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Title

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

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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 exhibits 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 ºC 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 ºC 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 ºC 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 *rol*B gene-specific primer (5'- ATGGATCCCAAATTGCTATTCCCCCACGA-3'and 5'-TTAGGCTTCTTTCATTCGGTTTACTGCAGC-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 *rol*B gene, the PCR amplification program was set up as follows: 94ºC for 5 min, 30 cycles of 94ºC for 60s, 60ºC for 60s and 72 ° C for 70s and followed by 72ºC 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±2ºC. 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 4ºC 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 4ºC 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= (final dry weight - weight at zero time)/ 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-glcosyl-transferase (*FcUFGT*) 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 cycler (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.

153 **2.7. Estimation of total phenolic content (TPC) and total flavonoids content (TFC)**

154 The powdered HRs (100 mg) were extracted in 3 mL of 80% methanol solvent. The mixture was shaken for 3-5 h,
155 bath sonicated for 30-60 min at 30ºC, centrifuged (4500 rpm, 20 min) and the collected supernatant was designated
156 as methanol or HR extract. TPC Measurement of HRs was based on Folin-Ciocalteu colorimetric method as described
157 by Singleton and Rossi (1965) with some modifications. The Folin- Ciocalteu solution containing 180 µL of distilled
158 water and 1200 µL of Folin-Ciocalteu reagent (10%) was added to 25 µL HRs extract. After addition of 960 µL of
159 7% sodium carbonate solution (Na_2CO_3), the tubes were allowed to stand in darkness at 25±2ºC for 30 min. Thereafter,
160 absorbance was recorded at UV/Vis absorption maxima λ_{max} = 760 nm by a spectrophotometry (Halo DB-20 UV-Vis
161 double beam spectrophotometer, Dynamica, UK). The TPC value was expressed as mg gallic acid equivalent (GAE)
162 per g of dry weight (DW) through use of gallic acid calibration curve ($y = 0.0144 x + 0.2303$; $r^2 = 0.996$).
163 Total flavonoids content was determined according to aluminium chloride ($AlCl_3$) method described by Bakar et al.
164 (2009). The 50 µL of HRs extract was added to 150 µL of 5% nitrite sodium. The mixture was maintained at room
165 temperature for 5 min followed by addition 300 µL of 10% $AlCl_3$. After 5-10 min, 1000 µL NaOH (1M) was added
166 and mixed well. The absorbance was read at λ_{max} = 510 nm by a spectrophotometer. Calibration curve was constructed
167 by use of quercetin ($y = 0.0094 x + 0.712$; $r^2 = 0.994$). The TFC value was calculated as mg of quercetin equivalent
168 per g of DW.

169 **2.8. Antioxidant assay**

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

2.8.1. DPPH assay

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

2.8.2. FRAP assay

FRAP reagent was prepared by mixing 300 µM acetate buffer (pH 3.6), 10 µM 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) solution in 40 µM HCl, and 20 µM $FeCl_3.6H_2O$ solution in a ratio of 10:1:1(v/v/v). FRAP reagent (3000 µL) was added to 25 µL of HRs extract and incubated in warm water bath (37ºC) for 30 min. The absorbance was recorded at λ_{max}= 593 nm. FRAP curve was calibrated by $FeSO_4.7H_2O$ (y = 0.0064 x + 0.2972; r^2 = 0.990). FRAP value was presented as 1 mol (M) $FeSO_4.7H_2O$ 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 (25-30ºC for 20 min), and centrifuged (3500 rpm, 15 min) for three times. The supernatant was collected and filtered using 0.22 µm membrane. The HPLC standards of phenolic compounds (purity >98%) were purchased from Merck (Darmstadt, Germany). HPLC analysis of nine phenolic compounds including gallic acid ($C_7H_6O_5$), cinnamic acid ($C_9H_8O_2$) and apigenin ($C_{15}H_{10}O_5$) at λ_{max}= 272 nm; chlorogenic acid ($C_{16}H_{18}O_9$), rosmarinic acid ($C_{18}H_{16}O_8$) and quercetin ($C_{15}H_{10}O_7$) at λ_{max}=250 nm; Caffeic acid ($C_9H_8O_4$), coumaric acid ($C_9H_8O_3$) and rutin ($C_{27}H_{30}O_{16}$) at λ_{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 × 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 ± 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 *rol*B 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 µg/ g DW) were obtained using elicitation with 2 % (v/v) of culture filtrate for 72 h incubation. The maximum content of quercetin (490.49 µg/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 µg/ 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 µg/ g DW, 80.5 times higher than control HRs), caffeic acid (828.58 µg/g DW, 26.2-times), coumaric acid (936.5 µg/g DW, 4.5-times), cinnamic acid (222.29 µg/g DW, 60.1-times) was obtained after 72 h elicitation with 2% culture filtrate. While the maximum level of chlorogenic acid (1812.74 µg/ 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 µg/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 be 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 us 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 *rol*B 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 Fc*MYB3* 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 routine), 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.

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550 **Tables**

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

Primer name	Sequence (5' to 3')	T_a (ºC)
ACT1-Forward	GCTGGTCGTGATCTCACTGAC	55 ºC
ACT1-Reverse	TCAGCACCGATTGTGATGACC	
FcPAL-Forward	GCAAGCCTTGAACTCTCCAC	56 ºC
FcPAL-Reverse	GGTTCTGCGAGAAGGATCTG	
FcCHS-Forward	CCGTGAAGTTGGGCFTTACAT	54 ºC
FcCHS-Reverse	AAACCACACTTGGCTTCCAC	
FcUFGT2-Forward	CAGTGTCGTTTGCTGCAGAT	60 ºC
FcUFGT2-Reverse	AAGGAAGTCAACGGCGAGTA	
FcMYB3-Forward	GCAATTGCATTCAAGGGTTT	54 ºC
FcMYB3-Reverse	GCCTTCCAGACACCAAATGT	

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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)	ΔDW (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	2	48	96.40±1.40^{bcde}	7.62±1.10^{j}
filtrate		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^{bcde}	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	2	48	93.00±10.00^{cde}	10.75±1.16^{ij}
extract		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^{fgh}
		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; ΔDW: g [$DW_{after\ elicitation}$ -$DW_{before\ elicitation}$]; GI: Growth index (ΔDW)/$DW_{before\ elicitation}$.

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

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Table 3. Phenolic compounds accumulation (µg/g DW) in *F. carica* HRs cultures elicited with different concentration of fungal elicitors

Elicitor	Concen (% v/v)	exposure time (h)	µg/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	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}
filtrate		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	2	48	134.82^{f}	195.26^{d}	1396.95^{d}	319.01^{d}	51.3^{g}	4.64^{i}	11.97^{g}	272.89^{c}	322.31^{b}
extract		72	177.82^{d}	154.80^{f}	1510.43^{c}	29.62^{n}	-	11.89^{g}	11.33^{gh}	154.83^{h}	62.93^{k}
		96	127.41^{g}	148.68^{g}	1583.23^{b}	346.88^{c}	-	6.42^{h}	10.39^{i}	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^{i}	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.

Figures legends

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

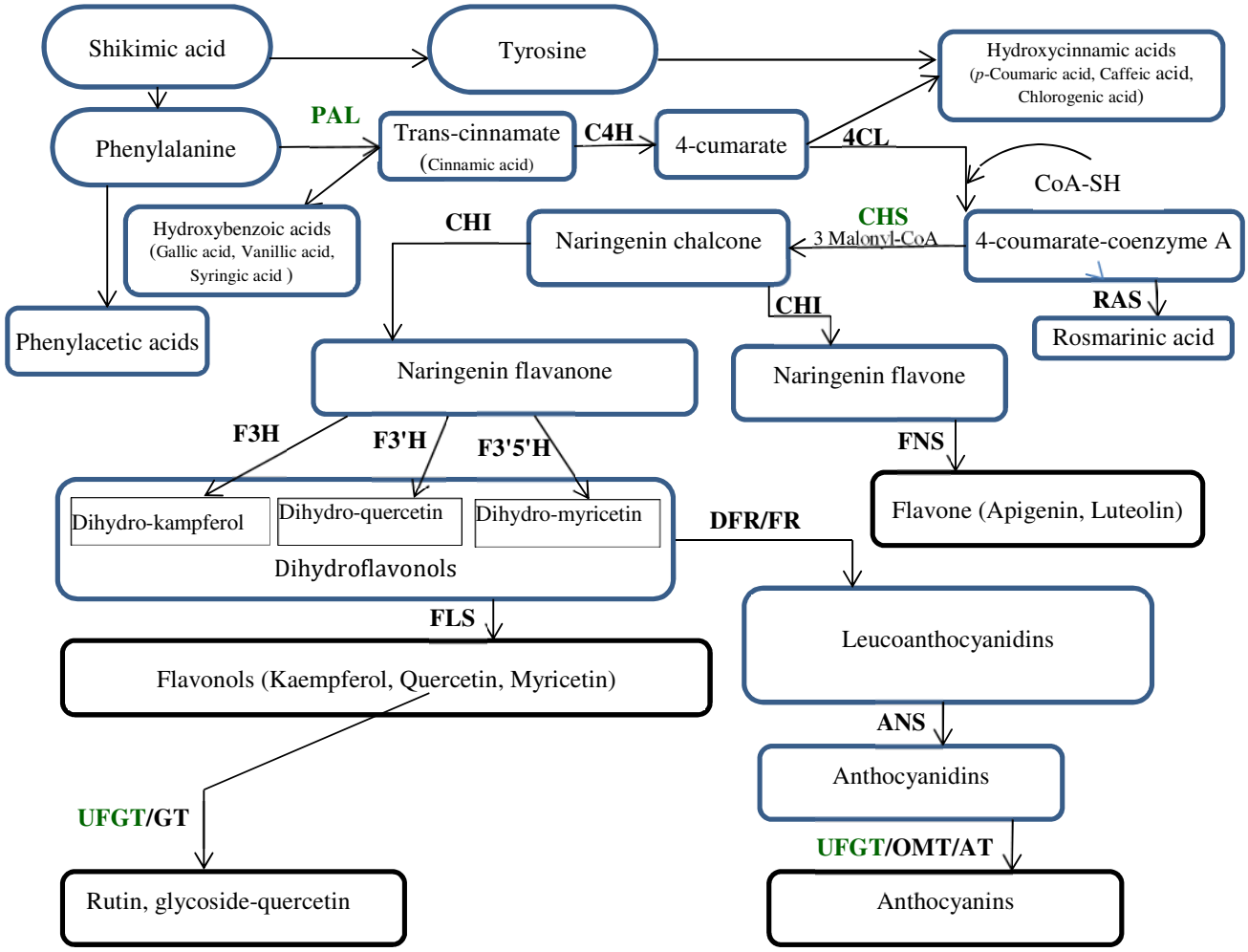
Fig 2. Hairy roots (HRs) establishment (A) and PCR-based confirmation of *rolB* gene presence (B) in *F. carica*; (A): Induced HRs at different sites of shoot explant (a and b), Established HRs on ½ MS solid medium (c), HRs cultures at exponential growth phase (d), HRs liquid cultures in late growth phase, before elicitation (e), HRs cultures after elicitation (f); Scale bar (a-c)= 5 mm, Scale bar (d-f)= 22 mm.
(B): L: 1 kb DNA marker ladder, HR: HR line, C^+: *Rhizobium rhizogenes* plasmid as positive control, C^-: normal root, C^0: PCR reaction without DNA.

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

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

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

579 **Figure 1**



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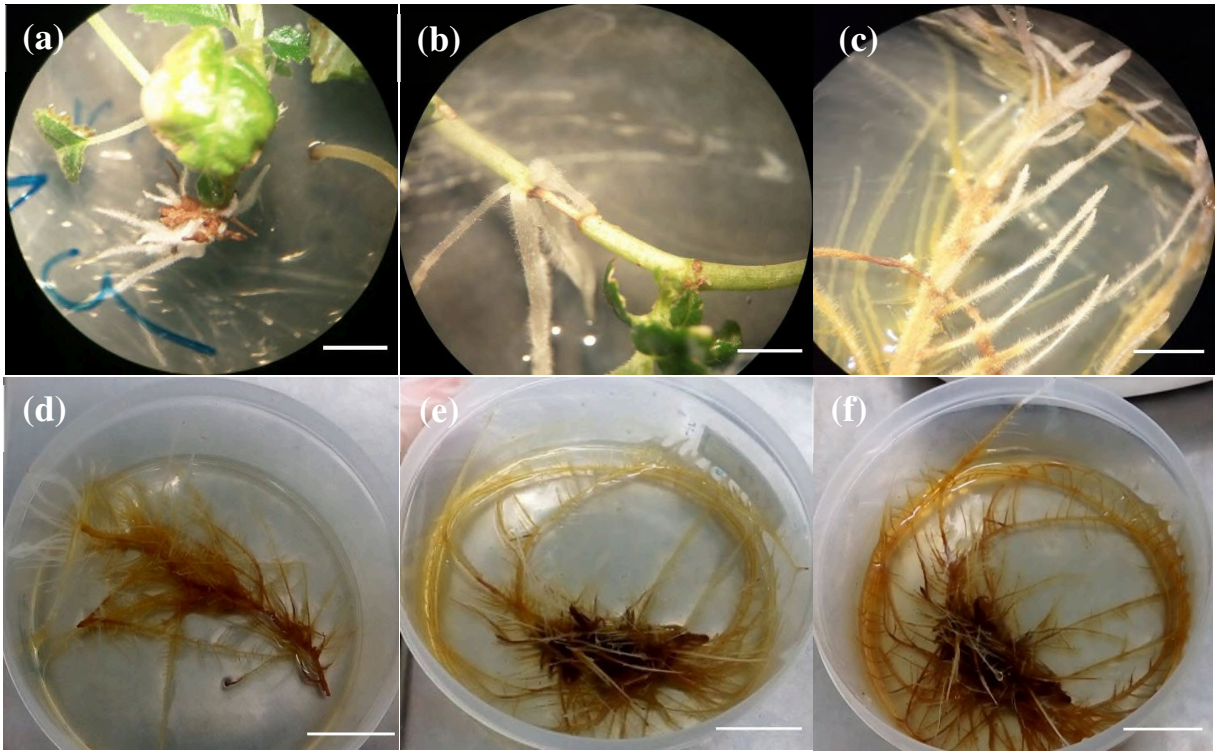
582 **Figure 2**

583 **A**



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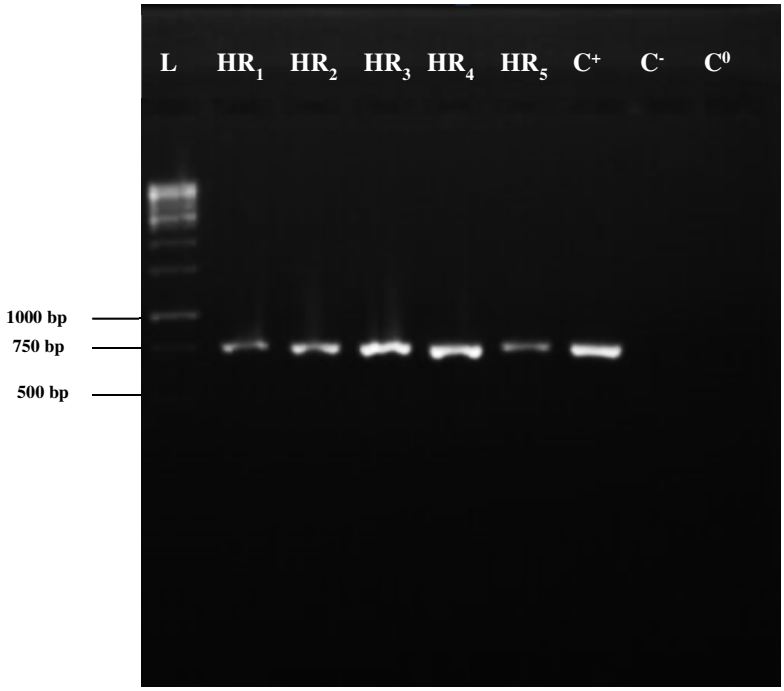

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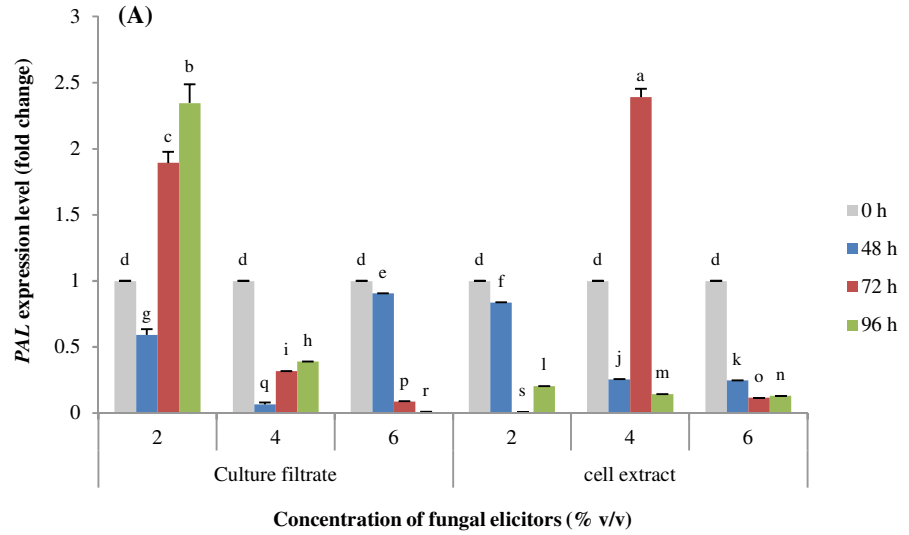
589 **Figure 3**



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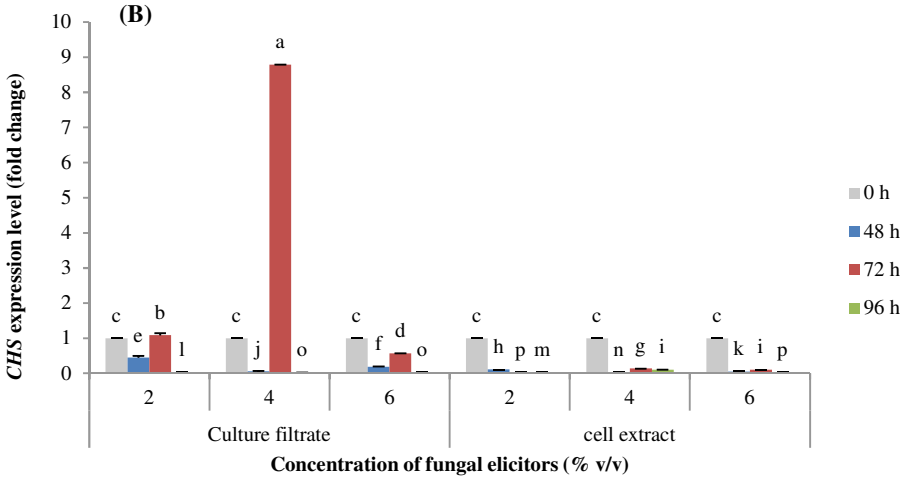

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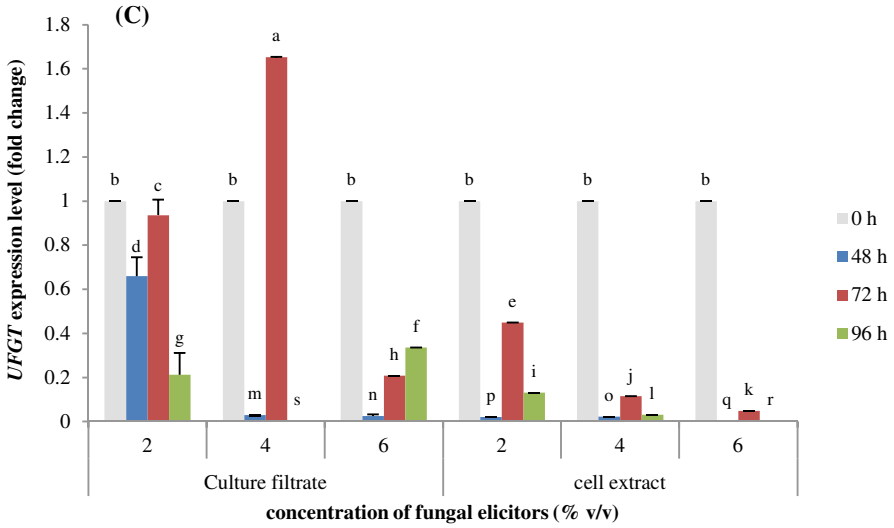

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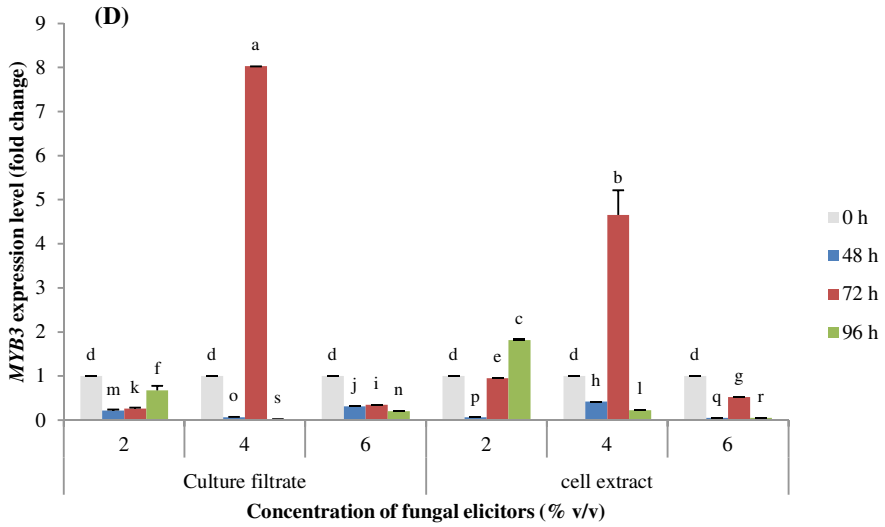

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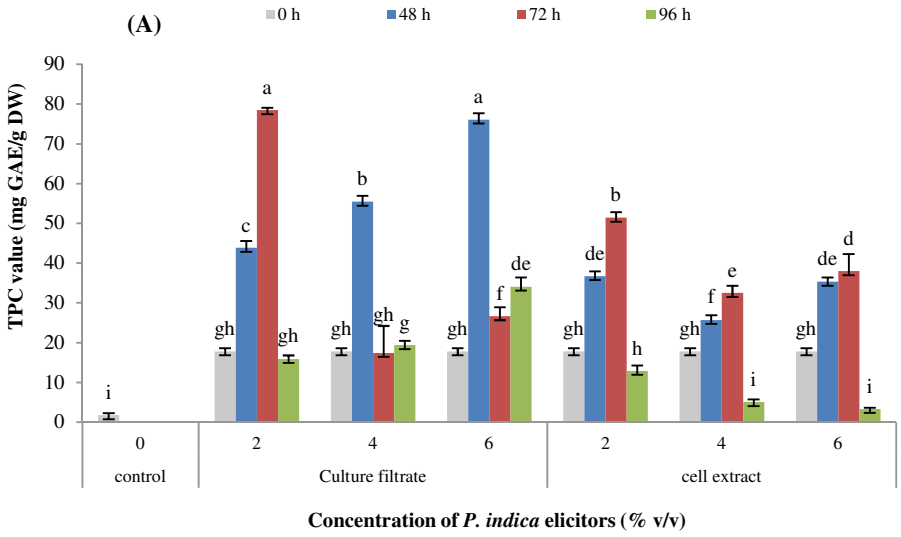
596 **Figure 4**



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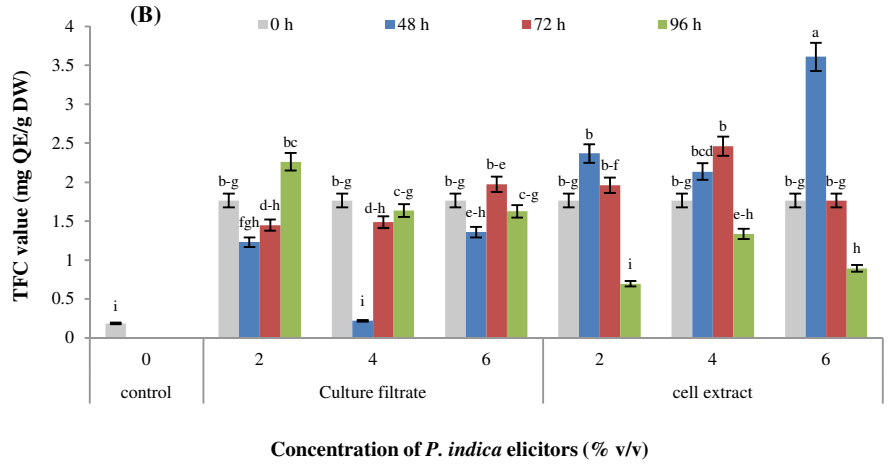

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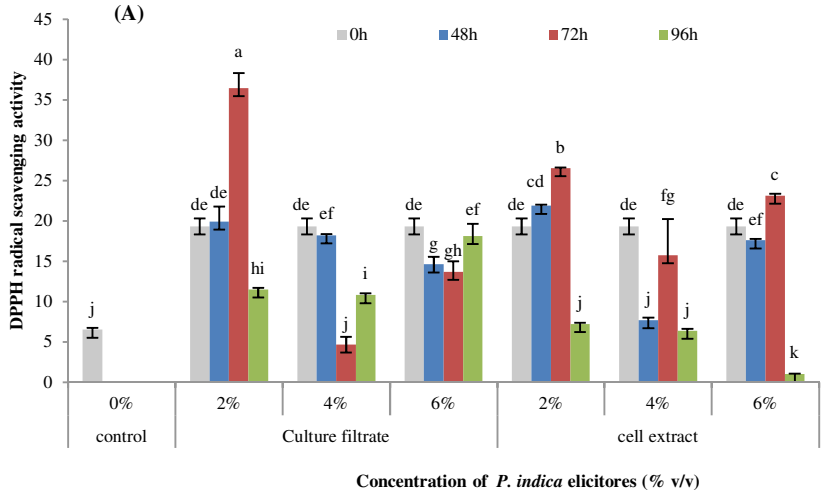
600 **Figure 5**



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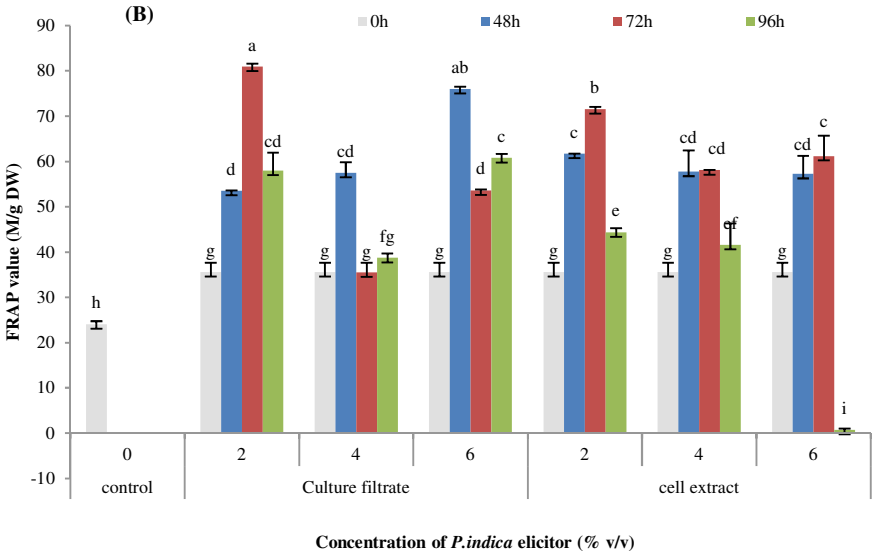

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Piriformospora indica based elicitation for overproduction of phenolic compounds by hairy root cultures of Ficus carica

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2020-12-30

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

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