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Photoautotrophic propagation of Brazilian ginseng [*Pfaffia glomerata* (Spreng.) Pedersen]

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Abstract *Pfaffia glomerata* (Spreng.) Pedersen is a medicinal species of great interest because it produces the phytoecdysteroid 20-hydroxyecdysone (20E). Generally, because of atypical growing conditions, in vitro propagated plants function less efficiently as autotrophs and have poorly developed morphological structures. This study analyzed the autotrophic potential of *P. glomerata* propagated in vitro and evaluated the influence that this has on 20E biosynthesis. Physiological and structural parameters of plants subjected to heterotrophic, photomixotrophic and photoautotrophic growth conditions were evaluated. Levels of 20E were measured by HPLC. Plants were acclimatized in a mixture of soil, sand and substrate, in a greenhouse. Conditions that provided higher carbon input led to an increase in plant growth, and the presence of sucrose was critical, in closure systems without a gas permeable membrane, for normal anatomical development of the micropropagated plants. The absence of sucrose increased photosynthesis and conditions that enhanced photoautotrophy induced greater levels of 20E. The increase of 20E levels by the photoautotrophic system offers new prospects for increasing the commercial production of this species, and for studies that could elucidate the biosynthetic pathway of phytoecdysteroids in plants.

Introduction

Species of *Pfaffia* (Amaranthaceae) are used by the medical, food and cosmetic industries (Corrêa et al. 2006), and *Pfaffia glomerata,* commonly known as Brazilian ginseng, has great economic value in both foreign and domestic markets as a medicinal plant (Vieira et al. 2002). Most of the wild collections of *P. glomerata* come from Brazil (Figueiredo et al. 2004); however, as with other medicinal plants (Sarasan et al. 2011), over-exploitation and unsustainable harvesting has caused a drastic decrease in natural populations and is leading to the genetic erosion of this species.

Pfaffia glomerata accumulates the phytoecdysteroid 20-hydroxyecdysone (20E), a compound that has been included in many commercial anabolic preparations for athletes (Lafont and Dinan 2003). Phytoecdysteroids are the major group isolated from *Pfaffia*, and 20E is the most frequently found and widely distributed phytoecdysteroid in this genus (Bakrim et al. 2008). They could have agrochemical, biotechnological, medicinal and pharmaceutical uses, and may potentially be involved in biochemical and physiological processes in plants (Festucci-Buselli et al. 2008a). It is possible that 20-hydroxyecdysone acts as a defense mechanism to protect plants against phytophagous insects (Dinan et al. 2009). Based on the fact that *P. glomerata* accumulates 20E, this species is an interesting model that could be used to determine the precise function of 20E in plants and to define the genes involved in the biosynthesis of this compound.

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Currently, the goal of some studies is to develop efficient propagation techniques to increase the production of *P. glomerata*, which will ensure a regular supply of high quality homogeneous raw material that is needed to meet the increasing demand for 20E (Festucci-Buselli et al. 2008a, b; Flores et al. 2010).

In vitro propagation is a promising alternative for providing a regular supply of high quality, disease-free and homogeneous plant material within a shorter period of time (Mosaleeyanon et al., 2004). A number of studies have evaluated factors that can affect the in vitro development of *P. glomerata* (Nicoloso et al. 2001, 2003; Russowski and Nicoloso 2003; Skrebsky et al. 2004; Maldaner et al. 2007; Alves et al. 2010; Flores et al. 2010).

Typically, in vitro propagated plants show peculiar characteristics, such as poorly developed shoots, less epicuticular and cuticular wax, tissues with low mechanical strength, higher water content, non-functional stomata and small, thin leaves with fewer trichomes and low photoautotrophic activity (Kozai and Kubota 2001; Cha-um et al. 2011; Xiao et al. 2011). The high relative humidity and CO_2 concentration inside a jar (because of reduced gas exchange) may influence anatomical, physiological and morphological plant characteristics, and can result in great losses during acclimatization (Kitaya et al. 2005).

In traditional in vitro cultivation methods, plants are supplied with exogenous carbohydrate sources to sustain growth and development because the CO_2 concentration inside culture containers is reduced, which limits photosynthesis (Zobayed 2006; Kozai 2010; Xiao et al. 2011). However, photoautotrophy in vitro can be induced by excluding carbohydrates from the medium and increasing gas exchange in the culture vessel. There are other advantages to removing carbohydrates from the culture medium. For example, this prevents the rapid growth of microorganisms in the cultures, reduces costs and increases plant survival during acclimatization (Mosaleeyanon et al. 2004; Xiao and Kozai 2006; Kozai 2010; Xiao et al. 2011).

The goal of this study was to analyze the autotrophic potential of *P. glomerata* propagated in vitro by comparing physiological and structural parameters of plants subjected to heterotrophic, photomixotrophic and photoautotrophic growth conditions, and evaluated how these conditions influence the biosynthesis of 20E.

Materials and methods

Plant material and treatments

Pfaffia glomerata shoot tips (about 9 mm in length) excised from 30-day-old in vitro plantlets with a pair of leaves, which were grown under heterotrophic conditions, were cultured in glass jars (12 cm high × 5 cm internal diameter) containing 50 mL of MS basal medium (Murashige and Skoog 1962) supplemented with 100 mg L^{-1} myo-inositol, two levels of sucrose (0 or 30 g L^{-1}), and 7 g L^{-1} granulated agar (Merck®, Darmstadt, Germany). The pH of medium was 5.7 ± 0.1 and the jars were autoclaved at 121 °C, 1.5 kgf cm^{-2}, for 20 min.

Four different closure systems were tested: 1. two layers of polyvinylchloride (PVC) transparent film (Alpfilm®, Curitiba, Brazil); 2. rigid polypropylene cap (RP); 3. RP with one vent hole (10 mm) covered with a polytetrafluoroethylene (PTFE) gas permeable membrane disk, 0.45 μm pore size (MilliSeal® Air Vent, Tokyo, Japan) (RP1); and 4. RP with two vent holes (10 mm) covered with PTFE membrane disks (RP2). The cultures were incubated in a growth room at 25 ± 2 °C, 16 h photoperiod, 60 μmol m^{-2} s^{-1} irradiance from two fluorescent tubes (Sylvania HO T12, Luz do Dia, 110 W, São Paulo, Brazil).

Growth parameters

The survival percentage; plant height; leaf number and nodal segments; fresh and dry weight of aerial parts and root system; and leaf area were assessed after 30 days of in vitro culture.

Photosynthetic rate and pigments

Photosynthetic rates of in vitro *P. glomerata* plants were measured using a closed system LCA2 infrared gas analyzer (Licor Inc., Lincoln, Nebraska, EUA). The reference air (500 μmol mol^{-1} CO_2) was pumped into culture jars at a constant flow rate (0.5 L min^{-1}). Plants were exposed to 500 μmol m^{-2} s^{-1} irradiance prior to and during the analysis. Determination of carotenoids and chlorophyll *a* and *b* followed Wellburn (1994). Six leaf disks (5 mm diameter) were taken from the third fully expanded leaf from the shoot tip and incubated in a 5 mL dimethyl sulfoxide solution (saturated with $CaCO_3$) at room temperature, for 48 h. Absorbances at 665, 645 and 480 nm were determined using a spectrophotometer (Hitachi U-2000, Tokyo, Japan).

Estimation of the number of gas exchanges per hour

The amount of gas exchange provided by each seal type, except for PVC, was determined as described by Fujiwara and Kozai (1995). Therefore, the headspace of each flask containing a different membrane type was saturated with a mixture of carbon dioxide (CO_2) at a concentration of 2.5 %. The readings of the inner CO_2 concentrations were performed with a Headspace Gas Analyzer 6600 (Illinois© Instruments, Johnsburg, IL, USA). The amount of gas

exchange per hour (N') for each type of seal was estimated by the following equation: $N' = \frac{1}{T}\ln\frac{C_1 - C_{out}}{C_2 - C_{out}}$ (Fujiwara and Kozai 1995). Where T represents the time (in hours) between two readings (1 and 2); C_1 and C_2 correspond to the inner CO_2 concentrations in the flasks at times 1 and 2; C_{out} is the CO_2 concentration in the environment external to the flask.

Anatomy and histochemical characterization

For the anatomical studies, samples from the middle region of fully expanded leaves were fixed in FAA (formalin: acetic acid: 50 % ethyl alcohol) for 24 h (Johansen 1940), washed in 70 alcohol, dehydrated through a graded series of ethyl alcohol and embedded in methacrylate (Historesin, Leica®). Cross and longitudinal sections (8 μm thick) were cut using a rotary automatic microtome (RM 2155—Leica), with disposable stainless steel blades, and stained with toluidine blue (O'Brien and McCully 1981) for 10 min, at pH 4.0. The sections were then mounted on glass slides with the synthetic resin Permount®.

For the hystochemical analyses, sections of the middle third section of the stems were cut with a hand microtome (LPC, Rolemberg & Bhering, Belo Horizonte, Brazil) and stained with phloroglucin acid to detect lignin (Johansen 1940) and ruthenium red (Johansen 1940) and coriphosphine to detect pectins (Ueda and Yoshioka 1976). Sections stained with neutral red were examined under UV light using a blue excitation filter (BP 450–490 nm). Observations and photography were carried out with an Olympus SZH stereo microscope (Olympus Optical, Tokyo, Japan) and an Olympus AX70TRF microscope (Olympus Optical, Tokyo, Japan) with a U-Photo Camera System (Spot Insight Color 3.2.0, Diagnostic Instruments Inc., USA).

Ultrastructural analysis: scanning electron microscopy (SEM)

Samples of the middle region of leaf blades (about 5 mm in width) were taken from leaves on the second node, fixed in Karnovsky solution (2.5 % glutaraldehyde and 2.5 % paraformaldehyde in 0.05 M cacodilate buffer, pH 7.2) and postfixed with 1 % osmium tetroxide. After dehydration through a graded series of ethyl alcohol, followed by critical point drying in a Bal-Tec CPD 030 (Bal-Tec, Balzers, Liechenstein), the samples were mounted on stubs and coated with gold using a FDU010 sputter coater (Bal-Tec, Balzers, Liechtenstein). Examinations and photography were carried out using a Leo 1430VP scanning electron microscope (Zeiss, Cambridge, England). All images were processed digitally.

HPLC determination of 20-hydroxyecdysone (20E)

Methanolic extracts from 3 independent samples were prepared with 100 mg of powdered plant material in 10 mL of methanol, and incubated at 25 °C under agitation for 7 days. Quantification of 20E in the methanolic extract was performed using High Performance Liquid Chromatography (HPLC) according to Festucci-Buselli et al. (2008b). The calibration curve was obtained by adding the standard 20-hydroxyecdysone (Sigma-Aldrich) to methanol at 20, 40, 60, 80, 100, 125, 150, and 200 mg L^{-1}. The data from this analysis are presented as percentages (mg for 100 mg of powdered plant dry matter).

Plant acclimatization

After 30 days in culture, the in vitro plantlets were transferred to 300 mL plastic cups containing a 2:2:1 mixture of soil, sand and the substrate Plantmax® (DDL Agroindústria, Betel Paulínia, Brazil), wrapped in 12×25 cm plastic bags for 15 days and then transferred to a greenhouse.

Experimental design and statistical analysis

The experiment was arranged in a 4^2 factorial, completely randomized design, with four types of seals (PVC, RP, RP1, and RP2) and two sucrose concentrations (0 and 30 g L^{-1}), with six replicates, each represented by 5 jars containing 5 plants each. All variables were examined by analysis of variance and means were compared with Tukey's test at 5 % of probability.

Data about dry root mass were analyzed using square root transformation. All statistical analyses were performed at a 5 % probability level, using the software GENES (Cruz 2006). The experiment was repeated twice.

Results and discussion

Conditions providing higher carbon input led to increased plant growth

In vitro plants grown in the presence or absence of sucrose and under different types of jar seals showed remarkable morphological differences (Fig. 1, Table 1). Treatments devoid of both sucrose and the venting PTFE membrane (Fig. 1f) had the lowest growth parameters, and this compromised plant development and led to mortality rates that were as high as 80 %. Likewise, lower leaf area indices were obtained in medium lacking sucrose in combination with the PVC film or RP seals without vent holes (Table 1). The highest leaf area indices were achieved in cultures
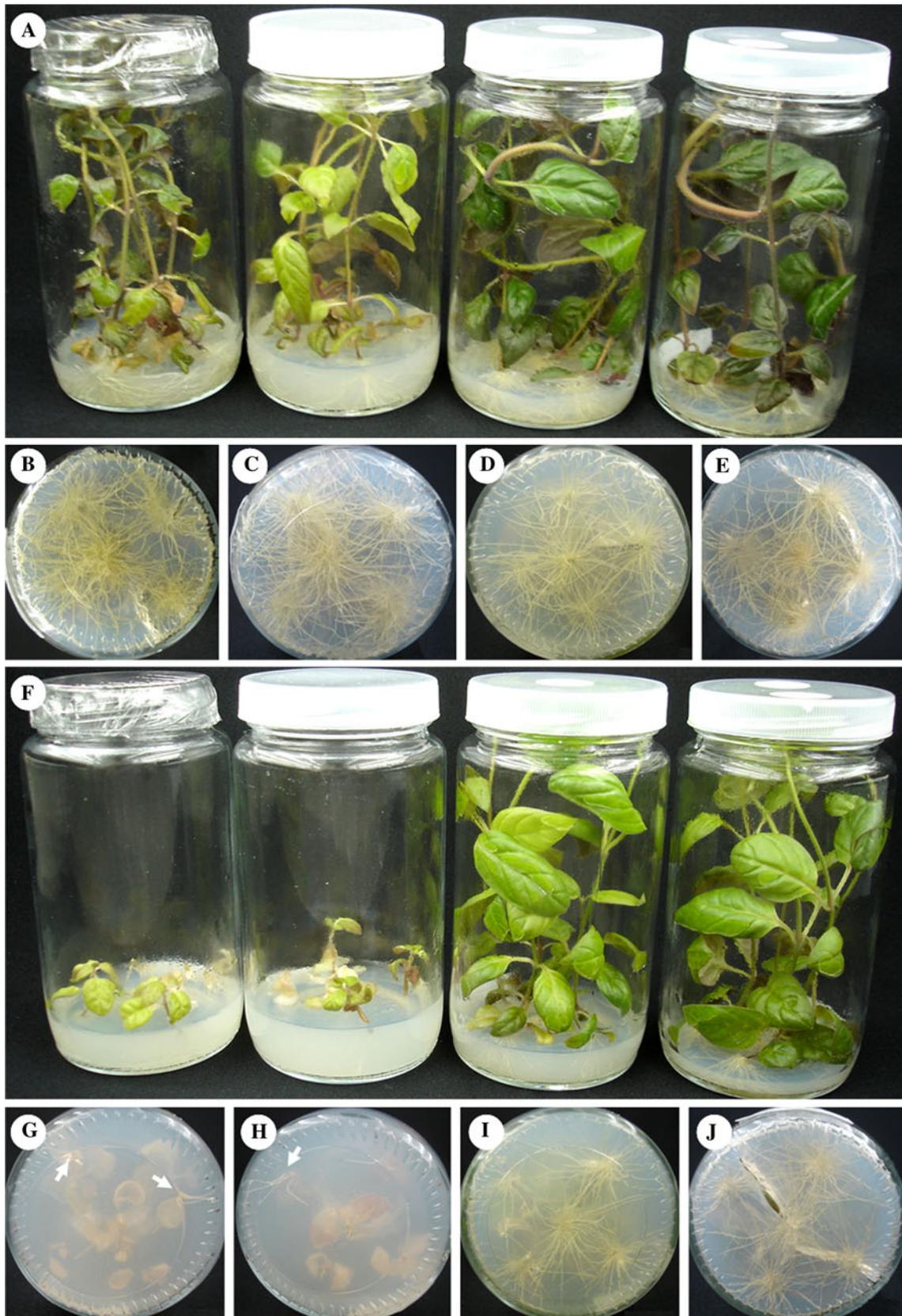
◄ **Fig. 1** *Pfaffia glomerata* plantlets propagated in vitro under different closure systems, at 32 days of culture: PVC transparent film; rigid polypropylene (*RP*) cap; RP cap with one gas permeable membrane disk (*RP1*); and RP cap with two gas permeable membrane disks (*RP2*) (from *left* to *right*, respectively), on medium supplemented with 30 g L^{-1} sucrose (**a**); roots of *P. glomerata* plantlets grown on sucrose-supplemented medium (**b–e**); plantlets grown on sucrose-free medium (**f**); roots of *P. glomerata* plantlets grown on sucrose-free medium (**g–j**). Arrows (**g**, **h**) indicate the development of a few roots

grown in jars closed with the PTFE membrane (RP1 and RP2), regardless of the presence of sucrose.

The amount of gas exchange per hour, estimated by the use of CO_2 in flasks sealed with RP, RP1 and RP2), was 0.03 (RP), 0.37 (RP1), and 0.86 (RP2). In the absence of sucrose and a porous membrane, the gas exchange was not enough to support plant growth (Fig. 1f; Table 1). The use of gas permeable membranes allowed for increased natural ventilation in the culture vessels, providing an adequate CO_2 concentration, which increased photosynthesis and the growth rate (Kitaya et al. 2005). In this study, biomass accumulation in shoots of *P. glomerata* was a function of the presence of the PTFE membranes in the caps of culture jars on both sucrose-supplemented and non-supplemented media. Nevertheless, growth responses as a function of gas exchange in the culture environment depend on the species, and the effect of gas exchange varies with the presence or absence of sucrose in the medium (Shim et al. 2003). Kozai and Kubota (2001) found that the growth of explants from most woody species was greater under photoautotrophic conditions when compared to photomixotrophic conditions.

The presence of sugar in the culture medium alters plant biochemistry, physiology and morphology of vitroplants (Badr et al. 2011). When plants are grown in vitro on

medium devoid of sucrose, there is the need to increase irradiance, CO_2 diffusion and humidity (Kozai and Kubota 2001) to enhance photosynthesis, transpiration, and dry mass accumulation (Aitken-Christie et al. 1995; Kitaya et al. 2005).

Greater development of the root system was observed in sucrose-supplemented medium (Fig. 1a–e). Structural and physiological analyses corroborated the variations observed. The absence of sucrose in the medium of jars sealed with the RP and PTFE membranes reduced fresh and dry root mass (Table 1). Root growth was reduced in the treatments without sucrose, which was possibly a result of the metabolic sink created by the investment in the photosynthetic apparatus (because photosynthesis became the only route for carbon uptake).

In general, the highest mean values for almost all of the tested variables were observed in the presence of sucrose, without any differences from the influence of the closure type (Table 1). The addition of the carbon source stimulated biomass production in *P. glomerata*. An increase in dry matter of in vitro propagated *P. glomerata* with increasing sucrose concentrations has been reported by other authors (Nicoloso et al. 2003; Skrebsky et al. 2004; Maldaner et al. 2006, 2007).

Absence of sucrose increased photosynthesis

Gas exchange was increased in the sucrose-free media, and plants grown without the carbohydrate had the highest photosynthetic rates (Table 2). Considering the effect of the closure type with the addition of sucrose, we found that only chlorophyll *a* and total chlorophyll differed

Table 1 Growth analysis of *Pfaffia glomerata* propagated in vitro under different closure systems and sucrose concentrations

Means followed by the same capital letter in the row and small letter in the column, for each variable, are not significantly different by the Tukey test at 5 % of probability

Variables	Sucrose (g L^{-1})	Treatments			
		PVC	RP	RP1	RP2
Leaf area (cm^2)	0	0.77Bb	0.97Bb	3.03Aa	3.21Aa
	30	1.51Aa	1.49Aa	2.04Ab	2.29Ab
Height (cm)	0	3.30Bb	3.78Bb	13.29Aa	14.21Aa
	30	14.10Aa	15.24Aa	13.47Aa	11.12Ab
Leaf number	0	1.73Bb	2.25Bb	10.73Aa	11.87Aa
	30	13.87Aa	14.47Aa	12.40Aa	9.80Aa
Number of nodal segments	0	2.17Ab	2.83Ab	4.73Aa	4.77Aa
	30	6.90Aa	6.80Aa	5.27Aa	4.57Aa
Fresh mass of aerial parts (g)	0	0.160Bb	0.205Bb	2.364Aa	2.868Aa
	30	2.291Aa	2.846Aa	2.756Aa	2.432Aa
Fresh mass of roots (g)	0	0.053Bb	0.095Bb	0.314Ab	0.319Ab
	30	1.027Aa	1.122Aa	1.280Aa	1.586Aa
Dry mass of aerial parts (g)	0	0.004Bb	0.005Bb	0.045Ab	0.058Ab
	30	0.062Aa	0.068Aa	0.091Aa	0.126Aa
Dry mass of roots (g)	0	0.006Bb	0.014Bb	0.049Ab	0.051Ab
	30	0.175Aa	0.167Aa	0.200Aa	0.194Aa

Table 2 Physiological parameters related to photosynthesis of *Pfaffia glomerata* propagated in vitro under different closure systems and sucrose concentrations

Variables	Sucrose (g L^{-1})	Treatments			
		PVC	RP	RP1	RP2
Photosynthesis (μmol CO$_2$ kg^{-1} s^{-1})	0	–[a]	–[a]	65.39Aa[b]	60.94Aa
	30	32.25Ab	20.14Ab	30.17Ab	24.14Ab
Chlorophyll *a* (μg cm^{-2})	0	7.11Bb	6.13Bb	15.80Ab	20.47Ab
	30	20.26Ba	18.99Ba	38.01Aa	44.51Aa
Chlorophyll *b* (μg cm^{-2})	0	2.50Ab	2.30Ab	4.78Ab	6.50Ab
	30	6.48Aa	5.86Aa	12.39Aa	14.65Aa
Chlorophyll total (μg cm^{-2})	0	9.61Bb	8.43Bb	20.58Ab	26.97Ab
	30	26.74Ba	24.86Ba	50.40Aa	59.16Aa
Carotenoids (μg cm^{-2})	0	1.76Ab	1.49Ab	3.01Ab	3.89Ab
	30	3.87Aa	3.65Aa	6.69Aa	7.78Aa

[a] Plants grown under these conditions did not grow, hence it was not possible to perform the analysis

[a] Means followed by same capital letter in the row and small letter in the column, for each variable, are not significantly different by the Tukey test at 5 % of probability

significantly (Table 2). The treatments without a gas permeable membrane, regardless of sucrose concentration, showed the lowest means for pigment content. But, the contents of chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids had the highest means when the medium contained sucrose (Table 2).

The CO$_2$ content in headspace and light intensity are the factors that limit photosynthesis in in vitro conditions (Galzy and Compan 1992).

The influence of the closure system, and the sucrose concentration, on the photosynthetic rate of *P. glomerata* reflected the heterotrophic condition in which the plants cultivated in vitro, using a traditional method (PVC and RP), was maintained. The higher rate of photosynthesis in *P. glomerata* grown in the absence of sucrose, compared with plants grown in the presence of sucrose with the same closure type, was also reported in grapevine; however, this occurred when the plants were subjected to higher gas exchange rates (2.5 and 4.4 h^{-1}) (Shim et al. 2003). When present in the culture medium, sucrose is preferentially absorbed by the plant as a carbohydrate source, inhibiting photosynthetic activity even when ventilation increases (Kozai and Sekimoto 1988). Plants grown without sucrose had a higher photosynthetic rate because this was the only route to carbon fixation.

No leaf senescence was observed in plants grown with the PTFE membranes, unlike treatments without both sucrose and a membrane. Chlorophyll loss is a phenomenon typically associated with senescence (Lu et al. 2003). The concentrations of total chlorophyll in sucrose-free medium combined with a membrane were similar to those in sucrose-added medium without a membrane. High concentrations of sucrose in the medium can inhibit

chlorophyll accumulation in vitro (Neumann and Bender 1987). Conversely, in *P. glomerata*, plants grown in treatments with both sucrose and a membrane had increased synthesis of photosynthetic pigments (chlorophyll *a*, *b* and carotenoids), corroborating the results obtained for *Vitis vinifera* and *Scrophularia yoshimurae* (Gribaudo et al. 2003; Tsay et al. 2006), in which gas-permeable culture conditions favoured the increase of chlorophyll content. However, for in vitro cultivation, there does seem to be a pattern of responses for the content of photosynthetic pigments as a function of sucrose concentration in the culture medium.

Sucrose is critical for normal development of *P. glomerata* plants micropropagated in closure systems without a gas permeable membrane

In vitro plants, in general, are poorly developed, with less epicuticular waxes, reduced supportive tissues, higher water content, non-functional stomata, thin leaves, few trichomes and low photoautotrophic activity (Kozai 1991; Kozai and Kubota 2001). These characteristics were not observed in *P. glomerata* grown using the traditional method of tissue culture, even in the treatments without sucrose; however, there were better results in the RP2 treatments (Fig. 2). Plants from all treatments without sucrose had a poorly developed vascular system and supportive tissues (Fig. 2d–f), showing a typical organization of cells and tissues found in plants grown heterotrophically. There was a visible reduction in the size of vascular bundles of the midrib in treatments without sucrose compared with treatments using the same closure systems and sucrose (Fig. 2f, l). Also, plants grown without both sucrose and a

Fig. 2 Cross sections of the middle portion of the leaf of *Pfaffia glomerata* propagated in vitro under different closure systems and sucrose concentrations. **a–f** Rigid polypropylene (*RP*) cap without membrane with (**a–c**) and without (**d–f**) sucrose; **g–l** RP cap with two membrane disks with (**g–i**) and without (**j–l**) sucrose. **a, d, g, j** Detail of leaf margin; **b, e, h, k** detail of leaf blade; **c, f, i, l** detail of midrib. *Ab* abaxial surface of the epidermis; *Ad* adaxial surface of the epidermis; *St* stomata; *Vb* vascular bundle; *Id* idioblast; *Pp* palisade parenchyma; *Sp* spongy parenchyma; *Su* supportive tissue; **+** (presence) and **−** (absence) of sucrose. *Arrowhead* indicates the presence of an idioblast containing a druse. *Bars* 50 μm
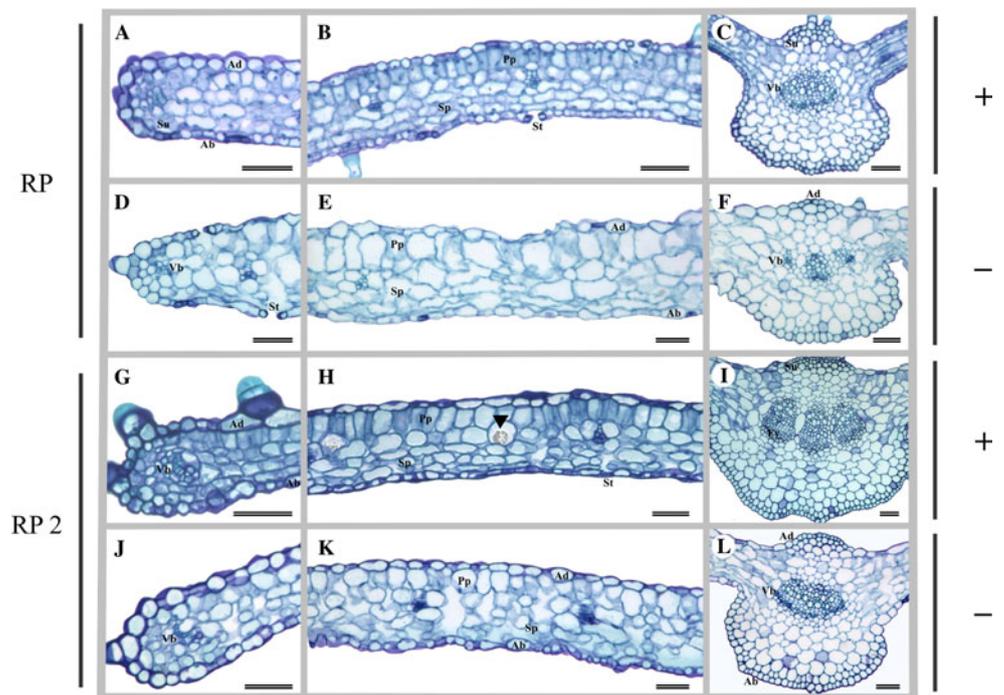
PTFE membrane lacked supportive tissue (collenchyma) in the subepidermal region of the midrib of the leaf (Fig. 2f). However, collenchyma formed in treatments with a membrane, and was more developed in treatments that used the RP2 membrane (Fig. 2l).

The percent reduction in dry weight of *P. glomerata* grown photoautotrophically, compared with the other treatments, was reflected in the leaf anatomy, because the leaf is the most susceptible organ to acclimatization. On the other hand, stem anatomical characteristics showed significant differences, which were mainly related to cell wall lignification and the size of vessel elements. Water accumulation in the plant results in weak organs and tissues with reduced mechanical support and thin cell walls (Donnelly and Tisdall 1993; Jausoro et al. 2010). This accumulation may be caused by the nutritional composition of the medium or the cultivation environment, inhibiting cell wall deposition and the formation of collenchyma and sclerenchyma (Donnelly et al. 1985) and restricting the development of the vascular system (Donnelly and Tisdall 1993). Such characteristics were observed in *P. glomerata* in treatments with lower biomass production, and without a membrane and sucrose.

The leaf of *P. glomerata* is amphistomatic, with a distinct dorsiventral structure in normally developed plants (Fig. 2). In the treatment with sucrose-free medium and RP2, the development of plants grown in vitro was typical, with large intercellular spaces and more globular palisade parenchyma (Fig. 2k), compared to plants grown on sucrose-supplemented medium with the same closure system (Fig. 2h). In treatments without both sucrose and a membrane, cells of the palisade parenchyma were larger and accounted for nearly half of the mesophyll volume (Fig. 2e).

Scanning electron microscopy showed that in plants grown with sucrose, regardless of the closure system used, or sucrose-free medium using RP2, the epidermal cells had sinuous walls (Fig. 3a, d, g, j) with epidermal appendages, such as non-glandular trichomes (Fig. 3h, j), which together with stomata (Fig. 3c, h, i) were more frequent on the abaxial surface. Stomata had a typical elliptic shape and were slightly raised above the other epidermal cells (Fig. 3). Non-glandular trichomes were uniseriate and multicellular with elongate cells, and had a surface ornamented with small warts, including the region of cell union/contact, which was typically dentate (Fig. 3f, g, l).

The high relative humidity, typical of the traditional method of tissue culture, reduces deposition of epicuticular waxes and alters the cuticle structure, mesophyll cells and the ability for leaf stomata to function. In this study, leaves
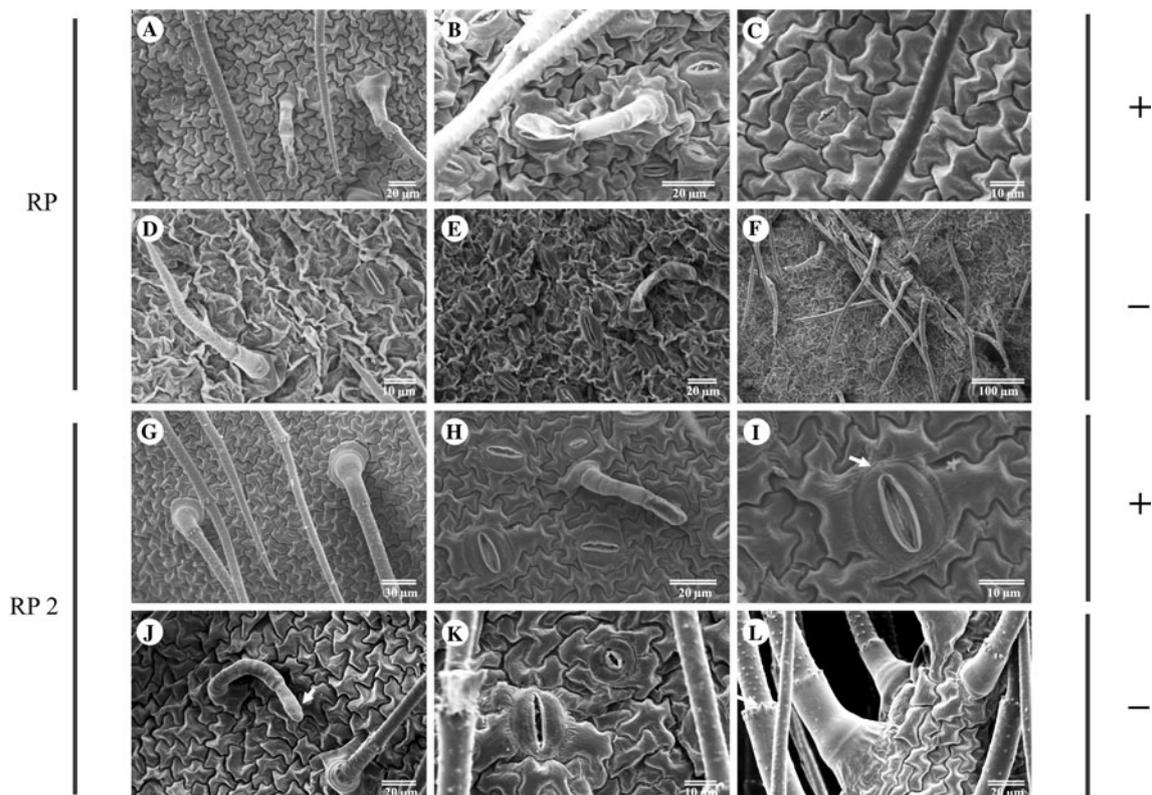
Fig. 3 Leaf surface scanning electron micrographs of *Pfaffia glomerata* propagated in vitro under different closure systems and sucrose concentrations. **a–f** Rigid polypropylene (*RP*) cap without membrane with (**a–c**) and without (**d–f**) sucrose; **g–l** RP cap with two membrane disks with (**g–i**) and without (**j–l**) sucrose. **a, d, g, j** Details the adaxial leaf surface; **b, e, h, k** details of the abaxial surface; **f** detail of malformed stomata showing bulged pores; **c, i, k** detail of normally developed stomata—*arrow* indicates initial formation of striations; **f** detail of a secondary vein, on the abaxial surface; *Y* detail of ornate trichomes on leaf margins

of plants grown in medium containing sucrose, regardless of the closure type, and in sucrose-free medium combined with caps with one or two membranes, showed normal structural development (Fig. 3a–c, g–i). Development of plants in sucrose-free medium without the PTFE membrane was affected (Fig. 3d–f). Epidermal cells with undefined walls prevented delimitation of cell boundaries; stomatal development was abnormal, most had bulged pores; trichomes were clearly reduced in number, and were broken or malformed (Fig. 3e, f).

Morphology of stomata and epidermal cells was more significantly different in plants grown with RP2 when compared with the other treatments, and it was possible to see the initial formation of striations in subsidiary cells, as well as at different developmental stages (Fig. 3g–l). Stomata that form under conventional conditions of tissue culture are often malformed and unable to function (Desjardins et al. 1987; Santamaria and Kerstiens 1994; Pospíšilová et al. 1999), making the plants susceptible to large transpiration losses, because they play a key role in the regulation of gas exchange and transpiration and are also involved with photosynthetic capacity and plantlet acclimatization. These characteristics were not observed in

P. glomerata from treatments that used sucrose or PTFE membranes, which provided better gas exchange.

Histochemical analyses confirmed the impairment of the structural development of plants grown in jars without sucrose and PTFE membranes (Fig. 4d–f), whereas in the other treatments plants showed normal development (Fig. 4a–c, g–l). The middle third stem section of *P. glomerata* plantlets grown in vitro showed a uniseriate epidermis with stomata and non-glandular trichomes. The subepidermal layers of the cortex (2-4) differentiated in angular collenchyma in treatments with sucrose (Fig. 4a, b, g, h) and sucrose-free medium with membrane disks (Fig. 4j, k), whereas plants grown without sucrose and a membrane did not develop supportive tissues in the cortical region (Fig. 4d, e). The collenchyma did not form a continuous layer/ring, and was interrupted in regions of the epidermis with stomata and/or trichomes by chlorenchyma, which formed the remaining cortical cell layers.

Pectins were detected in the cortical region using the coriphosphine reagent and fluorescence microscopy, which resulted in bright orange-red staining of collenchyma cells and weak staining of pith cells (Fig. 4a, d, g, j). The qualitative analysis showed that chlorenchyma cells were
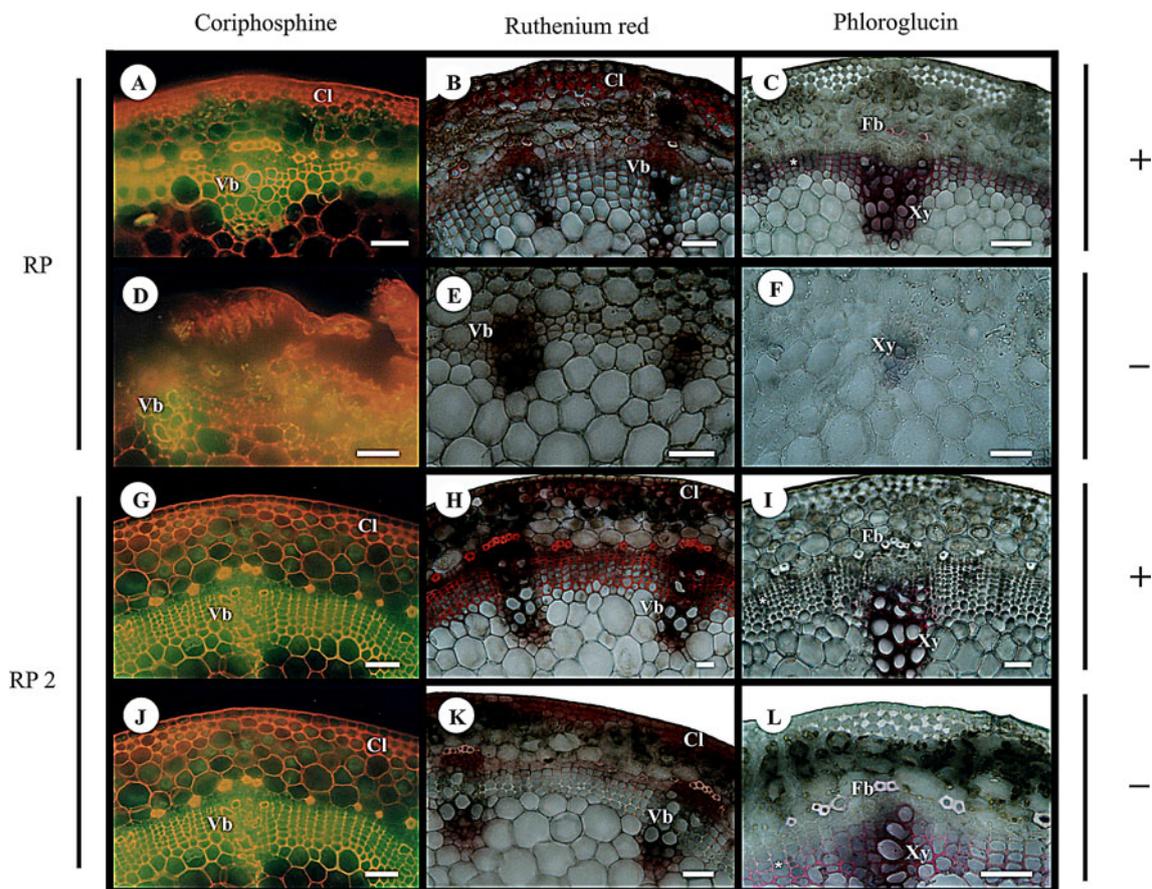
Fig. 4 Cross sections of the middle portion of the stem of *Pfaffia glomerata* propagated in vitro under different closure systems and sucrose concentrations, and stained with the following reagents: coriphosphine induced-fluorescence (**a**, **d**, **g**, **j**); ruthenium red (**b**, **e**, **h**, **k**); and phloroglucin (**c**, **f**, **i**, **l**). **a–c** Rigid polypropylene (*RP*) cap; **d–f** RP with two membrane disks (RP2). *Cl* collenchyma; *Fb* fiber; *Vb* vascular bundle, and *Xy* xylem; **+** (presence) and **−** (absence) of sucrose. *Bars* 50 μm

smaller and cell walls were thinner in the treatment supplemented with sucrose and without a membrane, when compared with plants grown in jars with two membrane disks, regardless of the addition of sucrose to the culture medium (Fig. 4).

Staining with phloroglucinol detected lignified structures in stem sections from the treatments with sucrose-supplemented medium (Fig. 4c, i). As observed for other reagents, plants grown in the RP treatment showed no cell wall thickening and incipient lignification of the vascular tissue (Fig. 4f). Interestingly, stem sections from plants grown in medium that lacked sucrose and flasks with membranes displayed an increased degree of lignification (Fig. 4l). An intense staining of vascular bundles in the remaining treatments indicated strong xylem lignification and cell wall thickening in the interfascicular region, forming a continuous ring (Fig. 2f). The reaction with phloroglucinol also revealed the presence of fibers attached to the bundles in the interfascicular region, which tended to be arranged in a circle. The largest number (5–9) of cell layers with cell wall thickening in the interfascicular region

was found in treatments that used membranes and media with or without sucrose (Fig. 4g–i, j–l). However, the plants grown with sucrose and two membrane disks (Fig. 4g–i) probably had a more intense lignification, because no reaction with phloroglucinol was observed in the interfascicular region or in the fibers. On the other hand, we observed fewer (3–5) cell layers with cell wall thickening, in the interfascicular region, in the treatments with sucrose-supplemented medium without a membrane (Fig. 4c) and treatments with sucrose-free medium using a membrane disks (Figs. 2f, l; 4l).

Conditions that enhance photoautotrophy induced greater levels of 20E

The development of a system to allow the commercial production of plant secondary metabolites in vitro has been proposed by various authors (Zhao et al. 2005; Chen et al. 2006; Lee et al. 2010; Zare et al. 2010; Savio et al. 2012).

Plants grown in the absence of sucrose and without a membrane did not developed under the experimental

Table 3 Production of 20E in the aerial parts of *Pfaffia glomerata* grown in vitro under different closure systems and sucrose concentrations after 30 days of culture

Variable	Sucrose (g L^{-1})	Treatments			
		PVC	RP	RP1	RP2
β-ecdisone	0	–[a]	–[a]	0.035Aa	0.031Aa
level (20E %)	30	0.017A	0.018A	0.019Ab	0.017Ab

Means followed by same capital letter in the row and small letter in the column are not significantly different by the Tukey test at 5 % of probability

[a] Plants grown under these conditions did not grow, hence it was not possible to perform the chemical analysis

conditions, and the dry matter produced was not enough to quantify. Also, 20E was not detected in the roots in any treatment. Thus, the comparative analyses described here excluded the treatments with sucrose-free medium without a membrane, as well as the root system.

When analyzing the factors in an isolated manner, considering the different closure systems and levels of sucrose, we found that in the absence of sucrose there was no statistical difference between the treatments using one or two membrane disks (Table 3). There was also no statistical difference among the treatments supplemented with 30 g L^{-1} sucrose (Table 3). However, when examining the different levels of sucrose within each type of closure, we found that sucrose-free medium associated with PTFE membranes had the highest levels of the active principle (20E), which was a significant accumulation (Table 3). These results differ from those described by Hao and Guan (2012), where the addition of sugars increased the accumulation of secondary metabolites. However, these authors worked with adventitious roots whereas we worked with the whole plant.

Dry matter production was higher in the presence of sucrose, but the percentage of the active principle of interest (20E) was greater in plants grown photoautotrophically.

The chemical nature and concentration of phytoecdysteroids in plants may vary according to the plant part, developmental stage and environmental conditions (Dinan 2001). In cultivation in vitro, plants do not form root tubers because of the reduced cultivation time (about 30 days), which is determined by the depletion of the medium and rapid plant growth. Subcultures are therefore required, but interfere directly with the presence of 20E, because its accumulation in the roots, as well as formation of root tubers, occurs gradually (Festucci-Buselli et al. 2008b).

Whether secondary metabolites are produced mainly in the roots and transported to leaves, or biosynthesized in the leaves and selectively transported from leaves to roots and whole plant, remains an unresolved question that needs to be further investigated. Therefore, it is noteworthy that

production of the active principle in the aerial part of *P. glomerata* grown in vitro was directly influenced by the photoautotrophic condition.

The increment level of 20E may be related to the adaptation to a new condition, such as when plants use secondary metabolites to rapidly and dynamically respond to an environmental stress (Metlen et al. 2009). The network between the primary metabolism and secondary metabolism is very complex and is balanced by their interconversion (Aharoni and Galili 2011).

The survival rate during acclimatization reached 100 %, except for the condition of absence of sucrose without a gas permeable membrane

Plants grown in the absence of sucrose and without a membrane (PVC and RP) did not grow under in vitro conditions. Therefore, ex vitro development was severely compromised and no plants survived the acclimatization phase. However, plants subjected to the other treatments showed a survival rate of 100 % when transferred from in vitro to ex vitro conditions. Plants from treatments with sucrose-supplemented medium and PTFE membranes showed greater resistance during the transfer, without the typical stress characteristics of this stage of in vitro growth.

In additon, Seon et al. (2000) observed that in plants from heterotrophic conditions the content of sugars decreased continuously after acclimatization, whereas plants from photoautootrophic conditions had better control of transpiration. Thirty days after transfer, the plants that survived showed uniform development, regardless of their condition during in vitro cultivation.

There are several advantages to reducing sugar in the culture medium, such as preventing the rapid growth of bacteria or fungi in the cultures, reducing costs and increasing plant survival during acclimatization (Kozai and Kubota 2001; Xiao and Kozai 2006). Photoautotrophic propagation improves in vitro, explant growth rates, physiological characteristics, plant photosynthethic competence and water relations, promoting plantlet hardening (Desjardins et al. 1987; Deng and Donnely 1993; Afreen et al. 2002). For *P. glomerata*, the removal of the carbohydrate source from the culture medium did not affect growth when associated with gas permeable PTFE membranes.

The important effect of increasing gas exchange in plant tissue culture vessels on morphogenesis has been studied (Kitaya et al. 2005; Mohamed and Alsadon 2010; Xiao et al. 2011). Our findings indicate that *Pfaffia* has a high potential for photoautotrophic propagation. The vitroplants in this study showed a vigorous growth pattern supported by increased gas exchange, which promoted the production of seedlings with desirable morphological and physiological characteristics needed for acclimatization. Moreover,

this is the first report in the literature that describes the influence of the photoautotrophic system on increasing the production of secondary metabolites. The increase in 20E levels provided by the photoautotrophic system offers new prospects, not only to increase commercial production of *P. glomerata*, but also for basic studies aiming at elucidate the biosynthetic pathway of phytoecdisteroids in plants. In vitro culture systems based on CO_2 enrichment may represent a promising alternative for mass propagation of this species and the enhancement of 20E levels.

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