



**PHENOLIC COMPOUNDS AND PARTHENOLIDE PRODUCTION FROM *in vitro* CULTURES OF *Tanacetum parthenium***

**PRODUCCIÓN DE COMPUESTOS FENÓLICOS Y PARTENOLIDA EN CULTIVOS *in vitro* DE *Tanacetum parthenium***

A. Nieto-Trujillo<sup>1</sup>, L. Buendía-González<sup>2</sup>, C. García-Morales<sup>2</sup>,  
A. Román-Guerrero<sup>1</sup>, F. Cruz-Sosa<sup>1\*</sup>, M.E. Estrada-Zúñiga<sup>3</sup>

<sup>1</sup>Departamento de Biotecnología, Universidad Autónoma Metropolitana-Iztapalapa. Av. San Rafael Atlixco N° 186, Col. Vicentina C.P. 09340, México D.F.

<sup>2</sup>Facultad de Ciencias, Universidad Autónoma del Estado de México, Campus El Cerrillo, Piedras Blancas. Carretera Toluca-Ixtlahuaca Km. 15.5, C.P. 50200, Toluca, Estado de México, México.

<sup>3</sup>Centro de Investigación en Recursos Bióticos-Facultad de Ciencias, Universidad Autónoma del Estado de México, Carretera Toluca-Ixtlahuaca Km 14.5, San Cayetano C.P. 50295, Toluca, Estado de México, México.

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**Abstract**

*Tanacetum parthenium* has gained attention for drug development due to its production of bioactive secondary metabolites, such as phenolic compounds (PHEC) and parthenolide (PTN). This work investigated the differences in the production of those compounds among *in vitro* cultures (organs from plantlets and roots cultures) of *T. parthenium*. Results showed that the *in vitro* cultures produced PHEC and PTN, which depended on the type of culture and the incubation time. The content of secondary metabolites was higher in shoots of plantlets (S-ivPL), followed by root cultures (R-ivC) and roots of plantlets (R-ivPL). The PHEC and PTN production was enhanced in plantlets by applying a combination of plant growth regulators (PGRs:  $\alpha$ -naphthalenacetic acid [NAA] at 0.27  $\mu$ M with kinetin [KIN] at 2.32  $\mu$ M). The highest content of secondary metabolites (62.54 mg gallic acid equivalents  $g^{-1}$ ; 4.261 and 4.973 mg chlorogenic acid  $g^{-1}$ ; 0.858 and 1.015 mg caffeic acid  $g^{-1}$ ; 1.931 mg salicylic acid  $g^{-1}$ ; 0.071 mg PTN  $g^{-1}$ ) was found in the PGRs-treated S-ivPL samples. Also, morphogenesis and growth of plantlets was enhanced by these PGRs.

**Keywords:** *Tanacetum parthenium*, parthenolide, phenolic compounds, *in vitro* cultures, bioactive secondary metabolites

**Resumen**

*Tanacetum parthenium* ha ganado la atención para el desarrollo de fármacos por su capacidad para producir metabolitos secundarios bioactivos, tales como compuestos fenólicos (CFEN) y partenolida (PTN). El presente trabajo investigó las diferencias en la producción de dichos metabolitos entre cultivos *in vitro* (órganos de plántulas y cultivos de raíces) de *T. parthenium*. Los resultados mostraron que los cultivos *in vitro* produjeron CFEN y PTN, la cual dependió del tipo de cultivo y del tiempo de incubación. El contenido de los metabolitos secundarios fue mayor en brotes de plántulas (B-ivPL), seguido de cultivos de raíces (R-ivC) y raíces de plántulas (R-ivPL). La producción de CFEN y PTN fue potenciada en las plántulas al aplicar una combinación de reguladores de crecimiento vegetal (RCVs: ácido  $\alpha$ -naftalenacético 0.27  $\mu$ M y cinetina 2.32  $\mu$ M). Los mejores contenidos de metabolitos secundarios (62.54 mg equivalentes de ácido gálico  $g^{-1}$  de fenoles totales; 4.261 y 4.973 mg ácido clorogénico  $g^{-1}$ ; 0.858 y 1.015 mg ácido cafeico  $g^{-1}$ ; 1.931 mg ácido salicílico  $g^{-1}$ ; 0.071 mg PTN  $g^{-1}$ ) fueron encontrados en B-ivPL tratados con RCVs. También, la morfogénesis y el crecimiento de las plántulas fueron potenciadas con los RCVs.

**Palabras clave:** *Tanacetum parthenium*, compuestos fenólicos, partenolida, cultivos *in vitro*, metabolitos secundarios bioactivos.

\* Corresponding author. E-mail: mail: cuhp@xanum.uam.mx  
Tel. 52-55-5804-4714; fax: 52-55-5804-4712

## 1 Introduction

*Tanacetum parthenium* (L.) Schultz-Bip (Asteraceae) is a species that has gained great attention for drug development, based on its outstanding uses in folk medicine against migraine, asthma, rheumatoid arthritis, gynecological problems, insect bites, psoriasis and fevers (Awang *et al.*, 1991; Pareek *et al.*, 2011). Pharmacological and phytochemical studies on this species have contributed to validate scientifically its medicinal potential (e.g. on cancer, migraine, inflammatory diseases, etc.) (Andonova *et al.*, 2016; Eardie *et al.*, 1992; Ernst and Pittler 2000; Ferro *et al.*, 2012; Pfaffenrath *et al.*, 2002). The phytochemical results have elucidate bioactive compounds belonging to terpenoid (essential oils and sesquiterpene lactones) and phenolic (flavonoids, phenolic acids and coumarins) secondary metabolites type (Majdi *et al.*, 2011; Pareek *et al.*, 2011; Wu *et al.*, 2006). Sesquiterpene lactones are one of the most important biologically active principles; more than 30 types of them have been identified, being PTN the most abundant (Pareek *et al.*, 2011; Cretnik 2005). PTN exerts cytotoxic activity against several human cancer cell lines (e.g. breast, leukemia, osteosarcoma, human cervical) (Al-Fatlawi *et al.*, 2015; D'Anneo *et al.*, 2013; Lesiak *et al.*, 2010; Pei *et al.*, 2012). Its mechanism of action has been associated with the inhibition of growth and the induction of apoptosis without having an effect on normal cells (Al-Fatlawi *et al.*, 2015; Cijo *et al.*, 2012; Mathema *et al.*, 2012). Additional biological effects have been described for PTN, which includes antimigraine (Tassorelli *et al.*, 2005), anti-inflammatory (Mathema *et al.*, 2012), antimyelomic (Cijo *et al.*, 2012), antimicrobial (Jamal *et al.*, 2014), radiosensitizer (Sun *et al.*, 2010) and antioxidant (Wu *et al.*, 2006). Another class of outstanding bioactive principles in *T. parthenium* are the phenolic compounds, such as caffeoyl derivatives (chlorogenic acid, chicoric acid, 1,5-3,5 and 4,5-di-O-caffeoylquinic acids) (Majdi *et al.*, 2011; Wu *et al.*, 2006) and flavonoids (luteolin, apigenin, quercetin, shrysoeriol, santin, jaceidin, centaureidin, quercetagenin 3,6-dimethyl ether and 6-hydroxykaempferol 3,6-dimethyl ether) (Pareek *et al.*, 2011; Wu *et al.*, 2006). Phenolic compounds have been associated with therapeutic effects due to their binding capability to proteins that are key in disease signal transduction (e.g. inflammation, apoptosis, proliferation) (Quideau *et al.*, 2011). Moreover, the antioxidant characteristic of the phenolic compounds has been related to protective

effects against cancer, diabetes, cardiovascular and neurodegenerative diseases (Pisoschi and Pop, 2015). In *T. parthenium*, the content of total phenolic compounds from extracts affects the antioxidant activity (Wu *et al.*, 2006). However, it is well known that the production of secondary metabolites in plants is regulated by different factors (Karuppusamy 2009; Verpoorte *et al.*, 2002). In *T. parthenium*, the production of parthenolide depends on the organ used to obtain extracts, stages of cultivation, environmental factors, genotype and developmental stage (Awang 1991; Fonseca *et al.*, 2005; Heptinstall *et al.*, 1992; Hojati *et al.*, 2016; Majdi *et al.*, 2013, 2015).

Plant tissue and organ culture has become a feasible biotechnological technique to produce bioactive compounds since it allows controlling the conditions to produce secondary metabolites and morphogenesis (Dias *et al.* 2016). Reports about *in vitro* cultures of *T. parthenium* have demonstrated that the production of parthenolide is affected by the type of cultured tissue, carbon source, PGRs and chemicals (El-Shamy *et al.*, 2007; Rateb *et al.*, 2007; Stojakowska and Kisiel 1997). Furthermore, it has been determined that *in vitro* cultures of *T. parthenium* can produce other types of secondary metabolites. In hairy root cultures, diacetylenes and coumarins (Stojakowska *et al.*, 2002, 2008) have been identified, while in callus coniferaldehyde derivatives (Sy and Brown 1999) and coumarin isofraxidin are detected (Banthorpe and Brown 1990). However, there is no evidence reporting the simultaneous production of parthenolide and phenolic compounds from *in vitro* cultures of *T. parthenium*. The aim of the present work was to determine the differences in the phenolic compounds and parthenolide production from *in vitro* cultures of *T. parthenium*.

## 2 Materials and methods

### 2.1 Plant material and establishment of aseptical cultures

Plants (15 cm height) and seedlings (4 cm height) of *Tanacetum parthenium* (L.) Sch. Bip. were collected from Apaxco, State of Mexico, Mexico on winter 2014. A sample of the collected material was deposited in the Ramón Riba y Nava Esparza Metropolitan Herbarium, Universidad Autónoma Metropolitana Iztapalapa (UAMIZ) for taxonomic identification. The species was registered with the number 78435. Plants and seedlings were maintained

in the UAMIZ greenhouse and fertilized to keep them healthy. Shoots of 8 cm height were removed from plants and sequentially immersed under shaking as follows: a) 5% (w/v) soap for 15 min; b) 70% (v/v) ethanol for 30 s; c) 1.2 or 1.8% (v/v) sodium hypochlorite for 30 min and d) finally rinsed four times with sterile distilled water under aseptic conditions. Afterwards, stem-nodes were obtained by segmenting the shoots and separating the leaves in a Petri dish containing an antioxidant solution (a mixture of 150 mg L<sup>-1</sup> ascorbic acid and 100 mg L<sup>-1</sup> citric acid). The sterile stem-nodes were used to develop plantlets while the sterile leaves were used to establish root cultures.

## 2.2 Culture medium and incubation conditions

Half-strength Murashige and Skoog (MS, Murashige and Skoog 1962) culture medium was added to 4% (w/v) D-glucose and used for all treatments. PRGs were added to culture medium to establish the *in vitro* cultures. 2.7 μM NAA was added for rooting of shoots to allow plantlet development; 16.2 μM NAA was used to establish root cultures from leaves; a PGRs-free culture medium was used as control treatment. The combination of 0.27 μM NAA with 2.32 μM KIN was tested on plantlets to determine its effect on secondary metabolite production, morphogenesis and growth. A mixture of 150 mg L<sup>-1</sup> ascorbic acid and 100 mg L<sup>-1</sup> citric acid was added to culture medium used to establish root cultures. Culture medium pH was adjusted to 5.8. Phytigel was added at 0.2% (w/v) to solidify the culture medium; this was required for almost all treatments, except those related to the proliferation of roots suspension cultures. Finally, the culture medium was sterilized by autoclaving at 121°C for 18 min. 15 mL culture medium were used in culture tubes (25 x 150 mm) for gelled culture medium, whilst 25 mL of liquid culture medium was put into a 125 mL Erlenmeyer flask. The incubation conditions for all the cultures were 25±2°C under warm-white fluorescent light (50 μmol m<sup>-2</sup> s<sup>-1</sup>) in a 16 h/8 h light/dark photoperiod for 30 days.

## 2.3 Regeneration of plantlets from stem node explants

Sterile stem nodes were grown in PGRs-free culture medium to allow the development of the axillary buds. The resulting shoots (1.5±0.4 shoots per stem-node) were removed from stem nodes, individualized

and transferred to culture medium containing 2.7 μM NAA for rooting (66.0±7.3% of the total shoots showed rooting); these plantlets were grown in PGRs-free culture medium for 30 days. Afterwards, the leaves from plantlets were removed. The leafless plantlets were transferred into PGRs-free medium and incubated for further 30 days. Harvesting procedure was made at 0, 9, 15, 20, 24, and 30 days of culture. Every harvest consisted on separating shoots from roots, following the measurement of length of the apical shoot and count of induced shoots and leaves. The harvested material was dried in an oven for 48 h and used to measure the corresponding dry weight (DW). The biomass, based on DW, was used to determine the corresponding growth parameters (specific growth rate,  $\mu$ ; time of duplication,  $t_d$ ; and growth index, GI) according to Estrada-Zúñiga *et al.*, (2009).

## 2.4 Establishment and proliferation of roots cultures

Sterile leaves obtained from the aseptic culture were treated with 16.2 μM NAA in order to promote the induction of adventitious root; a control treatment was simultaneously tested. The induced roots were transferred to fresh liquid medium (1.5 g fresh biomass weight per Erlenmeyer flask; same composition used to induce roots) in order to establish suspension cultures. Five subcultures were prepared every 30 days to proliferate biomass. Afterwards, the next three continuous subcultures were used to develop kinetic studies, which consisted on a period of incubation of 32 days with harvesting at day 0, 9, 15, 20, 24, 30 and 32. Every harvest consisted on separating biomass from residual culture medium by vacuum filtration; the biomass was washed with distilled water for three times then, roots were dried in an oven for 48 h and used to measure the corresponding DW which was used to determine growth parameters ( $\mu$ ;  $t_d$ ; GI) according to Estrada-Zúñiga *et al.*, (2009).

## 2.5 Effect of PGRs on *in vitro* plantlets

A comparison of the production of PHEC y PTN was carried out in order to select the *in vitro* culture showing the highest amounts. Since shoots of plantlets produced the highest content of secondary metabolites, other group of plantlets was regenerated according to the procedure described in the section 2.3. But, these leafless plantlets were grown in culture medium supplemented with 0.27 μM NAA and 2.32

$\mu\text{M}$  KIN. Growth, morphogenesis and phytochemical analysis data were recorded as indicated previously.

## 2.6 Processing of other sources of plant material for phytochemical analysis

To determine the potential of *in vitro* cultures in producing PHEC and PTN, each culture was compared to commercialized plants and collected samples of plants and seedlings. The collected material kept growing in a greenhouse for nine and one months, respectively. Afterwards, seedlings were harvested to obtain shoots and roots, whilst plants were harvested to obtain flowers, shoots and roots. On the other hand, the aerial part of commercialized material (shoots and flowers used for medicinal purposes) of *T. parthenium* was purchased from a market in Toluca, State of Mexico, Mexico. The vegetal material was dried in an oven for 48 h, following the determination of DW and phytochemical analysis.

## 2.7 Preparation of extracts and assessment of total phenol content

Dry biomass from *in vitro* cultures (shoots and roots of plantlets or roots suspension cultures) and from different sources of plant material (shoots, flowers or roots from greenhouse plants and seedlings or purchased plants) was used to obtain extracts by boiling with MeOH (200 mg of DW per 50 mL) during 30 min. The resulting extracts were used in two procedures: 1) The modified Folin-Ciocalteu method (Estrada-Zúñiga et al., 2016; 2012) to quantify total phenol content (TPC) and 2) HPLC analysis. Results were expressed as milligrams of gallic acid equivalents (mg GAE) per gram of biomass extracted based on DW ( $\text{mg GAE g}^{-1}$ ). The Folin-Ciocalteu and gallic acid reagents were acquired from Sigma-Aldrich Co., USA. Every sample was measured in duplicates.

## 2.8 Quantification of phenolic compounds and parthenolide by HPLC

The extracts, collected at 9, 20, 24, 30 or 32 days of culture, were concentrated (0.5-2 mL) in a rotatory evaporator to carry on HPLC analysis using an Agilent Technologies 1100 series HPLC system with a G1311A Quatpump (Alltech Co. USA) equipped with a Kromasil C18 column (250 mm x 4.6 mm,  $5\mu\text{m}$ ; Supelco, Sigma) and a G1315B DAD (Alltech Co. USA). Every extract sample was filtered through a nylon filter ( $0.45\mu\text{m}$ ) before the injection. Working

conditions consisted on a  $20\mu\text{L}$  injection volume;  $0.7\text{ mL min}^{-1}$  flow rate;  $30^\circ\text{C}$  column temperature; wavelength for DAD: 214 (for parthenolide), 245 (for salicylic acid) and 330nm (for the rest of phenolic compounds). The mobile phase was a mix of 0.3% (v/v) acetic acid solution (solvent A), acetonitrile (solvent B) and water (solvent C). Rosmarinic acid (Sigma-Aldrich Co., USA) was used as internal standard. The system was run in a gradient program as follows: 12 min, 100% to 75% A and 0% to 25% B; 30 min, 75% to 65% A and 25% to 35% B and kept for 2 min; 35 min, 65% to 0% A, 35% to 40% B and 0% to 60% C; 40 min, 40% to 55% B and 60% to 45% C; and finally 45 min, 55% to 100% A and 45% to 0% C. External standards of chlorogenic acid, caffeic acid, salicylic acid and parthenolide (Sigma-Aldrich Co., USA) were used to build the corresponding calibration curve (from 20 to  $200\mu\text{g mL}^{-1}$ ). All the standards and extracts were run in the same way. The CHEMSTATION chromatography software (Rev. A.08.03; Agilent Technologies) was used to acquire all data from the detector. The retention time and peak area showed in the chromatogram of each standard was used to identify and quantify this compound in extract samples. Every sample was injected two or three times.

## 2.9 Statistical analysis

All experiments corresponding to *in vitro* culture establishment were repeated twice with 10 experimental units ( $n=20$ ). The kinetic analysis was made by triplicate with 2 experimental units ( $n=6$ ). All experiments corresponding to phytochemical analysis from *in vitro* cultures and different sources of plant material were repeated twice with 3 experimental units ( $n=6$ ). Results were analyzed with a Variance analysis (ANOVA) and Tukey-Kramer's multiple range test using NCSS statistical software; significant differences were assumed with a  $p < 0.05$ . All the results were presented as mean  $\pm$  SD (standard deviation).

# 3 Results

## 3.1 Stability estimation of root cultures

No statistical differences among growth parameters ( $\mu = 0.0601 \pm 0.0033\text{ d}^{-1}$ ,  $t_d = 11.56 \pm 0.62\text{ d}$ , and  $\text{GI} = 2.31 \pm 0.23$ ) from the three continuous subcultures of R-ivC were found, suggesting metabolic stability. Subcultures showed an exponential growth phase from

culture day 0 to 20, and a stationary phase from day 20 onwards. The concentration of sugars in the residual culture medium at day 32 was 17.5 g L<sup>-1</sup>, while maximum biomass was 8.1 g L<sup>-1</sup> (data not shown).

### 3.2 TPC, phenolic acids and parthenolide from *in vitro* cultures and organs from different sources of plant material

Results revealed that the content of total phenols, phenolic acids and PTN depended on the type of *in vitro* culture from *T. parthenium*, as well as incubation

time. The content of secondary metabolites was higher in S-ivPL, followed by R-ivC and R-ivPL. Regarding incubation time, the highest secondary metabolites content in S-ivPL were: TPC and caffeic acid at day 9 (Figure 1a, Table 1); chlorogenic acid at day 9 and 20; salicylic acid and PTN at day 30 (Table 1). Chlorogenic acid was detected in all *in vitro* samples, and its concentration decreased after day 9 of incubation (Table 1). Caffeic acid was detected in some *in vitro* samples, whilst salicylic acid and PTN were detected in few *in vitro* samples collected at 30 or 32 days (Table 1).

Table 1. Content of secondary metabolites from *in vitro* cultures and from different sources of plant material of *T. parthenium*. All the results are presented as the means ± SDs (indicated by vertical bars). Means ± SDs followed by the same letter within the columns were not significantly different (*p* < 0.05). CP = commercialized plants; GP = Greenhouse plants; GS = Greenhouse seedlings.

| Source of plant material   | Content of the secondary metabolite (mg g <sup>-1</sup> ) |                            |                            |                             |
|--|---|----------------------------|----------------------------|-----------------------------|
| Samples collected from <i>in vitro</i> cultures at different incubation days | Phenolic acids  |                            |                            | Sesquiterpene lactones      |
|  | Chlorogenic acid  | Caffeic acid               | Salicylic acid             | Parthenolide                |
| <b>PGRs-free plantlets</b>   |   |                            |                            |                             |
| Shoots   |   |                            |                            |                             |
| 9  | 2.976±0.412 <sup>k</sup>                                  | -                          | -                          | -                           |
| 20   | 2.357±0.430 <sup>ij</sup>                                 | 0.559±0.108 <sup>g</sup>   | -                          | -                           |
| 24   | 1.833±0.236 <sup>g,h</sup>                                | 0.290±0.039 <sup>e</sup>   | -                          | -                           |
| 30   | 1.355±0.022 <sup>e,f</sup>                                | -                          | 0.254±0.026 <sup>a,b</sup> | 0.019±0.003 <sup>a,b</sup>  |
| Roots  |   |                            |                            |                             |
| 9  | 0.711±0.121 <sup>c,d</sup>                                | 0.056±0.016 <sup>c,d</sup> | -                          | -                           |
| 20   | 0.590±0.011 <sup>c</sup>                                  | 0.089±0.011 <sup>d</sup>   | -                          | -                           |
| 24   | 0.286±0.036 <sup>a</sup>                                  | -                          | -                          | -                           |
| 30   | 0.311±0.041 <sup>a</sup>                                  | -                          | 0.655±0.082 <sup>c</sup>   | 0.001±0.000 <sup>a</sup>    |
| <b>PGRs-treated plantlets</b>  |   |                            |                            |                             |
| Shoots   |   |                            |                            |                             |
| 9  | 4.973±0.756 <sup>l</sup>                                  | 0.858±0.118 <sup>h</sup>   | 0.589±0.079 <sup>b,c</sup> | 0.005±0.001 <sup>a</sup>    |
| 20   | 4.261±0.772 <sup>l</sup>                                  | 1.015±0.201 <sup>h</sup>   | -                          | -                           |
| 24   | 2.782±0.381 <sup>ij,k</sup>                               | 0.413±0.072 <sup>f,g</sup> | -                          | -                           |
| 30   | 1.881±0.291 <sup>g,h</sup>                                | 0.111±0.017 <sup>d</sup>   | 1.931±0.310 <sup>d</sup>   | 0.071±0.010 <sup>b,c</sup>  |
| Roots  |   |                            |                            |                             |
| 9  | 2.126±0.356 <sup>h,i</sup>                                | 0.312±0.056 <sup>c,f</sup> | 0.029±0.004 <sup>a</sup>   | -                           |
| 20   | 1.084±0.191 <sup>d,e</sup>                                | 0.473±0.061 <sup>f,g</sup> | 0.319±0.057 <sup>a,b</sup> | -                           |
| 24   | 0.714±0.088 <sup>c,d</sup>                                | -                          | -                          | -                           |
| 30   | 0.753±0.159 <sup>c,d</sup>                                | 0.246±0.048 <sup>c</sup>   | 0.177±0.041 <sup>a</sup>   | 0.005±0.001 <sup>a</sup>    |
| <b>Root cultures</b>   |   |                            |                            |                             |
| 9  | 2.223±0.111 <sup>h,i</sup>                                | 0.012±0.002 <sup>a</sup>   | -                          | -                           |
| 20   | 1.958±0.218 <sup>g,h</sup>                                | -                          | -                          | -                           |
| 24   | 1.656±0.196 <sup>f,g</sup>                                | -                          | -                          | -                           |
| 32   | 1.347±0.159 <sup>d,e</sup>                                | 0.851±0.099 <sup>h</sup>   | 0.190±0.028 <sup>a</sup>   | 0.0231±0.011 <sup>a,b</sup> |
| <b>Samples of organs from other sources of plant material</b>                |   |                            |                            |                             |
| Shoots – CP  | 6.657±0.111 <sup>m</sup>                                  | 0.434±0.021 <sup>f,g</sup> | 0.062±0.001 <sup>a</sup>   | 0.009±0.001 <sup>a</sup>    |
| Flowers – CP   | 2.311±0.393 <sup>ij</sup>                                 | -                          | -                          | 0.175±0.061 <sup>c</sup>    |
| Shoots – GP  | 4.342±0.041 <sup>l</sup>                                  | -                          | -                          | 0.001±0.000 <sup>a</sup>    |
| Flowers – GP   | 1.890±0.032 <sup>g,h</sup>                                | 0.023±0.004 <sup>b</sup>   | -                          | 0.085±0.017 <sup>c</sup>    |
| Roots – GP   | 0.759±0.094 <sup>c,d</sup>                                | -                          | -                          | -                           |
| Shoots – GS  | 2.991±0.041 <sup>k</sup>                                  | -                          | -                          | 0.015±0.002 <sup>a,b</sup>  |
| Roots – GS   | 0.400±0.069 <sup>a,b</sup>                                | 0.048±0.017 <sup>c</sup>   | -                          | -                           |



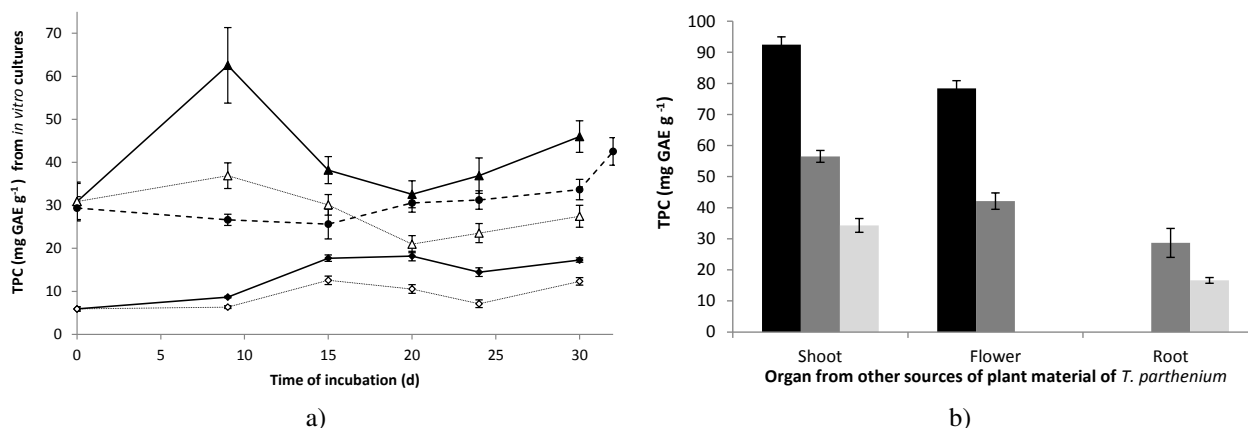


Fig. 1. Total phenol content (TPC) from *in vitro* cultures and different sources of vegetal material of *Tanacetum parthenium*. a) TPC in shoots and roots from plantlets PGRs-treated during 30 days (▲ shoots; ◆ roots) and non-treated control (△ shoots; ◇ roots); and root cultures (●) growth during 32 days, b) TPC in different sources of plant material (■ shoot and flower of commercialized plants; ■ shoot, flower and root of plants and ■ shoot and root of plantlets grown in greenhouse). All the results are presented as the means  $\pm$  the SDs (indicated by vertical bars)

On the other hand, comparison of secondary metabolite content among organs from different sources of plant material revealed that the higher values are found in commercialized plants (CP), followed by plants (GP) and seedlings (GS) grown in greenhouse.

### 3.3 PGR enhanced production of secondary metabolites, morphogenesis and growth of plantlets

Treatment of *T. parthenium* plantlets with PGRs enhanced the pattern profile of PHEC and PTN contents, morphogenesis and growth. Among all *in vitro* cultures, the S-ivPL treated with PGRs had the highest amounts of TPC, phenolic acid and PTN (Figure 1a, Table 1). When contents of PHEC and PTN from S-ivPL treated with PGRs were compared to those found in different sources of plants, they were similar to GP, higher than GS and lower than CP (Figure 1a-b, Table 1). Additionally, the PGRs significantly accelerated the development of new shoots, increased the number of induced shoots and leaves per plantlet, enhanced the length of the apical shoot and growth parameters, and increased the amount of biomass of shoots and roots (Figure 2a-d). However, the length of the exponential (from day 0 to day 24) and stationary (from day 24 to day 30) phases of growth were not affected by PGRs (Figure 2c), although the growth rate of roots during the exponential phase was affected by them (Figure

2d). The greatest response to PGRs on morphogenesis and growth were the induction of 13 shoots (showing a length from 1.5 to 2.0 cm) and 109 leaves per plantlet; a length of 7.4 cm apical shoot; a shoot DW of 97.4 mg and 51 mg DW of roots by day 24 of culture (Figure 2a-c).

## 4 Discussion

The results of the present work demonstrate that the production of phenolic compounds and parthenolide from *in vitro* cultures of *T. parthenium* depends on the type of *in vitro* culture, time of incubation and plant growth regulators. It has been reported that the phenolic compounds are naturally produced by *in vitro* cultures (Dias *et al.*, 2016). Secondary metabolites are not directly correlated with growth and development of the plant tissue (Buchanan *et al.*, 2015). However, the shikimic acid pathway is the main pathway for the biosynthesis of phenolic compounds (also known as phenylpropanoid pathway), which is also involved in the production of proteins, hence leading to competition between them (Cohen and Kennedy 2010; Karakaya 2004). Likewise, the production of secondary metabolites can be induced during stress conditions (Buchanan *et al.*, 2015). During the stationary growth phase of *T. parthenium in vitro* cultures, stress conditions may take place e.g. decreased of nutrients, which could activate the production of secondary metabolites.

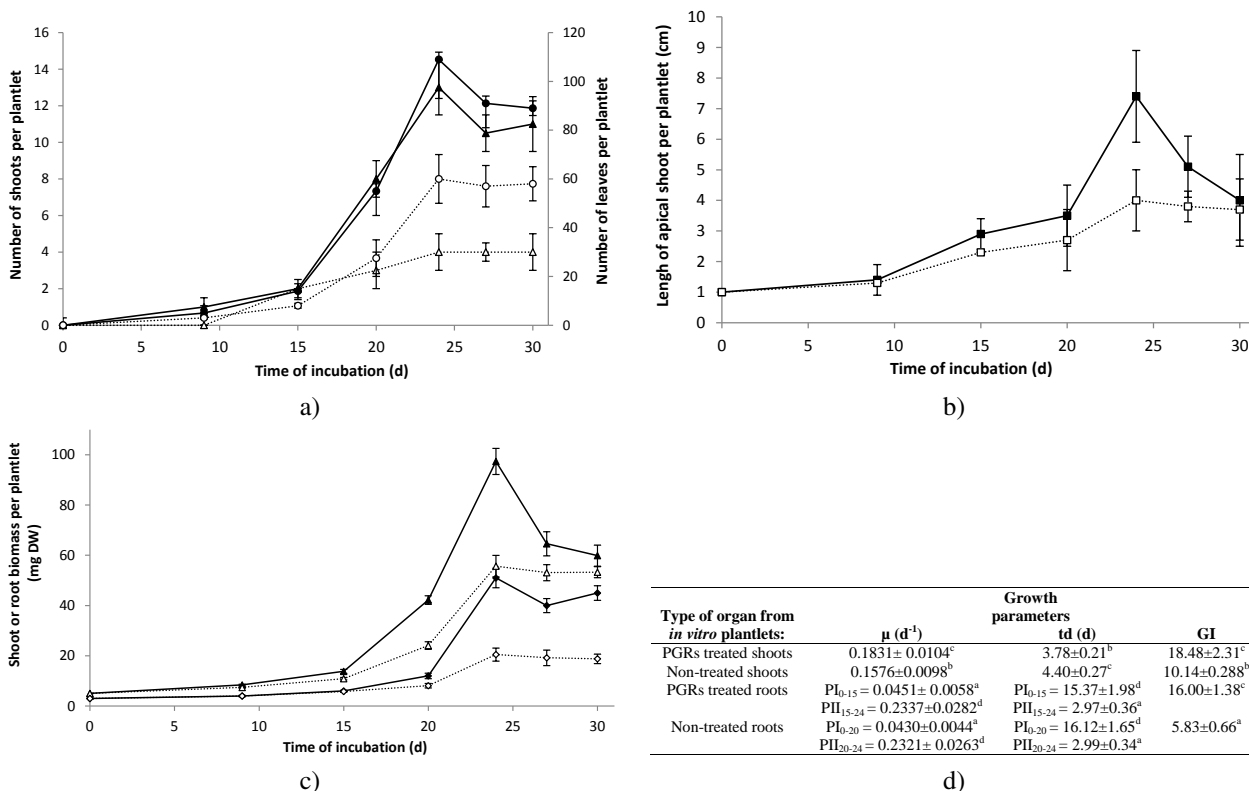


Fig. 2. Effect of PGRs on *T. parthenium* morphogenesis and growth during 30 days of culture. a) Number of induced shoots and leaves per PGRs-treated plantlet ( $\blacktriangle$  shoots;  $\bullet$  leaves) and non-treated control ( $\Delta$  shoots;  $\circ$  leaves), b) length of apical shoot per PGRs-treated plantlet ( $\blacksquare$ ) and non-treated control ( $\square$ ), c) biomass of shoots or roots per PGRs-treated plantlet ( $\blacktriangle$  shoots;  $\blacklozenge$  roots) and non-treated control ( $\Delta$  shoots;  $\diamond$  roots), d) growth parameters of induced shoots and roots of PGRs-treated plantlets and non-treated control during exponential phase of growth. All results are presented as the means  $\pm$  SDs (indicated by vertical bars). In “d)”, means  $\pm$  SDs followed by the same letter within a column were not significantly different ( $p < 0.05$ );  $PI_{X-Y}$  or  $PII_{X-Y}$  indicate phase I or phase II was from X to Y days.

Phenolic compounds, such as the chlorogenic acid, can also play a role during *de novo* organogenesis. This compound is involved in building the cell wall and the formation of roots. In *Hypericum perforatum*, chlorogenic acid participates in shoot, root and root hair development regulation (Franklin and Dias 2011). It may be possible that chlorogenic acid functions on growth and organogenesis during *in vitro* development of *T. parthenium*. In fact, there is evidence that indicates a coordinated mechanism to regulate phenylpropanoid and terpenoid biosynthesis (Hojati *et al.*, 2016; Kang *et al.*, 2014), that could explain the results of this work. Furthermore, salicylic acid is a phenolic compound that has been shown to act in plants as a PGR participating in biotic and abiotic stress (Davies 2010; Dong *et al.*, 2011; Rivas-San Vicente and Plasencia 2011). It has been reported that the accumulation of sesquiterpene lactones in hairy

roots of *Cichorium intybus* is affected by salicylic acid (Malarz *et al.*, 2007). The exogenous application of 1 mM salicylic acid activated the synthesis of parthenolide in leaves of *T. parthenium* after 24 h of exposure (Majdi *et al.*, 2015). The production of diacetylene in transformed roots of *T. parthenium* was affected by salicylic acid (Stojakowska *et al.*, 2002); all this evidence supports the idea that parthenolide and salicylic acid being produced at the end of the incubation period in *T. parthenium* may indicate a relationship with a stress condition.

It has been previously described that whether whole plants or *in vitro* cultures of *T. parthenium*, the production of parthenolide varies between different organs, being higher in aerial parts of the plant with the maximum content in flowers (Brown *et al.*, 1996, Stojakowska and Kisiel 1997). This study demonstrates that the production of parthenolide, as

well as phenolic compounds in *T. parthenium* is affected by the type of organ analyzed. In *Arnica montana* (Asteraceae), the composition of phenolic compounds was different between flowers and leaves. The flowers had higher contents of flavonoids and sesquiterpene lactones than leaves. But, the values of those contents showed variation attributed to environmental factors (Stefanache et al., 2016). The fact that the highest content of secondary metabolites was found in commercialized plants might be due to environmental factors. Genetics is another factor affecting the production of parthenolide (Stojakowska and Kisiel 1997); it probably contributes to determine low contents of parthenolide from *in vitro* cultures in this work, since not only plants used as source of explants had low amounts of parthenolide but also the commercialized plants used. In *Tanacetum vulgare* has been determined a variation between genotypes of 27% (Keskitalo et al., 1998). There are also reports describing that PGRs affect the production of parthenolide of *T. parthenium*. 6-benzylaminopurine (BAP) (~2.22, 4.44 and 8.88  $\mu\text{M}$ ) and NAA (~ 0.27, 0.54 and 1.08  $\mu\text{M}$ ) enhanced the production of parthenolide in shoot cultures (El-Shamy et al., 2007; Rateb et al., 2007). BAP, NAA and 2,4-dichlorophenoxyacetic acid (2,4-D), alone or in combination (at 0.5 or 1.0  $\text{mg L}^{-1}$ ), affected the production of parthenolide in callus (El-Shamy et al., 2007; Rateb et al., 2007). The production of parthenolide was increased with 2,4-D at 1.13 M even in flowers from 6 months-old plants grown in a greenhouse (Fonseca et al., 2005). In sprouts from *Fagopyrum esculentum* the auxin indole-3-acetic acid enhanced growth parameters and accumulation of phenolic compounds (Park et al., 2017). Auxins and cytokinins are the most important classes of PGRs used for *in vitro* cultures since they can modulate the induction and development of shoots and roots on explants growing in culture medium (Beyl, 2011). Our study revealed that PGRs increased the production of parthenolide and phenolic compounds and enhanced growth and morphogenesis of *T. parthenium* plantlets. The latest resulted in a higher formation of shoots compared to other reports of this species. 6 to 10 shoots were induced in leaves grown with 20  $\mu\text{M}$  BAP and 24  $\mu\text{M}$  NAA (Brown et al., 1996), and 6.6 shoots were observed in nodal explants from seedlings grown with 4.44  $\mu\text{M}$  BAP and 0.54  $\mu\text{M}$  NAA (Stojakowska and Kisiel 1997). It seems like the cytokinin BAP has a significant influence on the induction of shoots on explants of *T. parthenium*, and plantlets could be used as explants when shoot

propagation is desired. However, it is important to characterize the morphogenesis and growth from plantlets, especially if shoots are used for propagation purposes. In the present work the growth of shoots suddenly decreased after reaching the highest value.

Nowadays, the growing demand for natural products has focused the interest on *in vitro* culture, a biotechnological tool that allows the establishment of potential producers on an industrial scale for valuable phytochemicals, as well as other advantages such as continuous, reliable, predictable and independent production of environmental factors; and an easier extraction and purification of secondary metabolites compare to tissues or whole plants (Karuppusamy 2009). There are secondary metabolites from plants possessing outstanding biological activities which are produced biotechnologically (Korkina and Kostyuk 2012; Matkowski 2008). This work establishes the basis for future research on biotechnology topics for producing secondary metabolites bioactives from *T. parthenium*. The development of a large-scale application requires several strategies, for example, the screening of established cell lines producing high amounts of secondary metabolites, and the optimization of production media and assessment of growth (Verpoorte et al., 2002). However, *in vitro* systems reports about the production of parthenolide in *T. parthenium* and the effect on growth are limited. Brown et al., (1996) described that stem explants developed one shoot after 90 days, which increased its fresh weight from 16 to 1262 mg; these shoots formed roots that reached a fresh weight of 613 mg. The fact that the *in vitro* cultures produced secondary metabolites in similar amounts to that in the whole plants emphasizes the importance of investigating about enhancing their production (e.g. elicitation or establishment of hairy roots) (Karuppusamy 2009), identifying and isolating other bioactive secondary metabolites and performing biological assays. The greatest TPC values found in this work were higher to those reported for extracts of dried powders of *T. parthenium* (21.21  $\text{mg GAE g}^{-1}$ ; Wu et al., 2006). While the highest content of chlorogenic acid was lower than the reported for aerial parts of the plant (6.45  $\text{mg g}^{-1}$ ; similar to the value found here for shoots of CP) (Fraisie et al., 2011), but similar or higher than *in vitro* cultures of other species. Chlorogenic acid is the most abundant phenolic compound in extracts of some species belonging to Asteraceae family, and its production is higher than caffeic acid. Chlorogenic acid has shown antioxidant, anti-carcinogenic, anti-



inflammatory, analgesic, antipyretic, antilipidemic, antihypertensive and antidiabetic activities in *in vivo* and *in vitro* experiments (Dos Santos *et al.*, 2006; Santana-Gálvez *et al.*, 2017). Caffeic acid is used to develop new drugs due to its therapeutic potential based on its antioxidant, antibacterial and anti-inflammatory effects (Perumal *et al.*, 2015). Moreover, both phenolic acids have been attributed with the antioxidant activity since they are strong antioxidant compounds (Kenny *et al.*, 2014). The adventitious roots of *Echinacea purpurea* (Asteraceae) cultured in bubble bioreactors produced 4.4-5.2 mg<sub>chlorogenic acid</sub> g<sup>-1</sup> with a maximum biomass of 9.8 g DW L<sup>-1</sup> (Wu *et al.*, 2007); in this work, the root cultures of *T. parthenium* produced 8.1 g DW L<sup>-1</sup>. The production of chlorogenic acid was higher in shoot culture (0.066 mg g<sup>-1</sup>) followed by root culture (0.059 mg g<sup>-1</sup>) and roots of regenerated plantlets (0.013 mg g<sup>-1</sup>) from *in vitro* cultures of *Eryngium planum* (Thiem *et al.*, 2013);, a pattern similar to that observed in the present work. Callus of *Ephedra alata* produced 0.17 µg chlorogenic acid g<sup>-1</sup> (Hegazi and El-Lamey 2011). In cell suspension cultures of *Ipomoea batatas* the production of chlorogenic acid (0.86-8.9 mg/100 g FW) and caffeic acid (9.4-28.2 mg/100 g FW) depended on incubation length (Konczak-Islam *et al.*, 2003). In *Cuphea aequipetala* and *C. tanceolata* a positive correlation between the antioxidant activity and the content of antioxidant compounds was established (Cardenas-Sandoval *et al.*, 2012). It is reported that *in vitro* cultures allow obtaining uniformity in the production of desired compounds (Chaturvedi *et al.*, 2007) which can be a source of standardized extracts, while reducing the time of production of the bioactive compounds compared to the time required developing the whole plants (Karuppusamy 2009). The variability in the content of secondary metabolites can affect the therapeutic properties; therefore it is important to use standardized extracts of *T. parthenium* (Awang 1993; Heptinstall *et al.*, 1992). Natural products can exert a synergism in the treatment of cancer (Hemalswarya and Doble 2006). That synergism could be triggered by obtaining fractions of phenolic compounds and sesquiterpene lactones from *in vitro* cultures of *T. parthenium*; the derivatives of sesquiterpene lactones can be used as prodrugs (Woods *et al.*, 2013; Yip-Schneider *et al.*, 2013). Also, it is also important to optimize the extraction process of secondary metabolites. In *Lippia graveolens* it was found that temperature, solvent concentration and average particle size affected the concentrations of total

phenols and antioxidant capacity (Flores-Martínez *et al.*, 2016). Our results demonstrated that *in vitro* cultures of *T. parthenium* can be a controllable useful system for producing phenolic compounds and parthenolide metabolites possessing outstanding biological effects that could contribute to treat different diseases affecting world population such as cancer and diabetes.

## Conclusions

The *in vitro* cultures of *T. parthenium* had differences in producing phenolic compounds and parthenolide. The greatest production of most phenolic compounds happens during exponential growth phase while salicylic acid and parthenolide production occurs in the stationary phase. Plant growth regulators (NAA and KIN) enhance the production of phenolic compounds and parthenolide, morphogenesis and growth of plantlets. The source of plant material had differences in the content of secondary metabolites; being commercialized plants the ones with higher values, followed by plants and plantlets grown in greenhouse. *In vitro* cultures produced similar amounts of secondary metabolites to those in the whole plant used as source of explants. This system could represent a feasible way to obtain standardized extracts of bioactive compounds, capable of exerting biological effects on diseases such as cancer.

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