

Title

Piriformospora indica* based elicitation for overproduction of phenolic compounds by hairy root cultures of *Ficus carica

Authors:

Shahla Amani^a, Mehdi Mohebodini^{a*}, Shahram Khademvatan^b, Morad Jafari^c, Vinod Kumar^d

a. Department of Horticulture Sciences, University of Mohaghegh Ardabili, Ardabil, Iran

b. Cellular and Molecular Research Center & Department of Medical Parasitology and Mycology, Urmia University of Medical Sciences, Urmia, Iran

c. Department of Plant Production and Genetics, Urmia University, Urmia, Iran

d. Centre for Climate and Environmental Protection, School of Water, Energy and Environment, Cranfield University, Cranfield MK43 0AL, UK

* Corresponding author:

Dr. Mehdi Mohebodini

Department of Horticulture Sciences, University of Mohaghegh Ardabili, Ardabil, Iran

Tel and Fax: +98 45-33510140 and +98 45-33512204

Email: Mohebodini@uma.ac.ir

Abstract

Ficus carica L. is an important source of phenolic and flavonoid compounds with valuable pharmaceutical application across various diseases. The current study was carried out to investigate the influence of *Piriformospora indica* elicitation on growth, production of phenolic compounds, antioxidant capacity, and expression level of flavonoid biosynthetic pathway genes in hairy root (HR) cultures of *F. carica*. The maximum improvement in accumulation of phenolic compounds was observed when HR culture of *Ficus carica* L. was exposed to 2% culture filtrate of *P. indica* for 72 h: gallic acid (80.5-fold), caffeic acid (26.2-fold), coumaric acid (4.5-fold), and cinnamic acid (60.1-fold), apigenin (27.6-fold) and rutin (5.7-fold). While the highest levels of chlorogenic acid (4.9-fold) and quercetin flavonoid (8.8-fold) were obtained after 48 h elicitation with culture filtrate and cell extract of *P. indica* at 6% (v/v), respectively. The analysis of biosynthetic genes revealed that the exposure to fungal elicitors resulted in up-regulation of phenylalanine ammonia-lyase (*PAL*), chalcone synthase (*CHS*), UDP-glucose flavonoid 3-O-glucosyltransferase (*UFGT*) and MYB3 transcription factor. This study shows the potential of *P. indica* as an efficacious elicitor for enhancing the secondary metabolites production by *F. carica* HRs.

Key words: *Ficus carica* L.; Fungal elicitor; Phenolic compounds; *Piriformospora indica*; Elicitation, Gene expression

Abbreviations

PAL: phenylalanine ammonia-lyase; *CHS*: chalcone synthase; *UFGT*: UDP-glucose flavonoid 3-O-glucosyltransferase; *HR*: Hairy root; *PCR*: Polymerase chain reaction; *DW*: Dry weight; *GI*: Growth index; *TPC*: Total phenolic content; *TFC*: Total flavonoid content; *DPPH*: 2,2-diphenyl-1-picrylhydrazyl; *FRAP*: Ferric reducing antioxidant power; *HPLC*: High performance liquid chromatography

1. Introduction

Plants, in addition to the primary metabolites that are essential for their growth and development, also produce low-molecular-weight compounds known as secondary metabolites through biosynthesis pathways of fatty acid, alkaloid, phenylpropanoid and isoprenoid (Delgoda and Murray, 2017; Kumar and Goel, 2019). Although secondary metabolites are not essential for plant growth but they adapt the plant to different conditions and protect it against biotic and abiotic stresses (Delgoda and Murray, 2017). Because secondary metabolites as a major component of food and medicine are beneficial to human health, plants are considered as an important source for discovering new drugs (Delgoda and Murray, 2017). Phenolic compounds including phenolic acids (hydroxy-benzoic and hydroxy-cinnamic acid) and flavonoids have been widely used for cosmetic, medical, and food purposes (Havsteen, 2002; Kumar and Goel, 2019). These compounds exhibit a number of properties to serve as promising anticancer agents in the near future due to important biological activities including anti-proliferative activity, cell cycle-arresting function, apoptosis, differentiation induction, angiogenesis inhibiting ability, anti-oxidative and multidrug resistance reversal activity (Ren et al., 2003).

Ficus carica L. (Moraceae), as one of the richest sources of phenolic compounds, is well known to accumulate secondary metabolites with medicinal properties having importance in pharmaceutical industries (Barolo et al., 2014). The plant contains various natural compounds with anti-cancer, anti-diabetic, anti-hypertensive, anti-spasmodic, anti-platelet, anti-microbial, anti-oxidative and anti-obesogenic properties including polyphenols, flavonoids, anthocyanins, coumarins, terpenoids, tannins and sterols (Alamgeer et al., 2017; Barolo et al., 2014; Ghanbari et al., 2019; Gilani et al., 2008). The presence of phenolic acids including cinnamic acid, gallic acid, vanillic acid, coumaric acid, syringic acid, caffeic acid, and chlorogenic acid has been proven in *F. carica* (Jagtap and Bapat, 2020; Wang et al., 2017). Apigenin (flavone) and quercetin (Avicularin and isoquercitrin flavonols) are the bioactive flavonoids in *F. carica*, whose content changes throughout the course of growth and fruit maturity (Wang et al., 2017). The biosynthesis of phenolic acids and flavonoids take place through phenylpropanoid pathway with involvement of numerous enzymes (Figure 1) (Falcone Ferreyra et al., 2012; Huang et al., 2016; Jiang et al., 2013; Li et al., 2014; Tian et al., 2017; Wang et al., 2017).

Because of high demand of phenolic compounds, the improvement of production, extraction and purification methods of these compounds has been considered in scientific community. Establishment of hairy root (HR) cultures by *Rhizobium (Agrobacterium) rhizogenes*-mediated transformation of plants has provided an effective alternative option for high level production of plant secondary metabolites in comparison to other *in vitro* plant tissue culture systems (Singh et al., 2018). These HR lines (each individual HR induced by inoculation with *R. rhizogenes* is regarded as a line), not only shows high growth rates but are also genetically and biochemically stable (Fu et al., 2006; Hakkinen et al., 2016). They are able to produce large quantities of secondary metabolites in short time in comparison to whole plant which provides a basis for metabolic studies (Li et al., 2006; Singh et al., 2018). The positive effect of different elicitation techniques on the augmentation of secondary metabolites has been well proved in HR cultures (Yan et al., 2006). Elicitor is referred to any biotic/abiotic agent capable of inducing defensive responses in the plant and thereby increasing the synthesis of secondary metabolites for enhancing plant resistance to stress conditions (Kaur and Pati, 2018). The efficiency of elicitation depends on many factors as elicitor kind, elicitor concentration, age of the plant culture, method and exposure time of elicitation (Narayani and Srivastava, 2017). The axenic culture of fungi species can be used for biotic elicitation of HR cultures (Kumar et al., 2012). *Piriformospora indica* is a root endophytic fungus that can promote growth, yield and resistance to biotic and abiotic stresses of host plants (Jisha et al., 2018). The cell extract and culture filtrate of *P. indica* have been widely used to enhance the biosynthesis of secondary metabolites in suspension cultures of plant cells and HRs (Kumar et al., 2016; Kumar et al., 2012).

In this regard, accordance to high value of phenolic compounds in *F. carica*, the effects of cell extract and medium culture filtrate (two elicitors) derived from axenic culture of *P. indica* on some phenolic compounds and expression level of some genes in *F. carica* HR cultures was the prime purpose of the current study.

2. Materials and methods

2.1. Preparation of plant materials and bacterial strains

The seeds of Flora of Iran-identified *Ficus carica* L. Sabz cultivar (Azizian, 2001) were provided by Zarringiah Company, Urmia, Iran. Voucher specimens were deposited at Herbarium of Urmia University (Urmia, Iran). Seeds were sterilized using 70% ethanol (v/v) for 2 min and 2.5% sodium hypochlorite for 20 min. The seeds were cultured on woody plant medium (Lloyd and McCown, 1980) containing 20 g/L sucrose and 6 g/L agar. Cultures were incubated at 25±2 °C under 16/8 h (light/dark). Shoot explants were excised from 3-week-old plantlets for HRs induction with bacterial strain inoculation.

ATCC15834 strain of *R. rhizogenes* was provided by National Institute of Genetic Engineering and Biotechnology (Tehran, Iran). The single colony of bacterial strain was cultured on liquid Luria–Bertani (LB) medium (Bertani, 1951) supplemented with 50 mg/L rifampicin and maintained overnight on a rotary shaker at room temperature in darkness conditions.

2.2. Induction of HRs

The explants were wounded and inoculated with bacterial suspension for 10 min, placed on ½ Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 20 g/L sucrose, 100 µM acetosyringone, and 6 g/L agar, incubated in darkness at 25±2 °C for 3 days. To eliminate bacteria, co-cultivated explants were transferred to ½ MS solid media containing 500 mg/L cefotaxime. After 10-17 days, HRs were induced at wounded sites of explants. HR lines were excised from induction sites and sub-cultured in fresh solid media (½ MS medium with gradually decrease in cefotaxime concentration) every 2-3 weeks. After HR establishment and optimization of culturing (Amani et al., 2020), the 300 mg fresh weight of the hairy root line with the highest growth rate (superior HR line) were transferred to 30 mL hormone-free ½ MS liquid medium and placed on a rotary shaker (120 rpm) under darkness at 25±2 °C for further experiments.

2. 3. Molecular confirmation of HRs

The genomic DNA was extracted from HR lines and normal roots (natural roots cut from *in vitro* seedlings) by CTAB-based method (Pirttila et al., 2001). Molecular confirmation of HRs via polymerase chain reaction (PCR) analysis was carried out using *rolB* gene-specific primer (5'- ATGGATCCCAAATTGCTATTCCCCACGA-3' and 5'- TTAGGCTTCTTTTCATTTCGGTTTACTGCAGC-3') and super PCR Master Mix kit 2X (YTA, Yekta Tajhiz Azma, Tehran, Iran). The extracted Ri-plasmid DNA using alkaline lysis method (Sambrook and Russell, 2001) was used as positive control. To amplify 780 bp fragment of the *rolB* gene, the PCR amplification program was set up as follows: 94°C for 5 min, 30 cycles of 94°C for 60s, 60°C for 60s and 72 ° C for 70s and followed by 72°C for 10 min. PCR products were analyzed by 1.2% agarose gel electrophoresis.

2. 4. Elicitation

The fungus *Piriformospora indica* (ATCC® Number: 204458™) was cultured on liquid Kafer media (Kafer, 1977). The fungus culture was maintained on Kafer medium at pH 6.5, containing 10 g/L agar. After 8 days, fungus agar disks (8 mm) were sub-cultured into 100 mL liquid Kafer medium and incubated in shaker incubator (220 rpm) at 30±2°C. Culture filtrate and cell extract of *P. indica* was used as two biotic elicitors. The elicitors were prepared using submerged cultures of *P. indica* as described by Kumar et al. (2012) and Ahlawat et al. (2016). To prepare culture filtrate, *P. indica* culture was harvested during late growth phase (8-10 days old), paper filtered to separate fungus biomass, centrifuged (7500 rpm) for 15 min, filtered through 0.22 µm membrane filter and stored at 4°C until to use. To prepare cell extract elicitor, *P. indica* culture on 8th day of growth was autoclaved (15 min) and paper filtered under aseptic conditions to obtain fungal cell residue. The weighed fungal cells were washed with sterile water for three times and homogenized by mortar and pestle in sterile water (10 mg/mL). The obtained cell extract was stored at 4°C until to use. The elicitors were added at various concentrations (2.0, 4.0 and 6.0% v/v) to HRs cultures (liquid cultures of superior HR line) during late growth phase (30th day) with exposure times of 48 h, 72 h and 96 h. After the exposure, HRs cultures were harvested for biomass, molecular and phytochemical analysis. The untreated HRs cultures were

used as control samples. Dry weight (DW) was determined by drying the HRs in Behdad incubator model 50 (Tehran, Iran) at 30°C for four days followed by 1-2 days at 60°C to reach a constant weight. Growth index (GI) of HRs was determined by the formula: $GI = (\text{final dry weight} - \text{weight at zero time}) / \text{weight at zero time}$.

2.5. Total RNA extraction and first strand cDNA synthesis

Total RNA from elicited HRs and non-elicited HRs of *F. carica* was isolated using the cetyltrimethylammonium bromide (CTAB)-based RNA extraction protocol (Reid et al., 2006). Quality and quantity of RNA was determined by agarose gel (1.2%) electrophoresis and measuring absorbance at 260 nm and 280 nm in a nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). The first strand cDNA was synthesized with 2 µL of total RNA by RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Lithuania) accordance to the manufacturer's instructions.

2.6. Quantitative real-time polymerase chain reaction

Expression rate of phenylalanine ammonia-lyase (*FcPAL*), chalcone synthase (*FcCHS*) and UDP glucose-flavonoid 3-O-glucosyl-transferase (*FcUGT*) genes in start, middle and end point of flavonoids biosynthetic pathway and *FcMYB3* gene as transcription factor in HRs of *F. carica* (Fig. 1) were determined to investigate the impact of elicitation. Details of specific primer sets adopted from Wang et al. (2017) are given in Table 1. Prior to RT-PCR, the RT-PCR products of control HRs cDNA were checked by 1.2% agarose gel electrophoresis to confirm amplicon size of specific primer sets. Each three-step real-time RT-PCR reaction was performed on Rotor-Gene Q cyclor (QIAGEN, USA) using Thermo Scientific Maxima[®] SYBR-Green/ROX qPCR Master mix kit (Thermo Fisher Scientific, Lithuania) at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s, annealing (acquiring on green) at 54-60 °C for 40 s and extension at 72 °C for 45 s. The relative quantitative analysis by $2^{-\Delta\Delta CT}$ method was used to determine the fold change in expression level of genes. Actin as an endogenous housekeeping gene (*FcACT1*) (Ikegami et al., 2013) was used for normalization of real-time PCR data.

2.7. Estimation of total phenolic content (TPC) and total flavonoids content (TFC)

The powdered HRs (100 mg) were extracted in 3 mL of 80% methanol solvent. The mixture was shaken for 3-5 h, bath sonicated for 30-60 min at 30°C, centrifuged (4500 rpm, 20 min) and the collected supernatant was designated as methanol or HR extract. TPC Measurement of HRs was based on Folin-Ciocalteu colorimetric method as described by Singleton and Rossi (1965) with some modifications. The Folin- Ciocalteu solution containing 180 µL of distilled water and 1200 µL of Folin-Ciocalteu reagent (10%) was added to 25 µL HRs extract. After addition of 960 µL of 7% sodium carbonate solution (Na_2CO_3), the tubes were allowed to stand in darkness at $25\pm 2^\circ\text{C}$ for 30 min. Thereafter, absorbance was recorded at UV/Vis absorption maxima $\lambda_{\text{max}} = 760 \text{ nm}$ by a spectrophotometry (Halo DB-20 UV-Vis double beam spectrophotometer, Dynamica, UK). The TPC value was expressed as mg gallic acid equivalent (GAE) per g of dry weight (DW) through use of gallic acid calibration curve ($y = 0.0144 x + 0.2303$; $r^2 = 0.996$).

Total flavonoids content was determined according to aluminium chloride (AlCl_3) method described by Bakar et al. (2009). The 50 µL of HRs extract was added to 150 µL of 5% nitrite sodium. The mixture was maintained at room temperature for 5 min followed by addition 300 µL of 10% AlCl_3 . After 5-10 min, 1000 µL NaOH (1M) was added and mixed well. The absorbance was read at $\lambda_{\text{max}} = 510 \text{ nm}$ by a spectrophotometer. Calibration curve was constructed by use of quercetin ($y = 0.0094 x + 0.712$; $r^2 = 0.994$). The TFC value was calculated as mg of quercetin equivalent per g of DW.

2.8. Antioxidant assay

Antioxidant capacity of HRs cultures was evaluated by two colorimetric methods of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging (Argolo et al., 2004) and ferric reducing antioxidant power (FRAP) (Benzie and Strain, 1996) assays. The correlation between DPPH and FRAP measurements was assessed by Pearson correlation analysis.

2.8.1. DPPH assay

The DPPH (0.4 μ M) was dissolved in methanol (MeOH) and 2000 μ L of this solution was added to 5 μ L of HRs extract. The mixture was allowed to stand in darkness for 30 min. DPPH value was recorded at $\lambda_{\text{max}} = 517$ nm. The percentage of scavenged DPPH radical was calculated by the formula: Inhibition (%) = $100 \times (\text{Absorbance of the blank} - \text{Absorbance of the sample}) / \text{Absorbance of the blank}$.

2.8.2. FRAP assay

FRAP reagent was prepared by mixing 300 μ M acetate buffer (pH 3.6), 10 μ M 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) solution in 40 μ M HCl, and 20 μ M $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution in a ratio of 10:1:1(v/v/v). FRAP reagent (3000 μ L) was added to 25 μ L of HRs extract and incubated in warm water bath (37°C) for 30 min. The absorbance was recorded at $\lambda_{\text{max}} = 593$ nm. FRAP curve was calibrated by $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ($y = 0.0064x + 0.2972$; $r^2 = 0.990$). FRAP value was presented as 1 mol (M) $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ per g of DW.

2.9. HPLC analysis

To prepare extract for high-performance liquid chromatography (HPLC) analysis, the dried HRs (200 mg) were homogenized in 6 mL methanol 80% (HPLC grade), bath-sonicated (25-30°C for 20 min), and centrifuged (3500 rpm, 15 min) for three times. The supernatant was collected and filtered using 0.22 μ m membrane. The HPLC standards of phenolic compounds (purity >98%) were purchased from Merck (Darmstadt, Germany). HPLC analysis of nine phenolic compounds including gallic acid ($\text{C}_7\text{H}_6\text{O}_5$), cinnamic acid ($\text{C}_9\text{H}_8\text{O}_2$) and apigenin ($\text{C}_{15}\text{H}_{10}\text{O}_5$) at $\lambda_{\text{max}} = 272$ nm; chlorogenic acid ($\text{C}_{16}\text{H}_{18}\text{O}_9$), rosmarinic acid ($\text{C}_{18}\text{H}_{16}\text{O}_8$) and quercetin ($\text{C}_{15}\text{H}_{10}\text{O}_7$) at $\lambda_{\text{max}} = 250$ nm; Caffeic acid ($\text{C}_9\text{H}_8\text{O}_4$), coumaric acid ($\text{C}_9\text{H}_8\text{O}_3$) and rutin ($\text{C}_{27}\text{H}_{30}\text{O}_{16}$) at $\lambda_{\text{max}} = 310$ nm was carried out by a high-performance liquid chromatography system (Agilent 1100 Series HPLC Value System of Agilent Technologies American Manufacturing Company) as described in our previous study (Amani et al., 2020). Analysis of reverse phase liquid chromatographic was done using analytical column of octadecylsilane (Shim-pack VP-ODS 25 cm long $\times$ 4.6 mm

diameter, particle size 5 μm , Japan). The separation of each compound was performed based on the gradient ratio of acetonitrile to acetic acid (v/v) for 35 min. The obtained data was processed using Chemstation software.

2.10. Statistical analysis

The values were shown as the means $\pm$ standard deviation (SD) of three replicates of samples. Statistical analysis of data was performed by One-way analysis of variance (ANOVA) followed by Duncan's multiple range tests at significance level of $P < 0.05$ using IBM SPSS Statistic software version 22.

3. Results

3.1. Establishment of HRs

Inoculation of shoot explants with *R. rhizogenes* ATCC15834 strain suspension allowed to emerging HRs at wounded sites after 10-17 days of co-culture time (Fig. 2). As a result of previous study (Amani et al., 2020), SW8 HR line was screened as a superior HR line with the highest growth rate and high branching morphology and sub-cultured regularly over 10 months for further experiments. The observation of 780-bp DNA fragment with bacterial plasmid DNA as positive control confirmed the presence of *rolB* gene in HRs lines. No DNA band was detected in PCR products of the non-transformed roots as negative control (Fig. 2).

3.2. Effect of *P. indica* on HRs growth rate

To investigate the effect of fungal elicitors on HR growth, SW8 HR cultures were exposed to *P. indica* elicitors at the end of the growth phase. The growth index of HR cultures was significantly affected by culture filtrate and cell homogenate of *P. indica* as biotic elicitors, depending on concentration and exposure times (Table 2). Fungal elicitation with culture filtrate and as well as cell extract prolonged the progressive phase of growth and thus increased the growth rate of HRs in comparison with non-elicited cultures except for a few cases (72 h and 96 h in 4% cell extract (v/v) and 96 h in 6% cell extract (v/v)), (Table 2). The highest growth index was recorded after 72 h elicitation

with 2% fungal cell extract (40.82 %) after an exposure time of 72 h. The growth index of elicited HRs reduced with further increase in fungal cell extract concentration.

3.3. Expression analysis of some genes involved in flavonoid biosynthetic pathway

Investigation of fungal elicitation effect on expression level of some flavonoid biosynthetic pathway genes (*FcPAL*, *FcCHS*, *FcUFGT* and *FcMYB3*) in HRs cultures of *F. carica* cv. Sabz (Fig. 1) was performed by real time q-PCR analysis. As shown in Fig. 3, in depending of the concentration and elicitation time, expression levels of the target genes in *F. carica* HRs were significantly altered after exposure to fungal elicitors. In case of 2% culture filtrate, with increasing exposure time from 48 to 96 h, the expression of *FcPAL* gene increased significantly from 0.5 in 48 h to 1.89 in 72 h and then 2.34 at 96 h. At higher concentrations of culture filtrate, the highest expression level of PAL gene was 0.9 fold, which was recorded after 48 h of elicitation with 6% culture filtrate. In the case of fungal cell extract, although treatments of 2 and 6 % (v/v) did not induce a significant enhancement in *FcPAL* gene expression, but elicitation with 4% concentration and 72 h incubation, the *FcPAL* gene expression peaked to the highest level (2.4 fold) compared with control HRs (Fig. 3, A). In about of the expression of *FcCHS* gene, fungal culture filtrate at exposure time of 72 h was the most effective elicitor; the first peak of 8.78-fold was observed at 4% concentration and the second peak of 1.08 fold at 2% concentration of culture filtrate (Fig. 3, B). In similarly, after 72 h elicitation of *F. carica* HRs with 2 % (v/v) *P. indica* culture filtrate, the expression level of *FcUFGT* gene was 0.93 and, with increasing of concentration to 4%, it was significantly increased to 1.65 fold compared with control HRs (Fig. 3, C). The expression level of *FcMYB3* transcription factor gene was also strongly influenced by the fungal elicitation (Fig. 3, D). The maximum induction level of *FcMYB3* gene expression was observed after 72 h of 2 % culture filtrate elicitation, which showed an expression peak of 8.027 fold. On the other hand, the expression level of *FcMYB3* gene up-regulated after 96 h of 2% cell extract elicitation and reached to a peak of 4.65 fold at 72 h after 4% cell extract treatment.

3.4. Effect of *P. indica* on total phenolic and flavonoids content

The HRs cultures showed higher content of total phenolic and flavonoid compounds in comparison with normal roots. Fungal elicitation could affect the total phenolic content of HRs cultures with dependence on elicitor concentration and exposure time. It was found that the culture filtrate was more effective than cell extract in enhancing the total phenolic and flavonoids content. At low concentration of 2% (v/v), 72 h- exposure time yielded the best result, but at concentrations of 4% and 6% (v/v), 48 h-elicitation was more effective. The highest content of phenolic compounds in HRs cultures elicited with fungal cell extract (2.9-fold increase) was recorded at 2% concentration with exposure time of 72 h (Fig. 4). However, a significant increase of total flavonoid content in comparison to HRs control (2-fold increase) was found only by elicitation with cell extract concentration of 6% (v/v) for an exposure time 48 h (Fig. 4). Therefore, the optimum concentration of the fungal elicitor to induce high content of phenolic and flavonoid compounds in *F. carica* HRs was closely related to the elicitation time.

3.5. Effect of *P. indica* on antioxidant capacity

Besides affecting the content of total phenolic and flavonoid compounds, fungal elicitation exert significant effects on antioxidant capacity of HRs, depending on the concentration and exposure time of elicitor. Based on DPPH assay, HRs cultures elicited with low concentration (2 % v/v) of both culture filtrate and cell extract exhibited the highest DPPH radical scavenging activity after 72 h incubation (Fig. 5). FRAP antioxidant activity, as shown in Fig. 5, was significantly increased under elicitation treatments except for 96-h incubation with 6% cell extract (v/v).

3.6. Effect of *P. indica* on phenolic compounds

3.6.1. Apigenin, quercetin and rutin content

The HPLC analysis data indicated that fungal elicitation had a significant effect on the content of apigenin, quercetin and rutin in comparison with control HRs. Except for one case with 4% culture filtrate elicitor for 48 h, apigenin content increased remarkably in all treatments of elicitation (Table 3). The highest amount of apigenin (420.25 µg/g

DW, 27.6-fold greater than control) and rutin (45.84 $\mu\text{g/g}$ DW) were obtained using elicitation with 2 % (v/v) of culture filtrate for 72 h incubation. The maximum content of quercetin (490.49 $\mu\text{g/g}$ DW, 8.8 times more than control HRs) was recorded in 48 h incubation period with 6% (v/v) of cell extract. The highest content of rutin (45.84 $\mu\text{g/g}$ DW) was obtained after 72 h elicitation with 2 % (v/v) culture filtrate (Table 3).

3.6.2. Phenolic acids

To evaluate the effect of fungal elicitation on the synthesis of phenolic compounds in *F. carica* HRs cultures, six phenolic acids including gallic, caffeic, chlorogenic, coumaric, rosmarinic and coumaric acids were analyzed through HPLC. Fungal elicitation affected the phenolic compounds production depending on elicitor concentration and exposure time (Table 3). The highest stimulant effect was observed with fungal culture filtrate elicitation. The maximum content of gallic acid (7486.3 $\mu\text{g/g}$ DW, 80.5 times higher than control HRs), caffeic acid (828.58 $\mu\text{g/g}$ DW, 26.2-times), coumaric acid (936.5 $\mu\text{g/g}$ DW, 4.5-times), cinnamic acid (222.29 $\mu\text{g/g}$ DW, 60.1-times) was obtained after 72 h elicitation with 2% culture filtrate. While the maximum level of chlorogenic acid (1812.74 $\mu\text{g/g}$ DW, 4.8-times) was recorded with 6 % concentration of culture filtrate for 48 h incubation. Exposure to 2 % of culture filtrate for 96 h induced the maximum content of rosmarinic acid (2259.9 $\mu\text{g/g}$ DW, 95.8-times) in HRs cultures, whereas with other concentrations of culture filtrate except at 6 % (v/v) for 72 h, rosmarinic acid was not detected. Probably, esterified and/or glycosylated derivatives of rosmarinic acid coexisted with the free form and they haven't been detected in the assay.

In the case of cell extract, 2 % concentration was more effective on gallic, caffeic, chlorogenic and coumaric phenolic acids synthesis, while concentration of 6% on cinnamic acid synthesis and 4% on rosmarinic acid synthesis displayed higher stimulatory effect (Table 3). Based on results of this study, HR cultures equipped with *P. indica* cell extract/culture filtrate elicitors can be considered as an attractive tool for overproduction of phenolic compounds in *F. carica*.

4. Discussion

Induction of HR using *Rhizobium rhizogenes* and its successful establishment for extensive cultivation in order to use in pharmaceutical and other industries is one of the valuable achievements of the plant biotechnology field (Gai et al., 2015). Significant features of HRs include high growth rate, genetic stability, negative geotropism, high lateral branching and the lack of need for growth regulators in the culture medium (Hakkinen et al., 2016; Roychowdhury et al., 2015; Sharma et al., 2013). Accordance to genetic stability of HRs, molecular confirmation of bacterial transfer DNA (T-DNA) integration into the host genome by PCR analysis is the appropriate option to ensure transformation process. Although it remains unclear which *rol* genes in T-DNA are responsible for HR induction, it is clear that *rolB* gene plays the most important role (Nilsson and Olsson, 1997). It has been proved that transformation efficiency can be strongly influenced by the type of explants and strains of bacteria (Cao et al., 2009; Henzi et al., 2000; Vanhala et al., 1995). The successful induction of HRs by *R. rhizogenes* 15834 strain has been reported in many plant species, *Peganum harmala* (Zayed, 2011), peanut (Medina-Bolivar et al., 2007) and *Boerhaavia diffusa* (Gupta et al., 2016).

Currently, HR culture combined with the elicitation technique has been considered as helpful biotechnological tool for high production of secondary metabolites. Elicitors can influence the growth rate and metabolic profile in HR cultures (Narayani and Srivastava, 2017). The growth promoting effects of *P. indica* based elicitation have been reported in cell suspension culture of *Linum album* (Baldi et al., 2008). Our results are in agreement with Kumar et al. (2012) and Tashackori et al. (2016, 2018) where significant improvement in growth of HRs of *L. album* has been achieved using elicitors prepared from *P. indica*. Based on the results, lower concentration of fungal elicitor (2% v/v) at exposure time of 72 h was optimum in HRs growth of *F. carica*. The behavior of *P. indica* as an elicitor in the HRs cultures, in addition to type of plant species, depends on the concentration and exposure time. Hence, not necessarily, the higher concentrations will be induced the highest growth rate, perhaps the lowest biomass will be observed at higher concentrations. The mechanism of growth elicitation of *P. indica* is still not well-known, but it has been reported that culture filtrate and cell extract of *P. indica* as signaling molecules can induce plant immune responses and phenylpropanoid biosynthetic pathway, and so triggered the production of secondary metabolite such as phenolic and flavonoid compounds (Baldi et al., 2009; Tashackori et al., 2016). In most cases, the content of phenolic

compounds in the presence of *P. indica* elicitors showed a positive correlation with the accumulation of HRs biomass. The outcome of the fungal elicitation can be attributed to the combined or individual effects of extracellular fungal compounds including enzymes, chitin and disaccharides (Varma et al., 2013). Our results demonstrated that *P. indica* elicited the phenylpropanoid pathway to induce synthesis of phenolic acids and flavonoids in HRs cultures of *F. carica* L. and the correlation between phenolic compounds and genes expression level involved in the biosynthesis pathway of flavonoids was dependant on concentration and exposure time of treatment. As described in other studies (Huang et al., 2016; Nourozi et al., 2019; Tashackori et al., 2018), elicitation altered the transcript abundance of genes in elicited HRs cultures in comparison with control HRs. The complex of MYB, bHLH (basic helix-loop-helix) and WDR (WD-repeat proteins) transcription factors have been known as essential regulator factors in cell development and expression genes involved in the pathway of flavonoid biosynthesis (Wang et al., 2017). *MYB3* belongs to subgroup 4 of R2R3 MYB transcription factors. One of the important roles of *MYB3* transcription factor is the activation of some genes which responsible for the flavonoid biosynthesis such as *CHS*, *CHI*, *FLS*, *ANS* and *UFGT* genes (Vimolmangkang et al., 2013). In flowers of gentian plant it was reported that *MYB3* TF in complex with *bHLH1* displayed an activator role in expression of *F3'5'H* and *5AT* genes in blue flowers and *CHS*, *F3H*, *DFR*, *ANS* and *3GT* genes in orange flowers (Berman et al., 2016). Herein, along with the up-regulation of *MYB3* gene in response to 72 h elicitation with 4% culture filtrate, the highest co-expression of *FcCHS* and *FcUFGT* genes was also observed in HRs elicited. The expression level of *FcMYB3* and *FcCHS* genes were notably higher than *FcPAL* and *FcUFGT*. Therefore, it can be concluded that the application of *P. indica* culture filtrate elicitor in cultures of *F. carica* HRs was more effective compared to cell extract elicitor. On the other hand, since, higher content of phenolic compounds was recorded at 2% concentration of culture filtrate, it was expected that the highest expression level of flavonoid genes would also be obtained in the same treatment condition, but it was not. Hence, due to the complexity of the biosynthesis pathway for phenolic compounds, in order to provide a general result, all factors (other genes and transcription factors) involved in this process should be investigated. The positive effect of *P. indica* elicitation on expression level of genes responsible for secondary metabolites has also been reported in HRs and cell cultures of *Linum album* (Bahabadi et al., 2012; Tashackori et al., 2018) and cell cultures of *Withania somnifera* (Ahlawat et al., 2016).

In the line with genes expression, it has been determined that elicitation is able to increase the synthesis of secondary metabolites, especially phenolic compounds and, thereby increasing the antioxidant capacity of HR cultures (Jiao et al., 2018). By activating the plant defense system under efficient elicitation conditions, a significant increase in the production of phenolic compounds in HRs culture can be achieved (Tashackori et al., 2018). In our study, antioxidant activity of *F. carica* HRs showed a positive correlation with TPC values. The Pearson correlation coefficient of 0.777 ($p < 0.0001$) was found between DPPH and TPC and 0.825 ($p < 0.00001$) between FRAP and TPC. This positive correlation, which was also reported from previous studies (Michel et al., 2012; Zainol et al., 2003), suggested that phenolic compounds contributed considerably to enhance antioxidant activity.

Recently, the potential of pathogenic and root endophytic fungi (*P. indica*) have been confirmed as a good source of biotic elicitors in the culture of plant cells and HRs. In this regard, the enhancement production of lignans and phenolic compounds in *Linum* spp. (Bahabadi et al., 2012; Kumar et al., 2013; Tashackori et al., 2016), pentacyclic triterpenoids in *Lantana camara* L. (Kumar et al., 2016) and withaferins in *Withania somnifera* (Ahlawat et al., 2016; Ahlawat et al., 2017) has been reported as elicitation results of *in vitro* cultures with *P. indica*.

In natural conditions, phenolic acids are produced in all parts of plants, whereas flavonoids are often found in tissues susceptible to UV light such as leaves (epidermal layers) and apical meristems (Winkel-Shirley, 2002). In roots, flavonoids accumulate in the nuclei of meristematic cells and cytoplasm of epidermal and outer cortical cells of root tip and root cap as glycoside or aglycone forms that can release through root exudation, decomposition or leaching (Rao, 1990; Weston and Mathesius, 2013). Root flavonoids and other phenolic compounds as signaling molecules play crucial roles in symbiotic functions and plant protection from soil pathogens (Rao, 1990; Weston and Mathesius, 2013). Therefore, production of phenolic compounds is regulated by internal and environmental signals and it can be stimulated under abiotic and biotic stresses (Cheynier et al., 2013). Accordingly, unique morphological characters of HRs and elicitation with *P. indica* as simulated biotic stress condition cause to overproduction of phenolic acids and flavonoids in HR cultures. It seems that the elicitation resulted in activation or inactivation the expression of transcription factors and so key genes involved in biosynthesis pathway of phenolic compounds in HRs culture of *F. carica*. Flavonoids and phenolic acids are considered as the major classes of phenolic compounds which have different

biological activities in human health including carcinogen inactivation (Sharma et al., 2017). The major achievements of this study are the high production of dietary flavonoids (apigenin, quercetin and rutin), and important phenolic acids as well as rosmarinic acid, which has so far not been extracted from the plant natural tissues.

As a conclusion, the present study showed that, depending on concentration and treatment time, *P. indica* elicitation induced to change biomass, phenolic compounds accumulation and antioxidant activity of HRs. *P. indica* elicitation improved the expression rate of genes and increased the synthesis of apigenin, quercetin, rutin and phenolic acids (gallic, caffeic, chlorogenic, coumaric, rosmarinic and cinnamic acids) in *F. carica* elicited-HRs. Hence, HRs cultures of *F. carica* equipped with biotic elicitors could be presented as a new prospect to produce valuable biochemical compounds.

Conflict of Interest Statement

The authors clearly declare that the study was conducted in the absence of any relationship types that could be inferred as a conflict of interest.

References

- Ahlawat, S., Saxena, P., Ali, A., Abdin, M.Z., 2016. *Piriformospora indica* elicitation of withaferin A biosynthesis and biomass accumulation in cell suspension cultures of *Withania somnifera*. Symbiosis 69, 37-46. <https://doi.org/10.1007/s13199-015-0364-9>
- Ahlawat, S., Saxena, P., Ali, A., Khan, S., Abdin, M.Z., 2017. Comparative study of withanolide production and the related transcriptional responses of biosynthetic genes in fungi elicited cell suspension culture of *Withania somnifera* in shake flask and bioreactor. Plant Physiol. Biochem. 114, 19-28. <https://doi.org/10.1016/j.plaphy.2017.02.013>
- Alamgeer, Iman, S., Asif, H., Saleem, M., 2017. Evaluation of antihypertensive potential of *Ficus carica* fruit. Pharm. Biol. 55, 1047-1053. <https://doi.org/10.1080/13880209.2017.1278611>
- Amani, S., Mohebodini, M., Khademvatan, S., Jafari, M., 2020. *Agrobacterium rhizogenes* mediated transformation of *Ficus carica* l. for the efficient production of secondary metabolites. J. Sci. Food Agric. 100, 2185-2197. <https://doi.org/10.1002/jsfa.10243>
- Argolo, A., Sant'Ana, A., Pletsch, M., Coelho, L., 2004. Antioxidant activity of leaf extracts from *Bauhinia monandra*. Bioresour. Technol. 95, 229-233. <https://doi.org/10.1016/j.biortech.2003.12.014>

384 Azizian, D., 2001. Flora of Iran: Moraceae. Research Institute of Forests and Rangelands.

385 Bahabadi, S.E., Sharifi, M., Behmanesh, M., Safaie, N., Murata, J., Araki, R., Yamagaki, T., Satake, H., 2012. Time-
386 course changes in fungal elicitor-induced lignan synthesis and expression of the relevant genes in cell cultures of
387 *Linum album*. J. Plant Physiol. 169, 487-491. <https://doi.org/10.1016/j.jplph.2011.12.006>

388 Bakar, M.F.A., Mohamed, M., Rahmat, A., Fry, J., 2009. Phytochemicals and antioxidant activity of different parts
389 of bambangan (*Mangifera pajang*) and tarap (*Artocarpus odoratissimus*). Food Chem. 113, 479-483.
390 <https://doi.org/10.1016/j.foodchem.2008.07.081>

391 Baldi, A., Jain, A., Gupta, N., Srivastava, A., Bisaria, V., 2008. Co-culture of arbuscular mycorrhiza-like fungi
392 (*Piriformospora indica* and *Sebacina vermifera*) with plant cells of *Linum album* for enhanced production of
393 podophyllotoxins: a first report. Biotechnol. Lett. 30, 1671. <https://doi.org/10.1007/s10529-008-9736-z>

394 Baldi, A., Srivastava, A., Bisaria, V., 2009. Fungal elicitors for enhanced production of secondary metabolites in plant
395 cell suspension cultures. Symbiotic Fungi. Springer Berlin Heidelberg, pp. 373-380. https://doi.org/10.1007/978-3-540-95894-9_23

397 Barolo, M.I., Mostacero, N.R., López, S.N., 2014. *Ficus carica* L.(Moraceae): an ancient source of food and health.
398 Food Chem. 164, 119-127. <https://doi.org/10.1016/j.foodchem.2014.04.112>

399 Benzie, I.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the
400 FRAP assay. Anal. Biochem. 239, 70-76. <https://doi.org/10.1006/abio.1996.0292>

401 Berman, J., Sheng, Y., Gómez, L.G., Veiga, T., Ni, X., Farré, G., Capell, T., Guitián, J., Guitián, P., Sandmann, G.,
402 2016. Red anthocyanins and yellow carotenoids form the color of orange-flower gentian (*Gentiana lutea* L. var.
403 aurantiaca). PloS one 11, e0162410. <https://doi.org/10.1371/journal.pone.0162410>

404 Bertani, G., 1951. Studies on lysogenesis i.: The mode of phage liberation by lysogenic escherichia coli. J. Bacteriol.
405 62, 293. PMID: 14888646; PMCID: PMC386127.

406 Cao, D., Hou, W., Song, S., Sun, H., Wu, C., Gao, Y., Han, T., 2009. Assessment of conditions affecting
407 *Agrobacterium rhizogenes*-mediated transformation of soybean. Plant Cell, Tissue Organ Cult. 96, 45-52.
408 <https://doi.org/10.1007/s11240-008-9458-x>

409 Cheynier, V., Comte, G., Davies, K.M., Lattanzio, V., Martens, S., 2013. Plant phenolics: recent advances on their
410 biosynthesis, genetics, and ecophysiology. Plant Physiol. Biochem. 72, 1-20.
411 <https://doi.org/10.1016/j.plaphy.2013.05.009>

412 Delgoda, R., Murray, J., 2017. Evolutionary perspectives on the role of plant secondary metabolites. Pharmacognosy.
413 Elsevier, pp. 93-100. <https://doi.org/10.1016/B978-0-12-802104-0.00007-X>

414 Falcone Ferreyra, M.L., Rius, S., Casati, P., 2012. Flavonoids: biosynthesis, biological functions, and biotechnological
415 applications. Front. Plant Sci. 3, 222. <https://doi.org/10.3389/fpls.2012.00222>

416 Fu, C.-X., Xu, Y.-j., Zhao, D.-X., Ma, F.S., 2006. A comparison between hairy root cultures and wild plants of
417 *Saussurea involucreata* in phenylpropanoids production. Plant Cell Rep. 24, 750. <https://doi.org/10.1007/s00299-005-0049-6>

- Gai, Q.-Y., Jiao, J., Luo, M., Wei, Z.-F., Zu, Y.-G., Ma, W., Fu, Y.-J., 2015. Establishment of hairy root cultures by *Agrobacterium rhizogenes* mediated transformation of *Isatis tinctoria* L. for the efficient production of flavonoids and evaluation of antioxidant activities. PloS One 10, e0119022. <https://doi.org/10.1371/journal.pone.0119022>
- Ghanbari, A., Le Gresley, A., Naughton, D., Kuhnert, N., Sirbu, D., Ashrafi, G.H., 2019. Biological activities of *Ficus carica* latex for potential therapeutics in Human Papillomavirus (HPV) related cervical cancers. Sci. Rep. 9, 1013. <https://doi.org/10.1038/s41598-018-37665-6>
- Gilani, A.H., Mehmood, M.H., Janbaz, K.H., Khan, A.-u., Saeed, S.A., 2008. Ethnopharmacological studies on antispasmodic and antiplatelet activities of *Ficus carica*. J. Ethnopharmacol. 119, 1-5. <https://doi.org/10.1016/j.jep.2008.05.040>
- Gupta, R., Pandey, P., Singh, S., Singh, D.K., Saxena, A., Luqman, S., Bawankule, D.U., Banerjee, S., 2016. Advances in *Boerhaavia diffusa* hairy root technology: a valuable pursuit for identifying strain sensitivity and up-scaling factors to refine metabolite yield and bioactivity potentials. Protoplasma 253, 1145-1158. <https://doi.org/10.1007/s00709-015-0875-5>
- Hakkinen, S.T., Moyano, E., Cusido, R.M., Oksman-Caldentey, K.-M., 2016. Exploring the metabolic stability of engineered hairy roots after 16 years maintenance. Front. Plant Sci. 7, 1486. <https://doi.org/10.3389/fpls.2016.01486>
- Havsteen, B.H., 2002. The biochemistry and medical significance of the flavonoids. Pharmacol. Ther. 96, 67-202. [https://doi.org/10.1016/S0163-7258\(02\)00298-X](https://doi.org/10.1016/S0163-7258(02)00298-X)
- Henzi, M., Christey, M., McNeil, D., 2000. Factors that influence *Agrobacterium rhizogenes*-mediated transformation of broccoli (*Brassica oleracea* L. var. italica). Plant Cell Rep. 19, 994-999. <https://doi.org/10.1007/s002990000221>
- Huang, X., Yao, J., Zhao, Y., Xie, D., Jiang, X., Xu, Z., 2016. Efficient rutin and quercetin biosynthesis through flavonoids-related gene expression in *Fagopyrum tataricum* Gaertn. hairy root cultures with UV-B irradiation. Front. Plant Sci. 7, 63. <https://doi.org/10.3389/fpls.2016.00063>
- Ikegami, H., Habu, T., Mori, K., Nogata, H., Hirata, C., Hirashima, K., Tashiro, K., Kuhara, S., 2013. De novo sequencing and comparative analysis of expressed sequence tags from gynodioecious fig (*Ficus carica* L.) fruits: caprifig and common fig. Tree Genet. Genomes 9, 1075-1088. <https://doi.org/10.1007/s11295-013-0622-z>
- Jagtap, U.B., Bapat, V.A., 2020. Exploring Phytochemicals of *Ficus carica* L. (Fig). In: Murthy, H.N., Bapat, V.A. (Eds.), Bioactive Compounds in Underutilized Fruits and Nuts. Springer International Publishing, Cham, pp. 353-368. https://doi.org/10.1007/978-3-030-30182-8_19
- Jiang, X., Liu, Y., Li, W., Zhao, L., Meng, F., Wang, Y., Tan, H., Yang, H., Wei, C., Wan, X., 2013. Tissue-specific, development-dependent phenolic compounds accumulation profile and gene expression pattern in tea plant [*Camellia sinensis*]. PloS One 8, e62315. <https://doi.org/10.1371/journal.pone.0062315>
- Jiao, J., Gai, Q.-Y., Wang, W., Zang, Y.-P., Niu, L.-L., Fu, Y.-J., Wang, X., 2018. Remarkable enhancement of flavonoid production in a co-cultivation system of *Isatis tinctoria* L. hairy root cultures and immobilized *Aspergillus niger*. Ind. Crops Prod. 112, 252-261. <https://doi.org/10.1016/j.indcrop.2017.12.017>
- Jisha, S., Gouri, P., Anith, K., Sabu, K., 2018. *Piriformospora indica* cell wall extract as the best elicitor for asiaticoside production in *Centella asiatica* (L.) Urban, evidenced by morphological, physiological and molecular analyses. Plant Physiol. Biochem. 125, 106-115. <https://doi.org/10.1016/j.plaphy.2018.01.021>

456 Kafer, E., 1977. Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. Advances in
457 Genetics. Elsevier, pp. 33-131. [https://doi.org/10.1016/S0065-2660\(08\)60245-X](https://doi.org/10.1016/S0065-2660(08)60245-X)

458 Kaur, K., Pati, P.K., 2018. Stress-Induced Metabolite Production Utilizing Plant Hairy Roots. Hairy Roots. Springer
459 Singapore, pp. 123-145. https://doi.org/10.1007/978-981-13-2562-5_6

460 Kumar, N., Goel, N., 2019. Phenolic acids: Natural versatile molecules with promising therapeutic applications.
461 Biotechnology Reports 24, e00370. <https://doi.org/10.1016/j.btre.2019.e00370>

462 Kumar, P., Chaturvedi, R., Sundar, D., Bisaria, V., 2016. *Piriformospora indica* enhances the production of
463 pentacyclic triterpenoids in *Lantana camara* L. suspension cultures. Plant Cell, Tissue Organ Cult. 125, 23-29.
464 <https://doi.org/10.1007/s11240-015-0924-y>

465 Kumar, V., Rajauria, G., Sahai, V., Bisaria, V., 2012. Culture filtrate of root endophytic fungus *Piriformospora indica*
466 promotes the growth and lignan production of *Linum album* hairy root cultures. Process Biochem. 47, 901-907.
467 <https://doi.org/10.1016/j.procbio.2011.06.012>

468 Kumar, V., Sahai, V., Bisaria, V., 2013. Effect of *Piriformospora indica* on enhanced biosynthesis of anticancer drug,
469 podophyllotoxin, in plant cell cultures of *Linum album*. *Piriformospora indica*. Springer, pp. 119-137.
470 https://doi.org/10.1007/978-3-642-33802-1_7

471 Li, F.-X., Jin, Z.-P., Zhao, D.-X., Cheng, L.-Q., Fu, C.-X., Ma, F., 2006. Overexpression of the *Saussurea medusa*
472 chalcone isomerase gene in *S. involucrata* hairy root cultures enhances their biosynthesis of apigenin. Phytochemistry
473 67, 553-560. <https://doi.org/10.1016/j.phytochem.2005.12.004>

474 Li, S.-S., Wu, J., Chen, L.-G., Du, H., Xu, Y.-J., Wang, L.-J., Zhang, H.-J., Zheng, X.-C., Wang, L.-S., 2014.
475 Biogenesis of C-glycosyl flavones and profiling of flavonoid glycosides in lotus (*Nelumbo nucifera*). PLoS One 9,
476 e108860. <https://doi.org/10.1371/journal.pone.0108860>

477 Lloyd, G., McCown, B., (1980) Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use
478 of shoot-tip culture. International Plant Propagators Society, pp. 421-427.

479 Medina-Bolivar, F., Condori, J., Rimando, A.M., Hubstenberger, J., Shelton, K., O'Keefe, S.F., Bennett, S., Dolan,
480 M.C., 2007. Production and secretion of resveratrol in hairy root cultures of peanut. Phytochemistry 68, 1992-2003.
481 <https://doi.org/10.1016/j.phytochem.2007.04.039>

482 Michel, T., Destandau, E., Le Floch, G., Lucchesi, M.E., Elfakir, C., 2012. Antimicrobial, antioxidant and
483 phytochemical investigations of sea buckthorn (*Hippophaë rhamnoides* L.) leaf, stem, root and seed. Food Chem. 131,
484 754-760. <https://doi.org/10.1016/j.foodchem.2011.09.029>

485 Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures.
486 Physiol. Plant. 15, 473-497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>

487 Narayani, M., Srivastava, S., 2017. Elicitation: a stimulation of stress in *in vitro* plant cell/tissue cultures for
488 enhancement of secondary metabolite production. Phytochem. Rev. 16, 1227-1252. [https://doi.org/10.1007/s11101-](https://doi.org/10.1007/s11101-017-9534-0)
489 [017-9534-0](https://doi.org/10.1007/s11101-017-9534-0)

490 Nilsson, O., Olsson, O., 1997. Getting to the root: the role of the *Agrobacterium rhizogenes* *rol* genes in the formation
491 of hairy roots. Physiol. Plant. 100, 463-473. <https://doi.org/10.1111/j.1399-3054.1997.tb03050.x>

492 Nourozi, E., Hosseini, B., Maleki, R., Mandoulakani, B.A., 2019. Pharmaceutical important phenolic compounds
 493 overproduction and gene expression analysis in *Dracocephalum kotschyi* hairy roots elicited by SiO₂ nanoparticles.
 494 Ind. Crops Prod. 133, 435-446. <https://doi.org/10.1016/j.indcrop.2019.03.053>

495 Pirttilä, A.M., Hirsikorpi, M., Kamarainen, T., Jaakola, L., Hohtola, A., 2001. DNA isolation methods for medicinal
 496 and aromatic plants. Plant Mol. Biol. Rep. 19, 273-273. <https://doi.org/10.1007/BF02772901>

497 Rao, A.S., 1990. Root flavonoids. Bot. Rev. 56, 1-84. <https://doi.org/10.1007/BF02858531>

498 Reid, K.E., Olsson, N., Schlosser, J., Peng, F., Lund, S.T., 2006. An optimized grapevine RNA isolation procedure
 499 and statistical determination of reference genes for real-time RT-PCR during berry development. BMC Plant Biol. 6,
 500 27. <https://doi.org/10.1186/1471-2229-6-27>

501 Ren, W., Qiao, Z., Wang, H., Zhu, L., Zhang, L., 2003. Flavonoids: promising anticancer agents. Med. Res. Rev. 23,
 502 519-534. <https://doi.org/10.1002/med.10033>

503 Roychowdhury, D., Basu, A., Jha, S., 2015. Morphological and molecular variation in Ri-transformed root lines are
 504 stable in long term cultures of *Tylophora indica*. Plant Growth Regul. 75, 443-453. [https://doi.org/10.1007/s10725-](https://doi.org/10.1007/s10725-014-0005-y)
 505 [014-0005-y](https://doi.org/10.1007/s10725-014-0005-y)

506 Sambrook, J., Russell, D., (2001) Molecular cloning: A laboratory manual, the third edition. Cold spring harbor
 507 laboratory press, cold spring harbor, New York.

508 Sharma, A., Joshi, N., Kumar, R.A., Agrawal, P.K., Prasad, S., 2017. High performance liquid chromatographic
 509 analysis of phenolic compounds and their antioxidant properties from different cultivars of *Cyamopsis tetragonaloba*
 510 (L.) Taub. Microchem. J. 133, 622-628. <https://doi.org/10.1016/j.microc.2017.04.020>

511 Sharma, P., Padh, H., Shrivastava, N., 2013. Hairy root cultures: a suitable biological system for studying secondary
 512 metabolic pathways in plants. Eng. Life Sci. 13, 62-75. <https://doi.org/10.1002/elsc.201200030>

513 Singh, R.S., Chattopadhyay, T., Thakur, D., Kumar, N., Kumar, T., Singh, P.K., 2018. Hairy root culture for *in vitro*
 514 production of secondary metabolites: A promising biotechnological approach. Biotechnological Approaches for
 515 Medicinal and Aromatic Plants. Springer Singapore, pp. 235-250. https://doi.org/10.1007/978-981-13-0535-1_10

516 Singleton, V.L., Rossi, J.A., 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid
 517 reagents. Am. J. Enol. Vitic. 16, 144-158.

518 Tashackori, H., Sharifi, M., Chashmi, N.A., Behmanesh, M., Safaie, N., 2018. *Piriformospora indica* cell wall
 519 modulates gene expression and metabolite profile in *Linum album* hairy roots. Planta 248, 1289-1306.
 520 <https://doi.org/10.1007/s00425-018-2973-z>

521 Tashackori, H., Sharifi, M., Chashmi, N.A., Safaie, N., Behmanesh, M., 2016. Induced-differential changes on lignan
 522 and phenolic acid compounds in *Linum album* hairy roots by fungal extract of *Piriformospora indica*. Plant Cell,
 523 Tissue Organ Cult. 127, 187-194. <https://doi.org/10.1007/s11240-016-1041-2>

524 Tian, J., Zhang, J., Han, Z.-y., Song, T.-t., Li, J.-y., Wang, Y.-r., Yao, Y.-c., 2017. McMYB12 transcription factors
 525 co-regulate proanthocyanidin and anthocyanin biosynthesis in *Malus crabapple*. Sci. Rep. 7, 43715.
 526 <https://doi.org/10.1038/srep43715>

527 Vanhala, L., Hiltunen, R., Oksman-Caldentey, K.-M., 1995. Virulence of different *Agrobacterium* strains on hairy
528 root formation of *Hyoscyamus muticus*. Plant Cell Rep. 14, 236-240. <https://doi.org/10.1007/BF00233640>

529 Varma, A., Kost, G., Oelmüller, R., 2013. *Piriformospora indica*: Sebacinales and their biotechnological applications,
530 illustrated ed. Springer Science & Business Media.

531 Vimolmangkang, S., Han, Y., Wei, G., Korban, S.S., 2013. An apple MYB transcription factor, *MdMYB3*, is involved
532 in regulation of anthocyanin biosynthesis and flower development. BMC Plant Biol. 13, 176.
533 <https://doi.org/10.1186/1471-2229-13-176>

534 Wang, Z., Cui, Y., Vainstein, A., Chen, S., Ma, H., 2017. Regulation of fig (*Ficus carica* L.) fruit color: metabolomic
535 and transcriptomic analyses of the flavonoid biosynthetic pathway. Front. Plant Sci. 8, 1990.
536 <https://doi.org/10.3389/fpls.2017.01990>

537 Weston, L.A., Mathesius, U., 2013. Flavonoids: their structure, biosynthesis and role in the rhizosphere, including
538 allelopathy. J. Chem. Ecol. 39, 283-297. <https://doi.org/10.1007/s10886-013-0248-5>

539 Winkel-Shirley, B., 2002. Biosynthesis of flavonoids and effects of stress. Curr. Opin. Plant Biol. 5, 218-223.
540 [https://doi.org/10.1016/S1369-5266\(02\)00256-X](https://doi.org/10.1016/S1369-5266(02)00256-X)

541 Yan, Q., Shi, M., Ng, J., Wu, J.Y., 2006. Elicitor-induced rosmarinic acid accumulation and secondary metabolism
542 enzyme activities in *Salvia miltiorrhiza* hairy roots. Plant Sci. 170, 853-858.
543 <https://doi.org/10.1016/j.plantsci.2005.12.004>

544 Zainol, M., Abd-Hamid, A., Yusof, S., Muse, R., 2003. Antioxidative activity and total phenolic compounds of leaf,
545 root and petiole of four accessions of *Centella asiatica* (L.) Urban. Food Chem. 81, 575-581.
546 [https://doi.org/10.1016/S0308-8146\(02\)00498-3](https://doi.org/10.1016/S0308-8146(02)00498-3)

547 Zayed, R., 2011. Efficient *in vitro* elicitation of β -carboline alkaloids in transformed root cultures of *Peganum*
548 *harmala*. Bulletin of Faculty of Pharmacy, Cairo University 49, 7-11. <https://doi.org/10.1016/j.bfopcu.2011.07.002>

549

550 **Tables**

551 Table 1. Primer sequence information for real-time quantitative PCR analysis

Primer name	Sequence (5' to 3')	T _a (°C)
ACT1-Forward	GCTGGTCGTGATCTCACTGAC	55 °C
ACT1-Reverse	TCAGCACCGATTGTGATGACC	
FcPAL-Forward	GCAAGCCTTGA ACTCTCCAC	56 °C
FcPAL-Reverse	GGTTCTGCGAGAAGGATCTG	
FcCHS-Forward	CCGTGAAGTTGGGCFTTACAT	54 °C
FcCHS-Reverse	AAACCACACTTGGCTTCCAC	
FcUFGT2-Forward	CAGTGTCGTTTGCTGCAGAT	60 °C
FcUFGT2-Reverse	AAGGAAGTCAACGGCGAGTA	
FcMYB3-Forward	GCAATTGCATTCAAGGGTTT	54 °C
FcMYB3-Reverse	GCCTTCCAGACACCAAATGT	

552

553 Table 2. Impact of different concentrations and exposure time of *P. indica* based elicitors on growth of *F. carica* HRs

elicitor	Concentration (%)	exposure time (h)	Δ DW (mg)	GI (%)
Control	0	48	3.20±0.45 ^{ef}	0.3±0.043 ^k
		72	12.20 ± 1.10 ^{def}	1.15 ±0.10 ^k
		96	12.80 ±1.60 ^{def}	1.2 ±0.15 ^k
Culture filtrate	2	48	96.40±1.40 ^{bcd}	7.62±1.10 ^j
		72	402.80±1.50 ^a	35.81±0.13 ^b
		96	181.70±7.50 ^{bc}	25.60±1.05 ^{cd}
	4	48	213.70±2.00 ^{bc}	22.05±0.20 ^{de}
		72	204.20±10.00 ^{bc}	20.23±1.00 ^{def}
		96	104.20±6.00 ^{bcd}	10.06±0.56 ^{ij}
	6	48	216.00±3.28 ^b	13.69±2.10 ^{hij}
		72	132.90±14.10 ^{bc}	14.52±1.50 ^{ghi}
		96	163.10±8.00 ^{bc}	14.35 ±0.75 ^{ghi}
Fungal cell extract	2	48	93.00±10.00 ^{de}	10.75±1.16 ^j
		72	433.80±17.00 ^a	40.82±5.00 ^a
		96	13.70±3.00 ^{def}	1.22±0.17 ^k
	4	48	397.20±14.0 ^a	30.47±1.10 ^c
		72	(-)194.20±16.00 ^g	(-)13.49±1.11 ^l
		96	(-)1.20±0.11 ^{ef}	(-)0.12±1.20 ^k
	6	48	175.80±3.81 ^{bc}	17.84±3.90 ^{gh}
		72	123.70±10.00 ^{bcd}	12.62±1.00 ^{hi}
		96	(-)56.30±1.00 ^f	(-)3.85±0.34 ^k

554 DW: g dry weight; Δ DW: g [DW_{after elicitation} -DW_{before elicitation}]; GI: Growth index (Δ DW)/DW_{before elicitation}.

555 Data presented as mean ± SD of three replicates. The same letters in a column represent not significant difference at *P* = 0.05 level.

556

Table 3. Phenolic compounds accumulation ($\mu\text{g/g DW}$) in *F. carica* HRs cultures elicited with different concentration of fungal elicitors

Elicitor	Concen (% v/v)	exposure time (h)	$\mu\text{g/g}$								
			Gal	Caf	Chl	Cou	Ros	Cin	Rut	Que	Api
Control		48	92.90 ⁱ	31.50 ^o	377.59 ^q	208.19 ^g	23.33 ^h	3.66 ^j	8.00 ^k	54.87 ⁿ	15.13 ^q
		72	92.98 ⁱ	31.60 ^o	379.38 ^q	209.48 ^g	23.54 ^h	3.75 ^j	8.05 ^k	55.35 ⁿ	15.21 ^q
		96	93.18 ⁱ	31.87 ^o	379.55 ^q	209.88 ^g	23.91 ^h	3.86 ^j	8.08 ^k	56.06 ⁿ	15.36 ^q
Culture filtrate	2	48	121.57 ^h	231.67 ^b	1209.06 ^f	248.16 ^e	-	0.88 ^{mn}	15.81 ^e	87.09 ^l	57.95 ^l
		72	7486.3 ^a	828.58 ^a	316.62 ^s	936.50 ^a	-	222.29 ^a	45.84 ^a	212.57 ^e	420.25 ^a
		96	262.85 ^c	24.95 ^p	946.10 ^j	21.23 ^o	2259.9 ^a	1.27 ^{mn}	9.11 ^j	11.51 ^p	21.42 ^p
	4	48	83.66 ^j	210.75 ^c	1094.15 ^g	221.60 ^f	-	1.46 ^{lm}	22.91 ^c	66.77 ^m	11.23 ^r
		72	41.53 ⁿ	115.87 ^h	1380.44 ^e	150.25 ^h	-	6.76 ^h	18.89 ^d	442.37 ^b	247.72 ^c
		96	28.58 ^p	43.76 ⁿ	360.22 ^r	151.49 ^h	-	6.41 ^h	9.33 ^j	23.49 ^o	26.55 ^o
	6	48	382.44 ^b	24.86 ^p	1812.74 ^a	16.57 ^p	-	46.44 ^c	8.66 ^j	12.42 ^p	103.11 ^j
		72	62.33 ^l	181.60 ^e	1020.94 ⁱ	119.93 ^j	128.40 ^e	22.58 ^e	10.92 ^{hi}	255.61 ^d	199.38 ^e
		96	83.40 ^j	59.77 ^l	682.16 ^l	519.96 ^b	-	3.01 ^k	11.24 ^{gh}	118.99 ^j	53.38 ^m
Fungal cell extract	2	48	134.82 ^f	195.26 ^d	1396.95 ^d	319.01 ^d	51.3 ^s	4.64 ⁱ	11.97 ^g	272.89 ^c	322.31 ^b
		72	177.82 ^d	154.80 ^f	1510.43 ^c	29.62 ⁿ	-	11.89 ^g	11.33 ^{gh}	154.83 ^b	62.93 ^k
		96	127.41 ^g	148.68 ^g	1583.23 ^b	346.88 ^c	-	6.42 ^h	10.39 ^j	114.23 ^k	47.51 ⁿ
	4	48	63.74 ^{kl}	105.04 ⁱ	475.21 ⁿ	10.73 ^q	764.60 ^b	21.16 ^f	18.38 ^d	181.87 ^g	130.59 ^h
		72	53.73 ^m	98.81 ^j	877.64 ^k	21.78 ^o	-	0.84 ^{mn}	10.33 ⁱ	-	222.33 ^d
		96	165.48 ^e	31.69 ^o	620.44 ^m	39.37 ^m	137.10 ^d	0.72 ⁿ	6.66 ^l	213.02 ^e	136.25 ^e
	6	48	36.49 ^o	21.98 ^q	402.96 ^p	126.79 ⁱ	62.00 ^f	2.04 ^l	10.68 ^{hi}	490.49 ^a	148.82 ^f
		72	64.98 ^k	77.08 ^k	1071.13 ^h	52.56 ^l	381.00 ^c	27.92 ^d	14.48 ^f	145.39 ^j	116.81 ⁱ
		96	23.70 ^q	55.92 ^m	455.88 ^o	93.64 ^k	-	135.35 ^b	43.41 ^b	203.95 ^f	135.99 ^g

P. indica culture filtrate; *P. indica* cell extract; phenolic compounds: Gal, gallic acid; Caf, caffeic acid; Chl, chlorogenic acid; Cou, coumaric acid; Ros, rosmarinic acid; Cin, cinamic acid; Rut, rutin; Que, quercetin; Api, apigenin; -, not detected. Data presented as mean of three replicates. The same letters in a column represent not significant difference at $P = 0.05$ level.

562

563 **Figures legends**

564 **Fig 1.** Phenylpropanoid and flavonoid biosynthetic pathway in *Ficus carica*

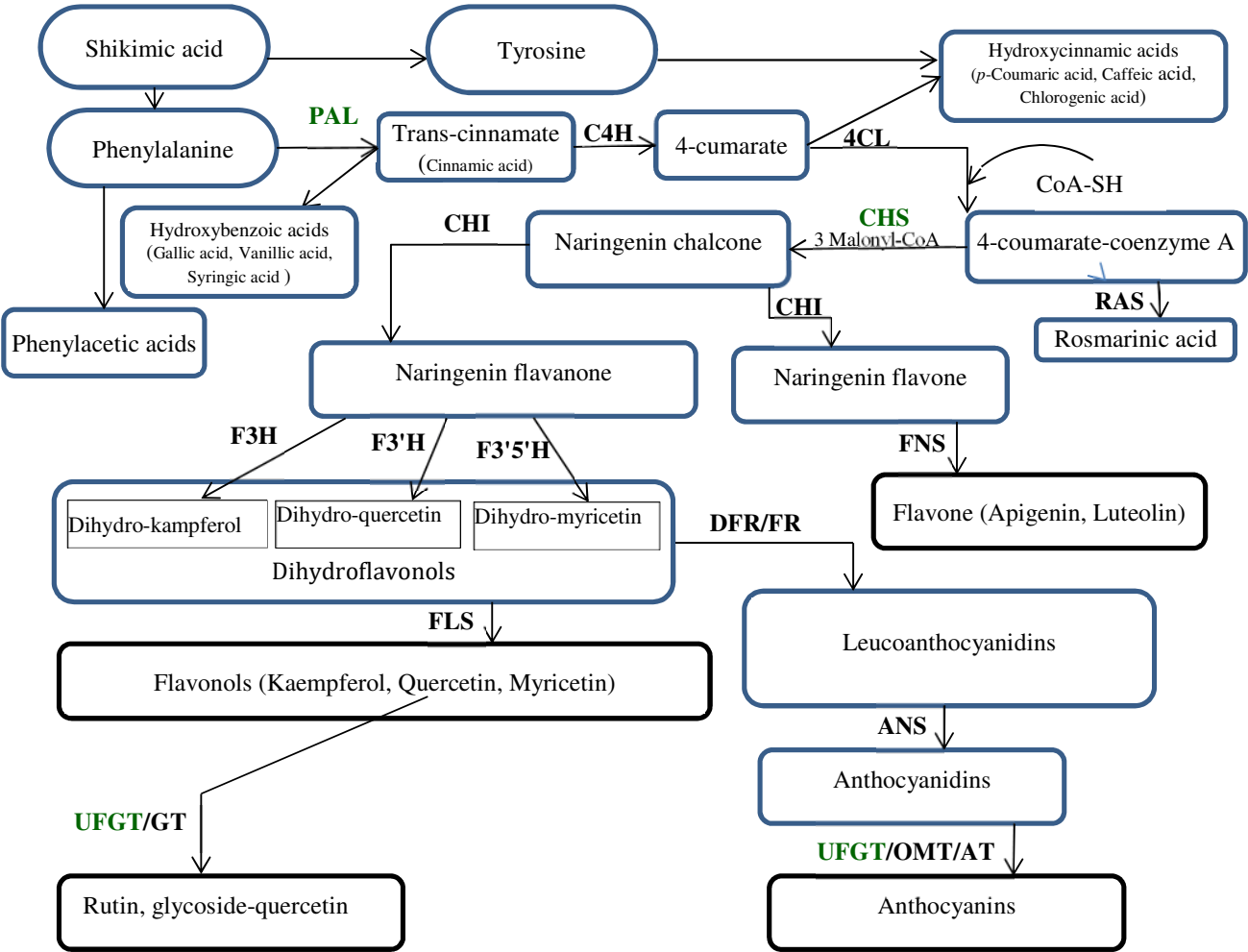
565 **Fig 2.** Hairy roots (HRs) establishment (A) and PCR-based confirmation of *rolB* gene presence (B) in *F. carica*; (A):
566 Induced HRs at different sites of shoot explant (a and b), Established HRs on ½ MS solid medium (c), HRs cultures
567 at exponential growth phase (d), HRs liquid cultures in late growth phase, before elicitation (e), HRs cultures after
568 elicitation (f); Scale bar (a-c)= 5 mm, Scale bar (d-f)= 22 mm.
569 (B): L: 1 kb DNA marker ladder, HR: HR line, C⁺: *Rhizobium rhizogenes* plasmid as positive control, C⁻: normal root,
570 C⁰: PCR reaction without DNA.

571 **Fig 3.** Effect of fungal elicitation on expression level of (A) *PAL* gene, (B) *CHS* gene, (C) *UFGT* gene and (D) MYB3
572 transcription factor in *F. carica* HRs cultures, The same letters represent not significant difference at *P* = 0.05 level.

573 **Fig 4.** Effects of different concentration of fungal elicitors on the amount of (A) total phenolic content and (B) total
574 flavonoids content in HR cultures of *F. carica*. Data presented as mean of three replicates. The same letters represent
575 not significant difference at *P* = 0.05 level.

576 **Fig 5.** Influence of different concentration of fungal elicitors on the antioxidant activity of HR cultures based on (A)
577 DPPH assay and (B) FRAP assay in HR cultures of *F. carica*. Data presented as mean of three replicates. The same
578 letters represent not significant difference at *P* = 0.05 level

579 **Figure 1**

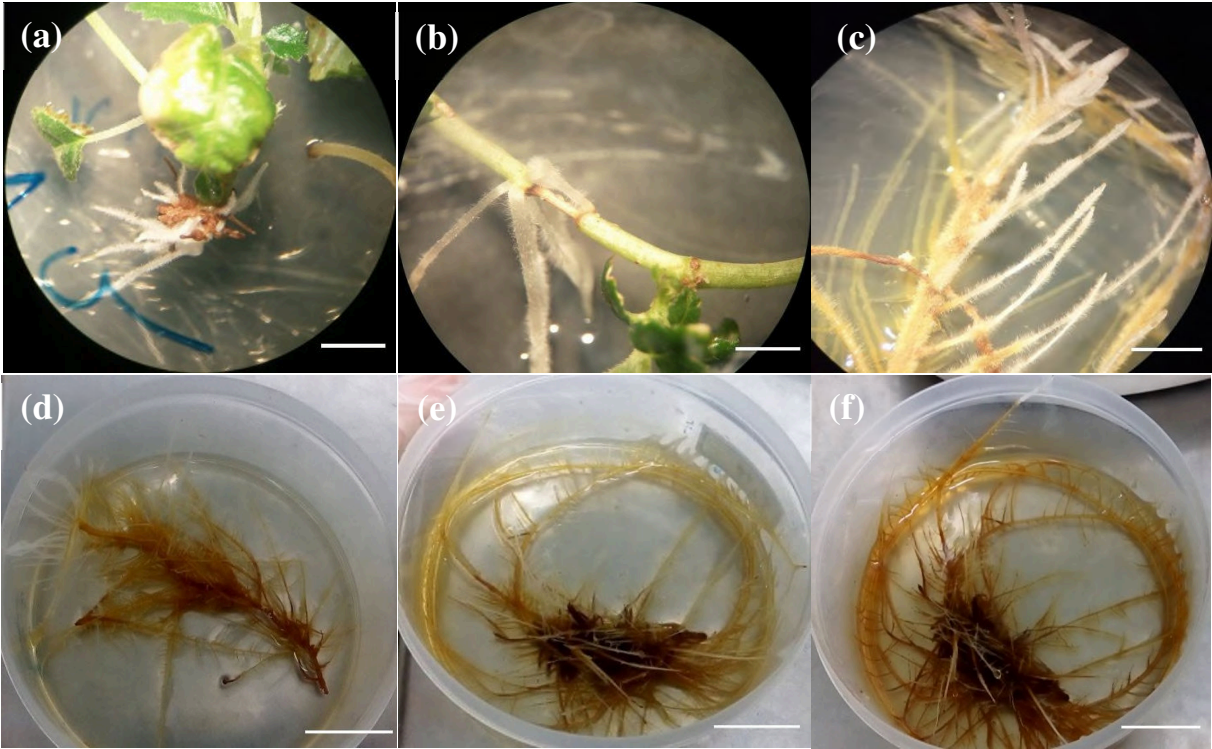


580

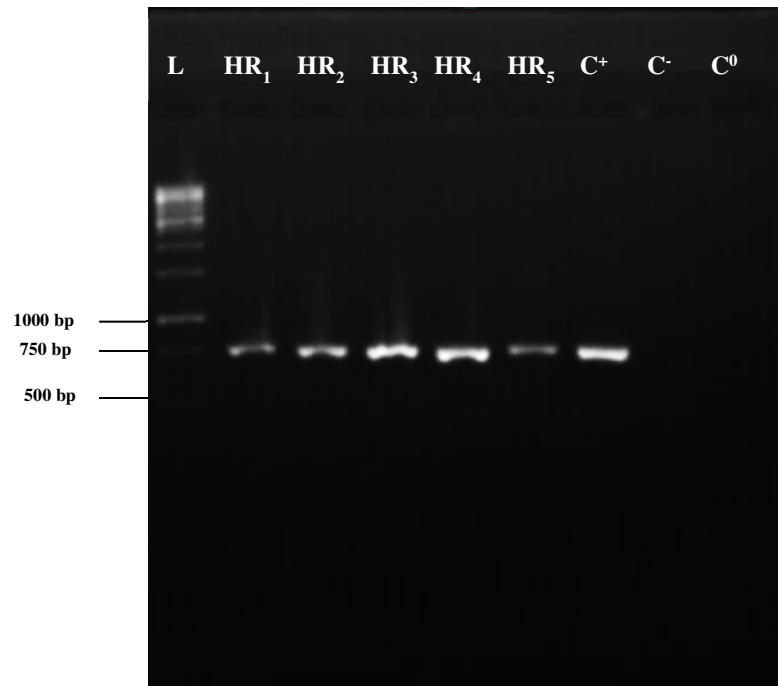
581

Figure 2

A



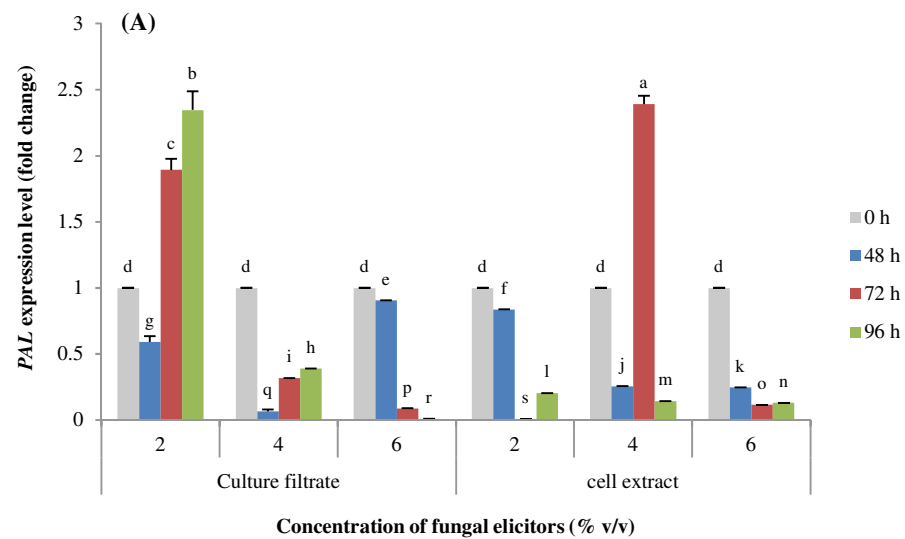
B



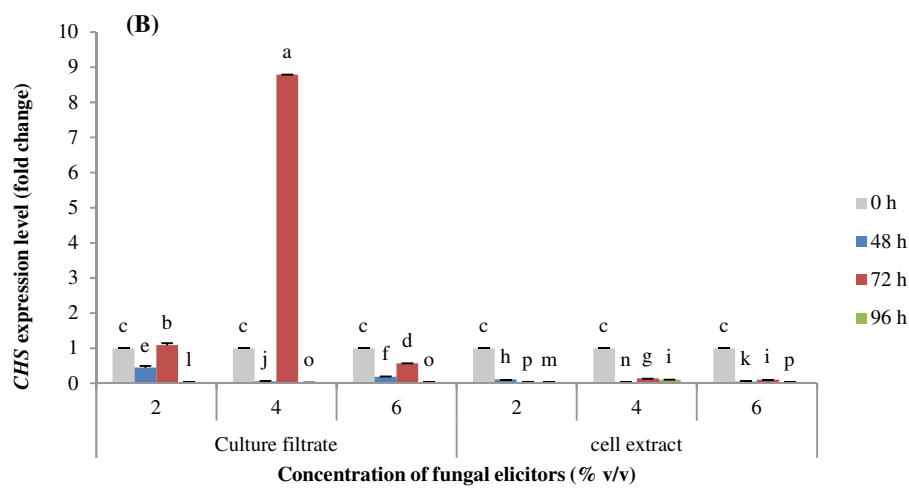
587

588

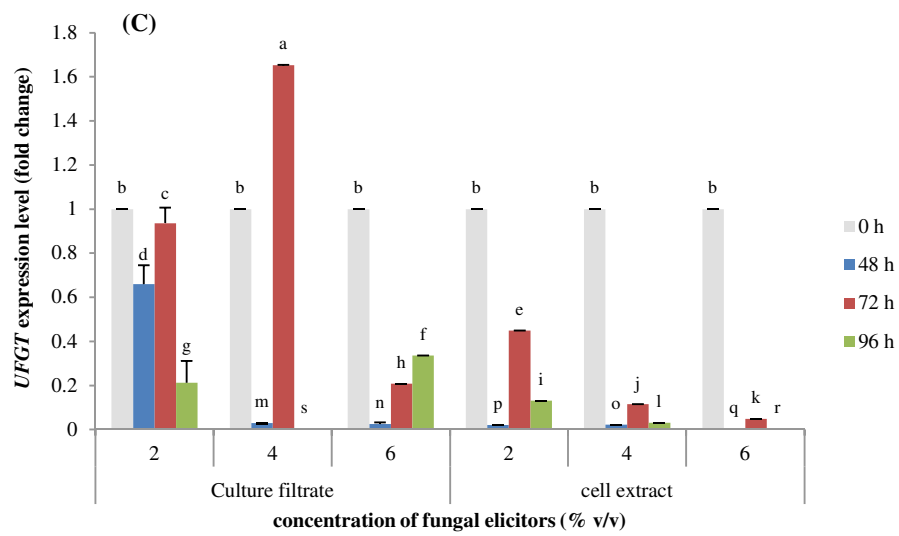
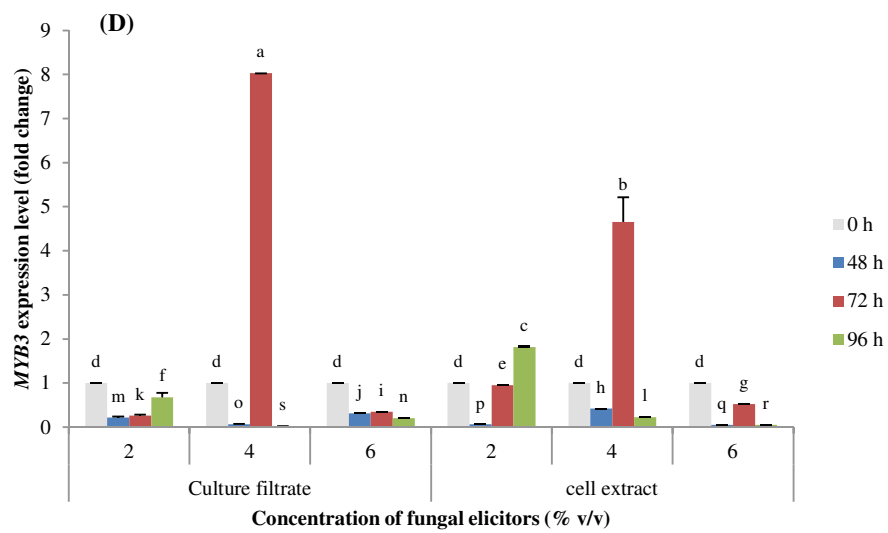
589 **Figure 3**



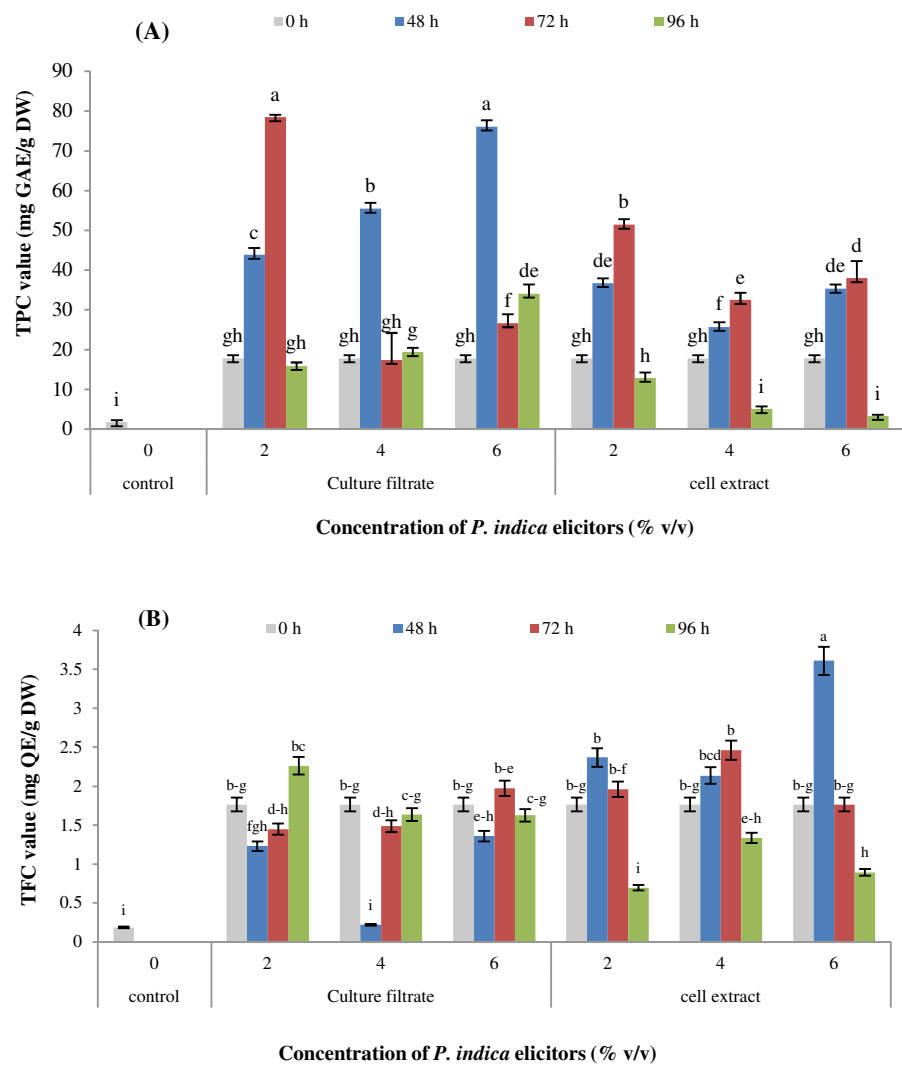
590



591



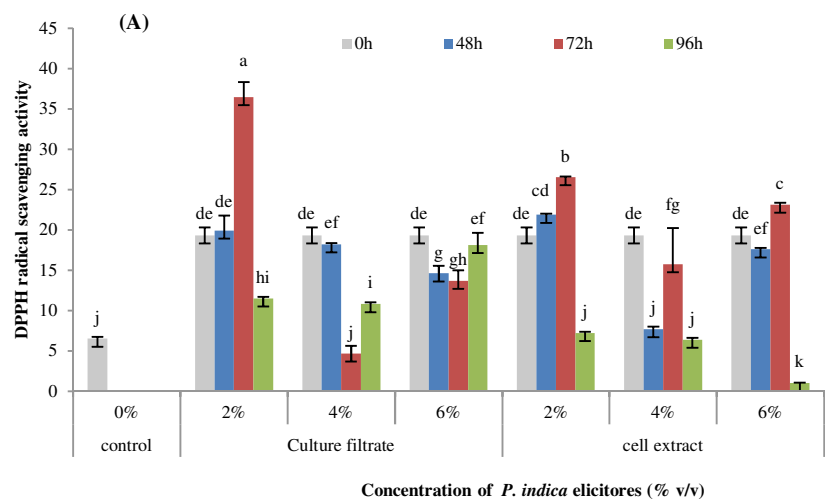
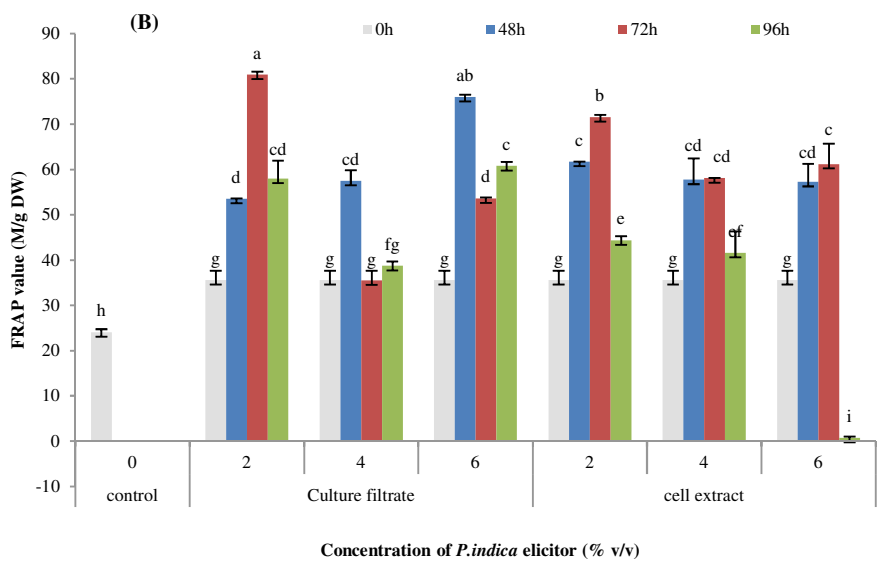
596 **Figure 4**



598

599

600 **Figure 5**





DSPACE

<https://dspace.org/>

Piriformospora indica based elicitation for overproduction of phenolic compounds by hairy root cultures of Ficus carica

Amani, Shahla; Mohebodini, Mehdi; Khademvatan, Shahram; Jafari, Morad; Kumar, Vinod

2020-12-30

Elsevier

<https://doi.org/10.1016/j.jbiotec.2020.12.015>

Amani S, Mohebodini M, Khademvatan S, et al., (2021) Piriformospora indica based elicitation for overproduction of phenolic compounds by hairy root cultures of Ficus carica. Journal of Biotechnology, Volume 327, February 2021, pp. 43-53