African Journal of Biotechnology

Volume 16 Number 52, 27 December, 2017

ISSN 1684-5315

Academic Journals

ABOUT AJB

The African Journal of Biotechnology (AJB) (ISSN 1684-5315) is published weekly (one volume per year) by Academic Journals.

African Journal of Biotechnology (AJB), a new broad-based journal, is an open access journal that was founded on two key tenets: To publish the most exciting research in all areas of applied biochemistry, industrial microbiology, molecular biology, genomics and proteomics, food and agricultural technologies, and metabolic engineering. Secondly, to provide the most rapid turn-around time possible for reviewing and publishing, and to disseminate the articles freely for teaching and reference purposes. All articles published in AJB are peer-reviewed.

Contact Us

Editorial Office: ajb@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJB

Submit manuscript online http://ms.academicjournals.me/

African Journal of Biotechnology

Table of Content: Volume 16 Number 52 27 December, 2017

ARTICLES

academicJournals

Vol. 16(52), pp. 2349-2354, 27 December, 2017
DOI: 10.5897/AJB2017.16209
Article Number: 0149EE355255
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Chemical composition of neem and lavender essential oils and their antifungal activity against pathogenic fungi causing ginseng root rot

Khalid Abdallah Hussein[1,2] and Jin Ho Joo[1,2*]

[1]Botany and Microbiology Department, Faculty of Science, Assiut University, 71516, Assiut, Egypt.
[2]Soil Biochemistry Laboratory, Department of Biological Environment, Kangwon National University, Republic of Korea.

Received 17 August, 2017; Accepted 20 December, 2017

The aim of this study was to assess *in vitro*, the antifungal activity and characterize the chemical constituents of the essential oils of *Azadirachta indica* (neem) and *Lavandula angustifolia* (lavender). The essential oils of *A. indica* and *L. angustifolia* plants were tested against several isolates of pathogenic fungal genera causing root rot disease of ginseng. Agar plate assay indicated that lavender oil at 10% exhibited the highest inhibition index of (86.0±0.7%) against *Sclerotinia nivalis* mycelial growth. Neem and lavender oils at 5% v/v showed inhibition index against *Alternaria panax* (72.9±2.1 and 45.0±1%, respectively). Lavender oil at 5% (v/v) inhibited growth of *S. nivalis* (83.1±0.2%) and *Cylindrocarpon destructans* (49.2±1%). The gas chromatography-mass spectrometry (GC-MS) analysis showed that the major constituents of neem oil were fatty acids (94.8%). However, sesquiterpenes were the dominant constituents of the lavender oil (57.6%). The antifungal indices demonstrated in this study are a clear evidence of the potentiality of neem and lavender essential oils to control plant diseases caused by phytopathogenic fungi.

Key words: Ginseng root rot, fungi, essential oils.

INTRODUCTION

Korean ginseng is well known as a very important economic herb plant cultivated and used in Asia. The medicinal value of Korean ginseng (*Panax ginseng*) has been discovered for over a thousand years (Baeg and So, 2013; Shishtar et al., 2014). Mainly, the pharmacological active compounds in ginseng are in the roots. Long cultivation time maximizes the concentrations of these vital compounds in root. Therefore, in Korea, ginseng plants are commonly cultivated for 4 or 6 years, usually in shady zones. However, continual cultivation in the same soil for a long duration leads to a decline in the fertility and physicochemical properties of the soil, and provide favorable conditions for infection by fungal soil-borne pathogens which cause severe damage in yield due to soil borne pathogens, such as *Fusarium* spp. and *Cylindrocarpon* spp., that can exist as highly virulent strains on ginseng and coniferous hosts (Seifert et al., 2013). *Cylindrocarpon* attacks only root, usually older

*Corresponding author. E-mail: jhjoo@kangwon.ac.kr. Tel: +82-33-250-6448. Fax: +82-33-241-6640.

ones (Davis and Persons, 2014). Chemical application of pesticides is a handy approach to control disease in ginseng plantations. However, the accumulation of undesirable pesticide residues in the surrounding soil and even in ginseng roots has become a hazardous environmental problem.

Currently, essential oils are considered as broad-spectrum pesticides, organic pesticides, and low-risk pesticides whereas high-risk pesticides cannot be conveniently applied such as in schools, greenhouses and homes (Lang and Buchbauer, 2012). Essential oils are volatile aromatic liquids obtained from plant origin, including flowers, leaves, fruits, seeds, bark, peel, wood, roots and whole plants (Hyldgaard et al., 2012). The ancient Egyptians utilized essential oils in perfumery and in the embalming and preparing bodies for burial through mummification. Many essential oils have antioxidant and antimicrobial properties (Dandlen et al., 2011; Lang and Buchbauer, 2012), but their use as food preservatives requires more knowledge of their properties, including their antimicrobial potency, the specific mode of action, and the sensitivity of the target microorganisms (Hyldgaard et al., 2012). Antifungal activities of different essential oils have been evaluated (Tabassum and Vidyasagar, 2013; Kamal et al., 2012). Tabassum and Vidyasagar (2013) classified and characterized antifungal essential oils on the basis of the family of origin plant. Basil oil was effective against the fungi, *Penicillium aurantiogriseum*, *Penicillium glabrum*, *Penicillium chrysogenum* and *Penicillium brevicompactum* (Kocić-Tanackov et al., 2012). Sweet basil oil at 0.6% v/v showed 100% inhibition of mycelia growth against the rice pathogenic fungi, *Pyricularia arisea* and *Fusarium moniliforme* and 50% inhibition against *Fusarium proliferratum*, but was not effective against *Rhizoctonia solani*. Basil oil at 0.8% v/v inhibited spore germination of *F. monoliforme* (90%) and *Alternaria brassicicola* (100%) (Piyo et al., 2009). The aims of the present study were to estimate the antimicrobial potential of neem and lavender essential oils on the growth of pathogenic fungal strains causing ginseng root rot and to characterize the oils chemical composition.

MATERIALS AND METHODS

Sampling and fungal strains

The fungi used for the bioassay test were collected from the infected ginseng roots in the farm of Soil Microbiology Laboratory, Department of Biological Environment, Kangwon National University, Korea. Infected plants were uprooted, placed into zipper bags, and transported to the laboratory. Fungal isolates were cultivated on potato dextrose agar (PDA) using a tiny isolation needle. Different species were isolated and purified based on their distinctive colonies' shapes using dilution plate technique on Czapek's solution agar (g/L) containing saccharose 20 g; sodium nitrate 3 g; dipotassium phosphate 1 g; magnesium sulfate 0.5 g; potassium chloride 0.5 g; ferrous sulfate 0.01 g; Agar 15 g. Stock cultures of fungi were maintained on 2% malt extract-agar (MEA) plates grown at 27°C and stored at 4°C.

Fungi identification

The fungal strains isolated from infected *P. ginseng* samples were identified by the microscopic examination and the culture features according to Domsch et al. (1980) and Moubasher (1993). Six fungal species were selected for this study (*Alternaria panax*, *Botrytis cinerea*, *Cylindrocarpon destructans*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum* and *Sclerotinia nivalis*). Their identification was confirmed as pathogenic strains by the Korean Agricultural Cultural Collection (KACC), Jeonju, Korea.

Agar dilution test

The experiment was conducted using 100% pure essential oil (EOs) of neem (*Azadirachta indica*) belonging to Meliaceae family and lavender belonging to Lamiacea family (*Lavandula angustifolia*) which were purchased from the manufacturer (Sydney Essential Oil Co. Pty. Ltd., Australia). Antifungal assays were conducted in three replicates and the average of the data was calculated. EOs were amended in PDA to make 5 and 10% concentration in Petri plates, respectively. Solidified agar plates were inoculated at the center with mycelial disc (6 mm) diameter of the pathogen and incubated at 27°C for 10 days. Plates without EOs served as control. The agar dilution test, a concentration gradient of the tested EOs, was used to evaluate the antifungal activity of tested substance (Lang and Buchbauer, 2012). The antimicrobial activities of EOs on the phytopathogenic fungi were examined after 10 days and the inhibition percentage was calculated according to the formula of Messgo-Moumene et al. (2015):

$$\text{Inhibition percentage (\%)} = (A_1-A_2)/A_1 \times 100$$

Where, A_1 is the colony area of untreated pathogenic fungus in the control and A_2 is the area of pathogenic fungus colony in dual culture.

Essential oil analysis

The essential oils composition was detected using GC-MS on a GC Agilent -7890A/MS GEOL JP/JMS-Q1050GC (GC/MS system) apparatus equipped with a DB-WAX column (30 × 0.32 mm, 0.5 µm film thickness). Helium was adopted as the carrier gas at 1.0 mL/min flow rate; column pulse pressure was 48.7 kPa; linear velocity was 36.0 cm/s; at a flow rate of 50 mL/min; the carrier flow rate was 24 mL/min; injector temperature was 250°C; detector temperature was 250°C; column temperature at 40°C (3 min) to 150°C (3 min) at 5°C/min, then 155 to 250°C at 10°C/min for 10 min. In the GC-MS detection, an electron ionization system was adopted with ionization energy of 75 eV. Samples were diluted 1/100 (v/v) in ethanol and 1.0 µL were injected in the splitless mode (Adams, 1995). The compounds were identified by matching their fragmentation patterns detected in the mass spectra with the patterns present in the NIST 98 mass spectrometry library (Central Lab., Kangwon National University, Korea). Quantification of the constituents was based on the peak area percentage of each constituent in relation to the total area of the ideal peaks in the chromatogram.

Statistical analysis

The statistical analysis was performed on all the data with SAS Institute (2011**)** using Tukey's test, version 11.0, to compare the means ($P > 0.05$).

Table 1. Antifungal indices of neem and lavender essential oils.

Species	Neem oil (%)		Lavander oil (%)	
	5	10	5	10
Alternaria panax	72.9 ± 2.1[a]	77.6 ± 0.9[a]	45 ± 1[c]	52.8 ± 0.7[c]
Sclerotinia sclerotiorum	52.9 ± 2.3[b]	69.7 ± 0.8[b]	5.6 ± 0.2[f]	11.5 ± 0.4[f]
Sclerotinia nivalis	50.3 ± 1.5[b]	66.4 ± 0.3[c]	83.1 ± 0.2[a]	86 ± 0.7[a]
Cylindrocarpon destructans	42.5 ± 1.7[c]	52.6 ± 0.6[d]	49.2 ± 1[b]	66 ± 0.8[b]
Botrytis cinerea	30.5 ± 0.9[d]	49.9 ± 0.6[e]	11.1 ± 0.1[e]	17.1 ± 0.5[e]
Fusarium oxysporum	24.6 ± 0.6[e]	31.6 ± 0.8[f]	14.8 ± 0.3[d]	31.1 ± 0.2[d]

Different letters (a-f) indicate significant difference (P<0.05) between control and treatments.

RESULTS AND DISCUSSION

At least five species of fungi have been involved in some form of ginseng root rot: *Alternaria*, *Botrytis*, *Cylindrocarpon*, *Fusarium* and *Sclerotinia* (Davis and Persons, 2014; Schnitzler et al., 2011; Silva et al., 2011; Oussalah et al., 2007). Therefore, to overcome this problem, most ginseng fields are treated with synthetic agricultural chemicals (Tawaha et al., 2007; Moreira et al., 2005). However, there is a strong anxiety about the safety aspects of chemical pesticides, since they are considered as carcinogenic and responsible for many diseases, as well as residual toxicity (Peng et al., 2005).

Antifungal assay of essential oil

Six representative strains of ginseng root rot fungi were selected to test the antifungal activity of neem and lavender essential oils. The antifungal indices demonstrated in Table 1 are a clear evidence of the excellent antifungal activities of the tested oils. The antimicrobial activity obtained from neem oil was relatively higher than lavender oil. Neem oil showed the highest antifungal activity against *Alternaria panax* (77.6±0.6%), followed by *S. sclerotiorum* (69.7±0.8%). Moderate antifungal activity was recorded against *S. nivalis* (66.4±1.5%), *C. destructans* (52.6±1.7%) and *B. cinerea* (49.9±0.6%). *F. oxysporum* was the least sensitive fungus (31.6±0.8%) at 10% v/v. Lavender oil showed distinctive antifungal activity against all the tested fungi. The highest index of inhibition (86±0.7%) and the lowest (11.5±0.4%) were recorded against *S. nivalis* and *S. sclerotiorum*, respectively. Moderate antifungal activity was recorded against *F. oxysporum* (31.1±0.2%) and *B. cinerea* (17.1±0.5%). *C. destructans* showed inhibition index of 66±0.8% at dose of 10% v/v (Figures 1 and 2). Kamal et al. (2012) studied the antifungal activity of the ajwain seeds volatile oil. The volatile components of ajwain seeds inhibited 70 to 90% growth of 10 fungi *Acrophialophora fusispora*, *Alternaria grisea*, *Alternaria tenuissima*, *Curvularia lunata*, *Drechslera tetramera, F. chlamydosporum*, *F. poae*, *Myrothecium roridum*, *Papulaspora* sp. and *Rhizoctonia solani*. The essential oil of *Chamaecyparis formosensis* wood possessed antifungal activity, with an antifungal index of 88.2 and 67.3% for the wood decay fungi, *Laetiporus sulphureus* and *Trametes versicolor* at a dose of 50 mg ml^{-1}, respectively (Wang et al., 2005). Leaves extracts of neem which are inexpensive and environmentally safe, are promising for protection of economic plants against fungal infection leading to improvement of the crop regarding yield and productivity (Mondall et al., 2009). The data revealed that fatty acids (5 compounds, 94.02%) are the main class in the neem oil. The gas chromatographic analysis of neem oil showed the presence of hexadecanoic acid with the highest percentage (78.25%), followed by tetradecanoic acid (7.24%), oleic acid (3.64%), octadecenoic acid (3.5%), and linoleic acid (1.39%) (Table 2). Figure 3 shows the main fatty acids skeletons of essential oil from neem. Many studies have reported that majority of the fatty acid content of neem (*A. indica*) extract ranges between 25 and 61.9% (Djenontin et al., 2012). Sandanasamy et al. (2013) showed the presence of nine fatty acids, including four major ones. These are oleic acid (41.91±0.69%), followed by linoleic acid (19.59±0.44%), stearic acid (18.71±0.46%) and palmitic acid (15.59±0.27%). The differences in the fatty acids percentage and their composition may be attributed to the extraction method, plant species and their cultivation conditions (Atabani et al., 2013). Among them, 1,6-octadien-3-ol, 3,7-dimethyl- (41.74%) and silane, triethylfluoro (36.7%) were the major components in the lavender essential oil. Table 3 shows the constituents of lavender essential oil. Oxygenated monoterpenes (4 compounds, 57.62%) are the main class in the lavender oil, of which 1,6-octadien-3-ol,3,7-dimethyl (41.74%) was the major compound followed by bicyclo [1.2.2] heptan-2-one, 1,7,7-trimethyl (6.91%) with lower amounts of eucalyptol (5.99%) and 3-cyclohexene-1-methanol, a,a4-trimethyl (2.99%). Figure 4 shows the main sesquiterpenes skeletons of these compounds, a lower percentage (42.38%) of aliphatic hydrocarbons (2 compounds), silane, triethylfluoro (36.71%) and methanesulfonyl chloride (5.67%). However, Hui et al. (2010) showed that dimethyl vinyl hexenylbutyrate was

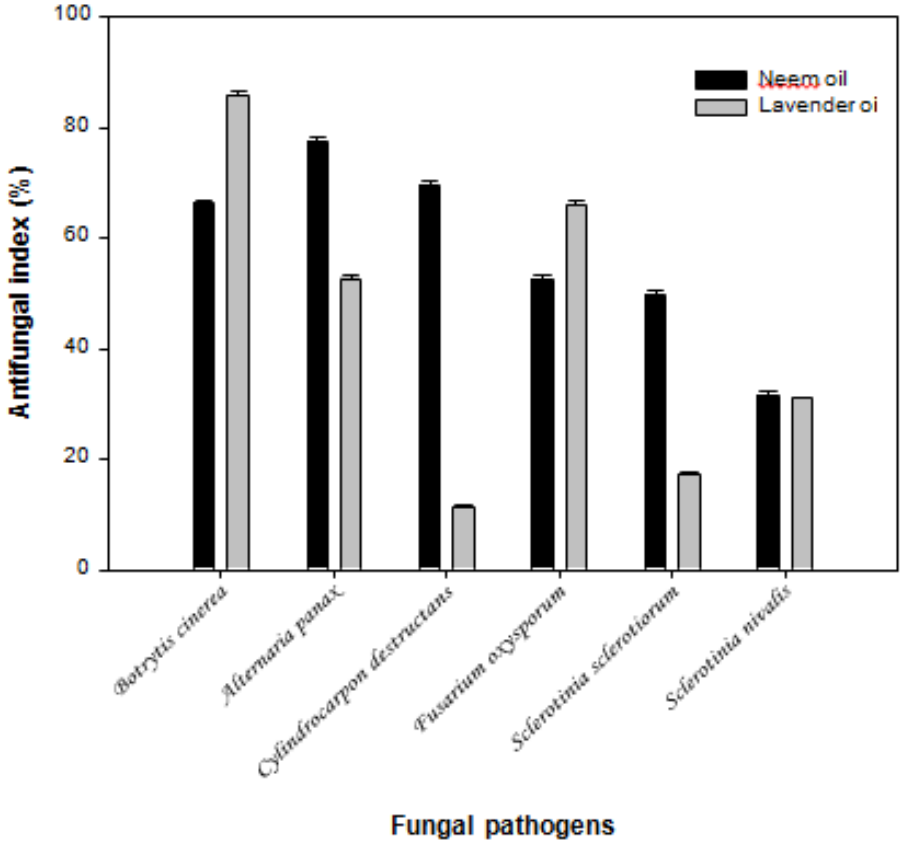

Figure 1. Antifungal activities of the essential oils neem (black bar) and lavender (grey bar) against ginseng root rot fungi at 10% v/v. The phytopathogenic fungi (agar dilution tests) were examined after 10 days and the inhibition percentages were calculated.

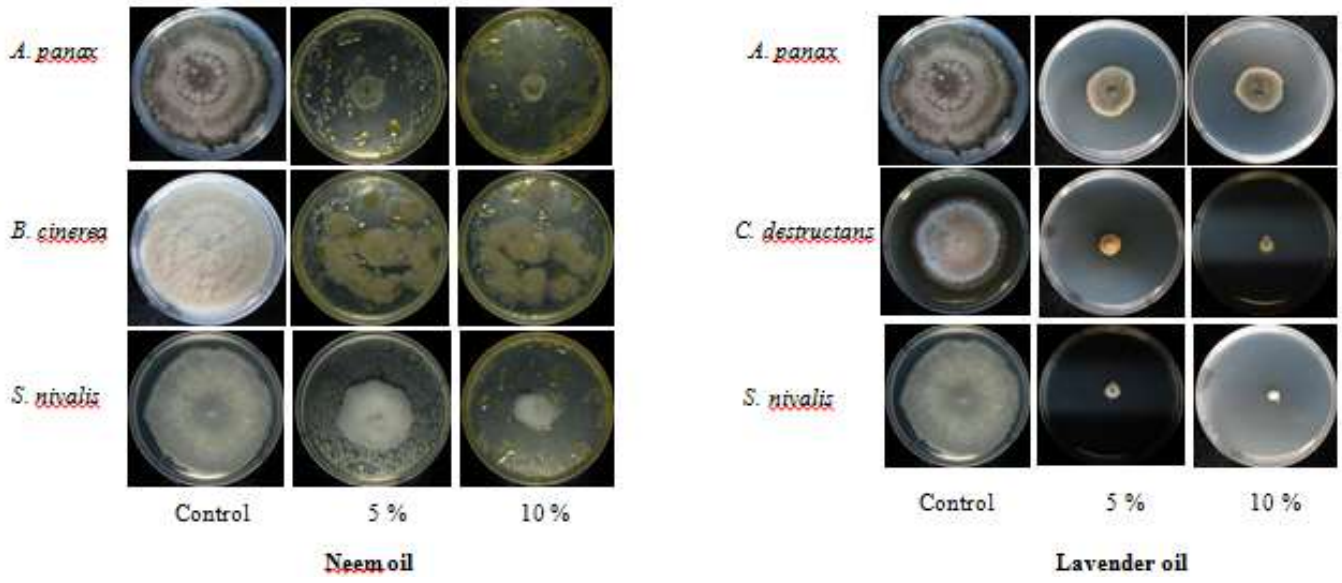

Figure 2. Antifungal activities of neem and lavender essential oils against the growth of fungal pathogens causing ginseng root rot, on agar dilution test, phytopathogenic fungi were examined after 10 days and the inhibition percentage was calculated according to the formula of Messgo-Moumene et al. (2015).

Table 2. Chemical compositions of neem essential oil.

Peak No.	Compound	Molecular formula	RT (min)	Concentration (%)
1	Silane, triethylfluoro-	$C_6H_{15}FSi$	8:44	3.96
2	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	33:23	3.5
3	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	33:37	0.87
4	Oleic Acid	$C_{18}H_{34}O_2$	33:58	3.64
5	Linoleic acid ethyl ester	$C_{20}H_{36}O_2$	34:14	1.39
6	Tetradecanoic acid	$C_{14}H_{28}O_2$	37:18	7.24
7	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	40:27	78.25
8	2-[2-[2-[2-[2-[2-[2-[2-[2-[2-(2-Hydroxyethoxy)ethoxy]ethoxy]	$C_{22}H_{46}O_{12}$	40:40	1.15

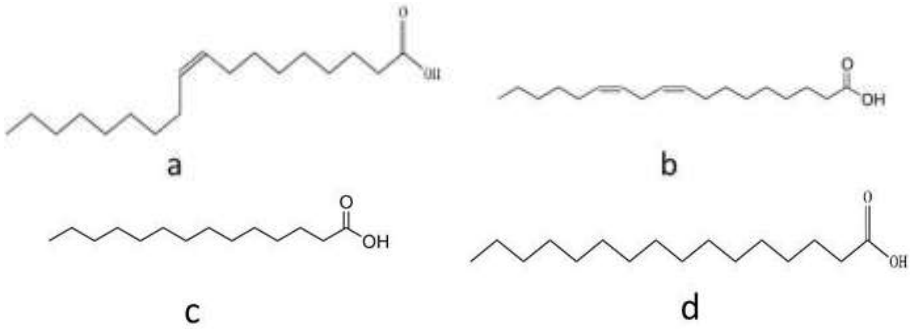

Figure 3. Main fatty acids skeletons of essential oil from neem (a) emery type, (b) linoleic type (c) myristic type (coconut oil) (d) palmitic type.

Table 3. Chemical compositions of lavender essential oil.

Peak No.	Compound	Molecular formula	RT (min)	Concentration (%)
1	Methanesulfonyl chloride	CH_3ClO_2S	6:57	5.67
2	Eucalyptol	$C_{10}H_{18}O$	7:03	5.99
3	Silane, triethylfluoro-	$C_6H_{15}FSi$	8:49	36.71
4	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (+)-	$C_{10}H_{16}O$	14:39	6.91
5	1,6-Octadien-3-ol, 3,7-dimethyl-	$C_{10}H_{18}O$	15:22	41.74
6	3-Cyclohexene-1-methanol, a,a4-trimethyl-	$C_{10}H_{18}O$	18:59	2.99

the main compound of essential oil (43.73%), followed by the octatriene dimethyl (25.10%), eucalyptol (7.32%) and camphor (3.79%). Some of the results in this study are in conformity with previous studies. The differences in chemical composition of plant essential oil may to some extent, due to the difference in maturity stages of growth, geographical sources and extraction techniques. Nevertheless, both investigated essential oils exhibited growth inhibition effect on the ginseng root rot fungal species tested in the present study. The results may assert the importance of essential oils of common plants in the preservation and protection of ginseng crop from fungal pathogens.

Conclusions

Searching for new antifungal agent from plant and detection of its ability to control plant diseases caused by phytopathogenic fungi are needed. *A. indica* (neem) and *L. angustifolia* (lavender) are among the most common plants used traditionally all over the world. The results on these plants showed their ability to be new sources of natural products used as antimicrobial agents. The results showed that their oils are active against the phytopathogenic fungal strains and opens the possibility for discovery of alternative clean method to control ginseng root rot.

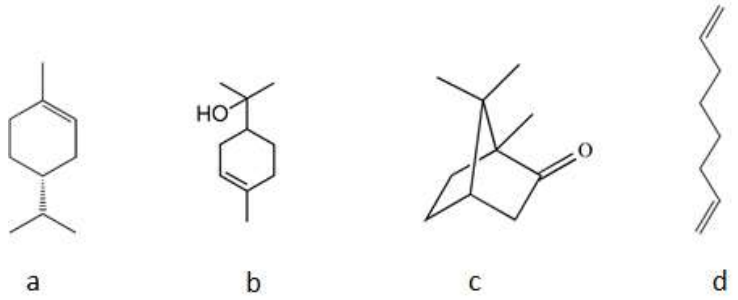

Figure 4. Main sesquiterpenes skeletons of essential oil from lavender (a) terpene type, (b) terpineol type, (c) camphore type, (d) octadien type.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

This work was carried out with the support of "Cooperative Research Program for Agriculture Science & Technology Development (Project no. PJ010848)" Rural Development Administration, Republic of Korea.

REFERENCES

Adams RP (1995). Identification of essential oil components by gas chromatography/mass spectrometry. Carol Stream: Allured Publishing Corporation 469p.

Atabani AE, Silitonga AS,Ong HC,Mahlia TMI, Masjuki HH, Irfan AB, Fayaz H (2013). Non-edible vegetable oils: A critical evaluation of oil extraction, fatty acid compositions, biodiesel production, characteristics, engine performance and emissions production. Renew. Sustain. Energy Rev. 18:211-245.

Baeg IH, So SH (2013). The world ginseng market and the ginseng (Korea). J. Ginseng Res. 37:1-7.

Dandlen SA, Lima A, Sofia M, Marta D, Miguel M, Graça FM, Sousa L,

Pedro MJ, Barroso LG, Figueiredo JG, Cristina A (2011). Antimicrobial activity, cytotoxicity and intracellular growth inhibition of Portuguese Thymus essential oils. Revista Brasileira de Farmacognosia, 21(6):1012-1024.

Davis J, Persons W S (2014). Growing and Marketing Ginseng, Goldenseal and other Woodland Medicinals. Printed in Canada. First printing June

Djenontin TS, Wotto VD, Avlessi F, Lozano P, Sohounhloue DKC, Pioch D (2012). Composition of *Azadirachta indica* and *Cara paprocera* (Meliaceae) seed oils and cakes obtained after oil extraction. Ind. Crops Prod. 38:39-45.

Domsch KH, Gams W, and Anderson T (1980). Compendium of soil fungi. Academic Press. London 1-2:405-859.

Hui L, He L, Huan L, Xiao L, Aiguo Z (2010). Chemical composition of *lavender* essential oil and its antioxidant activity and inhibition against rhinitisrelated bacteria. African J. Microbiol. Res. 4:309-313.

Hyldgaard M, Mygind T, Meyer RL (2012). Essential oils in food preservation: mode of action, synergies and interactions with food matrix components. Front Microbiol. 3:1-24.

Kamal J, Devi N, Thakur N, Tomar S, Shalit L, Thakur R (2012). *Trachyspermum ammi* (Ajwain): a comprehensive review. Int. Res. J. Pharm. 3:133-138.

Kocić-Tanackov SD, Dimić GR, Tanackov IJ, Pejin DJ, Mojović LjV, Pejin JD (2012). Antifungal activity of Oregano (*Origanum vulgare* L.) extract on the growth of *Fusarium* and *Penicillium* species isolated from food. Hemijska Industrija 66:33-41.

Lang G, Buchbauer G (2012). A review on recent research results on essential oils as antimicrobials and antifungals. A review. Flavour. Frag. J. 27:13-39.

Messgo-Moumene S, Li Y, Bachir K, Houmani Z, Bouznad Z, Chemat F (2015). Antifungal power of Citrus essential oils against potato late blight causative agent. J. Essen. Oil Res. 27:169-176.

Mondall NK, Mojumdar A, Chatterje SK, Banerjee A, Datta JK, Gupta S (2009). Antifungal activities and chemic al characterization of Neem leaf extracts on the growth of some selected fungal species in vitro culture medium. J. Appl. Sci. Environ. Manage. 13(1):49-53.

Moubasher AH (1993). Soil fungi in Qatar and other Arab countries. The Centre of Scientific and Applied Research, University of Qater, Doha, Qater.

Oussalah MS, Caillet L, Saucier M L (2007). Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: *E. coli* O157:H7, *Salmonella typhimurium*, *Staphylococcus aureus* and *Listeria monocytogenes*. Food Control 18:414-420.

Piyo A, Udomsilp J, Khang-Khun P, Thobunluepop P (2009). Antifungal activity of essential oils from basil (*Ocimum basilicum* Linn.) and sweet fennel (*Ocimum gratissimum* Linn.): Alternative strategies to control pathogenic fungi in organic rice. Asian J. Food Ag-Ind. (special issue), S2-S9.

Sandanasamy J, Nour AH, Bin Tajuddin SN, Nour AH (2013). Fatty acid composition and antibacterial activity of neem (*Azadirachta indica*) seed oil. The Open Conference Proceedings Journal. 4:43-48.

SAS Institute (2011). The SAS 10.2 software. Statistical Analysis System for Windows [Softaware]. Cary, N.C. SAS.

Schnitzler PA, Reichling J (2011). Screening for antiviral activities of isolated compounds from essential oils. 253643 J. Evid. Based. Complementary. Altern. Med. 25:36-43.

Seifert KA, McMullen CR, Yee D, Reeleder RD, Dobinson KF (2013). Molecular Differentiation and Detection of Ginseng-Adapted Isolates of the Root Rot Fungus *Cylindrocarpon destructans* Population Biology 93:1533-1542.

Shishtar E, Sievenpiper JL, Djedovic V, Cozma AI, Ha V, Jayalath VH (2014). The Effect of Ginseng (The Genus *Panax*) on Glycemic Control: A systematic review and meta-analysis of randomized controlled clinical trials. PLoS ONE 9(9): e107391.

Silva F S, Duarte A, Mendonça DI, Domingues FC (2011). Antifungal activity of *Coriandrum sativum* essential oil, its mode of action against candida species and potential synergism with amphotericin B. Phytomedicine. 19:42-47.

Tabassum N, Vidyasagar GM (2013). Antifungal investigations on plant essential oils: A review. Int. J. Pharm. Pharm. Sci. 5:19-28.

Wang SY, Wu CL, Chu FH, Chien SC, Kuo YH, Shyur LF, Chang ST (2005). Chemical composition and antifungal activity of essential oil isolated from *Chamaecyparis formosensis* Matsum. Wood. Holzforschung. 59:295-299.

academicJournals

Vol. 16(52), pp. 2355-2363, 27 December, 2017
DOI: 10.5897/AJB2017.16281
Article Number: 7C9AB0355257
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Plant cell suspension culture of *Thevetia peruviana* (Pers.) K. Schum. in shake flask and stirred tank reactor scale: A comparative study

Juan Pablo Arias[1,3*], Karol Zapata[2], Benjamín Rojano[2], Mariana Peñuela[3] and Mario Arias[1]

[1]Grupo de Biotecnología Industrial, Facultad de Ciencias, Universidad Nacional de Colombia Sede Medellín, Calle 59ª, No 63-20 Bloque 19A-313, Medellín, Colombia.
[2]Grupo de Química de los Productos Naturales y los Alimentos, Facultad de Ciencias, Universidad Nacional de Colombia Sede Medellín, Calle 59ª, No 63-20 Bloque 19A-211, Medellín Colombia.
[3]Grupo de Bioprocesos, Facultad de Ingeniería, Universidad de Antioquia, Calle 70 No. 52-21, Bloque 18-405 Medellín, Colombia.

Received 17 October, 2017; Accepted 22 December, 2017

The aim of this work was to evaluate the effect of changing the shake flask for a stirred-tank bench bioreactor on the performance of *Thevetia peruviana* cultures. Maximum cell growth of 18.59 and 8.46 g DW/L for shake flask and bench bioreactor at day 14 was obtained, respectively. The end of the exponential growth phase in shake flask was correlated with total sugar consumption, while this behavior was not observed in bench bioreactor. Preferential uptake of glucose over fructose was observed in both systems. Cell morphology was similar for both systems during culture time, exhibiting individual cells with cylindrical and elongated shape at day 0 and some aggregates with rounded cells at day 14. Polyphenol production was not affected by the system configuration and scale. Intra and extracellular antioxidant activity were directly related with phenol production in both systems. Guaiacol peroxidase activity (GPX) went from 1.87 to 7.97 µM/min/mg protein in shake flask and it correlated with low reactive oxygen species (ROS) production. This is an indication of a positive enzymatic cell response for culture in shake flask mediated by GPX. In contrast, the conditions in the bench bioreactor generated a higher stress environment, increasing ROS production without activating enzymatic or non-enzymatic responses led by guaiacol and phenolic compounds, respectively. In conclusion, bench stirred-tank bioreactor scale affects biomass production and sugars uptake negatively.

Key words: *Thevetia peruviana*, plant cell suspension culture, stirred-tank bioreactor, phenolic compounds, antioxidant activity.

INTRODUCTION

In vitro plant cell cultures have been considered over the last 50 years as an alternative of bioactive compounds production. Glycosides, alkaloids, terpenoids, phenolic acids and flavonoids are some of the bioactive metabolites that can be used as food and pharmaceutical additives (Dias et al., 2016), and could be produced in plant cell cultures. However, the establishment of *in vitro* plant cell cultures has technical difficulties, such as productivity loss related to scale-up from shake flask to stirred bioreactors (Ruffoni et al., 2010), among others.

Up to date, commercial processes involving plant cell cultures are limited to just a handful valuable secondary metabolites such as shikonin, ginseng, digoxin, sanguinarine and placlitaxel (Paek et al., 2014).

Thevetia peruviana is a shrub that belongs to the Apocynaceae family and it has been considered as an important source of biologically active compounds (Duke et al., 2002). The establishment of *in vitro* cultures of *T. peruviana* had been reported previously. Phenolic acids with significant antioxidant effects have been reported in callus cultures of *T. peruviana* at shake flask scale under regular (Rincón et al., 2016) and different light conditions (Arias et al., 2016), as well as *in vivo* cultures (Dixit et al., 2015). These studies show the growing importance of *T. peruviana* as a plant species with great potential in the production of secondary metabolites with biological activity. However, it is necessary to carry out further studies that allow to maintain or increase the productivity of such metabolites on a large commercial scale.

This work aims to evaluate the effect of scale change from shake flask to bench stirred-tank bioreactor on biomass, phenol production and antioxidant activity of suspension cultures of *T. peruviana* in order to establish a strong foundation for a successful scale-up.

MATERIALS AND METHODS

Establishment of plant and cell suspension cultures

The protocol for establishment of *in vitro* cultures of *T. peruviana* from fruit pulp has already been described in detail (Arias et al., 2009). Briefly, fruits were initially disinfected using ethanol (70% v/v), sodium hypochlorite (10% w/v), and sterile distilled water. Next, fresh explants were extracted aseptically and sowed in solid SH (Schenk and Hildebrandt, 1972) medium supplemented with 30 g/L sucrose, 2 mg/L of 2,4-D and 0.5 mg/L of kinetin. Friable callus (4 g) were transferred to 250 mL Erlenmeyer flask with SH liquid medium. Cell suspension cultures were maintained at 25°C constant agitation (110 rpm) and darkness condition. All cell suspension cultures used in the experiments were in exponential growth phase (6 days from the last subculture). Experiments started with an initial cell concentration of approximately 6 g DW/L.

Erlenmeyer flask cultures

Cell suspension cultures were inoculated in 100 mL of SH medium in 250 mL Erlenmeyer flasks. Cultures were incubated as described earlier and samples were taken every 2 to 3 days.

Bench bioreactor cultures

Cell suspension cultures were carried out in a 7 L stirred-tank bioreactor (Applikon, NL) with three baffle plates and a six-bladed disc turbine impeller. DO was maintained at 30% with air using a sterilizable DO probe (ColeParmer, USA), a Controller Alpha-DO2000PPG (Eutech Instruments, NL), and a stainless steel sparger (seven orifices) located below the turbine. pH was maintained at 5.8 using a sterilizable pH probe (ColeParmer, USA) and a Controller Alpha-pH 800 (Eutech Instruments, NL). Cells were inoculated in 5 L (working volume) of SH medium. Cultures were incubated at 25°C and rotational speed was set at 300 rpm. Samples were taken every 2 to 3 days.

Cell growth determination

Cell growth was determined by dry weight technique. Cell culture aliquots (5 mL) were subjected to vacuum filtration through a pre-dried filter paper Whatman 595. Next, the retained biomass was washed 3 times with distilled water and dried in a convection oven until it reached constant weight after 24 h at 60°C.

Viability

Cell membrane integrity was measured by the blue dye exclusion test, according to the protocol described by Gaff and Okong'o-ogola (1971) with some modifications. Briefly, 75 µL of cell suspension were incubated with 25 µL of Evan's blue (10 mg/mL) for 5 min and microscopically analyzed. The percent viability of samples was determined based on the number of non-stained cells (viable) respect to the number of total cells.

Morphology

Photographic recording of the cells at 10 and 40X was carried out using a Leica Microscope every 2 to 3 days. Circularity measurements (width/length) and cell aggregate size, if they were present, were made using the ImageJ software (https://imagej.nih.gov/ij/).

Sugar quantification

Extracellular sucrose, fructose and glucose concentrations were analyzed using a Shimadzu Prominence HPLC system with a pumping system LC-20 CE, coupled to a refractive index detector RID-10A and the Solution LC software was used. Separation and quantification of sugars were carried out using a reverse phase amino column RP-NH2 NUCLEODUR 100-5 and mobile phase acetonitrile/water (79:21 v/v ratio), with a 2 mL/min flow and a sample size of 10 µL at 35°C.

Metabolite extraction and analysis

Phenol production was determined every 2 to 3 days during 19 days of culture. Extracellular samples (phenolic content and antioxidant activity) were prepared by microfiltration (0.2 µm) of cells free medium. Intracellular phenolic content and antioxidant activity samples were obtained from 200 mg of fresh biomass collected by filtration and resuspended in 1 mL of phosphate buffer (pH 4). Cell extracts (extra and intracellular) were stored at -20°C until use.

*Corresponding author: E-mail: jpariase@unal.edu.co or marioari@unal.edu.co.

Phenolic content

The phenolic content was determined by the adapted Folin-Ciocalteu method (Singleton and Rossi, 1965). The extract (25 µL) was mixed with 70 µL of Folin-Ciocalteu solution and 200 µL of sodium carbonate solution (7.1% w/v). The resulting product was brought to 500 µL with distilled water. This solution was stirred and stored in darkness at 25°C for 30 min, and then the absorbance was measured at 760 nm. The absorbance values were compared with the standard (aqueous solutions of gallic acid) and the results were expressed as mg GAE/g DW and mg GAE/L for intra and extracellular samples, respectively.

Antioxidant activity

The antioxidant activity was evaluated by the 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) method, according to the protocol described by Re et al. (1999). This technique determines the capacity of a sample to trap the radical cation ABTS and cause its discoloration. The extract (20 µL) was added to 1980 µL of ABTS solution and the absorbance was measured after 30 min at 732 nm. The absorbance values were compared to the reference curve prepared with Trolox as a primary standard. The results were expressed as µmol Trolox Equivalent/g DW.

Production of reactive oxygen species (ROS)

Generation of ROS during the cell growth was evaluated using 2',7' dichlorofluorescein diacetate (DCFDA), a ROS-sensitive dye. The protocol employed was proposed by Martín-Romero et al. (2008). Aliquots of 30 µL of the sample were warmed to 37°C before addition of 270 µL of DCFDA (40 µmol/L in phosphate buffer). The kinetics of fluorescence emission at 530 nm was measured by excitation at 490 nm for 3000 s. The oxidation rate of DCFDA is equal to the slope of the kinetic curve. DCFDA oxidation rate can be assumed as an index of ROS production, that is, high oxidation rate indicates high ROS production.

Guaiacol peroxidase activity

Intracellular guaiacol peroxidase activity was measured according to the method described by Villegas et al. (2017) with some modifications. Briefly, 0.2 g of biomass, previously macerated with liquid nitrogen, were mixed with 1 mL of phosphate buffer 10 mM, pH 7.0 with 4% (w/v) of PVP. The mixture was centrifuged at 14000 g for 20 min at 4°C; the supernatant was used as crude extract. 60 µL of crude extract were mixed with 2340 µL of phosphate buffer 10 mM, pH 7.0, 600 µL of guaiacol (1% w/v) and 1.5 µL of H_2O_2. The absorbance was measured at 470 nm. Specific guaiacol peroxidase activity (GPX) activity was expressed as µM of TG/min/mg protein, where total intracellular proteins were measured by the Bradford (1976) method using the crude extract.

Statistical analysis

All data was reported as the mean values ± standard deviation (SD). All experiments were performed in duplicate (n=2). The difference between treatments was established via two-way analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$) for multiple group comparisons, using the Statgraphics Centurion XV version 15.2.05 software.

RESULTS AND DISCUSSION

Cell growth and substrate consumption

A maximum cell growth (Figure 1) of 18.59 and 8.46 g DW/L for shake flask and bench reactor were obtained at day 14, respectively. The end of the exponential growth phase in shake flask was correlated with the total sugar consumption, especially sucrose and glucose. This behavior was not observed in bench bioreactor, which did not show a clear exponential phase and accumulated fructose in the culture medium over the time.

The lower production of biomass in bench reactor corresponded to a lower degree of sucrose hydrolysis and total consumption of fructose; whereas in the shake flask on day 14, sucrose hydrolysis and glucose consumption were complete, while fructose consumption was close to completion. These behavioral differences in the dynamics of cell growth, sucrose hydrolysis, and sugar consumption between the two systems may be due to different mixing regimes and hydrodynamic stress. The type of agitation has been related to hydrodynamic stress especially in mechanically stirred-tank reactor (Kieran et al., 2000), which increases the shear stress in the cultures. Differences between maximum values of biomass in shake flask and reactor have been reported in other plant cell species. Cell cultures of *Rubia tinctorum* showed a 29% decrease in biomass concentration at reactor scale compared to shake flask (Busto et al., 2008). However, Villegas et al. (2017) observed a decrease in biomass production from shake flask (15.69 g DW/L) to reactor scale (10.53 g DW/L at 400 rpm) in cell suspension cultures of *Azadirachta indica*.

In our experiments, both systems showed preferential glucose uptake in contrast to fructose. This last one remained in higher concentration during the time of culture. Preferential glucose consumption has been reported for other plant species at shake flask (e.g., *Daucus carota* (Krook et al., 2000), *Taxus chinensis* (Li et al., 2003), and *Centella asiatica* (Omar et al., 2006), and bioreactor scale (e.g., *Elaeis guineensis* (Gorret et al., 2004)). This preferential glucose uptake could partially be explained by a higher affinity of the hexose carrier in the plasma membrane for glucose over fructose (Krook et al., 2000).

Cell viability (Figure 2) remained constant for shake flask cultures (85.78%±1.48). Regarding the bench reactor, a slight decrease in cell viability was observed in days 4 and 12; however, the value remains around 83.52%±3.60 during culture. These results suggest that the change in hydrodynamic conditions due to culture system does not affect cell integrity significantly, indicating that *T. peruviana* cells could be shear tolerant. Same behavior was observed in suspension cultures of *Beta vulgaris* (Rodríguez and Galindo, 1999) and *Uncaria tomentosa* (Trejo et al., 2005), where biomass production was affected at bench stirrer bioreactor scale while cell

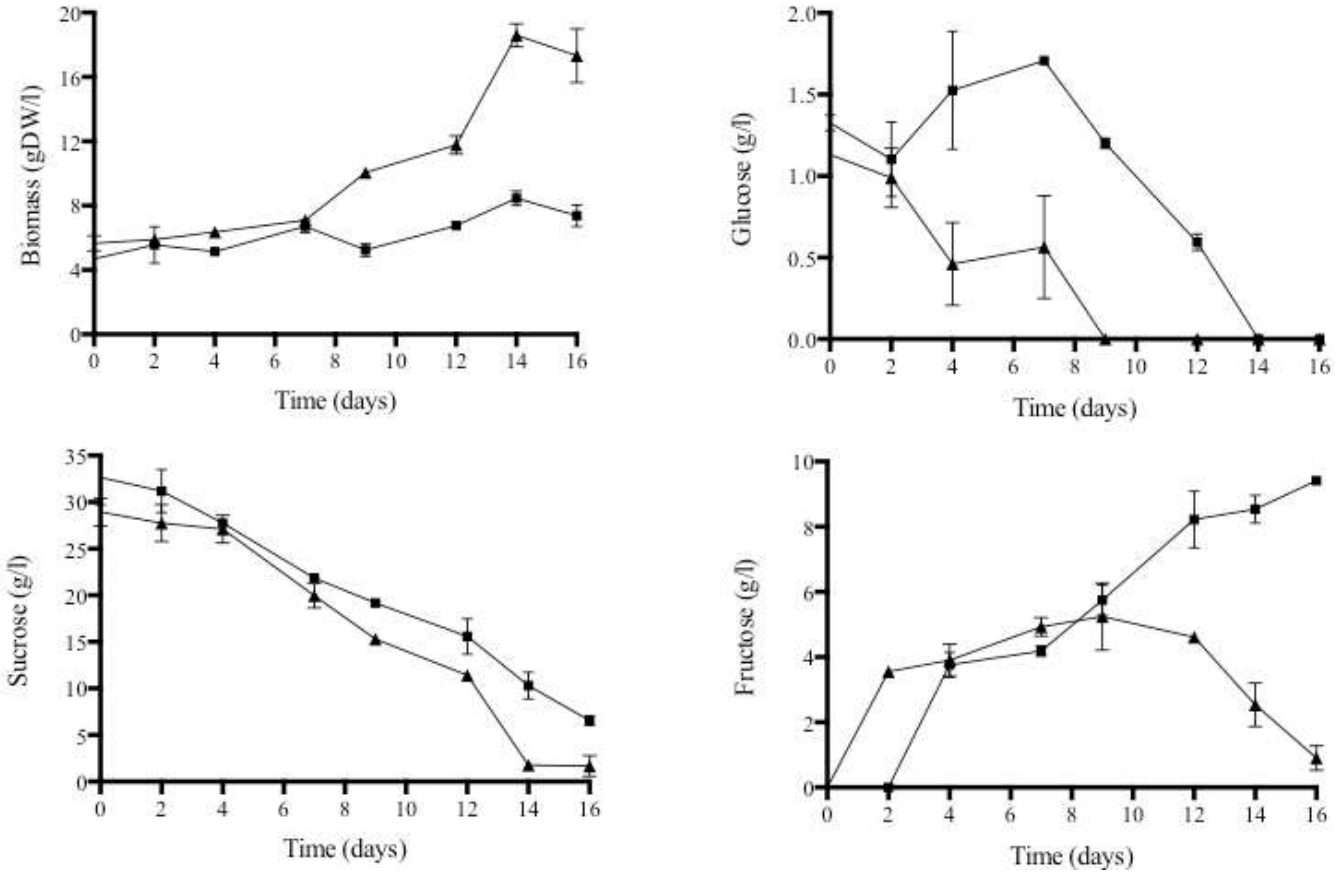

Figure 1. Cell growth and sugar uptake curves (sucrose, glucose and fructose) for cell suspension cultures of *T. peruviana* in shake flask (—▲—) and bench reactor (—■—).

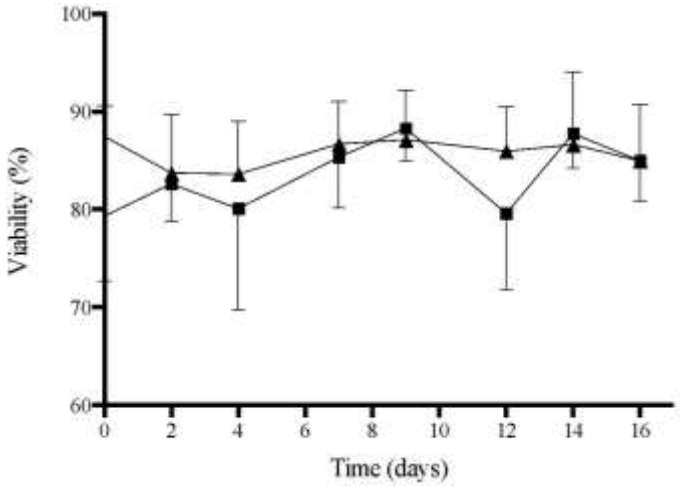

Figure 2. Viability of cell suspension cultures of *T. peruviana* in shake flask (—▲—) and bench stirred tank bioreactor scale (—■—).

viability remains equal. In contrast, suspension cultures of *R. tinctorum* (Busto et al., 2008) and *Centaurea calcitrapa* (Raposo and LimaCosta, 2006) presented a 6-fold reduction and 34% decrease on cell viability respectively, compared to shake flask.

Morphology

Shake flask cells presented a continuous increase of cell circularity from 0.44±0.19 at day 0 to 0.82±0.15 at day 16. Bench stirred tank bioreactor cells showed a slightly decrease from 0.38±0.09 at day 0 to 0.25±0.06 at day 4, and finally an increase to 0.78±0.15 at day 16 (Figure 3). Both systems tend to increase cell circularity throughout the culture. However, the circularity is greater in the shake flask than in the bench bioreactor. This behavior is most likely associated with the higher hydrodynamic stress present in the mechanically stirred bench reactor.

At the beginning of the cultures, cells show a cylindrical and elongated shape in both systems (Image 1A and C); this led to the formation of structures similar to fungal hyphae. Through the culture, cells are more rounded and tend to form some small agglomerates. Elongated cells have been reported for other plant cell species, e.g., *Nicotiana tabacum* (Curtis and Emery, 1993; Mcdonald et

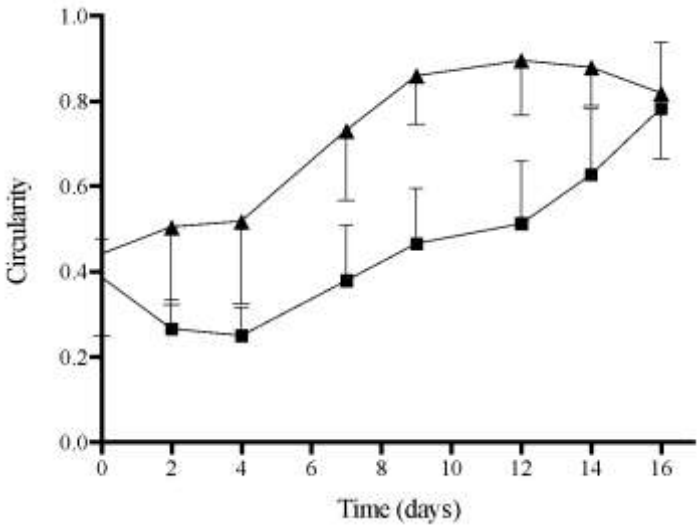

Figure 3. Cell circularity of cell suspension cultures of *T. peruviana* in shake flask (—▲—) and bench stirred tank bioreactor scale (—■—).

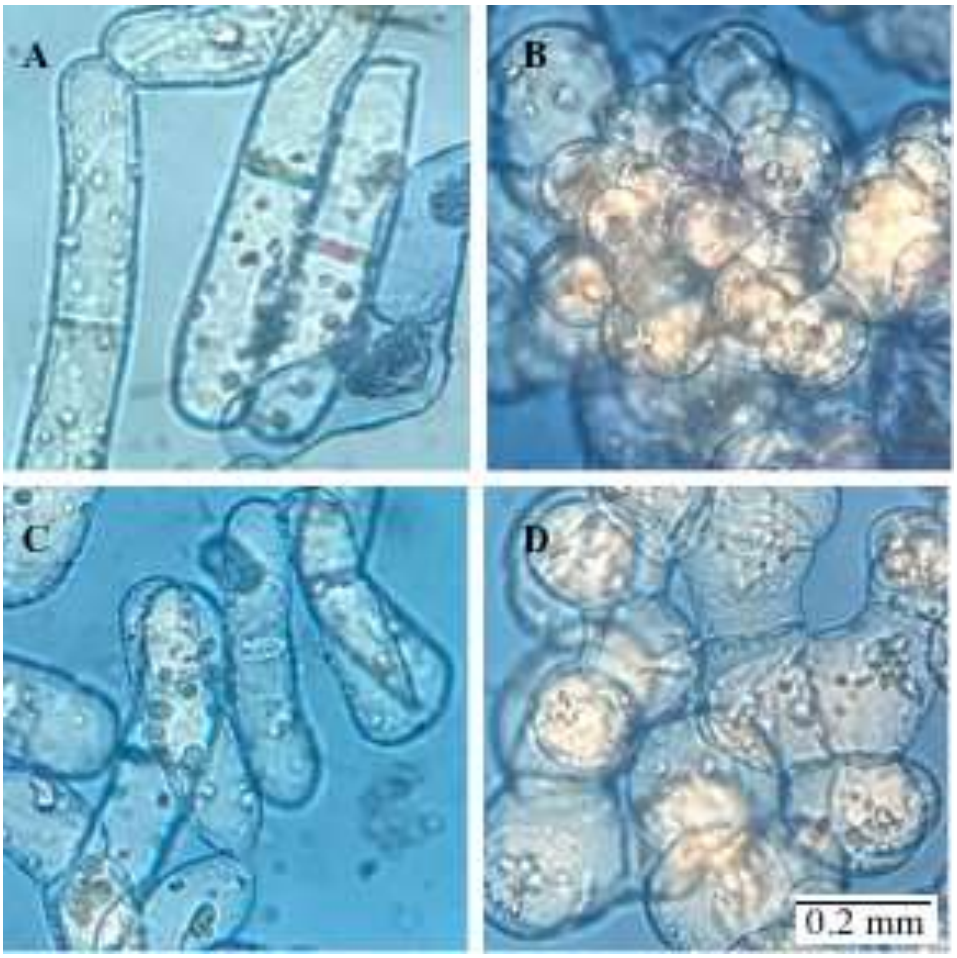

Image 1. Morphology of cell suspension cultures of *T. peruviana* in shake flask (A - day 0, B - day 16) and bench bioreactor scale (C - day 0, D - day 16).

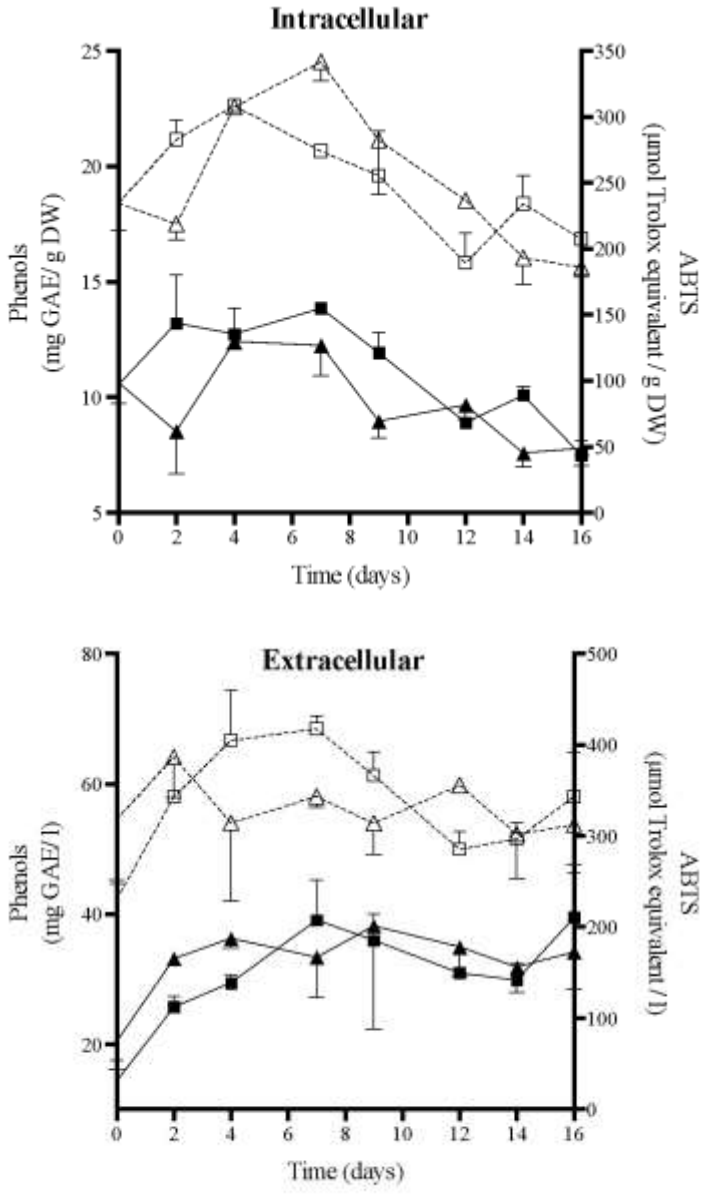

Figure 4. Intra and extracellular phenol production (close symbols) and ABTS activity (open symbols) in plant cell suspension cultures of *T. peruviana* in shake flask (—▲—) and bench reactor (—■—).

al., 2001) and *Solanum chrysotrichum* (Trejo et al., 2001). Similarly, rounded shape has been reported for *U. tomentosa* (Trejo et al., 2005) and *Trichosanthes kirilowii* (Mcdonald et al., 2001). The initial decrease in circularity and subsequent lower values for stirred tank bioreactor, compared to shake flask during culture time, could be a cell response to the change in hydrodynamic conditions. These results agree with other plant species report found in the literature. Suspension cultures of *S. chrysotrichum* at bench reactor scale showed a clear tendency to change from an elongated to a round form (Trejo et al., 2001). In contrast cell shape in *U. tomentosa* cultures changed from round to elongate in reactor culture (Trejo et al., 2005), indicating that this response is specific by species.

Table 1. The Pearson's Product-Moment Correlation Coefficients (PPMCC) between phenolic content and ABTS of the *T. peruviana* cells suspension cultures in shake flask and bench bioreactor scale.

Phenolic content vs. ABTS	PPMCC	
	Intracellular	Extracellular
Shake flask	0.975	0.926
Bench Reactor	0.979	0.916

Phenolic content and antioxidant capacity

The intra and extracellular phenolic contents and ABTS values of *T. peruviana* cell suspension cultures in shake flask and bench reactor are as shown in Figure 4. Results show that the production of polyphenols was not affected by the system configuration and scale. Only day 2 showed a significant difference. Kinetic behavior in phenols production indicates an increase until day 7, followed by a decrease. The highest intracellular phenol content was 13.86 and 12.24 mg GAE/g DW at day 7 in bench reactor and shake flask, respectively. The highest extracellular phenol content was 39.05 mg GAE/L at day 7 for bench reactor and 38.19 mg GAE/L at day 9 for shake flask.

Production of phenolic compounds in this study is higher than that obtained for other *T. peruviana* cultures, callus (2.34 mg GAE/g DW) and leaves (2.34 mg GAE/g DW) (Zibbu and Batra, 2012). Therefore, plant cell suspension cultures of *T. peruviana* are a feasible biotechnological platform for phenol production with important use in food industry.

Intra and extracellular ABTS values were directly related with phenol production in both systems. The correlation between phenolics and ABTS was calculated by the Pearson's Product-Moment Correlation Coefficient (PPMCC). PPMCC values were closed to one (Table 1) indicating positive and perfect correlation between the two variables. This behavior has been reported in previous studies (Arias et al., 2016), showing the consistency of the relationship between the production of phenolic compounds and the antioxidant capacity in plant cell suspension cultures of *T. peruviana*.

These results suggest that phenol production does not constitute an indicator of cell exposure to higher shear environment. To the best of our knowledge there is only one study showing a correlation between phenol accumulation in cell suspensions of *C. calcitrapa*, in stirred tank reactor, and different agitation rates. Therefore, suggesting that shear-sensitivity of these cells is responsible for an increased phenol production (Raposo and LimaCosta, 2006).

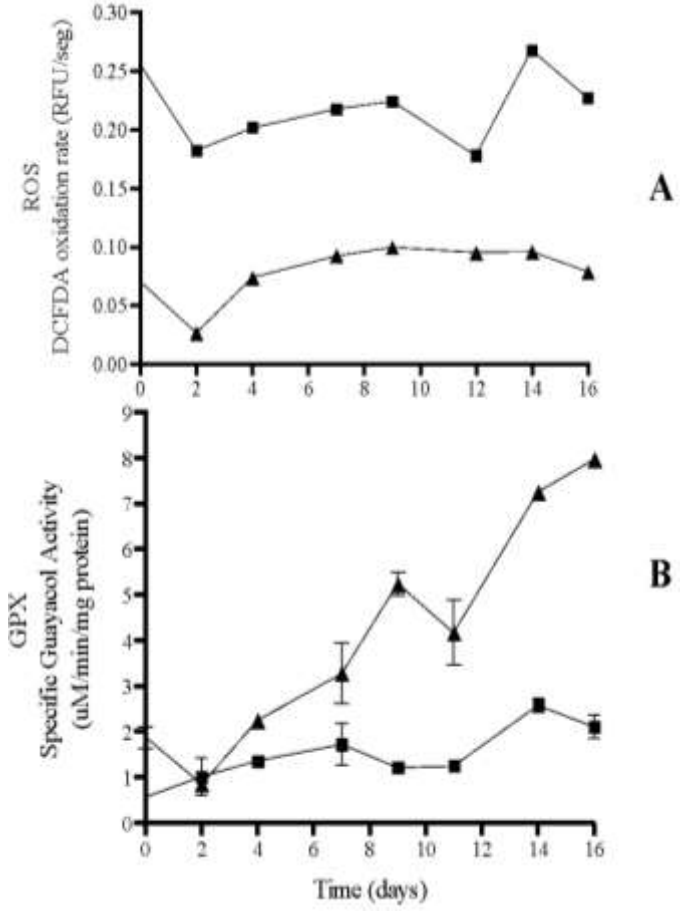

Figure 5. Intracellular reactive oxygen species (A) production and guaiacol peroxidase activity (B) in plant cell suspension cultures of *T. peruviana* in shake flask (—▲—) and bench reactor (—■—).

Other authors have reported shear-sensitivity in plant cell suspension cultures and changes in metabolite production as a response to shear rates. Suspension cultures of *R. tinctorum* showed an increase of 233% in anthraquinones content in bioreactor compared to Erlenmeyer flasks (Busto et al., 2008). Chen and Huang (2000) observed a 64% increase in L-3,4-dihydroxyphenylalanine (L-DOPA) production in *Stizolobium hassjoo* cells related to a rotational speed of 400 rpm. Shi et al. (2003) studied the shear stress applied in short term on cell suspension cultures of *T. chinensis* obtaining inhibition on phenylalanine ammonia-lyase (PAL) activity at low shear rate and high PAL activity at higher shear rate.

GPX and ROS production

The technique used in this work for ROS quantification depicts an overall view of the oxidative state of cells as response to culture conditions. Previous studies with other plant species have focused primarily on hydrogen peroxide (H_2O_2) as a ROS and its effect on cellular behavior (Busto et al., 2008; Chin et al., 2014; Trejo et al., 2007). However, the probe 2,7-dichlorodihydrofluorescein may be oxidized by the presence of other ROS besides hydrogen peroxide (Gomes et al., 2005), contributing to a broader assessment of ROS production. Figure 5 describes GPX activity and intracellular ROS production at shake flask and bench reactor scale in this study. GPX activity was higher at shake flask than in bench stirred tank bioreactor scale during the entire culture time. Increase in GPX from 1.87 to 7.97 µM/min/mg protein in shake flask correlated to low ROS production (0.09 RFU/seg). These results indicate a positive enzymatic cell response to culture condition at this scale mediated by GPX.

Figure 5A presents a higher intracellular ROS concentration for cell culture at bench stirred tank bioreactor scale compared to shake flask. This behavior was consistent throughout the culture time compared. Low GPX (Figure 5B) and high ROS production at bench reactor scale indicate that the antioxidative defense mechanism in *T. peruviana* cells is not responding or, at least, is not mainly mediated by GPX to counter the initial stressful condition; hence, leading to the observed decrease in cell growth and morphology changes without affecting cell viability. Other enzymes activities, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT), as well as non-enzymatic processes, could be taking place (Şen, 2012) and could be considered in future studies. The results reported in our study are consistent with those reported by Villegas et al. (2017) who used the same technique for ROS quantification in suspension cultures of *A. indica*. In this case, the enzymatic response to high shear stress condition in reactor scale was not mediated by guaiacol enzyme.

Finally, from our study it can be inferred that there was a stressful condition for the plant cells of *T. peruviana* cultivated at reactor scale, because their growth was considerably affected. However, the high cell viability, considerable sugar consumption, and observed morphology, in combination with the lack of enzymatic and non-enzymatic responses (by action of guaiacol peroxidase and production of phenolic compounds, respectively) suggest that the cell's metabolism could be affected to another level not identified yet.

Conclusion

This is the first study related to the change of plant cell suspension cultures of *T. peruviana* from shake flask to bench stirred tank reactor in the production of phenolic compounds. Bench reactor scale affected biomass production and sugars uptake negatively. Preferential consumption of glucose over fructose for cell suspension cultures of *T. peruviana* was found, while cell

morphology, viability, phenol production, and antioxidant activity were not significantly affected.

Increased ROS production, as an indicator of a metabolic response to a high stress condition in bench reactor scale, did not activate enzymatic and non-enzymatic responses led by guaiacol or phenolic compounds, respectively. Experimental observations indicate that the *T. peruviana* cell line is not as sensitive as other plant cell species (Busto et al., 2013; Raposo and LimaCosta, 2006; Shi et al., 2003) to shear forces under hydrodynamic stress environment. However, it is necessary to carry out further experiments in order to identify if other level of the cellular metabolism may be affected by hydrodynamic stress variations.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ABBREVIATIONS

ABTS, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); **GAE**, gallic acid equivalent; **DCFDA,** 2',7' dichlorofluorescein diacetate; **ROS,** reactive oxygen species; **GPX,** guaiacol peroxidase activity; **PVP,** polyvinylpyrrolidone; **TG,** tetraguaiacol.

REFERENCES

Arias JP, Zapata K, Rojano B, Arias M (2016). Effect of light wavelength on cell growth, content of phenolic compounds and antioxidant activity in cell suspension cultures of *Thevetia peruviana*. J. Photochem. Photobiol. B Biol. 163:87-91.

Arias M, Angarita MJ, Restrepo, JM, Caicedo LA, Perea M (2009). Elicitation with methyl-jasmonate stimulates peruvoside production in cell suspension cultures of *Thevetia peruviana*. *In vitro* Cell. Dev. Biol. Plant 46:233-238.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Busto VD, Calabró A, Rodríguez J, Giulietti AM, Merchuk JC (2013). Anthraquinones production in *Rubia tinctorum* cell suspension cultures: Down scale of shear effects. Biochem. Eng. J. 77:119-128.

Busto VD, Rodríguez J, Giulietti AM, Merchuk JC (2008). Effect of shear stress on anthraquinones production by *Rubia tinctorum* suspension cultures. Biotechnol. Prog. 24:175-181.

Chen SY, Huang SY (2000). Shear stress effects on cell growth and L - DOPA production by suspension culture of *Stizolobium hassjoo* cells in an agitated bioreactor. Bioprocess Biosyst. Eng. 22:5-12.

Chin WYW, Annuar MSM, Tan BC, Khalid N (2014). Evaluation of a laboratory scale conventional shake flask and a bioreactor on cell growth and regeneration of banana cell suspension cultures. Sci. Hortic. 172:39-46.

Curtis WR, Emery AH (1993) Plant cell suspension culture rheology. Biotechnol. Bioeng. 42:520-526.

Dias MI, Sousa MJ, Alves RC, Ferreira ICFR (2016). Exploring plant tissue culture to improve the production of phenolic compounds: A review. Ind. Crops Prod. 82:9-22.

Dixit A, Singh H, Sharma RA, Sharma A (2015). Estimation of antioxidant and antibacterial activity of crude extracts of *Thevetia peruviana* (Pers.) K. Schum. Int. J. Pharm. Pharm. Sci. 7:55-59.

Duke JA, Bogenschutz G MJ, DuCellier J, Duke PAK (2002). Handbook of Medicinal Herbs, Second. ed. CRC Press. Boca Raton, London, New York, Washington DC.

Gaff DF, Okong'o-ogola O (1971). The use of non-permeating pigments for testing the survival of cells. J. Exp. Bot. 22:756-758.

Gomes A, Fernandes E, Lima, JLFC (2005). Fluorescence probes used for detection of reactive oxygen species. J. Biochem. Biophys. Methods 65:45-80.

Gorret N, bin Rosli SK, Oppenheim SF, Willis LB, Lessard P, Rha C, Sinskey AJ (2004). Bioreactor culture of oil palm (*Elaeis guineensis*) and effects of nitrogen source, inoculum size, and conditioned medium on biomass production. J. Biotechnol.108:253-263.

Kieran PM, Malone DM, MacLoughlin PF (2000). Effects of hydrodynamic and interfacial forces on plant cell suspension systems. Adv. Biochem. Eng. Biotechnol. 67:139-177.

Krook J, Vreugdenhil D, Van Der Plas LHW (2000). Uptake and phosphorylation of glucose and fructose in *Daucus carota* cell suspensions are differently regulated. Plant Physiol. Biochem. 38:603-612.

Li C, Yuan Y, Wu J, Hu Z (2003). A structured kinetic model for suspension cultures of *Taxus chinensis* var. mairei induced by an oligosaccharide from Fusarium oxysporum. Biotechnol. Lett. 25:1335-1343.

Martín-Romero FJ, Miguel-Lasobras EM, Domínguez-Arroyo JA, González-Carrera E, Álvarez IS (2008). Contribution of culture media to oxidative stress and its effect on human oocytes. Reprod. Biomed Online 17:652-661.

Mcdonald KA, Jackman AP, Hurst S (2001). Characterization of plant suspension cultures using the focused beam reflectance technique. Biotechnol. Lett. 23:317-324.

Omar R, Abdullah MA, Hasan MA, Rosfarizan M, Marziah M (2006) Kinects and modelling of cell growth and substrate uptake in *Centella asiatica* cell culture. Biotechnol. Bioprocess Eng. 11:223-229.

Paek K, Murthy HN, Zhong J (2014). Production of Biomass and Bioactive Compounds Using Bioreactor Technology. Springer.

Raposo S, LimaCosta ME (2006). Rheology and shear stress of *Centaurea calcitrapa* cell suspension cultures grown in bioreactor. Biotechnol. Lett. 28:431-438.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 26:1231-1237.

Rincón J, Rodríguez L, Ruíz VM, Abud M, Luján MC, Ruiz N, González D, Gutiérrez FA (2016). Fatty acids profile, phenolic compounds and antioxidant capacity in elicited callus of *Thevetia peruviana* (Pers.) K. Schum. J. Oleo Sci. 318:311-318.

Rodríguez M, Galindo E (1999). Broth rheology, growth and metabolite production of *Beta vulgaris* suspension culture: A comparative study between cultures grown in shake flasks and in a stirred tank. Enzyme Microb. Technol. 24:687-693.

Ruffoni B, Pistelli L, Bertoli A, Pistelli L (2010). Plant cell cultures: bioreactors for industrial production. Adv. Exp. Med. Biol. 698:203-221.

Schenk RU, Hildebrandt AC (1972). Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50:199-204.

Şen A (2012). Oxidative Stress Studies in Plant Tissue Culture, In. Antioxidant Enzyme. pp. 59-88.

Shi Z, Yuan Y, Wu J, Shang G (2003). Biological responses of suspension cultures of *Taxus chinensis* var. mairei to shear stresses in the short term. Appl. Biochem. Biotechnol. 110:61-74.

Singleton VL, Rossi JJ (1965). Colorimetric of total phenolics with phosphomolybdic-phosptungstic acid reagents. Am. J. Enol. Vitic. 16:144-158.

Trejo G, Cerda CM, Rodríguez M, Ramos AC (2005). Monoterpenoid oxindole alkaloid production by *Uncaria tomentosa* (Willd) D.C. cell suspension cultures in a stirred tank bioreactor. Biotechnol. Prog. 21:786-792.

Trejo G, Jiménez A, Villarreal L, Rodríguez M (2001). Broth rheology and morphological analysis of *Solanum chrysotrichum* cultivated in a stirred tank. Biotechnol. Lett. 23:1943-1946.

Trejo G, Sepúlveda G, Trejo JL, Cerda CM, de la Torre M, Rodríguez M, Ramos AC (2007). Hydrodynamic stress induces monoterpenoid oxindole alkaloid accumulation by *Uncaria tomentosa* (Willd) DC cell

suspension cultures via oxidative burst. Biotechnol. Bioeng 98:230-238.
Villegas S, Martínez AD, Hoyos R, Rojano B, Orozco F (2017). Hydrodynamic stress and limonoid production in *Azadirachta indica* cell culture. Biochem. Eng. J. 122:75-84.
Zibbu G, Batra A (2012). *In vitro* and *in vivo* determination of phenolic contents and antioxidant activity of desert plants of Apocynaceae family. Asian J. Pharm. Clin. Res. 5:75-83.

African Journal of Biotechnology

Related Journals Published by Academic Journals

- *Biotechnology and Molecular Biology Reviews*
- *African Journal of Microbiology Research*
- *African Journal of Biochemistry Research*
- *African Journal of Environmental Science and Technology*
- *African Journal of Food Science*
- *African Journal of Plant Science*
- *Journal of Bioinformatics and Sequence Analysis*
- *International Journal of Biodiversity and Conservation*

academicJournals