Improvement of lignans production in hairy root culture of *Linum mucronatum* using abiotic and biotic elicitors

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Abstract

Elicitors could be used as the enhancer of plant secondary-metabolite synthesis and could play an important role in biosynthetic pathways of important medicinal compounds. The present study investigated the effect of abiotic elicitor (fungal extract of *Fusarium graminearum*) and two abiotic elicitors (methyl jasmonate and salicylic acid) at different concentrations in 18- and 30-day-old cultures of hairy roots infected with two strains of *Agrobacterium rhizogenes*, A13 and 9534. The hairy roots were harvested 48 and 96 h after inoculation. Polymerase chain reaction analysis (PCR) with specific primers *rol*B and *vir*D genes was performed to confirm the transgenic hairy roots production. Detection and identification of lignan was carried out by high performance liquid chromatography (HPLC) method. The results of PCR analysis showed diagnostic bands 780 bp in size related to specific reproduction of *rol*B gene. Also maximum lignan production for each elicitor was as follow: fungal extract at 1% v/v (12.87 ± 0.66 and 89.65 ± 3.9 mg/gr DW ± SD podophyllotoxin and 6-methoxypodophyllotoxin, respectively for), methyl jasmonat at 100 µm (11.37± 0.65 and 75.65 ± 3.9 mg/gr DW± SD for podophyllotoxin and 6-methoxypodophyllotoxin, respectively), and salicylic acid at 200 µm (7.97 ± 0.33 and 51.68 ±2.1 mg/gr DW ± SD for podophyllotoxin and 6-methoxypodophyllotoxin, respectively). Important factors such as strain of agrobacterium (A13), duration of exposure time (48 h), and age of culture (18-day-old culture) affected lignan accumulation.

Keywords: Fusarium graminearum; methyl jasmonate; hairy root; PCR analysis; salicylic acid

Abbreviations:
ACC: PCR: Polymerase chain reaction analysis; PTOX: Podophyllotoxin; MPTOX: 6-methoxypodophyllotoxin; HPLC: high performance liquid chromatography.

Samadi, S and Dini Torkamani, M. R. 2015. 'Improvement of Lignans production in hairy root culture of *Linum mucronatum* using abiotic and biotic elicitors. *Iranian Journal of Plant Physiology* 6 (1), 1535-1542.

Introduction

Elicitors are compounds of mainly microbial or non-biological origin which upon contact with higher plant cells trigger the increased production of pigments, flavones, phytoalexins, and other defense related compounds (Savitha et al., 2006). It is known that jasmonic acid and its derivative forms such as methyl jasmonate represent important plant

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Received: March, 2015
Accepted: October, 2015

signal transduction molecules involved in response to pathogen attack, and their exogenous addition to plant cell culture or intact plants stimulates biosynthesis of a wide range of secondary metabolites (Kuzmaa et al., 2009). Fungal elicitor has been reported to stimulate or decrease different secondary metabolites in many species such as syringin, valeportriates, and podophyllotoxin (Shams-Ardakani et al., 2005). Podophyllotoxin (PTOX) is a naturally occurring lignan with important antineoplastic and antiviral properties and is supported by detailed understanding of their mechanism of action, and facilitated by chemical manipulations that have amplified their bioactivity. Although elicitation is widely used in plant cell cultures to enhance the yield of compounds, there are only a few reported studies using this approach with PTOX. For instance, methyl jasmonate (MJ) and salicylic acid (SA) were found to induce PTOX production in cell culture of *L. album* (Van Furden et al., 2005). In the past years, numerous strategies such as cell line selection, establishment of cell suspension cultures of plant with a content of required phytochemicals, optimization of culture conditions, cell immobilization, the use of differentiated cells, elicitation, and more recently, metabolic engineering have been developed to improve the productivity of plant cell culture (Ahmadi Moghadam et al., 2013). Several studies have been done on the effect of different concentrations of MJ and other elicitors on lignan content in the hairy roots cultures but to our knowledge, the effect of elicitors on hairy root cultures inoculated with different strains of agrobacterium has not been studied. Therefore, in the present study a biotic elicitor derived from fungi namely *F. graminearum* and two abiotic elicitors, methyl jasmonate and salicylic acid, were tested for influencing the *in vitro* lignan production in hairy roots cultures of *L. mucronatum* infected with two different strains of *A. rhizogenes*.

Material and Methods

Plant material

L. mucronatum L. (*Linaceae*) seeds were collected from East Azarbaijan, Iran at an altitude of 1800 m. The plant material was carefully identified by Dr. Hassanzadeh, Agricultural and Natural Resource Research Center, West Azarbaijan, Iran; PTOX standard was provided by Prof. Randolph Arrow from Leicester School of Pharmacy De Montfort University, German; SA and MJ were purchased from Sigma Chemical Co and fungal elicitor was provided by Dr. Safaei, Tarbiat Modares University.

Molecular confirmation

Hairy roots with more than four lateral branches were obtained after transformation of *L. mucronatum* seeds with two different strains of *A. rhizogenes,* A13, and 9534. Maintenance conditions of hairy roots and their induction have been explained in a previous study (Samadi et al., 2012). The extraction of DNA from both hairy roots and untransformed roots (control) was carried out using the CTAB method (Khan et al., 2007). To confirm transformation, PCR analysis was performed using specific primers for *rolB* gene which are the main determinants for the development of hairy roots. The Ri plasmid of *A. rhizogenes* strains 'A13 and 9534' was used as a positive control. The primer sequences to amplify a 780-bp portion of the *rol*B genes were 5′-TGGATCCCAAATTGCTATTCCTTCCACGA3′ and 5-′TTAGGCTTCTTTCTTCAGGTTTACTGCAGC-3′ and the primers 5'-ATGCCCGATCGAGCTCAAGT-3' and 5'-CCTGACCCAAACATCTCGGCTGCCCA-3' were designed based on the *vir*D gene from the not-transferred virulence region of the *A. rhizogenes* Ri plasmid. The PCR reaction conditions have been explained in a previous study (Samadi et al., 2012).

Elicitation preparation

Stock solution of MJ was obtained by dissolving them in 96% ethanol after filter sterilization using 0.22 µ Sartorius filters. SA was dissolved in distilled water and the obtained stock solution PH was adjusted to 5.7 – 5.8 before autoclaving for 20 min at 121° C. Fungal elicitor was prepared as described by Esmaeilzadeh Bahabadi et al. (2011). One-week-old mycelia of *F. graminearum*, were harvested and rinsed with sterile distilled water. The collected mycelia were ground under liquid nitrogen and suspended with water to a final concentration of 250 mg ml^{-1}. The suspension was centrifuged at 10000 rpm for 10

min at 25° C, the supernatant was isolated and sterilized by autoclaving at 121° C and 1 atm over 20 min and used as elicitor (Esmaeilzadeh Bahabadi et al., 2011).

Addition of elicitors

Study of the effectiveness of three different elicitors was carried out with fungal elicitor (0.5, 1, 1.5 and 2 % v/v), MJ (50, 100, 150 and 200 µm), and SA (100, 200, 300 and 400 µm). For elicitation, each flask was filled with 50 ml MS (Murashige et al., 1962) medium and inoculated with 25 mg of fresh roots from 18-and 30-day-old shake-flask cultures for 48 and 96 h time periods of exposure. Each experiment was done three times to reach desired concentrations of elicitors. The control cultures received equal volumes of water on two age culture (18 and 30) day and all treatments were done in triplicate. All cultures were inoculated in the dark at 25° C in 50 ml medium in 125 ml shake-flasks and sub-cultured every two weeks so that sufficient biomass was obtained for lignan analysis.

Extraction of Lignans

Lignans were extracted from powdered plant cell material (200 mg) with MeOH (2 ml). The mixture was homogenized in an ultrasonic bath (2×30s) with intermediate cooling on ice. Distilled water (6 ml) was added and the pH was adjusted to 5.0 with 5% phosphoric acid. After adding β-glucosidase (1 mg) the sample was incubated at 35° C for 1 h in a water bath. MeOH (12 ml) was added and the mixture was incubated for another 10 min at 70° C in an ultrasonic bath. After centrifugation for 7 min at 4500 rpm, the volume of supernatant was determined. One milliliter of the supernatant was taken and centrifuged at 13,000 rpm for 5 min at 25° C. This final solution was used for HPLC analysis (Ionkova et al., 2009).

HPLC Analysis

HPLC determination was performed on a Thermo Quest (Agident, USA) equipped with a Spectra SYSTEM UV6000LP detector. The separation column was a GROM-SIL 120 ODS-5 ST (250 × 4.6 mm, particle size 3.5 µm). The gradient system was water with 0.01% phosphoric acid (85% (A) and acetonitrile (B) as follows: From 0 to 17 min from 40% to 67% B, from 17 to 18 min back to 40% B. The flow rate was 0.8 ml/min between 0 and 17 min 1 ml/min between 17 and 18 min, and again 0.8 ml/min after 24 min; detection was performed at 290 nm against reference standard.

Statistical analysis

The recorded data were evaluated and analyzed by one way ANOVA test using SPSS (version 16) at a 5% probability level and $n = 3$.

Results

Hairy root cultures of *L. mucronatum* were set up by infection of root tips with two different strains of *A. rhizogenes,* A13 and 9434. The successful transformation was confirmed by analyzing *rol*B and *vir*D genes using PCR analysis. Hairy root cultures were employed to study the formation of phenolic compounds such as PTOX and 6-MPTOX. In order to stimulate the biosynthesis of lignans, the transformed cultures were incubated with potential elicitors such as MJ, fungal extract, and salicylic acid. Then, the amounts of lignans were analyzed using HPLC analysis.

Effect of fungal extract

PTOX and 6-MPTOX content was enhanced to 12.87 ± 0.66 and 89.65 ± 3.9 mg/g DW ± SD by the addition of fungal extract at 48 h compared to 5.81 and 42.32 mg/g DW ± SD, respectively for the control culture in hairy roots culture incubated by A13 strain. The highest level of PTOX and 6-MPTOX in this study was obtained at fungal extract representing about 2.21 and 2.11-fold in comparison with control roots, respectively. Addition of *F. graminearum* mycelia extract at all doses during early stages of culture cycle (18^{th} day) caused an increased effect on lignan accumulation while low increase in lignan accumulation was observed at high concentration and 30-day-old culture (6.21 ± 0.19 and 44.16 ±1.1 mg/g DW ± SD, respectively for PTOX and 6-MPTOX) (Table 1).

Table 1
Different concentration effect of fungal treatments on lignan production in hairy roots culture of *L. mucronatum*

Strains	A13 strain		9534strain	
Concentration	PTOX mg/gr DW ± SD	MPTOX mg/gr DW ± SD	PTOX mg/gr DW ± SD	MPTOX mg/gr DW ± SD
Control	5.81 ± 0.61	42.32 ± 1.65	5.2 ± 0.58	35.42 ± 0.98
18th day- 48 h				
0.5	8.92 ± 0.43	58.34 ± 2.8	8.64 ± 0.48	57.34 ± 2.1
1	12.87 ± 0.66	89.65 ± 3.9	10.33 ± 0.52	75.43 ± 4.1
1.5	10.83 ± 0.64	59.56 ± 2.9	8.95 ± 0.51	57.87 ± 2.5
2	8.12 ± 0.63	47.21 ± 1.7	7.83 ± 0.32	45.21 ± 1.9
18th day- 96 h				
5	8.71 ± 0.25	53.52 ± 2.1	8.02 ± 0.29	49.42 ± 1.7
1	11.34 ± 0.54	81.38 ± 4.2	9.98 ± 0.57	47.76 ± 3.3
1.5	8.63 ± 0.35	59.83 ± 2.1	7.24 ± 0.43	48.27 ± 1.5
2	6.27 ± 0.18	44.31 ± 1.3	5.81 ± 0.27	39.12 ± 1.1
30th day- 48 h				
5	8.22 ± 0.27	54.32 ± 2.1	7.81 ± 0.33	51.34 ± 1.7
1	10.94 ± 0.65	76.56 ± 4.4	10.09 ± 0.63	75.65 ± 2.4
1.5	8.26 ± 0.38	51.43 ± 1.3	7.22 ± 0.32	48.46 ± 1.8
2	6.34 ± 0.27	45.30 ± 1.1	5.75 ± 0.17	37.28 ± 0.98
30th day- 96 h				
5	8.41 ± 0.30	59.44 ± 1.5	5.91 ± 0.23	45.23 ± 1.3
1	10.63 ± 0.71	73.85 ± 2.3	9.18 ± 0.77	68.78 ± 2.1
1.5	7.84 ± 0.46	47.31 ± 1.4	6.83 ± 0.24	42.23 ± 1.2
2	6.21 ± 0.19	44.16 ± 1.1	5.55 ± 0.11	36.14 ± 1.1

mg/g DW ± SD: mg/g dry weight ± standard error

Effect of methyl jasmonate

Table 2 shows the effect of different concentrations of MJ on PTOX and 6-MPTOX content after 48 and 96 h elicitation in hairy root cultures. The results of MJ treatment showed that different concentrations of this compound had significant effect on lignan production. Accumulation of the both lignans in the roots were enhanced by addition of 100 µm concentration of MJ that were about 1.95 and 1.78-fold in comparison with control roots, respectively while MJ, at the highest concentration after 96 h decreased the content of both lignan in comparison with control cultures (5.43± 0.21 and 39.36 ± 1.2 mg/g DW ± SD, respectively for PTOX and MPTOX).

Effect of salicylic acid

Fig. III shows the effect of different concentrations of salicylic acid on lignan content of hairy root cultures of *L. mucronatum* after 48 and 96 h elicitation in comparison with the control cultures. As Table 3 shows, salicylic acid was less effective in lignan production in *L. mucronatum* hairy roots cultures. Concentration of 200 µm of salicylic acid promoted the accumulation of lignan. However, no increase was observed by addition of very high concentration (400 µm) of salicylic acid after 96 h in hairy roots in comparison with un-treated roots 5.88 ± 0.22 and 43.26 ± 1.1 mg/g DW ± SD, respectively for PTOX and MPTOX lignans.

Detection of relevant transgenes in the selected hairy root lines

In the selected transgenic hairy root lines, a sharp band of 780-bp was amplified. All

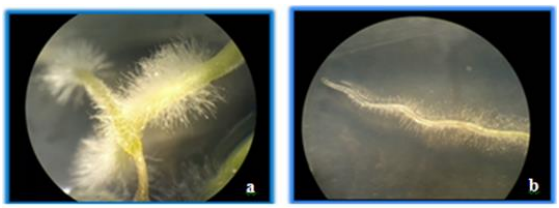

Fig. I. Variation in the type of emergence induced hairy roots on hypocotyl explants in *L. mucronatum* one week after inoculation by (a): 'A13' and (b): 9534 strain of *A. rhizogenes*

Table 2
Different concentration effect of methyl jasmonate treatments on lignan production in hairy roots culture of *L. mucronatum.*

Strains	A13 strain		9534 strain	
Concentration	PTOX mg/gr DW ± SD	MPTOX mg/gr DW ± SD	PTOX mg/gr DW ± SD	MPTOX mg/gr DW ± SD
Control	5.81 ± 0.61	42.32 ± 1.65	5.2 ± 0.58	35.42 ± 0.98
18th day- 48 h				
50	8.82 ± 0.33	53.34 ± 2.1	8.34 ± 0.22	51.34 ± 2.1
100	11.37 ± 0.65	75.65 ± 3.4	9.83 ± 0.64	71.43 ± 2.8
150	8.53 ± 0.54	67.56 ± 3.1	7.95 ± 0.52	55.87 ± 3.3
200	8.13 ± 0.31	45.21 ± 1.3	7.31 ± 0.22	40.21 ± 2.1
18th day- 96 h				
50	8.21 ± 0.12	50.52 ± 2.1	7.52 ± 0.28	49.42 ± 2.1
100	10.89 ± 0.64	69.38 ± 2.6	9.28 ± 0.69	67.76 ± 2.3
150	8.12 ± 0.34	59.83 ± 2.7	7.28 ± 0.53	51.27 ± 2.5
200	7.11 ± 0.21	40.11 ± 1.3	6.21 ± 0.21	31.12 ± 1.1
30th day- 48 h				
50	8.12 ± 0.26	51.32 ± 2.1	8.01 ± 0.13	50.34 ± 2.1
100	9.98 ± 0.85	67.56 ± 2.4	9.19 ± 0.53	61.65 ± 2.4
150	7.76 ± 0.43	50.43 ± 2.2	7.33 ± 0.22	49.46 ± 2.6
200	6.34 ± 0.12	36.30 ± 1.1	5.72 ± 0.16	32.28 ± 1.1
30th day- 96 h				
50	7.41 ± 0.22	47.44 ± 1.5	5.91 ± 0.16	44.23 ± 1.3
100	9.36 ± 0.64	63.85 ± 2.3	8.98 ± 0.38	58.78 ± 2.2
150	7.14 ± 0.32	51.31 ± 1.6	7.43 ± 0.53	42.23 ± 1.3
200	5.43 ± 0.21	39.36 ± 1.2	5.16 ± 0.16	33.14 ± 1.1

mg/g DW ± SD: mg/g dry weight ± standard error

transformants showed presence of amplified product of the 780 bp *rol*B and no *rol*B gene activity was found in control tissue (negative control) sample. The *vir*D gene, located outside the T-DNA, is diagnostic for the presence of any remaining agrobacteria in the root tissue. The negative results of PCR amplification for the *vir*D gene demonstrated that no bacterial DNA was involved in *rol*B amplification leading to false (Fig. II).

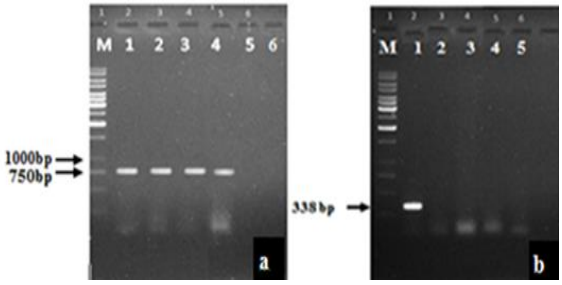

Fig. II. PCR amplification of the *rol*B and *vir*D genes using hairy root derived DNA. (a): M: 1 Kb DNA Ladder (Fermantas), 1: *A. rhizogenes* DNA (positive control); 2 to 4: transformed roots; 5: wild plant root as first negative control. (b) M: 1 Kb DNA Ladder, 1: *A. rhizogenes* DNA; 2, 3: Transgenic hairy roots 4: Wild plant root as first negative control, 5: Non-DNA template PCR reaction as second negative control

Discussion

Phenolic acids have antimicrobial properties and may be the constitutive defense compounds of the plants against microbial infection (Kumar et al., 2012). For improving a crop using genetic transformation, several parameters should be optimized.

In this study parameters such as specificity and concentration of elicitor, growth stage of the treated tissue, duration of exposure to elicitor, and for the first time, two strains of agrobacterium have been considered and the outcome was analyzed for lignan production. Our experimental results showed that the production of phenolic compounds in the *L. mucronatum* hairy root cultures can be stimulated by biotic (fungal extract) and abiotic elicitors (MJ and SA). Previous studies have also suggested this in the cell cultures of other plant species (Savitha et al., 2006; Kuzmaa et al., 2009; Shams-Ardakani et al., 2005).

Table 3
Different concentration effect of Salicylic acid treatments on lignan production in hairy roots culture of *L. mucronatum.*

Strains	A13 strain		9534strain	
Concentration	PTOX mg/gr DW ± SD	MPTOX mg/gr DW ± SD	PTOX mg/gr DW ± SD	MPTOX mg/gr DW ± SD
Control	5.81 ± 0.61	42.32 ± 1.65	5.2 ± 0.58	35.42 ± 0.98
18th day- 48 h				
100	6.22 ± 0.21	48.46 ± 1.1	5.82 ± 0.23	32.28 ± 1.1
200	7.97 ± 0.33	51.68 ± 2.1	6.99 ± 0.63	43.57 ± 2.1
300	6.96 ± 0.21	47.26 ± 1.6	6.59 ± 0.35	36.92 ± 1.7
400	5.82 ± 0.16	45.28 ± 1.1	5.35 ± 0.15	33.41 ± 1.3
18th day- 96 h				
100	6.11 ± 0.21	45.22 ± 1.3	5.12 ± 0.21	37.12 ± 1.1
200	7.22 ± 0.35	49.18 ± 2.2	6.78 ± 0.33	41.67 ± 1.4
300	6.32 ± 0.42	46.83 ± 1.6	6.13 ± 0.28	38.17 ± 1.2
400	5.73 ± 0.18	45.11 ± 1.1	5.21 ± 0.14	36.22 ± 1.2
30th day- 48 h				
100	6.02 ± 0.32	47.32 ± 1.2	5.21 ± 0.14	30.34 ± 1.2
200	7.34 ± 0.53	49.56 ± 1.4	6.79 ± 0.33	39.65 ± 1.2
300	6.16 ± 0.23	47.13 ± 1.2	7.13 ± 0.42	30.96 ± 0.9
400	5.83 ± 0.11	44.08 ± 1.1	5.4 ± 0.16	29.28 ± 0.8
30th day- 96 h				
100	6.15 ± 0.53	45.34 ± 1.2	5.41 ± 0.18	35.75 ± 1.1
200	7.23 ± 0.63	47.35 ± 1.7	6.58 ± 0.42	38.24 ± 1.3
300	6.04 ± 0.32	46.38 ± 1.2	6.59 ± 0.52	36.13 ± 1.2
400	5.88 ± 0.22	43.26 ± 1.1	5.31 ± 0.25	34.91 ± 1.1

mg/g DW ± SD: mg/g dry weight ± standard error.

Effect of elicitor concentration on lignan content

Fungal extract is composed of a variety of compounds, apart from amino acids, vitamins and minerals. All these compounds can produce an osmotic pressure and act as stress factors which cause rapid but transmitted activation of key enzymes in biosynthetic pathway (Kumar et al., 2012). The highest increased effect was showed in fungal elicitor treatment after 48 h exposure time in the 18-day-old culture while the highest decreased effect was shown in the MJ treatment after 96 h exposure time. On the other hands, high concentration and exposure time in all treatments was the reason for significant decrease in lignan content; however, SA and fungal extract elicitors have showed lower decreased effect at high concentration than MJ treatments. In all treatments smaller and excessive dosage did not show optimum results. High dosage of elicitor has been reported to induce hypersensitive response leading to cell death, whereas an optimum level was required for induction. MJ at l00 concentration showed the highest increased effect but two concentrations (150 and 200 µm)

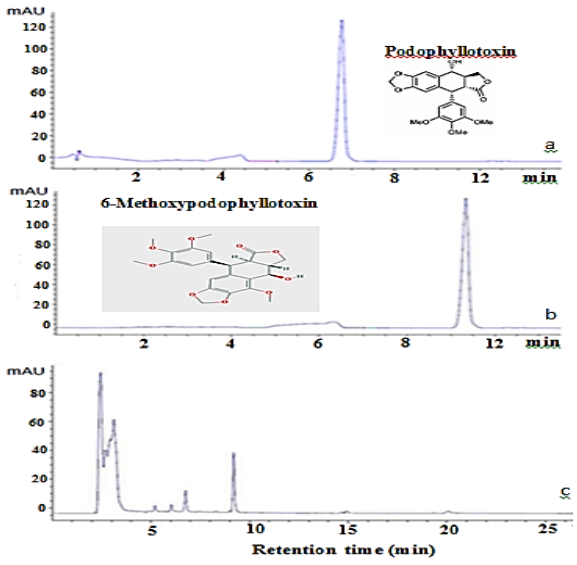

Fig. III. (a) and (b): HPLC chromatogram for PTOX and MPTOX standards. (c): HPLC chromatogram of the PTOX and MPTOX extracts from elicited roots by 1% fungal elicitor isolated of hairy roots grown in MS medium. Peaks indicate retention time for PTOX (6.76) and MPTOX (9.18).

had similar effect on lignan production and significant decrease in lignan production was not observed at 200 µm concentration. Also, in all of the treatments low concentration of elicitors did not have significant effect on lignin content and this might be due to non-availability of sufficient concentration to the growth promoting compounds present in the culture medium of hairy roots. In this case Kumar and et al. (2012) have reported that an elicitor with doses smaller than the optimum suggests that the elicitor binding sites in cells are not fully occupied for the activation of biosynthesis of secondary metabolites whereas excessive dose causes a deleterious effect on the biosynthetic capacity of the cells (Kumar et al., 2012).

Effect of agrobacterium strains on lignan content

Elicitation effect in hairy roots of two stains of agrobacterium was different. On the other hands, as different frequency and morphology in various strains, elicitation effect has also differed and varied depending on the type of bacteria (Fig. I). Our earlier findings on growth and lignan production in *in vitro* hairy roots cultures of *L. mucronatum* (Samadi et al., 2014) have also revealed an increased lignan content by A13 strain. This phenomenon has also been observed for podophyllotoxin production by cell suspension cultures of *Linum album* (Shams-Ardakani et al., 2005). Extensive morphological variation in individual hairy root cultures can be possibly due to differential expression of T-DNA genes present in the transferred T-DNA in the host genome (Akramian et al., 2008). It is seems that the strains of agrobacterium were the first elicitor for induction of defense response pathway and lignan production in which function of plants depend on virulence potential of strains. In latter step, other elicitors such as fungal extract, MJ, and SA acted as the second elicitor for induction of defense response pathway where concentration of elicitors played a better role than other factors such as exposure time and growth stages.

Effect of duration of elicitor exposure on lignan content

The results of this study showed that lignan production declined or did not increase by abiotic and biotic elicitors after 96 h exposure time in comparison with control roots culture (Tables 1, 2, and 3). These results could be due to conversion of lignans to other derivatives or due to their degradation and this is in agreement with the results of a study in which the effects of *Alternaria alternate* on the capsidiol accumulation in the fruits, the stems, and the leaves of *Capsicum annuum* were investigated (Koc et al., 2011). Also Kumar et al. (2012) have reported that the optimal time of addition of an elicitor depends on the right combination of the growth phase during which it is added and the exposure time.

Effect of age of culture on lignan production

Age of subculture plays an important roler in production of bioactive compounds by elicitation (Koc et al., 2011). 18-day-old culture showed a higher lignan content than the 30-day-old culture (Tables 1, 2, 3). Optimal induction occurs when the elicitors are added to cultures at late exponential or early stationary phase of plant cell growth. Therefore, the effect of any elicitor for maximum response depends on the age of culture, concentration of elicitor, and incubation time with the elicitor (Baldi et al., 2008).

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