In vitro culture of sweet basil: gas exchanges, growth, and rosmarinic acid production

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Abstract

Five *in vitro* culture systems with different ventilation rates were used to investigate the influence of vessel environment on photosynthesis, dark respiration, ethylene evolution, and rosmarinic acid (RA) production in sweet basil (*Ocimum basilicum* L.) micropropagated shoots. The systems under comparison were two bioreactors with either temporary (*RITA*™) or stationary (*Growtek*™) immersion, and three types of vessels (*Magenta*™, *Microbox* ECO_2™, and *PCCV25*™) that are largely used for plant micropropagation. Shoots of green-leaved cv. Genovese and purple-leaved cv. Dark Opal were cultured on a modified Murashige and Skoog medium containing 0.25 mg dm^{-3} 6-benzylaminopurine. The instantaneous rates of photosynthesis, dark respiration, and ethylene production were determined by gas chromatography measuring CO_2 and ethylene concentrations in vessel headspaces. The tissue RA content was determined by HPLC in HCl-methanol extracts. The explant growth and morphology were significantly affected by culture conditions and cultivars. The largest biomass production was observed under the photomixotrophic culture conditions provided by *Growtek*™, whereas the highest RA content in shoot tissues was found in the *RITA*™ photomixotrophic system, where ethylene accumulated to the greatest extent.

Additional key words: bioreactors, ethylene, mixotrophic culture, photosynthetetic rate, respiration rate, secondary metabolism.

Introduction

Sweet basil (*Ocimum basilicum* L.) belonging to the *Lamiaceae* family, is widely used as food flavouring agent and is cultivated all over the world (Makri and Kintzios 2007). This species is also used for cosmetic and pharmaceutic products as it contains large amounts of essential oils and rosmarinic acid (RA) (Jayasinghe *et al.* 2003, Petersen and Simmons 2003, Juliani *et al.* 2008).

Aromatic and medicinal plants, including sweet basil, are usually cultivated in open field which results in large year-to-year variability in both biomass production and the content of active principles (Bourgaud *et al.* 2001). For this reason, there is an increasing interest for *in vitro* cell and tissue cultures (Matkowski 2008, Karuppusamy 2009). The *in vitro* system is characterized by a strict control of the growing conditions and allows to obtain the all-year round production of uniform and high-quality plant material (Lucchesini and Mensuali-Sodi 2010). The possibility to obtain a high RA accumulation has been proven in different *in vitro* cultured species, such as *Lavandula vera*, *Coleus blumei*, and *Salvia officinalis* (Matkowski 2008, Park *et al.* 2008). Different basil species accumulate larger quantities of RA in cells, callus, hairy roots, and micropropagated shoots than *in vivo* plants (Kintzios *et al.* 2003, 2004, Rady and Nazif 2005, Hakkim *et al.* 2007, Kiferle *et al.* 2011).

Identifying and understanding the effects of *in vitro* environment on explant growth and secondary metabolism is essential for scaling-up the bioactive compound production by plant cell and tissue cultures (Georgiev *et al.* 2009). The conventional *in vitro* cultures are usually photomixotrophic: plantlets use both photosynthates and exogenous sugars as energy sources (Kozai 1991, Kozai and Kubota 2005, Xiao *et.al.* 2011, Dias *et al.* 2013). Plantlets with the increased photosynthetic rate (P_N) grow and develop better than those with low P_N (Lucchesini *et al.* 2001, Afreen *et al.* 2002, Xiao *et al.* 2011). The supply of CO_2 from external atmosphere is facilitated in vessels with a high ventilation rate which also reduces the accumulation of ethylene synthesized by plant tissues and/or released by

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Abbreviations: CAD - caffeic acid derivative; P_N - net phothosynthetic rate; PPFD - photosynthetic photon flux density; RA - rosmarinic acid; R_D - dark respiration rate
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abiotic sources, such as agar and plastic materials (Panizza *et al.* 1993, Xiao *et al*. 2011). A high ethylene concentration can influence plantlet morphogenesis and induce chlorophyll breakdown, thus affecting negatively their photosynthesis (Hazarika 2006). Many studies have been conducted to investigate the influence of ethylene and CO_2 concentrations in vessel atmosphere on growth and development of *in vitro* plantlets (Hazarika 2006, Xiao *et al.* 2011). However, much less attention has been paid to the effects of gas exchange on the production of secondary metabolites (Ikemeyer *et al.* 1989, Zobayed and Saxena 2004, Mosaleeyanon *et al.* 2005) and, to our knowledge, no paper focused on the RA production.

In this study, five *in vitro* culture systems differing in ventilation rates and physical properties of growing medium (solid or liquid) were used to investigate their influence on photosynthesis, dark respiration, ethylene accumulation and RA production in micropropagated shoots of two sweet basil genotypes differing in leaf pigmentation and RA content (Kiferle *et al.* 2011). The systems under comparison were two bioreactors with either temporary (*RITA*™) or stationary (*Growtek*™) immersion and three types of vessels (*Magenta*™, *Microbox ECO*$_2$™, and *PCCV25*™) largely used for plant micropropagation (Fig. 1, Table 1).

Fig. 1. Types of vessels used for the *in vitro* culture of sweet basil shoots: *A* - *MicroboxECO*$_2$™, *B* - *PCCV25*™, *C* - *GA-7* box *Magenta*™, *D* - temporary immersion system *RITA*™, *E* - cylindrical bioreactor *Growtek*™.

Table 1. Main characteristics of culture vessels and growing media used for the *in vitro* culture of sweet basil from nodal explants. The number of explants per vessel and the air exchange rate are also shown. The headspace volume was calculated as the difference between the total volume and the volume of growing medium. PSU - polysulfonate, PP - polypropylene, PC - polycarbonate, Ø - diameter, L - length, W - width, H - height, E - the number of gas exchanges of the vessel per hour.

Vessel type	Supplier	Material	Size [mm]	Medium [dm^3]	Headspace [dm^3]	Medium status	Explant number	E [h^{-1}]
ECO$_2$™	*Duchefa Micropoli*, Cesano Boscone, Italy	PP	L:125 W:65 H:80	0.100	0.450	solid	12	7.1
PCCV25™	*TQPL Company*, New Milton, UK	PP	Ø:90 H:70	0.050	0.092	solid	6	4.2
Magenta™	*Sigma Aldrich*, Milan, Italy	PC	L: 77 W: 77 H: 97	0.050	0.525	solid	6	0.7
Growtek™	*Scienceware*, Wayne, USA	PC + PP	Ø:11 H:160	0.150	1.028	liquid	12	1.0
RITA™	*Vitropic*, Saint-Mathieu-de-Tréviers, France	PSU	Ø:18 H:150	0.150	0.830	liquid	12	2.0

Materials and methods

Seeds of two sweet basil (*Ocimum basilicum* L.) cultivars, green-leaved Genovese (GE) and purple-leaved Dark Opal (DO), purchased from *SAIS* (Cesena, Italy) were germinated and then grown in hydroponics (floating system). Nodal segments were used as explants and cultured *in vitro* as previously described (Kiferle *et al.* 2011). Basil shoots were proliferated in *PCCV25*™ boxes (six explants per box) on a solid (7 g dm^{-3} of *Difco Bacto* agar) Murashige and Skoog (1962; MS) medium with 300 mg dm^{-3} reduced glutathione (GSH), 500 mg dm^{-3} 2-(N-morpholino) ethanesulfonic acid (MES), 30 g dm^{-3} sucrose, and 0.25 mg dm^{-3} 6-benzylamino-purine (BA). The pH was adjusted to 5.8 before autoclaving. From these stock cultures, explants were excised and sub-cultured in different types of vessels on either a solid or liquid medium with the same nutrient composition as previously described. *Magenta*™, *Microbox ECO*$_2$™, and *PCCV25*™ vessels were filled with the solid medium and equipped with a filter on their closures in order to ensure aseptic ventilation. *RITA*™

and *Growtek*™ bioreactors were employed for temporary and stationary immersion cultures, respectively. In *RITA*™ system, an air pump was used to push the liquid medium up and immerse temporarily the explants (1 min every 12 h). The air pressure was applied through 0.2 µm porous filters. *Growtek*™ was modified by fitting a round filter (a diameter of 19 mm) to the side tube for sterile ventilation. All culture vessels were equipped with a gas-sampling device to allow the analysis of headspace air (Lucchesini *et al.* 2001). The hourly number of gas exchanges (E) in each type of vessel was determined using ethylene as tracer gas. The cultures were incubated in a growth chamber at a temperature of 22 ± 1 °C, a 16-h photoperiod, and an irradiance of 100 µmol m^{-2} s^{-1}.

The fresh and dry masses were determined at the onset of the culture (0) and after 14, 21, and 28 d of culture, whereas the shoot height, leaf number, and tissue content of chlorophylls, anthocyanins, and RA were determined at the end of the experiment (28th day). Gas samplings were taken from the head-space of the culture vessels 14, 21, and 28 d after the onset of culture and analyzed for CO_2 and ethylene concentrations. Three 2-cm^3 air samples were consecutively withdrawn with a hypodermic syringe (three replicates, each consisting of an individual vessel). Air samples were collected at the end of the dark period and at the 1st, 3rd, 6th, and 8th hour of the photoperiod; the ethylene concentration was determined in air samples collected at the 6th hour of the photoperiod.

The ethylene and CO_2 concentrations were measured using an *HP 6890* gas chromatograph (*Hewlett Packard*, Milan, Italy) equipped with a stainless steel column (1.5 m length and 0.04 m i.d.) packed with *HaySep® T* (*Agilent Technologies*, Milan, Italy), a flame ionization detector (ethylene determination) and a thermal conductivity detector (CO_2 determination). Column and detector temperatures were 70 and 350 °C (the ethylene analysis) or 70 and 200 °C (the CO_2 analysis). Nitrogen was used as carrier gas at a flow rate of 30 cm^3 min^{-1} and as reference gas at 15 cm^3 min^{-1} for a thermal conductivity detector. The instantaneous P_N was determined as reported by Fujiwara *et al.* (1987): $P_N = (CO_{2in} - CO_{2out})_t \times K \times E \times V$ /d.m., where $(CO_{2in} - CO_{2out})$ is the difference between CO_2 concentration [cm^3 m^{-3}] inside and outside the culture vessel at time *t*, K is the molar conversion factor [41×10^{-3} µmol dm^{-3}], V [dm^3] is the headspace volume, E is the number of hourly gas exchanges (Table 1) of the vessel and d.m. [g] the shoot dry mass per culture vessel. The equation can be used for the estimation of dark respiration (R_D). In similar way, the rate of ethylene (C_2H_4) release from the culture [pmol g^{-1}(d.m.) s^{-1}] was calculated as follows: $C_2H_{4\ production} = (C_2H_{4in} - C_2H_{4out})_t \times K \times E \times V$ /d.m., where C_2H_{4in} is the ethylene concentration [mm^3 m^{-3}] inside the vessels at the time *t* and $C_2H_{4out} = 0$, taking into account that ethylene is present in a negligible concentration in the atmosphere outside the vessel. Moreover, the production of ethylene from the vessels with the solid medium in the absence of plants was subtracted from the measured values to exclude an abiotic contribution to the ethylene production (Mensuali-Sodi *et al*. 1992).

The content of chlorophylls [mg g^{-1}(f.m.)] and anthocyanins [mg(cyanidin-3-glucoside) g^{-1}(d.m.)] were determined spectrophotometrically in shoot samples that were extracted by the mixture of 95 % (v/v) ethanol and 80 % (v/v) methanol with 1.2 M HCl, respectively, in the dark at 4 °C overnight (Kiferle *et al.* 2011).

The content of RA and other caffeic acid derivatives (CADs; *e.i.*, caffeic acid, caftaric acid, chlorogenic acid, cicoric acid, cynarin, ferulic acid, *t*-cinnamic acid, and *p*-coumaric acid) were determined in samples consisting of one or two shoots from the same vessel. All the samples were rapidly washed in tap water, rinsed in deionised water, gently dried, frozen in liquid nitrogen, and stored at -80 °C before analyses which were performed within a few weeks after sampling. The samples were not dried in an oven, as desiccation prior to extraction was found to reduce markedly the RA content of sweet basil tissues (Kiferle *et al.* 2011). The content of RA and selected CADs in the HCl-methanol extracts was determined by HPLC as previously described (Kiferle *et al.* 2011) and expressed on a dry mass unit (the dry mass content was determined in sub-samples). Peak identification was accomplished with mass spectrometry (LC-MS) and tandem mass spectrometry (LC-MS-MS) as previously reported (Kiferle *et al.* 2011). The detection limit of the analytical method was 0.05 mg g^{-1}(d.m.).

A completely randomized design was adopted. Data were subjected to *ANOVA* and mean values were separated using the LSD test at the 5 % probability level. The statistical analysis was performed using the *Statgraphics Centurion XV.II* (*Manugistic Co*., Rockville, USA) software. The experiment was repeated twice with similar findings and the paper reports the results from a representative run.

Results

During the dark period, the headspace concentration of CO_2 ranged between 1 000 and 3 000 cm^3 m^{-3} in all the types of vessels with GE shoots except for the basil plantlets cultured into the *PCCV*™ vessel, where the CO_2 concentration rised up to 4 000 cm^3 m^{-3} at the end of the culture (Fig. 2*A*). DO plantlets showed similar CO_2 values also in *RITA*™ vessels (Fig. 2*B*). During daytime, all the solid cultures showed CO_2 concentrations below 500 cm^3 m^{-3}, whereas in *Growtek*™ and *RITA*™ bioreactors, the CO_2 concentrations were similar to those determined at the end of the night period (Fig. 2*C*,*D*). The highest CO_2 concentration was recorded in the DO

plantlets cultured in *RITA*™ system (Fig. 2*D*). The CO_2 concentration in the headspace depended on the plantlet P_N and R_D (Fig. 3). At the end of the dark phase (Fig. 3*A*,*B*), high R_D values were observed in *Magenta*™, *PCCV25*™, and especially in *Microbox ECO*$_2$™. The plantlets cultured in these vessels showed the highest CO_2 uptake during the light period (Fig. 3*C*,*D*). The CO_2 uptake in these cultures was evident just after one hour from the onset of photoperiod and showed constant values from the 3rd until the 8th hour (data not shown). The P_N (Fig. 3 *C*,*D*) increased with an increased gas exchange characterized by E value (Table 1). Basil shoots grown in *Microbox ECO*$_2$™ (E = 7.1 h^{-1}) exhibited the highest P_N during the whole culture period,whereas the lowest P_N was generally found in *Magenta*™ (E = 0.7 h^{-1}). The values of both R_D and P_N were very low in the explants grown in *RITA*™ and *Growtek*™ bioreactors (Fig. 3).

Analogous trends in terms of R_D and P_N were observed in both the sweet basil cultivars. By the way, the GE plantlets in *Magenta*™, *Microbox ECO*$_2$™, and *PCCV25*™ showed higher P_N values than the DO

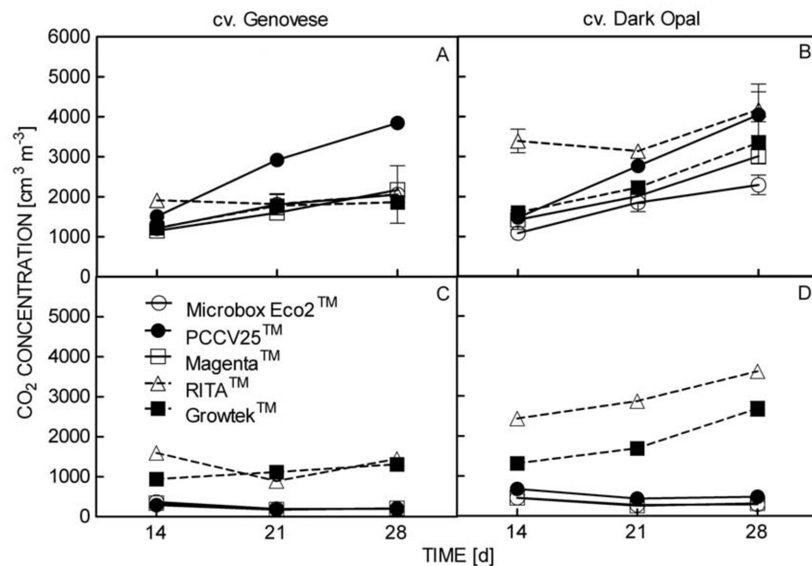

Fig. 2. The concentration of CO_2 in headspace of different culture vessels used for the *in vitro* cultivation for 14, 21, and 28 d of sweet basil shoots with green (cv. Genovese) or purple (cv. Dark Opal) leaves. The measurements were performed at the end of dark period (*A*, *B*) and during the daytime (average values of the 3rd, 6th and 8th hourof the photoperiod; *C*, *D*). Means ± SE, *n* = 3.

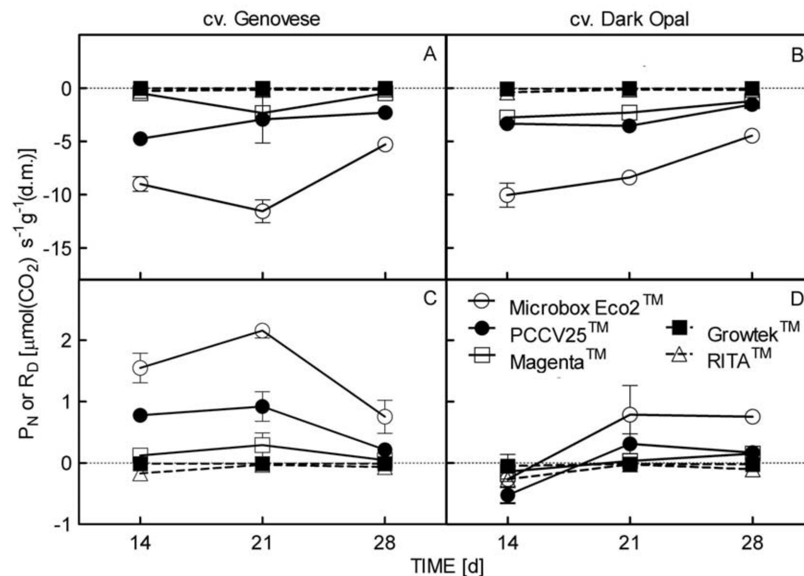

Fig. 3. The dark respiration rate (R_D) and net photosynthetic rate (P_N) in shoots of two sweet basil cultivars with green (Genovese) or purple (Dark Opal) leaves cultured *in vitro* for 14, 21, and 28 d using different types of vessels. The measurements were performed at the end of dark period (*A*, *B*) and during the daytime (average values of the 3rd, 6th and 8th hourof the photoperiod; *C*, *D*). Means ± SE, *n* = 3.

plantlets and acquired photosynthetic competence earlier than DO. In fact, a photosynthetic activity was evident after 14 or 21 d of culture in the GE and DO explants, respectively (Fig. 3*C*,*D*).

In the GE culture, the ethylene concentration in the vessel headspace (Fig. 4*A*) remained rather constant during the experiment regardless of the vessel type. In *RITA*™ bioreactor, the ethylene concentration was always significantly higher than in the other vessels (Fig. 4A). The ethylene concentration in the DO cultures showed a sharp increase only in *RITA*™ bioreactor after 21 d of culture (Fig. 4*B*). The rate of ethylene production from shoots of both the cultivars decreased gradually with time (Fig. 4*C*,*D*) except for DO in *RITA*™ that showed an increase after day 21. At the end of culture, the highest rate of ethylene production was determined in *RITA*™ bioreactors (Fig. 4*C*,*D*).

The longest shoots were observed in *Magenta*™ and *RITA*™ for GE, and in *PCCV*™ for DO (Table 2). The greatest number of leaves was recorded in the GE shoots cultured in *PCCV*™, whereas in DO, the lowest number of leaves was observed in the two bioreactors (Table 2). The shoot number per plantlet was quite low (it averaged 2.09) with no significant differences between the cultivars and among the vessel types (Table 2). The vessel type influenced significantly the accumulation of fresh and dry masses,but they were not affected by plant genotype (Table 2). In both the genotypes, the highest

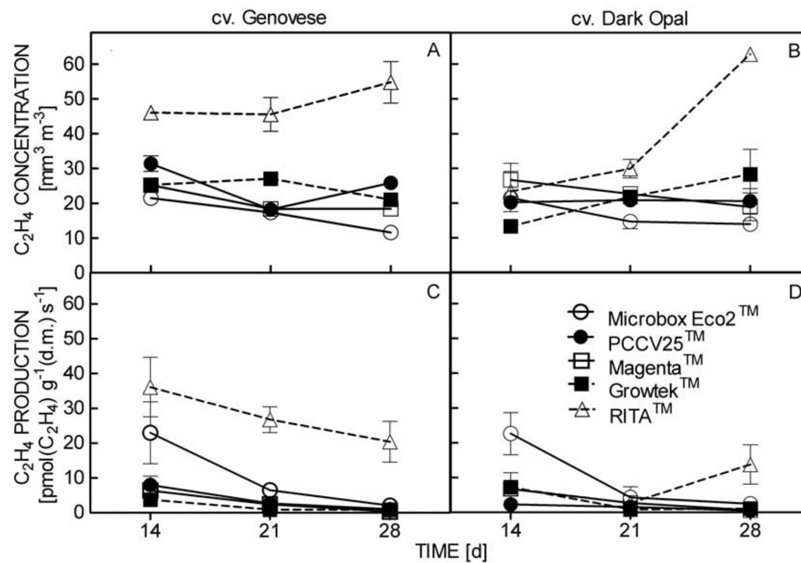

Fig. 4. The ethylene (C_2H_4) concentration in headspace at the 6th hour of photoperiod and the ethylene production in shoots of two sweet basil cultivars with green (Genovese) or purple (Dark Opal) leaves cultured *in vitro* for 14, 21, and 28 d using different types of vessels. Means ± SE, *n* = 3.

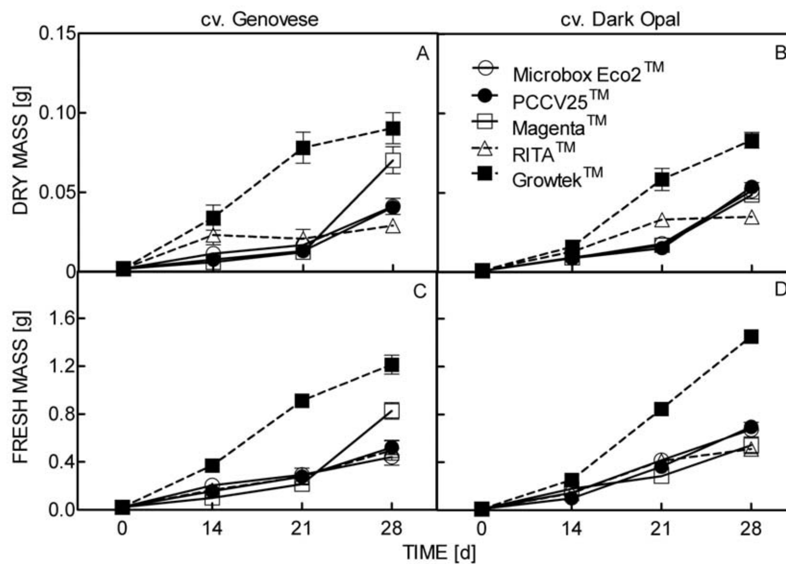

Fig. 5. The time courses of dry and fresh masses in shoots of two sweet basil cultivars with green (Genovese) or purple (Dark Opal) leaves cultured *in vitro* using different types of vessels. Means ± SE, *n* = 3.

fresh and dry masses were observed in *Growtek*™ (Fig. 5; Table 2). Moreover, the initial shoot fresh mass of GE and DO were 22 and 11 mg, respectively, and the shoot dry mass 1.9 and 0.9 mg. Shoot masses increased till the end of cultivation in all the vessels apart from the *RITA*™ system, where shoot growth stopped after 14 or 21 d of culture of GE and DO, respectively (Fig. 5).

The shoot chlorophyll content was similar in the DO and GE plantlets and was higher in shoots cultured on the solid medium than in those cultured in the liquid medium (Table 2). As expected, the DO explants contained much more anthocyanins than GE (Table 2). The vessel type did not influence the content of these pigments in the GE shoots. In contrast, the use of *Growtek*™ bioreactors reduced considerably the content of anthocyanins in DO. In these cultures, anthocyanins appeared to accumulate in the callus that developed at the base of the explants (Fig. 6*F*), whereas leaves did not show any reddish colour. Callus formation was not observed in the other vessels. The leaves of the GE plantlets maintained their typical oval shape and a dark green colour (Fig. 6*A*,*B*,*C*). The red leaves of DO showed a tendency to greening (Fig. 6*E*,*F*) in comparison to the *in vivo* plants (Fig. 6*D*). Some morphological alterations were also observed in the DO plantlets grown in *Growtek*™, as they did not exhibit the typical ruffled edge and showed hyperhydricity symptoms (Fig. 6*F*).

Among the CADs of interest, RA was the only compound found in all samples at concentrations higher than a detection limit. At the end of the culture period, the content of RA was significantly higher in the shoots grown in *RITA*™ compared to all the other vessel types (Fig. 7). Moreover, in this type of vessel, the GE and DO cultures showed a significant difference in the RA content: 154.8 and 100.2 mg g^{-1}(d.m.), respectively. No significant differences were evident in the RA content in all the other vessels, averaging 44 mg g^{-1}(d.m.) (Fig. 7).

Fig. 6. The *in vitro* shoot culture of two sweet basil cultivars with green (Genovese; *A*,*B*,*C*) or purple (Dark Opal, *D*,*E*,*F*) leaves and in different types of vessels: *PCCV*™ (*B*,*E*), *RITA*™ (*C*), or *Growtek*™ (*F*). The pictures were taken at the end of multiplication phase. *Arrows* in *F* show the conspicuous red callus masses.

Table 2. The shoot length (S_L) [cm], leaf number (L_N), shoot number (S_N), shoot fresh mass (S_{FM}) [g], shoot dry mass (S_{DM}) [g], and total chlorophyll (Chl) [mg g^{-1}(f.m.)] and anthocyanin (Anth) [mg g^{-1}(d.m.)] content in shoots of sweet basil cvs. Genovese and Dark Opal cultured *in vitro* for 28 d using different types of vessels. Data were subjected to *ANOVA* (** $P \leq 0.01$; *** $P \leq 0.001$; n.s. = not significant) and mean values (n = 12) were separated using the LSD test: mean values followed by different letters differ significantly ($P \leq 0.05$).

Cultivar	Vessel type	S_L	L_N	S_N	S_{FM}	S_{DM}	Chl	Anth
Genovese	*ECO2*™	0.87 b	9.5 b	1.92 a	0.442 b	0.035 cd	0.87 b	0.58 b
	PCCV25™	1.37 ab	12.3 a	2.25 a	0.522 b	0.035 cd	1.33 a	0.79 b
	Magenta™	2.00 a	9.5 b	2.33 a	0.826 b	0.070 b	0.81 b	0.48 b
	Growtek™	1.28 b	7.8 b	2.00 a	1.212 a	0.090 a	0.25 cd	1.47 b
	RITA™	2.00 a	7.0 b	2.25 a	0.507 b	0.029 c	0.39 cd	0.87 b
Dark Opal	*ECO2*™	1.37 ab	11.6 a	2.00 a	0.673 b	0.050 b	1.31 a	10.04 ab
	PCCV25™	2.04 a	14.3 a	2.00 a	0.697 b	0.053 bc	1.60 a	20.28 a
	Magenta™	1.25 b	11.3 a	2.00 a	0.543 b	0.043 bc	0.57 bc	19.12 a
	Growtek™	1.56 ab	9.3 b	2.18 a	1.458 a	0.083 a	0.10 d	1.49 b
	RITA™	1.39 ab	9.3 b	2.00 a	0.508 b	0.035 c	0.36 cd	9.25 ab
Cv. (A)		n.s.	**	n.s.	n.s.	n.s.	n.s.	***
Vessel (B)		***	***	n.s.	***	***	***	***
A × B		n.s.	n.s.	n.s.	n.s.	*	**	***

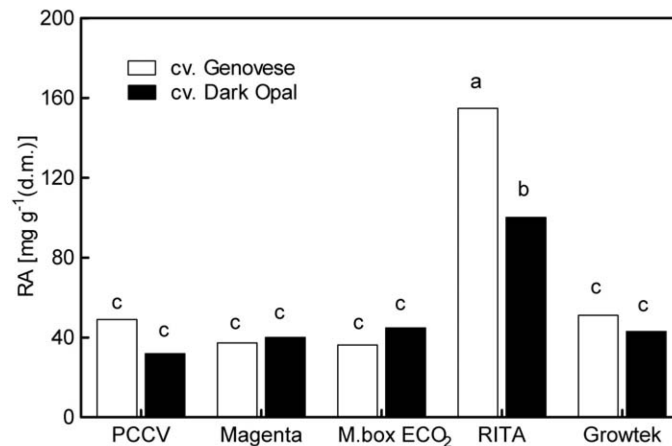

Fig. 7. The rosmarinic acid (RA) content in shoots of two sweet basil cultivars with green (Genovese) or purple (Dark Opal) leaves cultured *in vitro* using different types of vessels. The measurements were taken after 28 d of culture. *ANOVA* was performed and mean values were separated using the LSD test (*n* = 4). Different letters mark significant differences ($P \leq 0.05$) among vessel types.

Discussion

In our work, the type of vessel affected all measured growth parameters with the exception of the shoot number. In agreement with previous work (Kiferle *et al.* 2011), the proliferation aptitude of sweet basil explants was lower in comparison with previous findings in the same species (*e.g.*, Sahoo *et al.* 1997, Begum *et al.* 2002). Nevertheless, the adopted micropropagation protocols enabled a large mass production, which was about 80 mg(d.m.) per plantlet at the end of the multiplication phase in Magenta™ and *Growtek*™ (Table 2). Almost a 10-fold lower biomass production was obtained by Kintzios *et al.* (2004) from nodal explants of this species in bioreactors. A large biomass production in an *in vitro* culture may be ascribed to the favorable environmental conditions, in particular to vessel ventilation which can stimulate P_N reducing the degree of dependence on exogenous sugar (Kozai *et al.* 2000, Xiao *et al*. 2011).

In this work, the highest chlorophyll content was found in sweet basil shoots grown on the solid media, whereas the highest P_N was associated with the highest E of *Microbox ECO*$_2$™ and *PCCV*™ which were specifically designed to improve vessel aeration. A positive correlation between the degree of aeration in the culture vessels and chlorophyll content was reported by Pospíšilová *et al.* (2000). In contrast, we observed a negative relationship between P_N and growth. In fact, the largest dry mass accumulation was observed in *Magenta*™ [P_N = 0.15 µmol g^{-1}(f.m.) s^{-1}, E = 0.7 h^{-1}] and in Growtek™ [P_N = -0.01 µmol g^{-1}(f.m.) s^{-1}; E = 1.0 h^{-1}]. In many cases, photomixotrophic conditions can yield better results than photoautotrophic conditions (Hazarika 2006, Lucchesini *et al.* 2006). We may speculate that in the poorly-ventilated *Magenta*™ vessel, the reduction of explant P_N compared with the other two systems, *PCCV*™ and *Microbox ECO*$_2$™, allowed a better utilization of exogenous sugars, thus resulting in a larger mass accumulation. These results are in agreement with those reported by Chanemougasoundharam *et al.* (2004), who demonstrated that culture systems with low gas-permeable closures increase potato shoot growth.

The use of *Growtek*™ stimulated the shoot growth of both the cultivars, but it caused the modification of leaf shape, the proliferation of conspicuous callus mass and the occurrence of hyperhydricity, especially in the DO shoots. The use of a liquid medium offers several advantages for mass propagation but it may cause the occurrence of severe morpho-physiological disorders involving an apoplastic water accumulation due to the continuous immersion of tissue (Ziv 2000, Hazarika 2006, Savio *et al.* 2012). Compared to normal shoots of greenhouse-grown apple plants, the leaves of hyperhydric shoots cultivated in a bioreactor contain less chlorophyll and show a lower ribulose bisphosphate carboxylase activity resulting in reduced carbon assimilation (Chakrabarty *et al.* 2005). *In vitro* plants grown in a liquid medium are generally heterotrophic (Jackson 2003). These systems facilitate the sucrose uptake by plantlets in comparison with solid systems (Afreen *et al.* 2002, Dey 2005). It is known that higher sugar availability can decrease the photosynthetic ability of micropropagated plantlets (Kozai 1991, Lucchesini *et al.* 2006, Mingozzi *et al.* 2011). In this work, the basil plantlets, cultured in the liquid medium with sugars, showed a low chlorophyll content and low P_N. Moreover, in *RITA*™ and *Growtek*™, the temporary or constant submersion of the basil plantlets could alter the function of stomata, thus further affecting negatively their P_N as found by Afreen *et al.* (2002).

Dey (2005) reported that *Growtek*™ bioreactor facilitates the nutrients uptake by explants of several species due to the presence of a floating explant holder. In this work, the high availability of nutrients could explain the reduction of the anthocyanins content (greening) of the DO explants cultured in *Growtek*™, as

the synthesis of this class of pigments is inversely correlated to the availability of sugars and minerals in the medium (Steyn *et al.* 2002).

Vessel ventilation determines the whole gas composition of the headspace and can also influence the ethylene concentration (Mensuali-Sodi *et al.* 2007). In this work, a negative relationship between the ethylene accumulation and E was observed in all the culture vessels except *RITA*™, where the ethylene concentration in the headspace was affected by the high production of this hormone from the plant tissues. Ethylene is often synthesized by *in vitro* cultured tissues in response to stress conditions (Morgan and Drew 1997, Gaspar *et al.* 2002, Yasmin *et al.* 2013). In the *RITA*™ system, sweet basil was exposed to different kinds of stresses: *1*) the water accumulation in the apoplast which imposed a low oxygen content (Saher *et al.* 2004); *2*) the transient nutrient starvation due to the temporary immersion (Deng *et al.* 2012); and *3*) shoot stirring during the immersion due to bubbling the medium (Martre *et al.* 2001). All these stresses could increase ethylene biosynthesis. The ethylene accumulation in the *RITA*™ system was associated with a low dry mass, especially of the GE plantlets, but with a high RA content at the end of the culture. Ethylene may either stimulate or inhibit growth (Biddington 1992) and secondary metabolism in various plant tissue and cell cultures. For instance, ethylene promotes the production of alkaloids in cell suspension cultures of *Coffea arabica* and *Thalictrum rugosum* (Cho *et al.* 1988) and of *Thalictrum minus* (Kobayashi *et al.* 1991 a,b), and diosgenin in *in vitro* plantlets of *Dioscorea zingiberensis* (Diarra *et al.* 2013). The influence of plant growth regulators on production of secondary metabolites was also demonstrated in other studies (Patnaik *et al.* 1999, Lucchesini *et al.* 2009, Kiferle *et al.* 2011).

In experiments with different sweet basil cultivars, it was observed that *in vitro* cultured tissues or cells accumulate more secondary metabolites than donor *in vivo* grown plants (Kiferle *et al.* 2011, Bertoli *et al.* 2013). This may be ascribed to peculiar environmental conditions provided by the *in vitro* culture since *in vivo* sweet basil plants cultivated under greenhouse conditions or in hydroponics produced lower RA amounts (Juliani *et al.* 2008, Nguyen *et al.* 2010, Kiferle *et al.* 2013).

Kiferle *et al.* (2011) reported that among the CADs of interest, RA is the only compound found in basil shoot cultures at relatively high amounts: 40 - 50 mg g^{-1}(d.m.). Our experiments confirm the remarkable presence of RA [154.8 mg g^{-1}(d.m.)] in plantlets grown in *RITA*™ vessels exceeding by far the content reported in the literature for *in vitro*-grown sweet basil. For instance, the content of RA is 0.18 mg g^{-1}(d.m.) in nodal explants grown in a plastic airlift bioreactor (Kintzios *et al.* 2004), 10 mg g^{-1}(d.m.) in suspension cultures (Kintzios *et al.* 2003), and 20 mg g^{-1}(d.m.) in immobilized cells (Moschopoulou and Kintzios 2011). Despite the high RA content detected in plantlets growing in *RITA*™ bioreactor, we observed that DO accumulated less RA than GE. On the contrary, other authors showed a higher RA production in DO *in vitro* (Kiferle *et al.* 2011) than *in vivo* (Nguyen *et al.*, 2010). This red-leaved cultivar has been proven to accumulate a high amount of phenolics under greenhouse conditions with a more than double RA content as compared to other green-leaved sweet basil cultivars (Juliani *et al.* 2008). The different RA production of the two cultivars might be indeed related to the dissimilar evolution of ethylene and dry mass accumulation during the *in vitro* culture as observed in other plant tissue cultures (Mensuali-Sodi *et al.* 1989, Zhao *et al.* 2005). The stop of growth since the 14th day and the large ethylene production may be the result of an earlier shift from primary to secondary metabolism in GE than in DO, suggesting a different metabolic activity in the two cultivars.

In conclusion, the composition of medium and the vessel micro-environment influenced growth, morphology, and secondary metabolism of the *in vitro* grown basil shoots. The results obtained with *Magenta*™, *Microbox ECO$_2$*™, and *PCCV25*™ vessels suggest that photomixotrophic conditions coupled with positive P_N values influenced explants growth without any significant effects on the RA production. The largest amount of dry mass was produced in *Growtek*™; this system maintained daily negative P_N compensated by a facilitated sugar uptake from the medium. The poor growth and high RA content in the shoots cultured in *RITA*™ system were likely the result of a combination of factors including the large accumulation of ethylene released from the plant tissues and entrapped in the headspace, the tissue hyperhydration, the transient nutrient starvation, and the negative P_N values not compensated by an adequate sugar uptake.

The current study indicates that the RA production in the *in vitro* plants cultured in *RITA*™ bioreactor was up-regulated when low P_N was associated to a reduction of the biomass production. Our work confirmed the close relationship between the environment of the *in vitro* culture (the vessel, culture medium, exchange of air, *etc*.) and the plantlet metabolism which, when suitably modified, can lead to an increased production of secondary metabolites.

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