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Development of autotrophy and tolerance to acclimatization of *Myrtus communis* transplants cultured *in vitro* under different aeration

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Abstract

The behaviour of myrtle (*Myrtus communis* L.) plantlets during the last phase of *in vitro* culture before transplanting was studied. Myrtle plants were sampled from Mediterranean shrubland vegetation. *In vitro* growth of myrtle microcuttings was evaluated during the rooting phase using 500 cm^3 containers fitted with two different types of closures. The number of gas exchanges and time in which aerated and closed vessels lose half of their gas content were calculated. Both types of vessel closure allowed photosynthetic activity in myrtle cultures even though the higher aeration rate induced higher net photosynthetic rate (P_N) during all the culture. *In vitro* morphogenetic ability and rooting of myrtle microcuttings were affected by the different environment conditions inside the culture vessels: plantlet growth and root formation of myrtle explants increased in aerated vessels in comparison with closed ones. The well developed root system, the higher P_N and dry mass accumulation during the pre-acclimatization phase in aerated vessels induced a better ability to face the transplant stress.

Additional key words: CO_2, gas exchange, myrtle, micropropagation, net photosynthetic rate.

Introduction

The microclimate inside the culture vessel influences the growth of explants and the success of micropropagation just as well as the canopy microclimate affects physiology performance of plants growing in the field.

The physical environment which characterise the *in vitro* headspace of a micro-culture system is the result of interrelationships among temperature, light, pressure, gaseous diffusion and composition (Kozai 1991). The adoption of culture methods which allow us to manipulate the dynamics of these variables (Aitken-Christie *et al.* 1994, Kozai *et al.* 1995) could improve the quality and yield of the culture.

In the last years importance of gaseous environment inside tissue culture vessels on plant growth and development has been shown. Micropropagated plantlets usually need more CO_2 than usually occurring in conventional *in vitro* culture (Solárová and Pospíšilová 1997). Several methods to increase CO_2 concentration inside the culture vessels were proposed: *1*) by increasing CO_2 in the surrounding atmosphere, and *2*) by flushing CO_2 directly in the vessel (Buddendorf-Joosten and Woltering 1994, Desjardin *et al.* 1990, Figueira *et al.* 1991). Less attention has been paid to the modulation of CO_2 diffusion by the use of closures with improved gas permeability, *i.e.* microporous membranes and filter films (De Proft *et al.* 1985, Cournac *et al.* 1991, Mensuali *et al.* 1992, Matthijs *et al.* 1995, Marino *et al.* 1995). Ventilation rate is an important feature which assess the difference between the gaseous composition inside and outside the vessel.

The aim of this work was to determine to what degree the *in vitro* microenvironment, modified by the adoption of different closures, could affect the photoautotrophic growth and net photosynthetic rate of *in vitro* grown

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Abbreviations: Chl - chlorophyll; E - hourly gas exchange; P_N - net photosynthetic rate; RWC - relative water content; t_{50} - time in which vessels lose half of their gas content.
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microcuttings of myrtle and the subsequent acclimatization to *ex vitro* conditions. Micro-ecosystems of plantlets similar to the analogous *in vivo* cultures could be employed as a model for ecophysiological studies of myrtle mechanisms of tolerance to environmental stress (Tenhunen 1987, Gucci *et al.* 1999). Myrtle was selected on the basis of its importance on the ecology of the Mediterranean shrubland and in the ornamental and pharmaceutical fields.

Materials and methods

Plants and growth conditions: *In vitro* cultures were established from mature plants of *Myrtus communis* L. growing in a Mediterranean maquis vegetation on the coast of Tuscany as reported by Lucchesini and Mensuali-Sodi (2000). Stock cultures were grown on modified MS medium (Murashige and Skoog 1962) consisting of a full strength solution of micronutrients and vitamins and half strength solution of macronutrients added with 300 mg dm^{-3} glutathione (GSH) in reduced form, 0.25 mg dm^{-3} benzyladenine (BA), 30 g dm^{-3} sucrose, and 7 g dm^{-3} *Difco Bacto* agar. Apical shoots (2 cm) were excised from actively growing plantlets, stripped of their lower leaves and inserted vertically in the supporting rooting material (10 microcuttings in each vessel). Rooting was performed on perlite soaked with half strength MS mineral solution (100:50 v/v) added with 0.5 mg dm^{-3} indole-3-butyric acid (IBA) and 15 g dm^{-3} sucrose adjusting the pH to 5.8 before autoclaving. Rooting cultures were performed into autoclavable 500 cm^3 household glass jars (*Bormioli Rocco*, Parma, Italy) equipped with a gas sampling device.

Two types of closures were compared: *1*) metal screw caps (*Italcaps*, ∅ 70 mm) with an aluminium foil underneath pressed on the rim of the jar (closed vessels), and *2*) metal screw caps with a central hole corresponding to a porous membrane of an aluminium foil underneath pressed on the rim of the jar (aerated vessels). The gas sampling device consisted of a properly modified stainless steel joint (*Swagelok® Companies*, Solon, USA) through the vertical side of the vessel body. The joint was equipped with a caoutchouc (India rubber) septum (∅ 0.5 cm) for headspace sampling during the experiments. The cultures were maintained at a room temperature of 23 ± 1 °C, and a 16-h photoperiod [irradiance of 100 μmol(photon) m^{-2} s^{-1}].

Rooted plantlets were transferred into pots containing a unsterilised mixture (1:1 v/v) of perlite and soil. The plants were placed in a growth chamber at temperature of 20 ± 1 °C under a 16-h photoperiod at irradiance of 150 μmol m^{-2} s^{-1}. The relative humidity of the growth chamber was regulated to 55 %. To prevent water loss the potted plants were covered with transparent plastic bags for 7 d and the foliage was irrigated twice a day, avoiding to wet the substrate.

Gas exchange characteristic of vessels: Theoretically, the balance of gas concentration in the vessel at any given time t follows the Fick's First Law of gas diffusion so that Gas(t), is described by the differential equation:

$$dGas(t) / dt = -E \times Gas(t)$$

in which the term on the right hand side represents the gas loss from the vessel.

The air exchange characteristics of the vessel are expressed by the number of air exchanges per hour as defined by Kozai *et al.* (1986). The hourly number of gas exchanges of an empty vessel (E) were estimated using carbon dioxide or ethylene as a tracer gas. For this purpose, standard gases were injected into the vessel to an initial concentration of 600 cm^3 m^{-3} CO_2 and 300 cm^3 m^{-3} ethylene and samples were withdrawn for monitoring the gas course concentrations over time. Resolving the above equation for the initial $CO_{2(0)}$ concentration and for $CO_{2(t)}$ concentration at any given time t the following exponential equations were obtained:

$$CO_{2(t)} - CO_{2out} = (CO_{2(0)} - CO_{2out}) \times e^{-Et}$$

$$C_2H_{4(t)} = C_2H_{4(0)} \times e^{-Et}$$

for both carbon dioxide and ethylene, where ethylene is present in negligible concentration in the atmosphere outside the vessel.

The number of gas exchanges [h^{-1}] were determined by rate constant of non linear regressions of gas concentrations [cm^3 m^{-3}] over time [min].

The half time (t_{50}) that is the time in which the vessels lose half their gas content was computed as:

$$t_{50} = \ln 2 / E$$

Experimentally E were also calculated over 30 min and over 1 h periods from the beginning of CO_2 and C_2H_4 diffusions by the equation reported in Kozai *et al.* (1986):

$$E = -\ln [(CO_{2(t)} - CO_{2out}) / (CO_{2(0)} - CO_{2out})] \times 1 / t$$

$$E = -\ln [C_2H_{4(t)} / C_2H_{4(0)}] \times 1 / t$$

where $CO_{2(t)}$ and $CO_{2(0)}$ or $C_2H_{4(t)}$ and $C_2H_{4(0)}$ [cm^3 m^{-3}] are the carbon dioxide and ethylene concentrations inside the vessel at time t and time 0, respectively; CO_{2out} is the carbon dioxide concentration outside the vessel and t is the time interval [h] from time 0 to t.

Gas analysis and photosynthetic rate *in vitro* and *ex vitro*: For estimating ethylene and CO_2 concentrations

2 cm^3 air samples were withdrawn with hypodermic syringes from the culture vessels. Gas concentrations were estimated using an *HP 5890* gas chromatograph (*Hewlett Packard*, Milano, Italy) equipped with a stainless steel column packed with *HaySep T*, a flame ionisation detector (ethylene determination) and a thermal conductivity detector (CO_2 determination). Column and detector temperature were 70 and 350 °C for ethylene and 70 and 200 °C for CO_2 analysis. N_2 was used as a carrier at a flow rate of 30 cm^3 min^{-1} and 30 + 15 cm^3 min^{-1} for ethylene and CO_2, respectively.

Net photosynthetic rate (P_N) [mmol g^{-1}(d.m.) s^{-1}] was calculated during the *in vitro* rooting phase and acclimatization period after the method of Fujiwara *et al.* (1987):

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

where: (CO_{2in} - CO_{2out}) [mmol mol^{-3}] is the CO_2 difference between CO_2 concentration inside and outside the culture vessel, E is the number of hourly gas exchanges, and V (450 cm^3) is the headspace volume equivalent to the remain vessel volume excluding the culture medium. Analogous P_N estimations were performed over the *ex vitro* acclimatization period on samples consisted of two potted plants maintained in the closed vessels along 30 min under a photon irradiance of 150 µmol m^{-2} s^{-1}.

Chlorophyll content was detected on fresh leafy shoots at the end of the *in vitro* culture, extracted in 100 % methanol and determined according to the method of Lichtenthaler (1987).

Plant water status was characterized at day intervals during the *ex vitro* culture by measuring gravimetrically relative water content (RWC) and the water potential (Ψ_w) on fully expanded leaves of plantlets using a thermocouple psychrometer (*Decagon Devices*, Washington, USA). Analogous procedures were performed following sample freezing to determine osmotic potential. Measures were carried out at 25 °C and the instrument was calibrated with standard NaCl solutions.

Sampling and statistical analysis: Diffusion rates of CO_2 and ethylene out of the empty aerated and closed vessels were evaluated over one hour period (n = 3) and the experiment was run three times. Curves were fitted by non linear regressing gas concentrations over time and the fitting of data were assessed by F-test. The rate constants E, derived from the fitted curves of CO_2 diffusions from closed and aerated vessels, were compared by the Student's test ($P \leq 0.05$) as reported by Motulsky (1999). Morphological parameters were recorded at the end of the *in vitro* culture and after two weeks of acclimatization. The experiment was twice repeated.

The *in vitro* and *ex vitro* P_N evaluations were carried out by CO_2 measures: gas samples were withdrawn from five vessels for each treatment at day intervals during the *in vitro* and *ex vitro* culture. At the same time dry masses of *in vitro* explants and *ex vitro* plantlets were determined (n = 20 and n = 10, respectively). During the *in vitro* growth CO_2 was determined on the sampling days at the end of the dark period and at hour intervals after the onset of lights. Mean values related to morpho-physiological parameters and P_N were separate by using Student's test procedure. Percentage values were subjected to arcsine transformation before analysis.

Results

Gas exchange characteristics of vessels: No significant differences were observed between the ethylene and CO_2 diffusions from the closed ($y_{CO_2} = 100\ e^{-0.005\ t}$; $y_{C_2H_4} = 100\ e^{-0.003\ t}$) or aerated system ($y_{CO_2} = 100\ e^{-0.023\ x}$; $y_{C_2H_4} = 100\ e^{-0.016\ x}$). Fits from CO_2 data of the aerated and closed system were compared and the differences between the equation rate constants were highly significant ($P \leq 0.0001$). Hourly E and t_{50} values of aerated (1.4 and 0.49, respectively) and closed vessels (0.3 and 2.31, respectively) were chosen to describe the gas exchange characteristics of the two type of containers. Values E are very similar to those (data not shown) experimentally calculated according to the equations of Kozai *et al.* (1986) by CO_2 measurements at time intervals within 30 min after gas tracer injection.

***In vitro* plant development:** Myrtle microcuttings developed much better in aerated vessels showing a higher leaf area at the end of the culture period (35th day) (Table 1). Plantlet dry mass was almost twice as great in the aerated compared with the closed vessels. On the contrary, the closures did not have any effect on stem length, leaf number and root formation (Table 1). Nevertheless, aeration improved root development as demonstrated by the increase of root length and mass. Total chlorophyll content of rooted plantlets was similar in the aerated and closed vessels.

CO_2 evolution and net photosynthetic rate *in vitro*: The time course of the ratio between CO_2 inside and outside the vessels showed a different trend in the two types of micro-environment (Fig. 1*A*,*B*). At the end of the dark period the CO_2 concentration inside the closed vessel was approximately twice of the outside concentration. By contrast during the light period the CO_2 concentration inside the closed vessels became almost a

quarter of the outside CO_2 concentration. In these vessels, the ratio CO_{2in}/CO_{2out} gradually decreased as soon as the lights were turned on till the sixth hour of the photoperiod (Fig. 1*A*). On the 35th day of culture the CO_2 concentration inside the closed vessels suddenly decreased over the first hour of light period and low values became constant with time (Fig. 1*B*). In the aerated vessels the inside CO_2 was similar to that measured in the surrounding atmosphere at the end of the dark period. About one hour after the lights were turned on the ratio CO_{2in}/CO_{2out} stabilized around the value 0.7 on the first day of culture (Fig. 1*A*) and decreased to 0.4 at the end of the culture period (Fig. 1*B*).

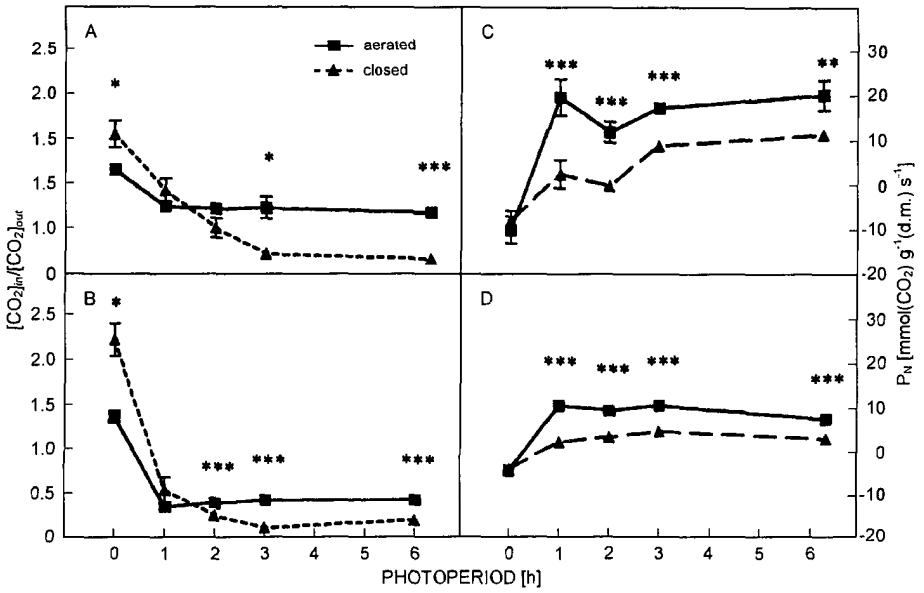

Fig. 1. Carbon dioxide concentration [CO_{2in} / CO_{2out}] ratio inside the aerated and closed vessels (*A*, *B*) and P_N (*C*, *D*) at the beginning (*A*, *C*) and at the end of the culture period (*B*, *D*) of myrtle microcuttings. CO_2 samples were withdrawn from vessels over six hours after beginning of photoperiod. Means ± SE (n = 5); * - $P \leq 0.05$; ** - $P \leq 0.01$; *** - $P \leq 0.001$ within each sampling time.

Table 1. Morphological parameters recorded at the end of the *in vitro* culture on myrtle rooted plantlets. Means ± SE (n = 30); differences were significant at * - $P \leq 0.05$, ** - $P \leq 0.01$, or *** - $P \leq 0.001$.

	Aerated vessels	Closed vessels
Leaf area [mm^2]	368.70 ± 27.26 **	247.00 ± 14.61
Stem length [cm]	2.60 ± 0.31	2.12 ± 0.23
Leaf number	9.10 ± 0.59	6.90 ± 1.06
Chl [mg g^{-1}(f.m)]	1.85 ± 0.22	1.55 ± 0.21
Root number	2.64 ± 0.30	3.00 ± 0.36
Rooting [%]	68.07 ± 7.31	63.64 ± 5.84
Root length [cm]	1.51 ± 0.14 ***	0.93 ± 0.08
Root f.m. [mg plant^{-1}]	47.38 ± 6.22 **	28.01 ± 2.65
Root d.m. [mg plant^{-1}]	8.07 ± 1.42 *	4.20 ± 0.59
Total f.m. [mg plant^{-1}]	103.30 ± 20.85 *	56.82 ± 4.38
Total d.m. [mg plant^{-1}]	24.59 ± 3.75 **	13.29 ± 0.98

Table 2. Morphological parameters estimated on *ex vitro* plantlets. Means ± SE (n =15) after two weeks of acclimatization and number of survived plants estimated (n = 30) at the end of the acclimatization phase (21st day); differences were significant at * - $P \leq 0.05$, or ** - $P \leq 0.01$.

	Aerated vessels	Closed vessels
Total survival %	68.70 ± 1.52*	57.00 ± 3.65
Stem length [cm]	2.73 ± 0.20*	1.36 ± 0.28
Leaf number	13.00 ± 2.50	6.00 ± 2.45
Leaf area [mm^2]	321.66 ± 26.94	248.66 ± 50.62
Root number	1.33 ± 0.41	2.33 ± 1.63
Root length [cm]	3.72 ± 0.35*	2.12 ± 0.27
Root f.m. [mg plant^{-1}]	60.00 ± 8.82	29.73 ± 14.25
Root d.m. [mg plant^{-1}]	11.60 ± 1.47	5.80 ± 2.57
Total f.m. [mg plant^{-1}]	106.06 ± 14.70*	54.63 ± 3.87
Total d.m. [mg plant^{-1}]	17.06 ± 2.85**	8.73 ± 4.12

At the end of the dark period, the respiration rate of plantlets growing in both the culture systems was similar. On the contrary, at the onset of the photoperiod and during the successive light hours myrtle rooting plantlets

growing under higher gas exchanges were able to assimilate significantly more CO_2 than plantlets cultured in relatively air-tight vessels (Fig. 1*C,D*).

P_N at the 1st, 3rd and 6th hour of light (Fig. 2) was significantly higher in the myrtle microcuttings rooting in aerated vessels than in closed ones. However, P_N values showed a decline with the progress of the culture and this was very evident after 6 h of photoperiod on the 35th day of culture (Fig. 2*C*).

***Ex vitro* plant development:** The myrtle *ex vitro* plants derived from aerated vessels had a higher survival percentage than transplants originated from the closed environment (Table 2). On the 14th day of acclimatization myrtle plants previously cultured in the aerated vessels showed a significantly higher stem length, root length and dry and fresh masses (Table 2) while the leaf number, the leaf area, the number of roots per explant, and dry and fresh masses of roots were not affected.

Water and solute potentials (Fig. 3*A,B*) showed a rapid decrease in both treatments in comparison with the end of the *in vitro* culture reaching average values of -2.0 MPa which corresponded to a decrease in the relative water content in both treatments (Fig. 3*C*). During the later days after *ex vitro* transfer all myrtle plants tended to recover their water content. At the same time solute potentials remained constant (below -1.5 MPa) in both treatments (Fig. 3*B*).

Rooted myrtle plantlets derived from the aerated *in vitro* micro-environment retained the higher photosynthetic ability during all the *ex vitro* growth even if the major difference was observed after the first week of acclimatization (Fig. 3*D*).

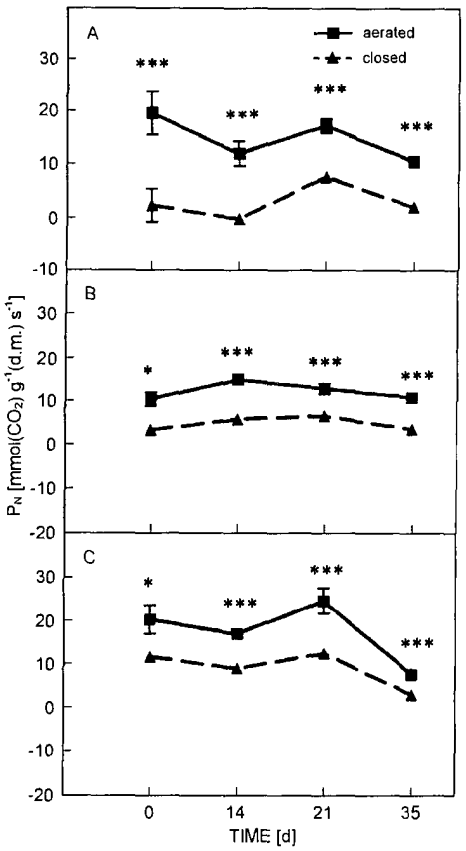

Fig. 2. P_N of *in vitro* myrtle plantlets developed in aerated and closed vessels during all the culture period. CO_2 samples were withdrawn from vessels at the 1st hour (*A*), 3rd hour (*B*) and 6th (*C*) hour after the beginning of photoperiod. Means ± SE ($n = 5$); * - $P \leq 0.05$; ** - $P \leq 0.01$; *** - $P \leq 0.001$ within each sampling time.

Discussion

The gas diffusion in aerated vessels maintain enough CO_2 to sustain carbon assimilation for all the culture period. By contrast 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 myrtle P_N: this condition is characteristic in the usual micropropagation protocols of the majority of the plant species (De Riek *et al.* 1991, Kozai 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 during all the culture. However, the efficiency of the gas permeability of the system was limited to the first three weeks of culture but, later, CO_2 supply was not enough related with the size of the explant and P_N.

The *in vitro* cultured myrtle microcuttings had similar Chl content as other plants growing in open field at analogous irradiance (Donelly and Vidaver 1984) and it was comparable in plantlets developed in aerated and closed vessels. Reduced gas exchanges inside closed containers could lead to the accumulation of toxic levels of ethylene and other oxidative products which directly affect the degradation of photosynthetic pigments (Righetti 1996, Cournac *et al.* 1991). In contrast, the rate of gas exchanges per hour (E = 0.3) performed by our system was just enough to prevent degradation or allow the regeneration of pigments in plantlets cultured in closed vessels.

As suggested by other authors (Kozai and Iwanami 1988, Cournac *et al.* 1991) a small amount of sugars (photo-mixotrophy) could improve the culture. The increase of photosynthetic activities observed in this work is the result of the right combination of gas exchange, light and sugar amount: but these conditions were species specific and could vary inside the same species since different genotypes have their own control on photosynthesis reactions and regulation of enzymatic activities (Galzy and Compan 1992).

Results showed in this work suggest that the well developed root system, the high P_N and the dry mass accumulation during the pre-acclimatization phase in aerated vessels can induce a better ability to face the transplant stress in myrtle plantlets as observed by other authors (Kozai 1991, Kirdmanee *et al.* 1995).

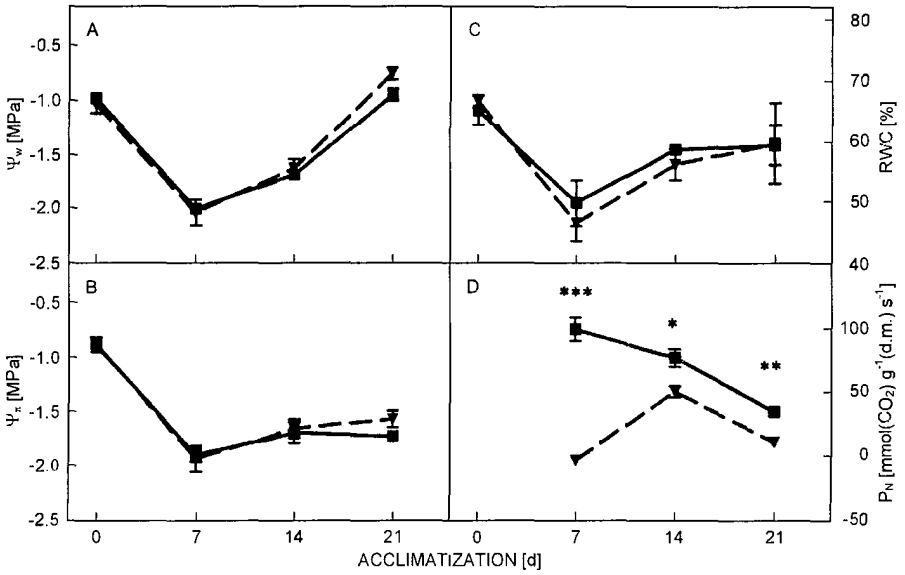

Fig. 3. Water potential, Ψ_w (*A*), osmotic potential Ψ_π (*B*), relative water content RWC (*C*) and P_N (*D*) of myrtle transplants derived from aerated and closed *in vitro* environments. Ψ_w, Ψ_π and RWC means ± SE (n = 10) and P_N means ± SE (n = 5); * - $P \leq 0.05$; ** - $P \leq 0.01$; *** - $P \leq 0.001$ within each sampling time.

Myrtle plants derived from aerated vessels had photosynthetic ability from the beginning of the *ex vitro* phase whereas, during the first two weeks of acclimatization, water losses and solute accumulation were observed. At the same time also the plants from closed vessels performed an increase in the water deficit, but in the absence of CO_2 assimilation. Capellades *et al.* (1990) suggested that changing environmental conditions during the *in vitro* culture caused anatomical modifications of plantlet stomata so that the efficiency of CO_2 assimilation and the quality of the *ex vitro* plants could be improved. At the 14th day of the acclimatization period it can be observed a tendency to develop a photosynthetic ability in the plants derived from the closed vessels and the partial re-hydration demonstrated the renewal of almost all the physiologic activities. At the end of pre-acclimatization phase myrtle plants developed in more aerated vessels triplicated the initial dry weight and duplicated the initial leaf area (data not shown) and, during the next *ex vitro* phase, they maintained this positive trend. This behaviour may be a consequence of larger reserve storage (Laforge *et al.* 1991, Van Huylenbroek and Debergh 1996) accumulated in myrtle plants which can develop autotrophy during the *in vitro* culture. From the results obtained, it is evident that the photosynthetic ability achieved during the *in vitro* phase was maintained also during acclimatization as reported on other species (Lees *et al.* 1991, De *et al.* 1993, Deng and Donelly 1993, Navarro *et al.* 1994). Moreover the extensive root apparatus developed in the artificial matrix support (perlite) combined with the aerated environment was as much as useful for facing the transplant stress likewise observed by Kirdmanee *et al.* (1995) on *Eucalyptus camaldulensis* shoots cultured photo-autotrophically on vermiculite as supporting material.

Finally it came out from this study that myrtle microcuttings were suitable explants to be also employed as *in vitro* model for ecophysiological studies. In fact, the development of photosynthetic activity *in vitro* can allow us to establish culture systems which tend to look like the analogous *in vivo* ones. Proper modulations of micro-environment conditions and medium compositions could bring towards *in vitro* simulations of environmental changes in order to make easier clonal selections in facing abiotic stress.

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