CHLOROPHYLL FLUORESCENCE IN *in vitro* CULTIVATED APPLE

*FLUORESCÊNCIA DE CLOROFILAS EM MACIEIRA CULTIVADA **in vitro***

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ABSTRACT

*Chlorophyll fluorescence is a useful tool because it allows the interpretation of the aspects related to the photosynthesis. The aim of this study was to examine the use of the fluorescence as an indicator of the photosynthetic efficiency of rootstock (**Malus domestica** - cv M9) cultivated **in vitro**. The explants (nodal sections with two lateral buds) were kept in growth chamber with 16 hours light and PF of 15 µmol $m^{-2}s^{-2}$. The plants were kept for 55 (T1) and 90 (T2) days, and the plants with 55 days were transferred to PF of 150 µmol $m^{-2}s^{-2}$ for 2 h before the determination (T3). The leaves were maintained in the dark for 15 minutes for determining the parameters of the dark adapted state. Then the leaves were submitted to the actinic light for 5 minutes for the determination of the parameters of the light adapted state. It was observed that T2 presented higher values of F_m; F_v/F_m and F_v/F_o, and lower F_o/F_m. The coefficients q_N and NPQ were higher in T2, indicating higher energy dissipation in non-photochemical processes; q_P did not differ between the treatments T2 and T3. Comparison of the quenching coefficients between T1 and T3 indicates that the **in vitro** culture conditions for T1 imposes limitations in the biochemical phase.*

*Key words: Photosynthetic apparatus, Quenching fluorescence coefficients, **Malus domestica**.*

RESUMO

*A fluorescência das clorofilas é uma ferramenta muito utilizada porque permite a interpretação de aspectos relacionados à fotossíntese. O objetivo deste estudo foi examinar o uso da fluorescência como indicador da eficiência fotossintética em brotos de macieira (**Malus domestica** - cv M9) cultivados **in vitro**. Os brotos foram mantidos em câmara de crescimento com fotoperíodo de 16 horas e fluxo de fótons de 15 µmol m^{-2} s^{-1} por 55 (T1) e 90 (T2) dias. Após 55 dias os brotos do T1 foram transferidos para fluxo de fótons de 150 µmol m^{-2} s^{-1} por 2 horas antes da determinação da fluorescência (T3). Em todos os tratamentos, para a determinação dos parâmetros do estado adaptado ao escuro e à luz, as folhas foram primeiramente mantidas no escuro por 15 minutos e em seguida submetidas à luz actínica por 5 minutos para determinação dos respectivos parâmetros. Verificou-se que T2 apresentou maiores valores de F_m; F_v/F_m e F_v/F_o e menor F_o/F_m. Os coeficientes q_N e NPQ foram maiores em T2, indicando maior dissipação de energia em processos não-fotoquímicos; q_P não apresentou diferenças entre os tratamentos T2 e T3. A comparação dos "quenching" entre T1 e T3 indicou que, para T1, as condições de cultivo **in vitro** impõem limitações na fase bioquímica.*

*Palavras-chave: aparato fotossintético, coeficiente de extinção, **Malus domestica**.*

Abbreviations: PS II - photosystem II; Q_A - primary acceptor of PS II; BAP – 6-benzyladeninepurine; MS – Murashige and Skoog (1962); DAS - dark state adapted; LAS - light state adapted; F_o - minimal fluorescence at DAS; F_m - maximal fluorescence at DAS; Φ_{Po} - maximal quantum yield photochemical of FSII [F_v/F_m]; F_v - variable fluorescence at DAS [F_m-F_o]; F_p - maximal fluorescence when actinic light; F_s - steady state fluorescence; F'_m - maximal fluorescence at LAS; F'_o - minimal fluorescence at LAS; F'_v - variable fluorescence at LAS [F'_m - F'_o]; q_n - photochemical quenching of F_v to F'_v [(F_v – F'_v) / F_v]; q_p - non-photochemical quenching of F'_m to F_s [1 – ((F_s – F'_o) / F_v)]; NPQ - total non-photochemical quenching [(F_m – F'_m) / F'_m]; Rfd - rate decrease fluorescence [(F_p – F_s) / F_s]; PF - flux photon; PAR - photosynthetic active radiation;

INTRODUCTION

Photon energy absorbed by photosynthetic pigments drives primary photochemical reactions. Energy conversion normally takes place with a high efficiency exceeding 90% of absorbed quanta (SCHREIBER et al., 2000). If charge separation does not occur, excited pigment molecules return to ground level and absorbed energy is released as heat (radiation-less deactivation) and/or chlorophyll fluorescence (KRAUSE & WEISS, 1991). Fluorescence is an easily measured signal, and different components of fluorescence, including photochemical and non-photochemical quenching, can be measured with the appropriate instrumentation. From these parameters, numerous aspects of the photosynthetic process can be analyzed (ROHÁCEK, 2002). Chlorophyll *a* fluorescence, though corresponding to a very small fraction of the dissipated energy from the photosynthetic apparatus, is widely accepted to provide an access to the understanding of its structure and function. There is a general agreement that at room temperature chlorophyll *a* fluorescence of plants in the spectral region 680-740 nm is almost exclusively emitted by PS II and it can therefore serve as an intrinsic probe of the fate of its excitation energy (ROHÁCEK, 2002; ROHÁCEK & BARTAK, 1999) . Both the spectra and the kinetics of chlorophyll *a* fluorescence have been proven to be powerful, non-invasive tools for these investigations (KRAUSE & WEISS, 1991, ROHÁCEK, 2002, BARBAGALLO et al., 2003).

Micropropagation has been extensively used for the rapid multiplication of many plant species (BOBROWSKI et al., 1996; DEL PONTE et al., 2001). Although this technique allows efficient multiplication of many plants, it is still confronted with many questions, especially the photosynthetic capacity of *in vitro* cultivated plants (SLAVTCHEVA & DIMITROVA, 2001). The physical and chemical characteristics, between their irradiance levels of the microenvironment, provided during proliferation phase affect the development of plant material *in vitro* (SMITH & SPOMER, 1995; KOZAY & GEONG, 1993). The relation between culture media and chlorophyll fluorescence of *Pinus radiata* cultivated *in vitro* demonstrated an increase of the efficiency photosynthetic of the sprouts when cultivated in sugar-free culture media

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(AITKEN-CHRISTIE et al., 1992). It was observed that *in vitro* plants have lower net photosynthetic and dark respiration rate than *ex vitro* plants (SLAVTCHEVA & DIMITROVA, 2001; SERRET et al., 2001). This is due to low light intensities, low CO_2 concentrations and inhibition of photosynthesis by low sugar concentration in the medium (AMANCIO et al., 1999; INFANTE et al., 1989). But research about *in vitro* culture demonstrated that many tissues with chlorophyll have photosynthetic capacity identical to the plant cultivated *ex vitro*, when cultivated on photoautotrophic conditions (KITAYA et al., 1995).

This study was carried out to evaluate the chlorophyll fluorescence in apple rootstoch (*Malus domestica* cv M9) cultivated *in vitro* for different periods.

MATERIALS AND METHODS

Plant material and in vitro establishment

Apple rootstock (*Malus domestica* - cv M9) *in vitro* originated culture was used for this experiment. To establish *in vitro* cultures, nodal sections with two lateral buds were cultured in a modified MS (MURASHIGE & SKOOG, 1962), with nitrogen at third strength; mio-inositol 100 mg L^{-1}; sucrose 30 g L^{-1}, BAP 0.8 mg L^{-1}, and agar 6 g L^{-1}. The media pH was adjusted to 5.8 before autoclaving. Environmental conditions during multiplication phase were 25±1 ºC (day) and 23±1 ºC (night); 16 h light and 15 µmol $m^{-2}s^{-2}$ of flux photon (PAR). The plants were kept in this condition for 55 (T1) and 90 (T2) days. Another treatment (T3) consisted in the transference of the plants with 55 days to higher light (150 µmol $m^{-2}s^{-1}$ flux photons - PAR) during 2 hours immediately prior to the fluorescence determination.

Chlorophyll Fluorescence

Chlorophyll *a* fluorescence parameters were measured in freshly detached leaves from *in vitro* culture. The leaves were kept for 15 minutes in the dark prior to measurements. Modulated fluorescence was measured using a pulse amplitude modulation system (FMS 2, Hansatech, King's Lynn, UK) chlorophyll fluorometer. Minimal fluorescence, F_o, was measured in 15 minutes dark-adapted leaves using light of < 0.1 µmol m^{-2} s^{-1} and maximal fluorescence (F_m), was measured after 0.8 s saturating pulse (> 3500 µmol m^{-2} s^{-1}) in the same leaves; variable fluorescence ($F_v = F_m - F_o$); the photochemical efficiency of PSII (F_v/F_m ou Φ_{Po}) were calculated for dark-adapted leaves (DAS); F_o/F_m (basal quantum production of the processes non-photochemical in PS II), F_v/F_o (maximum efficiency of the photochemical process in PS II). It was determined F_p (the maximum fluorescence when the actinic light is switched on); and, in actinic light continuum (LAS), steady state fluorescence yield (Fs) was measured after 15 minutes; maximal fluorescence in LAS (F'_m) after saturating pulse (> 3500 µmol m^{-2} s^{-1}) and minimal fluorescence in LAS (F'_o) measured when actinic light was turned off, were determined. The fluorescence values measured in LAS were used to calculate the following parameters: variable fluorescence in LAS ($F'_v = F'_m – F'_o$); photochemical quenching, $q_p = (F_v - F'_v)/F_v$ [3]; 1-qP (indicative of the state of reduction of Q_A in the light); non-photochemical quenching, $q_n = 1- (F_s - F'_o)/F_v$ [3]; total non-photochemical quenching (NPQ = $[(F_m - F'_m)] / F'_m$) (term indicative of excess of dissipation of energy in the form of heat in PS II) and ratio decreased to steady-state fluorescence (Rfd = $[(F_p - F_s)] / F_s$), also named "vitality index" (ROHÁCEK & BARTAK, 1999), and this parameter has been often used as an indicator of the "vitality" of plants and evolution of stress. The value of fluorescence parameters were normalized to F_o according (ROHÁCEK & BARTAK, 1999).

RESULTS AND DISCUSSION

When the leaves were adapted to the dark (DAS) all PS II reaction center and the electrons acceptor were oxidated (Table 1). T2 presented significantly higher values of F_m, F_v/F_m and F_v/F_o, associated to smaller values of F_o/F_m (basal quantum production of the processes non-photochemical in PS II) (ROHÁCEK, 2002). These results indicate that the plants cultivated *in vitro* for longer period presented greater absorption efficiency and exploitation light in DAS (acceptor PS II total open). These results indicate that when the plants were micropropagated longer, they presented a higher maximum photochemical efficiency associated to a smaller thermal energy dissipation of PS II (F_o/F_m) (ROHÁCEK, 2002). In *in vitro* plants of *Gardenia jasminoides* the F_v/F_m and F_v/F_o rate increased, whereas F_m and F_O significantly decreased, depending on the culture stage (multiplication and root induction) (SERRET et al., 1997). On the other hand, the values observed by these autors are similar to the ones determined in this work (F_v/F_m ranges between 0.710 and 0.806). These results can be attributed to the change in the capacity of exploitation of the photochemical energy in the dark adapted state.

Table 1 - Fluorescence parameters at dark adapted state in *in vitro* cultivated apple shoots.

Treatment	F_o	F_m	F_v/F_m	F_o/F_m	F_v/F_o
T_1 (55 days *in vitro*)	1 (714) [1]	3.70 b [2]	0.717 b	0.283 a	2.659 b
T_2 (90 days *in vitro*)	1 (380)	5.20 a	0.806 a	0.196 b	4.230 a
T_3 (55 days *in vitro* + additional light)	1 (785)	3.52 b	0.710 b	0.289 a	2.578 b

1 - F_o originals are in parentheses

2 - Different letters indicate significant differences among treatments, using Tukey test at 5% significance level.

The high value of F_m found for T2 was associated with the low relative values of the initial fluorescence for this treatment (380 relative unit). The higher values of F_o indicate obstruction in the transference of energy for the center of reaction of FS II. On the other hand, F_m is obtained when the PS II reaction center is closed (Q_A total reduced, i.e. $q_n = q_p = 0$) (VAN KOOTEN & SNEL, 1990) associated to the Q_A reoxidation rate (ROHÁCEK & BARTAK, 1999) and the photochemical process in FS II is completely saturated (VAN KOOTEN & SNEL, 1990). Higher values of F_o, and lower F_m can indicate a higher energy dissipation in PS II antenna system. Comparing treatments T1 and T3 (Table 1), the fluorescence parameters in DAS was not different, indicating that the supply of additional light for 2 hours, did not modify the absorption characteristic and the use of radiant energy, evidencing mainly by similar values of the relations F_v/F_m and F_v/F_o. The maximum photochemistry efficiency for the three treatments is a little inferior to the one in the literature, since

the ratio F_v/F_m typically ranges between 0.75 and 0.85 for non-stressed leaves *ex vitro* (BJÖRKMAN & DEMMING, 1987). However it must be highlighted that the literature is rich regarding field data, but limited for *in vitro* culture. In young apple tree leaves, PS II non-ciclic electron transport and F_v/F_m began to decrease at the PF of 900 µmol m^{-2} s^{-1} and 1200 µmol m^{-2} s^{-1}, respectively, and F_v/F_m was originally used to define photo-inhibition *in vitro* (HUSEN & DEQUAN, 2002).

The non-photochemical coefficients q_N and NPQ (Table 2) were higher in plants cultivated for long time (T2), indicating a greater dissipation of the energy absorbed in non-photochemical processes, but the photochemical quenching coefficient (q_p) was not different between the treatments T2 (0.649) and T3 (0.579), and higher than treatment T1 (0.382). This fact suggests that the supply of additional light for the plants cultivated for shorter period of time increases the efficiency of the use of energy absorbed and converted into NADPH and ATP, that will be used in CO_2 fixation. The relation between q_P/q_N (Table 2) allows an estimate of how the energy absorbed for PS II is being distributed between the photochemical and non-photochemical effect. This relation is lower in T2 treatment (1.081), but in this treatment, it was observed that q_N and q_P values were higher. This suggests that plants cultivated *in vitro* for a shorter time use more efficiently the energy. An increase was observed in q_N after transferring rose shootlets rooting *in vitro* to the greenhouse (GENOUD et al., 1999), suggesting that a part of the energy was used to protect photosystems, this dissipation process involved the xanthophylls cycle with the formation of zeaxanthin and indicating photoprotective non-radiative dissipation of excitation energy.

During the light period, the photosynthetic apparatus passes gradually from DAS to LAS. The LAS is characterized by continuos synthesis of ATP, NADPH, and concurrent fixation of CO_2. Chlorophyll fluorescence parameters in the light adapted state allows the accomplishment of the quenching of fluorescence, that is the change in fluorescence emission of maximal fluorescence to steady-state level, and these changes are intimately related to changes in both the rate of oxygen evolution (DEMMING & BJÖRKMAN, 1987) and carbon metabolism. Fluorescence may be quenched by photochemical and non-photochemical processes (ROHÁCEK, 2002; HUSEN & DEQUAN, 2002). The first occurs as a result of oxidation of PS II electron acceptors which allows an increase in the rate of dissipation of excitation energy via photochemistry. The second is an increase in the rate of chlorophyll deexcitation via radiationless decay (BRADBURY & BAKER, 1984). There are many interpretations about non-photochemical quenching in the literature, but it has been attributed mainly to the: generation of proton gradients across the tylakoid membranes, the possible involvement of reduced phaeophytin, cation electronchemical gradients and phosphorylation of thylakoid polypeptides (for review ROHÁCEK, 2002; BRADBURY & BAKER, 1984; KRAUSE et al.; 2002). The NPQ parameter (BILGER & BJÖRKMAN, 1990) derived from the Stern-Volmer equation based on a model of the antenna organization (Knox, 1975), and NPQ is often used as an indicator of the excess-radiant energy dissipation to heat in PS II antenna complexes in LAS (DEMMING-ADAMS et al., 1996).

The coefficient 1-qP (Table 2), in the T2 treatment (0.351) has value equal to T3 (0.421), but they were significantly different from the T1 (0.617). This fact could indicate that in new plants, without additional light, Q_A in LAS is more open (less reduced) than in other conditions. This coefficient can indicate the state of Q_A reduction or it can also tell the degree of closed PS II center of reaction (ROHÁCEK, 2002). The comparison of the quenching coefficients between the treatments that had differed in the additional supply of light (T1 and T3), can indicate that the *in vitro* culture conditions for T1 imposes limitations in the biochemical phase of the photosynthesis, associated to the limitation of the activity of the RUBISCO for the Rubisco ativase, indicating a reduction of the photosynthetic activity, fact that can be confirmed by the rise of the rate decrease of fluorescence (Rdf) verified in treatment T1 in comparison with T3, since this parameter is an indicative of the photosynthetic potential of the photosynthetic apparatus DEMMING & BJÖRKMAN, 1987) or direct measure of the photosynthetic activity (ROHÁCEK, 2002).

Table 2 - Fluorescence parameters at light adapted state in in *in vitro* cultivated apple shoots.

Treatment	q_N	q_P	q_P/q_N	$1-q_P$	NPQ	Rdf
T_1 (55 days *in vitro*)	0.283 c[1]	0.382 b	1.349 a	0.617 a	0.248 c	0.297 c
T_2 (90 days *in vitro*)	0.600 a	0.649 a	1.081 b	0.351 b	1.037 a	1.272 a
T_3 (55 days *in vitro* + additional light)	0.467 b	0.579 a	1.239 a	0.421 b	0.568 b	0.703 b

1 - Different letters indicate significant differences among treatments, using Tukey test at 5% significance level. (n=4)

The rate of decrease of fluorescence associated with the F_v/F_m, which was higher in old plant (90 days), allows to conclude that the plants cultivated by shorter period of time can present potential of use of the absorbed energy similar to plants cultivated for longer period of time, since they received higher light than the ones that were kept in the growth chamber.

CONCLUSION

Apple shoots grown *in vitro* have a functional photosynthetic apparatus. Sprouts of apple cultivated *in vitro* for shorter period of time present characteristics of the chlorophyll fluorescence in DAS, which indicate low efficiency of the use of the radiant energy. This fact could be associated to the limitations of the photosynthetic apparatus. The supply of additional light, higher than the used in growth chamber, increases the photosynthetic activity of the plants cultivated *in vitro* for shorter period of time.

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