EVALUATION OF THE RESPONSE OF MICROPROPAGATED PEACH ROOTSTOCK 'CADAMAN' AND CV. 'CRESTHAVEN' TO MYCORRHIZATION USING CHLOROPHYLL *A* FLUORESCENCE METHOD

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ABSTRACT

Micropropagation is used to produce certified stock for nurseries. The plants produced with this method are free from any microorganisms, including these beneficial. The technology of mycorrhization has been used during the last years in a number of micropropagated horticultural crops to introduce symbiotic microorganisms. The aim of the study was to evaluate the activity of photosynthetic apparatus of peach rootstock 'Cadaman' and cultivar 'Cresthaven' grafted on it to inoculation with native endomycorrhizal fungi isolated from Hungarian orchard (M109 and M116) or with commercial inoculum (BEG53). Response of photosynthetic apparatus to mycorrhization was determined by chlorophyll *a* fluorescence method with MINI-PAM apparatus. Growth and nutritional status of micropropagated peach rootstock 'Cadaman' and scion cultivar 'Cresthaven' were also determined.

During the three years of the experiment, phenological, physiological and morphological status of the plants changed from year to year. Whereas the young plants were effectively protected against photoinhibition by M116 inoculation, the older plants were most sensitive to BEG53 and M109 treatment. Area of leaves of the plants mycorrhized with M116 was larger than in the other treatments. Moderately positive effect of M116 was found in content of nitrogen in leaves of 'Cresthaven' cultivar.

It is concluded that a mix of native fungi with selected *Glomus* species (BEG53) could be used as a new formula of inoculum for peach nursery production.

Keywords: AMF inoculum, nutrients, growth, *Prunus persica,* rapid light curves (RLC)

INTRODUCTION

Among other applications, micropropagation is used to produce certified stock for nurseries. The plants produced with this method are free from any microorganisms, including these symbiotic which are accessory in nutrients and water uptake and provide some protection against certain biotic and abiotic stresses. Thus, a reintroduction of symbiotic micro-organisms is needed to facilitate a smooth plant transition from pro-tective *in vitro* conditions to the field (Sbrana et al., 1994; Monticelli et al., 2000; Taylor and Harrier, 2000; Borkowska, 2001).

The mycorrhization technology has been used during the last years in a number of micropropagated horticultural crops in order to improve their survival rate and growth during *post vitro* stages (Schubert and Lubraco, 2000; Rai, 2001; Borkowska, 2002, 2005). Mycorrhizal fungi help to recover the biological activity and physical properties of a soil and they are active in mobilising minerals (Camprubi et al., 1993; Diaz and Honrubia, 1993; Calvet et al., 2001). Thus, mycorrhizas are important in establishment and subsequent growth of horticultural crops, especially these cultured in intensive agriculture systems, where soil structure, chemistry and microflora is frequently degraded.

Colonization and penetration of plant roots with mycorrhizal fungi cause an increase in the demand for carbohydrates. Positive effects of mycorrhiza occur when the carbon costs of the association are balanced by photosynthetic activity of the host plant. Since photosynthetic activity of micropropagated plants is low, developing mycorrhiza could "down regulate" photosynthetic process and thus play temporary a role of a semiparasite. However, when the development of mycorrhizal symbiosis is balanced, an increase of net photosynthesis of plants colonised by arbuscular mycorrhizal fungi (AMF) is observed (Vodnik and Gogala, 1994; Martins et al., 1997; Staddon et al., 1999; Estrada-Luna et al., 2000; Mortimer et al., 2005; Wu and Xia, 2006). The photochemical performance of the host plants has also been activated after establishment of mycorrhizal symbiosis (Borkowska, 2002; Tsimili-Michael and Strasser, 2002; Borkowska, 2005; Pinior et al., 2005; Borkowska, 2006).

The measurements of chlorophyll *a* (Ch) fluorescence provide an opportunity for ecophysiological research through analysing the changes in activity of photosynthetic apparatus under biotic and abiotic stresses. If mycorrhization of micropropagated plantlets is recognized as a biotic stress, it may be expected that measurements of Ch fluorescence could help to determine effectiveness of mycorrhizal symbiosis (Bolhar-Nordenkampf et al., 1989; Rascher et

al., 2000; Lichtenthaler et al., 2005; Ralph and Gademann, 2005).

A MINI-PAM fluorometer (Waltz, Germany) based on the Pulse Amplitude Modulation (PAM)-/saturating flash method makes rapid measurements of basic (standard) parameters of dark-adapted and lighted samples: maximal (Fv/Fm) and effective quantum yield (Y) named also Genty parameter (Genty et al. 1989), respectively. Although the importance of these two parameters is well established in ecophysiology, MINI-PAM has been developed for measuring additional parameters as well, such as electron transport rate (ETR), saturating photosynthetically active radiation (PPFD) and other characteristics as given by rapid light-response curves (RLC) and rapid light-response curves plus recovery (RLC+REC.). Measurements of RLC and RLC+REC lead to a deeper insight into parameters which are not related to the momentary light conditions but to the range of physiological plasticity of a plant (White and Critchley, 1999; Rascher et al., 2000; Serodio et al., 2006; Belshe et al., 2007). MINI-PAM has also a program for quenching analysis. Photochemical quenching has a clear meaning and it refers to photosynthetic electron transport. It is marked commonly as qP. Non-photochemical quenching can be separated into several components. In the literature its analysis is confusing because of diverse nomenclature and a set of parameters termed "quenching coefficients" that has been used (Van Kooten and Snell, 1990; Baker and Rosenqvist, 2004; Zhu et al., 2004; Dreuw et al., 2005; Liu et al., 2007). MINI-PAM was programmed to distinguish non-photochemical quenching characterized by two parameters: qN and NPQ. NPQ has been referred as indicator of dissipation of light energy as heat.

The objectives of the experiment were to assess the reaction of micro-propagated 'Cadaman' peach rootstock and peach cultivar 'Cresthaven' grafted on 'Cadman', growing in the nursery, to inoculation with native and com-mercial arbuscular mycorrhizal fungi (AMF).

MATERIAL AND METHODS

Plant material and mycorrhizal inoculum

'Cadaman' is a patented Hun-garian-French-bred vegetative peach rootstock resistant to nematodes, which is widely used in Europe. Its shoot cultures were established from shoot apices isolated from virus-free mother plants at a time of intensive growth (May, 2000). Multiplication was carried out on modified Mura-shige-Skoog's medium (Mura-shige and Skoog, 1972). The shoots were rooted *in vitro*. Light and tempera-ture conditions during multi-plication were set up according to the standard method (22^{o}C, 16/8 hours photo-period, 25 µmol m^{-2} s^{-1} PPFD). Early in the spring 2002, the rooted shoots were removed from culture vessels and inoculated with various strains of AMF (*arbuscular mycor-rhizal fungi*). The plantlets were acclimatised in a greenhouse and in the early summer

moved outdoors and kept under moderate shade. They were regularly watered. In the spring of the next year (2003), the plants were transferred into the field. Planting density was 120 x 20 cm. In the summer 2003, the rootstocks were budded with cv. 'Cresthaven'. During 2004, crown of the cultivar was formed. Irrigation (sprinkler) was applied only in the first year of rootstock growing. Fertilization with 40 t/ha of organic manure was applied during soil cultivation (35 cm deep), prior to planting. Foliar fertilization with 0.5% "Biomit Plus", containing Ca, Mg and micro-elements as well as some plant extracts, was applied three times a year. Weeds were removed mechanically. No herbicides were used. In 2005, the trees were planted in the orchard on degraded sites classified as "replant disease soil". This part of experiment is not included in this publication.

Two of the inocula used (M116 and M109) were based on fungi selected from Hungarian orchards and they were prepared for the application in the Research Institute for Fruit-growing and Ornamentals. Inocula consisted of roots of host plants (*Tagetes sp.*) together with fungal body (propagules and spores). The fungi from both inocula were preliminarily identified as *Glomus spp.* The third inoculum was based on fungi from Glomales Gene Bank in France (BEG53). All the inocula were added to the substrate used for plantlet's acclimatization at 5% (v/v).

The growth of plants was measured two times during the experiment. The effect of mycorrhization on mineral status of the trees was evaluated in the third year of the experiment. Samples of the leaves of the grafted cultivar were collected and the content of N, P, K, Ca and Mg was determined using the standard methods.

Chlorophyll *a* fluorescence and chlorophyll content

Chlorophyll *a* (Ch) fluorescence was measured with the miniaturized pulse-amplitude-modulated photo-synthesis yield analyzer (MINI-PAM, Walz, Germany) equipped with the leaf clip holder. The MINI-PAM records all relevant fluorescent parameters, actinic irradiance and leaf temperature and calculates ETR by the formula:

ETR = Yield x PAR x 0.5 x 0.84, where:

- Yield (Y, Genty-parameter, marked also ΦPSII) – effective quantum yield;
- PAR – the actinic irradiance in μmol m^{-2} s^{-1} (measured with a sensor on the leaf holder);
- 0.5 is a multiplication factor because transport of a single electron requires the absorption of 2 light quanta;
- 0.84 is the specific fraction of incident quanta absorbed by the leaf (ETR-factor).

ETR is expressed as μmol of electrons m^{-2} s^{-1} (Hofstraat et al., 1994; White and Critchley, 1999).

Values of qP and qN vary between 0 an 1 whereas NPQ can assume values between 0 and 10.

The RLC, generated automatically by MINI-PAM, consists of 9 different and increasing actinic irradiances of 10 s duration, each separated by 0,8 s of saturating flash with actinic light from 0 to 1000 of μmol. m^{-2} s^{-1} in 2002 and from 0 to 800 μmol m^{-2} s^{-1} in 2003. Light was provided by internal halogen lamp using the fibre optic (5 mm in diameter) and the leaf clip holder. Kinetic of changes in value of ETR and quenching coefficients occurring in the leaves following changes in irradiance, were recorded automatically. Information on the dark-recovery was obtained by the function "rapid light curve plus recovery" (RLC+REC). The light program of RLC+REC is the same as for RLC but after termination of the last light period, recovery in the darkness is assessed. Six consecutive saturation pulses are applied at 10s, 30s, 60s, 2 min, 5 min and 10 min after switching off the light.

Shoots/leaves for measurements were brought from the field and acclimatized for 24 hours to standard laboratory conditions. Before starting the measurements the leaves were darkened at least for one hour.

Leaf chlorophyll content and leaf area were measured on the same leaves which were used for Ch fluorescence measurements. Chlorophyll content was estimated with Chlorophyll Content Meter CCM-200 (OPTI-SCIENCE, USA). This apparatus measures the chlorophyll absorbance and calculates CCI (chlorophyll content index), which is proportional to the concentration of chlorophyll in the sample. Leaf area was measured with portable Leaf Area Meter ADC (BioScientific Ltd).

For each of the treatments, 30 leaves (young but fully developed) were sampled at random from the upper part of the shoots from 15-30 plantlets/trees. Ch fluorescence measurements were conducted on the upper (adaxial) side of the leaf blade, between the main vein and the edge of the leaf.

Data were analysed with one-way ANOVA and Tukey's or LSD tests were performed to evaluate significance of differences at $p = 0.05$.

RESULTS

Characterization of the plant model

During the three years of the experiment, phenological, physiological and morphological status of plants changed from year to year. In the year of transferring the plantlets from *in vitro* to *ex vitro* conditions (2002), inoculation of sterile roots by mycorrhizal fungi could be recognized by the plants as a biotic stress. With development of a "dialog" between partners, mycorrhizal association was established and in the next year (2003) both partners become "biological unit". Biological status of the trees changed again in 2004 when 'Cresthaven' cv. developed from the grafted bud into the scion crown. Thus, evaluation of the host plant response to fungal partner has been done and assessed separately for each year.

Rapid light curves

In 2002 light harvesting effi-ciency increased with increasing PPFD reaching ETRmax at 150 µmol m^{-2} s^{-1}. Value of ETRmax, also called the light saturation parameter (Kim et al., 2006), was higher in M116-treated plants than in these inoculated with BEG53 and in the control (not-AMF plants). With irradiance further increasing, ETR value remained more or less on the same level (plateau) (Fig. 1). Decreasing phase was not measurable, what means that photo-synthetic apparatus was not photo-damaged. Low values of RLC-NPQ for plants mycorrhized with M116 show high ability of the photo-systems to utilize absorbed light energy (low portion of light energy was converted into heat). In contrast, mycorrhization with BEG53 increased photosystem's ability to dissipate the excess of light (Fig. 2). RLC-qP indicated higher photochemical quenching in the wide range of PPFD of mycorrhized plants than in these non-mycorrhized. At the end of the light period value of qP was about 0.200, which means that photosynthetic apparatus was still active (not photodamaged) (Fig. 3).

In 2003 the plants were growing in the field (they were planted in autumn 2002) and in July were budded with 'Cresthaven' cv. In this year, the trees mycorrhized with M109 were included into measurement schedule. Data presented on Fig. 4, 5 and 6 consist of two parts: A – RLC (light phase with increasing light irradiance) and B – REC (recovery during light period).

At the beginning of the light period RLC-ETR curves ran parallel for all the treatments and then rapidly diverged. Rising part of the slope (beginning) shows the high efficiency of light harvesting by plants mycorrhized with BEG53 and lower for these mycorrhized with M116. Decreasing slope at the end of the light period indicates photoinhibition; weaker for BEG53- and M109-inoculated plants, stronger for M116-inoculatd and control ones (Fig. 4A). Dark recovery of ETR was slow but in the fast phase (up to 60 s of darkness) was much more effective for BEG53- and M109-inoculated plants than for these inoculated with M116 and the control (Fig. 4B).

RLC-NPQ increased regularly with light intensity. The only tendency to more efficient heat dissipation showed BEG53-inoculated plants (Fig. 5A). Phase of fast recovery (up to 60 s of darkness) was efficient for each treatment, however at the end of the dark period none of them reached the initial value 0.0 (Fig. 5B).

RLC-qP decreased fast for all the treatments, going down to zero (Fig. 6A). After first 10 s of relaxation, the high dynamics of recovery exhibited plants treated with BEG53 and M109. After 60 s maximal value 1.000 was reached in all the treatments (Fig. 6B), what means reversion of photoinhibition.

Other measurements of Ch fluorescence parameters

Maximal (Fv/Fm) and effective (Y) quantum yields exhibited relatively consistent pattern during the three

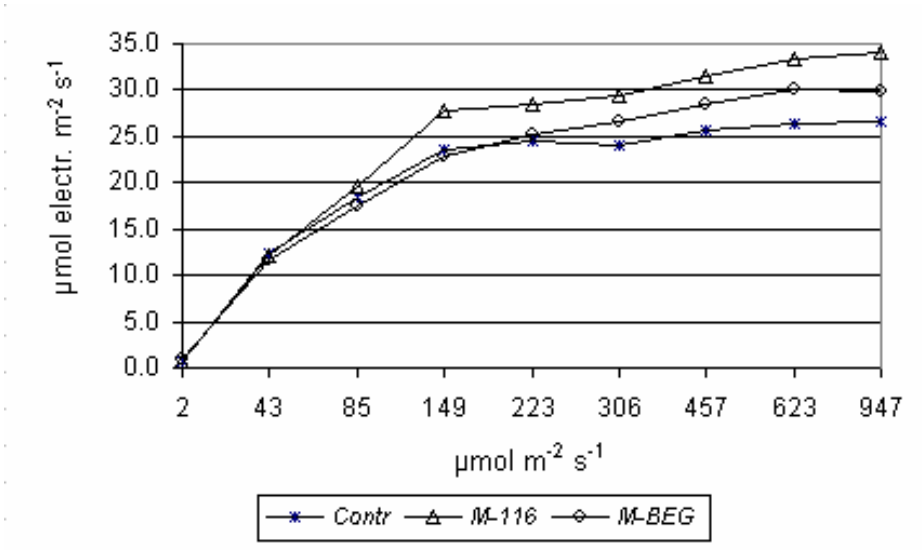

Figure 1. Rapid Light Curves (RLC) for ETR (electron transport rate) in the leaves of 'Cadaman' rootstock plantlets removed from *in vitro* cultures and treated with two mycorrhizal inocula (2002)

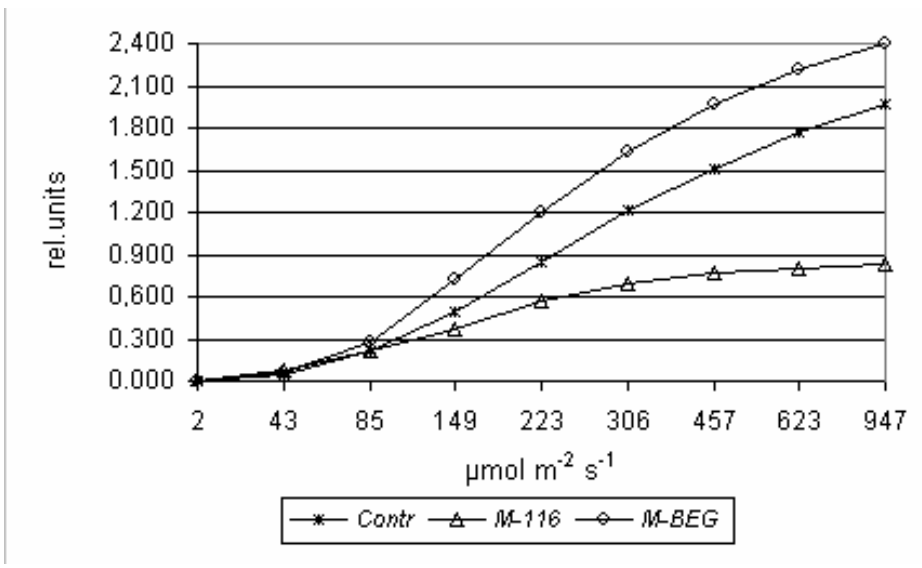

Figure 2. Rapid Light Curves (RLC) for non-photochemical quenching coefficient – NPQ, in the leaves of 'Cadaman' rootstock plantlets removed from *in vitro* cultures and treated with two mycorrhizal inocula (2002)

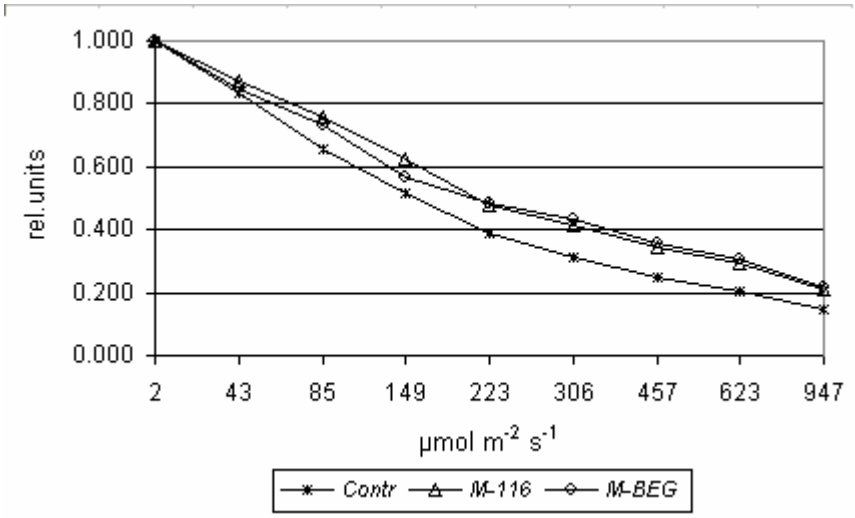

Figure 3. Rapid Light Curves (RLC) for photochemical quenching coefficient – qP in the leaves of 'Cadaman' rootstock plantlets removed from *in vitro* cultures and treated with two mycorrhizal inocula (2002)

years in all the mycorrhizal treatments. Only Y value measured on the grafted cultivar in 2004 was lower then the one measured earlier on the rootstock. Thus, it could indicate that scion cultivar 'Cresthaven' needs longer time to develop photosynthetic activity or it is an expression of poor compatibility between the rootstock and the cultivar.

Growth and minerals content

In the first year of the experiment, only the inoculum BEG53 stimulated extension growth of the plants. In the third year, grafted nursery trees inoculated with all kinds of mycorrhiza showed slight increase of trunk circumference (Tab. 1). Moderately positive effect was found in leaf mineral content of the grafted trees; nitrogen content was the highest for M116-treated trees. Calcium and magnesium was increased by each of the inocula used (Tab. 2).

Chlorophyll content and leaf area

Leaf chlorophyll content, estimated as ICC, of AMF-treated plants showed a tendency to be lower or was significantly lower in M116-treated than in the control plants (Tab. 3). Mycorrhizal fungi enlarged area of the leaves. The most distinct effect was for M116 treatment in the year of removing the plantlets from *in vitro* cultures (Tab. 3).

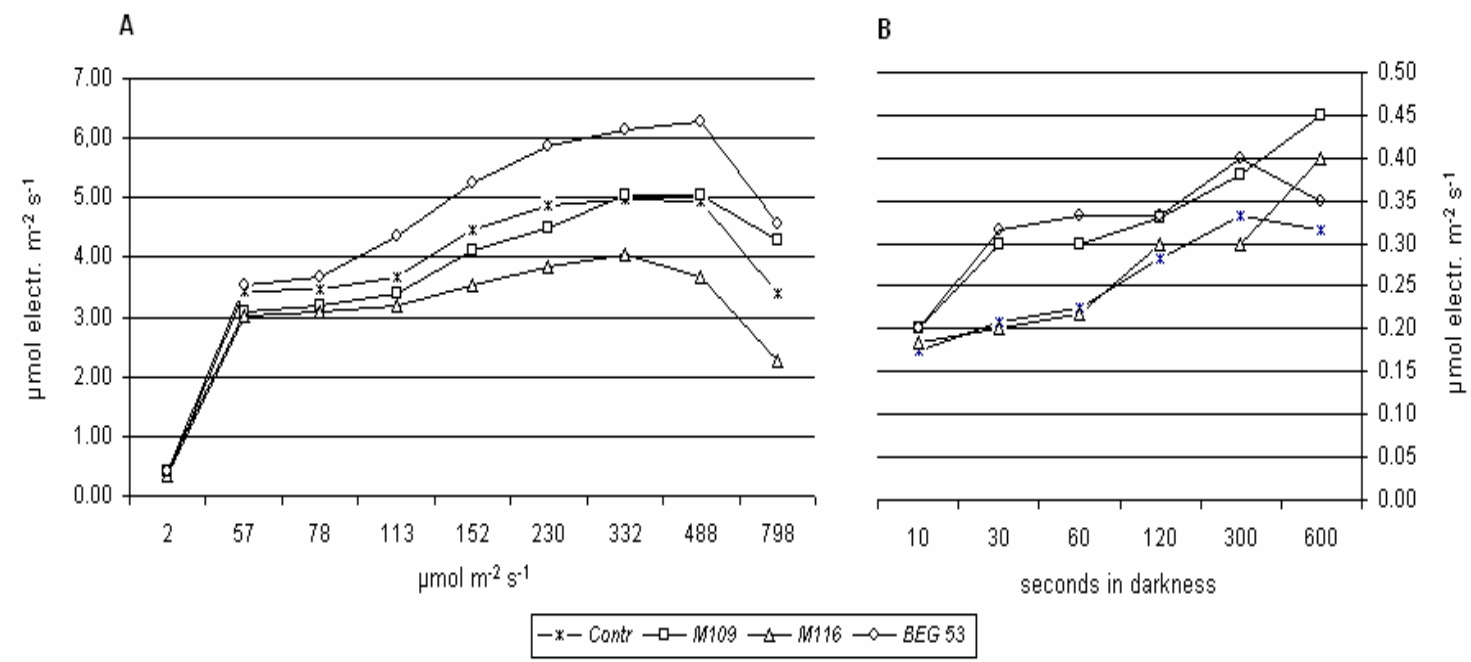

Figure 4. RLC+REC for ETR, in the first year of field cultivation and the year of budding with 'Cresthaven' cv. (2003). A – light phase (RLC); B – dark phase (recovery)

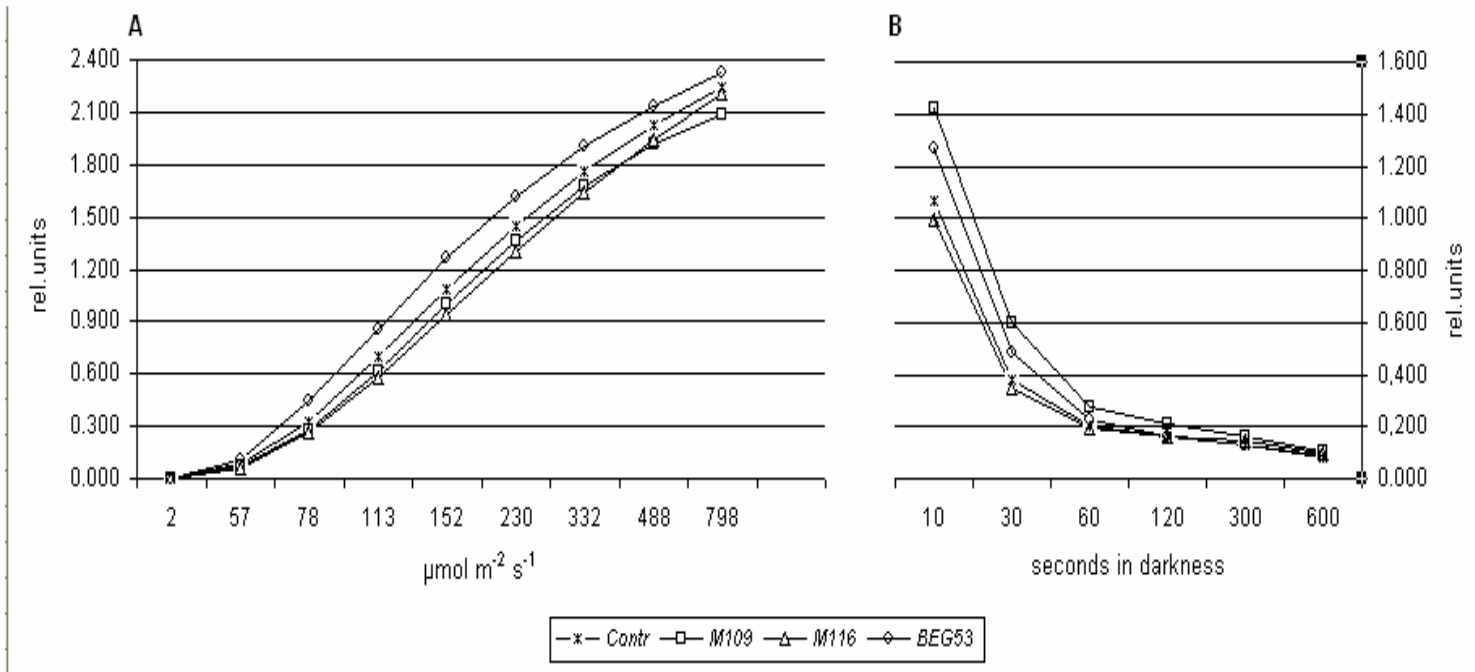

Figure 5. RLC+REC for non-photochemical quenching coefficient - NPQ, in the first year of field cultivation and the year of budding of the trees grafted with 'Cresthaven' cv. (2003). A – light phase (RLC); B – dark phase (recovery)

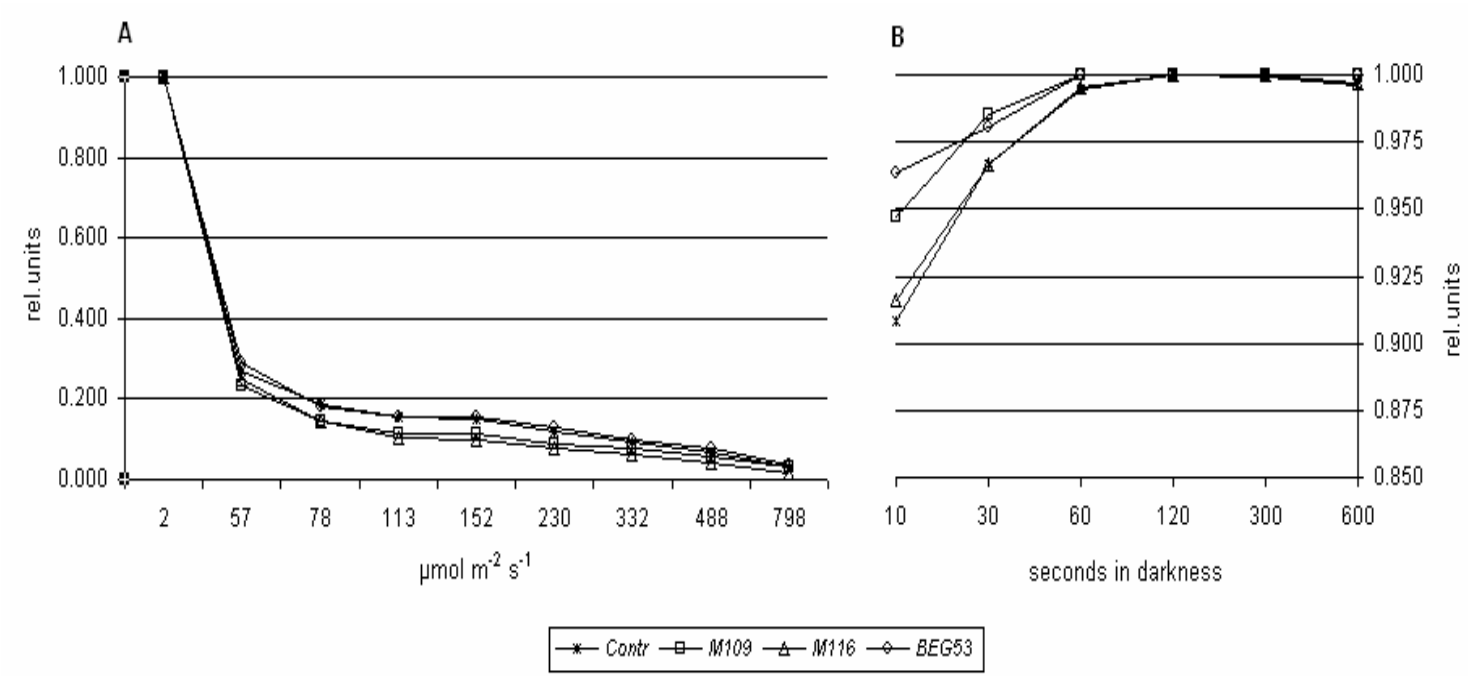

Figure 6. RLC+REC for photochemical quenching coefficient – qP, in the first year of field cultivation and the year of budding with 'Cresthaven' cv. (2003). A – light phase (RLC); B – dark phase (recovery)

Table 1. The effect of mycorrhization on growth of peach 'Cadaman' rootstock and scion cultivar 'Cresthaven'

Measurement	Control	M109	M116	BEG 53	$LSD_{5\%}$
Rootstock height in 2002 [cm]	32.8	31.6	29.3	42.6	7.9
Scion trunk circumference in 2004 [cm]	59.7	61.3	64.6	64.0	6.0

Table 2. The effect of mycorrhization on nutrient content in leaves of peach 'Cresthaven' trees grown in a nursery

Treatment	Nutrient content [% D.W.]				
	N	P	K	Ca	Mg
Control	4.22	0.28	1.86	1.44	0.56
M 109	4.39	0.28	1.68	1.68	0.68
M 116	4.71	0.28	1.52	1.68	0.72
BEG 53	3.89	0.28	1.68	1.68	0.72
$LSD_{p=0.05}$	0.83	0.02	0.40	0.90	0.19

Table 3. Chlorophyll content and area of the leaves from the trees inoculated with different kinds of mycorrhizal fungi

Year	Chlorophyll index (CCI)				Leaf area [cm^2]			
	Control	M109	M116	BEG 53	Control	M109	M116	BEG 53
2002	26.9 b*	-	28.1 b	21.3 a	13.2 a	-	21.3 b	12.2 a
2003-July	31.7 b	28.5 ab	25.9 a	31.5 b	-	-	-	-
2003-Sept.	29.3 b	26.5 ab	24.0 a	25.7 a	17.3 ab	19.8 b	17.6 ab	16.4 a
2004-Sept.	22.1 a	20.7 a	20.0 a	19.4 a	37.9 ab	39.7 ab	43.7 b	37.6 a

*values with the same letter within each line are not significantly different (Tukey's test)

DISCUSSION

Soil/plants inoculation is a practice for enhancing the growth and development of some agricultural crops and can be advantageous in sustainable agriculture. The usual method of introducing mycorrhizal fungi is to use commercially prepared products (Camprubi et al., 1993; Diaz and Honrubia, 1993; Bois et al., 2005). However, the success of this practice depends strongly on the effectiveness of the indigenous microorganisms. Therefore, the understanding of the interaction between the beneficial

microorganisms and host plant is essential. The spectacular method, allowing to evaluate plant performance to any stress, also mycorrhizal symbiosis, is an analysis of the Ch fluorescence kinetics (Waldhoff et al., 2000; Rivera-Becerril et al., 2002; Mielke et al., 2003; Baker and Rosenqvist, 2004; Pinior et al., 2005). This method was primarily designed for measuring potential and effective quantum yield of PSII under transient ambient light conditions. Although this provides important ecophysiological information, it is often necessary to learn more about the potential intrinsic capacities of leaves by measuring induction curve, termed JIP-test (Strasser and Strasser, 1995; Koves-Pechy et al., 1998; Calantzis et al., 1999; Ripley et al., 2004). A new instrument allowing to illustrate the acclimation of photosynthetic apparatus to a range of light intensities is a rapid light curve (RLC) measured with MINI-PAM. RLC are plots of Yield, ETR and quenching parameters versus increasing actinic irradiances. RLC was used by several authors to assess the actual state of photosynthetic apparatus of plants growing in different and changing conditions through years, seasons and days (White and Critchley, 1999; Rascher et al., 2000; Waldhoff et al., 2000; Belshe et al., 2007). Analyses of RLCs performed on ETR and quenching parameters show that young plants mycorrhized with M116 inoculum were rapidly adapted to light stress while for older plants the most active in adaptation processes was BEG53 strain. The analysis of RLC+REC confirmed high activity of BEG53 and M109 in the fast recovery of photochemical activity within 30 s of dark relaxation.

It is postulated that recognized plant reaction to light stress and its ability to recovery has more general meaning – showing plants' tolerance to different abiotic and biotic (including mycorrhization) stresses.

The effect of mycorrhiza on chlorophyll content is not clear. It was reported that mycorrhizal colonisation suppresses (Paradi et al., 2003; Borkowska, 2006) or increases chlorophyll content (Vodnik and Gogala, 1994). Lower chlorophyll index, which is proportional to chlorophyll content, determined in our experiments for M116-treated plants could be explained as the effect of "dilution", when amount of chlorophyll did not increase with the enlargement of leaf area.

The young plants during their adaptation to *in vivo* conditions reacted strongly to M116 inoculum based on native fungi isolated from Hungarian orchard. When trees were adapted to the field conditions, commercial inoculum BEG53 was the most active. Inoculum based on native fungi M109 was also highly promising. This suggests that that a mix of native fungi with selected *Glomus spp.* shall be used in new formulas of inoculum for peach nursery production.

The approach used for this study will be further applied for the comparison of the physiological effects of other candidate inocula myco-

symbionts, as well as for the study of the inter-relations between rootstocks and scions in presence of mycorrhizal symbiosis.

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OCENA REAKCJI NA MIKORYZACJĘ MIKROROZMNAŻANYCH PODKŁADEK BRZOSKWINI ‘CADAMAN’ I ZAOKULIZOWANEJ ODM. ‘CRESTHAVEN’, METODĄ FLUORESCENCJI CHLOROFILU *A*

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STRESZCZENIE

Mikrorozmnażanie jest stosowane do otrzymywania certyfikowanego materiału szkółkarskiego. Mikrorozmnażane rośliny są wolne od wszelkich mikroorganizmów, także współżyjących z roślinami. Technika mikoryzacji została wprowadzona jako element metody mikrorozmnażania, w celu wprowadzenia mikroorganizmów tworzących symbiozę z rośliną-gospodarzem. Celem prowadzonych badań była ocena reakcji aparatu fotosyntetycznego podkładki brzoskwini ‘Cadaman’ i zaokulizowanej odmiany ‘Cresthaven’ na inokulację izolatami grzybów tworzących endomikoryzę. Zastosowano dwie szczepionki oparte na izolatach z sadu węgierskiego (M109 i M116) oraz izolat *Globus spp.*, pochodzący z banku genów we Francji (BEG53). Odpowiedź aparatu fotosyntetycznego na mikoryzację oceniano pomiarami fluorescencji chlorofilu *a*, wykonanymi aparatem MINI-PAM. Aparat ten mierzy wszystkie podstawowe parametry, jak: Fv/Fm, yield, ETR, parametry wygaszania (qP, qN i NPQ). MINI-PAM posiada także program do pomiaru krzywych świetlnych (RLC) oraz krzywych świetlnych wraz z krzywymi pokazującymi odzyskiwanie

aktywności fotochemicznej (RLC+REC). Określano także wzrost, powierzchnię liści, zawartość chlorofilu i składników mineralnych mikrorozmnażanych podkładek ‘Cadaman’ i zaokulizowanej odm. ‘Cresthaven’.

Podczas trzech lat doświadczenia zmieniały się fazy fenologiczne i fizjologiczne oraz morfologia roślin. Zmieniała się również “odpowiedź” roślin na mikoryzację. Aparat fotosyntetyczny młodych roślin (w roku wyjęcia z kultur i*n vitro*) był skutecznie chroniony przed fotoinhibicją przez izolat M116. Rośliny starsze wykazywały podwyższoną tolerancję po inokulacji izolatami BEG53 i M109. Izolat M116 zwiększał powierzchnię liści tylko u młodych roślin. Wpływ badanych szczepionek na zawartość składników mineralnych w liściach odmiany ‘Cresthaven’ był umiarkowany: M116 podwyższał zawartość N, wszystkie szczepionki podwyższały poziom Mg i Ca.

Autorzy sugerują, że grzyby *Glomus spp.* naturalnie występujące w sadach węgierskich połączone z typem BEG53 (z banku genów), można połączyć jako jedną szczepionkę do zastosowania w produkcji podkładek dla brzoskwini.

Słowa kluczowe: endomikoryza (AMF), krzywe świetlne (RLC), parametry fluorescencji Ch, *Prunus persica*, makroelementy, wzrost