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Effect of Biotic Elicitors on Secondary Metabolite Production in Cell Suspensions of *Hypericum triquetrifolium* Turra

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Abstract. Many experiments were designed to induce and increase the production of some secondary metabolites in tissue cultures of *Hypericum triquetrifolium*Turra using some biotechnological approaches. Callus was initiated on leaf discs cultured on MS medium supplemented with thidazirion (TDZ) at the concentrations 1.0, 1.25, 1.5, 2.0, or 2.5 mgL^{-1} and indole-3-acetic acid (IAA) at 0.5 mgL^{-1}; callus was also initiated on stem explants on MS medium supplemented with 1.25 mgL^{-1} 6-benzyl-aminopurine (BAP) and 0.5 mgL^{-1} of IAA. HPLC was used to determine the type and quantity of secondary metabolites in comparison with standards. Phytochemical accumulation in suspension cultures derived from leaf (LCs), stem (SCs) and root (RCs) were investigated. Fungal extracts of *Aspergillus niger*, *Fusarium oxysporum* and commercial yeast were added to a liquid MS medium at 0.1, 0.25, 0.5 or 0.75 mgL^{-1}. Data showed that the yield of p-OH-benzoic acid and chlorgenic acid in LCs treated with 0.5 mgL^{-1} yeast extract increased significantly compared with the control. Caffeic acid and tannic acid decreased in LCs significantly after elicitation with all biotic elicitors, but catechin accumulation in LCs increased significantly, when *A. niger* extract was added at all concentrations. Rutin, hypersoid and quercitin production in SCs increased significantly when treated with the fungal elicitors*; A. niger*, *F. oxysporum* and yeast extracts. Chlorgenic decreased significantly in SCs, after the addition of all biotic elicitors at different concentrations.

Keywords: *Hypericum triquetrifolium* Turra, medicinal, secondary metabolite, elicitors, biotic stress

INTRODUCTION

The genus *Hypericum* encompasses approximately 460 different species of annuals, perennials, shrubs and small trees, widespread all over the world, but only sixteen species are found in Iraq; the most abundant herbs are *Hypericum perforatum* L. and *H. triquetrifolium* Turra (Al-Mukhtar, 1975). In this genus, some species have been used since ancient times as folk remedies and credited with a long list of medicinal uses, including antiviral, antimicrobial, antifungal, antitumor, analgesic, sedative and for the treatment of neurological disordered and depression (Barnes *et al*., 2001). Previous phytochemical studies on *H. perforatum* and other related species have led to the isolation and identification of several groups of phytochemicals including phenolic compounds (chlorgenic acid, tannic acid and caffeic acid), flavonoids (rutin, hypersoid, isoquercitrin, quercitrin, quercitin, and catechin), naphtodianthrones (hypericin and psudohypericin), and the phloroglucinols (hyperforin and adhypeforin) and essential oil (Hosni *et al*., 2011; Ghavamaldin *et al*., 2012).

The developing field of plant biotechnology may provide a better alternative for the large scale production of secondary metabolites; the use of biotechnological approaches, specifically plant cell and tissue cultures for the large-scale production of secondary metabolites has so far achieved only limited success due to the low and unreliable yields of the secondary products (Vijaya Sree *et al*., 2010). Various strategies have been developed to

improve the production of secondary metabolites. This means that the maximization of the production and accumulation of secondary metabolites by plant tissue cultured cells requires (i) manipulating the parameters of the environment and medium, (ii) selecting high yielding cell clones, (iii) precursor feeding, (iv) elicitation and (v) hairy root cultures (Mulabagal and Tsay, 2004). Elicitation is the induction of secondary metabolite production by either biotic or abiotic treatments. Nowadays, the use of pathogenic and non-pathogenic fungal preparations and chemicals as elicitors has become one of the most important and successful strategies to improve secondary metabolite production in plant cell culture (Baldi *et al*., 2009). A few studies are reported in the literature about medical usage of *H. triquetrifolium* in Iraq, this research work is aimed to quantify the secondary metabolites such as phenolic compounds (p-OH-benzoic acid, caffeic acid, chlorgenic acid and tannic acid), flavonoids (catechin, rutin, hypersoid and quercitin) after the exposure of cell suspension cultures derived from excised leaf and stem explants to fungal extracts as biotic elicitors.

MATERIALS AND METHODS

Plant material

Seeds of *H. triquetrifolium* Turra were collected from intact plant during December 2010 to January 2011 from wild population in the locality of Tasluja, in dry rocky soil of Sulaimania city, altitude 1000 m.a.s.l. Plant samples were dried at room temperature for 7 days. Dried plant material was then packed in paper bags and kept in a dark, dry and cool place.

Seed sterilization and Seed germination.

H. triquetrifolium Turra seeds were collected from Tasluja, Sulaimania city. They were surface sterilized by immersing in a 70% (v/v) ethanol for 30 sec, and then in a 20% (v/v) commercial bleach (5% NaOCl) plus 0.1 Tween 20 (polyoxythylene sorbitan monolaurete) for 25 min. Subsequently, they were washed 4 times with sterilized distilled water (Oluk *et al*., 2010). Seeds were germinated by two methods either by pre-soaking treatments with different GA_3, H_2SO_4 doses, and D.W. before placing in Petri dishes. They were soaked in 50, 100, and 150 mg/l GA_3, 1.5% H_2SO_4 and D.W. for 30 min. Treated seeds were placed individually in sterilized Petri dishes containing moisture-retaining paper liners. Paper liners in the Petri dishes were kept moist throughout the germination period; seedling was inoculated to test tubes (5 cm×9.5 cm) containing 25 ml of the ½ strength MS medium solidified with 0.8 w/v agar. Cultures were subjected to a photoperiod of 16/8 hrs (light/dark) in a growth chamber. Temperature was set at 25°C. Germination was measured after 20 days, seeds showed radical emergence and were recorded as germinated (Cirak, 2007); Or sterilized seeds were cultured on 0.8% (w\v) water-agar medium, after pre-soaking treatments. One seed placed in one test tube (5cm×9.5cm) containing 25 ml of water-agar. Cultures were maintained at 25°C under a photoperiod of 16/8 hrs (light/dark), and monitored every day for a period of 20 days (Oluk and Orhan, 2009).

Callus induction

Seedlings obtained from aseptically germinated seeds (14-20 days old) were cut into 1 cm long explants (leaf, stem and root) using surgical blade and forceps under aseptic conditions. They were inoculated into MS medium (10 ml/test tube) that supplemented with the auxin IAA (0.0 or 0.5) mgL^{-1} and the cytokinin TDZ (0.0, 1.0, 1.25, 1.5, 2.0 or 2.5) mgL^{-1} for leaf explants. For stem and root explants the auxin IAA (0.0 or 0.5) mgL^{-1} and the cytokinin BAP (0.0 or 1.25) mgL^{-1} were added to the culture medium. Those were used to investigate the response of callus induction on dissected explants. The cultures were

incubated at 25^{o}C for 16/8 hrs (light/dark) photoperiod at light intensity of 1000 lux. Callus fresh and dry weights were measured after 30 days (Oluk *et al*., 2010).

Cell suspension cultures.

H. triquetrifolum cell suspension cultures were initiated from 21-28 days callus cultures, approximately 5-10 g of callus fresh weight were cultured in 250 ml MS liquid medium supplemented with the same components as in callus maintenance medium without agar. Flasks were placed on a rotary shaker at 100-120 rpm, 27°C under 16/ 8 hrs light/dark with a light intensity of 1000 lux in a growth room for 14 days (Gopi and Vatsala, 2006).

The fungi extract of *Aspergillus niger* and *Fusarium oxysporum* were extracted according to the method described by Karwasara *et al*., (2010), but commercial yeast extracts were prepared by the method described by Hahn and Albersheim (1978). Fungal strains of *A. niger* and *F. oxysporum* were obtained from the Botanical garden, University of Babes Bolyai, Cluj-Napoca, Romania, which were identified by Prof. Porium Marcel. The fungi were maintained on Czapeck yeast extract agar (CYA) medium slant, transferred to 150 ml liquid potato dextrose agar (PDA) medium in 500-ml Erlenmeyer flasks, shaken at 100 rpm on a rotary shaker at 30°C for 14 days. After 14 days, the fungal suspension cultures were autoclaved at 1.04 kg/cm2, 121°C for 20 min. The autoclaved cultures were centrifuged and filtered through Whatman no.1 filter paper, then dried at room temperature for 48 hrs, and ground using pestle and mortar. Dried samples were stored at 4-8°C until further use. Yeast extracts were prepared from commercial yeast. Different concentrations of fungal and yeast extracts (0, 0.1, 0.25, 0.5 or 0.75 gL^{-1}) were added to liquid MS medium supplemented with 2 mg/l TDZ and 0.5 mgL^{-1} IAA containing calli derived from leaf; 1.25 mgL^{-1} BAP and 0.5 mgL^{-1} IAA for calli derived from stem and roots. Cultures were placed on a rotary shaker at 110-120 rpm for 16/8 hrs (light/dark) regime at 27±1°C for 14 days. Three replications were used for each treatment.

Preparation of plant extracts

Plant materials were dried at room temperature (25 ± 2 °C). A quantity of 0.5 g of the air dried plant material (L, S and R) that dissected from seedlings in *vitro*; LCs, SCs, and RCs was mechanically ground using a laboratory mill to obtain a homogenous powder. Extraction was carried out with 20 ml of methanol (HPLC grade) for 30 min. using ultrasonic cleaning bath, at a frequency of 35 KHz, to remove chlorophyll contents (Smelcerovic *et al*., 2006). The temperature of the water bath was regulated at 30°C. Extraction procedure was performed for 2 hrs with the assistance of magnetic stirrer under protection from day light, since it was covered with umber glass and aluminum foil. After decantation, the extract was separated and the residual sample was re-extracted with 10 ml of methanol for 1 hr (this process was repeated twice), centrifuged for 10 min. at 3000 rpm, and then concentrated using a rotary vacuum evaporator. Extracts were filtered through a Millipore unit with a pore size of 0.22 µm. The prepared extracts were kept in dark inside a refrigerator until use. Three readings were done for each sample using HPLC and the mean value was calculated (Hosni *et al*., 2011).

Separation and quantification of secondary metabolites

Secondary metabolites compounds were separated from extracted samples for the three plant parts (leaf, stem and root) of *H. triquetrifolium* Turrra using a method described by Hosni *et al*., (2011). A Shimaduz liquid chromatograph (Shimaduz corp, Kyoto, Japan) consisting of a LC-20AT quaternary pump, a DGU-20A3 degasser, an SPD-M20Adiode array detector and a manual rheodyne injector with a 20µl loop were used. Peaks were identified by comparison of their retention times with those of the reference standard and UV spectra in the range of 200-800 nm. The standard curves were obtained by plotting the peak areas of standard concentrations for phenolic acids (0.05, 0.1, 0.2, 0.3, or 0.5 $mgml^{-1}$), and for

flavonoids (0.0062, 0.0125, 0.025, 0.05, or 0.1 $mgml^{-1}$). Standard solutions were kept in dark at -20°C HPLC conditions, LC time program: 35 min., Total of mobile phase: 1ml/min, Injection volume: 20µl, Temperature of column: room temp. Pressure on pumps: (A, B, C) 7.9 – 9.0 milpaskal (Mpa) Mobile phase: A: water 99% - phosphoric acid 0.3%, B: Acetonitrile 100%, C: Methanol 100%.

Statistical analysis and experimental design

Statistical analysis was conducted using a completely randomized design (one-way) with fifteen replicates for tissue culture experiments were used. The same design was used for sample analysis but with three replicates by Duncan's multiple range test for mean comparison at ($P \leq 0.05$) using the Statistical Package for the Social Sciences (SPSS, version 17) (Duncan, 1995).

RESULTS AND DISCUSSIONS

H. triquetrifolium cell suspension cultures derived from leaves (LCs), stems (SCs) and roots (RCs) accumulated secondary metabolites like flavonoids and phenolic acids compounds as a response to *A. niger*, *F. oxysporum* and yeast extracts which acted as biotic elicitors (Tables 1, 2, and 3). Significant differences were recorded among the different treatments, LCs treated with 0.5 gL^{-1} of yeast extract increased p-OH-benzoic acid and chlorgenic acid significantly reached (0.0217, and 0.1223 mgg^{-1} dwt.) respectively compared with that of *A. niger* and *F. oxysporum*, while the highest concentration of p-OH-benzoic acid in SCs and RCs was recorded at a concentration 0.75 gL^{-1} yeast extract recording 0.0330 and 0.0070 mgg^{-1} dwt. respectively.

RCs cell primed with all elicitor treatments produced p-OH-benzoic acid which was not detected in control samples. This result is in agreement with those of Beerhues and Berger (1995) who reported that the *Centarium erythraea* and *C. littorale* accumulated and produced new phenolic acids and flavonoids after cell cultures were treated with methyl-jasmonate and yeast extract. Maximum content of chlorgenic acid in SC and RC was found in non-treated cell suspension cultures (control) with mean values 0.0970 and 0.0753 mgg^{-1} dwt. respectively. *Hypericum* cell suspension cultures treated with the all biotic elicitors were negatively affected in caffeic acid production in all plant parts, except SCs treated with 0.1 or 0.25 gL^{-1} of *F. oxysporum* since it produced 0.0517 and 0.0533 mgg^{-1} dwt. respectively.

Treatment with the a biotic elicitor *A. niger* at 0.1 gL^{-1} produced 0.8030 mgg^{-1} dwt. of catechin compound in LCs, while it reached 0.9313 mgg^{-1} dwt. when the concentration of elicitor increased to 0.75 gL^{-1}, and significantly increased in all concentrations, when compared with control. Despite the treatment with *F. oxysporum* as elicitor, it produced 0.8170 mgg^{-1} dwt. at 0.25 gL^{-1}. The addition of increasing concentrations of yeast extract (0.1-0.75 gL^{-1}) in the medium resulted in a decreased accumulation of catechin compound. The highest accumulation of catechin in SCs was recorded in cell suspension cultures lack elicitors (0.7930 mgg^{-1} dwt.) except treatment with yeast extract at 0.75 gL^{-1} which gave 0.7947 mgg^{-1} dwt. This effect of the fungal elicitor was not significantly different compared with the control. RCs treated with all elicitors displayed significant differences in catechin production compared to the control. Maximum accumulation of catechin (0.7197 mgg^{-1} dwt.) was recorded after the treatments with the elicitors *F. oxysporum* and *A. niger* at the concentrations of 0.25 and 0.75 gL^{-1} respectively which achieved a 3-fold increase in the production over control.

Tab. 1

Effect of different concentrations of fungal extracts on the production of phenolic compounds and flavonoids (mgg^{-1} dwt.) in cell suspension cultures initiated from leaf explants callus source.

Compound	Biotic elicitor	Control	Elicitor concentration (gL^{-1})			
			0.1	0.25	0.5	0.75
P-OH-benzoic acid	*A. niger*	0.0067 ^{b}	0.0070 ^{b}	0.000 ^{a}	0.000 ^{a}	0.000 ^{a}
	F. oxysporum	0.0067 ^{b}	0.000 ^{a}	0.000 ^{a}	0.0067 ^{b}	0.000 ^{a}
	Yeast extract	0.0067 ^{b}	0.0045 ^{b}	0.0077 ^{b}	0.0217 ^{d}	0.0153 ^{c}
Chlorgenic acid	*A. niger*	0.1067 ^{f}	0.0777 ^{d}	0.0910 ^{e}	0.0533 ^{bc}	0.0397 ^{a}
	F. oxysporum	0.1067 ^{f}	0.0753 ^{d}	0.0710 ^{d}	0.0753 ^{d}	0.0687 ^{d}
	Yeast extract	0.1067 ^{f}	0.0477 ^{ab}	0.0583 ^{c}	0.1223 ^{g}	0.0993 ^{ef}
Caffeic acid	*A. niger*	0.1455 ^{h}	0.0069 ^{a}	0.0074 ^{a}	0.0078 ^{a}	0.0076 ^{a}
	F. oxysporum	0.1455 ^{h}	0.0159 ^{d}	0.0183 ^{e}	0.0282 ^{g}	0.0211 ^{f}
	Yeast extract	0.1455 ^{h}	0.0111 ^{b}	0.0129 ^{c}	0.0132 ^{c}	0.0109 ^{b}
Catechin	*A. niger*	0.6250 ^{f}	0.8030 ^{h}	0.8913 ^{i}	0.7113 ^{g}	0.9313 ^{j}
	F. oxysporum	0.6250 ^{f}	0.6927 ^{g}	0.8170 ^{h}	0.000 ^{a}	0.000 ^{a}
	Yeast extract	0.6250 ^{f}	0.1390 ^{b}	0.1800 ^{c}	0.3660 ^{d}	0.5697 ^{e}
Rutin	*A. niger*	0.0067 ^{a}	0.1575 ^{l}	0.1372 j	0.1578 ^{l}	0.1475 ^{k}
	F. oxysporum	0.0067 ^{a}	0.0330 ^{c}	0.0488 ^{d}	0.0181 ^{b}	0.0517 ^{e}
	Yeast extract	0.0067 ^{a}	0.0641 ^{g}	0.0553 ^{f}	0.0902 ^{i}	0.0737 ^{h}
Hypersoid	*A. niger*	0.0382 ^{c}	0.0625 ^{g}	0.0611 ^{g}	0.0874 ^{i}	0.0752 ^{h}
	F. oxysporum	0.0382 ^{c}	0.0225 ^{a}	0.0216 ^{a}	0.0267 ^{b}	0.0264 ^{b}
	Yeast extract	0.0382 ^{c}	0.0564 ^{f}	0.0514 ^{e}	0.0610 ^{g}	0.0466 ^{d}
Quercitin	*A. niger*	0.0086 ^{a}	0.0160 ^{ab}	0.0187 ^{ab}	0.0155 ^{ab}	0.0188 ^{ab}
	F. oxysporum	0.0086 ^{a}	0.0788 ^{a}	0.0133 ^{ab}	0.0265 ^{ab}	0.0135 ^{ab}
	Yeast extract	0.0086 ^{a}	0.0324 ^{b}	0.0111 ^{ab}	0.0155 ^{ab}	0.0147 ^{ab}
Tannic acid	*A. niger*	0.0610 ^{h}	0.0167 ^{d}	0.0156 ^{cd}	0.0139 ^{ab}	0.0132 ^{a}
	F. oxysporum	0.0610 ^{h}	0.0151 ^{bc}	0.0249 ^{g}	0.0214 ^{f}	0.0127 ^{a}
	Yeast extract	0.0610 ^{h}	0.0197 ^{e}	0.0254 ^{g}	0.0256 ^{g}	0.0227 ^{f}

Notes: 1- Values followed by different small letters in the columns are significantly different ($P \leq 0.05$) according to Duncan's multiple ran test.

2- These compounds were analyzed separately in horizontal direction

Addition of *A. niger* fungal elicitor at 0.1 gL^{-1}, *F. oxysporum* at 0.75 gL^{-1} and yeast extract at 0.5 gL^{-1} resulted in a maximum yield of rutin yielding 23.5 times, 7.7 times and 13.4 times respectively, compared to the control culture of LCs. While in the case of SCs, biotic elicitors significantly increased rutin accumulation compared to the control, *A. niger* showed a maximum effect at a concentration of 0.25 gL^{-1}, and producing 4 times increase more than control. Also it was reported that *F. oxysporum* at a concentration of 0.75 gL^{-1}, raised the yield about 2-fold over control, but treatment with 0.25 gL^{-1} yeast extract recorded an increase of 1.7 times. Addition of *A. niger*, *F. oxysporum* and yeast extracts to the cell suspension cultures significantly decreased the accumulation of rutin in RCs at the end of the culture period, except for the yeast extract elicitor at a 0.5 and 0.75 gL^{-1} which showed a significant increase (4.4 and 2.2-fold) in rutin accumulation respectively. Hypersoid concentration in LCs gL^{-1} without any treatment gave 0.0382 mgg^{-1} dwt., while cell suspension cultures elicited with 0.5 gL^{-1} of *A. niger* produced maximum yield 0.0874 mgg^{-1} dwt. Hypersoid concentration reached 0.0874 mgg^{-1} dwt. When LCs was treated with 0.5 gL^{-1} of *A. niger* elicitor.

Tab. 2

Effect of different concentrations of fungal extracts on the production of phenolic compounds and flavonoids (mgg^{-1} dwt.) in cell suspension cultures initiated from stem explants callus source.

Compound	Biotic elicitor	Control	Elicitors concentration (gL^{-1})			
			0.1	0.25	0.5	0.75
P-OH-benzoic acid	*A. niger*	0.0123 ^{d}	0.0050 ^{b}	0.0093 ^{c}	0.000 ^{a}	0.0063 ^{b}
	F. oxysporum	0.0123 ^{d}	0.0127 ^{d}	0.0060 ^{b}	0.0057 ^{b}	0.000 ^{a}
	Yeast extract	0.0123 ^{d}	0.0217 ^{d}	0.0210 ^{e}	0.0183 ^{e}	0.0330 ^{f}
Chlorgenic acid	*A. niger*	0.0970 ^{g}	0.0570 ^{cd}	0.0477 ^{ab}	0.0413 ^{a}	0.0560 ^{cd}
	F. oxysporum	0.0970 ^{g}	0.0550 ^{c}	0.0690 ^{e}	0.0480 ^{b}	0.0720 ^{e}
	Yeast extract	0.0970 ^{g}	0.0733 ^{e}	0.0613 ^{d}	0.0823 ^{f}	0.0487 ^{b}
Caffeic acid	*A. niger*	0.0266 ^{b}	0.0066 ^{a}	0.0063 ^{a}	0.0043 ^{a}	0.0059 ^{a}
	F. oxysporum	0.0266 ^{b}	0.0517 ^{c}	0.0533 ^{c}	0.0259 ^{b}	0.0125 ^{a}
	Yeast extract	0.0266 ^{b}	0.0109 ^{a}	0.0102 ^{a}	0.0096 ^{a}	0.0073 ^{a}
Catechin	*A. niger*	0.7930 ^{f}	0.7430 ^{ef}	0.6180 ^{d}	0.6020 ^{d}	0.6353 ^{d}
	F. oxysporum	0.7930 ^{f}	0.6707 ^{de}	0.7900 ^{f}	0.5027 ^{c}	0.6620 ^{de}
	Yeast extract	0.7930 ^{f}	0.2608 ^{a}	0.3283 ^{ab}	0.3977 ^{b}	0.7947 ^{f}
Rutin	*A. niger*	0.0182 ^{a}	0.0690 ^{i}	0.0727 ^{j}	0.0443 ^{g}	0.0477 ^{h}
	F. oxysporum	0.0182 ^{a}	0.0236 ^{b}	0.0290 ^{d}	0.0247 ^{bc}	0.0373 ^{f}
	Yeast extract	0.0182 ^{a}	0.0232 ^{b}	0.0312 ^{e}	0.0258 ^{c}	0.0226 ^{b}
Hypersoid	*A. niger*	0.0144 ^{a}	0.0405 ^{g}	0.0527 ^{h}	0.0417 ^{g}	0.0417 ^{g}
	F. oxysporum	0.0144 ^{a}	0.0215 ^{d}	0.0255 ^{e}	0.0189 ^{c}	0.0173 ^{b}
	Yeast extract	0.0144 ^{a}	0.0226 ^{d}	0.0286 ^{f}	0.0224 ^{d}	0.0247 ^{e}
Quercitin	*A. niger*	0.0034 ^{a}	0.0123 ^{de}	0.0148 ^{f}	0.0135 ^{ef}	0.0136 ^{f}
	F. oxysporum	0.0034 ^{a}	0.0117 ^{cd}	0.0138 ^{f}	0.0278 ^{g}	0.0143 ^{f}
	Yeast extract	0.0034 ^{a}	0.0100 ^{b}	0.0109 ^{bc}	0.0110 ^{bcd}	0.0109 ^{bc}
Tannic acid	*A. niger*	0.0233 ^{ab}	0.0335 ^{b}	0.0166 ^{ab}	0.0157 ^{a}	0.0163 ^{ab}
	F. oxysporum	0.0233 ^{ab}	0.0166 ^{ab}	0.0167 ^{ab}	0.0177 ^{ab}	0.0165 ^{ab}
	Yeast extract	0.0233 ^{ab}	0.0167 ^{ab}	0.0207 ^{ab}	0.0209 ^{ab}	0.0229 ^{ab}

Notes: 1- Values followed by different small letters in the columns are significantly different at ($P \leq 0.05$) according to Duncan's multiple range test.

2- These compounds were analyzed separately in horizontal direction

Hypersoid concentration reached 0.0752 mgg^{-1} dwt. when LCs treated with 0.75 gL^{-1} of *A. niger*. LCs treated with *F. oxysporum* extract caused a decreased in hypersoid compound significantly after 14 days of elicitation compared to the control, LCs treated with yeast extract, significantly increase the production of hypersoid in all concentrations. In addition, the elicitation treatment of SCs significantly increased hypersoid accumulation. It was observed that *A. niger* extract was more effective stimulator than *F. oxysporum* and yeast extract, since the highest accumulation was recorded at 0.25 gL^{-1}, which was the highest among others, giving 0.0527 mgg^{-1} dwt. of hypersoid. The concentration of hypersoid in RCs increased significantly at different treatments except RCs treated with *A. niger* at 0.25 gL^{-1} which showed no significant differences compared with the control.

The influence of elicitor extracts on the quercitin contents in LCs after 14 days of elicitation resulted in non- significant differences among all biotic elicitors compared with non- elicited LCs, except the elicited cells treated with yeast extract at 0.1 gL^{-1} that gave 0.0324 mgg^{-1} dwt. It increased significantly 3.7 times over control, while the most effective stimulator was *F. oxysporum* extract at a concentration of 0.5 gL^{-1}, which gave 0.0278 mgg^{-1} dwt. of quercitin in SCs. However, it was found that the fungal elicitor *A. niger* at 0.1 gL^{-1} was higher among others, which produced 0.0444 mgg^{-1} dwt. of quercitin in RCs. Results showed that the incorporation of increasing concentrations of biotic elicitors homogenate of

A. niger, *F. oxysporum*, and yeast extract into the medium resulted in non-significant differences among the treatments and the control in tannic acid production, except LCs elicited with *A. niger* at a concentration of 0.1 gL^{-1}, which gave 0.0335 mgg^{-1} dwt. Tannic acid accumulation decreased significantly in all elicitor treated in LCs and SCs, but the accumulation of tannin in RCs elicited cells increased significantly treated with all biotic elicitors, but *A. niger* at a concentration of 0.5 gL^{-1} which was found the most effective biotic elicitor among others recording 0.0308 mgg^{-1} dwt.

Tab. 3

Effect of different concentrations of fungal extracts on the production of phenolic compounds and flavonoids (mgg^{-1} dwt.) in cell suspension cultures initiated from root explants callus source.

Compound	Biotic elicitor	Control	Elicitors concentration (gL^{-1})			
			0.1	0.25	0.5	0.75
P-OH-benzoic acid	*A. niger*	0.000 ^{a}	0.0333 ^{f}	0.0047 ^{bcd}	0.0050 ^{bcd}	0.0050 ^{bcd}
	F. oxysporum	0.000 ^{a}	0.0033 ^{b}	0.0053 ^{cde}	0.0047 ^{bcd}	0.0037 ^{bc}
	Yeast extract	0.000 ^{a}	0.0060 ^{de}	0.0050 ^{bcd}	0.0053 ^{cde}	0.0070 ^{e}
Chlorgenic acid	*A. niger*	0.0753 ^{g}	0.0443 ^{cd}	0.0450 ^{cd}	0.0380 ^{b}	0.0473 ^{d}
	F. oxysporum	0.0753 ^{g}	0.0320 ^{a}	0.0650 ^{f}	0.0613 ^{ef}	0.0390 ^{bc}
	Yeast extract	0.0753 ^{g}	0.0633 ^{f}	0.0497 ^{d}	0.0730 ^{g}	0.0563 ^{e}
Caffeic acid	*A. niger*	0.5320 ^{f}	0.0169 ^{b}	0.0159 ^{b}	0.0259 ^{e}	0.0258 ^{e}
	F. oxysporum	0.5320 ^{f}	0.0211 ^{d}	0.0211 ^{d}	0.0254 ^{e}	0.0189 ^{bc}
	Yeast extract	0.5320 ^{f}	0.0063 ^{a}	0.0061 ^{a}	0.0056 ^{a}	0.0039 ^{a}
Catechin	*A. niger*	0.2347 ^{a}	0.6910 ^{g}	0.5433 ^{d}	0.5243 ^{c}	0.7197 ^{h}
	F. oxysporum	0.2347 ^{a}	0.3060 ^{b}	0.7197 ^{h}	0.7587 ^{i}	0.3177 ^{b}
	Yeast extract	0.2347 ^{a}	0.5287 ^{c}	0.5470 ^{d}	0.6293 ^{f}	0.6077 ^{e}
Rutin	*A. niger*	0.0438 ^{c}	0.0167 ^{ab}	0.0216 ^{a}	0.0202 ^{abc}	0.0188 ^{abc}
	F. oxysporum	0.0438 ^{c}	0.0153 ^{ab}	0.0163 ^{ab}	0.0204 ^{abc}	0.0153 ^{ab}
	Yeast extract	0.0438 ^{c}	0.0440 ^{c}	0.0419 ^{bc}	0.1920 ^{e}	0.0974 ^{d}
Hypersoid	*A. niger*	0.0076 ^{a}	0.0112 ^{b}	0.0084 ^{a}	0.0132 ^{c}	0.0115 ^{b}
	F. oxysporum	0.0076 ^{a}	0.0103 ^{b}	0.0111 ^{b}	0.0133 ^{c}	0.0102 ^{b}
	Yeast extract	0.0076 ^{a}	0.0236 ^{e}	0.0257 ^{f}	0.0235 ^{e}	0.0199 ^{d}
Quercitin	*A. niger*	0.0023 ^{a}	0.0444 ^{f}	0.0202 ^{bcde}	0.0318 ^{def}	0.0258 ^{cde}
	F. oxysporum	0.0023 ^{a}	0.0262 ^{bcde}	0.0193 ^{bcde}	0.0336 ^{ef}	0.0293 ^{def}
	Yeast extract	0.0023 ^{a}	0.0114 ^{ab}	0.0127 ^{abc}	0.0185 ^{bcde}	0.0163 ^{abcd}
Tannic acid	*A. niger*	0.0134 ^{a}	0.0301 ^{f}	0.0214 ^{c}	0.0308 ^{f}	0.0282 ^{e}
	F. oxysporum	0.0134 ^{a}	0.0219 ^{c}	0.0261 ^{d}	0.0275 ^{de}	0.0213 ^{c}
	Yeast extract	0.0134 ^{a}	0.0169 ^{b}	0.0170 ^{b}	0.0171 ^{b}	0.0164 ^{b}

Notes: 1- Values followed by different small letters in the columns are significantly different at ($P \leq 0.05$) according to Duncan's multiple range test.

2- These compounds were analyzed separately in horizontal direction

CONCLUSION

H. triquetrifolium cell suspension cultures developed some secondary metabolites as a response upon *A. niger*, *F. oxysporum* and yeast extracts which acted as biotic elicitors, leading to an increase in phenolic compounds and flavonoids accumulation and the production of new constituents.

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