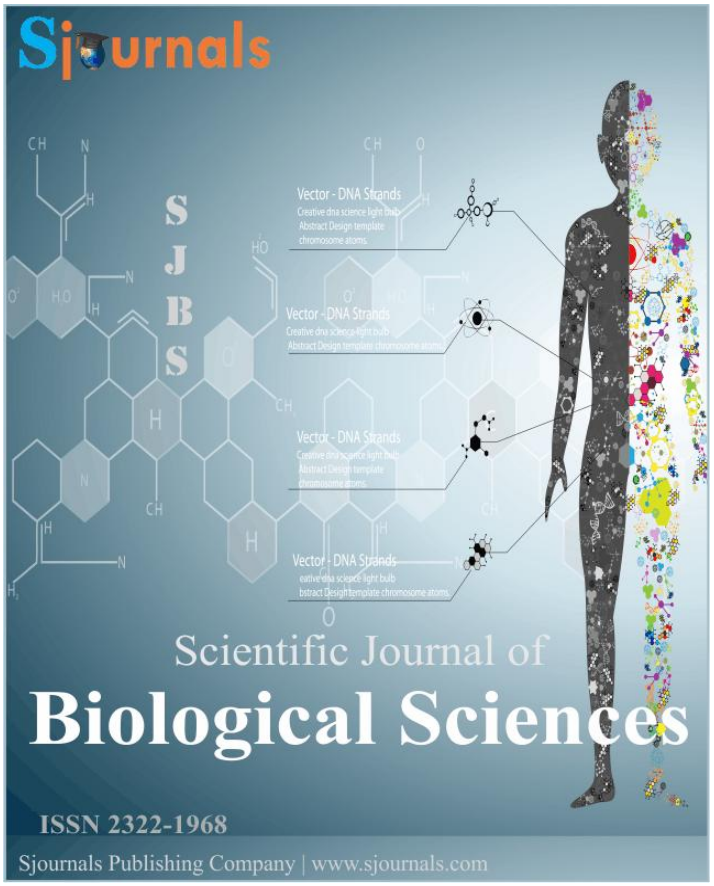

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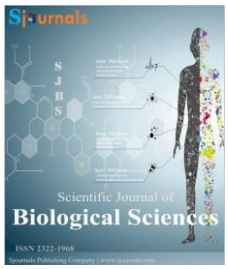

Original article

Response surface modeling of natural alizarin production in hairy root cultures of *Rubia tinctorum* L. upon elicitation with fungal mycelia

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ABSTRACT

The roots of *Rubia tinctorum* L., the common madder contain natural red dye known as alizarin. In the current study, central composite design of response surface methodology was employed for modeling of fungal elicitor treatment on natural alizarin production in uniform hairy root cultures of common madder in liquid 1/2 B5 medium. Upon fungal elicitation assay, using two fungal mycelia elicitors (*Aspergillus niger* and *Bipolaris maydis*) at three different times (0, 12 and 24h), the production of alizarin was determined. According to the results, after 24h; modeling and optimization conditions, including combination of 2 % of both elicitors for alizarin production equal to 10.0 $mg.g^{-1}$ DW was evaluated. Optimal process parameters have been determined by using a high desirability value of 1.00 in Design-Expert® software. Our results, altogether, offer a promising method regarding to the improvement of the alizarin production, as a pivotal natural dye in industrial applications.

1. Introduction

Common madder (*Rubia tinctorum* L.), as a family member of Rubiaceae, is a perennial plant usually distributed in Iran which has been used as a medicinal plant and natural dye (Zargari, 1992). *R. tinctorum* L.

cultivation and processing is deep-rooted in Iranian history, owing to its reputation in red dye making for Persian hand-woven carpets (Navaei et al., 2006). The root of this plant is well-known as a traditional herbal medicine used for treatment of kidney and bladder stones, mainly because of its important anthraquinones (Navaei et al., 2006). Roots of *R. tinctorum* L. produces various types of anthraquinone colorant, such as alizarin and purpurin, the first being the most abundant of which. Anthraquinones biosynthesis in elicited cultures would be an interesting tactic (Vasconsuelo et al., 2004), because of lower cost than synthetic production, renewed interest in natural dyes and lastly environmental concerns (Ozen et al., 2014). Hairy roots are generated by infecting plant tissue with *Agrobacterium rhizogenes* (Guillon et al., 2006), resulting in the transfer of T-DNA from the bacterial root inducing (Ri) plasmid into the plant genome and the genetic transformation into hairy root. Hairy roots show rapid growth in phytohormones-free media and promote the synthesis of phytochemicals whose biosynthesis needs differentiated root cell types (Oksman-Caldentey, 2002). Transformed *in vitro* root culture is reported to have a remarkable capacity to produce plant secondary metabolites (Hu and Du, 2006). They can also be used as model systems to scrutinize the symbiosis (Imanishi et al., 2011), the biosynthesis of secondary metabolites and ways to influence, for example by apply precursors and elicitors (Ono and Tian, 2011; Perassolo et al., 2011a; Zhou et al., 2011). The objective of this research was to elucidate response surface modeling for applying fungal elicitors in different times to increase the production of alizarin in hairy root cultures. To precisely identify and quantify alizarin production, it was necessary to employ Ultra-High Performance Liquid Chromatography (UHPLC).

2. Materials and methods

2.1. Establishment of hairy root cultures

Hairy root cultures are obtained as previously described (Ghorbani et al., 2014). The hairy root line C58C1 was selected on the basis of faster growth rate were excised from explants and transferred to solid B5 medium for more proliferation. After one month, genomic DNA was extracted from putative transformed hairy root using the (CTAB) method (Khan et al., 2007). The transgenic nature of this line was confirmed by PCR with *rolC* gene specific primers (F: 5'-CTCCTGACATCAAACTCGTC-3', R: 5'-TGCTTCGAGTTATGGGTACA-3'). To confirm the transgenic-free from any residual bacterial contamination, *virD* gene (F: 5'-ATGTCGCAAGGACGTAAGCCGA-3', R: 5'-GGAGTCTTTCAGCATGGAGCAA-3') specific primers were used. Meanwhile, the Ri-plasmid of *A. tumefaciens* strain C58C1 was used as a positive control. Amplification conditions for *rolC* and *VirD* described in previous study (Ghorbani et al., 2014).

2.2. Fungal elicitation

About 2-3 cm of transgenic hairy roots, which were grown on solid B5 medium, simultaneously transferred to the 33 Erlenmeyer flasks (250 ml) containing 50 ml of autoclaved hormone-free 1/2 B5 liquid medium pH 5.8, at 25ºC placed on a gyratory shaker set at 110 rpm in the dark conditions. The elicitation process was carried out with two fungal elicitors were prepared as described by Bahabadi et al. (2011). One-week-old mycelia of *Aspergillus niger* and *Bipolaris maydis* (Plant Protection Department, University of Tehran, Karaj, Iran) were harvested and rinsed with sterile distilled water. The collected mycelia were crushed under liquid nitrogen and suspended with water to an ultimate concentration of 250 mg/ml. The suspension was centrifuged at 10,000 g for 10 min, then the supernatant was autoclaved for 15 min at 120 ºC (Bahabadi et al., 2011). Three weeks after hairy root proliferation, 500 microliter (1 % v-v) and 1000 microliter (2 % v-v) of these elicitor solutions was then added to 50.0 ml of *R. tinctorum* L.'s C58C1 clones. The controls were treated with 500 and 1000 microliter of autoclaved distilled water. After the different elicitors had been added to the cultures, the amount of alizarin was determined by UHPLC system at various elicitation times (0, 12 and 24h).

2.3. Sample extraction and UHPLC conditions

Freeze-dried powdered root (50 mg) was immersed with 1 mL methanol at room temperature in 1.5 mL tube for 12h followed by ultrasonic extraction (WiseClean®) for 30 min at room temperature. The extract was centrifuged at 10000 g at room temperature for 10 min. Supernatant (900 µL) was transferred to a new tube and dried at 60°C. The residue was dissolved with 900 µL 25% (v-v) HCl-methanol and hydrolyzed at 90°C for 30 min and then brought to 1 mL using methanol. The extracts were analyzed by using a Knauer UHPLC/HPLC PLATIN Blue system (Knauer, Germany), equipped with a binary pump, an auto-sampler, column compartment and UV

detector. The samples were filtered through syringe membrane filter (PTFE, 0.22 µm), and 15 µL was injected into system. Analytical column was C18 column (Eurospher, 120 mm × 4.6 mm, 5 µm). The mobile phase consisted of methanol-acetonitrile (12:88 v/v). The flow rate was 0.8 mL/min with UV detection wavelength at 254 nm and the column temperature was 25°C. The quantification of the alizarin in hairy root samples was carried out using an external standard calibration method (Banyai et al., 2006).

2.4. Response surface methodology (RSM)

The experimental design protocol (Table S1) for RSM was developed using Design-Expert® software (Trial version 9, Stat-Ease Corporation, Minneapolis, MN). Central composite design (CCD) of RSM with 3 face centered points was employed for design of experiment. The analysis of variance (ANOVA) table was generated, and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significance of all the terms in the polynomial was judged statistically by computing the F value at a probability (p) of 0.05. Conceptual information about polynomial equation explained in previous study (Ghorbani et al., 2014).

3. Results and discussion

3.1. Establishment of hairy root cultures

The excised hairy root (About 3-4 cm), which was induced by *A. tumefaciens* strain C58C1 transferred to solid B5 medium for hairy root proliferation. The transgenic nature of hairy root was confirmed by PCR with the specific primer pairs of *rolC* gene and template DNA from hairy roots amplified the expected amplicon of 629 bp. Contamination confirmation was also performed by PCR analysis using *virD* (438 bp) gene specific primers, no amplicon was found in the hairy root under study, indicating the absence of bacterial contamination (Figure 1). Previously, we established transformed hairy roots of *R. tinctorum* with *A. tumefaciens* strain C58C1 from leaf explant. Our results showed that 1mM concentration of L-arginine could improve hairy root induction (Ghorbani et al., 2014). In this study, *R. tinctorum* hairy root clone was selected on the basis of faster growth rate, proliferated and elicited using fungal elicitors in liquid 1/2 B5 medium.

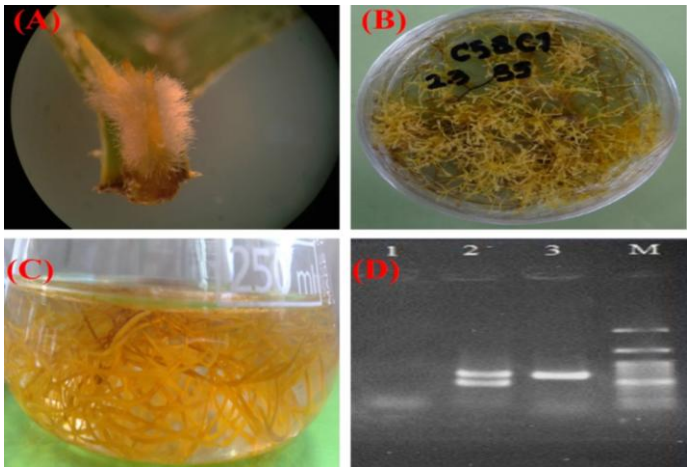

Fig. 1. Hairy root induction of *R. tinctorum* L. using *A. tumefaciens* strain C58C1 (A). Hairy root clone proliferation in solid B5 medium (B), Hairy root growth in liquid 1/2 B5 medium and fungal elicitation (C), (D): PCR amplification of *rolC* and *VirD* genes. 1: Water, 2: Ri-plasmid of *A. tumefaciens* strain C58C1. 3: Transgenic hairy root. M: Molecular size marker (100 bp ladder, SinaClone, Iran).

3.2. UHPLC analysis

During the investigated time period (0-24h), the alizarin content of the treated and control cultures were measured. Alizarin was identified using comparison of its retention time (0.6 minute) and absorption spectrum of its standard solution (Figure S2). The maximum alizarin content of elicited hairy root was raised to 2.40 fold of that in the control culture. On the subject of the time period, for both elicitors; a significant continuous increase in alizarin accumulation was detected between 0-24h.

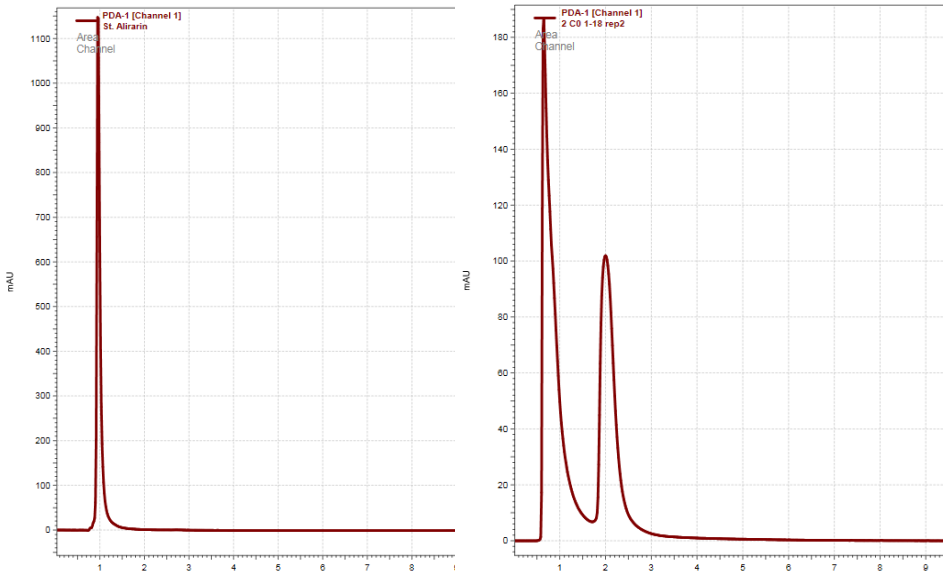

Fig. 2. HPLC chromatogram for Standard alizarin (Left) and hairy root sample (Right).

3.3. Response surface modeling

In this study, different elicitor concentrations (v-v), including 0-2 % of A*. niger* and *B. maydis* and elicitation time (0-24h) for production of alizarin (mg.g^{-1} DW) was studied (Table 1). According to the results, modeling and optimization conditions, including combination of 2 % of *A. niger* and 2 % of *B. maydis* elicitors for alizarin production equal to 10.0 mg.g^{-1} DW was evaluated (Table 2). Optimal process parameters have been determined by using a high desirability value of 1.000. The final equation obtained in terms of the coded factors for fungal elicitation is:

$$\text{Alizarin } (mg.g^{-1}\ \text{DW}) = +6.97 + 0.77\text{A} + 0.76\ \text{C} - 0.13\ \text{AB} + 0.06\ \text{AC} - 0.61\ \text{BC} \qquad (1)$$

Table 1
Factors and their coded levels chosen for CCD.

Factors	Units	Type	Factor level		
			-1	0	+1
A- *Aspergillus niger*	% (v:v)	Numerical	0	1	2
B- *Bipolaris maydis*	% (v:v)	Numerical	0	1	2
C- Time	h	Categorical	0	12	24

In the range of studied concentrations for both elicitors, the alizarin production goes through a maximum. Illustrated effects of elicitation parameters are shown in Figure 3. F-value of the model equal to 11.69 implies the model is significant. The model fitness was expressed with R^2, which was calculated to be 0.85, indicating that 85% of the variability in the response could be explained by the model. The value of the adjusted determination coefficient (adjusted R^2= 0.78) was also reasonably high, supporting a high significance of the model. The lack of fit F-value of 0.56 is not significant relative to true, pure error (Table 3). This proves that the equation as expressed above, provides a suitable model.

An elicitation method increased the synthesis of anthraquinone derivatives occurring in cell suspension cultures of *R. tinctorum* L. (Orbán et al., 2008). The highest alizarin content has been reported on solid 1/2 NMS medium in hairy root cultures of *R. tinctorum* L. (Banyai et al., 2006). Not only the concentration of fungal elicitor, also type and the origin of the elicitor seems to be important for alizarin production in *R. tinctorum* suspension culture (Bóka et al., 2002).

Table 2
Coded levels and experimental results of the independent variables in CCD.

				Alizarin (mg/g DW)	
Run	**A (%)**	**B (%)**	**Time (h)**	**Actual**	**Predicted**
1	1.0	1.0	0	4.29503	4.77709
2	1.0	2.0	12	5.78346	6.94313
3	0.0	1.0	24	5.86424	6.38592
4	1.0	0.0	24	5.85423	6.50280
5	2.0	2.0	0	4.17503	3.56247
6	0.0	2.0	12	5.79346	5.70473
7	1.0	0.0	0	4.18603	4.19578
8	1.0	1.0	24	6.84424	8.40708
9	1.0	1.0	0	4.20122	4.77709
10	2.0	1.0	24	9.8155	9.36633
11	1.0	1.0	24	9.8163	8.40708
12	0.0	1.0	12	5.70346	6.37091
13	0.0	0.0	12	5.18924	5.90812
14	2.0	1.0	12	6.98542	8.05629
15	1.0	1.0	12	9.89231	7.74455
16	2.0	2.0	24	9.8112	10.0063
17	2.0	1.0	0	4.19875	4.24536
18	0.0	0.0	24	4.75432	4.34641
19	1.0	0.0	12	7.81131	7.41700
20	0.0	0.0	0	4.11424	3.53037
21	0.0	1.0	0	4.20424	4.24693
22	1.0	1.0	12	9.3215	7.74455
23	2.0	0.0	24	7.77006	7.59730
24	1.0	1.0	12	7.38044	7.74455
25	2.0	0.0	0	4.10424	3.79929
26	1.0	1.0	24	8.76906	8.40708
27	2.0	0.0	12	7.37744	7.86399
28	1.0	2.0	24	8.76916	9.18239
29	1.0	2.0	0	4.16624	4.22945
30	2.0	2.0	12	7.37944	7.11962
31	0.0	2.0	0	4.16424	3.83453
32	1.0	1.0	0	4.16624	4.77709
33	0.0	2.0	24	7.83699	7.29648

Table 3
Analysis of variance results for the CCD.

Source	**Sum of Squares**	**df**	**Mean Square**	**F**	**p-value**
Model	117.35	11	10.67	11.69	< 0.0001
A-Aspergillus niger	10.88	1	10.88	11.92	0.0024
B-Bipolaris maydis	2.51	1	2.51	2.75	0.1123
C-Time	82.21	2	41.11	45.03	< 0.0001
AB	0.22	1	0.22	0.24	0.6289
AC	6.71	2	3.35	3.67	0.0428
BC	8.60	2	4.30	4.71	0.0204
A2	2.14	1	2.14	2.35	0.1404
B2	2.42	1	2.42	2.65	0.1183
Residual	19.17	21	0.91	-	-
Lack of Fit	11.15	15	0.74	0.56	0.8333
Pure Error	8.02	6	1.34	-	-

Secondary metabolite production in *R. tinctorum* L. has been enhanced with precursor treatments and altered growth medium compositions (Perassolo et al., 2011b). Moreover, tissue culture technique such as immobilization, using natural Lignocellulosic materials has been used in *R. tinctorum* L. suspension culture and can be shared with elicitation method (Nartop et al., 2013).

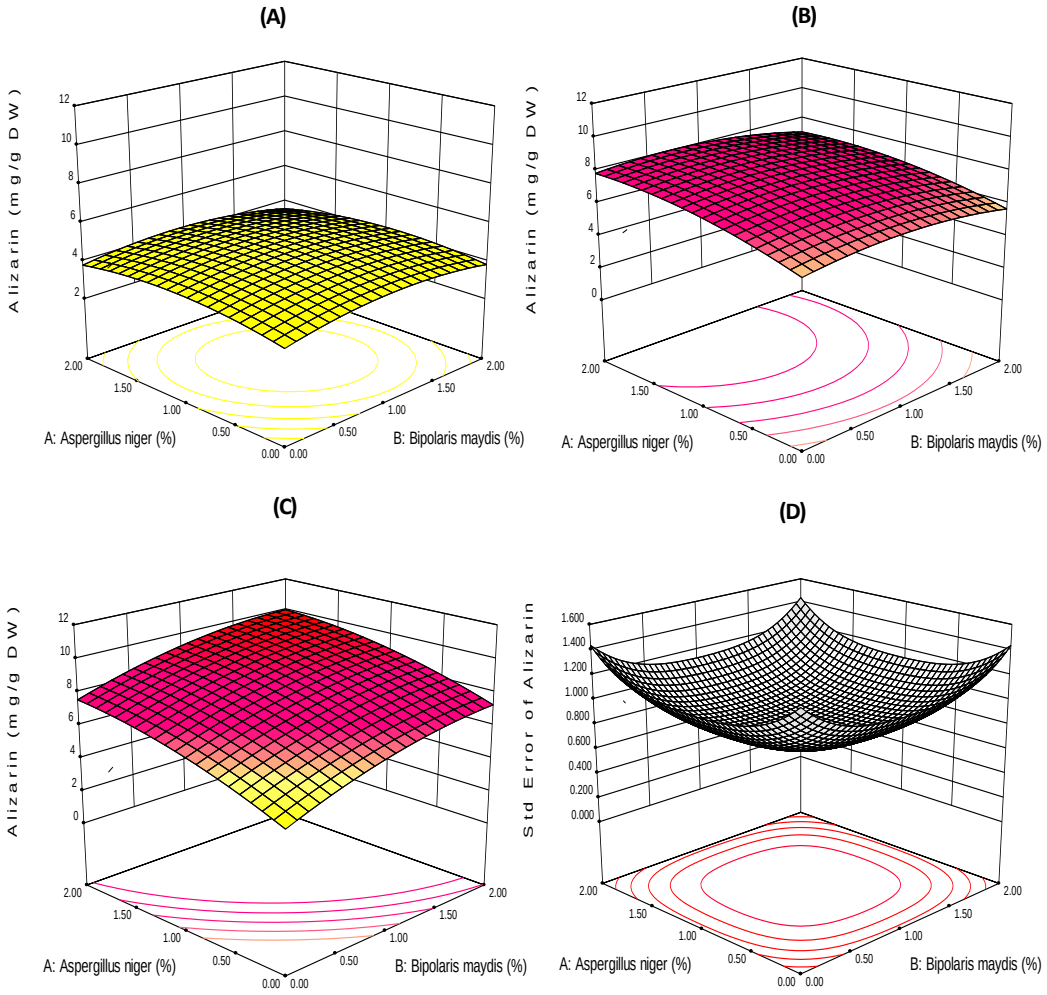

Fig. 3. 3D surface presentation for the effects of two fungal elicitors in coded conditions for alizarin production (mg.g^{-1}DW) in 0 (A), 12 (B) and 24h (C). Response Surface plots showing effects of two elicitor as a function of standard error (StdError) (D).

4. Conclusion

The major objective of this research was to development of a statistical approach to modeling and optimization of fungal elicitor application to better understanding of the hairy root-elicitor interactions for producing plant secondary metabolite alizarin as a natural dye. This research is the first report on alizarin assay in uniform transgenic hairy root clones of *R. tinctorum* L. elicited by fungal elicitors. Generally, based on our tryouts associated with two fungal elicitors, both types of elicitors have advantageous features. Our results, altogether, offer a promising avenue regarding the improvement of the alizarin production, as a pivotal natural dye in various applications.

Acknowledgments

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References

Bahabadi, S.E., Sharifi, M., Safaie, N., Murata, J., Yamagaki, T., Satake, H., 2011. Increased lignan biosynthesis in the suspension cultures of *Linum album* by fungal extracts. Plant. Biotechnol. Report., 5, 367-373.

Banyai, P., Kuzovkina, I.N., Kursinszki, L., Szoke, E., 2006. HPLC analysis of alizarin and purpurin produced by *Rubia tinctorum* L. hairy root cultures. Chromatographia, 63, S111-S114.

Boka, K., Jakab, J., Kiraly, I., 2002. Comparison of the effect of different fungal elicitors on *Rubia tinctorum* L. suspension culture. Biol. Plantarum., 45, 281-290.

Ghorbani, M., Omidi, M., Peighambari, S.A., 2014. L-Arginine optimization medium for hairy root induction of madder (*Rubia tinctorum* L.) based on one factor model of response surface methodology. Sci. J. Biol. Sci., 3, 117-125

Guillon, S., Trémouillaux-Guiller, J., Pati, P.K., Rideau, M., Gantet, P., 2006. Hairy root research: recent scenario and exciting prospects. Curr. Opin. Plant. Biol., 9, 341-346.

Hu, Z-B., Du, M., 2006. Hairy root and its application in plant genetic engineering. J. Integr. Plant. Biol., 48, 121-127.

Imanishi, L., Vayssières, A., Franche, C., Bogusz, D., Wall, L., Svistoonoff, S., 2011. Transformed hairy roots of discaria trinervis: A valuable tool for studying actinorhizal symbiosis in the context of intercellular infection. Mol. Plant. Microbe. Interact., 24, 1317-1324.

Khan, S., Qureshi, M.I., Alam, T., Abdin, M.Z., 2007. Protocol for isolation of genomic DNA from dry and fresh roots of medicinal plants suitable for RAPD and restriction digestion. Afr. J. Biotechnol., 6, 175-178.

Nartop, P., Akay, Ş., Gürel, A., 2013. Immobilization of *Rubia tinctorum* L. suspension cultures and its effects on alizarin and purpurin accumulation and biomass production. Plant Cell, Tissue and Organ Culture (PCTOC). 112, 123-128.

Navaei, M.N., Mirza, M., Dini, M., 2006. Chemical composition of the essential oils of *Rubia tinctorum* L. aerial parts from Iran. Flavour. Fragr. J., 21, 519-520.

Oksman-Caldentey, K.M., 2002. *Agrobacterium rhizogenes*-mediated transformation: Root cultures as a source of alkaloids. Plant. Med., 68, 859-868.

Ono, N.N., Tian, L., 2011. The multiplicity of hairy root cultures: Prolific possibilities. Plant. Sci., 180, 439-446.

Orbán, N., Boldizsár, I., Szűcs, Z., Dános, B., 2008. Influence of different elicitors on the synthesis of anthraquinone derivatives in *Rubia tinctorum* L. cell suspension cultures. Dyes and Pigments, 77, 249-257.

Ozen, E., Yeniocak, M., Goktas, O., Alma, M.H., Yilmaz, F., 2014. Antimicrobial and antifungal properties of Madder Root (*Rubia tinctorum*) colorant used as an environmentally-friendly wood preservative. Bio. Resour., 9, 1998-2009.

Perassolo, M., Quevedo, C.V., Busto, V.D., Giulietti, A.M., Talou, J.R., 2011a. Role of reactive oxygen species and proline cycle in anthraquinone accumulation in *Rubia tinctorum* cell suspension cultures subjected to methyl jasmonate elicitation. Plant. Physiol. Biochem., 49, 758-763.

Perassolo, M., Quevedo, C.V., Giulietti, A.M., Talou, J.R., 2011b. Stimulation of the proline cycle and anthraquinone accumulation in *Rubia tinctorum* cell suspension cultures in the presence of glutamate and two proline analogs. Plant Cell, Tissue and Organ Culture (PCTOC). 106, 153-159.

Vasconsuelo, A., Giulietti, A.M., Boland, R., 2004. Signal transduction events mediating chitosan stimulation of anthraquinone synthesis in *Rubia tinctorum*. Plant. Sci., 166, 405-413.

Zargari, A., 1992. Medicinal Plants vol 2. University of Tehran Press, Tehran.

Zhou, M.L., Zhu, X.M., Shao, J.R., Tang, Y.X., Wu, Y.M., 2011. Production and metabolic engineering of bioactive substances in plant hairy root culture. Appl. Microbiol. Biotechnol., 90, 1229-1239.

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