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Original article

L-Arginine optimization medium for hairy root induction of madder (*Rubia tinctorum L.)* based on one factor model of response surface methodology

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ABSTRACT

Medium optimization for hairy root cultures producing secondary metabolites was studied through statistical experimental design. In the following, one factor model of response surface methodology (RSM) was employed to formulate the L-Arginine amino acid levels alongside three categorical factors including bacterial strains (ATCC 15834, C58C1 and R1000), type of explant (Leaf and Stem) in co-cultivation media (B5 and MS) for hairy root induction of Madder (*Rubia tinctorum* L.). Design of experiment and data analysis was carried out by using Expert-Design® 7.1 software. According to the results, modeling and optimization conditions, including L-Arginine concentration 1.00 mM; bacterial strain; C58C1, leaf explant and B5 medium for HR induction frequency equal to 58% was evaluated (Desirability point=0.986). These optimal conditions predicted by RSM were confirmed to enhance hairy root induction as an application potential for biotechnological implementation to produce the anticipated compounds.

1. Introduction

Rubia tinctorum L., the common madder is an herbaceous perennial plant belongs to family Rubiaceae usually distributed in Iran, which has been used as a medicinal plant (Zargari, 1991). The root of this plant used for treatment of the kidney and bladder phosphate stones in traditional herbal medicine, mainly because of its important active compounds (Navaei et al., 2006). Anthraquinones are well-identified and main active compound present in madder which has been used in dye industry and medicine (Orban et al., 2008). Roots of R. tinctorum L. produce various types of anthraquinone natural dyes, such as alizarin, purpurin, xanthopurpurin and etc. (Navaei et al., 2006). Hairy roots (HR) are generated by infecting plant with Agrobacterium rhizogenes, resulting in the transfer of the root inducing (Ri) plasmid into the plant genomic DNA and hairy root formation (Guillon et al., 2006). HRs are organ culture technique produce secondary metabolites higher than in intact plant (Dehghan et al., 2012). Notwithstanding other tissue culture techniques, HRs show rapid growth in hormone free media and promote the synthesis of SMs (Zhao et al., 2014). The applicable age of madder roots for economic anthraquinone extraction is 3-5 years and harvesting consequences in the elimination of the established plants. Optimization of the cultivation conditions is important for achieving maximal SM production. Consequently, the culture medium compositions (carbon and nitrogen sources, vitamins, minerals, etc.) (Ryad et al., 2010; Srivastava and Srivastava, 2012) and the environmental conditions (temperature, agitation, time, etc.) (Pavlov et al., 2005; Maschke et al., 2015) affect the production of SMs. Response surface methodology (RSM) is a useful statistical method, which combines first and second-degree polynomial model to study complex processes, and it has been widely used in different fields (Khuri and Mukhopadhyay, 2010). The original perception was developed by Box and Wilson (Box and Wilson, 1951), and the basic and biological applications were reviewed by Mead and Pike (Mead and Pike, 1975). One factor RSM design allows us to develop up to a model for one numeric factor. The number of required levels depends on the order of the polynomial that we want to estimate. Categorical factors can be added to this design. The same number of levels of the numeric factor must be performed at every combination of the categorical factor(s) (Helseth, 2007). The objectives of this research were to optimize L-arginine amino acid in altered media for HRs induction of Madder, alongside different categorical factor using one factor RSM design.

2. Materials and methods

2.1. Plant material

Seeds of Rubia tinctorum L. purchased from Pakanbazr Company (Isfahan, Iran). The seeds were worn by topically 100 mesh abrasive paper and washed then surface sterilized with 70 % (v/v) ethanol for 1.5 min and 2.5 % (v/v) sodium hypochlorite solution. The seeds were germinated on 1/2 B5 medium at 25°C under a 16-8h (light-dark) photoperiod in a culture room.

2.2. Medium preparation

Two basic media formulations were tested at in vitro conditions: Murashige and Skoog (MS) (Murashige and Skoog, 1962) and Gamborg B5 (Gamborg et al., 1968) without phytohormones. Both media were supplemented with 100 mg/L myo-inositol, sucrose and agar at different concentrations according to the medium preparation protocols plus 0, 0.5, 1, 1.5 milliMolar (mM) L-arginine amino acid. The pH of the media was adjusted to 5.8 before autoclaving at 121°C for 20 min.

2.3. Co-cultivation of explants with agrobacterium strains

The leaf and stem explants were co-cultivated with Agrobacterium rhizogenes strains ATCC 15834, R1000 and Agrobacterium tumefaciens strain C58C1 with surgical scalpel immersed into the 12h old solid bacterial culture (LB medium containing 50 mg/L rifampicin) used to wound the explants and then were transferred in dark conditions (25˚C for 48h). Uninfected sterile explants were grown on the declared culture media as the control.

2.4. Elimination of bacteria

Bacteria was eliminated by sub-culturing in above-mentioned new media supplemented with 500 mg/L Cefotaxime which, antibiotic concentration decreased gradually in multiple subcultures until 100 mg/L constant concentration in the media. Sub-culturing in new media was repeated every 24h.

2.5. Pcr amplification

Genomic DNA was extracted from hairy roots, achieved from explants and natural root, obtained from seedlings by using Cetyl trimethylammonium bromide (CTAB) method (Khan et al., 2007). The transgenic nature of hairy roots was confirmed by PCR with specific primers of rolC gene. To confirm the transgenic-free nature of putative hairy roots from any bacterial contamination, virD gene specific primers were used (Table 1). Amplification conditions were 1 cycle at 94°C for 4 min followed by 32 cycles of amplification (45 s at 94°C, 45 s at 55°C and 45 s at 72°C) and 1 cycle at 72°C for 5 min. The PCR products after staining with GelRed™ were examined by electrophoresis on 1 % (w/v) agarose gel.

2.6. Statistical analysis

One factor model of response surface methodology used when there is only one continuous factor in the experiment and categorical factors can be added. Design for one numeric factor using 3 levels for a linear model, 5 levels for a quadratic model, 7 levels for a cubic model, plus some replicated points will be used. The design will be duplicated for every combination of the categorical factor levels (Helseth, 2007). The relationship between the response value (hairy root frequency) and the factors were explained by the following second-polynomial model:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i<j} \sum \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \epsilon \qquad \text{Model 1}$$

Where, Y is response factor (hairy root frequency), while, β0 and βi are the vector of regression coefficients and the design matrix X constitutes the linear, quadratic and interaction terms involving for the coded independent factors (Khuri and Mukhopadhyay, 2010).

Table 1
List of primer sequences used for PCR analysis in this study. F: Forward primer. R: reverse primer.

Primer name	Sequence	Expected band
rolC F	5'-CTCCTGACATCAAACTCGTC-3'	629 bp
rolC R	5'-TGCTTCGAGTTATGGGTACA-3'	
virD F	5'-ATGTCGCAAGGACGTAAGCCGA-3'	438 bp
virD R	5'-GGAGTCTTTCAGCATGGAGCAA-3'	

Table 2
Numeric and Categorical factors and their coded levels chosen for One Factor RSM Design.

Factor	Unit	Type	Factor level	
			-1	+1
A: L-Arginine	mM	Numeric	0	1.5
B: Medium	-	Categorical	B5	MS
C: Explant	-	Categorical	Leaf	Stem
D: Bacterial strains	-	Categorical	Control	ATCC 15834
			C58C1	R1000

The experimental design protocol (Table 2) for RSM was developed using Design-Expert® software (Version 7.1, Stat-Ease Corporation, Minneapolis, MN). The analysis of variance (ANOVA) table was generated, and the effect and regression coefficients of individual linear, quadratic, cubic 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.

3. Results

3.1. Hairy root growth

After about two weeks, the small calli were established in the site of infection, which were rapidly differentiated into hairy roots. The hairy roots of R. tinctorum commonly emerged from the calli sites after about 14-21 days of incubation on phytohormones-free MS and B5 media (Figure 1).

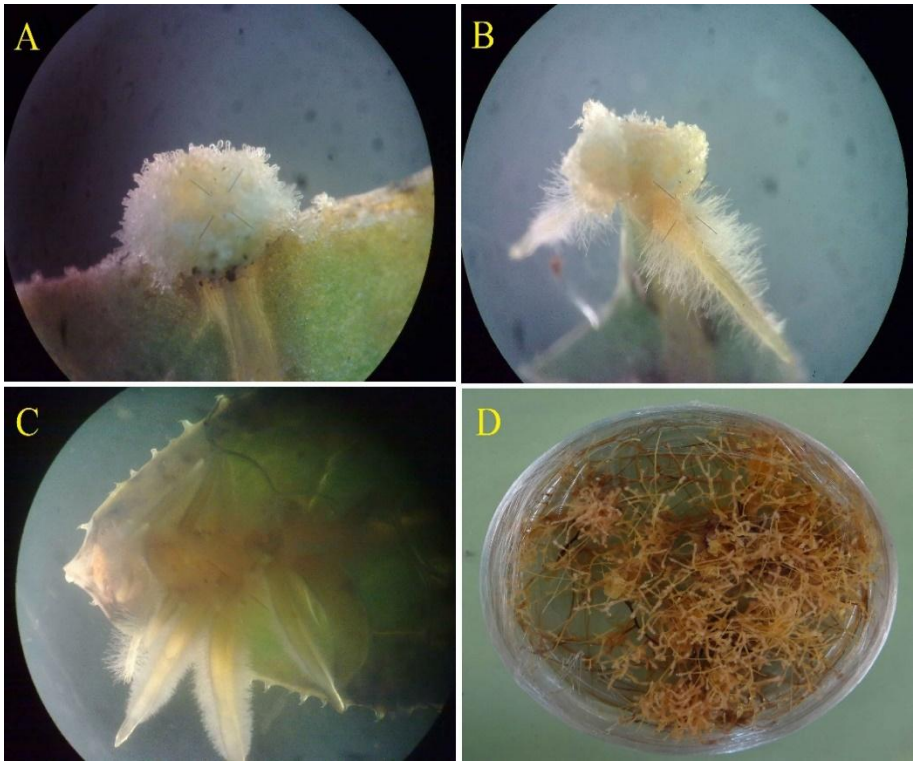

Fig. 1. Hairy root induction in Rubia tinctorum L. A: Callus induction. Hairy root emerging from callus in petiole (B) and Leaf explant (C), D: hairy root growth in B5 solid medium after one month.

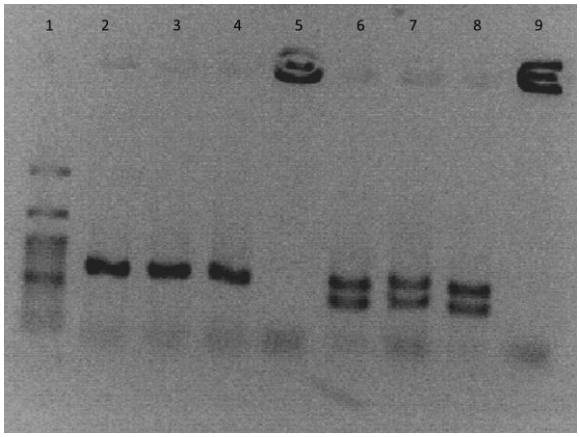

Fig. 2. Electrophoretogram of rolC and VirD genes PCR identification of hairy roots. Lane 1: molecular weight marker (100 bp ladder, SinaClone, Iran), lane 2-4: DNA from transformed hairy roots, lane 5: DNA from non-transformed root (negative control), lane 6-8: Agrobacterium Ri-plasmid (positive control), lane 9: Distilled water.

3.2. PCR analysis

The transgenic nature of hairy roots were confirmed by PCR with the specific primer pairs of rolC gene and template DNA from hairy roots amplified the expected amplicon of 629 bp. No amplicon (438 bp) of virD gene was

found in the hairy roots under study, representing the absence of bacterial contamination. Finally, on the subject of non-transformed root, no amplified fragment of the gene rolC was detected (Figure 2).

3.3. Bacterial strains and explant analysis

Two strains of A. rhizogenes (ATCC 15834 and R1000) and A. tumefaciens strain C58C1 were compared for their transformation efficiency. Hairy root induction ability of Agrobacterium strains according to the best explant for each strain was significantly different ($p<0.0001$). Leaf explants showed high rate of hairy root induction. On the other hand, the stem explants showed a low rate of hairy root induction (Figure 3). There was no significance difference between phytohormones-free MS and B5 media (Table 4). Also, interaction between media and each bacterial strain was significantly different.

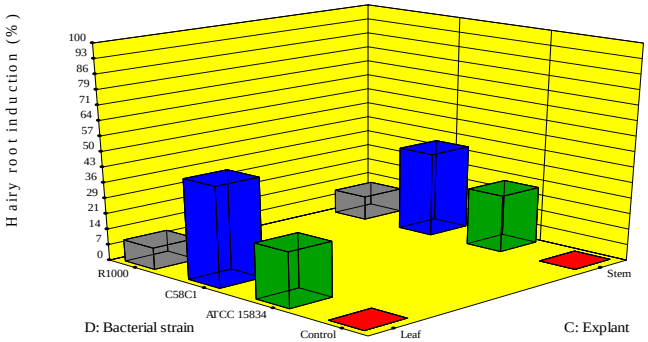

Fig. 3. Effect of different bacterial strains and explants on percentage of hairy root induction of the R. tinctorum L. in B5 medium containing 1 mM L-Arginine.

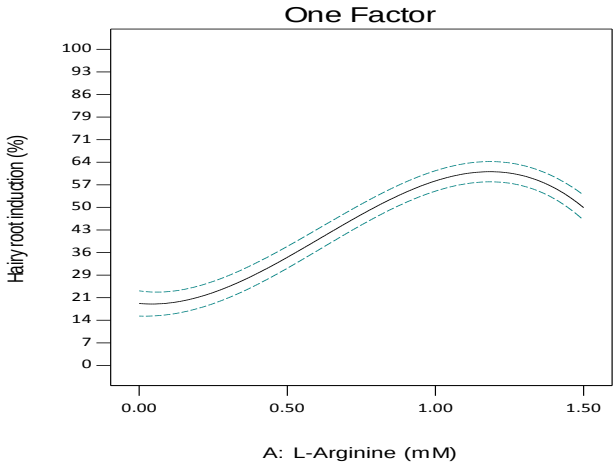

Fig. 4. One factor graph (Response Surface Plot). Effect of L-Arginine levels on hairy root induction (%). Conditions: C58C1 strain, B5 medium and Leaf explant. The dotted lines represent the 95% confidence.

3.4. Effect of l-arginine on frequency of hairy root induction

The addition of L-arginine to the co-cultivation media significantly improved the hairy root induction (Table 3). The highest frequency of hairy root induction was observed in co-cultivation medium complemented with 1 mM L-arginine, resulting in 2 fold increase in hairy root induction compared to the control medium (Figure 4).

3.5. Response surface optimization

In this study different factor treatment, including L-Arginine one factor levels, bacterial strain, type of explants and type of media for HR frequency (%) were studied (Table 3). According to the results (Table 4), modeling and optimization conditions, including L-Arginine concentration 1.00 mM; bacterial strain; C58C1, leaf explant and B5 medium for HR induction frequency equal to 58% was evaluated (Desirability= 0.986).

Table 3
Experimental design and results of One Factor RSM Design.

Run order	L-Arg (mM)	Medium	Explant	Strains	HR frequency	Run order	L-Arg (mM)	Medium	Explant	Strains	HR frequency
1	0.50	B5	Leaf	Control	0	57	0	MS	Stem	C58C1	20
2	0.50	B5	Stem	ATCC	20	58	0.5	B5	Leaf	ATCC	10
3	1.00	B5	Leaf	R1000	20	59	1.5	B5	Leaf	C58C1	50
4	0.00	B5	Stem	Control	0	60	1.5	B5	Stem	ATCC	50
5	0.00	MS	Leaf	C58C1	10	61	0	B5	Stem	ATCC	20
6	0.50	MS	Stem	ATCC	20	62	0.5	B5	Stem	C58C1	30
7	1.00	B5	Stem	R1000	20	63	1	MS	Leaf	Control	0
8	1.00	B5	Leaf	C58C1	60	64	0.5	MS	Stem	ATCC	20
9	0.00	B5	Leaf	C58C1	20	65	0	B5	Leaf	R1000	10
10	1.00	B5	Leaf	Control	0	66	0	MS	Leaf	R1000	10
11	0.50	MS	Stem	C58C1	30	67	0.5	B5	Leaf	C58C1	30
12	0.00	MS	Stem	Control	0	68	1	MS	Leaf	Control	0
13	0.00	B5	Leaf	C58C1	20	69	1	B5	Leaf	Control	0
14	0.00	B5	Stem	Control	0	70	1.5	MS	Leaf	R1000	20
15	0.00	B5	Stem	ATCC	20	71	0.5	MS	Leaf	ATCC	20
16	0.50	MS	Stem	R1000	10	72	1	MS	Stem	R1000	30
17	1.50	MS	Leaf	C58C1	40	73	1	MS	Leaf	C58C1	60
18	1.50	MS	Stem	R1000	30	74	1.5	B5	Leaf	C58C1	50
19	1.00	B5	Leaf	C58C1	60	75	0.5	B5	Stem	ATCC	20
20	1.50	B5	Stem	C58C1	50	76	0	B5	Leaf	Control	0
21	1.00	B5	Leaf	Control	0	77	1.5	MS	Stem	C58C1	50
22	0.00	MS	Stem	Control	0	78	0.5	MS	Leaf	C58C1	30
23	1.00	MS	Stem	Control	0	79	0	MS	Leaf	ATCC	10
24	1.00	B5	Leaf	R1000	20	80	1	MS	Stem	C58C1	60
25	0.50	B5	Stem	Control	0	81	1.5	B5	Stem	C58C1	50
26	0.50	MS	Stem	R1000	10	82	0.5	MS	Stem	Control	0
27	1.50	MS	Stem	Control	0	83	1.5	B5	Leaf	R1000	20
28	1.50	B5	Leaf	R1000	20	84	1	B5	Stem	R1000	20
29	0.50	B5	Leaf	R1000	0	85	0.5	MS	Stem	Control	0
30	0.00	B5	Stem	C58C1	30	86	0	B5	Leaf	R1000	10
31	1.50	MS	Stem	ATCC	40	87	1	MS	Leaf	R1000	30
32	0.00	B5	Stem	C58C1	30	88	0	B5	Leaf	ATCC	10
33	1.50	B5	Leaf	ATCC	40	89	1.5	MS	Stem	ATCC	40
34	0.50	MS	Leaf	Control	0	90	1.5	B5	Stem	R1000	30
35	1.50	MS	Leaf	C58C1	40	91	1.5	MS	Leaf	ATCC	30
36	0.50	B5	Stem	R1000	0	92	0	MS	Leaf	R1000	10
37	1.00	MS	Leaf	R1000	30	93	1	B5	Leaf	ATCC	40
38	0.50	B5	Stem	R1000	10	94	1.5	MS	Stem	R1000	30
39	1.00	MS	Leaf	C58C1	60	95	0.5	MS	Leaf	R1000	10
40	1.00	MS	Stem	C58C1	60	96	1.5	B5	Stem	R1000	30
41	0.50	B5	Stem	R1000	10	97	0	MS	Leaf	Control	0
42	1.00	B5	Stem	ATCC	40	98	1.5	MS	Leaf	R1000	20
43	0.00	MS	Stem	C58C1	20	99	1.5	B5	Stem	ATCC	50
44	0.50	B5	Leaf	Control	0	100	1	B5	Stem	C58C1	50
45	0.50	B5	Leaf	ATCC	10	101	1	MS	Stem	ATCC	40
46	1.50	B5	Leaf	ATCC	40	102	1	B5	Stem	Control	0
47	0.00	B5	Leaf	Control	0	103	1.5	MS	Stem	C58C1	50
48	0.50	MS	Leaf	Control	0	104	0	MS	Stem	ATCC	20

49	1.50	B5	Stem	Control	0	105	1	MS	Stem	R1000	40
50	0.00	MS	Leaf	C58C1	10	106	1	MS	Leaf	ATCC	50
51	0.00	MS	Stem	ATCC	20	107	0.5	MS	Stem	R1000	10
52	1.50	B5	Stem	Control	0	108	0.5	B5	Stem	Control	0
53	1.00	B5	Stem	C58C1	50	109	0	B5	Leaf	ATCC	10
54	1.50	MS	Stem	Control	0	110	1	MS	Leaf	Control	0
55	0.00	MS	Leaf	Control	0	111	1.5	MS	Leaf	ATCC	30
56	0.5	MS	Leaf	ATCC	20	112	0	MS	Leaf	ATCC	10

Table 4
Results of the regression analysis of the One Factor RSM Design.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	37828.67	35	1080.82	103.15	< 0.0001
A-L-Arginine	7165.08	1	7165.08	683.82	< 0.0001
B-Medium	3.57	1	3.57	0.34	0.5611
C-Explant	455.30	1	455.30	43.45	< 0.0001
D-Bacterial strain	21491.07	3	7163.69	683.68	< 0.0001
AB	4.34	1	4.34	0.41	0.5219
AC	10.48	1	10.48	1.00	0.3204
AD	3062.05	3	1020.68	97.41	< 0.0001
BC	3.61	1	3.61	0.34	0.5592
BD	169.54	3	56.51	5.39	0.0020
CD	52.62	3	17.54	1.67	0.1797
A2	529.55	1	529.55	50.54	< 0.0001
ABC	55.00	1	55.00	5.25	0.0247
ABD	160.99	3	53.66	5.12	0.0028
ACD	380.52	3	126.84	12.11	< 0.0001
BCD	104.12	3	34.71	3.31	0.0244
A2B	418.45	1	418.45	39.94	< 0.0001
A2C	440.48	1	440.48	42.04	< 0.0001
A2D	582.50	3	194.17	18.53	< 0.0001
A3	1785.78	1	1785.78	170.43	< 0.0001
Residual	796.33	76	10.48	-	-
Lack of Fit	679.67	24	28.32	12.62	< 0.0001
Pure Error	116.67	52	2.24	-	-

The final equation (Model 2) obtained in terms of the coded factors for culture conditions is:

$$\begin{aligned}\text{HR frequency } (\%) &= +23.49 + 29.53 \times A - 0.22 \times C + 6.36 \times D \\ &+ 6.96 \times AD - 1.20 \times BD - 4.99 \times A^2 + 1.003 \\ &\times ABC + 1.70 \times ABD + 6.03 \times ACD + 1.200 \times BCD \\ &- 4.71 \times A^2B + 5.67 \times A^2C + 2.63 \times A^2D \\ &- 23.73 \times A^3\end{aligned}$$

Mode l 1

The cubic model fitness was expressed with the coefficient of determination; R2, which was calculated to be 0.97, indicating that 97% of the variability in the response could be explained by the model and just 3% of the total variation for response were not explained by the respective model. The model F-value of 103.15 implies the model is significant. This proves that the model equation as expressed in Model 2 provides a suitable model (Table 4).

4. Discussion

In hairy root induction and tissue culture techniques, selection of Agrobacterium strains, type of explants and development medium compositions are critical factors (Danphitsanuparn et al., 2012). Varied response of different plant tissues to Agrobacterium transformation has been reported (Ercan et al., 1999; Lee et al., 2010). Our results showed that 1mM concentration of L-arginine could improve hairy root induction. There are reports that adding L-arginine not only resulted into increase root induction (Tabatabaei and Omidi, 2012), but also reduced necrosis of explants (Henzi et al., 2000; Sharafi et al., 2013). This is the first report on L-arginine amino acid optimization in hairy root induction of madder. The major objective of this research was the development of a statistical approach to better understanding of the relationship between the variables of the L-Arginine levels. In this method, the process can be optimized in order to save money and time, allowing an economically important tissue culture technique of high quality to be obtained.

5. Conclusion

Hairy root culture method have an application potential for biotechnological implementation to produce the anticipated compounds. Briefly, this research supports the possibility of an effective positive influence of L-Arginine on the hairy root induction of R. tinctorum L. and in next studies altering the compositions of the media will be needed.

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