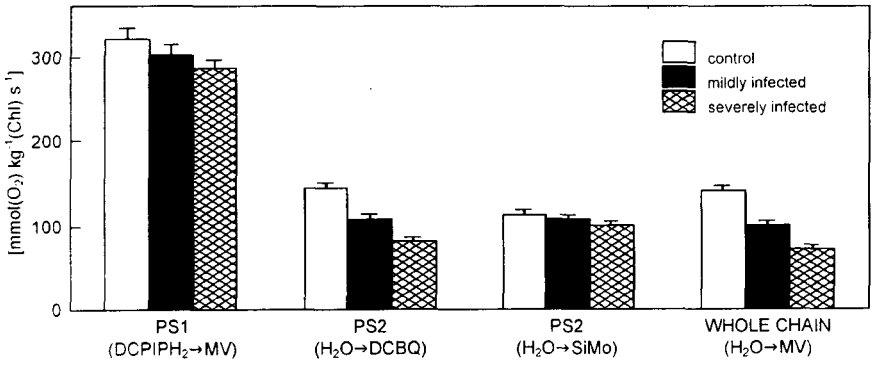

Fig. 1. Changes in the rates of whole chain (H_2O → MV), PS2 (H_2O → DCBQ; H_2O → SiMo), and PS1 ($DCPIPH_2$ → MV) electron transport activities in thylakoids isolated from control and phytoplasma infected leaves. Means ± standard errors of 5 replicates.

Changes in DCPIP photoreduction: PS2 activity was reduced by about 22 and 49 % in mildly and severely infected leaves, respectively, when water served as electron donor (Fig. 2). The artificial exogenous electron donors of DPC, NH_2OH, and $MnCl_2$ failed to restore the phytoplasma induced loss of PS2 activity in both mildly and severely infected leaves (Fig. 2).

Changes in Chl-protein complexes: To identify the specific complexes involved, Chl-protein complexes were separated on mild SDS-PAGE "green gel". Five Chl-containing bands (CP1, LHCP1, CPa, LHCP2, and FP) were resolved (Fig. 3) and were labeled according to the nomenclature of Anderson *et al.* (1978). The absorbance spectrum of CP1 contained a small amount of Chl *b* (not shown) and associated with the LHCP1 of PS1. CPa contains mainly PS2 Chl *a* complexes, but may contain a variable amount of LHCP1 under conditions that dissociate CP1a into CP1 and LHCP1 components (Metz *et al.* 1984). LHCP2 is the major light harvesting

Chl-protein complex of PS2. FP is the free pigment dissociated from the complexes during the detergent solubilization.

Quantification was performed by integration of gel scan peak areas. Contents of CPa and LHCP2 were decreased in phytoplasma-infected leaves without any effect on CP1 and LHCP1 (Table 3). As much as 21 and 10 % and 37 and 20 % of CPa and LHCP2 were reduced in mildly and severely infected leaves, respectively (Table 3).

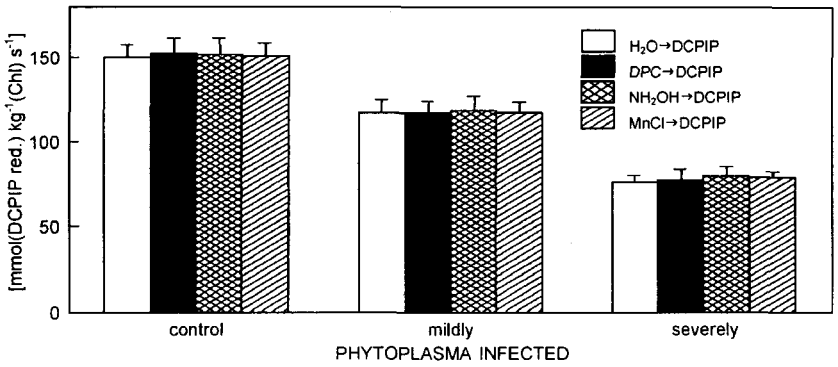

Fig. 2. Effect of various exogenous electron donors on photosystem 2 (PS2) activity (H_2O → DCPIP) in thylakoids isolated from control and phytoplasma infected leaves. Means ± SE of 5 replicates.

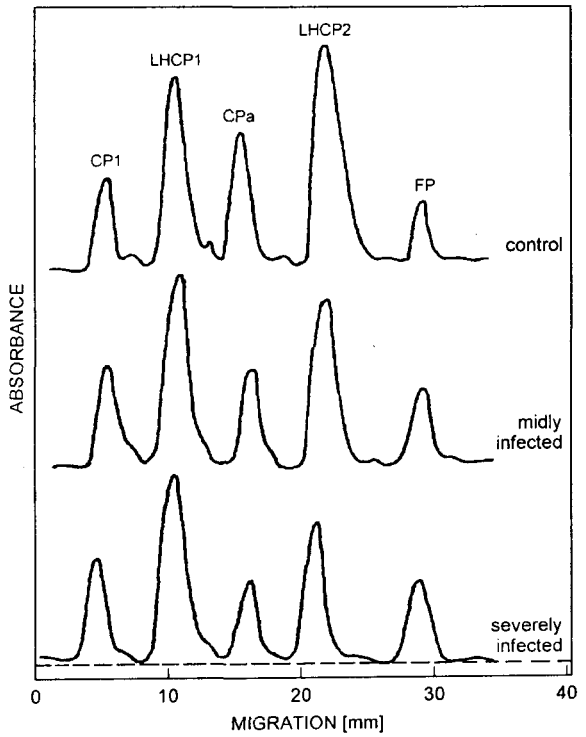

Fig. 3. Gel scan of Chl-protein complexes from isolated thylakoids of control and phytoplasma infected leaves. Lanes were scanned at 675 nm.

Changes in RuBPC activity and total soluble proteins in crude leaf extracts: When the enzyme activity was expressed on a protein basis, as much as 24 and 48 % reduction was noticed in mildly and severely infected leaves, respectively (Fig. 4). Similar trend was also found for total soluble proteins (Fig. 4).

Table 3. Relative area [%] after integration of the densitometric scans of chlorophyll-protein complexes of control and phytoplasma infected leaves. Means ± SE of 5 replicates.

Band	Control	Mildly infected	Severely infected
CP1	14 ± 0.62	16 ± 0.80	17 ± 0.81
LHCP1	27 ± 1.32	27 ± 1.36	28 ± 1.36
CPa	19 ± 0.92	15 ± 0.61	12 ± 0.56
LHCP2	31 ± 1.51	28 ± 1.40	25 ± 1.20
FP	9 ± 0.30	14 ± 0.70	15 ± 0.68

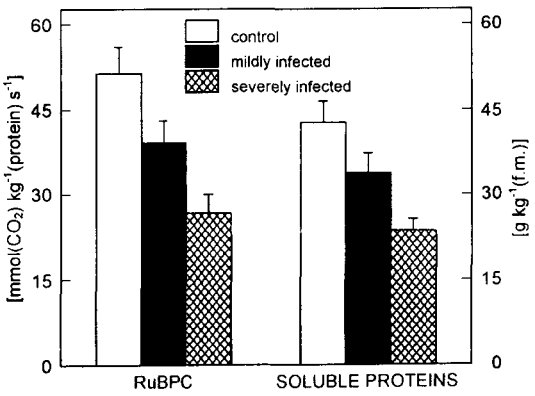

Fig. 4. Changes of RuBPC activity and content of soluble proteins in control and phytoplasma infected leaves. Means ± SE of 5 replicates.

Discussion

Phytoplasma infection reduced significantly the contents of Chl and Car in apple leaves. Both Chl *a* and Chl *b* contents were decreased and phytoplasma probably also enhanced the chlorophyllase activity in leaves. Chl *a* is a more exact characteristic of photosynthetic activity (Šesták 1966), the tendency towards a higher Chl *a/b* ratio might partially explain the higher photosynthetic rates found in control than infected leaves. We also found an increase of Car/Chl ratio in phytoplasma infected leaves. This was due to the relatively faster decrease of Chl than Car contents.

The control thylakoids showed a good PS2 activity, measured as the F_v/F_m ratio. Increase of infection in leaves lead to decrease in this ratio. The decrease in F_v/F_m ratio was mainly due to significant increase in F_o and insignificant decrease in F_v in both mildly and severely infected leaves. This is due to the phytoplasma infection induced changes on the acceptor side of PS2 in apple leaves (Allakhverdiev *et al.* 1987, Šetlík *et al.* 1990). Use of various electron transport acceptors for measurements in thylakoids isolated from infected leaves brought only a marginal effect on PS1 mediated reactions.

Phytoplasma did not significantly change the rate of PS1 activity in both mildly and severely infected leaves. Hence the phytoplasma must have action site(s) in the PS2 reaction. Analysis of electron transport in thylakoids isolated from mildly and severely infected leaves showed that O_2 evolution was significantly inhibited when using DCBQ as electron acceptor but not inhibited when the electron acceptor was SiMo. This indicates that thylakoids isolated from phytoplasma-infected leaves are affected at the reducing side of PS2.

Measurements of PS2-mediated DCPIP reduction in the presence of various artificial exogenous electron donors acting at the oxidizing side of PS2 (Wydrzynski and Govindjee 1975) were made to locate the possible site of phytoplasma-induced inhibition on PS2. Among the various electron donors $MnCl_2$, DPC, and NH_2OH were did not restore PS2 activity in both mildly and severely infected leaves. This indicates that phytoplasma affected the acceptor side of PS2, and perhaps close to the Q_B side of PS2.

Supporting evidence for the damage to PS2 activity was obtained from the analysis of Chl-protein complexes of apple thylakoid membrane: the appearance of CP bands in phytoplasma infected leaves revealed that phytoplasma inhibits Chl biosynthesis. This could be due to inactivation of certain enzymes in the Chl biosynthetic pathway. A comparison of thylakoids of phytoplasma infected leaves with those of the control showed specific loss of LHCP2 and CPa complexes. The loss was more pronounced in severely infected leaves. LHCP is one of the targets of several stress factors such as UV (Nedunchezhian and Kulandaivelu 1995), metals (Bornman 1989) and minerals (Nedunchezhian *et al.* 1997). In the present study, the content of LHCP2 was gradually decreased with increase of infection level. LHCP2 is the peripheral light-harvesting Chl *a/b* complex associated with PS2 (Bassi and Simpson 1987, Green 1988) and regulates the distribution of radiant energy between the two photosystems. This could be a reason for the observed marked loss of PS2 activity induced by phytoplasma in apple leaves. CPa is also most sensitive to many environmental effects and this supports the fact that the PS2 assembly is more sensitive than the PS1 complex. Hence the decrease in CPa complex was one of the reasons for the loss of O_2 evolution in infected leaves.

The content of total soluble proteins was reduced markedly in severely infected leaves. The relatively low content of soluble proteins may have been due to decrease in the synthesis of RuBPC, the major soluble protein of leaf. A loss of leaf protein in infected leaves partially accounts for chloroplast damage or is the result of inhibition of protein synthesis. The reduction in photosynthetic rates correlates well with the decrease in RuBPC activity in phytoplasma-infected leaves. A marked reduction of RuBPC activity was observed in severely infected leaves. Such reduction was due to inhibition of protein synthesis induced by phytoplasma.

We conclude that phytoplasma infection causes non-specific, general stress responses in apple leaves. The changes in contents and activities connected with photosynthesis of the infected leaf tissues are similar to those of induced senescence or ageing. A complicated interaction of damage to and degradation of the photosynthetic apparatus bring about the phytoplasma-induced reddish pattern in leaves.

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