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BRIEF COMMUNICATION

Effects of phytoplasma infection on growth and photosynthesis in leaves of field grown apple (*Malus pumila* Mill. cv. Golden Delicious)

M. BERTAMINI*, K. MUTHUCHELIAN**, M.S. GRANDO*, and N. NEDUNCHEZHIAN*,**,+

*Istituto Agrario di San Michele all' Adige, 38010 San Michele all' Adige, Italy**
*School of Energy, Environment and Natural Resources, Madurai Kamaraj University, Madurai - 625 021, India***

Abstract

The contents of chlorophyll (Chl), leaf biomass, and soluble proteins were markedly decreased in phytoplasma infected apple leaves. Similar results were also observed for ribulose-1,5-bisphosphate carboxylase, $^{14}CO_2$ fixation, and nitrate reductase activity. In contrast, the contents of sugars, starch, amino acids, and total saccharides were significantly increased in phytoplasma infected leaves. In isolated chloroplasts, phytoplasma infection caused marked inhibition of whole photosynthetic electron chain and photosystem 2 (PS2) activity. The artificial exogenous electron donor, diphenyl carbazide, significantly restored the loss of PS2 activity in infected leaves. Similar results were obtained when F_v/F_m was evaluated by *in vivo* Chl *a* fluorescence kinetic measurements.

Additional key words: amino acids; chlorophyll content and fluorescence; electron donor; nitrate reductase; photosystem; phytoplasma; proteins; ribulose-1,5-bisphosphate carboxylase; sugars; starch.

Mycoplasmas, prokaryotic microorganisms observed in plants and insect vectors, with the exception of spiroplasmas, resemble the mycoplasmas of the genus *Mycoplasma* in all morphological aspects. They lack cell wall, are bounded by a triple-layered "unit" membrane, and have cytoplasm, ribosomes, and strands of nuclear material. Their shape is usually spheroidal to ovoid or irregularly tubular to filamentous and their sizes are comparable to those of the typical mycoplasmas (Caudwell *et al.* 1971, Credi 1994).

Plant mycoplasma-like organisms recently termed phytoplasmas are restricted in trees to functional phloem sieve elements (Gunderson *et al.* 1994). They are associated with several hundreds of plant diseases, of which many are of considerable economic importance (Seemüller *et al.* 1998). Most phytoplasmas are transmitted from plant to plant by leafhoppers, but psyllids, and planthoppers (Frisinghelli *et al.* 2000) transmit some.

Apple proliferation (AP) is a serious disease of apple trees in Europe, associated with several genetically slightly different phytoplasmas (Kison *et al.* 1994, Jarausch *et al.* 2000). AP affects the vigour of the trees whose fruits cannot be commercialised because of their small size and poor taste. Witches broom, early leaf coloration in autumn, and incomplete coloration of fruit are other typical symptoms of AP. During the last 5 years a serious epidemic is spreading in the apple-growing areas of Trentino Alto Adige (Italy). As with other plant pathogenic phytoplasmas, all attempts to culture the AP phytoplasma in cell free media failed so far. Phytoplasma-diseased woody plants usually contain very low phytoplasma titres and they 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.

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+Author for correspondence; *permanent address*: Government Higher Secondary School, Vellimedupettai – 604 207, Tindivanam, India; e-mail: nedu2000@yahoo.com
Abbreviations: AP – apple proliferation; Chl – chlorophyll; DBMIB – dibromothymoquinone; DHQ – durohydroquinone; DPC – diphenyl carbazide; DTT – dithiothreitol; F_0 – initial level of Chl fluorescence after dark incubation; F_v – variable fluorescence; F_m – maximum fluorescence; MV – methyl viologen; NR – nitrate reductase; P_N – net photosynthetic rate; PS – photosystem; R_D – dark respiration rate; RuBPC – ribulose-1,5-bisphosphate carboxylase; SLA – specific leaf area.
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Phytoplasma infection damages severely the physiological and biochemical processes of plants (Catlin *et al.* 1975, Kartte and Seemüller 1991, Lepka *et al.* 1999). To our knowledge, the importance of phytoplasma infection in apple leaves has not been evaluated to date in the field. The present work was to investigate the effect of phytoplasma infection on growth and photosynthesis in field-grown apple leaves.

The apple (*Malus pumila* Mill. cv. Golden Delicious) leaves were collected from field-grown phytoplasma infected plants located in Istituto Agrario di San Michele all' Adige orchards, San Michele all' Adige, Italy. The phytoplasma-infected leaves (reddish symptomatic) were used for molecular detection that was described by Frisinghelli *et al.* (2000). The healthy control (symptom-less) leaves of the same cultivar were sampled at the same time and examined in the same way as the infected ones. Dry mass was determined after drying the plant material at 105 °C for 10 h. Chlorophyll (Chl) concentration was determined spectrophotometrically by the method of Lichtenthaler (1987). Starch, soluble proteins, amino acids, and sugars were extracted and their concentrations were determined following the methods of McCready *et al.* (1950), Bradford (1976), Hare (1977), and Hellubust and Craigie (1978), respectively. Net photosynthetic rate (P_N) and dark respiration rate (R_D) were determined using the closed gas-analytical system *LI 6000* (*Li-Cor*, U.S.A). For each measurement the leaves of 5-6 plants were put in the leaf chamber giving a total of 10 cm^2 leaf area. Leaf temperature was 20-30 °C, CO_2 concentration in the system was *ca.* 400 cm^3 m^{-3}, and photon flux density was 800 μmol m^{-2} s^{-1}. Before measurements of R_D the leaves were dark-adapted for 30 min. The procedure for Chl *a* fluorescence induction and decay measurement was essentially as described by Miles (1980).

Chloroplasts were isolated from 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→DBMIB, H_2O→DCPIP, DPC→DCPIP) or PS1 (DHQ→MV) were measured as described by Reinero and Beachy (1989) and Nedunchezhian *et al.* (1997), respectively. Fully expanded 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 centrifuged at 10 000×*g* for 10 min. The clear supernatant was decanted slowly and used for RuBPC analysis. The assay for RuBPC activity was carried out as described by Nedunchezhian and Kulandaivelu (1991). The rate of $^{14}CO_2$ fixation and nitrate reductase activity were measured according to the method of Muthuchelian *et al.* (1993).

Phytoplasma infection had reduced leaf fresh and dry masses and total Chl content (Table 1). The marked reductions of the leaf dry matter in infected plants were mainly due to reduction in leaf thickness and size. A decrease in leaf expansion and thickness of the infected leaves indicates that both cell division and enlargement were significantly affected. The leaf area and specific leaf area (SLA) decreased significantly in infected plants. This can be explained by a stronger phytoplasma effect on the linear leaf growth than on the accumulation of biomass. The infected leaves increased R_D and content of the amino acids in leaves, corresponding to the idea of accelerated leaf ageing in phytoplasma infected plants (Lepka *et al.* 1999).

The marked reduction of total Chl in phytoplasma-infected leaves was due to the decrease of both Chl *a* and Chl *b* contents (Table 1). Reinero and Beachy (1986) observed similar results in TMV-infected tobacco leaves. The lower F_v/F_m ratio observed in the infected leaves was due to a decrease in variable fluorescence (F_v) without increasing the F_0 level (Table 1). This is characteristic for inhibition of the donor side of PS2 (Allakhverdiev *et al.* 1987, Šetlík *et al.* 1990). Reinero and Beachy (1986) observed similar effects in TMV-infected tobacco leaves. The whole electron transport chain (H_2O→MV) measured in chloroplasts was markedly inhibited in phytoplasma-infected plants, much more than the PS1 activity (DHQ→MV). In contrast to PS1, the PS2 activity measured with DBMIB and DCPIP was significantly inhibited (Table 1). The reduction occurred prior to the site of electron donation by DHQ (see H_2O→DBMIB and DHQ→MV) thus implicating PS2 as the site where perturbation of electron transport occurred. DPC, an artificial electron donor for PS2, donates electrons close to the PS2 reaction centre (Packham *et al.* 1982). PS2 activity significantly restored in phytoplasma-infected leaves by an artificial donor (DPC) suggests that the damage might be located on the oxidising side of PS2, in the oxygen-evolving system (Table 1).

Phytoplasma increased the content of both sugars and total saccharides in leaf tissues (Table 1). Our results are in accordance with those of Lepka *et al.* (1999) in periwinkle and tobacco plants. The accumulation of saccharides could explain some of the symptoms observed after phytoplasma infection. The loss of Chl is accompanied by a general saccharide-mediated repression of genes involved in photosynthesis (Krapp *et al.* 1993, Lerchl *et al.* 1996). Phytoplasma infection increased amino acid contents in apple leaves. These increased amino acid contents in phytoplasma-infected plants are in contrast to the results of Catlin *et al.* (1975), who indicated that translocation of amino acids is impaired in affected pear trees. The reason for the blockage of phloem transport is not known. Catlin *et al.* (1975) and Braun and Sinclair (1978) showed that the blockage occurs before visible changes are seen in the phloem. In addition to physical problems due to the obliteration of sieve tubes in the phloem complex, subtler changes in the fine structure of bioenergetics of the phloem might occur.

Total soluble protein content and RuBPC activity were reduced markedly in phytoplasma-infected leaves (Table 1). The relatively low content of soluble proteins

Table 1. Effect of phytoplasma infection on growth, biochemical characteristics, chlorophyll (Chl) *a* fluorescence kinetics, and electron transport activities [whole chain ($H_2O \rightarrow MV$), PS2 ($H_2O \rightarrow$DBMIB; $H_2O \rightarrow$DCPIP; DPC→DCPIP), and PS1 (DHQ→MV)] [mmol(O_2) kg^{-1} (Chl) s^{-1}], and nitrate reductase and RuBPC activities of field grown apple leaves. Values in parentheses are percent reduction or increase with reference to healthy controls. Each value represents the mean of 10 independent measurements for growth and 5 replicates for biochemical assays ±SE.

Parameter		Control	Phytoplasma infected
Leaf fresh mass	[mg $plant^{-1}$]	724.3±30.2	478.0±18.9 (–34)
Leaf dry mass	[mg]	92.6±3.6	58.3±2.9 (–37)
Specific leaf area (SLA)	[m^2 kg^{-1}(leaf)]	68.4±3.1	57.5±2.4 (–16)
R_D	[μg(CO_2) kg^{-1} s^{-1}]	142.8±6.4	191.4±9.2 (+34)
P_N	[nmol(CO_2) kg(protein)$^{-1}s^{-1}$]	32.5±1.5	15.6±0.7 (–52)
Chl *a*	[g kg^{-1}(fr.m.)]	2.19±0.10	0.98±0.04 (–55)
Chl *b*	[g kg^{-1}(fr.m.)]	0.83±0.03	0.47±0.02 (–43)
Total Chl (*a*+*b*)	[g kg^{-1}(fr.m.)	3.02±0.12	1.45±0.06 (–52)
Chl *a*/*b*		2.62±0.11	2.08±0.09
Soluble proteins	[g kg^{-1}(fr.m.)]	52.8±2.1	29.0±1.5 (–45)
Sugars	[g kg^{-1}(fr.m.)]	38.4±1.8	53.0±2.4 (+38)
Starch	[g kg^{-1} (fr.m.)]	21.2±1.0	35.0±1.6 (+65)
Total saccharides	[g kg^{-1}(fr.m.)]	59.6±2.4	88.2±4.2 (+48)
Amino acids	[g kg^{-1} (fr.m.)]	10.8±0.5	13.5±0.6 (+25)
F_0		1.40±0.06	1.40±0.05
F_v		5.70±0.23	2.00±0.09 (–65)
F_v/F_m		0.80±0.03	0.60±0.02 (–27)
F_v/F_0		4.07±0.16	1.42±0.04 (–65)
Whole chain [$H_2O \rightarrow MV$]		142.4±6.8	39.8±1.5 (–72)
PS2 [$H_2O \rightarrow$DBMIB]		164.2±8.0	57.5±2.6 (–65)
PS2 [$H_2O \rightarrow$DCPIP]		158.8±7.2	60.3±2.4 (–62)
PS2 [DPC→DCPIP]		172.2±7.1	141.2±6.2 (–18)
PS1 [DHQ→MV]		426.2±20.4	383.6±18.6 (–10)
Nitrate reductase	[nmol(NO_2^-) kg^{-1}(fr.m.) s^{-1}]	68.16±3.10	42.25±1.90 (–38)
	[nmol(NO_2^-) kg^{-1}(protein) s^{-1}]	101.60±4.9	40.60±1.90 (–60)
	[nmol(NO_2^-) kg^{-1}(Chl) s^{-1}]	39.21±1.70	11.76±0.50 (–70)
-15 mM KNO_3	[nmol(NO_2^-) $kg^{-1}s^{-1}$(fr.m.)]	46.50±2.10	28.83±1.20 (–38)
+15 mM KNO_3	[nmol(NO_2^-) $kg^{-1}s^{-1}$(fr.m.)]	78.12±3.50	32.81±1.40 (–58)
RuBPC	[nmol(CO_2) kg^{-1}(protein) s^{-1}]	61.25±2.90	26.91±1.20 (–56)
	[nmol (CO_2) kg^{-1}(Chl) s^{-1}]	38.88±1.70	14.77±0.70 (–62)

in phytoplasma-infected plants may have been due to decrease in the synthesis of RuBPC, the major soluble protein of leaf. A loss of protein in phytoplasma-infected leaves would partially account for damaged chloroplasts or could be the result of inhibition of protein synthesis. The reduction in the overall photosynthetic rates correlates well with the decreased RuBPC activity in phytoplasma infected leaves. A marked reduction of RuBPC activity was observed in phytoplasma-infected leaves (Table 1). Such reduction was due to inhibition of protein synthesis induced by phytoplasma infection. The reduction in $^{14}CO_2$ fixation of phytoplasma-infected plants was probably an indirect effect due to the destruction of photosynthetic pigments (as evidenced by the present results) (Table 1).

Plants infected by phytoplasma had a relatively low nitrate reductase activity (Table 1). A drastic reduction of *in vivo* nitrate reductase activity of phytoplasma infected plants may reflect a balance between synthesis or inactivation 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 storage pool to an active cytoplasmic pool accessible to the enzyme. The inhibition of nitrate reductase activity might also be due to the inhibition of protein synthesis or to a decreased rate of photosynthetic supply in the phytoplasma-infected leaves.

Thus, our results suggest that the decrease of growth, chlorophyll, P_N, RuBPC and nitrate reductase activities, and the increase of contents of sugars, starch, and total saccharides in infected leaves was due to the phytoplasma inhibition induced rapid senescence or ageing in apple leaves.

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