Leaf ultrastructure, photosynthetic rate and growth of myrtle plantlets under different *in vitro* culture conditions

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Abstract

The *in vitro* rooting of myrtle (*Myrtus communis* L.) plantlets was performed in containers with gas permeable (V) and non-permeable (C) closures characterized by a different number of gas exchanges (1.4 and 0.3 h^{-1}, respectively). The rooting was induced on *Perlite*, soaked with half strength Murashige and Skoog (MS) medium with 0.5 mg dm^{-3} IBA, either with and without 15 g dm^{-3} of sucrose. During the rooting phase, it was demonstrated that C cultures without sucrose (C-) negatively affect the growth of myrtle plantlets. The net photosynthetic rate and the starch content showed the lowest values in C cultures with and without sucrose (C+ and C-) while chlorophyll *a* content did not vary among treatments, therefore it could not be considered an indicative parameter to evaluate the autotrophic metabolism in myrtle plantlets. Electron microscopy and image analysis were employed to evaluate the leaf ultrastructure at three sample dates. Plantlet rooted in vented vessels with and without sucrose (V+ and V-) showed chloroplasts with numerous starch inclusions, while several osmiophilic plastoglobules (frequently related with leaf senescence) were found in chloroplast of leaf cells of C- myrtle plantlets.

Additional key words: autotrophy, chlorophyll, chloroplast, gas exchange, mixotrophy, photosynthesis, starch.

Introduction

The structure and physiology performance of micro-propagated plants are modified by the special condition of the *in vitro* culture (*e.g.* Pospíšilová *et al.* 1999). The conventional micropropagated plants show leaves with less developed cuticle and stomata scarcely functioning in comparison with *in vivo* growing plants (Capellades 1990, Zobayed *et al.* 2001, Majada *et al.* 2002). Moreover, cells of micropropagated plants, which develop the hyperhydric symptoms, showed a lower chloroplast number, poorly developed thylakoid staking (Majada *et al.* 2002), a low amount of chlorophyll and disorganized grana. The *in vitro* headspace of closed systems is one of the most relevant factor affecting micropropagation because it is the site in which temperature, light, pressure, gaseous diffusion and composition interact to determine the vessel micro environment features (Kozai 1991). As defined by Kozai *et al.* (1986a), gas exchange is strictly related to the vessel closures and might be characterized by "ventilation rate" (number of complete exchanges of vessel atmosphere per hour). The high gas diffusion can be achieved by microporous membranes and filter films (De Proft *et al.* 1985, Cournac *et al.* 1991, Mensuali-Sodi *et al.* 1992, Matthijs *et al.* 1993, Marino *et al.* 1995).

The right combination of gas exchange, light and sugar amount can stimulate the photosynthetic activities of micropropagated plants (Kozai 1991). Moreover it has been reported that plantlets at rooting stage, cultivated under low or absent sugar supply, exhibit leaflets with photosynthetic characteristics similar to the *ex vitro* grown plants (Serret *et al.* 1996). It has been proved that, as the sucrose content decreases, the leaflet thickness increases and stomatal cells show a normal ultrastructure. Transmission electron microscopy (TEM) observations of mesophyll cells show developed chloroplasts also in cultures grown in presence of exogenous sugars, although

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Abbreviations: (C+) - closed with sucrose; (C-) - closed without sucrose; Chl - chlorophyll; E - number of gas exchanges; P_N - net photosynthetic rate; SM - starting material; (V+) - ventilated with sucrose; (V-) - ventilated without sucrose.
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the relative development of grana and the amount of starch vary from autotrophic cultures. The achievement of autotrophy, due to the variation in the culture conditions, will be useful in the improvement of the *in vitro* culture efficiency and plantlet physiological status.

In the literature, only few papers describe the leaf ultrastructural changes in relation with vessel gas exchange, hyperhydricity, sugar supply or irradiance (Lee *et al.* 1985, Serret *et al.* 1996, Majada *et al.* 2002). The aim of this work was to investigate how the number of gas exchanges with the surrounding atmosphere combined with different carbon supply could affect the leaf ultrastructure of myrtle (*Myrtus communis* L.) plantlets in relation with the growth and the photosynthetic ability. Myrtle was chosen as a model species because previous studies had demonstrated that proper modulations of culture conditions significantly affect the development of autotrophy and the adaptability in facing acclimatization (Lucchesini *et al.* 2001). Moreover, myrtle was selected on the basis of its economic value in the ornamental, food and pharmaceutical fields as well as on the importance in the Mediterranean ecology.

Materials and methods

Plants and growth conditions: Actively growing explants derived from mature plants of *Myrtus communis* L. provide the apical shoots (approximately 2 cm) for the establishment of rooting phase (10 microcuttings in each vessel; 5 vessels per treatment). The rooting was performed into autoclavable 500 cm^3 household glass jars (*Bormioli Rocco*, Parma, Italy) and comparing two types of closure, vented and tight closed, as described in Lucchesini *et al.* (2001). The culture support was *Perlite* soaked with half strength MS mineral solution (100:50 v/v) added with 0.5 mg dm^{-3} indole-3-butyric acid (IBA). The addition of sucrose (15 g dm^{-3}) or its absence in the media and the different closures defined four culture conditions: ventilated and closed vessels with sucrose (V+ and C+, respectively) and ventilated and closed vessels without sucrose (V- and C-, respectively). The pH was adjusted to 5.8 before autoclaving. The rooting phase lasted 30 d and plantlets were maintained at a temperature of 23 ± 1 °C, and a 16-h photoperiod with irradiance of 100 μmol $m^{-2}s^{-1}$.

Number of gas exchanges (E) and net photosynthetic rate (P_N) estimations: The number of air exchanges per hour (E), as defined by Kozai *et al.* (1986a), were estimated as E = 1.4 and E = 0.3, respectively; the rate constants of the non-linear regressions of gas concentrations over time were determined using CO_2 as gas tracer (Lucchesini *et al.* 2001). Glass jars were equipped with a gas-sampling device to allow headspace air analysis as described in Lucchesini *et al.* (2001).

Gas concentrations were estimated using an HP 5890 *(Hewlett Packard*, Pennsylvania, USA) gas chromatograph equipped with a stainless steel column packed with *HaySep*® T (*Agilent Technologies*, Milan, Italy) and a thermal conductivity detector (CO_2 determination). Column and detector temperatures were 70 and 200 °C for CO_2 analysis. N_2 was used as a carrier gas at a flow rate of 30 + 15 cm^3 min^{-1}.

Net photosynthetic rates (P_N), at the onset of the photoperiod and during the successive light hours (0, 3rd and 6th hour), were measured on the 15th and 30th day of the *in vitro* rooting phase from three vessels for each treatment relating values with corresponding plantlets dry mass (n = 30). P_N was calculated following the method of Fujiwara *et al.* (1987):

$$P_N = (CO_{2in} - CO_{2out})_t \times E \times V,$$

where $(CO_{2in} - CO_{2out})_t$ is the difference between CO_2 concentration inside and outside the culture vessel at the time t, E is the number of gas exchanges, V (450 cm^3) is the headspace volume equivalent to the residual vessel volume excluding the culture medium.

Chlorophyll and starch determinations: The content of chlorophylls (Chl *a* and Chl *b*) was detected at the end of the *in vitro* culture. Fresh leaves were extracted in 100 % methanol and pigments were determined according to the method of Lichtenthaler (1987) using UV/VIS spectrophotometer *Lambda 35* (*Perkin Elmer*, Wiesbaden, Germany).

The starch content determination was derived from an amount of 150 mg of lyophilized plant material subdivided in three replicates and weighed about 45 - 55 mg per treatment. Digestions of the extracts were performed dissolving the samples in 1.5 cm^3 of acetate buffer (pH 5.0) and incubating in a 100 °C sand bath for 1 h. The samples were allowed to cool and hydrolysed with an amyloglucosidase solution from *Aspergillus niger* with approximately 6 units per mg (*Sigma-Aldrich*, Milan Italy) at 55 °C for 16. Glucose was measured spectrophotometrically at 440 nm after enzymatic reactions based upon the glucose oxidase and peroxidase glucose kit (*Sigma-Aldrich*, Milan, Italy). Chlorophyll and starch spectrophotometric determinations are mean values ± SE of three measurements from each sample.

Sample preparation for TEM studies: Leaf explants at the beginning of the rooting phase (starting material = SM) and at the 15th and 30th day of culture were sampled. Pieces of tissue approximately 2 mm² were cut from similar positions in apical leaves during the photoperiod and immersed in phosphate buffer with 2 % glutaraldehyde and 3 % paraformaldehyde, postfixed in 1 % osmium tetroxide, and dehydrated in a graded

ethanol series. The specimens were embedded in *Epon* (*Fluka*, Milan, Italy) with propylene oxide as transition solvent and polymerized at 60 °C. Ultrathin sections were obtained using a diamond knife on a *LKB* ultratome (*Lab Extreme* Inc., Muskegon, Michigan USA) and mounted on Formvar-coated single hole copper grids. Finally, they were stained with uranyl acetate and lead citrate and investigated with a *Jeol* 100 Sx Transmission Electron Microscope (*Jeol*, Akishima, Tokyo, Japan) operating at 80 kV.

Ultrastructural images were manipulated using *Adobe Photoshop*® software (*Adobe System* Inc., Mountain View, CA, USA) after acquisition with *Coffee CupImage*® (*CoffeeCup* Software). Cells, chloroplasts, starch inclusions and plastoglobule cross sections areas and dimensions were determined at the beginning and at the end of the culture period for each images employing *Sigma Scan Pro* 5.0 software (*SPSS Science*^{TM}, Chicago USA). Known micrograph magnifi-cations of areas and linear dimensions were normalized through scale calibrations before the image analysis.

Statistical analysis: The parameters related to the plant growth on six plants per vessel (n = 30) (fresh, dry mass, height and root percentage) and the water status, estimated as the ratio of the difference between fresh and dry mass and the fresh mass, were measured at the end of the *in vitro* culture period (30th day). Mean values were separated using one-way ANOVA. In the tables values were presented as means ± SE. Percentage values were subjected to arcsine transformation before the analysis. All the experiments were repeated twice.

Results

Growth analysis and photosynthesis: The type of closures employed during the rooting phase of myrtle microcuttings had visible effects on plantlet development as demonstrated by differences in shoot length and dry mass at the end of the culture period (30th day) (Table 1). Especially dry matter of plantlets, grown in more aerated conditions, was almost twice as large as the initial (6.25 ± 0.9 mg). The lowest dry mass was in closed vessels without sucrose in the medium (C-) (Table 1).

The lowest water content was in V+ plantlet, differing significantly from the C- plantlets, which showed the highest value (Table 1). The root formation was achieved in every experimental condition after four weeks in culture even if the C- plantlets showed the lowest rooting percentage (Table 1).

The net photosynthetic rate of aerated plantlets (P_N) showed values very different from those of the closed ones at 15th and 30th day of culture (Fig. 1*A*,*B*). At the 3rd hour after the onset of the photoperiod, V- and C- plantlets showed higher P_N values than V+ and C+ plantlets, respectively. Furthermore, until the 6th hour of the photoperiod, the P_N of C plantlets remained near the compensation point. At the 6th hour of the 30th day (Fig. 1*B*) V- and V+ plantlets did not show differences in photosynthetic activity.

Table 1. Morphological parameters recorded at the end of the *in vitro* culture on myrtle plantlets cultured in the different conditions (V-, V+, C- and C+). Mean values (n = 30) were separated by one way *ANOVA*; different letters corresponded to significant differences ($P \leq 0.05$).

Culture conditions	Dry mass [mg]	Water content [%]	Height [cm]	Rooting [%]
V-	16.29ab	62.41bc	3.53a	56.95a
V+	16.86a	59.24c	3.01ab	68.41a
C-	8.02c	68.61a	2.01b	9.20b
C+	11.33bc	63.56b	2.02b	59.20a

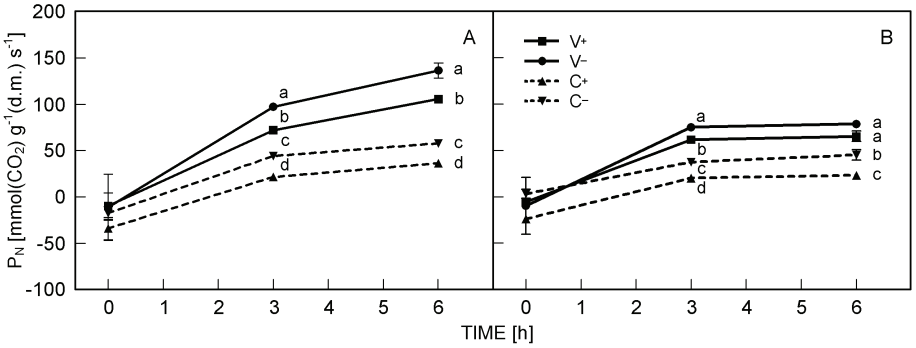

Fig. 1. Net potosynthetic rate, P_N [mmol (CO_2) mg^{-1}(d.m.) h^{-1}] of *in vitro* myrtle plantlets developed in ventilated and closed vessels growing with or without sucrose in the media (V+; V-; C+; C-) at the 15th (*A*) and 30th (*B*) day of the culture period. CO_2 samples were withdrawn from vessels at the end of the dark period, 3rd hour and 6th hour after the beginning of photoperiod. Mean values with the same letter are not different at P = 0.05 (n = 30). *Vertical bars* on data points represent ± SE (are not shown when smaller than the symbols).

Chlorophyll and starch: The lacking of sucrose from the medium and the tightly closed vessels resulted in a decrease of Chl *b* content for C- myrtle plantlets, that showed significantly different values from C+ and from the two types of V plantlets. Considering that Chl *a* value did not vary among the four conditions, the chlorophyll *a/b* ratio was significantly greater in C- plantlets (Table 2).

The analysis of starch content at the end of the culture period showed values four times higher in myrtle plantlets cultured in ventilated vessels with sucrose (V+) than in V- plantlets and almost ten fold higher than in those growing in the closed vessels (Table 2).

Table 2. Chlorophyll [mg g^{-1}(f.m.)] and starch[mg g^{-1}(d.m.)] contents at the end of the *in vitro* culture on myrtle plantlets cultured in the different conditions (V-, V+, C- and C+). Chlorophyll and starch mean values (n = 6) and (n = 3), respectively, were separated by one way *ANOVA*; different letters corresponded to significant differences ($P \leq 0.05$).

Culture	Chl *a*	Chl *b*	Chl *a*/*b*	Starch
V-	1.15a	0.41ab	2.82b	2.21b
V+	1.43a	0.55a	2.57b	8.84a
C-	1.38a	0.27b	4.67a	0.95b
C+	1.33a	0.57a	2.45b	0.94b

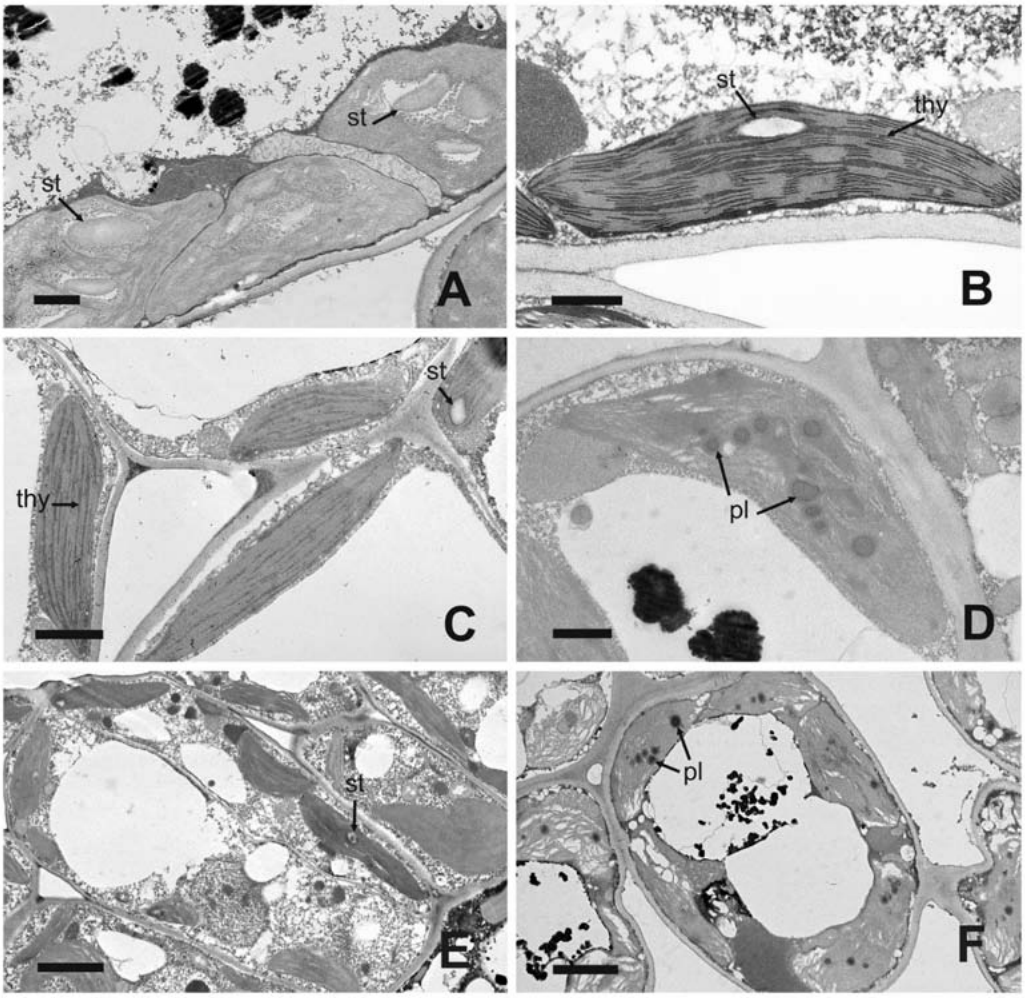

Fig. 2. Chloroplasts of *Myrtus communis* L. leaf palisade parenchyma cells. At the initial of rooting phase (SM), large chloroplasts showed abundant starch inclusions and some small plastoglobules (*A*). At the 15th day, chloroplast of a plantlet growing without sucrose (V-) showed thylakoids arranged into defined grana and lamellae and a starch grain (*B*). At the end of the rooting phase (30th day), V- chloroplasts presented little starch grains and arranged granal thylakoids (*C*), whereas C- chloroplasts had lightly unstacked thylakoids and many osmiophilic plastoglobules and the thylakoids were dilated (*D*). Cells of V+ plantlets had areas of dense cytoplasm with scattered vacuolar spaces and plastids well developed, sometimes with starch grain (*E*). Cell of C- plantlets exhibited extensive intercellular air spaces, prominent vacuoles that contained particulate and globular deposits and chloroplasts with many osmiophilic plastoglobules and dilated thylakoids (*F*). st - starch inclusion; thy - thylakoid; pl - plastoglobule; *scale bar* 1 µm.

Ultrastructure: At the TEM micrographs, SM samples exhibited a well-defined mesophyll layer, a compact palisade and a spongy parenchyma with many intercellular spaces. Great quantities of tannin and phenolic substance were often found in large vacuoles and the cytoplasm contained mitochondria, dictyosomes and chloroplasts. In chloroplasts, osmiophilic plastoglobules were occasionally observed showing a plastoglobule over chloroplast cross section area ratio of 6.03 ± 1.60 %; mainly strong starch accumulation appeared with a high starch grains over chloroplast cross section area ratio (14.50 ± 1.74 %), consequently thylakoids and grana stacking appeared somewhat disorganized (Fig. 2*A*).

After the 15th day of culture, the most important record was that chloroplasts in every condition has shown a well organized stroma and lamellar system with defined thylakoids, specially in plantlets grown in aerated vessels without sucrose in the medium (Fig. 2*B*).

At the end of the rooting phase (30th day), mesophyll cells of V+, V- and C+ plantlets were well differentiated; palisade parenchyma appeared compacted whereas spongy parenchyma presented extensive intercellular spaces. In chloroplasts, which were significantly smaller than those of SM cells, little starch deposits were occasionally found (Table 3), showing a starch inclusion over chloroplast cross section area ratio of 1.65 ± 0.95 % and 4.23 ± 1.31 % in V- and V+ plantlets, respectively. Moreover, osmiophilic plastoglobules disappeared and internal lamellar system had preserved, but sometimes it was lacked of a clear granal organization (Fig. 2*C*). On the contrary, chloroplasts of C- plantlets were deformed by thylakoid swelling and strong plastoglobule accumulation appeared (Fig. 2*D*), showing a plastoglobule over chloroplast cross section area ratio of 8.21 ± 2.18 %.

Table 3. Myrtle chloroplast dimensions (area, length and width) and chloroplast/cell area ratio measured on cross section at the beginning of the culture (SM) and after 30 d of rooting phase in the different culture conditions. In the tables values of SM plantlets were presented as averages ± SE (n = 10). Mean values (n = 10) of V-, V+, C- and C+ plantlets with different letters are significantly different ($P \leq 0.05$).

Culture	Area [μm^2]	Length [μm]	Width [μm]	Area [%]
SM	12.43±1.26	5.61±0.28	2.13±0.22	35.27±2.81
V-	2.94c	2.97b	1.17a	23.44a
V+	4.35b	4.01a	1.35a	27.91a
C-	7.05a	4.05a	1.34a	14.30b
C+	4.59b	3.98a	1.27a	30.91a

All cells V+, V- and C+ decreased in size (71.70 μm^2, 67.23 μm² and 51.97 μm², respectively), compared with those at the initial stage SM (230.6 μm²). Cells of palisade showed an extensive electron-dense cytoplasm area with organelles and mainly a scattered vacuolar space (Fig. 2*E*), whereas cells of spongy parenchyma had a prominent vacuoles containing globular material; for this reason, the cytoplasm was restricted to the parietal area. Even if the mesophyll of C- plantlets was well differentiated, it seemed disorganized, due to an increase in intercellular air spaces, both in palisade and spongy parenchyma. Cells presented the biggest cross section area (110.30 μm²). This fact was confirmed also by the lowest number of chloroplast over cell cross section area (Table 3) and a great amounts of electron-dense bodies in vacuoles (Fig. 2*F*).

Discussion

The reduced gas exchanges in the vessels with a conventional screw cap lead towards a progressive CO_2 depletion during the photoperiod, which could limit P_N: these conditions are characteristic in the usual micropropagation protocols of the majority of the plant species (Kozai 1991, De Riek *et al*. 1991).

Both types of vessel closure employed in these work could allow photosynthesis in myrtle cultures even though the higher aeration rates induced higher P_N for all the culture period. The efficiency of plantlet photosynthetic activity in the aerated V- system was the highest for the first two weeks of culture, but at the 30th day this efficiency became similar in both V- and V+ system. Likely, the CO_2 supply was not enough related with the size of the explants, as already verified in previous experiments (Lucchesini *et al*. 2001). The increase of photosynthetic activities observed in V- plantlets at the 15th day of culture is the result of the optimal combination of CO_2 and light in relation with the V- plantlets development. After 4 weeks this positive trend was verified only at the 3rd hour of the photoperiod. P_N values observed in cultures without sucrose were always higher throughout the culture period confirming the inverse correlation of sucrose with photosynthetic activity (Langford and Wainwright 1987). A possible role of sucrose on the down regulation of ribulose-1,5-biphosphate carboxylase (Rubisco) activity was suggested in cultures with reduced gas exchanges (Desjardin 1995).

As reported by other authors (Kozai *et al*. 1988, Cournac *et al*. 1991) a small amount of sugars (mixotrophy) could improve the culture. The sugar content in the final stages of micropropagation is particularly important for *in vitro* plantlets to overcome the stress period during the *ex vitro* acclimatization (Wilson *et al*. 2001). Positive effect of sucrose on cultivation of plantlets *in vitro* had been already described (Tichá *et al*. 1998, Hofman *et al*. 2002, Custódio *et al*. 2004). Sugars are considered as a source of metabolites and energy for the development of new leaves during the *ex vitro* accli-

matization (Cappellades *et al*. 1991, Van Huylenbroeck *et al*. 1996). The highest starch amount accumulated in V+ myrtle plants could be explained by the photosynthetic activity during the *in vitro* culture and by a simultaneous feeding of exogenous sugar from the medium (Laforge *et al*. 1991, Van Huylenbroeck *et al*. 1996). For this reason, V+ plantlets were the most developed with the highest dry matter and the highest rooting percentage: starch could be employed to supply energy to growth. The C- myrtle plantlets on the contrary showed a considerable decline in growth in absence of sucrose under sub-optimal CO_2 concentration. On the contrary, in other closed systems without sucrose in the medium, an important dry mass accumulation (Kozai *et al*. 1988) or an improvement of rooting (Fotopoulos and Sotiropoulos 2004) was observed. Myrtle V+ plantlets showed also lower water percentage than the C- plantlets. This feature might be due to the higher total osmotic potential of medium containing sucrose. Therefore, the presence of the sugar makes considerably different the osmotic potential of the culture medium employed for V+ and C+ in comparison with V- and C- systems. The presence of sucrose in the medium, which reduced water availability towards the shoots in culture, and a more aerated environment might cooperate to determine a decrease in water retention in V+ plantlets. In addition with the starch accumulation discussed above, the water status of myrtle plantlets could be very important for facing the successive acclimatization, which request plantlets more adapted to water stress (Lucchesini *et al*. 2001) as demonstrated also in other species (Kozai 1991, Kirdmanee *et al*. 1995). Moreover, less severe wilting in plants cultivated *in vitro* on sugar supplemented media could be connected with higher thickness of their leaves and/or with decrease of transpiration (Solárová *et al*. 1989).

The chlorophyll *a* contents were slightly affected by the tested culture conditions and they were similar as myrtle growing in open field [1.23 mg(Chl *a*) g^{-1}(f.m.) and 0.38 mg(Chl *b*) g^{-1}(f.m.), unpublished data] as described also in other species (Donelly *et al*. 1984). The rate of gas exchanges per hour (E = 0.3) in closed systems (C- and C+) was just enough to prevent degradation or to allow the regeneration of Chl *a* as observed also in tobacco plantlets growing in conventional glass or in Magenta vented vessels (Haisel *et al*. 1999). On the other hand, it has been frequently observed that the reduction of gas exchanges inside airtight cultivation vessels could lead to an accumulation of toxic levels of ethylene and other oxidative products, which directly affect the degradation of photosynthetic pigments (Cournac *et al*. 1991, Righetti 1996, Chanemougasoundharam *et al*. 2004). For what concern the chlorophyll *b*, the severe culture conditions that we had imposed in air-tight myrtle culture growing in absence of sucrose (C-), speeded up senescence processes. The Chl *b* degradation, and the consequent increase of the Chl *a/b* ratio, was observed also during the leaf senescence in other plant species (Ito *et al*. 1993). In leaflets of *Gardenia jasminoides* a high Chl *a/b* ratio was associated with low photosynthetic activity during the multiplication *in vitro* phase due to a less developed photosynthetic apparatus (Serret *et al*. 1996). In myrtle plantlets the absence of sucrose in presence of optimal aeration stimulated autotrophy but without significant variation in chlorophyll contents. Therefore chlorophyll contents alone did not seem to be good indicators of autotrophic development achieved by plantlets (Serret *et al*. 1996).

Very strong ultrastructural differences were detected between chloroplasts of starting material SM and that after 15th day of rooting. Chloroplasts at SM showed a round shape and a large cross section area in contrast with other reports, which observed flattened shapes (Lee *et al*. 1985). However, as reported by other authors (Majada *et al*. 2002, Serret *et al*. 1996, Jones *et al.* 1993), chloroplasts observed in myrtle plantlets at the beginning of the rooting phase showed a strong starch accumulation without a clear grana stacking. In this phase large and irregularly shaped starch grains appeared to disrupt the normal chloroplast structure but it did not affect photosynthetic activity (data not shown) even if an excess of starch accumulation in chloroplasts has been reported to result in chloroplast degeneration and chlorophyll decrease (Lee *et al*. 1985, Serret *et al*. 1996).

After 15 days, all the plantlets growing in the different culture conditions revealed well organized chloroplast membrane systems in accordance with the observed development of photosynthetic ability. This pattern was frequently reported during the rooting induction where the plantlets are photosynthetically more active (Serret *et al*. 1996). Moreover, the well formed membrane systems with defined thylakoids observed in V- plantlets (Fig. 2*B*) confirm a stronger development of photosynthetic machinery (Cournac *et al*. 1991, Kozai 1991, Kutík 1998).

With the progression of the culture, we observe an overall changing in the morphological and ultrastructural patterns as a result of the predominant effect of the growth conditions determining a prevalent decreasing of the cellular and chloroplast cross section area. Although two cell layers in the mesophyll were observed, the C- plantlets, at the end of rooting process, showed disorganized structures with an increase of the intercellular spaces. Similar results it was observed in other species belonging to the same plant family performing a heterotrophic metabolism or hyperhydric symptoms (Jones *et al*. 1993, Zobayed *et al*. 2001). Moreover in the differentiated palisade parenchyma cells of C- myrtle plantlets, the chloroplasts and others organelles were typically distributed around the periphery of the cells and bounded the vacuole indicating an older onthogenetic stage than cells of C+, V- and V+ plantlets, which showed typical features of an active proliferation.
At the 30th day of culture, the size of starch grains and the amount of thylakoid membranes in myrtle chloroplasts decreased in all treatments. In addition, a strong increase of plastoglobules (Desjardin *et al*. 1990) in chloroplast of

C- plantlets was observed as a response to the stress caused by lacking of sugar sources as confirmed by the increase of the plastoglobules to chloroplast cross section area ratio. The formation of the lipid particles is present in young chloroplasts as a normal thylakoid membrane turnover, but increases in number and cross section area of plastoglobules are unspecific responses due to different stress factors (Selga *et al*. 1996). Osmiophilic globules are involved in the synthesis of phenols and such substances have been stated to be promoters of plant development. The formation of plastoglobules is thought to be associated with the breakdown of thylakoids that accompanies senescence (Ghosh *et al.* 1994). Moreover, loss of the integrity of granal stacking and lumenal swelling in C- chloroplasts could confirm the decline of Chl *b* amount, which are principally associated with the photosystem 2 activity located in the stacked intragranal regions (Ghosh *et al*. 2001).

In conclusion, as suggested by the ultrastructural observations, a more aerated environment and the photoautotrophic or photomixotrophic induction, observed in V- and V+ plantlets respectively, seemed to delay the senescence symptoms that usually occur in conventional culture. The efficiency of the photosynthetic machinery depends on a regular chloroplast structure and in particular on a normal thylakoids stacking (Ghosh *et al*. 2001).

From this study it came out that proper modulations of micro-environment conditions and medium compositions could be useful tools to change the growth, the physiology and the cell structure of the *in vitro* myrtle plantlets. The employment of the two different closure apparatus either in presence or in absence of sucrose had resulted in four structurally and physiologically distinct plantlets types. These plantlets could be arranged in two main groups of photoautrophic (V- and C-) and photomixotrophic (V+ and C+) plantlets. The four plantlets variants could provide an interesting model to study their ability to overcome the stress of *ex vitro* acclimatization. In particular, the increased gas exchanges, the improved photosynthetic activity and the addition of a carbon source determined the best culture conditions allowing myrtle V+ explants to storage more starch. This status improve the quality of the myrtle plantlets making them more suitable to face the successive transfer from *in vitro* to *ex vitro* conditions.

References

Capellades, M.: Environment influences anatomy of stomata and epidermal cells in tissue-cultured *Rosa multiflora*. - J. amer. Soc. hort. Sci. **115**: 141-145, 1990.

Capellades, M., Lemeus, R., Debergh, P.: Effects of sucrose on starch accumulation and rate of photosynthesis in *Rosa* cultured *in vitro*. - Plant Cell Tissue Organ Cult. **25**: 21-26, 1991.

Chanemougasoundharam, A., Sarkar, D., Pandey, S.K., Al-Biski, F., Helali, O., Minhas, J.S.: Culture tube closure-type affects potato plantlets growth and chlorophyll contents. - Biol. Plant. **48**: 7-11, 2004.

Cournac, L., Dimon, B., Carrier, P., Lohou, A., Chagvardieff, P.: Growth and photosynthetic characteristic of *Solanum tuberosum* plantlets cultivated *in vitro* under different conditions of aeration, sucrose supply, and CO_2 enrichment. - Plant Physiol. **97**: 112-117, 1991.

Custódio, L., Martins-Loução, M.A., Romano, A.: Influence of sugars on *in vitro* rooting and acclimatization of carob tree. - Biol. Plant. **48**: 469-472, 2004.

De Proft, M.P., Maene, L.J., Debergh, P.C.: Carbon dioxide and ethylene evolution in the culture atmosphere of *Magnolia* cultured *in vitro*. - Physiol. Plant. **65**: 375-379, 1985.

De Riek, J., Van Cleemput, O., Debergh, P.C.: Carbon metabolism of micropropagated *Rosa multiflora* L. - *In Vitro* cell. dev. Biol. Plant **27**: 57-63, 1991.

Desjardin, Y.: Carbon nutrition *in vitro*- regulation and manipulation of carbon assimilation in micropropagated systems. - In: Aitken-Christie, J., Kozai, T., Smith, M.A.L. (ed.): Automation and Environmental Control in Plant Tissue Culture. Pp. 441-471. Kluwer Academic Publishers, Dordrecht 1995.

Donelly, D.J., Vidaver, W.E.: Pigment content and gas exchange of red raspberry *in vitro* and *ex vitro*. - J. amer. Soc. hort. Sci. **109**: 177-181, 1984.

Fotopoulos, S., Sotiropoulos, T.E.: *In vitro* propagation of the peach rootstock: the effect of different carbon sources and types of sealing material on rooting. - Biol. Plant. **48**: 629-631, 2004.

Fujiwara, K., Kozai, T., Watanabe, I.: Measurements of carbon dioxide gas concentration in stoppered vessels containing tissue cultured plantlets and estimates of net photosynthetic rate of the plantlets. - J. Agr. Meteorol. **43**: 21-30, 1987.

Ghosh, S., Hudak, K.A., Dumbroff, E.B., Thompson, J.E.: Release of photosynthetic protein catabolites by blebbing from thylakoids. - Plant Physiol. **106**: 1547-1553, 1994.

Ghosh, S., Mahoney, S.R., Penterman, J.N., Peirson, D., Dumbroff, E.B.: Ultrastructure and biochemical changes in chloroplasts during *Brassica napus* senescence. - Plant Physiol. Biochem. **39**: 777-784, 2001.

Haisel, D., Pospíšilová, J., Synková, H., Čatský, J., Wilhelmová, N., Plzáková, Š.: Photosynthetic pigments and gas exchange of *in vitro* grown tobacco plants as affected by CO_2 supply. - Biol. Plant. **42**: 463-468, 1999.

Hofman, P., Haisel, D., Komenda, J., Vágner, M., Tichá, I., Schäfer, C., Čapková, V.: Impact of *in vitro* cultivation conditions in thylakoid membrane proteins and pigments of tobacco during *ex vitro* acclimation. - Biol. Plant. **45**: 189-195, 2002.

Ito, H., Tanaka, Y., Tsuji, H., Tanaka, A.: Conversion of chlorophyll *b* to chlorophyll catabolites. - Arch. Biochem. Biophys. **306**: 148-151, 1993.

Jones, N.B., Drennan, P.M., Van Staden, J.: Leaf anatomy, chloroplast organization and photosynthetic rate of hyperhydric *Eucalyptus saligna* Sm. material. - S. Afr. J. Bot. **59**: 551-555, 1993.

Kirdmanee, C., Kytaia, Y., Kozai, T.: Effects of CO_2 enrichment and supporting material *in vitro* on photoautotrophic growth of *Eucalyptus* plantlets *in vitro* and *ex vitro*. - *In Vitro* cell. dev. Biol. Plant **31**: 144-149, 1995.

Kozai, T.: Controlled Environments in conventional and

automated micropropagation. - In: Vasil, I.K. (ed.): Scale-up and Automation in Plant Propagation. Pp. 213-230. Academic Press, San Diego 1991.

Kozai, T., Fujiwara, K., Watanabe, I.: Effects of stoppers and vessels on gas exchange rates between inside and outside of vessels closed with stoppers. - J. agr. Meteorol. **42**: 119-127, 1986a.

Kozai, T., Fujiwara, K., Watanabe, I.: Relation between the culture medium composition and water potential of liquid culture media. - J. agr. Meteorol. **42**: 1-6, 1986b.

Kozai, T., Iwanami, Y.: Effects of CO_2 enrichment and sucrose concentration under high photon fluxes on plantlet growth of carnation (*Dianthus caryophyllus* L.) in tissue culture during the preparation stage. - J. jap. Soc. hort. Sci. **57**: 279-288, 1988.

Kutík, J.: The development of chloroplast structure during leaf ontogeny. - Photosynthetica **35**: 481-505, 1998.

Laforge, F., Lussier, C., Desjardin, Y., Gosselin, A.: Effects of light intensity and CO_2 enrichment during *in vitro* rooting on subsequent growth of strawberry, raspberry and asparagus in acclimatization. - Scientia Hort. **47**: 259-269, 1991.

Langford, P.J., Wainwright, H.Y.: Effects of sucrose concentration on the photosynthetic ability of rose shoots *in vitro*. - Ann. Bot. **60**: 633-640.

Lee, N., Wetzstein, Y., Sommer, H.: Effect of quantum flux density on photosynthesis and chloroplast ultrastructure in tissue-cultured plantlets and seedling of *Liquidambar styraciflua* L. towards improved acclimatization and field survival. - Plant. Physiol. **78**: 637-641, 1985.

Lichtenthaler, H.K.: Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. - Methods Enzymol. **148**: 350-383, 1987.

Lucchesini, M., Mensuali-Sodi, A., Massai, R., Gucci, R.: Development of autotrophy and tolerance to acclimatization of *Myrtus communis* transplants cultured *in vitro* under different aeration. - Biol. Plant. **44**: 167-174, 2001.

Majada, J.P., Fall, M.A., Tadeo, F., Sànchez-Tamés, R.: Effects of natural ventilation on leaf ultrastructure of *Dianthus caryophyllus* L. cultured *in vitro*. - *In Vitro* cell dev. Biol. Plant **38**: 272-278, 2002.

Marino, G., Berardi, G., Ancherani, M.: The effect of the type of closure on the gas composition of the headspace and the growth of GF677 peach x almond rootstock cell suspension cultures. - *In Vitro* cell. dev. Biol. Plant **31**: 207-210, 1995.

Matthijs, D.G., Demeester, J.J., Pascat, B., Christiaens, K., Debergh, P.C.: Factors controlling the evolution of the gaseous atmosphere during *in vitro* culture. - In: Carre, F., Chagvardieff, P. (ed.): Proc. Int. Symp. "Ecophysiology and Photosynthetic *in Vitro* Cultures". Pp. 129-140. CEA, Cadarache, France 1995.

Mensuali-Sodi, A., Panizza, M., Tognoni, F.: Quantification of ethylene losses in different container-seal systems and comparison of biotic and abiotic contributions to ethylene accumulation in cultured tissues. - Physiol. Plant. **84**: 472-476, 1992.

Pospíšilová, J., Tichá, I., Kadleček, P., Haisel, D., Plzáková, Š,: Acclimatization of micropropagated plants to *ex vitro* conditions. - Biol. Plant. **42**: 481-497, 1999.

Righetti, B.: Chlorophyll, ethylene and biomass determination in *Prunus avium* cv. Victoria cultivated *in vitro* under different atmospheric conditions. - J. hort. Sci. **71**: 249-255, 1996.

Selga, T., Selga, M.: Response of *Pinus sylvestris* L. needles to electromagnetic fields. Cytological and ultrastructural aspects. - Sci. total Environ. **180**: 65-73, 1996.

Serret, M.D., Trillas, M.I., Matas J., Araus J.L.: Development of photoautotrophy and photoinhibition of *Gardenia jasminoides* plantlets during micropropagation. - Plant Cell Tissue Organ Cult. **45**: 1-16, 1996.

Solárová, J., Pospíšilová, J., Čatský, J., Šantrůček, J.: Photosynthesis and growth of tobacco plantlets in dependence on carbon supply. - Photosynthetica **23**: 629-637, 1989.

Tichá, I., Čáp, F., Pacovská, D., Hofman, P., Haisel, D., Čapková, V., Schäfer, C.: Culture on sugar medium enhances photosynthetic capacity and high light resistance of plantlets grown *in vitro*. - Physiol. Plant. **102**: 155-162, 1998.

Van Huylenbroeck, J., Debergh, P.: Impact of sugar concentration *in vitro* on photosynthesis and carbon metabolism during *ex vitro* acclimatization of *Spathiphyllum* plantlets. - Physiol. Plant. **96**: 298-304, 1996.

Wilson, S.B., Heo, J., Kubota, C., Kozai, T.: A forced ventilation micropropagation system for photoautotrophic production of sweetpotato plug plantlets in a scaled-up culture vessel: II. Carbohydrate status. - HortTechnology **11**: 95-99, 2001.

Zobayed., S.M.A., Afreen, F., Kozai, T.: Physiology of Eucalyptus plantlets grown photoautotrophically in a scaled-up vessel. - *In Vitro* cell. dev. Biol. Plant **37**: 807-813, 2001.