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The Effect of Different Elicitors on Hairy Root Biomass and Resveratrol Production in Wild *Vitis vinifera*

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Abstract

Introduction: Resveratrol is an antioxidant secondary metabolite which belongs to a specific phytoalexins called stilbenes. Grape has been considered as the main source of resveratrol in the human diet. Many studies have been conducted on genus *Vitis* due to the presence of high levels of polyphenolic compounds in different tissues. In recent years, a lot of effort has been made to increase resveratrol yield in *Vitis vinifera* via tissue and hairy roots culture.

Materials and Methods: In the present study, hairy roots have been produced by *Agrobacterium rhizogenes* strain ATCC15834 and successfully produced resveratrol in internode of grape genotype W16. Transformations of hairy roots were recognized by PCR using specific primers of *rolB* gene. After stimulation, the effect of abiotic elicitors with different concentrations including methyl jasmonate, sodium acetate, acetic acid and ammonium nitrate were tested on the production of hairy root biomass and resveratrol. Resveratrol content was measured by TLC and HPLC methods.

Results: Results showed that the capacity of hairy roots for resveratrol production is higher than natural roots. A significant difference was observed between different elicitors in terms of hairy root biomass and resveratrol amount. Findings revealed that treatment with 3 mM acetic acid and 50 µM methyl jasmonate led to the highest and lowest amount of hairy roots biomass and resveratrol content, respectively.

Conclusions: According to the fidings of the present study it can be stated that both hairy roots and different elicitors are effective in biomass and resveratrol production. This method can be used to increase the yield of resveratrol for large scale production via tissue culture.

Keywords: *Agrobacterium rhizogenes* , Resveratrol , T-DNA , *Vitis vinifera*

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Introduction

Phytoalexins are low molecular weight secondary metabolites produced by plants as a part of the defense system.[1] Resveratrol (trans-resveratrol, trans- 3, 4, 5- trihydroxystilbene), as one of the most important phytoalexins, is stimulated in some plants against different biotic and abiotic stresses due to their antifungal activity in plants.[2,3] This compound is considered as a secondary metabolite and has a powerful antioxidant activity.[4] Resveratrol exhibit many properties for human health including anti-inflammatory, chemo-preventive, modulation of lipoprotein metabolism and reducing cholesterol levels and has the potential to control many disorders such as types of cancers, cardiovascular disease, diabetes and neurodegenerative. Moreover, this compound can improve resistance to different biotic and abiotic stresses in plant .[5] Grapes and peanut are two important sources of resveratrol production.

The phenylpropanoides pathway is one of the most important routes in plant secondary metabolites in order to produce the phenolic compounds that contribute to the synthesis of a wide renge of natural and important plant products such as stilbenes. Phenylalanine ammonia-lyase (PAL), Cinnamic acid 4-hydroxylase (C_4H), 4-coumarate:coenzyme A (CoA) ligase (4CL) and stilbene synthase (STS) are key enzymes in the pathway. The production pathway of resveratrol is shown in Figure 1.

The grapes and its products, particulary red wine, have been reported as basic sources of resveratrol production. *Vitis vinifera* is an important source of phenolic compounds, especially stilbenes which are biosynthetized via shikimate pathway and have antioxidant activity and can consequently be used in medicine.[6,7]

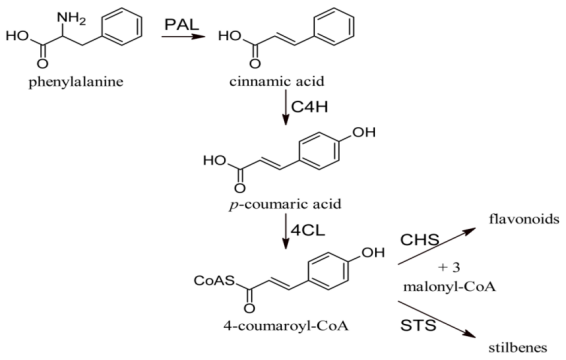

Figure 1. The Biochemical Pathway of Resveratrol Production. Phenylalanine ammonia-lyase (PAL), cinnamic acid 4-Hydroxylase (C_4H), 4-coumarate: coenzyme A (CoA) ligase (4CL) and stilbene synthase (STS) are key enzymes in the pathway.

Genetic engineering of medicinal plants by *Agrobacterium rhizogenes* with the aim of altering their metabolic pathway to increase desired compounds or decrease undesired compounds or production of novel compounds has been an interesting field of research in previous years.[8,9] In transformation by *A. rhizogenes*, the *rol* genes will transmit to plant cells. The *rolB* might function as a transcriptional coactivator/mediator and to affect expression of a particular stilbene-biosynthetic genes. In recent years, hairy roots have been used as an effective method to enhance the biosynthesis of effective compounds in medicinal plants.[10]

Resveratrol is known to be strongly stimulated by different environmental stresses, signaling molecules and drug transporters by mimicking a stress.[11-13] Different elicitors have been used to stimulate the production of secondary metabolites in hairy roots such as rays (UV), hormones (MeJA, JA, GA, SA), sugars (chitosan, cyclodextrins).[14] Previous studies showed that elicitors are effective in increasing the production of stilbenes in cell and tissue culture systems.[15,16]

In the present research the application of W16 genotype hairy roots were investigated for increasing resveratrol yield in the presence of different elicitors. The culture medium was initially evaluated for resveratrol productions based on this fact that a portion of the produced metabolites will be released into the medium. Up to now, several studies have been conducted on resveratrol production in grapes using acetic acid, ammonium nitrate, sodium acetate and methyl jasmonate elicitors.[17,18-20] In this study, different elicitors have been applied to investigate their effect on the production of resveratrol. Attempts to optimize the suitable way for higher production of resveratrol using hairy roots will be discussed.

Materials and Methods

Plant Materials

In order to carry out this research node explants of *V. vinifera* subsp. *sylvestris* were prepared. Explants were washed and sterilized as follows: keep in 70% (v v^{-1}) ethanol for 2 minutes and 1% (v v^{-1}) sodium hypochlorite for 10 minutes and distilled water in sterile condition. Explants were cultivated on 1/2 B5 culture medium supplemented with 1 mg L^{-1} BAP and 0.5 mg L^{-1} GA3. The plant cultures were kept at 25±2°C in the growth chamber. Four-week-old *in vitro* internode explants from genotype W16 were used for transformation.

Preparation of *Agrobacterium rhizogenesis* Strain

The bacterial strain ATCC 15834 was maintained on broth LB medium containing (tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, NaCl 10 g L^{-1}, pH 7). A colony of bacterial cultures was inoculated on 50 mL LB liquid medium containing rifampicin (50 mg L^{-1}) and kept at 28°C on a gyratory shaker at 180 rpm for 48 hours. The cells were centrifuged for 10 minutes at 5000 rpm, then this pellet was re-suspended to an OD 600 of 0.8 in 50 mL 1/2 B5 liquid containing 100 µM acetosyringone.

Transformation of Plant Cells by *Agrobacterium* ATCC 15834 Strain

Microcuttings (length: 0.8-1 cm) were prepared from 4-week-old cultures bearing two leaves with a blade scalpel to create wounded area for inoculation. Inoculation was carried out by immersion of wounded explants in bacterial suspension for 20 minutes. Then the explants were dried on filter paper.

Co-cultivation and Establishment of Hairy Root Cultures

Explants were transferred into 1/2 B5 culture medium containing 100 µM acetosyringone without phytohormones and antibiotics for 48 hours in darkness for co-cultivation in the plant growth chamber. To remove bacteria, the explants were washed twice with distilled water and then once with 1/2 B5 liquid medium containing cefotaxime (500 mg L^{-1}). After washing and during, the explants were cultured on solid 1/2 B5 medium containing 500 mg L^{-1} cefotaxime for hairy root stimulation and were then kept in the growth chamber in dark conditions. Uninfected explants (control) were cultured under the same conditions as well.

Genomic DNA Extraction and PCR

The DNA was extracted from 500 mg of plant material based on CTAB method.[19] Genomic DNA from roots and hairy roots were used as template. For checking transformation, the PCR amplification was carried out in a reaction containing 5 µL Mastermix, 3 µL distilled water, 1 µL genomic DNA (50 µg mL^{-1}), 0.5 µL of each primer. The PCR condition was as follows: 94°C for 5 minutes, 35 cycles of three steps [94°C for 45 seconds, 58°C for 45 seconds and 72°C for 45 seconds, and 72°C for 7 minutes. The *rolB* gene (500 bp) fragment was amplified using forward and reverse primers (5́-ATCCAACTCACATCACAATGG-3́ and 5́-TTCTAAATCAGGTTCCTCCG-3́). PCR yields were separated in 1% agarose gel for separation and the amplified fragments were compared with 1 kb DNA ladder.

Proliferation

Hairy roots were separated from different explants and during 2 months they were sub-cultured every 2 weeks on 1/2 B5 culture medium containing 500 mg L^{-1} cefotaxime in sterile condition. Approximately 3 cm long hairy roots tips were dissected and transferred into 50 mL of 1/2 B5 liquid medium supplemented with cefotaxime 100 mg L^{-1} and were maintained on an orbital shaker (120 rpm).

Elicitor Treatments

To identify the effective treatments on hairy roots biomass and also resveratrol stimulation, 20-day hairy roots were kept for 10 hours with methyl jasmonate and 24 hours with the other elicitors. The effect of different treatments were investigated on the amount of resveratrol. The tested elicitors were 5, 10 and 15 mM sodium acetate (NaOAc), 500, 750 and 1000 mg L^{-1} ammonium nitrate (NH_4NO_3), 1, 2 and 3 mM acetic acid, 50, 100 and 150 µM methyl jasmonate (MeJA).[17-20] The elicitors were added to fresh 1/2 B5 medium. Except for methyl jasmonate which was dissolved in EtOH 96%, other elicitors were dissolved in water.

Growth Measurement of Hairy Roots

The fresh and dry weight of the herbal substance was a criterion of their growth. The fresh weight of hairy roots was measured after treatment. To measure the dry weight, the hairy roots were kept in dark at 25°C for 48 hours until they were weighted.

Extraction of Resveratrol From Medium

Resveratrol can be easily recovered from the culture medium with relatively high efficiency. The media were centrifuged for 10 minutes in 5000 rpm. Ethyl acetate (EtOAc) was used as a solvent to recover resveratrol from the medium. Samples were kept on shaker at 100 rpm for 1 hour. The organic phase was recovered and partitioned with 10 mL ethyl acetate. The supernatant phase was recovered and completely dried at 25°C. Each of the dried samples were re-suspended in EtOAc (50 µL) for further analysis.

Extraction of Resveratrol From Hairy Roots

Hairy roots (frozen at –80°C) were lyophilized in a free drier. Powdered hairy roots were kept in 50 ml 96% EtOH for 72 hours in a thermoshaker at 25°C and 100 rpm. For complete extraction, samples were kept in ultrasonic for 1 hour and were filtered. To evaporate organic phases, samples were kept under vacuum at 40°C in a rotary evaporator (Rotavac-Heidolph) in 150 rpm at 25°C. Final extracts were kept at -20°C in dark. These dried extracts were re-suspended in EtOAc (50 µL) for further analysis.

TLC Analysis of the Extracted Metabolites of Medium and Hairy Roots

The extracts and standard of trans-resveratrol (Sigma, USA) (2 µL) were spotted on 15×15 cm silica gel (TLC) (thin layer chromatography) 0002/05563/ (Sigma). Mobile phase for analysis was EtOAc: AcOH: H_2O (17:1:2) as described. Plates were air dried and visualized under UV light (365 nm) using a Chromato-Vue C-75 (USA) in dark condition.

High-Performance Liquid Chromatography Analysis

The high-performance liquid chromatography (HPLC) device was used with the following conditions: Venusil MP C18 column (4.6 mm ×250 mm), solution of acetonitrile-water (15% v v^{-1}) for washing the column, mobile phase of MeOH in H_2O (80–100% MeOH) at a flow-rate of 0.5 mL min^{-1}. The solutions were filtered (0.45_m, Agilent) and chromatograms were recognized by a UV detector at 306 nm (Waters 512, USA). Peak of trans- resveratrol observated at retention time of about 2 minutes. The data was analyzed with the ChemStation® program var. 09 (Agilent Technologies, Germany).

Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) and least significant difference (LSD). The analysis were carried out by the SPSS 16 software. Three biological replications were used for each treatment.

Results

Hairy Root Analysis PCR

Different stages of plantlet production and hairy roots growth have been presented in Figure 2 (a, b, c and d). Results showed that hairy roots were emerged from internode explants of the W16 genotype (Figure 2b). The presence of a 500 bp fragment in hairy roots was confirmed using specific primers for *rolB* gene (Figure 2e). The pRi15834 plasmid was used as the positive control. No amplification was observed in natural roots (Figure 2e). All transformed roots showed the presence of 500 bp *rolB* amplified products.

Effect of Elicitors on Hairy Root Biomass

The production of hairy roots after treatment by elicitors have been presented in Figure 3 (a, b, c, d, e, f and g). The highest and lowest effects in the production of biomass were obtained in treatment with acetic acid and methyl jasmonate elicitors, respectively (Figure 3g). There is a significant difference between the different elicitors at 1% probability level (Figure 3g). The results of analysis of variance for dry (*a*) and fresh (*b*) weight of hairy roots in different concentrations of treatment have been presented in Table 1.

As shown in Figure 3g, the highest and lowest dry weight in hairy roots treated with acetic acid was observed at 3 mM and 1 mM concentration with 0.46 and 0.243 g DW, respectively. The highest and lowest dry weight in hairy roots treated with NH_4NO_3 was observed at 750 and 1000 mg L^{-1} concentration with 0.42 and 0.23 g DW, respectively (Figure 3g). The biomass of hairy roots treated with low concentration of NH_4NO_3 (500 and 750 mg L^{-1}) was higher than the hairy roots treated with high concentration (1000 mg l^{-1}). Among the three concentrations including 5, 10 and 15 mM sodium acetate, the latter concentration was most effective in enhancing fresh and dry weight (4.6 g FW, 0.4 g DW). The 5 mM concentration had a significant decrease (2.3 g FW, 0.2 g DW) in biomass production. The treated hairy roots with MeJA showed that 100 and 50 µM concentrations produced higher and lower hairy roots biomass, respectively (Figure 3g). The dry weight in 100 and 50 µM concentration were 0.3 and 0.14 g DW, respectively.

Effect of Different Elicitors on Accumulation of Resveratrol in Hairy Roots and Culture Medium by TLC and HPLC Methods

To assess to amount of resveratrol production the TLC was used which showed the presence of resveratrol. Subsequently HPLC method was used to quantify and measure the exact

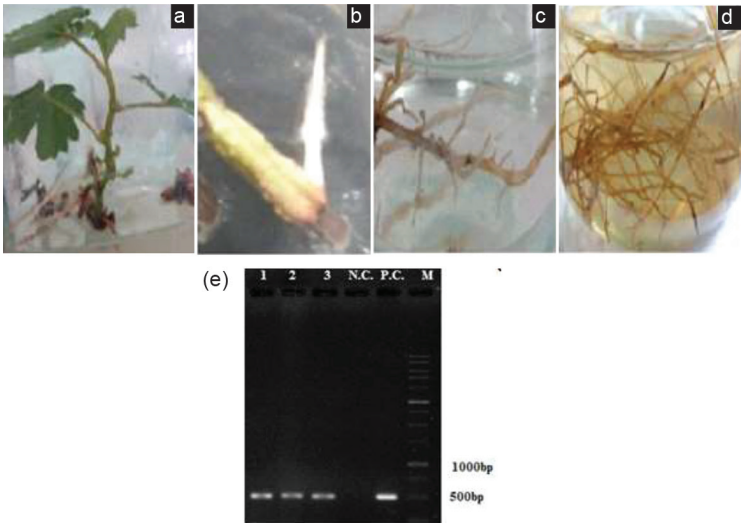

Figure 2. Hairy Roots Induction and Proliferation in 1/2B5 Solid And Liquid Mediums. **(a)** four-week-old *in vitro* plantlet production from nod explants, **(b)** hairy root initiation from internode explants, **(c)** growth of hairy roots, **(d)** proliferation of hairy roots in liquid medium. **(e)** PCR analysis of the *rolB* gene to confirm their integration into the transgenic hairy roots genome. P. C: Positive control (plasmid DNA). N. C: Negative control (plant natural roots DNA). M: Ladder 1 kb. Lane1- 3: transformed roots (hairy roots).

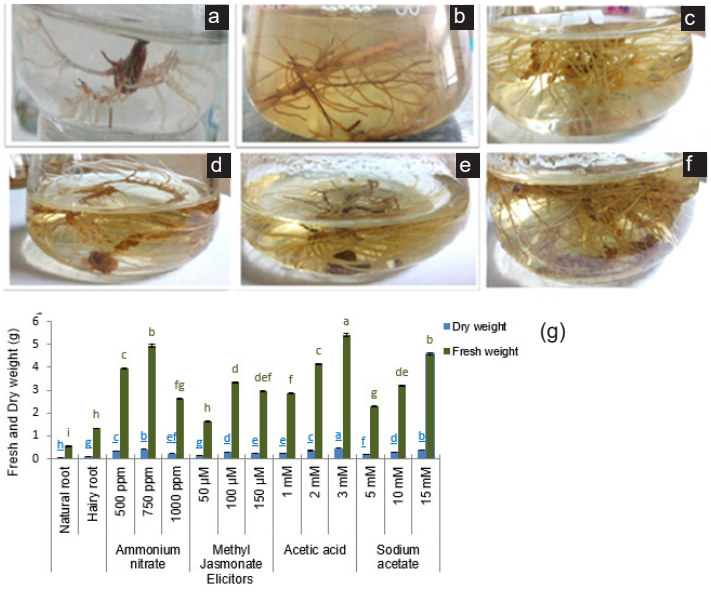

Figure 3. Effect of Different Elicitors on Hairy Roots Biomass. **(a)** natural roots, **(b)** hairy roots, **(c)** Sodium acetate 15 mM treated hairy roots, **(d)** ammonium nitrate 750 mg L^{-1} treated hairy roots, **(e)** acetic acid 3 mM treated hairy roots, **(f)** methyl jasmonate 100 µM treated hairy roots. (**g**) Effect of different elicitors on hairy root biomass compared to the control (natural root and hairy root) (P <0.01). Comparisons indicate significant difference between different elicitors based on Duncan test.

amount of resveratrol (Figures 4 and 5).

Quantitative and qualitative data of resveratrol were obtained from ethyl acetate extracts of medium and root tissue (Figure 4i and Figure 5f) which is based on expected blue fluorescence characteristic of resveratrol at k 365 nm (Figure 5f). As shown in Figures 4 and 5 all of the used elicitors enhanced resveratrol in the hairy roots, but a less amount of the resveratrol was secreted into the medium, and mostly remained in the hairy roots.

The intensity of fluorescence was higher when stimulated by acetic acid and ammonium nitrate elicitors than sodium acetate and methyl jasmonate, hence it was concluded that there is a significant difference between the different elicitors at the 1% probability level (Figures 4 and 5). Treatment with 3 mM acetic acid and 50 µM methyl jasmonate had the maximum and minimum effect on resveratrol production. The amount of resveratrol in elicited samples ranged from 1.04 to 14.23 fold higher than the control roots at culture medium. The natural root (control) showed lower fluorescent intensity than the hairy roots.

To validate and confirm the TLC results, a HPLC approach with RT ~2 minutes was used to show the exact amount of the compound in hairy roots. In this method, trans-resveratrol was used as an external standard (Figure 5a, b, c, d, e and f). Comparison of elicited hairy roots with natural roots showed that the maximum and minimum resveratrol was

Table 1 Analysis of Variance Dry Weight and Fresh Weight of Hairy Roots in Different Concentrations 0f Treatment

Source	Sig.	Mean Sequence	F
Dry Weight			
Concentration of elicitor	8	0.003	6.653**
Error	28	0	-
Fresh weight			
Concentration of elicitor	8	0.409	7.288**
Error	28	0.056	-

82.48 and 18.07 fold higher than natural roots, respectively (Figure 5f). According to the findings of the present study, applied elicitors were effective in increasing the production of resveratrol. Also, the resveratrol accumulation in roots depends on the concentration of elicitors. The highest level of resveratrol (181.45 µg g^{-1} hairy root DW or 798.38 nmol g^{-1} hairy root DW) was found in roots treated with the highest concentration of acetic acid (3 mM) (Figure 5f). The resveratrol content of hairy roots after treatment with ammonium nitrate was 148.15 µg g^{-1} hairy root DW or 651.9 nmol g^{-1} hairy root DW (Figure 5f). The resveratrol content of hairy roots after treatment with 15 mM sodium acetate was 90.74 µg g^{-1} hairy root DW or 26/399 nmol g^{-1} hairy root DW (Figure 5f). The latter treatment increased the resveratrol amount up to 41.25 fold higher than the control. As shown in Figure 5f, the resveratrol content of hairy roots after treatment with 100 µM methyl jasmonate was 39.75 µg g^{-1} hairy root DW or 174.9 nmol g^{-1} hairy root DW.

Based on these results, adding acetic acid, ammonium nitrate, sodium acetate and methyl jasmonate to *V. vinifera* genotype W16 hairy roots could induce hairy roots biomass and resveratrol amount. The culture conditions in this investigation was used to increase biomass and resveratrol yield in *V. vinifera* hairy roots. The highest accumulation of biomass and resveratrol was observed in hairy roots treated with 3 mM acetic acid.

Discussion

Results of this study showed that stilbene production in grapevine cell suspensions was 13.4 mg g^{-1} FW,[21] while this amount reached 18.1 mg g^{-1} FW in grapevine HRs, indicating that this system could be an essential tool for stilbenes production. In this study, all applied elicitors concentrations significantly stimulated biomass and resveratrol formation in roots. In this case, the resveratrol content depends on the concentration of elicitors. Based on the results obtained from this experiment, adding acetic acid to the culture media increased the biomass up to 4.56 to 8.69 fold higher compared to the normal roots. The application of acetic acid (3 mM) also increased the resveratrol both in tissue and media by 82.48 and 14.23 fold than the normal roots, respectively.

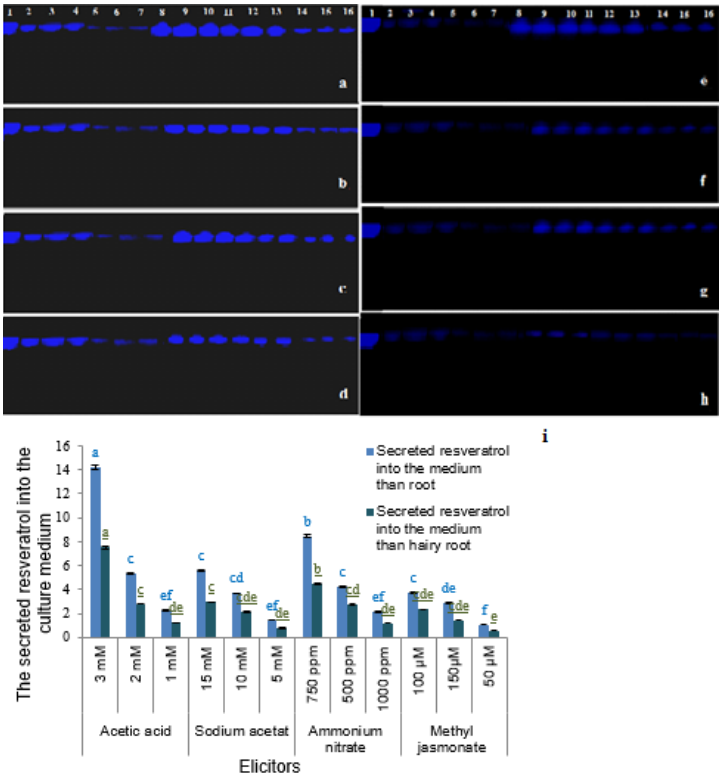

Figure 4. TLC of Resveratrol Extracts Respectively From The Tissues **(a, b, c, d)** and Culture Medium **(e, f, g, h)** of Hairy Roots Treated With Different Concentrations of Elicitors. Lanes (1) resveratrol standard, (2, 3 and 4) hairy root extract, (5, 6 and 7) natural root extract **a** and **e** acetic acid treatment, (8, 9 and 10) 3 mM, (11, 12 and 13) 2 mM, (14, 15 and 16) 1 mM concentration **b** and **f** sodium acetate treatment, lanes (8, 9 and 10) 15 mM, (11, 12 and 13) 10 mM, (14, 15 and 16) 5 mM concentration **c** and **g** ammonium nitrate treatment, (8, 9 and 10) 750 mg L^{-1}, (11, 12 and 13) 500 mg L^{-1}, (14, 15 and 16) 1000 mg L^{-1} **d** and **h** methyl jasmonate treatment, (8, 9 and 10) 100 µM, (11, 12 and 13) 150 µM, (14, 15 and 16) 50 µM. **i** The quantification of TLC for secreted resveratrol into the culture medium in different elicitors (P <0.01). Comparisons indicate a significant difference between different elicitors based on Duncan test.

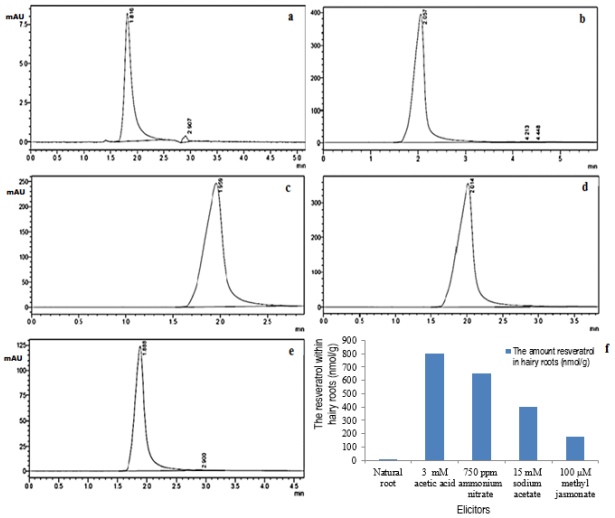

Figure 5. HPLC Chromatogrames of Extract of Induced Hairy Roots by (**a**) 3 mM Acetic Acid, (**b**) 750 mg L^{-1} Ammonium Nitrate, (**c**) 15 mM sodium acetate, (**d**) 100 µM Methyl Jasmonate and, (**e**) Natural Root (Control) with Fluorescence Detection (λ 306). **f** The amount resveratrol caused by the elicitors according to nmol/g DW hairy root (P <0.05). Comparisons indicate a significant difference between different elicitors based on Duncan test

Consistent with the results of the present research, in an experiment the effect of acetic acid (1mM) on production of resveratrol in *Arachis hypogaea* (peanut) hairy roots significantly increased resveratrol production as detected by TLC.[20] Adding ammonium nitrate increased the biomass up to 4.3 to 7.9 fold higher than the normal roots. Ammonium nitrate (750 mg L^{-1}) also increased the resveratrol in tissue and media up to 67.35 and 8.47 fold higher compared to the normal roots, respectively. The results of this study indicate that the growth of hairy roots might be prevented by increasing the concentrations of NH_4NO_3.[22] If ammonium accumulates too much into cell, it will become very toxic and can not be completely metabolized.[23] Consequently, various concentrations might have a direct or indirect effect in secondary metabolite production amounts.[23] In the investigation of ammonium nitrate effect with 500, 1000, 5000, and 10 000 mg L^{-1}, in *V. vinifera* cv. Pok Dum cell suspension culture, results showed that ammonium nitrate with concentration of 500 mg L^{-1} produced the highest biomass and resveratrol in cell and medium. The highest amount of biomass and resveratrol in cells was 86.6 g DW L^{-1} (8.8-fold), and 277.89 µg g^{-1} DW (5.6-fold), respectively.[18]

Sodium acetate treatment increased the biomass up to 3.69 to 7.44 fold higher than the normal root in the culture media. Concentration of 15 mM increased the resveratrol in tissue and media respectively up to 41.25 and 5.55 fold higher than normal roots. In *A. hypogaea* hairy roots this treatment also increased resveratrol content in roots and culture medium.[20] Treatment with this concentration in *V. vinifera* hairy roots also qualitatively increased resveratrol content significantly.[17]

Results indicate that MeJA significantly enhanced biomass formation compared to the control cultures. In this study, MeJA stimulated the biomass by up to 2.56 to 5.63 fold higher compared to normal roots. Methyl jasmonate (100 µM) also increased the resveratrol in tissue and media respectively up to 18.07 and 3.71 fold higher compared to the normal roots. Jasmonic acid ester and MeJA, have been proved to stimulate stilbene production.[24,25] Adding the concentration of 100 mM to MS culture medium in the hairy roots of *V. rotundifolia* increased resveratrol amount by 7 fold in 12-days old hairy roots and also increased secreted resveratrol into the medium.[26]

Results showed that *V. vinifera* subsp. *sylvestris* hairy root cultures are able to produce resveratrol, when treated with different elicitors such as ammonium nitrate, acetic acid, sodium acetate and methyl jasmonate, hence using these elicitors can be considered as an efficient strategy to increase resveratrol content in hairy roots culture. Treatment with different elicitors in hairy roots could increase biomass and resveratrol production. The highest amount of biomass and resveratrol was observed in hairy roots grown in the medium with acetic acid (3mM). However, the application of the elicitors at various concentrations have positive effects on the resveratrol contents in both tissue and culture medium. Based on the results of this experiment and previous experiments in this field, it can be concluded that different factors can have an influence on resveratrol production. Taking together the application of different elicitors in bioreactors could be considered as a useful strategy to increase the yield of effective compounds.

Conclusions

The applied elicitors in the present study can be used for increasing resveratrol production in tissue culture studies. Bioreactor technology could be considered for scale-up and commercialization of resveratrol. Both types of elicitors and concentrations are important for elevating effective compounds during tissue culture.

Authors' Contributions

BB and MM designed the research. BH conducted the experiment. DD and MA performed HPLC and TLC analysis. BH, BB and MM wrote the paper.

Conflict of Interest Disclosures

The authors declare no conflicts of interest regarding the publication of this article.

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