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Improving the Production of Total Phenolics and Flavonoids and the Antioxidant Capacity of *Echinacea purpurea* Callus through Biotic Elicitation

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Abstract

Echinacea (*Echinacea purpurea* L. Moench) is one of the most important medicinal plants worldwide with various pharmacological properties. In the current study, a biotic elicitation strategy using the fungal elicitors yeast extract (YE), *Aspergillus niger* and *Fusarium oxysporum* was employed to improve the productivity of callus biomass, total phenolics and total flavonoids in *E. purpurea* leaf-derived callus. Significant differences were recorded between biotic elicitors concerning callus production, total phenolics and flavonoids accumulation, DPPH scavenging activity, and antioxidant enzymes activity. Callus biomass, growth index, and relative growth rate were significantly enhanced in the elicited cultures. The highest content of total phenolics was recorded with 4 g/l YE, 1 g/l *A. niger* or 0.25 g/l *F. oxysporum*. However, 0.25 g/l of *F. oxysporum* was superior in enhancing the productivity of total phenolics, and the content and productivity of total flavonoids, representing 310.60%, 361.40%, and 649.50% increase of control, respectively. In general, callus extracts exhibited 79.02 – 89.51% scavenging activity of DPPH comparing with ascorbic acid (97.91%). YE at 4 g/l recorded the highest activity of catalase (CAT) and peroxidase (POD) enzymes. However, adding *A. niger* or *F. oxysporum* have a superior effect on polyphenol oxidase (PPO) activity. The current study revealed the possibility of using callus cultures and biotic elicitation as a promising tool for the *in vitro* production of phenols and flavonoids phytoconstituents with high antioxidant capacity from the high-value medicinal plant *E. purpurea*.

Keywords; Echinacea; yeast extract; fungal elicitors; phenolics; flavonoids; DPPH; antioxidant enzymes

1. Introduction

Plants are considered as chemical factories due to their ability to produce phytoconstituents of pharmaceutical and industrial importance which biosynthesized from simple building units [1]. *Echinacea purpurea* L. Moench is a perennial herb of Asteraceae found mostly in eastern North America. It is used for both medical applications and food industry [2]. The bioactive ingredients of *E. purpurea* are polysaccharides, phenolics, flavonoids, and alkylamides [3, 4]. The antioxidant capacity of extracts of echinacea plant can be attributed to the polyphenolic phytomolecules, such as phenolic acids and flavonoids [5]. Echinacea extract may enhance the monocytes and natural killer cells, which are the body's first line of immune defense against infection [6]. In the context of a dysregulated homeostasis of immune inflammation, echinacea could be one of the most suitable agents for potential use in COVID-19 [7, 8].

Chemical synthesis of bioactive phytochemicals is a challenging process due to the complex composition of the compounds and the high price of chemicals [9]. On the other hand, field cultivation is affected by ecotype, season, harvest date, soil fertility, water quality, and takes a long time, as well as the lack of standardization of the medicinal crop [10, 11]. The phytochemical constituents of echinacea fluctuate quantitatively and qualitatively during the growing season and growth cycle [12, 13].

In the search for an alternative solution to these problems faced by the plant pharmaceutical industries, biotechnology approaches, particularly plant tissue culture technique, have been reported to

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have a great potential to complement the conventional agriculture for the industrial and pharmaceutical production of plant bioactive metabolites [10, 14]. Among various biotechnology methods, callus cultures can be employed for large-scale and sustainable production of by-products in the pharmaceutical, cosmetics, and food industries [15, 16]. Calli cultures of medicinal crops synthesize and produce bioactive phytoconstituents with great antioxidant activity that can be used to treat a variety of diseases [17-20].

Plant cells show physiological and biochemical responses to physical, chemical, and microbial agents known as "elicitors". Elicitation is the process of inducing or stimulating the biosynthesis of phytomolecules by plant cells to ensure their viability and competitiveness [21]. Biotic and abiotic elicitors have been employed in plant tissue culture system to induce and improve different secondary metabolites production by reducing the time required to increase culture biomass to achieve high productivity [10, 22-24]. The strategy of fungal biotic elicitation, such as yeast extract, *Aspergillus niger*, and *Fusarium oxysporum* has been followed by several researchers in order to optimize the accumulation of different bioactive phytochemicals [20, 25-28]. Moreover, the antioxidant enzymes are activated to regulate the oxidative stress in cultures exposed to fungal biotic elicitation [26, 27, 29].

The present study aims to enhance the productivity of biomass and bioactive secondary metabolites, and antioxidant activity in *E. purpurea* callus cultures under biotic elicitation (yeast extract, *A. niger*, and *F. oxysporum*).

2. Experimental

The experiments were carried out in the Laboratory of Biotechnology, Horticulture Department, Faculty of Agriculture, Al-Azhar University, Nasr City, Cairo, Egypt.

2.1. Explant preparation and callus induction

Seeds of *E. purpurea* (Figure 1A) were obtained from Elnada Farms Company, Banha, Qalyubia, Egypt. Seeds were washed with commercial detergent and tap water and surface sterilized by ethanol (70%) for 30 sec, then immersed in 1% sodium hypochlorite solution containing few drops of Tween 20 for 20 min. After washing with sterile distilled water, the seeds were sown in culture jars containing Murashige and Skoog [30] basal medium (MS) and 30 g/l sucrose, solidified with 2 g/l phytagel (Plant BioLabs.inc. Ltd., England) and incubated for 10 days in the dark for germination (Figure 1B), then, seedling was transferred to 16-h light/8-h dark cycle with illumination from cool white fluorescence lights 40 $\mu mol^{-2}s^{-1}$ at 25±2°C. Leaves were excised from the *in vitro* seedling (8 weeks age) (Figure 1C) for callus induction.

Leaf segments (0.5 cm^2) were aseptically transferred to jars containing 25 ml of MS basal medium and 30 g/l sucrose, solidified with 2 g/l phytagel and supplemented with 2 mg/l benzyl adenine (BA) (Sigma-Aldrich Co. Ltd., Germany) and 0.5 mg/l α-naphthalene acetic acid (NAA) (Sigma-Aldrich Co. Ltd., Germany). Cultures were placed in a growth chamber for 5 weeks under 25±2°C and 16-h light/8-h dark cycle with illumination from cool white fluorescence lights 40 $\mu mol^{-2}s^{-1}$.

Figure (1). Seeds of *E. purpurea* (A), seed germination (B), and *in vitro* seedling (C)

2.2. Biotic elicitor treatments

A. niger and *F. oxysporum* fungi were prepared in Department of Agricultural Botany, Faculty of Agriculture, Al-Azhar University, Cairo. They were grown in malt extract medium (20 g/l) on shake flask (500 ml) with 100 ml medium on a rotary shaker (120 rpm) at room temperature. After 7 days, the cell suspension was autoclaved at 121°C for 20 min, and filtrated (Whatman no. 1). The mycelium was weighted and washed several times with sterilized distilled water and suspended in 25 ml water. This mixture was homogenized and autoclaved again [31].

Small pieces of callus fresh weight (0.25-0.30 g) were transferred to sterile jars containing 25 ml of MS medium and 30 g/l sucrose, solidified with 2 g/l

phytagel and supplemented with 0.5 mg/l NAA + 2 mg/l BA. YE (1, 2, and 4 g/l), *A. niger* (0.25, 0.5, and 1 g/l), and *F. oxysporum* (0.25, 0.5, and 1 g/l) were fortified to the culture medium as fungal elicitors.

The pH value of all tested media was adjusted to 5.8 with NaOH or HCl (1 N) prior to addition of phytagel. The cultures were placed in a growth chamber for 5 weeks under 25±2^{o}C and 16-h light/8-h dark cycle with illumination from cool white fluorescence lights 40 $\mu mol^{-2}s^{-1}$.

2.3. Measurements and determinations

2.3.1. Determination of callus biomass

Callus was collected after the incubation period (5 weeks) and the fresh weight (FW) was determined. Callus dry weight (DW) was recorded after drying at 45^{o}C for 2 days. Dry matter, growth index, and relative growth rate of callus were calculated as follows according to Ho *et al*. [32]:

Dry matter (%) = (final DW/final FW) x 100

Growth index = (final DW – initial DW)/initial DW

Relative growth rate = [(ln final DW) – (ln initial DW)]/incubation period,

where ln: natural log, and incubation period is 5 weeks

2.3.2. Determination of total phenolics

Callus tissue (100 mg DW) was immersed in ethanol (5 ml; 95%) for 48 h under 0^{o}C. The tubes were then homogenized and centrifuged for 10 min. Total phenolics content (TPC) was determined in the supernatants by following the Folin-Ciocalteu method as described by Singleton and Rossi [33] and Chandler and Dodds [34] with minor modifications. One ml of the extract supernatant was mixed with 1 ml of ethanol (95%), 5 ml of distilled water, 0.5 ml Folin-Ciocalteu reagent (50%) (bioWORLD Co., Dublin, USA). After 5 min, 1 ml of Na_2CO_3 (5%) was added and mixed well. The solution was incubated under 25±2^{o}C for 60 min. The absorbance was recorded at 725 nm against blank using JENWAY 6800 UV/Vis. spectrophotometer. Dilutions of gallic acid (Sigma-Aldrich Co. Ltd., Germany) were also read to draw the standard curve. TPC was expressed as mg gallic acid equivalents/g DW of callus.

Total phenolics productivity (mg/l of culture medium) = TPC (mg/g DW) x callus biomass yield (DW g/l of culture medium).

2.3.3. Determination of total flavonoids

Dried callus tissue (100 mg) was extracted with 5 ml ethanol (95%) for 24 h at room temperature. After filtration, total flavonoids content (TFC) in callus extract was determined by following the aluminum chloride colorimetric method as described by Chang *et al*. [35] and Madaan *et al*. [36]. Ethanol extract (0.5 ml) was mixed with 1.5 ml of ethanol (95%), 0.1 ml of $AlCl_3$ (10%), 0.1 ml of potassium acetate (1 M) and 2.8 ml of distilled water. The reaction mixture was incubated at 25±2^{o}C for 30 min. The absorbance was recorded at 415 nm against blank using JENWAY 6800 UV/Vis. spectrophotometer. Quercetin dilutions (Sigma-Aldrich Co. Ltd., Germany) were used to establish the calibration curve, and TFC was calculated and expressed as mg quercetin equivalents/g DW of callus tissue.

Total flavonoids productivity (mg/l of culture medium) = TFC (mg/g DW) x callus biomass yield (DW g/l of culture medium).

2.3.4. Determination of free radical scavenging activity

Samples of dried callus (100 mg) were extracted with 5 ml of ethanol (95%) for 24 h at room temperature. After filtration, the antioxidant activity of callus extract was assayed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) test according to Elateeq *et al*. [22]. Ethanol extract (0.7 ml) of the callus samples was mixed with 3 ml of DPPH ethanol solution (200 µM) (Phygene Biotechnology Co. Ltd., China). The mixture was shaken thoroughly and incubated for 30 min in the dark at 25±2^{o}C. The absorbance was recorded at 517 nm using a spectrophotometer (JENWAY 6800 UV/Vis. spectrophotometer). The percentage of DPPH radical scavenging activity was determined using the formula:

DPPH activity (%) = [(A control – A sample)/A control] x 100

where A control is the absorbance of the DPPH solution with 0.7 ml ethanol (95%) free sample, and A sample is the absorbance of callus extract mixed with DPPH solution.

2.3.5. Determination of antioxidant enzymes activity

Tissue preparation for enzymatic antioxidants

Fresh callus samples (200 mg) were ground and homogenized in an ice-bath in 4 ml of 50 mM potassium phosphate buffer solution (pH 7.8). The homogenate was centrifuged at 14,000 rpm at 4˚C for 10 min and the resulting supernatant was used for

Table (1). Effect of biotic elicitors YE, *A. niger* and *F. oxysporum* on callus production in *E. purpurea* after 5 weeks incubation period

Biotic elicitor (g/l)		Callus FW (g/explant)	Callus DW (g/explant)	Dry matter (%)	Growth index	Relative growth rate
Control	0	0.76 e	0.051 ef	6.95 bc	0.97 ef	0.13 c
YE	1	0.88 de	0.048 f	5.52 c	0.83 f	0.12 c
	2	0.92 de	0.077 bcd	8.44 ab	1.94 bcd	0.21 ab
	4	0.79 de	0.076 cd	9.62 a	1.88 cd	0.21 ab
A. niger	0.25	1.72 a	0.104 a	6.07 c	2.95 a	0.27 a
	0.5	1.38 b	0.095 abc	6.93 bc	2.62 abc	0.25 ab
	1.0	1.06 cd	0.074 cde	6.95 bc	1.81 cde	0.20 ab
F. oxysporum	0.25	1.35 b	0.091 abcd	6.77 bc	2.44 abcd	0.24 ab
	0.5	1.32 bc	0.100 ab	7.57 bc	2.81 ab	0.26 a
	1.0	1.07 cd	0.070 def	6.68 bc	1.66 def	0.19 b

Mean values followed by different letters in the column are significantly different according to DMRT at $P \leq 0.05$

enzymes assay. The absorbance was recorded using a spectrophotometer (JENWAY 6800 UV/Vis. spectrophotometer) for 60 s.

Assay of catalase activity

Catalase (CAT) action was precise according to Aebi [37]. Three ml reaction mixture containing 1.5 ml of 100 mM potassium phosphate classes buffer (pH = 7.2), 0.5 ml of 75 mM *hydrogen peroxide (*H_2O_2*)* (Aqua Chemicals, Egypt), 0.05 ml enzyme extraction. The distilled water was used to construct the volume up to 3 ml. Reaction in progress by the addition of H_2O_2. The absorbance recorded in decrease at 240 nm for 60s. The enzyme action was accounted by calculating the quantity of decomposed H_2O_2.

Assay of peroxidase activity

Peroxidase (POD) activity was measured by the method of Chance and Maehly [38]. The test solution was prepared by mixing 0.06 ml of enzyme solution with 1.07 ml of 100 mM potassium phosphate buffer, pH 6.0, 0.3 ml of 5% (w/v) pyrogallol solution (Riedel-De Haen AG, Seelze-Hannover, Germany), 0.1 ml of 0.5% (w/w) H_2O_2 and 0.7 ml distilled water. For blank, 3 ml of 100 mM potassium phosphate buffer pH 6.0 was added instead of enzyme solution. The absorbance was recorded at 420 nm.

Assay of polyphenol oxidase activity

The determination of polyphenol oxidase (PPO) activity was done according to Duckworth and Coleman [39] at 420 nm and 25°C. The test solution was prepared by mixing 0.05 ml of enzyme solution with 1.70 ml of 20 mM catechol solution (BDH Chemicals Ltd., Poole, England) (prepared in 50 mM potassium phosphate buffer, pH 6.8 at 25°C). The blank was prepared with the same amount of catechol and 3 ml of 50 mM potassium phosphate buffer.

The antioxidant enzymes activity was assayed as follow:

Enzyme activity (U(µmol)/ml) = $(\Delta A \times Vt \times 10^6)/(\Delta t \times l \times \varepsilon \times Vs \times 1000)$

Enzyme activity (U(µmol)/g FW) = $[(\Delta A \times Vt \times 10^6)/(\Delta t \times l \times \varepsilon \times Vs \times 1000)] \times$ dilution factor

where ΔA is the change in absorbance, Δt is the time of incubation (min), ε is the extinction coefficient (M^{-1} cm^{-1}), l is the cuvette diameter (1 cm), Vt is the total assay volume, and Vs is the enzyme sample volume (ml). ε240nm of H_2O_2 is 43.6 $M^{-1}.cm^{-1}$, ε420nm of pyrogallol is 2640 $M^{-1}.cm^{-1}$, and ε420nm of catechol is 2450 $M^{-1}.cm^{-1}$.

2.4. The statistical analysis

All experiments were conducted in a complete randomized design (CRD). Every treatment had 3 replicates, each replicate had 5 jars and each jar contained 3 explants. The analysis of variance of data was performed using Snedecor and Cochran [40] method. Computation was done using COSTAT computer package (CoHort software Monterey, California, USA), and means were compared according to Duncan's Multiple Range Test (DMRT) [41] at $P \leq 0.05$.

3. Results and Discussion

3.1. Effect of biotic elicitors on callus biomass weight

Significant differences were recorded between biotic elicitors concerning callus growth and biomass weight in general. Data presented in Table (1) and Figure (2A) show that YE at different levels had a low effect on callus fresh weight (FW) of *E. purpurea* with non-significant differences with control callus. It was reported that YE contains higher level of amino acids, vitamins and minerals which could improve biomass weight depending on plant species [42]. *A. niger* and *F. oxysporum* was more pronounced than YE in stimulating the callus biomass FW. The highest significant value of callus FW (1.72 g/explant; 206.92 g/l medium) was recorded by *A. niger* at 0.25 g/l compared to control (0.76 g/explant; 90.86 g/l medium), which represented 2.26-fold higher than control. Increasing levels of *A. niger* and *F. oxysporum* to 0.5 and 1 g/l decreased the biomass FW. These findings are consistent with Ajungla *et al*. [43] who found that increasing *A. niger* concentration in the culture medium of *Datura metel* reduced root growth. Moreover, El-Nabarawy *et al*. [44] on *Zingiber officinale* reported that only low levels of *A. niger* extract can enhance callus growth. Similarly, previous study mentioned that using fungal filtrate of *F. oxysporum* can be useful for improving callus size, but growth can be restrained at higher doses [45].

For callus biomass DW, data displayed in Table (1) and Figure (2B) indicate that using YE at 1 g/l failed to enhance callus DW (0.048 g/explant) of *E. purpurea* compared with control (0.051 g/explant). Increasing levels of YE to 2 and 4 g/l in callus medium significantly enhanced the DW of callus (0.077 and 0.076 g/explant; 9.32 and 9.14 g/l medium, respectively). Low and medium concentrations of *A. niger* and *F. oxysporum* were superior to YE levels. *A. niger* and *F. oxysporum* at 0.25 and 0.5 g/l resulted in the highest significant production of callus biomass DW (2.04, 1.86, 1.96, and 1.7-fold higher than control, respectively). The study of Manjula and Mythili [46] on *Marsilea quadrifolia* revealed that fungal elicitors improved plant growth and the content of protein and carbohydrates. This increase might be referred to the chemical structure of fungal mycelium which contain natural polymers such as chitin, cellulose, and proteins [47]. Higher dosage of *A. niger* and *F. oxysporum* (1 g/l) caused a decrease in callus FW and DW, which may be the result of stress on the cells by increasing the concentration of these natural polymers.

3.2. Effect of biotic elicitors on callus growth

The dry matter of *E. purpurea* callus was enhanced with YE application (Table 1). YE at moderate and high concentrations (2 and 4 g/l) achieved the highest significant value of callus dry matter (8.44 and 9.62%, respectively), which scored 1.21 and 1.38-fold increase than control (6.95 %), respectively. Application of *A. niger* and *F. oxysporum* did not significantly enhance the dry matter of *E. purpurea* callus as compared to the control.

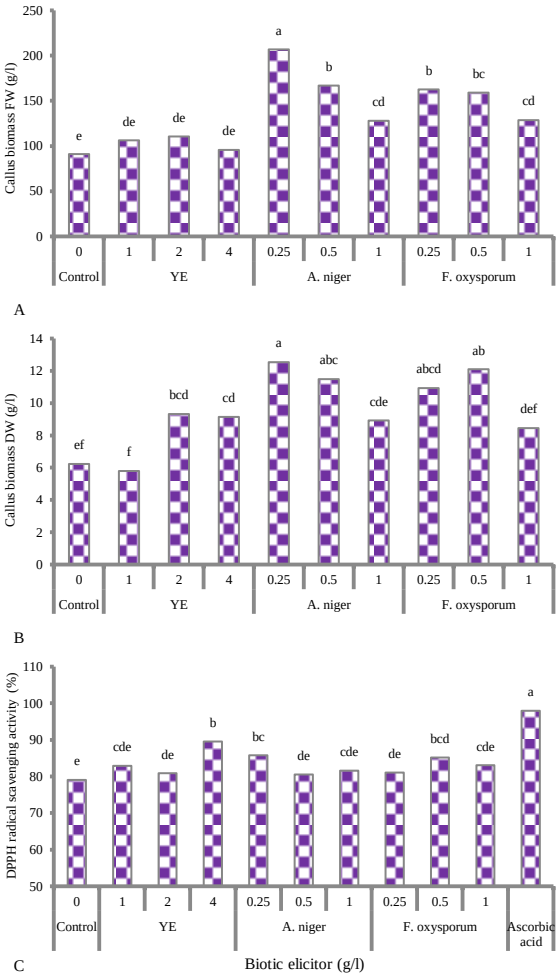

Figure (2). Effect of the biotic elicitors YE, *A. niger* and *F. oxysporum* on the productivity of biomass FW (A), biomass DW (B), and the antioxidant activity (C) of *E. purpurea* callus cultures after 5 weeks of elicitation. Columns with different letters are statistically different according to DMRT ($P \leq 0.05$)

In contrast, callus growth index was significantly

increased by fungal elicitors application except for low level of YE and high level of *F. oxysporum*. This was also proved by Ghorpade *et al*. [48] that elicitation with *F. oxysporum* extract at 750 and 1000 mg/l inhibited the growth of *Boswellia serrata* callus and concurrently enhanced the total boswellic acid content. Among all treatments of biotic elicitors, *A. niger* and *F. oxysporum* each at 0.25 and 0.5 g/l recorded the highest significant rate of growth index which estimated as 2.15 – 2.95-times of control. The relative growth rate of echinacea callus elicited with YE, *A. niger* and *F. oxysporum* at all concentrations (except for 1 g/l YE) was significantly enhanced recording values between 0.19-0.27 with non-statistical differences between them, compared to unelicited callus (0.13).

3.3. Effect of biotic elicitors on total phenolics production

After establishing *in vitro* callus cultures, further treatments are followed to enhance the accumulation of secondary metabolites by increasing the biosynthetic cellular capacity through several mechanisms [10, 49]. Elicitation had gained attention due to its important role in enhancing the production of various bioactive ingredients [22, 23, 50].

Data of Table (2) clearly show that fungal biotic elicitation of *E. purpurea* callus positively enhanced the total phenolics content (TPC). The TPC increased with an increase in the eliciting dose of YE and *A. niger* and vice versa with *F. oxysporum*. Higher level of YE (4 g/l) and *A. niger* (1 g/l) and low level of *F. oxysporum* (0.25 g/l) achieved the topmost significant value of TPC (5.19, 4.74, and 5.77 mg/g DW, respectively) which was higher than unelicited culture (3.32 mg/g DW) by 156.32, 142.77, and 173.79%, respectively. These findings are in accordance with the reports of Kordi *et al*. [51] on *Cicer spiroceras*, Park *et al*. [52] on *Agastache rugosa,* Zaman *et al*. [53] on *Ocimum basilicum*, and Arafa and Aly [24] on *Pimpinella anisum*. Al-Gendy *et al*. [54] also reported an enhancement in TPC in *Artemisia monosperma* callus upon elicitation with *F. oxysporum.* The addition of fungal elicitors like *A. niger* to the tissue culture media might affect genes expression by increasing the formation of endogenous signaling molecules including nitric oxide, ethylene, abscisic acid, jasmonic acid and salicylic acid which activate the biosynthetic pathways of secondary compounds [55, 56]. It was reported that cell wall extracts of fungi act as a polysaccharide elicitor, which induces levels of calcium in plant cells with the activation of various defense mechanism pathways leading to the accumulation of various secondary metabolites [57].

Table (2). Effect of biotic elicitors YE, *A. niger* and *F. oxysporum* on total phenolics production in *E. purpurea* callus cultures after 5 weeks incubation period

Biotic elicitor (g/l)		Total phenolics content (mg/g DW)	Increase rate (% of control)	Total phenolics productivity (mg/l medium)	Increase rate (% of control)
Control	0	3.32 d	100	20.08 g	100
YE	1	4.51 bc	135.84	25.79 f	128.43
	2	4.87 bc	146.68	44.53 cd	221.76
	4	5.19 ab	156.32	46.90 cd	233.56
A. niger	0.25	3.86 cd	116.26	48.02 c	239.14
	0.5	4.34 bc	130.72	49.60 bc	247.01
	1	4.74 abc	142.77	41.98 d	209.06
F. oxysporum	0.25	5.77 a	173.79	62.37 a	310.60
	0.5	4.57 bc	137.65	54.74 b	272.61
	1	4.29 bc	129.21	36.21 e	180.32

Mean values followed by different letters in the column are significantly different according to DMRT at $P \leq 0.05$

The productivity (yield) of total phenolics and total flavonoids was calculated by multiplying the content expressed in mg/g DW and the callus biomass DW expressed in g/l medium. The tabulated data in Table (2) reveal that total phenolics productivity was significantly improved in all elicited callus cultures, generally, as a result of the improvement in the callus DW and its phenol content. The study of Hoshyar and Kadhim [58] demonstrated that the yield of p-OH-benzoic acid and chlorogenic acid was increased in the suspension cultures of *Hypericum triquetrifolium* treated with

YE compared to the untreated cultures. Among fungal elicitors, *F. oxysporum* at 0.25 g/l recorded the highest significant yield of total phenolics (62.37 mg/l medium; 310.60% of control), followed significantly by 0.5 g/l (54.74 mg/l medium, 272.61% of control), and 0.5 g/l *A. niger* (49.60 mg/l medium, 247.01% of control) which considered the most appropriate elicitation for total phenolics production from *E. purpurea* calli.

3.4. Effect of biotic elicitors on total flavonoids production

The effect of different levels of fungal elicitors on the content and yield of total flavonoids in *E. purpurea* callus was investigated and the results are presented in Table (3). Significant differences were noticed between different types and levels of fungal elicitors added to callus media. The TFC was significantly increased in the elicited cultures. Likewise, the accumulation of rutin, hypersoid, and quercitin was enhanced in suspension cultures of *Hypericum triquetrifolium* elicited with YE, *A. niger* and *F. oxysporum* [58].

Increasing levels of *A. niger* significantly improved the accumulation of TFC compared to the control. These findings are in harmony with Tonk *et al*. [59] on *Catharanthus roseus*, Ebrahimi *et al*. [60] on *Peganum harmal*, and Shaikh *et al*. [61] on *Helicteres isora*. This positive influence might be associated with the endogenous phytohormones such as salicylic acid in the callus tissues, which increased by increasing the elicitation level with *A. niger* as reported by Ibrahim *et al*. [62]. In contrast to *A. niger*, increasing the elicitation dose of *F. oxysporum* significantly decreased the TFC in *E. purpurea* callus. It was reported that higher levels of *F. oxysporum* suppressed the plant growth and phytochemicals accumulation because of the higher oxidative stress which turned out from the high toxicity of *F. oxysporum* extract [63]. *F. oxysporum* at low concentration (0.25 g/l) achieved the maximal significant rate of TFC (10.77 mg/g DW) which estimated as 361.40% of control (2.98 mg/g DW). Al-Gendy *et al*. [54] and Ebad *et al*. [45] found that elicitation with *F. oxysporum* enhanced the TFC in *Artemisia monosperma* and *Solanum nigrum* in *in vitr*o cultures, respectively. Flavonoids had a vital role in osmotic arrangement, with stimulating anti-oxidative actions and defense mechanisms against biotic and abiotic elicitation or stress factors [63]. YE at 1 and 2 g/l also significantly enhanced the TFC (6.72 and 6.99 mg/g DW; 225.50% and 234.56% of control, respectively). In line with these results, Zhao *et al*. [64] reported that TFC was enhanced by the application of YE in hairy root cultures of *Fagopyrum tataricum*. Also, Mendhulkar and Vakil [65] on *Andrographis paniculata*, and Simic *et al*. [27] on *Hypericum perforatum* recorded the same findings. High activation of endogenous methyl jasmonate and/or jasmonic acid by YE supplementation could be the main reason that improving the total flavonoids production [66].

Table (3). Effect of biotic elicitors YE, *A. niger* and *F. oxysporum* on total flavonoids production in *E. purpurea* callus cultures after 5 weeks incubation period

Biotic elicitor (g/l)		Total flavonoid content (mg/g DW)	Increase rate (% of control)	Total flavonoid productivity (mg/l medium)	Increase rate (% of control)
Control	0	2.98 h	100	18.02 f	100
YE	1	6.72 bc	225.50	38.45 e	213.37
	2	6.99 b	234.56	64.40 b	357.38
	4	5.33 de	178.85	48.21 cd	267.53
A. niger	0.25	3.33 gh	111.74	41.42 de	229.85
	0.5	4.39 ef	147.31	50.20 cd	278.57
	1	5.91 cd	198.32	52.23 c	289.84
F. oxysporum	0.25	10.77 a	361.40	117.04 a	649.50
	0.5	4.74 ef	159.06	56.49 bc	313.48
	1	4.19 fg	140.60	35.34 e	196.11

Mean values followed by different letters in the column are significantly different according to DMRT at $P \leq 0.05$

From the data presented in Table (3) it appears that fungal elicitation significantly affected the total flavonoids productivity in *E. purpurea* callus compared to the control. *F. oxysporum* at 0.25 g/l yielded the highest significant total flavonoids (117.04 mg/l medium) representing 649.50% of

control (18.02 mg/l medium), followed by 2 g/l YE and 0.5 g/l *F. oxysporum* (64.40 and 56.49 mg/l medium, respectively). *A. niger* increased the total flavonoids productivity by 229.85%, 278.57%, and 289.84% of control when supplemented to the callus medium at 0.25, 0.5, and 1 g/l, respectively.

3.5. Effect of biotic elicitors on antioxidant activity

During plant stress exposure, reactive oxygen species (ROS) are turned out in plant biochemical pathways, potentially causing damage to plant cells, membrane lipids, and DNA. Plant secondary metabolites, such as terpenoids, phenolics, flavonoids, alkaloids, and others, are synthesised by plant cells and can act as antioxidant agents which protect plants from the harmful influences of oxidation [53]. In the present experiment, DPPH free radical scavenging activity was determined for extracts of *E. purpurea* callus elicited with the fungal elicitors. In general, callus extracts exhibited 79.02 – 89.51% scavenging activity of DPPH comparing to ascorbic acid (positive control) which exhibited 97.91% (Figure 2C).

The antioxidant capacity of the elicited callus was higher than that of unelicited one (79.02%). Within fungal elicitors treatments, 4 g/l YE recorded the highest significant percentage of antioxidant activity (89.51 %) followed by 0.25 g/l *A. niger* (85.80 %) and 0.5 g/l *F. oxysporum* (85.14 %). This may be related to the positive impact of YE on the accumulation of TPC and TFC which improved the antioxidant capacity. The positive correlation between TPC, TFC and antioxidant activity due to YE treatment was also proved by Nadeem *et al*. [67] on *Linum usitatissimum*. Although *A. niger* at 0.25 g/l gave low values of TPC and TFC, it displayed higher antioxidant capacity. This might be referred to other phytocompounds not determined in our study such as alkylamides, terpenoids, and alkaloids which located in *E. purpurea* plant and can affect the antioxidant activity [68, 69]. The same observation was also recorded for *F. oxysporum*-elicited callus. Other previous studies; Kordi *et al*. [51] on *Cicer spiroceras*, Rady *et al*. [70] on *E. purpurea*, and Zaman *et al*. [53] on *Ocimum basilicum* confirmed an increase in the concentration of various by-products in *in vitro* cultures treated with fungal biotic elicitors which was accompanied by an enhancement in their antioxidant capacities.

3.6. Effect of biotic elicitors on activity of some antioxidant enzymes

The CAT, PPOs and PODs are antioxidant enzymes, can act as defensive proteins and play a vital role in the plant response to various biotic and abiotic stressors [71-73]. The data of Figure (3) reveal that CAT, PPOs and PODs activities of the elicited callus were more activated compared to the control one. CAT activity reached the highest significant value at 4 g/l YE followed by 2 g/l YE (142.74 and 101.83 µm H_2O_2/g FW, respectively) (Figure 3A). On contrary, *A. niger* and *F. oxysporum* have a superior effect on PPO activity (Figure 3B). The higher level of *A. niger* (1 g/l), low and moderate levels of *F. oxysporum* (0.25 and 0.5 g/l), and moderate elicitation of YE (2 g/l) scored the highest significant rate of PPO activity (7.11, 6.90, 7.25, and 8.25 µm catechol/g FW, respectively). The rise in the concentration of each fungal elicitor in calli medium was accompanied by an increase in POD activity in general (Figure 3C). Hence, the maximal significant activity of POD enzymes was recorded for callus cultures elicited with 4 g/l YE, 1 g/l *A. niger*, and 1 g/l *F. oxysporum* (30.93, 22.15, and 23.90 µm pyrogallol/g FW, respectively).

Among biotic elicitors, YE at 4 g/l recorded the highest activity of CAT and POD (5.19 and 5.05-times of control, respectively). This may be related to the ability of higher concentrations of YE to induce a cellular stress which stimulated the gene expression of the oxidative enzymes [74]. Moreover, YE has been added to plant tissue culture media due to their capability to induce the defense mechanisms and antioxidant enzymes, which leads to stimulate the metabolic pathways of various bioactive metabolites [75]. This may explain why YE was superior to TPC accumulation in the callus tissue. However, it is not the best elicitor for TFC. The researchers proved that there are specific secondary metabolites that need specific elicitor to be stimulated [56].

Fungal extracts cause an oxidative stress which can increase the accumulation of endogenous abscisic acid, ethylene, salicylic acid and jasmonic acid in plant cells [55, 76]. These phytohormones enhancing the transcriptional regulation of biosynthesis PPO genes which increasing the activity of PPO [73]. Besides activating the oxidative enzymes, fungal elicitors stimulating the plant defense mechanism and induce metabolic changes like enhancing the pathways of secondary metabolites including phenols and flavonoids [53, 76].

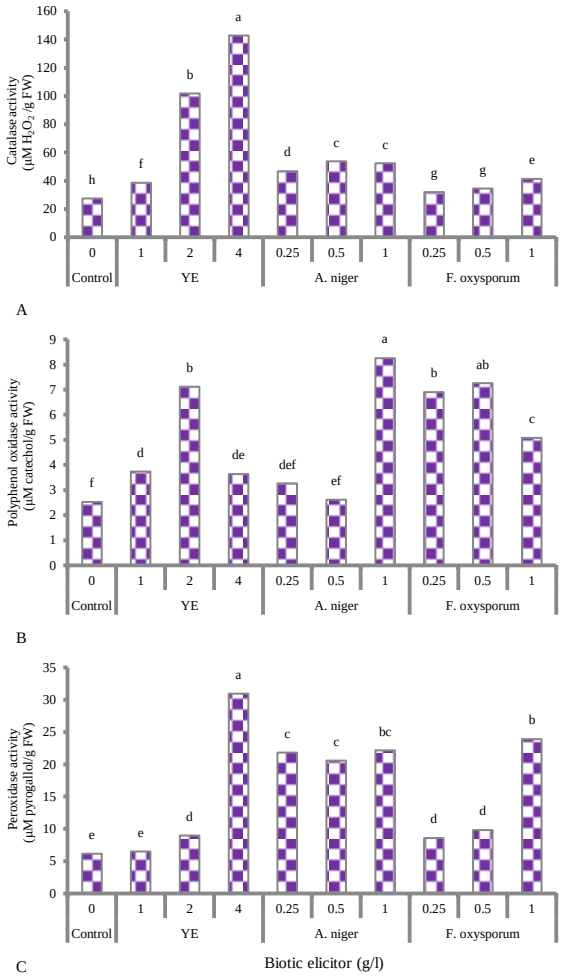

Figure (3). The activity of catalase (A), polyphenol oxidase (B), and peroxidase (C) enzymes in *E. purpurea* callus cultures after 5 weeks of elicitation with the fungal elicitors YE, *A. niger* and *F. oxysporum*. Columns with different letters are statistically different according to DMRT ($P \leq 0.05$)

4. Conclusions

Phenolics and flavonoids are among valuable bioactive metabolites in the medicinal plant *E. purpurea*. Using biotechnological methods, especially tissue culture system, to improve the productivity of such high-value phytochemicals is a promising approach to overcome limitations faced in the traditional cultivation. Biotic elicitation had an important role in increasing the accumulation of various by-products. In this study, fungal biotic elicitation with yeast extract, *A. niger* and *F. oxysporum* in callus cultures of *E. purpurea* represented an appropriate tool for the production of callus biomass having abundant of total phenolics and total flavonoids. Moreover, the antioxidant capacity of the fungal-elicited cultures was enhanced, which would expand its implementation in the pharmaceuticals and nutraceuticals fields. Further *in vitro* studies should explore the genes involved in the biosynthetic pathway of specific molecules of phenolics, flavonoids, and alkylamides in *E. purpurea* under elicitation.

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6. Conflicts of interest

"There are no conflicts to declare".

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