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ORIGINAL ARTICLE

Effect of light and natural ventilation systems on the growth parameters and carvacrol content in the in vitro cultures of *Plectranthus amboinicus* (Lour.) Spreng

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Abstract The aim of the current study is to investigate the influence of light intensity, quality of light and alternative membrane sytems on the growth and headspace-GC/MS chemical analysis of *Plectranthus amboinicus* cultivated in vitro. Nodal segments were grown under light intensities (26, 51, 69, 94 and 130 µmol m^{-2} s^{-1}) provided by cool-white fluorescent lamps. Apical segments were grown under light-emitting diodes blue; red; 1 blue/2.5 red; 2.5 blue/1 red; 1 blue/1 red and white fluorescent lamps. Apical and nodal segments were grown under alternative membrane and membrane-free systems. One, two or four PTFE membranes were used on the lid of the culture vessel. The membranes provided natural ventilation and worked as filters. The results have shown significant differences in the growth and carvacrol content, as well as in the content of carvacrol precursors (γ-terpinene and *p*-cymene) in different treatments. Among all tested light intensities, the significant increase in the dry weight and in the carvacrol content of plantlets derived from the nodal segments was recorded at 69 µmol m^{-2} s^{-1}. The monochromatic red led to greater shoot length and higher dry weight in plantlets derived from the apical segments, as well as to carvacrol accumulation greater than that provided by the fluorescent lamps. The culture vessel enclosure by one and two membranes led to higher dry weight in plantlets derived from the apical and nodal segments, respectively. They also showed higher carvacrol content. Thus, it is possible optimizing the growth and carvacrol content in *P. amboinicus* cultivated in vitro by adjusting these environmental parameters.

Keywords Irradiances · Light spectrum · Gas exchange · Medicinal plant · LEDs

Abbreviations

GC/MS	Gas chromatography/mass spectrometry
LED	Light emitting diodes
R	Red
B	Blue
F	Fluorescent
NMS	No membrane system
AMS	Alternative membrane system
AMS1	Alternative membrane system with one filter
AMS2	Alternative membrane system with two filters
AMS4	Alternative membrane system with four filters
PTFE	Polytetrafluoroethylene

Introduction

Plectranthus amboinicus (Lour.) Spreng belongs to the family Lamiaceae. From an agricultural standpoint, it has great medicinal potential, but is poorly studied (Arumugam et al. 2016). Recent studies have reported the species activity against breast cancer (Hasibuan et al. 2014). *Plectranthus amboinicus* essential oil is rich in thymol and carvacrol (Khare et al. 2011). These compounds besides having medicinal effect are used as natural preservative in the food industry (Trivellini et al. 2016). In addition, the species has environmental significance and can be used in the rhizofiltration of lead-contaminated water (Ignatius et al. 2014).

The micropropagation allows mass multiplication, species conservation, as well as the production of microorganisms-free

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plants (Coste et al. 2012; Kaur and Sandhu 2015). It is an important tool to studies about medicinal plants. Light is one of the environmental factors to be controlled according to the herein adopted technique. Light intensity is the main parameter affecting photosynthesis. The quality of light has effects on photosynthesis and affects photomorphogenesis: plant shape, development and flowering (Singh et al. 2015). Moreover, these light features may affect the secondary metabolism of plantlets, as it was shown by their effect on mono- and sesquiterpenes in *Achillea millefolium* (Alvarenga et al. 2015) and in *Salvia dolomitica* (Bassolino et al. 2015) cultivated in vitro.

However, the light effect changes from species to species. The intensity 100 µmol m^{-2} s^{-1} appeared to be the best for *Momordica grosvenori* growth in vitro (Zhang et al. 2009), whereas it was 60 or 120 µmol m^{-2} s^{-1} for ginger growth (Zhou et al. 2008). The blue spectrum proved best for *Remania glutinosa* growth in vitro (Manivannan et al. 2015), and the combination between the blue and red spectra was more suitable for *Alernanthera brasiliana* (Macedo et al. 2011). In addition, the culture vessels used in conventional micropropagation techniques allowed low gas exchange, reduced carbon dioxide (CO_2) concentration, as well as high humidity (Chen 2015) and high ethylene accumulation. This is a hormone able to inhibit plant growth (Fomenkov et al. 2015). Plantlets that are micropropagated under these conditions present abnormalities such as thin cuticle, poor stomatal control and reduced photosynthetic capacity. Such abnormalities difficult the acclimatization (Jiménez et al. 2015).

The use of light emitting diodes (LEDs) allows obtaining specific spectra and regulating the photosynthetically-active and photomorphogenic radiation levels required for the cultivation of each species in vitro (Gupta and Jatothu 2013). Moreover, natural ventilation enables gas exchange between the internal and external environments, reduces humidity and ethylene accumulation, as well as keeps the appropriate internal CO_2 concentration to stimulate photosynthesis (Saldanha et al. 2012). Thus, the ventilation may increase plant growth and affect its secondary metabolism due to the increased CO_2 availability. The increased CO_2 concentration in the environment has led to artemisinin increase in *Artemisia annua* (Zhu et al. 2015).

The aim of the current study is to investigate the influence of light intensity, quality of light and natural ventilation systems on the growth and chemical analysis of *P. amboinicus* species cultivated in vitro.

Materials and methods

General conditions of the experiments

Apical and nodal segments (1 cm) from plants grown in greenhouse were treated with sodium hypochlorite (1.25% active chlorine) for 20 min, under constant stirring, in order to establish the species in vitro. Next, they were washed in autoclaved distilled water (five times) and vertically inoculated in test tubes containing 12.5 mL of Murashige and Skoog (1962) medium (MS) with half of the salt concentration, without growth regulator, 30 g L^{-1} sucrose, 6 g L^{-1} agar (Himedia®, Type I) and pH adjusted to 5.7 ± 0.1, before autoclaving (for 20 min, at 121 °C).

After inoculation, the material was kept in growth room, at 26 ± 1 °C, under 16-h photoperiod and cool-white fluorescent lamps, with standard intensity of growth room for cultivation in vitro (39 µmol m^{-2} s^{-1}). The plantlets developing from these segments were replicated and subcultured in order to allow gathering the right number of plantlets to perform the experiments. The same medium used in the establishment of the species was used in the maintenance of plantlets in the experiments; the explants were also vertically inoculated in both cases. The experiment followed a completely randomized design (CRD).

Light intensity

Nodal segments (1 cm)—derived from plantlets cultivated in vitro—were inoculated in test tubes (25 × 150 mm) containing 12.5 mL of culture medium and kept under five different light intensities (26, 51, 69, 94 and 130 µmol m^{-2} s^{-1}) provided by cool-white fluorescent lamps (Osram©, Brazil). These segments and the headspace chemical analysis were used to assess the influence of light intensity on growth. Four (4) repetitions were performed with five plantlets per repetition.The plantlets were assessed for shoot length and length of the largest root (cm); number of shoots; leaf, stem, root and total (mg) dry weight; leaf area (cm^2); and photosynthetic pigment concentration (mg g^{-1} of fresh leaf); the chemical analysis of the leaves was performed through headspace-GC/MS, after 83 days. The growth features and leaf area analyses were carried out in 4 repetitions. The leaf area was measured in the WinFOLIA software using the EPSON PERFECTION V700 PHOTO scanner. Four (4) plantlets representing each treatment were chosen. The area of each leaf from the third pair (counting from the apex of the shoot to the root) was assessed.

The analyzed photosynthetic pigments were: chlorophyll *a*, chlorophyll *b*, b/a ratio, total chlorophyll and carotenoids. These photosynthetic pigments were extracted from 0.1 g of leaves through homogenization in 80% acetone, in the dark, and further analyzed in a spectrophotometer (Tecan Rchisto Infinite M200 PRO). The pigment content was calculated according to the methodology described by Lichtenthaler and Buschmann (2001). The leaves from 3 plantlets were analyzed in each treatment and the readings were performed in triplicate.

Quality of light

The apical segments (1 cm) were inoculated in 200 mL flasks containing 40 mL of culture medium. Next, they were cultivated under different LEDs (TECNAL© Piracicaba, Brazil), namely: B (monochromatic); R (monochromatic); 1B/2.5R; 2.5B/1R and 1B/1R, and under cool-white fluorescent lamps (Osram©, Brazil) at 42 µmol m^{-2} s^{-1} light intensity. The light was provided from the top. The plantlets were assessed for shoot length and length of the largest root (cm); leaf, stem, root and total (mg) dry weight; number of leaves; and leaf area (cm^2); and the chemical analysis was used to assess the leaves through headspace-GC/MS, after 50 days. Six repetitions were performed to assess the growth features, whereas 3 repetitions were performed to assess the leaf area. Three plantlets representing each treatment were chosen to have their leaf area assessed. They were measured as previously described in the light intensity experiment. The nodal segment was used to the light intensity and apical to the quality experiments due to the distinct growth of explants. The growth of the nodal segment was slower than the apical growth. Thus, the light quality experiment was assessed before the intensity experiment.

Alternative membrane system

Apical and nodal segments were excised from plantlets grown in vitro and inoculated in 200 mL flasks containing 40 mL of culture medium. A leave pairs were kept in both segments. The experiment was arranged according to a 4×2 factorial design; 4 cultivation systems, namely: NMS, AMS1, AMS2 and AMS4; using 2 explant types: apical and nodal. One, two or four PTFE membranes were used on the lid of the culture vessel. The membranes provided natural ventilation and worked as filters. They were arranged and manufactured according to Saldanha et al. (2012).

The plantlets were assessed for shoot length and length of the largest root (cm); number of shoots; leaf, stem, root and total (mg) dry weight; leaf area (cm^2); and the chemical analysis of the leaves was performed through headspace-GC/MS, after 30 days cultivation. Six (6) repetitions were performed in order to assess the growth features, whereas 5 repetitions were conducted to asses the leaf area. The leaf area measurement has followed the same procedures used in the previous experiment. Five (5) plantlets representing each treatment were used to assess this variable.

Chemical analysis of the leaves through headspace—GC/MS

The automatic headspace extractor CombiPAL Autosampler System (CTC Analytic AG, Switzerland) coupled to the CG/MS (gas chromatograph/mass spectrometer) system was used in the analyses. The following parameters were set after the operating conditions were optimized: sample incubation temperature at 110 °C for 60 min, and syringe temperature at 120 °C. The injection volume was 1000 µL of the steam phase, it was injected in split mode at the ratio 20:1.

The samples comprised 50 mg of dried *P. amboinicus* leaves packed in 20 mL vials sealed with PTFE/silicon septum. The analysis was carried out in an Agillent® 7890A gas chromatograph–mass spectrometer coupled to an Agillent® 5975C MSD mass selective detector (Agilent Technologies, California, USA) operated by electronic impact ionization at 70 eV, in scan mode, at rate 1 scan s^{-1}, with 40–400 mz material acquisition interval.

An HP-5-MS fused silica capillary column (30 m long × 0.25 mm internal diameter × 0.25 µm film thickness) (California, USA) was used in the current study. Helium was used as carrier gas at flow 1.0 mL min^{-1}; the temperature in the injector and in the transfer line to the MS was kept at 220 and 240 °C, respectively. The initial temperature in the oven was 50 °C. It was kept for 1 min and followed by temperature ramp of 3 °C per minute up to 200 °C, and by another temperature ramp of 10 °C per minute up to 280 °C, and then kept under isothermal condition for 1 min. The concentrations of constituents in the chemical analysis were expressed as the percentage of the relative area of the total ion chromatogram signals ± standard deviation (n = 3).

The chemical constituents were identified through the comparison of retention indexes in association with the coinjection of a standard *n*-alkane solution (C_8–C_{20}), Sigma-Aldrich®, St. Louis, USA, as well as through the comparison between the mass spectra in the NIST/EPA/NIH library database (National Institute of Standards and Technology—NIST 2008) and those in the literature (Adams 2007). The retention indexes were calculated through the equation by Dool and Kratz (1963).

Statistical analysis

The data were subjected to ANOVA through F test ($p < 0.05$), in the Sisvar® software, version 5.0 (Ferreira 2007). The significance of the variables was verified through the F test.The Scott-Knott average test was used to

analyze the growth variables, photosynthetic pigment concentrations and the levels of the major constituents of the volatile chemical composition.

Results and discussion

Light intensity

The different light intensities have significantly influenced *P. amboinicus* growth in vitro (Table 1). The 69 µmol m^{-2} s^{-1} treatment led to the best growth. The species cultivation under 26 µmol m^{-2} s^{-1} led to the lowest growth, whereas intensities higher than 69 µmol m^{-2} s^{-1} led to the lowest growth gain. The different intensities have affected growth and the photosynthetic pigment concentrations. The increased light intensity has led to reduced concentration of *a, b* and total chlorophyll, and carotenoids (Table 2).

Photosynthesis is inefficient under low light intensity, such as 26 µmol m^{-2} s^{-1}, and it reduces growth. On the other hand, light excess may damage the photosynthetic apparatus and lead to the interaction between light receptor pigments and oxygen. It produces free radicals, which may degrade these pigments (Darko et al. 2014; Taiz and Zieger 2004). It is possible inferring that there was light excess when *P. amboinicus* was cultivated under 130 µmol m^{-2} s^{-1}, because this treatment has resulted in growth reduction (Table 1) and in the concentration of photosynthetic pigments (Table 2).

The present growth results (Table 1) corroborate the findings by Fernandes et al. (2013), who have cultivated *Ocimum gratissimum* (Lamiaceae) in vivo, under different light intensities and found that the leaf weight of the species has linearly increased due to light intensity increase, whereas the dry weight, the number of leaves, the leaf area and the plant height increased under a certain intensity and decreased under the highest intensities.

The headspace-GC/MS analysis of *P. amboinicus* has shown qualitative and quantitative differences. Fourteen (14) or fifteen (15) chemical constituents were detected and they accounted for 97.55–99.32% of the total chemical composition, respectively. With respect to the qualitative differences in the chromatographic profile, camphene was just observed in plantlets grown under 69 µmol m^{-2} s^{-1}, whereas (*E*)-β-ocimene was observed in plantlets grown under 51 µmol m^{-2} s^{-1}. The chemical composition was characterized by monoterpenes (83.47–88.22%) and sesquiterpenes (10.99–15.85%). In addition, 45.57–52.22% of the monoterpenes were hydrocarbons, and 34.27–39.72% of them were oxygenates. The herein identified sesquiterpenes belonged to the hydrocarbon type, only. The oxygenate monoterpene carvacrol was the major constituent and presented the highest content (33.96–39.37%).

Six constituents accounted for 90.31–92.47% of the total volatile fraction (α-terpinene, *p*-cymene, γ-terpinene, carvacrol, (*E*)-caryophyllene and *trans*-α-bergamotene). The light intensity has significantly influenced the *p*-cymene, γ-terpinene and carvacrol levels. The treatment under 69 µmol m^{-2} s^{-1} provided the highest carvacrol (39.37 ± 0.60%) and *p*-cymene (19.97 ± 0.68%) contents. The lowest light intensity (26 µmol m^{-2}) led to the highest γ-terpinene content (Table 3).

Table 2 Photosynthetic pigments of *Plectranthus amboinicus* plantlets derived from nodal segments cultivated in vitro, under different light intensities, after 83 days

Light intensity (µmol m^{-2} s^{-1})	Chlorophyll (mg g^{-1} FM)				Carotenoids
	a	b	b/a	Total	
26	0.57^{a}	0.21^{a}	0.36^{a}	0.78^{a}	0.17^{a}
51	0.44^{b}	0.20^{a}	0.45^{a}	0.64^{a}	0.17^{a}
69	0.26^{c}	0.10^{b}	0.37^{a}	0.36^{b}	0.08^{b}
94	0.22^{c}	0.09^{b}	0.40^{a}	0.30^{b}	0.08^{b}
130	0.09^{d}	0.04^{c}	0.46^{a}	0.13^{c}	0.04^{c}

Means followed by the same letter in the column do not differ from each other according to the Scott-Knott test ($p < 0.05$)

Table 1 Growth of *Plectranthus amboinicus* plantlets derived from nodal segments cultivated in vitro, under different light intensities, after 83 days

Light intensity (µmol m^{-2} s^{-1})	SL (cm)	NS	LA (cm^2)	LDW (mg $plant^{-1}$)	SDW (mg $plant^{-1}$)	LLR (cm)	RDW (mg $plant^{-1}$)	TDW (mg $plant^{-1}$)
26	2.55^{a}	1.31^{b}	1.48^{b}	24.88^{c}	8.55^{c}	2.97^{b}	4.05^{d}	37.48^{c}
51	2.80^{a}	1.25^{b}	1.98^{a}	53.00^{b}	11.03^{b}	5.34^{a}	12.55^{a}	76.58^{b}
69	2.09^{b}	1.65^{a}	1.96^{a}	69.23^{a}	14.80^{a}	5.49^{a}	10.85^{b}	94.90^{a}
94	2.23^{b}	1.92^{a}	1.88^{a}	55.03^{b}	10.63^{b}	4.89^{a}	6.85^{c}	72.50^{b}
130	1.63^{c}	1.33^{b}	1.19^{b}	56.68^{b}	8.88^{c}	5.87^{a}	5.13^{d}	70.68^{b}

Means followed by the same letter in the column do not differ from each other according to the Scott-Knott test ($p < 0.05$)

SL shoot length, *NS* number of shoots, *LA* leaf area, *LDW* leaf dry weight, *SDW* stem dry weight, *LLR* length of the largest root, *RDW* root dry weight, *TDW* total dry weight

Table 3 *p*-Cymene, γ-terpinene and carvacrol contents in *Plectranthus amboinicus* leaves derived from nodal segments cultivated in vitro, under different light intensities, after 83 days

Constituents	Light intensities (μmol m^{-2} s^{-1})					
	RI[1]	26	51	69	94	130
p-Cymene	1022	13.70 ± 0.00[d]	19.23 ± 0.04[a]	19.97 ± 0.68[a]	16.43 ± 0.51[b]	14.83 ± 0.48[c]
γ-Terpinene	1056	25.24 ± 0.00[a]	22.30 ± 0.03[b]	18.89 ± 0.88[c]	21.62 ± 0.64[b]	21.35 ± 0.96[b]
Carvacrol	1305	34.17 ± 0.00[c]	33.96 ± 1.01[c]	39.37 ± 0.60[a]	37.84 ± 0.41[b]	38.36 ± 0.48[b]

Means followed by the same letter in the row do not differ from each other according to the Scott-Knott test ($p < 0.05$)

[1]Retention index of the *n*-alkane series (C_8–C_{20}) in HP5-5MS column, in order of elution. (Area % ± standard deviation, n = 3)

Table 4 Growth of *Plectranthus amboinicus* plantlets derived from apical segments cultivated in vitro, under different light qualities, after 50 days

Quality of light	SL (cm)	NL	LA (cm^2)	LDW (mg $plantlet^{-1}$)	SDW (mg $plantlet^{-1}$)	LLR (cm)	RDW (mg $plantlet^{-1}$)	TDW (mg $plantlet^{-1}$)
B	1.75[b]	5.50[e]	0.35[e]	8.40[e]	12.50[c]	0[c]	0.00[d]	20.90[e]
R	2.72[a]	11.50[b]	5.18[a]	80.28[a]	18.02[a]	6.65[a]	8.33[b]	106.63[a]
2.5B/1R	1.88[b]	8.00[d]	1.68[d]	34.12[d]	13.06[c]	4.07[b]	1.90[c]	49.08[d]
2.5B/1R	1.85[b]	13.17[a]	3.64[b]	63.67[b]	15.52[b]	7.95[a]	11.90[a]	91.08[b]
1R/1B	2.03[b]	9.67[c]	4.78[a]	77.33[a]	17.78[a]	5.83[a]	10.35[a]	105.47[a]
F	2.32[a]	14.33[a]	2.53[c]	45.12[c]	18.20[a]	3.20[b]	2.93[c]	66.25[c]

Means followed by the same letter in the column do not differ from each other according to the Scott-Knott test ($p < 0.05$)

B blue, *R* red, *F* fluorescent, *SL* shoot length, *NL* number of leaves, *LA* leaf area, *LDW* leaf dry weight, *SDW* stem dry weight, *LLR* length of the largest root, *RDW* root dry weight, *TDW* total dry weight

In addition, the 69 μmol m^{-2} s^{-1} light intensity has resulted in the lowest γ-terpinene content (18.89 ± 0.88%). According to the biosynthetic pathway proposed by Crocoll (2011), carvacrol is biosynthesized in plants belonging to family Lamiaceae (genera *Origanum* and *Thymus*) due to the hydroxylation reaction of α-terpinene or γ-terpinene in C2. Thus, it was possible seeing that the 69 μmol m^{-2} s^{-1} was the optimal intensity for growth and that it could also be the optimal intensity for γ-terpinene biotransformation into carvacrol.

Besides explaning the results through the biosynthetic route, the light intensity has affected the monoterpene content in *P. amboinicus* due to the function of this constituent class. Monoterpenes protect plants grown under environmental stress (such as excess of light) and may help stabilizing cell membranes against reactive oxygen species (Hartikainen et al. 2009; Holopainen 2011). Thus, *P. amboinicus* presented higher carvacrol content under the highest light intensities (69, 94 and 130 μmol m^{-2} s^{-1}) than it did under the lowest ones (26 and 51 μmol m^{-2} s^{-1}) (Table 3). The increased intensities have not resulted in carotenoid increase (Table 2). The main function of these pigments is the antioxidant action in cases of light excess (Raven et al. 2014). Another antioxidant mechanism, such as carvacrol increase, may have occurred.

Fig. 1 Overall appearance of *Plectranthus amboinicus* plantlets derived from apical segments cultivated in vitro under different light qualities, after 50 days. *B* blue, *R* red, *F* fluorescent

Quality of light

The growth of *P. amboinicus* plantlets was affected by the quality of light (Table 4; Fig. 1). Overall, R and the combinations 1R/1B and 2.5R/1B have improved plantlet growth. The R and 1R/1B treaments stood out, since they have provided the greatest leaf area, leaf, stem, and total dry weight, as well as the longest length of the

largest root. The monochromatic red has also provided longer shoot length and a larger number of leaves than the 1R/1B treatment, and it was the treatment that has most favored the species growth in vitro (Table 4). The improved plantlet growth provided by the red spectrum may be associated with the higher sensitivity of the phytochrome to such spectrum. The phytochrome absorbance peaks are mostly found under the red and far-red spectra (Runkle and Heins 2001). On the other hand, the blue LED has inhibited the growth of the species (Table 4).

The headspace-GC/MS analysis of *P. amboinicus* leaves from plantlets grown under different light qualities allowed observing the qualitative and quantitative differences. The number of detected constituents has ranged from 13 to 17. This variation results from the following chemical constituents: camphene, sabinene, (*E*)-β-ocimene and terpinolene. According to the quantitative analysis, 94.76–99.04% of the total chemical composition was elucidated. Of these percentage, 78.21–88.10% were characterized as monoterpenes and 9.32–16.99%, as sesquiterpenes. In addition, 42.33–56.05% of the monoterpenes were hydrocarbons and 32.05–38.78% of them were oxygenates. The herein identified sesquiterpenes belonged to the hydrocarbon type, only. The herein observed result was similar to that of the light intensity experiment. The constituents α-terpinene, *p*-cymene, γ-terpinene, carvacrol, (*E*)-caryophyllene and *trans*-α-bergamotene have accounted for 88.27–91.55% of the total chemical composition. Carvacrol has shown the highest content (31.81–38.67%) (Table 5).

The monochromatic blue light has enabled the largest carvacrol accumulation (38.67 ± 0.00%). It may have happened due to the high γ-terpinene biotransformation into carvacrol, as it was suggested by Crocoll (2011), or into *p*-cymene ortho-hydroxylation, according to Poulose and Croteau (1978). The blue spectrum has led to the accumulation of secondary metabolites related to the increased antioxidant capacity of plants, according to Manivannan et al. (2015) and Nascimento et al. (2013).

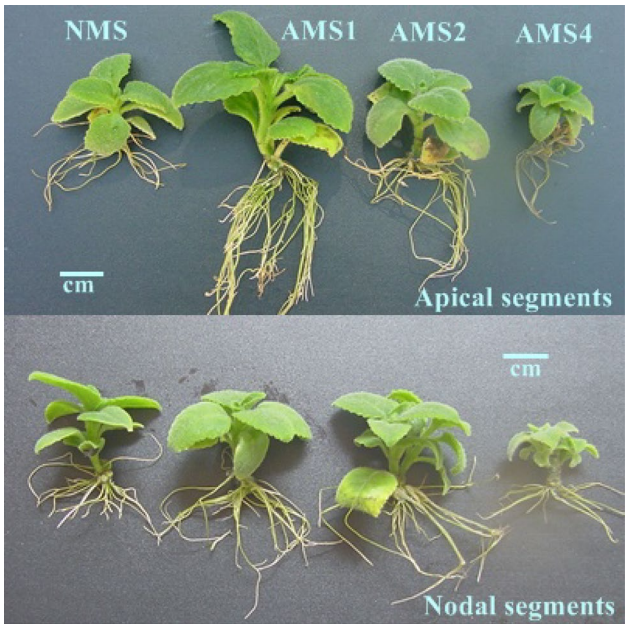

Fig. 2 Overall appearance of *Plectranthus amboinicus* plantlets derived from apical and nodal segments cultivated in vitro under no membrane system (NMS) and alternative membrane system—1 (AMS1), 2 (AMS2) and 4 membranes (AMS4) after 30 days

Similar result was found in the *P. amboinicus* investigated in the current study. The monochromatic blue light has led to carvacrol accumulation; carvacrol is an antioxidant substance (Cabello et al. 2015).

Another quantitative difference found in the current study concerns the content of the constituent *p*-cymene, which was higher under F (20.64 ± 0.24%) and followed by B (12.60 ± 0.00%), 2.5B/1R (12.04 ± 0.34%), 1R/1B (11.64 ± 0.30%), 2.5R/1B (10.80 ± 0.30%); and lower under R (9.09 ± 0.41%) (Table 5). The *p*-cymene content under F was approximately two times higher than that under R. However, the carvacrol content under R (35.67 ± 1.16%) was higher than that under F (31.81 ± 1.14%) (Table 5).

Table 5 *p*-Cymene, γ-terpinene and carvacrol contents in *Plectranthus amboinicus* leaves derived from apical segments cultivated in vitro, under different light qualities, after 50 days

Constituents	RI[1]	Light qualities					
		B	R	2.5B/1R	2.5R/1B	1B/1R	F
p-Cymene	1022	12.60 ± 0.00^{b}	9.09 ± 0.41^{e}	12.04 ± 0.34^{c}	10.80 ± 0.30^{d}	11.64 ± 0.30^{c}	20.64 ± 0.24^{a}
γ-Terpinene	1056	22.99 ± 0.00^{b}	24.12 ± 0.15^{a}	24.45 ± 0.50^{a}	25.45 ± 0.22^{a}	24.59 ± 1.20^{a}	22.80 ± 0.76^{b}
Carvacrol	1305	38.67 ± 0.00^{a}	35.67 ± 1.16^{b}	32.68 ± 1.16^{c}	33.09 ± 1.58^{c}	35.07 ± 0.00^{b}	31.81 ± 1.14^{c}

Means followed by the same letter in the row do not differ from each other according to the Scott-Knott test ($p < 0.05$)

[1]Retention index of the *n*-alkane series (C_8–C_{20}) in HP5-5MS column, in order of elution. *B* blue, R red, *F* fluorescent. (Area % ± standard deviation, n = 3)

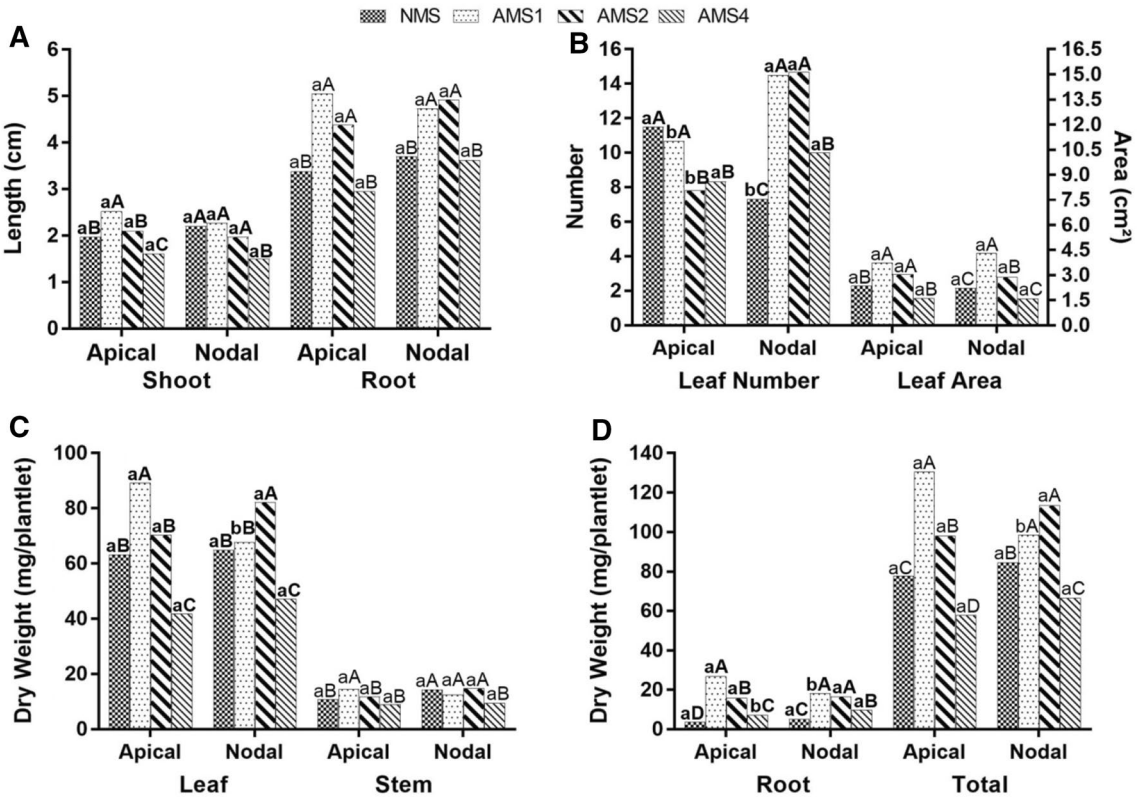

Fig. 3 Growth of *Plectranthus amboinicus* plantlets derived from apical and nodal segments cultivated in vitro under no membrane system (NMS), AMS1, AMS2, AMS4 (alternative membrane system—1, 2 and 4 membranes), after 30 days. The *lowercase letter* was used to compare the apical and nodal segments results to each growth parameter in the same culture system. The *uppercase letter* was used to compare the results from each culture system to each growth parameters and segment type. In both cases, the *same letter* do not differ from each other according to the Scott-Knott test ($p < 0.05$)

Alternative membrane system

The cultivation systems and the explant type have affected the growth of *P. amboinicus* plantlets in vitro (Figs. 2, 3). The AMS1 was the best cultivation system for the apical segments. The plantlets subjected to the AMS1 treatment have shown the highest value in all the assessed growth variables. As for the nodal segments, the AMS1 and AMS2 allowed the best plantlet growth. These systems have produced plantlets with higher values and statistically equal to each other concerning shoot length, number of leaves, length of the largest root, stem, root and total dry weight. They were different from each other with respect to the leaf area—which was larger under the AMS1 treatment—and to leaf dry weight—which was higher under the AMS2 treatment (Fig. 3). The AMS2 treatment was most beneficial for the cultivation of *P. amboinicus* nodal segments in vitro, due to leaf dry weight increase.

The present results may be associated with the increased photosynthesis caused by the increased CO_2 availability under AMS1 and AMS2 in comparison to that under NMS. According to Saldanha et al. (2012), the natural ventilation system with membranes keeps the CO_2 concentration, inside the culture vessel, suitable to stimulate photosynthesis. These authors have found that this system has improved the growth of *Pfaffia glomerata* in vitro. The results of the study conducted by Silva et al. (2014), who have cultivated pineapple in vitro under NMS and AMS, partly corroborates the findings in the current study. The AMS treatment in their study has caused positive effects on the growth of pineapple plantlets and it has overall provided longer shoot length and higher dry weight accumulation than the NMS in the presence of 30 g L^{-1} of sucrose.

The AMS4 has negatively affected the growth of both explant types (Fig. 3). It may have occurred due to water evaporation in the culture media, as well as to humidity decrease and to gas exchange increase in the microenvironment of the culture vessel. Humidity and gas exchanges are important factors for morphogenesis control in cultivations in vitro. The cultivation environment may affect the activity of many enzymes and lead to changes in the metabolic process and to responses similar to those of plants grown under stress conditions (Isah 2015).

Table 6 *p*-Cymene, γ-terpinene and carvacrol contents in *Plectranthus amboinicus* plantlets leaves cultivated in vitro, under no membrane system (NMS), AMS1, AMS2, AMS4, after 30 days

Constituents	RI[1]	Systems			
		NMS	AMS1	AMS2	AMS4
p-Cymene	1022	13.05 ± 0.32^{a}	14.15 ± 0.63^{a}	13.74 ± 0.55^{a}	13.13 ± 0.32^{a}
γ-Terpinene	1056	22.86 ± 0.14^{b}	25.17 ± 0.84^{a}	22.86 ± 0.23^{b}	20.92 ± 0.40^{c}
Carvacrol	1305	37.95 ± 0.49^{b}	34.30 ± 0.76^{c}	38.53 ± 0.42^{b}	41.04 ± 0.28^{a}

Means followed by the same letter in the row do not differ from each other according to the Scott-Knott test ($p < 0.05$)

[1]Retention index of the *n*-alkane series (C_8–C_{20}) in HP5-5MS column, in order of elution. (Area % ± standard deviation, n = 3). AMS (alternative membrane system—1, 2 and 4 membranes)

There was significant interaction between the systems and the explant types (Fig. 3). The apical segments have exceeded the nodal ones in number of leaves in cultivation under NMS, as well as in leaf, root and total dry weight accumulation under AMS1. The nodal segments have exceeded the apical ones in the number of leaves under AMS1 and AMS2. In addition, they have presented root dry weight under AMS4 higher than the apical segments. Overall, it was observed that the AMS treatments, mainly AMS1 and AMS2, have improved the rooting of *P. amboinicus* plantlets, in comparison to the NMS. Thus, these systems have favored the acclimatization process.

The different culture systems have affected the volatile chemical composition of *P. amboinicus* plantlets (Table 6). The chemical composition consisted of monoterpenes (84.29–86.29%) and sesquiterpenes (12.30–15.15%). In addition, 44.14–50.23% of the monoterpenes were hydrocarbons and 34.52–41.30% of them were oxygenates. The herein identified sesquiterpenes belonged to the hydrocarbon type, only. Overall, 97.55–99.42% of the chemical composition was elucidated, and 14–17 constituents were identified. The qualitative differences were attributed to the lack of chromatographic signals corresponding to camphene, sabinene, (*E*)-β-ocimene and/or terpinolene peaks in the treatments.

The main chemical constituents were *p*-cymene, γ-terpinene and carvacrol, which represented, 74.21% of the total chemical composition obtained by headspace-GC/MS, on average. The different cultivation systems have significantly influenced the content of these constituents (Table 6). The highest carvacrol content ($41.04\% \pm 0.28$) was observed in plantlets grown under AMS4, whereas the lowest γ-terpinene content was also found under this treatment (Table 6).

The lowest γ-terpinene content found under AMS4 may have occurred because of the γ-terpinene biotransformation into carvacrol (Crocoll 2011). Similarly, the AMS4 may also have enabled this process through *p*-cymene (Poulose and Croteau 1978). The AMS1 was the treatment that most hindered carvacrol accumulation in vitro. This treatment has not enabled the in vitro conversion of *p*-cymene and γ-terpinene into carvacrol, since the highest *p*-cymene and γ-terpinene contents were found under AMS1 (Table 6). It was possible seeing that the carvacrol concentration has increased in the following order: AMS1, AMS2 and AMS4 (Table 6). The γ-terpinene and *p*-cymene concentrations have decreased in the same order. The highest CO_2 concentration in the microenvironment has affected the monoterpenes production.

The low CO_2 availability has resulted in low photosynthetic rates. The reduced photosynthetic rate has decreased the availability of glyceraldehyde 3-phosphate, which is one of the substrates used in terpenes production (Schurgers et al. 2009). Moreover, as it was previously mentioned, the low humidity and the increased gas exchange may have triggered a metabolism process, which is characteristic of plants subjected to stress, and carvacrol biosynthesis is a form of defense. Thymol is reported in the literature as a major *P. amboinicus* constituent, but it was not found in the current study.

Conclusion

The light intensity and quality, as well as the natural ventilation of the culture vessel, should be controlled in *P. amboinicus* cultivation in vitro, since these factors affect growth and carvacrol content, as well as the content of carvacrol precursors (γ-terpinene and *p*-cymene). The intensity 69 μmol m^{-2} s^{-1} was optimal for *P. amboinicus* growth in vitro. Such light intensity changes the profile of volatile compounds and increases the carvacrol content. The monochromatic red was the best for optimal growth in vitro, whereas the monochromatic blue was the best for carvacrol content increase. The AMS1 and AMS2 natural ventilation systems have improved the species' rooting. The apical segments should be cultivated under AMS1 and the nodal ones should be cultivated under AMS2 in order to provide the best growth. Thus, it was also possible obtaining good quality plantlets regarding their carvacrol content. The assessed environmental factors appeared to have caused

changes in the γ-terpinene and *p*-cymene contents, which are carvacol precursors. The present study has contributed to the understanding about how light and ventilation affect terpenes in plants.

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