Plant Cell Tiss Organ Cult (2017) 131:15–26
DOI 10.1007/s11240-017-1257-9

ORIGINAL ARTICLE

Endophytic *Bacillus megaterium* and exogenous stimuli affect the quinonemethide triterpenes production in adventitious roots of *Peritassa campestris* (Celastraceae)

Marielle Cascaes Inácio[1] · Tiago Antunes Paz[1] · Ana Maria Soares Pereira[2] · Maysa Furlan[1]

Received: 17 April 2017 / Accepted: 12 June 2017 / Published online: 19 June 2017

Abstract The root bark of *Peritassa campestris* (Cambess.) A.C. Sm. (Celastraceae) accumulates quinonemethide triterpenes (QMTs), an important class of bioactive compounds that shows potent antitumor activity. The production of these metabolites is difficult by both chemical synthesis, because of the complex molecular structure, and extraction from plant resources, because of the low yield. Thus, the aim of this work was to evaluate the influence of some important factors on the synthesis of QMTs to increase their production in adventitious roots grown in vitro. The effects of luminosity, mechanical damage to the tissue, source and concentration of carbon, auxins, macronutrient and micronutrient concentrations and the elicitation with its endophytic microorganism, *Bacillus megaterium*, isolated from roots grown in vitro were evaluated. Additionally, we compared the production of QMTs of roots in vitro with that of *P. campestris* roots bark in natura. Our results showed that all stimulating agents affected the biosynthesis of QMTs, with the exception of luminosity. The pattern of QMTs produced was different for the in vitro and in natura roots, including the accumulation of the majority QMTs: the in vitro roots accumulated maytenin (**1**) and 22β-hydroxy-maytenin (**2**), and the in natura roots showed the accumulation of maytenin (**1**), 22β-hydroxy-maytenin (**2**), 20α-hydroxy-maytenin (**3**), and maytenol (**4**). Therefore, we concluded that the biosynthesis of QMTs by *P. campestris* roots is affected by biotic and abiotic factors.

Keywords Elicitation · Endophyte · Maytenin · 22β-Hydroxy-maytenin · 20α-Hydroxy-maytenin · Maytenol

Communicated by Ali R. Alan.

✉ Maysa Furlan
maysaf@iq.unesp.br

[1] Instituto de Química, Universidade Estadual Paulista - UNESP, Rua Prof. Francisco Degni, 55, Quitandinha, Araraquara, SP 14800-060, Brazil

[2] Departamento de Biotecnologia Vegetal, Universidade de Ribeirão Preto, Ribeirão Preto, SP 14096-900, Brazil

Introduction

Peritassa campestris (Cambess.) A.C. Sm. (Celastraceae) is a wild plant of the Brazilian savannah (Cerrado), a biodiversity hotspot region in which the species are under anthropic pressure and vulnerable to extinction. This species biosynthesizes quinonemethide triterpenes (QMTs), which is a class of secondary metabolites of pharmacological interest (Corsino et al. 2000; Buffa Filho et al. 2002; Rodrigues-Filho et al. 2002; Jeller et al. 2004; Carvalho et al. 2005; Paz et al. 2013).

The QMTs are restricted in occurrence to the Celastraceae and show a wide range of bioactivities, particularly anticancer activity against several cell lines, e.g., breast, lung, colon, prostate, esophagus, and others (Ngassapa et al. 1994; Carvalho et al. 2005; Sung et al. 2010; Almeida et al. 2010; Oramas-Royo et al. 2010; Seo et al. 2011; Salvador et al. 2014; Wang et al. 2015; Cevatemre et al. 2016; Hernandes et al. 2017).

Because QMTs are exclusively accumulated in the root bark of Celastraceae species, and their synthesis is not commercially viable, our research group has been dedicated to the study of QMT biosynthesis using some Celastraceae species in natura and in in vitro plant systems, which includes the use of suspension cells (Coppede et al. 2014) and adventitious roots (Paz et al. 2013; Pina et al.

2016b, a). The primary goals of this study are to develop scale production systems of these secondary metabolites, in addition to increasing the understanding of the ecophysiological aspects that interfere in these processes.

The use of effective strategies can optimize biotechnological production of plant secondary metabolites, which include elicitation to stimulate the biosynthesis of some plant defensive compounds. Among the elicitors, jasmonates, salicylic acid and also microorganism extracts have been used with success (Ramirez-Estrada et al. 2016).

Related to microorganisms as elicitors, bacterial or fungal endophytes are included, which live inside plants without causing pathogenic effects to the host (Gunatilaka 2006). In many cases, plant secondary metabolites are accumulated by induction or enhanced constitutively in plant tissues by these endophytes. The chemical signals of an endophyte can initiate a series of plant defensive responses that are similar to those of a plant–pathogen interaction (cascade of signal transduction), which can change and/or increase the production of secondary metabolites in plants (Zhi-lin et al. 2007).

In this study, we explored for the first time the endophytic microorganisms from in vitro adventitious root cultures of *P. campestris* and evaluated their ability to elicit increased production of QMTs. Additionally, we evaluated the influence of factors such as luminosity, mechanical damage to the tissue, source and concentration of carbon, auxins, and macronutrients and micronutrients on QMT accumulation by adventitious roots grown in vitro. We also compared the production of these compounds between roots grown in vitro and roots collected from natural habitats.

Materials and methods

Plant material and adventitious root culture

Adult plants of *P. campestris* (n = 6) were collected from the Brazilian savannah (Cerrado) in São Carlos, SP, Brazil, in May 2016. Dr. Julio Antonio Lombardi (Instituto de Biociências, UNESP, Rio Claro, SP) identified the specimens, and a voucher specimen, No. 1415, was deposited at the Herbarium of Medicinal Plants of the Universidade de Ribeirão Preto (HPM-UNAERP, Ribeirão Preto, SP). Soil samples were collected with each specimen and submitted for chemical analyses in the Agricultural Chemistry Laboratory, UNAERP. Soil was analyzed for organic matter (g dm^{-3}), pH, phosphorus (mg dm^{-3}), potassium ($mmol_c$ dm^{-3}), calcium ($mmol_c$ dm^{-3}), magnesium ($mmol_c$ dm^{-3}), sulfur (mg dm^{-3}), total bases ($mmol_c$ dm^{-3}), potential acidity ($mmol_c$ dm^{-3}), cation exchange capacity ($mmol_c$ dm^{-3}), base saturation (%), boron (mg dm^{-3}), copper (mg dm^{-3}), iron (mg dm^{-3}), manganese (mg dm^{-3}), and zinc (mg dm^{-3}).

The adventitious root cultures obtained were maintained in Woody Plant Medium (WPM) (Lloyd and McCown 1980) supplemented with 2% (w/v) sucrose, 0.01% (w/v) polyvinylpyrrolidone (PVP), and 0.004 g L^{-1} auxin indole-3-butyric acid (IBA). The media were adjusted to pH 6.0 and solidified with 0.25% (w/v) Phytagel® before autoclaving at 121 °C for 15 min (Paz et al. 2013). The cultures were incubated at 25 ± 2 °C in the dark. The WPM was used in all experiments, except for the experiment that evaluated different culture media. All in vitro assays were performed using roots with 60 days of cultivation as previously described by Paz et al. (2013). Details about initiation of in vitro root cultures, growth and dry mass yield of *P. campestris* can be seen in the same article.

Preparation of extracts

Whole in vitro adventitious roots (≈0.15 g) and in natura root bark (1 g) were dried and ground into a fine powder and then sonicated with dichloromethane (in vitro adventitious roots, 0.1 g dry weight mL^{-1} solvent) or chloroform (in natura roots) 3 times for 10 min at room temperature. The samples were filtered through filter paper and evaporated to dryness.

Preparation of samples for high performance liquid chromatography (HPLC) analysis

The root extracts were dissolved in methanol:water (MeOH:H_2O, 99:1, v/v) (0.1 g DW mL^{-1}), and a 1 mL aliquot of the resulting solution was submitted to solid-phase extraction (SPE) cartridges (Chromabond®) (3 mL) containing 0.5 g of C18 silica gel (particle size of 40 µm) preconditioned with MeOH and MeOH:H_2O (99:01, v/v). Cartridges were eluted with 3 mL of MeOH:H_2O (99:01, v/v). The interfering compounds were retained by the C18 silica gel, whereas the eluate was transferred to a 5 mL volumetric flask, adjusted to the mark with the same 99% MeOH. The resulting solution was filtered through a 0.22 µm membrane nylon filter, and an aliquot (20 µL) was analyzed by HPLC.

Quantitative and qualitative HPLC analysis of QMTs

The content of maytenin **(1)**, 22β-hydroxy-maytenin **(2)**, and maytenol **(4)** (Fig. 1) in root extracts (whole in vitro roots and root bark of in natura plants) was determined using an HPLC system from Shimadzu (Kyoto, Japan) with a series LC-20 AT pump, SPD-M20A UV/Vis detector, CTO-20 column oven, SIL-20A auto-sampler, DGU-20A3 degasser, CBM-20A system controller

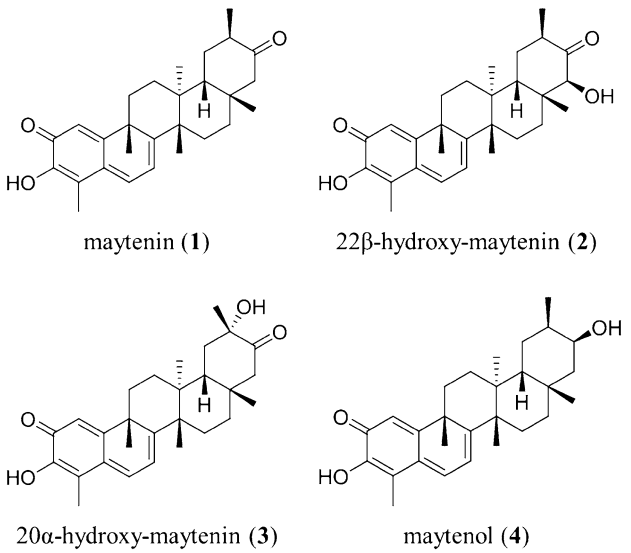

Fig. 1 Chemical structures of maytenin (**1**), 22β-hydroxy-maytenin (**2**), 20α-hydroxy-maytenin (**3**), and maytenol (**4**) identified in the root bark of *P. campestris*

and a Phenomenex (Torrance, CA, USA) Luna C18 column (250×4.6 mm i.d.; particle size of 5 µm). Data were acquired via LC Solution® software (Shimadzu, Kyoto, Japan). The chromatographic analysis was conducted in the gradient mode using a mobile phase consisting of H_2O (A) and MeOH:acetonitrile (B) (80:20, v/v). To both A and B mobile phases, formic acid 1% (v/v) was added. The gradient was programmed to start with a mobile phase condition of 15% A and 85% B (maintained for 10 min) to 0% A and 100% B after 13 min. This condition was maintained for 6 min before returning to the start condition. The flow rate was 1.2 mL min^{-1}; injection volume was 20 µL for the samples and the chemical standard. The detection was conducted at a wavelength of 420 nm. Maytenin (**1**), 22β-hydroxy-maytenin (**2**) and maytenol (**4**) (all HPLC purity >95%) were used as standard compounds previously isolated from the root bark of *P. campestris*. Their identities were determined by NMR and MS experiments and comparison with the literature (Gunatilaka 1996; Rodrigues-Filho et al. 2002; Lião et al. 2008; Paz et al. 2013). The absorbance measurements were integrated by comparison with an external standard calibration curve prepared at concentrations from 0.5 to 115 µg mL^{-1}, in triplicate. The analytical curves were considered acceptable when r (correlation) ≥0.99, consisting of at least 5 points.

The QMT 20α-hydroxy-maytenin (**3**) (Fig. 1) was identified in the root extracts by high-resolution mass spectrometry.

Analysis by HPLC coupled to high-resolution mass spectrometry (HPLC-MS)

High-resolution analysis by HPLC-MS was performed on a modular HPLC system from Shimadzu, which consisted of a communication bus module (CBM 20A), two pumps (LC-20AD), column oven (CTO 20A), auto-sampler (SIL-20AHT), and diode array detector (SPD-M20AV), coupled to a micrOTOFII (Bruker Daltonics) ESI-qTOF mass spectrometer. The LC conditions were the same applied for the quantitative and qualitative HPLC analysis of QMTs (item "Quantitative and qualitative HPLC analysis of QMTs"). The eluent was split at the HPLC column end to allow 30% eluent to flow into the mass spectrometer. HPLC-MS TIC chromatograms were recorded between 50 and 1300 m/z in the positive ionization mode. The spectrum was obtained in high resolution using a capillary and end plate with 3 kV and 25 V, respectively. Nitrogen was used as the nebulizer (5.5 bar). The flow of the drying gas was 10 L min^{-1}, and the drying gas temperature was 180 °C.

Isolation, cultivation and identification of endophytic microorganisms isolated from in vitro adventitious root bark of *P. campestris*

In a portion of the roots maintained in vitro for 5 years by periodic subculturing in fresh medium (WPM), which were maintained for 90 days without transfer to fresh medium, we observed the growth of microorganisms. Using a platinum loop, the microorganisms were transferred to petri dishes containing potato dextrose agar, Sabouraud agar, Czapek agar, nutrient agar, trypticase soy agar, Mueller Hinton agar or brain heart infusion agar media, which were stored at 28 °C for 24 h. Six microorganisms (MTS1, MTS2, MNA1, MNA2, MCZ1 and MMH) were isolated and kept in glycerol stock (15%) at −20 and −80 °C. The André Tosello Foundation (Fundação André Tosello, Campinas, São Paulo State, Brazil) identified the microorganisms as follow: total DNA from microorganisms was extracted using a commercial kit (UltraClean® Microbial DNA Isolation Kit—MoBio). Amplification of the target DNA through the polymerase chain reaction (PCR) was performed by the primer oligonucleotides 27F (5′-AGA GTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACG-GYTACCTTGTTACGACTT-3′) (Weisburg et al. 1991). After an electrophoretic run on TBE agarose gel 1% (w/v), the product was purified using an UltraClean PCR Clean-Up Kit (MoBio). Following a new electrophoretic run on TBE agarose gel 1% (w/v), the purified product was quantified, concentrated, aliquoted, and supplemented with the oligonucleotide (27F) used for sequencing, which

was performed on the ABI 3500 Genetic Analyzer (Life Technologies).

The taxonomic classification was based on the comparison between the obtained sequences and those deposited in GenBank (http://www.ncbi.nlm.nih.gov) via BLASTn. Sequences were aligned with the similar sequences available in the database, and a phylogenetic tree was constructed from the 16S rRNA gene using the MEGA 6.0 software by the neighbor-joining method with Kimura-2-parameter and bootstrap of 1000 replicates.

Scanning electron microscopy of in vitro adventitious roots of *P. campestris* colonized with endophytic microorganisms

One of the adventitious roots kept in vitro (without apparent contamination) was sectioned 0.5 cm from the cap, and a manual cross section was performed. The root fragment was placed directly on an optical microscope (Olympus-micronal BX41), and photographs were obtained using a DCE-5C USB 2.0 digital camera and scanning electron microscope (Hitachi TM3030PLUS).

Determination of QMTs production curve

Peritassa campestris adventitious roots were cultured in WPM medium supplemented with sucrose 2% (w/v), IBA 0.004 g L^{-1}, PVP 0.1 g L^{-1} and gelled with Phytagel® at 2.5 g L^{-1} (Paz et al. 2013). The roots (2 g) were inoculated into flasks containing 30 mL of culture medium; QMTs production was evaluated daily for 15 days using HPLC (item "Quantitative and qualitative HPLC analysis of QMTs").

Effect of mechanical damage

Roots of *P. campestris* cultured in vitro were transferred to new culture medium and sectioned in two ways: cut with bistoury and cut with tweezers (i.e., tearing the tissue). Non-sectioned roots were used as controls. For this experiment, and all of the following, 1 g of roots was inoculated per flask containing 15 mL of medium.

Luminosity effects

The effect of luminosity on the production of QMTs by *P. campestris* adventitious roots was evaluated in the presence or absence of light. The photoperiod used for the plants under light was 16:8 h, at a photosynthetic photon flux density of 20–70 mmol m^2 s^1.

Influence of carbon source and concentration

In this experiment, the roots of *P. campestris* cultured in vitro were transferred to culture media containing glucose or sucrose at concentrations of 0.5, 2, 3 or 4.5% (w/v).

Influence of auxins

The effect of auxins on the production of QMTs by in vitro roots was verified using indolebutyric acid (IBA), indoleacetic acid (IAA), naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) at the concentration of 0.004 g L^{-1}.

Influence of macronutrients, micronutrients and vitamins

In this experiment, we evaluated the production of QMTs by transferring the roots to MS culture medium (Murashige and Skoog 1962), B5 medium (Gamborg et al. 1968) or WPM supplemented with 2% (w/v) sucrose, 0.01% (w/v) PVP, and 0.004 g L^{-1} IBA. The following nutrients were evaluated (mg L^{-1}): KNO_3, NH_4NO_3, $(NH_4)_2SO_4$, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, $Ca(NO_3)_2{\cdot}4H_2O$, K_2SO_4, KH_2PO_4, $NaH_2PO_4{\cdot}H_2O$, $MnSO_4{\cdot}H_2O$, $MnSO_4{\cdot}4H_2O$, KI, H_2BO_3, $ZnSO_4{\cdot}7H_2O$, $CuSO_4$, $CuSO_4{\cdot}5H_2O$, $Na_2MoO_4{\cdot}2H_2O$, $CoCl_2{\cdot}6H_2O$, $FeSO_4{\cdot}7H_2O$, Sequestrene Fe®, $Na_2{\cdot}EDTA{\cdot}2H_2O$, myo-inositol, thiamine-HCl, nicotinic acid, glycine, pyridoxine-HCl, and sucrose.

Elicitation of in vitro adventitious roots with its endophytic microorganism, *Bacillus megaterium*

Elicitation of *P. campestris* roots cultured in vitro with *B. megaterium* was performed as described by Savitha et al. (2006) with few modifications. Initially, the microorganism kept in glycerol stock (item "Isolation, cultivation and identification of endophytic microorganisms isolated from in vitro adventitious root bark of *P. campestris*") was reactivated on a sterile petri plate containing nutrient agar medium and maintained in an oven at 28 °C for 24 h. Subsequently, the microorganism was subcultured on the same medium, maintained under the same conditions and time mentioned above and then transferred to the broth nutrient medium and maintained at 28 °C on a shaker at 120 rpm for 24 h. Microorganism quantification was performed using a spectrophotometer at 550 nm before (1.5678 ± 0.09) and after 24 h of growth in broth medium (2.1759 ± 0.02), indicating a growth of approximately 1.40-fold. After quantification, the microorganism was autoclaved for 15 min at 121 °C and 1 atm and then centrifuged at maximum power (26,300×*g*) for 1 h at room temperature. This material was then separated into culture filtrates (CFs, supernatant) and

dried cell powders (DCPs, sedimented). The DCPs were lyophilized, and the CFs were used without any processing.

The elicitation was performed in Erlenmeyer flasks (125 mL) using DCPs at concentrations of 0.10, 0.25 and 0.50% (w/v) and CFs at concentrations of 5, 7.5 and 10% (v/v). The culture medium was B5 (liquid) supplemented with sucrose 20 g L^{-1}, NAA 0.004 g L^{-1}, and PVP 0.1 g L^{-1}, with the pH adjusted to 6.0, followed by autoclaving. The roots were kept on a shaker at 100 rpm in the presence of light for 8 days. After this period, the in vitro elicited roots were dried and the extracts were obtained according to item "Preparation of extracts".

Statistical analyses

The experimental design was completely randomized with three repetitions and three replicates for each treatment. The evaluations were performed after 8 days, according to the result obtained in the experiment in which the peak production of QMTs was evaluated. The averages were analyzed by the Tukey or Scott Knott tests at 5% probability, using SISVAR software (Ferreira 2011).

A principal component analysis (PCA) was performed using (**1**) and (**2**) concentrations and the nutrients that varied between the WPM, MS and B5 media. We also performed a PCA analysis of soil nutrients for the plants collected using the (**1**), (**2**) and (**4**) concentrations and the nutrients that varied among the individual analyzed soils. The software used was Genes VS 2009.7.0 (Cruz 2013). After both PCA analyses, Pearson's simple correlation coefficient was calculated.

Results and discussion

Determination of QMTs production curve

The evaluation of the production of QMTs by *P. campestris* adventitious roots in vitro was restricted to maytenin (**1**) and 22β-hydroxy-maytenin (**2**), with the accumulation of (**1**) higher than that of (**2**) (Fig. 2a).

The highest production of (**1**) and (**2**) occurred at 8 days of cultivation (≈2484 and 700 µg g^{-1} dry weight, (DW), respectively). The accumulation of both compounds occurred in a similar way but was non-uniform during the period evaluated (Fig. 2a). Paz et al. (2013) found that the highest maytenin (**1**) production occurred at 7 days (≈972 µg g^{-1} DW) and that of 22β-hydroxy-maytenin (**2**) at 15 days in a 105-day evaluation period. In our work, we observed an accumulation 2.5-fold higher of (**1**) than that previously published data (Paz et al. 2013). However, the analysis performed by them was not daily. After 7 days of growth, the roots produced ≈ 741 µg g^{-1} DW, 1.31-fold lower than that obtained by Paz et al. (2013). The in vitro roots used by the authors were the same as those used in our studies. We also observed that the accumulation of QMTs varied according to the number of in vitro roots in the subculture, and that the production of these compounds might decrease over time; thus, further experiments are required to verify the stability and production capacity of these tissues over time.

Effect of mechanical damage

Mechanical damage caused by cuts to the in vitro adventitious roots did not influence the accumulation of (**2**). However, the content of (**1**) (1598.52 µg g^{-1} DW) increased significantly and was 4.78-fold greater in roots with a cut performed with tweezer than in cut performed with bistoury (334.89 µg g^{-1} DW). Although significant differences were not detected between the production of (**1**) in whole roots and roots cut with tweezers, we observed that roots drastically damaged accumulated more of this compound (Fig. 2b).

These injuries were similar to those that occur in nature, which lead to innumerable general and immediate defense responses to promote tissue healing and prevent infections when a plant tissue is damaged (Duran-Flores and Heil 2016). Several cytotoxic compounds involved in this process are constitutive, such as QMTs, and are stored in compartments with limited metabolic activity (Nosov 2012) for release upon injury.

Luminosity effects

Light did not change the production of QMTs (890.49 and 548.65 µg g^{-1} DW for (**1**) and (**2**), respectively) when compared with roots kept in the dark (1029.28 and 584.21 µg g^{-1} DW for (**1**) and (**2**), respectively, Fig. 2c).

In plants, terpenoids are biosynthesized by either MVA (mevalonate, in the cytosol) or MEP (2-C-methyl-d-erythritol 4-phosphate, in the plastids) pathways (Lipko and Swiezewska 2016), and the QMTs are biosynthesized through the MVA pathway (Pina et al. 2016b). The genes of both MVA and MEP pathways are regulated antagonistically by the circadian clock (Rodríguez-Concepción and Boronat 2015). In our experiments, the biosynthesis of QMTs in the roots occurred both in the presence and absence of light. However, a study conducted with seedlings of *Arapdopsis thaliana* demonstrated that in the dark, the genes that encode the enzyme HMG-CoA reductase, which catalyzes 3-hidroxy-3-methylglutaryl CoA in the MVA pathway, are overexpressed. Additionally, light can activate the MEP pathway (Rodriguez-Concepcion et al. 2004).

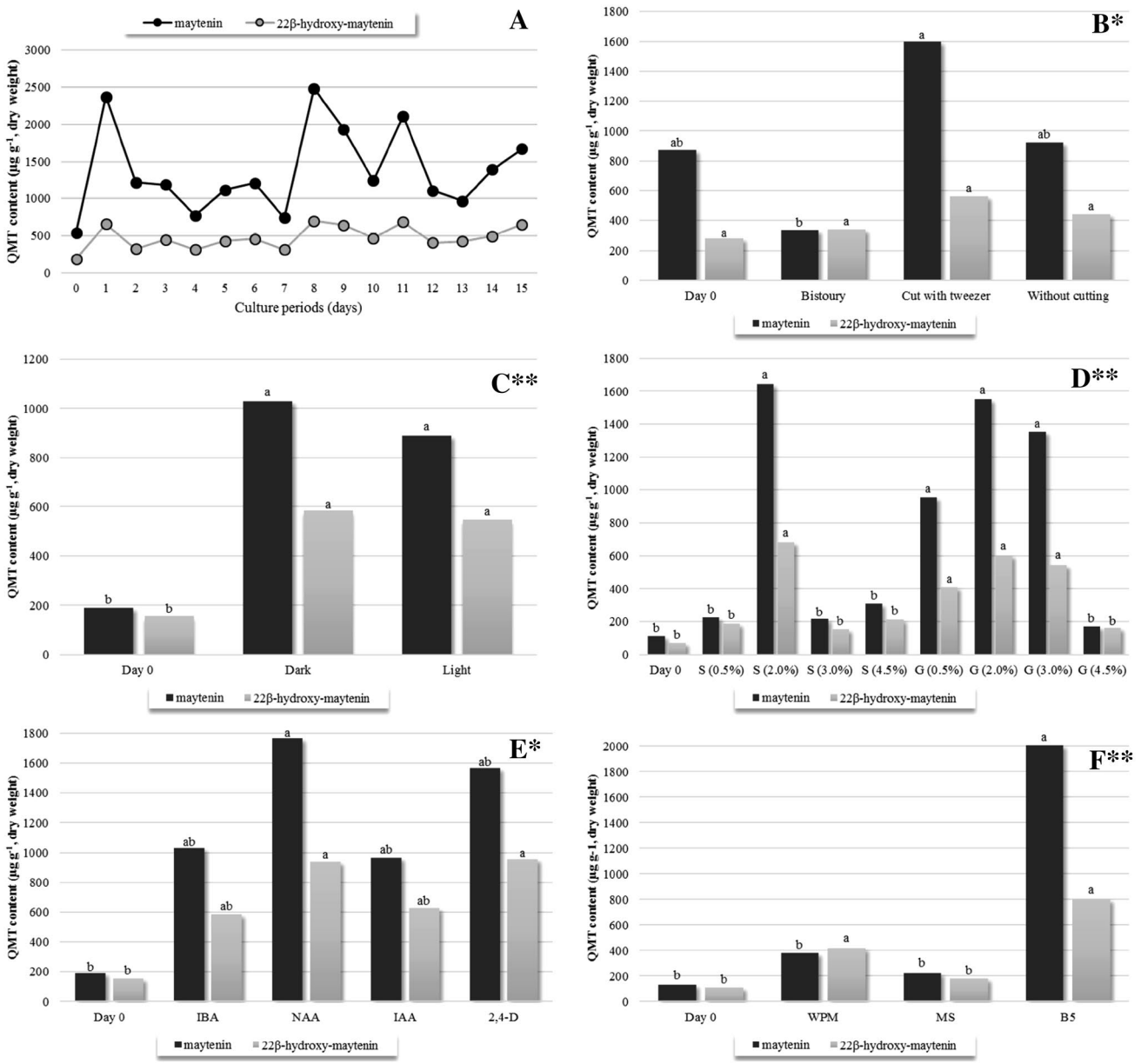

Fig. 2 Variation in content of maytenin (**1**) and 22β-hydroxy-maytenin (**2**) produced by adventitious roots of *P. campestris* grown in vitro **a** for the period of 15 days, **b** after 8 days of cultivation, without cutting, sectioned with bistoury or tweezer, **c** after 8 days of cultivation, in the absence and presence of light, **d** after 8 days of cultivation, according to the source and the carbon concentration in culture medium (*S* sucrose, *G* glucose), **e** after 8 days of cultivation, according to the source of auxin: *IBA* indolbutyric acid, *NAA* naphthaleneacetic acid, *IAA* indoleacetic acid and *2,4-D* 2,4-dichlorophenoxyacetic acid, **f** after 8 days of cultivation, according to the culture medium used: WPM, MS and B5. All analyses include the zero point (Day 0). Means followed by the *same letter* do not differ statistically by the Tukey (*) or Scott-Knott (**) test at a 0.5% probability, separately for (**1**) and (**2**)

In recent studies, crosstalk was demonstrated between common isoprenoid precursors from the MVA and MEP pathways (Rodriguez-Concepcion et al. 2004; Rodríguez-Concepción 2006; Liao et al. 2016), and the percentage of exchange of these precursors from the plastids to the cytosol can reach 100% (Liao et al. 2016). In our case, it is possible that the QMTs biosynthesized by in vitro adventitious roots in the presence of light could also be formed from the MEP-derived building blocks, which could be exported to the cytosol and used in the MVA pathway.

Influence of carbon source and concentration

In this experiment, we found that the highest concentration for both QMTs, (**1**) and (**2**), was achieved using sucrose at 2% as the carbon source (1643.59 and 682.87 µg g^{-1} DW

for (**1**) and (**2**), respectively) and glucose at 0.5% (951.80 and 411.14 μg g^{-1} DW for (**1**) and (**2**), respectively), 2% (1552.23 and 603.98 μg g^{-1} DW for (**1**) and (**2**), respectively), or 3% (1356.87 and 541.98 μg g^{-1} DW for (**1**) and (**2**), respectively) (Fig. 2d). Although sucrose at 2% resulted in a greater accumulation of QMTs by the *P. campestris* adventitious roots (when analyzing the graph), we found that the low glucose concentration in the medium was as efficient for the production QMTs as sucrose at 2% at statistically significant levels.

The glucose efficiency in the production of QMTs is certainly related to the direct availability of this carbon source in the plant respiratory pathway. In this pathway, sucrose is broken down into glucose and fructose by invertase and sucrose synthase enzymes and then glycolysis is initiated (Rolland et al. 2006; Ruan 2014). When we used glucose as the carbon source, the sucrose degradation step was not necessary; thus, the energy expenditure would be lower, and the generation of respiration by-products used in secondary metabolism would be favored. Additionally, exogenous sugars can modulate the metabolic flow, including the isoprenoid biosynthetic pathway, which forms QMTs (Lipko and Swiezewska 2016).

In addition to increasing the concentration of (**1**) and (**2**) in *P. campestris* roots in vitro, the addition of glucose in the culture medium led to the accumulation of another QMT, 20α-hydroxy-maytenin (**3**) ($C_{28}H_{36}O_4$, molecular mass = 436.26). The analysis of extracts by high resolution LC-MS confirmed the identity (Fig. 3). The specific absorption in the region of 420 nm, the retention time compared with its isomer (22β-hydroxy-maytenin) and the base peak at 201 m/z in the mass spectra for the formation of a tropylium ion indicated that this QMT was in the analyte (Paz et al. 2013).

Influence of auxins

The auxins affected the contents of (**1**) and (**2**) in *P. campestris* roots in vitro (Fig. 2e). The highest production of both QMTs occurred with NAA (1766.24 and 935.72 μg g^{-1} DW for (**1**) and (**2**), respectively), which yielded 1.72-fold (**1**) and 1.60-fold more (**2**) than IBA (1029.28 and 584.21, μg g^{-1} DW for (**1**) and (**2**), respectively). The production of QMTs by *P. campestris* roots was lower when natural auxins such as IBA and IAA were used than when the synthetic auxins 2,4-D and NAA were used, which stimulated higher production of QMTs (Fig. 2e).

The plant species will respond differently to each auxin, depending on the transporters and receptors specificity (Enders and Strader 2015). Given this diversity of effects, it is difficult to standardize responses related to the effects of auxins on different species, including their influences on secondary metabolism. However, some auxins can stimulate triterpene biosynthesis (Migas et al. 2006; Singh et al. 2015; Rasool and Mohamed 2016). Smolenskaya et al. (2007) found that 2,4-D induced mitosis in *Panax ginseng* cells, which resulted in a very low accumulation of ginsenosides (steroid glycosides and triterpene saponins), whereas the NAA induced low cell mitosis and promoted cellular elongation, nuclear DNA synthesis, and the biosynthesis of the same and different ginsenosides.

Additionally, studies show that auxins can influence cytochrome P450 enzymes (Chaban et al. 2003; Srivastava et al. 2015). This class of enzymes are monooxygenases that catalyze different oxygenation/hydroxylation reactions involved in detoxification, in addition to some biosynthetic pathways, including those for triterpenes in plants (Chaban et al. 2003; Rasool and Mohamed 2016). Previous investigations showed that the P450 enzymes catalyze the final reactions of the biosynthesis of QMTs (Corsino et al. 2000).

Influence of macronutrients, micronutrients and vitamins

Nutrients interfered with the production of QMTs by in vitro adventitious roots of *P. campestris*. We observed the highest accumulation of (**1**) and (**2**) in roots maintained in B5 medium (2006.05 and 803.23 μg g^{-1} DW for (**1**) and (**2**), respectively, Fig. 2f). The concentration of inorganic nutrients and salts in the B5 medium is much lower than that in the MS medium (Ling et al. 2011), and B5 is the most deficient of the media evaluated.

Principal components analysis showed high variation for $CaCl_2{\cdot}2H_2O$ and $CuSO_4$ (80.03% of the total variation). We observed highly positive correlations between the levels of (**1**) and (**2**) and the micronutrient $CuSO_4$ (0.99 and 0.94, respectively). High concentrations of copper ions can induce the plant defense system, including the biosynthesis of compounds from the isoprenoids pathway (Chmielowska et al. 2010), and have also been used in elicitation plant systems (Savitha et al. 2006).

Moreover, the concentration of copper ions in the B5 culture medium (156 μM) was higher than that in WPM and MS medium (both at 100.13 μM). Thus, we inferred that the copper excess in the B5 medium stimulated the plant defense system, inducing the accumulation of more (**1**) and (**2**).

Identification of endophytic microorganisms and scanning electron microscopy of colonized *P. campestris* roots in vitro

The isolated colonies named MCZ1, MMH1, MNA1, MTSA1 and MTSA2 were odorless, creamy in texture and in color, smooth, circular, convex and opaque with entire

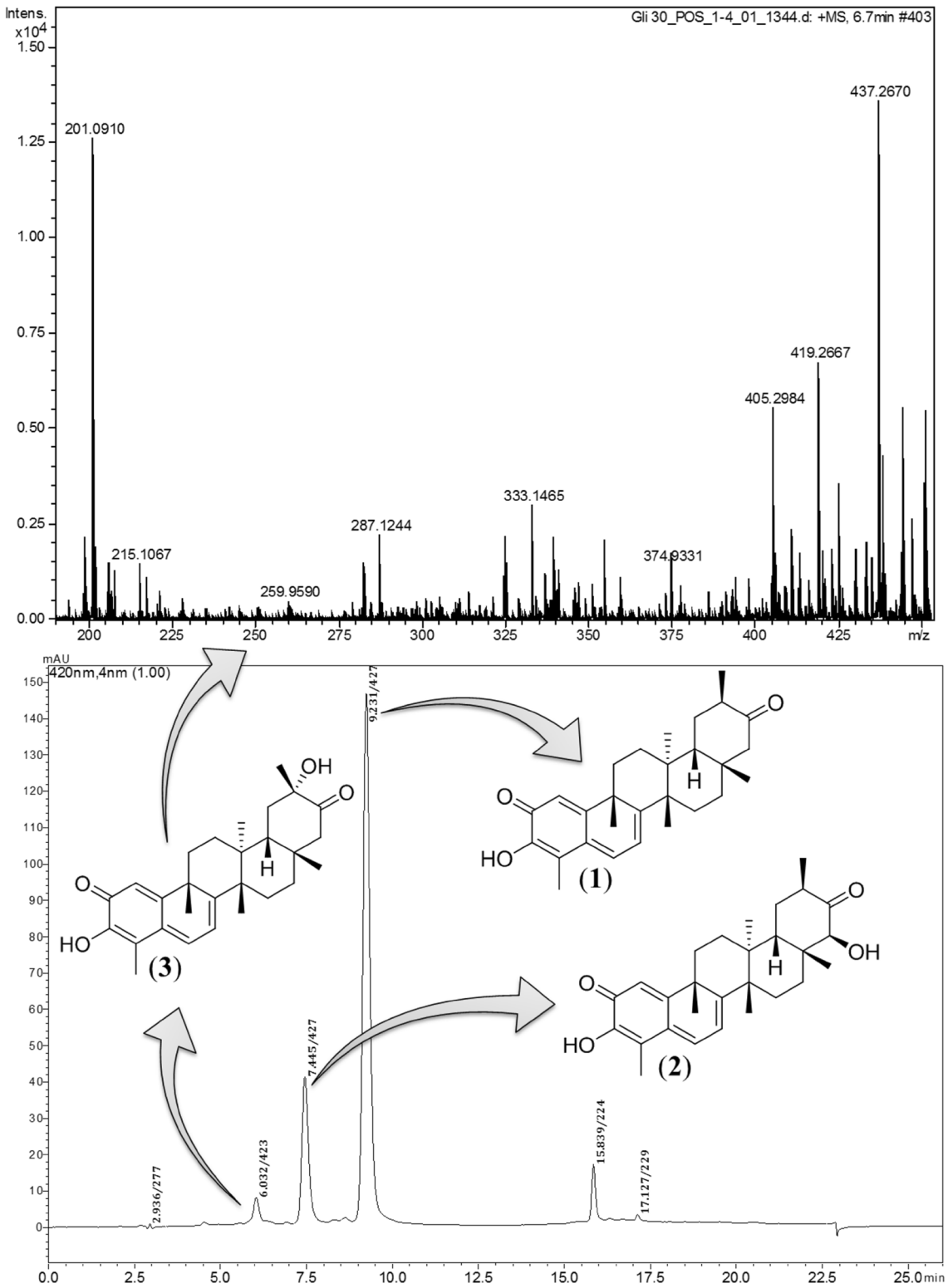

Fig. 3 Chromatogram demonstrating the presence of maytenin (**1**), 22β-hydroxy-maitenin (**2**) and 20α-hydroxy-maytenin (**3**), with their respective chemical structures and mass spectra confirming the presence of (**3**) in roots of *P. campestris* cultured in vitro in WPM medium supplemented with glucose at 3%, after 8 days of cultivation. Chromatographic conditions: item "Quantitative and qualitative HPLC analysis of QMTs"

margins. Cells were Gram-positive rods, occurring singly, in pairs or chains. Based on their sequence similarity (>99%, in GenBank), these strains were taxonomically classified as *B. megaterium*. The strain MNA2, occurring singly, (sequence similarity <90%) was classified as *Bacillus* sp.

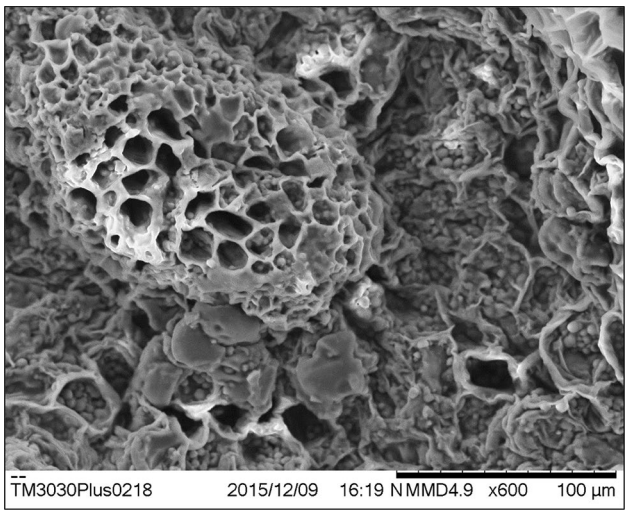

Fig. 4 Scanning electron microscopy of cross section of *P. campestris* root maintained in vitro showing the colonization by endophytic microorganisms

The electron microscopy image of *P. campestris* root culture in vitro showed the endophytic microorganisms located in the intracellular spaces. The colonies were in the parenchyma and vascular system of the root (Fig. 4). Endophytic microorganisms are those that interact intimately with plants and colonize their internal tissues (Mano and Morisaki 2008), living in symbiosis in different organs, particularly in the inter- and intracellular spaces (Qin et al. 2011). These microorganisms in plant tissue cultures were, until recently, disregarded, because the plant medium is aseptic and the cultures are considered axenic; however, they have been disregarded because endophytes can be observed primarily only by microscopy (Barrow et al. 2004).

Elicitation of adventitious roots with its endophytic microorganism, *B. megaterium*

The accumulation of QMTs in adventitious in vitro roots of *P. campestris* was influenced by elicitation with *B. megaterium*. In vitro roots elicited with CFs increased the production of both (**1**) and (**2**) at all concentrations evaluated (5, 7.5, and 10%), with 5% sufficient to stimulate the highest production: 2.31-fold of (**1**) when compared with

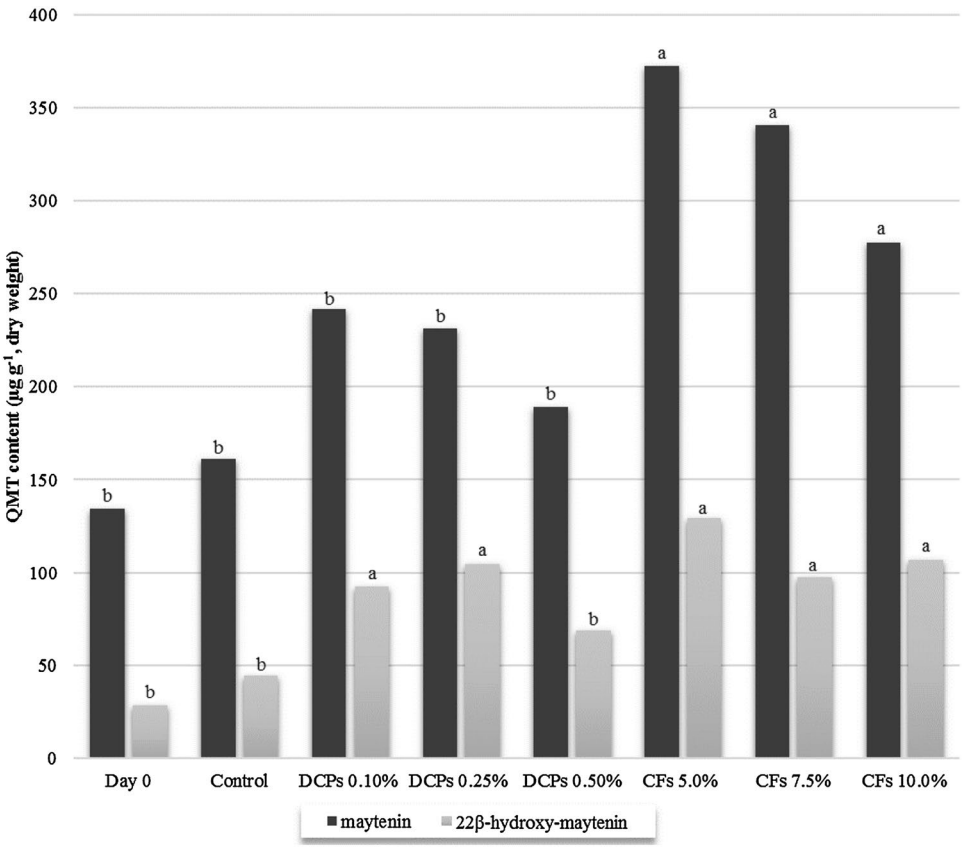

Fig. 5 Variation in content of maytenin (**1**) and 22β-hydroxy-maytenin (**2**) produced by in vitro roots of *P. campestris* elicited with endophytic bacterial *B. megaterium* culture filtrates (CFs) and dried cell powders (DCPs), after 8 days of cultivation, including the zero point. Means followed by the *same letter* do not differ statistically by the Scott Knott test at 0.5% probability, separately for (**1**) and (**2**)

the control (372.65 μg g^{-1} DW, Fig. 5) and almost threefold for (**2**) (129.22 μg g^{-1} DW, Fig. 5). Certainly, CFs are composed of thermostable chemicals, including peptides, molecules and fragments of secondary metabolites, polysaccharides, and others, among which are chemical signaling molecules for plant defense (Baenas et al. 2014). Using the DCPs, we found that only the content of (**2**) increased at 0.10 and 0.25% (Fig. 5). In this experiment, no other QMTs were identified.

Endophytic microorganisms and the substances produced by their primary and secondary metabolisms can stimulate the production of secondary metabolites in plants in different ways (Kusari et al. 2013), including the production of some secondary metabolites of interest that can only occur in symbiosis (Zhi-lin et al. 2007). In this work, we showed a chemical signaling provided by metabolites of the endophytic bacterium *B. megaterium* in *P. campestris* roots in vitro influenced the production of QMTs. However, as confirmed in the previous experiments, the content of QMTs is affected by several factors.

QMTs accumulation in in natura roots

In addition to (**1**) and (**2**), all in natura root bark also produced (**3**) and (**4**). The highest contents of (**1**) and (**2**) were found in specimen 4 (9037.95 and 987.84 μg g^{-1} DW for (**1**) and (**2**), respectively, Fig. 6) and that of (**4**) in specimen 2 (13,030.29 μg g^{-1} DW). This variation demonstrated that the production of QMTs is highly influenced by minimal environmental variations; because the distance between the specimens was a maximum of 10 m. Environmental influences were also demonstrated in experiments with roots grown in vitro.

The first two components of the PCA contained 81.78, 82.68 and 85.78% of the total variation for (**1**), (**2**) and (**4**), respectively: magnesium (Mg) in the first component and organic matter (OM) in the second. However, we observed a weak correlation for both Mg and OM (maximum 0.5).

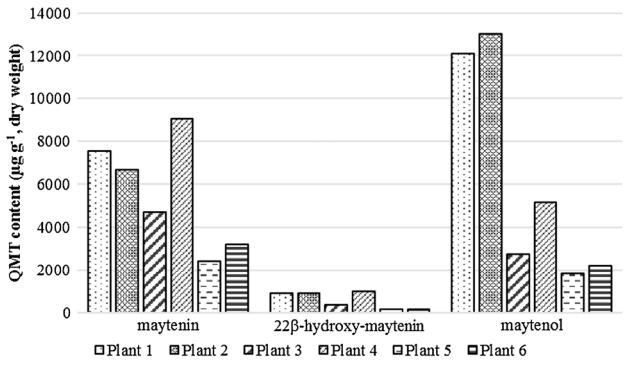

Fig. 6 Variation in content of maytenin (**1**), 22β-hydroxy-maytenin (**2**) and maytenol (**4**) in root bark of six specimens of *Peritassa campestris* collected in São Carlos, SP, Brazil

The profiles of QMTs produced by adventitious roots grown in vitro and roots collected in the natural habitat were different. In the in vitro system, the roots preferentially produced (**1**) and (**2**), although (**3**) was produced when glucose (at 0.5, 2 and 3%) was added to the medium, and for the in natura roots, some plants accumulated (**1**) and (**4**) as the primary compounds.

Studies conducted by our research group demonstrate that when a Celastraceae species is inserted into an in vitro culture system, the accumulated QMTs are predominantly (**1**) and (**2**), although in natura, the plant produces other major QMTs such as celastrol, pristimerin and/or (**4**). This specific accumulation pattern was observed with *P. campestris* (Paz et al. 2013), *Maytenus ilicifolia* (Coppede et al. 2014), *P. laevigata* (Pina et al. 2016b) and other species of Celastraceae (unpublished data). Thus, we hypothesized that the types of QMT accumulated by in vitro roots are potentially less toxic to their own cellular system, and that plants in vitro do not require the accumulation of other QMTs because they do not interact with all the fauna, flora and microbiota of the natural environment in which the plant normally develops.

We also observed that the amount of both (**1**) and (**2**) was much higher in roots collected in natural habitat than those grown in vitro. In previous studies we observed that *P. campestris* roots grown in vitro for 7 days produce 5.5 times more (**1**) than the seedling roots (1 year old) grown in greenhouse (Paz et al. 2013). However, the in natura adult roots used in this study were collected in a savannah area recently burned. Thereby, they were in a disturbed area and the amount of QMTs could not be compared with the roots grown in vitro.

Conclusions

Many environmental factors regulated the biosynthesis of QMTs in *P. campestris* roots, including the carbon source, auxins, and macro- and micronutrients, among others evaluated here. However, the higher concentration of copper in the B5 medium provided greater accumulation of both maytenin (**1**) and 22β-hydroxy-maytenin (**2**). In vitro adventitious roots of *P. campestris* produced mostly maytenin (**1**) and 22β-hydroxy-maytenin (**2**) but also produced 20α-hydroxy-maytenin (**3**) when glucose was used as a carbon source. However, in natura root bark produced maytenin (**1**), 22β-hydroxy-maytenin (**2**), 20α-hydroxy-maytenin (**3**), and maytenol (**4**).

Bacillus megaterium lives in symbiosis with *P. campestris* in in vitro roots and was an effective elicitor for the production of QMTs by those roots.

Acknowledgements The authors thank the São Paulo Research Foundation (FAPESP) for the CIBFar-2013/07600-3 Grant and Dr. José Carlos Tavares Carvalho (Biological Sciences and Health Department, Federal University of Amapá, Brazil) for the electron microscopy analysis. M. C. Inácio thanks FAPESP for her fellowship (2014/19362-2). T. A. Paz thanks CAPES for the provision of a scholarship. A. M. S. Pereira and M. Furlan would also like to thank the National Council for Scientific and Technological Development (CNPq) for their research fellowships.

Author Contributions MCI was responsible for the conception and design of all experiments, data analysis, and drafting and editing of the manuscript. TAP contributed with HPLC and MS analyses and revision of the manuscript. AMSP supervised the experiments and contributed to data interpretation and final revision of the manuscript. MF contributed to data interpretation, drafting and final revision of the manuscript and supervised all experiments.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Almeida MTR de, Ríos-Luci C, Padrón JM, Palermo JA (2010) Antiproliferative terpenoids and alkaloids from the roots of *Maytenus vitis-idaea* and *Maytenus spinosa*. Phytochemistry 71:1741–1748. doi:10.1016/j.phytochem.2010.06.023

Baenas N, García-Viguera C, Moreno D (2014) Elicitation: a tool for enriching the bioactive composition of foods. Molecules 19:13541–13563. doi:10.3390/molecules190913541

Barrow JR, Osuna-Avila P, Reyes-Vera I (2004) Fungal endophytes intrinsically associated with micropropagated plants regenerated from native *Bouteloua eriopoda Torr*. and *Atriplex canescens (Pursh) Nutt*. Vitr. Cell Dev Biol Plant 40:608–612. doi:10.1079/IVP2004584

Buffa Filho W, Corsino J, Bolzani V da S et al (2002) Quantitative determination of cytotoxic *Friedo-nor* -oleanane derivatives from five morphological types of *Maytenus ilicifolia* (celastraceae) by reverse-phase high-performance liquid chromatography. Phytochem Anal 13:75–78. doi:10.1002/pca.626

Carvalho PF, Silva DS, Bolzani V, Furlan M (2005) Antioxidant quinonemethide triterpenes from *Salacia campestris*. Chem Biodivers 2:367–372. doi:10.1002/cbdv.200590016

Cevatemre B, Botta B, Mori M et al (2016) The plant-derived triterpenoid tingenin B is a potent anticancer agent due to its cytotoxic activity on cancer stem cells of breast cancer in vitro. Chem Biol Interact 260:248–255. doi:10.1016/j.cbi.2016.10.001

Chaban C, Waller F, Furuya M, Nick P (2003) Auxin responsiveness of a novel cytochrome P450 in rice coleoptiles. Plant Physiol 133:2000–2009. doi:10.1104/pp.103.022202

Chmielowska J, Veloso J, Gutiérrez J et al (2010) Cross-protection of pepper plants stressed by copper against a vascular pathogen is accompanied by the induction of a defence response. Plant Sci 178:176–182. doi:10.1016/j.plantsci.2009.11.007

Coppede JS, Pina ES, Paz TA et al (2014) Cell cultures of *Maytenus ilicifolia Mart*. are richer sources of quinone-methide triterpenoids than plant roots in natura. Plant Cell Tissue Organ Cult 118:33–43. doi:10.1007/s11240-014-0459-7

Corsino J, Carvalho PRF de, Kato MJ et al (2000) Biosynthesis of friedelane and quinonemethide triterpenoids is compartmentalized in *Maytenus aquifolium* and *Salacia campestris*. Phytochemistry 55:741–748. doi:10.1016/S0031-9422(00)00285-5

Cruz CD (2013) GENES—a software package for analysis in experimental statistics and quantitative genetics. Acta Sci Agron 35:271–276. doi:10.4025/actasciagron.v35i3.21251

Duran-Flores D, Heil M (2016) Sources of specificity in plant damaged-self recognition. Curr Opin Plant Biol 32:77–87. doi:10.1016/j.pbi.2016.06.019

Enders TA, Strader LC (2015) Auxin activity: past, present, and future. Am J Bot 102:180–196. doi:10.3732/ajb.1400285

Ferreira DF (2011) Sisvar: a computer statistical analysis system. Ciência e Agrotecnol 35:1039–1042. doi:10.1590/S1413-70542011000600001

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158. doi:10.1016/0014-4827(68)90403-5

Gunatilaka AAL (2006) Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. J Nat Prod 69:509–526. doi:10.1021/np058128n

Gunatilaka AAL (1996) Triterpenoid Quinonemethides and related compounds (Celastroloids). In: Herz W, Kirby GW, Moore RE et al (eds) Fortschritte der chemie organischer naturstoffe/progress in the chemistry of organic natural products. Springer, Vienna, pp 1–123

Hernandes C, Pereira A, Severino P (2017) Compounds from *Celastraceae* targeting cancer pathways and their potential application in head and neck squamous cell carcinoma: a review. Curr Genomics 18:60–74. doi:10.2174/1389202917666160803160934

Jeller AH, Silva DHS, Lião LM et al (2004) Antioxidant phenolic and quinonemethide triterpenes from *Cheiloclinium cognatum*. Phytochemistry 65:1977–1982. doi:10.1016/j.phytochem.2004.03.039

Kusari S, Pandey SP, Spiteller M (2013) Untapped mutualistic paradigms linking host plant and endophytic fungal production of similar bioactive secondary metabolites. Phytochemistry 91:81–87. doi:10.1016/j.phytochem.2012.07.021

Liao P, Hemmerlin A, Bach TJ, Chye M-L (2016) The potential of the mevalonate pathway for enhanced isoprenoid production. Biotechnol Adv 34:697–713. doi:10.1016/j.biotechadv.2016.03.005

Lião LM, Silva GA, Monteiro MR, Rgio Albuquerque S (2008) Trypanocidal activity of quinonemethide triterpenoids from *Cheiloclinium cognatum* (*Hippocrateaceae*). Z Naturforsch 63:207–210. doi:10.1515/znc-2008-3-408

Ling APK, Ong SL, Sobri H (2011) Strategies in enhancing secondary metabolites production in plant cell cultures. Med Aromat Plant Sci Biotechnol 5:94–101

Lipko A, Swiezewska E (2016) Isoprenoid generating systems in plants—a handy toolbox how to assess contribution of the mevalonate and methylerythritol phosphate pathways to the biosynthetic process. Prog Lipid Res 63:70–92. doi:10.1016/j.plipres.2016.04.002

Lloyd G, McCown B (1980) Commercially-feasible micropropagation of mountain laurel, Kalmia latifolia, by use of shoot-tip culture. Int Plant Propag Soc Proc 30:421–427

Mano H, Morisaki H (2008) Endophytic Bacteria in the Rice Plant. Microbes Environ 23:109–117. doi:10.1264/jsme2.23.109

Migas P, Luczkiewicz M, Cisowski W (2006) The influence of auxins on the biosynthesis of isoprene derivatives in callus cultures of *Vaccinium corymbosum* var. bluecrop. Z Naturforsch C 61:565–570. doi:10.1515/znc-2006-7-816

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497. doi:10.1111/j.1399-3054.1962.tb08052.x

Ngassapa O, Soejarto DD, Pezzuto JM, Farnsworth NR (1994) Quinone-methide triterpenes and salaspermic acid from *Kokoona ochracea*. J Nat Prod 57:1–8. doi:10.1021/np50103a001

Nosov AM (2012) Application of cell technologies for production of plant-derived bioactive substances of plant origin. Appl Biochem Microbiol 48:609–624. doi:10.1134/S000368381107009X

Oramas-Royo SM, Chávez H, Martín-Rodríguez P et al (2010) Cytotoxic triterpenoids from *Maytenus retusa*. J Nat Prod 73:2029–2034. doi:10.1021/np100517u

Paz TA, dos Santos VAFFM, Inácio MC et al (2013) Production of the quinone-methide triterpene Maytenin by in vitro Adventitious Roots of *Peritassa campestris* (Cambess.) A.C. Sm. (Celastraceae) and rapid detection and identification by APCI-IT-MS/MS. Biomed Res Int 2013:1–7. doi:10.1155/2013/485837

Pina ES, Coppede JS, Contini SHT, et al (2016a) Improved production of quinone-methide triterpenoids by *Cheiloclinium cognatum* root cultures: possibilities for a non-destructive biotechnological process. Plant Cell, Tissue Organ Cult 128:705–714. doi:10.1007/s11240-016-1151-x

Pina ES, Silva DB, Teixeira SP et al (2016b) Mevalonate-derived quinonemethide triterpenoid from in vitro roots of *Peritassa laevigata* and their localization in root tissue by MALDI imaging. Sci Rep 6:22627. doi:10.1038/srep22627

Qin S, Xing K, Jiang J-H et al (2011) Biodiversity, bioactive natural products and biotechnological potential of plant-associated endophytic actinobacteria. Appl Microbiol Biotechnol 89:457–473. doi:10.1007/s00253-010-2923-6

Ramirez-Estrada K, Vidal-Limon H, Hidalgo D et al (2016) Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. Molecules 21:182. doi:10.3390/molecules21020182

Rasool S, Mohamed R (2016) Plant cytochrome P450s: nomenclature and involvement in natural product biosynthesis. Protoplasma 253:1197–1209. doi:10.1007/s00709-015-0884-4

Rodrigues-Filho E, Barros FAP, Fernandes JB, Braz-Filho R (2002) Detection and identification of quinonemethide triterpenes in *Peritassa campestris* by mass spectrometry. Rapid Commun Mass Spectrom 16:627–633. doi:10.1002/rcm.615

Rodriguez-Concepcion M, Forés O, Martinez-García JF, et al (2004) Distinct light-mediated pathways regulate the biosynthesis and exchange of isoprenoid precursors during *Arabidopsis* seedling development. Plant Cell Online 16:144–156. doi:10.1105/tpc.016204

Rodríguez-Concepción M (2006) Early steps in isoprenoid biosynthesis: multilevel regulation of the supply of common precursors in plant cells. Phytochem Rev 5:1–15. doi:10.1007/s11101-005-3130-4

Rodríguez-Concepción M, Boronat A (2015) Breaking new ground in the regulation of the early steps of plant isoprenoid biosynthesis. Curr Opin Plant Biol 25:17–22. doi:10.1016/j.pbi.2015.04.001

Rolland F, Baena-Gonzalez E, Sheen J (2006) Sugar sensing and signaling in plants: conserved and novel mechanisms. Annu Rev Plant Biol 57:675–709. doi:10.1146/annurev.arplant.57.032905.105441

Ruan Y-L (2014) Sucrose metabolism: gateway to diverse carbon use and sugar signaling. Annu Rev Plant Biol 65:33–67. doi:10.1146/annurev-arplant-050213-040251

Salvador JAR, Santos RC, Figueiredo SAC, Jing Y (2014) Antitumor effects of celastrol and semi-synthetic derivatives. Mini Rev Org Chem 11:400–407. doi:10.2174/1570193X1103140915113701

Savitha BC, Thimmaraju R, Bhagyalakshmi N, Ravishankar GA (2006) Different biotic and abiotic elicitors influence betalain production in hairy root cultures of *Beta vulgaris* in shake-flask and bioreactor. Process Biochem 41:50–60. doi:10.1016/j.procbio.2005.03.071

Seo HR, Seo WD, Pyun B-J et al (2011) Radiosensitization by celastrol is mediated by modification of antioxidant thiol molecules. Chem Biol Interact 193:34–42. doi:10.1016/j.cbi.2011.04.009

Singh J, Sabir F, Sangwan RS et al (2015) Enhanced secondary metabolite production and pathway gene expression by leaf explants-induced direct root morphotypes are regulated by combination of growth regulators and culture conditions in *Centella asiatica* (L.) urban. Plant Growth Regul 75:55–66. doi:10.1007/s10725-014-9931-y

Smolenskaya IN, Reshetnyak OV, Smirnova YN et al (2007) Opposite effects of synthetic auxins, 2,4-dichlorophenoxyacetic acid and 1-naphthalene acetic acid on growth of true ginseng cell culture and synthesis of ginsenosides. Russ J Plant Physiol 54:215–223. doi:10.1134/S1021443707020094

Srivastava S, Sangwan RS, Tripathi S et al (2015) Light and auxin responsive cytochrome P450s from *Withania somnifera Dunal*: cloning, expression and molecular modelling of two pairs of homologue genes with differential regulation. Protoplasma 252:1421–1437. doi:10.1007/s00709-015-0766-9

Sung B, Park B, Yadav VR, Aggarwal BB (2010) Celastrol, a triterpene, enhances TRAIL-induced apoptosis through the down-regulation of cell survival proteins and up-regulation of death receptors. J Biol Chem 285:11498–11507. doi:10.1074/jbc.M109.090209

Wang H, Teriete P, Hu A et al (2015) Direct inhibition of c-Myc-Max heterodimers by celastrol and celastrol-inspired triterpenoids. Oncotarget 6:32380–32395. doi:10.18632/oncotarget.6116

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16 S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703. doi:10.1128/jb.173.2.697-703.1991

Zhi-lin Y, Chuan-chao D, Lian-qing C (2007) Review: regulation and accumulation of secondary metabolites in plant-fungus symbiotic system. African J Biotechnol 6:1266–1271. doi:10.5897/AJB2007.000-2174